Cell Lineage Specification during Mouse Embryonic Gonad Development

by

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University Program in Genetics and Genomics
Duke University

Date: ___ April 3rd, 2017

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Debra Silver

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy
in the University Program in Genetics and Genomics
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ABSTRACT

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Abstract

The mouse embryonic gonad provides an outstanding model to study the complex mechanisms involved in cell fate specification and maintenance. At the bipotential stage, both XX and XY gonads are capable of becoming testes or ovaries upon specific molecular cues. The specification of the supporting cell lineage (as either Sertoli cells in the male or granulosa cells in the female) initiates the testis or ovary program, leading to male or female fate. However, there are significant gaps in our understanding of how the somatic cells in the gonad arise, are competent to differentiate, and determine and maintain their fates. In this dissertation, we addressed these questions.

We found that NUMB (an antagonist of Notch signaling) serves as competence factor for somatic cell differentiation during early gonadogenesis. The asymmetric allocation of NUMB to the basolateral domain of actively dividing coelomic epithelial (CE) cells is indispensable to (1) maintain the totipotent stem cell-like reservoir at the CE domain, and (2) give rise to progenitor cells that can further differentiate into supporting and interstitial cell lineages. Deletion of Numb; Numbl resulted in disruption of cell polarity in the CE domain as well as a reduction of multiple differentiated cell lineages within XX and XY gonads, including supporting cells and male steroidogenic cells, which were most severely affected. We detected elevated Notch downstream signaling in the Numb; Numbl mutant gonads. Moreover, treatment of DAPT (which blocks Notch signaling)
rescued the \textit{Numb; Numbl} mutant phenotypes, strongly suggesting that upregulation of Notch is responsible.

Previous experiments indicate that when supporting cells commit to the male (Sertoli) fate, they must repress the alternative female (granulosa) cell fate. In another line of experiments, we investigated the hypothesis that the Polycomb repressive complex 1 (PRC1) plays a critical role in repressing the female pathway during male gonad patterning. We found that loss of \textit{Ring1B} (a component of PRC1) led to the disruption of XY gonad development specific to the posterior region of male gonads. \textit{Sry}, the upstream driver of the male pathway, was not appropriately expressed in the posterior domain, which contained cells expressing female markers and, in some cases, small aggregates of undifferentiated cells. Using ChIP-Seq, we identified potential targets of PRC1 during male gonad development. Moreover, a key gene in the male pathway, \textit{SOX9}, interacts with RING1B, based on immunoprecipitation results, leading to the hypothesis that it may be involved in the recruitment of PRC1 to its target sites to execute the repression of female genes in male gonads.

Our findings provide insight into how somatic cell fate is determined and maintained during mammalian sex determination. Our results may be valuable for patients with disorders of sexual development with unidentified genetic contributions.
Dedication

I would like to dedicate this dissertation to my wonderful, loving parents, Sheng-Chieh Lin and Hwei-Yuen Tzou. Without their support, wisdom and encouragement, I wouldn’t have become who I am.
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1. Introduction

The gonads initiate as bipotential organs that can develop as testes or ovaries. All secondary sex characteristics that we associate with “maleness” or “femaleness” depend on whether testes or ovaries form. With multiple cell lineages present in the gonad, the cell fate decision of the “supporting cell lineage”, a somatic cell lineage, is the key to decide the fate of the gonads. Once supporting cell progenitors commit to Sertoli (male) or granulosa (female) fate, they propagate this decision to the other cells within the organ.

1.1 Formation of the gonad

Gonads form as paired, bilateral organs that are composed of several lineages of somatic cells as well as the population of germ cells. The SF1 (steroidogenic factor 1, aka NR5A1) -positive cells in the coelomic epithelium (CE) overlying the region of the intermediate mesoderm, the mesonephros, are the precursors of many of the somatic cells in the gonad. The CE begins to thicken in this region as SF1-positive cells proliferate at around embryonic day (E) 10.0. CE cells contribute to at least two distinct somatic precursor lineages that are bipotential: (1) supporting cell precursors, which give rise to Sertoli cells (male) or fetal granulosa cells (female), and (2) steroidogenic progenitors, which give rise to Leydig cells (male) or theca cells (female)(Karl and Capel, 1998; Schmahl et al., 2000). Genes including Wt1 (Wilms tumor 1 homolog)(Hammes et al.,
Lhx9 (LIM homeobox protein 9) (Birk et al., 2000), Emx2 (empty spiracles homeobox 2) (Kusaka et al., 2010), Sfl (Luo et al., 1994), M33 (Cbx2, chromobox 2) (Katoh-Fukui et al., 2012; Katoh-Fukui et al., 1998), Gata4 (Hu et al., 2013) and Six1/4 (sine oculis-related homeobox 1/4) (Fujimoto et al., 2013) are essential to establish the bipotential population of somatic cells in the gonad.

### 1.2 The bipotential stage

In the bipotential gonad, early somatic progenitors are capable of adopting either male or female fate. Previous work has shown that the transcriptomes of whole XX and XY gonads are nearly indistinguishable at E10.0 through E11.2 (Munger et al., 2013; Nef et al., 2005). At this bipotential stage, genes that are later associated with testis fate (i.e. Sox9 (Sry (sex determining region of the Y)-box 9) and Fgf9 (fibroblast growth factor 9)) and ovary fate (i.e. Wnt4 (wingless-type MMTV integration site family, member 4) and Rspo1 (R-spondin homolog 1)) are expressed at similar levels in XX and XY gonads (Jameson et al., 2012b; Munger et al., 2013). These results suggest that the bipotential plasticity of the mammalian gonad results from a transient balanced transcriptional state. Interestingly, although the gonad is poised to follow either pathway at this bipotential stage, the supporting cell lineage expresses more genes later associated with the female than the male pathway, suggesting a female biased program (Jameson et al., 2012b).
1.3 The first steps of male and female fate commitment

Sex determination initiates by tilting the balance in the transcription network toward the male or female fate. The Y chromosome-linked gene, Sry, is the switch to initiate the male pathway in the poised supporting cell progenitors. Accumulating evidence supports the crucial role of Sry. For example, an Sry transgene, driven in the XX gonad from its own promoter, caused differentiation of a testis (Koopman et al., 1991). This results suggested (1) Sry is the only Y chromosome gene that is required for male sex determination, and (2) the molecular environment of the XX gonad is fully competent for Sry activation and initiation of testis development. (reviewed in (Larney et al., 2014))

Based on an RNase protection study, the Sry gene is found to express just after E10.5 (10 tail somites (ts)) (Hacker et al., 1995). Using in situ hybridization, expression is detectable in the middle of the gonad at ts14 (~E11.0) and expands toward the anterior, then posterior poles (Bullejos and Koopman, 2001). The timing and level of expression of Sry are critical. Experiments using a heat shock promoter to drive Sry expression in XX gonads, suggested a requirement for Sry in the 6-hour time window between E11.0 and E11.25 (Hiramatsu et al., 2009). If expression is delayed, the testis pathway is aborted and ovarian development ensues. However, why the window of opportunity to initiate the male pathway closes at E11.25 remains unclear. XY mice carrying a weak allele of Sry
that shows a decrease/delay in expression are susceptible to male-to-female sex reversal as well (Albrecht et al., 2003; Nagamine et al., 1999; Wilhelm et al., 2009). Downstream of Sry expression, Sox9 is the earliest gene to be upregulated in the male pathway at E11.2, followed by Cited4 (Cbp/p300-interacting transactivator-4, with Glu/Asp-rich carboxy-terminal domain, and Sox13 (SRY-box 13) at E11.4, and a larger group of genes at E11.6 (Munger et al., 2013). Many of these genes are critical to establish male fate (Cui et al., 2004; Pierucci-Alves et al., 2001; Yao et al., 2002).

Genes associated with the female pathway become dimorphic slightly later, between E11.4-E11.6, including Wnt4, Rspo1, Irx3 (Iroquois related homeobox 3), Lhx9, Fst (follistatin), and Lef1 (lymphoid enhancer binding factor 1) (Jameson et al., 2012b; Munger et al., 2013). The downstream effect of WNT4/RSPO1 signaling is the stabilization of β-catenin (Tevosian and Manuylov, 2008; Tomaselli et al., 2011). β-catenin accumulates in the nucleus (Chassot et al., 2008; Liu et al., 2009) and interacts with the transcription factor LEF1 leading to the activation of downstream genes, as in other systems (Gottardi and Gumbiner, 2004). Stabilization of β-catenin in the XY gonad results in down-regulation of SOX9 and male to female sex-reversal (Maatouk et al., 2008). Antagonism between SOX9 and β-catenin may underlie the molecular decision in individual cells. Interestingly, loss of Wnt4 and/or Rspo1 and/or β-catenin does not lead
to complete female to male sex reversal until perinatal stages (Chassot et al., 2008; Manuylov et al., 2008; Tomizuka et al., 2008; Vainio et al., 1999).

Another key factor that regulates female fate is the transcription factor FOXL2 (forkhead box L2). FOXL2 co-operates with BMP2 (bone morphogenetic protein 2) to up-regulate the expression of Fst (Kashimada et al., 2011). In goats, loss of function of Foxl2 leads to female to male sex reversal in fetal life (Boulanger et al., 2014). However, in mice and humans, loss of Foxl2 does not lead to disruption of the female pathway until neonatal stages. Although loss of Foxl2 in combination with Rspo1 or Wnt4 slightly accelerates the sex reversal phenotype in mice (Auguste et al., 2011; Ottolenghi et al., 2007), no gene has been discovered whose loss leads to complete female to male sex reversal at early fetal stages. Since the bipotential gonad is initially biased toward the ovarian fate, a gene with a comparable role to Sry may not be required to initiate the female pathway. It may be sufficient not to initiate the male pathway.

Supporting cell precursors enter a quiescent state by E12.5 in XX gonads (Mork et al., 2012), consistent with the upregulation of negative regulators of the cell cycle observed in transcriptome studies (Bouma et al., 2007; Jameson et al., 2012b). The quiescent state of progenitor cells in the ovary may protect them from switching fate until proliferation resumes around the time of birth (Maatouk et al., 2013; Mork et al., 2012). In XY gonads, on the contrary, supporting cell progenitors initiate rapid
proliferation immediately after Sry expression, and blocking proliferation disrupts the male pathway (Schmahl and Capel, 2003; Schmahl et al., 2000). Whether proliferation is important for intracellular fate commitment or is required to establish a threshold population of Sertoli progenitors is still unclear.

Sexually dimorphic expression can result from activation in one sex, repression in the other sex, or a combination of both mechanisms. All of these patterns were evident in a study in which the gene expression profile for each gene was compared in XX and XY gonads at fine time points between E11.0 and E12.0 (Munger et al., 2013). Enrichment of male pathway genes occurred primarily through activation in the XY gonad. Interestingly, about half of the genes that became enriched in the female gonad are repressed in the XY gonad (Figure 1). This is a critical feature of the counterbalanced system that controls sex determination and gonadal fate: to establish a fate decision in the gonad, it is not sufficient to activate one of the alternative pathways – it is also necessary to repress the other.

1.4 Propagation of fate commitment across the gonad field

After the primary fate decision in both male and female pathways, feedback mechanisms are activated that canalize the chosen sexual fate. This occurs within individual cells and, also, across the gonad field.
XX<=>XY chimera experiments demonstrate that fate commitment can be propagated throughout the gonad field. Although XX<=>XY chimeras typically have a similar number of XX and XY cells in the fetal gonad (as in other organs), more than half of these gonads develop as testes. This result suggests that a threshold number of XY cells can establish the testis pathway throughout the organ, but in cases where this cell threshold is not met, the gonad develops as an ovary. In the adult testes of chimeras, most of the Sertoli cells are XY, interestingly, a small number of XX Sertoli cells can be found, indicating that XY supporting cells in the gonad can recruit XX cells (without a Y chromosome or Sry gene) to Sertoli fate, presumably through paracrine signals (Burgoyne et al., 1988; Palmer and Burgoyne, 1991). These results indicate that in addition to the fate determination step that occurs in each supporting cell, there is a “community decision” that takes place across the field of the gonad.

FGF9 and PGD2 (prostaglandin D2 synthase) are two paracrine signaling molecules downstream of SOX9 that contribute to propagation and maintenance of the male fate decision (Adams and McLaren, 2002; Kim et al., 2007; Kim et al., 2006; Malki et al., 2005; Moniot et al., 2009; Wilhelm et al., 2007; Wilhelm et al., 2005). Fgf9 is required to signal from the central part of the gonad and reinforce Sox9 expression toward the two ends of the gonad (Hiramatsu et al., 2010). The other crucial role of Fgf signaling is to antagonize Wnt signaling (Kim et al., 2006). Elimination of the expression of Fgf9 or its
receptor $Fgfr2$ (fibroblast growth factor receptor 2), leads to upregulation of the $Wnt$ pathway and male-to-female sex reversal (Bagheri-Fam et al., 2008; Colvin et al., 2001; Kim et al., 2007; Siggers et al., 2014). On the other hand, elimination of $Wnt4/Rspo1/\beta$-catenin leads to female-to-male sex reversal near birth (Chassot et al., 2008; Manuylov et al., 2008; Tomizuka et al., 2008; Vainio et al., 1999), suggesting that the $Fgf9$ and $Wnt4$ signaling pathways antagonize each other during sex determination. Deletion of both $Fgf9$ and $Wnt4$ or $Fgfr2$ and $Wnt4$ rescues the male-to-female sex reversal phenotype in the XY gonad. In double mutants both somatic cells and germ cells express markers associated with male development, strongly suggesting that the primary role of $Fgf$ signaling in XY gonad development is to repress the $Wnt$ pathway (Jameson et al., 2012a). Similar results were obtained by paired deletion of several other sets of antagonistic factors. For example, deletion of $Sox9$ leads to male to female sex reversal, but rescue of the male fate occurs when $Sox9$ and $Rspo1$ are simultaneously deleted (Lavery et al., 2012). In another case, when $Wnt4$ and $Activin\beta\beta B$ were simultaneously deleted, the female fate was rescued (Liu et al., 2010). These studies indicate a multi-layered input into sex determination and suggest that multiple coding genes and potentially microRNAs (Wainwright et al., 2013) all contribute to the balance between male and female fates.
How other somatic lineages across the gonad field commit to a testis or ovary fate is relatively unclear. However, it is known that this occurs downstream of the supporting cell fate commitment. The steroidogenic precursor lineage in XX and XY gonads shares an indistinguishable transcriptional profile at E11.5 (Jameson et al., 2012b). Not until E12.5 do XX and XY steroidogenic progenitors begin to show divergent expression patterns, after morphological changes that occur in the XY gonad, including the formation of the male-specific cord structures and vasculature. For Leydig cell development in the testis, \textit{Dhh} (Pierucci-Alves et al., 2001; Yao et al., 2002) and \textit{Pdgfra} (Brennan et al., 2003; Cool et al., 2011) are indispensable. However, it is still unclear whether their roles are direct, or indirectly mediated by effects on the vasculature. Disruption of the male-specific vasculature also disrupts Leydig cell development, likely through \textit{Notch/Jag1} signaling (Defalco et al., 2013). Steroidogenic cells in the ovary begin to produce hormones around birth. Like the steroidogenic lineage in the testis, they have a mixed origin. In the fetal mouse ovary, NR2F2 and MAFB label the progenitors of a cell type distinct from the supporting cell lineage (Maatouk et al., 2012; Rastetter et al., 2014) are likely to contribute to the later differentiated steroidogenic population. These progenitors differentiate in response to induction signals in their testis or ovary environment (DeFalco et al., 2011; Liu et al., 2012).
1.5 Germ cell fate commitment

Germ cells arise at the base of the allantois in the E6.25 embryos, migrate within the gut epithelium, and eventually arrive in the gonad during the early stages of gonadogenesis. During these stages, the germ cell genome is stripped of most of its methylation (Maatouk et al., 2006). Like ES cells, with bivalent histone marks on many developmentally regulated genes (Lesch et al., 2013), germ cells are highly pluripotent, and can readily give rise to embryonic germ cells (EG cells) (Labosky et al., 1994). Fate determination in the germ cell lineage involves repressing pluripotency and committing to differentiate as either male or female gamete precursors: spermatogonia or oogonia.

Germ cells differentiate as spermatogonia or oogonia based on cues from their somatic environment, irrespective of their genetic sex. In contrast to the supporting cell lineage, germ cells at the bipotential stage express more genes associated with a male fate (Jameson et al., 2012b), suggesting that female germ cells take the more divergent path at this stage of development. At E11.5, XX and XY germ cells have very similar transcriptomes: only a few Y- and X-linked genes have detectable differences (Munger et al., 2013). Regardless of these differences, both XX and XY germ cells are capable of responding to either male or female gonadal cues up until E12.5, after which their fate is fixed (McLaren and Southee, 1997).
XX and XY germ cells enter meiosis in an ovarian environment, or in cases where they are lost in the mesonephros or adrenal. In contrast, in a testis environment, germ cells arrest in G0 of the mitotic cell cycle (McLaren, 2003). The molecular explanation for this behavior is suggested by the presence of a repressor of meiosis in the XY gonad, as predicted by McLaren (McLaren and Southee, 1997), and an activator of meiosis in the XX gonad, as proposed by Bysmutantv (Byskov et al., 1993).

In XX gonads, retinoic acid (RA) signaling initiates meiotic entry via activation of Stra8 (stimulated by retinoic acid gene 8). RA is synthesized in the mesonephros, and may be transported by the mesonephric tubules that are physically connected with the anterior end of the gonad. This could explain the anterior-to-posterior wave of Stra8 expression that triggers meiotic entry between E13.5-E15.5 (Bullejos and Koopman, 2004; Menke et al., 2003; Yao et al., 2003). The timing of meiotic entry is controlled by polycomb complex 1 (PRC1), which represses expression of Stra8 prior to E13.5 in XX germ cells (Yokobayashi et al., 2013). Germ cells in the female gonad progress through leptotene, zygotene and pachytene, and arrest in diplotene near the time of birth.

RA is also produced in XY embryos, however, the P450 enzyme CYP26B1 (cytochrome P450, family 26, subfamily b, polypeptide 1) is synthesized early in the male pathway by somatic cells in the testis, and degrades RA, therefore blocking activation of Stra8 and meiosis (Bowles et al., 2006; Koubova et al., 2006). In mouse
genetic models with \textit{Cyp26b1} disruption, germ cells enter meiosis in the testis (Bowles et al., 2006; MacLean et al., 2007).

There are also additional factors that contribute to the divergence of male and female germ cell fates. The antagonistic signaling pathways, \textit{Wnt/Rspo} and \textit{Fgf}, also affect germ cell fate, in addition to their role in somatic sex determination. \textit{Wnt/Rspo} signaling acts through \textit{β-catenin} to regulate proliferation of XX germ cells and promote their entry into meiosis (Chassot et al., 2011). \textit{Wnt4} disruption leads to an anterior to posterior loss of female germ cells (Maatouk et al., 2013). In XY gonads, loss of \textit{Fgf9} leads to sex-specific apoptosis of germ cells and up-regulation of meiotic markers (Bowles et al., 2010; DiNapoli et al., 2006).

\textit{FGF9} is required for the up-regulation of \textit{Nanos2} (Barrios et al., 2010; Bowles et al., 2010), a key RNA-binding protein (RBP) that is critical for initiating the male pathway in germ cells. RBPs, including \textit{NANOS2/3} (Suzuki et al., 2010; Suzuki et al., 2014), TDRD1, a tudor domain containing RBP (Chuma et al., 2006; Hosokawa et al., 2007), DND1 (dead end homolog 1)(Cook et al., 2009; Cook et al., 2011), and PUMILIO (Xu et al., 2007) play prominent roles in male germ cells. In the absence of \textit{Nanos2}, male germ cells enter meiosis and/or undergo apoptosis (Suzuki and Saga, 2008). \textit{NANOS2} and \textit{NANOS3} associate in the CNOT complex to control adenylation of multiple mRNA targets and promote degradation of mRNAs encoding meiotic genes (Suzuki et al., 2010;
Suzuki et al., 2014). *Dnd1* also promotes translation of several negative regulators of the cell cycle, such as P21 (Cdkn1a, cyclin-dependent kinase inhibitor 1A) and P27 (Cdkn1b, cyclin-dependent kinase inhibitor 1B) (Cook et al., 2011; Kedde et al., 2007). Accumulation of negative cell cycle regulators is believed to bring male germ cells into mitotic arrest in G0, where they remain until perinatal stages when mitosis resumes prior to the establishment of the spermatogonial stem cell population (Western et al., 2008; Yoshida et al., 2006). Some evidence also suggests that cell cycle arrest in male germ cells is critical to repress pluripotency and establish spermatogonial fate. Germ cells that fail to enter G0 by E15.5 in the male gonad are susceptible to teratoma formation, an indication that germ cells have not repressed their pluripotent state (Bustamante-Marin et al., 2013; Cook et al., 2011; Heaney et al., 2012).

Although FGF9 also has been assigned a role in up-regulation of p15INK4B (Cdkn2b cyclin-dependent kinase inhibitor 2B) (Bowles et al., 2010), if both *Fgf9* and *Wnt4* are deleted, the XY gonad embarks on the testis fate, and XY germ cells fail to enter meiosis and express normal markers of the male pathway (Jameson et al., 2012a). This has been interpreted to mean that other Fgfs can compensate for this function of *Fgf9*, or that there are layers of antagonistic signals that control germ cell fate similar to those that control somatic cell fate.
1.6 Maintaining Sertoli or granulosa cell fate in adult life

Transdifferentiation from one committed fate to another is an unusual phenomenon that occurs naturally when germ cells are depleted from the adult ovary: in this situation, granulosa cells transdifferentiate to Sertoli cell fate (Guigon and Magre, 2006; Merchant, 1975). Several genes have been identified that are directly involved in active maintenance of supporting cell fate in adult life.

In the female gonad, FoxL2 plays a key role in maintaining ovarian fate postnatally. FOXL2 and ESR1/2 (estrogen receptors 1 and 2) cooperatively antagonizes the male pathway by direct binding to the TESCO regulatory region of Sox9 promoter to repress its expression. When FOXL2 is lost in adult life, granulosa cells transdifferentiate prior to germ cell loss, and theca cells also begin to produce testosterone (Uhlenhaut et al., 2009). Disruption of Esr1/Esr2, or P450 aromatase (Cyp19a1) also results in transdifferentiation in the postnatal ovary and germ cell loss (Britt et al., 2002; Couse et al., 1999). While it is difficult in these scenarios of transdifferentiation to disentangle the effects of germ cell loss, it is likely that they are due, at least in part, to direct effects of estrogen on maintenance of granulosa cell fate.

In the adult testis, DMRT1 (doublesex and mab-3 related transcription factor 1) binds to regulatory regions of testis- and ovary-promoting genes. DMRT1 activates testis-promoting genes such as Sox8, Sox9 and Ptgdr (prostaglandin D receptor), and represses
ovary-promoting genes such as FoxL2, Wnt4, and Rspo1. DMRT1 may be important to antagonize the influence of RA, which is produced in the adult testis to drive entry of spermatogonia into meiosis, and may have a feminizing influence on Sertoli cells (Minkina et al., 2014).

Commitment of the bipotential gonad to testis or ovary fate is the result of antagonistic male and female pathways that compete to control the differentiation of supporting cell precursors, likely through regulation of SOX9 and β-Catenin (Fig. 1). Sertoli or granulosa cell fate commitment involves not only the activation of one program but also the repression of the alternative pathway of development. The decision is then propagated across the gonad field, and controls the fate of the germ cell lineage as well as the other somatic lineages. In the adult testis and ovary, the initial fates decisions must be actively maintained, otherwise transdifferentiation can occur between Sertoli and granulosa cell fates. Many differentiated cells in other organs share a common bipotential progenitor, however, it is unclear whether the phenomenon of transdifferentiation between differentiated fates occurs naturally in other systems, or whether it is specific to gonadal cells. It could be an evolutionary remnant of the ability of some fish to switch sex in adult life (Wu and Chang, 2013) or of some vertebrates’ ability to function as “natural hermaphrodites” by maintaining a gonad with seasonally expanding ovarian and testicular regions such as the mole and alligator (Guillette et al.,
A study of dosage hypersensitive sites across the Sertoli progenitor genome at E13.5 revealed open chromatin near genes associated with both the male and female pathways (Maatouk et al., 2017). It will be interesting to see how this plasticity of cell fate is related to the epigenetic landscape at the bipotential stage, and in Sertoli and granulosa cells during fetal and adult life.

**Figure. 1** Mechanisms involve in commitment of the bipotential gonad to testis or ovary fate. Commitment and maintenance of gonadal cell fate. In XX and XY gonads at the bipotential stage, supporting cell precursors are exposed to male and female promoting signals that antagonize each other. In the XY gonad, expression of *Sry* triggers up-regulation of *Sox9* and *Fgf9*, which activate the male pathway and repress signals that promote the female pathway (WNT4/RSPO1 and β-catenin). Supporting cell precursors commit to Sertoli cell fate and orchestrate testis development by promoting Leydig cell differentiation from steroidogenic progenitors, and regulating mitotic arrest in germ cells. In the XX gonad, in the absence of *Sry* to initiate the male pathway, *Wnt4* and *Rspo1* maintain β-catenin signaling to promote the female pathway. Supporting cell precursors commit to granulosa cell fate and orchestrate ovary development by promoting Theca cell differentiation from steroidogenic progenitors, and regulating meiotic entry in germ cells. Reinforcing signals (dotted lines) exist between somatic and
germ cells in the developing testis and ovary. In the adult testis, \textit{Dmrt1} is required for maintaining Sertoli cell fate by repressing female promoting signals, while in the adult ovary, abolishing female promoting signals leads to loss of granulosa cell fate and up-regulation of \textit{Sox9} and other markers of Sertoli fate.

![Mechanisms involved in commitment of the bipotential gonad to testis or ovary fate.](image)

**Figure 1:** Mechanisms involved in commitment of the bipotential gonad to testis or ovary fate.
2. Materials and Methods

2.1 Mice strains and lines

The mouse line carrying a floxed allele of Numb, \textit{Numb}^{\text{tm1Ynj}} (Numb$^{\text{flox}}$) (Zhong et al., 2000) and a null allele for Numb-like, \textit{Numbl}^{\text{tm1Wmz}} (Numbl$^{-}$) (Petersen et al., 2002) was kindly provided by C. T. Kuo (Department of Cell Biology, Duke University) and maintained on a mixed background (B6/129). The ROSA-CreER (B6.129-Gt(ROSA)26Sor^{cre/ERT2}tm1(Jax Stock No: 008463) line was obtained from Jackson Laboratories. \textit{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 (also available from Jackson Laboratories: B6;129S6-Gt(ROSA)26Sor^{em9(CAG-tdTomato)Hze}tm1Jax Stock No: 007905). \textit{Sox9-ECFP} homozygous transgenic males (Kim et al., 2007) were bred to CD-1 (Charles River) females in timed matings to generate E13.5 embryos. For each antibody and 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.
**2.2 Timed mating**

Timed matings were performed by placing females in cages with males after 4 pm each day. The females were inspected and separated from the male the next morning. The presence of a vaginal plug was designated as embryonic day (E) 0.5.

**2.3 Tamoxifen administration**

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.

**2.4 Immunofluorescence**

Embryonic gonads at E11.0-E14.5 were dissected from embryos and fixed in 4% paraformaldehyde for 1-2 hours at room temperature. Fixed gonads were washed 3 x 20 minutes with TBS-Triton X-100 (0.1%), and stored in methanol at -20°C until use. Samples were re-hydrated and whole-mount immunostaining was performed as previously described (Jameson et al., 2012a). Primary antibodies used in this study are listed in Table 1. Secondary antibodies used included Alexa 647- and 488-conjugated secondary antibodies (Molecular Probes, Grand Island, NY), applied at 1:500, and Cy3-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) used at 1:500. Hoechst 33342 (Invitrogen, Eugene, OR, USA) was also used to label nuclei. Samples were mounted in 2.5% DABCO (Sigma-Aldrich) in 90% glycerol.
Table 1 List of antibodies used for immunofluorescence.

<table>
<thead>
<tr>
<th>Primary antibodies</th>
<th>Species</th>
<th>Cat#</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>NUMB</td>
<td>Rabbit</td>
<td>ab-14140</td>
<td>1:100</td>
<td>Abcam, Cambridge, MA</td>
</tr>
<tr>
<td>NOTCH2</td>
<td>Goat</td>
<td>sc-7423</td>
<td>1:250</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA</td>
</tr>
<tr>
<td>GATA4</td>
<td>Goat</td>
<td>sc-1237</td>
<td>1:500</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA</td>
</tr>
<tr>
<td>VCAM1</td>
<td>Goat</td>
<td>AF-643</td>
<td>1:1000</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>LHX9</td>
<td>Rat</td>
<td></td>
<td>1:50</td>
<td>kind gift of Ken-Ichiou Morohashi</td>
</tr>
<tr>
<td>DDX4</td>
<td>Rabbit</td>
<td>ab13840</td>
<td>1:250</td>
<td>Abcam, Cambridge, MA</td>
</tr>
<tr>
<td>SRY</td>
<td>Rabbit</td>
<td></td>
<td>1:250</td>
<td>kind gift of Dagmar Wilhelm</td>
</tr>
<tr>
<td>Laminin</td>
<td>Rabbit</td>
<td></td>
<td>1:500</td>
<td>kind gift of Harold Erickson</td>
</tr>
<tr>
<td>RFP</td>
<td>Rat</td>
<td>SF8</td>
<td>1:500</td>
<td>Chromotek, Germany</td>
</tr>
<tr>
<td>WT1</td>
<td>Rabbit</td>
<td>sc-192</td>
<td>1:100</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA</td>
</tr>
<tr>
<td>NR5A1</td>
<td>Rabbit</td>
<td></td>
<td>1:1500</td>
<td>kind gift of Ken-ichiou Morohashi</td>
</tr>
<tr>
<td>β1-Integrin</td>
<td>Rat</td>
<td>MAB1997</td>
<td>1:200</td>
<td>Millipore, Billerica, MA</td>
</tr>
<tr>
<td>3β-HSD</td>
<td>Goat</td>
<td>sc-30820</td>
<td>1:250</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA</td>
</tr>
<tr>
<td>FOXL2</td>
<td>Goat</td>
<td>NB100-1277</td>
<td>1:250</td>
<td>Novus Biologicals, Littleton, CO</td>
</tr>
<tr>
<td>MKI67</td>
<td>Rabbit</td>
<td>RM-9106-S</td>
<td>1:500</td>
<td>Neomarkers, Thermo Scientific, Waltham, MA</td>
</tr>
<tr>
<td>PECAM1</td>
<td>Rat</td>
<td>553370</td>
<td>1:500</td>
<td>BD Pharmingen, San Diego, CA</td>
</tr>
<tr>
<td>Ring1B</td>
<td>Mouse</td>
<td>D139-3</td>
<td>1:1000</td>
<td>MBL Life Science</td>
</tr>
<tr>
<td>SOX9</td>
<td>Rabbit</td>
<td>AB5535</td>
<td>1:2000</td>
<td>Millipore, Billerica, MA</td>
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<tr>
<td>cCASPASE3</td>
<td>Rabbit</td>
<td>9661S</td>
<td>1:250</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>phospho-Histone H3 (S10)</td>
<td>Rabbit</td>
<td>9701S</td>
<td>1:250</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>CADHERIN1</td>
<td>Rat</td>
<td>13-1900</td>
<td>1:500</td>
<td>Novus Biologicals, Littleton, CO</td>
</tr>
<tr>
<td>GFP</td>
<td>Chicken</td>
<td>GFP-1020</td>
<td>1:2000</td>
<td>Aves, Tigard, OR</td>
</tr>
</tbody>
</table>
2.5 Gonad culture with MitoTracker and DAPT

Embryonic gonads were dissected from embryos at E11.5 and cultured in agar blocks for 24 hours at 37°C with 5% CO2 in 2 ml Dulbecco's Minimal Eagle Medium (DMEM), supplemented with 10% fetal bovine serum and 50 μg/ml ampicillin (Martineau et al., 1997). For DAPT treatment, DAPT (N-[N-(3,5-difluorophenacyl-L-alanyl]-S-phenylglycine-t-butyl Ester, Cat No: 565784, 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 minutes in 1 μM MitoTracker Orange (CMTMRos, Invitrogen) diluted in culture, then washed extensively in culture medium. Gonads were placed on agar blocks and cultured for 24 hours as described above. To ensure that MitoTracker only labeled the first layer of the CE, sample gonads were collected, double-labeled with antibodies against LHX9, and imaged after 1 hour of culture. Remaining gonads were cultured for 24 hours prior to labeling and imaging. In all cases, gonads were fixed in 4% paraformaldehyde at room temperature for 1 hour and processed for immunofluorescence.

2.6 Quantitative RT-PCR

Isolated gonads were gone through the RNA preparation protocol or frozen at −80 °C. RNA was extracted as previously described (Munger et al., 2009). Verso cDNA synthesis kit (Thermo 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, Carlsbad, CA) using the following parameters for 45 cycles: 95°C for 15 sec, 59°C for 30 sec, and 72°C for 30 sec. Threshold cycle (Ct) values were calculated by using StepOne software (version 2.2.2; Applied Biosystems). ΔCt values were calculated by using the housekeeping gene Canx as an internal control (van den Bergen et al., 2009). The normalized expression values from the biological replicates were averaged to calculate mean normalized expression (MNE). A significant difference in normalized expression between genotypes was determined using Student t-tests. Primer sequences are listed in Table 2.

Table. 2 List of Primers used for qPCR. Primers of the genes used for qPCR. Gene symbol, name, NCBI gene ID and forward and reverse primer sequences are listed.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>NCBI Gene ID</th>
<th>Forward Primer (5’ to 3’)</th>
<th>Reverse Primer (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sax9</td>
<td>SRY (sex determining region Y)-box 9</td>
<td>20862</td>
<td>TCCAGCAAGAACAAGCCACAC</td>
<td>TCTGTCAGCAGCCTCCAG</td>
</tr>
<tr>
<td>FoxD2</td>
<td>forkhead box L2</td>
<td>26927</td>
<td>GCCAGGGAGGGAGGGGAGAACAC</td>
<td>GACGGGGAATGTCGCTATGAGT</td>
</tr>
<tr>
<td>Wnt4</td>
<td>wingless-type MMTV integration site family, member 4</td>
<td>22417</td>
<td>CTGTCCTTGGGAAATGGTGT</td>
<td>CATAGGCGATGTTGGTCAGAG</td>
</tr>
<tr>
<td>Stra8</td>
<td>stimulated by retinoic acid gene 8</td>
<td>20899</td>
<td>CAAAAGCCTTGGCTGTTGA</td>
<td>AAAAGTCTCCAGGGACTTCA</td>
</tr>
<tr>
<td>Ring1B</td>
<td>ring finger protein 2</td>
<td>19821</td>
<td>TTGAAAGTGGCAACAAAGAGTG</td>
<td>CGCTCTCATACTTCAGCAC</td>
</tr>
<tr>
<td>Hes1</td>
<td>hairy and enhancer of split 1</td>
<td>15205</td>
<td>ATAGCTTCGGGATCCCAAG</td>
<td>GOSCCGGCTATTCCGGCAAC</td>
</tr>
<tr>
<td>Hes5</td>
<td>hairy and enhancer of split 5</td>
<td>15208</td>
<td>GCTCGCTGCTGTAATGTTCT</td>
<td>CGGGCTTCGGGATCCGGGTTT</td>
</tr>
<tr>
<td>Hey1</td>
<td>hairy/enhancer-of-split related with YRPW motif 1</td>
<td>15213</td>
<td>GCGGCCGACGGAATGGAAA</td>
<td>TCGAGGTGATCCACAGCTCATCTG</td>
</tr>
<tr>
<td>Canx</td>
<td>calnexin</td>
<td>12230</td>
<td>GACATGACTCTTTCCGAACCT</td>
<td>CGTCCCATATGCTCATTCCCAC</td>
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<tr>
<td>Hoxa4</td>
<td>homeobox C4</td>
<td>15423</td>
<td>GAGCGCTACATATGCGCACCG</td>
<td>GGGCTGCACATTCTACGGGT</td>
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<td>Hoxa5</td>
<td>homeobox C5</td>
<td>15424</td>
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<td>CATTCGCGCGTAGCTGACC</td>
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<tr>
<td>Hoxd10</td>
<td>homeobox D10</td>
<td>15430</td>
<td>CTCGCAATTAGAATGGGGAGA</td>
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<tr>
<td>Hoxd11</td>
<td>homeobox D11</td>
<td>15431</td>
<td>CAACTCTCTGCGGATGCTCAAC</td>
<td>GACGGGCTCCCGCTTCCAGTCTT</td>
</tr>
</tbody>
</table>
2.7 Cell counts

Germ cells, Sertoli cells, granulosa cells, and Leydig 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 counted using the Fiji software suite. For each category, 3-7 biological replicates were counted. For E13.5 samples, two central Z sections separated by 50 µm were imaged on a Zeiss 710 inverted confocal microscope for each whole gonad. 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.) Finally, statistical significance was determined by unpaired t-tests.

2.8 ChIP-Seq

2.8.1 Collection of FACS sorted SOX9-positive cells

SOX9-CFP-positive Sertoli cells were collected from E13.5 XY gonads and FACS-purified. Sorted SOX9-CFP-positive cells were pelleted (4000 rpm 20 min at 4°C), resuspended in 360 µl PBS with 10 µl 37% formaldehyde to cross-link at room temperature with continuous rocking for 10 min. Cross-linking was stopped by addition of 46.3 µl of 1M glycine for 5 minutes at room temperature. Cells were then pelleted by removal of supernatant (5000 rpm 10 min at 4°C), and stored at -80°C.
2.8.2 ChIP-Seq and data analysis

SOX9-CFP-positive Sertoli cells from multiple sorts were pooled together and washed twice in 500 µl of PBS with protease inhibitors. Cells were then resuspended in 500 µl of lysis buffer (50mM Tris-HCL, 10mM EDTA, 1% SDS and protease inhibitors) and sonicated to obtain the optimal fragmentation of DNA length around 200-400 base pair by Branson 450 Sonicator (duty cycle of 30%, output power of 3, 16 cycles of 30 seconds with 1 min rest time between sonications, all the procedure performed on ice).

Bead-antibody complexes were prepared by incubating 30 µl of dynabeads (Protein A; Life Technologies 10002D) with 5 µg Ring1B antibody (monoclonal mouse anti-Ring1B; D139-3; MBL Life Science). The sonicated lysate was spun down at 4°C for 10 minutes at 10,000 rpm with 40 µl of the supernatant set aside as Input control and 200 µl transferred to tubes containing pre-incubated bead-antibody complexes. 700 µl of ChIP Dilution Buffer (CDB) (1% Triton X-100 (Sigma T8787), 2mM EDTA, 150mM NaCl, 20mM Tris (pH=8.0)) with protease inhibitors were added to IP tubes and incubated overnight at 4°C with continuous rotating on a mini tube rotator. For the Input control, 160µl of CDB and 8 µl of 5M NaCl were added to the tube and incubated at 65°C overnight.

The next day, IP tubes were washed as follows: Once with Wash Buffer I (50mM Tris HCl, 1mMEDTA, 150 mM NaCl, 0.1% SDS, 0.1% Triton X-100, 0.1% Sodium deoxycholate), twice with Wash Buffer II (50mM Tris HCl, 1mM EDTA, 500 mM NaCl,
0.1% SDS, 0.1% Triton X-100, 0.1% Sodium deoxycholate), once with Wash Buffer III (10mM Tris HCl, 1mM EDTA, 1% NP-40, 1% Sodium deoxycholate, 250mM LiCl), twice with Wash Buffer IV (50mM Tris HCl, 1mM EDTA, 500 mM LiCl, 1% NP-40, 0.7% Sodium deoxycholate), and finally, twice with TE buffer (pH=8.0). All washes were done in 1 ml, containing protease inhibitors, at 4°C for 5 minutes with continuous rotating. DNA-protein complexes were eluted twice from the beads with 100 µl elution buffer (100mM sodium bicarbonate, 1% SDS, 8mM NaOH). 8 µl of 5M NaCl was added to the eluates, and incubated at 65°C overnight. For the following day, ChIP samples and Input control were treated with 1 µl RNase-cocktail (Life Technologies AM2286) for 30 minutes at 37°C, then 4 µl of 0.5M EDTA, 8 µl of 1M Tris and 1µl of 10mg/ml Proteinase K was added for another 60 minutes at 45°C. Lastly, DNA was purified using PCR purification columns (Qiagen; 28104).

For library preparation for sequencing, DNA was concentrated using a speed vac to ~10µl. 10 µl of ChIP DNA and 1 µl of Input DNA was used in the library preparation using the Rubicon ThruPLEX FD kit according to the manufacturer’s protocol. Size selection of smaller size amplified DNA was done with SPRI beads (Agencourt AMPure XP A63880) at 0.6x concentration. Sequencing was performed at Duke’s Genome Sequencing and Analysis core facility on the Illumina HiSeq2000/2500 ChIP-seq reads were aligned with Bowtie (Langmead et al., 2009) with only uniquely aligning reads used for future processing. Peaks were called with SICER (Zang et al., 2009) with
enrichment called for the RING1B binding using the input track as the control. The window size was set to 150 fragment, effective genome fraction to 0.7, gap size to 600 and FDR to 0.01. Reads were first processed to remove low quality bases and adapter sequences from the 3’ end using the TrimGalore! Toolkit (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore). Reads that were at least 20nt in length after trimming were then aligned to the mouse genome (GRCm38 from ENSEMBL (Kersey et al., 2012)) by the Bowtie alignment algorithm(Langmead et al., 2009) (m 1 -v 3 --all --best --strata). The MACS2 peak finding algorithm (Zhang et al., 2008) was then used to identify regions of ChIP enrichment over the input control DNA (–nolambda --nomodel --extsize 200). Peaks that had an FDR-corrected p-value of <= 0.05 were reported as binding sites. Peaks were annotated according to GRCm38v73 version of the ENSEMBL transcriptome.

2.9 Co-immunoprecipitation

Gonads were dissected in phosphate-buffered saline (PBS), collected, lysed in IP Lysis buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.5% NP-40 (Nonidet P-40), proteinase inhibitor cocktail). Gonads were first dissociated by passing through 27 gavage needle for 5-10 times, then sit on ice for 30 min. Cell debris were removed by centrifuge at 2000rpm for 5 min at 4°C. The collecting supernatant were diluted with IP Lysis buffer to end the volume 400 μl (total lysate). Total lysate was precleared with 10 μl protein A-Sepharose beads (Pharmacia) by continuous rotating on a mini tube rotator
at 4°C for 2 hours. Meanwhile, 5 μl anti-RING1B antibody or Ms IgG control were conjugated to 50 μl of 50% A-Sepharose beads (diluted in IP lysis buffer) in μl 400 IP Lysis buffer by continuous rotating on a mini tube rotator at 4°C for 2 hours. Pre-conjugated beads were washed for 3 times by IP Lysis buffer. Beads in the pre-cleared lysate were removed by spinning down the beads at 2000 rpm 5 min centrifugation. For co-immunoprecipitation, the pre-cleared lysate was split to two parts and incubated with either anti-RING1B conjugated beads or Ms IgG control conjugated beads rotating overnight on a mini tube rotator at 4°C. 10% input were saved from the pre-cleared lysate for further analysis. After overnight co-immunoprecipitation, beads were pelleted and washed five times with IP lysis buffer, and subjected to SDS-PAGE and Western blot analysis with the SOX9 antibody and Ring1B antibody revealed with an ECL kit (Amersham).
3. NUMB regulates somatic cell lineage commitment during early gonadogenesis in mice

3.1 Summary

During early gonadogenesis, proliferating cells in the coelomic epithelium (CE) give rise to most 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 Numb-like 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 marker LHX9, and 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.

3.2 Introduction

The undifferentiated gonadal primordia arise within the intermediate mesoderm. Beginning about embryonic stage (E) 9.5, proliferation of cells in the region of the
coelomic epithelium (CE) that overlies the mesonephric ducts, leads to a 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).

Mammalian embryonic gonads are composed of various lineages of somatic cells that provide a niche for development of germ cells. 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). Abolishing any of these somatic cell lineages results in severe defects in testis or ovary development.

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). In previous studies, we and others deleted *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). Results of this experiment and subsequent results indicated 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 precocious or excessive 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. The 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 coactivator
mastermind-like 1 (MAML1), NICD converts CBF1 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 the localization of NOTCH 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 in (Knoblich, 2001, 2010)). For example, 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 cell (this cell becomes the signal-sending cell), while the other daughter cell that lacks NUMB becomes a pIIa cell (this cell becomes the signal-receiving cell) (Uemura et al., 1989). There are two Numb homologs in mice, Numb and Numb-like (Numbl), which often act redundantly (Petersen et al., 2002). Both Numb and Numbl 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 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 may be a direct or indirect
effect of loss of Numb/Numbl. These results reveal a new function for Numb and Notch signaling during mammalian gonadogenesis.

### 3.3 Results

#### 3.3.1 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 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 previously published microarray data from four sorted cell populations (supporting cells (Sertoli and granulosa cells), interstitial/stromal cells, endothelial cells, and germ cells) collected from XX and XY gonads from stages E11.5-13.5 (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, while male and female germ cells and endothelial cells expressed Notch2 at
slightly lower levels. Notch1 and Notch4 are specifically expressed in the endothelial lineages, while the expression of Notch3 is low in all tested lineages (Fig. 2).

Figure. 2 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.

Figure 2: Temporal transcriptional profiling of Notch receptor genes and *Numb*.

Figure 3 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’) NOTCH signal alone. (C, D) NUMB (green) is asymmetrically allocated to 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 signals 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 a
higher magnification of the boxed regions in E and F; E” and F” show the NUMB channel alone. Scale bars in A,B,C,D=5 μm; scale bars in E, E’, E” and F, F’, F”=25 μm.

Figure 3: NUMB is basolaterally localized, and Notch activity is high in CE cells of E11.5 gonads.
Expression of Notch2 was previously analyzed using a Notch2 reporter line (Notch2LacZ) (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). As reporter mice may not always reflect accurate expression of the endogenous protein, we re-investigated this pattern using antibodies against NOTCH2. Consistent with the microarray data (Fig. 2), NOTCH2 protein showed a broad expression pattern in gonadal cells (Fig. 3A,B and Fig. 4A,A”). Based on immunofluorescence, NUMB was also detected in almost all cell lineages at varying levels (Fig. 4A,A’). However, while NOTCH2 was distributed evenly in the CE cells (Fig. 3A,B), NUMB was asymmetrically allocated to the basolateral domain of CE cells in both E11.5 XX and XY gonads (Fig. 3C,D and Fig. 4A,A’). The asymmetric distribution of NUMB at the CE 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 4 copies of a consensus RBPJ (CBF/CBF1) binding site. In CBF:H2B-Venus Notch reporter mice, the activation of Notch signaling by any of the Notch receptors is reflected by expression of Venus protein. Despite wide expression of NOTCH2 protein in most cells in the gonad (Fig. 3A-B’ and
Fig. S4A,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. 3C-D’ and Fig. S4A,A’). Deeper inside the gonad field, where NUMB protein is present at high levels (Fig. 3E-F’), Notch signaling is activated in a small number of cells, very few of which are positive for the Sertoli cell marker SOX9 (Sry-box 9)(Fig.S4B-B”). Occasional cells double positive for the Notch reporter and SOX9 may be newly born Sertoli cells in which H2B-Venus has not yet degraded. Many H2B-Venus-positive cells are also positive for the endothelial marker PECAM1 (Platelet/Endothelial Cell Adhesion Molecule 1) (Fig. S4C-C2”).

**Figure. 4 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-C) 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’, C’, and C” 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) Notch activity is also absent from PECAM1-positive germ cells (arrowheads in C1’, C1”, C1”’), but is observed in PECAM1-positive vascular endothelial cells (arrow in C2’). C2’-C2”’ 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.
Figure 4: 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.
3.3.2 Temporal deletion of *Numb* in a *Numbl* mutant background led to severe morphological defects in the gonad

Homzygous *Numb*<sup>−/−</sup> 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 signaling in gonadogenesis, we deleted a conditional allele of *Numb* driven by a ubiquitously expressed, tamoxifen-inducible *ROSA-CreER* at E8.75. We determined that 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 to control littermates, E13.5 and E14.5 XX and XY mutant gonads conditionally deleted for *Numb* and also mutant for at least one allele of *Numbl* showed an irregular CE surface as well as variable morphological defects (Fig. 5A,B and Fig. 6). Antibodies against typical gonadal somatic cell markers, including PECAM1 (which labels vasculature and germ cells), NR5A1 (nuclear receptor subfamily 5, group A, member 1, also known as steroidogenic factor 1, SF1, which labels Sertoli cells, granulosa cells and steroidogenic cells), and vascular cell adhesion molecule 1 (VCAM1, which labels interstitial/stromal cells), failed to label large patches of cells within gonads of both sexes (Fig. 5C-F).
Figure. 5 *Numb/Numbl* mutant gonads have an irregular surface and interior pockets of undifferentiated cells. (A and 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 and D) In the E13.5 control XY gonad (C), cells were labeled with one or more of the following markers: PECAM1 labeled germ cells and endothelial cells; NR5A1 labeled Sertoli cells in regular cord structures as well as some interstitial cells; and VCAM1 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 and 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*\textsuperscript{flox/flox};*Numbl*\textsuperscript{−/−};ROSA-
CreER or Numb<sup>flm/flm</sup>; Numbl<sup>+/−</sup>; 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 in C-F=50 µm; scale bars in G-J=100 µm.

**Figure 5**: Numb/Numbl mutant gonads have an irregular surface and interior pockets of undifferentiated cells.
To determine whether these patches contained cells that retained markers of undifferentiated fate, we stained with an antibody against 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. 5G,I). In contrast, in mutant gonads LHX9 labeled cells throughout the gonad that lacked expression of markers for specific gonadal lineages, such as SOX9, which labels Sertoli cells, VCAM1, which labels interstitial progenitors (Fig. 5G,H), FOXL2 (Forkhead Box L2) which labels granulosa cells, and DDX4 (DEAD (Asp-Glu-Ala-Asp) box polypeptide 4, also known as MVH or VASA), which labels germ cells (Fig. 5I,J). These results suggested that LHX9-positive patches observed in mutant gonads are comprised of undifferentiated cells.

**Figure. 6** E13.5 Numb\(^{\text{flax/\text{flax}}}\);Numbl\(^{+/+}\);ROSA-CreER and Numb\(^{\text{flax/\text{flax}}}\);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\(^{\text{flax/\text{flax}}}\) or Numb\(^{\text{flax/\text{+}}}\) alleles in various combinations with wild-type (Numbl\(^{+/+}\)), heterozygous (Numbl\(^{+-}\)), or homozygous (Numbl\(^{-/-}\)) genotypes. Only Numb\(^{\text{flax/\text{flax}}}\) 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.

Figure 6: E13.5 Numb<sup>flx/flx</sup>;Numb<sup>+/−</sup>;ROSA-CreER and Numb<sup>flx/flx</sup>;Numb<sup>−/−</sup>;ROSA-CreER XY gonads display mutant phenotypes, whereas other segregating genotypes do not.
3.3.3 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-Feitz et al., 2008). Laminin and ITGB1 (also known as 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). Since 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 to their control littermates (Fig. 7). In controls, both Laminin and ITGB1 were allocated exclusively to the basolateral domain of surface epithelial cells (Fig. 7A,C). However, in *Numb/Numbl* mutants, Laminin and ITGB1 were no longer restricted to the basolateral domain of surface epithelial cells. Instead, Laminin and ITGB1 were distributed to all cell surfaces, including the apical domain of CE cells (Fig. 7B,D). These results indicated that depletion of NUMB disrupts the establishment of CE apical-basal polarity.

**Figure. 7 Polarity of CE cells in *Numb/Numbl* mutant gonads was disrupted.** 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), polarization of these makers is disrupted, and both Laminin and ITGB1 are 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. The region where the CE is expected to be is marked by a bracket in $B',B''$ and $D',D''$. Nuclei are labeled by Hoechst staining (blue). Scale bars in all images=5 µm.

**Figure 7:** Polarity of CE cells in *Numb/Numbl* mutant gonads was disrupted.
3.3.4 LHX9-positive patches are derived from the CE

LHX9 is a marker of undifferentiated cells in the gonad. To determine whether the LHX9-positive patches in mutant gonads arose via dedifferentiation 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 in 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 hour (Fig. 8A,C,E,G). Note that even in Numb;Numbl mutant gonads where 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 hours (Fig. 8B), the labeled cell population had expanded to the first 4-5 top cell layers of the gonad, consistent with previous findings that proliferation of the CE contributes to thickening of the gonad (Karl and Capel, 1998). LHX9-positive cells were restricted to the CE domain as shown previously (Fig. 8A,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 expression of LHX9 (Fig. 8C,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.

**Figure. 8 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 one hour, fixed and strained to confirm that MitoTracker had not penetrated beneath the CE layer during the labeling step (A,C,E,G). The other member of the gonad pair was cultured for 24 hours prior to fixation (B,D,F,H). Gonads were stained for SOX9 or FOXL2 (blue, Sertoli or granulosa cells) and LHX9 (green). After 24 hours of culture, several regular 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 hour of culture (C,G) but showed irregular labeling of cells deep within the gonad after 24-hour 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 in all images=50 µm.

Figure 8: Undifferentiated cells in Numb/Numbl mutant gonads derive from the CE, but are not in active cell cycle.
Cells in the CE are highly proliferative between E11.5-E13.5 (Schmahl et al., 2000). To investigate whether cells in the undifferentiated LHX9-positive patches are also in active cell cycle, we stained the mutant gonads and their control littermates with anti-MKI67 antibody (also known as KI67), a marker of all stages of the cell cycle except G0 (Fig. 8I-P). In both E13.5 (I-L) and E11.5 (M-P) mutant and control gonads, cells in the CE domain 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 also 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 this M phase marker in LHX9-positive patches or elsewhere in mutant gonads relative to controls (Fig. 9).

Figure. 9 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 pH3-positive cells in the CE domain and in the gonad field at both E11.5 and E13.5. pH3-positive cells were absent in LHX9-positive patches in mutant gonads (B,D,F and H). Scale bars in all images=50 µm.
Figure 9: No significant cell proliferation differences were observed between XX and XY E11.5 and E13.5 control and mutant gonads.

3.3.5 *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. 10A-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 images (Fig. 10I denoted with filled triangles). 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, and compared mutant to control samples. These data indicated a 60% reduction of SRY-expressing cells in mutant vs. control gonads (Fig. 10G).

**Figure. 10 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). In control gonads (A, C and 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 and F) had an irregular surface, multiple regions of LHX9-positive cells within the gonad (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. (H and 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 in mutants were double-labeled by SRY and LHX9 (filled triangles in I). All of the LHX9 positive cells are GATA4 positive (I’ and I’’). Scale bars in all images=50 µm.
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 *Numb^floxflox; Numbl^-/-; ROSAcreER* background. As shown in Fig. 11, ~50% of gonadal cells (in a patchy distribution across the gonad) were Tomato-
positive by E11.5, 3 days post-tamoxifen injection. While the majority of SRY-expressing cells were Tomato-negative, a few were double positive (carets, 11C-C”). It is unclear whether activation of the Cre reporter occurred before or after the activation of Sry.

**Figure. 11 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 *Numbfl/fl/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.

Figure 11: Tamoxifen injection at E8.75 led to activation of ROSACreER in ~50% of gonadal cells, unevenly distributed across the gonad field at E11.5.

However, expression of the RTR reporter involves recombination at only one allele, whereas loss of Numb activity requires recombination at 2 alleles in these crosses, and could show different kinetics. To obtain a more 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. 12A-C), but some samples already showed significant loss of NUMB (Fig. 12D). NUMB protein was strongly reduced in all mutants relative to controls by E11.5 in all cases where tamoxifen was administered at E8.75, although levels varied slightly among samples and across the field of the gonad (Fig. 12E-H). These findings indicate that NUMB protein is lost between E10.5 and E11.5 in most mutants.

**Figure. 12 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.
3.3.6 *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 using fluorescent immunocytochemistry (Fig. 5 and 13A-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 in control and mutant gonads (Fig. 13G, H, I). In XY mutant gonads, the Sertoli cell population was reduced by ~50% compared with control littermates. In XX mutant gonads, the number of granulosa cells was reduced by ~60% relative to controls. Leydig cells (HSD3B1-positive cells in XY gonads) 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 CASPASE3 (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 to controls (Fig. 14).

Figure. 13 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 staining for the steroidogenic enzyme HSD3B1 (green), the Leydig cell population was the most severely affected in mutant gonads, reduced to ~10% of controls. Scale bars in all images=50 µm.
Figure 13: Numbers of supporting cells in both XX and XY, and steroidogenic cells in XY E13.5 Numb/Numbl mutant gonads were reduced.

Figure. 14 Up-regulation of cell death pathways is unlikely to explain the loss of Sertoli or granulosa cells in mutants. Few eCASP3-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.
Figure 14: Up-regulation of cell death pathways is unlikely to explain the loss of Sertoli or granulosa cells in mutants.

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

The germ cell lineage was dramatically affected in both XX and XY mutants, with significantly fewer DDX4-positive germ cells present compared to controls (Fig. 15). Germ cell counts showed that, in both XX and XY mutant gonads, germ cells were reduced by 50-60% at E13.5 (Fig. 15A-F). Moreover, the germ cell defect was evident as early as E11.5 (Fig. 15G-L). To investigate whether loss of germ cells at E11.5 was associated with widespread apoptosis (as in Fgf9 mutants (DiNapoli et al., 2006)), we stained gonads at this stage with antibodies against cCASP3 and the germ cell markers Cadherin1 (CDH1, also known as E-Cad) (Fig. 15M,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.

**Figure. 15** 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 to controls. (G-L) The germ cell population was even more strongly reduced at E11.5, where numbers were 10-30% of controls. Antibodies against cCaspase3 (green), a marker of apoptotic cell death pathways, labeled very few cells in control gonads and in mutants (insets in M,N). 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. Scale bars in all images=50 µm.
Figure 15: Germ cells were lost in *Numb/Numbl* mutants as early as E11.5, but not via cell death within the gonad or mesonephros.
3.3.8 Blocking Notch signaling rescued the *Numb/Numbl* mutant phenotype

The canonical function of the NUMB protein is to antagonize Notch signaling. However, other functions have been reported for NUMB independent from 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 3 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 value =0.0012 in XY control versus mutant; P value =0.0117 in XX control versus mutant, asterisks apply to all three genes) (Fig. 16).

**Figure. 16 Three Notch downstream target genes are upregulated in E13.5 XY and XX mutant gonads.** Quantitative RT-PCR of *Hes1*, *Hes5* 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.
In a second line of experiments to test whether the accumulation of LHX9-positive cells within the gonad in Numb/Numbl mutants was due to over-activation of Notch signaling, we blocked Notch signaling in mutants using DAPT, an inhibitor of γ-secretase, the enzyme responsible for releasing the NOTCH intracellular domain (NICD) (Cheng et al., 2003). Gonads were collected at E11.5 and cultured with 100 µM DAPT or DMSO for 48 hours (Fig. 17). Control gonads treated with DAPT contained supporting and germ cells, although germ cell numbers were consistently reduced after culture with DAPT (Fig. 17A,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 over-activation of the Notch signaling pathway. Mutant gonads cultured with

![Graph showing fold change in gene expression]
DAPT often had more SOX9- or FOXL2-positive cells than those cultured with DMSO, but this result was also variable. Consistent with earlier studies (Tang et al., 2008) (data not shown), we also observed rescue of the Leydig cell population after DAPT treatment in XY mutant gonads. Interestingly, LHX9 expression in the CE was reduced in both controls and mutants (Fig. 17A-H), which could imply that Lhx9 is a target of Notch signaling.

Figure. 17 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 hours. (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 and 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 and F) Similar results were seen in XX gonads, where 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 and 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 in all images=50 µm.

Figure 17: Culture with DAPT rescued some aspects of the Numb/Numbl mutant phenotype.
3.4 Conclusion and 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 competent to differentiate as Sertoli cells in the testis or granulosa cells in the ovary, and interstitial cells. Here we show that deletion of Numb/Numbl disrupted CE cell polarity in both XX and XY gonads, and led to the presence 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). Cells in the CE domain 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.

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 absent from the apical domain and 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 hours, 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. 18). Our evidence based on deletion of Numb/Numbl is consistent with this hypothesis.

Figure. 18 NUMB is essential for asymmetric division of CE cells during gonadogenesis. Embryonic CE cells are the progenitors of Sertoli and interstitial cells. These “stem-like” progenitors are LHX9-positive and 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 may 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 similar, but continues until E14.5 (Mork et al., 2012).
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 patches of LHX9-positive cells 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 reductions in numbers of differentiated cells in gonads of both sexes based on markers for Sertoli cells (SRY and SOX9), granulosa cells (FOXL2), and Leydig cells (HSD3B1). 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 may 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 field of the gonad at E11.5. Using an antibody against NUMB, high levels of NUMB were still detectable in most samples at E10.5, but lost by E11.5. The timing of Numb deletion in different samples may 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) usually are located deeper in the gonad in mutants, whereas the LHX9-positive patches are typically located nearer the CE (Fig. 5).

Another possibility that is not mutually exclusive is that Numb also is required at a second step during Leydig cell differentiation. In a previous study, we used a Hes1 mutant mouse model, as well as DAPT treatments, to show that Notch signaling maintains Leydig progenitor cells, while 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, the over-activation 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 at least partially rescued by culturing with DAPT strongly suggests that the defect in Numb/Numbl 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 treatment with DAPT resulted in a reduction of 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 the case since 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. 2E) (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 lead to disruption of 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 where gonadal signals were disrupted (DiNapoli et al., 2006). These findings are consistent with the possibility that Numb 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/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, while the other daughter cell, which inherits NUMB, gains competence to differentiate as a supporting or interstitial cell (Fig. 18). 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 cells of different lineages at different stages of development. While 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.
4. *Ring1B* contributes to the patterning of XY gonad development

4.1 Summary

In mouse, sex determination depends on the balance between male and female programs. At E10.5, the gonad is bipotential and can differentiate into either a testis or an ovary. The expression level of genes involved in the male pathway (such as *Sox9* and *Fgf9*) and female pathway (such as *Wnt4* and *Rspo1*) are indistinguishable in the supporting cell precursors between XX and XY gonads (Fig. 1). The fate of the bipotential gonad is determined by a fate decision in the male or female supporting cell precursor (pre-Sertoli cells or pre-granulosa cells). In XY gonads, supporting cell fate is initiated by *Sry* (the sex determining gene on the Y chromosome), which activates *Sox9*. Emerging evidence suggested that repression of the opposing pathway is as crucial as activation of the male or female pathway for gonadal sex determination and maintenance. Based on the finding that loss of *Cbx2* (chromobox homolog 2, also known as *M33*), one of the components of the Polycomb Repressive Complex 1 (PRC1), leads to male to female sex reversal, I hypothesize that PRC1 contributes to female gene repression during male sex determination. In *Ring1B* (*Ring finger protein 2*, E3 ubiquitin ligase in the PRC1) mutant XY gonad, I observed a partial sex reversal phenotype in the posterior region of the gonad. Mis-expression of *FOXL2* (marker of female fate commitment) was detected at the posterior pole and female genes were up-regulated. In order to identify potential targets of PRC, I performed RING1B ChIP-Seq. Interestingly,
by using co-immunoprecipitation, I found that RING1B interacts with SOX9 \textit{in vivo} during male gonad formation.

\subsection{4.2 Introduction}

The presence of a repression mechanism in XY gonad development has been suggested by a study in which the transcriptome from isolated cell populations from E11.5, E12.5 and E13.5 gonads was analyzed (Jameson et al., 2012b; Nef et al., 2005). Microarray data from supporting cell populations of both XX and XY gonads revealed that bipotential cells are lineage-primed with a female bias. This work suggested that a repressive mechanism in the XY gonad is responsible for down-regulating the primed female pathway. \textit{Fgf9} has been demonstrated as one of the male factors that represses the female pathway and is essential for XY gonad development (Jameson et al., 2012a). In \textit{Fgf9} and \textit{Wnt4} double mutant XY gonads, the sex reversal phenotype of \textit{Fgf9} XY gonads was fully rescued by further deletion of \textit{Wnt4}, suggesting the main role of \textit{Fgf9} is to repress \textit{Wnt4} during male gonad formation. The important contribution of the repression mechanism during XY gonad development was further supported by a temporal transcription profiling project (Munger et al., 2013), in which, the gene expression profile was investigated in gonads at fine time points between E11.0 and E12.0. This analysis revealed that a group of female genes were repressed in XY gonads downstream of \textit{Sry} expression (Fig. 19).
Figure. 19 Changes in XX and XY gonads contribute to expression fold change between E11.0 and E12.0. Gene expression in XY and XX gonads was compared at the beginning and end of the 24-hour developmental window, demonstrating that enrichment in one sex is achieved by activation, repression, or both regulatory mechanisms. Genes that exhibited a 1.5-fold or greater change in expression in either sex between E11.0 and E12.0, log of the fold change in the XY gonad is plotted on the Y-axis, and log of the fold change in the XX gonad is plotted on the X-axis. Gray dots represent genes that are similarly up-regulated or down-regulated in both sexes. Blue dots represent genes that become enriched in XY gonads relative to XX. Red dots represent genes that become enriched in XX gonads relative to XY. Examples from each category are highlighted, and their expression patterns in XY (blue line) and XX (red line) gonads are displayed. (Adopted and modified from Munger et al., 2013)
The Polycomb Repressive Complex (PRC) is a plausible mechanism to execute the global female gene repression during male gonadogenesis. Polycomb-group (PcG) genes were originally identified in *Drosophila melanogaster*, where they are involved in the repression of *Hox* genes’ expression during body segmentation (Frei et al., 1985). In mammals, PcG proteins are involved in processes of development, differentiation and disease. In an oversimplified view, the gene repression mechanism mediated by PcG is a two-step process: first, Polycomb Repressive Complex 2 (PRC2) edits the target by tri-methylating at histone H3 lysine-27 (H3K27me3) which is followed by PRC1 recruitment and monoubiquination at histone H2A lysine-119 (H2AK119ub1), which results in the repression of gene expression (Fig. 20) (Vissers et al., 2012).
Figure. 20 Polycomb-repressive complexes (PRCs) and their enzymatic activities. The PcG transcriptional repressors are composed by two main PRCs, PRC1 and PRC2. PRC2 has four subunits: EZH 1 or EZH2 (the Su(var)3-9, enhancer-of-zeste, trithorax domain (SET) domain protein), SUZ12 (the zinc finger protein), EED (the WD-repeat protein) and RBAP46 and RBAP48 (the histone-binding proteins). EZH1 and EZH2 possess the main enzymatic activity of PRC2, which is to trimethylate histone H3 at lysine 27. Other substoichiometric complex components of PRC2, such as PHF1, has been described as well. PRC1 consists of RING1A or RING1B (the enzymatic subunit), a RING-domain cofactor of the PCGF family such as BMI1 (also known as PCGF2) or MEL18 (also known as PCGF4), one of the human polyhomeotic homologues (HPH1, HPH2 or HPH3) and one of the chromobox proteins (CBX2, CBX4, CBX6, CBX7 or CBX8). CBX proteins recognize the H3K27Me3 histone mark, then RING1A or RING1B catalyzes the final step of ubiquitin conjugation with their E3 ubiquitin ligase activity. Therefore, result in the repression effect of the target genes. (Vissers et al., 2012)
The link between the PRC and gonad sex determination is supported by the male to female sex reversal phenotype in Cbx2 (chromobox homolog 2) knockout mutant mice. Cbx2, also known as M33, is one of the main components of PRC1. It can interact with histones methylated by PRC2, and recruit the whole PRC1 complex to target sites, which eventually leads to repression of target genes’ expression. In mice, deletion of Cbx2 caused complete male-to-female sex reversal attributed to lack of Sry upregulation (Katoh-Fukui et al., 2012; Katoh-Fukui et al., 1998). Given the traditional role of CBX2 as part of the PcG repression complex, it seems unlikely that CBX2 directly upregulates Sry. Instead, downregulation of Sry may be an indirect effect of loss of Cbx2.

The role of Cbx2 in sex determination is supported by evidence in humans. A girl with a prenatal 46,XY karyotype with loss-of-function mutations in the Cbx2 gene was reported as a completely normal female phenotype, including uterus and histologically
normal ovaries (Biason-Lauber et al., 2009). This case revealed the functional conservation of CBX2 among mammals during sex determination.

Based on findings from mutants and our transcriptome analysis, we hypothesize that PRC is involved in male gonad development via an indirect mechanism involving global repression of female genes during establishment and maintenance of the male pathway.

4.3 Results

4.3.1 Fgf9 and Cbx2 synergistically repress the female pathway

During mammalian sex determination, male and female pathways antagonize each other to regulate gonadogenesis. Fgf9 is essential to establish the male pathway, and is known to repress Wnt4 (a “female gene”) during XY gonad formation (Jameson et al., 2012a). In order to test the hypothesis that Cbx2 acts synergistically with Fgf9 to repress the female pathway, I took advantages of Fgf9 and Cbx2 genetic depletion mice models. XY mice with homozygous null alleles of either Cbx2 or Fgf9 result in complete male to female sex reversal (Colvin et al., 2001; Katoh-Fukui et al., 1998). However, heterozygous mutants for Cbx2 or Fgf9 in the context of one wild type allele for both genes, develop normal testes. I characterized the phenotype of Cbx2 +/-; Fgf9+/- complex heterozygous XY gonads to investigate whether there is a synthetic phenotype (Fig. 21). In E13.5 control XY gonads, as well as Cbx2 +/- or Fgf9+/- single heterozygous XY gonads, Sertoli cells (SOX9-positive, red) were specified normally and no distinguishable phenotypic
differences were evident (Fig. 21A-C). Interestingly, I did observe a synthetic phenotype in Cbx2 +/-; Fgf9 +/- double heterozygous XY gonads. A significant number of granulosa cells (female supporting cells, FOXL2-positive, green) were present at both poles of Cbx2 +/-; Fgf9 +/- double heterozygous XY gonads. This result suggests that Fgf9 and Cbx2 act synergistically to repress the female pathway.

Figure. 21 XY Cbx2;Fgf9 double heterozygous gonads are sex-reversed at both of the gonadal poles. E13.5 XY control (A) and heterozygous of Cbx2 or Fgf9 (B,C) gonads were indistinguishable from each other with SOX9 stained Sertoli cells (red) and PECAM1 stained germ cells (blue). In Cbx2 and Fgf9 double heterozygotes (Cbx2 +/-; Fgf9 +/-) XY gonads (D), female granulosa cells labeled by FOXL2 antibody (green) were present at both poles of the gonads. Lower panel shows higher magnification images of anterior and posterior poles for each allelic combination. Scale bars are 50um. Images are representative of n>3.

Figure 21: XY Cbx2;Fgf9 double heterozygous gonads are sex-reversed at both of the gonadal poles.
4.3.2 Posterior specific partial sex reversal in XY *Ring1B* mutant gonads

Although XY *Cbx2*−/− mice display male to female sex reversal. This genetic model is not suitable to test our hypothesis that PRC is involved in male gonad development via an indirect mechanism involving global repression of female genes during establishment and maintenance of the male pathway. First, *Cbx2*−/− mutants show defects throughout the embryo, significant retardation of growth and gonadogenesis, and early lethality, which makes it difficult to determine whether sex-reversal in *Cbx2*−/− mice is a secondary effect of growth retardation. Second, in addition to the initial steps to determine male fate at the bipotential gonad stage, repression of the female pathway is required throughout development and in adulthood to maintain the male fate (Minkina et al., 2014). Because *Cbx2* regulates *Sry* gene expression and disrupts XY gonad development at a very early stage, by analyzing *Cbx2*−/− mice, we cannot investigate a potential role of PRC1 in male pathway maintenance. For these reasons, we built an alternative mouse model using a conditional allele of *Ring1B* (another component of the PRC1 complex) (Puschendorf et al., 2008), crossed with a tamoxifen inducible ubiquitously expressed transgenic Cre line, *ROSACreER*. To test the potential role of PRC at the initial stage as well as the maintenance stage of gonadogenesis, we induced CRE activity at various embryonic stages by Tamoxifen administration. Later stage deletion of *Ring1B* by injection of tamoxifen at E9.5, E11.75 or E13.0 did not result in activation of the female pathway or any evidence of a sex reversal phenotype (data not shown), suggesting that
*Ring1B* is not required at these stages or its activity is compensated by *Ring1A* or another member of the complex.

Strikingly, deletion of *Ring1B* at the initial steps of gonad formation did lead to a sex reversal phenotype, affecting the posterior end of the gonad (Fig. 22A-C). Compared to control E13.5 XY gonads, *Ring1B* mutant XY gonads contained a significant number of female granulosa cells (FOXL2-positive, green), usually located in the posterior region of the gonad. The severity of the sex reversal phenotype varied among XY *Ring1B* fl/fl; *ROSA-CreER* mutant gonads (Fig. 22B,C). In some cases, only the posterior third of the gonad was sex reversed. In other cases, the sex reversed region expanded across the midline and FOXL2-positive cells were present at the anterior pole of the mutant gonads. Notably, few cells co-expressed the male (Sertoli, SOX9-positive, red) and female (granulosa, FOXL2-positive, green) supporting cell markers simultaneously (arrows in Fig. 22C inset).

The sex reversal of *Ring1B* mutant gonads was male specific. In *Ring1B* mutant XX gonads, no mis-expressed SOX9-positive Sertoli cells were observed (Fig. 22D,E). The gonadogenesis retardation phenotype reported in *Cbx2*−/− gonads (Katoh-Fukui et al., 1998) were recapitulated in both XY and XX *Ring1B* mutant gonads. Moreover, in the female part of *Ring1B* mutant gonads (the XX *Ring1B* mutant and the sex reversed portion of XY *Ring1B* mutant) also had a significant reduction of germ cells number labeled by PECAM1 (blue) (Fig. 22A-E). This result is consistent with a previous study.
suggesting that Ring1B regulates the female germ cell population by maintaining expression of Oct4, to prevent premature induction of meiotic gene expression and entry into meiotic prophase (Yomutantbayashi et al., 2013).

To investigate whether more female genes were de-repressed in the posterior domain of XY Ring1B mutant gonads, we collected E13.5 control and mutant XY gonads, separated anterior and posterior portions, and performed quantitative RT-PCR (Fig. 22F). In both anterior and posterior XY Ring1B mutant gonad samples, we found a similar reduction of Ring1B mRNA. This result strongly suggested that the posterior specific sex reversal pattern in the XY mutant gonads was not due to residual Ring1B mRNA resulting from incomplete CRE recombination at the anterior end. RT-PCR showed that Sox9 expression was reduced along with a significant de-repression of female genes, Foxl2 and Stra8, in the posterior relative to the anterior portions of XY Ring1B mutant gonads. Given the fact that, in some severe mutant cases, sex reversal in XY Ring1B mutant gonads could expand to the anterior pole, we were not surprised to observe the de-repression of Foxl2 and Stra8 in the anterior half of XY Ring1B mutant samples, although at a lower level compared to the posterior portion (Fig. 22F)

Figure. 22 XY Ring1B mutant gonads displayed a partial sex reversal phenotype at the posterior pole at stage E13.5 but no sex reversal in XX mutant gonads. E13.5 control XY gonads (A) contained cord structures formed by Sertoli cells (SOX9-positive, red) and
germ cells (PECAM1-positive, blue). In Ring1B mutant XY gonads, comparable structures could be observed at the anterior portion (B). However, at the posterior end, the cord structures were deformed with significant reduction of Sertoli and germ cells. Female granulosa cells (FOXL2-positive, green) were present in this region (B and inset). In some severe cases, granulosa cells (green) could be found throughout the gonad field (C and inset). The affected region in Ring1B mutant XY gonads (B and C) also display a growth retardation phenotype with fewer cells compared to the controls. E13.5 control XX gonads (D) contained scattered granulosa cells (FOXL2-positive, green) along with germ cells (PECAM1-positive, blue). Ring1B mutant XX gonads also have growth retardation phenotype but no evidence of sex reversal (E). (F) Quantitative real-time PCR demonstrated a significant reduction of Ring1B mRNA in both anterior and posterior portions of E13.5 Ring1B mutant XY gonads compared to controls. The female genes Foxl2 and Stra8 were strongly upregulated in the posterior portion of Ring1B mutant XX gonads, and to a lesser degree, in the anterior portion. Scale bars are 50um.
Figure 22: XY Ring1B mutant gonads displayed a partial sex reversal phenotype at the posterior pole at stage E13.5 but no sex reversal in XX mutant gonads.

4.3.3 SRY-positive pre-Sertoli cells were missing at the Posterior portion of E11.5 XY Ring1B mutant gonads

To investigate whether the early specification of the SRY-positive pre-Sertoli cell population was also affected in XY Ring1B mutant gonads, we compared E11.5 mutants with their control littermates. In control XX gonads, no SRY-positive cells (red) could be detected (Fig. 23C), while in control XY gonads, SRY-positive cells were present throughout the gonad field (Fig. 23A). However, there was a strong reduction in SRY-positive cells at the posterior pole of E11.5 XY Ring1B mutant gonads. This data suggested that the posterior specific sex reversal in the E13.5 XY Ring1B mutant gonads was due to the failure of pre-Sertoli cell specification as early as E11.5. In previous work on Chx2 mutants, the authors concluded that Chx2 is responsible for the initiation of Sry
expression (Katoh-Fukui et al., 1998). Our data agree with the idea that PRC is involved in SRY-positive pre-Sertoli cell specification. However, whether it is through a direct mechanism to up regulate Sry expression, or through an indirect mechanism to inhibit the repression of Sry expression remains an open question.

**Figure.** 23 SRY-positive pre-Sertoli cells were missing at the posterior pole of E11.5 XY *Ring1B* mutant gonads. E11.5 control XY gonads (A) contained many pre-Sertoli cells (SRY-positive, red) throughout the gonad field (with slightly higher density in the middle and anterior regions, consistent with the mid->anterior->posterior sequence of Sry activation). (B) In E11.5 XY *Ring1B* mutant gonads, SRY-positive cells were localized in the anterior half of the gonad, with no positive cells in the posterior domain. (C) In E11.5 XX control gonads, no SRY-positive cells were present, as expected. Higher magnification images are shown in A’, A’’, B’ and B’’. Scale bars are 50um.
Figure 23: SRY-positive pre-Sertoli cells were missing at the posterior pole of E11.5 XY Ring1B mutant gonads.

4.3.4 Some LHX9-positive aggregates were present in XY Ring1B mutant gonads

In severe cases of XY Ring1B mutant gonads (Fig. 22C), some regions of the posterior domain of the gonads were not labeled by markers of differentiation, including SOX9, FOXL2 and PECAM1. To investigate the identity of this cell population, we stained mutant gonads with an antibody against LHX9, an marker of undifferentiated cells, typically restricted to the epithelial domain (Fig. 24A,A’). In mutant XY gonads, cells
that were not labeled by differentiated markers, DDX4 for germ cells (red) and GATA4 for supporting and interstitial cell lineages (blue), were positive for LHX9 staining. These small aggregates of LHX9-positive cells were absent from control gonads. These results suggested that, in severe cases, rather than a pool of cells expressing the female somatic cell marker, FOXL2, a pool of unspecified cells were present in the posterior pole of XY \textit{Ring1B} mutant gonads.

\textbf{Figure. 24 Small aggregates of LHX9-positive cells were present in E13.5 XY \textit{Ring1B} mutant gonads.} (A) In E13.5 control XY gonads, the marker of undifferentiated gonadal cells, LHX9 (green) was restricted to cells in the coelomic domain. Differentiated somatic cells (GATA4-positive, blue) and germ cells (DDX4-positive, red) were present in the interior of gonads. (B) In E13.5 XY \textit{Ring1B} mutant gonads, in addition to somatic cells (GATA4-positive, blue) and germ cells (DDX4-positive, red), small aggregates of LHX9-positive cells were present in the interior of the gonad field, with increasing frequency toward the posterior domain. Scale bars are 50um.
Figure 24: Small aggregates of LHX9-positive cells were present in E13.5 XY *Ring1B* mutant gonads.
4.3.5 Identification of potential PRC targets during male embryonic gonad formation

The canonical function of the PRC is to repress target genes. In order to identify the potential targets of PRC during male gonad formation, we performed ChIP-Seq using an anti-RING1B antibody.

In mammals, expression of the Sry gene initiates Sertoli differentiation in the supporting cell precursors. This process involves the activation of many genes associated with the male pathway, as well as the repression of many genes associated with the female pathway (Fig. 19) (Jameson; Munger, Natarajan). We hypothesized that PRC1 plays an important role in the establishment and maintenance of Sertoli cell fate by repressing the female pathway.

I took advantage of a SOX9-CFP transgenic line and used fluorescein activated cell sorting (FACS) to isolate E13.5 SOX9-CFP positive cells (Sertoli cells). I pooled batches of sorted SOX9-CFP positive cells collected from E13.5 embryonic XY gonads, and performed RING1B ChIP-Seq. I obtained 57066688 reads in total, and 52362302 of these passed the QC (quality control). Among these reads, 27232890 were mapped. Nearly 800 peaks were called using the input track as a control and setting the cut-off fold change to 2.

Based on the assumption that PRC binding sites would overlap regions of enhancers and promoters, I compared the RING1B ChIP-Seq peaks with a recent published DNaseI hypersensitivity (DHS) dataset, which also used sorted E13.5 SOX9-CFP positive cells
(Maatouk et al., 2017). This comparison yielded 59 peaks that were overlapping (Table 3). Next, I compared these 59 overlapping peaks with the expression profiles for E11.5-E13.5 Sertoli and granulosa cells (Jameson et al., 2012b). Among those peaks that mapped to genes expressed in the supporting cell lineage at E13.5, I found some interesting candidates. The most interesting one is *Axin2 (axis inhibition protein 2)*. Since *Axin2* is a well-characterized female gene, downstream of *Wnt* signaling (Lustig et al., 2002), the presence of PRC at its promoter is consistent with a repressive mechanism involved in silencing the female (granulosa) pathway during Sertoli differentiation. Another PRC peak mapped to *Pak3 (p21 protein (Cdc42/Rac)-activated kinase 3)*. This gene is associated with p21, which shows sustained expression during granulosa cell specification to maintain cell cycle arrest. Sertoli cells silence this pathway and exit cell cycle arrest as their differentiation is initiated. Three other genes were identified, including *Ppara (peroxisome proliferator activated receptor alpha)*, *Rreb1 (ras responsive element binding protein 1)* and *Fam172a (family with sequence similarity 172, member A)*, which may suggest novel regulatory mechanism(s).

My hits also included genes enriched in other lineages (interstitial/stromal cells, endothelial cells, germ cells) collected from XX and XY gonads from stages E11.5-13.5 (Jameson et al., 2012b)). For example, a peak was detected at *Jag1 (Jagged 1, also known as Serrate-1)*, which is expressed in the interstitial cell lineage, and *Kcna5 (potassium voltage-gated channel, shaker-related subfamily, member 5)*, which is expressed in the endothelial cell
lineage, as well as *Wasf3* (*WAS protein family, member 3*), which is expressed in the germ cell lineage. All of these candidates will need further validation and investigation regarding their regulation via PRC and their potential role in gonadogenesis.

**Table. 3 Overlapping hits from E13.5 SOX9-CFP DNaseI dataset and RING1B ChIP-Seq data.** 59 peaks were found to be overlapped in E13.5 SOX9-CFP DNaseI dataset and RING1B ChIP-Seq data. The expression profile of the most proximate gene to the mapped peak was investigated in the GUDMAP expression dataset (Jameson et al., 2012b).
### 4.3.6 Ring1B interacts with SOX9 in vivo

In *Drosophila*, several transcription factors have been proposed to be involved in mediating PRC1 recognition of the Polycomb response elements and H3K27me3 modification. Among these transcription factors only an ortholog of Pho, YY1, is preserved in mammals and has been found to interact with PRC1 (Lorente et al., 2006).

#### Table 3: Overlapping hits from E13.5 SOX9-CFP DNase I dataset and RING1B ChIP-Seq data.

<table>
<thead>
<tr>
<th>Chr</th>
<th>Start</th>
<th>Strand</th>
<th>Nearest Gene 1 Distance from TSS</th>
<th>Table 1</th>
<th>Cross comparison with GUMMAP data set</th>
<th>Nearest Gene 2</th>
<th>Cross comparison with GUMMAP data set</th>
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<td>1.25107</td>
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</table>

In *Drosophila*, several transcription factors have been proposed to be involved in mediating PRC1 recognition of the Polycomb response elements and H3K27me3 modification. Among these transcription factors only an ortholog of Pho, YY1, is preserved in mammals and has been found to interact with PRC1 (Lorente et al., 2006).
However, in mammals, the recruiting mechanism is inconclusive. The heterogeneity of PRC1 components has drawn significant research interest. Specificity of component selection enables the PRC1 to conduct distinct functions under different developmental conditions. With the advanced techniques of proteomics and genome-wide studies, various subsets of PRC complexes have been characterized. One recent study categorized PRC1 complexes in six major groups. Each group contains a distinct PCGF (Polycomb group RING finger) subunit, a RING1A/B ubiquitin ligase, and a unique set of associated polypeptides. Interestingly, each group occupies distinct sites in the genome (Gao et al., 2012). Coincidently, another study showed the non-overlapping functions of CBX family members in pluripotency maintenance and cell differentiation. Different CBX-associated PRC1 complexes regulate unique functions. In mouse embryonic stem cells (ESCs) CBX7 was shown to be present in pluripotent stem cells while CBX2 and CBX4 were shown be involved in lineage commitment (Morey et al., 2012). Thus, with specific and dynamic partner selection, PRC1 might be able to regulate the various subsets of genes at different developmental stages or in different cell lineages.

Transcription factors have been identified as a component of the PRC1 complex and might be involved in recruitment of PRC1 to its target loci: RUNX1/CBFβ has been shown to contribute to site-specific RING1B recruitment to target loci (Yu et al., 2012). SOX9 is a member of the HMG-box protein family, known to bind to cruciform DNA
structures (Bianchi et al., 1992). It also has been reported to interact with a SUMO-ligase RING-domain containing protein, PIAS (protein inhibitor of activated STAT-1)(Hattori et al., 2006). Since SOX9 is a critical male transcription factor immediately downstream of Sry, I hypothesized that SOX9 might interact with RING1B to target PRC1 to repress genes associated with the female pathway.

To test this hypothesis, I performed a co-immunoprecipitation assay using E13.5 XY gonad lysates (Fig. 25). In both the 10% Input lane as well as the RING1B antibody IP lane, we detected RING1B protein immunoprecipitated by the RING1B antibody (indicated by the lower asterisk). Compared with the Input lane, we observed a band present at the SOX9 molecular weight (indicated by the upper asterisk) suggesting an interaction between RING1B and SOX9 in E13.5 XY gonads.

**Figure. 25** RING1B interacts with SOX9 during XY gonad development. Co-immunoprecipitaion demonstrated that RING1B interacts with SOX9 in E13.5 XY gonads. RING1B was present in the 10% input control lane (10% Input), and IP lane after RING1B immunoprecipitation (IP: RING1B), but absent in the negative control lane using mouse IgG to perform the pull down (IP: Ms-IgG). SOX9 was present in the 10% input control lane (10% Input), and after RING1B immunoprecipitation (IP: RING1B) (IP: RING1B), but absent in the negative control lane using mouse IgG to perform the pull down (IP: Ms-IgG).
4.4 Conclusion and discussion

Antagonism between male and female pathways drives sex determination in the bipotential gonad (Lin and Capel, 2015). We hypothesized that PRC1, a protein complex with the ability to repress target genes, is involved in suppressing the female program during male gonad development. In \( Cbx2 \) and \( Fgf9 \) double heterozygous XY gonads
(Cbx2\(^{+/−}\); Fgf9\(^{+/−}\)), we observed a synthetic effect of sex reversal, evident at both of the gonad poles. In contrast, single null alleles for Cbx2 or Fgf9 (Cbx2\(^{+/−}\) or Fgf9\(^{+/−}\)) present as wild type phenotypes. Moreover, in Ring1B mutants, sex reversal was observed in XY gonads when we induced the deletion of Ring1B at early stages of gonad formation (Tamoxifen administration at E8.75). Our unpublished collaborative work with the Maatouk group also supports the idea that PRC is involved in repression of the female program. In this case, we found that simultaneous deletion of Cbx2 and Wnt4 (Cbx2\(^{+/−}\); Wnt4\(^{+/−}\)) rescued the Cbx2\(^{+/−}\) sex reversal phenotype, suggesting that the essential role of PRC is repression of the female program during XY gonadogenesis (Fig. 26, manuscript in progress). How does PRC achieve its mission to repress the female program? What are its targets, and how does PRC recruit to those target genes? Although all of these experiments require further validation, our RING1B ChIP-Seq and RING1B/SOX9 co-immunoprecipitation preliminary data might provide potential answers to these questions.

**Figure. 26 Sex reversal is rescued in Cbx2; Wnt4 double mutant embryos.**

Immunofluorescent analysis of control, Cbx2\(^{+/−}\) and Cbx2\(^{+/−}\); Wnt4\(^{+/−}\) embryonic gonads at E11.5 (A-D) and E13.5 (E-H) stained with the Sertoli-markers SRY (white) and SOX9 (red), and granulosa-marker FOXL2 (green). Brightfield images of embryonic gonads at E15.5 were shown in I-L. XY Cbx2\(^{+/−}\) gonads develop as ovaries (K) whereas XY Cbx2\(^{+/−}\);
Wnt4<sup>−/−</sup> gonads develop as testis (L); both are smaller compared to controls. Images are representative of n≥3. (Figure in progress, courtesy of Alexandra Garcia Moreno and Danielle Maatouk.)

Figure 26: Sex reversal is rescued in Cbx2; Wnt4 double mutant embryos.
An interesting alternative model, not exclusive with the original hypothesis, is that, 
Ring1B is involved in patterning of XY gonad formation. In flies, the canonical role of 
PRC is to define body segments by regulating Hox gene expression (Frei et al., 1985). In 
Drosophila, Hox gene domains define the position of the gonad (Greig and Akam, 1995). 
Although Hox genes are not currently known to specific the position of the gonad in 
mammals, it is possible that during mouse XY gonad formation, PRC regulates Hox gene 
domains, which determine where the gonad forms (or where Sry is expressed) in the 
mouse intermediate mesoderm. If this is true, then disrupting PRC expression could 
cause a pattern shift resulting in the posterior specific sex reversal phenotype we 
observed in Ring1B mutant gonads.

To compile a list of Hox genes that might be differentially expressed across the gonad 
field, I used the GUDMAP expression dataset to identify Hox genes expressed in the 
gonad at E11.5. I designed qRT-PCR primers against each of these genes 
(HoxC4,C5,D10,D11), isolated gonads at E11.5, divided them into an anterior and 
posterior half, extracted mRNA and analyzed the level of expression in each half. Based 
on this preliminary data, some Hox genes showed differential expression patterns in the 
anterior and posterior domains in wild type gonads (HoxC4 and HoxC5 were 
upregulated in the anterior half, whereas HoxD10 and HoxD11 were upregulated in the 
posterior half. Data not shown). These candidates require further validation.
5. Future directions

Embryonic mouse gonad, composed by multiple cell lineages, thus, served as a beautiful model to study the complexity of cell lineage specification, determination and maintenance. Starting from E10.5 as a bipotential gonad, XX and XY gonads are initially indistinguishable at molecular level, where later on female fate associated genes (such as \textit{Wnt4} and \textit{Rspo1}) as well as male fate associated genes (such as \textit{Sox9} and \textit{Fgf9}) are expressed at the similar level (Jameson et al., 2012b; Munger et al., 2013; Nef et al., 2005). The balance between female and male programs is then tilted by the presence or absence of \textit{Sry}, in a very narrow window of time (Hacker et al., 1995; Hiramatsu et al., 2009; Larney et al., 2014). With the initiation of male or female program, male and female gonads undergo dramatic morphological changes and eventually adopt their fates.

In this dissertation, we used mouse genetic mutation models of \textit{Numb}/\textit{Numbl} and \textit{Ring1B} to address questions about the early specification of the gonad and the fate determination decision in gonadal cells. Yet, findings always bring more questions. There are many directions to pursue in future studies.

We observed extensive germ cell loss in both male and female \textit{Numb}/\textit{Numbl} mutant gonads at the earliest stages of gonad formation. Since we used a ubiquitously expressed CRE line (\textit{ROSA-CreER}), where \textit{Numb} was deleted in all cell lineages upon tamoxifen induction, it was impossible to determine whether the germ cell loss phenotype was a cell autonomous or non-autonomous effect of losing NUMB. There is no known function
for Notch/Numb during specification and migration of germ cells in the mouse. Interestingly, NUMB has been suggested to contribute to directional cell migration in different developmental aspects (Nishimura and Kaibuchi, 2007). In on-going experiments, we are crossing the Numbr\textsuperscript{flox/flox};Numbl\textsuperscript{+/-} mice to a germ cell specific, tamoxifen inducible Cre line (Oct\textit{4-CreER}). Characterization of these gonads may shed lights on whether Numb is required cell autonomously in germ cells, or is non-cell autonomously involved in germ cell migration or survival.

Another open question that has interested the lab for a long time, is what differentiates the supporting cell lineage from the interstitial cell lineage, given the fact that both lineages are derived from common precursors, the CE cells (Karl and Capel, 1998). Originally we hypothesized that asymmetric distribution of NUMB could be responsible for distinguishing these two lineages. Our findings from the Numb/Numbl mutant gonads indicate that NUMB is required to maintain polarity of the stem-like cells in the CE reservoir, to suppress Notch signaling, and to give rise to precursor cells with competence to differentiate as both supporting and interstitial cell lineages. However, the original intention to unveil the KEY molecule that differentiates these two lineages has not been achieved. One possibility supported by my work in Numb mutants is that, although these two lineages arise from the same pool of precursors, they arise sequentially. Techniques such as single cell RNA-Seq at E11.0-E12.0 might be valuable to understand the transcriptome differences among cells derived from the CE.
Previous studies indicated that an important aspect of commitment to Sertoli or granulosa cell differentiation was repression of the alternative program (Kim et al., 2006; Munger et al., 2013). PRC1 is a known mediator of gene repression (Vissers et al., 2012). Although CBX2 (a component of PRC1) was known to be required for testis determination, studies had previously suggested that PRC1 acts as a positive regulator of Sry expression (Katoh-Fukui et al., 2012; Katoh-Fukui et al., 1998). We hypothesized that PRC1 plays a role in repression of the female pathway, such that loss of function would lead to upregulation of female genes and consequent repression of Sry and the male pathway. This might lead to failure to establish or maintain the male pathway, depending on the time of deletion of the gene, and the role it plays at different stages of sex determination.

When Ring1B deletion was induced at E8.75, we found disruption of XY gonad formation at E11.5, specifically in the posterior domain, where Sry expression was not detected. However, Sry expression levels seemed to be normal in the anterior domain, suggesting that PRC1 does not have a direct effect on Sry expression. Although, we saw expression of female genes in the posterior domain, the size and organization of the tissue, as well as the expression of the undifferentiated marker, LHX9, suggest this domain of the gonad was under-developed.

We are considering the possibility that the phenotype in Ring1B mutants is caused by a shift of patterning during early gonadogenesis. In fact, how and why Sry initially
expresses at the midline of male gonads is still an unanswered question in the field. It is possible that PRC regulates Hox genes that define the pattern/position of the gonad, just as they do during Drosophila body segment patterning (Jürgens G., 1985; Frei et al., 1985), and during Drosophila gonad positioning (Greig and Akam, 1995). With our preliminary data on Hox gene expression domains within the gonad field, this question might be answered in the future.

Loss of Ring1B at initial stage of gonad formation clearly led to an early phenotype in which the domain of Sry expression was disrupted. Recent evidence suggests that the fate of supporting cells in the testis must be actively maintained, even in adult life. Deletion of Dmrt1 in adult Sertoli cells leads to their transdifferentiation to granulosa cell fate (Minkina et al., 2014). Therefore, we hypothesize that later deletion of PRC1 would lead to a de-repression of the female pathway and a failure to maintain the male pathway.

To test this hypothesis, we tried to delete both Ring1A and Ring1B, at early or later stages of gonad formation using ROSA-CreER. However, when we administered tamoxifen at E8.75, E10.5, or E11.5 (stages at which we obtained viable embryos after deletion of Ring1B alone), we obtained no surviving embryos. The deletion of Ring1B alone at later stage did not disrupt the maintenance of Sertoli fate. We speculated that this result might be due to compensation by Ring1A (ring finger protein 1). In future experiments, a later stage induction (for example, tamoxifen administration at E12.25),
or crossing lineage specific Cre, such as Sf1-Cre, onto the \textit{Ring1A;Ring1B} conditional background are underway, which we hope will allow us to address this question in the future.

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**Biography**

Yi-Tzu Lin was born on June 14, 1979, in Taipei, Taiwan, to Sheng-Chieh Lin and Hwei-Yuen Tzou, as the first child of their two children. Her happy and colorful childhood was spent mostly in the big city Taipei, and, for numerous summer and winter vacations, in countryside, Jiaoxi, with her grandparents, chasing hens (or chased by hens) in the garden and baking sweet potato in the rice field. After graduate from Taipei Municipal Zhong Shan Girls High School, she matriculated at National Taiwan University, where he major was in Horticulture. Inspired and encouraged by her father, who worked as a scientist associate in Taiwan Forestry Research Institute, Yi-Tzu initiated her very first grand proposal in the very first summer as a freshman, which opened the door for her to all the fun part of doing research. In 2002, Yi-Tzu graduated
and started to pursue her master degree, majored in Biochemistry. In 2004, after graduating from the master program, Yi-Tzu got her first job offer as a lab technician in a male infertility lab, supervised Dr. Pauline Yen in Academia Sinica, Taiwan. Fascinated by the beauty of developmental and cell biology she learned in Pauline’s lab, Yi-Tzu decided to pursue her PhD in the US. In 2010, Yi-Tzu entered Duke through Developmental and Stem Cell Biology Program. She was then luckily welcomed by Dr. Blanche Capel to join her lab in Fall, 2011.

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**Scholarships and fellowships**

American Society for Cell Biology (ASCB) WICB travel award 2016

Government Scholarship to Studying Abroad, Ministry of Education, Taiwan 2012-2014

Hong Fellow, Institute of Biomedical Sciences, Academia Sinica, Taiwan 2010-2012