Dazl regulates mouse embryonic germ cell development

by

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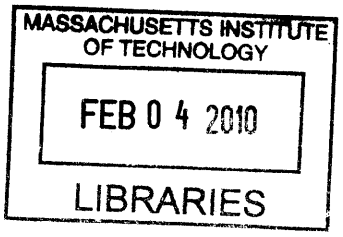

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ABSTRACT

In the mouse, germ cells can undergo differentiation to become either oocytes or spermatozoa in response to sex of their gonadal environment. The nature of the germ cell-intrinsic aspects of this signaling have not been well studied. The earliest known sex-specific difference in germ cells is the initiation of meiosis in female, but not male, embryonic germ cells. Experiments were performed showing that germ cells of both sexes transit through a state, the meiosis competent germ cell, that is required for initiation of meiosis. Acquisition of this state requires the function of the germ cell-specific RNA binding protein DAZL.

The sufficiency for the absence of meiosis to drive male germ cell differentiation was then tested by examining non-meiotic XX germ cells in the *Dazl*-deficient ovary. These cells did not exhibit male differentiation indicating that the absence of meiosis is not sufficient for male differentiation. XX *Dazl*-deficient germ cells also failed to exhibit normal female differentiation. In addition, XY *Dazl*-deficient germ cells do not display characteristics of either male or female germ cells. Taken together, these results indicate that germ cells must first undergo a sex non-specific differentiation step prior to acquiring sexual fate.

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Title: Professor of Biology

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Table of Contents

Chapter 1:

Germ cell development in the mouse

1. Introduction

Germ cells represent the physical link between generations of multi-cellular organisms. As such the development of these cells has been extensively studied. Germ cell development can be seen as a cycle composed of discrete stages leading from one generation to the next. In order to fully understand these individual stages they must be viewed within the context of the complete cycle (Fig. 1).

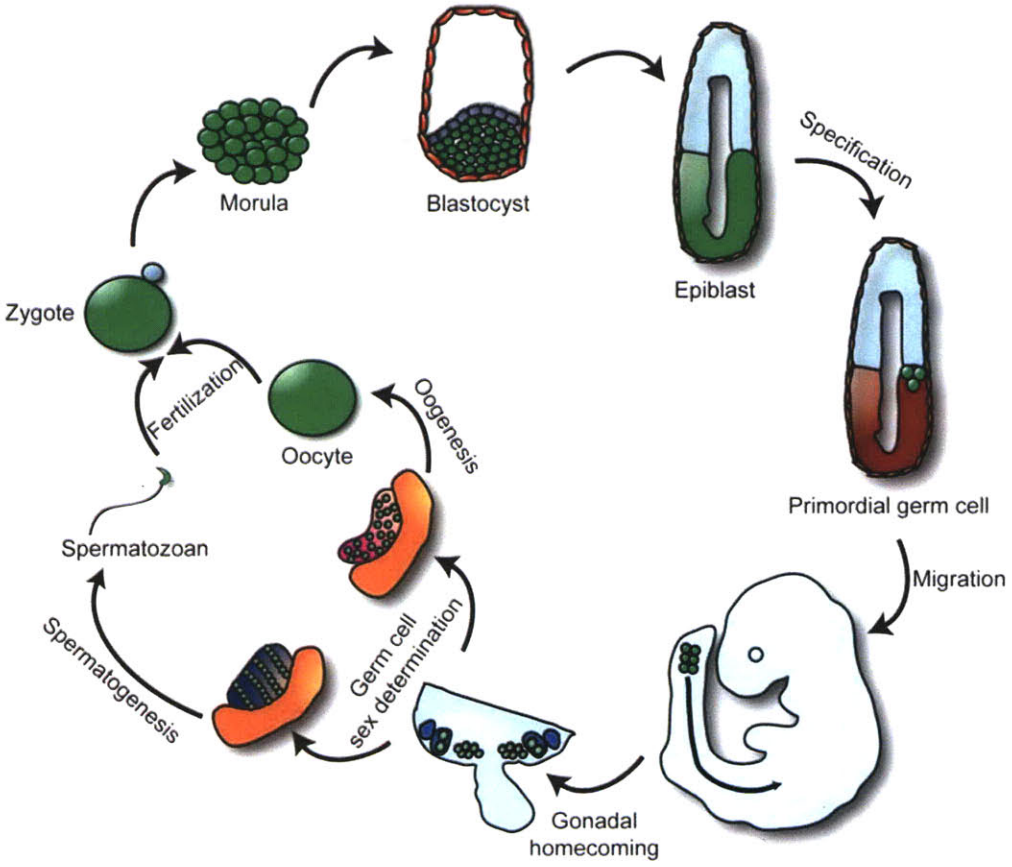

Fig 1. Cycle of the mammalian germ lineage. In this figure cells that have the potential to contribute to the germ cell lineage are labeled in green. Processes listed over arrows will be covered in greater detail in subsequent sections of this introduction.

The purpose of this introduction is to provide an overview of the developmental processes in the mouse leading from zygote to sexually mature gametes and back again.

The research described in Chapters 2 and 3 of this thesis is focused upon the development of the post-migratory embryonic germline, and as such this introduction will focus extensively on this area of development. For a more extensive treatment of lineage restriction during early mammalian embryogenesis I recommend (Rossant and Tam, 2009). Morelli and Cohen (2005) provide an excellent review of the details of mammalian meiosis in male and female germ cells. Russell et al. (1990) and Edson et al. (2009) provide a much more comprehensive review of postnatal spermatogenesis and oogenesis respectively.

2. Fertilization

While the development of the germline is cyclical and thus has neither a beginning nor an end, a logical place to begin considering the development of these cells is with fertilization. Fertilization is triggered by the binding of mature spermatozoa (sperm) to the zona pellucida (ZP), a glycoprotein coat surrounding the mature oocyte Fig. 2). The components of the ZP display significant sequence similarity with components of the vitelline envelope found in fish, birds and amphibians (Kubo et al., 1997; Tian et al., 1999). However, the exact structure of the ZP varies widely even between different species of mammals and is thought to be a major barrier against inter-specific fertilization events (Gwatkin, 1977; Yanagimachi, 1994). In mice, three genes encode the major components of the ZP, *Zp1, Zp2* and *Zp3*. *Zp3* encodes a glycoprotein capable of binding to the sperm head plasma membranes through its extensive oligosaccharide modifications (Bleil and Wassarman, 1980; Florman and Wassarman, 1985). While it is clear that ZP3 is the component of the oocyte that is bound by the

sperm, the identity of the complementary component within the sperm head has been elusive, with more than two-dozen different components being proposed to act as egg binding proteins (EBPs). The wide variety of species in which EBPs have been identified might have confounded these studies, as the means of sperm to ZP binding may not be conserved (Wassarman et al., 2001). Alternatively, while only a single sperm-binding component exists within the ZP, this function might be subdivided among many components in the sperm head.

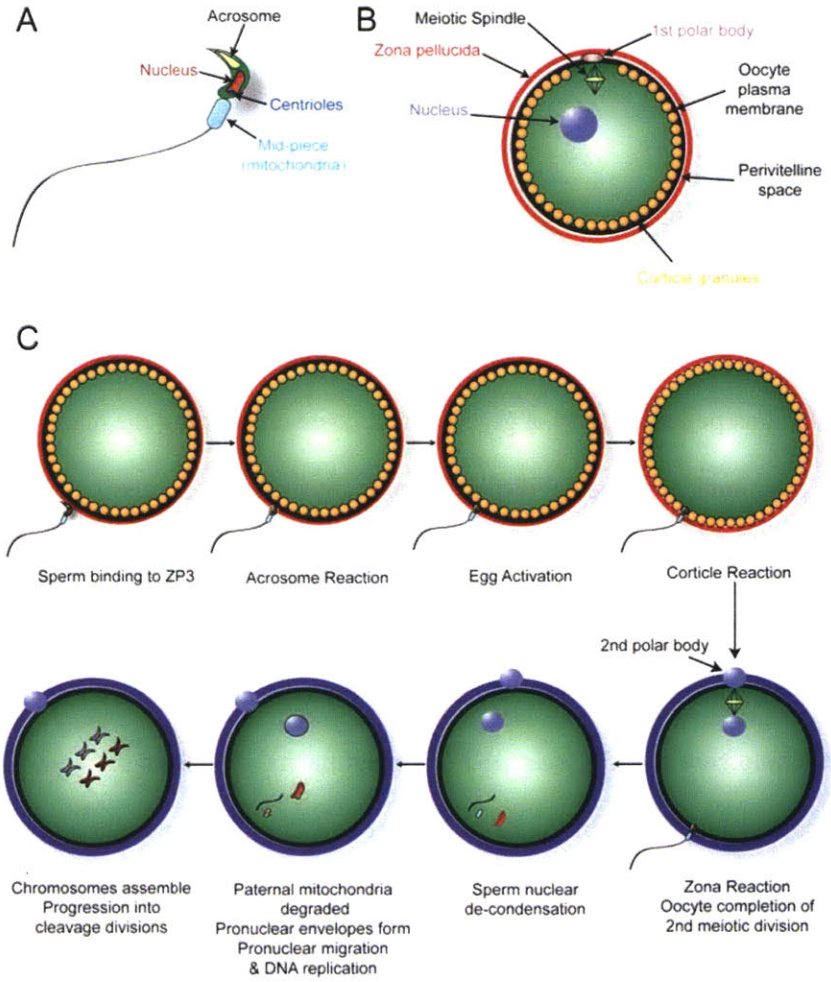

Figure 2. Fertilization in the mouse. (A) Structure of the mature spermatozoan. (B) Structure of a metaphase II oocyte. (C) Progression through fertilization from sperm binding to preparation for first cleavage division

Binding of the sperm to ZP3 results in the outer membrane of the acrosome, a secretory vesicle located on the sperm head, fusing with the sperm plasma membrane, an event known as the acrosome reaction (Florman et al., 2008). This results in release of the acrosomal contents, which include several proteases. These proteases then digest a portion of the ZP allowing the sperm entry to the peri-vitelline space, the area between the ZP and the oocyte plasma membrane. Once within the perivitelline space the sperm and egg plasma membranes fuse.

The fusion of the sperm and oocyte plasma membranes results in a process known as egg activation. This process results in an increase in intracellular levels of calcium, which in turn activate several signaling pathways (Swann and Yu, 2008). Additionally, egg activation results in the completion of the second meiotic division and extrusion of the second polar body. The fusion of the sperm and egg plasma membranes also triggers the cortical reaction, which helps to prevent polyspermy, the fertilization of one oocyte by more than one sperm (Gardner et al., 2007).

Cortical granules (CGs) exist just beneath the plasma membrane of the mature oocyte. CGs are secretory vesicles that, upon fusion with the sperm plasma membrane, are exocytosed into the perivitelline area. Exocytosis of the CGs activates a process known as the zona reaction (Sun, 2003). This reaction alters the ZP by eliminating the sperm-binding properties of ZP3 and cross-linking the glycoproteins.

Following fusion with the oocyte, the sperm head, midpiece and a portion of the sperm tail enter the oocyte cytoplasm. The midpiece of the sperm provides the zygote with paternal centrioles and mitochondria. These mitochondria, however, are actively degraded by factors within the cytoplasm and are eliminated before the development of

the eight cell stage embryo (Cummins, 2000). Following introduction of the sperm head into the oocyte cytoplasm, the nuclear lamina is disassembled and the sperm chromatin then undergoes extensive de-condensation (McLay and Clarke, 2003). Nuclear membranes then reform around the chromosomes and both maternal and paternal pronuclei move towards the center of the oocyte, undergoing DNA replication as they migrate. Once at the center of the oocyte, the nuclear membranes surrounding the pronuclei break down. The chromosomes then assemble on the spindle and the first mitotic division can commence.

3. Early embryogenesis

Once the chromosomes of the sperm and the oocyte have aligned, the zygote can undergo its first mitosis to form a two-cell embryo. Transcription, which has been inactive in the oocyte since its arrest at meiotic metaphase II, is reactivated in multiple waves starting in the two cell embryo and increasing dramatically in four- and eight-cell embryos (Hamatani et al., 2006). For the first three divisions of the embryo (up through the eight-cell stage) all cells of the embryo are considered equivalent. Following the eight-cell stage, embryos undergo a process known as compaction, developing into morulae with a polarized epithelium. At this stage the cells of the embryo undergo their first lineage specification. This decision separates the trophectoderm (TE), which will eventually develop into the placenta, from the primitive endoderm and epiblast. The earliest factor known to be required for TE development is the transcription factor *Tead4* (TEA domain family member 4) (Nishioka et al., 2008; Yagi et al., 2007). *Tead4* is required for expression of the transcription factor *Cdx2* (caudal type homeobox 2) (Yagi

et al., 2007). Cells located on the outer layer of the morula express on average higher levels of *Cdx2* than of the transcription factor *Oct4* (octamer binding 4, Mouse Genome Informatics: *Pou5f1*, POU domain, class 5, transcription factor 1) (Johnson and McConnell, 2004). Despite these differences in the eight- and sixteen-cell stages, all cells of the 16-cell embryo retain the capacity to produce all developmental lineages (i.e. they are totipotent) (Johnson and McConnell, 2004; Suwinska et al., 2008). Despite this capacity, lineage biases among blastomeres are seen based on the cleavage plane of the four-cell stage embryo (Piotrowska-Nitsche et al., 2005), suggesting that while an early lineage specification decision is made, cell fates are not firmly restricted until later in development.

Three and a half days after fertilization (E3.5) the embryo has developed into a ball of cells, called a blastocyst, containing a fluid filled cavity (the blastocoel). Cells that expressed high levels of *Cdx2* have developed into TE. *Cdx2* is not required for these cells to acquire trophoblast identity, but it is required down-stream of this decision in the TE lineage (Ralston and Rossant, 2008). These cells have deactivated expression of *Oct4* and continue to express high levels of *Cdx2*, whose expression is reinforced through the activity of the transcription factor *Elf5* (E74-like factor 5) (Ng et al., 2008).

While the trophectoderm forms the surface of the blastocyst, the cells of the inner cell mass are composed to two different cell lineages: the primitive endoderm (PE also known as the hypoblast) and the epiblast. The PE will form the yolk-sac of the embryo, while the epiblast will form the embryo proper (including the germ cells) as well as the amnion and the allantois. Prospective epiblast and PE cells can be distinguished by their expression of the transcription factors *Nanog* (Nanog homeobox) and *Gata6* (GATA

binding protein 6) respectively (Chazaud et al., 2006). Specification of PE appears to require active *Grb2* MAP kinase activity, as in the absence of *Grb2*, PE formation fails and only epiblast develops within the inner cell mass (Chazaud et al., 2006). During morula and early blastocyst development *Nanog* is expressed at varying levels in all blastomeres (Dietrich and Hiiragi, 2007). How its expression becomes restricted solely to the epiblast by late blastocyst stage remains unclear.

Five days after fertilization, the zona pellucida, which has continued to surround the developing embryo, is degraded by enzymes produced by cells of the TE (Wassarman, 1984). This process, known as hatching, is required for implantation of the embryo into the uterus (Enders and Schlafke, 1969). As the embryo is undergoing implantation the process of gastrulation begins.

4. Germ cell specification

The earliest studies attempting to identify the origins of embryonic germ cells utilized the enzymatic activity of alkaline phosphatase, which appears in a small number of embryonic cells by E7.25, to localize the primordial germ cells (PGCs) of the mouse (Chiquoine, 1954; Ginsburg et al., 1990). While alkaline phosphatase was and remains one of the most useful markers for identifying primordial germ cells, it is not required for their specification, survival or migration (MacGregor et al., 1995). Lineage tracing experiments using retroviral integration indicate that all primordial germ cells arise from at least three progenitor cells (Soriano and Jaenisch, 1986). These results were more recently verified using a multiple fluorescent imaging approach (Ueno et al., 2009).

In the most studied model systems the germ cell lineage can be traced from first embryonic cell division via the cytoplasmic segregation of a specialized cytoplasm known as germplasm (Cinalli et al., 2008). In mammals, despite extensive investigation, no germplasm appears to be present and germ cell identity is thought to be induced by extracellular signals. The first such signal identified in the mouse was BMP4 (bone morphogenetic protein 4), which is secreted from extraembryonic ectoderm (Lawson et al., 1999). Active BMP signaling can be detected in the epiblast cells most proximal to the extraembryonic ectoderm, however, transplantation experiments have shown that epiblast cells more distal to the extraembryonic ectoderm can also become PGCs and are only prevented from doing so *in vivo* by distance from the signaling source (Tam and Zhou, 1996).

Further studies have identified additional members of the BMP signaling family as having crucial functions in the specification of germ cells. BMP8b, also from the extraembryonic ectoderm (Ying et al., 2000), and BMP2 (Ying and Zhao, 2001), from the visceral endoderm, play critical roles in PGC specification. These signals are transduced via the down-stream signaling activities of the SMADs (MAD homologs (Drosophila)), SMAD1, SMAD4, and SMAD5, but not SMAD8 (Arnold et al., 2006; Chang and Matzuk, 2001; Chu et al., 2004; Hayashi et al., 2002; Tremblay et al., 2001). More recent studies have shown that the BMP4 signaling required for germ cell specification actually functions indirectly via activation of visceral endodermal signaling which in turn acts on epiblast cells to induce germ cell identity (de Sousa Lopes et al., 2004).

Following the discovery of extracellular regulation of germ cell specification, much effort was placed into identifying germ cell-intrinsic markers expressed

differentially from the very beginning of the specification process. Germ cells were thought to retain expression of genes associated with pluripotency such as *Oct4*, *Nanog* and *Sox2* for much longer in embryogenesis than somatic cells (Scholer et al., 1989; Western et al., 2005; Yamaguchi et al., 2005). More recently it has been suggested that expression of these genes is actually transiently lost in the PGC population and their expression is re-activated following crucial events in specification (Yabuta et al., 2006). However, these markers are difficult to study in the context of germ cell specification, as they are initially expressed quite broadly in the epiblast. One of the first markers identified that is expressed specifically in PGCs shortly after germ cell specification is *Dppa3* (developmental pluripotency associated gene 3; also known as *stella* or *Pgc7*) (Bortvin et al., 2003; Saitou et al., 2002; Sato et al., 2002). *Dppa3* was identified independently in three separate expression screens utilizing an *in silico* approach (Bortvin et al., 2003), a serial analysis of gene expression (SAGE) approach (Sato et al., 2002) and a single cell cDNA sequencing approach (Saitou et al., 2002). *Dppa3* has proven very useful as a marker of PGCs from E7.5 onwards, however, lineage-tracing experiments suggest that germ cells are specified significantly earlier than this (Soriano and Jaenisch, 1986), suggesting that other factors function upstream of *Dppa3* in germ cell specification. Additionally, *Dppa3* function is not required for germ cell specification (Bortvin et al., 2004; Payer et al., 2003), so while it is a useful marker, it has not provided significant insight into the mechanism of germ cell specification.

One of the studies that identified *Dppa3* as a PGC marker also identified an additional gene expressed more highly in PGCs than the surrounding epiblast known as *fragilis* (MGI: *Ifitm3*, interferon induced transmembrane protein 3) (Saitou et al., 2002).

fragilis is expressed throughout the proximal epiblast in more cells than are specified as germ cells. It has been proposed BMP signaling activates expression of *fragilis* which in turn makes proximal epiblast cell competent to respond to further specification signals. Unfortunately as with *Dppa3, fragilis* function is not required for germ cell specification (Lange et al., 2008).

Identification of a germ cell-intrinsic factor involved in germ cell specification finally occurred in 2005, when Ohinata et al. reported the discovery of role for *Blimp1* (B-lymphocyte induced maturation protein 1; MGI: *Prdm1*, PR domain containing 1, with ZNF domain) in germ cells (Ohinata et al., 2005). *Blimp1* is expressed in six *fragilis*-positive cells at E6.25. These cells then continue to proliferate and by E7.5 all *Blimp1* expressing cells also express alkaline phosphatase and *Dppa3*. Mice heterozygous for a *Blimp1* null allele exhibit decreased numbers of germ cells, while homozygous *Blimp1* null animals possess very few PGC-like cells. These PGC-like cells do not proliferate or migrate like normal PGCs and express *Hoxb1,* a somatic mesodermal marker normally not expressed in specified germ cells (Ohinata et al., 2005). These results suggest that one aspect of germ cell specification is prevention of somatic differentiation.

The protein encoded by *Blimp1* is a member of the PRDM family, members of which are characterized by the presence of a PR (PRDI-BF1 and RIZ) domain associated with a variable number of zinc fingers. PRDM family members function as transcriptional repressors via interactions with chromatin modifying enzymes. In PGCs *Blimp1* interacts with *Prmt5* (protein arginine N-methyltransferase 5) and this complex

induces repression of somatic mesoderm target genes via dimethylation of arginine 3 of histones H2A and H4 (Ancelin et al., 2006).

Given the phenotype of *Blimp1*-deficient mice, Yabuta et al. sought to determine if additional PRDM genes might be involved in germ cell specification (Yabuta et al., 2006). They examined the mRNA levels of the 16 PRDM genes in cDNA derived from single PGCs and somatic mesoderm cells from E6.75 through E8.5 embryos using quantitative RT-PCR. They found that *Prdm14* (PR domain containing 14) is expressed in PGCs but not somatic cells for all of the time points examined. In 2008, Yamaji et al. used transgenic reporter mice to show that *Prdm14* is expressed even more specifically in germ cells than *Blimp1* (which is also expressed in visceral endoderm) (Yamaji et al., 2008). They also showed that *Prdm14* is a critical player in early germ cell specification by generating *Prdm14*-deficient mice. Interestingly, *Prdm14*-deficient mice show a failure in the re-activation of expression of the pluripotency factor *Sox2* but unlike *Blimp1*-deficient mice do not ectopically express the somatic marker *Hoxb1*. Based on temporal expression and genetic data (Yabuta et al., 2006; Yamaji et al., 2008), a model for germ cell specification has emerged in which initially *Blimp1* serves to specify a small population of germ cells that in turn express *Prdm14* (Fig. 3A). These cells then require the activity of both *Prdm14* and *Blimp1* to activate expression of *Sox2* and *Dppa3*, and the activity of *Blimp1* alone to prevent expression of *Hoxb1*.

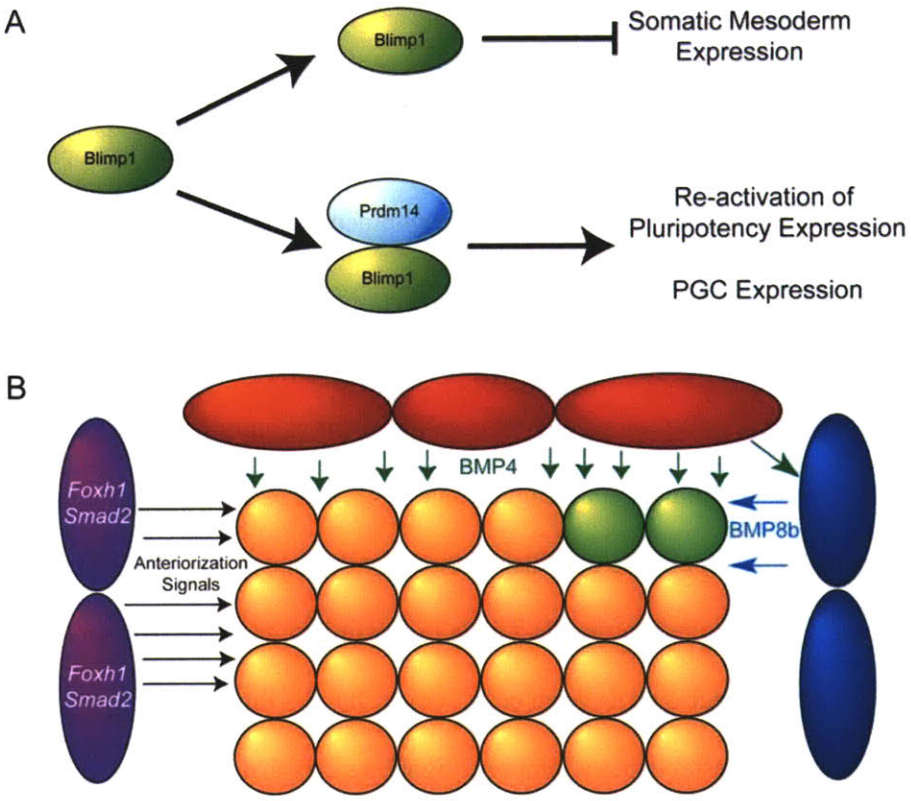

Figure 3: Regulation of primordial germ cell specification. (A) Model for the genetic regulation of PGC specification. Early in specification, *Blimp1* is expressed in nascent PGCs. These cells then induce expression of *Prdm14*. *Blimp1* alone represses somatic mesoderm fate, while *Blimp1* and *Prdm14* in conjunction induce expression of pluripotency genes (such as *Sox2* and *Oct4*) and induce expression of PGC markers (such as *Dppa3* and alkaline phosphatase). (B) Model for the extrinsic signaling involved in PGC specification. Extraembryonic ectodermal cells (red) secrete BMP4 to proximal epiblast cells (orange and green) and posterior visceral endodermal cells (PVE, blue). PVE cells in turn secrete BMP8b to proximal epiblast establishing highest BMP levels in posterior proximal epiblast. In addition anterior visceral endoderm (AVE) cells, determined by expression of *Smad2* and *Foxh1*, secrete anteriorization signals, which inhibit germ cell fate. All cells of the epiblast intrinsically express WNT3 between E6.0 and E6.25, making these cells responsive to BMP signaling. PGC identity (green) is specified by areas of highest BMP signaling and lowest anteriorization signals.

The model for germ cell specification was further refined in 2009 by the work of Ohinata et al. (Ohinata et al., 2009) (Fig. 3B). This group noted that only epiblast cells proximal to the extraembryonic ectoderm and in the posterior end of the embryo are specified to express *Blimp1* and *Prdm14*. BMP signaling provides a clear signal differentiating proximal from distal epiblast, but it provides no information as to the anterior or posterior identity of epiblast cells. Ohinata et al. therefore hypothesized the

existence of an additional signal operating on the anterior to posterior axis that would combine with BMP signaling to select a small number of cells for germ cell specification. Ohinata et al. examined embryos mutant for *Smad2* (MAD homolog 2 (Drosophila)) or *Foxh1* (forkhead box H1) in which anterior development is prevented and the entire epiblast acquires posterior fate and showed that in these mutants all proximal epiblast cells are specified as germ cells. The function of *Smad2* and *Foxh1* in establishing anterior identity occurs via their role in the establishment of anterior visceral endoderm (AVE). The results of Ohinata et al. suggest that AVE acts as an inhibitor of germ cell specification. Ohinata et al. also established that *Wnt3* (wingless-related MMTV integration site 3) is required for epiblast cells to acquire competence to respond to BMP signaling and thus be specified as germ cells. Given *Wnt3*'s limited timing of expression, it is thought that *Wnt3* establishes a temporal signaling axis that interacts with the two spatial signaling axes (BMP's and AVE) to specify a specific number of epiblast cells as PGCs only at the correct developmental time. These results have greatly enhanced understanding of which the cells of the epiblast will become PGCs, however, a detailed mechanism of how this process occurs remains to be seen.

5. Germ cell migration

PGCs are specified at a site in the embryo distant from the location where the somatic gonad that will house them develops. Early studies utilizing alkaline phosphatase activity suggested the migratory path of the PGCs from E8.0 until their arrival in the somatic gonad between E10.5 and E11.0. More recently, live-imaging studies using a GFP transgene driven by a modified *Oct4* promoter (Boiani et al., 2004)

have enabled a more detailed picture of the process and timing of PGC migration to emerge (Anderson et al., 2000; Molyneaux et al., 2001).

These live imaging studies have suggested that germ cell migration in the mouse can be divided into six discrete stages (Anderson et al., 2000; Molyneaux and Wylie, 2004; Molyneaux et al., 2001). Firstly germ cells migrate from their site of specification through the primitive streak and into the definitive endoderm, parietal endoderm and allantois (Anderson et al., 2000). Via an unknown mechanism, those germ cells that are present in the definitive endoderm are integrated into the hindgut pocket. Whether this is an active process or a passive by-product of embryonic morphogenesis remains unknown. Within the hindgut at E9.0 PGCs undergo a burst of motility that appears random with respect to direction (Molyneaux et al., 2001). About 12 hours later PGCs exit from the dorsal side of the hindgut and begin to migrate towards the urogenital ridges (the somatic gonadal anlagen). The PGCs then split into two streams of cells and migrate into the left and right genital ridges.

Studies of germ cell migration have been greatly aided and hindered by the need to perform experiments in modified PGC culture systems. Early studies of migration revealed that PGCs in culture are driven to migrate towards the developing E10.5 urogenital ridge (Godin et al., 1990). Continuation of these studies suggested that TGFB1 (transforming growth factor, beta 1) was produced by the urogenital ridge and possessed PGC chemo-attractant properties (Godin and Wylie, 1991). However, when embryos deficient for the TGFB1 receptor *Tgfbr1* (transforming growth factor, beta receptor I, also known as *Alk5*) were examined, it was found that PGC migration and

number was unaffected, suggesting that *in vivo* TGFB1 is not a crucial migratory cue for PGCs (Chuva de Sousa Lopes et al., 2005).

Identification of factors involved in germ cell migration is made particularly difficult by the fact that mis-migrating germ cells undergo apoptosis fairly shortly after mis-migration (Runyan et al., 2006). Thus most mutations that effect PGCs during their migration have been shown to affect germ cell survival and have not been shown to directly regulate migration. A list of genes affecting germ cells during their migration and their mutant phenotypes can be found in Table 1.

Table 1: Genes affecting PGCs during their migration to the somatic gonad.

Gene	Mutant Phenotype	References
Kit oncogene (*Kit*, also known as *W*)	Increased PGC apoptosis (*Bax*-dependent). Decreased PGC motility. Retention of PGCs in hindgut	Besmer et al. 1991. Godin et al. 1991. Runyan et al. 2006. Farini et al. 2007
Kit ligand (*Kitl*, also known as *Steel*)	Increased PGC apoptosis	Besmer et al. 1991
Nanos homolog 3 (*Drosophila*) (*Nanos3*)	Increased PGC apoptosis (both *Bax*-dependent and -independent)	Tsuda et al. 2003. Suzuki et al. 2008
Dead end 1 (*Dnd1*, also known as *ter*)	Increased PGC apoptosis. Teratoma formation on 129/Sv background	Noguchi et al. 1985. Youngren et al. 2005
Octamer binding protein 4 (*Oct4*, germ cell conditional mutation)	Increased PGC apoptosis	Kehler et al. 2004
Tia1 cytotoxic granule-associated RNA binding protein-like 1 (*Tial1*, also known as *Tiar*)	Increased PGC apoptosis	Beck et al. 1998
Chemokine (C-X-C motif) receptor 4 (*Cxcr4*)	Increased PGC apoptosis. Decreased PGC motilty in hindgut	Molyneaux et al. 2003

Of all factors required in PGCs during their migration, *Kit*, has the longest and perhaps most complex history. Mutations of the dominant white spotting locus (*W*, which was later found to encode the *Kit* receptor (Chabot et al., 1988)) were easily discernable because of their effect on coat color (Mintz and Russell, 1957). To date over 120 alleles of *Kit* have been isolated (MGI, 2009). The difficulty in analyzing the role of *Kit* in the migration of germ cells lies in the fact that *Kit* is also required for germ cell survival and proliferation during their migratory phase (Besmer et al., 1993; Godin et al., 1991). However a role for *Kit* as a direct migratory cue has been suggested by several studies. First, in mice homozygous for the W^e allele of *Kit*, PGCs are retained in the hindgut at E10.5, suggesting a role for this factor in migration of PGCs from the hindgut

into the urogenital ridge (Buehr et al., 1993). Consistent with this finding Runyan et al. showed that when *Kitl*-deficient PGCs are prevented from undergoing apoptosis by elimination of the pro-apoptotic *Bax* gene, they fail to migrate efficiently into the urogenital ridge (Runyan et al., 2006). Finally, Farini et al. using a novel feeder-free PGC culture system (Farini et al., 2005), established that PGCs are attracted to both urogenital ridges from E11.5 embryos and to purified recombinant KITL (Farini et al., 2007). These results suggest that in addition to providing pro-survival and proliferation cues to PGCs, activation of the KIT receptor by KITL provides directional cues to migrating PGCs. The exact nature and organization of the KITL signal in the PGC migratory path has not been determined with enough precision to fully understand how this process proceeds in greater detail.

While PGCs are migrating, they proliferate and undergo significant epigenetic modification. The early changes in PGC chromatin are thought to re-establish an epigenetic landscape more similar to that of an early embryo than the partially differentiated epiblast from which PGCs are specified (Hajkova et al., 2008; Seki et al., 2005; Seki et al., 2007; Surani et al., 2008). While there appears to be an orderly transition from one set of chromatin marks to another, no function for these changes has been shown.

One interesting epigenetic change in migrating PGCs involves the X chromosome. It has been known for more than 50 years that both X chromosomes are active in the female germline (Epstein, 1969). Early studies suggested that the reactivation of a previously inactive X chromosome occurred shortly before meiosis in ovarian germ cells (Monk and McLaren, 1981; Tam et al., 1994). Monk and McLaren

examined the enzymatic activity of X-linked HPRT (hypoxanthine phospho-ribosyl transferase) relative to autosomally encoded APRT (adenine phosphoribosyl transferase) and found a increase in the relative levels of HPRT at between E12 and E13 in XX but not XY or XO germ cells, suggesting that X reactivation occurs in germ cells starting around E13 (Monk and McLaren, 1981). Tam et al. utilized an X-linked beta-galactosidase transgene to examine X chromosome activity and found that expression was induced in all female germ cells by E13.5. Later results using XXY mice indicated that X chromosome re-activation occurs independently of the sex of the gonad in which PGCs find themselves (Mroz et al., 1999). Most recently, Chuva de Sousa Lopes et al., using an X-linked GFP transgene showed signals from XX but not XY urogenital ridges were required for X chromosome re-activation and that this reactivation occurred between E11.5 and E13.5 (Chuva de Sousa Lopes et al., 2008). These results were all limited by analysis of single X-linked loci as proxies for the behavior of the entire X chromosome.

To further study the regulation of X re-activation in germ cells, both Sugimoto and Abe and de Napoles et al. examined the expression of canonical markers of X inactivation, such as *Xist* (inactive X specific transcripts (Brockdorff et al., 1992)) and H3K27me3 (trimethylation of histone 3 on lysine 27 (Silva et al., 2003)) (de Napoles et al., 2007; Sugimoto and Abe, 2007). These groups found that as early as E7.75 these markers of X inactivation were missing from some PGCs. The number of PGCs lacking these markers increased with time such that by E9.5 70-90% of PGCs did not possess *Xist* RNA within their nuclei (de Napoles et al., 2007; Sugimoto and Abe, 2007). To further examine this process in detail Sugimoto and Abe examined loci across the entire X

chromosome and found that the timing of X re-activation was much more complex than previously thought (Sugimoto and Abe, 2007). Some X-linked loci were biallelically expressed even at E7.75 and many more became biallelically expressed by E10.5. Interestingly, even at E14.5 (the last time point examined) not all loci exhibited biallelic expression, suggesting that X re-activation is not complete even after germ cells initiate meiosis. These results highlight the difficulties in studying X chromosome re-activation and suggest the need to examine large number of loci across the entire chromosome in order to truly understand this process.

6. Gonadal homecoming

Around the time of their arrival in the developing somatic gonad, PGCs undergo several major changes: their nuclear diameter increases, they become connected into cysts via intracellular bridges, they display a significant loss of DNA methylation and they activate a program of gene expression specific to the post-migratory germline. The last two of these changes are linked, with DNA de-methylation likely a pre-requisite for the expression of post-migratory germ cell-specific genes.

The earliest difference between migratory PGCs and post-migratory germ cells to be discovered was an increase in the diameter of the nucleus of post-migratory germ cells relative to that of migratory PGCs. This difference is readily detectable by routine histological staining. Both the cause and consequence of this change is unknown.

Following their arrival in the embryonic gonad, germ cells are localized in clusters, referred to as cysts, throughout the gonad. These cysts are composed of synchronously dividing germ cells (Pepling and Spradling, 1998). These embryonic

germ cell cysts are connected by intracellular bridges containing the germ cell specific protein TEX14 (testis expressed 14) (Greenbaum et al., 2009). What if any role cyst formation plays in embryonic germ cell development has not been determined. However, germ cells deficient for TEX14 do not form intracellular bridges yet are capable of undergoing normal embryonic differentiation, suggesting that cyst formation is not essential for mouse germ cell development (Greenbaum et al., 2009).

While the specific timing of germline DNA de-methylation has not been determined, it is likely to occur around the time of germ cell arrival in the gonad. Studies assaying DNA de-methylation have yielded varying results, likely due to different experimental methodologies. While these studies are somewhat contradictory, certain aspects of the regulation of germline DNA methylation are generally agreed upon. Early in their development, primordial germ cells possess a DNA methylation pattern similar to that of somatic cells (Hajkova et al., 2002; Lee et al., 2002). Around the time of their arrival in the gonad, between E10.5 and E11.5, embryonic germ cell DNA methylation begins to decrease (Hajkova et al., 2002; Lee et al., 2002; Szabo and Mann, 1995; Walsh et al., 1998). Loss of DNA methylation occurs independently of the sex of the embryo (Hajkova et al., 2002; Lee et al., 2002; Szabo and Mann, 1995). Regulation of the timing of DNA de-methylation varies with particular classes of sequences and even particular loci of the same sequence class (Hajkova et al., 2002; Lee et al., 2002; Walsh et al., 1998). Finally, some level of variability exists within the population of germ cells with respect to the rate their of DNA de-methylation (Lee et al., 2002). Studies of DNA methylation in embryonic germ cells require the tissue microenvironment within which the germ cells reside to be disrupted. This disruption means that it is not currently

feasible to determine whether DNA de-methylation status correlates with the location of germ cells within the embryo.

In addition to the outstanding questions about the timing of germline genome de-methylation, very little is also known about the mechanism by which this occurs. Whether germline DNA de-methylation occurs passively by prevention of the activity of *Dnmt1* (DNA methyltransferase 1), the enzyme required for maintenance of DNA methylation during normal cell cycle progression (Hirasawa et al., 2008; Li et al., 1992), or is an active process is not known. However, the relative rapidity with which this process occurs suggests that an active mechanism may be involved (Hajkova et al., 2008). The absence of an enzyme encoded within the mammalian genome known to possess the capacity to catalyze the energetically unfavorable conversion of 5-methylcytosine to cytosine makes the study of this process particularly challenging. Recent evidence in plants shows that DNA de-methylation can be accomplished by utilizing DNA repair enzymes (Choi et al., 2002; Gong et al., 2002; Zhu et al., 2007). It remains to be seen whether DNA de-methylation in mammalian cells could utilize a similar mechanism.

Post-migratory germ cells are known to specifically express four genes. These include two germ cell specific RNA binding proteins: *Mvh* (mouse vasa homolog; MGI: *Ddx4)* (Fujiwara et al., 1994; Toyooka et al., 2000) and *Dazl* (deleted in azoospermia-like) (Seligman and Page, 1998), a component of the axial element of the synaptonemal complex: *Sycp3* (synaptonemal complex protein 3) (Di Carlo et al., 2000), and an antigen of unknown identity: GCNA1 (germ cell nuclear antigen 1) (Enders and May, 1994). It has been suggested that the continuing differentiation program of post-migratory germ

cells is coordinately regulated by the removal of DNA methylation (Maatouk and Resnick, 2003). For the three of these markers whose genomic loci are known, a region 5′ to their transcription start site containing DNA methylation has been identified (Maatouk et al., 2006). These regions display high levels of DNA methylation in migrating PGCs and somatic tissues, but significantly lower levels of DNA methylation in post-migratory germ cells. Functional evidence for DNA methylation playing a role in the control of these genes has been obtained by examining embryos with decreased *Dnmt1* function (Maatouk et al., 2006). These embryos display aberrant somatic expression of the normally germ cell specific GCNA1. In addition, migratory PGCs in these mutant embryos express *Mvh*, *Dazl*, and *Sycp3*. The long-term consequences of this aberrant gene expression have yet to be studied, as *Dnmt1* depleted embryos die shortly after germ cells arrive in the gonad (Li et al., 1992). Taken together these data suggest that the gene expression landscape of post-migratory germ cells is regulated by the loss of DNA methylation. In order to further characterize the transition of migratory PGCs to post-migratory germ cells, studies of the specific factors expressed in post-migratory germ cells have been carried out.

Mouse vasa homolog (*Mvh*)

The DEAD-box RNA helicase *Mvh* is the mouse homolog of the highly conserved germ cell gene *vasa* (Fujiwara et al., 1994). In a wide variety of metazoans *vasa* family members are required for germ cell development (Komiya et al., 1994; Noce et al., 2001; Schupbach and Wieschaus, 1986; Tsunekawa et al., 2000; Yoon et al., 1997). It is thus somewhat surprising that the mouse homolog of this family is only expressed in germ

cells following their arrival in the gonad (Fujiwara et al., 1994). Interestingly, expression of *Mvh* is not observed in cultured germ cells, unless those cells are cultured in the presence of gonadal somatic cells (Toyooka et al., 2000). However, presence or absence of *Mvh* expression *in vivo* does not appear to strictly correlate with distance from the urogenital ridge (Noce et al., 2001). Thus it is not clear whether *Mvh* expression is driven by germ cell-intrinsic or extrinsic environmental factors. It has not been determined whether the induction of *Mvh* in the culture system developed by Toyooka et al. is accompanied by de-methylation of the region 5′ of the *Mvh* transcriptional start site.

To understand the functional significance of *Mvh* expression in the mouse germline, *Mvh*-deficient mice were created (Tanaka et al., 2000). In these mice, the only defects detected thus far occur in male germ cells; female mice deficient for *Mvh* display normal fertility and fecundity (Tanaka et al., 2000). *Mvh*-deficient male germ cells exhibit significantly reduced proliferation after their arrival in the gonad at E11.5 and later undergo apoptosis in the zygotene stage of meiotic prophase I (Tanaka et al., 2000). The molecular mechanism behind both of these phenotypes remains unknown.

Deleted in azoospermia-like (*Dazl*)

Dazl encodes an RNA binding protein with an RRM type RNA binding domain and a DAZ domain, a domain of unknown function, which is characteristic of the DAZ family of proteins to which *Dazl* belongs. DAZ family proteins are germ cell specific RNA binding proteins that have been identified in all examined metazoans. In mice two members of this family are known, *Dazl* and *Boll* (boule-like; named for the *Drosophila* DAZ family member *boule* (Eberhart et al., 1996)). While *Dazl* is expressed in germ

cells beginning at the time of their arrival in the gonad (Seligman and Page, 1998), *Boll* is expressed only in postnatal male meiotic germ cells (Xu et al., 2001). The regulation of *Dazl*'s transcription and translation in the embryonic germ lineage has not been well studied. It is currently unknown whether the induction of *Dazl* expression is intrinsically timed or induced by the somatic environment.

All studies of *Dazl* function to date have utilized a single mutant allele (Ruggiu et al., 1997). All reports examining these *Dazl*-deficient mice have revealed an infertility phenotype (Haston et al., 2009; Lin and Page, 2005; McNeilly et al., 2000; Ruggiu et al., 1997; Saunders et al., 2003; Schrans-Stassen et al., 2001). However, mice deficient for *Dazl* exhibit a wide range of germ cell defects. Defects range from late embryonic germ cell apoptosis (Lin and Page, 2005; Ruggiu et al., 1997), to defects in postnatal male germ cell differentiation (Schrans-Stassen et al., 2001) and meiotic prophase of both sexes (Saunders et al., 2003). A major source of the variability observed between these phenotypes is thought to be due to genetic modifiers introduced by varying non-inbred strain backgrounds. *Dazl*-deficiency combined with an inbred genetic background appears to give a clear and completely penetrant embryonic germ cell defect (Lin and Page, 2005).

Germ cell nuclear antigen 1 (GCNA1)

An antibody against Germ Cell Nuclear Antigen 1 (GCNA1) was created by injecting mouse pachytene spermatocytes into rats and purifying antibodies displaying reactivity with mouse germ cells (Enders and May, 1994). GCNA1 is expressed in germ cells of both sexes beginning at E11.5 (Enders and May, 1994). Because the genomic

locus that encodes GCNA1 is still unknown, studies of its regulation and function are extremely limited. In one of the few studies to examine GCNA1 regulation, expression of GCNA1 was examined in mice deficient for the orphan nuclear receptor *Sf-1* (steroidogenic factor 1, MGI: *Nr5a1*)(Wang et al., 1997), whose expression is required for production of the somatic gonad (Luo et al., 1994). Wang et al. found that germ cells in *Sf-1*-deficient embryos express GCNA1 at the appropriate time despite the failure of somatic gonad development in these embryos (Wang et al., 1997). In addition, primary cultures of migratory PGCs exhibit GCNA1 expression despite lacking contact with gonadal somatic cells (Wang et al., 1997). These results suggest that the gonadal soma may not be required for the expression profile of post-migratory germ cells.

Synaptonemal complex protein 3 (*Sycp3*)

Sycp3 encodes a protein that is a component of the axial element of the synaptonemal complex (Dobson et al., 1994; Lammers et al., 1994). *Sycp3* is necessary for normal homologous chromosome synapsis in both male and female meiosis (Yuan et al., 2002; Yuan et al., 2000). However, *Sycp3* transcript and protein encoded can be detected in post-migratory embryonic germ cells of both sexes (Di Carlo et al., 2000). In embryonic male germ cells, this protein then disappears until the postnatal period (Di Carlo et al., 2000). Studies of gene expression in pre-meiotic male germ cells have identified expression of *Sycp3* in spermatogonia as well (Wang et al., 2001). The expression of *Sycp3* in embryonic male germ cells is particularly striking, as male germ cells do not initiate meiotic prophase until a week after birth. Why male embryonic germ cells produce this protein remains unclear. However, it has been suggested that *Sycp3*

may have a function beyond its role in the synaptonemal complex. This suggestion comes from observations that elimination of *Sycp3* in an *E2f1*-deficient background causes a severe pre-meiotic germ cell defect, which is greater than that observed in the testes of either single mutant (Hoja et al., 2004). Whether a similar pre-meiotic role for *Sycp3* exists in embryonic germ cells is unknown.

In all, the process of gonadal homecoming has not been studied in as great a depth as other processes in mammalian germ cell development. Several major questions remain surrounding this developmental transition. Firstly, what is the precise timing of genome-wide DNA de-methylation and is this correlated with germ cell age (i.e. number of divisions following specification) or location in the embryo? Secondly, how is genome-wide DNA de-methylation accomplished? Thirdly, does proximity to specific signals induce expression of post-migratory germ cell genes or does this expression program activate independently of cellular environment? Finally, is this developmental transition an important event in the cycle of the germline, and what are the consequences of failing in this transition?

7. Germ cell sex determination

Prior to their arrival in the developing somatic gonad germ cells do not exhibit sexually dimorphic characteristics. Even following their arrival in the gonad the early changes observed in germ cells (specifically DNA de-methylation and gene expression changes) also appear similar in both sexes. The first morphological difference observed between male and female germ cells is the presence at E13 of meiotic figures in female but not male germ cells (McLaren, 1983).

Meiotic figures are easily detectable by routine hematoxylin staining. The ease of this detection has led the field to use meiotic figures as the defining feature of female embryonic germ cells. Operating under a simple binary model, the field adopted the convention of defining embryonic germ cells without meiotic figures as male. This has led the field to essentially define the question of germ cell sex determination as the question of whether embryonic germ cells initiate meiosis or not.

In order to address the timing at which germ cells commit to a meiotic or non-meiotic fate, Adams and McLaren transplanted XX and XY germ cells into E12.5 developing gonads (Adams and McLaren, 2002). Germ cells from XX embryos E12.5 or younger do not produce meiotic figures if placed in a developing testis. On the other hand germ cells from XY embryos E11.5 or younger produce meiotic figures if placed within a developing ovary. These results suggest that between E11.5 and E12.5 the commitment to either a meiotic or non-meiotic fate is determined. More recently, a more comprehensive study was performed using germ cells isolated from E13.5 embryos (Iwahashi et al., 2007). This study cultured E13.5 germ cells with or without gonadal somatic cells for six days and showed that XX germ cells progressed into meiotic prophase, while XY germ cells did not even in the absence of further somatic gonadal cues. In addition this study also examined other characteristics of male and female germ cells (specifically establishment of imprinting and changes in the cell cycle) and found that germ cells do not require interaction with somatic cells after E13.5 to maintain their sexual identity (Iwahashi et al., 2007).

Studies of meiotic initiation in the mammalian germline have focused on two predominant models (Kocer et al., 2009). One of these models proposes that germ cells

autonomously initiate meiosis unless prevented from doing so by a substance present in the embryonic testis, but not the embryonic ovary, known as the meiosis preventing substance (MPS). The other predominant model suggests that germ cells must be induced to enter meiosis by the presence of a meiosis inducing substance (MIS) that is present in the embryonic ovary and absent from the embryonic testis.

Over a period of three decades data were presented suggesting that either one or the other of these models was correct. The earliest study of this process was performed by Byskov and showed that signals from the rete ovary (also known as the mesonephros) were capable of causing germ cells in embryonic testes to initiate meiosis (Byskov, 1974). McLaren and Southee suggested that if a MIS existed it must not be limited in its location to the embryonic ovary, but must instead be present in several different embryonic locations including the embryonic lung and adrenal (McLaren and Southee, 1997). They also showed that disruption of the structure of the embryonic testes was sufficient to cause male germ cells to initiate meiosis.

In 2006 work from Koubova et al., Bowles et al., and Baltus et al. revealed molecular identities to the previously theoretical substances proposed over the prior thirty years (Baltus et al., 2006; Bowles et al., 2006; Koubova et al., 2006). *Stra8* (stimulated by retinoic acid gene 8), was discovered to be required for early steps in embryonic female meiosis (Baltus et al., 2006). This gene was in turn discovered to require active retinoic acid signaling in order to be expressed in embryonic germ cells (Bowles et al., 2006; Koubova et al., 2006). Thus retinoic acid was characterized as an MIS. This discovery was consistent with the previous results of McLaren and Southee, which stated that the

MIS must be present broadly in the embryo, which retinoic acid is (McLaren and Southee, 1997).

In addition to identifying the MIS, the studies of Koubova et al. and Bowles et al. revealed how the sex specificity of gonadal retinoic acid signaling is established (Bowles et al., 2006; Koubova et al., 2006). Male but not female gonadal somatic cells express *Cyp26b1*, a member of the cytochrome p450 class of enzymes, whose molecular function is to alter retinoic acid and mitigate its signaling functions. Thus, *Cyp26b1* functions as the hypothesized MPS. These results suggested that both an MIS and an MPS existed to regulate the timing of meiotic initiation in embryonic germ cells.

It remains unclear whether meiotic initiation can be used as a proxy for the sex of embryonic germ cells. While the timing of meiotic initiation is different between male and female germ cells, the process of meiosis itself is not sex-specific. Many processes in germ cells that are sex-specific exist and the connection of these processes to meiosis has not been examined.

8. Spermatogenesis

Male germ cell sex determination is thought to occur by E12.5 (Adams and McLaren, 2002). However, the earliest male specific germ cell mutant phenotype (that of *Mvh*-deficient mice) occurs even earlier, at E11.5. Male *Mvh*-deficient mice fail to undergo normal proliferation after their arrival in the gonad (Tanaka et al., 2000). Whether the sex specificity of this phenotype is caused by somatic gonadal cues or by the sex chromosome constitution of the germ cells has not been determined.

Gene expression screens for male specific expression in embryonic gonads have discovered significant numbers of sex-specific somatic cell markers, but surprisingly few early germ cell markers (Bowles et al., 2000; Menke and Page, 2002). Among the earliest known genes expressed in male but not female germ cells is *Nanos2* (nanos homolog 2 (*Drosophila)*), which is detectable in male germ cells by E13.5 (Tsuda et al., 2003). *Nanos2* function is required in male germ cells to prevent meiosis after E15.5 (Suzuki and Saga, 2008). It is thought that this function in prevention of meiosis occurs as a back-up to the earlier role of *Cyp26b1* in preventing the activity of RA in activate *Stra8* expression (Saga, 2008).

Male embryonic germ cells, sometimes referred to as pro-spermatogonia, are characterized by entry into a period of cell cycle arrest. This process begins in some germ cells as early as E12.5 and has occurred in most germ cells by E14.5 (Western et al., 2008). This arrest continues until a few days after birth when male germ cells, now referred to as spermatogonia, resume mitotic divisions (de Rooij, 1998).

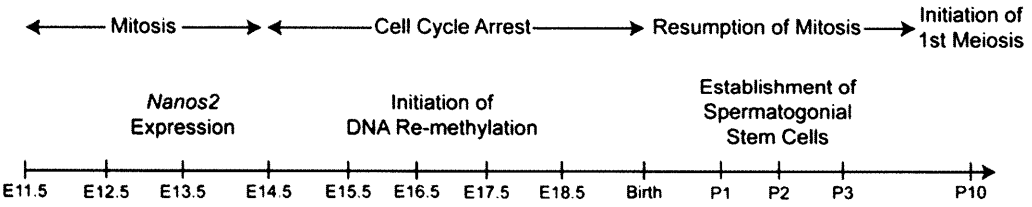

Figure 4: Events in early spermatogenesis. Timeline of events occurring in male germ cell development from the time of germ cell sex determination (at E11.5-E12.5) through the initiation of the first male meiosis (at P10).

Pro-spermatogonia also initiate the process of global DNA re-methylation. As such, pro-spermatogonia express enzymes of the DNMT3 (DNA methyltransferase 3) family, which possess *de novo* DNA methylation functions (Lees-Murdock et al., 2005; Okano et al., 1999). Interspersed repeat DNA, such as LINEs and IAPS, are partially de-

methylated in the germline of both sexes between E11.5 and E12.5, and are fully re-methylated in pro-spermatogonia beginning at E15.5 (Hajkova et al., 2002; Lees-Murdock et al., 2003). In addition paternally imprinted loci such as *H19, Rasgrf1* and *Gtl2l/Dlk* are biallelically methylated beginning by E14.5 (Davis et al., 2000; Li et al., 2004; Ueda et al., 2000). It has been proposed that the pro-spermatogonial cell cycle arrest is required for the initiation of male specific DNA re-methylation (Schaefer et al., 2007), however, this hypothesis has not been experimentally tested.

The population of spermatogonia existing after birth is composed of at least two different sub-populations. One population of spermatogonia will progress immediately into differentiation, while the other will form a pool of long-term spermatogonial stem cells (SSCs). Confirmation of the existence of these sub-populations was obtained by tracing the spermatogonial lineage expressing *Ngn3* (neurogenin 3) (Yoshida et al., 2006). It was found that sperm produced in the first round of spermatogenesis following birth do not pass through an *Ngn3*-expressing stage, while sperm produced in all other rounds of spermatogenesis do (Yoshida et al., 2006).

While it remains unclear how the SSC population is initially established, several studies have identified factors that are required for SSC self-renewal and differentiation. Within SSCs themselves two factors, *Plzf* (promyelocytic leukemia zinc finger protein, also known as *luxoid; Zfp145,* zinc finger protein 145; MGI: *Zbtb16*, zinc finger and BTB domain containing 16) (Buaas et al., 2004; Costoya et al., 2004) and *Taf4b* (TATA box-binding protein (TBP)-associated factor 4b) (Falender et al., 2005), have been shown to be required for SSC self-renewal.

In addition to SSC-intrinsic factors, two factors produced in testicular somatic cells have also been shown to be required for SSC self-renewal. While mice homozygous for mutations in *Gdnf* (glial cell line derived neurotrophic factor) die within a day after birth, heterozygotes display an eventual loss of spermatogenesis because of a loss of SSCs (Meng et al., 2000). In addition to showing that loss of *Gdnf* function caused eventual loss of SSCs, Meng et al. also showed that when *Gdnf* is over-expressed in the testis, SSCs accumulate. *Gdnf* functions by binding to the SSC expressed receptor *Gfra1* (glial cell line derived neurotrophic factor family receptor alpha 1) and activating the *Ret* signaling pathway (Naughton et al., 2006). The relevant down-stream targets of this pathway in SSCs remain unkown. The extrinsic factor that supports SSC self-renewal is *Erm* (Ets related molecule, MGI: *Etv5* (ets varient gene 5), which is produced by the supporting cells of the testis, the Sertoli cells (Chen et al., 2005). *Erm* is a transcription factor and the mechanism by which it regulates SSC differentiation or self-renewal remains unknown.

Once an SSC population has been established, spermatogenesis proceeds in an orderly and strictly timed manner. Spermatogenic cells form chains connected via intercellular bridges, containing the previously mentioned TEX14 protein (Greenbaum et al., 2006). Stem cell function (as assayed by transplantation assays) can be found within chains of cells that are composed of less than 32 cells (de Rooij and Russell, 2000). These cells, known collectively as the undifferentiated spermatogonia can be further sub-divided, based on chain length, into A_{single} (A_s, single unconnected cells), A_{paired} (A_{pr}, two connected cells) and $A_{aligned}$ (A_{al}, four to thirty-two connected cells). It has proven difficult to separate these cells from each other to determine which sub-population(s)

possesses SSC activity. It has been hypothesized that A_s represent functional stem cells *in vivo* and that A_{pr} and A_{al} possess stem cell activity only when placed into an artificially emptied niche, as is required for SSC transplantation assays (Yoshida et al., 2007). Without clear molecular markers to separate these cell types and lineage tracing experiments to track their *in vivo* development, it will not be possible to resolve this question.

In the adult mouse, SSCs initiate differentiation every 8.6 days (Oakberg, 1956). This commitment to differentiation begins with the transition of A_{al} cells into the first of six "differentiated" spermatogonial types, the A_1 cells. The A_{al} to A_1 transition represents a major regulatory point in spermatogenesis. Unlike many other transitions in spermatogenesis this transition is not accompanied by a mitotic cell division (de Rooij and Russell, 2000). This transition is sensitive to dietary vitamin A levels (van Pelt et al., 1995), temperature (de Rooij et al., 1999; Nishimune and Haneji, 1981) and perturbation of several genetic factors, including *Dazl* (Schrans-Stassen et al., 2001), *jsd* (de Rooij et al., 1999) and *Kitl* (de Rooij et al., 1999).

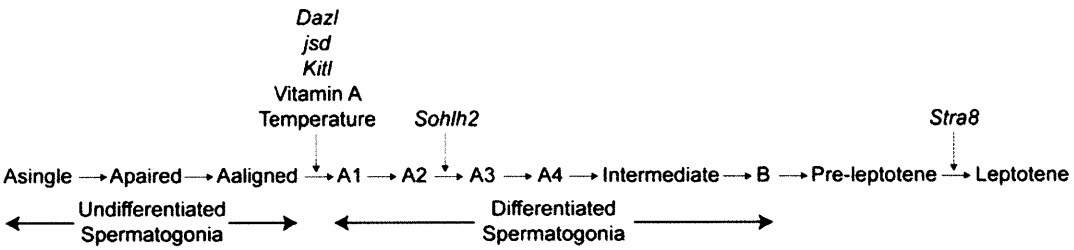

Figure 5: Pre-meiotic differentiation in adult spermatogenesis. Spermatogenic cell types of the adult testis from SSCs through entry into meiotic prophase. Factors listed above arrows regulate these transitions. Names listed below cell types refer to common groupings of types of spermatogonia.

Following the transition from A_{al} to A_1, A_1 cells undergo a series of five mitoses (de Rooij and Russell, 2000). The first three of these divisions produce A_2, A_3 and A_4

spermatogonia. These cells can only be distinguished from A_1 spermatogonia by the number of cells to which they are connected. A_4 spermatogonia divide to form Intermediate spermatogonia. These cells have a distinctive chromatin morphology, which can be determined by hematoxylin staining. Intermediate spermatogonia then divide to form Type B spermatogonia, which can also be distinguished by their chromatin morphology. Distinct molecular markers for the various sub-types of "differentiated" spermatogonia have not been discovered.

A recent series of genetic studies have suggested differences between sub-types of differentiated type A spermatogonia. Mice mutant for the helix-loop-helix transcription factor *Sohlh2* (spermatogenesis and oogenesis specific basic helix-loop-helix 2), show defects in spermatogenesis resulting in accumulation of M-phase cells described as resembling A_2 spermatogonia (Hao et al., 2008). This represents the first mutant phenotype suggested to affect the development of differentiated spermatogonia after the A_{al} to A_1 transition. A more recent study examining *Sohlh2*-deficient testes suggested a role for *Sohlh2* in regulating the transition of *Kit*-negative spermatogonia into *Kit*-positive spermatogonia (Toyoda et al., 2009). Given that this transition in gene expression occurs at the A_{al} to A_1 transition (Schrans-Stassen et al., 1999; Yoshinaga et al., 1991) and the difficulty in differentiating A_1 from A_2 spermatogonia (particularly in testes lacking more differentiated cell types), it is possible that the true defect in *Sohlh2*-deficient testes occurs at the A_{al} to A_1 transition. Further studies will be necessary to examine this possibility.

Type B spermatogonia divide to give rise to cells known as pre-leptotene spermatocytes. These cells will undergo pre-meiotic DNA replication and enter into the

leptotene stage of meiotic prophase. *Stra8,* a gene required for pre-meiotic DNA replication in embryonic ovarian germ cells (Baltus et al., 2006), has been shown to be required for the production of leptotene spermatocytes in juvenile mouse testes (Anderson et al., 2008). Interestingly, in the testis *Stra8*-deficient pre-leptotene germ cells undergo DNA replication (Anderson et al., 2008), while in the embryonic ovary *Stra8*-deficient germ cells appear to be arrested prior to DNA replication (Baltus et al., 2006). Thus while common factors regulate the earliest steps of meiosis in both male and female germ cells, there may be some mechanistic differences between the sexes.

Pre-leptotene spermatocytes progress into meiotic prophase proceeding through the characteristic stages of leptotene, zygotene, pachytene and diplotene without arrest. It is thought that during DNA replication in pre-leptotene spermatocytes, the cohesin complexes linking sister chromatids contain the meiosis-specific cohesin subunits *Stag3* (stromal antigen 3), *Rec8* (REC8 homolog (yeast)) and *Smc1b* (structural maintenance of chromosomes 1B) suggesting that these cells are prepared for meiotic prophase. (Revenkova and Jessberger, 2006). Interestingly, unlike in *S. cerevisiae* (Klein et al., 1999) or *C. elegans* (Pasierbek et al., 2001), mouse spermatocytes do not require meiosis-specific cohesins to initiate production of the synaptonemal complex (SC) (Bannister et al., 2004; Revenkova et al., 2004; Xu et al., 2005).

During leptotene, spermatocytes form the DNA double strand breaks (DSBs) necessary for meiotic recombination and begin loading components of the axial element of the SC (SYCP2 and SYCP3) onto their chromosomes (Eijpe et al., 2003). During early zygotene, homologous chromosomes pair and synapsis begins (Scherthan et al., 1996). At this point spermatocytes load the central element SC component SYCP1 (Eijpe

et al., 2003). Interestingly, the X and Y chromosomes contain SYCP3 along their lengths, but fail to load SYCP1 (Costa and Cooke, 2007).

Following zygotene spermatocytes progress into pachytene. In the mouse the pachytene stage of meiotic prophase is particularly long, lasting for over seven days (Oakberg, 1956). Beginning in pachytene transcription from the X and Y chromosomes is silenced via a mechanism known as meiotic sex chromosome inactivation (MSCI) (Turner et al., 2005). MSCI is thought to be a more specific example of the general phenomenon known as meiotic silencing of unpaired chromatin (MSUC) (Turner et al., 2005). During MSCI, the X and Y chromosomes are localized in a distinct nuclear partition known as the XY or sex body (McKee and Handel, 1993; Solari, 1974). In addition to its localization on the nuclear periphery, the XY body also possesses unique chromatin characteristics. In particular the phosphorylated histone variant γ-H2AX (phospho-Ser139 on histone 2A family, member X), localizes across the entirety of the XY body in pachytene cells (Mahadevaiah et al., 2001; Turner et al., 2005). MSCI persists through the diplotene stage of meiotic prophase and through the second meiotic division. Following meiosis X and Y chromosome genes exist in globally repressed state known as post-meiotic sex chromatin (PMSC) (Namekawa et al., 2006). It has been found that the repression found in PMSC is not complete and that in particular X-linked genes present in multiple copies are expressed in post-meiotic germ cells (Mueller et al., 2008).

In mouse the progression from meiotic metaphase I through the second meiotic division occurs relatively rapidly (i.e. in 10.4 hours (Oakberg, 1956)). The products of this division are four haploid germ cells known as spermatids. These cells must undergo

a series of characteristic changes known as spermiogenesis as they transition from their initial round appearance into an elongated form. This process takes 2 weeks to complete (Oakberg, 1956).

Spermiogenesis involves several male germ cell specific differentiation steps. One of the first steps in spermiogenesis is the initiation of acrosome formation (Russell, 1990). As a result of this process, the nucleus migrates to the surface of the cell, which establishes cellular polarity, separating the region which develop as the sperm head from that which will develop as the tail (Russell et al., 1983). Also during spermiogenesis histones in the spermatid chromatin are replaced first by transition proteins and then by protamines, resulting in a more compacted nucleus (Balhorn, 1982; Brewer et al., 2002). Nuclear volume likely also decreases because of elimination of nucleoplasm (Sprando and Russell, 1987). Perhaps as a consequence of this nuclear re-organization, transcription in spermatids is generally, but not universally, decreased (Steger, 2001).

Following the establishment of cellular polarity, spermatids begin the process of producing a flagellum from their centrioles, which have also migrated to the cellular surface (Russell, 1990). While the production of the flagellum is complete while the germ cell resides within the testis, it does not function within the testis and these cell are still non-motile (Russell, 1990). Spermiogenesis also requires the elimination of much of the spermatid's cytoplasmic volume. This results in at least 25% decrease in cellular volume from spermatid versus mature spermatozoa (Sprando and Russell, 1987), and in the formation of residual bodies within the testis (Fawcett, 1969).

Following their maturation in the testis, spermatozoa must be released from the testis and travel through the efferent ducts (also known as the rete testis), a process

known as spermiation. These cells still lack motility until their arrival in the epididymis. Once in the epididymis, the acrosome of these cells is coated with glycoproteins preventing the activation of the acrosome reaction. Interaction of spermatozoa with the female reproductive tract results in the process of capacitation, wherein this glycoprotein coat is eliminated and spermatozoa become hypermotile (Abou-haila and Tulsiani, 2009). These cells are now ready to interact with the zona pellucida of the oocyte and initiate fertilization.

9. Oogenesis:

The earliest gene known to be expressed in female but not male embryonic germ cells is *Stra8* (Menke et al., 2003). *Stra8* mRNA is detectable in the anterior most germ cells of the embryonic ovary at E12.5 and expression of *Stra8* sweeps through the ovary reaching the posterior most germ cells by E16.5. Interestingly, *Stra8* mRNA does not persist in ovarian germ cells, but instead is also down-regulated in an anterior to posterior wave, becoming undetectable after E16.5 (Menke et al., 2003).

As has previously been mentioned, *Stra8* plays a crucial role in the initiation of meiosis in embryonic ovarian germ cells (Baltus et al., 2006). This has some interesting implications for the overall system of female germ cell development. Many models for germ cell development in the embryonic ovary discuss an oogonial (mitotically dividing female-specific germ cell) state as the female counterpart to the male spermatogonial state. However, given that the earliest female-specific germ cell marker is in fact a meiotic marker, this state remains wholly theoretical.

After the events of germ cell sex determination, female germ cells initiate meiotic prophase. The events described earlier as occurring in male meiotic prophase I also occur in these cells, however, there are some differences between male and female prophase. Noticeably, the SC in female germ cells is twice as long as that of male germ cells (Tease and Hulten, 2004). It has also been noted that many mutations effecting meiosis result in more stringent infertility phenotypes in male germ cells than in the female (Hunt and Hassold, 2002). This difference in stringency may be caused by inherent differences in the systems of reproduction in male and female mammals. Sub-optimal meiotic conditions would likely result in less fit gametes (i.e. increased rates of aneuploidy). In males germ cells these cells can be replaced by subsequent rounds of spermatogenesis, presumably without the same defects. In females, since no subsequent meioses will occur, these sup-optimal gametes represent the only opportunity for reproduction.

Female germ cells progress through the leptotene, zygotene and pachytene stages of prophase and arrest at diplotene. The first oocytes to reach diplotene do so by E17.5 with all oocytes having done so by five days after birth (P5) (Borum, 1961). How the arrest of oocytes in diplotene is established remains largely uknown.

Little outside of meiosis is known to occur specifically in embryonic ovarian germ cells. These cells do not undergo global DNA re-methylation, as embryonic testicular germ cells do (Lees-Murdock et al., 2003; Lees-Murdock et al., 2005). One factor expressed specifically in female embryonic germ cells that is not involved in meiosis is the helix-loop-helix transcription factor *Figla* (factor in the germline alpha). *Figla* is expressed in female embryonic germ cells beginning at E13.5 and peaking at P2

(Soyal et al., 2000). While *Figla* is expressed in embryonic ovarian germ cells, no role for this gene has been found there. Microarray based expression studies of *Figla*-deficient embryonic ovaries found little to no expression differences when compared to wild-type embryonic ovaries (Joshi et al., 2007).

As previously mentioned, during their embryonic development germ cells of both sexes are connected in groupings known as cysts (Greenbaum et al., 2009). While the function of these cysts remains unknown, they break down in female germ cells within two to three days after birth (Pepling et al., 1999). This individualization of germ cells marks the beginning of a major differentiation program in female germ cell development: folliculogenesis (Pepling and Spradling, 2001). Follicles are structures composed of a single oocyte surrounded by layers of ovarian somatic cells.

The earliest type of follicle observed in the ovary is known as the primordial follicle. This structure consists of the oocyte surrounded by a single layer of flattened granulosa cells. Formation of primordial follicles requires the activity of the aforementioned transcription factor *Figla* (Soyal et al., 2000). *Figla* was originally identified as a direct regulator of zona pellucida (ZP) component gene expression (Liang et al., 1997). It is noteworthy that mice lacking ZP components are not defective in follicular development (Rankin et al., 1996; Rankin et al., 1999; Rankin et al., 2001). Thus the inability of *Figla*-deficient ovaries to form primordial follicles must be caused by some other defect. Gene expression studies suggest that in postnatal ovaries *Figla* regulates a large number of transcripts (Joshi et al., 2007). However, the relative contribution of these various mis-expressed genes to the *Figla* mutant phenotype remains unclear.

As oocytes are undergoing cyst breakdown many of these cells undergo apoptosis (Coucouvanis et al., 1993; Ratts et al., 1995). While the existence of this process, known as follicular atresia, has been known for many years, its function remains a mystery. Several hypotheses have been proposed to explain this seemingly wasteful process. Several groups have suggested that this process serves to eliminate those oocytes that are abnormal (and thus less fit) in some way (Morita and Tilly, 1999). Another group has suggested that in a process analogous to *Drosophila* oogenesis, atretic germ cells serve a nurse cell function to the surviving oocyte (Pepling and Spradling, 2001). How the ovary selects specific oocytes for atresia remains unknown. Without this information it is very difficult to test models for the function of this process.

Cohorts of primordial follicles are recruited into a growing oocyte pool, which will progress through the several stages of folliculogenesis, throughout the life of the animal (until menopause). What drives specific primordial follicles to enter the growing oocyte pool remains unknown. One factor involved in primordial follicle recruitment has been identified, *Foxo3* (forkhead box O3). Mice deficient for *Foxo3* undergo premature ovarian failure as all of their primordial follicles mature within the first several weeks after birth (Castrillon et al., 2003), while mice over-expressing *Foxo3* in the ovary are infertile due to failure in follicular recruitment (Liu et al., 2007). Recent studies have shown that *Foxo3* is down-regulated by the PI3K (phosphatidylinositol 3-kinase) pathway (John et al., 2008). This was confirmed *in vivo* by deletion of the PI3K inhibitor *Pten* (phosphatase and tensin homolog deleted on chromosome 10) in the ovary (John et al., 2008; Reddy et al., 2008). As expected, elimination of the inhibitory effects of *Pten* on the PI3K pathway leads to suppression of *Foxo3* and thus *Pten*-deficient oocytes

phenocopy *Foxo3*-deficient oocytes. While substantial ground has been made in understanding the regulation of *Foxo3*, how *Foxo3* functions to prevent recruitment of follicles remains unknown.

Other studies have attempted to identify factors involved in the progression of primordial follicles into primary follicles (oocytes surrounding by a single layer of cuboidal granulosa cells). Three factors involved in this stage of follicular development were identified using an *in silico* expression screen approach. Genes expressed specifically in germ cells of neonatal ovaries were identified using expressed sequence tag (EST) libraries. This approach identified two transcription factors *Nobox* (neonatal ovary homeobox gene) and *Sohlh1* (spermatogenesis and oogenesis helix-loop-helix 1) (Rajkovic et al., 2001; Suzumori et al., 2002). Subsequent *in silico* analysis revealed another transcription factor related to *Sohlh1* termed *Sohlh2* (spermatogenesis and oogenesis helix-loop-helix 2) (Ballow et al., 2006). Studies of mutants in each of these three genes showed defects specifically in the transition of primordial follicles into primary follicles (Choi et al., 2008b; Pangas et al., 2006; Rajkovic et al., 2004). Analysis of gene expression defects in the *Sohlh1*-deficient ovary revealed that *Sohlh1* regulates the transcription factor *Lhx8* (LIM homeobox protein 8) (Pangas et al., 2006). Further analysis has revealed that *Lhx8*-deficient ovaries phenocopy *Sohlh1*-deficient ovaries, suggesting that the regulation of *Lhx8* by *Sohlh1* represents a major portion of *Sohlh1*'s function (Choi et al., 2008a; Pangas et al., 2006).

While the effects of these factors on the histological development of follicles are clear, their genetic interactions are much more complex. While *Figla* is required in a developmental process upstream of *Sohlh1*, *Sohlh1* is required for normal *Figla*

expression in the developing ovary (Pangas et al., 2006). In addition, *Sohlh1* and *Sohlh2* appear to regulate each other's expression levels (Choi et al., 2008b). Thus even though factors required for this developmental transition and genes regulated by these factors have been identified, a comprehensive mechanistic model for the primordial to primary follicle transition remains elusive.

By P10-P12 the first cohort of growing follicles reaches the secondary follicle stage. This stage is characterized by the presence of multiple layers of granulosa cells surrounding a single oocyte. This phase of development involves significant signaling cross-talk between the oocyte and its surrounding granulosa layers. It has been found that oocytes provide the dominant cues in regulating follicular development (Eppig et al., 2002). One of the most important cues provided by oocytes in primary follicles to granulosa cells is *Gdf9* (growth differentiation factor 9) (McGrath et al., 1995). Mice deficient for *Gdf9* form primary follicles normally, but these follicles fail to progress into secondary follicles (Dong et al., 1996). Another factor involved in this transition is *Bmp15* (bone morphogenetic protein 15) (Dube et al., 1998). While *Bmp15* is not strictly required for secondary follicle formation *in vivo*, *Bmp15*-deficient *Gdf9* heterozygous ovaries fail to undergo normal secondary follicle formation, suggesting that these two factors act in a dose-dependent manner in this process (Yan et al., 2001). The mechanisms by which *Bmp15* and *Gdf9* function are unknown, however, one study has identified factors important for their regulation. The orphan nuclear receptor *Gcnf* (germ cell nuclear factor, MGI: *Nr6a1*) binds to sequences within the promoters of both *Gdf9* and *Bmp15*. Consequently, mice lacking oocyte-expressed *Gcnf* phenocopy *Gdf9*-deficient mice (Lan et al., 2003).

In addition to the *Gdf9*/*Bmp15* pathway, the neurotrophins *Ntf5* (neurotrophin 5) and *Bdnf* (brain derived neurotrophic factor) and their receptor *Ntrk2* (neurotrophic tyrosine kinase, receptor, type 2) are required for the primary to secondary follicle transition (Paredes et al., 2004). Interestingly, deletion of either *Ntf5* or *Bdnf* alone in the ovary is not sufficient to effect follicular development, but deletion of these two factors in combination causes loss of secondary follicles (Paredes et al., 2004).

Following secondary follicle formation, follicles begin to develop fluid-filled spaces known as antra and become known as antral follicles. These spaces coalesce into one large antrum as development proceeds. In contrast to preantral development, antral follicle development is governed in large part by hormones secreted by the pituitary gland, particularly by the gonadotropins *Fsh* (follicle stimulating hormone) and *Lh* (luteinizing hormone) (Edson et al., 2009).

In addition to hormonal signaling, connections between the oocyte and granulosa layers are essential for antral follicle development. *Cx37* (connexin 37; also known as *Gja4*) localizes to the gap junctions between oocytes and granulosa cells (Simon et al., 1997). In mice deficient for *Cx37,* follicles fail to develop past the preantral stages (Simon et al., 1997).

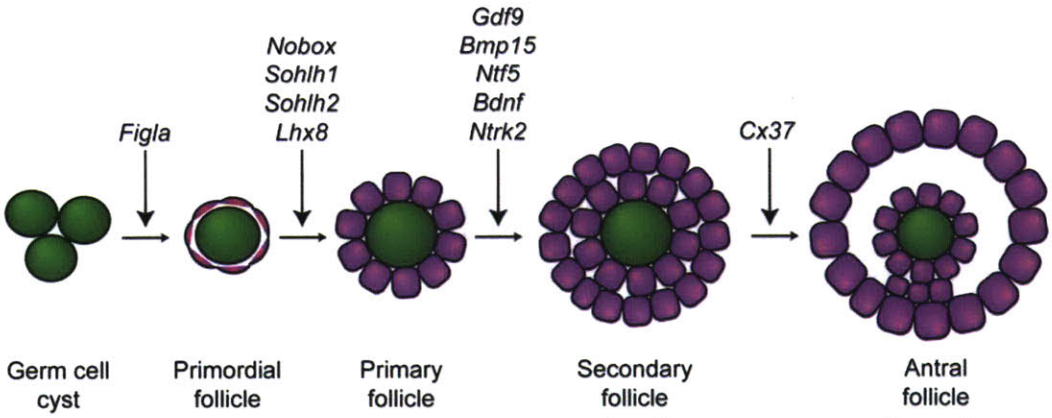

Figure 6: Follicular development during oogenesis. Cartoon representations of stages of follicle development in ovaries. Green cells represent oocytes, while purple cells represent granulosa cells. Factors listed above arrows are required for transitions indicated by arrows.

Around the time of antral follicle formation, oocytes produce a substance known as maturation-promoting factor (MPF). MPF is a composed of a complex containing the cyclin dependent kinase CDK1 and cyclin B (Mehlmann, 2005). Once the oocyte has produced a threshold level of MPF they are competent to exit from meiotic prophase arrest. They are prevented from doing so *in vivo*, however, by the presence of follicular somatic cells. It has been known for over seventy years that removal of an oocyte from an antral follicle *in vitro* results in rapid resumption of meiosis (which can be observed as the breakdown of the nuclear envelope (also known as the germinal vesicle)) (Edwards, 1965; Pincus and Enzmann, 1934).

How follicle cells prevent meiotic resumption in antral follicles has been studied for many years. It was suggested in the early 1980's that cyclic AMP (cAMP) levels in the oocyte were important for this process (Schultz et al., 1983a; Schultz et al., 1983b; Urner et al., 1983). It has been shown that in pre-ovulatory follicles cAMP levels are high and that this in turn leads to activation of PKA (protein kinase A). PKA in turn

inhibits CDC25B, an activator of CDK1 (Lincoln et al., 2002; Pirino et al., 2009; Zhang et al., 2008). PKA also activates WEE2, an inhibitor of CDK1 (Han et al., 2005).

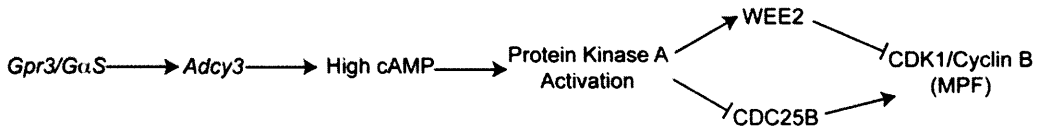

Figure 7: Genetic pathway controlling meiotic prophase arrest in antral follicles

Most of the oocytes recruited as primordial follicles into the growing oocyte pool undergo atresia prior to reaching the antral follicle stage. A small number of follicles survive to form the preovulatory follicles. How these oocytes are chosen is not known, however, it is thought that they survive because they are the most responsive to *Fsh* (Edson et al., 2009). As the initiation of ovulation proceeds, *Fsh* levels decrease and an increase in *Lh* levels (the so-called "LH surge") occurs. This results in follicular rupture and exit of the oocyte from the ovary into the oviduct.

One consequence of the LH surge is the resumption of meiosis in the oocyte. As previously mentioned, this occurs via a decrease in cAMP levels. These levels are thought to be regulated both through intrinsic oocyte based mechanisms and through communication with the granulosa cells. Mehlmann et al. showed that oocyte expressed stimulatory G proteins (Gas) were important for meiotic resumption (Mehlmann et al., 2002). In addition they identified *Gpr3* (G-protein coupled receptor 3) as the receptor that activates these G proteins (Mehlmann et al., 2004). Oocytes in mice deficient for *Gpr3* undergo premature resumption of meiosis and as a result these mice undergo premature ovarian failure (Ledent et al., 2005; Mehlmann et al., 2004). How then does *Gpr3* regulate cAMP levels in the oocyte? The adenylate cyclase *Adcy3* was found to be expressed highly in oocytes, and deletion of this gene results in premature meiotic

resumption in 50% of oocytes (Horner et al., 2003). This suggests that *Adcy3* plays a role in meiotic resumption, however, given the incomplete penetrance of this phenotype there are likely redundant pathways at play.

Following re-entry into meiosis, oocytes condense and segregate their chromosomes. The germinal vesicle breaks down and an asymmetric cell division occurs. One product of the first meiosis will be the oocyte, while the other product, the first polar body, will not form a gamete. Oocytes complete the first meiotic division, and immediately initiate meiosis II. The oocyte will then arrest in metaphase II (Mehlmann, 2005).

While MPF plays a role in the resumption of meiosis in diplotene arrested oocytes, it also plays a role in maintaining oocytes at metaphase II. A factor known as CSF (cytostatic factor) stabilizes MPF, arresting cells at metaphase. While the activity of CSF was known as early as 1971, it took many years to isolate the protein with this activity (Masui and Markert, 1971). The Moloney sarcoma oncogene (*Mos)* was found to have CSF activity and to be required for normal female fertility *in vivo* (Colledge et al., 1994; Hashimoto et al., 1994). In addition to *Mos*, *Emi2* (endogenous meiotic inhibitor 2, MGI: *Fbxo43,* F-box protein 43) has been shown to possess CSF activity, via its inhibition of the anaphase promoting complex, APC/C (Shoji et al., 2006).

At this point the oocyte is traveling through oviduct. It is ready for binding of spermatozoa to its zona pellucida and fertilization.

10. Conclusions:

Thus concludes a single transit of the germline cycle. While many different processes occur in this cycle one can see several commonalities in the study of mammalian germ cells and many areas where questions remain unanswered. The study of germ cell biology can be divided into four eras. The first was a purely descriptive age, in which the processes involved in germ cell development were laid out and categorized. The second involved further description as gene expression changes were identified, allowing more defined categorization of germ cell development to proceed. The third era involved the production of mutants in factors with interesting expression patterns. It is this third era that is (hopefully) coming to an end now. The fourth era, which has yet to truly begin, will involve connecting the phenotypes of multiple mutants to produce more defined pathways and determining the molecular functions of players known to be important in developmental transitions of the germ cell cycle.

This introduction places a great deal of emphasis on the arrival of germ cells in the somatic gonad and on germ cell sex determination. This is not by accident. These transitions (particularly the first) are not generally major points of study of the germline. The research presented in Chapters 2 and 3 of this thesis delves into the regulation of germ cell sex determination and the importance of the arrival of germ cells in the gonad as a regulator of the sex determination decision.

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Chapter 2:

Germ cell-intrinsic and –extrinsic factors govern meiotic initiation in mouse embryonic germ cells

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Retinoic acid (RA) is an essential extrinsic inducer of meiotic initiation in mammalian germ cells. However, RA acts too widely in mammalian development to account, by itself, for the cell-type and temporal specificity of meiotic initiation. We considered parallels to yeast, where extrinsic and intrinsic factors combine to restrict meiotic initiation. We demonstrate that, in mouse embryos, extrinsic and intrinsic factors together regulate meiotic initiation. The mouse DAZL RNA-binding protein, expressed by post-migratory germ cells, is a key intrinsic factor, enabling those cells to initiate meiosis in response to RA. Within a brief developmental window, *Dazl*-expressing germ cells in both XX and XY embryos actively acquire the ability to interpret RA as a meiosis-inducing signal.

Diploid eukaryotes generate haploid cells via meiosis, a program of two successive cell divisions preceded by one round of DNA replication. The onset of this program is referred to as meiotic initiation. In mammals, debate has focused on whether meiotic initiation is promoted by factors extrinsic or intrinsic to germline cells (Byskov, 1974; Byskov and Saxen, 1976; Di Carlo et al., 2000; McLaren, 1983; McLaren and Southee, 1997; Upadhyay and Zamboni, 1982). Meiotic initiation in female mice, commencing at embryonic day 12.5 (E12.5) (Baltus et al., 2006; Menke et al., 2003a), is induced by an extrinsic factor, retinoic acid (RA) (Baltus et al., 2006; Bowles et al., 2006; Koubova et al., 2006), but RA alone cannot account for the exquisite temporal and cell-type specificity of meiotic initiation. While diverse somatic cell types are exposed and respond to RA during mammalian development (Mark et al., 2006), meiotic initiation is limited to the germline. Indeed, embryonic germ cells do not respond specifically to RA

until their migration ends, at the developing gonad. Does meiotic initiation in mammals also require an intrinsic competence factor expressed in germ cells? Consider the yeast *S. cereviseae*, where meiosis is induced by a nutrient-depleted environment (Honigberg and Purnapatre, 2003). For an *S. cereviseae* cell to be competent to initiate meiosis in response to this extrinsic cue, the cell must express the a/α mating-type heterodimer (Hopper and Hall, 1975). We wondered whether an analogous interplay of extrinsic and intrinsic factors governs meiotic initiation in mammals.

We considered the possibility that the *Dazl* (*Deleted in azoospermia-like*) gene might be an intrinsic meiotic competence factor, given the location and timing of its expression. In both XX and XY mouse embryos, germ cells begin to express *Dazl* at about the time of their arrival at the gonad, between E10.5 and E11.5 (Seligman and Page, 1998). No somatic lineage has been shown to express *Dazl* (Cooke et al., 1996). Furthermore, *Dazl*-deficient mice are infertile due to germ cell differentiation defects (Lin and Page, 2005; Ruggiu et al., 1997b; Saunders et al., 2003; Schrans-Stassen et al., 2001). These defects are more consistent and pronounced in inbred C57BL/6 mice (Lin and Page, 2005) than in non-inbred mice (Ruggiu et al., 1997b; Saunders et al., 2003; Schrans-Stassen et al., 2001). Accordingly, we analyzed *Dazl* function in inbred C57BL/6 animals.

We began by testing whether germ cells survive in *Dazl*-deficient embryonic ovaries, as germ cells of *Dazl*-deficient C57BL/6 embryonic testes undergo apoptosis beginning by E14.5 (Lin and Page, 2005). We detected two germ cell markers – endogenous alkaline phosphatase (AP) activity (Ginsburg et al., 1990) and mouse *vasa* homolog (MVH) protein (Toyooka et al., 2000) – in the ovaries of wild-type and *Dazl*-

deficient embryos (Fig. 1, a and b). We also found MVH protein in wild-type and *Dazl*-deficient neonatal ovaries (Fig. 1c), indicating that *Dazl*-deficient ovarian germ cells survive embryonic development (Fig. 1, a and b) and persist through birth (Fig. 1c).

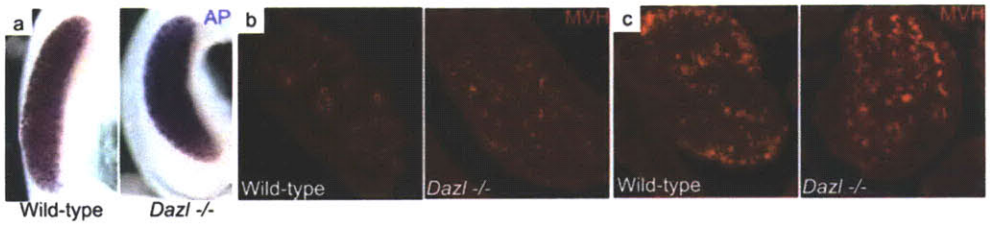

Figure 1: *Dazl* is not required for germ cell maintenance in C57BL/6 XX embryos. (a) Whole-mount staining for endogenous alkaline phosphatase activity in wild-type and *Dazl*-deficient E14.5 ovary-mesonephros complexes. (b, c) Immunohistochemical staining for MVH protein in sections from wild-type and *Dazl*-deficient ovaries. (b) E14.5. (c) P0 (neonatal).

We then compared the nuclear morphology of germ cells in wild-type and *Dazl*-deficient ovaries at E15.5. By this stage of development, many germ cell nuclei in wild-type ovaries exhibit the chromosome condensation that characterizes early meiotic prophase (Fig. 2a). By contrast, germ cells in *Dazl*-deficient ovaries do not display such condensation (Fig. 2b), suggesting that *Dazl* function might be required for meiotic prophase to occur. We then examined expression of *Stra8*, which is required for premeiotic DNA replication and the subsequent events of meiotic prophase in germ cells of embryonic ovaries (Baltus et al., 2006). As expected, *Stra8* is expressed abundantly in wild-type ovaries at E14.5 (Fig. 3, a and e). In *Dazl*-deficient ovaries, *Stra8* expression is dramatically reduced if not eliminated (Fig. 3, a and e), suggesting that *Dazl* might have an obligatory function upstream of meiotic initiation, which would account for the absence of meiotic chromosome condensation (Fig. 2b).

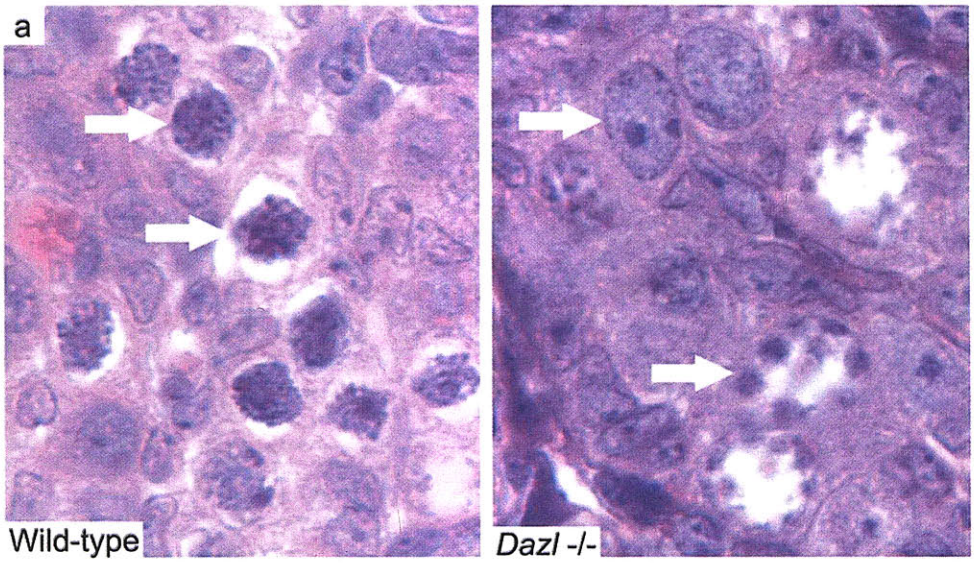

Figure 2. *Dazl* is required for meiotic chromosome condensation in C57BL/6 XX embryos. Photomicrographs of hematoxylin and eosin-stained ovarian sections from wild-type (a) and *Dazl*-deficient (b) E15.5 ovaries. Arrows indicate representative germ cell nuclei.

If *Dazl* is required for *Stra8* expression and meiotic initiation in embryonic ovaries, then germ cells in *Dazl*-deficient ovaries should not undertake meiotic recombination. We assayed whether *Dazl*-deficient female germ cells form DNA double-strand breaks (DSBs), which initiate meiotic recombination. Cells respond to DNA DSB formation by phosphorylating H2AX, a histone H2A variant, to generate γ-H2AX (Rogakou et al., 1998). As expected, immunostaining for γ-H2AX revealed the presence of DNA DSBs in many cells of wild-type ovaries at E15.5 (Fig. 3b). By contrast, *Dazl*-deficient ovaries are negative for γ-H2AX, indicating that DNA DSBs have not formed (Fig. 3b). In addition, we asked whether *Dazl*-deficient female germ cells express *Spo11* and *Dmc1*, which encode, respectively, a topoisomerase required to form meiotic DSBs and a recombinase functioning in meiotic DSB repair (Bannister and Schimenti, 2004). In previous studies, *Stra8* was shown to be required for expression of *Spo11* and *Dmc1* in

germ cells of embryonic ovaries (Baltus et al., 2006). We found that, in *Dazl*-deficient ovaries, expression of *Dmc1* and *Spo11* is markedly reduced if not eliminated (Fig. 3, c and e). The absence of H2AX phosphorylation and the absence of *Spo11* and *Dmc1* expression indicate that *Dazl*-deficient female germ cells do not engage in meiotic recombination.

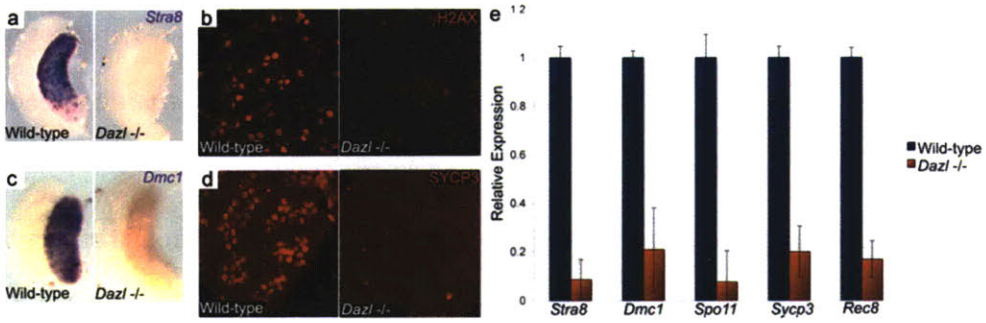

Fig. 3. *Dazl* is required for expression of meiotic prophase markers in C56BL/6 XX embryos. (a) Whole-mount *in situ* hybridization for *Stra8* mRNA in wild-type and *Dazl*-deficient E14.5 ovaries. (b) Immunohistochemical staining for γ-H2AX protein in sections of wild-type and *Dazl*-deficient E15.5 ovaries. (c) Whole-mount *in situ* hybridization for *Dmc1* mRNA in wildtype and *Dazl*-deficient E15.5 ovaries. (d) Immunohistochemical staining for SYCP3 protein in sections of wild-type and *Dazl*-deficient E15.5 ovaries. (f) Quantitative RT-PCR analysis of *Stra8*, *Dmc1*, *Spo11*, *Sycp3* and *Rec8* mRNA levels in wild-type and *Dazl*-deficient E14.5 ovaries. Plotted here are average fold changes, normalized to *Hprt*, in three independent biological replicates. Error bars represent standard deviations among biological replicates.

These findings are consistent with *Dazl* being required upstream of *Stra8*'s function in meiotic initiation. Does *Dazl* deficiency simply recapitulate the *Stra8* null phenotype (Baltus et al., 2006), or does it cause additional abnormalities? We assayed the expression in *Dazl*-deficient embryonic ovaries of the *Sycp3* and *Rec8* genes, which encode, respectively, a component of the synaptonemal complex and a meiosis-specific cohesin (Scherthan, 2003). Both SYCP3 and REC8 proteins function through their loading onto chromosomes. In *Stra8*-deficient female germ cells, as previously reported,

Sycp3 and *Rec8* are transcribed and translated, but the encoded proteins do not load onto chromosomes and therefore do not perform their meiotic functions (Baltus et al., 2006). We discovered that, in germ cells of *Dazl*-deficient embryonic ovaries, *Sycp3* function is disrupted at an even earlier step: SYCP3 protein and mRNA levels are markedly reduced as compared with those in wild-type ovaries (Fig. 3, d and e). Similarly, *Rec8* exhibited little or no expression in *Dazl*-deficient ovaries (Fig. 3e). Thus, *Dazl* is required for both *Stra8*-mediated initiation of meiosis in female germ cells, but also for *Stra8*-independent expression of *Sycp3* and *Rec8* there.

We propose a pathway by which embryonic germ cells advance from a primordial state to initiation of meiosis (Fig. 4). This pathway includes a newly posited cell state – the meiosis-competent gonocyte – whose derivation from a primordial germ cell requires the germ-cell-intrinsic factor *Dazl*, and whose progression to meiotic initiation and prophase in the female germline requires the extrinsic meiosis-inducing factor RA, and *Stra8*. We propose that this meiosis-competent cell state exists in both male and female embryonic germlines – despite the fact that meiosis does not initiate in male embryos. The posited meiosis-competent gonocyte contains SYCP3 protein not yet loaded onto chromosomes (Fig. 4), consistent with the observation (Di Carlo et al., 2000) that both male and female embryonic germ cells express SYCP3 protein before the sexes take different paths: female germ cells advance to meiotic prophase, where SYCP3 functions, while male germ cells down-regulate SYCP3 and arrest in G0.

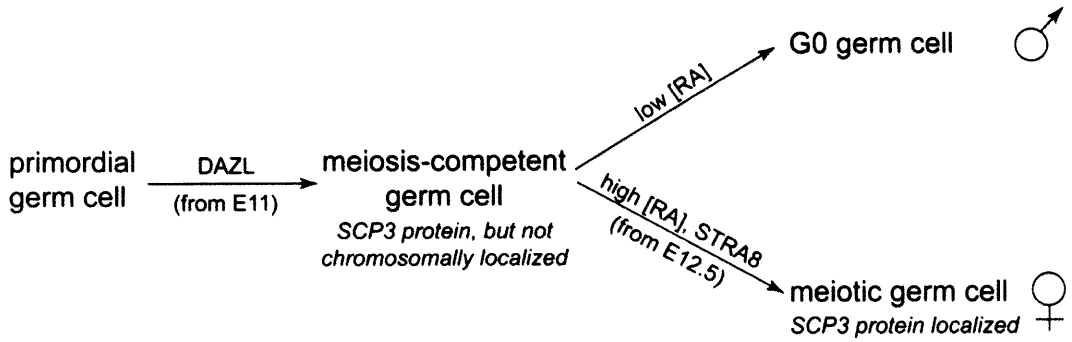

Figure 4: Embryonic germ cell states: A proposed path by which primordial germ cells acquire meiotic competence (in both XX and XY embryos) and subsequently initiate meiosis (in XX embryos) or undergo G0 arrest (in XY embryos). "low [RA]" and "high [RA]" are consequences of differential expression of RA-inactivating enzyme CYP26B1 in embryonic testes and ovaries (Bowles et al. 2006, Koubova et al. 2006).

Embryonic testicular germ cells express *Stra8* when exposed to exogenous RA, even though they normally express *Stra8* only after birth (Bowles et al., 2006; Koubova et al., 2006). Our model predicts that ectopic expression of *Stra8* in RA-treated embryonic testes should require *Dazl* function. We dissected testes from wild-type and *Dazl*-deficient E12.5 embryos, cultured them in the presence of 0.7 μM RA for 48 hours, and assayed *Stra8* expression by whole-mount *in situ* hybridization. As previously reported (Bowles et al., 2006; Koubova et al., 2006), RA induced robust expression of *Stra8* in wild-type testes (Fig. 5a). By contrast, in *Dazl*-deficient testes, no induction was observed (Fig. 5a). To confirm that this failure to induce *Stra8* expression in *Dazl*-deficient testes was not due to germ cell apoptosis (Lin and Page, 2005), we performed a control *in situ* hybridization for *Oct4* (MGI: *Pou5f1*), a gene expressed in embryonic germ cells but not in gonadal somatic cells (Pesce et al., 1998). We observed abundant *Oct4* expression in RA-cultured testes, both wild-type and *Dazl*-deficient (Fig. 5b). Thus, expression of *Stra8* in response to RA requires *Dazl* in embryonic testis and ovary alike, confirming that *Dazl* is a competence factor for meiotic initiation in embryos of both

sexes.

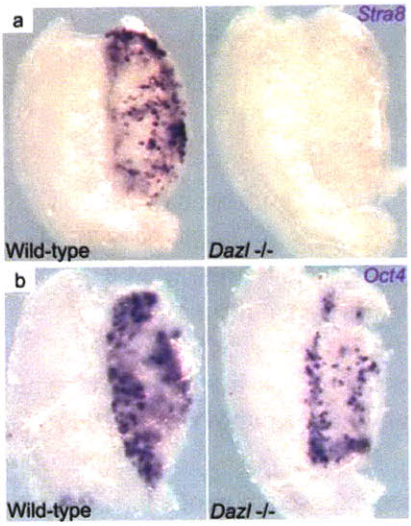

Figure 3: *Dazl* is required for RA-induced expression of *Stra8* in embryonic testes. Whole-mount *in situ* hybridization for (a) *Stra8* mRNA and (b) *Oct4* mRNA in wild-type and *Dazl*-deficient testes dissected at E12.5 and cultured for 48 hours in presence of 0.7μM RA.

In *S. cereviseae* cells, expression of the a/α mating-type heterodimer is a prerequisite to launching the meiotic initiation program in response to an extrinsic cue. Our findings demonstrate that *Dazl* plays an analogous role in embryonic mice. In both a unicellular eukaryote and a complex animal, meiotic initiation is governed by a cell-intrinsic competence factor and an extrinsic inducing signal.

Materials and Methods

Mice. Mice carrying the $Dazl^{TM1Hgu}$ allele (*1*) were generously provided by Howard Cooke, MRC Human Genetics Unit, Western General Hospital, Edinburgh, UK. As described previously (*2*), we crossed $Dazl^{TM1Hgu}$/+ mice to C57BL/6 mice (Taconic Farms Inc., Germantown, NY). All experiments were conducted using mice backcrossed to C57BL/6 between 7 and 16 generations, when 99.2% to >99.9% of the genome is expected to be of C57BL/6 origin; all Y chromosomes and mitochondria are of C57BL/6 origin. *Dazl*-deficient embryos were generated by mating heterozygotes. *Dazl* genotypes

were assayed by PCR as previously described (Ruggiu et al., 1997a). All experiments involving mice were

approved by the Committee on Animal Care at the Massachusetts Institute of Technology.

Embryo Collection and Sexing. To establish timed matings, female mice were housed with male mice overnight. Noon of the day when a vaginal plug was evident was considered E0.5. Embryonic gonads and mesonephroi were dissected into cold phosphate buffered saline. We determined the sex of the tissues by scoring the presence or absence of testicular cords.

Alkaline phosphatase staining. Dissected embryonic gonads and mesonephroi were fixed in 4% paraformaldehyde overnight and then treated for 4 minutes with methanol to permeabilize the tissue. Samples were then stained with NBT (nitroblue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt) (Roche).

Immunohistochemistry. Embryonic gonads were fixed in 4% paraformaldehyde overnight, embedded in paraffin and sectioned. Slides were de-waxed, rehydrated and autoclaved in 10 mM Sodium Citrate buffer, pH 6.0 for 5 minutes. Mouse monoclonal anti-γH2AX (Upstate Biotech) was used at a dilution of 1:1000. Rabbit anti-SYCP3 was a gift from Christa Heyting (Department of Genetics, Agricultural University, Wageningen, The Netherlands) and was used at a dilution of 1:700. Rabbit anti-MVH was a gift from Toshiaki Noce (Mitsubishi Kagaku Institute of Life Sciences (MITILS), Tokyo, Japan) and was used at a 1:1000 dilution. Texas Red-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories Inc.) were used at a dilution of 1:200.

Histology. Embryonic gonads were fixed in Bouin's solution at 4°C overnight,

embedded in paraffin and sectioned. Slides were then stained with hematoxylin and eosin.

Whole-mount *in situ* hybridization. Embryonic gonads and mesonephroi were fixed in 4% paraformaldehyde at 4°C overnight. Whole-mount in situ hybridizations were performed as previously described (Menke and Page, 2002; Wilkinson and Nieto, 1993). Riboprobes for *Stra8* and *Dmc1* were prepared as previously described (Menke et al., 2003b).

Quantitative RT-PCR. Embryonic gonads were dissected away from mesonephroi, placed in Trizol (Invitrogen), and stored at -80°C. Following genotyping, total RNAs were prepared according to the manufacturer's protocol. Total RNAs were then DNase treated using DNA Free Turbo (Ambion). 200 ng of total RNA was reverse transcribed using a RETROScript kit (Ambion). Quantitative PCR was performed using SYBR Green Core PCR Reagents (Applied Biosystems) on an ABI9700 Fast Real-time PCR machine (Applied Biosystems). Results were analyzed using the delta-delta Ct method using *Hprt* (hypoxanthine-guanine phosphoribosyltransferase) as a normalization control. RT-PCR primers for all genes were picked from the PrimerBank web site (Wang and Seed, 2003) and are listed below in Table 1.

Table 1: Primer sequences for Quantitative RT-PCR

Gene Symbol	Gene Name	PrimerBank ID	Forward Primer	Reverse Primer
Hprt	hypoxanthine guanine phosphoribosyl transferase	7305155a1	TCAGTCAACGGGGGACATAAA	GGGGCTGTACTGCTTAACCAG
Stra8	stimulated by retinoic acid gene 8	6678173a2	CTGTTGCCGGACCTCATGG	TCACTTCATGTGCAGAGATGATG
Dmc1	disrupted meiotic cDNA 1	6753650a1	CCCTCTGTGTGACAGCTCAAC	GGTCAGCAATGTCCCGAAG
Spo11	sporulation protein 11	6755624a1	CGTGGCCTCTAGTTCTGAGGT	GCTCGATCTGTTGTCTATTGTGA
Sycp3	synaptonemal complex protein 3	6755704a1	AGCCAGTAACCAGAAAATTGAGC	CCACTGCTGCAACACATTCATA
Rec8	REC8 homolog (yeast)	31982699a2	CTACCTAGCTTGCTTCTTCCCA	GCCTCTAAAAGGTGTCGAATCTG

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antisera; T. Noce for MVH antisera; and A. Amon, E. Anderson, A. Baltus, A. Bortvin, M. Carmell, G. Fink, A. Hochwagen, Y. Hu, J. Lange, J. Mueller, L. Okumura, and T. Orr-Weaver for advice and comments on the manuscript. Microscopy and image capture were conducted at the W.M. Keck Foundation Biological Imaging Facility at the Whitehead Institute. Supported by the Howard Hughes Medical Institute.

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Chapter 3:

Licensing gametogenesis: a novel transition in mouse embryonic germ cell development

Personal Contribution: Designed and performed all experiments (with the exceptions of 5-methylcytosine immunostaining (performed by Yueh-Chiang Hu) and *in situ* hybridizations for *Oct4* and *Dppa3* (performed by Yanfeng Lin)) and wrote the manuscript

Abstract:

Models for mammalian germ-line sex determination have assumed that embryonic germ cells are programmed to follow one of two fates: oogenic or spermatogenic. These models have utilized the embryonic initiation or prevention of meiosis as a surrogate as to whether female or male fate have been adopted. These models have not been tested genetically. We examined the sexual fate of germ cells from XX *Dazl*-deficient embryos, where meiotic initiation is prevented, and found that the absence of meiosis is not sufficient to drive mammalian embryonic germ cells into a male fate. We further characterized the germ cells of both XX and XY *Dazl*-deficient embryos and discovered that these cells are retained in a state reminiscent of primordial germ cells. The results presented here allow us to conclude that mammalian embryonic germ cells are not pre-programmed to adopt a sexual fate. We propose a model in which germ cells in embryos of both sexes undergo a developmental transition, which we term licensing, that enables these cells to become competent to respond to sex determining signals produced by the gonadal soma.

Mammalian germ cells are specified from a small population of pluripotent epiblast cells. They then migrate to the developing somatic gonad. These cells exist in a sexually undifferentiated state, and only acquire a sex-specific identity following their interaction with the sexually dimorphic somatic gonad. The first morphological difference between germ cells in XX and XY embryonic gonads is the chromosomal condensation associated with meiosis, which is observed in germ cells in XX, but not XY, gonads at approximately embryonic day (E)13.5 in the mouse (McLaren, 1984; McLaren, 2003). These observations have led to a model in which the decision of an embryonic germ cell to initiate or prevent meiosis also represents the decision of an embryonic germ cell to adopt a female or male sexual fate (Kocer et al., 2009). We set out to test this model genetically.

The gene encoding the germ cell specific RNA binding protein *Dazl,* is expressed prior to the onset of sexual differentiation in germ cells of both sexes (Seligman and Page, 1998). We have previously shown that germ cells in C57BL/6 XX *Dazl*-deficient embryos do not initiate meiosis (Chapter 2 of this thesis). In contrast to most mutants with phenotypes affecting meiosis, where some aspects of meiosis may be detected, XX *Dazl*-deficient germ cells exhibit none of the characteristics normally associated with early meiosis and thus allow us to cleanly study XX non-meiotic germ cells. Existing models predict the absence of embryonic meiotic initiation should lead embryonic germ cells to adopt a male sexual fate, and so we tested this prediction by examining XX *Dazl*-deficient embryonic germ cells for typically male characteristics.

An early gene expression difference between embryonic male and female germ cells is the expression of the *Nanos2* gene in embryonic male but not female germ cells

beginning at E13.5 (Tsuda et al., 2003). We examined mRNA levels of *Nanos2* transcript by RT-PCR, in gonads of wild-type and *Dazl*-deficient E13.5 embryos (Fig. 1a). We found no evidence for expression of *Nanos2* in XX *Dazl*-deficient gonads, suggesting that, despite not initiating meiosis, these cells were not undergoing male specific gene expression changes.

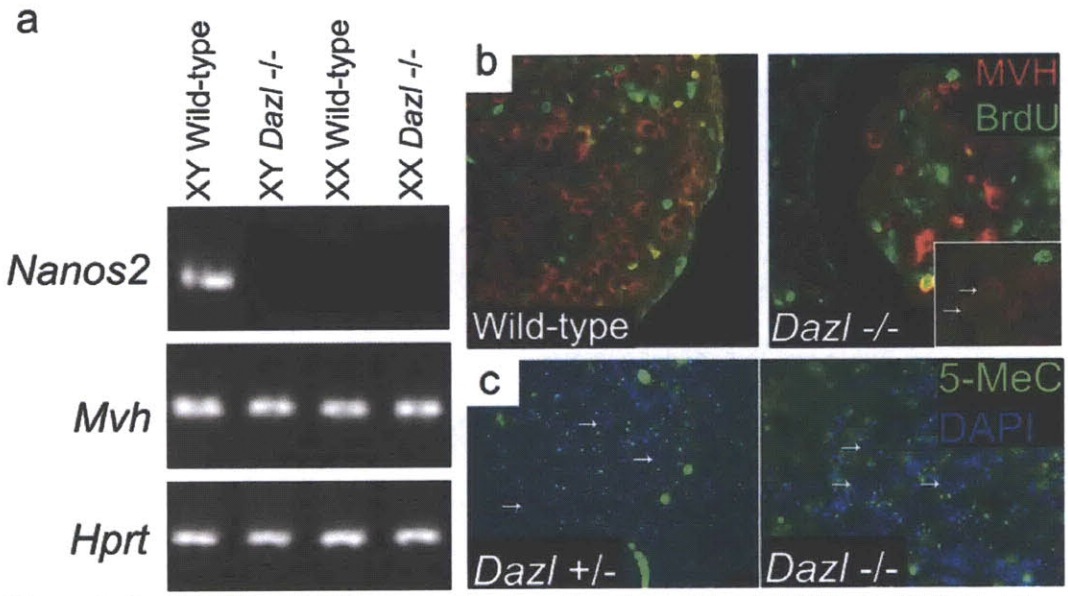

Figure 1: *Dazl*-deficient XX germ cells do not undergo "male" differentiation. (a) RT-PCR analysis of *Nanos2*, *Mvh* and *Hprt* mRNA on E13.5 wild-type and *Dazl*-deficient gonads. (b) Immunofluorescent staining for MVH protein and BrdU on sections of E18.5 wild-type and *Dazl*-deficient XX gonads. (c) Immunofluorescent staining for 5-methylcytosine on sections of E16.5 wild-type and *Dazl*-deficient XX gonads.

We next examined processes characteristic of male embryonic germ cell development. Male germ cells initiate a G0 cell cycle arrest, beginning between E12.5 and E14.5 (Western et al., 2008). We assayed whether XX *Dazl*-deficient germ cells were arrested in the cell cycle by examining the ability of these cells to continue replicating their DNA. We injected pregnant mice with bromodeoxyuridine (BrdU) at E18.5 and dissected embryonic gonads three hours later. We then immunostained sections of these gonads with antibodies against BrdU and MVH (mouse vasa homolog, a germ cell

marker; Mouse Genome Informatics: (MGI) DDX4 (Tanaka et al., 2000)). As expected, in wild-type XX gonads, where germ cells have progressed into meiotic prophase, BrdU incorporation was detected in somatic but not germ cells (Fig. 1b). In contrast, many germ cells in XX *Dazl*-deficient gonads were found to have incorporated BrdU (Fig. 1b) suggesting that these cells had not undergone the characteristically male G0 arrest.

During late embryogenesis male germ cells also initiate genome-wide DNA re-methylation (and expression of the *de novo* DNA methyltransferases of the DNMT3 family) (Marchal et al., 2004). We examined global levels of DNA methylation by immunostaining sections of gonads from E17.5 embryos with an antibody to 5-methylcytosine. We found that while wild-type germ cells in XY gonads exhibit clearly detectable levels of DNA methylation (data not shown), germ cells in wild-type and *Dazl*-deficient XX gonads do not (Fig. 1c). Thus *Dazl*-deficient XX germ cells do not exhibit any typical male embryonic germ cell characteristics. We conclude that the absence of meiotic initiation in embryonic germ cells is not sufficient to induce a male germ cell fate and that non-meiotic germ cells require additional cues to become male.

Given that XX *Dazl*-deficient germ cells are both non-meiotic (Chapter 2) and non-male (results above), we wondered whether these cells underwent non-meiotic female germ cell differentiation. The basic helix-loop-helix transcription factor *Figla* (factor in the germline alpha) is expressed in specifically in female germ cells by E13.5 (Soyal et al., 2000). This factor regulates the ability of female germ cells to recruit somatic granulosa cells to form primordial follicles (Soyal et al., 2000). We assayed *Figla* mRNA levels by RT-PCR on E14.5 gonads from wild-type and *Dazl*-deficient embryos. We found, as previously reported (Soyal et al., 2000), that *Figla* is expressed in

wild-type E14.5 XX, but not XY, gonads (Fig. 2). We could not detect *Figla* mRNA in XX *Dazl*-deficient gonads (Fig. 2), suggesting that in addition to failing to initiate male germ cell differentiation these cells had also failed to initiate female specific gene expression changes.

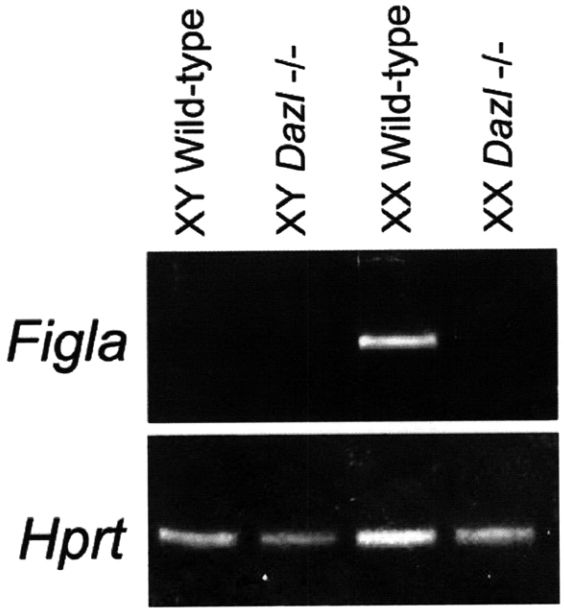

Figure 2: *Dazl*-deficient germ cells do not express *Figla*. RT-PCR analysis of *Figla* and *Hprt* mRNA on E14.5 wild-type and *Dazl*-deficient gonads.

As we have determined that embryonic germ cells in XX gonads can fail to exhibit sexually dimorphic characteristics, we wondered whether a similar phenomenon would be possible in germ cells in XY gonads. Given that *Dazl* is expressed prior to sexual differentiation in the germ cells of both sexes, we hypothesized that XY *Dazl*-deficient germ cells would also fail to undergo sexual differentiation. We assayed expression of *Nanos2* and *Figla* in XY *Dazl*-deficient germ cells by RT-PCR. We noticed that XY *Dazl*-deficient gonads do not express *Nanos2* or *Figla* (Fig. 1a&2). Thus both XX and XY *Dazl*-deficient germ cells fail to express genes normally associated with male and female specific development.

In order to extend the analysis of male differentiation in XY *Dazl*-deficient germ cells, we investigated the cell cycle status of XY *Dazl*-deficient germ cells, by examining expression of Ki-67 protein (MGI: MKI67) by immunohistochemistry. This protein is present in the nuclei of cells in the G1, S, G2 and M phases of the cell cycle, while being absent from arrested cells. The nuclei of XY wild-type germ cells from E16.5 embryos do not show significant levels of Ki-67, suggesting that they have largely entered cell cycle arrest (Fig. 3a). In contrast, XY *Dazl*-deficient germ cells are positive for Ki-67 (Fig. 3a), indicating that the cell cycle arrest normally characteristic of male germ development does not occur in the absence of *Dazl*.

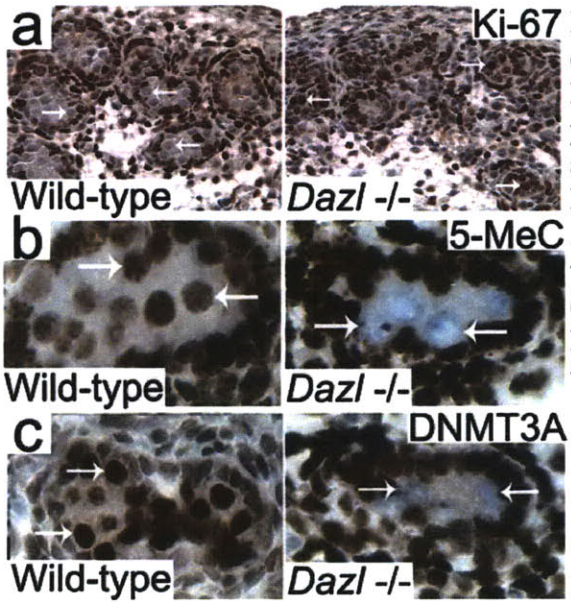

Figure 3: *Dazl* is required for "male" differentiation in XY germ cells. (a) Immunohistochemical staining for Ki-67 protein on sections of E16.5 wild-type and *Dazl*-deficient XY gonads. (b) Immunohistochemical staining for 5-methylcytosine on sections of E16.5 wild-type and *Dazl*-deficient XY gonads. (c) Immunohistochemical staining for DNMT3A protein on sections of E16.5 wild-type and *Dazl*-deficient XY gonads.

We next assayed XY *Dazl*-deficient germ cells for DNA methylation by immunostaining sections of E16.5 gonads with an antibody to 5-methylcytosine. We found that while staining for 5-methylcytosine was readily detectable in wild-type XY germ cells (Fig. 3b), XY *Dazl*-deficient germ cells did not display detectable levels of 5-methylcytosine (Fig. 3b). We also utilized immunohistochemistry with an antibody that

recognizes DNMT3A (a *de novo* DNA methyltransferase) on sections of XY E16.5 gonads. XY wild-type germ cells displayed significant levels of DNMT3A protein, while in XY *Dazl*-deficient germ cells the protein was undetectable (Fig. 3c). Thus, XY *Dazl*-deficient germ cells fail to undergo the developmental changes that normally occur in a male specific manner in embryonic germ cells.

Given the failure of germ cells in both XX and XY *Dazl*-deficient gonads to undergo sexual differentiation, we conclude that germ cells are not pre-programmed to adopt a sexual fate but must be induced to do so via that action of the *Dazl* gene. We propose a model in which upon expression of *Dazl,* germ cells initiate a cellular state, which we term the gametogenesis competent cell (GCC), that is primed to respond to the gonadal somatic environment by initiating oogenesis or spermatogenesis (Fig. 4). We believe that the acquisition of the GCC state, which we term licensing for gametogenesis, represents a heretofore uncharacterized developmental transition required for the production of functional sexually dimorphic gametes.

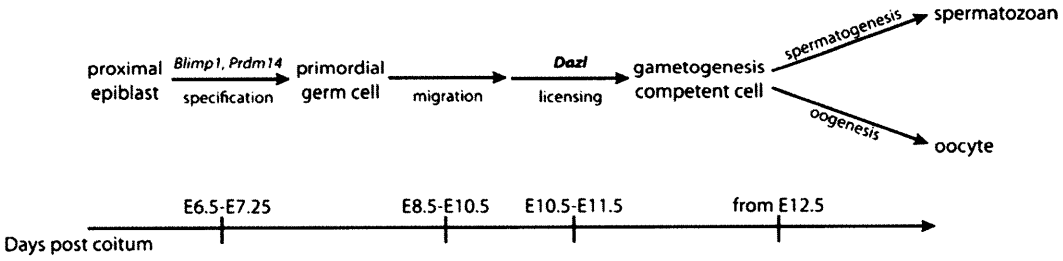

Figure 4: Model for the development of embryonic germ cells. Germ cells are specified from proximal epiblast cells, migrate to the somatic gonad and are licensed to undergo gametogenesis depending upon the sex of the surrounding gonadal tissue.

Our model predicts that embryonic germ cells that fail to become GCCs would become developmentally blocked and thus retain the characteristics of early embryonic

germ cells, known as primordial germ cells (PGCs). In the mouse, PGCs are characterized by retained expression of genes normally associated with early embryos and pluripotency, including *Oct4, Nanog, Sox2* and *Dppa3* (also known as *stella*). XX germ cells down-regulate expression of these genes beginning around E14.5 (Pesce et al., 1998; Western et al., 2005; Yamaguchi et al., 2005). In XY mouse embryos, gene expression levels of these genes are down-regulated slightly later, with *Oct4* and *Sox2* transcripts being detectable in postnatal undifferentiated spermatogonia (Pesce et al., 1998; Shi et al., 2006; Western et al., 2005; Yamaguchi et al., 2005). We tested the prediction of our model that germ cells that fail to attain the GCC state would retain a PGC state, by examining expression of *Oct4, Sox2, Nanog* and *Dppa3* in the gonads of XX and XY *Dazl*-deficient embryos. We examined the mRNA levels of these genes in XX embryos from E12.5 through E16.5 by quantitative RT-PCR and *in situ* hybridization. As expected, in XX wild-type embryos levels of these transcripts show a decrease between E13.5 and E15.5 (Fig. 5a). In XX *Dazl*-deficient embryos the high levels of these transcripts are retained through E16.5 (Fig. 5a-c). We confirmed the presence of OCT4, NANOG and SOX2 protein in E16.5 XX *Dazl*-deficient, but not wild-type, gonads by immunohistochemistry (Fig. 5d-f). Because of XY germ cell apoptosis in C57BL/6 *Dazl*-deficient mice (Lin and Page, 2005), it was not possible to examine global mRNA levels by quantitative RT-PCR in the same manner in XY *Dazl*-deficient germ cells as we used in XX *Dazl*-deficient germ cells. We therefore used immunohistochemistry to examine the levels of OCT4 and NANOG protein in germ cells remaining in E16.5 XY embryonic gonads. We found that while germ cells in XY wild-type gonads exhibit weak staining for OCT4 and NANOG at E16.5 (Fig. 5g&h), germ

cells in XY *Dazl*-deficient gonads display very strong nuclear staining for both of these proteins (Fig 5g&h). Thus as predicted by our model failure to undergo licensing for gametogenesis leads to germ cells that retain a PGC state.

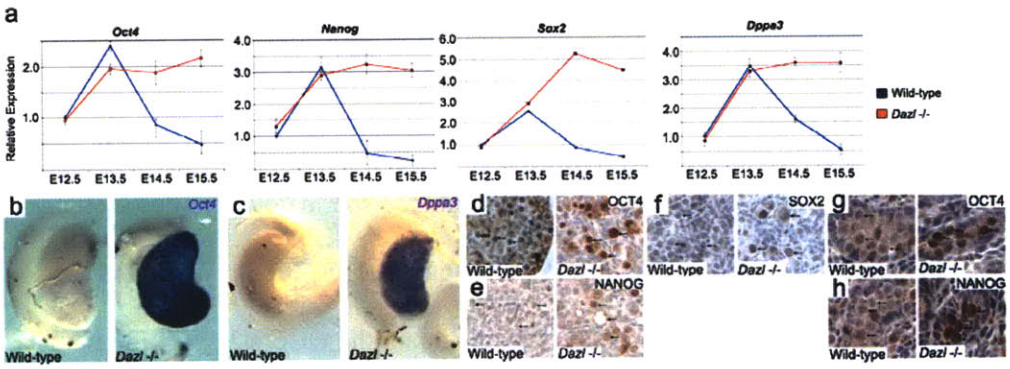

Figure 5: *Dazl*-deficient germ cells retain PGC characteristics. (a) Quantitative RT-PCR analysis of *Oct4, Nanog, Sox2* and *Dppa3* mRNA in E12.5-E15.5 wild-type and *Dazl*-deficient XX gonads. (b) Whole-mount in situ hybridization for *Oct4* mRNA in E16.5 wild-type and *Dazl*-deficient XX gonads. (c) Whole-mount in situ hybridization for *Dppa3* mRNA in E15.5 wild-type and *Dazl*-deficient XX gonads. (d-f) Immunohistochemistry for OCT4 (d), NANOG (e) and SOX2 (f) protein in E16.5 wild-type and *Dazl*-deficient XX gonads. (g&h) Immunohistochemistry for OCT4 (g) and NANOG (h) in E16.5 wild-type and *Dazl*-deficient XY gonads.

The phenotype reported here is striking for several reasons. First, while many genes involved in germ cell differentiation have been discovered, many of these genes have been characterized for their roles in germ cell survival. While *Dazl*-deficient germ cells do eventually undergo apoptosis (Lin and Page, 2005), they do not do so until several days (in the case of XY germ cells) or more than a week (in the case of XX germ cells) after a primary differentiation defect can be detected. This window of germ cell survival has enabled us to characterize a previously unreported phenotype, failure in PGC differentiation.

Our results suggest a re-examination of several aspects of mammalian germ cell development. Models for sex determination in germ cells are generally built around a

binary decision. These models suggest that germ cells that fail to adopt one sexual fate would automatically attain an alternative fate. This idea is often stated as suggesting a "default" state for germ cell sex. Our results suggest that upon their arrival in the gonad, germ cells are not pre-determined as either male or female, but must be induced to become competent to undergo the sex determination decision.

These results leave several interesting questions unanswered. What drives the expression of *Dazl* specifically in germ cells around the time of their arrival in the gonad? A hypothesized intrinsic "clock" activated by germ cell specification could drive licensing for gametogenesis; alternatively, the cellular environment in which germ cell find themselves could be the driving force. Further studies will be necessary to determine which of these hypotheses are true.

Materials and Methods:

Mice. Mice carrying the *Dazl*^{TM1Hgu} allele (Ruggiu et al., 1997) were generously provided by Howard Cooke, MRC Human Genetics Unit, Western General Hospital, Edinburgh, UK. As described previously (Lin and Page, 2005), we crossed *Dazl*^{TM1Hgu}/+ mice to C57BL/6 mice (Taconic Farms Inc., Germantown, NY). All experiments were conducted using mice backcrossed to C57BL/6 between 10 and 22 generations, when >99.9% of the genome is expected to be of C57BL/6 origin; all Y-chromosomes and mitochondria are of C57BL/6 origin. *Dazl*-deficient embryos were generated by mating heterozygotes. *Dazl* genotypes were assayed by PCR as previously described (Ruggiu et al., 1997). The Committee on Animal Care at the Massachusetts Institute of Technology approved all experiments involving mice.

Embryo Collection and Sexing. To establish timed matings, female mice were housed with male mice overnight. Noon of the day when a vaginal plug was evident was considered E0.5. Embryonic gonads and mesonephroi were dissected into phosphate buffered saline. Sex of the tissues was determined by scoring the presence or absence of testicular cords.

Immunohistochemistry. Embryonic gonads were fixed in either 4% paraformaldehyde or Bouin's solution overnight, embedded in paraffin and sectioned. Slides were de-waxed, rehydrated, heated in 10 mM Sodium Citrate buffer, pH 6.0 for 10 minutes and blocked for 30 minutes in 2.5% horse serum. Slides were then incubated with primary antibody overnight at 4°C. Primary antibody was then washed off and samples were incubated with Rabbit or Mouse ImmPress Reagent (Vector Labs) and developed using DAB Substrate Kit, 3,3'-diaminobenzidine (Vector Labs). Samples were then counter-stained with hematoxylin, dehydrated and mounted with Permount (Fisher Scientific). Mouse anti-OCT4 (BD Transduction Labs) was used at a concentration of 1:100; rabbit anti-NANOG (Bethyl Laboratories) was used at a concentration of 1:200; mouse anti-$5^{me}C$ (Calbiochem) was used at a concentration of 1:500 and mouse anti-DNMT3A (Imgenex) was used at a concentration of 1:100.

Whole-mount *in situ* hybridization. Embryonic gonads and mesonephroi were fixed in 4% paraformaldehyde at 4°C overnight. Whole-mount in situ hybridizations were performed as previously described (Menke and Page, 2002; Wilkinson and Nieto, 1993).

Riboprobes for *Oct4* and *Dppa3* were prepared as previously described (Lin and Page, 2005; Menke et al., 2003).

Quantitative RT-PCR. Embryonic gonads were dissected away from mesonephroi, submerged in Trizol (Invitrogen), and then stored at -80°C. Following genotyping, total RNA was prepared according to the manufacturer's protocol. Total RNA was then DNase treated using DNA Free Turbo (Ambion). 200 ng of total RNA was reverse transcribed using a RETROScript kit (Ambion). Quantitative PCR was performed using SYBR Green Core PCR Reagents (Applied Biosystems) on an ABI9700 Fast Real-time PCR machine (Applied Biosystems). Results were analyzed using the delta-delta Ct method using *Hprt* (hypoxanthine-guanine phosphoribosyltransferase) as a normalization control. RT-PCR primers for all genes were picked from the PrimerBank web site (Wang and Seed, 2003), and their sequences are available in Table 1.

Table 1: Primer sequences for Quantitatve RT-PCR

Gene Symbol	Gene Name	PrimerBank ID	Forward Primer	Reverse Primer
Hprt	hypoxanthine guanine phosphoribosyl transferase	7305155a1	TCAGTCAACGGGGGACATAAA	GGGGCTGTACTGCTTAACCAG
Oct4 (Pou5f1)	POU domain, class 5, transcription factor 1	7305399a3	CAGCCAGACCACCATCTGTC	GTCTCCGATTTGCATATCTCCTG
Nanog	Nanog homeobox	31338864a3	CCTGATTCTTCTACCAGTCCCA	GGCCTGAGAGAACACAGTCC
Sox2	SRY box protein 2	31543759a1	GCGGAGTGGAAACTTTTGTCC	CGGGAAGCGTGTACTTATCCTT
Dppa3 (Stella)	developmental pluripotency associated 3	21218416a1	GACCCAATGAAGGACCCTGAA	GCTTGACACCGGGGTTTAG

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Chapter 4:

Conclusions and Future Directions

In this thesis I have explored the status of mouse embryonic germ cells prior to the onset of their sexual differentiation, by examining the role of a gene first expressed in germ cells during this developmental window, *Dazl*. I have found that prior to the onset of meiosis in the ovary, germ cells of both sexes pass through a meiotically competent state allowing them to respond to environmental cues, including retinoic acid levels. Expanding on these results, I have found that meiotic competence is one aspect of a broader cellular state, that of the gametogenesis competent cell. These cells are primed to respond to the gonadal environment in which they find themselves by initiating either oogenesis or spermatogenesis. These results challenge some basic assumptions about the development of the germ lineage and allow us to draw a more refined model as to how germ cells acquire sexual fate in mammals.

With respect to meiosis, the results presented here resolve at least one long-standing debate about the nature of mammalian meiotic initiation. Substantial work has been devoted to determining whether meiotic initiation is driven by extrinsic environmental signals or germ cell intrinsic factors (Byskov, 1974; Byskov and Saxen, 1976; McLaren, 1983; McLaren and Southee, 1997; Upadhyay and Zamboni, 1982). The discovery that retinoic acid (RA), produced outside of embryonic germ cells, is an essential driver of meiotic initiation in the embryonic germline established that at least one extrinsic cue plays a role in meiotic initiation (Bowles et al., 2006; Koubova et al., 2006). The results presented in Chapter 2 of this thesis, reveal that both this extrinsic signal must be coupled with expression of the germ cell intrinsic *Dazl* gene in order to produce functional meiotic initiation. Thus the answer to the long-standing debate as to

the nature of the meiosis initiating signal is that both intrinsic and extrinsic factors are necessary for meiotic initiation in embryonic germ cells.

Analysis of the meiotic phenotype of *Dazl*-deficient embryos enabled the conclusion that germ cells transit through a sex non-specific cellular state that is competent to begin meiosis. The nature of this cellular state remains rather poorly characterized. We have identified expression (but not chromosomal loading) of the synaptonemal complex component SYCP3 as a molecular marker of this meiosis competent state. It will be interesting to further examine the expression profile of this cellular state to determine if other synaptonemal complex (SC) or meiotic cohesin complex components are expressed as well. Studies of SYCP1, a component of the central element of the SC is not expressed in embryonic male and female germ cells, suggesting heterogeneity in the expression of SC components (Di Carlo et al., 2000).

Meiosis competent germ cells are characterized by their ability to respond to RA by inducing expression of the *Stra8* gene. The molecular nature of this transition remains completely unknown. Two major hypotheses about the RA responsiveness of meiosis competent germ cells are possible. Firstly, germ cells that do not become meiosis competent could be completely irresponsive to environmental RA signals. Alternatively, the failure of expression of *Stra8* in response to RA in non-meiosis competent germ cells, could represent a failure to respond to RA signaling in a germ cell-typical manner. To test these hypotheses, I examined expression of the retinoic acid receptor (RAR) genes in wild-type and *Dazl*-deficient embryonic ovaries. RARs are nuclear receptors, which serve to potentiate the RA signal, and *Rarb* in particular is a canonical direct target of RA signaling. I found that while all three RAR isoforms were expressed in the wild-type

embryonic ovary, their expression is decreased in *Dazl*-deficient ovaries (Fig.1, a&b). In particular *Rarb* expression is not detectable in the E14.5 *Dazl*-deficient ovary (Fig. 1b). These results suggest that *Dazl*'s role in regulating germ cell response to RA is likely a general effect on RA signaling and does not only effect germ cell specific RA targets.

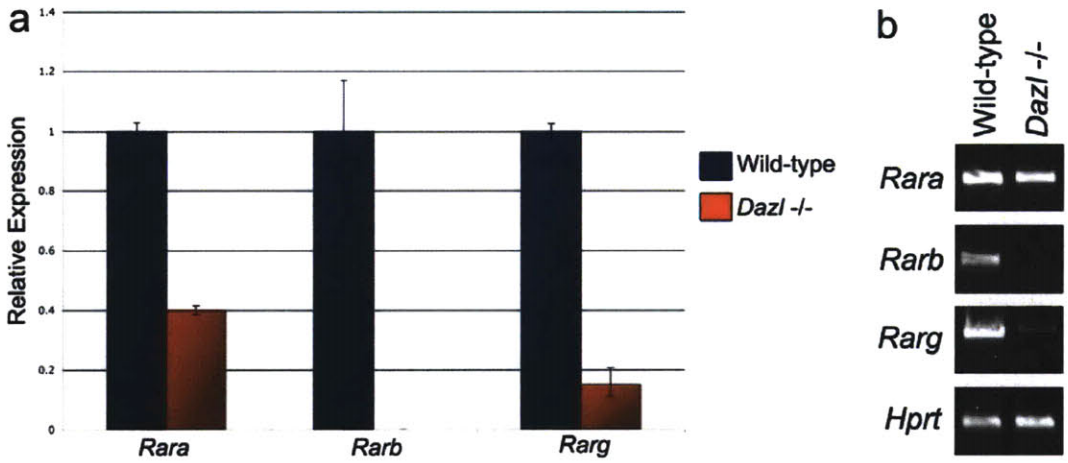

Fig 1. *Dazl* is required for embryonic ovarian expression of retinoic acid receptors. (a) Quantitative RT-PCR analysis of *Rara*, *Rarb* and *Rarg* mRNA levels in wild-type and *Dazl*-deficient E14.5 ovaries. Plotted here are average fold changes, normalized to *Hprt*, in three independent biological replicates. Error bars represent SD among biological replicates. (b) Agarose gel of conventional RT-PCR of *Rara*, *Rarb*, *Rarg* and *Hprt* in wild-type and *Dazl*-deficient E14.5 ovaries.

Several questions remains regarding the role of *Dazl* in establishing meiotic competence. The nature of the molecular interaction between *Dazl* and the RA signaling pathway remains wholly un-elucidated. The mouse DAZL protein is an mRNA-binding protein with putative translational regulatory functions. This makes it unlikely that DAZL is directly involved in the transcriptional regulatory role of the RA signaling pathway and likely functions through a more indirect means. One approach to identify the mechanism of this interaction would be to identify DAZL-interacting mRNAs. Prior studies that have done this have not identified any known components of nuclear receptor signaling pathways as direct interactors of DAZL (Reynolds et al., 2005). This is likely

due to the fact that these studies have focused on genes expressed specifically in adult testicular germ cells. It seems likely that some DAZL interacting mRNAs may be either unique to the embryonic germline or may even be ubiquitously expressed. Thus a more systematic approach to identifying DAZL interacting mRNAs would be more illuminating.

An additional question involves the sufficiency of RA to induce meiosis in embryonic germ cells. While it has been shown that RA is required for embryonic ovarian germ cells to initiate meiosis, the sufficiency of RA to drive all embryonic germ cells into, and through, meiosis has not been clearly determined. It will be interesting to determine if *Dazl*'s role in the regulation of RA signaling is its only role in the initiation of meiosis or if additional factors involved in meiotic initiation might also be *Dazl* dependent.

Chapter 3 of this thesis extends the analysis of *Dazl*'s role in the embryonic germline beyond meiosis into a more general study of sexual differentiation. Disentangling the role of meiotic initiation in male and female fate determination is an interesting, and overlooked, question. Our results reveal that the lack of meiotic initiation is not sufficient to induce male germ cell fate. It has not been determined whether the presence of meiotic initiation is sufficient to prevent male differentiation or whether meiotic initiation is necessary for female differentiation. These questions are likely best answered by analyzing mutants other than *Dazl*, as the role of *Dazl* at its extreme upstream position within the germline sex determination pathway would likely make interpretation of these results difficult. *Stra8*-deficient embryonic ovarian germ cells do not initiate meiosis, but are still able to respond to retinoic acid signaling and so would

provide one interesting mutant for this sort of analysis. *Cyp26b1*- or *Nanos2*-deficient mice would allow the analysis of this process in male embryonic germ cells where meiosis is initiated.

The other major question in the study of germline sex determination is what are the signals that drive germ cells to become male or female. RA serves as one example of a pathway likely driving female differentiation. However, examination of non-meiotic female differentiation (specifically the expression of *Figla*) suggests that RA is not sufficient to fully feminize embryonic germ cells (Fig 2). Thus it would be interesting to examine what factors drive expression of *Figla* in the embryonic ovary.

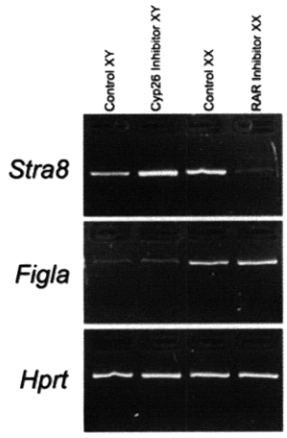

Figure 2: RA is neither necessary or sufficient for *Figla* expression in embryonic gonads. E12.5 XY gonads were cultured for 48 hours in the presence of Cyp26 inhibitor (R115866) or vehicle control. E12.5 XX gonads were cultured for 48 hours in the presence of RAR inhibitor (BMS-204493) or vehicle control. RNA was extracted from gonads and RT-PCR for *Stra8*, *Figla* and *Hprt* was performed.

In addition, while we know that the absence of RA signaling is one important regulator of male germ cell differentiation (Bowles et al., 2006; Koubova et al., 2006), the results in Chapter 3 indicate that signaling pathways exist within the developing testis. The nature of these pathways remains unknown. Given the absence of sex-specific differentiation in the *Dazl* mutant, one might hypothesize that these gonads lack responsiveness to these factors in addition to RA. One approach to identify these unknown pathways would be to examine signaling pathways that fail to activate (or be

inactivated) in *Dazl*-deficient germ cells. These pathways would be particularly good candidates for being involved in germ cell sex determination.

Ultimately examining the function of the DAZL protein *in vivo* in the mouse is one of the most obvious and fundamental questions raised by this research. Even the simple question of whether *in vivo* DAZL's function in the mouse involves translational regulation has not been answered. All experiments to date examining mammalian DAZL's molecular function have utilized heterologous systems (Lee et al., 2006; Reynolds et al., 2007; Reynolds et al., 2005). Examining endogenous DAZL in the context of a mouse germ cell where its endogenous target genes are expressed will be very important in determining its function. A system to enable this process has recently become available with the long-term culture of germline stem cells (GSCs) (Kanatsu-Shinohara et al., 2003). GSCs endogenously express high levels of DAZL as well as two of its candidate mRNA interactors, MVH and SYCP3 (Reynolds et al., 2007; Reynolds et al., 2005). Using this system should allow analysis of DAZL function in a more *in vivo* relevant manner.

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Appendix 1:

Additional meiotic and PGC expression defects in *Dazl*-deficient embryonic ovaries

In Chapter 2 of this thesis, the necessity of normal *Dazl* function for the process of meiotic initiation in the mouse embryonic ovary was discovered. These conclusions are based upon the discovery that *Dazl* is required for the expression of *Stra8*, a key meiotic regulator, as well as its down-stream functions, namely expression of the topoisomerase-like gene *Spo11*, the meiotic recombinase *Dmc1*, and the modified histone variant γH2AX. In addition, independently of *Stra8* function, *Dazl* is also required for the expression of *Sycp3*, a component of the synaptonemal complex (SC) and *Rec8*, a meiosis specific component of the cohesin complex. In order to extend and confirm the conclusions of Chapter 2, the expression of additional genes involved in meiosis was investigated in E14.5 wild-type and *Dazl*-deficient XX gonads by quantitative RT-PCR.

Sycp1 (synaptonemal complex protein 1), *Sycp2* (synaptonemal complex protein 2), *Syce1* (synaptonemal complex central element protein 1) and *Tex12* (testis expressed gene 12) encode components of the SC (Bolcun-Filas et al., 2009; de Vries et al., 2005; Hamer et al., 2008; Yang et al., 2006). As with the previously examined SC component, *Sycp3,* expression of these genes was significantly decreased in E14.5 *Dazl*-deficient XX gonads, relative to wild-type controls (Figure 1).

Stag3 (stromal antigen 3) and *Smc1b* (structural maintenance of chromosomes 1B) encode meiotic specific components of the cohesin complex (Pezzi et al., 2000; Revenkova et al., 2004). As with *Rec8*, these genes also depend upon *Dazl* function for their normal expression in the E14.5 embryonic XX gonad (Figure 1).

Msh4 (mutS homolog 4 (*E. coli*)) and *Msh5* (mutS homolog 5 (*E. coli*)) encode proteins of the mismatch repair pathway that are required for normal meiosis I synapsis

(Edelmann et al., 1999; Kneitz et al., 2000). Normal expression of both of these genes requires the activity of *Dazl* in the embryonic XX gonad (Figure 1).

Mei1 (meiosis defective 1) is a vertebrate specific gene required for meiotic progression that is upstream of *Dmc1* expression in the mouse (Reinholdt and Schimenti, 2005). It was first identified in a genetic screen for defects in gametogenesis (Munroe et al., 2000). As with its downstream target *Dmc1*, *Mei1* expression is dependent on *Dazl* in the embryonic ovary (Figure 1).

Finally, *Hormad1* (HORMA domain containing 1) encodes a protein with sequence similarity to the *Saccharomyces cerevisiae Hop1* protein, which is involved in early meiosis in yeast, and which localizes to unsynapsed meiotic chromosomes (Fukuda et al.; Wojtasz et al., 2009). In E14.5 *Dazl*-deficient XX gonads expression of *Hormad1* is significantly reduced relative to wild-type controls (Figure 1). Thus expression of all 15 examined meiotic genes requires the activity of the *Dazl* gene, confirming the importance of *Dazl* in the initiation of meiosis.

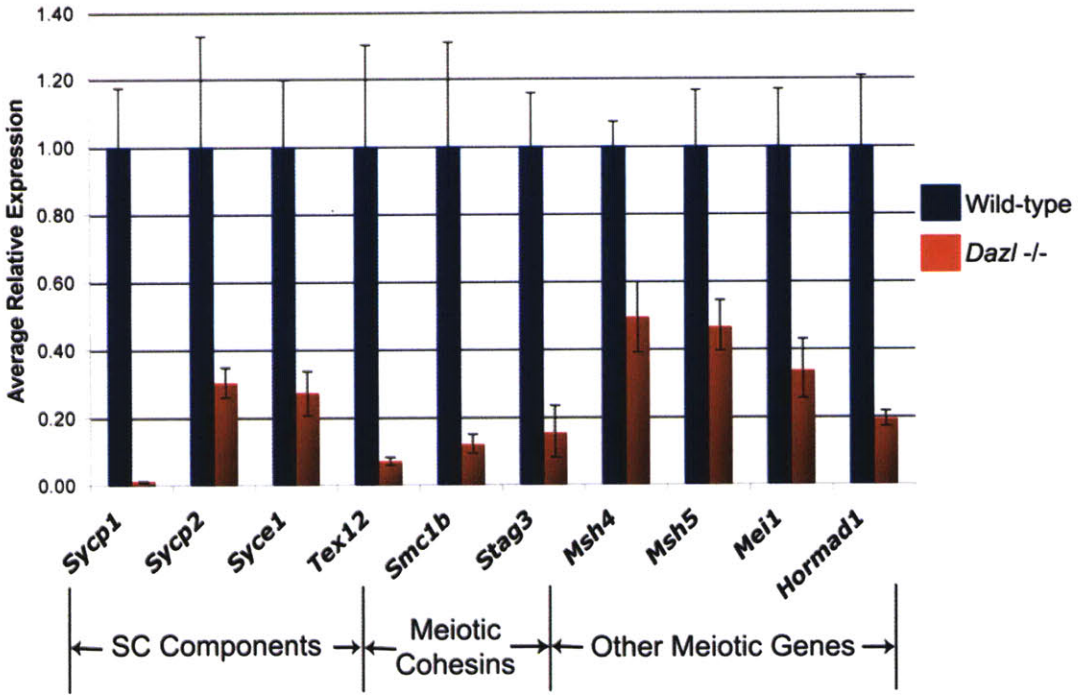

Figure 1: Expression of meiotic genes in wild-type and *Dazl*-deficient embryonic ovaries. Quantitative RT-PCR analysis of *Sycp1, Sycp2, Syce1, Tex12, Smc1b, Stag3, Msh4, Msh5, Mei1 and Hormad1* mRNA levels in E14.5 wild-type and *Dazl*-deficient XX gonads. All data are normalized to *Hprt*. Plotted here are average fold changes of three independent biological replicates. Error bars represent standard deviations among biological replicates.

In Chapter 3 of this thesis, *Dazl*-deficient germ cells were shown to retain a primordial germ cell like expression profile. This profile was shown by examining *Oct4*, *Sox2, Nanog* and *Dppa3* expression. In addition to being expressed in primordial germ cells these markers are also associated with cellular pluripotency. To extend the analysis of the cellular state of *Dazl*-deficient embryonic germ cells, more markers of pluripotent cells were examined in E14.5 wild-type and *Dazl*-deficient XX gonads by quantitative RT-PCR.

In 2003, Bortvin et al. characterized several genes whose expression pattern was similar to *Oct4*, using an *in silico* approach. One of these genes *Dppa3* was examined in detail in Chapter 3. An additional four genes from this screen, *Dppa2* (developmental

pluripotency associated gene 2), *Dppa4* (developmental pluripotency associated gene 4), *Dppa5* (developmental pluripotency associated gene 5; also known as *Esg1*) and *Ndp52* (nuclear domain 10 protein 52, also known as *Calcoco2*) were found to be expressed in the embryonic germline. Expression of all of these genes were found to be significantly higher in E14.5 *Dazl*-deficient XX gonads relative to controls (Figure 2).

fragilis (also known as *Ifitm3* (interferon induced transmembrane protein 3)) was first identified on the basis of its selective upregulation in early specified primordial germ cells relative to surrounding epiblast cells (Saitou et al., 2002). It has been hypothesized to play a role in sensitizing germ cells to signaling factors from their environments (Saitou et al., 2002). As with other markers of early primordial germ cells, expression of *fragilis* was elevated relative to wild-type in E14.5 *Dazl*-deficient XX gonads (Figure 2).

Utf1 (undifferentiated embryonic cell transcription factor 1) is a transcription factor that is expressed specifically in early embryos, pluripotent cells in culture and germ cells (Okuda et al., 1998; van Bragt et al., 2008). It has been proposed to be a crucial downstream target of *Oct4* in pluripotent cells (Nishimoto et al., 2005). As with its upstream regulator *Oct4,* expression of *Utf1* is significantly upregulated in the XX gonads of *Dazl*-deficient mice (Figure 2).

Mitsui et al. 2003 identified genes whose expression in the blastocyst was restricted to the inner cell mass. This screen identified the key pluripotency factor *Nanog* (Mitsui et al., 2003). In addition two other factors were identified, *AB211064* (now known as *L1td1* and *Fbxo15* (F-box protein 15). As with *Nanog* both *L1td1* and *Fbxo15* expression are maintained in *Dazl*-deficient E14.5 XX gonads (Figure 2). Thus in addition to the retention of expression of the four markers of primordial germ cells

outlined in Chapter 3, expression of an additional eight marker genes, associated with pluripotency or primordial germ cells, is also retained in E14.5 *Dazl*-deficient XX germ cells.

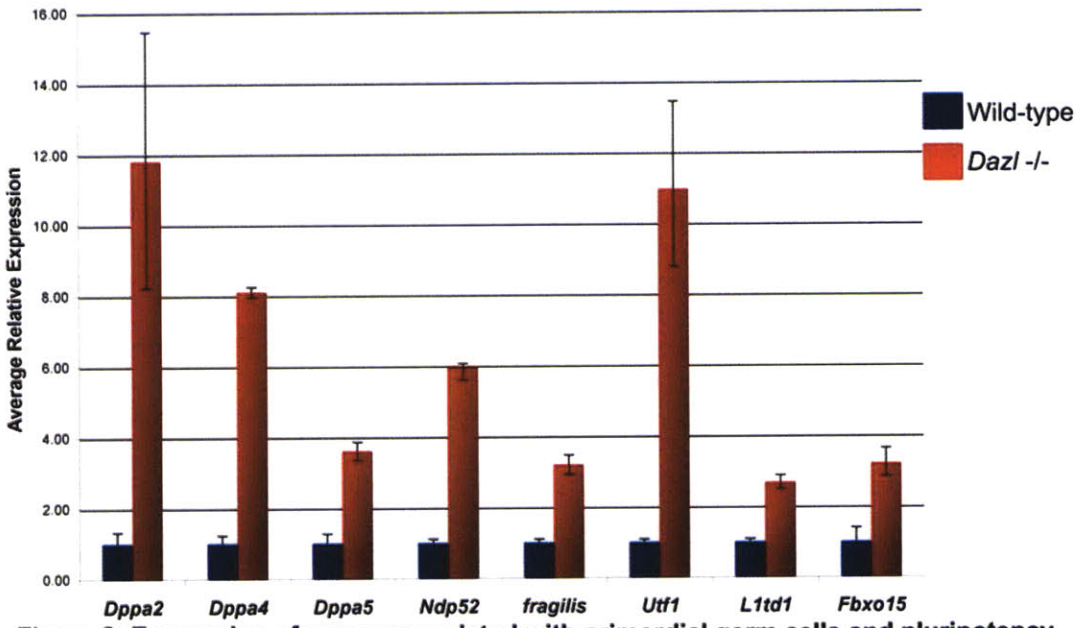

Figure 2: Expression of genes associated with primordial germ cells and pluripotency in wild-type and *Dazl*-deficient embryonic ovaries. Quantitative RT-PCR analysis of *Dppa2, Dppa4, Dppa5, Ndp52, fragilis, Utf1, L1td1*, and *Fbxo15* mRNA levels in E14.5 wild-type and Dazl-deficient XX gonads. All data are normalized to *Hprt*. Plotted here are average fold changes of three independent biological replicates. Error bars represent standard deviations among biological replicates.

Materials and Methods:

Quantitative RT-PCR. Embryonic gonads were dissected away from mesonephroi, placed in Trizol (Invitrogen), and stored at -80°C. Following genotyping, total RNAs were prepared according to the manufacturer's protocol. Total RNAs were then DNase treated using DNA Free Turbo (Ambion). 200 ng of total RNA was reverse transcribed using a RETROScript kit (Ambion). Quantitative PCR was performed using SYBR Green Core PCR Reagents (Applied Biosystems) on an ABI9700 Fast Real-time PCR

machine (Applied Biosystems). Results were analyzed using the delta-delta Ct method using *Hprt* (hypoxanthine-guanine phosphoribosyltransferase) as a normalization control. RT-PCR primers for all genes were picked from the PrimerBank web site (Wang and Seed, 2003) and are listed below in Table 1.

Gene Symbol	Gene Name	PrimerBank ID	Forward Primer	Reverse Primer
Sycp1	synaptonemal complex protein 1	735904a1	TGAGGGGAAGCTCACGGTT	CGAACAGTGTGAAGGGCTTTTG
Syce1	synaptonemal complex central element 1		CGAAGAAGACAGCTCATGGA	ATTAGGTCCTGCTTGATGGG
Tex12	testis expressed gene 12	13385150a1	TGGCAAACCACCTTGTAAAACC	TGCTCATATCGCTCAAATCCTTC
Smc1b	structural maintenance of chromosomes 1B	17978290a3	GAAGATGGCATACGAGCCTTAG	CTTCCGTCTGACTTGCTCCT
Stag3	stromal antigen 3	8394370a2	ACAGAGACTCACAGGGAACTG	TCTTCCGATGTTTTGCTGCTG
Msh4	mutS homolog 4 (E. coli)	13994197a1	CTGCGCGATTACAGCACTG	GTGGCTTCGAGCACTCCAA
Msh5	mutS homolog 5 (E. coli)	12856483a1	CCGGAAACTACTCCTTCATCTCA	CCAATTCTTCTTCGACTCAGGAA
Mei1	meiosis defective 1		CATCAAGTGAGCATGCTGTG	ACTGGGTGTGGTCTGATGAA
Hormad1	HORMA domain containing 1	12853078a2	GGCTCCTAGCTGTTTCAGTATCT	GCATCCACTTCACTAGCTGTG
Dppa2	developmental pluripotency associated 2	12846402a1	TCAACGAGAACCAATCTGAGGA	GCGTAGCGTAGTCTGTGTTTG
Dppa4	developmental pluripotency associated 4	21312074a3	GAGGAACGTCCCTTACTCAGC	CAGAGGAACTGTCACCTCAGA
Dppa5 (Esg1)	developmental pluripotency associated 5	27228963a1	ATGATGGTGACCCTCGTGAC	ACCTCGATAAGTTCTTCGGGAG
Ndp52	nuclear domain 10 protein 52	12846522a1	GCCCCATACCTACCTTGCTG	TCGAGGGATGAACTTTTCAGTG
fragilis (Ifitm3)	interferon induced transmembrane domain containing 3	21539593a1	CCCCCAAACTACGAAAGAATCA	ACCATCTTCCGATCCCTAGAC
Utf1	undifferentiated cell transcription factor 1	6678525a1	TGTCCCGGTGACTACGTCT	CCCAGAAGTAGCTCCGTCTCT
L1td1 (AB211064)	LINE-1 type transposase domain containing 1		CCGTCAGTTTACCAGCAGAA	TTACCCAATCTTTCCCGAAG
Fbxo15	F-box protein 15	6456112a1	TCGTGGGACTGAGCACAACTA	TGACAGATGAGCCTCTAACAAAC

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