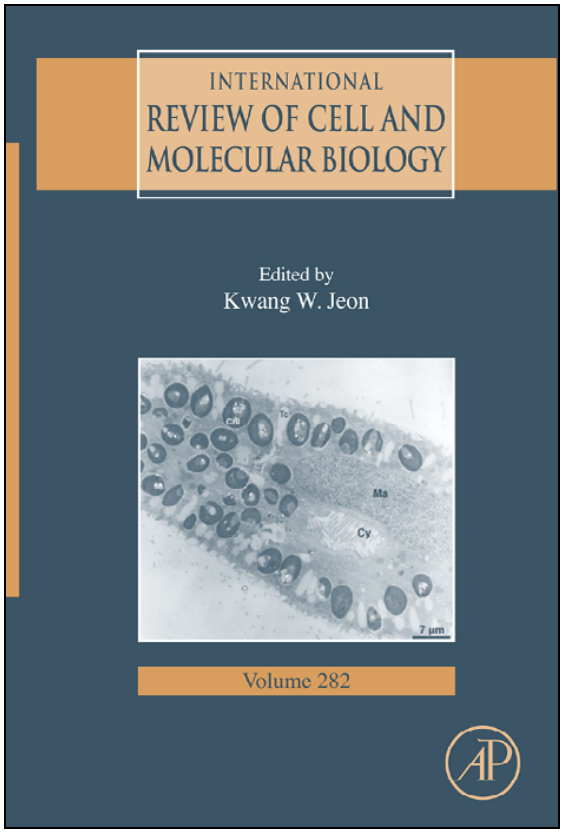

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CHAPTER TWO

MEIOTIC SILENCING IN *CAENORHABDITIS ELEGANS*

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Contents

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Abstract

In many animals and some fungi, mechanisms have been described that target unpaired chromosomes and chromosomal regions for silencing during meiotic prophase. These phenomena, collectively called "meiotic silencing," target sex chromosomes in the heterogametic sex, for example, the X chromosome in male nematodes and the XY-body in male mice, and also target any other chromosomes that fail to synapse due to mutation or chromosomal rearrangement. Meiotic silencing phenomena are hypothesized to maintain genome integrity and perhaps function in setting up epigenetic control of embryogenesis. This review focuses on meiotic silencing in the nematode, *Caenorhabditis elegans*, including its mechanism and function(s), and its relationship to other gene silencing processes in the germ line. One hallmark of meiotic silencing in *C. elegans* is that unpaired/unsynapsed chromosomes and chromosomal regions become enriched for a repressive histone modification, dimethylation of histone H3 on lysine 9 (H3K9me2). Accumulation and proper targeting of H3K9me2 rely on activity of an siRNA pathway, suggesting that histone methyltransferase activity may be targeted/regulated by a small RNA-based transcriptional silencing mechanism.

Key Words: Meiotic silencing, Germ line, H3K9me2, Chromatin, RNA-directed RNA polymerase, Histone modification, X chromosome, RNAi.

1. Introduction

The term "meiotic silencing" refers to the silencing of unpaired/unsynapsed chromosomes and chromosomal regions during prophase of meiosis I. Meiotic silencing has been studied in many animal species (e.g., mammals, birds, nematodes, insects) as well as certain fungi (Baarends et al., 2005; Cabrero et al., 2007; Kelly et al., 2002; Mahadevaiah et al., 2009; Schoenmakers et al., 2009; Shiu et al., 2001; Turner et al., 2005). In animals, meiotic silencing is thought to include the silencing of sex chromosomes in the heterogametic sex, a process called meiotic sex chromosome inactivation (MSCI) (Handel, 2004). Subsequent to the discovery of MSCI, researchers used mutations and chromosomal rearrangements to examine the regulation of unpaired autosomes and found them to be regulated in a similar fashion. Hence, meiotic silencing appears to be a general process not unique to the heterogametic germ line or

chromosomes. Meiotic silencing phenomena have different characteristics in different species, as their organism-specific names reflect. Collectively, these phenomena have been referred to as meiotic silencing (e.g., Kelly and Aramayo, 2007), although the functional relationships among them are not completely clear.

Meiotic silencing in animals typically occurs at the chromatin level and involves accumulation of histone modifications that are thought to promote a closed chromatin configuration and transcriptional repression. In addition to transcriptional repression, these changes in chromatin structure may contribute to meiotic chromosomal events such as chromosome disjunction. In *Caenorhabditis elegans*, where failure of chromosomes to pair and synapse triggers accumulation of histone silencing modifications on the unpaired chromatin, the process is referred to as meiotic silencing of unpaired chromatin (MSUC; Maine et al., 2005). In mouse, the process is referred to as meiotic silencing of unsynapsed chromatin (MSUC; Schimenti, 2005) because the failure of chromosomes or chromosomal regions to synapse is the trigger for accumulation of histone silencing marks and transcriptional repression.

A fundamentally different meiotic silencing mechanism is at work in the fungus, *Neurospora crassa*, where the presence of unpaired chromatin triggers silencing not only of that unpaired region but also of homologous paired DNA elsewhere in the genome. This phenomenon is termed meiotic silencing by unpaired DNA (MSUD; Shiu et al., 2001) and appears to occur strictly at a posttranscriptional level.

2. Chromatin Regulation in the Germ Line

Meiotic silencing can be considered within the context of germ line development. Animal germ cells undergo specific differentiation programs to produce gametes that have the capacity, upon fusion, to give rise to a new individual. Hence, the chromatin in haploid gametes must have the flexibility to reorganize during early embryogenesis and support the development of diverse cell lineages. Germ line development requires mechanisms that allow the formation of gametes while also protecting germ cells from the expression of gene products that might decrease progeny viability. The misregulation of gene expression during germ line development can have disastrous consequences for fertility and for the health and development of offspring. One important mechanism of gene regulation in all tissues, including the germ line, is the modulation of chromatin structure to promote or repress transcription. Chromatin structure in the germ line must also accommodate special features of germ cell biology, including homolog pairing, synapsis, and recombination.

The mechanisms of chromatin regulation have been discussed in several recent excellent reviews (e.g., Gelato and Fischle, 2008; Kouzarides, 2007; Rando and Chang, 2009; Wu et al., 2009) and will be discussed only briefly here. The basic unit of chromatin is the nucleosome, which includes DNA wrapped (twice) around an octamer of histone proteins. Chromatin is compacted to differing degrees, both locally and at the level of the entire chromosome. Chromosome condensation is critical for chromosome segregation during the mitotic and meiotic divisions. Local chromatin regulation modulates the ability of nonhistone proteins to contact the DNA and, thereby, regulate transcription. Intense effort has identified a large collection of conserved histone modifications that correlate with transcriptional states and are thought to alter chromatin structure, thereby promoting or preventing transcription (Kouzarides, 2007). Early studies identified certain modifications as associating with expressed or repressed chromatin based on indirect immunofluorescence labeling. For example, H3K9me3 was observed to be enriched in constitutive heterochromatin in mouse, *Drosophila*, and mealy bug cells, including at centromeres, some telomeres, the inactive X chromosome in female mouse cells, and the highly condensed X and Y chromosomes (together with associated factors referred to as the XY-body) in male meiotic germ cells (Cowell et al., 2002). More recently, genome-wide mapping studies have begun to provide detailed information about the fine-structure distribution of specific modifications (Barski et al., 2007; Kolasinska-Zwierz et al., 2009; Rando and Chang, 2009). In general, active genes tend to have a nucleosome-free region at the transcription start site. Some histone modifications (also called histone marks) such as trimethylation of histone H3 on lysine 4 (H3K4me3) are found on nucleosomes immediately flanking the transcription start site of active genes. Other marks such as H3K36me3 are found on nucleosomes within exons of active genes. In contrast, for silent genes, marks such as H3K27me3 are found on nucleosomes extending several kilobase pairs up- and downstream of the transcription start site. Interestingly, although many modifications appear to be associated primarily with active or silent loci (e.g., H3K4me3 or H3K27me3, respectively), the correlations for other marks are not so clear, and many questions remain about how (or whether) specific histone modifications influence transcription.

A situation directly relevant to the analysis of meiotic silencing is the fact that although the presence of certain histone marks correlates with increased or decreased transcription, it has been a challenge to show cause and effect in most cases. For example, it is not clear whether histone ''activation'' marks change chromatin structure in such a manner as to allow transcription or whether they arise as a consequence of transcription. The story is simpler at the level of phenotype in the sense that defects in the ability to make specific chromatin modifications in the germ line are known to reduce or

eliminate fertility in a variety of species, as discussed below. Moreover, the inheritance of inappropriately modified chromatin can contribute to developmental defects and increased susceptibility to disease (Chong et al., 2007; Kimmins and Sassone-Corsi, 2005; Strome and Kelly, 2007; Turner, 2007; Zamudio et al., 2008).

Unique aspects of chromatin biology in the germ line include not only meiotic silencing but also the chromatin reorganization that occurs at diagnostic times during development, imprinting of maternal or paternal alleles, and the extreme condensation of sperm chromatin via histone replacement by protamines (Allegrucci et al., 2005; Hajkova et al., 2008; Kimmins and Sassone-Corsi, 2005). This review focuses on the mechanism and function of meiotic silencing in *C. elegans*, and discusses this phenomenon within the larger context of meiotic silencing processes in general. Meiotic silencing is of special interest as a chromatin regulatory mechanism with functions in both the male and female germ lines in addition to its sex-specific roles in male germ line development.

3. Repressive Mechanisms in *C. elegans* Meiotic Germ Line

C. elegans is a hermaphroditic species where the predominant sex, XX hermaphrodites, produce both sperm and oocytes. XX animals have a female soma and a germ line that is male (produces sperm) during larval development and becomes female (produces oocytes) at approximately the time of the molt preceding the adult stage. Males are XO and produce sperm; they typically result from fertilization of a nullo-X gamete (produced as a result of meiotic nondisjunction). This mode of sexual reproduction requires that XX and XO germ cells undergo spermatogenesis. The X chromosome receives different histone marks in XX versus XO germ lines, hence spermatogenesis can accommodate these different chromatin states.

The organization of the mature gonad is shown in Fig. 2.1. The distal end of the gonad contains a population of proliferating germ cells that function like stem cells and are maintained in mitosis via signals from the somatic gonad. More proximally, the germ cells are arranged in sequential stages of meiotic prophase (leptotene–zygotene, pachytene, and diplotene), which can be distinguished based on nuclear morphology and analysis of stage-specific markers. Both males and hermaphrodites store mature, haploid sperm in the proximal gonad. Adult hermaphrodites contain growing in the proximal gonad. Oocytes have progressed to diakinesis stage by the time they are ovulated into the spermatheca and immediately fertilized by stored sperm.

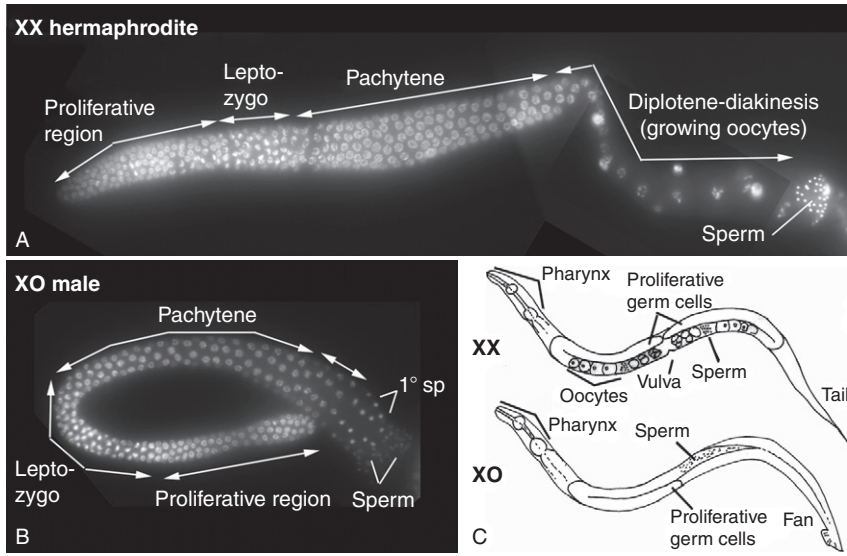

Figure 2.1 Organization of the mature *C. elegans* germ line. Photomicrgraphs of dissected (A) hermaphrodite and (B) male adult gonads are shown. Tissue was fixed and stained with the DNA dye, DAPI. Proliferative germ cells are located at the distal end of each gonad arm. (A) Proliferating, leptotene–zygotene (lepto–zygo), pachytene, diplotene–diakinesis, and sperm nuclei are indicated. (B) Proliferating, leptotene–zygotene (lepto–zygo), pachytene, diplotene, primary spermatocyte (1° sp), and sperm nuclei are indicated. (C) Schematic representation of the hermaphrodite (XX) and male (XO) body.

Relatively few X-linked genes are expressed in the *C. elegans* germ line. In both male and hermaphrodite mitotic and meiotic germ cells, active RNA polymerase II (RNA pol II) is associated with autosomes and absent from the X chromosome, suggesting that few X-linked genes are expressed (Kelly et al., 2002). The presence of repressive marks on the X chromosome in both sexes correlates with gene expression profiling data indicating germ line expression of many autosomal genes but relatively little germ line expression of X-linked genes (Reinke et al., 2000, 2004). Ooi et al. (2006) obtained further evidence of X chromosome silencing in their analysis of histone H3 replacement during meiosis. Typically, the H3.1 isoform is replaced by H3.3 during transcription. H3.3 is not detected on the hermaphrodite X chromosomes until late pachytene stage and fails to appear on the male X at all (Ooi et al., 2006).

The pattern of histone modifications in the germ line is consistent with all of the above observations (Kelly et al., 2002; Reuben and Lin, 2002). In both the XX and XO germ line, autosomes are relatively highly enriched for histone modifications associated with transcriptional activation, such as

H3K4me2 (Kelly et al., 2002; Reuben and Lin, 2002). In contrast, germ line X chromosomes have relatively low levels of histone activation marks and are enriched for a mark that correlates strongly with transcriptional silencing, H3K27me3 (Bender et al., 2004). Superimposed on this regulation, during early pachytene stage the single male X becomes enriched for H3K9me2 (Kelly et al., 2002), a mark loosely correlated with silencing (e.g., see Barski et al., 2007; Rando and Chang, 2009). Transient H3K9me2 foci appear on (perhaps all) hermaphrodite chromosomes during a very narrow window in late meiotic prophase, but this process seems to be independent of the male X enrichment. To date, no other marks besides H3K9me2 and H3K27me3 have been reported to accumulate preferentially on the X chromosome in the *C. elegans* germ line although it is certainly possible that such marks exist. The general pattern of chromatin marks is diagrammed in Fig. 2.2 and summarized in Table 2.1. A detailed discussion of the observed chromatin modifications is presented below.

3.1. Extrachromosomal transgenic arrays

The pattern of histone modifications in the *C. elegans* germ line was first investigated by researchers studying the phenomenon of transgene silencing (Kelly et al., 2002; Reuben and Lin, 2002). In *C. elegans*, transgenes are often produced via a method that results in production of highly repetitive, extrachromosomal arrays. DNA is injected into the germ line syncytium, where it does not readily integrate into an endogenous chromosome, but instead forms a long concatemer called an extrachromosomal transgene array (Stinchcomb et al., 1985). Extrachromosomal arrays are mitotically transmitted in a quasi-stable manner, perhaps due at least in part to the holocentric structure of *C. elegans* chromosomes. Genes present in highly repetitive arrays typically express in somatic tissues but often fail to express in germ cells (Kelly et al., 1997). Better germ line expression of transgenes is often observed when arrays are less repetitive, suggesting that the germ line silencing system is especially sensitive to repetitive sequences (Kelly et al., 1997). This silencing may be analogous to the silencing of centromere repeats and other repetitive DNA sequences that have been observed in other organisms.

Using indirect immunofluorescence to detect specific histone modifications, Kelly et al. (2002) and Reuben and Lin (2002) demonstrated that germ line-silenced transgenic arrays lack H3K4me2 and are enriched for H3K9me2. Moreover, Kelly et al. surveyed several other histone modifications known to correlate with transcriptional activity, including H3K9/K14ac, H3S10phos, H4K8ac, and H4K16ac, and none was present on silent arrays. In contrast, histone activation marks were observed on a germ line-expressed transgenic array, consistent with the hypothesis that transgene expression depends on, or at least correlates with, chromatin state.

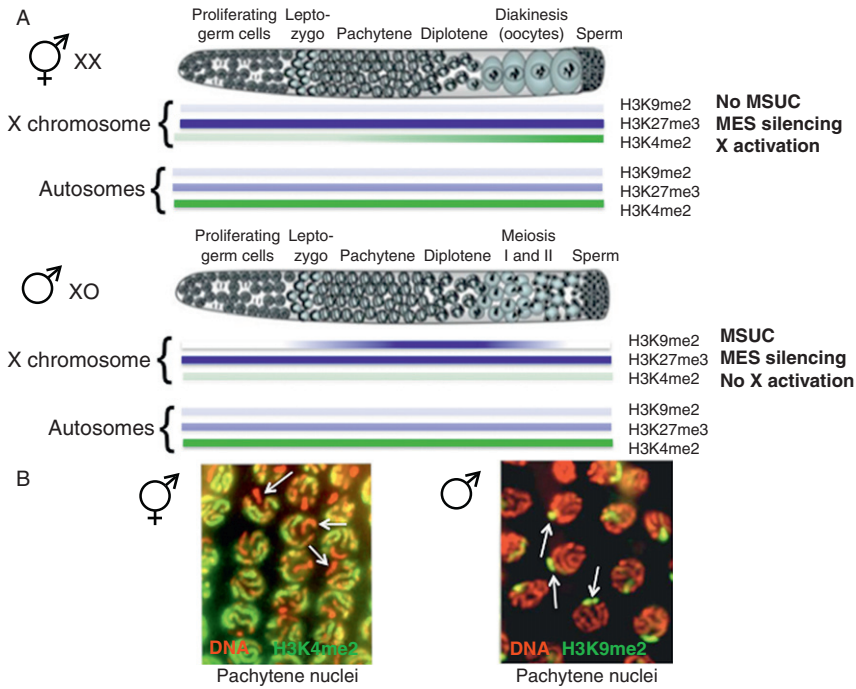

Figure 2.2 The dynamic pattern of chromatin regulation in the germ line. (A) Schematic diagram of XX (upper) and XO (lower) germ cells as they enter and progress through prophase of meiosis I (see Fig. 2.1). Shaded bars indicate the relative level of three histone marks, H3K9me2, H3K27me3, and H3K4me2, on X chromosomes and autosomes. (B) Photomicrographs show H3K4me2 distribution in XX mid-pachytene nuclei and H3K9me2 distribution in XO mid-pachytene nuclei. In each image, DNA is labeled in red and the chromatin mark is labeled in green. Arrows indicate the X chromosomes. H3K4me2, a mark associated with actively expressed chromatin, is concentrated on the autosomes and not visible on the X chromosomes. Note that XO pachytene nuclei would have a similar H3K4me2 distribution at this time, which is prior to the late-pachytene X-linked gene activation observed in XX germ lines. H3K9me2, a mark associated with unpaired chromatin, is concentrated on the male X chromosome and barely detectable on the autosomes and hermaphrodite X chromosomes. H3K27me3, a mark associated with silent chromatin, is observed on X chromosomes and autosomes, but is particularly concentrated on Xs (adapted with permission from Strome and Kelly (2007). Copyright Cold Spring Harbor Laboratory Press).

This is supported by the observation that transgenes that are normally repressed in germ cells are activated in mutants with defective H3K27 methylation in the germ line (Holdeman et al., 1998; Kelly and Fire, 1998; Korf et al., 1998).

Table 2.1 Histone modifications in the *C. elegans* germ line

Chromosome	Activation marks (mitosis; meiotic prophase)	H3K27me3 (mitosis; meiotic prophase)	H3K9me2 (meiotic prophase)
Wild type			
Paired autosomes	*High*	Low	Low
Paired Xs (hermaphrodite)	Transient	*High*	Low
Single X (male)	Not detected	Moderate	*High*
Other unpaired chromatin			
Unpaired autosomes and autosomal duplications	*High*	Low	*High*
Unpaired Xs (hermaphrodite)	Transient	*High*	*High*
Germ line-active arrays	*High*	NA	Low
Germ line-silenced arrays	Not detected	NA	*High*

Indirect immunofluorescence was used to evaluate histone modifications. Relative intensity of labeling is indicated as high, moderate, low, or not detected. "Transient" indicates a brief period of labeling, as described in the text. NA, not assayed. Data are from Kelly et al. (2002), Reuben and Lin (2002), Fong et al. (2002), Bender et al. (2004), and Bean et al. (2004). Activation marks that were assayed include H3K4me2, H3K9/K14ac, H3S10phos, H4K8ac, and H4K16ac.

3.2. X Chromosome silencing

The same indirect immunofluorescence approach revealed that the chromatin state of the X chromosome is different from that of autosomes throughout the germ line. Critical to these studies was the ability to distinguish the X chromosome from autosomes. Kelly et al. (2002) employed various methods to identify the X chromosome, including analysis of X chromosome::autosomal fusions, fluorescent *in situ* hybridization (FISH) with X-specific probes, and deconvolution microscopy. Reuben and Lin (2002) tentatively identified the X chromosome based on length, as it was known to be the shortest of the six *C. elegans* chromosomes. Both studies observed differential accumulation of histone activation marks within germ line nuclei. In mitotic and meiotic nuclei, a relatively high level of activation marks was consistently detected on autosomes. In contrast, activation marks were only obvious on paired (hermaphrodite) X chromosomes in late pachytene through diakinesis stages of meiotic prophase, and the single male X chromosome lacked activation marks altogether. Consistent with the pattern of histone activation marks, the activated form of

RNA pol II was detected in association with autosomes but not with the X chromosome (Fong et al., 2002; Kelly et al., 2002). Analysis of histone modifications associated with transcriptional repression revealed a relatively high level of H3K9me2 marks on the male X, and a moderate enrichment for H3K27me3 on all (hermaphrodite and male) X chromosomes. In contrast, H3K27me2 does not appear preferentially associated with any particular chromosome (Bender et al., 2004; Kelly et al., 2002).

3.3. Genome-wide analysis of germ line gene expression

X chromosome gene expression in the *C. elegans* germ line has also been explored using gene expression profiling and functional genomic analysis (Maeda et al., 2001; Piano et al., 2000, 2002; Reinke et al., 2000, 2004). Gene expression profiling has identified large sets of transcripts that are enriched in the germ line relative to the soma; these genes can be grouped into several categories based on expression pattern, including (i) germ line-intrinsic genes expressed in the XX and XO germ line, (ii) spermatogenesis-specific genes expressed in the XO and larval XX germ lines, and (iii) oogenesis-specific genes expressed in the female and adult hermaphrodite germ line (Reinke et al., 2000, 2004). These studies revealed that germ line-intrinsic and spermatogenesis-specific genes are underrepresented on the X chromosome relative to autosomes. In contrast, oogenesis-specific genes are not underrepresented on the X (Reinke et al., 2000, 2004), although essential ovary-expressed genes tend not to be X-linked (Maeda et al., 2001; Piano et al., 2000, 2002). A trend away from germ line-expressed genes on the X was borne out by subsequent genetic studies showing that, among sets of duplicated genes, those that are expressed in the germ line tend to be located on autosomes while those expressed in the soma may be located on the X chromosome (Maciejowski et al., 2005; Ohmachi et al., 2002). Taken together, these data are consistent with the pattern of H3K4me2 and other activation marks observed by Kelly et al. (2002) in the germ line: there is little X chromosome expression during mitosis, male meiosis, and spermatogenesis, but there is a burst of X-linked expression in oogenesis.

In an initial attempt to determine the functional significance of the observed patterns of dynamic chromatin modifications, Kelly et al. (2002) compared the average transcript level for all genes versus oogenesis-expressed genes on each chromosome. They found that genes whose expression remains high during meiosis tend to be located on autosomes. In contrast, the average X chromosome transcript levels were two- to three-fold lower than autosomal transcript levels in the germ line. In the soma, no significant difference in autosomal versus X chromosomal transcript level was observed. These data were consistent with X-linked transcription occurring in only a small subset of germ cells. Consistent with this hypothesis, when *in situ* hybridization analysis was used to visualize transcript

distributions, X-linked transcripts were observed during the late-pachytene/diplotene window (Kelly et al., 2002). For each gene examined, mRNA was first visible in late pachytene nuclei, consistent with the appearance of chromatin activation marks on the X chromosomes at that stage.

3.4. Enrichment of H3K9me2 on male X and other unpaired chromosomes

Germ line H3K9me2 distribution is dynamic and chromosome specific (Kelly et al., 2002; Reuben and Lin, 2002). In XO nuclei, the single X chromosome does not have a pairing partner during meiosis. Goldstein (1982) had previously observed that the male X is highly condensed in pachytene nuclei and remains so through the rest of spermatogenesis. In the male, H3K9me2 marks are detected during pachytene–diplotene stages. The X chromosome contains a relatively high level of H3K9me2 marks while the autosomes contain a low ("basal") level. In the XX hermaphrodite, H3K9me2 is not preferentially associated with the X chromosomes. Instead, a focus of H3K9me2 labeling is visible in early pachytene stage at the end of what may be an autosome (W. Kelly, personal communication). As nuclei progress to late pachytene, H3K9me2 foci are observed at many chromosomal sites (Kelly et al., 2002). These foci persist into diplotene stage before disappearing. Based on these data, H3K9me2 appears to have a major role in regulation of the single male X chromosome but a more limited role with respect to the pair of hermaphrodite X chromosomes. The relationship between these transient H3K9me2 foci and the H3K9me2 marks on the male X chromosome is unclear.

Bean et al. (2004) later demonstrated that H3K9me2 can become enriched on autosomes and X chromosomes if they fail to pair in either sex. In their studies, Bean and colleagues took advantage of mutations that disrupt pairing and synapsis of homologous chromosomes. For example, they evaluated H3K9me2 labeling in *him-8* mutants, where the hermaphrodite X chromosomes fail to pair and synapse. In *him-8* hermaphrodites, H3K9me2 was enriched on both X chromosomes from early pachytene through diplotene stages, similar to what was observed for the male X. Hence, the disruption of pairing and/or synapsis appears to allow or trigger H3K9me2 accumulation.

Bean et al. (2004) obtained consistent data when they evaluated the chromatin state of unattached (free) autosomal duplications, such as the unattached chromosome III duplications, *sDp1*, *sDp2*, and *sDp3*. In any given nucleus, a free duplication can remain unpaired or pair with an intact chromosome, forcing a portion of the intact homolog to be unpaired (see Fig. 2.3). Bean and colleagues detected one to two H3K9me2 foci in nuclei carrying a free duplication, and the timing of H3K9me2 accumulation was similar to that observed in *him-8* mutants. These data are consistent with a mechanism that (i) senses the presence of unpaired/unsynapsed

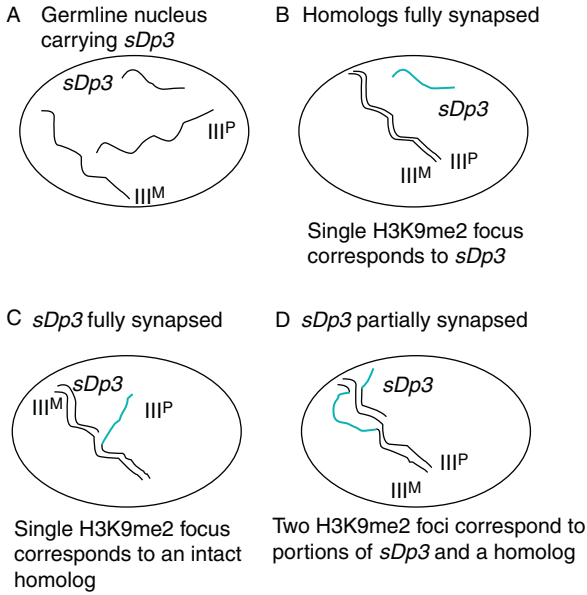

Figure 2.3 Inferred pairing and synapsis in nuclei carrying a chromosomal duplication. (A) Maternal (M) and paternal (P) copies of chromosome III are indicated, as well as the free (unattached) duplication, *sDp3*. *sDp3* corresponds to approximately the left half of chromosome III. (B–D) Meiotic nuclei. Heavy line indicates H3K9me2 enrichment. (B) Maternal and paternal homologs are fully paired and synapsed. *sDp3* is unpaired and unsynapsed, and becomes enriched for H3K9me2. (C, D) Examples where full pairing of the intact homologs is disrupted by pairing with *sDp3*.

chromosomes and chromosomal regions and (ii) targets histone methyltransferase (HMTase) activity to those regions. Presumably the (relatively low level of) HMTase activity is targeted to paired homologs via another mechanism.

MET-2, a candidate HMTase, is required for all H3K9me2 marks in the germ line (Bessler et al., 2010). Proteins that methylate histone lysine residues typically contain a SET (*S*u(var)3–9, *E*nhancer of zeste, *T*rithorax) domain, which is required for catalytic activity. *met-2* was originally analyzed as a part of a systematic survey of the 38 *C. elegans* SET domain protein-coding genes and their potential role in somatic (vulval) development (Andersen and Horvitz, 2007). By indirect immunofluorescence, H3K9me2 is not detected in *met-2* mutant germ lines, even in mutant backgrounds where ectopic H3K9me2 would normally be present (Bessler et al., 2010; E. Maine, unpublished data). However, germ line H3K9me3 is normal in *met-2* mutants (Bessler et al., 2010).

Interestingly, Andersen and Horvitz (2007) implicated MET-2 in H3K9 *trimethylation* in the soma. They identified MET-2 as partially redundant with another SET domain protein, MET-1, in vulval development. MET-2 is an ortholog of human SETDB1, an H3K9 methyltransferase; and MET-1 is an ortholog of *S. cerevisiae* Set2, an H3K36 methyltransferase. Using quantitative protein blots, the authors demonstrated that trimethylation of H3K9 and H3K36 were both reduced in *met-1* and *met-2* mutant embryos, although H3K9me3 was more severely reduced in *met-2* mutants, and H3K36me3 was more severely reduced in *met-1* mutants. The authors concluded that MET-2 was primarily responsible for H3K9me3 and MET-1 was primarily responsible for H3K36me3 in the embryo. However, they did not examine H3K9 dimethylation in the *met-2* soma, therefore it is not known if MET-2 promotes this mark. Presumably the specificity of MET-2 action, that is, as a di- versus trimethyltransferase, is modulated via interactions with other factors, which may be tissue (e.g., germ line) specific.

3.5. Targeting mechanisms

Genetic and molecular studies have identified some of the factors responsible for H3K9me2 (Table 2.2) and H3K27me3 accumulation in the *C.elegans* germ line. At present, completely independent mechanisms appear to be responsible for targeting these modifications to appropriate sites. One common feature of the two mechanisms is that disruption of each can result in inappropriate deposition of silencing marks on autosomes, as described below.

3.5.1. Regulation of germ line H3K9me2 by siRNA-mediated pathway

How is MET-2 activity targeted to unpaired chromosomes during meiosis? One important regulatory mechanism involves small RNAs. The specific accumulation of H3K9me2 on unpaired chromosomes requires activity of a small RNA-mediated pathway whose members include: EGO-1, an RNA-directed RNA polymerase (RdRP); CSR-1, an Argonaute protein; DRH-3, a DEAH/D-box helicase; and EKL-1, a Tudor domain protein (Maine et al., 2005; She et al., 2009). These four proteins function in small RNA-mediated processes such as RNAi and cosuppression (Aoki et al., 2007; Duchaine et al., 2006; Kim et al., 2005; Robert et al., 2005; Smardon et al., 2000; Yigit et al., 2006) and have been shown to interact genetically with components of the Ras/Raf and GLP-1/Notch signaling pathways (Qiao et al., 1995; Rocheleau et al., 2008; She et al., 2009). Recently, they were shown to participate in a biochemical network that produces and utilizes a subclass of small-interfering RNAs (siRNAs) (Claycomb et al., 2009; Gu et al., 2009; van Wolfswinkel et al., 2009). Directly relevant to meiotic silencing, this pathway is required for mitotic chromosome segregation in

Table 2.2 Regulators of H3K9me2 accumulation in the *C. elegans* germ line

Gene	Product	H3K9me2 phenotype	References
met-2	Histone methyltransferase	H3K9me2 absent	Bessler et al. (2010)
ego-1	RdRP family	No H3K9me2 enrichment on unpaired chromatin in XX or XO germ line	Maine et al. (2005)
csr-1	Argonaute family	H3K9me2 reduced on unpaired chromatin and elevated/ectopic at many paired sites	She et al. (2009)
drh-3	DEAH-box helicase	H3K9me2 reduced on unpaired chromatin and elevated/ectopic at many paired sites	She et al. (2009)
ekl-1	Tudor domains; methyl-binding	H3K9me2 reduced on unpaired chromatin and elevated/ectopic at many paired sites	She et al. (2009)
chk-2	Kinase	No H3K9me2 enrichment on male X; delayed H3K9me2 accumulation in XX germ line	Bessler et al. (2007)
him-17	Chromatin-binding protein	Reduced/delayed H3K9me2 accumulation	Reddy and Villeneuve (2004)
rha-1	RNA helicase A	H3K9me2 reduced/absent across genome	Walstrom et al. (2005)
sin-3	HDAC complex assembly	No H3K9me2 enrichment on unpaired chromatin in XX germ line	She et al. (2009) and X. She and E. Maine (unpublished data)

the embryo (Claycomb et al., 2009) (see below). All known components of this functional pathway are essential for fertility (Duchaine et al., 2006; Qiao et al., 1995; She et al., 2009; van Wolfswinkel et al., 2009; Yigit et al., 2006).

Loss of EGO-1, CSR-1, EKL-1, and DRH-3 activity has drastic effects on H3K9me2 accumulation during meiosis. Interestingly, two different defects are observed. In *ego-1* mutants, H3K9me2 does not become enriched on unpaired DNA, and the overall level of germ line H3K9me2 is very low (Maine et al., 2005). EGO-1 activity is not absolutely required for H3K9me2 deposition in germ cells, because the basal level of H3K9me2 normally observed on autosomes is still present in *ego-1* mutants (Maine et al., 2005). Loss of H3K9me2 enrichment on unpaired chromosomes was observed in both *ego-1* null [*ego-1(0)*] mutants and in animals carrying one copy of a null mutation and one copy of a point mutation at a conserved residue in the putative RdRP catalytic domain [*ego-1*(*RdRP/null*)]. Therefore, RdRP activity seems to be specifically required for H3K9me2 enrichment. Cellular RdRPs are responsible for synthesis of siRNAs from RNA templates (Aoki et al., 2007; Makeyev and Bamford, 2002; Pak and Fire, 2007; Sijen et al., 2001, 2007). An obvious hypothesis is that EGO-1 may be responsible for synthesis of siRNAs that target MET-2 and/or other chromatin modifiers to unpaired DNA. A second H3K9me2 phenotype is observed in *csr-1*, *ekl-1*, and *drh-3* mutants. In mutant males, the H3K9me2 level is partially reduced on the X chromosome and is elevated on the autosomes (She et al., 2009). Here, unlike in *ego-1* mutants, H3K9me2 is inappropriately deposited on paired chromosomes. In this study, She and colleagues identified the X chromosome based on an absence of histone activation marks and a condensed morphology. In contrast to wild-type gonads, when dissected *csr-1*, *drh-3*, and *ekl-1* gonads were colabeled for H3K9me2 and an activation mark, H3K4me2, the two marks were observed to colocalize at many autosomal sites. This phenotype is consistent with MET-2 HMTase activity being mistargeted in *csr-1*, *ekl-1*, and *drh-3* mutants and suggests that the activity of CSR-1, EKL-1, and DRH-3 ultimately attracts MET-2 to unpaired chromosomes or excludes MET-2 from paired chromosomes. She and colleagues performed careful analysis of meiotic pairing and synapsis in these mutants to rule out the possibility that pairing or synapsis defects were responsible for the autosomal H3K9me2 foci. All chromosomes appeared to be synapsed based on distribution of HIM-3, an axial component, and SYP-1, an inner component of the synaptonemal complex. When homolog pairing was analyzed using FISH to visualize the 5S ribosomal RNA gene cluster located on LGV, a minor pairing defect was observed in *drh-3* and *ekl-1* mutants. However, the frequency of nuclei where chromosome V was unpaired was much lower than the frequency of nuclei with ectopic H3K9me2, indicating H3K9me2 was abnormally present at paired chromosomal sites in *csr-1*, *ekl-1*, and *drh-3* mutants.

H3K9me2 accumulation was also abnormal in *csr-1*, *ekl-1*, and *drh-3* mutant hermaphrodites (XX) germ lines if unpaired chromosomes or a

chromosomal duplication was present. Normally, H3K9me2 marks are enriched on the chromosomal duplication, *sDp3*, and on unpaired X chromosomes in *him-8* XX mutant hermaphrodites (Bean et al., 2004). This enrichment is reduced or absent in *csr-1*, *ekl-1*, and *drh-3* XX mutants, and H3K9me2 is elevated on other chromosomes (She et al., 2009). These findings suggest a role for the small RNA machinery in directing MET-2 HMTase activity to unpaired chromosomes and/or away from paired chromosomes. One can ask whether H3K9me2 accumulation is actively targeted to unpaired chromosomes or, in contrast, is simply unable to occur when chromosomes are paired. Ectopic H3K9me2 accumulation on paired chromosomes in certain mutant backgrounds provides evidence for a mechanism to actively prevent H3K9me2 accumulation on those chromosomes.

Interestingly, the activity of another RdRP, called RRF-3, is also important for regulation of H3K9me2 levels during meiosis (Maine et al., 2005). Normally, H3K9me2 enrichment disappears from the X chromosome as germ cells undergo spermatogenesis such that very little enrichment is observed in primary spermatocytes. In *rrf-3* mutants, in contrast, H3K9me2 foci are clearly visible in primary spermatocytes. Therefore, H3K9me2 appears to turn over more slowly in these mutants, and H3K9me2 levels may be more highly elevated on the X chromosome, as well. RRF-3 activity is known to be required for activity of the so-called ERI (enhanced RNAi) pathway and for biogenesis of certain siRNAs (Gent et al., 2009; Han et al., 2009; Pavelec et al., 2009; Simmer et al., 2002; and references therein). Evidence suggests that EGO-1 and RRF-3 RdRPs have both interrelated and independent functions (see Gent et al., 2010 and references therein). Prolonged H3K9me2 enrichment in *rrf-3* mutants may indicate that ERI pathway activity directly limits H3K9me2 enrichment. Alternatively, when RRF-3 is absent, EGO-1 may be more readily available to participate in chromatin regulation.

3.5.2. SiRNA functional pathways in the *C. elegans* germ line

Multiple siRNA-mediated pathways are active in the *C. elegans* germ line (Claycomb et al., 2009; Gent et al., 2009; Gu et al., 2009; Han et al., 2009; Pak and Fire, 2007; Ruby et al., 2006; van Wolfswinkel et al., 2009). A number of recent studies have combined genetic and deep sequencing approaches to elucidate interrelated small RNA-mediated pathways in the germ line and soma. In the germ line, two classes of 22G-RNAs (22 nucleotide siRNAs containing a 5′ guanosine) have been described, both of which required DRH-3, EKL-1, and RdRP activity for synthesis but function in conjunction with different Argonaute proteins (Gu et al., 2009). One class of 22G-RNA corresponds to expressed, protein-coding genes and loads specifically onto CSR-1/Argonaute. EGO-1 is responsible for biogenesis of this class of 22G-RNAs. This functional pathway participates in H3K9me2 regulation during meiosis, presumably by regulating MET-2

complex activity (Maine et al., 2005; She et al., 2009). Intriguingly, this pathway participates in kinetochore formation and mitotic chromosome segregation in the early embryo, and has been shown to associate with mitotic chromosomes (Claycomb et al., 2009). An obvious hypothesis is that these factors also associate with chromatin in the meiotic germ line. A second class of 22G-RNA produced by EGO-1 and another RdRP, RRF-1, is loaded on WAGO-class Argonautes. This pathway targets transposase transcripts, aberrant transcripts, and pseudogene transcripts, presumably for degradation via a conventional posttranscriptional silencing mechanism.

Additional components of these two pathways have been identified, including the β-nucleotidyl transferases CDE-1 (CSR-1 pathway) and RDE-3 (WAGO pathway), and MUT-7/RNaseD (WAGO pathway) (Gu et al., 2009; van Wolfswinkel et al., 2009). Evidence from *Schizosaccharomyces pombe* suggests that β-nucleotidyl transferase activity determines whether or not an RdRP complex can recognize a particular RNA as substrate (Motamedi et al., 2004). The existence of distinct β-nucleotidyl transferases required for recognition of distinct sets of RNA templates is consistent with a need to recruit different RdRP complexes to different templates.

How does this regulatory network influence chromatin regulation? In the absence of EGO-1 activity, very few CSR-1 class 22G-RNAs are produced. In the absence of EKL-1 activity, very few 22G-RNAs of either class are produced. In the absence of DRH-3 activity, fewer 22G-RNAs of both classes are produced and they are biased toward the 3′ end of the gene (as if DRH-3 is critical for RdRP processivity). In the absence of CSR-1 activity, 22G-RNAs may be inappropriately loaded onto other Argonautes. It would be useful to know if the 22G-RNA pool is altered in mutants with unpaired chromosomes, for example, *him-8* or *zim-2* mutants. In other words, does the presence of unpaired chromosomes lead to altered RdRP activity? Also, we do not know if there are differences between the X chromosome-derived 22G-RNAs in XO versus XX animals.

Interestingly, many components of the CSR-1 and WAGO siRNA pathways are reported to localize to germ line P granules (Claycomb et al., 2009; Gu et al., 2009), and P granule morphology is altered in germ lines mutant for *ego-1*, *csr-1*, *drh-3*, *cde-1*, and *ekl-1* (Claycomb et al., 2009; Updike and Strome 2009; Vought et al., 2005). An emerging model, based on recent systematic searches for P granule components, is that the P granule serves as a site where the quality of RNAs is assessed as they are exported from the nucleus (Updike and Strome 2009, 2010).

3.5.3. Other germ line regulators of H3K9me2

The function of several other proteins has also been linked to meiotic H3K9me2 accumulation, including RNA helicase A (RHA-1; Walstrom et al., 2005), HIM-17 (a THAP domain chromatin-binding protein; Reddy and Villeneuve, 2004), CHK-2 (related to csd1/chk2 kinases; Bessler et al.,

2007), and SIN-3 (a putative component of type III histone deacetylase (HDAC) complexes; She et al., 2009; X. She and E. Maine, unpublished data). Reduction or loss of function of each of these proteins changes the distribution of H3K9me2 in the meiotic germ line.

Loss of RHA-1 function severely reduces H3K9me2 accumulation on transgenic arrays and the male X chromosome in meiotic germ cells in mutants raised at high temperatures (Walstrom et al., 2005; X. She and E. Maine, unpublished data). In addition, activation marks (e.g., H3K4me2 and H4K16ac/H4K8ac) appear on all chromosomes, including the condensed X chromosome (Walstrom et al., 2005). Some aspects of the *rha-1 (null)* phenotype are temperature sensitive: germ line development and meiotic H3K9me2 accumulation are disrupted in *rha-1* mutants at restrictive temperature but not at permissive temperatures. The *rha-1(0)* mutant also is partially defective in germ line RNAi, a phenotype that is not subject to temperature. One model is that RHA-1 is partially redundant for function with another RNA helicase capable of substituting for RHA-1 at lower temperatures but incapable of doing so at higher temperatures.

HIM-17 is a chromatin-binding protein implicated in H3K9me2 enrichment in both the XX and XO germ line. H3K9me2 enrichment on the male X and at localized regions in the XX genome is reduced and delayed in *him-17(null)* mutants (Reddy and Villeneuve, 2004). In contrast, the basal level of H3K9me2 present across the genome seems to be normal. Genetic analysis indicated that HIM-17 is required for double-strand break formation but not for synapsis. These findings suggest a link between chromatin regulation and double-strand break formation.

CHK-2, a member of the checkpoint kinase family, is required for cell cycle arrest and apoptosis in the germ line in response to UV-induced DNA damage (Stergiou et al., 2007). CHK-2 activity is also required for pairing (MacQueen and Villeneuve, 2001) and, consistent with this observation, CHK-2 has been shown to phosphorylate several target proteins during leptotene/zygotene stage of meiotic prophase I (Penkner et al., 2009). H3K9me2 accumulation is severely reduced in *chk-2* males and delayed in *chk-2* hermaphrodites (Bessler et al., 2007). Perhaps one or more targets of CHK-2 kinase activity promote meiotic H3K9me2 accumulation.

SIN-3 is the sole *C. elegans* ortholog of mammalian Sin3A and SIN3B proteins. Various studies in mammals and yeast have shown that Sin3 proteins bind HDACs as well as proteins involved in nucleosome remodeling, DNA methylation, *N*-acetyl-glucosamine transferase activity, histone methylation, and transcriptional control (Cunliffe, 2008; Silverstein and Ekwall, 2005). Evidence suggests that *C. elegans* SIN-3 activity may be particularly critical for meiotic silencing of chromosomes other than the male X (X. She and E. Maine, unpublished data). One model is that SIN-3 deacetylase activity may allow or promote H3K9 methylation. An

alternative model is that SIN-3 recruits chromatin or nucleosome remodelers whose activity promotes MET-2 activity.

3.6. Enrichment of H3K27me3 on X chromosomes

The accumulation of H3K9me2 on unpaired chromosomes during early meiotic prophase is superimposed on other chromatin marks that may be present in the mitotic germ line. As described above, there is a general lack of histone activation marks on X chromosomes in mitotic nuclei in both the XX and XO germ line. In addition, the X chromosome is enriched for H3K27me3, a histone modification strongly associated with transcriptional repression. This general X-specific enrichment for H3K27me3 was discovered by Strome and colleagues as a consequence of their analysis of the maternal effect controls on germ line development. Capowski et al. (1991) isolated mutations in a set of *mes* (*m*aternal-*e*ffect *s*terile) genes whose expression in the maternal germ line promotes survival and development of germ cells in their progeny. Four genes, *mes-2*, *-3*, *-4*, and *-6*, promote germ cell survival in the larva; in the absence of MES-2, -3, -4, and -6 function, germ cells tended to degenerate during larval development and rarely produced gametes (Capowski et al., 1991; Garvin et al., 1998; Paulsen et al., 1995).

The initial indication that MES protein activity might regulate X chromosome function in the germ line was provided by genetic analysis of *mes* phenotypes in XO, XX, and XXX animals (Garvin et al., 1998). The severity of the *mes-2*, *-3*, *-4*, and *-6* mutant phenotypes was strongly influenced by X chromosome dose; among progeny of *mes* mutant mothers, XO animals had (on average) the mildest phenotype while XXX animals had the more severe phenotype. Using sex determination mutants, Garvin et al. (1998) demonstrated that germ cell survival was linked to X chromosome number rather than sexual identity. One interpretation of these data was that elevated X-linked gene expression in *mes* mutants caused germ cell degeneration; the greater the number of X chromosomes, the stronger the degenerative phenotype. Overall, genetic data were consistent with the hypothesis that MES-2, -3, -4, and -6 function in a mechanism to limit the expression of X-linked genes in the germ line.

Molecular studies revealed a role for the MES system in regulating X chromatin in the germ line. Initially, two different lines of evidence pointed in this direction. MES-2 and MES-6 proteins were shown to be members of the Polycomb group (PcG) family (Holdeman et al., 1998; Korf et al., 1998), proteins later shown to have histone-modifying activity. At the same time, Kelly and Fire (1998) identified *mes-2*, *-3*, *-4*, and *-6* as regulators of transgene silencing: an extrachromosomal transgenic array, normally silenced in the germ line, was expressed (''desilenced'') in *mes* mutant germ lines. Indirect immunofluorescence analysis indicated that H3K9me2

distribution was normal in the *mes* mutants; hence, the MES system appeared to regulate transgenes independently of the meiotic silencing process (Fong et al., 2002). Later studies showed that MES-2, -3, and -6 promote histone H3 lysine 27 di- and trimethylation in the germ line (Bender et al., 2006). In wild type, these two marks are present on all germ line chromosomes, but H3K27me3 is particularly enriched on X chromosomes (Bender et al., 2006). In addition, MES-2, -3, and -6 function to limit the accumulation of histone activation marks on the X chromosome (Fong et al., 2002).

3.7. Regulation of germ line H3K27me3 by Polycomb Repressive Complex 2 (PRC2) system

Three of the four MES proteins, MES-2, -3, and -6, appear to function in a protein complex required for H3K27 methylation in the germ line. MES-2 and MES-6 are orthologs of PcG proteins Enhancer of zeste [E(z)] and extra sex combs, respectively (Holdeman et al., 1998; Korf et al., 1998). MES-3 is a novel protein found in complex with MES-2 and MES-6 (Paulsen et al., 1995; Xu et al., 2001a,b). MES-2 has H3K27 methyltransferase activity, and MES-6 and MES-3 are required for this activity *in vitro* (Ketel et al., 2005). Consistent with the hypothesis that MES-2/-3/-6 complex is responsible for depositing H3K27me3 onto the X chromosome, these proteins are detected in the nucleoplasm (Holdeman et al., 1998; Korf et al., 1998; Xu et al., 2001a). They presumably act directly on chromatin, although a direct association has not been observed.

In contrast to other identified components of the MES system, MES-4 has H3K36 methyltransferase activity and does not physically interact with MES-2, -3, or -6 (Bender et al., 2006; Xu et al., 2001a). MES-4 localizes to the autosomes and active transgene arrays and is largely absent from X chromosomes and silenced transgene arrays (Fong et al., 2002). In the embryo, activity of the MES-2/-3/-6 complex prevents MES-4 from associating with the (oocyte derived) X chromosome (Fong et al., 2002), suggesting that inappropriate MES-4 activity on the X may cause the germ line defects observed in *mes-2*, *-3*, and *-6* mutants. Interestingly, MES-4 is never observed to associate with the sperm-derived X, perhaps due to the imprint described by Bean et al. (2004) (discussed in Section 4.3).

Data suggest that MES-4 is not simply a positive regulator of gene expression. Typically, H3K36me3 marks are found within the exons of active genes (Kolasinska-Zwierz et al., 2009). However, MES-4 does not colocalize with activated RNA pol II, and microarray analysis of *mes-4* mutants revealed little change in autosomal gene expression; instead, a subset of X-linked genes was upregulated, suggesting that the primary function of MES-4 activity on the autosomes is to limit X chromosome gene expression (Bender et al., 2006). Alternatively, MES-4 activity on autosomes may limit MES-2/-3/-6 complex activity to the X, ensuring

H3K27me3 enrichment on the X. Another function for MES-4 may be to mark genes that should be activated in the germ line. In a mutant where MES-4 abnormally associates with the X, some X-linked genes would be inappropriately marked and therefore expressed, in turn impairing germ line function.

In addition to the known MES proteins, other regulators are clearly involved in X chromosomal repression because the pattern of H3K27me3 enrichment across the genome is grossly normal in the germ line of *mes-4* mutants. For example, the absence of MES-4 activity does not lead to elevated H3K27me3 on autosomes or to a visible loss of H3K27me3 on the X chromosome (Bender et al., 2004). The mechanisms targeting MES-2/-3/-6 and MES-4 are unclear, although one obvious model is that noncoding RNA (ncRNA) may participate in the targeting process given the recent data demonstrating interactions between ncRNAs and PRC2 in mammalian tissues (discussed in Section 6, below).

Another regulator of X chromosome silencing, the chromodomain protein MRG-1, shares many features in common with MES-4. MRG-1, like MES-4, ensures survival of primordial germ cells, promotes silencing of X-linked genes in the XX germ line, promotes silencing of extrachromosomal arrays in the (XX and XO) germ line, and localizes to autosomes (Fujita et al., 2002; Takasaki et al., 2007). Similarly, the *mrg-1* phenotype is much less severe in males than in hermaphrodites. The relationship between MRG-1 and MES-4 is unclear at present, although MRG-1 can associate with autosomes even in the absence of MES-4.

Other regulators of X chromosome silencing in the germ line include histone-modifying enzymes and histone variants. For example, the histone variant, HIS-24/H1.1, and SIR-2.1 deacetylase appear to promote H3K27me3 deposition in the germ line (Jedrusik and Schulze, 2007; Wirth et al., 2009). Based on indirect immunofluorescence analysis of mutants, the loss of SIR-2.1 activity correlates with increased H3 acetylation, and methylated H3 lysine 9 does not appear on repetitive transgene arrays in *his-24* mutants. These findings suggest a link between H3K9 deacetylation, H3K9 and H3K27 methylation, and HIS-24 activity. Analysis of the H3K9me2 distribution in *his-24* males has not been reported. It would clearly be of interest to determine whether HIS-24 activity is linked to H3K9me2 enrichment on unpaired chromosomes. As another example, *mes-3* and *mes-4* phenotypes are enhanced by the loss of SET-2 function. SET-2 is a putative H3K4 methyltransferase related to mammalian SET1/MLL (Simonet et al., 2007; Xu and Strome, 2001). Presumably inappropriate H3K4 methylation of active genes on the X chromosome in *mes-3* and *mes-4* mutants contributes to their phenotype. Therefore, it is not immediately clear why the loss of H3K4 methylation exacerbates the *mes* phenotype. Perhaps loss of SET-2 function causes a widespread misregulation of germ line gene expression that exacerbates the ill health of *mes-3* and *mes-4* mutant germ cells.

3.8. Summary

In summary, two distinct mechanisms are known to regulate the X chromosome in the germ line. (i) The meiotic silencing system is active during early meiotic prophase, and targets the H3K9me2 modification to the single (male) X chromosome. In mutant backgrounds where other chromosomes are unpaired/unsynapsed, the meiotic silencing system targets H3K9me2 to those chromosomes. (ii) The MES system is active in mitotic and meiotic germ cells and ensures survival/function of the germ line. MES activity is responsible for histone modification across the genome, although the X appears to be a preferential target. H3K27me3 is well documented to correlate with transcriptional quiescence in a variety of organisms, whereas the link between H3K9me2 and transcriptional repression is less firm. In the *C. elegans* germ line, chromosomal regions can receive H3K9me2 yet remain active, raising interesting questions as to the function of this modification. Perhaps H3K9me2 has a different effect in different chromatin contexts, for example, when accumulating on chromosomal regions entering meiosis in an active state (autosomes) or in a silent state (X chromosomes).

Although progress has been made toward understanding the mechanisms for targeting H3K27me3 and H3K9me2 to appropriate sites, many questions still remain. Clearly, identification of the relevant histone modifiers is an important step. The next step is to understand how these modifiers are directed to appropriate chromosomal locations. Although the mechanisms described above are distinct, one intriguing parallel is both mechanisms appear to maintain a balance between silencing of the correct targets and incorrect targets. For example, CSR-1, EKL-1, or DRH-3 activity allows MET-2, preferentially active on the male X, to have elevated activity on paired/synapsed autosomes. Similarly, loss of MES-2/-3/-6 activity allows MES-4, normally active on autosomes, to associate with the X.

4. Meiotic Silencing and Germ line Development in *C. elegans*

The function of H3K9me2 enrichment is unclear, however it does not appear simply to correlate with the repression of gene expression. Unpaired homologs remain transcriptionally active as evidenced by the presence of histone activation marks (Bean et al., 2004; Jaramillo-Lambert and Engebrecht, 2010) and by extensive genetic data. Mutations such as *him-8* and *zim-2* do not cause major germ line developmental defects; hence, expression of essential maternal effect genes in the germ line is not grossly abnormal in these mutants (Brenner et al., 1979; Phillips and Dernburg,

2006; see other examples discussed below). Moreover, free chromosomal duplications are routinely used in as balancers and in gene dosage studies to demonstrate that a mutation with a germ line phenotype is strictly recessive loss of function. For example, the recessive sterile phenotype of *glp-1* mutants (*glp-1(−/−)*) is rescued in *glp-1(−/−); mnDp37* animals by expression of the wild-type copy of *glp-1* present on *mnDp37* (Austin and Kimble, 1987). Such data suggest that chromosomal regions containing expressed genes can also be enriched for H3K9me2, consistent with the hypothesis that H3K9me2 may accumulate on unpaired chromosomes for a purpose other than—or in addition to—silencing transcription. One caveat is the likelihood that the duplication is paired with an intact homolog in some germ line nuclei (as depicted in Fig. 2.3), and expression of the gene in question in those nuclei may be sufficient to rescue the mutant phenotype. Relevant here is the observed repression of X-linked genes in XO oogenesis (Bean et al., 2004; Jaramillo-Lambert and Engebrecht, 2010; see Section 4.4), which seems to indicate that the regulation of the single X chromosome is different from that of other unpaired chromosomes. Perhaps the meiotic silencing system cannot shut down expression of genes that enter meiosis in an active state (e.g., autosomal genes) but is able to maintain repression of the X, which is silent prior to pachytene.

There is considerable speculation in the literature as to the function(s) of meiotic silencing in animals species. In *C. elegans*, the situation is obviously complicated by the presence of the MES system, a general repressive mechanism that appears to be responsible for repression of X-linked gene expression *per se*. Moreover, the appearance of H3K9me2 on unpaired homologs does not correlate with wholesale reduction in transcription of genes on those chromosomes. In the male, where MES activity does not seem to be essential for germ line survival, H3K9me2 accumulation on the X may provide partial functional redundancy in silencing gene expression, allowing germ line survival and function. However, it seems very likely that H3K9me2 enrichment on unpaired chromosomes, *per se*, has a different function.

Part of the difficulty in understanding the importance of regulating unpaired chromatin is that we do not know the biological consequences of the H3K9me2 mark (or most other chromatin marks, for that matter). Certain modifications correlate with increased or decreased transcription, but cause and effect is rarely clear (e.g., see Wu et al., 2009). During *C. elegans* meiosis, chromatin structure may be controlled for other purposes besides or in addition to transcription, that is, to prevent unpaired chromatin from tripping a checkpoint, for example, or to allow chromosomes to progress through pachytene. Another observation to bear in mind is that the loss of H3K9me2 accumulation on the X chromosome in *ego-1* mutant males did not prevent the chromosome from undergoing its typical condensation or allow activation marks to accumulate (Maine et al., 2005). Hence, the male X is subject to more than one regulatory process. Perhaps

H3K9me2 deposition on the male X, made within the context of other regulatory mechanisms active on that chromosome, has a different outcome than it does within the context of actively expressed autosomal and hermaphrodite X chromosomes. The various proposed functions of meiotic silencing in animals are discussed below in terms of their applicability to *C. elegans*.

In addition to these considerations, H3K9me2 accumulation is not strictly required for production of normal sperm, because *met-2* mutant males are fertile (Bessler et al., 2010). It is not yet clear if these mutants have reduced fertility, particularly over time. *met-2* mutant hermaphrodites are reported as having a mortal germ line (Mrt) phenotype whereby homozygous mutants become sterile after 18–24 generations (Andersen and Horvitz, 2007). The Mrt phenotype is more severe in *met-1; met-2* double mutants, suggesting that the simultaneous loss of multiple histone marks leads to gradual loss of germ line viability and function. It seems surprising that the complete loss of a conserved histone modification would have little apparent effect on tissue function. One explanation for the relatively mild *met-2* phenotype might be that H3K9me2 marks are partially redundant with other histone marks. Alternatively, H3K9me2 accumulation might be more critical in certain environmental conditions not commonly found in the laboratory.

4.1. Repression of DNA insertions

Meiotic silencing in *N. crassa* represses expression of transposons and other DNA insertions. Might *C. elegans* meiotic silencing have a similar function? For example, transcription of DNA insertions might be reduced as a consequence of H3K9me2 accumulation. This function would limit both the detrimental expression of foreign DNA during gametogenesis and the mutagenic effect of transposons. However, transposon activity is repressed via a posttranscriptional mechanism active throughout the germ line (Girard and Hannon, 2008; Golden et al., 2008; Sijen and Plasterk, 2003), which might obviate any need for chromatin-based repression that the meiotic silencing system could provide. Furthermore, many questions remain about how chromatin-based transposon silencing would work in *C. elegans*. As discussed above, unpaired chromosomal duplications are not transcriptionally silenced despite the presence of H3K9me2 marks. Hence, there is no evidence that an unpaired transposon or DNA insertion would be transcriptionally silenced. On a related point, nothing is known about the lower size limit of the H3K9me2 enrichment phenomenon, that is, we do not know how large an unpaired region must be in order to receive the mark. It is not clear whether a single unpaired transposable element could be detected and targeted for H3K9me2 accumulation. These questions will be addressed by the identification of specific sites that become enriched for H3K9me2 during meiotic prophase.

4.2. Maintenance of genome integrity

Another hypothesis is that meiotic silencing mechanisms function in maintaining genome integrity during gametogenesis. The stability and segregation of unpaired chromosomes during meiotic prophase and the meiotic divisions, respectively, may require establishment of particular chromatin structure. A related idea is that meiotic silencing may be important for checkpoint control. There is a meiotic checkpoint in place to detect delays in pairing/synapsis, however in XO individuals of either sex the X chromosome is not subject to this control (Jaramillo-Lambert and Engebrecht, 2010). In other words, the single X chromosome somehow escapes detection by the checkpoint system. One intriguing idea is that the specialized chromatin structure of the single X shields it from detection as unsynapsed (see Gartenberg, 2009; Handel 2004; Kelly and Aramayo 2007). Such functions would be critical for completion of meiosis and, consequently, fertility. The effect may be minor in most nuclei, but the cumulative effect over generations might be large. For example, *met-2* mutant hermaphrodites (which lack germ line H3K9me2) exhibit a variety of germ line defects, each only weakly penetrant (Bessler et al., 2010) but which may contribute to the mortal germ line phenotype observed over many generations (Andersen and Horvitz 2007).

4.3. Epigenetic control of embryogenesis

Meiotic silencing may contribute to the establishment of heritable chromatin marks during spermatogenesis that persist in the early embryo (imprinting). In many organisms, differences between activation of maternally versus paternally inherited genes have been observed. Such differences in gene expression are thought to reflect the patterns of histone marks inherited from each parent, for example, the paternal versus maternal X chromosomes may contain different patterns of histone marks. Such marks might promote embryonic viability and development, including eventual fertility of the offspring (Bean et al., 2004; Huynh and Lee, 2003; Strome, 2005; Turner, 2007; Turner et al., 2006). Bean et al. (2004) compared the chromatin state of the maternal and paternal X chromosomes in XX *C. elegans* embryos. They observed differential regulation of a single chromosome from the sperm pronucleus, which failed to label with antibodies against the activation marks H3K4me2 and H3K9/K14ac. Most sperm-derived chromosomes accumulated these activation marks prior to the first cell division; however, these activation marks did not appear on the sperm-derived X chromosome until the embryo had undergone several rounds of cell division. Interestingly, late activation of the sperm X chromosome was observed regardless of whether the sperm was derived from an XO male or an XX hermaphrodite, suggesting an X chromatin imprint is

characteristic of sperm *per se*. However, the parental origin of the X chromosome did seem to influence the timing of imprint loss: H3K4me2 marks were detected on the hermaphrodite sperm-derived X by 10–14 cell stage, whereas they were not visible on the male sperm-derived X until the 12–20 cell stage. Bean and colleagues hypothesize that the late loss of the X chromatin imprint is related to the unpaired status of the male-derived X chromosome. Consistent with this model, the X chromatin imprint is lost early in the progeny of XX *tra-2* males—where the X chromosomes are paired throughout meiosis as they are in normal hermaphrodites.

Recently, Hammoud et al. (2009) showed that chromatin marks present in human sperm are transmitted to offspring. These marks include activation (H3K4me2, H3K4me3) and silencing (H3K27me3) modifications enriched at developmentally important loci. Similarly, there is also some evidence that histone activation marks established in the *C. elegans* germ line persist in sperm and are transmitted to the embryo (J. Arico and W.G. Kelly, personal communication). Hence, the transcriptional history of genes in the adult germ line, as reflected in the chromatin state of gametes, may have a direct impact on the epigenetic information inherited by the embryo. We do not yet know the physiological consequences of this differential regulation of maternal versus paternal X chromosomes in the *C. elegans* embryo.

4.4. Transcriptional regulation of single X

Does the meiotic silencing system function in transcriptional repression in the XO nucleus? One way to address this question is to examine the effect of H3K9me2 accumulation on oogenic gene expression in XO hermaphrodites. Mutations in the *her-1* gene transform XO soma and germ line to hermaphrodite development (Hodgkin, 1980). In *her-1* XO loss-of-function mutants, oocyte production is reduced and average self-progeny viability is only 1% of wild type (Hodgkin, 1980, 1983). Embryonic lethality can be partially explained by the observation that ~25–30% of the progeny of XO *her-1* hermaphrodites are nullo-X due to loss of the single X chromosome at meiosis (Hodgkin, 1983). However, additional factors must further reduce progeny viability and oocyte production. Bean et al. (2004) analyzed H3K9me2 accumulation in XO null mutants and found that the single X was enriched for H3K9me2 in pachytene–diplotene stages, that is, at the time when there is normally a burst of X-linked transcription. Moreover, the levels of at least some X-linked transcripts are lower in XO versus XX hermaphrodite germ lines as analyzed by *in situ* hybridization, suggesting that the elevated H3K9me2 may limit transcription. XO *her-1(null)* hermaphrodites produced oocytes but no viable progeny, leading Bean and colleagues to hypothesize that inappropriate silencing of the single X chromosome during meiosis had impaired oogenesis and resulted in embryonic lethality. It is not clear if the difference

in brood sizes between the different *her-1* alleles is important, however the data are consistent with the hypothesis that H3K9me2 accumulation on the single X may reduce gene expression. In their analysis of checkpoint controls, Jaramillo-Lambert and Engebrecht (2010) showed that transcription from the single X chromosome in XO *fem-3* hermaphrodites is also inappropriately silenced during late pachytene. Hence, the single X chromosome is subject to unique regulation regardless of germ line sex.

4.5. Chromosome evolution

Over time, differential regulation of the heterogametic sex chromosomes may also have influenced chromosome evolution. In nematodes, X-linked essential genes often have an autosomal paralog that is active throughout germ line development (Maciejowski et al., 2005; Ohmachi et al., 2002). In males, in particular, very few X-linked genes are expressed in the germ line. The dearth of X-linked gene expression in the germ line may be an adaptation to prevent silencing of essential genes. Meiotic silencing may aid in repressing/ensuring the low level of X-linked gene expression; as described above, *in situ* hybridization experiments indicate that X-linked oogenic genes fail to express properly in the feminized XO germ line, an outcome that suggests the single X chromosome is transcriptionally silenced (Bean et al., 2004; Jaramillo-Lambert and Engebrecht, 2010). It is difficult to know which came first, migration of germ line-essential genes off of the X or establishment of repressive X chromatin structure.

4.6. Summary

Hypotheses abound as to the possible function(s) of meiotic silencing. To resolve this question for *C. elegans*, we need to identify the targets of H3K9me2 modification and better understand the effect of such modifications on chromatin structure. Another consideration to bear in mind is the possibility that meiotic H3K9me2 enrichment on the single X chromosome in the XO germ line may produce a different—or partially overlapping—set of outcomes than does H3K9me2 enrichment on homologs (autosomes or Xs) that remain unpaired/unsynapsed due to mutation.

5. Mechanistic and Functional Comparison of Meiotic Silencing Phenomena in Different Species

As discussed in Section 1, the term meiotic silencing is applied to a variety of phenomena in diverse species. As these processes have been studied, researchers have uncovered similarities and differences among them with

regard to the mechanisms involved and effects on gene expression. Many mechanistic details of meiotic silencing seem to be integrated with other, simultaneous processes occurring in the germ line, as described below.

5.1. Meiotic silencing in *N. crassa*

Meiotic silencing is best understood in the fungus, *N. crassa*, where it targets expression of individual unpaired genes and paired genes whose homology to each other is lower than a particular threshold. In marked contrast to *C. elegans* and other animal models, evidence suggests that meiotic silencing in *N. crassa* occurs at a posttranscriptional level. For example, meiotic silencing elicited by a chromosomal deletion will target transcripts produced from paired copies of the deleted region (present as transgenes) that are located at a distinct site (Lee et al., 2004; Shiu et al., 2001). Similarly, the presence of a chromosomal duplication will trigger silencing of transcripts produced from the corresponding paired regions. Components of the silencing pathway also are required for successful meiosis and gametogenesis, perhaps because they function in developmental gene regulation (Shiu and Metzenberg, 2002; Shiu et al., 2001). Meiotic defects may reflect a failure to silence unpaired regions and/or a failure of the MSUD machinery to engage in other regulatory activities. For example, one component is an RdRP whose activity may promote the biogenesis of siRNAs required for developmental regulation of gene expression. Similarly, *C. elegans* meiotic silencing mutants also tend to be sterile or subfertile.

There are striking parallels between meiotic silencing in *N. crassa* and posttranscriptional mechanisms of transposable element silencing that have been described in *C. elegans* and other animal species (see Girard and Hannon, 2008). These mechanisms utilize the core RNAi machinery. For example, meiotic silencing in *N. crassa* utilizes an RdRP (SAD-1), an Argonaute protein (SMS-2), and a Dicer-like protein (SMS-3) (Alexander et al., 2008; Lee et al., 2003; Shiu et al., 2001). Indeed, the most obvious difference described so far between small RNA-mediated transposable element silencing in animal germ lines and MSUC in *N. crassa* is simply the timing of onset: transposition in animals is repressed throughout the proliferating *and* meiotic germ line, whereas MSUD specifically initiates in early meiotic prophase. The mechanistic similarities may reflect a common origin for these posttranscriptional silencing processes as adaptations of an ancestral genome defense mechanism.

5.2. Meiotic silencing in insects

It has been unclear whether sex chromosomes are silenced during insect meiosis. In *Drosophila*, X-linked gene expression is similar in male and female germ lines (Gupta et al., 2006). Moreover, synapsis—which

commonly prevents meiotic silencing—does not occur in the male germ line (although homologs do align) (see Zickler, 2006). Hence, the current thinking is that sex chromosomes are not silenced during meiosis in *Drosophila* males. In contrast, recent analysis of X chromosome regulation in grasshopper has revealed some features in common with XY-body regulation in mouse (Cabrero et al., 2007). Like *C. elegans*, the grasshopper *Eyprepocnemis plorans* lacks a Y chromosome; males are XO. In *E. plorans*, histone activation marks (e.g., acetylation of H3K9) are observed on the male X chromosome in mitotic germ cells, suggesting some X-linked genes are expressed prior to meiotic entry. These activation marks are lost at the very onset of meiotic prophase (early leptotene stage). By late zygotene stage, the male X chromosome accumulates histone silencing marks and histone variants similar to those associated with MSCI in mouse, including H2AX phosphorylation (gamma-H2AX) (Cabrero et al., 2007). Based on these results, meiotic silencing (or a related process) does appear to occur in *E. plorans*. Many questions remain to be addressed, including the identity of the silenced genes and whether the silencing mechanism also targets unpaired autosomes or autosomal duplications.

5.3. Meiotic silencing in mammals

Chromatin-based silencing of the male sex chromosomes has been described in both eutherians such as mouse (Turner, 2007) and marsupials such as opossum (Franco et al., 2007; Namekawa et al., 2007). The process is best characterized in mouse, where several histone marks accumulate on the XY-body, including H3K9me2 (Khalil et al., 2004), H2A ubiquitination (Baarends et al., 2005; de Vries et al., 2005), and phosphorylation of the histone 2A variant, H2AX (Handel 2004). In addition, some marks of active chromatin are absent from the male sex chromosomes (e.g., H3K9ac, Khalil et al 2004; Namekawa et al., 2006), while other marks are present (e.g., H3K4me2; Khalil et al., 2004). Furthermore, some histones are replaced by variants; for example, H3.1 and H3.2 are replaced with H3.3 (van der Heijden et al., 2007). Interestingly, *C. elegans* has the reverse situation wherein the single X remains devoid of histone variant H3.3 while paired/synapsed chromosomes accumulate it (Ooi et al., 2006).

Disruption of the meiotic silencing process in both mice and opossum leads to elevated expression of X-linked genes as assayed by real-time PCR and by association of active RNA pol II with the sex chromosomes (see Turner, 2007; Zamudio et al., 2008). In normal development, expression of some Y- and X-linked genes decreases as early as the onset of first meiotic prophase (Wang et al., 2005). Reminiscent of the situation in *C. elegans*, many X-linked essential genes have an autosomal paralog that is active during male meiosis (Wang, 2004). In mouse, disruptions in male sex chromosome regulation

correlate with meiotic progression defects and sterility (Handel, 2004; Mahadevaiah et al., 2008; Turner, 2007; Zamudio et al., 2008).

As in *C. elegans*, meiotic silencing in mammals naturally targets the male sex chromosomes, but also targets large unpaired chromosomal regions such as chromosomal translocations that may be present in either the XX or XY germ line. Analysis of mouse strains carrying chromosomal rearrangements or synapsis mutants has shown that unsynapsed autosomes and autosomal translocations are targeted for H3K9me2 and other diagnostic silencing marks during meiotic prophase in the male and female germ line (Baarends et al., 2005; Turner et al., 2005). Histone replacement occurs on both the XY-body and asynapsed autosomes (van der Heijden et al., 2007). Based on the presence of histone variants associated with transcriptional repression and the absence of RNA pol II (as assayed by indirect immunofluorescence), these unsynapsed autosomal regions appear to be silenced. Therefore, a general MSUC phenomenon was judged to be present in mouse, and MSCI is considered to be the "natural" result of MSUC.

Recent studies indicate that the meiotic silencing machinery can be overloaded by the presence of multiple unsynapsed chromosomes or translocations, leading to reduced efficiency in silencing the XY-body (Homolka et al., 2007; Kouznetsova et al., 2009; Mahadevaiah et al., 2008). In mouse, defective autosomal synapsis is associated with meiotic arrest and infertility, perhaps due at least in part to a failure to repair double strand breaks (Turner, 2007). The presence of unpaired chromosomal duplications is linked to male meiotic prophase arrest, and defects in pairing or synapsis generally result in inappropriate silencing of the affected regions, which presumably accounts for the subsequent meiotic failure and/or germ cell death (Turner, 2007). It is unclear whether silencing of autosomal genes also contributes to the observed meiotic defects. Clearly, mutations in the MSCI machinery lead to meiotic arrest, however cause and effect is not clear (see discussion in Turner, 2007). It has been suggested that meiotic silencing in this context may function to abort production of sperm with DNA insertions. Alternatively, meiosis may fail because the meiotic silencing machinery may be diverted from regulation of the sex chromosomes to regulation of other unpaired regions (Homolka et al., 2007; Schimenti, 2005; see Turner, 2007). This characteristic of meiotic silencing is distinct from the situation in *C. elegans*, where unpaired/unsynapsed regions (with the exception of the single X) do not appear to be transcriptionally silenced.

The mechanism of meiotic silencing in mouse requires activity of a set of DNA-damage recognition and repair proteins (Turner, 2007 and references therein). In addition, the XY-body accumulates H2A variants associated with the DNA damage response (Turner, 2007). Meiotic silencing is thought to be triggered by the association of BRCA1 protein with unsynapsed chromosomes, in turn recruiting the ATR checkpoint kinase, which

then phosphorylates H2AX (Fernandez-Capetillo et al., 2003; Turner et al., 2004, 2005). Other unsynapsed regions that may be present (e.g., translocations) also accumulate these factors (van der Heijden et al., 2007). This aspect of the meiotic silencing machinery is apparently not shared with *C. elegans* because orthologs of many XY-body-associated proteins and histone variants are either absent from *C. elegans* (e.g., H2AX; http://www.wormbase.org) or have no apparent role in meiotic silencing (e.g., BRCA1; W.G. Kelly, personal communication).

5.4. Meiotic silencing in birds

In chicken, where females are the heterogametic sex, the single Z and W chromosomes are subject to MSCI (Schoenmakers et al., 2009). The observation of MSCI in birds is important because it indicates that this phenomenon is not specific to spermatogenesis, but also functions in oogenesis. As in XX/XY species, meiotic silencing in chicken occurs during pachytene–diplotene stages of first meiotic prophase. The major evidence for MSCI at present is the absence of activated RNA pol II from the ZW chromosome pair and a reduction in Z-linked mRNA transcript levels during meiotic prophase (Schoenmakers et al., 2009). In addition, the ZW pair becomes enriched for H3K9me3 and other histone silencing marks, as well as specialized histone variants typically associated with nontranscribed loci. Very recently, it was shown that a specialized chromosome present in a single copy in male zebra finch (the "germ line restricted chromosome") is silenced during male (ZZ) meiosis, indicating the presence of a general mechanism for silencing unsynapsed chromatin (Schoenmakers et al., 2010).

The processes of MSCI in chicken and mouse show several differences with respect to the relative timing of events, for example, the meiotic stage at which gamma-H2AX associates with each sex chromosome. Moreover, the mechanisms of MSCI are different in terms of the importance of the synaptonemal complex. In chicken, Z and W are fully synapsed during meiosis, hence MSCI occurs despite the presence of a synaptonemal complex. This situation is distinct from that in the mouse where failure to synapse is thought to be the trigger for MSCI (see Turner, 2007). Perhaps the failure of homologs to pair (or subsequent nonhomologous synapsis) is the trigger for MSCI in birds. Given these results, different species appear to rely on synapsis-dependent or -independent sensing/triggering mechanisms.

5.5. Other meiotic transsensing phenomena in vertebrates

Small RNAs have not yet been linked to meiotic silencing in vertebrates, but they have been implicated in other meiotic transsensing phenomena. For example, the mouse MAELSTROM protein is known to physically interact with two Argonaute proteins and is required for transposon repression and

fertility (Soper et al., 2008). Although initially reported to localize to the XY-body (Costa et al., 2006), subsequent analysis indicated that it is not required for meiotic silencing and does not localize to the XY-body (Soper et al., 2008). More importantly, an epigenetic silencing phenomenon related to paramutation in plants has been reported in mouse (Grandjean et al., 2009; Herman et al., 2003; Rassoulzadegan et al., 2002, 2006; Worch et al., 2008). Paramutation is a phenomenon whereby one allele induces a heritable change in another naive allele present in *trans* (Chandler et al., 2000). Most of the documented cases involve repression of the naive allele by a "paramutator" allele; the induced allele remains silent even in individuals who do not inherit the original paramutator allele (Chandler et al., 2000). Paramutation does not involve a change in DNA sequence and is presumed to be an epigenetic phenomenon. In plants, paramutation has been shown to require RdRP activity, indicating a probable role for small RNA in establishing the heritable change in gene expression (Alleman et al., 2006, Sidorenko and Chandler, 2008). It is early days for the analysis of paramutation-related phenomena in mammals, however data suggest that small RNA participates in the process (Cuzin et al., 2008). Hence, epigenetic regulation during mammalian meiosis may involve small RNA.

5.6. Summary

Based on current data, the importance of meiotic silencing to germ line function in *C. elegans* is unclear. While many intriguing hypotheses can be proposed, a clear understanding of the biological consequences of H3K9me2 accumulation on unpaired chromosomes awaits further molecular analysis.

6. Noncoding RNA and Chromatin Structure

Although the small RNA machinery was first described as regulating gene expression at a posttranscriptional level, these pathways have now been implicated as regulators of chromatin structure, chromosome segregation, and chromosome stability in plants, animals, fungi, and ciliated protozoa (Moazed, 2009; Peters and Meister, 2007; Zaratiegui et al., 2007). Small RNAs participate in posttranscriptional silencing by acting as guides to target an Argonaute protein-containing complex to specific mRNA targets (Hammond, 2005; Peters and Meister, 2007). siRNAs are thought to serve a similar guide function during transcriptional control where they participate in a transcriptional silencing complex.

The best-studied example of siRNA-mediated chromatin regulation is the transcriptional silencing of centromeric repeats in *S. pombe*, which utilizes components of the RNAi machinery to target H3K9me2 marks

(Djupedal and Ekwall, 2009; Verdel et al., 2009; Volpe et al., 2002; White and Allshire, 2008). This machinery shares many components with the meiotic silencing machinery described in *C. elegans*, although some important differences exist. In the *S. pombe* system, an RdRP complex is proposed to convert RNA pol II-derived single strand RNA, generated at centromeric repeats, to dsRNA; components of the RdRP complex include RdP1 (RdRP), Hrr1 (helicase), and Cid12 (poly(A) polymerase). DsRNA is processed by Dicer to form siRNAs, which seed the assembly of an RNA-induced transcriptional silencing complex (RITS) whose components include Ago (Argonaute protein), Chp1 (chromatin-binding protein), and Tas3 (a protein responsible for ''spreading'' H3K9me2 marks along the chromosome). RITS in turn is hypothesized to recruit chromatin-modifying enzymes, for example, Clr4 HMTase (Buhler and Moazed, 2007; Buhler et al., 2007; Cam et al., 2005; Iida et al., 2008; Zhang et al., 2008). In this system, Chp1 must physically bind existing H3K9me2 marks in order for the machinery to deposit additional H3K9me2 marks on adjacent nucleosomes (Schalch et al., 2009). Although the details are unclear, it is hypothesized that activity of the siRNA pathway establishes a ''self-amplifying'' loop at centromeric DNA, leading to further recruitment of HMTase and further deposition of H3K9me2 marks (Buhler et al., 2007; Iida et al., 2008; Schalch et al., 2009; Zhang et al., 2008; see also Buhler and Moazed, 2007). In contrast to this mechanism, Dicer is not required for meiotic silencing in *C. elegans* (Maine et al., 2005), presumably because EGO-1-dependent 22G RNAs are generated in a Dicer-independent manner (Gu et al., 2009).

Recent studies have implicated ncRNAs—both large and small—in heterochromatin assembly in mammals. A mammalian RdRP has recently been identified (Maida et al., 2009), and antisense transcripts are implicated in epigenetic regulation in mouse (Yu et al., 2008) and humans (Han et al., 2007; Morris et al., 2008). For example, Yu et al. (2008) report that inappropriate production of antisense transcripts due to mutation can result in silencing of genes that should be active, for example, tumor suppressor genes. Hence, inappropriate production of antisense transcripts due to mutation may repress gene expression at the chromatin level, leading to tumor growth and perhaps other disease conditions. It is estimated that antisense RNAs may be present for the majority of mouse transcripts, hence antisense transcripts may normally promote chromatin-based silencing of some genes. The mechanism of heterochromatin formation in this case is not understood, except that Dicer is not required, and therefore microRNA and other small RNAs whose biogenesis requires Dicer activity are not involved. Recent work in mammals has also demonstrated the association of chromatin-modifying complexes, such as PRC2 and an H3K9me2 methyltransferase complex called G9, with large intergenic noncoding (linc) RNAs (Khalil et al., 2009; Nagano et al., 2008; Ponting et al., 2009; Zhao et al., 2008). At present,

the relative importance of different classes of ncRNAs in recruiting chromatin modifiers to specific genomic sites is unclear. However, it seems likely that small RNA-mediated mechanisms of chromatin regulation in mammals will share some features with regulatory mechanisms being uncovered in *C. elegans* and *S. pombe*.

7. Conclusions and Future Prospects

Many unresolved questions remain regarding the mechanism and function of meiotic silencing in *C. elegans* and other species. Outstanding issues include the mechanism for sensing chromosome targets (i.e., unpaired/unsynapsed chromosomes), mechanisms for targeting H3K9me2 to them, and the function of H3K9me2 enrichment on those chromosomes. In addition, it is important to understand how H3K9me2 enrichment on unpaired chromosomes is related to the basal H3K9me2 accumulation detected across the genome and the physiological H3K9me2 enrichment occurring in the XX germ line during late meiotic prophase.

7.1. Mechanism

What characteristic of a chromosome makes it a target for H3K9me2 enrichment? Based on studies in *C. elegans* and other organisms, two likely possibilities are that the cell assesses homolog alignment and/or synapsis. For example, the histone modifying machinery might be recruited via activity of a factor that is associated with unpaired or unsynapsed chromosomes but lost upon pairing or synapsis. Alternatively, it might be excluded via the activity of a factor that associates with chromosomes during pairing or synapsis. Recent evidence suggests that the XX and XO germ lines may use (at least partially) different mechanisms to identify chromatin for H3K9me2 accumulation (A. Fedotov and W.G. Kelly, personal communication).

It is important to resolve the mechanistic details of how the EGO-1/CSR-1 siRNA pathway, RHA-1, and other factors implicated in H3K9me2 regulation in fact act to target MET-2 activity to unpaired chromosomes. For example, does the siRNA machinery act directly on chromatin as is the case for centromeric regulation in *S. pombe*? Which component(s) confer specificity with respect to the target sites? How are the activities of these different factors integrated? A related question is how the mechanisms of basal and physiological H3K9me2 deposition are related to meiotic silencing. Based on its mutant phenotype (general loss of H3K9me2), RHA-1 may promote MET-2 activity, perhaps functioning as a component of the MET-2 complex. Are the ectopic H3K9me2 foci

observed in *csr-1*, *ekl-1*, and *drh-3* mutants the result of upregulated modification of what are normally sites of basal or physiological H3K9me2?

7.2. Function

A central remaining question is how H3K9me2 modifications function at target sites. One strategy to address this question is to identify those targets and determine how loss of H3K9me2 (e.g., in an *ego-1* mutant) might alter their expression—if indeed those sites are within coding regions. According to recent reports, EGO-1, DRH-3, and EKL-1 are responsible for the biogenesis of different classes of 22G-RNAs, one of which functions in posttranscriptional gene silencing (of transposons, pseudogenes, etc.) and another of which functions in mitotic chromosome segregation (Claycomb et al., 2009; Gu et al., 2009). The former class of siRNA acts as guide molecules for RISC complexes containing a set of partially redundant WAGO Argonautes, whereas the latter class of siRNA guides RISC complexes containing CSR-1/Argonaute (Claycomb et al., 2009; Gu et al., 2009). One hypothesis is that CSR-1 activity, targeted by EGO-1-generated siRNA, guides MET-2 to unpaired chromosomes and/or prevents MET-2 from acting on paired chromosomes. Ectopic H3K9me2 observed in *csr-1*, *drh-3*, and *ekl-1* mutants may reflect the inappropriate interaction of EGO-1-generated siRNAs with another RISC/Argonaute complex that normally is responsible for basal and/or physiological H3K9me2 accumulation on autosomes.

Critical to understanding the role of the chromatin-based meiotic silencing observed in animals is to determine the lower size limit for regions targeted by meiotic silencing in each species. For example, if regulation of individual genes is important, then meiotic silencing may act at the level of individual genes. If establishing chromatin structure across the chromosome is important, then meiotic silencing may act on a larger scale, targeting transcribed and nontranscribed regions. *C. elegans* has a robust posttranscriptional mechanism for repressing transposon activity, hence there may not be much selection pressure to repress transposons at the transcriptional level.

Does meiotic silencing function in speciation? We know that heterologous pairing in *C. elegans* leads to incomplete synapsis, and that H3K9me2 is elevated on regions lacking a synaptonemal complex. It could be informative to determine whether meiotic chromosomes are enriched for H3K9me2 in interspecies hybrids, in particular males. Does elevated autosomal H3K9me2 correlate with male sterility in these cases? The answer may be yes: evidence from studies in *Neurospora* indicates that hybrid sterility can be partially alleviated when meiotic silencing is impaired (Shiu et al., 2001). On the flip side, evidence from mammals suggests that (at least some) components of the meiotic silencing machinery are limiting such that meiotic silencing of the X chromosome is less efficient when multiple unsynapsed regions are present (Homolka et al., 2007; Kouznetsova et al., 2009).

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