THE DEVELOPMENT OF SEXUAL DIMORPHISM IN THE *DROSOPHILA* GONAD

by

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ABSTRACT

Throughout the animal kingdom, sex-specific development is used to create different forms in males and females. Sexual dimorphism is important for successful reproduction both on social and biological levels, but is especially vital in the gonad, which must be sexually dimorphic so it can support germline differentiation into sperm and eggs.

In *Drosophila*, adult testes and ovaries are highly specialized organs that can serve as good models for studying sex-specific gonadogenesis, however, it is not well understood how sexual dimorphism is initially established in the embryo. In this thesis I present an analysis of how differences between the male and female somatic gonad are brought about during early development. I have observed that the *Drosophila* gonad is already sexually dimorphic at the time of its initial formation, and have characterized two sex-specific cell types termed the male-specific somatic gonadal precursors (msSGPs) and the pigment cell precursors. msSGPs and pigment cells give rise to specific adult testis cell types and express *Sox100B*, a homolog of *Sox9*, a factor required for mammalian sex determination. These two cell types employ different cellular mechanisms, such as apoptosis and cell-cell signaling, to ensure sexual dimorphism in the gonad. Sex-specific gonad development relies on positional information provided by the homeotic genes and proper sexual identity downstream of the sex determination gene *doublesex*. The sexually dimorphic behavior of msSGPs and pigment cells appears to be controlled non-autonomously, which is distinct from cell-autonomous sex determination that has been reported for most other *Drosophila* somatic tissues. Finally, I have analyzed the function of *Sox100B* in gonad development, and have found a role in adult

testis formation, suggesting that there is a conserved molecular mechanism for regulating sexual dimorphism between flies and mammals.

These results demonstrate many common features between *Drosophila* and mammalian gonadogenesis. Thus, despite vast differences in initial sex determination between species, these data strongly support a hypothesis that the downstream regulation of sexual dimorphism in the gonad is an evolutionarily conserved process at the cellular and molecular levels.

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CHAPTER 1

INTRODUCTION

An interesting topic in developmental biology is why sexual dimorphism exists and how it has been shaped by evolution. An obvious question that needs to be answered is why are there two sexes? Furthermore, why are they morphologically distinct from one another? An examination of the animal kingdom will show that taxonomic groups without sexes (e.g., parthenogenetic and self-fertilizing animals) are in the minority. In fact, no large group is made up of only parthenogenetic species, even though, in the short run, it may conserve energy having a "unisex" species, since males usually constitute half of the biomass but only contribute their genes to the next generation (Hodgkin, 1992). It may even seem contradictory to evolutionary theory that sexual reproduction has survived because asexual populations show a twofold advantage in fitness as compared to their sexual counterparts (Agrawal, 2001). Not only is half the population unable to produce offspring, but anisogamy (gamete sexual dimorphism) renders sperm contribution minimal to the zygote even though 50% of the genes are paternally derived (Agrawal, 2001). The effects of sexual selection upon males created by anisogametic sexual reproduction counteracts the "cost" of sex through a lower equilibrium frequency of deleterious mutations and an increased probability of new, beneficial mutations (Agrawal, 2001; Barton and Charlesworth, 1998; Whitlock, 2000). As evolution has demonstrated, the variation provided by sexual reproduction is likely a key element to survival in a dynamic, constantly changing world. Sex-specific dimorphism may also have created an added level of diversity on the species level and, in the process, aided in withstanding adversity in the environment.

Sexual dimorphism in the gonad is arguably the most important sexual dimorphism, given that it is required in order to support spermatogenesis and oogenesis.

Ultimately, this will ensure successful sexual reproduction and the passage of genetic material to progeny. Therefore it is of great interest and importance to study the genetic and cellular mechanisms that are involved in ensuring male- and female-specific gonad morphogenesis.

The process of establishing sexual dimorphism in the embryonic gonad involves many factors in addition to merely determining the initial sexual identity of a cell. Many different cell types are required to form the reproductive system, including the somatic and germline components of the gonad, along with extra-gonadal reproductive organs and tracts. Distinct interactions between the soma and germ line of the gonad are especially critical for the production of sperm and eggs (Kierszenbaum and Tres, 2001), thus the creation and maintenance of a functional, sexually dimorphic reproductive organ is ongoing throughout the life of the animal. These interactions depend on proper gonadal fates, which are brought about via complex genetic mechanisms that pattern cellular identities. Additionally, the somatic gonad must not only be patterned properly, but clearly must also possess a sexual identity that is compatible with its germ line in order to produce viable gametes.

These tasks are common to and required of virtually all animals, yet the mechanisms used in different species to determine sexual fate in the embryo have diverged rapidly in the animal kingdom. While the molecular and genetic factors used for other basic developmental processes, such as anterior-posterior patterning by the homeotic genes (see below), have been evolutionarily conserved, there seems to be little such conservation in the fundamental process of establishing male and female identity. However, growing evidence suggests that, downstream of initial sex determination, there

is some conservation of molecular and cellular mechanisms in the generation of sexual dimorphism.

The study of sexual dimorphism in model systems, such as the fruit fly *Drosophila melanogaster*, can help us to better understand the genetic and cellular basis for sex-specific phenotypes. *Drosophila* shows extensive sexual dimorphism throughout development, but differences between male and female adults are striking. Size is one difference, in which females are larger than males. The pigmentation of the distal part of the abdomen has distinct sex-specific patterns, wherein males show a dark pigmentation throughout the posterior-most segments of the body. External bristle sensory structures also differ between males and females; males possess a cluster of extremely large bristles on the foreleg termed the "sex comb," which aids in the mating process. In addition to these dimorphisms, there is also a large degree of sex-specificity in the reproductive system. The external genitalia show dramatic differences in the two sexes, in addition to the sexual dimorphism in the gonad. The adult testis is a long, coiled tube in which developing spermatocytes and spermatids mature, whereas the adult ovary is a collection of ovarioles, each of which is an assembly line of developing egg chambers.

Given that many aspects of gonad formation are common in *Drosophila* and vertebrates, it is a model system that is well suited for studying gonadogenesis. Due to the wide complement of genetic tools available in *Drosophila*, we are able to use the fruit fly model to understand elements of sex determination and sexual dimorphism that may be common among different animal species. Since proper sexual dimorphism in the gonad is directly linked to fertility and reproduction, this research has potential clinical

and medical relevance, and may shed some light on how evolution has had an effect on this fundamental aspect of development.

Gonad formation

While sex determination mechanisms have diverged rapidly, the formation and structure of the gonad is similar among animal species. The gonad is formed from the interaction of germ cells with a specific subset of mesodermal cells called the somatic gonad. The germ cells will give rise to future sperm or eggs, while the somatic gonad will form tissues that intimately associate with germ cells and nurture and support gametogenesis. The origins of these two cell types are distinct and they exhibit unique genetic profiles and behaviors. These basic features of the gonad are present in both *Drosophila* and the mouse *Mus musculus*, the most well studied mammalian model organism.

Germ cell specification

Germ cells in *Drosophila*, like those in most other animals, are segregated from the somatic cell lineage early in embryonic development. Furthermore, the germ cells are initially located at a distance from the somatic gonad and must therefore migrate through the embryo to eventually reach their target cells. In *Drosophila*, as in other model animals such as nematodes, zebrafish, and frogs, the germ cells arise from a specialized cytoplasm, termed the germ plasm, which is formed maternally and is deposited in the oocyte prior to fertilization (reviewed by Starz-Gaiano and Lehmann, 2001). In *Drosophila*, the transplantation of germ plasm to the anterior of the embryo is sufficient to induce ectopic germ cells in the anterior of the embryo (Illmensee and Mahowald,

1974). The germ plasm contains mitochondrial ribosomal RNA, proteins such as VASA and TUDOR, and mRNAs such as *oskar, nanos*, and *germ cell-less* (Boswell and Mahowald, 1985; Ephrussi et al., 1991; Hay et al., 1988; Jongens et al., 1992; Kobayashi et al., 1993; Kobayashi et al., 1996; Lehmann and Nüsslein-Volhard, 1991). The distinction between the germ cell and the somatic fate seems to be regulated in part by *nanos*. *nanos* mutant germ cells fail to express the germ cell marker Vasa, have the potential to incorporate into somatic tissue and express somatic cell markers (Hayashi et al., 2004), and also fail to form the germ cell-specific spectrosome organelle (Wawersik and Van Doren, 2005).

Germ cell specification in the mouse seems to act through a fundamentally different mechanism than in *Drosophila*. While some of the *Drosophila* germ cell gene homologs, such as *nanos* and *vasa*, are expressed in the mouse germ line and may show some conserved function in germline development (Fujiwara, 1994; Tanaka et al., 2000; Toyooka et al., 2000; Tsuda et al., 2003), at present there is no evidence for preformation of the germ line via a germ plasm like in *Drosophila* or some other vertebrates (Ciemerych et al., 2000; Zernicka-Goetz, 1998). The precursors to germ cells arise from a subset of extraembryonic mesoderm near the border of the epiblast (embryo) and extraembryonic tissue (Lawson and Hage, 1994). Somatic cells of the extra-embryonic ectoderm may induce cells of the proximal epiblast to acquire a germ cell fate (Tam and Zhou, 1996). Results from tissue transplantation experiments indicate that even distal epiblast cells can give rise to germ cells if they are transplanted to a proximal epiblast location, and conversely, proximal epiblast cells take on a somatic cell fate if transplanted distally, suggesting an induction by signaling from the adjacent extra-embryonic

ectoderm (Tam and Zhou, 1996). The induction to a germline identity is likely mediated by members of the Bone Morphogenetic Protein (BMP) family belonging to the TGF-β superfamily of signaling factors. *Bmp-4* and *Bmp-8b* knockout mice show decreased numbers of germ cells, even as heterozygotes, and it has been shown that *Bmp-4* is expressed in the extra-embryonic ectoderm and is required in that tissue for proper germline specification (Lawson et al., 1999; Ying et al., 2000).

Somatic gonad specification and initial gonad formation in Drosophila

The somatic gonad, whose embryonic precursors are called somatic gonadal precursors (SGPs) in *Drosophila,* is comprised of specialized mesodermal cells associated with germ cells. SGPs will give rise to the cell types in the adult gonad responsible for nurturing germ cells to produce gametes. While the somatic gonad can actually form somewhat normally in the absence of a germline in flies (Aboïm, 1945; Boyle and DiNardo, 1995; Brookman et al., 1992), the SGPs are required for the formation of the gonad. In genetic backgrounds in which SGPs are absent, germ cells scatter throughout the embryo and are lost.

In *Drosophila*, the mesoderm is divided into 14 parasegments (PS), due to the action of gap and pair-rule genes that pattern the anterior-posterior (A-P) axis of the embryo. PS are subdivided into domains by the pair-rule genes *even-skipped* (*eve*) anteriorly and *sloppy-paired* (*slp*) posteriorly (in addition to other pair-rule genes) (reviewed in Riechmann, et al., 1998). SGPs are specified as three bilateral clusters of 10-12 cells each in the dorsolateral mesoderm of the *eve* domain in PS10-12, in a mechanism dependent on the genes *tinman* (*tin*) and *zinc finger homeodomain protein 1*

(*zfh-1*)(Broihier et al., 1998). SGPs are first observed during stages 11 and 12 of embryogenesis (staging after Campos-Ortega and Hartenstein, 1985), and were initially characterized by their expression of the retrotransposon 412, the *lacZ* enhancer trap 68-77, and the transcription factor Clift/Eyes absent (Eya) (Boyle et al., 1997; Boyle and DiNardo, 1995; Brookman et al., 1992; Simon et al., 1990). *eya* is not required for the specification of the SGPs, but is necessary for the maintenance of SGP identity; in *eya* mutants, germ cells initially undergo proper migratory movements, but later are dispersed throughout the posterior of the embryo (Boyle et al., 1997). In PS4-9, dorsolateral mesodermal cells will form the fat body, the fly equivalent of the liver. The fat body fate is repressed in PS10-12 by the homeotic gene *abdominal-A* (*abd-A*) (Moore et al., 1998a; Riechmann et al., 1998), which serves to repress *serpent* (*srp*), a transcription factor that is necessary and sufficient for fat body development (Rehorn et al., 1996; Sam et al., 1996). The fat body develops in other subsets of the mesoderm and will form a lattice-like structure throughout the embryo, and wraps around the gonad during development. The role of the fat body in gonad formation is not known, although given its proximity to the gonad, it is likely that it interacts with the gonad to aid in morphogenesis and/or differentiation.

After initial specification of the SGP clusters, the germ cells and SGPs must recognize one another and undergo "coalescence" from a loose association of cells into a rounded organ during stage 13. The interaction between SGPs that drives the morphogenetic movements responsible for gonad coalescence requires the zinc transporter Fear of intimacy (FOI) (Mathews et al., 2005; Moore et al., 1998b; Van Doren et al., 2003) and the cell adhesion molecule E-cadherin, encoded by the *shotgun* (*shg*)

locus (Jenkins et al., 2003). In *foi* and *shg* mutants, the cellular identities of SGPs and germ cells are unaffected, but the two cell types fail to condense into a round organ and instead remain as a loose array of cells. In *foi* mutant gonads, E-cadherin expression is reduced relative to wild-type, suggesting that E-cadherin-mediated cell adhesion is involved in gonad compaction.

Upon the initial formation of the rounded gonad by stage 15, different subpopulations of somatic cells are already present and readily distinguishable from each other. An *Abdominal-B* (*Abd-B*) regulatory element *lacZ* reporter, bluetail, is only expressed in the posterior of the embryonic gonad (Boyle and DiNardo, 1995; Galloni et al., 1993), while a *lacZ* reporter for the *escargot* (*esg*) gene labels anterior SGPs (Boyle and DiNardo, 1995; Gonczy et al., 1992). These observations suggest that there are already anterior and posterior SGP identities established in the early embryonic gonad. These differences in the gonad are likely mediated by the action of the homeotic genes of the bithorax complex (see below), which render unique identities to cells along the A-P axis. Both *Ultrabithorax* (*Ubx*) and *abd-A* are expressed in the mesoderm of PS10-12 before and after gonad formation (Karch et al., 1990; Macias et al., 1990; White and Wilcox, 1985). *Abd-B* expression, however, is dynamic over the time range of initial gonadogenesis. Prior to SGP specification (stage 11), *Abd-B* is expressed only in the mesoderm of PS13-14, but approximately at the time SGPs arise, transcripts can be detected in PS11-12 (Boulet et al., 1991; Celniker et al., 1989; DeLorenzi and Bienz, 1990; Kuziora and McGinnis, 1988). While a role for *Ubx* in the gonad has not been reported, *abd-A* is absolutely required for SGP specification (Boyle and DiNardo, 1995; Broihier et al., 1998; Cumberledge et al., 1992; Greig and Akam, 1995). In the absence

of *abd-A*, the dorsolateral mesoderm of PS10-12 becomes fat body due to the ectopic expression of *srp* (see above) (Moore et al., 1998a; Riechmann et al., 1998). In addition, *abd-A* is sufficient to induce ectopic SGPs in other PS (Boyle et al., 1997; Boyle and DiNardo, 1995; Greig and Akam, 1995). The role of *Abd-B* is unclear, however, in that different gonad phenotypes have been described for *Abd-B* mutant embryos. It appears that a rudimentary gonad that includes germ cells and some SGPs does form (Brookman et al., 1992; Greig and Akam, 1995). Boyle and DiNardo found that while a somatic gonad is not observed in *abd-A* mutants, in the absence of *Abd-B*, posterior SGP identity is absent, as assayed by *eya* expression (Boyle and DiNardo, 1995). Conversely, ectopic *abd-A* expression induced ectopic anterior *esg*-expressing SGPs. These data support a model in which *abd-A* works to determine anterior SGP identity, while a combination of *abd-A* and *Abd-B* work together to specify posterior SGP identity (Boyle and DiNardo, 1995). The data indicate that there are anterior and posterior cell types in the embryonic gonad, it is thought that the newly formed embryonic gonad is bipotential and does not yet show sexual dimorphism.

Sexual dimorphism in the Drosophila gonad

While sexual dimorphism in the adult testis and ovary has been characterized (see below), sexual dimorphism in the embryonic gonad is not as well understood. However, some observations suggest that there are already sex-specific differences in the early embryo. By the time of gonad coalescence, germ cell expression of *male germline marker-1* (*mgm1*) is sex-specific (Staab et al., 1996). Since the sex of the soma influences sex determination of *Drosophila* germ cells (Staab et al., 1996; Steinmann-

Zwicky, 1994; Steinmann-Zwicky et al., 1989; Waterbury et al., 2000), the sex-specific expression of *mgm1* suggests that SGPs are sexually dimorphic. In addition, it has been documented that male embryos contain slightly more germ cells than female embryos (Poirié et al., 1995; Sonnenblick, 1941), and by the beginning of the first instar larval stage, male gonads are larger than female gonads (Kerkis, 1931). Germ cell division is male-specific in the embryonic gonad, mediated by JAK/STAT signaling from the soma (Wawersik et al., 2005); thus it is likely that increased incorporation of germ cells into the gonad and increased cell division is responsible for sexually dimorphic gonad size in embryonic and larval stages. These data indicate that some properties of the male and female SGPs are already distinct by the end of embryogenesis.

Some reports have suggested that somatic cells are sexually dimorphic in the embryonic gonad. A group of somatic cells in the posterior of the male pupal gonad were termed *cellules des canaux* ("channel cells") due to their function later in development, which was to connect the testis and the rest of the reproductive tract (Geigy, 1931). Cells in the posterior of the embryonic and larval gonad that had similar morphology to *cellules des canaux* were found to be mesodermally derived and present in only some gonads during all stages of development, suggesting that these cells were male-specific (Aboïm, 1945).

Interestingly, signs of spermatogenesis are already visible in the first instar larval male gonad (Aboïm, 1945; Sonnenblick, 1941), suggesting that the gonadal cell types required for spermatogenesis in adults are already functional in early larval development. Throughout all of larval development, the size of the gonad is sexually dimorphic, with the male gonad larger than the female gonad. By the end of larval development, male

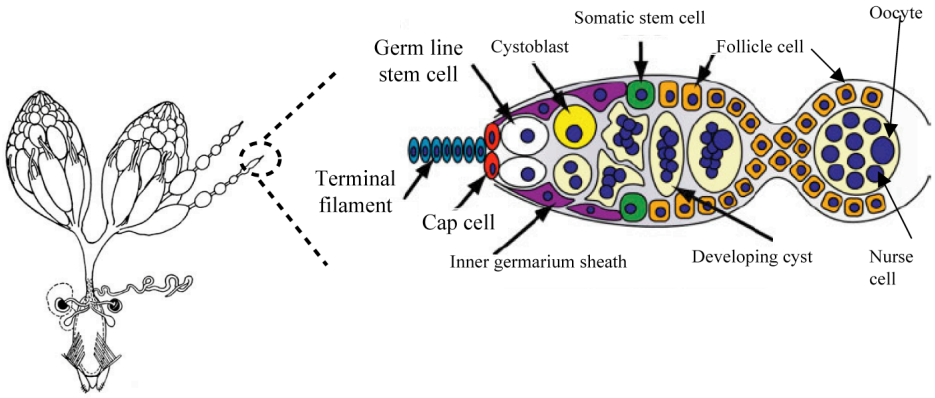
germ cells have undergone mitotic divisions to produce large numbers of spermatocyte cysts that fill the testis, while in the female third instar larval ovary, germ cells have not yet differentiated. Although it is not known exactly which cells are the precursors to specific cell types in later stage testes and ovaries, it is likely that they are specified in the embryo, especially in the case of the male.

Adult Drosophila *testis*

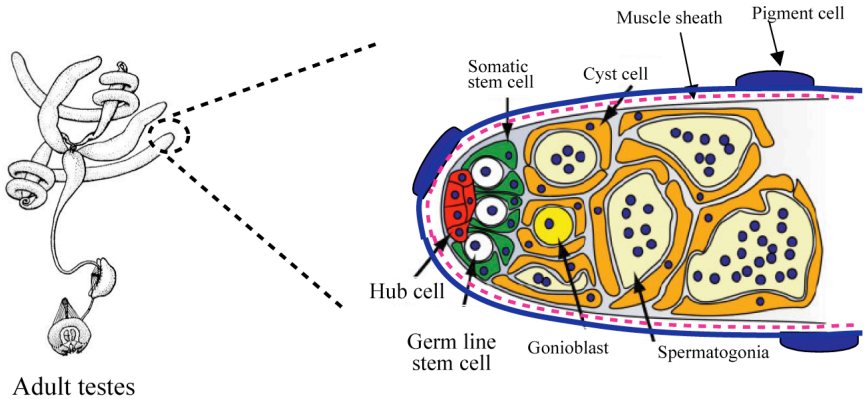
In the adult testis, multiple sex-specific cell types are important for the maintenance of a fertile individual (Fig. 1.1). The "hub" is located in the anterior tip of the adult testis, forming part of the germline stem cell (GSC) niche (Hardy et al., 1979). The hub is a cluster of 8-12 non-dividing somatic cells that is responsible for keeping the neighboring 5-9 germline stem cells from undergoing differentiation into spermatocytes. This control over stem cell identity is regulated by JAK/STAT and BMP signaling from hub cells to the GSCs (Kawase et al., 2004; Kiger et al., 2001; Tulina and Matunis, 2001). The plane of GSC division is oriented such that one daughter cell is displaced away from hub signaling, thus initiating differentiation and spermatogenesis (Yamashita et al., 2003). This daughter cell undergoes 4 mitotic divisions to produce a 16-cell cyst that will eventually undergo meiosis to produce 64 haploid spermatids. During all these steps in spermatogenesis, the germ cells are ensheathed by a pair of somatic cyst cells, which undergo drastic cell shape changes to accommodate the growing cyst. The somatic cyst cell population is maintained by a somatic stem cell population in the anterior of the testis, adjacent to the hub and GSCs (Gonczy and DiNardo, 1996). Once the 64-cell cyst is ready to undergo the last steps of spermiogenesis to become fully motile sperm, the

Figure 1.1. *Drosophila* adult gonads are dimorphic organs with multiple sex-specific cell types.

Drawings of adult *Drosophila* testis and ovary. Apical tips of ovariole and testis are highlighted on right side, with testis- and ovary-specific cell types labeled. Testis terminal epithelium and ovarian sheath are not shown. Drawings are adapted from (Miller, 1950) and (Xie et al., 2005).



Adult ovaries



Adult testes

encasing somatic cyst cells must interact with the terminal epithelium in the basal area of the testis. The terminal epithelium acts to degrade the cyst cells and the waste bag after the liberation of the 64 individualized sperm (Tokuyasu, 1974). This is the final step in sperm differentiation, after which the sperm pass into the seminal vesicle, a structure that is derived not from the gonad itself, but rather the genital disc.

In addition to the hub, somatic cyst cells, and terminal epithelium, there are other supporting cell types that are required for the establishment of testis architecture. One such cell type is the testis muscle sheath, which is thought to be genital disc-derived (Kozopas et al., 1998). This layer of cells contacts the posterior of the gonad during pupal stages, eventually migrates over the entire length of the testis, and is thought to be responsible for driving the transformation from a round gonad to a coiled tubule. In addition to a muscle sheath layer coating the testis, the outermost cell layer of the adult testis consists of pigment cells, which are cells with large nuclei that ensheath the entire length of the testis and seminal vesicle. Aside from rendering a yellow pigment to the wild-type testis (the pigmentation itself is not required for a functional testis), pigment cells are thought to be required for the interaction between the genital disc muscle sheath and the testis. In *Wnt2* mutant adult testes, pigment cells are absent in the late larval and adult testis, and the adult testis shows defects in muscle sheath wrapping and extension; this results in a small, round gonad phenotype, instead of the wild-type coiled phenotype (Kozopas et al., 1998). It has been hypothesized that the pigment cells serve as the substrate for the muscle sheath to migrate along the gonad. It is not known what cell type gives rise to pigment cells, nor at what stage *Wnt2* is required for pigment cell development. Pigment cells are not required for the formation of other testis cell types,

nor are they required for survival of germ cells through spermatogenesis; rather it has been proposed that the disruption of proper pigment cell-muscle sheath interactions causes structural and mechanical defects in the testis that hinder the exit of motile sperm from the reproductive system (Kozopas et al., 1998).

Adult Drosophila ovary

The adult *Drosophila* ovary is very different from the adult testis in both shape and function (Figure 1.1). Whereas the male is a single coiled lumen, the ovary consists of 16-20 ovarioles, each of which represents the basic unit for egg chamber production (King, 1970; Spradling, 1993). The ovariole is encased by an epithelial sheath and a muscle layer, which serve to separate ovarioles and provide structure and support. Egg chambers are present along the length of the ovariole, at different stages of development. As egg chambers mature, they progress posteriorly through the ovariole in 14 morphological stages of development. The undifferentiated GSC are present in the germarium, the anterior-most region of the ovariole; once GSCs leave the GSC niche, they undergo 4 mitotic divisions to produce a 16-cell cyst, which will mature into 15 nurse cells and one oocyte. By the end of oogenesis, the nurse cells will eventually die and dump their contents into the oocyte. Each egg chamber, upon exit from the germarium, will be coated by follicle cells, and will be connected to its neighbor follicle by interfollicular stalk cells. By the end of stage 14, oogenesis and vitellogenesis are complete, with the chorion and dorsal appendages (which aid embryonic respiration) present in the egg.

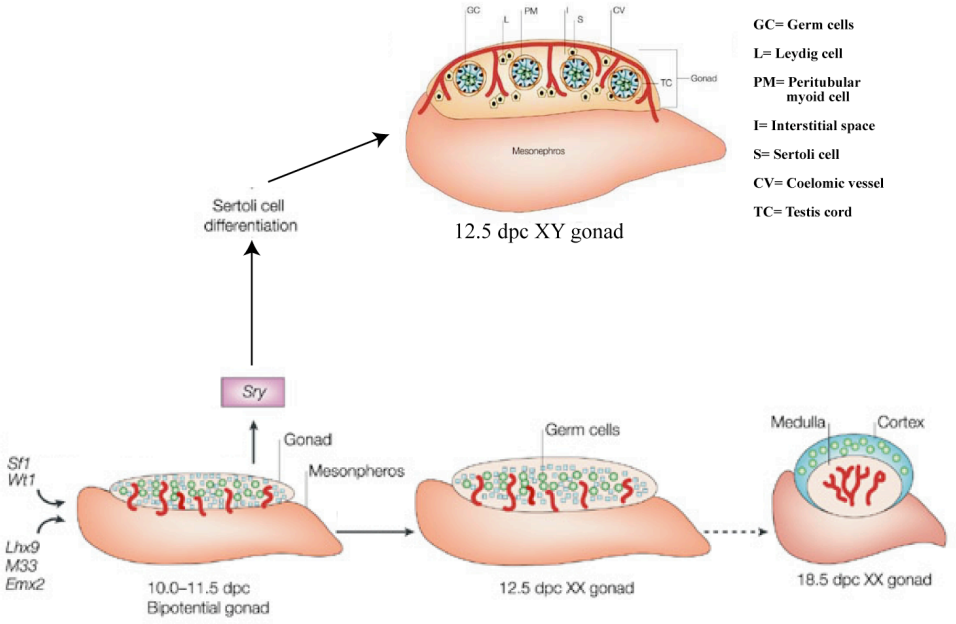
As in the testis, the ovary contains multiple tissue-specific cell types that promote development of the oocyte. The ovarian GSC niche is located at the anterior tip of the germarium, and consists of terminal filament cells, cap cells, and inner germarium sheath (IGS) cells (Fig. 1.1). As in males, the niche acts in part through BMP signaling to repress differentiation of 2-3 germline stem cells in the niche (Song et al., 2004; Xie and Spradling, 1998). A somatic stem cell population is also present in the germarium, which will supply the follicle cells that will ensheath the developing germline cysts. A recent study has identified "escort stem cells" (ESCs) that encase early developing cysts in the germarium (Decotto and Spradling, 2005), which are reminiscent of testis cyst cells. ESCs are similar to testis cyst cells in their morphology and requirement for JAK/STAT signaling (Decotto and Spradling, 2005; Kiger et al., 2001; Tulina and Matunis, 2001).

Mouse gonad formation and sexual dimorphism

In mice, the somatic gonad is visible by day e10, as a structure termed the genital ridge, which is a swelling of the coelomic epithelial layer that lines the body cavity. Specifically, the genital ridge lies in the area adjacent to the mesonephros (Fig. 1.2), one of the three primordia that give rise to the urogenital tract (pronephros, mesonephros, and metanephros). In addition, the primordia for both female and male ductal system are present in both sexes, as the Müllerian and Wolffian ducts, respectively. Knockout analysis has shown that several genes are required for genital ridge formation and expansion, including *Wilms tumor homolog 1* (*Wt1*), *Steroidogenic factor-1* (*SF-1*), *Empty spiracles homolog 2* (*Emx2*), and *LIM homeobox protein 9* (*Lhx9*) (Kreidberg et al., 1993; Luo et al., 1994; Miyamoto et al., 1997; Shawlot and Behringer, 1995). The

Figure 1.2. Time course of mouse gonad formation.

Depiction of gonad development in mouse. Prior to sex determination at e10, the gonad lacks sexual dimorphism, however, at e12.5 the male gonad exhibits morphological arrangement of testis cords. Male-specific gonad cell types are labeled at right. Genes required for steps in gonadogenesis and sexual dimorphism are noted. Adapted from (Brennan and Capel, 2004).


GC
L
PM
I
S
CV
TC
Gonad
Mesonephros
GC= Germ cells
L= Leydig cell
PM= Peritubular myoid cell
I= Interstitial space
S= Sertoli cell
CV= Coelomic vessel
TC= Testis cord
Sertoli cell differentiation
12.5 dpc XY gonad
Sry
Gonad
Mesonpheros
Sf1
Wt1
Lhx9
M33
Emx2
10.0–11.5 dpc Bipotential gonad
Germ cells
12.5 dpc XX gonad
Medulla
Cortex
18.5 dpc XX gonad

genital ridge is thought to be bipotential at day e10, able to be diverted down either the female or male pathway of development, and does not yet show any morphological sexual dimorphism.

The trigger for initiating sexual dimorphism is mediated by the *Sex determining region on Y* (*Sry*) gene (Gubbay et al., 1990; Lovell-Badge and Robertson, 1990), which is first expressed in the somatic cells of the gonad between e10.5 and e12.5 (Albrecht and Eicher, 2001; Bullejos and Koopman, 2001; Hacker et al., 1995). *Sry* encodes a DNA binding protein containing a high-mobility group (HMG) box motif, which is thought to affect gene transcription by bending DNA and altering local chromatin structure (Ferrari et al., 1992). Misexpression of *Sry* is sufficient to induce XX embryos to develop as males (Koopman et al., 1991), presumably through activation of male-specific genes. However, similar to the sex determination gene *doublesex* (*dsx*) in flies (see below), the targets of this master sex determination gene are largely unknown.

The expression of *Sry* induces somatic cells of the gonad to become Sertoli cells, a supporting cell type in the testis. In the absence of *Sry*, it is thought that somatic precursors become the supporting cells of the ovary, granulosa cells (Albrecht and Eicher, 2001). Sertoli cells secrete anti-Müllerian hormone (AMH; also called Müllerian inhibiting substance, MIS) by e12.5, which is one of the first sexually dimorphic features of the fetal gonad. AMH is a secreted protein of the TGF-β family that acts in males to promote the degradation of the Müllerian ducts, which would otherwise form the internal reproductive structures of the female. Male mice lacking the *Amh* gene or its receptor fail to show regression of the Müllerian duct, however, testis formation is normal (Behringer et al., 1994; Mishina et al., 1996). AMH likely acts by binding to cells near the Müllerian

duct and inducing them to release a factor that promotes apoptosis of the Müllerian duct epithelium (Roberts et al., 1999).

The specification of sex-specific cells leads to a visible morphological change in the gonad by e12.5, which is the formation of testis cords in males. A testis cord is a linear structure composed of germ cells (which have already migrated into the genital ridge during the bipotential phase) and surrounding Sertoli cells. The testis cords will eventually hollow out during puberty and become the adult seminiferous tubules that will contain developing sperm. In the female, the germ cells will remain around the periphery of the gonad, and will be part of structures called the cortical sex cords. A cortical sex cord consists of a cluster of granulosa cells surrounding a single germ cell that will become an ovum, however, cortical sex cords do not penetrate as deeply into the gonad as the testis sex cords. The interior mesenchymal cells of the ovary will give rise to steroid-producing cells (see below) that, in conjunction with granulosa cells, will eventually form follicles in the mature ovary (reviewed in Gilbert, 2000).

The formation of testis cords also depends on *Sry*-dependent cellular migration from the adjacent mesonephros into the testis (Buehr et al., 1993a; Martineau et al., 1997). The migrating cells appear to be endothelial, perivascular, and peritubular myoid cells, which help to partition the gonad into testis cord structures and set up vasculature that will aid in signaling via hormone circulation in the gonad. In addition, the testis cord serves to segregate germ cells and Sertoli cells from other cell types, such as hormone-secreting, vascular, and interstitial cells, via the deposition of a basal lamina by peritubular myoid cells. This testis architecture is common to most vertebrates, and is also similar to what is observed in *Drosophila*. As noted earlier, in *Drosophila*, myoid

cells (muscle sheath cells) from the male genital disc migrate over the testis, along with pigment cells, to segregate germ cells and supporting somatic cyst cells from other cells.

Other important cell types in the mammalian fetal gonad are hormone-secreting cells, called Leydig cells in the testis and thecal cells in the ovary. Leydig cells secrete the steroid testosterone, which promotes the male-specific differentiation of tissues outside of the gonad. Specifically, testosterone stimulates the Wolffian ducts to develop into the vas deferentia, seminal vesicles, and epididymides (whereas AMH causes the degeneration of the Müllerian duct; see above). Testosterone can also be transformed into dihydrotestosterone (DHT), via the enzyme 5α-ketosteroid reductase, in the external genitalia to promote the differentiation of the male urethra, prostate, penis, and scrotum. The majority of secondary sexual development in the male, e.g., body and facial hair, is also mediated by the action of testosterone, as is the maintenance of sperm production throughout adult life. In addition, testosterone can be aromatized to the female hormone estradiol (a derivative of estrogen), which also is required for fertility (Eddy et al., 1996), via its function in the efferent tubules of the testis. Estrogen receptor-α knockout mice show reduced reabsorption of efferent tubule luminal fluid, which is required to concentrate sperm and to improve their survival and maturation during storage, thus resulting in abnormal sperm and low sperm counts (Hess et al., 1997).

Aside from roles in the male, estrogen is known as the hormone responsible for promoting the female phenotype. The thecal cells secrete estrogen that is required for the Müllerian ducts to undergo their differentiation into female adult reproductive organs, such as the uterus, fallopian tubes, and cervix. In contrast to males, the influence of estrogen promotes the survival of the Müllerian duct, and the lack of testosterone causes

the Wolffian duct to degenerate and disappear (Gilbert, 2000). Certain chemical contaminants in the environment are estrogen analogs, and can disrupt proper development of the female reproductive tract, and may also contribute to male infertility. In some cases, these pollutants can cause feminization of wildlife. These chemical compounds may disrupt endocrine function by binding to estrogen receptors or may inhibit steroid production (dicussed in Stone, 1994). Therefore, even external factors such as the environment can have a significant impact on gonad formation, sexual dimorphism, and fertility.

Sex determination and sexual dimorphism

Sexual dimorphism is established via regulatory mechanisms that divert an initially "bipotential" embryo towards a male or female path of development. Although the benefits of sexual reproduction and sexual dimorphism are evident, there is still the interesting question of why sex determination pathways are not conserved throughout the animal kingdom. It appears that that both the initial sex determination "switches" that trigger this process and the downstream pathways that must respond to these switches are widely varied among different species.

Sex determination switches

Sex determination "switches" are signals that can be genetic or environmental cues and can take many different forms. In genetic-based mechanisms, sex chromosomes can contain a single dominant master controller of sex determination, such as the *Sry* on the Y chromosome of mammals, which is thought to regulate a wide array of gene targets

required for male-specific development (Gubbay et al., 1990; Lovell-Badge and Robertson, 1990). Alternatively, there may be X-linked genes, such as in nematode *Caenorhabditis elegans* and *Drosophila* (see Cline and Meyer, 1996), which are responsible for initiating sex-specific development by "sensing" sex chromosome ratio, i.e., if an embryo is XX or not.

Environmental sex determination mechanisms can utilize temperature, social structure, or population density. For example, in some turtles and in crocodilian reptiles, temperature of the embryo during a critical window of development is the deciding factor in male- or female-specific differentiation. In the case of the American alligator *Alligator mississippiensis*, the temperature-sensitive time is a span of ten days during embryogenesis (after which sex determination cannot be reversed), in which a warmer egg incubation temperature gives rise to all male progeny while a cooler one produces all female progeny (Ferguson and Joanen, 1982). Interestingly, temperature has the opposite effect in the red-eared slider turtle *Trachemys scripta*, in which a warmer incubation temperature leads to female development (Bull et al., 1982). This discrepancy of the effect of temperature on sex determination further demonstrates that sex determination is highly diverged and plastic. Even within a single species, more than one sex determination scheme may be used for different tissues. One example is in marsupials, in which gonadal sex is regulated by the presence of a Y chromosome, but the decision of making either a female pouch or a male scrotum depends on X-chromosome dosage and is independent of the Y chromosome (Graves, 1996).

Drosophila *sex determination*

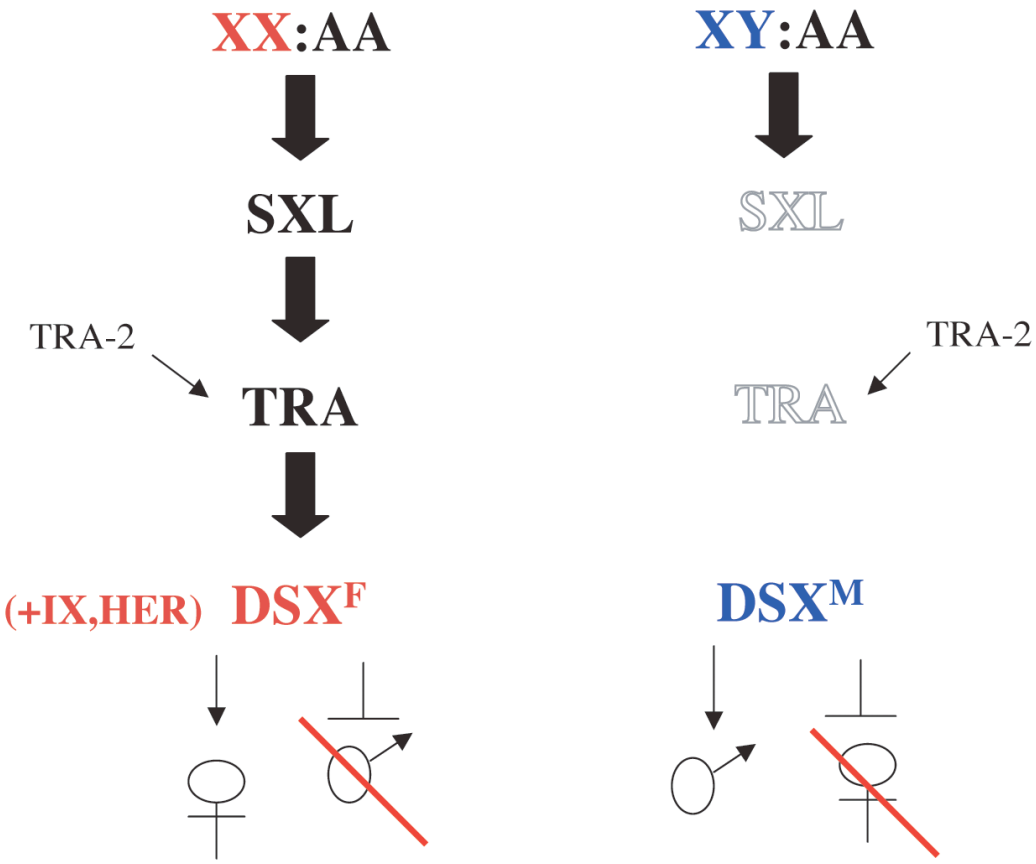
In *Drosophila*, the sex chromosome to autosome ratio (X:A ratio) is the signal that triggers the sex determination pathway (reviewed in Cline and Meyer, 1996). High X:A levels (1.0 for XX individuals) appear to be responsible for turning on the master regulator gene *Sex lethal* (*Sxl*), which is the gene promotes the female path of development (Fig. 1.3). The X:A ratio is gauged by the competitive activity of X-chromosome-encoded "numerator" proteins that will activate the *Sxl* gene, such as Sisterless-a and Sisterless-b, versus the autosome-encoded "denominator" proteins that will repress the *Sxl* promoter, such as Deadpan and Extramacrochaetae. Therefore, a low X:A ratio (0.5 for XY males) keeps the *Sxl* promoter inactive during early stages of embryogenesis, while a high X:A ratio results in the production of an "early"-type *Sxl* mRNA that is only found during the first four hours of development. Once this early mRNA is produced, *Sxl* remains active. After early *Sxl* mRNA is transcribed, a "late" *Sxl* promoter is then activated in both sexes, but studies of late *Sxl* mRNA show that it appears in different forms in males and females (Bell et al., 1988). This variation is due to differential mRNA splicing in the presence or absence of SXL protein (which is a splicing factor) that results in a termination codon in different places in the male- and female-specific transcripts. In males, the *Sxl* pre-mRNA gets spliced in the "default" manner to encode for a non-functional SXL protein, which leads to the male developmental pathway (Keyes et al., 1992).

Active female SXL protein aids in the sex-specific splicing of *transformer* (*tra*), the next gene in the somatic sex determination pathway. Similar to *Sxl*, *tra* transcript is

Figure 1.3. The *Drosophila* sex determination pathway implements X:A ratio to ensure sex-specific development of the soma.

In the *Drosophila* sex determination pathway, the X chromosome to autosome (X:A) ratio is responsible for turning on *Sxl*, the master regulator of female development. Downstream of *Sxl*, *tra* and *tra-2* are responsible for the female-specific splicing of *dsx*, which encodes DSX-F, a transcription factor thought to activate female-specific genes and repress male-specific genes. In males, the lack of *Sxl* and *tra* function results in the default male-specific splicing of *dsx* to encode for DSX-M, which is thought to promote male development and repress female development.

Sex determination in *Drosophila*



spliced in a sex-specific manner. In males, the termination codon is early in the *tra* transcript, encoding a truncated, non-functional protein, while in the female a full-length active TRA protein is made (Boggs et al., 1987). TRA, which is also a splicing factor, then acts in concert with Transformer-2 (TRA-2) to promote female development (McKeown et al., 1988; Nagoshi et al., 1988; Ryner and Baker, 1991).

Sexual determination in *Drosophila* is controlled in most of the soma by the gene *doublesex* (*dsx*). The gene is transcribed in both sexes, but is also spliced in a sex-specific manner (Burtis and Baker, 1989). The alternative splicing is controlled by TRA working together with TRA-2; if the female-specific TRA protein is present, then the *dsx* mRNA is spliced in a female-specific fashion, to encode DSX-F protein. The DSX-F protein is thought to activate a multitude of genes needed for female somatic development and also to repress male-specific genes. DSX-F sometimes requires binding to a partner protein, such as Intersex or Hermaphrodite, in order to induce female differentiation in certain tissues, such as in the female external terminal genitalia (Garrett-Engele et al., 2002; Li and Baker, 1998). If there is no female-specific TRA protein, then the *dsx* mRNA is spliced in the default manner to encode for the male DSX isoform DSX-M. DSX-M is thought to activate genes necessary for male development and to repress female-specific genes. In the absence of any *dsx* transcript (as in a dsx^{1} mutant), an "intersex" phenotype occurs and the resultant fly may have rudiments (or a mixture) of both male and female gonads, genitalia, and cuticular structures (Hildreth, 1965).

Both DSX-F and DSX-M are functional proteins, and share a common N-terminus that contains a DM zinc finger-type DNA binding domain (Erdman and Burtis,

1993). The DNA binding domain has been characterized to bind to the sequence AC(A/T)A(T/A)GT (Erdman et al., 1996; Yi and Zarkower, 1999). It has been shown that the two isoforms possess identical DNA binding activity, and both proteins are believed to be able to act as either transcriptional activators or repressors. DSX-F is 427 amino acids long and DSX-M is 549 amino acids long, with a common N-terminus of 397 residues. The unique C-termini of DSX-F and DSX-M are thought to help mediate protein-protein interactions with transcriptional co-regulators.

While DSX is a key regulator of downstream factors needed for sex-specific development and is involved in the onset of sexual dimorphism, surprisingly, only one case is known in which DSX directly binds to DNA to regulate sex-specific transcription. DSX regulates the *yolk protein* (*yp*) genes, which are expressed in the female fat body and ovarian follicle cells, and whose function is to provide amino acids to the developing oocyte via the formation of yolk storage granules. The *yp1* and *yp2* genes are adjacent to each to each other on the X chromosome and are oriented in opposite directions, sharing a common 1200 bp upstream 5' region. Within this region, there is a 125-bp region termed the "fat body enhancer" (FBE). In the FBE, 4 DSX binding sites are present (Burtis et al., 1991), as demonstrated by DNA footprinting and promoter-*lacZ* fusion assays. In addition, within the FBE, there are binding sites for transcriptional co-factors that render tissue specificity to *yp* gene expression (i.e., fat body versus ovary). These factors include the CCAAT/enhancer binding protein (C/EBP), which is a transcriptional activator; adult enhancer factor-1 (AEF-1), which has been shown to inhibit C/EBP binding and repress its activating function; and box-binding factor-2 (BBF-2), which activates transcription of other genes in the fat body (Abel et al., 1992; Falb and Maniatis,

1992). The transcriptional activators and repressors likely interact with DSX and/or compete with DSX to promote *yp* gene expression in the female fat body but not in the male fat body.

Autonomous vs. non-autonomous sex determination

In addition to a great variety of sex determination switches in the animal kingdom, the mechanism by which a sexual phenotype is established may also differ between animal species. Within a given sex determination scheme, the exual identity of an individual cell may be brought about via a cell-autonomous or a non-autonomous mechanism. In a cell-autonomous mechanism, every cell must decide its sexual fate on its own, completely independent of the state of neighboring cells. This process is often called "primary sex determination," of which one example in is the mammalian somatic gonad, specifically in the supporting cells of the testis called the Sertoli cells. In experiments in which chimeric mice containing a mixture of XX and XY cells were used to examine the genetic component of gonadal cells, Sertoli cells were the only cells in the testis that were almost exclusively XY. All other cells in the testis were about 50% XX and 50% XY cells (Burgoyne et al., 1988; Palmer and Burgoyne, 1991). These results led researchers to propose that Sertoli cells are the only cells for which presence of the Y chromosome is critical for their development and differentiation, whereas in other testis cell lineages, the Y chromosome and *Sry* are not initially required. Therefore, the precursors to Sertoli cells in the somatic gonad must normally detect the presence or absence of the *Sry* gene cell-autonomously, and subsequently undergo the morphogenetic and molecular changes required for either female- or male-specific gonadal development.

Prior studies using *Drosophila* sexual mosaics have led to the dogma that most somatic fruit fly tissues undergo a cell-autonomous sex determination mechanism. These studies, first performed by Thomas Hunt Morgan, utilized genetically mosaic flies that were a mixture of male and female cells, termed "gynandromorphs." The mosaic flies are the product of X-chromosome loss only during the earliest divisions in the XX embryo, which results in clones of XX (female) cells and XO (male) cells. By using an X-chromosome-linked genetic marker, it is possible to visualize the genotype and subsequent sexual phenotype of every cell and determine if the genotype and the phenotype correlate perfectly. In these gynandromorphs, the phenotype of "female" and "male" cells was consistent with the genotype, suggesting that each cell was autonomously regulating its development. More recently, Baker and Ridge used X-ray-induced mitotic recombination to create random clones of cells that were mutant for *tra*, *tra-2*, or *dsx*. In the case of adult cuticular structures, like the abdominal cuticle and the foreleg, every cell's sexual fate is linked to its genotype, demonstrating a cell-autonomous mechanism for the sex determination genes in these tissues in *Drosophila* (Baker and Ridge, 1980).

In a non-autonomous sex determination mechanism, the sexual identity of a cell is dictated by external signals originating from other cells. In the case of mammals, this is termed "secondary sex determination," in which hormones secreted from the gonad circulate through the bloodstream to dictate the sexual phenotype of all other tissues. This mechanism was discovered when Jost removed gonads from fetal rabbits before the time of sexual differentiation, which resulted in all fetuses having a female phenotype (Jost, 1953). This result demonstrated that the gonad was the source of the determining

factor for the sexual fate of other cells, thus forming the dogma of non-autonomous sex determination for all non-gonadal tissues in mammals. While the majority of tissues in *Drosophila* have been shown to undergo cell-autonomous sex determination, instances of non-autonomous control have been documented. In the nervous system, male neurons can cause female muscle precursor cells to become the male-specific muscle of Lawrence (Lawrence and Johnston, 1986), which is required for proper copulatory behavior. Also, it has been previously shown that a male genital disc transplanted into a female host can cause female cells to produce male testis pigment (Fung and Gowen, 1957).

Positional information interacts with the sex determination pathway to establish sexual dimorphism

While proper sex determination is clearly necessary for specifying sexually dimorphic tissues, positional information is also a key input for establishing sex-specific cell types. Cells must not only know their sexual identity, but also must have the ability to respond to cues from the sex determination pathway. The capability of undertaking a sex-specific response comes from cellular identity, which is derived partly from homeotic genes that are responsible for patterning the anterior-posterior axis of the animal. In addition to their roles in patterning the embryo, homeotic genes also play a role in establishing sex-specific cell types in the gonad.

Homeotic genes

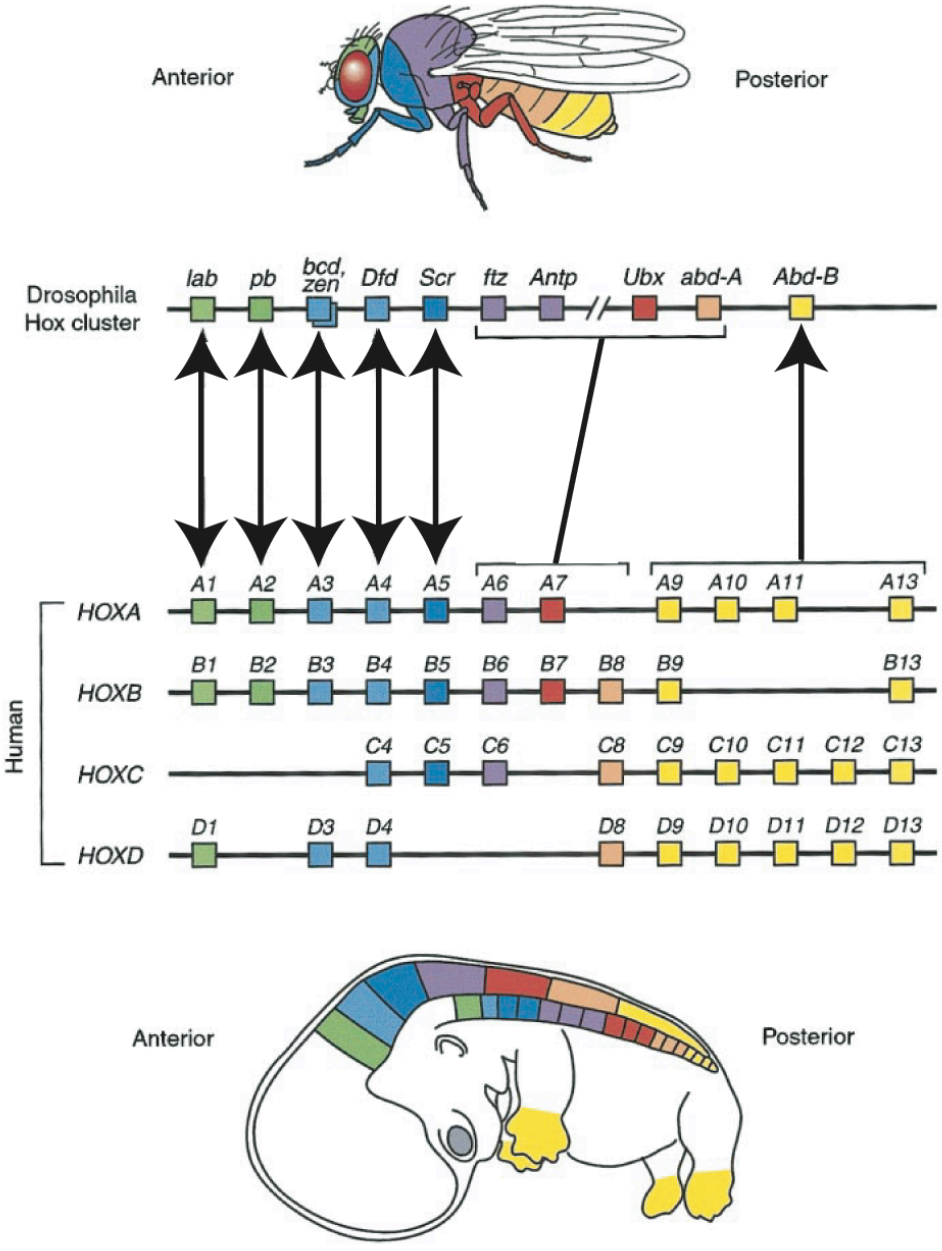
The homeotic genes are responsible for specifying cellular fates along the anterior-posterior (A-P) axis of the embryo. Homeotic genes encode transcription factors that are

characterized by the presence of a homeobox, a 60-residue alpha-helical DNA-binding domain. It is believed that this family of transcription factors is responsible for activating downstream genes that will promote a specific fate to cells in a given parasegment (PS). As the DNA-binding targets among the different homeotic genes are likely similar, co-factors are required to render binding specificity or tissue specificity that will create a unique cellular identity in each PS. Such co-factors include Extradenticle, which interacts with Ubx (and other homeotic proteins) to pattern segments with the correct identity (Peifer and Wieschaus, 1990).

In *Drosophila* homeotic genes are grouped near to one another on the chromosome and are physically arranged in the same order as their expression pattern in the embryo, whereby more anteriorly expressed homeotic genes are located closer to the centromere of the chromosome (Figure 1.4). Five homeotic genes control the anterior structures of the fly (i.e., mouth, head, and thoracic segments): *labial*, *Proboscipedia*, *Deformed*, *Sex combs reduced*, and *Antennapedia*. These genes are grouped together on the chromosome into what is termed the Antennapedia complex. Separately on the chromosome, a cluster of three other genes controls the development of the posterior region of the fly (i.e., posterior thoracic and abdominal segments): *Ubx*, *abd-A*, and *Abd-B*. This group of genes collectively is called the bithorax complex, and like those of the Antennapedia complex, are spatially arranged on the chromosome in the same order as their expression pattern (Lewis, 1978). The initial expression pattern of the homeotic genes is regulated by gap and pair-rule genes (Harding and Levine, 1988; Irish et al., 1989) however, the expression of gap and pair-rule genes is transient in the embryo. Therefore, once stable expression patterns of the homeotic genes have been established,

Figure 1.4. Homeotic genes have been evolutionarily conserved between flies and humans.

Cartoon of *Drosophila* homeotic gene and human *HOX* gene chromosomal organization and expression patterns. While chromosomal and gene duplications have resulted in an increased number of human genes, aspects of the general expression pattern and organization of gene clusters is similar between the two species. Figure adapted from (Veraksa et al., 2000).


Anterior
Posterior
Drosophila
Hox cluster
lab
pb
bcd,
zen
Dfd
Scr
ftz
Antp
Ubx
abd-A
Abd-B
Human
HOXA
A1 A2 A3 A4 A5 A6 A7 A9 A10 A11 A13
HOXB
B1 B2 B3 B4 B5 B6 B7 B8 B9 B13
HOXC
C4 C5 C6 C8 C9 C10 C11 C12 C13
HOXD
D1 D3 D4 D8 D9 D10 D11 D12 D13
Anterior
Posterior

their expression must be maintained throughout life. Permanent maintenance of homeotic gene expression is achieved through the action of the Trithorax (Trx) and Polycomb (Pc) families of proteins. Trx and Pc proteins are thought to act by altering the chromatin state of the homeotic genes in either an open or closed configuration. The Trx proteins serve to maintain activation of homeotic gene transcription in the proper segments, whereas the Pc proteins repress homeotic genes in the segments where the homeotic genes should not be expressed (Kennison and Tamkun, 1988; Simon et al., 1992; Struhl, 1981; Struhl, 1982).

In mammals, anterior-posterior patterning is also regulated by a group of homeobox-containing transcription factors termed *Hox* genes, which are the homologs of the homeotic genes in *Drosophila* (rewiewed in Veraksa et al., 2000) (Figure 1.4). In contrast to *Drosophila*, which contains a single set of homeotic genes (made up of the Antennapedia complex and the bithorax complex), mammals possess four copies of the *Hox* complex (*Hoxa,b,c,* and *d*), and each copy is found on a separate chromosome; the multiple copies are thought to have arisen from chromosomal duplications during evolution. In addition to having multiple copies of the *Hox* complex, mammals also have more than one homolog of each of the fly genes, likely caused by gene duplication or the gain or loss of ancestral *Hox* genes. The *Hox* genes are labeled 1 to 13, where 1 is most anteriorly expressed and 13 is most posteriorly expressed (although not all four complexes contain 13 genes). The expression of these mammalian *Hox* genes are similar to what is observed in flies; the homologs of the Antennapedia complex are expressed in the anterior of the body, and the bithorax complex-like genes are expressed in the posterior. For example, the group of *Hox7* genes corresponds to the *Ubx* gene, the *Hox8*

genes to *abd-A*, and the *Hox9-13* genes to *Abd-B* (Veraksa et al., 2000). Although chromosomal and gene duplication has increased the number of homeotic genes in mammals, it appears that the basic blueprint for patterning the A-P axis has not changed much over time. Some mammalian homeotic genes, when introduced into flies, can even produce similar phenotypes to their fly homologs, suggesting a functional molecular conservation between the two species (Malicki et al., 1990).

Homeotic genes pattern sexually dimorphic tissues in *Drosophila*

Prior studies have shown that homeotic genes interact with the sex determination pathway and are required for sexual dimorphism in certain tissues (Kopp et al., 2000; Sanchez et al., 2001). Studies that examine the interaction between the posterior homeotic gene *Abd-B* and the sex regulatory gene *dsx* in the sex-specific patterning of the *Drosophila* genital disc and adult abdomen highlight the necessity of both positional information and sexual identity in the establishment of sexual dimorphism.

Homeotic genes pattern the sexually dimorphic genital disc via Wg and Dpp signaling

The genital disc is one of the imaginal discs, epithelial clusters of cells that are specified via invagination of the embryonic ectoderm and which will eventually give rise to most adult tissues (e.g., wing, legs, antennae, etc.). In contrast to imaginal discs, most larval structures will be histolyzed during the process of metamorphosis (not the gonad, however). In the case of the genital disc, it will form all the non-gonadal reproductive organs and terminal structures in the adult organism. These structures include the uterus, oviduct, seminal receptacle, and spermatheca in the female, and the ejaculatory duct,

ejaculatory bulb, accessory gland, and vas deferens/seminal vesicle in the male; in addition, it also gives rise to the external genitalia and analia (which are also sexually dimorphic) in both sexes. The genital disc is initially formed in the posterior-most segments of the embryo, and remains there throughout larval stages. During pupal stages, the genital disc grows, undergoes final differentiation, and extends to meet the gonad (which is derived from embryonic tissue) so that the two tissues connect together to form the adult reproductive system.

The genital disc is unique in that is derived from 3 different segments, abdominal segments A8-A10 (roughly equivalent to PS13-15), whereas most other imaginal discs arise from a single segment (Figure 1.5A). In the embryo, the A8 portion of the disc represents the female primordium and A9 is the male primordium. In each sex, only one of two primordia will give rise to adult terminalia. In a female, DSX-F will promote the female primordium to grow and differentiate into various reproductive structures, and the male promordium will go into a "repressed" state (repressed male primordium; RMP) with limited growth. Conversely, in males, DSX-M will promote the growth and differentiation of the male primordium and causes the female primordium to go into a "repressed" state (repressed female primordium; RFP). These repressed primordia originally were not thought to contribute to the adult fly, but it has been shown that the RMP gives rise to the parovaria in the female, and the RFP gives rise to the eighth tergite (cuticle segment) in the male (Keisman and Baker, 2001). The anal primordium, in A10, will develop in both sexes, but depending on its sexual identity will form either male- or female-specific analia (Estrada et al., 2003).

Given the posterior region in which the genital disc precursors arise, the homeotic gene *Abd-B* is responsible for patterning the genital disc, not only in its initial specification in the embryo, but also for its sex-specific differentiation. *Abd-B* is required for genital disc specification; in *Abd-B* mutants, the genital primordia form ectopic leg appendages (Estrada and Sanchez-Herrero, 2001). The differential expression of two

isoforms of Abd-B proteins, "Abd-Bm" and "Abd-Br" (Kuziora and McGinnis, 1988; Sanchez-Herrero and Crosby, 1988), which are expressed in the female and male primordia, respectively, work with DSX to regulate growth and differentiation by regulating Wingless (Wg) and Decapentaplegic (Dpp) signaling (Figure 1.5B). In females, this combinatorial action of Abd-B and DSX serves to enhance Wg signaling in the female primordium and repress Dpp signaling in the RMP, while in males, the converse takes place. In the absence of DSX, both male and female primordia develop, but only at a low level, due to a basal level of *dpp* and *wg* expression. Signaling by Wg and Dpp likely exert their effects by regulating *dachshund*, a key regulator of genital disc differentiation (Estrada and Sanchez-Herrero, 2001; Keisman and Baker, 2001; Sanchez et al., 2001). The differential control of signaling gives rise to sex-specific patterns of growth and development in the genital disc that result in the sexual dimorphism of the adult terminal and reproductive structures. Another interesting aspect of regulation by Wg and Dpp signaling is that it is controlled sex-specifically in a non-autonomous manner. It has been shown that there is an "organizer" region in the border of anterior and posterior compartments of each PS of the genital disc (as defined by *engrailed* expression), and that the sexual identity of the organizer region is sufficient to impose sex-specific development upon the entire disc (Keisman et al., 2001).

Figure 1.5. Homeotic genes and sex determination cues are both required for patterning of sexually dimorphic tissues in *Drosophila*.

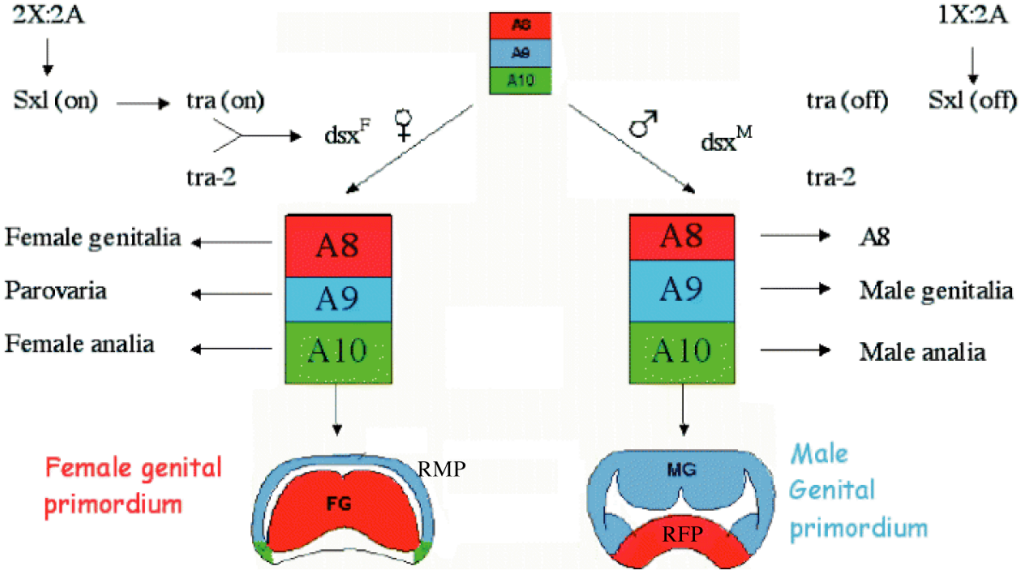
A) Schematic of genital disc development. The genital disc arises from abdominal segments A8-A10 and shows a sexually dimorphic pattern of differentiation, downstream of *dsx*. In females, the A8 primordium gives rise to the female genitalia (FG) and the A9-derived repressed male primordium (RMP). Conversely, in males, A8 forms the repressed female primordium (RFP) and A9 produces the male genitalia (MG). Adapted from (Estrada et al., 2003).

B) Diagram outlining the role of *Abd-B* and *dsx* in the sexually dimorphic development of the genital disc. The differential isoforms of Abd-B (m and r) combine with DSX sex-specific isoforms to regulate Wingless (Wg) and Decapentaplegic (Dpp) signaling responsible for growth and maturation of the male and female genital disc primordia (FGP and MGP). Adapted from (Sanchez et al., 2001).

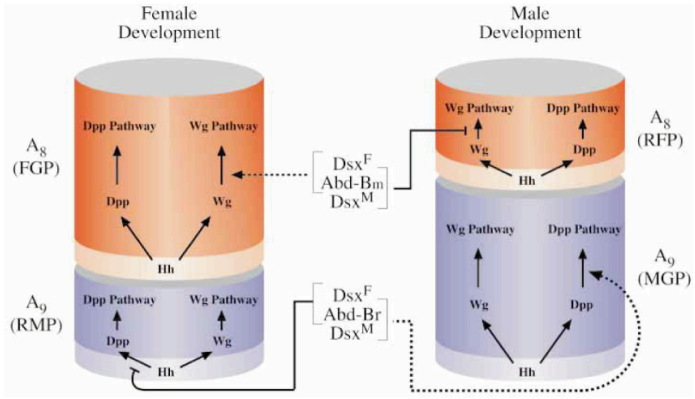
C) Picture of female and male adult abdominal cuticles, which display a sexually dimorphic pattern of pigmentation. Adapted from (Kopp et al., 2000).

D) Mechanism for *dsx* and *Abd-B* regulation of *bric à brac* (*bab*) in abdominal pigmentation. *bab* normally acts to inhibit pigmentation. In males, Abd-B represses *bab* and allows pigmentation, whereas in females, DSX-F overrides Abd-B's repression of *bab* and *bab* subsequently blocks pigmentation. Adapted from (Kopp et al., 2000).

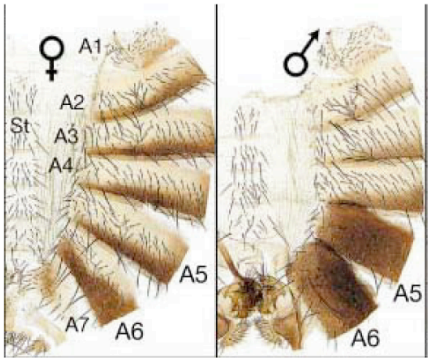
A


2X:2A
Sxl (on)
tra (on)
dsx^F
tra-2
1X:2A
tra (off)
Sxl (off)
dsx^M
tra-2
A8
A9
A10
Female genitalia
Parovaria
Female analia
A8
Male genitalia
Male analia
Female genital primordium
FG
RMP
MG
RFP
Male Genital primordium

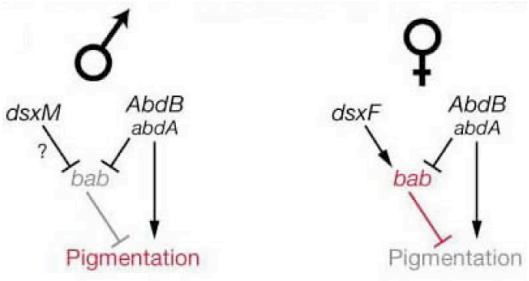
B


Female Development
Male Development
A8 (FGP)
A9 (RMP)
Dpp Pathway
Wg Pathway
Dpp
Wg
Hh
DsxF Abd-Bm DsxM
DsxF Abd-Br DsxM
A8 (RFP)
A9 (MGP)

C


A1
A2
St
A3
A4
A5
A7
A6
A5
A6

D


dsxM
AbdB
abdA
?
bab
Pigmentation
dsxF
AbdB
abdA
bab
Pigmentation

Homeotic genes and the sex determination pathway regulate bric à brac to pattern the abdominal cuticle

Another example of the sex determination pathway and homeotic genes interacting to regulate sexual dimorphism involves the sex-specific pigmentation of the adult cuticle. The 5th and 6th dorsal cuticle segments in the abdomen, A5 and A6, are present in both sexes, but show only partial pigmentation in females (non-sex-specific pigmentation), while in males they are completely pigmented (Figure 1.5C). *Abd-B* is necessary and sufficient for the male-specific pattern, and in the cuticle *Abd-B* is only expressed in segments A5 and A6. In *Abd-B* loss-of-function mutants where *Abd-B* expression is absent from A5 and A6, only the non-sex-specific pigmentation is observed. In gain-of-function mutants where *Abd-B* is ectopically expressed in A3 and A4, the male-specific pigmentation pattern is expanded anteriorly along the abdomen (Kopp et al., 2000). The sex determination gene *dsx* also has been shown to be responsible for sexual dimorphism of the cuticle; in *dsx* mutant XX adults, a predominantly male pattern of pigmentation is observed in the cuticle (Baker and Ridge, 1980; Hildreth, 1965; Kopp et al., 2000).

Abd-B and *dsx* act to regulate pigmentation via the gene *bric à brac* (*bab*). Normally, *bab* is expressed in all of the abdomen in females. In contrast, in males, it is absent in A5 and A6, suggesting that *bab* acts to repress male-specific pigmentation. In *Abd-B* loss-of-function mutants, *bab* expression is expanded to the entire abdomen of males and in *Abd-B* gain-of-function mutants, *bab* expression is ectopically repressed, demonstrating that *Abd-B* represses *bab*. In *dsx* mutants, *bab* is expressed in a male-like pattern and is excluded from A5 and A6, indicating that DSX-F is necessary to activate *bab* in A5 and A6.

Therefore, inputs from both *Abd-B* and *dsx* are required to establish sexually dimorphic pigmentation of the abdomen. In the model proposed, *bab* is a repressor of pigmentation, and *Abd-B* promotes pigmentation in both sexes via its repression of *bab* (Figure 1.5D). In males, this results in the absence of *bab* in A5 and A6, allowing *Abd-B* to induce pigment formation. However, the presence of DSX-F in females blocks the repression of *bab* by *Abd-B*, and thus the expression of *bab* in A5 and A6 blocks *Abd-B*'s ability to promote pigmentation (Kopp et al., 2000). The sexually dimorphic pattern of pigmentation was shown to be important for female attractiveness to males in mating (Kopp et al., 2000), demonstrating a requirement for sexual dimorphism of pigmentation at the social level.

Role of homeotic genes in mammalian sexual dimorphism

In mammals, given the gene duplication and gene redundancy in the Hox gene complex, it may prove difficult to find a severe phenotype with a single gene mutation, as is observed in *Drosophila*. However, two specific Hox genes, *Hoxa11* and *Hoxc10*, both of which are *Abd-B* homologs, are required for sex-specific differentiation of the mammalian gonad and reproductive system. Mouse *Hoxa11* is not expressed in the embryonic gonad, but is expressed in both the Müllerian and Wolffian ducts, which will give rise to the non-gonadal structures in the reproductive tract (see below). *Hoxa11* mutant mice are viable, but both female and male adult homozygous mutants are sterile. Female infertility is thought to arise from uterine defects that render them unable to support embryonic development to term, however, analysis of mutant ovaries and embryo transfer experiments show that oogenesis is normal (Hsieh-Li et al., 1995). Male

knockout mice show defects in the epididymis consistent with a homeotic transformation into vas deferens, and testes are smaller than wild-type mice. Examination of adult mutant testes indicates that, although somatic gonadal cell types are unaltered, spermatogenesis is disrupted and many germ cells die by apoptosis (Hsieh-Li et al., 1995). Similarly, *Hoxa10* is required for male and female fertility (Satokata et al., 1995). Mutant female uteri exhibit an inability to support implantation of embryos, but embryo transfer experiments show that eggs can give rise to viable progeny. The mutant defect was characterized as a transformation of the upper uterus into oviduct-like tissue (Benson et al., 1996). Male mutant testes also show defects in spermatogenesis, in which mature sperm are absent in spite of normal numbers of somatic cells in the testis (Satokata et al., 1995). However, some male phenotypes in the male testis of the *Hoxa10* and *Hoxa11* knockout mice may be due to the fact that testes are undescended, and mammalian spermatogenesis likely requires the exit of the testes from the abdominal cavity into the scrotum.

Interestingly, the mouse homolog of the *Drosophila* gene *Polycomb*, *M33*, is also required for sexual dimorphism (Katoh-Fukui et al., 1998). *M33*, like its fly counterpart, is involved in patterning A-P polarity by regulating Hox gene expression. *M33* knockout XY (male) and XX (female) adults are both sterile, but XY animals show a male to female sex reversal in the gonad and external genitalia. All these observations in the mouse indicate that there may be a sex-specific role for certain Hox genes in the process of gonad formation and gametogenesis, and that sex determination and positional information are both required for specifying sexually dimorphic tissues.

Evolutionary conservation of mechanisms to establish sexual dimorphism

Even though the establishment of sex-specific phenotype is a fundamental process that has the same essential requirements for all animals, sex determination switches appear to have evolved rapidly in the animal kingdom. Even among mammals, the use of *Sry* is not absolutely universal, as some vole species lack *Sry* DNA sequences (O'Neill and O'Neill, 1999). However, there are more downstream effectors that regulate sexual dimorphism that are expected to be less likely to diverge over the course of evolution. These effectors may be genes that directly interact with and control the ovary and testis morphogenesis machinery. Due to this critical role, these genes should be under selective pressure to be conserved, and aspects of their function may be similar in distantly related animal species. Two examples of this evolutionary conservation are the *Dmrt* family of genes and the gene *Sox9*.

As discussed above, the *dsx* gene in *Drosophila* is the terminal gene in the somatic sex determination pathway in non-central nervous system tissues and plays an essential role in establishing sexual dimorphism. Given this critical function for *dsx*, it is possible that homologs in other animal species also play a role in sex determination. Homologs of DSX share amino acid similarity in the zinc-finger type DNA binding domain, and are collectively called the DSX and Male abnormal-3 (MAB-3) related transcription factor (DMRT) family, after the founding members in *Drosophila* and *C. elegans*. In fact, the *C. elegans* homolog of DSX, MAB-3, is involved in the sexually dimorphic development of the male V-rays, a structure required for proper copulation. A study by Raymond and colleagues demonstrated that DSX-M can substitute for MAB-3

and can rescue the V-ray phenotype in *mab-3* mutants (Raymond et al., 1998), indicating a functional conservation of DMRT genes in regulating sexual dimorphism.

Studies of another factor may provide insight into the evolution of sex determination. The *Sry*-related HMG box gene 9 (Sox9*)* protein is a member of the Sox family of proteins, which have diverse roles in development and are characterized by their similarity in the HMG box. The *Sox* gene family seems to be restricted to the animal kingdom, and homologs have been discovered in nematodes, flies, and mammals (Bowles et al., 2000).

Sox proteins are further divided into groups (Groups A-J) by similarity to each other in the HMG box domain (Bowles et al., 2000). Whereas there are no Group E genes in *C. elegans*, the mammalian Group E genes contain the genes *Sox8, Sox9*, and *Sox10*. Human *SOX9* has a role in sex determination, since 75% of *SOX9* heterozygous XY patients show male to female sex reversal, and, furthermore, a duplication of *SOX9* is sufficient to induce XX female to male sex reversal in both humans and mice (Bishop et al., 2000; Foster et al., 1994; Huang et al., 1999; Vidal et al., 2001; Wagner et al., 1994). Expression of *SOX9* is male-specific in fetal human somatic gonad (de Santa Barbara et al., 2000), and is concomitant with the expression of *SRY*, suggesting that *SOX9* is a downstream target of *SRY* function. Interestingly, the expression of *Sox9* is male-specific in the gonads of mouse, chick, turtle, and trout (Kent et al., 1996; Moreno-Mendoza et al., 1999; Takamatsu et al., 1997), suggesting that *Sox9* may play a conserved role in the regulation of sexual dimorphism despite the variety of sex determination mechanisms in those species. While loss-of-function data is not available for all species, it was recently shown that *Sox8* and *Sox9* are required for mouse male sex determination; *Sox8/Sox9*

knockout mice no longer show testis formation, and mutant gonads ectopically express female-specific gonadal markers (Chaboissier et al., 2004). The data support the hypothesis that *Sox9* is an ancestral sex determination gene that may have a conserved role in testis formation.

The lone *Sox* Group E gene in *Drosophila* is *Sox100B*, whose HMG box shows 80% amino acid similarity to that mouse *Sox9* (Loh and Russell, 2000). A recent study has described the embryonic expression pattern of Sox100B in *Drosophila*. Sox100B is expressed in different compartments of the digestive and excretory system, and is also strongly expressed in the somatic gonad before and after initial gonad formation (Loh and Russell, 2000). Expression in the gonad indicates that the role of *Sox100B* in sex determination or sexual dimorphism may extend to insects in addition to vertebrates.

CHAPTER 2

Sex-specific apoptosis regulates sexual dimorphism in the *Drosophila* embryonic gonad

SUMMARY

Sexually dimorphic development of the gonad is essential for germ cell development and sexual reproduction. We have found that the *Drosophila* embryonic gonad is already sexually dimorphic at the time of initial gonad formation. Male-specific somatic gonadal precursors (msSGPs) contribute only to the testis, and express a *Drosophila* homolog of Sox9 (Sox100B), a gene essential for testis formation in humans. The msSGPs are specified in both males and females, but are only recruited into the developing testis. In females, these cells are eliminated via *doublesex*-dependent programmed cell death. Our work furthers the hypotheses that a conserved pathway controls gonad sexual dimorphism in diverse species, and that sex-specific cell recruitment and programmed cell death are common mechanisms for creating sexual dimorphism.

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INTRODUCTION

A key issue in developmental biology is how individuals of a species take on different sexual phenotypes, male vs. female. Sexual dimorphism in somatic tissues controls the development of germ cells into eggs or sperm, and provides the mechanism for bringing gametes of opposite sexes together. Sexual dimorphism is initiated by primary sex determination switches that vary widely between different animal species. However, there is increasing evidence that the pathways these switches activate to produce sexual dimorphism may be more highly conserved than the switches themselves (Zarkower, 2001). A prime example is the *Sox9* gene, which is essential for testis development in humans (Foster et al., 1994; Wagner et al., 1994), and is expressed in a male-specific manner in the gonads of vertebrates with different mechanisms of primary sex determination (Kent et al., 1996; Moreno-Mendoza et al., 1999). The somatic cells of the gonad play an essential role in nurturing sexual dimorphism in the developing gametes. In mammals, the somatic cells of the gonad are the only cells that respond to the sex determination switch, and these cells then control the sex-specific development of the rest of the organism indirectly. Thus, if the pathway promoting sexual dimorphism is conserved between animal species, this should be most evident in the developing gonad.

Although little is understood about the development of sexual dimorphism in the *Drosophila* gonad, a considerable amount is known about the cells that contribute to this organ. The gonad is formed from a combination of germ cells and specialized somatic cells known as somatic gonadal precursors (SGPs). The germ cells form initially as the pole cells at the posterior of the blastoderm embryo, and migrate through the embryo to contact the SGPs (reviewed in (Starz-Gaiano and Lehmann, 2001). SGPs are specified

within the *eve* domain of the dorsolateral mesoderm in parasegments (PS) 10, 11 and 12 (Boyle et al., 1997; Boyle and DiNardo, 1995; Brookman et al., 1992; Moore et al., 1998a; Riechmann et al., 1998). The actions of *tinman* and *zfh-1* allow the formation of the dorsolateral mesoderm (Broihier et al., 1998), while expression of *eyes absent (eya)* specifically in SGPs is required for their proper specification (Boyle et al., 1997). The homeotic gene *abdominal A* (*abd-A*) is required to promote SGP formation in PS 10-12 (Brookman et al., 1992; Cumberledge et al., 1992; Moore et al., 1998a; Riechmann et al., 1998). Once the germ cells and SGPs have properly associated, they then coalesce into a tightly compacted and organized embryonic gonad, in a process that requires the genes *shotgun/E-cadherin* and *fear of intimacy* (Jenkins et al., 2003; Van Doren et al., 2003).

In *Drosophila*, the primary sex determination switch is controlled by the X chromosome to autosome ratio, which leads to the expression of Sex lethal (Sxl) protein specifically in females. Sxl allows expression of Transformer (Tra) protein which, along with Transformer-2, leads to female-specific splicing of the *doublesex* (*dsx*) transcript (Burtis and Baker, 1989). In males, *dsx* is spliced in a male-specific (default) pattern. The sex-specific *dsx* mRNAs encode for either Dsx^M (male isoform) or Dsx^F (female isoform), which are transcription factors responsible for regulating sex-specific gene expression. *dsx* is required for virtually all aspects of sexual dimorphism in the soma outside the central nervous system (Baker and Ridge, 1980), although the only direct targets known for the Dsx proteins are the *yolk protein* genes (Burtis et al., 1991; Coschigano and Wensink, 1993). Since both Dsx^M and Dsx^F play an active role in promoting their respective sexual pathway, most tissues acquire an intersexual phenotype in *dsx* mutant adults (Hildreth, 1965). Recently, progress has been made in

understanding how *dsx* controls the sexually dimorphic development of some tissues. The sex-specific pattern of pigmentation on the posterior cuticle of the adult fly is controlled by *dsx* acting through *bric à brac* (Kopp et al., 2000). Furthermore, *dsx* controls the sex-specific development of the genital imaginal disc, the tissue that forms many of the non-gonadal reproductive structures, by regulating both patterning (*dachshund*, *wingless*, *decapentaplegic*) and cell migration (*branchless*) (Ahmad and Baker, 2002; Keisman and Baker, 2001; Sanchez et al., 2001).

Although it is not known when sexual dimorphism is initially established in the somatic gonad, the germ cells show dimorphism at very early stages. A slightly larger number of germ cells enter into the male embryonic gonad as it forms (Poirié et al., 1995; Sonnenblick, 1941). By early larval stages, the difference in germ cell number becomes more dramatic, and the sex of the embryo can be clearly identified by gonad size (Aboïm, 1945; Kerkis, 1931). This difference in gonad size requires *dsx* (Steinmann-Zwicky, 1994), but since *dsx* is not required in the germ cells (Schüpbach, 1982), *dsx* is likely to be acting in the soma to control germ cell number. By later stages, it is also clear that *dsx* acts to control development of the somatic gonad itself (Hildreth, 1965). One downstream factor controlling sexual dimorphism in the somatic gonad is *Wnt2*, which is expressed in a male-specific manner in the larval gonad, and is required for proper testis development (Kozopas et al., 1998).

Here, we extend our understanding of sexually dimorphic development of the somatic gonad in *Drosophila*. We have found that the gonad is already dimorphic at the time of gonad coalescence, and we have identified a group of cells, termed the male-specific SGPs (msSGPs), that becomes part of the developing testis, but not the ovary.

These cells express SGP markers such as Eya, but also exhibit expression of a Sox9 homolog, Sox100B. msSGPs are initially specified in both males and females, and we demonstrate that sex-specific programmed cell death determines the sexually dimorphic development of these cells.

MATERIALS AND METHODS

Fly stocks

The following stocks were used in our analyses: *foi*$^{20.71}$ (Moore et al., 1998b), *tin*^{GC14} (M. Frasch), *zfh-1*$^{75.26}$ (Moore et al., 1998b), *eya*$^{cli\text{-}IID}$, J3B9^{RV12} (a deficiency spanning the *Sox100B* locus; S. Russell), *abd-A*^{MX1}, *bluetail* (W. Bender), 68-77 (D. Godt), *tra*^{1}, *dsx*^{1}, *dsx*^{23}, *dsx*^{D}, Df(3L)H99 (H. Steller), *hid*^{A206}(A. Bergmann) (Grether et al., 1995), UAS-*tra*^{F}-20J7, UAS-*p35*-BH3, UAS-*mCD8::GFP* (L. Luo) (Lee and Luo, 1999), *twist*-GAL4 (Baylies and Bate, 1996), 24B-GAL4 (Brand and Perrimon, 1993), *tubulin*-GAL4-LL7. *ru st faf-lacZ e ca* flies were used as wild-type controls (Moore et al., 1998b), in addition to Canton-S, *w*^{1118}, and *Dfd-lacZ*-HZ2.7 (W. McGinnis) (Bergson and McGinnis, 1990). Agametic embryos were the progeny of *osk*^{301}/*osk*^{CE4} mothers, raised at 18°C (Lehmann and Nüsslein-Volhard, 1986). Any unspecified stocks were obtained from the Bloomington Stock Center and information on these lines can be found at Flybase (http://flybase.bio.indiana.edu).

Whole-mount antibody stainings

Embryos were fixed and devitellinized as previously described (Patel, 1994), with the following modifications: all rinses were done with PT (PBS with 0.1% Triton X-100), embryos were dechorionated in 50% bleach for 5 minutes, and fixative was 8 mL

heptane, 0.25 mL 37% formaldehyde, and 1.75 mL PEMS (100 mM PIPES, 2 mM $MgSO_4$, 1 mM EGTA [pH 6.9]). Embryos were immunostained as previously described (Patel, 1994), with the following modifications. Tween-20 was always used in place of Triton X-100, blocking with PBT+NGS was done for 60 minutes in 1 ml volume, and last 2 wash series after secondary antibody incubation did not include BSA. After staining, embryos were mounted on slides in 2.5% DABCO (Sigma) in 70% glycerol and viewed with a Leica NTS or Zeiss 510 Meta confocal microscope.

The following primary antibodies (sources) were used: chicken-anti-Vasa (K. Howard) at 1:5,000 or 1:10,000; rabbit-anti-Vasa (R. Lehmann) at 1:10,000; rabbit-anti-β-gal (Cappel) at 1:10,000; mouse-anti-β−gal (Promega) at 1:10,000; rabbit-anti-GFP (Torrey Pines) at 1:2,000; mouse-anti-Eya10H6 (Developmental Studies Hybridoma Bank [DSHB]; N. Bonini) at 1:25; rabbit-anti-Sox100B (S. Russell) at 1:1,000; mouse-anti-En4D9 (DSHB; C. Goodman) at 1:2; and mouse-anti-SxlM18 (DSHB; P. Schedl) at 1:25 or 1:50. The following secondary antibodies were used, all at 1:500: Cy5 goat anti-chicken (Rockland), Cy5 goat anti-rabbit (Amersham Pharmacia), Alexa 594 goat anti-chicken, Alexa 594, 546, or 488 goat anti-rabbit, Alexa 568 or 488 goat anti-mouse. All Alexa antibodies are from Molecular Probes.

Whole-mount in-situ hybridization

Embryos were fixed and devitellinized as described above. Whole mount in-situ hybridization with digoxigenin-labeled riboprobes was performed as previously described (Lehmann and Tautz, 1994), except HNPP/Fast Red (Roche) was used as a fluorescent substrate for the alkaline phosphatase reaction. The *Wnt-2* antisense riboprobe was

synthesized by digesting plasmid pBSDWnt-2 (a gift from R. Nusse) with *Not*I and transcribing with T3 RNA polymerase (Promega) using digoxigenin-labeled UTP (Boehringer-Mannheim). Antibody staining was performed (as above) after in-situ hybridization and before incubation with the Fast Red substrate. Samples were mounted on slides in 2.5% DABCO (Sigma) in 70% glycerol and visualized by confocal microscopy.

Genotyping and sexing of embryos

In our experiments, we used balancer chromosomes containing *lacZ* or *GFP* transgenes in order to distinguish homozygous mutant embryos from siblings carrying balancer chromosomes.

Sexing of embryos was performed using a female-specific anti-Sxl antibody or using an X-chromosome carrying a *lacZ* or *GFP* transgene: P[*Dfd-lacZ*-HZ2.7] or P[*Kr*GAL4, UAS-GFP-Fm7c]. When using labeled X-chromosomes, crosses were set up in which males carrying the labeled chromosome were mated to wild-type virgin *faf-lacZ* females. Only the female progeny of such crosses contain a labeled X-chromosome whose β-gal or GFP expression pattern can be detected by immunostaining or fluorescence microscopy.

Electron microscopy

Embryos were collected and dechorionated as described above. Embryos were sorted under a fluorescent dissecting microscope to distinguish males and females by X-

chromosome GFP expression (see above). Transmission electron microscopy was performed as described in (Jenkins et al., 2003).

Sox100B scoring system for msSGPs

To quantitate msSGP development, we created a scale based on the appearance and number of Sox100B-immunoreactive cells in the posterior of the coalesced gonad (stage 15 or later). The range of scores is from –2 to +2, in which –2 represents an absence of Sox100B staining, -1 represents one Sox100B-positive nuclear-stained cell or faint diffuse posterior staining, 0 represents three to five nuclear-stained cells, +1 represents roughly seven to twelve Sox100B-positive cells, and +2 represents a large cluster of more than twelve nuclear-stained cells. At least 30 gonads were scored for each genotype.

RESULTS

Sexual dimorphism in the embryonic gonad

To investigate when sexual dimorphism is first manifested in the somatic gonad, we analyzed expression of SGP markers in embryos whose sex could be unambiguously identified, at a developmental stage (stage 15) soon after gonad coalescence has occurred. Analysis of Eya expression reveals anti-Eya immunoreactivity throughout the female somatic gonad (Figure 2.1A), though Eya expression is somewhat stronger in the posterior, as was previously reported (Figure 2.1A) (Boyle and DiNardo, 1995). In males, anti-Eya immunoreactivity is also found throughout the somatic gonad. However, the expression at the posterior of the gonad is much more intense than in females, as there appears to be a cluster of Eya-expressing cells at the posterior of the male gonad that is

not present in females (Figure 2.1B). In blind experiments, the sex of an embryo could be accurately identified by the Eya expression pattern in the gonad. Thus, sexual dimorphism is already apparent in the somatic gonad soon after initial gonad formation. A sex-specific expression pattern is also observed with *Wnt-2* at this stage. As is observed with Eya, *Wnt-2* is expressed in the SGPs of the female gonad (Figure 2.1C), but its expression is greatly increased at the posterior of the male gonad (Figure 2.1D). The SGP marker *bluetail* (Galloni et al., 1993) exhibits a similar sex-specific pattern as Eya (data not shown); however, the SGP marker *68-77* (Simon et al., 1990) is expressed equally in both sexes (see below). Thus, the somatic gonad is sexually dimorphic by stage 15, but only a subset of SGP markers reveals this sexual dimorphism.

Sox100B, a homologue of Sox9, is sexually dimorphic in the gonad

During *Drosophila* embryogenesis, Sox100B is expressed in a number of cell types, including the gonad (Loh and Russell, 2000). Since Sox100B is closely related to Sox9, an important sex determination factor in humans and mice (Foster et al., 1994; Kent et al., 1996; Wagner et al., 1994), we tested whether Sox100B expression is sexually dimorphic in *Drosophila*. Interestingly, we find that after gonad coalescence (stage 15), Sox100B expression in the gonad is male-specific. Sox100B immunoreactivity is not observed in the coalesced female gonad (Figure 2.1E), whereas it is detected in a posterior cluster of SGPs in the male gonad (Figure 2.1F). While we see this expression pattern in most wild-type backgrounds (including Canton-S and *faf-lacZ*), in certain "wild-type" lines, such as w^{1118}, we observe a few Sox100B-positive cells in the posterior of the coalesced female gonad (however, this is still clearly distinguishable

from the number of Sox100B positive cells in the male; data not shown). Unlike Eya and *Wnt-2*, Sox100B is not expressed in all SGPs, since it is usually absent from female gonads and from the anterior region of the male gonad, and does not colocalize with the SGP marker *68-77* (inset, Figure 2.1F). Sox100B expression appears restricted to the posterior cluster of SGPs that is observed only in the male gonad. Thus, like Sox9 expression in vertebrates, Sox100B exhibits a male-specific pattern of expression in the Drosophila embryonic gonad, suggesting that it may indeed be an ortholog of Sox9.

Sexual dimorphism does not require gonad coalescence or the presence of germ cells

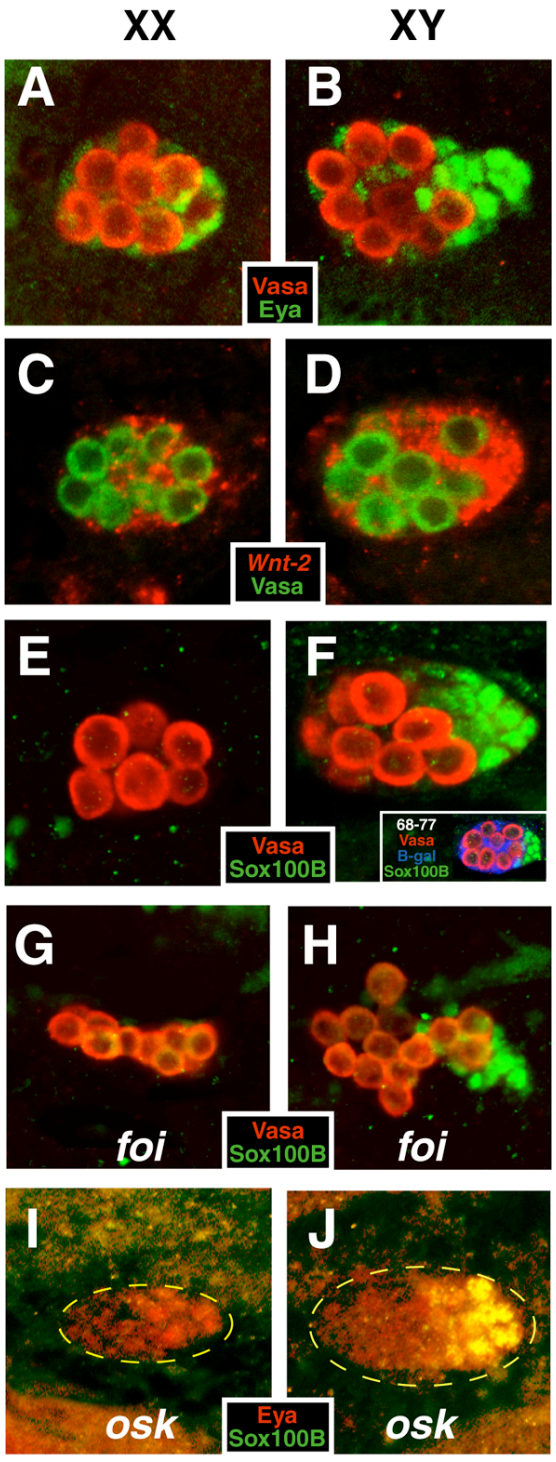
After having identified sexually dimorphic markers of the embryonic gonad, we wanted to use these markers to investigate how sexual dimorphism is established. We asked whether proper gonad formation is necessary for the establishment of sexual dimorphism by examining Sox100B expression in *fear-of-intimacy* (*foi*) mutant embryos. In *foi* mutants, germ cells migrate and associate normally with the SGPs but these two cell types fail to coalesce into a round and compact gonad (Van Doren et al., 2003). Despite the failure of gonad coalescence, we still observe a cluster of Sox100B-expressing cells at the posterior of the male gonad (Figure 2.1H), while no Sox100B-expressing cells are observed in the female at this stage (Figure 2.1G).

We also investigated whether the presence of germ cells is necessary for the establishment of sexual dimorphism in the embryonic gonad. We examined embryos that lack germ cells due to a hypomorphic mutation in *oskar*, a gene required for germ cell formation (Lehmann and Nüsslein-Volhard, 1986). Other aspects of embryonic development occur normally in these embryos, including the formation and coalescence

of the SGPs (Boyle and DiNardo, 1995; Brookman et al., 1992). We observe that agametic gonads show identical sexual dimorphism to wild-type embryos. Sox100B is co-expressed with Eya in the cluster of somatic cells in the posterior of the male gonad (Figure 2.1J), but Sox100B expression is not observed in the female gonad (Figure 2.1I). Thus, sexual dimorphism of the embryonic somatic gonad does not require proper gonad morphogenesis, or the presence of germ cells.

Figure 2.1. Sexual dimorphism in the embryonic gonad.

Drosophila stage (st.) 15 embryonic female (A,C,E,G,I) and male (B,D,F,H,J) gonads. Anterior is to left in all panels. Vasa, Eya, and Sox100B were revealed with immunofluorescence. *Wnt-2* was analyzed with fluorescent in-situ hybridization. Colors of stainings are as indicated in panels. (A-F) wild-type *faf-lacZ* embryos. (F, inset) *68-77* enhancer trap expressing β-galactosidase in the SGPs. (G,H) *foi* mutant embryos. (I,J) *osk* mutant agametic embryos. Note increased staining in posterior of male gonads. Magnification in (G,H) is 0.75x relative to all other panels.


XX
XY
A
B
Vasa
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C
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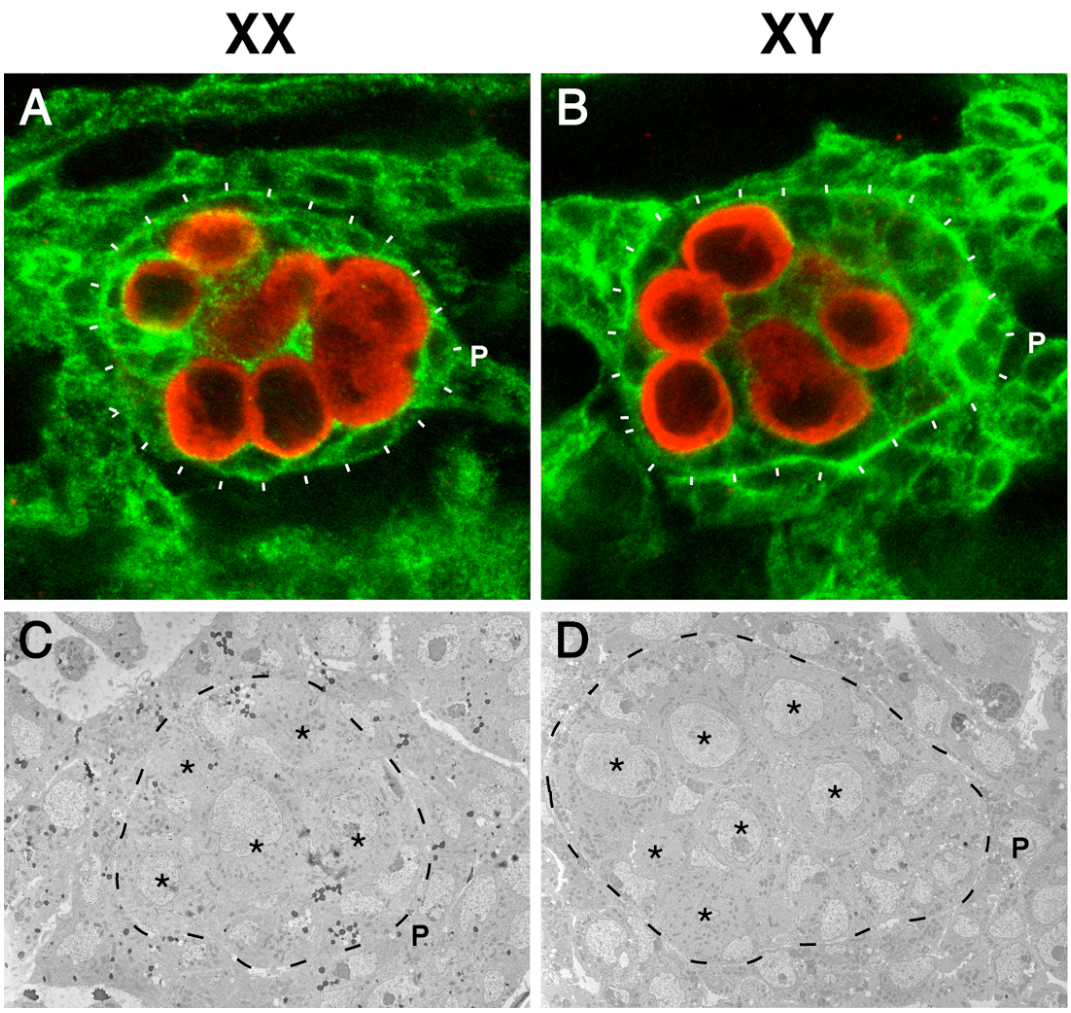
E
F
Vasa
Sox100B
68-77
Vasa
B-gal
Sox100B
G
H
foi
Vasa
Sox100B
foi
I
J
osk
Eya
Sox100B
osk

Sexual dimorphism results from the presence of male-specific somatic gonadal precursors

The posterior cluster of Eya and Sox100B co-expressing cells could result from sex-specific differences in gene expression within the cells of the gonad. Alternatively, it could reflect a difference in gonad morphology, in which these cells are only present in males and not in females. To distinguish between these possibilities, we analyzed the morphology of the male and female coalesced (stage 15) gonad, using approaches that do not depend on cell type-specific SGP markers. First, we expressed a CD8-GFP fusion protein broadly in the mesoderm. The fusion of the extracellular and transmembrane regions of mouse CD8 with GFP allows for visualization of cell and tissue morphology (Lee and Luo, 1999). We consistently observe a cluster of mesodermal cells attached to the posterior of the male gonad (Figure 2.2B) that is not observed in the female (Figure 2.2A). In blind experiments, the sex of the embryo can be predicted based on the presence of this posterior cluster of cells. We also examined male and female gonads by transmission electron microscopy (TEM). Male and female embryos were first sorted using an X-chromosome-linked GFP expression construct, and then processed separately for TEM (see Experimental Procedures). In this analysis, we again observe a cluster of cells at the posterior of the male gonad (Figure 2.2D) that is not present in the female gonad (Figure 2.2C). The size and morphology of these cells indicates that they are somatic cells rather than germ cells. Thus, the observed sexual dimorphism reflects a change in gonad morphology, not just a change in gene expression. Since the additional cells at the posterior of the male gonad express at least some markers in common with SGPs (e.g., Eya), we refer to these cells as male-specific SGPs (msSGPs).

Figure 2.2. Male and female gonads are morphologically distinct

The posterior of the gonad is as indicated (P) in each panel. (A,B) St. 15 female (A) and male (B) gonads expressing CD8-GFP in the mesoderm (UAS-mCD8::GFP x *twist*-GAL4). Anti-Vasa (red) labels germ cells, and anti-GFP (green) marks the cell surface of somatic mesodermal cells. Outline of gonad is indicated by dashed lines. (C,D) Sagittal TEM sections of a st. 15 female (C) and a male (D) gonad. Dashed lines mark the boundaries of each gonad. Germ cells in (C,D) are indicated by asterisks (*).

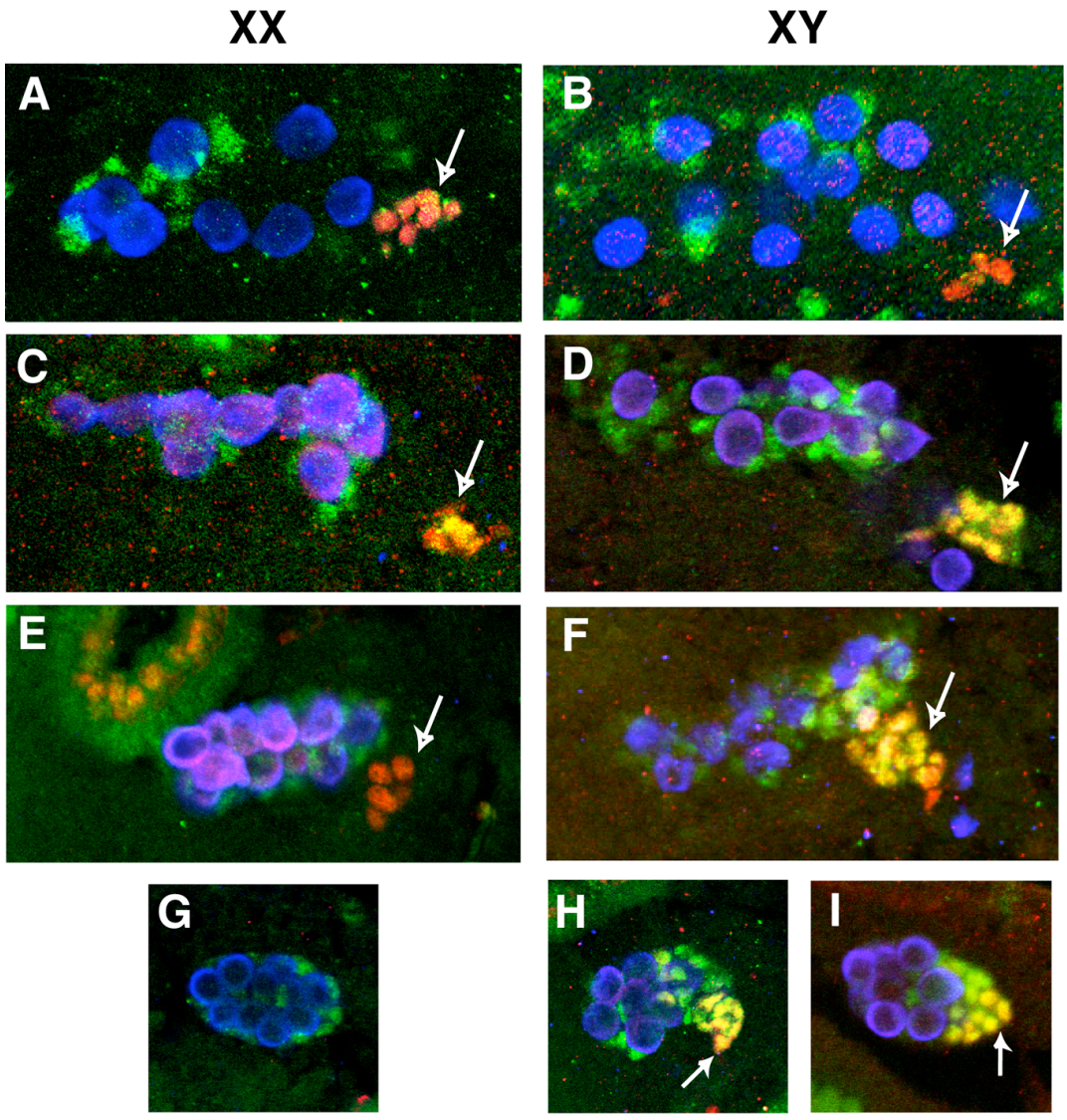

XX
XY
A
B
P
P
C
D
P
P

msSGPs form in both males and females and are recruited into the developing testis

We next wanted to determine the origin of these male-specific cells. Since we have not observed sex-specific differences in SGP proliferation in the gonad (data not shown), it seems unlikely that the SGPs are dividing to produce the msSGPs. Therefore, we used Sox100B as a marker for the msSGPs to determine where and when these cells are first specified. At stages prior to gonad coalescence (stages 12 and 13) a cluster of Eya/Sox100B double-immunopositive cells is observed posterior and ventral to the developing clusters of SGPs, which express Eya alone (Figure 2.3). Interestingly, this cluster of Eya/Sox100B double-positive cells is initially observed in both males and females and appears identical (Figures 2.3A and 2.3B), although Eya expression may be somewhat lower in the female cluster (compare Figure 2.3C to Figure 2.3D). During stage 13, as the SGPs and germ cells associate closely along PS 10-12, the Eya/Sox100B double-positive cells move toward the gonad in both sexes (Figure 2.3C and 2.3D). In males, these cells join the posterior of the coalescing gonad (Figures 2.3F, 2.3H and 2.3I). In contrast, these cells do not join the gonad in females (Figure 2.3E), and only Eya-positive, Sox100B-negative cells are found in the coalesced gonad (Figure 2.3G). We conclude that the Eya/Sox100B double-positive cells are the msSGPs, and that they form separately from the SGPs. These cells are initially specified in both males and females, and move anteriorly to join the gonad in males. In females, these cells do not form part of the gonad, as judged by the above morphological analysis, and are no longer detected using available markers.

Figure 2.3. Time course for establishment of sexual dimorphism in the embryonic gonad

Wild-type female (A,C,E,G) and male (B,D,F,H,I) embryos undergoing gonad coalescence. Anterior is to left in all panels. Vasa (blue), Sox100B (red) and Eya (green) were revealed by immunofluorescence. (A,B) St. 12 gonads with Sox100B-immunopositive cell cluster (arrows) posterior to germ cells. (C,D) St. 13 gonads. (E,F) Early st. 14 gonads. (G) St. 15 female gonad. Note that the Sox100B-immunopositive cell cluster is not observed in females at this stage. (H) Late st. 14 male gonad. (I) St. 15 male gonad. Sex of embryos was determined using an X-chromosome linked transgene (P[*Dfd-lacZ*-HZ2.7], see Materials and Methods).


XX
XY
A
B
C
D
E
F
G
H
I

The msSGPs are under distinct regulatory control from the SGPs

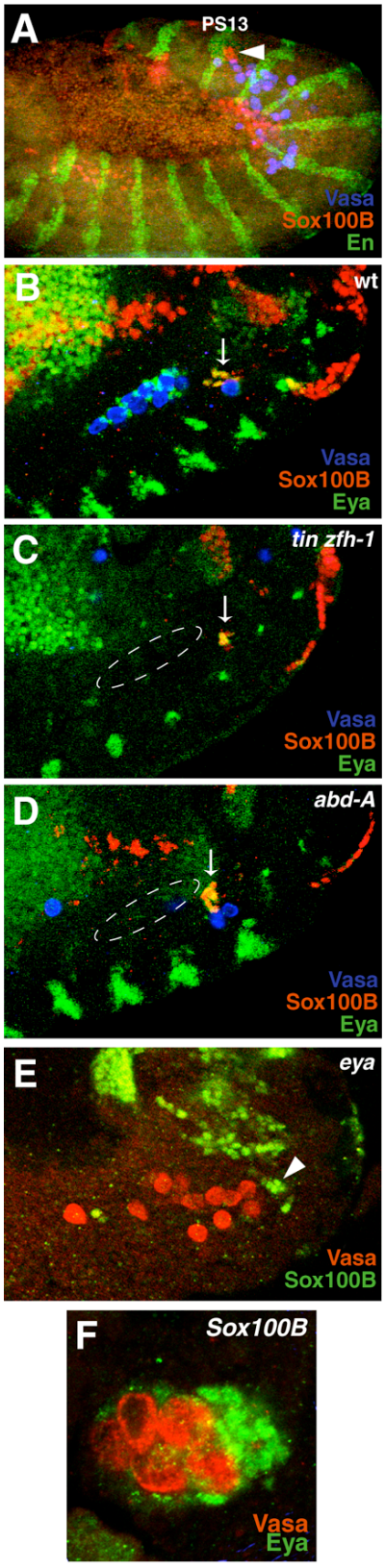
Since the msSGPs develop separately from the SGPs we wanted to address where the msSGPs arise and what controls their specification. By marking the anterior of each parasegment using an antibody against Engrailed (Patel et al., 1989), we determined that the msSGPs are specified in PS13 (Figure 2.4A, arrowhead). This observation is consistent with these cells arising posteriorly to the SGPs, which form in PS 10, 11 and 12. Other Sox100B expression is observed in non-gonadal tissues, as previously described (Loh and Russell, 2000). We also addressed whether, like the SGPs, the msSGPs are specified in the dorsolateral domain of the mesoderm (Boyle et al., 1997; Brookman et al., 1992). Mesodermal cell types that form in this region, such as the SGPs and the fat body, require the homeodomain proteins Tinman and Zfh-1 for their specification (Broihier et al., 1998). However, in embryos double mutant for *tinman* and *zfh-1,* we find that the msSGPs are still specified, even though the SGPs fail to develop (Figure 2.4C, compare to Figure 2.4B). Thus, msSGPs do not arise from the dorsolateral domain, consistent with the fact that the msSGPs are first observed in a position ventral to the SGPs. The msSGPs also differ from the SGPs in terms of their requirements for the homeotic gene *abd-A*. SGP specification absolutely requires *abd-A* (Cumberledge et al., 1992), while msSGPs are still present in these mutants (Figure 2.4D). Thus, despite the fact that the msSGPs and the SGPs share expression of some molecular markers such as Eya and *Wnt-2*, their specification is under independent control.

Since the msSGPs express both Eya and Sox100B, we investigated the requirements for each of these genes in msSGP specification. In *eya* mutants, Sox100B-positive cells are still observed posterior to the germ cells at early stages, in a position

where the msSGPs normally develop (Figure 2.4E). Since the SGPs are not maintained in these mutants (Boyle et al., 1997), the germ cells disperse and the gonad does not coalesce. Therefore, it is impossible to tell if the msSGPs would join the posterior of the male gonad in *eya* mutants. However, initial msSGP specification does not require *eya*. Similarly, in a deletion that removes the *Sox100B* locus, we still observe a large cluster of Eya-positive cells at the posterior of the male gonad that does not appear in females (Figure 2.4F). Thus, the initial development of the msSGPs does not require Sox100B. Expression of Eya and Sox100B are mutually independent, and are likely to be downstream of factors controlling initial msSGP specification.

Figure 2.4. The msSGPs are specified separately from the SGPs

Anterior is to left in all panels. All markers were revealed using immunofluorescence. Colors of stainings are as indicated in panels. (A) Wild-type st. 12 embryo. Engrailed staining marks the parasegmental boundaries. Note Sox100B-expressing mesoderm (arrowhead) arising under PS13 En stripe. Sox100B staining is also observed in non-gonadal tissues, such as anterior midgut primordium, posterior midgut primordium, and Malpighian tubules, as previously reported (Loh and Russell, 2000). (B) Wild-type st. 13 embryo, showing Sox100B/Eya double-positive msSGPs (arrow). (C) *tin zfh-1* mutant st. 13 embryo, with msSGPs visible (arrow) and PS10-12 SGPs disrupted (outline). (D) *abd-A* mutant st. 13 embryo, with msSGPs visible (arrow) and PS10-12 SGPs disrupted (outline). (E) *eya* mutant st. 13 embryo. Note presence of msSGPs (arrowhead). Other Sox100B expression is observed in Malpighian tubules and hindgut, as previously described (Loh and Russell, 2000). (F) *Sox100B* mutant st. 15 male gonad. Sex of embryo in (F) was determined using an anti-Sxl antibody. Magnification in (F) is 4x relative to all other panels.


A
PS13
Vasa
Sox100B
En
B
wt
Vasa
Sox100B
Eya
C
tin zfh-1
Vasa
Sox100B
Eya
D
abd-A
Vasa
Sox100B
Eya
E
eya
Vasa
Sox100B
F
Sox100B
Vasa
Eya

Sexual dimorphism in the embryonic gonad requires *tra* and *dsx*

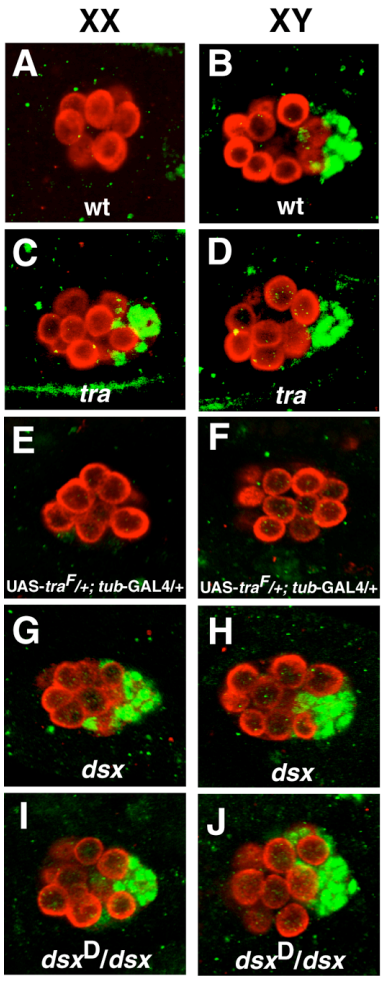
Since the msSGPs are initially specified in both males and females, we determined how these cells receive information about their sexual identity that allows them to behave differently in the two sexes. *tra* plays a key role in the sex determination pathway in *Drosophila*, and is required to promote female differentiation in somatic tissues. We examined *tra* mutant gonads to test if *tra* function is required for gonad sexual dimorphism and found that XX embryos are masculinized by mutations in *tra*. Sox100B-immunopositive cells are observed in the posterior somatic gonad of both XX and XY *tra*-mutant embryos (Figures 2.5C and 2.5D) in a manner comparable to wild-type males (Figure 2.5B). Analysis of the Sox100B expression pattern in the gonad reveals that there are no differences between XX and XY *tra* mutants, or between either of these genotypes and wild type males (Figure 2.5K). Conversely, when Transformer is expressed in XY embryos (UAS-tra^F, *tubulin*-GAL4), we no longer observe Sox100B-immunopositive cells in these gonads (Figure 2.5F), and they now appear similar to wild-type females.

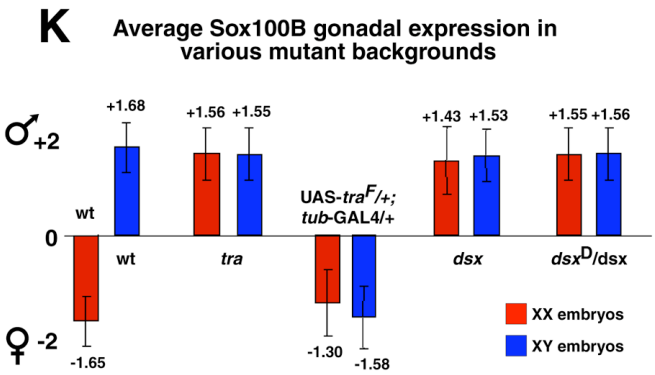
In most somatic tissues, the principle sex determination factor downstream of *tra* is *dsx* (Baker and Ridge, 1980). Unlike *tra, dsx* is required for both the male and female differentiation pathway, since both XX and XY *dsx* mutant adults show an intersexual phenotype (Hildreth, 1965). However, we find that, in the somatic gonad, *dsx* mutant XY embryos are indistinguishable from wild-type males and show no change in Sox100B expression (Figure 2.5H). Thus, unlike in most somatic tissues, this early characteristic of male development does not require *dsx*. In XX embryos that are mutant for *dsx*, we observe a completely masculinized phenotype (Figure 2.5G), in which Sox100B

expression in the gonad is similar to a wild-type male (Figure 5K). When a dominant allele of *dsx*, dsx^D (Nagoshi and Baker, 1990), is used to express Dsx^M (dsx^D/dsx) in XX embryos (Figure 2.5I), we find that these gonads are no more masculinized than *dsx* null XX gonads. Therefore, while Dsx^F is required for the proper female phenotype in XX gonads, it appears that the male Sox100B expression pattern is the "default" state in the absence of *dsx* function.

Figure 2.5. Sexual dimorphism in the embryonic gonad requires *tra* and *dsx*

(A-J) St. 15 XX (A,C,E,G,I) and XY (B,D,F,H,J) embryonic gonads. Vasa (red) and Sox100B (green) were revealed using immunofluorescence. Genotypes of embryos are as indicated in panels. Genotypes were determined by using GFP-labeled balancer chromosomes, and sex of embryos was determined using an anti-Sxl antibody. (K) Quantitation of average Sox100B staining in various mutant backgrounds. A scale of -2 to +2 was created (see Experimental Procedures), with -2 denoting a complete absence of gonadal Sox100B staining at st. 15 (e.g., A) and +2 denoting a large cap of bright posterior gonadal Sox100B staining (e.g., B). Average values for each genotype are indicated adjacent to bars. Red bars indicate XX embryos and blue bars indicate XY embryos. Error bars denote standard deviations. Genotypes: wt=*faf-lacZ*, *tra*=tra^{1}/ tra^{1}, *dsx*=dsx^{1}/dsx^{23}, dsx^{D}/*dsx*=dsx^{D}/dsx^{1}.


XX
XY
A
wt
B
wt
C
tra
D
tra
E
UAS-traF/+; tub-GAL4/+
F
UAS-traF/+; tub-GAL4/+
G
dsx
H
dsx
I
dsxD/dsx
J
dsxD/dsx


K
Average Sox100B gonadal expression in various mutant backgrounds
♂ +2
0
♀ -2
+1.68
+1.56
+1.55
+1.43
+1.53
+1.55
+1.56
-1.65
-1.30
-1.58
wt
wt
tra
UAS-traF/+; tub-GAL4/+
dsx
dsxD/dsx
XX embryos
XY embryos

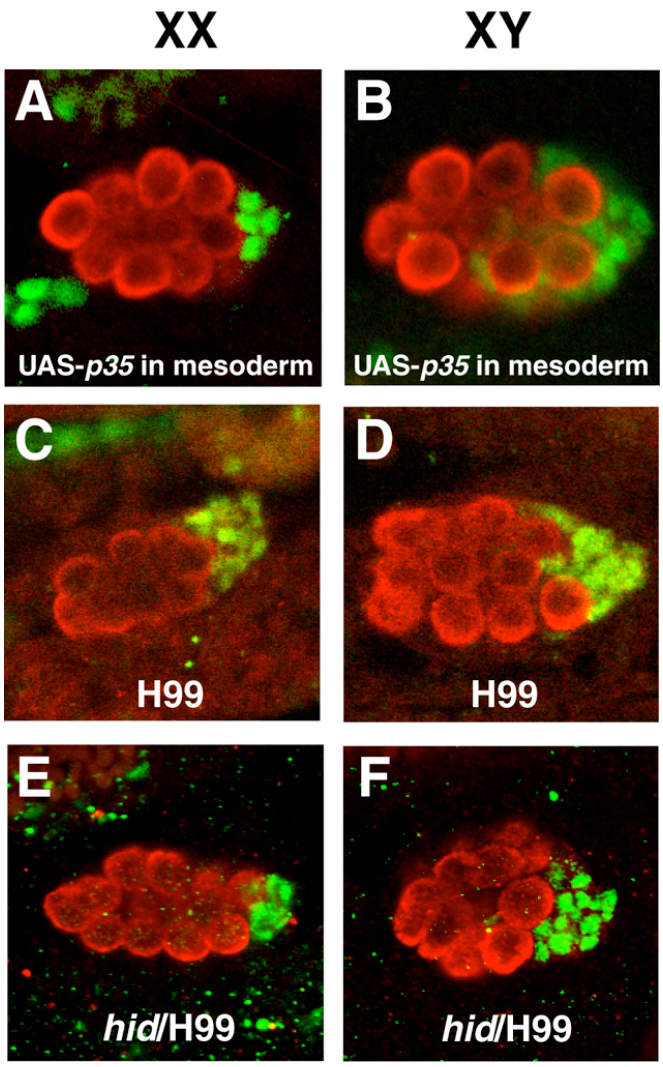
Sexual dimorphism in the embryonic gonad results from sex-specific apoptosis

Since the msSGPs join the posterior of the male gonad but are no longer detected in the female, we investigated the basis for the sexually dimorphic behavior of these cells. In the female, these cells could turn off Sox100B and Eya and contribute to some other tissue, or they might be eliminated altogether. To test this latter hypothesis, we addressed whether msSGPs are eliminated by sex-specific programmed cell death in the female. Since programmed cell death occurs in a caspase-dependent manner, we examined the gonad phenotype in embryos in which caspase activity was inhibited by expressing the baculovirus p35 protein (Hay et al., 1994) in the mesoderm (Figures 2.6A and 2.6B). In these embryos, we find that XX gonads now appear masculinized; Sox100B-positive cells (msSGPs) persist and join the posterior of female gonads (Figure 2.6A), and coexpress Eya (data not shown), as in wild-type male embryos. There are not as many Sox100B-positive cells in females as in males, suggesting that p35 may not be completely suppressing cell death. The presence of such cells in the female gonad does not appear to drastically affect ovary formation or oogenesis, since embryos of the genotype in Figure 2.6A develop into fertile adult females. To investigate how programmed cell death might be controlled in the msSGPs, we examined the genes of the H99 region (*head involution defective (hid), reaper (rpr)* and *grim*), which are regulators of apoptosis in *Drosophila* (Chen et al., 1996; Grether et al., 1995; White et al., 1994). A small deletion (DfH99) removes all three of these genes and blocks most programmed cell death in the *Drosophila* embryo (White et al., 1994). In DfH99 mutants, we observe an equivalent cluster of Sox100B-positive cells in both males and females (Figure 2.6C and Figure 2.6D). Again, these posterior cells are also Eya-positive (data not shown).

Furthermore, we observed that XX embryos mutant for *hid* alone also contained Sox100B-positive cells in the posterior of the gonad (Figure 2.6E), although the posterior cluster of cells was not as large as in male *hid* mutant siblings (compare Figure 2.6E and 2.6F). We conclude that the msSGPs are normally eliminated from females through sex-specific programmed cell death, controlled by *hid* and possibly also other genes of the H99 region. However, if cell death is blocked in females, these cells can continue to exhibit the normal male behavior of the msSGPs, including proper marker expression and recruitment into the gonad. Therefore, the decision whether or not to undergo apoptosis is likely the crucial event leading to the sexually dimorphic development of these cells at this stage.

Figure 2.6. msSGPs undergo sex-specific programmed cell death

St. 16 embryonic female (A,C,E) and male (B,D,F) gonads. Vasa (red) and Sox100B (green) were analyzed with immunofluorescence. (A,B) Gonads in embryos ectopically expressing p35 in the mesoderm (UAS-p35 x *twist*-GAL4, 24B-GAL4). (C,D) H99 deficiency (DfH99) homozygous mutant gonads. (E,F) hid^{A206}/DfH99 mutant gonads.


XX
XY
A
UAS-p35 in mesoderm
B
UAS-p35 in mesoderm
C
H99
D
H99
E
hid/H99
F
hid/H99

DISCUSSION

Sexual dimorphism in the *Drosophila* gonad

We have found that the somatic gonad in *Drosophila* is sexually dimorphic at the time of initial gonad formation, far earlier than previously suspected. One manifestation of this dimorphism results in the presence of the male-specific somatic gonadal precursors (msSGPs), which can be identified by their expression of the Sox9 homolog Sox100B. These cells are initially specified in both males and females, and move anteriorly to become part of the gonad in males. In females, these cells are eliminated by sex-specific programmed cell death. Through our analysis of the msSGPs, we have begun to understand how positional information is combined with sex determination signals to produce sexual dimorphism in the somatic gonad.

msSGP specification

It was previously thought that the SGPs made up the entire somatic gonad in *Drosophila*. However, we have found that the msSGPs are a distinct cell type that contributes to the male gonad. The SGPs and msSGPs are similar in some respects; both cell types express molecular markers such as Eya and *Wnt2* and coalesce with the germ cells to form the embryonic gonad. Both SGPs and msSGPs also express increased levels of the adhesion molecule *D*E-cadherin, and require *D*E-cadherin for their ability to properly contribute to the gonad (Jenkins et al., 2003). However, the identities of msSGPs and SGPs are specified quite differently. While the SGPs are specified in PS10, 11 and 12, and require *abd-A*, the msSGPs form in PS13 and are independent of *abd-A*. Furthermore, the SGPs develop in the dorsolateral domain of the mesoderm and require *tin* and *zfh-1* (Broihier et al., 1998), while the msSGPs appear to form more ventrally and

are independent of these genes. Thus, while the SGPs and msSGPs share some aspects of their identity, they acquire this identity through unique mechanisms.

msSGP development is regulated by the sex determination pathway

Proper information from the sex determination pathway is required to control the sexually dimorphic behavior of the msSGPs. The female phenotype in the embryonic gonad is dependent on both *tra* and *dsx*. Interestingly, it seems that the male phenotype is the default state; in the absence of any *tra* or *dsx* function, msSGPs in both XX and XY embryos behave as in wild-type males. This is a different situation than in most other tissues, in which *dsx* is required in both sexes to promote proper sexual differentiation. In particular, while we find no role for Dsx^M in this process, Dsx^F is positively required either to establish the female fate in the posterior somatic gonad or to repress the male fate. This role for Dsx^F in msSGP development is analogous to its role in the genital disc, in which Dsx^F is required to block recruitment of *btl*-expressing cells into the disc (Ahmad and Baker, 2002); in both cases, *dsx* female function serves to repress incorporation of a male-specific cell type. Since the msSGPs are initially specified in a sex-independent manner, this may account for the fact that the persistence of these cells (the male phenotype) is the default state. It will be of interest in the future to address the role of the msSGPs in testis development, and how genes such as *dsx*, *eya* and *Sox100B* act in this process.

Sex-specific apoptosis as a mechanism for creating sexual dimorphism

We have demonstrated that sexual dimorphism in the gonad relies on apoptosis of msSGPs in females immediately prior to gonad coalescence. This apoptosis is caspase-

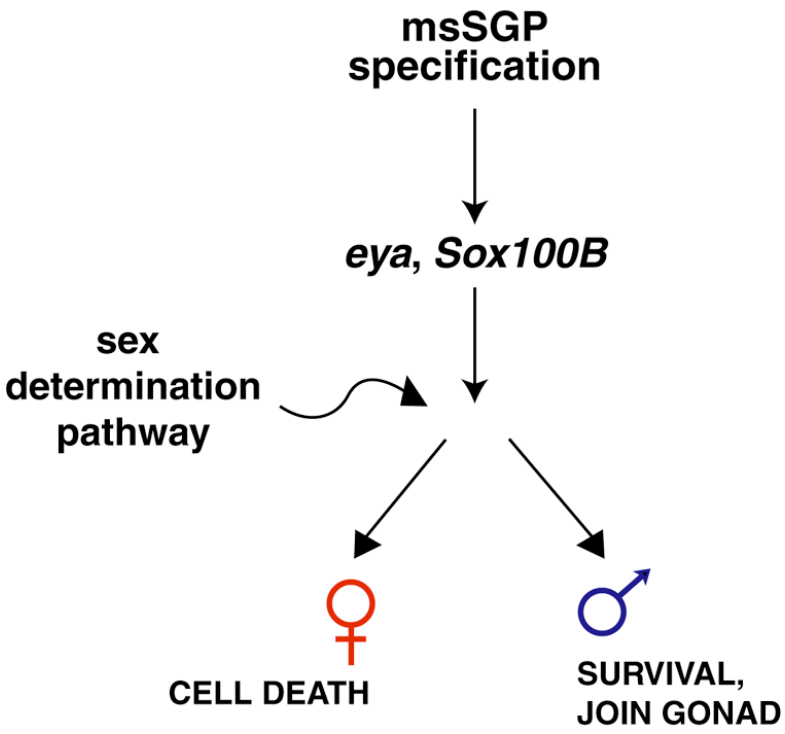
dependent, as sex reversal is observed when the caspase inhibitor p35 is introduced in females. In addition, our work indicates that *hid* is required for the apoptosis observed in females, perhaps in combination with one or more other genes of the H99 region. msSGPs that survive in *hid* or DfH99 mutant females, or in females expressing ectopic p35, still incorporate into the gonad and express msSGP-specific markers, such as Sox100B. Since female msSGPs behave like male msSGPs when they are allowed to survive, it appears that all msSGPs have the potential to join the gonad, and it is exclusively female-specific cell death that establishes the sex-specific phenotype of these cells.

A molecular model for sex-specific apoptosis

Sexual dimorphism requires the integration of positional information with sexual identity. In the case of the msSGPs, positional information specifies this unique cell type within PS13 of the mesoderm which, when combined with a female identity, results in programmed cell death (Figure 2.7). One possibility is that this sexually dimorphic output is manifested at the transcriptional level of *hid*, *grim*, or *reaper*, since up-regulation of these genes has been shown to induce apoptosis in other cell types (Chen et al., 1996; Grether et al., 1995). For example, *hid* would be specifically expressed in msSGPs in females, under the control of regulatory regions with binding sites for both positional factors (i.e., homeotic proteins or mesoderm-specific factors) and sex determination factors (e.g., Dsx^{F}). This model provides a molecular framework for understanding how the integration of positional and sexual identity controls sexual dimorphism in the somatic gonad.

Figure 2.7. Positional information and sexual identity are integrated to create sexual dimorphism

Model for how msSGPs arise in PS13 and how their behavior is regulated in a sex-specific manner. Parasegment-specific and mesoderm-specific factors are required for msSGP specification, while sexual identity determines whether or not the msSGPs undergo apoptosis.


msSGP
specification
eya, Sox100B
sex
determination
pathway
CELL DEATH
SURVIVAL,
JOIN GONAD

A general mechanism for promoting sexual dimorphism

Sex-specific apoptosis has been observed in a few cases in other species. In *C. elegans*, male-specific programmed cell death of hermaphrodite-specific neurons (HSNs) is required for sexual dimorphism in the nervous system, and is mediated by the sex-determination factor Tra-1a and regulatory elements of the cell-death gene *egl-1* (Conradt and Horvitz, 1999). In mammals, sex-specific programmed cell death has been observed in the sexually dimorphic nucleus of the preoptic area (SDN-POA) of the rat hypothalamus, which results in a smaller size of SDN-POA in females. Sex-specific cell death in the SDN-POA is a secondary sex characteristic, since testosterone is sufficient to block apoptosis in these cells in castrated males (Davis et al., 1996).

Our work demonstrates how apoptosis can be used as a mechanism for promoting sexual dimorphism in the somatic gonad. Recent observations of apoptosis in the developing zebrafish gonad (Uchida et al., 2002) suggest that this may also occur in other species, including vertebrates. Furthermore, other reproductive tissues clearly utilize sex-specific programmed cell death. In mammals, the Mullerian duct, which produces much of the female reproductive tract, is thought to degenerate in the male via programmed cell death (Price et al., 1977; Roberts et al., 1999). Germ cells are also observed to undergo apoptosis and are sensitive to survival and apoptotic factors produced in the somatic cells of the gonad (Kierszenbaum and Tres, 2001). Therefore, programmed cell death may be used extensively as a tool to mediate sexual dimorphism, both in the gonad and throughout the body.

Evolutionary conservation and sex determination

Although sex determination schemes vary widely in the animal kingdom, there is evidence that the molecular and cellular pathways used to control sexual dimorphism may be conserved, even between vertebrates and invertebrates. One example is *Sox9*, which has been implicated as an ancestral sex-determining gene in vertebrates given its male-specific gonad expression in diverse species such as human, mouse, turtle, and chicken (Foster et al., 1994; Kent et al., 1996; Moreno-Mendoza et al., 1999). In this study, we demonstrate that a potential *Drosophila* ortholog of Sox9, Sox100B (Loh and Russell, 2000), is expressed in a male-specific manner in the embryonic somatic gonad. The manner of Sox100B expression is reminiscent of that in the mouse; *Sox9* is initially expressed in both sexes, but is maintained and up-regulated in the male gonad (Kent et al., 1996). It will be very interesting to compare the role that *Sox100B* plays in the development of the *Drosophila* testis to the one played by *Sox9* in vertebrates.

Molecular conservation is also observed amongst the members of the Dsx/Mab-3 Related Transcription Factor (DMRT) family. DMRT family members have been shown to be essential for sex-specific development in *Drosophila* (Dsx), *C. elegans* (mab-3), medaka fish (DMY), and mice (DMRT1), and have been implicated in human sex reversal (Hildreth, 1965; Matsuda et al., 2002; Raymond et al., 2000; Raymond et al., 1999; Raymond et al., 1998). We demonstrate here that *dsx* is essential for proper sex-specific development of the msSGPs. Thus, increasing evidence indicates that DMRT family members are also conserved regulators of sexual dimorphism.

In addition to molecular conservation, there is also conservation of cellular mechanisms in mediating sexual dimorphism in different species. As discussed above,

apoptosis may represent one example of such a conserved mechanism. Even more striking is the use of cell migration and sex-specific cellular recruitment to produce sexual dimorphism. We have demonstrated that in *Drosophila*, the msSGPs are specifically recruited to join to the male gonad. An analogous situation is found during mouse testis development, as neighboring mesonephric cells are induced to migrate into the testis in an SRY-dependent manner, and are required for testis cord formation (Capel et al., 1999; Tilmann and Capel, 1999). This mechanism is also used to promote sexual dimorphism in other tissues, as sex-specific cell migration contributes to sexually dimorphic development of the *Drosophila* genital disc (Ahmad and Baker, 2002). Therefore, there is evolutionary conservation of both molecular and cellular mechanisms to promote sexual dimorphism in diverse species. Perhaps this is not so surprising given the ancient relationship between a sexually dimorphic somatic gonad and the production of sex-specific gametes.

CHAPTER 3

Abdominal-B is Essential for Proper Sexually Dimorphic Development of the *Drosophila* Gonad

SUMMARY

Sexual dimorphism requires the integration of positional information in the embryo with the sex determination pathway. Homeotic genes are a major source of positional information responsible for patterning along the anterior-posterior axis in embryonic development, and are likely to play a critical role in sexual dimorphism. Here we investigate the role of homeotic genes in the sexually dimorphic development of the gonad in *Drosophila*. We have found that Abdominal-B (ABD-B) is expressed in a sexually dimorphic manner in the embryonic gonad. Furthermore, *Abd-B* is necessary and sufficient for specification of a sexually dimorphic cell type, the male-specific somatic gonadal precursors (msSGPs). In *Abd-B* mutants, the msSGPs are not specified and male gonads now resemble female gonads with respect to these cells. Ectopic expression of *Abd-B* is sufficient to induce formation of extra msSGPs in additional segments of the embryo. *Abd-B* works together with *abdominal-A* to pattern the non-sexually dimorphic somatic gonad in both sexes, while *Abd-B* alone specifies the msSGPs. Our results indicate that *Abd-B* acts at multiple levels to regulate gonad development and that *Abd-B* class homeotic genes are conserved factors in establishing gonad sexual dimorphism in diverse species.

This work has been reprinted and modified from the published manuscript: DeFalco, T., Le Bras, S., Van Doren, M. (2004). *Abdominal-B* is Essential for Proper Sexually Dimorphic Development of the *Drosophila* Gonad. *Mech. Dev.* **121**: 1323-1333.

INTRODUCTION

Embryonic gonad formation involves the association of two cell types, germ cells and somatic gonadal precursors (SGPs). In *Drosophila*, SGPs are groups of mesodermal cells specified in abdominal parasegments (PS) 10-13 and can be identified by stage 11 of embryonic development (stages as in (Campos-Ortega and Hartenstein, 1985). SGPs form in bilateral clusters within PS10, 11 and 12 (Boyle et al., 1997; Boyle and DiNardo, 1995; Brookman et al., 1992; Riechmann et al., 1998), while in PS13, a distinct subset of mesoderm gives rise to the male-specific SGPs (msSGPs) (Chapter 2). During stage 13, germ cells and SGPs begin to closely associate and coalesce, and by stage 15, coalescence is complete and the rounded embryonic gonad is formed. By this time, distinct anterior-posterior (A-P) cellular identities are already present in the embryonic gonad. Certain markers such as *escargot* label anterior SGPs (Gonczy et al., 1992), while others, such as *Wnt-2, bluetail*, and *eyes absent (eya*) identify posterior SGPs (Boyle et al., 1997; Galloni et al., 1993; Kozopas et al., 1998). Those three posterior markers also label msSGPs, which in addition express SOX100B (Chapter 2).

The abdominal region of the embryo which gives rise to the gonad is patterned by the homeotic genes *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*) and *Abdominal-B* (*Abd-B*) (Lewis, 1978). *Ubx* and *abd-A* are expressed in the mesoderm of PS10-12 during all stages of embryonic gonad formation (Karch et al., 1990; Macias et al., 1990; White and Wilcox, 1985), while *Abd-B* has a more dynamic expression pattern. Prior to SGP specification, the anterior limit of mesodermal *Abd-B* expression is PS13, but, beginning at stage 11, it is also observed in PS12 and weakly in PS11. By stage 13, *Abd-B* mesodermal expression is strongest in PS11-14, with weak expression in PS10 (Boulet et

al., 1991; Celniker et al., 1989; DeLorenzi and Bienz, 1990; Kuziora and McGinnis, 1988). However, the expression of homeotic genes relative to the distinct cell types of the developing gonad has not been fully addressed.

abd-A is required in the soma for proper SGP specification and gonad coalescence, and is sufficient to specify SGPs (Boyle et al., 1997; Boyle and DiNardo, 1995; Brookman et al., 1992; Cumberledge et al., 1992; Greig and Akam, 1995). *abd-A* restricts SGP formation to PS10-12 by blocking the action of *serpent*, which promotes fat body development in other PS of the embryo (Moore et al., 1998a; Riechmann et al., 1998). While some reports describe SGP specification defects in *Abd-B* mutants, others observe no gonad phenotype. However, it is clear that many SGPs do form in *Abd-B* mutants, and germ cells associate with these SGPs to form a gonad (Brookman et al., 1992; Greig and Akam, 1995). It is thought that *Abd-B* further works with *abd-A* to pattern the A-P axis of the formed gonad (Boyle and DiNardo, 1995). No role for *Ubx* in the gonad has yet been found (Greig and Akam, 1995).

The specification of sexually dimorphic tissues is dependent not only upon proper sexual identity, but also upon positional information provided in part by the homeotic genes. In *Drosophila,* genital disc differentiation and adult abdomen pigmentation both require the proper combination of *doublesex* (*dsx*) sex determination signals and the homeotic gene *Abd-B* (Ahmad and Baker, 2002; Estrada and Sanchez-Herrero, 2001; Keisman and Baker, 2001; Kopp et al., 2000; Sanchez et al., 2001). In the somatic gonad, msSGPs represent the earliest known sexual dimorphism (Chapter 2). msSGPs are initially specified in both sexes, but only join the posterior of the coalesced male gonad. In females, msSGPs die by apoptosis prior to coalescence, in a mechanism

involving the cell death gene *head involution defective (hid)*. Similar to the genital disc and the adult abdomen, msSGPs require proper *doublesex* signals for their sex-specific behavior (Chapter 2).

In this study we examine the role of homeotic genes in the development of sexual dimorphism in the somatic gonad. We find that *Abd-B* is expressed in a sexually dimorphic manner in the *Drosophila* embryonic gonad. Furthermore, *Abd-B* is necessary and sufficient for specification of a sexually dimorphic cell type, the msSGPs. Our results indicate that patterning by *Abd-B* class homeotic genes is part of a common mechanism for creating gonad sexual dimorphism in diverse species.

MATERIALS AND METHODS

Fly stocks

The following mutant alleles were used for analyses: $Abd\text{-}B^{M5}$ (M. Akam); Df(3L)H99 (H. Steller); dsx^{1}; dsx^{23}; In(3R)Pc^{3}, Pc^{3}; Pc^{XT109} (R. Paro); tra^{1}; Ubx^{1}. The following transgenic lines were used: UAS-*abd-A* 21.9, UAS-*Abd-B* 2 (M. Akam) (Castelli-Gair et al., 1994; Greig and Akam, 1993), *twist*-GAL4 (Baylies and Bate, 1996). The following *lacZ* enhancer trap lines were used: *68-77* (D.Godt) (Simon et al., 1990), HCJ200 (W. Bender) (Bender and Hudson, 2000), esg^{G66} (Whiteley et al., 1992). *ru st fat facets-lacZ e ca (faf-lacZ)* (Moore et al., 1998b), *Dfd-lacZ*-HZ2.7 (W. McGinnis) (Bergson and McGinnis, 1990) outcrossed to *faf-lacZ* , and esg^{G66} outcrossed to w^{1118} were used as wild type controls. Unspecified fly stocks were obtained from the Bloomington Stock Center and information about these alleles can be found at Flybase (http://flybase.bio.indiana.edu).

Whole-mount antibody stainings

Embryos were fixed, devitellinized, and immunostained as previously described (Patel, 1994), with modifications as in Chapter 2. Following staining, embryos were mounted in 2.5% DABCO (Sigma) on slides and viewed with a Leica NTS or Zeiss 510 Meta confocal microscope.

The following primary antibodies were used: chicken anti-VASA (K. Howard) at 1:5,000 or 1:10,000; rabbit anti-SOX100B (S. Russell) at 1:1,000; rabbit anti-β-GAL (Cappel) at 1:10,000; mouse-anti-β-GAL (Promega) at 1:10,000; rabbit-anti-GFP (Torrey Pines) at 1:2,000; mouse anti-UBX mAb-5C.2B (V. Corces; Lopez and Hogness, 1991) at 1:50; mouse anti-ABD-A mAb Dmabd-A.1 (D. Mattson-Duncan) at 1:2,000; mouse anti-ABD-B 1AE129 (E. Lewis) at 1:50; mouse anti-EYA 10H6 (Developmental Studies Hybridoma Bank [DSHB]; N. Bonini) at 1:25; and mouse-anti-SXL M18 (DSHB; P. Schedl) at 1:25 or 1:50. The following secondary antibodies were used, all at 1:500: Cy5 goat-anti-chicken (Rockland); Alexa 594 or 568 goat anti-chicken; Alexa 594, 546, or 488 goat anti-rabbit; and Alexa goat 568 or 488 anti-mouse. All Alexa antibodies are from Molecular Probes.

Genotyping and sexing of embryos

In our experiments, we used balancer chromosomes carrying a *GFP* or *lacZ* transgene: P[*hb-lacZ*-TM3], P[*Ubx-lacZ*-TM3], P[*Kr-GFP*-CyO], or P[*Kr-GFP*-TM3], in order to distinguish homozygous mutant embryos from balancer-carrying siblings (Casso et al., 2000).

To determine sex of embryos, we used anti-SXL antibody or X-chromosomes carrying a *lacZ* or *GFP* transgene: P[*Dfd-lacZ*-HZ2.7] (Bergson and McGinnis, 1990) or P[*Kr-GAL4*,UAS-*GFP*-Fm7c] (Casso et al., 2000). When using tagged X-chromosomes, males carrying the labeled chromosome were crossed to wild type virgin females. Only female progeny contain the tagged X-chromosome whose β–GAL or GFP expression pattern is detectable by antibody staining and fluorescence microscopy.

RESULTS

msSGPs express ABD-B but not ABD-A

To investigate how the homeotic genes regulate gonad formation, we examined the expression pattern of ABD-A and ABD-B, the key homeotic factors in the posterior of the *Drosophila* embryo. ABD-A is expressed in the SGPs and also in a group of cells more ventral to the gonad, as previously described (Fig. 1A) (Boyle and DiNardo, 1995). The expression of ABD-A in SGPs is weaker than in the ventral cells, but is clearly visible using either the ABD-A antibody (inset, Fig. 3.1A) or the HCJ200 *abd-A* enhancer trap (data not shown) (Bender and Hudson, 2000). ABD-A expression in SGPs is uniform along the A-P axis of the gonad. We also find that msSGPs (identified by expression of SOX100B) do not express ABD-A (Fig. 3.1A), consistent with our previous observation that *abd-A* is not genetically required for msSGP specification (Chapter 2).

In contrast, msSGPs express high levels of ABD-B at the time of gonad formation (Fig. 3.1B, 3.1C). msSGPs are specified in PS13 in both males and females, and ABD-B is expressed in early msSGPs in both sexes (stage 12, data not shown). After gonad

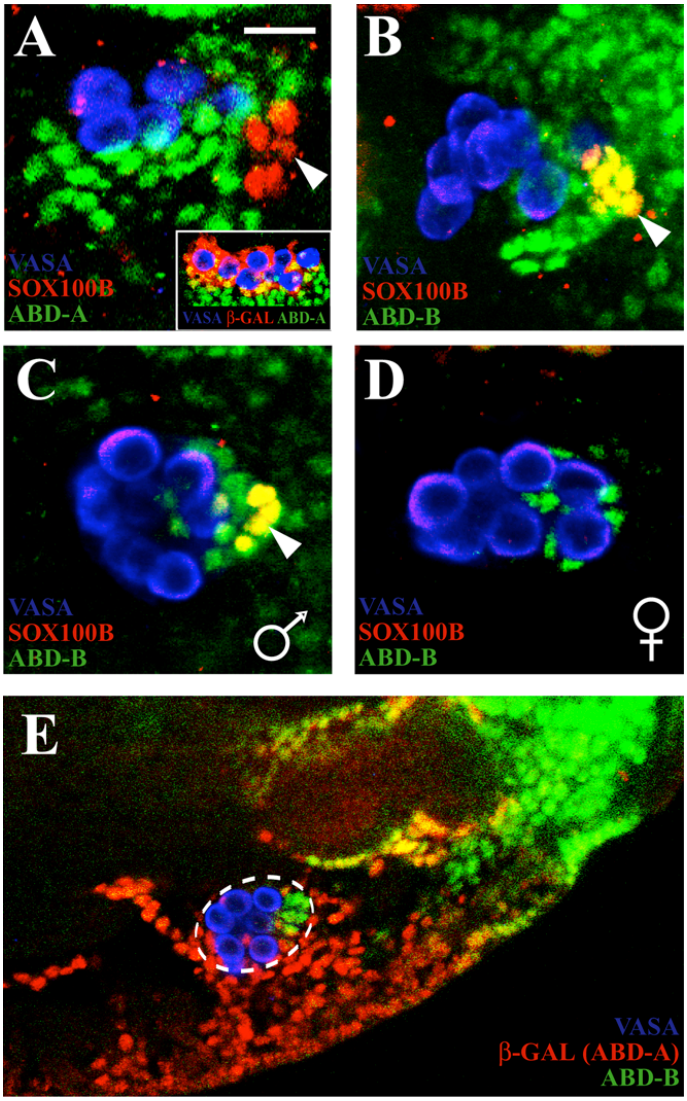
coalescence, msSGPs have joined to form the posterior portion of the male gonad, and continue to express both ABD-B and SOX100B (stage 15, Fig. 3.1C). In the female, SOX100B-positive msSGPs are no longer present, and many fewer ABD-B-positive cells are observed in the coalesced gonad (Fig. 3.1D). Thus, msSGPs express ABD-B at all stages we have examined; they arise in an embryonic region of high ABD-B expression (PS13), and maintain this expression even though the gonad will coalesce in PS10, a region where most cells express high levels of ABD-A and only low levels of ABD-B (Fig. 3.1E).

We also see ABD-B expression in posterior SGPs that is distinct from the msSGP expression (Fig. 3.1C, 3.1D) (DeLorenzi and Bienz, 1990). Posterior SGPs are observed as ABD-B-positive cells associating with germ cells in coalesced gonads in females (Fig. 3.1D), and as ABD-B-positive, SOX100B-negative cells in coalesced male gonads (Fig. 3.1C). These are likely to represent SGPs that are originally specified in PS 12, and their expression of ABD-B is consistent with a role for *Abd-B* in patterning posterior SGPs (see below).

We do not observe any role for the homeotic gene *Ubx* in initial gonad formation; we do not detect expression of UBX in SGPs or msSGPs, and Ubx^{1} mutant embryos exhibit normal SGP specification, gonad coalescence, and sexually dimorphic msSGP behavior (data not shown).

Figure 3.1. Homeotic gene expression pattern in the gonad

Immunostainings of *Drosophila* stage 13 (A,B) and stage 15 (C-E) embryonic gonads. Anterior is to left in all panels. Colors of stainings are as indicated in each panel; VASA is used to label germ cells. (A,B) Wild type (*faf-lacZ*) msSGPs (arrowheads), identified by expression of SOX100B, do not express ABD-A (A), but do express high levels of ABD-B (B). (A, inset) *68-77* enhancer trap line expressing β-galactosidase in SGPs (Simon et al., 1990). Stage 15 male (C) and female gonads (D) express ABD-B in posterior SGPs and in msSGPs (arrowhead). Although msSGPs remain at the posterior of the male gonad, ABD-B-expressing SGPs associate with germ cells similarly in males and females. (E) Wild type embryo expressing β-galactosidase from the HCJ200 *abd-A* enhancer trap. The coalesced gonad (outline) resides in PS10, an area of high ABD-A expression and low ABD-B expression. Embryos in (C,D) were sexed using a GFP-expressing X chromosome (see Experimental Procedures). Scale bar represents 10 μm in (A-D) and 20 μm in (E).


A
VASA
SOX100B
ABD-A
VASA β-GAL ABD-A
B
VASA
SOX100B
ABD-B
C
VASA
SOX100B
ABD-B
♂
D
VASA
SOX100B
ABD-B
♀
E
VASA
β-GAL (ABD-A)
ABD-B

Regulation of sexually dimorphic ABD-B expression

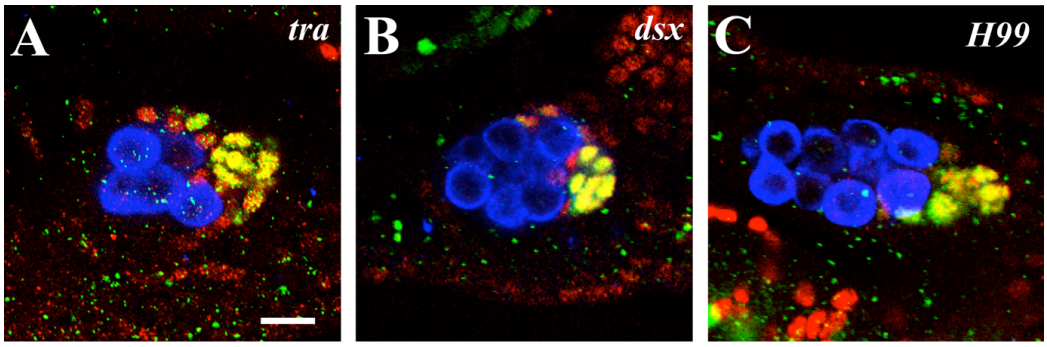
The coalesced gonad exhibits sexual dimorphism with regard to ABD-B expression: there are many more ABD-B-positive cells in the male gonad than in the female (Fig. 3.1C, 3.1D). We addressed whether this is due solely to the programmed cell death of the msSGPs in females, or if ABD-B expression is regulated independently by the sex determination pathway. Programmed cell death of msSGPs in females is regulated by the sex determination genes *tra* and *dsx*. In *tra* and *dsx* mutants, msSGPs survive in both XX and XY embryos and continue to express the msSGP marker SOX100B (Chapter 2). We find that these msSGPs are also able to express ABD-B; in 100% of *tra* (n=28) and *dsx* (n=26) mutant embryos (i.e., both XX and XY), msSGPs exhibit ABD-B expression (Fig. 3.2A, 3.2B, cf. Fig. 3.1C). Interestingly, *dsx* mutant embryonic gonads initially appear fully masculinized, as previously described (Chapter 2), despite having an intersexual phenotype in larval stages (Steinmann-Zwicky, 1994).

It is possible that expression of ABD-B in msSGPs is normally repressed in females. To test this possibility, we examined ABD-B expression in msSGPs in females where programmed cell death was blocked, by using a deficiency (H99) that removes three genes (*hid*, *grim*, and *reaper*) thought to be required for virtually all apoptosis in the *Drosphila* embryo (White et al., 1994). We have shown previously that the genes in this deficiency are required for apoptosis of msSGPs in females, and the surviving msSGPs still express SOX100B (Chapter 2). In 100% (n=25) of H99 mutant embryos (i.e., males and females), msSGPs express ABD-B in addition to SOX100B (Fig. 3.2C). Thus, ABD-B expression is not directly regulated by the sex determination pathway, and sexually

dimorphic expression of ABD-B in the gonad is a result of sex-specific programmed cell death of msSGPs.

Figure 3.2. Regulation of sexually dimorphic ABD-B expression

(A-C) Stage 15 embryonic gonads. Anterior is to left in all panels. Immunofluorescence was used to reveal VASA (blue), ABD-B (red), and SOX100B (green). (A) *tra*^{1} mutant. (B) *dsx*^{1}/*dsx*^{23} mutant. (C) H99 deficiency mutant. msSGPs are distinguishable by SOX100B/ABD-B co-expression. Scale bar represents 10 μm.


A
tra
B
dsx
C
H99

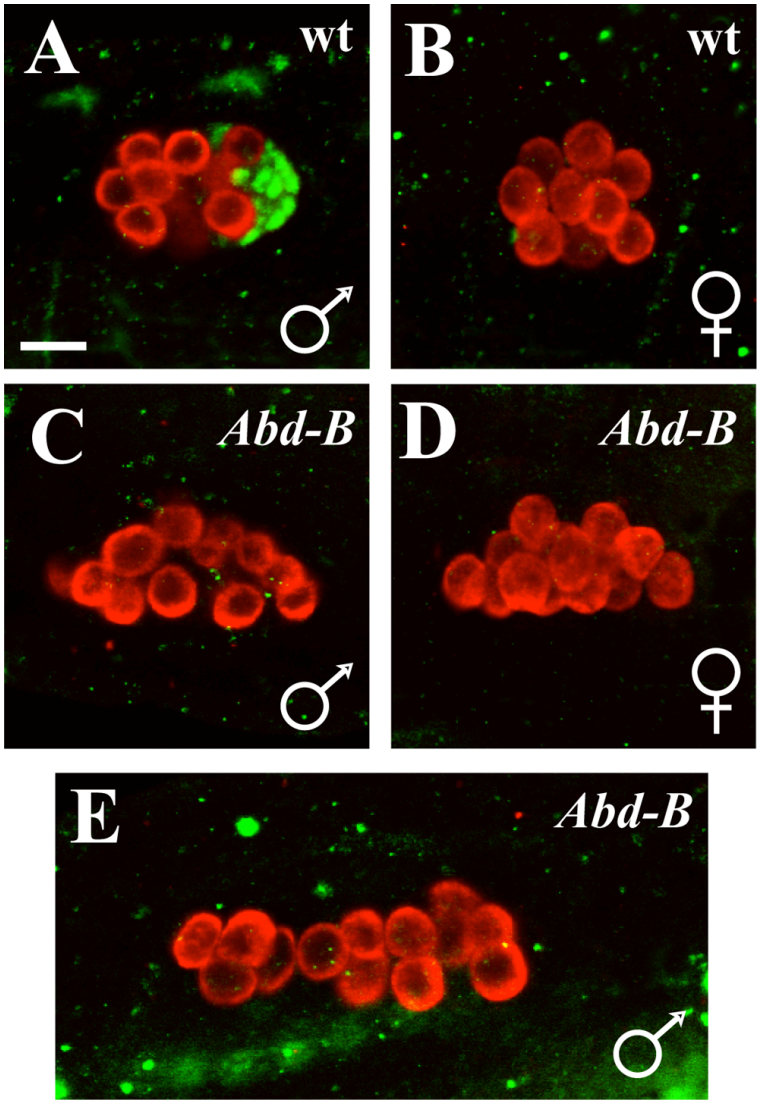
Abd-B is required for msSGP specification

Previously, it has been shown that SGPs require *abd-A* for their specification (Boyle and DiNardo, 1995; Brookman et al., 1992; Cumberledge et al., 1992), while msSGPs do not (Chapter 2). Since we observe ABD-B expression in msSGPs, we tested whether *Abd-B* is required for msSGP specification. After gonad coalescence, when msSGPs are normally present in males (Fig. 3.3A), no SOX100B expressing msSGPs are observed in *Abd-B* mutants of either sex (Fig. 3.3C, 3.3D). msSGPs are also no longer detectable by EYA expression (data not shown). In effect, sex reversal (with regard to msSGPs) has been achieved through the removal of *Abd-B* function since male gonads now lack msSGPs and resemble female gonads. Examination of the SGPs reveals that normal numbers of SGPs are specified in *Abd-B* mutants (average of 31.2 EYA-positive SGPs in *Abd-B*$^{-}$ vs. 30.2 in wild type, n=5 gonads for each genotype), and they associate with the migrating germ cells. However, the gonads are often not as tightly compacted as in wild type.

We next examined earlier stages in *Abd-B* mutants to determine if msSGPs fail to be initially specified or, instead, undergo programmed cell death in both sexes. At stage 13, when msSGPs are present in both males and females in wild type embryos, we never observe SOX100B-positive cells in *Abd-B* mutants (Fig. 3.3E; data not shown). We conclude that *Abd-B* is required for initial specification of msSGPs.

Figure 3.3. *Abd-B* is required for msSGP specification

Immunostainings of stage 15 (A-D) and stage 13 (E) embryonic gonads for VASA (red) and SOX100B (green). Anterior is to left in all panels. (A,B) Wild type *faf-lacZ* coalesced male (A) and female (B) gonads. (C,D) *Abd-B*^{M5} mutant male (C) and female (D) coalesced gonads. (E) *Abd-B*^{M5} mutant male gonad prior to gonad coalescence. Note absence of SOX100B staining in gonads in (C) and (E). All embryos were sexed with anti-SXL antibody. Scale bar represents 10 μm.


A
wt
B
wt
C
Abd-B
D
Abd-B
E
Abd-B

ABD-B is sufficient to induce msSGP specification

We also tested whether *Abd-B* is sufficient to induce msSGPs in more anterior regions of the embryo. Normally, msSGPs (identified by co-expression of EYA and SOX100B) are only specified in PS13 (Fig. 3.4A) (Chapter 2). However, if *Abd-B* is ectopically expressed throughout the mesoderm using the GAL4/UAS system (Brand and Perrimon, 1993), additional clusters of EYA and SOX100B co-expressing cells are observed in more anterior regions (Fig. 3.4B). As many as 10 total clusters of msSGPs are observed, spanning from PS4-13. Thus, each of these PS contains a subset of mesoderm that is competent for msSGP specification in the presence of ABD-B.

As has been previously shown, ectopic expression of *Abd-B* blocks the specification of SGPs, likely by repressing *abd-A* (Greig and Akam, 1995; Karch et al., 1990; Macias et al., 1990). Consequently, when *Abd-B* alone is expressed throughout the mesoderm, no gonads form and germ cells are either scattered or associated with msSGPs (Fig. 3.4B). However, when *abd-A* is co-expressed with *Abd-B* (Fig. 3.4C), we observe a rescue of EYA-positive SGPs along with the ectopically induced msSGPs. Ectopic *abd-A* also induces additional SGPs anterior to the gonad, as has previously been described (Boyle et al., 1997; Boyle and DiNardo, 1995; Greig and Akam, 1995). A large group of SGPs and msSGPs associates with most of the germ cells in the region where the gonad would normally be coalescing, and ectopic SGPs and msSGPs also associate with one another in more anterior regions. Thus, *Abd-B* alone induces ectopic msSGPs, while expression of *abd-A* and *Abd-B* together induces both SGPs and msSGPs.

To determine if the additional EYA/SOX100B co-expressing cells are truly msSGPs, we wanted to determine if these cells would join the gonad in males, and

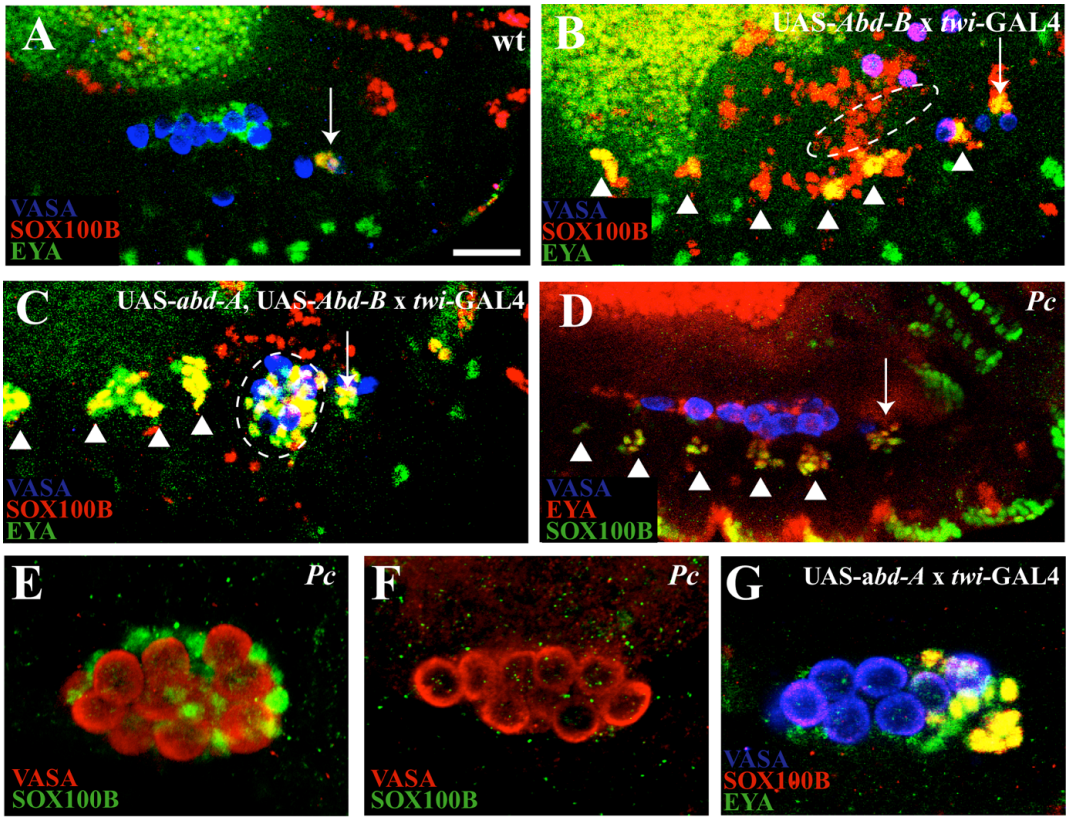
undergo programmed cell death in females, since this is the proper behavior for the endogenous msSGPs. However, since embryos of the genotypes utilized above die just before the time of gonad formation, we could not assess the later fates of the ectopic msSGPs. Instead, we examined embryos mutant for *Polycomb (Pc)*, a negative regulator of homeotic gene expression. In *Pc* mutants, ABD-A and ABD-B are ectopically expressed in anterior parasegments (Celniker et al., 1989; Simon et al., 1992) (data not shown) and ABD-A appears downregulated in the area of the gonad, but is not eliminated (data not shown). These embryos survive to late embryogenesis (Celniker et al., 1989; Kuziora and McGinnis, 1988), well beyond the time of gonad formation. In *Pc* mutants before gonad coalescence, ectopic msSGPs (EYA/SOX100B double-positive) are observed in both males and females (Fig. 3.4D), similar to what we observe using ectopic *Abd-B* expression. We also observe normal and ectopic SGP specification (EYA-positive only cells), likely due to remaining ABD-A present in this background. At later stages in males, a large number of ectopic msSGPs associate with the SGPs and germ cells to form a gonad (Fig. 3.4E; data not shown). In contrast, in most female embryos, msSGPs are no longer observed at later stages (Fig. 3.4F), although a few SOX100B-positive cells persist in some embryos and associate with germ cells. This suggests that the ectopic msSGPs in females undergo programmed cell death similar to endogenous msSGPs. Thus, the additional EYA/SOX100B-expressing cells induced by ectopic ABD-B behave as msSGPs in both males and females.

The above data also indicate that expression of *abd-A* does not inhibit msSGP formation, even though msSGPs normally do not express ABD-A (Fig. 3.1). To confirm this observation, we over-expressed *abd-A* alone to determine the effects on endogenous

msSGP specification. Under these conditions, no change in the msSGPs is observed: these cells are still present, and join the posterior of the male gonad as in wild type (Fig. 3.4G). At earlier stages, msSGPs are observed in both males and females under these conditions, and disappear in females (data not shown). Over-expression of *abd-A* does induce ectopic SGP formation, seen as additional EYA-positive cells anterior to the normal position of the gonad, as has been observed previously with other SGP markers (Boyle et al., 1997; Boyle and DiNardo, 1995; Greig and Akam, 1995). We conclude that msSGPs are regulated by *Abd-B* alone, and are not affected by mutations in *abd-A* (Chapter 2) or forced expression of *abd-A*.

Figure 3.4. *Abd-B* is sufficient to specify msSGPs

Immunostainings of embryonic gonads. Anterior is to left in all panels. Colors of stainings are as indicated in each panel. (A) Stage 13 wild type *faf-lacZ* with EYA/SOX100B co-expressing msSGPs (arrow). (B) Stage 13 embryo expressing *Abd-B* throughout the mesoderm, with ectopic clusters of EYA/SOX100B co-expressing cells in anterior segments (arrowheads) in addition to PS13-derived msSGPs (arrow). Outline denotes absence of gonad in this background. (C) Stage 13 embryo expressing both *abd-A* and *Abd-B* throughout the mesoderm, with ectopic SGP/msSGP clusters (arrowheads) and PS-13 derived msSGPs (arrow). Outline denotes region where gonad would normally be forming. (D) Stage 13 Pc^{3}/Pc^{XT109} mutant, with ectopic msSGP clusters in anterior segments (arrowheads) and PS13-derived msSGPs (arrow). (E,F) Stage 15 Pc^{3}/Pc^{XT109} mutant male (E) and female (F) gonads. (G) Stage 15 male embryo expressing *abd-A* throughout the mesoderm. Embryos in (E,F) were sexed with anti-SXL antibody. Scale bar represents 23 μm in (A-D) and 10 μm in (E-G).


A
wt
VASA
SOX100B
EYA
B
UAS-Abd-B x twi-GAL4
VASA
SOX100B
EYA
C
UAS-abd-A, UAS-Abd-B x twi-GAL4
VASA
SOX100B
EYA
D
Pc
VASA
EYA
SOX100B
E
Pc
VASA
SOX100B
F
Pc
VASA
SOX100B
G
UAS-abd-A x twi-GAL4
VASA
SOX100B
EYA

Regulation of SGP identity by *abd-A* and *Abd-B*

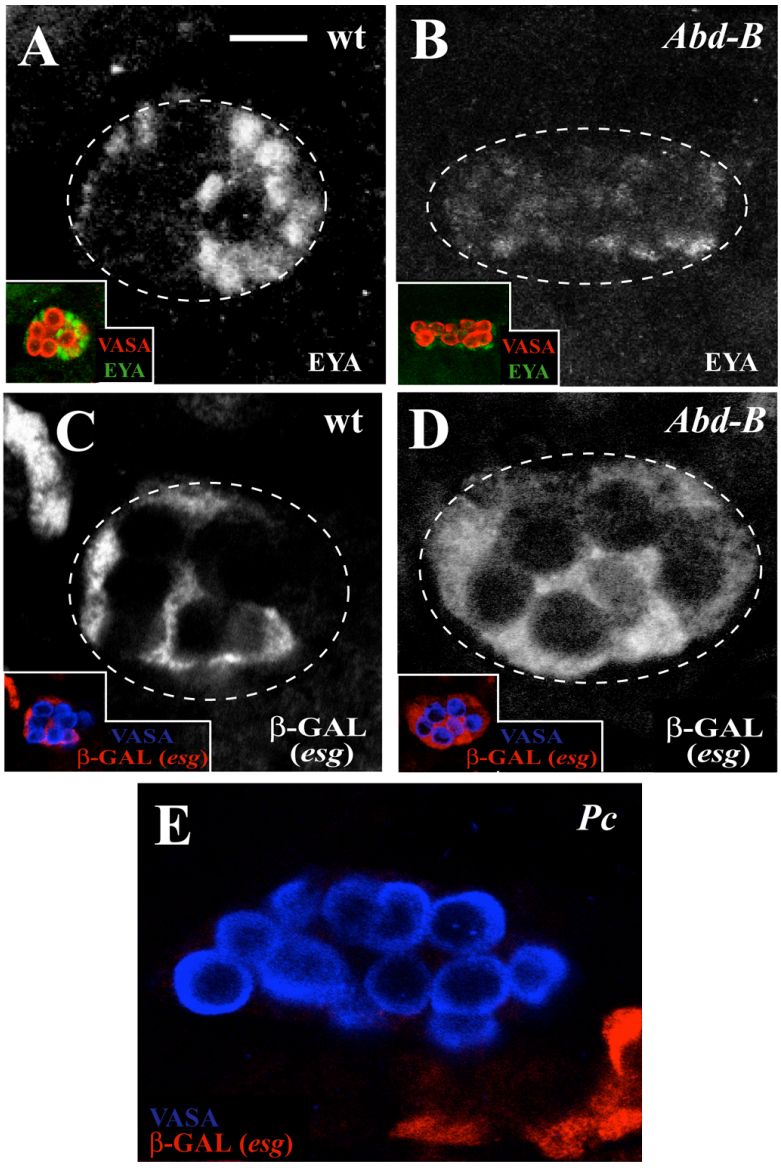
Abd-B is also likely to play a critical role in patterning the SGPs. Boyle and DiNardo (1995) have previously proposed a model in which anterior SGP identity is specified by *abd-A* alone, while posterior SGP identity is specified by the combination of *abd-A* and *Abd-B*. These original studies were conducted prior to the discovery of the msSGPs, and since the SGPs and msSGPs express many markers in common, it was not possible to distinguish between SGPs and msSGPs in those experiments. Since *Abd-B* is critical for specification of the msSGPs (above), it is important to verify that *Abd-B* also has an independent role in specifying posterior SGPs. After gonad formation, EYA is expressed at higher levels in posterior SGPs than in anterior SGPs (Boyle et al., 1997). We find that this is true even in females (Fig. 3.5A), where no msSGPs are present after gonad formation to interfere with analysis of the SGPs. In *Abd-B* mutant embryos, EYA is no longer expressed at higher levels in posterior SGPs (Fig. 3.5B), and these cells express a low level of EYA similar to that observed in anterior SGPs. Thus, independent of its effects on the msSGPs, *Abd-B* also plays a role in specifying posterior vs. anterior SGP identity, strongly supporting the model of Boyle and DiNardo (1995).

This model makes two further predictions that we have tested. First, *Abd-B* should be completely dispensable for anterior SGP identity. *escargot (esg)* is a marker for anterior SGP identity (Gonczy et al., 1992), and we utilized an enhancer trap in the *esg* locus as a reporter for *esg* expression (Whiteley et al., 1992). This marker is expressed in SGPs in the anterior region of the coalesced gonad in wild type (Fig. 3.5C). In an *Abd-B* mutant background, this marker is still expressed in the anterior gonad, indicating that *Abd-B* is not required for anterior SGP identity (Fig. 3.5D). We also find gonads in which *esg* is now expressed more posteriorly throughout the entire gonad,

suggesting that more of the gonad now has adopted an anterior fate. The model also predicts that ectopic expression of *Abd-B* in the anterior should repress anterior SGP identity. We again used the *Pc* mutant background to examine gonads in which ABD-A and ABD-B were broadly co-expressed. In *Pc* mutants, the *esg* reporter is no longer expressed in the gonad (Fig. 3.5E), suggesting that ectopic ABD-B is indeed capable of repressing anterior SGP identity.

Figure 3.5. Regulation of SGP identity by *Abd-B*

(A-E) Immunostainings of stage 15 embryonic gonads. Anterior is to left in all panels, and outline denotes boundary of gonad. (A,B) EYA expression in a wild type *faf-lacZ* female (A), with increased expression in posterior SGPs, and in an $Abd\text{-}B^{M5}$ mutant (B), with no increase of EYA in posterior SGPs. (A,B, insets) Same gonads showing both the VASA (red) and EYA (green) channels. (C,D) *esg-lacZ* β-GAL expression in a wild type (C) and $Abd\text{-}B^{M5}$ mutant (D) gonad. (C,D, insets) Same gonads showing both the VASA (blue) and β-GAL (red) channels. (E) Pc^{3}/Pc^{XT109} mutant gonad stained for VASA (blue) and *esg-lacZ* β-GAL (red). Progeny of *esg-lacZ* males and w^{1118} females were used for wild type in (C), and sex of embryo in (A) was determined by using a β-GAL-expressing X chromosome (see Experimental Procedures). Scale bar represents 10 μm in (A-E) and 37 μm in insets.


A
wt
VASA
EYA
EYA
B
Abd-B
VASA
EYA
EYA
C
wt
VASA
β-GAL (esg)
β-GAL (esg)
D
Abd-B
VASA
β-GAL (esg)
β-GAL (esg)
E
Pc
VASA
β-GAL (esg)

DISCUSSION

We have analyzed the role of the homeotic genes in establishing distinct identities amongst the cells that make up the somatic portion of the gonad. This includes the specification of a unique cell type, the msSGPs, which produces the earliest known difference between the male and female somatic gonad. The gonad provides a fascinating example of how the homeotic genes can be used at different stages, and in different combinations, to create the cellular diversity required to form a single organ.

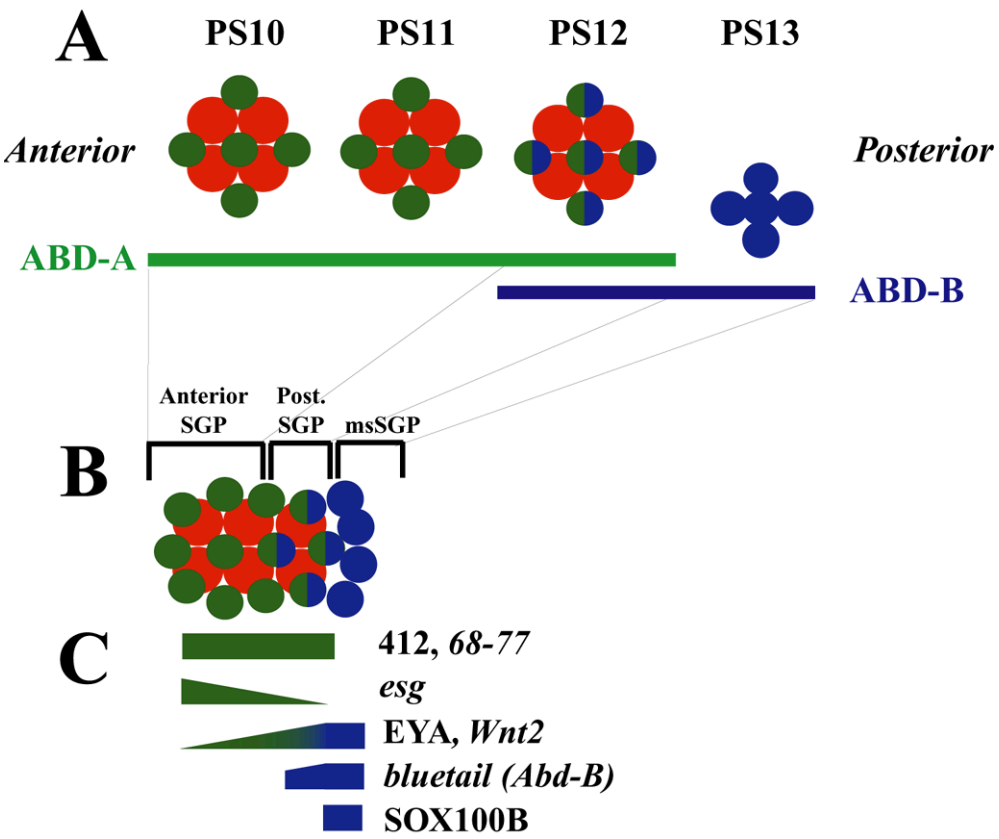
Homeotic genes and gonad development

Specification of SGPs and msSGPs. The homeotic genes initially work to specify the distinct types of somatic cells that will contribute to the gonad. We have demonstrated that *Abd-B* is necessary for the specification of msSGPs in PS13, and is sufficient to induce msSGP clusters in ectopic positions. Thus, *Abd-B* appears to restrict msSGP development to PS13. Consistent with this idea, the anterior limit *of Abd-B* expression is initially in PS13, and only later extends into more anterior regions (Boulet et al., 1991; Celniker et al., 1989; DeLorenzi and Bienz, 1990; Kuziora and McGinnis, 1988).

In a similar manner, *abd-A* is required for the specification of SGPs in PS10, 11 and 12. *abd-A* acts to promote SGP development by blocking *srp* and fat body development in these parasegments (Moore et al., 1998a; Riechmann et al., 1998). *abd-A* is also sufficient to induce ectopic SGPs when expressed in more anterior regions (Boyle et al., 1997; Boyle and DiNardo, 1995; Greig and Akam, 1995). Thus, the first stage where the homeotic genes act in patterning the somatic gonad is in restricting SGP and msSGP development to their proper parasegments.

Figure 3.6. A model for homeotic gene regulation of cell identity in the somatic gonad

Red cells represent germ cells, while green color represents SGPs with ABD-A expression and blue color represents SGPs with ABD-B expression. (A) Expression of ABD-A and ABD-B in the precursors of the somatic gonad, spread out over PS10-13 at stage 11-12, prior to gonad coalescence. (B) A stage 15 coalesced embryonic gonad. The somatic gonad can be divided into three cell types: anterior SGPs, posterior SGPs, and msSGPs. (C) Cellular markers are differentially expressed in anterior and posterior gonadal cell types. Bars represent uniform expression throughout an area of the gonad, while wedges represent differences in expression along the A-P axis. Markers are as indicated in text, except for 412 retrotransposon, which marks SGPs (Brookman et al., 1992).


A
PS10
PS11
PS12
PS13
Anterior
Posterior
ABD-A
ABD-B
Anterior SGP
Post. SGP
msSGP
B
C
412, 68-77
esg
EYA, Wnt2
bluetail (Abd-B)
SOX100B

Patterning SGP identity. The homeotic genes next act to pattern distinct identities within the somatic gonad (Fig. 3.6). *abd-A* alone specifies anterior SGP identity, a combination of *abd-A* and *Abd-B* specifies posterior SGP identity, and *Abd-B* alone is required to specify msSGP identity. This role for the homeotic genes is greatly facilitated by the fact that the cells of the somatic gonad are originally specified in four different parasegments of the embryo, allowing these cells to acquire unique homeotic gene expression profiles, or Hox codes, that will determine A-P identities. These Hox codes are maintained as the SGPs and msSGPs move anteriorly and coalesce with the germ cells to form a gonad in PS10, as clearly evidenced by the maintenance of ABD-B expression in the msSGPs and posterior SGPs in the coalesced gonad (Fig. 3.1).

The precursors for the dorsal vessel, the *Drosophila* heart, are similarly specified in separate parasegments (4-13), allowing distinct identities to be patterned along the A-P axis by *Ultrabithorax, abd-A,* and *Abd-B* (Lo et al., 2002; Lovato et al., 2002; Ponzielli et al., 2002). This is also true in other tissues, such as the visceral mesoderm and fat body (Bienz, 1994; Marchetti et al., 2003). Thus, it is a common theme that organ precursors are specified in a spatially segregated manner, allowing the cells to acquire distinct identities that are preserved during organogenesis.

Sexual dimorphism. The last stage where homeotic genes act is in the development of sexual dimorphism in the gonad. The unique identity of the msSGPs, provided in part by *Abd-B*, allows these cells to behave differently in males and females. In males these cells join the posterior of the coalescing gonad, while they are removed by programmed cell death in the female (Chapter 2). Furthermore, we have found that the anterior SGPs also

behave differently in males vs. females (SLB and MVD, unpublished), indicating that the unique SGP identity conferred by *abd-A* also allows cells to respond differently to distinct sexual identities. How cell identity in the gonad, regulated by the homeotic genes, interacts with the sex determination pathway to produce distinct outputs is a fascinating area for future study.

The relationship between *dsx* and *Abd-B*

There appears to be a common regulatory link between cell types specified by *Abd-B* and sex-specific regulation by the sex determining gene *dsx*. We have shown that *Abd-B* is critical for specifying msSGP identity (this work) and that *dsx* is critical for causing these cells to behave differently in males and females (Chapter 2). The *head involution defective (hid)* gene is essential for female-specific programmed cell death of the msSGPs (Chapter 2), and is a candidate for being differentially regulated by *Abd-B* and *dsx* in the two sexes.

A similar relationship between *Abd-B* and *dsx* has been observed in several other examples. It has been shown that these genes interact to control the pattern of sex-specific pigmentation in the *Drosophila* abdomen, and that *bric à brac (bab)* integrates positional and sexual inputs in this tissue (Kopp et al., 2000). The combination of *Abd-B* and female identity allows *bab* to act in blocking pigment formation, whereas in males, *Abd-B* can repress *bab* in order to allow pigment formation to occur.

Abd-B and *dsx* also cooperate in sex-specific development of the genital disc, which gives rise to the non-gonadal structures that must eventually join with the gonad to form the functional adult reproductive system. *Abd-B* and *dsx* act through the signaling

molecules *wingless* and *decapentaplegic* to pattern the genital disc, and through the FGF ligand *branchless* to regulate mesodermal cell migration into the disc (Ahmad and Baker, 2002; Estrada and Sanchez-Herrero, 2001; Keisman and Baker, 2001; Sanchez et al., 2001). The expression of a key regulator of genital disc development, *dachshund,* has been shown to be affected by both *Abd-B* and *dsx* (Estrada and Sanchez-Herrero, 2001; Keisman and Baker, 2001; Sanchez et al., 2001).

Thus, *Abd-B* and *dsx* are used in combination to pattern several independent tissues during development. Other cell-type-specific factors must be involved, since these tissues exhibit distinct responses to *Abd-B* and *dsx*. However, *Abd-B* and *dsx* clearly form a common regulatory network used multiple times in development to create sexual dimorphism.

Abd-B may be a conserved factor regulating somatic gonad development

Data from studies on *C. elegans* and mice suggest that regional identities conferred by homeotic genes are required for the proper development and sexual dimorphism of the gonad in these species. An *Abd-B* homolog in *C. elegans*, *egl-5,* is expressed in the somatic gonad and is required for SGP development (Chisholm, 1991; Ferreira et al., 1999). Furthermore, in a certain percent of *egl-5* mutant males it appears as if the somatic gonad takes on a hermaphrodite-like morphology (Chisholm, 1991). This sex-specific phenotype may be analogous to what we see in *Drosophila*, in which *Abd-B* mutant male gonads take on a partial female phenotype (as characterized by an absence of msSGPs). Due to a great deal of gene expansion in the mammalian homeotic complex resulting in potential gene redundancy or overlapping function, it may prove

difficult to find a single mouse gene with a similar phenotype to *Abd-B* or *egl-5*. However, *Hoxa10* male knockout mice exhibit blocks in spermatogenesis, while the female gonad can produce functional eggs (Satokata et al., 1995), demonstrating a sexually dimorphic role for posterior Hox genes in mouse gonad development.

In addition, studies of the *Polycomb* (*Pc*) group of homeotic regulators are also consistent with a role for homeotic genes in establishing sexual dimorphism. *C. elegans Pc* homologs *mes-2, mes-3, and mes-6* have been shown to regulate homeotic gene expression, in particular *egl-5* (discussed above) and *mab-5*, the latter of which is necessary for sexually dimorphic male V-ray sense organs (Ross and Zarkower, 2003). Knockouts of the mouse *Pc* homolog *M33* have altered expression of *Hox* genes resulting in sterility and male-to-female sex reversal (Katoh-Fukui et al., 1998).

These results indicate that the regulation of homeotic gene expression is important for gonad development and sexual dimorphism in diverse organisms. Although methods of initial sex determination have widely diverged among animal species, many lines of evidence strongly suggest that mechanisms to promote sexual dimorphism in the gonad are conserved. Positional information provided by the homeotic genes is likely to be a key conserved element in creating sexual dimorphism.

CHAPTER 4

The sex determination pathway regulates *Wnt2* signaling in order to specify sexually dimorphic pigment cells

SUMMARY

The adult testis and ovary of *Drosophila* are highly specialized organs that function to support sex-specific gametogenesis. While they differ greatly in their morphology, not much is known about sexual dimorphism in the embryonic stages of gonad formation. We have examined the embryonic gonad and have characterized a sexually dimorphic cell type which ensheaths the male gonad and expresses Sox100B, a *Drosophila* homolog of *Sox9*, a factor involved in mammalian sex determination. Results indicate that these cells are the precursors to adult testis pigment cells (PCs), which are important for proper adult testis development. PC precursors express fat body markers and require *serpent* for their specification, suggesting that they are recruited to the gonad from the neighboring fat body. The signaling molecule Wnt2 is responsible for sexually dimorphic PC specification, regulated by the *doublesex* branch of the somatic sex determination pathway. Ectopic Wnt2 is sufficient to induce PCs in female embryos, suggesting that a cell non-autonomous mechanism regulates PC formation. Our results indicate that secondary sex determination plays a role in *Drosophila* gonad sexual dimorphism, and that gonad formation in flies shows many similarities to what is observed in the mammalian system.

INTRODUCTION:

Sexual dimorphism is a result of events that are downstream of initial sex determination, which renders a male or female sexual identity to cells in the organism. The initial sex determination "switch" that launches sex-specific differentiation can be a genetic or environmental mechanism, and varies widely in the animal kingdom. In *Drosophila*, the X chromosome to autosome (X:A) ratio is the trigger for male or female development. An X:A ratio of 1 (e.g., in XX female embryos) turns on *Sex lethal* (*Sxl*), which is responsible for initiating the cascade of regulatory events that will promote female differentiation, including the induction of Transformer (TRA), a splicing factor that acts in conjunction with Transformer-2 (TRA-2) to sex-specifically splice the transcript of the *doublesex* (*dsx*) gene. In the presence of Tra, *dsx* pre-mRNA is spliced into the female isoform encoding the DSX-F transciption factor, however, in the absence of TRA or TRA-2, *dsx* pre-mRNA is spliced in the default male-specific manner to encode for the DSX-M protein (Burtis and Baker, 1989). DSX transcription factors are thought to be activators and repressors for genes that are responsible for establishing sexual dimorphism, however, the only characterized transcriptional target of DSX are the *yolk protein* (*yp*) genes, which are expressed in the female fat body and ovary, and are repressed in the male (Burtis et al., 1991; Coschigano and Wensink, 1993). In *dsx* mutant adults, an intersex phenotype results, in which somatic tissues contain aspects of both male and female development (Baker and Ridge, 1980; Hildreth, 1965). Some aspects of central nervous system development have been shown to be *dsx*-independent (Finley et al., 1997; Taylor, 1992).

The manner in which switches act to establish sexual dimorphism also varies in different sex determination systems. Specifically, the induction of sexual fate can be controlled either cell-autonomously or non-autonomously. In cell-autonomous sex determination, which is the mechanism for primary sex determination in the mammalian somatic gonad, cells independently assess their sexual identity. Experiments with chimeric mice composed of XX (female) and XY (male) cells showed that Sertoli cell precursors were the only cells that were predominantly XY (Burgoyne et al., 1988; Palmer and Burgoyne, 1991). This result indicates that only Sertoli cells sense the presence of *Sry* and therefore all other cells undergo non-autonomous sex determination. In non-autonomous sex determination, the genetic or chromosomal sexual makeup of a cell is subordinate to a signal received from the external environment. Sexual dimorphism in all mammalian non-gonadal tissues is thought to be mediated via a non-autonomous mechanism, termed secondary sex determination. Experiments by Jost showed that all gonadectomized fetal rabbits develop as females (Jost, 1953), revealing the gonad as a source of a signal that is necessary to promote sexually dimorphic development, which is now known to be hormones that control secondary sex characteristics in all extra-gonadal tissues. Some gonadal tissues in the mouse also undergo non-autonomous sex determination, as the migration of mesonephric cells into the gonad depends on the presence of *Sry* in the gonad (Capel et al., 1999; Martineau et al., 1997). It is thought that most *Drosophila* tissues undergo a cell-autonomous sex determination mechanism (Baker and Ridge, 1980), however, non-autonomous signaling may play a role in regulating sexual dimorphism in flies. In the central nervous system, it has been demonstrated that male neurons can induce female muscle precursors to become

the male-specific muscle of Lawrence (Lawrence and Johnston, 1986). It also has been shown that a male genital disc can cause female cells to produce male pigment when transplanted into a female host (Fung and Gowen, 1957).

The *Drosophila* embryonic gonad, like in most animals, is formed from the association of germ cells with mesodermal cells termed somatic gonadal precursors (SGPs), which must be sexually dimorphic to support spermatogenesis and oogenesis. We have previously shown that the gonad is already sexually dimorphic at the time of its initial formation, as male-specific SGPs (msSGPs) only join the male gonad and die by apoptosis in females (Chapter 2). msSGPs express SOX100B, a homolog of Sox9, a factor required for human and mouse sex determination and which has been considered an ancestral sex determination gene. *SOX9* heterozygous humans show frequent XY sex reversal (an XY karyotype with a female phenotype), while duplications of *SOX9* have been implicated in XX sex reversal (Foster et al., 1994; Huang et al., 1999; Wagner et al., 1994). Consistent with a hypothesis that *Sox9* plays a conserved role in sex determination, *Sox9* is expressed sex-specifically in the testis in a wide array of animal species, such as human, mouse, chick, turtle, and trout (de Santa Barbara et al., 2000; Kent et al., 1996; Moreno-Mendoza et al., 1999; Takamatsu et al., 1997). Given its expression pattern and role in mammalian sex determination, SOX100B is likely a good marker for further studying sexual dimorphism in the *Drosophila* gonad.

In addition to msSGPs, there is evidence for sexual dimorphism in both SGPs and germ cells soon after initial gonad formation by stage 15 (stages as in (Campos-Ortega and Hartenstein, 1985)). By the beginning of the first instar larval stage, male gonads are larger than female gonads (Kerkis, 1931), and it has been shown that the number of germ

cells initially incorporated into the gonad is higher in males than females (Poirié et al., 1995). The expression of *male germ-line-marker-1* (*mgm1*) is sex-specific in germ cells, and is dependent on the somatic sex of the embryo (Staab et al., 1996). In addition, germ cell division is male-specific in the embryonic gonad, mediated by signals from the male soma (Wawersik et al., 2005). During stage 17, we have also determined that hub cells, which will form part of the testis germline stem cell niche, are specified in embryonic male gonads (S. Le Bras and M. Van Doren, submitted), demonstrating that the properties of the male and female SGPs are already distinct by the end of embryogenesis.

While germ cells will give rise to sperm or oocytes, the SGPs will give rise to multiple adult cell types that are crucial for sex-specific testis morphogenesis and fertility (Fuller, 1993), although it is currently unclear which subsets of SGPs give rise to those adult testis cell types. In the anterior of the adult testis resides the germline stem cell (GSC) niche, a cluster of 8-12 somatic cells (Hardy et al., 1979), which is required for maintaining GSCs in an undifferentiated state via JAK/STAT and BMP signaling from niche cells (Kawase et al., 2004; Kiger et al., 2001; Tulina and Matunis, 2001). Throughout spermatogenesis, germ cells are encased by a pair of somatic cyst cells, which are maintained by a somatic stem cell population adjacent to the GSC niche (Gonczy and DiNardo, 1996). Upon the completion of spermatogenesis, the cyst of developing sperm interacts with the terminal epithelium, a tissue at the base of the testis that aids in final sperm differentiation by degrading the cyst cells and waste bag that holds discarded sperm cytoplasm (Tokuyasu, 1974). A layer of muscle cells, which is derived from the genital disc (Kozopas et al., 1998), ensheaths the entire length of the testis and is thought to be essential for proper gonad-genital disc interactions. Over the

muscle layer are pigment cells, which represent the outermost cell layer of the testis. Pigment cells are thought to be required for adult testis morphogenesis as a substrate for muscle sheath migration via a *Wnt2*-mediated mechanism; in *Wnt2* mutant adults, pigment cells are absent and the muscle sheath fails to cover the entire testis (Kozopas et al., 1998). The fat body, an adipose tissue believed to be the equivalent of the mammalian liver, also is intimately associated with the gonad and envelops it throughout development (Kerkis, 1931). However, it is not known what specific role the fat body plays in gonad development and morphogenesis.

In this study, we continue our examination of the establishment of sexual dimorphism in the *Drosophila* gonad. We show that two sexually dimorphic cell types in the embryonic gonad give rise to testis terminal epithelium and pigment cells, demonstrating that testis tissues are already well-defined during embryogenesis. We also show that nearby fat body cells are recruited to the gonad in a sexually dimorphic manner, dependent on the signaling factor Wnt2, in order to initiate one aspect of male-specific gonad development. Furthermore, the sexual dimorphism of pigment cells is regulated via a non-autonomous mechanism in which the sex of the cells is not critical for their sexually dimorphic fate, similar to what is seen in a subset of the mammalian testis. These data suggest that the *Drosophila* gonad undergoes secondary sex determination, and that regulation of sexual dimorphism in flies shares many features with mechanisms observed in mammals.

MATERIALS AND METHODS

Fly Stocks

The following stocks were used: *HCJ199*(*ry*$^{-}$) (W. Bender) (Bender and Hudson, 2000), *Wnt2*^{1}, *Wnt2*^{O}, *svp*^{07842} (*svp-lacZ*), *srp*^{3}, *ems*^{1}, *tra*^{1}, *dsx*^{1}, *dsx*^{23}, Df(3L)*H99*, UAS-*GAL4*12B, UAS-*GFP.nls*8, UAS-*GFP.nls*14, UAS-*tra*^{F}-20J7, UAS-*dTCFΔN* (J.Treisman) (van de Wetering et al., 1997), UAS-*sgg*^{S9A} (J. Treisman) (Hazelett et al., 1998), *even-skipped stripe3+7*-GAL4 (S. Small), *paired*-GAL4 (Brand and Perrimon, 1993), *nanos 3'UTR*::VP16-GAL4 (*nos*-GAL4) (Van Doren et al., 1998), *lacZ*[857] (M.Fuller via E. Matunis), *lacZ*[B-57] (E. Matunis). *Kr*-GAL4, UAS-*GFP* used was present on both TM3 and CyO balancer chromosomes (TM3, P[GAL4-*Kr*.C]DC2, P[UAS-*GFP*.S65T]DC10 and CyO, P[GAL4-*Kr*.C]DC3, P[UAS-*GFP*.S65T]DC7) (Casso et al., 2000) and showed similar gonad expression with both chromosomes. Lineage tracing using the UAS-*GAL4* construct has been successfully used in the nervous system (Hassan et al., 2000). *w*^{1118} was used as a wild-type control. Information about unspecified stocks can be found on Flybase (www.flybase.org).

Antibody Stainings:

Embryos were fixed and stained as described in Chapter 2. Stage 17 embryos were subjected to a single three-second pulse with a Branson Sonifier 250 in order to facilitate antibody penetration through cuticle. Following staining, embryos were mounted in 2.5% DABCO (Sigma) and mounted on slides for imaging on a Zeiss 510 Meta confocal microscope.

Adult testes were dissected in PBS, followed by a 30-minute room temperature fixation in 4.5% formaldehyde in PBS containing 0.1% Triton X-100 (PBTx). After several washes in PBTx, testes were blocked for 60 minutes in PBTx containing 5% normal goat serum (BBTx). Testes were then incubated with primary antibodies overnight at 4 degrees C. After several washes in PBTx, testes were blocked with BBTx for 60 minutes. Secondary antibodies were incubated with testes for 2 hours at room temperature, followed by several PBTx washes. Testes were then rinsed twice with PBS and mounted in 2.5% DABCO on slides.

The following antibodies were used: chicken anti-VASA (K.Howard) at 1:5,000 or 1:10,000; rabbit anti-VASA (R. Lehmann) at 1:10,000; rabbit anti-SOX100B at 1:1,000 (S. Russell); mouse anti-ABD-B 1A2E9 (Development Studies Hybridoma Bank: DSHB) at 1:50; mouse anti-EYA 10H6 (DSHB) at 1:25; rabbit anti-GFP (Torrey Pines) at 1:2,000; mouse anti-GFP (Santa Cruz) at 1:50; mouse anti-FAS3 7G10 (DSHB) at 1:30; rabbit anti-β-GAL (Cappel) at 1:10,000; mouse anti-β-GAL (Promega) at 1:10,000; mouse anti-SXL M18 (DSHB); and rabbit anti-SRP (R. Reuter) at 1:1,000.

The following secondary antibodies were all used at 1:500: Cy5 goat anti-chicken (Rockland), Alexa 546 goat anti-chicken, Alexa 546 or 488 anti-rabbit, and Alexa 546 or 488 goat anti-mouse. All Alexa antibodies are from Molecular Probes.

In situ hybridization:

Embryos were fixed as decribed in Chapter 2. Whole-mount in situ hybridization with digoxigenin-labeled riboprobes was performed as in Chapter 2, with HNPP/Fast Red (Roche) used as a fluorescent substrate for the alkaline phosphatase reaction. The *Wnt2*

antisense riboprobe was synthesized by digesting plasmid pBSDWnt2 (a gift from R. Nusse) with NotI and transcribing with T3 RNA polymerase (Promega) using digoxigenin-labeled UTP (Boehringer-Mannheim). The *dsx* antisense riboprobe was synthesized by cutting cDNA GH08308 in pOT2 vector (BACPAC resources at Children's Hospital Oakland Research Institute) with MluI and transcribing with Sp6 RNA polymerase (Promega). The template for *ems* antisense riboprobe was created via PCR on wild-type genomic DNA and TOPO TA cloning into pCR2.1 vector (Invitrogen). Primers used were: emsF: GATCGCCGTAGAGCACAAGTC and emsR: CTTGAGAGAGAAGTGTGGATG. Template was then digested with NcoI and transcribed with T7 RNA polymerase (Promega). Antibody staining followed after in situ developing reaction, with protocol above, except with blocking steps and normal goat serum omitted.

Genotyping and sexing of embryos:

In these experiments, *GFP* or *lacZ*–expressing balancer chromosomes were used to distinguish homozygous mutant embryos from balancer-containing heterozygous siblings. To determine sex of embryos, we used a female-specific anti-SXL antibody or X chromosomes containing a *lacZ* transgene *Dfd-lacZ*-H2.7 (Bergson and McGinnis, 1990). When using *Dfd-lacZ*, males carrying the *Dfd-lacZ* X chromosome were crossed to wild-type virgin females. Only female progeny of this cross will possess the *lacZ*-expressing X chromosome whose β-GAL expression pattern is detectable via antibody staining and fluorescence microscopy.

RESULTS:

Two male-specific SOX100B-expressing cell types are present in the embryonic gonad

Using an antibody for the SOX9 homolog SOX100B, which marks msSGPs in the stage 15 male gonad, we looked for sexual dimorphism during the last stage of embryogenesis (stage 17) when further male- and female-specific differences may be observed. Whereas anti-SOX100B antibody does not label any cells in the female gonad (Fig. 4.1A), we observe a SOX100B-positive population of cells around the male gonad (Fig. 4.1B). These cells have nuclei with a flattened morphology, ensheath the outside of the gonad, and appear to be distinct from msSGPs. Given the common expression of SOX100B in msSGPs and in the ensheathing cells, it is possible that msSGPs give rise to the ensheathing population of cells. Therefore, we looked at expression of embryonic msSGP markers in the stage 17 gonad to compare gene expression profiles of the two cell types. While msSGPs maintain expression of Eyes Absent (EYA) and Abdominal-B (ABD-B), the ensheathing population of SOX100B-positive cells does not express either of these msSGP markers (Fig. 4.1C,D). In addition, we find that a *Kr*-GAL4, UAS-*GFP* construct present on balancer chromosomes is expressed male-specifically in the stage 17 gonad, co-expresses with SOX100B around the outside of the gonad (although not always in every cell at this stage), and does not express in msSGPs (Fig. 4.1E,F). Therefore, we conclude that there is a molecular difference between msSGPs and stage 17 ensheathing cells.

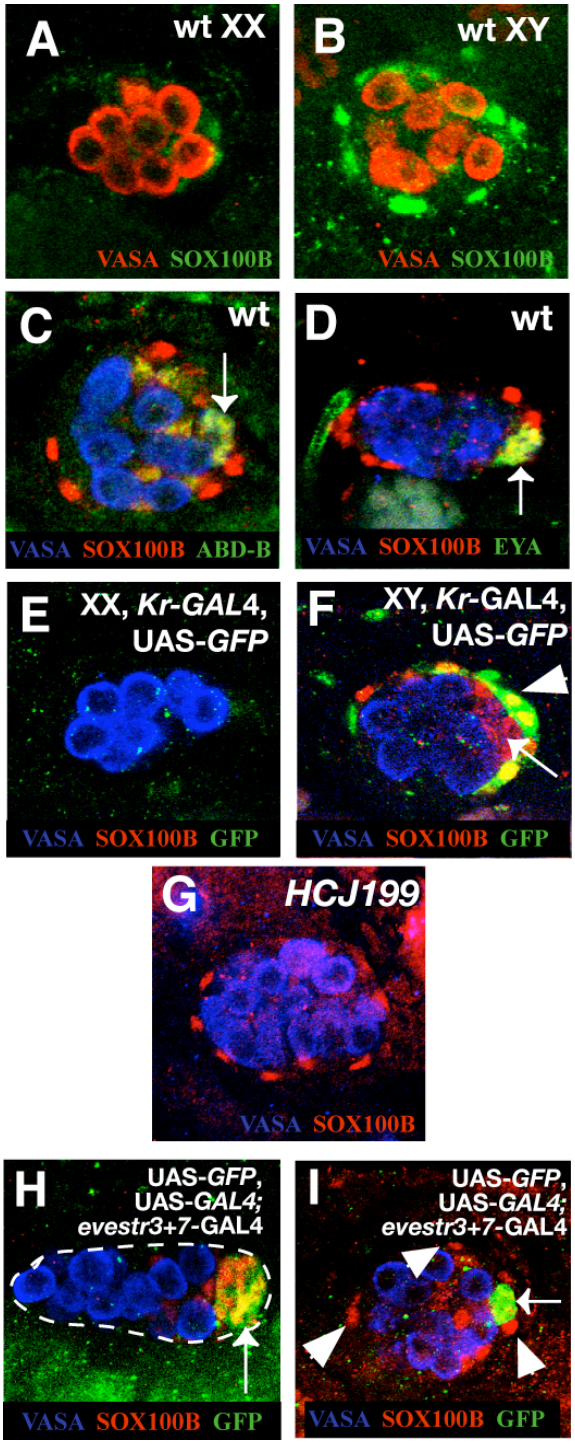
To test the hypothesis that the two cell types are different and independent more definitively, we examined gonads that lack msSGPs. In *Abd-B* mutant gonads, msSGPs

are absent (Chapter 3), yet the ensheathing population of SOX100B-expressing cells are still observed at stage 17 (Fig. 4.1G), suggesting that msSGPs are not required for the ensheathing cells to be present. Furthermore, in certain mutant backgrounds in which msSGPs sometimes fail to join the gonad, such as *shotgun*/*E-cadherin* mutants (Jenkins et al., 2003), SOX100B-positive cells are still observed around the gonad at stage 17 (data not shown).

To assess which cell types give rise to the two embryonic cell types, we implemented lineage tracing using the GAL4/UAS system (Brand and Perrimon, 1993). Since msSGPs arise in parasegment 13 (PS13), we used an *even-skipped stripe 3+7*-GAL4 driver (*evestr3+7*-GAL4; S. Small), which specifically expresses in PS13 cells in the forming gonad, to express UAS-*GFP* in the msSGPs (Fig. 4.1H). In addition, we used a UAS-*GAL4* construct that generates permanent GAL4 expression (Hassan et al., 2000) to permanently express UAS-*GFP* in msSGPs.. Thus, any cell that ever turns on GAL4 cannot turn it off, allowing us to use GFP as a lineage tracer. We observe that, when we express GFP initially in msSGPs, GFP is only expressed in the posterior of the stage 17 gonad (msSGPs) and not in the ensheathing layer of cells (Fig. 4.1I), indicating that msSGPs do not give rise to any other cells in the embryonic gonad. Without the amplification provided by the UAS-*GAL4* construct, *evestr3+7*-GAL4 levels of expression are too low to be detected in later embryonic stages (data not shown), demonstrating that these constructs can be successfully used for lineage analysis in the gonad.

Figure 4.1. SOX100B marks two sexually dimorphic gonad cell types.

Immunostainings of st. 17 (A-G,I) and st.13 (H) embryonic gonads. Anterior is to left in all panels. Colors of stainings are as indicated in each panel. VASA is used to label germ cells. A,B) Wild-type XX A) and XY B) stage 17 gonads. SOX100B labels a group of male-specific cells that ensheaths the gonad. C,D) Wild-type st. 17 male gonads. msSGPs express ABD-B and EYA (arrows), whereas the SOX100B-positive population of ensheathing cells does not. E,F) St. 17 XX E) and XY F) gonads from embryos expressing *Kr*-GAL4, UAS-*GFP* constructs on a TM3 balancer chromosome. GFP is only expressed around male gonads (arrowhead), but not in msSGPs (arrow). G) *HCJ199* (*Abd-B* enhancer trap) mutant st. 17 gonad, which lacks msSGPs but still exhibits ensheathing SOX100B-positive cells. H) St. 13 gonad in UAS-*GFP,* UAS-*GAL4*; *evestr3+7*-GAL4 embryo. GFP is only expressed in msSGPs in gonad (arrow). I) St. 17 gonad in UAS-*GFP,* UAS-*GAL4*; *evestr3+7*-GAL4 embryo. GFP is only expressed in msSGPs (arrow), and is not observed in ensheathing SOX100B-expressing cells (arrowheads). Embryos in (A-F) were sexed using an X-chromosome *Dfd-lacZ* construct (see Materials and Methods).


A
wt XX
VASA SOX100B
B
wt XY
VASA SOX100B
C
wt
VASA SOX100B ABD-B
D
wt
VASA SOX100B EYA
E
XX, Kr-GAL4,
UAS-GFP
VASA SOX100B GFP
F
XY, Kr-GAL4,
UAS-GFP
VASA SOX100B GFP
G
HCJ199
VASA SOX100B
H
UAS-GFP,
UAS-GAL4;
evestr3+7-GAL4
VASA SOX100B GFP
I
UAS-GFP,
UAS-GAL4;
evestr3+7-GAL4
VASA SOX100B GFP

msSGPs give rise to testis terminal epithelium

To determine the fate of the msSGPs in the adult, we followed the *evestr3+7* GFP lineage tracer throughout development. In the adult testis, GFP labels the terminal epithelium (Fig. 4.2A), which is the proximal-most cell layer in the testis that interacts with somatic cyst cells and is required for proper sperm differentiation (see (Fuller, 1993) for review). Without any UAS-*GAL4* construct, adult testis labeling was not detected (data not shown). As a positive internal control, the *evestr3+7*-GAL4 also expresses in the segments of the embryonic genital disc precursor, and, in the adult, labels the genital disc-derived reproductive structures, such as the seminal vesicle (Fig. 4.2A). A similar experiment using a *paired*-GAL4 driver that expresses in embryonic msSGPs shows GFP expression in the adult terminal epithelium (data not shown). Consistent with msSGPs giving rise to terminal epithelium, weak *Abd-B* mutants that live to adulthood but do not have embryonic msSGPs appear to exhibit a disruption of the terminal epithelium, as determined by altered Fasciclin 3 staining in the base of the testis and loss of Eyes Absent (EYA) staining in the terminal epithelial region (data not shown). Furthermore, these testes do not show a loss of somatic cyst cells or hub cells (data not shown). These data strongly suggest that the msSGPs give rise to the terminal epithelium of the adult testis.

Ensheathing SOX100B-positive cells give rise to testis pigment cells

To determine the fate of the SOX100B-positive cells that ensheath the stage 17 gonad, we used the *Kr*-GAL4, UAS-*GFP* construct to label these cells in the adult testis. We find that GFP expression is located along the entire length of the testis and seminal vesicle (Fig. 4.2B,B'). When we create a three-dimensional reconstruction, we find that

GFP expression is restricted only to the outer layer of the organ (inset, Fig. 4.2B') and is not expressed in internal structures of the testis. These cells are reminiscent of testis pigment cells (see Fuller, 1993), which are the outermost testis cell layer and are important for proper testis morphogenesis (Kozopas et al., 1998). Thus, the *Kr*-GAL4-expressing cells appear to be pigment cells.

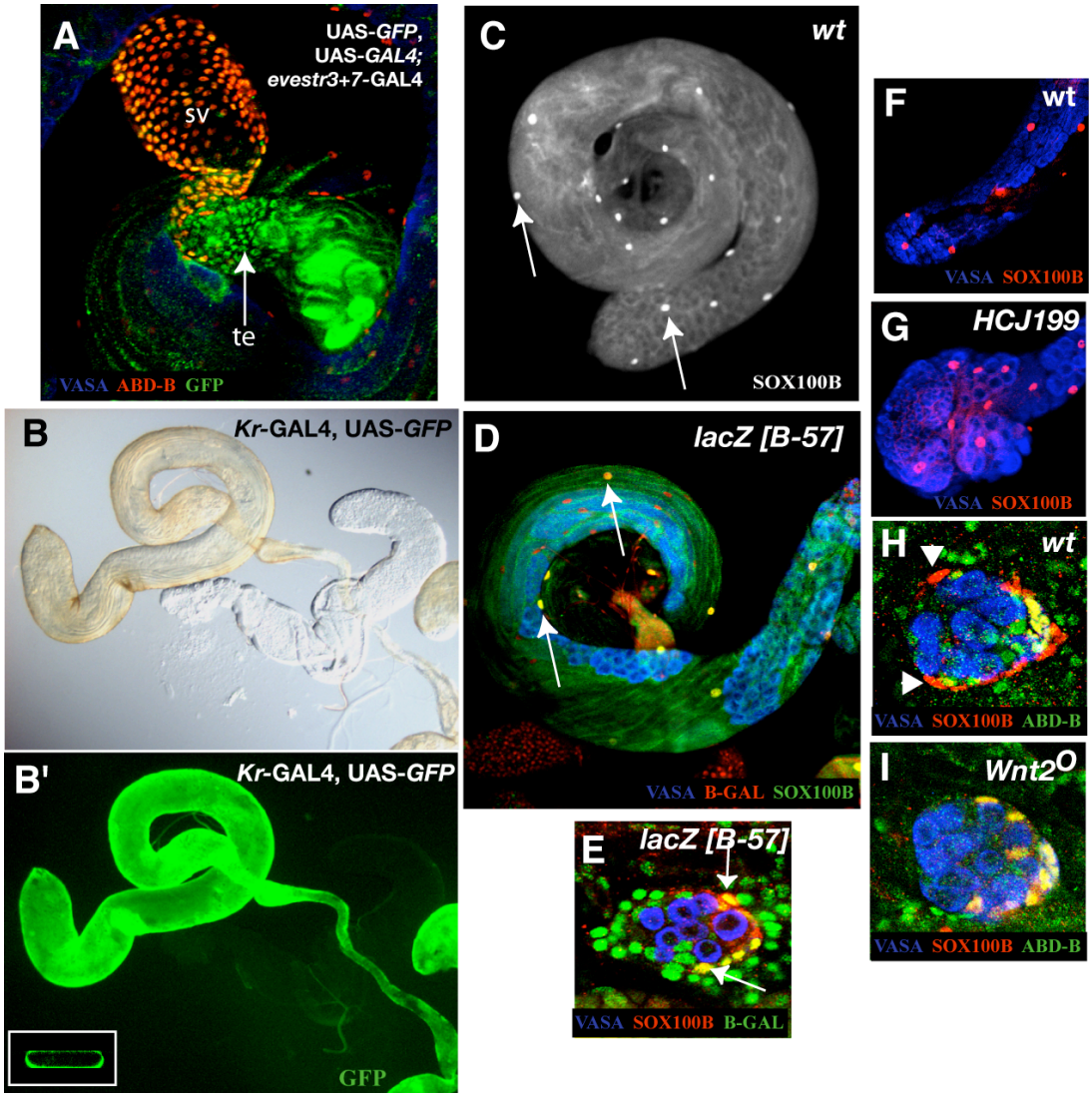
In adult testes, SOX100B labels a population of cells with large nuclei that coat the outside of the testis (Fig. 4.2C), which is also suggestive of pigment cells. We used two pigment cell reporter constructs, *lacZ [B-57]* and *lacZ [857]*, and found that SOX100B co-expresses with β-GAL from these pigment cell markers both in adult testes and in stage 17 gonads (Fig. 4.2D,E and data not shown). The *Kr*-GAL4, UAS-*GFP* construct expression also is coincident with the adult pigment cell layer that gives a yellow color to the adult wild-type testis (Fig. 4.2B,B'). In addition, in *Abd-B* mutant adult escapers, in which msSGPs are absent in the embryo, we still observe a wild-type number of SOX100B-positive pigment cells in the adult testis (Fig. 4.2F,G). These data indicate that the stage 17 SOX100B-positive and *Kr*-GAL4-expressing ensheathing cells are embryonic pigment cell precursors.

Larval and adult testis pigment cells require *Wnt2*, one of the members of the *Wnt/wingless* family of signaling molecules (Russell et al., 1992). However, it is not known what particular function *Wnt2* plays in the development of pigment cells, nor at which stage in development it is necessary. We looked at gonad development in *Wnt2* mutant embryos, and in our analysis of stage 17 gonads we did not observe any SOX100B-positive pigment cell precursors (Fig. 4.2H,I). msSGPs are unaffected in

Wnt2 mutants and still die in females (data not shown). These results suggest that *Wnt2* is specifically required for the embryonic recruitment of pigment cells.

Figure 4.2. SOX100B-expressing cells give rise to testis terminal epithelium and pigment cells.

Immunostainings of st. 17 gonads and adult testes. Colors of stainings are as indicated in panels. A) UAS-*GFP,* UAS-*GAL4*; *evestr3+7*-GAL4 adult testis. GFP labels terminal epithelium, in addition to seminal vesicle and other genital disc-derived structures. B,B') *Kr*-GAL4, UAS-*GFP* testis. GFP is present along the length of the testis and seminal vesicle. Inset) Slice of three-dimensional reconstruction of testis. GFP is only observed in the outer layer of the testis. C) Wild-type testis. SOX100B is observed in large nuclei reminiscent of pigment cells. D,E) Adult testis D) and st. 17 gonad E) expressing the pigment cell marker [B-57], which co-localizes with SOX100B in both stages. F,G) Wild-type F) and *HCJ199* mutant G) testes stained with SOX100B. Despite a lack of msSGPs, pigment cells are present in the *HCJ199* testis. H,I) Wild-type H) and *Wnt2* mutant I) st. 17 gonads. In *Wnt2* mutants, msSGPs (ABD-B and SOX100B co-expressing cells) are present, but pigment cell precursors (SOX100B-only-positive cells) are not observed.


A
UAS-GFP,
UAS-GAL4;
evestr3+7-GAL4
SV
te
VASA ABD-B GFP
B
Kr-GAL4, UAS-GFP
B'
Kr-GAL4, UAS-GFP
GFP
C
wt
SOX100B
D
lacZ [B-57]
VASA B-GAL SOX100B
E
lacZ [B-57]
VASA SOX100B B-GAL
F
wt
VASA SOX100B
G
HCJ199
VASA SOX100B
H
wt
VASA SOX100B ABD-B
I
Wnt2O
VASA SOX100B ABD-B

Pigment cell precursors are recruited to the gonad from surrounding fat body

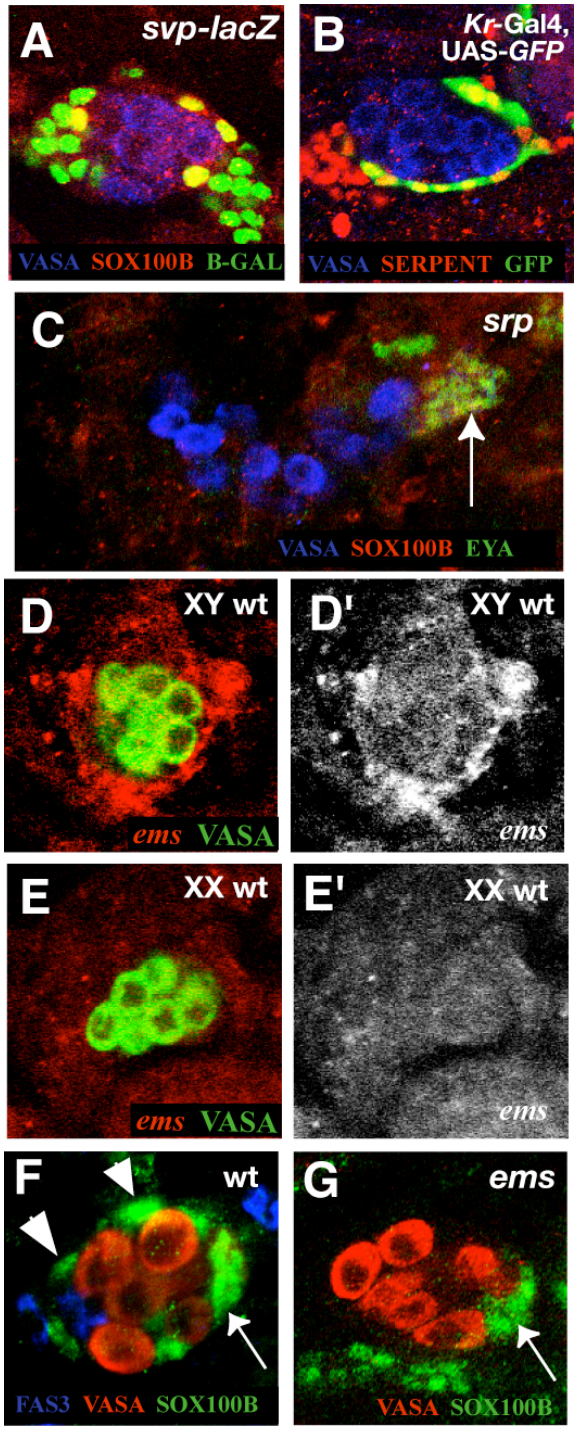
One possibility is that the SOX100B-positive cells that ensheath the stage 17 gonad are derived from the neighboring fat body, an adipose tissue adjacent to the gonad throughout development. It has long been known that the fat body is intimately associated with the gonad by the end of embryogenesis and start of larval stages (Kerkis, 1931), and therefore may play a role in gonad morphogenesis. We examined markers of fat body identity and characterized their expression in tissues immediately surrounding the late embryonic gonad. Two genes that are required for fat body development and are often used as markers for fat body identity are *seven up* (*svp*) and *serpent* (*srp*) (Hoshizaki et al., 1994; Sam et al., 1996). We observe that the SOX100B and *Kr*-GAL4 double-positive cells express both *svp* (as assayed with a *svp-lacZ* enhancer trap*)* and SRP (as determined by an anti-SRP antibody) (Fig. 4.3A,B). In addition, the gene *Kr* itself is potentially also a fat body marker, given that *Kr* expression in the larval fat body has been previously reported (Hoshizaki, 1994). To further investigate the role of the fat body in the development of these male-specific cells, we examined SOX100B expression in gonads of *srp* mutant embryos, which have defects in fat body morphogenesis (Sam et al., 1996). In stage 17 *srp* mutant gonads, while msSGPs are present (as identified by SOX100B and EYA co-expression), the outer population of SOX100B positive cells is not observed (Fig. 4.3C), suggesting that fat body is required for these cells to be specified. These data indicate that gonad-proximal fat body cells are precursors for a sexually dimorphic cell type that gives rise to pigment cells.

Another gene expressed in the tissue ensheathing the gonad is *empty spriracles* (*ems*). Interestingly, the mammalian homolog *Emx2*, is required for gonad and kidney

formation in the mouse and is expressed in the precursors of the gonad and urogenital system (Miyamoto et al., 1997). *In situ* hybridization shows that expression of *ems* around the *Drosophila* gonad is present by the beginning of stage 17, and analysis on embryos whose sex we can unambiguously identify reveals that gonad-associated *ems* expression is male-specific (Fig. 4.3D,E). We also have observed that *ems* mutant gonads, similar to *Wnt2* mutants, do not contain pigment cell precursors (Fig. 4.3F), but do have msSGPs and morphological markers of late embryonic male gonad development, such as anterior-most germ cell orientation around the putative embryonic hub cells (S. Le Bras and M. Van Doren, submitted). Staining with anti-SRP antibody reveals that fat body identity and general organization around the gonad is normal in *ems* mutants, but the morphology of the fat body cells ensheathing the male gonad is different from pigment cell precursors; cells are far from the gonad and do not show a flattened nuclear morphology (data not shown). This data suggests that *ems* is involved in regulating the ability of the fat body to become pigment cell precursors.

Figure 4.3. Pigment cell precursors are specified from fat body cells surrounding the male gonad.

Immunostaining (A-C,F-G) and in situ hybridization (D-E) on st. 17 gonads. Anterior is to left in all panels. Colors are as indicated in panels. A) *svp-lacZ* (fat body marker) expressing gonad, which labels SOX100B-positive pigment cell precursors. B) *Kr*-GAL4, UAS-*GFP* expression gonad, in which GFP and SRP co-label pigment cell precursors. C) *srp* mutant gonad, lacking pigment cell precursors, but maintaining msSGPs (arrow). D,E) In situ hybridization for *ems* on wild-type XY D) and XX E) gonads, showing male-specific *ems* gonad expression in cells surrounding the gonad. D',E') *ems* channel only. F,G) Wild-type F) and *ems* mutant G) gonads. Note absence of pigment cell precursors (arrowheads) in G), but presence of msSGPs (arrows). Fas3 labels embryonic hub cells in F, showing germ cell orientation towards hub cells that occurs in F) and G). Embryos in D,E) were sexed with a X-chromosome *Dfd-lacZ* construct.


A
svp-lacZ
VASA SOX100B B-GAL
B
Kr-Gal4,
UAS-GFP
VASA SERPENT GFP
C
srp
VASA SOX100B EYA
D
XY wt
ems VASA
D'
XY wt
ems
E
XX wt
ems VASA
E'
XX wt
ems
F
wt
FAS3 VASA SOX100B
G
ems
VASA SOX100B

The sex determination genes *transformer* and *doublesex* regulate sexually dimorphic pigment cell specification

The somatic sex determination pathway in *Drosophila* acts through a regulatory cascade responsive to the X chromosome to autosome ratio to ensure sex-specific development. Two genes, *transformer* (*tra*) and *doublesex* (*dsx*), are downstream genes that are required for sexual dimorphism of the soma, and we have previously shown these two genes are necessary for proper development of msSGPs in the embryonic gonad (Chapter 2). In *tra* mutants, we expect XX (female) gonads to be masculinized, and in stage 17 we observe SOX100B-positive pigment cell precursors in both XX and XY gonads (Fig. 4.4A,B). Furthermore, when we express Tra ubiquitously in the soma, both XX and XY gonads lack pigment cells (Fig. 4.4C,D). Dowstream of *tra*, *doublesex* is thought to be the terminal sex regulatory gene in the soma outside of the nervous system (Baker and Ridge, 1980), and relies on *tra* for splicing of its transcripts into male- and female-specific isoforms that act to promote sexual development (Burtis and Baker, 1989). In *dsx* mutant embryos, both XX and XY gonads contain pigment cells and exhibit a masculinized phenotype (Fig. 4.4E,F). This indicates that *dsx* is not required in males to specify pigment cells, but rather is only necessary in females to repress pigment cell specification. This role for *dsx* in regulating sexual dimorphism in the gonad is similar to what has been observed for msSGPs (Chapter 2), as well as embryonic hub cells (S. Le Bras and M.Van Doren, submitted).

We also wanted to determine whether *dsx* acts to regulate pigment cell specification or pigment cell survival. Perhaps, similar to the cellular mechanism of msSGPs, pigment cell precursors are specified in both sexes, but can only survive and

turn on SOX100B in males. To test this hypothesis, we examined *H99* deficiency homozygous mutant gonads that are deficient for programmed cell death (White et al., 1994). Previously, we have shown that msSGPs can survive in *H99* mutant female gonads (Chapter 2). However, we find that in *H99* mutant stage 17 gonads, sexual dimorphism is normal; pigment cell precursors are absent in XX embryos and are observed only in XY embryos (Fig. 4.4G,H). Thus, although msSGPs and pigment cells both depend on *dsx* for sex-specific development, the cellular mechanisms employed to ensure sexual dimorphism of the two cell types are different.

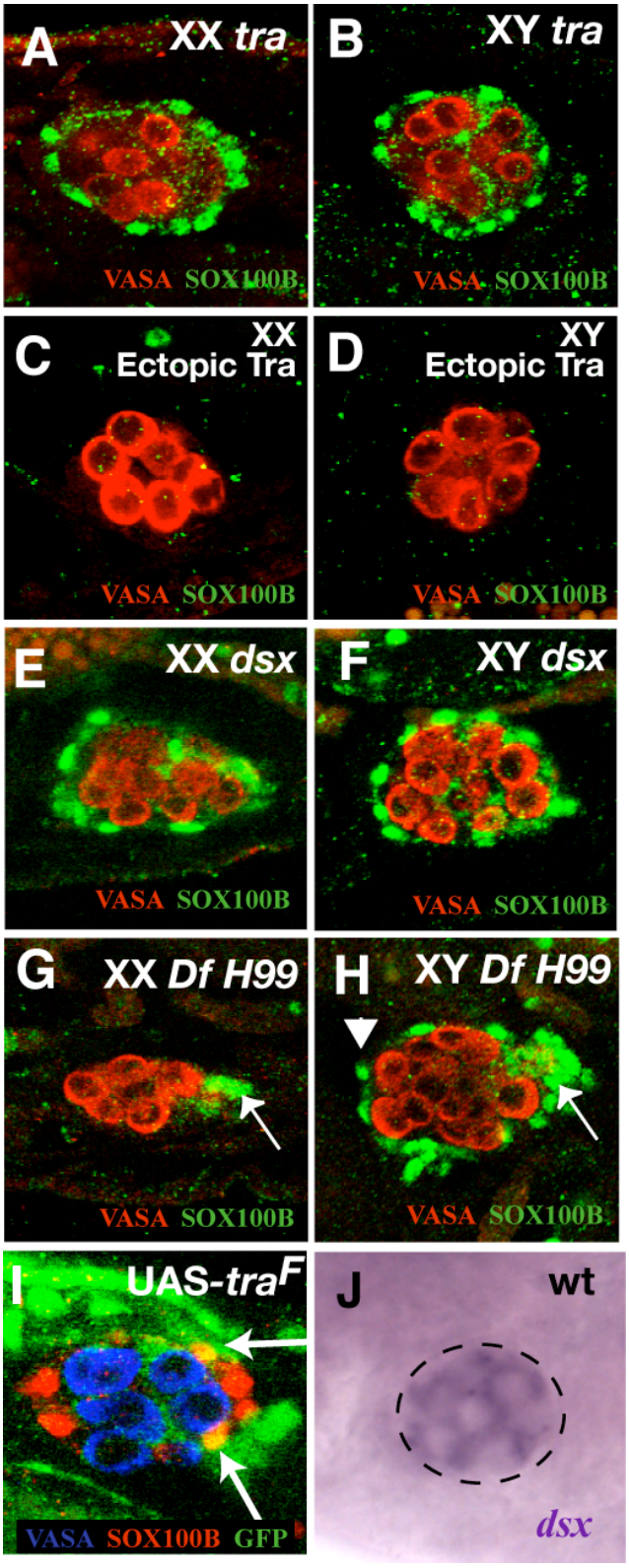
To elucidate further how sexual dimorphism in the gonad is regulated, we wanted to determine the mechanism of action of the sex determination genes, i.e., if the sexual fate of the gonad-associated fat body is controlled cell-autonomously or non-autonomously. To test if *tra* acts cell-autonomously, we used a *paired*-GAL4 (*prd*-GAL4) driver to express a UAS-tra^F construct only in a subset of fat body cells in XY embryos (Brand and Perrimon, 1993), thus creating a sexually "mosaic" tissue in which female cells are present in a male background. To assess in which cells TRA is expressed, we simultaneously used a UAS-*GFP* construct to label the *prd*-GAL4-expressing cells directly with GFP. If *tra* is acting cell-autonomously, then we expect no TRA-expressing cell to become a pigment cell and turn on SOX100B. Interestingly, we find that GFP- and TRA-expressing (feminized) cells can express SOX100B and become pigment cell precursors (Fig. 4.4I), indicating that a male-specific fate can be induced in cells with a female identity, and that the fat body exhibits a non-autonomous mechanism of sex determination. Therefore, we hypothesize that there is a signal coming from

adjacent tissues, potentially the somatic gonad, that acts to control the sexual fate of the fat body.

Given that sexual dimorphism of pigment cells requires the function of *dsx*, we examined the expression of *dsx* in the gonad at stage 17, the time when pigment cells are observed in the gonad. *dsx* mRNA is only highly expressed in the SGPs and does not appear to be expressed in the surrounding fat body or other tissues (Fig. 4.4J). This expression pattern is similar to what has been reported in earlier stages (Berkeley *Drosophila* Genome Project in situ expression database). This suggests that the sex determination pathway acts through SGPs to non-autonomously regulate sexual dimorphism of pigment cells in the surrounding fat body.

Figure 4.4. Sex determination in pigment cells acts through *dsx* in a non-autonomous mechanism.

Immunostaining (A-I) and in situ hybridization (J) on st. 17 gonads. Anterior is to left in all panels. Colors are as indicated in panels. A,B) tra^{1} mutant XX A) and XY B) gonads. C,D) UAS-tra^{F}; *tubulin*-GAL4 XX C) and XY D) gonads. E,F) dsx^{1}/dsx^{23} mutant XX E) and XY F) gonads. G,H) *Df H99* homozygous mutant XX G) and XY H) gonads. I) UAS-tra^{F}; *prd*-GAL4, UAS-*GFP* gonad. Note co-expression of SOX100B and GFP in pigment cells. J) In situ hybridization for *dsx* on wild-type gonad. Note highest expression in SGPs. Embryos in (A-H) were sexed with an anti-SXL antibody.


A
XX tra
VASA SOX100B
B
XY tra
VASA SOX100B
C
XX
Ectopic Tra
VASA SOX100B
D
XY
Ectopic Tra
VASA SOX100B
E
XX dsx
VASA SOX100B
F
XY dsx
VASA SOX100B
G
XX Df H99
VASA SOX100B
H
XY Df H99
VASA SOX100B
I
UAS-traF
VASA SOX100B GFP
J
wt
dsx

***Wnt2* acts non-automonously to regulate development of sex-specific pigment cells**

Given the role of *Wnt2* in pigment cell specification ((Kozopas et al., 1998) and this work), a likely hypothesis is that *Wnt2* is the non-autonomous signaling factor regulating sexual dimorphism in the late embryonic gonad and fat body. Therefore, we wanted to examine the expression of *Wnt2* at stage 17, the time when pigment cells are first observed. Earlier in embryogenesis, *Wnt2* is expressed in the posterior of the embryonic gonad (Kozopas et al., 1998; Russell et al., 1992), specifically in msSGPs and posterior SGPs (Chapter 2). However, we find that in stage 17, *Wnt2* is expressed specifically throughout male gonads and is only present at background levels in females (Fig. 4.5A,B). Co-labeling experiments with the *Kr*-GAL4, UAS-*GFP* pigment cell marker shows that *Wnt2* is only expressed in SGPs and not in the surrounding fat body itself (data not shown). Given the role of *dsx* in pigment cell specification, we looked at *Wnt2* expression in *dsx* mutants and observe that *Wnt2* is expressed in 100% of *dsx* mutants (n=25), i.e., in both XX and XY gonads (Fig. 4.5C). This result shows that, in the absence of *dsx*, the male phenotype is the "default" state of the gonad, and is consistent with our observations that pigment cell precursors are present in all *dsx* mutant gonads. A prior study has shown that *Wnt2* expression is sufficient to induce pigment cell formation in larval and adult female gonads (Kozopas et al., 1998). To see if *Wnt2* expression in embryos is sufficient to induce pigment cell specification in embryonic stages, we expressed *Wnt2* in XX gonads and examined SOX100B expression at stage 17. We observe that XX *Wnt2*-expressing gonads contained SOX100B-positive cells (Fig. 4.5D), although nuclear morphology of these ectopic pigment cells does not appear

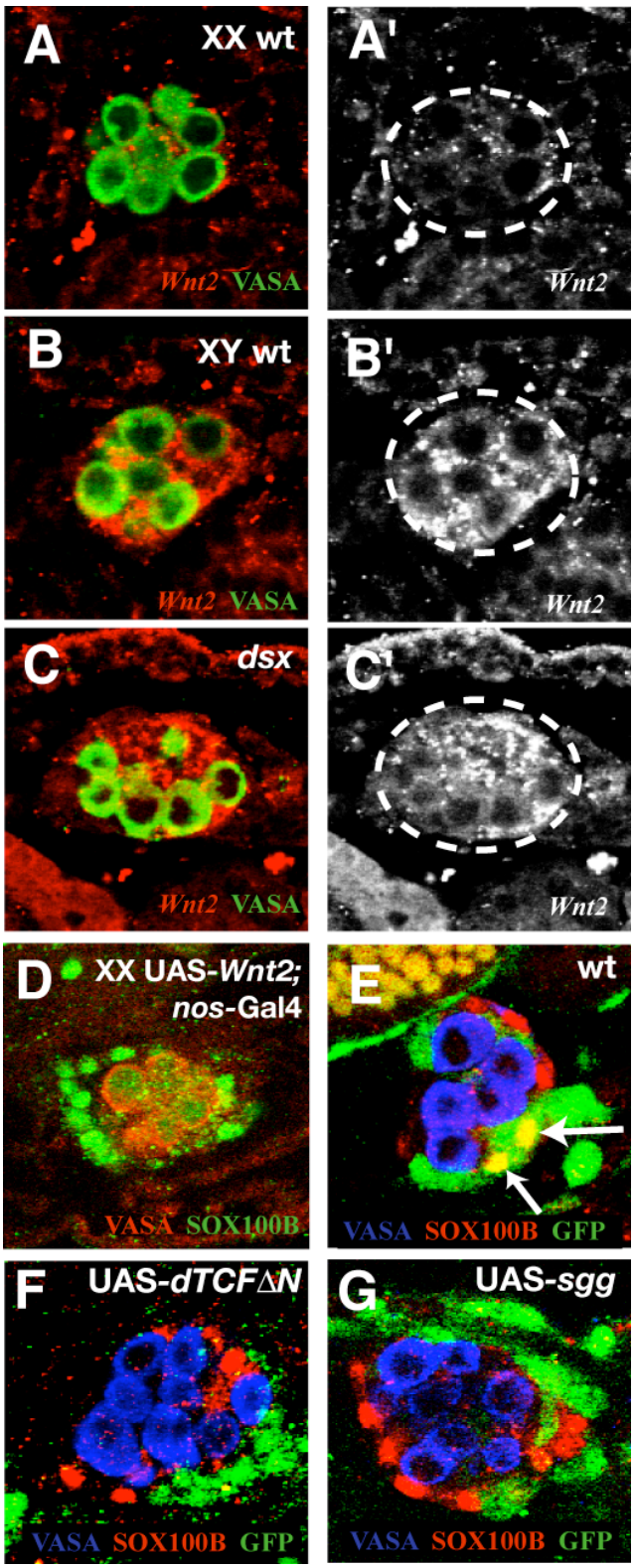
wild-type; the cells seem detached from the gonad, suggesting that complete pigment cell fate and morphogenesis is not determined solely by *Wnt2*.

To assess if *Wnt2* acts directly on the fat body to induce pigment cell specification, we again used *prd*-GAL4 to drive expression in a subset of fat body. We used dominant negative *pangolin/TCF* (UAS-*dTCFΔN*) and constitutively active *shaggy* (*Zw3* glycogen synthase kinase) components of the *Wnt* signaling pathway to cell-autonomously inhibit *Wnt* signal transduction in *prd*-GAL4-expressing cells. If *Wnt* signaling is required in fat body cells for pigment cell specification, then we expect that cells that cannot transduce *Wnt*-signaling will not express SOX100B and become pigment cells. To directly assess in which cells we have blocked *Wnt* signaling, we again employed a UAS-*GFP* construct in the experiment to label those cells of interest. In control embryos expressing solely *prd*-GAL4 and UAS-*GFP*, we observe 100% of male gonads (n=27 gonads) containing pigment cells that co-express SOX100B and GFP (Fig. 4.5E). However, when we use either a UAS-dominant negative *TCF* or a UAS-*shaggy* construct, we fail to observe any SOX100B expression in GFP-expressing cells near the gonad (n=26 gonads) (Fig. 4.5F,G), although immediately adjacent cells can express SOX100B, suggesting that canonical *Wnt* signaling in the fat body is cell-autonomously required for pigment cell specification. Using an anti-SRP antibody to assess fat body identity, we find that blocking *Wnt* transduction with *prd*-GAL4 in these experiments does not block the ability of the fat body to form (data not shown), suggesting that the dominant negative *Wnt* constructs are specifically blocking pigment cell specification. We conclude from these data that the gonad uses a non-autonomous sex-determination mechanism in which the sex of the fat body itself does not influence the sex-specific fate

of becoming testis pigment cells. Instead, the sex of the SGPs dependent upon *dsx* (and resultant expression of *Wnt2*) is the determining factor in pigment cell formation, along with the transduction of the *Wnt2* signal within the fat body.

Figure 4.5. *Wnt2* acts non-automonously to regulate sex-specific pigment cell specification

In situ hybridization (A-C) and immunostaining (D-G) on st. 17 gonads. A,B) In situ hybridization for *Wnt2* on wild-type XX A) and XY B) gonads. Note sex-specific expression. C) dsx^{1}/dsx^{23} mutant gonad. D) XX UAS-*Wnt2*; *nos*-GAL4 gonad. Note ectopic SOX100B expression in cells around the gonad. E) *prd*-GAL4, UAS-*GFP* control gonad. Note co-expression of SOX100B and GFP in pigment cells (arrows). F) UAS-*dTCFΔN; prd*-GAL4, UAS-*GFP* gonad. G) UAS-sgg^{S9A}*; prd*-GAL4, UAS-*GFP* gonad. Note absence of SOX100B and GFP co-expression in F,G). Embryos in A,B) were sexed with an X-chromosome *Dfd-lacZ* construct. Embryo in D was sexed with an anti-SXL antibody.


A
XX wt
Wnt2 VASA
A'
Wnt2
B
XY wt
Wnt2 VASA
B'
Wnt2
C
dsx
Wnt2 VASA
C'
Wnt2
D
XX UAS-Wnt2;
nos-Gal4
VASA SOX100B
E
wt
VASA SOX100B GFP
F
UAS-dTCFΔN
VASA SOX100B GFP
G
UAS-sgg
VASA SOX100B GFP

DISCUSSION:

Wnt signaling is involved in non-autonomous sex determination of pigment cell precursors

A role in sexually dimorphic reproductive organ development has been shown for multiple members of the mammalian family of *Wnt* signaling genes. *Wnt4*, *Wnt7a*, and *Wnt9b* are all involved in the sex-specific embryonic development of the mouse reproductive tract or gonad (Parr and McMahon, 1998; Vainio et al., 1999; Jeays-Ward et al., 2004; Carroll et al., 2005). In this study we have demonstrated that SGP *Wnt* signaling is also necessary to create sexual dimorphism in the *Drosophila* embryonic gonad. Specifically, we find that transduction of *Wnt* signaling derived from SGPs is required in the fat body for pigment cell specification. It appears that either sex has the potential to respond to *Wnt* signaling, given that *Wnt2* expression in XX embryos is sufficient to induce SOX100B expression. Therefore, it is unlikely that the *Wnt* machinery is sexually dimorphic, for example, at the level of Frizzled or Frizzled2 receptors on the membrane of fat body cells. However, given that the morphology of "pigment cells" in XX embryos induced by ectopic expression of *Wnt2* is not completely wild-type (the nuclei fail to show a flattened morphology and do not show a tight association with the gonad), it appears that some sex-specific factor downstream of *Wnt* signaling regulates the complete morphogenesis of pigment cells. Our data suggest that *ems* may be a regulator of this process, as *ems* mutant fat body nuclei fail to show a flattened morphology. It is possible that the male gonad is the proper "substrate" for pigment cell morphogenesis and, despite SOX100B expression, XX pigment cells cannot undergo a full transformation to a male fate while in contact with a female gonad. We

have not been able to determine if such ectopic pigment cells can affect female ovary morphogenesis. XX embryos that express *Wnt2* from germ cells develop into fertile females with normal ovary morphology (data not shown), however, one caveat with this result is that the germ cell driver *nos*-GAL4 does not express during adulthood in females.

A role for secondary sex determination in Drososphila gonad formation.

Sexual dimorphism in the animal kingdom can be regulated via two different mechanisms: autonomous (primary) or non-autonomous (secondary) sex determination. In the cells that adopt a sexually dimorphic fate, they determine their sexual identity cell-autonomously, or they must rely on non-autonomous signals from nearby or distant cells. The current dogma for most *Drosophila* somatic tissues is that of cell-autonomous sex determination, which is the result of prior studies of cuticular and other adult structures in sexually mosaic animals (Baker and Ridge, 1980). In this study, we have shown that secondary sex determination may also play a key role in the sexual dimorphism of the *Drosophila* gonad, and is similar to what is seen in the mouse gonad (see below). We have been able to create "sexually mosaic" gonads and observe the role of gonad and fat body sexual identity in the process of specifying male-specific pigment cells. Our analyses indicate that for fat body-derived pigment cells, the sexual identity of the fat body itself is not critical, but rather it is the sexual identity of the SGPs and their *Wnt2* expression that dictates the sexual fate of the fat body. We have found a similar result with msSGPs; in gonads in which TRA is expressed in msSGPs (but not in adjacent SGPs), msSGPs still exhibit a male-specific phenotype and survive to join the gonad (N.

Crnkovich, M. Hoang, TJD, MVD, unpublished). This suggests that the sexual identity of msSGPs alone is insufficient to regulate their sexually dimorphic behavior. The data support a model in which non-autonomous, secondary sex determination plays an essential role for regulating sexual dimorphism in two important testis cell types, the terminal epithelium (i.e., msSGPs) and pigment cells. Recent work has also shown that sexual dimorphism in *Drosophila* germ cells is regulated non-autonomously, as JAK/STAT signaling from the male somatic gonad controls sex-specific cell division and gene expression in the germ line (Wawersik et al., 2005). Outside of the gonad, similar phenomena have been reported. In the nervous system, male neurons can induce the formation of the male-specific muscle of Lawrence from female muscle precursors (Lawrence and Johnston, 1986). Additionally, the sexually dimorphic development of the genital disc is regulated via *dsx*-mediated Wingless and Decapentaplegic signaling from "organizer" regions to the rest of the disc (Keisman et al., 2001).

Much of the somatic gonad does not come from SGPs

Prior work has established that the precursors of the somatic gonad arise from a bilateral cluster of cells from parasegments (PS) 10-12 (Boyle and DiNardo, 1995; Brookman et al., 1992). However, we have previously shown that a separate cluster of cells, termed male-specific SGPs (msSGPs), arises in PS13 and contributes to the male gonad (Chapter 2). We have demonstrated that these cells become the terminal epithelium in the adult testis (this work), which is essential for final sperm differentiation. We have also found that pigment cells, which are important for fertility and testis morphogenesis (Kozopas et al., 1998), are derived from fat body cells adjacent to the gonad during embryogenesis.

The testis muscle sheath may also represent another somatic gonad component that arises from non-SGP cells. Although it has been previously shown that the muscle sheath comes from the ectodermally-derived genital disc, the pupal and adult muscle sheath cells express Twist (Kozopas et al., 1998), a known muscle and mesodermal marker (Bate et al., 1991; Thisse et al., 1988). Therefore, it is possible that the muscle sheath cells are a subset of the mesodermal cells recruited into the genital disc during late larval development that also give rise to the vas deferens and paragonia (Ahmad and Baker, 2002). Interestingly, the interaction of the muscle sheath with the gonad likely requires pigment cells (Kozopas et al., 1998), whereby pigment cells serve as a substrate for muscle sheath migration over the length of the testis. Although the terminal epithelium and pigment cells are derived from non-SGP cell types, we have also shown that a subset of SGPs in PS10-12 give rise to embryonic hub cells (S. Le Bras and M. Van Doren, submitted).

Multiple roles for fat body in gonad development

The tissue immediately adjacent to the gonad throughout embryonic, larval, and adult development is the fat body. Aside from proximity to each other in the embryo, the gonad and the fat body share a common origin. The SGPs and the fat body both arise from dorsolateral mesoderm, and the action of *abd-A* in PS10-12 serves to repress *srp*, which otherwise specifies fat body (and blocks SGP specification) in other PS (Moore et al., 1998a; Riechmann et al., 1998). In this study, we have demonstrated that a subset of gonad-proximal fat body cells serve as precursors to a specific testis cell type, the adult pigment cells. Pigment cells have been shown to be an important cell type for proper testis morphogenesis (Kozopas et al., 1998). Given the unique ability of the fat body to

secrete steroid-like compounds, the fat body-derived pigment cells are likely a crucial intermediary between the surrounding fat body and the testis. The fat body also intimately associates with the female gonad throughout development. However, the role of the gonad-proximal fat body in the ovary is not yet known, although expression of the *yolk protein* genes in the fat body is important for oogenesis. Given the steroidogenic capability of the fat body and its proximity to the gonad, the fat body likely serves both in supporting and signaling capacities.

Mouse and *Drosophila* gonad formation share common features

We have shown that non-autonomous, secondary sex determination may play a role in the *Drosophila* gonad, demonstrating an aspect of development that is similar to what is seen in mammals. In mammalian tissues outside of the gonad, secondary sex determination is the model for the regulation of sexual dimorphism, controlled by the systemic delivery of androgens via the circulatory system. In addition, within the gonad itself, non-autonomous sex determination plays a role in sexual dimorphism on multiple levels. Cells from the mesonephros must migrate into the gonad, and this migration is required for the formation of testis cords (Martineau et al., 1997; Tilmann and Capel, 1999). The presence of *Sry* in the gonad is sufficient to induce this migration of cells, even from an XX mesonephros (Capel et al., 1999). The non-gonadal duct systems of the mammalian male reproductive tract also employ non-autonomous mechanisms, as the female Müllerian duct precursors must be degraded during embryogenesis via the action of anti-Müllerian hormone (AMH). Male knockout mice for the *Amh* gene or its receptor fail to show regression of the Müllerian duct (Behringer et al., 1994; Mishina et al., 1996), and

AMH is thought to act by binding nearby cells and inducing them to release a factor that promotes apoptosis of the Müllerian duct (Roberts et al., 1999). In addition, the germ cells of the mouse gonad rely on the somatic cells around them to induce sex-specific differentiation of sperm and egg; for example, XX germ cells can be induced to enter spermatogenesis in a male somatic gonad environment (Adams and McLaren, 2002; Palmer and Burgoyne, 1991).

In addition to similarities in non-autonomous sex determination described above, there are also common features in the cellular mechanisms of gonadogenesis in *Drosophila* and mice. For example, the recruitment of pigment cells to the male gonad from the fat body is reminiscent of mesonephric cells migrating into the embryonic testis. Just as mesonephric cell migration occurs independently of the sex of the mesonephros (Capel et al., 1999), *Drosophila* pigment cell specification is independent of the sex of the fat body cells. In addition, a subset of the migrating mesonephric cells in the mouse appear to be peritubular myoid cells, which help to organize the gonad into testis cords that will act to segregate germ cells and supporting Sertoli cells from other cell types. This testis architecture is similar to what is we observe in *Drosophila*, in which myoid cells (i.e., muscle sheath) from the male genital disc, along with pigment cells, migrate over the testis in order to segregate germ cells and supporting somatic cyst cells from other cell types. Therefore, it appears the similar structure of the fly and mouse testes are brought about via similar cellular mechanisms. However, it is currently unknown to what extent the *Drosophila* fat body is equivalent to the mouse mesonephros, except for its proximity to the gonad during embryogenesis.

We have shown that the regulation of sexual dimorphism in the *Drosophila* gonad shares many aspects in common with mammalian systems. This is evident at the cellular level, with cell migration and recruitment aiding in the establishment of sexually dimorphic development in both mice and flies, and at the molecular level, in which genes such as *Sox9*/*Sox100B*, *Emx2*/*ems*, and *Wnt2* play roles in gonad differentiation. Further study of the cellular and molecular mechanisms of fly gonad development will likely provide more insight into the evolution of sex determination and sexual dimorphism, and may also add to growing evidence that the regulation of sex-specific gonad development is an evolutionarily conserved process.

CHAPTER 5

The *Sox9* homolog *Sox100B* is required for testis development in *Drosophila*

SUMMARY

Throughout the animal kingdom, sexual dimorphism is evident in a vast majority of species, and is critical for successful sexual reproduction. While sexual dimorphism is a common theme in animal species, sex determination methods in the embryo widely vary, with a range of genetic and environmental mechanisms. However, growing evidence suggests that the downstream regulation of sexual dimorphism is an evolutionarily conserved process at cellular and molecular levels. One example of this conservation is the gene *Sox9*, which is expressed male-specifically in the gonads of human, mouse, chick, rainbow trout, and turtle, and has been implicated in mammalian sex determination. *Sox100B*, a *Drosophila* homolog of *Sox9* and the only *Sox* Group E gene in *Drosophila*, also has been previously shown to have male-specific gonad expression. Given this expression pattern, it is possible that *Sox100B*/*Sox9* may represent a conserved molecular factor controlling male-specific differentiation. Consistent with this hypothesis, we have found that *Sox100B* is required for adult testis development. *Sox100B* mutant testes are severely atrophic, lacking proper size and morphology, due to lack of both somatic and germline lineages. Adult mutant females do not show a gonad phenotype, suggesting that *Sox100B* is acting only in the testis. Results indicate that *Sox100B* plays a role during pupal stages in the function of pigment cells, a sexually dimorphic cell type that is required for proper adult testis morphogenesis. These data support a model in which the regulation of sexual dimorphism in animals is conserved at the molecular level, despite a lack of conservation in upstream sex determination.

This work was carried out in collaboration with Dr. Steven Russell and Shreeya Nanda at the University of Cambridge (U.K.), who shared initial findings and pertinent reagents. However, all data and images presented in this chapter were collected from experiments carried out by the author.

INTRODUCTION

Differences between males and females are necessary for successful reproduction, both at the social and biological level. The establishment of male and female phenotypes in the gonad is especially vital for the development of germline cells into sperm and eggs, which must be properly differentiated in order to pass on genetic material to progeny.

While the sex-specific nature of the gonad has been well studied for adult stages, sexual dimorphism in the gonad is initially established in embryogenesis, during which sex-specific cell types begin to give rise to tissues that support gametogenesis in the adult. We have previously demonstrated that male-specific somatic gonadal precursors (msSGPs) represent an early sexual dimorphism in the somatic gonad, whereby msSGPs only join the male gonad and die by programmed cell death in females (Chapter 2). In addition, it has been previously shown that the somatic gonad employs sex-specific JAK/STAT signaling to regulate sexual dimorphism in the embryonic germ line (Wawersik et al., 2005), indicating that male- and female-specific development of the somatic gonad is indeed already present in embryonic stages.

Adult *Drosophila* reproductive organs, the ovaries and testes, exhibit dramatic sexual dimorphism in morphology and function. Ovaries consist of 16-20 ovarioles, each of which is an assembly line of developing egg chambers (reviewed in Spradling, 1993).

Each egg chamber is composed of somatic follicle cells that coat the egg chamber, 15 germline-derived nurse cells, and a single oocyte. At the distal tip of each ovariole resides the germarium, in which the germline stem cell (GSC) niche is located. In contrast, the adult testis is a coiled tubular organ containing a single lumen, in which cysts of developing spermatocytes, each ensheathed by a pair of somatic cyst cells, differentiate into sperm. At the base of the testis, the terminal epithelium interacts with the cyst cells and allows the liberation and final differentiation of individual sperm. Along the outside length of the testis runs a layer of pigment cells and a genital disc-derived muscle sheath, both of which aid in the morphogenesis of the testis into its coiled shape upon contact with the genital disc during metamorphosis (reviewed in Fuller, 1993).

Sexual dimorphism is brought about via the integration of sexual determination cues and positional information in order to turn on effector genes that regulate sex-specific differentiation. The sex determination machinery has evolved rapidly in the animal kingdom, as different animal classes employ a variety of genetic or temperature-dependent signals to launch male- or female-specific developmental pathways. While these upstream sex determination "switches" have diverged, it is possible that the downstream effector genes have been evolutionarily conserved. One such family of effector genes is the *Doublesex and Mab-3 related transcription factor* (*DMRT*) family, which encode transcription factors that regulate various aspects of sexual dimorphism in flies (*doublesex*), nematodes (*mab-3*), medaka fish (*DMY*), mice (*Dmrt*1), and humans (*DMRT1*) (Hildreth, 1965; Matsuda et al., 2002; Raymond et al., 2000; Raymond et al., 1999; Shen and Hodgkin, 1988).

Another effector gene that is a candidate for being an ancestral sex determination gene is *Sry-related HMG box gene 9* (*Sox9*), which is required for human and mouse sex determination (Chaboissier et al., 2004; Foster et al., 1994; Wagner et al., 1994). It has been shown that *Sox9* is expressed sex-specifically in the embryonic testes of multiple animal species, such as human, mouse, chick, trout, and turtle (de Santa Barbara et al., 2000; Kent et al., 1996; Moreno-Mendoza et al., 1999; Takamatsu et al., 1997), which utilize a variety of sex determination switches. Recently, it was shown that *Sox100B*, a *Drosophila* homolog of *Sox9*, is expressed in the somatic gonad prior to and after initial gonad formation (Loh and Russell, 2000), although it is not known what role *Sox100B* plays in the development of the gonad. We have shown that Sox100B is expressed male-specifically in the *Drosophila* embryonic gonad, in the precursors of the testis terminal epithelium and pigment cells (Chapters 3,4). The expression of *Sox9* homologs in the embryonic testes of species as diverse as flies and humans suggests that *Sox9* plays a central role in sexual dimorphism, and further study may provide insight into evolutionarily conserved aspects of sex-specific gonad formation.

In this study, we undertake an investigation into the role of *Sox100B* in *Drosophila* gonad development. We find that *Sox100B* is expressed in multiple testis cell types in embryonic, larval, and adult stages. Similar to *Sox9* homologs in other species, we find that *Sox100B* is required for adult testis formation. *Sox100B* mutant adults have atrophic testes that lack germline and somatic cell types, whereas mutant ovaries show no apparent phenotype. In late larvae, *Sox100B* mutant testes appear wild-type, suggesting that *Sox100B* acts during pupal stages to regulate testis differentiation. Potentially, *Sox100B* exerts its effects by maintaining pigment cells, which are implicated in testis

morphogenesis. These data strongly support the hypothesis that *Sox9* and its homologs play an evolutionarily conserved role in male-specific gonadogenesis and sexual dimorphism.

MATERIALS AND METHODS

Fly Stocks

The following fly stocks were used: UAS-*GAL4*12B, UAS-*GFP.nls*14, *even-skipped stripe3+7*-GAL4 (S. Small), UAS-*Sox100B* A4 (S. Russell), UAS-*Sox100B* A10 (S. Russell), *nanos 3'UTR*::VP16-GAL4 (*nos*-GAL4) (Van Doren et al., 1998), *c587*-GAL4 (A. Spradling), lacZ[842] (M. Fuller through E. Matunis); $J3B9^{rv12}$ (a P-element excision removing only *dco* and *Sox100B*; S.Russell), P[*dco*+], Df *tll-e* (a *dco* rescue construct recombined with a large deficiency). $J3B9^{rv12}$ and P[*dco*+], Df *tll-e* are used as transheterozygotes to produce *Sox100B* mutants, which survive to pharate stages. *Kr*-GAL4, UAS-*GFP* is on either TM3 and CyO balancer chromosomes (TM3, P[GAL4-*Kr*.C]DC2, P[UAS-*GFP*.S65T]DC10 and CyO, P[GAL4-*Kr*.C]DC3, P[UAS-*GFP*.S65T]DC7) (Casso et al., 2000) and shows similar gonad expression on both chromosomes. The TM3 construct was used for adult analysis of pigment cells. See Chapter 4 for GFP lineage tracing of msSGPs with UAS-*GAL4* and *even-skipped stripe3+7*-GAL4. w^{1118} was used a wild-type control.

Antibody Stainings:

Embryo and adult testis fixation and immunostainings were performed as in Chapter 4. After staining, samples were mounted in 2.5% DABCO (Sigma) on slides and imaged

with a Zeiss 510 Meta confocal microscope. Adult ovary staining protocol is identical to testis protocol, except that fixation time is 20 minutes. Incubations with DNA dye (either TOTO3 or Oligreen [both from Molecular Probes]) were done as final steps prior to mounting. TOTO3 was used at a 1:3,000 dilution in PBS containing 0.1% Triton X-100 (PBTx) for 12-15 minutes after a 30-minute preincubation in PBTx with 0.4 mg/ml RNaseA. Oligreen was used at a 1:10,000 dilution in PBTx for 12-15 minutes. After incubation with DNA dye, 3 quick PBTx rinses and 2 quick PBS rinses were performed prior to mounting on slides in DABCO.

Third instar larvae were decapitated and inverted in PBS prior to fixation in 4.5% formaldehyde for 20 minutes. Rest of staining protocol for third instar larvae is identical to testis staining protocol. After final washes, larvae were further dissected in a drop of PBS on slides in order to isolate testes and ovaries. Dissected samples were mounted in DABCO.

Primary antibodies used were: chicken anti-VASA (K. Howard) at 1:10,000; rabbit anti-SOX100B (S. Russell) at 1:1,000; mouse anti-GFP (Santa Cruz) at 1:50; mouse anti-ABD-B 1A2E9 (Developmental Studies Hybridoma Bank [DSHB]) at 1:50; rabbit anti-ZFH-1 (R. Lehmann) at 1:5,000; rabbit anti-β-GAL (Cappel) at 1:10,000; mouse anti-EYA 10H6 (DSHB); mouse anti-ORB 4H8 (DSHB) at 1:30.

The following secondary antibodies were all used at 1:500: Cy5 goat anti-chicken (Rockland), Alexa 633 goat anti-rabbit, Alexa 546 goat anti-chicken, Alexa 546 or 488 goat anti-rabbit, and Alexa 546 or 488 goat anti-mouse. All Alexa antibodies are from Molecular Probes.

Genotyping and sexing of embryos

In these experiments, *GFP* or *lacZ*–expressing balancer chromosomes were used to distinguish homozygous mutant embryos from balancer-containing heterozygous siblings. To determine sex of embryos, we used a female-specific anti-SXL antibody or X chromosomes containing a *lacZ* transgene *Dfd-lacZ*-H2.7 (Bergson and McGinnis, 1990). When using *Dfd-lacZ*, males carrying the *Dfd-lacZ* X chromosome were crossed to wild-type virgin females. Only female progeny of this cross will possess the *lacZ*-expressing X chromosome whose β-GAL expression pattern is detectable via antibody staining and fluorescence microscopy.

RESULTS:

Sox100B is expressed dynamically in the gonad throughout development

SOX9/*Sox9* is required for human and mouse sexual dimorphism, and shows male-specific gonad expression in a wide array of species with divergent sex determination schemes. In a further attempt to characterize *Sox9*'s evolutionarily conserved role in regulating sexual dimorphism, we examined the expression of its *Drosophila* homolog *Sox100B* in the gonad throughout development. We previously observed that SOX100B is expressed in a subset of embryonic SGPs posterior to the developing gonad that is initially present in both sexes, but only joins the posterior of the male gonad (Fig. 5.1A). This group of cells is termed the male-specific SGPs, or msSGPs, and we have shown that msSGPs die in females prior to gonad formation (Chapter 2).

In late stages of embryogenesis, SOX100B expression is occcasionally observed in other SGPs. These SGPs are likely to be derived from PS12, as they co-express the

posterior homeotic factor ABD-B (data not shown), and are not in the putative stem cell niche in the extreme anterior of the gonad (S. Le Bras and Van Doren, submitted). We have confirmed this by lineage tracing msSGPs (which are derived from PS13). When lineage-tracing experiments are performed to label PS13-derived cells in the male gonad with GFP, only a subset of SOX100B-positive cells express GFP (Fig. 5.1B). This result suggests that multiple SGP cell types derived from different primordia express SOX100B.

While SOX100B expression in msSGPs diminishes by the end of embryogenesis, a new population of cells turns on expression of SOX100B in males. These cells have been characterized as the embryonic precursors of the adult testis pigment cells (Chapter 4), which ensheath the outside of the gonad and exhibit a flattened nuclear morphology (Fig. 5.1C). Both lineage-tracing and genetic experiments indicate that the msSGPs and the pigment cell precursors are different, independent cell types. In *Abd-B* mutant gonads, which lack msSGPs (Chapter 3), we find that pigment cells are still present (Chapter 4). In addition, in our GFP-labeling experiment to mark PS13 cells, we also find that pigment cells do not express GFP, indicating that msSGPs do not give rise to pigment cells. The pigment cells precursors are derived from the fat body, and the expression of SOX100B in these cells is dependent on the signaling molecule Wnt2 (Chapter 4).

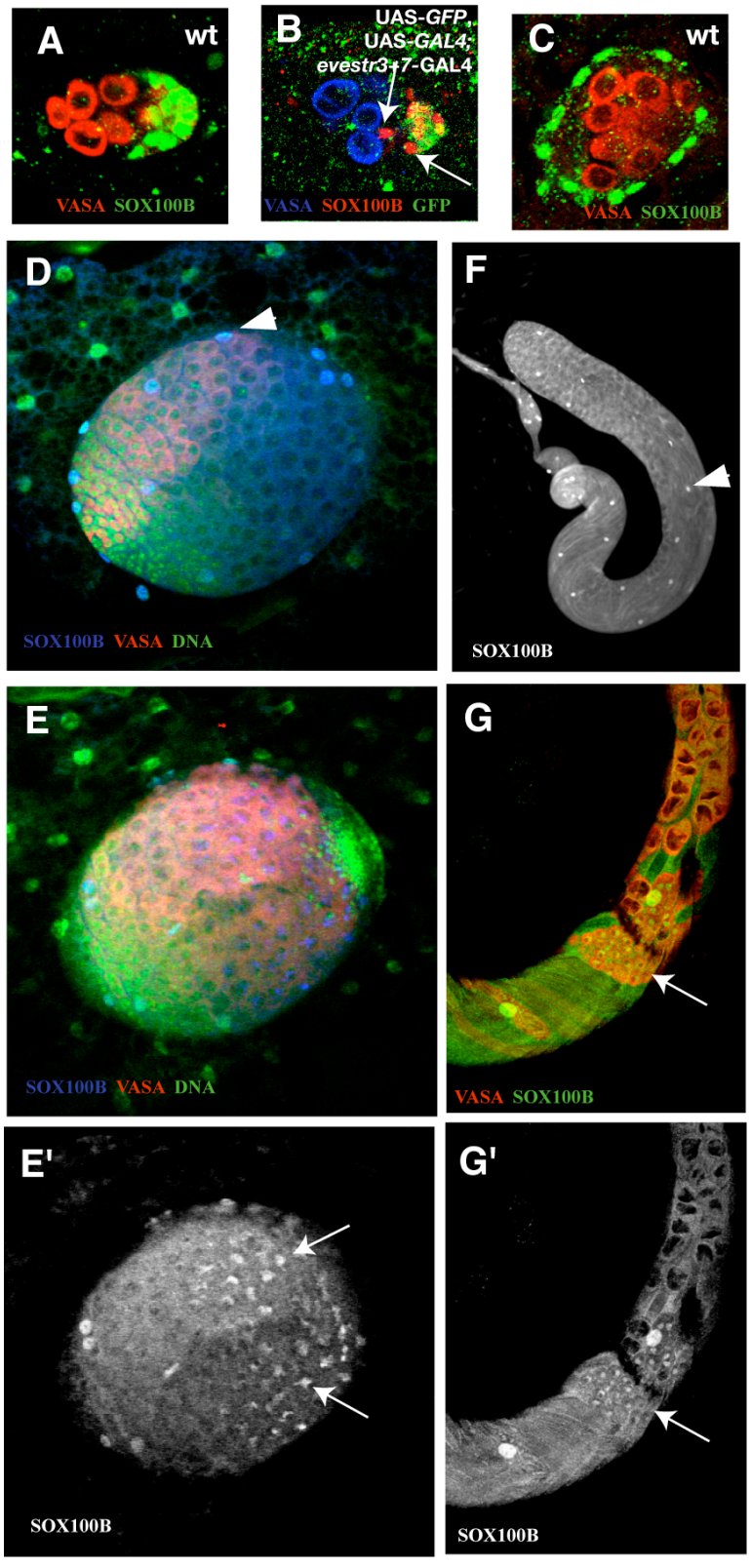
In the third larval instar stage, SOX100B expression is strongly maintained in the nuclei of pigment cells in the male gonad (Fig. 5.1D, data not shown). In addition, at this stage, expression in germ cells is observed. Nuclei of the posterior-most (and presumably the most "differentiated") cysts of spermatocytes are labeled with SOX100B,

while younger, smaller cysts do not show any staining (Fig. 5.1E,E'). It appears that the SOX100B-positive cysts are 16-cell cysts, which have ended their mitotic proliferation and will enter meiosis later on during pupal and adult stages. Additionally, SOX100B is weakly expressed in the nuclei of fat body cells throughout the body (data not shown).

In the adult testis, SOX100B is most prominently expressed in the nuclei of the pigment cells, which comprise the outermost cell layer of the testis and have characteristically large nuclei (Fig. 5.1F). In addition, diffuse staining is observed throughout somatic cyst cells along the length of the testis, however, as this staining is cytoplasmic and excluded from the nucleus, it is not apparent if this SOX100B expression is significant, as the activity of the mammalian homolog Sox9 is shown to be dependent on subcellular localization (de Santa Barbara et al., 2000; Argentaro et al., 2003). Cytoplasmic germ cell expression is also present in mitotic and early meitotic spermatocytes, and staining is consistently excluded from nuclei. Interestingly, nuclear germ cell expression is not seen in 16-cell cysts as was observed in the third instar larva, but rather in the adult testis, nuclear SOX100B is seen in onion-stage spermatids (Fig.5.1G,G'), which are undergoing meiosis and are beginning to streamline their cytoplasm for spermiogenesis. It is difficult to ascertain if SOX100B is maintained in mature sperm, given that the nuclei of sperm after this stage are small and extremely condensed.

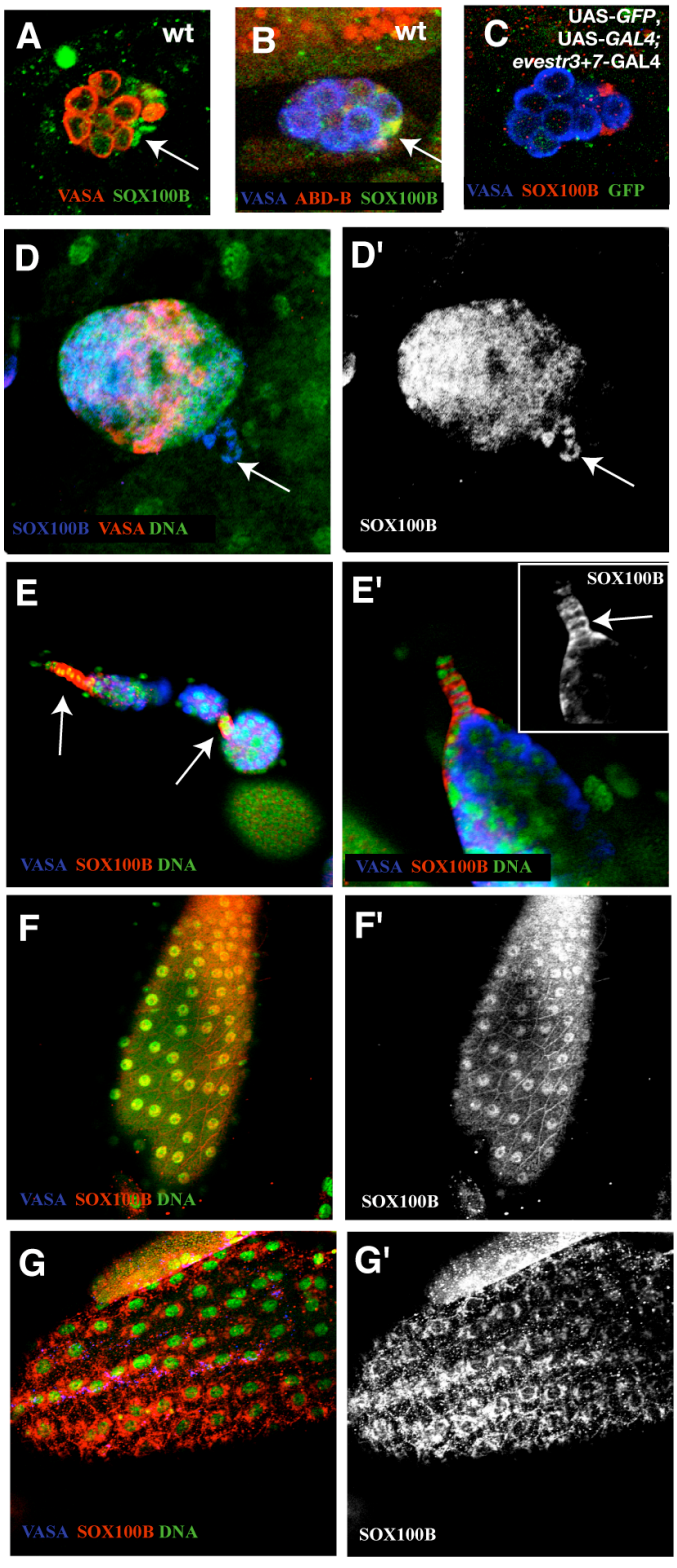
Figure 5.1. SOX100B is expressed in the male gonad throughout development in various cell types.

Immunolabeling of embryonic gonads, larval testes, and adult testes. A) St. 15 wild-type male gonad. Note SOX100B expression in msSGPs. B) St. 15 male UAS-*GFP,* UAS-*GAL4*; *evestr3+7*-GAL4 gonad that expresses GFP in PS13. Note expression of SOX100B in non-PS13 cells (arrows). C) St. 17 wild-type male gonad. SOX100B is expressed in pigment cell precursors that ensheath the gonad, but is not observed in msSGPs. D,E) L3 male testis. Note SOX100B expression in pigment cells (arrowhead) and in spermatoctyes (arrows). E') SOX100B channel only. F,G) Adult wild-type testes. SOX100B is observed in pigment cells (arrowhead) and in spermatid nuclei (arrow). G') SOX100B channel only.


A
wt
VASA SOX100B
B
UAS-GFP,
UAS-GAL4;
evestr3+7-GAL4
VASA SOX100B GFP
C
wt
VASA SOX100B
D
SOX100B VASA DNA
F
SOX100B
E
SOX100B VASA DNA
G
VASA SOX100B
E'
SOX100B
G'
SOX100B

While *Sox9* expression in the female mammalian gonad has not been reported, we do observe some SOX100B expression in the female *Drosophila* gonad. In embryos, in certain genotypes, SOX100B is often present in posterior SGPs (Fig. 5.2A), and lineage tracing and ABD-B expression indicate they are derived from PS12 and not from PS13 (Fig. 5.2B,C). This expression is often not as bright as in males and is often cytoplasmic rather than nuclear. In third instar larvae, SOX100B is expressed in somatic cells of the ovary, in the group of cells that will organize to give rise to the terminal filament, the anteriormost structure of the adult ovary (Fig. 5.2D,D'). In addition, SOX100B is observed in cells adjacent to the posterior of the ovary, whose fate we have not been able to determine (Fig. 5.2D,D'). In the adult ovary, SOX100B is observed in the terminal filament and in interfollicular stalk cells that join consecutive cysts (Fig. 5.2E), however, the majority of this staining is cytoplasmic (Fig. 5.2E'), unlike the staining in the male gonad (Fig. 5.2E' inset). SOX100B is also expressed in follicle cells themselves, with staining being cytoplasmic early on, and then during the penultimate stage of oogenesis (stage 13), SOX100B transiently becomes nuclear in the follicle cells (Fig. 5.2F,F'). By the final stage of oogenesis (stage 14), SOX100B is again excluded from the nucleus (Fig. 5.2G,G').

Figure 5.2. SOX100B is expressed in the female gonad throughout development. Immunolabeling of embryonic gonads, larval ovaries, and adult ovaries. A,B) St. 15 wild-type female gonads. Note ABD-B expression in posterior SOX100B-positive cells (arrows). C) St. 15 female UAS-*GFP,* UAS-*GAL4*; *evestr3+7*-GAL4 gonad that expresses GFP in PS13. Note absence of GFP expression. D) L3 larval ovary. SOX100B expression is strongest in the anterior region, and is also present in posterior cells (arrow). D') SOX100B channel only. E,E') Adult wild-type ovaries. SOX100B is observed in terminal filaments, cap cells, and interfollicular stalk cells, although staining is mostly cytoplasmic (E' inset). F) Stage 13 egg chamber, in which SOX100B staining is nuclear in follicle cells. F') SOX100B channel only. G) Stage 14 egg chamber, in which SOX100B is excluded from follicle cell nuclei. G') SOX100B channel only.


A
wt
VASA SQX100B
B
wt
VASA ABD-B SOX100B
C
UAS-GFP,
UAS-GAL4;
evestr3+7-GAL4
VASA SOX100B GFP
D
SOX100B VASA DNA
D'
SOX100B
E
VASA SOX100B DNA
E'
SOX100B
VASA SOX100B DNA
F
VASA SOX100B DNA
F'
SOX100B
G
VASA SOX100B DNA
G'
SOX100B

SOX100B is not required for embryonic or larval gonad morphogenesis and sexual dimorphism.

Given the expression pattern of SOX100B in the male gonad, we hypothesized that it plays a role in the specification or maintenance of either msSGPs (terminal epithelium) or pigment cells. Therefore, we examined the development of these two tissues in the embryonic gonad of *Sox100B* mutants. msSGPs express Abdominal-B (ABD-B), Zinc finger homeodomain protein-1 (ZFH-1) and Eyes Absent (EYA), and we used these markers to determine the presence of msSGPs in the *Sox100B* mutant embryonic gonad. In *Sox100B* mutant embryos, msSGPs are initially observed in both sexes prior to gonad formation and are present only in male gonads after gonad formation (Fig. 5.3A,B; data not shown).

To assay for pigment cell specification, we used *Kruppel* (*Kr*)-GFP reporter expression (*Kr*-GAL4, UAS-*GFP*), which is male-specifically expressed in the fat body cells that will give rise to pigment cell precursors in the embryo, and which is expressed in adult pigment cells (Chapter 4). In *Sox100B* mutant embryos, *Kr*-GFP is still expressed around the outside of the gonad male-specifically (Fig. 5.3C-F), indicating that pigment cell precursors are specified.

To test for the function of *Sox100B* in SGP formation, we used EYA, ABD-B, and the enhancer trap line lacZ[842] to assay for SGP specification in embryos. All these markers are expressed in the *Sox100B* mutant gonad in a manner similar to wild-type embryos (Fig. 5.3A,B, data not shown). Additionally, the lacZ[842] marker labels the SGPs that may eventually become cyst cells and hub cells, as does the cell-cell adhesion molecule Fasciclin 3 (Fas 3) (Fig. 5.3G; S. Le Bras and M. Van Doren, submitted). In

Sox100B mutants, lacZ[842] and Fas 3 expression is unperturbed, indicating that cyst cells and stem cell niche precursors form normally (Fig. 5.3H; G. Garcia, unpublished observations).

Given that embryonic gonad morphogenesis is unaffected by *Sox100B* mutations, *Sox100B* may be required for the maintenance of gonad cell types into later larval stages. Therefore we examined *Sox100B* mutant testes in the third instar larval (L3) stage. Overall, the morphology of both male and female gonads appears wild-type, and sexually dimorphic size is maintained in the *Sox100B* mutant background, suggesting that sex reversal has not occurred.

To assay for male SGP function, we used EYA to label late larval-stage somatic cyst cells. *eya* is expressed in somatic cyst cells and is required for their function in adult testes (Fabrizio et al., 2003). EYA protein is present in *Sox100B* mutant larval testes, and the organization of the somatic cyst cells around growing cysts of spermatocytes appears wild-type (Fig. 5.3I,J). In addition, EYA antibody labels the terminal body in the posterior of the wild-type larval testis (Fig 5.3I), which is derived from the msSGPs and gives rise to the terminal epithelium (Chapter 4). In *Sox100B* mutant larvae, EYA staining is present in the posterior of the testis in a manner similar to wild-type larvae (Fig. 5.3J), suggesting that the terminal body is present and is of a wild-type size and morphology

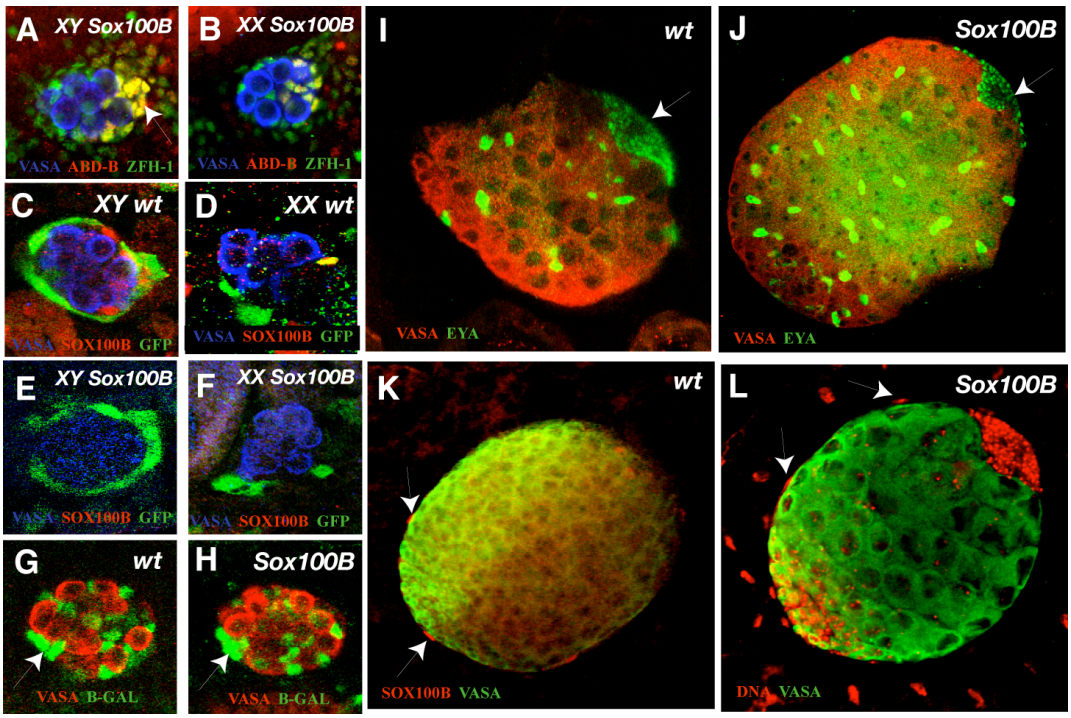
While we have not been able to directly assay for pigment cells in *Sox100B* mutants at this stage with a specific molecular marker, DNA dye experiments indicate that pigment cells are unaffected in L3 *Sox100B* mutants, as characterized by the large size of nuclei, flattened nuclear morphology, and position adjacent to the gonad (Fig.

5.3K,L). However, in 50% of *Sox100B* mutant L3 testes (n=20), DNA staining suggests that pigment cells may be disrupted or fail to be maintained.

Given the normal size of the L3 testis and the wild-type organization of spermatocyte cysts, we do not see any defects in the stem cell niche or hub of *Sox100B* mutant males. Using theVASA antibody to label germ cells, we notice no significant difference between wild-type and mutant germ cell number in L3 larvae. We also note that the rosette structure of germ cells around the hub is observed in *Sox100B* mutant testes (data not shown).

Figure 5.3. *Sox100B* is not required for male embryonic and larval gonad development.

Immunolabeling of embryonic st. 15 (A,B), st.17 (C-H), and L3 larval (I-L) testes. Anterior is to left in all panels. Colors of stainings are as indicated in panels. A,B) *Sox100B* mutant XY A) and XX B) gonads. msSGPs (arrow) are still observed in A) but not in B). C,D) Wild-type XY C) and XX D) gonads expressing *Kr*-GAL4, UAS-*GFP*. Note sexually dimorphic GFP expression pattern. E,F) *Sox100B* mutant XY E) and XX F) gonads expressing *Kr*-GAL4, UAS-*GFP*, which still maintain sex-specific GFP expression around the gonad. G,H) Male wild-type G) and *Sox100B* mutant H) gonads expressing *lacZ*[842]. I,J) Male L3 larval wild-type I) and *Sox100B* mutant J) testes. Terminal cells are labeled with EYA staining (arrows). K,L) Male L3 larval wild-type K) and *Sox100B* mutant L) testes. Pigment cells are distinguishable as large, flat nuclei coating the gonad (arrows). Embryos in A,B) were sexed with an X-chromosome *Dfd-lacZ* construct, and embryos in C-F) were sexed with an anti-SXL antibody.


A XY Sox100B
VASA ABD-B ZFH-1
B XX Sox100B
VASA ABD-B ZFH-1
I wt
VASA EYA
J Sox100B
VASA EYA
C XY wt
VASA SOX100B GFP
D XX wt
VASA SOX100B GFP
E XY Sox100B
VASA SOX100B GFP
F XX Sox100B
VASA SOX100B GFP
K wt
SOX100B VASA
L Sox100B
DNA VASA
G wt
VASA B-GAL
H Sox100B
VASA B-GAL

***Sox100B* is required for adult testis formation.**

Sox100B mutants die as pharate adults (a stage just prior to exit from the pupal case), therefore we cannot assay them for fertility, however, this is at the point by which the morphogenesis of both external and internal adult male reproductive structures is complete in wild-type backgrounds. Mutant external genitalia are still sexually dimorphic and wild-type in morphology, indicating that *Sox100B* is not required for the development of non-gonadal-derived (ectodermal) reproductive organs. Internal genital disc-derived reproductive organs in the male, such as the seminal vesicle, accessory glands, ejaculatory duct, and ejaculatory bulb, are undisturbed in *Sox100B* mutants. However, adult mutant testes are small and atrophic. The size of *Sox100B* mutant testes is less than 10% of wild-type, and the testes fail to show proper coiling as in wild-type adults (Fig, 5.4A,B). We also note that the larval fat body of the mutants fails to histolyze properly and in adults is less structured than in wild-type (data not shown), suggesting that *Sox100B* is required for fat body development.

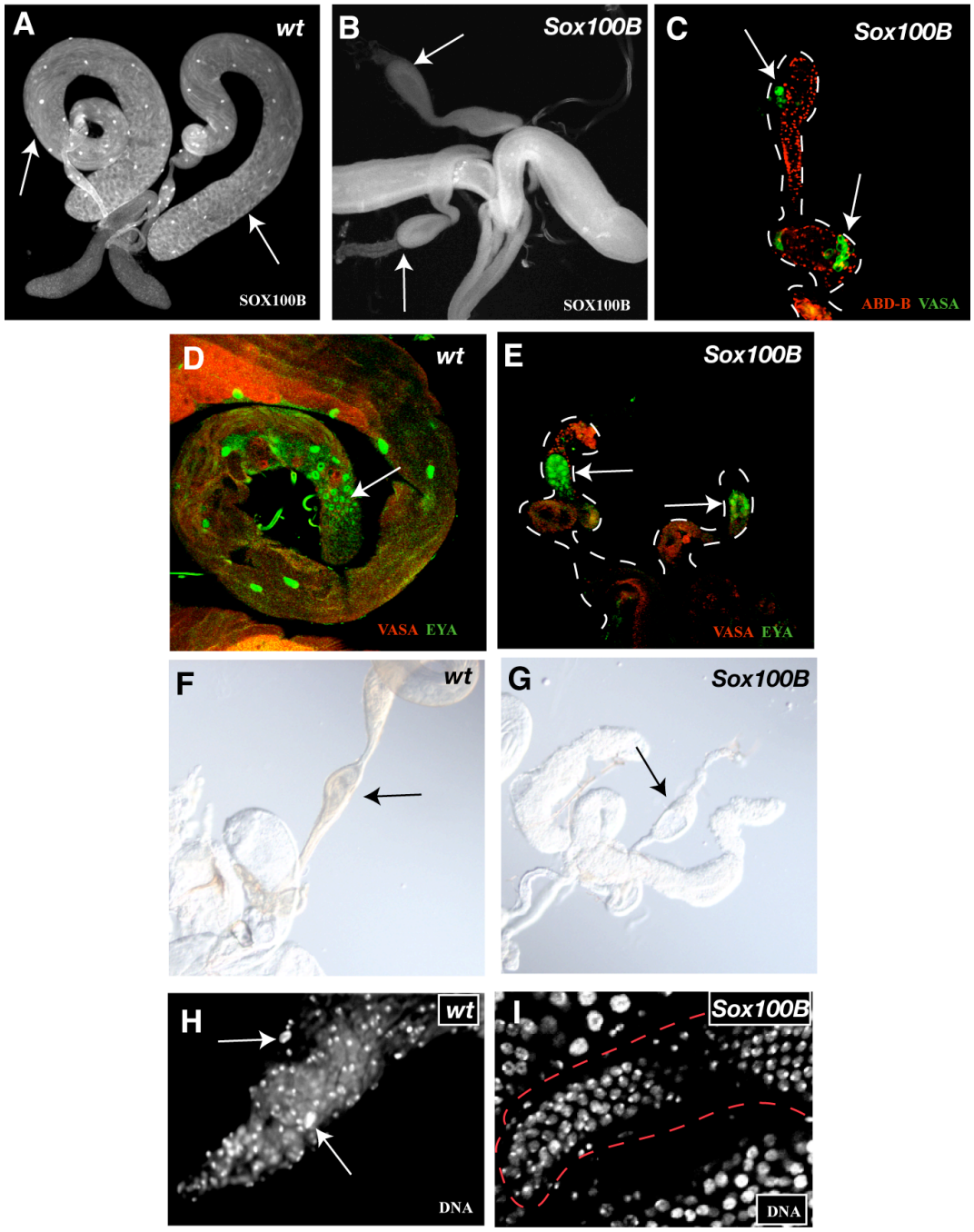
To determine the cause of the *Sox100B* mutant phenotype, we assayed for different adult testis cell types. Using the VASA antibody to label germ cells, we do not observe a differentiated germ line (Fig. 5.4C). Only 5-10 undifferentiated germ cells are present, perhaps indicating that a population of supporting somatic cells is disturbed in mutant testes during pupal and adult development. We used EYA antibody to label somatic cyst cells, which associate with the developing germ line during spermatogenesis (Fabrizio et al., 2003) (Fig. 5.4D) and find that cyst cells are not present in the mutant testis. However, we do find some EYA-positive cells at the base of the testis adjacent to the seminal vesicle (Fig. 5.4E). The position and morphology of these cells indicate that

they are terminal epithelial cells, however, there are not as many cells as in wild-type testes. Given the presence of terminal epithelial cells in the L3 and adult testis, one possibility is that the testis initially joins the genital disc, but atrophies thereafter.

Pigment cells, in addition to aiding in morphogenesis, provide a yellow color to the adult wild-type testis. The pigment cell layer covers the entire length of the testis and ensheaths the genital disc-derived seminal vesicle ending at the junction with the anterior ejaculatory duct (Fig. 5.4F). Adult *Sox100B* mutant testes are unpigmented, as assayed by light microscopy (Fig. 5.4G). Furthermore, DNA dye staining fails to reveal any large nuclei characteristic of pigment cells, suggesting that pigment cells are not present in the mutant testis (Fig. 5.4H,I).

Figure 5.4. *Sox100B* is required for adult testis maintenance.

Immunolabeling of pharate adult testes. Colors are as indicated in panels. A,B) Wild-type A) and *Sox100B* mutant B) reproductive systems. Arrows point to testes. *Sox100B* mutant testes are atrophic, while other reproductive structures are normal in appearance. Magnification of B) is twice that of A). C) *Sox100B* mutant testis, showing a severe decrease in germ cell number (arrows). ABD-B labels the testis muscle sheath. D,E) Wild-type D) and *Sox100B* mutant E) testes. EYA labels terminal epithelial cells (arrows). F,G) Wild-type F) and *Sox100B* mutant G) testes. *Sox100B* mutant testes are unpigmented. Arrow marks seminal vesicle, which is normally a pigmented structure in wild-type testes. H,I) Wild-type H) and *Sox100B* mutant I) testes labeled with DNA dye. Arrows in H) point to large nuclei indicative of pigment cells, which are not observed in *Sox100B* mutants.


A
wt
SOX100B
B
Sox100B
SOX100B
C
Sox100B
ABD-B VASA
D
wt
VASA EYA
E
Sox100B
VASA EYA
F
wt
G
Sox100B
H
wt
DNA
I
Sox100B
DNA

Sox100B is not required for egg chamber formation

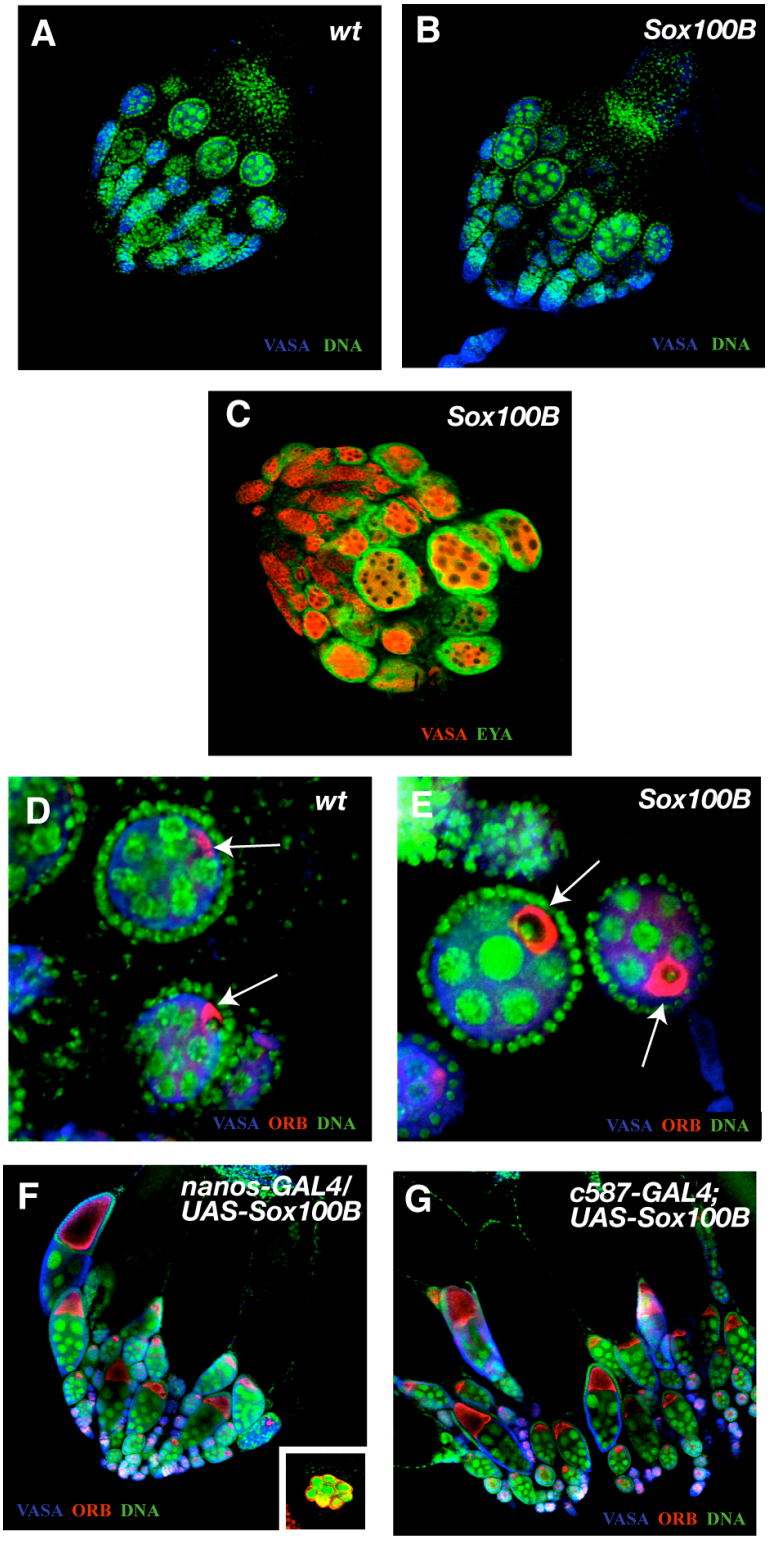
Sox9, the mammalian homolog of *Sox100B*, is required for male sex determination in mice and humans, but a role has not been attruibuted to this gene in females. To determine if *Sox100B* functions in female gonadogenesis, we examined pharate adult mutant ovaries for any defects in oogenesis. DNA staining reveals a normal overall ovarian morphology, with 15-20 ovarioles present with an assembly line of developing egg chambers (Fig. 5.5A,B). The mutant germ line expressed the germline-specific protein VASA and, furthermore, DNA dyes showed that a wild-type number of 16 nuclei are present in developing egg chambers. To test for somatic follicle cell development, we utilized DNA dyes and EYA staining. By these assays, follicle cells appear to be normally specified and encase developing oocytes as in wild-type ovaries (Fig. 5.5C). To assess oocyte specification, we implemented oo18 RNA-binding protein (ORB) antibody labeling, which normally is enriched in the lone cell that will become the oocyte (Lantz et al., 1992) (Fig. 5.5D). In *Sox100B* mutant egg chambers, ORB staining is observed in one posterior germline cell (Fig. 5.5E), indicating that *Sox100B* is not required for formation of the oocyte. Unfortunately, *Sox100B* mutants die as pharate adults, precluding examination of *Sox100B* mutations of late stage oocytes, since vitellogenesis and oogenesis are not completed until after eclosion.

We also determined whether *Sox100B* could be a testis "switch" that induces male-specific development downstream of sex determination. To test this hypothesis, we ectopically expressed *Sox100B* in females, either in the germ line or in the somatic gonad, using the GAL4/UAS system. We utilized *nanos*-GAL4 to drive expression of a UAS-

Sox100B transgene in germ cells (Van Doren et al., 1998) and a *c587*-GAL4 driver to express UAS-*Sox100B* in the somatic cells of late embryonic and larval ovaries (data not shown; Zhu and Xie, 2003). Expression of *Sox100B* produced no phenotype in adult female ovaries; both germline and somatic components were wild-type in appearance, and oocyte specification as assayed by ORB staining was not disturbed (Fig. 5.5F,G).

Figure 5.5. *Sox100B* is not necessary for egg chamber formation.

Immunolabeling of pharate adult ovaries. Colors are as indicated in panels. A,B) Wild-type A) and *Sox100B* mutant B) ovaries. DNA staining suggests overall normal ovariole and egg chamber organization in the mutant ovary. C) *Sox100B* mutant ovary. EYA is expressed in follicle cell nuclei coating developing egg chambers. D,E) Wild-type D) and *Sox100B* mutant E) egg chambers. ORB antibody labeling is enriched in a single oocyte nucleus (arrows). F) *nanos*-GAL4/UAS-*Sox100B* ovary in which *Sox100B* has been expressed in germline. F, inset) St. 17) *nanos*-GAL4/UAS-*Sox100B* st. 17 XX embryo, stained for VASA (red) and SOX100B (green), showing strong SOX100B expression in germ cells. G) *c587*-GAL4; UAS-*Sox100B* ovary, in which *Sox100B* has been expressed in the somatic cells of the gonad. Embryo in F, inset) was sexed with an anti-SXL antibody.


A
wt
VASA DNA
B
Sox100B
VASA DNA
C
Sox100B
VASA EYA
D
wt
VASA ORB DNA
E
Sox100B
VASA ORB DNA
F
nanos-GAL4/
UAS-Sox100B
VASA ORB DNA
G
c587-GAL4;
UAS-Sox100B
VASA ORB DNA

DISCUSSION:

Sox100B is expressed in sexually dimorphic cell types of the gonad

Sox9 has been shown to be male-specifically expressed in the embryonic gonads of a number of vertebrate species, such as human, mouse, trout, fish, and alligator. In this study and in Chapters 2 and 4, we have observed that the expression of SOX100B, a *Drosophila* homolog of Sox9, is also largely male-specific in the embryonic gonad, and is expressed in two sexually dimorphic cell types that will give rise to the testis terminal epithelium and pigment cells. This expression pattern is striking given that the sex determination mechanisms used by these species vary widely, from chromosomal to temperature-sensitive sex determination. Even in animals that utilize temperature-sensitive sex determination, *Sox9* is responsive to temperature levels, indicating a central role in regulating sexual dimorphism (Moreno-Mendoza et al., 1999).

Therefore, while the initial upstream sex determination switches in different animal classes are distinct from one another, such as *Sry* in mammals, *Sex lethal* in *Drosophila*, and temperature for crocodilian and other reptiles, more downstream regulators of sexual dimorphism, such as *Sox9*, appear to be conserved. Our data on SOX100B expression suggests that indeed Sox9 and its homologs are downstream regulators of sexual dimorphism in the gonad and have been evolutionarily conserved in different species.

Sox100B is required for adult *Drosophila* testis development

In this study, we have demonstrated that *Sox100B* is required for adult testis formation and acts during pupal stages to aid in the morphogenesis, maintenance, or survival of the

adult testis. Whereas *Sox100B* embryonic and larval gonads are wild-type in appearance, *Sox100B* mutant adult testes are atrophic. We have shown that there is an absence of germline and somatic components in the testes of *Sox100B* mutant adults. While it is possible that the gonad merely has not fused to the genital disc derivatives during metamorphosis, the presence of germ cells and terminal epithelium (which are derived from the gonad), suggests that at least some contact has occurred between the testis and the genital disc. In addition, the muscle sheath has migrated over the mutant testis, also suggesting that testis-genital disc interactions have taken place. However, it is possible that once contact has formed, the gonad is lost or degraded.

The adult testis phenotype in *Sox100B* mutants may be due to the absence of pigment cells observed in a percentage of L3 mutant gonads. However, a lack of pigment cells is unlikely to give rise to such a severe phenotype. In adult *Wnt2* mutants, which do not posess pigment cells (Chapter 4 and Kozopas et al., 1998), most testis cell types are still preserved in the adult, such as spermatocytes, terminal epithelium, hub cells, and somatic cyst cells (Kozopas et al., 1998). The *Sox100B* mutant phenotype is much more drastic, with only a few germ cells and terminal epithelial cells present. Perhaps the fat body phenotype in *Sox100B* mutants contributes to the degradation of the adult testis. While in *Sox100B* mutant larvae the fat body is normal in appearance, in pharate adults, the fat body appears to lack normal structure (data not shown). Given that there is SOX100B expression in the larval fat body, *Sox100B* may potentially play a role in fat body development. It is not currently known exactly how the the fat body influences the development of the testis, although pigment cells are specified from embryonic fat body cells (Chapter 4). However, since the fat body is intimately associated with the gonad

beginning in embryonic stages (Kerkis, 1931), it is not surprising that the fat body is involved in testis development. An interesting observation is that the female fat body is also affected in *Sox100B* mutants, yet ovary formation is largely wild-type, suggesting that lack of structural support provided by the fat body is not sufficient to explain the *Sox100B* mutant testis phenotype. A possibility is that the fat body is acting through its steroid-producing capacity to influence the maintenance of the adult testis. While the role of steroids in *Drosophila* gonadogenesis have not been thoroughly characterzed, during metamorphosis, large pulses of hormones are responsible for the pupal to adult transition (Riddiford, 1993). Perhaps the fat body produces a hormone that is required by the male gonad to proceed from pupal stages to the fully formed adult testis. Consistent with this hypothesis, in embryonic stages, the pigment cells express *seven-up*, which encodes a putative hormone-binding nuclear receptor (Mlodzik et al., 1990), suggesting that the pigment cells mediate fat body-gonad hormone signaling. Further studies are needed to determine any potential roles for steroid involvement in the *Sox100B* mutant phenotype.

Sox Group E genes are an evolutionarily conserved group of testis formation genes

Members of the *Sox* Group E family of genes, which includes *Sox8*, *Sox9*, and *Sox10* in mammals and *Sox100B* in *Drosophila* (Bowles et al., 2000), have been implicated in sex determination. *SOX9* heterozygosity in humans is responsible for the bone and cartilage disease campomelic dysplasia, and it has been observed that 75% of heterozygous *SOX9* XY individuals also display a male to female sex reversal phenotype (Foster et al., 1994; Wagner et al., 1994). In addition, duplications of *SOX9* have been linked to XX female

to male sex reversal (Huang et al., 1999), indicating that *SOX9* is necessary and sufficient for male sex determination. In mice, *Sox9* is also necessary and sufficient for male development, with *Sox8* acting with conjunction with *Sox9* to initiate the testis formation pathway (Bishop et al., 2000; Chaboissier et al., 2004; Vidal et al., 2001). While there is no loss-of-function data for the role of *Sox9* in other vertebrates, given the nearly universal expression pattern of *Sox9* in the male gonad, it implies that *Sox9* may also be involved in other species.

In this study, we have found that the *Drosophila Sox* Group E gene *Sox100B* is required for male adult gonad formation. While we have not observed a complete sex reversal in *Sox100B* mutants (we observed normal egg chamber formation in the *Sox100B* mutant ovary), a role in testis formation is consistent with the hypothesis that *Sox9* and its homologs are conserved factors that regulate sexual dimorphism and testis morphogenesis. While *Sox9* is involved in Sertoli cell differentiation in the mammal, we have found that *Sox100B* may be required for the maintenance of pigment cells, which likely mediate fat body-gonad interactions in *Drosophila*. These findings add to growing evidence that *Sox9* is an ancient sex determination gene that regulates testis development in multiple animal species.

CHAPTER 6

CONCLUSIONS

Successful sexual reproduction requires sexual dimorphism on many levels. In particular, sexual dimorphism in the gonad is critical for the proper differentiation of germ cells into viable gametes, given that a failure in the process of regulating sexual dimorphism of the somatic gonad invariably leads to infertility. Therefore, a study of male- and female-specific gonadogenesis is potentially a rich area of research for questions of biological and clinical importance.

In this thesis, I have presented an analysis of the establishment of sexual dimorphism in the *Drosophila* somatic gonad. I have discovered that the *Drosophila* gonad is already sexually dimorphic at the time of its initial formation, as evidenced by msSGPs and pigment cells present only in the male gonad. The sexually dimorphic behavior of msSGPs is regulated via the combination of sex determination, acting through the *dsx* gene, and positional information, provided by the posterior homeotic gene *Abd-B*. In addition, the msSGPs express *Sox100B*, the *Drosophila* homolog of *Sox9*, a factor required for mammalian sex determination. I have found another sexually dimorphic cell type that expresses *Sox100B*, the embryonic precursor to the adult testis pigment cell, which is specified from fat body cells ensheathing the gonad. Interestingly, sexual dimorphism of these cells is regulated via a cell non-autonomous mechanism involving *dsx* and the signaling molecule Wnt2. A non-autonomous mechanism of sex determination in pigment cells is reminiscent of mammalian systems, rather than the cell-autonomous mechanism that has been reported for other *Drosophila* tissues (Baker and Ridge, 1980). Finally, I have described a role for *Sox100B* in *Drosophila* testis formation, in which *Sox100B* may act in the pigment cells or fat body to control the morphogenesis and maintenance of the adult testis. The data in this thesis highlight the

common features of mammalian and fly gonad formation and sexual dimorphism, and also supports the hypothesis that sex-specific gonad formation has been evolutionarily conserved at cellular and molecular levels.

Sexual dimorphism is evident upon initial gonad formation

In mice, it is believed that upon initial interaction of germ cells and somatic gonadal cells, the gonad primordium is in a bipotential phase, in which the gonad can be driven towards either male or female development. Prior to this thesis, it was unclear as to whether the *Drosophila* gonad also existed in a bipotential state in which sexual dimorphism was not yet evident. Some evidence suggested that the somatic gonad was already sexually dimorphic in the embryo. The germline expression of the *mgm1* enhancer trap only in male embryos at the time of initial gonad formation is dependent on the somatic sex of the embryo (Staab et al., 1996), suggesting that the somatic gonad is already sexually dimorphic at this stage. Additionally, the JAK/STAT pathway is active only in male embryonic germ cells, depending on signals from the male soma (Wawersik et al., 2005). However, it is unclear how the somatic gonad is different at this stage. In this thesis, I have confirmed that the somatic gonad exhibits a great deal of sexual dimorphism during embryogenesis. The male embryonic gonad contains two sexually dimorphic cell types, the msSGPs and pigment cell precursors, which give rise to two testis-specific tissues, the terminal epithelium and pigment cells. In agreement with these findings, it has also been documented that *esg*-expressing precursors to hub cells are specified in the male embryonic gonad only (S. Le Bras and M. Van Doren, submitted), indicating that sexual dimorphism is evident immediately upon formation of the gonad.

One difference between the mouse and *Drosophila* gonad that leads to a bipotential phase only in one species is likely the expression pattern of the sex determination machinery. In mice, the gonad is formed around day e10, but the *Sry* gene is not expressed there until about one day later (Hacker et al., 1995; Koopman et al., 1990). In *Drosophila*, *dsx* gene expression is observed in somatic gonadal precursors prior to gonad formation and is consistently expressed in the somatic gonad throughout development (N. Crnkovich and M. Van Doren, unpublished; Chapter 4; Berkeley Drosophila Genome Project in situ expression database).

Different cellular mechanisms are used in establishing sexual dimorphism

While the genetic or environmental mechanisms for sexual determination are varied, the cellular consequences that are the manifestation of sex determination are also very diverse. In this thesis, I have shown that the cellular mechanisms used to establish sex-specific cell types downstream of *dsx* are different in msSGPs and pigment cells. Even though *dsx* is required for the two cell types, msSGPs exhibit cell death as a response whereas pigment cell precursors respond by being specified and recruited to the gonad.

Apoptosis as a cellular mechanism to create sexual dimorphism

Programmed cell death is a common method used in different animal species to establish sexually dimorphic development (Conradt and Horvitz, 1999; Uchida et al., 2002). I have shown that the msSGPs rely on a *dsx*- and caspase-dependent mechanism acting through the cell death gene *hid* to ensure female-specific apoptosis. In zebrafish, apoptosis has been also been linked to sex-specific gonad development. In

undifferentiated zebrafish gonads, the tissue is ovary-like and contains oocytes, and in males these oocytes and certain somatic tissues must be removed. It was observed that, in the transition from ovarian-like undifferentiated gonadal tissue to testes, apoptosis was observed in males during the time period of sex determination (Uchida et al., 2002), suggesting that apoptosis is the trigger for testis formation in zebrafish. In *C. elegans*, the central nervous system is highly sexually dimorphic and also is controlled by programmed cell death. Hermaphrodite-specific neurons (HSNs) are initially specified in both sexes (similar to msSGPs), but die by apoptosis in males, by a mechanism in which the feminizing gene *tra-1* represses cell death in females (Conradt and Horvitz, 1999). Finally, in mammals, the ductal systems of the reproductive tract are regulated by apoptosis. The precursors to the male and female reproductive ducts, the Wolffian and Müllerian ducts, respectively, are initially present in both sexes, but one of the two precursors must be degraded during embryogenesis. It has been shown that anti-Müllerian hormone (AMH) is responsible for initiating caspase-dependent apoptosis of the Müllerian duct in males (Roberts et al., 1999).

Cell recruitment and migration as sex-specific cellular responses

In addition to cell death, cell recruitment and migration represent other cellular responses that aid in the establishment of sexual dimorphism. I have shown that the pigment cells in the *Drosophila* gonad are initially neighboring fat body cells that are recruited to the gonad. msSGPs are also recruited to the male gonad, although the migration and recruitment are not sex-specific, since female msSGPs whose cell death is blocked can still join the gonad (Chapter 2). The recruitment of pigment cells to the gonad involves

transduction of a *Wnt* signal sent by SGPs to the surrounding fat body, and the subsequent morphological change of the fat body cells to a flattened morphology. In addition to a role for *Wnt2* in signaling, the flattened morphology of fat body cells likely requires the action of *ems*, whose mammalian homolog is necessary for mouse gonad formation (Chapter 4). This recruitment of cells from neighboring tissue to the male fly gonad has parallels with mouse testis formation, in which mesonephric cells migrate into the gonad (Buehr et al., 1993b; Martineau et al., 1997). The mesonephric cells are required for the formation of testis cords and for proper Sertoli cell function (Tilmann and Capel, 1999).

Non-autonomous, secondary sex determination in *Drosophila* gonad

While cellular responses to sex determination signals may be similar between different species, another interesting aspect of development is how the sex determination information is relayed to sexually dimorphic cells. There are two basic mechanisms: cell-autonomous and non-autonomous. In mammals, only Sertoli cells in the gonad cell-autonomously sense the presence of *Sry* (Burgoyne et al., 1988; Palmer and Burgoyne, 1991). All other tissues rely on hormones to influence their sex-specific development. For most sexually dimorphic *Drosophila* tissues examined, such as the adult cuticle and foreleg, the sex determination genes act cell-autonomously; each cell independently assesses its sexual identity (Baker and Ridge, 1980). There have been instances in which non-autonomous control of sexual dimorphism has been reported in *Drosophila*, however. In the nervous system, male neurons can induce a male-specific muscle fate to

female cells (Lawrence and Johnston, 1986), and a male genital disc transplanted into a female can induce ectopic male pigment formation (Fung and Gowen, 1957).

In this study, I have observed that male-specific cell types in the gonad undergo non-autonomous sex determination, in a manner more closely resembling mammalian systems rather than *Drosophila* tissues. In gonads where msSGPs are feminized via the expression of a *tra* transgene, those feminized cells survive and join the gonad, as in wild-type males (Chapter 4; N.Crnkovich, M. Hoang, M. Van Doren, unpublished). When the fat body is feminized, it also can express *Sox100B*, indicative of male pigment cell fate (Chapter 4). Supporting this hypothesis of a non-autonomous mechanism, I have observed that *dsx* is only highly expressed in the SGPs and not the fat body during embryogenesis, suggesting that the sex of the fat body is not critical for its sexually dimorphic behavior. This situation is similar to the mouse gonad, in which the sex of the gonad is sufficient to induce the migration of mesonephric cells (Capel et al., 1999).

Multiple aspects of sex-specific gonadogenesis are evolutionarily conserved

Although I have shown that cellular and mechanistic aspects of sexual dimorphism are similar in different species, a topic in this field that has interested researchers is the apparent lack of molecular conservation in sex determination. A recent study has examined the conservation of sex-determining genes, in that *dsx* could restore sexual dimorphism in a *C. elegans mab-3* sex determination mutant (Raymond et al., 1998). Thus, *dsx* and *mab-3* represent the founding members of the DM domain gene family whose mammalian homologs have also been shown to be required for male development (Raymond et al., 2000; Raymond et al., 1999).

Another gene that has been implicated as an ancestral sex determination gene is the *Sox* Group E gene *Sox9*. *Sox9* is necessary and sufficient for mammalian sex determination (Bishop et al., 2000; Chaboissier et al., 2004; Foster et al., 1994; Huang et al., 1999; Vidal et al., 2001; Wagner et al., 1994). In addition, it is expressed male-specifically in the embryonic gonad of multiple animal species (de Santa Barbara et al., 2000; Kent et al., 1996; Moreno-Mendoza et al., 1999; Takamatsu et al., 1997). This specific pattern of *Sox9* expression is striking given that these diverse species (e.g., mammals, chick, turtle, trout) use different sex determination switches. The *Drosophila* homolog of *Sox9*, *Sox100B*, was also reported to be expressed in the somatic gonad (Loh and Russell, 2000). In Chapter 3, I observed that *Sox100B* is male-specific in the embryonic somatic gonad, and in Chapter 5, I show that *Sox100B* is required for adult testis formation. While a functional role in sex determination has not yet been assigned to *Sox9* in non-mammalian vertebrates, this role for *Sox100B* in an invertebrate species is consistent with a universal role for *Sox*9 and its homologs in the animal kingdom.

This analysis has shown that *Drosophila* gonad formation and sexual dimorphism share many common aspects with mammals at both cellular and molecular levels. Therefore, the study of sexual dimorphism in model organisms is likely to lead to more insights into the process of sex determination and how an initial sex determination signal is responsible for bringing about a male- or female-specific phenotype. Given the importance of a sexually dimorphic gonad for sexual reproduction, this area of research is likely to have importance for both basic developmental biology and the study of fertility.

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BIRTHPLACE
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EDUCATION:
Ph.D. in Biology 2000-present
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Bachelor of Arts in Biology, Bachelor of Arts in Spanish 1996-2000
University of Virginia, Charlottesville, VA
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GRADUATE RESEARCH:
Graduate research, Johns Hopkins University, Department of Biology 2001-present
Principle investigator: Mark Van Doren
Thesis: "Development of sexual dimorphism in the *Drosophila* gonad"

- Discovered and characterized sex-specific gonad cell types in the embryo
- Investigated genetic and cellular mechanisms for establishment of sexual dimorphism in *Drosophila* gonad
- Established a collaboration to determine a role for *Drosophila* gene (*Sox100B*) whose homolog is involved in human sex determination (Dr. Stephen Russell, Cambridge, UK)
- Trained and mentored an undergraduate student for a research project investigating *BMP/decapentaplegic* signaling in embryonic germ cells

RESEARCH EXPERIENCE:
Undergraduate research, University of Virginia, Department of Biology 1999-2000
Principle investigator: Robert Kretsinger

- Investigated physical and biological properties of crystalline cytoplasmic inclusions in *Paramecium tetraurelia*.

PEER-REVIEWED PUBLICATIONS:

DEFALCO, T., Van Doren M. The sex determination pathway regulates *Wnt2* signaling in order to establish sexually dimorphic pigment cell specification. Manuscript in preparation.

DEFALCO, T., Le Bras, S., Van Doren M. (2004). *Abdominal-B* is essential for proper sexually dimorphic development of the *Drosophila* gonad. *Mech Dev*. 121(11): 1323-1333.

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Creutz, C.E., Mohanty, S., **DEFALCO, T.**, Kretsinger, R.H. (2002). Purine composition of the crystalline cytoplasmic inclusions of *Paramecium tetraurelia*. *Protist* 153(1): 39-45.

PRESENTATIONS:

Talks:

DEFALCO, T., Verney, G., and Van Doren M. Sex-specific apoptosis regulates sexual dimorphism in the *Drosophila* somatic gonad. Society for Developmental Biology 62nd Annual Meeting, July 2003.

DEFALCO, T. and Van Doren, M. Sexual dimorphism in the *Drosophila* embryonic gonad. Baltimore Developmental Biology Research Interest Group, April 2003.

Posters:

DEFALCO, T. and Van Doren, M. Regulation of sexual dimorphism in the *Drosophila* gonad. 46th Annual *Drosophila* Research Conference, March 2005.

DEFALCO, T. and Van Doren, M. Regulation of sexual dimorphism in the *Drosophila* somatic gonad. Cold Spring Harbor Laboratory Germ Cells Meeting, October 2004.

TEACHING EXPERIENCE:

Mentor of Undergraduate Research Project	Summer 2004 -present
Teaching Assistant: Graduate Advanced Genetics and Development	Spring 2004
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AWARDS:

Dupont Teaching Award for Excellence in Teaching, Genetics	Fall 2001
Election into Phi Beta Kappa Honor Society	Spring 2000
Hudd Fund Award (Undergraduate research support)	Summer 1999
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