# Oocyte differentiation is genetically dissociable from the meiotic program in mice

by

Gregoriy A. Dokshin

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Signature of Author:

Department of Biology

Certified by:

David C. Page
Professor of Biology
Thesis Supervisor

Accepted by:

Stephen P. Bell
Professor of Biology
Chair, Committee for Graduate Students

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## ABSTRACT:

Oogenesis is a developmental program by which a gametogenesis-competent germ cell becomes a fertilization-competent egg. During oogenesis, growth and differentiation of oocytes are closely coordinated with initiation and progression through meiosis. In mammals, the timing of meiotic initiation is sexually dimorphic, with only ovarian and not testicular germ cells initiating meiosis during fetal development. Consequentially, fetal meiotic initiation is thought to be prerequisite to subsequent growth and differentiation of the ovarian germ cell into a fully grown oocyte. Here I present evidence that meiotic initiation and prophase I are genetically separable from oocyte growth and differentiation, thereby, demonstrating that oogenesis consists of two independent processes under separate regulation. This represents a novel view of the oogenesis program and revises the current model of germ cell commitment to oogenesis in mice. The proposed revised model accounts for independent commitment of a germ cell to meiosis and differentiation. This model may provide insights into previously unexplained cases of female infertility and has practical implications for *in vitro* oogenesis strategies.

Thesis Supervisor: David C. Page
Title: Professor of Biology

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## Introduction

In sexually reproducing animals, sex cells (called gametes) serve as a link between generations. In species with heterogametic sexes, the large female gamete is called an egg, while the small motile male gamete is called a sperm. Despite their stark differences, sperm and eggs come from the same pool of precursor cells called the primordial germ cells (PGCs). How PGCs choose to become one of these highly specialized cell types is a fundamental question in germ cell development. There are ongoing efforts to answer this question in a number of different model systems; here I will limit my discussion to the best-studied mammalian model, the mouse.

In recent years a major advance in our understanding of the sperm/egg decision in mouse came when a new developmental state that follows the PGC state but precedes sexual differentiation was described. Germ cells in this state are called gametogenesis competent cells (GCCs) and possess the competence to undergo sexual differentiation as sperm or eggs, but have not yet done so (Lin et al. 2008). The developmental program by which a GCC becomes an egg is called oogenesis and its counterpart in males is called spermatogenesis. Thus the problem of the sperm/egg decision is currently modeled as a single choice made by a GCC: whether to embark on oogenesis or spermatogenesis. This model assumes that spermatogenesis and oogenesis are cohesive programs under a single regulatory switch. Alternatively, a GCC could face not one but multiple decisions when committing to oogenesis.

At fertilization a sperm cell and an egg cell fuse to yield a one-cell embryo called a zygote. An egg must supply the zygote with exactly one copy of each chromosome (a haploid maternal genome) and must also be capable of undergoing fertilization and thereby giving rise to a cleaving embryo. The former is accomplished through meiosis, and the latter through oocyte differentiation. These two components of oogenesis are closely coordinated and generally

believed to be interdependent. The work presented in this thesis is the first direct test of the functional relationship between initiation of meiosis and oocyte differentiation. Herein I demonstrate that meiotic initiation and prophase I are genetically separable from oocyte differentiation in mouse. This new understanding necessitates a revision of the current model of the sperm/egg decision to account for the independent commitment to meiosis and to oocyte differentiation. Thus commitment to oogenesis requires not one but at least two decisions on the part of the ovarian germ cell.

In this chapter I begin by reviewing the origins of the germ line in mouse. I then introduce two components of oogenesis: meiosis and oocyte differentiation, focusing on their individual roles in preparing a functional egg. Finally, I examine the available data on the regulation of the sperm/egg decision, much of which was generated under the assumption of the single decision model. In chapter 2 I will present the work that demonstrates genetic independence of oocyte differentiation from meiotic initiation and prophase. In the final chapter I will discuss the revised model of oogenesis, its implications for our understanding of the sperm/egg decision, its practical applications to *in vitro* oogenesis, and potential future experiments this work motivates.

**Origins of the germ line: induction, migration, and pluripotency**

In many animals, such as worms, flies, fish, and frogs, the germ line is specified by "preformation", meaning germ cell fate determinants (also known as the germ plasm) are inherited by a subset of cells in the embryo, designating them as the germ line precursor cells. By contrast in mouse and other mammalian embryos, no cells are committed to the germ cell lineage through early embryogenesis. Instead the germ cell precursors are induced after the

blastocyst stage (McLaren 2003; Lesch and Page 2012; Hayashi, de Sousa Lopes, and Surani 2007; Extavour and Akam 2003). Supported by bone morphogenic protein (BMP) signaling from the extra-embryonic ectoderm, the precursors of germ cells arise as a cluster of *fragilis* (also known as *Ifitm3* [interferon induced transmembrane protein 3]) mRNA-expressing cells. These cells arise in the proximal epiblast (at the base of the allantois structure) of the pre-gastrulation embryo (Saitou, Barton, and Surani 2002). As the somatic cells of the embryo differentiate and lose their pluripotency, a subset of these precursor cells in the posterior of the *fragilis*-positive cluster maintains expression of pluripotency markers *Oct4* (octamer-binding transcription factor 4; also known as *Pou5f1*), *Nanog*, *Sox2* (SRY-box containing gene 2), and *Tnap* (tissue non-specific phosphatase) and represses activation of somatic genes (Vincent et al. 2005; Ohinata et al. 2005). These cells become the PGCs identifiable by expression of *Dppa3* (developmental pluripotency associated 3; also known as *Stella*) at mouse embryonic day (E) 7.0 (Hayashi, de Sousa Lopes, and Surani 2007; McLaren 2003; Lesch and Page 2012). PGCs eventually give rise to either oocytes or sperm. During gastrulation PGCs actively migrate from their origin through the hindgut towards the urogenital ridge, where they colonize the newly formed gonads (Kunwar, Siekhaus, and Lehmann 2006). Once in the gonads PGCs are called gonocytes.

In mouse, PGCs arrive in the gonads around E10.5, when somatic sex is beginning to be determined. (Prior to sex determination the developing gonad is bipotential and capable of becoming either a testis or an ovary.) Somatic sex determination is initiated around E10.5, when *Sry* (sex determining region of chromosome Y) becomes expressed in the somatic cells of the XY gonad and drives it to differentiate into a testis. In the absence of *Sry* the XX gonad differentiates into an ovary (Brennan and Capel 2004). As I will discuss later, the phenotypic

sex of the somatic gonad controls the germ cells' decision to enter meiosis during fetal development, and may play an instructive role in the sperm/egg decision. The earliest events of gonocyte development, however, unfold identically between the ovarian (XX) and testicular (XY) germ cells.

Upon arrival in the gonads, both XX and XY germ cells express gonocyte markers including *Dazl* (deleted in azospermia like), *Mvh* (mouse vasa homolog), and *Gcna1* (germ cell nuclear antigen 1) and down regulate *Oct4*, *Nanog*, *Sox2*, *Tnap*, and *Dppa3*. Expression of *Dazl* marks a major developmental transition termed licensing, as gonocytes become GCCs (Fig. 1). Both XX and XY GCCs divide mitotically several times and express *Sycp3* (synaptonemal complex protein 3) characteristic of the pre-meiotic state (Chuma and Nakatsuji 2001; Di Carlo, Travia, and De Felici 2000). Without *Dazl*, germ cells fail to down regulate pluripotency genes, fail to express *Sycp3*, do not initiate meiosis, and do not express sex-specific markers such as *Figla* (factor in the germline alpha) and *Nanos2* (nanos homolog 2) (Gill et al. 2011; Lin et al. 2008).

In the pre-meiotic state (in mouse at approximately E12.5), GCCs face an important developmental decision: whether to become sperm or eggs. Ovarian (XX) GCCs initiate meiosis, as they enter oogenesis to become eggs. By contrast, testicular (XY) GCCs down regulate *Sycp3* and arrest proliferation. They will embark on spermatogenesis and initiate meiosis only after birth. Thus, the earliest difference between ovarian (XX) and testicular (XY) GCCs is fetal meiotic initiation in the ovary (McLaren 1984, 2003; Lesch and Page 2012) (Fig. 1).

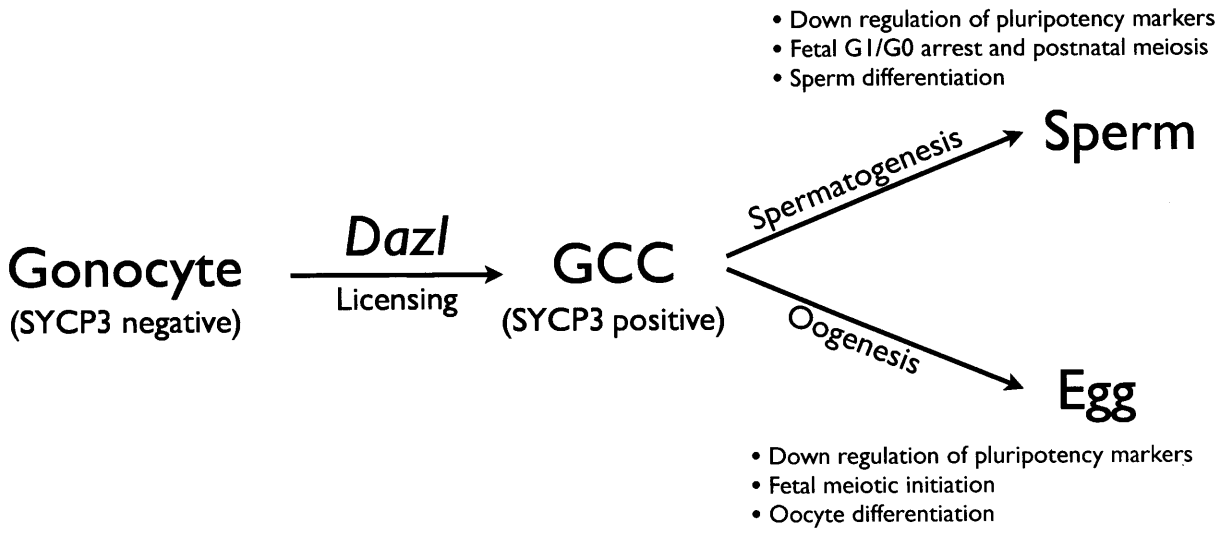



**Figure 1: Licensing gametogenesis**
*Dazl* mediates the germ cell's transition form a naïve gonocyte to a gametogenesis competent cell (GCC), a process termed licensing. During licensing germ cells enter pre-meiotic state characterized by expression of SYCP3. After licensing GCCs face a choice of whether to undergo spermatogenesis or oogenesis. During both programs germ cells down regulate pluripotency markers, initiate meiosis and undergo sex-specific differentiation. Note that the timing of meiotic initiation differs between sexes.

**What is meiosis?**

In animals most somatic cells are diploid, meaning they contain two copies of each chromosome. These cells proliferate by mitosis, a cell cycle in which one round of chromosome replication is followed by one cell division (Fig. 2a). Prior to replication each chromosome consists of one chromatid. During replication the chromatids are copied, such that after replication each chromosome consists of two identical chromatids. During the mitotic division sister chromatids separate and segregate, such that exactly one chromatid of each chromosome goes to a given daughter cell. The result is that each daughter cell receives the same chromosomes (in number and identity) as the mother cell, thus preserving the diploid state.

Unlike somatic cells, gametes are haploid, meaning they contain just one copy of each chromosome. These haploid cells arise from diploid precursors via meiosis. During meiosis one round of chromosome replication is followed by two rounds of cell division (Fig. 2b). Unlike in mitosis, during the first meiotic division homologous chromosomes segregate, such that one homolog (consisting of two chromatids) goes to each daughter cell, thereby reducing ploidy by half. During the second division, sister chromatids segregate, much like in mitosis. By the time they complete the second meiotic division, gametes contain half the number of chromosomes of the mother cell and only one copy of each chromatid (note: the egg does not complete the second meiotic division until fertilization and, therefore, unfertilized eggs contain two copies of each chromatid). In order to ensure proper segregation during both meiotic divisions sister chromatids must be cohesed and homologous chromosomes must be paired. This is accomplished during a specialized meiotic prophase I that follows chromosome replication and precedes the first division. To avoid chromosome mis-segregation a number of events must occur during meiotic

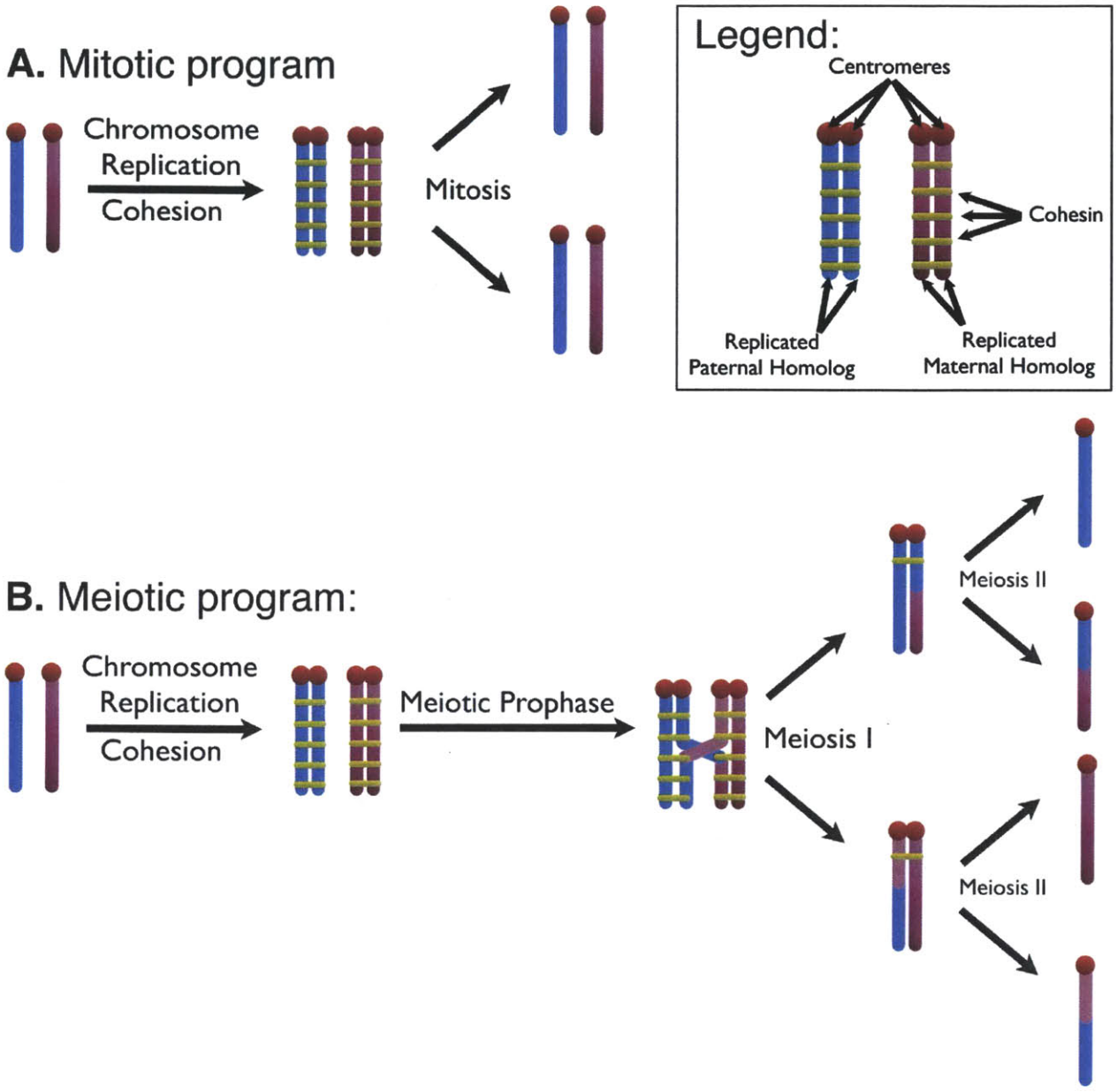



**Figure 2: Comparison of the mitosis and meiosis programs**
During mitosis one round of chromosome replication is followed by one division, preserving the diploid state. During meiosis one round of replication is followed by two divisions, reducing ploidy. A specialized prophase I and sequential removal of cohesin from chromosome arms and pericentric regions during meiosis allows for accurate segregation of homologous chromosomes during the first division and of the sister chromatids during the second division.

prophase I: sister chromatid cohesion, homologous chromosome pairing and synapsis, and (in most cases) homologous recombination.

**Molecular events that define the meiotic prophase**

Historically, in the field of germ cells sex determination, meiotic initiation has been assayed via prophase-associated chromatin condensation detected by hematoxylin staining (Adams and McLaren 2002; McLaren 1984, 2003; McLaren and Southee 1997). However, this approach provided very limited information about the extent of meiotic progression observed. With the development of molecular markers for all functional aspects of the meiotic prophase, we can now unambiguously assay meiotic initiation and progression through all the stages of meiotic prophase I (Fig. 3), giving researchers the ability to study perturbations in meiotic initiation and progression with a high degree of precision. This set of molecular assays should be considered the gold standard for convincingly demonstrating meiotic initiation *in vitro* or *in vivo*. I will now discuss the molecular events of prophase I and their markers in detail.

Meiotic prophase I is subdivided into four stages named after their chromosome morphology (in Greek): leptotene, zygotene, pachytene, and diplotene. The synaptonemal complex (SC) (a proteinaceous structure that forms along the length of paired homologous chromosomes) can be used to visualize and stage meiotic prophase more precisely (Fig. 4). Specifically, one can visualize the assembly of two key components of the SC: SYCP3 (synaptonemal complex protein 3) (forming the axial elements [AEs] along the axis of each homolog) and SYCP1 (forming the central elements [CEs] between the axes of the two homologs). Synapsis is defined by the formation of the SYCP1 CEs. During leptotene, AEs

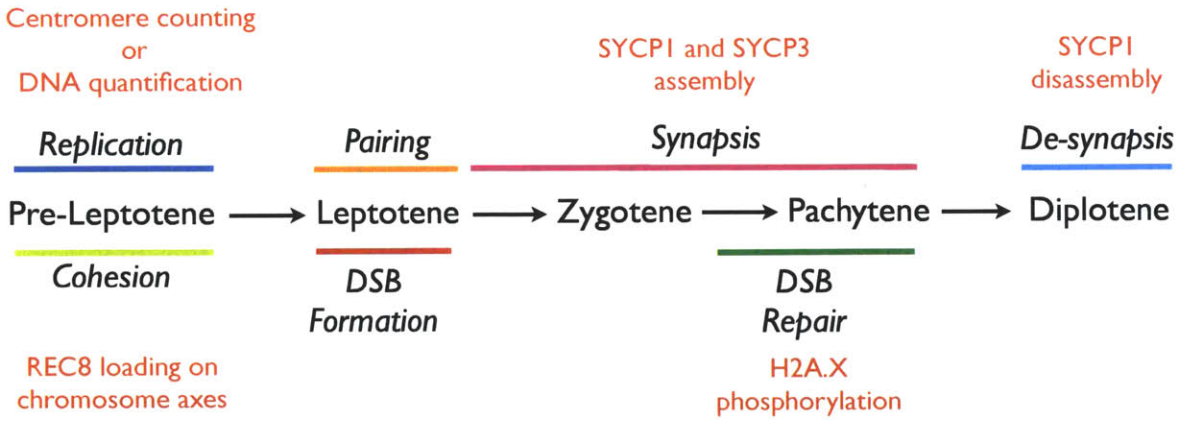



**Figure 3: Functional events of the meiotic prophase**
Major events of pre-meiotic chromosome replication and meiotic prophase overlaid on the stages of meiotic prophase. Molecular assays for these events are shown in red.

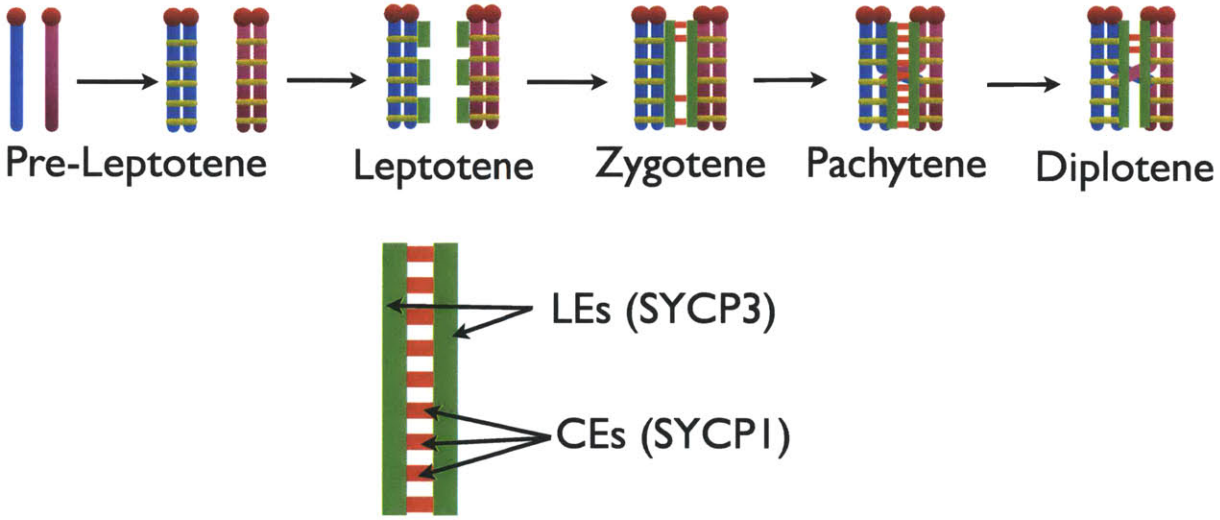



**Figure 4: Behavior of the synaptonemal complex during the meiotic prophase**
Chromosomes are replicated during pre-leptotene. Axial elements (AE) marked by SYCP3 begin to assemble during leptotene and complete assembly by zygotene. Synapsis (formation of lateral elements [CEs] begins during zygotene and is complete in pachytene. (Synapsed AEs are called lateral elements [LEs]. Transition from pachytene to diplotene is marked by initiation of desynapsis (dissolution of the CEs).

begin to form along each chromosomal axis. In zygotene, AE formation is completed and CEs elongate, "zipping up" the axes of the homologs. Pachytene is defined as a stage where no "bubbles" (or areas of asynapsis) exist between the AEs of any two homologs and the CEs are continuous along the axes of each homologous chromosome pair. (Note: synapsed AEs are called lateral elements [LEs]). During diplotene, homologous chromosomes desynapse, losing the CEs (Zickler and Kleckner 1999). Only SYCP3 and SYCP1 localization should be used to assay progression through meiosis, as expression of these proteins is not restricted to meiosis (Fig. 3) (Di Carlo, Travia, and De Felici 2000).

While the SC is specific to meiosis, the cohesin complex holds sister chromatids together in both mitosis and meiosis. However, in addition to the standard mitotic cohesin complex subunits (which are also present during meiosis), the meiotic cohesin complex utilizes a few substitutes, including *Rec8* (recombination 8), *Stag3* (stromal antigen 3), and *Smc1b* (structural maintenance of chromosomes 1B) (Watanabe 2005). The cohesin complex is present prior to chromosome replication, and becomes cohesive as chromatids are replicated, thereby holding the newly replicated chromatids together (Uhlmann and Nasmyth 1998). During the first meiotic division, the REC8 subunit of the cohesin complex is cleaved along the chromosome arms, but remains protected at centromeres (Watanabe 2005). This allows homologs to separate while keeping sister chromatids attached. During the second meiotic division, pericentric REC8 is cleaved, releasing sister chromatids from each other. Meiotic cohesion is most commonly assayed by chromosomal localization of REC8 (Fig. 3). Like *Sycp3*, *Rec8* expression alone is not a marker of meiosis, as it is expressed prior to meiotic initiation (Prieto et al. 2004). Again, protein localization to the chromosomal axes must be assayed.

Homologous recombination is a process of exchange of genetic material between two homologous chromosomes, and can result in a structure termed chiasma that functions to hold together homologous chromosomes. During recombination, double-strand breaks (DSBs) are generated throughout the genome by an enzyme *Spo11* (sporulation protein 11) (Keeney, Giroux, and Kleckner 1997). *Spo11* mutants are deficient for meiotic DSBs and fail to generate crossovers. In addition, *Spo11* function is necessary for completion of synapsis, although the AEs of the SC are fully assembled and some CEs begin to form (Baudat et al. 2000; Romanienko and Camerini-Otero 2000). During repair of *Spo11*-induced DSBs, a meiosis-specific *Rad51* (radiation sensitive 51) homolog *Dmc1* (disrupted meiotic cDNA1) facilitates strand invasion, a key early step in the repair pathway (Bishop et al. 1992). *Dmc1*-mutant oocytes fail to repair *Spo11*-induced DSBs and die (Yoshida et al. 1998; Pittman et al. 1998). Expression of *Dmc1* and *Spo11* can be used to confirm the presence of the recombination machinery. However, some level of these transcripts is present in non-meiotic germ cells, and it is best to use functional assays of recombination.

A simple way to detect the onset of recombination is to assay a cellular response to DSBs. This can be done by staining for γH2A.X, a phosphorylated version of histone variant H2A.X, which is phosphorylated at DSB sites (Rogakou et al. 1998) (Fig. 3). It was recently reported that H2A.X is also phosphorylated in response to asynapsis in later stages of meiosis, thus highlighting the importance of sampling at the right time points (Wojtasz et al. 2012).

The elaborate choreography of chromosomes during the meiotic prophase, including cohesion, synapsis and recombination, is absolutely essential for proper segregation of chromosomes during meiotic divisions. In order to demonstrate meiotic initiation and progression, each of these aspects should be assayed with molecular markers.

**Oocyte differentiation in mouse**

Prior to the onset of meiosis, ovarian germ cells divide mitotically. At least some of these mitotic divisions result in incomplete cytokinesis, creating clusters of interconnected germ cells (Pepling and Spradling 1998; Mork et al. 2012). These clusters are reminiscent of ovarian germ cell cysts in the fruit fly *Drosophila melanogaster* (*D. melanogaster*). (In *D. melanogaster* cysts result from incomplete cytokinesis during the last four mitotic divisions of ovarian germ cells.) It has been proposed that like the *D. melanogaster* ovarian germ cell cysts, the mouse germ cell clusters play a role later in ovarian development, specifically in selecting oocytes that will form the animal's follicle pool (Pepling and Spradling 2001). In mouse, bridges between germ cells are marked by TEX14 (testis expressed gene 14) protein and exist in both males and females. Disruption of *Tex14* ablates the bridges but does not have any detectable effects on female fertility (Greenbaum et al. 2009). Thus, despite their importance in *D. melanogaster* gametogenesis, the role of germ cell cysts in mouse oogenesis remains unclear.

The first functional oocyte-differentiation event in mouse happens when oocytes recruit squamous granulosa cells of the ovarian soma to form primordial follicles, which happens within the first several days after birth (Fig. 5). Animals deficient for oocyte-specific transcription factor *Figla* fail to form primordial follicles, and their oocytes die soon after birth, suggesting a role for *Figla* in primordial follicle formation (Soyal, Amleh, and Dean 2000). However, the mechanism by which *Figla* might be involved in primordial follicle formation remains a mystery.

Primordial oocytes constitute the entire oocyte supply of the female and become recruited to grow and differentiate throughout the female's life. The molecular details of how primordial oocytes are recruited for growth and differentiation remain poorly understood. It is known that

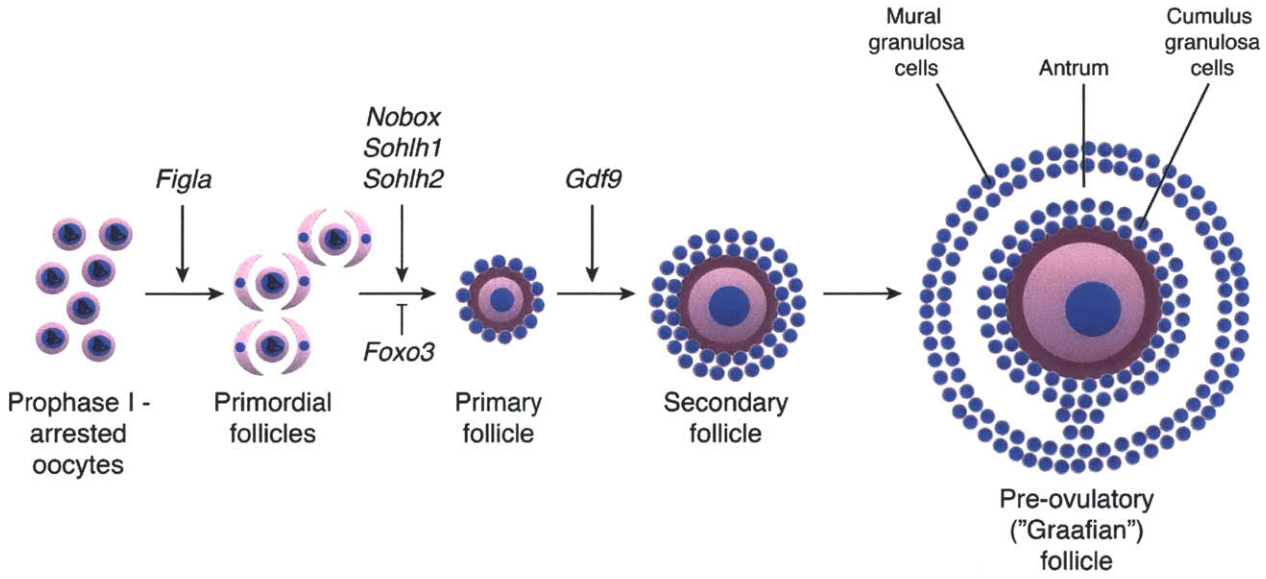



**Figure 5: Oocyte growth, differentiation, and regulation of folliculogenesis**
Oocytes grow and differentiate while driving re-organization of the ovarian soma in a process called folliculogenesis. *Figla*, *Nobox*, *Sohlh1*, *Sohlh2* and *Gdf9* are oocyte-expressed genes required for growth and folliculogenesis. When mutated these genes have oocyte differentiation and folliculogenesis phenotypes at indicated transitions. *Foxo3* is required to repress recruitment of follicles from the primordial follicle pool.

mutation in transcription factor *Foxo3* (forkhead box O3) causes premature recruitment of all primordial follicles. This in turn leads to quick depletion of the primordial follicle pool and premature ovarian failure (Castrillon et al. 2003). Furthermore, *Foxo3* overexpression blocks recruitment of any follicles for growth (Liu et al. 2007). Thus, *Foxo3* somehow restricts recruitment of primordial oocytes from the primordial follicle pool but the molecular mechanism of action is not known.

Once recruited to grow, oocytes induce surrounding granulosa cells to transition from squamous to cuboidal shape, transitioning from primordial to primary follicles (Fig. 5). Three transcription factors – *Nobox* (newborn ovary homeobox), *Sohlh1* (spermatogenesis and oogenesis specific basic helix-loop-helix 1) and *Sohlh2* – are known to be required for primordial to primary follicle transition (Rajkovic et al. 2004; Pangas et al. 2006; Choi, Yuan, and Rajkovic 2008). As oocytes transition from primordial to primary, they begin to undergo extensive cytoplasmic and nuclear growth. A fully grown oocyte's nucleus is called the germinal vesicle (GV) and it is many times the size of a somatic nucleus (Fig. 6). Growing oocytes also begin to generate glycoprotein coats called zonae pellucidae, which consist of oocyte-synthesized glycoproteins ZP1-3 (zona pellucida 1-3) (Bleil and Wassarman 1980). It has been demonstrated that the same oocyte-transcription factor FIGLA that is required for primordial follicle formation binds directly to promoters of the zona pellucida genes *Zp1-3* and drives their expression (Liang, Soyal, and Dean 1997).

As oocytes continue to grow, their surrounding granulosa cells proliferate and become multilayered, forming secondary follicles. Eventually, a fluid-filled cavity called an antrum forms between layers of granulosa cells separating them into the inner (cumulus) and outer

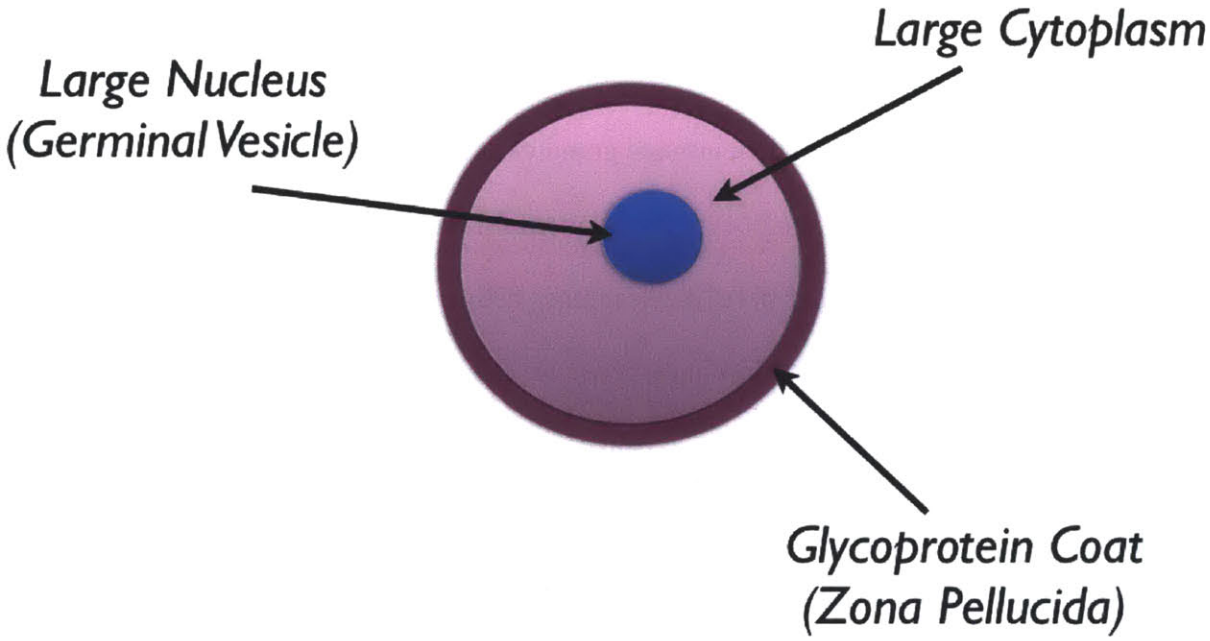



**Figure 6: Defining morphological features of a fully grown oocyte**
Fully grown oocytes feature a large cytoplasm, a large nucleus (germinal vesicle) and a glycoprotein coat (zona pellucida).

(mural) granulosa cells. At this point the follicle is considered antral. The antrum expands to form pre-ovulatory follicles (also known as Graafian follicles) (Fig.5). Progression beyond primary follicles requires an oocyte-expressed TGF-beta (transforming growth factor beta) family growth factor *Gdf9* (growth differentiation factor 9) (Dong et al. 1996). The presence of FSH (follicle stimulating hormone) and its receptor in the ovary are also required (Dierich et al. 1998; Kumar et al. 1997).

Fully grown pre-ovulatory follicles are ovulated in response to an LH (luteinizing hormone) surge (Hawkins and Matzuk 2008). During ovulation, the follicle ruptures and the oocytes are released together with the cumulus granulosa cells, called the COC (cumulus oocyte complex). *Gdf9* signaling from the oocyte is important for the formation and maintenance of the COC (Varani et al. 2002). Furthermore, the granulosa cells mass, also known as cumulus oophorus, is expanded during ovulation in response to signals from the oocyte. Oocyte competence to support cumulus oophorus expansion is gained late in differentiation (Eppig, Wigglesworth, and Chesnel 1993).

During ovulation, oocytes undergo an asymmetric cell division to produce a mature egg cell and a small polar body. They then undergo one more asymmetric division at fertilization to yield a fertilized egg and a second polar body. From then on, the newly formed zygote will cleave symmetrically. Early development is driven by materials (such as mRNAs) stored during oocyte growth (Schultz and Heyner 1992). In mouse, zygotic transcription is activated at the two-cell stage (Schultz 2002).

**Meiotic initiation and prophase precede oocyte differentiation**

Meiosis is closely coordinated with oocyte differentiation events. Once ovarian germ cells initiate meiosis (in mouse around E13.5), they progress through the meiotic prophase I until the diplotene stage, where they enter primary arrest. In mouse, all germ cells are arrested in prophase around the time of birth, prior to forming primordial follicles (Fig. 7).

One study in rat suggests that completion of meiotic prophase I may be functionally necessary for formation of primordial follicles. When researchers manipulate levels of SYCP1, a component of the synaptonemal complex required for synapsis, they observe that oocytes with less SYCP1 desynapse their chromosomes sooner. Desynapsis is a hallmark of progression to diplotene and completion of prophase I. Correlated with early desynapsis, they observe early formation of primordial follicles (Paredes et al. 2005). Although this correlation is intriguing, it does not directly address whether meiotic prophase I is functionally necessary for initiation of folliculogenesis and oocyte differentiation.

Oocytes remain arrested in prophase I while in the primordial follicle pool, and further remain arrested while undergoing growth and differentiation to become fully grown oocytes in pre-ovulatory follicles (Fig. 7). Although the exact point of arrest during prophase I varies among species, growth and differentiation of oocytes during prophase I arrest is a conserved feature among a number of species, including humans, flies, worms, and frogs, among others (Page and Orr-Weaver 1997; Von Stetina and Orr-Weaver 2011). During ovulation, oocytes resume meiosis, progress through metaphase I, and segregate one of each pair of homologous chromosomes to the first polar body. They then align the remaining chromosomes on the second metaphase plate and enter secondary arrest. (Although the phenomenon of secondary arrest is

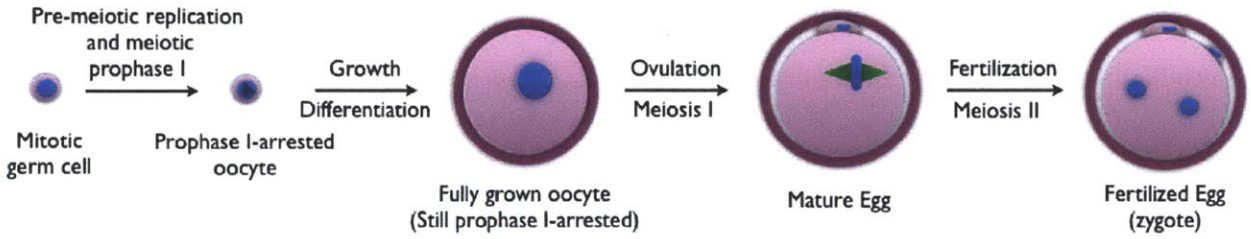



**Figure 7: Coordination of meiotic progression with oocyte differentiation**
Meiotic initiation and prophase I precede oocyte growth and differentiation, which happen during prophase I arrest. Meiosis is resumed at ovulation and completed at fertilization. During meiosis I one of each pair of homologous chromosomes is segregated to the first polar body, while during meiosis II one of each pair of sister chromatids is segregated to the second polar body.

evolutionarily conserved, it does not always occur at metaphase II (Page and Orr-Weaver 1997; Von Stetina and Orr-Weaver 2011). Oocytes that have reached secondary arrest are considered eggs. Eggs will complete meiosis only once activated, such as when fertilized by a sperm. Upon progressing through metaphase II, they segregate one of each pair of sister chromatids to the second polar body (Fig 7).

**Role of sex chromosomes in commitment to oogenesis**

A germ cell's decision to embark on oogenesis could theoretically be made cell autonomously, or could be environmentally induced. Germ cells could rely on their own sex chromosome constitution and become eggs if they were XX or sperm if they were XY. Alternatively, germ cell sex could be instructed by the sex of the gonadal soma. (The decision could also be made via some combination of intrinsic sex chromosome constitution and extrinsic somatic signals.) In theory the question of the source of sex-determining information could be resolved *in vivo* using XX/XY chimeras, which differentiate as either males or females depending on the contribution of the XY cells to the gonadal somatic lineage (Mystkowska and Tarkowski 1968; Palmer and Burgoyne 1991). Some such chimeras would contain germ cells with sex chromosome content that is inconsistent with the animal's phenotypic sex (XY germ cells in phenotypic females and XX germ cells in phenotypic males). One could then simply assay the sex of these germ cells during fetal development, and ask whether their behavior was consistent with the sex chromosome content or with the animal's phenotypic sex.

Such XX/XY chimera experiments were performed in the 1960s and 1970s, but the results were challenging to interpret. Through breeding, examination of spermatids, and direct analysis of spermatogonia and spermatocytes, it was established that XX germ cells were not

present postnatally in the phenotypically male chimeras' germ lines (Mystkowska and Tarkowski 1968; Ford et al. 1975; McLaren 1975; Ford et al. 1974). (This was an inconclusive result, since sperm or oocyte differentiation could not be directly assayed.) Researchers did, however, observe meiotic germ cells in fetal chimeric testes, suggesting perhaps XX germ cells are able to initiate meiosis in the testis on the oogenic timetable. This observation could mean that fetal meiotic initiation is determined by the XX chromosome constitution. Unfortunately, uncertainty confounded this interpretation. At the time it was not possible to determine whether these meiotic cells were XX or XY, and whether the reason for meiotic initiation was germ cell-intrinsic or due to a feminized microenvironment (Mystkowska and Tarkowski 1970). Subsequently, chimeras were made using genetically marked XX and XY cells, such that in the adult the sex chromosome identity of any given cell could be identified. This experiment demonstrated that XX germ cells form pro-spermatogonia in chimeric testes, suggesting that the environment was sex determining. However, the evidence for this claim was based on lack of meiotic prophase chromosomal morphology, and spermatogenesis could not be assayed directly (Palmer and Burgoyne 1991). Thus it is reasonable to conclude that XX germ cells fail at meiotic initiation in the testis, but whether they become truly sex reversed could not be determined.

The converse experiment proved to be even more challenging because only a minority of the chimeras develops as female (Mystkowska and Tarkowski 1968; McLaren 1975). Indeed, during the early experiments only two chimeric females were informative. In 1975 an XX/XY chimeric female was reported to yield a pup with an XXY karyotype, which, researchers concluded, was a result of an XY-bearing oocyte being fertilized by an X-bearing sperm (Ford et al. 1975). Subsequently, a single XY oocyte in a chimeric female was reported (Evans, Ford,

and Lyon 1977). A decade later another set of chimeric experiments produced a hermaphrodite with XY oocytes (Burgoyne and McLaren 1988). These studies support the idea that commitment to oogenesis is independent of the germ cell's own sex chromosome content (XY germ cells could become oocytes in ovarian environment). Thus, commitment to oogenesis either relies on inductive cues from the ovarian soma, or oogenesis is the default pathway in both XX and XY germ cells. In the latter scenario, commitment to oogenesis would have to be prevented by somatic signals from the testis. Whether the testis environment is sufficient to masculinize XX germ cells is not clear.

**The "default pathway" model of commitment to oogenesis**

An important insight into whether the oogenesis pathway is the default pathway came from an observation that germ cells that mis-migrate to the adrenal gland instead of the gonad in wild-type animals (and thus reside in neither a testis or an ovary) differentiate as oocytes regardless of the animal's phenotypic and chromosomal sex (Upadhyay and Zamboni 1982; Zamboni and Upadhyay 1983). These cells appear to initiate meiosis during fetal development and grow during the first few postnatal weeks into oocyte-like cells, complete with zonae pellucidae (Zamboni and Upadhyay 1983). This observation strongly suggests that oogenesis is the default pathway, and that germ cells must be deterred from oogenesis by a testis factor. However, an important caveat to the "oogenesis is default" interpretation is that an oogenesis-inducing signal could exist in the adrenal as well as the ovary. As we will see later this turned out to be exactly the case for the meiosis-inducing signal, retinoic acid.

Pioneering studies by McLaren and colleagues set out to define a window during which meiosis could be prevented by testicular factors. To do this, they aggregated XY testicular germ

cells with XX ovaries at various embryonic time points and observed that prior to E12.5 germ cells would enter meiosis but not thereafter. They also observed meiotic entry when aggregating XY germ cells with fetal lung tissue; reinforcing the idea that oogenesis is the default pathway. McLaren and colleagues concluded that, prior to E12.5, all germ cells would differentiate as oocytes by default and that a testicular meiosis-preventing substance (MPS) blocked this potential (McLaren and Southee 1997).

The converse experiment was designed to test whether the MPS in testicular soma could also block meiotic initiation in XX germ cells. By aggregating XX ovarian germ cells with XY testes at various embryonic time points, they were able to establish that XX germ cells could be prevented from entering meiosis prior to E13.5, but not thereafter (Adams and McLaren 2002). Based on lack of fetal meiotic initiation, they concluded that the testicular soma had driven XX germ cells into spermatogenesis.

Taking the aggregation data together, McLaren and colleagues were able to define the window during which the hypothesized testicular MPS could prevent meiotic initiation (Kocer et al. 2009; McLaren 2003). An important caveat to these interpretations is that, in the aggregation studies, meiosis was assayed by prophase-associated chromosome condensation and, therefore, it is not known how far the ectopic meiosis would progress, and whether it would do so normally. Furthermore, unlike in the Zamboni and Upadhyay adrenal gland observations, sexual differentiation could not be directly assayed in the aggregate studies, and all conclusions about sexual differentiation were made based on the presence or absence of meiosis-associated chromosome condensation. Thus, in these early studies and many studies since, fetal meiotic initiation became a proxy for oocyte differentiation, and the study of germ cell sex determination became the study of fetal meiotic initiation. To state it more explicitly: in these and many

subsequent experiments, interpretations of germ cell sexual fate rely on the assumption that if meiosis is initiated during fetal development, oocyte differentiation will necessarily follow, and if fetal meiosis is blocked, spermatogenesis will follow (Adams and McLaren 2002; Kocer et al. 2009; Krentz et al. 2011; McLaren 1984, 2003; McLaren and Southee 1997).

A competing model to McLaren's MPS was pioneered by Byskov and colleagues. In 1974, Byskov made the observation that, if E12.5 ovaries with the adjacent mesonephroi were transplanted in nude mice for 14 days, their germ cells would initiate meiosis and grow and differentiate as oocytes. However, if mesonephroi were removed prior to transplantation, germ cells would fail at both meiotic initiation and oocyte differentiation and instead resemble the morphology of pre-meiotic oogonia (Byskov 1974). This observation suggested that oogenesis did not occur by default, but instead a signal from the mesonephros was required to induce oogenesis. This hypothetical signal was named the meiosis-inducing substance (MIS). Subsequent experiments showed that co-culture of fetal testes with fetal ovaries induced meiosis in the testis, suggesting the MIS was diffusible and could act in trans (Byskov and Saxen 1976).

It is possible to reconcile the MIS and the MPS models in a joint model that employs both substances (Kocer et al. 2009). In such a model, a germ cell would need an MIS to initiate meiosis, and the MPS in the testis would prevent germ cells from responding to the MIS. To reconcile this model with the adrenal gland and lung aggregate observations (McLaren and Southee 1997; Zamboni and Upadhyay 1983; Upadhyay and Zamboni 1982), the MIS would also have to be present in these two tissues. In considering this joint model it is important to once again note that evidence for existence of an MPS is limited to a block of meiotic initiation, and it is not known (although it is often assumed) that the MPS would also block oocyte differentiation. MIS seems to be required for both meiotic initiation and oocyte differentiation

(Byskov 1974), although it is not known whether it is sufficient to induce oocyte differentiation in the testis (Byskov and Saxen 1976). It is important to keep in mind that, formally, the MIS might consist of several independent signals required for oogenesis. Some such signals might induce meiosis, while others induce oocyte differentiation. These complexities are becoming more apparent as we gain molecular understanding of meiotic initiation.

**Intrinsic competence and extrinsic signals are required for meiotic initiation**

Since the time of the pioneering McLaren and Byskov experiments (McLaren and Southee 1997; Adams and McLaren 2002; Byskov 1974; Byskov and Saxen 1976), we have begun to understand murine meiotic initiation on a molecular level. Several important molecular players have been identified:

- intrinsic meiotic-competence factor – *Dazl* (Lin et al. 2008)
- external meiosis-inducing signal – RA (Bowles et al. 2006; Koubova et al. 2006)
- testicular meiosis-preventing factor – *Cyp26b1* (Bowles et al. 2006; Koubova et al. 2006; MacLean et al. 2007)
- RA-responsive, meiosis-essential gene – *Stra8* (Baltus et al. 2006)

We will now consider these factors in detail (Fig. 8).

In thinking about meiotic initiation in mouse, it may be useful to consider meiotic initiation in budding yeast, *Saccharomyces cerevisiae* (*S. cerevisiae*), where the process is best understood. *S. cerevisiae* require both an intrinsic competence cue (mating type heterodimer that tells a cell it is a diploid) and an environmental signal (nutrient starvation) to initiate meiosis (van Werven and Amon 2011). Although the exact components are not conserved, an analogous mechanism plays out in mouse. Like *S. cerevisiae's* mating-type heterodimer, mouse gene *Dazl*

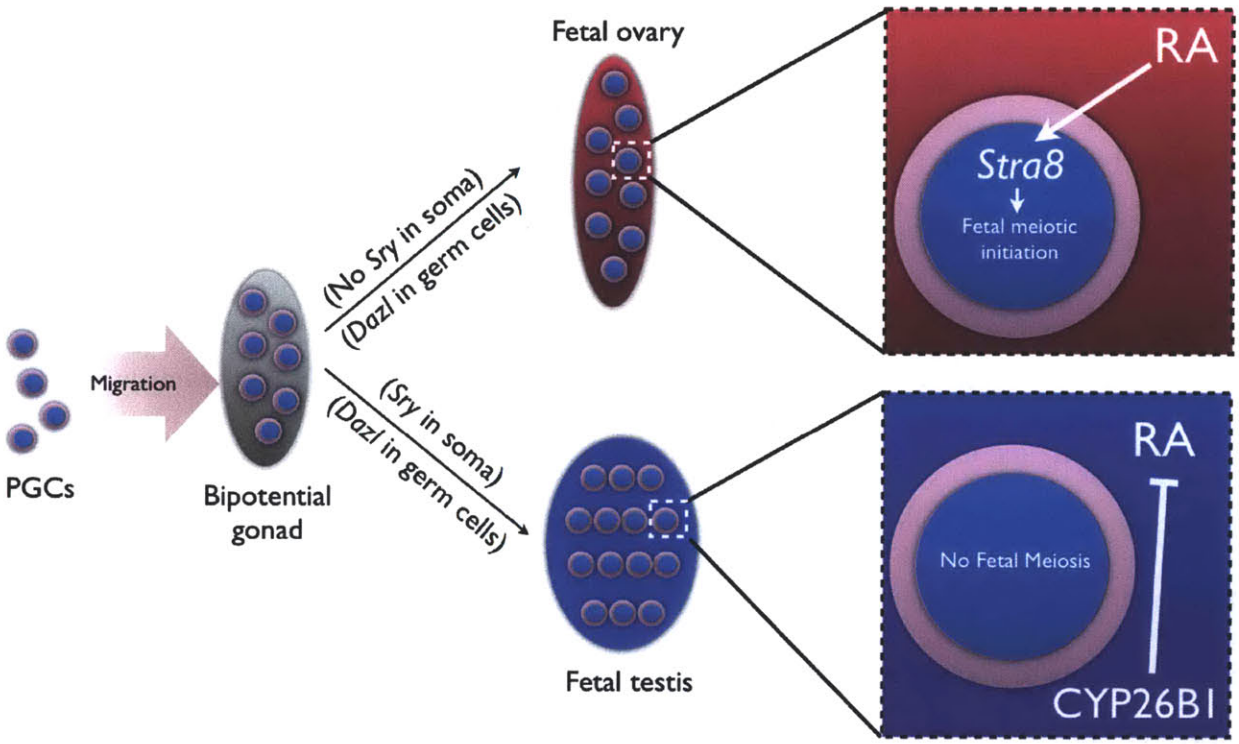



**Figure 8: Molecular mechanism of ovary-specific fetal meiotic initiation in mouse**
*Sry* expression in the soma drives the gonad to differentiate as a testis. Without *Sry* the gonad differentiates as an ovary. *Dazl* is the intrinsic meiosis competence factor expressed in both testicular and ovarian germ cells. In the ovary RA triggers expression of *Stra8*, which leads to fetal meiotic initiation. In the testis RA is degraded by CYP26B1, protecting germ cells from meiosis-inducing RA signal. Without RA testicular germ cells do not express *Stra8* and do not initiate meiosis.

is required as a competence factor for meiotic initiation. Without *Dazl*, both testicular and ovarian germ cells fail to express *Sycp3* mRNA, fail to enter the pre-meiotic state, and cannot respond to the extrinsic meiosis-inducing factor (Lin et al. 2008). Analogous to the nutrient starvation in *S. cerevisiae*, the meiosis-inducing signal in mouse is retinoic acid (RA) (Bowles et al. 2006; Koubova et al. 2006). The diffusible nature of RA makes it a strong candidate for Byskov's MIS, and its presence in the adrenal and the fetal lung nicely accounts for ectopic meiotic initiation in these two locales.

An RA-responsive gene, *Stra8* (stimulated by retinoic acid gene 8), is the intrinsic trigger of meiotic initiation in the mouse ovary (Baltus et al. 2006) and also regulates meiotic initiation in the postnatal testis (Anderson et al. 2008). *Stra8* mutants of both sexes fail to initiate meiosis. Molecularly, *Stra8*-deficient germ cells fail to localize REC8-containing cohesin, fail to load SYCP3 protein to initiate SC formation and synapsis, and fail to make recombination essential DSBs. Furthermore, in ovaries, *Stra8*-deficient germ cells fail to undergo pre-meiotic chromosome replication, demonstrating that the decision to enter meiosis precedes the replication event and that *Stra8*-deficient ovarian germ cells are truly non-meiotic (Baltus et al. 2006).

The molecular mechanism by which fetal meiotic initiation is prevented in the male was also elucidated (Fig. 8). Specific expression of a cytochrome P450-family gene *Cyp26b1* (cytochrome P450, family 26, subfamily b, polypeptide 1) in the fetal testis is thought to cause degradation of the RA signal, thus preventing up regulation of *Stra8* (Bowles et al. 2006; Koubova et al. 2006) (Fig. 8). To prevent meiotic initiation when *Cyp26b1* expression decreases, *Nanos2* independently represses *Stra8* expression specifically in the male (Suzuki and Saga 2008). Additionally, *Dmrt1* (doublesex and mab-3 related transcription factor 1) has been

reported to increase *Stra8* expression in the ovary (Krentz et al. 2011), and *Fgf9* (fibroblast growth factor 9) is thought to decrease *Stra8* expression in the testis (Bowles et al. 2010).

A number of questions remain. First, much remains to be learned about the establishment of the pre-meiotic GCC state, including how the pluripotency program is down regulated and what defines gametogenesis competence on the molecular level. Some of these questions may be answered as we learn more about the molecular function of the DAZL protein. Second, whether *Stra8* is a direct target of RA signaling remains to be determined. Third, it is not known whether RA signaling has germ cell targets independent of *Stra8*. Although *Stra8* is absolutely required for meiosis, it may not be sufficient to drive the program. It is also not clear whether RA is sufficient to drive meiosis or whether other signals may act in parallel, since progression through meiosis has never been assayed in detail in ectopic models (Bowles et al. 2006; Koubova et al. 2006; MacLean et al. 2007). Finally, despite their role in meiosis, we do not know whether *Stra8* and its inducer RA are required for oocyte differentiation.

**Is meiotic initiation necessary for oocyte differentiation?**

Since fetal meiotic initiation is the first event specific to ovarian germ cells, it is tempting to use it as a proxy for female differentiation. In fact, this was done systematically for more than thirty years (Adams and McLaren 2002; McLaren 1984, 2003; McLaren and Southee 1997). Following in that tradition, when the molecular basis of meiotic initiation was elucidated and it was clear that *Stra8* was required for fetal meiotic initiation in the ovary, fetal *Stra8* expression became synonymous with female fate in the writings of some investigators (Bowles et al. 2006; Krentz et al. 2011; Suzuki and Saga 2008; Bowles et al. 2010), although the sex differentiation phenotype of the mutant had not been directly examined.

In the work presented here, we genetically dissect the program of oogenesis. From the characterization of the *Dazl* mutant we know that a PGC to GCC transition licenses both meiotic initiation and sexual differentiation, but it remained a mystery whether these were part of the same process or independent (Gill et al. 2011). By directly characterizing the oocyte differentiation and postnatal meiotic phenotypes of the *Stra8* mutant we were able to solve this mystery.

**References:**


Adams, I. R., and A. McLaren. 2002. Sexually dimorphic development of mouse primordial germ cells: switching from oogenesis to spermatogenesis. *Development* 129 (5):1155-64.

Anderson, E. L., A. E. Baltus, H. L. Roepers-Gajadien, T. J. Hassold, D. G. de Rooij, A. M. van Pelt, and D. C. Page. 2008. Stra8 and its inducer, retinoic acid, regulate meiotic initiation in both spermatogenesis and oogenesis in mice. *Proc. Natl. Acad. Sci. U.S.A.* 105 (39):14976-80.

Baltus, A. E., D. B. Menke, Y. C. Hu, M. L. Goodheart, A. E. Carpenter, D. G. de Rooij, and D. C. Page. 2006. In germ cells of mouse embryonic ovaries, the decision to enter meiosis precedes premeiotic DNA replication. *Nat. Genet.* 38 (12):1430-4.

Baudat, F., K. Manova, J. P. Yuen, M. Jasin, and S. Keeney. 2000. Chromosome synapsis defects and sexually dimorphic meiotic progression in mice lacking Spo11. *Mol. Cell* 6 (5):989-98.

Bishop, D. K., D. Park, L. Xu, and N. Kleckner. 1992. DMC1: a meiosis-specific yeast homolog of E. coli recA required for recombination, synaptonemal complex formation, and cell cycle progression. *Cell* 69 (3):439-56.

Bleil, J. D., and P. M. Wassarman. 1980. Structure and function of the zona pellucida: identification and characterization of the proteins of the mouse oocyte's zona pellucida. *Dev. Biol.* 76 (1):185-202.

Bowles, J., C. W. Feng, C. Spiller, T. L. Davidson, A. Jackson, and P. Koopman. 2010. FGF9 suppresses meiosis and promotes male germ cell fate in mice. *Dev. Cell* 19 (3):440-9.

Bowles, J., D. Knight, C. Smith, D. Wilhelm, J. Richman, S. Mamiya, K. Yashiro, K. Chawengsaksophak, M. J. Wilson, J. Rossant, H. Hamada, and P. Koopman. 2006. Retinoid signaling determines germ cell fate in mice. *Science* 312 (5773):596-600.

Brennan, J., and B. Capel. 2004. One tissue, two fates: molecular genetic events that underlie testis versus ovary development. *Nat. Rev. Genet.* 5 (7):509-21.

Byskov, A. G. 1974. Does the rete ovarii act as a trigger for the onset of meiosis? *Nature* 252 (5482):396-7.

Byskov, A. G., and L. Saxen. 1976. Induction of meiosis in fetal mouse testis in vitro. *Dev. Biol.* 52 (2):193-200.

Castrillon, D. H., L. Miao, R. Kollipara, J. W. Horner, and R. A. DePinho. 2003. Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. *Science* 301 (5630):215-8.

Choi, Y., D. Yuan, and A. Rajkovic. 2008. Germ cell-specific transcriptional regulator sohlh2 is essential for early mouse folliculogenesis and oocyte-specific gene expression. *Biol. Reprod.* 79 (6):1176-82.

Chuma, S., and N. Nakatsuji. 2001. Autonomous transition into meiosis of mouse fetal germ cells in vitro and its inhibition by gp130-mediated signaling. *Dev. Biol.* 229 (2):468-79.

Di Carlo, A. D., G. Travia, and M. De Felici. 2000. The meiotic specific synaptonemal complex protein SCP3 is expressed by female and male primordial germ cells of the mouse embryo. *Int. J. Dev. Biol.* 44 (2):241-4.

Dierich, A., M. R. Sairam, L. Monaco, G. M. Fimia, A. Gansmuller, M. LeMeur, and P. Sassone-Corsi. 1998. Impairing follicle-stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. *Proc. Natl Acad. Sci. U.S.A.* 95 (23):13612-7.

Dong, J., D. F. Albertini, K. Nishimori, T. R. Kumar, N. Lu, and M. M. Matzuk. 1996. Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature* 383 (6600):531-5.

Eppig, J. J., K. Wigglesworth, and F. Chesnel. 1993. Secretion of cumulus expansion enabling factor by mouse oocytes: relationship to oocyte growth and competence to resume meiosis. *Dev. Biol.* 158 (2):400-9.

Evans, E. P., C. E. Ford, and M. F. Lyon. 1977. Direct evidence of the capacity of the XY germ cell in the mouse to become an oocyte. *Nature* 267 (5610):430-1.

Extavour, C. G., and M. Akam. 2003. Mechanisms of germ cell specification across the metazoans: epigenesis and preformation. *Development* 130 (24):5869-84.

Ford, C. E., E. P. Evans, M. D. Burtenshaw, H. Clegg, R. D. Barnes, and M. Tuffrey. 1974. Marker chromosome analysis of tetraparental AKR leads to CBA-T6 mouse chimaeras. *Differentiation* 2 (6):321-33.

Ford, C. E., E. P. Evans, M. D. Burtenshaw, H. M. Clegg, M. Tuffrey, and R. D. Barnes. 1975. A functional 'sex-reversed' oocyte in the mouse. *Proc. Royal Soc. London. Series B* 190 (1099):187-97.

Gill, M. E., Y. C. Hu, Y. Lin, and D. C. Page. 2011. Licensing of gametogenesis, dependent on RNA binding protein DAZL, as a gateway to sexual differentiation of fetal germ cells. *Proc. Natl. Acad. Sci. U.S.A.* 108 (18):7443-8.

Greenbaum, M. P., N. Iwamori, J. E. Agno, and M. M. Matzuk. 2009. Mouse TEX14 is required for embryonic germ cell intercellular bridges but not female fertility. *Biol. Reprod.* 80 (3):449-57.

Hawkins, S. M., and M. M. Matzuk. 2008. The menstrual cycle: basic biology. *Ann. N. Y. Acad. Sci.* 1135:10-8.

Hayashi, K., S. M. de Sousa Lopes, and M. A. Surani. 2007. Germ cell specification in mice. *Science* 316 (5823):394-6.

Keeney, S., C. N. Giroux, and N. Kleckner. 1997. Meiosis-specific DNA double-strand breaks are catalyzed by Spo11, a member of a widely conserved protein family. *Cell* 88 (3):375-84.

Kocer, A., J. Reichmann, D. Best, and I. R. Adams. 2009. Germ cell sex determination in mammals. *Mol. Hum. Reprod.* 15 (4):205-13.

Koubova, J., D. B. Menke, Q. Zhou, B. Capel, M. D. Griswold, and D. C. Page. 2006. Retinoic acid regulates sex-specific timing of meiotic initiation in mice. *Proc. Natl. Acad. Sci. U.S.A.* 103 (8):2474-9.

Krentz, A. D., M. W. Murphy, A. L. Sarver, M. D. Griswold, V. J. Bardwell, and D. Zarkower. 2011. DMRT1 promotes oogenesis by transcriptional activation of Stra8 in the mammalian fetal ovary. *Dev. Biol.* 356 (1):63-70.

Kumar, T. R., Y. Wang, N. Lu, and M. M. Matzuk. 1997. Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. *Nat. Genet.* 15 (2):201-4.

Kunwar, P. S., D. E. Siekhaus, and R. Lehmann. 2006. In vivo migration: a germ cell perspective. *Annu. Rev. Cell Dev. Biol.* 22:237-65.

Lesch, B. J., and D. C. Page. 2012. Genetics of germ cell development. *Nat. Rev. Genet.* 13 (11):781-94.

Liang, L., S. M. Soyal, and J. Dean. 1997. FIGalpha, a germ cell specific transcription factor involved in the coordinate expression of the zona pellucida genes. *Development* 124 (24):4939-47.

Lin, Y., M. E. Gill, J. Koubova, and D. C. Page. 2008. Germ cell-intrinsic and -extrinsic factors govern meiotic initiation in mouse embryos. *Science* 322 (5908):1685-7.

Liu, L., S. Rajareddy, P. Reddy, C. Du, K. Jagarlamudi, Y. Shen, D. Gunnarsson, G. Selstam, K. Boman, and K. Liu. 2007. Infertility caused by retardation of follicular development in mice with oocyte-specific expression of Foxo3a. *Development* 134 (1):199-209.

MacLean, G., H. Li, D. Metzger, P. Chambon, and M. Petkovich. 2007. Apoptotic extinction of germ cells in testes of Cyp26b1 knockout mice. *Endocrinol.* 148 (10):4560-7.

McLaren, A. 1975. Sex chimaerism and germ cell distribution in a series of chimaeric mice. *J. Embryol. Exp. Morphol.* 33 (1):205-16.

———. 1984. Meiosis and differentiation of mouse germ cells. *Symp. Soc. Exp. Biol.* 38:7-23.

———. 2003. Primordial germ cells in the mouse. *Dev. Biol.* 262 (1):1-15.

McLaren, A., and D. Southee. 1997. Entry of mouse embryonic germ cells into meiosis. *Dev. Biol.* 187 (1):107-13.

Mork, L., H. Tang, I. Batchvarov, and B. Capel. 2012. Mouse germ cell clusters form by aggregation as well as clonal divisions. *Mech. Dev.* 128 (11-12):591-6.

Mystkowska, E. T., and A. K. Tarkowski. 1968. Observations on CBA-p-CBA-T6T6 mouse chimeras. *J. Embryol. Exp. Morphol.* 20 (1):33-52.

———. 1970. Behaviour of germ cells and sexual differentiation in late embryonic and early postnatal mouse chimeras. *J. Embryol. Exp. Morphol.* 23 (2):395-405.

Ohinata, Y., B. Payer, D. O'Carroll, K. Ancelin, Y. Ono, M. Sano, S. C. Barton, T. Obukhanych, M. Nussenzweig, A. Tarakhovsky, M. Saitou, and M. A. Surani. 2005. Blimp1 is a critical determinant of the germ cell lineage in mice. *Nature* 436 (7048):207-13.

Page, A. W., and T. L. Orr-Weaver. 1997. Stopping and starting the meiotic cell cycle. *Curr. Opin. Genet. Dev.* 7 (1):23-31.

Palmer, S. J., and P. S. Burgoyne. 1991. In situ analysis of fetal, prepuberal and adult XX----XY chimaeric mouse testes: Sertoli cells are predominantly, but not exclusively, XY. *Development* 112 (1):265-8.

Pangas, S. A., Y. Choi, D. J. Ballow, Y. Zhao, H. Westphal, M. M. Matzuk, and A. Rajkovic. 2006. Oogenesis requires germ cell-specific transcriptional regulators Sohlh1 and Lhx8. *Proc. Natl. Acad. Sci. U.S.A.* 103 (21):8090-5.

Paredes, A., C. Garcia-Rudaz, B. Kerr, V. Tapia, G. A. Dissen, M. E. Costa, A. Cornea, and S. R. Ojeda. 2005. Loss of synaptonemal complex protein-1, a synaptonemal complex protein, contributes to the initiation of follicular assembly in the developing rat ovary. *Endocrinol.* 146 (12):5267-77.

Pepling, M. E., and A. C. Spradling. 1998. Female mouse germ cells form synchronously dividing cysts. *Development* 125 (17):3323-8.

———. 2001. Mouse ovarian germ cell cysts undergo programmed breakdown to form primordial follicles. *Dev. Biol.* 234 (2):339-51.

Pittman, D. L., J. Cobb, K. J. Schimenti, L. A. Wilson, D. M. Cooper, E. Brignull, M. A. Handel, and J. C. Schimenti. 1998. Meiotic prophase arrest with failure of chromosome synapsis in mice deficient for Dmc1, a germline-specific RecA homolog. *Mol. Cell* 1 (5):697-705.

Prieto, I., C. Tease, N. Pezzi, J. M. Buesa, S. Ortega, L. Kremer, A. Martinez, A. C. Martinez, M. A. Hulten, and J. L. Barbero. 2004. Cohesin component dynamics during meiotic prophase I in mammalian oocytes. *Chromosome Research* 12 (3):197-213.

Rajkovic, A., S. A. Pangas, D. Ballow, N. Suzumori, and M. M. Matzuk. 2004. NOBOX deficiency disrupts early folliculogenesis and oocyte-specific gene expression. *Science* 305 (5687):1157-9.

Rogakou, E. P., D. R. Pilch, A. H. Orr, V. S. Ivanova, and W. M. Bonner. 1998. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J. Biol. Chem.* 273 (10):5858-68.

Romanienko, P. J., and R. D. Camerini-Otero. 2000. The mouse Spo11 gene is required for meiotic chromosome synapsis. *Mol. Cell* 6 (5):975-87.

Saitou, M., S. C. Barton, and M. A. Surani. 2002. A molecular programme for the specification of germ cell fate in mice. *Nature* 418 (6895):293-300.

Schultz, G. A., and S. Heyner. 1992. Gene expression in pre-implantation mammalian embryos. *Mutat. Res.* 296 (1-2):17-31.

Schultz, R. M. 2002. The molecular foundations of the maternal to zygotic transition in the preimplantation embryo. *Hum. Reprod. Update* 8 (4):323-31.

Soyal, S. M., A. Amleh, and J. Dean. 2000. FIGalpha, a germ cell-specific transcription factor required for ovarian follicle formation. *Development* 127 (21):4645-54.

Suzuki, A., and Y. Saga. 2008. Nanos2 suppresses meiosis and promotes male germ cell differentiation. *Genes Dev.* 22 (4):430-5.

Uhlmann, F., and K. Nasmyth. 1998. Cohesion between sister chromatids must be established during DNA replication. *Curr. Biol. : CB* 8 (20):1095-101.

Upadhyay, S., and L. Zamboni. 1982. Ectopic germ cells: natural model for the study of germ cell sexual differentiation. *Proc. Natl. Acad. Sci. U.S.A.* 79 (21):6584-8.

van Werven, F. J., and A. Amon. 2011. Regulation of entry into gametogenesis. *Philos. Transact. Royal Soc. London. Series B, Biol. Sci.* 366 (1584):3521-31.

Varani, S., J. A. Elvin, C. Yan, J. DeMayo, F. J. DeMayo, H. F. Horton, M. C. Byrne, and M. M. Matzuk. 2002. Knockout of pentraxin 3, a downstream target of growth differentiation factor-9, causes female subfertility. *Molec. Endocrinol.* 16 (6):1154-67.

Vincent, S. D., N. R. Dunn, R. Sciammas, M. Shapiro-Shalef, M. M. Davis, K. Calame, E. K. Bikoff, and E. J. Robertson. 2005. The zinc finger transcriptional repressor Blimp1/Prdm1 is dispensable for early axis formation but is required for specification of primordial germ cells in the mouse. *Development* 132 (6):1315-25.

Von Stetina, J. R., and T. L. Orr-Weaver. 2011. Developmental control of oocyte maturation and egg activation in metazoan models. *Cold Spring Harb. Perspect. Biol.* 3 (10):a005553.

Watanabe, Y. 2005. Sister chromatid cohesion along arms and at centromeres. *Trends Genet. : TIG* 21 (7):405-12.

Wojtasz, L., J. M. Cloutier, M. Baumann, K. Daniel, J. Varga, J. Fu, K. Anastassiadis, A. F. Stewart, A. Remenyi, J. M. Turner, and A. Toth. 2012. Meiotic DNA double-strand breaks and chromosome asynapsis in mice are monitored by distinct HORMAD2-independent and -dependent mechanisms. *Genes Dev.* 26 (9):958-73.

Yoshida, K., G. Kondoh, Y. Matsuda, T. Habu, Y. Nishimune, and T. Morita. 1998. The mouse RecA-like gene Dmc1 is required for homologous chromosome synapsis during meiosis. *Mol. Cell* 1 (5):707-18.

Zamboni, L., and S. Upadhyay. 1983. Germ cell differentiation in mouse adrenal glands. *J. Exp. Zool.* 228 (2):173-93.

Zickler, D., and N. Kleckner. 1999. Meiotic chromosomes: integrating structure and function. *Annu. Rev. Genet.* 33:603-754.

# Chapter 2

# Oocyte differentiation is genetically dissociable from the meiotic program in mice

**Author contributions for chapter 2:** Gregoriy Dokshin, Andrew Baltus, John Eppig, and David Page designed the experiments. Gregoriy Dokshin performed the experiments. Gregoriy Dokshin and David Page wrote the manuscript.

**Abstract**

Oogenesis is the process by which ovarian germ cells undertake meiosis and differentiate to become eggs. In mice, *Stra8* is required to initiate the meiotic program, but its role in differentiation remains unknown. Here we report *Stra8*-deficient ovarian germ cells that grow and differentiate into oocyte-like cells that synthesize zonae pellucidae, organize surrounding somatic cells into follicles, are ovulated in response to hormonal stimulation, undergo asymmetric cell division to produce a polar body, and cleave to form two-cell embryos upon fertilization. These events occur without pre-meiotic chromosomal replication, sister chromatid cohesion, synapsis, or recombination. Thus, oocyte growth and differentiation are genetically dissociable from the meiotic program.

## Introduction

Oogenesis is the process by which an ovarian germ cell becomes a mature female gamete, an egg. A functional egg must contribute to the zygote exactly one copy of each chromosome (a haploid maternal genome), and it must sustain the early embryo from fertilization through the activation of zygotic genes. The former is achieved through meiosis, while the latter is achieved through growth and differentiation of the cell. These chromosomal and cellular events of oogenesis are closely coordinated, but whether they are interdependent remains unknown.

In mice, the earliest stages of germ cell development unfold identically in XX and XY embryos. In both sexes, germ cells originate in the epiblast and then migrate to the developing gonad(McLaren 2003). Upon arriving there, both XX and XY germ cells undergo a developmental transition from primordial germ cells (PGCs) to gametogenesis-competent cells (GCCs). Both XX and XY GCCs are competent to respond to signals from the gonadal soma to initiate meiosis and undergo sexual differentiation (Gill et al. 2011; Lin et al. 2008).

The first morphological difference between germ cells in XX and XY gonads appears when meiotic chromosome condensation – a defining feature of meiotic prophase – occurs in ovarian (XX) germ cells during fetal development (McLaren 1984, 2003). (Testicular germ cells enter meiotic prophase after birth). Thus, the fetal onset of meiotic prophase marks the onset of oogenesis. By extension, fetal meiotic initiation is commonly used as a proxy for oocyte growth and differentiation, and the absence of meiotic initiation is conventionally taken as evidence that fetal germ cells have adopted a spermatogenic fate (McLaren 1984; McLaren and Southee 1997; Adams and McLaren 2002; McLaren 2003; Bowles et al. 2006; Kocer et al. 2009; Bowles and Koopman 2010; Krentz et al. 2011). Of course, this interpretation assumes that fetal meiotic

initiation and oocyte growth and differentiation are inseparable components of the oogenesis program – an assumption that remains to be tested.

Genetically, meiotic initiation is governed by the retinoic acid (RA)-responsive gene *Stra8* (Baltus et al. 2006; Anderson et al. 2008). Upon receiving the RA signal and expressing *Stra8*, ovarian germ cells replicate their chromosomes and enter meiotic prophase. Oocytes then progress through meiotic prophase until the diplotene stage of prophase I, arrest, and form primordial follicles – the first functional differentiation event. Throughout the reproductive life of the female, these prophase-arrested primordial oocytes are recruited to grow and differentiate into fully grown oocytes. (Meiosis is resumed at ovulation and completed at fertilization.) Thus, in mice, oocytes grow and differentiate only after they reach the diplotene stage of prophase I. Indeed, oocyte growth and differentiation during arrest in meiotic prophase I is conserved in humans, frogs, flies, and worms, among other animals (Page and Orr-Weaver 1997; Von Stetina and Orr-Weaver 2011). Furthermore, in rats the timing of primordial follicle formation can be altered by manipulating chromosome desynapsis (a hallmark of progression to diplotene and completion of prophase)(Paredes et al. 2005). Together these observations support the assumption that meiotic initiation and prophase I are a part of the same pathway as oocyte differentiation. However, this has not been directly tested, and whether oocytes can grow and differentiate without meiotic initiation and prophase I remains to be determined.

In recent years, advances in embryonic stem (ES) and induced pluripotent stem (iPS) cell technologies have motivated efforts to derive oocytes *in vitro* from these and other undifferentiated cell types. Several laboratories have reported derivation *in vitro* of cells that morphologically resemble oocytes (Hubner et al. 2003; Lacham-Kaplan, Chy, and Trounson 2006; Qing et al. 2007; Kerkis et al. 2007; Salvador et al. 2008; Nicholas et al. 2009; White et al.

2012). Some reports of *in vitro*-derived oocyte-like cells include no direct assessment of the state of the chromosomes, drawing criticism from meiosis researchers (Novak et al. 2006; Oatley and Hunt 2012). In other cases the oocyte-like cells have been reported to reduce their DNA content to 1C *in vitro*, in the absence of fertilization (White et al. 2012); normally this would occur in female germ cells only upon fertilization. These difficulties in achieving proper coordination of oocyte differentiation and meiosis *in vitro* may reflect the underlying biology of oogenesis; they could be taken as evidence that differentiation and meiosis are under independent regulation.

In this study we sought to investigate the functional relationship between the two aspects of oogenesis (meiosis and oocyte differentiation) *in vivo* using genetic analysis. We find that *Stra8*-deficient germ cells, which do not initiate the meiotic program, can differentiate into oocyte-like cells. Morphological and functional analysis reveals that *Stra8*-deficient oocyte-like cells can synthesize zonae pellucidae, organize surrounding somatic cells into follicles, be ovulated in response to hormonal stimulation, undergo asymmetric cell division to produce a polar body, and cleave to form two-cell embryos upon fertilization. By analyzing these oocyte-like cells directly, we demonstrate that they develop without passing through meiotic prophase or even undergoing pre-meiotic chromosome replication. We conclude that the oogenesis program can be genetically dissociated into two independent components: the meiotic program and oocyte growth and differentiation. Based on this finding we re-evaluate the current model of germ cell commitment to oogenesis and postulate the existence of a *Stra8*-independent pathway, yet to be identified, that governs germ cells commitment to oocyte growth and differentiation.

## Results

Our prior studies of *Stra8*-deficient ovarian germ cells had been conducted in mice of mixed genetic background (not inbred) (Baltus et al. 2006). To ensure the reproducibility of our previous findings, and of those reported here, we backcrossed the *Stra8* mutant allele onto an inbred genetic background (C57BL/6) and characterized the meiotic defect in these mice. In agreement with our earlier findings, we observed that C57BL/6 *Stra8*-deficient ovarian germ cells fail to properly assemble the synaptonemal complex, as evidenced by DAPI staining of DNA and aberrant localization of the protein SYCP3 (Fig. 1a). The meiotic cohesin REC8 is also not properly localized (Fig. 1b). Thus the synapsis and cohesin loading that are characteristic of meiotic prophase do not occur (Scherthan 2003; Baltus et al. 2006). Based on the absence of staining for γH2A.X (Fig. 1c), C57BL/6 *Stra8*-deficient ovarian germ cells do not form the DNA double-strand breaks (DSBs) essential for meiotic recombination (Rogakou et al. 1998). These findings confirmed that *Stra8* is necessary for meiotic prophase in C57BL/6 females, as we previously reported for females of mixed genetic background(Baltus et al. 2006). We conducted all subsequent experiments using C57BL/6 animals.

As we reported previously, fetal and postnatal loss of germ cells is accelerated in *Stra8*-deficient females; their ovaries have reduced numbers of germ cells at birth and are completely devoid of germ cells by six or eight weeks of age (Fig. 2) (Baltus et al. 2006). Nonetheless, some *Stra8*-deficient germ cells evidently survive embryonic and fetal development despite the meiotic initiation block at E13.5 - E14.5 (Baltus et al. 2006). We confirmed this by comparing ovarian histology from wild-type and *Stra8*-deficient C57BL/6 animals between E14.5 and E16.5 (Fig. 1d). To corroborate our prior conclusion

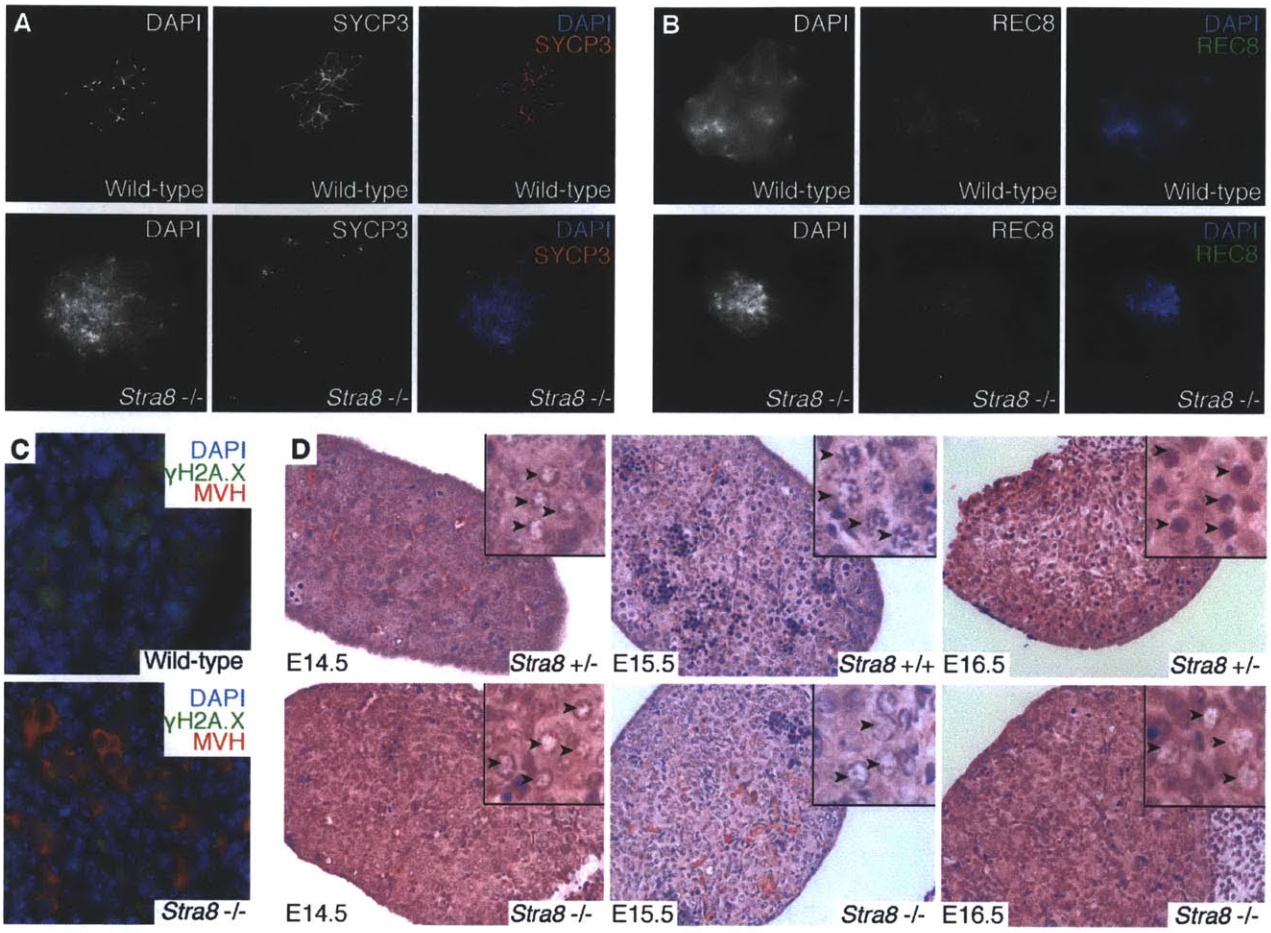



**Figure 1: Stra8 is required for meiotic initiation in ovarian germ cells on C57BL/6 inbred background employed throughout this study.**

(A, B) Immunofluorescent staining of chromosome spreads of E16.5 wild-type and *Stra8*-deficient germ cells for (A) SYCP3 and (B) REC8 proteins. In merged images, DAPI is shown in blue, SYCP3 in red, and REC8 in green.

(C) Immunohistochemical staining for double-strand break response marker γH2A.X (nuclear), in green, and germ cell marker mouse vasa homolog (MVH; cytoplasmic), in red, on sections of wild-type and *Stra8*-deficient E16.5 ovaries. In the wild-type ovary, γ H2A.X signal is present in MVH-positive cells, while in the mutant the γ H2A.X signal is absent from MVH-positive cells.

(D) Photomicrographs of sections from control (wild-type or *Stra8*-heterozygous) and *Stra8*-deficient ovaries at E14.5, E15.5, and E16.5 stained with hematoxylin and eosin. Insets show higher magnification and arrows indicate representative germ cells. While wild-type germ cells condense their chromosomes as they progress through meiotic prophase at E15.5 and E16.5, *Stra8*-deficient germ cells maintain pre-meiotic nuclear morphology.

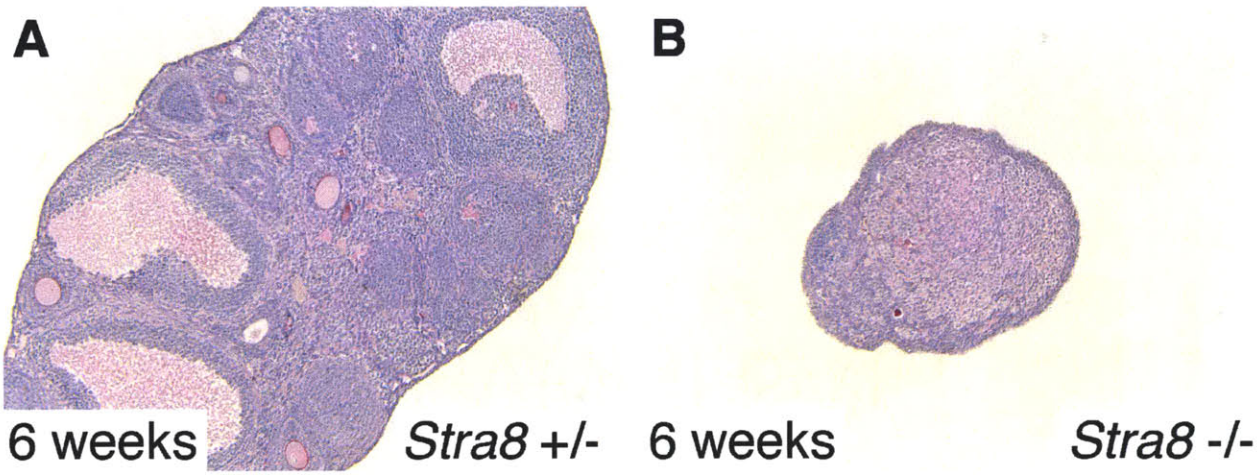



**Figure 2: *Stra8*-deficient ovaries are depleted of germ cells by six weeks of age.**

Photomicrographs of sections from control (*Stra8*-heterozygous) (A) and *Stra8*-deficient (B) ovaries at P30 stained with periodic acid-Schiff (PAS) and hematoxylin. *Stra8*-deficient ovaries are significantly smaller than control ovaries and contain no germ cells or follicle structures.

(Baltus et al. 2006) that some *Stra8*-deficient germ cells survive postnatally, we stained postnatal day 2 (P2) ovarian sections for MVH (mouse vasa homolog; DDX4) protein, a marker of germ cells; we observed MVH-positive germ cells in both wild-type and *Stra8*-deficient C57BL/6 ovaries (Fig. 3). We went on to examine the oogenic potential of these surviving germ cells.

**Surviving *Stra8*-deficient Germ Cells Differentiate into Oocyte-like Cells**

If entry into meiotic prophase is necessary for oocyte growth and differentiation, then the latter processes should not occur in *Stra8*-deficient ovarian germ cells. To test this, we compared ovarian histology of wild-type and *Stra8*-deficient females.

In a wild-type ovary, during the first postnatal week, a cohort of germ cells is recruited to grow and differentiate synchronously (Fig. 4a,c,e). Surprisingly, in *Stra8*-deficient ovaries, we observed that the surviving germ cells also began to grow and differentiate on a timetable similar to that of the first cohort in wild-type ovaries (Fig. 4b,d,f).

We next examined histological sections of *Stra8*-deficient ovaries at P21, when, in wild-type ovaries, growth and differentiation of the first cohort of oocytes are pronounced. In wild-type ovaries, we observed fully grown oocytes with large nuclei, called germinal vesicles (GVs), that feature prominent nucleoli. The perimeters of these large cells stain brightly using periodic acid-Schiff (PAS) reagent, indicating the presence of an oocyte-specific glycoprotein coat called the zona pellucida (Fig. 5a). We examined sections of ovaries from nine different *Stra8*-deficient animals and found that all ovaries examined contained germ cells whose size and morphology were comparable to those of the wild-type oocytes. Like wild-type oocytes, these large *Stra8*-deficient germ cells featured GVs and zonae pellucidae (Fig. 5b). the latter of which we confirmed by staining sections for the zona pellucida protein ZP2 (Fig. 5c,d).

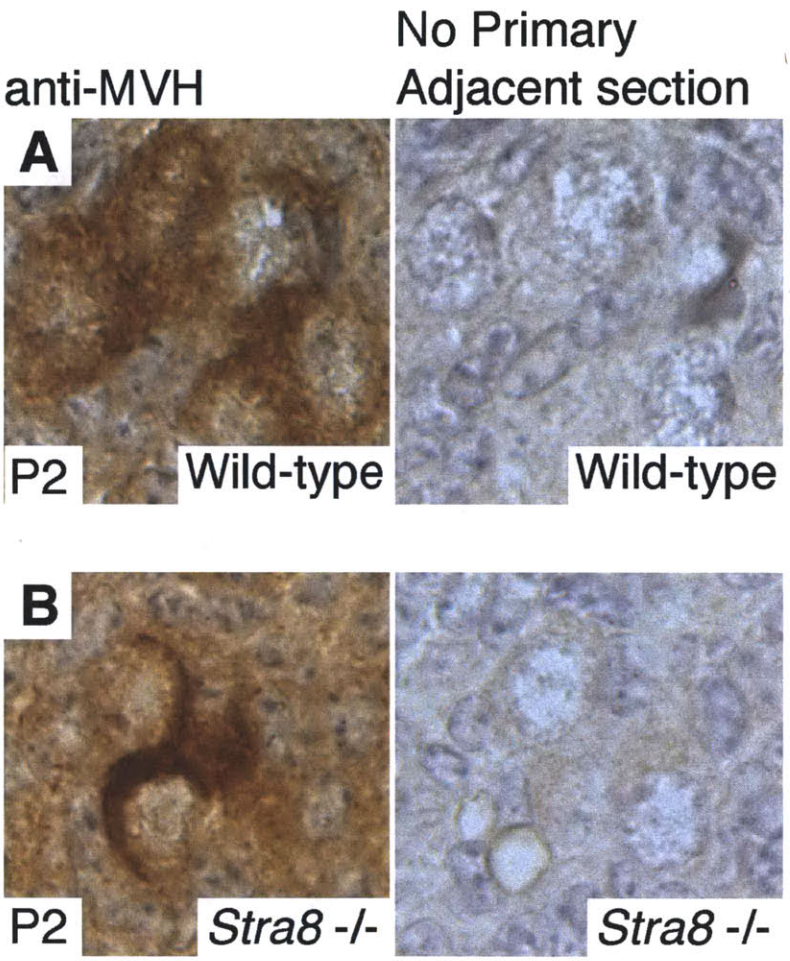



**Figure 3: *Stra8*-deficient ovarian germ cells survive postnatally.**

Immunohistochemical staining of P2 wild-type (A) and *Stra8*-deficient (B) ovary sections for germ cell marker MVH; counter-stained with hematoxylin. As controls, sections adjacent to those stained for MVH were counter-stained with hematoxylin to reveal germ cell morphology but were not treated with primary antibody.

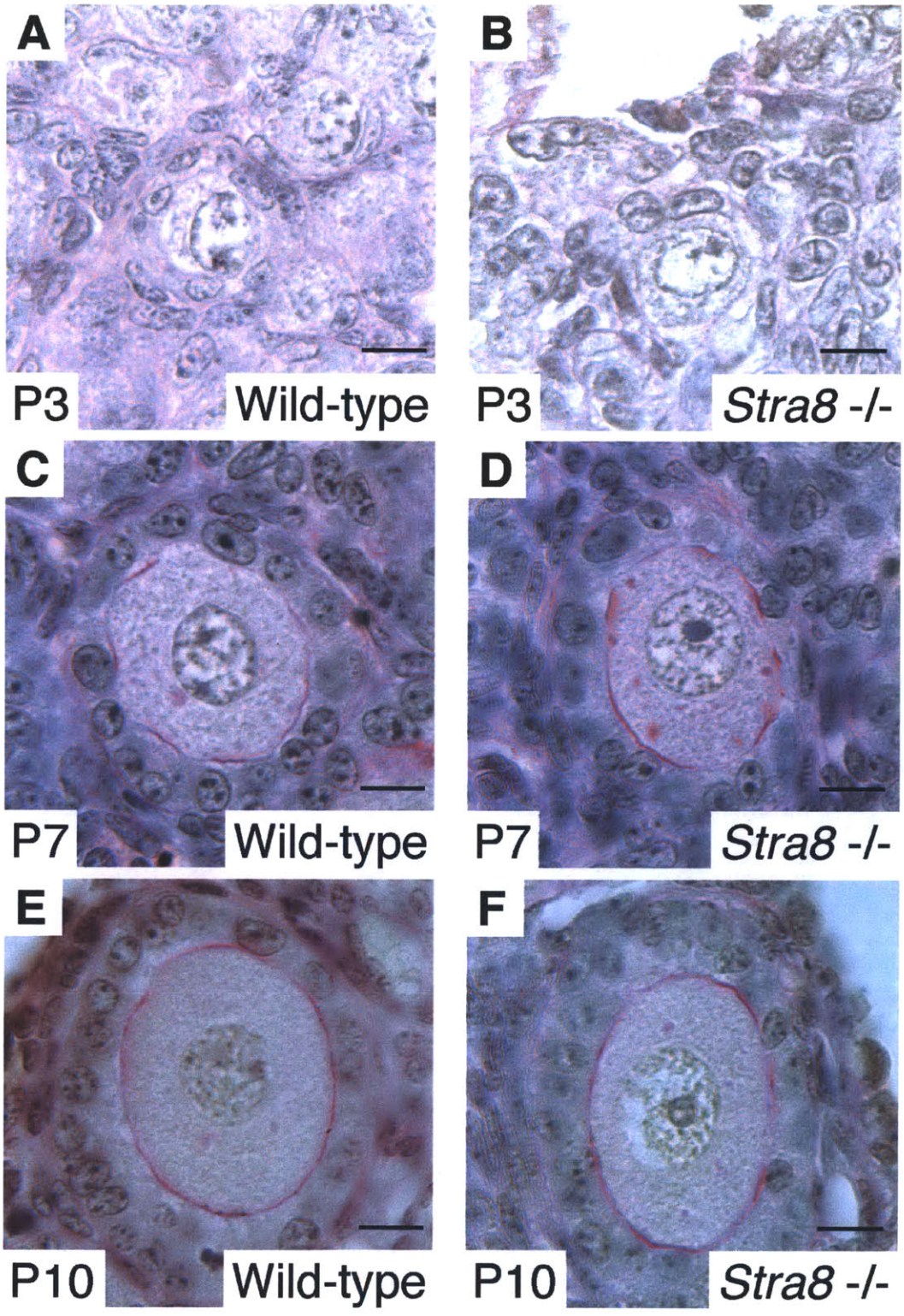



**Figure 4: In *Stra8*-deficient ovaries, germ cells grow and differentiate during first postnatal week.**

Photomicrographs of sections from wild-type (A, C, E) and *Stra8*-deficient (B, D, F) ovaries at P3, P7, and P10 stained with PAS and hematoxylin. Scale bars represent 10 μm.

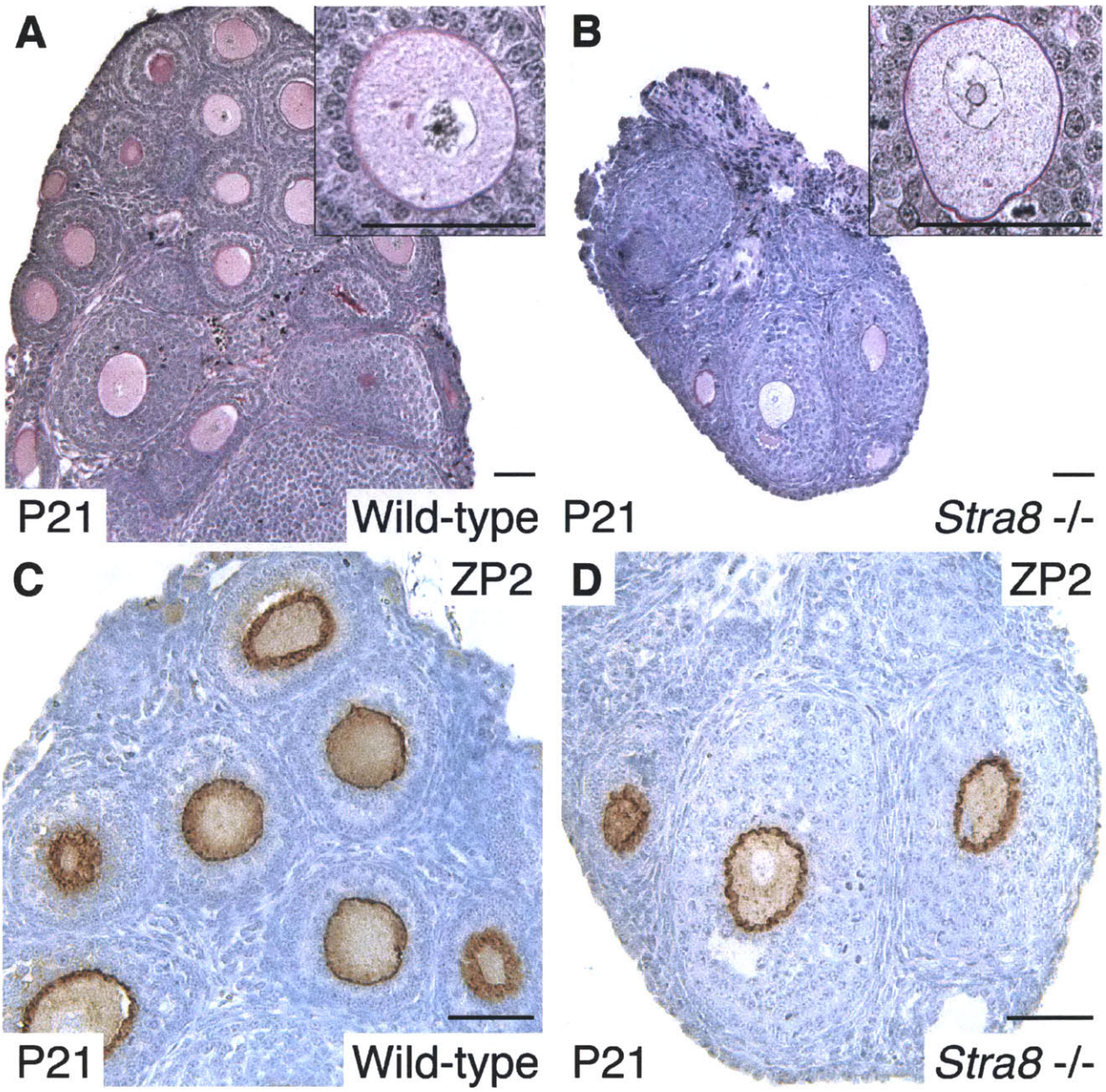



**Figure 5: *Stra8*-deficient ovarian germ cells differentiate into oocyte-like cells.**

Photomicrographs of sections from wild-type (A) and *Stra8*-deficient (B) ovaries at P21 stained with periodic acid-Schiff (PAS) and hematoxylin. Insets show higher magnification of individual germ cells. Insets show higher magnification of individual germ cells. Immunohistochemical staining of sections of P21 wild-type (C) and *Stra8-deficient* (D) ovary for zona pellucida protein ZP2; counter-stained with hematoxylin. Scale bars represent 50 μm. For a developmental time course also see Figure 4.

**Double Checking that *Stra8*-deficient Oocyte-like Cells have not Entered Meiotic Prophase**

Despite the evidence that germ cells in *Stra8*-deficient fetal ovaries fail to initiate meiosis (Fig. 1) (Baltus et al. 2006), the small number of germ cells surviving postnatally (Fig. 5) raised the possibility that these *Stra8*-deficient survivors had differentiated as oocytes after entering meiotic prophase. We excluded this possibility through a double-mutant (epistasis) experiment involving *Stra8* and *Dmc1*. *Dmc1*-deficient ovarian germ cells die perinatally because *Dmc1* is required for repair of the DSBs that arise during meiotic prophase (Bishop et al. 1992; Pittman et al. 1998; Yoshida et al. 1998). This death of *Dmc1*-deficient germ cells does not occur if DSB formation is prevented, for example in animals lacking a gene (*Spo11*) required for meiotic DSB formation (Keeney, Giroux, and Kleckner 1997; Baudat et al. 2000; Di Giacomo et al. 2005). We reasoned that if the surviving *Stra8*-deficient germ cells had entered meiotic prophase and formed meiotic DSBs, then no double-mutant (*Stra8*-deficient / *Dmc1*-deficient) germ cells should survive postnatally. However, if *Stra8* deficiency stringently blocked meiotic initiation and DSB formation, then germ cell survival in the double mutant should resemble that in the *Stra8* single mutant. We observed, as expected (Pittman et al. 1998; Yoshida et al. 1998; Di Giacomo et al. 2005), that *Dmc1*-deficient ovaries at P30 contained no germ cells (Fig. 6b). Double-mutant (*Dmc1*-deficient / *Stra8*-deficient) ovaries, however, contained oocyte-like cells similar to those of *Stra8*-deficient single-mutant ovaries (Fig. 6a,c). As the survival of *Stra8*-deficient oocyte-like cells is unaffected by the presence or absence of *Dmc1*, we conclude that these *Stra8*-deficient cells have not formed meiotic DSBs, reinforcing the previous evidence that they have not entered meiotic prophase. Instead, their oocyte-like characteristics formed independently from meiosis.

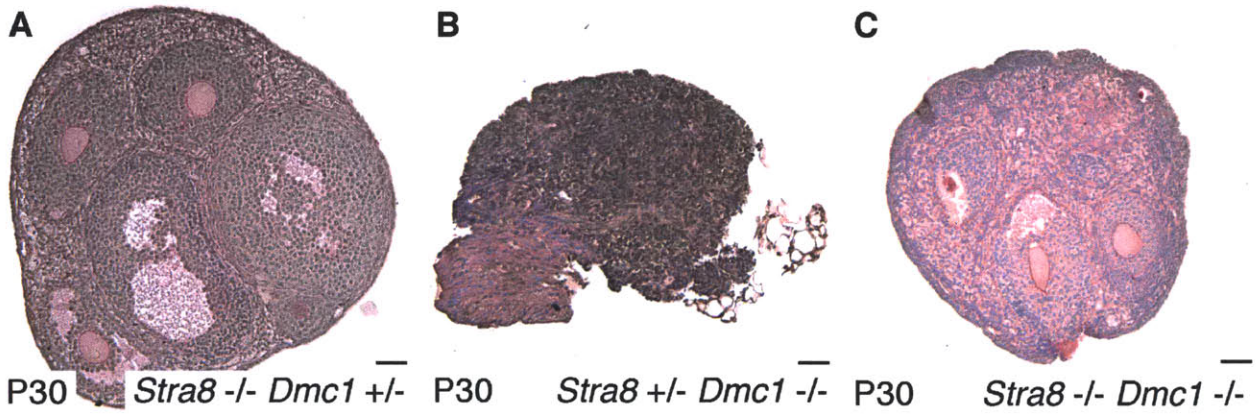



**Figure 6: *Stra8*-deficient oocyte-like cells grow and differentiate without meiotic prophase.**

Sections from P30 *Stra8*-deficient (A), *Dmc1*-deficient (B) and *Stra8* and *Dmc1* double-deficient (C) ovaries, stained with PAS and hematoxylin. *Dmc1*-deficient ovaries (B) were small and dense, lacking germ cells or follicles. Scale bars represent 50 μm.

### *Stra8*-deficient Oocyte-like Cells Organize Soma into Follicles that Support Ovulation

A critical function of wild-type oocytes is to actively organize somatic cells of the fetal and postnatal ovary into follicles, the hallmark of ovarian structure and function (Su, Sugiura, and Eppig 2009). Like many wild-type oocytes, *Stra8*-deficient germ cells were enclosed in large follicles composed of multiple layers of cuboidal granulosa cells (Fig. 5), demonstrating their ability to drive folliculogenesis. By P30, large, preovulatory follicles were observed in 11 of 12 ovaries from six different *Stra8*-deficient animals (data not shown; see also Fig. 6a and 7e,f).

To extend this observation to the molecular level, we examined *Stra8*-deficient ovaries for expression of NOBOX, an oocyte-specific protein required for primary follicle formation (Rajkovic et al. 2004). We detected NOBOX protein in germ cells of all three *Stra8*-deficient and five control (wild-type or *Stra8*-heterozygous) ovaries examined by immunohistochemistry at P5 and P7 (Fig. 7a,b).

To confirm at the molecular level that proper granulosa cell differentiation had occurred in response to *Stra8*-deficient oocyte-like cells, we stained sections of wild-type and *Stra8*-deficient ovaries for FOXL2 protein, a key factor in granulosa cell differentiation and identity (Crisponi et al. 2001; Schmidt et al. 2004). We found that FOXL2 was expressed in the granulosa cells of both wild-type and *Stra8*-deficient ovaries at P10, when secondary follicles are present, and at P30, when large antral follicles are present. These findings confirm that *Stra8*-deficient oocyte-like cells support proper granulosa cell specification and differentiation (Fig. 7c-f).

We then asked whether the oocyte-like cells in *Stra8*-deficient ovaries, together with the surrounding somatic (cumulus) cells, can be ovulated. Because *Stra8*-deficient germ cells are lost before the animals reach sexual maturity, we could not assay natural ovulation. Instead, we

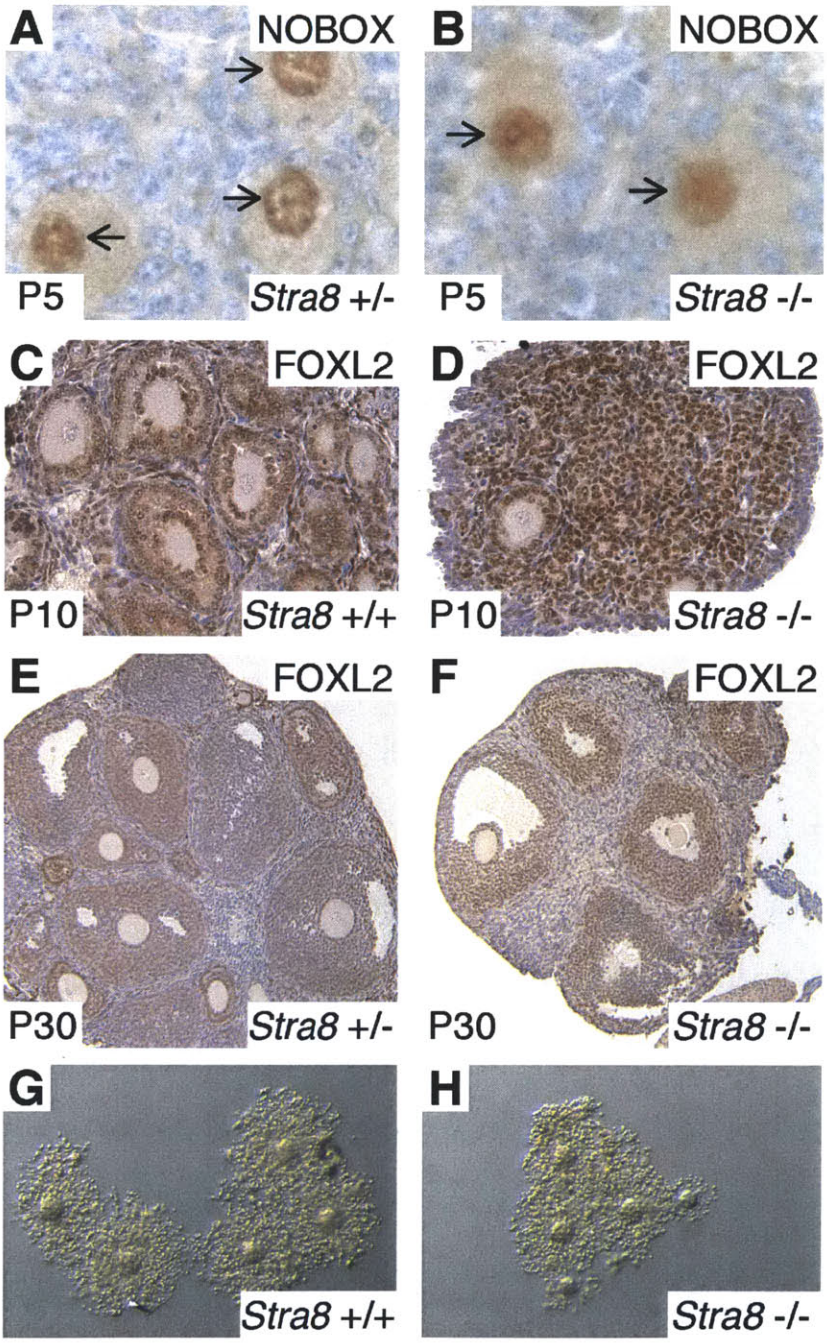



**Figure 7: *Stra8*-deficient oocyte-like cells organize ovulation-competent follicles.**

Immunohistochemical staining of P5 control (*Stra8*-heterozygous) (A) and *Stra8*-deficient (B) ovary sections for folliculogenesis-essential protein NOBOX; counter-stained with hematoxylin. Arrows indicate representative germ cells. Immunohistochemical staining of P10 (C and D) and P30 (E and F) control (C and E) and *Stra8*-deficient (D and F) ovary sections for granulosa cell marker FOXL2. Cumulus-oocyte complexes isolated from wild-type (G) and *Stra8*-deficient (H) superovulated animals.

hormonally stimulated wild-type and *Stra8*-deficient females at P20 to accelerate ovulation ("superovulation") and then flushed their oviducts to identify any ovulated cumulus-oocyte complexes (COCs). We isolated ovulated COCs from wild-type and from all five *Stra8*-deficient females subjected to this regiment. In wild-type females, the cumulus cells of the preovulatory follicle secrete hyaluronic acid, causing expansion of the cumulus cell mass (the "cumulus oophorus") that surrounds the oocyte. This process is dependent on the oocyte, and competence to support cumulus oophorus expansion is acquired late in oogenesis (Eppig, Wigglesworth, and Chesnel 1993). Examination of wild-type and *Stra8*-deficient ovulated COCs revealed that the cumuli oophori were fully expanded in both groups (Fig. 7g,h). We conclude that *Stra8*-deficient oocyte-like cells can be ovulated with expanded cumuli oophori, implying that they can interact with the ovarian soma in a way that resembles wild-type oocytes.

**_Stra8_-deficient Oocyte-like Cells Undergo an Asymmetric Division to Produce Polar Bodies**

In wild-type females, fully grown GV-stage oocytes are arrested in diplotene of the first meiotic prophase. At ovulation, wild-type GV-stage oocytes resume meiosis, breaking down the nuclear envelope, condensing their chromosomes, and dividing asymmetrically to form a large secondary oocyte and a much smaller polar body. Competence to undergo this division is acquired in late stages of oocyte growth and differentiation, making it a defining functional feature of a fully grown oocyte (Sorensen and Wassarman 1976). Several mutants defective in individual aspects of meiotic prophase I (such as synapsis or recombination) have been reported to produce polar bodies (Woods et al. 1999; Kouznetsova et al. 2007), but it is not known whether blocking meiotic initiation (preventing the meiotic program in its entirety) would preclude this asymmetric

division. We, therefore, asked whether *Stra8*-deficient oocyte-like cells could produce polar bodies.

Fully grown wild-type oocytes can be induced to undergo maturation by removing them from follicles (Donahue 1968). We extracted cumulus-oocyte complexes from P22 wild-type and *Stra8*-deficient ovaries by follicle puncture and cultured them overnight. We then mechanically removed the cumulus cells and visually assayed polar body formation. We found that most *Stra8*-deficient cells underwent germinal vesicle breakdown (GVB) – the first step on the path to polar body formation. Of the cells that underwent GVB, 28 of 35 wild-type oocytes and 11 of 42 *Stra8*-deficient oocyte-like cells formed polar bodies (Fig. 8), demonstrating that *Stra8*-deficient cells can undergo maturation and an asymmetric division. (We also observed polar body formation *in vivo*, following superovulation [Fig. 9], corroborating our findings *in vitro*.)

The failure of many *Stra8*-deficient oocyte-like cells to produce polar bodies was likely due to a failure of spindle assembly in the absence of paired homologous chromosomes. Of the 31 *Stra8*-deficient cells that underwent GVB but did not produce polar bodies, seven cells fragmented, while the other 24 cells remained arrested without a visible GV or polar body. Closer examination of the latter group revealed that they contained aberrant spindles reminiscent of those observed in *Mlh1* mutant oocytes, which fail to maintain homologous chromosome pairing (Fig. 10) (Woods et al. 1999).

### *Stra8*-deficient Oocyte-like Cells Develop without Pre-meiotic Chromosome Replication

We have previously shown that fetal germ cells in *Stra8*-deficient females fail to undergo pre-meiotic chromosome replication (Baltus et al. 2006). Accordingly, we tested whether the *Stra8*-

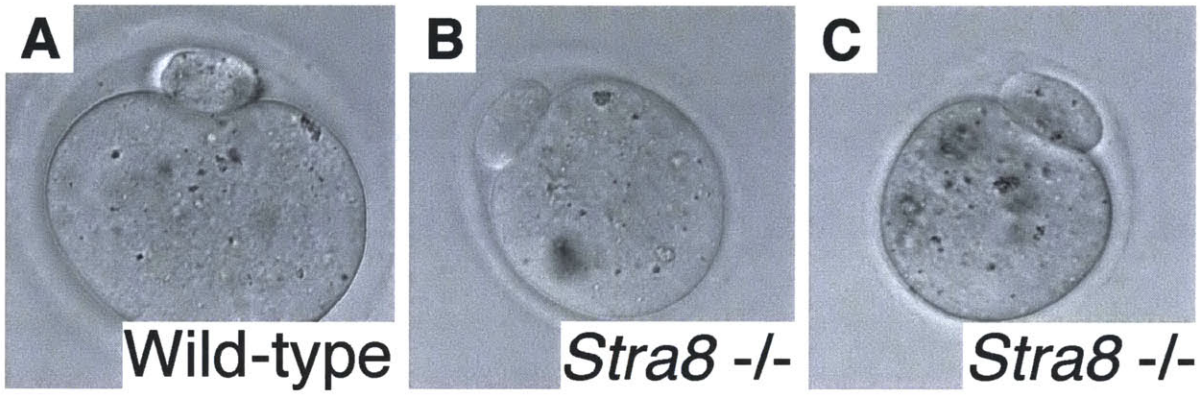



**Figure 8: *Stra8*-deficient oocyte-like cells divide asymmetrically upon maturation.**

Differential-interference-contrast photomicrographs of *in vitro*-matured wild-type oocytes (A) and *Stra8*-deficient oocyte-like cells (B and C). For *in vivo* polar body formation see also Fig. 9.

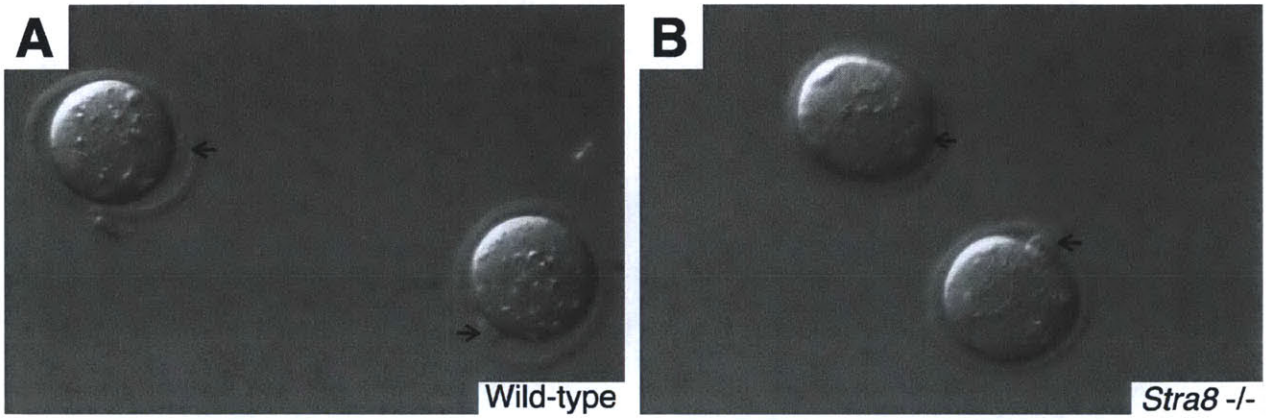



**Figure 9: *Stra8*-deficient oocyte-like cells form polar bodies upon ovulation.**

Photomicrographs of ovulated wild-type oocytes and *Stra8*-deficient oocyte-like cells following cumulus cell removal by hyaluronidase treatment. Arrows indicate polar bodies. Hyaluronidase treatment is necessary to free ovulated oocytes from the sticky cumulus cell mass but has been suspected to induce artificial polar body extrusion (Woods et al., 1999). To exclude the possibility of artificial polar body extrusion we also performed *in vitro* maturation experiments (Fig. 8).

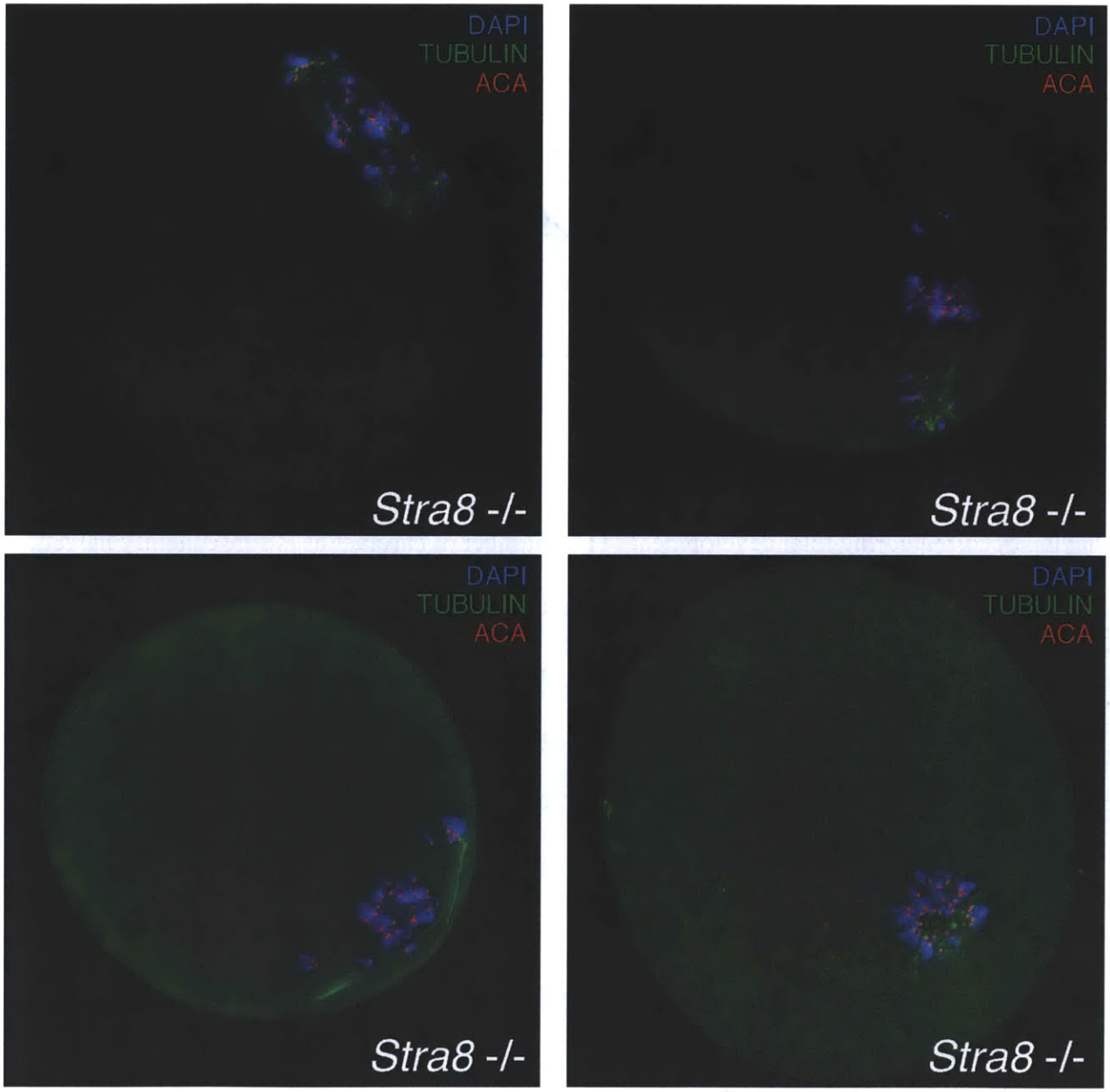



**Figure 10: Some *Stra8*-deficient oocyte-like cells exhibit spindle assembly defects resulting from absence of meiotic prophase.**

Deconvolved, projected Z-stacks of images of *in vitro*-matured *Stra8*-deficient oocyte-like cells that underwent GVB but failed to extrude a polar body after overnight culture. Cells were immunofluorescently labeled with anti-tubulin antibody (green) and anti-centromere antibody (ACA) (red). Chromosomes stained with DAPI (blue). In these oocytes the spindles are abnormally elongated with chromatids scattered along the spindle. By contrast, after overnight culture, wild-type cells arrest at metaphase II (see Fig. 12a).

deficient oocyte-like cells had replicated their chromosomes. We first addressed this question by comparing DAPI intensity in GV-stage wild-type oocytes and *Stra8*-deficient oocyte-like cells. Using this semi-quantitative approach, we found that wild-type oocytes, with replicated chromosomes, contained significantly more DNA than *Stra8*-deficient oocyte-like cells (Fig. 11).

To confirm this observation independently, and quantitatively, we examined the configuration of chromosomes in *Stra8*-deficient oocyte-like cells that had matured to produce polar bodies. Haploid mouse gametes have 20 chromosomes, and diploid cells have 40 chromosomes. In wild-type females, postnatal GV-stage oocytes have 40 cohesed pairs of sister chromatids – 80 chromatids in all – as a consequence of pre-meiotic chromosome replication having occurred during fetal development. (The number of chromatids is subsequently halved, during the first meiotic division, and halved again during the second meiotic division.)

Following the first division, chromatids (more precisely, their centromeres – one per chromatid) can be visualized and counted in the oocyte and polar body. We stained *in vitro*-matured cells with DAPI, anti-tubulin antibody, and anti-centromere antibody (ACA) to visualize, respectively, DNA, the spindle, and centromeres. We mounted the stained cells on slides in a manner that preserved three-dimensional structure, and we collected Z-stacks of images through the entire volume. We then counted centromeres and visualized the configurations of sister chromatids in both the oocyte (or oocyte-like cell) and its adjoining polar body.

As expected, in each of 16 wild-type samples, we observed a total of 80 chromatids: 20 pairs of cohesed chromatids in the matured oocyte and another 20 pairs of cohesed chromatids in the polar body (Fig. 12a,b). By contrast, in each of 11 *Stra8*-deficient samples, we observed a total of 40 uncohesed chromatids distributed unevenly between the oocyte-like cell and the polar

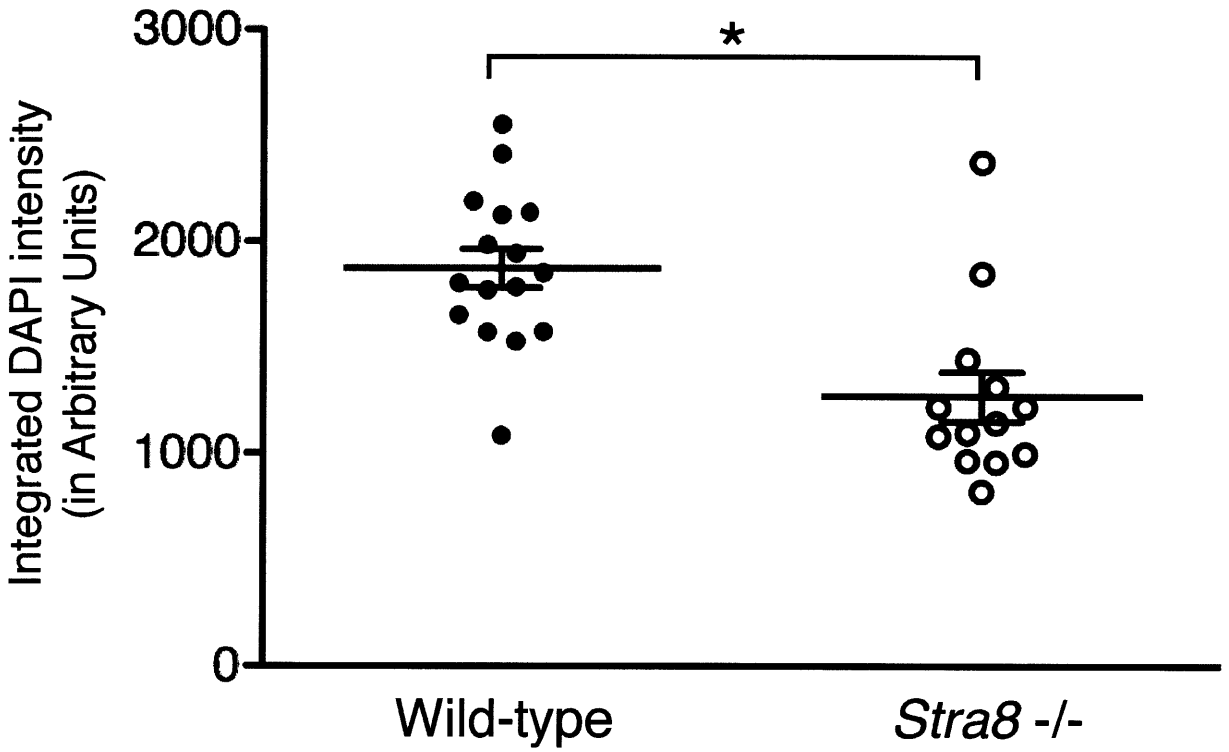
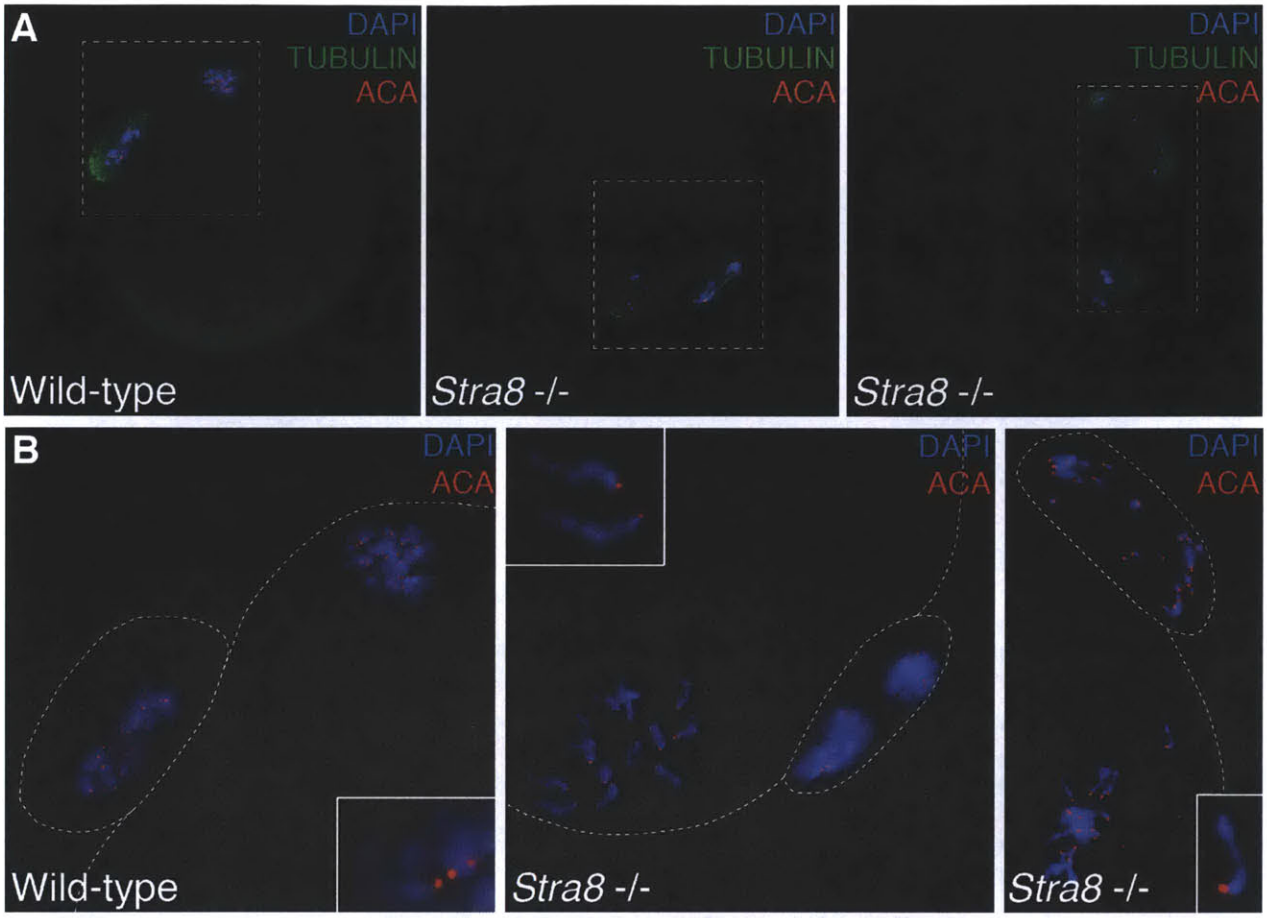



**Figure 11: *Stra8*-deficient oocyte-like cells contain significantly less DNA than wild-type oocytes at GV stage.**

Integrated DAPI intensity (in arbitrary units) of GV-stage wild-type oocytes and *Stra8*-deficient oocyte-like cells. Error bars represent standard error of the mean (SEM).
*$p < 0.001$ (Wilcoxon test).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ACA
Wild-type
Stra8 -/-
Stra8 -/-

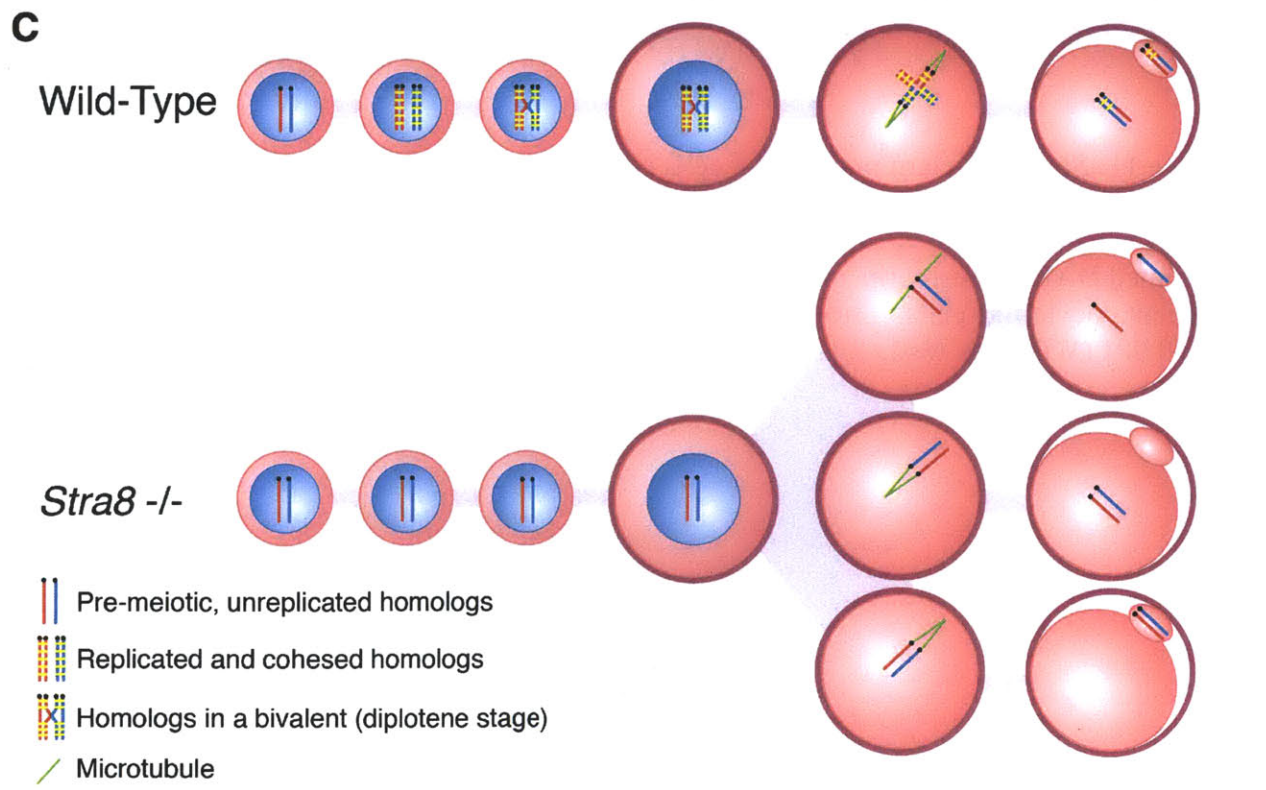
C
Wild-Type
Stra8 -/-
Pre-meiotic, unreplicated homologs
Replicated and cohesed homologs
Homologs in a bivalent (diplotene stage)
Microtubule

**Figure 12: Pre-meiotic chromosome replication is dispensable for oocyte differentiation.**

(A) Deconvolved, projected Z-stacks of images of *in vitro*-matured wild-type and *Stra8*-deficient oocyte-like cells. Cells were immuno-fluorescently labeled with anti-tubulin antibody (green) and anti-centromere antibody (ACA) (red). Chromosomes stained with DAPI (blue). (B) High-magnification views of boxed areas in (A); tubulin channel deleted. In each image, the polar body and oocyte or oocyte-like cell are outlined with dashed line. Insets provide ultra-high-magnification views of representative chromatids. Wild-type oocytes contain pairs of sister chromatids (as a result of chromosome replication), while mutants contain unreplicated single chromatids. Brightness and contrast of channels were adjusted independently. (C) Schematic interpretation of data presented in (A) and (B). At top: wild-type oocytes progress through meiotic prophase, with homologous chromosomes in bivalents. During growth and differentiation, chromosomes remain in bivalents. Upon maturation, homologous chromosomes segregate in an orderly fashion, one to the main cell and the other to the polar body. By contrast, during growth and differentiation of *Stra8-deficient* oocyte-like cells, chromosomes remain in an unreplicated, pre-meiotic configuration. Upon maturation of *Stra8-deficient* oocyte-like cells, the univalent homologous chromosomes segregate chaotically, with one, both, or neither going to the polar body.

body (Fig. 12a,b). Examination at high magnification confirmed that while wild-type oocytes contained cohesed sister chromatids, *Stra8*-deficient oocyte-like cells contained single chromatids (Fig. 12a,b insets). The only explanation for this chromosomal configuration is that, prior to maturation and polar body formation, these *Stra8*-deficient oocyte-like cells had 40 uncohesed chromatids – as would be found in an ordinary diploid cell that had not replicated its chromosomes. In the absence of meiotic prophase, bi-polar spindle attachment of homologous chromosomes would not have occurred. This evidently led to chaotic chromosome segregation, with one, both, or neither member of each homologous chromosome pair being apportioned to the polar body (Fig. 5c).

Taken together, these results argue strongly that *Stra8*-deficient germ cells completed oocyte-like differentiation and maturation without having undergone pre-meiotic chromosome replication and, by extension, without having entered meiotic prophase.

### *Stra8*-deficient Cells Cleave to Yield Two-cell Embryos upon Fertilization

Since *Stra8*-deficient oocyte-like cells have only 40 chromatids (as opposed to 80 in wild-type oocytes), which they segregate in an apparently haphazard manner, the probability of achieving a euploid egg that could yield a live-born pup is vanishingly small. Nevertheless, we wondered whether these oocyte-like cells could undergo fertilization and support early events of embryogenesis. To address this question we performed *in vitro* fertilization (IVF) experiments using control (wild-type or *Stra8*-heterozygous) oocytes and *Stra8*-deficient oocyte-like cells harvested by superovulation of juvenile females as described earlier. Six hours after IVF, we observed that control oocytes and *Stra8*-deficient oocyte-like cells had extruded a second polar body and contained two pronuclei, indicating successful fertilization (Fig. 13a). At 22 hours, 52

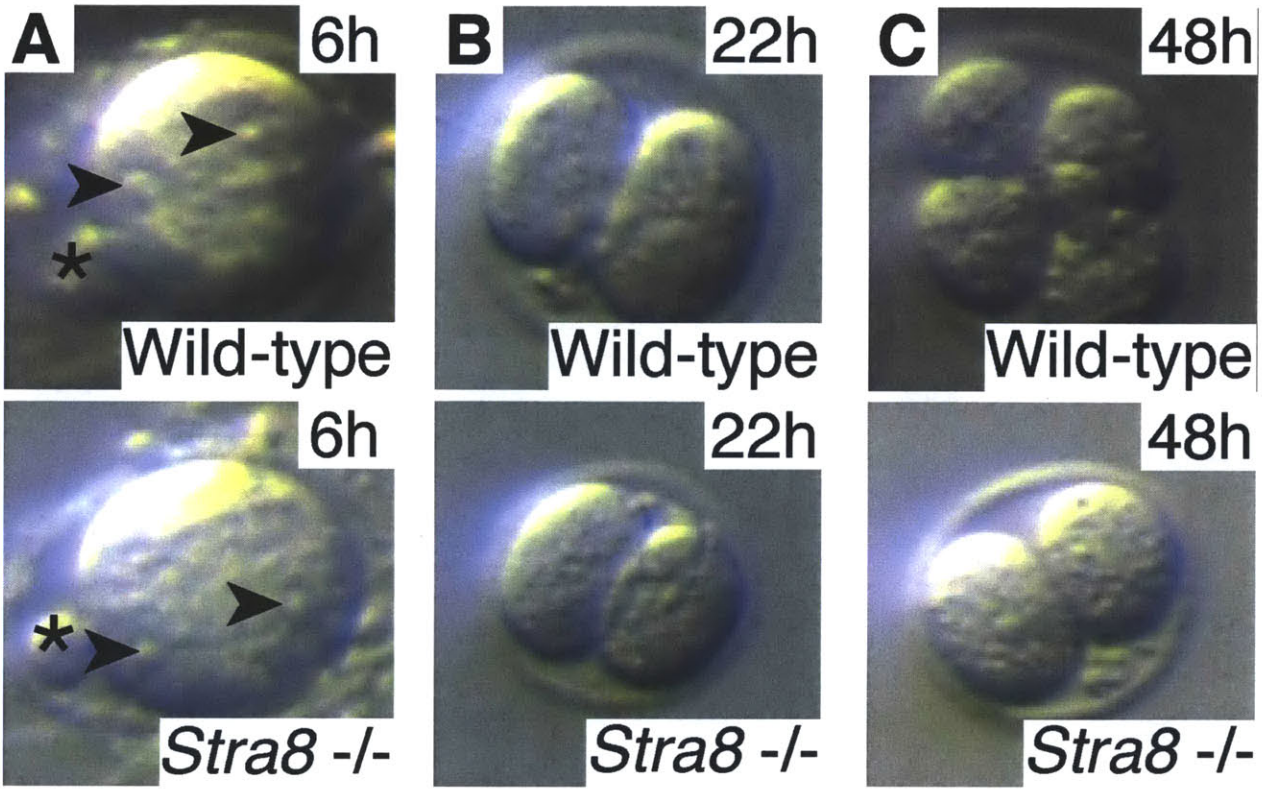


**Figure 13: *Stra8*-deficient oocyte-like cells cleave to yield two-cell embryos upon fertilization.**

Wild-type oocytes and *Stra8*-deficient oocyte-like cells at 6 (A), 22 (B), and 48 (C) hours after IVF. Arrowheads indicate paternal and maternal pro-nuclei. Asterisks indicate second polar bodies.

of 68 control oocytes and 7 of 39 *Stra8*-deficent oocyte-like cells had progressed to become two-cell embryos (Fig. 13b). At 48 hours, the control four-cell embryos had progressed to the four-cell stage, but all *Stra8*-deficent embryos remained arrested at the two-cell stage (Fig. 13c). The two-cell arrest in the mutant may be due to gross chromosomal deficiencies (hypoploidy) and imbalance as zygotic transcription begins to play a role in development (Schultz 2002). These results demonstrate that meiotic prophase is not required for the formation of a fertilization-competent egg-like cell.

## Discussion

### Oocyte growth/differentiation and meiosis are genetically separable processes

In this study we investigated the functional relationship between two components of the oogenesis program: cytoplasmic growth and differentiation and the chromosomal events of the meiotic prophase I. Using genetic analysis, we demonstrated that these two components are separable *in vivo*. We conclude that oogenesis in mice consists of two processes – differentiation and meiosis – that unfold in parallel, and that meiotic initiation and prophase I are not prerequisite to oocyte differentiation (Fig. 14).

### Practical ramifications of oocyte-like differentiation without meiosis: infertility and *in vitro* gametogenesis

Our finding that oogenesis can be genetically separated into oocyte differentiation and meiotic progression, has implications beyond basic understanding of egg development. Oocyte-like differentiation without meiosis may explain some cases of infertility, especially where women cannot achieve or sustain pregnancy despite the presence of cells that histologically resemble oocytes, and perhaps even in cases where IVF yields two-cell embryos. Similarly, some recent reports of mouse or human oocytes derived from cells grown in culture (Hubner et al. 2003; Lacham-Kaplan, Chy, and Trounson 2006; Kerkis et al. 2007; Qing et al. 2007; Salvador et al. 2008; Nicholas et al. 2009; White et al. 2012) may actually be demonstrating oocyte-like differentiation without meiosis (Novak et al. 2006). Our observation that oocyte-like morphology and functionality can arise in absence of meiotic prophase, clearly illustrates why claims of successful oogenesis *in vitro* cannot rest solely on evidence of oocyte-like differentiation. Meiotic initiation and progression must be documented directly.

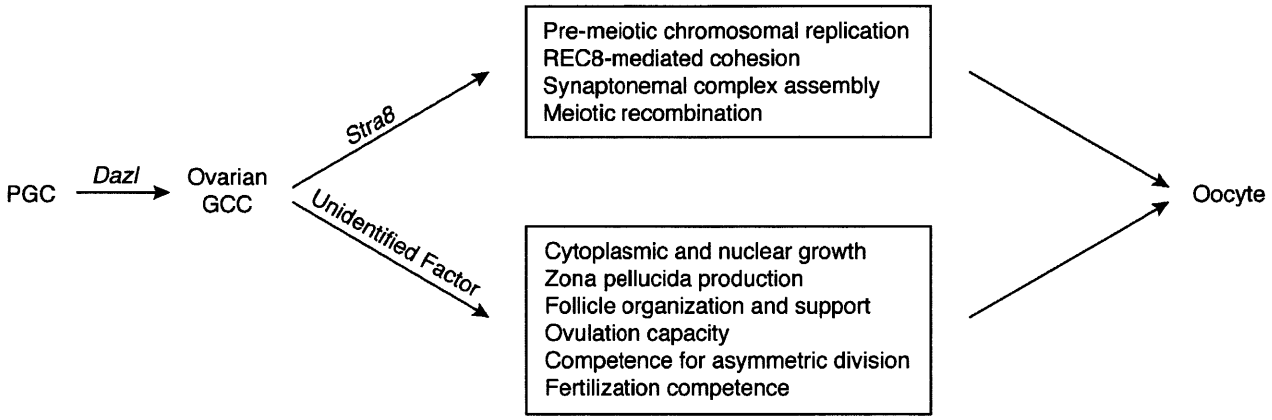


**Figure 14: A proposed model for parallel regulation of (upper arrows) meiotic initiation and (lower arrows) oocyte growth and differentiation in the mouse ovary.**

The gametogenesis- competent cell (GCC), which derives from a primordial germ cell (PGC), embarks on the meiotic program (above) through the action of the meiotic initiation factor *Stra8,* and it embarks on the *Stra8-independent* program of growth and differentiation through the action of one or more factors yet to be identified. The two processes thus set in motion constitute oogenesis.

**Meiotic initiation is not required for germ cell sex determination**

Our findings challenge the prevailing view of germ cell sex determination in mammals, which claims that if meiosis begins during fetal development, the germ cell is committed to oogenesis, while postponing meiosis commits it to spermatogenesis (Adams and McLaren 2002; Bowles et al. 2010; Bowles et al. 2006; Bowles and Koopman 2010; Kocer et al. 2009; McLaren 1984, 2003; McLaren and Southee 1997). On the contrary, we have shown that oocyte differentiation can occur in the absence of fetal or even postnatal initiation of meiosis. We suggest that fetal germ cells confront two decisions: whether to initiate meiosis, and whether to initiate oogenic differentiation. We therefore postulate the existence of a meiosis-independent pathway, yet to be identified, that regulates oocyte growth and differentiation (Fig. 14).

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## Methods

### Mice

*Stra8*-deficient mice were generated and backcrossed as previously described (Baltus et al. 2006; Anderson et al. 2008). All *Stra8* animals used were backcrossed to C57BL/6NtacfBR for at least 18 generations. *Dmc1*-deficient mice (Pittman et al. 1998), which had been backcrossed to C57BL/6J for at least 10 generations, were purchased from Jackson Laboratory. *Stra8*-deficient females were generated by mating heterozygotes. *Dmc1*-deficient females and *Stra8 / Dmc1* double-deficient females were generated by mating *Stra8 / Dmc1* double heterozygotes. All experiments involving mice were approved by the Committee on Animal Care at the Massachusetts Institute of Technology.

### Histology

Dissected ovaries were fixed in Bouin's solution (Polysciences) overnight at 4°C, embedded in paraffin and sectioned. Slides were stained with periodic acid-Schiff (PAS) reagent (Sigma) according to the manufacturer's protocol and counter-stained with Mayer's modified hematoxylin (Invitrogen). For every genotype at least four samples were analyzed.

### Immunocytochemistry

Ovarian germ cell spreads were prepared according to a published protocol (Susiarjo, Rubio, and Hunt 2009). Cells were permeabilized with 0.5% Triton-X100 for 5 minutes and blocked with 1% BSA for 1 hour. Rabbit anti-SYCP3 (Abcam) was used at a 1:1000 dilution. Rabbit anti-

REC8 (courtesy of C. Heyting, Agricultural University, Wageningen, Netherlands) was used at a 1:250 dilution.

**Immunohistochemistry**

Dissected ovaries were fixed in Bouin's solution or 4% paraformaldehyde overnight at 4°C, embedded in paraffin and sectioned. Slides were de-waxed in xylenes, re-hydrated through an ethanol gradient and boiled in sodium citrate buffer (pH 6.0) for 15 minutes. For colorimetric immunohistochemistry, slides were pre-treated for 10 minutes with 0.3% $H_2O_2$. Mouse monoclonal anti-γH2A.X (Millipore) was used at a 1:100 dilution. Goat anti-hVASA (R&D Systems) was used at a 1:250 dilution. Rabbit polyclonal anti-NOBOX (Abcam) was used at a 1:1000 dilution. Goat anti-FOXL2 (Abcam) was used at 1:500 dilution.

**Superovulation and Isolation of Ovulated COCs**

Female mice were stimulated with 5 international units (IU) of pregnant mare serum gonadotropin (PMSG) (Sigma) at postnatal day 20 (P20). Forty-four to 48 hours after PMSG injection, the animals were stimulated with 5 IU of human chorionic gonadotropin (hCG) (Sigma). Fifteen to 16 hours after the second injection, the animals were sacrificed and their oviducts were dissected away from the uterus and ovaries. Oocytes were released by slicing open the oviducts and allowing the COCs to spill into a drop of KSOM (potassium simplex optimized medium) (Millipore) under oil. Cumulus cells were removed by treating COCs with hyaluronidase (Sigma) for 5 minutes and washing twice in KSOM.

### Germ Cell *In Vitro* Maturation

Female mice were stimulated with 5 IU of PMSG at P20. Forty-four to 48 hours after injection, animals were sacrificed and ovaries dissected. COCs were isolated by follicle puncture and cultured overnight in MEM-α supplemented with 0.3% BSA. The next day, cumulus cells were removed mechanically, and polar body formation was assessed.

### DAPI Intensity Integration

GV-stage oocytes were isolated by follicle puncture, mechanically denuded and spread by drying down on slides wetted with 1% parafarmaldehyde. Slides were imaged using constant exposure time across all samples. DAPI intensity was quantified using CellProfiler software (Carpenter et al. 2006).

### Whole Oocyte Immunocytochemistry

*In vitro*-matured oocytes were fixed in 4% parafarmaldehyde for 45 minutes, permeabilized with 0.1% Triton-X100 and blocked with 10% donkey serum. Monoclonal rat anti-tubulin [YL/2] (Abcam) was used at a 1:250 dilution. Human anti-centromere antibody (ACA) (Antibodies, Inc.) was used at a 1:100 dilution.

### Microscopy

Images were acquired using a DeltaVision deconvolution microscope (AppliedPrecision). For oocytes and oocyte-like cells, Z-stacks were collected at 0.5 μm spacing. All images within the stacks were deconvolved using DeltaVision softWoRx software. Z-stack projections were

generated using the maximum intensity method in ImageJ (Abramoff 2004). ACA signals were counted on projected image. Individual images from the Z-stacks were used to resolve centromeres that were superimposed in the Z plane.

### *In Vitro* Fertilization

Superovulated COCs were collected as described earlier and maintained in MEM-α (minimum essential medium alpha) (Invitrogen) supplemented with 5% fetal bovine serum (FBS) to prevent zona pellucida hardening. To collect sperm, epididymi were dissected from adult males and cut to release sperm in serum-free MEM-α supplemented with 0.3% bovine serum albumin (BSA) (Sigma). COCs were washed out of serum into MEM-α supplemented with 0.3% BSA and added to sperm, which had been diluted 1:90 in MEM-α supplemented with 0.3% BSA under oil. Fertilization dishes were incubated for 6 hours before washing away sperm and then incubated overnight. Two-cell embryos were transferred to KSOM for further culture.

## References


Abramoff, M.D. 2004. Image Processing with ImageJ. *Biophotonics International* 11 (7):36-42.

Adams, I. R., and A. McLaren. 2002. Sexually dimorphic development of mouse primordial germ cells: switching from oogenesis to spermatogenesis. *Development* 129 (5):1155-64.

Anderson, E. L., A. E. Baltus, H. L. Roepers-Gajadien, T. J. Hassold, D. G. de Rooij, A. M. van Pelt, and D. C. Page. 2008. Stra8 and its inducer, retinoic acid, regulate meiotic initiation in both spermatogenesis and oogenesis in mice. *Proc. Natl. Acad. Sci. U.S.A.* 105 (39):14976-80.

Baltus, A. E., D. B. Menke, Y. C. Hu, M. L. Goodheart, A. E. Carpenter, D. G. de Rooij, and D. C. Page. 2006. In germ cells of mouse embryonic ovaries, the decision to enter meiosis precedes premeiotic DNA replication. *Nat. Genet.* 38 (12):1430-4.

Baudat, F., K. Manova, J. P. Yuen, M. Jasin, and S. Keeney. 2000. Chromosome synapsis defects and sexually dimorphic meiotic progression in mice lacking Spo11. *Mol. Cell* 6 (5):989-98.

Bishop, D. K., D. Park, L. Xu, and N. Kleckner. 1992. DMC1: a meiosis-specific yeast homolog of E. coli recA required for recombination, synaptonemal complex formation, and cell cycle progression. *Cell* 69 (3):439-56.

Bowles, J., C. W. Feng, C. Spiller, T. L. Davidson, A. Jackson, and P. Koopman. 2010. FGF9 suppresses meiosis and promotes male germ cell fate in mice. *Dev. Cell* 19 (3):440-9.

Bowles, J., D. Knight, C. Smith, D. Wilhelm, J. Richman, S. Mamiya, K. Yashiro, K. Chawengsaksophak, M. J. Wilson, J. Rossant, H. Hamada, and P. Koopman. 2006. Retinoid signaling determines germ cell fate in mice. *Science* 312 (5773):596-600.

Bowles, J., and P. Koopman. 2010. Sex determination in mammalian germ cells: extrinsic versus intrinsic factors. *Reproduction* 139 (6):943-58.

Carpenter, A. E., T. R. Jones, M. R. Lamprecht, C. Clarke, I. H. Kang, O. Friman, D. A. Guertin, J. H. Chang, R. A. Lindquist, J. Moffat, P. Golland, and D. M. Sabatini. 2006. CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biol.* 7 (10):R100.

Crisponi, L., M. Deiana, A. Loi, F. Chiappe, M. Uda, P. Amati, L. Bisceglia, L. Zelante, R. Nagaraja, S. Porcu, M. S. Ristaldi, R. Marzella, M. Rocchi, M. Nicolino, A. Lienhardt-Roussie, A. Nivelon, A. Verloes, D. Schlessinger, P. Gasparini, D. Bonneau, A. Cao, and

G. Pilia. 2001. The putative forkhead transcription factor FOXL2 is mutated in blepharophimosis/ptosis/epicanthus inversus syndrome. *Nat. Genet.* 27 (2):159-66.

Di Giacomo, M., M. Barchi, F. Baudat, W. Edelmann, S. Keeney, and M. Jasin. 2005. Distinct DNA-damage-dependent and -independent responses drive the loss of oocytes in recombination-defective mouse mutants. *Proc. Natl. Acad. Sci. U. S. A.* 102 (3):737-42.

Donahue, R. P. 1968. Maturation of the mouse oocyte in vitro. I. Sequence and timing of nuclear progression. *J. Exp. Zool.* 169 (2):237-49.

Eppig, J. J., K. Wigglesworth, and F. Chesnel. 1993. Secretion of cumulus expansion enabling factor by mouse oocytes: relationship to oocyte growth and competence to resume meiosis. *Dev. Biol.* 158 (2):400-9.

Gill, M. E., Y. C. Hu, Y. Lin, and D. C. Page. 2011. Licensing of gametogenesis, dependent on RNA binding protein DAZL, as a gateway to sexual differentiation of fetal germ cells. *Proc. Natl. Acad. Sci. U.S.A.* 108 (18):7443-8.

Hubner, K., G. Fuhrmann, L. K. Christenson, J. Kehler, R. Reinbold, R. De La Fuente, J. Wood, J. F. Strauss, 3rd, M. Boiani, and H. R. Scholer. 2003. Derivation of oocytes from mouse embryonic stem cells. *Science* 300 (5623):1251-6.

Keeney, S., C. N. Giroux, and N. Kleckner. 1997. Meiosis-specific DNA double-strand breaks are catalyzed by Spo11, a member of a widely conserved protein family. *Cell* 88 (3):375-84.

Kerkis, A., S. A. Fonseca, R. C. Serafim, T. M. Lavagnolli, S. Abdelmassih, R. Abdelmassih, and I. Kerkis. 2007. In vitro differentiation of male mouse embryonic stem cells into both presumptive sperm cells and oocytes. *Cloning Stem Cells* 9 (4):535-48.

Kocer, A., J. Reichmann, D. Best, and I. R. Adams. 2009. Germ cell sex determination in mammals. *Mol. Hum. Reprod.* 15 (4):205-13.

Kouznetsova, A., L. Lister, M. Nordenskjold, M. Herbert, and C. Hoog. 2007. Bi-orientation of achiasmatic chromosomes in meiosis I oocytes contributes to aneuploidy in mice. *Nat. Genet.* 39 (8):966-8.

Krentz, A. D., M. W. Murphy, A. L. Sarver, M. D. Griswold, V. J. Bardwell, and D. Zarkower. 2011. DMRT1 promotes oogenesis by transcriptional activation of Stra8 in the mammalian fetal ovary. *Devel. Biol.* 356 (1):63-70.

Lacham-Kaplan, O., H. Chy, and A. Trounson. 2006. Testicular cell conditioned medium supports differentiation of embryonic stem cells into ovarian structures containing oocytes. *Stem Cells* 24 (2):266-73.

Lin, Y., M. E. Gill, J. Koubova, and D. C. Page. 2008. Germ cell-intrinsic and -extrinsic factors govern meiotic initiation in mouse embryos. *Science* 322 (5908):1685-7.

McLaren, A. 1984. Meiosis and differentiation of mouse germ cells. *Symp. Soc. Exp. Biol.* 38:7-23.

———. 2003. Primordial germ cells in the mouse. *Dev. Biol.* 262 (1):1-15.

McLaren, A., and D. Southee. 1997. Entry of mouse embryonic germ cells into meiosis. *Dev. Biol.* 187 (1):107-13.

Nicholas, C. R., K. M. Haston, A. K. Grewall, T. A. Longacre, and R. A. Reijo Pera. 2009. Transplantation directs oocyte maturation from embryonic stem cells and provides a therapeutic strategy for female infertility. *Hum. Mol. Genet.* 18 (22):4376-89.

Novak, I., D. A. Lightfoot, H. Wang, A. Eriksson, E. Mahdy, and C. Hoog. 2006. Mouse embryonic stem cells form follicle-like ovarian structures but do not progress through meiosis. *Stem Cells* 24 (8):1931-6.

Oatley, J., and P. A. Hunt. 2012. Of mice and (wo)men: purified oogonial stem cells from mouse and human ovaries. *Biol. Reprod.* 86 (6):196.

Page, A. W., and T. L. Orr-Weaver. 1997. Stopping and starting the meiotic cell cycle. *Curr. Opin. Genet. Dev.* 7 (1):23-31.

Paredes, A., C. Garcia-Rudaz, B. Kerr, V. Tapia, G. A. Dissen, M. E. Costa, A. Cornea, and S. R. Ojeda. 2005. Loss of synaptonemal complex protein-1, a synaptonemal complex protein, contributes to the initiation of follicular assembly in the developing rat ovary. *Endocrinol.* 146 (12):5267-77.

Pittman, D. L., J. Cobb, K. J. Schimenti, L. A. Wilson, D. M. Cooper, E. Brignull, M. A. Handel, and J. C. Schimenti. 1998. Meiotic prophase arrest with failure of chromosome synapsis in mice deficient for Dmc1, a germline-specific RecA homolog. *Mol. Cell* 1 (5):697-705.

Qing, T., Y. Shi, H. Qin, X. Ye, W. Wei, H. Liu, M. Ding, and H. Deng. 2007. Induction of oocyte-like cells from mouse embryonic stem cells by co-culture with ovarian granulosa cells. *Differentiation* 75 (10):902-11.

Rajkovic, A., S. A. Pangas, D. Ballow, N. Suzumori, and M. M. Matzuk. 2004. NOBOX deficiency disrupts early folliculogenesis and oocyte-specific gene expression. *Science* 305 (5687):1157-9.

Rogakou, E. P., D. R. Pilch, A. H. Orr, V. S. Ivanova, and W. M. Bonner. 1998. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J. Biol. Chem.* 273 (10):5858-68.

Salvador, L. M., C. P. Silva, I. Kostetskii, G. L. Radice, and J. F. Strauss, 3rd. 2008. The promoter of the oocyte-specific gene, Gdf9, is active in population of cultured mouse embryonic stem cells with an oocyte-like phenotype. *Methods* 45 (2):172-81.

Scherthan, H. 2003. Knockout mice provide novel insights into meiotic chromosome and telomere dynamics. *Cytogenet. Genome Res.* 103 (3-4):235-44.

Schmidt, D., C. E. Ovitt, K. Anlag, S. Fehsenfeld, L. Gredsted, A. C. Treier, and M. Treier. 2004. The murine winged-helix transcription factor Foxl2 is required for granulosa cell differentiation and ovary maintenance. *Development* 131 (4):933-42.

Schultz, R. M. 2002. The molecular foundations of the maternal to zygotic transition in the preimplantation embryo. *Hum. Reprod. Update* 8 (4):323-31.

Sorensen, R. A., and P. M. Wassarman. 1976. Relationship between growth and meiotic maturation of the mouse oocyte. *Dev. Biol.* 50 (2):531-6.

Su, Y. Q., K. Sugiura, and J. J. Eppig. 2009. Mouse oocyte control of granulosa cell development and function: paracrine regulation of cumulus cell metabolism. *Semin. Reprod. Med.* 27 (1):32-42.

Susiarjo, M., C. Rubio, and P. Hunt. 2009. Analyzing mammalian female meiosis. *Methods Mol. Biol.* 558:339-54.

Von Stetina, J. R., and T. L. Orr-Weaver. 2011. Developmental control of oocyte maturation and egg activation in metazoan models. *Cold Spring Harb. Perspect. Biol.* 3 (10):a005553.

White, Y. A., D. C. Woods, Y. Takai, O. Ishihara, H. Seki, and J. L. Tilly. 2012. Oocyte formation by mitotically active germ cells purified from ovaries of reproductive-age women. *Nat. Med.* 18 (3):413-21.

Woods, L. M., C. A. Hodges, E. Baart, S. M. Baker, M. Liskay, and P. A. Hunt. 1999. Chromosomal influence on meiotic spindle assembly: abnormal meiosis I in female Mlh1 mutant mice. *J. Cell Biol.* 145 (7):1395-406.

Yoshida, K., G. Kondoh, Y. Matsuda, T. Habu, Y. Nishimune, and T. Morita. 1998. The mouse RecA-like gene Dmc1 is required for homologous chromosome synapsis during meiosis. *Mol. Cell* 1 (5):707-18.

# Chapter 3

# Rethinking oogenesis

The work presented in chapter 2 invites a new way of thinking about the developmental program of oogenesis. Instead of a single program, we can now think of oogenesis as two independent processes under separate regulation. This new understanding invites a revision of the current "single decision" model of commitment to oogenesis. It also has practical implications for *in vitro* oogenesis strategies. In this chapter we will review the major conclusions of the findings presented thus far, discuss their implications, and pose some remaining unanswered questions.

### *Stra8* is necessary for meiotic initiation in the ovary

It was previously reported that *Stra8*-deficient ovarian germ cells fail to initiate meiosis during fetal development (Baltus et al. 2006). Here we confirmed these findings on a controlled genetic background, using inbred C57BL/6 mice. We also extended the previously reported observations by directly analyzing postnatal *Stra8*-deficient germ cells during the first wave of oogenesis. Through study of *Stra8/Dmc1* double mutants, we demonstrated that *Stra8*-deficient cells develop without making meiotic DSBs and hence without entering meiotic prophase I at any point in development. Furthermore, by examining the chromosomes of *Stra8*-deficient postnatal germ cells directly, we showed that weeks after wild-type germ cells initiate meiosis, the chromosomal content and configuration of *Stra8*-deficient germ cells remains identical to that of a pre-meiotic germ cell. This observation highlights an important difference between the *Stra8* mutant and meiotic mutants such as *Spo11, Dmc1, Rec8, Sycp1, Sycp3,* or *Mlh1*. Unlike the *Stra8* mutant, meiotic mutants initiate meiosis and carry out many aspects of meiotic prophase I. Their defects are instead limited to a specific subset of meiotic chromosomal processes (Baudat et al. 2000; Romanienko and Camerini-Otero 2000; Pittman et al. 1998; Yoshida et al. 1998; Kouznetsova et al. 2007; Xu et al. 2005; Bannister et al. 2004). In contrast,

the *Stra8* mutant does not undergo any of the chromosomal events associated with prophase of meiosis I and therefore is non-meiotic. Thus the *Stra8* mutant represents a unique and powerful tool with which to study ovarian germ cell differentiation in the absence of the meiotic program.

**Oocyte differentiation can occur in absence of *Stra8* function**

Following our original report that *Stra8* is required for meiotic initiation in the fetal ovary, other investigators inferred – given prevailing models – that *Stra8* must also be required for oocyte differentiation (Bowles et al. 2010; Bowles et al. 2006; Bowles and Koopman 2010; Krentz et al. 2011; Suzuki and Saga 2008). To the contrary, *Stra8* function, and hence meiotic initiation and prophase I, is not required for oocyte differentiation, as judged by morphological criteria, molecular markers, and functional assays. In the absence of the meiotic program, *Stra8*-deficient ovarian germ cells can develop a large cytoplasm, synthesize zonae pellucidae, organize surrounding somatic cells into follicles, undergo asymmetric cell divisions to form polar bodies, be ovulated in response to hormonal stimulation and cleave to yield two-cell embryos upon fertilization. We conclude that oogenesis in mice is a genetically dissociable union of two concurrent processes – meiosis and differentiation. While the former depends on *Stra8*, the latter does not.

**Meiosis and oocyte differentiation in other species**

Oocyte growth and differentiation during meiotic prophase I arrest is broadly conserved (Page and Orr-Weaver 1997; Von Stetina and Orr-Weaver 2011). Therefore, it would be interesting to know whether meiotic initiation is necessary for oocyte differentiation in other animals, such as

*Caenorhabditis* elegans (*C. elegans*), *D. melanogaster*, and *Xenopus*. The *Stra8* gene is present in the *Xenopus* genome, but its role in meiotic initiation and oocyte differentiation has not been established. In other animals it may also be possible to conduct similar studies, once meiosis-essential genes are identified.

A study in rat reported that manipulating the oocyte's rate of progression to diplotene could modulate the timing of primordial follicle formation (Paredes et al. 2005). Based on the relatively short evolutionary distance between mouse and rat, one would anticipate that in rat meiotic initiation would also be dispensable for oocyte differentiation. If that is the case, then an alternate explanation for early primordial follicle formation in this system will be needed.

**Implications for germ cell sex determination**

A basic question in germ cell development is: how does a gametogenesis competent germ cell choose to become an egg or a sperm? Until now, this problem has been viewed as a single choice between oogenesis and spermatogenesis. Consequentially, the prevailing view of germ cell sex determination is the "single decision" model. This model maintains that once a germ cell chooses to initiate meiosis during fetal development it commits to oogenesis, and oocyte differentiation will necessarily follow. Conversely, if fetal meiotic initiation is prevented, spermatogenesis will follow (McLaren 1984, 2003; Bowles et al. 2006; Bowles and Koopman 2010; Kocer et al. 2009; Adams and McLaren 2002; McLaren and Southee 1997). This model depends on the assumption that all of oogenesis is under single regulation; an assumption we challenged. We have shown that oocyte differentiation can occur in the absence of fetal or even postnatal initiation of meiosis. We, therefore, suggest that fetal germ cells confront not one but two decisions: whether to initiate meiosis, and whether to initiate oocyte differentiation. We

postulate the existence of a *Stra8*-independent pathway, yet to be identified, that regulates oocyte growth and differentiation. In the absence of meiotic initiation, this pathway can still drive oocyte differentiation, but the resultant oocyte-like cells lack the proper chromatid number and organization and resultant chromosome segregation is chaotic.

**Identity of the oocyte-determining signal**

Pioneering studies by McLaren and colleagues were intended to shed light on the mechanism of sperm/egg decision (Adams and McLaren 2002; McLaren 1984; McLaren and Southee 1997). However, given the findings reported in chapter 2 it is now clear that McLaren and colleagues were not studying commitment to oogenesis or spermatogenesis, but rather meiotic initiation alone. Thus, although their results remain relevant for ovary-specific fetal meiotic initiation, the implications of their data for oocyte differentiation are not clear. By contrast, Zamboni and Upadhyay, as well as Byskov were able to assay oocyte differentiation directly, and it is important to interpret their findings in light of the new understanding of oogenesis (Byskov 1974; Upadhyay and Zamboni 1982; Zamboni and Upadhyay 1983). To do so, it may be necessary to reproduce the Updahyay and Zamboni experiments with modern molecular tools. With the set of markers described in chapter 1, we can characterize in detail the extent of meiotic progression and oocyte differentiation accomplished by the germ cells in the adrenal. Such characterization would allow us to identify the aspects of oogenesis (if any) that are dependent on the ovarian environment. Detailed comparison of XX and XY adrenal germ cells may provide novel insights into the role of sex chromosomes in oogenesis.

Zamboni and Upadhyay's observations suggested that oogenesis is the default developmental pathway because germ cells in the adrenal gland undergo oogenesis (Upadhyay

and Zamboni 1982; Zamboni and Upadhyay 1983). The McLaren experiments partially support this idea, by demonstrating that meiosis could be blocked by a testicular MPS. However, knowing that the McLaren MPS data only pertain to meiotic initiation and not to oocyte differentiation, one wonders whether there is an oocyte-differentiation preventing substance as well. To investigate this further, one could make use of recently developed aggregation and transplantation technologies to reproduce the McLaren experiments and assay not only meiotic initiation but oocyte or sperm differentiation as well (Hayashi et al. 2011; Adams and McLaren 2002; Hayashi et al. 2012; McLaren and Southee 1997).

Is it still possible that oocyte differentiation is the default pathway? Byskov's experiments of culturing ovaries with and without the mesonephroi argue against this model (Byskov 1974). In her study, Byskov did not see oocyte differentiation without the mesonephros, suggesting oocyte differentiation must be induced by a mesonephric signal. However, her finding is a negative result, and the idea of oocyte differentiation as the "default pathway" may require further investigation.

If oocyte differentiation is not default and must be induced, what could be an oocyte differentiation inducing substance? For one, if it exists, it must be present in the adrenal. Could it be RA signaling though a *Stra8*-independent pathway? That remains a possibility, although we have no evidence to support the RA hypothesis over any other signal. A candidate approach may be useful to identifying genes in the oocyte differentiation pathway. These genes are likely to be expressed specifically in fetal female germ cells, and unlike meiotic genes, should not be expressed postnatally in males. Perhaps in the future an efficient *in vitro* oogenesis system could facilitate a chemical or RNAi screening approach to identifying oocyte differentiation factors.

**Practical implications for in vitro oogenesis strategies**

Recently, successful oogenesis from ES and iPS cells was achieved in mouse by culturing induced PGC-like cells within gonadal aggregates followed by transplantation to the ovary of an adult female (Hayashi et al. 2012). This study presents a major advance for the *in vitro* oogenesis field, in that it demonstrates the ability to derive functional PGC-like cells, capable of oogenesis, entirely *in vitro*. The authors of this study are also able to as achieve some degree of synaptonemal complex assembly (one marker of meiotic prophase) in aggregate cultures of the PGC-like cells with fetal gonads. However, completion of meiosis and oocyte differentiation in this study required both aggregation of PGC-like cells with fetal gonads and transplantation into a surrogate ovary. Thus, neither meiosis nor oocyte differentiation has been achieved *in vitro*. Understanding the functional dependencies, if any, between meiosis and oocyte differentiation may be critical to moving this technology entirely *in vitro*. For example, our discovery that *Stra8* is not required for oocyte differentiation, may explain why *in vitro* differentiation strategies that select for a STRA8-GFP marker fail to achieve growth and differentiation despite demonstrating some aspects of meiotic initiation (Nicholas et al. 2009). In light of our findings, it is reasonable to expect that the next major *in vitro* oogenesis breakthrough will require identification of the independent oocyte growth and differentiation signal.

**Open questions**

The work presented in chapter 2 raises several important questions, which remain to be investigated. How does the non-meiotic genome of the *Stra8*-deficient ovarian germ cell drive oogenic growth and differentiation? What are the functional consequences, if any, of having 2C DNA content versus the wild-type 4C DNA content during oogenesis? These questions might be

addressed in the future, at least in part, by assessing the epigenetic and transcriptional status of the *Stra8*-deficient oocyte-like cells. It is important to keep in mind that these experiments may present some challenges, since it is difficult to obtain *Stra8*-deficient oocyte-like cells in substantial numbers. It will be critical to analyze *Stra8*-deficient germ cells at fully grown GV stage, before the chaotic chromosome segregation causes heterogeneity among oocyte-like cells. Additionally, analysis at earlier stages of oocyte-like development may provide novel developmental insights.

Would the cytoplasm of a non-meiotic, *Stra8*-deficient oocyte-like cell be capable of supporting embryogenesis beyond the two-cell stage if presented with a normal complement of chromosomes? The answer to this question would provide a more stringent test of the limits of oogenic differentiation in the absence of a meiotic program. This might in the future be tested by nuclear transplantation or other means.

From fetal and postnatal histological analysis, we know that the absence of *Stra8* function accelerates the germ cell loss that is a prominent feature of the wild-type ovary. We do not know the cause(s) of this germ-cell death in either the *Stra8*-deficient or wild-type ovaries. It seems unlikely that it would be due to any of the checkpoints usually associated with progression through meiotic prophase, since DSBs and asynapsed axial elements, recognized by DNA damage or asynapsis checkpoints respectively, are not present in the *Stra8* mutant (Wojtasz et al. 2012; Di Giacomo et al. 2005). This suggests that an oocyte quality control mechanism that assesses aspects of oogenesis outside of meiotic progression may exist, but remains to be elucidated.

## References


Adams, I. R., and A. McLaren. 2002. Sexually dimorphic development of mouse primordial germ cells: switching from oogenesis to spermatogenesis. *Development* 129 (5):1155-64.

Baltus, A. E., D. B. Menke, Y. C. Hu, M. L. Goodheart, A. E. Carpenter, D. G. de Rooij, and D. C. Page. 2006. In germ cells of mouse embryonic ovaries, the decision to enter meiosis precedes premeiotic DNA replication. *Nat. Genet.* 38 (12):1430-4.

Bannister, L. A., L. G. Reinholdt, R. J. Munroe, and J. C. Schimenti. 2004. Positional cloning and characterization of mouse mei8, a disrupted allelle of the meiotic cohesin Rec8. *Genesis* 40 (3):184-94.

Baudat, F., K. Manova, J. P. Yuen, M. Jasin, and S. Keeney. 2000. Chromosome synapsis defects and sexually dimorphic meiotic progression in mice lacking Spo11. *Mol. Cell* 6 (5):989-98.

Bowles, J., C. W. Feng, C. Spiller, T. L. Davidson, A. Jackson, and P. Koopman. 2010. FGF9 suppresses meiosis and promotes male germ cell fate in mice. *Dev. Cell.* 19 (3):440-9.

Bowles, J., D. Knight, C. Smith, D. Wilhelm, J. Richman, S. Mamiya, K. Yashiro, K. Chawengsaksophak, M. J. Wilson, J. Rossant, H. Hamada, and P. Koopman. 2006. Retinoid signaling determines germ cell fate in mice. *Science* 312 (5773):596-600.

Bowles, J., and P. Koopman. 2010. Sex determination in mammalian germ cells: extrinsic versus intrinsic factors. *Reproduction* 139 (6):943-58.

Byskov, A. G. 1974. Does the rete ovarii act as a trigger for the onset of meiosis? *Nature* 252 (5482):396-7.

Di Giacomo, M., M. Barchi, F. Baudat, W. Edelmann, S. Keeney, and M. Jasin. 2005. Distinct DNA-damage-dependent and -independent responses drive the loss of oocytes in recombination-defective mouse mutants. *Proc. Natl. Acad. Sci. U.S.A.* 102 (3):737-42.

Hayashi, K., S. Ogushi, K. Kurimoto, S. Shimamoto, H. Ohta, and M. Saitou. 2012. Offspring from Oocytes Derived from in Vitro Primordial Germ Cell-Like Cells in Mice. *Science.* 338(6109):971-5

Hayashi, K., H. Ohta, K. Kurimoto, S. Aramaki, and M. Saitou. 2011. Reconstitution of the mouse germ cell specification pathway in culture by pluripotent stem cells. *Cell* 146 (4):519-32.

Kocer, A., J. Reichmann, D. Best, and I. R. Adams. 2009. Germ cell sex determination in mammals. *Mol. Hum. Reprod.* 15 (4):205-13.

Kouznetsova, A., L. Lister, M. Nordenskjold, M. Herbert, and C. Hoog. 2007. Bi-orientation of achiasmatic chromosomes in meiosis I oocytes contributes to aneuploidy in mice. *Nat. Genet.* 39 (8):966-8.

Krentz, A. D., M. W. Murphy, A. L. Sarver, M. D. Griswold, V. J. Bardwell, and D. Zarkower. 2011. DMRT1 promotes oogenesis by transcriptional activation of Stra8 in the mammalian fetal ovary. *Dev. Biol.* 356 (1):63-70.

McLaren, A. 1984. Meiosis and differentiation of mouse germ cells. *Symp. Soc. Exp. Biol.* 38:7-23.

———. 2003. Primordial germ cells in the mouse. *Dev. Biol.* 262 (1):1-15.

McLaren, A., and D. Southee. 1997. Entry of mouse embryonic germ cells into meiosis. *Dev. Biol.* 187 (1):107-13.

Nicholas, C. R., K. M. Haston, A. K. Grewall, T. A. Longacre, and R. A. Reijo Pera. 2009. Transplantation directs oocyte maturation from embryonic stem cells and provides a therapeutic strategy for female infertility. *Hum. Mol. Genet.* 18 (22):4376-89.

Page, A. W., and T. L. Orr-Weaver. 1997. Stopping and starting the meiotic cell cycle. *Curr. Opin. Genet. Dev.* 7 (1):23-31.

Paredes, A., C. Garcia-Rudaz, B. Kerr, V. Tapia, G. A. Dissen, M. E. Costa, A. Cornea, and S. R. Ojeda. 2005. Loss of synaptonemal complex protein-1, a synaptonemal complex protein, contributes to the initiation of follicular assembly in the developing rat ovary. *Endocrinol.* 146 (12):5267-77.

Pittman, D. L., J. Cobb, K. J. Schimenti, L. A. Wilson, D. M. Cooper, E. Brignull, M. A. Handel, and J. C. Schimenti. 1998. Meiotic prophase arrest with failure of chromosome synapsis in mice deficient for Dmc1, a germline-specific RecA homolog. *Mol. Cell* 1 (5):697-705.

Romanienko, P. J., and R. D. Camerini-Otero. 2000. The mouse Spo11 gene is required for meiotic chromosome synapsis. *Mol. Cell* 6 (5):975-87.

Suzuki, A., and Y. Saga. 2008. Nanos2 suppresses meiosis and promotes male germ cell differentiation. *Genes Dev.* 22 (4):430-5.

Upadhyay, S., and L. Zamboni. 1982. Ectopic germ cells: natural model for the study of germ cell sexual differentiation. *Proc. Natl. Acad. Sci. U.S.A.* 79 (21):6584-8.

Von Stetina, J. R., and T. L. Orr-Weaver. 2011. Developmental control of oocyte maturation and egg activation in metazoan models. *Cold Spring Harb. Perspect. Biol.* 3 (10):a005553.

Wojtasz, L., J. M. Cloutier, M. Baumann, K. Daniel, J. Varga, J. Fu, K. Anastassiadis, A. F. Stewart, A. Remenyi, J. M. Turner, and A. Toth. 2012. Meiotic DNA double-strand breaks and chromosome asynapsis in mice are monitored by distinct HORMAD2-independent and -dependent mechanisms. *Genes Dev.* 26 (9):958-73.

Xu, H., M. D. Beasley, W. D. Warren, G. T. van der Horst, and M. J. McKay. 2005. Absence of mouse REC8 cohesin promotes synapsis of sister chromatids in meiosis. *Dev. Cell* 8 (6):949-61.

Yoshida, K., G. Kondoh, Y. Matsuda, T. Habu, Y. Nishimune, and T. Morita. 1998. The mouse RecA-like gene Dmc1 is required for homologous chromosome synapsis during meiosis. *Mol. Cell* 1 (5):707-18.

Zamboni, L., and S. Upadhyay. 1983. Germ cell differentiation in mouse adrenal glands. *J. Exp. Zool.* 228 (2):173-93.