There and Back Again: Regulation of Stem Cell Homeostasis and Dedifferentiation in The *Drosophila* Male Germline by *me31B*

by

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LIST OF ABBREVIATIONS

Add	Adducin
bam	*bag-of-marbles*
bgcn	*benign gonial cell neoplasm*
BMP	Bone Morphogenic Protein
CC	Cyst Cell
CySC	Cyst Stem Cell
dpp	*decapentaplegic*
FasIII	Fasciclin 3
GB	Gonialblast
GFP	Green Fluorescence Protein
GSC	Germline Stem Cell
hsp70	*heat shock protein 70*
JAK-STAT	Janus Kinases-Signal Transducer and Activator of Transcription proteins
JNK	c-Jun N-terminal Kinases
me31B	*maternally expressed at 31B*
nos	*nanos*
osk	*oskar*
PGC	Primordial Germ Cell

pMad	Phosphorylated Mothers against decapentaplegic
pum	*pumilio*
RIP-seq	RNA-Immunoprecipitation and sequencing
RNAi	RNA interference
RP49	Ribosomal Protein 49
SC	Spermatocyte
SG	Spermatogonia
STAT92E	Signal-transducer and activator of transcription protein at 92E
tkv	*thickveins*
tre1	*trapped in endoderm 1*
upd	*unpaired*
zfh-1	*Zinc finger homeodomain 1*

ABSTRACT

Tissue-specific stem cells maintain tissue homeostasis by providing a continuous supply of differentiated cells throughout the life of organisms. However, even stem cells can be damaged or lost, leading to a tissue that cannot be properly replenished if stem cells are not replaced or recreated. One method of recreating stem cells is dedifferentiation, the process of a more differentiated/specialized cell reverting back to a stem cell identity. In some cases, dedifferentiation of differentiated/differentiating cells help maintain the stem cell pool beyond the lifetime of individual stem cells. Although dedifferentiation is important to maintain the stem cell population, it is also speculated to underlie tumorigenesis and several previous studies have identified dedifferentiated cells as the source of cancerous growths. Therefore, this process must be tightly controlled. Here, we show that a translational regulator *me31B* plays a critical role in preventing excess dedifferentiation in the *Drosophila* male germline: in the absence of *me31B*, spermatogonia (SGs) dedifferentiate into germline stem cells (GSCs) at a dramatically elevated frequency. Our results show that the excess dedifferentiation is likely due to misregulation of *nos,* a key regulator of germ cell identity and GSC maintenance, which is both necessary and sufficient for dedifferentiation. Moreover, our data also reveal new details about *nos* transcription and translational regulation, a pattern that likely contributes to the gradual rate of differentiation in the spermatogonia. Taken together, our data reveal a method in the testis to balance stem cell maintenance with differentiation: by negatively regulating dedifferentiation

while maintaining a gradual pace for differentiation, a pool of differentiating germ cells stands ready for recall as germline stem cells.

CHAPTER 1:
Introduction

STEM CELL FUNCTION, TISSUE HOMEOSTASIS, AND DEDIFFERENTIATION

As multicellular organisms, humans (and other animals) are made up of roughly 40 trillion cells working together (Bianconi et al. 2013). The vast majority of these are 'terminally differentiated' and thus severely limited in their potency/plasticity. Additionally, the lifespan of most differentiated cells is much less than the lifespan of the whole organism, as they age and/or wear out over time, resulting in loss. The cells primarily responsible for replacing differentiated cells are stem cells: small populations of (typically tissue-specific) cells that have retained a greater degree of potency and capacity for proliferation in order to produce and replenish differentiated cell populations (He, Nakada, and Morrison 2009; Morrison and Spradling 2008). This division of labor is also protective: specialized/differentiated cells lack potentially dangerous potency but are safely replaced when they become worn out or damaged because proliferation is restricted to a small subset of stem cells instead (Morrison and Spradling 2008). By constantly generating a supply of new cells to replace those that are lost to age and damage, stem cell populations form the foundation of physiological homeostasis.

However, this system as described so far fails to acknowledge that stem cell populations are also subject to damage, loss, and aging (He, Nakada, and Morrison 2009). Therefore, it is vital that strategies for their replenishment and replacement also be available if needed. One such strategy for replenishing stem cell populations is dedifferentiation, the process where

differentiated cells revert back into a stem or progenitor cell identity within the same lineage (Merrell and Stanger 2016). Such changes in cell fate should not be undertaken lightly, especially since undergoing dedifferentiation could expose an organism to the risks typically avoided by strictly delineating stem cell identity from differentiated cell identity in the first place. But the reward, a full and active pool of dividing stem cells, must be worth the risk if it means prolonging the overall function of the tissue.

Indeed, much of the recent work on dedifferentiation occupies two prongs of focus: observing its endogenous employment and characterizing its role in tumorigenesis (He, Nakada, and Morrison 2009; Friedmann-Morvinski and Verma 2014; Merrell and Stanger 2016; Jopling, Boue, and Belmonte 2011). Multiple studies have observed a great diversity of animal species using dedifferentiation in a variety of contexts to their benefit while among cancer biologists, dedifferentiation is commonly used as a hallmark of malignant tumors (Niu, Mercado-Uribe, and Liu 2017; Friedmann-Morvinski and Verma 2014; Akao et al. 1995). This dichotomy (that is, dedifferentiation as a critical aspect of stem cell homeostasis *and* tumorigenesis) begs the question: How is a beneficial result achieved over a harmful one? I suspect that one answer can be found by fostering a greater knowledge of how dedifferentiation is endogenously regulated, which genes are involved, and how their interactions strike the balance between risk and reward. Thus, my thesis' main focus is to deepen our understanding of how endogenous dedifferentiation in *Drosophila* testes is regulated to ensure maintenance of a functional population of germline stem cells in the face of loss.

DROSOPHILA MELANOGASTER SPERMATOGENESIS

To investigate dedifferentiation, we have chosen to use testes from *Drosophila melanogaster*. The *Drosophila* germline offers a unique combination of an easily accessible stem

cell niche with single cell resolution paired with the immense versatility of genetic tools (Decotto and Spradling 2005). These qualities make it ideal for studying a variety of stem cell functions, including homeostasis of the germline stem cell population.

The *Drosophila* testis is a tubular tissue with a group of somatic cells constituting the "hub" at the apical blind end (Figure 1.1). Attached to the hub cells are the germline stem cells (GSCs) and cyst stem cells (CySCs). The hub cells produce two primary niche-signaling factors received by the GSCs and CySCs that maintain these stem cell populations: *unpaired* (*upd*) and *decapentaplegic* (*dpp*). These are the *Drosophila* homologs for the cytokine-like JAK-STAT receptor ligand and the BMP ligand, respectively (de Cuevas and Matunis 2011). Of these, *upd* and the downstream JAK-STAT cascade in the GSCs is responsible for keeping them attached to the hub, as loss of STAT signaling will result in GSC loss (Brawley and Matunis 2004; Tulina

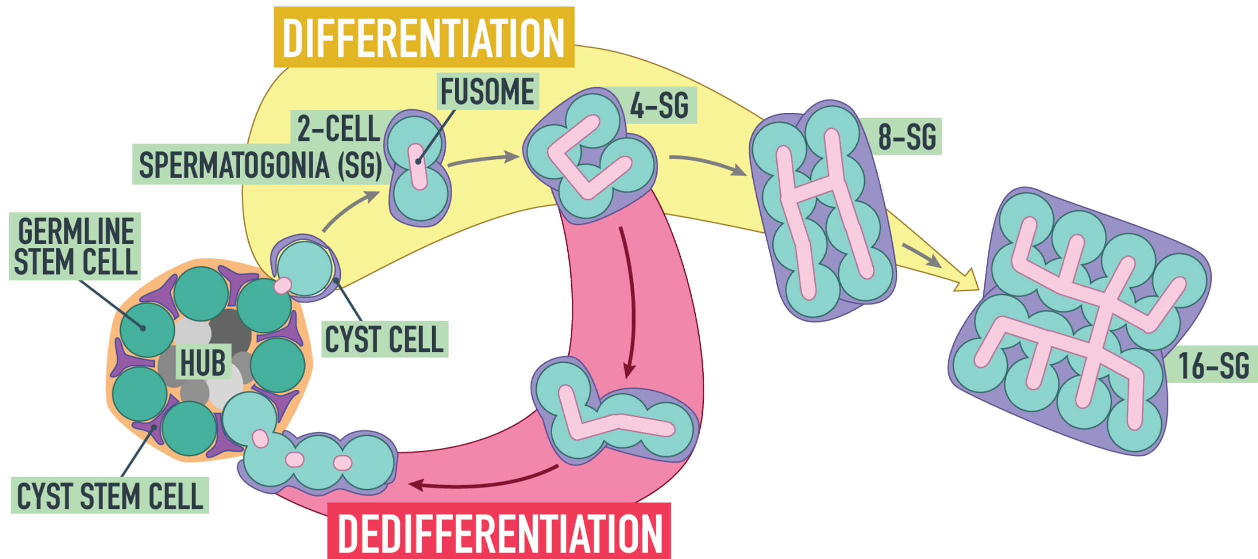

Figure 1.1: Model of Differentiation and Dedifferentiation in The *Drosophila* Testis
To the left is the apical tip of the testis, where the hub and the niche (in orange) are situated. The hub is surrounded by germline stem cells (teal) and cyst stem cells (purple). These divide asymmetrically to produce a germ cell (light teal, called a gonialblast) which is surrounded by two cyst cells (light purple). Along the top in yellow, the typical process of differentiation is shown. Gonialblasts undergo incomplete transit-amplifying divisions and remain connected by a fusome (light pink). The bundle of germ cells is numbered and called spermatogonia cyst. In the lower-center in red, a depiction of dedifferentiation is shown. In the SG cyst touching the hub, the fragmented fusome is present, which allows me to identify dedifferentiating cysts.

and Matunis 2001). BMP signaling is important for GSC identity via its suppression of the differentiation factor *bag of marbles (bam)* (D. M. McKearin and Spradling 1990). In order to further ensure that their daughter cells (called gonialblasts, ‘GBs’) will not receive or co-opt any *dpp* signal meant for GSCs, GSCs primarily receive *dpp* through microtubule-based nanotubes that extend into the hub (Inaba, Buszczak, and Yamashita 2015). This nanotube contains the cell’s receptors for *dpp* on the membrane, ready to directly bind niche signals released by the hub cells (Inaba, Buszczak, and Yamashita 2015).

Under typical circumstances, GSCs continuously divide asymmetrically in order to sustain spermatogenesis. As they divide, GSCs displace their daughter cells away from the niche where they will begin to undergo differentiation first as spermatogonia (SG), then as spermatocytes (SC) upon initiating meiosis, and eventually leading to the production of sperm (Fabian and Brill 2012). In order to maintain the number of GSCs, asymmetric division must preserve one cell in the niche while displacing the other. This is accomplished through two main features: 1) the GSC is physically attached to the hub on one side of the cell via E-cadherin and; 2) during interphase the centrosomes are aligned with this physical connection, ensuring that the plane of division runs parallel with the hub (Yamashita, Jones, and Fuller 2003; C. Chen and Yamashita 2021). If completed as intended, one daughter cell will remain as a GSC, occupying a spot in the niche while the other will have been driven as far away from the niche signals as a single cell division can push.

The somatic CySCs are also attached to the hub and encapsulate GSCs, regulating their identity together with hub cells (Decotto and Spradling 2005). Much like GSCs, *upd* and JAK-STAT signaling is required for CySCs to sustain their identity and place within the niche, as CySCs will prematurely differentiate without JAK-STAT signaling (Zoller and Schulz 2012).

However, JAK-STAT function in CySCs is partially for GSCs as well: The transcriptional repressor *zfh-1* is activated downstream of JAK-STAT and its expression in CySCs is required for non cell-autonomous maintenance of GSCs (Leatherman and Dinardo 2008). Thus, GSC and CySC maintenance and identity is sustained through multiple sources of signal and cells, creating a web that limits stem cell identity to a strict spatial area.

As each GSC divides, so do the CySCs to either side of it. The daughter cells of CySCs, called cyst cells (CCs), encapsulate GBs throughout the process of differentiation, ensuring two somatic cells accompany each germ cell cyst (Zoller and Schulz 2012). Disruption of the encapsulation can lead to the accumulation of germ cell cysts that do not proceed with differentiation and result in tumor-like testes, directly affecting fertility (Fairchild et al. 2016; Fairchild, Smendziuk, and Tanentzapf 2015). The establishment of a germ-soma barrier during gametogenesis is commonplace among most animals, but in *Drosophila* testis, the initial encapsulation by the cyst cells does not yet establish this permeability barrier. Using a fluorescent dye, Fairchild et al 2015 found that although encapsulation is immediate, the cyst cells do not form a permeability barrier with septate junctions until at least the 4-cell SG stage. Formation of this barrier also correlates precisely with the rise in expression of differentiation factor *bam* in the SGs, and disrupting the barrier also disrupts the expression pattern of *bam* (Fairchild, Smendziuk, and Tanentzapf 2015). These results show that further differentiation is dependent on the cyst cells forming a permeability barrier.

During differentiation, GBs undergo four mitotic divisions while cytokinesis remains incomplete, ultimately resulting in a group of 16 interconnected germ cells. These SG grow together as a cyst, sharing cytoplasm and a subcellular organelle called the fusome that runs through the cytoplasmic bridges of all the cells. As SG differentiation progresses and the number

of cells in the cyst increases, the fusome expands as well to maintain the connections (Fig. 1.1, light pink). Thus, the branching morphology of the fusome serves as a precise indicator of the differentiation stage for each SG cyst. After finishing all transit-amplifying divisions, the 16-cell SG cyst will grow in size in preparation for meiosis and are now called spermatocytes. The spermatocyte cyst will then divide twice (i.e. meiosis), producing a bundle of 64 immature spermatids for each asymmetric GSC division. As this process occurs for all GSCs in the niche, each new SG displaces the previous one, meaning the physical progression of cells from the apical tip down the testis is roughly temporal.

***NOS* AND *BAM* AS RNA REGULATORS OF STEM CELL MAINTENANCE AND DIFFERENTIATION**

As SGs move away from the hub and divide, they also initiate differentiation. While multiple regulatory networks function in parallel to achieve this, two genes in particular, *nanos* (*nos*) and *bag of marbles* (*bam*), play key roles in both transcriptional and translational changes to gene expression. Additionally, their roles are often dictated by the relationship they have with other genes and their relationship with each other.

At its core, the function of *nos* is to prevent premature differentiation, a goal it accomplishes through translational repression (Deshpande et al. 1999). Most of the work characterizing *nos* function occurred in the female germline and the embryo. In the embryo, maternally-deposited *nos* is critical for successful primordial germ cell (PGC) migration (Z. Wang and Lin 2004; Kugler and Lasko 2009; Gavis and Lehmann 1992; 1994). Without it, PGCs will prematurely differentiate and fail to migrate to the proper location in the embryo (Kunwar et al. 2008). In the ovaries, *nos* works as a prolific translational repressor with its

partner *pumilio* (*pum*) (Li et al. 2012). Together with *pum*, *nos* targets a swath of mRNA transcripts containing *pumilio* response elements (formerly *nos* response elements) in the 3'UTRs (Asaoka-Taguchi et al. 1999; Sonoda and Wharton 1999). In ovarian GSCs, *nos* expression is required to prevent GSCs from differentiating and starting oogenesis (Li et al. 2009).

Less is known about the molecular action of *nos* in the male germline, but we do know its expression is just as critical. Similar to the female germline, *nos* is known to be required for maintaining stem cell identity in the GSCs. In the male germline, GFP labeling shows active protein expression found in germ cells from GSCs through to the 4-cell SG stage, where it abruptly falls off (see Figure 2.5, A and A'). Interestingly, no studies have yet been published describing a role for *pum* in the testis, implying that the work *nos* does in the testis is either done independently or with a repressional partner yet to be identified.

In contrast to the role that *nos* plays in preventing premature differentiation, *bam* primarily functions as a promoter of differentiation (D. M. McKearin and Spradling 1990). In the ovaries, mutations in *bam* lead to tumorous ovaries, with germ cells packed in and failing to continue differentiation (D. Chen and McKearin 2003; D. McKearin and Ohlstein 1995). A similar phenotype can be observed in the testis when driving the overexpression of *dpp*, the BMP ligand in *Drosophila*. Unsurprisingly, the BMP signaling pathways directly antagonizes *bam* in order to maintain GSC identity in the female germline (D. Chen and McKearin 2003; Song et al. 2004). In particular, the gradient of received *dpp* is critical for determining which cells will continue to function as GSCs. If the cells receive an insufficient amount of BMP signaling, they can no longer maintain *bam* repression and the cell is pushed towards differentiation (D. Chen

and McKearin 2003). Similarly, bam expression in male GSCs will also drive differentiation (Sheng, Brawley, and Matunis 2009).

The relationship between *bam* and *nos* is also antagonistic. In the male germline, Bam protein signal rises in the 4-cell SG stage as soon as Nos protein signal ceases, and continues to maintain high expression for all SG stages beyond. While the protein expression of Nos and Bam in the male germline is clearly reciprocal, the direct interactions between *nos* and *bam* have been more thoroughly explored in the female germline. In the ovaries, *bam* forms a complex with *benign gonial cell neoplasm* (*bgcn*) and directly binds to *nos* mRNA to repress translation in order to promote differentiation (Li et al. 2009; Kim, Lee, and Kim 2010; Malik et al. 2020). Although a mutation in either *bam* or *bgcn* results in the accumulation of undifferentiated germ cells in the testis (Gönczy, Matunis, and DiNardo 1997), there has been no direct evidence yet that these two genes also directly bind *nos* mRNA in the testis like they do in the ovary.

DEDIFFERENTIATION IN *DROSOPHILA*

Dedifferentiation was first observed in both the male and female germline of *Drosophila* in 2004. In both cases, the relationship between the differentiation factor Bam and the BMP signaling needed to maintain GSC identity played a central role (Kai and Spradling 2004; Brawley and Matunis 2004).

Kai and Spradling first observed dedifferentiation in the female germline in response to prematurely inducing differentiation in the larval ovary. In the larval ovary, *dpp* signaling maintains not just one or two GSCs, but a whole pool of premature germ cells, as differentiation and multi-cell cyst formation only begins in pupae. When Kai and Spradling forcibly differentiated these premature germ cells into multi-cell cysts using heat-shock driven *bam*

expression, removing the flies from heat-shock did not prompt the cysts to undergo apoptosis. Instead, the cysts broke down into individual cells; each one reverting back into individual germ cells that functioned just as well as any other GSC upon reaching adulthood.

To test if dedifferentiation was also possible in the adult ovary, where only one or two GSCs sit within a niche maintained by somatic cells, the authors first overexpressed *dpp* and then *bam* to generate many adult GSCs and then forcibly differentiate them into multi-cell cysts respectively. The result was an adult ovary filled with multi-cell cysts and no single germ cells or GSCs. When no longer driving heat-shock *bam*, the cysts in the adult ovary underwent exactly the same process observed in the larval ovary: the fusomes thinned, ring canals closed, and each cell in the cysts reverted back into an individual GSC.

In the testis, dedifferentiation was first observed following forcible depletion the GSCs using a heat-induced STAT92E mutation (Brawley and Matunis 2004). Since STAT92E is a crucial member of the JAK-STAT signaling pathway necessary to maintain stem cell identity, loss of function caused all stem cells to differentiate, leaving an empty hub in their wake (Brawley and Matunis 2004). When flies were shifted back to a temperature that allowed STAT92E to function again, the multicellular SG cysts (which were previously the GSCs) crowded the bare hub and broke apart in order to repopulate the niche (Brawley and Matunis 2004).

Subsequently, dedifferentiation was found to occur in unperturbed tissue, showing for the first time that it is implemented endogenously for maintaining the tissue homeostasis, not just a response to the artificially-induced catastrophic loss of GSCs (Cheng et al. 2008). By permanently labeling SGs that have made it to the 4-cell SG stage with LacZ, Cheng et al was also able to observe the frequency of dedifferentiation over the lifetime of the flies. Shockingly,

nearly half of all GSCs in the testis were the result of SG dedifferentiation by 50 days of age (Cheng et al. 2008). These results strongly implied that not only was dedifferentiation used endogenously, it is also specifically employed to maintain tissue homeostasis against the stress of age (Cheng et al. 2008).

In a 2009 study, Sheng et al investigated forcibly induced dedifferentiation, this time using a more targeted approach by inducing dedifferentiation in only GSCs (as opposed to all stem cells around the hub) via temperature-regulated overexpression of the differentiation factor *bam*. Since leaving CySCs in the niche resulted in no delay or altered morphology in dedifferentiating cysts, these results may suggest that dedifferentiation requires neither proximity to the hub nor available space in the niche to occur, implying that signaling is responsible for initiating dedifferentiation (Sheng, Brawley, and Matunis 2009). Using live imaging in 2011, the authors also observed that dedifferentiating cysts migrating back to the hub have cytoplasmic projections towards the niche. These filopodia imply that the cells are likely following some kind of cell signal in order to navigate back to the niche, once again lending support to the hypothesis that dedifferentiation is an active process initiated by external signaling rather than by available space (Sheng and Matunis 2011).

In 2018, Hererra and Bach tested if dedifferentiation of late-stage SGs were indeed necessary for GSC maintenance over time in the face of stressful conditions. They inhibited dedifferentiation in late-stage SGs in young males, then compared the GSC numbers of males subjected to chronic stress to those of males aged in stress-free conditions (Herrera and Bach 2018). Indeed, the males undergoing starvation cycles had far fewer GSCs and reduced levels of sperm production as they aged, implying that dedifferentiation is specifically responsible for maintaining GSCs during adverse conditions (Herrera and Bach 2018). Furthermore, GSCs that

arose from dedifferentiation seemed to shoulder more of the burden than native GSCs once they arrived: GSCs derived from dedifferentiation were more proliferative and these males actually produced 45% more offspring than wildtype males (Herrera and Bach 2018). Lastly, the authors investigated the role of JNK signaling, a pathway known to be involved in both stress response in the testis and regeneration/cellular reprogramming in other organisms, in the process of dedifferentiation (Herrera and Bach 2018). Using lineage tracing of cells with upregulated JNK signaling, they observed an uptick in labeling during refeeding, the same period in which they see increased dedifferentiation (Herrera and Bach 2018). Blocking the JNK pathway in late-stage SGs resulted in a large reduction in late-stage SG dedifferentiation, showing that JNK signaling activation is required for late-stage cysts to dedifferentiate in adverse conditions (Herrera and Bach 2018). Although this study was unable to completely prevent dedifferentiation from early-stage SGs, these results show that dedifferentiation is essential to recover and maintain fertility in adverse environments (Herrera and Bach 2018).

In summary, SGs can and will dedifferentiate under specific circumstances, allowing one or more cells per cyst to revert into GSCs. When SGs dedifferentiate, the cyst gains mobility and migrates back into the stem cell niche. Once there, one or more of the cells in the cyst will contact the hub and the fusome will break down, generating unconnected GSCs that will quickly become indistinguishable from GSCs not generated via dedifferentiation. As the fusome disintegrates, it assumes a round morphology (now called "spectrosome"), which can be used as a reliable marker for dedifferentiation. For the duration of this body of work, detecting dedifferentiation relies on observing the presence of an SG cyst with one or more cells touching the hub while still remaining connected to other germ cells in its SG cyst via the spectrosome (so before the connections are completely severed). Spermatogonial cysts at any stage can undergo

dedifferentiation, though there is some evidence that less differentiated SGs are more likely to do so than more differentiated SGs (Sheng and Matunis 2011). It is very likely that GBs and 2-cell SG cysts dedifferentiate the most frequently, but these events are not currently detectable as GBs will instantly blend in with surrounding GSCs and dedifferentiating 2-cell SG cysts are indistinguishable from normal asymmetric GSC division. Thus, all dedifferentiation detected in this study is the result of SGs containing four or more cells in the cyst.

DEDIFFERENTIATION IN HOMEOSTASIS AND TUMORIGENESIS

In many organisms, dedicated stem cell populations use dedifferentiation as a way to maintain homeostasis, compensate for stem cell loss, and as a stepping-stone for regeneration. The presence of dedifferentiation in early-diverging metazoan lineages, such as cnidarians and poriferans, implies that dedifferentiation is likely a strategy that animals have been employing for at least ~550 million years (Gold and Jacobs 2013; Lavrov and Kosevich 2018; Borisenko et al. 2015). The degree to which it is used and how it is executed across animal species, however, varies greatly depending on context. For example, in zebrafish hearts and many amphibious species, dedifferentiation of existing non-stem cells is the primary method through which a pool of progenitor cells is generated in response to damage and amputation (Poss, Wilson, and Keating 2002). This mass of dedifferentiated cells, called a “blastema”, forms at the site of amputation and divides to provide ample cells needed to regenerate the missing limb or tissue (Poss, Wilson, and Keating 2002).

In fruit flies and mammals, dedifferentiation of more specialized cells is most often observed as a way to replenish existing stem cell populations rather than generate a new pool of them (Tata et al. 2013; Cheng et al. 2008; Hsu, Pasolli, and Fuchs 2011). In mice lung

epithelium, an otherwise stable population of basal cells functions as the stem cell population and gives rise to both secretory cells and ciliated cells (Tata et al. 2013). However, when Tata et al 2013 ablated the basal cells, secretory cells were able to dedifferentiate to reform missing basal cells, restoring function and maintaining the tissue, much like the spermatogonia do in the *Drosophila* testis. Interestingly, secretory cells adjacent to existing basal cells are strictly inhibited from dedifferentiating, suggesting that basal cells themselves may suppress this function in secretory cells and/or the position that basal cells occupy in the niche may also be of importance (Tata et al. 2013).

However, the cell-fate plasticity associated with dedifferentiation is not without risk. The presence of dedifferentiated cells is often referred to as a hallmark of cancer and dedifferentiation features heavily in both initiation and promotion of cancer (Niu, Mercado-Uribe, and Liu 2017; Friedmann-Morvinski and Verma 2014; Landsberg et al. 2012; Schwitalla et al. 2013; Friedmann-Morvinski et al. 2012; Puri, Folias, and Hebrok 2015). While inducing cancer in multiple models, such as leukemia and aggressive brain cancer, multiple studies have observed that these cancerous cells arise from dedifferentiated non-stem cells rather than their corresponding stem cell populations. In a model for colon cancer in mice, the most common source for tumors is not the stem cells in the crypts, but the fully differentiated intestinal epithelium cells (Schwitalla et al. 2013). In order to initiate tumorigenesis, these epithelial cells first undergo dedifferentiation, caused by inflammation-driven Wnt signaling (Schwitalla et al. 2013). Suppression of immune-response signaling (and thus lower Wnt signaling) in only the intestinal epithelial cells greatly reduces the proliferation observed, reaffirming that this phenotype arises from previously differentiated cells (Schwitalla et al. 2013; de Sousa E Melo and de Sauvage 2019).

On top of the danger of dedifferentiation giving rise to tumors, Landsberg et al 2012 made another alarming observation in already-established cancer: some melanoma cells will dedifferentiate in response to the inflammation signals that follow immunotherapy treatment, thereby using the process to evade detection by T-cells. The authors strongly suggest that this unrecognizable plasticity is at least partially responsible for the delayed failure of immunotherapy observed in some patients (Landsberg et al. 2012).

These studies serve as warnings that dedifferentiation in animals must be strictly regulated. Either the absence of dedifferentiation or mistakes in the process can send an animal to an early grave. However, our knowledge about the regulatory networks involved in dedifferentiation remains slim. Understanding the signaling networks that control dedifferentiation could be key to furthering our understanding of regeneration, long-term tissue homeostasis, and aging.

SUMMARY OF DISSERTATION

Although tissue-specific stem cells are responsible for the bulk of maintaining tissue homeostasis by providing a continuous supply of differentiated cells throughout the life of organisms, differentiated cells can revert back to a stem cell identity via dedifferentiation to help maintain the stem cell pool beyond the lifetime of individual stem cells (Sheng, Brawley, and Matunis 2009; Herrera and Bach 2018; Cheng et al. 2008). While dedifferentiation is important to maintain the stem cell population, it is speculated to underlie tumorigenesis (Landsberg et al. 2012; Schwitalla et al. 2013; Friedmann-Morvinski and Verma 2014; Niu, Mercado-Uribe, and Liu 2017). Therefore, this process must be tightly controlled. Despite previous work, endogenous regulators of dedifferentiation have remained largely elusive.

Here I show that a translational regulator *me31B* plays a critical role in preventing excess dedifferentiation in the *Drosophila* male germline: in the absence of *me31B*, spermatogonia dedifferentiate into germline stem cells at a dramatically elevated frequency. Our results show that the excess dedifferentiation is likely due to misregulation of *nos*, a key regulator of germ cell identity and GSC maintenance. Taken together, these data reveal negative regulation of dedifferentiation balances stem cell maintenance with differentiation.

CHAPTER TWO:

me31B Regulates Stem Cell Homeostasis by Preventing Excess Dedifferentiation in The *Drosophila* Male Germline

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ABSTRACT

Tissue-specific stem cells maintain tissue homeostasis by providing a continuous supply of differentiated cells throughout the life of organisms. Differentiated/differentiating cells can revert back to a stem cell identity via dedifferentiation to help maintain the stem cell pool beyond the lifetime of individual stem cells. Although dedifferentiation is important to maintain the stem cell population, it is speculated to underlie tumorigenesis. Therefore, this process must be tightly controlled. Here we show that a translational regulator *me31B* plays a critical role in preventing excess dedifferentiation in the *Drosophila* male germline: in the absence of *me31B*, spermatogonia (SGs) dedifferentiate into germline stem cells (GSCs) at a dramatically elevated frequency. Our results show that the excess dedifferentiation is likely due to misregulation of

nos, a key regulator of germ cell identity and GSC maintenance. Taken together, our data reveal negative regulation of dedifferentiation to balance stem cell maintenance with differentiation.

INTRODUCTION

Tissue-specific adult stem cells play a critical role in sustaining tissue homeostasis by continuously providing differentiated cells throughout the life of organisms (He, Nakada, and Morrison 2009; Nystul and Spradling 2006). The loss of stem cells or their functions underlie tissue degeneration under physiological and pathological conditions. The stem cell pool is primarily maintained by self-renewal. In addition, dedifferentiation, a process whereby differentiated and/or differentiating cells revert back to a stem cell identity, also helps to maintain the stem cell population beyond the lifetime of individual stem cells (de Sousa E Melo and de Sauvage 2019; Merrell and Stanger 2016). However, the misregulation of dedifferentiation has been implicated to underlie tumorigenesis (Schwitalla et al. 2013; Landsberg et al. 2012). Therefore, dedifferentiation must be tightly controlled to ensure stem cell maintenance, while preventing transformation. However, the molecular mechanisms that regulate dedifferentiation are not well understood.

The *Drosophila* testis serves as an excellent model system to study dedifferentiation. Notably, this model offers unambiguous identification of stem cells (germline stem cells (GSCs)) and their differentiating progeny (Fuller and Spradling 2007; Yamashita 2018). GSCs are attached to post-mitotic somatic hub cells, which function as a major component of the stem cell niche (Figure 1.1, 2.2A). The hub cells secrete two major signaling ligands that promote GSC self-renewal: a cytokine-like ligand Upd that activates the JAK-STAT pathway, and a BMP ligand Dpp that activates the downstream Tkv receptor (Shivdasani and Ingham 2003; Kawase et

al. 2004; Schulz et al. 2004; Kiger et al. 2001; Tulina and Matunis 2001). Upon GSC divisions, daughter cells that are displaced away from the hub initiate differentiation as gonialblasts (GBs), which then continue with proliferative mitotic divisions (or transit-amplifying divisions) as spermatogonia (SGs) before entering meiotic program as spermatocytes (SCs). SG divisions are characterized by incomplete cytokinesis, connecting all sister cells as a cluster (i.e. cyst). A membranous organelle called the fusome runs through the stabilized contractile ring, called ring canals, connecting SGs within a cyst (Figure 2.2A) (Yamashita 2018).

Although GSCs are maintained relatively stably through consistent asymmetric divisions, which generate one GSC and one GB (Yamashita, Jones, and Fuller 2003), GSCs can occasionally be lost (Wallenfang, Nayak, and DiNardo 2006). Upon GSC loss, SGs can respond to niche vacancy, and dedifferentiate to replenish the GSC pool. During dedifferentiation of SGs, the fusome that connects SGs fragments into a more spherical structure, referred to as 'spectrosome' as typically observed in GSCs (Figure 2.2A) (Brawley and Matunis 2004). Fragmenting fusomes in >2 cell SGs are observed only during dedifferentiation, not during differentiation, and these features can be used to unambiguously identify dedifferentiating SGs without lineage tracing (Brawley and Matunis 2004; Sheng and Matunis 2011; Sheng, Brawley, and Matunis 2009). Dedifferentiation was first shown in an experiment that artificially removed all GSCs via transient overexpression of Bam, a master regulator of differentiation (Brawley and Matunis 2004; Sheng, Brawley, and Matunis 2009; Sheng and Matunis 2011). While temporally controlled overexpression of Bam induced all GSCs to differentiate, withdrawal of Bam allowed SGs to repopulate the stem cell niche and produce GSCs. Subsequently, it was shown that SG dedifferentiation occurs naturally and increases during aging in unperturbed tissues (Cheng et al., 2008), suggesting that dedifferentiation is likely a mechanism that helps to maintain the GSC

population throughout the lifetime of organisms, particularly with age. More recent work showed that dedifferentiation is important to sustain the GSC population under conditions that repeatedly induce GSC replenishment and challenge tissue homeostasis, such as cycles of starvation and refeeding (Herrera and Bach, 2018). SG dedifferentiation under these conditions required JNK signaling (Herrera and Bach, 2018). However, whether mechanisms exist to prevent excess dedifferentiation remain poorly understood.

Maternally expressed at 31B (me31B) encodes an RNA helicase of the DEAD-box family that regulates translation (Nakamura et al., 2001, Kugler et al., 2009, Kugler and Lasko, 2009). In particular, Me31B silences the translation of oocyte-localizing mRNAs, such as *oskar,* in nurse cells prior to their transport to the oocyte (Nakamura et al., 2001, McDermott et al., 2012). Me31B was also shown to repress translation of *nanos (nos*) (Gotze et al., 2017, Jeske et al., 2011), a translational regulator that is critical for germ cell specification and maintenance of GSCs (Li et al., 2009, Wang and Lin, 2004). Here, we show that *me31B* is a critical negative regulator of dedifferentiation in the *Drosophila* testis. In the absence of *me31B*, SGs frequently dedifferentiated even in the absence of known triggers, such as the induced removal of GSCs. We further show that *me31B* suppresses SG dedifferentiation by repressing *nos*. Our study reveals that dedifferentiation is actively repressed under normal conditions, likely to protect the native GSC population, and identifies *me31B* as a previously unknown negative regulator of dedifferentiation.

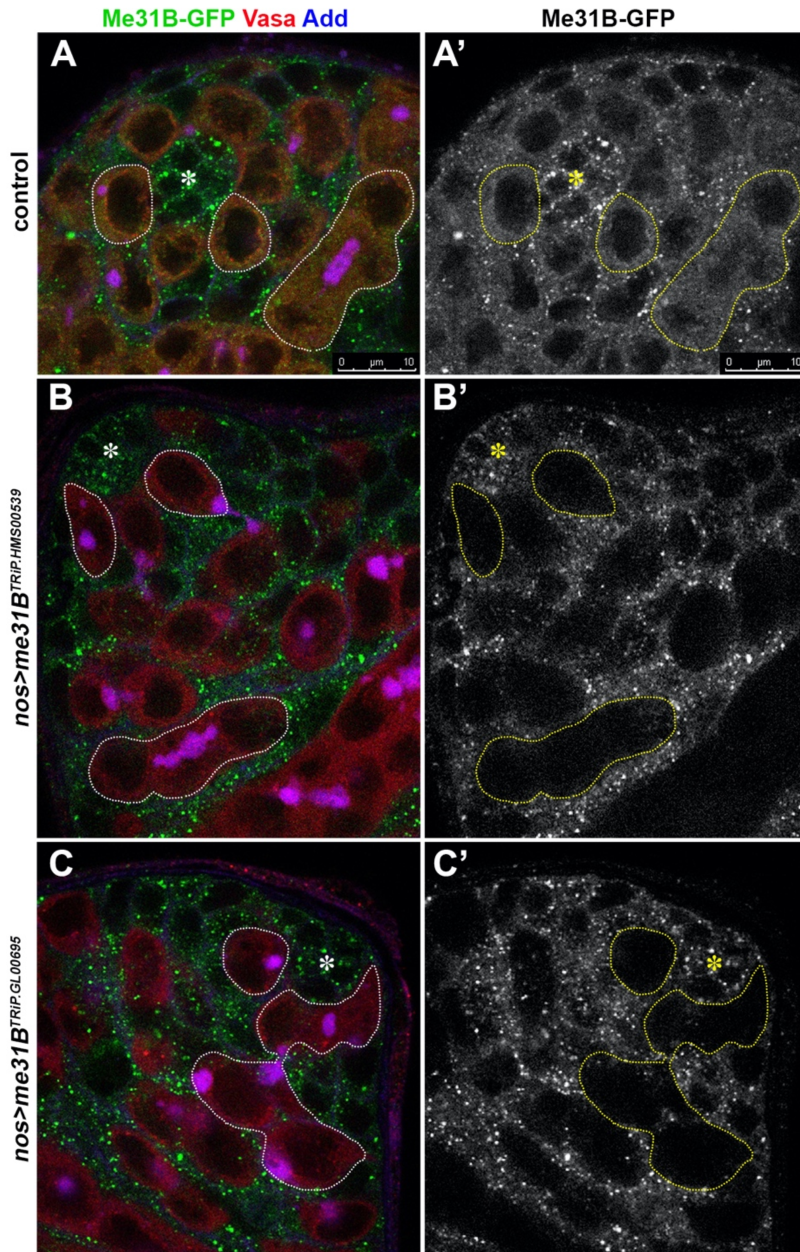

Figure 2.1: Germline-Specific Knockdown of *me31B*
Two independent *me31B* knockdown constructs were expressed using the *nos-gal4* driver in the Me31B-GFP protein trap line. GFP signal was diminished in germ cells upon expression of *me31B* knockdown constructs, leaving the GFP signal in the somatic cyst cells.
A. control testis, **B.** *nos>me31B*^{TRiPHMS00539}, **C.** *nos>me31B*$^{TRiP.GL00695}$. Examples of germ cell cysts are indicated by dotted lines. The hub is indicated by asterisks. Red: Vasa (germ cells, nuage), Blue: Adducin-like (Add, fusome, note that Blue appears to be magenta in the figure due to overlap with Vasa signal in red), Green: Me31B-GFP

RESULTS

me31B prevents excess dedifferentiation of SGs in *Drosophila* testes.

To study the role of *me31B* in the testis, we used two independent RNAi constructs (*UAS-me31B*$^{TRiP.GL00695}$ and *UAS-me31B*$^{TRiP.HMS00539}$, available from Bloomington Stock Center, see methods). Using these constructs and the *nos-gal4* driver, we knocked down *me31B* in germ cells (Figure 2.1, *nos-gal4 >UAS-me31B*$^{TRiP.GL00695}$ and *nos-gal4>UAS-me31B*$^{TRiP.HMS00539}$, hereafter *nos>me31B*$^{TRiP.GL00695}$ and *nos>me31B*$^{TRiP.HMS00539}$, respectively, or simply *nos>me31B*^{RNAi} as essentially the same results were obtained with both RNAi constructs). We

found that Me31B-GFP was expressed in both germline and somatic cells in the testis, and the GFP signal was substantially reduced in the germline upon expression of the RNAi construct using *nos-gal4*, confirming the efficiency of these RNAi constructs (Figure 2.1). Although Me31B has been reported to be a component of nuage (germ granules) (DeHaan et al., 2017, Thomson et al., 2008, Liu et al., 2011), we observed diffuse cytoplasmic localization of Me31B-GFP in germ cells in the adult testis and Me31B-GFP did not co-localize with the nuage marker Vasa in control flies. Moreover, *me31B* knockdown did not affect nuage morphology (Figure 2.1).

As expected, GSCs in control testes surrounded the hub and were either single cells or connected to their immediate daughter cells (GBs) prior to completion of cytokinesis (Figure 2.2B). Intriguingly, *nos>me31B*^{RNAi} testes often contained dedifferentiating SG cysts that were attached to the hub cells (Figure 2.2B). Their identity as dedifferentiating SG cysts is based on the fact that they contained ≥3 germ cells that were connected to each other (Figure 2.2C-D) (see details for identifying dedifferentiating cysts). The fusomes in these SG cysts at the hub in *nos>me31B*^{RNAi} testes were fragmented (Figure 2.2C-D), a well-established hallmark of dedifferentiating SGs (Brawley and Matunis, 2004, Sheng et al., 2009, Sheng and Matunis, 2011), rather than continuous as in differentiating SGs (Figure 2.2E).

We observed dedifferentiating SG cysts, identified by their fragmented fusomes and attachment to the hub, in about 80% of *nos>me31B*^{RNAi} testes but not in any control testes (Figure 2.2F). The number of SGs within dedifferentiating SG cysts was not always 2^n: often they contained 3 SGs, indicating that some SGs might have already dedifferentiated into single GSCs or died during dedifferentiation.

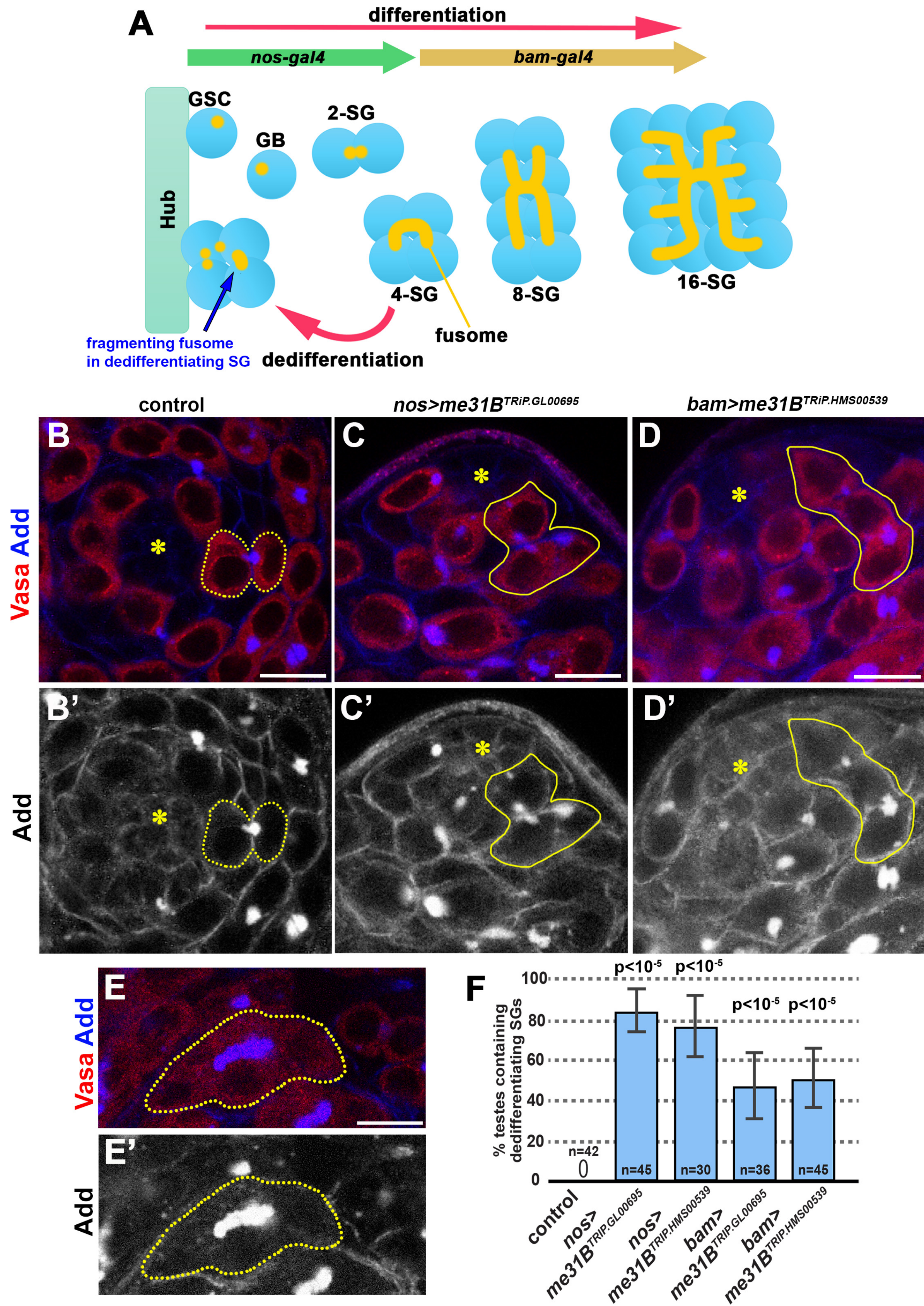
A
differentiation
nos-gal4
bam-gal4
Hub
GSC
GB
2-SG
4-SG
8-SG
16-SG
fusome
fragmenting fusome
in dedifferentiating SG
dedifferentiation
control
nos>me31B^TRiP.GL00695
bam>me31B^TRiP.HMS00539
B
C
D
Vasa Add
B'
C'
D'
Add
E
E'
F
% testes containing dedifferentiating SGs
p<10^-5
n=42
n=45
n=30
n=36
n=45
control
nos> me31B^TRiP.GL00695
nos> me31B^TRiP.HMS00539
bam> me31B^TRiP.GL00695
bam> me31B^TRiP.HMS00539

Figure 2.2: *me31B* Knockdown Leads to Excessive Dedifferentiation in the *Drosophila* Testis

A. *Drosophila* spermatogenesis. Germline stem cells (GSCs) are attached to the hub cells, which provide signaling ligands required for GSC self-renewal. Asymmetric GSC division generates a GSC and a gonialblast (GBs) that undergo 4 rounds of mitotic divisions to create 2-, 4-, 8-, and 16-cell spermatogonia (SGs). 16-cell SGs then proceed to spermatocyte stage, then to meiosis to produce sperm (not depicted). SGs can revert back to the GSC identity via dedifferentiation. During dedifferentiation, a cytoplasmic organelle called the fusome, which is normally a continuous structure that connects SGs, breaks apart. The fragmenting fusome in the dedifferentiating SG is indicated by a blue arrow. The *nos-gal4* driver is expressed in GSCs until the 4-cell SGs, whereas *bam-gal4* is expressed after the 4-cell SG stage. Note that RNAi initiated by *nos-gal4* typically perdures after *nos-gal4* expression ceases, due to persistence of RNAi (Bosch et al., 2016).

B-D. Apical tip of the testis stained for Vasa (red, germ cells) and Adducin-like (Add, blue, fusome) in controls (B), and *nos>me31B*$^{TRiP.GL00695}$ (C), and *bam>me31B*$^{TRiP.HMS00539}$ (D) knockdown lines. Note that both RNAi lines were similarly effective, and experiments were conducted using both RNAi lines (unless the genetics crosses were too complicated to generate a desired genotype). Throughout the manuscript, examples may be shown only with one RNAi construct, but the results were confirmed by using both constructs unless otherwise noted. Yellow dotted lines indicate GSC-GB pair (B), and yellow solid lines indicate dedifferentiating SG cyst (C, D). Note that fusomes are fragmented in dedifferentiating SG cysts (C, D). Bar: 10 µm. Hub is indicated by the asterisks.

E. An example of a continuous fusome observed in differentiating SGs (a 4-cell cyst). Bar: 10 µm.

F. Frequency of testes (%) containing dedifferentiating SG cysts attached to the hub with ≥3 germ cells and fragmented fusomes in control vs. *me31B* knockdown testes. n = number of testes scored. p-value from Fisher's exact test is provided compared to control.

We considered two possibilities that could explain this phenotype. First, *me31B* may be required in SGs to directly prevent their dedifferentiation. Second, *me31B* may be required to maintain GSCs in the niche, which would indirectly prevent SG dedifferentiation. To determine if *me31B* acts directly in SGs, we used the *bam-gal4* driver to deplete *me31B* only in the 4-cell SG and later stages (Chen and McKearin, 2003b). We found that about 50% of *bam>me31B*^{RNAi} testes contained dedifferentiating SG cysts (Figure 2.2D, F). These results demonstrate that *me31B* is required in SGs in a cell autonomous manner to prevent their dedifferentiation; however, we note that the frequency of dedifferentiation is higher when RNAi constructs were

driven by *nos-gal4* than by *bam-gal4*, suggesting that *me31B* may have additional functions in early germ cells to indirectly prevent dedifferentiation (see below).

Dedifferentiating SGs activate BMP signaling.

GSC identity in the *Drosophila* testis is specified by JAK-STAT and BMP signaling (Shivdasani and Ingham, 2003, Kawase et al., 2004, Schulz et al., 2004, Kiger et al., 2001, Tulina and Matunis, 2001). We examined whether the activation of these pathways was altered upon knockdown of *me31B*.

In wild-type testes, activation of BMP signaling triggers phosphorylation of Mad (pMad) in GSCs and in GBs that are still connected to GSCs (Kawase et al., 2004) (Figure 2.3A). We found that knockdown of *me31B*, either by *nos-gal4* or *bam-gal4*, resulted in a high pMad signal in germ cells outside GSCs and GBs (Figure 2.3B, C). Moreover, in $me31B^{RNAi}$ testes, we observed high pMad signal in all the germ cells within a dedifferentiating SG cyst attached to the hub (Figure 2.3B, C) and even in SGs that were not yet attached to the hub (Figure 2.3B). We observed pMad-positive germ cells outside the niche in only 7.7% of control testis (n=39 testes), but in over 50% of $me31B^{RNAi}$ testes (91.7% in *nos>*$me31B^{TRiP.HMS00539}$, n=48, 66.7% in *nos>*$me31B^{TRiP.GL00695}$, n=18, 58.8% in *bam>*$me31B^{TRiP.HMS00539}$, n=34, 54.8% in *bam>*$me31B^{TRiP.GL00695}$, n=31). These results indicate that the activation of BMP signaling precedes the re-acquisition of GSC identity during dedifferentiation in $me31B^{RNAi}$ testes, and may mediate dedifferentiation. Indeed, we found that overexpression of constitutively active Tkv (Tkv*) (Nellen et al., 1996), the receptor of BMP ligands, either by *nos-gal4* or *bam-gal4,* was sufficient to induce dedifferentiation (Figure 2.3D). Taken together, we propose that *me31B* may prevent dedifferentiation of SGs by directly or indirectly downregulating BMP signaling.

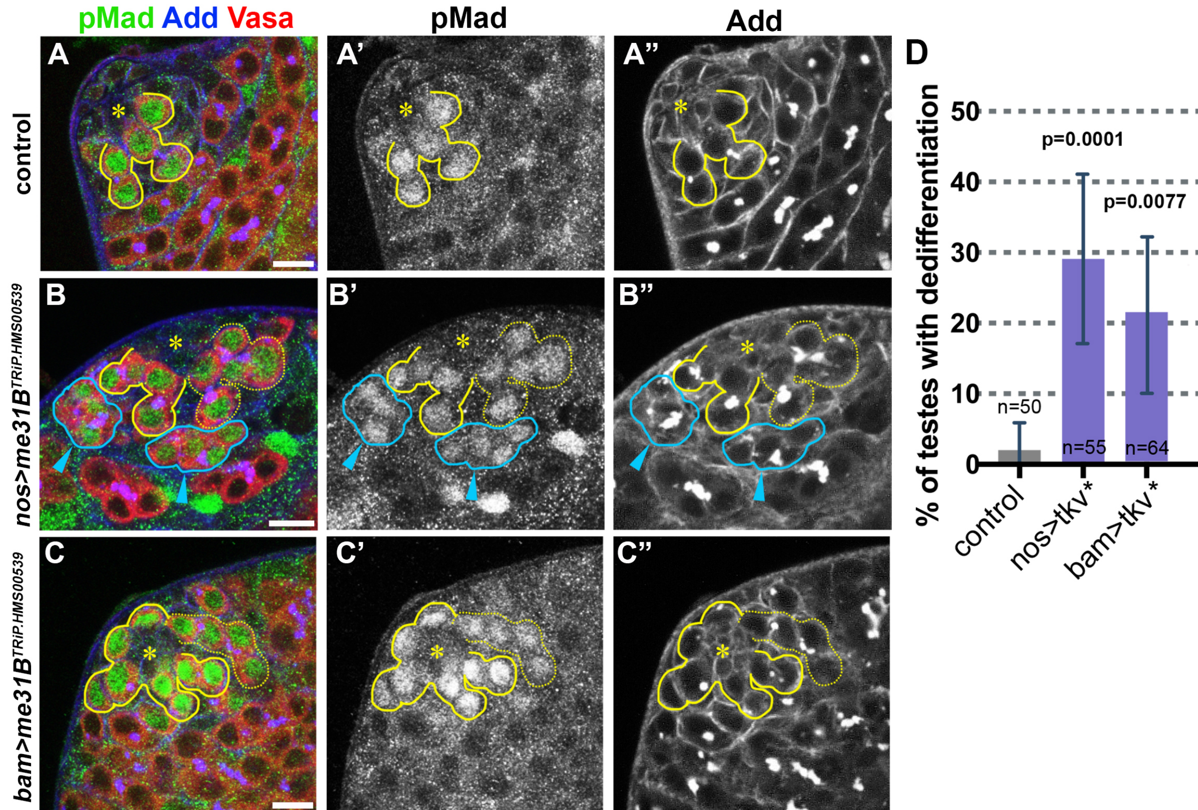

Figure 2.3: BMP Signaling Is Upregulated Upon Knockdown of *me31B*
A-C. Apical tip of the testes in control **(A)**, *nos-gal4>me31B*$^{TRiP.HMS00539}$ **(B)**, or *bam-gal4> me31B*$^{TRiP.HMS00539}$ **(C)** stained for pMad (green), Vasa (red), and Adducin-like (blue). Bar: 10µm. Hub is indicated by the asterisks. GSCs and connected GBs are indicated by yellow lines. Dedifferentiating cysts that are attached to the hub are indicated by yellow dotted lines. Dedifferentiating cysts that are not yet attached to the hub are indicated by blue lines and arrowheads.
D. Ectopic expression of constitutive active Tkv (Tkv*) either by *nos-gal4* driver or *bam-gal4* driver results in elevated dedifferentiation. n=number of testes scored. p-value from Fisher's exact test is provided compared to control.

In contrast to the deregulation of BMP signaling upon knockdown of *me31B*, we found that GSCs in *bam>me31B*^{RNAi} testes had similar STAT expression as control testes (Figure 2.4A-B), suggesting that dedifferentiation induced in *bam>me31B*^{RNAi} testes is not due to altered STAT signaling. However, STAT expression was reduced in GSCs of the *nos>me31B*^{RNAi} testes compared to control (Figure 2.4C-D), suggesting that *me31B* may have an additional role in GSCs to maintain STAT activation (see Discussion).

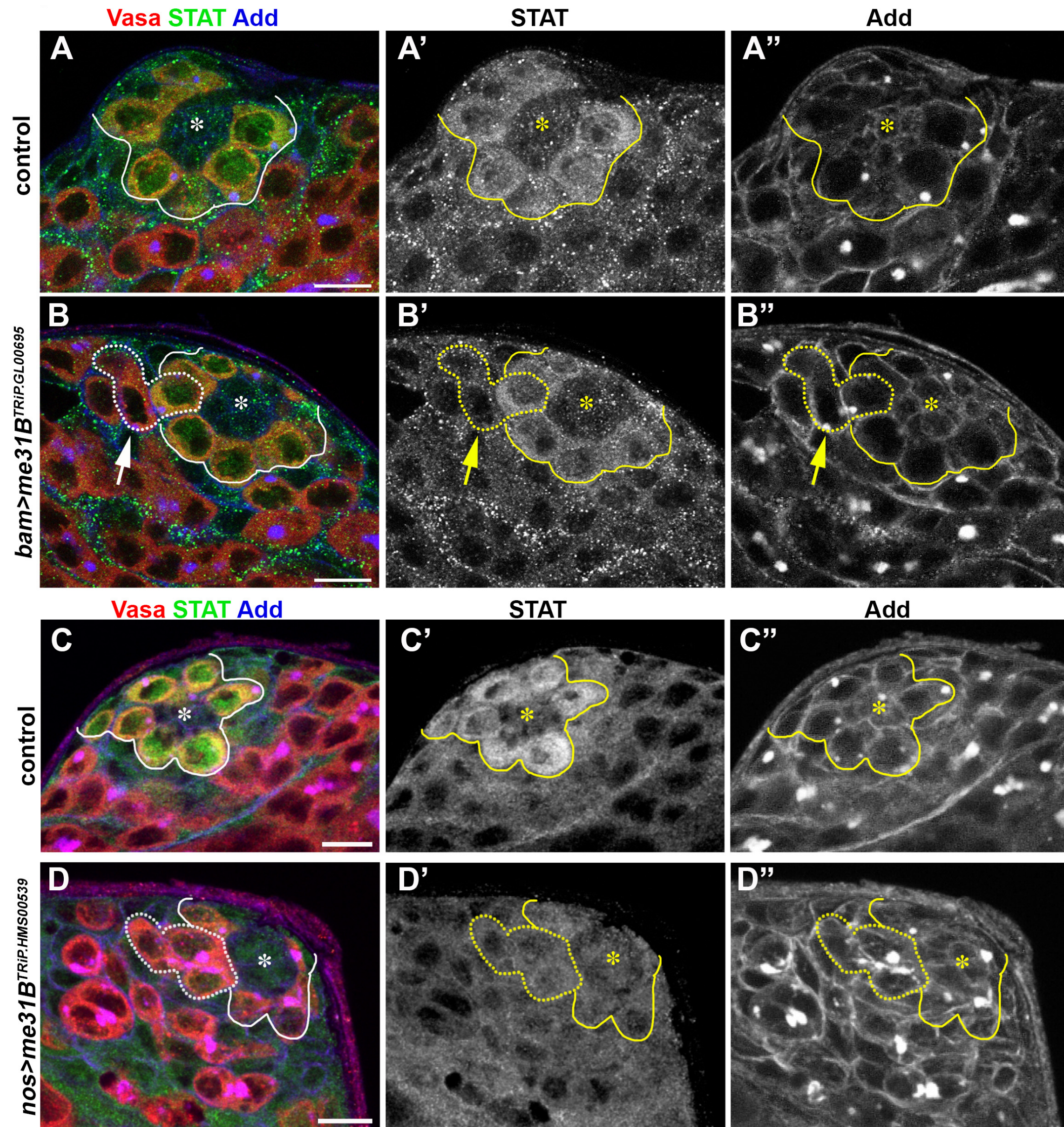

Figure 2.4: STAT Expression Upon *me31B*$^{TRiP.GL00695}$.
A-B. STAT signal in GSCs was reduced upon RNAi-mediated knockdown of *me31B* by *bam-gal4* driver. Control **(A)**, and *bam>me31B*$^{TRiP.GL00695}$ **(B)** testes. In **B**, a dedifferentiating cyst is indicated by the arrow, where only the germ cell that is attached to the hub has a high STAT signal, whereas the remaining germ cells do not have a high STAT signal. STAT level was monitored by anti-STAT antibody. GSCs are indicated by solid line. Dedifferentiating cysts, identified by fragmented fusomes connecting ≥3 germ cells and attachment to the hub, are indicated by dotted line. Hub is indicated by asterisk. Bar: 10μm. n= 47 for control, n= 44 for *bam>me31B*$^{TRiP.GL00695}$, n= 39 for *bam>me31B*$^{TRiP.HMS00539}$ (100% of testes exhibited normal STAT in all genotypes)
C-D. STAT signal did not change upon RNAi-mediated knockdown of *me31B* by *nos-gal4* driver. Control **(C)** and *nos>me31B*$^{TRiP.HMS00539}$ **(D)** testes. n= 10 for control, n= 36 for *nos>me31B*$^{TRiP.GL00695}$(81% of testes exhibited downregulated STAT), n= 17 for *nos>me31B*$^{TRiP.HMS00539}$ (100% of testes exhibited downregulated STAT).

Knockdown of *me31B* leads to misregulation of *nos* expression.

Previous work showed that Me31B silences *nos* mRNA translation during embryonic development of *Drosophila* (Gotze et al., 2017, Jeske et al., 2011). In the adult germline, Nos instructs germ cell identity and GSC maintenance via translational repression of critical targets, such as Bam (Li et al., 2009, Wang and Lin, 2004) and a regulatory feedback exists between *nos*, Mad and *bam* to control germ cell differentiation (Harris et al., 2011).

To investigate whether Me31B might regulate *nos* mRNA translation during spermatogenesis, we examined Nos protein levels upon knockdown of *me31B*. In control testes, we detected Nos protein in early-stage germ cells (GSC to 4-cell stage SGs) (Figure 2.5A). In contrast, upon knockdown of *me31B* either by *nos-gal4* or *bam-gal4*, we observed Nos protein even in 8-cell SGs (Figure 2.5B, C, D), consistent with Me31B downregulating *nos* mRNA translation in the *Drosophila* testis. Nos and Bam, a master regulator of differentiation (McKearin and Ohlstein, 1995, McKearin and Spradling, 1990), are expressed in a reciprocal manner and act antagonistically in stem cell maintenance and differentiation in the *Drosophila* germline (Li et al., 2009, Chen and McKearin, 2005). Indeed, *me31B* knockdown in the testes led to delayed Bam expression and a dramatic increase in the frequency of 4-cell SGs that lacked Bam protein (Figure 2.6).

To determine if Me31B regulates *nos* mRNA levels, we conducted single molecule RNA in situ hybridization to quantify *nos* mRNA levels (see Methods). We did not detect any difference in *nos* mRNA levels comparing control vs. *nos*>*me31B*^{RNAi} testes, either in GSCs or SGs (Figure 2.5E-J), suggesting that Me31B does not regulate *nos* mRNA levels. Taken

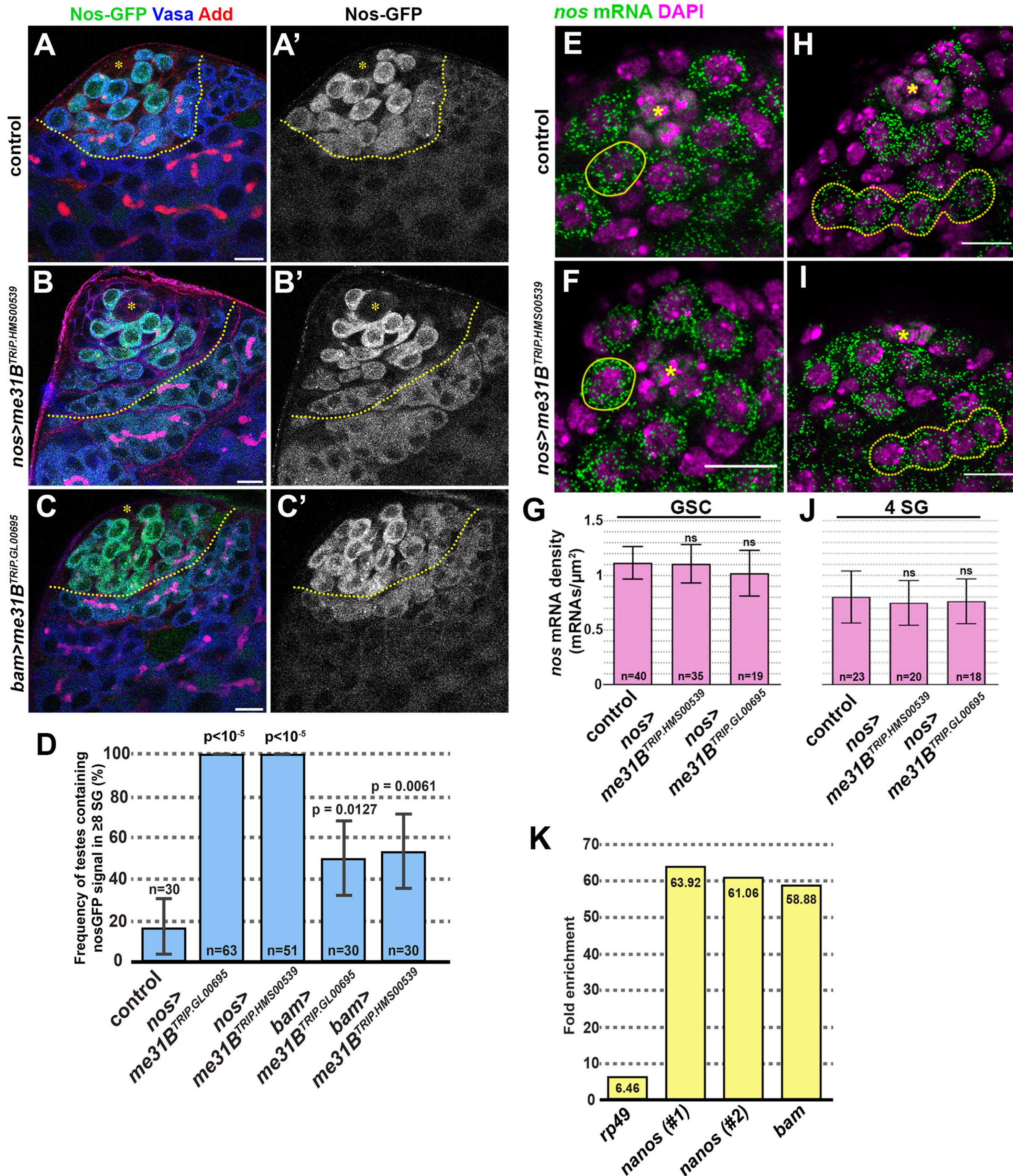

Figure 2.5: Me31B Binds to *nos* and *bam* mRNA to Promote SG Differentiation.
A-C. Apical tip of the testes expressing *nos-GFP* under the control of endogenous promoter and 3'UTR, stained for Vasa (blue) and Adducin-like (red). Control **(A)**, *nos >me31B$^{TRiP.HMS00539}$* **(B)**, or *bam>me31B$^{TRiP.GL00695}$* **(C)**. Bar: 10µm. Hub is indicated by the asterisks. The boundary between 4-cell and 8-cell SGs is indicated by yellow dotted lines.
D. The frequency of the testes that contains Nos-GFP-positive ≥8-cell SGs. n=number of testes scored. p-value from Fisher's exact test is provided compared to control.

E, F. Apical tip of the testes from control **(E)** or *nos>me31B*$^{TRiP.HMS00539}$ **(F)** probed for *nos* mRNA with single molecule RNA *in situ* hybridization with representative GSCs encircled. *nos* mRNA (green), DNA (magenta). The hub is indicated by the asterisks. Bar: 10µm.
G. The quantification of *nos* mRNA signals in GSCs from control and *nos>me31B*^{RNAi} (*nos* mRNA molecules/µm^2 at the central cross section). The indicated number of GSCs have been quantified from 6-9 testes of biological duplicates for each genotype. Error bars show SD, P values of t-tests are indicated.
H, I. Apical tip of the testes from control **(H)** or *nos>me31B*$^{TRiP.HMS00539}$ **(I)** probed for *nos* mRNA with single molecule RNA *in situ* hybridization with representative 4-cell SGs encircled. *nos* mRNA (green), DNA (magenta). The hub is indicated by the asterisks. Bar: 10µm.
J. The quantification of *nos* mRNA signals in 4-cell SGs from control and *nos>me31B*^{RNAi} (*nos* mRNA molecules/µm^2 at the central cross section). The indicated number of GSCs have been quantified from 5 testes of biological duplicates for each genotype. Error bars show SD, P values of t-tests are indicated.
K. Me31B-GFP RIP-qPCR probed for two sets of primers for *nos* mRNA and a primer set for *bam* mRNA, demonstrating that both *nos* mRNA and *bam* mRNA are highly enriched upon pulldown of Me31B-GFP protein. Standard deviation for each primer set is as following. *RP49*: 0.049237, *nos* #1: 0.046968, *nos* #2: 0.026151, *bam*: 0.065409.

together, these results suggest that Me31B regulates *nos* mRNA translation but not mRNA levels, consistent with other contexts where Me31B acts as a regulator of translation (Peter et al., 2019, Wang et al., 2017, Nakamura et al., 2001).

To determine if Me31B might regulate *nos* mRNA translation via direct binding, we performed RNA immunoprecipitation (RIP)-qPCR with testes expressing Me31B-GFP or GFP as a control (see methods: note that we also ectopically expressed Dpp to cause SG overproliferation (Schulz et al., 2004, Kawase et al., 2004, Shivdasani and Ingham, 2003) to increase the starting material). We found that *nos* mRNA co-immunoprecipitated with Me31B-GFP (Figure 2.5K). Interestingly, *bam* mRNA also co-immunoprecipitated with Me31B-GFP (Figure 2.5K). These results indicate that *nos* mRNA is likely a direct target of Me31B in the testis, and identify *bam* mRNA as a potential additional target. Overall, we conclude that *me31B* prevents dedifferentiation of SGs by reducing Nos protein levels and potentially increasing Bam protein levels.

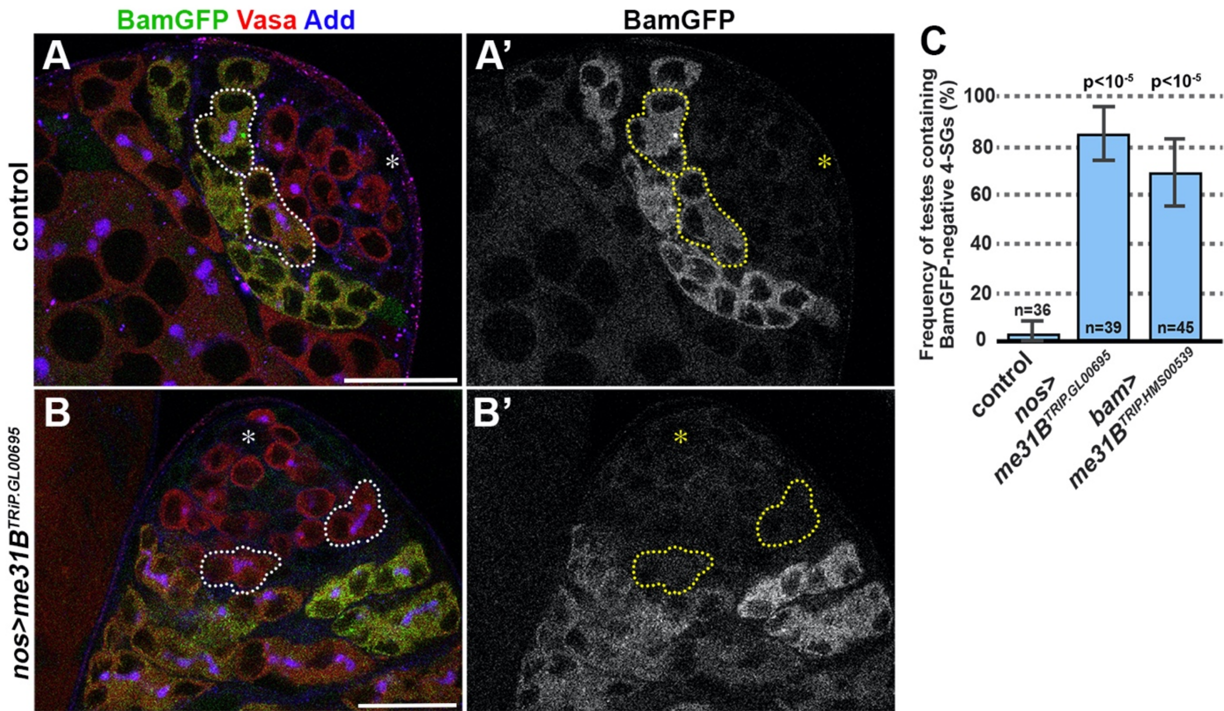

Figure 2.6: Bam Expression Is Delayed Upon Knockdown of *me31B*.
A. In control testis, germ cells start expressing Bam-GFP in 4-cell SG stage (indicated by dotted lines). **B.** Upon knockdown of *me31B*, 4-cell SGs often lacks Bam-GFP expression (dotted lines). Hub is indicated by asterisk. Bar: 25μm. **C.** Frequency of testes containing 4-cell SG without Bam expression in control vs. *me31B*^{RNAi} testes. n = number of testes scored. p-value from the Fisher's exact test is provided.

***nos* is necessary and sufficient for dedifferentiation.**

Based on the results described above, we hypothesized that Me31B prevents dedifferentiation in late SGs by silencing *nos* mRNA translation. This hypothesis predicts that *nos* downregulation would rescue the elevated dedifferentiation caused by knockdown of *me31B*. Indeed, we found that simultaneous knockdown of *nos* and *me31B* greatly reduced dedifferentiation to the level of the control (Figure 2.7A, Figure 2.8). These data suggest that *nos* is the major functional target of *me31B* in preventing dedifferentiation. To verify that the reduced dedifferentiation in the double knockdown lines is not due to the presence of two UAS-driven transgenes and thus dilution of the gal4 driver, we tested a control genotype expressing

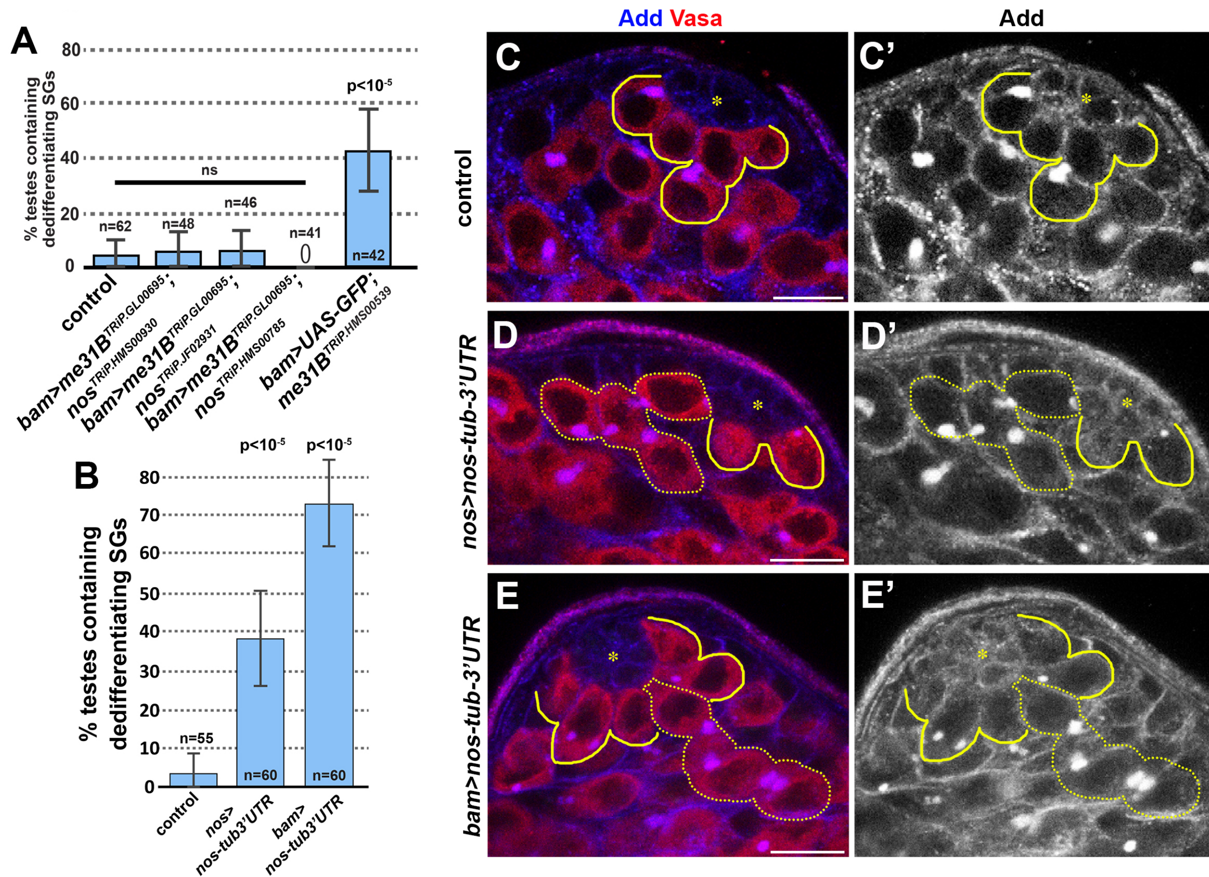

Figure 2.7. *nos* Is Necessary and Sufficient for Dedifferentiation
A. Frequency of testes containing dedifferentiating cysts in the indicated genotypes. Knockdown of *nos* diminishes dedifferentiation due to *me31B* knockdown. n=number of testes scored. p-value from Fisher's exact test is provided compared to control. ns: not statistically significant (p>0.5)
B. Frequency of testes containing dedifferentiating cysts upon ectopic expression of *nos* with *tubulin 3'UTR* (*nos-tub3'UTR*) driven by *nos-gal4* or *bam-gal4*. p-value from Fisher's exact test is provided compared to control.
C-E. Apical tip of testes from control testis **(C)**, testis expressing *nos-tub3'UTR* by *nos-gal4* **(D)** or *bam-gal4* **(D)**. GSCs and connected GBs are indicated by solid yellow lines, and dedifferentiating cysts are indicated by dotted yellow lines. Bar: 10µm. Hub is indicated by the asterisks.

$me31B^{RNAi}$ and a GFP transgene under the control of UAS. This genotype maintained the high frequency of dedifferentiation (Figure 2.7A, Figure 2.8). These results support that *nos* is necessary for the dedifferentiation induced by depletion of *me31B*.

Moreover, we found that upregulation of *nos* was sufficient to induce dedifferentiation. We employed a *nos* transgene in which the 3'UTR is replaced by the *tubulin* 3'UTR (*UAS-nos-*

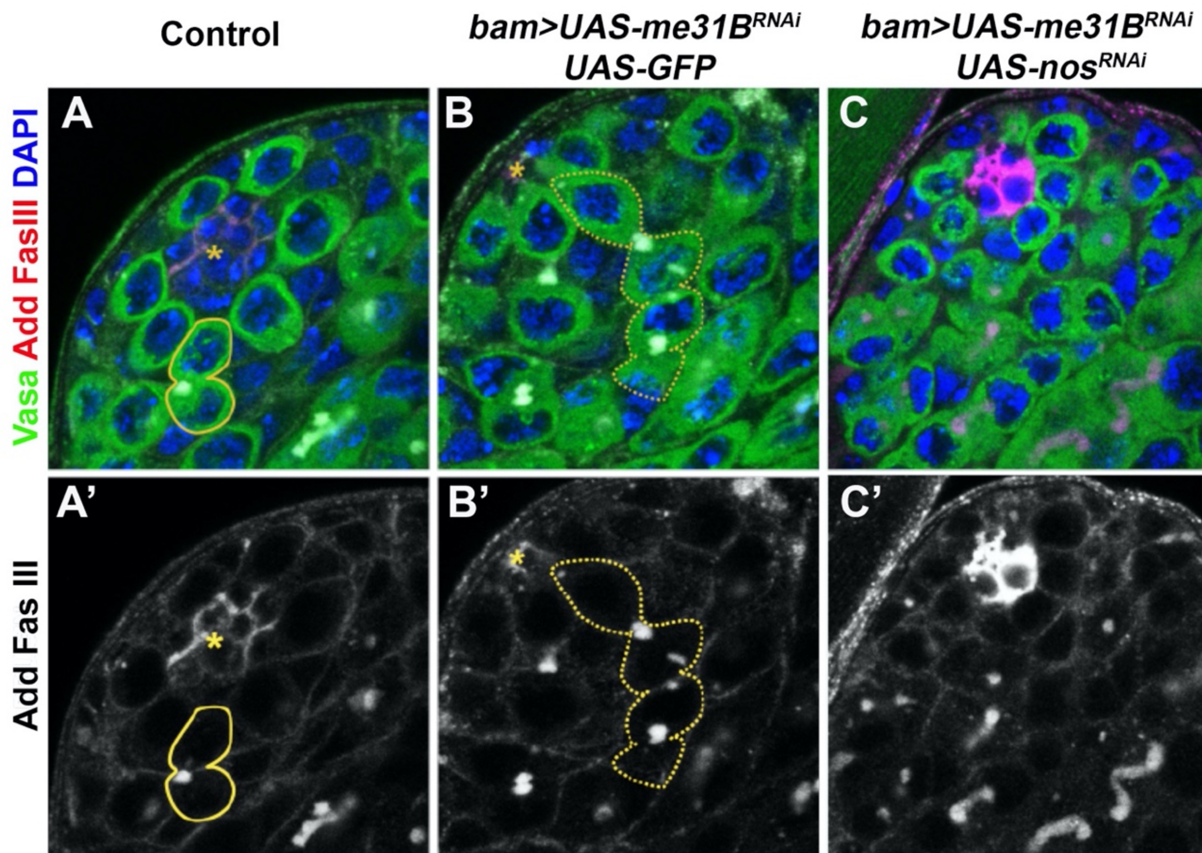

Figure 2.8: *nos* Is Required for Spermatogonial Dedifferentiation Induced by *me31B* Depletion.
A. Control, **B.** *bam>UAS-me31B^{RNAi}, UAS-GFP. UAS-GFP* was expressed to control for the number of transgenes driven by bam-gal4. **C.** *bam>UAS-me31B^{RNAi}, UAS-nos^{RNAi}*. *nos* depletion prevented dedifferentiation induced by *me31B^{RNAi}*.

tub3'UTR), which disrupts the regulation of *nos* by translational repressors such as Me31B (Gavis and Lehmann, 1994). When the *UAS-nos-tub3'UTR* transgene was expressed with the *nos-gal4* driver, we found that ~40% of testes contained dedifferentiating SGs, as opposed to ~3% in control (Figure 2.7B, C, D). Moreover, when the *UAS-nos-tub3'UTR* transgene was driven by *bam-gal4*, we observed an even higher frequency of dedifferentiation (~70%) (Figure 2.7B, E). These results suggest that upregulation of *nos* is sufficient to induce dedifferentiation.

Interestingly, when *me31B* knockdown was combined with *nos-tub3'UTR* expression under the control of the *nos-gal4* driver, it led to a near complete block of differentiation

(*nos>nos-tub3'UTR, me31B*$^{TRiP.HMS00539}$) (Figure 2.9). The differentiation block was so severe that our criteria of dedifferentiation used above (i.e. connected cells at the hub with fragmented fusomes) was not applicable, although we frequently observed cysts with fragmenting fusomes, indicative of dedifferentiation. 29% of testes (n=45 testes) contained SGs but never progressed to SC differentiation (which can be recognized by growth in cell size) (Figure 2.9B). In addition, 91% of testes (n=45 testes) contained SG cysts with ≥32 cells, further suggesting the failure in

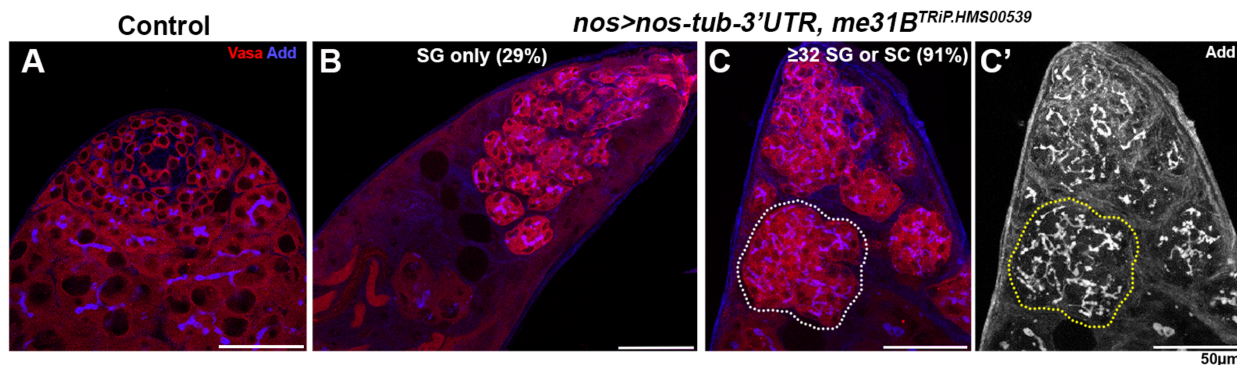

Figure 2.9: Combination of *nos* Upregulation and *me31B* Knockdown Blocks Differentiation.
A. Apical tip of the testes stained for Vasa (red) and Adducin-like (blue) in control **(A)**, or *nos>nos-tub3'UTR, me31B*$^{TRiP.HMS00539}$ **(B, C)**. A cyst that contains >>16 SGs is indicated by dotted lines in **C**. Bar: 50µm.

differentiation into SC stage (Figure 2.9C). It cannot be determined whether these SGs continue to proliferate (e.g. to 64 SG, 128 SG etc), as such cysts may also break apart by dedifferentiation. The fact that *nos* overexpression enhances *me31B*-knockdown phenotype implies that additional targets of *me31B* cooperate with misregulated *nos* to enhance the phenotype. Alternatively, further upregulation of endogenous *nos* due to *me31B* depletion and the *nos-tub3'UTR* transgene may enhance the effect.

Nos expression is dynamically regulated at multiple levels during differentiation in the male germline.

Regulation of *nos* mRNA translation has been well documented and intensively studied, particularly in the context of germ cell specification (Gavis and Lehmann, 1992, Gavis and Lehmann, 1994, Kugler and Lasko, 2009). The regulation of mRNA translation is critically important during oocyte development: the mRNAs that specify germ cell fate in the embryos, including *nos* and *osk* mRNA, are transcribed in nurse cells, transported into developing oocytes, and stored in mature oocytes to be translated later (Lehmann, 2016). Accordingly, mRNA synthesis (transcription) is spatially and temporally separated from protein production (translation), making it critically important to control the timing of translation by both translational repression and activation.

Whether *nos* transcription is spatiotemporally distinct from Nos protein production during the development of male germ cells in the testis is not known. To address this question, we generated a *nos* promoter reporter by driving a destabilized GFP (d2EGFP) fused to the *hsp70 3'UTR* from the *nos* promoter (Figure 2.10A). Because neither the mRNA nor protein products are stable in this reporter, the GFP signal closely recapitulates the activity of the *nos* promoter. Interestingly, we found that the *nos* promoter is active only in GSCs and GBs that are still connected to GSCs (Figure 2.10B), suggesting that *nos* is transcribed only in these early germ cells. These data suggest that Nos protein that is observed in 2- to 4-cell stage SGs is primarily produced by translation of *nos* mRNA inherited from GSCs and GBs (Figure 2.10C). In addition, stable Nos protein generated in GSCs and GBs may contribute to its persistence through to the 4-cell SG stage.

These results reveal dynamic regulation of *nos* expression through multiple layers (Figure 2.10C): 1) GSCs and GBs actively transcribe *nos* mRNA, which is translated to produce Nos protein. 2) 2- and 4-cell SGs no longer transcribe *nos* but inherit *nos* mRNA, which is translated

to produce Nos protein. 3) ≥8-cell SGs to not transcribe *nos* mRNA, and translation of inherited *nos* mRNA is inhibited by Me31B, leading to overall downregulation of Nos protein. Loss of

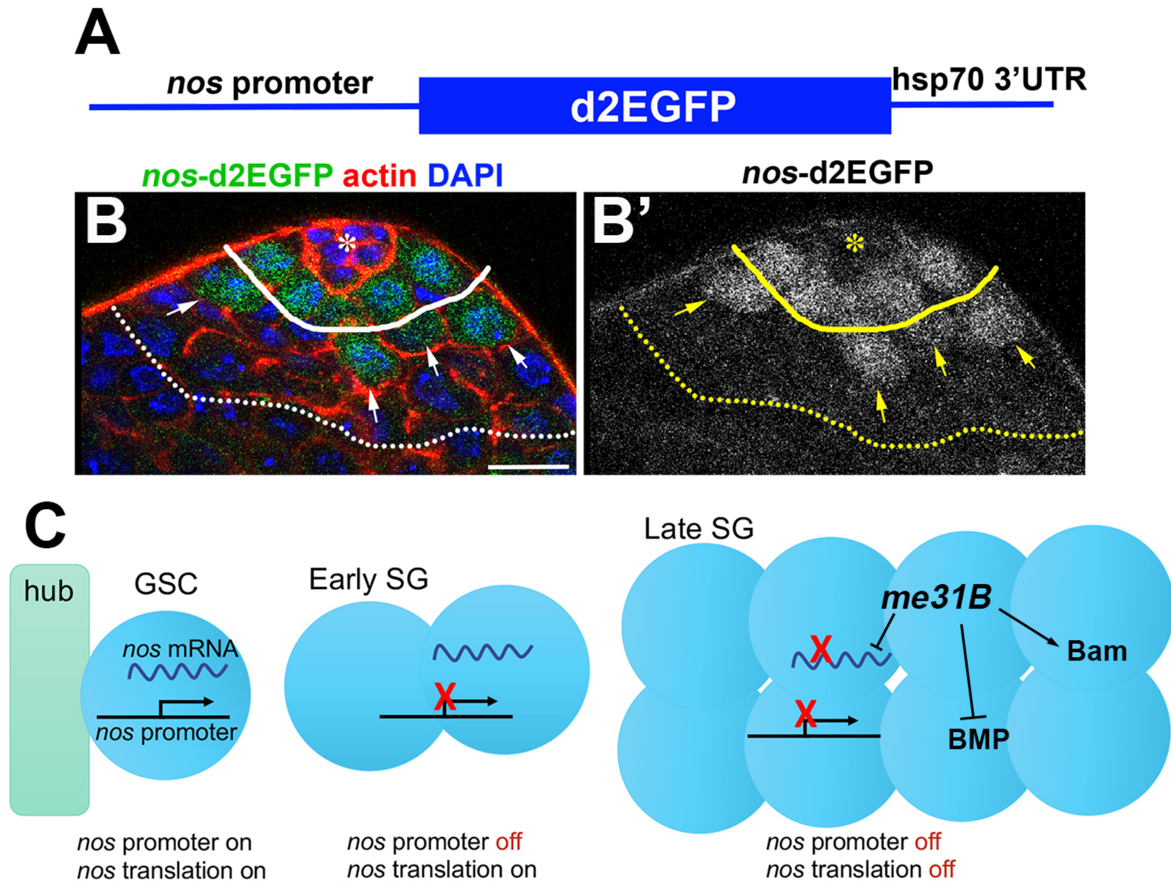

Figure 2.10: *nos* Is Transcriptionally and Translationally Regulated During *Drosophila* Spermatogenesis

A. Diagram of *nos* transcription reporter, where *nos* promoter drives unstable GFP protein and 3'UTR sequence from *hsp70*, which makes mRNA short-lived.

B. Apical tip of the testis expressing nos transcription reporter. GSC-GB boundary is indicated by solid line, and 4-cell/8-cell SG boundary is indicated by dotted line. GBs that are still connected to GSCs, thus still expressing *nos* transcription reporter are indicated by arrows. Bar: 10µm. Hub is indicated by the asterisk.

C. Model of *nos* regulation during germ cell development. In GSCs, the *nos* gene is transcribed and its mRNA is translated, leading to high Nos protein level and thus GSC maintenance. In early SGs, the *nos* gene is no longer transcribed, but Nos protein is produced via translation of inherited *nos* mRNA. In late SGs, the *nos* gene is no longer transcribed, and translation *nos* mRNA is inhibited by *me31B*. This leads to disappearance of Nos protein in these cells, promoting their differentiation. Interactions between *me31B* and its targets indicated by arrows may be direct or indirect.

Me31B leads to increased translation of *nos* mRNA, thus increased levels of Nos protein, promoting dedifferentiation at later stages.

DISCUSSION

Stem cell maintenance is critically important for long-term tissue homeostasis. Despite their ability to self-renew, stem cells are not immortal and their life span is often shorter than that of the organism. Dedifferentiation can replenish stem cell pools via conversion of more differentiated cells back into stem cell identity. However, uncontrolled dedifferentiation can lead to tumorigenesis (Schwitalla et al., 2013, Landsberg et al., 2012), thus proper control of dedifferentiation must be essential. Despite its importance, the mechanisms that regulate dedifferentiation are poorly understood.

This study identified *me31B* as a previously unknown and key negative regulator of dedifferentiation through its ability to regulate *nos* mRNA. Both *nos* and *bam* mRNAs co-immunoprecipitated with Me31B-GFP (Figure 2.5G). Me31B may reinforce the known antagonistic relationship between *nos* and *bam* in the germline (Li et al., 2009, Chen and McKearin, 2005) by independently regulating these transcripts (Figure 2.10C). In addition to extending Nos protein expression to 8-cell SGs and delaying Bam protein expression during germline development, depletion of *me31B* resulted in upregulation of BMP signaling, leading to an increased frequency of dedifferentiating SG cysts (Figure 2.3). It remains unknown whether *me31B* directly regulates any components of BMP signaling. However, given the antagonistic relationship between *nos* and *bam*, and that BMP signaling represses *bam* expression (Li et al., 2009, Wang and Lin, 2004, Harris et al., 2011, Song et al., 2004, Li et al., 2012, Chen and

McKearin, 2003a, Chen and McKearin, 2005, Chen and McKearin, 2003b)*,* it is possible that BMP upregulation can be explained as a downstream effect of misregulated *nos* and/or *bam.*

In contrast to the deregulation of BMP signaling upon knockdown of *me31B*, STAT does not appear to be a relevant target of *me31B* in inducing dedifferentiation (Figure 2.4). *bam>me31B*^{RNAi} testes did not detectably alter STAT signaling. Importantly, when a cyst of dedifferentiating *bam>me31B*^{RNAi} SGs was attached to the hub cells, only the germ cells that were in direct contact with the hub had high STAT levels (Figure 2.4B, arrow). These results indicate that germ cells in ≥4-cell SG cysts can reestablish STAT signaling upon homing into the niche during dedifferentiation triggered by depletion of *me31B*. Although downregulation of JAK-STAT signaling is reported to prevent SG dedifferentiation (Sheng et al., 2009), our data suggest that the dedifferentiation induced by depletion of *me31B* does not directly involve the activation of the JAK-STAT pathway. We speculate that JAK-STAT signaling might help maintain GSCs that were generated by dedifferentiation, instead of inducing dedifferentiation *per se*. Interestingly, however, STAT expression was reduced in GSCs of the *nos>me31B*^{RNAi} testes compared to controls (Figure 2.4C-D), suggesting that *me31B* has an additional role in GSCs to maintain STAT activation. Reduced STAT in *nos>me31B*^{RNAi} testes, which may deplete native GSCs, might explain why we observe a higher frequency of dedifferentiation with *nos-gal4*-driven *me31B*^{RNAi} compared to *bam-gal4*-driven *me31B*^{RNAi} (Figure 2.2F).

It remains elusive what controls *me31B* to promote differentiation and/or prevent dedifferentiation. Is *me31B* downregulated by conditions that trigger dedifferentiation? We did not observe any changes in Me31B-GFP protein level or localization when dedifferentiation was artificially induced by transient expression of Bam (not shown). In future studies, it will be of

interest to investigate whether and how Me31B senses niche vacancy (missing GSCs) to trigger dedifferentiation of SGs.

The right balance of differentiation and dedifferentiation must be achieved to ensure maintenance of the stem cell pool, while minimizing the risk of tumorigenesis. The results presented in this study suggest that SGs are in a state of transitioning from stem cell identity to full commitment to differentiation (SC). Whereas GSCs produce Nos protein via *nos* mRNA transcription and its translation, 2- and 4-cell SGs produce Nos protein only via translation of inherited *nos* mRNA. We propose that 2- and 4-cell SGs represent a critical cell population/developmental time window that is not yet fully committed to differentiation but maintains the potential to dedifferentiate, as they still have Nos protein like GSCs, but no longer transcribe *nos* unlike GSCs (Figure 2.10C). These SGs may hit a perfect balance of Nos protein that maintains their potential to dedifferentiate into GSCs as necessary, but prevents tumorigenesis by shutting down *nos* transcription. Indeed, 2- and 4-cell SGs are known to be most potent for dedifferentiation (Sheng and Matunis, 2011): although this was speculated to be mostly due to their physical proximity to the hub cells, it is also possible that their 'Nos production state' (actively producing Nos protein from inherited mRNA) is more suited for dedifferentiation than later SGs. We propose that stepwise transitions from the stem cell state to the differentiated state are key for maintaining the stem cell pool while preventing tumorigenesis. In summary, the present study provides a new insight into how gradual commitment to differentiation is ensured by transcriptional and translational control of a key regulator of cell fate.

MATERIALS AND METHODS

Fly husbandry and strains

Unless otherwise stated, all flies were raised on standard Bloomington medium at 25°C, and young flies (1- to 3-day-old adults) were used for all experiments. See Table 2.1 for the list of stocks used in this study.

Table 2.1: List of *Drosophila* Stocks in this Study

Symbol/name used in publication	Source information
nos-gal4 (on Chr2)	PMID: 9501989
nos-gal4 (on Chr3)	FBti0012410/ PMID: 9501989
bam-gal4	PMID: 12571107, Gift from Dennis McKearin
UAS-me31B P{TRiP.HMS00539}attP2	FBst0033675
UAS-me31B P{TRiP.GL00695}attP40	FBst0038923
UAS-me31B P{TRiP.HM05052}attP2	FBst0028566
*UAS-tkv**	FBst0036537
STAT-GFP	FBst0038670
nos-GFP	FBal0339106
hs-FLP, nos-FRT-stop-FRT-gal4	PMID: 24465278
nos-dE2EGFP	see methods
UAS-nos-tub3'UTR	FBal0141015/ PMID: 12091303
UAS-nos P{TRiP.JF02931}attP2	FBst0028300
UAS-nosP{TRiP.HMS00785}attP2	FBst0032985
UAS-nosP{TRiP.HMS00930}attP2	FBst0033973
Me31B-GFP	FBst0051530
Bam-GFP	FBal0144433/ PMID: 12571107
UAS-GFP	FBti0013987
UAS-Dpp	FBst0001486

Immunofluorescence staining and microscopy

Immunofluorescence staining was performed as described previously (Cheng et al., 2008). Briefly, tissues were dissected in the phosphate-buffered saline (PBS), transferred to 4% formaldehyde in PBS and fixed for 30 min. Tissues were then washed in PBS-T (PBS containing 0.1% Triton-X) for at least 30 min (three 10 min washes), followed by incubation with primary antibody in 3% bovine serum albumin (BSA) in PBS-T at 4°C overnight. Samples were washed for 60 min (three 20 min washes) in PBS-T, incubated with secondary antibody in 3% BSA in PBS-T at 4°C overnight, washed as above, and mounted in VECTASHIELD with DAPI (Vector Labs). The antibodies used are described in Table 2.2. Images were taken using a Leica TCS SP8 confocal microscope with 63x oil-immersion objectives (NA = 1.4). Images were processed using Adobe Photoshop and ImageJ software.

Dedifferentiating SG cysts were identified as the cysts containing ≥3 SGs that are connected to each other by the fragmented fusome (Figure 2.2C, D). In contrast, normally-differentiating SGs contain continuous fusome that connects all cells within the cyst (Figure 2.2E). Thus, the morphology of the fusome distinguishes differentiating SGs vs. dedifferentiating SGs. The connectivity of cells within the dedifferentiating SGs was determined by the presence of fusome fragments between two cells within a cyst: for example, the connection between cell #1 and cell #2 can be confirmed by the presence of the fusome fragment between these two cells. Cell #2 may be then connected to cell #3 with another fragment of the fusome, establishing the connectivity of cell #1, #2 and #3, and so on. In rare cases, when two cells clearly shared the cytoplasm by continuous Vasa staining, such cell pairs may be determined as connected without the presence of fusome in between. When ≥3 cells were determined to be connected to each other with this method, and found at the hub cells, such SG cysts were scored as

‘dedifferentiating’. Significance was determined using a Fischer’s Exact Test in comparison to a control.

Table 2.2: List of Antibodies Used in this Study

Anti-pSmad	Cell Signaling: Phospho-Smad1/5 (Ser463/465) (41D10) Rabbit mAb #9516	1:100 dilution for immunofluorescence (IF) staining
Anti-GFP	Fisher Scientific, mouse monoclonal antibody (3E6)	Used for RIP (see methods)
Anti-STAT	PMID: 26131929	1: 5000 dilution (from original serum)
Anti-Add/Hts	DSHB	1:20 dilution for IF
Anti-Vasa	DSHB	1:20 dilution for IF

RNA Fluorescent in situ hybridization

To detect *nos* mRNA, single molecule fluorescent in situ hybridization (smFISH) was conducted by following a previously described protocol (Fingerhut et al., 2019). All solutions used for smFISH were RNase free. Testes from 2–3 day old flies were dissected in 1X PBS and fixed in 4% formaldehyde in 1X PBS for 30 minutes. Then testes were washed briefly in PBS before being rinsed with wash buffer (2X saline-sodium citrate (SSC), 10% formamide) and then hybridized overnight at 37°C in hybridization buffer (2X SSC, 10% dextran sulfate (sigma, D8906), 1mg/mL E. coli tRNA (sigma, R8759), 2mM Vanadyl Ribonucleoside complex (NEB S142), 0.5% BSA (Ambion, AM2618), 10% formamide). Following hybridization, samples were washed three times in wash buffer for 20 minutes each at 37°C and mounted in VECTASHIELD with DAPI (Vector Labs). Images were acquired using an upright Leica TCS SP8 confocal microscope with a 63X oil immersion objective lens (NA = 1.4) and processed using Adobe Photoshop and ImageJ software. Fluorescently labeled probes were added to the hybridization buffer to a final concentration of 50nM (for satellite DNA transcript targeted probes). Probe set against *nos* exons was designed using the Stellaris® RNA FISH Probe Designer (Biosearch Technologies, Inc.) available online at www.biosearchtech.com/stellarisdesigner. The

Stellaris® RNA FISH (Biosearch Technologies, Inc.) probes were labeled with Quasar 670. Probe set was added to the hybridization buffer in 50nM final concentration. For smFISH probe sequences, see Table 2.3.

Table 2.3: List of Probe Sequences for *nos* RNA *in situ* Probes (Stellaris ®). Probes Were Conjugated to Quasar 670.

5'-tccaagttgctgcggaacat-3'/ 5'-aaagttatctgctgctgcgc-3'/ 5'-ctcctctggcgtgaaaagca-3'/ 5'-tgcaggcccagaatgttgag-3'/ 5'-ccactggtatccaaatacat-3'/ 5'-gtaatgggcggactcaaagt-3'/ 5'-tcggccagaaaagggaagtg-3'/ 5'-cataaggagcgaattggcgg-3'/ 5'-caagtggtagtggtactgtc-3'/ 5'-ttgctggtgactcgcactag-3'/ 5'-aaggatcgcgcaatctcgtc-3'/ 5'-cgtcacctgcgcaaagattt-3'/ 5'-catagccattggtcgcgaac-3'/ 5'-taggacatgcgaccgagatc-3'/ 5'-cattaagttgccgccattgg-3'/ 5'-agtgggtggcgagtggaatg-3'/ 5'-cacacgttgttcagatgctc-3'/ 5'-ggctggtatatacgacatgt-3'/ 5'-ctgcaaacccattgtattgg-3'/ 5'-cgagattggtggacacagtg-3'/ 5'-tactggaattggaagctccg-3'/ 5'-ttgctgttgtaacgcttgta-3'/ 5'-aaaagacgcagtggcggctg-3'/ 5'-tctggttcgttgttattctc-3'/ 5'-gcactgagtggctattgata-3'/ 5'-cacagcactcggttaaagtt-3'/ 5'-acacgtaggtgcgtagtttg-3'/ 5'-cagtacttaatcgtgtgcgc-3'/ 5'-atggtgatgatcggcttctt-3'/ 5'-gaacgattccgccttgatcg-3'/ 5'-agtaactgctcttggctagg-3'/ 5'-taaaccttcatctgttgctt-3'

RNA immunoprecipitation (RIP)-qPCR

Samples were collected from two genotypes, a control (*nos-gal4>UAS-GFP, UAS-dpp*) and an experimental (*nos-gal4>UAS-dpp, me31B-GFP*) and processed in pairs. Dpp overexpression (*UAS-dpp*) was introduced to increase SGs in the sample. ~200 testes per sample were collected into RNAse-free PBS, frozen in liquid nitrogen after removing excess liquid, and stored at -80°C until extraction. Lysis was completed by grinding the tissue in 400 µL of lysis buffer (150 mM KCl, 20 mM HEPES pH 7.4, 1mM $MgCl_2$ with 1x c0mplete™ EDTA-free Protease Inhibitor Cocktail and 1U/µl RNasin® Plus RNase Inhibitor from Promega added right before the use) and incubating for 30 minutes on ice with pipetting every 10 minutes. After centrifugation at 12,000xg for 5 minutes, pelleted cell debris were discarded. At this point, a 10% pre-IP input sample was removed and saved to serve as a control. For precipitation of Me31B-GFP and control GFP, GFP-conjugated magnetic beads were prepared by incubating 10 µg of mouse anti-GFP antibodies (Fisher Scientific) with 50 µL of Protein G Dynabeads™ in 200 µL of Ab Binding and Washing Buffer (provided in the kit) for 10 min at room temperature

on a rotator. After antibody conjugation, beads were magnetically separated and washed once with 200 µL of Ab Binding and Washing Buffer. The antibody-conjugated beads were then incubated with the lysate for 10 minutes at room temperature (samples tubes were tumbled end-over-end during incubation). After magnetic separation of the beads, 10% of the supernatant was taken as non-bound fraction sample. The beads were washed with the Dynabeads Protein G kit Washing Buffer 3 times, and were resuspended in TRIzol (the 10% pre-IP and 10% post-IP samples were also processed with TRIzol at this time) according to the manufacturer's instructions. cDNA was generated using SuperScript III® Reverse Transcriptase (Invitrogen) followed by qPCR using *Power* SYBR Green reagent (Applied Biosystems). 10% inputs were diluted to a 1% input before RT was run. The fold enrichment was calculated by the ΔΔCt method. First, Ct values from each IP sample were normalized to their respective 1% input for each primer (ΔCt) to account for RNA sample preparation differences.

ΔCt [normalized RIP] = Ct [RIP] – (Ct [Input] – $Log_2$100)

Then, the ΔΔCt (Me31B-GFP/control GFP) was obtained to compare these normalized values between the Me31B-GFP sample versus the UAS-GFP control for each primer set.

ΔΔCt [Me31B-GFP/control GFP] = ΔCt [normalized Me31B-GFP RIP] - ΔCt [normalized control GFP RIP]

Finally, the fold enrichment was obtained by the following formula.

Fold enrichment = $2^{-\Delta\Delta Ct}$

Experiments were done in technical triplicates with three biological replicates. Primers used are the following: *rp49*, forward 5'-TACAGGCCCAAGATCGTGAA-3', reverse 5'-TCTCCTTGCGCTTCTTGGA-3'. *nanos* set #1, forward 5'-CAGTACCACTACCACTTGCTG-3', reverse 5'-AAAGATTTTCAAGGATCGCGC-3'. *nanos* set #2, forward 5'-

CACCGCCAATTCGCTCCTTAT-3', reverse 5'- GCTGGTGACTCGCACTAGC-3'. *bam*, forward 5'-TGACGTTACTGCACCACTCC-3', reverse 5'-CGAACAGATAGTCCGAGGGC-3'.

Acknowledgements

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CHAPTER THREE:

Implications, Future Directions, and Conclusions

SUMMARY OF RESULTS

The key insight of the work described here is the identification of the translational regulation pathway in place to balance dedifferentiation endogenously. This axis, the direct suppression of *nos* mRNA by Me31B and their subsequent interaction with the differentiation factor Bam, is the first time a pathway for suppressing male dedifferentiation has been described. Not only is *nos* necessary and sufficient for dedifferentiation, but the spatial landscapes for *nos* transcription and Nos protein activity proved to be radically different from one another. These results imply that transcriptional choices in GSCs coupled with translational control in the SGs can be a means of setting the pace of differentiation for the next several cell divisions. We also found that not only did SGs in the process of dedifferentiating have increased BMP signaling, but overexpressing *dpp* receptors in SGs is sufficient to significantly increase the rate of dedifferentiation, both of which suggest that *dpp* and BMP signaling is an important pathway to activate in cysts returning to the niche and possibly responsible for initiating dedifferentiation. Taken together, these results indicate that keeping a population of daughter cells only partially differentiated and thus ready for easy dedifferentiation (via translational control of *nos* mRNA by Me31B) can be a vital resource for the niche in times of need (a need potentially communicated and/or answered by BMP signaling).

However, this model is by no means complete, nor does it address many pressing outlying questions that need to be answered. Below, I will explore some of these questions and, in some cases, outline other pieces of data generated to begin answering them.

REGULATION OF *NOS* IN SPERMATOGONIA CREATES A "TIMER" FOR DEDIFFERENTIATION.

If dedifferentiation is a strategy for sustaining stem cell populations but carries the risk of tumorigenesis, then I wish to reframe a key introductory question for the reader: how do the data presented thus far help us understand how a beneficial result is achieved over a harmful one? In short, these results point to the unique "molecular signature" — that is, the differences in transcription and translation of certain genes over time — of the spermatogonia, which allows these cells to both differentiate and retain vestiges of gene expression related to GSC identity, as a key factor in managing risk and reward.

In the *Drosophila* testis the SGs are transit-amplifying cells: the stage in which a germ cell (not a *germline stem cell*, which divide asymmetrically to create a differentiating germ cell; see Fig 1.1, page 3) divides symmetrically to rapidly create several more cells moving towards meiosis. Thus far, previous work has regarded the creation transit-amplifying cell populations as method of reducing the overall burden on GSCs by outsourcing the creation of additional cells to their daughters. This also keeps GSC populations smaller and more manageable, which is important with proliferative populations in order to reduce risk of tumorigenesis (see introduction, page 12). Beyond their characterization as a 'transitory' state, little attention is paid to unique identity of these populations. However, the work presented in this thesis suggest that it

is precisely the ‘transitory nature’ of the molecular signaling in transit amplifying cells that can also provide additional opportunities to benefit an organism’s fitness.

By characterizing the unique molecular signature of transit-amplifying cells over time, we found that SGs maintain a steady pace of differentiation ***and*** mimic GSC identity at the same time for a significant window of development. In addition to Nos protein production and *nos* mRNA, true GSCs also have active *nos* transcription (Fig 2.10B and Fig 3.1), which sets them apart from all other germ cells in the testis. In contrast, the transit amplifying cells (SGs) do not have active *nos* transcription, instead relying on already existing *nos* mRNA for production of Nos protein (Fig 2.5E-J and Fig 3.1). Ultimately, the loss of *nos* mRNA and protein likely represents terminal commitment to meiosis.

These differences in molecular signature become more than just a ‘transitional phase’ in the context of dedifferentiation. If perpetual Nos protein expression is required for GSC identity but only GSCs transcribe *nos* mRNA, then SGs are running out over time and the remaining *nos* mRNA serves as a “timer”. Although presence of Nos protein may mean SG identity is similar to GSC identity, this identity cannot be fully “claimed” by SGs without additional transcription, which can only be achieved by moving into the niche, the only place where they can undergo additional *nos* transcription. Thus, some level of Nos protein expression in SGs is required for dedifferentiation to be possible and some outside queue/event (e.g., a signal that will prompt dedifferentiation, see next section) must be received by the SG before the Nos levels are depleted to the point that the timer ‘expires’.

Conceptually, this paradigm reveals a new use for transit-amplifying cell population: by setting an expiration date on the same gene necessary for reversion, these cells are forced into binary choice. If we imagine the process of differentiation as a slope, the cells are capable of

moving in either direction (dedifferentiate or differentiate) so long as they abide by two simple rules: 1) they must always be actively transitioning (e.g., moving along the slope) and 2) the presence or absence of a queue determines their direction. Essentially, the testis ensures dedifferentiation is beneficial by holding an entire pool of cells in a gradually transitory but directionally binary state: if these cells are not actively dedifferentiating, they ***must*** differentiate.

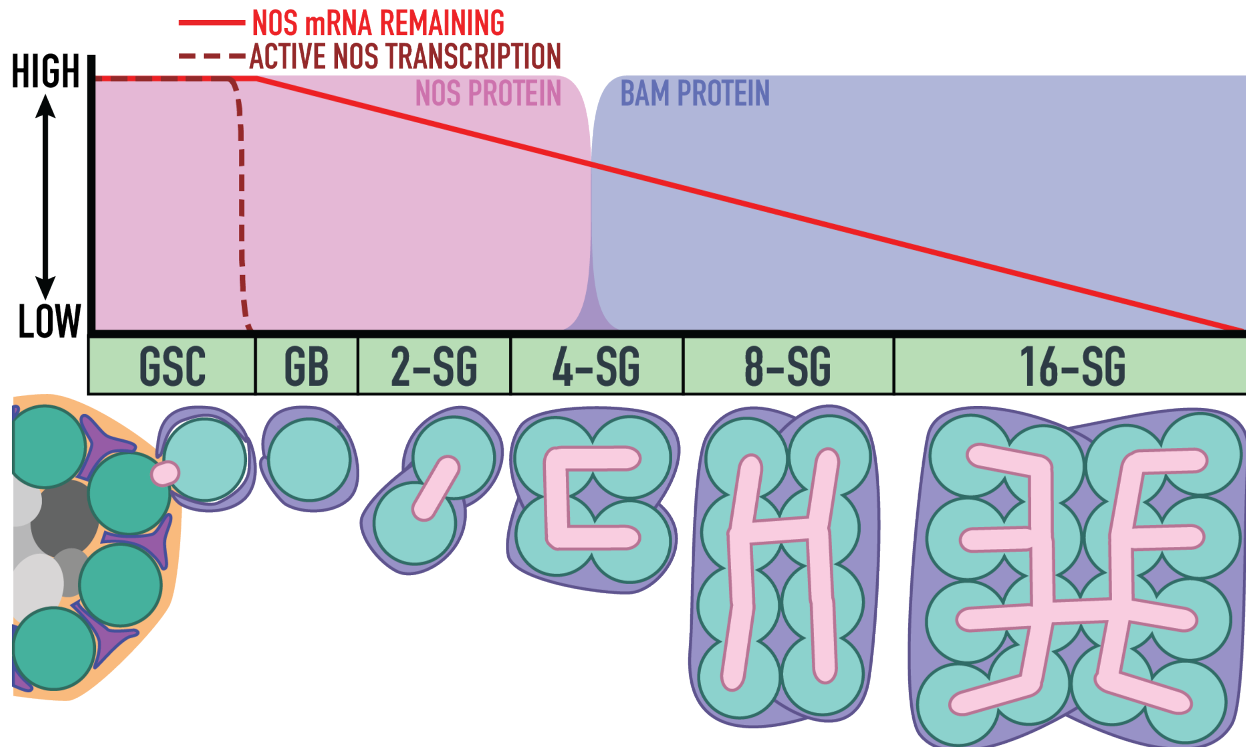


Figure 3.1: Representation of *nos* Transcription, mRNA Levels, and Protein Levels in The Germ Cells.
Taken together, this model shows a theoretical distribution of *nos* active transcription, *nos* mRNA levels, Nos protein expression, and Bam protein expression in a wildtype testis with no cysts currently dedifferentiating. Nos and Bam protein levels are shown as pink and lavender curves respectively, based on GFP protein labeling seen in Fig 2.5A and 2.6A. *nos* mRNA levels are denoted by the bright red line, present but decreasing in abundance as shown in Fig 2.5E-J. Active nos transcription is denoted by the maroon dotted line, which is only present in the GSCs and their attached daughter cells as seen in Fig 2.10B.

SENSING NEED AND/OR NICHE VACANCY: INITIATION OF DEDIFFERENTIATION.

Given that dedifferentiation is a method of maintaining stem cell homeostasis but requires a balance between GSC replenishment and the possibility of cancer, dedifferentiation must be carefully regulated to create only enough stem cells to fill the loss. How then is the loss of GSCs detected in the niche? After loss is sensed, how exactly is dedifferentiation initiated endogenously? Does the niche sense that it needs GSC replenishment and thus sends out a signal that SGs respond to? Or do SGs sense a spatial vacancy and then move to occupy them? How this process is initiated and achieved is a fascinating question, but remains virtually uninvestigated. Here I will discuss several possibilities, integrating our results.

Detecting GSC loss, the "leaky niche" model, and *dpp*

Since all recent research on dedifferentiation in the testis confirm that it is a mechanism for replenishing lost GSCs, the first question to ask should be, "Is GSC loss actively detected?" After all, cells need not necessarily be "called" in order to find and remain in a freshly available space. In this case, that could mean the SG cysts might simply slip into place of a lost GSC via chance and proximity. The alternative hypothesis would be that GSC loss is actively detected (likely by the hub cells in the niche).

Multiple pieces of data from several previous studies support the latter model, including the data presented in this thesis. Although live imaging has shown that the likelihood of dedifferentiating decays as SGs get further and further from the niche (Sheng and Matunis 2011), I would expect the rate of dedifferentiation to become nearly nonexistent for SGs at the 4-cell stage or beyond if dedifferentiation were primarily a passive function of filling a space. Instead, live imaging from previous work and our own work frequently observe dedifferentiation from

SGs that have already begun expressing *bam*—meaning they have already put two or more cell lengths between themselves and the niche (Sheng and Matunis 2011).

Indeed, Sheng et al. 2009 also argues that their data implies GSC loss is actively detected. They conclude that since their method of forcibly differentiating GSCs leaves the cyst stem cells (CySCs) behind, the niche is not truly empty when they observe dedifferentiation (Sheng, Brawley, and Matunis 2009). However, this argument fails to account for the multiple ways that GSCs both adhere to and communicate with the hub cells, both of which could be methods to sense vacancy outside of physical presence. Given that the adherens junctions and the nanotubes facilitate constant close-range niche signaling between the two cell populations, it is difficult to believe that any GSC could be lost without notice (Inaba et al. 2010; Inaba, Buszczak, and Yamashita 2015). Clearly, there are multiple functions already in place that the niche could conceivably play a part in sensing the presence/absence of GSCs.

Instead, the more compelling piece of evidence from Sheng et al. 2009 in support of this model are their observations of the behavior of dedifferentiating cysts themselves. Fluorescent labeling of the membrane in dedifferentiating SGs revealed long cytoplasmic protrusions reaching out from the cyst towards the hub as it crawled back towards the apical tip of the testis (Sheng, Brawley, and Matunis 2009). The implication of these data is twofold: 1) dedifferentiating cells are actively moving towards a goal, and 2) they are likely following an extrinsic signal. Thus far, the niche is by far the most likely source for any type of external signaling ligand.

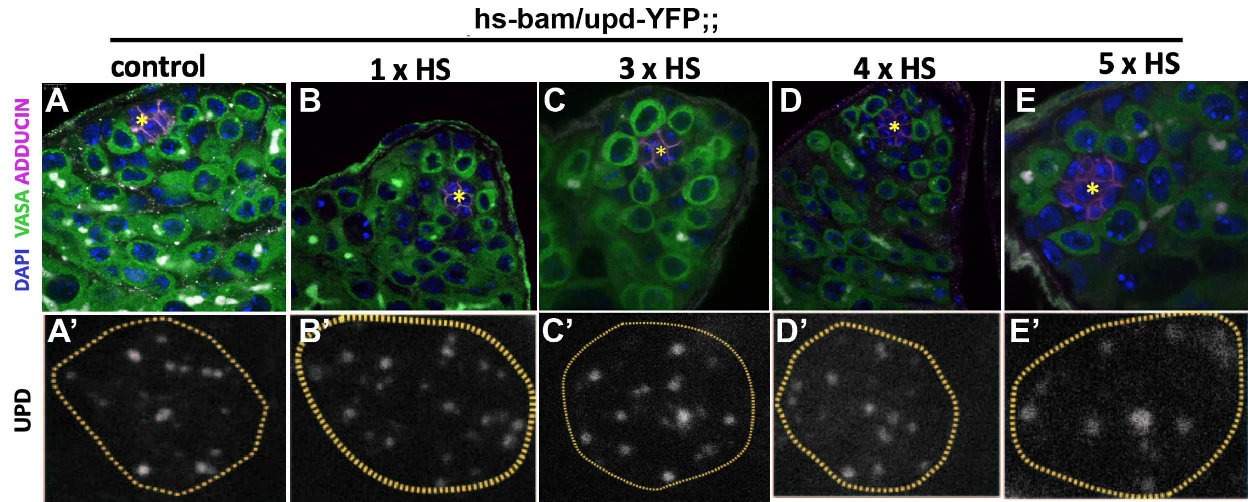
Then what is the signal? There are two primary possibilities: niche ligands we already know exist in the testis or a completely new and unknown ligand. Here, I will discuss the possibility that the ligand responsible for initiation is either *dpp, upd,* or perhaps both. The main

questions associated with this option are 1) does niche behavior change or not? And; 2) is one or the other ligand responsible or is it both?

The simplest hypothesis is that the “lack of change” in the niche combined with stem cell loss triggers dedifferentiation. Under typical circumstances, the niche and the GSCs are bound tightly together and niche signaling factors are exchanged in very close, highly controlled quarters using cytoplasmic nanotubules (Inaba, Buszczak, and Yamashita 2015). Should GSC loss disrupt this connection and the niche carries on with producing the ligand (e.g., there is a ‘lack of change’ in niche behavior), then niche signaling ligands will theoretically diffuse or “leak” outwards. These ligands are then possibly picked up by the differentiating SGs, who interpret them as a signal to assume GSC identity and return to the niche.

Although the niche uses two ligands to communicate with GSCs to sustain their identity (*dpp* and *upd*, BMP signaling and JAK-STAT signaling respectively) (Morrison and Spradling 2008; de Cuevas and Matunis 2011), our data imply that they are not equally likely to be responsible for initiating dedifferentiation after leaking. Our data already show that BMP signaling is upregulated in dedifferentiating SG cysts, while STAT signaling is only established upon physical contact with the hub. Similarly, our data show that overexpression of BMP receptors in the SGs leads to a significantly higher rate of dedifferentiation in comparison to overexpression of STAT receptors. These results imply that if one of the niche ligands is responsible for initiating dedifferentiation, it is likely to be *dpp* and the BMP signaling pathway.

Ultimately these data prompt another important question: If the disrupting of GSC connections to the hub causes niche ligand diffusion, then why would only Dpp be the ligand diffusing? Theoretically, it would be equally likely that Upd would also diffuse from the niche during GSC loss, so why don’t we see evidence of it? There are two primary explanations: 1)



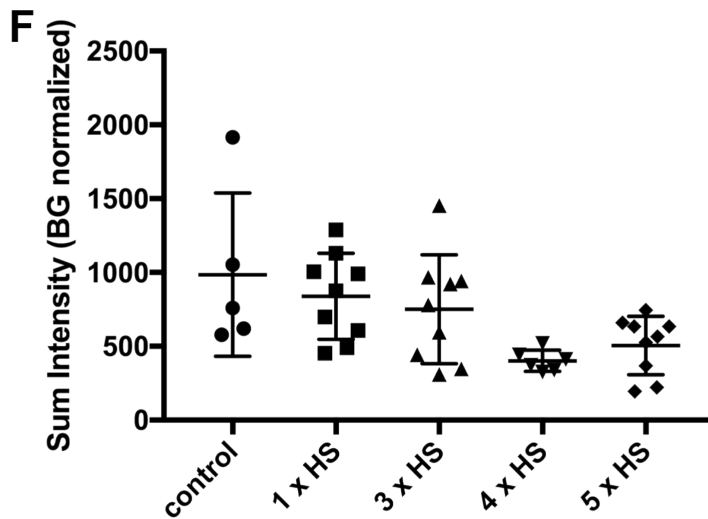


Figure 3.2: GSC Disruption via *hs-bam* Caused No Observable Change in Upd-YFP.
A-E) Staining and morphology of testes that have undergone between 0 and 5 separate, two-hour heat-shock sessions at 37C, with a period of rest at 29C in between. A'-E') Corresponding images of Upd-YFP in the hub of each treatment. The dots are the Upd, and the total signal intensity for each hub was summed up and graphed in **F**. Although a modest decrease is visible, an ANOVA did not find a significant decrease is total signal across treatments. Data was taken and analyzed by Bitarka Bisai.

Upd is prevented from diffusing by some specific mechanism within the niche, or 2) SGs themselves are insulated from/unresponsive to the Upd signal. The latter case is not necessarily a farfetched idea: SG's are already insulated from external signaling due to the encapsulation of the cyst cells (Fairchild, Smendziuk, and Tanentzapf 2015), a yet unaddressed factor in this work (discussed on page 53). But there is also evidence of the former case as well. A visiting master's student, Bitarka Bisai, attempted to visualize the possible diffusion of Upd-GFP signal in the niche before and after disrupting the GSCs. Unsurprisingly, we saw no noticeable increase in either spread or loss/gain of signal, implying that Upd remains stable and localized within the

niche even during GSC loss (Fig 3.2). These results suggest that the niche is likely not just passively leaking all niche ligands into testis, and that the niche does have some level of control over its release of niche-signaling factors.

Assuming the niche does actively release a signal to initiate dedifferentiation and that signal turns out to be Dpp, what does this mean for its place in the signaling network with Me31B and Nos? At first glance, this hypothesis seems to imply that upon receiving Dpp, Me31B is suppressed through BMP signaling, allowing the SG cyst to dedifferentiate. However, our data show that pMad (and therefore BMP signaling) rises in dedifferentiating cysts when *me31B* is knocked down, which implies that BMP signaling is downstream of Me31B, as it is an important aspect of reasserting GSC identity. Given these data, it is more likely that Me31B remains upstream of BMP signaling and perhaps its deactivation is required before SG cysts can "listen to" the Dpp signal they receive.

However, I ultimately remain unconvinced that Dpp truly is responsible for initiation of dedifferentiation until more compelling evidence is collected. Firstly, very little information is known as to what extent Tkv receptors are expressed on the SGs, if at all. What would be the point of broadcasting a signal like Dpp if no cells are listening for it? Perhaps, similar to what I mentioned previously, it is Me31B that prevents the presence of Tkv receptors on the membrane until it is deactivated. However, in such a case, what is responsible for signaling to Me31B that it is time to stop repressing *nos* translation? Possibly a third, yet unidentified signal? At this point, the data presented in this thesis do little to resolve the question of how dedifferentiation is initiated. What is clear, however, is that future investigations into initiation should likely begin by either confirming or eliminating Dpp as a candidate.

The last possibility for initiation of dedifferentiation is that the signal responsible has not been yet been identified. For example, GSCs may send a signal to constantly notify the niche of their presence and, when this signal goes absent, it is the responsibility of the hub to express a ligand specific to recruiting new GSCs. The transient presence of this molecule could easily explain its lack of identification and detection thus far.

This hypothesis neatly dovetails the hypothesis of Dpp diffusion with the prospect of cyst cell involvement (discussed more in the following section). As further detailed below, cyst cells encapsulate the SGs and would make it very unlikely that a diffusing signal would reach the germ cells easily. It is possible that the cyst cells may respond to a yet unidentified signal that causes the cyst cells to unseal. Perhaps an unknown ligand is the signal intended to crack open the cyst encapsulation, exposing the germ cells within to Dpp diffusing from the niche. Whether dedifferentiation is initiated with two ligands or one, known or unknown, must be a top priority for future work.

The neglected but necessary role of cyst cells in dedifferentiation

Despite the appeal, the model I discussed above is insufficient for explaining the initiation of dedifferentiation. This model, and indeed this entire study, fails to account for a critical aspect of testis morphology: germ cells are encapsulated from external signals by somatic cyst cells (Fairchild, Smendziuk, and Tanentzapf 2015; Decotto and Spradling 2005; Zoller and Schulz 2012).

Assuming that dedifferentiation is prompted by the diffusion of niche ligands resulting from the disruption of GSC at-niche attachment, additional questions still remain. Thus far, our results have mostly addressed the internal genetic signaling of the germ cells. However, our

model cannot encompass the entirety of how this process is completed simply because it does not acknowledge the physiology of the tissue itself. After asymmetric cell division, the gonialblasts are likely encapsulated within two somatic cyst cells (Zoller and Schulz 2012). These cells continue to surround the germ cells even as they undergo transit-amplifying divisions, playing a larger role in preserving and gatekeeping germ cells than one might initially expect. Fairchild et al 2015 does a spectacular job of uncovering the process and function of cyst cell encapsulation. Most interestingly, their results suggest that while cyst cell encapsulation happens immediately, the cyst cells do not establish a barrier impermeable to external inputs until the SG has reached the 4-cell stage (Fairchild, Smendziuk, and Tanentzapf 2015).

However, initiation of SG dedifferentiation is observed among all stages of SG cysts, regardless of an established permeability barrier (Sheng, Brawley, and Matunis 2009). Not only have I witnessed and imaged instances of 8-cell and 16-cell SGs dedifferentiating during RNAi-knockdown of *me31B*, but live imaging data from Sheng et al 2011 also observed 8-cell SGs and beyond dedifferentiating to the niche. These observations clearly demonstrate that cyst cell encapsulation is not a barrier to dedifferentiation and neither is the establishment of an impermeability barrier.

Combined with the observations from the Sheng et al 2009 and 2011 works and the insights into cyst cell behavior brought to light in Fairchild et al (2015), our results heavily imply that some level of signaling to or involvement of the somatic cyst cells is necessary for dedifferentiation. Cyst cells must be the first respondents to the niche's call for dedifferentiated germ cells. It is possible, perhaps, that cyst cells themselves are facilitating the migration, or they are opening up in response to a signal from the niche, allowing SGs to directly receive a signal.

While our knowledge of cyst cell encapsulation means that these cells absolutely must be involved in dedifferentiation (indeed, they may turn out to be the main player), it is still important to emphasize that nearly all the data presented in this study serves to show that the germ cells are by no means passively shepherded back to the niche by soma alone during dedifferentiation. Indeed, further investigation into the possible communication between the soma and germ cells will likely be a critical and extremely interesting aspect of our understanding dedifferentiation. Ultimately, whether they are tested in isolation or in conjunction with their germ cell communication, the role of cyst cells must be addressed in future work on dedifferentiation in the testis.

Initiation of dedifferentiation via the primordial germ cell migration pathway

Although *nos* is a translational repressor critically required for adult germ cell identity and has been shown to regulate GSC maintenance, it is also key for proper primordial germ cell (PGC) migration post-gastrulation (Lehmann and Richardson 2010). In wildtype flies, Nos suppresses premature differentiation in PGCs while PGCs migrate toward the future gonad by navigating through the posterior midgut (Kunwar et al. 2003). In this process, Nos regulates a GPCR, *trapped in endoderm 1 (tre1)*, which is ultimately required for PGC migration toward the future gonad. Activation of the GPCR Tre1 is known to be a critical regulator of PGC migration (Kunwar et al. 2003). Without Tre1, migration of the PGCs through the posterior midgut does not occur. Considering that our data show *nos* is required for dedifferentiation and that the observed migratory behavior of dedifferentiating SGs back to the stem cell niche closely resembles that of migrating PGCs, it is possible that dedifferentiating SGs reactivate developmental program of PGC migration as a mechanism for moving back to the GSC niche.

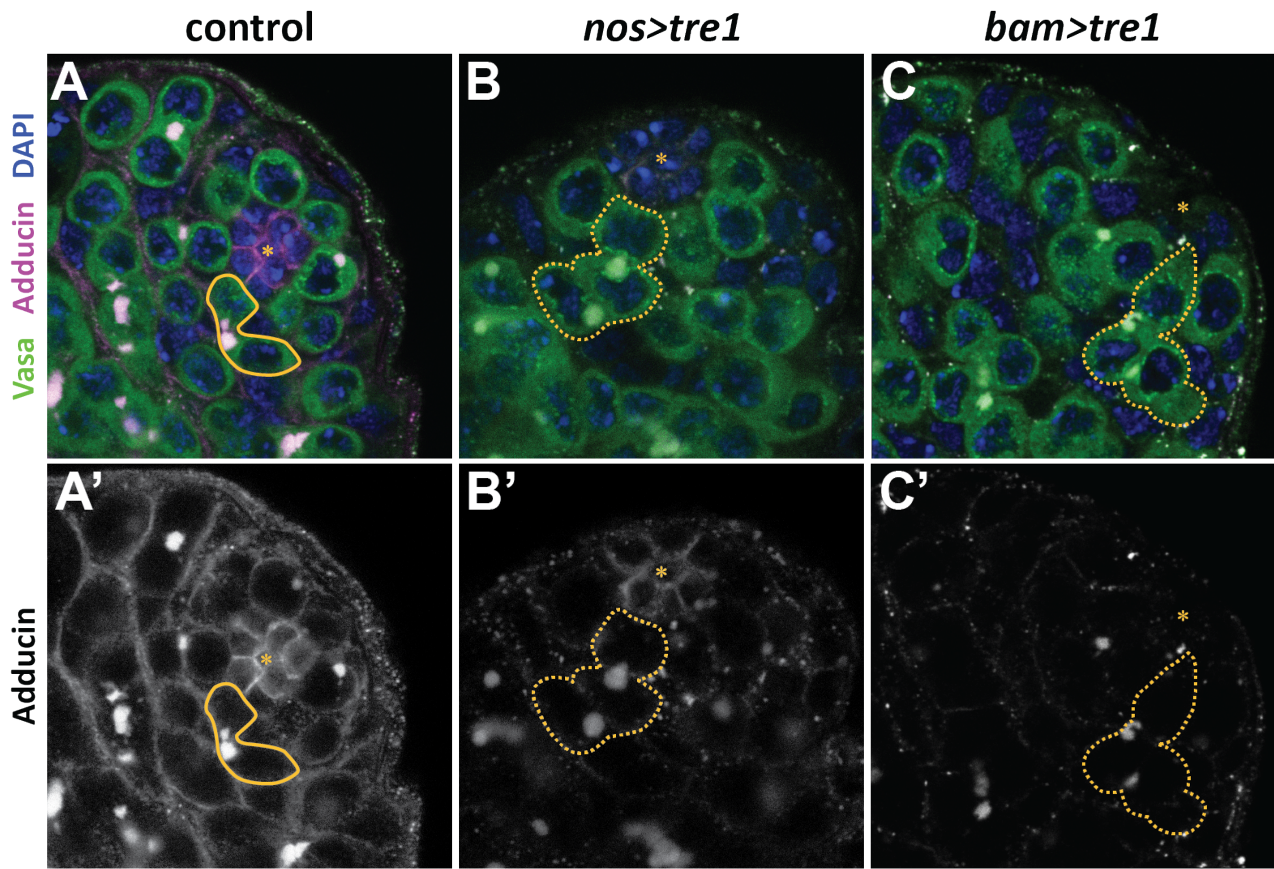
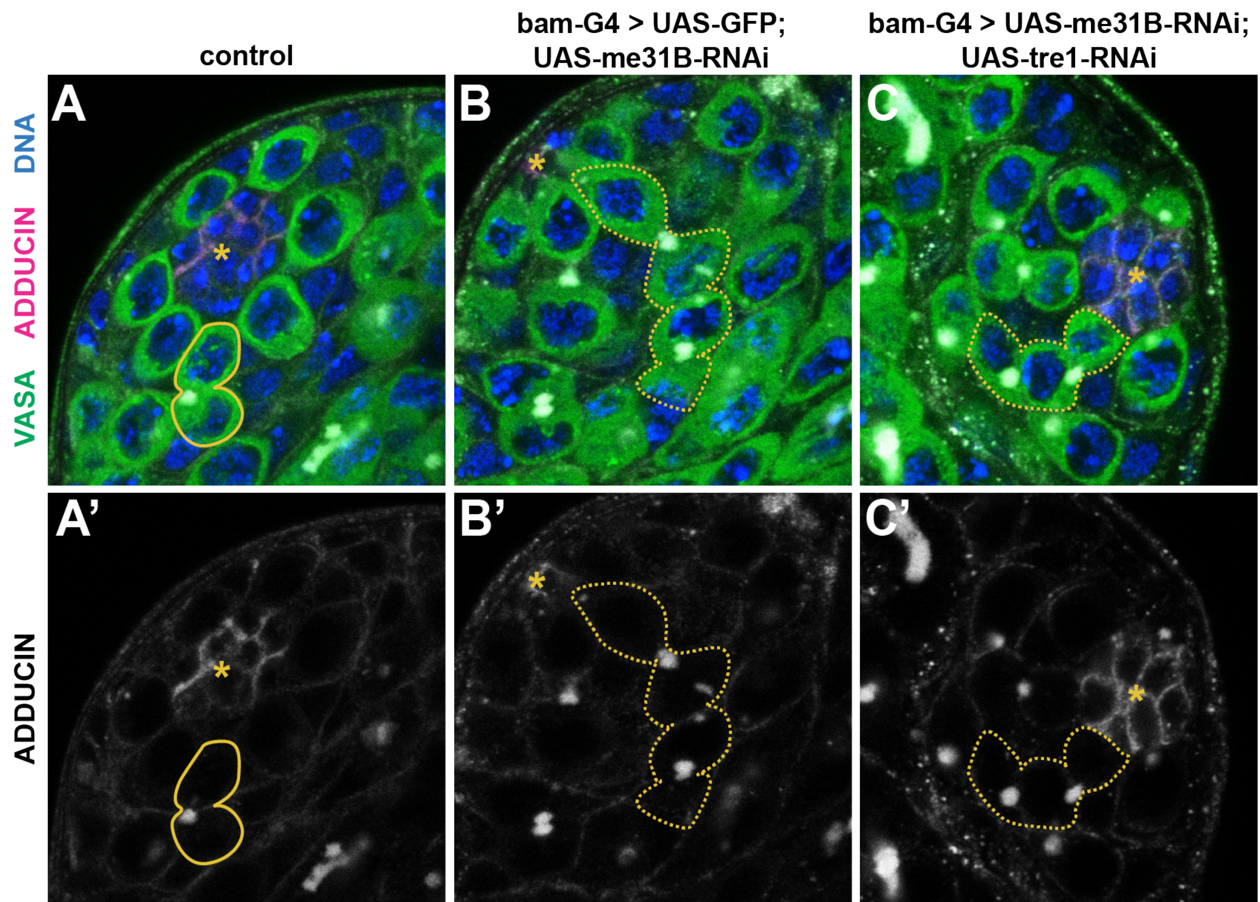


Figure 3.3: Overexpression of Tre1 in Early and Late-Stage Spermatogonia Is Sufficient to Drive a Modest Increase in Dedifferentiation Rate. **A-C.** Apical tip of the testis showing *nos>tre1* (A), and *bam>tre1* (B) with dedifferentiating cysts (yellow dotted lines). Contrast these cysts to a GSC-GB pair in the control (A), outline with a solid line. Hub is indicated by the asterisks. A’-C’. Adducin-only channel, so that fragmented fusomes are visible. **D.** Frequency of testes (%) containing dedifferentiating SG cysts. n = number of testes scored. p-value from Fisher’s exact test is provided compared to control.

To test this hypothesis, I examined the potential role of Tre1 in SG dedifferentiation in the adult testis. First, overexpression of Tre1 was sufficient to induce SG dedifferentiation (Figure 3.3), suggesting that SG dedifferentiation may indeed reactivate PGC program to migrate



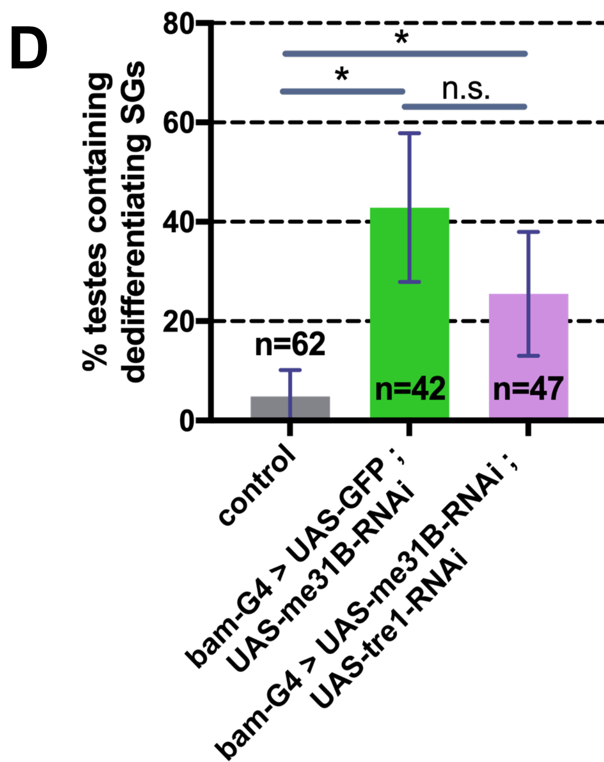


Figure 3.4: Tre1 Is Not Necessary for Dedifferentiation.
A. Control, **B.** *bam>UAS-me31B^{RNAi}, UAS-GFP*. *UAS-GFP* was expressed to control for the number of transgenes driven by bam-gal4. **C.** *bam>UAS-me31B^{RNAi}, UAS-tre1-RNAi*. *tre1* depletion did not prevent dedifferentiation induced by *me31B^{RNAi}*. **A'-C'.** Adducin-only channel, so that fragmented fusomes are visible.
D. Frequency of testes (%) containing dedifferentiating SG cysts. n = number of testes scored. p-value from Fisher's exact test is provided compared to control.

toward the GSC niche. However, *tre1* does not appear to be a physiological effector of SG dedifferentiation in the adult testis, as its knockdown did not influence the frequency of SG dedifferentiation induced by *me31B* knockdown (Fig 3.3), with the caveat that *tre1*^{RNAi} used in this experiment might not be effective. However, in situ hybridization to detect *tre1* mRNA in adult testis or during dedifferentiation revealed no signal (data not shown), suggesting that *tre1* may not play any role in adult testes.

One possible explanation is that it is not Tre1 but another GPCR that is responsible for adult dedifferentiation. Foremost of these candidates is *moody*, another GPCR closely related to *tre1* responsible for cell polarization and organization in establishment of the blood-brain barrier (Bainton et al. 2005). Interestingly, a poster abstract from 2000 noted that overexpression of *moody* reportedly led to "super-migratory" behavior in germ cells (Starz-Gaiano 2000), making it an appealing substitute for *tre1.* If this is the case, Tre1 overexpression may function as dominant active inducer of dedifferentiation in adult testis, but its knockdown may not have any effect on dedifferentiation. Investigating such possibility and looking for adult testis-specific GPCR responsible for adult SG dedifferentiation (e.g. moody) awaits future studies.

MAMMALIAN PARALLELS OF DEDIFFERENTIATION

In mammals, most studies of dedifferentiation focus on its role in tumorigenesis whereas there seem to be comparatively fewer examples of endogenous dedifferentiation employed to maintain tissue homeostasis dedifferentiation (Schwitalla et al. 2013; Niu, Mercado-Uribe, and Liu 2017; Friedmann-Morvinski et al. 2012; Friedmann-Morvinski and Verma 2014; Tata et al. 2013). However, there are mammalian examples of dedifferentiation that are similar in overall structure and purpose to the one described here in *Drosophila* testis. Although the genes and

their relationships to one another may not be the same, they still share of the major physiological trends we observe, such as: a stem cell population is established and continuously proliferative; stem cell identity is restricted to a niche defined by the signals produced from nearby cells; differentiating daughter cells undergo transit-amplifying divisions where they remain dedifferentiation competent; and dedifferentiation assists in maintaining tissue homeostasis in response to damage. Here, I mean to describe what is known about the analogous mammalian system and highlight where gaps of in our knowledge may benefit from similar lines of investigation and logic shown in this study.

Spermatogenesis in the mouse testis offers the most direct corollary to what we observe in the Drosophila testis. In the mouse testis, we once again observe that the transit-amplifying population retains some level of potential for stem cell identity and, when the testis is spurred to regenerate, these spermatogonial cysts fragment into single-cell spermatogonia that act as stem cells and replenish the tissue, arguably dedifferentiating in the process (Nakagawa, Nabeshima, and Yoshida 2007; Nakagawa et al. 2010). However, there are a few large differences in the physiology of the tissues that make this parallel less fitting.

Most prominent among them are 1) the germline stem cells in the mouse testis are less easy to define because there does not seem to be a defined niche and 2) the number of cells in a germ cell cyst is not a reliable indicator of their differentiation (de Rooij 2017). As a result, the spermatogonia are split into two primary populations: the “undifferentiated” and the “differentiated” (de Rooij 2017). Both of these population undergo incomplete mitotic divisions and form cysts of multiple, interconnected germ cells, but are delineated by differential gene expression that limits their stem cell potential (de Rooij 2017). Although it is clear that the cell population functioning as germline stem cells lies within the undifferentiated spermatogonia, it is

not entirely clear which cells are strictly defined as the stem cells (Nakagawa, Nabeshima, and Yoshida 2007; de Rooij 2017). While the cells acting as stem cells for normal spermatogenesis are almost certainly the single-celled stage of the undifferentiated spermatogonia, evidence suggests that not all these cells function as stem cells (Nakagawa, Nabeshima, and Yoshida 2007; de Rooij 2017).

How then is it determined which undifferentiated cells serve more as stem cells and which are transit-amplifying cells? And, subsequently, what changes do the transit-amplifying cells undergo in order to fulfill their "potential" to function as stem cells during a time of regeneration? Here the similarities to *Drosophila* return: two genes expressed in a reciprocity of one another are key to this decision. Cells positive for *GDNF family receptor alpha-1* (*Gfrα1*) expression tend to be smaller cysts and more likely to assume the role of a stem cell whereas cells expressing *Neurogenin-3* (*Ngn3*) are typically part of a larger cyst and more likely to transition onwards into differentiated spermatogonia (Nakagawa et al. 2010). As undifferentiated spermatogonia of any stage differentiate, their GFRα1 expression reduces as their NGN3 expression increases and the vice versa hold true during dedifferentiation. These expression patterns are at least partially due to the release of the GFRα1 ligand *Glial cell-derived neurotrophic factor* (*Gdnf*) by the nearby Sertoli cells (Meng et al. 2000), but the ligand alone fails to account for the heterogeneity of expression and morphology of the spermatogonial cysts when there is little spatial organization restricting the access of differentiating cysts to the ligand. This makes GFRα1 a prime candidate for a gene functioning in the mouse testis much like Nanos functions in the fly testis, as perhaps it is the gradual scarcity of the receptor that dictates the speed of differentiation.

It is curious, however, that one half of the paradigm that is seemingly essential in restricting GSC identity and enforcing the differentiation of transit-amplifying cells in the *Drosophila* testis (the niche) is seemingly absent here in the mouse testis. With time, perhaps a study will either identify a gene regulatory network that maintains an overall trend towards differentiation in the majority of the spermatogonia that retain the competency for dedifferentiation or propose a model for why the mouse testis is not so negatively impacted by playing it fast and loose with stem cell identity among a larger population of competent cells.

FUTURE DIRECTIONS

As this work is the first study to identify a key regulatory pathway in *Drosophila* male germline dedifferentiation, it has prompted multiple avenues for future investigation and there are several major outlying questions that this study raises and does not yet address. Chief among them is expanding our understanding of the role of BMP signaling in dedifferentiation and its relationship with *me31B*. Overexpression of active Tkv (thus artificially inducing BMP signaling in SG cysts) increases dedifferentiation rates and, from our data in Figure 2.3, we also know that pMad signaling is upregulated in dedifferentiating cysts. However, one aspect that remains partially unanswered is whether or not BMP signaling is required for initiating SG migration back to the hub during dedifferentiation. Although we know that establishing GSC identity requires upregulating BMP signaling, it is not clear if upregulated BMP signaling is required for getting back to the niche in the first place. Testing the rate of dedifferentiation using the late-stage driver to knock down *me31B* (*bam>me31B*^{RNAi}) while simultaneously repressing BMP signaling by driving a dominant negative *tkv* receptor may begin to answer this question. Ideally, I would expect that a lower frequency of dedifferentiation is the result of SGs failing to

dedifferentiate because BMP signaling is necessary for dedifferentiation. However, it is also possible that a lower rate of dedifferentiation could be due to lacking the Tkv necessary to establish a physical connection upon reaching the hub (thus the SGs are not necessarily failing to dedifferentiate because dedifferentiation requires BMP signaling, they are failing to dedifferentiate because they cannot assume GSC identity once reaching the hub). It is possible that using only a late-stage SG driver (*bam-GAL4*) could circumvent this issue, but it is likely that live-imaging should be done to verify that this is the case. In that case, the FLP-induced *bam-GAL4* in conjunction with *me31B*^{RNAi} and the dominant negative *tkv* could assist with observing the behavior of the labeled SGs. Overall, these results may lend further credibility to *dpp* acting as the ligand initiating dedifferentiation.

In a similar vein, the potential for a physical and direct relationship between Me31B and BMP signaling also remained mostly unaddressed. Me31B already partially interfaces with the BMP pathway according to the work done here, as we showed that Me31B directly targets *bam* mRNA (a gene that directly antagonizes BMP signaling) and we also showed that BMP signaling increases during *me31B* knockdown. It is possible that Me31B binds *bam* mRNA to promote its expression (thereby indirectly inhibiting BMP signaling) but this would be contrary to the previous work done on Me31B that overwhelmingly characterizes its function as inhibitory to the targets it binds. In order to test whether or not Me31B directly inhibits BMP signaling, however, I think it would be appropriate to conduct RIP-qPCR for the mRNA of a few genes in the BMP signaling pathway using testes enriched for spermatogonia.

Additionally, it may also be beneficial for us to identify the main protein partner that Me31B is working with in the Drosophila testis. We know from previous studies that Me31B does not work alone nor is it the protein that determines which mRNAs to target in a given tissue

context (instead, Me31B is typically recruited to coat and bind an mRNA by another protein). Thus, there are two main reasons for why identifying which protein partner Me31B is working with would be worthwhile in the future: 1) Unlike the vast number of mRNA targets that Me31B is known to bind, the number of protein partners it has been documented to work with is relatively manageable in size and 2) Since many of these proteins tend to have smaller pools of preferred mRNA targets and/or they have binding motifs present in the 3'UTRs of their mRNA targets, identifying Me31B's protein partner in the testis could narrow our search for other mRNA targets of Me31B involved in dedifferentiation. Conducting a co-IP western blot using Me31B for the following proteins in the testis would be worthwhile: Cup, Tral, Smaug, Imp, Belle, Pcm, Not1, and Aub. These results, followed up with conformational analyses on the microscope, have the potential to greatly expand our understanding of how the regulatory network that Me31B is a part of functions in the testis.

On top of investigating its protein partners, both previous work and data presented in this study demonstrate that other mRNA targets of Me31B should be investigated for a potential role in dedifferentiation. In the *Drosophila* embryo, Me31B binds to the mRNA of half of all expressed genes (M. Wang et al. 2017), suggesting that understanding its role in any one specific phenotype will require careful strategies to narrow the scope of relevant targets. Among our own data, Figure 2.10 on page 35 shows that when Nos protein is overexpressed in conjunction with $me31B^{RNAi}$, differentiation grinds to a halt. This result shows that the effects of these genetic manipulations are additive, rather than mimicking the results we see when either of these constructs are used alone (which is what we would expect if these two worked in isolation in this pathway). The most likely explanation is that during $me31B^{RNAi}$, another Me31B target is no longer repressed in order to block excess dedifferentiation, or is antagonistic to differentiation.

As such, identifying additional Me31B targets in the testis specifically involved in dedifferentiation must also be a priority for future experiments. One way to start towards that goal would be to conduct RNA immunoprecipitation sequencing (RIP-seq). RIP-seq is a technique that uses immunoprecipitation to pull down a specific RNA-binding protein while its transcriptional targets are still attached. Me31B's RNA targets can be pulled down with the protein by using a transgenic line containing a GFP attached to Me31B, then freeing the mRNA from the protein and sequencing. Processing the subsequent reads with DEseq2 (Love, Huber, and Anders 2014, 2) to identify enriched bound mRNA in comparison to a background signal control should yield a ranked list of Me31B mRNA targets in the testis.

However, Me31B protein seems to be widely expressed across the multiple cell types of the *Drosophila* testis (see Figure 2.1A) and, if whole testes are collected for RIP-seq samples, the results will be confounded by information about Me31B targets in cells outside the germ cells. On top of that, I expect that the results will also contain an overwhelming number of enriched transcripts, (similar to what is found in other studies), thus drowning candidates in deluge of targets not relevant to either the cells or the phenotype of interest. As such, methods for parsing these data and identifying a pool of interesting candidates small enough to make testing feasible must be developed.

One solution is to cross-compare the candidates identified in the RIP-seq with another bioinformatics dataset that is specific to the SG and dedifferentiation. I suggest using an RNA-seq dataset generated via differential expression analysis between samples with and without *me31B*^{RNAi} using the *bam-GAL4* driver. The driver will ensure that the differential expression is due to changes in the SG specifically. Although the results will include changes in direct targets as well as genes targeted by Me31Bs targets, the specificity can be made up by the RIP-seq. RIP-

seq will be useful for identifying which mRNA are actually physically targeted by Me31B, while comparative RNA-seq will be needed in order to see which genes are regulated specifically in the SG as a result of knock-down. When the datasets are compared, they should yield a smaller pool of gene candidates that can be tested more readily.

If even that pool of candidates proves too large, the Gene Ontology Annotation database has a well-curated list of genes known to be involved in germline stem cell and stem cell identity or renewal. Investigative priority should be given to genes that crop up on all three of these lists. Once a pool of interesting targets is established, then mRNA in situ during *me31B*^{RNAi} should be done to verify the bioinformatics data and see if further testing should be done.

Lastly, as previously mentioned on page 54, any future study into dedifferentiation in the testis would be remiss to not address the role of the somatic cyst cells that encapsulate each SG cyst and eventually form an impermeable barrier, cutting the germ cells off from any outside signal. So if some kind of external signal is responsible for initiating dedifferentiation (and the data from both this study and previous work in Sheng et al 2009 and 2011), then there are two likely explanations: 1) somatic cyst cells dissolve the permeability barrier in response to the signal initiating dedifferentiation in order to allow the signal to be received by the germ cells or 2) somatic cyst cells receive the signal initiate dedifferentiation and communicate with the germ cells. On top of these complex questions about the role of cyst cells in the initiating of dedifferentiation, there are basic and yet unanswered questions about what happens to the cyst cells during dedifferentiation. For example, it is very likely that cyst cells must stop encapsulating a germline cyst at some point in order for the germ cell to form the necessary physical connections with the hub and assume GSC identity. However, we are unsure when this

might happen, either at the beginning or the end of the germ cells migration to the niche, as there have been no attempts thus far to observe the cyst cells during dedifferentiation.

Unfortunately, a combination of several issues make this area of investigation particularly challenging. It is not a simple task to try and observe the specific cyst cells of a single SG cyst; although it's not impossible to find the nucleus of a cyst cell, the rest of a cyst cell surrounding the SG is very flat and pressed closely against the cyst cells of neighboring SG's. Clonal labeling can help visually isolate just a single set of cyst cells, but the likelihood of being able to induce labeling of the cyst cells in a dedifferentiating SG is very low.

One method that skirts some of these problems and make some basic morphology observations would be to use the fluorescent dye used in Fairchild et. al. 2015 to visualize the establishment of the impermeability barrier. The dye, which consists of dextran (a polysaccharide) conjugated to Alexa Fluor 647 at a concentration of 0.2 μg/μl, is visible between the individual germ cells in SG cysts that have not yet established a permeability barrier. At around the 4-cell stage when the permeability barrier is established, the dye remains outside the cyst. One aspect that might begin to answer some of our questions might be to use this assay in conjunction with *bam>me31B*^{RNAi}, where we could observe an increased number of late-stage cysts without permeability barriers as a sign that their permeability barrier is deconstructed during dedifferentiation. Alternatively, I would also like to do the same experiment but induce dedifferentiation via the starvation and refeeding protocol outline in Herrera and Bach 2018, to test if the external initiation for dedifferentiation has an effect on the permeability barrier (as opposed to driving dedifferentiation internally via transgenic changes to the germ cells). Lastly, if the *bam>me31B*^{RNAi} has clear disturbances of the permeability barrier, I would like to see if

clonally inducing knock down of me31B in a single late-stage cyst will allow me to make some observations of cyst cell morphology in the process of dedifferentiation.

CONCLUSION

Stem cell populations serve as a foundational component of long-term tissue function; as such, their health and safety cannot be taken for granted. While we had previously known that dedifferentiation is one such method of replenishing stem cells, we had yet to identify genes with major regulatory roles or develop a model for how dedifferentiation is accomplished without tumorigenesis.

Although this study is not the first to induce dedifferentiation in the testis, it is the first to do so without needing to remove stem cells. This was accomplished by identifying *me31B* as a negative regulator of dedifferentiation, making it the first study to also pinpoint a key regulatory mechanism for preventing excess dedifferentiation. These data also show that Me31B accomplishes this by physically preventing translation of *nos* mRNA, a gene both necessary and sufficient for dedifferentiation. By identifying these genes and establishing that this is a direct and physical pathway, we have cracked open the door for future work to further characterize the pathway and genes involved in this process.

Moreover, our further investigations into *nos* revealed a new landscape for *nos* transcription, translation, and expression, all of which allowed us to describe a paradigm for how dedifferentiation is accomplished with minimal risk of tumorigenesis. While GFP-tagging has shown Nos protein in GSCs through to 4-cell SGs, we show that active transcription of *nos* only occurs in GSCs or their attached daughter cells; SGs are left to create Nos protein from whatever limited amount of *nos* mRNA remains. The net impact is that while SGs may resemble GSC

identity, they continue to slide slowly towards differentiation as their supplies run dry. Thus, the SGs act as a 'dedifferentiation competent pool' of cells: they have not ceased differentiating, but their slowed rate of commitment means that they can be easily prompted into dedifferentiating during GSC loss. These transit-amplifying cells exist in a sustained extension of a developmental window in order to specifically allow for regeneration, even while *me31B* safeguards against unnecessary transformation. These results serve as an excellent demonstration of how an organism endogenously manages the risk and vs. reward of employing dedifferentiation as a strategy for tissue homeostasis.

In some ways, reading through the previous studies on dedifferentiation of the last two decades gave me the distinct feeling of listening in on two distinct conversations: one that considers dedifferentiation primarily as an endogenous phenomenon important for the sustaining tissue homeostasis and another that views the process as a biological mistake that can give rise to cancer. It is my opinion that these conversations are simply two sides of the same coin; the reality is that many organisms use dedifferentiation endogenously to replenish their tissues ***and*** they somehow mitigate the risk of tumorigenesis whilst doing so. By having these conversations separately, I believe we risk neglecting the critical question of, "How exactly is such a task accomplished?" And, as we continue to investigate this process in other organisms, "Are there cross-species similarities to be found in how dedifferentiation is regulated?" For example, the same genes need not necessarily be present for another species to use a paradigm similar to the one described here that uses limited *nos* transcription to create and maintain a pool of cells primed for dedifferentiation. A great deal of recent biomedical research focuses on 1) preventing tumorigenesis and 2) extending the health and function of a tissue. Here, we have described an endogenous system for accomplishing both these tasks at once, albeit in a narrow context.

Nonetheless, these results help expand our understanding of stem cell homeostasis and have several implications for our progress towards future disease treatments.

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