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Characterization of sterility and germline defects caused by *Smed-boule* RNA-interference

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

By

JESSICA KATHRYNE STEINER
B.S., Wright State University, 2013

2016
Wright State University
WRIGHT STATE UNIVERSITY

GRADUATE SCHOOL

April 25, 2016

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY
SUPERVISION BY Jessica Kathryne Steiner ENTITLED Characterization of Sterility and
Germline Defects Caused by Smed-boule RNA-Interference BE ACCEPTED IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
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ABSTRACT

Steiner, Jessica Kathryn. M.S. Department of Biological Sciences, Wright State University, 2016. Characterization of sterility and germline defects caused by \textit{Smed-boule} RNA-interference.

Evolutionarily conserved molecular processes involved in construction of the germline and embryonic development are essential for the procreation of many species. Infertility affects 15% of couples in the world and can be caused by dysfunctions during egg and sperm development, anatomic defects, as well as faulty embryonic development. Although there are some infertility disorders that are genetically defined, such as Turner and Klinefelter syndromes, many clinical infertility cases are diagnosed as idiopathic due to the lack of understanding of basic fertility mechanisms. \textit{Schmidtea mediterranea} is a freshwater planarian species that has the ability to regenerate complete organisms, including germ cells and reproductive structures, from small tissue fragments containing pluripotent somatic stem cells. The developmental plasticity of planarians provides a wonderful opportunity to investigate the molecular mechanisms behind the differentiation and development of specialized cells, including gametes. \textit{Smed-boule} encodes for an RNA-binding protein and is the most ancestral member of the \textit{Deleted in AZoospermia} (\textit{DAZ}) gene family. \textit{DAZ} family genes function in different aspects of germ cell development and fertility in species ranging from sea anemone to humans. Whole-mount \textit{in situ} hybridization experiments revealed \textit{Smed-boule} expression is enriched in the testes and ovaries of planarian flatworms. Interestingly, \textit{Smed-boule} RNA-interference (RNAi)
planarians lost the ability to produce gametes, yet still were able to deposit sterile egg capsules. Virgin *Smed-boule(RNAi)* and control planarians maintained in isolation also continuously produced sterile egg capsules. Altogether these results demonstrate that egg capsule production in *S. mediterranea* occurs independently of ovulation, fertilization, and mating events. In addition, detailed analysis of gametogenesis defects revealed that *Smed-boule* functions at different stages during male and female germline development. These findings provide novel information about the evolution of *boule* and *DAZ*-family gene expression and function in sexual reproduction.
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Chapter 1

Introduction
1.1 Mechanisms involved in germline development

Sexually reproducing species depend on the proper differentiation of their germ cells in order to procreate. In humans, 15% of couples in the world suffer from infertility and are often diagnosed as idiopathic due to the lack of understanding and scientific knowledge that describe the molecular events for generation of sperm and ova (Matzuk & Lamb, 2002). Gametes (sperm and ova) arise from primordial germ cells (PGCs), a type of stem cell that holds all the information to give rise to all of the cells in the germline. The PGCs divide and proliferate to create germline stem cells (GSCs), cells that will either continue to self-renew as stem cells or carry out gametogenesis, the process of germ cell differentiation into gametes, oocytes and sperm (Seydoux & Braun, 2006). By better understanding the genetics and molecular mechanisms responsible for the creation of these cells, scientists will gain many advantages for biomedical and therapeutic applications, as well as answers to how these cells have evolved over time.

Germline stem cells are characterized by their expression of a group of core genes that are specific to cells in the germline. This includes *vasa, piwi* and *nanos*, all known as the core group of genes that are conserved and used across metazoans (Seervai & Wessel, 2013). PGCs are stem cells that expand their population through multiple rounds of mitosis and migrate to the site of the developing gonad during embryogenesis. These cells decide to either maintain their stemness and continue to divide, or to differentiate into mature gametes (Spradling et al., 2011). In many animals, and all mammals analyzed thus far, the differentiation potential of PGCs relies on signals from the surrounding
environment, and such mechanisms vary widely across many species (Lesch & Page, 2012).

There are two molecular mechanisms that direct germline formation; inherited and inductive determination (Figure 1.1). These mechanisms specify PGCs that will give rise to the rest of the germline. The inheritance (preformation) based mechanism involves maternal mRNAs and proteins (germ plasm) that accumulate in specific regions during oogenesis and early embryogenesis. (Extavour & Akam, 2003). The term preformation refers to preformed germ plasm, material that present in the egg cytoplasm and serve as initial material for germline determination. This method of germline determination is utilized and well-studied in organisms such as *Drosophila melanogaster, Caenorhabditis elegans, and Xenopus laevis*. The germ plasm is a mass of specialized cytoplasm that is abundant in RNAs and proteins and involved in translational regulation (Lesch & Page, 2012). Their germ plasm is set aside during oogenesis and is segregated asymmetrically, at one pole of the oocyte (Figure 1.2B). The pole cells present in that region acquire the germ plasm, becoming PGCs and later carry out gametogenesis. In *C. elegans*, germline precursor distribution is initiated after sperm fertilizes the egg. Once the zygote is formed, the germ plasm becomes asymmetric after a few cellular divisions, incorporates into blastomeres, and eventually become PGCs (Figure 1.2A) (Lesch & Page, 2012; Nakamura & Seydoux, 2008).

In contrast to the inheritance based mechanism, the inductive germline determination mechanism does not use localized maternal products to drive germline differentiation. Instead, the inductive mechanism involves cellular interactions and positional information to assign the fate of their germline and takes place later in
embryogenesis (Figure 1.2C) (Seervai & Wessel, 2013). All mammals studied to date use the inductive method to specify their germline (Lesch & Page, 2012). Most studies of inductive germline differentiation have been done in the mouse. PGCs in mice are first apparent after gastrulation, when the blastula forms the epiblast, endoderm, mesoderm and ectoderm. Embryonic stem cells in the epiblast give rise to PGCs in response to cellular signaling from extraembryonic tissues (Tam & Zhou, 1996). The recruitment and signaling to induce cells to become germ cells are position dependent. Cells from the distal region of the epiblast of a mouse embryo were transplanted into the proximal region of the epiblast and later turned into PGCs (Tam & Zhou, 1996). This finding shows that germline stem cell fate in mice is site specific and is influenced by cell signals from tissues in the developing embryo.
Figure 1.1. An image presenting inherited versus inductive germline formation. Red represents expression of the core germ line determination genes. These methods are used by metazoans, yet each organism differs when it comes to when and how the genes are utilized during germ line development. During the inherited mechanism, localized maternal products accumulate in specific regions of an egg or embryo and the cells present there absorb the material and begin germline determination. This mechanism is used by *C. elegans* and *Drosophila* and is the most widely studied. The inductive mechanism relies on cellular interactions that occur in the late stages of embryogenesis (post embryonically). Figure adapted from (Seervai & Wessel, 2013).
Figure 1.2. Early germ cell development in *Drosophila, C. elegans* and mice. A) Germ plasm (pink) in *C. elegans* is segregated asymmetrically in the zygote and eventually distributes to germline blastomeres. Primordial germ cells (PGCs) eventually migrate inside the embryo. B) In *Drosophila*, germ plasm is gathered and preformed during oogenesis at the posterior pole of oocytes. The germ plasm is acquired by cells present at the posterior pole, becoming PGCs, and is carried inside the embryo to form embryonic gonads. C) In mammals, namely mice, during embryonic development, signals coming from the extraembryonic ectoderm to epiblast cells leads to gene expression. The activated epiblast cells proliferate and form PGCs that migrate to the somatic gonad. Figure adapted from (Nakamura & Seydoux, 2008).
1.2 Conserved core gene set required for germline determination

The viability of metazoan PGCs relies on three core genes, *vasa*, *piwi*, and *nanos*, for development. The expression of these genes varies widely amongst different organisms, but they are found in all animals during the development or maintenance of germline cells (Seervai & Wessel, 2013). Vasa, a DEAD-box RNA helicase, functions by regulating the translation of specific mRNAs. DEAD box proteins have many cellular functions, one of which is unwinding RNA duplexes so that other molecular interactions can occur (Gustafson & Wessel, 2010). In *Drosophila*, Vasa was characterized and found to function in embryonic patterning and germ-cell specification. *Drosophila* is known to use pole granules for germ cell determination and preformation of their germline. Pole cells in *Drosophila* accumulate at the posterior pole of the embryo, inherit the pole plasm, and become PGCs (Extavour & Akam, 2003). Vasa protein localized in the pole granules in *Drosophila* embryos interacts with initiation factors to regulate the translation of mRNAs that are required for germline differentiation and embryonic patterning. Mutations of *vasa* resulted in the failure of *Drosophila* to develop germ cells, as the polar granules and pole cells were absent, which are crucial for germline development. The *vasa* mutants also caused developing embryos to have defects in their abdominal segments (Hay et al., 1988; Schüpbach & Wieschaus, 1986).

Piwi is a member of the Argonaute family of small RNA-binding proteins and was originally identified in *Drosophila* to be required for germline stem cell differentiation (Lin & Spradling, 1997). These proteins bind non-coding RNAs called piwi interacting RNAs (piRNAs) that are enriched in sequences from transposons (Ma et
This is critical during germline development, as mutations could be carried on with future generations. piRNAs have sequences that are antisense orientation to transposon transcripts and thus, can hybridize and form complexes with them (Ishizu et al., 2012). Like Vasa, Piwi proteins are present in the pole cells of *Drosophila* embryos. In adult *Drosophila*, Piwi is also present in somatic cells of male and female gonads (Juliano et al., 2011). Mutations in *piwi* caused severe defects in spermatogenesis and oogenesis in *Drosophila* (Lin & Spradling, 1997), which is consistent with the idea that Piwi proteins are required for GSCs maintenance and gametogenesis.

*Nanos*, the third key germline determinant, has also been well characterized as a gene required for germ cell development and is also expressed in PGCs. In *Drosophila*, *nanos* is required for germ cell maintenance (Hayashi et al., 2004) and differentiation (Forbes & Lehmann, 1998). In PGCs that lack *nanos*, cells have mitotic defects and fail to migrate to somatic gonads, resulting in the loss of stem cell identity. *Cyclin B* mRNA translation is required for cells to enter mitosis and to carry out cell division (Castedo et al., 2002). Nanos functions to repress *Cyclin B* mRNA in PGCs after formation in posterior poles of the *Drosophila* embryo (Asaoka-Taguchi et al., 1999), so it makes sense that mutations in *nanos* would have defects in the cell cycle process and have improperly formed PGCs and other cells associated with the germline. These germline requirements for *nanos* are also conserved in *C. elegans* (Subramaniam & Seydoux, 1999), zebrafish (Köprunner et al., 2001), planarians (Wang et al., 2007), the mouse (Tsuda et al., 2003) and other animals.

*Vasa, piwi, and nanos* have conserved germline determination functions that are used across metazoans. However, the way these genes are utilized are different from
species to species because there is a wide range of reproductive strategies, as well as alterations in developmental pathways, utilized by each organism. These core germline determinants are found in all metazoans during development of a germline and some have a molecular function described, but this list is certainly not limited (Lesch & Page, 2012). There are many genes whose identity has yet to be discovered that are necessary for germline construction. Many genes and proteins are required for successful construction of the germline, and it is obvious that genes and gene products do not function in isolation, especially when these genes are characterized as RNA binding proteins. Often times genes and gene products work in protein networks and serve as components of a complex of macromolecules that carry out normal cellular functions (Zhong et al., 2009), for example construction of a germline. By losing function of one gene, a loss of interaction with normal binding partners must be associated to cause significant phenotype such as infertility. Many genes are necessary for constructing a germline and their germ cells, and identifying these unknown genes and their interacting partners will be crucial for understanding and improving current untreatable, idiopathically diagnosed illnesses, such as human fertility disorders.
1.3 Gamete Development: Meiosis and Cellular Maturation

The development of an embryo begins with a single cell that contains all of the information that is correctly expressed in daughter cells and eventually divides to form the correct tissues in the correct locations to form an organism. In order to gain an understanding of how an embryo develops, it is crucial to have an understanding of the development of gametes and the process of fertilization, as the first step of development is dependent on sperm and egg uniting.

1.4 Meiosis

The genetic content within each gamete is distributed during meiosis. The goal of meiosis is to create haploid gametes that each contain one copy of each chromosome, for most organisms. Meiosis is ultimately the division of chromosomes by the standard phases of cell division: prophase, metaphase, anaphase, and telophase. During gametogenesis, there are 2 rounds of meiotic cell divisions that will give rise to haploid daughter cells. Primordial germ cells (PGCs) divide and produce spermatogonia or oogonia. These cells replicate their DNA and become primary spermatocytes/oocytes. After the first meiotic division, they are called secondary spermatocytes/oocytes. Completion of the second meiotic division yields spermatids and eggs (Kalthoff, 1996). This process is fundamental so that upon fertilization, the fusion of egg and sperm will restore the diploid chromosome number needed to create an organism (Von Stetina & Orr-Weaver, 2011).
In humans, male and female meiosis occurs at different time periods. In females, meiosis I begins before the embryo is born, and the oocytes are arrested in meiotic prophase I until it matures years later during a menstrual cycle. Arrest during prophase I ensures growth, differentiation and stockpiling of maternal components in the oocyte that are crucial for a developing embryo (Von Stetina & Orr-Weaver, 2011). This step is conserved across metazoans, oocytes are in limited supply and the investment into their production is crucial for reproductive success. Depending on the organism, the arrest during prophase I can be maintained for a few days (*Drosophila*), to up to 50 years (humans) (Kalthoff, 1996; Von Stetina & Orr-Weaver, 2011). In human males, meiosis begins after birth, specifically during puberty, and continues throughout a lifespan. For males, each primary spermatocyte will produce four sperm and the germline stem cell population constantly renews itself after puberty. On the other hand, the human female oocyte population number is established before they are born, and each primary oocyte will yield one mature oocyte (Sweeney, 1998).

1.5 Cellular maturation of gametes: Oogenesis vs. spermatogenesis

1.5.1 Oogenesis

Oogenesis differs from spermatogenesis in that it is a single cellular process that combines both steps of meiosis and cell maturation. In female mammals, oogenesis begins *in utero* in the developing embryo. PGCs first differentiate and migrate to gonadal tissue, where they are then referred to as oogonia. Here the oogonia will begin the first round of meiosis and proliferate, giving rise to primary meiotic oocytes. The primary
meiotic oocytes are arrested in prophase I, ending female germ cell production, and remain arrested throughout most of the adult life (Figure 1.3). These oocytes mature upon reaching puberty, and only one oocyte matures at a time and gives rise to a single egg. As the follicular cells increase in number, the oocyte increases in volume and matures, yet is still arrested in prophase of meiosis I (Gilbert, 2000; Tilly, 2001). During this growth phase, the oocyte is actively synthesizing building blocks such as RNAs and proteins that are maternally provided and crucial for the early development of the embryo. These maternal resources are integrated in the oocyte during the meiotic arrest (Kalthoff, 1996). In many animals, yolk also accumulates inside the oocyte during meiotic arrest during a process called vitellogenesis. Animals such as birds and reptiles have very large eggs and need to synthesize more yolk to support embryogenesis after oviposition, in contrast to mammalian eggs, which are continuously provided with nutrients from the mother after fertilization (Kalthoff, 1996).

Upon arrest in prophase I, the oocyte is considered fully grown and can now prepare for the second meiosis to generate the fully mature oocyte (Figure 1.3). Maturation involves chromosome condensation, nuclear envelope breakdown, and release of the oocyte from the ovary, or ovulation. The oocyte becomes an egg only once it is released from the oocyte and ovulated. Meiosis I is complete upon ovulation, and the egg is then ready for fertilization. The egg is arrested in metaphase II until it is fertilized, and only then will meiosis be complete (Figure 1.3) (Kalthoff, 1996).
Figure 1.3. Human oogenesis. Primary oocytes arrest during prophase I of meiosis. In response to hormone signaling during menstruation, primary oocytes will continue through meiosis. Image adapted from Young (2006).
1.5.2 Spermatogenesis

Meiosis in males occurs during spermatogenesis and ends with a processes of cellular maturation for meiotic products, referred to as spermiogenesis. Meiosis and spermiogenesis ultimately form a motile nucleus that will inject genetic content into an egg. In mammals, the development of sperm is initiated at puberty and begins with the primordial germ cells that are located in the seminiferous tubules of the testes (Figure 1.4). The cells in the earliest stages of spermatogenesis are found within the periphery of each tubule, and the more advanced cell stages will be found toward the interior (Kalthoff, 1996). Spermatogenesis begins with PGCs becoming mitotically active and producing spermatogonia. The spermatogonia initiates meiosis I by replicating their DNA, forming primary spermatocytes (Figure 1.4). Meiosis I concludes as the primary spermatocytes divide, forming two daughter cells termed secondary spermatocytes. These cells divide during the second meiotic division, producing four round haploid cells, the spermatids (Figure 1.4). Each cell division corresponds to the maturation of the particular cell involved in spermatogenesis. Thus, as the cells move towards the center of the lumen, the maturation process from spermatogonial stem cell to mature, flagellated sperm progresses (Figure 1.4) (Sweeney, 1998).

Spermiogenesis is the process of each haploid spermatid maturing and differentiating into an elongated spermatid, or spermatozoa. During this stage a number of cellular processes are performed to produce a compact propulsion system that ultimately fuses with an egg and delivers DNA. First, an acrosomal vesicle forms around the head of the spermatid, essentially capping the nucleus (Kalthoff, 1996). The
acrosomal vesicle is essentially a giant lysosome whose contents will secrete and digest surrounding materials, crucial for sperm penetration of the egg of interest during fertilization (Berruti & Paiardi, 2011). Opposite of the acrosome exists a growth of microtubules that forms the flagellum, which is surrounded by mitochondria. The last steps of spermiogenesis includes condensing of the nucleus and the bulk of the cytoplasm pinches off (Gilbert, 2000). Ultimately, spermiogenesis creates mature sperm with a head, midpiece and tail. The flagellated tail will propel the sperm that is destined to reach the egg. The midpiece is surrounded by mitochondria and glycogen which provide the energy for propelling the sperm and making it motile. Lastly, the acrosomal vesicle surrounds and protects the nucleus (Sweeney, 1998).
Figure 1.4. Human spermatogenesis. A section of the seminiferous tubule shows the process of spermatogenesis. As cells mature and differentiate, they advance towards the lumen. Image adapted from (Gilbert, 2000).
1.6 Planarians as a model for sexual reproduction

The planarian flatworm *Schmidtea mediterranea* is an exceptional model organism to study developmental and reproductive biology. *S. mediterranea* has the ability to continuously regenerate their germ cells and accessory reproductive structures from tissue fragments that lack reproductive organs (Newmark et al., 2008). Their regenerative abilities are made possible through the use of their neoblasts, a pool of pluripotent somatic stem cells that are maintained throughout the lifespan of each planarian. Importantly, like mammals, the germline of the planarian flatworm is specified through inductive signaling (Newmark & Sanchez Alvarado, 2002; Wang et al., 2007), in contrast to *C. elegans* and *Drosophila*, who specify their germlines via preformation (Lesch & Page, 2012).

In the laboratory, *S. mediterranea* is maintained as two strains: sexual and asexual. In the wild, the planarians can alternate between the two modes of reproduction depending on the season (Newmark & Sanchez Alvarado, 2002) (Figure 1.5). Asexual animals reproduce by transverse fission; by adhering themselves to a substrate and stretching until breaking into two fragments. Each fragment regenerates into two complete animals (Figure 1.5A). Sexual planarians are cross-fertilizing hermaphrodites and deposit egg capsules. Depending on the species, each egg capsule has the potential to hold up to twenty hatchlings (Newmark & Sanchez Alvarado, 2002) (Figure 1.5B). Upon reaching a certain size of maturity, the planarians develop ovaries, testes, gametes, accessory glands and a copulatory apparatus. They have two ovaries located ventrally at the base of the brain, and numerous testes which are located on the dorsal side and run bilaterally (Figure 1.5A) (Newmark & Sanchez Alvarado, 2002). The developmental
plasticity of planarians that allows them to regenerate their entire reproductive system provides a wonderful opportunity to investigate the mechanisms behind the differentiation and development of the germline post embryonically.
Figure 1.5. Planarians have two modes of reproduction. A) Asexual planarians reproduce by transverse fission. This process involves adhering to a substrate and stretching into two pieces. Each piece regenerates into a complete organism within a week. B) Sexually mature planarians are hermaphrodites and reproduce by cross fertilization. Developing embryos are deposited in egg capsules, which release up to 20 hatchlings depending on the species (Newmark & Sanchez Alvarado, 2002).
1.7 Vitellocytes (yolk cells) and Egg Capsule Production

The planarian reproductive system is sophisticated and parental contributions to the embryo not limited to products from the testes and ovaries. The vitelline glands, or yolk glands, are also part of the reproductive system that contribute to development of the embryo(s) in planarians. The vitelline cells are present on the outside of the embryo during development which makes these organisms ectolecithal. Ectolecithy is defined by an organism’s germline separation into oocytes and yolk cells. These glands deposit yolk cells, or vitelline cells, on the egg as it travels down the oviduct for deposition (Figure 1.5B) (Newmark & Sanchez Alvarado, 2002). The vitellocytes are a very important structure for reproduction in Platyhelminthes and serves two main functions: egg shell formation, and to provide nutrition for the developing embryo (Swiderski & Xylander, 2000). These events are due to the vitellocyte’s high rate of metabolic activity, specifically protein synthesis and glycogenesis (Swiderski & Xylander, 2000).

Upon fertilization, depending on the species, potentially up to 20 zygotes are formed and surrounded by an abundance of yolk cells. As the egg moves down the oviduct for deposition, yolk is deposited on the outside (Newmark & Sanchez Alvarado, 2002). The embryo will ingest the surrounding encapsulated yolk cells throughout its development via the provisional pharynx. This pharynx is eventually replaced by the definitive pharynx, the permanent structure that is used for food intake once emerging from the egg capsule (Sanchez Alvarado, 2003).
1.8 Parthenogenesis and matrotrophy

Many species have reproduction strategies that allows the development of an embryo where no sperm is involved at all. Parthenogenesis is defined as a reproductive strategy where no sperm is needed to prompt embryonic development in an oocyte. The mother can create viable offspring with no paternal inheritance. In other words, sperm does not fertilize the oocyte, yet an embryo still develops and offspring is produced (Bos-Mikich et al., 2016). Parthenogenesis in flatworms has also been documented. *Schmidtea polychroa*, a freshwater planarian is a simultaneous hermaphrodite that can reproduce sexually or by parthenogenesis (Pongratz et al., 2003). This phenomenon has been documented in vertebrates such as whiptail lizards (Crews et al., 1986), boa constrictors (Booth et al., 2011), and avians such as quails, turkeys and chickens (Parker & McDaniel, 2009; Yao & Olsen, 1955). In turkeys, it has been found that in some cases unfertilized eggs laid have resulted in the formation of embryonic tissues, and in some extreme cases, the contents inside the egg reached full development and the embryos were viable, with beating hearts and even fully developed vascular systems (Olsen & Marsden, 1953). The haploid oocyte is activated by an unknown stimulus, undergoes cell division and along the way becomes diploid (Cassar et al., 1998). Not all of these eggs are viable, yet the tissue that develops in these infertile eggs somehow become diploid, making it tasking to differentiate normal and parthenogenic embryos under the microscope (Yao & Olsen, 1955).

One possible explanation for an activating factor in these parthenogenic eggs could be the vitelline cells, a feature present across cestode flatworms and is essential for
egg capsule development (Swiderski & Xylander, 2000). Egg capsule development has been attributed to vitellogenesis in cestodes, and has been shown to be a key process in the formation of oocytes in tapeworms, namely the parasitic Neodermata (Yoneva et al., 2015). Vitellogenesis has also been shown to contribute to embryonic nutrition in lizards and fish (Stewart & Thompson, 1993). The use of vitelline cells during embryonic development is termed matrotrophy, described as the distribution of extra-vitelline nutrients during gestation, also referred to as extraembryonic nutrition (EEN) (Ostrovsky et al., 2015). Similarly, placentotrophy occurs in humans and other mammals and vertebrates, where nutrition is provided through the placenta (Ostrovsky et al., 2015). It is clear that these features are important in the viability of many organisms’ offspring and therefore is a likely candidate as an activating factor in parthenogenic eggs.

1.9 Planarian flatworms as a model for studying germline development

It is important to decipher the mechanisms involved in germ cell specification, as many genetic and molecular mechanisms still need to be understood. We face limitations in this field as most of the information to date explaining the mechanisms behind germ cell specification has been done in Drosophila and C. elegans, both of which use the preformation method. Studies deciphering epigenesis, genetic expression influenced by the environment, has been limited to the mouse model (Seervai & Wessel, 2013). In order to investigate the epigenetic mechanism of germ cell specification, to question whether these mechanisms are conserved amongst organisms or diverged, it is crucial to analyze and compare germ cell specification in many organisms.
Planarian flatworms have been well documented as organisms for studying germline development. *nanos* is well known to have functions in germ cell maintenance and are conserved throughout metazoans. Wang et al identified an ortholog of *nanos* in *S. mediterranea*. Whole mount *in situ* hybridization (WISH) revealed *nanos* was expressed in the testes and ovaries of planarians (Wang et al., 2007). Genetic loss of function by RNA interference (RNAi) is performed in planarians by administering double stranded RNA during feedings (Rouhana et al., 2013). This results in the disruption and maintenance of the tissue where the gene of interest is expressed. This method has proved success for genetic characterization of the germline in planarians (Chong et al., 2013; Wang et al., 2010; Wang et al., 2007). The outcome of *nanos* RNAi was that the planarians lacked their reproductive structures, germline stem cells, and consequently their gametes (Wang et al., 2007).

The absence of a germline in *nanos*(RNAi) planarians lead the authors to identify the underrepresented genes by microarray analyses comparing the transcript expression profiles of *nanos*(RNAi) juvenile sexual planarians to control planarians (Wang et al., 2010). They were able to identify 178 underrepresented genes that have homologs in other organisms. Genes underrepresented in *nanos*(RNAi) are likely to play a role in germ cell development. They validated expression patterns of 93 genes in the testes and 3 more in the ovaries. RNAi was performed to identify a function for the genes with germline expression and revealed 13 of the genes tested were required for various stages of planarian germ cell development including testis formation, maintenance, spermatid elongation or meiotic progression (Wang et al., 2010).
Altogether, Wang et al identified genes required for major stages of male germ cell development in planarian flatworms. The genes identified for germline development in their results demonstrates that planarians are a very valuable model organism to study epigenesis, germ cell specification from soma. The work from Wang et al pioneered the future using planarians as a model for screening a large number of genes, and ultimately a great resource to better understand the mechanisms behind germline development and germ cell differentiation.
Chapter 2

Genetic screen of candidates identified through RNAseq analysis of \textit{CPEB1(RNAi)} animals
Cytoplasmic Polyadenylation Element Binding Protein (CPEB) controls the translation of mRNAs during germ cell development and maturation by either repressing or activating translation (Mendez & Richter, 2001; Rouhana & Wickens, 2007). The mRNAs are translationally repressed when CPEB recruits translational repressor proteins such as Maskin, which binds to the cap-binding protein eIF4E and prevents translation initiation (Cao & Richter, 2002). mRNAs can be translationally activated by CPEB when it recruits GLD2, a cytoplasmic poly(A) polymerase that catalyzes the synthesis of a poly(A) tail (Rouhana & Wickens, 2007). Two planarian homologs of CPEB have been shown to have separate sex functions in the development of male and female germlines and sexual maturation (Rouhana et al., unpublished). Preliminary research for this proposal identified a homolog of CPEB; Smed-CPEB1, which is required for oogenesis and yolk gland development in planarians. Decreased expression through RNAi of Smed-CPEB1 left animals without oocytes and yolk glands. The results of the Smed-CPEB1(RNAi) experiment suggests Smed-CPEB1 is likely regulating the expression of genes that are required for the development of cells that make up the female reproductive system, more specifically, oocytes and yolk glands.

Due to the advancement of science techniques, next-generation sequencing technology was used instead of previously used microarray analyses (Wang et al., 2010) to create transcription expression profiles of Smed-CPEB1(RNAi) planarians and control(RNAi) planarians (Rouhana et al., unpublished). RNaseq analysis was used to compare the gene expression profile of control planarians, (sexually mature planarians that contain their gonads and reproductive structures), and Smed-CPEB1(RNAi) planarians, (animals that lack oocytes and yolk glands). Hundreds of genes were
identified that were significantly underrepresented \((p < 0.05)\) (Rouhana et al., unpublished). These down regulated genes had at least 2-fold higher expression in control animals when compared to \textit{Smed-CPEB1(RNAi)} animals, suggesting that these genes are likely expressed in the oocytes and/or yolk glands and are involved in the development of those missing tissues. Over half of the genes identified have orthologs in other organisms, which set the direction of the study to focus on genes required in oocyte development and function across metazoans.

It is hypothesized these underrepresented genes in \textit{Smed-CPEB1(RNAi)} animals are normally expressed in the ovaries or yolk glands and that they may be required for the development of these tissues. To test this hypothesis I set out to characterize a subset of the underrepresented genes by verifying their expression in the germline and testing their function by RNAi. My first task was to sort the underrepresented genes by their fold change expression and conducted BLAST searches on NCBI comparing \textit{S. med} sequences to characterized proteins of \textit{Mus musculus}. I noted how similar the sequences were to each other by E-value, and also details of each sequence in terms of what has been published on the gene. Overall, I formed an Excel file that included the gene name, the BLAST top hit in the mouse, fold change expression, E value, and gene details based on published literature. After organizing the Excel file, I picked the top 56 hits to study (Table 1).
Table 1. Selected group of top candidates from sexual Smed-CPEB1(RNAi) planarians vs. control RNAseq analysis. Excel file.
The top hits were chosen based on larger decrease in fold change expression, as these are the genes that are hypothesized to likely to have a role in development of the ovaries and yolk glands since they were missing in *Smed-CPEB1(RNAi)* animals. I designed primers for the 50 top hits and amplified each gene by PCR. The PCR products were then purified, ligated into a vector and transformed into bacterial cells for cloning. Of the 50 genes, I was able to successfully transform 13 into bacterial cells and verified that the sequence was correct by BLAST comparison of the cloned sequence to the *Schmidtea mediterranea* sequenced genome (Table 2).

Interestingly, many of these 13 genes have various cellular functions such as cytoskeletal transport, transcription, translation, and also functions in the reproductive systems and the germline development of other organisms (Table 2). For example, BOLL (also known as BOULE, or BOULE-LIKE), is an RNA binding protein that has been shown to express during oogenesis in the fetal mouse ovary. BOLL proteins are encoded by the *Deleted in Azoospermia (DAZ)* gene family, which have been shown to be essential for germ cell development (He et al., 2013). Another candidate was a member of the B cell lymphoma-2 (BCL2) family proteins that regulate cell death and survival and are involved in apoptotic cellular processes. In female mice harboring a hypomorphic allele of *Bcl2l1*, the offspring were born with a depleted number of primordial and primary follicles, caused by germ cell apoptosis. Interestingly, when primordial germ cells of mice were transfected with a BCL2L1L expression vector, the germ cells had an increased survival rate. Consistent with this, when BCL2 was overexpressed in fetal oocytes, mice were born with increased numbers of primordial follicles. (Boumela et al., 2011). These genetic similarities further reiterates that planarians are an innovative model
organism to study germ cell development at the genetic level and will provide more insight in the mechanisms of germ cell development in the metazoan phylum.
Table 2. Candidate genes chosen for expression and phenotypic analysis. Excel File.

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2.1 Verification of candidate genes with germline expression

To validate the RNAseq results from the CPEB1(RNAi) screen, I verified the expression of 13 genes (Table 2) by whole mount in situ hybridization. A hermaphroditic strain of the planarian species Schmidtea mediterranea was used for all experiments (See figure 2.1 for flowchart). Gene sequences were identified in a reference transcriptome of S. mediterranea cDNA sequences (See Appendix A; (L. Rouhana et al., 2012)). Total RNA from sexually mature planarians was extracted and used to synthesize cDNA. Primers were designed for each of the sequences identified based on contigs from reference transcriptome (See Appendix B), from the contig sequences assembled from RNAseq (Rouhana et al., unpublished). Genes were PCR amplified, ligated, and inserted into pJC53.2 (Collins et al., 2010). The plasmid was then transformed into bacterial cells, purified, and sequenced. The sequences were verified by BLAST and the sexual S. mediterranea cDNA library (Zayas et al., 2005). The sequences inserted into pJC53.2 and transformed into DH5α served as the template for riboprobe synthesis and dsRNA for RNAi (Figure 2.1).
Figure 2.1. Flow chart describing the steps taken to analyze the candidate genes.
To examine the expression patterns of the genes, riboprobes were generated for whole mount in situ hybridization (WISH) by in vitro transcription of PCR templates with Digoxigenin-12-UTP (Roche) and either SP6 or T3 RNA polymerase. WISH was performed using the formaldehyde fixation method that has been previously described (Pearson et al., 2009) and optimized for planarians (King & Newmark, 2013). Gene expression was compared to germline markers such as CPEB1, expressed in the oocytes and yolk glands, and gH4 for neoblasts and germ cells surrounding the ovaries and testes. Expression patterns in specific cell and tissue types were analyzed with bright field microscopy. I performed WISH five times on each of the genes, using 10 planarians for each gene each time. I looked at the most consistent expression patterns for each gene; 7 out of 13 genes had the most consistent expression patterns in the germline (Table 3). Six genes showed ovarian expression (Figure 2.2) and three showed enrichment in the testes (Figure 2.3). Therefore, my screening of candidate genes from the RNAseq analysis of Smed-CPEB1 RNAi planarians was effective at identifying factors expressed in the germline.
Figure 2.2. Candidate genes expressed in the ovaries of sexual planarians. Six candidate genes were identified and expression was validated in the ovaries by WISH. Scale bars = 1mm.
Figure 2.3. Candidate genes expressed in testes of sexual planarians. Three candidate genes were identified and expression was validated in the testes by WISH. Scale bars = 1mm.
Table 3. Genes with germline expression identified in this work.

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<th>ovary expression</th>
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<td>transcription factor ii-i</td>
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<td>protein encoding histone</td>
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<td>+</td>
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<tr>
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<td>cre-tars-1 protein</td>
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<tr>
<td>Contig9714</td>
<td>glutamate metabotropic 4</td>
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2.2 Functional characterization of genes required for germline development

To define a function for germ cell development, RNAi experiments were performed on the seven genes that showed germline enrichment (Figures 2.2 and 2.3, Table 3). Genetic loss of function can be facilitated by feeding double stranded RNA (dsRNA) to adult planarians. RNAi functions by decreasing the expression of transcripts (Rouhana et al., 2013; Wang et al., 2007). dsRNA was diluted in homogenized beef liver and water to a final concentration of ~100ng/µL. Sexually immature planarians were fed twice a week until they reached maturity, indicated by the presence of a gonopore, or when controls laid eggs for 2 weeks (Figure 2.4). Sexually mature planarians were fed twice per week for a three month period. The fertility of these animals was monitored during this time period by observing and documenting the number of egg capsules that were laid, and how many of them hatched (Figure 2.4). I also observed any physical abnormalities during the RNAi treatment such as lesions and head regression. As a negative control for all experiments, animals were fed dsRNA corresponding to CCDB, a bacterial gene with no homology to genes in the planarian genome. For all RNAi experiments, as a positive control animals were fed double stranded CPEBI RNA, resulting in the loss of oocytes, yolk glands, and the ability to produce egg capsules as shown previously (Rouhana et al., unpublished).
Figure 2.4. Synthesis and administration of dsRNA for genetic function. A) PCR amplification of a gene of interest cloned into vector pJC53.2 (Collins et al., 2010) is done by using primers, recognizing two T7 promoters on the plasmid that flank a sequence. Double-stranded RNA (dsRNA) is in vitro transcribed using T7 RNA polymerase. B) Organic beef liver puree is mixed with water and food dye and dsRNA is mixed into each tube. The liver:dsRNA mixture is then fed to the planarians twice a week. The number of egg capsules produced and the number of egg capsules hatched were monitored for each group every week for two months.
Among the seven RNAi experiments, three had phenotypes that were abnormal. *Histone-encoding* was abundantly expressed in the testes and ovaries. *Histone-encoding(RNAi)* planarians formed lesions around the gonopore and eventually the tail detached from the upper portion of the body (Figure 2.5A). This phenotype was observed in both sexually mature and immature planarians. In sexually mature planarians, they stopped laying eggs at the 9th week of RNAi. 6/6 sexual planarians had tails that lysed at the gonopore region, but all animals regenerated. The same phenotype was seen in juvenile planarians raised to sexual maturity. 6/6 juvenile planarians had tails that lysed at their gonopore region. 2/6 of these animals died, 4/6 of them regenerated and survived. A replicate experiment was set up to verify the first result. This time, testing 3 groups of 7 sexually mature planarians, and 3 groups of 7 juvenile planarians. By the 6th week the sexually mature planarians had lesions appearing. I noticed before animals lost their tail region, a mass of eggs were laid (8 egg capsules within 4 days in 2/3 groups). This time, 14/21 planarians lost their tails, regenerated and survived to the end of the experiment. 7/21 lost their tails and did not survive the remainder of the RNAi experiment. Sexual juveniles also had the same phenotype, where 15/21 planarians lost their tails, regenerated and survived the experiment. 2/21 never lost their tails, and 4/21 did not survive to the end of the experiment.

*Cre-tars-1* was expressed in the ovaries of sexual planarians. RNAi knockdown of *Cre-Tars-1(RNAi)* planarians resulted in the animals to form lesions, and eventually dying soon after they were observed in both sexually mature and immature planarians.
(Figure 2.5B). 7/7 sexually mature planarians formed lesions and died by the 10\textsuperscript{th} week of RNAi experiments, while 7/7 juveniles died by week 8.
Figure 2.5. Abnormalities observed from *Histone* encoding and *Cre tars-1* RNAi experiments. **A)** *Histone* encoding RNAi experiments resulted in lesion formation around the gonopore region and eventual tail detachment. Blue arrows are pointing to both events. **B)** *Cre tars-1* RNAi resulted in full body lesion formations and death.
Smed-boule was expressed in both the testes and ovaries of sexually mature planarians. Most interestingly, the first round of RNAi for Smed-boule I observed that planarians continued to lay eggs, but after the 6th week they no longer hatched. I replicated the experiment to verify this phenotype by performing RNAi with 3 groups of 7 planarians each, for both sexually mature and juvenile planarians. As expected, this time, 21/21 planarians continued to deposit egg capsules throughout the 3 month experiment (Figure 2.6A) and were unable to hatch after the first month, that is 100/108 egg capsules (Figure 2.6B). Thus, only the RNAi phenotype described above in Smed-boule(RNAi) animals appeared to be germline specific, as the phenotype resulting showed a characteristic of infertility. This result showing a germline specific defect warranted further investigation.
Figure 2.6. *Boule* is required for production of hatchlings but disposable for capsule formation. A liver:dsRNA mixture was fed to planarians twice a week over a 3 month period. The number of egg capsules laid and the number of capsules that hatched were scored. A) RNAi and control planarians continued to lay capsules throughout the experiment. *CPEB1* (RNAi) ceased capsule production. B) The percentage of capsules that hatched per capsules laid calculated for each RNAi group. After the first month, *boule* (RNAi) animals showed a significant reduction in hatched capsules compared to controls. ** = p ≤ 0.05, *** ≤ 0.001
2.3 DAZ gene family

In humans, half of infertility cases are attributed to male partners, and some men are infertile because of defects in sperm production resulting in azoospermia (lack of sperm) (Vangompel & Xu, 2011). A subset of these men commonly have microdeletions on the Y chromosome (Reynolds & Cooke, 2005; Vangompel & Xu, 2011). These Y chromosomal deletions were first discovered in six azoospermic men, all of whom displayed a loss of the distal portion of their Y chromosome. This finding lead to the hypothesis that an “Azoospermia Factor” (AZF) exists, controls spermatogenesis, and is located in the distal portion of the long arm to areas of the Y chromosome (Tiepolo & Zuffardi, 1976). AZF was eventually broken down to three regions in the Y chromosome, AZFa, AZFb, AZFc, by identifying deletions in each of these areas in a population of infertile, azoospermic men (Vogt et al., 1996). This lead to the identification of Deleted in Azoospermia (Daz), a gene required for normal testicular function and spermatogenesis (Reijo et al., 1995).

DAZ is a gene family that consists of three genes: Daz, Dazl and Boule, all of which encode RNA-binding proteins and are reproduction specific (Vangompel & Xu, 2011). Boule is the most ancestral member of the DAZ family and is conserved throughout metazoans, and germ cell expression patterns varies upon each organism (VanGompel & Xu, 2010; Xu et al., 2001) (Figure 2.7A). All three DAZ family genes have a RNA-Recognition Motif (RRM) in common and at least one copy of a DAZ repeat that is 24 amino acids long and abundant in N, Y and Q residues (Shah et al., 2010; Xu et al., 2001) (Figure 2.7B). In Xenopus, XDazl is expressed in the germplasm, ovaries,
spermatogonia and spermatocytes of their testes (Houston et al., 1998). Loss of XDazl caused defects in the migration and differentiation of PGCs, and a reduction in tadpoles and reproduction (Houston & King, 2000). In zebrafish, localized expression of Zdazl mRNA is in the cortex of oocytes and during embryogenesis expression becomes more restricted to the vegetal poles and to blastomeres (Maegawa et al., 1999). Boule homologs in C. elegans and Drosophila have opposite gametogenic requirements. In C. elegans, Boule is sex specific and is detected in the cytoplasm of mitotically active germ cells and expression is maintained throughout oogenesis. Loss of boule function in C. elegans caused the organisms to become sterile, as oogenesis ceased at the pachytene stage of meiosis and the cells undergoing meiosis had altered morphologies (Karashima et al., 2000; Maruyama et al., 2005). In Drosophila, boule is expressed in the testes and loss of function results in azoospermic males by arrest during spermatogenesis (Eberhart et al., 1996). Similarly, boule is expressed in the testes of mice. In knockout mice, spermiogenesis is arrested at the round spermatid stage suggesting boule is required for late stages of male germ cell differentiation in mammals (VanGompel & Xu, 2010; Xu et al., 2001).

In non-vertebrates, the vast majority of studies on the DAZ gene family members have been studied extensively in D. melanogaster and C. elegans, only two of the major bilaterian phylums Ecdysozoa and Deuterostomia. Until recently, there has been no other information on the function of boule in non-vertebrates. In Macrostomum ligano, three orthologues of boule were identified, and for the first time the gene was characterized in a lophotrochozoan. Macbol1 was shown to block spermatocyte differentiation and Macbol3 was required for oogenesis, as egg maturation ceased (Kuales et al., 2011).
Historically, *DAZ* and *DAZL* have been characterized to be involved in early germ cell development while *boule* is responsible for directing meiosis (Xu et al., 2001). In the next chapter I focused on the detailed characterization of *Smed-boule* in planarian flatworms to provide more knowledge in Deuterostomia.
Figure 2.7. Evolution of DAZ gene family and structural components. A) Members of the DAZ gene family consist of boule, DAZL, and DAZ. boule is the earliest ancestor of the gene family. boule, DAZL, and DAZ all have germ cell specific expression, but their functions vary across metazoans. Image adapted from (VanGompel et al., 2011). B) DAZ family members encode for proteins that contain conserved RNA-binding domain, and also contain 1-18 DAZ repeats that are rich in N/Y/Q amino acids and involved in protein-protein interactions.
2.4 References


Chapter 3

Germline defects caused by Smed-boule RNA-interference reveal that egg capsule deposition occurs independently of fertilization, ovulation, mating, or the presence of gametes in planarian flatworms.

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3.1 ABSTRACT

Few animals are known to lay eggs in the absence of ovulation or copulation, as it is presumably energetically wasteful and subjected to negative selection.

Characterization of Smed-boule, a member of the DAZ family of germline RNA-binding proteins, revealed that egg capsule (or capsule) production and deposition occurs independently of the presence of gametes in the planarian flatworm Schmidtea mediterranea. Reduction of Smed-boule expression by RNA-interference (RNAi) causes ablation of spermatogonial stem cells and the inability of ovarian germline stem cells to undergo oogenesis. Although animals subjected to Smed-boule RNAi lose their gametes and become sterile, they continue to lay egg capsules. Production of sterile capsules is even observed in virgin Smed-boule(RNAi) and control planarians maintained in complete isolation, demonstrating that egg production in S. mediterranea occurs independently of ovulation, fertilization, or mating. Evidence suggests that this is a conserved feature amongst Platyhelminthes, and therefore relevant to the pathology and dissemination of parasitic flatworms. These findings demonstrate that Smed-boule functions at different stages during male and female germline stem cell development, and also demonstrate that egg capsule production by planarian flatworms occurs independently of signals produced by mating or ova.
3.2 AUTHOR SUMMARY

Our work shows that production and deposition of egg capsules by planarian flatworms does not require fertilization, mating, ovulation, or even the existence of gametes. We also uncovered evidence for the existence of gender-specific germline stem cells in *Schmidtea mediterranea*, a hermaphroditic species of flatworm that develops germ cells post-embryonically. These findings surfaced from the characterization of *Smed-boule*, a member of the *Deleted in AZoospermia* gene family of RNA-binding proteins required for germline development in a broad range of animals. These findings lead to a better appreciation of the evolutionary diversity in approaches to oviparity. Additionally, discovering that egg capsule production occurs independently of germline or mating activities may carry a potential applied aspect with regards to regulating the dissemination and pathology of parasitic flatworms (such as blood flukes and tapeworms), if conserved in these organisms.
3.3 INTRODUCTION

The characterization of developmental processes involved in sexual reproduction has important implications towards reproductive medicine, stockbreeding, farming, and for controlling the dissemination of infectious disease. Evolutionarily conserved molecular processes involved in metazoan germline development have been identified through decades of research using model organisms. For example, post-transcriptional regulation of gene expression by conserved germline-specific RNA-binding proteins is one of the conserved molecular processes that ensure development of gametes (Kimble, 2011; Spradling et al., 2011; Voronina et al., 2011). On the other hand, there is great diversity in the processes that occur during and after fertilization, many of which are the outcome of speciation events (Blackburn, 1999; Vacquier, 1998).

Planarian flatworms belong to the phylum Platyhelminthes, and are well known for their extraordinary regenerative abilities, which are founded in the availability of a pluripotent stem cell population throughout their life (Elliott and Sanchez Alvarado, 2013; Newmark and Sanchez Alvarado, 2002; Rink, 2013; Shibata et al., 2010). The evolutionary history of these organisms has yielded extreme divergence of reproductive strategies, both between and within populations of different planarian species. For example, there are planarians that rely exclusively or temporally on asexual reproduction, which involves transverse fission and stem cell driven regeneration (Grasso and Benazzi, 1973; Newmark and Sanchez Alvarado, 2002). There are also populations of planarians that reproduce predominantly through parthenogenesis (Pongratz et al., 2003). However, the default reproductive strategy of turbellarians is believed to be hermaphroditic sexual reproduction (Rieger, 1986), more specifically for planarians through cross-fertilization.
and oviparity (Newmark et al., 2008). By contrast, some parasitic flatworms (i.e. schistosomes or blood flukes) have complex life cycles that involve dioecious and asexual reproductive phases during transitions between vertebrate and invertebrate hosts, respectively (Basch, 1991). Since the complex life cycle of schistosomes complicates husbandry and experimentation in laboratory settings, researchers have begun to use planarian flatworms as a model to dissect the molecular mechanisms behind the extensive lifespan and reproduction of their parasitic cousins (Collins and Newmark, 2013). One aspect of particular interest is the continuous production of thousands of eggs that both facilitate dissemination and sustain the pathology of schistosomes by populating organs of their host (Basch, 1991; Collins and Newmark, 2013).

Planarian flatworms have become useful models for the study of metazoan germline development (Newmark et al., 2008; Wang et al., 2010). In general, the specification of germline stem cells can occur through mechanisms that involve: 1) inherited material deposited in the cytoplasm of the maturing oocyte (preformation); or 2) embryonic stem cell differentiation in response to inductive cell-to-cell interactions (epigenesis) (Extavour and Akam, 2003; Seydoux and Braun, 2006). Inductive determination occurs in mice and is also observed in planarians, both initially and during regeneration of fragments that lack germ cells, and it occurs through differentiation of pluripotent somatic stem cells called neoblasts (Wang et al., 2007). In the planarian species *Schmidtea mediterranea*, germline stem cells are first detected as dorsolateral clusters in the area where testes develop (Wang et al., 2007). In other planarian species, such as *Dugesia ryukyuensis*, germline stem cells are first detected in the area of the ovaries (Kobayashi and Hoshi, 2002; Nakagawa et al., 2012). Upon feeding and growth,
planarians that reproduce sexually develop a hermaphroditic reproductive system and their gonads begin continuous production of gametes (Kobayashi and Hoshi, 2002, Newmark et al., 2008). Germline stem cells in the ovary enter oogenesis and produce oocytes of approximately 40 µm diameter that exit the ovary and are fertilized by sperm deposited in the tuba (Wang et al., 2010; Newmark et al, 2008). Even-though oocytes are large in comparison to other planarian cells (e.g. neoblasts are ~8 µm diameter) these do not hold the nutrients necessary for embryonic development, as is normally observed in eggs of insects, amphibians or fish (to name a few). Instead, planarian yolk glands (vitellaria in other flatworms) produce separate cells that provide material required for egg capsule shell formation and nurturing embryonic development (Gremigni and Domineci, 1974; Cardona et al., 2006).

The development of planarian germline stem cells depends on conserved post-transcriptional regulators such as Nanos and Bic-C (Wang et al., 2010; Wang et al., 2007). Boule is an RNA-binding protein encoded by the basal member of the Deleted in AZoospermia (DAZ) gene family, which is required for germ cell development in species ranging from sea anemone to humans (Kee et al., 2009; Vangompel and Xu, 2011). How DAZ family homologs contribute to germline development in planarians remains unknown. In this study, we characterize a Boule homolog in the planarian Schmidtea mediterranea and demonstrate that it functions at different stages during male and female germline development. Functional analyses by RNA-interference (RNAi) revealed that Smed-boule is required for development and maintenance of spermatogonial stem cells, but disposable for the existence of their oogonial counterparts, uncovering the presence of sex-specific germline stem cells in planarian hermaphrodites. Long-term analysis of
Smed-boule knockdowns revealed that egg capsule deposition in planarians is not triggered by gametogenesis, ovulation, oocyte activation, fertilization, or mating. These results demonstrate that egg capsule formation occurs regardless of signals from sexual activity or germ cell activity in S. Mediterranea. These findings also provide a unique opportunity to identify internal mechanisms that influence capsule production in Platyhelminthes, which is central in the dissemination and pathology of parasitic members of this phylum.
3.4 RESULTS

3.5 Smed-boule is required for male and female germline development

We identified a boule homolog in the planarian flatworm *S. mediterranea* with a region of amino acid sequence 55% identical with that of the RNA recognition motif of human BOLL (E-value = 1e-23; Fig 3.1A). The protein encoded by this gene shared highest homology with members of the Boule-like subfamily, as compared with other members of the DAZ family of proteins (Fig 3.1B). Expression of this gene (from here on referred to as *Smed-boule* or *boule*) was detected by whole-mount *in situ* hybridization (ISH) in testes and ovaries of sexually mature planarians that are actively laying egg capsules (Fig 3.2A-D). *Smed-boule* expression was also detected in testis primordia of hatchlings and sexually immature animals (Fig 3.2D and 3.2D’). To better understand the distribution of *Smed-boule* expression in testes and ovaries, we performed detailed analysis by double fluorescent *in situ* hybridization (FISH) with the germline stem cell marker *Smed-nanos* (Wang et al., 2007) (Fig 3.3). Detection of *Smed-boule* mRNA overlapped with that of *Smed-nanos* in testes (Fig 3.3A’’) and partially in ovaries (Fig 3.3B’’’). The presence of *Smed-boule* mRNA was also robustly detected in the spermatogonial layer of the testes (Fig 3.3A’’’). Detection of *Smed-boule* expression was not apparent in the soma, and thus we conclude that expression of this gene is restricted to the germline in *S. mediterranea*. 
Figure 3.1. *Smed-boule* encodes for a member of the germline-specific Boule-Like subfamily of proteins. (A) Illustration of human Boule-Like (BOLL) protein architecture, which includes a RNA-recognition motif (RRM; yellow) and a DAZ domain (blue). The amino acid sequence conservation within the RRM of *Schmidtea mediterranea* and *Homo sapiens* homologs is shown. (B) Neighbor-joining phylogenetic tree depicting the closer association of *Smed-boule* predicted amino acid sequence with members of the Boule-Like (green) subfamily of DAZ proteins, than with members of the DAZ (blue) and DAZ-Like (magenta) subfamilies. Phylogenetic analysis was performed using Clustal Omega with default parameters (Sievers et al., 2011) and sequences obtained from NCBI accession NP_932074.1, XP_001169371.2, XP_001086915.2, XP_005640556.1, NP_001095585.1, NP_083543.2, XP_006245003.1, XP_004942650.1, NP_001261614.1, XP_315505.3, NP_001005785.1, XP_001138045.3, XP_002803072.1, NP_034151.3, NP_001102884.1, NP_989549.1, NP_571599.1, NP_989079.1, NP_004072.3, XP_003319020.2, CCD81039, NP_011092, NP_001177740.1. Abbreviations used for species names included Anopheles gambiae (Ag), Bos taurus (Bt), Canis lupus familiaris (Cl), Danio rerio (Dr), Drosophila melanogaster (Dm), Gallus gallus (Gg), Homo sapiens (Hs), Macaca mulatta (Mmul), Mus musculus (Mmus), Pan troglodytes (Pt), Rattus norvegicus (Rn), Saccharomyces cerevisiae (Sc), Schmidtea mediterranea (Smed), Schistosoma mansoni (Sm), and Xenopus tropicalis (Xt). Scale bar represents 0.05 substitutions per amino acid position.
Figure 3.2. *Smed-boule* expression is restricted to the planarian germline. (A) Simplified depiction of the reproductive anatomy of planarian hermaphrodites. Reproductive organs located dorsally (top) and ventrally (bottom) are illustrated separately. (B-D) *Smed-boule* expression is detected in testes and ovaries by whole mount in situ hybridization in dorsal (B and D) and ventral (C) views of sexually mature *S. mediterranea*, respectively. Arrows in (B) indicate individual testis lobes within testes regions found dorsolaterally in the animal. Magnified view of signal from ovaries (open arrowheads) is shown in (C’) inset. *Smed-boule* expression is also detected in germline stem cells (black arrowheads) of 1 week-old hatchlings in the dorso-lateral region where testes develop (D’), as well as continuously in maturing animals (D). Scale bars = 1 mm.
Figure 3.3. Smed-boule is expressed in germline stem cells of testes and ovaries. Detailed analysis by FISH and confocal microscopy of testes (A) and ovaries (B) of *S. mediterranea* reveals *Smed-boule* mRNA (magenta) is detected in germline stem cells, which are recognized with expression of *nanos* (green). *Smed-boule* mRNA was detected in all *nanos* (+) cells of the testes (yellow arrows) (A”-A”). *Smed-boule* mRNA was also detected in some (yellow arrows) but not all (orange arrows) *nanos* (+) cells of the ovary (B’-B”). DAPI staining (A and B) and merged images (A” and B””) show the nuclei of all cells present in the imaged frames. Scale bars = 25 µm.
To test the function of Smed-boule in planarian germline development and sexual reproduction we subjected planarians to three months of RNAi. Planarians continuously turn over all cells in their body from a continuous population of pluripotent stem cells, which allowed us to assess whether Smed-boule is required for normal germline development in sexually mature adults using germ cell markers (Fig 3.4A-F; Supplementary Fig S1 and S2). Groups of seven sexually mature planarians were fed liver supplemented with 100 ng/µl of double-stranded RNA (dsRNA) twice per week. Smed-boule knockdowns (Smed-boule(RNAi)) were compared to control(RNAi) planarians. DsRNA corresponding to a planarian Cytoplasmic Polyadenylation Element Binding Protein 1 homolog, which is required for yolk gland development and egg capsule production (below), was administered to an additional group (CPEB1(RNAi)) as readout of RNAi effectiveness overtime. At the end of three months of RNAi, we observed that both oocytes (Fig 3.4B; Supplementary Fig S2B) and sperm (Fig 3.4E; Supplementary Fig S2B”) were absent in Smed-boule(RNAi). No defects in oocyte or sperm development were observed in control(RNAi) planarians (Fig 3.4A and 3.4D; Supplementary Fig S2A-A”). The testes of CPEB1(RNAi) samples were fully developed (Fig 3.4F), but their ovaries displayed abnormal morphology and distribution of oocyte marker expression (Fig 3.4C). From these results we concluded that Smed-boule is required for development of sperm and ova in S. mediterranea.

Neoophoran flatworms rely on a particular approach to oviparity in which nutrients for the developing embryo (yolk) are not accumulated in the developing ova. Instead, nutritional support is contributed by yolk cells (vitellocytes), which are transferred from yolk glands to the planarian uterus and encapsulated with embryos during egg capsule
deposition. We checked for the presence of yolk glands using the yolk cell marker Smed-surfactantB (Supplementary Fig S1B), which proved to be of comparable abundance and distribution in control(RNAi) and Smed-boule(RNAi) animals (Fig 3.4G and 3.4H). However, the presence of yolk glands in CPEB1(RNAi) animals was severely reduced (Fig 3.4I). We looked for other possible defects in the development of somatic reproductive structures but were unable to find any abnormalities other than the absence of accumulated sperm in the seminal vesicles of Smed-boule(RNAi) (Fig 3.5), which is due to their inability to produce sperm (Fig 3.4E). There was also a noticeable difference in size of CPEB1(RNAi) planarians, which were on average 30.8% larger than control animals maintained under the same conditions (n = 14; unpaired two-tailed t-test, p < 0.05; Supplementary Fig S3). The normality and functionality of the accessory reproductive system in Smed-boule(RNAi) was further supported by quantitative analyses of egg capsule production (below).
3.6 Continuous production of sterile egg capsules by *Smed-boule*(RNAi)

As expected, from planarians with underdeveloped yolk glands (Fig 3.4I), *CPEB1*(RNAi) ceased laying eggs within a month of RNAi (Fig 3.4J). The rate of egg production in *Smed-boule*(RNAi) was unaffected during the three months of RNAi treatment (Fig 3.4J). Both the *control*(RNAi) and *Smed-boule*(RNAi) groups continuously laid eggs for the entirety of the experiment (Fig 3.4J). In fact, an increase of 33% and 30% in egg capsule production was observed in *Smed-boule*(RNAi) when compared to *control*(RNAi) planarians during the second and third months of RNAi treatment, respectively (unpaired two-tailed *t*-test, *p* < 0.05; Fig 3.4J). Given the surprising result that planarians devoid of gametes continued to deposit egg capsules, we monitored and quantified the number of fertile capsules (yielding progeny) produced by the different knockdown groups for two months after capsule deposition. From this, we discovered that egg capsules produced by animals subjected to two months of *Smed-boule* RNAi completely ceased to hatch (Fig 3.4K). Egg capsules produced by *control*(RNAi) groups hatched 22% to 48% of the time (Fig 3.4K). From these results, we concluded that *Smed-boule* function is required for germline development and sexual reproduction in *S. mediterranea*, but dispensable for production of egg capsules. Furthermore, the continuous production of egg capsules by groups of *Smed-boule*(RNAi) planarians (Fig 3.4J) suggested that production and deposition of egg capsules do not require fertilization, contributions from sperm, ovulation, or the presence of oocytes.
Figure 3.4. *Smed-boule* is required for germline maintenance and production of fertile capsules. (A-I)
The reproductive anatomy of RNAi animals monitored in (J-K) was analyzed by whole mount *in situ* hybridization using *synaptotagminXV* (A-C) or *surfactant b* (G-I) as oocyte and yolk gland markers, respectively (Supplementary Fig S1). DAPI staining was used to visualize sperm development in the testes (D-F). The fraction of animals displaying the phenotype represented by the image is shown at the bottom-left corner of each frame. (J-K) Capsule production (J) and hatching (K) from groups of sexually mature planarians subjected to continuous control, *Smed-boule*, or *CPEB1* RNAi treatments for three months (first, second, and third month represented by column from left to right in each group). Quantification of the number of capsules laid (J) and the number of fertile capsules (K) show that capsules deposited by *Smed-boule(RNAi)* animals ceased being fertile and that *CPEB1(RNAi)* ceased capsule production as a result of RNAi. Asterisks (*) represent statistically significant results compared to controls of same month by unpaired two-tailed *t*-test (*p > 0.05*). Scale bars = 1 mm.
Smed-boule RNAi does not affect development of somatic reproductive structures. Whole-mount *in situ* hybridization (ISH) analysis of planarians subjected to three months of control (A, C, E, and G) or Smed-boule (B, D, F, H) RNAi revealed no differences between these two samples in development of oviducts (black arrows in A-B), atrium (asterisks in A-B), tuba (pink arrows in A-B), spermducts (white arrow heads in G'-G'' and H'-H''), or seminal vesicle development (black arrowheads in G-H). DAPI staining of these samples revealed that development of the bursal canal (white arrow in C-D) and gonopore (pound sign in E-F) were indistinguishable. However, DAPI staining only revealed sperm in testes and seminal vesicles (black arrowheads) of control animals (E-F). The fraction of animals displaying the phenotype represented by the image is shown at the bottom-left corner of each frame. Scale bars = 1 mm.
3.7 Continuous egg production in planarians regardless of isolation and absence of gametes

Given the fact that Smed-boule(RNAi) planarians were capable of producing sterile egg capsules in the absence of germ cells (and therefore fertilization events) lead us to hypothesize that control animals would also produce sterile egg capsules in the absence of fertilization events. To test this hypothesis, we obtained ≤ 1 week-old hatchlings (which lack ovaries, testes, yolk glands, and accessory reproductive organs) and maintained them in isolation for four months under continuous RNAi regimens. Planarians were maintained in isolation throughout the experiment, which allowed us to test whether egg capsule production is independent of signals produced during mating or the presence of potential mates altogether. Since planarians in this experiment were subjected to Smed-boule RNAi within a week of being born, which is a point when no sperm has developed, this approach also allowed us to verify that lingering sperm in adult knockdowns used in the previous experiment was not contributing to egg capsule production. Two categories of isolated virgins were maintained on either liver containing Smed-boule dsRNA or control dsRNA and were fed twice per week. These animals were expected to grow and eventually reach sexual maturity under these husbandry conditions. The production of egg capsules would only occur if independent from stimuli produced during mating, fertilization, embryonic development and, in the case of Smed-boule(RNAi), the absence of gametes.
Indeed, both control and *Smed-boule(RNAi)* isolated animals produced egg capsules during the third and fourth months of the experiment (Fig 3.6A). The number of capsules produced during the length of the experiment by isolated individuals from each category ranged from none to six (Fig 3.6B). The average number of capsules deposited by individuals in the control category was slightly, but not significantly higher than those of *Smed-boule(RNAi)* (unpaired two-tailed *t*-test, *p* = 0.25) (Fig 3.6B). As expected from results observed in animals subjected to RNAi in the presence of potential mates (Fig 3.4J and 3.4K), none of the egg capsules produced by *Smed-boule(RNAi)* individuals were fertile (n = 0/28 capsules). Capsules produced by control RNAi animals were also completely sterile (n = 0/43 capsules), suggesting that the production of egg capsules in these animals were not due to self-fertilization or parthenogenesis. We verified that normal gamete development was present in control animals at the end of the isolation experiment (Fig 3.6C and 3.6E) and absent in *Smed-boule(RNAi)* flatworms (Fig 3.6D and 3.6F), which was expected from analyses of knockdowns not maintained in isolation (Fig 3.4A, 3.4B, 3.4D and 3.4E). We also validated successful development of yolk glands in control and *Smed-boule(RNAi)* planarians raised in isolation (Fig 3.6G and 3.6H). Collectively, these results demonstrate that production of egg capsules in *S. mediterranea* occurs in response to internal triggers that do not require the presence of a mate, mating, or fertilization events. Furthermore, the production of egg capsules by *Smed-boule(RNAi)* planarians suggests that this trigger is detached from signals originating from sperm and oocyte development or ovulation.
Figure 3.6.
Egg capsules are produced in the absence of gametes or mating events.

(A-B) Egg capsules produced per month by planarian hatchlings (≤ 1 week old) raised in isolation on a diet of liver containing control (left) and Smed-boule (right) dsRNA for four months. The average number of egg capsules deposited per month per isolated individual (A), as well as the total number of capsules deposited per each isolated individual (B), show no significant difference (unpaired two-tailed t-test, p > 0.05) in egg capsule production between control and Smed-boule(RNAi) isolated virgins. Dashed lines in (B) represent the mean of total number of capsules produced per animal. Vertical lines represent standard deviation (C-H) The reproductive anatomy of isolated planarian virgins monitored in (A and B) was analyzed using synaptotagminXV as an oocyte marker (C-D), DAPI staining for testes (E-F) and surfactant b to assess yolk gland development (G-H). Smed-boule(RNAi) lacked oocytes (D) and sperm (F) seen in control animals (C and E), but developed yolk glands comparably (G-H). Scale bars = 1 mm.
3.8 Spermatogonial stem cells are lost and oogonial stem cells display early oogenesis defects after Smed-boule RNAi

We decided to evaluate the severity of germline development defects caused by Smed-boule RNAi. The most severe phenotype would be the loss of germline stem cells, which are specified and maintained post-embryonically through neoblast differentiation (Wang et al., 2007). Germline stem cells in the planarian ovaries and testes can be identified by the characteristic expression of nanos (Handberg-Thorsager and Salo, 2007; Nakagawa et al., 2012; Sato et al., 2006; Wang et al., 2007). We tested for the presence of germline stem cells in control(RNAi) and Smed-boule(RNAi) by nanos ISH after 3 - 4 months of RNAi (at the end of experiments in Fig 3.4J-K and 3.6A-B). Whole-mount ISH analysis of germinal histone H4 and nanos expression revealed the presence of germline stem cells in the testes region of control(RNAi) animals (Fig 3.7A’’ and 3.8A). However, germline stem cells were completely absent from the testes region of Smed-boule(RNAi) planarians (Fig 3.7B’’ and 3.8B). Surprisingly, germline stem cells in the ovary region of both control(RNAi) and Smed-boule(RNAi) planarians were readily detectable (Fig 3.7A’ and 3.7B’, Fig 3.8C and 3.8D). Identical results were observed from hatchlings raised subjected to Smed-boule RNAi while maintained in isolation (Supplementary Fig S4). Furthermore, analysis of germline stem cells in presumptive testis primordia present in asexual strains of S. mediterranea (Wang et al., 2007) were also lost after Smed-boule RNAi (Supplementary Fig S5). From these results, we conclude that spermatogenesis defects in Smed-boule(RNAi) are due to the absence of male germline stem cells, whereas defects in oogenesis occur further downstream in the differentiation pathway.
To better evaluate the progression of oogenesis in *Smed-boule*(RNAi) ovaries, we analyzed control and *Smed-boule* knockdowns stained with DAPI by confocal microscopy (Fig 3.8E and 3.8F; Supplementary Movie File S1 and S2). DAPI is retained by DNA and allowed for the visualization of numerous large oocytes with condensed chromosomes in the ovaries of control samples (Fig 3.8E; Supplementary Movie File S1). In contrast, neither oocytes, cells with condensed chromosomes, or otherwise recognizable mid- to late-oogenic intermediates, were detectable in ovaries of *Smed-boule*(RNAi) flatworms (Fig 3.8F; Supplementary Movie File S2). From these results we conclude that *Smed-boule* function is required during the initial stages of oogenesis, sometime before development of primary oocytes, but after specification of ovarian germline stem cells.

The different outcomes observed on germline stem cells of testes and ovaries following *Smed-boule*(RNAi) reveal that these are two fundamentally distinct germline stem cell populations that require *Smed-boule* function at different developmental stages. *Smed-boule* function is necessary for neoblast differentiation into male germline stem cells and/or maintenance of male germline stem cells, whereas ovarian germline stem cells only require *Smed-boule* for progression through early stages of oogenesis (Fig 3.8G). Furthermore, the severe defects in germline development observed after *Smed-boule* RNAi further support the hypothesis that egg capsule production and deposition occur independently of gametes, ovulation, parthenogenesis, fertilization, mating, or embryonic development in *S. mediterranea*. 
Figure 3.7. *Smed-boule* is required for maintenance of testicular germ cells, but not for all ovarian germ cells. (A-B) Whole-mount *in situ* hybridization (ISH) analysis of neoblast and germ cell (GC) distribution through detection of germinal histone H4 (*gH4*) mRNA in planarians subjected to three months of control (A) or *Smed-boule* (B) RNAi. GCs were detected in the ovary region (pink arrowheads) of both controls (A’) and *Smed-boule*(RNAi) (B’). Testicular GCs (white arrowheads) detected in control samples (A”) were absent in *Smed-boule*(RNAi) samples (B”). The fraction of animals displaying the phenotype represented by the image is shown at the bottom-left corner of each frame. Scale bars = 1 mm.
Figure 3.8. *Smed-boule* is required for maintenance of testicular germline stem cells, but not for ovarian germline stem cells. (A-D) Whole-mount *in situ* hybridization analysis of germline stem cell (GSC) distribution visualizing *nanos* expression in the testis (A-B) and ovary (C-D) regions of planarians subjected to three months of control or *Smed-boule* RNAi. GSCs were specifically absent in the testes region of *Smed-boule* knockdowns (B), but present in the ovary region of both control (C) and *Smed-boule* (D) knockdowns. Counter-stain with DAPI (A’-D’) shows the presence of sperm in testes of control samples (A’), but not *Smed-boule(RNAi)* (B’). The fraction of animals displaying the phenotype represented by the image is shown at the bottom-left corner of each frame. (E-F) Confocal images of control and *Smed-boule(RNAi)* animals stained with DAPI reveal normal development of oocytes (arrowheads) in control ovaries (E) and the absence of oocytes in *Smed-boule(RNAi)* ovaries (F). Scale bars = 1 mm (A-D) and 50 µm (E-F). (G) Schematic representation of the current model for *Smed-boule* function in differentiation of female GSCs and development and/or maintenance of male GSCs.
3.9 DISCUSSION

Collectively, the data presented demonstrate that production and deposition of the egg capsules that ensure development of planarian embryos occurs independently of fertilization events. Rather, it seems that egg capsule deposition, at least in *S. mediterranea*, is driven by intrinsic signals that are activated once these flatworms grow past a certain size and develop their yolk glands and other accessory reproductive organs. Given these findings, conclusions regarding planarian “fecundity” previously calculated from the rate of capsule production (Greeff et al., 1999; Weinzierl et al., 1999), may need to be re-evaluated. Additionally, knowledge of the separation between capsule deposition and fertility should assist in the study of planarian germline and embryonic development, as well as in generation of methodologies for transgenesis, which have proven elusive to this point.

Planarian reproduction can occur asexually through transverse fission, or sexually through post-embryonic development of a hermaphroditic reproductive system (Newmark and Sanchez Alvarado, 2002). In planarians committed to sexualization, the development of gonads and gametes precedes formation of the oviducts, sperm ducts, and copulatory organs (Kobayashi and Hoshi, 2002). Yolk gland development in *S. mediterranea*, which is essential for production of egg capsules, is initiated towards the end of sexual development depending on sufficient nutritional intake and growth. The rate of egg capsule production observed in our experiments (1 to 5 egg capsules per animal per month; Fig 3.4J) is comparable to those observed in different planarian species both in their natural habitat and raised under laboratory conditions following a similar liver-only diet (Jenkins and Brown, 1963; Kostelecky et al., 1989). Therefore, we
believe that the conditions used for husbandry of *S. mediterranea* in the laboratory are conducive to normal egg capsule production rates, and that this is not the limiting factor in reproductive output. However, the low yield of fertile egg capsules observed from control animals in our experiments (22% to 48%; Fig 3.4J and 3.4K) suggests that optimal laboratory husbandry conditions need to established to promote oocyte production, ovulation, or mating (either of which may be rate limiting in actual reproductive output).

How can triggering egg production independently of fertilization be an efficient approach to survival of planarian populations? First, we must consider that in terms of sexual reproduction, *S. mediterranea* performs rather poorly under laboratory conditions. This is supported by the studies of Jenkins and Brown (Jenkins and Brown, 1963) who observed *D. dorotocephala* yield an average of 16.5 hatchlings per egg (approximately 10-fold higher from what is observed in our laboratory for fertile egg capsules of *S. mediterranea*). Studies in *S. polychroa* have shown that siblings emerging from a single egg result from different fertilization events, which is possible because sperm from one or more partners can be stored for at least a month after insemination (Pongratz and Michiels, 2003). The ability to store sperm for an extended period of time after insemination, combined with the delay in development of yolk glands in comparison to the rest of the reproductive system, presents a scenario that would benefit from a mechanism that triggers capsule formation independently of copulation. In fact, it would be optimal if the activation of capsule formation also triggers ovulation of the many fully-grown oocytes present in ovaries of sexually mature planarians (Consequential Model; Supplementary Fig S6A). Since sperm can be stored in the tuba, massive ovulation could
maximize the number of hatchlings generated per capsule. Alternatively, encapsulation of multiple embryos in a single egg capsule could also be facilitated by extended storage of zygotes prior to capsule deposition (Complete Autonomy Model; Supplementary Fig S6B). We are currently unable to differentiate between these two possibilities, or the possibility that passage of oocyte precursors (e.g. oogonial or female germline stem cells) may activate capsule formation. Indeed, oocytes were not detected in Smed-boule(RNAi) planarians (n = 0/20; Fig 3.4B and 3.6D; Supplementary Fig S2B), but ovaries and oogonial stem cells were readily observed (n = 14/15; Fig 3.7B, 3.8D and 3.8F; Supplementary Fig S4D; Supplementary Movie S2). It is possible that the release of early oocyte precursors from the ovary triggers capsule formation. However, this hypothesis is challenged by the fact that dozens of hatchlings often emerge from single capsules of different planarian species, and the observation that the rate of capsule production was not compromised in Smed-boule(RNAi) when compared to control planarians (which contained both oocytes and precursors). Nevertheless, current and previous observations do support a model by which a sustainable approach to oviparity could rely on a trigger for capsule formation that is independent of mating, fertilization, or ovulation.

Inside the phylum Platyhelminthes, free-living species (such as S. mediterranea) are evolutionary distant from members of parasitic groups (Trematoda, Monogenea, and Cestoda). However, the non-causative relationship between ovulation/fertilization and capsule production appears to be conserved in some cestodes and trematodes, whose dissemination and pathology depend on the continuous production of egg capsules. Parasitic flatworms of the genus Schistosoma have been reported to produce egg capsules
from females after single-sex infections of mammalian hosts (Shaw and Erasmus, 1981; Shaw, 1987). Although female schistosomes depend on interactions with a mate to fully grow and develop their gonads, they are also able to develop some vitelline cells and immature ovaries on their own. Shaw (1987) observed the production of infertile capsules from females without male stimuli, probably through mechanisms conserved with those reported here for planarian flatworms. Similarly, parasitic flatworms belonging to the class Cestoda (tapeworms), have been reported to produce unviable egg capsules in the absence of fertilization events when cultured in vitro (Smyth, 1946; Smyth, 1947). Thus, given that continuous production and deposition of egg capsules is central to dissemination and pathology of different types of parasitic flatworms, the molecular machinery involved in egg capsule production (and not germline development alone) becomes a desirable target for therapeutic developments.
3.10 MATERIALS AND METHODS

Planarian Culture

A laboratory sexual strain of *Schmidtea mediterranea* (Zayas et al., 2005) was used all experiments, except for those presented with asexual planarians (Cebria and Newmark, 2005) in Supplementary Fig S5. Planarian cultures were maintained in 0.75x Montjuïc Salts at 18°C under dark conditions, whereas 1x Montjuïc Salts and 21°C were used for asexuals as per (Cebria and Newmark, 2005). Planarians were exposed to room temperature and light during weekly feedings of pureed organic beef liver (Vantage USA, Oak Park, Illinois). Experimental animals were starved at least seven days before experimentation.

**cDNA constructs**

All *S. mediterranea* contig sequences (Appendix A) were assembled from RNAseq and conventional cDNA expressed sequence tag reads ((Rouhana et al., 2012); [https://www.ideals.illinois.edu/handle/2142/28689](https://www.ideals.illinois.edu/handle/2142/28689)). PCR products were amplified from oligo(dT)-primed total RNA cDNA using corresponding forward and reverse primers (Appendix B) and ligated to pJC53.2 (Collins et al., 2010) after Eam1105I restriction digest.

**in situ hybridization and DAPI staining**

Fixation and preparation of samples for whole-mount *in situ* hybridization and DAPI staining were performed as per King and Newmark (2013). Colorimetric development for visualization of riboprobes was performed as described by Pearson et al. (2009). *Smed-boule* riboprobes were synthesized using SP6 RNA Polymerase. All other riboprobes...
synthesized for the genes in Table 2 were done with T3 RNA Polymerase. *Smed-CPEB1* (NCBI accession number KU990884), *Smed-nanos*, were also synthesized from a pJC53.2-based construct (Collins et al., 2010), whereas *Smed-synaptotagminXV, Smed-granulin* (*grn*), *Smed-surfactant b, germinal histone H4*, and a tuba/oviduct marker were synthesized from pBluescript-based clones (PL04017B1F10, PL05005A1F08, PL010001001D12, pBS-*gH4*, and PL04015A2A02, respectively (Zayas et al., 2005; Wang et al., 2007; Chong et al., 2011)) using T3 RNA Polymerase. Colorimetric and low magnification analyses of DAPI signals from testes were imaged on a Zeiss Axio Zoom.V16 stereoscope. Confocal images were captured on an Olympus FluoView FV1000 confocal microscope hosted at Wright State University’s Microscopy Core Facility.

**RNAi**

Double-stranded RNAi feedings were performed twice every seven days and the protocol was followed as previously described (Rouhana et al., 2013). DsRNA corresponding *Escherichia coli ccdB* sequence, which does not affect planarian development or behavior was used for unaffected control groups. For isolated RNAi samples, each planarian was fed individually and in isolation. For other experiments, planarians were maintained in groups of seven animals.

**Analysis of egg capsule production and hatching**

Groups of seven sexual planarians of 0.5 to 0.7 cm size and with a visible gonopore were maintained in glass Petri dishes and subjected to dsRNA feedings as described above. For isolated experiments, single ≤ 1 week-old hatchlings were maintained in isolation in glass petri dishes throughout the experiment, under the husbandry conditions described
above. Isolated planarians were fed liver containing control or *Smed-boule* dsRNA twice per week, at which point any capsules present were collected and isolated. DsRNA corresponding to *E. coli ccdB* sequence was used for control samples. Egg capsules were monitored for hatchling events weekly for a period of three months after deposition.
3.11 Supporting Information
Supplementary Figure S1. Markers used in the analysis of oocytes and yolk gland development. (A) Double-fluorescence in situ hybridization (ISH) and confocal microscopy detect synaptotagmin XV (A’) and Smed-CPEB1 (A’’) mRNAs in oocytes of S. mediterranea. Oocytes are distinguished by DAPI staining (A) as large cells with condensed chromosomes in the ovary. (A’’) Merged image. (B) Whole-mount ISH on sexual strain specimens of S. mediterranea reveals timing and distribution of Smed-surfactant b expression resembling that of yolk glands in the largest animal. Scale bars = 25 µm in (A) and 1 mm in (B).
Supplementary Figure S2. *Smed-boule* is required for oocyte development. *CPEB1* whole-mount *in situ* hybridization (ISH) in sexual planarians subjected control RNAi reveals the presence of oocytes (A), which are absent in *Smed-boule* RNAi planarians (B). Images of DAPI staining in these individuals show the ventral side of control (A’) and *Smed-boule(RNAi)* animals (B’), and the presence of testes in the dorsal side of control animals (A”), but not in *Smed-boule(RNAi)* (B”). The fraction of animals displaying the phenotype represented by the image is shown at the bottom-left corner of each frame. Scale bars = 1 mm.
Supplementary Figure S3. A size increase is observed in $CPEB1(RNAi)$ planarians. Average size (cm) of sexually mature sized planarians after three months of RNAi treatment reveal a significant enlargement (*) using unpaired two-tailed $t$-test ($p < 0.05$) in $CPEB1(RNAi)$ animals compared to the size of control or $Smed$-$boule(RNAi)$. 
Supplementary Figure S4. *Smed-boule* is required for development of testicular germline stem cells, but not for ovarian germline stem cells. (A-D) Whole-mount *in situ* hybridization analysis of germline stem cell (GSC) distribution visualizing *nanos* expression in the testes (A-B) and ovary (C-D) regions of planarian hatchlings raised in isolation and subjected to continuous control or *Smed-boule* RNAi. GSCs were specifically absent in the testes region of *Smed-boule* knockdowns (B), but present in the ovary region of both control (C) and *Smed-boule* (D) knockdowns. The fraction of animals displaying the phenotype represented by the image is shown at the bottom-left corner of each frame. Scale bars = 1 mm.
Supplementary Figure S5. *Smed-boule* is required of development of germline stem cells in presumptive testis primordia in asexual strain samples of *S. mediterranea*. Detection of germline stem cell (GSC) clusters (white arrows) in presumptive testis primordia of asexual planarians subjected to three weeks of control RNAi (left) or *Smed-boule* (right). Magnified views show detection of GSC clusters in control samples, but not in *Smed-boule*(RNAi). The fraction of animals displaying the phenotype represented by the image is shown at the bottom-left corner of each frame. Scale bars = 1 mm.
Supplementary Figure S6. Egg capsules are produced in the absence of gametes or mating events. Models of planarian oviparity that could rely on a shared upstream trigger (A), or on separate and independent pathways (B), for initiating ovulation/fertilization and capsule deposition.
3.12 ACKNOWLEDGEMENTS

We thank Phil Newmark and Tracy Chong for providing DNA constructs for the analysis of somatic reproductive structures, as well as Scott Baird, Mill Miller, Andrew Voss, and Laura Rouhana for comments on the manuscript.
3.13 References


Chapter 4

Concluding Remarks and Appendices
4.1 Future Directions

Many questions arise from the analysis of Smed-boule(RNAi). My data disproves a very fundamental idea: that deposition of egg capsules in the sexual mode of hermaphroditic planarian flatworms is the result of a mating event by cross-fertilization. Egg capsules, or egg shells, have vast evolutionary significance and are not uncommon structures across metazoans. They are maternally derived structures that have broad differences functionally and structurally, yet all exist to surround eggs and/or embryos (Shu et al., 2015). Some egg structures are known for mediating fertilization while other egg-derived structures have several roles during embryonic development such as protection from biotic (predators) and abiotic (environmental) dangers. For example in mammals, the zona pellucida is a type of egg coat that is required for egg-sperm recognition and protects a developing embryo until it implants on the wall of a uterus (Monné et al., 2006).

I showed sexual S. mediterranea planarians continued to deposit egg capsules (Figure 3.4J) in the absence of the male germline (Figures 3.4E, 3.6F), oocytes (Figures 3.4B, 3.6D), and sex (3.6A-B), which is novel to the field and has not been documented before. I also showed animals that lacked yolk glands (Figure 3.4I) failed to deposit egg capsules (Figures 3.4J). Therefore, the production and deposition of egg capsules in S. mediterranea occurs by an unknown signal, or internal trigger, that occurs in the absence of a mate, mating, and gametes. There were only two cell types present in animals that continued to deposit egg capsules: vitelline cells (yolk glands) (Figures 3.4G-H, 3.6 G-H) and female germline stem cells (Figures 3.7A’-B’, 3.8C-D Supplementary figure 4).
Currently there is no ovulation cycle known about planarians, and there is also no knowledge of specific triggers, or signals, that causes egg capsule production and deposition in planarians, and other flatworm biology. Across metazoans, it is well known that yolk is important for nourishing the developing embryo. In planarian flatworms yolk cells are packaged with the embryo as the egg/zygote travels down the oviduct for deposition (Newmark & Sanchez Alvarado, 2002). In lizards and fish, vitelline cells have also been shown to support embryonic development (Stewart & Thompson, 1993). In cestodes and tapeworms, vitelline cells have been associated with egg capsule development and also in the formation of oocytes (Swiderski & Xylander, 2000).

Clearly, the vitelline cells are a very important structure and should be further investigated, as they are present in planarians actively laying egg capsules without gametes. Therefore it is likely they play a role in ovulation, activation and deposition of egg capsules in planarian flatworms and the embryonic function may be conserved across metazoans. We know SurfactantB is expressed in the yolk glands of planarians (Figure 3.6G-H, 3.4G-H, Supplementary Figure 1). SurfactantB(RNAi) should be performed, and the potential phenotype due to the absence of vitelline cells would explain whether or not that structure is indispensable for the deposition of egg capsules.

We also need to define cellular differences between the deposited egg capsules of Smed-boule(RNAi) and control egg capsules. It will be very helpful to the lab in the future to have an established protocol for dissecting egg capsules and staining the contents. We can see what stages an embryo has reached during development, if any at all. We know that planarians lay egg capsules that sometimes don’t hatch, so it would be interesting and useful to know what is being expressed in those egg capsules.
The female germline stem cells present in *Smed-boule(RNAi)* planarians actively depositing egg capsules is also intriguing. These cells could also be responsible for being the activating signal that causes planarians to deposit egg capsules. Evidence in turkeys showed a haploid oocyte is activated by an unknown stimulus and the deposited egg develops embryonic, diploid tissues (Cassar et al., 1998). *nanos(RNAi)* should be performed in sexual planarians, as *nanos* is expressed in female and male germline stem cells and is required for germline development and maintenance (Wang et al., 2007). It is not known whether the animals during this experiment continued to deposit egg capsules. In addition to this, whether there is a presence of yolk glands in *nanos(RNAi)* animals is also unknown. This information should be obtained and will help explain the mechanism that triggers the deposition of egg capsules.

My data also showed evidence for the presence of gender specific germline stem cell populations in *S. mediterranea*. Evidenced by *nanos* WISH, *Smed-boule(RNAi)* planarians lost male germline stem cells (Figure 3.8B, Supplementary figure 4B), but retained female germline stem cell populations around the ovaries (Figure 3.8D, Supplementary figure 4D). No *nanos* positive cells were detected in any *Smed-boule(RNAi)* testes. I showed that *Smed-boule* functions through initial stages of oogenesis, as oogonia are the only cell type present. This would be before any meiotic division, so this cell type should be diploid and in the future should be confirmed. In contrast, *Smed-boule* is required for male germline stem cell development. Altogether this data suggests *Smed-boule* is required for male germline stem cell development, and for oogenesis, specifically the meiotic stages. Furthermore, this data suggests the
possibility of gender specific germline stem cell populations (Figure 3.8; Supplementary Figure 4). To investigate this interesting possibility, RNAseq analysis comparing Smed-boule(RNAi) animals to Smed-nanos(RNAi) animals should reveal the genes expressed in the female germline stem cell population.

In humans, most cases of infertility are diagnosed as idiopathic due to the lack of understanding of mechanisms and genes required to make sperm and ova. In addition to this, male contraceptives remain nonexistent and therapies for sterility are lacking. The data uncovered from the characterization of Smed-boule, a member of the Deleted in AZoospermia gene family of RNA binding proteins confirmed that it is required for germline development in planarians. The DAZ gene family is known to be required for germline development across metazoans from sea anemone to humans (Vangompel & Xu, 2011). Evolutionarily speaking, this is very helpful as it is important to understand and identify the genetic mechanisms that have remained conserved between diverse organisms. This information is one more additional piece to the puzzle of germline development and there is much more to learn.

This thesis supports the notion S. mediterranea as a brilliant model organism for identifying and functionally characterizing genes that are required for inductive germ cell specification, germline development, and reproduction. Importantly, the genome of S. mediterranea has extensive similarity to vertebrates. The entire S. mediterranea genome has been sequenced and uploaded to a public database (Robb et al., 2015) and interestingly, of the planarian ESTs uploaded to GenBank that are homologous with other GenBank entries, 64% of them encode proteins that are more similar to vertebrates as opposed to invertebrate model organisms (Alvarado et al., 2002).
*S. mediterranea* is a member of the Platyhelminthes phylum and possesses an important amongst Metazoans. These flatworms have an abundance of developmental properties that make them an attractive organism to study, namely, their sophisticated anatomy, regenerative capacities, as well as their reproductive strategies (Newmark & Sanchez Alvarado, 2002). From a medicinal standpoint, Platyhelminthes serve as a wonderful opportunity to study mechanisms underlying cell fate due to their large population of pluripotent stem cells, their neoblasts. These cells allow them to regenerate any tissue and cell type throughout their lifespan, including their germ cells. Information resulting from my and future studies will contribute to research in biomedical fields such as tissue regeneration, contraception, gene therapies focusing on the reproductive system, as well as infertility and sterility.

In addition to regeneration and stem cell biology studies, we can use *S. mediterranea* as a model organism to better understand their parasitic family members of the genus *Schistosoma*. Schistosomiasis is a disease that sickens more than 200 million people in the world annually (Chitsulo et al., 2000). These parasites are attracted to fatty acids of human skin and burrow through it, lodging themselves into veins and in some cases organs, where they feed on blood and reproduce (Collins & Newmark, 2013). Schistosomes produce hundreds to thousands of eggs every day, and these eggs prolong infection by continuing to invade tissues and veins. These eggs cause serious inflammatory responses that lead to severe pathologies and even death (Collins & Newmark, 2013). Put simply, the entire schistosome infection is attributed solely to their reproduction cycle; the presence of their eggs and the inflammation response that follows. Despite the differences between free-living and parasitic Platyhelminthes,
planarians being simultaneous hermaphrodites and schistosomes having separate sexes, there are very important similarities that make them worth studying for therapeutic potentials. Neoblast-like stem cells have been identified in *Schistosoma mansoni* (Collins et al., 2013). Importantly, both schistosomes and planarians use yolk glands (planarians) or vitellaria (schistosomes) for egg shell production, and both species’ eggs are ectolecithal in that the yolk cells produced by these organs surround the zygote upon a fertilization event (Collins & Newmark, 2013). The molecular machinery involved in their egg capsule production is an obvious target for therapies against this parasitic disease, and the results from my work provides future directions to investigate this.

The results from these studies revealed novel information about *Boule* and *DAZ*-family gene expression and function in sexually reproducing animals, and attest planarians as an exceptional model system to study and identify genes required for germline development and function that will be relevant to the health of mankind. There are many genes whose identity has yet to be discovered that are necessary for germline construction. Due to the availability of genomic resources for studies in *S. mediterranea*, it will be advantageous to identify and characterize genes and their interacting partners to help scientists define germ cell development and reproduction. In addition to this, these future studies will have evolutionary advantages to explain what genes and molecular mechanisms have remained conserved throughout Metazoans in terms of their diversity, development, and stem cell activity.
4.2 List of Appendices

4.2.1 APPENDIX A: Sequences of candidate genes analyzed

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Reference Contig:
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CCGTGAGA
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CATTCCAA
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CCCTTACT
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Reference Contig:
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ATCGTTGAA
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AGAAGGCC
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AATAGTA
GAGATGATGATGAAAACATGATATTTAATCTGCAATTGGACTATGAAA
AATATTT
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AACGCTG
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TTTCAAA
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AGAAGG
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109
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