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DIFFERENTIAL BMP SIGNALING INITIATES CELL COMPETITION IN *DROSOPHILA* FEMALE GERMLINE STEM CELLS

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Abstract

In multi-cellular organisms, cell competition serves as a quality control machinery to eliminate suboptimal cells and to maximize tissue fitness. Failure of this process has been implicated in conditions as diverse as aging and tumor progression. The phenomenon and some of the mechanisms underlying cell competition are conserved from *Drosophila* to mammals. Cell competition occurs both within tissue primordia and also among adult stem cells, which function to maintain organismal homeostasis. Most adult stem cells reside in a specific microenvironment, called a niche, that both anchors the stem cell and produces signals to ensure continued stem cells maintenance. While adult stem cells compete with each other for niche anchorage and niche signaling, the relationship between niche secreted maintenance signals and stem cell competition is poorly understood.

In this thesis, I used *Drosophila* female Germline Stem Cells (GSCs) as a model system to study whether the GSC maintenance factors, members of the Bone Morphogenetic Protein (BMP) family, also play a role in GSC competition. I demonstrated that differential BMP signaling between neighboring GSCs directly triggers stem cell competition. My data indicate that there are two thresholds for controlling distinct GSCs behaviors. A very low level of BMP signaling in GSCs is necessary to maintain the GSCs in an undifferentiated state, irrespective of the state of neighboring GSCs. A second, higher threshold of BMP signaling is involved in GSC competition. In between these two thresholds, a single GSC will be outcompeted by a GSC with wild-type signaling, but will be maintained if all other GSCs have a similar level of signaling. Thus, an absolute low value of BMP signaling is necessary for GSC maintenance, while differences in BMP signaling between GSCs serve as a read out of the relative fitness of the GSC. GSC competition may have important evolutionary implications, as GSC fitness can be

markedly affected by novel germline mutations that could have deleterious consequences for future generations.

CHAPTER I: INTRODUCTION

I.1. Introduction to Cell Competition

In multi-cellular organisms, cells are known not only to coordinate, but also to compete, with each other. Cell competition, a process in which cells with suboptimal fitness are eliminated by their neighbors with higher fitness (Figure I.1A), was implicated as a surveillance system to maximize tissue fitness (Moreno et al., 2002). Interestingly, the idea of cells competing for limited space and survival factors is in parallel to the concept of Darwinian selection at the scale of individual, and also results in the outcome of selection, survival of the fittest (Moreno and Rhiner, 2014).

Cell competition was first identified in the *Drosophila* primordial wing tissues in 1975 (Morata and Ripoll, 1975). Morata and Ripoll discovered that cells lacking one copy of a *Minute* gene, which encodes a ribosomal protein (Marygold et al., 2007), were viable in a homotypic *Minute* heterozygous context, but were eliminated through apoptosis when the surrounding tissue had a wild-type genotype (Morata and Ripoll, 1975; Moreno et al., 2002), suggesting a context-dependent phenomenon, which has come to be the hallmark of cell competition (Figure I.1A-B). A much later study showed cells with extra copies of *dMyc*, a growth promoting transcription factor, became supercompetitors (Figure I.1C), excluding wild-type neighbors from the imaginal disc (Cova et al., 2004; Moreno and Basler, 2004). These experimentally induced cell competition assays defined the idea of canonical cell competition, in which less fit cells lose the capability to compete with wild-type cells, whereas supercompetitors have the ability to outcompete wild-type cells. In both these scenarios, the final organ size remains constant; cell competition was thus proposed to remove viable, but aberrant, cells while maintaining tissue homeostasis. The relative anabolic properties, *i.e*., the growth and proliferative rate that are

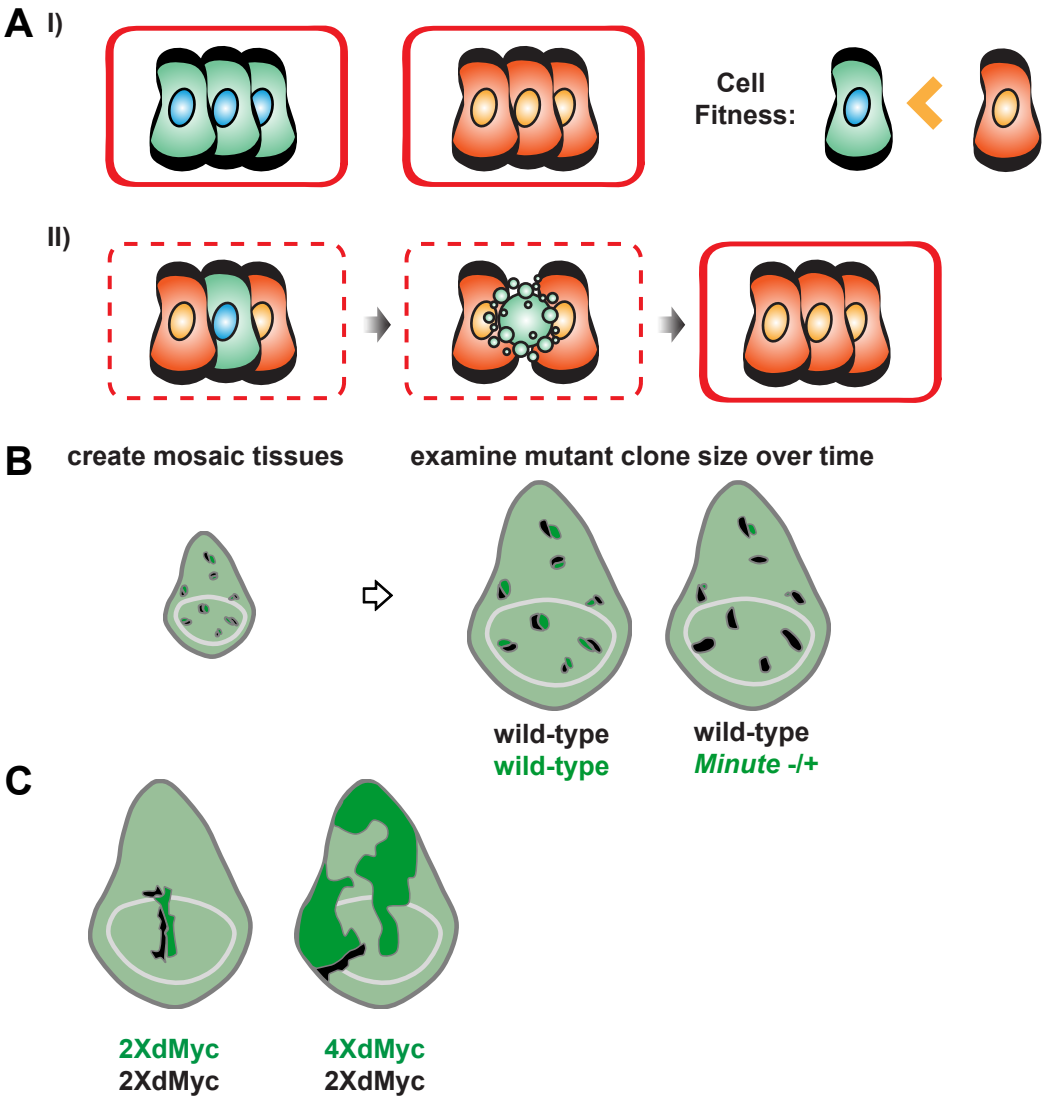


Figure I.1. Cell competition eliminates cells with lower fitness.
(A) Cell competition happens within populations of cells with different fitness. Cells of similar fitness are maintained (i), while cells that encounter neighbors with different fitness undergo competition, which results in the elimination of cells of lower fitness (ii). (B) Cell competition was identified in *Drosophila* by genetic manipulations that created mosaic wing imaginal discs that contained cells with different doses of a *Minute* gene during development. Over time in control discs, clones that had the same *Minute* gene dosage grew to the same size, while in experimental discs, clones with fewer *Minute* gene copies were largely eliminated from the disc. (C) Supercompetition was identified in mosaic wing discs that contained cells with extra copies of the *dMyc* gene. In control imaginal discs, unlabeled and labeled clones that contained same dose of the *dMyc* gene grew to the same size. In experimental imaginal discs, labeled clones with four copy of *dMyc* became supercompetitors and largely eliminated neighboring wild-type clones with two copy of *dMyc.*

regulated by *Minutes* and *dMyc*, were thus interpreted to define the “fitness” of individual cells involved in cell competition.

I.1.1. The established triggers of cell competition

Cell competition can be best described based on the trigger that initiates competition. Recently, Torres’s group described two types of triggers (Clavería and Torres, 2016). The first class of trigger is metabolic; in these cases, cell competition is driven by the differential anabolic ability of neighboring cells. Triggers for this form of competition can include differences in the regulators of biosynthetic genes, such as *Minutes* and *Myc*, the signaling pathways necessary for cell survival, such as the Bone Morphogenetic Protein (BMP)/ Decapentaplegic (Dpp) pathway, the Wnt/ Wingless pathway, the JAK-STAT pathway, the Ras/ MAPK pathway, and the Hippo pathway. The second class of trigger is structural; in these cases, cell competition is triggered within epithelial cells when their apical-basal polarity is altered.

Survival signals can serve as triggers for cell competition only when neighboring cells receive or transduce different levels of the signal. In the *Drosophila* wing disc, cells with compromised Wg signaling survive when they are surrounded by other less fit cells in the same compartment, but are eliminated through apoptosis when they encounter wild-type cells. In addition, cells with elevated Wg signaling outcompete neighboring wild-type cells by activating apoptosis in the loser cells, a phenomenon similar to the *dMyc* induced supercompetition (Vincent et al., 2011). Similarly, imaginal disc cells lacking *State92E*, the *Drosophila* STAT transcription factor in the JAK-STAT pathway, are outcompeted by wild-type neighbors through apoptosis and this elimination is suppressed if neighboring cells are heterozygous for a *Minute* mutation, suggesting that both types of competition triggers affect a common output whose relative level is

measured during competition. Cells with constitutive active *State92E* become supercompetitors, and induce apoptosis in neighboring wild-type cells (Rodrigues et al., 2012). Lastly, loss of function mutations in the Hippo (Hpo) pathway or hyperactivated Yki, the transcriptional co-activator whose activity is suppressed by Hippo pathway genes, cause hyperplasia. In the *Drosophila* wing disc and during eye development, the loss of *Minute* heterozygous cells is suppressed by inactivation of the Hpo pathway (Tyler et al., 2007), suggesting that the loss of the Hpo pathway activity compensates for the decrease in the anabolic state caused by the Minute mutations. Similarly, cells with loss-of-function Hpo pathway mutations or Yki overexpression upregulate *dMyc* level transcriptionally and become supercomptitors, resulting in clonal cells gaining a growth advantage over their wild-type neighbors (Neto-Silva et al., 2010; Ziosi et al., 2010). However, tissues lacking survival signals cannot be maintained over time, which is not consistent with the original strict definition of loser cells, as viable, but less-fit cells.

Structural cell competition, on the other hand, is triggered by mutations in the neoplastic tumor suppressor genes, *scribble* (*scrib*), *disc large* (*dlg*), *lethal giant larvae* (*lgl*), and its associated protein *mahjong* (*mahj*). Scrib, Dlg, and Lgl function as a complex; mutation in any one of which results in disruption of apical-basal polarity and neoplastic overgrowth (Humbert et al., 2003). While massive overgrowth of imaginal discs are observed in homozygous *scrib* and *lgl* larvae, clones of mutant *scrib* or *lgl* cells are eliminated through JNK-mediated apoptosis when juxtaposed to wild-type neighbors, indicative of a context-dependent competition (Brumby and Richardson, 2003; Chen et al., 2012; Grzeschik et al., 2007; Menéndez et al., 2010). *mahj* works downstream of *lgl* during competition; mosaic clones mutant for *mahj* are eliminated via apoptosis, while overexpression of *mahj* is sufficient to suppress the elimination of *lgl* mutant clones (Tamori et al., 2010). Interestingly, unlike metabolic cell competition, the loser cells in

structural cell competition are cells with the capability to grow faster, raising the question whether cell competition is triggered solely by differences in the anabolic properties of neighboring cells. I will revisit this idea briefly in the discussion using an example of BMP signaling.

I.1.2. The established mechanisms underlying cell competition

Cell competition depends on elimination of loser cells and accompanied compensatory proliferation of winner cells. The capability of loser cells to sense their differential fitness compared to their neighbors is crucial. As the understanding of cell competition has expanded, the data do not support a common mechanism to explain the complexity of this process. To date, at least three different models has been proposed for the recognition and elimination of loser cells (Merino et al., 2016).

I.1.2.1. Competition for survival factors

The first hypothesis concerning the mechanism of cell competition originated from the idea of neurotrophic theory for culling excess neurons during development (Levi-Montalcini, 1987); specifically, it was proposed that cells compete with their neighbors for the limited availability of secreted survival signals, for example, Dpp in the *Drosophila* wing disc (Moreno et al., 2002). Evidence supporting this model included the observation that BMP signaling is differentially active in winner and loser cells in the contexts of *Minute* and *dMyc* induced competition, while ectopic upregulation of BMP signaling via expressing a constitutive active form of the Dpp receptor, Thickveins (Tkv) or enhancing the endocytosis of activated Dpp-receptor complexes by overexpressing the Rab5 GTPase is sufficient to suppress the elimination of less fit cells (Moreno and Basler, 2004; Moreno et al., 2002). In mammals, two populations of T cell

progenitors compete for the cytokine IL7 in the thymus: older thymus resident progenitors and younger bone marrow derived progenitors. The competition results in the elimination of the older population, and confers the replacement of T cell progenitors (Martins et al., 2014). Thus, it is possible that competition for survival factors is a general phenomenon of cell competition across evolution (Martins et al., 2014; Moreno, 2014). However, clones of cells homozygous for null mutations in *tkv*, which are normally eliminated from the disc, are viable if all surrounding tissues are heterozygous for a *Minute* mutation, suggesting that BMP signaling contributes to the determination of fitness, rather than acting as an output of competition (Amoyel and Bach, 2014; Burke and Basler, 1996). In addition, ectopic activation of BMP signaling suppresses elimination of loser cells more completely in the peripheral region of the wing disc, where BMP signaling is low, compared to the center of the wing pouch where endogenous *dpp* is expressed and BMP signaling is high, suggesting that the relative difference in BMP signaling, but not BMP signaling itself, plays a role in cell competition (Baillon and Basler, 2014; Moreno and Basler, 2004). Competition for survival factors thus provides a simple explanation for the loss of less fit cells; however, there is increasing evidence that challenges the idea that competition is mediated by a limited supply of survival factors. In the *Drosophila* imaginal disc, cells near where Dpp is secreted are more likely to be outcompeted than those in peripheral regions (Moreno, 2008). In addition, research in mouse ESCs showed that changes in the amount of available ligand does not alter the outcome of competition (Sancho et al., 2013). Thus, it seems the competition for survival factors may not be sole mechanism that causes the elimination of loser cells during cell competition.

I.1.2.2. Elimination through a fitness fingerprint

A second mechanism states that less fit cells are labeled as loser cells by the presence of a cell surface protein, which serves as a fitness fingerprint. This surface marker, together with other

downstream regulators, form the fitness fingerprint machinery that labels winner and loser cells and that results in the elimination of loser cells. The machinery, which is composed of three genes, *flower* (*fwe*), *sparc*, and *azot*, was first identified in the *Drosophila* imaginal disc as genes that became expressed in wild-type cells after exposure to supercompetitor cells with elevated *dMyc* expression (Rhiner et al., 2010). The fitness fingerprint, *fwe*, has different isoforms, one ubiquitous and two loser isoforms, and the isoform that is displayed on the cell surface demarcates the relative fitness status of the cell. A cell expressing either of the loser isoforms is eliminated due to apoptosis. *fwe* is required for cell competition; reducing *fwe* transcripts prevents elimination of loser cells. Clonal lack of expression of the ubiquitous *fwe* isoform causes cells to be eliminated, while differential expression of one of the *fwe* loser isoforms causes elimination of cells expressing the higher level of the loser isoform suggesting it is the relative levels of the *fwe* isoforms between neighboring cells that determines the fitness status of cells (Rhiner et al., 2010). Counteracting the function of the flower fingerprint is the protective protein, Sparc, which is upregulated in loser cells to transiently prevent loser cells from apoptosis and elimination. Loser cells with downregulated *sparc* have accelerated apoptosis, while ectopic expression of *sparc* is sufficient to prevent competition-dependent apoptosis (Portela et al., 2010). Integrating these two fitness inputs is *azot*. *azot* is transcribed in loser cells, and is required for their elimination, while an extra copy of *azot* causes accelerated elimination of loser cells. Azot functions as the fitness checkpoint, as it computes the relative level of Fwe[lose] and Sparc and determines whether a cell will undergo elimination (Merino et al., 2015). However, so far the Fwe system has not been reported to be involved in other systems of canonical competition. Although the mammalian orthologs of both Fwe and Sparc have been proposed to confer higher competitiveness to tumor cells in mice and humans, respectively (Petrova et al., 2011; Petrova et al., 2012), there needs to be a more rigorous study of the roles of these orthologs in *Minute* or *Myc* triggered competition assays.

I.1.3. The conservation of cell competition

So far, the process of cell competition is believed to be evolutionarily conserved, as the triggers and mediators of cell competition identified in *Drosophila* were also found to play similar roles in mammals. In chimera mice carrying both wild-type cells and cells heterozygous for mutations in *Belly Spot and Tail* (*Bst*), the ortholog of the *Drosophila Minute* gene encoding the ribosomal protein L24, the *Bst*/+ cells have a disadvantage for growth and survival, similar to *Minute* heterozygous cells in mosaic flies (Oliver et al., 2004). Moreover, clonal overexpression of c-Myc, the ortholog of *Drosophila* Myc, in the mouse epiblast resulted in the expansion of cells with higher Myc levels, while many wild-type cells underwent apoptosis and were eliminated from the tissue (Clavería et al., 2013). Similarly, in vitro culture of mouse embryonic stem cells (ESCs) that lack activity of the BMP receptor 1a, the ortholog of *Drosophila thickveins (tkv)*, are eliminated when surrounded by wild-type cells, but survive when cultured separately, suggesting the elimination is context dependent, and is likely due to cell competition (Sancho et al., 2013). On the other hand, Mahj, which functions downstream of Lgl during cell competition in the *Drosophila* imaginal disc, was shown to have a conserved role in mammalian cell culture, such that cells in which the ortholog of Mahj was downregulated undergo apoptosis only when confronted with wild-type cells (Tamori et al., 2010). Together, these data suggest not only the conservation of the phenomenon of cell competition throughout evolution, but also that some genetic manipulations that result in cell competition in *Drosophila* also trigger cell competition in mammalian cells.

I.1.4. The physiological roles of cell competition

The conservation of cell competition suggests it plays an essential role in all animals. However, the in vivo physiological cues that trigger cell competition are now just beginning to be understood. Some processes that have been implicated include quality control during development or aging, tumor suppression, and tumor progression during cancer development.

Although cell competition has long been proposed as a quality control mechanism to eliminate aberrant cells in order to maximize tissue fitness, the role of cell competition in development and aging has not been proven until recently. During *Drosophila* retinal development, neuronal culling eliminates unwanted neurons via apoptosis. Studies on the Fwe fitness fingerprint links the developmental culling process with cell competition, as peripheral photoreceptors expressing *fwe[lose-B]* are predetermined for elimination, while removal of the loser *fwe* isoform suppresses this process (Merino et al., 2013). Accordingly, *azot* is upregulated in the cells that will be culled, and flies homozygous for an *azot* null mutation retain the unwanted neurons (Merino et al., 2015). Interestingly, lack of *azot* in *Drosophila* not only causes developmental abnormalities, but also results in malfunctioning cells accumulating in adult tissues, causing tissue degeneration, accelerated aging, and a shortened lifespan. Thus, cell competition is implicated in maintaining the health of tissues and prolonging adult lifespan (Merino et al., 2015). The role of cell competition in optimizing progenitor pools during development is also conserved in mammals. Heterogenous Myc levels are observed in the mouse epiblast and result in natural competition, with lower Myc levels corresponding to a higher tendency of a cell to undergo apoptosis (Clavería et al., 2013).

In addition, cell competition has been associated with regulation of tumor growth. As mentioned before, mutations in genes involved in structural cell competition resulted in neoplastic lesions in

a homotypic environment, while if these cells are adjacent to wild type neighbors they are outcompeted, suggesting cell competition could play a role in preventing the progression of pre-cancerous tissues. Similarly, as described before, cell competition contributes to T cell progenitor replacement in the mouse thymus; however, the outcome of lack of cell competition is more severe than just the failure of cell replacement. Without a supplement of fresh bone marrow derived progenitors, mice develop tumors resembling human T cell acute lymphoblastic leukemia within months (Martins et al., 2014), highlighting that involvement of cell competition in tumor suppression is an evolutionarily conserved phenomenon.

Conversely, supercompetition resulting from mutations in oncogenes or tumor suppressor genes could be thought of as a mechanism to promote tumor growth. One of the early stages of epithelial cancers is the presence of genetic alternations that lead to formation of adenomas or small tumors without causing gross morphological malformation (Barker et al., 2009; Rhiner and Moreno, 2009; Snippert et al., 2014). In *Drosophila,* the supercompetitor phenotype caused by an increase in the copy number of *dMyc* results in the expansion of a pre-cancerous lesion harboring oncogenic mutations without overt morphological change (Cova et al., 2004; Moreno and Basler, 2004) mimicking the early stages of cancer development. Similarly, studies in mice show that oncogenic mutations in intestinal stem cells confer a competitive advantage to these stem cells over their wild-type neighbors (Snippert et al., 2014; Vermeulen et al., 2013). Such clonal expansion of genetically altered cells throughout a tissue increases the likelihood of subsequent mutations that directly lead to cancer development, which is consistent with the concept of ‘field cancerization,’ proposed after observation of multiple tumor foci in oral squamous cell carcinoma (Rhiner et al., 2009; Slaughter et al., 1953). In conclusion, the acquisition of the ability of supercompetition in pre-cancerous tissues highlights the importance of cell competition during cancer development.

I.2. Stem cell competition

While cell competition occurs between cells in somatic primordia, it has also been shown to occur among adult stem cells, undifferentiated cells that divide to give rise to most differentiated tissues in the body. Adult stem cells are maintained in a microenvironment called a “niche”, and it has been proposed that niche-secreted signals are involved in regulating stem cell competition. In the next section, I will first briefly describe the characteristics and biology of adult stem cells. I will then briefly review what is known about the mechanisms of cell competition among adult stem cells.

I.2.1. General characteristics of adult stem cells and the niche

Adult stem cells are characterized by longevity and their potency, such that they are able to persist during the individual’s lifetime while continually generating differentiated cells with specific identities and functions (Lander et al., 2012). The number and activity of adult stem cells must be carefully regulated to ensure organismal homeostasis: reducing stem cell number can lead to tissue loss over time, while increasing stem cell activity can result in aberrant overgrowth. Stem cells either undergo asymmetric divisions, producing one daughter cell that remains as a stem cell and one that differentiates, or symmetric divisions, producing two daughter cells of the same type. While a few types of adult stem cells primarily undergo asymmetric divisions, the majority of adult stem cells can quickly switch between asymmetric and symmetric divisions to maintain a fixed ratio of stem cells to differentiated cells, in a phenomenon called “population asymmetry.” Interestingly, even in stem cells that primarily undergo asymmetric division, such as the *Drosophila* male Germline Stem Cells (GSCs) or the *Drosophila* midgut Intestinal Stem Cells (ISCs), population asymmetry is also observed especially during tissue regeneration, suggesting division asymmetry and population asymmetry

may be coordinated. During population asymmetry, it is likely that extrinsic signals from the microenvironment of the stem cell play a fundamental role in specifying the type of division the stem cell will undergo.

Most adult stem cells are present in a niche that is required for stem cell self-renewal, maintenance or survival. A niche can be composed of a single cell type or multiple cell types, or a combination of cells and extracellular structures, such as the matrix. The niche maintains most, if not all, adult stem cells by generating restricted amounts of secreted or cell surface factors that are essential for stem cell function. Furthermore, the niche itself could be thought of as a component in feedback control in order to coordinate tissue status with stem cell function by varying niche signals, so that the stem cell pool can be maintained in fluctuating environmental conditions (Lander et al., 2012).

I.2.2. The established mechanisms of stem cell competition

Stem cells compete with each other in the niche for limited space and survival signals (Stine and Matunis, 2013). Two types of stem cell competition have been observed. The first is neutral competition, which happens among genetically equivalent stem cells. While the underlying basis of neutral competition likely has a variety of causes, it has been proposed that transient fluctuations in niche signaling reception could initiate neutral competition. The second is non-neutral competition, which occurs among genetically distinct stem cells, or among stem cells that differ in their exposure to stress. Disrupting stem cell competition has been shown to result in imbalanced stem cell divisions, and has been implicated in disease states, such as tumor generation (Stine and Matunis, 2013). Thus, understanding the mechanisms underlying stem

cell competitions could provide valuable therapeutic insights. In this section, I will briefly outline what is known about the different categories of stem cell competition.

I.2.2.1. Neutral competition

As described above, neutral competition happens among genetically identical stem cells, and the phenomenon is observed in both the invertebrate and mammalian intestine, the mammalian testis, and in other systems. Neutral competition is characterized by the random loss and replacement of stem cells to maintain a stable stem cell pool. During neutral competition, no one stem cell has a long-term advantage, thus the lifetime of an individual stem cell is determined stochastically. The best-characterized example of neutral competition occurs between mammalian intestinal stem cells (ISCs), where crypt-based Lgr5-positive stem cells compete for the limited contact with Paneth cells, their niche. Each intestinal crypt is a closed unit, containing around 15 stem cells and 15 Paneth cells intermingled with each other. If a multi-color labeling cassette is used to label the ISCs, initially the crypt is composed of ISCs of many colors. However, over time, all ISCs in the crypt become labeled with the same color, indicating that the progeny of a single stem cell now populates the crypt. The observed monoclonality occurs because of the natural process of loss and replacement of stem cells within each intestinal crypt over time. As no ISC has a long-term advantage over any other ISC, the color of the clone that is retained is stochastic. This short-term scaling process is the hallmark of neutral competition within a closed system. In comparison, the *Drosophila* midgut represents an open stem cell system in which ISCs are scattered along the posterior midgut without a geographic boundary between each ISC unit. Modeling of the phenomenon of neutral competition within an open system matches the experimental observations: the number of genetically marked ISCs clones decrease, but the size of each clone increases over time, a phenomenon of long-term scaling (de Navascués et al., 2012).

I.2.2.2. Non-neutral competition

Non-neutral competition, on the other hand, is a biased competition. The winner stem cells gain a long-term advantage and over time completely occupy the niche, while the loser stem cell population is removed from the niche and differentiates. Non-neutral competition has been examined in many systems, both in mammals and in *Drosophila*.

Non-neutral competition in mammalian stem cell systems

A competitive transplantation/ repopulation assay has been used to examine non-neutral competition among mammalian hematopoietic stem cells (HSCs) and spermatogonial stem cells. In this assay, the relative competitiveness of stem cells from two different populations is measured by co-transplanting these cells into a recipient host whose stem cell niche has been emptied, usually by irradiation, allowing for the occupation by the exogenous stem cells. In mouse HSCs, DNA damage induced by mild irradiation results in damaged stem cells that are outcompeted by wild-type stem cells in this competitive transplantation assay, while these damaged stem cells are able to occupy the HSC niche and maintain a stable stem cell pool if they are transplanted alone, suggesting competition selects for stem cells with less damage. Lowering the genetic dosage of the stress sensor p53 in the irradiated HSCs suppresses the competition, while differential p53 levels, in otherwise wild-type HSCs, does not confer any selective advantage, thus suggesting HSC competition is p53-dependent. Interestingly, it is the relative, but not absolute level, of p53 in competing HSCs that determines the outcome of competition, further confirming the non-autonomous competitive interaction between stem cell populations (Bondar and Medzhitov, 2010). On the other hand, mouse spermatogonial stem cells lacking p27, a cyclin dependent kinase inhibitor, are outcompeted by wild-type stem cells, while stem cells from a p27 knock out testis alone are capable of producing normal germ cell

colonies in recipient mice, suggesting normal spermatogonial stem cell activity (Kanatsu-Shinohara et al., 2010). Thus, in the mammalian systems in which it has been tested, stem cell competition selects the healthiest stem cells.

Non-neutral competition in *Drosophila* stem cell systems

Non-neutral stem cell competition in *Drosophila*, which has been studied by generating clones of mutant stem cells and examining the behavior of the mutant cells compared to their wild-type neighbors, has been examined in the midgut and in the gonad. In the *Drosophila* midgut, differential dose of a *Minute* gene can trigger competition in which *Minute* heterozygous cells undergo apoptosis and eliminated from the tissues only when confronted with wild-type cells. To replenish the lost ISCs, the winner wild-type ISCs undergo more rapid symmetric and asymmetric divisions, via activation of JAK-STAT pathway (Kolahgar et al., 2015). Possibly the best known examples of stem cell competition, however, are from studies of the *Drosophila* gonad, in which GSCs compete with each other, or with neighboring somatic stem cells, for niche occupancy (Issigonis et al., 2009; Rhiner et al., 2009). In the male testis, GSCs and somatic Cyst Stem Cells, CySCs, share the same niche, the hub cells. The hub cells maintain both stem cell populations via the BMP pathway and the JAK-STAT pathway, respectively (Matunis et al., 2012). One report concluded that the JAK/STAT maintenance pathway is involved in stem cell competition both among CySCs and between CySCs and GSCs (Issigonis et al., 2009). In these experiments, clones of CySCs mutant for a negative regulator of the JAK/STAT pathway, Socs36E outcompeted neighboring GSCs or wild-type CySCs for niche occupancy. However, this conclusion was challenged by later publications that demonstrated that lack of Socs36E in the CySCs also upregulates the activity of the EGFR pathway, and it is the increased activity of the EGFR pathway, and not the JAK/STAT pathway, that promotes Socs36E mediated stem cell competition (Amoyel et al., 2014; Amoyel et al., 2016). Thus,

although these findings remain controversial, these results are the only existing published observations that suggest that differences in the level of a stem cell maintenance signals of adult stem cells can play a role in triggering stem cell competition, which is the main conclusion of this thesis.

I.3. Competition among female germline stem cells in *Drosophila*

Similar to the *Drosophila* male GSCs, *Drosophila* female GSCs are associated with and maintained by their niche, primarily the somatic, non-dividing cap cells (Xie and Spradling, 1998). Cap cells maintain the GSCs in an undifferentiated state by secreting maintenance factors of the BMP family (Song et al., 2004), and providing niche anchorage, which are the two components that female GSCs have been shown to compete for. I will briefly describe the anatomy of the female germline, and then detail the experiments that have been performed to look at the relationship between maintenance signaling and GSC competition.

I.3.1. The *Drosophila* female GSCs and the niche

Each *Drosophila* ovary is composed of 16-20 ovarioles. Within each ovariole, around two to three GSCs are located at the anterior tip of the germinal center, the germarium (Figure I. 2A). The GSC niche is composed of cap cells, terminal filament cells, and escort cells. Cap cells directly contact the GSCs, and maintain the GSCs by both providing anchorage via adherens junctions (AJs) and secreting BMP ligands to activate BMP signaling within the GSCs (Song et al., 2002; Xie and Spradling, 1998). Female GSCs undergo an asymmetric division that produces one daughter cell, which is retained in the niche and inherits stem cell identity, and a second daughter cell, a Cystoblast (Cb), which is born outside the niche, has low BMP

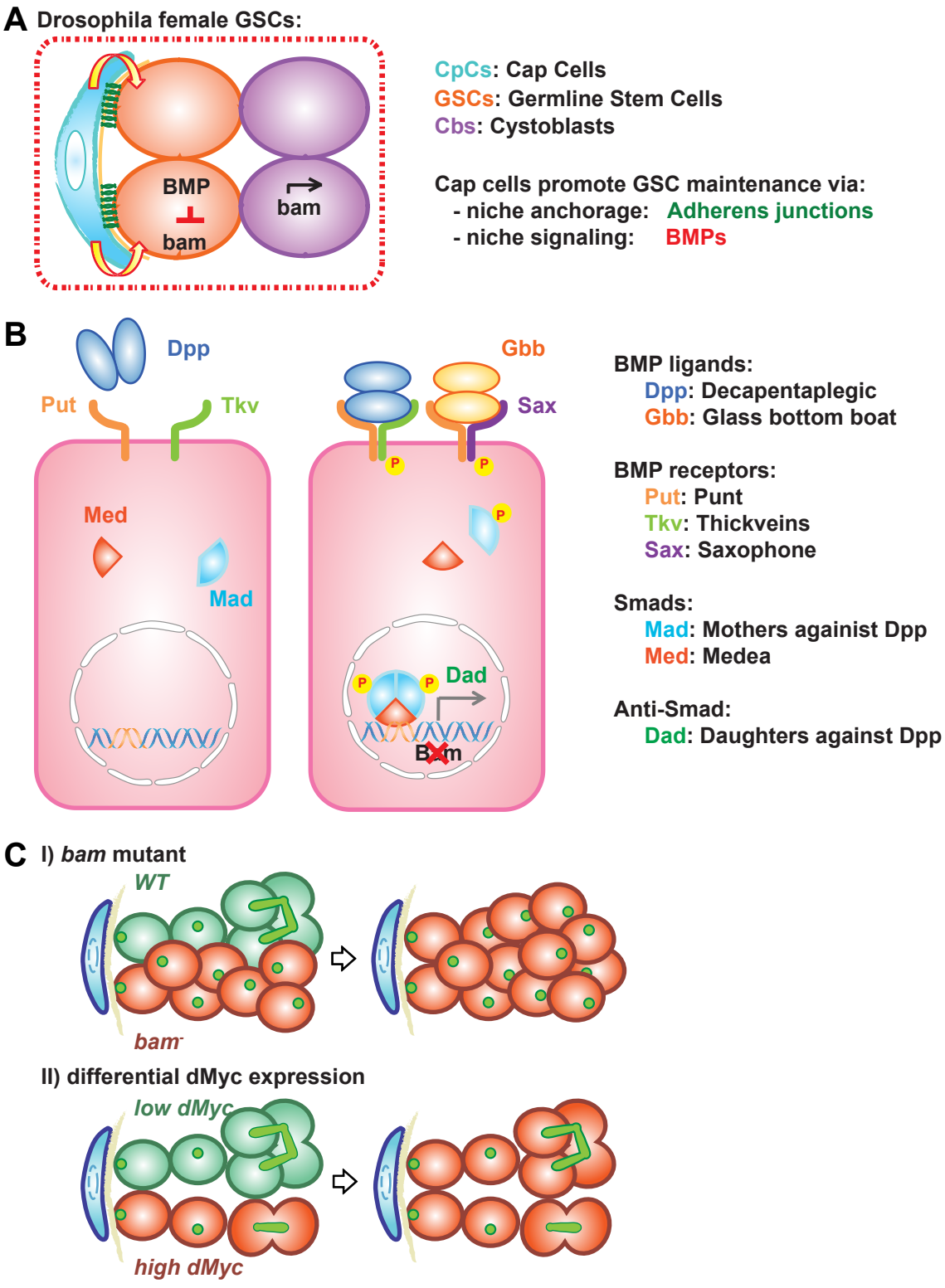


Figure I.2. Differential BMP signaling between GSCs results in GSC competition.

Figure I.2. Continued.
(A) Cap cells directly contact *Drosophila* female GSCs and act as one of the somatic niche cells that maintain GSCs in an undifferentiated state. Cap cells provide anchorage to the niche through Adherens Junctions and secrete maintenance signals of the BMP family. GSCs undergo an asymmetric self-renewal division to produce one GSC and one Cb. BMP signaling is high in GSCs, but low in Cbs. Lack of BMP signaling in Cbs results in the transcriptional activation of the differentiation factor, Bag of marbles (Bam). (B) BMP signaling pathway in *Drosophila* female GSCs. BMP signaling is activated by Dpp or Gbb binding to their corresponding type I receptors, Tkv or Sax, resulting in phosphorylation by the type II receptor, Put. The activated type I receptor phosphorylates the downstream Smad protein, Mad. Phosphorylated Mad forms a trimer with Medea, and the complex translocates into the nucleus to control downstream gene transcription, such as activating *Dad* transcription, and repressing *bam* expression. (C) Previous examples of GSC competition. (i) GSC competition for niche anchorage. Differentiation defective *bam* GSCs outcompete neighboring wild-type GSCs via upregulating DE-cadherin to increase niche anchorage. (ii) GSC competition for niche signaling. Differential *dMyc* dosage among GSCs causes competition in which GSCs with higher *dMyc* outcompete GSCs with lower *dMyc*, and this competition is correlated with a decrease in BMP responsiveness in the loser GSCs.

signaling, and becomes committed to differentiation (Chen and McKearin, 2003a; Deng and Lin, 1997). Each Cb undergoes four rounds of divisions with incomplete cytokinesis to produce a 16-cell cyst, in which one cell eventually differentiates as oocyte, while the others become polyploid nurse cells that support oocyte development. While GSCs have been reported to undergo a symmetric division to replenish an empty niche, the normal pattern of their division is asymmetric with one GSC centrosome located at the niche-GSC interface and the resulting mitotic plane parallel to the interface (Deng and Lin, 1997; Lu et al., 2012; Xie and Spradling, 2000). This plane of division determines the asymmetric daughter fates, as the division plane strongly correlates with the differential activation of BMP signaling in the two daughter cells due to their geometric location after division.

I.3.2. The mechanism of BMP signaling and its role in maintenance of GSCs

In *Drosophila* female GSCs, BMP signaling is activated by binding of the BMP ligands, Decapentaplegic (Dpp) or Glass-bottom boat (Gbb) to their corresponding combination of type I and type II receptors, Tkickveins (Tkv) and Punt (Put) or Saxophone (Sax) and Put, respectively (Figure I. 2B). The ligand binding results in the phosphorylation and activation of the type I receptor by the type II receptor. Once activated, the type I receptor phosphorylates the BMP signal transducer protein, the Smad homolog Mothers against Dpp (Mad), which forms a trimer with the Smad4 homolog Medea and translocates into nucleus to control transcription of downstream genes. In GSCs, BMP signaling transcriptionally represses the differentiation factor, *bag of marbles* (*bam*), thereby maintaining the GSC fate (Chen and McKearin, 2003b; Song et al., 2004). Within Cbs, *bam* is expressed, and Bam activity in turn suppresses BMP signaling, by restricting nuclear accumulation of activated, phosphorylated Mad. In Cbs, Bam associates with Benign gonial cell neoplasm (Bgcn), and promotes Cb commitment to differentiation (Chen and McKearin, 2003a; Lavoie et al., 1999; Li et al., 2009; McKearin and

Ohlstein, 1995; Ohlstein and McKearin, 1997). Clonal GSCs lacking components of BMP signaling, *tkv*, *put*, or *Mad*, differentiate prematurely, while *bam* or *bgcn* deficient flies contain germaria filled with GSC-like cells, reflecting the roles of these genes in GSC maintenance and differentiation, respectively (McKearin and Ohlstein, 1995; Xie and Spradling, 1998).

I.3.3. Previous examples of GSC competition within *Drosophila* ovary

Neutral competition has not yet been identified in female GSCs; however, two studies provide evidence that non-neutral competition is sufficient to trigger differential niche anchorage and niche signaling among competing GSCs or GSC-like cells (Figure I. 2C), thereby causing exclusion of loser stem cells from the niche (Jin et al., 2008; Rhiner et al., 2009).

I.3.3.1. GSC competition for niche anchorage

The progeny of GSCs lacking either *bam* or *bgcn* do not differentiate. Thus, in both mutant genotypes, the germaria is filled with undifferentiated GSC-like cells. Interestingly, differentiation defective *bam* or *bgcn* GSCs have a competitive advantage over neighboring wild-type GSCs that is mediated through differential adhesion to the niche. *bam* or *bgcn* GSCs have more niche contacts and accumulate more DE cadherin at the interface with the niche, compared to their wild-type neighbors. Moreover, downregulation of *shotgun*, the gene encoding DE cadherin, in *bgcn* GSCs is sufficient to eliminate their competitive advantage over wild-type GSCs. Thus, *bam* and *bgcn* mediated GSC competition results from differences in niche anchorage because *bam* activity suppresses DE-cadherin accumulation (Jin et al., 2008).

I.3.3.2. GSC competition for niche signaling

Similar to what was observed in *Drosophila* imaginal discs, GSCs with elevated *dMyc* dosage outcompete GSCs with lower *dMyc* dosage for niche occupancy without altering the total number of GSCs at the niche. As the winner GSCs display a relative higher level of BMP signaling, as measured by staining for the active, phosphorylated form of Mad (pMad), the authors proposed that differential *dMyc* results in a differential ability to uptake BMP ligands, with resulting imbalances in BMP signal transduction, which is required to maintain a GSC fate. Under this scenario, the large decrease in BMP responsiveness causes the loser cells to undergo differentiation. In support of this hypothesis, the authors also demonstrated that expression of a constitutively active form of the Tkv receptor is sufficient to suppress *dMyc*-mediated GSC competition. These data thus suggest that the level of the BMP maintenance signal is a response to the initiation of GSC competition as a quality control mechanism to ensure the stem cell with highest metabolic efficiency is maintained (Rhiner et al., 2009).

However, these data raise the issue of whether the differential level of a stem cell maintenance signal could initiate competition among a group of stem cells in a niche. This hypothesis is appealing, as a single absolute threshold of a maintenance signal to determine stem cell residency at the niche may not be sufficient to ensure that the retained stem cells are the healthiest ones. The reduced level of maintenance signaling within a damaged stem cell could still be sufficient to maintain that stem cell at the niche, which would lead to the generation of suboptimal tissues over time. Thus, competition based on the relative level of maintenance signals among niche-resident stem cells could play a large role to exclude non-healthy stem cells from the niche and to prevent stem cells from producing non-optimal tissues. However, this idea has not been documented in any stem cell system to date. In particular, in the case of *Drosophila* female GSCs, it has not been tested whether differential BMP signaling is sufficient to trigger GSC competition.

I.4 Overview of the thesis

In my thesis, I used the *Drosophila* female GSC as model system to examine the question of whether a stem cell maintenance signal can also initiate competition between niche resident stem cells. In Chapter II, I report the development of a platform where gene activity can be disrupted in one or all GSCs in a tunable fashion. I then used this platform with RNA interference constructs against the BMP receptors to examine whether differential BMP signaling among GSCs can trigger GSC competition. In Chapter III, I report the results of experiments using the CRISPR/Cas9 system to examine BMP receptor distribution and to confirm that BMP signaling is required for GSC maintenance. I discuss the implications of this research in Chapter IV. In the Appendices, I report the results of various other experiments I conducted during the course of this research. In Appendix I, I report the results of experiments that demonstrate that another Smad family member, Smox, is not involved in either GSC maintenance or competition. In Appendix II, I report the results of experiments that sought to examine whether elevated BMP signaling could confer GSCs with supercompetitor identity. I also report the results of experiments seeking to identify genes that mediate BMP-initiated competition. My data indicate BMP signaling regulates two distinct behaviors of GSCs, GSC maintenance and GSC competition. There are two thresholds for BMP signaling in the GSCs: a low level for GSC maintenance and a higher level for GSC competition. Specifically, GSC with a level of BMP signaling in between these two thresholds will be eliminated from the niche only when they are surrounded by wild-type GSCs. These data formally demonstrate that differences in the level of a stem cell maintenance signal can directly initiate competition among niche-resident stem cells.

CHAPTER II: DIFFERENTIAL BMP SIGNALING INITIATES CELL COMPETITION IN *DROSOPHILA* FEMALE GERMLINE STEM CELLS

II.1. Abstract

To ensure organismal homeostasis, adult stem cells compete with each other for niche occupancy. While many adult stem cells require niche secreted maintenance signals to keep them in an undifferentiated state, the relationship between these maintenance signals and cell competition is poorly understood. In the *Drosophila* ovary, niche cells secrete Bone Morphogenetic Proteins (BMPs) to maintain the Germline Stem Cells (GSCs). We now demonstrate that a difference in BMP signaling between neighboring GSCs directly triggers competition for niche occupancy. Specifically, a GSC with a low level of BMP signaling will be outcompeted by wild-type GSCs, but will be maintained if all neighboring GSCs have similar low levels of signaling. Competition is manifest downstream of, or in parallel to, transcriptional control of the GSC differentiation factor Bag-of-marbles, Bam. This study reveals the level of BMP signaling can act as a measure of cell fitness under normal physiological conditions. Moreover, by assaying their relative levels of BMP signaling, GSCs are capable of continuously occupying the niche under conditions where the levels of BMP signals are globally reduced, for example, in aged individuals.

II.2. Introduction

Stem cells maintain tissue homeostasis throughout adult life (Biteau et al., 2011; Simons and Clevers, 2011). Most adult stem cells occupy a specific microenvironment, a niche, and there is accumulating evidence that competition among stem cells for niche occupancy is a fundamental feature of stem cell biology (Morrison and Spradling, 2008; Stine and Matunis, 2013). The hallmark of cell competition, which was identified in the larval *Drosophila* wing disc epithelium, is

that if one cell is less fit than another cell it will be replaced, but if two cells are equally fit, both will remain (Amoyel and Bach, 2014; Vincent et al., 2013). Experimental evidence indicates that cell competition can be triggered by differential dosage of either *Minute* genes, which encode ribosomal proteins, or *dMyc*, which promotes cell growth (Cova et al., 2004; Morata and Ripoll, 1975; Moreno and Basler, 2004; Moreno et al., 2002). However, little is known about how stem cells measure their relative fitness to initiate competition.

The *Drosophila* ovary provides a powerful system to investigate competition between stem cells. At the anterior tip of each ovariole, two to four GSCs occupy a niche primarily composed of non-dividing cap cells that anchor GSCs through adherens junctions and secrete Bone Morphogenetic Proteins (BMPs) to maintain GSCs in an undifferentiated state (Song et al., 2002; Song et al., 2004; Xie and Spradling, 1998). GSCs divide asymmetrically such that one daughter remains GSC identity with high BMP signaling, while the other daughter, the Cystoblast (Cb), is born outside of the niche, has low BMP signaling (Chen and McKearin, 2003b) and begins to differentiate. In GSCs, BMP ligands bind to the type I receptors, Thickveins (Tkv) and Saxophone (Sax), causing their phosphorylation by the type II receptor Punt (Put). Activated type I receptors phosphorylate the SMAD homolog, Mothers against Dpp (Mad), which accumulates in the nucleus to control BMP target gene expression. BMP signaling promotes GSC maintenance in part by repressing *bag of marbles* (*bam)* transcription in GSCs (Chen and McKearin, 2003b; Song et al., 2004). Low levels of BMP signaling in Cbs and descendant cyst cells result in *bam* expression, which promotes germline differentiation, in part by repressing Nanos (Nos) translation in 2-4 cell cysts (Li et al., 2009).

The role of BMP signaling in initiating cell competition has been investigated in other systems. Initial investigators proposed that outcompeted *Drosophila* wing imaginal disc cells had reduced ability to compete for BMP ligands, which act as cell survival factors during competition (Moreno and Basler, 2004; Moreno et al., 2002). Similarly, Rhiner et al. (2009) showed that GSCs with extra copies of *dMyc* outcompete wild-type GSCs and that outcompeted GSCs have lower levels of pMad staining, suggesting that a greater ability to capture BMP ligands underlay GSC competition. Conversely, recent studies in wing imaginal discs have used differential BMP signaling as a trigger for cell competition (Merino et al., 2015; Portela et al., 2010; Rhiner et al., 2010). Likewise, mouse ESCs with reduced BMP signaling were outcompeted by wild-type ESCs (Sancho et al., 2013). However, in these systems, the reduced level of BMP signaling used to initiate cell competition was not sufficient to support normal development in vivo.

Here we examine whether differential BMP signaling initiates competition among *Drosophila* GSCs. We show that if a GSC with low levels of BMP signaling is adjacent to a GSC with wild-type levels of signaling, it is outcompeted, while if all neighboring GSCs have similar low levels of BMP signaling, they are maintained. Thus, differential BMP signaling can directly drive GSC competition, highlighting that a single extrinsic signal can play multiple roles in stem cell homeostasis.

II.3. Results and discussion

II.3.1. A tunable system to control BMP signaling in GSCs

To test the hypothesis that differential BMP signaling can initiate GSC competition, we developed a tunable system, similar to one recently published (Salzmann et al., 2013), that allowed us to reduce activity of a BMP receptor in either one or all GSCs. We then measured

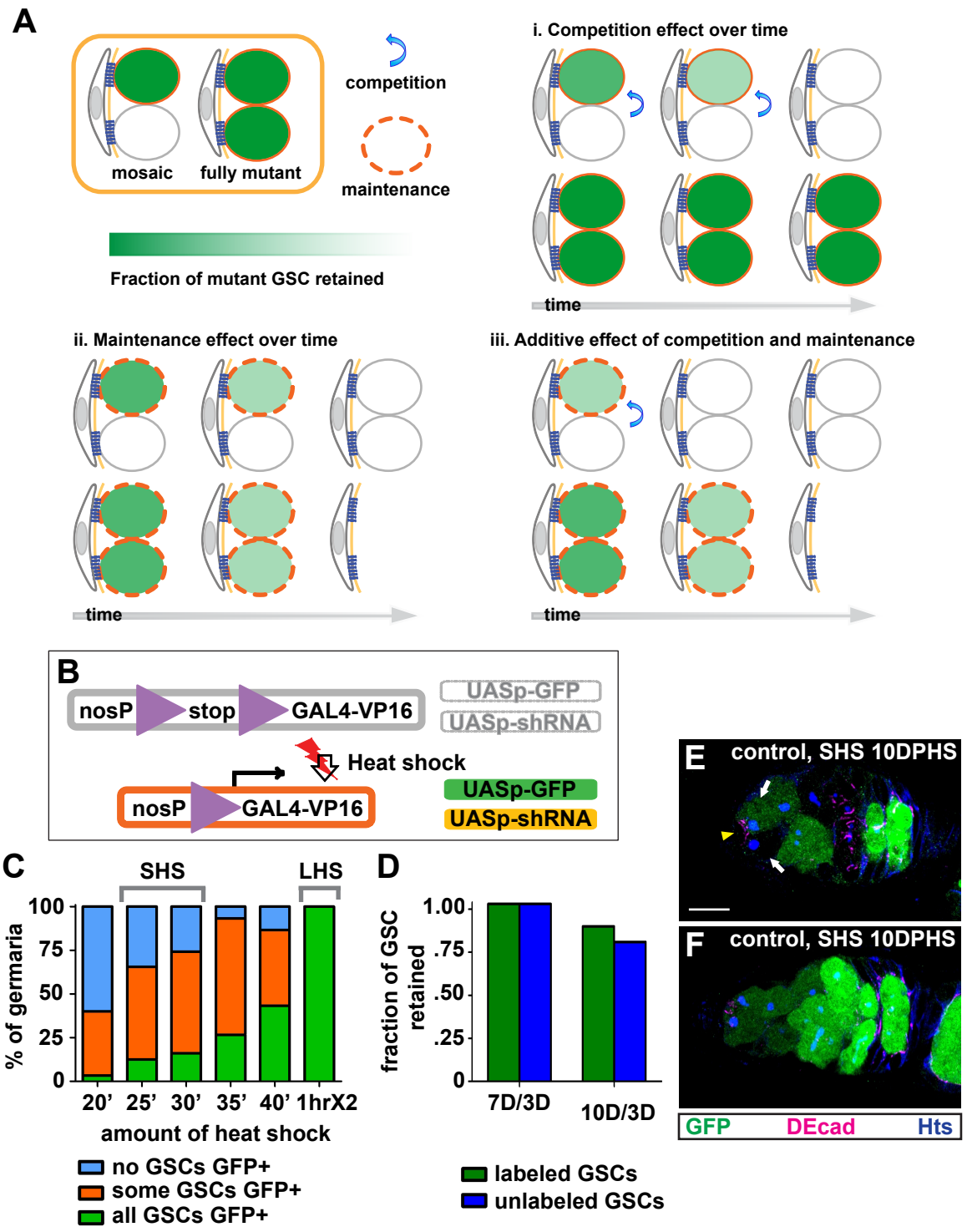


Figure II.1. A strategy to study GSC competition.
(A) Distinguishing gene involvement in GSC competition vs. maintenance. Elongated grey cell with nucleus, somatic cap cells, a stromal niche cell for GSCs. Cell with grey outline, wild-type GSC. Cell with orange outline, mutant GSC. Clustered blue lines, adherens junctions.

Figure II.1. continued
(B) The *nos*-FLPout cassette. FRT element, purple arrowhead.
(C-F) Females heterozygous for hsFLP, *nos*-FLPout, and UASp-GFP transgenes subject to different amounts of heat shock. (C) The percentage of germaria at 3 days post heat shock with no, some ("mosaic"), or all GFP-labeled GSCs as function of amount of heat shock. SHS, short heat shock; LHS, long heat shock. (D) Fraction of labeled and unlabeled GSCs retained at 7 and 10 days (D) post SHS, compared to 3 days. (E,F) Germaria 10 days post SHS. (E) Mosaic germarium containing labeled and unlabeled GSCs. (F) Fully labeled germarium. Yellow arrowhead, adherens junctions between cap cells and GSCs; white arrows, GSCs. All panels: anterior to left; scale bar, 10μm.

the fraction of BMP signaling impaired GSCs retained over time in “mosaic” germaria that contain both wild-type and mutant GSCs and in “fully mutant” germaria where all GSCs are signaling impaired.

A stem cell’s ability to remain in its niche can be affected by both cell competition and maintenance signals. Cell competition measures relative fitness of a population of cells: therefore, if differential BMP signaling between GSCs only initiates cell competition, mutant GSCs with low BMP signaling should be lost in mosaic germaria, but retained in fully mutant germaria (Figure II.1Ai). Conversely, transduction of a maintenance signal is a cell intrinsic event necessary for stem cell survival: if BMP signaling only promotes GSC maintenance, mutant GSCs should be lost at an equal rate in both mosaic and fully mutant germaria (Figure II.1Aii). However, if BMP signaling is involved in both GSC competition and maintenance, mutant GSCs should be lost faster in mosaic germaria than in fully mutant germaria (Figure II.1Aiii).

We engineered flies carrying a *nos*-FLPout transgene that contains a FLPout cassette placed between the *nanos* (*nos*) promoter, which drives expression in all germ cells, and the GAL4-VP16 activator. After heat-shock mediated FLPout recombination in a GSC, GAL4 expression drives both GFP and an shRNA against a specific gene; the former labeling the GSC, the latter permitting manipulation of BMP signaling (Figure II.1B). Because the FLPout event is independent within each GSC, increasing the amount of heat shock increases the fraction of labeled GSCs. A short heat shock (25-30’, SHS) produced 40 - 50% mosaic germaria, while a long heat shock (two, one-hour exposures, LHS) produced 95-100% fully labeled germaria (Figure II.1C).

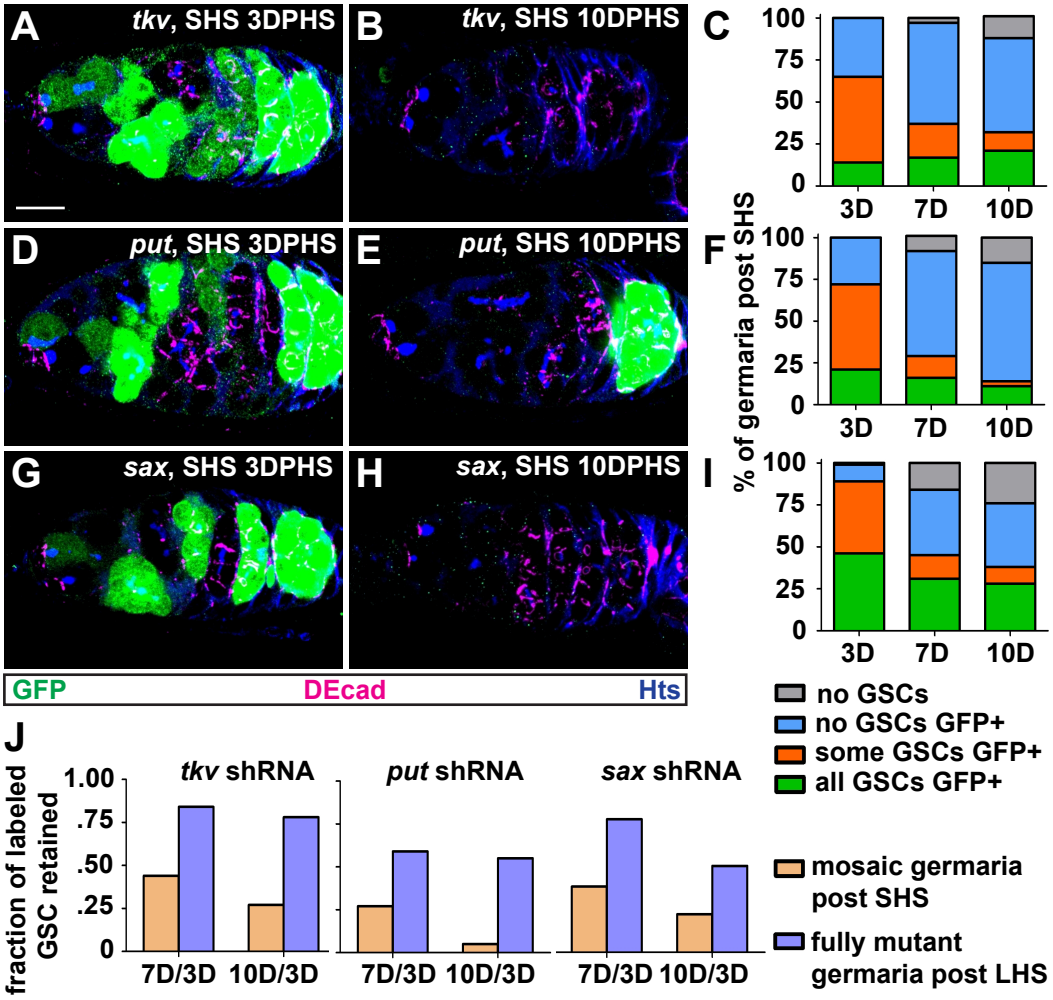


Figure II.2. Differential BMP signaling between GSCs results in GSC competition. (A-I) In germaria post SHS, behavior of GSCs expressing shRNAs against different BMP receptors, *tkv* (A-C), *put* (D-F), *sax* (G-I). (A,D,G) Presence of labeled GSCs in germaria 3 days post heat shock (DPHS). (B,E,H) Absence of labeled GSCs 10 DPHS. (C,F,I) Percentages of germaria of different classes at increasing number of days (D) post SHS. (J) Histogram from Table II.1A: for each receptor shRNA, fraction of labeled GSCs retained at 7 and 10 DPHS compared to 3 DPHS.

Table II.1. Average number of labeled GSCs per germarium and characterization of shRNA GSCs over time.

A — Average number of GFP labeled GSCs per germarium[a,b]

		3DPHS	7DPHS	10DPHS
tkv shRNA	SHS	0.64	0.28	0.17
	LHS	2.45	2.05	1.90
put shRNA	SHS	0.80	0.22	0.04
	LHS	2.25	1.32	1.22
sax shRNA	SHS	0.92	0.35	0.20
	LHS	1.60	1.23	0.79

B — Characterization of shRNA GSCs over time[c,d]

	20°C			22->28°C					
	30DPE			3DPTS			10DPTS		
	av. # GSCs / germarium	% germaria w/ # of GSCs		av. # GSCs / germarium	% germaria w/ # of GSCs		av. # GSCs / germarium	% germaria w/ # of GSCs	
		>=2	0-1		>=2	0-1		>=2	0-1
control	2.20	92	8	2.64	95	5	2.37	95	5
tkv shRNA	n.t.	n.t.	n.t.	2.28	84	16	2.28	90	10
put shRNA	2.04	86	14	0.30	11	89	n.a.	n.a.	n.a.

a, A minimum number of 57 germaria were analyzed per time point per condition.

b, *nos*-FLPout females carrying a shRNA construct against a BMP receptor.

c, A minimum number of 86 germaria were analyzed per time point per condition.

d, *nos*-GAL4 females with or without a shRNA construct against a BMP receptor.

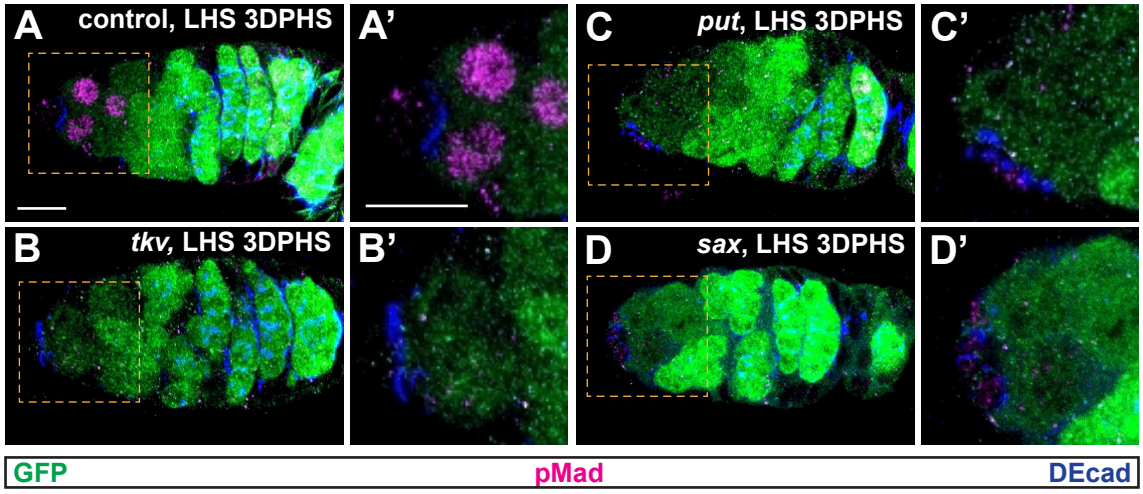
SHS, short heat shock; LHS, long heat shock; DPHS, days post heat shock.

DPE, days post eclosion; DPTS, days post temperature shift (22°C to 28°C); n.t., not tested; n.a., not applicable.

To validate the system, we conducted two experiments. We first confirmed that FLPout-mediated GFP expression does not affect GSC retention by performing a control experiment without an shRNA. In all experiments, we deemed a germ cell a GSC if it was adjacent to cap cells and had a round spectrosome, marked by the adducin homolog Hu li tai shao (Hts), closely apposed to cap cells. When adult flies were subject to short heat shock, a similar fraction of labeled and unlabeled GSCs were retained over the ten-day time course (Figure II.1D-F), indicating GFP expression is not deleterious. We then confirmed the efficiency of shRNAs against each BMP receptor by demonstrating that nuclear phosphorylated Mad (pMad), the readout of BMP signaling, was markedly reduced in GSCs in fully mutant germaria (Figure II.3A-D).

II.3.2. Differential BMP signaling promotes GSC competition

To assay the ability of differential BMP signaling to initiate GSC competition, we first subjected flies carrying a BMP receptor shRNA to short heat shock and examined changes over time in the percentages of germaria that had none, some, or all GSCs labeled (Figure II.2A-I). As expected, the frequency of mosaic germaria, those with some GSC labeled, rapidly declined starting seven days post heat shock (Figure II.2C,F,I), as did the average number of labeled GSCs per germarium (Table II.1A). We then subjected females of identical genotypes to long heat shock and calculated the average number of labeled GSCs per germarium at different time points (Table II.1A). Lastly, for each procedure, we calculated the ratio of the average number of labeled GSCs at seven and ten days post heat shock to the average number of labeled GSCs at three days post heat shock (Materials and Methods, Figure II.2J). For each shRNA, this ratio was greater in fully mutant germaria than in mosaic germaria. Data from a second independent replicate (Figure II.3E,F) were similar. These results indicate that an individual GSC with low BMP signaling will perdure longer in the niche if all neighboring GSCs also have low signaling,



E **Average number of GFP labeled GSCs per germarium**

		3DPHS	7DPHS	10DPHS
tkv shRNA	SHS	0.70	0.36	0.06
	LHS	2.47	2.06	2.01
put shRNA	SHS	0.63	0.25	0.06
	LHS	2.33	1.65	1.23
sax shRNA	SHS	0.52	0.09	0.09
	LHS	1.72	0.70	0.73

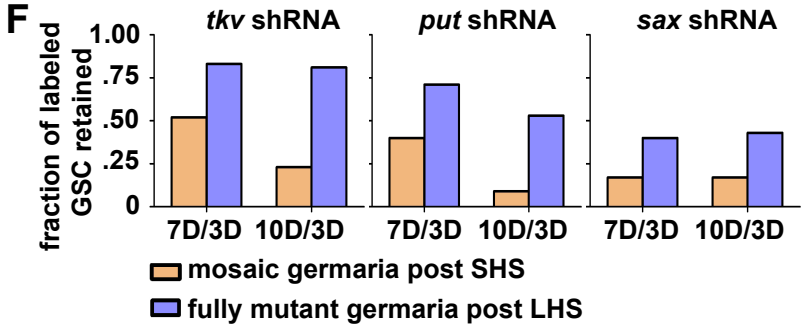
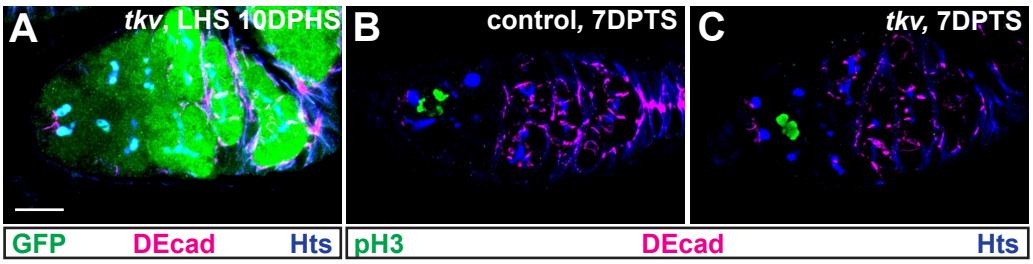


Figure II.3. Functionality of the shRNA constructs; differential BMP signaling in GSCs leads to competition

(A-D) Anti-pMad staining of a *nos*-FLPout control germarium (A) and germaria from females carrying shRNA constructs against *tkv* (B), *put* (C), and *sax* (D) after *nos*-FLPout driven expression 3 days post LHS. (A'-D') Enlargement of the boxed regions in (A-D).
(E, F) A second replicate of the experiment in Table II.1A and Figure 3J. (E) The average number of labeled GSCs per germarium at 3, 7, and 10 days post SHS or LHS. A minimum number of 60 germaria were analyzed per time point per condition. (F) Histogram derived from (E) showing the fraction of labeled GSCs retained at 7 and 10 DPHS compared to 3 DPHS.



D

Characterization of GSC divisions

	pH3+ GSCs	eggs/ female/ day
control	4.1%	11.8
tkv shRNA	2.5%	9.3

Figure II.4. GSCs in *tkv* shRNA expressing females are maintained and continue to divide.

(A) A germarium from a *nos*-FLPout *tkv* shRNA female 10 days post LHS with 2GSCs in the niche.

(B and C) Anti-phospho Histone H3 staining of a *nos*-GAL4 control germarium (B) and a germarium from a *nos*-GAL4 *tkv* shRNA female (C) grown at 22°C and shifted to 28°C. In both germaria, a single mitotic GSC is stained.

(D) Characterization of GSC divisions in *nos*-GAL4 *tkv* shRNA females. Percentage of GSCs stained with anti-phospho Histone H3 antibody (n = 460, control; n = 271, *tkv* shRNA). Average number of eggs laid per female per day by control and *tkv* shRNA females.

than if it has wild-type GSC neighbors. Thus, differential BMP signaling between GSCs initiates cell competition among GSCs, such that those with lower signaling are more rapidly lost from the niche (Figure II.1Aiii).

Strikingly, *tkv* shRNA GSCs were lost from mosaic germaria but largely retained in fully mutant germaria (Figure II.2J, Figure II.3F, Figure II.4A), suggesting that this level of reduction in *tkv* activity initiates GSC competition but does not cause appreciable maintenance defects. To quantitate this difference, we calculated the decay time, the number of days post heat shock before the average number of labeled *tkv* shRNA GSCs per germarium fell to half of its initial value (Rhiner et al., 2009). In both replicates, the decay time of *tkv* shRNA GSCs in mosaic germaria was less than half the decay time in fully mutant germaria (6-7 days *vs.* 16-17 days), further highlighting the role of GSC competition in accelerating loss of BMP signaling defective GSCs.

To see whether we could recapitulate the *tkv* phenotype with shRNAs against the other receptors, we drove *put* and *sax* shRNAs at lower temperatures using a *nos*-GAL4 transgene, which is expressed throughout germ cell development. *nos*-Gal4 *tkv* shRNA females grown at 22^{o}C and shifted to 28^{o}C after eclosion retained GSCs over a ten day period post temperature shift, and their GSCs displayed very little pMad staining one day post temperature shift (Table II.1B and Figure II.5A-D). In addition, these GSCs continued to divide with a rate only slightly slower than wild-type GSCs and these females continued to lay eggs over the experimental time course (Figure II.4B-D). Newly eclosed *nos*-GAL4 *sax* shRNA females lacked GSCs even when grown continuously at 18^{o}C (data not shown), possibly because of the requirement for BMP signaling in primordial germ cell maintenance (Deshpande et al., 2014; Gilboa and Lehmann, 2004). *nos*-GAL4 *put* shRNA females either grown continuously at 22^{o}C, or grown at 22^{o}C and

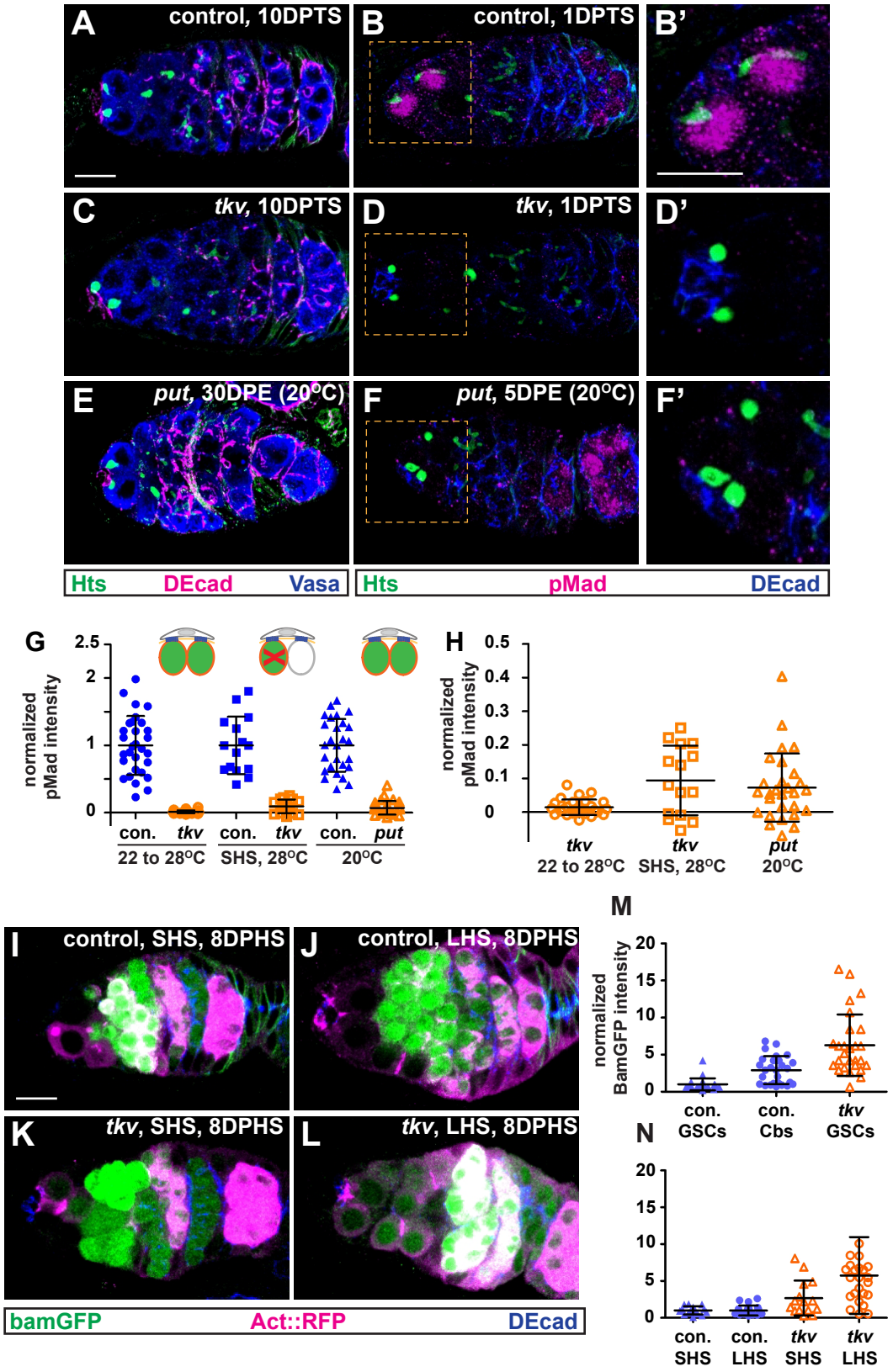
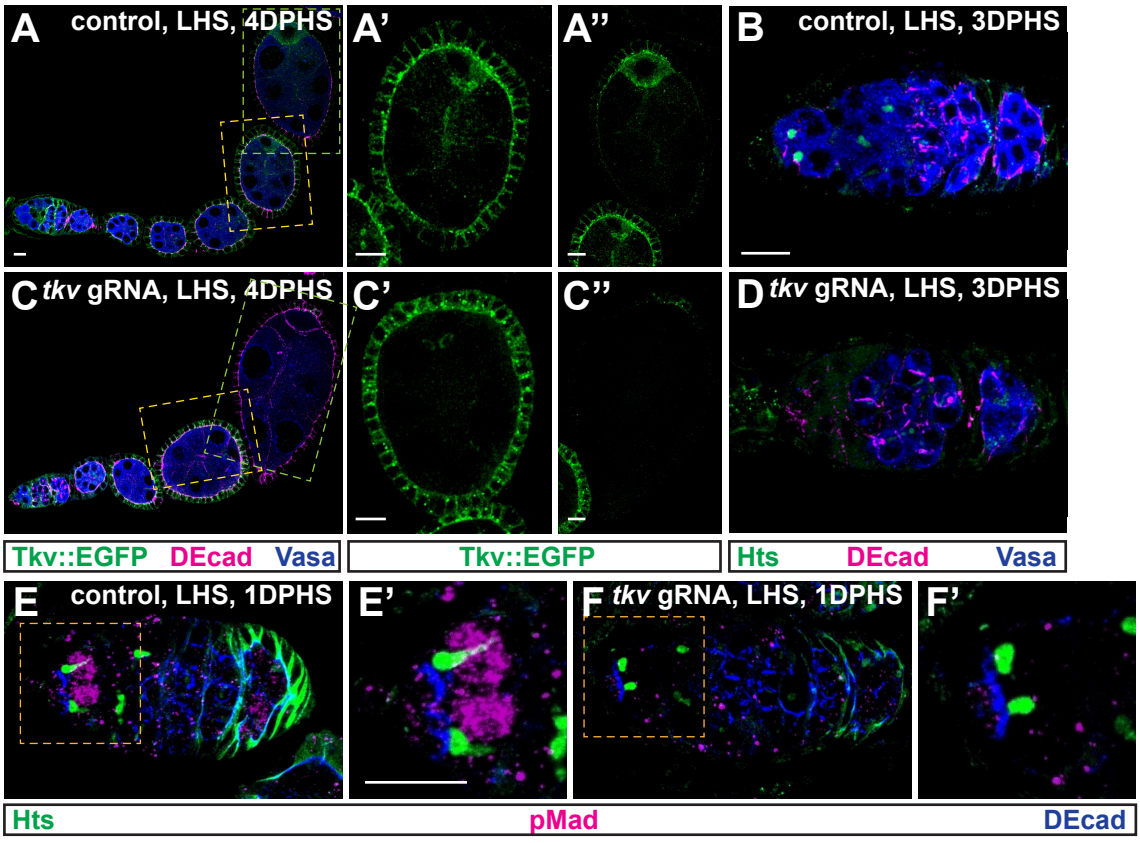


Figure II.5. GSCs with low levels of pMad are maintained in the niche and express bam.

Figure II.5. continued
(A-F) Germaria from *nos*-GAL4 females. (A,C,E) Maintenance of GSCs, as evidenced by anti-Hts staining of niche-associated spectrosomes, in *nos*-GAL4 control (A) and *tkv* shRNA (C) germaria 10 DPTS, and in *put* shRNA germarium 30 DPE at 20°C (E). (B,D,F) Anti-pMad staining in control (B) and *tkv* shRNA (D) germaria 1 DPTS, or in *put* shRNA germarium 5 DPE at 20°C (F). (B',D',F') Enlargement of boxed regions in (B,D,F).
(G-H) Nuclear pMad intensities in GSCs from *nos*-Gal4 *tkv* shRNA females at 1 day post temperature shift (DPTS) from 22°C to 28°C (orange circles: 1.37±2.32% of control, n=28), labeled GSCs in mosaic germaria from *nos*-FLPout females with *tkv* shRNA 4 days post SHS (orange squares: 9.38±10.34% of control, n = 15), or *nos*-GAL4 *put* shRNA females grown continuously at 20°C for 5 days (orange triangles: 7.25±10.13% of control, n=28), normalized to pMad intensities in control GSCs raised under identical conditions (corresponding controls; blue circles: 100±43.60, n=30; blue squares: 100±42.65, n=15; blue triangles: 100±39.03, n=27). Data are represented as mean ± s.d. (H) Enlargement of normalized pMad intensities in BMP signaling defective GSCs from (G).
(I-L) *nos*-FLPout UAS-Act::RFP females carrying a *bam*-GFP reporter after either SHS (I,K) or LHS (J,L), and aged for 8 DPHS at 28°C.
(M) *bam*-GFP intensity was greater in *nos*-GAL4 control Cbs (blue circles: 2.91±1.87 fold, n=27) and *nos*-GAL4 *tkv* shRNA GSCs at 10 DPTS (orange triangles: 6.28±4.15 fold, n=27), compared to normalized *bam*-GFP intensity in control GSCs (blue triangles: 1.00±0.81, n=23). GSCs and Cbs were distinguished by their pattern of Hts staining.
(N) *bam*-GFP intensity was greater in labeled *nos*-FLPout; *tkv* shRNA GSCs 8 days post SHS (orange triangles, 2.72±2.38 fold, n=15) and LHS (orange circles, 4.83±2.67 fold, n=23), compared to normalized *bam*-GFP intensity in control GSCs (corresponding controls: blue triangles: 1.00±0.55, n=15; blue circles: 1.00±0.67, n=21). Data are represented as mean ± s.d. *bam*-GFP expression is higher in *tkv* shRNA GSCs after LHS than in *tkv* shRNA GSCs after SHS (p =0.016).

shifted to 28°C as adults, lost GSCs by seven days post eclosion (data not shown) or three days post temperature shift (Table II.1B), respectively. In contrast, females of identical genotype grown continuously at 20°C retained GSCs for at least thirty days post eclosion and displayed very low levels of pMad in GSCs five days post eclosion (Table II.1B, Figure II.5E,F). Thus, like *tkv* shRNA GSCs, GSCs with strongly reduced *put* activity and pMad staining can be maintained over time in the absence of wild-type GSCs.

The initial experiments that demonstrated a cell-autonomous role for BMP signaling in GSC maintenance used FLP-mediated mitotic recombination to inactivate BMP signaling components in individual GSCs in germaria that also contained wild-type GSCs (Xie and Spradling, 1998). Thus, the observed decrease in the half-life of mutant GSCs reflected the combined roles of BMP signaling in GSC competition and GSC maintenance. Given that *tkv* shRNA GSCs with greatly reduced BMP signaling can be maintained in the niche, we wished to formally demonstrate that *tkv* activity is required for GSC maintenance. We therefore combined the *nos*-FLPout system with CRISPR-Cas9 methodology to induce mutations in *tkv* within each GSC. CRISPR-mediated activity of either of two *tkv* gRNAs eliminated expression of an endogenously EGFP-tagged Tkv (Materials and Methods) in the ovary (Figure II.6A,C, data not shown). In *tkv* gRNA expressing ovarioles dissected one day post heat shock, GSC morphology was largely normal, but pMad was greatly reduced (Figure II.6E,F), indicating that the great majority of GSCs had CRISPR-induced mutations in *tkv*. Furthermore, *tkv* gRNA GSCs were lost after three days, while GSCs expressing a functional gRNA against *yellow* (Port et al., 2014) were maintained for the full ten-day period (Figure II.6B,D,G,H). Thus, if all GSCs in a germarium lack *tkv* activity, GSCs are rapidly lost, indicating *tkv* is absolutely required for GSC maintenance.



G

Average number of GSCs per germarium

	1DPHS	3DPHS	7DPHS	10DPHS
control	2.31	2.28	n.t.	n.t.
y gRNA	2.10	1.69	2.10	2.04
tkv gRNA	1.97	0.29	n.a.	n.a.

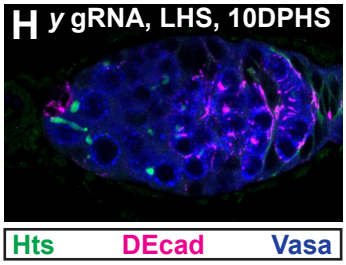


Figure II.6. CRISPR-mediated loss of *tkv* function in all GSCs results in GSC loss.
(A-F) Ovarioles from females heterozygous for hsFLP, *nos*-FLPout, UAS-Cas9 transgenes without (A, B, and E) and with (C, D, and F) a *tkv* gRNA transgene. In control ovarioles with a endogenously tagged Tkv::EGFP gene (A), EGFP is expressed in the follicle cells and accumulates in the oocyte in stage 6 (A') and stage 7 (A'') egg chambers at 4 days post LHS. The oocyte accumulation of Tkv::EGFP is eliminated in females of similar genotype that contain a *tkv* gRNA (C' and C'' at corresponding stages). At 3 days post LHS, GSCs with niche associated spectrosomes, marked by Hts staining, are present in control (B) but not in *tkv* gRNA (D) germaria. (E and F) Anti-pMad staining of a control germarium (E) and a germarium from a female carrying *tkv* gRNA transgene (F) 1 day post LHS. (E' and F') Enlargement of the boxed regions in (E and F).
(G) Average number of GSCs per germarium from females heterozygous for hsFLP, *nos*-FLPout and UAS-Cas9 transgenes without any guide RNA (control) or with a *tkv* gRNA or a *y* gRNA at different time points post LHS. The minimum number of ovarioles scored per time point was 88. n.t., not tested; n.a., not applicable.
(H) Germarium of a female heterozygous for hsFLP, *nos*-FLPout, UAS-Cas9, and *y* gRNA transgenes at 10 days post LHS.

II.3.3. Two thresholds for BMP signaling in GSC maintenance and competition

To determine a level of BMP signaling that permits continued niche occupancy in absence of GSC competition, we quantitated pMad levels in BMP receptor knockdown GSCs compared to normalized wild-type GSCs (Figure II.5G,H). GSCs from *nos*-GAL4 *tkv* shRNA females had 1.37% ($p<0.0001$) of wild-type levels of pMad at one day post temperature shift to 28^{o}C, and GSCs from *nos*-GAL4 *put* shRNA females grown continuously at 20^{o}C had 7.25% ($p<0.0001$) of wild-type levels of pMad at five days post eclosion. In comparison, in mosaic *nos*-FLPout *tkv* shRNA germaria, labeled GSCs, which would later be lost due to competition, displayed 9.38% ($p<0.0005$) of wild-type levels of pMad at four days post short heat shock. Thus, the level of BMP signaling that permits GSC retention in fully mutant germaria of genotype *nos*-GAL4 *tkv* shRNA is less than the level that initiates competition in mosaic germaria of genotype *nos*-FLPout *tkv* shRNA ($p =0.0003$).

II.3.4. GSC competition acts downstream of, or in parallel to, *bam* expression

To determine whether *bam* was expressed in *tkv* shRNA GSCs, we used a *bam-GFP* construct, in which the *bam* promoter drives GFP expression. The level of GFP in *nos*-GAL4 *tkv* shRNA GSCs was significantly higher than in wild-type GSCs or their Cb progeny ($p<0.0001$ and $p=0.0003$, respectively), suggesting that the level of *bam* expression in these GSCs could be sufficient to initiate GSC differentiation (Figure II.5M). Similarly, *bam*-GFP intensity was elevated in *nos*-FLPout *tkv* shRNA GSCs in mosaic germaria, as well as in fully mutant germaria; however, the level of GFP expression was not higher in the former GSCs compared to the latter (Figure II.5I-L,N), suggesting that GSC competition triggered by differential BMP signaling does not further increase *bam* transcription. However, unlike in wild-type cysts, *bam* expression in *tkv* shRNA GSCs did not inhibit Nos translation (Figure II.7A,B). Although Bam levels in these

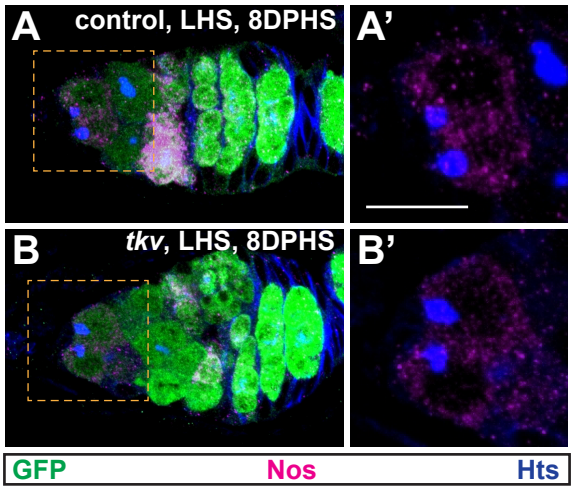


Figure II.7. Nos protein is present in tkv shRNA expressing GSCs.
(A-B) Anti-Nos staining of a *nos*-FLPout control germarium (A) and a germarium from a *nos*-FLPout *tkv* shRNA female 8 days after LHS. Nos is expressed in GSCs of both genotypes. (A' and B') Enlargements of boxed regions in (A and B).

GSCs could have been insufficient to suppress Nos translation, we note that *bam*-expressing GSCs can be maintained for at least ten days. These data indicate that in the absence of GSC competition, *bam* is expressed but does not drive premature GSC differentiation. Thus, BMP-mediated competition could cause GSC differentiation by increasing Bam translation or activity, or by a Bam-independent mechanism (Xi et al., 2005).

II.4. Discussion

While BMP signaling has long been known to be essential for GSC maintenance, we have now shown that differential BMP signaling between GSCs directly triggers GSC competition, leading to loss of GSCs with lower BMP signaling. Our data formally separate two roles of BMP signaling in controlling GSC maintenance and competition. A very low level of BMP signaling is necessary to maintain a GSC in an undifferentiated state. A second, higher threshold of BMP signaling is involved in GSC competition. In between these two thresholds, a single GSC will be outcompeted by a GSC with wild-type levels of signaling, but will be maintained, and will continue to divide, if all other GSCs have a similar level of signaling. We propose that such a mechanism could contribute to GSC homeostasis under conditions in which the overall level of BMP maintenance signals decreases, for example, under adverse environmental conditions or in aged individuals (Zhao et al., 2008).

II.5. Material and Methods

Drosophila strains and culture

Fly stocks were maintained at 18-25^{o}C on standard cornmeal molasses medium. Experimental crosses were set up at 22^{o}C and transferred to 28^{o}C either after eclosion or after heat shock

treatment, unless otherwise noted. Some experimental crosses designed to reduce the efficiency of shRNA knockdown were grown at 18^{o} or 20^{o}C. *tkv* shRNA (BL#35653), *put* shRNA (BL#35195), *sax* shRNA (BL#57319), and UASt-Cas9 P2 (BL#58985) were obtained from The Bloomington *Drosophila* stock center (Bloomington, IN). The *y*-gRNA transgenic line was a gift from Dr. Simon Bullock.

Generation of the *nos*-FLPout construct

The *nos*-FLPout (*nos* promoter and 5'UTR-FRT-stop-FRT-tubulin 3'UTR) construct was constructed in pBluescript and then cloned into pCaSpeR4 (DGRC#1213). The *nos* promoter and 5'UTR region and the GAL4::VP16 sequences were derived from the vector *nos*GAL4-MW-pBacns, DGRC#1290, from the *Drosophila* Genomics Resource Center (Bloomington IN).

Target selection and primer design for CRISPR experiments

Two experiments used CRISPR methodology: NHEJ-mediated targeting of *tkv* in the germline, and tagging of endogenous Tkv protein with EGFP by homologous recombination. For NHEJ targeting of *tkv*, gRNA target site #1 (GCCATCGCAGTAGCAGGTTAGGG) and gRNA target site #2 (TGAGACGACCGGGATGTACGAGG), both of which target sequences that encode the extracellular ligand-binding domain, were used. For tagging the endogenous Tkv protein, gRNA target site #3 (GCTGTTTGTAGTCTCGTTTTAGG), which targets sequences at the 3'UTR region, was used. The PAM sites are underlined. Primers (Invitrogen) were annealed and ligated into pCFD#3 vectors, according to a standard protocol (Port, Chen, Lee, & Bullock, 2014) and information from http://www.crisprflydesign.org.

tkv-gRNA#1 sense: 5'–GTCGCCATCGCAGTAGCAGGTTA

tkv-gRNA#1 anti-sense: 5'–AAACTAACCTGCTACTGCGATGG

tkv-gRNA#2 sense: 5'–GTCGTGAGACGACCGGGATGTACG

tkv-gRNA#2 anti-sense: 5'–AAACCGTACATCCCGGTCGTCTCA

tkv-gRNA#3 sense: 5'–GTCGCTGTTTGTAGTCTCGTTTT

tkv-gRNA#3 anti-sense: 5'–AAACAAAACGAGACTACAAACAG

Generation of transgenic flies

P element transformation for the *nos*-FLPout construct was performed by Genetic Services (Sudbury, MA). The *tkv* gRNA transgenes, pCFD#3-tkv gRNA, were incorporated into the docking site attP2 by integrase-mediated site-specific integration.

Immunohistochemistry

Ovaries were dissected in PBS, fixed for 12 min in 4% paraformaldehyde solution (Electron Microscopy Services, Hatfield, PA) with PBTF as previously described (Lu et al., 2012). Ovaries were then washed 6 times with PBT, blocked 1hr in NBT, and stained with primary antibody in NBT at 4^{o}C overnight. After washing, ovaries were stained with secondary antibody at 4^{o}C overnight. Samples were washed again and were mounted in SlowFade antifade Gold reagent with DAPI (Invitrogen, Waltham, MA). Images were acquired using either a Zeiss LSM 510 or 880 confocal microscope, and were processed or analyzed by Image J. The following primary antisera were used: 1:3 mouse anti-Hts (DSHB 1B1 from the Developmental Studies Hybridoma Bank, Iowa City, IA), 1:3 rat anti-DEcad (DSHB DCAD2), 1:3 mouse anti-FasIII (DSHB 7G10), 1:100 rabbit anti-Vasa (Santa Cruz d-260 from Santa Cruz Biotechnology, Dallas, TX), 1:500 chicken anti-GFP (Abcam ab13907 from Abcam, Cambridge, UK), 1:100 rabbit anti-pMad

(Abcam ab52903), and 1:50 rabbit anti-phospho Histone H3 (Ser 10) (Millipore 06-570 from EMD Millipore, Billerica, MA) Secondary antibodies were obtained either from Jackson labs (Bar Harbor, ME) or Invitrogen and were used at a 1:500 dilution.

Temperature shift and heat shock protocols

For the temperature-shift experiments, flies heterozygous for a *tkv*, *put* or *sax* shRNA transgene and the *nos*-GAL4 transgene were grown at 22^{o}C. Newly-eclosed progeny were transferred to 28^{o}C and aged for 1, 3, 7 or 10 days. For the heat shock experiments, newly eclosed female flies heterozygous for the hsFLP, *nos*-FLPout and UASp-GFP transgenes, with or without a UASp-shRNA transgene, were collected, aged for 3-4 days, and subjected to different doses of heat shock using a water bath apparatus. Between 30 - 40 flies of a given genotype were heat shocked in the same vial at the same time. About 10 flies were dissected for analysis at each of the three time points – 3, 7, and 10 days post heat shock. For the long heat shock treatment, the two 1-hour heat shocks were separated by 8-12 hours.

Control experiments to tune the nos-FLPout system and test the effect of UASp-GFP on GSC maintenance

Adult female flies heterozygous for the hsFLP, *nos*-FLPout and UASp-GFP transgenes were exposed to 37^{o}C heat shock for increasing periods of time. A wild-type germarium has 2-4 GSCs (2.66±0.55, n=101); thus, heat shock produces germaria with 0-4 labeled GSCs. Heat-shocked females were dissected at 3 DPHS and the percentages of germaria with no labeled GSCs, some labeled GSCs, “mosaic germaria,” and germaria in which all GSCs were labeled, “fully-labeled germaria,” were determined. The time points that gave the maximal fraction of mosaic germaria with the least number of fully labeled germaria were used in future short heat

shock (SHS) experiments, while the time point that gave the maximal percentage of fully labeled germaria without deleterious effects on GSC survival was used for the long heat shock (LHS) experiments.

The fraction of labeled and unlabeled GSCs that were retained in mosaic germaria over time was determined by calculating the average number of labeled and unlabeled GSCs per germarium at 3, 7, and 10 days post SHS and dividing the 7 day and 10 day averages by the 3 day base line average.

Analysis of fraction of labeled GSCs retained in SHS vs. LHS germaria

Females heterozygous for hsFLP, *nos*-FLPout, UASp-GFP, and BMP receptor shRNAs transgenes were subjected to a SHS or a LHS. Although SHS generates a large percentage of mosaic germaria, some fully mutant germaria are generated. Because the biological condition of interest in the SHS experiments was a mosaic germarium that contained both wild-type and signaling-defective GSCs, fully mutant germaria were not included in the SHS analysis at any time point. Similarly, because the biological condition of interest in the LHS experiments was a fully mutant germarium, mosaic germaria were not included in the LHS analysis at any time point.

The ovaries of the females after heat shock treatment were dissected at 3, 7, and 10 days post heat shock (DPHS), and the total number of GSCs, and the number of GFP-labeled GSCs was determined for each germarium that met the above conditions. These data were used to calculate the average number of GFP-labeled GSCs per germarium. To determine the fraction

of the labeled GSCs retained after heat shock under each condition, the average number of GFP-labeled GSCs per germarium at 7 and 10 DPHS was divided by the average number of GFP-labeled GSCs at 3 DPHS. The difference in the fraction of GSC retained over time between mosaic and fully mutant germaria (after SHS and LHS, respectively) is an indication of the amount of competition that a BMP-signaling defective GSC is subject to from its wild-type neighboring GSCs. Two independent replicates of each experiment were performed.

Measurement of decay time of *tkv* shRNA GSCs in mosaic and fully mutant germaria

The decay time of *tkv* shRNA GSCs in the two independent replicates (Table II.1A and Figure II.3E) was determined via linear regression according to previously published methodology (Rhiner et al., 2009). The linear regression of y=ax+b (x, days; y, average number of labeled *tkv* shRNA GSCs per germarium) was calculated for three individual time points after heat shock, 3 days, 7 days, and 10 days for each replica. The decay time for each replica was calculated as the time to reach half of the y value at day 0. Linear regression on the first replicate (Table II.1A) gave rise to y=-0.0684x + 0.8192 for SHS (mosaic) germaria, and y = -0.0797x + 2.6649 for LHS (fully mutant) germaria, with decay times of 6.0 days and 16.7 days, respectively. Linear regression on the second replicate (Figure II.3E) gave rise to y = -0.0911x + 0.9805 for SHS germaria, and y = -0.0677x + 2.6314 for LHS germaria, with decay times of 7.2 days and 16.5 days, respectively.

Quantification of rate of egg laying

Flies heterozygous for the *nos*-GAL4 and *tkv* shRNA transgenes and control flies of genotype *nos*-GAL4 were raised at 22^{o}C. Newly eclosed flies of both genotypes were collected and incubated at 28^{o}C for the experimental time course. Three days after the temperature shift, 25

females and 20 w^{1118} males were placed in a cage. Quantification of egg laying was done daily between six and nine days post temperature shift to assure that all eggs laid were generated post temperature shift, and the average number of eggs per female per day was calculated for each genotype.

Image processing and comparative analyses for nuclear pMad and bam-GFP intensities

Both control flies and experimental flies in which BMP signaling was knocked down in the germline were aged and dissected in parallel. Control ovaries were pre-stained overnight with mouse anti-FasIII antibody, which strongly marks the somatic polar cells, washed and then combined with experimental shRNA ovaries in the same tube for further primary and secondary antibody staining. To increase the permeability of the nuclear membrane for pMad detection, the concentration of Triton X in the PBT solution was increased to 1%. Both control and experimental ovaries were mounted on the same slide, and imaged in a single confocal session with identical imaging parameters. Z-series images were obtained using a Zeiss 510 or a Zeiss 880 confocal microscope.

To measure nuclear pMad intensity, DAPI staining was used to create a mask for each nucleus. A series of images that covered the height of each GSC nucleus was flattened using the SUM projection tool in ImageJ, and the intensity of the anti-pMad staining was calculated within each GSC nuclear volume. The level of background staining was then calculated by performing the same measurement on a nearby non-Cb germ cell and was subtracted from the intensity in the GSC. The pMad intensities of individual shRNA GSCs were then normalized to the mean intensity of control GSCs within a given replicate. Multiple independent replicates for each

genotype were then combined to perform statistical analyses (unpaired Student's *t* test) using Prism.

Because *bam*-GFP is localized to both the nuclei and cytoplasm, *bam*-GFP intensities were calculated in a single section only within the GSC or Cb nucleus, which were marked by DAPI staining. Normalization and comparison were performed as above, with the exception that there was no background subtraction.

CHAPTER III: THE APPLICATION OF CRISPR TECHNOLOGY TO EXAMINE BMP RECEPTOR DISTRIBUTION AND TO INVESTIGATE WHETHER BMP SIGNALING IS REQUIRED FOR GSC MAINTENANCE

III.1. Abstract

In this chapter, I describe the use of the CRISPR/Cas9, the most popular technique for genomic editing, to investigate the roles of the BMP type I receptors, Tkv and Sax, during embryogenesis and oogenesis. I applied CRISPR/Cas9 technology both to create loss of function phenotypes in a tissue-specific manner and to insert a fluorescent tag at C-terminus of each protein. Transgenic flies carrying Tkv::EGFP and Sax::EGFP were constructed and validated. Interestingly, the distribution of each receptor has a distinct pattern within the germaria. While Sax predominantly accumulates at the membrane of germline cells, Tkv is more evenly distributed throughout the cells. In addition, high levels of Tkv are present at the membrane of somatic escort cells. I also used these constructs to examine receptor distribution during embryogenesis in an attempt to investigate the positive feedback regulation of BMP signaling in the specification of dorsal cell fates. Live imaging does not detect large alternations in the distribution of either type I receptor during the refinement of BMP signaling gradient. Finally, to investigate the role of Tkv and Sax in GSC maintenance, I constructed several gRNA transgenic lines targeting either Tkv and Sax. I successfully validated at least one line for each receptor by showing that the gRNA is efficient in eliminating Tkv or Sax accumulation. Using these gRNAs in the *nos*-FLPout system, I demonstrated that both Tkv and Sax are required for GSC maintenance.

III.2. Introduction

DNA sequences known as clustered regularly interspaced short palindromic repeats (CRISPR) coupled with the RNA-guided endonuclease Cas9 comprise the adaptive immune machinery in bacteria and archaea that guards against foreign nucleic acids (Wright et al., 2016). Recently, the CRISPR/Cas9 system has been modified to act as a binary system that allows precise genomic editing in a wide range of organisms. The binary components consist of a chimeric gRNA and the Cas9 protein, which are required for target recognition and endonuclease activity, respectively. Once a gRNA recognizes a complementary target sequence, Cas9 creates a double strand break at the defined sequence via RNA guided endonuclease activity. Cells can repair the resulting DNA lesion via nonhomologous end joining (NHEJ), which introduces small insertion or deletions, or homology-directed repair (HDR). If plasmid DNA, which has been concurrently introduced to the cell, contains DNA of the investigator's choosing flanked by sequences with homology to those on either side of the double strand break, HDR can precisely integrate this DNA into the cell's genome. NHEJ frequently results in frame-shift mutations or small deletions, while HDR is capable of incorporating an exogenous DNA fragment, such as a fluorescent protein tag or FRT sequences, at a desired position in the genome.

Among the many uses of the CRISPR/Cas9 methodology has been its use to create tissue specific gene knockouts in systems as diverse as *Drosophila*, zebrafish, and mouse. This protocol creates biallelic gene disruptions in the locus of interest with different degrees of mosaicism depending on the expression profile of the CRISPR/Cas9 and the degree of each gRNA efficiency (Ablain et al., 2015; Carroll et al., 2016; Port et al., 2014). In *Drosophila*, tissue-specific biallelic gene targeting has been successfully applied to generate loss of function phenotypes in genes that control body color and wing morphology, and has also been used to disrupt Wg signaling (Port et al., 2014).

In my thesis, I applied NHEJ to create tissue-specific loss of function mutations and HDR to EGFP tag the C-terminus of two BMP type I receptors, Tkv and Sax. These reagents were used to study the distribution of each type I receptor during oogenesis and embryogenesis and to investigate the roles of each receptor in the maintenance of female GSCs.

III.3. Results

III.3.1. Target selection of gRNAs to create transgenic gRNAs targeting Tkv and Sax

In the previous chapter, I characterized two different phenotypes when different BMP receptors were knocked down by shRNAs. While *tkv* shRNA germaria retained the majority of GSCs, *put* and *sax* shRNAs caused strong GSC loss. In order to determine whether *tkv* functions in GSC maintenance, similar to the other BMP receptors, I used the CRISPR/Cas9 technique to both tag the endogenous receptor and to create somatic, biallelic *tkv* mutations within female GSCs.

The type I receptors, Tkv and Sax share structural similarities with other TGF-beta receptors. Both type I receptors contain an N-terminal extracellular domain with a 6 Cysteine cluster required for ligand recognition, a single transmembrane domain, a juxtamembrane region with a Gly/Ser-enriched GS domain that is the target for type II receptor phosphorylation and an intracellular C-terminal Ser/ Thr kinase domain (Brummel et al., 1994; Huse et al., 1999; Twombly et al., 2009). The approach to select different sequences for gRNAs against the *tkv* gene was described in the previous chapter (and also see Figure III.1a). Similarly, several gRNA target sequences in *sax*, both in regions encoding the 6 Cys cluster and in the C-terminus UTR, were chosen to create loss of function mutations and to tag the C-terminus with EGFP, respectively (Figure III.1b). Together, two targets in *tkv* and three targets in *sax* were used to

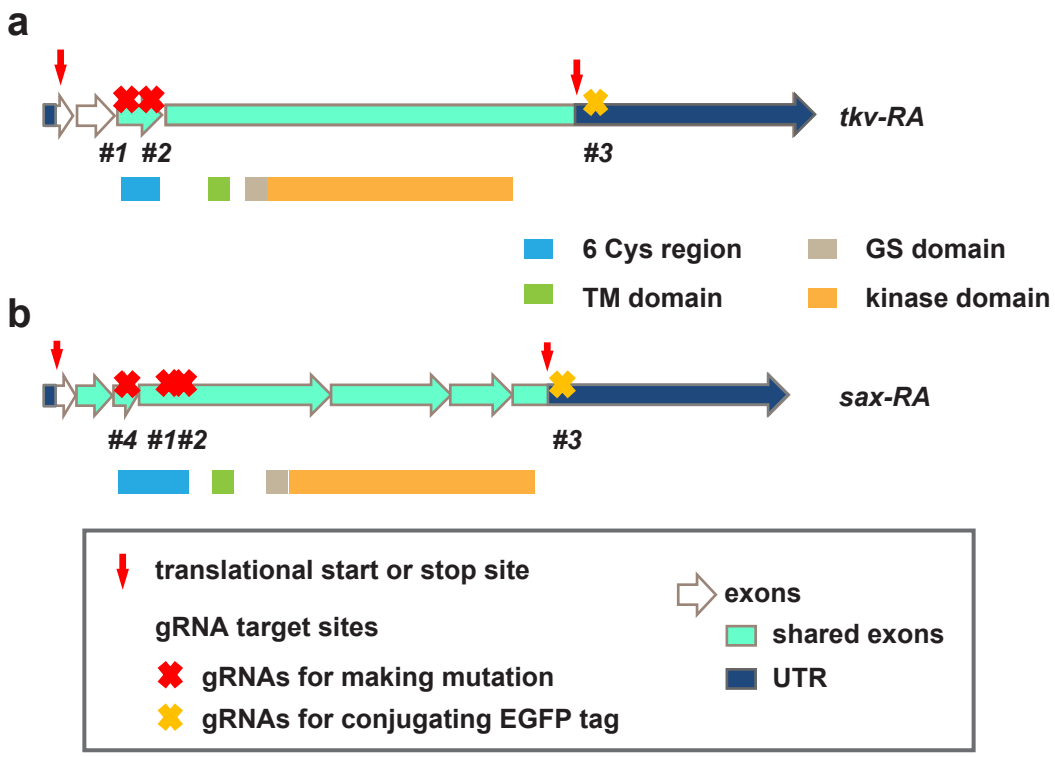


Figure III.1. Selection of gRNA target sequences in *tkv* and *sax*.
(a-b) RA transcripts of *tkv* (a) and *sax* (b), with the gRNA target sequences (cross icons in red or yellow, for making biallelic mutations or for creating a fluorescent tag, respectively) along each transcript. Each exon is represented by a horizontal arrow. The exons shared between all transcripts are mint green, while the untranslated regions are teal. The vertical red arrows demarcate either translation start or stop sites. The boxes underneath each transcript represent functional domains of the protein.

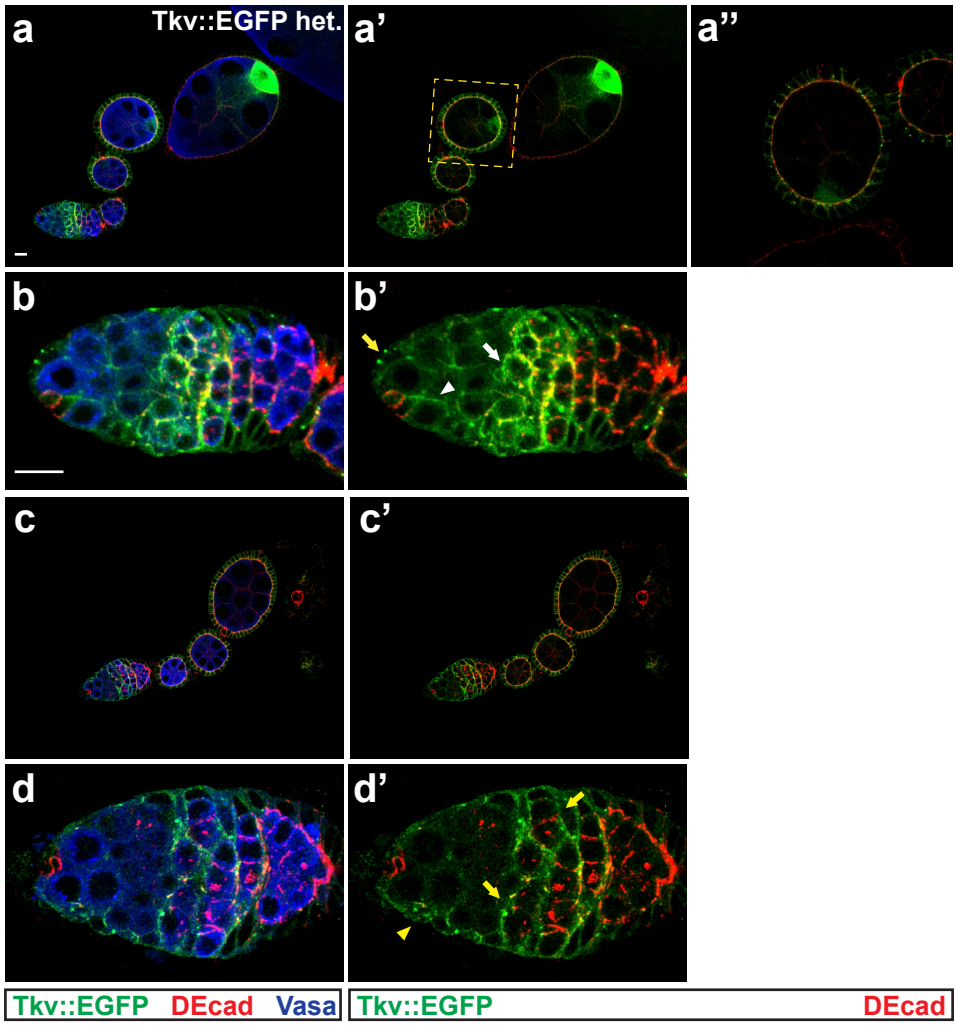


Figure III.2. Visualization of Tkv in Drosophila ovaries by C-terminal EGFP tagging. (a-b) Ovarioles from females heterozygous for Tkv::EGFP transgenes showing Tkv distribution throughout the ovariole (a, a'), in an early stage egg chamber (a'') and in a germarium (b). (a'') In the egg chamber, Tkv accumulates in both follicle cells and the oocyte, the latter is more evident in a later stage egg chamber (a). (b) Tkv is expressed throughout the germaria in both germline (labeled with Vasa) and somatic cells. Note the relatively strong outline of GSCs (white arrowhead) and of more differentiated cyst cells (white arrow), and also vesicle-like puncta in the somatic escort cells (yellow arrow). (c-d) Ovarioles from *nos*-GAL4 EGFP shRNA females heterozygous for Tkv::EGFP. (c, c') Expression of Tkv::EGFP in the germline is greatly downregulated, and the strong oocyte accumulation is eliminated. (d, d') In the germarium, eliminating Tkv::EGFP expression in the germline leaves strong accumulation of Tkv::EGFP in the somatic escort cells (yellow arrowhead), especially in their extended membranes that wrap around interconnected cysts (yellow arrows). All figure panels: anterior to left; scale bar, 10μm.

generate transgenic gRNA flies. To incorporate C-terminal EGFP tags, one target site in the 3'-UTR was selected each type I receptor.

III.3.2. The differential distributions of Tkv and Sax in the female ovary

Gbb and Dpp mediated BMP signaling have long been known to work synergistically for patterning in *Drosophila* wing imaginal disc, and it has been proposed that the relative ratio of Dpp to Gbb signaling, which is determined by activating of their corresponding type I receptors, Tkv and Sax, is essential for cell fate determination (Bangi and Wharton, 2006; Haerry et al., 1998). In the *Drosopihila* ovary, both Tkv-mediated Dpp and Sax-mediated Gbb signaling is required for GSC maintenance; however, Dpp, but not Gbb, signaling is sufficient to trigger ectopic GSC-like cells throughout the germaria (Song et al., 2004). This result suggests a certain degree of synergistic cooperation between Dpp and Gbb signaling, but also implies the possibility of functional or expressional divergence between Tkv and Sax within the ovary. To investigate this possibility, I incorporated DNA sequences encoding fluorescent proteins into the endogenous *tkv* and *sax* genes using CRISPR/Cas9.

The distribution of Tkv::EGFP in the ovary was roughly described in the previous chapter. Tkv and Sax proteins show similar distributions throughout an ovariole. Within developing egg chambers, Tkv has strong homogenous accumulation within the cytosol of the oocyte, and, in the follicle cells that surround the nurse cells and oocyte, accumulates at their apical and lateral membranes (Figure III. 2a-a"). Sax::EGFP has the same distribution as Tkv::EGFP in follicle cells (Figure III. 3a). However, unlike Tkv::EGFP, Sax::EGFP is mostly enriched at the membrane of the oocyte (Figure III. 3a"), with a relatively weak accumulation in the nurse cells.

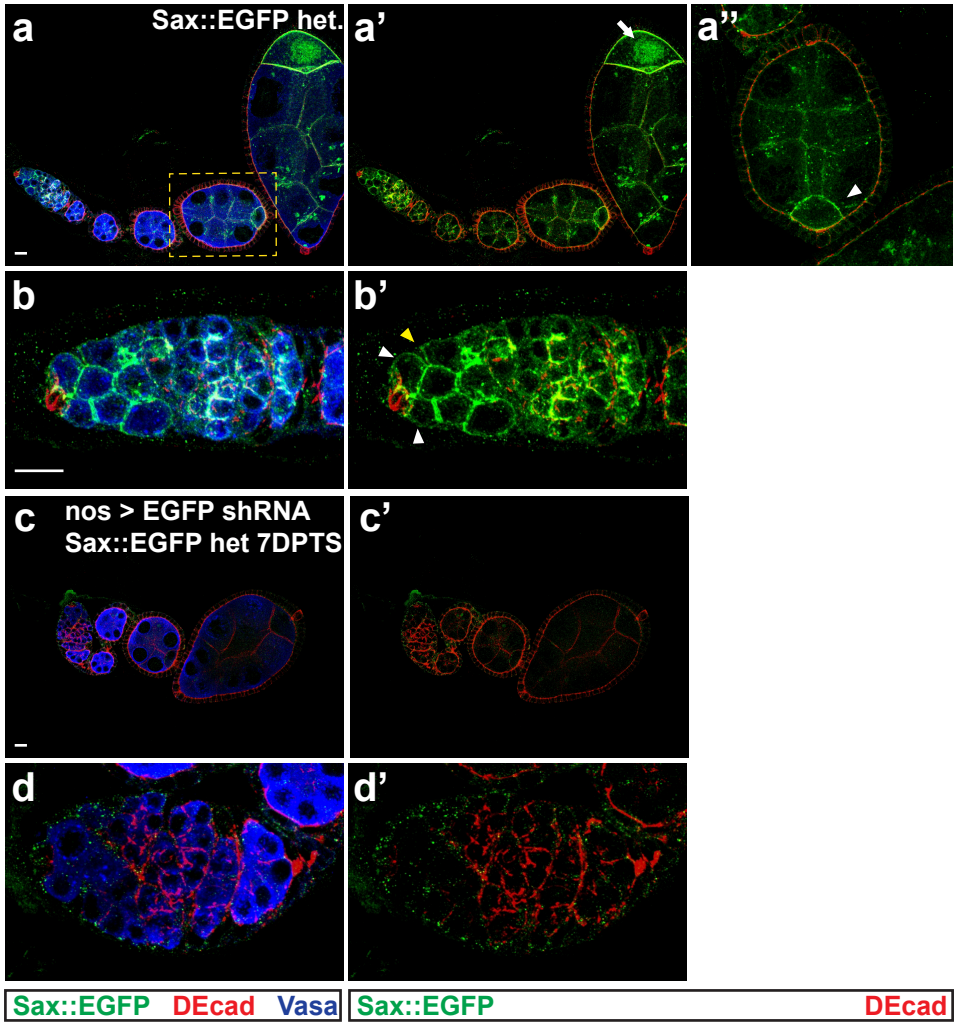


Figure III.3. Visualization of Sax in Drosophila ovaries by C-terminal EGFP tagging. (a-b) Ovarioles from females heterozygous for Sax::EGFP transgenes showing Sax distribution throughout the ovariole (a, a'), in an early stage of egg chamber (a'') and in a germarium (b). (a'') In the egg chamber, Sax accumulates in the membrane of oocyte (white arrowhead), while there is relatively weak accumulation in the membranes of the follicle cells and nurse cells. In later stage egg chambers, Sax::EGFP occasionally forms aggregates within the oocyte (white arrow in a'). (b) Sax::EGFP accumulates primarily at the membrane of germline cells (labeled with Vasa). Note that Sax::EGFP outlines the membrane of two GSCs (white arrows) and the lack of Sax::EGFP where the somatic escort cells are located (yellow arrowhead). (c-d) Ovarioles from nos-GAL4 EGFP shRNA females heterozygous for Sax::EGFP. There is almost no detectable EGFP in the overiole (c), and within the germarium (d), suggesting that Sax::EGFP accumulates in germline cells.

In later stage egg chambers, Sax::EGFP sometimes forms aggregates within the oocyte (Figure III. 3a').

In the germarium, close examination reveals differences between the distribution of these two type I receptors. Tkv::EGFP is present throughout the cortex of GSCs, their immediate progeny, and neighboring somatic cells. Interestingly, it is particularly enriched at the extended membranes of escort cells that wrap around the GSCs and their differentiated cyst cells (Figure III. 2b), demonstrated by knocking down Tkv::EGFP in the germ line lineage via shRNA against EGFP, whose efficiency was validated by observing elimination of oocyte accumulation of Tkv::EGFP (Figure III. 2c). In germaria lacking germline Tkv::EGFP accumulation, Tkv::EGFP still outlined the interconnected cyst cells, which is the expected pattern if Tkv::EGFP is present on the extended membrane of escort cells (compare Figure III. 2d to b).

Conversely, Sax::EGFP was mainly distributed at the membrane of GSCs and their decedents in the germaria (Figure III. 3b). Knocking down Sax::EGFP in the germ line lineage via EGFP shRNA resulted in germaria with no detectable Sax::EGFP, confirming that the primary distribution of Sax is within germ cells, and that Sax::EGFP may not be present in the somatic cells of the germarium (Figure III. 3c & d). Together, these data suggest that the two type I receptors have overlapping but distinct distribution patterns, which may provide some insights into their functional divergence in regulating GSC behavior.

III.3.3 Attempt to visualize positive feedback regulation by examining Sax and Tkv distribution during the refinement of BMP gradient in *Drosophila* embryos.

In pre-gastrula *Drosophila* embryos, BMP signaling is required for the formation of amnioserosa. Embryos lacking the BMP ligand Dpp are ventralized, while excess Dpp causes extra aminoserosa specification (Ferguson and Anderson, 1992; Sutherland et al., 2003). Visualization of BMP signaling by pMad staining shows that BMP signaling is dynamic through early embryogenesis. At the mid-blastoderm stage, the pMad domain is broad and has low intensity, while at the onset of gastrulation, the pMad domain has refined to become a sharp stripe with high intensity in dorsal nuclei that will differentiate as amnioserosa cells (Dorfman and Shilo, 2001; Ross et al., 2001).

Many efforts from a variety of labs have identified a general outline of the mechanism responsible for the refinement of the pMad domain. The extracellular proteins, Short gastrulation (Sog) and Twisted gastrulation (Tsg), and the metalloprotease Tolloid (Tld), have been shown to be involved in this process. Sog and Tsg together bind Dpp and allow it to diffuse within the perivitelline space. Dorsally-located Tld cleaves Sog when it is bound to Dpp, thereby releasing Dpp from the complex and permitting it to bind to its receptor. Thus, this extracellular system concentrates Dpp in the dorsal regions of the embryo. In addition, Scw, another BMP ligand, together with Dpp, synergistically activates the Tkv/ Sax heterodimer, boosting BMP signaling intensity (Neul and Ferguson, 1998; Shimmi et al., 2005; Wang and Ferguson, 2005). Lastly, a positive feedback circuit that enhances BMP receptor/ ligand interactions as a function of previous BMP signaling has been proposed to be an integral component of the refinement of the pMad stripe by the onset of gastrulation (Wang and Ferguson, 2005). Specifically, local injection of an activated version of the Tkv receptor recruits Dpp-GFP ligand to the site of injection, and decreases its binding to other regions. However, the mechanism of this positive feedback circuit is not known. In summary, the combination of dorsally directed facilitated Dpp diffusion, coupled

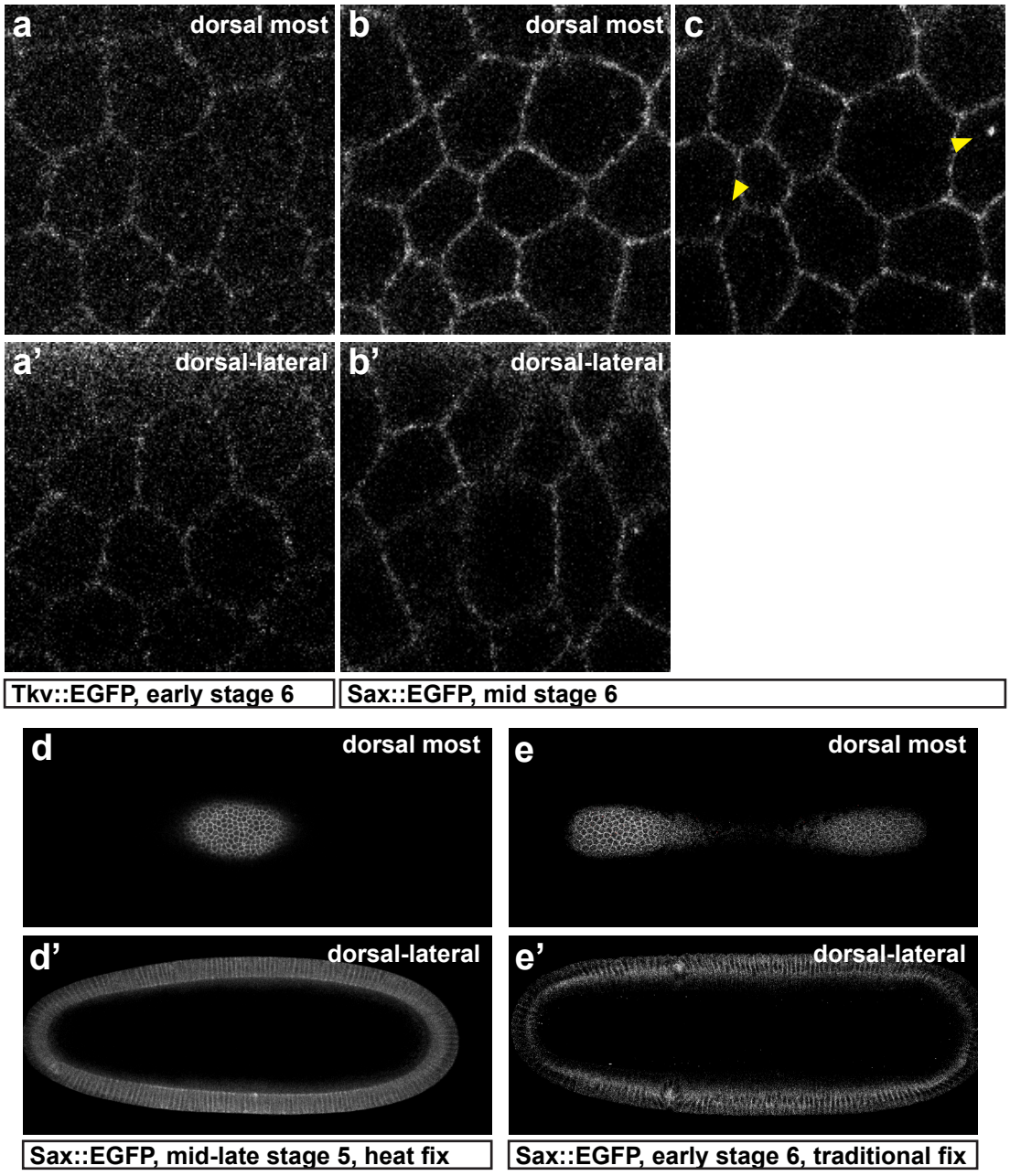


Figure III.4. Examination of of Tkv::EGFP and Sax::EGFP distribution during refinement of the BMP gradient in *Drosophila* embryos.
(a-c) Live imaging of maternal Tkv::EGFP (a) and Sax::EGFP (b & c) embryos at either early stage 6 or mid stage 6. (a) and (b) show optical sections of the dorsal-most tissues, while (a') and (b') show optical sections from the dorsal-lateral region. Yellow arrow heads in (c) indicate vesicle-like structures in the Sax::EGFP embryo.

Figure III.4. continued:
(d-e) Maternal Sax::EGFP embryos fixed with either heat fixation (d) or traditional (Heptane) fixation (e), and stained with anti-EGFP antibody. (d) and (e) show optical sections encompassing the dorsal most tissues, while (d') and (e') show optical sections encompassing the dorsal-lateral region. In all cases (a-e), there is no large change in the intensity or subcellular distribution of Tkv::EGFP and Sax::EGFP between dorsal most and dorsal-lateral regions.

with the positive feedback circuit, transforms a broad low-intensity pMad gradient, into a bistable high-intensity stripe of pMad in the dorsal most region of the embryo.

One possibility for the mechanism underlying the positive feedback circuit would be if previous BMP signaling could cause the cell surface localization of additional BMP receptors, potentially by recycling these receptors to the cell surface after bulk endocytosis. EGFP-tagged forms of Tkv and Sax might therefore by able to provide some insight into the feedback mechanism, because these reagents allow visualization of endogenous receptor distribution with high resolution in real time.

To examine the distribution of the endogenous Tkv and Sax receptors, I performed live imaging on stage 5 to stage 6 embryos, the time during which pMad refines to become a dorsal midline stripe (Dorfman and Shilo, 2001). Throughout the time window I examined, EGFP-tagged Tkv and Sax had similar expression patterns: each receptor was distributed uniformly around the circumference of the syncytial embryo. Frequently, I observed vesicle-like structures within the blastoderm (Figure III. 4c), presumably as a result of vesicle trafficking of the receptors. Due to the curvature and auto-fluorescence of the embryonic vitelline membrane, as well as the relatively weak endogenous expression of both EGFP-tagged type I receptors, it was hard to perform statistical analyses on either the intensity or quantity of these vesicle-like structures from the images I obtained, especially at the apical cortex of the embryo. However, for both Tkv and Sax, I did not observe large changes to either the pattern of membrane localized receptor or the pattern of internalized receptor, when I compared images from the dorsal-most and dorsal-lateral embryonic regions (compare Figure III. 4a to a’ and 4b to b’), suggesting the

feedback-mediated increase in BMP signaling may not be due to changes in spatial rearrangement or subcellular localization of either type I receptor.

As an alternative strategy, I performed either traditional embryo fixation or heat fixation, which preserves the membrane structure of embryos, followed by standard immunohistochemical staining in Sax::EGFP embryos. Although the curvature of embryos still impeded detailed comparisons of Sax::EGFP at apical surfaces of the dorsal-most and dorsal-lateral regions, my data indicated that Sax::EGFP seems to be distributed evenly in both regions during the formation of the pMad stripe (Figure III. 4d and e).

III.3.4. Validating different gRNAs and Cas9 transgenes

The loss of function phenotype of either *tkv* and *sax* in adult GSCs could be examined in flies carrying hsFLP, *nos*-FLPout, UAS-Cas9, and a specific gRNA transgene targeting either *tkv* or *sax*. As the efficiency of individual gRNAs in generating double strand break varies, I first performed experiments to validate each gRNA by using Cas9 transgenes that have restricted expression in either the germline, *nos*Cas9 and *vasa*Cas9, or the soma, *act*Cas9. It is worth noting that *vasa*Cas9 was reported to have strong somatic leakiness, while *act*Cas9 is maternally active in the germline and early embryo (Port et al., 2014). Flies carrying *nos*Cas9 with different gRNA transgenes were generated for the initial tests. BMP signaling is involved in primordial germ cell (PGC) establishment and maintenance in advance of PGC maturation into adult GSCs (Deshpande et al., 2014; Gilboa and Lehmann, 2004); thus, germ cell-less ovaries were expected when functional gRNAs against a BMP receptor were paired with a *nos*Cas9 transgene. Across assays, each gRNA resulted in a consistent phenotypic severity. While *tkv*

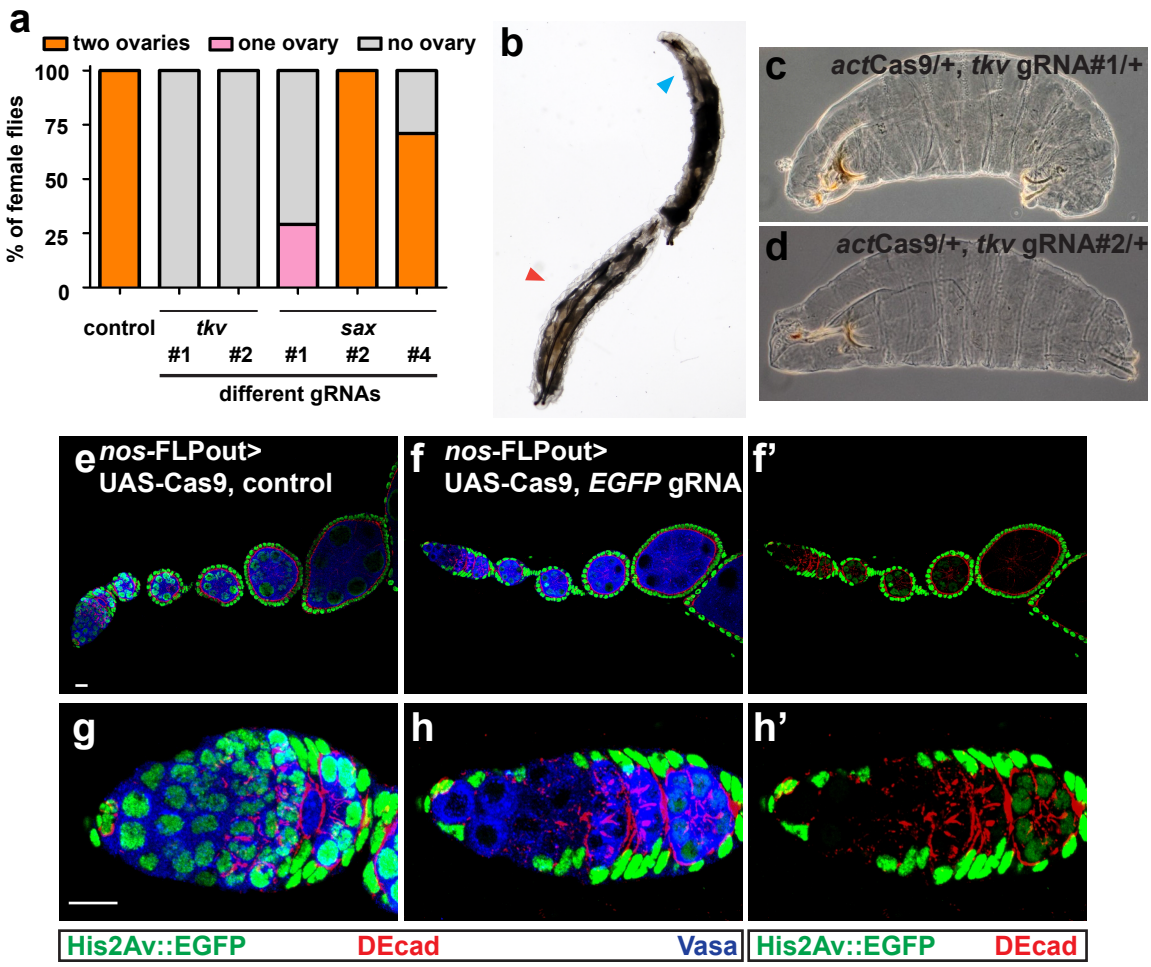


Figure III.5. Investigation of the efficiency and specificity of the *tkv* and *sax* gRNAs. (a) The percentage of female flies carrying *nos*Cas9 and an individual gRNA transgene targeting either *tkv* or *sax* with either no ovary, a single ovary, or the normal two ovaries. The different severities of the ovarian loss phenotype indicate the potential effectiveness in creating a double strand break at the target site (n=7-15). (b) L3 Larvae with *vasa*Cas9 and different *sax* gRNAs. *sax* gRNA #1 results in semi-transparent larvae (red arrowhead), while *sax* gRNA #2 (blue arrowhead) results in normal larval appearance (more than 10 larvae were examined for each construct). (c-d) Cuticle preparations of embryos carrying *act*Cas9 with either *tkv* gRNA#1 or #2. (c) *tkv* gRNA#1 results in a tail-up phenotype, resembling the phenotype caused by zygotic loss of a weak *tkv* allele, while the cuticle from a *tkv* gRNA#2 embryo is comparatively normal (d). (e-h) Ovarioles from flies carrying heterozygous *nos*-FLPout, histone2Av::EGFP, and UASt-Cas9 P2 with (f-h) or without (e-g) an EGFP gRNA 3 days post long heat shock. EGFP gRNA causes elimination of nuclear EGFP in the majority of germline cells in egg chambers (compare f to e) and in germaria (compare h to g). (20/24 EGFP gRNA bearing germaria contained no GFP labeled GSCs, compared with 0/16 from the control).

gRNA#1, *tkv* gRNA#2, and *sax* gRNA#1 resulted in significant ovary loss in more than half of the females, the great majority of *sax* gRNA #2 females had normal ovaries (Figure III. 5a), confirming that different gRNAs have different efficiencies in guiding the creation of double strand breaks in their target gene DNA.

Lack of Gbb signaling during larval development causes defects in fat body formation, thus resulting in larval transparency (Ballard et al., 2010; Sekelsky et al., 1995; Twombly et al., 2009). (In fact, Gbb stands for glass bottom boat, in honor of this phenotype). Larvae with *vasa*Cas9, which has the greatest amount of Cas9 leakiness in somatic tissues, and *sax* gRNA #1 displayed semi-transparency (Figure III. 5b), consistent with what has been reported for *sax* mutant larvae that are trans-heterozygous for a null allele and a deficiency of the locus (Twombly et al., 2009). Substitution of *act*Cas9 for *vasaCas9* in these crosses caused similar phenotypes (data not shown), thus confirming the specificity and efficiency of *sax* in generating loss of function phenotypes.

To validate the *tkv* gRNAs, I made cuticle preparation from embryos carrying *act*Cas9, and *tkv* gRNAs #1 or #2. I found out the majority of *tkv* gRNA#1 expressing embryos displayed a tail-up phenotype (Figure III. 5c), which is the zygotic phenotype of weak *tkv* allele, indicative of the involvement of *tkv* in embryonic dorsal closure (Affolter et al., 1994). However, no cuticles displayed a complete dorsal open phenotype, the zygotic null phenotype for *tkv*, possibly reflecting the inability of *act*Cas9 to cause null mutations in all embryonic cells, thus resulting in a mosaic *tkv* phenotype (Affolter et al., 1994; Penton et al., 1994). However, all *tkv* gRNA#1 embryos died, suggesting that *tkv* gRNA#1 was still very effective. Conversely, no abnormalities were observed in cuticles prepared from *tkv* gRNA#2 expressing embryos (Figure III. 5d), while

all flies carrying *tkv* gRNA#2 died during pupation, possibly reflecting its relatively weak efficiency, but also highlighting the central role *tkv* plays in elaboration of imaginal disc pattern, and the relatively small number of precursor cells that make up the primordia of each disc.

To successfully perform gRNA targeting within the germline, a functional UAS-Cas9 construct that is active in, and specific to, the adult germline lineages is required. Thus, I examined the Cas9 activity of two available UASt-Cas9 constructs, UASt-Cas9 P, which contains a *Drosophila* codon optimized Cas9, and UASt-Cas9 P2, which contains a human codon optimized Cas9. To maximize Cas9 activity within the germline lineage, I constructed transgenic flies carrying UASp-Cas9, which contains human codon-optimized Cas9 within the backbone of UASp expression vector that is optimized for germline expression. Strains of flies carrying the hsFLP, *nos*-FLPout, histone2Av::EGFP, functional EGFP gRNA, and various UAS-Cas9 constructs were generated to examine the efficiency and specificity of nuclear EGFP elimination within the adult germline lineage. Only UASt-Cas9 P2 resulted in loss of EGFP in germline lineage, inclusive of the GSCs, at high frequency, although a low degree of somatic leakiness was observed (Figure III. 5e-h). In contrast, UASt-Cas9 P1 displayed strong somatic leakiness (data not shown), and UASp-Cas9 was not sufficient to cause loss of EGFP, nor did it result in a decrease of nuclear pMad activity when paired with validated gRNAs targeting different BMP pathway components (data not shown).

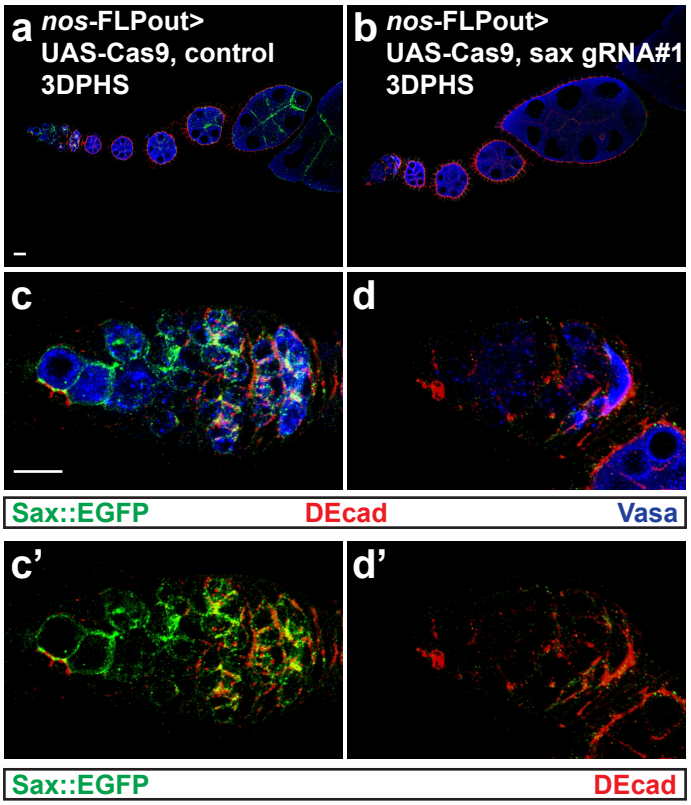
In conclusion, I successfully validated gRNA transgenes for *tkv* and *sax* targeting, and identified a functional UAS-Cas9 transgene that displays strong and restrictive germline activity. Validation of these reagents allowed me to determine how individual BMP components function to regulate adult GSC behaviors.

III.3.5. Targeting Sax by CRISPR technique results in GSC loss.

Data and methodology concerning the efficiency of *tkv* gRNA targeting in causing adult GSC loss were described in Chapter 2. *sax* gRNA targeting in adult GSCs was performed using the same methodology as *tkv* gRNA targeting. To confirm the efficiency and efficacy of *sax* gRNA transgenic lines in creating loss of function alleles within adult GSCs, I subjected Sax::EGFP heterozygous adult flies carrying hsFLP, *nos*-FLPout, UASt-Cas9 P2, and one of the validated *sax* gRNA transgenes to a long heat shock treatment. At three days post heat shock, more than half of the females examined had no Sax::EGFP staining both in their germaria and in developing egg chambers (Figure III. 6a-d), suggesting the *sax* gRNAs successfully eliminated Sax protein espression within the germline lineage. These data also confirmed the predominantly germline accumulation of Sax within the germarium, consistent with what I observed in germaria using EGFP shRNAs to knockdown Sax::EGFP in the germline lineage. To investigate whether Sax is required for GSC maintenance, I examined the average number of GSCs retained within each germarium (Figure III. 6e). Two different *sax* gRNA transgenic lines were assayed, and both resulted in germaria with extremely low number of GSCs present at 3 and 7 DPHS, indicative of the requirement for Sax in maintaining female GSCs. Together with the *tkv* gRNA data (Chapter 2), these data confirmed the initial report that used GSC mitotic clones to establish that both *tkv* and *sax* are required for GSC maintenance in the ovary (Xi et al., 1998).

III.4. Discussion

I have validated endogenously-tagged EGFP Tkv and Sax receptors constructed using CRISPR/ Cas9 mediated HDR. Examination of the distribution of both type I receptors within ovarioles revealed similar, but distinct, patterns of localization. For example, both Tkv and Sax



e

Average number of GSCs per germarium

	3DPHS	7DPHS
control	3.00	1.89
sax gRNA #1	0.53	0.41
sax gRNA #4	0.87	0.21

Figure III.6. *sax* gRNA targeting results in GSC loss.
(a-d) Ovarioles from flies heterozygous for *nos*-FLPout and UASt-Cas9 P2 with (b and d) or without (a and c) *sax* gRNA#1 three days post LHS treatment. *sax* gRNA#1 caused elimination of Sax::EGFP in the majority of germline cells within egg chambers (compare b and a) and germaria (compare d' and c') (15/29 and 15/18 for *sax* gRNA#1, and #4, respectively). Germaria lacking GSCs were observed in *sax* gRNA#1 expressing flies, as no Vasa stained germline cells were present near the niche (d), suggesting *sax* is required for GSC maintenance. (e) Average number of GSCs per germarium calculated from females carrying *nos*-FLPout and UASt-Cas9 P2 without (control) or with *sax* gRNA#1 or *sax* gRNA#4 at 3 days or 7 days post LHS treatment. Both *sax* gRNAs result in rapid GSC loss (n= 15, 15, 8 for 3DPHS and n= 29, 43, 46 for 7DPHS of *sax* gRNA#1, #4, and control).

are present in germline cells. However, in the germarium, Tkv is largely localized to the membrane protrusions of somatic escort cells that envelop and support GSCs and their descendant, while Sax is primarily distributed at the membranes of GSCs and differentiated germ cells.

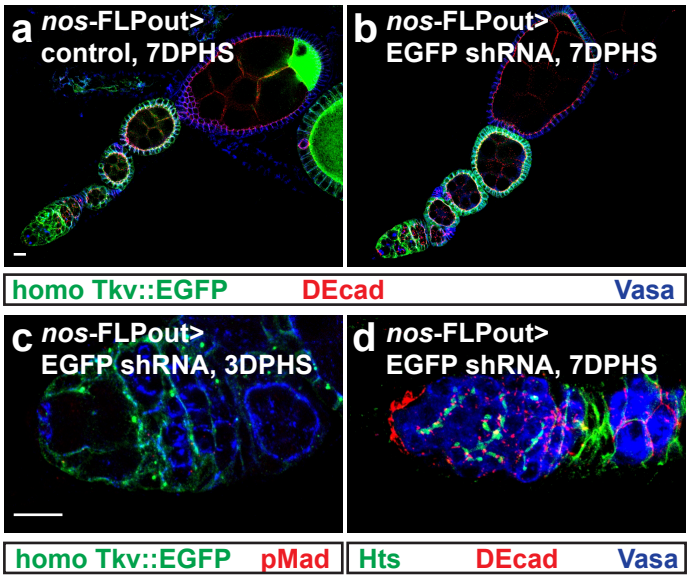
Their differential expression patterns in germaria suggests functional divergence of the type I receptors in regulating germ cell behavior. Consistent with this hypothesis, a recent study proposed Tkv functions as a sink for the Dpp ligand in escort cells to prevent BMP signaling from expanding throughout the germarium (Luo et al., 2015), which is in agreement with my result that there is a high level of Tkv accumulation in escort cells. Interestingly, in the somatic escort cells, some Tkv::EGFP accumulates in large vesicle like structures, while smaller vesicle like puncta were observed in GSCs using live imaging (data not shown). It will be interesting to examine whether these puncta result from the endocytosis of activated Tkv or whether there is any intrinsic difference between the functions of these vesicle-like structures. One approach to this question would be to examine the co-localization of Tkv puncta with the activated Tkv sensor (Michel et al., 2011). If the vesicular Tkv in the escort cells come from Tkv acting as a sink for sink for excess BMP ligands, it would be expected that the activated Tkv sensor would not localize to these vesicles, compared with expected localization of the sensor in neighboring GSC vesicles.

The results from examination of Tkv and Sax distribution in the blastoderm embryo do not lend support to a model in which positive feedback regulation of the interaction between BMP ligands and their receptors occurs by increasing cell-surface receptor concentration. Conversely, the data suggest that another mechanism facilitates increased ligand capture. However, there are

extensive caveats associated with this conclusion. First, the technical difficulties that interfered with conducting a direct comparison of high-resolution receptor distribution in dorsal most and dorso-lateral regions could have resulted in the failure to detect a difference in receptor distribution. To solve the problem of weak expression of fluorescent protein tagged type I receptor, one could increase the intensity of fluorescence by replacing EGFP monomer with a multimeric version. On the other hand, the curvature of the embryo could be overcome by taking thin images only within small regions of interest, and comparing the intensities of type I receptor::EGFP in different regions by using another ubiquitously expressed protein as a standard. For example, one could first take images at the dorsal-most region of embryos carrying maternal EGFP-tagged type I receptor and actin::RFP transgene, rotate the embryos to a lateral view, and take similar images for the dorsal-lateral region. The comparison of actin::RFP would serve to normalize image differences. It likely impossible to eliminate the autofluorescence from the vitelline membrane; thus, heat fixation followed by standard immunostaing may substitute for live imaging.

On the other hand, I was surprised to discover that localization of the receptor to putative intracellular, endocytic vesicles does not appear to be higher in the dorsal regions compared to the lateral regions, as it is known that TGF-β/Smad signaling takes place in the early endosome (Chen, 2009). Although it is possible that technical difficulties limited my ability to identify endocytosed receptors, which could be overcome with one of the approaches described above, these unexpected findings raise the question whether those vesicle-like structure represent endocytosed activated Tkv receptor or whether they label some other intracellular compartment. To test this idea, one could examine the colocalization of these vesicle-like structures visualized by EGFP-tagged type I receptors with markers that label various vesicular compartments, such as a rab5::RFP to label the early endosomes.

To examine the requirement of both type I receptors in adult GSC maintenance, I successfully constructed and validated at least two gRNA transgenes against each type I receptor that eliminate the accumulation of endogenous fluorescently tagged receptors in the germline. My results indicate targeting either *tkv* or *sax* is sufficient to cause the differentiation of GSCs at extremely high frequency, resulting in germaria lacking all germ cells over time. As an alternative approach to examine the role of *tkv* in adult GSCs, I used EGFP shRNA to knock down the endogenously-tagged Tkv::EGFP in the germline lineage. Female flies carrying the hsFLP, *nos*-FLPout, UASp-EGFP shRNA transgenes and homozygous for Tkv::EGFP were subjected a long heat shock treatment at adult stage. The ability of EGFP shRNA to knock down *tkv* activity was demonstrated by the lack of oocyte Tkv::EGFP accumulation at seven days post heat shock (Figure III. 7a and b). In addition, nuclear pMad was undetectable in the GSCs, confirming the efficiency of EGFP shRNA in knocking down BMP activity (Figure III. 7c). In these female flies, GSC were lost rapidly as, by ten days post heat shock, the average number of GSCs per germaria dropped to less than a quarter of the average at the initial time point (Figure III. 7d and e). Thus, my data confirmed that both *tkv* and *sax* are involved in two different aspects of GSC behavior, GSC competition and GSC maintenance.



e

Average number of GSCs per germarium

	3DPHS	7DPHS	10DPHS
control	1.67	1.24	1.26
EGFP shRNA #1	1.76	0.46	0.41
EGFP shRNA #4	2.09	0.54	0.19

Figure III.7. Confirmation that *tkv* is required for GSC maintenance by shRNA knock down of Tkv::EGFP in GSCs.

(a-d) Ovarioles from homozygous Tkv::EGFP flies carrying heterozygous *nos*-FLPout, with (b and c) or without (a) EGFP shRNA 7 days post LHS treatment. (b) The oocyte accumulation of Tkv::EGFP was eliminated in flies carrying EGFP shRNA (compare b to a), suggesting the efficiency of this shRNA transgene in knocking down Tkv::EGFP expression. (c) Germarium at 3 days post LHS showing strong membrane retention of Tkv::EGFP in somatic escort cells. (d) Germarium at 7 days post LHS treatment lacking GSCs and with a niche occupied by differentiating cyst cells characterized by branched fusomes. (e) Average number of GSCs per germarium of homozygous Tkv::EGFP females carrying *nos*-FLPout without (control) or with different EGFP shRNA transgenes. Both EGFP shRNA #1 and #4 caused strong GSC loss observed at 7 days post LHS (n= 103, 101, and 99 for EGFP shRNA , and n= 101, 100, 101 for control germaria at 3DPHS, 7DPHS, and 10DPHS).

CHAPTER IV: DISCUSSION AND FUTURE DIRECTIONS

IV.1. Proposed model for BMP signaling in regulating GSC maintenance and competition

In this thesis, I demonstrated that BMP signaling, which is required for GSC maintenance, is sufficient to induce GSC competition when a GSC with low BMP signaling is juxtaposed in the niche with a GSC with wild-type levels of signaling. Specifically, I provided evidence that signaling downstream of the type I receptors, Tkv and Sax, and the type II receptor, Put, can elicit either of the two distinct GSC behaviors, depending on the activity level of the pathway. Quantifying nuclear pMad intensity in BMP signaling defective GSCs in the presence or absence of competition, I showed that GSCs undergoing competition and destined for elimination can show higher BMP signaling than GSCs not undergoing competition and are retained at the niche. I thus proposed that there are two thresholds to BMP signaling and GSCs below each of the two thresholds face different consequences. Below a certain threshold of BMP signaling, GSCs will be eliminated from the niche, irrespective of the state of the neighboring GSCs. Conversely, if BMP signaling in a GSC is between the two thresholds, the GSC will be eliminated if other GSCs in the niche have wild-type levels of signaling, but the GSC will be retained if all GSCs have a similar level of signaling. Thus, my data is the first demonstration that niche maintenance signaling can also initiate stem cell competition.

However, while I was able to show that reduction in the activities of either *tkv* or *put* was sufficient to identity dual thresholds of BMP signaling activity, I was not able to formally demonstrate that reduction in the activity of the BMP signal transducer, *Mad*, displayed the same phenotypes. Specifically, my data suggest that both Tkv and Sax work through Mad, but not Smox, to regulate GSC maintenance, as loss of *Mad*, but not *smox*, activity caused defects in GSC maintenance. Unfortunately, due to the unavailability of the proper reagent, I was unable

to assay whether appropriately knocking down *Mad* activity results in a residual level of activity sufficient to trigger GSC competition, but one that allows for GSC maintenance. Thus, it remains formally possible that Mad is not involved in GSC competition, but only contributes to GSC maintenance. In this scenario, for any residual level of *Mad* activity, I would not expect to observe the accelerated loss of *Mad* shRNA GSCs in mosaic germaria, compared to the rate of loss in fully-labeled germaria. In other words, in mosaic germaria, *Mad* shRNA GSCs would be retained at the niche even in conditions in which the nuclear pMad level is lower than the competition threshold identified in *tkv* shRNA GSCs.

For this scenario to be plausible, one would have to postulate a second Smad-independent signaling pathway downstream of both receptors that is involved in competition. Reduction in BMP receptor activity would reduce the level of both pathways, which would be assayed as a reduction in nuclear pMad activity. However, it seems unlikely that Mad is not involved in GSC competition as Smad-independent signaling pathways downstream of the BMP receptors have not been identified, at least in flies. My data excluding *smox* from GSC maintenance further supports this idea. In addition, in the wing imaginal disc cells, both activated BMP signaling and enhanced endocytosis were sufficient to rescue the loss of loser cells, suggesting it is BMP signaling transduction, but not the receptors themselves, that play a role during cell competition.

Although it seems unlikely, one can still propose that other competitive regulators work downstream of these BMP receptors in a Smad independent manner. To formally exclude this possibility, one could perform competition assays with an appropriate *Mad* shRNA. An alternative strategy would be to use *dad* overexpression to perform the competition assay, as Dad acts as a repressor of Mad-mediated BMP signaling. However, this leaves open the

possibility that *dad* overexpression could block both putative pathways. A second possible approach would be to rescue BMP signaling in a receptor independent manner during *tkv* shRNA-mediated GSC competition. However, the only pathway activator available is a constitutive active form of Tkv; there are no constitutively-active forms of the Smad proteins, Mad and Medea. Thus, using currently available regents, it is not possible to perform receptor-independent activation of the BMP signaling pathway. On the whole though, I consider this formal possibility unlikely. The most likely scenario is that there is a linear, non-branched pathway of BMP signaling components, extending from the type I and type II receptors to the Smad proteins, whose differential output controls both GSC maintenance and GSC competition.

IV.2. Identifying mechanisms underlying BMP mediated GSC competition.

Although I showed that differential BMP signaling can trigger GSC competition, I was unable to determine how GSCs assess their relative levels of BMP signaling, or what are the underlying mechanisms by which GSC competition results in elimination of loser GSCs from the niche. I did demonstrate that the trigger for GSC competition is not differential transcription of the differentiation factor, *bam*. Furthermore, nuclear pMad intensity is not further decreased in *tkv* shRNA GSCs that will be outcompeted compared with those GSCs in fully mutant germaria that will be retained (data not shown). Thus, there is no feedback regulation of BMP signaling that is triggered by GSC competition. I also demonstrated that two other GSC differentiation factors, Bgcn and Sxl, which function in Bam-dependent and Bam-independent manners, respectively, are not involved in delaying differentiation of *tkv* shRNA GSCs in germaria where the absence of competition allows for retention of GSCs with very low levels of BMP signaling.

However, I did not formally test whether GSC competition could be triggered by the differential protein activities of Bam, Bgcn, or Sxl. Future experiments could focus on examining the activity of these differentiation promoting factors, as described in the discussion of Appendix II. In addition, I examined whether several regulators of competition identified in other systems could be involved in GSC competition. My data suggest that neither the Toll pathway nor Dp53 are involved in BMP-mediated GSC competition. Another potential candidate that I did examine is dMyc, as differential Myc activity has been shown in a variety of systems to initiate stem cell competition, including *Drosophila* GSCs (see Introduction), raising the possibility that dMyc may act as a central regulator of stem cell competition. I first tested whether dMyc activity is reduced in loser GSCs as a consequence of BMP-mediated GSC competition. However, due to the uncertainty of whether available anti-dMyc antisera faithfully captured dMyc distribution within germaria, my experiments were inconclusive. To resolve this issue, it would be necessary to obtain a better anti-dMyc antibody or to develop a fluorescently tagged dMyc. I then attempted to test whether increased dMyc levels could suppress cell competition within mosaic germaria carrying *tkv* shRNA GSCs; however, the transgene I used to overexpress dMyc caused cell lethality, making the experiment impractical to perform. In conclusion, a closer examination of whether dMyc levels are altered as a result of BMP mediated GSC competition would provide insights into whether Myc proteins work as central regulators of cell competition in different stem cell systems.

I have demonstrated that differential BMP signaling is sufficient to initiate GSC competition within the *Drosophila* ovary. So far, the majority of studies on cell competition have focused on genes that are downregulated within loser cells; however, much less is known about how neighboring cells respond when they acquire winner cell status. In this section, I will propose experiments to identify genes that when downregulated in wild-type GSCs could block the

elimination of neighboring loser GSCs that have lower levels of BMP signaling. Such genes may work in at least three different ways. First, they may be involved in the mechanism that GSCs use to communicate their relative level of BMP signaling. This mechanism is completely unknown. Second, these genes could function in wild-type GSCs to promote the elimination of the loser BMP signaling impaired GSCs, *e.g.,* by potentially sending a differentiation signal to the loser GSCs. Third, and potentially the broadest class of genes, loss of gene function could "equalize" the fitness of the otherwise wild-type GSCs and the BMP-signaling impaired GSCs, so that competition does not occur.

It is impractical to identify such genes through random mutagenesis. However, I will describe an shRNA screening system that could be used on a large number of candidate genes to test their involvement in the processes described above. The system is designed to express one of the following two classes of genes in each adult GSC; either *tkv* shRNA or an shRNA against a candidate gene. I propose to adopt a construct, iMOS (Clavería et al., 2013), that was first used to differentially express Myc in mouse epiblast cells. This construct has two variants of loxP sequences, lox2272 and lox5171, each of which is capable of recombining with its homotypic partner, but not the heterotypic lox sequence. The resulting transgene would be as follows: *nos*- lox2272- lox5171- stop- lox2272- *tkv* shRNA- stop- lox5171- GAL4. In this case, loxP mediated recombination in a GSC would result in the GSC and its descendants expressing either *tkv* shRNA or GAL4. GSCs with Gal4 would be marked by GFP using a UASp-GFP construct and would also express shRNA against a candidate gene. To express the CRE recombinase that acts upon the lox sites, an inducible adult germline-expressing Cre construct, such a *nos*- FRT- stop- FRT- Cre transgene coupled with a hs-FLP construct, could be used.

Flies carrying these constructs will be subjected to a long heat shock treatment to activate Cre in every GSC. Assuming Cre is uniformly functional, then each GSC would express either *tkv* shRNA or GAL4, hopefully with equal probability. Thus, assuming that there are 2 GSCs in each germarium, one-half of the germaria would be mosaic for GSC genotype, *i.e.*, one would express *tkv* shRNA and one would express GAL4 driven GFP and an shRNA. In the other half of germaria, all GSCs would have the same genotype, and these germaria would not be useful. In mosaic germaria, the *tkv* shRNAs would be subject to competition. If the candidate shRNA had no effect, then over time there should be no mosaic ovarioles: 75% of the ovarioles should have all labeled germ cells (25% having both GSCs initially labeled, and 50% having lost the outcompeted non-labeled GSC), while 25% of the ovarioles should have all labeled germ cells. However, if expression of the candidate shRNA in a wild-type GSC eliminates its ability to outcompete a BMP signaling impaired GSC, then 50% of the ovarioles will remain mosaic. Thus, immediately after heat shock, a few ovarioles will be examined to verify the degree of mosaicism. The remaining flies will be aged for an appropriate time that will allow the loss an outcompeted GSC and all of its differentiated progeny egg chambers, which is around 10 days. If GSC competition is eliminated, egg chambers will continued to be produced by both classes of GSCs, resulting in mosaicism throughout the ovariole. Screening ovarioles for continued mosaicism would be a relatively quick process, facilitating screening of a large quantity of candidate genes. However, to confirm the involvement of any given candidate, a careful examination of continued mosaicism at the level of the GSC would be required.

One caveat of this experiment is the application of Cre-LoxP system in *Drosophila* may not be as efficient as observed in mammals. If this would be the case, I propose to replace the loxP containing transgene with another construct that is adopted from a system that allows multi-color labeling in *Drosophila* cells, the Raeppli construct (Kanca et al., 2013). In this scenario,

the construct would have the following elements: *nos*- FRT- stop- FRT- integrase- attP- *tkv* shRNA- stop- attP- Gal4. As both the flippase and integrase systems are widely used in *Drosophila*, I would expect this screening platform to be functional.

As mentioned above, the obtained hits will fall into three classes: genes that communicate differential BMP signaling between GSCs, genes that are expressed in winner GSCs to cause elimination of loser GSCs, or genes that when mutated reduce the fitness of the otherwise wild-type GSCs to be equal to the BMP signaling impaired genes. To distinguish among these classes of genes, one could apply the *nos*-FLPout platform to examine whether mosaic germaria expressing only the shRNA against the candidate gene will result in GSC elimination. If the GSC is eliminated, I would interpret that candidate as belonging to the third class of genes, as loss of its function alone reduces GSC fitness and causes competition. To distinguish between members of the first two classes of genes, one could determine whether overexpression of the gene in one GSC promotes loss of the neighboring GSC. If so this gene is a candidate for being involved in the production of a “winner signal”.

IV.3. Functionality of stem cells competing for maintenance signals

My data suggest the maintenance signals serve as quality control machinery to ensure the retention of GSCs with higher BMP signaling. One of the central questions raised by this study is why stem cells would use the differential level of their maintenance signals as a measurement of cell fitness. I propose and discuss two different hypotheses. First, in an adverse environment, the overall level of a stem cell maintenance signal could be greatly reduced compared to an unstressed environment. Having the ability to compare the level of maintenance signaling across all stem cells could guard against rapid stem cell loss under such adverse conditions.

Second, normal fluctuations in the level of a maintenance signal could serve to initiate neutral competition among a group of stem cells, thus contributing to cell fate determination by defining winner and loser stem cells. Aged female flies have reduced BMP signaling within their GSCs. Although GSC activity, as measured by the rate of stem cell division, progressively declines over time, the number of GSCs remain stable during the aging process (Zhao et al., 2008). In addition, increasing BMP signaling in the GSCs was sufficient to rescue the decline in the rate of GSC division, indicating that the effect of aging on the rate of GSC divisions is reversible if the level of secreted maintenance signals is increased. Thus, it is possible that by establishing an extremely low threshold for stem cell maintenance, the organism is able to retain the majority of stem cells in the niche to mitigate the effect of adverse conditions, including aging, nutrient starvation, or other environmental perturbations.

Conversely, it was proposed that fluctuations of niche secreted maintenance signals could drive neutral competition by influencing the identity of the loser and winner stem cells (Stine and Matunis, 2013). In the *Drosophila* testis, an increase in the number of symmetric replacement divisions compensates for lost GSCs. For example, male flies suffering from nutrient deprivation displayed a reduced production in the level of the maintenance signal and an increase in the number of GSCs undergoing symmetric divisions, implying transient fluctuations of niche signals may regulate GSC daughter fate determination through stem cell competition for niche signals (Ballard et al., 2010; Sheng and Matunis, 2011). In addition, in multiple stem cell systems, heterogeneity of maintenance signals and cell competition regulators among populations of stem cells lead to differences in cell fate determination. For example, stochastic fluctuation in the expression of the pluripotent factor, Nanog, in mouse ESCs, was associated with distinct cell fates. Specifically, ESCs with low Nanog expression had less self-renewal potential and had the tendency to differentiate (Abranches et al., 2014; Torres-Padilla and Chambers, 2014). In

the mouse epiblast, cells show heterogenous Myc levels and the level of Myc correlates with the outcome of competition: cells with higher Myc level survive, while cells with lower Myc undergo apoptosis.

Both my data and studies from another lab have demonstrated that, even under optimal conditions, BMP signaling is variable between GSCs in the same niche (Zhao et al., 2008). My observations that differential BMP signaling is sufficient to initiate GSC competition during non-neutral competition assay, and the heterogeneity of BMP signaling among GSCs leads me to raise the question, and propose experiments to address, whether the differential level of BMP signaling under physiological conditions is capable of initiating GSC competition.

In order to investigate whether heterogeneity of BMP signaling is sufficient to initiate competition and whether lower BMP activity in a GSC is always associated with loser identity, a tool that enables the quantification of BMP activity in real time fashion is required. Thus, I propose to monitor the nuclear distribution of Mad by tagging endogenous Mad with EGFP using CRISPR/Cas9 and examining its nuclear to cytoplasmic ratio in flies carrying a histone::CFP transgene that labels the nucleus. To further enhance the visualization of Mad::EGFP, an EGFP multimer tag could be used. The distribution of Mad::EGFP would be examined in GSCs from *nos*-GAL4 expressing female flies with either *tkv*$^{[Q]}$ or *tkv* shRNA transgenes to determine the range of the ratio of nuclear to cytosolic Mad intensity that is compatible with continued niche occupancy. The first use of this construct would be to confirm the heterogeneity of BMP signaling that was observed as differences in nuclear pMad intensity among wild-type GSCs by examining the nuclear/cytoplasmic ratio of Mad::EGFP using live imaging. I would then test whether random fluctuations in the level of activated Mad among the GSCs are sufficient to

induce GSC competition. As the process of cell competition takes time, I propose to use a lineage tracing system instead of continuous time series imaging to capture the elimination of loser GSCs. Mad::EGFP expressing female flies carrying *nos*-FLPout platform with a UASp-RFP transgene to mark GSC clones would be treated with short heat shock to create mosaic germaria. The flies would be aged to allow for the maturation of RFP marker, and then lineage traced with live imaging up to 14 hours, which is the maximal time of live imaging that has been reported within germaria (Morris and Spradling, 2011). The experiment would be performed with three different time points with initial time point used to identify a germarium carrying an RFP marked GSC with extremely low nuclear Mad::EGFP, while neighboring GSCs have high nuclear Mad::EGFP intensity. The germarium would be incubated in vitro and would be imaged at additional two time points separated by several hours to record whether the marked GSC is eliminated from the niche over the observational window.

One could argue that the Mad::EGFP intensity that is used to define a GSC with low BMP activity for lineage tracing is based on its value at a certain time, but not its average intensity over time. It is possible that BMP signaling within the GSCs undergo dynamic changes, for example, due to cell cycle regulation. However, for technical reasons, a continuous time series experiment is not practical; it not only causes photobleaching, but also likely decreases the health of the germarium. Thus, I would propose to perform lineage tracing for a relatively large quantity of germaria simultaneously by using the multi-position software installed on the Zeiss confocal. If large number of GSCs with both high and low levels of pMad would be followed simultaneously, the increase in sample size would allow a statistical analysis of the likelihood that a GSC with high BMP signaling would be lost over time compared to a GSC with low signaling. If the level of the BMP fluctuates over time in a normal GSC, but a small percentage of GSCs have long periods of low BMP signals, they would likely be captured in this analysis.

If the loss of a GSC with low nuclear Mad activity is observed, one can further examine whether surrounding GSCs with higher BMP signaling undergo a symmetric replacement division. Although it would be extremely hard to capture such GSC divisions, this process could still be examined by using red fluorescent tagged-anillin (Kitazawa et al., 2014), which marks the contractile ring during cytokinesis, or beta-tubulin (Ayeni et al., 2014; Basto et al., 2008), which marks the mitotic spindles, to define the mitotic division plane during time lapse observations. If division plane is in parallel to niche-GSC interface, it would be the sign of a symmetric replacement division.

Although time consuming, the examination of heterogeneity of Mad::EGFP among GSCs and investigation of its role in GSC competition would be informative, perhaps providing a direct linkage between maintenance signals and stem cell competition under a physiological condition.

IV.4. Revisiting BMP signaling and cell competition in primordial cells

Although the function of most cell competition regulators is believed to rely on their abilities to promote cell proliferation and cell growth, this hypothesis has not been formally proven. A recent study from the Gibson group could help address this issue. They engineered a *dpp*$^{[FLP\text{-}off]}$ (*dpp*$^{[FO]}$) strain in which the endogenous *dpp* locus was replaced by *dpp*$^{[FO]}$, a conditional knockout construct that is inducible by flippase activity. The authors demonstrated that if the *dpp* gene is flipped out in imaginal discs at the L3 larval stage, there is loss of nuclear pMad and loss of expression of BMP downstream target genes; however, the cells in the disc continue to proliferate normally, suggesting BMP signaling is not required for cell proliferation in the later stage of larval development (Akiyama and Gibson, 2015; Harmansa et al., 2015). Using this knowledge, one could attempt to separate the distinct roles of BMP signaling in cell competition

and cell proliferation by examining whether differential BMP signaling between adjacent cells would be sufficient to trigger cell competition during the stage in the imaginal discs when BMP signaling is not required for cell proliferation.

One simple way to test this idea would be to knockout BMP signaling mosaically in imaginal discs at the L3 stage using strains similar to *dpp*$^{[FLP\text{-}off]}$, such as *tkv*$^{[FLP\text{-}off]}$ or *mad*$^{[FLP\text{-}off]}$, that have been treated with a short heat shock to create mosaic imaginal discs and a long heat shock to create fully mutant discs. One caveat to this general approach, however, is contained within the original publication. The authors cannot rule out the possibility that residual Dpp coming from a separate source, potentially from the peripheral region of the wing disc, could be sufficient to support cell proliferation in the entire imaginal disc (Akiyama and Gibson, 2015). If this is the case, strong cell proliferation defects would still be expected in *tkv*$^{[FLP\text{-}off]}$ or *mad*$^{[FLP\text{-}off]}$ fully mutant imaginal discs.

Nevertheless, the application of *tkv*$^{[FLP\text{-}off]}$ or *mad*$^{[FLP\text{-}off]}$ in studying the requirement of BMP signaling in cell proliferation and the capability of initiating cell competition would be informative. If differential BMP signaling is sufficient to initiate cell competition at a period of time during which BMP is not required for cell proliferation, this would suggest BMP mediated cell competition does not depend on its capability to also promote cell proliferation. As all the regulators of metabolic cell competition are involved in cell proliferation or growth control, this could change our current views of how metabolic cell competition is regulated.

IV.5. Similarities and differences in cell competition between primordial cells and stem cells

Cell competition has been mostly studied within primordial cells and stem cell systems. Major initiators of cell competition identified in the imaginal discs, such as *Minute* or *dMyc* mutations, have been also reported to trigger stem cell competition in the *Drosophila* midgut and ovary (Kolahgar et al., 2015; Rhiner et al., 2009), although the effect of cell competition in the midgut is not restricted to stem cells. In addition to BMP signaling, several cell survival signaling pathways have been reported to initiate competition in imaginal disc cells, including the JAK-STAT and Hpo pathways; however, none of these pathways trigger *Drosophila* female GSC competition, as clonal GSCs with elevated or reduced pathway activity do not have alterations in the rate of GSC retention at the niche over time (Decotto et al., 2005; Huang and Kalderon, 2014; López-Onieva et al., 2008; Shcherbata et al., 2007; Sun et al., 2008; Wang et al., 2008). My preliminary data using using the *nos*-FLPout system with various shRNAs that knocked down specific components in these different signaling pathways suggested a similar result (data not shown). Although the JAK-STAT pathway has been reported to regulate stem cell competition within the *Drosophila* midgut and testis (Issigonis et al., 2009; Kolahgar et al., 2015), cell competition within the intestine is not stem cell restricted as described above (Kolahgar et al., 2015), and the involvement of JAK-STAT signaling in competition among somatic cyst stem cells has been recently challenged (Amoyel et al., 2014; Amoyel et al., 2016). These contradictory observations raise the question whether there are common regulators of cell competition in these two different systems.

In addition, loser cells in each system are eliminated via different mechanisms from each tissue, as I described in the Introduction. Since stem cells are maintained and regulated mainly by the microenvironment of their niche, the control of stem cell competition is likely to be niche dependent. In addition, individualized niche maintenance signals and niche anchorage mechanisms regulate stem cells in each niche; thus it may be possible that there are no

common mechanisms that trigger competition among stem cells. It would thus be interesting to investigate the role of maintenance signals in initiating competition in different stem cell systems in the future.

CHAPTER V: MATERIALS AND METHODS

Drosophila strains and culture

Most fly stocks and culture conditions are described in Chapter II.5. Additional stocks are listed as follows: *smox*$^{[MB388]}$ (BL#44384), EGFP shRNA #1(BL#41551), EGFP shRNA#4(BL#41558), *nos*Cas9 (BL#54591), *vasa*Cas9 (BL#52669), *act*Cas9 (BL#54590), *smox* shRNA (BL#43138, and BL#41670), *dad* shRNA (BL#33759), *smurf* shRNA (BL#40905) were obtained from The Bloomington *Drosophila* stock center (Bloomington, IN). UASp-*dad* and UASp-*Mad* KD were gifts from Dr. Dahua Chen. Bgcn::GFP and p53 R-nls were gifts from Dr. Benjamin Bolival and Dr. John Abrams, respectively.

Target selection and primer design for CRISPR experiments

Target selection and primer design for CRISPR/Cas9 experiments for *sax* and *Mad* was similar to what was done to generate the Tkv::EGFP and *tkv* gRNA transgenes. To tag endogenous Sax protein with EGFP by homologous recombination, gRNA target site #3: (TAAATTACCGCTTACAGCTAAGG), which targets sequences at the 3'UTR region, was used. The PAM sites are underlined. For generating gRNA transgenes targeting *sax*, gRNA target site #1(GGGCCCAGTAAACGCAATACGGG), gRNA target site #2 (TACGGGGAAGTTCGTGAATGTGG), and gRNA target site #4 (ATCCCGACAGGGCGGCTCGCAGG) were used. For targeting *Mad,* gRNA target site #2 (GCTGGACGGACGATTACAGGTGG), gRNA target site #3 (TGGCGAGGAACGAGTACTGGCGG), and gRNA target site #4 (CAATTCGCCGTACGACAGCTTGG) were used.

Integrase-mediated site-specific integration was used for generating different gRNA transgenes with the selection of docking site, attP2, the same as described in Chapter II.5.

Primers for generating *sax*-gRNAs

sax-gRNA#1 sense: 5'– GTCGGGCCCAGTAAACGCAATAC

sax-gRNA#1 anti-sense: 5'– AAACGTATTGCGTTTACTGGGCC

sax-gRNA#2 sense: 5'– GTCGTACGGGGAAGTTCGTGAATG

sax-gRNA#2 anti-sense: 5'– AAACCATTCACGAACTTCCCCGTA

sax-gRNA#3 sense: 5'– GTCGTAAATTACCGCTTACAGCTA

sax-gRNA#3 anti-sense: 5'– AAACTAGCTGTAAGCGGTAATTTA

sax-gRNA#4 sense: 5'– GTCGATCCCGACAGGGCGGCTCGC

sax-gRNA#4 anti-sense: 5'– AAACGCGAGCCGCCCTGTCGGGAT

Primers for generating *Mad*-gRNAs

Mad-gRNA#2 sense: 5'– GTCGCTGGACGGACGATTACAGG

Mad-gRNA#2 anti-sense: 5'– –AAACCCTGTAATCGTCCGTCCAG

Mad-gRNA#3 sense: 5'– GTCGTGGCGAGGAACGAGTACTGG

Mad-gRNA#3 anti-sense: 5'– AAACCCAGTACTCGTTCCTCGCCA

Mad-gRNA#4 sense: 5'– GTCGCAATTCGCCGTACGACAGCT

Mad-gRNA#4 anti-sense: 5'– AAACAGCTGTCGTACGGCGAATTG

Immunohistochemistry

The detailed immunohistochemistry protocols for *Drosophila* ovaries were described in Chapter II.5. The immunohistochemistry protocols for *Drosophila* embryos were performed by either heat fixation or traditional Heptane fixation (Gavin-Smyth et al., 2013). Eggs were collected from an hour deposition of Tkv::EGFP or Sax::EGFP heterozygous females, and were then aged for two and half hours to obtain the correct embryonic stage. For heat fixation, embryos were dechorionated with bleach, and heat fixed for 30 seconds with boiling embryo wash solution (7% NaCl and 0.5% TritonX in PBS). Vitelline membranes were then removed from these embryos

using a needle. After post-fixation wash with a 3.7% formaldehyde/ PBT solution, embryos were washed and stained for the desired antibody using a standard protocol (Gavin-Smyth et al., 2013). Additional primary antisera for ovary staining that were used in this thesis includes: 1:3 mouse anti-Sxl (DSHB M114), 1:3 mouse anti-Dorsal (DSHB 7A4), 1:3 mouse anti-Cactus (DSHB 3H12). In addition, 1:500 rabbit anti-GFP (Abcam 6556), chicken anti-GFP (Abcam ab13907), or 1:500 rabbit anti-pMad (Abcam ab52903) were used for embryo staining.

Live imaging for *Drosophila* embryos

For examining the distribution of each BMP type I receptor during the refinement of the BMP signaling domain, eggs derived from homozygous Tkv::EGFP or Sax::EGFP females were collected and aged for 2 hours at 25^{O}C. The resulting embryos were dechorionated in bleach, washed, and submerged into halocarbon oil 700. The embryos that were undergoing cellularization were picked on a dissecting microscope and transferred to slides; multiple embryos were placed on the same slide mounted with halocarbon oil for live imaging. For embryos at the onset of gastrulation, thick Z-series sections (with a 1um interval thickness) were taken at the dorsal view with 40X oil lens from dorsal-most tissues to dorsal-lateral tissues.

Examination of gRNA efficiency

To test individual gRNA efficiency, female flies carrying Cas9 transgenes were crossed to males of individual gRNA lines. Crosses were set up at 25^{O}C and were aged until the desired stage. To examine the zygotic *tkv* weak allele phenotype, cuticle preparation of *act*Cas9 *tkv* gRNA embryos were performed by using a standard protocol (Decotto and Ferguson, 2001). Flies containing *act*Cas9 or *vasa*Cas9 with either *Mad* shRNA or *sax* shRNA were aged to late L3 larval stage to examine the larvae for transparency, which is characteristic of *gbb* mutations.

Female flies carrying *nos*Cas9 and individual gRNAs were aged until 3 to 10 days after eclosion to examine loss of germ cells in the ovary.

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APPENDIX I: IN GSCS, *MAD*, BUT NOT *SMOX*, IS INVOLVED IN BMP RECEPTOR MEDIATED SIGNALING

A I.1. Abstract

Drosophila has two R-Smad proteins that transduce BMP signaling. Although the role of Mad is well-characterized during embryogenesis, larval development, and oogenesis, little is known about Smox, nor whether these two R-Smads coordinate. In this appendix, inspired by a recent study detailing two distinct functions of BMP signaling within adult stem cells of the *Drosophila* intestine, I addressed whether different Smads are involved in BMP signaling to regulate two different GSC behaviors – maintenance and competition. Using mitotic clones and the CRISPR/Cas9 technique, my results suggest that Mad is the main, if not sole, *Drosophila* Smad whose activity is involved in GSC maintenance.

A I.2. Introduction

Activation of the TGF-β pathway is regulated by the phosphorylation of R-Smad proteins. In vertebrates, activation of the BMP2/4 pathway results in phosphorylation of the Smads 1, 5, and 8, while activation of Activin pathway results in phosphorylation of the Smads 2 and 3. Thus, versatility in TGF-β signaling is controlled by different type I receptors and their downstream R-Smad proteins. In *Drosophila*, there are two R-Smad proteins, Mad, and Smox. While Mad couples with Tkv and Sax for Dpp and Gbb signaling, it is not clear whether Smox can couple with either of these two type I receptors.

Smox/ dSmad2 was characterized as the *Drosophila* Smad 2/3 homolog (Brummel et al., 1999; Henderson and Andrew, 1998). Initially, Smox was found to work downstream of Baboon (Babo), another type I receptor, to regulate cell proliferation in larval imaginal discs (Brummel et

al., 1999). However, work in the *Drosophila* testis suggested *sax* functions upstream of *smox* in somatic cyst cells to restrict spermatogonial proliferation (Li et al., 2007). Interestingly, a more recent study in the regenerating *Drosophila* intestine showed Dpp signaling plays two distinct roles in response to infection. While Sax-mediated phosphorylation of Smox works in the early phase to induce Intestinal Stem Cell (ISC) proliferation, Tkv-mediated phosphorylation of Mad restores the quiescence status of the ISCs during the later phase of infection. It is the differential level of signaling downstream of Sax and Tkv that determines whether an ISC undergoes Smox or Mad mediated Dpp signaling at each phase during regeneration (Ayyaz et al., 2015). Knowing that two different sets of type I receptor/ Smad proteins work in coordinating opposite effects in ISC proliferation, I wondered whether female GSCs utilize similar machinery to regulate their two distinct behaviors, competition and maintenance. More specifically, I asked whether Smox functions downstream of Sax in regulating GSC maintenance.

In addition, my attempts to knockdown *Mad* by using currently available shRNA transgenic lines (Wang et al., 2011) failed to eliminate nuclear pMad in female adult GSCs, as nuclear pMad intensity was not completely eliminated 7 day post long heat shock treatment (data not shown), demonstrating the inefficiency of these RNA interference constructs. Although GSC clones homozygous for *Mad* null mutations were lost from the niche in mosaic germaria (Xie and Spradling, 1998), my finding that BMP components are involved in GSC competition raised the question: what is the mechanism underlying the loss of *Mad* mutant GSCs - induction of GSC competition or a maintenance defect? To address this question, I utilized the CRISPR/Cas9 technique to examine whether adult GSCs with biallelic targeted *Mad* mutations were maintained in the niche.

III.3. Results

To investigate whether Smox works downstream of Sax to control GSC maintenance, I first assayed whether GSCs lacking Smox activity are lost from the niche. Germline specific knockdown of *smox* was performed with two different shRNA transgenic lines, and both resulted in no detectable GSC loss at 7 days post temperature shift compared with the control (Figure AI.1a). In addition, I generated mitotic clones by using $smox^{[MB388]}$, a missense mutation that results in Glu to Lys mutation in MH2 domain and causes neuronal remodeling defects (Zheng et al., 2003). Although a slight decrease of retention of *smox* mutant GSCs was observed by calculating the percentages of mosaic germaria carrying both mutated *smox* GSCs, which were marked by lack of histone::RFP, and wild-type GSCs (Figure AI.1b) -- from 39% at 4DPHS to 27% at 14DPHS (n=41 and n=37, respectively), the rate of reduction is far less than that was observed in mutant GSC clones lacking *Mad* (Figure AI.1c) or *sax* (Xie and Spradling, 1998). Thus, I conclude that Smox is not required for GSC maintenance.

As described previously, none of the *Mad* shRNAs resulted in the reduction of nuclear pMad activity in GSCs (data not shown), indicating that they were not effective. Thus, I constructed gRNA transgenic flies that targeted endogenous *Mad*. Mad is an intracellular protein that is composed of an N-terminal MH1 domain, which is responsible for DNA binding, a C-terminal MH2 domain, which is responsible for transcriptional activation of downstream targets, and a SSXS motif at the extreme C-terminus, which is phosphorylated by the type I receptor, thereby activating the protein (Raftery and Sutherland, 1999). In order to examine the role of *Mad* in GSC maintenance, three target sequences were chosen that corresponded to regions either within the MH1 domain or in the linker region between the MH1 and MH2 domains (Figure AI.2a).

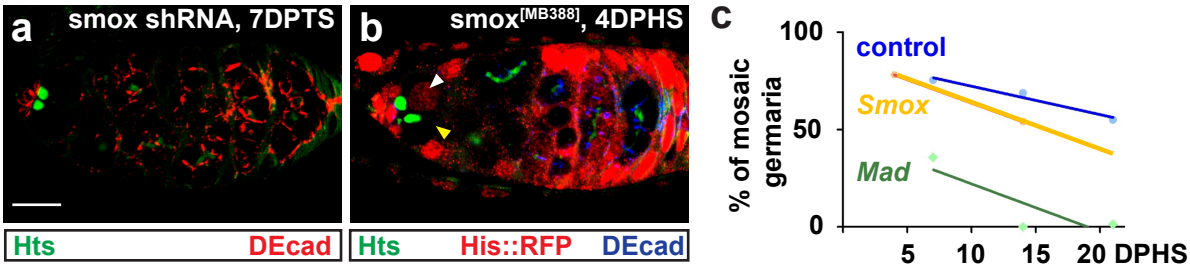


Figure AI.1. *smox* is not required for female GSC maintenance.
(a) Germarium from *nos*-GAL4 *smox* shRNA (GL10476) expressing female 7 days post temperature shift with two GSCs. Two different *smox* shRNA constructs were used, and the germaria are indistinguishable to those of control (n= 15 for each shRNA construct and for control) (b) Mosaic germarium with homozygous *Smox*$^{[MB388]}$ GSC (yellow arrowhead), which is marked by lack of nuclear histone::RFP, and wild-type GSC (white arrowhead), which has nuclear histone::RFP, at 4 days post heat shock (n= 41). (c) Percentage of mosaic germaria retained over time. Data of wild-type control and *Mad*$^{[12]}$ GSCs expressing mosaic germaria were adopted from Xie et al., 1998. The rate of loss of mosaic germaria carrying *Smox*$^{[MB388]}$ is not as strong as that observed in mosaic *Mad*$^{[12]}$ germaria.

To validate the obtained gRNA transgenes, I first crossed each gRNA line with *nos*Cas9, which expresses Cas9 in the germline. *Mad* gRNAs #2, #3 resulted in loss of ovaries with germ cells in more than half of the females, while the great majority of *Mad* gRNA #4 females had normal ovaries, indicating gRNAs #2 and #3 efficiently created double strand breaks in their target gene (Figure AI. 2b). As described before, larvae with mutations in the *gbb* signaling pathway are transparent (Ballard et al., 2010; Sekelsky et al., 1995; Twombly et al., 2009). Larvae heterozygous for *vasa*Cas9, or *act*Cas9, which has Cas9 activity in somatic tissues, and *Mad* gRNAs #2 or #3 transgenes, gave rise to fully transparent larvae (Figure AI. 2c and data not shown), in agreement with the previously described phenotype (Ballard et al., 2010). In conclusion, I validated two *Mad* gRNA transgenes for their ability to create biallelic mutations.

Following the same protocol I used to target type I receptors (see Chapter III), I then used the functional *Mad* gRNAs to target *Mad*. Specifically, I generated female flies carrying hsFLP, *nos*-FLPout, UASt-Cas9 P2, and an efficient *Mad* gRNA transgene, gRNA#2. At 4 DPHS, all females examined lacked GSCs in their niche (Figure AI.2e), suggesting rapid GSC loss, while all control flies with an inefficient *Mad* gRNA#4, had normal germaria (Figure A1.2d). To confirm the specificity of *Mad* targeting, I obtained the same GSC loss phenotype with a second *Mad* gRNA, gRNA#3 (data not shown). Based on these data, I conclude that *Mad*, but not *Smox*, is required for GSC maintenance, and thus Sax is likely to work upstream of Mad, given that the same phenotype was observed in both *Mad* and *sax* mutant germaria.

III.4. Discussion

In this appendix, I examined the roles of *Smox* and *Mad* in BMP signaling in female GSC maintenance. Specifically, I explored the hypothesis, which has since been discarded (see

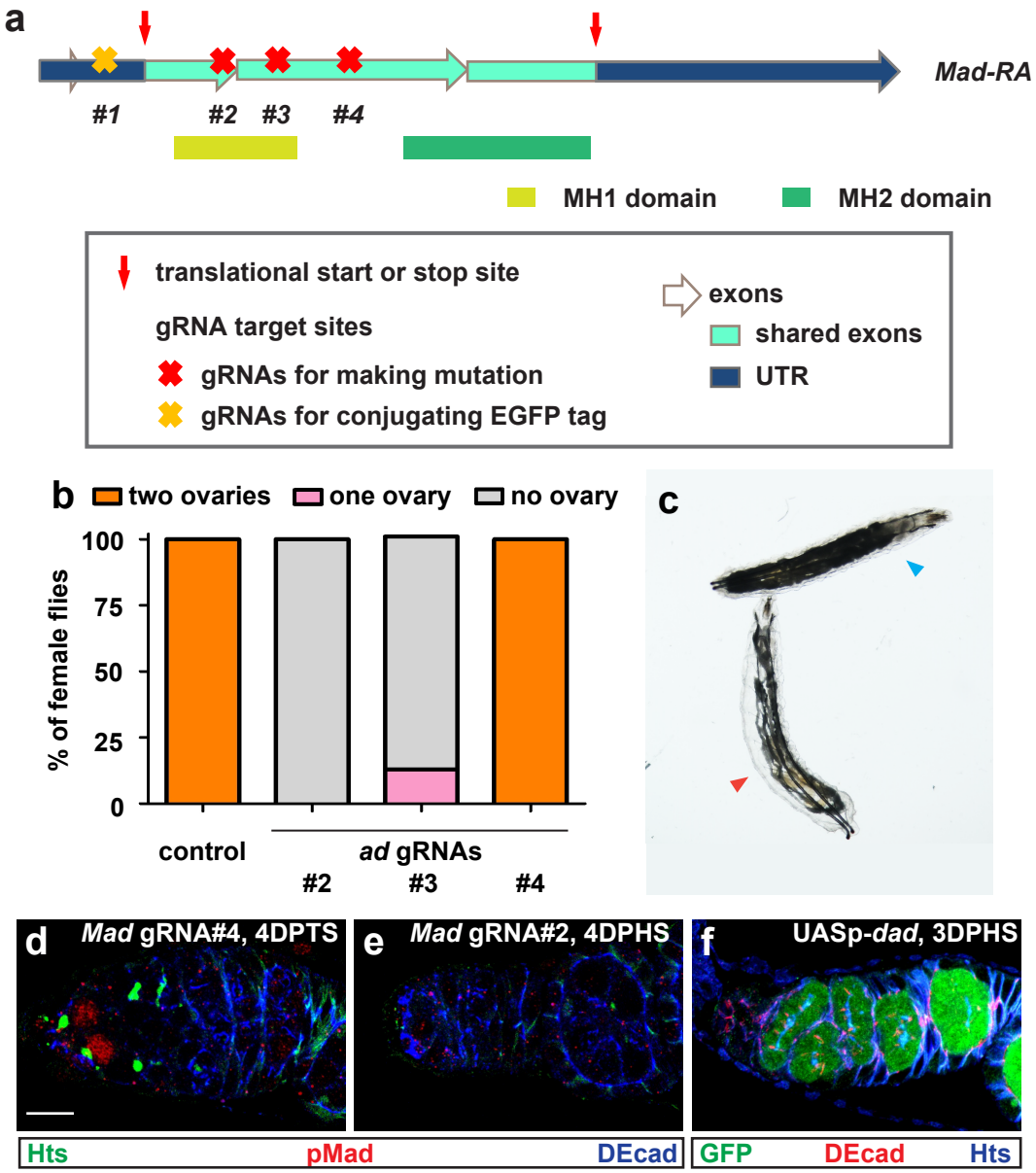


Figure AI.2. Mad-mediated BMP signaling is required for GSC maintenance in female ovary.
(a) *Mad* gRNA target sequences selection: diagram shows RA transcript of *Mad* and the locus of target sequences (cross icons in red or yellow for making biallelic mutations or for creating fluorescent tag, respectively) along the transcript. Each exon is represented by a horizontal arrow. The exons shared between all transcripts are mint green, while the untranslated regions are teal. The vertical red arrows demarcate either translation start or stop sites. The boxes underneath each transcript represent functional domains of the protein.

Figure AI.2. continued:
(b) The percentage of female flies carrying *nos*Cas9 and individual gRNA transgenes targeting *Mad* with either no ovaries, a single ovary, or two normal ovaries. The differing phenotypic severity indicates the potential effectiveness of the gRNA (n=15-16 for each gRNA construct). (c) L3 Larvae with *vasa*Cas9 and different *Mad* gRNAs. *Mad* gRNA #2 results in larvae transparency (red arrowhead), while *Mad* gRNA #4 (blue arrowhead) leads to normal larval appearance. *Mad* gRNA#3 gave rise to the same phenotype as *Mad* gRNA#2. More than 10 larvae were scored for each gRNA construct and the effective constructs displayed 100% penetrance. (d-e) Germaria from females heterozygous for *nos*-FLPout and UASt-Cas9 P2 with either *Mad* gRNA#2 (b) or *Mad* gRNA#4 (a) 4 days post LHS. While *Mad* gRNA#4 GSCs still retain high nuclear pMad activity (a, n=10), females with *Mad* gRNA#2 GSCs failed to maintain pMad positive germ cells at the niche. *Mad* gRNA#3 gave the same phenotype as *Mad* gRNA#2 (n=15 for both *Mad* gRNAs#2 and #3). (e) Germarium from female carrying *nos*-FLPout, UASp-GFP and UASp-*dad* transgenes, showing no GSCs at 3 days post LHS (33/105 had no GSCs at 3DPHS, and the severity of the phenotype increased to 86/102 at 7DPHS).

Chapter II), that Tkv works through Mad while Sax couples with Smox in transducing a BMP signal, and that Tkv/Mad signaling regulates GSC competition while Sax/Smox signaling regulates GSC maintenance. My data, however, suggested that both Tkv and Sax work through Mad, as lack of any of these proteins in the adult germline lineage results in strong GSC loss (compare data shown in this appendix to data in Chapters II and III). In addition, overexpressing *dad*, which plays a dual role as both a downstream target and a negative regulator of Mad mediated BMP singling, results in adult GSC loss (Figure AI.2f), confirming the linear relationship of these BMP components in regulating GSC maintenance.

However, these data do not absolutely demonstrate that GSC competition proceeds through a *Mad*-dependent pathway. Specifically, all available *Mad* shRNAs failed to work in adult GSCs, making it difficult to create a state in which a GSC has a partial loss of *Mad* activity, as was done for Tkv and Put (Chapter II). Thus, my results do not formally prove that *Mad* is involved in GSC competition. In addition, since my attempts to detect a Smox-Flag fusion protein in the germaria by antiserum against the Flag tag were not successful (data not shown), I am uncertain whether Smox is present in the germline, and whether Smox mediated signaling functions within female GSCs. One could still postulate that Smox works downstream of either type I receptor to control the initiation of GSC competition. However, my data argue against this possibility, as GSC clonal loss of *smox* does not result in a strong GSC loss over time. My current data thus support, but do not formally prove, a model in which Mad mediated BMP signaling is responsible for GSC competition. To confirm this model, future experiments would need to focus on developing *Mad* shRNA that will work in adult GSCs to significantly reduce BMP signaling.

APPENDIX II: OTHER ASPECTS OF BMP-MEDIATED GSC COMPETITION

AII.1. Abstract

Supercompetition, which was first identified in the *Drosophila* wing disc, refers to the observation that cells with extra copy of *dMyc* will outcompete wild-type cells. Since this observation, additional data have indicated that clonal activation of any of several survival pathways, including the Wg or JAK-STAT pathways, or elevation of the activity of the Hippo component Yki results in a similar phenotype, in that neighboring wild-type cells are eliminated via apoptosis. As my data, as well as that in previous studies, have indicated that in various contexts cells with compromised BMP signaling are eliminated by cell competition, I examined whether elevated BMP signaling in GSCs would be sufficient to initiate supercompetition. In addition, the mechanism underlying BMP- mediated GSC competition is not known. In this appendix, I also present data that test various hypotheses concerning this mechanism.

AII.2. Introduction

A previous study on GSC competition showed that a GSC with either extra copies of the *dMyc* gene will outcompete wild-type GSCs, while a GSC with less *dMyc* activity will be outcompeted by a wild-type GSC (Rhiner et al., 2009). The data in Chapter II have shown that a GSC with less BMP signaling is outcompeted by wild-type GSCs. Studies in the *Drosophila* wing imaginal disc showed that an increase in BMP signaling can suppress the loss of loser cells after competition (Moreno and Basler, 2004). However, whether an increase in BMP signaling can behave like *dMyc* to create supercompetitor cells is not clear. If BMP signaling is capable of creating supercompetitor cells, then clonally-elevated BMP signaling could be sufficient to trigger GSC competition against wild-type GSCs. In the first part of this appendix, I present three different strategies I used to potentially elevate BMP signaling within GSCs. I then

summarize my examination of GSC competitiveness in mosaic germaria carrying these genetic manipulations.

In the second half of the appendix, I will describe my attempts to study the molecular mechanisms underlying BMP mediated GSC competition. The question of how BMP mediated GSC competition is regulated could be assayed in two ways: what are the genes that facilitate the elimination of *tkv* shRNA expressing GSCs in mosaic germaria, and how differentiation of *tkv* shRNA GSCs is delayed in fully mutant germaria. As my data suggest that transcriptional control of *bam* is not involved in either process, I searched for other genes whose activities are known to play a role in germ cell differentiation (Perinthottathil and Kim, 2011). Benign gonial cell neoplasm (Bgcn) and Sex-lethal (Sxl) are known as differentiation promoting factors that work in a *bam*-dependent and *bam*-independent manner, respectively. Thus, I investigated whether either factor is involved in BMP mediated GSC competition.

On the other hand, knowledge of cell competition from somatic tissues or from other stem cell systems could provide insights in searching for potential regulators of GSC competition. As described in Chapter I, classical regulators that contribute to metabolic cell competition include proteins that regulate biogenesis, such as Minute proteins and dMyc, as well as various survival pathways, while structural cell competition involves several cell-polarity genes. In addition, a recent study determined that the innate immunity pathways can contribute to cell competition, depending on the cellular context. Specifically, the Toll pathway regulates canonical competition while the IMD pathway is required during supercompetition (Meyer et al., 2014). In mammalian hematopoietic stem cells, the stress mediator, p53 can play an essential role during competition

(Bondar and Medzhitov, 2010). In this appendix, I assayed the role both of the Toll pathway and of p53 in BMP mediated GSC competition.

AII.3. Results

AII.3.1. Examination of whether mosaic elevation of BMP signaling results in supercompetition.

I. *tkv*$^{[Q]}$ GSCs

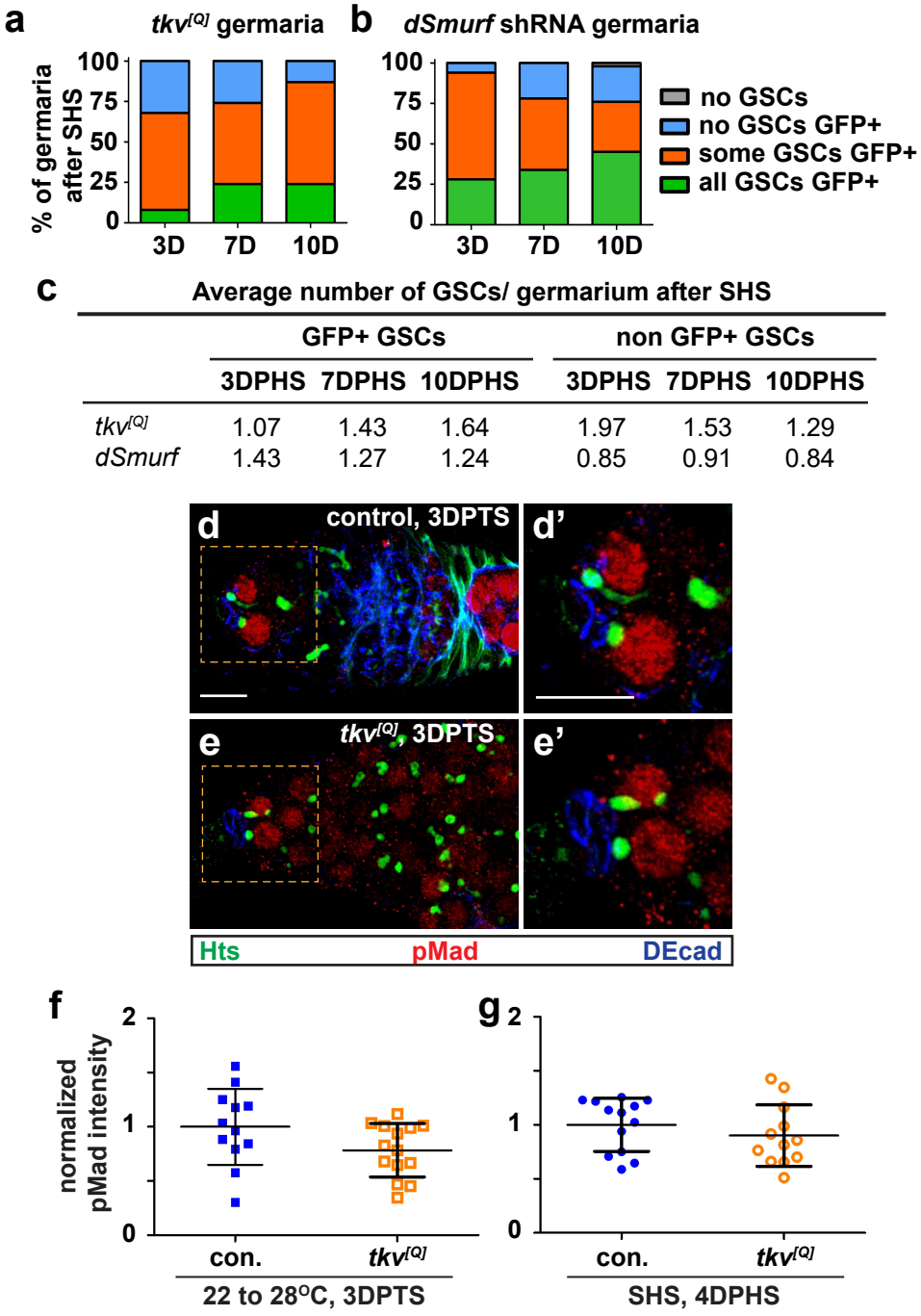
The assay to determine whether elevated BMP signaling in a GSC is sufficient to transform that GSC into a supercompetitor was performed using the same *nos*-FLPout platform previously described. Instead of using different shRNAs against BMP type I receptors, transgenic constructs that cell-autonomously elevated BMP signaling were used. Adult flies of the appropriate genotypes were subjected to a short heat shock treatment and the ability of GSCs with higher BMP signaling to outcompete their wild-type neighbors was scored in mosaic germaria. The constitutively active form of *tkv* (*tkv*$^{[Q]}$), which contains a single amino acid replacement of glutamine to aspartate in the juxtamembrane GS domain, thereby generating a phosphomimetic form of Tkv, is able to ectopically activate BMP signaling in *Drosophila* (Nellen et al., 1996). In the *Drosophila* ovary, germline expression of *tkv*$^{[Q]}$ is sufficient to block GSC differentiation, resulting in ectopic GSC-like cells with strong nuclear pMad staining (Casanueva and Ferguson, 2004).

In an attempt to elevate BMP signaling and to examine whether it can trigger GSC supercompetition, I applied a short heat shock treatment to flies carrying *nos*-FLPout and *tkv*$^{[Q]}$ transgenes to generate mosaic germaria carrying GFP-labeled, *tkv*$^{[Q]}$ expressing GSCs. The

resulting germaria, which were assayed as described in Chapter II at three time points post heat shock, were classified into three classes dependent on GFP expression -- no GSCs GFP+, some GSCs GFP+, or all GSCs GFP+. Note that in this experiment, although all *tkv*$^{[Q]}$ expressing germ cells have a GSC-like morphology, I defined a “GSC” using criteria identical to those I used in Chapter II, *i.e*., a germ cell adjacent to the niche with a round spectrosome closely apposed to cap cells.

In *tkv*$^{[Q]}$ expressing germaria, I observed that the class of all GSCs GFP+ germaria increased from 8% to 24% from 3DPHS to 10DPHS, and this increase is likely, but not certainly, attributed to the loss of wild-type GSCs in some GSCs GFP+ germaria. In most cases, germaria carrying no GFP labeled GSCs cannot become fully labeled over time (Figure AII.1a); however, a differentiating germ cell that expresses *tkv*$^{[Q]}$ can revert back to the GSC-like morphology. Thus, there is the possibility that a non-labeled GSC germarium could over time contain a labeled GSC. In addition, scoring the average number of labeled GSCs per germaria showed an increase in the average number of GFP labeled GSCs (from 1.07 at 3DPHS to 1.64 at 10 DPHS), at the expense of non GFP labeled wild-type GSCs (from 1.97 at 3DPHS to 1.29 at 10 DPHS), further raising the possibility of competition between *tkv*$^{[Q]}$ GSCs and wild-type GSCs (Figure AII.1c).

Interestingly, I did notice a decline of no GSCs GFP+ germaria over time, with a slight increase in the percentage of germaria with some GSCs GFP+ (Figure AII.1a). While the change seems subtle and I cannot rule out the possibility of normal sampling variability, the decline of the no GSCs GFP+ class of germaria was not observed in any previous experiments, either in experimental or control germaria. As described above, I hypothesize that the increase in



c

Average number of GSCs/ germarium after SHS

	GFP+ GSCs			non GFP+ GSCs		
	3DPHS	7DPHS	10DPHS	3DPHS	7DPHS	10DPHS
tkv[Q]	1.07	1.43	1.64	1.97	1.53	1.29
dSmurf	1.43	1.27	1.24	0.85	0.91	0.84

Figure AII.1. *tkv*[Q] or d*Smurf* shRNA GSCs do not gain a competitive advantage. (a-b) Flies carrying *nos*-FLPout, UASp-GFP, and either *tkv*[Q] or *dSmurf* shRNAs, were treated with short heat shock to create mosaic germaria. Percentage of different categories of germaria in flies expressing *tkv*[Q] (a) or *dSmurf* shRNA (b) at three different time points after short heat shock. (n=101, 100, 111 for *tkv*[Q] germaria. n=47, 64, 49 for *dSmurf* shRNA germaria).

Figure AII.1. continued
(c) Average number of GFP+ GSCs or non GFP+ GSCs per germarium. Over time, *tkv*$^{[Q]}$ expression results in an increase in the average number of GFP+ GSCs, and a decrease in the number of non GFP+ GSCs per germarium. (d-e) pMad staining in germaria from *nos*-GAL4 *tkv*$^{[Q]}$ expressing females (e) or control females (d). Ectopic nuclear pMad staining was observed throughout germaria in *tkv*$^{[Q]}$ germaria. (d' and e') Enlarged boxed regions in (d and e). (f) Nuclear pMad intensities of GSCs from *nos*-Gal4 *tkv*$^{[Q]}$ expressing females at 3 days post temperature shift (DPTS) from 22°C to 28°C (orange squares: 78.3 ± 26.1% of control, n=14) normalized to pMad intensities in control GSCs (blue squares: 100 ± 35.1, n=12). There is no significant difference between *tkv*$^{[Q]}$ and control GSCs (p=0.0772). Data are represented as mean ± s.d. (g) Nuclear pMad intensities of labeled GSCs in mosaic germaria from *nos*-FLPout *tkv*$^{[Q]}$ expressing females 4 days post SHS (orange circles: 90.0 ± 28.4% of control, n=12), normalized to pMad intensities in control GSCs (blue circles: 100 ± 24.6, n=13). There is no significant difference between *tkv*$^{[Q]}$ and control GSCs (p=0.3557). Data are represented as mean ± s.d.

germaria with some GSCs GFP+ may be due to the replacement of wild-type GSCs by *tkv*$^{[Q]}$ expressing germ cells. These *tkv*$^{[Q]}$ germ cells dedifferentiate, occupy the germaria, and could possibly push wild-type GSCs away from niche. In this scenario, the observed incremental loss of wild-type GSCs could be caused by a mechanical force, and not due to being outcompeted by *tkv*$^{[Q]}$ GSCs.

I then examined whether *tkv*$^{[Q]}$ did, in fact, elevate the level of BMP signaling in a GSC. Control germaria and germaria carrying fully mutant *tkv*$^{[Q]}$ GSCs were stained for pMad; however, there was no obvious difference of nuclear pMad level within GSCs in germaria of these two genotypes (Figure AII.1d and e). To examine BMP activity more carefully, I quantitated nuclear pMad intensity of *tkv*$^{[Q]}$ GSCs in two classes of experimental germaria, one of which had all *tkv*$^{[Q]}$ GSCs (Figure AII.1f), the other of which was mosaic for *tkv*$^{[Q]}$ GSCs (Figure AII.1g). In both classes of germaria, the normalized nuclear pMad intensity within *tkv*$^{[Q]}$ GSCs is not different from that of control GSCs. These data suggest that *tkv*$^{[Q]}$, contradictory to our expectations, does not elevate the level of BMP signaling within a GSC. It is likely that BMP signaling in the GSC is at a maximal level, possibly due to a limiting step downstream of receptor activation. Thus, the *tkv*$^{[Q]}$ reagent cannot be used to determine whether elevation of BMP signaling in a GSC can cause the GSC to act as a supercompetitor.

In addition, I also performed a similar experiment with an active form of *sax*, UASp-*sax**, (Pan et al., 2007). Females with germline expression of *sax** had normal germaria (data not shown), suggesting *sax** cannot ectopically activate BMP signaling in the germline. Because of this, no further GSC competition assays or quantification of nuclear pMad intensity were performed with the *sax** construct.

II. *dSmurf* or *dad* shRNA GSCs

I then sought other genetic means to increase BMP signaling in GSCs. dSmurf and Dad are negative regulators of BMP signaling. dSmurf is an ubiquitin E3 ligase. In both the wing imaginal disc and in the embryo, dSmurf antagonizes BMP activity by targeting pMad for degradation (Liang et al., 2003; Podos et al., 2001). In the *Drosophila* germaria, dSmurf was proposed to downregulate BMP signaling in order to promote Cb differentiation. Loss of *dSmurf* delays the differentiation of Cbs, causing additional GSC-like cells with high BMP signaling (Casanueva and Ferguson, 2004). Later research revealed a surprising role for dSmurf in the germarium: dSmurf forms a complex with Fu, a Serine/ Threonine kinase that physically interacts with the activated form of Tkv to ubiquitinate the activated form of Tkv in Cbs (Xia et al., 2010). On the other hand, in female GSCs lack of *dSmurf* increases the amount of cytoplasmic pMad (Lu et al., 2012). Thus, it is possible that lack of *dSmurf* could upregulate BMP signaling in the GSCs.

To knockdown *dSmurf*, I used the only available *dSmurf* shRNA construct, and I performed a competition assay, using a protocol identical to that for the $tkv^{[Q]}$ assay. After short heat shock, both control and *dSmurf* shRNA expressing flies showed an increase in the percentage of germaria containing all GSCs GFP+ from 28% at 3DPHS to 45% at 10DPHS, coupled with a reduction in the percentage of germaria with some GSCs GFP+ (Figure AII.1b and c). However, the average number of GFP labeled GSCs and non GFP labeled GSCs per germaria stayed relatively constant over time (Figure AII.1c), suggesting there is no gain of competitiveness in GSCs lacking *dSmurf* activity. However, there was no independent verification that the *dSmurf* shRNA was functional; thus, for now these conclusions must be considered tentative.

I also attempted to answer the same question using shRNA against *dad*, which encodes the *Drosophila* Inhibitory Smad, which binds to the type I receptors Tkv and Sax to prevent phosphorylation of Mad (Kamiya et al., 2008; Tabata et al., 1997). Germaria carrying *dad* shRNA constructs had a slight increase of number of germ cells with activated BMP signaling, suggesting that the *dad* shRNA construct was functional. However, I did not observe an increase in the percentage of germaria that contained all GSCs GFP+ at 7DPHS compared to 3DPHS (data not shown). Together, these results from *dSmurf* and *dad* shRNAs suggest there is no gain of competition within these genetically manipulated GSCs.

AII.3.2. Investigation of whether Bgcn or Sxl plays a role in BMP mediated GSC competition

So far, my data do not shed light on the mechanisms by which BMP-mediated GSC competition occurs. In Chapter 2, I showed that competition does not occur by elevating the level of *bam* transcription. In this section, I test whether two known differentiation-promoting factors could play a role in BMP-mediated GSC competition.

Bgcn physically interacts with Bam to promote Cb differentiation (Lavoie et al., 1999; Li et al., 2009; Ohlstein et al., 2000), thus serving as one potential candidate for a regulator of BMP mediated GSC competition. Moreover, *bgcn* activity is necessary for *bam* activity to promote GSC differentiation. I therefore examined whether *tkv* shRNA GSCs in fully mutant germaria have normal levels of Bgcn by using a Bgcn::GFP transgene (Li et al., 2009). However, no detectable difference of Bgcn intensity was observed between control and *tkv* shRNA GSCs at 10DPTS (Figure AII.2a and b), suggesting transcriptional control of *bgcn* does not play a role in delaying the differentiation of *tkv* shRNA GSCs in fully mutant germaria.

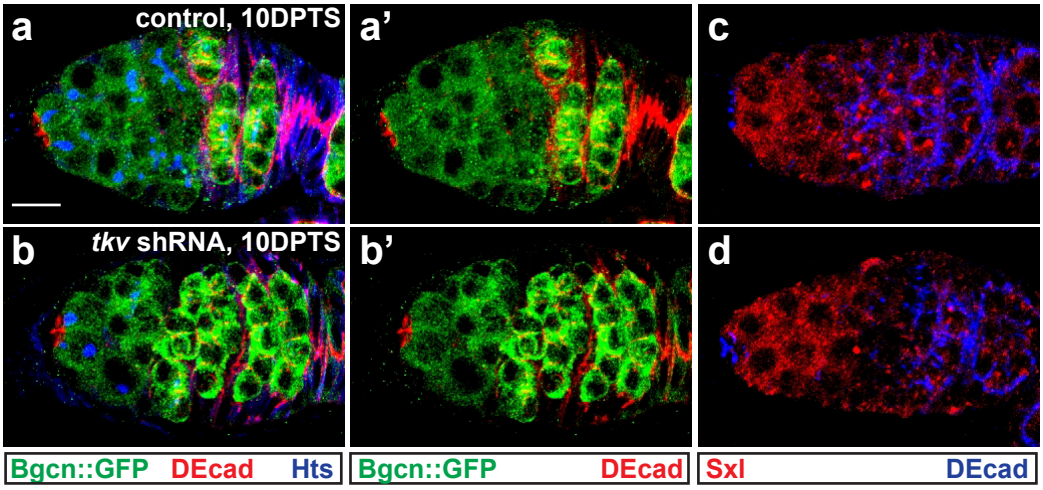


Figure AII.2 The distributions of Sxl and Bgcn proteins are not altered in mosaic tkv knockdown GSCs.
(a-d) Germaria from females carrying *nos*-GAL4 and Bgcn::GFP transgenes, with (b and d) or without (a and c) *tkv* shRNA 10 DPTS. (a and b) Bgcn::GFP is expressed in the germline cells throughout germaria in both control flies (a and a') and *tkv* shRNA expressing flies (b and b'). (c and d) Sxl is mostly enriched in GSCs and Cbs, while downregulated in more differentiated cysts in both control females (c) and *tkv* shRNA expressing females (d).

Another potential candidate for a gene involved in BMP mediated competition is *Sxl*. Sxl is a RNA binding protein that is necessary for female sex determination (Salz, 2012). Females lacking *Sxl* activity in their germline develop a tumor that misexpresses male-specific germline markers (Wei et al., 1994). Sxl is also known to promote GSC differentiation in a Bam-independent manner by translationally repressing Nanos (Chau et al., 2009; Chau et al., 2012). However, the pattern of Sxl detected by anti-Sxl antiserum was similar in *tkv* shRNA GSCs within fully mutant germaria and in control GSCs at 10DPTS (Figure AII.2c and d). Based on the results from Bgcn and Sxl protein distribution, I conclude that there is no evidence suggesting either differentiation-promoting factor is involved in delaying the differentiation of *tkv* shRNA expressing GSCs in fully mutant germaria. However, whether these two proteins are involved in other aspects of BMP-mediated GSC competition, for example, to facilitate the loss of *tkv* mutant GSCs in mosaic germaria, is still uncertain.

AII.3.3. Examination of the role of other factors involved in cell competition in BMP mediated GSC competition

As my attempts to determine whether other genes involved in GSC differentiation could play a role in BMP mediated GSC competition were not successful, I therefore investigated the contributions of two other pathways/genes, Toll signaling and p53, that are known to be involved in cell competition in other tissues.

Maternally-supplied components of the Toll signaling pathway specify the embryonic dorsal/ ventral axis of the *Drosophila* embryo. The output of the pathway is a nuclear gradient of the NF-κB transcription factor homolog, Dorsal, along the ventral to dorsal axis. The pathway is activated by localized ventral activation of Toll receptor by ligand binding. Toll signaling results

in the degradation of I-κB inhibitor protein, Cactus, (Reach et al., 1996) and phosphorylation of Dorsal (Drier et al., 1999). Once Dorsal is released from Cactus, Dorsal enters the nucleus and controls the transcription of downstream target genes. As described earlier, Toll signaling was shown to play a role in canonical competition in the *Drosophila* imaginal disc. To investigate whether the Toll pathway is activated during BMP mediated GSC competition, I examined whether nuclear translocation of Dorsal could be detected within germaria carrying GSCs undergoing BMP mediated competition. BMP-mediated GSC competition assays were performed as described in Chapter 2 by creating mosaic germaria containing both *tkv* shRNA GSCs and wild-type GSCs. These germaria were immunostained with antiserum against Dorsal; however, I did not observe any cytoplasmic or nuclear accumulation of Dorsal protein (Figure AII.3a). In addition, germaria stained with anti-Cactus antibodies revealed that Cactus was present in the muscle sheath that surrounds the germarium, but not within germ cells (Figure AII.3b). These data together suggest that the Toll pathway is not activated during BMP mediated GSC competition.

As described above, mammalian p53 has been shown to be involved in stem cell competition. *Drosophila* P53 (Dp53), on the other hand, is expressed within the GSCs and Cbs of germaria exposed to stress as a protective machinery to fight against genome instability (Wylie et al., 2014). Lack of Dp53 results in aberrant mitosis of mutated cells within the stressed germaria. Knowing that Dp53 is able to be activated in GSCs, and mammalian p53 is involved in hematopoietic stem cell competition, I tested whether Dp53 is activated within GSCs undergoing BMP-mediated competition. Specifically, I examined whether the Dp53 sensor, p53R-GFPnls, is turned on in GSCs in *tkv* shRNA mosaic germaria. Unfortunately, the majority of mosaic germaria expressing *tkv* shRNA had no expression of the p53 sensor in the germaria (3/15 germaria expressed the sensor, compared to 1/15 of control germaria; Figure AII.3c and d), nor

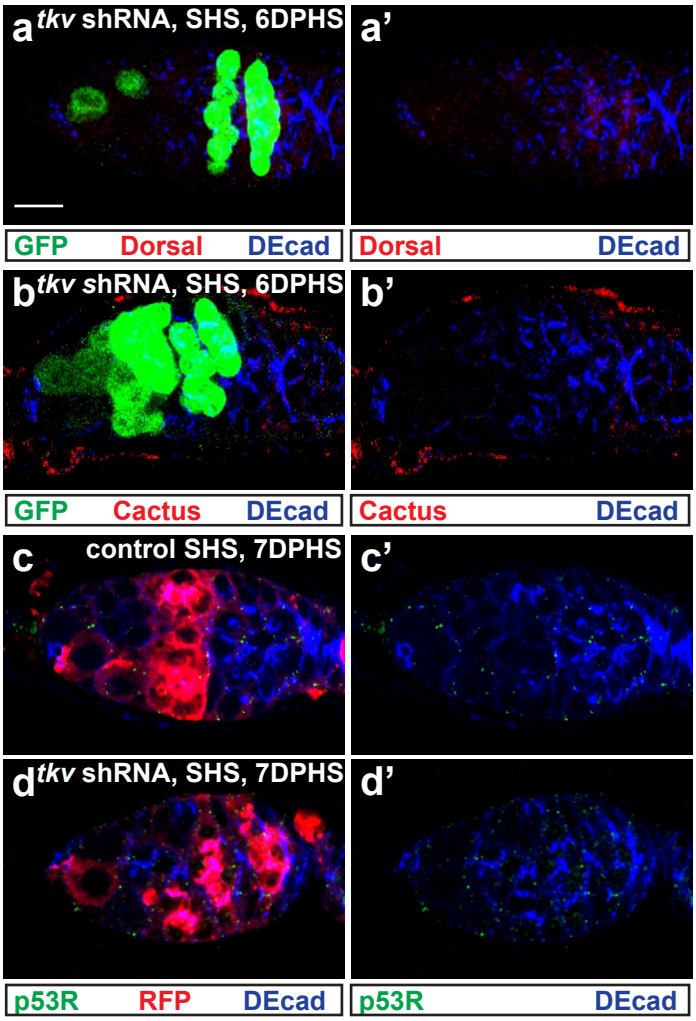


Figure AII.3 Other competitive regulators do not have differential expression in GSCs undergoing BMP-mediated competition.
(a-b) Expression of Toll pathway components in germaria from *nos*-FLPout,UASp-GFP, and *tkv* shRNA expressing females 6 days post short heat shock treatment. Germaria stained with anti-Dorsal (a) and anti-Cactus (b) antibodies, showing that Toll pathway components are not expressed within *tkv* shRNA GSCs that are labeled with GFP.
(c-d) Germaria from females carrying *nos*-FLPout and UASp-actin::RFP, with (d) or without a (c) *tkv* shRNA transgene 7 days post short heat shock treatment. Both control germaria and *tkv* shRNA expressing germaria show no activation of Dp53 in RFP labeled GSCs, as indicated by the Dp53 sensor, Dp53R-GFPnls, suggesting BMP-mediated competition does not turn on Dp53 in GSCs (12/15 *tkv* shRNA expressing germaria have no Dp53 sensor expression, compared to 14/15 of control germaria).

was the expression of the p53 reporter restricted to *tkv* shRNAs GSCs undergoing competition, suggesting that unlike mammalian p53, *Drosophila* Dp53 is not involved in BMP mediated GSC competition.

AII.4. Discussion

AII.4.1. Can BMP activity be elevated in GSCs by genetic manipulation?

In an attempt to examine whether upregulated BMP signaling causes GSCs to become supercompetitors, I tested different transgenes including $tkv^{[Q]}$, and *dSmurf* and *dad* shRNAs. Although there was no detectable gain of competitiveness in GSCs expressing *dSmurf* or *dad* shRNAs compared with their wild-type neighbors, a reduction over time in the niche occupancy of wild-type GSCs was observed within mosaic $tkv^{[Q]}$ germaria. However, my data suggested competition does not happen between GSCs. Instead, I postulate that the observed decrease in wild-type GSCs was due to their replacement by neighboring GSC-like cells, thus resulting in decreasing number of germaria with no GSCs over time. In addition, I found that there is no upregulation of BMP activity, as quantified by nuclear pMad intensity, in $tkv^{[Q]}$ expressing GSCs. Thus, it is still uncertain whether BMP signaling could be further elevated by genetic manipulation in the GSCs. Perhaps it is not possible to determine whether a GSC with elevated BMP signaling could act as a supercompetitor against wild-type GSCs.

AII.4.2. How does replacement of wild-type GSCs occur in mosaic germaria expressing $tkv^{[Q]}$?

It is unclear how wild-type GSCs are replaced over time in $tkv^{[Q]}$ expressing germaria. Possible mechanisms include one in which $tkv^{[Q]}$ GSC-like cells gain competitiveness, for example, by

having more niche contact over wild-type GSCs, which was observed in differentiation defective *bam* mutant GSC-like cells (Jin and Xie, 2007). Alternatively, the large numbers of GSC-like cells could simply exert mechanical force to ultimately push wild-type GSCs out of the niche. One possible experiment to test this hypothesis would be to increase the adhesion between wild-type GSCs and the niche by overexpressing *shotgun (shg)*, which encodes *Drosophila* E-cadherin, in all GSCs, but knocking down *shg* with a shRNA construct in $tkv^{[Q]}$ GSCs. While this experiment does have many caveats, it could examine whether increased niche contact of the $tkv^{[Q]}$ GSCs might be the reason for the apparent increase in competition by these cells. Alternatively, in Chapter IV, I describe a novel protocol for creating two classes of non wild-type GSCs in the niche. This protocol could also be applied here to examine GSC behavior in germaria in which one GSC overexpresses *shg*, while the other GSC expresses $tkv^{[Q]}$. One additional caveat of these experiments, however, is that boosting GSC DE-cadherin is known to increase the lifetime of GSCs and to delay the progressive aging of the niche (Pan et al., 2007); thus, it might be possible that one could not tell whether the suppression of GSC loss is caused by lowering competition for niche contact or simply due to increased lifetime of the DE-cadherin overexpressing GSCs.

One could also test the more general hypothesis that mechanical forces can push wild-type GSCs out of the niche by creating a crowded germarium containing wild-type GSCs and over-proliferating differentiated cyst cells using a *bam*-independent methodology. If the hypothesis were true, one might expect the niche would become occupied by mutated differentiated cyst cells. Bruno 1, which is required for differentiation of Cbs, translationally represses *Sxl* by binding to its UTR. Germaria lacking *Bruno 1* activity or expressing a mutated *Sxl* transcript lacking the Bruno Response Element (BRE) develop a mild tumorous phenotype due to

overproliferating differentiated cysts (Wang and Lin, 2007). Thus, one could construct flies carrying the *nos*-FLP-out system and an shRNA against *bruno1* or a UASp-*sxl* ΔBRE construct.

Traditionally, a standard short heat shock treatment protocol is designed to create germaria in which half of the GSCs are labeled. In the experiment to create germaria filled with differentiated cysts, I would examine ovarioles with mutated differentiated cysts, but not GSCs, to eliminate the possibility of competition between GSCs of different genotypes and to investigate whether wild-type GSCs could be pushed away from the niche over time. Thus, a modified heat shock treatment to create germaria with *bruno1* shRNA or UASp-*sxl* ΔBRE expression only in differentiated cysts cells would need to be developed. One such methodology could employ a *bam*-FLPout construct in which the *bam* promoter drives GAL4 after a heat shock-mediated Flip-out event. A standard long heat shock treatment for flies carrying the *bam*-FLPout, an shRNA against *bruno1* or a UASp-*sxl* ΔBRE construct would be sufficient to create germaria filled with overproliferating differentiated cysts, but containing wild-type GSCs. If mechanical forces due to overcrowding are sufficient to remove a wild-type GSC from the niche, then the wild type GSCs in the above experiment will also be lost over time.

AII.4.3. What are the potential mechanisms underlying BMP mediated GSC competition?

Immunohistochemical examination of the pattern of Bgcn and Sxl expression suggested that neither differentiation-promoting factor is involved in delaying differentiation of *tkv* shRNA GSCs in fully mutant germaria. Two additional experiments could be performed in the future to further test the roles of these genes. First, it would be interesting to determine whether the expression level of either of these proteins is increased in GSCs undergoing competition by examining distribution within GSCs that are being outcompeted. Second, these proteins could be

overexpressed in *tkv* shRNA GSCs in mosaic germaria to determine whether the elimination of the *tkv* shRNA GSC is suppressed.

In addition, my data suggest that Toll pathway and Dp53 are not involved in BMP mediated GSC competition. The Toll pathway is known to be activated in response to differential *dMyc* dosage in wing disc (Meyer et al., 2014), and *dMyc* induced stem cell competition has been observed in female GSCs; however, my results suggest that BMP-mediated GSC competition likely acts via a mechanism distinct from that that mediates cell competition in the wing disc. The difference may be caused by the use of different triggers for cell competition. A simple test of this hypothesis would be to examine whether Toll signaling is involved in dMyc-induced GSC competition. On the other hand, it might be interesting to determine whether BMP-mediated competition in the wing disc is sufficient to trigger Toll pathway activation. Although mammalian p53 has been implicated in mediating hematopoietic stem cell competition, this conclusion was based on examination of the relative competitiveness of wild-type HSCs and HSCs exposed to stress at the DNA level. In addition, it was the role of p53 as a general stress sensor that drove the investigators to examine its role as a regulator of HSC competition. The characteristic of "stress-induced' competition may explain why I did not observe upregulation of the p53 sensor in my experiment.

Together, my data rule out the involvement in BMP-mediated GSC competition of some genes and pathways that have been previously involved in cell competition in various other systems. Future studies should focus on screening for the underlying regulators, and I elaborate strategies to find such genes in Chapter IV.