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Epigenetic Regulation of Germline Development in *C. elegans*

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DEVELOPMENTAL BIOLOGY

by

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ABSTRACT

Epigenetic Regulation of Germline Development in *C. elegans*

Laura Gaydos

To retain cell identity during development, cells must remember patterns of gene expression through cell division. The MES proteins in *Caenorhabditis elegans* are key regulators of gene expression in the germline and are necessary in parents for fertility in the next generation. MES-2, MES-3, and MES-6 form the *C. elegans* Polycomb Repressive Complex 2 (PRC2) and generate a repressive histone modification, methylation on Lysine 27 of Histone H3 (H3K27me), on genes repressed in the germline. MES-4 generates a different histone modification, H3K36me, on genes expressed in the germline. To further define the targets of MES protein regulation, determine if they work together to regulate gene expression, and investigate when they are important during germline development, I used genomics, genetics, and microscopy approaches. Transcript profiling of dissected mutant germlines revealed that MES-2/3/6 and MES-4 cooperate to promote expression of germline genes and repress somatic genes and genes on the X chromosome. Loss of MES-4 from germline genes causes H3K27me3 to spread to germline genes, resulting in reduced H3K27me3 elsewhere on the autosomes and especially on the X. This finding supports the model that methylation of H3K36 antagonizes methylation of H3K27 on the same histone tails. My finding that loss of MES-2, 3, or 6 results in expression from the X chromosomes and sterility, unless the X chromosome is repressed by other means, showed that the essential role of MES-2/3/6 in worms is

repression of the X chromosomes in germ cells. I determined that repressive H3K27me3 is transmitted to embryos by both sperm and oocytes. By generating embryos containing some chromosomes with and some lacking H3K27me3, I showed that in embryos lacking MES-2/3/6 function, H3K27me3 is transmitted to daughter chromatids through several rounds of cell division. In embryos with MES-2/3/6 function, the mosaic pattern of H3K27me3 is perpetuated through embryogenesis. Subsequently, during germline proliferation in larval stages, H3K27me3 accumulates on all chromosomes. During germline proliferation in larvae is also when I found MES-3 to be most important for germline development in the next generation. These latter two findings suggest that germline memory is reset during germline proliferation. Taken together, my findings support a "germline memory model" in which MES-4 and H3K36me act as a memory of germline gene expression and help concentrate MES-2/3/6 repression on the X chromosome. MES-2/3/6 and H3K27me3 act as a memory of germline gene repression, most importantly on the X chromosomes. The germline memory generated by the MES proteins is epigenetically transmitted across generations and is critical for the proper development of nascent germ cells.

ACKNOWLEDGMENTS

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Members of the Strome Lab have created a great working environment during my time in the lab and I am grateful to have worked with them. My friends and family have all helped in their own ways and I thank all of them for their loving support.

CHAPTER 1: Introduction

Germline Development in *C. elegans*

Germ cells differentiate into eggs and sperm and must maintain different characteristics than somatic cells. These special germ cell characteristics allow for maintenance of totipotency and immortality. For germ cells, this means that in the next generation all cell types are generated (totipotent) and this can continue for generation after generation (immortal) (SEYDOUX and BRAUN 2006; CINALLI *et al.* 2008). To retain these special characteristics, germ cells must regulate gene expression so that genes needed for germ cell development are expressed and genes that promote somatic differentiation are repressed (STROME and LEHMANN 2007). Mechanisms used by germ cells to regulate gene expression are the focus of this dissertation.

Though germ cells across species share the properties of immortality and totipotency, the mechanisms that set germ cells apart from somatic cells vary across phyla and fall into two main categories: "induction" and "preformation" (EXTAVOUR and AKAM 2003; SEYDOUX and BRAUN 2006). In mammals, primordial germ cells arise through "induction"; embryonic cells in a specific location in the epiblast-stage embryo are induced to develop as germ cells (TAM and ZHOU 1996). In *C. elegans*, *Drosophila*, *Xenopus* and zebrafish, PGCs are specified by "preformation"; germ cells are set apart from somatic cells during early development and inherit specialized "germ plasm" supplied by the oocyte (SAFFMAN and LASKO 1999).

In *C. elegans* embryos, a series of four asymmetric divisions progressively separate the germline from the soma (Figure 1-1). This separation can be traced by the segregation of cytoplasmic granules in the germ plasm. These granules segregate asymmetrically at the first cell division to the germline blastomere (P1 cell) and hence were named P granules (STROME and WOOD 1982; UPDIKE and STROME 2010). P granules continue to be asymmetrically segregated to the P-cell daughter (P2, P3, P4) at each of the following three asymmetric divisions (Figure 1-1). At the ~100-cell stage, the P4 cell divides symmetrically to form the two primordial germ cells (PGCs) Z2 and Z3 (SULSTON *et al.* 1983) (Figure 1-1). Z2 and Z3 remain quiescent until the embryo hatches as an L1 larva, when the germline program is turned on and the PGCs begin proliferation.

Though specification of germ cells varies in different organisms, it is common for PGCs to repress somatic programs of gene expression (SEYDOUX and BRAUN 2006; STROME and LEHMANN 2007). *C. elegans* germline blastomeres (P0, P1, P2, P3, and P4) repress a somatic fate by globally repressing transcription (SEYDOUX and FIRE 1994; WANG and SEYDOUX 2013). PIE-1 accumulates specifically in the P cells and mediates transcriptional repression in those cells by inhibiting transcription elongation until Z2 and Z3 are born at the ~100-cell stage (MELLO *et al.* 1996; SEYDOUX *et al.* 1996; SEYDOUX and DUNN 1997; ZHANG *et al.* 2003). Although PIE-1 becomes undetectable shortly after Z2 and Z3 are born, those cells remain largely transcriptionally repressed through the rest of embryogenesis. Since the decrease in PIE-1 coincides with a change in histone modifications, it is thought that this

transition marks a switch from PIE-1 repression to chromatin-based repression (SCHANER *et al.* 2003). Once the embryo hatches and feeds, the PGCs turn on the germline transcription program and acquire histone modifications associated with transcription (SCHANER and KELLY 2006).

The PGCs proliferate during larval development to generate either a hermaphrodite adult with ~2,000 germ cells organized in two gonad arms (Figure 1-1) or a male with ~1,000 germ cells organized in one gonad arm (KIMBLE and WHITE 1981). The PGCs begin mitotic proliferation at the L1 stage, and mitotic proliferation continues throughout adulthood. Germ cells enter meiosis in the L3 stage. During the L4 stage, the initial cells to undergo meiosis differentiate into sperm. In hermaphrodites, sperm are stored in the spermatheca, after which adults switch to making oocytes, which are fertilized as they pass through the spermatheca (HIRSH *et al.* 1976). Males produce sperm through adulthood. Male sperm transferred to hermaphrodites during mating are able to fertilize hermaphrodite oocytes (KLASS *et al.* 1976). Production of both sperm and oocytes are required for fertility of hermpahrodites, and mutations in any part of these processes result in sterile worms.

A group of proteins necessary for germ cell proliferation and production of oocytes and sperm (gametes) were discovered in a screen for maternal-effect sterile (*mes*) mutants and are called the MES proteins. A maternal supply of *mes*(+) gene product is required for development of the germline in the next generation (CAPOWSKI *et al.* 1991) (Figure 1-2). A heterozygous *mes*/+ mother contributes maternally synthesized *mes*(+) gene products to oocytes and produces homozygous

mes/mes embryos, which inherit the maternally contributed MES(+) protein but do not make their own MES(+) protein. This initial M+Z- *mes/mes* generation (M+ for maternal load, Z- for no zygotic synthesis) is fertile due to the maternal contribution. However, the next generation of *mes/mes* animals has no maternal MES protein (M-) or zygotically produced MES protein (Z-) and such M-Z- mutants develop into sterile adults (Figure 1-2). In the M-Z- generation, germ cells undergo only a few rounds of cell division after L1s hatch and then degenerate midway through larval development to produce a sterile but otherwise healthy adult (CAPOWSKI *et al.* 1991; GARVIN *et al.* 1998). Some exceptions to this phenotype exist in worms with only one X chromosome (males), which are discussed in Garvin et al. (1998) and further investigated in Chapter 3. The MES proteins regulate gene expression in the germline by generating histone modifications, as introduced in the next section.

Chromatin Regulation by the MES Proteins

Many proteins are involved in regulating the compaction and accessibility of DNA in the nucleus. At the first level of DNA compaction, DNA is wrapped around protein complexes composed of histones. A histone octamer with DNA wrapped around it is a nucleosome and is the repeating unit of chromatin (KORNBERG 1974). Differential packing of DNA is associated with regulation of gene expression. Active genes were initially found to be associated with less dense packing of nucleosomes, based on their accessability to digestion with DNase 1, while repressed regions were found to be less accessible to digestion by DNase 1 (WEINTRAUB and GROUDINE

1976). This early observation and years of subsequent work led to the view that the genome is made up of regions or domains of expressed genes and domains of repression and that nucleosome presence and positioning influences these states (ELGIN 1990).

Nucleosomes contain two each of histone H2A, H2B, H3 and H4, which have tails extending from their core. These tails can have various post-translational modifications (LUGER *et al.* 1997; LUGER and RICHMOND 1998) (Figure 1-3). There are several different types of histone modifications that vary in location (histone subunit and amino acid) (KOUZARIDES 2007; BANNISTER and KOUZARIDES 2011). The initial finding that demonstrated a histone modification could control gene expression was in *Tetrahymena*; a protein that was previously found to control transciption was discovered to have histone acetyltransferase (HAT) activity (BROWNELL *et al.* 1996). The hypothesis that a combination of localized histone modifications inform different downstream functions, such as gene expression or repression, was termed the "histone code hypothesis" (REA *et al.* 2000; STRAHL and ALLIS 2000; JENUWEIN and ALLIS 2001). The histone code hypothesis spurred intense research aimed at understanding which proteins generate, read, and erase each histone modification and what downstream function(s) is associated with each modification (GARDNER *et al.* 2011).

Two well-studied groups of proteins that generate and associate with histone marks are the Polycomb group (PcG) and trithorax group (trxG). These groups were identified by their ability to maintain the silent (PcG) or active (trxG) states of the

Hox genes in *Drosophila* (KENNISON 1995). Polycomb Repressive Complex 2 is a complex made of a subset of the PcG proteins and is composed of four main components: E(Z), ESC, SU(Z)12 and NURF-55 (MARGUERON and REINBERG 2011). The enzymatic subunit E(Z) is a histone methyltransferase (HMT) that methylates histone H3 on lysine 27 (H3K27me), which is associated with repression (CZERMIN *et al.* 2002; MULLER *et al.* 2002). ASH-1 is a trxG protein that methylates histone H3 on lysine 36 (H3K36me), which is associated with active transcription (TANAKA *et al.* 2007). Antagonism between H3K36me and H3K27me has been shown *in vitro* and *in vivo*. *In vitro*, H3K36 methylation of histone tails prevents H3K27 methylation on the same histone tails (SCHMITGES *et al.* 2011; YUAN *et al.* 2011). *In vivo*, ASH-1 prevents H3K27 methylation by PRC2 on the Hox gene *Ultrabithorax* (KLYMENKO and MULLER 2004; PAPP and MULLER 2006). H3K27 and H3K36 methylation are generated by orthologous protein complexes in mammals and *C. elegans* (CAO *et al.* 2002; KUZMICHEV *et al.* 2002; KETEL *et al.* 2005; RECHTSTEINER *et al.* 2010).

The MES proteins generate H3K27 and H3K36 methylation in the *C. elegans* germline. MES-2, MES-3 and MES-6 form a trimeric PRC2 complex that generates H3K27 di and tri methylation (me2 and me3) (XU *et al.* 2001a; BENDER *et al.* 2004) (Figure 1-3). MES-2 is the H3K27 HMT and is homologous to E(Z) in *Drosophila* and EZH2 in mammals (HOLDEMAN *et al.* 1998). MES-6 is homologous to *Drosophila* ESC and mammalian EED (KORF *et al.* 1998), and MES-3 is a *C. elegans*-specific subunit of worm PRC2 (PAULSEN *et al.* 1995) (Figure 1-4). MES-4 generates H3K36me2 and me3 (BENDER *et al.* 2006) (Figure 1-3). MES-4 is

homologous to *Drosophila* MES-4 (dMES-4) and mammalian NSD proteins, NSD-1, NSD-2 and NSD-3, which are important in human development and have been linked to several developmental disorders and to cancer (FONG *et al.* 2002; RAYASAM *et al.* 2003; WANG *et al.* 2007; NIMURA *et al.* 2009; RECHTSTEINER *et al.* 2010) (Figure 1-4). The similar role of MES-2/3/6 and MES-4 in germline development and their opposing histone marks led me to investigate a potential antagonism between H3K36 and H3K27 methylation in *C. elegans*, discussed in Chapters 2 and 4.

Epigenetic Inheritance of Histone Marks

The field of epigenetics stemmed from the study of events that occur during development to form vastly different cells from one original founding cell. This field evolved with studies on DNA packaging and deployment into epigenetics being a mode of inheritance that involves changes in gene function that are not associated with changes in DNA sequence, but may instead reflect changes in DNA marking, packaging, and availability (FELSENFELD 2014). Many studies on epigenetics have focused on inheritance of chromatin states, factors, and phenotypes through cell divisions (from parent to daughter cells) and transgenerationally (from parent to offspring). Early work on X-chromosome inactivation in mammals provided evidence for DNA methylation serving as an epigenetic mark that can be transmitted from parent to daughter cells (HOLLIDAY and PUGH 1975; RIGGS 1975). DNA methylation is also involved in transgenerational epigenetic inheritance, as some genes require parent-specific DNA methylation (or imprinting) for proper

development of offspring (BARLOW and BARTOLOMEI 2014). Compared to DNA methylation, histone modifications are not as closely associated with DNA, since the modifications are not directly on DNA but instead on the histones associated with DNA. That said, histone modifications have been heavily investigated as transmitters of epigenetic inheritance and have proven to be as influential for gene function as DNA methylation.

Some of the earliest evidence for histone modifications being involved in epigenetic inheritance was the discovery that the protein HP1 is recruited to H3K9 methylation on chromatin and could recruit an enzyme that propagates H3K9 methylation (BANNISTER *et al.* 2001; LACHNER *et al.* 2001; NAKAYAMA *et al.* 2001). These findings offered a possible mechanism for transmission of histone marks through cell divison: a protein that can recognize a histone modification recruits an enzyme that can generate more of that histone modification on surrounding nucleosomes (FELSENFELD 2014). PRC2 appears to incorporate both functions into one complex, as PRC2 can both associate with H3K27me3 and generate new H3K27me3 (HANSEN *et al.* 2008; MARGUERON *et al.* 2008; MARGUERON *et al.* 2009). Numerous studies have addressed how repression by PRC2 is inherited through cell division (MARGUERON and REINBERG 2011). Studies have shown that PRC2 and H3K27me3 are enriched at repressed genes immediately before replication and remain associated with DNA during cell division (HANSEN *et al.* 2008; LANZUOLO *et al.* 2011). Recently, PRC2 proteins were found at the replication fork by sequential re-ChIP of DNA that immunoprecipitated with a protein at the replication fork

(PCNA), but H3K27me3 was not found on newly replicated chromatin (PETRUK *et al.* 2012). These findings demonstrate that PRC2 can be epigenetically transmitted through cell divisions and suggest that H3K27me3 may not be directly inherited but that after DNA replication PRC2 newly generates H3K27me3 on chromatin. My work in Chapter 3 provides evidence that H3K27me3 can itself be transmitted through cell division.

Numerous studies in *C. elegans* have shown that histone marks are involved in transgenerational epigenetic inheritance. H3K4me is associated with active genes and is on the X chromosomes in oocytes, but not in sperm (KELLY *et al.* 2002; ARICO *et al.* 2011) (Figure 1-5). In early embryos, H3K4me remains off the sperm-contributed X chromosome for a few cell divisions (BEAN *et al.* 2004). This shows that differential marking of the X chromosome can be transmitted transgenerationally from the parent germline to progeny. H3K4me has also been shown to be involved in transgenerational inheritance from studies of an H3K4me2 demethylase mutant. In this mutant, H3K4me2 accumulates progressively over generations and the frequency of sterile worms increases over generations (KATZ *et al.* 2009). Furthermore, the balance between active H3K4me and repressive H3K9me was recently proposed to regulate this progressive sterility phenotype (GREER *et al.* 2014).

The MES proteins are also involved in transgenerational epigenetic inheritance in *C. elegans*. MES-4 generates H3K36me on genes that are expressed in the germline (i.e. germline genes) and has been proposed to act as a memory of germline gene expression in the next generation (RECHTSTEINER *et al.* 2010). MES-4

and H3K36me remain on germline genes during embryogenesis even though many of these genes are not transcribed in embryos and lack Pol II (FURUHASHI *et al.* 2010; RECHTSTEINER *et al.* 2010) (Figure 1-6). Since *mes-2, mes-3* and *mes-6,* like *mes-4,* are needed maternally for the next generation to be fertile, they may also have a role in transgenerational epigenetic inheritance. Chapters 2 and 3 show that MES-2/3/6 and H3K27me are important transgenerational epigenetic factors for X-chromosome repression.

X-Chromosome Repression in the Germline

All diploid organisms have two of each chromosome except for the chromosome(s) that determines sex. While most organisms have at least one X chromosome, the number of X chromosomes varies between sexes. Dosage compensation is the mechanism used to compensate for the different number of X chromosomes in the different sexes. Dosage compensation adjusts transcription from the X chromosome(s) in one sex to match transcription in the other sex (STRAUB and BECKER 2007) (Figure 1-7). In female mammals (XX), one X chromosome is randomly inactivated by a mechanism that involves PcG proteins, leaving one functional X like in males (XY) (HEARD 2005; ZHAO *et al.* 2008). In *Drosophila* and *C. elegans*, the X chromosome is not silenced, but either up- or down-regulated. *Drosophila* males (XY) up-regulate their single X chromosome by two-fold (GELBART and KURODA 2009), and *C. elegans* hermaphrodites (XX) down-regulate both their X chromosomes by two-fold (MEYER and CASSON 1986; JANS *et al.* 2009;

MEYER 2010). Transcriptome studies have shown that dosage compensation is not as simple as matching transcript levels from the X chromosome(s) in both sexes, but also involves matching transcription of the X chromosome(s) relative to the autosomes, as originally proposed by Ohno ~50 years ago (GUPTA *et al.* 2006; NGUYEN and DISTECHE 2006; DENG *et al.* 2011).

The above mechanisms are what somatic cells do to compensate for X-chromosome dosage differences. How germ cells deal with different X dosages is less well understood. In *Drosophila* germ cells, genome-wide analyses of gene expression levels from the X chromosome vs. autosomes provide evidence for dosage compensation (GUPTA *et al.* 2006; DENG *et al.* 2011), even though the somatic dosage compensation complex is not found in germ cells (RASTELLI and KURODA 1998). Female mammals reactivate the inactive X chromosme in early germ cell development (SAITOU and YAMAJI 2012), but it is not known whether a dosage compensation mechanism is employed later in germ cell development (NGUYEN and DISTECHE 2006). *C. elegans* germ cells have been well-studied and found to employ a completely different mechanism from somatic cells to accomplish "dosage compensation".

Germ cells in *C. elegans* do not express several of the proteins involved in the dosage compensation mechanism that operates in somatic tissues. *C. elegans* germ cells instead accomplish dosage compensation by near silencing of the X chromosomes in both sexes: both X chromosomes are repressed in XX hermaphrodites, and the single X is repressed in XO males (STROME *et al.* 2014).

Two lines of evidence demonstrate the near silencing of the X chromosomes in the germline. First, the X chromosomes in the germline lack marks of active chromatin, such as H3K4me2, through mitosis and most of meiosis and in sperm (KELLY *et al.* 2002; OOI *et al.* 2006; ARICO *et al.* 2011) (Figure 1-5). Second, most genes expressed in the germline are located on the autosomes and many fewer than expected by chance are located on the X chromosome. However, genes involved in oogenesis go against this trend and many are on the X chromosome (REINKE *et al.* 2000; REINKE *et al.* 2004). Indeed, the X chromosomes acquire active histone modifications and turn on transcription during late-stage oogenesis (KELLY *et al.* 2002). The current view is that in most stages of germ cell development (except oogenesis) the X chromosomes are globally repressed and the MES proteins participate in this repression.

The current model is that the MES-2/3/6 complex directly represses the X chromosomes and MES-4 indirectly represses the Xs. Several findings support the direct role of MES-2/3/6. By immunostaining, H3K27me3 is visibly enriched on the X-chromosome pair in meiosis pachytene (BENDER *et al.* 2004) (Figure 1-5), and when H3K27me3 is lost in a *mes* mutant, the X chromosomes acquire marks of active chromatin (FONG *et al.* 2002). By genome-wide chromatin immunoprecipitation (ChIP-chip), H3K27me3 is enriched on the X chromosome in early embryos, which likely reflects the state of the X chromosome inherited from the parental germline (LIU *et al.* 2011). The involvement of MES-4 in X-chromosome repression is likely indirect. By immunostaining, MES-4 is concentrated on the autosomes and absent

from all but the left tip of the X. MES-4 generates H3K36me, which is associated with active chromatin, and H3K36me2 is on the autosomes but absent from the X-chromosome pair in meiosis pachytene (Figure 1-5). Given the focus of MES-4 action on the autosomes, it was surprising to learn that X-chromosome genes are up-regulated in *mes-4* mutant germlines (BENDER *et al.* 2006). Chapter 2 includes my investigations of transcription from the X chromosomes in *mes* mutants, Chapter 3 looks at the transmission and propagation of MES-2/3/6 repression, and Chapter 4 explores when during development repression by MES-2/3/6 is needed. Both Chapters 2 and 4 include a model of how MES-4 on the autosomes may participate in repressing the X chromosomes by antagonising MES-2/3/6 and H3K27me3 and helping to focus their action on the X.

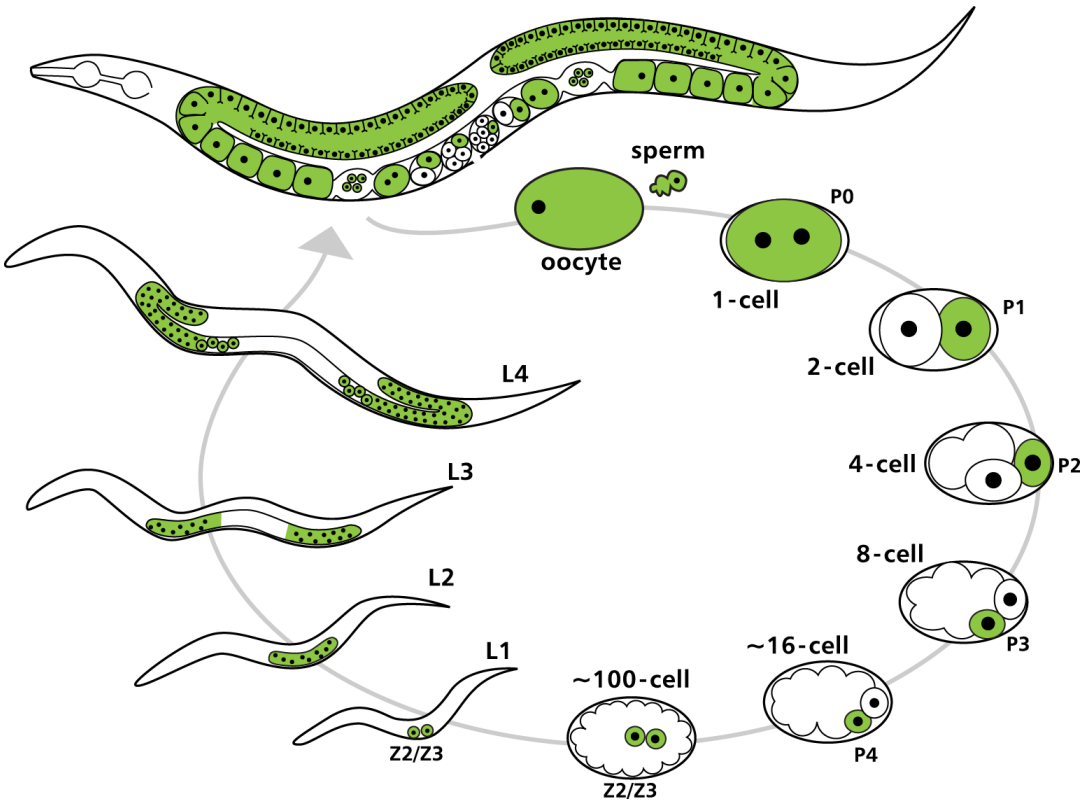

Figure 1-1. The *C. elegans* life cycle.
Germ cells (green) are set apart from somatic cells (white) during early embryo cell divisions (germline blastomeres P0-P4, primordial germ cells Z2/Z3) and are quiescent through embryogenesis until the worm hatches and starts feeding. During the larval stages (L1-L4), the primordial germ cells (Z2 and Z3) proliferate into the full adult germline in hermaphrodites (shown here) and males (not shown). In hermaphrodites, sperm are made at the L4 stage and stored in the spermatheca and during adulthood oocytes are made and pass through the spermatheca for fertilization.

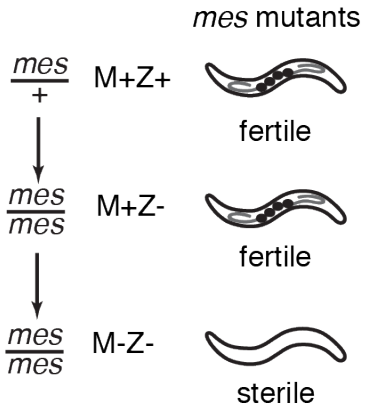

Figure 1-2. The Maternal Effect Sterile (MES) phenotype.
The first generation of *mes* mutants (*mes/mes*) inherit wild-type maternal (M+) MES products, but do not produce any zygotic MES products (Z-). Such M+Z- hermaphrodites develop into fertile adults. The second generation of *mes/mes* mutants does not inherit maternal or synthesize zygotic MES products, so is M-Z-. M-Z- hermaphrodites develop into sterile but otherwise healthy adults. *mes* genotype on left, phenotype on right. M, maternal; Z, zygotic.

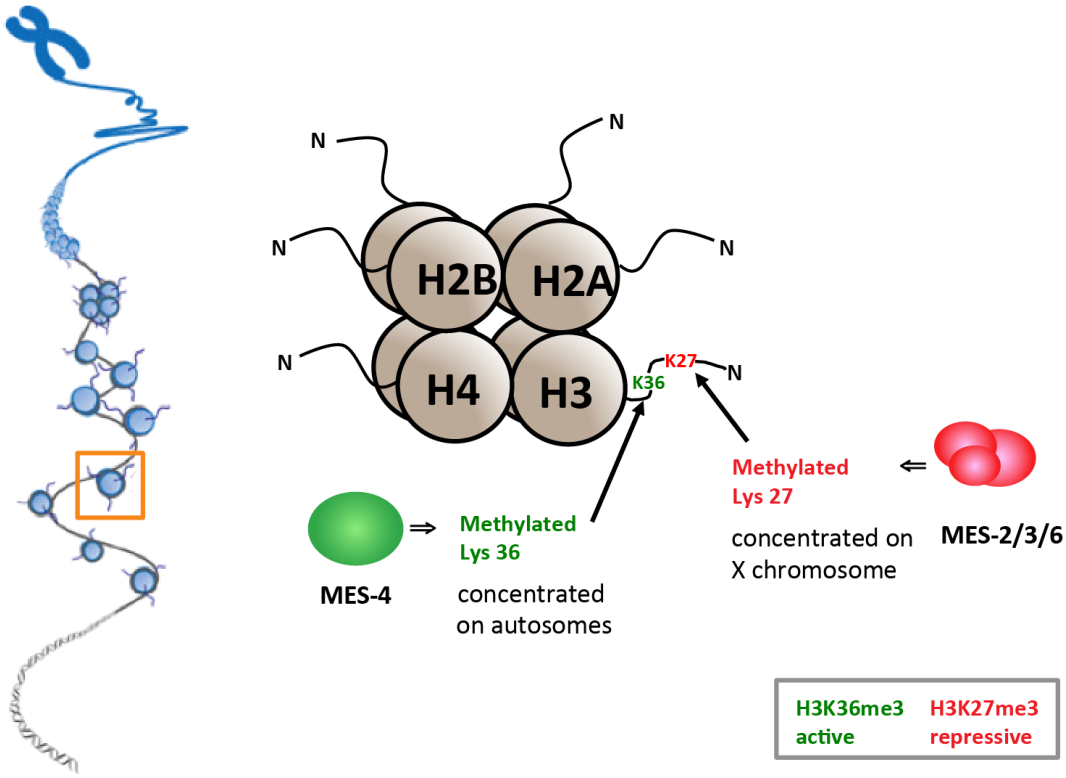

Figure 1-3. The MES proteins methylate the tail of histone H3.
DNA is wrapped around histone octamers, as shown on the left with a histone octamer or "nucleosome" boxed in orange. Close-up of a histone octamer showing the histone tails and methylation of histone H3 at Lys 36 (K36) and Lys 27 (K27). H3K36me2 and me3 are associated with actively transcribed genes, and H3K27me3 is associated with repressed genes. MES-4 and MES-2/3/6 generate H3K36me2,me3 and H3K27me3, respectively.

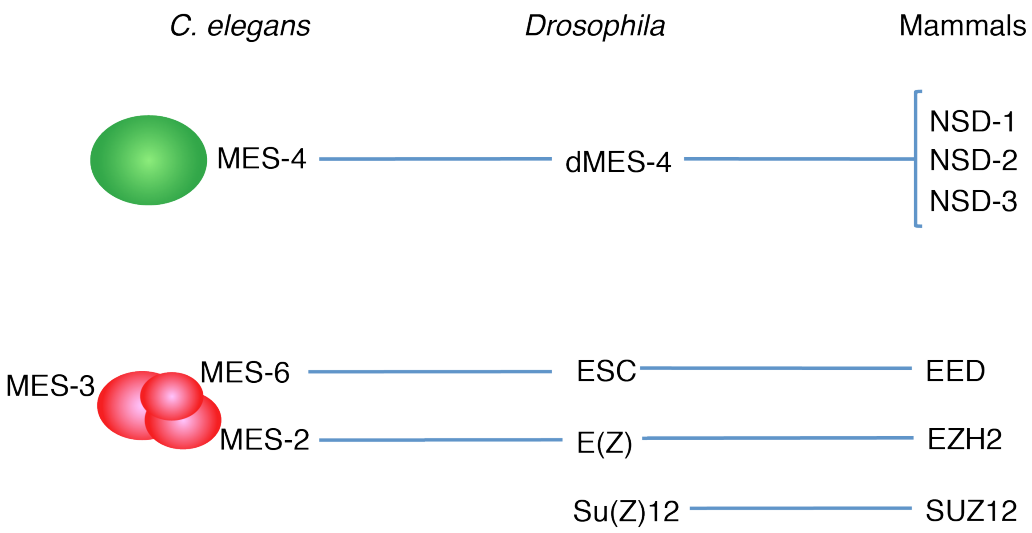

Figure 1-4. *C. elegans* MES protein orthologs in *Drosophila* and mammals. The ortholog of *C. elegans* MES-4 (green) in *Drosophila* is dMES-4, and the homologs in mammals are the NSD (for nuclear receptor SET domain) proteins. The orthologs of *C. elegans* MES-2 and MES-6 (red) are E(Z) and ESC in *Drosophila* and EZH2 and EED in mammals. MES-3 is a worm-specific subunit. MES-2/3/6 and the related complexes form Polycomb Repressive Complex 2.

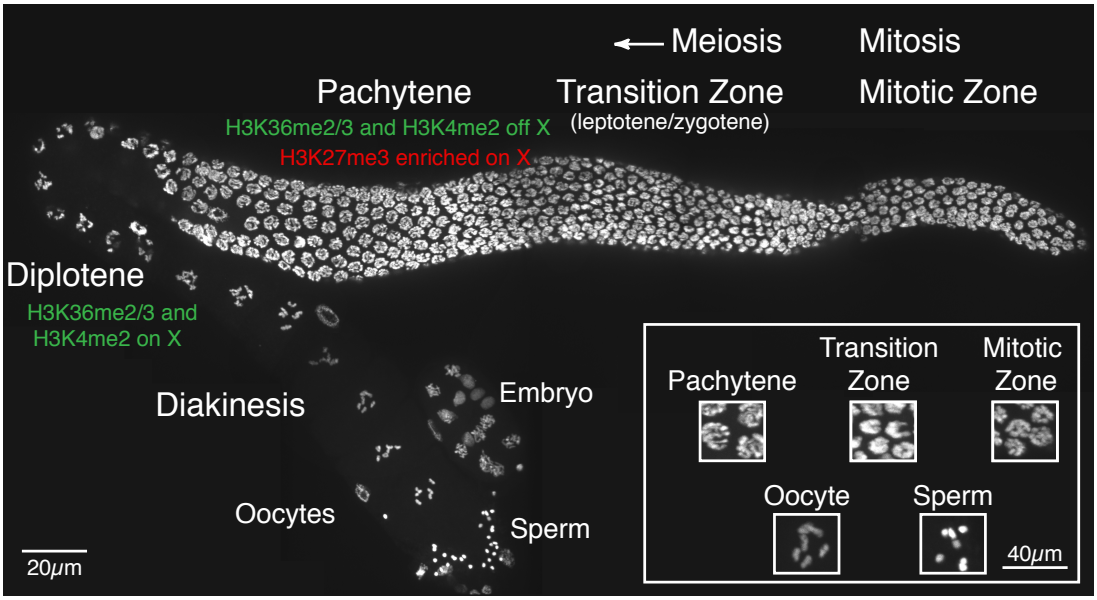

Figure 1-5. Stages of hermaphrodite germline development.
Mitosis of germline stem cells occurs in the mitotic zone. Meiosis includes the transition zone (leptotene and zygotene), pachytene, diplotene, and diakinesis and results in formation of oocytes and sperm. Zoomed view of nuclei in each stage shown in bottom right. Histone mark patterns associated with pachytene and diplotene in green (active marks) and red (repressive marks).

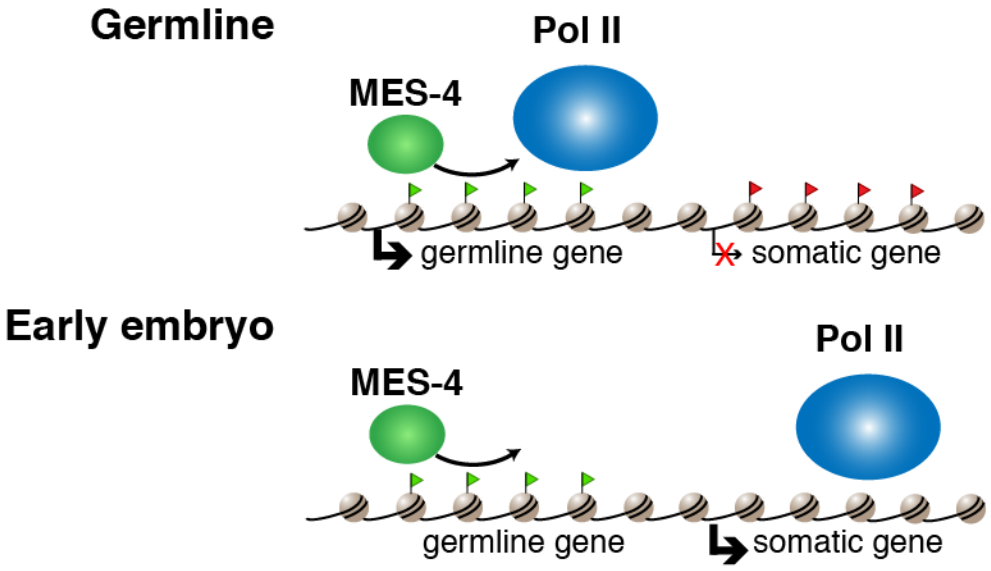

Figure 1-6. MES-4 memory model.
MES-4 in green, RNA Polymerase II (Pol II) in blue. Green flags are active marks put down by MES-4, red flags are repressive marks. MES-4 marks genes that are transcribed in the germline of adults and maintains that marking in early embryos, even when those genes are no longer being transcribed.

Organism	Male	Female or hermaphrodite
C. elegans	XO	XX
Drosophila	X_Y	XX
Mammals	XY	$X_{Xinactive}$

Figure 1-7. Dosage compensation in somatic cells of different organisms. Hermaphrodite *C. elegans* down-regulate X-chromosome expression by 1/2. *Drosophila* males up-regulate X-chromosome expression by 2. Mammalian females inactivate one X chromosome (in red).

CHAPTER 2: Antagonism between MES-4 and Polycomb Repressive Complex 2 Promotes Appropriate Gene Expression in C. elegans Germ Cells.[1]

INTRODUCTION

A major goal for understanding how cell fates are specified and how cells can be reprogrammed to new fates is defining how chromatin states influence gene expression. Tissue-appropriate patterns of gene expression require that genes needed for tissue development reside in chromatin that can be accessed by transcription factors and transcribed by polymerases, while other genes must be kept in a repressed chromatin state. Two histone marks that are signatures of expressed and repressed chromatin are histone H3 tri-methylated on Lys 36 (H3K36me3) and on Lys 27 (H3K27me3), respectively. H3K36me3 is introduced co-transcriptionally upon the passage of RNA Polymerase II through genes; this mark can also be epigenetically maintained on genes in the absence of ongoing transcription (KROGAN *et al.* 2003; LI *et al.* 2003; FURUHASHI *et al.* 2010; RECHTSTEINER *et al.* 2010). H3K27me3 is a well-established mark of repressed chromatin that can be propagated in an epigenetic manner (HANSEN *et al.* 2008; LANZUOLO *et al.* 2011; MARGUERON and REINBERG 2011). H3K36me3 and H3K27me3 marks generally occupy non-overlapping regions of genomes (ERNST and KELLIS 2010; KHARCHENKO *et al.* 2011; LIU *et al.* 2011).

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The notion that these marks may regulate each other's distribution is supported by two types of evidence. First, prior methylation of H3K36 prevents methylation of K27 on the same histone tails *in vitro* (SCHMITGES *et al.* 2011; YUAN *et al.* 2011). Second, *Drosophila ash1* mutants, which are thought to be defective in H3K36 methylation, show spreading of H3K27me3 into and silencing of the *Ultrabithorax* gene (KLYMENKO and MULLER 2004; PAPP and MULLER 2006; TANAKA *et al.* 2007). These *in vitro* and single-gene studies suggest that H3K36 methylation antagonizes H3K27 methylation, and that H3K27 methylation is otherwise a default modification. We sought to test this model in vivo on a genome-wide scale, and to examine the effects on gene expression patterns of removing H3K36me3 or H3K27me3 or both.

The *C. elegans* MES proteins are essential chromatin regulators in germ cells (CAPOWSKI *et al.* 1991). MES-2, MES-3, and MES-6 form the *C. elegans* version of the widely conserved Polycomb Repressive Complex 2 and generate repressive H3K27me3 (XU *et al.* 2001a; BENDER *et al.* 2004; KETEL *et al.* 2005). MES-4, a homolog of the vertebrate NSD proteins, generates H3K36me3 on genes expressed in the germline (FURUHASHI *et al.* 2010; RECHTSTEINER *et al.* 2010). Loss of any of the four MES proteins causes germ cells to die. MES regulation is maternal effect: maternally provided MES(+) product promotes development of a fertile germline, while absence of maternal MES(+) product leads to death of nascent germ cells and sterile adults (CAPOWSKI *et al.* 1991). Previous studies focused attention on MES regulation of the X chromosomes. The X chromosomes in XX hermaphrodites and XO males are considered to be globally "silenced" during most stages of germ cell

development. This is supported by the finding that histone marks associated with active gene expression decorate the autosomes but are not detected on the X chromosomes in mitotic, early meiotic, and spermatogenic germ cells, and by the low expression of X-linked genes compared to autosomal genes in dissected germlines (KELLY *et al.* 2002; WANG *et al.* 2009). Perhaps as a consequence of X silencing, germline-expressed genes are significantly under-represented on the X compared to the five autosomes (REINKE *et al.* 2004). The MES proteins participate in X silencing, as illustrated by the spread of marks of active chromatin to the Xs in immunostained *mes-2, mes-3* and *mes-6* mutant germ nuclei and the up-regulation of X-linked genes in dissected *mes-4* germlines (FONG *et al.* 2002; BENDER *et al.* 2006). The apparently similar involvement of the four MES proteins in X silencing is puzzling given their strongly asymmetric distributions: MES-4 and H3K36 methylation are strikingly enriched on the five autosomes and nearly absent from the X, while MES-2/3/6-generated H3K27me3 is modestly enriched on the X (FONG *et al.* 2002; BENDER *et al.* 2004; BENDER *et al.* 2006).

This chapter explores how autosomally concentrated MES-4 and X-enriched MES-2/3/6 activity contribute to silencing the Xs, identifies autosomal targets of MES regulation, and tests the model that H3K36 methylation generated by MES-4 repels H3K27me3 from germline-expressed genes. Our findings reveal how antagonistic histone modifiers can shape genome organization and tissue-appropriate gene expression patterns, and lay the foundation for understanding how loss of MES

regulation and the resulting altered chromatin landscape renders germ cells susceptible to conversion to somatic cells (PATEL *et al.* 2012).

The work in this chapter was initiated by Coleen Carroll and pursued by me, in collaboration with Andreas Rechtsteiner and Thea Egelhofer. Coleen Carroll performed microarray analysis of *mes-2, mes-3* and *mes-4* germlines on amplicon microarrays; I repeated microarray analysis on a new platform (long oligonucleotide microarrays), as described in this chapter. Coleen Carroll also contributed analysis of the *mes* double mutant phenotype shown in Figure 2-5. Thea Egelhofer performed all of the ChIP-chip experiments. I worked with Andreas Rechtsteiner on bioinformatic analysis and comparison of microarray and ChIP-chip data.

RESULTS AND DISCUSSION

MES-4 and MES-2/3/6 cooperate to silence the X chromosomes in the germline.

To better understand when and how maternal MES(+) function promotes development of a functional germline, we determined how long maternally encoded MES proteins persist in the absence of zygotically produced protein in *mes/mes* mutants with a maternal load (M+) and no zygotic expression (Z-) of *mes* gene product. During larval development, MES-4 generates all detectable H3K36me2 and some of the H3K36me3 in germ cells. Consequently, we focused on H3K36me2 for assessing disappearance of MES-4-generated histone marks. In confocal images, MES-4 and H3K36me2 were undetectable by the L2 and L3 stage, respectively (Figure 2-1 and 2-3A). MES-2 and H3K27me3 were undetectable by the L3 and L4

stage, respectively (Figure 2-2 and 2-3A). Quantification of immunostaining pixel intensity in L4 germ nuclei showed that in *mes-4* mutants, MES-4 was reduced to 3.4% and H3K36me2 to 9.5% of wild type, and in *mes-2* mutants, MES-2 was reduced to 1.3% and H3K27me3 to 1.9% of wild type (Figure 2-3B). These results reveal that in homozygous *mes* mutants, maternal MES protein and their histone modifications persist through activation of transcription in the primordial germ cells, commencement of germ cell proliferation in late L1s, and, for H3K27me3, initiation of meiosis in L3s. The resulting adult germlines can produce oocytes and progeny but display compromised health (CAPOWSKI *et al.* 1991; XU and STROME 2001). These adults offered an opportunity to examine gene expression changes that follow larval loss of MES proteins and their methyl marks (Figure 2-3A).

We compared transcript accumulation in dissected germlines from *mes-4*, *mes-2*, and wild-type control adults. We previously compared *mes-4* and wild-type germlines using amplicon microarrays (BENDER *et al.* 2006). For this study we switched to long oligonucleotide microarrays, which have been reported to have greater specificity than amplicon arrays while maintaining sensitivity (ZHU *et al.* 2005). Expression analysis of *mes-4* mutant germlines on oligonucleotide arrays identified 276 significantly mis-regulated genes in *mes-4* compared to wild type: up-regulation of 154 X-linked genes and 66 autosomal genes, and down-regulation of 56 autosomal genes (Figure 2-4A). To verify these and subsequent microarray results, mRNA levels were measured by quantitative PCR for a subset of genes (Table 2-1). Expression analysis of *mes-2* mutant germlines identified 183 significantly mis-

regulated genes in *mes-2* compared to wild type: up-regulation of 16 X-linked genes and 142 autosomal genes, and down-regulation of 1 X-linked gene and 24 autosomal genes (Figure 2-4A). Thus, larval decline of maternal MES-2 led to predominantly up-regulation of genes in the adult germline, consistent with the repressive role of MES-2 orthologs and H3K27 methylation in other systems (KIRMIZIS *et al.* 2004; LEE *et al.* 2006; TOLHUIS *et al.* 2006). Larval decline of maternal MES-4 also led to predominantly up-regulation of genes, with a strong bias for up-regulation of genes on the X.

We compared the X-linked genes mis-regulated in *mes-2* and *mes-4* mutants, to assess whether MES-2 and MES-4 influence expression of the same genes. Ten of the 16 up-regulated genes in *mes-2* mutants were also up-regulated in *mes-4* mutants (Figure 2-4B). Additionally, among the X-linked genes significantly up-regulated in *mes-4* mutants, most show some up-regulation in *mes-2* mutants even though they were not scored as significant (i.e. FDR < 0.05). These results suggest that MES-4 and MES-2 cooperate to down-regulate expression of some of the same X-linked genes.

MES-4 and MES-2/3/6 catalyze antagonistic histone modifications, but both promote development of healthy germ cells in a maternal-effect fashion. To investigate the interplay between MES-4 and the MES-2/3/6 complex, we analyzed double mutants. If they operate in the same pathway, we expected double mutants to resemble single mutants. If they serve antagonistic roles in the same process, similar to *Drosophila* ASH1 and E(Z) (KLYMENKO and MULLER 2004), double mutants

might display a less severe phenotype than single mutants. If they control a common process via parallel pathways or control different processes, we expected double mutants to display a more severe phenotype than single mutants. We saw the latter result: *mes-2; mes-4* and *mes-3; mes-4* double mutants display sterility a generation earlier than single mutants (Figure 2-5A,B). As a control, *mes-2; mes-3* double mutants resemble the single mutants. Sterile *mes-2; mes-4* and *mes-3; mes-4* double mutants display a range of germline phenotypes (Figure 2-5B). 33% possess a well-proliferated and healthy-appearing germline, offering an opportunity to examine gene expression in germlines lacking both MES-2 and MES-4. This analysis identified 464 mis-regulated genes in *mes-2; mes-4* compared to wild type: up-regulation of 210 X-linked genes and 177 autosomal genes, and down-regulation of 3 X-linked genes and 74 autosomal genes (Figure 2-4A). Compared to *mes-4* single mutants, *mes-2; mes-4* double mutants up-regulated more genes on the X and showed elevated up-regulation of X-linked genes (Figure 2-4C). Thus, even though MES-2/3/6 and MES-4 operate independently of each other (XU *et al.* 2001a), they cooperate at some level to repress expression of genes on the X.

MES-4 and MES-2/3/6 promote gene expression patterns appropriate for germ cells.

Since mis-regulation of gene expression in *mes* mutant germlines is likely to contribute to sterility, an important question is whether particular classes of genes are mis-regulated. We categorized genes according to their expression in published

microarray studies (REINKE *et al.* 2004) and SAGE (Serial Analysis of Gene Expression) studies (MEISSNER *et al.* 2009; WANG *et al.* 2009). The germline-enriched category includes genes whose expression is enriched in germline tissue based on comparing adults with and without a germline (REINKE *et al.* 2004). Our germline-specific category includes genes with SAGE tags in dissected germlines and not in FACS-sorted intestine, muscle, or nerve cells. Our soma-specific category includes genes with SAGE tags in at least one somatic tissue (intestine, muscle, and/or nerve) and not in the germline. Our ubiquitously expressed category includes genes with SAGE tags in germline, intestine, muscle, and nerve. We also looked at X-linked genes up-regulated in worms defective in somatic dosage compensation (JANS *et al.* 2009). We determined whether genes mis-regulated in *mes-4, mes-2,* and *mes-2; mes-4* mutant germlines are enriched for genes in these categories.

X-linked genes up-regulated in *mes* mutant germlines are not enriched for genes that are dosage compensated in the soma (Figure 2-6A), establishing that the focus of MES regulation in the germline is different than the focus of dosage compensation in somatic tissues. In all three *mes* genotypes analyzed, up-regulated genes on the X are enriched for genes in the ubiquitously expressed category (Figure 2-6A). Ubiquitously expressed genes are often among the most highly expressed (WANG *et al.* 2009), but the X chromosome is considered to be nearly silent in most regions of the wild-type adult germline (KELLY *et al.* 2002; REINKE *et al.* 2004). This conundrum raised the question: are X-linked genes that are up-regulated in *mes* mutants normally expressed at low or high levels in the germline? We found that they

are among the more highly expressed X-chromosome genes in wild-type germlines (as well as in somatic tissue), based on two independent transcript profiling studies on single-color (Affymetrix) microarrays (SPENCER *et al.* 2011; TABUCHI *et al.* 2011) (Figure 2-6B). Thus, the MES proteins dampen germline expression of ubiquitously and robustly expressed genes on the X.

Autosomal genes up-regulated in *mes* mutant germlines are enriched for genes whose expression is normally restricted to somatic tissues (Figure 2-6C). Conversely, autosomal genes down-regulated in *mes* mutant germlines are enriched for genes whose expression is normally restricted to germ cells (Figure 2-6D). We conclude that in adult germ cells the MES proteins participate in repressing expression of genes associated with somatic development and promoting expression of genes associated with germline development.

Taken together, transcription profiling and gene class analysis reveal that the MES proteins influence gene expression in a manner appropriate for germline development, enhancing expression of certain germline genes, repressing somatic genes, and dampening expression of X-chromosome genes that are not silent in wild-type germlines, but instead are expressed at appreciable levels and in numerous tissues. These findings establish the importance of MES-4 and MES-2/3/6 in guiding gene expression patterns appropriate for germ cells, but raise the question how proteins that generate antagonistic histone modifications cooperate at a molecular level.

Methylated H3K36 and H3K27 occupy mutually exclusive domains, and methylated H3K27 is strikingly enriched on the X.

To investigate how MES-4 and MES-2/3/6 contribute to regulation of gene expression in the germline, we compared the genome-wide distributions of the histone marks they generate, H3K36me3 and H3K27me3, using chromatin immunoprecipitation followed by hybridization to microarrays (ChIP-chip). We previously established that early embryos retain a germline distribution of at least some histone modifications. In particular, we showed that MES-4 maintains H3K36me3 on germline-expressed genes throughout embryogenesis and independently of ongoing transcription, and that embryo-expressed somatic genes have no to low H3K36me3 in early embryos (FURUHASHI *et al.* 2010; RECHTSTEINER *et al.* 2010). Figure 2-7 and 2-8 extend this analysis to H3K27me3 and confirm that early embryo chromatin retains germline signatures: germline-specific genes display elevated H3K36me3 and low H3K27me3, while soma-specific genes display low H3K36me3 and elevated H3K27me3. These findings validate performing ChIP analysis from early embryos to gain insights into germline chromatin.

We found that the autosomes are composed of alternating clusters of H3K36me3-bound genes and H3K27me3-bound genes (Figure 2-7A). These define mutually exclusive domains of these two opposing histone modifications, with a negative correlation coefficient r = -0.82 (comparing H3K36me3 and H3K27me3 on 1 kb segments across the genome). The X chromosome is strikingly different. With the exception of the leftmost 300 Mb, the X displays very few regions of H3K36me3

enrichment, and is marked by H3K27me3 along its length (Figure 2-7A). These ChIP-chip results in early embryos are consistent with the striking under-representation of germline-expressed genes on the X chromosome and immunostaining results showing absence of marks of active chromatin and concentration of repressive H3K27me3 on X chromosomes in the germline (KELLY *et al.* 2002; BENDER *et al.* 2004; REINKE *et al.* 2004). The results strongly suggest that MES-2/3/6 participates directly in X repression by concentrating a repressive chromatin mark on X-linked genes. The results also raised the possibility that MES-4 and/or methylated H3K36 repel MES-2/3/6 from autosomal genes that should be expressed in the germline.

MES-4-generated H3K36 methylation antagonizes H3K27 methylation on germline-expressed genes on the autosomes and concentrates H3K27me3 on the X chromosome.

To test the model that MES-4 repels MES-2/3/6 from germline-expressed genes on the autosomes, we analyzed the distribution of H3K27me3 in early embryos whose mothers were depleted of MES-4 by RNAi. Confirming that RNAi was effective, MES-4 and H3K36me3 were depleted to below detectable levels from genes with germline-specific expression, which lack transcription in early embryos (RECHTSTEINER *et al.* 2010). Ubiquitously expressed genes with detectable transcription in early embryos, such as *ama-1,* retained some H3K36me3; this H3K36me3 signal is likely catalyzed by the other H3K36 HMT MET-1, which

becomes active at the ~40-cell stage of embryogenesis and which is thought to methylate H3K36 cotranscriptionally (RECHTSTEINER *et al.* 2010). Examination of germline-specific genes revealed that loss of H3K36me3 in *mes-4(RNAi)* embryos was accompanied by acquisition of H3K27me3 (Figure 2-7B,C, 2-8C and 2-9). Genes that retained H3K36me3, such as *ama-1*, remained devoid of H3K27me3 (Figure 2-7C and 9-2). We conclude that MES-4 activity repels MES-2/3/6 repressive activity from genes whose expression is associated with germline development.

If acquisition of H3K27me3 is responsible for down-regulation of germline-expressed genes on the autosomes in *mes-4* mutant germlines, then those genes should be restored to closer to wild-type levels when H3K27me3 is lost in *mes-2; mes-4* double mutant germlines. Eight of 33 autosomal genes analyzed were restored to closer to normal levels in *mes-2; mes-4* compared to *mes-4* mutants (Figure 2-10). Two possible explanations for the remaining 25 genes showing similar or enhanced down-regulation in *mes-2; mes-4* compared to *mes-4* are: 1) early action of maternally supplied MES-2 and H3K27me3 in homozgyous *mes* mutants is sufficient to maintain repression in adults, or 2) MES-4 promotes expression of at least some germline genes independently of repelling MES-2/3/6.

We previously hypothesized that MES-4 participates in X silencing by repelling a repressor from the autosomes and focusing its repressive activity on the X (BENDER *et al.* 2006). The above analysis suggested that MES-2/3/6 is the repressor that MES-4 repels. In support of this scenario, H3K27me3 levels were strikingly reduced on a majority of X-linked genes in *mes-4(RNAi)* embryos (Figure 2-8A and

2-9). Importantly, X-linked genes up-regulated in *mes-4* mutant germlines displayed markedly reduced H3K27me3 in *mes-4(RNAi)* early embryos (Figure 2-8B and 2-9). X-linked genes with reduced H3K27me3 in *mes-4(RNAi)* are particularly enriched for ubiquitously expressed genes (Figure 2-8C). We conclude that MES-4 activity helps concentrate MES-2/3/6 repressive activity on the Xs to dampen X gene expression.

We wondered if MES-4 repulsion of MES-2/3/6 activity helps concentrate H3K27me3 elsewhere on the autosomes. In wild-type early embryos, autosomal genes whose expression is specific to somatic cells generally lack H3K36me3 and possess H3K27me3 (Figure 2-9). In *mes-4(RNAi)* early embryos, those genes displayed reduced H3K27me3, ranging from modest to strong reduction (Figure 2-9). Importantly, autosomal genes up-regulated in *mes-4* mutant germlines generally showed reduced H3K27me3 in *mes-4(RNAi)* embryos (Figure 2-8B), and both autosomal genes up-regulated in *mes-4* mutants and autosomal genes with significantly reduced H3K27me3 in *mes-4(RNAi)* are enriched for soma-specific genes (Figure 2-6C and 2-8C). These findings support the view that MES-4 and MES-2/3/6 contribute not only to promoting expression of germline genes but also to repressing somatic genes in the germline.

CONCLUSIONS

Genome-wide ChIP-chip analysis and transcription profiling have advanced our understanding of antagonistic chromatin modifications and how they influence gene expression patterns during development. We show that loss of H3K36

methylation causes global redistribution of H3K27me3 and parallel changes in gene expression. This work provides mechanistic insight into how the proteins that catalyze these histone modifications cooperate to ensure germ cell survival and development in *C. elegans*: MES-4 function repels MES-2/3/6 repressive activity from germline genes on the autosomes and concentrates their repressive action on other autosomal regions, including somatic genes, and on the X chromosomes (Figure 2-11). This antagonism ensures proper patterns of gene expression in germ cells, which includes repression of somatic genes and the Xs (Figure 2-11). Loss of both MES-4 and MES-2/3/6 results in loss of H3K36me3 from germline genes and loss of H3K27me3 from somatic genes and the X. This likely explains the enhanced gene mis-regulation and earlier sterility of *mes-2; mes 4* double mutants compared to single mutants, although both mutant and RNAi approaches to elimination of gene function suffer from gradual loss of protein and uncertainty about which effects are primary and which are secondary. Loss of MES-4 or MES-2/3/6 is not sufficient to allow expression of tested somatic proteins in the germline ((PATEL *et al.* 2012) and our unpublished results). However, MES loss enables germ cells to be converted to neural or muscle fates upon ectopic expression of terminal selector transcription factors (PATEL *et al.* 2012). Similarly, embryos lacking MES-2 display prolonged developmental plasticity and susceptibility to cell fate conversion compared to wild-type embryos (YUZYUK *et al.* 2009). These studies reveal how proper chromatin states can protect cell fates and how altering chromatin context can enable reprogramming of cell fate.

EXPERIMENTAL PROCEDURES

Strains and Culture

C. elegans were cultured (BRENNER 1974) at 20°C.

The following balancers were used:

mnC1[dpy-10(e128)unc-52(e444)] II

hT2-GFP*[bli-4(e937)let(q782)qIs48] (I;III)*

DnT1-GFP*[unc(n754) let qIs51] (IV;V)*

The following strains were used:

N2 variety Bristol

SS0352 *spe-11(hc90) unc-11(e47) dpy-5(e61)/sDp2 (I)*

SS0818 *mes-3(bn35)/hT2-GFP (I;III)*

CB0120 *unc-4(e120) (II)*

SS0186 *mes-2(bn11) unc-4(e120)/mnC1 (II)*

SS0803 *mes-4(bn85)/DnT1-GFP (IV;V)*

SS1095 *mes-4(bn73)/DnT1-GFP (IV;V)*

SS0864 *mes-3(bn35)/hT2-GFP(I;III); mes-2(bn11) unc-4(e120)/mnC1 (II)*

SS1122 *mes-3(bn35)/hT2-GFP(I;III); mes-4(bn85)/DnT1-GFP (IV;V)*

SS0965 *mes-2(bn11) unc-4(e120)/mnC1 (II); mes-4(bn85)/DnT1-GFP (IV;V)*

SS1109 *mes-2(bn11) unc-4(e120)/mnC1 (II); mes-4(bn73)/DnT1-GFP (IV;V)*

Immunocytochemistry

Whole larvae and dissected germlines were immunostained and processed as described (PETRELLA *et al.* 2011).

Primary antibodies and dilutions used for immunostaining were: 1:30,000 mouse anti-H3K27me3 (Active Motif 39535 Lot #174, SS#29), 1:2500 mouse anti-H3K36me2 (Kimura MAb 2C3, SS#69), 1:100 rabbit anti-MES-2 (SS#581) (HOLDEMAN *et al.* 1998), 1:400 rabbit anti-MES-4 (SS#554) (BENDER *et al.* 2006), 1:50,000 rabbit anti-PGL-1 (SS#596)(KAWASAKI *et al.* 1998), 1:50 mouse MAb OIC1D4 (STROME 1986), and 1:500 rat anti-SPE-11 (BROWNING and STROME 1996). Mouse monoclonal antibodies against H3K27me3 (1E7) and H3K36me2 (2C3) were gifts from Hiroshi Kimura (Osaka University) (KIMURA *et al.* 2008). Secondary antibodies conjugated to Alexa Fluor 488 or 594 (Molecular Probes) were used at 1:300.

Quantification of Immunostaining

Germlines from *spe-11* (used as wild type (WT) for this analysis) and M+Z- *mes* mutant (*mes-4(bn73)* or *mes-2(bn11)*) L4 larvae were fixed and stained on the same slide with antibodies to MES proteins or histone marks and imaged using identical exposure times. Costaining with rat anti-SPE-11 (BROWNING and STROME 1996) was used to identify WT and *mes* mutant germlines. The boundaries of germline nuclei and were identified by DAPI staining, and the average pixel intensity within nuclei minus surrounding germline cytoplasm was calculated using Volocity

software (Release 5.1.0; http://www.improvision.com). Nuclear signal remaining in the germ nuclei of *mes* mutant L4s may represent persistent maternal product or background.

Microarray Analysis

Adult hermaphrodite germlines were dissected and cut at the spermatheca with 30 1/2 gauge needles in egg buffer (27.5mM HEPES, 130mM NaCl, 2.2mM $MgCl_2$, 2.2mM $CaCl_2$ and 0.528mM KCl) containing 0.5% tween-20 and 1mM levamisole. For wild type (N2 or *unc-4(e120)*), *mes-2(bn11) unc-4(e120),* and *mes-4(bn85)*, germlines were dissected from adults containing no more than a few fertilized embryos. For *mes-2(bn11) unc-4(e120); mes-4(bn85)* double mutants, well proliferated germlines with oocytes were collected. For each replicate, 100 gonad arms were collected and total RNA was isolated using Trizol (Invitrogen). One round of RNA amplification was performed on isolated RNA using the Message Amp II aRNA amplification kit (Ambion). aRNA was purified using the RNeasy kit (Qiagen). RNA quality was measured on an Agilent 2100 bioanalyzer and was between 250 and 5500 nucleotides. aRNA was labeled with Cy3 or Cy5 with a ULS labeling kit (Kreatech). Differentially labeled mutant and wild-type samples (2μg of each) were pooled. Samples were fragmented using fragmentation reagents (Ambion) and blocked with 25% KREAblock (Kreatech) in 1X formamide hybridization buffer (Genisphere) for 10 min at 80°C. Samples were hybridized to microarrays at 42°C for 15 hrs. Microarrays, obtained from Washington University, were printed on Corning

Epoxy slides and contained 60mer oligonucleotides representing 18,470 genes. After hybridization, microarrays were washed in 2x SSC with 0.2% SDS at 42°C for 10 min, 2x SSC at room temp for 10 min and 0.2x SSC at room temp for 10 min. Slides were dried by centrifugation at 130xg for 4 min and scanned on a GenePix 4000B scanner using GenePix 6.0 software.

The two-color microarray data were normalized using print-tip Loess normalization in the marray Bioconductor package (YANG *et al.* 2002). Statistical analysis for mis-expression was performed using a moderated t-test from the package limma (SMYTH 2004). All genes with a false discovery rate of ≤5% ($q \leq 0.05$) (STOREY and TIBSHIRANI 2003) were selected for further analysis.

Single color Affymetrix microarray and tiling array expression data for dissected germlines were obtained from (TABUCHI *et al.* 2011) and (SPENCER *et al.* 2011), respectively. In addition, tiling array expression data were obtained for L4 *glp-1* larvae (soma) from (SPENCER *et al.* 2011). The data were $\log_2$ transformed and quantile normalized.

Quantitative RT-PCR

PCR was done as described (PETRELLA *et al.* 2011) with total RNA from three or four biological replicates of young adult germlines as prepared for microarrays.

ChIP-chip Experiments and Normalization

Collection of wild-type and *mes-4(RNAi)* early embryos, preparation of extracts, anti-H3K36me3 and anti-Pol II antibodies used, ChIP methods, and data processing and analysis are described in (RECHTSTEINER *et al.* 2010). Anti-H3K27me3 antibody was MAb 1E7 from H. Kimura.

MES-4 and H3K36me3 ChIP-chip normalization in both wild type and *mes-4(RNAi)* was performed as described in detail (Rechtsteiner et al., 2010). Briefly, for wild type all $\log_2$ ChIP/Input probe values were standardized to mean 0 and standard deviation 1 genome wide. MES-4 and H3K36me3 are largely absent from the X chromosome in wild type. Therefore, in *mes-4(RNAi)* both factors were standardized so $\log_2$ ChIP/Input probe values had the same mean and standard deviation as on the X chromosome in wild type. H3K27me3, which is a broad histone mark occupying more than half the genome, was standardized so $\log_2$ ChIP/Input probe values within the gene boundaries of the 50% top expressed genes had mean 0 and standard deviation 1.

Microarray and ChIP Accession Numbers

Microarray and ChIP data were deposited to Gene Expression Omnibus. The super series accession number is GSE38160.

Gene Category Analysis

Gene categories were defined using previously published microarray (REINKE *et al.* 2004) and SAGE studies (MEISSNER *et al.* 2009; WANG *et al.* 2009). Ubiquitous includes genes with at least one SAGE tag in muscle, neural, gut and germline. Germline-enriched includes genes with transcripts enriched in adults with a germline compared to adults lacking a germline by microarray analysis (REINKE *et al.* 2004). Germline-specific includes genes with at least one SAGE tag in germline and zero SAGE tags in muscle, neural, and gut. Soma-specific includes genes with at least two SAGE tags in muscle, neural, and/or gut and zero SAGE tags in germline. Additional gene categories used in Figure 2-7 include muscle-enriched and ASE-enriched. Muscle-enriched genes have at least three times more SAGE tags in muscle than neuron and gut and no SAGE tags in germline (MEISSNER *et al.* 2009; WANG *et al.* 2009). Gustatory neuron (ASE)-enriched genes were defined similarly to (ETCHBERGER *et al.* 2007), genes with at least three times more SAGE tags in ASE neurons than in thermosensory neurons (AFD). In addition, we required them to have zero SAGE tags in germline (WANG *et al.* 2009). Any genes with no signal in microarray or ChIP experiments were excluded from the analysis. The expected number of genes from microarray or ChIP analysis in each gene category was calculated as [(total number of genes in category on X or autosomes)/(total number of genes on X or autosomes)]*[number of genes up- or down-regulated on X or autosomes (for microarray analysis) OR number of genes with increased or decreased H3K27me3 in *mes-4(RNAi)* (for ChIP analysis, see below)].

Identification of Genes with a Significant Increase or Decrease in H3K27me3 in *mes-4(RNAi)*

Standardized $\log_2$ ChIP/Input ratios (z-scores) for H3K27me3 in wild type and *mes-4(RNAi)* were median smoothed over 1kb overlapping windows along the genome. A 1kb window was identified as showing a significant increase in H3K27me3 in *mes-4(RNAi)* compared to wild type if its median H3K27me3 value in wild type had $z < 0$ and in *mes-4(RNAi)* $z > 0.5$. A window was identified as showing a significant decrease in H3K27me3 if its median H3K27me3 value in wild type had $z > 0.5$ and in *mes-4(RNAi)* $z < 0$. Overlapping windows with increased or decreased H3K27me3 were strung together. A gene showed an increase or decrease in H3K27me3 if its gene body overlapped by at least 300 bp with a window that was identified as having an increase or decrease in H3K27me3 in *mes-4(RNAi)*.

qPCR Primer Sequences

C05E11.1, f-tcgaatgcggacatcttaatc; C05E11.1, r-ggacgagtcaacggaatttg

C07G3.9, f-aatctcgtcctgggttcca; C07G3.9, r-gagcacacggcttctttcac

C24G6.4, f-aagctcgagacttttgtgtgg; C24G6.4, r-ctcgaattgtttgcacttgg

C41D11.7, f-cctgcgcttgtttttactga; C41D11.7, r-tccaatatacgatccatcacaga

C46F4.1, f-tgcgaaccccaataaacatc; C46F4.1, r-tcggaccataataattgaacaca

F13B10.1, f-acaccgaccaccaaagaaat; F13B10.1, r-tcttggtccaccgatgct

F20D1.1, f-ggaacacgatccgaatgc; F20D1.1, r-cgaagaatagggagaggaaacc;

F28H6.4, f-gctcacaactgcgaaaacac; F28H6.4, r-cgatggagatgaagtcgatg;

F52H3.3, f-tgttggcaaagcttcagga; F52H3.3, r-tgatcgctttaacgtcggata

F57G4.8, f-gtctataaattgggccaggag; r-gggttgaatctccattcactg

F59A1.7, f-atgccattggacatcattca; F59A1.7, r-caaacttttcgaagagaaagtcg

R03A10.4, f-tgtcgtcctcatttgctc; R03A10.4, r-tgaactcaacccaaatagatg

T04C12.5, f-cgtcatcaaggagtcatggtc; T04C12.5, r-catgtcgtcccagttggtaa

T04D3.8, f-cagaagccgttattgcacag; T04D3.8, r-tccatttgatggtcttcgtg

Y37H2A.4, f-acggtcgctgatgctattaaa; Y37H2A.4, r-aacacaatgatagttccgctgtt

Y48G8AL.11, f-aagttgtcgcactttgtggtc; Y48G8AL.11, r-cgagcagatgggttattgtg

Y48G9A.11, f-tgtggcaaaaccaagaattg; Y48G9A.11, r-ttagagtgtttggcctgatgc

Y49E10.4, f-agcttgagccagagtggaaa; Y49E10.4, r-gatggactcgtgagcagttg

Y50D4C.3, f-ccacgtggaatgggaaat; Y50D4C.3, r-ttgttggtgggattgttgaa

ZC434.8, f-gcaatggatttgggagagaa; ZC434.8, r-gggtatccttgcagagaacg

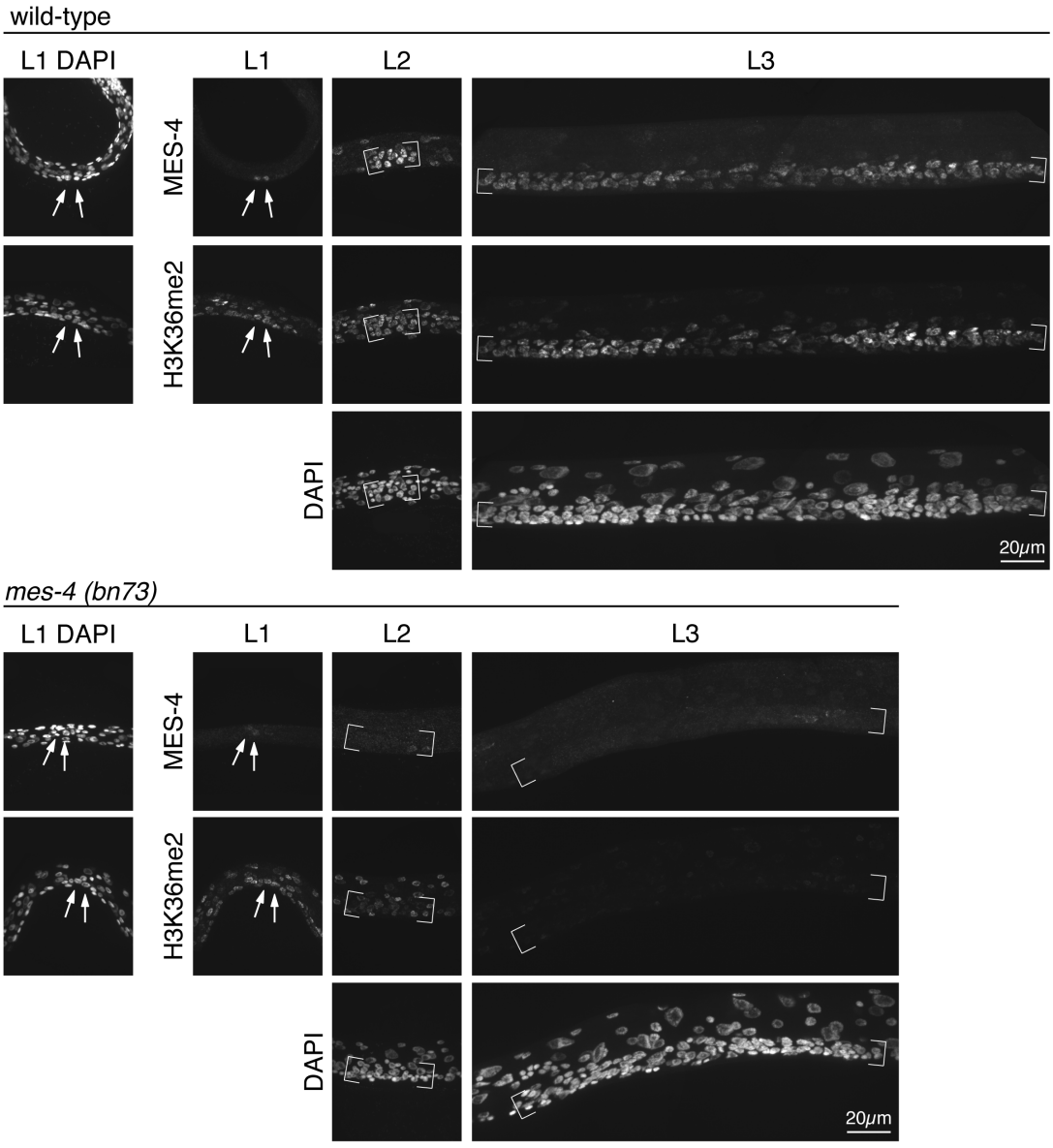


Figure 2-1. Perdurance of maternal MES-4 and H3K36me2 in M+Z- *mes-4* mutants. DAPI staining of nuclei and antibody staining of MES-4 and H3K36me2 (Kimura MAb 2C3, SS#69) in wild type (WT) and *mes-4*(*bn73)* mutants at larval stages L1-L3. Images show part of the larvae including all germ cells. Germ cells are indicated by arrows and brackets and were identified by PGL-1 staining at the L1 stage. Very weak H3K36me2 and MES-4 staining were observed in nuclei in the proximal adult germline of all *mes-4* alleles tested. This likely represents background staining. Identical exposures and image processing were used for wild type and *mes* mutants at each stage.

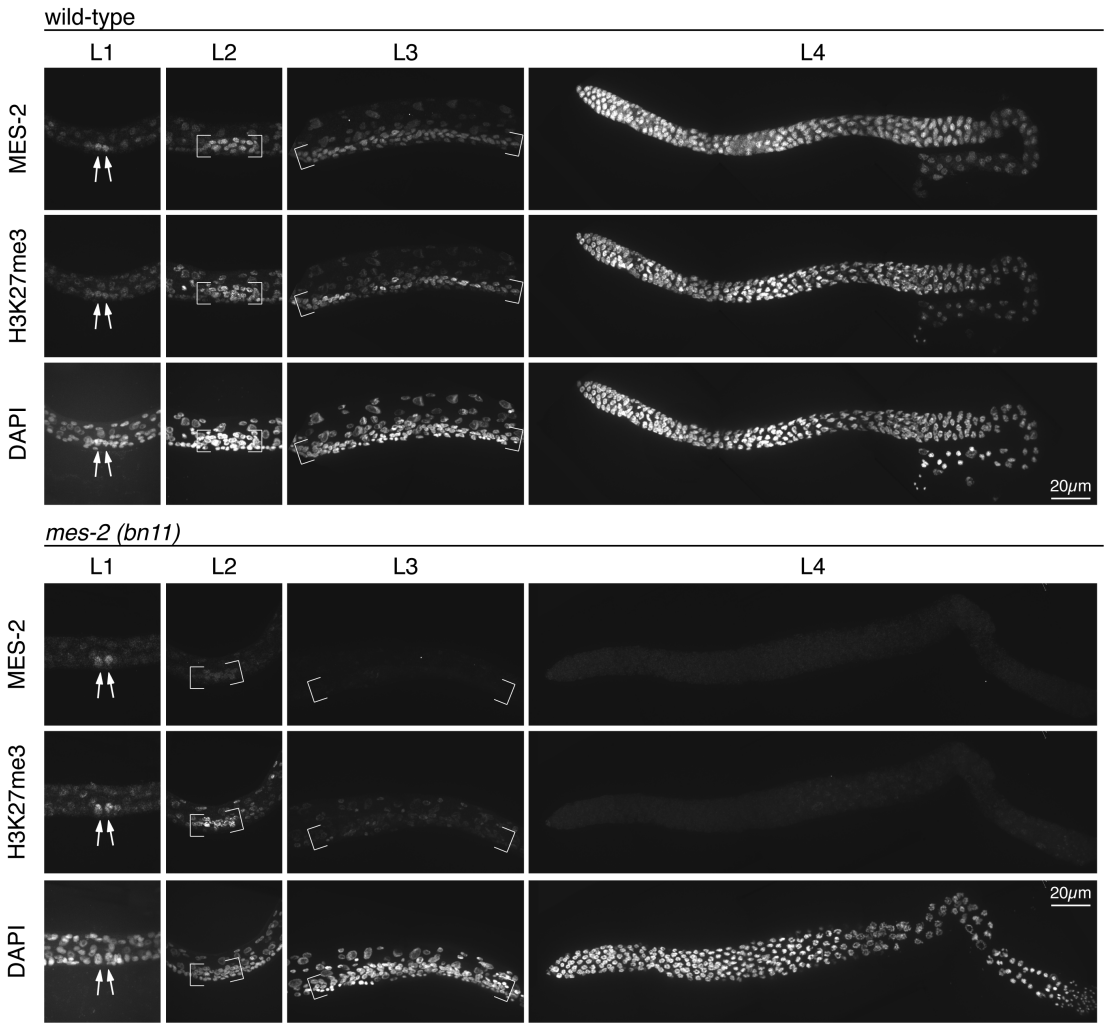


Figure 2-2. Perdurance of maternal MES-2 and H3K27me3 in M+Z- *mes-2* mutants. DAPI staining of nuclei and antibody staining of MES-2 and H3K27me3 (Active Motif 39535 Lot #174, SS#29) in wild type (WT) and *mes-2(bn11)* mutants at larval stages L1-L4. L1-L3 images show part of the larvae including all germ cells. Germ cells are indicated by arrows and brackets and were identified by PGL-1 at the L1 stage. L4 images show dissected germlines. Identical exposures and image processing were used for wild type and *mes* mutants at each stage. Some H3K27me3 staining was observed in the proximal germline of M+Z- *mes-2* adults with the anti-H3K27me3 antibody used by (BENDER *et al.* 2004) and with an antibody from Upstate (07-449 Lot#24440, SS#41) but not with two other antibodies (Active Motif 39535 Lot #174, SS#29 and Kimura MAb 1E7, SS#70).

A

	MES-2		H3K27me3		MES-4		H3K36me2	
Stage	WT	*mes-2*	WT	*mes-2*	WT	*mes-4*	WT	*mes-4*
L1	+++	+++	+++	+++	+++	++	+++	+++
L2	+++	++	+++	+++	+++	-	+++	+
L3	+++	-	+++	+	+++	-	+++	-
L4	+++	-	+++	+	+++	-	+++	-
Adult	+++	-	+++	-	+++	-	+++	-

B

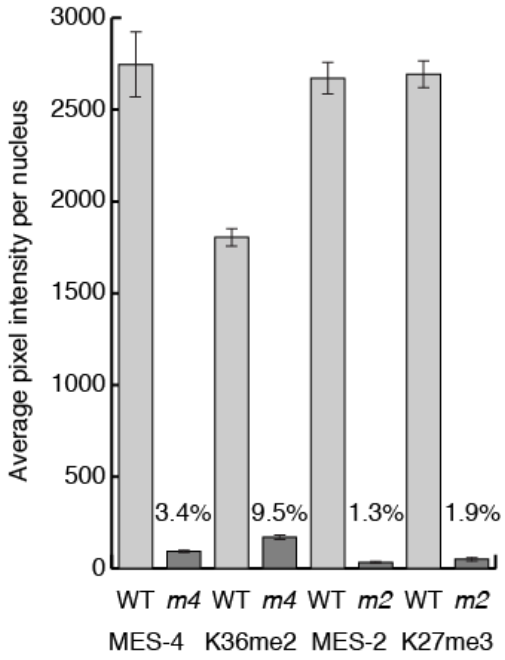


Figure 2-3. Summary and quantitation of MES proteins and histone mark perdurance in the M+Z- generation.
(A) Summary of the perdurance (+) of maternal MES proteins and histone marks in *mes-2* and *mes-4* mutants from Figures 2-1 and 2-2. **(B)** Quantification of MES-4, H3K36me2, MES-2, and H3K27me3 immunostaining pixel intensity in L4 wild-type (WT) and M+Z- *mes* mutant germlines. Light grey bars represent wild type and dark grey bars represent *mes* mutants (*m4* is *mes-4(bn73)*, *m2* is *mes-2(bn11)*). Values above the bars are percentages of average pixel intensity in mutants compared to wild type. Histogram bars are each based on at least 100 nuclei collectively within 4-5 germlines; error bars indicate s.e.m. The average pixel intensity of DAPI staining was not significantly different between wild type and *mes* mutants (data not shown).

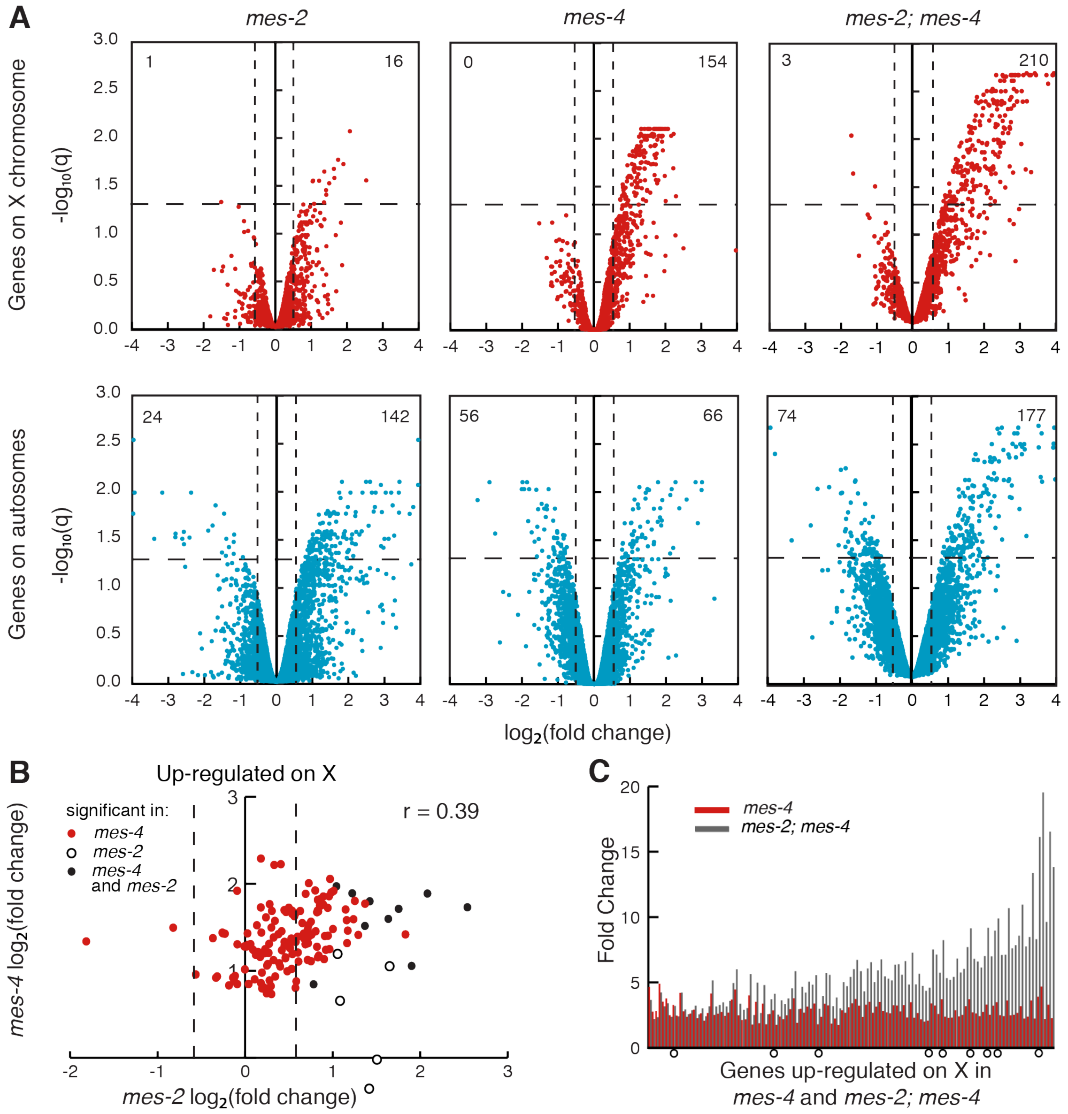


Figure 2-4. Microarray analysis of germlines from *mes-2*, *mes-4,* and *mes-2; mes-4* mutants compared to wild type.
(A) Volcano plots showing $\log_2$ of the fold change (FC) between *mes* and wild-type expression and the false discovery rate ($-\log_{10}$ q) of all genes on the microarray. Dashed lines mark the significance cut-off of q=0.05 and 1.5-fold up- or down-regulation. The numbers of genes significantly up- or down-regulated are in the top quadrants. Genes with $\log_2$(FC) >4 or <-4 are represented as 4 or -4. For validation of data by quantitative PCR, see Table 2-1. **(B)** Comparison of $\log_2$(FC) of X-linked genes significantly mis-regulated in *mes-4* only (red circles), *mes-2* only (white circles), and both *mes-4* and *mes-2* (black circles). Dashed lines show 1.5-fold up- or down-regulation. Correlation coefficient is 0.39. **(C)** Fold changes of genes significantly up-regulated in *mes-4* (red bars) and *mes-2; mes-4* (black bars). Open circles, genes significantly up-regulated in *mes-2* as well.

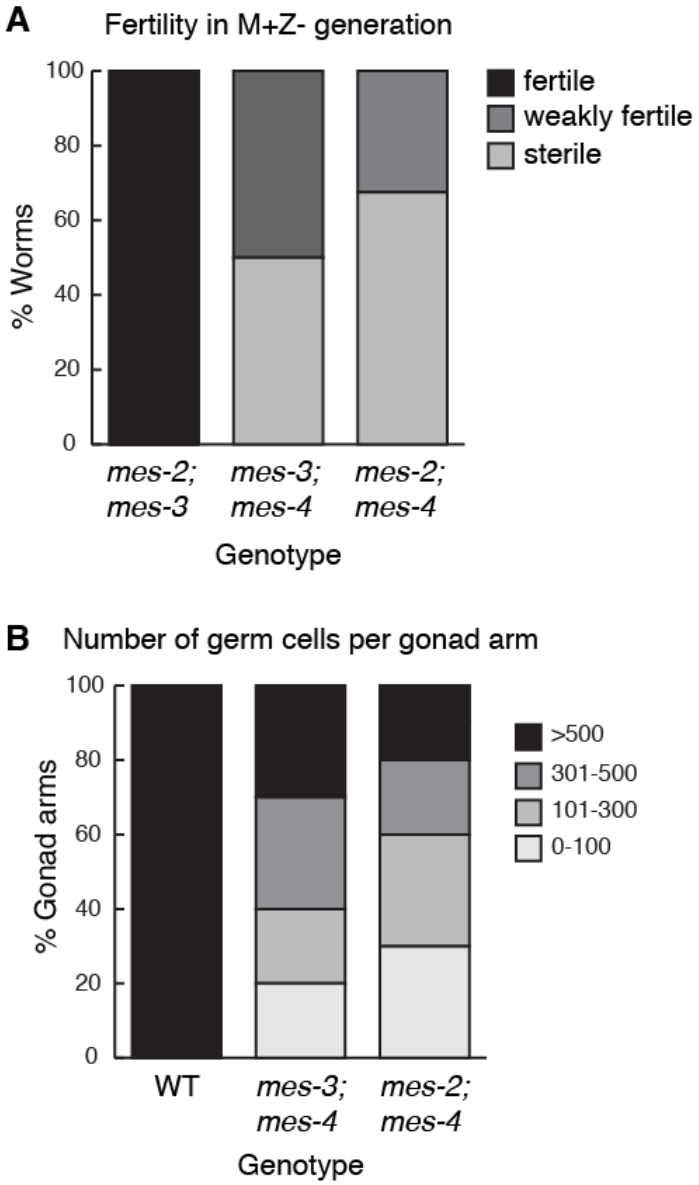


Figure 2-5. Phenotype of *mes* double mutants.
(A) Fertility of *mes* double mutants in the M+Z- generation. Genotypes: *mes-2(bn11); mes-3(bn35), mes-3(bn35); mes-4(bn85), mes-2(bn11); mes-4(bn85).* Fertile (more than 100 progeny), weakly fertile (1-18 progeny), sterile (no progeny). *mes-2(bn11) and mes-3(bn35)* are null mutations that result in no detectable protein. *mes-4(bn85)* is a large deletion in the SET domain, but protein is still detectable. *mes-4(bn73)*, a null allele with no detectable protein, showed similar reduced fertility in combination with *mes-2(bn11)* (data not shown). **(B)** Number of germ cells in gonads of *mes* double mutant young adults in the M+Z- generation. Nuclei from 6 wild-type and 10 mutant gonad arms were counted.

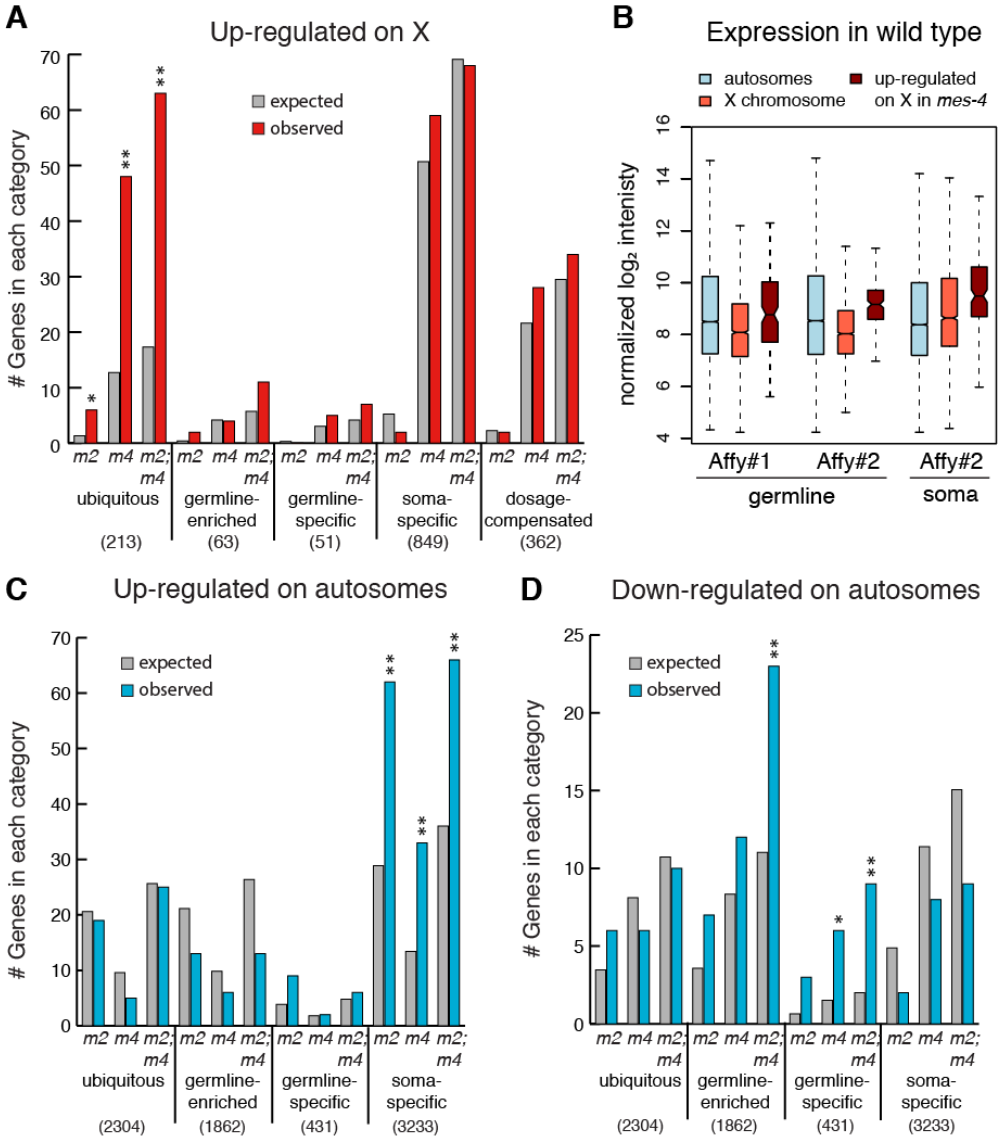


Figure 2-6. Genes mis-regulated in *mes* mutant germlines are enriched for particular expression categories.

(A,C,D) Expected and observed numbers of genes in different expression categories among genes up-regulated on the X **(A)**, genes up-regulated on autosomes **(C)**, and genes down-regulated on autosomes **(D)** in *mes-2* (*m2*), *mes-4* (*m4*) and *mes-2; mes-4* (*m2; m4*) compared to wild type (WT). See text and experimental procedures for definitions of expression categories. Numbers in parentheses are the total number of genes in each category on the X or on the autosomes. Asterisks indicate significantly more genes than expected (hypergeometric test p-value < 0.01 (*) or < 0.001 (**)). See also Figure 2-8C. **(B)** Comparison of autosomal and X-linked transcript levels in wild-type germlines and somatic tissue analyzed on single-color Affymetrix microarrays (Affy #1; (TABUCHI *et al.* 2011)) and tiling arrays (Affy #2; (SPENCER *et al.* 2011)).

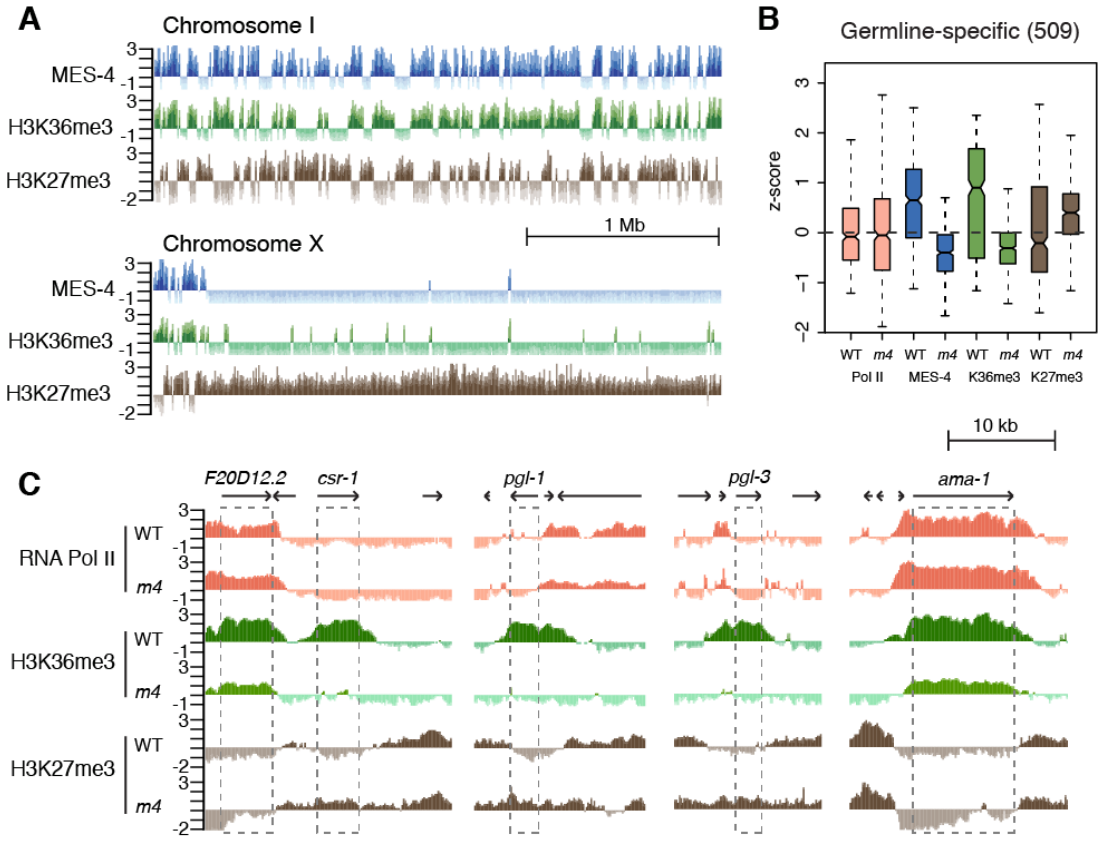


Figure 2-7. Genomic distributions of MES-4, H3K36me3, and H3K27me3. **(A)** ChIP-chip z-scores (standardized $\log_2$ ratios of ChIP/Input signals) of MES-4, H3K36me3, and H3K27me3 across the leftmost 3 Mb of ChrI and ChrX. **(B)** Levels of RNA Polymerase II, MES-4, H3K36me3, and H3K27me3 on germline-specific genes in wild type (WT) and *mes-4(RNAi)* (*m4*). Each box extends from the 25th to 75th percentile of the z-scores in the set. Whiskers extend to the 2.5th and 97.5th percentile. Wedges around the median indicate 95% confidence interval for the medians. See also Figure 2-9. **(C)** Genome browser views of germline genes (*csr-1, pgl-1,* and *pgl-3*), showing absence of Pol II in wild type, and loss of H3K36me3 and acquisition of H3K27me3 in *mes-4(RNAi)*. F20D12.2 and *ama-1* have Pol II, and do not lose H3K36me3 or acquire H3K27me3 in *mes-4(RNAi)*.

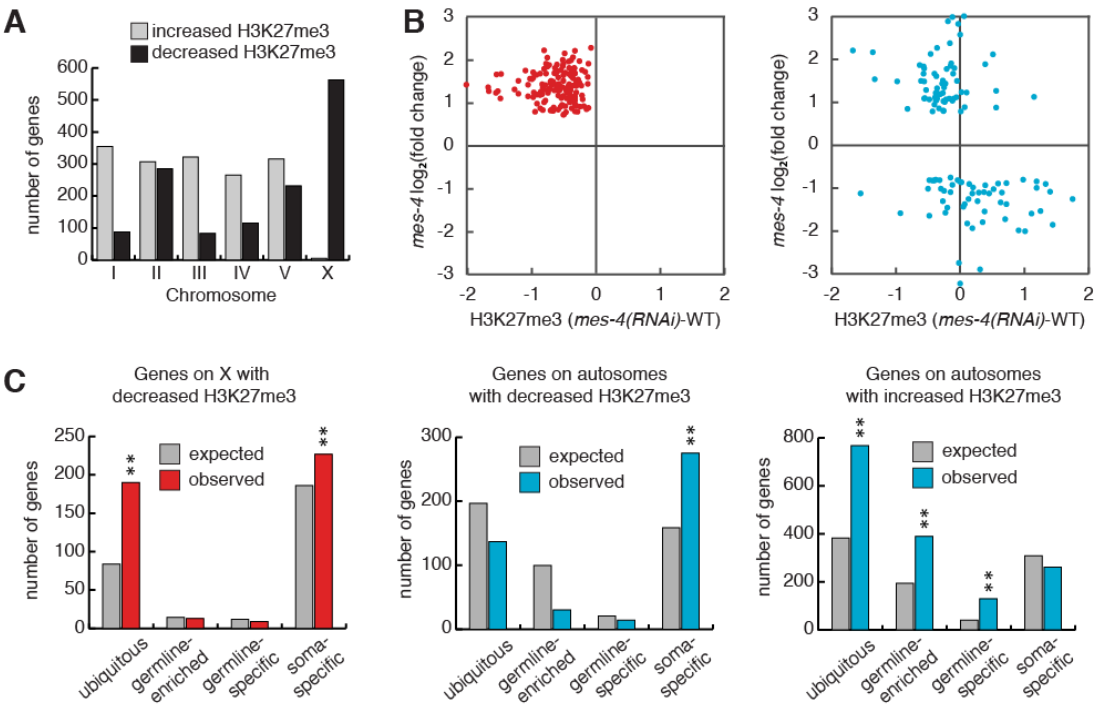


Figure 2-8. Assessment of redistribution of H3K27me3 upon depletion of MES-4. **(A)** Number of genes on each chromosome with significantly increased or decreased H3K27me3 in *mes-4(RNAi)* compared to wild type. **(B)** For 276 genes mis-regulated in *mes-4* mutant germlines, comparison of gene mis-expression (X genes, red circles; autosomal genes, blue circles) versus change in H3K27me3 levels between *mes-4(RNAi)* and wild-type early embryos. **(C)** Expected and observed numbers of genes in different expression categories among genes with significantly increased or decreased H3K27me3 in *mes-4(RNAi)* compared to wild type.

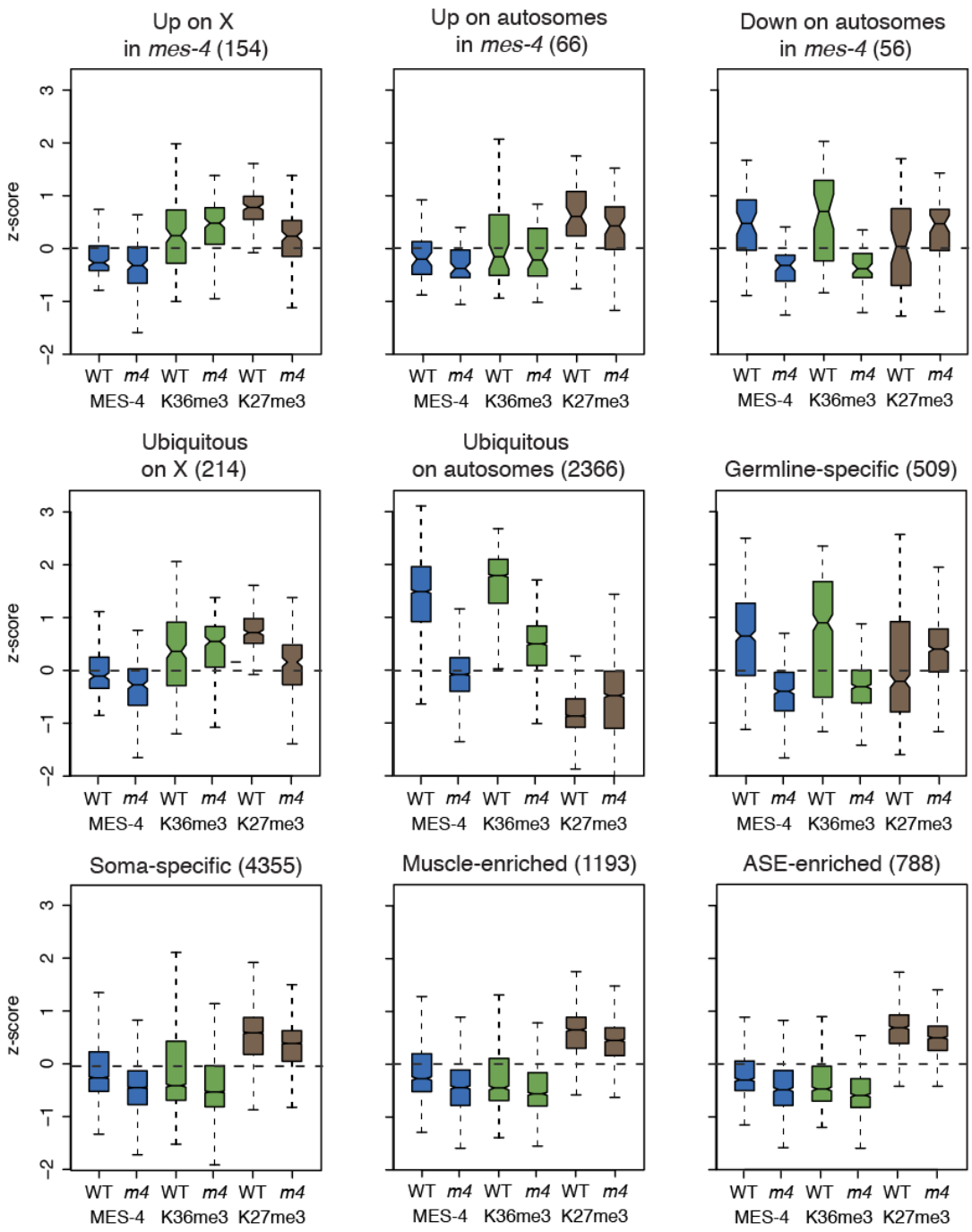


Figure 2-9. Levels of MES-4, H3K36me3, and H3K27me3 on different gene sets in wild-type (WT) and *mes-4(RNAi)* (*m4*) early embryos.
The number of genes in each gene set is in parentheses. Each box extends from the 25th to 75th percentile of the z-scores in the set. Whiskers extend to the 2.5th and 97.5th percentile. Wedges around the median indicate 95% confidence intervals for the medians.

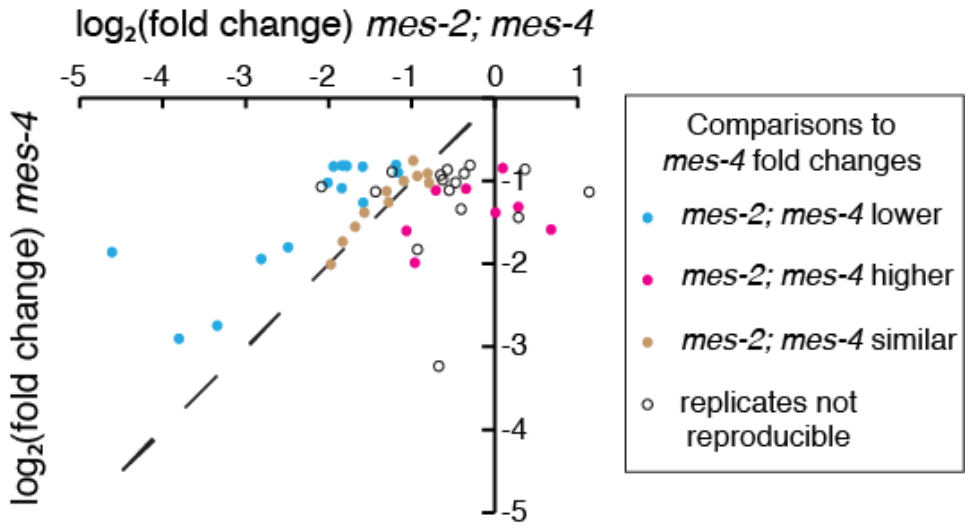


Figure 2-10. Log_2 fold change (FC) of down-regulated genes in *mes-4* and *mes-2; mes-4* germlines.
All genes were significantly down-regulated in *mes-4* mutants. Colored dots represent genes that had reproducible replicates in *mes-2; mes-4* (at least three of four replicates with raw *mes-2; mes-4* log_2 FC between -0.585 and 0.585, log_2 FC <-0.585, or log_2 FC >0.585). Blue, log_2 FC for *mes-2; mes-4* is lower than log_2 FC for *mes-4* by more than 0.25. Pink, log_2 FC for *mes-2; mes-4* is higher than log_2 FC for *mes-4* by more than 0.25. Tan, log_2 FC for *mes-2; mes-4* is within 0.25 of log_2 FC for *mes-4*. Open circles, replicates were not reproducible or signal on microarrays was too low.

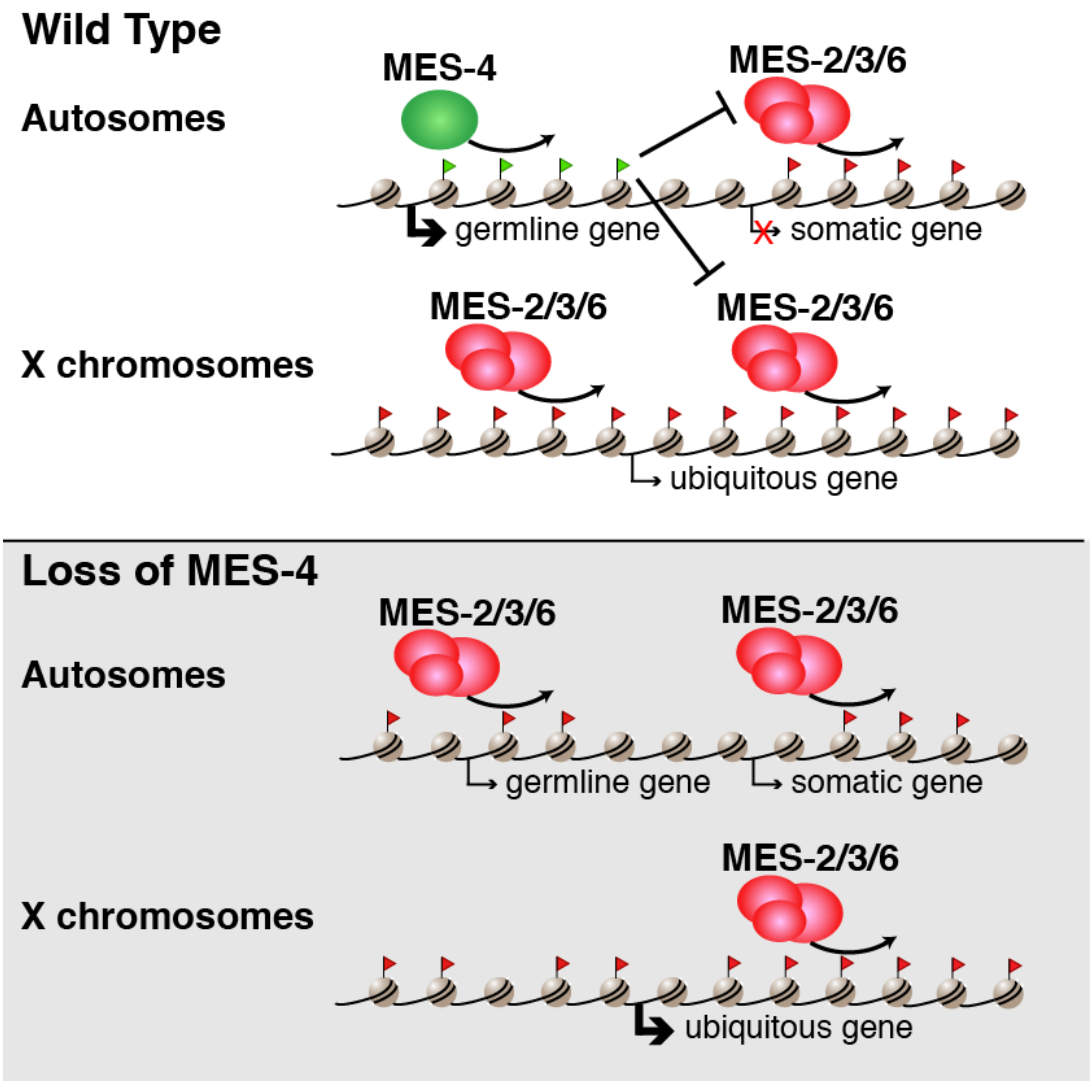


Figure 2-11. Model of MES-2/3/6 and MES-4 regulation of gene expression in the germline with and without MES-4.
DNA wrapped around histones is shown with MES protein regulation on germline genes, somatic genes, and ubiquitous genes. Colored flags represent histone marks generated by MES proteins, H3K27me3 in red and H3K36me3 in green. Arrows represent gene expression levels; bold arrows represent high expression, thin arrows represent low expression, and arrows with a red X represent no expression.

		Microarray		Quantitative PCR	
Gene	Genotype	Fold change	q-value	Fold change	p-value
Up-regulated on X					
F20D1.1	*mes-2*	2.7	0.039	3.3	0.007
	mes-4	3.5	0.027	8.8	0.000
	mes-2; mes-4	9.8	0.002	12.3	0.008
C46F4.1	*mes-2*	2.1	0.028	2.8	0.023
	mes-4	3.9	0.008	8.2	0.009
	mes-2; mes-4	16.1	0.002	40.6	0.001
C05E11.1	*mes-2*	2.3	0.028	2.6	0.019
	mes-4	3.7	0.009	4.4	0.011
	mes-2; mes-4	9.1	0.002	12.4	0.021
R03A10.4	*mes-2*	1.2	0.673	1.7	0.039
	mes-4	3.2	0.008	7.9	0.008
	mes-2; mes-4	7.1	0.004	22.2	0.002
F28H6.4	*mes-2*	2.6	0.022	2.3	0.018
	mes-4	2.9	0.018	4.4	0.017
	mes-2; mes-4	4.1	0.032	10.8	0.001
Up-regulated on autosomes					
F52H3.3	*mes-2*	3.1	0.0169	2.7	0.010
	mes-4	2.0	0.0346	3.3	0.014
	mes-2; mes-4	4.3	0.0071	12.0	0.004
C07G3.9	*mes-2*	2.4	0.0284	3.3	0.025
	mes-4	2.7	0.0102	6.9	0.048
	mes-2; mes-4	2.4	0.0175	3.5	0.056
C24G6.4	*mes-2*	3.4	0.0343	3.9	0.003
	mes-4	2.1	0.0277	5.0	0.007
	mes-2; mes-4	2.5	0.0448	12.7	0.003
F13B10.1	*mes-2*	2.6	0.0578	3.1	0.027
	mes-4	1.7	0.034	3.8	0.010
	mes-2; mes-4	3.6	0.0391	16.4	0.015

		Microarray		Quantitative PCR	
Gene	Genotype	Fold change	q-value	Fold change	p-value
Down-regulated on autosomes					
Y49E10.4	*mes-2*	0.111	0.010	0.102	0.003
	mes-4	0.275	0.008	0.307	0.040
	mes-2; mes-4	0.041	0.002	0.055	0.000
Y37H2A.4	*mes-2*	0.164	0.031	0.119	0.006
	mes-4	0.301	0.026	0.463	0.108
	mes-2; mes-4	0.281	0.019	0.696	0.202
Y48G9A.11	*mes-2*	0.168	0.026	0.123	0.032
	mes-4	0.149	0.027	0.033	0.036
	mes-2; mes-4	0.099	0.032	0.003	0.005
F59A1.7	*mes-2*	0.065	0.010	0.150	0.000
	mes-4	0.500	0.043	0.807	0.135
	mes-2; mes-4	0.470	0.034	0.971	0.965
C41D11.7	*mes-2*	0.559	0.202	0.791	0.202
	mes-4	0.342	0.015	0.442	0.161
	mes-2; mes-4	0.312	0.029	0.555	0.095
ZC434.8	*mes-2*	0.693	0.142	0.684	0.017
	mes-4	0.470	0.015	0.432	0.014
	mes-2; mes-4	0.279	0.006	0.227	0.011
Y48G8AL.11	*mes-2*	0.143	0.030	0.113	0.001
	mes-4	0.287	0.009	0.206	0.020
	mes-2; mes-4	0.178	0.065	0.195	0.002
F57G4.8	*mes-2*	0.168	0.035	0.097	0.000
	mes-4	0.403	0.014	0.537	0.038
T04D3.8	*mes-2*	1.072	0.859	0.450	0.024
	mes-4	0.261	0.009	0.238	0.086
	mes-2; mes-4	0.143	0.022	0.051	0.001
Y50D4C.3	*mes-2*	0.566	0.223	0.529	0.027
	mes-4	0.384	0.025	0.418	0.131
	mes-2; mes-4	0.337	0.008	0.323	0.035

Table 2-1. Validation of microarray analysis by quantitative PCR.
Significantly mis-regulated genes from the microarray analysis were tested by quantitative PCR to verify their mis-regulation. p-values were calculated using a one sample t-test.

CHAPTER 3: H3K27 Methylation and PRC2 Epigenetically Transmit a Memory of Repression Across Generations and During Development

INTRODUCTION

Proper development depends on regulation of gene expression by packaging the genome into expressed and repressed chromatin domains. Our understanding of how that packaging is achieved and maintained is incomplete. Methylation of histone H3 on Lys 27 (H3K27me) is a well-established mark of repressed chromatin that is generated by Polycomb Repressive Complex 2 (PRC2) in diverse phyla. In *Drosophila,* PRC2 and H3K27me maintain repression of important genes, including the Hox genes during somatic development and cell cycle genes during germline development (CAO *et al.* 2002; IOVINO *et al.* 2013; PENGELLY *et al.* 2013). In mammals, H3K27me is also present on developmentally important genes in somatic and germ cells (BERNSTEIN *et al.* 2006; HAMMOUD *et al.* 2009; MOCHIZUKI *et al.* 2012), and PRC2 serves numerous roles during development, including participation in X inactivation and differentiation of embryonic stem cells (HEARD 2005; PASINI *et al.* 2007). In *C. elegans*, PRC2 is required only in germ cells (CAPOWSKI *et al.* 1991) and, as shown in this chapter, only for repression of the X chromosomes.

A critical question is how H3K27me-repressed chromatin states are passed from mother to daughter cells. One model is that H3K27 methylated histones are passed locally to the two daughter chromatids during DNA replication (LANZUOLO *et al.* 2011). Another model is that PRC2, but not methylated histones, is passed locally to daughter chromatids and newly establishes patterns of H3K27me after each round

of DNA replication (PETRUK *et al.* 2012). The limited requirement for PRC2 in *C. elegans* allowed me to test these models by examining cells with differentially H3K27-methylated chromosomes and containing or lacking PRC2 activity. I present evidence that H3K27-methylated histones transmit the memory of repression transgenerationally and short-term in embryos and that PRC2 promotes long-term memory during development.

C. elegans PRC2 is composed of MES-2 (ortholog of E(Z)/EZH2), MES-6 (ortholog of ESC/EED), and MES-3 (worm-specific subunit), and is essential for germline development but not somatic development (CAPOWSKI *et al.* 1991; XU *et al.* 2001a; BENDER *et al.* 2004). PRC2 is maternally supplied to progeny and needed for the progeny's primordial germ cells to survive and proliferate (CAPOWSKI *et al.* 1991; PAULSEN *et al.* 1995). In both naturally occurring sexes in *C. elegans*, hermaphrodites with two X chromosomes (XX) and males with one X chromosome (XO), germline development requires near silencing of the X chromosomes (STROME *et al.* 2014). Several findings have shown that PRC2-generated H3K27me3 participates in repressing the X chromosomes in XX germ cells: repressive H3K27me3 is on all chromosomes but is concentrated on the X chromosomes in germ cells (BENDER *et al.* 2004), and loss of PRC2 function causes germlines to up-regulate numerous genes on the X (GAYDOS *et al.* 2012). Whether XO germlines require PRC2 repression has been unclear (GARVIN *et al.* 1998). I demonstrate that PRC2 is the main mode of X repression in XO worms, but that PRC2 function can be

bypassed if the single X in XO worms experienced two other repressive conditions in the parental germline: spermatogenesis and H3K9me.

The work in this chapter investigates a finding initially made by Wenchao Wang, a graduate student in the Strome lab at Indiana University. He found that *mes* males were fertile if they inherited their X chromosome from the sperm, but not if they inherited their X chromosome from the oocyte. I repeated Wenchao Wang's experiments and confirmed his findings and then followed up by performing a thorough analysis of different genotypes and alleles of *mes* males and by investigating X-chromosome repression and inheritance, as described in this chapter.

RESULTS AND DISCUSSION

Loss of maternal PRC2 causes sterility in all XX offspring but affects XO offspring in a manner that depends on the gamete source of the X chromosome.

mes mutant hermaphrodites from homozygous *mes/mes* mothers display highly penetrant maternal-effect sterility. Studies of *mes* mutants with different doses of the X chromosome led to the hypothesis that over-expression of the X chromosome may underlie the maternal-effect sterility of *mes* mutants. Compared to *mes* mutant hermaphrodites (XX), mutants with an extra X chromosome (XXX hermaphrodites) display more severe sterility, and mutants with fewer X chromosomes (XO males) produce gametes and can be fertile (GARVIN *et al.* 1998). It was subsequently observed that XO *mes* mutant males generated by a different method than used by Garvin et al. are always sterile. This prompted me to test if the

fertility of XO *mes* males depends on the gamete source of their single X chromosome.

The X chromosomes in germ cells are globally repressed during most stages of development except during late oogenesis (KELLY *et al.* 2002; STROME *et al.* 2014). Thus, an X chromosome inherited from the sperm (X^{sp}) has a repressed transcriptional "history", while an X chromosome inherited from the oocyte (X^{oo}) lost repression prior to fertilization. I analyzed XO *mes* mutant males that inherited their X chromosome from these two different backgrounds. To generate XO (X^{oo}) *mes* males, XX hermaphrodites were mated to XO males. Male offspring resulted from the union of an X-bearing oocyte from the hermaphrodite with a nullo-X sperm from the male (Figure 3-1A). XO (X^{sp}) *mes* males were generated using *him-8* XX mothers that undergo non-disjunction of their X chromosomes predominantly during oogenesis (HODGKIN *et al.* 1979) and produce male offspring from the union of a nullo-X oocyte with an X-bearing sperm (Figure 3-1A). To unambiguously identify the gamete source of the X in XO *mes-3* males, one or the other parent contributed to progeny an X chromosome that expressed an X-linked GFP-tagged nuclear lamin transgene (shown as a green X) (Figure 3-1B,C).

I found that XO *mes-3* mutants that inherited their X from the oocyte (X^{oo}) had a severely underproliferated germline, lacked sperm, and were usually sterile. 0% of X^{oo} males with a non-marked X chromosome were fertile, and 1% of X^{oo} males with an X chromosome marked by *lmn-1*::GFP expression were fertile (Figure 3-1C). In striking contrast, XO *mes-3* mutants that inherited their X from the sperm (X^{sp})

generally had a well-proliferated germline. 73% of X^{sp} males with a non-marked X chromosome were fertile, and 82% of X^{sp} males with an X chromosome marked by *lmn-1*::GFP expression were fertile (Figure 3-1C). Similar results were observed for *mes-2* and *mes-6* mutants with respect to germline proliferation (Figure 3-2A), but *mes-2* mutants were always sterile (Figure 3-2B). MES-2 is in the same complex as MES-3 and MES-6, and all three mutants were expected to show similar phenotypes. I looked into possible explanations for *mes-2* mutants being sterile despite having well proliferated germlines. The sperm generated by *mes-2; him-8* males were competent to be activated and extend pseudopods *in vitro* (Figure 3-2D), and should be able to fertilize oocytes. I tested for the ability of *mes-2; him-8* males to transfer sperm to hermaphrodites during mating by labeling sperm with MitoTracker red and looking for the presence of red sperm in hermaphrodites. I did not see evidence of sperm transfer from *mes-2* males to hermaphrodites, while control males effectively transferred MitoTracker-labeled sperm. I hypothesize that the failure to transfer sperm, not defective germ cell proliferation, underlies the sterility *mes-2; him-8* males.

MES-4 operates distinctly from the MES-2/3/6 complex and displays more severe mutant phenotypes. Only 10% of *mes-4; him-8* males were fertile and 0% of *mes-4* males generated by mating were fertile (Figure 3-2A,B). Most *mes-4; him-8* males had under-proliferated germlines with no sperm. This disagrees with a previous study that reported that the majority of *mes-4; him-8* males had a well proliferated germline with sperm (GARVIN *et al.* 1998). This discrepancy could be

due to growth conditions that differed between past and present experiments. For this reason, I focused on *mes-2*, *mes-3*, and *mes-6*, whose protein products form a trimeric complex that resembles Polycomb Repressive Complex 2 and generates repressive H3K27me3. Despite *mes-2* XO males always being sterile (see preceding paragraph), *mes-2, mes-3* and *mes-6* all show the same basic phenotype for germ cell proliferation in XO males and XX hermaphrodites. In hermaphrodites XX worms inherit X chromosomes from both sperm and oocyte and require PRC2 for germline health and fertility. Since XO worms that inherit a sperm X do not require PRC2, the only essential role of PRC2 in worms is repression of the X chromosome in germ cells.

Loss of maternal PRC2 and PRC2-generated H3K27 methylation is tolerated in XO worms with a sperm-contributed X because H3K9 methylation provides an alternative mechanism of X repression.

I sought to determine the mechanism by which a sperm-inherited X chromosome allows a *mes-2, mes-3*, or *mes-6* mutant male to have a proliferated and healthy germline and in the case of *mes-3* and *mes-6* to become a fertile adult. MES-2/3/6-generated H3K27me3 is associated with repression and is enriched on the X chromosomes in the *C. elegans* germline (BENDER *et al.* 2004; MARGUERON and REINBERG 2011). I reasoned that another repressive mark enriched on the X in the germline, specifically in the sperm-contributing parent, might suffice to repress the X in male offspring. H3K9me2 is a repressive mark enriched on chromosomes that lack a pairing partner during meiosis, including the single X chromosome in males (BEAN

et al. 2004). When the X chromosome has a pairing partner, such as in wild-type hermaphrodites and *tra-2* males (OKKEMA and KIMBLE 1991), H3K9me2 is not enriched on the X but is present at lower levels on all chromosomes (BEAN *et al.* 2004). To test if H3K9 methylation, either enriched on the X chromosome or not enriched, is an alternative mechanism of X repression in the absence of MES-2/3/6-generated H3K27me3, I analyzed mutants that lack H3K27me3 and have either low levels of H3K9me2 or no H3K9me2.

I found that males whose parents lacked both repressive H3K27 and H3K9 methylation are sterile even if their X chromosome was inherited from the sperm, but are fertile even with low levels of H3K9me2. To analyze complete loss of H3K27me3 and H3K9, I used *mes-3* mutants (lacking H3K27me3), *met-2 set-25* double mutants (lacking H3K9me1, me2 and me3) (TOWBIN *et al.* 2012), and *mes-3; met-2 set-25* triple mutants (lacking both H3K27me and H3K9me). To analyze loss of H3K27me3 and low levels of H3K9me2, I used *mes-3; tra-2* XX males. *tra-2* males with 2 X chromosomes do not have H3K9me2 enriched on the Xs (Figure 3-3A). I compared the fertility of males with a sperm-derived X chromosome from these four genotypes. If either H3K27me3 or H3K9me2 can serve as a mechanism of X repression, I expected loss of either mark to be tolerated and loss of both marks to have a severe effect on male fertility. Indeed, *mes-3* males had well proliferated germlines and 73% were fertile, and *met-2 set-25* males had well proliferated germlines and 38% were fertile, while triple mutant males had severely under-proliferated germlines and 0% were fertile (Figure 3-3A). If enrichment of H3K9me2

on the X is required for fertility, I expected low levels of H3K9me2 on the X in male parents to have an effect on the fertility of male progeny as well. Males that inherited their X chromosome from sperm from *mes-3; tra-2* males were 68% fertile, similar to the 82% fertility of males that inherited their X from sperm with enriched H3K9me2 (Figure 3-3A,B). Thus, the X in the sperm-contributing parent must bear some H3K9me but need not be enriched for H3K9me to ensure fertility of *mes-3* males. This reveals that without repressive H3K27me3, H3K9 methylation provides an alternative mode of X repression.

Enrichment of H3K9me on the X chromosomes allows even XX offspring of PRC-2-lacking mothers to be fertile.

Our previous results suggest that, in the absence of PRC2 and H3K27me, H3K9me can act as an alternate mechanism of X-chromosome repression even without being enriched on the X chromosome. This allows *mes* XO males to be fertile if their X chromosome was inherited via sperm from a parent with H3K9me2 in their germline. When the X chromosome was inherited via the oocyte, XO males were usually sterile (Figure 3-1C and 3-2B), but if the parent had X-enriched H3K9me2 (*him-8*), 7% of XO males were fertile (data not shown). I tested if hermaphrodites can be fertile when both their inherited X chromosomes had X-enriched H3K9me2. To test this, I analyzed *mes* M-Z-*; him-8* hermaphrodite progeny from *mes* M+Z-*; him-8* mothers with enriched H3K9me2 on both X chromosomes during gametogenesis (Figure 3-4A). Normally 100% of *mes* M-Z- hermaphrodites

are sterile, but in a *him-8* background a small percent are fertile. 13% of XX *mes-2; him-8*, 2% of XX *mes-3; him-8* and, 6% of XX *mes-6; him-8* were fertile (Figure 3-4B). Consistent with this finding, from DAPI staining of whole worms, I found that a small percentage of worms resembled wild type with well proliferated germlines containing gametes, some had well proliferated germlines but lacked gametes, and some had under-proliferated germlines with no gametes (Figure 3-4C). As expected, most worms lacked germ cells (Figure 3-4C). To test if the *him-8* mutation causes some fertility independent of enriched H3K9me2 on the X, I tested the fertility of *mes-3; meDf2* hermaphrodites. These hermaphrodites carry a deficiency of part of the X chromosome that disrupts the ability of the X chromosomes to pair during meiosis, resulting in X-enriched H3K9me2 (VILLENEUVE 1994; LAMELZA and BHALLA 2012). In the M-Z- generation 7% of *mes-3; meDf2* mutants were fertile; similar to the fertility seen with *mes-3; him-8* mutants (Figure 3-4B). These results suggest that even though enrichment of H3K9me2 on the X is not required for fertility of XO *mes* mutants, such enrichment may confer upon the X chromosome a slightly more repressed state even during oogenesis, allowing for some fertility of XX *mes* hermaphrodites.

Next I tested if a higher percent of hermaphrodites are fertile if their inherited oocyte X had enriched H3K9me and their sperm X had both H3K27 and H3K9 methylation. This was achieved by mating *mes; him-8* hermaphrodites to wild-type males and analyzing XX hermaphrodite cross progeny. Both *mes-2* and *mes-3* mutants had an increase in the % fertile worms in the M-Z+ generation compared to

the M-Z- generation. 19% of *mes-2* and 6% of *mes-3* M-Z+ hermaphrodites were fertile (Figure 3-4B). Without the *him-8* mutation, 0% of *mes* M-Z+ hermaphrodites were fertile (Figure 3-4B). Some fertility of *mes-3; meDf2* M-Z+ hermaphrodites was also expected, but none was observed. This could be due to a complication of hermaphrodite progeny being heterozygous for *meDf2*; the analysis should be redone by crossing *mes-3; meDf2* M+Z- hermaphrodites to *meDf2* mutant males instead of wild-type males. These results emphasize my previous conclusion that fertility of *mes* mutants requires that the inherited X chromosome(s) was in a repressed state during gametogenesis.

Derepression of the X chromosome correlates with sterility in XO *mes* mutants.

I tested whether the gamete source of the X chromosome correlates with subsequent repression or expression of the X in XO male germlines, using an X-linked *lmn-1*::GFP transgene that in wild-type worms is well expressed in somatic cells and silenced in the germline (LIU *et al.* 2000). Widespread *lmn-1*::GFP expression was seen in 95% of germlines from XO (X^{oo}) *mes-3* mutants, but in only 9% of germlines from XO (X^{sp}) *mes-3* mutants (Figure 3-5A,B). These values are similar to the percentages of XO (X^{oo}) and XO (X^{sp}) *mes-3* mutants that were sterile (Figure 3-1C). *lmn-1*::GFP expression was not observed in wild-type germlines, *met-2 set-25* germlines lacking H3K9me, or under-proliferated germlines of *glp-4(ts)* mutants raised at the restrictive temperature (BEANAN and STROME 1992) (Figure 3-5B). Thus, X derepression correlates with sterility of *mes* mutants and is not a

consequence simply of under-proliferation of the germline or loss of other repressive histone marks. Our findings suggest that fertility depends on continued X-chromosome repression in the germline, which requires inheriting a repressed X chromosome.

Repressive H3K9me2 and H3K27me3 are transmitted to embryos on sperm chromosomes.

My data suggest that the memory of X-chromosome repression can be inherited through sperm. As sperm mature in *C. elegans*, many histones are exchanged for sperm-specific histone variants and putative protamine-like proteins (CHU *et al.* 2006). H3K4me has been detected in *C. elegans* sperm (ARICO *et al.* 2011), but repressive histone marks were previously reported to be absent from sperm (SCHANER and KELLY 2006). I tested if repressive histone marks are present on chromatin in mature sperm and if they are transmitted to embryos via sperm. Immunostaining of mature sperm revealed that H3K27me3 and H3K9me2 are both present in sperm chromatin (Figure 3-6).

To track the inheritance of sperm histone modifications in embryos, I analyzed early embryos from the union of mutant oocytes (lacking the histone mark of interest on maternal (M) chromosomes) with sperm from wild-type males (that contribute the histone mark on paternal (P) chromosomes). Such M-P+ embryos also lacked the maternally inherited histone methyltransferase (HMT) of interest and were not able to generate new histone marks during early embryogenesis. In M-P+ 1-cell

embryos from oocytes lacking H3K27me3 (*mes-3*) fertilized by wild-type sperm with H3K27me3, I observed H3K27me3 on the sperm-contributed autosomes and X chromosome but not the oocyte-contributed chromosomes (Figure 3-7A). In M-P+ 1-cell embryos from oocytes lacking H3K9me (*met-2 set-25*) fertilized by wild-type sperm, I observed H3K9me2 lightly on the sperm-contributed autosomes and heavily on the sperm-contributed X, similar to its pattern in the male germline (BEAN *et al.* 2004) (Figure 3-7B). These results demonstrate that the repressive histone marks H3K27me3 and H3K9me2 are transmitted to embryos by wild-type sperm.

In the absence of MES-2/3/6 and MET-2 SET-25 histone methyltransferase activity, H3K9me2 and H3K27me3 are transmitted through cell divisions.

My generation of embryos with H3K27me3 only on paternal chromosomes (*mes-3* M-P+) offered an opportunity to test whether H3K27me3 can be passed to daughter chromatids in the absence of HMT activity. In such M-P+ embryos, H3K27me3 persisted at detectable levels on chromatin until the 16-24-cell stage, through at least 4 rounds of DNA replication (Figure 3-7A,C). H3K27me3 remained associated with a subset of chromosomes, which I infer to be the sperm-derived chromosomes, and did not detectably spread to all chromosomes in each diploid nucleus (Figure 3-7A,C). A similar pattern was observed for H3K9me2 in M-P+ embryos lacking MET-2 and SET-25 HMT activity tracked through the 2-cell stage (Figure 3-7B). It has been reported that HMTs but not modified histones remain associated with DNA through DNA replication (PETRUK *et al.* 2012). My results

separate histone and HMT inheritance and demonstrate that modified histones can remain associated with DNA through several rounds of replication. These results also suggest that the memory of repression inherited on sperm chromosomes is transmitted through early embryo development.

PRC2 maintains the memory of repression during embryogenesis.

To investigate the propagation of repressive marks by ongoing methylation in the embryo, I generated embryos with HMT activity (maternally supplied in early embryos and transcribed from the embryonic genome in later stage embryos) and with H3 methyl marks on some but not all chromosomes. In this case I analyzed M+P- embryos with H3K27 methylation on the oocyte chromosomes but not on the sperm chromosomes. In such M+P- embryos, MES-2/3/6 maintained high levels of H3K27me3, and remarkably H3K27me3 remained restricted to one set of chromosomes, likely the oocyte-derived chromosomes, throughout embryo development (Figure 3-8A). I verified that H3K27me3 persisted on oocyte-derived chromosomes by analyzing embryos with a maternally contributed III;X;IV fusion chromosome; H3K27me3 remained on the fusion chromosome through multiple cell divisions (Figure 3-8B). In the germline of larvae, I observed H3K27me3 gradually become detectable on all chromosomes as larval development progressed (Figure 3-8A). This spreading of H3K27me3 to all chromosomes is probably the result of germ cells turning on their transcriptional program and establishing repressed chromatin

domains *de novo*. I speculate that H3K27me3-repressed chromatin is newly established during larval germline development each generation.

As discussed above, like HK27me3, H3K9me2 is also a mark of repression. In contrast to H3K27me3, maternally inherited H3K9me2 in the presence of H3K9 HMT activity spread to all chromosomes by the 2-cell stage (Figure 3-9A). This difference in histone mark propagation may underlie *C. elegans'* reliance on PRC2 for transgenerational regulation of repression. PRC2 methylation of H3K27, but not MET-2 SET-25 methylation of H3K9me2, provides long-term epigenetic memory to embryos.

Inheriting a repressed X chromosome and propagation of repression through embryogenesis are both important for germ cell proliferation in XO males.

After finding that inheritance of a repressed X chromosome is needed for fertility and that PRC2 can propagate a memory of repression through H3K27me, I sought to determine if propagation of H3K27me by PRC2 is critical for fertility. I tested the fertility of males that inherited their X chromosome from sperm with H3K9me, H3K27me, both, or neither and varied whether or not they also inherited maternal HMTs, produced zygotic HMTs, or had both. First I analyzed males that inherited a repressed X chromosome from sperm with H3K9me, H3K27me, or both and did not inherit maternal HMT for either H3K9me (*met-2 set-25*) or H3K27me (*mes-3*). Next I analyzed males that inherited an X chromosome from sperm without H3K9me or H3K27me repression, but did inherit maternal HMTs.

Males that inherit their X chromosome from sperm, but do not inherit maternal HMTs have H3K9me and H3K27me histone marks associated with their paternally inherited chromosomes through early stages of embryogenesis, but are not able to propagate this mark into later stages (Figures 3-7 and data now shown). To test their fertility, I analyzed males that inherited paternal H3K9me or H3K27me without maternal HMTs. Males that inherited paternally contributed H3K9me, but not H3K27me, were fertile if they inherited maternal H3K9 HMTs (Figure 3-10 line B), but not if they only produced zygotic H3K9 HMTs (Figure 3-10 line G). Males that inherited paternally contributed H3K27me, but not H3K9me, were fertile if they inherited maternal H3K27 HMT (Figure 3-10 line C) or if they only produced zygotic H3K27 HMT (Figure 3-10 line F).

Since most zygotic genes are not expressed until after the 40-cell stage of embryogenesis (BAUGH *et al.* 2003), it is unknown whether paternally inherited H3K27me3 is propagated by zygotic HMT in later stages without maternal HMT activity in early embryo stages. To test if zygotic HMT is needed for fertility I tested males that had paternally inherited H3K9me and H3K27me but lacked both maternal and zygotic HMTs. These males were not fertile (did not produce cross progeny), but surprisingly did contain well-proliferated germlines with sperm (Figure 3-10 line E). The fertility of these males may be hindered by the lack of H3K9me HMTs, since *met-2 set-25* mutant males have well proliferated germlines with sperm but were often not fertile in my mating tests (Figure 3-10 line C).

By testing the fertility of males without maternal HMT, I discovered that without H3K27me, propagation of H3K9me is needed for fertility (Figure 3-10 line G), but that without H3K9me, propagation of inherited H3K27me may not be needed for fertility (Figure 3-10 line F). This is supported by my result showing that inheritance of paternally contributed H3K9me and H3K27me with no HMT activity (maternal or zygotic) allows for germ cell proliferation and gamete production (Figure 3-10 line E). My finding that propagation of H3K27me3 by PRC2 is not needed for germ cell proliferation does not rule out the possibility of a repressive mechanism downstream of H3K27me3 propagating the memory of H3K27me3 repression.

My finding that propagation of inherited X-chromosome repression by PRC2 may not be needed for fertility, led me to investigate the importance of maternal and zygotic HMTs. Without PRC2 and H3K27me3, the X chromosome can be sufficiently repressed if it has H3K9me and is inherited from sperm (Figure 3-3 and Figure 3-10 line B). I tested if males that inherited an X chromosome from sperm without H3K9me or H3K27me, but inherited maternal HMT and autosomes with H3K27me3 were fertile. Males that only had maternally contributed H3K27me and HMT were usually sterile (Figure 3-10 line H), but if they also had zygotically expressed H3K27 HMT they were usually fertile and almost 100% had well proliferated germlines with sperm (Figure 3-10 line I). H3K9me did not fare as well in this test; only about 50% of males that had maternally contributed H3K9me and both maternal and zygotic H3K9 HMTs had well proliferated germlines and were

fertile (Figure 3-10 line J). These results suggest that inheritance of an X chromosome with H3K9me or H3K27me is not necessary for fertility as long as H3K9me or H3K27me repression on autosomes is inherited maternally and can be propagated in the next generation by maternal and zygotic HMTs (Figure 3-10 line H vs. lines I and J). With maternally contributed H3K27me and PRC2 HMT, repression of the X chromosome may be reset during germ cell proliferation in larval stages by zygotically expressed PRC2. Perhaps the X chromosome inherited from sperm lacking both H3K9me or H3K27me is nevertheless in a repressed state during early embryogenesis since it was not expressed during spermatogenesis in the parent germline.

These results highlight the importance of H3K27me repression, since H3K9me is not always a sufficient replacement. H3K9me is only a sufficient replacement for H3K27me when the X chromosome is inherited from sperm with H3K9me and there are maternal and zygotic HMTs (Figure 3-3 and Figure 3-10 line B). When the X chromosome is inherited from sperm with H3K9me or H3K27me, but no maternal HMTs, males with H3K27me are fertile, while males with H3K9me are not (Figures 3-10 lines F vs. G). When the X chromosome is inherited from sperm without H3K9me or H3K27me, but maternal and zygotic HMTs are present, males with H3K27 HMT are more often fertile than males with only H3K9 HMTs (Figure 3-10 lines I vs. J). While having PRC2/H3K27me or MET-2/SET-25/H3K9me repression at multiple stages contributes to fertility, maternal PRC2 and

X chromosome repression by PRC2 and H3K27 methylation are the most important for fertility.

CONCLUSIONS

My findings demonstrate that PRC2-generated H3K27 methylation is epigenetically transmitted across generations and in *C. elegans* is important for transmitting the memory of X-chromosome repression in the germline. H3K27me3 is transmitted to embryos on the X chromosome and autosomes from both oocyte and sperm and remains associated with parent-of-origin chromosomes through several rounds of DNA replication in the absence of HMT activity (Figure 3-11). In the presence of HMT activity, H3K27me3 is propagated through embryogenesis on parent-of-origin chromosomes (Figure 3-11). These findings reveal that histones are themselves carriers of epigenetic information and suggest that PRC2 activity perpetuates paternally and maternally inherited patterns of repression through embryogenesis. I hypothesize that, similar to H3K36me and MES-4 transmitting the memory of gene expression from the parent germline to primordial germ cells (PGCs) in progeny (RECHTSTEINER *et al.* 2010), H3K27me3 and PRC2 transmit the memory of gene repression from the parent germline to PGCs in progeny.

My studies in worms provide precedents for transmission of sperm H3K27me3 patterns to embryos and for important development consequences of paternal marking. Paternal inheritance could be similarly important in mammals where H3K27me3 is present on sperm chromatin in a pattern that suggests

involvement in embryogenesis (HAMMOUD *et al.* 2009; BRYKCZYNSKA *et al.* 2010). Many of these H3K27me3 targets are also repressed in PGCs (BRYKCZYNSKA *et al.* 2010) where H3K27me3 increases just prior to epigenetic reorganization of the genome and may facilitate reacquisition of pluripotency by germ cells (SEKI *et al.* 2005; SAITOU and YAMAJI 2012). Thus, two common themes for PRC2 function in germ cells may be transmission of a memory of repression from parents to offspring and protection of the immortal and totipotent properties of germ cells.

EXPERIMENTAL PROCEDURES

Strains and culture

C. elegans were maintained on NGM (Nematode Growth Medium) agar plates using *Escherichia coli* OP50 as a food source at 15°C and experiments were carried out at 20°C or 24°C.

The following balancers were used:

mnC1[dpy-10(e128)unc-52(e444)] II

hT2-GFP*[bli-4(e937)let(q782)qIs48] (I;III)*

DnT1-GFP*[unc(n754) let qIs51] (IV;V)*

The following strains were used:

N2 variety Bristol

SS0104 *glp-4(bn2) I*

SS1178 *glp-4(bn2) I; ccIs4811 [lmn-1*::GFP + *dpy-20(+)] X*

SS0818 *mes-3(bn35) I/hT2*-GFP *(I;III)*

SS1167 *mes-3(bn35) I/hT2*-GFP *(I;III); fem-2(b245ts) III/hT2*-GFP *(I;III)*

SS1099 *mes-3(bn35) I/hT2*-GFP *(I;III) ; him-8(e1489) IV*

SS1133 *mes-3(bn35) I/hT2*-GFP *(I;III); met-2(n4256) set-25(n5021) III/hT2*-GFP *(I;III); him-8(e1489) IV*

SS1171 *mes-3(bn35) I/hT2*-GFP *(I;III); ccIs4811 [lmn-1*::GFP + *dpy-20(+)] X*

SS1169 *mes-3(bn35) I/hT2*-GFP *(I;III); otls181* [*dat-1*::mCherry + *ttx-3*::mCherry] *III/hT2*-GFP *(I;III); ccIs4811 [lmn-1*::GFP + *dpy-20(+)] X*

SS1172 *mes-3(bn35) I/hT2*-GFP *(I;III); him-8(e1489) IV; ccIs4811 [lmn-1*::GFP + *dpy-20(+)] X*

SS1170 *mes-3(bn35) I/hT2*-GFP *(I;III); otls181* [*dat-1*::mCherry + *ttx-3*::mCherry] *III/hT2*-GFP *(I;III); him-8(e1489) IV; ccIs4811 [lmn-1*::GFP + *dpy-20(+)] X*

SS0186 *mes-2(bn11)unc-4(e120)/mnC1 II*

SS1026 *unc-4(e120) II; him-8(e1489) IV*

SS1027 *mes-2(bn11)unc-4(e120)/mnC1 II; him-8(e1489) IV*

DH0245 *fem-2(b245ts) III*

unc-119(ed3) III

SS1148 *met-2(n4256) set-25(n5021) III/hT2*-GFP *(I;III)*

CB1489 *him-8(e1489) IV*

SS0784 *mes-6(bn38) him-8 IV/DnT1-GFP (IV;V)*

SS0782 *mes-6(bn38) IV/DnT1*-GFP *(IV;V); dpy-11(e224) V/DnT1*-GFP *(IV;V)*

AV311 *dpy-18(e364) unc-3(e151) meT7 (III;X;IV)*

Fertility assays

To test XO males for fertility, single L4-stage XO males were put on a plate with at least 6 L4 *unc-119* hermaphrodites and allowed to mate at 20°C for 4 days. Males were scored as fertile if the plate contained at least 4 non-Unc progeny (cross progeny).

Staining germline nuclei

L4 XO males were separated from hermaphrodites and incubated overnight at 20°C to allow for build up of sperm. The next day, to remove bacteria, the males were allowed to crawl on blank plates for a few minutes. Males were picked into 1µl drops of water on a gelatin chrom alum-coated slide. The slide was waved over an alcohol burner flame until dry, and worms were mounted in 4',6-diamidino-2-phenylindole (DAPI) at a final concentration of 0.05µg/ml in Gelutol mounting medium. Germline nuclei were counted, and images were taken on a Zeiss Axioskop using a QImaging Retiga 2000R camera and ImageJ AquireQCam plug-in software.

Immunocytochemistry

Worms were fixed using methanol/acetone (STROME and WOOD 1983). L1 larvae were obtained by hatching embryos in the absence of food in M9 buffer and fixed after feeding for ~5 hours. L3 and L4 larvae were obtained by hatching L1s in the absence of food, then transferring L1 larvae to a plate with food for ~24 hours

(L3) or ~36 hours (L4). Embryos and larvae were fixed whole. Adults were dissected to isolate germlines, embryos, and sperm. Dissection of germlines and embryos was done in Egg Buffer (25mM HEPES pH 7.4, 118mM NaCl, 48mM KCl, 2mM $CaCl_2$, 2mM $MgCl_2$) with 1mM levamisole. Dissection to release sperm was done in Sperm Salts (50mM HEPES pH7.5, 1mM $MgSO_4$, 25mM KCl, 45mM NaCl, 5mM $CaCl_2$) with 1mM levamisole. Primary antibodies and dilutions used for immunostaining were: 1:30,000 mouse anti-H3K27me3 (Kimura mAb 1E7, SS#70), 1:30,000 mouse anti-H3K9me2 (Kimura mAb 6D11, SS#157), 1:10,000 rabbit anti-H3K4me2 (Millipore 07030, SS#86), 1:750 mouse anti-GFP (Roche 11814460001, SS#401), 1:30,000 rabbit anti-PGL-1 (SS#596) (*31*). Mouse monoclonal antibodies against H3K27me3 (1E7) and H3K9me2 (6D11) were gifts from Hiroshi Kimura (Osaka University) (KIMURA *et al.* 2008). Secondary antibodies conjugated to Alexa Fluor 488 or 594 (Molecular Probes) were used at 1:300 with 0.05μg/ml 4',6-diamidino-2-phenylindole (DAPI) for 2 hours at room temperature. Images were acquired with a Volocity spinning disk confocal system (Perkin Elmer/Improvision, Norwalk, CT, USA) fitted on a Nikon Eclipse TE2000-E inverted microscope.

Analysis of *lmn-1*::GFP expression in germlines

XO males with the *lmn-1::*GFP transgene were dissected as adults, and their germlines were fixed and stained for GFP as described for immunocytochemistry. Images were acquired on a compound microscope as described for staining germline nuclei.

Generation of M-P+ and M+P- embryos and worms

To generate M-P+ embryos, *mes-3* M+Z-; *fem-2* feminized hermaphrodites were mated with wild-type males, or *met-2 set-25* hermaphrodites were mated with wild-type males containing MitoTracker red-labeled sperm (ZHOU *et al.* 2011) and only embryos showing traces of MitoTracker red were analyzed. M+Z- animals inherited a maternal load of gene product but did not produce gene product from the zygotic genome. To generate M+P- embryos and larvae, *fem-2* feminized hermaphrodites were mated with *mes-3* M+Z- or *met-2 set-25* M+Z- males. To generate M+P- embryos with fused M+ chromosomes, *dpy-18 unc-3 meT7 (III;X;IV)* hermaphrodites were mated with *mes-3* M+Z- males. Only cross progeny showing mosaic H3K27me3 were analyzed.

Sperm activation

In vitro sperm activation was carried out using pronase as previously described (SINGARAVELU *et al.* 2011).

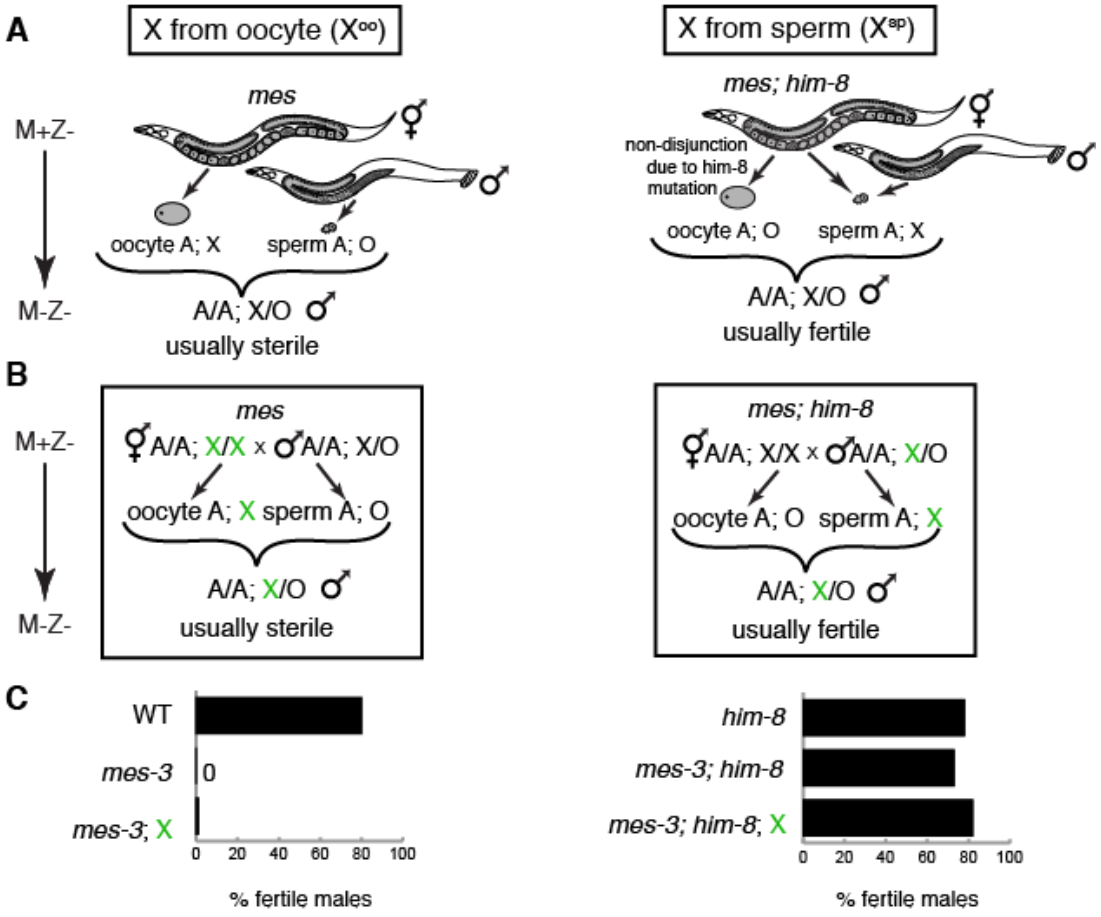


Figure 3-1. Germline health and fertility of XO *mes* mutant males depend on the gamete source of the X chromosome.
(A) Diagrams of crosses used to generate XO *mes* males that inherited their X chromosome from the oocyte (X^{oo}) or the sperm (X^{sp}). M is maternal supply of *mes*(+) gene product, Z is zygotic expression of the *mes*(+) gene. **(B)** Diagrams of crosses used to unambiguously identify the gamete source of the X in *mes-3* males. One or the other parent contributed to progeny an X chromosome that expressed an X-linked GFP-tagged nuclear lamin transgene (shown as a green X). **(C)** Fertility of control (wild-type (WT) and *him*-8) and *mes-3* males generated by the different methods described in A and B. Fertility was tested by mating single males with *unc-119* hermaphrodites and scoring for the production of non-Unc outcross progeny.

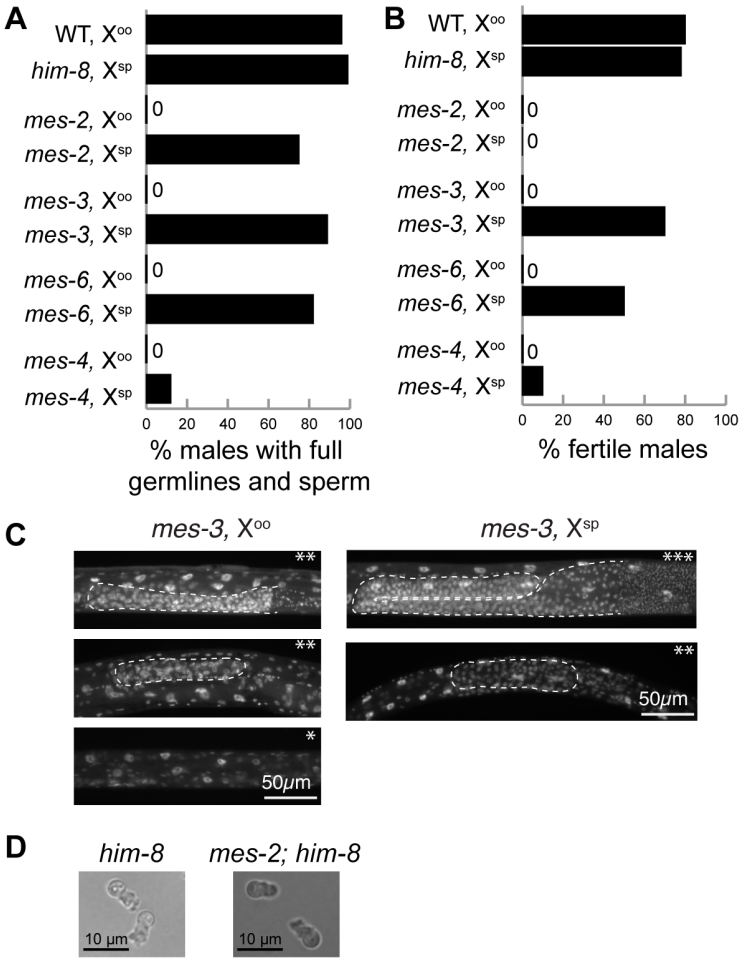


Figure 3-2. As in *mes-3* XO mutant males, germline health in *mes-2* and *mes-6* XO mutant males depends on the gamete source of the X.
(A) Percent of XO *mes* mutants with well proliferated germlines and sperm, as determined by DNA staining. *mes-3,* X^{oo} and *mes-4,* X^{oo} were produced by mating *mes* hermaphrodites with *mes* males. *mes-2,* X^{oo} and *mes-6,* X^{oo} were produced by mating *mes* hermaphrodites with wild-type males. **(B)** Fertility of control (wild-type (WT) and *him*-8) and *mes* males generated by the different methods described in Figure 3-1A. Fertility was tested by mating single males with *unc-119* hermaphrodites and scoring for the production of non-Unc outcross progeny. **(C)** Images of germ cell proliferation phenotypes in XO (X^{oo}) and (X^{sp}) *mes-3* males by DAPI (DNA) staining (germlines outlined by white dashed lines). Worms had either well proliferated germlines and sperm (***), under-proliferated germlines (**), or no germ cells (*). **(D)** Because (X^{sp}) *mes-2* mutants had well proliferated germlines with sperm but were sterile (panels A and B), sperm were scored for their ability to be activated and extend pseudopods *in vitro* after treatment with pronase. Sperm from *mes-2 unc-4; him-8* mutant males were compared to sperm from control *unc-4; him-8* males.

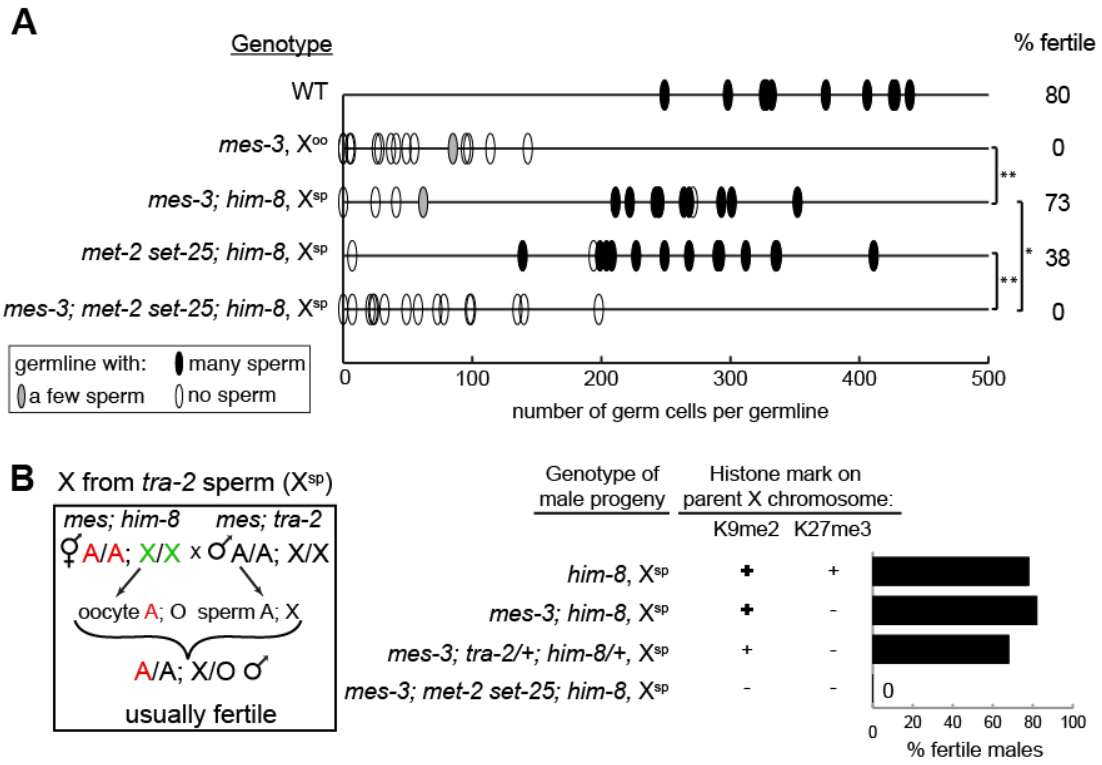


Figure 3-3. H3K9 methylation provides an alternative mechanism of X repression**.** **(A)** Analysis of germline proliferation and presence of sperm in XO males: wild type (WT), *mes-3* mutants (lacking H3K27me), *met-2 set-25* double mutants (lacking H3K9me), and triple mutants that inherited their X from the oocyte (X^{oo}) or X from the sperm (X^{sp}). Statistically significant differences between genotypes, $p<0.01$ (*) Mann-Whitney test. Fertility tests, $n>65$ for each genotype. **(B)** Diagram of crosses used to generate XO *mes* males that inherited their X chromosome from *mes-3; tra-2* male parents (X^{sp}) and the % fertile males from *mes* X^{sp} genotypes for comparison to *mes-3; tra-2/+* males. Males inherited their X chromosome from sperm with either enriched (bold +), low levels (+) or no (-) H3K9me2 and with (+) or without (-) H3K27me3.

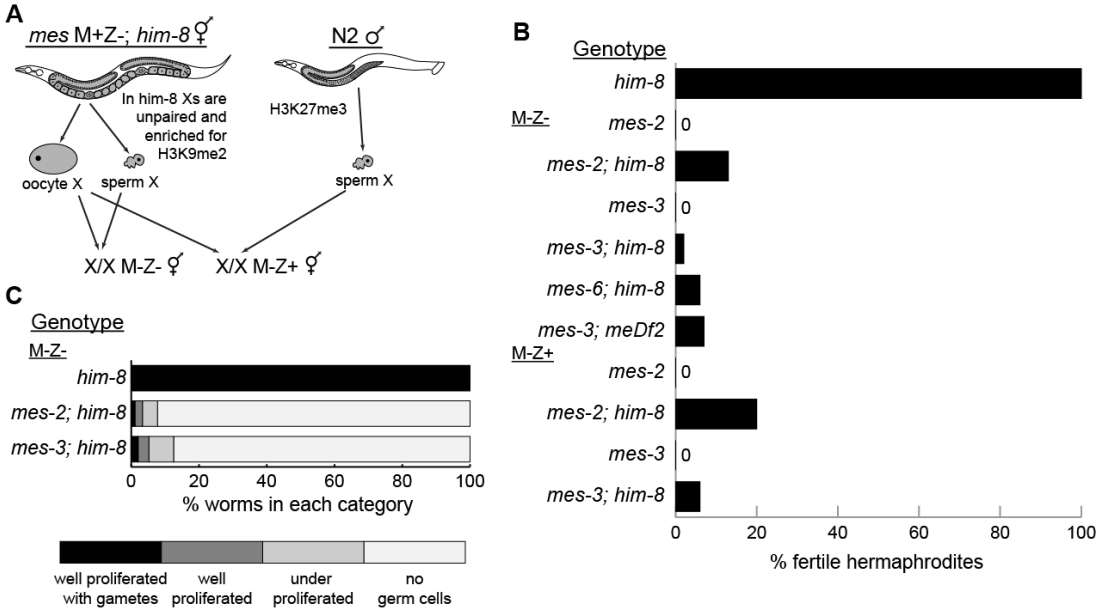
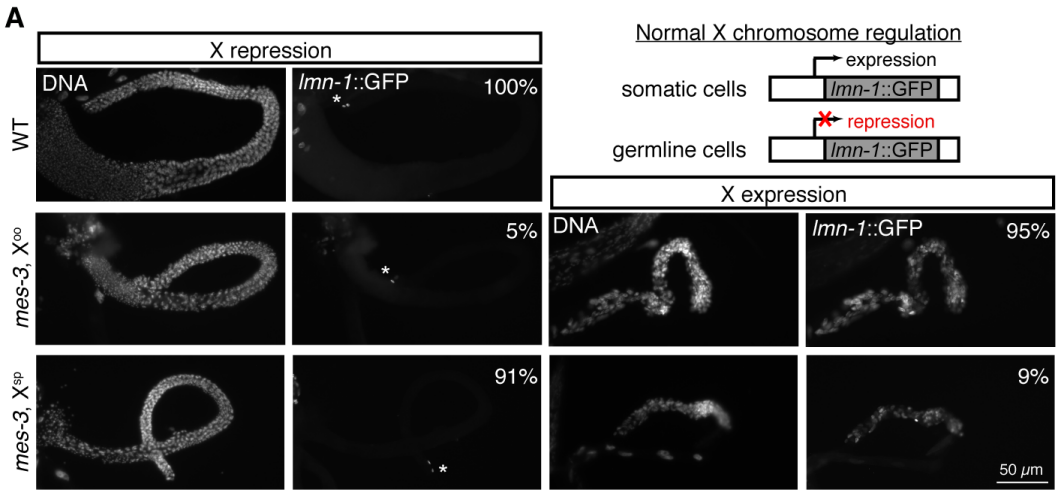


Figure 3-4. *mes* XX mutant hermaphrodites can be fertile if both X chromosomes come from a history of repression in the germline.
(A) Diagrams of crosses used to generate XX M-Z- or M-Z+ *mes* hermaphrodites with X-enriched H3K9me2. M is maternal supply of *mes*(+) gene product, Z is zygotic expression of the *mes*(+) gene. **(B)** Fertility of control (*him-8*) hermaphrodites and M-Z- and M-Z+ *mes* hermaphrodites generated by the different methods shown in A. Fertility was tested by picking individual hermaphrodites and scoring for production of self progeny. **(C)** Percent of XX *mes* mutants with no germline, under-proliferated germlines, well proliferated germlines, and well proliferated germlines with gametes, as determined by DNA staining. n>65 for each genotype.



B

	% with germline *lmn-1*::GFP	n=
WT, X^{oo}	0	41
WT, X^{sp}	0	37
glp-4, X^{oo}	0	81
met-2 set-25 M-Z-, X^{oo}	0	36
met-2 set-25 M-Z-, X^{sp}	0	35
mes-3 M-Z-, X^{oo}	95	57
mes-3 M-Z-, X^{sp}	9	70

Figure 3-5. Derepression of the X chromosome in XO (X^{oo}) *mes-3* males, as reported by an *lmn-1*::GFP transgene.
(A) Germline repression of *lmn-1*::GFP (left) and expression (right), with percentages of germlines showing each pattern in the top right corner. The somatic gonad distal tip cells (*) at the end of each gonad arm express the transgene. **(B)** Percent germlines showing expression of *lmn-1*::GFP for different XO male genotypes with the X chromosome from the oocyte (X^{oo}) or sperm (X^{sp}) (n=number of germlines counted).

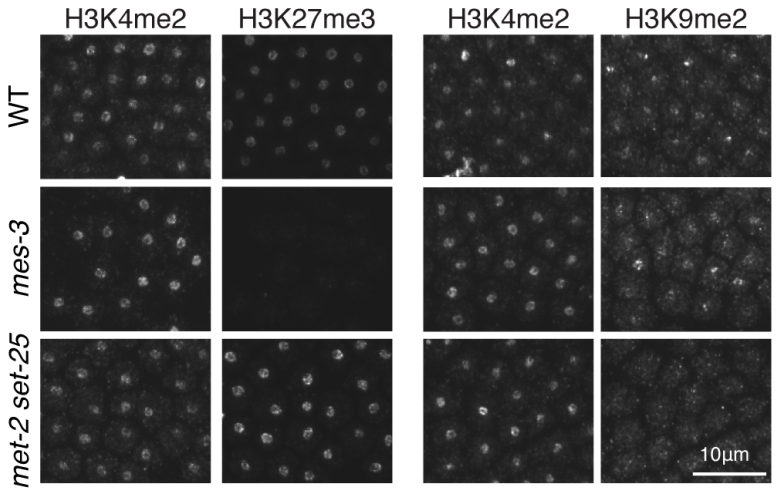


Figure 3-6. H3K27 methylation and H3K9 methylation are present in mature sperm. H3K27me3 and H3K9me2 are present in mature sperm in wild-type (WT) males and absent from sperm in mutant males lacking the respective HMTs, *mes-3* for H3K27me3 and *met-2 set-25* for H3K9me2.

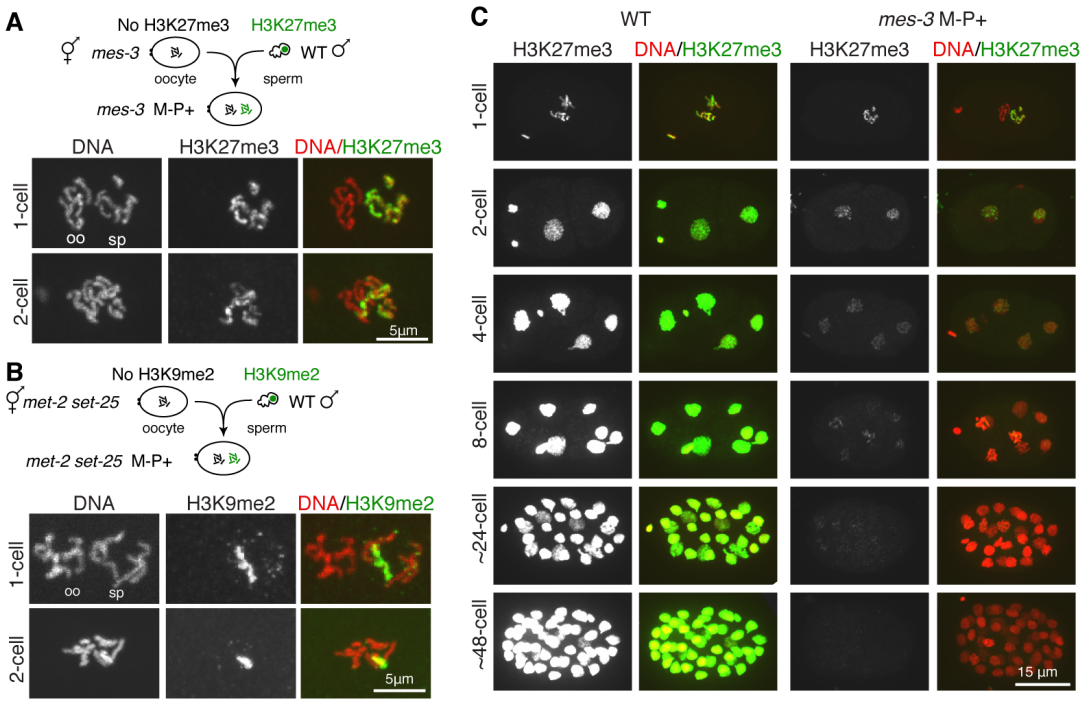


Figure 3-7. H3K27me3 and H3K9me2 are epigenetically transmitted from sperm to embryos and through cell divisions.
(A) Diagram of oocyte and sperm union to generate 1-cell M-P+ embryos with H3K27me3 inherited from the sperm (P+) but not the oocyte (M-) and lacking maternal HMT. Wild type is shown in Figure 3-8C. Images are of the two pronuclei in a 1-cell embryo, and a diploid nucleus in a 2-cell embryo, with DNA in red and H3K27me3 in green in merge panels. The assignment of oocyte chromosomes (oo) and sperm chromosomes (sp) is based on the location of the polar bodies (shown in the diagrams, not in the images). Note that while H3K27me is most important for X-chromosome repression, it is present on the autosomes as well (BENDER *et al.* 2004). **(B)** Diagram of oocyte and sperm union to generate embryos with H3K9me2 on the sperm chromosomes but not on the oocyte chromosomes (M-P+). Images are of two pronuclei in a 1-cell embryo and a diploid nucleus in a 2-cell embryo. In the 1-cell, H3K9me2 is concentrated on the X inherited from male-contributed sperm (as observed in the adult male germline; BEAN *et al.* 2004). In the 2-cell, H3K9me2 inherited from the sperm persists on one chromosome, likely the X. In the merge panel, DNA is in red and H3K9me2 in green. **(C)** Perdurance of paternally inherited H3K27me3 on a subset of chromosomes until the ~24-cell stage. Identical acquisition settings were used within each genotype.

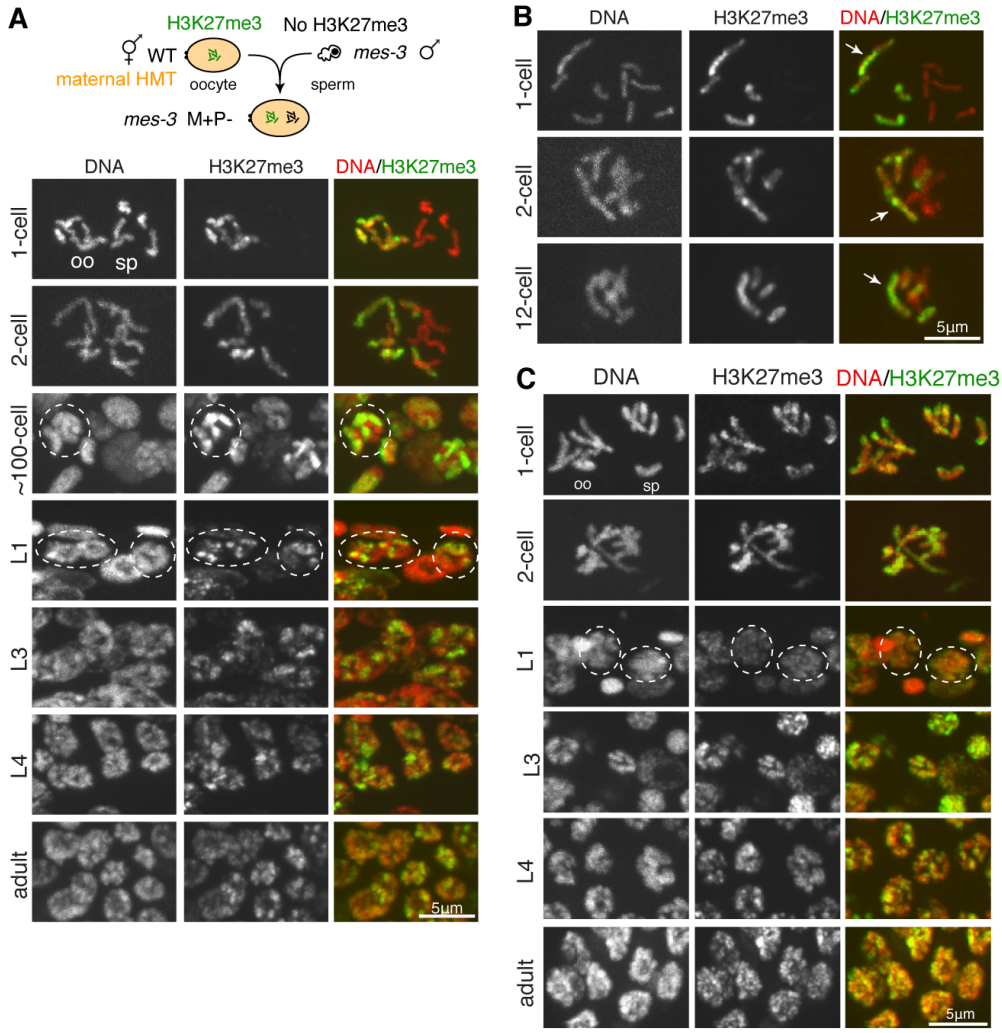


Figure 3-8. Maternal PRC2 maintains H3K27me3 on parent-of-origin chromosomes during embryogenesis.
(A) Diagram of oocyte and sperm union to generate 1-cell M+P- embryos with H3K27me3 inherited from the oocyte (M+) but not the sperm (P-) and containing maternal HMT. Images are of the two pronuclei in a 1-cell embryo, and a diploid nucleus in a 2-cell embryo and extend to later-stage embryos (one nucleus circled), L1 larvae (germ nuclei, identified by staining for germ granules, circled), and L3, L4, and adult germlines. The assignment of oocyte chromosomes (oo) and sperm chromosomes (sp) is based on the location of the polar bodies (shown in the diagrams, not in the images). H3K27me3 on only a subset of chromosomes persists until at least L1. Merge: DNA in red and H3K27me3 in green. **(B)** M+P- embryos bearing a III-X-IV chromosome fusion in the oocyte (M+) chromosome set (arrow). H3K27me3 persists on the oocyte-derived fusion chromosome through cell divisions. **(C)** Images of H3K27me3 in wild-type embryos and larvae as described for panel A.

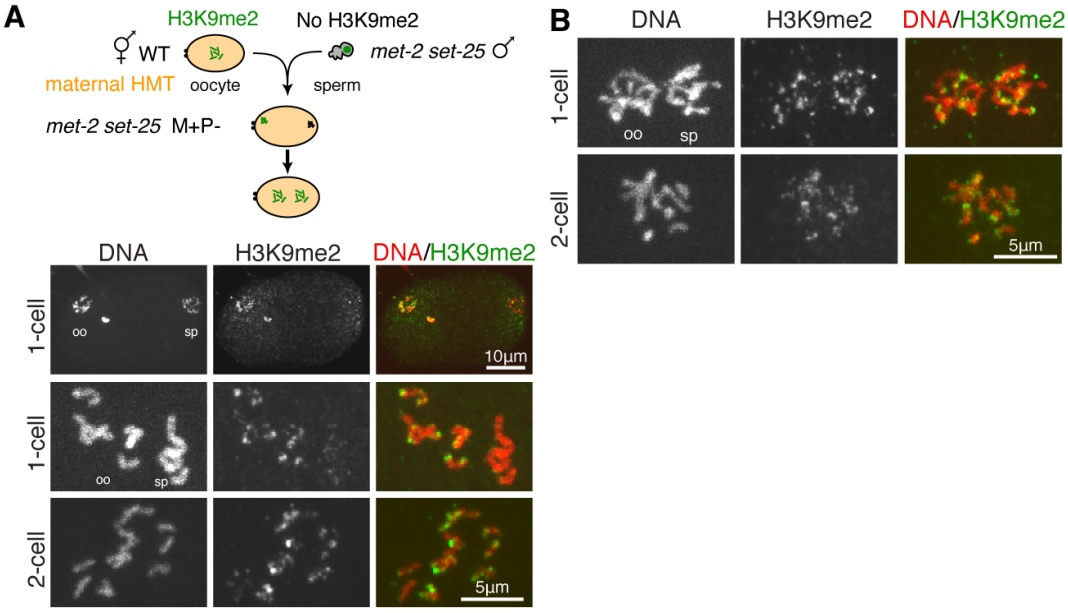


Figure 3-9. In the presence of maternal HMT, H3K9me2 does not stay restricted to parent-of-origin chromosomes.
(A) Diagram of oocyte and sperm union to generate 1-cell M+P- embryos with H3K9me2 inherited from the oocyte (M+) but not the sperm (P-) and containing maternal HMT. Images of two pronuclei before and after meeting in a 1-cell embryo and a diploid nucleus in a 2-cell embryo show that H3K9me2 spreads to all chromosomes. The assignment of oocyte chromosomes (oo) and sperm chromosomes (sp) is based on the location of the polar bodies (shown in the diagrams, not in the images). **(B)** Images of H3K9me2 in wild-type 1-cell and 2-cell embryos.

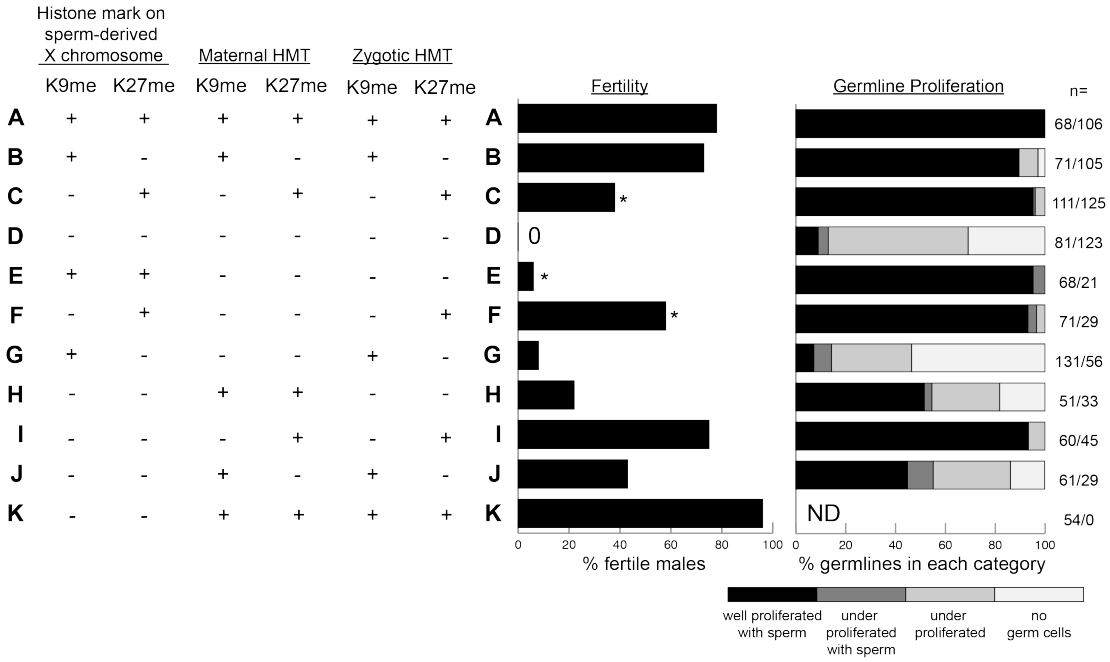


Figure 3-10. Analysis of XO (X^{sp}) mutant male fertility with and without maternal and zygotic histone methyltransferase (HMT).
(A-K) Fertility and germline proliferation phenotypes of males that inherited their X chromosome from sperm with (+) or without (-) H3K9me and H3K27me, inherited (+) or did not inherit (-) maternal HMT for H3K9me (MET-2 and SET-25) and H3K27me (MES-3), and zygotically express (+) or do not express (-) HMT for H3K9 and H3K27me. To facilitate genotype interpretation:

A-D are controls
E is paternal only
F and G are paternal and zygotic
H is maternal only
I-K are maternal and zygotic

All male genotypes and parent genotypes are listed in Table 3-1. Some genotypes (*) have mostly well proliferated germlines with sperm but show unexpectedly low fertility. Number of males scored for fertility/germline proliferation (n).

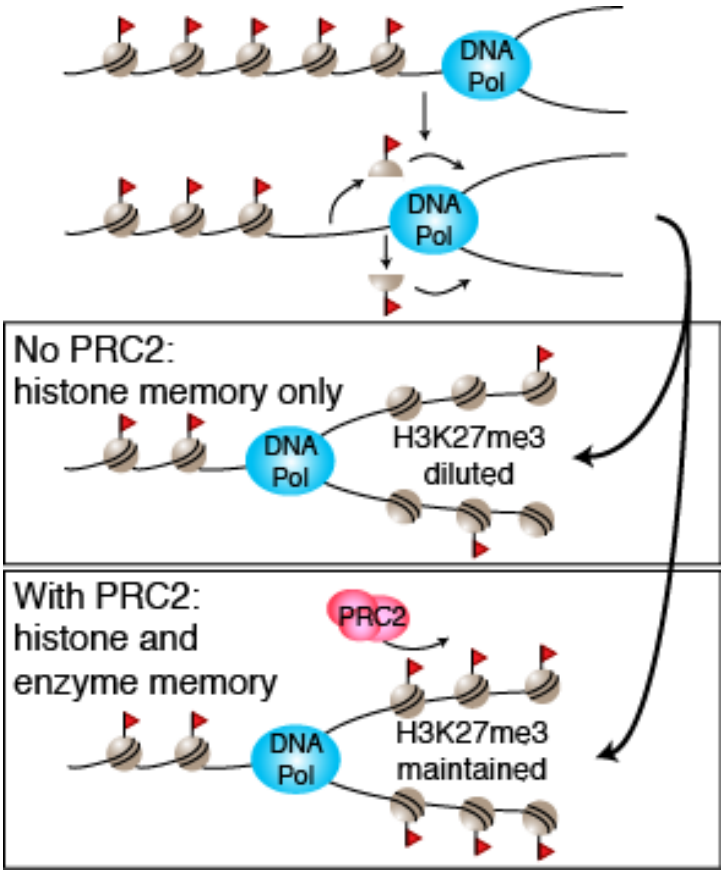


Figure 3-11. Model for transmission of the memory of repression through DNA replication.
Without PRC2, local passage of H3K27me3 (red flags) to daughter chromatids transmits short-term memory, for example on P+ chromosomes in M-P+ embryos (Figure 3-7A,C). With PRC2, passage of H3K27me3 and new methylation by PRC2 transmit long-term memory, for example on M+ chromosomes in M+P- embryos (Figure 3-8A,B).

	Father	Mother	Male offspring
A	N/A	*him-8* (IV)	*him-8* (IV)
B	N/A	*mes-3* M+Z- (I); *him-8* (IV)	*mes-3* M-Z- (I); *him-8* (IV)
C	N/A	*met-2 set-25* M+Z- (III); *him-8* (IV)	*met-2 set-25* M-Z- (III); *him-8* (IV)
D	N/A	*mes-3* M+Z- (I); *met-2 set-25* M+Z- (III); *him-8* (IV)	*mes-3* M-Z- (I); *met-2 set-25* M-Z- (III); *him-8* (IV)
E	*mes-3*/hT2g (I); *met-2 set-25*/hT2g (III); *him-8* (IV); *lmn-1*::GFP (X)	*mes-3* M+Z- (I); *met-2 set-25* M+Z- (III); *him-8* (IV)	*mes-3* M-P+Z- (I); *met-2 set-25* M-P+Z- (III); *him-8* (IV); *lmn-1*::GFP (X)
F	*met-2 set-25* M+Z- (III); *him-8* (IV); *lmn-1*::GFP (X)	*mes-3* M+Z- (I); *met-2 set-25* M+Z- (III); *him-8* (IV)	*mes-3* M-P+Z+ (I); *met-2 set-25* M-P-Z- (III); *him-8* (IV); *lmn-1*::GFP (X)
G	*mes-3* M+Z- (I); *dat-1*::mCherry+*ttx-3*::mCherry (III); *him-8* (IV); *lmn-1*::GFP (X)	*mes-3* M+Z- (I); *met-2 set-25* M+Z- (III); *him-8* (IV)	*mes-3* M-P-Z- (I); *met-2 set-25* M-P+Z+ (III); *him-8* (IV); *lmn-1*::GFP (X)
H	*mes-3* M+Z- (I); *met-2 set-25* M+Z- (III); *him-8* (IV); *lmn-1*::GFP (X)	*mes-3/hT2g* (I); *met-2 set-25/hT2g* (III); *him-8* (IV)	*mes-3* M+P-Z- (I); *met-2 set-25* M+P-Z- (III); *him-8* (IV); *lmn-1*::GFP (X)
I	*mes-3* M+Z- (I); *met-2 set-25* M+Z- (III); *him-8* (IV); *lmn-1*::GFP (X)	*met-2 set-25* M+Z- (III); *him-8* (IV)	*mes-3* M+P-Z+ (I); *met-2 set-25* M-P-Z- (III); *him-8* (IV); *lmn-1*::GFP (X)
J	*mes-3* M+Z- (I); *met-2 set-25* M+Z- (III); *him-8* (IV); *lmn-1*::GFP (X)	*mes-3* M+Z- (I); *him-8* (IV)	*mes-3* M-P-Z- (I); *met-2 set-25* M+P-Z+ (III); *him-8* (IV); *lmn-1*::GFP (X)
K	*mes-3* M+Z- (I); *met-2 set-25* M+Z- (III); *him-8* (IV); *lmn-1*::GFP (X)	*him-8* (IV)	*mes-3* M+P-Z+ (I); *met-2 set-25* M+P-Z+ (III); *him-8* (IV); *lmn-1*::GFP (X)

Table 3-1. Genotypes of male offspring in Figure 3-10 and their parents' genotypes.

CHAPTER 4: Studies with a *mes-3* Temperature-Sensitive Mutant Highlight the Importance of H3K27me3 Repression and Antagonism by MES-4

INTRODUCTION

MES-3 is an essential component of the H3K27me3-generating PRC2 complex in *C. elegans* along with MES-2 and MES-6. A temperature-sensitive (ts) allele of *mes-3* (*mes-3(bn21)*) was isolated in the original screen for maternal-effect sterile mutants. At the restrictive temperature (25°C), the ts allele behaves like the non-conditional *mes* allele; it causes 100% maternal-effect sterility (CAPOWSKI *et al.* 1991). MES-3, like the other MES proteins, normally accumulates in nuclei during early embryogenesis and is then enriched in primordial germ cell nuclei by the comma stage (XU *et al.* 2001b). In *mes-3* ts worms at the permissive temperature (15°C), MES-3 is somewhat enriched in nuclei but also present in the cytoplasm, and homozygous mutants are fertile (XU *et al.* 2001b). At the restrictive temperature, MES-3 is mainly cytoplasmic and homozygous mutants are sterile (XU *et al.* 2001b).

To determine when MES-3 is needed during development, *mes-3* ts mutant mothers and progeny were upshifted or downshifted at different stages during development (XU *et al.* 2001b). Upshift of early embryos resulted in sterility of that generation, while upshift of later embryos and newly hatched L1 larvae did not cause sterility of that generation; these results suggested that functional MES-3 is needed during early to mid-embryogenesis (XU *et al.* 2001b). Upshift of mothers at the L4 stage followed by downshift of their early embryos resulted in mainly fertile progeny, while upshift of mothers at the L1 stage followed by downshift of their early embryos

resulted in sterile progeny; these results suggested that function MES-3 is needed during larval development of the parent (XU *et al.* 2001b). Taken together, these studies suggested that MES-3 is needed in the parent between the L1 and L4 stage and in the embryo when the primordial germ cells are generated (XU *et al.* 2001b). I conducted refined temperature shift experiments to determine which larval stages are most important for MES-3 activity.

Results of previous studies and in Chapters 2 and 3 have shown that MES-3 is important for H3K27me3 and repression of the X chromosome (BENDER *et al.* 2004). I performed immunostaining of histone marks associated with repressed or active chromatin on upshifted worms to look for evidence of X-chromosome derepression. From analysis of *mes-3* and wild-type worms at the permissive temperature, I noticed that a difference in the level of H3K27me3 signal on the X vs. autosomes in *mes-3* mutants compared to wild type. This observation prompted me to analyze *mes-3* ts worms at the permissive temperature. My analysis highlighted the importance of H3K27me3 repression and antagonism of H3K27me3 by MES-4.

RESULTS AND DISCUSSION

MES-3 is most important during mid-larval stages to generate X-enriched H3K27me3.

At the L1 stage there are two primordial germ cells, which proliferate during the larval stages to form the adult germline (KIMBLE and WHITE 1981). A previous study found MES-3 to be most important during larval stages in the parent generation

and in early embryos (XU *et al.* 2001b). To determine at which larval stage in the parent (P0) it is most important to have functional MES-3, I performed temperature upshifts and downshifts at different larval stages in P0 and allowed the next generation (F1) to develop at the permissive temperature (Figure 4-1A). As a negative control, I did not upshift P0 worms, which resulted in mostly fertile F1 progeny. As a positive control, I upshifted P0 worms as L1s and did not downshift the F1 generation, which resulted in fertile P0 adults and all sterile F1 progeny. In the upshift and downshift experiments, P0 larvae were at the restrictive temperature for approximately the time it takes to develop to the next larval stage. The temperature shift that resulted in the most sterile worms in the F1 generation was upshifting L2/L3s and downshifting L4/YA (Figure 4-1A). A smaller percentage of F1 worms grew up to be sterile when the P0 upshift and downshift occurred earlier (up as L1 and down as L2/L3) or later in larval development (up as L3 and down as young adult). These results suggest that the L2 and early L3 stages are the most important for MES-3 function. This window is when germ cells are undergoing mitotic proliferation (from ~10 to ~50 cells) and before meiosis begins (HIRSH *et al.* 1976; BEANAN and STROME 1992). This may be an important window for germline gene expression patterns to be established.

Since MES-3 is necessary for H3K27me3 and X-chromosome repression, I analyzed H3K27me3 (repressive mark) and H3K36me2 (active mark) staining in the adult germline after upshifting those worms as L1s and L4s, to see if repression by H3K27me3 is compromised soon after upshift. After upshifting L4s, H3K27me3 and

H3K36me2 had normal patterns in the pachytene region of the germline; H3K27me3 was enriched on the Xs, which lacked H3K36me2 (BENDER *et al.* 2004; BENDER *et al.* 2006) (Figure 4-1B). After upshifting L1s, H3K27me3 was no longer enriched on the Xs and H3K36me2 was on all chromosomes (Figure 4-1B). These results suggest that after upshift, H3K27me3 is depleted from the X chromosome and the X gains marks of active chromatin. Based on results from Chapter 2 and 3, H3K27me3 is likely diluted during germ cell division in the absence of active MES-3 in the MES-2/3/6 histone methyltransferase complex. More cell divisions occur between the L1 and adult stage than between the L4 and adult stage; this may explain why H3K27me3 is depleted by the adult stage after upshifting L1s, but not after upshifting L4s. These results correlate with the larval up/downshift experiments that showed that MES-3 is important during germ cell proliferation in larval stages (XU *et al.* 2001b). One hypothesis is that when P0 larvae are upshifted before germ cell proliferation commences (L1s), the F1 generation inherits incorrect H3K27me3 localization and is sterile. When P0 larvae are upshifted as L4s, the majority of germ cells contain and are able to transmit a repressed X chromosome to the next generation.

Partial loss of MES-3 function results in lower H3K27me3 on autosomes.

In wild-type embryos as revealed by immunostaining, MES-3 is highly enriched in nuclei. In *mes-3* ts mutant embryos, MES-3 is less enriched in nuclei and a substantial proportion is located in the cytoplasm. This is especially pronounced in

embryos generated at the restrictive temperature, but is also apparent in embryos generated at the permissive temperature (XU *et al.* 2001b). I investigated whether *mes-3* ts mutants are defective in H3K27 methylation at the permissive temperature. By immunostaining, H3K27me3 appears dimmer on the autosomes in *mes-3* ts mutants at the permissive temperature than in wild type (Figure 4-2A). I quantified the average intensity of H3K27me3 staining across the X chromosomes and autosomes, and found similar intensities of H3K27me3 on the X chromosomes and a significant decrease in H3K27me3 on the autosomes in *mes-3* ts mutants compared to wild type ($p=0.0001$) (Figure 4-2B).

The microarray analysis presented in Chapter 2 showed that the MES proteins are important for repressing expression of somatic genes on the autosomes. This finding and the low level of H3K27me3 on autosomes in *mes-3* ts mutants at the permissive temperature suggest that *mes-3* ts mutants are less effective at repressing genes on the autosomes at the permissive temperature. In Chapter 3, I established that the sole essential role of MES-3 is repression of the X chromosomes in the germline. Observing that H3K27me3 is reduced on autosomes but remains elevated on the Xs in fertile *mes-3* ts mutants reinforces the idea that X-chromosome repression, but not autosomal repression, is necessary for fertility.

Less functional MES-3 results in a mortal germline phenotype.

After working with a homozygous *mes-3* ts strain for about a month, I noticed that it became harder to find fertile progeny. This phenotype is similar to the mortal

germline (Mrt) phenotype in which populations of mutants become progressively sterile over multiple generations (AHMED and HODGKIN 2000). To assay for a Mrt phenotype, the *mes-3* ts mutation was placed over a balancer chromosome, and the strain was maintained at the permissive temperature as heterozygotes. Homozygotes were newly picked or "cloned" to assay for sterility over progressive generations. By scoring the percent sterile progeny at each generation from 51 cloned hermaphrodites, I determined that *mes-3* ts at the permissive temperature indeed has a Mrt phenotype. The parental *mes-3* ts/balancer generation (P0) was composed of all fertile adults. A small percent of first generation (F1) *mes-3/mes-3* homozygotes were sterile. However, by generation F5, the average percent of progeny that were sterile was about 50%, and by generation F9, 100% of progeny were sterile (Figure 4-3B). I also analyzed the intensity of H3K27me3 on autosomes and the X chromosomes in the germlines of fertile adults at different generations. The intensity of H3K27me3 on the Xs remained high, but the intensity on autosomes declined over multiple generations (Figure 4-3A). These results suggest that even at the permissive temperature MES-3 is not as efficient at generating H3K27me3 and as a result H3K27me3 is lower on autosomes, which may underlie the Mrt phenotype. While results from Chapter 3 show that autosomal repression by MES-3 is not necessary for fertility of the next generation, the *mes-3* ts results suggest that progressive loss of autosomal repression over multiple generations compromises germline health.

MES-4 helps keep H3K27me3 concentrated on the X chromosome.

In Chapter 2 we investigated the antagonism between MES-4/H3K36me3 and MES-2/3/6(PRC2)/H3K27me3. We found by ChIP-chip analysis that without MES-4, H3K27me3 spreads onto germline genes in the early embryo (GAYDOS *et al.* 2012). In *mes-4* mutant germlines, there is no apparent change in the distribution of H3K27me3 compared to wild type (unpublished observation). Since there is an observable difference between H3K27me3 on the X chromosomes vs. autosomes in the *mes-3* ts mutant, I used this sensitized background to see if lack of MES-4 changes the distribution of H3K27me3 in the adult germline. I compared H3K27me3 localization in a *mes-3* ts; *mes-4* double mutant to that in a *mes-3* ts single mutant and in wild type. In the *mes-3* ts single mutant, H3K27me3 was concentrated on the X chromosomes and lower on the autosomes, as discussed earlier in this chapter. In the *mes-3* ts; *mes-4* double mutant, H3K27me3 was visibly lower on the X chromosomes (Figure 4-4A). I quantified the staining intensity of H3K27me3 in wild type, *mes-3* ts single mutants, and *mes-3* ts; *mes-4* double mutants. This revealed lower levels of H3K27me3 on the X chromosomes in the double mutant compared to the single mutant ($p=0.049$) (Figure 4-4B). The slightly higher level of H3K27me3 on the autosomes in the double mutant compared to the single mutant was not statistically significant (Figure 4-4B). These findings suggest that normally MES-4 and H3K36me3 on the autosomes block H3K27me3 from binding these regions, which helps concentrate H3K27me3 on the X chromosome. Upon loss of MES-4, H3K27me3 is redistributed on the autosomes and is not as concentrated on the X

chromosome. These results from adult germlines strengthen the early embryo findings described in Chapter 2 and Gaydos et al. 2012 and allow us to conclude that MES-4 and H3K36 methylation antagonize methylation of H3K27 by MES-2/3/6 to concentrate H3K27me3 on the X chromosome.

CONCLUSIONS

MES-3 is important for generating H3K27me3, which represses transcription of soma-specific genes on the autosomes and is essential for repressing the X chromosome and maintaining a memory of germline repression for the next generation (Chapters 2 and 3). In this chapter, I determined when MES-3 is most important for fertility in the next generation. I also analyzed a new phenotype, a mortal germline or Mrt phenotype, displayed by *mes-3* ts mutants at the permissive temperature. The Mrt phenotype appears to be associated with the X chromosome retaining a repressed state and the autosomes having diminished repression. I was also able to use the *mes-3* ts mutants to investigate repression of the autosomes and the antagonism between MES-4/H3K36me and MES-2/3/6(PRC2)/H3K27me3 in the adult germline.

By doing temperature shifts at different larval stages during germline development, I found that MES-3 is most important during larval stages L2-L3. These stages may represent a window when germline gene expression patterns need to be set before the germline initiates meiosis. From analysis in Chapter 3 of *mes-3* mutants showing persistence of H3K27me3 on maternally but not paternally inherited

chromosomes until larval stages, I hypothesized that when germ cell proliferation occurs during larval stages, H3K27me3 is reset from the parental pattern to the pattern appropriate to a new generation of germ cells. Together these results suggest that the important window for resetting H3K27me3 repression and memory for the next generation is during the L2 and L3 stages. If the memory is not reset correctly, then the next generation is sterile.

In Chapter 2, my microarray analysis showed that H3K27me3 is important for repression of soma-specific genes on the autosomes. When MES-3 function is partially compromised, there is less H3K27me3 on the autosomes compared to wild type. This result suggests that MES-3 still effectively represses gene expression from the X chromosome, but is not as effective at repressing transcription from the autosomes. I speculate that inheritance of progressively less H3K27me3 on the autosomes generation after generation leads to expression of soma-specific genes in the germline, eventually causing germ cell death and sterility (the Mrt phenotype described in this chapter). These results highlight the importance of somatic gene repression for survival of germ cells.

The ChIP-chip data presented in Chapter 2 showed antagonism between MES-4/H3K36me and MES-2/3/6(PRC2)/H3K27me3 in early embryos. Chapter 4 shows similar results, but in adult germlines and looking at whole chromosomes instead of specific genes. Together they show that with loss of MES-4, during larval stages H3K27me3 spreads to germline genes on the autosomes, leaving less on the X chromosomes. This memory of repression/expression is passed on to progeny, in

which impairment of H3K27me3 repression of the X chromosomes leads to defects in germ cell proliferation and survival during larval stages.

EXPERIMENTAL PROCEDURES

Strains and Culture

C. elegans were maintained on NGM (Nematode Growth Medium) agar plates using *Escherichia coli* OP50 as a food source at 15°C. Experiments were carried out at 15°C and 24°C. *mes-3(bn21)* mutants were always obtained from the balanced heterozygous strain and either analyzed in the first homozygous mutant generation (parents) or second generation (progeny), except for the Mrt experiments where multiple generations were analyzed.

The following balancers were used:

hT2-GFP[bli-4(e937)let(q782)qIs48] (I;III)

DnT1-GFP[unc(n754) let qIs51] (IV;V)

DnT1[unc(n754) let] (IV;V)

The following strains were used:

N2 variety Bristol

SS0990 *mes-3(bn21ts) (I)*

SS1024 *mes-3(bn21ts) (I)/hT2-GFP (I;III)*

SS1098 *mes-3(bn21ts) (I)/hT2-GFP (I;III); mes-4(bn73)dpy-11(e224) (V)/DnT1 (IV;V)*

Temperature shifts

mes-3 ts worms were maintained at 15°C as heterozygotes and homozygotes were upshifted to 24°C or downshifted from 24°C to 15°C at various stages. To stage worms, adults were put in 100µl M9 on a gelatin chrom alum (GC) slide for 36-48 hours to obtain starved L1s. L1s were placed on plates with food and allowed to grow at 15°C or upshifted to 24°C. Stages were determined by analysis under a dissecting microscope of worm size and vulva morphology.

Immunocytochemistry

Dissected germlines were immunostained and processed as described (PETRELLA *et al.* 2011). Primary antibodies and dilutions used for immunostaining were: 1:30,000 mouse anti-H3K27me3 (Active Motif 39535 Lot #174, SS#29), 1:50,000 mouse anti-H3K27me3 (Kimura mAb 1E7, SS#70) (KIMURA *et al.* 2008), 1:1,000 rabbit anti-H3K36me2 (Abcam ab9049, SS#57), 1:10,000 rabbit anti-H4K16Ac (Millipore 07-329, SS#86). Secondary antibodies conjugated to Alexa Fluor 488 or 594 (Molecular Probes) were used at 1:300. Images within each figure were captured with the same acquisition settings.

Quantitation of H3K27me3 staining

Nuclei for quantitation were chosen based on the presence of X-chromosome enrichment of H3K27me3 and visual separation of the X chromosomes and autosomes (determined by co-staining with H4K16Ac). Using ImageJ, the average

pixel intensity on lines across the X chromosomes and the autosomes in the H3K27me3 channel were measured. This was repeated for 10 nuclei in at least 5 germlines. The pixel measurements in each germline were averaged, then each germline was treated as a replicate. The average pixel intensity on a line across a background region was also measured. The average background was subtracted from the average X-chromosome and average autosome measurements. Box plots were generated with these averages. p-values were calculated in Microsoft excel using a two-sample equal variance Student's t-Test.

Assay for Mortal germline (Mrt) phenotype

From heterozygous *mes-3* ts / balancer mothers, 51 fertile *mes-3* homozygous adults (P0) were placed on individual plates (cloned) at 15°C. Adults were removed from the plates after 21 hours of egg laying. F1 adults were counted and scored as clean sterile, grotty sterile, or fertile. One fertile adult F1 from each P0 worm was cloned, and the scoring procedure described above was repeated for each generation until there no fertile progeny were produced.

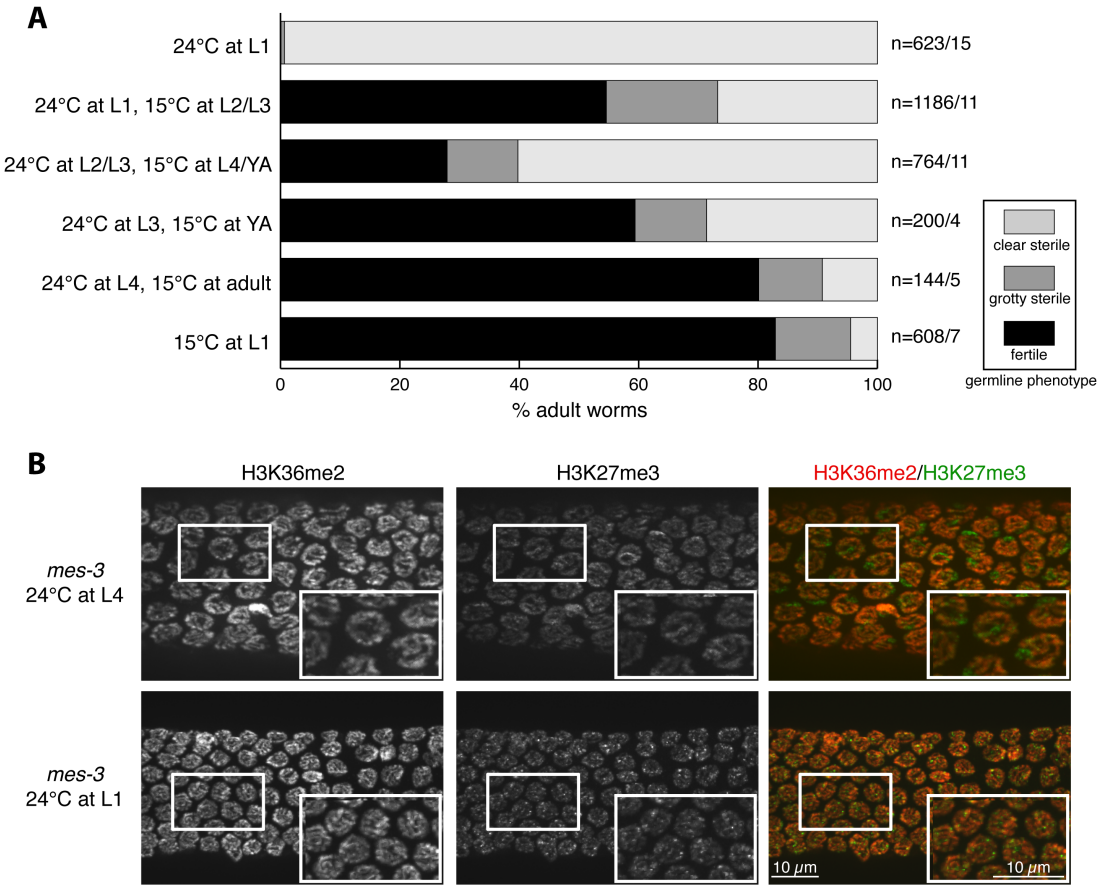


Figure 4-1. MES-3 functions during larval germline development to concentrate repressive H3K27me3 on the X chromosomes.
(A) Temperature shifts of *mes-3* ts worms from 15°C up to 24°C (restrictive temperature) and back down to 15°C (permissive temperature) at various larval (L1, L2, L3, L4), young adult (YA) or adult stages. The top and bottom histograms show controls: L1s upshifted and not downshifted, and L1s not upshifted. The progeny of temperature shifted animals were scored as sterile lacking gametes in their uterus (clear sterile – light grey), sterile with some gametes in their uterus (grotty sterile - dark grey), and fertile (fertile - black). n=number of worms scored/number of plates contributing to number of worms scored. **(B)** Antibody staining showing histone mark localization in *mes-3* ts adult germlines after upshift to 24°C at the L4 or L1 stage. H3K36me2 (red) and H3K27me3 (green) in merge.

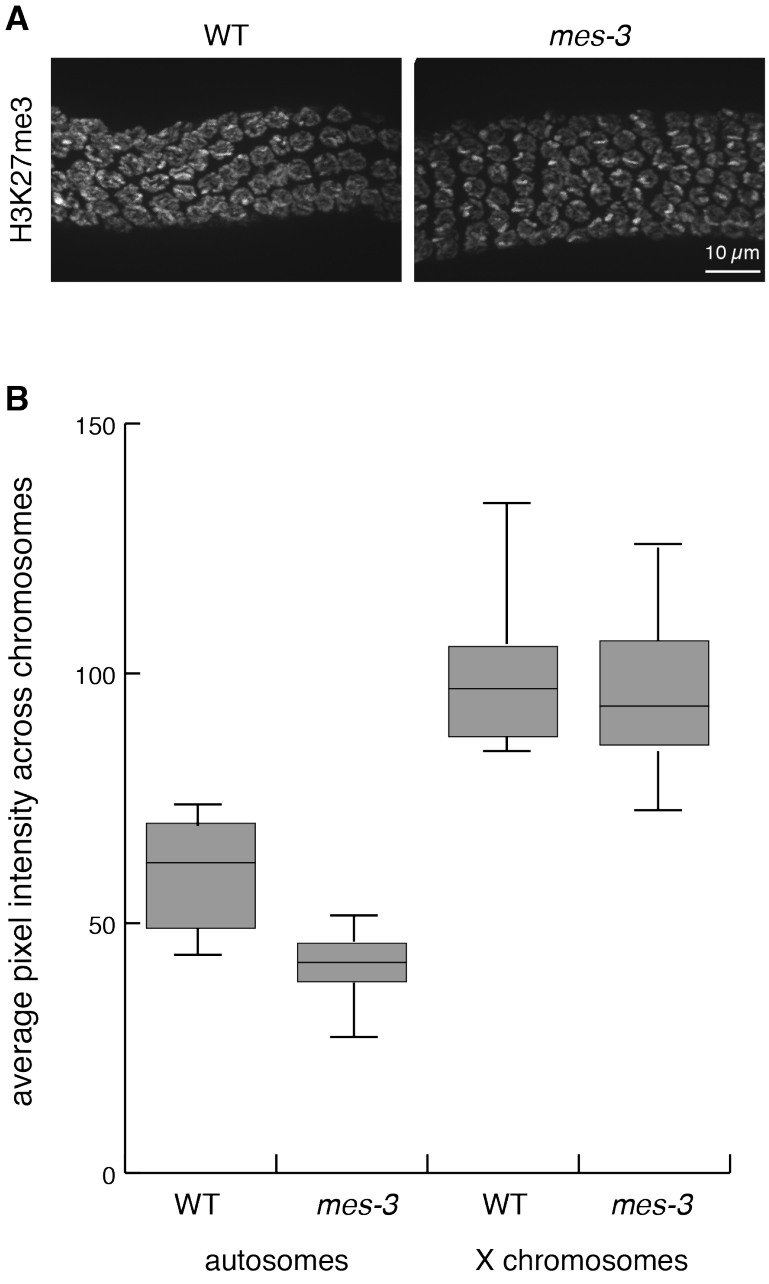


Figure 4-2. H3K27me3 is reduced on autosomes in *mes-3* ts mutants maintained at the permissive temperature.
(A) Antibody staining of H3K27me3 at the permissive temperature (15°C). **(B)** Box plot showing the average pixel intensity across the autosomes and the X chromosomes. Wild type (WT) and *mes-3* autosomes have a significantly different average number of pixels, p=0.0001. n = 5 germlines (10 nuclei from each germline) for each genotype.

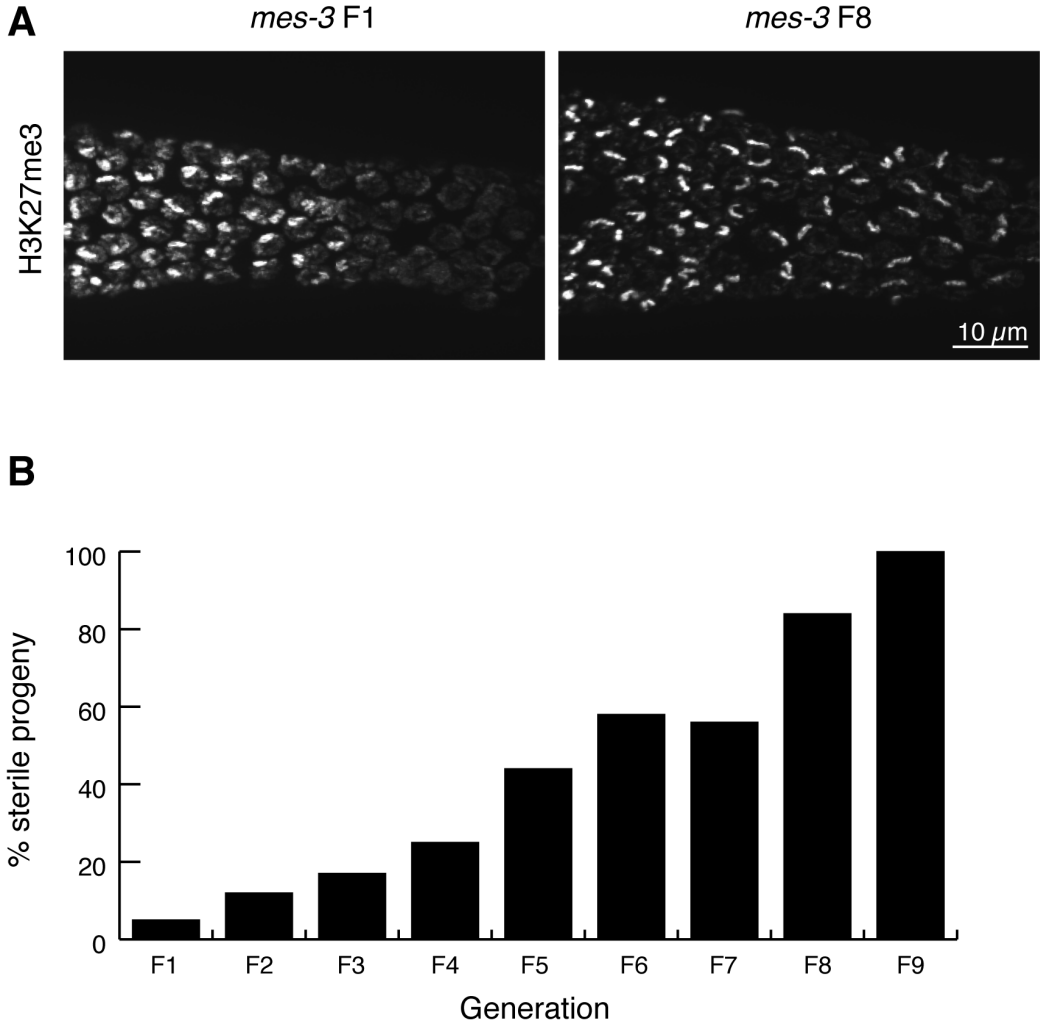


Figure 4-3. Over multiple generations of growth of *mes-3* ts worms at the permissive temperature, H3K27me3 becomes progressively depleted from the autosomes and sterility increases.
Worms were maintained as *mes-3* ts/balancer heterozygotes. The analyses shown in this figure were performed on the 1st through 9th generation (F1-F9) of *mes-3* ts homozygous worms. **(A)** Antibody staining of H3K27me3 at the permissive temperature **(**15°C**),** after one generation (F1) and eight generations (F8). **(B)** The percent clear sterile adult progeny at each generation for 9 generations (F1-F9). Percent sterile progeny represents an average from following progeny from 51 cloned fertile adult worms each generation.

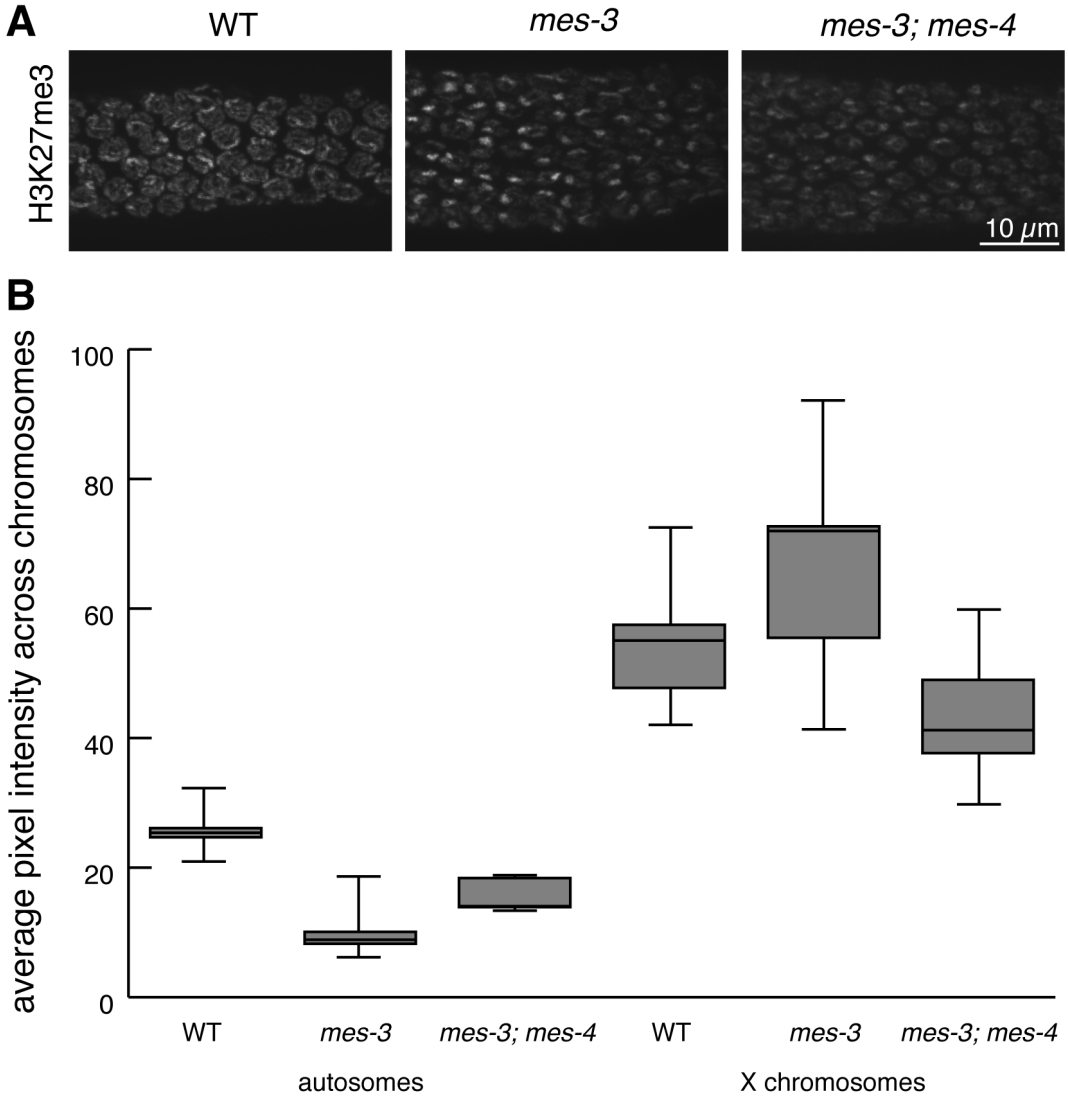


Figure 4-4. MES-4 antagonizes H3K27me3 on autosomes and helps concentrate it on the X chromosomes.
(A) Antibody staining of H3K27me3 at the permissive temperature **(**15°C**)** in wild type, *mes-3* ts single mutants, and *mes-3* ts; *mes*-4 double mutants. **(B)** Box plots showing average pixel intensity across the autosomes and the X chromosomes. WT and *mes-3* autosomes have a significantly different average number of pixels, p=0.0006. *mes-3* and *mes-3; mes-4* X chromosomes have a significantly different average number of pixels, p=0.049. For all genotypes, n = 5 germlines (10 nuclei from each germline).

REFERENCES

Ahmed, S., and J. Hodgkin, 2000 MRT-2 checkpoint protein is required for germline immortality and telomere replication in C. elegans. Nature 403**:** 159-164.

Arico, J. K., D. J. Katz, J. van der Vlag and W. G. Kelly, 2011 Epigenetic patterns maintained in early Caenorhabditis elegans embryos can be established by gene activity in the parental germ cells. PLoS Genet 7**:** e1001391.

Bannister, A. J., and T. Kouzarides, 2011 Regulation of chromatin by histone modifications. Cell Res 21**:** 381-395.

Bannister, A. J., P. Zegerman, J. F. Partridge, E. A. Miska, J. O. Thomas *et al.*, 2001 Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. Nature 410**:** 120-124.

Barlow, D. P., and M. S. Bartolomei, 2014 Genomic imprinting in mammals. Cold Spring Harb Perspect Biol 6.

Baugh, L. R., A. A. Hill, D. K. Slonim, E. L. Brown and C. P. Hunter, 2003 Composition and dynamics of the Caenorhabditis elegans early embryonic transcriptome. Development 130**:** 889-900.

Bean, C. J., C. E. Schaner and W. G. Kelly, 2004 Meiotic pairing and imprinted X chromatin assembly in Caenorhabditis elegans. Nat Genet 36**:** 100-105.

Beanan, M. J., and S. Strome, 1992 Characterization of a germ-line proliferation mutation in C. elegans. Development 116**:** 755-766.

Bender, L. B., R. Cao, Y. Zhang and S. Strome, 2004 The MES-2/MES-3/MES-6 complex and regulation of histone H3 methylation in C. elegans. Curr Biol 14**:** 1639-1643.

Bender, L. B., J. Suh, C. R. Carroll, Y. Fong, I. M. Fingerman *et al.*, 2006 MES-4: an autosome-associated histone methyltransferase that participates in silencing the X chromosomes in the C. elegans germ line. Development 133**:** 3907-3917.

Bernstein, B. E., T. S. Mikkelsen, X. Xie, M. Kamal, D. J. Huebert *et al.*, 2006 A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell 125**:** 315-326.

Brenner, S., 1974 The genetics of Caenorhabditis elegans. Genetics 77**:** 71-94.

Brownell, J. E., J. Zhou, T. Ranalli, R. Kobayashi, D. G. Edmondson *et al.*, 1996 Tetrahymena histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation. Cell 84**:** 843-851.

Browning, H., and S. Strome, 1996 A sperm-supplied factor required for embryogenesis in C. elegans. Development 122**:** 391-404.

Brykczynska, U., M. Hisano, S. Erkek, L. Ramos, E. J. Oakeley *et al.*, 2010 Repressive and active histone methylation mark distinct promoters in human and mouse spermatozoa. Nat Struct Mol Biol 17**:** 679-687.

Cao, R., L. Wang, H. Wang, L. Xia, H. Erdjument-Bromage *et al.*, 2002 Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science 298**:** 1039-1043.

Capowski, E. E., P. Martin, C. Garvin and S. Strome, 1991 Identification of grandchildless loci whose products are required for normal germ-line development in the nematode Caenorhabditis elegans. Genetics 129**:** 1061-1072.

Chu, D. S., H. Liu, P. Nix, T. F. Wu, E. J. Ralston *et al.*, 2006 Sperm chromatin proteomics identifies evolutionarily conserved fertility factors. Nature 443**:** 101-105.

Cinalli, R. M., P. Rangan and R. Lehmann, 2008 Germ cells are forever. Cell 132**:** 559-562.

Czermin, B., R. Melfi, D. McCabe, V. Seitz, A. Imhof *et al.*, 2002 Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. Cell 111**:** 185-196.

Deng, X., J. B. Hiatt, D. K. Nguyen, S. Ercan, D. Sturgill *et al.*, 2011 Evidence for compensatory upregulation of expressed X-linked genes in mammals, Caenorhabditis elegans and Drosophila melanogaster. Nat Genet 43**:** 1179-1185.

Elgin, S. C., 1990 Chromatin structure and gene activity. Curr Opin Cell Biol 2**:** 437-445.

Ernst, J., and M. Kellis, 2010 Discovery and characterization of chromatin states for systematic annotation of the human genome. Nat Biotechnol 28**:** 817-825.

Etchberger, J. F., A. Lorch, M. C. Sleumer, R. Zapf, S. J. Jones *et al.*, 2007 The molecular signature and cis-regulatory architecture of a C. elegans gustatory neuron. Genes Dev 21**:** 1653-1674.

Extavour, C. G., and M. Akam, 2003 Mechanisms of germ cell specification across the metazoans: epigenesis and preformation. Development 130**:** 5869-5884.

Felsenfeld, G., 2014 A brief history of epigenetics. Cold Spring Harb Perspect Biol 6.

Fong, Y., L. Bender, W. Wang and S. Strome, 2002 Regulation of the different chromatin states of autosomes and X chromosomes in the germ line of C. elegans. Science 296**:** 2235-2238.

Furuhashi, H., T. Takasaki, A. Rechtsteiner, T. Li, H. Kimura *et al.*, 2010 Trans-generational epigenetic regulation of C. elegans primordial germ cells. Epigenetics Chromatin 3**:** 15.

Gardner, K. E., C. D. Allis and B. D. Strahl, 2011 Operating on chromatin, a colorful language where context matters. J Mol Biol 409**:** 36-46.

Garvin, C., R. Holdeman and S. Strome, 1998 The phenotype of mes-2, mes-3, mes-4 and mes-6, maternal-effect genes required for survival of the germline in Caenorhabditis elegans, is sensitive to chromosome dosage. Genetics 148**:** 167-185.

Gaydos, L. J., A. Rechtsteiner, T. A. Egelhofer, C. R. Carroll and S. Strome, 2012 Antagonism between MES-4 and Polycomb repressive complex 2 promotes appropriate gene expression in C. elegans germ cells. Cell Rep 2**:** 1169-1177.

Gelbart, M. E., and M. I. Kuroda, 2009 Drosophila dosage compensation: a complex voyage to the X chromosome. Development 136**:** 1399-1410.

Greer, E. L., S. E. Beese-Sims, E. Brookes, R. Spadafora, Y. Zhu *et al.*, 2014 A Histone Methylation Network Regulates Transgenerational Epigenetic Memory in C. elegans. Cell Rep.

Gupta, V., M. Parisi, D. Sturgill, R. Nuttall, M. Doctolero *et al.*, 2006 Global analysis of X-chromosome dosage compensation. J Biol 5**:** 3.

Hammoud, S. S., D. A. Nix, H. Zhang, J. Purwar, D. T. Carrell *et al.*, 2009 Distinctive chromatin in human sperm packages genes for embryo development. Nature 460**:** 473-478.

Hansen, K. H., A. P. Bracken, D. Pasini, N. Dietrich, S. S. Gehani *et al.*, 2008 A model for transmission of the H3K27me3 epigenetic mark. Nat Cell Biol 10**:** 1291-1300.

Heard, E., 2005 Delving into the diversity of facultative heterochromatin: the epigenetics of the inactive X chromosome. Curr Opin Genet Dev 15**:** 482-489.

Hirsh, D., D. Oppenheim and M. Klass, 1976 Development of the reproductive system of Caenorhabditis elegans. Dev Biol 49**:** 200-219.

Hodgkin, J., H. R. Horvitz and S. Brenner, 1979 Nondisjunction Mutants of the Nematode CAENORHABDITIS ELEGANS. Genetics 91**:** 67-94.

Holdeman, R., S. Nehrt and S. Strome, 1998 MES-2, a maternal protein essential for viability of the germline in Caenorhabditis elegans, is homologous to a Drosophila Polycomb group protein. Development 125**:** 2457-2467.

Holliday, R., and J. E. Pugh, 1975 DNA modification mechanisms and gene activity during development. Science 187**:** 226-232.

Iovino, N., F. Ciabrelli and G. Cavalli, 2013 PRC2 controls Drosophila oocyte cell fate by repressing cell cycle genes. Dev Cell 26**:** 431-439.

Jans, J., J. M. Gladden, E. J. Ralston, C. S. Pickle, A. H. Michel *et al.*, 2009 A condensin-like dosage compensation complex acts at a distance to control expression throughout the genome. Genes Dev 23**:** 602-618.

Jenuwein, T., and C. D. Allis, 2001 Translating the histone code. Science 293**:** 1074-1080.

Katz, D. J., T. M. Edwards, V. Reinke and W. G. Kelly, 2009 A C. elegans LSD1 demethylase contributes to germline immortality by reprogramming epigenetic memory. Cell 137**:** 308-320.

Kawasaki, I., Y. H. Shim, J. Kirchner, J. Kaminker, W. B. Wood *et al.*, 1998 PGL-1, a predicted RNA-binding component of germ granules, is essential for fertility in C. elegans. Cell 94**:** 635-645.

Kelly, W. G., C. E. Schaner, A. F. Dernburg, M. H. Lee, S. K. Kim *et al.*, 2002 X-chromosome silencing in the germline of C. elegans. Development 129**:** 479-492.

Kennison, J. A., 1995 The Polycomb and trithorax group proteins of Drosophila: trans-regulators of homeotic gene function. Annu Rev Genet 29**:** 289-303.

Ketel, C. S., E. F. Andersen, M. L. Vargas, J. Suh, S. Strome *et al.*, 2005 Subunit contributions to histone methyltransferase activities of fly and worm polycomb group complexes. Mol Cell Biol 25**:** 6857-6868.

Kharchenko, P. V., A. A. Alekseyenko, Y. B. Schwartz, A. Minoda, N. C. Riddle *et al.*, 2011 Comprehensive analysis of the chromatin landscape in Drosophila melanogaster. Nature 471**:** 480-485.

Kimble, J. E., and J. G. White, 1981 On the control of germ cell development in Caenorhabditis elegans. Dev Biol 81**:** 208-219.

Kimura, H., Y. Hayashi-Takanaka, Y. Goto, N. Takizawa and N. Nozaki, 2008 The organization of histone H3 modifications as revealed by a panel of specific monoclonal antibodies. Cell Struct Funct 33**:** 61-73.

Kirmizis, A., S. M. Bartley, A. Kuzmichev, R. Margueron, D. Reinberg *et al.*, 2004 Silencing of human polycomb target genes is associated with methylation of histone H3 Lys 27. Genes Dev 18**:** 1592-1605.

Klass, M., N. Wolf and D. Hirsh, 1976 Development of the male reproductive system and sexual transformation in the nematode Caenorhabditis elegans. Dev Biol 52**:** 1-18.

Klymenko, T., and J. Muller, 2004 The histone methyltransferases Trithorax and Ash1 prevent transcriptional silencing by Polycomb group proteins. EMBO Rep 5**:** 373-377.

Korf, I., Y. Fan and S. Strome, 1998 The Polycomb group in Caenorhabditis elegans and maternal control of germline development. Development 125**:** 2469-2478.

Kornberg, R. D., 1974 Chromatin structure: a repeating unit of histones and DNA. Science 184**:** 868-871.

Kouzarides, T., 2007 Chromatin modifications and their function. Cell 128**:** 693-705.

Krogan, N. J., M. Kim, A. Tong, A. Golshani, G. Cagney *et al.*, 2003 Methylation of histone H3 by Set2 in Saccharomyces cerevisiae is linked to transcriptional elongation by RNA polymerase II. Mol Cell Biol 23**:** 4207-4218.

Kuzmichev, A., K. Nishioka, H. Erdjument-Bromage, P. Tempst and D. Reinberg, 2002 Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. Genes Dev 16**:** 2893-2905.

Lachner, M., D. O'Carroll, S. Rea, K. Mechtler and T. Jenuwein, 2001 Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. Nature 410**:** 116-120.

Lamelza, P., and N. Bhalla, 2012 Histone methyltransferases MES-4 and MET-1 promote meiotic checkpoint activation in Caenorhabditis elegans. PLoS Genet 8**:** e1003089.

Lanzuolo, C., F. Lo Sardo, A. Diamantini and V. Orlando, 2011 PcG complexes set the stage for epigenetic inheritance of gene silencing in early S phase before replication. PLoS Genet 7**:** e1002370.

Lee, T. I., R. G. Jenner, L. A. Boyer, M. G. Guenther, S. S. Levine *et al.*, 2006 Control of developmental regulators by Polycomb in human embryonic stem cells. Cell 125**:** 301-313.

Li, B., L. Howe, S. Anderson, J. R. Yates, 3rd and J. L. Workman, 2003 The Set2 histone methyltransferase functions through the phosphorylated carboxyl-terminal domain of RNA polymerase II. J Biol Chem 278**:** 8897-8903.

Liu, J., T. Rolef Ben-Shahar, D. Riemer, M. Treinin, P. Spann *et al.*, 2000 Essential roles for Caenorhabditis elegans lamin gene in nuclear organization, cell cycle progression, and spatial organization of nuclear pore complexes. Mol Biol Cell 11**:** 3937-3947.

Liu, T., A. Rechtsteiner, T. A. Egelhofer, A. Vielle, I. Latorre *et al.*, 2011 Broad chromosomal domains of histone modification patterns in C. elegans. Genome Res 21**:** 227-236.

Luger, K., A. W. Mäder, R. K. Richmond, D. F. Sargent and T. J. Richmond, 1997 Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature 389**:** 251-260.

Luger, K., and T. J. Richmond, 1998 The histone tails of the nucleosome. Curr Opin Genet Dev 8**:** 140-146.

Margueron, R., N. Justin, K. Ohno, M. L. Sharpe, J. Son *et al.*, 2009 Role of the polycomb protein EED in the propagation of repressive histone marks. Nature 461**:** 762-767.

Margueron, R., G. Li, K. Sarma, A. Blais, J. Zavadil *et al.*, 2008 Ezh1 and Ezh2 maintain repressive chromatin through different mechanisms. Mol Cell 32**:** 503-518.

Margueron, R., and D. Reinberg, 2011 The Polycomb complex PRC2 and its mark in life. Nature 469**:** 343-349.

Meissner, B., A. Warner, K. Wong, N. Dube, A. Lorch *et al.*, 2009 An integrated strategy to study muscle development and myofilament structure in Caenorhabditis elegans. PLoS Genet 5**:** e1000537.

Mello, C. C., C. Schubert, B. Draper, W. Zhang, R. Lobel *et al.*, 1996 The PIE-1 protein and germline specification in C. elegans embryos. Nature 382**:** 710-712.

Meyer, B. J., 2010 Targeting X chromosomes for repression. Curr Opin Genet Dev 20**:** 179-189.

Meyer, B. J., and L. P. Casson, 1986 Caenorhabditis elegans compensates for the difference in X chromosome dosage between the sexes by regulating transcript levels. Cell 47**:** 871-881.

Mochizuki, K., M. Tachibana, M. Saitou, Y. Tokitake and Y. Matsui, 2012 Implication of DNA demethylation and bivalent histone modification for selective gene regulation in mouse primordial germ cells. PLoS One 7**:** e46036.

Muller, J., C. M. Hart, N. J. Francis, M. L. Vargas, A. Sengupta *et al.*, 2002 Histone methyltransferase activity of a Drosophila Polycomb group repressor complex. Cell 111**:** 197-208.

Nakayama, J., J. C. Rice, B. D. Strahl, C. D. Allis and S. I. Grewal, 2001 Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. Science 292**:** 110-113.

Nguyen, D. K., and C. M. Disteche, 2006 Dosage compensation of the active X chromosome in mammals. Nat Genet 38**:** 47-53.

Nimura, K., K. Ura, H. Shiratori, M. Ikawa, M. Okabe *et al.*, 2009 A histone H3 lysine 36 trimethyltransferase links Nkx2-5 to Wolf-Hirschhorn syndrome. Nature 460**:** 287-291.

Okkema, P. G., and J. Kimble, 1991 Molecular analysis of tra-2, a sex determining gene in C.elegans. EMBO J 10**:** 171-176.

Ooi, S. L., J. R. Priess and S. Henikoff, 2006 Histone H3.3 variant dynamics in the germline of Caenorhabditis elegans. PLoS Genet 2**:** e97.

Papp, B., and J. Muller, 2006 Histone trimethylation and the maintenance of transcriptional ON and OFF states by trxG and PcG proteins. Genes Dev 20**:** 2041-2054.

Pasini, D., A. P. Bracken, J. B. Hansen, M. Capillo and K. Helin, 2007 The polycomb group protein Suz12 is required for embryonic stem cell differentiation. Mol Cell Biol 27**:** 3769-3779.

Patel, T., D. Rahe and O. Hobert, 2012 Removal of a Polycomb repressor complex makes C. elegans germ cells susceptible to direct conversion into specific somatic cell types. Cell Reports.

Paulsen, J. E., E. E. Capowski and S. Strome, 1995 Phenotypic and molecular analysis of mes-3, a maternal-effect gene required for proliferation and viability of the germ line in C. elegans. Genetics 141**:** 1383-1398.

Pengelly, A. R., Ö. Copur, H. Jäckle, A. Herzig and J. Müller, 2013 A histone mutant reproduces the phenotype caused by loss of histone-modifying factor Polycomb. Science 339**:** 698-699.

Petrella, L. N., W. Wang, C. A. Spike, A. Rechtsteiner, V. Reinke *et al.*, 2011 synMuv B proteins antagonize germline fate in the intestine and ensure C. elegans survival. Development 138**:** 1069-1079.

Petruk, S., Y. Sedkov, D. M. Johnston, J. W. Hodgson, K. L. Black *et al.*, 2012 TrxG and PcG proteins but not methylated histones remain associated with DNA through replication. Cell 150**:** 922-933.

Rastelli, L., and M. I. Kuroda, 1998 An analysis of maleless and histone H4 acetylation in Drosophila melanogaster spermatogenesis. Mech Dev 71**:** 107-117.

Rayasam, G. V., O. Wendling, P. O. Angrand, M. Mark, K. Niederreither *et al.*, 2003 NSD1 is essential for early post-implantation development and has a catalytically active SET domain. EMBO J 22**:** 3153-3163.

Rea, S., F. Eisenhaber, D. O'Carroll, B. D. Strahl, Z. W. Sun *et al.*, 2000 Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature 406**:** 593-599.

Rechtsteiner, A., S. Ercan, T. Takasaki, T. M. Phippen, T. A. Egelhofer *et al.*, 2010 The histone H3K36 methyltransferase MES-4 acts epigenetically to transmit the memory of germline gene expression to progeny. PLoS Genet 6.

Reinke, V., I. S. Gil, S. Ward and K. Kazmer, 2004 Genome-wide germline-enriched and sex-biased expression profiles in Caenorhabditis elegans. Development 131**:** 311-323.

Reinke, V., H. E. Smith, J. Nance, J. Wang, C. Van Doren *et al.*, 2000 A global profile of germline gene expression in C. elegans. Mol Cell 6**:** 605-616.

Riggs, A. D., 1975 X inactivation, differentiation, and DNA methylation. Cytogenet Cell Genet 14**:** 9-25.

Saffman, E. E., and P. Lasko, 1999 Germline development in vertebrates and invertebrates. Cell Mol Life Sci 55**:** 1141-1163.

Saitou, M., and M. Yamaji, 2012 Primordial germ cells in mice. Cold Spring Harb Perspect Biol 4.

Schaner, C. E., G. Deshpande, P. D. Schedl and W. G. Kelly, 2003 A conserved chromatin architecture marks and maintains the restricted germ cell lineage in worms and flies. Dev Cell 5**:** 747-757.

Schaner, C. E., and W. G. Kelly, 2006 Germline chromatin. WormBook**:** 1-14.

Schmitges, F. W., A. B. Prusty, M. Faty, A. Stutzer, G. M. Lingaraju *et al.*, 2011 Histone methylation by PRC2 is inhibited by active chromatin marks. Mol Cell 42**:** 330-341.

Seki, Y., K. Hayashi, K. Itoh, M. Mizugaki, M. Saitou *et al.*, 2005 Extensive and orderly reprogramming of genome-wide chromatin modifications associated with specification and early development of germ cells in mice. Dev Biol 278**:** 440-458.

Seydoux, G., and R. E. Braun, 2006 Pathway to totipotency: lessons from germ cells. Cell 127**:** 891-904.

Seydoux, G., and M. A. Dunn, 1997 Transcriptionally repressed germ cells lack a subpopulation of phosphorylated RNA polymerase II in early embryos of Caenorhabditis elegans and Drosophila melanogaster. Development 124**:** 2191-2201.

Seydoux, G., and A. Fire, 1994 Soma-germline asymmetry in the distributions of embryonic RNAs in Caenorhabditis elegans. Development 120**:** 2823-2834.

Seydoux, G., C. C. Mello, J. Pettitt, W. B. Wood, J. R. Priess *et al.*, 1996 Repression of gene expression in the embryonic germ lineage of C. elegans. Nature 382**:** 713-716.

Singaravelu, G., I. Chatterjee, M. R. Marcello and A. Singson, 2011 Isolation and in vitro activation of Caenorhabditis elegans sperm. J Vis Exp.

Smyth, G. K., 2004 Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 3**:** Article3.

Spencer, W. C., G. Zeller, J. D. Watson, S. R. Henz, K. L. Watkins *et al.*, 2011 A spatial and temporal map of C. elegans gene expression. Genome Res 21**:** 325-341.

Storey, J. D., and R. Tibshirani, 2003 Statistical significance for genomewide studies. Proc Natl Acad Sci U S A 100**:** 9440-9445.

Strahl, B. D., and C. D. Allis, 2000 The language of covalent histone modifications. Nature 403**:** 41-45.

Straub, T., and P. B. Becker, 2007 Dosage compensation: the beginning and end of generalization. Nat Rev Genet 8**:** 47-57.

Strome, S., 1986 Establishment of asymmetry in early Caenorhabditis elegans-embryos: visualization with antibodies to germ cell components in *Gametogenesis and the Early Embr*, edited by J. G. Gall. Alan R. Liss, Inc., New York.

Strome, S., W. G. Kelly, S. Ercan and J. D. Lieb, 2014 Regulation of the X Chromosomes in Caenorhabditis elegans. Cold Spring Harb Perspect Biol 6.

Strome, S., and R. Lehmann, 2007 Germ versus soma decisions: lessons from flies and worms. Science 316**:** 392-393.

Strome, S., and W. B. Wood, 1982 Immunofluorescence visualization of germ-line-specific cytoplasmic granules in embryos, larvae, and adults of Caenorhabditis elegans. Proc Natl Acad Sci U S A 79**:** 1558-1562.

Strome, S., and W. B. Wood, 1983 Generation of asymmetry and segregation of germ-line granules in early C. elegans embryos. Cell 35**:** 15-25.

Sulston, J. E., E. Schierenberg, J. G. White and J. N. Thomson, 1983 The embryonic cell lineage of the nematode Caenorhabditis elegans. Dev Biol 100**:** 64-119.

Tabuchi, T. M., B. Deplancke, N. Osato, L. J. Zhu, M. I. Barrasa *et al.*, 2011 Chromosome-biased binding and gene regulation by the Caenorhabditis elegans DRM complex. PLoS Genet 7**:** e1002074.

Tam, P. P., and S. X. Zhou, 1996 The allocation of epiblast cells to ectodermal and germ-line lineages is influenced by the position of the cells in the gastrulating mouse embryo. Dev Biol 178**:** 124-132.

Tanaka, Y., Z. Katagiri, K. Kawahashi, D. Kioussis and S. Kitajima, 2007 Trithorax-group protein ASH1 methylates histone H3 lysine 36. Gene 397**:** 161-168.

Tolhuis, B., E. de Wit, I. Muijrers, H. Teunissen, W. Talhout *et al.*, 2006 Genome-wide profiling of PRC1 and PRC2 Polycomb chromatin binding in Drosophila melanogaster. Nat Genet 38**:** 694-699.

Towbin, B. D., C. Gonzalez-Aguilera, R. Sack, D. Gaidatzis, V. Kalck *et al.*, 2012 Step-wise methylation of histone H3K9 positions heterochromatin at the nuclear periphery. Cell 150**:** 934-947.

Updike, D., and S. Strome, 2010 P granule assembly and function in Caenorhabditis elegans germ cells. J Androl 31**:** 53-60.

Villeneuve, A. M., 1994 A cis-acting locus that promotes crossing over between X chromosomes in Caenorhabditis elegans. Genetics 136**:** 887-902.

Wang, G. G., L. Cai, M. P. Pasillas and M. P. Kamps, 2007 NUP98-NSD1 links H3K36 methylation to Hox-A gene activation and leukaemogenesis. Nat Cell Biol 9**:** 804-812.

Wang, J. T., and G. Seydoux, 2013 Germ cell specification. Adv Exp Med Biol 757**:** 17-39.

Wang, X., Y. Zhao, K. Wong, P. Ehlers, Y. Kohara *et al.*, 2009 Identification of genes expressed in the hermaphrodite germ line of C. elegans using SAGE. BMC Genomics 10**:** 213.

Weintraub, H., and M. Groudine, 1976 Chromosomal subunits in active genes have an altered conformation. Science 193**:** 848-856.

Xu, L., Y. Fong and S. Strome, 2001a The Caenorhabditis elegans maternal-effect sterile proteins, MES-2, MES-3, and MES-6, are associated in a complex in embryos. Proc Natl Acad Sci U S A 98**:** 5061-5066.

Xu, L., J. Paulsen, Y. Yoo, E. B. Goodwin and S. Strome, 2001b Caenorhabditis elegans MES-3 is a target of GLD-1 and functions epigenetically in germline development. Genetics 159**:** 1007-1017.

Xu, L., and S. Strome, 2001 Depletion of a novel SET-domain protein enhances the sterility of mes-3 and mes-4 mutants of Caenorhabditis elegans. Genetics 159**:** 1019-1029.

Yang, Y. H., S. Dudoit, P. Luu, D. M. Lin, V. Peng *et al.*, 2002 Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Res 30**:** e15.

Yuan, W., M. Xu, C. Huang, N. Liu, S. Chen *et al.*, 2011 H3K36 methylation antagonizes PRC2-mediated H3K27 methylation. J Biol Chem 286**:** 7983-7989.

Yuzyuk, T., T. H. Fakhouri, J. Kiefer and S. E. Mango, 2009 The polycomb complex protein mes-2/E(z) promotes the transition from developmental plasticity to differentiation in C. elegans embryos. Dev Cell 16**:** 699-710.

Zhang, F., M. Barboric, T. K. Blackwell and B. M. Peterlin, 2003 A model of repression: CTD analogs and PIE-1 inhibit transcriptional elongation by P-TEFb. Genes Dev 17**:** 748-758.

Zhao, J., B. K. Sun, J. A. Erwin, J. J. Song and J. T. Lee, 2008 Polycomb proteins targeted by a short repeat RNA to the mouse X chromosome. Science 322**:** 750-756.

Zhou, Q., H. Li and D. Xue, 2011 Elimination of paternal mitochondria through the lysosomal degradation pathway in C. elegans. Cell Res 21**:** 1662-1669.

Zhu, B., G. Ping, Y. Shinohara, Y. Zhang and Y. Baba, 2005 Comparison of gene expression measurements from cDNA and 60-mer oligonucleotide microarrays. Genomics 85**:** 657-665.