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Epigenetic Regulation of *Drosophila* Germline Stem Cell Maintenance and Differentiation

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Abstract

Gametogenesis is one of the most extreme cellular differentiation processes that takes place in *Drosophila* male and female germlines. This process begins at the germline stem cell, which undergoes asymmetric cell division (ACD) to produce a self-renewed daughter that preserves its stemness and a differentiating daughter cell that undergoes epigenetic and genomic changes to eventually produce haploid gametes. Research in molecular genetics and cellular biology are beginning to take advantage of the continually advancing genomic tools to understand: (1) how germ cells are able to maintain their identity throughout the adult reproductive lifetime, and (2) undergo differentiation in a balanced manner. In this review, we focus on the epigenetic mechanisms that address these two questions through their regulation of germline-soma communication to ensure germline stem cell identity and activity.

Keywords

Drosophila; germline stem cells; somatic gonadal cells; signaling pathways; transcription; chromatin regulator; epigenetics

1. Introduction

Germ cells have the unique ability to differentiate into gametes that will give rise to a new organism upon fertilization. *Drosophila* gametogenesis is intensively studied to understand cellular differentiation in adult stem cell lineages. This is due to the linear organization of germ cells according to their differentiation status in the gonads, which is conducive for visualization (Spradling et al., 2011). The complex cellular differentiation processes that produce gametes originate from the germline stem cells (GSCs) in both sexes (Fuller and Spradling, 2007; Matunis et al., 2012; Spradling et al., 2011). GSCs must self-renew to

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retain their stemness and produce a differentiating daughter cell (Figure 1) (Chen and McKearin, 2003a; Davies and Fuller, 2008; Kiger et al., 2000; Yamashita and Fuller, 2005). However, both female and male GSCs are capable of symmetric cell division (SCD) to increase GSC number, albeit at low frequency under physiological conditions (Salzmann et al., 2013; Sheng and Matunis, 2011; Xie and Spradling, 2000). In addition, partially differentiated mitotic germ cells can return to the niche and become GSC-like cells under particular conditions, such as pathological and stress-induced conditions, or during aging, a process called dedifferentiation (Brawley and Matunis, 2004; Cheng et al., 2008; Herrera and Bach, 2018; Kai and Spradling, 2004; Lim et al., 2015; Liu et al., 2015; Sheng et al., 2009). The ACD of GSCs is controlled by both cell-autonomous and non-cell-autonomous mechanisms [reviewed in (Gleason et al., 2018; Kahney et al., 2019)]. The somatic gonadal cells, such as the cyst stem cells (CySCs), cyst cells, and hub cells in testes (Figure 1A), and the cap cells, escort cells and follicle cells in ovaries (Figure 1B), contribute to the control of proper germ cell maintenance and differentiation through signaling pathways in a non-cell autonomous manner (Decotto and Spradling, 2005; Gonczy and DiNardo, 1996; Hardy et al., 1979; Nystul and Spradling, 2007; Xie and Spradling, 2000).

In recent years, broad epigenetic mechanisms that alter gene expression due to heritable chromatin structure change, but no primary DNA sequence change, have been shown to regulate germ cell maintenance and differentiation (Buszczak et al., 2009; Casper et al., 2011; Davies et al., 2013; Maines et al., 2007; Xi and Xie, 2005). Such epigenetic mechanisms include the modification of DNA-associated proteins or RNAs which can create chromatin structural changes or recruit transcription regulators. These actions subsequently allow gene expression changes, such as repression of non-lineage genes or activation of germline differentiation genes.

The basic structure of chromatin is comprised of DNA and histones. A single nucleosome consists of 147 bp of DNA wrapping around a canonical histone octamer, composed of an H3-H4 tetramer and two H2B and H2A dimers. The main epigenetic mechanisms known to control germline fate and regulate germ cell differentiation are (1) DNA methylation, (2) the incorporation of histone and (3) histone variants, (4) the post-translational modifications of histones by different histone modifying enzymes, (5) the repositioning of nucleosomes by chromatin remodelers, and (6) the regulation of non-coding RNA-mediated chromatin landscape by piRNAs and miRNAs. This review focuses on recent discoveries of epigenetic mechanisms in maintaining GSCs and controlling differentiation of early-stage germline cells using *Drosophila* as the model organism.

2. Epigenetic Mechanisms Regulating GSC Maintenance and Self-Renewal

2.1. DNA modifications

DNA methylation is an epigenetic mechanism conserved from plants to mammals. It functions through the covalent modification of cytosine and adenine residues (Luo et al., 2015). Methylation at the fifth position of the cytosine base (5mC) is established and maintained by a conserved enzyme family called DNA methyltransferases (DNMTs). 5mC modifications normally at the promoter region silence the transcriptional activity of target

genes, as well as repressing transcription at transposable elements and other repetitive sequences (Suzuki and Bird, 2008; Wu and Zhang, 2014).

There are two main DNMT families, normally classified by their distinct functions to either regulate *de novo* methylation (DNMT3) or maintain existing methylation (DNMT1) (Law and Jacobsen, 2010). Although DNMT2 shows structural similarities to DNMT1 and DNMT3 family proteins, its function and substrate remain unclear. For example, DNMT2 family proteins show transposon silencing in the *Drosophila* early embryo cells and weak methyltransferase activity to methylate ribonucleic acids (tRNAs) (Goll et al., 2006; Phalke et al., 2009; Schaefer et al., 2010). *Drosophila* belongs to the "DNMT2 only" species by retaining the DNMT2 homolog Mt2 but loses both DNMT1 and DNMT3 (Raddatz et al., 2013). In *Drosophila,* the level of 5mC has been shown to be exceptionally low compared to other organisms, with only 0.034% 5mC out of total cytosines in the adult fly genome. Additionally, this methylation appears to be independent of DNMT2 activity, further indicating that DNMT2 may not act as a DNA methylase in *Drosophila* (Capuano et al., 2014; Raddatz et al., 2013; Takayama et al., 2014). DNMT2 is involved in non-random sister chromatid inheritance during ACD of *Drosophila* male GSCs (Yadlapalli and Yamashita, 2013). Therefore, the scarcity of 5mC in the *Drosophila* genome and the unclear molecular function of DNMT2 suggest that other epigenetic mechanisms may play more important roles in the *Drosophila* germline.

2.2. Canonical histones

Due to the lack of DNA methylation in the fly genome, histones are likely the main source of epigenetic information in *Drosophila*. Histones closely interact with DNA and carry extensive post-translational modifications (PTMs), which can recruit factors, such as histone modifying enzymes and chromatin remodelers, to either activate or repress local gene expression (Allfrey et al., 1964; Bannister and Kouzarides, 2011; Boros, 2012; Dawson et al., 2009). In the *Drosophila* male germline, pre-existing (old) histone H3 is found to segregate to the GSC, while the newly synthesized (new) histone H3 is preferentially inherited by the differentiating daughter cell (Tran et al., 2012). This asymmetric inheritance pattern is specific to histones H3 and H4, which have the most known PTMs that regulate gene expression and whose incorporation is DNA replication-dependent. Histone variants such as histone H3.3, which are incorporated independently of replication, do not show this asymmetric inheritance pattern (Tran et al., 2012). Therefore, it was hypothesized and later shown that this old versus new H3 and H4 asymmetry between sister chromatids is established during DNA replication (Wooten et al., 2019). Additionally, to achieve the asymmetric segregation pattern, the mitotic machinery ensures that old histone H3-enriched sister chromatids are segregated to the GSC while new histone H3-enriched sister chromatids are segregated to differentiating daughter cell. Differential phosphorylation at Threonine 3 of H3 (H3T3ph) is shown in the mitotic GSCs, where old H3 is more enriched with H3T3ph than new H3. Mis-regulation of this phosphorylation using either a dominant negative H3T3A mutant or a phosphomimetic H3T3D mutant leads to randomized segregation of old- versus new-histone enriched sister chromatids, as well as cellular defects such as stem cell loss and progenitor germline tumor (Xie et al., 2015). Recently, a GSC-specific 'mitotic drive' phenomenon has been identified. Sister chromatids have different

amounts of the histone variant that specifies the centromere region, the CENP-A homolog Centromere IDentifier (CID). This asymmetric distribution of CID between sister centromeres coordinates with the dynamic activities of the spindle microtubules and temporal breakdown of the nuclear envelop, first at the proximal side toward the stem cell niche followed by the distal side. Together, the spatiotemporally regulated events ensure preferential recognition and attachment of the asymmetric sister centromeres by dynamic microtubes. Loss of either the asymmetric CID distribution between sister chromatids, by compromising the activity of the CID-specific histone chaperone CAL1, or temporally asymmetric microtubule activities using microtubule depolymerization drug Nocodazole, causes randomized sister chromatid segregation (Ranjan et al., 2019). Together, spatially asymmetric CID and H3T3ph between sister chromatids and the temporally asymmetric microtubule activity provide the cellular basis for asymmetric histone inheritance during ACD of *Drosophila* male GSCs. Therefore, in light of the phenotypes caused by disrupting asymmetric histone inheritance, this process likely maintains GSC identity by retaining old histone while allow differentiation of the daughter germ cell by resetting its chromatin structure. However, it remains unclear how histones are inherited during SCD of GSCs or during spermatogonial dedifferentiation. As SCD normally occurs at a low frequency, it is likely that old and new H3/H4 as well as the H3 variant CID display more symmetric inheritance patterns during SCD, which are also detectable at a relatively low frequency under normal conditions (Ranjan et al., 2019; Tran et al., 2012; Wooten et al., 2019). In order to understand histone inheritance during dedifferentiation, certain technical caveats need to be solved. For example, spermatogonial dedifferentiation under physiological conditions during aging often results in GSC-like cells with misoriented centrosome (Cheng et al., 2008), which tend to be arrested and fail to proceed with active mitosis. Even though pathological conditions or genetic depletion of *bona fide* GSCs could induce more efficient dedifferentiation, whether and how dedifferentiated GSC-like cells undergo a balanced self-renewal *versus* differentiation is unclear. Combination of these treatments with precise lineage tracing to locate these GSC-like cells at single cell resolution, along with histone labeling strategies and live cell imaging tools, will illuminate these intriguing questions.

Canonical histone H1, the linker histone, regulates proper female GSC self-renewal. In the *Drosophila* ovary, the Bone Morphogenic Protein (BMP) ligands Dpp and Gbb are expressed from the niche and regulate GSC identity and activity. These ligands activate BMP signaling mediated by phosphorylated Mothers against Dpp (pMad), which subsequently activates Daughters against Dpp (Dad) expression in order to repress the transcription of the main differentiation factor called Bag of marbles (Bam) in GSCs (Figure 2B) (Casanueva and Ferguson, 2004; Chen and McKearin, 2003a; Chen and McKearin, 2003b; Eliazer and Buszczak, 2011; Kawase et al., 2004; McKearin and Ohlstein, 1995; McKearin and Spradling, 1990; Song et al., 2004; Xie and Spradling, 1998). Knockdown of histone H1 in the early-stage germline results in the loss of GSCs and premature differentiation due to precocious Bam expression. Interestingly, loss of H1 also results in an increase of transcription activating histone modification H4K16ac in the GSCs. This is similar to the effect caused by overexpression of MOF, the acetyltransferase for H4K16 (Akhtar and Becker, 2000). Collectively, these results suggest that H1 and MOF act antagonistically to

maintain H4K16ac at a level that prevents ectopic expression of differentiation genes in *Drosophila* female GSCs (Sun et al., 2015).

2.3. Histone variants

In addition to canonical histones, histone variants also regulate many cellular processes including transcriptional activity, heterochromatin formation, DNA repair and cell identity (Swaminathan et al., 2005; Talbert and Henikoff, 2017; Tanabe et al., 2008). In *Drosophila*, the only H2A variant is H2Av, which has the functions of both mammalian H2A variants in DNA damage repair and transcriptional regulation (Baldi and Becker, 2013; van Daal et al., 1988). In the *Drosophila* male germline, H2Av is required for both GSC and CySC maintenance, where knockdown of H2Av *via* RNAi or compromising function using *H2Av* mutants results in declined GSCs and CySCs over time. In the testicular niche, the hub cells secrete Unpaired (Upd), which activates JAK-STAT signaling with the downstream phosphorylation of the Stat92E transcription factor (pStat92E) in both GSCs and CySCs (Harrison et al., 1998; Kiger et al., 2001; Tulina and Matunis, 2001). In CySCs, pStat92E activates CySC and GSC self-renewing factors, Zfh1 and Chinmo (Flaherty et al., 2010; Leatherman and Dinardo, 2008). In GSCs, pStat92E ensures the expression of adhesion molecules for their attachment to the hub cells (Figure 2A) (Herrera and Bach, 2019; Leatherman and Dinardo, 2010). However, the role of H2Av in GSC self-renewal seems to be independent of the JAK-STAT signaling pathway, as the loss of H2Av does not affect pStat92E levels and mutation of the *stat92E* gene does not affect H2Av expression, either. Furthermore, differentiation of both progenitor germ cells and cyst cells appear to be unaffected, suggesting that H2Av regulates solely GSC self -renewal by an unknown mechanism, independent of the JAK-STAT pathway (Morillo Prado et al., 2013).

2.4. Histone-modifying enzymes

Histone modifications play profound roles in regulating chromatin landscape and function (Allfrey et al., 1964; Bannister and Kouzarides, 2011). For example, the addition of an acetyl group to the lysine side chains by acetyltransferases neutralizes the positive charge of the lysine, weakening the interaction between the histones and DNA (Hodawadekar and Marmorstein, 2007). Modifications can recruit remodeling enzymes that reposition nucleosomes to regulate many processes such as DNA replication, repair and recombination (Bartke et al., 2010; Vermeulen et al., 2010). The histone modifications are catalyzed by specific enzymes that can generate, or “write”, these covalent changes. Additional factors can recognize, or “read”, these modifications and recruit other factors to lay down additional modifications that either promote or repress transcription. Another class of enzymes can remove, or “erase”, histone modifications. The histone modifying enzymes are often encoded by a single gene in *Drosophila,* making it an ideal model organism to study the roles of histone modifications and their modifiers without complications caused by redundant genes (Bannister and Kouzarides, 2011; Boros, 2012; Hennig and Weyrich, 2013).

Polycomb Group (PcG) and Trithorax Group (TrxG): Both PcG and TrxG protein complexes consist of histone modifying enzymes, as well as additional components that assist their functions through chromatin compaction, inhibition of chromatin remodeling, etc. (Kassis et al., 2017). Several PcG complexes have been characterized, including

Polycomb Repressive Complex 1(PRC1) and Polycomb Repressive Complex 2 (PRC2). In *Drosophila*, PRC1 consists of Polycomb (Pc), Polyhomeotic (Ph), Posterior sex combs (Psc) or Suppressor of Zestes 2 [Su(z)2], and Sex combs extra (Sce/dRing) (Francis et al., 2001). Within PRC1, Pc has a chromodomain that allows it to recognize and bind to the H3K27me3 modification; Ph has a SAM domain important for the PRC1-mediated repression of target genes; Psc and Su(z) 2 have a common C-terminal region that mediates chromatin compaction or remodeling; Sce or dRing has the H2A ubiquitin ligase activity to generate the H2AK118ub modification, which promotes PRC2 activity in generating more H3K27me3 (Francis et al., 2004; Gambetta and Muller, 2014; Kalb et al., 2014; King et al., 2005; Lo et al., 2009; Messmer et al., 1992; Wang et al., 2004). For PRC2, Enhancer of Zestes [E(z)] is the core histone methyl-transferase that generates the H3K27me3 mark. Other PRC2 components include Extra Sex combs (Esc), Suppressor of Zestes 12 [Su(z) 12], and Caf1-55 (Table 1) (Czermin et al., 2002; Muller et al., 2002). The main function of Esc and Su(z)12 is to stimulate the enzymatic activity of E(z), but the biochemical role of Caf1-55 remains unclear (Anderson et al., 2011; Cao et al., 2002; Czermin et al., 2002; Kassis et al., 2017; Ketel et al., 2005; Margueron et al., 2009; Muller et al., 2002; Tie et al., 2007; Wen et al., 2012; Xu et al., 2010).

PRC1 components regulate male GSC self-renewal. In the *Drosophila* testis, compromising Psc and Su(z) 2 function by mutations or RNAi results in CySC-like tumors. Moreover, these overpopulated CySC-like cells outcompete and prevent GSCs from attaching to the hub, leading to GSC loss. This CySC overproliferation phenotype can be attributed to ectopic expression of a homeobox gene *Abdominal B* (*Abd-B*), as forced expression of *Abd-B* in wild-type testes results in a similar tumor phenotype (DeFalco et al., 2004). Therefore, Psc and Su(z) 2 repress *Abd-B* expression in the adult germline, in order to maintain CySC identity and activity in the adult testes (Morillo Prado et al., 2012). This inadvertently allows for the maintenance of GSCs in a non-cell-autonomous nature by preserving the attachment of GSCs to the hub and preventing CySC encroachment.

In addition to PRC1, PRC2 component E(z) maintains the *Drosophila* male germline through a cell-autonomous mechanism. In the germline, *E*(*z*) mutations cause GSC depletion over time, due to defective dedifferentiation of spermatogonial cells. Knockdown of *E*(*z*) by RNAi specifically in spermatogonial cells also induces GSC loss, confirming that dedifferentiation defect contributes to the GSC loss phenotype. In *E*(*z*) mutant germ cells, ChIP experiments show increased H3K4me3 at 39% of genes that lose H3K27me3, suggesting that a global chromatin change in the spermatogonial cells undermines their dedifferentiation potential. Therefore, E(z) maintains the global chromatin state of spermatogonial cells that is conducive for dedifferentiation to replenish lost GSCs due to aging or degeneration (Eun et al., 2017).

On the other hand, TrxG acts antagonistically to PcG by activating gene expression through a variety of mechanisms, including histone modifications, ATP-dependent chromatin remodeling, chromosome cohesion, and recruitment of RNA polymerase II to the promoters of target genes (Kassis et al., 2017). In *Drosophila*, three classes of Complex of Proteins Associated with SET1 (COMPASS) have been identified, each containing a distinct histone methyltransferase [Trithorax (Trx), SET domain containing 1 (Set1), or Trithorax related

(Trr)], Ash2, and three additional subunits (Mohan et al., 2011). Set1 is responsible for generating the majority of H3K4me2 and H3K4me3 in *Drosophila*, while the role of Ash2 is to stabilize the methyltransferase activity of the COMPASS complexes (Table 1) (Ardehali et al., 2011; Dou et al., 2006; Hallson et al., 2012; Kassis et al., 2017). Recently, Set1 has been shown to act with an E3 ubiquitin ligase Bre1 to generate the majority of H3K4me3 modification and regulate GSC maintenance and differentiation in the *Drosophila* ovary (Bray et al., 2005). Mutation of *bre1* or knockdown of *set1* by RNAi cause female GSC loss and decreased expression of BMP signaling components, such as pMad or Dad. In the germaria, Dally restricts Dpp diffusion to ensure that only the GSCs receive Dpp signal (Akiyama et al., 2008; Guo and Wang, 2009). In addition, DE-cadherin ensures GSC adhesion to the niche cells (Song and Xie, 2002; Song et al., 2002) (Figure 2B). Inactivation of *bre1* or *set1* in the terminal filament and cap cells results in female GSC loss with reduced Dally and DE-cadherin expression, leading to precocious GSC differentiation. Moreover, compromising *bre1* or *set1* in the escort cells induces overproliferation of GSC-like pMad-positive cells due to increased Dpp and Dally. Therefore, Bre1 and Set1 act together to control female GSC maintenance and differentiation through multiple mechanisms (Xuan et al., 2013). It is possible that in the GSCs and the niche, the addition of H3K4me3 by Set1 at the genomic loci of BMP pathway components promotes their expression for the maintenance of GSCs. In addition, Set1 may act in the escort cells to promote the expression of a negative regulator of Dpp and Dally. However, both of these possibilities have yet to be determined by identifying direct targets of H3K4me3 and Set1 in a cell-type-specific manner. In addition, a RNAi screen study identified Set1 as a self-renewal factor in female GSCs (Yan et al., 2014).

Su(var)3-9 and Heterochromatin Protein 1 (HP1): Telomeric and pericentric chromosomal regions are enriched with transcriptionally repressed heterochromatin, whose formation is regulated by H3K9me2/3 “writer” [Su(var)3-9] and H3K9me2/3 “reader” Heterochromatin Protein 1 (HP1) (Table 1) (Eissenberg et al., 1990; James et al., 1989; James and Elgin, 1986; Schotta et al., 2002; Schotta et al., 2003). The recognition of H3K9me3 by HP1 facilitates heterochromatin formation by recruiting more Su(var)3-9 (Bannister et al., 2001; Grewal and Jia, 2007; Lachner et al., 2001). HP1 has a variety of functions in addition to repressing gene expression, such as telomere capping and maintaining telomere length (Fanti et al., 1998; Perrini et al., 2004; Vermaak and Malik, 2009). In *Drosophila,* HP1 regulates both male and female GSC maintenance. In the male germline, knockdown of HP1 or Su(var)3-9 induces ectopic Bam expression in the GSCs and GBs, resulting in GSC loss. These phenotypes in the early-stage germline are concomitant with the loss of two germ cell markers, Vasa and Escargot (Esg) (Streit et al., 2002; Voog et al., 2014). On the other hand, overexpression of HP1 or Su(var)3-9 in the early-stage germline results in the opposite phenotypes, shown as loss of Bam expression and overexpression of Vasa and Esg. Overexpression of HP1 also partially rescues the GSC loss phenotype in *hopscotch* (*hop*) mutant testes. Since Hop is the *Drosophila* Receptor Tyrosine Kinase (RTK), mutations in *hop* result in the loss of proper JAK-STAT signaling in male GSCs and GSC loss (Herrera and Bach, 2019; Kiger et al., 2001; Tulina and Matunis, 2001). Based on these results, HP1 seems to enhance GSC self-renewal by promoting the JAK-STAT signaling (Xing and Li, 2015). Interestingly, in GSC-like cells in the Upd-

overexpressing testes, overexpression of HP1 induces differentiation by regaining Bam expression. Therefore, HP1 reverts the GSC tumor phenotype upon hyperactivating the JAK-STAT signaling pathway, suggesting a negative regulation of STAT signaling by HP1 (Loza-Coll et al., 2019). However, it is unclear whether these seemingly contradictory results are due to different genetic backgrounds carrying distinct JAK-STAT signaling activities. For example, HP1 could act as a "rheostat" to upregulate the activity of JAK-STAT signaling when it is low, but downregulate it when it is too high. The molecular mechanisms will await more investigations.

In the female germline, HP1 is also important for GSC maintenance and differentiation. Knockdown of HP1 results in germaria containing only GSC-like cells or only differentiated cells, suggesting an imbalance between GSC self-renewal and differentiation at the cellular level. At the molecular level, both differentiation gene *bam* and genes promoting stemness as well as germline identity, such as *nanos*, *cup*, *p-element induced wimpy testis* (*piwi*), and *vasa* are all downregulated in *HP1* mutant ovaries (Forbes and Lehmann, 1998; Gonzalez et al., 2015; Wang and Lin, 2004). CLIP-PCR and ChIP experiments demonstrate that HP1 protein binds to *nanos*, *cup* and *piwi* mRNAs. Therefore, HP1 regulates female GSC maintenance and differentiation though post-transcriptional stabilization of stemness and differentiation gene mRNA by forming an hnRNP nuclear complex (Casale et al., 2019). However, how HP1 balances between these two group of mRNAs for proper GSC maintenance and differentiation remains to be addressed.

Eggless/ dSETDB1: Eggless/dSETDB1 (Egg) is another histone lysine methyltransferase that catalyzes tri-methylation of H3K9 and regulates *Drosophila* oogenesis (Table 1) (Clough et al., 2007). Mutations of *egg* gene causes two main phenotypes in the ovary: accumulation of GSC-like tumor cells and loss of differentiated germ cells (Clough et al., 2014; Wang et al., 2011). Dad expression is lost in the *egg* mutant GSCs; however, both Bam and Benign gonial cell neoplasm (Bgcn), another germline differentiation factor, are found to be normally expressed, suggesting that Egg maintains GSCs in a Bam-Bgcn independent manner (Clough et al., 2014; Li et al., 2009; Wang et al., 2011). It is possible that Egg represses differentiation genes in GSC, but it is unknown whether this repression is through direct regulation of Dad expression.

Enoki mushroom (Enok): Enok is a Monocyticleukaemia zinc finger, Ybf2/ Sas3, Sas2 and Tip60 (MYST) family histone acetyltransferase that preferentially acetylates Lys23 on histone H3 tail and promotes transcription (Table 1) (Huang et al., 2014). Compromised Enok in female GSCs causes rapid GSC loss through premature differentiation due to ectopic expression of Bruno, an RNA Recognition Motif (RRM)-containing RNA binding protein that targets Sxl for translational repression, in order to promote CB differentiation and female germline cyst formation (Wang and Lin, 2007). Additionally, loss of Enok in the cap cells results in impaired BMP and Notch signaling, leading to GSC loss due to premature differentiation and niche size reduction, respectively. Therefore, Enok maintains *Drosophila* female GSCs through both cell-autonomous and non-cell-autonomous mechanisms. This indicates that Enok positively regulates BMP and Notch signaling in the cap cells but negatively regulates Bruno in the female GSCs (Xin et al., 2013).

Ubiquitously transcribed tetratricopeptide repeat, X chromosome histone demethylase (dUTX): The *Drosophila* UTX (dUTX) is a H3K27me2/me3-specific demethylase that can form a complex with an acetyltransferase CREB-binding protein (CBP), a chromatin remodeler and TrxG component Brahma (Brm), in order to antagonize PcG-mediated gene silencing (Table 1) (Holowatyj et al., 2015; Smith et al., 2008; Tie et al., 2012). In the male germline, dUTX maintains active transcription of *Suppressor of cytokine signaling at 36E* (*Socs36E*) gene, which encodes a negative regulator of the JAK-STAT pathway, by ensuring the transcription start site is deprived of the repressive H3K27me3 mark. Socs36E is predominantly detected in the hub cells and CySCs to maintain proper JAK-STAT signaling activity and the 1:2 ratio of GSCs: CySCs (Figure 2A) (Herrera and Bach, 2019; Issigonis et al., 2009; Terry et al., 2006). Compromising dUTX in the CySCs induces increased Zfh1-positive cells inside and around the hub area as well as ectopic pStat92E in the more differentiated germ cells. In addition, knockdown of *dUTX* in the germline results in enhanced DE-cadherin expression, leading to increased hub size and hub cell number. These defects can be suppressed by either overexpressing Socs36E or halving Stat92E. Together, dUTX controls JAK-STAT signaling activity and regulates the feedback from stem cells (i.e. GSCs and CySCs) to the niche, in order to maintain niche morphology and regulate proper gene expression non-cell-autonomously (Tarayrah et al., 2013).

Little imaginal Disc (Lid): Lid is a H3K4me3-specific demethylase that maintains male GSC self-renewal and prevents premature differentiation (Eissenberg et al., 2007; Holowatyj et al., 2015; Lee et al., 2007; Tarayrah et al., 2015). Inactivation of Lid *via* RNAi knockdown or mutations in testes results in GSC loss and the presence of more differentiated spermatogonial cysts next to the hub, in place of GSCs. In addition, *lid* mutant testes have ectopic Bam and reduced Stat92E expression in the GSCs. Furthermore, enhanced expression of Stat92E or DE-Cadherin can partially rescue the GSC loss phenotype in *lid* mutant testes. Therefore, Lid acts cell-autonomously to regulate JAK-STAT signaling in the germline to maintain proper GSC activity and proliferation potential (Tarayrah et al., 2015).

2.5. Chromatin remodeling factors

For biological processes such as DNA replication, recombination, repair, and transcription, chromatin remodeling enzymes change the packaging state of the chromatin (Narlikar et al., 2013). Chromatin remodeling factors use energy from ATP hydrolysis to move, eject or restructure nucleosomes to allow certain factors to either gain or lose access to specific genomic regions. There are four families of chromatin remodeling enzymes: Switch Defective/Sucrose Nonfermenting (SWI/SNF), Imitation Switch (ISWI), Chromodomain-Helicase-DNA-binding protein (CHD), and Inositol Requiring 80 (INO80). All four families have a conserved ATPase subunit that belongs to the helicase superfamily 2 (SNF2), but each has unique flanking domains that specifically regulate its corresponding functions and activities (Eisen et al., 1995). For example, the SWI/SNF family ATPase contains a C-terminal bromodomain that assists in sliding and ejecting nucleosomes (Hassan et al., 2002). The ISWI family ATPase contains a SANT domain that optimizes nucleosomal spacing for chromatin assembly and transcriptional repression activities (Corona et al., 2002; Corona and Tamkun, 2004; Deuring et al., 2000). The CHD family ATPase contains a chromodomain that recognizes modified nucleosomes to be ejected or moved, allowing for

transcriptional activity changes (Marfella and Imbalzano, 2007; Woodage et al., 1997). Lastly, the INO80 family has a split ATPase domain, which is responsible for promoting transcriptional activation and DNA repair (Bao and Shen, 2007; Clapier and Cairns, 2009; Narlikar et al., 2013; Shen et al., 2000).

Nucleosome remodeling factor (NURF): In the ISWI family, NURF is an ATP-dependent complex that plays important roles in both *Drosophila* male and female early-stage germline (Table 2) (Badenhorst et al., 2002; Xi and Xie, 2005). In the male, the NURF complex components ISWI, Nurf55 and Nurf301 are expressed in the testicular niche, including hub cells, GSCs, and CySCs (Figure 1A). Nurf301, a specific NURF complex component, is required cell-autonomously for both GSC and CySC maintenance. Compromising Nurf301 results in the loss of both GSCs and CySC over time, due to premature differentiation caused by ectopic Bam expression and the loss of Stat92E expression. Increasing Stat92E levels partially rescues the CySC loss phenotype in *nurf301* mutant testes. In addition, downregulation of Nurf301 results in partial rescue of the GSC loss phenotype in *socs36E* mutant. These results indicate that NURF interacts with JAK-STAT pathway components to ensure proper stem cell maintenance in testes, by promoting Stat92E expression and preventing precocious Bam expression (Cherry and Matunis, 2010).

In addition, the NURF complex interacts with the Ecdysone (Ecd) steroid hormone pathway in female GSCs and male CySCs to maintain stem cell self-renewal. Binding of Ecd to the Ecdysone Receptor (EcR) results in the dimerization between EcR and Ultraspecle (Usp), which initiates a transcriptional cascade including E74, E75, and Broad (Br) (Schwedes and Carney, 2012). In the ovary, loss of Usp, E74, Ecd, or EcR results in female GSC loss. Additionally, *Usp* or *E74* mutant female GSCs display decreased ISWI expression and compromised BMP signaling. Therefore, Ecdysone signaling acts through the NURF chromatin remodeling complex to promote female GSC maintenance by regulating BMP signaling (Ables and Drummond-Barbosa, 2010). In the testes, Ecdysone signaling components are expressed in hub cells and the CySC lineage. Inactivation of Ecd in the CySCs results in both GSC and CySC loss. Additionally, EcR has been shown to interact with Nurf301 to maintain both stem cell populations. Together, these results show that EcR and Nurf301 interact to regulate the transcription of stemness factors to promote the self-renewal of both GSCs and CySCs in the testis (Li et al., 2014).

Polybromo-containing Bap (PBAP): In *Drosophila*, Brm is the only SWI/SNF chromatin remodeling ATPase and is present in both SWI/SNF complexes: Brm-associated protein (BAP) complex and PBAP complex (Table 2) (Mohrmann et al., 2004; Moshkin et al., 2007). In the *Drosophila* ovary, loss of PBAP components Bap180 and Brm in the germline or niche has been shown to result in GSC loss over time, suggesting that PBAP complex is required for female GSC maintenance (He et al., 2014).

Domino (Dom): In the INO80 family, Dom is a SWR1-like ATPase that is linked to the chromatin incorporation of the histone variant H2Av in both male and female germline (Table 2) (Kobor et al., 2004; Mizuguchi et al., 2004; Ruhf et al., 2001). In the male, clonal induction using *dom* mutations results in both GSC and CySC loss over time, suggesting that Dom is required for GSC and CySC maintenance cell-autonomously. Additionally,

reduced H2Av incorporation is detected in *dom* mutant GSCs. Therefore, both Dom and H2Av are required to maintain GSCs and CySCs, likely by repressing differentiation genes and/or maintaining active expression of self-renewal genes (Morillo Prado et al., 2013). In the female, Dom is identified as a GSC self-renewal factor (Yan et al., 2014)

Nucleosome Remodeling and Deacetylase (NuRD): In the CHD family, NuRD is the ATPase complex that functions antagonistically to the DNA replication-related element factor (DREF), in order to regulate male GSC maintenance (Table 2) (Hirose et al., 2002; Hirose et al., 1996). Compromising DREF leads to precocious GSC differentiation and GSC loss over time. GSCs without DREF display impaired Dad expression and ectopic Bam expression due to compromised BMP signaling. Genetic interaction experiments show that mutations in NuRD complex components *Mi-2* and *Caf1* suppress the GSC loss phenotype in the *DREF* mutants. Therefore, NuRD antagonizes DREF while DREF allows GSCs to self-renew, likely as a transcription factor to promote expression of BMP pathway genes to inhibit differentiation (Angulo et al., 2019).

2.6. RNA- binding proteins and non-coding RNAs

MicroRNAs (miRNAs) are small RNAs that negatively regulate gene expression post-transcriptionally. It has been demonstrated that miRNAs regulate stemness, cell division, differentiation, and homeostasis of multiple *Drosophila* stem cells (Hatfield et al., 2005; Park et al., 2007; Shcherbata, 2019). Primary miRNAs (pri-miRNAs) are expressed in the nucleus by RNA polymerase II. The hairpin structure of pri-miRNAs is recognized by the RNase II enzyme, Drosha, which along with its partner, Pasha/DGCR8, cleaves the pri-miRNAs into precursor miRNAs (pre-miRNAs). Subsequently, another RNase II enzyme, Dicer-1 (Dcr-1), which along with its partner, Loquacious, cleaves pre-miRNAs into a 22-nucleotide RNA duplex followed by interacting with Argonaute 1 (Ago1). With Ago1, miRNAs form the RNA-induced silencing complex (miRISC), which cleave and degrade target mRNAs, as well as repress their translation (Shcherbata, 2019).

Mutations in genes encoding components for miRNA biogenesis, such as *dcr-1*, *ago1*, *loquacious*, and *mei-P26*, all lead to GSC loss phenotypes (Azzam et al., 2012; Forstemann et al., 2005; Jin and Xie, 2007; Li et al., 2012; Yang et al., 2007). In *mei-P26* mutant ovaries, GSC loss can be attributed to disrupted BMP signal transduction, which results in the impaired Dad and pMad expression in GSCs. In addition, ectopic Bam and Brain tumor (Brat) expression is detected in the *mei-P26* mutant germaria. In wild-type GSCs, *brat* mRNA is the target of the Nanos-Pumilio complex: when Nanos expression decreases in the late-stage germline, Brat expression increases and in turn, represses the translation of Mad. When pMad is not present, *bam* expression is de-repressed, which initiates germline differentiation (Figure 2B) (Harris et al., 2011). These results suggest that Mei-P26 cooperates with Nanos and miRISC to repress the translation of specific mRNA targets such as *brat*, with which pMad is upregulated and *bam* is repressed, a condition for promoting GSC self-renewal (Li et al., 2012; Li et al., 2013).

In addition, miRNAs themselves regulate GSC maintenance in both male and female gonads. In the testis, malfunction of the *miRNA-310/313* cluster results in abnormal

germline and somatic gonadal cell differentiation, which can be suppressed by reducing Armadillo (Arm, or β-catenin) levels. Indeed, *miR-310/313* recognizes the 3' UTR of *arm* mRNA and targets it for degradation. Arm is an important effector of the Wnt signaling pathway and a key cell-cell adhesion molecule contributing to attachment of GSCs to the niche cells (Song et al., 2002) (Figure 2). These results indicate that *miR-310/313* controls Wnt signaling and regulates GSC-hub cell adhesion in testes (Pancratov et al., 2013).

Piwi interacting RNA (piRNAs) are the most abundant small non-coding RNA in the *Drosophila* male and female gonads. The main role of piRNAs is to protect genomes of both germline and somatic gonadal cells by repressing transposons (Brennecke et al., 2008; Hermant et al., 2015). The piRNAs act in a complex with the PIWI family proteins, which includes Piwi, Aubergine (Aub), and Argonaute 3 (Ago3) (Brennecke et al., 2007; Saito et al., 2006). Together, this complex silences transposons at both the transcriptional and post-transcriptional levels (Le Thomas et al., 2013; Lim et al., 2009; Vagin et al., 2006; Yang and Xi, 2017). Piwi is the founding member of the PIWI family and is present in both germline and somatic gonadal cells, while Aub and Ago3 are only required for piRNA production in the germline (Brennecke et al., 2007). In the ovaries, Piwi protein has been shown to interact with PcG components in the terminal filament cells, cap cells and GSCs to regulate female GSC maintenance and differentiation (Peng et al., 2016). Loss of Piwi in the early germline results in GSC loss, but *Pc* or *E*(*z*) mutations suppress this phenotype in *piwi* mutant ovaries. Piwi also physically interacts with PcG components Su(z)12 and Esc; E(z) has a higher enrichment at its target genes in *piwi* mutant ovaries. As Su(z)12, Esc and E(z) are all PRC2 components, Piwi may interact with Su(z)12 and Esc to sequester E(z) away from the chromatin, which may lead to reduced H3K27me3 but increased RNA Polymerase II at target genes to activate transcription. Therefore, the negative regulation of PRC2 and H3K27me3 by Piwi may be necessary for transposon suppression, proper female GSC activity, and oogenesis (Peng et al., 2016).

3. Epigenetic Mechanisms Regulating GSC Differentiation

3.1. Canonical Histones

Canonical linker histone H1 regulates proper female GSC differentiation. In the ovary, H1 is required for escort cell maintenance and germ cell differentiation. Knockdown of H1 in the escort cells leads to accumulation of GSC-like cells with upregulated BMP signaling, ectopic expression of cap cell-specific genes, as well as elevated transposon activity and DNA damage, resulting in escort cell death. Overall, these results demonstrate that H1 acts cell-autonomously in escort cells to repress cap cell identity and transposon activity, which are necessary to maintain escort cell identity and regulate proper germ cell differentiation (Yang et al., 2017).

3.2. Histone Modifying Enzymes

Polycomb Group (PcG) and Trithorax Group (TrxG): In addition to Su(z)2 and Psc, Pc has been shown to regulate *Abd-B* expression in the CySCs. Knockdown of Pc in the CySCs leads to Zfh1-positive CySC-like tumor cells, overproliferation of early-stage germ cells at the expense of differentiation. The overproliferative CySCs phenotype can be

partially rescued by compromising *Abd-B*, suggesting mis-expressed *Abd-B* in the CySCs underlies these cellular defects (Zhang et al., 2017).

In the *Drosophila* ovary, knockdown of PRC1 components Sce, Ph, or Psc/Su(z) 2 in the escort cells results in a GSC-like tumor cells, which express the BMP pathway components pMad and Dad, but lack Bam expression. Upregulated BMP signaling is responsible for this tumor phenotype, as compromising Dpp results in partial rescue. Finally, the TrxG component Brm activates Dpp expression in the escort cells, as compromising Brm also rescues this tumor phenotype (Dingwall et al., 1995). Therefore, PRC1 acts in the escort cell to repress Dpp to allow for the proper differentiation of GSCs. Either inactivation of PRC1 or hyperactivation of Brm induces ectopic Dpp expression, which prevents germline differentiation with accumulation of GSC-like tumor cells in the *Drosophila* ovary (Li et al., 2016). Therefore, PRC1 acts non-cell autonomously in the escort cells to restrict active BMP signaling in GSCs.

Eggless/ dSETDB1: In addition to maintaining GSCs, Egg is required for GSC differentiation. Loss of *egg* results in escort cell death, increased BMP signaling in the germaria, and GSC differentiation defects (Wang et al., 2011). Egg also acts in escort cells to regulate GSC differentiation non-cell autonomously, likely by repressing BMP regulators, such as Dally.

Su(var)3-3/ lysine -specific demethylase 1 (Lsd1): Lsd1 is a demethylase for H3K4 mono- and di methylation and is a regulator for heterochromatin spreading and GSC niche size in the *Drosophila* ovary (Table 1) (Di Stefano et al., 2007; Eliazer et al., 2011; Holowatyj et al., 2015; Rudolph et al., 2007). Lsd1 also acts non-cell-autonomously to regulate female GSC differentiation. Inactivation of *lsd1* by mutation or RNAi in escort cells induces escort cell death and formation of GSC-like tumors, due to ectopic Dad expression and loss of Bam expression. ChIP experiments suggest that Engrailed is a direct target of Lsd1, suggesting that BMP signaling is indirectly repressed by Lsd1. In wild-type germaria, Engrailed, a homeobox transcription factor, acts in the terminal filament and cap cells to maintain female GSCs, but in *lsd1* mutant germaria, Engrailed is detected in the escort cells (Bolivar et al., 2006; Desplan et al., 1985; Kornberg, 1981; Morata and Lawrence, 1975; Rojas-Rios et al., 2012). Moreover, compromising Engrailed in *lsd1* mutant ovaries rescues the GSC-like tumor phenotype and overexpression of Engrailed phenocopies *lsd1* mutant phenotype. Knockdown of *lsd1* in the escort cells also causes cell fate changes, shown by ectopic expression of Hedgehog (Hh), which is normally expressed exclusively in the terminal filament and cap cells to promote expression of Dpp for the neighboring GSCs (Rojas-Rios et al., 2012). In conclusion, Lsd1 acts in the escort cells to inhibit Engrailed, which prevents hyperactive Hh signaling from activating Dpp expression. These functions restrict Dpp expression solely in the cap cells and terminal filament cells, which in turn ensures BMP signaling is only received by the female GSCs (Eliazer et al., 2014; Eliazer et al., 2011).

Tat interactive protein 60kDa (Tip60): The *Drosophila* Tip60 complex acetylates lysines 5, 8, 12, and 16 on histone H4 to regulate gene expression, chromatin packaging, and DNA repair (Kusch et al., 2004; Schirling et al., 2010). It is comprised of the histone

acetyltransferase Tip60, the ATPase Domino, Nipped-A, Enhancer of Pc [E(Pc)] and a few other components (Table 1) (Clapier and Cairns, 2009; McCarthy et al., 2018). Among them, E(Pc) and Nipped-A are shown to regulate male and female GSC differentiation, respectively. Knockdown of E(Pc) in the CySC and early cyst cells in testes causes overproliferation of CySC-like cells and GSC-like cells, and ectopic expression of somatic cell markers in germline tumors. Cell-type-specific ChIP experiments show that E(Pc) directly binds to genes encoding components of multiple signaling pathways, including JAK-STAT and EGF. The EGF signaling pathway has been previously shown to control encapsulation, proper differentiation and mitotic divisions of early-stage germ cells by the cyst cells (Kiger et al., 2000; Parrott et al., 2012; Schulz et al., 2002). Inactivation of Tip60 acetyltransferase function in the CySC lineage results in similar phenotypes as seen in *E*(*Pc*) mutant testis. Therefore, E(Pc) and Tip60 act synergistically to promote CySC differentiation cell-autonomously and to regulate proper germ cell differentiation and maintain germline identity non-cell-autonomously (Feng et al., 2017).

In the male germline, E(Pc) is required for proper germ cell activity, as the germline-specific knockdown of *E*(*Pc*) results in germline tumor and GSC cell death phenotypes. In addition, compromising E(Pc) in the germline results in increased CycB expression in the late-stage germ cells and enhanced γH2Av, a marker for DNA double-strand break (DSB) and cell death (Lake et al., 2013). Finally, knocking down E(Pc) in the germline reduces Bam expression, responsible for the germline tumor phenotype. In summary, in the male germline E(Pc) regulates transcription of *cycB* through H4 acetylation, accumulation of Bam protein, and DNA repair for proper GSC differentiation and the mitosis-to-meiosis transition (Feng et al., 2018).

In the female germline, knockdown of *Nipped-A* results in reduced Bam expression, leading to the accumulation of GSC-like tumor cells. Inactivation of Tip60 complex components, such as Bap55, Domino, E(Pc), YL-1 and Tip60, also results in GSC-like tumors in the ovaries. Through ChIP experiments, *bgcn* is detected as a direct target gene of Nipped-A. Since Bgcn regulates GSC differentiation, overexpressing Bgcn could rescue differentiation defects of *Nipped-A* knockdown germ cells. In conclusion, the Tip60 complex is required cell autonomously to ensure Bgcn expression in the pre-cystoblast to promote female GSC differentiation (McCarthy et al., 2018).

Ovaries absent (Ova): A recent study identified Ova and its bridging role between HP1 and Lsd1 to silence heterochromatic genes in the *Drosophila* ovary. The *ova* mutant ovaries show either germline tumor or germ cell loss phenotypes. Specific knockdown of *ova* in the somatic gonadal cells results in GSC-like tumor cells, accompanied with ectopic pMad and Dad expression but decreased Bam levels. Biochemical assays show that Ova directly binds to Lsd1 and HP1α *in vitro* and expression of the Ova protein fragment that interacts with both HP1α and Lsd1 is sufficient to rescue the *ova* mutant defects in ovaries. Moreover, ChIP experiments show increased H3K4me2 at telomeric transposons in *ova* mutant ovaries compared to the control ovaries. Given that Lsd1 is an H3K4me2 demethylase, these results suggest that Ova is indispensable for the H3K4 demethylation activity of Lsd1 in heterochromatic gene silencing. Therefore, Ova is necessary for heterochromatin formation

as well as HP1- and Lsd1-mediated gene silencing of GSC self–renewal genes to ensure proper differentiation of female GSCs (Yang et al., 2019).

3.3. RNA- binding proteins and noncoding RNAs

miRNAs and piRNAs have also been shown to regulate GSC differentiation. Mutations of *miR-9a* result in decreased GSC division and failure in spermatogenesis. RNA-seq experiments have shown increased N-cadherin expression in *miR-9a* mutant testes. Consistently, overexpression of N-cadherin phenocopies while compromising N-cadherin levels suppresses *miR-9a* mutant phenotypes. Since N-cadherin is a cadherin family member for adherens junction between GSCs and hub cells (Figure 2), these results suggest that normal functions of *miR-9a* downregulate N-cadherin to allow for detachment of GSCs from the niche for differentiation (Epstein et al., 2017).

In the ovary, Vret, a Tutor domain-containing protein, is associated with Piwi and Aub. Knocking down Vret in the germline or the somatic gonadal cells results in mis-localized Piwi to the nucleus in both cell types and loss of Aub in the germline nuage structure. In addition, *vret* mutant ovaries show reduced piRNA levels and enhanced transposons mobilization, as well as somatic cell death and GSC differentiation defects. These result in germaria filled with GSC-like cells, but these phenotypes can be suppressed when wild-type *Vret* is expressed in the somatic gonadal cells. Based on these data, Vret is likely an essential component of the Piwi- and Aub-containing RISC complexes in the germline, as well as Piwi-containing RISC complexes in the somatic gonadal cells, to regulate transposon levels and biogenesis of primary piRNAs in the ovary (Zamparini et al., 2011).

4. Conclusions and Perspective

In this review, we focus on distinct epigenetic regulatory mechanisms in both female and male GSC maintenance and differentiation using *Drosophila* as the model organism. The commonalities and differences between these two GSC systems provide insight into some general scenario as well as sexual dimorphism. Here, we summarize these highlights and comment on existing caveats as well as future perspectives:

1. Both GSC systems achieve a balanced ratio between stem cells and differentiating cells through ACD, which greatly facilitate studying epigenetic mechanisms in endogenous stem cells at single-cell resolution. This feature, combined with cell-type-specific labeling and cutting-edge imaging methods, such as live cell imaging and superresolution imaging, will provide more important insight on how epigenetic regulators play biological functions *in vivo*.
2. Both GSC systems have well characterized niche structure, critical signaling pathways emanated from the niche, cellular features including cell cycle progression and markers for inter- and intra-cellular components. Interestingly, studies exploring the roles of epigenetic factors in regulating GSC self-renewal and differentiation mainly reveal their control on the activity of two signaling pathways: JAK/STAT and BMP pathways. These could be due to two reasons: First, it is likely because of the key roles these two signaling pathways play in determining the balance between stem cell fate and differentiation cell fate. For

example, epigenetic regulators tend to change the expression of stemness genes, such as *dad* and *stat92E*, or the differentiation genes, such as *bam* and *bgcn*. Second, the convolution of the results on a few downstream targets could be due to detection methods. Given the abundant reagents available to analyze abnormal expression or localization of these known key factors, it is not surprising that they serve as the top candidates when designing experiments to explore the "read-outs" of abnormal epigenetic regulation. However, rapid development on single-cell genomics and epigenomics techniques will now or soon allow characterization of the direct targets of these epigenetic regulators in pure cells with distinct identities, leading to a comprehensive understanding of their roles in adult stem cell systems. On the other hand, due to their intricate regulation on signaling pathways, it is intriguing to see that many of these epigenetic regulations have both cell-autonomous and non-cell-autonomous roles. Co-existence of GSCs with somatic gonadal cells in both systems provides a unique opportunity to dissect these relationships *in vivo*, given their reliable anatomy and morphological distinctions, as well as the relative less complicated tissue architecture compared to the mammalian tissue/organ.

3. Interestingly, disruption of epigenetic regulation often results in multiple cellular defects. Characterization of these pleiotropic phenotypes often reveal gene misexpression (i.e. expression levels are too high, too low, or are ectopic in the wrong cell and/or at the wrong timing), suggesting a rather confused cell fate. Given that it is very plausible that each epigenetic regulator has many targets and even the same epigenetic regulator has different targets in different cells, the nature of the cell fate state in their loss-of-function mutants could be quite complicated. Furthermore, most of the "rescuing" results when genetic suppression has been detected are mainly based on morphological recovery but not on functional recovery, the later requires more rigorous assay including the ability to regain stem cell activity for both successful self-renew and full differentiation to become functional egg or sperm. Therefore, to completely understand their molecular and cellular defects, new technology such as single-cell RNA-seq could help elucidate the genome-wide transcriptome change; and lineage tracing could reveal their differentiation footage. Finally, given our current knowledge that certain chromatin changes could precede actual transcription change, such as the phenomena of "poised" RNA Pol II and bivalent histone modifications, the downstream effects could comprise both actual gene expression changes and alterations only at the chromatin level. The later will require chromatin structure profiling using small number of cell or even at the single-cell level, which is becoming more and more feasible given the quick advancement of single-cell epigenome techniques.

4. Finally, even though results using both female and male GSC systems demonstrate many common themes, distinct feature have also been identified, as discussed throughoutin this review. Many of these distinctions could reflect the intrinsic differences between these two adult stem cell lineages. These include findings from many fly germ cell laboratories, such as the different Bam

expression patterns, the distinct roles of the JAK-STAT pathway, position and function differences of certain organelles (e.g. spectrosome), germline-soma interactions at the niche, etc. As their abnormalities are often found upon compromising certain epigenetic regulator using genetic mutations or RNAi knockdown, which often takes some time for the phenotypes to manifest. It is still challenging to know the dynamics of the functions of epigenetic regulators. More acute disruption methods especially the reversible ones, such as the Auxin-inducible degron (AID) technology, combined with high-temporal resolution analyses of cellular defects, could greatly facilitate understanding of their primary roles.

In summary, our understandings of epigenetic regulation in early-stage germ cells at single-gene resolution have greatly improved in recent years. Moreover, the advanced techniques will pave the way for better understanding of the dynamic epigenetic regulation in the *Drosophila* gonads, at both genome-wide level and individual gene locus, as well as in real-time with single cell resolution.

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Highlights:

Note—Since this is a review but not a research article, we do not think this requirement applies. Please let us know if you think otherwise. Thanks!

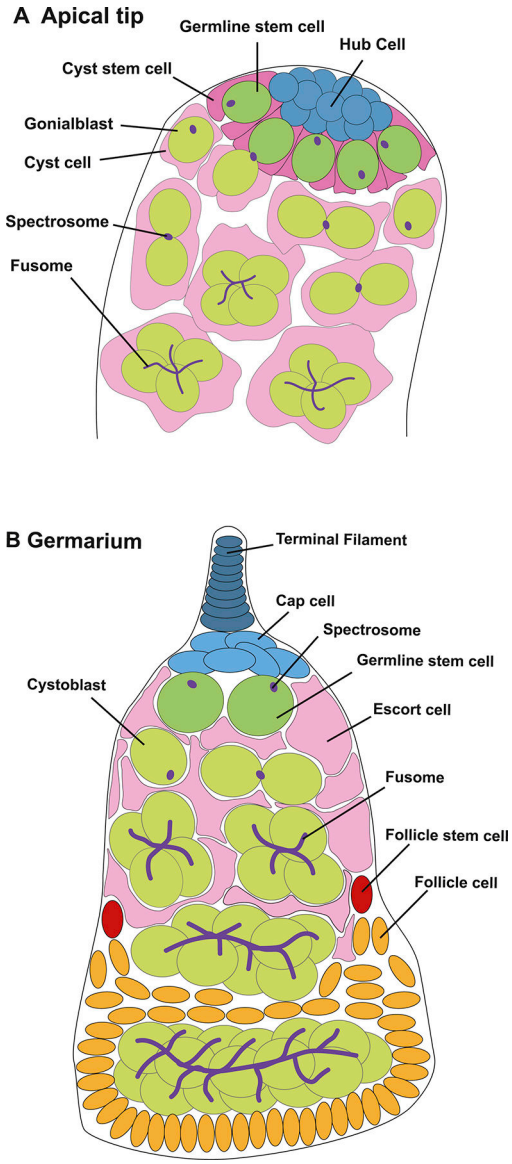

Figure 1: Niches, early-stage germline and somatic gonadal cells in *Drosophila* male and female gonads.

(**A**) The *Drosophila* testis is a pair to two coiled tubes, each containing a single stem cell niche at the apical tip. The stem cell niche is called the hub (dark blue), which is a cluster of 10–12 densely packed somatic cells. The germline stem cells (GSCs) (dark green) and the cyst stem cells (CySCs) (dark pink) are radially positioned around the hub. There are 10–15 GSCs arranged around the hub with two CySCs enveloping each GSC. The GSCs undergo an asymmetric division to produce a self-renewed daughter GSC and a gonialblast (GB) (light green). The GB is displaced from the hub and undergoes 4 rounds of miotic divisions with incomplete cytokinesis to create cysts of interconnected germ cells. The differentiating germline cysts continue to be encapsulated by two post mitotic cyst cells (light pink). The 16-spermatogonial cell cyst then undergoes meiosis as spermatocytes to produce 64 spermatids. (**B**) The *Drosophila* ovary consist of 16–20 tubular structures called ovarioles. An ovariole consists of a germarium at the apical tip and a linear progression of

differentiating egg chambers that produce eggs, located at the base. The germarium is comprised of the GSC niche and the proliferating germ cells. The GSC niche includes a stack of 8–10 post-mitotic somatic cells called the terminal filament (dark blue) and 5–7 epithelial cells called the cap cells (light blue) that directly interact with the 2–3 GSCs (dark green). Female GSCs divide asymmetrically to produce a self-renewed GSC that stays in contact with the cap cells and a posteriorly displaced daughter cell that leaves the niche and differentiates into a cystoblast (CB) (light green). The CB then undergoes 4 rounds of synchronous mitotic cell divisions with incomplete cytokinesis to create 16 interconnected cystocytes. Interspersed between the early germline cells are 4 −6 escort cells (pink) that surrounded the GSCs and the dividing CBs. The interconnected germ cell cysts then begin to associate with somatic follicle cells. Follicle cells (orange) are derived from two follicle stem cells (FSCs) (red) that are located between the escort cell and follicle cell transition zone. Once the 16-cell germ cyst is surrounded by follicle cells, it becomes an egg chamber, buds off from the germarium, and continues to mature and undergoes meiosis to develop into an oocyte and 15 interconnected nurse cells. Adapted from Gleason et al., Genetics 2018.

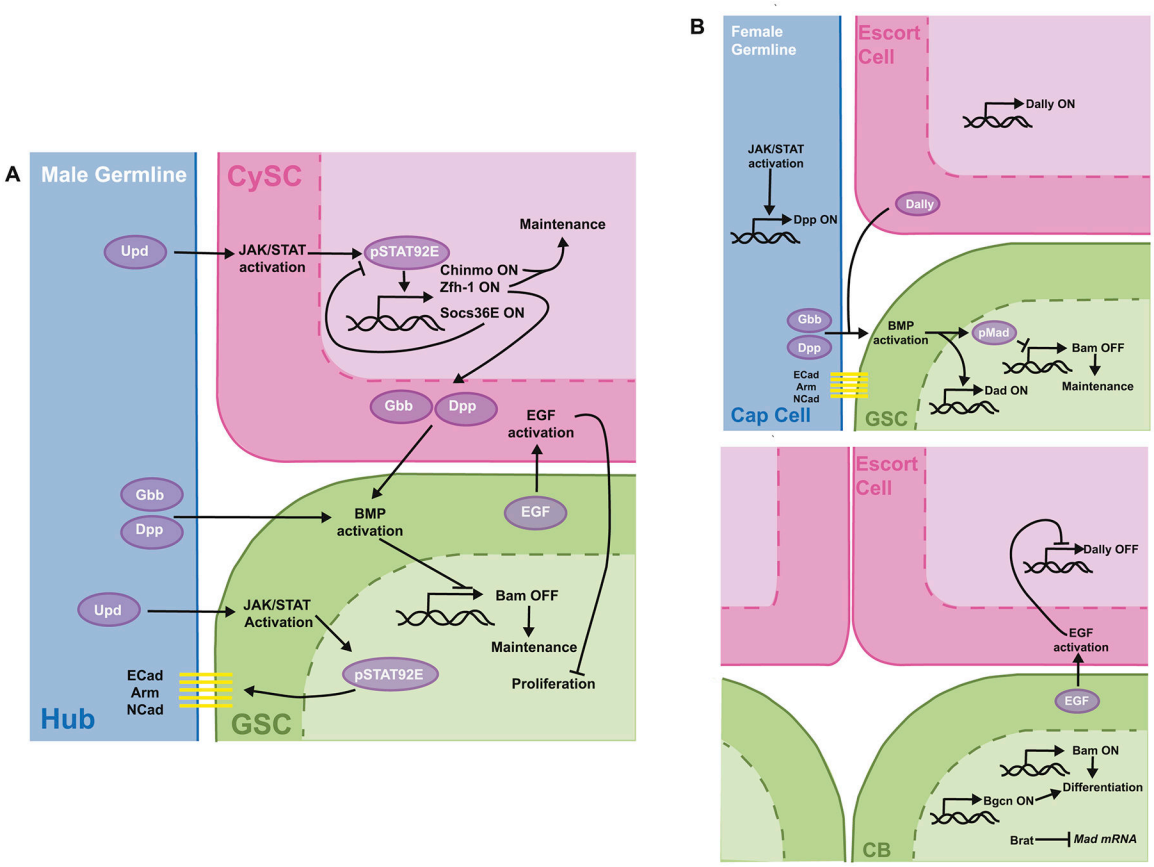

Figure 2: Signaling pathways regulating GSC self-renewal and differentiation at niches in *Drosophila* male and female gonads.

(**A**) In the *Drosophila* testis, Hub cells secrete the ligand Unpaired (Upd), which activates Janus Kinase and Signal Transducer and Activator of Transcription (JAK-STAT) signaling in the GSCs and CySCs. In the CySCs, JAK-STAT activation is sufficient for stem cell self-renewal, while the GSCs are maintained by signaling from both the hub cells and CySCs. Activated pStat92E in the CySCs activates expression of *zn finger homeodomain 1* (*zfh1*) and *chronologically inappropriate morphogenesis* (*chinmo*), which are important for CySC self-renewal and identity. Activation of Zfh1 turns on expression of the BMP ligands *glass bottom boat* (*gbb*) and *decapentaplegic* (*dpp*) in the CySCs, which activate BMP signaling in the GSCs, important for GSC self-renewal and maintenance. Activation of BMP signaling in the GSCs by the hub cells and CySCs also inhibits expression of *bag of marbles* (*bam*) to prevent differentiation. Activation of pStat92E in the GSCs promotes GSC adhesion to the hub. Activation of pStat92E in the CySCs also activates expression of suppressor of cytokine signaling at 36E (*socs36E*), a negative regulator of the JAK-STAT pathway. In addition, EGF from the GSCs to the CySCs regulates their abilities to encapsulate germ cells and GSC divisions. (**B**) In the *Drosophila* ovary, JAK-STAT acts in the cap cells to activate BMP ligand, *dpp*. Dpp/Gbb activates BMP signaling in the GSCs, resulting in phosphorylation of Mothers against dpp (pMad) and activated transcription of *dads against dpp* (*dad*). pMad and its partner Medea translocate to nucleus and repress transcription of *bam*, inhibiting GSC differentiation (Hudson et al., 1998). This repression is relieved once the GSC daughter cell

leaves the niche. Brain tumor (Brat) reduces BMP responsiveness in the CB. EGF from the CB activates EGF signaling in the neighboring escort cells, where it represses the transcription of *dally* (Liu et al., 2010). This ensures that BMP ligands Dpp/Gbb are restricted to the cap cells and BMP activation only occurs in the GSCs. Adapted from Matunis et al., Spermatogenesis 2012.

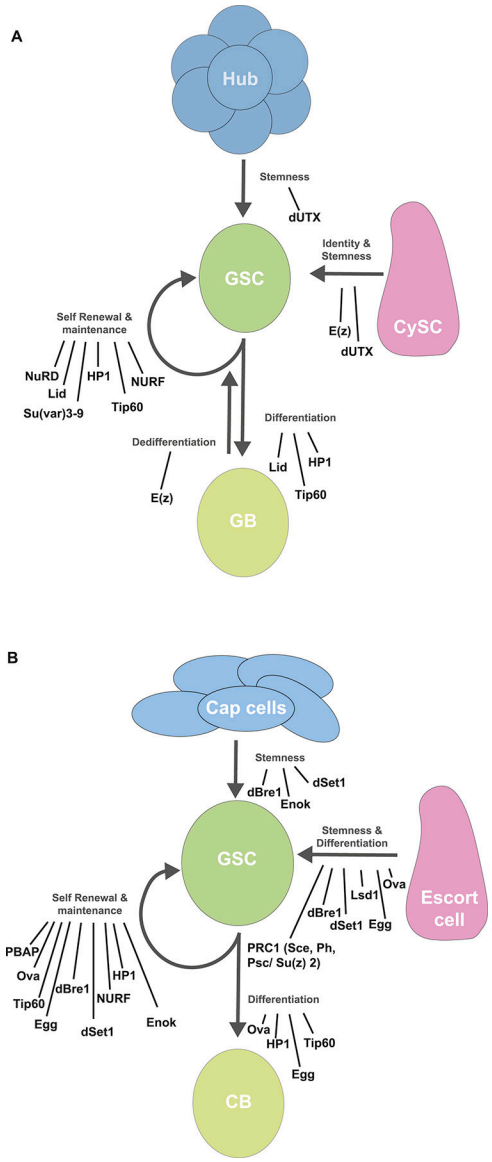

Figure 3: Epigenetic Regulation of GSC maintenance and differentiation in the male and female *Drosophila* germline.

Chromatin regulators and histone modifying enzymes are placed where they regulate GSC maintenance or differentiation in the male (**A**) and female (**B**) early germline. Schematic drawings of niche and somatic cells regulating GSC maintenance, GSC self-renewal, and GSC differentiation.

Table 1:

Histone PTMs and Histone Modifying Enzymes in the *Drosophila* Male and Female Gonads

PTM	Writer	Eraser	Reader/additional components	Function	Germline loss-of-function phenotypes	Gonad studied & cell types present	Phenotype due to loss of Enzymatic activity
H3K27me3	E(z)	dUTX	Pc/PRC2 (Esc, Su(z)12, & Caf1-55)	Present at inactive gene promoters, pericentric heterochromatin, and inactive euchromatic regions	Overproliferation of early cyst cells; germ cells expressing somatic cell markers; increased hub size; defective germ cell dedifferentiation	Male germline; germ cells & cyst cells	N.D.
H2AK118ub	Sce/dRing	PR-DUB	PRC1 (Pc, Ph, Psc/ Su(z) 2)	Promotes chromatin repression	N.D.	N.D.	N.D.
H3K4me3	Trx, Trr, dSet1	Lid	Ash2, Rbbp5, Wdr5, Dpy30	Present at promoters of actively transcribing genes	GSC loss; overproliferation of GSC-like cells	Male & female germline germ cells, escort cells & cap cells	N.D.
H3K9m3	Su(var)3–9, Egg	Kdm4B	HP1	Present at constitutive heterochromatin and maintains pericentric heterochromatin	GSC loss or overproliferation of GSC-like cells; escort cell death	Male & female germline; germ cells & escort cells	N.D.
H3K4me1/2	N. D.	Su(var)3–3/ Lsd1	HP1/Ova	Present at promoters of actively transcribing genes	Overproliferation of GSC-like cells; germ cell loss; escort cell death	Female germline; germ cell & escort cells	N.D.
H4K(5, 8, 12, 16)ac	Tip60	N.D.	Domino, Nipped-A, E(pc)	Regulates gene expression, chromatin packaging and DNA repair	GSC cell death; overproliferation of CySC-like cells & GSC-like cells; germ cells express somatic cell markers; spermatogonial tumors	Male & female germline; germ cells & cyst cells	Yes
H3K23ac	Enok	N.D.	N.D.	Promotes gene expression	GSC loss	Female germline; germ cells & cap cells	N.D.

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Table 2:

Chromatin Remodelers in the *Drosophila* Male and Female Gonads

Family	Complex	ATPase	other subunits	Function	Germline loss of function phenotype	Gonad studied & cell types present
SWI/SNF	PBAP	BRM/ Brahma	Polybromo / BAP180, BAP170	Slides and ejects nucleosomes for diverse processes including DNA damage repair and recruitment and initiation of transcription	GSC loss	Female germline; cap cells & germ cells
ISWI	NURF	ISWI	Nurf301, Nurf55, Nurf38	Optimize nucleosome spacing for chromatin assembly; promote H1 loading for chromatin compaction; regulates Dosage compensation; randomizes spacing for RNA Pol II activation; transcriptional repression	GSC and CySC loss	Male and female germline; hub cells, germ cells & cyst cells
CHD	Mi-2/NuRD	dMi-2	dMBD2/3, dMTA, dRPD3, p55, p66/68	Slides or ejects nucleosomes to promote transcription elongation	N.D.	Male germline; germ cells
INO80	Tip60	Domino	dTip60, E(Pc)	INO80 complexes promote transcriptional activation and DNA repair; promotes H2Av incorporation	GSC and CySC loss; cystoblast cell differentiation defects	Male and female germline; germ cells, cyst cells, escort cells, cap cells, & follicle cells