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Epigenetic dynamics during germline development: insights from *Drosophila* and *C. elegans*

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Abstract

Gametogenesis produces the only cell type within a metazoan that contributes both genetic and epigenetic information to the offspring. Extensive epigenetic dynamics are required to express or repress gene expression in a precise spatiotemporal manner. On the other hand, early embryos must be extensively reprogrammed as they begin a new life cycle, involving intergenerational epigenetic inheritance. Seminal work in both *Drosophila* and *C. elegans* has elucidated the role of various regulators of epigenetic inheritance including (1) histones (2) histone modifying enzymes, and (3) small RNA (sRNA)-dependent epigenetic regulation in the maintenance of germline identity. This review highlights recent discoveries of epigenetic regulation during the step-wise changes of transcription and chromatin structure that takes place during germline stem cell self-renewal, maintenance of germline identity, and intergenerational epigenetic inheritance. Findings from these two species provide precedence and opportunity to extend relevant studies to vertebrates.

Introduction

During gametogenesis, extraordinary cellular differentiation processes produce morphologically and functionally distinct gametes, i.e., oocytes and sperm. Gonads in both *Drosophila melanogaster* and *Caenorhabditis elegans* have a linear organization of germ cells with germline stem cells (GSCs) located toward one end and differentiated cells at the other. Such a spatial organization ensures that all stages of oogenesis and spermatogenesis can be visualized (Figure 1). In both systems, GSCs respond to extracellular signals emanated from the niche, which play important roles in maintaining GSC identity, in the absence of which GSCs undergo precocious differentiation (see reviews [1–4]).

In *Drosophila*, both male and female GSCs can undergo asymmetric cell division (ACD) to produce a self-renewed GSC and a cystoblast (CB, in the female) or a gonialblast (GB, in

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the male), which subsequently undergo transit amplification of four mitoses before entering meiosis and terminal differentiation (Figure 1A and B). Differential gene expression has been detected in GSC vs. GB/CB [5–8]. Meanwhile, in *C. elegans* a pool of 150–200 germ cells in the 'progenitor zone' at the distal end of the gonad are maintained by a single cell niche called the distal tip cell (DTC) [9,10]. Enwrapping of GSCs by the DTC functions to extend the range of niche signaling to promote stem cell fate in a large pool of cells [11]. A recent study examined germ cell divisions at the interface between the DTC and the adjacent somatic gonad cell, the Sh1 cell[12]. Live-cell imaging revealed the dividing cells polarize their spindles between the DTC and Sh1 and divide asymmetrically, giving rise to two distinct daughter cells with one daughter remaining anchored to the DTC, , while the other daughter cell becomes enwrapped by the Sh1 cell and is set on a path to differentiation [12](Figure 1C). Two recent reports further investigate the role of the distal somatic gonad architecture and its relationship with the underlying germline stem cell population [13,14]. Differences between these studies and their conclusions highlight the importance for further research in this area, particularly in determining whether the Sh1 cell is necessary or sufficient for germ cell exit from the niche, asymmetric cell division, and germ cell differentiation.

In this review, we focus on the recent discoveries of various epigenetic mechanisms in regulating extensive cellular differentiation during gametogenesis and protecting germline identity during *Drosophila* and *C. elegans* oogenesis and spermatogenesis. We start by discussing recently discovered epigenetic dynamics during the asymmetric GSC divisions in *Drosophila* male and female germlines. We next proceed to an examination of the role epigenetics plays in maintaining germline identity in *Drosophila* and *C. elegans*. Finally, we explore the developing field of intergenerational epigenetic inheritance.

Chromatin dynamics during GSC self-renewal and differentiation in *Drosophila*

In eukaryotic nuclei, DNA is compacted into chromatin, a regular repeated structure in which the nucleosome represents the basic unit. The nucleosome provides information that affects nuclear functions including DNA replication, repair and transcription. This information is conveyed through numerous combinations of histone post-translational modifications (PTMs) and the incorporation of histone variants (For a review on the role of histones in distinct chromatin states see [15]). During ACD of *Drosophila* male GSCs, preexisting (old) histones H3 and H4 are selectively retained in the self-renewing stem cell, whereas newly synthesized H3 and H4 are enriched in the differentiating daughter cell (Figure 2) [16–18]. In *Drosophila* female GSCs, histone asymmetry occurs locally at differentially expressed genes, shown as subdomains of non-overlapping old *versus* new H3 [19]. A three-step model has been proposed wherein old and new histones are first asymmetrically distributed on sister chromatids during DNA replication (Step 1), then differentially segregated during ACD (Step 2), and regulate distinct cellular events in the resulting two daughter cells (Step 3). First, it has been demonstrated that old histones are preferentially recycled to the leading strand, whereas new histones are incorporated by the lagging strand. Moreover, unidirectional fork progression occurs with

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a high incidence in early-stage germ cells. Together with the strand bias between old and new histones, unidirectional fork movement could explain how asymmetric histone incorporation at individual replication forks could generate a more global asymmetry between sister chromatids [18]. Additionally, a stem cell 'mitotic drive' phenomenon has been reported in asymmetrically dividing GSCs in both female [20] and male [21]. A series of asymmetric components in the mitotic machinery coordinate in a sequential manner to ensure preferential attachment and segregation of the epigenetically distinct sister chromatids to define distinct daughter cell fates. Furthermore, these cellular mechanisms are critical for maintaining the stem cell fate [21,22]. Finally, several unique features for old versus new histone-enriched chromosomal regions have been identified, including different nucleosome density, differential chromosomal condensation, and discrete H3 Ser10 phosphorylation. These distinct features likely lead to their differential association with Cdc6, an essential component of the pre-replication complex, and asynchronous entry into the subsequent G1/S phase between the two daughter cells. Disruption of asymmetric histone inheritance using either a microtubule depolymerizing drug or a mutant histone compromise these differences and result in synchronous cell cycle progression between the two daughter cells. Furthermore, stem cell defects are detectable under these conditions, suggesting a connection among histone inheritance, cell cycle progression and cell fate decision or maintenance. Together, these studies reveal a novel 'readout' of asymmetric histone inheritance, which advance our understanding of this phenomenon and may have implications in other stem cells or asymmetrically dividing cells [23].

Studies in *Drosophila* GSC systems demonstrate that asymmetric H3 and H4 distribution could be global (e.g., male GSCs) or local (e.g., female GSCs). It is possible that the epigenetic differences between sister chromatids could be at the genome or chromosome level, or at specific genomic regions. In the male GSCs, it has been shown that sex chromosomes (i.e., X and Y) have a nonrandom segregation pattern while the major autosomes (e.g., 2nd and 3rd) have a random but co-segregation pattern [24]. Recently, both the *cis*-elements (i.e., the rDNA sequences) and the *trans*-factor (i.e., an rDNA binding protein Indra) are demonstrated to be required for this nonrandom segregation of the sex chromosomes. Intriguingly, these biased segregation of sex chromosomes maintain rDNA copy number in the GSCs to antagonize age-dependent loss and deterioration of GSC activity [25].

In addition, in *Drosophila* female GSCs, knockdown of the linker histone H1 leads to stem cell loss and premature differentiation. H1 acts with MOF, the acetyltransferase for H4K16, in an antagonistic manner for proper H4K16ac level to prevent ectopic expression of germline differentiation genes [26]. H1 is also required in the escort cells as compromising H1 in the escort cells leads to accumulation of GSC-like cells and escort cell death [27]. In *Drosophila* male germline, the germ cell-specific histone H1 (dBigH1) is expressed in both GSCs and meiotic spermatocytes. Interestingly, dBigH1 and a germline differentiation factor Bag-of-marbles (Bam) have a reciprocal repression for each other in a stage-specific manner: dBigH1 suppresses Bam in GSCs to prevent their precocious differentiation, then in the mitotic spermatogonial cells Bam represses dBigH1. However, at the transition from mitosis to meiosis, upregulated dBigH1 downregulates Bam again for the proper transition.

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Together, these results demonstrate a highly orchestrated series of events for *Drosophila* spermatogenesis [28].

Chromatin regulators and small RNA pathways maintain germline identity in *Drosophila* and *C. elegans*

Germ cells must maintain expression of germline-specific genes as well as repress genes that would lead to somatic differentiation. Repressive chromatin modifications play an essential role in maintaining germline identity by repressing somatic gene expression in both *Drosophila* and *C. elegans* gonads. The Polycomb group (PcG) components constitute chromatin repressive complexes PRC1 and PRC2. In the *Drosophila* testis, the PRC2 component Enhancer of zeste [E(Z)] [29], the PRC1 component Polycomb [30] and Enhancer of Polycomb [E(Pc)] [31] are all required in the somatic gonadal cyst cells to prevent ectopic expression of a somatic transcription factor, Zfh-1, in the germ cells. These non-cell-autonomous roles of PcG are required to maintain normal germline identity and proper differentiation. In the *Drosophila* ovary, the PcG components have a sequential expression and function to regulate germ cell differentiation. In female GSCs, a Polycomb-like protein (Pcl) is highly expressed and antagonizes the canonical PcG-mediated gene silencing. Contrastingly, in the differentiated nurse cells, Pcl is downregulated with a concomitant upregulation of another PcG component Scm, which cooperates for the PcG-mediated gene silencing. These data indicate that distinct components of the PcG complex are developmentally programmed to regulate oogenesis [32]. Additionally, dSETDB1, a histone methyltransferase for generating the repressive H3K9me3 modification, has been shown to repress male germ cell-specific gene expression in the female germline to maintain the proper sexual identity [33]. Moreover, it has been shown that multiple signaling pathways interplay to regulate female GSC differentiation through a glypican Dlp. Both Hh and Wnt signaling repress *dlp* transcription through the H3K9me3 modification-mediated gene silencing together with a transcription repressor. These activities are essential to counteract BMP signaling to ensure proper differentiation of female GSCs [34].

PRC2 also plays a role in repressing somatic fate and maintaining germline identity in *C. elegans*. In the *C. elegans* germline, H3K27me3 is generated by the conserved PRC2 complex containing the E(z) homolog, MES-2 [35]. Loss of H3K27me2/3 leads to germline degeneration and loss of fertility [36]. Transcript profiles of dissected mutant germlines revealed that H3K27me2/3 and H3K36me2/3, a histone PTM associated with expressed genes, occupy mutually exclusive domains on the autosomes, while H3K27me2/3 is enriched on the X chromosome [37]. Interestingly, loss of MES-4, a H3K36me2/3 methyltransferase, results in the genome-wide redistribution of H3K27me3 to germline genes, resulting in reduced H3K27me3 elsewhere on autosomes and on the X chromosome [37]. Recently, a key transcription factor, LIN-15B, has been identified as a major cause of X misexpression and germline death observed in *mes-4* and *mes-3* (a PcG member) mutants [38]. Analysis of gene expression defects in primordial germ cells (PGCs) lacking Nanos also revealed that upregulation of LIN-15B leads to sterility due to upregulation of X-linked genes [39]. Furthermore, transgenerational sterility occurs over multiple generations in animals with mutations in certain histone-modifying enzymes, including

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the H3K4 methyltransferase, SET-2/COMPASS [40]. Transcriptional profiling over multiple generations in *set-2* mutant germlines revealed that down-regulation of germline genes, and misexpression of somatic transcriptional programs, including the progressive deregulation of LIN-15B, among other transcription factors, leads to loss of germline [41]. Taken together, these findings demonstrate germ cell fate is maintained by multiple protective mechanisms to antagonize germline expression of LIN-15B. The prevention of the germline to undergo somatic differentiation has also been demonstrated in *C. elegans*, which is correlated with the absence of H3K27 methylation [42,43]. Disruption of H3K27 methylation patterns, either by knockdown of PRC2 subunits or through the genome redistribution by knocking down *mes-4*, can induce trans-differentiation of germ cells into neurons or muscle cells following the ectopic expression of either the neuronal transcription factor (TF) CHE-1, or the MyoD orthologue, HLH-1, the muscle TF. Notably, this cell fate conversion depends on the ectopic expression of lineage-specific TFs, since compromising PRC2 activity alone is not sufficient. Additionally, LIN-53, a histone chaperone and component of many chromatin complexes, was also shown to prevent TF-induced germline reprogramming [42]. Thus, chromatin organization is crucial to maintain germline identity and prevent cellular reprogramming. Recently, an RNAi screen showed that knockdown of *mrg-1* results in a permissive condition for the conversion from germ cells to neurons under overexpression of CHE-1 [44]. MRG-1 was found to associate predominantly with genomic loci carrying active histone marks, including H3K36me3, H3K4me3, and H3K9ac, using ChIP-seq analysis. It was recently discovered that upon loss of MRG-1, a histone acetyltransferase, CBP-1, is released from euchromatin and mistargeted to heterochromatin, leading to derepression of silent genes and mis-organization of chromatin [45]. Together, these findings show that MRG-1 protects germ cells by maintaining chromatin integrity and ensuring expression of germline genes.

Lineage specification of the germline from the soma is one of the earliest lineage specifications among metazoan organisms, including *Drosophila* and *C. elegans*. In both flies and worms, the germline is specified through a continuous mode, termed preformation, where the germline is present at all stages and the PGCs inherit the maternally supplied germ plasm. In both organisms, perinuclear ribonucleoprotein (RNP) condensates form germ granules, which are maternally loaded and actively partitioned to the PGCs during early embryonic cleavages [46]. Germ granules are thought to have many roles, including post-transcriptional regulation, germ cell fate determination, transmission of epigenetic information, and distinguishing between self-transcripts and foreign nucleic acid sequences [47,48]. In *Drosophila*, loss of germ granules results in PGC loss in embryos [49]. In *C. elegans,* the absence of germ granules (i.e., P granules) leads to improper expression of somatic genes in the germline and results in sterile adults [50–52]. Consistently, spontaneous trans-differentiation of *C. elegans* germ cells into somatic cell types, including neurons and muscle cells, was frequently detectable when compromising P granule formation and perinuclear localization [52]. Taken together, these results suggest that P granules maintain germline identity and antagonize somatic cell fate. Further studies are required to link P granules and the epigenetic landscape to the repression of somatic cell fate, which is possibly mediated by small RNAs and P-granule-associated argonaute proteins.

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Indeed, several argonaute proteins implicated in sRNA silencing pathways, including CSR-1 and the PIWI-class argonaute PRG-1, as well as proteins involved in the production and maintenance of sRNAs, are found in *C. elegans* germ granules [53]. Similarly, in *Drosophila*, germ granule components include Aubergine (Aub), a Piwi family piRNA-binding protein, Vasa, Nanos, Pumilio, and Tudor, among others [47]. In both fly and worm, maternally deposited piRNAs are important for transposon silencing in the next generation [54–56]. In *C. elegans*, the germline chromatin landscape is shaped by the interplay between two sRNA pathways that localize to germ granules. PRG-1 accumulates in germ granules that overlay the nuclear pores and associates with roughly 15,000 piRNAs (21U sRNAs in *C. elegans*) encoded in the genome [57–59]. Binding of PRG-1 to piRNAs initiates germline silencing of non-self transcripts, including transposons and endogenous genes, which is mediated by the nuclear argonaute, HRDE-1 [59,60]. HRDE-1 is capable of inducing chromatin silencing through the recruitment of several factors, including H3K9 methyltransferases and methylated H3K9 reader HP1, at genes downstream of piRNAs [60–62]. Studies in *Drosophila* have also implicated the PIWI/piRNA complex in heterochromatin formation through recruiting HP1 to silence transposons in the germline [63,64]. On the other hand, CSR-1 binds to widely expressed genes and thought to have an 'activating' role that distinguishes self-genes and protects them from silencing [65]. It was recently reported that the decreased expression of CSR-1 target genes upon loss of *csr-1* correlates with ectopic elevation of silencing chromatin marks including H3K9me3 [66]. One model for CSR-1 and PRG-1 regulation of the germline transcriptome has been referred to as a "genome-wide surveillance" mechanism, which functions to route germline transcripts through the germ granules, where they can be recognized by CSR-1 (memory of self/licensed transcripts) or PRG-1 (memory of silenced, or unlicensed transcripts)[67]. Surprisingly, considering their opposing roles, both *csr-1* and *prg-1* mutants result in a reduction of histone mRNA [68,69]. It was proposed that one function of CSR-1 and PRG-1 is to directly process histone transcripts through the endonucleolytic cleavage of their 3'UTRs [68,70]. It is possible that the reduction in histone levels leads to changed histone PTM density, which has been shown to be critical for the inheritance of heterochromatin, specifically H3K9me3, in fission yeast [71]. Further studies are needed to determine how the change in histone levels in the *csr-1* and *prg-1* mutants influences the chromatin structure in the germline and early embryo in *C. elegans*.

Regulation of chromatin during intergenerational epigenetic inheritance in *C. elegans*

Genetic and molecular studies have identified two major pathways involved in intergenerational epigenetic inheritance, including chromatin architecture and sRNAs. In both *C. elegans* and *Drosophila*, elegant genetic experiments demonstrate that H3K27me3 is intergenerationally inherited in a parent-of-origin manner, suggesting that both sperm and oocytes pass epigenetic information to the early embryo [72,73]. Furthermore, H3K27me2/3 is propagated through embryonic cell divisions by the histone methyl-transferase activity of MES-2. Similar effects were also observed for a heterochromatin PTM, H3K9me2 [73]. Inheritance of H3K27me3-deficient sperm results in derepression of somatic genes in the germline, especially neuronal genes, priming the germ cells to lose their identity and

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adopt a neuronal fate [74]. Notably, *C. elegans* sperm also retain high level of euchromatin-associated histone PTMs, including H3K36me3 and H3K4me3 [75]. Similar to H3K27me3, H3K36me3 is also transmitted across generations through both sperm and oocytes, and is maintained through multiple embryonic cell divisions once inherited [76]. Recent results demonstrate that maternally loaded MES-4 is sufficient for germline development, while the intergenerational inheritance of H3K36me3 was not necessary [38,76].

Recent studies highlight both sRNAs, including piRNAs, and chromatin modifications in the regulation of epigenetic memory that can be inherited across multiple generations. Transgenerational silencing has been observed in response to double-stranded RNA (dsRNA) and for piRNAs [77]. Silencing in response to exogenous dsRNA is called RNAi (RNA interference), while RNAe (RNA-induced epigenetic silencing) is a stable gene silencing pathway triggered by piRNAs produced from endogenous loci. Both RNAi and RNAe can direct transcriptional gene silencing by stalling RNA polymerase II during transcriptional elongation and result in repressive histone PTMs at targeted loci (Figure 3) [78–80]. In *C. elegans*, RNAe is one of the sturdiest forms of epigenetic silencing that can last multiple generations and relies on very similar nuclear machinery as germline nuclear RNAi, including the germline nuclear argonautes HRDE-1, NRDE-2, and NRDE-3 [61,78,81,82] In *C. elegans*, both H3K27me3 and H3K9me3 deposition can be triggered at targeted loci by RNAe and RNAi pathways through HRDE-1, NRDE-2, and NRDE-3 [78,82]. Recently, a chromodomain and kinase-like domain protein, HERI-1, was identified as a negative regulator of transgenerational epigenetic inheritance, which prevents transgenerational effects from persisting indefinitely [83]. HERI-1 is recruited to chromatin in a HRDE-1- and SET-32 (an H3K23me3-writer) dependent manner [83] [84]. Taken together, epigenetic information can be inherited between generations through both RNAi and RNAe pathways, and maintained through stable histone PTMs even after the initiating conditions have changed.

There is growing evidence that epigenetic alterations in gene expression can be initiated by stress in *C. elegans* and *Drosophila*. For example, mild heat stress in the nematode results in a maternally heritable, sRNA-dependent signal that alters gene expression for three to four generations [85]. Heat exposure also causes increased expression of somatically expressed transgenes and repetitive elements, which persists for up to seven generations [86]. This derepression response correlated with the reduction of H3K9me3 along the transgene arrays when comparing progenies from grandparents grown at different temperatures. In *Drosophila*, heat exposure can also lead to disruption of heterochromatin, and can be inherited by the next generation [87] Similar to heat, starvation can also alter expression levels of many endogenous sRNAs involved in nutrition, which persists in fed worms for three generations in an HRDE-1-dependent manner [88]. More studies are needed to understand how different epigenetic mechanisms are initiated through environmental alterations and cooperate to ensure proper transgenerational inheritance.

Concluding remarks and outlook

In this review, we have discussed the inheritance of epigenetic regulators, such as histones, upon asymmetric cell divisions, as well as histone PTMs across generations. We also

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highlight the growing evidence that histone modifications, and sRNAs, are crucial for the epigenetic memory of germline identity and maintenance. As new techniques emerge, such as CRISPR/Cas9-mediated genome editing, in combination with improved temporal and superresolution imaging, they will allow us to trace distinct molecules, such as RNAs and proteins, as well as organelles and subcellular structures in order to gain new insights into germ cell differentiation at individual developmental stages. Furthermore, with recent advances in genomic analysis, including single cell transcriptome analysis, CUT&RUN/CUT&Tag, and Hi-C, we anticipate rapid progress over the next few years to resolve dynamic epigenetic regulation mediated by histone modifications, sRNAs, and other epigenetic regulators of germ cell differentiation at single-cell resolution, in real-time, and at both genomic and specific gene loci in *C. elegans* and *Drosophila*.

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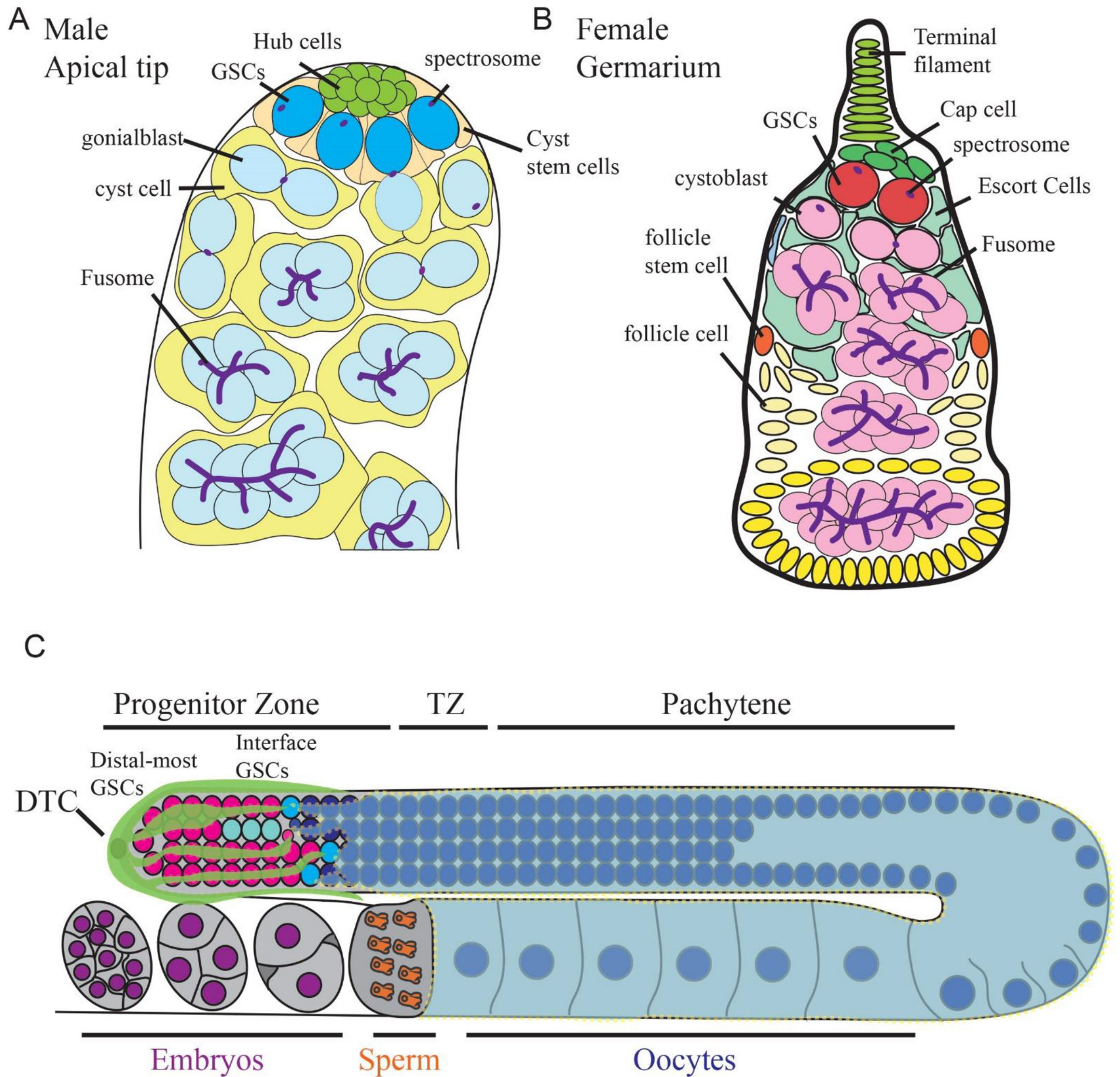

Figure 1. Anatomy of early-stage *Drosophila* male and female gonads, as well as the *C. elegans* hermaphrodite germline.

(A) The *Drosophila* testis is a pair of two coiled tubes, each containing a single stem cell niche at the apical tip. The stem cell niche is called the hub (green nuclei), which is a cluster of 10–12 densely packed somatic cells. The GSCs (dark blue) and the cyst stem cells (CySCs) (light orange) are positioned around the hub. 10–15 GSCs are arranged around the hub with two CySCs enveloping each GSC. GSCs undergo asymmetric divisions to produce a self-renewed daughter GSC and a gonialblast (GB) (yellow). Which subsequently undergo transit amplification of four mitoses before entering meiosis and terminal differentiation. **(B)** A *Drosophila* ovariole consists of a germarium at the apical tip. The germarium is comprised

of the GSC niche and the proliferating germ cells. The GSC niche includes a stack of post-mitotic somatic cells called the terminal filament (light green) and cap cells (dark green) that interact with the GSCs (red). As female GSCs divide asymmetrically they leave the niche and differentiate into cystoblasts (CBs, pink). The CB then undergoes 4 rounds of synchronous mitotic cell divisions with incomplete cytokinesis to create interconnected cystocytes. Once the cyst is surrounded by follicle cells, it becomes an egg chamber, buds off from the germarium, and continues to mature. (*Drosophila* apical tip, and germarium illustrations were adapted from Gleason et al., Genetics, 2018 [89]) **(C)** Illustration of a *C. elegans* hermaphrodite gonad. Germline nuclei are arranged in a spatiotemporal pattern progressing from the Distal tip cell (DTC, green) towards the somatic gonadal sheath cells in cyan (Sheath cell pairs 1–5 represented as superficial cell of each pair shown in cyan, and the Sh1 cell boundary represented by the dotted lines within the progenitor zone). The Sh1 cell and the DTC cell intercalate in the distal gonad. Germ cells nuclei in contact only with the DTC are in magenta, germ cells in DTC/Sh1 compartment are in light blue, and germ cells which contact neither are in turquoise, all other germline nuclei are in dark blue. The transition zone (TZ) contains meiotic leptotene/zygotene stages. Oocytes become fully cellularized by late diakinesis, pass through the spermatheca filled with sperm (orange), and undergo early embryonic development in utero.

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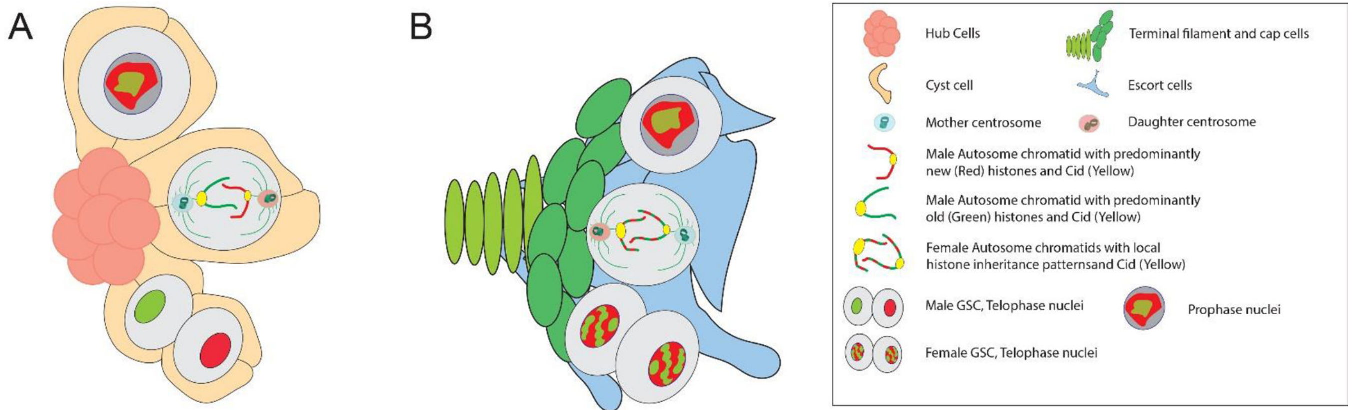

Figure 2. Illustration of the Drosophila male and female germline stem cell (GSC) asymmetric cell division (ACD).

Asymmetric GSC divisions give rise to two daughter cells: a self-renewed GSC that remains in proximity to the niche (male: pink hub cells, female: green terminal filament and cap cells), and a differentiating daughter cell that migrates away from the niche. During this ACD in the male (A); (1) mother and daughter centrosomes are asymmetrically inherited, (2) old histone H3 is predominantly retained in the self-renewed GSC (Green nuclei), whereas newly synthesized H3 is enriched in the differentiating daughter cell (red nuclei). In the female (B); (1) the mother and daughter centrosomes are asymmetrically inherited, (2) local histone inheritance patterns are detected at large-scale domains that are distinctly enriched for old and new histones. In both male and females, the chromatids enriched for Cid (yellow) are inherited by the self-renewing daughter cell.

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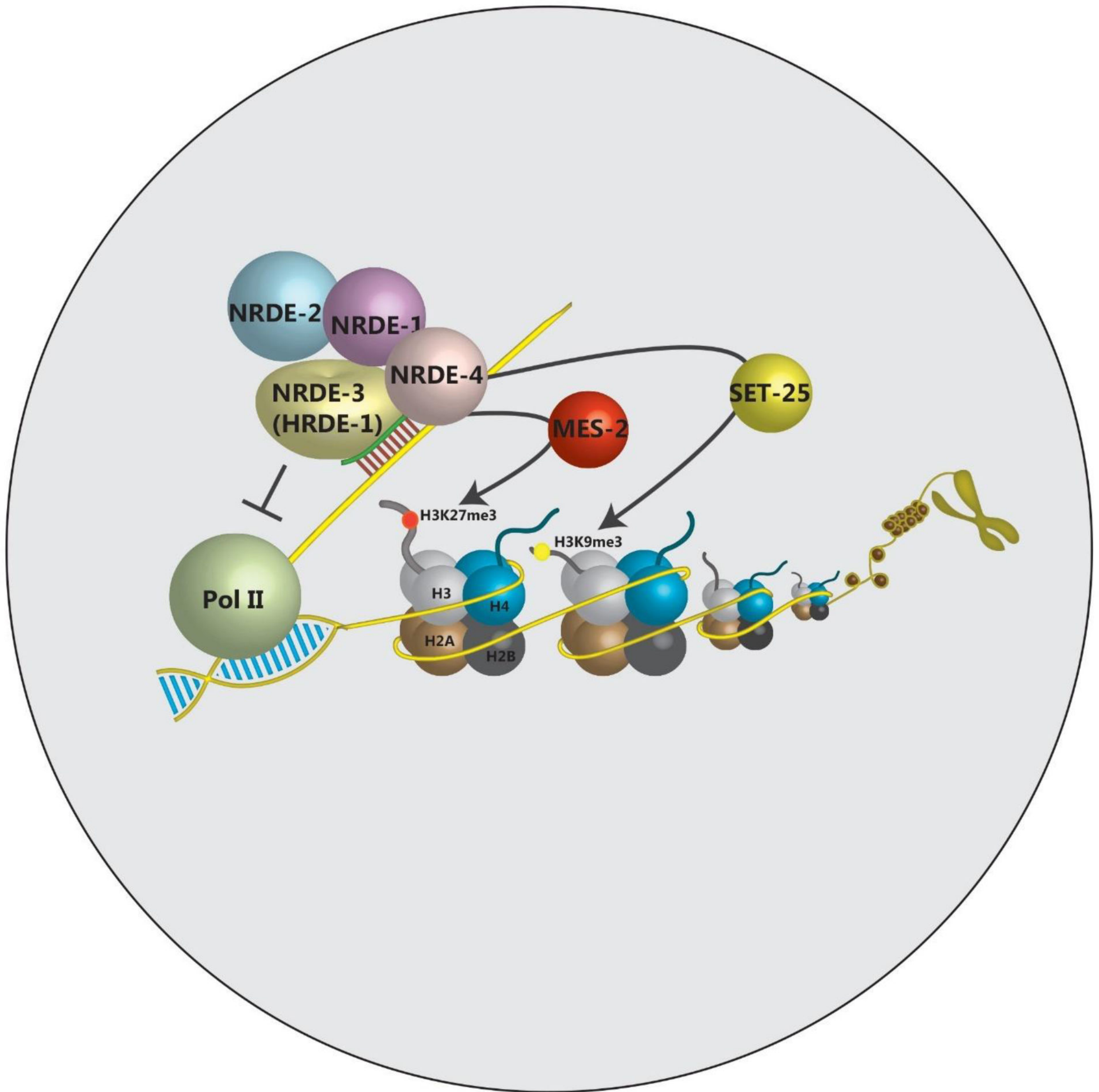

Figure 3. sRNA-directed H3K27me3 and H3K9me3 histone H3 post-translational modifications (PTMs) through the NRDE pathway in *C. elegans*.

HRDE-1 and NRDE-3 use sRNAs as guides to recognize and bind pre-mRNAs with sequence homology to the sRNAs, in the germline and soma, respectively. HRDE-1 then recruits downstream silencing factors, such as the NRDEs and chromatin modifying enzymes to deposit H3K9me3 and H3K27me3. Moreover, NRDE factors directly impair the activity of RNA Pol II.