BMP Regulation of Stem Cell Development During Drosophila Testis Formation

Ashley P. Fidler
College of William and Mary

Follow this and additional works at: https://scholarworks.wm.edu/honorstheses

Part of the Cell and Developmental Biology Commons

Recommended Citation
https://scholarworks.wm.edu/honorstheses/87

This Honors Thesis is brought to you for free and open access by the Theses, Dissertations, & Master Projects at W&M ScholarWorks. It has been accepted for inclusion in Undergraduate Honors Theses by an authorized administrator of W&M ScholarWorks. For more information, please contact scholarworks@wm.edu.
BMP Regulation of Stem Cell Development During *Drosophila* Testis Formation

A thesis submitted to the Department of Biology at
The College of William & Mary

by

Ashley Fidler

Accepted for

Honors

(Honors)

Dr. Matthew Wawersik, Advisor

Dr. Mark Forsyth

Dr. Shanta Hinton

Dr. Douglas Young

Williamsburg, VA
May 1, 2014
TABLE OF CONTENTS

ABSTRACT ........................................................................................................................................4

INTRODUCTION ..............................................................................................................................5

STEM CELLS – ESSENTIAL FOR DEVELOPMENT AND TISSUE HOMEOSTASIS ........................................5
THE FRUIT FLY DROSOPHILA MELANOGASTER A MODEL ORGANISM ................................................7
THE MATURE DROSOPHILA TESTIS STEM CELL NICHE ........................................................................10
CELL SIGNALING IN THE ADULT DROSOPHILA TESTIS .....................................................................12
THE DROSOPHILA TESTIS THROUGHOUT DEVELOPMENT ......................................................................15
THE BMP PATHWAY IN THE DEVELOPING TESTIS ............................................................................17

METHODS .......................................................................................................................................18
FLY STOCKS .................................................................................................................................18
COLLECTION OF EMBRYONIC, LARVAL, AND ADULT ORGANISMS ................................................18
IMMUNOSTAINING OF EMBRYOS AND LARVAE ...........................................................................19
IMMUNOSTAINING OF ADULT TESTES ..........................................................................................21
CONFOCAL MICROSCOPY ..............................................................................................................21
ANALYSIS AND GENOTYPING ..........................................................................................................21

RESULTS ........................................................................................................................................23
BMP SIGNALING IS DYNAMICALLY ACTIVATED DURING TESTIS DEVELOPMENT ................................23
BMP SIGNALING IS NECESSARY AND SUFFICIENT FOR GSC MAINTENANCE IN THE DEVELOPING TESTIS .........26
GERMLINE-SPECIFIC BMP HYPERACTIVATION RESULTS IN LESS SEVERE PHENOTYPES ............28
PERTURBATION OF BMP SIGNALING IN THE SOMA RESULTS IN ABERRANT SPERMATOGENIC DIFFERENTIATION ..........................................................................................................................30
SOMATIC ACTIVATION OF JAK-STAT SIGNALING PROMOTES GSC MAINTENANCE THROUGH THE BMP SIGNALING PATHWAY ..................................................................................................................32

DISCUSSION ...................................................................................................................................34
DYNAMIC ACTIVATION OF BMP SIGNALING BEFORE HUB FORMATION MAY INDICATE UNEXPLORIED ROLES OF BMP SIGNALING IN THE TESTIS ..................................................................................35
A SECOND PATHWAY MAY WORK IN TANDEM WITH THE BMP PATHWAY TO PROMOTE GSC MAINTENANCE LATER IN DEVELOPMENT ..........................................................................................36
COMMUNICATION BETWEEN THE SOMA AND THE GERMLINE IS CRITICAL IN THE REGULATION OF GERM CELL BEHAVIOR ...........................................................................................................38
FUTURE DIRECTIONS ...........................................................................................................................40

ACKNOWLEDGMENTS ......................................................................................................................41

REFERENCES ....................................................................................................................................43

FIGURES ..........................................................................................................................................47
FIG. 1: ASYMMETRIC DIVISION IS A HALLMARK OF STEM CELL BEHAVIOR .........................................47
FIG. 2: THE DROSOPHILA MELANOGASTER LIFE CYCLE ....................................................................48
FIG. 3: THE GAL4/UAS GENE OVEREXPRESSION SYSTEM IS UTILIZED TO INDUCE TISSUE-SPECIFIC GENE EXPRESSION ..............................................................................................................49
FIG. 4: SCHEMATIC OF THE ADULT DROSOPHILA TESTES ................................................................50
FIG. 5: THE JAK-STAT SIGNALING PATHWAY ....................................................................................51
FIG. 6: THE BONE MORPHOGENETIC PROTEIN (BMP) SIGNALING PATHWAY ....................................52
FIG. 7: CURRENT MODEL OF STEM CELL REGULATION IN THE ADULT DROSOPHILA TESTIS ............53
FIG. 8: SCHEMATIC SHOWING TESTIS DEVELOPMENT AROUND THE TIME OF HUB FORMATION ..........54
FIG. 9: BMP SIGNALING IS DYNAMICALLY EXPRESSED DURING EARLY TESTIS DEVELOPMENT.......................... 56
FIG. 10: p-SMAD POSITIVE SOMATIC CELLS CORRESPOND WITH A SUBPOPULATION OF TRAFFIC JAM-
EXPRESSING POSTERIOR CYST CELLS........................................................................................................ 57
FIG. 11: p-SMAD EXPRESSION IN THE GERMLINE IS DETECTED LESS OFTEN AS TESTES AGE..................... 59
FIG. 12: BMP SIGNALING IS NECESSARY AND SUFFICIENT FOR GSC MAINTENANCE IN THE DEVELOPING TESTIS.
................................................................................................................................................................ 61
FIG. 13: TESTES EXPERIENCING GERMLINE-SPECIFIC BMP HYPERACTIVATION DUE TO A CONSTITUTIVELY
ACTIVE THICKVEINS RECEPTOR RECOVER LATE DURING THE SECOND INSTAR. ........................................ 62
FIG. 14: GENERALIZED DPP OVER-EXPRESSION IMPACTS GSC MAINTENANCE MORE SEVERELY THAN GERMLINE
SPECIFIC HYPERACTIVATION. .......................................................................................................................... 64
FIG. 15: PERTURBATION OF BMP SIGNALING IN THE SOMA RESULTS IN ABERRANT SPERMATOGENIAL
DIFFERENTIATION........................................................................................................................................... 66
FIG. 16: PERTURBED SPERMATOGENIAL DIFFERENTIATION IS A REPRODUCIBLE RESULT OF SOMA-SPECIFIC
BMP INHIBITION. ............................................................................................................................................... 68
FIG. 17: EXPRESSION OF THE BMP PATHWAY IN THE GERMLINE IS EXPANDED UNDER CONDITIONS OF CYSC
EXPANSION DUE TO SOMATIC JAK-STAT HYPERACTIVATION................................................................ 69
FIG. 18: TRAFFIC JAM (TJ) CAN BE USED AS A MARKER FOR CYSC EXPANSION AFTER SOMATIC JAK-STAT
HYPERACTIVATION......................................................................................................................................... 70
FIG. 19: EXPANSION OF BMP ACTIVATION IN THE GERMLINE CORRELATES WITH THE PROLIFERATION OF
TRAFFIC JAM (TJ) – EXPRESSING CYST CELLS. .......................................................................................... 71
ABSTRACT

Stem cells are vital for organogenesis, tissue regeneration, and tissue homeostasis. These asymmetrically dividing cells provide the functional cell types necessary for organogenesis while maintaining a stem cell population that continuously replaces damaged and dying cells. Despite the fundamental importance of stem cells to living systems, the mechanisms regulating stem cell development are not well understood. One of the most thoroughly studied systems for examining stem cell behavior is the stem cell niche in the adult Drosophila testis. This niche is composed of two populations of stem cells: the sperm-producing germline stem cells (GSCs) and the somatic cyst stem cells (CySCs). Both of these populations are anchored around a cluster of non-mitotic somatic cells found at the testis apex, called the hub. Within this stem cell niche, the Bone Morphogenetic Protein (BMP) pathway has been shown to regulate GSC maintenance in the adult organism. In this thesis, we examine the role of BMP signaling during the dynamic process of development. Specifically, we characterize the pattern of BMP activation in the testis throughout development, determine the role of BMP signaling in the maintenance of GSCs in larval testes, assess the role of BMP signaling on cyst cell function, and evaluate interactions between the BMP pathway and Jak-STAT, another pathway known to regulate CySC behavior. We find that BMP signaling expresses a dynamic pattern of activation in developing primordial germ cells (PGCs) during embryogenesis, and that BMP activation becomes restricted to GSCs and germ cells in the testis anterior after hub formation. Additionally, we find that BMP signaling is both necessary and sufficient for the maintenance of undifferentiated GSCs in the early larval testis and that in the soma it is required for proper spermatogonial differentiation. Finally, we show that soma-specific Jak-STAT hyperactivation results in expanded expression of BMP signaling, indicating interactions between the CySCs and the GSCs using the BMP pathway. Thus, our results show that BMP signaling from the CySCs promotes GSC
maintenance and demonstrates the importance of soma-germline communication in spermatogonial differentiation. Furthermore, the research presented in this thesis suggests other possible roles for BMP signaling in testis development, including a possible function in GSC establishment, as well as the existence of a second pathway that helps promote GSC maintenance during late stages of testis development.

INTRODUCTION

At some point during its life cycle, every organism on the planet exists as a single cell. Through the process of development, many organisms, including human beings, grow from a single cell into multicellular beings composed of a variety of different organs and tissue types. Composed of many cells specified for explicit functions, these organs and tissues operate in concert to perform all the processes required to maintain life. But how exactly does a single cell develop into dynamic, complex body of trillions of cells vastly different in appearance and function? Although this question in and of itself encapsulates the fundamental principle of modern developmental biology, a sizable portion of its answer lies in stem cells, or unspecialized cells capable of differentiating into functional cell types. In this thesis, I will examine the mechanisms controlling stem cell maintenance in developing organs further through the model system of the *Drosophila melanogaster* testis.

**Stem cells – essential for development and tissue homeostasis**

In multicellular organisms, stem cells are responsible for organogenesis and tissue homeostasis. Early in development, the cells that ultimately produce organs develop from unspecialized pluripotent stem cells capable of differentiating into a variety of specific cell types under the proper conditions. However, in addition to being able to differentiate into functional, tissue-specific cell types, stem cells must also be able to replicate themselves in order to
regenerate the population of stem cells for tissue homeostasis. Asymmetric division, a hallmark of stem cell behavior, provides the mechanism by which stem cells are able to perform both of these functions (Fig. 1). When a stem cell divides asymmetrically, one of its daughters retains the stem cell identity of the parent while the other, displaced from the microenvironment that provides the proper conditions for stem cell maintenance, differentiates. Thus, these asymmetrically dividing stem cells provide the cells necessary for organ development and homeostasis while maintaining a stem cell population that continuously replaces damaged and dying cells.

Because of their important role in organogenesis and tissue maintenance, many physicians and scientists hoping to better understand and treat human disease have become interested in stem cells and their regulation. Failure to appropriately regulate stem cell behavior has been linked to numerous medical conditions, including developmental disorders, cancer, and senescence (Kuhn, 2011; Martin-Belmonte and Perez-Moreno, 2012; Warren and Rossi, 2009). Furthermore, due to their regenerative capacity, therapies involving stem cells are being explored in hopes of reversing or minimizing injury and disease (Daley, 2012). As of 2012, there were over 4,000 listed clinical trials attempting to utilize stem cells to fight diseases such as Parkinson’s disease and Diabetes mellitus (Daley, 2012).

Despite their relevance in human disease and tremendous potential in therapeutics and drug design, we still know very little about the processes by which these stem cell populations are first formed and regulated. Stem cell development tends to be difficult to study as developing organs can be difficult to obtain, stem cells are often present in tissues in low numbers, and many stem cells share qualities with the surrounding tissues complicating identification and isolation.
(Xie and Spradling, 2000). Fortunately, these difficulties can be largely overcome by employing the common fruit fly, *Drosophila melanogaster*, as a model organism for stem cell development.

**The fruit fly *Drosophila melanogaster* as a model organism**

First propagated in the laboratory in 1901, the common fruit fly *Drosophila melanogaster* soon became the model organism of choice for laboratory genetics due to the pioneering work of Thomas Hunt Morgan and his students at Columbia at the turn of the century (Sang, 2001). Shortly after uncovering his first mutant, a white-eyed fly, in 1910, Morgan and his colleagues embarked on a process of discovery that would fundamentally alter the field of genetics (Rubin and Lewis, 2000). Using *Drosophila* as a model organism, they soon formulated the chromosome theory of heredity, constructed the first genetic map of an organism’s genome, and showed that ionizing radiation could introduce mutations into the genetic code, which revolutionized mutant-based genetic analysis. Today, even though other organisms, such as *Caenorhabditis elegans*, *Saccharomyces cerevisiae*, and *Xenopus laevis*, are widely available as models, *Drosophila* is still regularly utilized as a model organism for many different systems, including vision, sex determination, neuronal maintenance, and muscle development (Raghu et al., 2000; Wawersik, 2006; Moraru et al., 2012; Enriquez et al., 2012). The *Drosophila* genome was sequenced in 2000, illustrating both its historical and contemporary importance to the field of genetics (Adams et al., 2000).

*Drosophila melanogaster*’s small size, quick generation time, and large brood size have ensured its continued popularity in the laboratory (Sang, 2001). Upon being laid externally, a fertilized egg undergoes the process of embryogenesis during the first ~24 hours after egg laying (AEL). Following embryogenesis are three larval stages, or instars, separated by molting that encompass the time periods 24 – 48 hours AEL, 48 - 72 hours AEL, and 72 – 96 hours AEL.
respectively. Larvae consume nutrients to prepare themselves for pupation, a roughly four-day period during which the larval tissues are destroyed and replaced by adult tissues. After pupation, a morphologically mature fly emerges from the pupal case in a process called eclosion (Fig. 2). The mature female fly, which can be easily distinguished from the male by examining abdominal pigmentation and presence of external genitalia, can lay up to 100 fertilized eggs a day at peak fertility, or approximately 2000 eggs total throughout her lifetime, leading to substantial increases in the population over a relatively short period of time (Sang, 2001). Because mature flies are only 3 mm in length and 2 mm in width and do not required expensive equipment to maintain, these exponential population increases are easily accommodated in the laboratory and can be utilized effectively in screens and experiments (Manning, , 2010; Patterson, 1943).

The accessibility of the Drosophila genome only augments the usefulness of Drosophila melanogaster as a model organism. At 5% the size of an average mammalian genome, the Drosophila genome is better suited for genetic analysis when compared to its vertebrate counterparts (Rubin, 1988). In part because of its manageable size, many tools exist to manipulate and study the Drosophila genome. In 1918, one of Thomas Hunt Morgan’s students, H.J. Muller introduced balancer chromosomes into Drosophila genetics to ensure that genetic lines could be maintained entirely by self-mating (Rubin and Lewis, 2000). Balancer chromosomes contain multiple inversions that prevent normal paring and recombination between chromosomes during metaphase of meiosis. In addition, many balancer chromosomes also contain homozygous lethal alleles to remove any fly from the breeding pool that obtained balancer chromosomes from both parents. Thus, balancer chromosomes ensure that only organisms heterozygous for a particular gene of interest are maintained in a stock population.
To induce tissue-specific expression of a particular gene, researchers using *Drosophila* have yet another tool – the Gal4/UAS gene overexpression system. This system utilizes the *Saccharomyces cerevisiae* transcription factor Gal4 to transcribe a gene under the control of an Upstream Activating Sequence (UAS) element (Duffy, 2002). In *Drosophila* specifically, a fly with a Gal4 driver expressed in a particular tissue can be mated to a fly that possesses a gene of interest regulated by a UAS element. In the resulting progeny, the Gal4 transcription factor will bind the UAS element in cells of the tissue where Gal4 is expressed, resulting in tissue-specific transcription of the gene of interest (Fig. 3). By allowing a researcher to express any gene they desire in a specific tissue, this tool greatly expands the numbers and types of assays *Drosophila* researchers are able to perform. *UAS – LacZ* and *UAS – GFP* transgenes can be employed to map gene expression and cell morphology. To assess the role of particular protein or cell signaling pathway within a tissue, this system can also be used to overexpress the gene encoding that particular protein or one of its known inhibitors. Furthermore, because a gene of interest can only be expressed in cells that possess both a Gal4 driver and a UAS element, the Gal4/UAS system eliminates the possibility of parental infertility caused by transgene-induced disruptions in reproduction and lethality due to overexpression of the desired gene in another tissue.

Not only are fruit flies easy and inexpensive to maintain and manipulate in the laboratory environment, but they also possess many genes that are conserved in higher vertebrates, including humans. In particular, many of the genes encoding molecules fundamental to basic life-maintaining processes are homologous to genes found in vertebrates (Rubin, 1988). Numerous studies have shown that this homology extends to the function of the gene products as well. For example, studies have shown that, although streamlined in *Drosophila*, the fundamental cell signaling cascades, including the Jak-STAT pathway, are conserved between
*Drosophila* and vertebrates (reviewed in Arbouzova and Zeidler, 2006). In addition to gene conservation, many developmental paradigms are also conserved (Jemc, 2011). Therefore, the knowledge derived from research in *Drosophila* can often be used to understand similar systems in human beings.

While many of the basic mechanisms employed in the development and maintenance of organs and tissues in *Drosophila* are conserved in higher vertebrates, often *Drosophila* biological systems are highly simplified in structure. The structural simplicity of its organ systems, in conjunction with its homology to vertebrate systems and general practicality in the laboratory, make *Drosophila* an ideal model organism for the study of organogenesis. For example, the development of the *Drosophila* eye, a highly ordered epithelial tissue consisting of approximately 750 ommatidia arranged in hexagonal patterns, has been extensively studied to better understand the role of intercellular cross-talk within developing organs and tissues (reviewed in Voas and Rebay, 2004). In a similar vein, *Drosophila* wing development has been employed to explore the influence of pattern regulators on organ growth and the role of competitive interactions between cells in tissue homeostasis (reviewed in Neto-Silva et al., 2009). Just as the developing eye and wing are great model systems for intercellular communication and pattern regulators, the *Drosophila* testis is a particularly good model for the analysis of stem cell development because of its relatively simple structure (reviewed in de Cuevas, 2011).

*The mature Drosophila testis stem cell niche*

Male fruit flies possess a pair of testes (Fig. 4). In the adult organism, each testis forms a coiled tube with a single blind end where the stem cell niche is situated (reviewed in de Cuevas, 2011; Fuller, 1993). The stem cell niche contains two populations of stem cells: the germline
stem cells (GSCs) and the somatic cyst stem cells (CySCs) (Fig. 4). The GSCs are responsible for producing sperm, while the CySCs produce cyst cells that support spermatogenesis. Both of the stem cell populations are arrayed around a cluster of ~10 to 15 non-mitotic somatic cells known as the hub (Hardy et al., 1979). Located at the apex of the testis, the hub acts as a source of local signals that dictate stem cell fate. To maintain the appropriate number of stem cells in the niche, the hub forms polarized cell adhesions to orient stem cell divisions away from the hub. When the GSCs divide asymmetrically, the daughter cell that remains docked at the hub maintains its stem cell identity while the other, referred to as a gonialblast, differentiates and divides further to produce spermatogonia and, later, sperm. Thus, the orientation of division in relation to the hub helps ensure both stem cell population maintenance and gonialblast differentiation.

Two CySCs flank each GSC. Like GSCs, CySCs divide asymmetrically to maintain the CySC population and produce progeny called cyst cells, which encase gonialblasts and promote spermatogenic differentiation. Each gonialblast, supported by enlarging cyst cells, undertakes four rounds of transit-amplifying division to produce a 16-cell spermatogonia. After achieving the 16-cell stage, the cells within the spermatogonia undergo meiosis to create sperm. Once formed, sperm are released from the open end of the testis into the seminal vesicle where they are stored (de Cuevas, 2011).

The differentiation status of a germ cell can be easily visualized by immunostaining for specific cellular marker. One such marker is the fusomes, a germline-specific organelle that forms cytoplasmic extensions between differentiating germ cells that undergo incomplete cytokinesis. GSCs and newly made gonialblasts both possess spherical fusomes, while 2-cell spermatogonia are connected by an elongated fusome. As spermatogonia continue to divide with
incomplete cytokinesis, the fusomes become increasingly elongated and branched as the spermatogonium reaches the 16-cell stage. Therefore, the morphology of the fusomes can be employed as an indicator of differentiation status of germ cells.

Cell signaling in the adult Drosophila testis

Communication between the different cell types of the testis is critical for the regulation of stem cell maintenance and spermatogonia differentiation (Tulina and Matunis, 2001; Kiger et al., 2001). Cell signaling is paramount to this intercellular communication. Two of the most important cell signaling pathways to stem cell regulation in the Drosophila testis are the Janus kinase-Signal transducer and activator of transcription (Jak-STAT) signaling pathway and the Bone Morphogenic Protein (BMP) signaling pathway (de Cuevas, 2011).

The Jak-STAT Signaling Pathway

The Jak-STAT signaling pathway was initially identified in vertebrates (Darnell, 1997; Heinrich et al., 1998). Soon after, it was also found to exist in invertebrates (Dearolf, 1999). Although the Jak-STAT pathway is highly conserved, in comparison to the vertebrate pathway, which has multiple isoforms of each of the major signaling components, the Drosophila Jak-STAT pathway is greatly simplified (Arbouzova and Zeidler, 2006). In Drosophila, the Jak-STAT ligand, Unpaired (UPD), binds to a transmembrane receptor called Domeless (DOME) (Fig. 5). This receptor undergoes a conformational change that results in the activation of Hopscotch (HOP), a JAK kinase associated with the receptor. Once activated, the two HOP kinases on neighboring domains of the receptor cross phosphorylate each other, generating the docking sites for the SH2 domains of Signal Transducers and Activators of Transcription (STAT) protein. Once the binding sites on the receptor are available, STAT monomers are
recruited from the cytoplasm and then are phosphorylated. The phosphorylated STAT monomers dimerize and travel to the nucleus where the dimer acts as a transcription factor, regulating gene expression (Arbouzova and Zeidler, 2006).

In the *Drosophila* testis specifically, the Jak-STAT signaling pathway has been shown to be critical for stem cell maintenance and positioning (Leatherman and Dinardo, 2010). The hub secretes the Jak-STAT ligand UPD, resulting in the phosphorylation of STAT and thus changes in gene expression in both the GSCs and the CySCs (Flaherty et al., 2010; Kiger et al., 2001; Leatherman and Dinardo, 2008; Leatherman and Dinardo, 2010; Tulina and Matunis, 2001). In the somatic stem cells, activation of the Jak-STAT signaling pathway promotes the maintenance of CySC stem cell identity by inducing the expression of Zinc finger homeodomain-1 (ZFH-1) and Chinmo (Flaherty et al., 2010; Leatherman and Dinardo, 2008). In the GSCs, Jak-STAT signaling is responsible for the formation of cell adhesions with hub (Leatherman and Dinardo, 2010).

*The BMP Signaling Pathway*

Proteins in the BMP pathway are members of the larger transformation growth factor beta (TGFβ) superfamily (Zhang and Li, 2005). Like the Jak-STAT pathway, the BMP pathway is conserved in invertebrates such as *Drosophila*, as well as vertebrates. In *Drosophila*, three different ligands for the BMP pathway exist – Decapentaplegic (DPP), Glass Bottom Boat (GBB), and Screw (Scw) (Newfeld et al., 1999; reviewed in O'Connor et al., 2006) (Fig. 6). These ligands can form either homo- or hetero- dimers in order to activate a common set of transmembrane receptors. These serine/threonine kinase receptors include Thickveins (Tkv), Saxophone (Sax), and Punt (Shi and Massague, 2003; O'Connor et al., 2006). Upon activation by a ligand, the receptors phosphorylate Mothers Against Decapentaplegic (p-MAD), which then
dissociates from the receptor and complexes with another p-MAD protein and a co-SMAD called Medea. This complex translocates to the nucleus where it acts as a transcription factor (O'Connor et al., 2006). One of the genes activated by this transcription factor encodes Daughters Against Decapentaplegic (DAD), which participates in a direct negative feedback loop that inhibits the BMP pathway after activation by preventing the phosphorylation of MAD (Tsuneizumi et al., 1997). The HECT domain ubiquitin E3 ligase SMAD ubiquitination regulatory factor (SMURF) also negatively regulates the BMP pathway when activated in *Drosophila* (Chang et al., 2013; Kavsak et al., 2000; Lin et al., 2000; Zhang et al., 2001; Zhu et al., 1999).

Because it is heavily involved in the regulation of cell growth and differentiation, the BMP pathway is essential in many developmental processes (Zhang and Li, 2005; O'Connor et al., 2006). In the *Drosophila* ovary, the BMP pathway is responsible for ensuring that GSCs remain stem cells and divide appropriately (Xie and Spradling, 2000). Studies in the *Drosophila* testis indicate that BMP signaling plays a similar role in maintaining GSC identity in the adult male. Because GSCs simply need to be in contact with CySCs in order to maintain their stem cell identity, the current model for BMP regulation of GSC identity in the testis is that CySCs secrete the BMP ligands DPP and GBB, activating the BMP pathway in adjacent GSCs (Leatherman and Dinardo, 2010; Kawase et al., 2004; Shivdasani and Ingham, 2003; Wang et al., 2008; Zheng et al., 2011) (Fig. 7).

Recent research, however, suggests the role of BMP signaling in the testis may be more complex than depicted in the current model. A study in late larval, pupal, and adult *Drosophila* male testis demonstrates that activation of the BMP pathway in the germline attenuates dramatically during the course of late testis development (Chang et al., 2013). In comparison with late first instar larvae, both p-MAD and DAD levels are significantly decreased in second
larval instar and pupal testes and are nearly undetectable in adults. Meanwhile, over the same time period, cyst cell p-MAD expression remains strong. The germline-specific attenuation of BMP signaling over the course of development has been attributed to SMURF-mediated proteolysis of MAD (Chang et al., 2013). While a dramatic attenuation of BMP activation in the germline over time does not necessarily preclude BMP signaling from promoting GSC maintenance, this data casts doubt on the BMP pathway’s accepted role as the primary regulator of GSC maintenance in the developing testis.

A second study more directly challenges the current model for GSC maintenance in the adult testis (Lim and Fuller, 2012). After ablation of CySCs and cyst cells in the adult testis via activation of a temperature-sensitive apoptotic activator, cyst cells loss resulted in severe defects in spermatogonial differentiation while CySC loss appeared to have little effect on anterior germ cells. Without CySCs, the germ cells arrayed around the hub continued to exhibit spherical fusomes consistent with GSCs and newly made gonialblasts. Interestingly, elevated level of p-MAD were observed in the germ cells of these testes, despite being undetectable in the GSCs and spermatogonia of control testes. These data suggest that not only are the CySCs not responsible for transducing the signal that promotes GSC maintenance, but that they are also not responsible for BMP activation in the GSCs. Together, the results from these two studies indicate that a reexamination of the current model of stem cell maintenance and differentiation in the Drosophila testis is sorely needed.

The Drosophila testis throughout development

With potentially some exceptions, the bulk of the literature implicates the BMP pathway in GSC maintenance in the adult Drosophila testis. However, the mechanisms controlling processes such as differentiation, proliferation, and apoptosis in the dynamic and highly complex
environment of a developing organ may vary substantially from those observed in the adult. Therefore, to determine the mechanism governing GSC maintenance during the critically important period of organogenesis, the developing testis needs to be examined explicitly.

Development itself is a highly dynamic process (Fig. 8). Embryogenesis, which occurs during the first 24 hours after egg laying (AEL), consists of 17 stages. The embryonic testis consists of two populations of cells: primordial germ cells (PGCs) that ultimately develop into the germ cells of the larval and adult testis and somatic gonadal precursors (SGPs), which becomes the somatic cells of the testis (reviewed in Jemc, 2011). During the first 15 stages of embryogenesis, the steps leading to the creation of the gonads is not sexually dimorphic. In both males and females, the PGCs arise from maternally derived factors in the posterior of the embryo at stages 4-5 of embryogenesis, and remain in this location until they are pulled into the interior of the embryo by invagination of somatic tissues at stage 7 of embryogenesis (Kunwar et al., 2008). The SGPs are not specified until stage 11 when they are formed in the mesoderm in three clusters located bilaterally within the embryo. A fourth cluster contains male-specific SGPs (msSGPs), or SGPs that ultimately undergo apoptosis in females (DeFalco et al., 2003). At stage 12, actively migrating PGCs reach the bilateral SGP clusters and begin to intermingle with them (Boyle and DiNardo, 1995). Subsequently, at stage 13, cytoplasmic extensions from the SGPs surround the PGCs in a process called ensheathment (Decotto and Spradling, 2005; Gonczy and DiNardo, 1996; Jenkins et al., 2003). In males, the msSGPs join this cluster at stage 14 (DeFalco et al., 2003).

Thus, by gonad coalescence at stage 15 of embryogenesis (~15 hours AEL), the testis is distinguishable from the ovary (reviewed in Jemc, 2011). Late into stage 17, 10-12 anterior SGPs in which Notch signaling has been induced from the hub and begin to express multiple different
adhesion proteins, including Fasciclin 3 (Fas 3) (Le Bras and Van Doren, 2006; Okegbe and DiNardo, 2011; Kitadate and Kobayashi, 2010, Sinden et al., 2012). Following hub formation, the PGCs give rise to GSCs established in the stem cell niche by adhesion to the hub (Sheng et al., 2009). The SGPs that encased the PGCs at this point give rise to the CySCs that continue to surround the GSCs in pairs (Sinden et al., 2012). Therefore, by the start of the first larval instar (L1; ~23 hours AEL) the architecture of the stem cell niche observed in the adult testis has been constructed. Once the stem cell niche forms, both the GSCs and the CySCs divide asymmetrically to maintain the stem cell population within the niche and to produce spermatogonia (reviewed in Jemc, 2011). While Jak-STAT signaling has been shown to be responsible for CySC maintenance in the larval testis (Sinden et al., 2012), the mechanism governing GSC maintenance in the developing gonad remains unknown.

**The BMP pathway in the developing testis**

Given the BMP pathway’s crucial role in GSC maintenance in the adult testis, experiments establishing its role in the developing testis have the potential to generate novel insights into stem cell regulation within the dynamic microcosm of a developing organ. In addition to revealing whether the BMP pathway’s role in GSC maintenance is conserved in the developing testis, these experiments may implicate BMP signaling in other functions, such as establishment or cyst cell regulation of spermatogenesis. Furthermore, a complete developmental perspective on BMP signaling in the testis may provide some clarity to the BMP pathway’s role in the adult, addressing some of the concerns that have been raised by the recent literature.

The goal of this thesis is to provide this much needed developmental perspective on BMP signaling in during testis development. Specifically, we examined the role of BMP signaling in the regulation of stem cell development within the developing testis. In order to examine this
role, we have (1) characterized BMP activation throughout development, (2) assessed whether BMP signaling is responsible for GSC maintenance during development, (3) examined the role of BMP signaling on cyst cell function, and (4) evaluated potential interactions between the BMP pathway and the Jak-STAT pathway. Here, we show that BMP is dynamically activated before and during hub formation before restricting to the anterior GSC as observed in the adult and that BMP signaling is necessary and sufficient for GSC maintenance in the L1 testis. Furthermore, our results indicate that BMP activation in the soma is critical for proper spermatogonial differentiation. Finally, we find that hyperactivation of the Jak-STAT pathway results in an expansion of BMP activation within the testis, suggesting extant interactions between these two important signaling pathways.

METHODS

Fly Stocks

Fly Stocks

y, w1118 flies were used as controls. w1118; P{GAL4::VP16-nos.UTR}CG6325MVDD (Lehmann; Van Doren et al., 1998) was employed to induce germline expression of UAS-transgenes. A third line, w;P[w+, B10.2nos-Gal4], was used in experiments requiring a reporter of relative transgene expression. c587-Gal4 (Spradling; Kai and Spradling, 2003) drove UAS-transgene expression in the soma. UAS-lines used include: UAS-dpp (no tag) J3, UAS-dad (U16), UAS-tkv activated (-HA) 1b, UAS-tkv ΔG (1F2 + 1F3), UAS-punt ΔI (4B2 + 6B2), and UAS-hopTumL (Harrison; Hanratty and Dearolf, 1993). All fly stocks were obtained from the Bloomington Stock Center (http://flystocks.bio.indiana.edu/), unless otherwise specified.

Collection of embryonic, larval, and adult organisms

The control y, w1118 embryos and larvae were collected at time increments of 0-16 (embryos), 16-24 (embryos/early L1), 24-36 (early to mid L1), 36-48 (mid/late L1), 48-60
(early/mid L2), and 60-72 (mid /late L2) hours after egg laying (AEL) at 24°C. To obtain larvae for the germline BMP inhibition and hyperactivation experiments, \textit{P\{GAL4::VP16-nos.UTR\CG6325\}} virgin females were mated to \textit{UAS-dpp}, \textit{UAS-dad}, \textit{UAS-tkv activated (-HA) 1B-}, or \textit{UAS-tkv AG} males. Embryos from this mating were collected from 0 to 24 hours AEL and aged at 24°C for 24 or 48 hours to yield separate larval samples aged 24-48 and 48-72 hours AEL respectively. The same procedure was employed for matings between \textit{c587-Gal4} virgin females and \textit{UAS-dpp}, \textit{UAS-dad}, \textit{UAS-tkv activated (-HA) 1B-}, \textit{UAS-tkv AG}, or \textit{UAS-punt \Delta I} males. To obtain larvae with ectopic somatic \textit{hopTumL} expression, \textit{c587-Gal4} virgin females were mated with \textit{UAS-hopTumL} males. Embryos were collected from 0 to 7 hours AEL at 24°C and then incubated at 29°C for 48 (early L2) or 55 (mid L2) hours. All adult testes were dissected out of male flies 5 to 7 days after eclosion.

\textit{Immunostaining of embryos and larvae}

Immunostaining of embryos and larvae was performed as described (Fidler et al, 2013). Briefly, collected embryonic and larval samples were fixed in formaldehyde and stored for immunostaining in methanol. To prepare for the addition of antibodies, samples were rehydrated in 50% methanol/PBTw solution and washed with BBTw. Larval samples were then sonicated repeatedly for 2 seconds until the larval cuticle that prevents antibody permeation had been effectively removed. Following sonication, the sample was blocked in a 5% normal goat serum (NGS)/BBTw solution to increase staining efficiency. Primary antibodies were then added and allowed to incubate overnight at 4°C and then for minimum of 4 hours at ~24°C. After additional washes to remove the primary antibodies and a second block, fluorophore-labeled secondary antibodies were added to the sample for ~48 hours at 4°C. Immunostained samples were stored in DABCO solution (Sigma) at -20°C. This protocol results in the staining of an average of
72.3% of larvae, although exact staining efficiency is inversely proportional to the age of sample (Fidler et al., 2013).

The following primary antibodies were used: chick anti-Vasa at 1:3000 (K. Howard); rabbit anti-Vasa at 1:5000 (R. Lehmann); rat anti-Vasa at 1:20; rabbit anti-p-SMAD at 1:100; mouse anti-Fasciclin 3 at 1:10 (C. Goodman; Developmental Studies Hybridoma Bank [DSHB]); mouse anti-1B1 at 1:4 (H. Lipshitz; DSHB); guinea pig anti-Traffic Jam at 1:2500 (D. Godt); rat anti-BAM at 1:1000; rabbit anti-ZFH1 at 1:5000 (R. Lehmann). The following secondary antibodies were used: goat anti-chick 546, goat anti-mouse 633, goat anti-mouse 488, goat anti-rabbit 488, goat anti-rabbit 546, goat anti-rat 488, goat anti-rat 546, and goat anti-guinea pig 633. All secondary antibodies were used at 1:500. Nuclei were stained with DAPI at 1:1000 (Roche) for 3 minutes after the removal of secondary antibodies.

Modifications were made to this protocol to enhance the staining quality of particular antibodies. In experiments assessing the state of BMP activation in the testis, anti-p-SMAD was allowed to incubate alone overnight at 4°C and then for an additional 4 hours at room temperature. Anti-p-SMAD was then removed and any other primary antibodies necessary were added for at least 4 hours at room temperature. For anti-p-SMAD and anti-Traffic Jam co-stains, anti-p-SMAD was again allowed to incubate alone overnight at 4°C and then for an additional 4 hours at room temperature. After the appropriate washes and block, the secondary antibody was added to the sample for ~24 hours at 4°C. Following removal of the secondary antibody, the anti-Traffic Jam antibody was added to incubate alone overnight at 4°C and then for an additional 4 hours at room temperature. The secondary antibody was then added overnight at 4°C. The secondary was then removed and any other primary antibodies necessary were added for at least
4 hours at room temperature. The same procedure was performed for anti-Zfh-1 and anti-Traffic Jam co-stains.

**Immunostaining of adult testes**

Immunostaining of dissected adult testes was performed as described (Matunis et al., 1997). In brief, testes dissected in Ringers solution were fixed in a 4% formaldehyde solution, washed in PBTx and blocked in a 5% normal goat serum/BBTx solution. The anti-p-SMAD antibody was then added at 1:100 in PBTx and NGS and allowed to incubate overnight at 4°C and then at room temperature for 3 hours. After removal of the anti-p-SMAD antibody, all other primary antibodies were added for 4 hours at room temperature. After additional washes and a block, the secondary antibodies were added overnight at 4°C. The stained testes were stored in DABCO solution at -20°C.

**Confocal microscopy**

Embryonic and larval samples were mounted and staged on a glass microscope slide in a DABCO/p-phenylenediamine (PPD) anti-fade agent solution (Sigma). Embryos were staged according to morphology apparent under a light microscope (Campos-Ortega and Hartenstein, 1985) whereas larvae were staged by physical size. Appropriate staging for wildtype embryos and larvae was confirmed by testis morphology. Samples were examined with an Olympus BX51 microscope equipped with a DSU spinning disc confocal system and a Q-imaging RETIGA-SRV CCD camera. Images were captured and analyzed using Slidebook 5.0 software by 3i. All images presented are 3 plane-projections of the three-dimensional capture.

**Analysis and genotyping**

To compare the relative prevalence of p-SMAD staining between first instar larval and adult testes, testes with p-SMAD staining in the posterior cyst cells were scored based on the
presence of p-SMAD staining in the germline. Percentages were calculated by dividing the number of testes in which germline p-SMAD expression was absent but somatic p-SMAD activation was present by all testes that showed positive somatic expression. Significance was determined using a standard two-tailed z-test.

To examine the impact of altered BMP signaling on testis development, two factors were considered: (a) germ cell differentiation status, and (b) gonad size. Germ cell differentiation status was assessed through an examination of the fusomes. Testes experiencing germline BMP inhibition were scored positively for a non-wildtype differentiation phenotype if fusome elongation was observed in germ cells located near the hub. In testes undergoing BMP hyperactivation, failure to achieve spermatogonia differentiation beyond the 4-cell stage by early L2 was considered indicative of a mutant differentiation phenotype. For testes experiencing either somatic BMP hyperactivation or inhibition, the presence of a spherical or elongated fusome equidistant from the hub as branched fusomes was used as an indicator of a mutant differentiation phenotype. Prevalence of each of these phenotypes is recorded as the percentage of testes with disturbed differentiation patterns out of all testes with clear 1B-1 staining analyzed.

Size was assessed by measuring the length of each testis from the hub to the farthest posterior germ cell using Slidebook 5.0 (ruler tool). The length of all testes oriented longitudinally within the imaging plane were measured and compared to a set of wildtype testes of comparable age (n = 13). The significance of differences in gonad size was determined with a standard two-tailed student t-test.

For the progeny of the mating between nanos-Gal4 and UAS-tkv ΔG flies, we also examined vacuolization, or the formation of regions that appear to lack cells, within early second instar (L2) testes. A testis was scored positive for vacuolization if it contained a region larger
than 3 germ cells in diameter that contained no DAPI-stained nuclei. The prevalence of this vacuolization phenotype is recorded as the percentage of testes that scored positive for vacuolization out of all testes with clear Vasa staining analyzed.

The mating between c587-Gal4 virgin females and heterozygous UAS-hop\textsuperscript{Tuml} males performed to hyperactivate Jak-STAT signaling in the testis’ somatic cells produces both hop\textsuperscript{Tuml}-positive progeny and sibling controls. To genotype testes from this mating, we initially examined the testes for the presence of spermatogonial clusters, which would indicate a wildtype phenotype. Of testes with spermatogonial clusters, the percentage of those with restricted BMP activation was reported. The percentage of testes with expanded germline BMP expression was reported for testes without spermatogonial clusters. The significance of the difference between these two proportions was determined using a two-tailed $z$ test. After verifying Traffic Jam expansion in hop\textsuperscript{Tuml}-positive testes, the same analysis procedure was performed, instead using Traffic Jam expansion as an indictor of hop\textsuperscript{Tuml}-positive progeny. Again, a two-tailed $z$-test was employed to determine significance.

RESULTS

BMP signaling is dynamically activated during testis development

Studies in the adult Drosophila testis have suggested that the BMP pathway is responsible for GSC maintenance (Leatherman and Dinardo, 2010; Kawase et al., 2004; Shivdasani and Ingham, 2003; Wang et al., 2008; Zheng et al., 2011). Although a substantial body of work appears to validate this conclusion, recent findings that report a dramatic attenuation of BMP signaling in pupal and adult testes call the accepted role of BMP signaling in the testis into question (Lim and Fuller, 2012; Chang et al., 2013). To address this contradiction, as well as understand the role of BMP signaling during testis development, we first sought to
determine the pattern of BMP activation during wildtype testis formation. To do so, we utilized phosphorylated MAD, a downstream target of BMP signaling, as an indicator of BMP activation in wildtype embryonic and larval testes. The presence of phosphorylated MAD was assessed using an antibody for its mammalian homolog, phospho-SMAD (p-SMAD). The efficacy of the mammalian p-SMAD antibody has been demonstrated in several different Drosophila tissues, including the embryonic endoderm and the adult gonads and muscles (Casas-Tinto et al., 2008; Leatherman and Dinardo, 2010; Goldstein et al., 2011).

In the adult testis, p-SMAD expression is restricted to GSCs and germ cells residing in close proximity to the hub at the anterior of the testis and to cyst cells (Leatherman and Dinardo, 2010). Early in testis development, we find that p-SMAD expression is less static. After a period of initial activation in the newly formed PGCs at the embryo posterior (~5 hours AEL; n = 5, data not shown), p-SMAD is not detected in the PGCs during germ band extension (data not shown) and through the process of germ cell migration (~10 hours AEL; n = 27, Fig. 9B). However, after the coalescence of PGCs into a tight cluster with the SGPs (~15 hours AEL), p-SMAD is detected in a subset of PGCs at the posterior of the gonad (n = 21, Fig. 9C). p-SMAD expression then segregates to a single row of cells that extends toward the anterior of the testis as the somatic hub forms (~23 hours AEL; n = 18, Fig. 9D, 9E). Around the embryo to larval transition (~24 hours AEL), p-SMAD becomes restricted to anterior germ cells, including the GSCs localized immediately adjacent to the hub, as is observed in the adult organism (n = 63, Fig. 9F). Once the architecture of the stem cell niche is established, the gonad increases in size due to germ cell division and spermatogonial divisions. Around the onset of germ cell differentiation (~36 hours AEL; mid-L1), we also observe p-SMAD expression in the cyst cells encasing the differentiating gonialblasts and spermatogonia (n = 55). p-SMAD expression in the
testis remains restricted to the anterior germline and the cyst cells consistently throughout these larval stages (Fig. 9G). These observations indicate that BMP signaling is highly dynamic before and during hub formation, but that it restricts to the GSCs and anterior germ cells in pattern similar to the adult testis after a functional GSC niche is established in the early larval testis.

As noted above, p-SMAD expression is regularly observed in cyst cells within the adult Drosophila testis (Leatherman and Dinardo, 2010). In order to verify the cyst cell identity of the p-SMAD expressing somatic cells observed after the onset of differentiation in the larval testis, we added a cyst cell marker, Traffic Jam (TJ), to testes stained with p-SMAD (Fig. 10). We found that the somatic p-SMAD expressing cells co-express TJ (100%, n = 9), confirming that the cyst cells strongly express p-SMAD in the larval testis just as they do in the adult.

Although p-SMAD is expressed in the late first instar larval (L1) testis in a manner characteristic of the adult, p-SMAD expression in GSCs and their immediate daughter cells is often more difficult to detect in older testes. Interestingly, in some adult testes, no p-SMAD expression is observed in the germline despite strong p-SMAD staining in the posterior cyst cells (Fig. 11A). A similar pattern can be observed earlier in some second larval instar (L2) testes (Fig. 11B). While the cyst cells express p-SMAD strongly, p-SMAD expression is not discernible in the GSCs and their germ cell daughters. To quantify this observation, we compared the percentage of adult testes that failed to exhibit germline p-SMAD staining in the presence of discernable cyst cell p-SMAD staining to the percentage of L1 testes that showed a similar discrepancy in p-SMAD staining. Of adult testes that show p-SMAD expression in the cyst cells, 80% (n = 5) fail to exhibit any p-SMAD staining in the germline, as compared to 30% (n = 23) of first instar larvae (Fig. 11C). This result suggests that the levels of BMP activation decrease over the course of development (p = 0.0394). Taken together, these data indicate that
BMP activation is dynamic in the *Drosophila* testis both before hub formation and after restriction to the GSCs early in the first larval instar.

**BMP signaling is necessary and sufficient for GSC maintenance in the developing testis**

The pattern of BMP activation in larval *Drosophila* testes suggests that BMP signaling plays a role in GSC maintenance during development much like it does in the mature gonad. To determine whether the BMP pathway is responsible for GSC maintenance in the developing testis, we sought to assess whether BMP signaling is necessary and sufficient to repress GSC differentiation. Using the GAL4/UAS gene overexpression system (Duffy, 2002), known activators and repressors of BMP signaling were induced in larval germ cells shortly after GSC establishment. We assessed the state of germ cell differentiation in early second larval instar testes by examining the fusomes, which form cytoplasmic extensions due to incomplete cytokinesis during spermatogonial division. In wildtype testes, GSCs docked at the hub and germ cells that have yet to undergo division possess spherical fusomes (Fig. 12A). As the germ cells divide and form spermatogonia, fusomes become increasingly elongated and form branched structures in 4-, 8-, and 16-cell spermatogonia at the testis posterior.

In order to determine whether BMP signaling is necessary to prevent GSC differentiation, we examined the impact of BMP inhibition in the germline on germ cell differentiation. We first overexpressed *daughters against decapentaplegic* (*dad*), a gene that encodes a negative feedback inhibitor of BMP signaling, in the germline using the *nanos*-Gal4 driver (Fig. 12B). Germline BMP inhibition with DAD results in the formation of elongated fusomes between the germ cells localized adjacent to the hub and adjacent germ cells (100%, n = 5), indicating a loss of GSC identity. Furthermore, the testis failed to achieve the size expected of a wildtype testis. As
continuous production of spermatogonia relies the maintenance of GSC in the testis anterior, reduced testis size is consistent with the loss of GSC maintenance. Although the exact dimensions of the testes experiencing germline BMP inhibition vary substantially, on average, they are decreased in size by roughly 30% (s.d. = 2.7, p = 6.16 × 10^{-5}) when compared to the wildtype (n = 13).

To validate these results, we also examined germline BMP inhibition resulting from the induction of a defective Tkv receptor expressed in the germline using the nanos-Gal4 driver (Fig. 12C). Like testes experiencing germline-specific DAD hyperactivation, these testes also exhibited elongated fusomes in germ cells docked localized adjacent to hub (100%, n = 8) and a decrease in testis size by ~17% in comparison with the wildtype (n = 8, s.d. = 2.7, p = 0.00021). In addition to a loss of GSC identity and decreased size, 37.5% of testes (n = 8) experiencing germline BMP inhibition due to a defective Thickveins receptor exhibited vacuolization, or nuclei-less holes in the testis body. The exact cause of vacuolization within the testis remains unknown. Although we are currently unable to explain the exact cause of vacuolization, the decrease in size and, more importantly, loss of stem cell identity exhibited by testes undergoing germline BMP inhibition demonstrates that BMP signaling is necessary for GSC maintenance.

To determine whether the BMP signaling is sufficient to promote GSC maintenance in the developing testis, we next examined the impact of BMP hyperactivation in the germline. We first used the somatic c587-Gal4 driver to express Decapentaplegic (DPP), one of the primary Drosophila BMP ligands (Fig. 12D). Under these conditions of generalized BMP hyperactivation in both the germ and somatic cells, germ cells fail to differentiate properly, as evidenced by the lack of fusome elongation or branching in the testis posterior (93%, n = 15).
Like testes undergoing BMP inhibition, testes experiencing generalized BMP hyperactivation are ~40% smaller than wildtype testes of a comparable age (n = 15, s.d. = 2.4, p = 1.71 × 10⁻⁹).

To examine the impact of BMP hyperactivation on the germline specifically, we also assessed the impact of overexpressing a constitutively active tkv allele with the germline driver nanos-Gal4 (Fig. 12E). In these testes, germ cells failed to differentiate past the two-cell stage, showing no fusome branching and only elongated fusomes in the posterior (74%, n = 10). This failure to proceed past the two-cell stage by the start of the second larval instar results in testes roughly 25% smaller than wildtype testes (n=10, s.d. = 3, p = 6.47 × 10⁻⁵). These results, supported by the strong phenotype produced by generalized BMP hyperactivation in the testis, indicate that BMP signaling is sufficient for GSC maintenance in the early second larval instar (L2) testis. Therefore, the BMP pathway is both necessary and sufficient for GSC maintenance in L2 testes.

**Germline-specific BMP hyperactivation results in less severe phenotypes**

While germline specific BMP hyperactivation results in dramatically reduced germ cell differentiation in early L2 testes (Fig. 13A), many testes appear to recover wildtype morphology by the end of the second larval instar (Fig. 13B). At ~72 hours AEL, germ cells in the posterior of the testis have progressed beyond the two-cell stage of differentiation and now contain eight-cell spermatogonia with branched fusomes (71% recover, n = 11). Although these testes have begun to exhibit a more normalized pattern of germ cell differentiation, they still do not match their wildtype age counterpoints in size. Even late in the second larval instar, testes experiencing germline specific BMP hyperactivation are not different in size from an early L2 testis of the same genotype (s.d. = 6.19; p = 0.31765). The decreased size of the testes, in conjunction with restored germ cell differentiation patterns in the testis posterior, suggests that germline specific
BMP hyperactivation using a constitutively active tkv allele and the nanos-Gal4 driver merely delays germ cell differentiation, rather than halting it entirely. This result, in conjunction with our data suggesting that BMP activation may attenuate later in development (Fig. 11), potentially indicates that although the BMP pathway is sufficient to prevent GSC maintenance during the dynamic first and early second larval instars, a second factor may support the BMP pathway in promoting maintenance in later in development.

Several other plausible explanations besides a complimentary pathway can be provided for the eventual recovery of germline-specific BMP hyperactivated testes, including inherent heterogeneity in the germline nanos-Gal4 driver and germ cell competition for positions at the hub. To better distinguish between these possibilities, we over-expressed DPP to hyper-activate BMP signaling throughout the testis using the germline driver nanos-Gal4. Because BMP signaling should be generally hyperactivated throughout the testes in this assay, we initially expected to observe a similar phenotype to c587-Gal4 driven BMP hyperactivation. However, upon analysis we found that these testes exhibited a much weaker phenotype (Fig. 14C) than testes experiencing c587-Gal4 driven BMP hyperactivation (Fig. 14B). Branched fusomes connecting germ cells in eight-cell spermatogonia are present in the posterior, indicating that BMP hyperactivation has failed to suppress germ cell differentiation (100%, n = 19). In addition to evidence of substantial germ cell differentiation, these testes are not statistically different in size from their wildtype age counterparts (s.d. = 2.6, p = 0.54862). Therefore, like the germline specific BMP hyperactivation mutant testes at ~72 hours AEL, testes experiencing generalized BMP hyperactivation using a germline specific driver exhibit essentially wildtype morphology and growth. Although there is not enough information yet to eliminate the possibility of weakness or extreme heterogeneity in the nanos-Gal4 driver, the dissimilarity between the
germline and soma-driven generalized BMP hyperactivation phenotypes may suggest the presence of a regulatory mechanism that prevents the secretion of the DPP ligand from the germline.

_Perturbation of BMP signaling in the soma results in aberrant spermatogonial differentiation_

Strong p-SMAD staining in the cyst cells after the embryo to larval transition in wildtype testes, as well as the severe phenotype produced by generalized BMP hyperactivation using a somatic driver, suggests that BMP signaling may be involved in cyst cell function. In order to probe the role of the BMP pathway in the soma, we assessed the impact of hyperactivating and inhibiting BMP signaling in the cyst cells of the gonad. To hyperactivate the BMP pathway specifically in the cyst cells, we expressed a constitutively active _tkv_ allele using the somatic driver _c587-Gal4_. In early second larval instar testes, germ cell differentiation is disrupted (Fig. 15B). While fusome elongation in the posterior demonstrates that differentiation is able to occur under conditions of somatic BMP hyperactivation, spherical fusomes located the same distance from the hub as differentiating spermatogonia indicate a failure in the regulation of germ cell differentiation (90%, _n_ = 10). This phenotype corresponds with a roughly 25% decrease in testis size compared to wildtype gonads of a comparable age (_n_ = 8, s.d. = 3.2, _p_ < 0.00015), indicating that inconsistent regulation of spermatogonia differentiation interrupts the proper progression of spermatogonia through regular cell divisions, delaying growth.

To ascertain the impact of BMP inhibition in the soma, we expressed a defective _tkv_ allele also using the somatic _c587-Gal4_ driver (Fig. 15C). Interestingly, these testes exhibit a phenotype very similar to that observed after somatic BMP hyperactivation. Elongated and branched fusomes are present in the testis posterior, and germ cells in close proximity to the hub
exhibit spherical fusomes as in the wildtype testis. However, spherical fusomes can also be observed in the testis posterior among the differentiating spermatogonia (82%, n = 11). Furthermore, these testes were also small for their age, showing a ~20% decrease in size as compared to similarly aged wildtype testes (n = 17, s.d. = 2.5, p = 0.00017). As the cyst cells support the development of spermatogonia, the disruption of germ cell differentiation patterns observed after both the hyperactivation and inhibition of somatic BMP signaling phenotypes emphasizes the importance of soma-germline communication. Tampering with the levels of BMP activation in the cyst cells appears to interfere with their ability to communicate with the dividing germ cells and thus appropriately support the process of spermatogonial differentiation.

To validate that BMP inhibition interrupts soma-germline communication, we examined two other parallel systems for BMP signaling inhibition in the soma. We first overexpressed DAD in the soma using the c587-Gal4 driver (Fig.16B). As expected, we detected a phenotype very similar somatic BMP inhibition phenotype we had observed previously. Germ cells with spherical fusomes can be seen approximately the same distance from the hub as spermatogonia that possess elongated or branched fusomes (100%, n = 24). Perhaps even more striking than disrupted spermatogonial differentiation in these testes is their small size. On average, testes experiencing somatic DAD overexpression were ~35% smaller than their wildtype counterparts (n = 32, s.d. = 1.9, p = 1.09 × 10^{-14}). In the second assay, we expressed a defective allele for punt, another component of the receptor that transduces BMP signaling, in the somatic cells using c587-Gal4 (Fig. 16C). Again, germ cells with spherical fusomes are found throughout the testes despite evidence of more extensive spermatogonial differentiation (59%, n = 17), and the testes are decreased in size by 39% from wildtype controls (n = 19, s.d. = 2.3, p = 3.79 × 10^{-10}). Thus, regardless of the mechanism employed to repress the BMP pathway, alterations in BMP
signaling in the soma result in the disrupted spermatogonial differentiation and reduced growth in the *Drosophila* testes. Taken together, these data indicate that BMP activation in the soma is critical for soma-germline communication and is therefore not only responsible for promoting GSC maintenance in the testis anterior, but also for ensuring appropriate spermatogonial differentiation in the posterior.

**Somatic activation of Jak-STAT signaling promotes GSC maintenance through the BMP signaling pathway**

In the current model for stem regulation in the adult *Drosophila* testis (Fig. 7), the CySCs are maintained by the Jak-STAT pathway and in turn secrete the BMP ligands DPP and GBB, activating the BMP pathway in the germline to ensure GSC (Leatherman and Dinardo, 2010; Kawase et al., 2004; Shivdasani and Ingham, 2003; Wang et al., 2008; Zheng et al., 2011. We have already demonstrated the not only is the BMP pathway active in the developing testis, but also that it promotes GSC maintenance, supporting the current model. However, we have yet to assess directly whether the CySCs are responsible for BMP activation in the germline. To evaluate this, we examined BMP activation in the germline under conditions of CySC expansion. Specifically, we overexpressed a temperature-activated Jak allele, *hop<sup>TumL</sup>*, with *c587-Gal4* to hyperactivate Jak-STAT signaling in the somatic cells of first and second larval instar testes. Hyperactivation of Jak-STAT signaling in the soma has previously been shown to cause CySC over-proliferation at the expense of differentiating cyst cells coupled with expansion of the undifferentiating germ cell population (Sinden et al., 2012). To determine whether somatic Jak-STAT hyperactivation under these circumstances prevents GSC differentiation through germline BMP activation, we stained the testes for p-SMAD. Under the current model, we would expect BMP signaling to be more broadly expressed in the testis after CySC expansion, especially in undifferentiated germ cells.
Results are consistent with the current model of stem cell regulation in the testis. Testes experiencing somatic Jak-STAT hyperactivation (hop\textsuperscript{TumL}-positive) were distinguished from sibling controls (hop\textsuperscript{TumL}-negative) by the absence of visible spermatogonial clusters in the testis posterior. In hop\textsuperscript{TumL}-positive testes, p-SMAD expression is extended to include a majority of the germ cells in the gonad (87%, n = 10; Fig. 17B) as compared to sibling controls in which p-SMAD expression is restricted to GSCs docked at the hub and proximal germ cells (100%, n = 8; Fig. 17A). Therefore, somatic Jak-STAT hyperactivation in the testis inhibits germ cell differentiation by activating the BMP pathway in the germline (p = 0.00024). This result confirms that the CySCs employ the BMP pathway to promote GSC maintenance and thus supports the current model of stem cell regulation in the \textit{Drosophila} testis.

Without markers to assess the differentiation state of the germ or cyst cells, it can be difficult to discern hop\textsuperscript{TumL}-expressing testes from their phenotypically wildtype siblings. In previous studies, expanded expression of ZFH-1, a CySC marker, and a corresponding loss of the cyst cell marker EYA in the gonad’s somatic cells designated the testes as hop\textsuperscript{TumL}-positive (Sinden et al, 2012). However, because the ZFH-1 and p-SMAD antibodies are incompatible as they are made in the same species, we developed a new way to genotype our samples. We chose to examine another cyst cell marker, Traffic Jam (TJ), for expansion due to somatic Jak hyperactivation. In phenotypically wildtype testes with restricted ZFH-1 expression, TJ-expressing cells are clustered in the anterior of the testes and become more disperse as the spermatogonia become larger (100%, n = 2) (Fig. 18A). However, when ZFH-1 is expanded, TJ-expressing cyst cells are more uniformly distributed throughout the testes because there are no expanding spermatogonia to separate them (100%, n = 4) (Fig. 18B). Thus, Traffic Jam
expression in the cyst cells can be used as an effective marker for CySC over-proliferation and GSC maintenance due to somatic Jak-STAT hyperactivation.

To verify that we were able to effectively differentiate between hop<sup>TumL</sup>-positive testes and the sibling controls, we co-stained testes with p-SMAD and TJ. When the TJ expression pattern indicates a phenotypically wildtype testes, p-SMAD is observed in the GSCs docked at the hub and nearby germ cells (100%, n = 2), but not in the clearly defined spermatogonia in the testis posterior (Fig. 19A). On the other hand, in testes exhibiting a greater density of TJ expressing cells in the posterior, p-SMAD expression is expanded to include most of the germ cells with in the testes (100%, n = 3, p = 0.0251). Because the somatic Jak–STAT hyperactivation promotes GSC maintenance, pronounced spermatogonia are not detected in these testes. Taken together, these data validate our conclusion that BMP signaling from CySCs to GSCs plays a critical role in the maintenance of GSCs in the developing testis.

**DISCUSSION**

Our data indicate that the BMP pathway plays a critical role in stem cell regulation within the developing *Drosophila melanogaster* testis. After testis coalescence, the BMP pathway is activated in a subset of PGCs in the testis posterior. After extending toward the anterior around the time of hub formation, BMP activation restricts to GSCs and germ cells in close proximity to the hub and to cyst cells surrounding differentiating spermatogonia in the larval testis. While levels of BMP activation in the cyst cells remain constant as the gonads age, the levels of BMP activation in the germline attenuate over the course of development. Furthermore, in early second larval instar (L2) testes, we found that BMP signaling is necessary and sufficient for GSC maintenance. However, many testes appear to recover wildtype morphology after BMP hyperactivation in the germline in late L2. In addition to its role in GSC maintenance, disruptions
of BMP signaling in the soma result in aberrant spermatogonial differentiation, indicating that appropriate levels of somatic BMP activation are necessary for the soma-germline communication. Finally, we found that hyperactivation of the Jak-STAT pathway in the soma promotes GSC maintenance through the BMP pathway, demonstrating interactions between the Jak-STAT and BMP pathway. Taken together, these data support the current model for stem cell regulation in the testis (Fig. 8) while providing some potentially interesting insights into the system.

**Dynamic activation of BMP signaling before hub formation may indicate unexplored roles of BMP signaling in the testis**

Although BMP activation in the germline restricts to the testis anterior by first larval instar (L1) in a pattern resembling the adult, the BMP pathway is active in the developing testis as early as gonad coalescence (~15 hours AEL). Given its numerous and varied functions in other developmental systems, it is possible that the BMP pathway performs other roles besides promoting GSC maintenance in the developing testis, especially before it enters the first larval instar.

The patterns of BMP activation observed in the testis during embryogenesis suggest several novel roles for BMP signaling. Following a period of inactivation in the PGCs during migration, BMP activation can be observed in a subset of PGCs in what will become the posterior of the testis shortly after testis coalescence. The timing of BMP reactivation as well as these cells’ relative distance from the position of the future hub may indicate that the BMP pathway performs a similar function in these PGCs as it does in the GSCs later in development. After the PGCs form in the posterior of the embryo, transcriptional repression mediated by the localized RNAs *germ cell-less (gcl)*, *nanos (nos)*, and *polar granule component (pgc)* prevents differentiation (Santos and Lehmann, 2004). At gonad coalescence, these PGCs make contact
with clusters of SGPs, relieving transcriptional repression in the PGCs. Thus, after gonad coalescence, another mechanism must be employed to prevent PGC differentiation. Given that the SGPs appear to control gonad coalescence and that the somatic cells are crucial to GSC maintenance in the adult, it is possible that signal repressing PGC differentiation originates from the hub precursors at the anterior of the testis. However, since the posterior PGCs are relatively far from the position where the hub forms, perhaps these cells require another mechanism, such as the BMP pathway, to prevent their differentiation. More research is needed to understand the dynamics of this post-testis coalescence system.

In addition to this possible role in preventing PGC differentiation in the testis posterior, the BMP pathway may also be actively involved in GSC establishment at the hub around the time of hub formation. Late during stage 17 of embryogenesis, the pattern of BMP activation in the testis extends from the posterior PGCs along a line of PGCs upward toward the anterior of the testis. Around this same time period, the hub forms and both stem cell populations are established in their positions near the hub (Le Bras and Van Doren, 2006; Okegbe and Dinardo, 2011; Kitadate and Kobayashi, 2010; Sinden et al., 2012; Sheng et al., 2009). Given the timing of this change in BMP expression, perhaps the BMP pathway is involved in establishing the new GSCs in their positions around the hub. Potentially, BMP signaling could be responsible for mediating the movement of germ cells into the correct positions around the hub or for increasing the germ cells’ receptiveness toward interactions with the hub. Again, more research is necessary to explore this possibility further.

A second pathway may work in tandem with the BMP pathway to promote GSC maintenance later in development

Our data indicate that BMP signaling is required for GSC maintenance in the developing testis (Fig. 12). However, the recovery of normal differentiation patterns late in the second larval
instar (L2) after germline-specific BMP hyperactivation (Fig. 13) indicate that, while BMP signaling is still necessary for GSC maintenance in later in development, it does not appear to be sufficient to promote GSC maintenance after the second larval instar. Indeed, experiments attempting to induce ectopic GSCs through BMP hyperactivation alone in the adult testis have failed to produce sufficient results (Leatherman and Dinardo, 2010). This inability to produce ectopic GSCs, in conjunction with our findings that show that testes experiencing germline-specific BMP hyperactivation can recover wildtype morphology, suggests that the BMP pathway may not be the only factor involved in GSC maintenance. We postulate that a second pathway supports the BMP pathway in its role promoting GSC maintenance, thus explaining how the BMP pathway is certainly necessary, but possibly not sufficient for GSC maintenance.

The attenuation of BMP signaling over the course of the late larval and pupal stages of development is consistent with this hypothesis (Fig. 11; Chang et al., 2013). Decreasing levels of BMP activation in the germline may indicate that the testis is less reliant on BMP signaling alone to maintain GSCs. Especially given the claim that both p-SMAD and DAD expression are nearly undetectable in the GSCs and anterior-most germ cells of the adult testis (Fuller et al, 2013), the data seems to suggest the existence of another pathway that supplements the BMP pathway and helps promote the maintenance of the GSCs.

Despite the evidence supporting the existence of a second pathway that promotes GSC maintenance, there are other alternatives that may explain the recovery of wildtype morphology in testes experiencing germline-specific hyperactivation. The first is heterogeneity in the germline driver we employed, nanos-Gal4. If variation exists in the expression the Gal4 transcription factor between germ cells, then certain cells will have lower levels of transcription of the target gene than others. Therefore, some cells may produce insufficient levels of the
constitutively active Tkv receptor to prevent differentiation. However, we do not observe any recovery in testes experiencing germline-specific BMP inhibition with the same nanos-Gal4 driver, implying that the impact of insufficient target gene produced due to heterogeneity in the driver is negligible.

Competition for space at the hub could also partially explain the recovery of testes after germline-specific BMP hyperactivation. The ability of differentiating germ cells to dedifferentiate and establish themselves in the niche under the appropriate conditions has been documented in the adult testis (Brawley and Matunis, 2004; Sheng et al., 2009). When BMP signaling is hyperactivated in the germline and the GSCs subsequently fail to differentiate, germ cells with higher levels BMP activation due to heterogeneity in the nanos-Gal4 driver may outcompete germ cells with lower levels of BMP activation for positions at the hub, thus erecting essentially the same gradient of BMP activation found in the wildtype testis with simply a higher initial level of activation. Furthermore, this hypothesis may also be able to explain why we do not observe recovery in testes after germline-specific BMP inhibition. While competition for positions at the hub is imaginable when the germ cells experience variable increases in BMP activation, this phenomenon would seem less likely under conditions of variable BMP inhibition since a majority of the germ cells already do not experience BMP activation in the wildtype testis. While this model of germ cell competition is attractive, more research on the heterogeneity of the nanos-Gal4 driver and on competition in response to variable levels of BMP activation is needed to substantiate it.

*Communication between the soma and the germline is critical in the regulation of germ cell behavior*

Our data emphasizes the importance of soma-germline communication in both the maintenance of GSCs and in the regulation of spermatogonial differentiation. We show that
when Jak-STAT signaling is hyperactivated in the soma, the expression of the BMP pathway in the germline is significantly expanded as well (Fig. 17), indicating that the CySCs interact with the germline to prevent GSC differentiation through the BMP pathway. While the importance of the CySCs to GSC maintenance has been challenged recently in the literature (Lim and Fuller, 2012), our data strongly supports the current accepted model of stem cell regulation, which identifies the CySCs as the pivotal transducers of the signal promoting GSC maintenance.

In this thesis, we also demonstrate that communication between the somatic cyst cells and the spermatogonia is critical for appropriate spermatogonial differentiation in the developing testis (Fig. 15 and 16). Alterations in the levels of BMP signaling in the soma resulted in disruptions in the progression of spermatogenesis. The importance of the cyst cells surrounding the spermatogonia in supporting proper spermatogonial differentiation is consistent with our current understanding of cyst cell function in the testis (Lim and Fuller, 2012; Sinden et al., 2012; Buszczak et al., 2007).

In addition to communication from the cyst cells to the differentiating spermatogonia, studies have also suggested that the germline communicates back to the cyst cells (Gonczy and DiNardo, 1996). However, while the BMP pathway is activated in the cyst cells, the germ cells do not appear to be the source of the activating ligands. Our data shows that generalized DPP overexpression originating from the germline has a minimal effect on the developing gonad (Fig. 14) despite the strong phenotype observed when DPP was overexpressed in the soma (Fig. 12). Because the disparity between these two phenotypes, we postulate that some regulatory mechanism prevents secretion of BMP ligands from germ cells. Thus, it seems unlikely that the germline is the source of the BMP ligands activating the BMP pathway in the soma. The source of this somatic BMP activation remains unknown.
**Future Directions**

The data presented in this thesis provide an interesting developmental perspective on the role of BMP signaling in the testis. Furthermore, it highlights the need for more research into stem cell development and regulation. In addition to exploring the possibility of a second pathway supporting BMP signaling in promoting GSC maintenance and finding the source of somatic BMP activation, three other questions that can be immediately examined are provided below.

*Does the BMP pathway play a role in GSC establishment?*

To determine whether BMP signaling plays a role in the establishment of functional GSCs at the hub, the impact of inhibiting the BMP pathway in the developing testis before hub formation can be examined. Specifically, the GAL4/UAS gene expression system can be used to over-express RNAi constructs against components of the BMP pathway in PGCs prior to stem cell establishment. These RNAi constructs eliminate both zygotically transcribed and maternally inherited mRNA transcripts, more effectively inhibiting the BMP pathway at this early time point when maternally inherited mRNA may still be present. Samples can then be stained to assess for presence of cell adhesion molecules indicative of the polarized GSC adhesions with the hub. If the BMP signaling pathway is involved in GSC establishment, the embryonic germ cells that develop into GSCs in wildtype organisms will fail to assume key GSC characteristics, such as asymmetric division.

*Do germ cells with higher levels of BMP activation more successfully compete for positions at the hub?*

To assess whether germ cells with higher levels of BMP activation more successfully compete for positions at the hub, a fly containing a heterogeneously expressed germline driver
with an incorporated Green Fluorescent Protein (GFP) element can be mated to flies with either a UAS-
tkv act transgene or a y,w^{1118} transgene as a control. The progeny from the control mating with y,w^{1118} flies should express the driver stochastically throughout the germline as assessed by relative amounts of GFP staining. However, if germ cells with higher levels of BMP activation outcompete other germ cells for positions at the hub, then those germ cells with greater expression of the germline driver, and thus of the UAS-tkv act transgene, will be found near the hub more often. Therefore, when the testis of these flies are stained for GFP, the GFP expression pattern will no longer be stochastic, but rather form a gradient with the strongest staining cells adjacent to the hub.

How is the nanos-Gal4 driver expressed within the developing testis?

It is of critical importance that we understand both the time period and the degree of heterogeneity of expression of the nanos-Gal4 driver within the testis throughout development. In order to examine nanos-Gal4 expression throughout development, we can mate flies that possess the driver to flies with a UAS – LacZ transgene. The progeny produced in the mating can be collected at discrete time points and stained with an anti-βGal antibody to reveal the expression of the driver. Extreme heterogeneity in the expression of the driver may require the utilization of another germline driver with more consistent expression in the testis to examine GSC maintenance.

ACKNOWLEDGMENTS

I would like to sincerely thank all the members of the Wawersik lab for their support throughout this project. In particular, I would like to acknowledge the two other members of the BMP signaling project, Emma Rudebusch and T.A. Nguyen, for their assistance in gathering
some of the data presented in this thesis as well as Lauren Boulay, Tyler Jones, and Andrea Lin for their friendship and many words of support over the past several years. Beyond the Wawersik lab, I would recognize the assistance of the entire Drosophila community in obtaining fly lines and antibodies, including the Bloomington Stock Center, the Developmental Studies Hybridoma Bank, Dr. Mark Van Doren, Dr. Ruth Lehman, and Dr. Doug Harrison. In addition, I very much appreciate the grants I have received to fund this project from the Howard Hughes Medical Research Institute (HHMI) Freshman Research Program, the Charles Center, and the Biology Department. Furthermore, I would like to thank all the members of my committee – Dr. Mark Forsyth, Dr. Shanta Hinton, Dr. Douglas Young, and Dr. Matthew Wawersik – for all the time and energy they have devoted to this thesis. Finally, I would like to especially thank my advisor, Dr. Matthew Wawersik, for not only for his invaluable support during the whole of this project, but also for helping me discover my love of research.
REFERENCES


Manning, G. The WWW Virtual Library: Drosophila.


Fig. 1: Asymmetric division is a hallmark of stem cell behavior.
When a stem cell divides, it produces two progeny: a daughter that retains its stem cell identity and a daughter that is fated to undergo differentiation into a specific cell type. Through this mechanism, stem cell are able to both replenish the stem cell population and replace differentiated cells in a tissue.
**Fig. 2: The Drosophila melanogaster life cycle**

At 25°C, a fertilized egg can develop into adult *Drosophila* organism capable of reproduction in 11 days. For the first ~24 hours after egg laying, the organism undergoes embryogenesis.

Following embryogenesis are three larval instars, referred to as L1, L2, and L3. After the third larval instar, the organism enters the pupal stage during which it undergoes metamorphosis. Four days later, it emerges as an adult fly in a process called eclosion.

Source: Scientific Frontiers in Developmental Toxicology and Risk Assessment (2000)
Fig. 3: The Gal4/UAS gene overexpression system is utilized to induce tissue-specific gene expression.

A fly with a Gal4 driver expressed in a particular tissue is mated to a fly of the opposite sex with a gene regulated by an upstream UAS element. In their progeny, the Gal4 transcription factor will bind to the UAS element, resulting in the transcription of the target gene.
Fig. 4: Schematic of the adult Drosophila testes

The adult testis forms a coiled tube with one blind end where the stem cell niche is located. Within the stem cell niche, two stem cell populations, the GSCs (yellow) and CySC (green) are arrayed around the hub (blue), a cluster of 10-12 non-mitotic cells. The gonialblasts resulting from GSC divisions (white) differentiate to become sperm. The CySCs surround the GSC in pairs produce the cyst cells (light green) that support spermatogonial differentiation. Fusomes (purple) connect the dividing germ cells within the spermatogonial clusters. The other side of the structure opens into the seminal vesicle, which collects and stores the sperm created in the testis.
The Jak-STAT signaling pathway

The Jak-STAT ligand Unpaired (UPD) binds to the receptor Domeless (DOME), resulting in a conformational change that activates the Jak kinase, Hopscotch (HOP). After the Jak kinases cross phosphorylated each other, STAT binds the receptor and is phosphorylated. Two phosphorylated STAT monomers dissociated from the receptor and dimerize with each other. The STAT dimer then enters the nucleus and changes gene expression.
Fig. 6: The Bone Morphogenic Protein (BMP) Signaling Pathway

When the BMP ligand Decapentaplegic (DPP) binds to the dimerized receptor, receptor-associated Mothers Against Decapentaplegic (MAD) is phosphorylated. Phosphorylated MAD (p-MAD) dissociates from the receptor, dimerizes, and then binds with the co-SMAD, MEDEA, creating a transcription factor that enters the nucleus and influences gene expression. One of the genes expressed is Daughters Against Decapentaplegic (DAD), which inhibits the BMP receptor.
**Fig. 7: Current model of stem cell regulation in the adult Drosophila testis**

In the adult, the hub excretes the Jak-STAT ligand Unpaired (UPD), activating the Jak-STAT pathway in both the CySCs and the GSCs. In the CySCs, Jak-STAT activation promotes CySC maintenance. In the GSCs, the Jak-STAT pathway is responsible for GSC adhesion to the hub. The CySCs excrete BMP ligands, which activate BMP signaling in the GSC. The BMP pathway in the GSC acts to promote GSC maintenance.

Fig. 8: Schematic showing testis development around the time of hub formation

In stage 15 (~12 hours AEL), the PGCs (yellow) and the SGPs (blue) intermingle, forming the testis. The hub (also blue) forms in late stage 17 (~23 hours AEL). At the time of hub formation, the PGCs give rise to GSCs (also yellow) and the SGPs give rise to the CySCs (also blue) that surround them in pairs. The testis then increases in size due to the continuous incomplete divisions of germ cells (white) within spermatogonial clusters supported by cyst cells (light green).
**Fig. 9: BMP signaling is dynamically expressed during early testis development.**

Wildtype embryonic and larval testes immunostained with anti-Vasa (red) to detect germ cells, anti-Fasciclin 3 (Fas 3; blue) to detect the hub, and anti-p-SMAD (green) to reveal BMP activation. The testis anterior, if determined (C-G), is oriented toward the left of the image. Testes are outlined (white dotted lines) and the hub is indicated by an asterisk when present. (A) Animated schematic of testis development. (B, B’) Migrating germ cells exhibit no p-SMAD (D, D’, E, E’) p-SMAD segregates to a single row of cell extending between the posterior and the anterior germ cells. (F, F’) p-SMAD restricts to anterior GSCs. (G, G’) p-Smad remains restricted to the anterior during spermatogonial differentiation. Some cyst cells show p-Smad staining. Scale bars at 20 µm.
**Fig. 10: p-SMAD positive somatic cells correspond with a subpopulation of Traffic Jam-expressing posterior cyst cells.**

Wildtype larval testes immunostained with anti-Vasa (red) to detect germ cells, anti-Traffic Jam (TJ; blue) to detect the somatic gonadal cells, including the cyst cells, and anti-p-SMAD (green) to reveal BMP activation. The testis anterior is oriented toward the left of the image. Testes are outlined (white dotted lines) and the hub is indicated by an asterisk. (A) A TJ-expressing cyst cell exhibits p-SMAD expression (white arrow) in a first larval instar testis. Definitive staining for p-Smad (A’) and for TJ (A’’) can be established individually. Scale bars at 20 µm.
Fig. 11: p-SMAD expression in the germline is detected less often as testes age.
Wildtype larval and adult testes immunostained with anti-Vasa (red) to detect germ cells, and anti-p-SMAD (green) to reveal BMP activation in the germline and somatic cells. The testis anterior is oriented toward the left of the image. Testes are outlined (white dotted lines) and the hub is indicated by an asterisk. (A, A’) No p-Smad is expressed in the GSCs surrounding the hub (white arrow) while p-SMAD is clearly expressed in the cyst cells (yellow arrow) in a second larval instar testis. (B, B’) In an adult testis, definitive p-Smad staining is observed in the cyst cells (yellow arrow), but not in the GSCs (white arrow). Scale bars at 20 µm. (C) Graph showing the percentage of first larval instar and adult testes exhibiting p-SMAD in the cyst cells that also exhibit p-SMAD staining in the GSCs. Result is statistically significant at a 95% confidence level (0.0394).
**Fig. 12: BMP signaling is necessary and sufficient for GSC maintenance in the developing testis.**

Larval testes aged 48 hours AEL immunostained with anti-Vasa (red) to detect germ cells, anti-Fasciclin 3 (Fas 3; green) to detect the hub, and anti-1B-1 (green) to detect fusomes. The testis anterior is oriented toward the left of the image. Testes are outlined (white dotted lines) and the hub is indicated by an asterisk. (A, A’) A control (c587-Gal4/+; Cyo/) demonstrates age-appropriate spherical fusomes in the testis anterior (white arrow) and fusome elongation and branching in the posterior (yellow arrow). (B, B’) Elongated fusomes between germ cells docked at the hub and adjacent cells (yellow arrow). (C, C’) Anterior germ cells with elongated fusomes (yellow arrows) and morphological defects (white arrowheads) corroborate results of BMP inhibition in second genotype. (D, D’) Spherical fusomes in the testis posterior (white arrows) indicate that BMP hyperactivation prevents differentiation. (E, E’) Posterior spherical fusomes (white arrows) corroborate results of BMP hyperactivation in second genotype. Scale bars for (A) at 25 µm and for (B-E) at 20 µm.
Fig. 13: Testes experiencing germline-specific BMP hyperactivation due to a constitutively active Thickveins receptor recover late during the second instar.

*nanos*-Gal4; UAS-*tkv act*. larval testes immunostained with anti-Vasa (red) to detect germ cells, anti-Fasciclin 3 (Fas 3; green) to detect the hub, and anti-1B-1 (green) to detect fusomes. The testis anterior is oriented toward the left of the image. Testes are outlined (white dotted lines) and the hub is indicated by an asterisk. (A, A’) Spherical fusomes in the posterior (white arrows) indicate a lack of germ cell differentiation in early second larval instar testes. (B, B’) Late in the second larval instar however, branched fusomes indicate spermatogonial differentiation (yellow arrows). Scale bars at 20 µm.
Fig. 14: Generalized DPP over-expression impacts GSC maintenance more severely than germline specific hyperactivation.

Larval testes aged 48 hours AEL immunostained with anti-Vasa (red) to detect germ cells, anti-Fasciclin 3 (Fas 3; green) to detect the hub, and anti-1B-1 (green) to detect fusomes. The testis anterior is oriented toward the left of the image. Testes are outlined (white dotted lines) and the hub is indicated by an asterisk. (A, A’) A control (c587-Gal4/+; Cyo/+) demonstrates age-appropriate spherical fusomes in the testis anterior (white arrow) and fusome elongation and branching in the posterior (yellow arrow). (B, B’) Although spherical fusomes in the testis posterior (white arrows) indicate that BMP hyperactivation prevents differentiation when dpp is generally overexpressed by a somatic driver, the phenotype is substantially less pronounced when DPP is specifically overexpressed in the germline (C, C’). Highly branched fusomes can be observed in the posterior (yellow arrow). Scale bars for (A) at 25 µm and for (B, C) at 20 µm.
**Fig. 15: Perturbation of BMP signaling in the soma results in aberrant spermatogonial differentiation.**

Larval testes aged 48 hours AEL immunostained with anti-Vasa (red) to detect germ cells, anti-Fasciclin 3 (Fas 3; green) to detect the hub, and anti-1B-1 (green) to detect fusomes. The testis anterior is oriented toward the left of the image. Testes are outlined (white dotted lines) and the hub is indicated by an asterisk. (A, A’) A control (c587-Gal4/+; Cyo/+) demonstrates age-appropriate spherical fusomes in the testis anterior (white arrow) and fusome elongation and branching in the posterior (yellow arrow). (B, B’) Extensive spermatogonial differentiation (yellow arrows) and spherical fusomes (white arrows) are present equidistant from the hub. (C, C’) Germ cells with spherical fusomes (white arrows) are observed farther from the hub than spermatogonial clusters (yellow arrows). Scale bars for (A) at 25 µm and for (B,C) at 20 µm.
**Fig. 16: Perturbed spermatogonial differentiation is a reproducible result of soma-specific BMP inhibition.**

Larval testes aged 48 hours AEL immunostained with anti-Vasa (red) to detect germ cells, anti-Fasciclin 3 (Fas 3; green) to detect the hub, and anti-1B-1 (green) to detect fusomes. The testis anterior is oriented toward the left of the image. Testes are outlined (white dotted lines) and the hub is indicated by an asterisk. (A, A’) A control (c587-Gal4/+; Cyo/+) demonstrates age-appropriate spherical fusomes in the testis anterior (white arrow) and fusome elongation and branching in the posterior (yellow arrow). (B, B’) Germ cells with spherical fusomes (white arrows) are observed farther from the hub than spermatogonial clusters (yellow arrows) as a result of DAD overexpression. (C, C’) Elongated fusomes indicative of spermatogonial differentiation (yellow arrows) and spherical fusomes (white arrows) are present equidistant from the hub due to expression of a defective Punt receptor. Scale bars for (A) at 25 µm and for (B,C) at 20 µm.
Fig. 17: Expression of the BMP pathway in the germline is expanded under conditions of CySC expansion due to somatic Jak-STAT hyperactivation.

Larval testes aged 60 hours AEL immunostained with anti-Vasa (red) to detect germ cells and anti-p-SMAD (green) to assess BMP activation. The testis anterior is oriented toward the left of the image if distinguishable. Testes are outlined (white dotted lines) and the hub is indicated by an asterisk. (A- A’’) A control (c587-Gal4/+; Cyo/+) as determined by the presence of spermatogonial clusters in the testis posterior exhibits restricted BMP activation in the germline. (B-B’’) In hopTumL-positive testes that shows no spermatogonial clusters in the testis posterior, the expression of BMP signaling has expanded in the germline. Scale bars at 20 µm.
**Fig. 18: Traffic Jam (TJ) can be used as a marker for CySC expansion after somatic Jak-STAT hyperactivation.**

Larval testes aged 48 hours AEL immunostained with anti-Vasa (red) to detect germ cells, anti-ZFH-1 to detect the CySCs (green), and anti-Traffic Jam (TJ, blue) to detect CySCs and cyst cells. The testis anterior is oriented toward the left of the image if distinguishable. Testes are outlined (white dotted lines) and the hub is indicated by an asterisk. (A- A’’) A control (c587-Gal4/+; Cyo/+) demonstrates that TJ expressing cells are distantly spaced in the testis posterior while ZFH-1 remains restricted to CySCs in the testis anterior. (B-B’’) In hopTumL-positive testes, ZFH-1 expressing cells are found throughout the testis. In addition, TJ expressing cyst cells are evenly spaced throughout the testis. Scale bars at 20 µm.
**Fig. 19: Expansion of BMP activation in the germline correlates with the proliferation of Traffic Jam (TJ) –expressing cyst cells.**

Larval testes aged 48 hours AEL immunostained with anti-Vasa (red) to detect germ cells, anti-Traffic Jam to detect the CySCs and cyst cells (TJ, blue), and anti-p-SMAD (green) to assess BMP activation. The testis anterior is oriented toward the left of the image if distinguishable. Testes are outlined (white dotted lines) and the hub is indicated by an asterisk. (A- A’’’) A control (c587-Gal4/+; Cyo/+) in which TJ expressing cells are distantly spaced in the testis posterior exhibits restricted BMP activation in the germline. (B-B’’’) In hop\textsuperscript{TumL}-positive testes, TJ expressing cyst cells are evenly spaced throughout the testis and the expression of BMP signaling has expanded in the germline. Scale bars at 20 µm.