Chapter 13
Epigenetic Control of Germline Development

Priscilla M. Van Wynsberghe and Eleanor M. Maine

Abstract Dynamic regulation of histone modifications and small noncoding RNAs is observed throughout the development of the *C. elegans* germ line. Histone modifications are differentially regulated in the mitotic vs meiotic germ line, on X chromosomes vs autosomes and on paired chromosomes vs unpaired chromosomes. Small RNAs function in transposon silencing and developmental gene regulation. Histone modifications and small RNAs produced in the germ line can be inherited and impact embryonic development. Disruption of histone-modifying enzymes or small RNA machinery in the germ line can result in sterility due to degeneration of the germ line and/or an inability to produce functional gametes.

Keywords Epigenetic • *C. elegans* • Germ line • Chromatin • MES complex • Histone • Small RNA • siRNA • piRNA • Meiotic silencing

13.1 Introduction

The term epigenetics is commonly used to describe mechanisms that regulate gene expression in a heritable manner without altering the DNA sequence. That said, different writers interpret this rather vague definition more or less broadly when
deciding what mechanisms to classify as epigenetic (see Bird 2007). One point of debate is that some researchers consider heritable to mean a change that persists through cell generations (i.e., passed through mitotic divisions), while other researchers more narrowly define it to mean a change that persists through organismal generations (i.e., passed through meiosis). Two generally accepted mechanisms of epigenetic inheritance are histone modification and DNA modification, both of which may persist during mitotic cell division and during gametogenesis. In addition, gene regulation via noncoding RNA is sometimes described as epigenetic because many noncoding RNAs are heritable. In writing this chapter, we chose to discuss both chromatin modification and small RNA function.

Studies in many organisms demonstrate the importance of epigenetic regulation in development. Examples of epigenetic phenomena include imprinting, X chromosome dosage compensation, and gene silencing. X chromosome dosage compensation mechanisms, for example, utilize chromatin modifications and noncoding RNAs to heritably inactivate one X chromosome in female placental mammals and up-regulate the male X chromosome in *Drosophila* (Arthold et al. 2011; Ilik and Akhtar 2009). Phenomena such as position effect variegation and paramutation exemplify how epigenetic mechanisms can be inappropriately triggered to silence gene expression (Erhard and Hollick 2011; Eissenberg and Reuter 2009). Position effect variegation, for example, arises when a chromosomal rearrangement places what should be an active gene into or near a region of transcriptionally inactive chromatin (heterochromatin).

A dynamic chromatin structure accompanies germline development (Sasaki and Matsui 2008; Feng et al. 2010; Schaner and Kelly 2006). In the very early embryo, the chromatin state of the newly formed germ cell precursors (primordial germ cells, PGCs) is thought to be important for maintaining totipotency and preventing these cells from taking on a somatic fate. Epigenetic changes observed as PGCs proliferate to form the germ line may be important for rapid proliferation and/or for subsequent gametogenesis. During gametogenesis, distinct patterns of chromatin modifications are observed in male vs female germ cells. In this chapter, we describe what is known about the epigenetic regulation of the developing *Caenorhabditis elegans* germ line and compare this process to what occurs in two other model organisms, mouse and *Drosophila*.

### 13.2 Epigenetic Regulation by Histone Modifications

Histone modifications influence many biological processes by altering chromatin structure or the recruitment of nonhistone proteins. These changes ultimately determine the transcription state of the gene and thus a particular biological outcome. Multiple classes of histone modifications have been identified including: acetylation, methylation, phosphorylation, deimination, ubiquitylation, sumoylation, ADP ribosylation, and the non-covalent structural modification proline isomerization (Kouzarides 2007). Considerable effort has been made to describe the distributions of histone marks across the genome, and differential distribution of histone marks
Epigenetic Mechanisms has been observed at both the transcription start sites and internal introns and/or exons of expressed vs non-expressed genes (Barski et al. 2007; Gerstein et al. 2010; Li et al. 2007, Andersson et al. 2009; Kolasinska-Zwierz et al. 2009). Here, we describe the types and sites of histone modifications in the *C. elegans* germline as well as their modes of regulation.

13.2.1 *Descriptions and Sites of Modifications*

Histone methylation is a common type of modification that can cause distinct outcomes on gene expression depending on the extent or location of methylation (see Table 13.1). Methylation of lysine 4 on histone 3 (H3K4me) is associated with transcriptional activation, while methylation of lysine 9 or lysine 27 on histone 3 (H3K9me or H3K27me) is most often associated with transcriptional repression (Kouzarides 2007). In contrast, histone acetylation, another common modification, is associated with transcriptionally active genes (Fig. 13.1) (Kouzarides 2007).

With the advent of chromatin immunoprecipitation coupled deep sequencing (ChIP-seq) and microarray analysis (ChIP-chip) technology, multiple studies have analyzed the sites of specific histone modifications throughout the *C. elegans* genome (Gu and Fire 2010; Liu et al. 2011; Gerstein et al. 2010). As part of the *C. elegans* modENCODE (*Model Organism Encyclopedia Of DNA Elements*) project, Liu et al. (2011) and Gerstein et al. (2010) examined chromatin isolated from the embryo, which contains primarily somatic cells, and L3 larvae, where the germline is substantially smaller than the soma. In contrast, Gu and Fire (2010) examined chromatin isolated from young adults, where germ cells are more abundant but nevertheless comprise fewer than half the cells in the body. In order to analyze germ cells in a focused way, researchers have relied primarily on antibody labeling

---

**Fig. 13.1** Histone and DNA modifications regulate chromatin compaction. Highly simplified drawing depicts nucleosomes, each of which contains DNA (line) wrapped around a histone octamer core (circles). The presence of certain histone modifications and/or DNA methylation promote tighter compaction (lower drawing) and transcriptional repression, whereas the presence of certain histone modifications and the absence of DNA methylation promote a more open configuration allowing transcriptional activation.
experiments (as described below). Overall, global studies found the common activation marks H3K27ac and H3K4me2/3 to be enriched and displaying nearly identical profiles on the promoter regions of highly expressed genes (Liu et al. 2011; Gu and Fire 2010). The common repressive marks H3K27me3 and H3K9me1/2/3 were enriched in transcriptionally silent regions (Liu et al. 2011; Gu and Fire 2010).

On a global scale, the autosomal arms and left arm of the X, regions enriched for repetitive sequences and transposable elements, were enriched for H3K9me marks, while chromosome centers and the right arm of the X, regions enriched for expressed genes, tended to be enriched for H3K4 methylation and H3K27 acetylation marks (Liu et al. 2011; Gu and Fire 2010; Gerstein et al. 2010). Enrichment for H3K9me was even higher in the vicinity of the meiotic pairing center on each chromosome (Liu et al. 2011; Gu and Fire 2010). However, it should be noted that these broad domains do not have sharp boundaries, and the transition from an H3K9 methylation-poor to methylation-rich state, for example, happens gradually over many hundred kilobases (Liu et al. 2011; Gu and Fire 2010). At the level of the individual gene, H3K79me2/3 and H3K36me3 were, respectively, enriched in regions near the transcription start site or throughout the body of highly expressed genes, respectively (Liu et al. 2011).

To balance X-chromosome gene expression in male and hermaphrodite somatic tissues, *C. elegans* uses a process called dosage compensation to reduce gene expression from both hermaphrodite X chromosomes (Meyer 2010). However, dosage compensation is not active in the germ line, and instead other mechanisms regulate X chromosome expression. Overall, germline-expressed genes are underrepresented on the X in both males and hermaphrodites (see below) (Reinke et al. 2000, 2004). Consistent with a low level of X-linked gene expression in the germ line, global analysis of histone modifications found that active marks were more enriched on autosomal genes while repressive marks were more enriched on X chromosome genes (Fig. 13.2) (Liu et al. 2011; Gerstein et al. 2010). In somatic cells, H3K27me1 and H4K20me1, two marks associated with transcriptional activation (Barski et al. 2007),

<table>
<thead>
<tr>
<th>Modification</th>
<th>Transcriptional state of associated chromatin*</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3K4me2/3</td>
<td>Active</td>
</tr>
<tr>
<td>H3K9me1/2/3</td>
<td>Inactive</td>
</tr>
<tr>
<td>H3K9ac</td>
<td>Active</td>
</tr>
<tr>
<td>H3S10phos</td>
<td>Active</td>
</tr>
<tr>
<td>H3K27me1</td>
<td>Active</td>
</tr>
<tr>
<td>H3K27me2/3</td>
<td>Inactive</td>
</tr>
<tr>
<td>H3K27ac</td>
<td>Active</td>
</tr>
<tr>
<td>H3K36me2/3</td>
<td>Active</td>
</tr>
<tr>
<td>H3K79me2/3</td>
<td>Active</td>
</tr>
<tr>
<td>H4K20me1</td>
<td>Active</td>
</tr>
</tbody>
</table>

*Table lists the typical transcriptional state as described by genome-wide studies. See text for further details. Me methyl; ac acetyl; phos phosphate.*

### Table 13.1  Histone modifications discussed in this chapter

<table>
<thead>
<tr>
<th>Modification</th>
<th>Transcriptional state of associated chromatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3K4me2/3</td>
<td>Active</td>
</tr>
<tr>
<td>H3K9me1/2/3</td>
<td>Inactive</td>
</tr>
<tr>
<td>H3K9ac</td>
<td>Active</td>
</tr>
<tr>
<td>H3S10phos</td>
<td>Active</td>
</tr>
<tr>
<td>H3K27me1</td>
<td>Active</td>
</tr>
<tr>
<td>H3K27me2/3</td>
<td>Inactive</td>
</tr>
<tr>
<td>H3K27ac</td>
<td>Active</td>
</tr>
<tr>
<td>H3K36me2/3</td>
<td>Active</td>
</tr>
<tr>
<td>H3K79me2/3</td>
<td>Active</td>
</tr>
<tr>
<td>H4K20me1</td>
<td>Active</td>
</tr>
</tbody>
</table>

*Table lists the typical transcriptional state as described by genome-wide studies. See text for further details. Me methyl; ac acetyl; phos phosphate.*
Epigenetic Mechanisms tend to accumulate at sites where the dosage compensation proteins accumulate (Liu et al. 2011; Gerstein et al. 2010). Moreover, these two marks tend to be more highly enriched on transcribed regions of highly expressed X-linked compared to autosomal genes. The two marks show different patterns of enrichment over developmental time, perhaps reflecting different functions. H4K20me1 marks were

Fig. 13.2 Distribution of histone modifications in the adult *C. elegans* germ line. (a, b) Dissected male and hermaphrodite germ lines labeled with DAPI to visualize DNA. Proliferating germ cells are present at the distal end of the gonad arm (\*); as they exit mitosis and move proximally, germ cells progress through meiosis/gametogenesis in assembly line manner. *Inset in panel A* contains a set of pachytene nuclei from a male germline labeled via indirect immunofluorescence to visualize H3K9me2 (red) and H3K4me2 (green). H3K9me2 is enriched on the X, and H3K4me2 is enriched on the autosomes. DNA is labeled in blue. (c) Table summarizes the differential distribution of certain histone modifications across the developing germ line. Column headings correspond to labeled regions in panels (a) and (b).

tend to accumulate at sites where the dosage compensation proteins accumulate (Liu et al. 2011; Gerstein et al. 2010). Moreover, these two marks tend to be more highly enriched on transcribed regions of highly expressed X-linked compared to autosomal genes. The two marks show different patterns of enrichment over developmental time, perhaps reflecting different functions. H4K20me1 marks were
particularly enriched in (L3) larvae where they were also present on silent genes, while H3K27me1 marks were strongly enriched on highly expressed X-linked genes in embryos. It was suggested that embryonic H3K27me1 marks may be remnants of germline X chromosome regulation that persist into embryogenesis, while H4K20 methylation may be linked to the somatic dosage compensation process that is fully established by L3 stage (Liu et al. 2011).

In general, genes specific to the soma or germline display intermediate levels of active and repressive histone marks compared to ubiquitously expressed genes and silent genes, which respectively show high levels of active or repressive marks (Liu et al. 2011). One exception to this pattern is the distribution of H3K27me1, which was more enriched on soma- and germline-specific genes than on ubiquitously expressed genes (Liu et al. 2011). This distribution suggests a role for H3K27 methylation in tissue-specific gene expression (Liu et al. 2011).

Examination of histone marks specifically in the germline via indirect immunofluorescence reveals the presence of many marks of active chromatin, e.g., H3K4 methylation and H3K9 acetylation, on the autosomes and the near absence of those marks on the X in all or most germ cells (Kelly et al. 2002; Fong et al. 2002). Conversely, marks associated with gene silencing are observed on both autosomes and X chromosomes, although certain marks are enriched on hermaphrodite and male X chromosomes or specifically on the male X (Kelly et al. 2002; Bender et al. 2004). This general pattern correlates well with microarray data indicating expression of few X-linked genes in the germ line (Reinke et al. 2000, 2004).

13.2.2 Mechanisms of Regulation

Multiple proteins and protein complexes modify histones. Addition or elimination of a histone mark can dramatically alter the ability of effector proteins to interact with a particular histone residue causing a change in the transcriptional state of a gene. Below we discuss some of the main protein complexes and histone methyltransferases (HMTases) responsible for histone modification in the C. elegans germ line.

13.2.2.1 MES Proteins and H3K27 Methylation

During germ cell mitosis and early meiosis both X chromosomes in hermaphrodites and the single X chromosome in males are silenced by histone modification in the germline (Kelly et al. 2002). The MES complex, composed of the Polycomb group chromatin repressors MES-2 and MES-6 as well as the MES-3 protein, is responsible for this silencing (Bender et al. 2004). Together these MES proteins cause silencing via H3K27me2/me3 in the adult germline and early embryos (Bender et al. 2004). H3K27me3 marks are concentrated on the X chromosome, and loss of MES protein function causes maternal-effect sterility due to germ cell underproliferation and death (Bender et al. 2004; Capowski et al. 1991; Garvin
et al. 1998). The SET domain of MES-2 is crucial for the HMTase activity of the MES complex (Bender et al. 2004). Together, the MES-2/-3/-6 complex functions as the C. elegans PRC2 (Polycomb repressive complex 2). Parenthetically, we note that C. elegans apparently lacks a PRC1.

13.2.2.2 MES Proteins and H3K36 Methylation

Another SET domain protein, MES-4, also has HMTase activity that it uses to di- and tri-methylate lysine 36 on histone 3 (H3K36me2, H3K36me3) in mitotic and early meiotic germline nuclei and early embryos (Bender et al. 2006; Furuhashi et al. 2010). In the embryonic soma, H3K36 methylation also depends on activity of the SET domain protein, MET-1 (ortholog of yeast Set2) (Furuhashi et al. 2010). Genome-wide analysis of histone modifications in a variety of species has determined that H3K36 methyl marks tend to be enriched in the body of expressed genes (Li et al. 2007; Shilatifard 2008). In the C. elegans germ line, H3K36 methylation is enriched on autosomes and at the left end of the X chromosome, and very low elsewhere on the X chromosome, consistent with autosomal linkage of most germline-expressed genes (Bender et al. 2006). In the C. elegans germ line, H3K36 methylation is enriched on autosomes and at the left end of the X chromosome, and very low elsewhere on the X chromosome, consistent with autosomal linkage of most germline-expressed genes (Bender et al. 2006). Germline MES-4 protein is concentrated at the sites of H3K36me accumulation, as expected for a protein with a direct role in depositing the mark (Bender et al. 2006). This pattern of MES-4 activity is in striking contrast the MES-2/3/6 complex, which is active across the X chromosome (Bender et al. 2006). Intriguingly, exclusion of MES-4 activity from (most of) the X chromosome depends on the MES-2/3/6 complex since MES-2/3/6 mutants contain both MES-4 and H3K36me2/3 marks across the X chromosome (Bender et al. 2006). Moreover, despite the main presence of MES-4 on autosomes, MES-4 activity is important for silencing X-linked genes in the germline, and mes-4 mutants exhibit a maternal-effect sterile phenotype similar to that of mes-2, mes-3, and mes-6 mutants (Bender et al. 2006; Capowski et al. 1991). Interestingly, the memory of genes last expressed in the parental germline, and marked by H3K36me, is transferred from parent to offspring by MES-4 activity (Rechtsteiner et al. 2010; Furuhashi et al. 2010). This epigenetic inheritance is crucial for germline viability (Rechtsteiner et al. 2010).

13.2.2.3 The MLL Complex and H3K4 Methylation

As described above, H3K4 methyl marks are commonly associated with actively transcribed genes of multiple species (Kouzarides 2007). The MLL complex is responsible for H3K4 methylation in C. elegans and many other species. The canonical MLL complex contains four components: MLL, a SET domain protein with HMTase activity; WDR-5; ASH-2/Ash2L; and RBBP-5. In C. elegans, MLL complexes containing different sets of components are responsible for H3K4 di- and tri-methylation in the embryo and in much of the germ line. In the early embryo, the MLL complex components WDR-5.1, RBBP-5, and ASH-2 are essential for H3K4
methylation (Li and Kelly 2011; Xiao et al. 2011). Though wdr-5.1 and rbbp-5 mutants do not exhibit strong phenotypes, when grown at 25°C successive generations have progressively smaller brood sizes and exhibit a variety of germline developmental defects (Li and Kelly 2011; Xiao et al. 2011). The HMTase responsible for H3K4 trimethylation in the embryo is SET-2, while the HMTase responsible for H3K4 dimethylation is unknown (Li and Kelly 2011; Xiao et al. 2011). Though little RNA polymerase II transcription occurs in early dividing blastomeres and germline precursors of C. elegans embryos, high H3K4me2 levels, but not H3K4me3 levels, are maintained throughout multiple cell divisions by the MLL complex (Li and Kelly 2011).

In adult germ cells, H3K4me2 and H3K4me3 marks are present in high abundance across all autosomes, but not on the X chromosome (Kelly et al. 2002; Reuben and Lin 2002). The presence of H3K4me3 marks in the germline stem cells (GSCs) depends on SET-2, WDR-5.1, and RBBP-5 (Li and Kelly 2011; Xiao et al. 2011). H3K4me2 marks in the mitotic germ line clearly depend on WDR-5.1 and RBBP-5 activity; however, there is debate about the importance of SET-2 activity in deposition of these marks. In the absence of SET-2 activity, Li and Kelly (2011) observed moderately reduced H3K4me2 levels while Xiao et al. (2011) observed virtually no H3K4me2 signal. Interestingly, maintenance of H3K4 methylation in the GSCs is independent of active transcription (Li and Kelly 2011). In contrast to the GSCs, maintenance of H3K4 methylation in meiotic germ cells is partially independent of SET-2, WDR-5.1, RBBP-5, and ASH-2 activity (Li and Kelly 2011; Xiao et al. 2011). Taken together, these results suggest that MLL complexes containing WDR-5.1 and RBBP-5 are required for H3K4 di- and tri-methylation in early embryos and GSCs, while other proteins are required for H3K4 methylation during meiosis (Li and Kelly 2011; Xiao et al. 2011).

13.2.2.4 SET Domain Proteins Implicated in H3K9 Methylation

As previously described, H3K9 methylation commonly correlates with heterochromatin and silenced genes (Kouzarides 2007). However, despite only a single methyl difference between H3K9me2 and H3K9me3, in C. elegans these two marks exhibit distinct localization patterns, functions, and require different HMTases (Bessler et al. 2010). H3K9me2 is present in a gradient pattern throughout the adult meiotic germline with low levels found in early pachytene and progressively higher levels found throughout late pachytene and diplotene stages (Kelly et al. 2002; Bessler et al. 2010). The H3K9me2 mark is highly enriched on the unpaired X chromosome in XO males and in him-8 mutant hermaphrodites, as well as other unpaired regions such as free chromosomal duplications and extrachromosomal arrays (Fig. 13.3) (Kelly et al. 2002; Bean et al. 2004). The HMTase MET-2 is essential for germline H3K9 dimethylation in both males and hermaphrodites (Bessler et al. 2010; Andersen and Horvitz 2007). MET-2 is homologous to the SETDB1 family of histone methyltransferases that have been shown to methylate lysine 9 of histone 3 (Schultz et al. 2002).
**Fig. 13.3** Accumulation of H3K9me2 on unpaired homologs in the hermaphrodite germ line. Pachytene nuclei from a dissected *him-8* hermaphrodite germ line with histone modifications labeled as indicated. HIM-8 protein (not shown) associates with the X chromosome pairing center and promotes pairing/synapsis of the homologous chromosomes. In the *him-8* mutant, X chromosomes typically fail to pair or synapse. (a) DNA stained with DAPI. (b) H3K9ac marks are enriched on autosomes and not detected on the X chromosomes (*arrowheads*), as also observed in wild-type hermaphrodites. Two X homologs are visually distinct from each other in many nuclei, while in other nuclei only one X is visible in this focal plane. (c) The two unpaired X chromosomes are enriched for H3K9me2 marks. (d) Merged H3K9ac and H3K9me2 images.
Although H3K9 methylation marks generally correlate with heterochromatin and reduced transcription, this correlation is not as strong as for H3K27 methylation (e.g., see Lienert et al. 2011). Nonetheless, evidence suggests the accumulation of H3K9me2 on the single X may have a transcriptional repressive effect. During spermatogenesis, essentially no X-linked genes are transcribed while, during oogenesis in XX animals, a number of X-linked genes are transcribed in late pachytene/diplo-tene stage (as monitored by in situ hybridization). Interestingly, in XO hermaphrodites or females (produced as a result of her-1 or fem-3 mutations, respectively), the single X accumulates H3K9me2 and the burst of oogenesis-specific X-linked transcription is not observed (Bean et al. 2004; Jaramillo-Lambert and Engebrecht 2010). Hence, enrichment for H3K9me2 on the single X correlates with transcriptional repression.

In contrast to H3K9me2 marks, H3K9me3 marks are present on all chromosomes in all germ cells throughout the C. elegans gonad (Bessler et al. 2010). The H3K9me3 mark is also enriched on high-copy transgene arrays present in germ cell nuclei (Bessler et al. 2010). The HMTase MES-2, but not MET-2, is required for germline accumulation of H3K9me3 in conjunction with at least one additional, unidentified enzyme (Bessler et al. 2010). These results suggest that the same MES complex required for H3K27 methylation, described above, may also be needed for acquiring or maintaining the H3K9me3 mark (Bessler et al. 2010).

### 13.2.2.5 The Small RNA Pathway and H3K9 Methylation

In addition to MET-2, activity of the RNA-dependent RNA polymerase (RdRP) EGO-1 is required for H3K9me2 enrichment on unpaired chromosomes (Maine et al. 2005). Male worms mutant for the Argonaute protein CSR-1, the Tudor domain protein EKL-1, or the DEAH/D-box helicase DRH-3 also exhibit reduced H3K9me2 enrichment on unsynapsed chromosomes and increased H3K9me2 accumulation on synapsed autosomes (She et al. 2009). Mutation of ego-1, csr-1, ekl-1, or drh-3 causes various germline defects, and double mutants display even stronger phenotypes in both hermaphrodites and male worms (She et al. 2009). Because of the roles that EGO-1, CSR-1, DRH-3, and EKL-1 play in small RNA production and function (Gu et al. 2009; Claycomb et al. 2009), these results suggest that H3K9me2 enrichment on unpaired X chromosomes may be driven by small RNAs (She et al. 2009). It is unclear at present whether the small RNA pathway is involved in the initiation and/or maintenance of H3K9me2 marks (see Future Directions, below).

### 13.2.3 Erasure of Histone Modifications

The removal of specific histone modifications is just as important as their addition for eliciting the appropriate gene expression response. However, until recently many histone modifications, including methylation, were presumed irreversible. RBR-2,
the *C. elegans* homolog of the Jumonji C domain-containing JARID1 protein, specifically demethylates H3K4me3 and H3K4me2 in vitro (Christensen et al. 2007). In addition, *rbr-2* mutants displayed increased H3K4me3 levels at all developmental stages and vulval defects, suggesting that RBR-2 regulates vulval development through demethylation of H3K4me3 (Christensen et al. 2007).

H3K4 dimethylation has been proposed to act as an epigenetic memory mark of transcriptional activity. This would allow stable transmission of gene expression patterns in developing somatic cells, but could cause inappropriate expression in the germline. The *C. elegans* homolog of the H3K4me2 demethylase, LSD1/KDM1, is SPR-5. Mutants for *spr-5* exhibit phenotypes, an egg-laying defect and reduced brood size, which become progressively worse over multiple generations (Katz et al. 2009). H3K4me2 levels also increase after many generations, and most spermatogenesis-expressed genes are misregulated in *spr-5* mutants (Katz et al. 2009). These results suggest that SPR-5 normally demethylates H3K4me2 to prevent transmission of this mark to successive generations and thus inappropriate overexpression of spermatogenesis-expressed genes and sterility (Katz et al. 2009) (see below).

Histone modifications appear to be removed in germ cell precursors and reestablished as the germline develops. The *C. elegans* germline precursor cell, P4, forms at the fourth embryonic cleavage division, and later divides once to form two primordial germ cells, Z2 and Z3 (see Wang and Seydoux 2012, Chap. 2). The levels of many histone modifications associated with active chromatin, e.g., H3K4 methylation and H4K8 acetylation, are severely reduced (or masked) in Z2 and Z3 such that they cannot be detected by indirect immunofluorescence (Schaner et al. 2003).

### 13.2.4 Transgenerational Maintenance of Germline Viability

Maintenance of germline size and viability over succeeding generations is critical for continuation of the species. In *C. elegans*, progressive germline loss (termed a mortal [Mrt] germ line defect) occurs over succeeding generations in animals with mutations in many DNA damage response and repair proteins (Ahmed and Hodgkin 2000; Meier et al. 2009). In some cases, e.g., *mrt-1* and *mrt-2*, telomere shortening occurs suggesting that the Mrt phenotype is triggered by the loss of genome integrity (Ahmed and Hodgkin 2000; Meier et al. 2009). Interestingly, an Mrt germline is also associated with mutations in certain histone-modifying enzymes, e.g., the demethylase SPR-5/LSD1, the HMTase MET-2, and components of the MLL complex described above (Bessler et al. 2010; Katz et al. 2009; Li and Kelly 2011; Xiao et al. 2011). In these mutants, there is no evidence of telomere shortening or other chromosomal abnormalities, e.g., chromosome segregation defects. Therefore, inappropriate gene expression levels, resulting from incorrect patterns of H3K4me2 and H3K9me2 marks may be responsible for the Mrt phenotype. The Mrt phenotype has not been described in other study organisms, e.g., *Drosophila* and mouse, perhaps because of their longer generation times. Nonetheless, it has...
provided an opportunity to identify mechanisms essential for long-term fertility and highlights the critical importance of histone regulation in the establishment of the germline.

### 13.3 Epigenetic Regulation by Small RNA-Mediated Silencing

Different classes of small interfering RNAs (siRNAs) are commonly used by RNAi-like pathways in *C. elegans* to guide sequence-specific regulation of gene silencing and chromatin structure (see Table 13.2). The siRNA molecule provides specificity by interacting with an Argonaute (AGO) protein and targeting it to specific RNAs based on sequence complementarity (Ketting 2011). Classes of siRNAs differ in their source, structural features like size and 5’ or 3’ modifications, mechanism of biogenesis, and means of function. They also differ in their associated AGO. Exogenous siRNAs (exo-siRNAs) are ~22 nt siRNAs made from processed exogenous dsRNAs, while endogenous siRNAs (endo-siRNAs) are mainly formed from short RdRP transcripts. RdRP products are produced as part of the response to exogenous dsRNA (exogenous RNAi) and as part of endogenous gene-regulatory mechanisms. Endo-siRNAs in *C. elegans* are mainly classified as 22G-RNAs or 26G-RNAs (Han et al. 2009). 22G-RNAs are ~22 nt in length and have a triphosphorylated 5’G, while 26G-RNAs are ~26 nt in length and have a monophosphorylated 5’G (Han et al. 2009). These two classes of siRNAs are produced by related, but distinct mechanisms and function together with different Argonuate proteins. *C. elegans* produce another class of small RNA, 21U-RNA, which function together with associated Argonaute proteins to silence certain transposons. 21U-RNAs are 21 nucleotides in length and contain a 5’ U (Ruby et al. 2006). 21U-RNAs are analogous to piRNAs described in other organisms (Castaneda et al. 2011) and, like piRNAs, their mechanism of biogenesis appears to be very distinct from that of other siRNAs. In this section we discuss the role of endo-siRNAs, 21U-RNAs, and their associated Argonaute proteins in transposon silencing and germline development.

<table>
<thead>
<tr>
<th>Type</th>
<th>Origin</th>
<th>Associated argonaute</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>22G-RNAs</td>
<td>EGO-1/RRF-1 RdRP activity</td>
<td>WAGOs</td>
<td>Transposon, pseudogene, aberrant RNA silencing</td>
</tr>
<tr>
<td>22G-RNAs</td>
<td>EGO-1 RdRP activity</td>
<td>CSR-1</td>
<td>MSUC; embryonic chromosome segregation</td>
</tr>
<tr>
<td>26G-RNAs, class I</td>
<td>RRF-3 RdRP and DCR-1 activity</td>
<td>T22B3.2, ZK757.3</td>
<td>Translational repression (sperm)</td>
</tr>
<tr>
<td>26G-RNAs, class II</td>
<td>RRF-3 RdRP and DCR-1 activity</td>
<td>ERGO-1, other?</td>
<td>Translational repression (oocytes, embryos)</td>
</tr>
<tr>
<td>21U-RNAs (piRNAs)</td>
<td>Genome-encoded; biogenesis unknown</td>
<td>PRG-1, -2</td>
<td>Transposon silencing</td>
</tr>
</tbody>
</table>

See text for further details.
13.3.1 Role of 22G-RNAs in Transposon Silencing

22G-RNAs can associate with two distinct AGOs: WAGO-1 or CSR-1 (Gu et al. 2009; Claycomb et al. 2009; Maniar and Fire 2011). In addition to WAGO-1, *C. elegans* contains an additional 11 WAGO proteins (members of the worm-specific Argonaute clade) that also can associate with 22G-RNAs. The WAGO-dependent 22G-RNAs are essential for silencing their target genes, which include transposable elements, pseudogenes, and aberrant transcripts (Gu et al. 2009). Most 22G-RNAs are expressed in the germline and many are maternally inherited. WAGO-1 is also expressed in the germline where it localizes to perinuclear foci called P granules (Gu et al. 2009). P granules are ribonucleoprotein particles located on the cytoplasmic side of nuclear pores and enriched in polyadenylated mRNAs (Updike and Strome 2010). WAGO-1 mutants contain reduced germline 22G-RNAs levels, and worms mutant for all WAGO proteins express no detectable germline 22G-RNAs, suggesting that WAGO-1 is essential for 22G-RNA production and function (Gu et al. 2009). Nearly identical populations of germline 22G-RNAs were also depleted in *mut-16, mut-7* or *rde-3*, but not *rde-4* mutants, suggesting that MUT-16, MUT-7 and RDE-3 also function in the 22G-RNA silencing pathway (Gu et al. 2009; Zhang et al. 2011). All transposon classes were depleted of 22G-RNAs in *rde-3, mut-7* or WAGO mutants, and these 22G-RNAs apparently associate with WAGO-1 as they are recovered in anti-WAGO-1 co-immunoprecipitation experiments (Gu et al. 2009). Consequently, protein complexes composed of WAGO, RDE-3, and MUT-7 use 22G-RNAs to guide transposon silencing in the germline (Gu et al. 2009).

Levels of WAGO-associated 22G-RNAs are also reduced in *rrf-1 ego-1* double mutant worms, but not single *rrf-1* or *ego-1* mutants (Gu et al. 2009). Thus, these RdRPs likely function redundantly in the germline to produce the class of 22G-RNAs that associates with WAGOs (Gu et al. 2009). Both RRF-1 and EGO-1 can physically interact with the Dicer-related helicase (DRH-3), and germline 22G-RNAs are absent in *drh-3* mutant worms (Gu et al. 2009). Consistent with a germline function, *drh-3* mutants exhibit a variety of phenotypes including sterility, embryonic lethality, and high incidence of males (Nakamura et al. 2007; She et al. 2009; Claycomb et al. 2009). DRH-3 also physically interacts with the Tudor-domain protein EKL-1 (Gu et al. 2009), and *ekl-1* mutants also exhibit phenotypes similar to *drh-3* mutants (She et al. 2009; Claycomb et al. 2009). Thus, DRH-3, EGO-1, and EKL-1 likely interact to form a core RdRP complex essential for 22G-RNA biogenesis, while WAGO, RDE-3 and MUT-7 participate with a subset of these 22G-RNAs in the germline to mediate transposon silencing (Gu et al. 2009).

13.3.2 Role of 22G-RNAs in Germline Development

As discussed above, 22G-RNAs can associate with two distinct types of AGO protein to mediate different functions regulating development. 22G-RNAs associated
with the AGO CSR-1 are expressed in the germline and target germline-expressed protein-coding genes, not transposons or pseudogenes (Claycomb et al. 2009, Maniar and Fire 2011). Like WAGO-1, CSR-1, DRH-3, EGO-1, and EKL-1 all colocalize with P granules, which are normally located on the cytoplasmic side of nuclear pores and are enriched in polyadenylated mRNAs (Gu et al. 2009; Claycomb et al. 2009, E. Maine and X. Xu, unpublished data). However, though WAGO-1 has no effect on the intracellular position of P granules, CSR-1, EGO-1, DRH-3, and EKL-1 are important for perinuclear localization of P granules (Vought et al. 2005; Claycomb et al. 2009; Updike and Strome 2009). Further evidence that EGO-1, DRH-3, EKL-1, and CSR-1 function in a common pathway is provided by genetic analysis of siRNA level. ego-1, drh-3, and ekl-1 mutant worms have reduced levels of CSR-1-associated 22G-RNAs (Claycomb et al. 2009; Maniar and Fire 2011). Accordingly, these mutants have phenotypes similar to csr-1 mutants (Claycomb et al. 2009; She et al. 2009; Rocheleau et al. 2008).

There is debate in the literature about the extent to which interaction of CSR-1-associated 22G-RNAs with their antisense target sequences causes target mRNA degradation (Claycomb et al. 2009, Maniar and Fire 2011). Claycomb et al. (2009) observe little change in target mRNA levels and instead posit that CSR-1-associated 22G-RNAs interact with targets to mediate proper organization of the holocentric chromosomes of C. elegans during metaphase (Claycomb et al. 2009). In the absence of csr-1, worms exhibit defects in chromosome segregation that cause various abnormalities including aberrant chromosome numbers, a high incidence of males (him) phenotype, and ultimately reduced fertility (Claycomb et al. 2009). In contrast, Maniar and Fire (2011) observe an increase in the level of most mRNAs targeted by EGO-1-dependent 22G-RNAs and CSR-1, and they hypothesize that EGO-1 activity is critical for negative regulation of developmentally important genes in the germ line.

To limit the accumulation of CSR-1-associated 22G-RNAs and thus inappropriate gene silencing and chromosome segregation defects, the nucleotidyl transferase CDE-1 localizes to mitotic chromosomes in an EGO-1- and CSR-1-dependent manner where it uridylates these RNAs at the 3’ end triggering their degradation (Claycomb et al. 2009; van Wolfswinkel et al. 2009). CDE-1 physically associates with EGO-1, and its activity may be critical for targeting specific EGO-1 products to CSR-1 (Van Wolfswinkel et al. 2009). It is unclear what mechanism targets CDE-1 activity to a particular subset of EGO-1 products. Nonetheless, multiple proteins function with CSR-1 and CSR-1-associated 22G-RNAs to mediate proper chromosome segregation (Claycomb et al. 2009).

### 13.3.3 Role of 26G-RNAs in Germline Development

26G-RNAs are enriched in the germline of C. elegans where they regulate gene expression of mature mRNAs during spermatogenesis (Han et al. 2009). Moreover, maternally inherited 26G-RNAs regulate gene expression in the zygote (Han et al. 2009).
There are two subclasses of 26G-RNAs whose pattern of expression and associated Argonautes differ (Han et al. 2009). Class I 26G-RNA expression in sperm coincides with spermatogenesis in the L4 and young adult stages. Class II 26G-RNAs are expressed in oocytes and embryos. Both types of 26G-RNAs are perfectly complementary to their target genes.

26G-RNAs are produced via a different mechanism than 22G-RNAs. Their expression depends on the RdRP RRF-3, the exonuclease ERI-1, DCR-1/Dicer, and the dsRNA-binding protein RDE-4 (Han et al. 2009; Vasale et al. 2010). Together, this biogenesis pathway is referred to as the ERI (enhanced RNAi) pathway because exogenous RNAi is enhanced when the ERI pathway is disabled (e.g., Simmer et al. 2002). Consistent with a role for some 26G-RNAs in spermatogenesis, both rrf-3 and eri-1 single mutant worms are temperature-sensitive sterile due to spermatogenesis defects (Gent et al. 2009; Simmer et al. 2002; Kennedy et al. 2004). Class I 26G-RNAs associate with AGOs T22B3.2 or ZK757.3 that are enriched during spermatogenesis, while class II 26G-RNAs use the AGO ERGO-1 (Han et al. 2009; Conine et al. 2010; Vasale et al. 2010).

The relationship between 26G- and 22G- RNAs is complex. Many more 22G-RNA species have been described than 26G-RNA species, and distinct RdRPs are linked to production of 22G- vs 26G- RNAs. However, 26G-RNAs appear to function in a two-step mechanism that also involves 22G-RNAs. In the soma, certain mRNAs are targeted by both 26G- and 22G-RNAs, and activity of the 26G-RNA machinery promotes accumulation of this particular subset of 22G-RNAs (Gent et al. 2010). Hence, production of these particular 26G- and 22G- RNAs is coordinated. A similar relationship is observed in the germ line between certain 26G- and 22G-RNAs (Vasale et al. 2010). These results are consistent with a coordinated mechanism in the germ line whereby certain mRNAs are targeted first by 26G-RNA/ERGO-1 activity and later by 22G-RNA/WAGO activity (Vasale et al. 2010).

### 13.3.4 Role of 21U RNAs in Transposon Silencing and Development

Members of the Piwi subfamily of Argonaute proteins function in germline development and transposon silencing in diverse animals. In most systems, Piwi proteins interact with 24–30 nucleotide piRNAs (Castaneda et al. 2011). In *C. elegans*, a different class of small RNA, initially termed the 21U-RNA, physically interacts with the Piwi protein, PRG-1 (Wang and Reinke 2008; Batista et al. 2008). The vast majority of 21U-RNA/piRNA sequences are present in clusters located in intergenic or intronic regions on chromosome IV (Ruby et al. 2006; Batista et al. 2008; Kato et al. 2009). Although biogenesis of 21U-RNAs is not clear, it appears to require substantially different machinery than does biogenesis of miRNAs and siRNAs (Batista et al. 2008; Das et al. 2008). 21U-RNAs are largely absent in *prg-1* mutants, suggesting that they are stabilized by PRG-1 activity (Wang and Reinke 2008; Batista et al. 2008). PRG-1 and PRG-2, an Argonaute sharing ~90% amino acid
sequence identity with PRG-1, are implicated in transposon silencing: excision rates of Tc3 elements are elevated approximately 100-fold in prg-1 prg-2 double mutants (Das et al. 2008). Evidence suggests that the 21U-RNA pathway acts upstream of MUT-7 (Das et al. 2008), hence this pathway may feed into the WAGO pathway described above.

PRG-1 activity is required for fertility at elevated temperatures, although the loss of prg-1 function partially impairs germline development at a range of culture temperatures (Batista et al. 2008; Wang and Reinke 2008). Mutations in prg-1 are associated with many germline defects, particularly in mitotic proliferation (Batista et al. 2008) and spermatogenesis (Wang and Reinke 2008). PRG-1 associates with P granules, although there is debate about whether this occurs strictly in the spermatogenic germline (Wang and Reinke 2008) or also in the oogenic germline (Batista et al. 2008). In prg-1 mutants, the levels of many spermatogenesis-enriched mRNAs are reduced while the levels of other germline-enriched mRNAs are not substantially changed (Wang and Reinke 2008; Batista et al. 2008).

13.4 Epigenetic Regulation of Germline Development in Other Animals

Epigenetic regulation during germline development in other animals shares some broad similarities with C. elegans, including the importance of histone modifications and small RNAs and sex-specific reorganization of chromatin structure during gametogenesis. A major mechanistic difference in many organisms is the use of DNA methylation, in addition to histone modification, to limit transcription. Here, we discuss the general features of epigenetic regulation in the developing germ lines of two common study organisms, mouse and Drosophila.

13.4.1 Mechanisms of Epigenetic Regulation in the Murine Germ Line

Chromatin regulation in the mouse germ line involves extensive DNA methylation in addition to histone modifications (Feng et al. 2010; Zamudio et al. 2008, 2011; Sasaki and Matsui 2008). Methylation occurs at cytosine residues via one of the three different mechanisms that are active in different DNA sequence contexts (Feng et al. 2010). In mammals, methylation at CG sites is maintained by a DNA methyltransferase called DNMT1 in conjunction with a co-factor, UHRF1. At some sites, additional DNA methyltransferases, DNMT3A and Dnmt3b, are required to maintain CG methylation. Methylation can also occur at CHG and CHH sites (where H represents A, T, or G), although in animals CG is by far the most common site of methylation. Certain genes are methylated (“imprinted”) during either oogenesis or
spermatogenesis to ensure expression of only the paternal or maternal allele in the embryo (Feng et al. 2010; Hudson et al. 2010). This regulation is developmentally important: in at least some cases, defects in imprinting adversely impact development and health of the offspring (Surani et al. 1986; Feng et al. 2010).

The extensive chromatin reorganization in the developing mouse germline involves the removal/reestablishment of DNA methylation and many histone modifications (Hajkova et al. 2008; Farthing et al. 2008; Sasaki and Matsui 2008; Feng et al. 2010). When the PGCs form at embryonic day 7.25 (E7.25), their pattern of repressive chromatin marks (DNA methylation, H3K27me3, and H3K9me2) resembles that in adjacent somatic cells. The levels of these repressive marks begin to change very soon thereafter, with DNA methylation and H3K9me2 decreasing and H3K27me3 increasing. There is a brief period of time (E7.5-E8.25) when the levels of repressive marks are relatively low and transcription might be expected to initiate; however, RNA polymerase II is inactive during this time due to another (unknown) mechanism. During this period, X chromosome dosage compensation is reversed in the PGCs, as described below.

Once the migrating PGCs reach the developing somatic gonad, they proliferate and, in females, eventually initiate gametogenesis. In males, gametogenesis does not initiate until after birth. Changes in chromatin modifications continue through this period, including removal of parental imprints (Sasaki and Matsui 2008; Feng et al. 2010). This reprogramming is important for regulating PGC-specific gene expression and for the eventual establishment of sex-specific chromatin modifications.

During gametogenesis, sex-specific patterns of de novo DNA methylation and histone modification are observed, presumably reflecting the very extensive differences in sperm vs oocyte formation (Sasaki and Matsui 2008; Feng et al. 2010). In males, DNA methylation imprints are established in mitotic germ cells prior to entry into meiosis. Upon entry into meiosis, widespread changes in histone modification are observed, as well as incorporation of numerous histone variants (Godman et al. 2009; Kageyama et al. 2007; Sasaki and Matsui 2008). In female germ cells, DNA methylation imprinting occurs during diplotene stage of meiotic prophase I when oocytes are in the growth phase. Global analysis of histone modifications revealed a general increase in the number of histone modifications in female germ cells as oogenesis proceeds, as well as incorporation of a histone H1 variant, although overall the observed chromatin reorganization during meiosis is much less dramatic than that observed in males (Gu et al. 2010; Sasaki and Matsui 2008). The most obvious pattern of altered histone modifications is an increase in acetylated H3 and H4 during prophase of meiosis I, which is then reversed later as oocytes proceed through the meiotic divisions at fertilization.

Genetic analysis has underscored the functional importance of chromatin reorganization during murine gametogenesis: mutations in components of the chromatin regulatory machinery are associated with sterility. For example, mutations in Prdm9 H3K4 tri-methyltransferase and Ehmt2 H3K9 mono- and di- methyltransferase, two HMTases normally active in the male and female germ line, cause extensive defects including meiotic arrest and an incorrect pattern of gene expression (Sasaki and Matsui 2008).
13.4.1.1 Meiotic Sex Chromosome Inactivation

In mouse, the male sex chromosomes are transcriptionally silenced for a portion of first meiotic prophase (Turner 2007). This process, called meiotic sex chromosome inactivation (MSCI), is an example of a larger phenomenon called meiotic silencing of unpaired chromosomes (MSUC) that also targets asynapsed autosomes and large chromosomal translocations (Schimenti 2005; Turner 2007). MSUC is thought to be analogous to meiotic silencing of unpaired chromatin in *C. elegans*. In male germ cells, the X and Y chromosomes form a distinct structure called the XY-body. During first meiotic prophase, the XY-body accumulates a specific set of histone variants, e.g., H3.1 and H3.2 are replaced with H3.3, and an altered pattern of histone modifications (Turner 2007; Sasaki and Matsui 2008). Changes in histone modification include elevated H3K9me2, H2A ubiquitination, and H2AX phosphorylation, and reduced H3K9ac (Turner 2007; Payer et al. 2011). Similar histone modifications and replacement also occur on asynapsed autosomes (van der Heijden et al. 2007). Interestingly, the reverse situation is observed in *C. elegans* where synapsed chromosomes accumulate H3.3 and the single X does not (Ooi et al. 2006). Global analysis of gene expression in the mouse identified a phase during early meiosis where X-linked gene expression is down-regulated relative to autosomal gene expression (Wang et al. 2005). Disruption of MSCI results in up-regulation of X-linked genes and arrest of male meiosis suggesting that the differential regulation of XY chromatin silences gene expression in a manner necessary for meiosis (Turner 2007; Zamudio et al. 2008; Royo et al. 2010).

The mechanism of meiotic silencing in mouse differs (at least to some extent) from that in nematodes, although asynapsis appears to trigger the process in each species (Turner 2007; Maine 2010). MSCI in mouse requires components of the DNA damage response machinery and accumulation of H2A variants that are associated with the DNA damage response (Turner 2007; Sasaki and Matsui 2008; Payer et al. 2011). Histone variant H2AX, which localizes to meiotic double strand breaks (DSBs), also localizes to the XY-body. Initial steps in the meiotic silencing process include the association of BRCA1 protein with asynapsed chromosomes and subsequent recruitment of the checkpoint kinase, ATR, which then phosphorylates H2AX at late zygotene/early pachytene stage (Payer et al. 2011). H2AX located at DSBs is also phosphorylated, although this occurs earlier, and these marks are no longer detected at pachytene when synapsis is complete. In *C. elegans*, components of the DNA damage response machinery apparently do not have a role in meiotic silencing (Maine 2010). Numerous other histone regulatory proteins either associate with or are excluded from the XY-body, and mutations in many of these factors lead to defects in XY-body formation (Sasaki and Matsui 2008). A role for the small RNA machinery in meiotic silencing in mouse has not been ruled out, and it will be very interesting to see if this aspect of the process is conserved.

Relevant to X chromosome regulation in the germ line is the process of X chromosome dosage compensation. Ultimately, one X chromosome is randomly inactivated in cells of the early female embryo. In order for X chromosome inactivation
to be random, the inherited paternal X that was silenced in the male germline by MSCI must be activated. There has been substantial debate about the details of paternal X regulation in the female embryo, and the bulk of the evidence in mouse now suggests a complex series of events, as follows. MSCI is reversed after fertilization and then the paternal X is quickly re-silenced such that only the maternal X is expressed in extraembryonic tissues. The paternal X is reactivated in the inner cell mass of the blastocyst, and subsequently one X chromosome is randomly inactivated in each cell of the early embryo (epiblast) by the dosage compensation machinery (Payer et al. 2011). The inactive X is eventually reactivated in the early PGCs (Sasaki and Matsui 2008).

13.4.1.2 Transposon Silencing and Other siRNA-Mediated Mechanisms in the Germ Line

In mouse, the repression of transposon activity is especially important in males where actively dividing germline stem cells are maintained. Moreover, the global reduction in silencing marks in early PGCs might provide an opportunity for elevated transposon activity. In mouse, as in C. elegans, transposon activity is limited in the germ line via a small RNA-mediated mechanism involving Argonaute proteins of the Piwi clade and associated piRNAs (analogous to C. elegans 21U RNAs) (Castaneda et al. 2011; Sasaki and Matsui 2008). PiRNA pathway activity leads to degradation of transposon-encoded mRNA and methylation of transposable element DNA. Accordingly, inactivation of the piRNA pathway is associated with very high expression of transposable elements during meiosis, in turn leading to myriad meiosis defects and eventual sterility (Castaneda et al. 2011). In addition to regulating transposons, there is some evidence that the small RNA machinery may directly regulate developmental gene expression in the germ line. For example, endogenous siRNAs are produced from dsRNA in developing oocytes and limit the accumulation of cognate mRNAs (Watanabe et al. 2008).

13.4.2 Mechanisms of Epigenetic Regulation in the Drosophila Germ Line

In Drosophila, as in C. elegans and mouse, a repressive chromatin structure is responsible for maintaining transcriptional quiescence in the PGCs (often called pole cells) (Nakamura and Seydoux 2008). In Drosophila, as in nematodes, DNA methylation is absent and chromatin modifications strictly involve histones. Global analysis of histone modifications in Drosophila indicates that active histone marks are absent from (or present at very low levels in) newly formed pole cells. These studies were performed by indirect immunofluorescence analysis of histone modifications in pole cells of female embryos (Schaner et al. 2003; Rudolph et al. 2007).
and ChIP-chip and ChIP-seq analysis of undifferentiated germ cells derived from *bam* (bag of marbles) mutant males (Gan et al. 2010a, b). Activity of the H3K4 demethylase, SU(VAR)3-3, restricts accumulation of H3K4 methyl (activation) marks in pole cells and is required for accumulation of H3K9me2 silencing marks and formation of heterochromatin (Rudolph et al. 2007). In comparison, marks of active chromatin are present in the *C. elegans* germ line founder cell, P4, but are globally removed as P4 divides during embryogenesis to form the initial PGCs (Z2 and Z3) (Schaner et al. 2003). No further PGC divisions occur until larval development, at which time active modifications are detected on all chromosomes except the X (Schaner et al. 2003; Nakamura and Seydoux 2008). This regulation resembles the situation in mouse described above where the global pattern of chromatin marks in the initial PGCs is similar to that of the surrounding somatic cells, but quickly becomes distinctive as PGCs begin to divide and migrate.

In *Drosophila*, chromatin regulation is critical for maintenance of the GSCs in the adult gonad. Scrawny (Scny) is a ubiquitin-specific protease that deubiquitylates H2B and is essential for maintenance of several types of stem cells, including GSCs (Buszczak et al. 2009). In *scny* mutants, GSCs have elevated levels of ubiquitylated H2B and H3K4me3 and, as a consequence, transcription. It is hypothesized that Scny activity maintains the stem cell fate by preventing expression of differentiation genes. Sex-specific histone modifiers function in the GSCs, as well. In the female germline, H3K9 methyltransferase activity is critical for fertility. The activities of three distinct H3K9 methyltransferases, dSETDB1/Eggless, SU(VAR)3-9, and dG9a, produce H3K9me3 modifications during oogenesis (Yoon et al. 2008; Lee et al. 2010). However, dSETDB1/Eggless activity in GSCs and early in oogenesis is required for female fertility, while SU(VAR)3-9 activity later in oogenesis is nonessential for fertility (Clough et al. 2007; Yoon et al. 2008). Expression of dG9a is required in germline support cells (nurse cells) during oogenesis (Lee et al. 2010). Genetic studies suggest partial functional redundancy among these three H3K9 MTases (Lee et al. 2010 and references therein). H3K9me3 levels also depend on activity of the heterochromatin-associated protein, Stonewall (Stwl) (Yi et al. 2009). Stwl activity maintains the female GSC fate and prevents premature germ cell differentiation (Maines et al. 2007; Yi et al. 2009). In *stwl* mutants, levels of H3K9me3 and H3K27me3 are reduced, and these changes presumably contribute to the inappropriate expression of differentiation genes and loss of the GSC fate in *stwl* mutants (Yi et al. 2009).

Male-specific regulators of GSC chromatin also have been identified. Nclb (No child left behind) is a chromatin-binding protein whose function is essential for maintaining the GSC fate in males but not in females (Casper et al. 2011). Levels of H3S10 phosphorylation, a histone modification associated with transcriptional elongation, are very reduced in *nclb* mutants. This and other evidence suggest that transcription is reduced in *nclb* mutants, and therefore Nclb activity is likely to ensure transcription of genes necessary for maintenance of the GSC fate in males.

As in mouse and *C. elegans*, chromatin regulation is critical during *Drosophila* gametogenesis and changes in male germ cell chromatin are more substantial than those in female germ cell chromatin. As in other species, the incorporation of histone
variants into germ cell chromatin during meiosis is important for chromosome condensation. In the absence of H3.3 expression, visible defects in chromosome morphology are observed beginning in meiosis; chromosomes fail to condense properly and later fail to segregate correctly during the meiotic divisions (Ooi et al. 2006; Sakai et al. 2009). Chromatin compaction in the mature sperm head late in spermatogenesis (during spermiogenesis) requires accumulation of histone H4 acetylation marks, which then promote the replacement of histones by small basic proteins called protamines (Awe and Renkawitz-Pohl 2010).

13.4.2.1 Meiotic Sex Chromosome Inactivation

There is contradictory evidence as to whether meiotic sex chromosome inactivation occurs in *Drosophila*. One difficulty in answering this question may have to do with technical problems in examining nuclei of the correct meiotic stage in the *Drosophila* testis, which includes a heterogeneous population of somatic and germ cells. In addition, autosomes do not synapse during meiosis in *Drosophila* males, so such a distinction between autosomes and sex chromosomes is not present. Nonetheless, several studies have examined global analysis of gene expression in the testis. Gene expression analysis of spermatogenic arrest mutants and developing (wild-type) testes failed to detect evidence of MSCI (Sturgill et al. 2007; Mikhaylova and Nurminsky 2011), whereas analysis of dissected regions of the testis found a very mildly reduced level of X-linked relative to autosomal gene expression in cells enriched for meiotic as opposed to mitotic or post-meiotic cells (Vibranovski et al. 2009). The best evidence for silencing of the *Drosophila* male X chromosome was provided by transgene studies showing that expression of autosomal spermatogenesis genes becomes down-regulated when these genes are incorporated into the X chromosome as transgenes (Hense et al. 2007; Meiklejohn et al. 2011). This phenomenon was initially interpreted as a sign that X-linked genes are silenced during male meiosis (Hense et al. 2007). However, recent evidence indicates that transcription from the male X is reduced relative to autosomes even prior to meiotic entry (i.e., in mitotic germ cells), and therefore the observed transgene silencing is not strictly meiotic (Meiklejohn et al. 2011). Indeed, genes with a male-biased expression pattern are severely underrepresented on the X, and therefore X-linked gene expression should be relatively low in the male germ line (Parisi et al. 2003; Sturgill et al. 2007). So far, there are no reports suggesting that unsynapsed regions other than the male X and Y, e.g., autosomes or translocations, are silenced, as would be expected for general meiotic silencing of unpaired chromatin.

13.4.2.2 Transposon Silencing and Other siRNA-Mediated Processes in the Germ Line

As in other organisms, repression of transposon activity in the *Drosophila* germ line is important for maintenance of genome integrity. A piRNA pathway analogous to
that present in mouse functions to repress transposon expression (Khurana and Theurkauf 2010). This pathway is active in both the male and female germline, and Piwi/piRNA complexes are transmitted maternally in order to prevent transposable element activity in the progeny. Up-regulation of transposon production in the female germline can cause sterility, possibly by triggering a checkpoint that would normally eliminate germ cells with a high potential for carrying mutations (Chen et al. 2007). Interestingly, activity of dSETDB1/Eggless is required for transcription of piRNA clusters (Rangan et al. 2011). Since dSETDB1 activity promotes heterochromatin assembly, and piRNA clusters are located within heterochromatic regions, it is hypothesized that expression of piRNAs is triggered in some way by the presence of heterochromatin (Rangan et al. 2011). In addition, piRNAs (originally called repeat-associated RNAs, rasiRNAs) appear to be important for germline development as mutations that disrupt their accumulation cause female sterility (Pane et al. 2007).

13.5 Implications for the Embryo

Evidence from many organisms suggests that the epigenetic state of gamete chromatin directly influences gene expression in the embryo. Imprinted DNA methylation marks regulate the expression of maternal vs paternal genes in the early embryo prior to global erasure of such imprinted marks. Moreover, although chromatin modifications are removed from many sites in the early embryo, other marks are reported to escape removal. Therefore, epigenetic regulation established in the germ line can be inherited by and influence gene expression in the offspring. This phenomenon, termed transgenerational epigenetic inheritance, has been described in C. elegans, mouse, and Drosophila as well as in many other animals and in plants (Daxinger and Whitelaw 2010). Mechanisms of epigenetic transgenerational inheritance involve histone modifications and small RNAs. In C. elegans, gene activity in the parental germ line contributes to epigenetic regulation in both of the embryonic germ cell precursors (Rechtsteiner et al. 2010; Furuhashi et al. 2010) and in the soma (Arico et al. 2011). Moreover, effects can be observed well beyond embryogenesis: the loss of H3K4 methylation complex activity in the parent (caused by mutations in set-2, ash-2, or wdr-5) will extend lifespan in descendants for up to three generations (Greer et al. 2011). This effect is suppressed by the loss of RBR-2 demethylase activity. Therefore, in this case, the longevity phenotype is presumably caused by insufficient H3K4 methylation.

Heritability of RNAi via either maternally or paternally transmitted factors was demonstrated more than 10 years ago (Grishok et al. 2000), and RNAi was recently shown to trigger the heritable expression of siRNAs in the progeny of animals treated with dsRNA (Burton et al. 2011). Other recent studies suggest that inherited 26G RNAs regulate gene expression during embryogenesis (Gent et al. 2009; Han et al. 2009). Gent et al. (2009) demonstrated that RRF-3 activity during spermatogenesis is required for normal embryonic development, suggesting that
paternally inherited 26G RNAs may function in embryogenesis. In a complementary study, Han et al. (2009) demonstrated maternal inheritance of 26G RNAs whose presence correlates with reduced expression of target genes. Using genetic assays, Alcazar et al. (2008) demonstrated the ability of RNAi to persist over multiple generations in the absence of the original targeted allele. This effect could be passed through both oocytes and sperm and was independent of the original targeted allele. These data are consistent with the inherited factor being an siRNA and with the ability of inherited 26G RNAs to repress gene expression. Similar to siRNA, antiviral RNAs (viRNAs) produced in response to viral infection can be inherited (Rechavi et al. 2011). ViRNAs function in silencing viral gene expression via an RNAi-like mechanism (Rechavi et al. 2011). Individuals who lack the machinery to generate viRNAs can mount an antiviral response utilizing inherited viRNAs. These observations further substantiate the hypothesis that inherited small RNAs are critical regulators of gene expression during development.

In their analysis of heritable gene silencing, Burton et al. (2011) demonstrated that RNAi triggers the heritable expression not only of siRNAs but also of H3K9me3 marks. Accumulation of H3K9me3 marks was observed at the target locus, a somatically expressed gene called dpy-11, in dsRNA-treated (P0) animals and in their F1 progeny. Evidence suggests that dpy-11 siRNAs are inherited, while H3K9me3 marks at the dpy-11 locus are not inherited and instead are reestablished in the F1 progeny. Activity of the nuclear RNAi (NRDE) pathway in the F1 progeny is necessary for expression of dpy-11 siRNAs and reestablishment of H3K9me3. The nuclear RNAi mechanism involves the Argonaute NRDE-3 and siRNAs generated by RdRP activity in the exogenous RNAi process (termed secondary [2° siRNAs]). The NRDE-3/2° siRNA complex enters the nucleus and recruits two other factors, NRDE-1 and NRDE-3, to chromatin-associated transcripts having homology to the 2° siRNAs (Guang et al. 2008, 2010). A fourth component, NRDE-4, associates with NRDE-1 in the nucleus, and together the NRDE proteins repress transcription of the RNAi-targeted locus by inhibiting RNA polymerase II elongation and directing the deposition of H3K9me3 marks (Guang et al. 2010; Burkhart et al. 2011). In their analysis of heritable RNAi, Burton et al. (2011) observed NRDE-dependent RNAi inheritance phenotypes for only a single generation, whereas other groups have described examples of inherited RNAi of germline-expressed genes extending over many generations (e.g., Grishok et al. 2000; Alcazar et al. 2008; Vastenhouw et al. 2006). It is not yet known if the NRDE pathway functions to maintain the inheritance of germline RNAi and if only germline-expressed genes can be heritably silenced over many generations. Moreover, the function of heritable RNAi is not known, although Burton and colleagues suggest it may be a way for the individual to transmit an environmental, gene-regulatory signal from one generation to the next. In addition, these findings demonstrate that siRNAs can participate in the establishment/maintenance of a heritable pattern of histone modifications at a specific locus.

Recently, Johnson and Spence (2011) described a new phenomenon, termed epigenetic licensing, whereby the presence of a maternally inherited transcript is
essential for expression of the cognate gene in the embryonic germ line. This phenomenon was described with respect to fem-1, a gene expressed in XO animals and in the larval XX germ line to allow development of the male fate. Johnson and Spence (2011) observed that the inheritance of either a complete or partial fem-1 mRNA was required for transcription of embryonic fem-1 in the PGCs. Even inheritance of a non-protein coding, partial transcript was sufficient to allow transcription of embryonic fem-1; hence, RNA appears to be the critical inherited factor. The authors propose that fem-1 may be subject to a form of epigenetic silencing in the PGCs (but not the soma), and the presence of inherited transcripts may override this silencing. The mechanism of this regulation is unknown; however, epigenetic licensing appears to be a new form of regulation distinct from previously described mechanisms.

13.6 Future Directions

Many questions remain as to the mechanisms and developmental importance of epigenetic control in the C. elegans germ line. Despite global chromatin analysis that has already been done, in most cases we do not yet know the specific sites of histone modification, and more importantly, the mechanisms responsible for differential distribution of chromatin marks. For example, what mechanisms ensure preferential H3K27me3 accumulation on the X chromosome and H3K9me2 accumulation on unpaired chromosomes? Do all genes on a chromosome receive these marks or only a subset of genes? Another task is to identify the protein complexes responsible for recognizing specific histone modifications and responding to them, e.g., to control gene expression. A related question is the extent to which certain modifications function to regulate chromatin on a chromosomal level, e.g., in order to establish structure important for chromosome segregation. Finally, what is the developmental importance to the embryo of inherited chromatin marks and small RNAs from the sperm and/or oocyte?

Different mechanisms are likely to target MET-2 activity to unpaired chromosomes vs MES2/3/6 activity to X chromosomes. Because MET-2 activity is highest on unpaired chromosomes, it may be recruited by a factor associated with unpaired/unsynapsed chromosomes or excluded by a factor associated with synapsed chromosomes. MES-2/3/6 activity, in contrast, associates with the X chromosome independent of its pairing status and is presumably regulated via a different mechanism that requires MES-4 activity. In other organisms, PRC2 targets regions containing Polycomb response elements (PRE), several DNA-binding proteins have been shown to promote PRC2 binding, and long noncoding RNAs have been implicated as regulators of PRC2 function (Margueron and Reinberg 2011). C. elegans MES-2/3/6 activity may likewise be governed by a complex interplay of cis-regulatory sites, trans-acting proteins, and noncoding RNAs. Recent work also indicates that H3K27 methylation is inhibited by the presence of modifications associated with
active chromatin (Schmitges et al. 2011). Perhaps the low density of active marks on the X, reflecting the paucity of germline-essential genes, creates an appropriate environment for H3K27 methylation.

The developmental importance of histone modifications and histone variants will become clearer as researchers identify the factors that “read” and respond to specific patterns of modifications. The extensive chromatin biology literature has established two distinct roles for histone modifications in (1) directly regulating structural conformation of the chromatin, e.g., physically blocking chromatin compaction, and (2) functioning as binding sites for proteins such as histone-modifying enzymes, transcription factors, and chromatin remodeling proteins (Oliver and Denu 2011). Many modifications appear to function in a context-dependent manner, and a major goal of the field now is to understand the dynamic interactions occurring among chromatin-binding proteins and histones in different chromatin domains. H3K27me3 accumulation at promoter regions is widely observed to correlate with transcriptional repression (Justin et al. 2010). Therefore the elevated level of H3K27me3 marks on C. elegans germline X chromosomes presumably reflects the lower numbers of actively expressed genes on the X as compared with autosomes. In contrast, H3K9me2 is broadly distributed over genomic DNA corresponding to facultative heterochromatin regions and, while absent from active regions in some cell types (e.g., mouse ES cells and neuronal stem cells), is detected within the bodies of some active genes in differentiated cell types (Wen et al. 2009; Lienert et al. 2011). This pattern is interesting given the situation in the C. elegans germ line where the presence of elevated H3K9me2 marks on unpaired autosomes, e.g., on unpaired chromosome V in zim-2 mutants, does not disrupt development and, therefore, presumably does not indicate a dramatic reduction in transcription. This result is consistent with accumulation of H3K9me2 marks that do not disrupt expression of active genes. Instead, these marks might serve another function, for example relevant to segregation of non-synapsed homologs. An additional level of complexity is added by the recent report that MET-2 activity, and hence H3K9me2 accumulation, is important for repressing the pachytene checkpoint in XO animals, but not in him-8 hermaphrodites (Checchi and Engebrecht 2011). Hence, the chromatin state of heterogametic sex chromosomes may serve a purpose distinct from that of unsynapsed homologs.

Although the embryo clearly inherits histone modifications, the developmental importance of these marks is not completely understood. As already discussed, MES-4 function (H3K36 methylation) is important for setting up the correct pattern of transcription in the embryo. The importance of other inherited histone modifications to embryonic development, including differentially distributed H3K27me3 and H3K9me2 marks, remains to be determined.

Acknowledgments We thank Xingyu She for the images included in Fig. 13.3, and Bill Kelly, Michael Cosgrove, Yiqing Guo, and Tim Schedl for comments on the manuscript. This work was supported by NIH funding (1R01GM089818) to EMM.
References


Casteneda J, Genzor P, Bortvin A (2011) piRNAs, transposon silencing, and germ line genome integrity. Mutation Res 714:95–104


