ABSTRACT Gametogenesis represents the most dramatic cellular differentiation pathways in both female and male flies. At the genome level, meiosis ensures that diploid germ cells become haploid gametes. At the epigenome level, extensive changes are required to turn on and shut off gene expression in a precise spatiotemporally controlled manner. Research applying conventional molecular genetics and cell biology, in combination with rapidly advancing genomic tools have helped us to investigate (1) how germ cells maintain lineage specificity throughout their adult reproductive lifetime; (2) what molecular mechanisms ensure proper oogenesis and spermatogenesis, as well as protect genome integrity of the germline; (3) how signaling pathways contribute to germline-soma communication; and (4) if such communication is important. In this chapter, we highlight recent discoveries that have improved our understanding of these questions. On the other hand, restarting a new life cycle upon fertilization is a unique challenge faced by gametes, raising questions that involve intergenerational and transgenerational epigenetic inheritance. Therefore, we also discuss new developments that link changes during gametogenesis to early embryonic development—a rapidly growing field that promises to bring more understanding to some fundamental questions regarding metazoan development.

KEYWORDS oogenesis; spermatogenesis; germline stem cells; somatic gonadal cells; mitosis; meiosis; signaling pathways; piRNA; transcription; chromatin regulator; epigenetics; FlyBook

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**Mechanisms Regulating GSC Self-Renewal vs. Differentiation**

**DNA methylation and demethylation**

DNA methylation is a widely conserved epigenetic mechanism that functions through the covalent and heritable modification of genomic DNA at both cytosine and adenine residues (Suzuki and Bird 2008; Luo et al. 2015). DNA methylation of the fifth position of cytosine (5-methylcytosine, 5mC) is established and maintained by a conserved family of enzymes called DNA methyltransferases (DNMTs), which have been found to function in transcriptional silencing of promoters, transposable elements, and other repetitive sequences in most plant, animal, and fungal species (Wu and Zhang 2014). DNMTs are divided into three subfamilies based on sequence conservation and function. DNMT3 functions in the de novo methylation of cytosine. DNMT1 maintains DNA methylation postreplication on the newly synthesized DNA strands, and DNMT2 exhibits a weak catalytic activity on DNA compared to DNMT3 (Hermann et al. 2003). DNMT2-mediated methylation of multiple transfer ribonucleic acids (tRNAs) has also been documented (Goll et al. 2006; Schaefer et al. 2010). Drosophila belongs to the “DNMT2 only” category of organisms based on loss of the canonical DNA methyltransferases (DNMT1 and DNMT3), and retention of the DNMT2 homolog DNMT2/Mt2. In addition, 5mC levels in Drosophila have been found to be very low compared to other organisms (Lyko et al. 2000a; Phalke et al. 2009; Krauss and...
which studies have revealed that mammalian DNA, between 2 and 10% of all cytosine residues are modified to 5mC, whereas Drosophila DNA contains only 0.1–0.6% of modified 5mC of all cytosine residues (Gowher et al. 2000; Zemach et al. 2010). While the mechanism by which DNMT2 functions in the germline remains obscure, recent studies have revealed that DNMT2 is involved in multiple processes, including sister chromatids' segregation in the male germline, retrotransposon silencing in the early embryo, and gene silencing (Phalke et al. 2009; Yadlapalli and Yamashita 2013). Expression of DNMT2 was first observed in the ovaries, as well as during early embryogenesis through RNA in situ hybridization, and more recently in the male germline (Lyko et al. 2000b; Gan et al. 2010a). DNMT2 function was found to be necessary for proper segregation of X and Y

Figure 1 Anatomy of Drosophila female and male gonads. (A) Anatomy of germarium and oogenesis. The Drosophila ovaries are made up of 16–20 tubule structures, called ovarioles, that resemble linear assembly lines of progressively differentiating egg chambers to produce eggs. The germanium, designated by the dashed rectangular outline, where the egg chamber originates, is located at the anterior tip of each ovariole. The germarium consists of the GSC niche and the proliferative germ cells that remain active, producing eggs throughout adulthood. At the anterior tip of each germanium resides the niche, which consists of a stack of 8–10 postmitotic somatic cells, called the terminal filament (light green), five to seven squamous epithelial cells, and cap cells (dark green) that literally cap the underlying two to three GSCs (red). Female GSCs divide asymmetrically such that the anteriorly positioned daughter cell remains in contact with the cap cells and maintains GSC identity, while the posteriorly displaced daughter cell leaves the niche and differentiates into cystoblasts (CBs) (pink). Following the asymmetric cell division, the daughter CB undergoes four rounds of synchronous mitotic cell divisions with incomplete cytokinesis to give rise to 16 interconnected cystocytes (pink). During early germ cell development, early germ cells associate intimately with neighboring somatic cells, including escort cells and follicle cells (yellow). Interspersed between the GSCs are four to six escort cells (blue cells), which cover most of the GSC and dividing CBs, isolating early germ cells from each other, but not from the cap cells. Next, the interconnected germ cell cyst associates with another somatic cell type, the somatic follicle cells. These somatic follicle cells are derived from two somatic follicle stem cells (FSCs) (orange), which are maintained at the boundary between escort cells and the follicle cells. When the 16-cell cyst is surrounded by follicle cells, it becomes an egg chamber, buds from the germanium, and continues to mature (Dawring and Sunner 1973). One of the 16 cells will progress through meiosis and develop into the oocyte, while the other cells will develop into polyplid nurse cells that will support oocyte growth. A single egg chamber consists of the single oocyte connected to 15 nurse cells via a system of intercellular bridges and a surrounding monolayer of up to 650 somatic follicle cells (King 1970; Spradling 1993). The nurse cells deliver their cytoplasm into the oocytes and undergo apoptosis during the latest stage of oogenesis to produce a mature egg (Foley and Cooley 1998). Meiotic divisions in the egg are only accomplished after sperm entry, leading to one female pronucleus and three polar bodies, which subsequently undergo degeneration. The female and male pronuclei appose each other, followed by fusion, which labels the formation of a zygote and the onset of a new life cycle. (B) Anatomy of testis and spermatogenesis. The adult testis of Drosophila melanogaster is a pair of coiled tubes ~2 mm in length, each composed of a single stem cell niche at the apical end designated by the dashed gray outline (Hardy et al. 1979). The apical cells are assembled into a centrally located structure having GSCs (dark blue), and CySCs (orange) are radially positioned around a cluster of 10–12 small densely packed somatic cells called the hub (Green). Six to nine GSCs are arranged around the hub, while two CySCs fully envelope one GSC such that GSC-to-GSC contact never occurs. Spermatogenesis initiates with the asymmetric division of the GSC to produce one self-renewed daughter cell and a gonialblast (GB) cell (light blue). Upon division, the GB is displaced from the hub and undergoes four transit-amplifying divisions with incomplete cytokinesis, generating a cyst of interconnected germ cells joined by cytoplasmic bridges (light blue). After transit-amplification, the cyst of 16 interconnected spermatogonia synchronously undergoes meiotic DNA synthesis. During meiotic prophase I as spermatocytes, each cell grows ~25-fold and initiates a robust gene expression program that enables meiotic division and spermatid differentiation. After two meiotic divisions, 64 haploid spermatids are produced, as designated by the dashed blue outline.

Reuter 2011; Raddatz et al. 2013; Capuano et al. 2014; Takayama et al. 2014; Zhang et al. 2015). For example, in mammalian DNA, between 2 and 10% of all cytosine residues are modified to 5mC, whereas Drosophila DNA contains only 0.1–0.6% of modified 5mC of all cytosine residues (Gowher et al. 2000; Zemach et al. 2010). While the mechanism by which DNMT2 functions in the germline remains obscure, recent studies have revealed that DNMT2 is involved in multiple processes, including sister chromatids' segregation in the male germline, retrotransposon silencing in the early embryo, and gene silencing (Phalke et al. 2009; Yadlapalli and Yamashita 2013). Expression of DNMT2 was first observed in the ovaries, as well as during early embryogenesis through RNA in situ hybridization, and more recently in the male germline (Lyko et al. 2000b; Gan et al. 2010a). DNMT2 function was found to be necessary for proper segregation of X and Y

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sister chromatids during asymmetric male GSC divisions (Yadlapalli and Yamashita 2013).

Until recently, DNA methylation of the sixth position of Adenine (6mA) was thought to be restricted to bacteria, archaea, protists, and fungi (Wion and Casadesus 2006). However, recent studies have identified 6mA to be present in 0.07–0.001% of all adenine residues in the Drosophila genome during early- and late-stage embryogenesis, respectively (Zhang et al. 2015). Demethylation of 6mA is regulated by the Drosophila Tet homolog, DNA 6mA demethylase (Dmad), during embryogenesis and oogenesis. During oogenesis, loss of Dmad results in an increase of 6mA in the ovaries and accumulation of GSC-like cells. On the other hand, overexpression of Dmad leads to a significant loss of germ cells, including GSCs. Furthermore, Dmad-mediated 6mA demethylation correlates with transposon suppression, indicating that Dmad actively removes 6mA to suppress transposon expression (Zhang et al. 2015). These results are consistent with the role Dmad plays in the demethylation of 6mA to promote GSC differentiation during oogenesis. Together, these results indicate that 5mC has a limited or spatiotemporally specific role in Drosophila, likely independent of DNMT2/ Mt2 whose enzymatic role is yet to be defined. On the other hand, recent data demonstrate important roles of 6mA and its demethylase Dmad in Drosophila oogenesis and embryogenesis, even though the corresponding methyltransferase has not been characterized. Given the clear biological functions of 5mC in mammals, it is possible that fly and mammals use distinct DNA methylation mechanisms for their epigenomes.

**Chromatin remodeling factors**

The chromatin structure of GSCs and somatic stem cells (SSCs) is regulated by ATP-dependent chromatin remodeling enzymes in both males and females in order to maintain self-renewal and prevent differentiation. These enzymes utilize the energy of ATP hydrolysis to establish and maintain a particular chromatin state during development. The different subfamilies of chromatin-remodeling enzymes catalyze a remarkable range of chromatin modifications that include histone exchange, translocating the histone octamer and changing the conformation of nucleosomal DNA (Narlíkar et al. 2013). Common across all ATP-dependent chromatin remodeling enzymes is the ATPase subunit belonging to the

### Table 1 Cell-type-specific Gal4 drivers in Drosophila gonads

| Cell-Type Expression | Name | Description | References |
|----------------------|------|-------------|------------|
| Male germline | nos-Gal4-VP16 | GSCs and early germline cysts | Van Doren et al. (1998) |
| | bam-Gal4-VP16 | Initiates expression during transit-amplification divisions and expressed in early spermatocytes | Chen and McKearin (2003b) |
| Hub cells | vas-Gal4 | Most germline cells | Zhao et al. (2013) |
| | upd-Gal4 | Hub cells | Zeidler et al. (1999) |
| | hh-Gal4 | Hub cells | Tanimoto et al. (2000) |
| | fasIII-Gal4 | Hub cells | Wolfstetter and Holz (2012) |
| CySCs and somatic cells | tj-Gal4 | CySCs, early cyst cells, and hub cells | Hayashi et al. (2002) |
| | C587-Gal4 | CySCs, early cyst cells, and hub cells | Kaid and Spradling (2003), Zhu and Xie (2003) |
| Somatic cells | ptc-Gal4 | CySCs and cyst cells | Tazuke et al. (2002) |
| | eya-gal4 | CySCs and cyst cells, weakly expressed in hub cells | Leatherman and Dinardo (2008) |
| | arm-Gal4 | Most somatic cells including Hub cells, CySCs, and cyst cells | Sanson et al. (1996) |
| | Hsp83-Gal4 | Ubiquitously expressed | Arama et al. (2003) |
| Female germline | nos-Gal4 [pBac (GreenEye.nos-Gal4-VP16)] | Stem cells, young egg chambers, and increased expression at stage 5 | Holtzman et al. (2010) |
| | nos-Gal4-VP16 | Stem cells, young egg chambers, and increased expression at stage 5 | Van Doren et al. (1998) |
| Maternal Triple Driver (MTD)-Gal4 | Bam-Gal4-VP16 | Uniform expression in the germinarium and throughout oogenesis, including GSCs | Petrella et al. (2007) |
| | bam-Gal4-VP16 | Germ cell expression starting at the two-cell cyst stage or CB cells | Chen and McKearin (2003b) |
| Escort cells | pCOG-Gal4-VP16 | Moderate levels throughout oogenesis | Rorth (1998) |
| | C587-Gal4 | Escort cells | X. Song et al. (2004) |
| Terminal filament and Cap cells | bab-1-Gal4 | Terminal filament and cap cells | Cabrera et al. (2002) |
| | hh-Gal4 | Terminal filament and cap cells | Tanimoto et al. (2000) |
| Follicle stem cells | 10S-30-Gal4 | Follicle stem cells and early follicle lineage | Hartman et al. (2010) |
| | CH16-Gal4 | All follicle cells starting in gerarium | Ward et al. (2002) |
| Somatic cells | tj-Gal4 | All follicle, follicle stem cells, escort cells, and cap cells | Hayashi et al. (2002) |
| Ubiquitous expression | tub-Gal4 | Ubiquitous expression in all cells | Lee and Luo (1999) |
helicase superfamily 2 (SNF2) (Eisen et al. 1995). This SNF2 family of proteins can be further classified on the basis of distinct domains conserved among the subfamilies, such as the bromodomain shared by the SWI2/SNF2 (SWI2/Sucrose NonFermentable) family, the chromodomain shared by the CHD (Chromodomain-Helicase-DNA-binding protein) family, and the SANT domain shared by the ISWI (Imitation SWI) family (Hota and Bruneau 2016).

**Imitation switch (ISWI):** In *Drosophila*, ISWI serves as an ATP-dependent motor that governs transcriptional regulation through catalyzing changes in nucleosomal assembly and composition (Deuring et al. 2000; Badenhorst et al. 2002; Corona et al. 2002). In both males and females, ISWI is essential for GSC maintenance, suggesting a common epigenetic mechanism employed by both sexes to maintain a chromatin configuration for stem cell maintenance. In females, ISWI is present at high levels in all cell types, including GSCs and PSCs (Xi and Xie 2005). Mitotic recombination techniques (Xu and Rubin 1993) were used to generate marked *iswi* mutant GSC clones, most of which were lost from the niche owing to premature differentiation. Similar to ISWI, bone morphogenetic protein (BMP) signal transduction is essential for GSC maintenance. Upon signal transduction, the BMP signaling cascade is mediated by phosphorylated MAD (pMAD), which activates the target *Daughters against dpp (Dad)* transcription, and results in transcriptional repression of the differentiation marker *bag of marbles (bam)* (Chen and McKearin 2003a; X. Song et al. 2004). Since cystoblasts (CBs) do not receive enough BMP ligand, they begin the differentiation process by the increased expression of *bam* (Y. Li et al. 2009). Significant premature upregulation of *bam* was found in *iswi* mutant GSCs when compared to wild-type GSC clones. *Dad* transcription was also aberrantly regulated in the absence of *iswi*. These results demonstrate that ISWI maintains GSC self-renewal through BMP signaling-mediated gene expression.

**Nucleosome remodeling factor (NURF) complex:** In *Drosophila*, ISWI is a component of three chromatin remodeling complexes, including NURF (Nucleosome Remodeling Factor), ACF (ATP-utilizing Chromatin assembly and remodeling Factor), and CHRAC (CHRomatin Accessibility Complex). In males, GSC self-renewal is specifically regulated by the NURF complex (Cherry and Matunis 2010), which is composed of ISWI, NURF301, NURF55, and NURF38. Similar to the *iswi* mutant female GSC phenotype, inactivation of *iswi* and *Nurf301* leads to loss of male GSCs from premature differentiation by precocious expression of Bam. Clonal analysis revealed that *Nurf301* mutant CySCs, similar to GSCs, are lost rapidly as a result of premature differentiation.

Similar to the NURF complex, JAK/STAT signaling is also required for the maintenance of both GSCs and CySCs. In the male, the hub cells secrete the signaling ligand Unpaired (Upd) to support stem cell self-renewal of both GSCs and CySCs, as well as adhesion of GSCs to the hub cells (Kiger et al. 2001; Tulina and Matunis 2001; Leatherman and Dinardo 2008, 2010). Loss-of-function of either the Janus kinase (JAK), encoded by *hopsatch (hop)*, or the signal transducer and activator of transcription (STAT), encoded by *Stat92E*, in the germline leads to rapid loss of GSCs and early germ cells (Kiger et al. 2001; Tulina and Matunis 2001). Consistent with the role of the JAK/STAT pathway in GSC self-renewal, ectopic expression of Upd in early germ cells leads to a dramatic increase in the number of GSC-like cells with a concomitant decrease in the number of cells undergoing differentiation. To test whether the NURF complex regulates the maintenance of GSCs and CySCs through mediating JAK-STAT signaling from the niche, JAK-STAT activity was monitored in *Nurf301* null clones by measuring STAT92E expression levels. Loss of *Nurf301* resulted in decreased STAT92E, suggesting that *Nurf301* promotes the maintenance of GSCs, at least in part, through positively regulating the JAK-STAT pathway. Furthermore, *suppressor of cytokine signaling 36E (Socs36E)* is a conserved target of the JAK/STAT pathway in CySCs that functions in a negative feedback loop by downregulating JAK/STAT activity (Issigonis et al. 2009). Similar to loss of JAK/STAT signaling, downregulation of NURF301 partially rescued the *Socs36E* phenotype. These studies highlight that the chromatin remodeling complex, the NURF complex, functions as a positive regulator of JAK/STAT signaling in both GSCs and CySCs in the testis.

Recent studies have also revealed that the ecdysone steroid hormone pathway acts through the NURF complex in female GSCs and in male CySCs (Ables and Drummond-Barbosa 2010; Li et al. 2014). The ecdysone receptor (EcR) is expressed throughout the ovary in multiple cell types (Buszczak et al. 1999). Upon binding of ecdysone to EcR, EcR dimerization occurs with Ultraspiracle (Usp), initiating a transcriptional cascade that includes *E74, E75*, and *broad (br)* as targets (Riddiford et al. 2000). Analysis of GSC clones homozygous for *usp* and *E74* in female and temperature-sensitive alleles of ecdysone and EcR demonstrated that ecdysone signaling promotes GSC maintenance. Interestingly, genetic interactions were discovered between the NURF complex genes *iswi* and *Nurf301* and the Ecdysone pathways genes *usp* and *E74*. Additionally, *loss-of-function* mutations in *usp* and *E74* result in reduced levels of nuclear ISWI. As mentioned earlier, *iswi* mutations result in aberrant BMP signaling and premature *bam* expression. Consistent with the decrease in ISWI, BMP signaling levels are reduced in *usp* and *E74* null clones. The ecdysone signaling pathway acts with the NURF chromatin remodeling complex to promote female GSC maintenance. Intriguingly, purified NURF physically interacts with EcR in an ecdysone-dependent manner, and expression of EcR target genes is significantly reduced in *Nurf* mutants, suggesting that Nurf is a coactivator of EcR (Badenhorst et al. 2002).

In the male, ecdysone signaling components are expressed in both hub cells and the CySC lineage, and they are required for CySC maintenance (Li et al. 2014). Loss of ecdysone signaling in CySCs results in loss of GSCs, as well as CySCs,
suggesting that EcR signaling contributes to both stem cell populations in the testis. It currently remains unknown whether GSC maintenance requires an ecdysone-dependent or -independent signal from CySCs.

**Domino (dom):** This SWR1-like ATP-dependent chromatin remodeling factor functions in both male and female gonads for stem cell self-renewal (Xi and Xie 2005; Morillio Prado et al. 2013). Unlike the chromatin remodeling factor ISWI, which is essential for GSC self-renewal, DOM is only essential for FSC self-renewal in the female. Clonal analysis of dom mutant GSCs reported no change in their division rate. In contrast, female FSCs marked for loss of dom demonstrated that DOM specifically controls FSC self-renewal, but not survival (Xi and Xie 2005). In the female, these studies revealed that different stem cell types, GSCs and FSCs, depend on distinct chromatin remodeling factors, ISWI and DOM, respectively, to control their self-renewal.

In the male, clonal analysis revealed that DOM is required cell autonomously for both GSC and CySC maintenance, and may regulate the incorporation of the histone variant H2Av (Morillio Prado et al. 2013). H2Av is the *Drosophila* sole homolog of mammalian H2A.Z and H2A.X (Talbert and Henikoff 2010; Baldi and Becker 2013). Although the expression of H2Av is ubiquitous, its function is dispensable for germline and cyst cell differentiation, suggesting a specific role for maintaining the stem cell state in these lineages. H2Av, which is incorporated by SWR1-like remodeling complexes, regulates transcriptional control, formation of heterochromatin boundaries, lineage commitment, and DNA repair throughout development (Henikoff et al. 2004; Creyghton et al. 2008; Venkatesh and Workman 2015). Dom has been purified from S2 cells as part of a 16-subunit assembly, and this complex has been shown to exchange H2Av in vitro (Kusch et al. 2004). Because Dom is required for H2Av incorporation, loss of dom function reduced H2Av levels in male GSCs. Furthermore, a recent study has highlighted specific roles for a distinct dom splicing variant required for the incorporation and removal of H2Av during oogenesis (Borner and Becker 2016). Similar to dom, H2Av is required for both male GSC and CySC maintenance independent of the JAK/STAT pathway, and it has been implicated in both transcriptional repression and activation (Morillio Prado et al. 2013). Lack of H2Av does not result in global changes in H3K4me3 or H3K27me3 immunostaining pattern. However, it is possible that the H2Av mutation disrupts H3K9me2/3-enriched heterochromatin structure in GSCs, as previously shown in somatic cells (Swaminathan et al. 2005). Therefore, DOM and H2Av may be required to maintain GSC and CySCs by facilitating repression of differentiation genes and/or maintaining activation of genes necessary for GSC self-renewal. Finally, dom mutants can be partially rescued by the human ortholog, SRCAP (Eissenberg et al. 2005).

**Brahma:** Brahma (Brm), a bromodomain protein, is the sole member of the *Drosophila* SWI/SNF-type ATPase chromatin remodeler. It has cell-autonomous, as well as non-cell-autonomous, roles in regulating female GSC self-renewal (Brizuela et al. 1994; Elfring et al. 1998; Zraly et al. 2003; He et al. 2014). Brahma is a member of two protein complexes, BAP and PBAP, and it is expressed in all cell types in the gerarium and follicle cells. Both complexes share seven subunits, including Brm, and differ in three subunits. OSA is a member of the BAP complex, while Polybromo and BAP170 are members of the PBAP complex (Mohrmann et al. 2004). Using both clonal analysis of brm mutant and tissue-specific RNAi knockdown, a cell-autonomous role of brm in sustaining the GSC population has been revealed. Furthermore, knocking down brm in the niche cells showed a non-cell-autonomous role for brm in regulating GSC self-renewal. To distinguish whether a specific Brm complex, BAP or PBAP, regulates GSC self-renewal, loss of osa and polybromo/bap180 was tested individually. This revealed that mutations in polybromo/bap180, rather than osa, cause similar GSC loss phenotype. These studies indicate that Brm functions in the PBAP complex for GSC maintenance.

**Nclb:** A novel chromatin factor encoded by no child left behind (nclb) specifically regulates male, but not female, GSC maintenance (Casper et al. 2011). Nclb is enriched at chromatin regions with active transcription. In nclb mutant GSCs, Stat92E has decreased transcription or protein accumulation (Casper et al. 2011), suggesting that Nclb acts via signaling pathways to determine GSC fate.

**Histones**

The principal components of epigenetic information, histones, are uniquely distributed with pre-existing (old) histone H3 segregating to the stem cell and newly synthesized (new) H3 localizing to the differentiating daughter cell during *Drosophila* male GSC asymmetric division [Figure 2, (Tran et al. 2012)]. The histone variant H3.3, which is incorporated in a replication-independent manner, does not exhibit such an asymmetric pattern. Therefore, it is likely that DNA replication plays an important role in establishing histone asymmetry between sister chromatids. Furthermore, asymmetric H3 inheritance occurs specifically in the asymmetrically dividing GSCs, but not in the symmetrically dividing progenitor germ cells, suggesting that polarized mitotic machinery could contribute to recognizing the sister chromatid asymmetry established by replication. Cellular specificity exhibited by H3 suggests that global asymmetric histone inheritance occurs uniquely in a cell type (GSC) where the mother cell must divide to produce two daughter cells, each with a unique cell fate. However, more research is required to investigate whether the observed H3 asymmetry occurs at all chromosomes, particular chromosomes, or at specific genomic regions. It has also been shown that differential phosphorylation at Threonine 3 of H3 (H3T3P) distinguishes old vs. new histones in dividing GSCs. The H3T3P is enriched at the pericentric region and is only detectable from prophase to early anaphase. The tight spatiotemporal regulation of this phosphorylation likely ensures...
that it acts at the right location and with the precise timing. Misregulation of this phosphorylation, using either a dominant negative mutant or a phosphomimetic form, leads to randomized segregation of old vs. new histones, as well as stem cell loss and germline tumors. This finding sheds light on the biological significance of asymmetric histone inheritance, which may help maintain GSC identity and reset chromatin structure in the other daughter cell for proper differentiation (Xie et al. 2015).

Utilizing CO-FISH (chromosome orientation fluorescence in situ hybridization) probes, which allow strand-specific hybridization of sister chromatids, it has been demonstrated that both X and Y sister chromatids exhibit an ~85:15 bias during male GSC asymmetric division. Autosomes, specifically chromosome 2 and 3, display a remarkable segregation pattern, but do show a remarkable cosegregation mode (i.e., WW: CC instead of WC: CW, W, Watson strand; C, Crick strand) (Figure 2, Yadlapalli and Yamashita 2013). An earlier study using the nucleoside analog 4-bromo-2-deoxyuridine (BrdU) incorporation assay demonstrated that male GSCs do not follow the immortal strand model (Yadlapalli et al. 2011)—a model that hypothesizes that stem cells retain a template copy of DNA, specifically the sister chromatid that contains the oldest strand as a template, to avoid accumulation of DNA replication-induced mutations (Cairns 1975). Since sister chromatids are identical DNA copies of each other, the distribution of distinct information for asymmetric inheritance likely occurs through epigenetic mechanisms, which is consistent with an alternative hypothesis (Klar 2007; Lansdorp 2007).

**Histone-modifying enzymes and factors that affect histone modification(s)**

**Cell autonomous mechanisms:** Recent research has identified a set of specific enzymes and factors that generate (“write”), recognize (“read”), and remove (“erase”) histone modifications, provoking studies of their in vivo functions during development (Sarmiento et al. 2004; Seligson et al. 2005). Post-translational modifications that decorate canonical histones (i.e., H2A, H2B, H3, and H4), as well as histone variants, such as H3.3 and H2Av, can serve as molecular memory bookmarks to maintain, or reestablish, transcriptional activation or repression after mitosis. Indeed, different histone modifications are very robust in the male germline (Hennig and Weyrich 2013). The study of histone-modifying enzymes in *Drosophila* offers a great opportunity because many of them encode the sole ortholog in *Caenorhabditis elegans* (Ringrose and Paro 2004). It is generally agreed that both PcG and TrxG complexes employ epigenetic mechanisms that alter chromatin state to either repress or activate gene expression (Surface et al. 2010). PcG proteins act in at least two distinct, but interacting, protein complexes, Polycomb Repressive Complex 1 (PRC1) and PRC2 (Schwartz and Pirrotta 2007). PRC2 contains an enzymatic component, Enhancer of Zeste [E(z)], which methylates...
hormone H3 at Lys27 (H3K27me3) (Cao et al. 2002; Czermin et al. 2002; Kuzmichev et al. 2002; Muller et al. 2002). This methylated histone recruits PRC1, which binds to the H3K27me3 epigenetic mark through the chromodomain of the Polycomb (Pc) protein (Fischle et al. 2003; Min et al. 2003), leading to the nucleation of the entire PcG complex. It has been shown that mutation on the H3 Lys27 residue phenocopies loss-of-function mutants of PRC2 components, indicating that H3K27 is indeed the critical in vivo substrate of PRC2 histone methyltransferase activity (Pengelly et al. 2013). In addition, the dRing in the PRC1 complex acts as an E3 ubiquitin ligase, which ubiquitylates histone H2A at Lys119 (H2AK119ub) (Wang et al. 2004). H2AK119ub may affect transcription by blocking efficient elongation (Stock et al. 2007). Phosphorylation of the H2A variant H2Av (γH2Av), which serves as a specific marker for double-strand DNA breaks, often indicates an early response to DNA damage. Consistently, it was reported that mutations in H2Av enhance *Drosophila* male germline defects caused by DNA damage that initially results from mutations in the PcG gene multi sex combs (msc) (Landaïs et al. 2014).

On the other hand, the active H3K4me3 mark is generated by the TrxG complex (Byrd and Shearn 2003; Klymenko and Muller 2004; Ringrose and Paro 2004), and opposes the PcG function. In the female GSCs, it has recently been shown that the global level of H3K4me3 is decreased upon loss-of-function of the *Drosophila* ortholog of Ctr9, a component of the Pa1 complex normally required for transcriptional initiation and polyadenylation. However, the functional readout of this global H3K4me3 loss remains unclear (Chaturvedi et al. 2016).

In *Drosophila*, not only the maintenance of GSCs, but also the formation of primordial germline cells during embryogenesis, depends on the cell-autonomous function of Piwi (Megas et al. 2006; Ma et al. 2014). Piwi promotes GSC differentiation nonautonomously through somatic gonadal cells in both male and female gonads (Ma et al. 2014; Gonzalez et al. 2015). The necessity of Piwi function for ovarian GSC maintenance, however, does not seem to be relevant to biogenesis of Piwi-interacting RNAs (piRNAs). The Piwi-piRNA complex is required for transposon silencing in the nucleus; however, Piwi in cytoplasm is shown to be sufficient for GSC maintenance (Klenov et al. 2011). The 3R-TAS1 piRNA is a specific Piwi-bound piRNA and is involved in female GSC maintenance. However, its role and the mode of action remain elusive (Yin and Lin 2007). Piwi function in GSC maintenance is conserved in other organisms, including mouse and zebrafish, where PIWI-family proteins are also involved in GSC maintenance and/or differentiation (Houwing et al. 2007, 2008; Unhavaithaya et al. 2009). Recently, it has been demonstrated that Piwi physically interacts with PRC2 components, and restricts the accessibility of PRC2 to chromatin (Peng et al. 2016).

**Eggless**: *eggless/dSETDB1* encodes a H3K9 methyltransferase which plays multiple roles in *Drosophila* oogenesis (Clough et al. 2014). The eggless mutants show female GSC self-renewal defects. However, the GSC loss defect is not caused by loss-of-function in the well-known BMP signaling pathway, suggesting a potential H3K9me3-dependent, but BMP-independent, mechanism for GSC maintenance (Wang et al. 2011). Another study showed that eggless mutant female flies have a defective egg chamber owing to its requirement for FSC proliferation and survival of both FSCs and germ cells (Clough et al. 2007). In addition, it has been shown that Eggless functions in the transcriptional regulation of piRNA clusters (Rangan et al. 2011). Activated piRNAs act

### Table 2 PTMs and the corresponding enzymes in *Drosophila* gametogenesis

| PTM         | Writer | Reader | Eraser         | Function                                                                 | Reference                      |
|-------------|--------|--------|----------------|---------------------------------------------------------------------------|--------------------------------|
| H3K4(me)    | dset1, trr, trx, and ash1 | Phf7   | lid, Su(var)3-3 (Isd1) | Commonly associated with promoters of actively transcribed gene           | Beisel et al. (2002), Sedkov et al. (2003), Di Stefano et al. (2007), Hallson et al. (2012), Yang et al. (2012), Tarayrah et al. (2015) |
| H3K9(me)    | Su(var)3-9, eggless/dSETDb1, dG9a | Rhino (HP1d), HP1a, HP1b, HP1e | dKDM4B | A mark of constitutive and facultative heterochromatin, functions in the maintenance of pericentric heterochromatin | Tschiersch et al. (1994), Ebert et al. (2006), Mis et al. (2006), Stabell et al. (2006), Levine et al. (2015) |
| H3K23(acetyl) | enok   | N.D.   | N.D.           | Promotes gene expression                                                  | Scott et al. (2001), Huang et al. (2014), Min et al. (2003), Ebert et al. (2004), Tarayrah et al. (2013) |
| H3K27(me)   | E(z)   | Pc     | dUTX           | Associated with inactive gene promoters, indexing both pericentric heterochromatin and inactive euchromatic domains | Wang et al. (2004), Scheuermann et al. (2010) |
| H2A (Ub)    | dRing  | N.D.   | PR-DUB (calypso/Asx) | Promotes repressive chromatin                                              | Buszczak et al. (2009), Xuan et al. (2013) |
| H2B(Ub)     | dBre1  | N.D.   | Scrawny        | Enhances nucleosome stability and mediates H3K4(me)                       |                                |
with the PIWI protein to protect germline genome by preventing transposable element activity, which may be a conserved mechanism for germline genome integrity across species (Malone et al. 2009; Sienski et al. 2012; Huang et al. 2013; Le Thomas et al. 2013; Rozhkov et al. 2013).

**Scrawny**: Scrawny encodes a deubiquitylating enzyme and targets the mono-ubiquitylation H2B, which normally serves as an active mark for transcription. Therefore, the normal function of Scrawny is to make chromatin more compact to repress gene expression. Interestingly, Scrawny is required for stem cell maintenance in multiple adult stem cell systems, including both female and male GSCs, as well as FSCs in the ovary, and intestinal stem cells, suggesting some common chromatin feature among different stem cell types. It is likely that Scrawny maintains stem cells by repressing transcription of the differentiation genes in the corresponding lineages. In addition, inactivation of Scrawny leads to global changes of the chromatin landscape, including increased levels of H3K4me3 and acetylated H3, suggesting crosstalk among different histone modifications, likely through their modifying enzymes. Another intriguing feature is that Scrawny has highly enriched nucleolar localization in both female and male germ-line (Buszczak et al. 2009). In another study, enhanced ribosomal RNA transcription at nucleolus was shown to maintain normal female GSC proliferation and avoid precocious differentiation (Q. Zhang et al. 2014). It is unclear whether Scrawny is required for proper chromatin structure at rDNA genes for their upregulated transcription.

**Little imaginal disc (Lid)**: In adult testis, the H3K4me3-specific histone demethylase Lid has cell-autonomous roles to maintain GSC self-renewal and prevent GSCs from undergoing precocious differentiation. When the function of Lid is compromised in early-stage germ cells, the niche is deprived of GSCs, but occupied by differentiating spermatogonial cysts. The key downstream effector of Lid is the Stat92E transcription factor of the JAK-STAT signaling pathway. Lid is required for both normal Stat92E transcript level and protein accumulation. Removing one copy of Stat92E greatly enhanced lid mutant phenotype, and expression of a Stat92E cDNA in early-stage germ cells rescued lid loss-of-function phenotype completely. Therefore, Lid acts through Stat92E in regulating male GSC activity (Tarayrah et al. 2015).

**Stonewall (Stwl)**: Stwl encodes a DNA-binding protein, which was originally predicted as a putative transcription factor. More recent work suggests that Stwl normally represses expression of many target genes, likely through making the chromatin structure more compact. Stwl is both necessary and sufficient for female GSC cell fate (Maines et al. 2007), as well as the transit amplification of CBs (Akiyama 2002). It has been shown that both H3K9me3 and H3K27me3, two histone modifications to silence gene expression, are decreased in Stwl mutants (Yi et al. 2009), suggesting that Stonewall maintains normal heterochromatin structure, as one of its functions.

**longitudinals lacking (lola)**: In the male GSC lineage, a transcriptional regulator of the BTB-Zinc finger family encoded by lola was reported to have pleiotropic roles in adult testis. Lola is ubiquitously expressed and is required cell-autonomously for both GSC and CySC maintenance, likely independent of the known JAK-STAT and BMP signaling pathways. In addition, lola is required for proper mitosis-to-metiosis transition, and lola mutant spermatogonial cysts have >16 cells, likely owing to faster cell cycle progression. Finally, lola is also needed for meiosis and terminal differentiation of sperm. Intriguingly, lola has 19 alternative spicing isoforms. With the possibility that Lola may act as a dimer, the combination among different isoforms could give rise to its pleiotropic roles (Davies et al. 2013). By contrast, in female GSCs, lola is repressed by Stwl and is dispensable for female GSC maintenance (Maines et al. 2007), suggesting sex-specific roles of Lola in gametogenesis.

**Non-cell-autonomous mechanisms**: A fundamental question in stem cell biology is how extrinsic signaling pathways and intrinsic epigenetic mechanisms cooperate to determine and maintain stem cell fate. Recent findings provide new insights into the non-cell-autonomous roles of different histone-modifying enzymes, many of which are acting through signaling pathways and required for crosstalk among multiple cell types within the stem cell niche.

**Lysine-specific histone demethylase 1 (Lsd1)**: The lsd1 gene encodes the H3K4me1/2-demethylase in Drosophila (Di Stefano et al. 2007). Lsd1 acts in escort cells to regulate a diverse group of genes, including both BMP-related and BMP-unrelated genes (Eliazer et al. 2014). Lsd1 regulates germline differentiation by preventing ectopic BMP signaling outside of the niche (Eliazer et al. 2011, 2014), as well as using BMP-independent mechanisms.

**Enhancer of zeste [E(z)]**: E(z) is a key PRC2 component, which is an H3K27me3-specific methyltransferase (Muller et al. 2002). In Drosophila testis, E(z) acts in somatic gonadal cells to prevent expression of a somatic lineage transcription factor encoded by zinc-finger homeodomain protein 1 (zfh-1) in the germline. Consensus holds that germ cells maintain their unique identity after being specified early in embryogenesis, which is essential for proper gametogenesis. Using complementary somatic and germline lineage-tracing experiments, Zfh-1 was shown to ectopically express in the germ-line in adult testes when E(z) is inactivated in the somatic cells, suggesting the importance of cell–cell communication in maintaining germ cell identity. Furthermore, only early-stage germ cells, including GSCs, retain the ability to express zfh-1. In contrast, further differentiated spermatogonial cells lose this ability, suggesting that chromatin undergoes structural changes during GSC differentiation that may lock their cell fate choice (Eun et al. 2014).

**Posterior sex combs (Psc) and suppressor of zeste 2 [Su(z)2]**: Both Psc and Su(z)2 are PRC1 components which have some redundant functions. Loss of both genes in ovary leads to overproliferation and “metastasis” of FSCs, likely from misregulation of the canonical Wnt signaling and planar polarity pathways (Li et al. 2010). In testis, both Psc and Su(z)2 act in the CySC lineage to maintain their identity.
and restrict excess proliferation. Loss of both Psc and Su(z)2 leads to tumors that arise from overproliferative CySCs, which also physically displace GSCs from their niche (Morillo Prado et al. 2012).

**Utx histone demethylase (dUTX):** The dUTX gene encodes an H3K27me3-specific histone demethylase (Herz et al. 2010). In the testis niche, dUTX removes the repressive H3K27me3 histone modification near the transcription start site of Socs36E and allows active transcription of Socs36E, which encodes an inhibitor of the JAK-STAT signaling pathway. JAK-STAT plays an essential role in the testis niche; as such, dUTX is critical for maintaining the balance between GSCs and CySCs. Loss of dUTX function in either GSCs or CySCs leads to niche identity and morphological defects in a non-cell-autonomous manner. These defects can be fully rescued by either overexpression of Socs36E or removal of one copy of the downstream transcription factor-encoding gene, Stat92E, of the JAK-STAT pathway. Therefore, through direct control of JAK-STAT signaling, dUTX coordinates crosstalk among different cell types within the Drosophila testis niche (Tarayrah et al. 2013).

**Enoki mushroom (Enok):** The enok gene encodes a putative MYST family histone acetyltransferase that controls female GSC maintenance, both cell-autonomously and non-cell-autonomously (Xin et al. 2013). Loss of enok in female GSCs leads to rapid GSC loss. Enok maintains GSCs through regulating Bruno, which encodes an RNA-binding protein and targets mRNAs in the ovary for translational repression. Furthermore, compromised enok in cap cells impairs niche size and BMP signaling output, thereby causing defective GSC maintenance through a parallel non-cell-autonomous pathway.

**dbre1:** dbre1 encodes an E3 ubiquitin ligase required for mono-ubiquitination of H2B. The dbre1 controls both GSC maintenance and germ cell differentiation via distinct mechanisms (Xuan et al. 2013). Loss of dbre1 leads to both GSC loss and a significant reduction in H3K4me3. Further analysis revealed that dbre1 regulates GSC maintenance through modulating BMP signaling response. In addition, dbre1 has a non-cell-autonomous role to maintain GSCs via DE-cadherin-mediated adhesion of GSCs to the niche, as well as the BMP signaling pathway. Finally, dbre1 functions in escort cells to control female germ cell differentiation in a non-cell-autonomous manner through limiting BMP signaling output by downregulating the BMP ligand (Dpp) and Dally—a regulator of BMP ligand diffusion. Interestingly, loss of dSet1—an H3K4 methyltransferase—results in phenotypes similar to those observed in dbre1 mutant ovaries. Genetic analysis suggests that dbre1 interacts with dSet1 to control both female GSC maintenance and germ cell differentiation.

**RNA-binding proteins and noncoding RNAs**

RNA-binding proteins, such as Musashi (Msi) (Siddall et al. 2006), Held-out-wings (HOW) (Monk et al. 2010), and the IGF-II mRNA-binding protein (Imp) (Toledano et al. 2012) are all required for male GSC maintenance, suggesting an important role of post-transcriptional regulation in the testis niche.

Epigenetic regulation is also controlled by noncoding RNAs, such as microRNAs (miRNAs) that regulate gene expression post-transcriptionally. Mature miRNAs are ~22 nt, and they are processed from primary miRNAs by a set of evolutionally conserved enzymes, such as RNase III type endonucleases Partner of drosha (Pasha), Loquacious (Loq), Dicer-1 (Dcr-1), and Argonaute-1 (Ago-1) (Filipowicz et al. 2008) in Drosophila. By base-pairing to the 3’ untranslated regions (3’UTR) of target miRNAs (Gu et al. 2009), mature miRNAs either control target mRNA stability or interfere with its translation (Vasudevan et al. 2007; Filipowicz et al. 2008). Mutations of miRNA biogenesis components dcr-1, loquacious, argonaute 1, and mei-P26 lead to the loss of female GSCs (Forstemann et al. 2005; Jin and Xie 2007; Park et al. 2007; Yang et al. 2007; Li et al. 2012, 2013). A couple of miRNAs have also been found to regulate GSC maintenance and differentiation in female gonads. For example, mir-184 (Iovino et al. 2009) and bantam (Yang et al. 2009) are both required to balance GSC maintenance vs. differentiation in ovaries. Another example is male germline-specific regulation of the Wnt signaling pathway by miRNAs. Both β-catenin and the downstream transcription factor TCF are downregulated by miRNAs in Drosophila. Loss of this antagonization leads to male germline differentiation defects and decreased fertility (Pancratov et al. 2013).

Another class of small noncoding RNAs, called piRNAs, is present in the both male and female gonads. The piRNAs are the most abundant class of small RNAs in gonads. The piRNAs repress transposons, provide immunity against transposons to protect the next generation, and function in maternal-to-zygotic transmission during early embryogenesis (Brennecke et al. 2008; Barckmann et al. 2015; Hermant et al. 2015; Iwasaki et al. 2015; Vourekas et al. 2016). Their biogenesis and functions will be described in detail in the following sections.

**Mechanisms Controlling Mitotic Germ Cell Proliferation, Transition to Meiosis, and Dedifferentiation**

**Transit-amplification stage and mitosis-to-meiosis transition**

The transit-amplification stage stage ensures that limited GSCs and their divisions have a high-throughput outcome for producing gametes. However, in both female and male germlines, this process needs to be tightly controlled since genetic lesions or epigenetic misregulation of gene expression may trap them as ever-dividing mitotic cells, and block entry into meiosis, leading, in turn, to either germline tumors or infertility (Clarke and Fuller 2006).

**Cell-autonomous mechanisms:** A key differentiation factor encoded by the bam gene is expressed in transit-amplifying cells in both female and male gonads (McKearin and Spradling...
1990; Gonczy et al. 1997). In GSCs from both sexes, BMP signaling activated by somatic cells in the niche represses bam transcription (Shvidrasani and Ingham 2003; Kawase et al. 2004; Schulz et al. 2004). It is important that bam remain silenced in GSCs (Schulz et al. 2004; Insco et al. 2009; Monk et al. 2010). Ectopic expression of bam in GSCs induces precocious differentiation or cell death and, hence, loss of GSC phenotype (Ohlstein and McKearin 1997; Schulz et al. 2004; Sheng et al. 2009). A recent study shed light on the biochemical activity of Bam protein by showing that it assists a deubiquitinating enzyme and protects CycA from degradation (Ji et al. 2017).

In the female GSC lineage, Bam is absent in the GSC, but it is expressed in its immediate daughter cell CB. This abrupt change of Bam expression is regulated by a steep gradient of response to BMP signaling, both by a cis-acting transcriptional slencer repressing bam transcription in GSCs (Chen and McKearin 2003a,b; X. Song et al. 2004) and by a trans-acting mechanism, such as the CB-specific degradation of Thickveins—a BMP signaling receptor (Xia et al. 2010, 2012). In addition, post-transcriptional regulation via different RNA-binding proteins contributes to sharpening the change of Bam expression and the decision between GSC self-renewal and CB differentiation (Chen and McKearin 2005). Further differentiation of the CB relies on the homolog of human Ataxin 2-Binding Protein 1 (A2BP1), which is expressed immediately after Bam in 4- to 16-cell germline cysts. Mutations in the A2BP1 gene lead to germline cyst differentiation defects, giving rise to germline tumors that result from mitosis-to-mitosis transition defects (Tastan et al. 2010). In addition, two H3K9 methyltransferases encoded by eggless/dSETDB1 and Su(var)3-9 act in a sequential manner with dSETDB1 in GSCs and early-stage germline, as discussed previously, while Su(var)3-9 mainly works in germ cells at the later stage. This temporal difference could underlie their distinct loss-of-function phenotypes with eggless mutants showing severe germline differentiation defects, whereas Su(var)3-9 mutants undergo normal oogenesis (Yoon et al. 2008).

In the male GSC lineage, Bam is required for the transition from mitotic spermatogonia to meiotic spermatocytes (McKearin and Spradling 1990; Gonczy et al. 1997). Bam protein is detectable in four- to 16-cell spermatogonia with a peak level in eight-cell spermatogonia (Gonczy et al. 1997). Examples of post-transcriptional regulation of bam include the HOW RNA-binding protein (Monk et al. 2010) and mir-7, both of which have been implicated in binding to bam mRNA and downregulating bam expression (Pek et al. 2009). Another RNA binding protein, Maelstrom (Mael), is required in spermatogonia to repress mir-7 and upregulate bam expression so that the transit-amplification can proceed normally (Pek et al. 2009). The transition from mitotic spermatogonia to meiotic spermatocyte is regulated by the accumulation of Bam to a threshold level. Expediting Bam accumulation or slowing down the transit-amplifying cell cycle results in insufficient proliferation before the transition to meiosis, as demonstrated by spermatocyte cysts with <16 cells. On the other hand, inhibition of Bam accumulation, or facilitating the transit-amplifying cell cycle, results in extra round(s) of mitosis before the transition to meiosis, as shown in spermatocyte cysts with >16 cells (Insco et al. 2009). Therefore, expression of Bam needs to be tightly controlled during the transit-amplification stage in the male germline.

Another differentiation gene, benign gonial cell neoplasm (bgcn), has mutant phenotypes similar to bam in both male and female germlines (Gonczy et al. 1997). Studies in the female germline demonstrate that Bam and Bgcn form a protein complex to antagonize GSC self-renewal factors, and promote differentiation gene expression in transit-amplifying cells (Y. Li et al. 2009). It has been demonstrated that the Trim-NHL tumor suppressor homolog Mei-P26 has a reciprocal regulation with Bam whereby Mei-P26 initially promotes Bam protein accumulation in early transit-amplifying cells. Increased Bam acts with Bgcn to bind the 3’UTR of Mei-P26, and, consequently, repress translation of Mei-P26 in late transit-amplifying cells (Insco et al. 2012). Recent studies have revealed another RNA-binding protein, Tumorous testis protein complex to antagonize GSC self-renewal factors, and promote differentiation gene expression in transit-amplifying cells (Y. Li et al. 2009). It has been demonstrated that the Trim-NHL tumor suppressor homolog Mei-P26 has a reciprocal regulation with Bam whereby Mei-P26 initially promotes Bam protein accumulation in early transit-amplifying cells. Increased Bam acts with Bgcn to bind the 3’UTR of Mei-P26, and, consequently, repress translation of Mei-P26 in late transit-amplifying cells (Insco et al. 2012). Recent studies have revealed another RNA-binding protein, Tumorous testis (Tut), that acts in synergy with Bam-Bgcn for the translational repression of Mei-P26 (Chen et al. 2014). Indeed, post-transcriptional regulation of gene expression is a widely used mechanism, particularly in the germline. A very recent study reports the generality of this mechanism in the male germline (Shan et al. 2017). In addition, post-translational regulation also directly, or indirectly, regulates Bam protein function. Specifically, a Drosophila homolog of the highly conserved LAMMER/Cdc2-like kinase (CLK), called Doa, has been shown to regulate the mitosis-to-meiosis switch in the male germline through regulating Bam protein (Zhao et al. 2013).

In order to study the transcriptional profile and chromatin state in transit-amplifying cells, bam or bgcn mutant testes were used for transcriptome profiling because they are enriched with overproliferative spermatogonial cells (Terry et al. 2006; Gan et al. 2010a; Chen et al. 2011). High-throughput mRNA sequencing (RNA-seq) studies reveal that both chromatin remodeling factors and histone-modifying enzymes have enriched transcription in bam testes compared to wild-type testes (Gan et al. 2010a). Furthermore, ChIP followed by high-throughput sequencing (ChIP-seq), revealed a distinct chromatin structure in bam testes (Gan et al. 2010b). In mouse embryonic stem cells, differentiation genes have both repressive H3K27me3 and active H3K4me3 modifications (i.e., “bivalent” chromatin signature), as well as stalled RNA polymerase II (Pol II, i.e., “poised” genes), at their promoter regions (Bernstein et al. 2006; Buszczak and Spradling 2006; Guenther et al. 2007). By contrast, differentiation genes required for spermatocyte maturation and spermiogenesis are either enriched with H3K27me3 only, or deprived of both H3K4me3 and H3K27me3, in bam testes, and they are not associated with stalled Pol II (Gan et al. 2010b). This distinct chromatin structure may prevent ectopic transcription of the differentiation genes in transit-amplifying cells. On the other
hand, it suggests that dramatic changes at the promoter region of differentiation genes are needed to turn on their robust transcription in spermatocytes.

In addition to these genome-wide studies, it was reported that an epigenetic reader-coding Plant Homeodomain Finger 7 (PHF7) gene is specifically expressed in GSCs and transit-amplifying cells. PHF7 recognizes active H3K4me2 histone modification and is required for GSC maintenance and proper spermatogonial differentiation (Yang et al. 2012). Further studies to identify the target genes of PHF7, which should be enriched with H3K4me2 or H3K4me3, will shed light on its in vivo roles.

Non-cell-autonomous mechanisms: The Epidermal growth factor (Egf) signaling pathway plays an important role in the regulation of the mitosis-to-metosis switch. The Egfr (Egf receptor) ligand Spitz is processed by Stet—a transmembrane protease—in germ cells (Schulz et al. 2002). Activated Spitz then acts on Egfr expressed in somatic cells (Kiger et al. 2000). Egf signaling acts through the guanine nucleotide exchange factor (GEF) Vav to activate Rac-type small GTPases, which are antagonized by the Rho-type small GTPases (Sarkar et al. 2007). Egfr signaling acts in cyst cells to restrict GSC self-renewal and spermatogonial proliferation, while promoting GSC-to-GB and spermatogonia-to-spermatocyte transitions (Kiger et al. 2000). Egfr signaling decreases the frequency of GSC divisions in the adult, but not larval, testes, suggesting a temporal mode of Egfr regulation (Parrott et al. 2012). In addition, mutations in a serine/threonine kinase signal transducer encoded by raf result in phenotypes similar to the Egfr mutant, suggesting that the receptor tyrosine kinase (RTK) pathway is, in general, required in cyst cells for proper transit-amplification (Tran et al. 2000). The direct target genes for the Egfr/Raf pathway have not been identified; however, because compromised Egf signaling leads to defects in germline-soma interaction and overproliferation of spermatogonial cells, it is possible that the target genes regulate proper encapsulation of germ cells by cyst cells (Schulz et al. 2002; Sarkar et al. 2007). A recent study has shown that a chromatin factor encoded by the Enhancer of Polycomb [E(PC)] gene acts in the CySC lineage to regulate multiple signaling pathways, including both EGF and JAK-STAT pathways, in order to promote both CySC and GSC differentiation. In addition, consistent with biochemical data showing E(Pc) as a component of the NuA4 (nucleosome acetyltransferase of H4) histone acetyltransferase (HAT) complex (Galarneau et al. 2000; Boudreault et al. 2003; Chittluru et al. 2011), inactivation of the Drosophila NuA4 homolog, Tip60, in the CySC lineage resembles E(Pc) loss-of-function phenotype, suggesting that they may act together in vivo (Feng et al. 2017). Another recent study revealed that the endocytic process in the CySC lineage is required to prevent overproliferation of transit-amplifying germ cells in testis, which is accomplished through both JNK and BMP signaling pathways (Tang et al. 2017).

Furthermore, a nuclear envelope component, Nucleoporin98-86, regulates proper GSC-to-GB and spermatogonia-to-spermatocyte transitions, and functions upstream of BMP, JAK-STAT, and Egfr signaling pathways (Parrott et al. 2011). Interestingly, another study showed that nuclear lamina regulates specific nucleoporin distributions and promotes nuclear localization of phosphorylated ERK—the downstream effector of the Egf pathway (Chen et al. 2013). These results highlight the importance of nuclear structure in regulating cellular differentiation during spermatogenesis.

Cell-autonomous mechanisms: In both female and male GSC lineages, partially differentiated mitotic germ cells could undergo a dedifferentiation process to return to the niche and become GSC-like cells (Brawley and Matunis 2004; Kai and Spradling 2004). During aging (Wallenfang et al. 2006; Cheng et al. 2008) and tissue regeneration (Sheng et al. 2009), lost GSCs could be replenished by dedifferentiation to maintain tissue homeostasis. However, once the meiotic program is initiated, as in spermatocytes, dedifferentiation could no longer be detected (Brawley and Matunis 2004; Wallenfang et al. 2006; Sheng et al. 2009), suggesting that the mitotic spermatogonial cells have unique characteristics permissive for dedifferentiation. Similar irreversible commitment may also apply to female meiotic germ cells because only four- to eight-cell transit-amplifying cells have been reported to undergo dedifferentiation in the ovary (Kai and Spradling 2004). It has recently been shown that RNA-binding Fox 1 (Rbfox1) represses pumilio mRNA translation. Because of the essential roles of Pumilio in early-stage germ cells, including GSCs, ectopic Pumilio is expressed in Rbfox1 mutants, and promotes dedifferentiation of germline cysts to become GSC-like cells in the ovary (Carreira-Rosario et al. 2016).

Non-cell-autonomous mechanisms: Using live cell imaging, it has been observed that the dedifferentiated spermatogonial cyst undergoes fragmentation to become individual cells that form actin-based protrusions to make initial contact with the stem cell niche (Sheng et al. 2009), suggesting potential extrinsic cues from the niche to guide dedifferentiation. Indeed, it has been reported that the aminopeptidase Slamdance is highly expressed in the hub cells. Slamdance is both necessary and sufficient to promote dedifferentiation during homeostasis and regeneration, and such activity depends on its enzymatic function. These data showed that cells and molecules in the stem cell niche regulate the dedifferentiation process. Slamdance also has a cell-type-specific expression in the female GSC niche cells, and may play a similar role for the dedifferentiation process in the female germline (Lim et al. 2015).

Despite increasing knowledge about the intrinsic factors and extrinsic cues for dedifferentiation, the extent to which dedifferentiated GSC-like cells behave like bona fide GSCs remains to be elucidated. For example, it has been shown that dedifferentiated GSC-like cells tend to have misoriented...
centrosomes, which lead to cell cycle arrest because of a centrosome orientation checkpoint in male GSCs (Cheng et al. 2008; Inaba et al. 2010, 2015; Yuan et al. 2012). Therefore, it is unclear whether dedifferentiated GSC-like cells could reenter differentiation and give rise to fully functional gametes without any defects. Addressing this intriguing question would need double lineage tracing to trace those GSC-like cells arising from dedifferentiation and reentering differentiation, as well as functional analyses of those differentiated gametes from dedifferentiated GSC-like cells.

**piRNAs and piRNA Pathway Function to Protect the Germline Genome**

Transposons are autonomous elements present in all eukaryotic organisms. Their content in the genome of higher eukaryotes varies between 10 and 80%; they constitute 23% of the *D. melanogaster* genome. Transposons are involved in the regulation of gene expression, as well as both evolution and speciation. However, the ability of transposons to transpose from one site to another in the genome demands tight regulation of their movements. Transposition in the genome is particularly important for transposons to propagate in a population. Previously, piRNAs present in both the male and female gonads were introduced as another class of small noncoding RNAs. It is this unique class of small RNAs that carefully safeguard the germline genome, ensuring fitness of the offspring. Genome-wide screens in *Drosophila* have revealed that piRNA biogenesis requires 69 or more genes and that their distinct subsets are expressed in the germline and somatic gonadal cells (Czech et al. 2013; Handler et al. 2013). In this section, we focus on the biogenesis of piRNAs in germ cells, as well as somatic gonadal cells.

**Transposons in Drosophila**

*D. melanogaster* possesses >49 families of long terminal repeat (LTR) transposons (Kaminker et al. 2002). The transposition mechanisms for many transposon families have been studied. The most studied example among the LTR family members is gypsy, which is composed of three parts: a Gag-like protein containing a nucleocapsid region, a protease-polymerase fusion protein, and the envelope (Mejilumian et al. 2002). With the encoded coat proteins, gypsy is capable of exiting the follicle cells and infecting the neighboring oocyte. Another LTR retrotransposon called ZAM has a replicative cycle similar to that of gypsy, but it can be transmitted to the oocyte by the vitellogenin secretion pathway (Leblanc et al. 2000; Brasset et al. 2006). LTR families are often activated in ovarian somatic cells and transmitted to the oocyte, threatening the genome stability of the oocyte. By contrast, non-LTR families tend to be activated in nurse cells, and are deposited to the oocyte via the cytoplasmic bridges called ring canals (Chambeyron et al. 2008). Hence, active transposon mechanisms function both in the germline and somatic gonadal cells.

In *Drosophila*, though transposons threaten the genome, they are also essential for the integrity of both centromeres and telomeres (Pardue and DeBaryshe 2003; Wong and Choo 2004). In most other species, telomeres are composed of simple repeats and are maintained by telomerase, but, in *Drosophila*, telomeres consist of three non-LTR transposons, namely HeT-A, TART, and TAHERE (Pardue and DeBaryshe 2003, 2011; Abad et al. 2004). To properly maintain the telomere, the copy number of these transposons at telomeres is strictly regulated (Fanti et al. 1998; Perrini et al. 2004; Frydrycova et al. 2008; Pardue and DeBaryshe 2011).

**piRNAs and PIWI proteins**

Studies on the piRNA pathway in *Drosophila* ovaries have expanded our knowledge about piRNA pathway function (Iwasaki et al. 2015). The piRNAs in *Drosophila* are 23 to 29 nucleotides in length and are the most abundant small RNAs in gonads (Balakireva et al. 1992; Aravin et al. 2001, 2003, 2004). The piRNAs were first recognized for their role in suppressing the Stellate protein in the male germ cells (Aravin et al. 2001, 2004). Shortly thereafter, it was reported that *Drosophila* ovaries and embryos contain abundant repeat-associating small RNAs called rasiRNAs (Vagin et al. 2006). Later, they were renamed as piRNAs, as they are produced by and associate with PIWI-family proteins to suppress transposons (Saito et al. 2006; Brennecke et al. 2007; Gunawardane et al. 2007).

The piRNA pathway in *Drosophila* is active in both the germline and somatic gonadal cells to counter transposons that threaten to invade the germline through distinct routes. The piRNA pathway silences transposons post-transcriptionally by triggering degradation of their transcripts, as well as transcriptionally by silencing transposon loci (Vagin et al. 2006; Lim et al. 2009; Le Thomas et al. 2013; Rozhkov et al. 2013). Although a different subset of proteins function in piRNA biogenesis in germline and somatic cells, PIWI-family proteins—a subclade of the Argonaute family—are central in the piRNA pathway. Piwi, the founder member of PIWI-family proteins, is present in both germline and somatic cells. Two other PIWI-family proteins, namely Aubergine (Aub) and Argonaute3 (Ago3), are required for piRNA production in germ cells. These proteins contain PAZ (Piwi-Argonaute-Zwille) and PIWI domains. The PAZ domain also harbors an oligonucleotide/oligosaccharide-binding-fold, which binds to single-stranded nucleic acids (Lingel et al. 2003; Yan et al. 2003). The PIWI domain is structurally similar to that of RNase H enzymes (J. Song et al. 2004). Crystal structure analysis suggested that the PAZ domain forms a pocket for the target RNA, while the PIWI domain cleaves its bound RNA (Yan et al. 2003; J. Song et al. 2004).

The piRNAs are processed from longer precursor molecules. The piRNA precursor transcripts are produced from discrete loci, termed piRNA clusters (Brennecke et al. 2007). These clusters are composed of fragmented copies of transposons in the genome, and serve as heritable sequence repositories for transposon repression. In the gonadal somatic
cells, piRNAs are processed from piRNA cluster transcripts, which are in antisense orientation, to active transposons, in a linear mode called primary processing. While in germ cells, piRNAs are generated from both cluster and transposon transcripts in two different modes: primary processing and secondary amplification cycle (Brennecke et al. 2007; C. Li et al. 2009; Malone et al. 2009). The piRNAs in both cell types are loaded onto Piwi to form the Piwi-piRNA complex, which subsequently translocates into the nucleus for transcriptional silencing of transposons.

**Source of piRNAs—piRNA clusters and transcription of piRNA precursors**

Generally, piRNA clusters can be classified on the basis of transcription of precursors. Most piRNA clusters that participate in piRNA biogenesis in ovarian somatic cells are transcribed in one direction, and thus called uni-strand clusters. By contrast, active clusters in germ cells are transcribed in a convergent manner from both directions, and thus called dual-strand clusters (reviewed by Hirakata and Siomi 2016).

**Active piRNA clusters in germ cells and generation of piRNA precursors**: Most clusters active in germ cells are located in pericentric or subtelomeric regions, which are heterochromatic in nature, and yet they are readily transcribed and processed into piRNAs (Klattenhoff et al. 2009; Malone et al. 2009; Mohn et al. 2014; Z. Zhang et al. 2014). Although piRNA clusters are transcribed by RNA Pol II, majority of piRNA precursors are reported as nonpolyadenylated and lack capping at the 5′-end (Mohn et al. 2014; Z. Zhang et al. 2014; Chen et al. 2016) (Figure 3). Notably, piRNA precursor transcription depends on H3K9me3, and loss of a histone methyltransferase encoded by setDB1/egg causes severe reduction in cluster transcript levels from both uni- and bidirectional clusters (Rangan et al. 2011). A recent study showed that loss of rpp30, which encodes a subunit of RNase P for tRNA processing, leads to reduction of H3K9me3 at piRNA clusters and reduction in levels of cluster transcripts, supporting the importance of H3K9me3 for cluster transcription (Molla-Herman et al. 2015).

Transcription from piRNA clusters requires specialized complexes because many such clusters are present in gene-poor regions and lack canonical promoters, as well as canonical intron-exon boundaries. In the germline, the RDC (Rhino-Deadlock-Cutoff) protein complex licenses transcription from piRNA clusters (Mohn et al. 2014; Z. Zhang et al. 2014) (Figure 3). RDC is composed of Rhino (Rhi), a homolog of Heterochromatin Protein 1a (HP1a), Deadlock (Del) without any conserved domain, and Cutoff (Cuff), an Ral1/Dom3Z-family protein (Chen et al. 2007; Klattenhoff et al. 2009; Mohn et al. 2014; Z. Zhang et al. 2014). The chromodomain on Rhino recognizes H3K9me3 marks at clusters for the binding of RDC complex (Mohn et al. 2014; Z. Zhang et al. 2014; B. Yu et al. 2015). However, the detailed mechanism underlying the establishment of H3K9me3 at clusters is not known. The RDC complex binds to all dual-strand piRNA clusters, which are active in the germline, but not to the clusters active in somatic cells. The RDC complex prevents transcription termination of adjacent genes to allow for transcription of the clusters. The RDC complex also licenses transcription from noncanonical promoters in piRNA clusters (Le Thomas et al. 2014; Mohn et al. 2014; Z. Zhang et al. 2014). Loss of RDC complex leads to an increase in splicing of cluster transcripts, which could potentially destabilize these cluster transcripts (Mohn et al. 2014; Z. Zhang et al. 2014; Chen et al. 2016). Indeed, it was shown that RDC tethering to a transgene leads to intron stabilization and transcription beyond the polyA site. Aravin and colleagues suggest that Cuff in the RDC complex is necessary to prevent binding of Cleavage and Polyadenylation Specific Factor (CPSF) for a continuous transcription throughout the piRNA cluster (Chen et al. 2016). Cuff is also required for stabilizing the noncapped cluster transcripts, by antagonizing cluster transcript destabilization by 5′-3′ exonuclease dRat1 (Chen et al. 2016). The RDC complex is important not only for transcription from the piRNA clusters, but also for transcription and piRNA production from transgenes (Z. Zhang et al. 2014). In addition, RDC complex also participates in channeling the cluster transcripts to the piRNA processing site for piRNA generation.

Recently, it was reported that Tho5 and other THO subunits of the Transcription/Export (TREX) complex are recruited to piRNA clusters by Cuff, and are loaded onto cluster transcripts (Hur et al. 2016) (Figure 3). The proteins are required for accumulation of nascent cluster transcripts in a splicing-independent manner in nucleus. Another protein, UAP56, a component of the nuclear pore complex, binds to cluster transcripts in a Rhi-dependent manner. Through interaction with a nuage component, Vasa, an RNA helicase, UAP56 likely functions to export cluster transcripts to nuage, the site of piRNA processing (Zhang et al. 2012) (see below).

**Transcription of piRNA clusters active in the ovarian somatic cells**: Somatic piRNA clusters do not require the RDC complex for their transcription. Details about their transcription remain limited. In contrast to germline cluster transcripts, somatic piRNA cluster transcripts are polyadenylated. A piRNA cluster, flamenco, located near the pericentric region of X-chromosome, is particularly active in the ovarian follicle cells. The flamenco locus has fragmented copies of transposons expressed in somatic cells, such as ZAM and gypsy (Prud’homme et al. 1995; Sarot et al. 2004; Desset et al. 2008). The flamenco-derived transcripts are in antisense orientation to the active transposons. Transcription from the flamenco locus is reported to be dependent on the transcription factor Cubitus interruptus (Ci). The flamenco-derived transcripts are alternatively spliced, probably for diversity (Goriaux et al. 2014).

**piRNA biogenesis in somatic gonadal cells via primary processing**

The somatic piRNA cluster transcripts are processed into piRNAs in the cytoplasm (Haase et al. 2010; Saito et al.
Mechanisms of transport of somatic piRNA precursors from the nucleus to cytoplasmic processing sites remain elusive. Most primary piRNA processing components are localized to mitochondria. Yb-body and Zucchini (Zuc)-associated proteins have been shown to coordinate piRNA processing and loading of piRNAs to Piwi (Haase et al. 2010; Saito et al. 2010; Handler et al. 2011; Qi et al. 2011). The 5' end of piRNAs is generated by an exonuclease, Zucchini (Zuc), which localizes to the mitochondrial surface (Ipsaro et al. 2012; Nishimasu et al. 2012). Zuc generates 5'-ends of piRNAs, the resultant ends of which are not enriched with U (Uracil). However, the 5'-ends of mature piRNAs are remarkably biased for U. In vitro study of the silkworm Piwi homolog Siwi suggested that this bias for U at the 5'-end could be introduced during piRNA loading to Siwi (Kawaoka et al. 2011). The 3'-ends of piRNAs are speculated to be generated while piRNAs are being loaded to PIWI proteins. Zuc is thought to function in 3'-end formation of piRNAs, although the resultant products have additional nucleotides at the 3'-end. These extra nucleotides are proposed to be trimmed by another exonuclease (Kawaoka et al. 2011). A Tudor-domain protein, PAPI, has also been suggested to be important for 3'-end trimming of piRNAs, although PAPI does not have any nuclease domain (Honda et al. 2013). The piRNAs are loaded to Piwi at Yb-bodies present at the outer mitochondrial surface close to Zuc (Olivieri et al. 2010; Saito et al. 2010). Piwi forms a complex with Yb-body proteins, such as Vretreno (Vret), Shutdown (Shu), and Armitage (Armi). Severe reduction of piRNAs in vret, shu, and armi mutants leads to cytoplasmic accumulation of Piwi, which likely results from the failure of piRNA loading on Piwi (Haase et al. 2010; Olivieri et al. 2010, 2012; Saito et al. 2010; Handler et al. 2011; Qi et al. 2011). In addition, Shu functions for piRNA loading onto Piwi with the help of Hsp 83 (Handler et al. 2011).

The Yb-body, where many proteins required for primary piRNA processing are localized, is named after the Yb protein containing both an RNA-helicase and a Tudor domain (King et al. 2001; Szakmary et al. 2009). Yb proteins bind to the flamenco-derived piRNA precursors through their DEAD box domain, channel them to the Yb-body, and stabilize the piRNA processing apparatus at Yb-bodies (Murota et al. 2014). Although all Yb-body components are required for piRNA biogenesis, their exact function remains unknown.

**piRNA biogenesis in germ cells**

In addition to primary piRNA processing, germ cells have an additional piRNA processing machinery called secondary amplification or the ping-pong cycle. This piRNA biogenesis mechanism allows more robust piRNA production against transposons and provides more flexibility to adapt for newer transposon threats.

**Primary piRNA biogenesis in germ cells:** The mechanistic details of primary piRNA biogenesis in germ cells remain unknown. However, many components required for primary piRNA processing in ovarian somatic cells, such as Zuc, Armi, Gasz, Shu, Mino, and HSP90, are expressed in germ cells and suggested to function for primary piRNA processing in germ cells.

**Secondary piRNA processing in germ cells; ping-pong amplification:** Secondary piRNA processing is a feed-forward amplification loop involving two PIWI-family proteins, Aub and Ago3, and it takes place at the nuage in germ cells where the key components of this secondary processing are localized. The Aub-bound antisense piRNAs target the transposon transcripts, and piRNA-loaded Aub harboring slicer activity cleaves the transposon transcript, generating the 5'-end of transposon-derived sense piRNA. The 3'-end of the piRNAs is generated either by slicer activity or by Zucchini. Ultimately the 3’ end generated by both the mechanisms requires trimming by an exonuclease Nibbler, generating mature Ago3-bound sense piRNAs of correct size. (Nishimasu et al. 2012; Hayashi et al. 2016). In turn, piRNA-bound Ago3 targets and cleaves cluster transcripts to generate more antisense piRNAs loaded onto Aub or Piwi, and the processing cycle amplifies piRNAs in a feed-forward loop. This processing leads to a significant 10-nt overlap between Aub and Ago3-bound piRNA sequences, with a U at position 1 of Aub-bound piRNAs and an
revealed by a series of recent studies. For example, Vasa may not always be dependent on sDMA (Patil and Kai 2010).

The secondary piRNAs also trigger production of Zuc-dependent, 3’-directed phased piRNAs. Phasing is triggered by so-called responder piRNAs, which result from Ago3-piRISC activity in the ping-pong cycle. The piRNAs downstream of responder piRNAs are associated with Piwi. These piRNAs are designated as trailer piRNAs. The responder piRNAs and trailer piRNAs show marked phasing with a ~27 nt interval and a striking bias for U at the 5’-end. Production of these trailer piRNAs depends on Zuc. These findings also suggest that piRNA 3’-ends are defined by Zuc endonucleolytic activity (Han et al. 2015; Mohn et al. 2015) (Figure 4). Biogenesis of such phased piRNAs spreads piRNA production beyond the target cleavage sites of Ago3 and Aub, thus allowing sequence diversification in the piRNA pool, which could target transposon threat in an adaptive manner by both TGS (Transcriptional Gene Silencing) and PTGS (Post-Transcriptional Gene Silencing) mechanisms (Han et al. 2015; Homolka et al. 2015; Mohn et al. 2015; Sato et al. 2015; Senti et al. 2015; Wang et al. 2015; Webster et al. 2015).

**Nuage as a site for ping-pong cycle in germ cells:** Both PIWI-family proteins, Aub and Ago3, involved in ping-pong amplification are found at the perinuclear foci in the cytoplasmic face called nuage (Brennecke et al. 2007). Nuage is an amorphous, electro-dense structure present at the cytoplasmic face of nuclear membrane (reviewed in Eddy 1975). Nuage has been widely recognized, albeit occasionally by different names, as a hallmark of germ cells in animals, and yet its function remained unknown for a long time.

Studies have shown that different kinds of proteins localize to nuage and participate in piRNA processing, including RNA helicase Vasa and Spindle-E (SpnE). Most nuage components are Tudor domain proteins, such as Tudor (Tud), Qin/Kumo, Tejas (Tej), Tapas (Tap), Krimp (Krimp), and SpnE. Other proteins include HMG box protein Mael and nucleases, such as Zuc and Squash (Squ) (Lim and Kai 2007; Pane et al. 2007; Malone et al. 2009; Patil and Kai 2010; Zhang et al. 2011; Anand and Kai 2012; Senti et al. 2012; Patil et al. 2014). Tudor domains preferably bind to symmetrical demethylation of Arginine (sDMA) sites on PIWI-family proteins (Nishida et al. 2009). Tud binds to Aub and Ago3 in an sDMA-dependent manner and ensures proper binding of piRNAs to Aub and Ago3 (Nishida et al. 2009). However, the interaction between Tudor domains and PIWI-family proteins may not always be dependent on sDMA (Patil and Kai 2010).

The details of function of many nuage proteins have been revealed by a series of recent studies. For example, Vasa interacts with UAP56 and likely functions for transport of piRNA precursors to the nuage (Zhang et al. 2012). The Tudor domain protein Krimp maintains Aub and Ago3 on the nuage for proper ping-pong amplification. Krimp interacts with Ago3, which does not require piRNA loading on Ago3 or Arginine methylation. However, the binding of Krimp to Aub requires arginine methylation of Aub. Krimp promotes arginine methylation on Ago3, and prevents the loading of antisense piRNA on it (Sato et al. 2015; Webster et al. 2015) (Figure 4). The nuage components Tej and Tap function synergistically for piRNA production, and are required for maintenance of all other piRNA pathway components at the nuage (Patil and Kai 2010; Patil et al. 2014). Another Tudor domain protein, Qin/Kumo, is also required for proper maintenance of Aub and Ago3 at nuage, and it prevents the loading of sense piRNAs to Piwi and Aub, thereby enforcing heterotypic Aub: Ago3 ping-pong (Zhang et al. 2011; Anand and Kai 2012; Wang et al. 2015). The function of piRNA pathway proteins explains, to some extent, the observed mutual dependence for their localization to nuage based on a genetic hierarchical relationship (Lim and Kai 2007; Anand and Kai 2012; Patil et al. 2014). For example, Vasa, which is genetically farther upstream, also becomes functionally upstream. Similarly, Krimp, Qin/Kumo, Tej, and SpnE support ping-pong, and, therefore, also support Aub and Ago3 on nuage. The piRNA biogenesis requires a multistep mechanism, and, as such, a number of proteins assist Aub and Ago3 for piRNA generation and piRNA-mediated degradation of transposon transcripts.

**piRNA-mediated transcriptional silencing of transposons**

Transposon transcripts are post-transcriptionally silenced by piRNAs at the nuage, possibly at cytoplasmic processing bodies where piRNA pathway components are localized in later stages of oogenesis (Lim et al. 2009). In addition, piRNAs transcriptionally repress transposons, and piRNA loss leads to concomitant loss of repressive histone marks at transposons in the *Drosophila* female germline and somatic cells (Klenov et al. 2007, 2014; Sienski et al. 2012; Le Thomas et al. 2013; Rozhkov et al. 2013).

**Piwi is a key mediator of transcriptional silencing:** The piRNA loading on Piwi is important for its entry in to the nucleus. Piwi-piRISC enters the nucleus to transcriptionally silence the transposons in both somatic and germ cells (Le Thomas et al. 2013; Rozhkov et al. 2013). Piwi loss does, indeed, cause an increase in RNA polymerase II occupancy at promoter regions of transposons, as well as reduction of H3K9me3 levels (Sienski et al. 2012; Czech et al. 2013; Huang et al. 2013; Le Thomas et al. 2013; Rozhkov et al. 2013; Klenov et al. 2014). Although the enrichment of Piwi at transposon loci has still not been established, predominant loading of antisense piRNAs to Piwi led to the speculation that Piwi-piRISC scans for nascent transposon transcripts to enforce transcriptional repression (Han et al. 2015; Homolka et al. 2015; Mohn et al. 2015; Sato et al. 2015; Senti et al. 2015; Wang et al. 2015; Webster et al. 2015). Notably, although Piwi is equipped with a slicer domain, this domain is dispensable for piRNA production and transposon silencing (Darricarrere et al. 2013).
Distinct components act together with Piwi: Piwi-piRISC interacts with proteins to enforce transposon silencing. Recent work has identified several downstream components of Piwi-piRISC for transposon transcriptional silencing in both the germline and somatic cells. Many proteins have been shown to act downstream of Piwi-piRISC for H3K9me3 enrichment at transposon loci.

The double CHHC zinc finger protein, gametocyte-specific factor 1 (GTSF1), has been shown as a downstream Piwi-piRISC partner in both germline and somatic cells in the ovary (Dönertas et al. 2013; Ohtani et al. 2013). GTSF1 interacts with Piwi to establish H3K9me3 at the transposon loci for repression of transposons (Dönertas et al. 2013; Ohtani et al. 2013). The downstream nature of GTSF1 is suggested by unchanged piRNA levels upon GTSF1 loss, but transposon derepression profile and loss of H3K9me3 at transposon loci mimic what has been observed upon Piwi loss (Dönertas et al. 2013; Ohtani et al. 2013). However, the precise molecular mechanisms that explain how GTSF1 engages Piwi in TGS remain elusive. Another protein, Panoramix/Silencio (Panx), is vital for transposon repression through transcriptional silencing (TS) in germ cells (Sienski et al. 2015; Y. Yu et al. 2015). Panx interacts with Piwi and recruits the methyltransferase Egg to deposit H3K9me3 for heterochromatin formation at transposon loci. The loss of Panx leads to global transposon derepression without any reduction of piRNAs, suggesting its role as a downstream partner of Piwi-piRISC (Sienski et al. 2015; Y. Yu et al. 2015). In addition, Piwi-piRISC may silence transposons through the removal of H3K4me2 (Fadloun et al. 2013; Klenov et al. 2014). Depletion of the Lsd1 demethylase in Drosophila ovaries resulted in derepression of a subset of transposons, which is independent of Panx (Czech et al. 2013).

The function of Piwi-RISC for TGS of transposons is through not only histone modifications but also mediated
by chromatin binding proteins. For example, Piwi loss leads to reduction of HP1α at few transposons in both germline and somatic cells in the ovary (Ohtani et al. 2013; Kloenov et al. 2014). In germ cells, HP1α loss leads to derepression of transposons (Wang and Elgin 2011). However, in somatic cells, transposon derepression resulting from HP1α loss is not necessarily correlated with that caused by Piwi loss (Ohtani et al. 2013). This suggests that other chromatin regulators could repress some transposons independent of Piwi. Functions of these proteins also overlap, for example, HP1α likely enforces transposon silencing downstream of Piwi-piRISC by recruiting SetDB1 via interaction with Piwi-RISC tethered at the transposon loci (Brower-Toland et al. 2007; Sienski et al. 2015). Recently, histone H1 was also shown to be one of the downstream components of Piwi-piRISC for transposon repression in ovarian somatic cells, functioning in parallel with HP1α. However, unlike HP1α, H1 function is independent of H3K9Me3 marks (Iwasaki et al. 2016). Hence, it is possible that Piwi recruits different downstream factors, such as HP1α and H1, for more efficient transposon repression.

Current studies suggest that Piwi acts with different downstream factors to repress different sets of transposons. Although the underlying mechanism is not fully understood, the transposon location, type and evolutionary age are speculated to contribute to this. Indeed, the evolutionarily older transposons are shown to be enriched at pericentric regions and are targeted by fewer piRNAs (Koller et al. 2012; Kelleher and Barbash 2013). It has been suggested that evolutionarily older transposons are more likely to be silenced transcriptionally, while the evolutionarily younger transposons depend more on the post-transcriptional silencing mechanism (Senti et al. 2015). In summary, the piRNA pathway incorporates a wide variety of partners to maintain piRNA clusters, transport, TGS and PTGS, for effective silencing of transposon globally and better sustainability of species in an evolutionary arms race with transposons.

**Mechanisms that Regulate Meiotic Cell Maturation**

The maturation of both male and female gametes is a stepwise developmental process that requires the coordinated control of the cell cycle, cellular morphology, and cellular positioning. The coordination of these processes are absolutely essential, and require exquisite transcriptional, as well as translational, regulation of a diverse set of genes.

**Spermatocyte maturation**

In the male germline, the transition from spermatogonia to spermatocytes is accompanied by a series of transcriptional, epigenetic, and morphological changes. After transition amplification, germ cells undergo the last S phase followed by an extended G2 phase that initiates the spermatocyte stage. Spermatocytes grow 25 times in volume and turn on a robust transcription program to activate genes required for spermatocyte maturation, as well as genes needed for meiotic divisions and terminal differentiation (White-Cooper et al. 1998).

**Transcriptional regulators**: Many genes required for meiotic divisions and terminal differentiation are under translational repression until a later time when their encoded proteins are required (Schafer et al. 1995). The G2/M transition in meiosis I requires Cyclin B, Boule (a RNA-binding protein) and Twine (Cdc25 homolog), all transcribed in spermatocytes (Alphey et al. 1992; Courtot et al. 1992; White-Cooper et al. 1998). Boule translocates from the nucleus to the cytoplasm to trigger the G2/M transition in meiosis I by allowing translation of Twine (Maines and Wasserman 1999). At this point in time, Cyclin B also escapes from translational repression and accumulates Cyc B protein in the cytoplasm of spermatocytes (White-Cooper et al. 1998). In both boule and twine mutant testes, spermatid differentiation occurs in a manner independent of meiotic cell cycle progression, suggesting that these two processes can be uncoupled (Alphey et al. 1992; Eberhart et al. 1996). However, the discovery of two classes of genes expressed in early spermatocytes reveals a high degree of coordination between meiotic divisions and spermatid differentiation (Lin et al. 1996). Mutations in any of these genes arrest meiosis and block spermatid differentiation, leading to testes filled with immature spermatocytes. These genes are named “meiotic arrest” genes, which are further classified into “aly-class” and “can-class” based on morphological differences of the chromosomal structure in the mutant spermatocytes (Lin et al. 1996; White-Cooper et al. 1998) and their distinct target genes (Lin et al. 1996; White-Cooper et al. 1998, 2000; Hiller et al. 2001, 2004; Ayyar et al. 2003; Jiang and White-Cooper 2003; Perezgasga et al. 2004; Beall et al. 2007; Jiang et al. 2007; Chen et al. 2011). For example, transcription of meiotic cell cycle genes, such as Cyclin B, Boule, and Twine, rely on aly-class, but not can-class, genes (White-Cooper et al. 1998). However, Boule protein accumulation requires the can-class genes (Chen et al. 2005). Since meiotic arrest genes regulate transcription or translation of meiotic cell cycle genes, the meiotic cell cycle cannot proceed until terminal differentiation genes are robustly transcribed (Lin et al. 1996; White-Cooper et al. 1998).

The six known aly-class genes are always early (aly), cookie monster (comr), matotopetli (topi), tombola (tomb), achin-tya/vismay (achi/vis), and Caf1 (Beall et al. 2007). All of the aly-class genes, except achi/vis, are expressed exclusively in primary spermatocytes (Ayyar et al. 2003; Jiang and White-Cooper 2003; Wang and Mann 2003; Perezgasga et al. 2004; Jiang et al. 2007; White-Cooper 2009). Four aly-class proteins have putative DNA-binding domains, including Comr, which contains a winged helix; Topi, which contains multiple Zn-finger motifs; Tomb, which has a CXC domain; and Achi/Vis, products from a gene duplication, which have homeodomains. Thus, it is thought that these proteins regulate the transcription of target genes by directly binding to DNA sequences, even though their direct target genes have not been identified. Immunoaffinity purification studies have revealed that Aly and Tomb proteins are copurified with Mip40 (Myb interacting protein, 40 kDa) to form...
the testis meiotic arrest complex tMAC, which also contains Topi, Comr, and CAF1 (Beall et al. 2007). A second form of tMAC contains Aly, Comr, and Achi/Vis (Wang and Mann 2003). The tMAC resembles the MIP/dREAM complex in mammals and the SynMuv complexes in C. elegans (White-Cooper et al. 1998, 2000; Ayyar et al. 2003; Jiang and White-Cooper 2003; Perezgasga et al. 2004; Beall et al. 2007; Jiang et al. 2007). Studies using the DamID method profiled ~300 direct target genes of Comr in testis, most of which have decreased expression in the comr mutant, suggesting that it functions mainly as a transcriptional activator (Laktionov et al. 2014). This is consistent with earlier results demonstrating that expression of Achi/Vis fused with a strong transactivation domain, VP16, rescued the achi/vis mutant phenotype, while the fusion of Achi/Vis with a repression domain, EnR, failed to rescue (Wang et al. 2008). Consistent with these findings, all tMAC subunits have been found to colocalize with euchromatin in primary spermatocytes (White-Cooper et al. 2000; Jiang and White-Cooper 2003; Wang and Mann 2003; Jiang et al. 2007).

The can-class genes encode testis-specific homologs of ubiquitously expressed subunits of the general transcription factor II D (TFIID). TFIID is one of the general transcription factors that constitute the RNA Pol II preinitiation complex composed of TATA-binding protein (TBP) and 13–14 TBP-associated factors (TAFs) (Tora 2002; Matangkasombut et al. 2004; Cler et al. 2009). TFIID coordinates the interaction between RNA Pol II and gene promoter regions. The characterized can-class genes include cannonball (can, TAF5L), meiosis I arrest (mia, TAF6L), no hitter (nht, TAF4L), ryan express (rye, TAF12L), and spermatoocyte arrest (sa, TAF8L). Among the five TAF homologs, four, including Mia, Nht, Rye, and Sa, share similar structural domains called histone folding motifs for protein-protein interaction, while Can is a WD40-repeat-containing protein (Hiller et al. 2001). Indeed, Nht and Rye form a heterodimer in vitro (Hiller et al. 2004). These testis-specific TAFs (tTAFs) are thought to form a testis-specific complex required for transcriptional activation of the terminal differentiation genes (Hiller et al. 2001, 2004). Such predicted functions of tTAFs suggest that they localize to the euchromatin in spermatocyte nuclei. However, while a proportion of the total protein of each tTAF associates with chromosomes in spermatocytes, most tTAF protein is localized to a subcompartment within nucleolus (Chen et al. 2005; Metcalf and Wassarman 2007). Interestingly, Polycomb and other components of PRC1 are colocalized to the same nucleolar subcompartment with tTAFs in spermatocytes. Furthermore, localization of PRC1 components to the spermatocyte nucleolus is coincident with tTAF expression and dependent on wild-type tTAF function (Chen et al. 2005). These results suggest that tTAFs act as derepressors by sequestering PRC1 to the spermatocyte nucleolus to counteract PcG-induced repression. However, removing PcG activity is not sufficient to turn on terminal differentiation genes in the absence of tTAFs (Chen et al. 2011), suggesting that chromatin-associated tTAFs are required to activate terminal differentiation genes. Consistent with these observations, tTAFs were reported to turn on transcription of >1000 genes, many of which are required for spermatid differentiation (White-Cooper et al. 1998; Chen et al. 2011). Among the tTAF-dependent genes, three are shown to be direct target genes of tTAF by ChIP assay: fuzzy onions (fso), which encodes a protein required for mitochondrial fusion in early spermatids (Hales and Fuller 1997); mst87F, which encodes a component of the sperm tail (Schafer et al. 1993) and don juan (dj), which encodes a sperm-specific DNA-binding protein that also localizes to mitochondria (Santel et al. 1998). ChIP analysis at the promoter regions of these three genes directly targeted by tTAF showed that levels of the repressive H3K27me3 mark and paused Pol II are high, while levels of the active H3K4me3 mark are low in can and aly mutant testes (Chen et al. 2011). These data suggest that tTAFs and tMAC might recruit TrxG, whose activities antagonize PcG, to methylate H3K4 at promoters of terminal differentiation genes and activate robust transcription (Chen et al. 2005).

Although the mode of interaction between tMAC components (aly-class) and tTAFs (can-class) is not fully understood, the transcription coactivator Mediator likely acts to coordinate tMAC and tTAFs. The tMAC recruits Mediator components to spermatocyte chromatin, and Mediator subsequently helps proper tTAF localization. Together, tMAC, tTAFs and Mediator coregulate a cohort of spermatid differentiation gene expression (Lu and Fuller 2015). It was also reported that the function of aly is required for the binding of TAF8L to target gene promoters. Aly is also required for the proper nucleolar localization of several tTAFs and Polycomb in spermatocytes, suggesting that tMAC acts upstream of tTAFs (Chen et al. 2011). This is consistent with assays using Northern blot, in situ hybridization, and microarray analysis (White-Cooper et al. 1998; Hiller et al. 2001; Chen et al. 2011). In addition, while Mip40 is coimmunoprecipitated with tMAC components, loss of mip40 results in spermatocytes with condensed chromosomes, a phenotype similar to mutants of can-class genes (Beall et al. 2007), suggesting that Mip40 might mediate the interaction between tMAC and tTAFs. Both tMAC and tTAFs have their canonical counterparts that act generally in other tissues, as well as in spermatocytes, probably by regulating target genes distinct from the testis-specific forms. Similarly, the canonical chromatin remodeler NURF has a germline-specific function in regulating meiotic divisions and spermatocyte differentiation (Kwon et al. 2009), most likely through using an alternatively spliced isoform.

Five other meiotic arrest genes, which cannot be classified as either aly-class or can-class, were identified and characterized. Wake-up-call (Wuc) was identified by its physical interaction with Aly in a yeast-two-hybrid screen (Jiang et al. 2007). In spermatocytes, the Wuc protein is highly expressed and associated with chromatin, similar to other tMAC components. However, unlike tMAC or tTAF mutants, loss of wuc does not abolish expression of either meiotic cell cycle genes or spermatid differentiation genes (Doggett et al. 2011).
Another study showed that disruption of a component of the THO complex, THOC5, led to the meiotic arrest phenotype. The THO complex is known to export mRNAs from nucleus to cytoplasm. However, no mRNA export defects were detectable in the thoc5 mutant. Moreover, neither meiotic cell cycle genes nor spermatid differentiation genes have decreased transcription in the thoc5 mutant, even though a more comprehensive study is needed. THOC5 is localized to a perinucleolar region, and loss of thoc5 function leads to disrupted nucleolar structure and the localization of tTAFs, which could contribute to its mutant phenotype (Moon et al. 2011). A more recent study identified Ntx1, another mRNA export machinery component, as required for accumulation of many spermatogenesis-specific mRNAs. However, the dependence of these transcripts on Ntx1 has a distinct mode compared to tMAC- or tTAF-dependent genes, which is regulated by the primary transcript structure (Caporilli et al. 2013). Moreover, through characterization of a meiotic arrest mutant magellan (magn), the Ubi-p63E gene encoding polyubiquitin has been shown to regulate proper spermatocyte chromatin structure, meiotic cell cycle progression, and spermiogenesis. However, the different phenotypes caused by loss-of-function of proteasome subunits suggest that Ubi-p63E acts in a protein degradation-independent manner in spermatocytes (Lu et al. 2013). Finally, a very recent study identified a novel meiotic arrest gene kumgang (kmg), which encodes a zinc finger-containing protein. The kmg gene is specifically turned on in early spermatocytes, independent of either tMAC or tTAF. Interestingly, Kmg is required to maintain germline identity by suppressing the expression of hundreds of somatic genes. Genetic, genomic, and biochemical analyses reveal that Kmg acts with the chromatin remodeler dMi-2 to restrict the tMAC component Aly from helping to fire transcription from cryptic promoters of a cohort of somatic genes, which are normally turned on in somatic tissues, such as gut and brain (Kim et al. 2017). Both identification of wuc, thoc5, Ntx1, magn, and kmg mutants, and detailed characterization of their phenotypes and mechanisms demonstrate the existence of meiotic arrest genes other than aly- and cand-class. Further understanding of their molecular and cellular mechanisms will lead to new information about spermatocyte maturation.

**MicroRNAs:** In males, bam mRNA is detectable, but Bam protein is undetectable in the meiotic spermatocytes. It has been shown that a specific miRNA, miR-275, represses Bam protein accumulation through 3′UTR in spermatocytes. If this repression of Bam protein accumulation in spermatocytes is misregulated, spermiogenesis cannot proceed properly, and this will lead to decreased male fertility (Eun et al. 2013). Therefore, although Bam is an important differentiation factor to initiate GSC differentiation, its downregulation is also critical for proper spermatid terminal differentiation. Furthermore, this post-transcriptional regulation of Bam protein accumulation does not occur in the female germline, again suggesting sex-specific modes in the regulation of meiotic germ cell maturation.

**Oocyte specification and maturation**

Oocyte development begins with oocyte specification in the germarium; once specified, the oocyte migrates to the posterior region of the cyst, which will be enclosed by follicle cells and buds off as an egg chamber. While the egg chamber is growing, the polarity of the oocyte is established. These processes involve both signal transduction pathways, as well as the cytoskeletal machinery. Furthermore, the piRNA pathway has also been reported to have a significant role in establishing oocyte polarity and proper oocyte development.

**Oocyte specification and polarity establishment in the germarium:** As an extremely specialized cell type, oocyte specification requires a series of developmentally regulated processes to break the symmetry and give rise to this highly polarized, gigantic cell (Roth and Lynch 2009). Polarity formation begins in the germarium with the specification of oocytes, and this polarity is already established as early as the first division of the CB (de Cuevas and Spradling 1998). In regions 2a and 2b (Figure 1A), mRNA transport allows accumulation of specific markers in the developing oocyte (Suter and Steward 1991; Lantz et al. 1994; Mach and Lehmann 1997). After completing the transit-amplification stage, the microtubule organizing center (MTOC) appears in one or two cells inside the 16-cell cyst, which has four ring canals connected to other cells in the cyst. These two cells, called pro-oocytes, start meiosis, which is characterized by the appearance of double-strand breaks (DSBs) and the formation of synaptonemal complex (SC). Later, one of them will be committed to an oocyte. Upon the specification of oocyte, DSBs, which have been seen in both pro-oocytes, become restricted to one oocyte in region 2a/2b, and are repaired thereafter (Jang et al. 2003). In piRNA pathway mutants, such as armitage (armi) and aub, DSBs persist longer and are accumulated in the oocyte during later stages of development (Klattenhoff et al. 2007). The upregulation of transposons, and their increased transpositions in the oocyte, are believed to cause accumulation of DSBs in the oocyte, although no study has explicitly proven this. At region 2b/3 (Figure 1A), oocyte determination is evident in that the SC remains in only one germ cell determined to be an oocyte (Huynh and St Johnston 2000; Page and Hawley 2001). In addition, MTOC appearance is more pronounced, and gurken (grk) mRNA is localized posteriorly (Neuman-Silberberg and Schupbach 1993, 1996). Egg chambers (Stage 1–14) are assembled posterior to the germarium, which contains the nurturing nurse cells and the developing oocyte ensheathed by follicle cells derived from FSCs.

**Oocyte determination and polarity formation during oocyte maturation:** As oogenesis proceeds toward region 3, the oocyte meiotic chromosomes form a compact spherical structure called the karyosome, which is mainly transcriptionally silent (Parfenov et al. 1989; Bastock and St Johnston 2008; Lancaster et al. 2010). At this stage, the oocyte development is also determined by the coordinated activity of both...
cell cycle genes and polarity genes (Lilly and Spradling 1996; Mach and Lehmann 1997; Mata et al. 2000; Huynh et al. 2001a; Hong et al. 2003).

Microtubule networks, as organized by spectrosome/fusome structure (Grieder et al. 2000), play important roles in the formation and maintenance of oocyte polarity. The MTOCs shift from the anterior to the posterior side of the oocyte (Theurkauf et al. 1992, 1993; Huynh et al. 2001b; Vaccari and Ephrussi 2002). The reorganized microtubule network is important for proper localization of maternal components, such as bicoid and oskar (osk), as polarity determinants, and define the anterior and posterior sides of the developing oocyte, respectively (Ephrussi et al. 1991; Kim-Ha et al. 1991; Brendza et al. 2000; Januschke et al. 2002). This polarity within the oocyte also defines the embryonic anterior—posterior axis. Live imaging of Osk particle movement during oogenesis showed that the mRNA is actively transported along microtubules in all directions, with a slight bias toward the posterior (Zimyanin et al. 2008). Meanwhile, grk mRNA is found at the posterior of oocyte in the gerarium, while in the later stages, grk mRNA is repositioned at the dorsal anterior corner of the oocyte (Neuman-Silberberg and Schupbach 1996; Van Buskirk and Schupbach 1999; St Johnston 2005). Grk accumulation defines the dorsal—ventral axis of the oocyte, as well as the embryos (Schupbach 1987; Neuman-Silberberg and Schupbach 1993; Nilson and Schupbach 1999; Moussian and Roth 2005). Grk localization and oocyte nucleus migration are mediated by microtubules (reviewed by Roth and Lynch 2009). Although the exact mechanism for grk mRNA localization is not known, microtubules from MTOC and dynein are important for Grk localization in the oocyte and mediate communication with follicle cells (Brendza et al. 2000, 2002; Duncan and Warrior 2002; Januschke et al. 2002). Together, polarized localization of bicoid, osk, and grk mRNAs defines both the AP and DV axes of the oocyte and embryo. However, imaging data revealed that the overall microtubule network is actually much less polarized than previously expected (MacDougall et al. 2003; Zimyanin et al. 2008), provoking more studies using new techniques such as live cell imaging.

By midoogenesis, a specialized cytoplasm, termed as pole plasm, assembles at the posterior end of oocyte (Hay et al. 1988; Lasko and Ashburner 1988; Ephrussi et al. 1991; Golumbeski et al. 1991; Ephrussi and Lehmann 1992; Harris and Macdonald 2001; Megosh et al. 2006).

**Function of Pcg in oocyte specification:** While the determined oocyte will initiate extraordinary cell growth and meiotic cell cycle, the 15 nurse cells in the Drosophila ovary will enter the endocycle and become polyploid cells to provide RNAs and proteins to the developing oocyte. Transdetermination from oocyte to nurse-like cells was observed when PRC2 components E(z) and Su(z)12 were knocked down in the Drosophila female germline. This cell fate change results from derepression of Cyclin E and cyclin-dependent kinase inhibitor Dapaco upon loss of the repressive H3K27me3 mark (Iovino et al. 2013). However, such cell fate switch does not occur in the male germline. In males, all 16 spermatogonial cells enter meiosis simultaneously after mitosis and differentiate into mature sperm synchronously. This phenomenon suggests that differences between the female and male germline differentiation pathways require distinct epigenetic regulators.

**piRNA pathway components for polarity formation:** In addition to cytoskeletal machinery and RNA-binding proteins, loss of many piRNA pathway proteins results in discernible defects in polarity formation. For example, in arni, spnE, zuc, mael, and krmp mutants, Grk and Osk proteins fail to localize to the dorsal—anterior region and to the posterior region, respectively (Findley et al. 2003; Cook et al. 2004; Chen et al. 2007; Klattenhoff et al. 2007; Lim and Kai 2007; Pane et al. 2007). Failure of microtubule network polarization in piRNA pathway mutants is believed to cause mislocalization of these components. In addition, Osk is precociously translated in some of the piRNA pathway mutants (Cook et al. 2004; Lim and Kai 2007; Pane et al. 2007). The piRNA pathway component Mael interacts with MTOC components, including centrosomin, mini spindles, and γ—tubulin (Sato et al. 2011). The interaction of Mael with cytoskeletal structure further strengthens the role of piRNA pathway components in oocyte polarity formation. Interestingly, upregulation of I-element transposon is known to perturb the localization of grk and bcd mRNAs (Van De Bor et al. 2005). However, some piRNA pathway mutants, such as tej, and qin/kumo, do not show defects in the localization of grk or polarity formation of oocyte, despite the severe depression of transposons including I-element (Patil and Kai 2010; Anand and Kai 2012; Patil et al. 2014), suggesting that derepression of transposons alone is not sufficient to cause polarity defects in oocytes.

Consensus has still not formed around the role of piRNA pathway proteins in DNA damage response or polarity determination. In some piRNA pathway mutants, ablation of DNA damage checkpoint components, such as mei41 and chkl2, could suppress oocyte polarity defects (Klattenhoff
Mechanisms in Regulating Intergenerational and Transgenerational Epigenetic Inheritance

Traditionally, heritability is a characteristic feature of the genetic material of an organism, notably its DNA. Nonetheless, many phenomena and mechanisms of non-DNA sequence-based inheritance of vastly different phenotypes have been described from one generation to the next in multiple organisms ranging from plants to vertebrates (Youngson and Whitelaw 2008; Heard and Martienssen 2014). This inheritance of information beyond the primary DNA sequence is known as epigenetic. Direct epigenetic inheritance from parent to offspring is termed intergenerational epigenetic inheritance (IEI), and it is distinguished from transgenerational epigenetic inheritance (TEI), which is observed in generations that were not exposed to the initial signal or environment that triggered the acquired change.

One of the earliest reports of TEI in *Drosophila* was uncovered studying the Fab-7 chromosomal boundary element (Cavalli and Paro 1998). The Fab-7 boundary element, also a member of Polycomb Response Elements, is derived from the bithorax complex (BX-C) and is required to prevent crosstalk between adjacent regulatory regions, *iab-6* and *iab-7*, which control the spatial expression of the *Abd-B* gene (Hagstrom et al. 1996). To determine the function of the defined Fab-7 element, transgenic reporter strains were engineered to carry the Fab-7 element upstream of a GAL4 UAS-inducible *lacZ* reporter and a *mini-white* gene. The Fab-7 element was found to act as a strong silencer, repressing expression of both *lacZ* and the distantly located *mini-white* gene (Zink and Paro 1995). Increased GAL4 expression could stably activate both *lacZ* reporter and *mini-white* gene. Furthermore, a short, single pulse of GAL4 expression, regulated by a heat-shock promoter (*hs-GAL4*), during embryogenesis was sufficient to induce activation of the *mini-white* gene throughout development, resulting in adult flies with red eyes (Cavalli and Paro 1998). This continued expression of the reporter suggests a loss of silencing that is mitotically inheritable over many cell divisions and not dependent on the duration of the GAL4 protein. Surprisingly, GAL4-independent transmission of the active *mini-white* gene could be propagated through the female gametes for four generations. Inheritance of the expression pattern was not observed in the male germ-line. This was tested under conditions where offspring did not inherit the *hs-GAL4*, demonstrating that a short pulse of GAL4 induced during early embryogenesis alters the epigenetic landscape of a gene in a way that is stably inherited during both mitosis and meiosis. The molecular carrier for the maintenance of these patterns of expression through meiosis remains to be determined. Despite the fact that the mechanistic basis underlying both IEI and TEI is largely unknown and under intense investigation, three epigenetic information carriers have, in fact, been identified, including DNA methylation, chromatin structure, and RNAs.

**DNA methylation in intergenerational and transgenerational epigenetic inheritance**

DNA methylation may function as a molecular carrier during IEI in *Drosophila*. Inheritance of 5mC DNA methylation has been well documented in both mammalian and plant models of epigenetic inheritance (Heard and Martienssen 2014). Although the full extent to which DNA methylation participates in IEI in *Drosophila* remains elusive, investigations of sister chromatid inheritance, as well as tumor susceptibility, have highlighted two separate cases of intergenerational epigenetic effects in genetically compromised backgrounds.

During ACD of male GSCs, sister chromatids of the X and Y chromosome are distinguished and segregated in a nonrandom manner (Yadlapalli and Yamashita 2013). Loss of *DNMT2* leads to randomized sister chromatid segregation of both X and Y chromosomes, suggesting that *DNMT2* confers epigenetic information to the X and Y chromosomes that leads to distinct sister chromatid segregation. Furthermore, systematic crosses between homozygotic and heterozygotic *DNMT2* parents revealed that parental *DNMT2* function is necessary for proper segregation of X and Y sister chromatids in the next generation in a parent-dependent manner, very much like the imprinting phenomenon. These data suggest that parental *DNMT2* functions during gametogenesis in both males and females to transmit heritable information on the X and Y chromosomes, and such information is maintained during early embryogenesis (Yadlapalli and Yamashita 2013).

Little is known about epigenetic reprogramming of DNA methylation during gametogenesis and early embryogenesis in *Drosophila*. Studies of an oncogenic JAK kinase encoded by *hopscotch*<sup>1-1</sup> (hop<sup>1-1</sup>) demonstrated that this temperature-sensitive hypermorphic allele is able to antagonize a cellular program that erases DNA methylation of parental origin, allowing the epigenetic alterations to be maintained in the absence of the original mutation (Xing et al. 2007). Tumorous-lethal (Tum-1) is a dominant temperature-sensitive mutation in the *hop* locus that leads to overproliferation of hemocytes and formation of melanotic tumors, which are black masses of hemocytes correlated with lethality. In a genetic approach to identify genes important for *hop<sup>1-1</sup>*-induced tumorigenesis, 37 modifiers that either enhanced or suppressed *hop<sup>1-1</sup>* tumorigenesis were identified (Shi et al. 2006). Interestingly, many of the identified mutations exhibited paternal-effect modification of *hop<sup>1-1</sup>* tumor
susceptibility. For example, one of the modifiers, Kruppel (Kr), enhances hop\textsuperscript{hptum-1} tumorigenicity. When hop\textsuperscript{hptum-1} heterozygotic females were mated with male heterozygotes for Kr, tumorigenesis associated with hop\textsuperscript{hptum-1} was enhanced in the F1 generation, irrespective of the inheritance of the modifier mutation itself. This enhancement persisted into the F2 generation, but diminished by the F3 generation. Further studies indicate that the Kr mutation establishes DNA methylation at promoters during early embryogenesis. Furthermore, the epigenetic alterations induced by Kr are normally erased in the next generation. However, in the presence of the hop\textsuperscript{hptum-1} allele, the increased DNA methylation induced by Kr was transmitted to the next generation.

**Chromatin structure in intergenerational and transgenerational epigenetic inheritance**

Histones and histone variants have become primary candidates for mediating germline epigenetic inheritance. Histone modifications and variants are capable of transmitting epigenetic information through mitosis and meiosis to the next generation (Gaydos et al. 2014). A major barrier to TEI or TE is the epigenetic reprogramming during gametogenesis and early embryo development, during which global changes in histone modifications and variants occur (Harrison and Eisen 2015). An extreme example of histone replacement is the transition from nucleosome-based to protamine-based chromatin structure during Drosophila spermatogenesis (Rathke et al. 2007). Upon fertilization, chromatin undergoes dramatic remodeling again when the paternal genome is remodeled, replacing protamines with the histone variant H3.3 (Loppin et al. 2005). The Drosophila sesame mutant exhibits lesions at the HIRA gene encoding H3.3/H4 replication-independent nucleosome assembly chaperone. Characterization of sesame revealed that protamines are replaced by maternal H3.3 prior to the first S phase during embryogenesis. Upon fertilization, H3.3 is used to remodel paternal chromatin. Despite these sweeping changes, recent proteomic analysis of whole sperm mass spectrometry has revealed that all four canonical histones, as well as histone variants, are retained in mature sperm (Dorus et al. 2006). This retention in mature sperm raises the possibility that they retain epigenetic information for transmission across generations.

The centromere-specific histone variant Centromere identifier (Cid) is also present in mature sperm (Raychaudhuri et al. 2012). In Drosophila, nucleosomes with Cid, instead of other histone H3 variants, are stably incorporated exclusively at the centromeric region. Analysis of centromere identity has indicated that the centromere is specified epigenetically (Black and Cleveland 2011). Cid is retained in mature sperm and during the protamine-to-histone transition after fertilization. This paternally inherited Cid is required for the maintenance of paternal chromosomes in the next generation. In the absence of paternally inherited Cid, paternal chromosomes fail to recruit the maternally provided Cid and cannot generate functional kinetochores during the first mitosis.

Paternal genome stability in the embryo also relies on a heterochromatin-associated protein 1 (HP1) paralog, HP1E. The Drosophila genome encodes five HP1 paralogs, HP1A–E. The genes that encode HP1A, HP1B, and HP1C are expressed in all tissues and localize primarily to heterochromatin, with the exception of HP1C, which localizes exclusively to euchromatin (Smotherns and Henikoff 2001). HP1D and HP1E have special roles in the female and male germline, respectively (Volpe et al. 2001; Vermaak and Malik 2009). HP1D is required for transposon silencing in the female germline, and loss of HP1D results in female sterility (Volpe et al. 2001; Klatthenhoff et al. 2009). Expression of HP1E is developmentally restricted within the male germline where it localizes to the developing spermatids and functions in heterochromatin integrity. Specifically, HP1E localizes to developing spermatids subsequent to the completion of Meiosis II, but it is not detectable in mature sperm. In the absence of HP1E in males, embryos have defects in paternal chromatin condensation and fail to separate chromosomes during mitosis, resulting in “chromatin bridges” and lethality (Levine et al. 2015). Unlike Cid, which is inherited from the previous generation, HP1E is not inherited from sperm; instead, HP1E primes paternal chromosomes during spermatogenesis to ensure proper segregation in the next generation.

**Role of piRNA pathway in maternal deposition of transcripts and their clearance**

The oocyte provides transcripts and proteins to embryos for their early development. Transcripts of many genes, including those required for early development, are maternally deposited through the ring canals from nurse cells to the oocyte during oogenesis (reviewed by Laver et al. 2015). Transcription from zygotic genome starts at 2 hr postfertilization, and concurrently, a subset of maternally deposited materials is eliminated. This process is referred to as Maternal to Zygotic Transition (MZT; reviewed by Laver et al. 2015). During MZT, the transition of gene expression is tightly regulated in several different ways, including clearance of the maternally deposited transcripts. Recent high-throughput analyses identified that a significant number of transcripts, from 7000 to 10,000, are maternally transmitted to embryos (Lecuyer et al. 2007; Thomsen et al. 2010). Approximately two-thirds of them are either degraded, or significantly reduced, within 3 hr postfertilization (Thomsen et al. 2010; Laver et al. 2015). An RNA-binding protein, Smaug, triggers degradation of those RNAs by deadenylation through the CCR4/POP2/NOT4 deadenylase complex. Several studies revealed that piRNA pathway proteins and piRNAs promote the decay of a subset of posteriorly localizing maternally deposited RNAs in the bulk of embryo, possibly via the deadenylase complex, leading to the enrichment of germline determinants at pole plasm (Rouget et al. 2010; Barckmann et al. 2015; Vourekas et al. 2016).

**Enrichment of nos transcript at pole plasm:** Simonelig and colleagues first reported that piRNA pathway proteins and piRNAs promote the deadenylation and decay of maternally
deposited *nanos* (*nos*) transcripts (Rouget et al. 2010). Maternally deposited *nos* mRNA is present throughout embryos at very early stages, but it is translationally repressed in the somatic part and degraded in a deadenylation-dependent manner involving Smaug (Dahanukar and Wharton 1996). Osk, the key component of pole plasm formation, prevents deadenylation of *nos* transcript, and promotes its translation at the posterior pole, which helps to form Nos gradient at the posterior region (Santos and Lehmann 2004; Zaessinger et al. 2006). In addition to Osk, the piRNA pathway components *aub*, *ago3*, *spinE*, and *piwi* were shown to be required for deadenylation and decay of the maternal *nos* transcript (Rouget et al. 2010). Aub and Ago3 are present throughout embryos and likely trigger deadenylation of *nos* mRNA by recruiting the deadenylation complex. The piRNAs arising from 412 and 800 transposons target *nos* 3’UTR for *nos* mRNA decay. Those piRNAs bound to Aub and Ago3 likely recruit Smaug and the CCR4-NOT adenylation complex to *nos* transcript and degrade it in the bulk embryo, but not at the pole, forming the Nos gradient (Rouget et al. 2010).

**Decay and anchoring of maternal transcripts en masse:** Recent genome-wide analyses of Aub-bound RNAs using CLIP (crosslinking and immunoprecipitation) experiments by two groups further revealed the functions of piRNAs in anchoring and enriching maternally deposited transcripts at the posterior pole (Konig et al. 2010; Barckmann et al. 2015; Vourekas et al. 2016). While both small RNAs and long RNAs are found in the Aub-CLIP libraries, the small RNAs, mostly comprised of piRNAs, are more abundant than long ones. Almost all Aub-bound long RNAs bind to Aub in a piRNA-dependent manner, and do not contain transposon sequences, suggesting that RNAs bound to Aub are unrelated to piRNA biogenesis (Barckmann et al. 2015).

Both studies also reported that Aub-bound transcripts are derived from genes involved in diverse functions (Barckmann et al. 2015; Vourekas et al. 2016). Posterior localization of Aub does not seem to be necessary for binding with these transcripts, except for some localized at the posterior pole (Barckmann et al. 2015; Vourekas et al. 2016). A large number of posteriorly localizing transcripts, including *osk*, *germ cell-less* (*gcl*), *polar granule component* (*pgc*), *hsp83*, and *nos*, depend on Aub for their degradation in the bulk of embryos (Barckmann et al. 2015; Vourekas et al. 2016). Many of them are degraded by the deadenylation-dependent pathway because they are stabilized in the embryos with mutations of deadenylation complex components (Rouget et al. 2010; Barckmann et al. 2015). In addition, Simonelig and colleagues found very few secondary piRNAs pairing with Aub-bound transcripts, suggesting that the ping-pong mechanism contribute to the clearance of fewer maternal transcripts (Barckmann et al. 2015). Hence, Aub is likely acting for MZT by its endonucleolytic activity and through the deadenylation-dependent pathway. It is possible that Smaug and the downstream components are involved in degradation of these transcripts, possibly triggered by piRNAs.

Extensive computational analysis by Zissimos and colleagues further elucidated the importance of piRNAs in anchoring transcripts to Aub in embryos. Aub-bound piRNAs exhibit rather weak complementarity to mRNA in a manner reminiscent of miRNA–mRNA interaction (Vourekas et al. 2016). The transcripts localized at the posterior pole are enriched with such piRNA binding sites, suggesting that the Aub-piRNA complex in the pole granules may serve as nucleation sites for the proper localization of these transcripts. It is speculated that the same Aub-piRNA complex could also scan the transposon transcripts to maintain the fitness of species in the next generation.

Overall, piRNAs are important for maintaining mRNAs in Aub-RNA complexes. Aub functions to degrade transcripts of germline determinants in the bulk of cytoplasm, possibly by either endonucleolytic activity of Aub itself or triggering deadenylation, in turn leading to their enrichment in the pole plasm. In addition, Aub-bound piRNAs play an important role in anchoring the transcripts involved in posterior localization and germline development. Further studies are needed to elucidate the molecular mechanism that underlies the spatiotemporal regulation of Aub-piRNA complex/mRNAs interaction.

**Perspectives**

In summary, to understand both oogenesis and spermatogenesis, developmental genetics and cell biology approaches can take advantage of the reliable developmental processes and distinct morphologies for each stage of germ cell differentiation. Formation of both male and female gametes requires the interaction between the germline and the somatic gonadal cells. During this process, the germline identity is protected, while GSC self-renewal, differentiation, and meiotic cell cycle genes are tightly regulated by the sequential changes of the chromatin structure in germ cells (Figure 5). On the other hand, mature gametes carry both genetic and epigenetic information from one generation to the next.

As described in this review, the germline genome must be protected against transposable elements (TE), or transposons, which are DNA sequences that can alter the genetic identity of a cell by changing their position in the genome. Charged with this task is the piRNA pathway, which is directly involved with the silencing of transposons. Both the steady state of TE repression, and the dynamics of the piRNA pathway during germline development are better understood today. Newly introduced transposons initially escape from repression via the piRNA pathway, but germ cells quickly acquire adaptation to new invasion of transposons by producing piRNAs in a single generation (Khurana et al. 2011). Repression of evolutionarily older transposons needs fewer piRNAs, while recent transposon insertions attract a higher number of piRNAs (Kelleher and Barbash 2013). Certainly, the study of piRNA pathway function from a population-wide perspective might shed light on the evolutionary nature and adaptive events in the piRNA population over generations. While studies have
provided a glimpse of piRNA precursor transcription and processing, a proper understanding of events prior to piRNA processing remain enigmatic.

In the future, we can expect a better understanding of the unique features of germ cells, which will greatly facilitate applying them for the treatment of diseases and regenerative medicine. For example, piRNA pathway proteins are reported for transposon repression of embryonic stem cells in mammals (Darricarrere et al. 2013; Marchetto et al. 2013; Peng et al. 2016). Recently, the piRNA pathway proteins have also been shown to support survival and proliferation of cancer cells from flies to human (Janic et al. 2010; Fagegaltier et al. 2016; Ng et al. 2016; Sumiyoshi et al. 2016). In higher vertebrates, PIWIs are implicated in somatic stem cell functions and/or regeneration of the tissues (Rizzo et al. 2014). Based on the conserved nature of the piRNA pathway across species and during development and disease, understanding the relationship between piRNAs and transposons during early development may provide insight into the development of tumors, highlighting the importance of studying noncoding RNA regulation, and in turn, leading to the identification of new therapeutic targets.

However, it remains challenging to study epigenetic regulation at the individual gene in germ cells at particular stages, such as PGCs in embryos and GSCs in adults. A major technical hurdle involves obtaining a sufficient number of homogenous cells to investigate their chromatin structure. However, technological advances have significantly reduced the required cell number for such studies, thus providing unprecedented opportunities to understand germ cell identity and activity. This step will be invaluable for treating diseases associated with defects in germ cell differentiation, such as infertility and germ cell tumors, as well as applying germ cells in regenerative medicine. Also, new imaging techniques, such as live cell imaging and superresolution imaging, in combination with genomic engineering, will allow us to trace distinct molecules, such as mRNAs and proteins, as well as organelles and subcellular structures in order to gain new insights into germ cell differentiation at individual developmental stages (Cheng et al. 2008, 2011; Sheng et al. 2009; Morris and Spradling 2011; Spradling 2011; Lenhart and DiNardo 2015; Shalaby and Buszczak 2017). Furthermore, new advances in genomic analyses, including RNA-seq, ChIP-seq, and Hi-C, are beginning to reveal chromatin structure during germline development in a sequence-specific manner. We anticipate rapid progress in the near future to resolve dynamic epigenetic regulation of germ cell differentiation at
single-cell resolution, in real-time, and at both genomic and specific gene loci in Drosophila.

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