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The Role of Histone Variants, Histone Modifications, and Germline Transcription on Imprint Establishment and Epigenetic Inheritance in *Caenorhabditis elegans*

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By

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Bachelor of Science, University of Notre Dame, 2005

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An abstract of a dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in the Graduate Division of Biological and Biomedical Sciences
Biochemistry, Cell, and Developmental Biology
2011

Abstract

The Role of Histone Variants, Histone Modifications, and Germline Transcription on Imprint Establishment and Epigenetic Inheritance in *Caenorhabditis elegans*

By Jackelyn Kay Arico

Epigenetic information such as parental imprints can be transmitted along with genetic information through the germ line from parent to offspring. Recent reports show that histone modifications marking developmentally regulated loci can be transmitted through sperm as a component of this information transfer. How the information that is transferred is established in the parent and maintained in the offspring is poorly understood. Our lab had previously described a form of imprinted X inactivation in *C. elegans* where dimethylation on histone 3 at lysine 4 (H3K4me2), a mark of active chromatin, is excluded from the paternal X chromosome (Xp) during spermatogenesis and persists through early cell divisions in the embryo. We first examined the role of histone variants and histone modifications in imprint establishment. Although the histone variants examined were not required for imprint establishment, and the histone modifications characterized were not unique to the Xp, these studies reinforced the observation that the Xp (unlike the maternal X or any autosome) is largely transcriptionally inactive in the paternal germ line. Based on this observation, we hypothesized that transcriptional activity in the parent germ line may influence epigenetic information inherited by and maintained in the embryo. We observed that chromatin modifications and histone variant patterns assembled in the germ line can be retained in mature gametes. Furthermore, despite extensive chromatin remodeling events at fertilization, the modification patterns arriving with the gametes are retained in the early embryo. Using transgenes, we observed that expression in the parental germline correlates with differential chromatin assembly that is replicated and maintained in the early embryo. Expression in the adult germ cells also correlates with more robust expression in the somatic lineages of the offspring. These results suggest that chromatin environments established in the parental germ lines may provide a potential mechanism for the inheritance of parent of origin epigenomic content that can be maintained and heritably affect gene expression in the offspring.

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ACKNOWLEDGEMENTS

I'd like to thank Bill for his support and enthusiasm towards my research and allowing me the freedom to pursue my interests inside and outside of the lab and my committee for their advice and support. I'd also like to thank the Kelly Lab for their scientific expertise and discussions, as well as their camaraderie and support throughout my graduate career. I'd especially like to thank my husband, Steve, for all his love and support through all our adventures during our time here in Atlanta and for being the best simultaneous househusband and primary bread earner ever. Michael, Gregory, and baby; thank you for the love, diversions, and incentives to finish my degree. I thank my family for love and support from afar. And finally, I thank God for the beautiful and complex world in which we live and all the people and circumstances which have allowed me the opportunity to study His creation.

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Chapter 1

Introduction

Epigenetics is defined as encompassing processes that induce a heritable change in gene expression that does not affect the underlying DNA sequence. The most striking example of epigenetics is the development of a multicellular organism from a single celled zygote. Despite the fact that nearly all cells in the human body contain the same DNA sequences, differences in gene expression from one cell to the next allow for different tissues and cell types to arise. Transcription factors and chromatin are both involved in differential gene expression. The known epigenetic effectors of changes in gene expression are those that regulate chromatin structure, histone modifications, DNA methylation and non-coding RNAs (ncRNAs). Although epigenetic changes may be acquired during the parents' lifetime in a manner similar to Lamark's acquired traits, in order for inheritance of these marks to occur epigenetic changes must be established in the parents' germline and passed onto the offspring. While evidence suggests that epigenetic inheritance does occur, a mechanism for inheritance of histone modifications has not been directly demonstrated across generations or even through cell divisions.

Chromatin and Histone Modifications

DNA does not exist alone in the nucleus of a cell but is organized into a higher order structure called chromatin. DNA is wound around an octamer of four proteins, histones H2A, H2B, H3 and H4 (Figure 1.1 A). A tetramer of two molecules each of histones H3 and H4, along with two dimers each containing one molecule of histones H2A and H2B make up the nucleosome, around which 147 base pairs (bp) of DNA is wound (Luger et al., 1997). DNA entering and exiting the nucleosome interacts with the linker histone, H1. Nucleosomes are spaced roughly 50 bp apart in the simplest chromatin conformation, termed the 'beads on a string' structure (Olins and Olins, 1974),

and can be further folded into higher order structures such as the 30 nm fiber and up to the highly condensed structure observed in mitotic chromatids. The four core histones are highly conserved throughout evolution, with a globular histone fold domain comprising the core of the nucleosome, and unstructured N-terminal "tails" which protrude from the nucleosome (Figure 1.1 A). The N-terminal tails of histones can be highly modified with various posttranslational modifications, such as methylation, acetylation, phosphorylation, and ubiquitination (Strahl and Allis, 2000) (Figure 1.1 B). These modifications of histone tails can act alone or together to have positive or negative effects on gene expression by recruiting additional histone modifying factors or chromatin remodeling enzymes as well as directly influencing chromatin structure. A "histone code hypothesis" has been proposed that posits that different combinations of post-translational modifications, can have distinct effects on gene expression (Strahl and Allis, 2000). However, although there are correlations that provide evidence in support of the histone code, examples of specific combinations of individual modifications strictly determining biological outputs have not been demonstrated.

Two of the most highly studied post-translational modifications are histone lysine acetylation and methylation. Acetylation of histone lysines correlates with transcriptionally active chromatin. Acetylation of lysines, carried out by histone acetyltransferases (HATs), reduces the positive charge of the histone, reducing its affinity for negatively charged DNA, and potentially leading to a more open chromatin state. In addition to directly affecting DNA-histone interactions, acetylation of H4K16 may lead to a more open chromatin state by several different mechanisms. Acetylation on H4K16 can directly affect the inter-nucleosome contacts between H4 and an acidic patch on

H2A, as well as by directly repelling Sir3 to prevent heterochromatin spreading at chromatin boundaries (Carmen et al., 2002; Shogren-Knaak et al., 2006; Shogren-Knaak and Peterson, 2006; Suka et al., 2002; Vaquero et al., 2007; Zhou et al., 2007). HATs, such as the SAGA complex in yeast (STAGA in humans), are recruited to DNA by physically associating with RNA polymerase (Nagy and Tora, 2007). Acetylation by such complexes may recruit ATP dependent chromatin remodelers such as the SWI/SNF complex, leading to further histone displacement, and keeping the chromatin in recently transcribed regions in an "open" state (Chandy et al., 2006; Hassan et al., 2001; Huang et al., 2003). Conversely, histone de-acetylases (HDAC's), remove acetyl groups by a simple hydrolysis reaction, leading to more compact, less accessible chromatin. In budding yeast, hypoacetylation of H4K16 can be recognized by Sir4p and Sir3p which interact with the HDAC Sir2 and leads to spreading of silent chromatin (Hecht et al., 1995).

While histone acetylation is clearly associated with active chromatin, and its absence mostly associated with silent chromatin, histone methylation can be repressive, or activating, depending on which histone residue is modified. Histone tails can be mono-, di- or tri- methylated on lysines and mono- or di- methylated on arginines by three classes of histone methyltransferases (HMTs). Arginines in histone tails can be mono- or di- methylated (Rme1 and Rme2, respectively), either symmetrically or asymmetrically by the PRMT family of HMTs (Wysocka et al., 2006), but this class of enzymes will not be discussed further. The DOT1 family comprises non-SET domain HMTs that specifically catalyze methylation of histone H3 at lysine 79 (H3K79me) (Ng et al., 2002; van Leeuwen et al., 2002). A third family of HMTs,which contain the

conserved SET-domain, carries out all other known histone lysine methylation. Histone methylation can be correlated with silent chromatin, in the case of H3K9me3, H3K27me2/3 and H4K20me3. Alternatively, this mark can correlate with active chromatin in the case of H3K4me2/3, H3K36me2/3, or H3K79me2. These histone modifications are not known to directly affect intrinsic chromatin structure, as histone acetylation does, but rather provide a platform for specific binding of downstream effector proteins, including transcription factors, chromatin remodeling enzymes, adaptor proteins, and additional chromatin modifying enzymes. Methylated lysine residues can be bound by at least four protein domains, including plant-homeodomain (PHD), chromodomains, Tudor domains, and WD40 repeats. Proteins bearing these motifs can apparently distinguish between methylation at different lysine residues, and in some cases di- and tri- methylation, with their interactions resulting in different outputs (Adams-Cioaba and Min, 2009).

Methylation on lysine nine of histone H3 (H3K9me2/3), for example, has been shown to correlate with transcriptionally inactive or silenced chromatin. In humans, fission yeast, and flies H3K9me3 is catalyzed by Su(var)3-9 homologs (Czermin et al., 2002; Nakayama et al., 2001; Rea et al., 2000; Schotta et al., 2002). H3K9me3 can be bound by heterochromatin protein 1 (HP1) via its chromodomain, which recruits Su(var)3-9. This has been proposed to provide a self-reinforcing loop in which H3K9me3 spreads and further silences adjacent regions (Aagaard et al., 1999). Alternatively, methylation of lysine four on histone H3 (H3K4me2/3) has been correlated with transcriptionally active chromatin, and in fact physically associates with the RNA polymerase II holoenzyme in budding yeast (Krogan et al., 2003; Li et al., 2003; Ng et

al., 2003; Xiao et al., 2003). H3K4me3 binding has been observed with two different proteins, WDR5 and Chd1, although WDR5 has recently been shown to interact with H3 lysine four independent of methylation (Li and Kelly PloS Genetics 2011 in press). WDR5, which preferentially binds H3K4me2 through its WD40 repeat domain, is a component of the MLL complex that possesses H3K4me3 activity (Wysocka et al., 2005). It has been proposed that MLL recruitment converts H3K4me2 to H3K4me3 (Wysocka et al., 2005). The chromodomain of Chd1 interacts directly with H3K4me2/3 and is a component of the SAGA histone acetyltransferase (Pray-Grant et al., 2005; Sims et al., 2007). The interaction of Chd1 with H3K4me2/3 is proposed to recruit the SAGA complex to acetylate nearby histones and further open and activate chromatin.

Until recently, methylation was thought to be a permanent post-translational modification based on the stable nature of the C-N bond and the low turnover rate of this mark (Byvoet et al., 1972). However the discovery of lysine demethylases KDMs (LSD1 and the Jumonji domain containing KDMs) has brought to light the dynamic nature of this mark. LSD1 (lysine-specific demethylase 1) is the founding member of this group of enzymes (Shi et al., 2004). LSD1 is an H3K4me1/2 de-methylase with homologues in *Arabidopsis*, *Drosophila*, *C. elegans* and *S. pombe*, however it is not found in *S. cerevisiae*. More recently LSD1 has been reported to also demethylate H3K9me2 at androgen receptor target genes (Metzger et al., 2005). Demethylation by LSD1 homologues is thought to be restricted to di- or mono- methylated lysines, while different Jumonji-class demethylases can remove mono-, di-, and tri- methylation (Mosammaparast and Shi, 2010).

Interestingly, lysine demethylases typically exist in complexes, which include other chromatin modifying enzymes. For example, LSD1 is a member of both the CoRest complex and NuRD complexes, which deacetylate histones (Mosammaparast and Shi, 2010). In these cases demethylation of H3K4me2 complements deacetylation towards the common goal of repressing chromatin. Similarly, the Jumonji domain containing protein UTX has been reported to operate in a complex with MLL (an HMT that methylates H3K4), and MOF (a HAT), where it demethylates H3K27me3, to create a more accessible, transcriptionally active chromatin environment (Mosammaparast and Shi, 2010).

Histone modifications are not only co-regulated by distinct enzymes existing in the same complexes, but also by influencing the establishment of other modifications in *cis*. For example phosphorylation of H3 serine 10 recruits HMT Gcn5, which establishes H3K14Ac (Walter et al., 2008). As another example, HMT Set2, which tracks along with RNA polymerase during transcription, lays down H3K36 dimethylation. This H3K36me2 recruits Rpd3S (an HDAC) leading to deacetylation of H3 and H4, preventing inappropriate internal initiation within mRNA coding regions of recently transcribed genes (Lee and Shilatifard, 2007).

Histone Variants

Adding another layer of complexity to chromatin regulation are histone variants. While histones H4 and H2B are largely invariant, Histones H3 and H2A have multiple variant forms that are conserved across species. The canonical form of histone H3 conserved among all metazoans, H3.2, is a replication dependent variant, that is incorporated into replicating DNA during S-phase (Elsaesser et al., 2010). This histone is

enriched in heterochromatin and thus is highly modified with K27me2/3 as well as H3K9me2/3 (Johnson et al., 2004; McKittrick et al., 2004). Histone H3.1 is an additional replication-dependent variant, which differs from H3.2 by only one amino acid; amino acid 96 is a cystine in H3.1 and a serine in H3.2 (Franklin and Zweidler, 1977). While H3.2 is the evolutionarily more conserved H3 variant, there is only one gene expressing this variant in the human genome. With eleven genes, H3.1 is the major H3 variant in humans (Marzluff et al., 2002). Each of these copies is simultanteously expressed to facilitate bulk chromatin assembly during S-phase. H3 incorporation into replicating chromatin requires the histone chaperone Caf-1 (Tagami et al., 2004). Another class of H3 histones the CenH3's, are centromere specific variants. The function of CenH3s is highly conserved; they recruit to the centromere, kinetochore proteins that serve as a nucleating cite for microtubules during mitosis (Blower and Karpen, 2001; Blower et al., 2002; Buchwitz et al., 1999; Howman et al., 2000; Stoler et al., 1995). Centromeres and CenH3 patterns are stably maintained through both mitosis and meiosis. Another variant, H3.3, distinguishes itself from canonical H3.2/H3.1 in that it can be expressed in a replication independent fashion, allowing for incorporation of this variant outside of DNA replication (Ahmad and Henikoff, 2002; Wu et al., 1982). H3.3 differs from H3.2 by 4 amino acids and is associated with euchromatin and enriched in active histone modifications such as K4me2/3, K9Ac, and K14Ac (Chow et al., 2005; Johnson et al., 2004; McKittrick et al., 2004). Based on these observations H3.3 is thought to be incorporated into chromatin after transcription mediated nucleosome displacement via its chaperone HIRA (Tagami et al., 2004). The definition of HIRA as an H3.3 chaperone is due to its ability to assemble nucleosomes independent of DNA synthesis (Loyola and

Almouzni, 2004; Ray-Gallet et al., 2002; Tagami et al., 2004). When H3.3 is unavailable, however, HIRA can incorporate canonical H3 at active genes by replication-independent nucleosome replacement (Sakai et al., 2009). H3.3 is not exclusively coupled to transcription, nor does it appear to be essential for normal transcription since transcriptional outputs from flies lacking H3.3 were indistinguishable from wild-type animals (Hodl and Basler, 2009). In fact, when H3.3 is knocked out in Drosophila, H3.1 expression is up-regulated, demonstrating that H3.1 is capable of fulfilling H3.3's role in transcription (Sakai et al., 2009). However, H3.3 plays key roles in the germline and early development, and has been recently described in heterochromatin as well. While flies lacking H3.3 are viable and superficially normal they are sterile (Hodl and Basler, 2009). This sterility cannot be rescued by H3.2 or an H3.3 mutant where lysine four is changed to alanine, further suggesting that methylation on H3.3 lysine four is more important for development than for incorporation of H3.3 in facilitating transcription. Flies lacking the H3.3-specific chaperone HIRA are maternal effect embryonic lethal because maternally provided HIRA is required for H3.3 incorporation into the decondensing paternal pronucleus after fertilization (Loppin et al., 2005). *Tetrahymena* provides another example where H3.3s play a role in development, rather than in active transcription; H3.3 is required for the production of viable sexual progeny, but is not necessary for cell growth (Cui et al., 2006). For a comparison of histone H3 variants see Table 1.1.

In mouse zygotes the decondensing paternal pronucleus is also low in H3.1 and accumulates H3.3 and HIRA (Torres-Padilla et al., 2006; van der Heijden et al., 2005). Additionally, early mouse development specifically requires H3.3 with lysine at residue

27 for normal heterochromatin formation in the embryo, implying a role for K27 methylation of H3.3 in this process (Santenard et al., 2010). Furthering H3.3's potential role in silencing are reports of its involvement in silencing of the inactive X chromosome. H3.3, HIRA, and an H3.3 chaperone, Daxx, are enriched on the transcriptionally silent XY body during mammalian spermatogenesis (Rogers et al., 2004; van der Heijden et al., 2007). Daxx exists in a complex with the SNF2 family chromatin remodeler ATRX (Lewis et al., 2010). Furthermore, ATRX is found on both the imprinted and randomly inactivated X chromosome, and imprinted X inactivation is lost in the absence of ATRX (Baumann and De La Fuente, 2009; Garrick et al., 2006). This association, along with the recent data showing H3.3 enrichment at telomeres and pericentric heterochromatin suggest that H3.3 may have a role in transcriptional silencing (Drane et al., 2010; Goldberg et al., 2010; Lewis et al., 2010; Wong et al., 2009).

In addition to their roles in development, histone variants also directly impact inherent chromatin structure. Nucleosomes containing H3.3 are less stable than those containing H3.1 or H3.2, which may further enhance transcription in regions containing this nucleosome (Jin and Felsenfeld, 2007). H3.3 containing nucleosomes can be further destabilized by the inclusion of the histone variant H2A.Z (Jin and Felsenfeld, 2007). Histone H2A.Z is the major H2A variant comprising 5-10% of the H2A in the mammalian genome, and it varies significantly from H2A (Abbott et al., 2001; Leach et al., 2000). It has a conserved extended acidic patch, which may cause the observed instability in nucleosomes with H3.3 (Fan et al., 2004; Suto et al., 2000). In some studies H2A.Z correlates with active chromatin and is enriched at transcription start sites, yet in others it has been shown to play a role in pericentric heterochromatin maintenance

(Adam et al., 2001; Dhillon and Kamakaka, 2000; Mizuguchi et al., 2004; Santisteban et al., 2000). Another H2A variant, H2A.Bbd, is excluded from the inactive X chromosome, destabilizes nucleosomes, and is associated with active genes (Chadwick and Willard, 2001; Gautier et al., 2004; Zhou et al., 2007). H2A.Bbd was also recently described as being expressed in testis during spermatogenesis (Ishibashi et al., 2010). In contrast another H2A variant, MacroH2A, characterized by it large "macro" C-terminal domain, is associated with heterochromatin, the inactive X chromosome, and DNA methylation (Chadwick and Willard, 2001; Chadwick and Willard, 2002; Choo et al., 2006; Choo et al., 2007; Costanzi and Pehrson, 1998; Costanzi et al., 2000; Grigoryev et al., 2004; Pehrson and Fried, 1992; Zhang et al., 2005). For a comparison of histone H2A variants see Table 1.2.

DNA methylation

In mammals, DNA is methylated primarily on CpG dinucleotides and this methylation is mediated by DNA-methyltransferases. Dnmt1 is an essential maintenance methyltransferase required for methylating hemi-methylated DNA after replication (Bestor, 1992; Li et al., 1992). Dnmt3a and Dnmt3b are responsible for *de novo* methylation during development (Hsieh, 1999; Lyko et al., 1999; Okano et al., 1999). Dnmt3a and 3b also interact with HDACs, furthering their ability to silence chromatin (Burgers et al., 2002). CpGs are less abundant in the genome than expected, due to the fact that methylated cytosine is mutagenic. If an un-methylated cytosine is deaminated, it becomes uracil, which does not belong in DNA, and will be removed by the base excision repair pathway. However, if a methylated cytosine is deaminated it becomes a thymine and the miss-paired T-G may be correctly repaired to a C-G or, incorrectly

'repaired' to a T-A by the mismatch repair system. Over evolutionary time cytosines in CpGs have been converted to T's, depleting CpGs throughout the genome (Bird, 1980). Regions remaining rich in CpG dinucleotides are called CpG islands and are enriched in gene promoters across the human genome (Jones and Baylin, 2002). CpG methylation is extensive in heterochromatin, which is also rich in H3K9 dimethylation (Stancheva, 2005). The first link between DNA methylation and histone methylation was demonstrated in *Neurospora*, where a mutation in an H3K9 HMT leads to the loss of all DNA methylation in the genome (Tamaru and Selker, 2001). In *Arabidopsis* H3K9me3 recruits HP1, which can go on to recruit the DNA methyltransferase Cmt3b, which catalyses DNA methylation at CpNpG's (Jackson et al., 2002). DNA methylation can mediate silencing by inhibiting transcription factor binding, and indirectly through recruitment of Methyl-CpG binding proteins (MECPs). Many of these MECPs are associated with histone deacetylases and histone modifying enzymes, which go on to further silence chromatin. For example, MECP MBD2 (methyl binding domain 2) is associated with the NuRD complex, which contains HDACs as well as an ATP-dependent chromatin remodeling enzyme, Mi-2 (Wade et al., 1999; Zhang et al., 1999). DNA methylation has not been observed in all organisms; exceptions include *C. elegans*, *S. cerevisiae*, and adult *Drosophila* (Lyko et al., 2000). DNA methylation is present in mature gametes, but is largely erased genome wide at fertilization, allowing for reprogramming and reactivation of previously silenced genes in the totipotent zygote (Bourc'his et al., 2001; Hata et al., 2002; Howlett and Reik, 1991; Kafri et al., 1992; Mayer et al., 2000; Oswald et al., 2000; Reik and Walter, 2001; Rougier et al., 1998).

Mammalian genomic imprints however, as discussed below, are resistant to this initial wave of erasure.

Imprinting

One well-studied epigenetic phenomena is genomic imprinting, where genes are expressed from one or another, but not both alleles, in a parent-of-origin dependent manner. This deviation from Mendelian genetics is an excellent model for studying epigenetic inheritance. The importance of genomic imprinting is illustrated by diseases, such as Prader-Willi and Angelman syndromes, where imprinted gene regulation is disrupted (Amor and Halliday, 2008). Genomic imprinting has been most closely studied in mammals, although examples in fish, worms, flies, and other insects exist as well.

Helen Crouse first defined imprinting in 1960 in Sciara, the fungus gnat, where the paternal X chromosome(s), or even the entire paternal genome, is eliminated depending on sex and tissue type (Crouse, 1960; Goday and Esteban, 2001). This imprinting phenomenon is primarily understood from a cell biological perspective, and the mechanism for distinguishing the paternal chromosomes has not been characterized. In lecanoid Coccids, or mealybugs, the entire paternally inherited chromosome set becomes heterochromatic and genetically inert (Bongiorni and Prantera, 2003; Brown and Nelson-Rees, 1961). In contrast to mammals where DNA methylation correlates with gene silencing, in mealybugs the heterochromatic paternal genome is hypomethylated compared to the euchromatic maternal genome (Bongiorni et al., 1999). The paternally inherited genome of the mealybug is also enriched in H3K9 tri-methylation and HP1 (Cowell et al., 2002). Bongiorni et al. recently proposed that H3K9me2/3 is the signal used to establish the paternal imprint in coccids, as it is present

in mature sperm and persists in the paternal pronucleus (Bongiorni et al., 2009). In flies, imprinting is a heterochromatin-based phenomena; the imprint is manifest when chromosomal rearrangements cause marker genes to juxtapose heterochromatin. These genes are silenced in a manner similar to position effect variegation, however the silencing can be dependent on the parent of origin (Lloyd, 2000). These studies reveal that imprinting in insects is manifest in a broad variety of ways.

There is evidence of transgene imprinting in zebrafish as well. Transgene methylation patterns may be affected by sex as well as environmental influences (Martin and McGowan, 1995). However, if parental imprinting exists in zebrafish, it does not result in essential genes being inactivated when their expression is essential for development, as androgenic progeny are viable (Corley-Smith et al., 1996). Dnmt3A and Dnmt3b, the *de novo* DNA maintenance methyltransferases responsible for establishing methylation are conserved in fish; however a regulatory cofactor required for establishment of DNA methylation at mammalian imprints, Dnmt3L, is not conserved (Yokomine et al., 2006). Dnmt3L is however conserved in opossum/marsupials.

In mammals, imprinted loci generally lie in clusters regulated by DNA methylation of an imprint control region (ICR) and are often associated with a non-coding RNA (ncRNA) antisense to the protein coding genes in the cluster. Reciprocal DNA methylation is seen based on the parent of origin. For example, at the Igf2r locus the promoter of the ncRNA Air serves as the ICR and is methylated on the maternal allele, and is unmethylated on the paternal allele. DNA methylation on the maternal allele silences Air expression, allowing for the genes in the opposite orientation to be transcribed (Sleutels et al., 2002; Smilinich et al., 1999). Conversely, the ICR is

unmethylated on the paternal allele, allowing for transcription of Air, which represses transcription of the rest of the genes in the cluster on the paternal chromosome. While the consequences of imprinting have been closely examined, the mechanisms by which imprints are established in the parents' germline are unknown.

A dramatic example of imprinting in mammals is imprinted X chromosome inactivation (iXi), in which the paternal X is preferentially inactivated prior to implantation in mammals (Mak et al., 2004). iXi is sustained only in the placental tissues of eutherians, while it is replaced by random X inactivation in the post-implantation embryo (Mak et al., 2004). In marsupials however, iXi is maintained in both the embryonic and placental lineages (Sharman, 1971). The first well-characterized step in iXi, as in random X inactivation, is *Xist* expression. The ncRNA *Xist* is expressed preferentially from the X chromosome that will be silenced; i.e., the Xp in iXi and either X in random Xi. Xist spreads *in cis* and coats the inactive X chromosome, leading to silencing of most of the genes on the chromosome. Similar to differentially methylated ICR at genomic imprinting loci, in female somatic cells where random Xi occurs, the promoter for the ncRNA *Xist* is methylated on the transcriptionally silent X, but not the X that expresses *Xist* (Norris et al., 1994). However, this differential methylation is not observed in the embryo until the blastocyst stage and unlike most genomic imprints, iXi does not require the maintenance DNA methyltransferase Dnmt1 (Lewis et al., 2004; McDonald and Kay, 1997; Sado et al., 2000; Sado et al., 2004). It does, however, require repressive histone modifications such as H3K9me3 and H3K27me3 established by the polycomb group histone methyltransferases (Mak et al., 2002; Silva et al., 2003; Wang et al., 2001). These features are consistent with the theory that histone modifications are the

more conserved imprinting mark, as DNA methylation is not associated with imprinting phenomena in worms or flies for example, and yet epigenetic imprinting phenomena have been observed in these organisms (Bean et al., 2004; Lloyd, 2000; Sha and Fire, 2005). How and when iXi is initiated is a hotly debated topic with two main schools of thought. iXi is certainly established by the 4-cell stage, but whether iXi initiates upon zygotic genome activation and XIST expression in the embryo, or arrives at fertilization in a pre-inactivated state resulting from meiotic sex chromosome inactivation (MSCI) is less clear (Heard and Disteche, 2006; Huynh and Lee, 2003).

Meiotic Sex Chromosome Inactivation

In addition to random and imprinted X chromosome inactivation, a third form of X inactivation occurs in the germline of mammalian males, termed meiotic sex chromosome inactivation (MSCI). The X and Y sex chromosomes are unpaired in meiosis except for along a small homologous region; the pseudo-autosomal region (PAR). During the pachytene stage of spermatogenesis, the autosomes and the PAR pair and synapse. The unpaired X and Y regions are transcriptionally silenced and compartmentalized into a condensed structure termed the XY body. This MSCI persists throughout the rest of meiosis. Silencing of the X and Y continuing beyond meiosis into spermatids was reported by several groups and termed postmeiotic sex chromatin (Namekawa et al., 2006; Turner et al., 2006). MSCI was proposed to persist through fertilization, leading to an Xp inherited in a pre-inactivated state, and evidence of PMSC seemed strengthen this argument (Huynh and Lee, 2003). However, there are X-linked genes that are reactivated in postmeiotic cells (Mahadevaiah et al., 2009; Namekawa et al., 2006). This postmeiotic activation argues against repression being continuous

between MSCI and iXi. Additionally, apparent transcriptional activity from the Xp at the 2 cell stage further weakens the pre-inactivation argument (Okamoto et al., 2004). Nevertheless, more recent evidence has shown that initiation of iXi occurs in the absence of XIST expression and silencing of repetitive elements occurs by the two cell stage, independent of XIST (Kalantry et al., 2009; Namekawa et al., 2010). As XIST is not necessary to initiate iXi some other process must be at work, reopening the possibility that despite some postmeiotic reactivation of certain genes, the Xp may arrive in the embryo in a pre-inactivated state.

Sperm Chromatin

One of the final steps of spermatogenesis in many organisms is histones removal and replacement with highly basic proteins called protamines. Although the presence of histones in mature mammalian sperm has been demonstrated for some time, the replacement of histones with protamines was thought to be near complete, negating major consequences of histone or histone modification inheritance through the male gamete (Ward and Coffey, 1991; Wykes and Krawetz, 2003). While histone replacement with protamines in mammals and flies is extensive, histone replacement with protamines varies greatly among animals and even between genera of the same order (Frehlick et al., 2006). In *C. elegans*, GFP-tagged H3.3 histone variants can be visualized in mature sperm (Ooi et al., 2006). In the blue swimming crab *Portunus pelagicus*, H2B and H3 remain in sperm and histone replacement by protamines appears minimal. Sperm nuclei are not very condensed, and reports of the presence or absence of histones in other species of crab vary from complete loss to significant retention of histones (Chevaillier, 1967; Kurtz et al., 2009; Kurtz et al., 2008; Langreth, 1969; Stewart et al., 2010; Vaughn

and Thomson, 1972). During spermatogenesis in amphibians histone retention can occur as one of three variations. In *Rana catesbeiana* there is no evidence of histone loss or replacement. In *Xenopus laevis,* H2A and H2B are lost while H3 and H4 are retained and chromatin is compacted with the aid of protamine like sperm nuclear basic proteins. *Bufo marinus* sperm contain typical vertebrate protamines (Frehlick et al., 2006). Additionally, the model plant *Arabidopsis* contain an apparently high level of H3.3 in the male gamete (Ingouff et al., 2007). Most recently, a study in zebra fish has shown that there is no apparnt histone repacement during spermatogenesis, and histones retain bivaltent chromatin patterns at developmental loci, much like humans and mice (Wu et al., 2011).

Two groups recently demonstrated that histones, as well as modifications associated with both transcription and silencing, are retained in the mature sperm of mice and humans. Although histones are retained at low levels genome wide (4-10% in human sperm and <1% in mouse sperm) they are retained as nucleosomes at certain developmental promoters (Brykczynska et al., 2010; Hammoud et al., 2009). Hammoud et al. showed that H3K4me3 is enriched at HOX gene clusters, which regulate anterior-posterior body patterning in development, as well as at paternally expressed, but not paternally repressed, imprinted loci. Alternatively, H3K27me3 was enriched at developmental promoters repressed in early embryos. Within those H3K27me3-enriched loci there was a subset also modified with H3K4me3. Such bivalant domains largely overlapped with previously characterized bivalent promoters in embryonic stem cells (Bernstein et al., 2006; Brykczynska et al., 2010; Hammoud et al., 2009). Brykczynska et al. showed that H3K4me2 was enriched on spermatogenesis and housekeeping genes,

while H3K27me3 was enriched on developmental regulators such as HOX genes, and genes that are not expressed during male or female gametogenesis or preimplantation in the embryo (Brykczynska et al., 2010). An independent study showed by immunofluorescence that sperm derived histones may be incorporated into zygotic chromatin in humans (van der Heijden et al., 2008). These data along with evidence in other organisms of modest to significant inheritance of histones via sperm allows for the inheritance of histone modifications across generations that could allow for inheritance of epigenetic information.

Transgenerational Inheritance

Erasure of epigenetic information between parent and offspring is necessary for the transition from terminally differentiated gametes to the totipotent zygote. In mouse, Drosophila, and *C. elegans* erasure of epigenetic information in primordial germ cells between generations has been demonstrated, and likely exists in other organisms as well (Schaner et al., 2003; Seki et al., 2005; Seki et al., 2007). In order for gametic epigenetic inheritance to occur, epigenetic changes must be established in the parents germline, packaged into gametes and be resistant to erasure mechanisms normally in place in the embryo. Instances of loci that escape from erasure mechanisms include genomicly imprinted genes in mammals, mating type loci in yeast and paramutation in plants. However, resistance to erasure in these examples is not a trait acquired in the parents' lifetime. Therefore they do not occur in response to experiences of the parent and are not adaptive, but rather a normal part of development (Youngson and Whitelaw, 2008). Whether changes in chromatin and gene expression that occur during the parents' lifetime can be inherited with phenotypic consequences is less well established. Evidence of fetal

programming in humans where parental or even grandparental nutrition affects offspring health have been well documented, however, the molecular mechanisms behind this inheritance are as yet unknown (Bateson, 2001; Kaati et al., 2002; Kaati et al., 2007; Lumey, 1992; Pembrey et al., 2006; Stein and Lumey, 2000). Apparent transgenerational inheritance through the maternal line up to the F2 generation could be due to F1 prenatal exposure or even exposure of the F2 genome/epigenome as developing germ cells while the F1 is developing *in utero* (Youngson and Whitelaw, 2008). Studies showing epigenetic inheritance through the male line could therefore be more telling, however, even when examined in mice, the molecular mechanisms of gametic inheritance events are still unknown (Carone et al., 2010). One study of note demonstrated that during gonadal sex determination exposure to the endocrine disruptor vinclozolin leads to a variety of abnormalities in the offspring. These abnormalities are seen at high penetrance (90%) and are inherited through the male line out to at least three generations (Anway et al., 2005; Anway and Skinner, 2006). These studies are of note because abnormal DNA methylation was observed and inherited, suggesting a mechanism for inheritance of epigenetic marks over several generations (Anway et al., 2005; Anway and Skinner, 2006). A question still unanswered from these studies is how these marks escape epigenetic erasure over several generations.

C. elegans

Our research focuses on transgenerational epigenetic inheritance. As research in humans is impractical and unethical, and even model systems such as mouse have a long life cycle limiting their utility in generational studies, we chose *C. elegans* as our model system. *C. elegans* is a 1mm long non-parasitic nematode (roundworm). Their relatively

short three-day life cycle along with their large brood size (between 250-350 offspring per hermaphrodite) makes them particularly amenable to generational research questions. Upon hatching, *C. elegans* proceed through four larval stages (L1-L4) prior to developing into adults and typically live around 2 weeks. Their genome is comprised of 6 holocentric chromosomes, five autosomes and one sex chromosome, the X, making genetic and chromosomal cell biology approachable. Additionally, there is no reported DNA cytosine methylation, simplifying studies on chromatin and epigenetics. *C. elegans* has two sexes, males and hermaphrodites and sex is determined by the number of X chromosomes inherited; hermaphrodites have two X's while males have only one. Dosage compensation of the X's in hermaphrodite *C. elegans* is achieved by down-regulating expression from both X chromosomes by half. Hermaphrodites can mate with males and are also self-fertile as they make sperm as L4 larvae, and then switch exclusively to oogenesis as adults. In hermaphrodites, sperm are stored in the spermatheca where oocytes are fertilized in adults. Males only make sperm and therefore require hermaphrodites to reproduce.

Germ cells are the only players in the transfer of epigenetic information between and across generations. Studying germ cells in *C. elegans* is particularly attractive due their high number and cytological accessibility in adults, and the fact that the entire cell lineage including the germline has been mapped (Sulston et al., 1983). From fertilization, the one cell zygote divides asymmetrically to give rise to the AB somatic blastomere and the germ cell precursor P1. The embryonic germline continues to divide asymmetrically for three subsequent cell divisions, giving rise to a somatic blastomere and a P-lineage cell at each division until P4 is born. P4 then divides symmetrically to

give to rise to the primordial germ cells Z2 and Z3 (Figure 1.2). The P-lineage is transcriptionally repressed by the maternally supplied protein Pie-1 (Seydoux and Dunn, 1997). Z2 and Z3 are maintained in a transcriptionally repressed state by chromatin-based mechanisms until they re-enter the cell cycle at hatching (Furuhashi et al., 2010; Schaner et al., 2003).

Germ cells develop within the somatic germ line, which is comprised of two U-shaped ovary arms in the hermaphrodite and a single testis in the male (Figure 1.3). *C. elegans* is an excellent model system for studying germ cell to zygote transitions as germ cells comprise over half the cells in an adult animal; adults have 959 somatic cells and over 1000 germ cells are produced by each gonad arm. The gonads are syncytial; nuclei are partially enclosed by the plasma membrane, but share a contiguous cytoplasm. Gametogenesis proceeds in linear fashion through the gonad from distal to proximal, allowing for simultaneous examination of each stage of germ cell maturation in a single animal. At the distal end the somatic distal tip cells create a stem cell niche under the control of the Notch-like receptor GLP-1 (Kimble and Crittenden, 2007; Kimble and Simpson, 1997). When germ cells lose contact with the distal tip cells or Notch signaling is otherwise disrupted, they enter meiosis. During male meiosis in L4 larval hermaphrodites and in males, the germ cells progress through the pachytene stage of meiotic prophase I, where they transition into 4N primary spermatocytes, then divide to give rise to 2N secondary spermatocytes, followed by rapid progress through meiosis II giving rise to 1N spermatids. Male gamets are stored as spermatids in the seminal vesicle until mating in males, and as spermatozoa in the spermatheca in hermaphrodites. In hermaphrodites, meiotic cells proceed through pachytene, diplotene and are held in

diakinesis in mature oocytes until fertilization, which occurs after ovulation into the spermatheca. Embryos then begin the germline cycle again, re-establishing the P-lineage in the first asymmetric division of the zygote.

Upon fertilization the two parental pronuclei are not equivalent at the chromatin level, even in self-fertilized offspring of hermaphrodites. Our lab has previously shown that the X chromosome that passed through spermatogenesis, whether in a male or a hermaphrodite, lacks active histone modifications on histone H3 (H3K4me2, H3K9Ac, H3K14Ac) while accumulating diacetylation on histone H4. In contrast, the maternally inherited Xm chromosome, as well as all of the autosomes, are intensely decorated with active histone modifications, even at the one cell stage when transcription has not yet initiated. This paucity of these active histone H3 modifications on the Xp persists through several rounds of cell division in the early embryo, but seems to weaken through subsequent cell divisions, so that the Xp is indistinguishable from the other chromosome with regards to active histone modifications beyond the 24-cell stage (Bean et al., 2004). The persistent absence of active histone modifications on the Xp is what is referred to as a chromatin based imprint in *C. elegans*. The means by which this imprint is established in the parental germ cells and further maintained in the embryo is the focus of my dissertation.

Rationale

I aim to determine how the Xp imprint is established in *C. elegans*. I first proposed that histone variants or modifications incorporated during spermatogenesis could render the Xp refractive from acquiring active histone modifications in the early embryo. While demonstrating that a range of histone variants was not required for

establishment of the imprint, and that certain histone modifications are the same on both the Xp and the rest of the chromosomes (Chapter 2), I made the following observations. 1) Histones and histone modifications are maintained in mature sperm. 2) Genomic segments sharing the transcriptional quiescence characteristic of the Xp while passing through gametogenesis are similarly imprinted, while those regions that are transcriptionally active during gametogenesis acquire and maintain active histone modifications. 3) Inherited chromatin environments may impact embryonic expression (Chapter 3). From these observations we went on to test whether similar observations could be made with regards to the expression of imprinted non-coding RNAs in mammals (Chapter 4).

The X chromosome in *C. elegans* is noticeably depleted of germline essential and spermatogenesis specific genes (Reinke et al., 2000). The inactive state of the X chromosome in male meiosis, and maintenance of that state during spermatogenesis, may be a passive consequence of the lack of genes required for meiosis or spermatogenesis from this chromosome. Indeed, genes with demonstrated functions in meiosis and germline sex determination in both sexes are absent from the X, as are genes that have been either defined as exhibiting sperm-enriched expression, or mapped mutations causing sperm defects (*spe;* Table 1.3; (Reinke et al., 2000). Furthermore, cell cycle genes that are not germline specific but are important for germ cell proliferation are also absent from the X (Table 1.3). Finally, a number of examples have been reported in which essential loci with X and autosomal linked paralogs exhibit germ-cell specific defects when only the autosomal copy is defective, suggesting only the autosomal copy is active in germ cells (L'Hernault and Arduengo, 1992; Maciejowski et al., 2005). Thus,

the consensus of published data suggests there is little need for transcription from the X chromosome during spermatogenesis.

In this thesis I demonstrate that imprint establishment is not unique to the paternally inherited X chromosome, but rather is characteristic of large genomic fragments that pass through gametogenesis in a transcriptionally inactive state. More interestingly, chromatin that was transcriptionally active in the parent's germline, whether it be a transgene or an entire chromosome accumulates active histone modifications in the germline. These modifications can be packaged into mature gametes and carried into the embryo where the inherited histone modification patterns can be maintained and perhaps propagated in the absence of transcription in the early embryo as well as possibly influencing gene expression in the offspring.

Significance

Evidence for inheritance of epigenetic modifications across generations is implied, but direct mechanisms for this inheritance are not well established. The imprinting phenomenon in *C. elegans* is a useful model system for studying epigenetic inheritance as the fundamental elements of this phenomenon, histones and histone modifying machinery, is highly conserved. Epigenetic regulation plays a pivotal role in mammalian imprinting and gene regulation throughout the genome. When these processes are disrupted diseases such as cancer and Prader-Willi/Angelman Syndrome, as well as other imprinting diseases can result. Studying the imprinting phenomena in *C. elegans* will further our understanding of the mechanisms involved in these processes, expand our knowledge of chromatin regulation in general, and provide a foundation that could lead to treatment of disease in the future.

Table 1.1 Comparison of Histone H3 variants. Canonical histone H3, H3.2, associated marks, timing of expression and chaperon is compared other histone H3 variants, H3.1, H3.3 and human CenH3, CENPA. Amino Acid changes are a comparison of the human proteins.

Table 1.1 Comparison of Histone H3 Variants

	Associated with	Expression/incorporation	Chaperone	Amino Acid Changes
H3.2	Heterochromatin K27me2/3, K9me2/3	S-phase	Caf-1	canonical
H3.1	Heterochromatin K27me2/3, K9me2/3	S-phase	Caf-1	96: S → C
H3.3	Euchromatin Transcription H3K4me2/3 K9Ac K14Ac Heterochromatin Inactive X	S-phase Throughout cell cycle Fertilization	HIRA Daxx	31: A → S 87: S → A 89: V → I 90: M → G
CENPA	Centromere			50% identity 63% homology

Table 1.2 Comparison of Histone H2A variants. Canonical histone H2A associations and amino acid conservation is compared with other histone H2A variants, H2A.Z, H2A.Bbd and MacroH2A. Amino Acid changes are a comparison of the human proteins.

Table 1.2 Comparison of H2A Variants		
	Associated with…	Amino Acid comparison with H2A
H2A		canonical
H2A.Z	Active transcription Pericentric heterochromatin	65% identity 76% homology
H2A.Bbd	Excluded from inactive X Destabilizes nucleosomes Active genes Expressed in testis	49% identity 70% homology
MacroH2A	Heterochromatin Inactive X DNA methylation	Large (254 amino acid) unique C-terminal Macro Domain N-terminal 66% identity 75% homology

Table 1.3. Chromosomal Distributions of Genes that Express in Germ Cells

Chromosomal linkages of common germline expressing genes (assembled from Wormbase.org) are restricted to autosomes. None appear on the X with one exception; cdk-4 is only required for larvae.

Table 1.3 Germline-expressing loci chromosomal linkage

Spermatogenesis genes (45)	Linkage Group	**Meiosis genes (53)**	Linkage Group	**Cell cycle genes (26)**	Linkage Group	**Germline sex determination (21)**	Linkage Group
fer-1	I	air-2	I	cdc-25.1	I	fbf-1	II
fog-3	I	atl-1	V	cdk-1	III	fbf-2	II
gsp-3	I	brc-1	III	cdk-4	X*	fem-1	IV
gsp-4	I	brc-2	III	cdk-7	I	fem-2	III
htas-1	IV	chk-1	V	cep-1	I	fem-3	IV
k08c7.3	IV	chk-2	V	cki-1	II	fog-1	I
msp-142	II	dpy-28	III	clk-2	III	fog-2	V
msp-19	IV	dyp-26	IV	cul-1	III	fog-3	I
msp-31	II	glp-3	III	cyd-1	II	gld-1	I
msp-33	II	him-1	I	cye1	I	gld-3	II
msp-40	II	him-10	III	dpl-1	II	her-1	V
msp-45	II	him-11	III	efl-1	V	mog-1	III
msp-49	II	him-12	IV	emb-27	II	mog-2	II
msp-50	III	him-13	I	emb-30	III	mog-3	III
msp-53	IV	him-14	II	hus-1	I	mog-4	II
msp-55	IV	him-15	III	lin-23	II	mog-5	II
msp-57	IV	him-2	I	lin-35	I	mog-6	II
msp-77	IV	him-3	IV	lin-36	III	nos-3	II
msp-79	IV	him-5	V	lin-9	III	tra-1	III
msp-81	IV	him-6	IV	mat-1	I	tra-2	II
smz-1	IV	him-7	V	mat-2	II	tra-3	IV
smz-2	I	him-8	IV	mat-3	III		
spch-1	IV	him-9	II	mdf-1	V		
spch-2	I	hop-1	I	mrt-2	III		
spch-3	I	htp-1	IV	san-1	I		
spe-1	II	htp-2	IV	wee-1.2	II		
spe-10	V	htp-3	I				
spe-11	I	mei-1	I				
spe-12	I	mei-2	I				
spe-13	I	mes-2	V				
spe-14	IV	mex-1	II				
spe-15	I	mex-3	I				
spe-16	III	mre-11	V				
spe-17	IV	msh-2	I				
spe-19	V	msh-4	II				
spe-2	II	msh-5	IV				
spe-3	II	msh-6	I				
spe-4	I	pie-1	III				
spe-5	I	rad-51	IV				
spe-6	III	rad-54	I				
spe-7	II	rec-8	IV				
spe-8	I	smc-2	II				
spe-9	I	smc-3	III				
ssp-10	IV	spo-11	IV				
Y59e9ar.1	IV	sun-1	V				
		syp-1	V				
		syp-2	V				
		syp-3	I				
		zhp-3	I				
		zim-1	IV				
		zim-2	IV				
		zim-3	IV				
		zyg-12	II				

Figure 1.1 Nucleosome Schematic and Histone H3 N-terminal Modifications (A) Schematic of a typical nucleosome is shown with canonical histones H2A (blue), H2B (orange), H3 (green), and H4 (pink), globular domains, DNA (red) and linker histone H1 (gray). N-terminal histone "tails" protrude from the nucleosome and can be highly modified. (B) N-terminal histone H3 modifications examined in this work: Amino acid residues modified are in black below the tail. Circles represent possible modifications at that residue. Methylation (blue), Acetylation (purple), Phosphorylation (green)

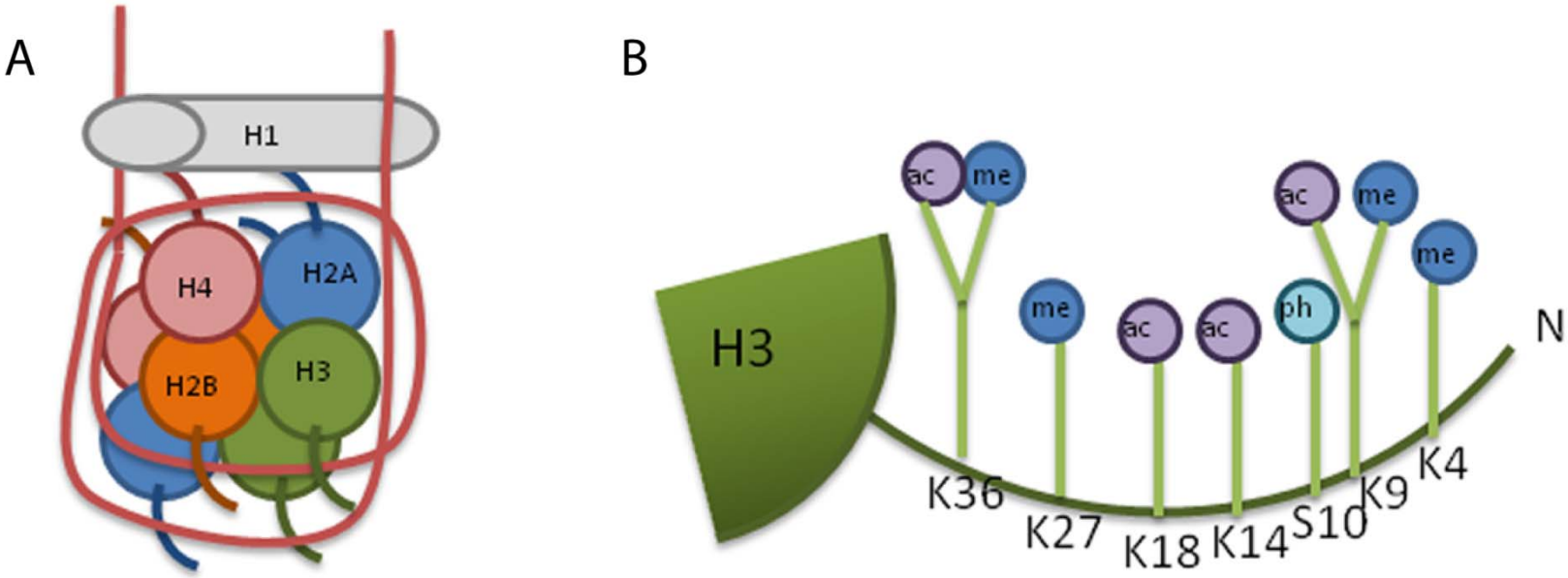
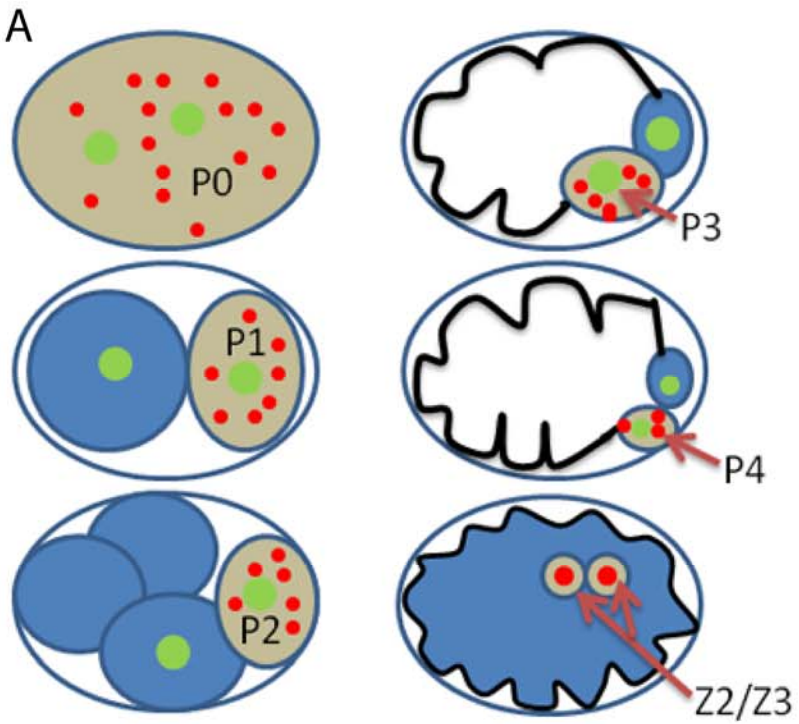

A
H1
H4
H2A
H2B
H3
B
H3
ac
me
me
ac
ac
ph
me
me
N
K36
K27
K18
K14
S10
K9
K4

Figure 1.2 Germ cell lineage and histone modifications in embryogenesis (A) Asymmetric cell divisions give rise to the primordial germ cells Z2/Z3. P-cells (P0-P4 gray) are primordial germ cell precursors. Transcription is repressed in P-cells by pie-1 (red). P-cell nuclei are decorated with H3K4me2 (green). Pie-1 and H3K4me2 are lost in the nuclei of Z2/Z3. (B) H3K4me2 is present in both somatic lineages and germ cell precursors prior to the birth of Z2/Z3. In primordial germ cell precursors (P0-P4) transcription is repressed by pie-1. In Z2/Z3 Pie-1 is degraded and H3K4me2 is lost.


A
P0
P1
P2
P3
P4
Z2/Z3

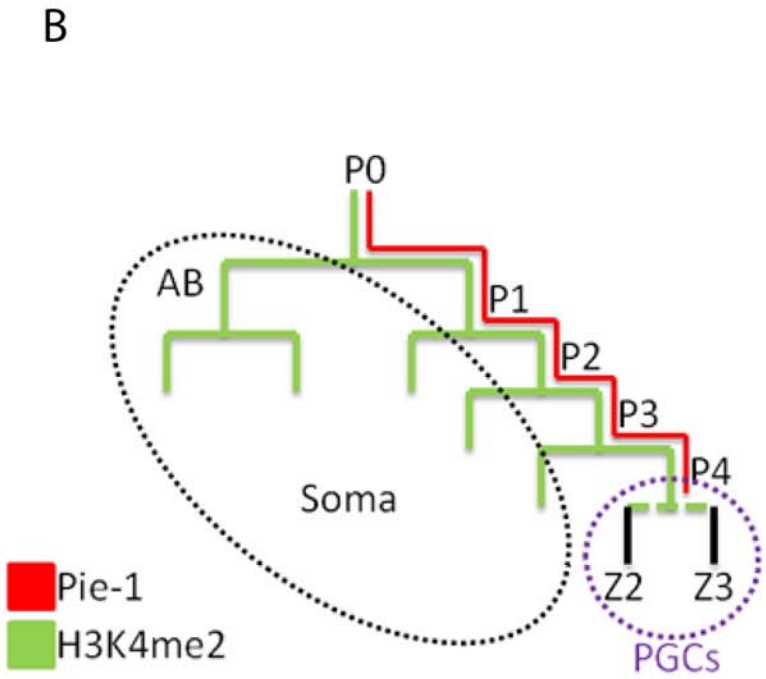
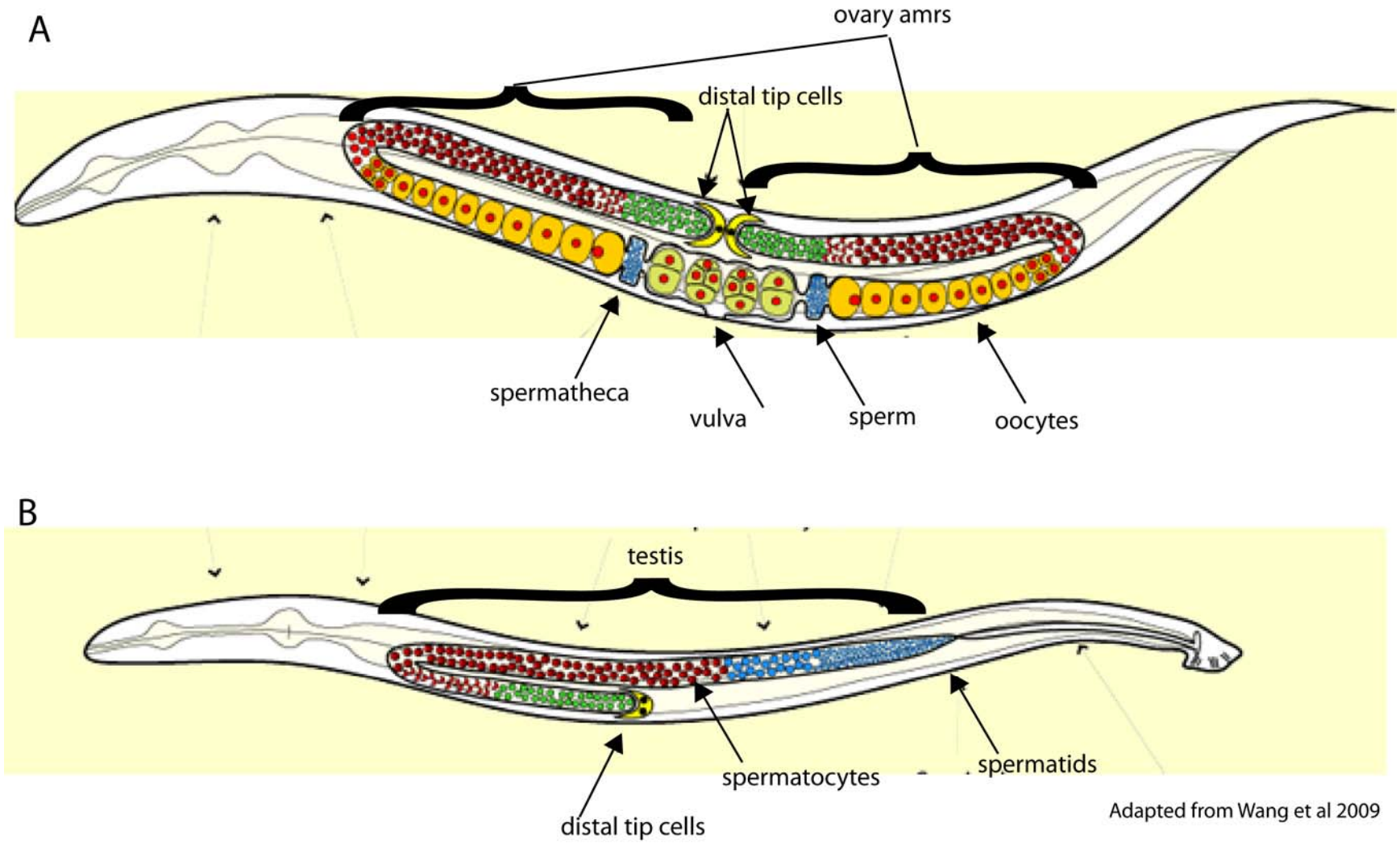

B
P0
AB
P1
P2
P3
P4
Soma
Z2
Z3
PGCs
Pie-1
H3K4me2

Figure 1.3 *C. elegans* germline anatomy Adapted from (Wang et al., 2009) (A) Adult hermaphrodite with two U-shaped ovary arms. Distal tip cells (yellow). Mitotically dividing germ cell nuclei (green). Meiotic germ cell nuclei (red). Diakinetic oocytes (yellow). Sperm, produced as larvae, are stored in the spermatheca (blue). Embryos (light green). (B) Adult male. Distal tip cells (yellow). Mitotically dividing germ cell nuclei (green). Meiotic germ cell nuclei (red). Postmeiotic sperm (blue).


A
ovary amrs
distal tip cells
spermatheca
vulva
sperm
oocytes
B
testis
spermatocytes
spermatids
distal tip cells
Adapted from Wang et al 2009

Chapter 2

Examining the Role of Histone Variants and Histone Modifications in Imprint Establishment in *C. elegans*

INTRODUCTION

X inactivation, the silencing of an X chromosome to achieve dosage compensation in female mammals, is an epigenetic event, in that the silencing is dependent on mechanisms other than DNA sequence. Epigenetic phenomena, as discussed in Chapter 1, include DNA methylation, non-coding RNAs, histone modifiers and remodelers, histone modifications and histone variants. Of these, the ncRNAs *Xist* and *Tsix* are well established as playing key roles in X inactivation in the mammalian embryo and roles for histone variants and histone modifications in X inactivation are emerging as well. There are two types of X inactivation in placental mammals. Imprinted X inactivation (iXi) is the preferential silencing of the paternally inherited X chromosome. iXi is initiated by as early as the two cell stage at some loci and chromosome wide by the four-cell stage and persists in the extra-embryonic tissue (Namekawa et al., 2010; Okamoto et al., 2004). iXi is lost in the inner cell mass of the embryo, and replaced by random inactivation of either X chromosome in the embryo proper. The first well-characterized step in both iXi and random X inactivation is *Xist* expression. The ncRNA *Xist* is expressed preferentially from the X chromosome that will be silenced; the Xp in iXi and either X randomly in random X inactivation. *Xist* goes on to coat the inactive X chromosome silencing it in *cis* (Penny et al., 1996). Subsequently, active chromatin modifications such as H3K4me and H3K9Ac are lost (Heard et al., 2001). This is followed by hypoacetylation of H3K4, and enrichment of marks associated with heterochromatin such as H3K27me3, H3K9me2, H4K20me1 and H2AK119Ub (de Napoles et al., 2004; Heard et al., 2001; Kohlmaier et al., 2004; Plath et

al., 2003; Silva et al., 2003). However, mechanisms establishing imprints in mammals, or any organism, remain to be identified.

The inactive X chromosome in mammals has a distinct chromatin environment with regards to histone modifications and histone variant makeup. Typical of silent chromatin, the inactive X chromosome is hypoacetylated on nearly all histones as well as being hypomethylated on H3K4 (Boggs et al., 2002; Heard et al., 2001; Jeppesen and Turner, 1993). iXi shares many similarities with genomic imprinting, where allelic gene expression is determined based on parent of origin, and the two are often paralleled. Based on the association between iXi and genomic imprinting, as well as the fact that hypomethylation of H3K4 is one of the first chromatin signatures of the inactive X, it is interesting to note that establishment of maternal genomic imprints requires KDM1B, a H3K4 demethylase (Ciccone et al., 2009). However a direct link between active H3K4 demethylation and iXi has not been demonstrated. The inactive X chromosome is enriched with and in fact requires heterochromatic markers H3K9me2 and H3K27me3, as well as components of the polycomb H3K27 histone methyl transferase complexes (Mak et al., 2002; Silva et al., 2003; Wang et al., 2001). DNA methylation on the inactive X chromosome is one of the final events of random X inactivation, and is not required for iXi in the eutherian trophectoderm or in marsupials (Heard and Disteche, 2006). Additionally, with H3K27me3 and H3K9me2, iXi can persist in the absence of the DNA maintenance methyl transferase, Dnmt1 (Mak et al., 2002; Plath et al., 2003; Silva et al., 2003; Wang et al., 2001).

H3K27me3 established by polycomb repressive complex 2 (PRC2) is capable of recruiting the Polycomb repressive complex 1 (PRC1) (Sarma et al., 2008). PRC1

catalyzes ubiquitination of H2A on lysine 119 (UbH2A) in mammals (Wang et al., 2004). The E3 ligase responsible for ubiquitination in PRC1, Ring1B, as well as UbH2A itself are transiently enriched on both the imprinted inactive X in trophoblast stem cells, as well as the randomly inactivated X chromosomes in differentiating embryonic stem cells (Fang et al., 2004). This transient association, along with the accumulation of UbH2A upon induction of a transgene expressing *Xist*, implied that UbH2A could play a role in initiation of X inactivation (de Napoles et al., 2004). However, it was subsequently shown that neither initiation nor maintenance of silencing at an inducible *Xist* transgene requires Ring1B/UbH2A (Leeb and Wutz, 2007). One further modification, H4K20me1, catalyzed by the HMT Prset7, coincides with *Xist* expression on an inducible *Xist* transgene as well (Kohlmaier et al., 2004; Oda et al., 2009). A summary of histone modifications and histone variants associated with the inactive X chromosome in mammals is provided in Table 2.1.

In addition to a unique chromatin modification signature, the inactive X chromosome has a unique histone variant makeup. Both the imprinted and random inactive X chromosomes are enriched for the H2A variant MacroH2A (Costanzi and Pehrson, 1998; Costanzi et al., 2000). MacroH2A has a large C-terminal domain, which inhibits transcription initiation *in vitro* and is associated with heterochromatin (Costanzi et al., 2000; Doyen et al., 2006). Further suggesting a role in gene silencing, MacroH2A is found at DNA-methylated inactive imprint control regions in genomic imprinting loci (Choo et al., 2006; Choo et al., 2007). While MacroH2A is enriched on the inactive X, its presence is not required for maintenance of X inactivation and is unlikely to be required for initiation of X inactivation, as imprinted X inactivation begins by the two or

four-cell stage depending on the loci, prior to when Macro H2A is detected, beginning at the eight-cell stage (Csankovszki et al., 1999).

A histone H3 variant, H3.3, can be expressed outside of S-phase, and incorporated into chromatin independent of replication. H3.3 was initially thought to be involved in enhancing an active chromatin environment, due to it's enrichment at active genes, promoters, and regulatory elements, and its ability to be incorporated outside of S-phase (Ahmad and Henikoff, 2002). Recently, a more nuanced view of this variant suggests it may have important roles independent of gene activation, in development and heterochromatin. H3.3, as well as its chaperone HIRA and histone remodeler CHD1 are required for paternal pronuclear decondensation and fertility in flies (Hodl and Basler, 2009; Loppin et al., 2005; Sakai et al., 2009). H3.3, its classic chaperone HIRA, as well as its recently described chaperone Daxx, localize to the transcriptionally silent XY body during mammalian spermatogenesis (Rogers et al., 2004; van der Heijden et al., 2007). Daxx exists in a complex with the SNF2 family chromatin remodeler ATRX (Lewis et al., 2010). ATRX is found on both the imprinted and random inactive X chromosome, and iXi is lost in the absence of ATRX (Baumann and De La Fuente, 2009; Garrick et al., 2006). This association, along with the recent data showing H3.3 at telomeres and pericentric heterochromatin suggest that H3.3 may play a role in X inactivation (Drane et al., 2010; Goldberg et al., 2010; Lewis et al., 2010; Wong et al., 2009). Enrichment and absence of histone variants on the mammalian inactive X are summarized in Table 2.1.

Imprinted X inactivation (iXi) in *C. elegans* is a chromatin-based phenomenon, where active histone modifications such as H3K4me2 and acetylation on histone H3 are absent from the paternally inherited X chromosome (Xp) in the early embryo. At the

outset of this project my goal was to further characterize chromatin modifications associated with the inactive X chromosome in the germline and embryo, reasoning that histone modifications established in the parents' germline, if carried to the zygote, could impact acquisition of histone modifications on the Xp in the embryo. I also examined whether enrichment of a specific histone variant on the X chromosome during spermatogenesis could be responsible for the Xp's apparent resistance to acquiring active histone modifications in the early embryo as well.

Based on the unique chromatin signature of the inactive X in mammals, we first investigated whether histone variants or additional histone modifications could establish the unique chromatin environment of the paternally inherited X chromosome. We observed that, in addition to the active histone modifications previously described, tri-methylation on lysine 4 of histone H3 (H3K4me3) was also absent from the Xp. We also found that unmodified H3K4 (H3K4me0), was enriched on the X during spermatogenesis and the paternal pronucleus, but this enrichment did not persist through the first cell division. Similarly, differences in H3.1 and H3.3 accumulation on the X during spermatogenesis were observed, however, these differences did not persist upon fertilization. Additionally, all other histone variants examined did not impact the presence of the imprint in *C. elegans*. Other histone modifications examined were either the same among autosomes, the maternally inherited X (Xm) and the Xp, or were the same between the Xm and the Xp, while different from the autosomes, and therefore these modifications do not play an obvious role in imprint establishment in *C. elegans*. These results suggested that the imprint is established by means other than chromatin

modifications, and that the paucity of H3K4me2/3 and H3K9/14Ac are a consequence of another mechanism establishing the imprint, which we explored further in Chapter 3.

RESULTS

Characterization of Histone H3 Variants in the Germline and Early Embryo

As discussed in the introduction, the histone H3 variant, H3.3 can be expressed outside of S-phase, and in many studies correlates with transcriptional activity. However associations of H3.3 with heterochromatin and the inactive X in mammals, as well as a specific role for this variant in development and heterochromatin formation, have recently been described (Baumann and De La Fuente, 2009; Drane et al., 2010; Garrick et al., 2006; Goldberg et al., 2010; Lewis et al., 2010; Rogers et al., 2004; van der Heijden et al., 2007; Wong et al., 2009). The *C. elegans* genome includes two H3.3s and several H3.3-like variants (Table 2.2). H3 variants HIS-71 and HIS-72 have the conserved H3.3-specific residues AIG at positions 87, 89, and 90, respectively, as well as a potentially phosphorylateable threonine at position 31, which is a serine in other H3.3s (Ooi et al., 2006). Residues 87-90 have been demonstrated to be important for replication-independent incorporation of H3.3, and may be responsible for the chaperone HIRA's higher affinity for H3.3 over H3.1 (Ahmad and Henikoff, 2002). *his-69, his-70, his-73,* and *his-74* have also been identified as H3.3-like variant genes (Pettitt et al., 2002). The presence of HIS-69, HIS-70, HIS-71, HIS-72 and HIS-74 protein were detected in both spermatogenic and oogenic germlines by a proteomic screen (Chu et al., 2006). Although not identified in the proteomics screen and characterized as a pseudogene, we also observed sperm specific expression of *his-73*, which has a highly divergent N-terminal

domain and conserved histone fold domain. We first asked whether a histone variant enriched on the X chromosome during spermatogenesis could be responsible for the unique chromatin structure of the Xp. Several variants were particularly attractive as they either lacked lysine 4 or had amino acid changes adjacent to lysine 4, which could perhaps render them resistant to acquiring any methylation on that residue (Table 2.2).

To test for a requirement for these H3 variants in X imprint establishment, we tested deletion mutations of *C. elegans* H3.3-related variants *his-69* and *his-70*. No defects in X chromatin dynamics were observed in either the adult germline or embryo (Figure 2.1 A-Bi). Additionally, *his-69* was confirmed to be a pseudogene by RT-PCR, as previously reported (Figure 2.1 G and (Ooi et al., 2006)). *his-73* was also annotated as a pseudogene, however a *his-73* transcript is detected by RT-PCR (Figure 2.1 G), and preliminary studies showed *his-73* transcript in spermatocytes and a HIS-73 antibody showed staining in the same region (C. Bean unpublished). A GFP-tagged construct of *his-73* with its native promoter initially showed a spermatogenesis specific expression pattern (Figure 2.1 E). With its highly divergent N-terminal domain, lacking a lysine at position 4, this variant seemed a likely candidate for a sperm specific histone variant that could specifically be enriched on the Xp. However, there was no evidence for the presence of the GFP-tagged HIS-73 histone on the Xp or any other chromosomes in the early embryo by immunofluorescence (Figure 2.1 D). Additionally, animals that were homozygous mutation for a *his-73* (*tm1643*) deltion, which takes out most of a large portion of the unique N-terminal region are viable, although with a reduced brood size (T. M. Edwards and W. G. Kelly, unpublished), and the imprint is intact in these animals (Figure 2.1 C). *his-74* was not pursued; due to its high homology to H3 it was not

anticipated to have any unique variant properties influencing the imprint. These H3.3 variants are thus unlikely to be specifically accumulating on the X chromosome or contributing to imprint establishment during spermatogenesis.

We also examined the localization of the *C. elegans* centromeric H3 variant HCP-3. In eukaryotes centromeres are characterized by enrichment for Centromere-specific H3 histones (CenH3s) essential components of the kinetochore (Ekwall, 2007). *C. elegans* chromosomes are holocentric; they do not have a focused centromeric constriction and microtubules attach along the length of the chromosome during mitosis (Albertson and Thomson, 1993). The functional CenH3 in *C. elegans* is HCP-3, is an H3-like variant that decorates mitotic chromosomes and is necessary for proper chromosome segregation (Buchwitz et al., 1999). Like H3.3, CenH3 incorporation is independent of DNA replication, although the exact timing varies among organisms (Torras-Llort et al., 2009). Although the C-terminal histone-fold core region of CenH3 is well conserved, the N-terminal region is highly divergent (Torras-Llort et al., 2009). Based on its characterization as a histone variant and it's highly divergent N-terminal domain, HCP-3 could play a unique or additional role in chromatin formation in *C. elegans*, independent of or in addition to its function in chromosome segregation. We therefore asked whether the Xp could be distinguished by enrichment of HCP-3. Anti-HCP-3 antibody did not show enrichment or depletion from any particular chromosome in either the germline or the embryo (Figure 2.1 F).

We next tested whether there was a difference in H3.1 dynamics on the Xp relative to other chromosomes. We obtained a monoclonal antibody, which can specifically recognize canonical histone H3.1, by recognition of the alanine at position

31, which is a serine on H3.3s in mammals and a threonine in H3.3 in *C. elegans* (Table 2.2). We observed that H3.1 was enriched on the X during pachytene of both sexes and this enrichment continued during spermatogenesis into mature sperm (Figure 2.1 H). The difference between H3.1 levels on the Xp and the autosomes in sperm was lost after fertilization (Figure 2.1 I). In contrast, H3.1 was depleted from all chromosomes during the course of oogenesis, and was detected at very low levels in mature oocytes. Little to no H3.1 was detected in early embryos until they had gone through several rounds of replication (Figure 2.1 I).

To complement our H3.1 antibody studies, we examined a strain expressing a GFP-tagged version of *C. elegans* H3.3, HIS-72, which we obtained from the Henikoff lab (Ooi et al., 2006). Reciprocal of our H3.1 data, H3.3::GFP did not accumulate on the X at high levels during pachytene in either spermatogenesis or oogenesis. H3.3::GFP became enriched on all chromosomes, including the X during oogenesis, but did not accumulate on the X at any point during spermatogenesis ((Ooi et al., 2006) and Figure 2.1 J). In contrast to H3.1, H3.3::GFP decorated all chromosomes shortly after fertilization ((Ooi et al., 2006)and Figure 2.1 K). The absence of any significant differences in the levels of H3.1 and H3.3::GFP on the Xp compared to other chromosomes in the early embryo suggest that these variants do not play a substantial role in imprint establishment in *C. elegans*. The implications of these results are further discussed within the context of Chapter 3. These experiments failed to identify any histone variants that may be playing a significant role in imprint establishment in *C. elegans*.

Examination of Histone Modifications in the Germline and Early Embryo

Active Histone Modifications H3K4me3, H3K18Ac, and H3K36me3

As histone variants did not seem to play a role in iXi establishment, we chose to further characterize histone modifications that could potentially influence iXi. The imprint previously described is characterized by the absence of active histone modifications H3K4me2, H3K9Ac, and H3K14Ac. We further examined active histone modifications on the X chromosome during gametogenesis and early embryogenesis.

While H3K4me2 methylation patterns largely overlap, H3K4me3 is more strongly correlated with ongoing transcription and is enriched at transcription start site of expressed genes, whereas H3K4me2 is found in the coding region and may reside at euchromatic genes that are not being expressed (Liu et al., 2005; Pokholok et al., 2005). We examined H3K4me3 patterns in the germline and early embryo, and did not observe a difference between these two marks. H3K4me3 was scarce on the X chromosome in pachytene in both sexes, accumulated on the Xm during oogenesis, and was absent from the Xp while robustly present on the Xm and autosomes in the early embryo (Fig 2.2 A and B).

H3K18 Acetylation, like other acetyl histone marks is correlated with active chromatin. Like other active histone modifications, this mark was low on the X chromosome during pachytene in both sexes (Figure 2.2 C). Unexpectedly, in the early embryo all chromosomes seemed to be equally decorated with this modification (Figure 2.2 D).

The HMT SET2 mediates H3K36 methylation and associates with elongating RNA polymerase II phosphorylated on serine 2 of the hepta-peptide repeat (Krogan et al.,

2003; Xiao et al., 2003). Due to its association with active RNA polymerase II, H3K36 methylation is closely associated with active chromatin. The SET2 homologue mediating H3K36me2 in the *C. elegans* germline is MES-4 and the methyltransferase as well at H3K36me2 is concentrated on the autosomes and excluded from the X in oogenesis, spermatogenesis, and the early embryo (Bender et al., 2006). We examined H3K36me3 in the germline and early embryo and also noted that this mark was enriched on autosomes and not the X during gametogenesis of either sex (Figure 2.2 E). In the early embryo, both X chromosomes had lower levels of H3K36me3 than autosomes, however one of the X chromosomes may have an intermediate level of H3K36me3 (Figure 2.2 F). I speculate this is the maternally inherited Xm, from which some oogenesis genes are expressed after pachytene, possibly allowing for a low accumulation of this mark during oogenesis, and inheritance by the early embryo.

Ubiquitination

Ubiquitination of H2A on lysine 119 (UbH2A), is established by the PCR1 complex, comprised of polyhomeotic (Ph), polycomb (Pc), Psc (posterior sex combs) and Ring1b, the E3 ubiquitin ligase, and is strongly linked to gene silencing in mammals (de Napoles et al., 2004; Wang et al., 2004). UbH2A blocks H3K4me2/3 mediated by the HMT MLL-3, while de-ubiquitination allows H3K4 to be methylated by MLL-3 (Nakagawa et al., 2008). However chromatin containing UbH2A along with an H3 with a lysine to arginine mutation at position 4 (H3K4R) can be transcribed, suggesting along with the above data, that UbH2A may block transcription indirectly by preventing H3K4me2/3 (Nakagawa et al., 2008). UbH2A is also enriched on the inactive X in mammals, as discussed above. Based on this information we wanted to ask whether

enrichment of UbH2A could be preventing the accumulation of H3K4me2/3 on the Xp in *C. elegans*. While there is no clear Ubiquitin E3 ligase homologues in *C. elegans* UbH2A is present in *C. elegans*, perhaps by a non-homologous E3 (de Napoles et al., 2004). We examined UbH2A by immunofluorescence. Although UbH2A antibody staining was present in the germline, it did not overlap with DAPI dense regions of pachytene nuclei, nor was it enriched on the Xp (Figure 2.3 A). We were unable to detect any UbH2A on DNA in early embryos (Figure 2.3 B). Based on these data we did not find any role for UbH2A in preventing H3K4me2/3 on the Xp.

In yeast H2B ubiquitination by the ubiquitin E3 ligase Rad6/Bre1 is required for H3K4me by the HMT complex COMPASS (Dover et al., 2002; Sun and Allis, 2002; Wood et al., 2003). In *Arabidopsis*, mutations in the de-Ubiquitination enzyme SUP32/UBP26 lead to a decrease in H3K9me2, an increase in H3K4me3, and inappropriate release of silencing (Sridhar et al., 2007). In yeast a mutation in the ubiquitin hydrolyase Ubp8, which de-ubiquitinates H2B leads to an increase in H3K4me3 (Henry et al., 2003). In general H2B ubiquitination is correlates with active transcription (Zhang, 2003). Based on this information we hypothesized that a de-ubiquitination enzyme targeted to the Xp during spermatogenesis could prevent it from acquiring active chromatin marks like H3K4me2/3. As no ubiquitin H2B antibody was commercially available at the time of these studies, we began to examine this question with a pan-ubiquitin antibody, to see if ubiquitin was absent from the Xp or not. However, the pan-ubiquitin antibody proved uninformative, as we had high non-specific background staining throughout the animal as well as the germline and embryos (Figure 2.3 C and D). We next tried to address this question by examining animals with a deletion of the *C.*

elegans E2 ubiquitin ligase homologue *ubc-1*, with the rationale that if this enzyme is typically excluded from the Xp and then if removed H3K4me2/3 would be lost form the autosomes as well. However, this deletion had no effect on H3K4me2/3 in the germline or the imprinted Xp in the embryo (Figure 2.3 E and F). As we lacked the tools to further address the role of ubiquitination in establishment of the imprint in *C. elegans*, we did not pursue these questions further.

Unmodified H3K4

Histone H3 Serine 10 phosphorylation is present on all chromosomes in the early embryo, confirming that N-terminal tail cleavage, that has been recently reported, is not noticeably enhanced on H3 on the Xp (Bean et al., 2004; Duncan et al., 2008). Further confirming that the Xp was not deficient in intact histones H3 tails, nor enriched in a form of H3 that is inaccessible to the antibodies against H3K4me2/3, binding of an antibody specific for unmodified H3K4 (H3K4me0) was enriched on the X in pachytene of both sexes, throughout spermatogenesis, and even persisted into mature sperm and the paternal pronucleus (Figure 2.4 A and B). However after pronuclear fusion all chromosomes became highly decorated with H3K4me0, and the Xp became indistinguishable from autosomes with this antibody by the two-cell stage (Figure 3.4 Fi). The results of this experiment are detailed further in Chapter 3.

H3K27 Methylation

In *C. elegans* H3K27 di- and tri- methylation in the germline and early embryo is mediated by the MES-2/3/6 complex (Bender et al., 2004). MES-2 is a SET-domain containing histone methyltransferase homologous to the Drosophila PRC2 HMT E(z) (Bender et al., 2004; Holdeman et al., 1998). MES-2/3/6-dependent H3K27me2/3 is

found on all chromosomes in the germline and early embryo. H3K27me3 is enriched on the X in pachytene, although this enrichment does not seem to perdure into the embryo (Bender et al., 2004). In our experiments we also observed H3K27me2 on all chromosomes of in the germline and early embryo (Figure 2.3 C and D). However in contrast to a previous report, we did not detect noticeable enrichment of H3K27me3 on the X during pachytene with our antibody (Figure 2.4 E). As no enrichment of these marks has been linked to the Xp in either the germline or the embryo by either study (Figure 2.4 C-F), it is unlikely that methylation of H3K27 is contributing directly to the imprint in *C. elegans*.

H3K9 tri-methylation

Like H3K27me2/3, methylation of H3K9 is strongly associated with heterochromatin. In particular H3K9me2 is enriched on unpaired chromatin, such as the unpaired X chromosome during XO male meiosis, and much lower levels on paired chromosomes in hermaphrodites by immunofluorescence in *C. elegans* ((Bean et al., 2004) and Alex Fedotov in preparation). Enrichment of H3K9me2 is a characteristic of meiotic silencing of unpaired chromatin (MSUC), first described in neurospora (Shiu et al., 2001). The silencing of the unpaired sex chromosomes in male meiosis, termed Meiotic sex chromosome inactivation (MSCI), is found across many species (Turner, 2007). Although H3K9me2 is enriched on the Xp as it progresses in an unpaired state during male meiosis in *C. elegans*, H3K9me2 does not remain enriched on the Xp in early embryos(Bean et al., 2004). H3K9me3 in particular is typically associated with constitutive heterochromatin. It was recently reported that H3K9me3 is enriched on repetitive transgenes carrying somatic genes in the germline of *C. elegans* (Bessler et al.,

2010). As such transgenes are typically silenced in the germline of *C. elegans*, the presence of H3K9me3 was perhaps unsurprising. However, we observed H3K9me3 accumulation on transgenes in general, including a non-repetitive transgene that is transcriptionally active in the germline (Figure 3.10 Gi). H3K9me3 is thus not a reliable marker for repressed transgenes in germ cells. Interestingly, H3K9me3 is not enriched on the Xp in either the germline or in early embryos, thus separating its role on transgenes from the imprinting phenomena in *C. elegans* (Figure 2.4 G and H). These results, with particular attention to H3K9me3 accumulating on transgenes, are discussed in further detail in Chapter 3. A comparison of histone modifications and histone variant patterns on the Xp vs. the Xm and autosomes is found in (Table 2.3).

In summary, we did not observe any additional unique histone variants or unique histone modifications that correlated with the imprinted X in *C. elegans*. However, our results further reinforced the idea that the imprint is a consequence of the distinctive lack of transcriptional activity of the X chromosome passing through spermatogenesis, which serves as the basis of our hypothesis outlined in Chapter 3.

DISCUSSION

We have shown that although the pattern of some additional histone modifications and histone variants in the adult germline differ between the spermatogenic X chromosome and the oogenic X chromosome as well as the autosomes, these differences do not persists beyond the zygote. The differences we did observe seemed to correlate with the transcriptional status of the different chromosomes as the germ cells developed in the different (germline) sexes. Indeed, most histone modifications and histone variants are indistinguishable by immunofluorescence between the spermatogenic X chromosome

and the oogenic X or autosomes (Summarized in Table 2.3). These results suggest that the histone modifications and histone variants examined are unlikely to influence iXi in *C. elegans*.

In mammals, X chromosome inactivation is characterized by a unique chromatin environment (Table 2.1). While the absence of active histone modifications such as H3K4me and H3Ac are conserved between the inactive X in *C. elegans* and mammals, many of the modifications characteristic of iXi in mouse were not found on the imprinted Xp in *C. elegans* (See Tables 2.1 and 2.3). In particular, repressive histone modifications H3K9me and H3K27me while not enriched on the Xp in *C. elegans*, are enriched on the inactive X in mammals, and preservation of imprinted X inactivation is dependent on H3K27 HMTs. The strong association of these marks with the inactive X in mammals may have more to do with maintenance of inactivation rather than the underlying mechanism biasing the imprinted X chromosome for silencing.

Similarly, our observation of H3.3 not accumulating on the X during spermatogenesis is in contrast to reports in mammals, where the variant is enriched on the transcriptionally silenced XY sexbody and the entire paternal pronucleus (van der Heijden et al., 2007; van der Heijden et al., 2005). Indeed, our results showing H3.3 failing to accumulate on the transcriptionally silent X during pachytene fall more inline with the traditional view of H3.3 as a variant associated with active chromatin. Perhaps enrichment of this variant on the sex chromosomes in mammals is necessary for re-activation of MSCI repressed chromatin upon fertilization. This perspective aligns that data with the role of H3.3 in development and fertilization in other metazoans. However the role of the newly described H3.3 chaperone, Daxx, on maintenance of iXi is just now

being described and may create a more complex picture of this variants role in transgenerational transitions.

The imprint in *C. elegans* is characterized by the absence of active histone modifications from the paternally inherited X chromosome. As our examination was not exhaustive the possibility for cross talk between another histone modification or histone variant that could prevent the accumulation of H3K4me2/3 or H3K9/K14Ac in *C. elegans* is formally possible. Evidence for one chromatin modification influencing another has been proposed and established before. For example H3K9me3 is dependent on H3K27me/MES-2 HMT in the germline of *C. elegans*. The HMT responsible for H3K9me3 in the *C. elegans* germline has not been identified. Bessler et al. proposed that MES-2 may directly methylate H3K9, as the MES-2 homologue in Drosophila, E(z), has H3K27 as well as H3K9 HMT activity in vitro (Bessler et al., 2010; Czermin et al., 2002; Muller et al., 2002). However, it seems more likely that recruitment of the unidentified H3K9 tri-methyl HMT is dependent on H3K27me, as has been suggested in other systems (Erhardt et al., 2003; Liu et al., 2007). The correlation between H2B ubiquitination and H3K4me is very consistent (Zhang, 2003). However, the tools for examining the role of H2BUb in *C. elegans* are limited, so this modification or some other we did not examine could in fact influence the imprint. However as discussed in the next chapter, the histone modifications characterizing the imprint may be reflective of, rather than causative of the mechanism truly establishing iXi in *C. elegans.*

Nevertheless, our studies on histone variants and histone modifications reinforced an important observations; the transcriptional history of the Xp versus the Xm in the germline is vastly different, as reflected in their different chromatin makeup. Although

not all of the chromatin differences between the Xp and the Xm are inherited by the zygote, H3K4me2/3 and H3K9/14 Ac are inherited by the Xm and autosomes and not the Xp. These marks are well correlated with transcription and are likely laid down as a result of it. Based on these observations, we continued to pursue the mechanism establishing iXi in *C. elegans* and reasoned, that these marks were laid down as a consequence of transcriptional history, rather than anything inherent about the Xp. We pursued the role of transcription in imprint establishment in Chapter 3.

MATERIALS AND METHODS

Strains We used standard techniques for worm maintenance and handling. We carried out all crosses and grew all worms at 20° C. We used the following strains: wild-type N2 (Bristol), zuIs178 [(his-72^{1kb}::HIS-72::GFP); *unc-119(ed3),* gift from S. Henikoff], *his-69(gk394), his-70* (gift from D. Chu), *his-73 (tm1643), ubc-1(gk14),* KW1864 (ckEx2 his-73^{1kb}::HIS-73::GFP; rol-6) randomly integrated on X.

RT-PCR RNA was amplified with RiboMax large scale RNA production kit (Promega) (a kind gift from Matt Edwards). Reverse transcription was carried out using SuperScript III reverse transcriptase (Invitrogen) with random and oligo-dT primers. PCR was performed for 35 cycles with Genescript taq cycles. PCR program was as follows: 1) 94˚ 3 mins. 2) 94˚ 45 secs. 3) 50˚ 30 secs 4) 72˚ 1 mins. 5) go to 2) 34 times. 6) 72˚ 7 mins. 7) 4˚ forever. The following primers were used: his-69 a) (gaaagaacgcaatcgtcgtc and agaagaagatcagtggactttt), his-69 b) (AACGCAATCGTCGTCGGAGCC and TTAGTTGGGCTAGTTGAAT), his-73 c) (atggttccgcaccagcgca and ttaagcgcgctctcctcgg).

Immunocytochemistry Whole-mount fixation and antibody staining of worms and embryos with methanol/acetone fixation was done as previously described(Strome and Wood, 1983). For detection of specific histone modifications, we used the following primary antibodies at the specified dilutions: rabbit antibody to H3K4me2 (1:500; Millipore), mouse antibody to GFP (1:200, Millipore), mouse monoclonal #34 against H3.1 (van der Heijden et al., 2005), mouse monoclonal CMA301 specific for unmodified H3K4 (H3K4me0; 1:500; a gift from H. Kimura, Osaka University, Japan), rabbit polyclonal to HCP-3 (1:1000; a gift from Abby Dernberg), Mouse Monoclonal H2AUb IgM (Millipore 1:50), Mouse monoclonal antibody to Ubiquitin (1:500 Covance), Mouse Monoclonal to H3Ser10P (1:1000 Upstate), Rabbit polyclonal to H3K27me2 (1:100 Abcam), Mouse monoclonal to H3K27me3 (1:100 Abcam), Mouse monoclonal to H3K9me2 (1:500 Abcam), Rabbit polyclonal to H3K9me3 (1:1000 Abcam). Rabbit polyclonal to H3K36me3 (1:500 Abcam), Rabbit polyclonal to H3K18Ac (1:500), Rabbit polycolonal to H3K4me3 (1:1000 Abcam). Secondary antibodies were used at 1:500: Alexaflour™;594 donkey anti-rabbit IgG, Alexaflour™; 488 donkey anti mouse IgG (Molecular Probes).

Immunofluorescence Analysis We observed images using a Leica DMRA microscope outfitted with a Q-imaging Retiga-SRV Fast 1394 Camera. We acquired and processed the images with Simple PCI software. For detection of unique chromosomes lacking or enriched for a particular modification in the embryo, embryonic nuclei were optically sectioned at intervals of 0.1-0.2 micrometers and analyzed by Z-stacks.

Table 2.1 Histone Variants and Histone Modifications Associated with the Inactive X in Mammals

Histone modification, histone variant, or histone chaperone patterns of enrichment or deficiency on the imprinted X chromosome (or genomicly imprinted loci) as indicated. rXi= random X inactivation, iXi= imprinted X inactivation

Table 2.1 Histone Modifications and Variants Associated with X-inactivation in Mammals		
Histone modification	**iXi, rXi, or genomic imprints**	***Citation***
Hypo acetylated H4	rXi	(Keohane et al., 1996) (Csankovszki et al., 2001) (Heard et al., 2001) (Jeppesen and Turner, 1993)
Hypo methylated H3K4	rXi iXi KDMB1 demethylase required for maternal genomic imprints	(Boggs et al., 2002) (Heard et al., 2001) (O'Neill et al., 2003) (Costanzi et al., 2000) (Ciccone et al., 2009)
H3K9 hypo acetylation	rXi iXi	(Heard et al., 2001) (Costanzi et al., 2000)
H3K9me2	rXi iXi genomic imprinting	(Heard et al., 2001) (Peters et al., 2002) (Mermoud et al., 2002) (Boggs et al., 2002) (Okamoto et al., 2004) (Umlauf et al., 2004)
H3K27me3	rXi (not necessary for maintenance) iXi (required for maintenance) genomic imprinting Inducible XIST transgene	(Plath et al., 2003) (Silva et al., 2003) (Erhardt et al., 2003) (Lewis et al., 2004) (Kohlmaier et al., 2004)
Eed/Enx1/Ezh2 G9a	iXi (required for maintenance) rXi (not necessary for maintenance) Genomic imprinting not required for iXi	(Wang et al., 2001) (Mak et al., 2002) (Silva et al., 2003) (Kalantry et al., 2009) (Erhardt et al., 2003) (Plath et al., 2003)
H4K20me1	Accumulates upon induction of XIST transgene (models rXi)	(Kohlmaier et al., 2004)
H2AK119Ub	Enriched on iXi and rXi in ES cells Acquired upon inducible XIST expression (rXi model) Not required for initiation or maintenance of X inactivation	(Fang et al., 2004) (de Napoles et al., 2004) (Leeb and Wutz, 2007) (Plath et al., 2004)
Histone Variants and Chaperones		
MacroH2A enriched	rXi iXi	(Mermoud et al., 1999) (Csankovszki et al., 1999) (Choo et al., 2006; Choo et al., 2007) (Costanzi and Pehrson, 1998; Costanzi et al., 2000)
H3.3 chaperons enriched	Daxx on XY body HIRA on XY body ATRX on rXi and iXi iXi lost without ATRX	(Rogers et al., 2004) (van der Heijden et al., 2007) (Baumann and De La Fuente, 2009) (Garrick et al., 2006)
H2ABbD deficient	Excluded from rXi	(Chadwick and Willard, 2001)
H2A.Z deficient	Enriched on X or Y in 1N sperm Excluded from inactive X	(Greaves et al., 2006) (Rangasamy et al., 2003)

Table 2.2 Alignment of Histone H3 Protein Sequences in *C. elegans*

H3 variants in worms, along with an S-Phase histone from fly (DmH3.3a). HIS-17 is a representative H3.1 from *C. elegans*. HIS-71 and HIS-72 are *C. elegans* H3.3s. Red letters indicate differences between either H3.1 or H3.3. Blue letters indicate residues found in H3.3. Lysine 4 is highlighted in yellow. Residues 31 and 32 are highlighted in green. Residues 87-90 are highlighted in pink.

Table 2.2 Alignment of H3 Histones

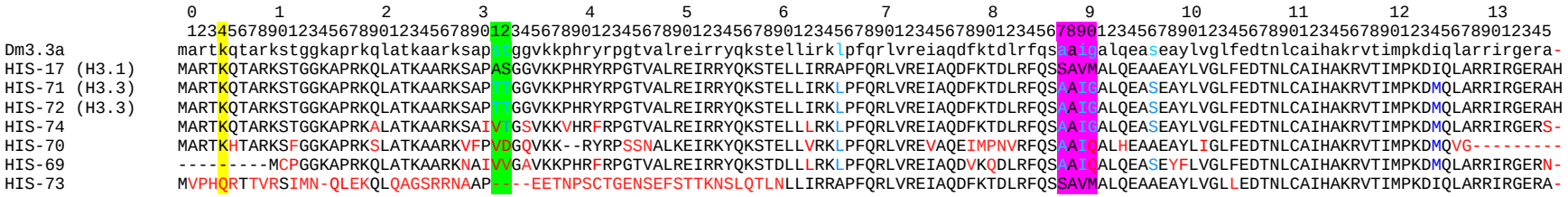


Table 2.3 Histone Variants and Modification Patterns on the Autosomes, the Xm, and the Xp in *C. elegans* Embryos. Summary of histone variant and histone modification patterns in the early *C. elegans* embryo from experiments in this work and in published literature. The only characterized instances where the Xp is different from the Xm and autosomes are highlighted in yellow. The Xp is not decorated with high levels of H3K4me2/3 or H3K9/14Ac2, while the Xm and autosomes are. All other modifications are either present or absent on all chromosomes including the Xp, or are absent from both the Xp and the Xm while enriched on the autosomes (H3K36me2/3), however, the Xm does seem to accumulate a low level of H3K36me3 intermediate of the Xp and autosomes (see Figure 2.2 F).

Table 2.3 Histone Variant and Modification Patterns in the Early *C. elegans* Embryo				
Variant/Modification	Autosomes	Xm	Xp	Reference
H3.1	-	-	-	Figure 2.1 Ii
H3.3	+	+	+	Figure 2.1 Ki
HCP-3	+	+	+	Figure 2.1 Fi
H3K4me2/3	+	+	-	(Bean et al., 2004) and Figure 2.2 B
H3K9/K14 Ac2	+	+	-	(Bean et al., 2004)
H3Ser10P	+	+	+	(Bean et al., 2004)
H4K18/K16 Ac	+	+	+	(Bean et al., 2004)
H3K18Ac	+	+	+	Figure 2.2 D
H3K36me2/3	+	-	-	(Bender et al., 2006) and Figure 2.2 F
UbH2A	-	-	-	Figure 2.3 Bi
Ub	+	+	+	Figure Di
H3K4me0	+	+	++	Figure 2.4 Bi
H3K27me2/3	+	+	+	(Bender et al., 2004) and Figure 2.4 D and Fi
H3K9me2	-	-	-	Fedotov personal communication
H3K9me3	+	+	+	Figure 2.4 Hi

Figure 2.1 Histone Variants Not Affecting the Imprint in *C. elegans*

The X chromosome lacks H3K4me2 in *his-69* deletion animals as usual in both pachytene (A) and early embryos (Ai) (one cell embryo shown). The X chromosome lacks H3K4me2 in *his-70* deletion animals in both pachytene (B) and early embryos (Bi) (nuclei from a 4 cell embryo shown). The X chromosome lacks H3K4me2 in *his-73* deletion animals in both pachytene (C) and early embryos (Ci) (one cell embryo shown). A-Ci DAPI (red), H3K4me2 (green). HIS-73::GFP does not accumulate on the Xp in the early embryo (D) (one cell embryo shown) arrow indicates Xp, DAPI (red), H3K4me2 (green), (Di) anti-GFP staining. HIS-73::GFP is expressed in mature sperm (E) DAPI (red), H3K4me2 (green), (Ei) anti-GFP staining. HCP-3 staining does not colocalize with DNA in pachytene (F), but does stain mitotic chromatids (Fi) (nuclei from a two cell embryo shown). *his-69* is not expressed in *C. elegans*, while *his-73*, which is also classified as a pseudogene, is (G) a) RT-PCR products for *his-69* primer set. No product is seen in either RT+ or RT- reactions indicate no transcript is expressed. b) RT-PCR products for another *his-69* primer set. No product see in either RT+ or RT- reactions indicate no transcript is expressed. c) RT-PCR products for *his-73* primer set. Product in RT+ lanes only indicates this transcript is expressed. + indicates reactions with reverse transcriptase, - indicates reactions without reverse transcriptase, g indicates PCR product amplified from genomic DNA.

H3.1 is enriched on the X during pachytene (Hi), however this enrichment is lost upon fertilization, and very little H3.1 decorates chromatin in a one cell embryo (Ii). His-72::GFP is depleted from the X during pachytene (Ji), however all chromosomes including the Xp contain His-72::GFP in the early embryo (Ki) (one cell embryo shown).

(A-K) DAPI (red), H3K4me2 (green), H3.1 (Hi and Ii), anti-GFP in his-72::GFP animals in (Ji and Ki).

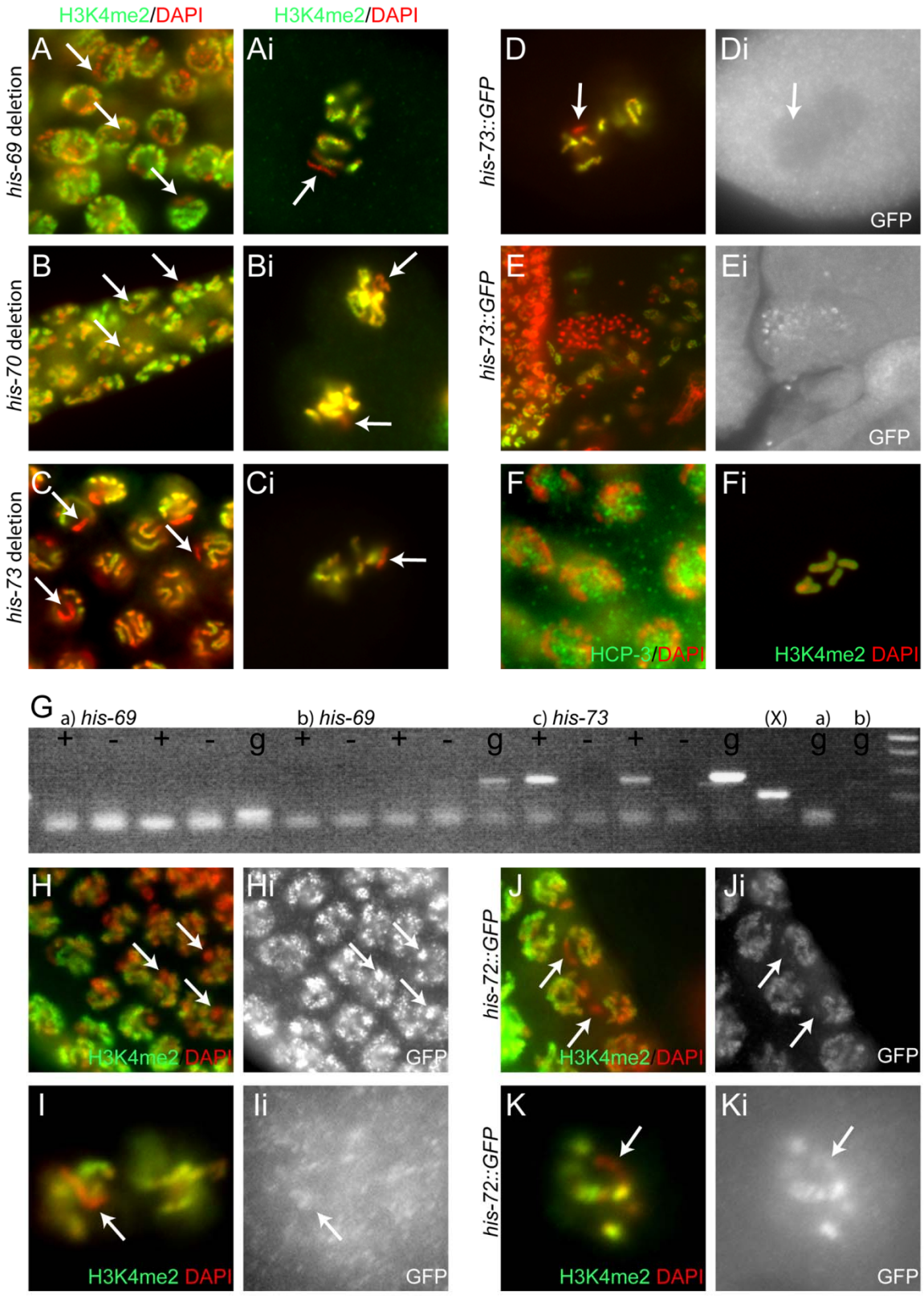

H3K4me2/DAPI
H3K4me2/DAPI
A
Ai
his-69 deletion
B
Bi
his-70 deletion
C
Ci
his-73 deletion
D
Di
his-73::GFP
GFP
E
Ei
his-73::GFP
GFP
F
HCP-3/DAPI
Fi
H3K4me2 DAPI
G
a) his-69
b) his-69
c) his-73
(X)
a)
b)
+ - + - g + - + - g + - + - g g g
H
H3K4me2 DAPI
Hi
GFP
J
his-72::GFP
H3K4me2 DAPI
Ji
GFP
I
H3K4me2 DAPI
Ii
GFP
K
his-72::GFP
H3K4me2 DAPI
Ki
GFP

Figure 2.2 H3K4me3, H3K18Ac and H3K36me3 in Pachytene and Embryos

H3K4me2 is absent from the X during pachytene (A), as well as from the Xp in the early embryo (B). H3K18Ac is low on the X in pachytene (C), but decorates all chromosomes in the early embryo (D). H3K36me3 is low the X during pachytene (E), as well as on both X chromosomes in the early embryo (F). The Xp has very low levels of H3K36me3 (F arrow), while the Xm (F arrowhead) has a low level of H3K36me3 intermediate to the Xp and the autosomes. Antibodies as indicated (green) with DAPI counterstain (red).

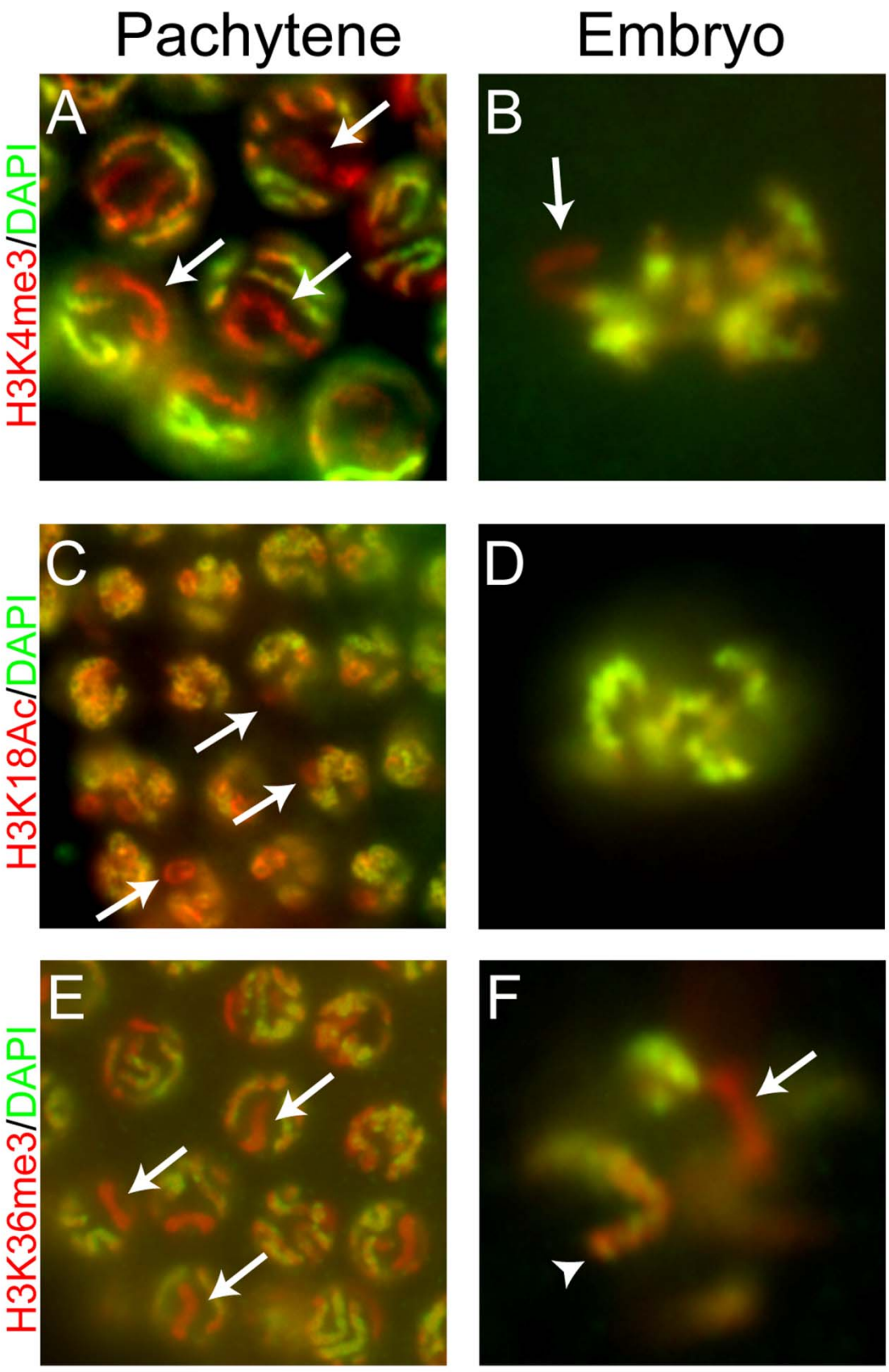

Pachytene
Embryo
A
B
C
D
E
F
H3K4me3/DAPI
H3K18Ac/DAPI
H3K36me3/DAPI

Figure 2.3 Ubiquitination in the Germline and Early Embryo.

Antibody against UbH2A showed non-specific staining in pachytene (Ai) and did not decorate chromatin in the early embryo (Bi) (nuclei from a two cell embryo shown). A pan-ubiquitin antibody showed non-specific background staining in both pachytene (Ci) and the embryo (Di). Deletion mutants for *ubc-1* had normal H3K4me2 staining in both pachytene (E) and the embryo (F) (one cell embryo shown). (A-F) DAPI (red), H3K4me2 (green). (Ai and Bi) DAPI (red), anti-H2Aub (green). (Ci and Di) DAPI (red) anti-Ubiquitin (green). Arrows indicate the X chromosome in pachytene and the Xp in embryos.

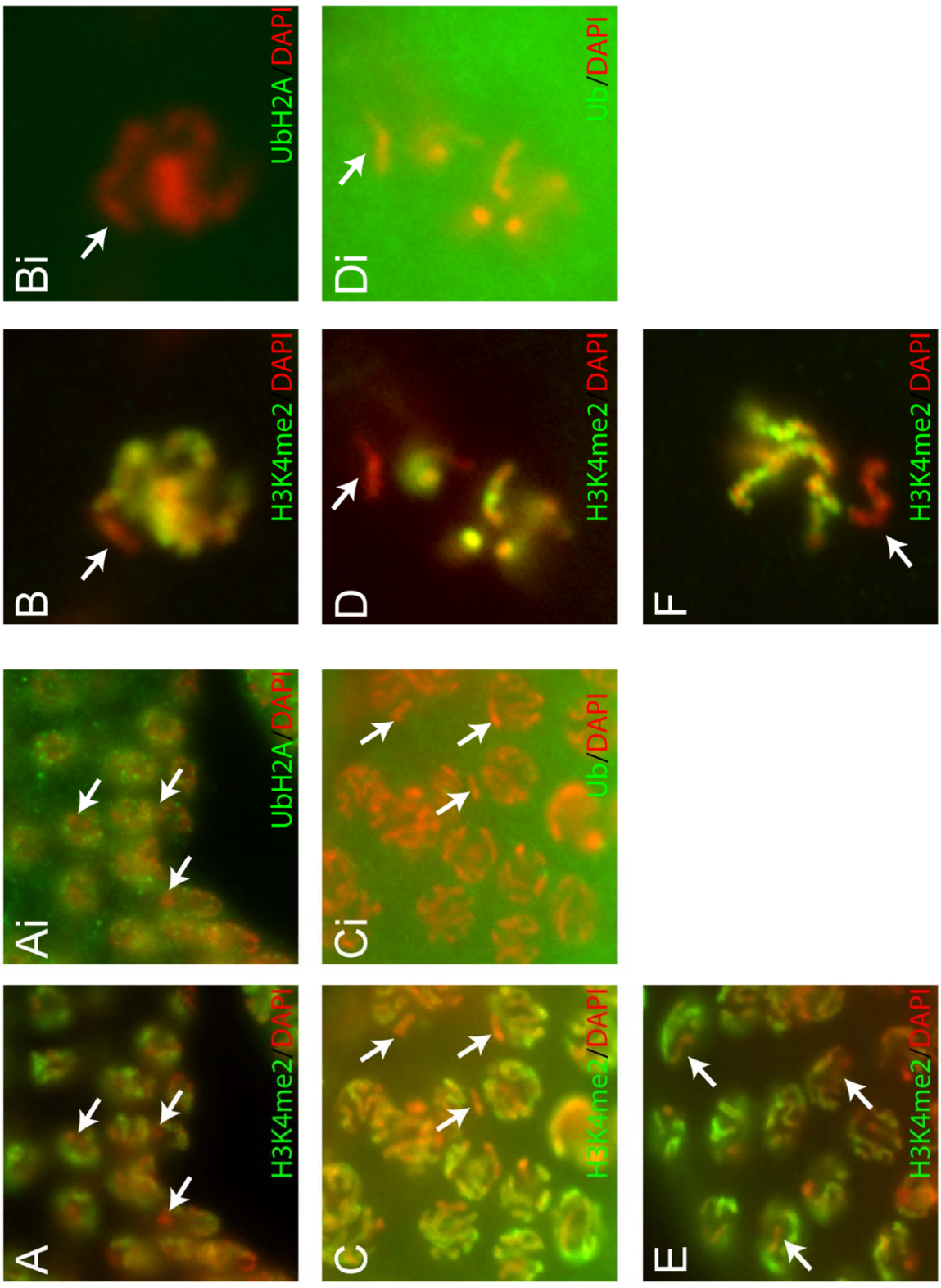
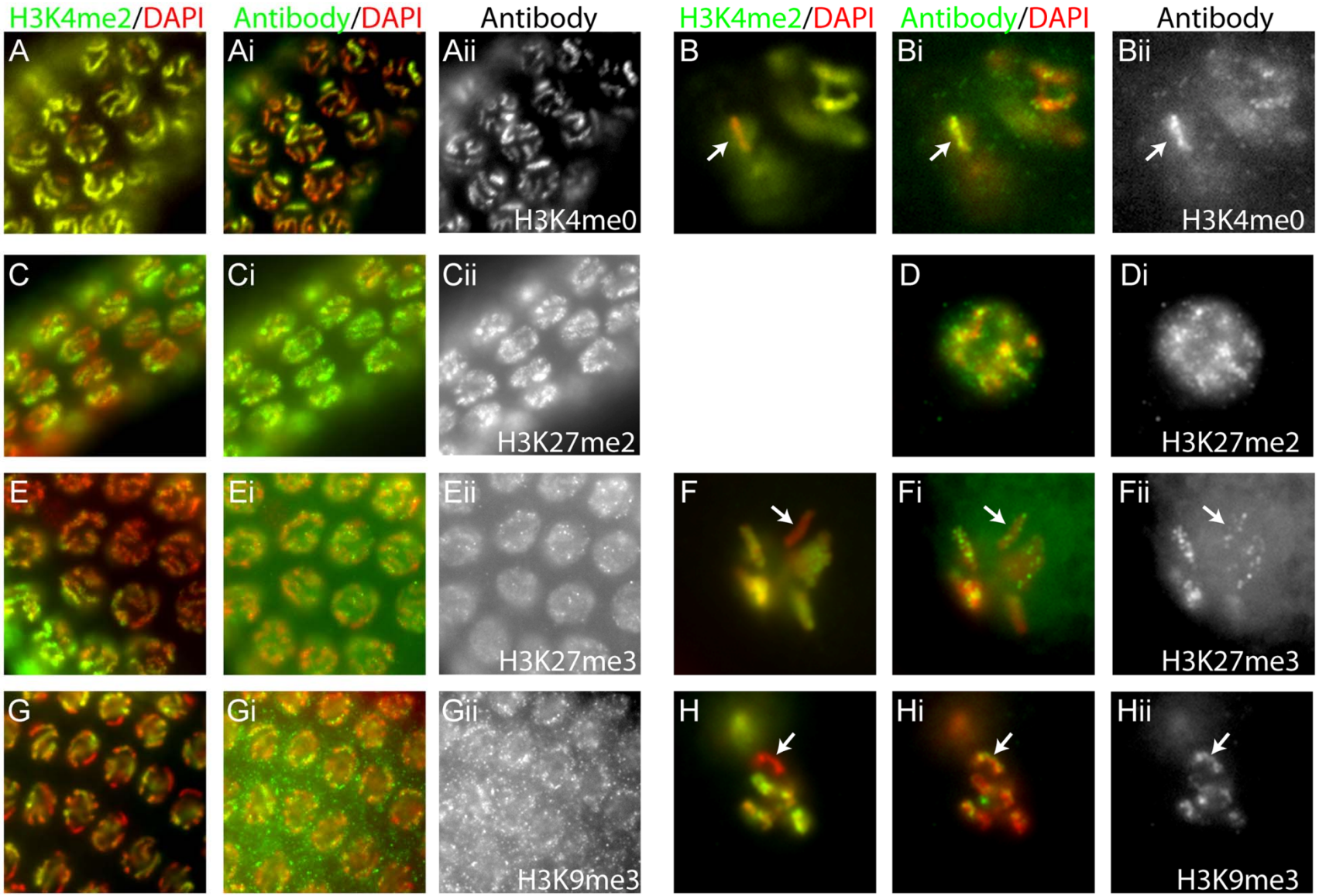

A
H3K4me2/DAPI
Ai
UbH2A/DAPI
B
H3K4me2/DAPI
Bi
UbH2A DAPI
C
H3K4me2/DAPI
Ci
Ub/DAPI
D
H3K4me2/DAPI
Di
Ub/DAPI
E
H3K4me2/DAPI
F
H3K4me2/DAPI

Figure 2.4 H3K4me0, H3K27me2/3, and H3K9me3 in Pachytene and Early Embryo. Antibody staining for H3K4me0 (Ai and Bi), H3K27me2 (Ci and Di), H3K27me3 (Ei and Fi), and H3K9me3 (Gi and Hi), compared to H3K4me2. (A-H). Antibodies (green) with DAPI (red). H3K4me0 is enriched on the X in pachytene as identified by lack of H3K4me2 (Ai). Some enrichment for this mark can still be observed in a one cell embryo (Bi). H3K27me2 decorates all chromosomes including the X in pachytene (Ci) and the early embryo (Di) (nuclei form a two cell embryo). H3K27me3 decorates all chromosomes in pachytene (Ei) and the early embryo (Fi) (one cell embryo shown). H3K9me3 decorates all chromosomes in a pachytene(G) and the early embryo (Hi) (one cell embryo shown).


H3K4me2/DAPI
Antibody/DAPI
Antibody
H3K4me2/DAPI
Antibody/DAPI
Antibody
A
Ai
Aii
H3K4me0
B
Bi
Bii
H3K4me0
C
Ci
Cii
H3K27me2
D
Di
H3K27me2
E
Ei
Eii
H3K27me3
F
Fi
Fii
H3K27me3
G
Gi
Gii
H3K9me3
H
Hi
Hii
H3K9me3

Chapter 3

Epigenetic Patterns Maintained in Early *C. Elegans* Embryos Can Be Established by Gene Activity in the Parental Germ Cells

As Submitted to PLoS Genetics with additions

INTRODUCTION

The information transferred by gametes from parent to offspring is not limited to that encoded in DNA; epigenetic information is also an important component of cross-generation inheritance (Youngson and Whitelaw, 2008). How this information is established in the parent and stably maintained in the offspring is poorly understood. The importance of this information is revealed in developmental diseases that result from defective genomic imprinting, in which defective epigenetic information establishment in the parental germ line can cause abnormal somatic gene expression in the offspring (Amor and Halliday, 2008). Although this is limited to parent-to-offspring inheritance, recent studies suggest that epigenetic abnormalities in the parental germ line can cause heritable defects across many generations (Anway et al., 2005; Katz et al., 2009). The germ line therefore not only protects and distributes genetic information, but may also identify and regulate what epigenetic information is "proper" and heritable through subsequent generations.

A dramatic example of imprinting in mammals is imprinted X chromosome inactivation (iXi), in which the paternal X is preferentially inactivated prior to implantation in mammals (Mak et al., 2004). iXi is sustained only in the placental tissues of eutherians, but is also observed in embryonic lineages in marsupials (Mak et al., 2004; Sharman, 1971). Unlike most genomic imprints, iXi does not require the maintenance DNA methyltransferase Dnmt1 (Lewis et al., 2004; Sado et al., 2000; Sado et al., 2004). It does however require repressive histone modifications such as H3K9me and H3K27me established by the polycomb group histone methyltransferases (Mak et al., 2002; Silva et al., 2003; Wang et al., 2001). These features are consistent with the theory that histone

modifications are the more conserved imprinting mark, as DNA methylation is not associated with imprinting phenomena in worms or flies for example, and yet epigenetic imprinting phenomena have been observed in these organisms (Bean et al., 2004; Lloyd, 2000; Sha and Fire, 2005).

What "marks" the paternal X for iXi? One mechanism that is unique to the paternal X is meiotic sex chromosome inactivation (MSCI). MSCI targets the XY chromosome pair for significant transcriptional repression during male meiosis. This is thought to be due to the largely unpaired/unsynapsed status of these chromosomes, which renders the X and Y targets for a process generally termed Meiotic Silencing (Turner et al., 2004). This correlation between MSCI and iXi has not gone unnoticed, and highly debated models linking these processes have been proposed (Heard and Disteche, 2006; Huynh and Lee, 2003). Regardless, it is clear that in mice and marsupials, passage through spermatogenesis imparts an imprint that selectively renders the X prone to early repression in the offspring, while passage through oogenesis prevents this from occuring.

The X chromosome is also condensed and transcriptionally inert during *C. elegans* spermatogenesis, and, as in some mammals, the paternal X (Xp) is initially inactive in the early embryo (Bean et al., 2004; Goldstein, 1982; Kelly et al., 2002). This iXi in *C. elegans* consists of a near complete absence of most "active" histone H3 modifications on the Xp, a unique status that is stable through early cell divisions, becoming less obvious by ~24 cells (Bean et al., 2004). The absence of specific H3 modifications on the Xp implies that *de novo* zygotic chromatin assembly on the Xp is somehow uniquely and heritably refractory to addition of these marks during early stages. This information is clearly epigenetic in nature: it is only imparted to an X that has

passed through spermatogenesis and not to a genetically identical chromosome encountering oogenesis. Interestingly, the Xp is "imprinted" whether it passed through spermatogenesis in an XX hermaphrodite or an XO male. That is, the pairing status of the X going through spermatogenesis does not affect imprint establishment. This seems to omit a contiguous linkage between this process and meiotic silencing mechanisms. The only determinant seems to be whether the X chromosome went through spermatogenesis before arriving in the zygote, independent of whether that spermatogenesis occurred in an XX hermaphrodite or an XO male.

A key difference between the Xp and the rest of the chromosomes in the embryo, including the oocyte-derived Xm, is a significant difference in their respective transcriptional activities in the parental germ lines. The X chromosome appears largely transcriptionally inactive in pre-meiotic and early to middle stages of meiosis in both sexes. This is likely due to the paucity of X-linked genes expressed in germ cell stages common to both sexes, which itself may be an evolved consequence of meiotic silencing mechanisms (Kelly and Aramayo, 2007; Reinke et al., 2000). The X also lacks spermatogenesis specific genes, and thus remains largely inactive throughout sperm development (Kelly et al., 2002; Reinke et al., 2000). In contrast, oogenesis-enriched loci are well represented on the X and this chromosome becomes active during oogenesis (Kelly et al., 2002; Reinke et al., 2000). Thus the X's from egg and sperm arrive into the zygote with significantly different transcriptional histories.

A histone modification that is associated with transcription is histone H3 lysine 4 methylation (H3K4me). H3K4me deposition can result from active transcription, and it has been implicated in providing a heritable and trans-generational memory of where

transcription has occurred in the genome (Muramoto et al., 2010). H3K4me has been implicated as playing an important role in differential DNA methylation, as this mark can interfere with *de novo* methylation *in vitro,* and mutations in the H3K4 demethylase KDM1b lead to defective maternal imprint establishment *in vivo* (Ciccone et al., 2009; Ooi et al., 2007). It is thus possible that transcription-coupled addition of H3K4me in the parental germ line can influence the establishment of epigenetic imprints inherited by the offspring. Likewise, the absence of this mark could also have consequences for epigenetic inheritance. Importantly, H3K4me is largely absent from Xp chromatin in *C. elegans* spermatogenic germ cells, and its continued absence in the embryo is what defines iXi in this organism (Bean et al., 2004).

We therefore hypothesized that the epigenetic imprint of the Xp in the offspring is a passive consequence of its transcriptional quiescence, which implies the equally interesting idea that transcriptional activity in the adult germ line may influence the epigenetic information that is inherited by the offspring. This also implies that differential assembly of chromatin, such as that imposed by transcription in the parental germ cells, may survive gametogenesis, be transferred intact into the offspring, and be maintained in early embryogenesis. In order to test this, we further characterized Xp chromatin assembly in the adult germ line and the heritability of epigenetic information through sperm, and studied the connections between gene activity in the parental germ line and the patterns of chromatin modifications that are maintained in the zygote.

RESULTS

Sex Body Formation and Imprint Establishment are X DNA Autonomous

The X chromosome in *C. elegans* is largely devoid of genes that are expressed in spermatogenic germ cells as well as genes that are enriched for expression in both oogeneic and spermatogenic germ cells (Reinke et al., 2000). Additionally, a number of examples have been reported in which essential loci with X- and autosomal-linked paralogs exhibit germ cell-specific defects when only the autosomal copy is defective, suggesting that only the autosomal copy is active in germ cells (L'Hernault and Arduengo, 1992; Maciejowski et al., 2005). Thus the consensus in published data suggests there is little need for transcription from the X chromosome during any stage of spermatogenesis. Indeed, few histone marks found in active chromatin are detected on X chromatin during spermatogenesis, and the X chromosome(s) become highly condensed relative to autosomes during both XO and XX spermatogenesis (Figure 3.1A, male pachytene nuclei with X chromosome (arrow), and (Goldstein, 1982; Kelly et al., 2002)). This premature condensation, along with the absence of H3 histone modifications correlating with transcription, is reminiscent of XY-sex body formation in mammalian spermatogenesis (Handel, 2004). If X inactivity during spermatogenesis is a passive consequence of X chromosome sequence content, then attachment of X sequence to an active autosome should not affect either condensation or accumulation of active histone marks, such as dimethylated H3K4 (H3K4me2). This is indeed the case, as animals carrying a fusion of chromosomes IV and X (*mnT12*) exhibit a chromosome with both autosome and X chromosome structural characteristics that are limited to each respective half. In wild-type males (or wild-type hermaphrodite L4 larvae) undergoing

spermatogenesis, the X chromosome in pachytene lacks H3K4me2 and forms a condensed ball reminiscent of the XY body in mammalian spermatogenesis (Figure 3.1A arrow and (Goldstein, 1982; Kelly et al., 2002)). Strikingly, in *mnt12* hermaphrodite L4 larvae undergoing spermatogenesis, one half of the fusion chromosome lacks H3K4me2 and is highly condensed (Figure 3.1C arrow), while the other half remains elongated and is decorated by H3K4me2 (Figure 3.1C arrowhead). These data suggest that the transcriptionally quiescent sex body-like structure formed during spermatogenesis is autonomous to the DNA content of the X-chromosome.

We next looked at this fusion chromosome in the early embryo. The *mnt12* chromosome is easily identifiable cytologically because it is approximately twice the length of the rest of the chromosomes (Sigurdson et al., 1986). When paternally inherited, this chromosome is "half-imprinted"; i.e., exhibits the characteristic absence of H3K4me2 along half its length (one cell embryo; Figure 3.1D). Furthermore, combined FISH-antibody analyses indicate that there is little or no spreading of H3K4me2 into the X chromosome sequence (Figure 3.1 E). In contrast, the *mnT12* chromosome encountering oogenesis becomes fully decorated with H3K4me2, consistent with the normal representation of X-linked oocyte-expressed genes (Kelly et al., 2002), and heritably maintains this status in the zygote (Figure 3.1 F). These data suggest that the imprint in the zygote is specific for and limited to X DNA, and correlates with sex-specific gene activity in the parental germ lines.

Mature Sperm Chromatin Retains Epigenetic Information that Correlates with Spermatogenic Transcription

Histone variant H3.3 incorporation correlates with transcriptionally active chromatin and other modes of chromatin remodeling, and is reported to be enriched on the XY body and unpaired chromatin targeted by MSCI in mammals (Ahmad and Henikoff, 2002; van der Heijden et al., 2007). In contrast, and as previously reported, a *C. elegans* H3.3::GFP (HIS-72::GFP (*zuIs178*)) expressed in germ cells is largely absent from the X but accumulates on the autosomes in pachytene nuclei during both oogenesis and spermatogenesis in *C. elegans* (Ooi et al., 2006). Additionally, HIS-72::GFP accumulates on the X in mature oocytes ((Ooi et al., 2006) and Figure 3.2 B-D). HIS-72::GFP is known to be present in haploid spermatids and thus survives sperm chromatin condensation (Ooi et al., 2006). We further observed, however, that whereas the rest of the chromosomes contain H3.3 in mature sperm, the X remains devoid of this variant (Figure 3.3A). The genomic distribution of H3.3 within sperm chromatin thus grossly overlaps with the general pattern of its distribution during gametogenesis.

Although HIS-72::GFP is largely and uniquely absent from X chromosomes in spermatocytes, this does not rule out an enrichment for other H3 variants that exist in the *C. elegans* genome (Ooi et al., 2006). Incorporation of H3 variants outside of DNA replication necessarily involves the replacement of the canonical S-phase specific histone H3.1. We therefore tested whether H3.1 was being replaced on the X at any stage in germ cells of either sex. Using a monoclonal antibody specific for H3.1 (van der Heijden et al., 2005) we observed that H3.1-specific staining was enriched on all chromosomes in the proliferating region of the gonad, as expected (Figure 3.2E). In the pachytene region

of the hermaphrodite gonad H3.1 was progressively lost from the autosomes but persisted on the X chromosome (Figure 3.2F). However, as the germ cells progressed into oogenesis, H3.1 on the X chromosomes became reduced to levels indistinguishable from autosomes (Figure 3.2G). This is consistent with transcriptional quiescence of the X during meiosis and its activation during oogenesis, as previously reported (Bean et al., 2004; Kelly et al., 2002). In spermatogenesis, we saw similar staining in the mitotic (not shown) and pachytene regions of the gonad (Figure 3.2H). In contrast to oogenesis, H3.1 persisted beyond pachytene, and could be detected in a specific region in the nuclei of mature sperm (Figure 3.3B). We presume this to be the X, since in addition to enrichment in H3.1 the region also lacked H3K4me2 (below). These data are reciprocal of the H3.3 data, and are consistent with X chromatin being transcriptionally inactive and comparatively free of large-scale H3 remodeling throughout spermatogenesis. The male X thus uniquely retains substantial levels of H3.1 that were incorporated during pre-meiotic replication. In contrast, during oogenesis the X becomes transcriptionally active, and H3.1 is largely replaced, presumably by H3.3. Therefore the general deposition pattern of histone H3.3, as a mark of chromatin activity, is retained during spermatogenesis and is carried into the offspring with the paternal DNA.

It is possible that the enrichment of other H3.3-like variants may not have been detected in our assay. We tested deletion mutations of *C. elegans* H3.3-related variants *his-69* and *his-70* and observed no defects in X chromatin dynamics in either the adult or embryo (not shown). These H3.3 variants are thus unlikely to be specifically accumulating on the X chromosome or contributing to imprint establishment during spermatogenesis.

These data show that an X going through spermatogenesis is largely refractory to transcription-dependent or other H3 remodeling activities, presumably as a passive consequence of the paucity of genes on this chromosome that are active in spermatogenic germ cells. As a result, histone H3.1 incorporated during pre-meiotic S-phase persists in Xp chromatin in sperm. Conversely, patterns of histone variant incorporation that result from transcription or other processes appear to persist in other regions of the genome in sperm.

We next asked whether histone modifications such as H3K4me2 are present in mature *C. elegans* sperm, as has been recently reported in mammals (Brykczynska et al., 2010; Hammoud et al., 2009). We detected substantial levels of both H3K4me2 and unmodified H3K4 (H3K4me0) in purified haploid spermatids by immunofluorescence (Figure 3.3). We were also able to detect substantial levels of histone H3, H3K4me2, H3K4me0 as well as H2A in sperm by western blot (Figure 3.3 D-G). Furthermore, we found that the H3K4me2 that is retained in sperm nuclei is strikingly excluded from one region in spermatid nuclei (Figure 3.3, arrow). Co-staining with an antibody that specifically recognizes unmodified H3K4 (H3K4me0) gave the reciprocal pattern (Figure 3.3C). As this pattern is consistent with what is observed for the X chromatin in all earlier stages of spermatogenesis (Figure 3.2L), we conclude that this region is the X chromosome. This indicates that histone H3 not only remains associated with DNA during sperm chromatin compaction, but also retains chromosomal epigenetic patterns placed during spermatogenesis. Thus, the X chromosome arriving from spermatogenesis is enriched, relative to both autosomes and an X going through oogenesis, in histone H3.1 molecules that contain few marks of transcriptional activity. These experiments also

show that the failure of the Xp to be recognized by antibodies against modified H3K4 is not due to epitope masking or enrichment for N-terminally cleaved forms of H3 that have recently been reported (Duncan et al., 2008).

Histone H3 and its marks remain highly abundant in purified sperm chromatin, and thus the paternal chromatin enters the egg carrying substantial epigenetic information (Figure 3.3D-G). However, extensive post-fertilization remodeling of the paternal pronucleus by H3.3 occurs in many organisms, and such remodeling could significantly interrupt the heritability of sperm chromatin (Loppin et al., 2005; van der Heijden et al., 2005). Indeed, H3.3 (e.g., HIS-72::GFP) that is retained in mature sperm of *C. elegans* appears to disperse after fertilization, presumably through substantial incorporation of maternal H3.3 into the sperm pronucleus (Ooi et al., 2006). This indicates that as in other organisms, there is dynamic histone H3 mobilization into and out of the paternal chromatin during sperm pronuclear decondensation in the *C. elegans* zygote. These dynamics appear to precede DNA replication in the zygote. Importantly, little H3.1 is detected on any chromosome by immunofluorescence in early (1- 8 cell) embryos (Figure 3.4Aii and Bii). This suggests that the bulk of the H3 dynamics in the zygote involve deposition of H3.3, as observed in other species, and is largely replication independent and maternal in origin (Ooi et al., 2006). Importantly, the Xp is not noticeably resistant to H3.3 incorporation, and thus not refractive to post-fertilization histone dynamics (Figure 3.4Cii and Dii). Despite the similar H3 dynamics, the Xp maintains its relative enrichment for H3K4me0 through sperm decondensation and the first S-phase, when the autosomes also become increasingly enriched in H3K4me0 due to substantial incorporation of unmodified H3/H3.3 in all chromosomes (Figure 3.4Eii). Furthermore,

after subsequent rounds of DNA replication and histone incorporation (by the 2-cell stage and beyond), anti-H3K4me0 signals on the Xp are indistinguishable from the other chromosomes (Figure 3.4Fii). However, in spite of substantial *de novo* incorporation of H3, the Xp remains uniquely devoid of H3K4me2. Importantly the autosomes and Xm maintain their H3K4me2 enrichment, as established in the parental germ line, despite the significant post-fertilization H3 dynamics that we observe for these chromosomes. The H3K4me2 we observe at these early stages is likely due to maintenance of parental chromatin patterns, rather than *de novo* transcription-dependent establishment, as there is little or no zygotic transcription at these early stages (Guven-Ozkan et al., 2008; Seydoux and Dunn, 1997; Seydoux and Fire, 1994)**.**

These observations indicate that the chromatin assembled *de novo* in the zygote does not grossly perturb a significant amount of the epigenetic information that is carried by pre-existing histones in gamete chromatin. This implies that the unique chromatin status of the Xp could be due to an absence of an instructive template that is present on the Xm and autosomes, i.e. the epigenetic information retained during assembly of chromatin in the zygote is guided by pre-existing information present in the gamete from which it arrived. The establishment of this template could conceivably be influenced by transcriptional activity in the parent, and retained in the zygote by transcription-independent processes.

Transcriptional Activity in Adult Correlates with Chromatin Status in the Embryo

Transcriptional regulation in the adult germ line is poorly understood in most organisms including *C. elegans*, presenting a major challenge to experimentally modulating the transcriptional activity of endogenous loci. We therefore tested

transgenes that exhibit differential germ line transcription properties and chromosome linkage to correlate epigenetic status in adult germ cells with that in the early embryo. We first examined a strain carrying an X-linked repetitive transgene with a GFP reporter driven by a soma-specific promoter [*him-5(e1490);*axIs36 (pes-10::GFP, *dpy-20(e1282)*] (Montgomery et al., 1998). This reporter is not normally active in the germ line of either sex, and as reporter transgenes are typically silent in the germ line of *C. elegans*, no aberrant or ectopic GFP expression was detected in germ cells of this strain (not shown). This transgene is devoid of H3K4me2 during meiosis in the parental germ cells (Figure 3.6 A). Combined H3K4me2 antibody/DNA fluorescence *in situ* hybridization (FISH) analyses showed that H3K4me2 remained strikingly depleted from the transgene region on the Xm in embryos (Figure 3.5A). Importantly, the absence of H3K4me2 on the Xm transgene was observed through multiple rounds of cell division in the embryo until GFP became detectable in lineages where the *pes-10* promoter is active (8-24 cells; not shown). H3K4me2 remained depleted from the transgene in lineages where the promoter is not active until at least the 50-cell stage (not shown). The establishment of a heritable chromatin state in the embryo that correlates with transgene expression in the parental germ line is thus not specific to the sex of the germ line.

We next asked if the absence of H3K4me2 on germ line silent transgenes is limited to X-linked loci by examining a repetitive transgene construct carrying a similar GFP reporter to the transgene above, but integrated on chromosome V (*mIs10* V). In early embryos from this strain we could identify the imprinted Xp, which lacked H3K4me2 (Figure 3.5B arrowhead), as well as two autosomes exhibiting stripes lacking H3K4me2, which overlapped with the FISH signals detecting the transgene (Figure 3.5B

arrows). We observed similar results for several other germline inactive transgenes (Figure 3.3 F and G, and Table 3.1). These and the above data together indicate that the absence of H3K4me2 in embryonic chromatin correlates with a lack of adult germline expression rather than germline sex or chromosome linkage.

Repetitive transgenes are subjected to a number of silencing mechanisms, and could be targeted for assembly of repressive chromatin assembly in the embryo by mechanisms independent of template establishment (Kelly and Fire, 1998). We therefore tested whether embryonic chromatin assembled on a non-repetitive transgene correlated with its expression in adult germ cells. The KW1336 strain is homozygous for a mutation in the essential gene, *let-858*, but is rescued to viability and fertility by a non-repetitive, or "complex", transgene carrying a *let-858::gfp* construct (Kelly and Fire, 1998). The germline expression of this transgene is maintained by selection, since failure to express *let-858* in germ cells results in sterile animals (Kelly and Fire, 1998). Importantly, in the absence of selection this transgene can become heritably silenced in germ cells, which allowed us to directly compare zygotic chromatin assembly on an identical transgene that was either active or repressed in parental germ cells.

In the line with expression maintained by selection, H3K4me2 was detectably present on the transgene array in 71% (n=126) of embryos, of which many exhibited high levels of H3K4me2 comparable to autosomes (Figure 3.5C). We next outcrossed this line to wild-type males to remove the selective pressure to maintain germline expression, and followed the outcrossed line for several generations (Figure 3.8). Germline expression was maintained in the majority of outcross progeny in the first two generations; that is, the majority (93-100%) of offspring from germline-expressing adults

initially also expressed the transgene in their germ line. By the F3 generation, however, only 69% of the offspring of germline-expressing F2 animals showed germline GFP, and germ cell expression was not detected in any of the offspring (F4) of the germline-expressing F3 animals (Figure 3.8). In all cases the silencing of the GFP reporter in germ cells, once established, was heritable in all subsequent generations. Note that germline silencing of the transgene was not linked to any genotype, as it eventually occurred in all descendants of all randomly selected outcross progeny.

We examined, in parallel, whether H3K4me2 was detected at significant levels on the transgene in the embryos of each generation (Figure 3.8). In all cases in which the parents exhibited germline GFP expression, 64-78% of the embryos showed H3K4me2 on the transgene. Note that the adult germline is syncytial, thus it is possible that some transgenes inherited by offspring from adults exhibiting germline GFP were not active in adult germ cells. The frequency of H3K4me2 on the transgene in embryos from germline-silenced adults dropped to ~30%, independent of which generation the silencing occurred. A similar correlation was also observed for H3K4me3 (Figure 3.7). Oddly, this ~30% frequency was stably maintained in silenced animals for many (>20) generations. This may suggest that a background level of H3K4me2 is stably, or stochastically, maintained on this transgene array long after removal of selection for germline expression. Importantly, the KW1336 array is composed of relatively few copies of the *let-858::gfp* reporter construct embedded in random fragments of *C. elegans* genomic DNA (Kelly and Fire, 1998; Kelly et al., 1997). Since we do not know the composition of the array other than the reporter sequences, we cannot know if this represents sporadic transcription in the adult germ line from the embedded genomic

fragments, or DNA elements that may attract this modification through some other process. Importantly, none of the embryos from a different line carrying the same *let-858::gfp* reporter transgene, but in a germline-silenced array that lacks embedded genomic sequences, showed any detectable H3K4me on the transgene array (strain PD7271; Figure 3.5 F (Kelly and Fire, 1998)).

We further tested a total of 13 transgenes linked to various chromosomes and with various expression in adult germ cells, several of which correspond to genes that are normally expressed in all tissues (e.g., *let-858, his-24, lmn-1*), but in some cases exhibit germline silencing (Table 3.1). The persistence of H3K4me2 in the transgene chromatin in embryos generally follows the same pattern for all: transgene expression status in the parental germ cells is predictive for heritable and persistent H3K4me2 status on the transgene in the embryo. We observed three transgenes that did not fit this pattern. Two transgenes, the KW1864 and TJ375 strains, respectively, exhibited no detectable H3K4me2 in parental germ cells, although both show unusual germline expression patterns. The KW1864 transgene is expressed post-meiotically during spermatogenesis, as detected by both RNA *in situ* and antibody staining (T. M. Edwards and W. Kelly, manuscript in preparation). TJ375 carries a heat-shock promoter that drives GFP expression in all somatic lineages, but its germline expression is also unusual. After heat shock, GFP RNA is only detected in a narrow band of late pachytene cells, and the RNA is not detectably translated and is short-lived(Sheth et al., 2011).

We confirmed that transcription occurs in this limited pattern, but did not detect significant accumulation of H3K4me in transgene chromatin (not shown). The nature of the heat shock promoter, or the late timing of expression, or the very limited period of

activity may impinge upon the window of opportunity for establishing active chromatin modifications in the germline. The correlation between these unusual expression characteristics and their inconsistent correlation with germline expression and H3K4me2 is not understood. The transgene in the third strain, PD4251, was selected for minimal mosaicism in its expression in muscle cells, and this selection for more efficient mitotic transmission and somatic expression may be connected to its higher "background" of H3K4me2 in the zygote ((Sha and Fire, 2005), A. Fire, personal communication). Nevertheless, these data show that a DNA segment can be differentially targeted for chromatin assembly in the embryo in a manner that appears to depend largely on its activity in the parental germ line, although other modes of H3K4me insertion in the parental germ cells may also participate. It is again important to note that chromatin assembly in the one cell embryo has no connection to zygotic transcription, as chromatin assembly and the H3K4me pattern maintenance occurs prior to any known zygotic genome activation (Guven-Ozkan et al., 2008; Seydoux and Dunn, 1997; Seydoux and Fire, 1994).

We next tested whether transcription activity on the Xp in adult germ cells was able to establish a region of heritable H3K4me2 maintenance on this chromosome in the offspring. The IN373 *(dtIs372)* strain carries a multi-copy *his-24::gfp* transgene that is integrated on the X chromosome. *his-24* encodes a ubiquitously expressed *C. elegans* H1 linker histone (Hatzold and Conradt, 2008; Jedrusik and Schulze, 2007). The *dtIs372* transgene initially showed robust HIS-24::GFP expression throughout the soma, and weak expression in the germ line during both oogenesis and spermatogenesis. In embryos from these animals we initially observed a distinct stripe of H3K4me2 on the

Xp, which was largely limited to the transgene FISH signal (Figure 3.5E). During the course of these studies, this transgene became heritably silenced in adult germ cells, and thereafter the stripe of H3K4me2 coinciding with the transgene in embryonic Xp chromatin was no longer detected in this strain (Figure 3.5 H).

We also tested whether inheritance of H3K4me2 that was established in the parental germ line can affect expression of the transgene when it becomes active in the offspring. We compared somatic expression of the LET-858::GFP reporter in offspring from the parental line, in which germline expression is under selection, to that of outcrossed animals, in which the selection was removed and germline expression is lost (e.g., Figure 3.8) . In both the parental line and outcrossed animals, inheritance frequency of the transgene is ~70% as determined by DNA FISH**,** and the LET-858::GFP reporter is expressed in most if not all of the somatic tissues in adult animals. Most (over 90 %) of the embryos from germline expressing parents (either parental or outcrossed strain) had easily detected GFP-positive nuclei in most if not all somatic cells, whereas few (14%; n=92) of embryos from germline silent parents had detectable GFP expression (Figure 3.9 C and D). This is far below the frequency of array inheritance in these offspring. GFP expression was significantly weaker among embryos from the germline silent outcrossed line, and strong mosaicism of expression was also often observed (Figure 3.9 C). This basal level of expression was consistent in offspring from the germline silenced animals for >20 generations (not shown). The increased expression frequency in embryos from the germline-expressing parents, which exceeded the inheritance rate of the transgene, indicated that at least part of the GFP detected in these embryos was provided maternally. We therefore compared GFP fluorescence of the two

sets of transgenic offspring at larval and adult stages. L2 larvae through adult stage offspring from germline-expressing parents still showed significantly higher levels of GFP expression compared to the germline-silenced offspring both immediately after germline silencing occurs, and after many generations of the transgene being shut down in the germline (data not shown and Figure 3.9). These results indicate that, at least for this transgene, expression in the adult germ line strongly correlates with enhanced somatic expression in the offspring, even in late stages of development.

It has been reported that repetitive transgenes, including several that we examined in this study, accumulate H3K9me3 during meiosis (Bessler et al., 2010). We examined H3K9me3 to see if this mark, accumulated in the parent, is also inherited by the offspring. The presence of this mark could prevent the addition of H3K4me2 to inherited chromatin in the zygote, and thus be causal to its absence. Importantly, this correlation does not exist for the Xp, which lacks both H3K9me3 and H3K4me2 in both adult germ cells and in the zygote (Figure 3.10). In addition, although H3K9me3 may also be inherited from gamete chromatin, there was no obvious correlation between H3K9me3 presence and H3K4me2 absence on transgenes in either adult germ cells or in embryonic chromatin. On all transgenes examined, including those that expressed in adult germ cells, we observed an enrichment of H3K9me3 on transgene chromatin in pachytene and oocyte nuclei, and this mark persisted in the embryo (Figure 3.10 Gii-Iii). The presence of H3K9me3 in transgene chromatin did not correlate with either expression in the parent, or the presence or absence of H3K4me2 in the embryo.

The lack of correlation between expression in the germ line and appearance or absence of H3K9me3 on transgenes, and the absence of H3K9me3 on the imprinted Xp

at any stage, indicates that H3K9me3 accumulation on transgenes is due to a mechanism distinct from, and has little detectable influence on, the heritable chromatin assembly we report here. In addition, we did not observe either enrichment for, or absence of, other repressive marks (e.g., H3K27me2 or H3K27me3) on either germline-silent or germline-expressed transgenes. Indeed, abundance of these marks was indistinguishable between transgenes and autosomes in both germ line and embryo (data not shown).

These data show that the establishment of heritable epigenetic information that guides chromatin assembly in the zygote is likely gene autonomous, independent of chromosomal location, and that the information established can be guided by transcriptional activity in the adult germ line.

DISCUSSION

Our data shows that epigenetic information in the form of histone modifications, imposed during transcription and other chromatin-modifying processes in the adult germ line, can be stably carried by gametes into the offspring. This information appears to be grossly impervious to *de novo* chromatin assembly and histone dynamics in the embryo, and is maintained in the embryo in the absence of significant levels of zygotic transcription. These results suggest that sex-specific activities, including gamete-specific transcription, in the parental germ line can create parent-of-origin specific epigenetic content that may determine or guide the epigenetic information that is maintained in embryonic chromatin during development. This information content could directly affect transcriptional regulation in later development or may provide a bias for stochastic aspects of epigenetic regulation.

C. elegans spermatids retain histones, histone variants, and histone modification patterns that were established in the parental germ line. During spermatogenesis in plants and a wide range of animals, histones are largely replaced by protamines, small basic proteins synthesized in late stage spermatids, to facilitate sperm compaction (Balhorn, 2007; Frehlick et al., 2006). While the extent of histone displacement can vary between organisms, histone retention in spermatid chromatin is now recognized as being substantial in many organisms, as histones have been observed in the mature sperm of mammals, amphibians, *Drosophila*, and *C. elegans* (Dorus et al., 2006; Frehlick et al., 2006; Gatewood et al., 1990; Ooi et al., 2006; Palmer et al., 1990; Palmer et al., 1991). Importantly, histones and their modifications are retained at developmental promoters and imprinted loci in human and mouse sperm (Brykczynska et al., 2010; Hammoud et al., 2009). Histones, and any accompanying histone modifications, surviving protamine replacement therefore have the capacity to transmit epigenetic information across generations. Our results show that sperm chromatin can retain epigenetic information that reflects prior genetic activity in the parental germ cells, and which appears to template epigenetic patterns maintained in the early zygote. Inheritance of modified histones is thus likely to be a conserved mode of epigenetic information transferred between generations, and parental transcription may therefore contribute to the content of this information (Katz et al., 2009; van der Heijden et al., 2005).

Recent data provides evidence for such a system. Histone H3K36 methylation, like H3K4me, is normally considered a consequence of transcriptional activity. In budding yeast, the Set2 methyltransferase tracks along with the elongating RNA polymerase holoenzyme, adding this mark within the body of transcribed genes (Li et al.,

2007). Unlike yeast, however, metazoans have more than one H3K36 methyltransferase: in *C. elegans* this additional activity is encoded by the *mes-4* gene, a gene required for germline viability (Capowski et al., 1991). Unlike Set2, MES-4-dependent H3K36 methylation appears to be a maintenance activity--- it can maintain H3K36me in the bodies of genes that are transcriptionally inert(Furuhashi et al., 2010; Rechtsteiner et al., 2010). Amazingly, the genes marked by MES-4 dependent H3K36me in embryos are specifically those that are expressed in adult germ cells, and exclude genes expressed solely in somatic lineages (Furuhashi et al., 2010; Rechtsteiner et al., 2010). MES-4 system is thus maintaining, in the embryo, an "epigenetic memory" of transcription in genes that were last transcribed in adults. Intriguingly, we recently found that the conserved MLL H3K4-specific methyltransferase complex is essential for transcription-independent activity in the early embryo, and this activity, like MES-4, is largely required for the maintenance of H3K4me in early embryonic chromatin (T. Li and W. Kelly, Plos Genetics, in press).

It is clear that not all of the epigenetic information transferred into the zygote is stable; indeed there is extensive chromatin remodeling and epigenetic reprogramming observed after fertilization in most species. However, some information survives and is maintained; how this maintenance is achieved is only now beginning to be identified. Whereas DNA methylation has a clear propagation intermediate and activity for maintenance (e.g., hemi-methylated DNA and DNMT1 methyltransferase, respectively), mechanisms that can maintain a memory composed of histone modifications have been less clear. The inheritance of repressive histone marks, however, has received significant attention. The well-known mitotic inheritance and propagation of H3K27 methylation by

the PRC2 complex, for example, requires the H3K27me3-recognition domain of the EED subunit (Sarma et al., 2008). It is thus likely that marks such as H3K4me2 could also have analogous propagation mechanisms. Histones and their marks that are inherited from parental chromatin could "seed" an epigenetic template that is recognized and propagated in the absence of transcription in the zygote. This would presumably involve H3K4me recognition proteins capable of recruiting methyltransferase activities. The epigenetic signature thus retained or re-established may be maintained with meta-stable fidelity in the germ line epigenome across generations to guide and maintain germ cell function and pluripotency. In somatic lineages at each generation, however, this information would become increasingly altered during tissue-specific zygotic transcription, thereby leading to decreased pluripotency in differentiated cells.

Our results predict that epigenetic information involving H3K4me2, if ectopically presented to the zygote, could become stabilized through germline passage and have functional consequences for transcription patterns in the offspring. H3K4 methylation has been directly implicated in the heritable maintenance of transcriptional activity in *Drosophila* via the Trithorax system, and more recently in *Dictyostelium* (Muramoto et al., 2010; Schuettengruber et al., 2007). Indeed, lysine 4 of H3 has been specifically implicated in developmental epigenetic "programming", and this residue in H3.3 has been reported to be essential for fertility in *Drosophila*, although the specific role of K4 in the fertility defects has been questioned (Hodl and Basler, 2009; Ng and Gurdon, 2008; Sakai et al., 2009). We have shown that mutations in *spr-5*, a *C. elegans* ortholog of the H3K4me2 demethylase Lsd1/Kdm1, cause a generation-dependent build-up of H3K4me2 levels in germ cell chromatin, resulting in sperm-specific transcriptional defects and

ultimately germ cell failure (Katz et al., 2009). This is strong evidence that histone modifications can become heritably stable across generations, and that the ectopic presence of H3K4me in the germline epigenome can have lasting consequences on transcriptional regulation in subsequent generations. Removal of H3K4me2 acquired during spermatogenesis from spermatid or sperm pronuclear chromatin may thus be required to prevent its inappropriate templating in the zygote and subsequent generations. How the information established at any locus is targeted for maintenance or erasure is not known in any system and remains an important question.

It is interesting that the somatic expression of the KW1336 transgene in the offspring appeared to be influenced by its activity in the parental germ cells. We have not been able to test directly whether *ectopic* activation of a transgene in the germ line can cause heritable ectopic activation in offspring. However initiation of the repressed state, e.g., the spontaneous silencing of active transgenes in adult germ cells, is always heritable and appears to be very stable: such silenced transgenes have never been observed to reactivate in our lab under normal conditions, even after many generations (these studies and W. Kelly, unpublished). It is unclear whether parental germ line activity can be as stably and heritably instructive as parental germline repression, and this question clearly requires further inquiry. Indeed, the germ line appears to be preferentially poised for repression in *C. elegans*, with numerous interrelated RNAi and chromatin based mechanisms targeting exogenous DNA for silencing (e.g.,(Kelly and Fire, 1998)). The persistence of H3K4me2 on the outcrossed *KW1336* transgene (in the absence of detectable expression in the adult) could reflect persistence of a heritable chromatin state in transgene chromatin that is either unrelated to the regulation of the

GFP reporter, or is insufficient to overcome other modes of repression that target the transgene in both soma and germ line. Expression in the adult germ cells and the consequential increased inheritance of "active chromatin" may help overcome or prevent these modes of repression and may favorably bias expression in the offspring.

The relationship of iXi in the offspring to transcriptional repression in the adult germ line in both worms and some mammals is striking, irrespective of the issue of continuity of the inactive state. As in *C. elegans*, iXi in mice is meta-stable, in that it is ultimately reversed: it is stabilized in the first lineages to differentiate, the extra-embryonic tissues, but (as in *C. elegans*) becomes unstable in embryonic cells and is ultimately replaced by another dosage compensation process, random X inactivation (Mak et al., 2004; Okamoto et al., 2004). In metatherians that have been tested, iXi occurs despite the absence of an XIST locus (Duret et al., 2006; Hore et al., 2007; Shevchenko et al., 2007). Some forms of iXi could thus have arisen or been adapted from a mechanism similar to the templated chromatin assembly we observe in *C. elegans.* Marsupial X-linked loci silenced during meiosis are activated in round spermatids, yet still subjected to iXi in the zygote (Mahadevaiah et al., 2009). This may suggest a temporal restriction for the establishment of a chromatin state that can be interpreted by the zygote.

iXi in mice does not require the maintenance DNA methyltransferase Dnmt1, but does involve repressive histone modifications (Lewis et al., 2004; Mak et al., 2002; Sado et al., 2000; Sado et al., 2004; Silva et al., 2003; Wang et al., 2001). These features are consistent with the theory that histone modifications may be the more ancient imprinting mark. Indeed, epigenetic imprinting phenomena have been observed in many organisms

independent of DNA methylation, such as *C. elegans* and numerous insect species (Bean et al., 2004; Bongiorni and Prantera, 2003; Lloyd, 2000; Sha and Fire, 2005). Furthermore, H3K4 methylation has been proposed to affect the establishment of *de novo* DNA methylation, such as imprinted DNA methylation (Ooi et al., 2007). Mammalian imprinting may thus be an adaptation imposed upon more ancient, DNA methylation-independent modes of imprinted gene regulation. We propose that sex-specific incorporation of epigenetic information via transcription-linked processes (or lack thereof) in the adult germ line may be a conserved process that could guide imprinted chromatin assembly in the offspring. A sex-specific difference in transcriptional activity during gametogenesis could thus provide an underlying mechanism that contributes to imprint establishment in the germ line, and perhaps influence imprinted gene regulation during embryonic development.

MATERIALS AND METHODS

Strains. We used standard techniques for worm maintenance and handling. We carried out all crosses and grew all worms at 20° C. We used the following strains: wild-type N2 (Bristol), mnt12 (X;IV fusion), zuIs178 [(his-72^{1kb}::HIS-72::GFP); *unc-119(ed3)*, gift from S. Henikoff], *his-69(gk394), his-70* (gift from D. Chu), *him-5(e1490);*axIs36 (pes-10::GFP, *dpy-20(e1282)*), mIs10 (myo-2::GFP, pes-10::gfp, F22B7.9::gfp, V), mes-4(bn85)/DnT1 (*unc-?(n754), let-?* qIs50(myo-2::GFP, pes-10::GFP, F22B7.9::GFP), gift from S. Strome, KW1336 *unc-4(e120) let-858(cc500)* II; unc-4(e120)let-858 Ex1336 [pBK48.1 (let-858::GFP)];pRF4), Ex1336 [pBK48 (let-858::GFP)], PD7271 ((*pha-1(e2123)* III; *ccEX7271*(pBK48.1[*let-858::GFP*] and pC1 [*pha-1*(+)])), *dtIs372* (*his-24*::HIS-24::GFP, X), gift from B. Conradt, PD3861 (*pha-1(e2123ts) III; ccIn3861 (pha-*

1 (+) *unc-54p*::GFP)) gift from A. Fire, KW1864 (*ckIn2 his-73*::GFP, *rol-6*), PD4251 (*ccIs4251* (*myo-3p*::nuclear GFP,*myo-3p*::mitochondrial GFP, *dpy-20*) *dpy-20(e1282)*), DG1575 (*tnIs6 lim-7*::GFP, *rol-6), ccIn4810* lamin::GFP (X). TJ375 (*hsp-16-2::GFP::unc-54 3' UTR*) gift from J. Priess,

Immunocytochemistry. Whole-mount fixation and antibody staining of worms and embryos with methanol/acetone fixation was done as previously described (Strome and Wood, 1983). For detection of specific histone modifications, we used the following primary antibodies at the specified dilutions: rabbit antibody to H3K4me2 (1:500; Millipore), mouse antibody to GFP (1:200, Millipore), mouse monoclonal #34 against H3.1 (1:300) (van der Heijden et al., 2005), mouse monoclonal CMA301 specific for unmodified H3K4 (H3K4me0; 1:500; a gift from H. Kimura, Osaka University, Japan), rabbit polyclonal antibody to H3K4me3 (1:1000 Abcam), rabbit polyclonal to H3K9me3 (1:100 Abcam). Secondary antibodies were used at 1:500: Alexaflour^{TM};594 donkey anti-rabbit IgG, Alexaflour^{TM}; 488 donkey anti mouse IgG Alexaflour^{TM};594 donkey anti rat IgG (Molecular Probes). Fluorescein-labeled antibody to digoxigenin was used at 1:200 (Roche).

Immunofluorescence Analyses. Images were obtained using a Leica DMRA microscope outfitted with a Q-imaging Retiga-SRV Fast 1394 Camera. We acquired and processed the images with Simple PCI software. For analyses of H3K4me2 in transgene chromatin in the embryo, embryonic nuclei were optically sectioned at intervals of 0.1-0.2 micrometers to detect transgene arrays. Array chromatin in one to four cell embryos

was scored blind to the genetic background of the animals as one of the following: None (-), Low (+) or Hi (++) relative to the autosomes and imprinted Xp in the same embryo.

FISH analysis. For combined histone antibody and DNA FISH experiments, we did sequential antibody staining, and DNA FISH with a digoxigenin-labeled probe detected by a fluorescein-labeled anti-digoxigenin antibody (Roche), as previously described(Bean et al., 2004). We carried out FISH using the digoxigenin-labeled (Roche) probe L4054 (Fire Lab vector Kit) that recognizes the sequence for GFP in transgenic worms. X-paint probe (Figure 3.1 E and F) was generated by digoxigenin labeling of a mixture of X-chromosome YACs (generous gift from G. Csankovszki). We observed the prepared samples and recorded and processed images as described above.

Protein Preparation and Western Blot. Mixed stage embryos were harvested from gravid adults by standard procedure (Epstein and Shakes, 1995). Purified sperm were a kind gift of S. L'Hernault. To each 150 ul sample 450 ul of grinding buffer (15 mM HEPES, 10 mM KCl, 5 mM MgCl2, 0.5 mM EGTA, 15% Glycerol, 1 mM DTT, 1X complete mini EDTA-free protease inhibitor cocktail [Roche]) were added, frozen in liquid nitrogen, homogenized, re-frozen in liquid nitrogen, and thawed on ice for 15 mins. 600 ul of 2X extraction buffer (500mM NaCl, 0.8% NP40, 10 mM HEPES (pH 7.5), 1mM MgCl2, 1mM DTT, 1X protease inhibitor) were added to samples, vortexed and rocked for 30 mins. at 4°C. Samples were centrifuged at 8000g for 5 mins. at 4°C, and supernatant was removed. Pellet was re-suspended in 222 ul digestion buffer (0.4% NP40, 10 mM HEPES, 1 mM MgCl2, 1 mM DTT, 1X protease inhibitor cocktail). 30 ul

of sample was sonicated in a sonicating water bath for 15 mins. and DNA concentration was quantified with a BioRad VersaFluor Fluoremeter using the BioRad Fluorescent DNA Quantitation kit. Equivalent amounts of DNA for each sample were loaded and run on a 15% SDS-PAGE gel, transferred and probed with antibodies: H3 (1:5000; Abcam ab1791), H3K4me2 (1:5000; Abcam), H3K4me0 (1:5000, a gift from H. Kimura, Osaka University, Japan) H2A (1:5000 Upstate). Primary antibodies were detected by Goat anti-rabbit IgG HRP conjugated secondary antibody (1:5000; Upstate Biotechnology, Inc.) and detected by chemiluminescence with the Amersham ECL Plus Detection kit.

GFP expression analysis. KW1336 or outcrossed animals (F20+) rolling adults carrying the Ex1336 transgene were cloned, and mixed stage embryos were harvested from gravid adults by a brief (20 second) hypochlorite treatment and either harvested or allowed to develop. Embryos were then transferred to painted-well slides and examined at 100X by phase contrast and GFP using a Leica DMRA microscope outfitted with a Q-imaging Retiga-SRV Fast 1394 Camera. 1.5- to 3- fold embryos were scored + or – for the presence of detectable nuclear LET-858:GFP fluorescence. Animals were allowed to develop at 20 degrees for 36hrs (L2's), 60 hrs (L3/L4's) or 84hrs (adults) and mounted on 5% agrose in microbeads and images were captured at 40X by phase contrast and GFP as above. 5-10 gut nuclei and 5 background areas were measured per animal. The average nuclear GFP intensity minus the average background intensity for each animal was calculated to give average adjusted GFP intensity. 17-22 animals were scored for each background at each time point.

Table 3.1 Germline Expression and H3K4 Methylation in Different Transgenes.

Expression of the GFP reporter in adult germ cells is compared to the enrichment of H3K4me2 in the transgene chromatin in the offspring.

Table 3.1 Germline Expression and H3K4 Methylation in Different Transgenes

Strain	**Transgene Name**	**Transgene Constructs**	**Chromosome**	**Germline Expression**	**H3K4me2 in Pachytene**	**H3K4me2 in Embryos**
N2 (XO)	*n.a.*	n.a.	X	no	no	no
N2	*n.a.*	n.a.	A	yes	yes	yes
SP646 (mnt12)	*n.a.*	n.a.	X/A	no/yes	no/yes	no/yes
JH103	*axIs36*	*pes-10::GFP, dpy-20*	X	no	no	no
PD4793	*mIs10*	*pes-10::GFP, myo-2::GFP, gut::GFP*	V	no	no	no
KW1336 (*let-858*)	*Ex1336*	*let-858::GFP, rol-6*	Ex	yes	yes	yes (71%)
KW1336 outcross	*Ex1336*	*let-858::GFP, rol-6*	Ex	no	no	no (72%)
IN373	*dtIs372*	*his-24::GFP, rol-6*	X	yes	yes	yes
PD3861	*ccIn3861*	*pha-1 (+) unc-54p::GFP*	V	no	no	no
mes-4 (bn85)/DnT1	*qIs50*	*myo-2::GFP, pes-10::GFP, F22B7.9::GFP*	V	no	no	no
PD7271	*ccEx7271*	*let-858::GFP, pha-1 (+)*	Ex	no	no	no
KW1864*	*ckIn2*	*his-73::GFP, rol-6*	X	Low sperm only*	no	no
TJ375		*hsp-16-2::GFP::unc-54 3′ UTR*	A	late pachytene in ~20 nuclei	no	N.D.
PD4251**	*ccIs4251*	*myo-3p::nuclear GFP,myo-3p::mitochondrial GFP, dpy-20*	I	no	35% yes **(N=65)	47% yes **(N=38)
DG1575	*tnIs6*	*lim-7::GFP, rol-6*	X	no	no	no
CB1489	*ccIn4810*	*lmn-1::GFP*	X	no	no	no

*The GFP expression in the KW1864 transgene is only detected in post-meiotic sperm (T.M. Edwards and W. Kelly, unpublished)

**The PD4251 transgene has been selected for non-mosaic expression, and does not display gamete-of-origin imprinting effects ([14], and A. Fire, pers. communication)

Figure 3.1 Sex Body Formation and Imprint Establishment are X DNA Autonomous. The male X chromosome lacks H3K4me2 and forms a dense, sex body-like, ball in wild-type pachytene nuclei (A, arrow), and continues to lack H3K4me2 after chromatin assembly in the zygote (B, arrow) (one cell embryo shown). In L4 hermaphrodite spermatogenesis *mnt12* pachytene nuclei (C), one half of the X:IV fusion chromosome lacks H3K4me2 and forms a dense ball (arrow) while the other half remains elongated and accumulates H3K4me2 (arrowhead). In *mnt12* zygotes (D), only half of the fusion chromosome lacks H3K4me2 (arrow) while the other half accumulates H3K4me2 (arrowhead) (one cell embryo shown). (E) Pachytene nucleus in adult animal carrying *mnT12* IV:X fusion. H3K4me2 on IV chromatin does not appreciably spread into X sequences. (F) X-chromosome DNA in an *mnt12* IV:X fusion oocyte has H3K4me2 at a level indistinguishable from the attached autosomal DNA. H3K4me2 (green), DAPI (red). DNA FISH (blue) Scale bars, 5um.

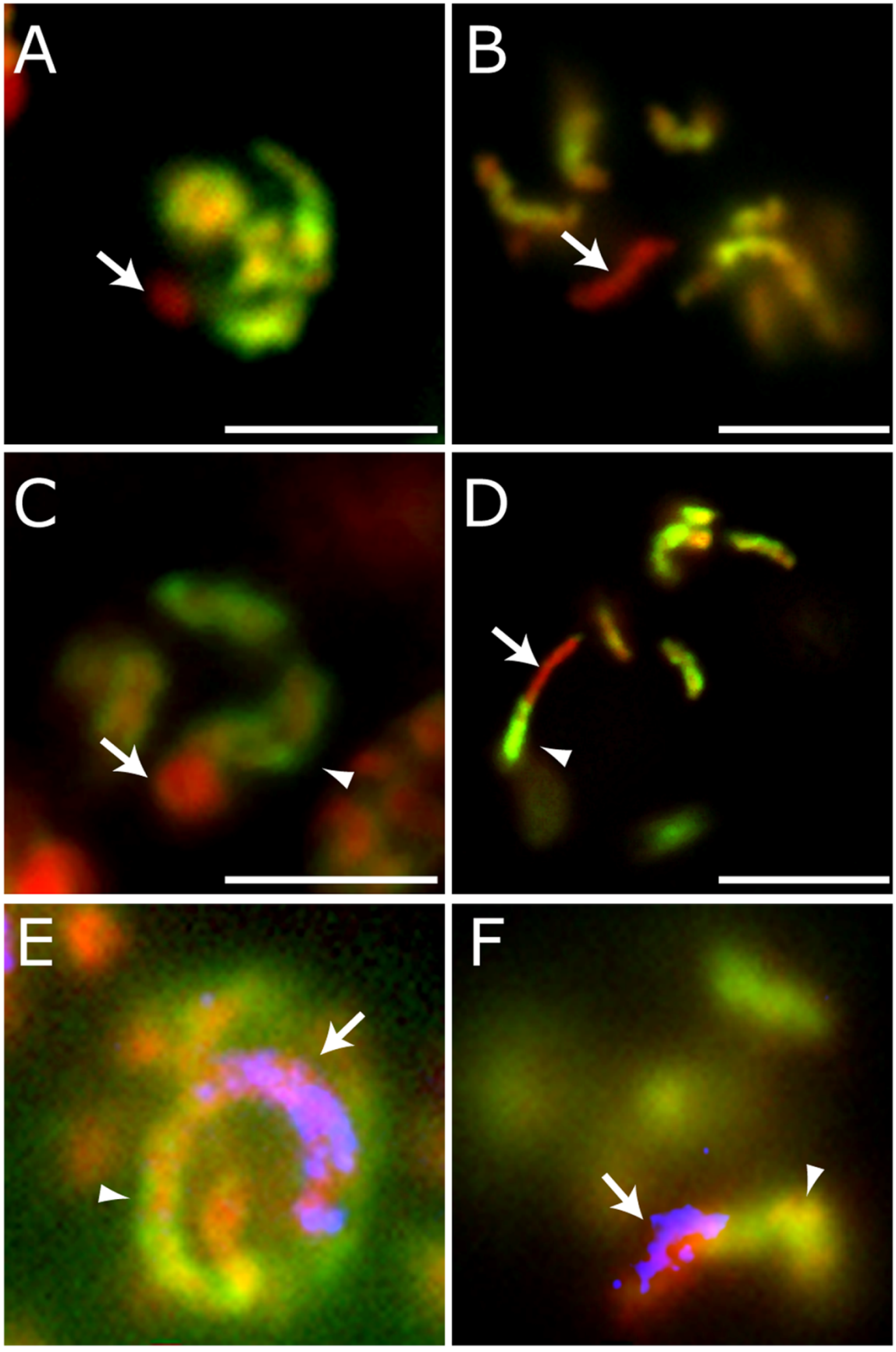

A
B
C
D
E
F

Figure 3.2 H3.3, H3.1, and H3K4me0 Dynamics in Oogenesis and Spermatogenesis. (A-H) Comparison of H3.3::GFP (A, Bi-Di) to H3K4me2 (Bii-Dii), and H3.1 (E, Fi-Hi) to H3K4me2 (Fii-Hii), in hermaphrodite germ cells. (A) H3.3 is low in the distal nuclei of the gonad (d), and accumulates as nuclei progress towards the proximal (p) end. H3.3::GFP is absent from X in pachytene nuclei (Bi; arrow), identified by lack of H3K4me2 (Bii). H3.3::GFP is present on all chromosomes in mature oocytes (Ci), as is H3K4me2 (Cii). H3.3::GFP is absent from X's in larval hermaphrodite (spermatogenic) pachytene nuclei (Di; arrow), coincident with the absence of H3K4me2 (Dii). (E) H3.1 is high in the distal region of the gonad (d), and is depleted as nuclei progress towards the proximal (p) end. H3.1 is enriched on the X in pachytene nuclei (Fi; arrow) as identified by lack of H3K4me2 (Fii). H3.1 is low on all chromosomes in mature oocytes (Gi), while H3K4me2 is abundant (Gii). H3.1 is enriched on the paired X's in larval spermatogenic pachytene nuclei (Hi; arrows) as identified by the absence of H3K4me2 (Hii).

(I, Ji-Li) X chromosome enrichment for H3K4me0 in male germ cells. (I) In both male and hermaphrodite (not shown) germ cells, H3K4me0 is high in the distal region of the gonad (d) and is depleted as nuclei progress towards the proximal (p) end. H3K4me0 is enriched on the X in pachytene hermaphrodite nuclei (Ji; arrow), as identified by lack of H3K4me2 (Jii). H3K4me0 is low on all chromosomes in mature oocytes (Ki) while H3K4me2 is abundant (Kii). H3K4me0 is enriched on the X's in larval hermaphrodite (spermatogenic) pachytene nuclei (Li; arrow) as identified by lack of H3K4me2 (Lii). Antibodies as indicated (green) with DAPI counterstain (red). Scale bars, 5um.

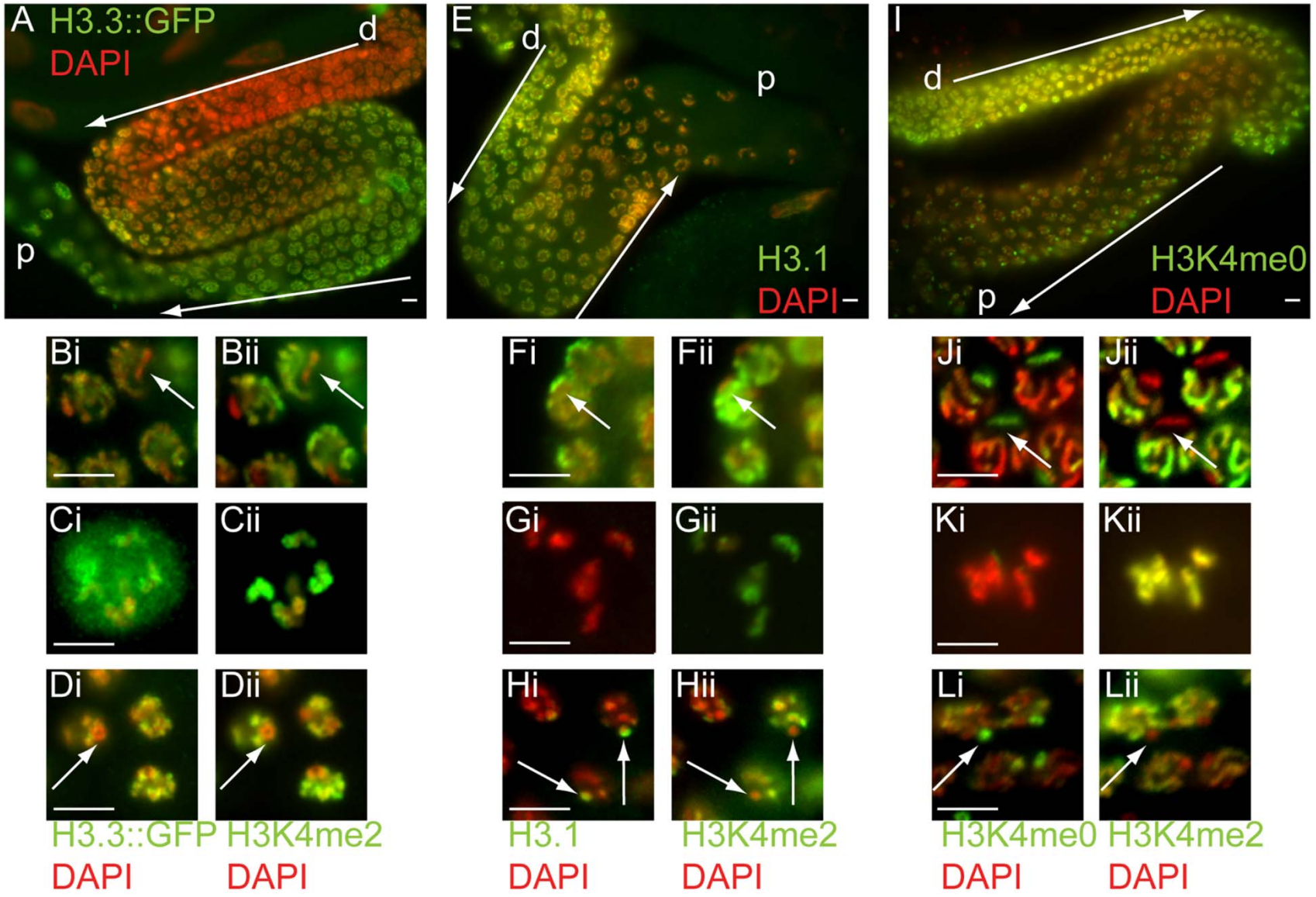

A H3.3::GFP
DAPI
d
p
E
d
p
H3.1
DAPI
I
d
p
H3K4me0
DAPI
Bi
Bii
Ci
Cii
Di
Dii
H3.3::GFP
DAPI
H3K4me2
DAPI
Fi
Fii
Gi
Gii
Hi
Hii
H3.1
DAPI
H3K4me2
DAPI
Ji
Jii
Ki
Kii
Li
Lii
H3K4me0
DAPI
H3K4me2
DAPI

Figure 3.3 Mature Sperm Chromatin Retains Epigenetic Information Established in Meiosis. Antibody staining for H3.3::GFP, H3.1 and H3K4me0 compared to antibody staining for H3K4me2 in mature hermaphrodite spermatids. (A) H3.3::GFP has substantial overlap with H3K4me2, but is depleted from the X region (arrow), which lacks H3K4me2. In contrast, both H3.1(B) and H3K4me0 (C) are enriched on the X chromosome, marked by a lack of H3K4me2 (arrows). Antibodies (green) with DAPI (red). Scale bars, 5um. (D-G) Western blot analysis of histone modifications in mature sperm and embryos. H3 is abundant in mature sperm chromatin at levels near that of embryonic chromatin(D). H3K4me2 is abundant in mature sperm chromatin at levels near that of embryonic chromatin (E). H3K4me0 is detected in both mature sperm and embryonic chromatin (F). H2A is detected in mature sperm chromatin at levels near that of embryonic chromatin, however there is some degradation of the sperm sample (G). Lanes were loaded with DNA equivalents from left to right as follows 1) embryo 1X DNA, 2) embryo 2X DNA, 3) sperm 1X DNA, 4) sperm 2X DNA.

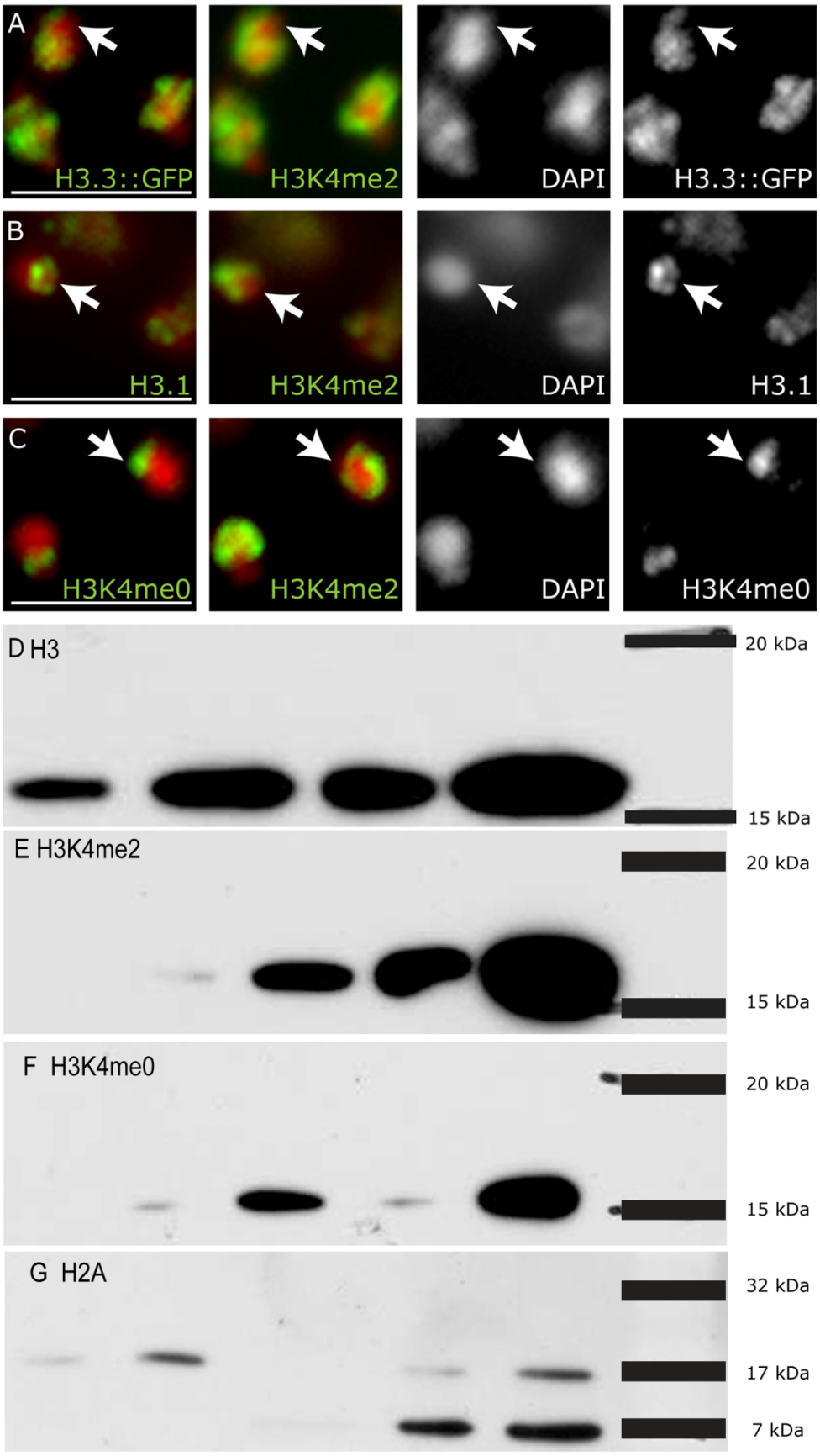

A
H3.3::GFP
H3K4me2
DAPI
H3.3::GFP
B
H3.1
H3K4me2
DAPI
H3.1
C
H3K4me0
H3K4me2
DAPI
H3K4me0
D H3
20 kDa
15 kDa
E H3K4me2
20 kDa
15 kDa
F H3K4me0
20 kDa
15 kDa
G H2A
32 kDa
17 kDa
7 kDa

Figure 3.4 H3.1, H3.3, and H3K4me0 Dynamics in the Early Embryo. In all image sets, a merged image of H3K4me2 (green) and DAPI (red) are shown, while the adjacent gray scale image shows a separate channel corresponding to co-staining with antibodies against H3.1, H3.3, or H3K4me0 as indicated. (Ai) In a one-cell embryo, all chromosomes except the Xp (arrow) incorporate H3K4me2 (green), but very little H3.1 is present on any chromosome (Aii). In 2 cell embryos low levels of H3.1 are detected on all chromosomes, including the Xp (Bi; arrow). Maternally provided H3.3:GFP is immediately incorporated into all sperm chromosomes, including the Xp, in the one cell stage (Cii; arrow), and thereafter (Dii nuclei from two cell embryo). Despite the incorporation of maternal H3.3, the autosomes retain H3K4me2 and the Xp (Ei; arrow) still lacks this modification, and is enriched for H3K4me0 relative to the autosomes (arrow; Eii). By the 2 cell stage and thereafter (Fii and not shown) all chromosomes (including the Xp; arrow), have incorporated high levels of H3K4me0 (Fii), but the Xp still excludes H3K4me2 (Fi; arrow). Scale bars, 5um.

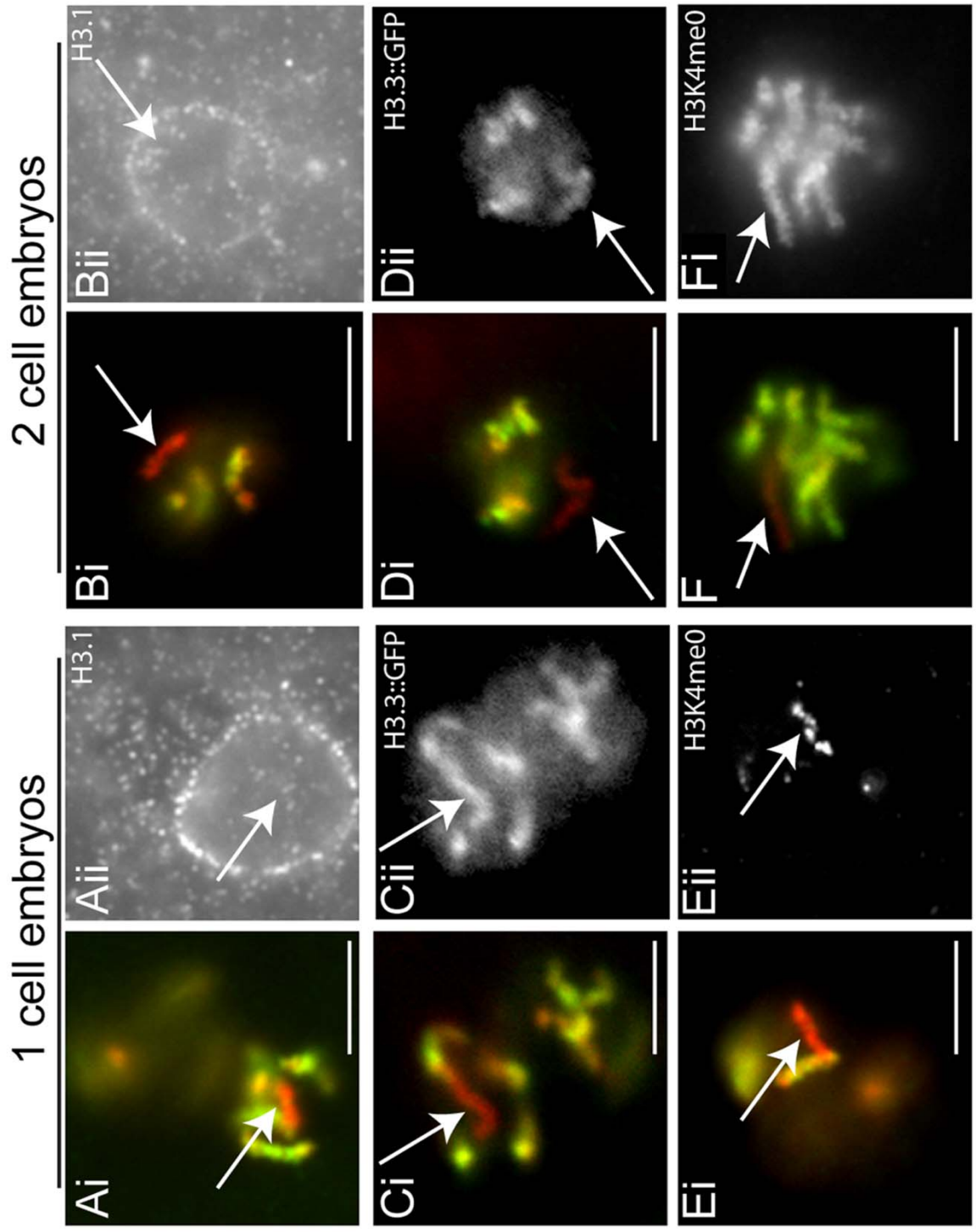

1 cell embryos
2 cell embryos
Ai
Aii
H3.1
Bi
Bii
H3.1
Ci
Cii
H3.3::GFP
Di
Dii
H3.3::GFP
Ei
Eii
H3K4me0
F
Fi
H3K4me0

Figure 3.5 Transcriptional Activity in Parental Germ Cells Influences Chromatin Assembly in the Zygote. (A-E) Chromosomes and transgenic arrays in 1-2 cell embryos with DAPI (red), antibody against H3K4me2 (green), and DNA FISH marking the transgene (blue). Arrowheads in all panels mark the Xp; arrows mark the transgenes. (A) X-linked, germline silent *pes-10::GFP* transgene lacks H3K4me2 on Xm (arrow) in addition to Xp (arrowhead) in embryos. (B) LG V-linked, germline silent *mIs10* transgene (arrow) lacks H3K4me2 in embryos. (C) Germline expressing Ex1336 extra-chromosomal transgene (arrows) accumulates H3K4me2 in embryos. (D) Same transgene as in (C), but lacking adult germline expression and lacks H3K4me2 in embryo (arrow). (E) X-linked, germline expressing *his-24::GFP* transgene accumulates H3K4me2 on Xp in embryo (arrow). (F) Germline silent PD7271 extrachromosomal transgene (arrow) lacks H3K4me2 in the embryo. (G) LG V-linked, germline silent CCIn3861 transgene (arrow) lacks H3K4me2 in embryos. (H) X-linked, germline silent his-24::GFP transgene lacks H3K4me2 in the embryo. Scale bars, 5um.

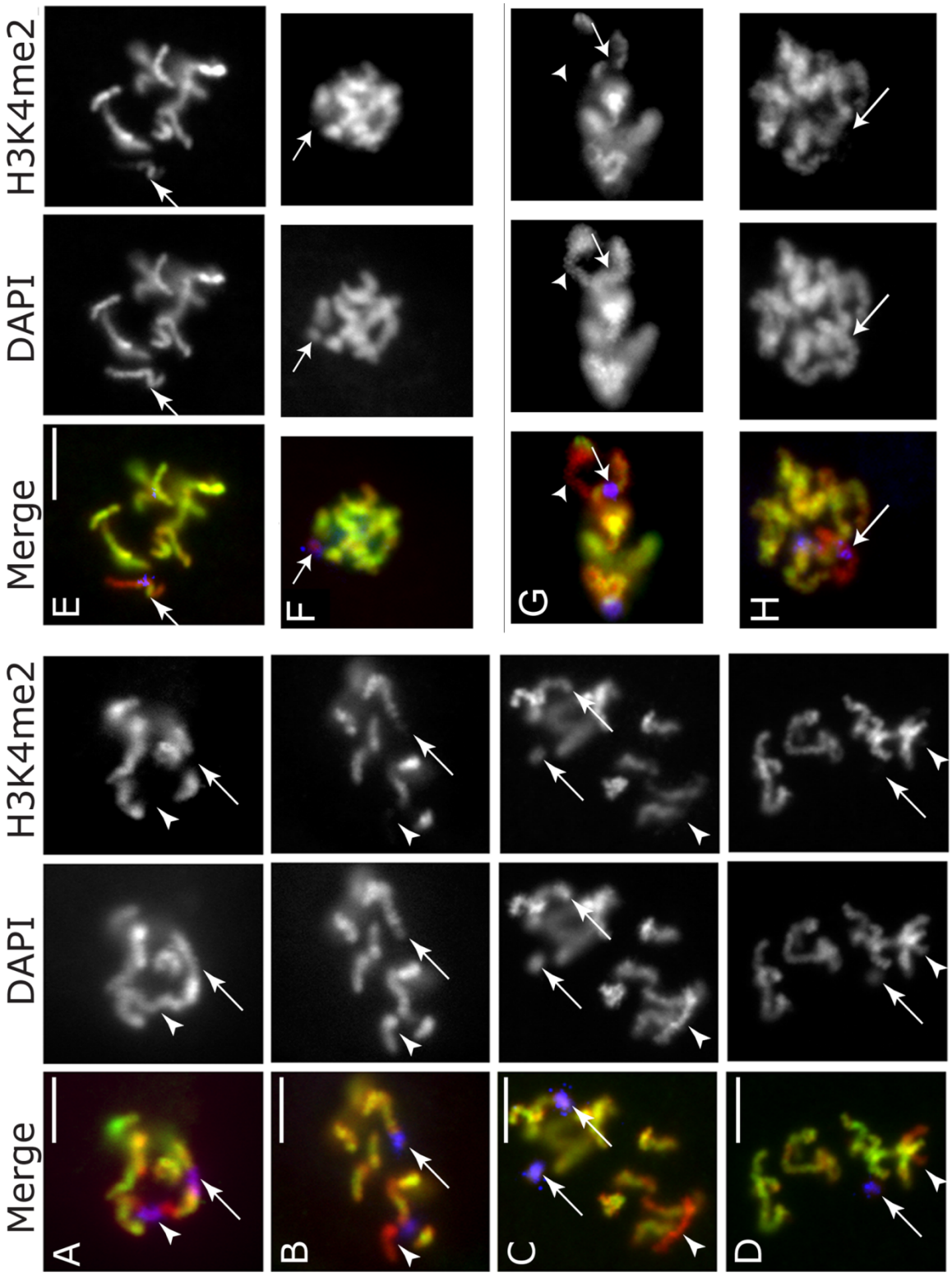

Merge
DAPI
H3K4me2
A
B
C
D
E
F
G
H

Figure 3.6. H3K4me2 in Transgene Chromatin in Germ Cells Correlates with Germline Transcription. (A-E) Pachytene nuclei from adult hermaphrodites with DAPI (red), antibody against H3K4me2 (green), and transgene DNA FISH (blue). (A) X-linked, germline silent *pes-10::GFP* transgene (arrows) lacks H3K4me2, as does the rest of the X chromosome, in pachytene nuclei. (B) LG V-linked, germline silent *mIs10* transgene (arrows) lacks H3K4me2 in pachytene nuclei. (C) Germline expressing Ex1336 extrachromosomal transgene (arrows) accumulates H3K4me2 in pachytene nuclei. (D) Germline silent Ex1336 extrachromosomal transgene (arrow) in wild-type background lacks H3K4me2 in pachytene nuclei. (E) X-linked, germline expressing *his-24::GFP* transgene accumulates H3K4me2 on X in pachytene nuclei (arrow) (FISH not shown). (F) X-linked, germline silent his-24::GFP transgene does not accumulate H3K4me2 on X in pachytene nuclei (arrow).

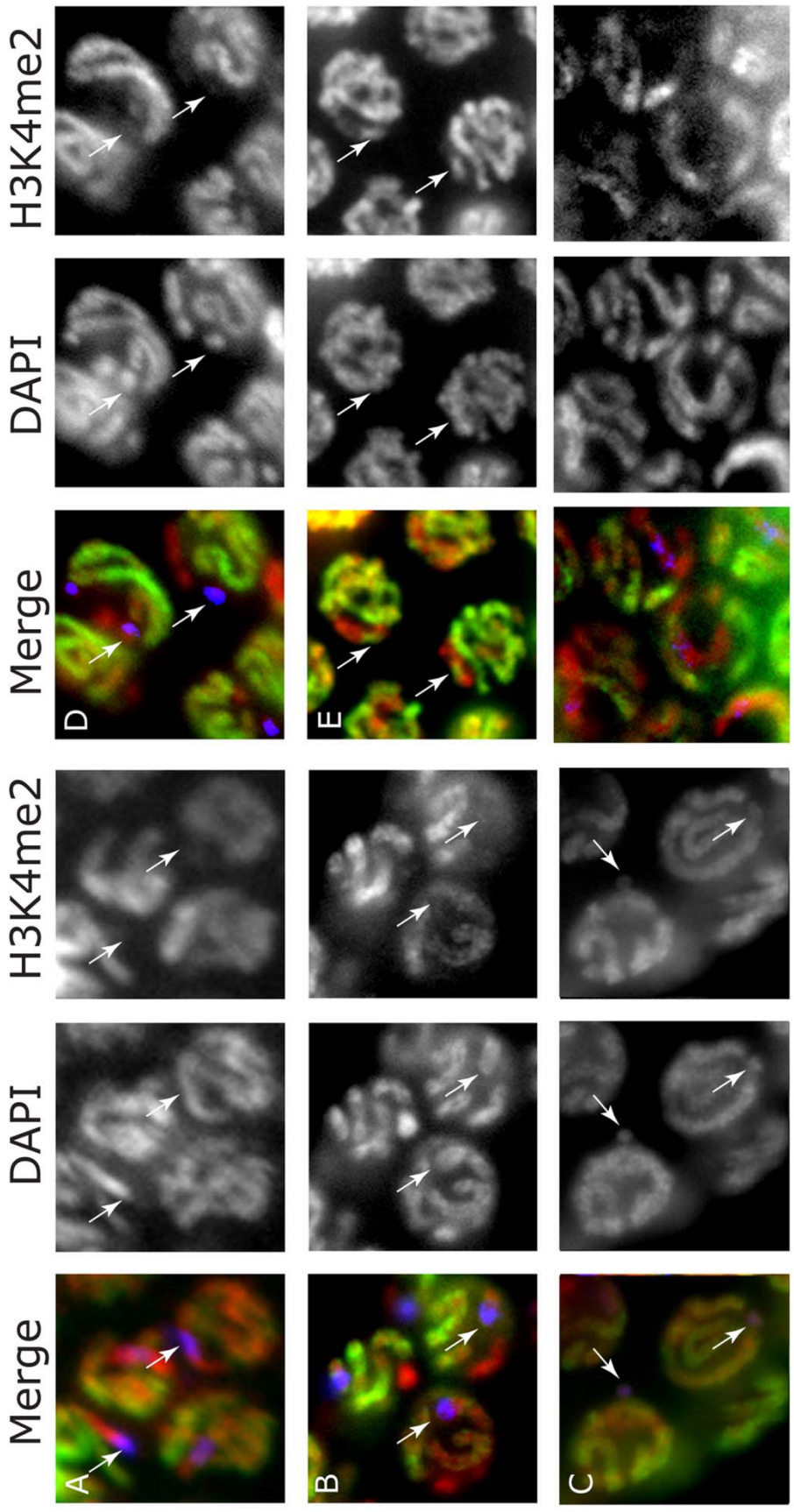

Merge
DAPI
H3K4me2
Merge
DAPI
H3K4me2
A
B
C
D
E

Figure 3.7 H3K4me3 of Transgenes in Germ Cells and Early Embryo Correlates with Germline Transcription. (A-J) Pachytene nuclei from adult hermaphrodites or one to two cell embryos with DAPI (red), antibody against H3K4me2 (green), and DNA FISH (blue). (A) X-linked, germline silent *pes-10::GFP* transgene lacks H3K4me3, as does the rest of the X chromosome, in pachytene nuclei. (B) X- linked *pes-10::GFP* transgene also lacks H3K4me3 on the Xm in a two cell embryo. (C) LG V-linked, germline silent *mIs10* transgene (arrow) lacks H3K4me3 in pachytene nuclei. (D) LG V-linked, germline silent *mIs10* transgene (arrow) also lacks H3K4me3 in a one cell embryo. (E) Germline expressing Ex1336 extrachromosomal transgene (arrows) does not appear to have H3K4me3 in pachytene nuclei. (F) Germline expressing Ex1336 extrachromosomal transgene (arrows) does accumulate H3K4me3 in a one cell embryo. (G) Germline silent Ex1336 extrachromosomal transgene (arrow) in wild-type background lacks H3K4me3 in pachytene nuclei. (H) Germline silent Ex1336 extrachromosomal transgene (arrow) in wild-type background lacks H3K4me3 in a one cell embryo. (I) X-linked, germline silent *his-24::GFP* transgene lacks H3K4me3 on X in pachytene nuclei (arrow). (J) X-linked germline silent *his-24::GFP* transgene lacks H3K4me3 on Xp in one cell embryo.

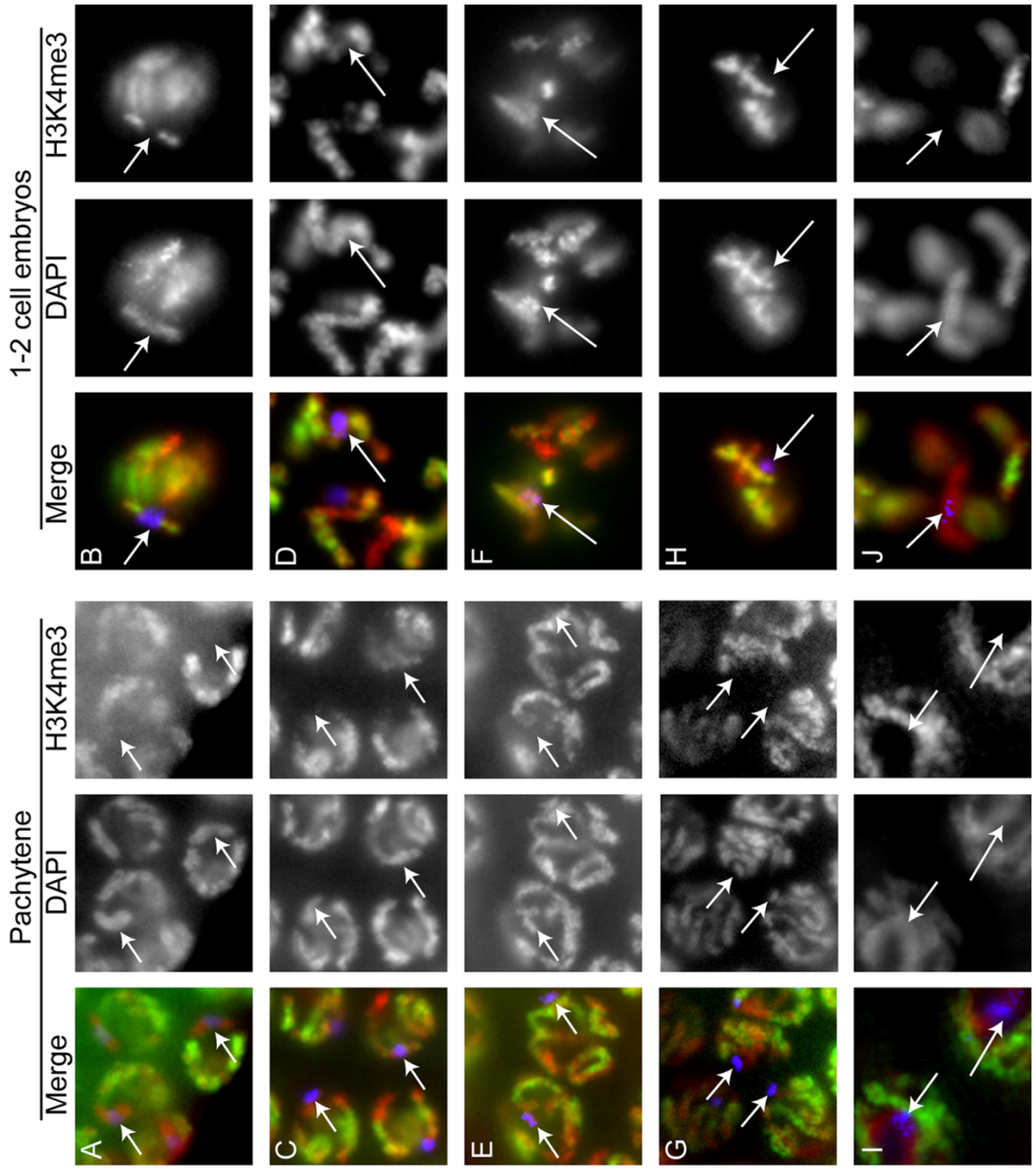

Pachytene
1-2 cell embryos
Merge
DAPI
H3K4me3
A
B
C
D
E
F
G
H
I
J

Figure 3.8 Transgene Germline GFP Expression vs. Transgene H3K4me2 in Embryos (A) Schematic of selection of animals during this experiment. At each generation animals were cloned out and allowed to lay. Half of the animals were scored live for germline GFP expression and the remaining animals were dissected to retrieve offspring for DNA FISH and anti-H3K4me2 immunofluorescence (B) % of animals expressing let-858::GFP in the germline as adults at each generation (blue bars) vs. % of offspring from each generation retaining H3K4me2 on the transgene as embryos (red bars). Number of animals scored is indicated in parentheses.

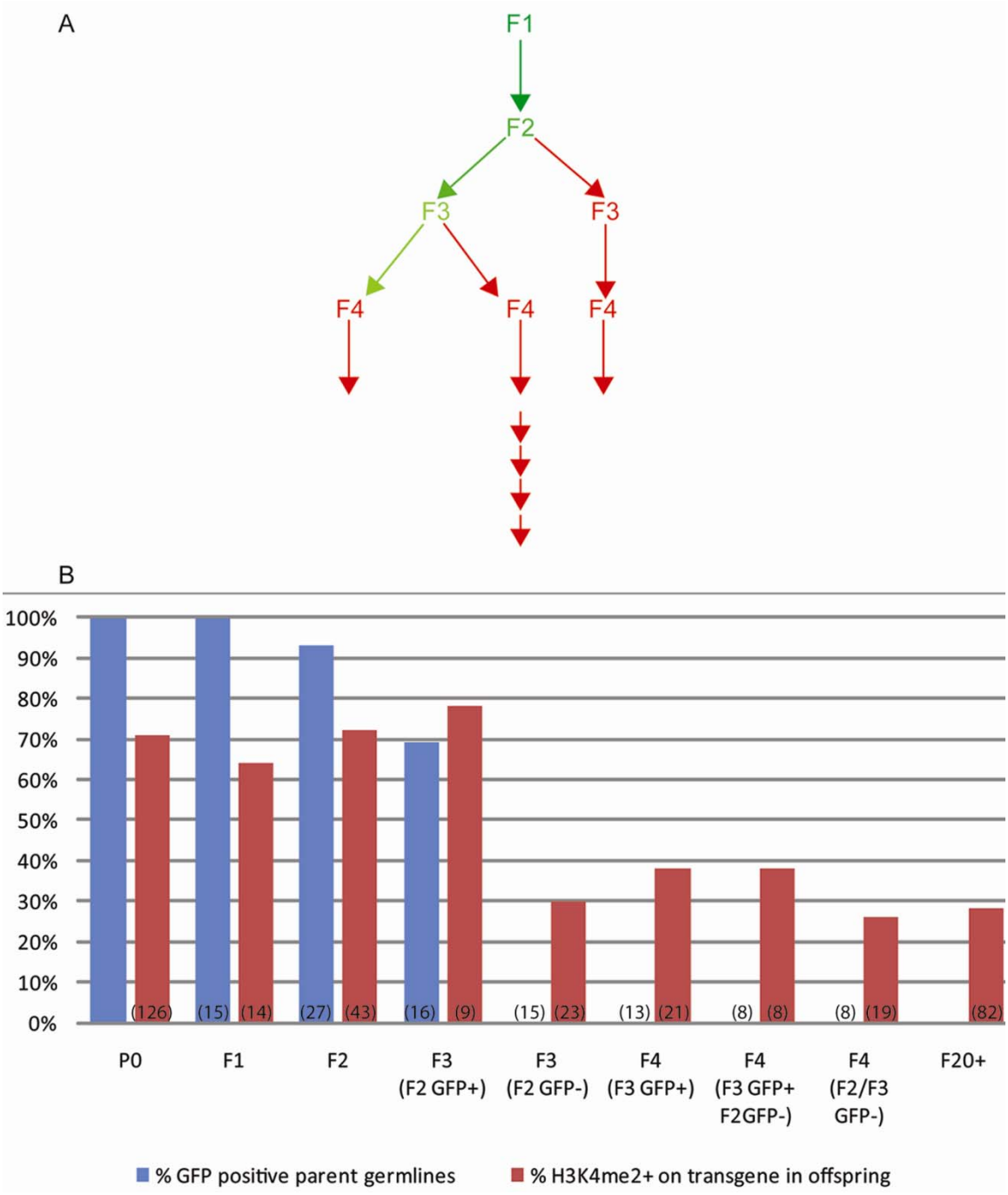
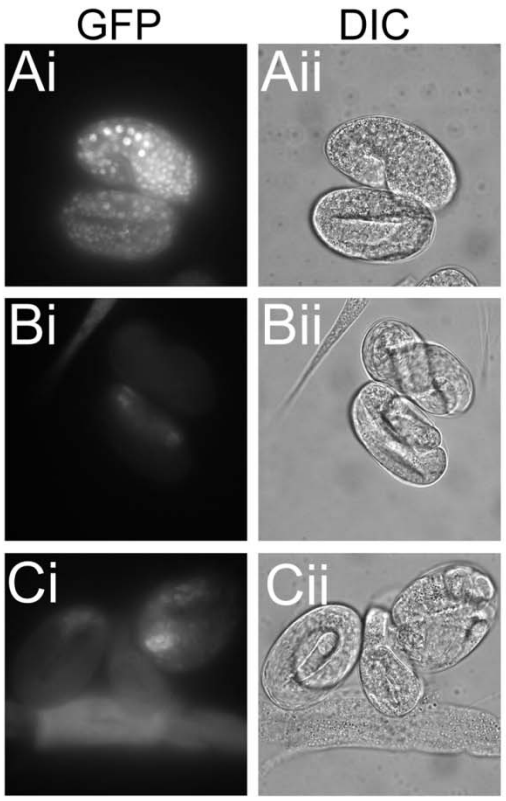
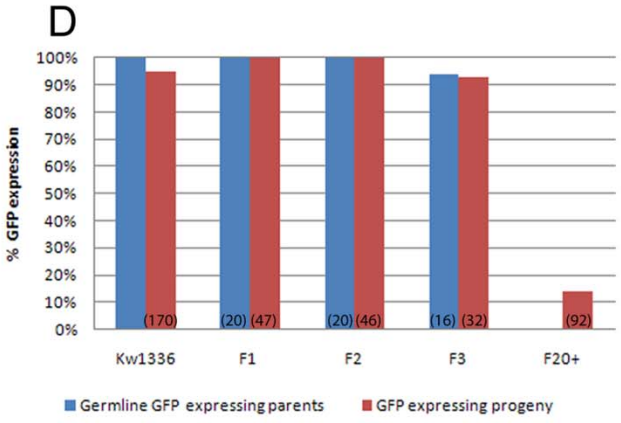

A
F1
F2
F3
F3
F4
F4
F4
B
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
(126)
(15)
(14)
(27)
(43)
(16)
(9)
(15)
(23)
(13)
(21)
(8)
(8)
(8)
(19)
(82)
P0
F1
F2
F3 (F2 GFP+)
F3 (F2 GFP-)
F4 (F3 GFP+)
F4 (F3 GFP+ F2GFP-)
F4 (F2/F3 GFP-)
F20+
% GFP positive parent germlines
% H3K4me2+ on transgene in offspring

Figure 3.9 Embryonic and Somatic GFP Intensity is Higher in Offspring From Germline Expressing than in Offspring from Germline Silent Parents. GFP fluorescence (Ai-Ci) or DIC (Aii-Cii) microscopy of 1.5-fold to 3–fold stage live embryos . (Ai) 93% (n=27) of embryos from KW1336 offspring from parents which express *let-858:gfp* in the germline show robust GFP expression in all nuclei. (Bi) 87% (n=47) of offspring from outcrossed animals where germline expression of *let-858:gfp* was lost lacked any GFP positive nuclei (approximately 40% of offspring inherit the array). (Ci) Rare (13% n=47) embryos from outcrossed parents where germline expression of *let-858:gfp* was lost with GFP positive nuclei (far right) have weaker and more variegated GFP expression than offspring from germline expressing parents (Ai). (D) % of animals expressing let-858::GFP in the germline as adults at each generation (blue bars) vs. % of offspring expressing somatic GFP as embryos (red bars). Number of animals scored is indicated in parentheses. (E) Average GFP intensity (arbitrary units) of somatic nuclei in offspring from germline GFP expressing (KW1336) parents vs. germline GFP silenced (N2 outcrossed 20+ generations) parents at larval stages (L2 and L3/L4) and adults. Number of animals scored is indicated in parentheses.


GFP
DIC
Ai
Aii
Bi
Bii
Ci
Cii


D
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% GFP expression
(170)
(20) (47)
(20) (46)
(16) (32)
(92)
Kw1336
F1
F2
F3
F20+
Germline GFP expressing parents
GFP expressing progeny

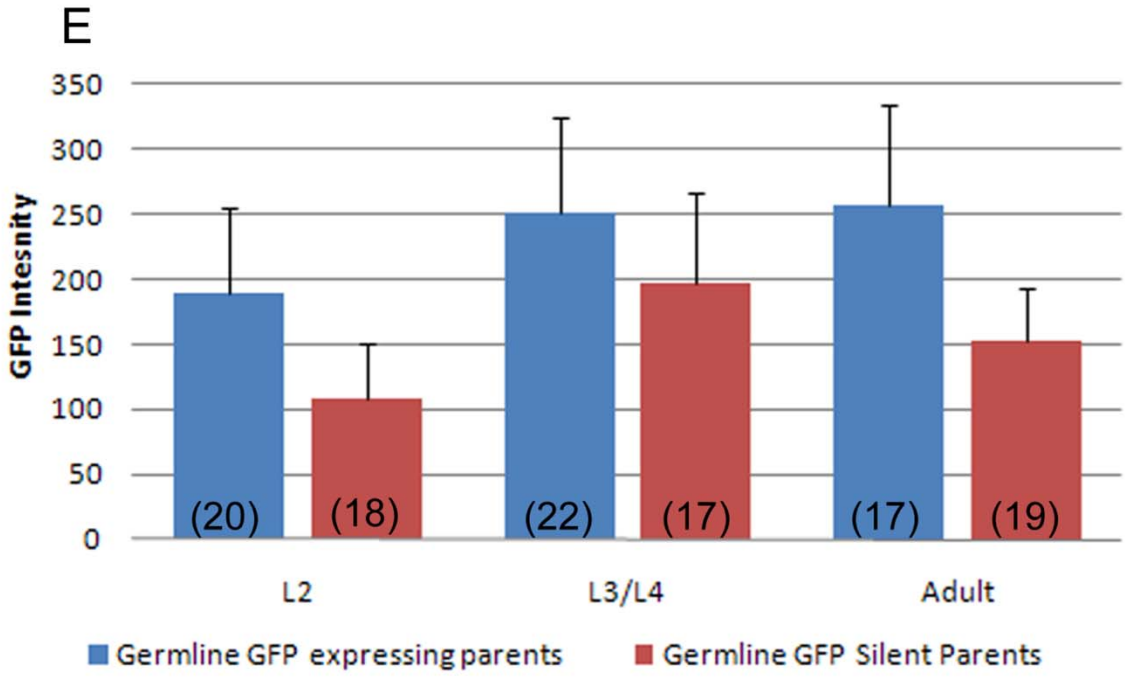
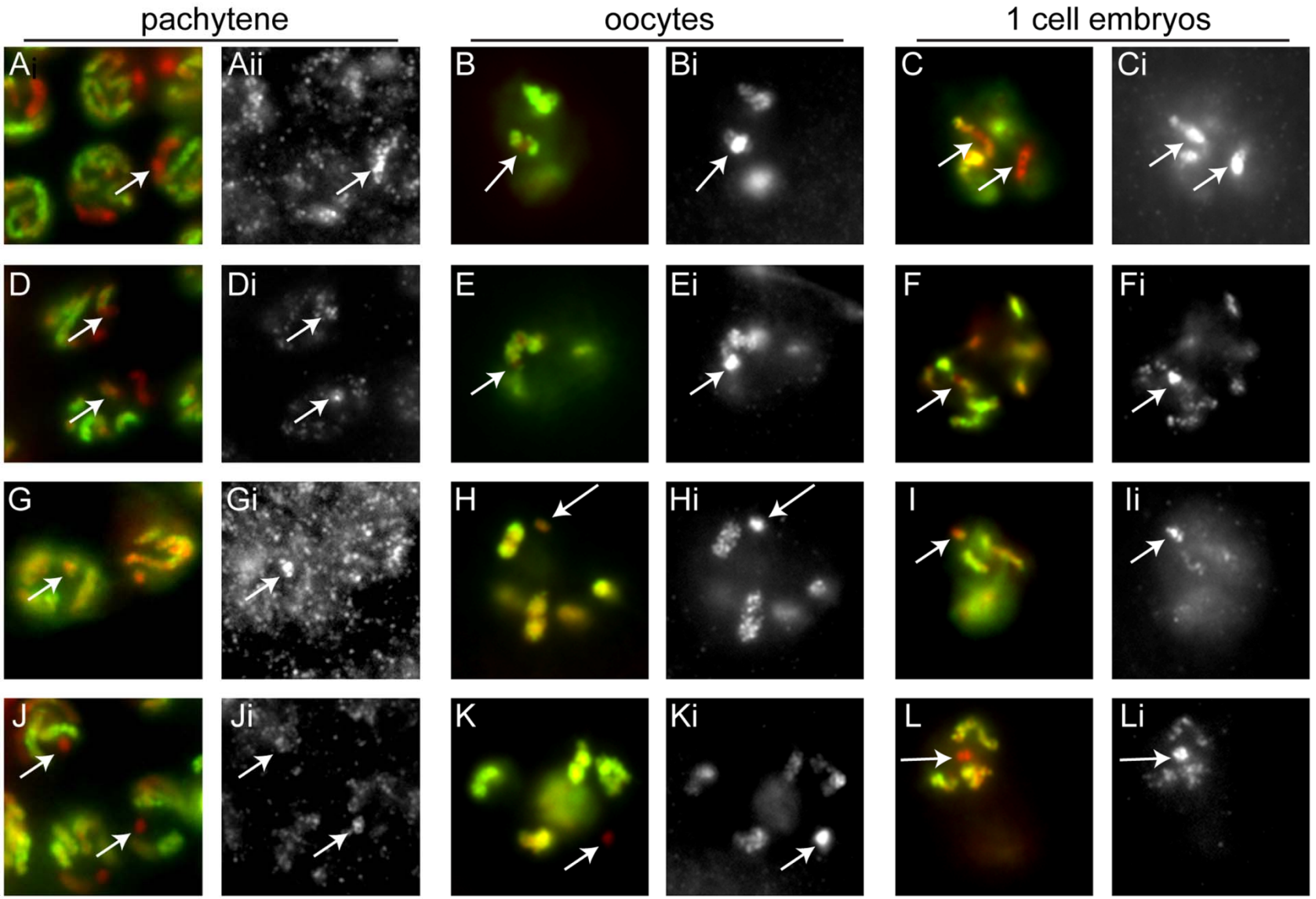

E
350
300
250
200
150
100
50
0
GFP Intesnity
(20)
(18)
(22)
(17)
(17)
(19)
L2
L3/L4
Adult
Germline GFP expressing parents
Germline GFP Silent Parents

Figure 3.10 Transgenes are Enriched for H3K9me3 in Pachytene, Oocytes, and Early Embryos Independent of Transcription in the Germline. Pachytene nuclei, oocytes, and nuclei from one or two cell embryos with H3K4me2 (green) and DAPI (red) (Ai-Li) or H3K9me3 (Aii-Lii). (Ai-Ci) X-linked, germline silent *pes-10::GFP* transgene (arrow) is enriched for H3K9me3 over autosomes and surrounding X-chromatin(Aii-Cii), (Di-Fi) LG V-linked, germline silent *mIs10* transgene identified by lack H3K4me2 (arrow) is enriched for H3K9me3 over autosomes and surrounding chromatin (Dii-Fii). (Gi-Ii) Germline expressing Ex1336 extrachromosomal transgene (arrows) is enriched for H3K9me3 over autosomes, particularly in oocytes and early embryos (Gii-Iii). (Ji-Li) Germline silent extrachromosomal array PD7271 (arrow) is enriched for H3K9me3 over autosomes, particularly in oocytes and early embryos (Jii-Lii).


pachytene
oocytes
1 cell embryos
Ai
Aii
B
Bi
C
Ci
D
Di
E
Ei
F
Fi
G
Gi
H
Hi
I
Ii
J
Ji
K
Ki
L
Li

Chapter 4

Transcription of Imprinted Non-Coding RNAs in Mammalian Spermatogenesis

INTRODUCTION

Genomic imprinting, biasing or monoallelic gene expression based on whether the allele was inherited maternally or paternally, was discovered in mammals by elegant experiments by McGrath and Solter and independently by Surani et al in 1984 (McGrath and Solter, 1984; Surani et al., 1984). Although it had been known for some time that uniparental gynogenesis or androgenesis was embryonic lethal, this was originally thought to be due to the homozygosity of recessive lethal genes. Experiments in the 1980's showed that bi-parental gynogenesis or androgenesis was also embryonic lethal and further that uniparental disomy of particular genomic regions had opposite phenotypes, depending on which parent the duplication was inherited from (Cattanach and Kirk, 1985; McGrath and Solter, 1984; Surani et al., 1984). These experiments suggested that something unique and necessary was independently contributed by the maternal and paternal genomes. Evidence of a specific gene subjected to genomic imprinting was described in 1991 by Barlow *et al.* and Dechiara *et al.* (Barlow et al., 1991; DeChiara et al., 1991). Together these groups demonstrated that a functional copy of Igf2 was contributed maternally while a functional copy of Igf2r was contributed paternally in mouse (Barlow et al., 1991; DeChiara et al., 1991). To date 144 imprinted genes have been confirmed in mouse (see: http://www.har.mrc.ac.uk/research/genomic_imprinting/) and many of these are imprinted in the same pattern in humans (http://igc.otago.ac.nz/home.html).

Imprinted genes typically occur in clusters and are CpG rich, with an imprint control region (ICR) that is differentially methylated depending on parent of origin. Long non-coding RNAs (ncRNAs) are expressed from loci near the ICR in many

imprinted clusters, and are often transcribed in the opposite orientation of the protein coding genes that are repressed on the same allele indicating roles for these ncRNAs in gene regulation in *cis* (Sleutels et al., 2002; Smilinich et al., 1999). While DNA methylation and histone modifications regulate or maintain imprinted gene expression in the offspring, how the imprints are established in the parental germline in mammals, or any system, is unknown.

As discussed in previous chapters, one well-studied example of imprinting in mammals is imprinted X chromosome inactivation (iXi), in which the entire paternal X chromosome is inactive prior to implantation (Mak et al., 2004). iXi is maintained in only the placental tissues of eutherians, but persists in embryonic lineages in marsupials (Mak et al., 2004; Sharman, 1971). Unlike most genomic imprints, iXi does not require the maintenance DNA methyltransferase Dnmt1 (Lewis et al., 2004; Sado et al., 2000; Sado et al., 2004). It does however require repressive histone modification H3K9me2 as well as H3K27me3 established by the polycomb group histone methyltransferases (Mak et al., 2002; Silva et al., 2003; Wang et al., 2001). DNA methylation at gene promoters on the inactive X chromosome is one of the final events of random X inactivation, however, DNA methylation does not appear to be necessary for iXi in the eutherian trophectoderm nor in marsupials (Kaslow and Migeon, 1987; Sado et al., 2000). These features are consistent with the theory that histone modifications are the more ancient imprinting mark.

Our lab has previously described a chromatin based imprinting phenomenon in *C. elegans* (Bean et al., 2004). This work (described in Chapter 3) suggests a model by which transcription in the parents' germline in *C. elegans* may establish a chromatin

environment that is inherited by the offspring. Based on these findings, we hypothesized that transcription of imprinted non-coding RNA's in the parents' germline in mice may set up a chromatin environment that could provide the basis for differential DNA methylation and ultimately imprint establishment in mouse. We would predict that paternally expressed imprinted ncRNAs would be expressed during spermatogenesis and not oogenesis, and reciprocally that maternally expressed imprinted ncRNAs would be expressed during oogenesis and not spermatogenesis.

The long non-coding RNA that is essential for X inactivation in eutherians is named *Xist* (X-inactive specific transcript). *Xist* along with its antisense transcript *Tsix* (X-inactive specific transcript, antisense) are expressed from the Xic (X-inactivation center) and control both iXi discussed above, and the random X inactivation process that governs dosage compensation in placental mammals. *Xist* is expressed from the X chromosome that will become inactive and coats that chromosome silencing it *in cis*, in part by recruiting Polycomb group proteins involved in heterochromatin formation. (de Napoles et al., 2004; Mak et al., 2002). *Tsix* expression from the active X represses *Xist* expression, thereby preventing inactivation *in cis* (Lee, 2000; Lee and Lu, 1999; Sado et al., 2001).

Air is another well-studied long ncRNA involved in mammalian genomic imprinting at the *Igf2r* locus. The ICR at the *Igf2r* locus is methylated on the maternally inherited chromosomes, which represses *Air* transcription, and allows for expression of the adjacent imprinted genes at this locus. The paternally inherited allele is un-methylated at the ICR, allowing for transcription of *Air* which correlates with repression of the imprinted protein coding genes in *cis* (Sleutels et al., 2002). Deletion of either the

Air promoter or truncation of the transcript results in expression of the normally silenced imprinted genes from the paternal allele at this locus, suggesting that either transcription of the ncRNA or the transcript itself functions to silence these genes. G9a, a histone methyl-transferase responsible for di-methylation at H3K9, has been shown to interact with *Air* and to be required for repression of the imprinted gene *Slc22a3*, which is antisense to *Air* (Nagano et al., 2008). However, G9a was not necessary for silencing of Igf2r (Nagano et al., 2008). Additionally, the chromatin region that becomes enriched with *Air* lacks RNA polymerase II and active histone modifications, and colocalizes with PRC 1 and 2 components (Terranova et al., 2008). Due to these associations, *Air* is believed to function in a manner similar to *Xist* by recruiting histone modifying and remodeling enzymes to the imprinted region to mediate silencing.

Another long ncRNA *Kcnq1ot1* has also been shown to regulate an imprinted cluster in *cis*. Like *Air*, the *Kcnq1ot1* promoter is methylated on the maternal allele and un-methylated on the paternal allele, and is antisense to the major protein coding transcript in the cluster *Kcnq1*. The absence of methylation on the paternal allele allows for transcription of the long non-coding RNA, which leads to silencing of the imprinted protein coding transcripts at this locus in *cis* (Mancini-Dinardo et al., 2006; Shin et al., 2008; Thakur et al., 2004). Methylation of the DMR ICR on the maternal allele represses *Kcnq1ot1* and allows for transcription of the imprinted genes including *Kcnq1* (Mancini-DiNardo et al., 2003; Pandey et al., 2004). Similar to *Xist*, *Kcnq1ot1* can interact with repressive histone methyltransferases Ezh2 and G9a (Pandey et al., 2008; Terranova et al., 2008).

One of the first imprinted gene clusters discovered, and one of the best characterized is the Igf2/H19 locus (Bartolomei et al., 1991). The Igf2/H19 locus is distinctive in that the ICR at this locus is methylated on the paternal allele; only 2 other paternally methylated ICRs have been described, Meg3 and Rasgrf1 (http://igc.otago.ac.nz/home.html). CpG methylation on the paternal allele prevents the insulator protein CTCF from binding, allowing a distal enhancer to stimulate the Igf2 promoter. On the maternal allele, the ICR is un-methylated, allowing CTCF to bind and act as an insulator, preventing Igf2 expression, while allowing expression of the ncRNA *H19* (Bell and Felsenfeld, 2000; Hark et al., 2000; Kanduri et al., 2000; Szabo et al., 2000). Loss of the ICR and *H19,* or loss of the *H19* transcriptional until alone, results in phenotypically normal mice, even though this results in loss of imprinting of the maternal allele and biallelic expression of Igf2r (Leighton et al., 1995; Thorvaldsen et al., 1998). How *H19* may play a role in repressing Igf2 is not established. There is no evidence that the *H19* transcript interacts with polycomb group proteins and in fact, the RNA is typically located in the cytoplasm (Schoenfelder et al., 2007). However, the H19 promoter interacts with cohesins and may modulate expression of imprinted genes at the locus by an insulator function (Nativio et al., 2009; Stedman et al., 2008). *H19* has been implicated as a tumor suppressor (Hao et al., 1993) as well as having other regulatory functions, but how the ncRNA mediates regulation is not understood.

In Chapter 3 we formed a model, which suggests that transcription in the germline of *C. elegans* can establish an epigenetic pattern that is passed into gametes, is inherited by the embryo, and may affect transcription in the embr offspring. Based on this model, we asked whether this hypothesis could be extended to imprinting in

mammals. We hypothesized that transcription of imprinted long ncRNAs in the parents germline could establish a chromatin environment at that locus that could be inherited and establish imprinted expression of the ncRNA in the offspring in a parent of origin fashion. In order to test this we examined the expression of the four ncRNAs discussed above in staged spermatocytes and spermatids from mice by quantitative RT-PCR. In contrast to our hypothesis, we observed that paternally expressed imprinted ncRNAs were expressed at negligible levels compared to a major sperm transcript in developing mouse spermatocytes and spermatids. Similarly contradicting our hypothesis, we readily detected the presence of the maternally expressed H19 transcript in spermatocytes and spermatids, in line with previously published results. These data suggest that, unlike our model for *C. elegans*, imprint establishment in mice may not be regulated by germline transcription in a parent of origin fashion, leaving the mechanisms still unknown.

RESULTS

If parental germ line expression of ncRNAs were involved in establishing imprints in mammals, then we would predict that ncRNAs that are expressed from the paternal, and not the maternal allele, in the offspring would be expressed during spermatogenesis, and not oogenesis. Conversely, we would predict that ncRNAs expressed from the maternal allele, and not the paternal allele, in the offspring would be expressed during oogenesis and not spermatogenesis, according to our model. In order to test this hypothesis we began by examining transcript levels of *Xist*, *Air*, *Kcnq1ot1*, and *H19* in spermatocytes and spermatids.

In mice, oogenesis initiates at 13.5 days post coitus, making retrieval of developing oocytes from embryos technically difficult (Adams and McLaren, 2002). We

therefore chose to begin with spermatocytes and spermatids provided by the McCarrey lab for the pilot experiments. Pgk-2 (phosphoglycerate kinase two), an autosomal housekeeping gene expressed exclusively in meiotic and post meiotic testis, was used as a positive control (VandeBerg et al., 1973; Yoshioka et al., 2007). During iXi *Xist* ncRNA is expressed from and coats the paternally inherited X chromosome. *Xist* has also been previously been detected by (35 cycles of) RT-PCR in spermatogenesis (McCarrey and Dilworth, 1992). We therefore predicted *Xist* would be expressed in developing spermatocytes and spermatids, based on both published data and our model. Similarly, we predicted paternally expressed *Air* and *Kcnq1ot1* would be expressed during spermatogenesis. The *Pgk-2* transcript was readily detected in oligo-dT primed and random primed reverse transcriptase (RT) plus spermatocytes and spermatid PCR samples. *Pgk-2* expression was higher in spermatids in both samples and there was no detectable *pgk-2* transcript in the RT-minus negative control (Figure 4.1 A). However, no significant expression of any of the paternally expressed transcripts was observed (Figure 4.1 A). Of the ncRNAs, *Kcnq1ot1* had the highest expression level, although the levels were negligible compared to *pgk-2*. *Xist* and primer sets to three different regions of the *Air* transcript did not produce significant levels of product by quantitative RT-PCR (Figure 4.1 A).

H19 is a maternally expressed transcript, and we would not expect to detect its expression during spermatogenesis based on our model. Contrary to our model, *H19* transcript was detected in both spermatocytes and spermatids (Figure 4.1 B). Although sperm RNA samples had been treated with DNAse, the RT- samples also produced some product, perhaps amplified off of environmental contamination, as a similar intensity

band was amplified in the blank (no template added) control. Despite the contamination in the RT- samples, RT+ samples had much more product. However, these samples could not be quantified due to the contamination. Strengthening our result, the *H19* transcript has also been detected in mouse and rat spermatogenic cells previously by other groups (McCarrey et al., 1999; Shima et al., 2004).

These results show that paternally expressed imprinted ncRNAs are not significantly expressed in either spermatocytes or spermatids compared to a major sperm transcript, *pgk-2* (Figure 4.1 A), and that the maternally expressed ncRNA *H19* is expressed during spermatogenesis (Figure 4.1 B). These findings do not fit with our hypothesis and suggest that genomic imprints in mice are not established by germline transcription in the parent, as may be the case in *C. elegans*.

DISCUSSION

We have shown that the paternally expressed ncRNAs *Xist*, *Kcnq1ot* and *Air* are not significantly expressed in mouse spermatocytes or spermatids compared to a major sperm specific transcript, *pgk-2*. Additionally, we have been able to detect the maternally expressed ncRNA *H19* in mouse spermatocytes and spermatids. In Chapter 3 we formed a model for iXi in *C. elegans*, where transcription in the parents germline establishes a chromatin environment that is packaged into mature gametes and maintained in early embryo, and could influence gene expression in the offspring. We speculated that this model might also hold true for genomically imprinted genes in mouse. Based on that model we hypothesized that expression of ncRNAs in the parent could establish a chromatin environment that was inherited by the offspring and could potentiate biased expression of that ncRNA from that parental allele in the offspring. However our results

suggest that our hypothesis in correct, and a more complicated mechanims is likely involved in regulating imprint establishment in mammals. In contrast to our hypothesis, paternally expressed ncRNAs, such as *Air* and *Kcnq10t*, were not expressed in developing sperm while the maternal ncRNAs *H19* was.

Another group has studied the role of transcription in imprint establishment in mammalian oocytes. They showed that transcription of the coding gene *Nesp* across the differentially methylated region (DMR) within the ICR in oocytes is necessary for acquisition of methylation at the maternal DMR (Chotalia et al., 2009). As this transcript, as well as other coding transcripts that cross DMRs, was found shortly prior to and during the time when genomic DMR methylation appears, the authors suggested that transcription across a DMR maybe generally required for acquisition of maternal germline methylation at those loci. The authors suggest that this transcription leads to a chromatin state that allows access to the DNA methyltransferase Dmnt3a and its cofactor Dmnt3L which are necessary for *de novo* germline methylation at imprinted loci (Chotalia et al., 2009). This speculation is confusing due to previous observations that 1) transcription is closely associated H3K4me2/3 in general and 2) the Dnmt3L component of *de novo* DNA methylation specifically interacts with histones un-methylated on H3K4 (Ooi et al., 2007). Pursuing these studies at paternally methylated DMR during spermatogenesis is needed to broaden the implication of these results. Finally, this model still leaves unanswered the question; what is the event establishing imprints in the parents' germline? Why does transcription at these loci lead to recruitment of *de novo* DNA methyltransferases, while other non-imprinted loci expressed during gametogenesis remain unaffected by imprinting?

In mice the DNA-methylated vs. un-methylated ICR have distinct histone modification patterns in somatic tissue. H4K20me3 and H3K9me3 are enriched on the methylated ICR in somatic cells, while the un-methylated allele is characterized by H3K4me2 and H3K9/K14Ac (Reviewed in (Koerner et al., 2009)). These patterns are also observed in spermatogenesis before protamine exchange, suggesting that inheritance of these histone modifications may allow for imprints to be carried across generations (Delaval et al., 2007). In particular, the presence of H3K4me2/3 on the ICR inherited though sperm could indirectly prevent DNA methylation on the paternal allele (Delaval et al., 2007). More recent in-depth analysis of histones and histone modifications in mature sperm has revealed that although present at low levels genome wide, histones are retained at developmentally regulated loci such as HOX gene clusters and imprinted loci (Brykczynska et al., 2010; Hammoud et al., 2009). Consistent with the above study, Hammoud *et al* found H3K4me3 at paternally expressed imprinted loci (Hammoud et al., 2009). This chromatin status could either prime these loci for expression in the offspring, or indirectly prevent DNA methylation from silencing them.

It's possible that the antithesis of our hypothesis is true: that parental expression of the ncRNA somehow predisposes it to a repressed state in the offspring, as suggested by one study (Chotalia et al., 2009) However this seems unlikely, as the tendency to observe similar transcriptional activity at the same locus, at least between cycles of mitotic replication is well supported, but the tendency for expression leading to silencing is unprecedented. The only exception to conflicting chromatin signals are bivalent domains in embryonic stem cells. Bernstein *et al*. first described bivalent domains, where both the repressive histone modification H3K27me3 as well as the active histone

modification H3K4me3 were found at the same locus in mouse embryonic stem cells in 2006. Initial reports suggested that bivalent domains predisposed those genes for activation (Azuara et al., 2006; Bernstein et al., 2006). However, more recent reports showed that in mouse embryonic fibroblasts only half of the biallelic domains are resolved as highly expressed H3K4me2 regions (Mikkelsen et al., 2007). The remaining domains were either silenced or expressed at a low level. It is possible that germline transcription of these ncRNAs establishes an H3K4me2/3 positive domain that is additionally enriched in H3K27me3 and allows for additional factors to impact imprinted expression in the embryo based on parent of origin.

MATERIALS AND METHODS

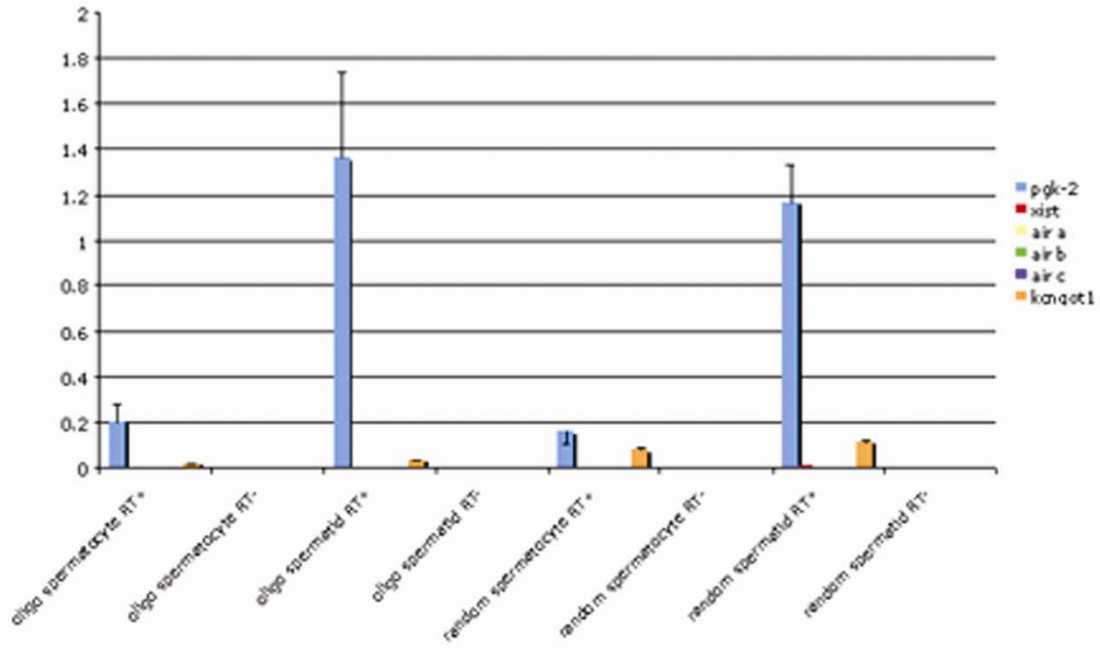
Quantitative RT-PCR spermatocytes and spermatid RNA were kind gifts from John McCarrey. Reverse transcription was carried out using SuperScript III reverse transcriptase (Invitrogen) with random and oligo-dT primers. The qPCR analyses were performed using a real-time PCR instrument (Applied Biosystems 7300 Real-time PCR system) with supplied reaction mix (LightCycler 480 SYBR Green 1 Master Mix; Roche Applied Sciences). Input cDNA was amplified under conditions of 95°C for 10 min followed by 40 cycles of steps 95°C for 15 seconds, annealing at specified temperatures for 1 minute and 72°C for 30 seconds. PCR primers and annealing temperatures are listed in Table 4.1.

Table 4.1 Primers Used for Q-RT-PCR

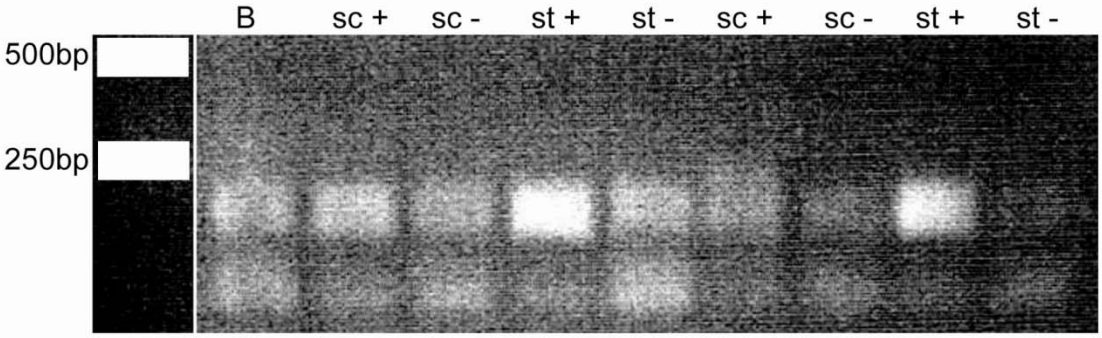
primer name	Primer sequence	ncRNA	size	annealing temperature
JKA13	TGGACCGAGTGATAAGAACTAC	Air a Fwd	113	62
JKA14	GGCTATTGCTAAGTGGCTACTAC	Air a Rev		
JKA15	ACTTTGACAGAACAATCGGCTCAG	Air b Fwd	145	62
JKA16	GAACATTTGCAAAGGACAGTCGAG	Air b Rev		
JKA17	GACCAGTTCCGCCCGTTT	Air c Fwd	79	62
JKA18	GCAAGACCACAAAATATTGAAAAGAC	Air c Rev		
JKA19	TTGGATTACTTCGGTGGGCT	kcnq10t1 Fwd	119	60
JKA20	ACACGGATGAAAACCACGCT	kcnq10t1 Rev		
JKA21	CATCCAGCCTTCTTGAACACC	H19 Fwd	82	62
JKA22	GGGAAAAGTGAAAGAACAGACGG	H19 Rev		
JKA23	CATGGCCTTCCGTGTTCCTA	Gap DH Fwd	82	
JKA24	TGTCATCATACTTGGCAGGTTT	Gap DH Rev		
JKA25	TGCCTGGATTTAGAGGAGTGAAG	Xist Fwd	74	60
JKA26	TCCTTGAGTCTCACATAGGGATTG	Xist Rev		
JKA27	TGGTGGTGGAATGGCTTACACCTTCCTG	Pgk-2 Fwd	85	68
JKA28	TCGTGGCTCCCTCTTCATCAAACAAGG	Pgk-2 Rev		
JKA31	TCGCAGCAGAATGGCACATA	H19 fwd	149	62
JKA32	GGCAACCCTGCACCTCTTCT	H19 REV		

Figure 4.1 Expression of Imprinted ncRNAs in Spermatocytes and Spermatids. A) ncRNA's expressed from the paternal allele in the offspring are not expressed in spermatocytes or spermatids. Relative expression of ncRNA transcripts in spermatocytes and spermatids by RT-Q-PCR compared to an exclusively sperm expressed transcript *pgk-2*. B) imprinted ncRNA *H19*, which is expressed from the maternal allele in the offspring, is expressed in both spermatocytes and spermatids. RT-PCR products from H19. B(blank, no template added), - (no reverse transcriptase), + (reverse transcribed), sc(spermatocytes), st(spermatids). The first four samples were reverse transcribed with oligodT primers. The last four samples were reverse transcribed with random primers.

A


2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
pgk-2
xist
air a
air b
air c
kcnqot1
oligo spermatocyte RT+
oligo spermatocyte RT-
oligo spermatid RT+
oligo spermatid RT-
random spermatocyte RT+
random spermatocyte RT-
random spermatid RT+
random spermatid RT-

B


B
sc +
sc -
st +
st -
sc +
sc -
st +
st -
500bp
250bp

Chapter 5

Future Directions

In this work we have put forth a model for imprint establishment in *C. elegans*, where transcription in the parental germline establishes a chromatin environment that is inherited by the early embryo and presented evidence that inheritance of these marks may influence gene expression in the offspring. This imprint, or templated chromatin assembly, is not limited to the paternally inherited X chromosome, or even one sex or another, but appears to be the result in differences in germline transcription alone. Although we were unable to extend this model to mammals, this work nevertheless adds insight to the strongly implied, but still unproven mechanisms behind epigenetic inheritance of histone modifications across cell cycle divisions, as well as across generations.

Model

We have described the dynamics of histone modifications and histone variants during gametogenesis and the differences in these dynamics between the X during spermatogenesis compared to the X during oogenesis or the autosomes in either sex. Futher we have presented evidence that these modification patterns established in the parents' germlines are packaged into mature gametes and inherited by the zygote. Our results suggest the following model of events. In the mitotic region of the germline in both sexes, canonical histone variant H3.1 is incorporated on all chromosomes. In the pachytene region of the gonad, transcription on the autosomes leads to replacement of H3.1 with H3.3 and accumulation of H3K4me2/3, while the transcriptionally inactive X chromosome retains H3.1 and remains unmodified in both sexes. As oogenesis proceeds transcription of oogenesis specific genes from the X chromosome leads to displacement of H3.1 by H3.3 and accumulation of H3K4me2/3. During spermatogenesis however, the

X chromosomes remains transcriptionally inactive and retains H3.1 and H3K4 remains unmodified (Figure 5.1 A). These patterns, enrichment of H3.3/H3K4me2/3 on the Xm and autosomes and persistence of H3.1/H3K4me0 on the Xp, are packaged into mature gametes and inherited by the zygote. Upon fertilization H3.3 is incooporated into the paternal genome (Figure 5.1 B). However in spite of this influx of new H3.3 the patterns of H3K4me2/3 enrichment on the autosomes and not the Xp persists, and is maintained, perhaps by an unknown histone methyl transferase (Figure 5.1 C). This model of establishment of chromatin patterns established in the parents germline, and inherited by and maintained in the offspring could be generally applied to other systems as well.

Future Directions

CHIP-Seq of Histone Modifications at Gene-by-Gene Resolution

Our conclusions are somewhat limited by the resolution of our assay. We used immunofluorescence to examine accumulation or absence of histone modifications and variants at a global chromosome wide level in the early embryo. One can imagine that on a gene-by-gene basis, a more distinct pattern would develop. We observed H3K4me2/3 evenly across entire autosomes in the germline and early embryo, but of course every loci on the autosomes is not expressed during gamogenesis. In order to observe where in the genome H3K4me2/3 accumulates, and whether there are any genes on the paternally inherited Xp that escape the imprint and accumulate H3K4me2/3, a chromatin immunoprecipitation (CHIP) approach is necessary. In these studies we chose to examine chromatin modifications by immunofluorescence, as we had no way to purify large numbers of early 1-2 cell embryos that would be necessary to biochemically distinguish the difference between H3K4me2/3 levels between the Xp and the autosomes

at the stage at which those differences are most distinct. However, in order to answer questions about accumulation and inheritance of histone modifications on a gene-by-gene bases purification of 1-2 cell embryos as well as adult germ cells and gametes is necessary. New techniques for sorting early *C. elegans* embryos have recently been described, and optimization of this technique is underway in our lab (Stoeckius et al., 2009). Once a pure population of 1-2 cell embryos can be collected, we can do chromatin-immunoprecipitation followed by DNA sequencing (Chip-Seq) to determine more precisely where H3K4me2/3 is inherited on a genome wide basis.

By comparing H3K4me2/3 levels genome wide by Chip-Seq in the germline and early embryos we can begin to answer questions about establishment, erasure, and inheritance of histone modifications across generations. Essential house keeping genes that are expressed in both germline and soma may have high, or consistent H3K4me2/3 levels across generations, allowing for robust early zygotic expression of these genes. The status of germline-expressed loci would be highly interesting. These genes would be predicted to acquire high levels of H3K4me2/3 in the germline. However, unlike the essential housekeeping genes just mentioned, inheritance of H3K4me2/3 at germline-specific genes in the embryo could potentiate inappropriate expression there. Examination of germlines and early embryos may reveal erasure of germline-gene specific H3K4me2/3. Indeed, our lab has previously characterized the KDM1 homologue *spr-5* in *C. elegans* and saw increased expression and accumulation of H3K4me2 on sperm specific loci in mutant animals, suggesting that spr-5 is normaly responsible for erasure of H3K4me2 at these loci (Katz et al., 2009). This accumulation led to a germline-mortality defect gradauly leading to sterility over many generations.

Evidence of erasure at meiosis or oogenesis genes could point to the possibility of distinct demethylases acting at these loci.

Alternatively, the histone modification patterns at germline silent somatic loci could be addressed. There are a plethora of autosomal linked genes that are not expressed in the germline; neuronal, or muscle cell specific transcription factors for example. Small regions lacking H3K4me2/3 across the autosomes have not been detected by immunofluorescence due perhaps to the abundance of or high transcriptional levels of housekeeping and germline specific genes on these chromosomes. Although it is likely that germline silent genes dispersed across the genome lack H3K4me2/3, it is also possible that they may have H3K4me2/3. If this proved to be true, such loci should be examined for the presence of repressive histone modifications.

In addition to examining H3K4me2/3 alone, additional histone modifications, both active and repressive could be examined in the germline and early embryo. Unique patterns of chromatin modifications comprised of both active (H3K4me2/3) and repressive (H3K27me3) modifications, termed ‘bivalent domains’, characterize developmental genes in both sperm and ES cells in mammals (Bernstein et al., 2006; Brykczynska et al., 2010; Hammoud et al., 2009). Resolution of bivalent domains into either active or repressed chromatin drives to cell fate decisions and lineage restriction. If germline silent somatic loci are decorated by H3K4me2/3, this may be balanced by H3K27me3 as well. Another modification that would be interesting to examine is H3K36 methylation. H3K36 methylation has been generally correlated with active chromatin, however recent reports have shown that this modification can negatively affect transcription when ectopically recruited to promoters, or cryptic promoters

(Kouzarides, 2007). A recent study for our lab has shown that H3K36me3 is maintained independent of transcription in primordial germ cells and may maintain germline promoters in a "poised" state (Furuhashi et al., 2010). Examining H3K36me3 at the germ cell to zygote transition may reveal a similar bivalent domain as above with H3K36 methylation along with another mark that resolves as H3K36me3 positive loci in the germline lineage, and resolves as repressed chromatin in the somatic lineages. Resolution of inherited chromatin modifications upon fertilization could control the transition between gamete and progeny, while maintenance of bivalent chromatin could allow for perpetuation of the immortal state of the germline in the germ cell progenitors. Additionally a distinct combination of chromatin modifications could differentially mark germline versus housekeeping/cell-cycle/essential loci providing a basis for determining which inherited marks should be maintained and which should be erased across generations.

Candidate RNAi screen for Modifiers of the Imprint

A question that remains to be answered is whether there are any consequences of inheriting histone modifications across generations. Preliminary data presented in Chapter 3 suggest that offspring who inherit active histone modifications may have higher or more robust expression of those genes post-embryonically. In order to test the consequences of inheriting modification patterns established in the parent, it would be necessary to use inducible gene expression to establish these marks at specific loci or disruption of the X-imprint. In our studies two transgenes showing expression late in gametogenesis (HIS-73::GFP and TJ375) did not accumulate H3K4me2/3 in the germline or in the embryo. These results suggest that timing or level of expression in the germline

may restrict the establishment of H3K4me2/3, or mark it for erasure. As robust inducible transgenes escaping germline silencing have not been described, finding mutants that disrupt the imprint may be a more viable route. An RNAi screen for candidate genes involved in chromatin regulation could be carried out, where candidates could be screened for a loss of the imprint (accumulation of H3K4me2/3) on the Xp by immunoflourecence. If our model of germline transcription establishing the imprint were correct, mutants of this class could fall in to at least two categories. If H3K4me2/3 accumulates on the X in pachytene, mutants of this class could potentially mis-express oogenesis genes from the X chromosome during spermatogenesis, perhaps resulting in totally or partially sexually transformed individuals, which would likely be sterile. Additionally if animals accumulating H3K4me2/3 on the Xp in the germline are fertile, embryos inheriting an Xp decorated with H3K4me2/3 may be defective in dosage compensation early in the embryo before the zygotic dosage compensation occurs. Alternatively, if the imprint is not essential for development, there may be no phenotypic consequence of disrupting the imprint. However, aberrant H3K4me2/3 accumulated during spermatogenesis on the Xp could be erased by an unknown mechanism, resulting in embryos with normal imprints despite accumulation of H3K4me2/3 on the X during spermatogenesis.

My work suggests that the X chromosome imprint in *C. elegans* is a result of a paucity of transcription from that chromosome during spermatogenesis. In *C. elegans* a number of examples have been reported in which X/autosomal paralog pairs exhibit germ cell-specific defects when only the autosomal copy is defective, suggesting that only the autosomal copy is active in germ cells (L'Hernault and Arduengo, 1992; Maciejowski et

al., 2005). It is interesting to speculate what the effect on the imprint would be if expression from the X-linked paralogue occurred during spermatogenesis. We observed H3K4me2 accumulating on a transgene expressing a GFP tagged version of the linker histone HIS-24 integrated on the X chromosome during gametogenesis in both sexes. *his-24* itself is located on the X chromosome and the native gene could potentially be an escaper of X chromosome silencing, possibly owning to the transgenes ability to escape germline silencing as well as the repressive chromatin environment of the X chromosome during gametogenesis. However, like other X-linked housekeeping genes described, there are autosomal paralogues of *his-24* (*hil-1, hil-2, hil-4, hil-5, hil-6*), which may fulfill its function in the germline while it is silent during gametogenesis. To examine the consequences of expression of X linked loci during spermatogenesis, a candidate RNAi screen looking for accumulation of H3K4me2/3 on the Xp in the early embryo at these particular loci could be preformed. This approach could be strengthened if it were performed in animals carrying mutations in one of the X-linked genes with an autosomal paralogue. For many of the X-linked genes with autosomal homologues that have been described, deletion of the autosomal homologue leads to viable, but sterile animals suggesting that the X-linked copied is silenced in the germline and cannot rescues. Mutations in X-linked paralogues, on the other hand, have a range of defects specific to each gene, which are not rescued by the autosomal homologue in the soma (Maciejowski et al., 2005). Reactivation of the X-linked homologues could be used to screen for loss of the imprint at least at that loci in those animals.

An alternative screen that could be done would be one for mutants where H3K4 methylation is either not established in the germline or not maintained on the autosomes

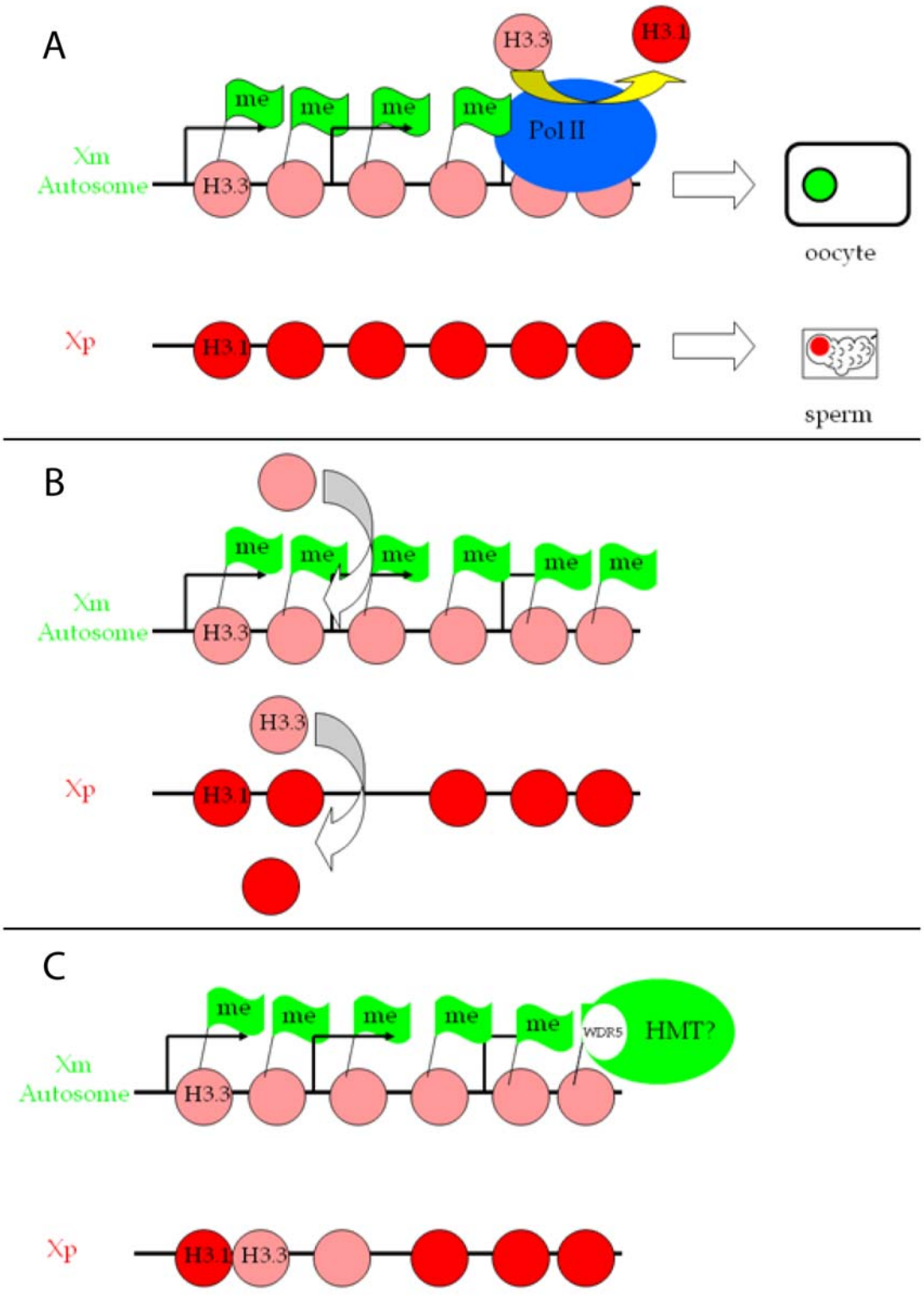
in the embryo. If establishment and inheritance of these marks plays an important functional role, we would expect to see consequences for not maintaining this mark. Also, mutants that have a loss of H3K4me2/3 in the germline, even in the presence of transcription, could reveal enzymes responsible for reading histone modification and determining which ones are to be maintained and which one are to be erased. Alternatively, if H3K4me2/3 is simply inherited as a consequence of transcription in the parents' germline, and plays no functional role, then there should be no consequence of loss of this mark. Indeed our lab has recently shown that wdr-5.1 mediated H3K4 methylation is maintained in adult germline stem cells as well as early embryos independent of transcription, while a separate wdr-5.1 independent mode of H3K4 methylation correlates more directly with transcription in adult germ cells and embryos (Li and Kelly PloS Genetics 2011, in Press). The fact that some of these marks do seem to be actively maintained in the absence of transcription suggests that a reading and writing machinery is in place to preserve them. While these maintenance enzymes could have no essential role in the embryo, but rather only be available for a future time point when maintenance of other marks is necessary, it seems energetically wasteful to express them so early in development without a function or purpose. A screen for mutants where H3K4me2/3 is inherited but not maintianed would address the importance of maintenance of these marks in the early embryo.

Conclusions

Highly specialized, terminally differentiated gametes give rise to the totipotent zygote. My data and that of others demonstrate that epigenetic information is passed between parents and offspring. This inheritance suggests that there must be a) readers of

this information if it is important and b) erasers of this information where it is irrelevant, unwanted or unimportant. The zygote gives rise to the embryo, with further and further restricted lineages, whereupon there is known to be another epigenetic erasure events in the primordial germ cells in mouse, *Drosophila* and *C. elegans* (Schaner et al., 2003; Seki et al., 2005; Seki et al., 2007). Clearly inheritance of active epigenetic modifications on germline expressed genes will not be necessary or even wanted in most embryonic lineages, unless they are house keeping genes. How these marks are are read and acted upon, maintained, or erased are essential questions still needing to be addressed. This work creates a foundational model from which these questions can be approached and answered. Once the mechanisms involved in establishing and properly maintaining epigenetic chromatin modifications across cell divisions and generations have been addressed, methods for repairing these processes when they go awry in diseases such as cancer can begin to be addressed.

Figure 5.1 Chromatin Dynamics in the C. elegans Germline and Early Embryo. (A) Transcription during pachytene on the autosome in both sexes and the X during oogenesiss (Xm) leads to replacement of H3.1 with H3.3 and accumulation of acitive histone modifications such as H3K4me2/3. In spermatogenesis the X (Xp) remains transcriptionally inactive and retains H3.1 unmodified on H3K4. The patterns of histone variants and histone modifications are packaged into mature gametes. (B) Upon fertilization H3.3 is integrated on all chromosomes, displacing H3.1 on the Xp. (C) Despite the influx of new H3.3, histone modification patters of H3K4me2/3 on the Xm and autosome and the absence of H3K4me2/3 on the Xp persists, and are maintained, perhaps by an unknown histone methyl transferase. H3.1 (red), H3.3 (pink) H3K4me2/3 (green me flags), RNA polymerase II (blue), unknown histone methyl transferase (HMT, green).


A
H3.3
H3.1
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Pol II
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oocyte
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H3.1
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WDR5
HMT?
Xm
Autosome
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Xp
H3.1
H3.3

References

Aagaard, L., Laible, G., Selenko, P., Schmid, M., Dorn, R., Schotta, G., Kuhfittig, S., Wolf, A., Lebersorger, A., Singh, P. B., *et al.* (1999). Functional mammalian homologues of the Drosophila PEV-modifier Su(var)3-9 encode centromere-associated proteins which complex with the heterochromatin component M31. Embo J *18*, 1923-1938.

Abbott, D. W., Ivanova, V. S., Wang, X., Bonner, W. M., and Ausio, J. (2001). Characterization of the stability and folding of H2A.Z chromatin particles: implications for transcriptional activation. J Biol Chem *276*, 41945-41949.

Adam, M., Robert, F., Larochelle, M., and Gaudreau, L. (2001). H2A.Z is required for global chromatin integrity and for recruitment of RNA polymerase II under specific conditions. Mol Cell Biol *21*, 6270-6279.

Adams-Cioaba, M. A., and Min, J. (2009). Structure and function of histone methylation binding proteins. Biochem Cell Biol *87*, 93-105.

Adams, I. R., and McLaren, A. (2002). Sexually dimorphic development of mouse primordial germ cells: switching from oogenesis to spermatogenesis. Development *129*, 1155-1164.

Ahmad, K., and Henikoff, S. (2002). The histone variant H3.3 marks active chromatin by replication-independent nucleosome assembly. Mol Cell *9*, 1191-1200.

Albertson, D. G., and Thomson, J. N. (1993). Segregation of holocentric chromosomes at meiosis in the nematode, Caenorhabditis elegans. Chromosome Res *1*, 15-26.

Amor, D. J., and Halliday, J. (2008). A review of known imprinting syndromes and their association with assisted reproduction technologies. Hum Reprod *23,* 2826-2834.

Anway, M. D., Cupp, A. S., Uzumcu, M., and Skinner, M. K. (2005). Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science *308,* 1466-1469.

Anway, M. D., and Skinner, M. K. (2006). Epigenetic transgenerational actions of endocrine disruptors. Endocrinology *147,* S43-49.

Azuara, V., Perry, P., Sauer, S., Spivakov, M., Jorgensen, H. F., John, R. M., Gouti, M., Casanova, M., Warnes, G., Merkenschlager, M., and Fisher, A. G. (2006). Chromatin signatures of pluripotent cell lines. Nat Cell Biol *8,* 532-538.

Balhorn, R. (2007). The protamine family of sperm nuclear proteins. Genome Biol *8,* 227.

Barlow, D. P., Stoger, R., Herrmann, B. G., Saito, K., and Schweifer, N. (1991). The mouse insulin-like growth factor type-2 receptor is imprinted and closely linked to the Tme locus. Nature *349,* 84-87.

Bartolomei, M. S., Zemel, S., and Tilghman, S. M. (1991). Parental imprinting of the mouse H19 gene. Nature *351,* 153-155.

Bateson, P. (2001). Fetal experience and good adult design. Int J Epidemiol *30,* 928-934.

Baumann, C., and De La Fuente, R. (2009). ATRX marks the inactive X chromosome (Xi) in somatic cells and during imprinted X chromosome inactivation in trophoblast stem cells. Chromosoma *118,* 209-222.

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

Bell, A. C., and Felsenfeld, G. (2000). Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene. Nature *405*, 482-485.

Bender, L. B., Cao, R., Zhang, Y., and Strome, S. (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., Suh, J., Carroll, C. R., Fong, Y., Fingerman, I. M., Briggs, S. D., Cao, R., Zhang, Y., Reinke, V., and Strome, S. (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., Mikkelsen, T. S., Xie, X., Kamal, M., Huebert, D. J., Cuff, J., Fry, B., Meissner, A., Wernig, M., Plath, K., *et al.* (2006). A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell *125*, 315-326.

Bessler, J. B., Andersen, E. C., and Villeneuve, A. M. (2010). Differential localization and independent acquisition of the H3K9me2 and H3K9me3 chromatin modifications in the Caenorhabditis elegans adult germ line. PLoS Genet *6*, e1000830.

Bestor, T. H. (1992). Activation of mammalian DNA methyltransferase by cleavage of a Zn binding regulatory domain. Embo J *11*, 2611-2617.

Bird, A. P. (1980). DNA methylation and the frequency of CpG in animal DNA. Nucleic Acids Res *8*, 1499-1504.

Blower, M. D., and Karpen, G. H. (2001). The role of Drosophila CID in kinetochore formation, cell-cycle progression and heterochromatin interactions. Nat Cell Biol *3*, 730-739.

Blower, M. D., Sullivan, B. A., and Karpen, G. H. (2002). Conserved organization of centromeric chromatin in flies and humans. Dev Cell *2*, 319-330.

Boggs, B. A., Cheung, P., Heard, E., Spector, D. L., Chinault, A. C., and Allis, C. D. (2002). Differentially methylated forms of histone H3 show unique association patterns with inactive human X chromosomes. Nat Genet *30*, 73-76.

Bongiorni, S., Cintio, O., and Prantera, G. (1999). The relationship between DNA methylation and chromosome imprinting in the coccid Planococcus citri. Genetics *151*, 1471-1478.

Bongiorni, S., and Prantera, G. (2003). Imprinted facultative heterochromatization in mealybugs. Genetica *117*, 271-279.

Bongiorni, S., Pugnali, M., Volpi, S., Bizzaro, D., Singh, P. B., and Prantera, G. (2009). Epigenetic marks for chromosome imprinting during spermatogenesis in coccids. Chromosoma *118*, 501-512.

Bourc'his, D., Xu, G. L., Lin, C. S., Bollman, B., and Bestor, T. H. (2001). Dnmt3L and the establishment of maternal genomic imprints. Science *294*, 2536-2539.

Brown, S. W., and Nelson-Rees, W. A. (1961). Radiation Analysis of a Lecanoid Genetic System. Genetics *46*, 983-1007.

Brykczynska, U., Hisano, M., Erkek, S., Ramos, L., Oakeley, E. J., Roloff, T. C., Beisel, C., Schubeler, D., Stadler, M. B., and Peters, A. H. (2010). Repressive and active histone methylation mark distinct promoters in human and mouse spermatozoa. Nat Struct Mol Biol *17*, 679-687.

Buchwitz, B. J., Ahmad, K., Moore, L. L., Roth, M. B., and Henikoff, S. (1999). A histone-H3-like protein in C. elegans. Nature *401*, 547-548.

Burgers, W. A., Fuks, F., and Kouzarides, T. (2002). DNA methyltransferases get connected to chromatin. Trends Genet *18*, 275-277.

Byvoet, P., Shepherd, G. R., Hardin, J. M., and Noland, B. J. (1972). The distribution and turnover of labeled methyl groups in histone fractions of cultured mammalian cells. Arch Biochem Biophys *148*, 558-567.

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

Carmen, A. A., Milne, L., and Grunstein, M. (2002). Acetylation of the yeast histone H4 N terminus regulates its binding to heterochromatin protein SIR3. J Biol Chem *277*, 4778-4781.

Carone, B. R., Fauquier, L., Habib, N., Shea, J. M., Hart, C. E., Li, R., Bock, C., Li, C., Gu, H., Zamore, P. D., *et al.* (2010). Paternally induced transgenerational environmental reprogramming of metabolic gene expression in mammals. Cell *143*, 1084-1096.

Cattanach, B. M., and Kirk, M. (1985). Differential activity of maternally and paternally derived chromosome regions in mice. Nature *315*, 496-498.

Chadwick, B. P., and Willard, H. F. (2001). A novel chromatin protein, distantly related to histone H2A, is largely excluded from the inactive X chromosome. J Cell Biol *152*, 375-384.

Chadwick, B. P., and Willard, H. F. (2002). Cell cycle-dependent localization of macroH2A in chromatin of the inactive X chromosome. J Cell Biol *157*, 1113-1123.

Chandy, M., Gutierrez, J. L., Prochasson, P., and Workman, J. L. (2006). SWI/SNF displaces SAGA-acetylated nucleosomes. Eukaryot Cell *5*, 1738-1747.

Chevaillier, P. (1967). [DNA-histone relations during spermatogenesis of decapod crustaceans]. C R Seances Soc Biol Fil *161*, 435-436.

Choo, J. H., Kim, J. D., Chung, J. H., Stubbs, L., and Kim, J. (2006). Allele-specific deposition of macroH2A1 in imprinting control regions. Hum Mol Genet *15*, 717-724.

Choo, J. H., Kim, J. D., and Kim, J. (2007). MacroH2A1 knockdown effects on the Peg3 imprinted domain. BMC Genomics *8*, 479.

Chotalia, M., Smallwood, S. A., Ruf, N., Dawson, C., Lucifero, D., Frontera, M., James, K., Dean, W., and Kelsey, G. (2009). Transcription is required for establishment of germline methylation marks at imprinted genes. Genes Dev *23*, 105-117.

Chow, C. M., Georgiou, A., Szutorisz, H., Maia e Silva, A., Pombo, A., Barahona, I., Dargelos, E., Canzonetta, C., and Dillon, N. (2005). Variant histone H3.3 marks promoters of transcriptionally active genes during mammalian cell division. EMBO Rep *6*, 354-360.

Chu, D. S., Liu, H., Nix, P., Wu, T. F., Ralston, E. J., Yates, J. R., 3rd, and Meyer, B. J. (2006). Sperm chromatin proteomics identifies evolutionarily conserved fertility factors. Nature *443*, 101-105.

Ciccone, D. N., Su, H., Hevi, S., Gay, F., Lei, H., Bajko, J., Xu, G., Li, E., and Chen, T. (2009). KDM1B is a histone H3K4 demethylase required to establish maternal genomic imprints. Nature *461*, 415-418.

Corley-Smith, G. E., Lim, C. J., and Brandhorst, B. P. (1996). Production of androgenetic zebrafish (Danio rerio). Genetics *142*, 1265-1276.

Costanzi, C., and Pehrson, J. R. (1998). Histone macroH2A1 is concentrated in the inactive X chromosome of female mammals. Nature *393*, 599-601.

Costanzi, C., Stein, P., Worrad, D. M., Schultz, R. M., and Pehrson, J. R. (2000). Histone macroH2A1 is concentrated in the inactive X chromosome of female preimplantation mouse embryos. Development *127*, 2283-2289.

Cowell, I. G., Aucott, R., Mahadevaiah, S. K., Burgoyne, P. S., Huskisson, N., Bongiorni, S., Prantera, G., Fanti, L., Pimpinelli, S., Wu, R., *et al.* (2002). Heterochromatin, HP1 and methylation at lysine 9 of histone H3 in animals. Chromosoma *111*, 22-36.

Crouse, H. V. (1960). The Controlling Element in Sex Chromosome Behavior in Sciara. Genetics *45*, 1429-1443.

Csankovszki, G., Nagy, A., and Jaenisch, R. (2001). Synergism of Xist RNA, DNA methylation, and histone hypoacetylation in maintaining X chromosome inactivation. J Cell Biol *153*, 773-784.

Csankovszki, G., Panning, B., Bates, B., Pehrson, J. R., and Jaenisch, R. (1999). Conditional deletion of Xist disrupts histone macroH2A localization but not maintenance of X inactivation. Nat Genet *22*, 323-324.

Cui, B., Liu, Y., and Gorovsky, M. A. (2006). Deposition and function of histone H3 variants in Tetrahymena thermophila. Mol Cell Biol *26*, 7719-7730.

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

de Napoles, M., Mermoud, J. E., Wakao, R., Tang, Y. A., Endoh, M., Appanah, R., Nesterova, T. B., Silva, J., Otte, A. P., Vidal, M., *et al.* (2004). Polycomb group proteins Ring1A/B link ubiquitylation of histone H2A to heritable gene silencing and X inactivation. Dev Cell *7*, 663-676.

DeChiara, T. M., Robertson, E. J., and Efstratiadis, A. (1991). Parental imprinting of the mouse insulin-like growth factor II gene. Cell *64*, 849-859.

Delaval, K., Govin, J., Cerqueira, F., Rousseaux, S., Khochbin, S., and Feil, R. (2007). Differential histone modifications mark mouse imprinting control regions during spermatogenesis. Embo J *26*, 720-729.

Dhillon, N., and Kamakaka, R. T. (2000). A histone variant, Htz1p, and a Sir1p-like protein, Esc2p, mediate silencing at HMR. Mol Cell *6*, 769-780.

Dorus, S., Busby, S. A., Gerike, U., Shabanowitz, J., Hunt, D. F., and Karr, T. L. (2006). Genomic and functional evolution of the Drosophila melanogaster sperm proteome. Nat Genet *38*, 1440-1445.

Dover, J., Schneider, J., Tawiah-Boateng, M. A., Wood, A., Dean, K., Johnston, M., and Shilatifard, A. (2002). Methylation of histone H3 by COMPASS requires ubiquitination of histone H2B by Rad6. J Biol Chem *277*, 28368-28371.

Doyen, C. M., An, W., Angelov, D., Bondarenko, V., Mietton, F., Studitsky, V. M., Hamiche, A., Roeder, R. G., Bouvet, P., and Dimitrov, S. (2006). Mechanism of polymerase II transcription repression by the histone variant macroH2A. Mol Cell Biol *26*, 1156-1164.

Drane, P., Ouararhni, K., Depaux, A., Shuaib, M., and Hamiche, A. (2010). The death-associated protein DAXX is a novel histone chaperone involved in the replication-independent deposition of H3.3. Genes Dev *24*, 1253-1265.

Duncan, E. M., Muratore-Schroeder, T. L., Cook, R. G., Garcia, B. A., Shabanowitz, J., Hunt, D. F., and Allis, C. D. (2008). Cathepsin L proteolytically processes histone H3 during mouse embryonic stem cell differentiation. Cell *135*, 284-294.

Duret, L., Chureau, C., Samain, S., Weissenbach, J., and Avner, P. (2006). The Xist RNA gene evolved in eutherians by pseudogenization of a protein-coding gene. Science *312*, 1653-1655.

Ekwall, K. (2007). Epigenetic control of centromere behavior. Annu Rev Genet *41*, 63-81.

Elsaesser, S. J., Goldberg, A. D., and Allis, C. D. (2010). New functions for an old variant: no substitute for histone H3.3. Curr Opin Genet Dev *20*, 110-117.

Epstein, H., and Shakes, D. (1995). Methods in Cell Biology, Vol 48 (London: Academic Press, Inc.).

Erhardt, S., Su, I. H., Schneider, R., Barton, S., Bannister, A. J., Perez-Burgos, L., Jenuwein, T., Kouzarides, T., Tarakhovsky, A., and Surani, M. A. (2003). Consequences of the depletion of zygotic and embryonic enhancer of zeste 2 during preimplantation mouse development. Development *130*, 4235-4248.

Fan, J. Y., Rangasamy, D., Luger, K., and Tremethick, D. J. (2004). H2A.Z alters the nucleosome surface to promote HP1alpha-mediated chromatin fiber folding. Mol Cell *16*, 655-661.

Fang, J., Chen, T., Chadwick, B., Li, E., and Zhang, Y. (2004). Ring1b-mediated H2A ubiquitination associates with inactive X chromosomes and is involved in initiation of X inactivation. J Biol Chem *279*, 52812-52815.

Franklin, S. G., and Zweidler, A. (1977). Non-allelic variants of histones 2a, 2b and 3 in mammals. Nature *266*, 273-275.

Frehlick, L. J., Eirin-Lopez, J. M., Jeffery, E. D., Hunt, D. F., and Ausio, J. (2006). The characterization of amphibian nucleoplasmins yields new insight into their role in sperm chromatin remodeling. BMC Genomics *7*, 99.

Furuhashi, H., Takasaki, T., Rechtsteiner, A., Li, T., Kimura, H., Checchi, P. M., Strome, S., and Kelly, W. G. (2010). Trans-generational epigenetic regulation of C. elegans primordial germ cells. Epigenetics Chromatin *3*, 15.

Garrick, D., Sharpe, J. A., Arkell, R., Dobbie, L., Smith, A. J., Wood, W. G., Higgs, D. R., and Gibbons, R. J. (2006). Loss of Atrx affects trophoblast development and the pattern of X-inactivation in extraembryonic tissues. PLoS Genet *2*, e58.

Gatewood, J. M., Cook, G. R., Balhorn, R., Schmid, C. W., and Bradbury, E. M. (1990). Isolation of four core histones from human sperm chromatin representing a minor subset of somatic histones. J Biol Chem *265*, 20662-20666.

Gautier, T., Abbott, D. W., Molla, A., Verdel, A., Ausio, J., and Dimitrov, S. (2004). Histone variant H2ABbd confers lower stability to the nucleosome. EMBO Rep *5*, 715-720.

Goday, C., and Esteban, M. R. (2001). Chromosome elimination in sciarid flies. Bioessays *23*, 242-250.

Goldberg, A. D., Banaszynski, L. A., Noh, K. M., Lewis, P. W., Elsaesser, S. J., Stadler, S., Dewell, S., Law, M., Guo, X., Li, X., *et al.* (2010). Distinct factors control histone variant H3.3 localization at specific genomic regions. Cell *140*, 678-691.

Goldstein, P. (1982). The synaptonemal complexes of Caenorhabditis elegans: pachytene karyotype analysis of male and hermaphrodite wild-type and him mutants. Chromosoma *86*, 577-593.

Greaves, I. K., Rangasamy, D., Devoy, M., Marshall Graves, J. A., and Tremethick, D. J. (2006). The X and Y chromosomes assemble into H2A.Z-containing [corrected] facultative heterochromatin [corrected] following meiosis. Mol Cell Biol *26*, 5394-5405.

Grigoryev, S. A., Nikitina, T., Pehrson, J. R., Singh, P. B., and Woodcock, C. L. (2004). Dynamic relocation of epigenetic chromatin markers reveals an active role of constitutive heterochromatin in the transition from proliferation to quiescence. J Cell Sci *117*, 6153-6162.

Guven-Ozkan, T., Nishi, Y., Robertson, S. M., and Lin, R. (2008). Global transcriptional repression in C. elegans germline precursors by regulated sequestration of TAF-4. Cell *135*, 149-160.

Hammoud, S. S., Nix, D. A., Zhang, H., Purwar, J., Carrell, D. T., and Cairns, B. R. (2009). Distinctive chromatin in human sperm packages genes for embryo development. Nature.

Handel, M. A. (2004). The XY body: a specialized meiotic chromatin domain. Exp Cell Res *296*, 57-63.

Hao, Y., Crenshaw, T., Moulton, T., Newcomb, E., and Tycko, B. (1993). Tumour-suppressor activity of H19 RNA. Nature *365*, 764-767.

Hark, A. T., Schoenherr, C. J., Katz, D. J., Ingram, R. S., Levorse, J. M., and Tilghman, S. M. (2000). CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus. Nature *405*, 486-489.

Hassan, A. H., Neely, K. E., and Workman, J. L. (2001). Histone acetyltransferase complexes stabilize swi/snf binding to promoter nucleosomes. Cell *104*, 817-827.

Hata, K., Okano, M., Lei, H., and Li, E. (2002). Dnmt3L cooperates with the Dnmt3 family of de novo DNA methyltransferases to establish maternal imprints in mice. Development *129*, 1983-1993.

Hatzold, J., and Conradt, B. (2008). Control of apoptosis by asymmetric cell division. PLoS Biol *6*, e84.

Heard, E., and Disteche, C. M. (2006). Dosage compensation in mammals: fine-tuning the expression of the X chromosome. Genes Dev *20*, 1848-1867.

Heard, E., Rougeulle, C., Arnaud, D., Avner, P., Allis, C. D., and Spector, D. L. (2001). Methylation of histone H3 at Lys-9 is an early mark on the X chromosome during X inactivation. Cell *107*, 727-738.

Hecht, A., Laroche, T., Strahl-Bolsinger, S., Gasser, S. M., and Grunstein, M. (1995). Histone H3 and H4 N-termini interact with SIR3 and SIR4 proteins: a molecular model for the formation of heterochromatin in yeast. Cell *80*, 583-592.

Henry, K. W., Wyce, A., Lo, W. S., Duggan, L. J., Emre, N. C., Kao, C. F., Pillus, L., Shilatifard, A., Osley, M. A., and Berger, S. L. (2003). Transcriptional activation via sequential histone H2B ubiquitylation and deubiquitylation, mediated by SAGA-associated Ubp8. Genes Dev *17*, 2648-2663.

Hodl, M., and Basler, K. (2009). Transcription in the Absence of Histone H3.3. Curr Biol.

Holdeman, R., Nehrt, S., and Strome, S. (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.

Hore, T. A., Koina, E., Wakefield, M. J., and Marshall Graves, J. A. (2007). The region homologous to the X-chromosome inactivation centre has been disrupted in marsupial and monotreme mammals. Chromosome Res *15*, 147-161.

Howlett, S. K., and Reik, W. (1991). Methylation levels of maternal and paternal genomes during preimplantation development. Development *113*, 119-127.

Howman, E. V., Fowler, K. J., Newson, A. J., Redward, S., MacDonald, A. C., Kalitsis, P., and Choo, K. H. (2000). Early disruption of centromeric chromatin organization in centromere protein A (Cenpa) null mice. Proc Natl Acad Sci U S A *97*, 1148-1153.

Hsieh, C. L. (1999). In vivo activity of murine de novo methyltransferases, Dnmt3a and Dnmt3b. Mol Cell Biol *19*, 8211-8218.

Huang, Z. Q., Li, J., Sachs, L. M., Cole, P. A., and Wong, J. (2003). A role for cofactor-cofactor and cofactor-histone interactions in targeting p300, SWI/SNF and Mediator for transcription. Embo J *22*, 2146-2155.

Huynh, K. D., and Lee, J. T. (2003). Inheritance of a pre-inactivated paternal X chromosome in early mouse embryos. Nature *426*, 857-862.

Ingouff, M., Hamamura, Y., Gourgues, M., Higashiyama, T., and Berger, F. (2007). Distinct dynamics of HISTONE3 variants between the two fertilization products in plants. Curr Biol *17*, 1032-1037.

Ishibashi, T., Li, A., Eirin-Lopez, J. M., Zhao, M., Missiaen, K., Abbott, D. W., Meistrich, M., Hendzel, M. J., and Ausio, J. (2010). H2A.Bbd: an X-chromosome-encoded histone involved in mammalian spermiogenesis. Nucleic Acids Res *38*, 1780-1789.

Jackson, J. P., Lindroth, A. M., Cao, X., and Jacobsen, S. E. (2002). Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. Nature *416*, 556-560.

Jedrusik, M. A., and Schulze, E. (2007). Linker histone HIS-24 (H1.1) cytoplasmic retention promotes germ line development and influences histone H3 methylation in Caenorhabditis elegans. Mol Cell Biol *27*, 2229-2239.

Jeppesen, P., and Turner, B. M. (1993). The inactive X chromosome in female mammals is distinguished by a lack of histone H4 acetylation, a cytogenetic marker for gene expression. Cell *74*, 281-289.

Jin, C., and Felsenfeld, G. (2007). Nucleosome stability mediated by histone variants H3.3 and H2A.Z. Genes Dev *21*, 1519-1529.

Johnson, L., Mollah, S., Garcia, B. A., Muratore, T. L., Shabanowitz, J., Hunt, D. F., and Jacobsen, S. E. (2004). Mass spectrometry analysis of Arabidopsis histone H3 reveals distinct combinations of post-translational modifications. Nucleic Acids Res *32*, 6511-6518.

Jones, P. A., and Baylin, S. B. (2002). The fundamental role of epigenetic events in cancer. Nat Rev Genet *3*, 415-428.

Kaati, G., Bygren, L. O., and Edvinsson, S. (2002). Cardiovascular and diabetes mortality determined by nutrition during parents' and grandparents' slow growth period. Eur J Hum Genet *10*, 682-688.

Kaati, G., Bygren, L. O., Pembrey, M., and Sjostrom, M. (2007). Transgenerational response to nutrition, early life circumstances and longevity. Eur J Hum Genet *15*, 784-790.

Kafri, T., Ariel, M., Brandeis, M., Shemer, R., Urven, L., McCarrey, J., Cedar, H., and Razin, A. (1992). Developmental pattern of gene-specific DNA methylation in the mouse embryo and germ line. Genes Dev *6*, 705-714.

Kalantry, S., Purushothaman, S., Bowen, R. B., Starmer, J., and Magnuson, T. (2009). Evidence of Xist RNA-independent initiation of mouse imprinted X-chromosome inactivation. Nature *460*, 647-651.

Kanduri, C., Pant, V., Loukinov, D., Pugacheva, E., Qi, C. F., Wolffe, A., Ohlsson, R., and Lobanenkov, V. V. (2000). Functional association of CTCF with the insulator upstream of the H19 gene is parent of origin-specific and methylation-sensitive. Curr Biol *10*, 853-856.

Kaslow, D. C., and Migeon, B. R. (1987). DNA methylation stabilizes X chromosome inactivation in eutherians but not in marsupials: evidence for multistep maintenance of mammalian X dosage compensation. Proc Natl Acad Sci U S A *84*, 6210-6214.

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

Kelly, W. G., and Aramayo, R. (2007). Meiotic silencing and the epigenetics of sex. Chromosome Res *15*, 633-651.

Kelly, W. G., and Fire, A. (1998). Chromatin silencing and the maintenance of a functional germline in Caenorhabditis elegans. Development *125*, 2451-2456.

Kelly, W. G., Schaner, C. E., Dernburg, A. F., Lee, M. H., Kim, S. K., Villeneuve, A. M., and Reinke, V. (2002). X-chromosome silencing in the germline of C. elegans. Development *129*, 479-492.

Kelly, W. G., Xu, S., Montgomery, M. K., and Fire, A. (1997). Distinct requirements for somatic and germline expression of a generally expressed Caernorhabditis elegans gene. Genetics *146*, 227-238.

Keohane, A. M., O'Neill L, P., Belyaev, N. D., Lavender, J. S., and Turner, B. M. (1996). X-Inactivation and histone H4 acetylation in embryonic stem cells. Dev Biol *180*, 618-630.

Kimble, J., and Crittenden, S. L. (2007). Controls of germline stem cells, entry into meiosis, and the sperm/oocyte decision in Caenorhabditis elegans. Annu Rev Cell Dev Biol *23*, 405-433.

Kimble, J., and Simpson, P. (1997). The LIN-12/Notch signaling pathway and its regulation. Annu Rev Cell Dev Biol *13*, 333-361.

Koerner, M. V., Pauler, F. M., Huang, R., and Barlow, D. P. (2009). The function of non-coding RNAs in genomic imprinting. Development *136*, 1771-1783.

Kohlmaier, A., Savarese, F., Lachner, M., Martens, J., Jenuwein, T., and Wutz, A. (2004). A chromosomal memory triggered by Xist regulates histone methylation in X inactivation. PLoS Biol *2*, E171.

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

Krogan, N. J., Kim, M., Tong, A., Golshani, A., Cagney, G., Canadien, V., Richards, D. P., Beattie, B. K., Emili, A., Boone, C., *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.

Kurtz, K., Ausio, J., and Chiva, M. (2009). Preliminary study of sperm chromatin characteristics of the brachyuran crab Maja brachydactyla. Histones and nucleosome-like

structures in decapod crustacean sperm nuclei previously described without SNBPs. Tissue Cell *41*, 334-344.

Kurtz, K., Martinez-Soler, F., Ausio, J., and Chiva, M. (2008). Histones and nucleosomes in Cancer sperm (Decapod: Crustacea) previously described as lacking basic DNA-associated proteins: a new model of sperm chromatin. J Cell Biochem *105*, 574-584.

L'Hernault, S. W., and Arduengo, P. M. (1992). Mutation of a putative sperm membrane protein in Caenorhabditis elegans prevents sperm differentiation but not its associated meiotic divisions. J Cell Biol *119*, 55-68.

Langreth, S. G. (1969). Spermiogenesis in Cancer crabs. J Cell Biol *43*, 575-603.

Leach, T. J., Mazzeo, M., Chotkowski, H. L., Madigan, J. P., Wotring, M. G., and Glaser, R. L. (2000). Histone H2A.Z is widely but nonrandomly distributed in chromosomes of Drosophila melanogaster. J Biol Chem *275*, 23267-23272.

Lee, J. S., and Shilatifard, A. (2007). A site to remember: H3K36 methylation a mark for histone deacetylation. Mutat Res *618*, 130-134.

Lee, J. T. (2000). Disruption of imprinted X inactivation by parent-of-origin effects at Tsix. Cell *103*, 17-27.

Lee, J. T., and Lu, N. (1999). Targeted mutagenesis of Tsix leads to nonrandom X inactivation. Cell *99*, 47-57.

Leeb, M., and Wutz, A. (2007). Ring1B is crucial for the regulation of developmental control genes and PRC1 proteins but not X inactivation in embryonic cells. J Cell Biol *178*, 219-229.

Leighton, P. A., Ingram, R. S., Eggenschwiler, J., Efstratiadis, A., and Tilghman, S. M. (1995). Disruption of imprinting caused by deletion of the H19 gene region in mice. Nature *375*, 34-39.

Lewis, A., Mitsuya, K., Umlauf, D., Smith, P., Dean, W., Walter, J., Higgins, M., Feil, R., and Reik, W. (2004). Imprinting on distal chromosome 7 in the placenta involves repressive histone methylation independent of DNA methylation. Nat Genet *36*, 1291-1295.

Lewis, P. W., Elsaesser, S. J., Noh, K. M., Stadler, S. C., and Allis, C. D. (2010). Daxx is an H3.3-specific histone chaperone and cooperates with ATRX in replication-independent chromatin assembly at telomeres. Proc Natl Acad Sci U S A *107*, 14075-14080.

Li, B., Carey, M., and Workman, J. L. (2007). The role of chromatin during transcription. Cell *128*, 707-719.

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

Li, E., Bestor, T. H., and Jaenisch, R. (1992). Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell *69*, 915-926.

Liu, C. L., Kaplan, T., Kim, M., Buratowski, S., Schreiber, S. L., Friedman, N., and Rando, O. J. (2005). Single-nucleosome mapping of histone modifications in S. cerevisiae. PLoS Biol *3*, e328.

Liu, Y., Taverna, S. D., Muratore, T. L., Shabanowitz, J., Hunt, D. F., and Allis, C. D. (2007). RNAi-dependent H3K27 methylation is required for heterochromatin formation and DNA elimination in Tetrahymena. Genes Dev *21*, 1530-1545.

Lloyd, V. (2000). Parental imprinting in Drosophila. Genetica *109*, 35-44.

Loppin, B., Bonnefoy, E., Anselme, C., Laurencon, A., Karr, T. L., and Couble, P. (2005). The histone H3.3 chaperone HIRA is essential for chromatin assembly in the male pronucleus. Nature *437*, 1386-1390.

Loyola, A., and Almouzni, G. (2004). Histone chaperones, a supporting role in the limelight. Biochim Biophys Acta *1677*, 3-11.

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

Lumey, L. H. (1992). Decreased birthweights in infants after maternal in utero exposure to the Dutch famine of 1944-1945. Paediatr Perinat Epidemiol *6*, 240-253.

Lyko, F., Ramsahoye, B. H., and Jaenisch, R. (2000). DNA methylation in Drosophila melanogaster. Nature *408*, 538-540.

Lyko, F., Ramsahoye, B. H., Kashevsky, H., Tudor, M., Mastrangelo, M. A., Orr-Weaver, T. L., and Jaenisch, R. (1999). Mammalian (cytosine-5) methyltransferases cause genomic DNA methylation and lethality in Drosophila. Nat Genet *23*, 363-366.

Maciejowski, J., Ahn, J. H., Cipriani, P. G., Killian, D. J., Chaudhary, A. L., Lee, J. I., Voutev, R., Johnsen, R. C., Baillie, D. L., Gunsalus, K. C., *et al.* (2005). Autosomal genes of autosomal/X-linked duplicated gene pairs and germ-line proliferation in Caenorhabditis elegans. Genetics *169*, 1997-2011.

Mahadevaiah, S. K., Royo, H., Vandeberg, J. L., McCarrey, J. R., Mackay, S., and Turner, J. M. (2009). Key Features of the X Inactivation Process Are Conserved between Marsupials and Eutherians. Curr Biol.

Mak, W., Baxter, J., Silva, J., Newall, A. E., Otte, A. P., and Brockdorff, N. (2002). Mitotically stable association of polycomb group proteins eed and enx1 with the inactive x chromosome in trophoblast stem cells. Curr Biol *12*, 1016-1020.

Mak, W., Nesterova, T. B., de Napoles, M., Appanah, R., Yamanaka, S., Otte, A. P., and Brockdorff, N. (2004). Reactivation of the paternal X chromosome in early mouse embryos. Science *303*, 666-669.

Mancini-DiNardo, D., Steele, S. J., Ingram, R. S., and Tilghman, S. M. (2003). A differentially methylated region within the gene Kcnq1 functions as an imprinted promoter and silencer. Hum Mol Genet *12*, 283-294.

Mancini-Dinardo, D., Steele, S. J., Levorse, J. M., Ingram, R. S., and Tilghman, S. M. (2006). Elongation of the Kcnq1ot1 transcript is required for genomic imprinting of neighboring genes. Genes Dev *20*, 1268-1282.

Martin, C. C., and McGowan, R. (1995). Genotype-specific modifiers of transgene methylation and expression in the zebrafish, Danio rerio. Genet Res *65*, 21-28.

Marzluff, W. F., Gongidi, P., Woods, K. R., Jin, J., and Maltais, L. J. (2002). The human and mouse replication-dependent histone genes. Genomics *80*, 487-498.

Mayer, W., Niveleau, A., Walter, J., Fundele, R., and Haaf, T. (2000). Demethylation of the zygotic paternal genome. Nature *403*, 501-502.

McCarrey, J. R., and Dilworth, D. D. (1992). Expression of Xist in mouse germ cells correlates with X-chromosome inactivation. Nat Genet *2*, 200-203.

McCarrey, J. R., O'Brien, D. A., and Skinner, M. K. (1999). Construction and preliminary characterization of a series of mouse and rat testis cDNA libraries. J Androl *20*, 635-639.

McDonald, L. E., and Kay, G. F. (1997). Methylation analysis using bisulfite genomic sequencing: application to small numbers of intact cells. Biotechniques *22*, 272-274.

McGrath, J., and Solter, D. (1984). Completion of mouse embryogenesis requires both the maternal and paternal genomes. Cell *37*, 179-183.

McKittrick, E., Gafken, P. R., Ahmad, K., and Henikoff, S. (2004). Histone H3.3 is enriched in covalent modifications associated with active chromatin. Proc Natl Acad Sci U S A *101*, 1525-1530.

Mermoud, J. E., Costanzi, C., Pehrson, J. R., and Brockdorff, N. (1999). Histone macroH2A1.2 relocates to the inactive X chromosome after initiation and propagation of X-inactivation. J Cell Biol *147*, 1399-1408.

Mermoud, J. E., Popova, B., Peters, A. H., Jenuwein, T., and Brockdorff, N. (2002). Histone H3 lysine 9 methylation occurs rapidly at the onset of random X chromosome inactivation. Curr Biol *12*, 247-251.

Metzger, E., Wissmann, M., Yin, N., Muller, J. M., Schneider, R., Peters, A. H., Gunther, T., Buettner, R., and Schule, R. (2005). LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. Nature *437*, 436-439.

Mikkelsen, T. S., Ku, M., Jaffe, D. B., Issac, B., Lieberman, E., Giannoukos, G., Alvarez, P., Brockman, W., Kim, T. K., Koche, R. P., *et al.* (2007). Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. Nature *448*, 553-560.

Mizuguchi, G., Shen, X., Landry, J., Wu, W. H., Sen, S., and Wu, C. (2004). ATP-driven exchange of histone H2AZ variant catalyzed by SWR1 chromatin remodeling complex. Science *303*, 343-348.

Montgomery, M. K., Xu, S., and Fire, A. (1998). RNA as a target of double-stranded RNA-mediated genetic interference in Caenorhabditis elegans. Proc Natl Acad Sci U S A *95*, 15502-15507.

Mosammaparast, N., and Shi, Y. (2010). Reversal of histone methylation: biochemical and molecular mechanisms of histone demethylases. Annu Rev Biochem *79*, 155-179.

Muller, J., Hart, C. M., Francis, N. J., Vargas, M. L., Sengupta, A., Wild, B., Miller, E. L., O'Connor, M. B., Kingston, R. E., and Simon, J. A. (2002). Histone methyltransferase activity of a Drosophila Polycomb group repressor complex. Cell *111*, 197-208.

Muramoto, T., Muller, I., Thomas, G., Melvin, A., and Chubb, J. R. (2010). Methylation of H3K4 Is required for inheritance of active transcriptional states. Curr Biol *20*, 397-406.

Nagano, T., Mitchell, J. A., Sanz, L. A., Pauler, F. M., Ferguson-Smith, A. C., Feil, R., and Fraser, P. (2008). The Air noncoding RNA epigenetically silences transcription by targeting G9a to chromatin. Science *322*, 1717-1720.

Nagy, Z., and Tora, L. (2007). Distinct GCN5/PCAF-containing complexes function as co-activators and are involved in transcription factor and global histone acetylation. Oncogene *26*, 5341-5357.

Nakagawa, T., Kajitani, T., Togo, S., Masuko, N., Ohdan, H., Hishikawa, Y., Koji, T., Matsuyama, T., Ikura, T., Muramatsu, M., and Ito, T. (2008). Deubiquitylation of histone

H2A activates transcriptional initiation via trans-histone cross-talk with H3K4 di- and trimethylation. Genes Dev *22*, 37-49.

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

Namekawa, S. H., Park, P. J., Zhang, L. F., Shima, J. E., McCarrey, J. R., Griswold, M. D., and Lee, J. T. (2006). Postmeiotic sex chromatin in the male germline of mice. Curr Biol *16*, 660-667.

Namekawa, S. H., Payer, B., Huynh, K. D., Jaenisch, R., and Lee, J. T. (2010). Two-step imprinted X inactivation: repeat versus genic silencing in the mouse. Mol Cell Biol *30*, 3187-3205.

Nativio, R., Wendt, K. S., Ito, Y., Huddleston, J. E., Uribe-Lewis, S., Woodfine, K., Krueger, C., Reik, W., Peters, J. M., and Murrell, A. (2009). Cohesin is required for higher-order chromatin conformation at the imprinted IGF2-H19 locus. PLoS Genet *5*, e1000739.

Ng, H. H., Feng, Q., Wang, H., Erdjument-Bromage, H., Tempst, P., Zhang, Y., and Struhl, K. (2002). Lysine methylation within the globular domain of histone H3 by Dot1 is important for telomeric silencing and Sir protein association. Genes Dev *16*, 1518-1527.

Ng, H. H., Robert, F., Young, R. A., and Struhl, K. (2003). Targeted recruitment of Set1 histone methylase by elongating Pol II provides a localized mark and memory of recent transcriptional activity. Mol Cell *11*, 709-719.

Ng, R. K., and Gurdon, J. B. (2008). Epigenetic memory of an active gene state depends on histone H3.3 incorporation into chromatin in the absence of transcription. Nat Cell Biol *10*, 102-109.

Norris, D. P., Patel, D., Kay, G. F., Penny, G. D., Brockdorff, N., Sheardown, S. A., and Rastan, S. (1994). Evidence that random and imprinted Xist expression is controlled by preemptive methylation. Cell *77*, 41-51.

O'Neill, L. P., Randall, T. E., Lavender, J., Spotswood, H. T., Lee, J. T., and Turner, B. M. (2003). X-linked genes in female embryonic stem cells carry an epigenetic mark prior to the onset of X inactivation. Hum Mol Genet *12*, 1783-1790.

Oda, H., Okamoto, I., Murphy, N., Chu, J., Price, S. M., Shen, M. M., Torres-Padilla, M. E., Heard, E., and Reinberg, D. (2009). Monomethylation of histone H4-lysine 20 is involved in chromosome structure and stability and is essential for mouse development. Mol Cell Biol *29*, 2278-2295.

Okamoto, I., Otte, A. P., Allis, C. D., Reinberg, D., and Heard, E. (2004). Epigenetic dynamics of imprinted X inactivation during early mouse development. Science *303*, 644-649.

Okano, M., Bell, D. W., Haber, D. A., and Li, E. (1999). DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell *99*, 247-257.

Olins, A. L., and Olins, D. E. (1974). Spheroid chromatin units (v bodies). Science *183*, 330-332.

Ooi, S. K., Qiu, C., Bernstein, E., Li, K., Jia, D., Yang, Z., Erdjument-Bromage, H., Tempst, P., Lin, S. P., Allis, C. D., *et al.* (2007). DNMT3L connects unmethylated lysine 4 of histone H3 to de novo methylation of DNA. Nature *448*, 714-717.

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

Oswald, J., Engemann, S., Lane, N., Mayer, W., Olek, A., Fundele, R., Dean, W., Reik, W., and Walter, J. (2000). Active demethylation of the paternal genome in the mouse zygote. Curr Biol *10*, 475-478.

Palmer, D. K., O'Day, K., and Margolis, R. L. (1990). The centromere specific histone CENP-A is selectively retained in discrete foci in mammalian sperm nuclei. Chromosoma *100*, 32-36.

Palmer, D. K., O'Day, K., Trong, H. L., Charbonneau, H., and Margolis, R. L. (1991). Purification of the centromere-specific protein CENP-A and demonstration that it is a distinctive histone. Proc Natl Acad Sci U S A *88*, 3734-3738.

Pandey, R. R., Ceribelli, M., Singh, P. B., Ericsson, J., Mantovani, R., and Kanduri, C. (2004). NF-Y regulates the antisense promoter, bidirectional silencing, and differential epigenetic marks of the Kcnq1 imprinting control region. J Biol Chem *279*, 52685-52693.

Pandey, R. R., Mondal, T., Mohammad, F., Enroth, S., Redrup, L., Komorowski, J., Nagano, T., Mancini-Dinardo, D., and Kanduri, C. (2008). Kcnq1ot1 antisense noncoding RNA mediates lineage-specific transcriptional silencing through chromatin-level regulation. Mol Cell *32*, 232-246.

Pehrson, J. R., and Fried, V. A. (1992). MacroH2A, a core histone containing a large nonhistone region. Science *257*, 1398-1400.

Pembrey, M. E., Bygren, L. O., Kaati, G., Edvinsson, S., Northstone, K., Sjostrom, M., and Golding, J. (2006). Sex-specific, male-line transgenerational responses in humans. Eur J Hum Genet *14*, 159-166.

Penny, G. D., Kay, G. F., Sheardown, S. A., Rastan, S., and Brockdorff, N. (1996). Requirement for Xist in X chromosome inactivation. Nature *379*, 131-137.

Peters, A. H., Mermoud, J. E., O'Carroll, D., Pagani, M., Schweizer, D., Brockdorff, N., and Jenuwein, T. (2002). Histone H3 lysine 9 methylation is an epigenetic imprint of facultative heterochromatin. Nat Genet *30*, 77-80.

Pettitt, J., Crombie, C., Schumperli, D., and Muller, B. (2002). The Caenorhabditis elegans histone hairpin-binding protein is required for core histone gene expression and is essential for embryonic and postembryonic cell division. J Cell Sci *115*, 857-866.

Plath, K., Fang, J., Mlynarczyk-Evans, S. K., Cao, R., Worringer, K. A., Wang, H., de la Cruz, C. C., Otte, A. P., Panning, B., and Zhang, Y. (2003). Role of histone H3 lysine 27 methylation in X inactivation. Science *300*, 131-135.

Plath, K., Talbot, D., Hamer, K. M., Otte, A. P., Yang, T. P., Jaenisch, R., and Panning, B. (2004). Developmentally regulated alterations in Polycomb repressive complex 1 proteins on the inactive X chromosome. J Cell Biol *167*, 1025-1035.

Pokholok, D. K., Harbison, C. T., Levine, S., Cole, M., Hannett, N. M., Lee, T. I., Bell, G. W., Walker, K., Rolfe, P. A., Herbolsheimer, E., *et al.* (2005). Genome-wide map of nucleosome acetylation and methylation in yeast. Cell *122*, 517-527.

Pray-Grant, M. G., Daniel, J. A., Schieltz, D., Yates, J. R., 3rd, and Grant, P. A. (2005). Chd1 chromodomain links histone H3 methylation with SAGA- and SLIK-dependent acetylation. Nature *433*, 434-438.

Rangasamy, D., Berven, L., Ridgway, P., and Tremethick, D. J. (2003). Pericentric heterochromatin becomes enriched with H2A.Z during early mammalian development. Embo J *22*, 1599-1607.

Ray-Gallet, D., Quivy, J. P., Scamps, C., Martini, E. M., Lipinski, M., and Almouzni, G. (2002). HIRA is critical for a nucleosome assembly pathway independent of DNA synthesis. Mol Cell *9*, 1091-1100.

Rea, S., Eisenhaber, F., O'Carroll, D., Strahl, B. D., Sun, Z. W., Schmid, M., Opravil, S., Mechtler, K., Ponting, C. P., Allis, C. D., and Jenuwein, T. (2000). Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature *406*, 593-599.

Rechtsteiner, A., Ercan, S., Takasaki, T., Phippen, T. M., Egelhofer, T. A., Wang, W., Kimura, H., Lieb, J. D., and Strome, S. (2010). The histone H3K36 methyltransferase MES-4 acts epigenetically to transmit the memory of germline gene expression to progeny. PLoS Genet *6*, e1001091.

Reik, W., and Walter, J. (2001). Genomic imprinting: parental influence on the genome. Nat Rev Genet *2*, 21-32.

Reinke, V., Smith, H. E., Nance, J., Wang, J., Van Doren, C., Begley, R., Jones, S. J., Davis, E. B., Scherer, S., Ward, S., and Kim, S. K. (2000). A global profile of germline gene expression in C. elegans. Mol Cell *6*, 605-616.

Rogers, R. S., Inselman, A., Handel, M. A., and Matunis, M. J. (2004). SUMO modified proteins localize to the XY body of pachytene spermatocytes. Chromosoma *113*, 233-243.

Rougier, N., Bourc'his, D., Gomes, D. M., Niveleau, A., Plachot, M., Paldi, A., and Viegas-Pequignot, E. (1998). Chromosome methylation patterns during mammalian preimplantation development. Genes Dev *12*, 2108-2113.

Sado, T., Fenner, M. H., Tan, S. S., Tam, P., Shioda, T., and Li, E. (2000). X inactivation in the mouse embryo deficient for Dnmt1: distinct effect of hypomethylation on imprinted and random X inactivation. Dev Biol *225*, 294-303.

Sado, T., Okano, M., Li, E., and Sasaki, H. (2004). De novo DNA methylation is dispensable for the initiation and propagation of X chromosome inactivation. Development *131*, 975-982.

Sado, T., Wang, Z., Sasaki, H., and Li, E. (2001). Regulation of imprinted X-chromosome inactivation in mice by Tsix. Development *128*, 1275-1286.

Sakai, A., Schwartz, B. E., Goldstein, S., and Ahmad, K. (2009). Transcriptional and developmental functions of the H3.3 histone variant in Drosophila. Curr Biol *19*, 1816-1820.

Santenard, A., Ziegler-Birling, C., Koch, M., Tora, L., Bannister, A. J., and Torres-Padilla, M. E. (2010). Heterochromatin formation in the mouse embryo requires critical residues of the histone variant H3.3. Nat Cell Biol.

Santisteban, M. S., Kalashnikova, T., and Smith, M. M. (2000). Histone H2A.Z regulats transcription and is partially redundant with nucleosome remodeling complexes. Cell *103*, 411-422.

Sarma, K., Margueron, R., Ivanov, A., Pirrotta, V., and Reinberg, D. (2008). Ezh2 requires PHF1 to efficiently catalyze H3 lysine 27 trimethylation in vivo. Mol Cell Biol *28*, 2718-2731.

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

Schoenfelder, S., Smits, G., Fraser, P., Reik, W., and Paro, R. (2007). Non-coding transcripts in the H19 imprinting control region mediate gene silencing in transgenic Drosophila. EMBO Rep *8*, 1068-1073.

Schotta, G., Ebert, A., Krauss, V., Fischer, A., Hoffmann, J., Rea, S., Jenuwein, T., Dorn, R., and Reuter, G. (2002). Central role of Drosophila SU(VAR)3-9 in histone H3-K9 methylation and heterochromatic gene silencing. Embo J *21*, 1121-1131.

Schuettengruber, B., Chourrout, D., Vervoort, M., Leblanc, B., and Cavalli, G. (2007). Genome regulation by polycomb and trithorax proteins. Cell *128*, 735-745.

Seki, Y., Hayashi, K., Itoh, K., Mizugaki, M., Saitou, M., and Matsui, Y. (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.

Seki, Y., Yamaji, M., Yabuta, Y., Sano, M., Shigeta, M., Matsui, Y., Saga, Y., Tachibana, M., Shinkai, Y., and Saitou, M. (2007). Cellular dynamics associated with the genome-wide epigenetic reprogramming in migrating primordial germ cells in mice. Development *134*, 2627-2638.

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

Sha, K., and Fire, A. (2005). Imprinting capacity of gamete lineages in Caenorhabditis elegans. Genetics *170*, 1633-1652.

Sharman, G. B. (1971). Late DNA replication in the paternally derived X chromosome of female kangaroos. Nature *230*, 231-232.

Sheth, U., Pitt, J., Dennis, S., and Priess, J. R. (2011

). Perinuclear P granules are the principal sites of mRNA export in adult C. elegans germ cells. Development *137*, 1305-1314.

Shevchenko, A. I., Zakharova, I. S., Elisaphenko, E. A., Kolesnikov, N. N., Whitehead, S., Bird, C., Ross, M., Weidman, J. R., Jirtle, R. L., Karamysheva, T. V., *et al.* (2007). Genes flanking Xist in mouse and human are separated on the X chromosome in American marsupials. Chromosome Res *15*, 127-136.

Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J. R., Cole, P. A., Casero, R. A., and Shi, Y. (2004). Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell *119*, 941-953.

Shima, J. E., McLean, D. J., McCarrey, J. R., and Griswold, M. D. (2004). The murine testicular transcriptome: characterizing gene expression in the testis during the progression of spermatogenesis. Biol Reprod *71*, 319-330.

Shin, J. Y., Fitzpatrick, G. V., and Higgins, M. J. (2008). Two distinct mechanisms of silencing by the KvDMR1 imprinting control region. Embo J *27*, 168-178.

Shiu, P. K., Raju, N. B., Zickler, D., and Metzenberg, R. L. (2001). Meiotic silencing by unpaired DNA. Cell *107*, 905-916.

Shogren-Knaak, M., Ishii, H., Sun, J. M., Pazin, M. J., Davie, J. R., and Peterson, C. L. (2006). Histone H4-K16 acetylation controls chromatin structure and protein interactions. Science *311*, 844-847.

Shogren-Knaak, M., and Peterson, C. L. (2006). Switching on chromatin: mechanistic role of histone H4-K16 acetylation. Cell Cycle *5*, 1361-1365.

Sigurdson, D. C., Herman, R. K., Horton, C. A., Kari, C. K., and Pratt, S. E. (1986). An X-autosome fusion chromosome of Caenorhabditis elegans. Mol Gen Genet *202*, 212-218.

Silva, J., Mak, W., Zvetkova, I., Appanah, R., Nesterova, T. B., Webster, Z., Peters, A. H., Jenuwein, T., Otte, A. P., and Brockdorff, N. (2003). Establishment of histone h3 methylation on the inactive X chromosome requires transient recruitment of Eed-Enx1 polycomb group complexes. Dev Cell *4*, 481-495.

Sims, R. J., 3rd, Millhouse, S., Chen, C. F., Lewis, B. A., Erdjument-Bromage, H., Tempst, P., Manley, J. L., and Reinberg, D. (2007). Recognition of trimethylated histone H3 lysine 4 facilitates the recruitment of transcription postinitiation factors and pre-mRNA splicing. Mol Cell *28*, 665-676.

Sleutels, F., Zwart, R., and Barlow, D. P. (2002). The non-coding Air RNA is required for silencing autosomal imprinted genes. Nature *415*, 810-813.

Smilinich, N. J., Day, C. D., Fitzpatrick, G. V., Caldwell, G. M., Lossie, A. C., Cooper, P. R., Smallwood, A. C., Joyce, J. A., Schofield, P. N., Reik, W., *et al.* (1999). A maternally methylated CpG island in KvLQT1 is associated with an antisense paternal transcript and loss of imprinting in Beckwith-Wiedemann syndrome. Proc Natl Acad Sci U S A *96*, 8064-8069.

Sridhar, V. V., Kapoor, A., Zhang, K., Zhu, J., Zhou, T., Hasegawa, P. M., Bressan, R. A., and Zhu, J. K. (2007). Control of DNA methylation and heterochromatic silencing by histone H2B deubiquitination. Nature *447*, 735-738.

Stancheva, I. (2005). Caught in conspiracy: cooperation between DNA methylation and histone H3K9 methylation in the establishment and maintenance of heterochromatin. Biochem Cell Biol *83*, 385-395.

Stedman, W., Kang, H., Lin, S., Kissil, J. L., Bartolomei, M. S., and Lieberman, P. M. (2008). Cohesins localize with CTCF at the KSHV latency control region and at cellular c-myc and H19/Igf2 insulators. Embo J *27*, 654-666.

Stein, A. D., and Lumey, L. H. (2000). The relationship between maternal and offspring birth weights after maternal prenatal famine exposure: the Dutch Famine Birth Cohort Study. Hum Biol *72*, 641-654.

Stewart, M. J., Stewart, P., Soonklang, N., Linthong, V., Hanna, P. J., Duan, W., and Sobhon, P. (2010). Spermatogenesis in the blue swimming crab, Portunus pelagicus, and evidence for histones in mature sperm nuclei. Tissue Cell *42*, 137-150.

Stoeckius, M., Maaskola, J., Colombo, T., Rahn, H. P., Friedlander, M. R., Li, N., Chen, W., Piano, F., and Rajewsky, N. (2009). Large-scale sorting of C. elegans embryos reveals the dynamics of small RNA expression. Nat Methods *6*, 745-751.

Stoler, S., Keith, K. C., Curnick, K. E., and Fitzgerald-Hayes, M. (1995). A mutation in CSE4, an essential gene encoding a novel chromatin-associated protein in yeast, causes chromosome nondisjunction and cell cycle arrest at mitosis. Genes Dev *9*, 573-586.

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

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

Suka, N., Luo, K., and Grunstein, M. (2002). Sir2p and Sas2p opposingly regulate acetylation of yeast histone H4 lysine16 and spreading of heterochromatin. Nat Genet *32*, 378-383.

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

Sun, Z. W., and Allis, C. D. (2002). Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast. Nature *418*, 104-108.

Surani, M. A., Barton, S. C., and Norris, M. L. (1984). Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. Nature *308*, 548-550.

Suto, R. K., Clarkson, M. J., Tremethick, D. J., and Luger, K. (2000). Crystal structure of a nucleosome core particle containing the variant histone H2A.Z. Nat Struct Biol *7*, 1121-1124.

Szabo, P., Tang, S. H., Rentsendorj, A., Pfeifer, G. P., and Mann, J. R. (2000). Maternal-specific footprints at putative CTCF sites in the H19 imprinting control region give evidence for insulator function. Curr Biol *10*, 607-610.

Tagami, H., Ray-Gallet, D., Almouzni, G., and Nakatani, Y. (2004). Histone H3.1 and H3.3 complexes mediate nucleosome assembly pathways dependent or independent of DNA synthesis. Cell *116*, 51-61.

Tamaru, H., and Selker, E. U. (2001). A histone H3 methyltransferase controls DNA methylation in Neurospora crassa. Nature *414*, 277-283.

Terranova, R., Yokobayashi, S., Stadler, M. B., Otte, A. P., van Lohuizen, M., Orkin, S. H., and Peters, A. H. (2008). Polycomb group proteins Ezh2 and Rnf2 direct genomic contraction and imprinted repression in early mouse embryos. Dev Cell *15*, 668-679.

Thakur, N., Tiwari, V. K., Thomassin, H., Pandey, R. R., Kanduri, M., Gondor, A., Grange, T., Ohlsson, R., and Kanduri, C. (2004). An antisense RNA regulates the bidirectional silencing property of the Kcnq1 imprinting control region. Mol Cell Biol *24*, 7855-7862.

Thorvaldsen, J. L., Duran, K. L., and Bartolomei, M. S. (1998). Deletion of the H19 differentially methylated domain results in loss of imprinted expression of H19 and Igf2. Genes Dev *12*, 3693-3702.

Torras-Llort, M., Moreno-Moreno, O., and Azorin, F. (2009). Focus on the centre: the role of chromatin on the regulation of centromere identity and function. Embo J *28*, 2337-2348.

Torres-Padilla, M. E., Bannister, A. J., Hurd, P. J., Kouzarides, T., and Zernicka-Goetz, M. (2006). Dynamic distribution of the replacement histone variant H3.3 in the mouse oocyte and preimplantation embryos. Int J Dev Biol *50*, 455-461.

Turner, J. M. (2007). Meiotic sex chromosome inactivation. Development *134*, 1823-1831.

Turner, J. M., Aprelikova, O., Xu, X., Wang, R., Kim, S., Chandramouli, G. V., Barrett, J. C., Burgoyne, P. S., and Deng, C. X. (2004). BRCA1, histone H2AX phosphorylation, and male meiotic sex chromosome inactivation. Curr Biol *14*, 2135-2142.

Turner, J. M., Mahadevaiah, S. K., Ellis, P. J., Mitchell, M. J., and Burgoyne, P. S. (2006). Pachytene asynapsis drives meiotic sex chromosome inactivation and leads to substantial postmeiotic repression in spermatids. Dev Cell *10*, 521-529.

Umlauf, D., Goto, Y., Cao, R., Cerqueira, F., Wagschal, A., Zhang, Y., and Feil, R. (2004). Imprinting along the Kcnq1 domain on mouse chromosome 7 involves repressive histone methylation and recruitment of Polycomb group complexes. Nat Genet *36*, 1296-1300.

van der Heijden, G. W., Derijck, A. A., Posfai, E., Giele, M., Pelczar, P., Ramos, L., Wansink, D. G., van der Vlag, J., Peters, A. H., and de Boer, P. (2007). Chromosome-wide nucleosome replacement and H3.3 incorporation during mammalian meiotic sex chromosome inactivation. Nat Genet *39*, 251-258.

van der Heijden, G. W., Dieker, J. W., Derijck, A. A., Muller, S., Berden, J. H., Braat, D. D., van der Vlag, J., and de Boer, P. (2005). Asymmetry in histone H3 variants and lysine methylation between paternal and maternal chromatin of the early mouse zygote. Mech Dev *122*, 1008-1022.

van der Heijden, G. W., Ramos, L., Baart, E. B., van den Berg, I. M., Derijck, A. A., van der Vlag, J., Martini, E., and de Boer, P. (2008). Sperm-derived histones contribute to zygotic chromatin in humans. BMC Dev Biol *8*, 34.

van Leeuwen, F., Gafken, P. R., and Gottschling, D. E. (2002). Dot1p modulates silencing in yeast by methylation of the nucleosome core. Cell *109*, 745-756.

VandeBerg, J. L., Cooper, D. W., and Close, P. J. (1973). Mammalian testis phosphoglycerate kinase. Nat New Biol *243*, 48-50.

Vaquero, A., Sternglanz, R., and Reinberg, D. (2007). NAD+-dependent deacetylation of H4 lysine 16 by class III HDACs. Oncogene *26*, 5505-5520.

Vaughn, J. C., and Thomson, L. A. (1972). A kinetic study of DNA and basic protein metabolism during spermatogenesis in the sand crab, Emerita analoga. J Cell Biol *52*, 322-337.

Wade, P. A., Gegonne, A., Jones, P. L., Ballestar, E., Aubry, F., and Wolffe, A. P. (1999). Mi-2 complex couples DNA methylation to chromatin remodelling and histone deacetylation. Nat Genet *23*, 62-66.

Walter, W., Clynes, D., Tang, Y., Marmorstein, R., Mellor, J., and Berger, S. L. (2008). 14-3-3 interaction with histone H3 involves a dual modification pattern of phosphoacetylation. Mol Cell Biol *28*, 2840-2849.

Wang, H., Wang, L., Erdjument-Bromage, H., Vidal, M., Tempst, P., Jones, R. S., and Zhang, Y. (2004). Role of histone H2A ubiquitination in Polycomb silencing. Nature *431*, 873-878.

Wang, J., Mager, J., Chen, Y., Schneider, E., Cross, J. C., Nagy, A., and Magnuson, T. (2001). Imprinted X inactivation maintained by a mouse Polycomb group gene. Nat Genet *28*, 371-375.

Wang, X., Zhao, Y., Wong, K., Ehlers, P., Kohara, Y., Jones, S. J., Marra, M. A., Holt, R. A., Moerman, D. G., and Hansen, D. (2009). Identification of genes expressed in the hermaphrodite germ line of C. elegans using SAGE. BMC Genomics *10*, 213.

Ward, W. S., and Coffey, D. S. (1991). DNA packaging and organization in mammalian spermatozoa: comparison with somatic cells. Biol Reprod *44*, 569-574.

Wong, L. H., Ren, H., Williams, E., McGhie, J., Ahn, S., Sim, M., Tam, A., Earle, E., Anderson, M. A., Mann, J., and Choo, K. H. (2009). Histone H3.3 incorporation provides a unique and functionally essential telomeric chromatin in embryonic stem cells. Genome Res *19*, 404-414.

Wood, A., Schneider, J., Dover, J., Johnston, M., and Shilatifard, A. (2003). The Paf1 complex is essential for histone monoubiquitination by the Rad6-Bre1 complex, which signals for histone methylation by COMPASS and Dot1p. J Biol Chem *278*, 34739-34742.

Wu, R. S., Tsai, S., and Bonner, W. M. (1982). Patterns of histone variant synthesis can distinguish G0 from G1 cells. Cell *31*, 367-374.

Wu, S. F., Zhang, H., and Cairns, B. R. (2011). Genes for embryo development are packaged in blocks of multivalent chromatin in zebrafish sperm. Genome Res.

Wykes, S. M., and Krawetz, S. A. (2003). The structural organization of sperm chromatin. J Biol Chem *278*, 29471-29477.

Wysocka, J., Allis, C. D., and Coonrod, S. (2006). Histone arginine methylation and its dynamic regulation. Front Biosci *11*, 344-355.

Wysocka, J., Swigut, T., Milne, T. A., Dou, Y., Zhang, X., Burlingame, A. L., Roeder, R. G., Brivanlou, A. H., and Allis, C. D. (2005). WDR5 associates with histone H3 methylated at K4 and is essential for H3 K4 methylation and vertebrate development. Cell *121*, 859-872.

Xiao, T., Hall, H., Kizer, K. O., Shibata, Y., Hall, M. C., Borchers, C. H., and Strahl, B. D. (2003). Phosphorylation of RNA polymerase II CTD regulates H3 methylation in yeast. Genes Dev *17*, 654-663.

Yokomine, T., Hata, K., Tsudzuki, M., and Sasaki, H. (2006). Evolution of the vertebrate DNMT3 gene family: a possible link between existence of DNMT3L and genomic imprinting. Cytogenet Genome Res *113*, 75-80.

Yoshioka, H., Geyer, C. B., Hornecker, J. L., Patel, K. T., and McCarrey, J. R. (2007). In vivo analysis of developmentally and evolutionarily dynamic protein-DNA interactions regulating transcription of the Pgk2 gene during mammalian spermatogenesis. Mol Cell Biol *27*, 7871-7885.

Youngson, N. A., and Whitelaw, E. (2008). Transgenerational epigenetic effects. Annu Rev Genomics Hum Genet *9*, 233-257.

Zhang, R., Poustovoitov, M. V., Ye, X., Santos, H. A., Chen, W., Daganzo, S. M., Erzberger, J. P., Serebriiskii, I. G., Canutescu, A. A., Dunbrack, R. L., *et al.* (2005). Formation of MacroH2A-containing senescence-associated heterochromatin foci and senescence driven by ASF1a and HIRA. Dev Cell *8*, 19-30.

Zhang, Y. (2003). Transcriptional regulation by histone ubiquitination and deubiquitination. Genes Dev *17*, 2733-2740.

Zhang, Y., Ng, H. H., Erdjument-Bromage, H., Tempst, P., Bird, A., and Reinberg, D. (1999). Analysis of the NuRD subunits reveals a histone deacetylase core complex and a connection with DNA methylation. Genes Dev *13*, 1924-1935.

Zhou, J., Fan, J. Y., Rangasamy, D., and Tremethick, D. J. (2007). The nucleosome surface regulates chromatin compaction and couples it with transcriptional repression. Nat Struct Mol Biol *14*, 1070-1076.