GENOMIC IMPRINTING IN *Drosophila melanogaster*: EPIGENETIC REGULATION OF THE *Dp(1;f)LJ9* IMPRINTED DOMAIN

by

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Abstract

Genomic imprinting is an epigenetic phenomenon whereby the expression of a gene, chromosomal region, or entire chromosome, depends on the sex of the transmitting parent. Imprinting results in an otherwise fully functional gene being transcriptionally silenced when transmitted by one parent, yet the same gene, with identical DNA sequence, is active when transmitted by the other. Thus, the gene retains an imprint or "memory" of its genetic history, which is reversible and reset each successive generation by passage through the germline. Within this thesis, I present my findings that show genomic imprinting in *Drosophila* is regulated by distinct epigenetic mechanisms at different stages of embryogenesis, suggesting the requirement of a transitional stage to stabilize the imprint between establishment in the germline and maintenance in the soma. I futher show that *Drosophila* utilize epigenetic mechanisms that are involved in regulating genomic imprinting in mammals and plants, such as DNA methylation, histone modification, antisense RNA, and chromatin insulators. These findings demonstrate convergence of the epigenetic mechanisms that regulate genomic imprinting in diverse organisms.

List Of Abbreviations Used

5-azaC	5-azacytidine
AEL	After Egg Lay
asRNA	Antisense RNA
CTCF	CCCTC-binding factor
CpG	Cytosine-Guanine nucleotide linear sequence
DMD	Differentially Methylated Domain
DMR	Differentially Methylated Region
Dnmt	DNA Methyltransferase
dRSF	Remodeling and Spacing Factor
dsRNA	Double Stranded RNA
E(var)	Enhancer of Variegation
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
ICR	Imprint Control Region
MBD	Methyl-DNA Binding Protein
miRNA	Micro Interfering RNA
modENCODE	model organism ENCyclopedia Of DNA Elements
ncRNA	Non-Coding RNA
PEV	Position-Effect Variegation
piRNA	Piwi Interfering RNA
rasiRNA	Repeat Associated Short Interfering RNA
RISC	RNA-Induced Silencing Complex
RT-PCR	Reverse Transcription Polymerase Chain Reaction
siRNA	Short Interfering RNA
Su(var)	Suppressor of Variegation

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Chapter 1 Introduction

1.1 Epigenetics

A broad definition of epigenetic processes encompasses all the regulatory and structural modifications that alter gene expression, without any changes being made to the DNA sequence of a gene. This includes aspects of genome integrity, such as chromosome structure, telomeres, centromeres, as well as the processes involved in cellular determination and differentiation. As these processes are critically important, alterations to epigenetic mechanisms can lead to developmental arrest or changes in cell fate, including tumorigenesis. Epigenetic mechanisms direct the creation of either active or silent transcriptional states and this epigenetic regulation is achieved by multiple mechanisms, notably, DNA methylation, histone modifications and RNA interference (RNAi). Nearly a decade after the human genome was first sequenced, the first profiles of the mammalian 'epigenome' are starting to emerge. High-resolution mapping of genome-wide DNA methylation patterns and histone modification profiles are providing detailed maps of the epigenome (Celniker et al. 2009; Lister et al. 2009). Widespread differences in DNA methylation patterns are present between pluripotent and differentiated cell types (Lister *et al.* 2009), while similar differences in histone modifications are found in distinct cell types (Mikkelsen *et al.* 2007; Zhao *et al.* 2007; McEwen and Ferguson-Smith 2010), corresponding to stage specific or tissue specific gene expression. The term 'epigenetics' is now commonly used to describe the study of epigenetic inheritance, including the parental influence of gene expression in progeny.

1.2 Genomic Imprinting

Genomic imprinting is a phenomenon in which the expression of a gene depends on the sex of the transmitting parent. Imprinting results in an otherwise fully functional gene being transcriptionally silenced when transmitted by one parent, yet remaining unaffected when transmitted by the other. Thus, the gene retains an imprint or "memory" of its genetic history, which is reversible and reset within each successive generation by passage through the germline. Genomic imprinting depends on epigenetic processes. The

differential demarcation of parental chromosomes results in the independent recruitment of transcriptional regulators to the maternal and paternal chromosomes, resulting in the maintenance of parent-specific expression in somatic cells. Genomic imprints are reset in the germ line, where all previous epigenetic marks are erased from chromosomes and new parent-specific imprints are established (Figure 1.1). Such parent-specific gene regulation produces a marked departure from the norm of Mendelian inheritance in which the genomes of both parents participate equally and, provides valuable insights into epigenetic mechanisms of gene regulation.

Genomic imprinting has been widely reported in eutherian mammals, with the number of imprinted genes identified approaching 100 in both mice and humans (Reik and Walter 2001; McEwen and Ferguson-Smith 2009). Imprinted genes are also present in marsupials (Renfree *et al.* 2009) and plants (Garnier *et al.* 2008), while imprinted chromosomes and chromosomal regions are present in insects (Lloyd 2000), fish (McGowan and Martin 1997) and, nematodes (Bean *et al.* 2004; Sha and Fire 2005). Imprinted regions are contained as distinct structural domains, facilitating the regulatory isolation of imprinted genes or multi-gene clusters. While the function and characteristics of imprinted loci vary, both between and within organisms, there are some features that are common such as the asynchronous replication of imprinted regions (Gribnau *et al.* 2003) and a close association with regions of the chromosome containing tandem repeats or transposable elements (Lloyd 2000; Suzuki *et al.* 2007; Garnier *et al.* 2008; Pask *et al.* 2009). These distinct imprinted domains may also serve as sites that contribute to cellular functions, such as meiotic chromosomal pairing, which could account for the prevalence of imprinting in diverse organisms (Pardo-Manuel de Villena *et al.* 2000).

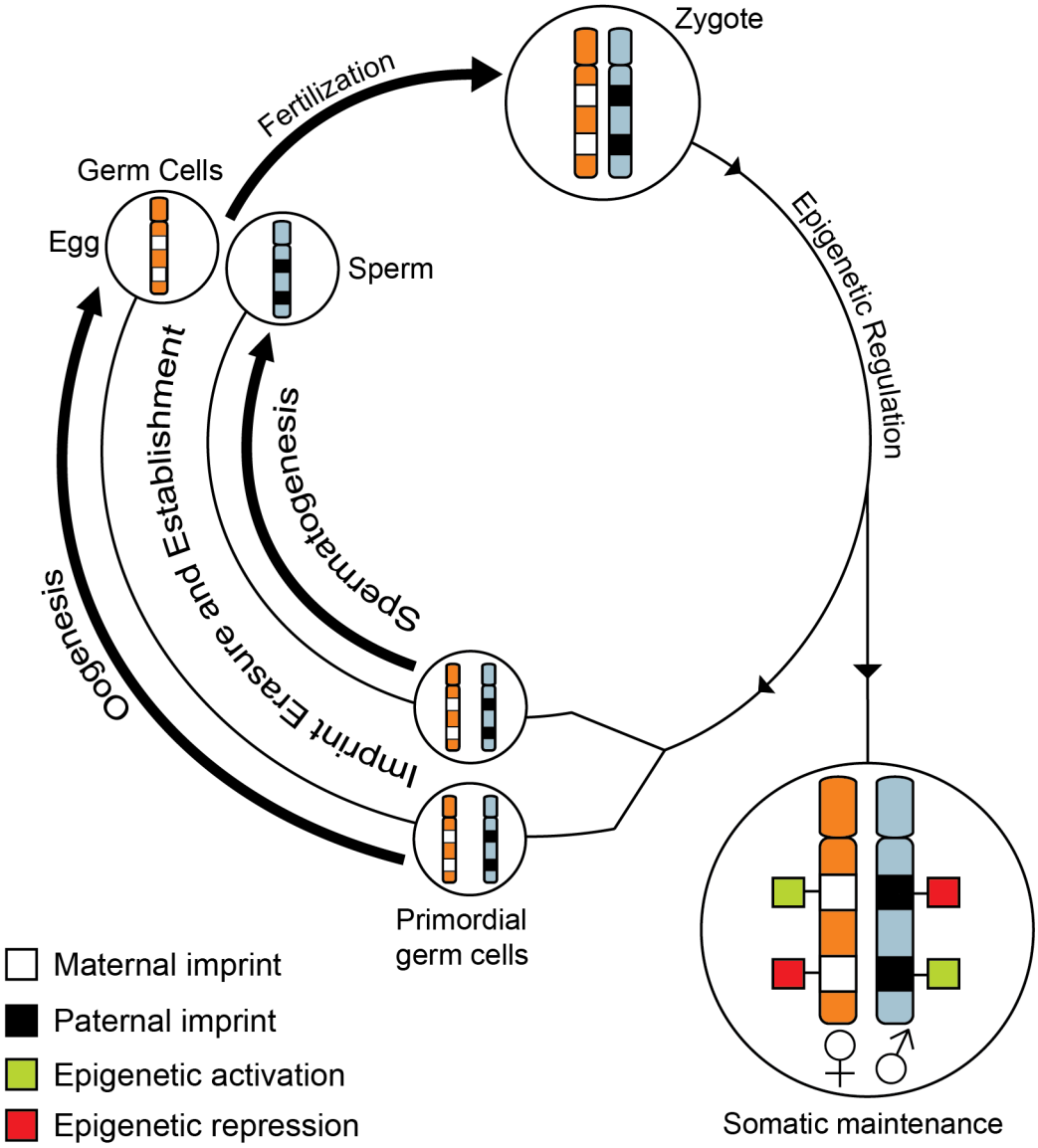

Figure 1.1 - Genomic Imprint Establishment And Maintenance.
During the production of germ cells, the imprint is erased and re-established in accordance with the sex of the genome. After fertilization, epigenetic regulators are differentially recruited to imprinted genes in a parent-specific manner that results in differential expression of imprinted genes in somatic cells. Figure adapted from Renfree *et al*. 2009.

1.3 Epigenetic Mechanisms Of Genomic Imprinting

Imprinted regions of the genome are regulated from distinct sequences called imprint control regions (ICRs), which act as nucleation sites for the differential recruitment of epigenetic factors on maternal and paternal chromosomes. DNA methylation and modifications to histone N-terminal tails, such as methylation and acetylation, are the primary epigenetic factors associated with ICRs (Figure 1.2A). Modifications to histones H3 and H4 have been well documented; with phosphorylation and acetylation typically being marks of active chromatin, while methylation is generally associated with silent chromatin (Figure 1.2B). Parent-specific histone modifications are a common feature associated with genomic imprinting. Histone H3 lysine 9 methylation (H3K9), a silencing modification, is associated with imprinted silencing in mammals, plants and insects (Joanis and Lloyd 2002; Garnier *et al.* 2008; Bongiorni *et al.* 2009; McEwen and Ferguson-Smith 2010). Likewise, the activating histone modification of H3 lysine 4 methylation (H3K4) is associated with active mammalian imprinted domains (McEwen and Ferguson-Smith 2010) and demethylation of H3K4 is required for imprinted silencing in *Drosophila* (Joanis and Lloyd 2002). Histone modifications at imprinted regions facilitate the recruitment of secondary silencing mechanisms such as heterochromatic components. The parent-specific marking of DNA by cytosine methylation is also common at many ICRs, defining differentially methylated regions (DMRs) (Figure 1.2C). Gene regulation by DNA methylation and histone modification is intertwined; each epigenetic mark mutually aids in the other's recruitment to reinforce differential epigenetic states (Tariq and Paszkowski 2004; Cedar and Bergman 2009).

Polycomb group proteins, along with Trithorax group proteins, are involved in somatic memory and homeotic gene regulation throughout the genome. Polycomb group proteins and the Polycomb-specific histone modification H3 lysine 27 methylation (H3K27) have been associated with the maintenance of expression from imprinted domains in plants (Takeda and Paszkowski 2005), while in mammals they are involved at only some imprinted domains (Delaval and Feil 2004; McEwen and Ferguson-Smith 2010). In *Drosophila*, Polycomb group protein binding and heterochromatic protein binding generally do not overlap (de Wit *et al.* 2007); however, Polycomb group proteins

may have a broader function in gene silencing as they share some specific binding domains with heterochromatic proteins (Fanti *et al.* 2008). Parent-specific recruitment of Polycomb group proteins and H3K27 methylation are not associated with those imprinted regions so far characterized in *Drosophila*, suggesting that heterochromatin-specific factors are required for imprinted silencing (Joanis and Lloyd 2002).

Heterochromatic protein 1 (HP1) is a highly conserved heterochromatic protein associated with imprinted domains (Joanis and Lloyd 2002; Riclet *et al.* 2009). HP1 is a non-histone chromatin protein that self-associates and recruits several other heterochromatic proteins to construct heterochromatic blocks (Figure 1.2D) (James and Elgin 1986; Grewal and Elgin 2002). Heterochromatin has been traditionally thought of as 'junk DNA' due to its lack of genes and highly compacted state, that generally inhibits access to transcription factors and promotes transcriptional silencing. However, it is now apparent that there are a number of genes and a remarkable amount sequence diversity within heterochromatin, suggesting that it is an important and dynamic chromosomal component (Dimitri *et al.* 2005; Dimitri *et al.* 2009). Heterochromatin is essential for proper chromosomal function as it is involved in the formation of centromeres (Ahmad and Henikoff 2001a) and assists chromosomal segregation during meiosis and mitosis (Wines and Henikoff 1992; Dialynas *et al.* 2008). Heterochromatic regions also share a trait common to imprinted genes, in that both undergo late replication during the S phase of the cell cycle (Dimitri *et al.* 2009). Thus, it is not unexpected that many imprinted regions either recruit heterochromatic components or are segregated to heterochromatic areas of the chromosome (Lloyd 2000; Kacem and Feil 2009).

The production of non-coding RNA has been described at multiple imprinted regions in both mammals and plants (Royo and Cavaillé 2008; Zhang and Qu 2009). In many organisms, components of the RNAi silencing pathway are found to be involved in the recruitment of factors that facilitate higher order chromatin structure (Figure 1.2E) (Djupedal and Ekwall 2009). A recent survey in mice found extensive transcription of non-coding RNA at multiple imprinted loci, with many of these transcripts extending beyond the previously established boundaries of imprinted regions (Babak *et al.* 2008).

As more imprinted domains in diverse organisms become characterized, RNAi may be found to have a significant role in the regulation of genomic imprinting. A detailed review of the epigenetic mechanisms, their inter-relationship, and role in regulating imprinting in model organisms is presented in Chapter 2.

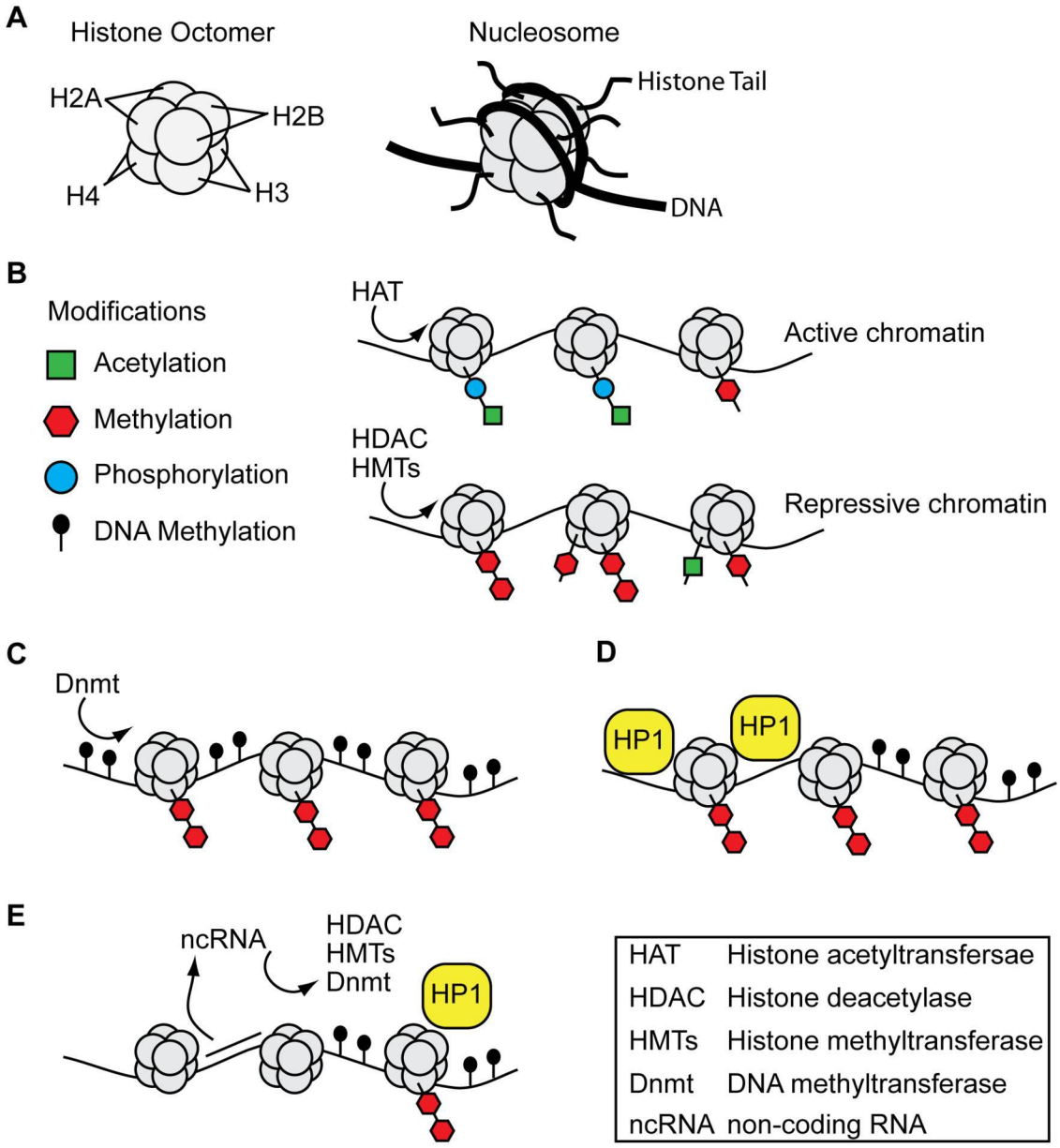

Figure 1.2 - Chromatin Epigenetics.
A) DNA is wrapped around nucleosomes, which are comprised of histones octomers (one pair each of histone H2A, H2B, H3, and H4), to form the basic structure of chromatin.
B) Common epigenetic chromatin modifications; Histone phosphorylation and acetylation are primarily activating modification (H4K12 acetylation is an exception), histone methylation is primarily a repressive modification (H3K4 methylation is an exception).
C) DNA methyltransferases modify DNA directly, converting cytosine to 5-methyl-cytosine.
D) Recruitment of heterochromatic components, such as HP1, stabilize silent domains through higher order chromatin structures.
E) Non-coding RNA and RNAi machinery assist in the recruitment of chromatin remodelers and the formation of heterochromatic domains.

1.4 Developmental Significance Of Imprinting

The unequal contribution of male and female genomes was first demonstrated in mice, where embryos with either two male pronuclei (androgenic) or two female pronuclei (gynogenic) failed to develop and resulted in embryonic lethality (McGrath and Solter 1984; Surani *et al.* 1984). Five years after these experiments, Prader-Willi and Angelman syndromes became the first genetic disorders linked to genomic imprinting (Nicholls *et al.* 1989). Many of the known imprinted genes in mammals have developmentally significant roles in both fetal growth and neural development and, as a result, their disrupted imprint regulation can lead to diseases (Peters and Beechey 2004). A global loss of imprinting can have deleterious results, as evidenced by imprinting loss being one of the most prevalent occurrences in cancer cells (Jelinic and Shaw 2007). The prevalence of cancer associated with uniparental disomy is often a consequence of failed imprinted regulation of specific genes arising from the inheritance of sister chromosomes bearing imprints from only one parent (Tuna *et al.* 2009). Compounding the fact that many imprinted genes are developmentally important is that imprinted genes are functionally haploid; only one copy of a gene pair is expressed and the inactive gene copy cannot compensate for mutations in the active gene. Thus, the developmental importance of maintaining proper regulation of imprinted genes likely contributes to the extensive epigenetic control of imprinted regions.

Imprinted regulation of developmentally important genes contributes to the inviability in mammalian embryos generated through parthenogenesis or certain inter-species hybrids. The imprinted gene *H19* and the *Dlk1-Dio3* imprinted region are necessary and sufficient to block development of bi-maternal embryos in mice and likely contribute to failed parthenogenesis (Kawahara *et al.* 2007). Mis-expression of imprinted genes has similarly been linked to hybrid incompatibility in mammals (Vrana *et al.* 2000), plants (Josefsson *et al.* 2006) and, possibly in *Drosophila* (Menon and Meller 2009). Together, these results demonstrate that the proper regulation of imprinted regions is critical for maintaining genomic integrity.

1.5 Genomic Imprinting In Insects

The descriptive term "imprint" came originally from the study of chromosome elimination in the fungus gnat, *Sciara* (Crouse 1960). Crouse attributed the somatic loss of paternally derived X chromosomes to a genomic imprint that ensures only one female X chromosome remains in the gametes. Similarly, in coccids, paternal chromosomes are silenced by heterochromatinization or eliminated from the genome (Nur 1990) and whole chromosome imprinting is well characterized in *Planococcus citri*, where the silencing of paternal chromosomes coincides with histone methylation and DNA hypomethylation (Bongiorni *et al.* 2009).

Imprinting was first documented in *Drosophila* as 'parental effects' in a series of experiments in the 1930's and 1940's by N.I. Noujdin and A.A. Prokofyeval-Belgouskaya, who described increased paternal-specific silencing in the X chromosome (reviewed in Lloyd 2000). Imprinted domains in *Drosophila* have since been identified in the X chromosome (Lloyd *et al.* 1999a), the Y chromosome (Maggert and Golic 2002), and autosomes (Cohen 1962; Lloyd 2000). A common feature of all *Drosophila* imprinted domains is that they reside within heterochromatic chromosomal regions (Lloyd 2000). A possible explanation for this is the relatively compact genome of *Drosophila*, where the isolation of imprinted domains within restrictive heterochromatic regions serves to constrain the influence of imprinted domains, thus preventing deleterious parent-specific effects (Anaka *et al.* 2009).

While endogenously imprinted chromosomal regions have been described in *Drosophila*, individually imprinted genes have yet to be discovered. Embryos generated through parthenogenesis are viable and, as such it can be assumed that imprinted genes do not act to block parthenogenesis in *Drosophila.* Nevertheless, genomic imprinting does appear to have a regulatory role in the development of *Drosophila* in general, as the regulation of dosage compensation on the paternal X chromosome relies on imprinted regions within the Y chromosome (Menon and Meller 2009).

1.6 Position-Effect Variegation

Drosophila has been extensively drawn upon as a model animal for the study of epigenetics, owing to its history as a model research organism, particularly in the areas of histone modification and chromatin regulation. Early experiments by Muller describe a red and white mottled eye color resulting from X-ray induced inversions of the X chromosome, *In(1)w^{m4}*, placing the *white* (*w*) gene into the "inert" centric regions of the chromosome (Muller 1930). It was further noted that multiple genes were similarly affected by these rearrangements and that the degree of mottled silencing could be altered by factors influencing the "inert" heterochromatic regions of the chromosome (Gowen and Gay 1934; Schultz 1936; Baker 1967; Spofford 1967). These early experiments established that higher-order chromatin structure is a critical component of eukaryotic chromosomal organization and gene regulation, one that could be modulated by the availability of euchromatic or heterochromatic components. This dynamic form of silencing is termed position-effect variegation (PEV).

PEV is an important tool in the genetic dissection of chromatin remodeling and the heterochromatic regulation of gene expression. An early model of PEV proposed that silencing was established early and, subsequently, maintained throughout development (Baker 1967; Spofford 1976). This model was later confirmed by experiments that showed developmentally stable variegated expression is a consequence of heterochromatin occluding transcriptional binding sites (Ahmad and Henikoff 2001b). For a gene to be silenced by heterochromatin, the local recruitment of heterochromatic components must reach a threshold necessary for disrupting and blocking transcriptional binding sites (Vogel *et al.* 2009). Cell-to-cell variations in chromatin component levels and transcriptional occupancy result in the characteristic "mottled" eye phenotype (Figure 1.3A).

Mutational screens for the modulation of PEV in *Drosophila* have contributed greatly towards the identification of heterochromatic proteins and our current understanding of heterochromatic domains. Several large-scale genetic screens utilizing *In(1)w^{m4}* identified mutations that either suppressed variegation (Su(var) mutations), or

enhanced variegation (E(var) mutations), as a result of affecting heterochromatic components or active transcriptional regulators, respectively (Schotta *et al.* 2003). Some of the prominent Su(var) mutations include: *Su(var)3-9* encoding the chromo and SET domain protein responsible for H3K9 methylation; *Su(var)205* encoding HP1; and *Su(var)3-3* encoding a histone H3K4 demethylase (James and Elgin 1986; Eissenberg *et al.* 1990; Schotta *et al.* 2002; Rudolph *et al.* 2007). The E(var) mutation *Jil-1* encodes a histone phosphatase responsible for the phosphorylation of histone H3 serine 10 (H3S10), a histone modification associated with active chromatin (Bao *et al.* 2007). Pigment assays (Figure 1.3B) and the visual scoring of eye phenotypes are used as an effective and established method for quantifying changes in PEV (Real *et al.* 1985; Joanis and Lloyd 2002).

While PEV has been best characterized in *Drosophila*, it is a conserved phenomenon also found in mammals (Dobie *et al.* 1997; Hiragami-Hamada *et al.* 2009), plants (Matzke and Matzke 1998) and yeast (De Rubertis *et al.* 1996; Grunstein 1997; Bühler 2009). This suggests the molecular properties of heterochromatin formation and stability required for gene silencing is functionally similar in diverse eukaryotes. And, as imprinted regions in *Drosophila* are located in heterochromatin, PEV can be used as a tool to identify the parent-specific epigenetic mechanisms that direct heterochromatic formation at imprinted domains.

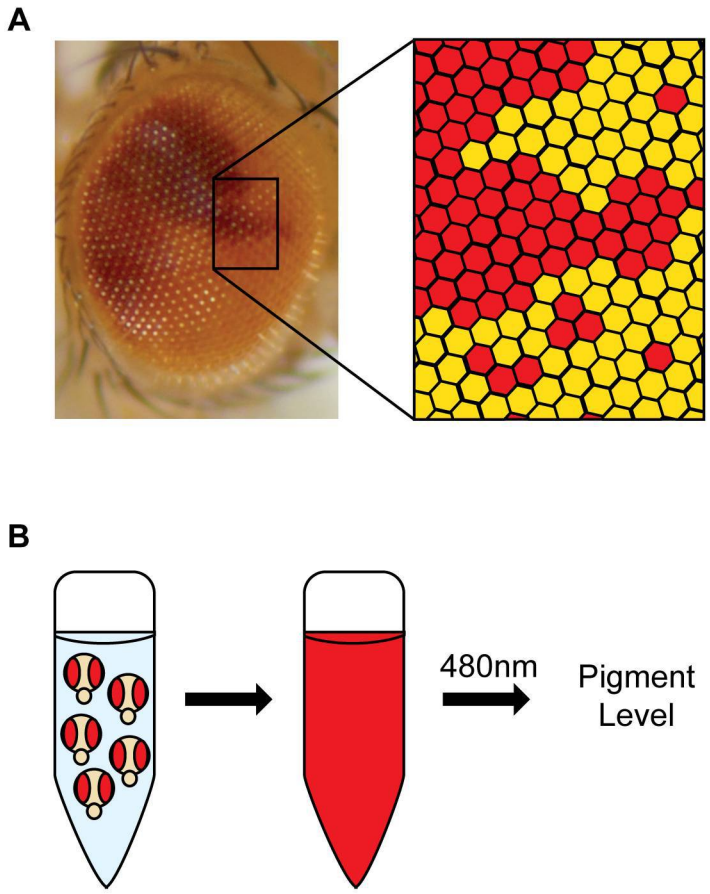

Figure 1.3 - Position-Effect Variegation (PEV) In *Drosophila*.
A) The mottled eye phenotype is generated within regions of the eye where gene expression for ommatidia pigmentation is either active (red) or silent (yellow). Cell-to-cell variations in the availability of heterochromatic components and occupation of transcriptional promoters determine if the gene will be active or silenced within a cell.
B) Pigment assays involve placing *Drosophila* heads in acidified ethanol on an orbital shaker to extract pigments from the eyes. Pigment levels are then quantified with a spectrophotometer at a wavelength of 480 nm.

1.7 The *Drosophila Dp(1;f)LJ9* Imprinting Model System

The *Dp(1;f)LJ9* mini-X chromosome is the product of a chromosomal inversion, *In(1)sc*^{29}, followed by the X-ray deletion of a large portion of euchromatin as well as the distal end of centric heterochromatin (Hardy *et al.* 1984). As a consequence of the deletion, a region containing euchromatic genes is abutted to the centric heterochromatic breakpoint and consequently falls under regulatory control of an endogenous imprinted domain (Figure 1.4A) (Lloyd *et al.* 1999a). One of the translocated genes is the *garnet* gene, which encodes a subunit of an adaptin complex transporter that is expressed in all cells and tissues throughout development and is involved in transporting the proteins responsible for pigmentation to the eye pigment cells (Lloyd *et al.* 1999b). The gene *garnet* is positioned approximately 130kb from the predicted heterochromatic breakpoint and serves as a marker gene for imprint regulation (Hardy *et al.* 1984; Anaka *et al.* 2009). Maternally inherited *Dp(1;f)LJ9* results in full and stable *garnet* expression, while paternally inherited *Dp(1;f)LJ9* results in variegated *garnet* expression (Figure 1.4B).

1.7.1 Maintenance And Establishment Of The *Dp(1;f)LJ9* Imprinted Domain

Silencing of *garnet* from the paternal *Dp(1;f)LJ9* ICR is relaxed by mutations in HP1 and the H3K9 histone methyltransferase genes, illustrating the importance of heterochromatin formation in maintaining the paternal imprint (Joanis and Lloyd 2002). The stable *garnet* expression from maternally inherited *Dp(1;f)LJ9* is not affected by mutations in these heterochromatic components and is also unaffected by mutations in the *trithorax* group transcriptional activators (Joanis and Lloyd 2002). These results suggest that the maternal ICR produces a robust domain that promotes expression independently of *trithorax* group activators and counteracts heterochromatin formation.

At the commencement of this research, the epigenetic processes involved in establishing the *Dp(1;f)LJ9* imprint in the germline were not known. The same factors that disrupted the maintenance of the *Dp(1;f)LJ9* imprint, such as histone modifications and heterochromatin formation, had no affect on the establishment of either the maternal or paternal imprint (Lloyd *et al.* 1999a; Joanis and Lloyd 2002). The generation of cloned

Drosophila through embryonic nuclear transfer results in a loss of paternal-specific silencing, suggesting that passage through gametogenesis is critical for the resetting and establishment of *Dp(1;f)LJ9* imprint (Haigh and Lloyd 2006). Recently, it has been determined that the linker histone H1 is involved in establishing the *Dp(1;f)LJ9* maternal imprint (Kent 2008).

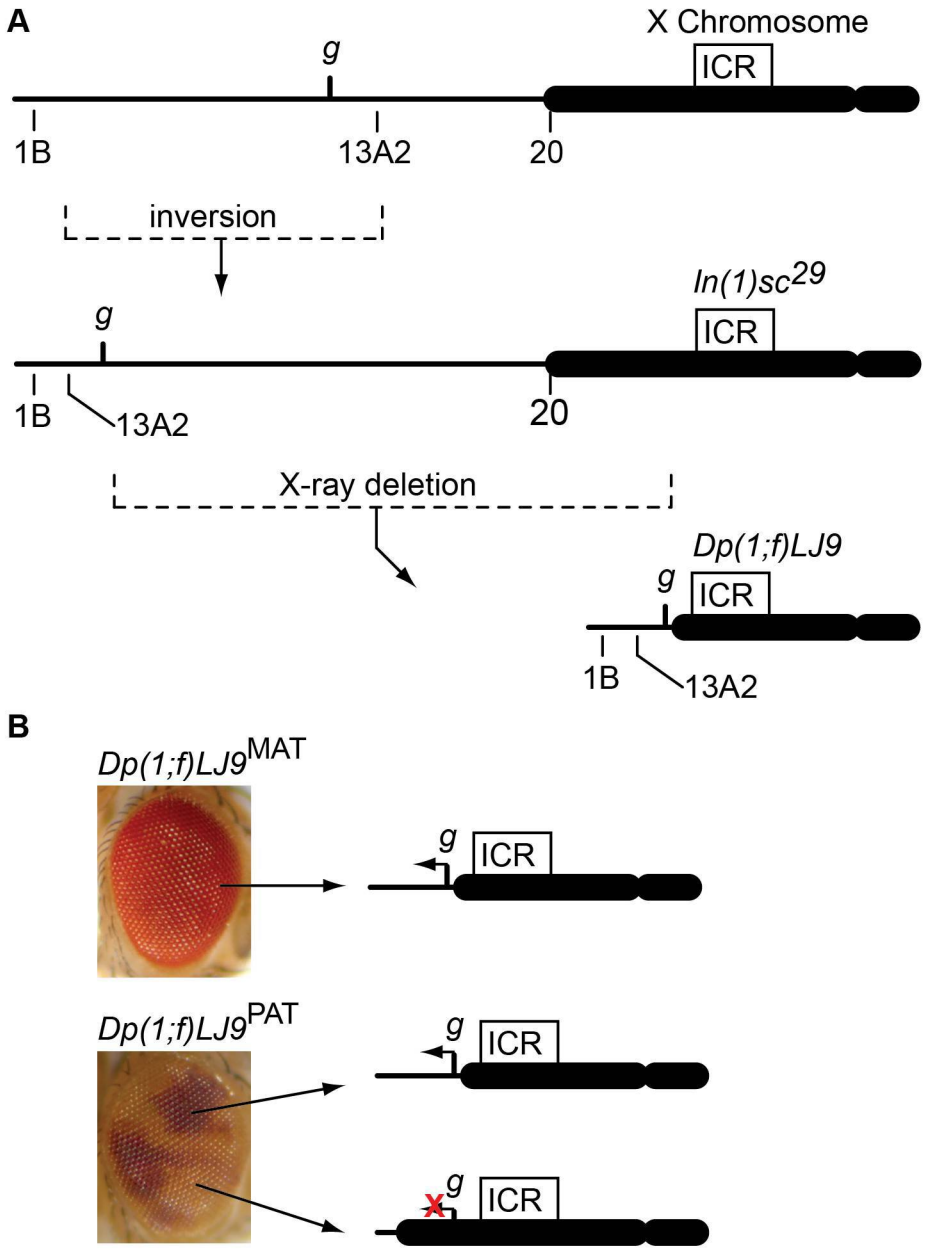

Figure 1.4 - The *Drosophila Dp(1;f)LJ9* Mini-X Chromosome.
A) *In(1)sc*^{29} is the product of an X chromosome inversion between regions 13A2 and 1B, positioning the *garnet* (*g*) gene near the end of the X chromosome. X-ray irradiation induced a large deletion, eliminating the area between rearranged region 12A10 and the distal end of heterochromatin, producing the *Dp(1;f)LJ9* mini-X chromosome and positioning *g* next to a heterochromatic imprint control region (ICR).
B) Maternal inheritance of *Dp(1;f)LJ9* (*Dp(1;f)LJ9*^{MAT}) generates full *garnet* expression, while paternal inheritance of *Dp(1;f)LJ9* (*Dp(1;f)LJ9*^{PAT}) generates variegated *garnet* expression as a consequence of heterochromatic silencing.

1.7.2 Epigenetic Regulation Of The *Dp(1;f)LJ9* Imprinted Domain

The work presented in this thesis outlines my research into the epigenetic mechanisms that regulate expression from the *Dp(1;f)LJ9* imprinted domain. Heterochromatin formation is responsible for maintaining silencing from paternally inherited *Dp(1;f)LJ9* and this finding served as a starting point for my research. Heterochromatin is the structure ultimately formed after a prior series of histone modifications and chromatin remodeling events. Understanding the epigenetic factors recruited to the imprinted domain early in development will assist in determining the processes that maintain stable expression from maternally inherited *Dp(1;f)LJ9* and the paternal-specific silencing from paternally inherited *Dp(1;f)LJ9*.

The majority of my findings presented in this thesis are the result of testing mutations in genes encoding components of specific epigenetic mechanisms for their effects on *garnet* expression from both maternally and paternally inherited *Dp(1;f)LJ9*. Any change in *garnet* expression, resulting from mutations, is reflected in altered eye pigment levels, quantified visually and through pigment assays. Most mutations that affect PEV do so as dominant modifiers that alter PEV when in a heterozygous state (Schotta *et al.* 2003), this is experimentally beneficial as many mutations affecting components of epigenetic regulation are homozygous lethal. The complete *Drosophila* stock list for all mutations tested in this thesis is provided in Appendix A.

Through collaboration with the laboratory of Dr. V.H. Meller, who generated a mutant *CTCF* allele, I was able to test the effects of the conserved boundary element protein CTCF. In mammals, CTCF is involved in maintaining active expression from several imprinted domains (Filippova 2008). I found a similar role for CTCF in protection of imprinted regions in *Drosophila*; *CTCF* mutations disrupted the expression normally maintained by the stable boundary formed when the *Dp(1;f)LJ9* is maternally inherited. These results are published in BMC Biology (MacDonald 2010) and presented in Chapter 3. Prior to working with the *CTCF* mutant alleles, I determined that a methyl-DNA binding protein *MBD2/3* null mutation suppressed paternal-specific silencing of *garnet*. Building on my results from the *CTCF* and *MBD2/3* mutant alleles, I investigated

whether the DNA methyltransferase (Dnmt) inhibitor, 5-azacytidine, disrupted the establishment or maintenance of the *Dp(1;f)LJ9* imprint. 5-azacytidine acts to block DNA methylation by interfering with the binding of Dnmts to DNA and effectively blocks DNA methylation in *Drosophila* (Kunert *et al.* 2003). The results of these trials, presented in Chapter 4, implicate DNA methylation and MBD2/3 binding in stabilizing the paternal imprint early in embryogenesis and contribute to the silencing of *garnet* from paternally-inherited *Dp(1;f)LJ9*.

In Chapter 5, I present my findings from testing mutations of core histone proteins, histone variants and, histone modifiers. The results demonstrated that differential recruitment and modifications of histones distinguish the paternal from maternal *Dp(1;f)LJ9* imprinted domain. Of particular interest are the results for the histone variant H2Av: replacement of H2A with H2Av has been identified as an early step promoting the formation of heterochromatin (Swaminathan *et al.* 2005), yet an *H2Av* null mutation enhanced silencing of *garnet* from paternally-inherited *Dp(1;f)LJ9* and disrupted the stable boundary of maternally-inherited *Dp(1;f)LJ9*. This finding suggests that H2Av is a dynamic component of heterochromatin, and that the *Dp(1;f)LJ9* imprinted domain is a chromatin region distinct from other heterochromatin domains.

Chapter 6 details my work exploring the involvement of RNAi processes in the regulation of the *Dp(1;f)LJ9* imprint. Mutations affecting components of the short-interfering RNA (siRNA), micro RNA (miRNA) and piwi-interacting RNA (piRNA) RNAi pathways were tested. Significantly, the results showed there are distinct patterns of regulation for each RNAi pathway. I found that dedicated miRNA pathway mutations had no affect on imprint regulation, while siRNA mutations disrupted silencing from the paternal *Dp(1;f)LJ9* imprint. Many mutations from the piRNA pathway enhanced silencing from both paternally and maternally inherited *Dp(1;f)LJ9*. These results suggest different roles for the RNAi pathways in regulating the *Dp(1;f)LJ9* imprinted domain. The RNAi component mutations that enhanced silencing are all part of the recently defined repeat-associated siRNA (rasiRNA) pathway (Huisinga and Elgin 2009), which may account for their collective function at the *Dp(1;f)LJ9* imprinted domain.

The results presented in this thesis further the understanding of imprint regulation in *Drosophila*. I have shown that within the first few hours of development, DNA methylation stabilizes silencing of the paternal *Dp(1;f)LJ9* imprint, while CTCF is involved in isolating the maternal *Dp(1;f)LJ9* imprint and protecting maternal-specific expression. Differential chromatin remodeling between the maternal and paternal imprinted domains is achieved through histone modifications and RNAi pathways, generating a transcriptionally repressive paternal imprinted domain and a transcriptionally active maternal imprinted domain. Together, these results demonstrate that *Drosophila* use a complex series of epigenetic mechanisms, similar to those reported in mammals and plants, to regulate imprinted domains.

1.8 Transition To Chapter 2

The following chapter is a comprehensive review of the literature pertaining to the epigenetic mechanisms that regulating genomic imprinting in model organisms. This review focuses attention on the similarities of epigenetic regulation between mammals, plants and insects. This chapter was originally drafted for my preliminary examination and, has since been updated to include recent advances in the field, including my own research.

Chapter 2 The Regulation Of Genomic Imprinting In Model Organisms: Common Epigenetic Mechanisms Act At Diverse Imprinted Genes

2.1 Abstract

Genomic imprinting produces a form of epigenetic inheritance that can cause the expression of a gene to be dependent on the sex of the transmitting parent. During gametogenesis, imprinted regions of DNA are differentially marked in accordance to the sex of the parent, resulting in parent-specific expression. Genes regulated through genomic imprints have been described in a broad variety of organisms, including mammals, plants and insects. Each of these organisms employs multiple, inter-related, epigenetic mechanisms such as DNA methylation, histone modification, chromatin structure and RNA interference to maintain parent-specific expression. While the imprinted genes and their control regions are species and locus-specific, the same suites of epigenetic mechanisms are used to achieve imprinted gene expression. This review summarizes our current understanding of the epigenetic mechanisms responsible for genomic imprinting in mammals, plants and insects.

2.2 Introduction

Epigenetic regulation of the genome is a critical facet of development. Epigenetic control of gene expression allows heritable changes in gene expression without the need for alterations in DNA sequence. This is achieved through the recruitment of molecular processes that assist transcription, block transcription, or degrade existing transcripts. Genomic imprinting is an epigenetic process that marks DNA in a sex-dependent manner, resulting in the differential expression of a gene depending on its parent of origin. Achieving an imprint requires establishing meiotically stable male and female imprints during gametogenesis and maintaining the imprinted state through DNA replication in the somatic cells of the embryo. Erasure of the preceding generation's imprint occurs in the germ line, followed by imprint reestablishment, in accordance with the sex of the organism. Each step in this imprinting process requires epigenetic marks to be interpreted by the genome and acted upon accordingly to result in parent-specific gene expression.

Imprinting has been reported in a variety of organisms including; mammals (Reik and Walter 2001), plants (Alleman and Doctor 2000), fish (McGowan and Martin 1997), insects (Lloyd 2000; Normark 2003), and other invertebrates (Bean *et al.* 2004). Traditionally, the fact that different genes are imprinted in different species has meant that the epigenetic processes leading to genomic imprinting in each organism have been considered in isolation. Yet, strikingly similar mechanisms are employed in organisms as diverse as mammals, plants and insects. In this review, the epigenetic mechanisms involved in the regulation of imprinted genes in mice, *Arabidopsis* and *Drosophila* are explored. This select group represents model organisms in which the epigenetic mechanisms responsible for imprinting are best understood. The evolutionary history and genomic structure of an organism directs how imprinted regions are organized and which epigenetic mechanisms are employed. Common themes are apparent in the different epigenetic mechanisms utilized and the multiple levels of regulation required to execute this parent-dependent mode of inheritance.

2.3 The Anatomy Of A Genomic Imprint – Common Epigenetic Mechanisms

Genomic imprinting, as an epigenetic process, alters gene expression without altering DNA sequence. However, DNA sequences are important in demarcating an imprinted domain. Imprinting control regions (ICRs) are often composed of repetitive DNA sequences found flanking, or internal to, imprinted genes, and in most cases removal of an ICR will result in a loss of imprinting. Epigenetic modifiers of gene expression such as DNA methylation, histone modification, RNA interference, and heterochromatin protein recruitment act directly within ICRs to establish and maintain the imprinted state. ICRs act as nucleation sites for gene silencing or activation, and are able to regulate expression of a single gene or an entire gene cluster. Enhancers and boundary elements are often associated with ICRs to restrict imprinted regulation to specific domains.

2.3.1 Histone Modification

Histone proteins and the modifications applied to them are highly conserved and comprise the most pervasive elements of imprinting across all taxa. Nuclear DNA is wrapped around nucleosomes, histone octamers composed of histones H2A, H2B, H3 and, H4, to form the basic repeating unit of chromatin. Various epigenetic modifications can be applied to the histones that affect chromatin conformation. Histone acetylation generally creates an accessible chromatin conformation while histone deacetylation, often coupled to histone methylation, initiates a compressed chromatin conformation that promotes silencing and the formation of heterochromatin (Berger 2002). Histone methylation can confer an active or repressed transcriptional state depending upon which lysine is methylated. For example, histone 3 lysine 9 (H3K9), histone 4 lysine 20 (H4K20) and, histone 3 lysine 27 (H3K27), are silencing modifications, while histone 3 lysine 4 (H3K4) methylation produces active chromatin (Cheung and Lau 2005). An active imprinted allele is typically characterized by histone acetylation and H3K4 methylation. Repressed imprinted alleles often recruit histone deacetylases followed by histone methyltransferases to silence transcription.

2.3.2 DNA Methylation

DNA methylation, the first epigenetic mechanism to be associated with imprinting, is the only epigenetic modification that is applied directly to a strand of DNA (Reik *et al.* 1987; Sapienza *et al.* 1987). DNA methyltransferases (Dnmt) are highly conserved classes of enzymes that transfer methyl groups onto cytosine-C5, which are essential for both mammal and plant genome stability (Xiao *et al.* 2006; Spada *et al.* 2007). CpG dinucleotide methylation is typically associated with the silenced imprinted allele, and has been identified with both imprint establishment and maintenance in mammals. Dmnt3A and Dmnt3B are the major classes of *de novo* methyltransferases, which have been identified as establishing parent-specific DNA methylation marks at some mammalian imprinted domains (Hata *et al.* 2002; Kaneda *et al.* 2004). Dmnt1 is considered to be a maintenance methyltransferase as it shows a preference for hemi-methylated strands (Scarano *et al.* 2005). Consistent with its molecular function, it plays

a role in imprint maintenance but not establishment. Parent-specific DNA methylation is lost if Dnmt1 function is disrupted (Hirasawa *et al.* 2008) and, Dnmt1 cannot reestablish parent-specific DNA methylation patterns if prior methylation marks are lost (Tucker *et al.* 1996).

The Dnmt2 DNA methyltransferase is highly conserved yet is the least characterized methyltransferase in the Dnmt family. Dnmt2 is the only DNA methyltransferase found in *Drosophila*, where it is responsible for non-CpG methylation at CpA and CpT dinucleotides, but is not essential for viability (Lyko *et al.* 2000; Kunert *et al.* 2003). In humans, Dmnt2 has limited activity (Hermann *et al.* 2004) and Dnmt2 mutant phenotypes are not evident in mice or plants (Goll *et al.* 2006). Non-CpG methylation has been identified in mammalian embryonic stem cells (Ramsahoye *et al.* 2000), and mammalian Dmnt2 is able to direct non-CpG methylation in transgenic *Drosophila* (Marhold *et al.* 2004b), but it remains unclear if Dmnt2 assists in producing non-CpG methylation in mammals.

The level of dependence on DNA methylation for non-imprinted epigenetic regulation varies widely between taxa; mammalian and plant genomes have heavily methylated DNA, while the *Drosophila* genome has low levels of DNA methylation primarily restricted to the early embryo, and the genome of the nematode *C.elegans* has no known DNA methylation. Not surprisingly, there appears to be a close correlation between the native level of DNA methylation in an organism and the subsequent involvement of DNA methylation in the regulation of an imprint.

2.3.3 Heterochromatin And Polycomb Group Protein Silencing

Maintaining transcriptional inactivation of an imprinted allele often involves the formation of heterochromatin, a compacted chromatin structure that can spread in *cis* and generally impose transcriptional silencing. Heterochromatic regions remain stable throughout development and are propagated through cell division by late replication in S phase of the cell cycle (Kamakaka 2003). The first characterized heterochromatic protein, heterochromatic protein 1 (HP1), is a highly conserved non-histone chromatin protein

that binds to H3K9 methylated histones (James and Elgin 1986; Grewal and Elgin 2002). HP1 is able to recruit other heterochromatic proteins and accessory factors, such as histone methyltransferases, to reinforce the structure of heterochromatin and initiate spreading in *cis* (Eissenberg and Elgin 2000).

Homeotic gene silencing is achieved by Polycomb group proteins, which form a silencing pathway largely parallel to heterochromatic silencing (Orlando 2003). The establishment of Polycomb group silencing also involves histone deacetylases and histone methyltransferases, while the central structural protein, Polycomb (Pc), contains a chromo-domain similar to that of HP1. The Polycomb group protein *suppressor of zeste 12* (*Su(z)12*) links HP1 to other Polycomb group proteins, indicating the potential for Polycomb group silencing to propagate heterochromatic silencing (Yamamoto *et al.* 2004). Despite these parallels between Polycomb group and heterochromatic silencing, there is only modest overlap between the two silencing pathways.

2.3.4 Non-coding RNA, Antisense RNA And RNA Interference

The presence of non-coding antisense transcripts in some imprinted domains has raised questions about the role of RNA interference (RNAi) in imprint regulation. RNAi is a highly conserved post-transcriptional silencing mechanism in which double stranded RNA (dsRNA) acts as a trigger for the destruction of complementary RNA transcripts. The conserved RNaseIII nuclease, Dicer, cleaves dsRNA into short 21-26-nucleotide RNAs (Bernstein *et al.* 2001), which are then fed into an RNA silencing complex to guide further degradation of complementary mRNAs (Volpe *et al.* 2002). Additionally, RNAi machinery is linked to the recruitment of factors involved in epigenetic mechanisms, such as DNA methyltransferases, chromatin remodelers and, heterochromatic factors, which can induce transcriptional silencing (Huisinga and Elgin 2009).

2.4 Imprinting In Mammals

Mammals have dominated much of the research on genomic imprinting. About 100 imprinted genes have been reported in mice and humans (McEwen and Ferguson-Smith 2009), with many being developmentally important (Delaval and Feil 2004). Typical features of mammalian genomic imprinting include clusters of imprinted genes regulated from the same ICRs and the involvement of DNA methylation in the parent-specific silencing of gene expression. Mammalian ICRs are often referred to as differentially methylated regions (DMR), due to the pervasiveness of parent-specific DNA methylation at these sites. Increasingly, the importance of additional epigenetic mechanisms for regulating mammalian imprinting is being recognized and, as such, DNA methylation is a component of mammalian imprinting, but not always a requirement. For example, a survey of human and mouse genomes found more tandem repeats in methylated regions of imprinted genes than methylated regions of non-imprinted genes (Hutter *et al.* 2006). This may indicate the presence of additional structural elements in imprinted regions that could nucleate compacted chromatin formations, or recruit additional epigenetic mechanisms.

2.4.1 DNA Methylation And *Igf2-H19* Imprinting In Mammals

The mouse *insulin-like growth factor 2* (*Igf2*) and *H19* genes were among the first imprinted genes to be characterized in detail (Bartolomei *et al.* 1991; DeChiara *et al.* 1991). Subsequently, the same imprinting pattern was found for the human *Igf2* and *H19* genes (Zhang and Tycko 1992; Giannoukakis *et al.* 1993), leading to the imprinted status of *Igf2* becoming a standard assay for determining the presence of genomic imprinting in other vertebrates such as fish, birds, marsupials and cattle (Nolan *et al.* 2001; Dindot *et al.* 2004; Lawton *et al.* 2005; Suzuki *et al.* 2005).

The reciprocal imprinting of the *Igf2* and *H19* genes is mechanistically coupled. *H19* is maternally expressed and *Igf2* paternally expressed (Figure 2.1A). Two ICRs exist for *Igf2* and both are paternally methylated. DMR1, which is upstream of *Igf2* promoter 1, is a silencer that is inactivated by methylation (Constancia *et al.* 2000). DMR2 is

located in exon 6 of *Igf2* and is an enhancer activated by methylation (Murrell *et al.* 2001). *H19* has one ICR which is located upstream of the *H19* gene and is also paternally methylated (Bartolomei *et al.* 1993). Regulation of the *Igf2* and *H19* imprinted domains is dependent on paternal-specific DNA methylation within the DMRs to maintain monoallelic expression; deletions of the *H19* DMR and *Igf2* DMR1, or alterations to Dnmts, result in biallelic expression of both *H19* and *Igf2* (Arney 2003). Passage through the germline is required to establish *Igf2*/*H19* DMD methylation (Tucker *et al.* 1996), which is carried out by the Dnmt3a methyltransferase assisted by the Dnmt cofactor, Dnmt3L (Kaneda *et al.* 2004; Suetake *et al.* 2004). Once established, paternal-specific methylation is then identified and maintained in somatic cells by Dnmt1 (Hirasawa *et al.* 2008).

While the *Igf2* and *H19* imprinted domains remain one of the most studied examples of imprinting, there is still much to be learned about the additional epigenetic events contributing to the imprinting of *Igf2/H19*. Recently, it was shown that the DMRs of *Igf2* and *H19* can physically interact, potentially inducing parent-specific chromosome loops separating the two domains into active or repressed nuclear compartments (Murrell *et al.* 2004). The isolation of maternal and paternal alleles into different nuclear compartments may result in asynchronous replication late is S phase of the cell cycle, a hallmark of heterochromatin (Gribnau *et al.* 2003). Such replication asynchrony is not dependent on the methylation state of a DMR and does not affect the establishment of the imprint, but differential nuclear localization and late replication timing may provide additional reinforcement for the maintenance of parent-specific expression (Cerrato *et al.* 2003; Gribnau *et al.* 2003).

In mammals, both paternal and maternal genomes undergo extensive demethylation a few hours after fertilization. However, imprinted DMRs escape this process and recruit maintenance methyltransferases to retain their methylated status (Bartolomei 2009). In comparison to mice, sheep embryos have lower levels of genome reprogramming through DNA demethylation (Beaujean *et al.* 2004), and only limited levels of active paternal genome demethylation (Hou *et al.* 2008). An investigation into

the epigenetic regulation of imprinted genes in sheep has found that parent-specific gene expression is not initiated until after the blastocyst stage, suggesting a later embryonic onset of DNA methylation patterns (Thurston *et al.* 2008). Furthermore, the imprinted genes *Igf2* and *H19* remain the only imprinted genes in sheep that have identifiable germline DMR methylation, the DMRs of other investigated imprinted genes only acquire parent-specific methylation marks later in embryonic development (Thurston *et al.* 2008; Colosimo *et al.* 2009). Together, these results demonstrate that DNA methylation can be recruited to maintain silencing at imprinted regions that lack germline parent-specific DMRs, and that species-specific differences in genome regulation are associated with differences in epigenetic regulation of imprinted expression.

2.4.2 Chromatin Domains And The CTCF Insulator

The evolutionarily conserved CCCTC-binding factor (CTCF) is also involved in *Igf2* and *H19* imprinting. Within the *H19* ICR there is a CCCTC domain that is only functional on the unmethylated maternal allele. When CTCF binds the maternally unmethylated *H19* ICR, it acts as an insulator, blocking access of the *Igf2* promoter to enhancers (Szabo *et al.* 2004). Paternal methylation of the *H19* ICR inhibits CTCF binding, allowing enhancers access to the *Igf2* promoter on the paternal chromosome (Bell and Felsenfeld 2000; Hark *et al.* 2000). Silencing of the *Igf2* maternal allele is also facilitated by CTCF, which insulates maternal DMR1 and DMR2 from methylation when bound to the maternal *H19* ICR (Lopes *et al.* 2003). A loss of CTCF function results in *de novo* methylation of the maternal H19 ICR, which effectively erases imprinted expression of *H19* and *Igf2* (Fedoriw *et al.* 2004). Recent phylogenetic and mutational analysis has shown that the CTCF binding sites, and not DNA methylation of ICRs, are the more reliable predictor of the imprinted expression of *Igf2*. CTCF binding sites are conserved in humans, mice and, marsupials, which all have imprinted *Igf2* and *H19*, while they are lacking in monotremes that do not imprint *Igf2* or *H19* (Weidman *et al.* 2004). Furthermore, *Igf2* DMR2 is biallelically methylated in both marsupials and monotremes, even though it is only biallelically expressed in monotremes, showing that methylation alone does not cause imprinted expression (Weidman *et al.* 2004).

CTCF binds numerous sites within mammalian genomes, where it is identified both as a transcriptional regulator and a chromatin insulator able to block the spread of heterochromatin and mediate long-range chromosomal interactions (Filippova 2008). CTCF-directed intrachromosomal loops are thought to contribute to parent-specific expression of *Igf2* and *H19* (Figure 2.1B). Self-association between CTCF proteins bound to ICRs can initiate a chromosomal loop that isolates *H19* to maintain maternal expression, while reinforcing *Igf2* silencing through the creation of a repressive domain (Li *et al.* 2008). Disruption of CTCF binding to the maternal *H19* ICR results in *de novo* DNA methylation of maternal *Igf2* DMR1 and DMR2, suggesting that intrachromosomal looping mediates regulation of the entire maternal *Igf2*/*H19* imprinted region (Kurukuti *et al.* 2006). Isolation of imprinted alleles by CTCF has been reported at various other mammalian imprinted domains, where parent-specific binding of CTCF is critical for maintaining active expression from an imprinted allele (Wan and Bartolomei 2008). However, it remains to be determined if the initiation of higher-order chromatin structures via CTCF-mediated intrachromosomal looping is a common feature of these other imprinted domains.

2.4.3 Histone Modification And Mammalian Imprinting

Although DNA methylation has been the focus of the majority of studies on genomic imprinting in mammals, it is becoming clear that histone modification and RNA-based processes also play a role. The receptor of Igf2, *Igf2r*, is another well-characterized imprinted gene (Barlow *et al.* 1991). Rodents and marsupials imprint their *Igf2r* gene, while monotremes, birds and primates (including humans) do not, and thus have biallelic *Igf2r* expression (Wilkins and Haig 2003). In Mice, *Igf2r* is maternally expressed, displaying a reciprocal pattern of imprinting to that of *Igf2* (Figure 2.1C). Two ICRs are present in *Igf2r*; the first, DMR1, is located in the *Igf2r* promoter region and is paternally methylated, and the second, DMR2, lies within the second intron of *Igf2r* and is maternally methylated. DMR2 corresponds to the promoter of an antisense RNA transcript *Air*, a large transcript that overlaps the promoter region of *Igf2r* (Sleutels *et al.* 2002). The *Air* RNA transcript is exclusively paternally expressed, and not only

contributes to the silencing of paternal *Igf2r*, but also to the silencing of the genes which are in the same region as *Igf2r*, yet do not overlap the *Air* transcript (Sleutels *et al.* 2002).

Histone methylation patterns are critical components of the parent-specific expression of *Igf2r* and *Air* genes. In mice, the expressed maternal *Igf2r* allele and paternal *Air* allele are both marked by H3K4 di- and trimethylation marks, while the repressed paternal *Igf2r* allele and maternal *Air* allele are both marked by H3K9 trimethylation within the promoter region (Vu *et al.* 2004). Indeed, histone methylation marks are more reflective of the imprinted state of *Igf2r* than the presence of *Air* transcripts or DNA methylation patterns. In the mouse brain, *Igf2r* is biallelically expressed. This correlates with the presence of activating H3K4 methylation in both the paternal and maternal *Igf2r* DMR1 promoter region, despite retention of paternal *Air* transcription (Vu *et al.* 2004). In humans, activating H3K4 methylation is present within both the maternal and paternal *Igf2r* promoter regions (Figure 2.1D), yet is absent from the *Air* promoter region, eliminating *Air* expression while facilitating biallelic *Igf2r* expression (Vu *et al.* 2004). Recently, H3K4 demethylation is shown as a requirement for establishing imprinted silencing at some maternally repressed genes in mice, where the disruption of H3K4 demethylation prevented *de novo* DNA methylation of DMRs (Ciccone *et al.* 2009). H3K4 demethylation appeared critical for imprinted genes that undergo *de novo* DNA methylation at later stages in embryonic development, suggesting the interaction between histone modifications and DNA methylation may be dependent on the developmental timing of epigenetic regulatory activity.

A comprehensive survey of the histone modification present at imprinted regions compared to non-imprinted regions in mice determined three modifications characterized all imprinted genes tested; repressed alleles contained H3K9 trimethylation and H3K20 trimethylation, while active alleles contained H3K4 trimethylation (McEwen and Ferguson-Smith 2010). The chromatin state of imprinted regions was found to closely resemble heterochromatin, and may be distinct from the general developmental silencing of genes, as H3K27 trimethylation was not present at all imprinted genes. This epigenetic signature was present in imprinted genes regardless of whether the gene contained a

DMR within its IRC, demonstrating both the importance and consistency of histone modification at imprinted domains.

2.4.4 Antisense Transcripts In Mammalian Imprinting

The presence of non-coding RNA transcripts, such as the *H19* and *Air* RNAs, are associated with imprinted regions in mammals. Deletion of the DMR2 *Air* promoter (Wutz *et al.* 1997), or the truncation of the *Air* transcript (Sleutels *et al.* 2002), results in paternal activation and biallelic expression of *Igf2r* and the neighboring gene clusters. Additionally, the *Air* transcript is capable of maintaining paternal silencing in this gene cluster even if the paternal *Igf2r* promoter is experimentally activated (Sleutels *et al.* 2003). Whether RNA transcripts, such as those from *Air* and *H19*, are incorporated into the RNAi pathway or involved in the recruitment of additional epigenetic silencing complexes remains to be determined.

In human cells, synthetic short interfering RNAs (siRNAs) are capable of initiating DNA and histone methylation at a targeted gene sequence (Kawasaki and Taira 2004), and in mice the proper nucleation of heterochromatin is dependent on the RNA binding activity of HP1 in addition to H3K9 methylation (Muchardt *et al.* 2002). Thus, RNAi in mammals is capable of inducing changes in DNA methylation, histone modification and, heterochromatin recruitment, all of which are involved in imprint regulation. In mice the *Air* transcript is able to maintain *Igf2r* silencing when DNA methylation of DMR2 is lost (Barlow *et al.* 1991), but cannot establish silencing when H3K4 methylation is present within the *Igf2r* promoter (Aagaard *et al.* 1999). RNAi-directed heterochromatin formation initiated from the *Air* transcript could explain this discrepancy. The *Air* transcript could stabilize and spread heterochromatin formation to regions that are lacking DNA methylation, yet may not be able to recruit histone methyltransferases to change existing H3K4 histone methylation patterns and initiate silencing.

MicroRNAs (miRNAs) are endogenous 21-25nt RNAs that target complementary sequences for silencing (Zeng *et al.* 2003). Two miRNA genes, *miR-127* and *miR-136*,

have been shown to be part of an imprinted domain responsible for the imprinted expression of the retrotransposon-like gene *Rtl1* in mice and the orthologous *PEG11* gene in sheep and humans (Charlier *et al.* 2001; Seitz *et al.* 2003). Imprinted expression is associated with an unmethylated maternal ICR, leading to the miRNA genes only being maternally expressed which drives maternal-specific silencing of *Rtl1* (Lin *et al.* 2003). In sheep, *PEG11* produces a functional protein as well as an antisense *PEG11* transcript (Byrne *et al.* 2010). Imprinted silencing is directed by maternally produced antisense miRNA acting as guides for RISC-mediated destruction of maternal *PEG11* transcript (Davis *et al.* 2005). However, complex modulations of maternal miRNA generation suggest that maternal gene expression levels are balanced for dosage and not completely silenced (Davis *et al.* 2005; Byrne *et al.* 2010). It is unclear if RNAi processing of *PEG11* transcripts by RNAi machinery recruits additional chromatin remodelers to regulate expression from the maternal allele.

Genomic imprinting has been linked to dosage compensation in some mammals, where the silencing is directed towards the paternal X chromosome (Latham 2005). In female mice, the paternal X chromosome is selectively silenced in extraembryonic tissues, in part by the production of the non-coding RNA *Xist*. Transcription of *Xist* spreads from an initial transcription site to cover most of the paternal X chromosome, leading to the recruitment of additional epigenetic silencing factors, such as histone methyltransferases and heterochromatic proteins (Andersen and Panning 2003). Preferential silencing of the paternal X chromosome still occurs if *Xist* non-coding RNA is lost; however, silencing is destabilized (Kalantry *et al.* 2009). This may be related to the finding that the RNAi component Dicer is required for the spread of *Xist* and recruitment of the H3K27 trimethylation silencing in somatic cell X inactivation (Ogawa *et al.* 2008). It is possible that imprinted silencing of the paternal X chromosome in extraembryonic mouse tissues originates from the imprinted silencing of specific target genes or regions, which then act as nucleation sites for RNAi-directed spreading of silencing across the whole chromosome.

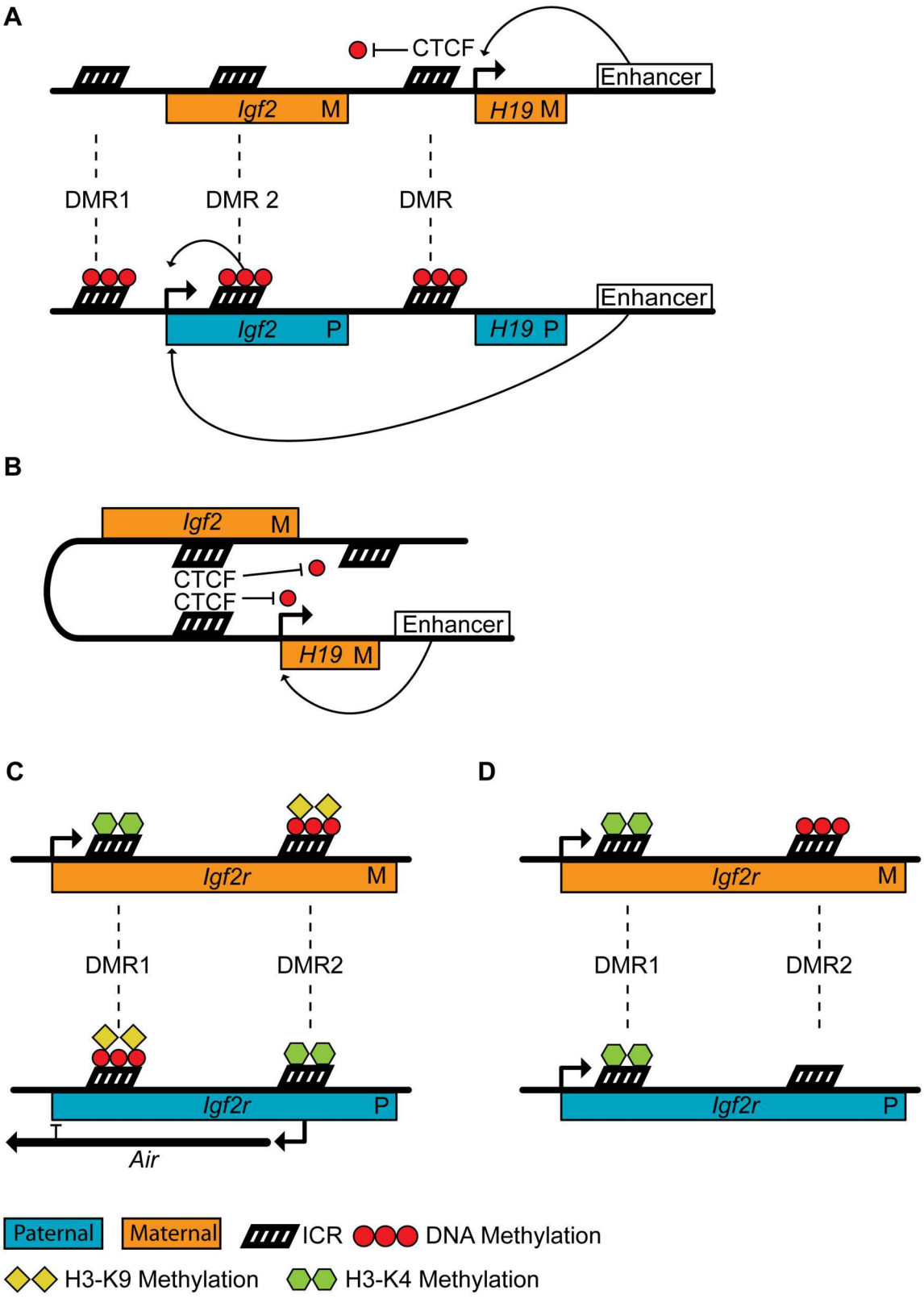

Figure 2.1 - Imprinted Regulation Of *Igf2/H19* And *Igf2r/Air* In Mice And Humans.

(Figure 2.1 continued)

A) The *Igf2* and *H19* genes are reciprocally imprinted, with *H19* and *Igf2* being expressed maternally and paternally, respectively. CTCF binds the maternal *H19* ICR and acts as an insulator sequestering enhancers to initiate maternal *H19* transcription while also protecting the *H19* ICR from methylation. Methylation on the paternal *H19* ICR prevents CTCF binding and silences paternal transcription. *Igf2* is only expressed paternally as a lack of CTCF binding in the paternal *H19* ICR allows enhancers to activate the *Igf2* promoter. DMR1 is a silencer that is inactivated by methylation while DMR2 is an enhancer that is activated by methylation. DRM1 and DRM2 are both methylated on the paternal allele, facilitating paternal *Igf2* transcription and blocking maternal transcription.
B) CTCF mediate an intrachromosomal loop, which prevents DNA methylation of the *H19* DMR and *Igf2* DMRs, while facilitating *H19* expression.
C) In mice, *Igf2r* is maternally expressed while the overlapping *Air* antisense transcript is paternally expressed. Histone H3K4 methylation in the maternal *Igf2r* promoter (DRM1) initiates transcription, while DNA methylation and histone H3K9 methylation in the downstream *Air* promoter region (DRM2) silences maternal *Air* transcription. Activating H3K4 methylation at the paternal *Air* promoter region initiates paternal transcription of the *Air* transcript. The *Air* transcript overlaps the *Igf2r* promoter and contributes to the silencing of the paternal *Igf2r* allele along with DNA methylation and histone H3K9 methylation.
D) In humans, *Ifg2r* is biallelically expressed. Activating H3K4 methylation is found in both the maternal and paternal promoter regions of *Igf2r*. While maternal specific DNA methylation of DMR2 is maintained, there is no H3K4 methylation of paternal DRM2, preventing the transcription of the *Air* transcript.

2.5 Imprinting In Plants

Imprinting in plants was first documented in 1970, when it was found that a gene in maize produced fully colored kernels when maternally inherited and variegated kernels when paternally inherited (Kermicle 1970). In more recent years, genomic imprinting in angiosperms has been investigated extensively in *Arabidopsis*. Angiosperms experience double fertilization, with one sperm fusing the egg cell to produce the embryo proper, and the other fusing with the central cell to produce endosperm. The endosperm acts largely as support structure of the developing embryo and is terminally differentiated.

2.5.1 DNA Methylation In *Arabidopsis* FWA And FIS2 Imprinting

The *Arabidopsis* gene FWA encodes a homeodomain-containing transcription factor involved in the regulation of flowering, and is a well-characterized imprinted gene expressed solely from the maternal allele (Kinoshita *et al.* 2004). FWA imprinting involves DEMETER (DME), a DNA glycosylase able to excise modified nucleotide bases and the MET1 DNA methyltransferase (Figure 2.2A). It is proposed that DME acts to protect the maternal FWA allele from MET1, which methylates tandem repeats in the FWA promoter of the paternal allele (Kinoshita *et al.* 2004). If DME protection is lost, the imprint is also lost as both maternal and paternal FWA alleles become methylated by MET1 (Kinoshita *et al.* 2004). This scenario implies methylation is the default state and active protection from methylation is required to imprint an allele. DME is primarily expressed in the female central cell before fertilization and is not expressed until long after fertilization or in the male sporophyte (Choi *et al.* 2002). This disparity in DME expression provides a window during which the imprint can be established on the maternal FWA allele prior to fertilization, but requires additional mechanisms to maintain expression after fertilization. Like FWA, FERTILIZATION INDEPENDENT SEED 2 (FIS2) is also maternally expressed and is regulated through the antagonistic action of DME and MET1 (Figure 2.2B). A distinct 200bp region upstream from FIS2 acts as the nucleation center for FIS2 paternal methylation but, unlike the MET1 methylation site in the FWA gene, there are no tandem repeats in this region (Jullien *et al.* 2006b). For both

FWA and FIS2, active MET1 methylation is required during male gametogenesis to produce paternal-specific silencing (Jullien *et al.* 2006b).

2.5.2 RNAi And Heterochromatin Formation In *Arabidopsis* FWA Imprinting

RNA-directed DNA methylation (RdDM) is a process that produces locus-specific heterochromatin formation in angiosperms, and is attributed to the need to silence transposons. Initially, dsRNA is processed by RNAi machinery into small interfering RNAs (siRNA). These siRNA then guide site-specific DNA methylation and heterochromatinization (Lippman and Martienssen 2004). Methylation produced by RdDM does not spread significantly in *cis* so silencing is precisely targeted to the region producing the dsRNA (Wang *et al.* 2001). Heterochromatin formation arising from the RdDM pathway involves the ATPase chromatin-remodeling factor DECREASE IN DNA METHYLATION1 (DDM1), a SWI/SNF homologue involved with the maintenance of H3K9 histone methylation and DNA methylation (Lippman and Martienssen 2004).

The FWA promoter contains tandem repeats that produce dsRNA from the paternal FWA allele, which guides DDM1 methylation and heterochromatin formation (Lippman and Martienssen 2004). The function of DDM1 is exclusively in the maintenance of silencing as FWA methylation cannot be re-established by DDM1 after siRNA or DNA methylation is lost (Lippman *et al.* 2003). Mutations in genes involved in the RNAi pathway of *Arabidopsis*, including *dicer-like3* and *argonaute4*, result in a loss of paternal FWA methylation (Chan *et al.* 2004). It has been proposed that the siRNA generated from the FWA promoter tandem repeats also guide DOMAINS REARRANGED METHYLTRANSFERASE (DRM), a Dmnt3 homologue, to perform *de novo* methylation (Chan *et al.* 2004). This shows that the RNAi pathway in *Arabidopsis* can initiate silencing of targeted imprinted domains.

2.5.3 Histone Methylation And Polycomb Group Silencing In *Arabidopsis* Imprinting

The *Arabidopsis* Polycomb group protein MEDEA (MEA) gene is imprinted, resulting in expression exclusively from the maternal allele in the endosperm (Figure 2.2C). Similar to FWA and FIS2 imprinting, MEA regulation also involves DME activation and MET1 DNA methylation (Xiao *et al.* 2003). However, while DNA methylation is found in the promoter region of the paternal MEA allele, it likely does not play a large role in the initial regulation of the imprint (Luo *et al.* 2000). Transcriptional activation of maternal MEA is maintained in the female central cell by DME (Choi *et al.* 2002), while the paternal MEA allele is silenced by H3K27 histone methylation (Jullien *et al.* 2006b). Paternal MEA silencing is maintained by a Polycomb group complex, which includes FERTILIZATION INDEPENDENT ENDOSPERM (FIE), FIS2 and, the maternally produced MEA (Jullien *et al.* 2006a; Jullien *et al.* 2006b). This Polycomb group complex is able to initiate a self-reinforcing loop of silencing, maintaining H3K27 methylation and recruiting additional Polycomb complexes.

MEA not only assists in regulating its own imprinted expression, but also causes a cascade of imprinted expression in the genes that it regulates. The gene PHERES1 (PHE1) is regulated by the imprinted MEA protein and, as a consequence, is also imprinted (Kohler *et al.* 2005). PHE1 encodes a type I MADS-box protein, a protein family typically involved in DNA binding, and leads to uncontrolled endosperm proliferation when over expressed. MEA, acting as part of a multi-protein complex with other polycomb group proteins, forms condensed chromatin structures at its binding site within the PHE1 promoter which silences the PHE1 gene (Figure 2.2D) (Kohler *et al.* 2003). As only the maternal MEA allele is active prior to fertilization in the endosperm, PHE1 Polycomb silencing is also limited to the maternal allele (Kohler *et al.* 2005). The imprinting of both MEA and PHE1 demonstrate that the imprinting of a regulatory gene can produce a cascade of parent-specific gene expression.

2.5.4 The mee1 Gene Is Imprinted In The Maize Embryo

While all imprinted genes in *Arabidopsis* have so far been found to be monoallelically expressed only in the endosperm, a gene in maize, *maternally expressed in embryo 1* (*mee1*), is reported to have parent-specific expression in both the endosperm and embryo (Jahnke and Scholten 2009). Maternal-specific expression of *mee1* in the endosperm is regulated in a manner similar to that described for *Arabidopsis,* with maternal-specific active DNA demethylation and protection from DNA methyltransferases. The paternal *mee1* allele is methylated in gametes and remains methylated at all stages of development, preventing paternal transcription. The maternal allele is also methylated DNA in gametes; however, active demethylation of a DMR located near the transcriptional start site of *mee1* occurs after fertilization, suggesting the initial parent-specific demarcation of the alleles is independent of DNA methylation. During gamete production, the maternal allele regains DNA methylation within the DMR. It remains to be determined what epigenetic mark establishes the maternal imprint but, it appears as though the *mee1* DMR is in fact a differentially demethylated region, which may be a reflection of species-specific epigenetic reprogramming dynamics. Regardless, this finding illustrates the ability of the maize genome to maintain parent-specific demarcation of genes in the developing embryo, and predicts the identification of further genes with imprinted embryonic expression in plants.

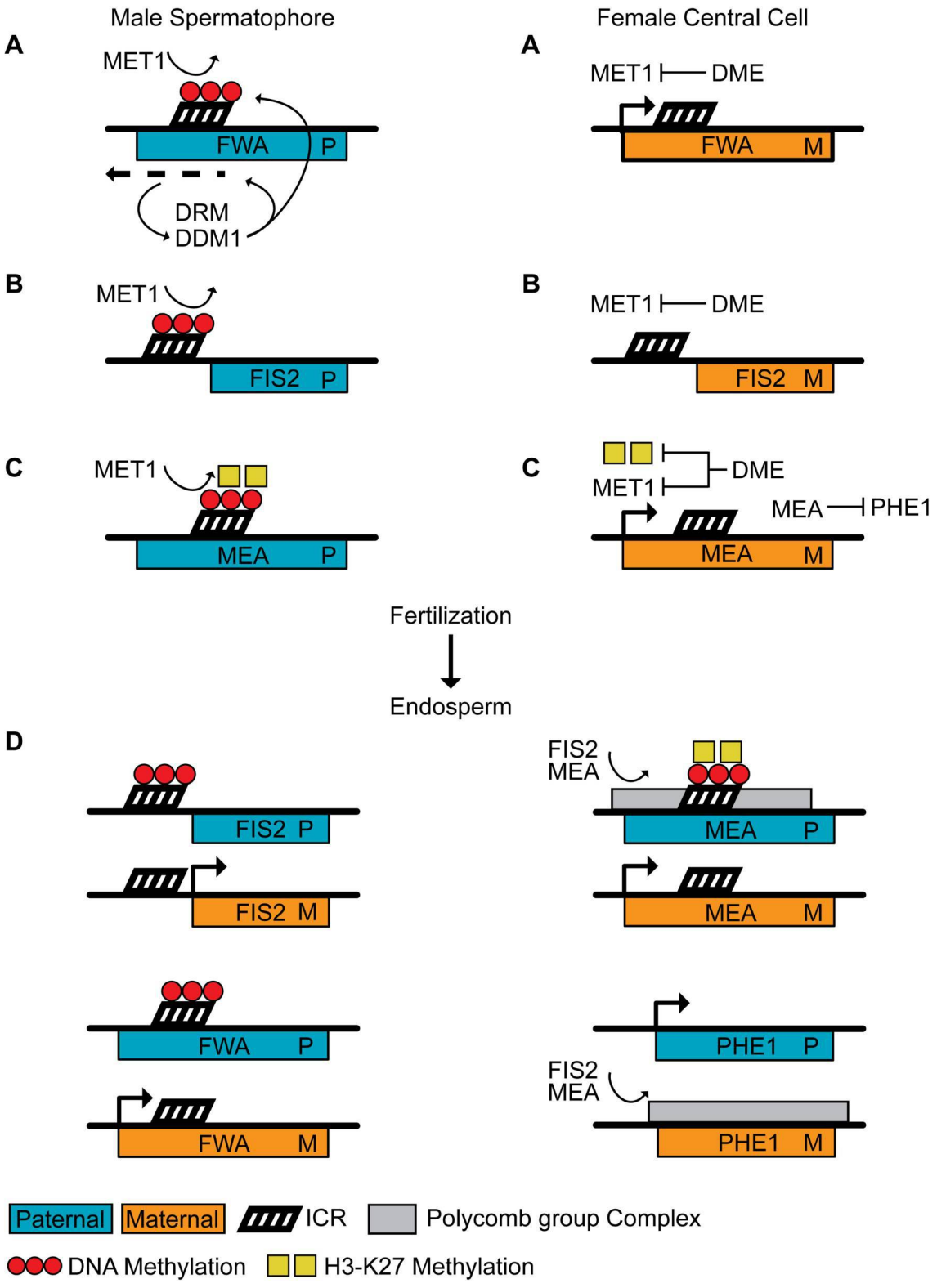

Figure 2.2 - Imprinted Regulation Of The *Arabidopsis* Genes FWA, FIS2, MEA, And PHE1.

(Figure 2.2 continued)

A) Imprinted FWA is only expressed from the maternal allele. Prior to fertilization, MET1 methylates the paternal FWA promoter. In the male spermatophores, tandem repeats in the promoter produce siRNA (represented by the dashed arrow), which recruit DRM and DDM1 to the promoter region to maintain the methylated state. In the female central cell, DME protects the FWA promoter from MET1 methylation, facilitating maternal expression.
B) The antagonistic relationship between MET1 and DME is also involved in the imprinting of FIS2. MET1 methylates a region upstream of the paternal FIS2 allele that initiates silencing. DME protects the maternal allele from methylation.
C) The imprinted regulation of MEA also involves MET1 and DME; however, histone modification plays a key role in initiating parent-specific expression. Histone H3K27 methylation is present in the promoter region of the paternal MEA allele in addition to DNA methylation. DME protects the maternal promoter from both DNA and histone methylation. Transcribed maternal MEA, which encodes a member of the Polycomb group silencing complex, initiates the parent-specific silencing of maternal PHE1.
D) In the endosperm, the Polycomb group gene MEA contributes to its own imprinted expression in the endosperm, with maternally produced MEA involved in the silencing of the paternal MEA allele. FIS2, which is also part of a Polycomb silencing complex, contributes to silencing the paternal MEA allele. PHE1, which is regulated by Polycomb group silencing, is only expressed from the paternal allele. Maternally produced FIS2 and MEA combine to maintain the silencing of the maternal PHE1 allele.

2.6 Imprinting In Insects

The investigation of imprinting in insects has progressed quietly since early studies in *Sciara* and *Coccids* revealed that gene silencing induced by whole chromosome heterochromatinization was dependent on the parental origin of the chromosome (Schrader 1921; Metz 1925). It was the study of chromosome elimination in the fungus gnat, *Sciara,* which lead to the use of the descriptive term "imprint" (Crouse 1960). Crouse reported that X chromosomes acquire an "imprint" which directs paternally derived X chromosomes to be eliminated from somatic cells and ensures that only the female X chromosomes remain in the gametes. This work provided explicit evidence of parent-specific silencing. Whole chromosome imprinted regulation such as this is not uncommon in insects (Normark 2003); however, parent-specific transcriptional silencing of smaller chromosome regions, similar to that found in mammals and plants, has also been described in *Drosophila.*

2.6.1 Genomic Imprinting In *Drosophila*

Thus far, all imprinted domains in *Drosophila melanogaster* have been found only in chromosome regions that are heterochromatic (Lloyd 2000). In *Drosophila*, most heterochromatin is compartmentalized into large blocks such as those flanking the centromeres, the entire Y chromosome, and in a few discrete regions that are developmentally controlled. The relegation of imprinted domains to gene poor, largely uncharacterized, chromosomal regions is selectively advantageous as it limits parent-specific silencing to relatively few genes (Anaka *et al.* 2009). But this property has also has made identifying endogenous imprinted genes in *Drosophila* difficult. Most known imprinted domains in *Drosophila* have been detected through position-effect variegation (PEV), which causes variegated transcriptional silencing of gene clusters placed adjacent to heterochromatic regions. Using transgenes or reporter genes placed into heterochromatic regions, imprinted domains have been identified by the display of parent-specific PEV silencing of the marker gene. The majority of the *Drosophila* Y chromosome is imprinted, as inserted transgenes are silenced in a parent-specific manner (Haller and Woodruff 2000; Maggert and Golic 2002), while distinct imprinted domains

have been reported in heterochromatic regions of the X chromosome and the autosomes (Cohen 1962; Lloyd 2000).

2.6.2 Imprinting Of The *Drosophila Dp(1;f)LJ9* Mini-X Chromosome

The *Drosophila Dp(1:f)LJ9* mini-X chromosome is the result of an X chromosome inversion and deletion which juxtaposes euchromatic genes to a heterochromatic *Drosophila* imprinting center (Figure 2.3A) (Hardy *et al.* 1984; Lloyd *et al.* 1999a). One of the euchromatic genes that becomes imprinted is the eye color gene *garnet.* This gene is uniformly expressed when maternally inherited and exhibits variegated silencing when paternally inherited, and so acts as a reporter for the imprint. Mutations which alter PEV by either enhanced silencing (*E(var*)) or suppressed silencing (*Su(var)*) do so by affecting proteins and accessory factors involved in heterochromatin formation. An extensive screen of the effects of *Su(var)* mutations on imprinted *garnet* expression revealed that both HP1 (*Su(var)2-5*) and the H3K9 histone methyltransferase (*Su(var)3-9*) were required for the maintenance of the paternal imprint (Figure 2.3B) (Joanis and Lloyd 2002). Additionally, a mutation of *Su(var)3-3*, responsible for H3K4 demethylation (Rudolph *et al.* 2007), also disrupted the silencing of the paternally inherited *Dp(1:f)LJ9* (Joanis and Lloyd 2002). This suggests active removal of the activating H3K4 methylation mark is required before H3K9 methylation can direct HP1 recruitment and the formation of heterochromatin. While Polycomb group proteins have been implicated in the regulation of both mammalian and plant imprinting (Delaval and Feil 2004; Takeda and Paszkowski 2005), they do not appear to have any role in epigenetic regulation from the *Dp(1:f)LJ9* imprinting center. Mutations in Polycomb group genes, including *Enhancer of zeste E(z)*, which initiates H3K27 methylation, has no affect on paternal-specific silencing (Joanis and Lloyd 2002).

None of the *Su(var)* mutations tested on *Dp(1:f)LJ9* had any affect on the stability of the maternal imprint, demonstrating that maternal inheritance of *Dp(1:f)LJ9* allows a stable boundary to form between the marker gene and the ICR to counteract heterochromatinization. The compact *Drosophila* genome utilizes many insulator proteins to create regulatory domains, but only the CTCF insulator protein is highly

conserved (Schoborg and Labrador 2010). Similar to the role of CTCF in maintaining mammalian imprinted domains, CTCF also acts to protect maternally-inherited *Dp(1:f)LJ9* by acting as a boundary element against the spread of heterochromatin (Figure 2.3B) (MacDonald *et al.* 2010). Other *Drosophila*-specific insulator proteins have not yet been tested for their involvement in the *Dp(1:f)LJ9* maternal-specific boundary; however, many of these insulator proteins depend on PcG and Trx group proteins for proper function (Gerasimova and Corces 1998). The failure of PcG and Trx group mutations in modify maternal *Dp(1:f)LJ9 garnet* expression suggests other insulators would not be involved.

The role of heterochromatin at the *Dp(1:f)LJ9* imprint center is limited to imprint maintenance; no *Su(var)* mutations, Polycomb group protein mutations, or chemical heterochromatin modifiers impacted either the maternal or paternal establishment of the imprint (Lloyd *et al.* 1999a; Joanis and Lloyd 2002). Similarly, CTCF is not involved in establishment of the maternal imprint (MacDonald *et al.* 2010), mirroring its role in mammalian imprinting, in which it is also not required for imprint establishment (Schoenherr *et al.* 2003; Szabo *et al.* 2004). These findings illustrate the fact that distinct epigenetic mechanisms are used for the establishment and maintenance of parent-specific expression from the *Dp(1:f)LJ9* ICR. Establishment of the imprint requires correct passage through the germline, as evidenced by the loss of the *Dp(1;f)LJ9* paternal imprint in cloned *Drosophila* (Haigh and Lloyd 2006).

Regulation of the *Dp(1;f)LJ9* imprinting center demonstrates features of both discrete mammalian ICRs and whole chromosome imprinting characteristics found in other insects. Paternal inheritance of the disrupted imprinting region results in the spreading of heterochromatic silencing to proximal areas; a similar spreading of silencing from an imprinted region has also been described in mammals (Greally *et al.* 1999). However, a secondary effect of the exposed paternal *Dp(1;f)LJ9* ICR is a chromosome-wide decrease in transcription (Anaka *et al.* 2009), similar to the imprinted silencing of whole chromosomes in *Coccids*. The stable maternal boundary generated from the *Dp(1;f)LJ9* ICR prevents both the local spreading of heterochromatin and the

chromosome-wide reduction of transcription (Anaka *et al.* 2009). This finding demonstrates that silencing initiated from a heterochromatic ICR is able to impose chromosome-wide alterations in regulation when not properly insulated within a heterochromatic region.

2.6.3 RNAi And Imprinting In *Drosophila*

RNAi is likely to be involved in laying the initial foundation for heterochromatin formation in *Drosophila* as disruption of RNAi machinery leads to a reduction in H3K9 methylation and the dispersal of HP1 (Pal-Bhadra *et al.* 2004). The *Drosophila* dodeca centromeric binding protein 1 (DDP1) binds a conserved sequence within centric heterochromatin and is capable of binding RNA through a single-stranded nucleic acid binding motif. DDP1 has been found to be an active component in early heterochromatin formation and may act to facilitate RNA-DNA interactions in heterochromatic domains (Birchler *et al.* 2004).[1]

Drosophila dosage compensation involves an increase in male X chromosome expression instead of the silencing of one female X chromosome, as occurs in mammals (Deng and Meller 2006a). Increased transcription of the male X chromosome coincides with the binding of the male-specific lethal complex (MSL), which is recruited to specific chromosome sites by the non-coding RNAs *roX1* and *roX2* (Deng and Meller 2006a). Deletion of both *roX* genes eliminates compensated expression from genes on the X chromosome, resulting in male lethality (Deng and Meller 2006b). Similar to the stabilization role of *Xist* in spreading of X chromosome silencing in mice, the MSL complex is still able to colocalize to specific X chromosome sites and direct limited activation in the absence of roX (Deng and Meller 2006a). The spreading of MSL transcriptional activation is dependent on *roX* RNA transcription (Kelley *et al.* 2008). Recently, it has been reported that experimental manipulation causing maternal inheritance of the Y chromosome significantly relieves male lethality caused by *roX*

[1] The results presented in Chapter 6 suggest that distinct RNAi pathways are involved in the establishment and maintenance of the *Dp(1;f)LJ9* imprint, and that paternal silencing involves RNAi guided by antisense RNA transcripts.

mutations, suggesting imprinted regions on the Y chromosome augment *roX* expression (Menon and Meller 2009). This suggests correct passage of the Y chromosome through the male germline is requited to establish the correct male imprints that influence dosage compensation in *Drosophila*.

2.6.4 DNA Methylation And Imprinting In Insects

There is a precedent for the involvement of DNA methylation in insect imprinting in the mealybug *Planococcus citri*. Complete silencing of paternally inherited chromosomes in males is associated with DNA hypomethylation (Bongiorni *et al.* 1999). In this case, hypomethylated chromosomes, which have been inherited paternally, become silenced in males, while chromosomes inherited maternally remain hypermethylated and active. The epigenetic imprint marking paternal chromosomes for silencing appears to be H3K9 di- and trimethylation, which is established during gametogenesis, while the lack of H3K9 di- and trimethylation on the maternal chromosomes may simply reflect a default imprinted state (Bongiorni *et al.* 2009). Heterochromatic spreading reinforces the silent state of paternal chromosomes, as HP1-like and HP2-like complexes are recruited to chromosomes with H3K9 di- and trimethylated histones (Bongiorni *et al.* 2007). It is proposed that silencing of entire paternal chromosomes is nucleated from discrete ICRs marked by H3K9 di- and trimethylation, which escape early embryonic activation signals and propagate chromosomal silencing (Bongiorni *et al.* 2009). Such spreading of silencing, originating from discrete ICRs to cover the entire chromosome, corresponds to the mechanisms guiding parent-specific chromosomal regulation described in *Drosophila* and mouse extraembryonic tissues.

Drosophila possesses a single DNA methyltransferase, Dnmt2, which primarily methylates CpA and CpT dinucleotides early in embryogenesis (Kunert *et al.* 2003). In the developing embryo nuclear concentrations of DNA methylation peak early embryogenesis then begin to decline as development progresses (Schaefer *et al.* 2008; Phalke *et al.* 2009). DNA methylation in *Drosophila* is not critical for development, as no observable phenotype results from *Dnmt2* disruption (Lyko *et al.* 2000). However,

Dnmt2 has been found to have a role in the genomic regulation of retrotransposons, suppressing retrotransposon transcription in somatic cells of the early embryo (Phalke *et al.* 2009). Loss of *Dnmt2* resulted in the mislocalization of the H3K20 methyltransferase, resulting in the elimination of H3K20 trimethylation and reduced retrotransposon repression (Phalke *et al.* 2009), illustrating the potential for DNA methylation to assist in the recruitment and stabilization of heterochromatic factors in *Drosophila*.

The role of Dnmt2 in retrotransposon repression does not extend to the germline (Phalke *et al.* 2009). This finding is supported by research involving transgenic *Drosophila* with mammalian Dnmts. Flies over-expressing mammalian Dnmts are not viable (Lyko *et al.* 1999); yet, germline specific expression of mammalian Dnmts does not affect fertility or the viability of progeny (Weyrich *et al.* 2008). Together, these findings suggest that genomic regulation by DNA methylation in *Drosophila* is restricted to somatic cells and, unlike mammals and plants, does not have an essential role in the germline. While research in this area is still in its infancy, these findings would suggest that DNA methylation is not be involved in establishing an imprint epigenetic mark in *Drosophila*.[2]

2.6.5 Conservation Of Imprinting Elements Between Mammals And *Drosophila*

Various transgenic *Drosophila* lines have been produced which contain either mouse or human ICRs (Lyko *et al.* 1997; Lyko *et al.* 1998; Erhardt *et al.* 2003). These ICRs function as silencers in *Drosophila* but do not confer parent-specific silencing. Similar experiments involving human ICRs introduced into transgenic mice also resulted in a loss of parent-specific regulation (Blaydes *et al.* 1999; Jones *et al.* 2002). Transgenic studies involving the mouse *H19* ICR exemplify remarkable conservation of epigenetic function between the mouse and *Drosophila* genomes. A specific region of the upstream *H19* ICR was identified as a silencing element in mice by first being identified as a

[2] The results presented in Chapter 4 demonstrate that DNA methylation is not involved *Dp(1;f)LJ9* imprint establishment, but is required to maintain the paternal-specific silencing during the early stages of embryogenesis.

required sequence for silencing in transgenic *Drosophila* (Brenton *et al.* 1999). Furthermore, the production of non-coding RNA transcripts from the upstream *H19* ICR was also first discovered in transgenic *Drosophila*, where non-coding RNA production from the transgenic insert was associated with reporter gene silencing (Schoenfelder *et al.* 2007). The upstream *H19* ICR is necessary for proper repression of paternal *H19* expression in mice (Drewell *et al.* 2000), where the non-coding transcripts are thought to be involved in the recruitment of other silencing mechanisms (Schoenfelder *et al.* 2007). Both of these studies involving the transgenic mouse *H19* ICR identified endogenous silencing mechanisms using a transgenic system, demonstrating epigenetic regulatory fidelity between two distinct organisms.

The *Drosophila* insulator protein, *suppressor of hairy wing* (*Su(Hw)*), and Polycomb group proteins, *Enhancer of zeste* (*E(z)*) and *Posterior sex combs* (*Psc*), were found to regulate the transgenic *Igf2/H19* ICR construct (Schoenfelder and Paro 2004). These results show that imprinted transgenes are able to recruit histone modifiers and chromatin remodelers to direct silencing of a chromosomal domain. The binding of *Su(Hw)* to the transgenic *Igf2/H19* ICR construct is reminiscent of CTCF binding to the endogenous *H19* ICR in mice (Szabo *et al.* 2004). In mice CTCF protects *H19* from methylation and silencing, whereas in *Drosophila Su(Hw)* binding to the *H19* ICR initiates downstream silencing, possibly by the recruitment of heterochromatic factors.

Interestingly, the ICR for the human imprinted *SNRPN* locus also acts as a silencer element in *Drosophila*; however, its silencing abilities are not dependent on *Su(Hw)* (Schoenfelder and Paro 2004). The differential regulation of separate mammalian ICRs in transgenic *Drosophila* suggests the ICRs retain a measure of epigenetic functionality and are not ubiquitously silenced by regulators of the *Drosophila* genome.

An intriguing finding from the mammal-*Drosophila* transgenic imprinting experiments is that silencing activity is often maintained, but the insulator/ boundary activity necessary for maintaining gene expression is lost. Expression from an imprinted domain requires the parent-specific recruitment of both silencing and activating

chromatin remodelers, which includes insulators. Binding of *Su(Hw)* to the transgenic *H19* ICR did not produce the same insulator properties as endogenous CTCF binding provides, but, rather, acted as a silencer (Schoenfelder and Paro 2004). Furthermore, multiple transgenic constructs, produced from sections of both human and mouse *H19* ICRs, all acted as silencing elements in *Drosophila*, but did not retain any of their insulator functions (Arney *et al.* 2006). This suggests that the maintenance of the active component of imprinted regions might be more complex than the silenced component and may require species-specific recognition of epigenetic marks. The default state may be bi-allelic silencing due to the location of imprinted genes in repressive chromosomal areas or large imprinted domains. Robust regulatory mechanisms would then be required to ensure the maintenance of active imprinted alleles, exemplified by the complex intrachromosomal folding associated with maternal activation of *H19* (Figure 2.1B). Together, these transgenic experiments show that the epigenetic mechanisms capable of silencing genes are highly conserved; however, the elements that superimpose the parental specificity of silencing are more specialized and tailored to the regulatory needs of each species.

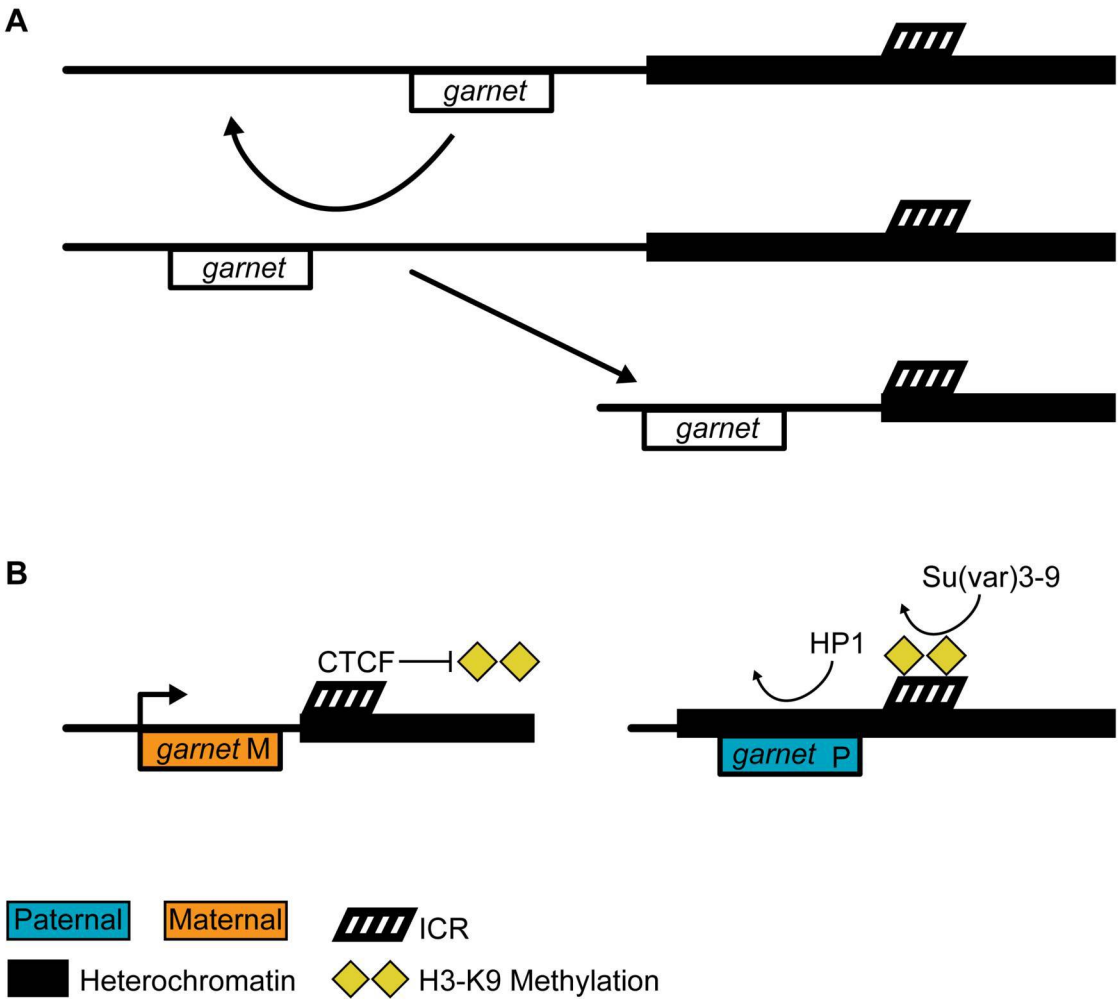

Figure 2.3 - Creation Of The *Drosophila* Mini-X Chromosome And The Resulting Imprinted Expression Of The *garnet* Marker Gene.
A) The *Dp(1:f)LJ9* mini-X chromosome was generated through an inversion followed by a large deletion by X-ray irradiation. In the resulting mini-X chromosome, *garnet* is placed next to a region centric of heterochromatin containing an imprinting center.
B) Maternal transmission of the mini-X chromosome results in active transcription of the *garnet* gene, maintained by CTCF counteracting heterochromatin formation. Paternal transmission of the mini-X chromosome results in variegated silencing of *garnet*, as a result of H3K9 methylation and heterochromatin formation.

2.7 Shared Epigenetic Regulation And The Evolution Of Genomic Imprinting

Various hypotheses have been developed to explain the evolutionary selective forces acting on genomic imprinting, and most focus on the phenotypic consequences of selective silencing of specific imprinted genes (de la Casa-Esperón and Sapienza 2003). Possibly the most debated hypothesis of this class is the parental conflict hypothesis, which proposes that competing interests between maternal and paternal genomes favor the conservation of imprinted genes that regulate fetal growth and development (Moore and Haig 1991). The parental conflict hypothesis predicts that paternally-directed fetal growth would be advantageous to the father while equal growth of all offspring would be advantageous to the mother. The oppositely imprinted genes *Igf2* (paternally expressed) and *Igf2r* (maternally expressed) are the primary example used to illustrate parental conflict, as *Igf2* expression encourages fetal growth, while *Igf2r* expression clears circulating *Igf2*, thus managing growth. While parental conflict may account for the conservation of imprinting of a subset of genes, it does not address the evolutionary advent of imprinting or the selection for the imprinting of other classes of genes (Hurst and McVean 1998; Pardo-Manuel de Villena *et al.* 2000).

2.7.1 Host Defense Hypothesis

Similarities between the epigenetic mechanisms that regulate imprinted domains and foreign DNA elements, such as retrotransposons, have led of the development of the 'host defense' hypothesis (Barlow 1993; McDonald *et al.* 2005). In mammals, the suppression of retrotransposons, especially through DNA methylation, parallels many of the characteristics of imprinted gene repression. During DNA demethylation of the male pronucleus in mice, imprinted genes escape demethylation, as do some retrotransposons (Gehring *et al.* 2009b). The Dnmt cofactor Damt3L is essential for the proper methylation of imprinted gene DMRs and elimination of Dnmt3L in mice results in not only the reduction, or complete loss, of DNA methylation within imprinted gene DMRs but also decreased DNA methylation of retrotransposons (Kato *et al.* 2007). One of the imprinted genes devoid of DMR methylation in Dnmt3L mutants is *Rasgrf*, a gene for

which imprinted expression has been attributed to the acquisition of repetitive elements (Pearsall *et al.* 1999).

The *Dlk1-Dio3* domain provides recent evidence in support of the host defense theory. This domain is present in all vertebrates but is only imprinted in eutherian mammals, suggesting the occurrence of a mammalian-specific retrotransposition event within the *Dlk1-Dio3* domain initiated the imprint (Edwards *et al.* 2008). Analysis of the monotreme genome, which as yet has no identified imprinted genes, was found to have fewer repetitive DNA elements within regions orthologous to those regulated by imprinting in therian mammals (Pask *et al.* 2009).Further support comes from the comparison of conserved genomic regions in therian mammals and monotremes. Incorporation of the retrotransposon-derived gene *Peg10* in therian mammals has resulted in the imprinted expression of the *Peg10* gene in marsupials, and the formation of an imprinted locus in mice (Suzuki *et al.* 2007). The orthologous region in monotremes is not imprinted and is also lacking the retrotransposon-derived *Peg10* gene. This suggests that the presence of retrotransposon elements has implanted imprinted epigenetic marks to the *Peg10* gene, which eventually extended to generate an imprinted locus (Suzuki *et al.* 2007).

The analysis of imprinted regions in plants has revealed a similar relationship between the location and regulation of transposons and imprinted genes. In the *Arabidopsis* endosperm, DNA demethylation by DME occurs not only at imprinted genes but also at repetitive elements and transposons (Gehring *et al.* 2009a; Hsieh *et al.* 2009). Screening for regions with reduced endosperm DNA methylation has lead to the identification of additional novel imprinted genes, which either contain, or are flanked by, repetitive elements (Gehring *et al.* 2009a). It remains to be determined if the host defense theory could account for the presence of genomic imprinting in *D. melanogaster*. However, imprinted chromosomal regions identified in *D. melanogaster*, such as the Y chromosome (Maggert and Golic 2002) and pericentric heterochromatin (Lloyd 2000), are all areas known to have high levels of repetitive DNA and transposable elements

(Pimpinelli *et al.* 1995; Steinemann and Steinemann 2005; Bergman *et al.* 2006), which are silenced, in part, by DNA methylation (Phalke *et al.* 2009).[3]

2.8 Conclusion: Common Epigenetic Mechanisms Regulate Diverse Imprinted Genes

Producing parent-specific expression requires independent regulation of the maternal and paternal alleles. Histone modification and DNA methylation, leading to heterochromatin formation, are common regulators of imprinted silencing. Non-coding RNA and RNAi and are emerging as critical components for the early recruitment of silencing mechanisms to ICRs. Boundary elements have also been shown to be necessary to maintain discrete regulatory domains by protecting active alleles, in a parent-specific manner, from silencing by blocking either the recruitment or spreading of silencing mechanisms. In all cases, genomic imprinting relies on multiple epigenetic mechanisms acting in concert to maintain and reinforce silencing.

Regulation of an imprint is a multi-step process that may employ many of the available epigenetic processes to ensure parent-specific expression of a gene. The recent identification of H3K4, H3K9, and H3K20 trimethylation as epigenetic marks across all imprinted genes tested in mice (McEwen and Ferguson-Smith 2010), is a significant step in understanding the epigenetic code that constitutes the establishment of a genomic imprint. As high-throughput screening of genome-wide epigenetic modifications is explored in more organisms, it will be interesting to see if a similar, concise pattern of epigenetic modifications emerges. It is encouraging that in *Drosophila*, both H3K9 and H3K4 methylation are associated with the *Dp(1;f)LJ9* ICR. However, while the role of H3K20 remains to be determined (Joanis and Lloyd 2002). The finding that H3K27 trimethylation was found at some, but not all, imprinted genes in mice (McEwen and Ferguson-Smith 2010), yet is the primary histone modification associated with imprinting

[3] The finding that DNA methylation is involved in imprint regulation in somatic cells, but not in the germline (Chapter 4), highlights the similarities between the genomic regulation of retrotransposons and imprinted regions in *Drosophila*.

in *Arabidopsis*, may reflect the role of H3K27 trimethylation as a ubiquitous epigenetic modification in *Arabidopsis* (Zhang *et al.* 2007).

While the same suite of epigenetic processes are seemingly involved in mammal, plant and insect imprinting, species-specific variations in the robustness of epigenetic regulators such as DNA methylation or RNAi, will be reflected in how an imprinted region is regulated. Variation in the structure of an imprinted domain, and the organism in which it found, will result in differential reliance on specific epigenetic mechanisms and, possibly, the order in which they are recruited. Evolutionary pressures and the species-specific arrangement of chromosomes also factor into the construction of large imprinted domains or novel genes acquiring imprinted regulation Nevertheless, in all species examined here, the same suite of epigenetic processes appears to be employed.

The study of genomic imprinting has progressed for the better part of a century but it is still very much in its infancy. It is clear that genomic imprinting is a form of inheritance found in many diverse organisms. Multiple epigenetic mechanisms, largely conserved between different taxa, are required for maintenance of the imprint. Recognizing the common themes in the process of genomic imprinting will aid our understanding of the mechanisms required to distinguish maternal and paternal genomes in both model and non-model organisms.

2.9 Transition To Chapter 3

The following chapter details my research, using a mutation supplied and characterized by the Dr. V.H. Meller laboratory (Wayne State University), on the effects of *Drosophila CTCF* mutations on the regulation of the *Dp(1;f)LJ9* imprint. CTCF was found to be exclusively involved in maintaining the chromatin boundary specific to the maternal imprint, which is required to counteract heterochromatin formation and preserve full maternal expression. This chapter is published in *BMC Biology*.

Chapter 3 The *Drosophila* Homolog Of The Mammalian Imprint Regulator, CTCF, Maintains The Maternal Genomic Imprint In *Drosophila melanogaster*

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3.1 Abstract

Background: CTCF is a versatile zinc finger DNA-binding protein that functions as a highly conserved epigenetic transcriptional regulator. In mammals, CTCF is known to act as a chromosomal insulator, bind promoter regions, and facilitate long-range chromatin interactions. CTCF is active in the regulatory regions of some genes that exhibit genomic imprinting, acting as insulator on only one parental allele to facilitate parent-specific expression. In *Drosophila*, CTCF acts as a chromatin insulator and is thought to be actively involved in the global organization of the genome.

Results: To determine if CTCF regulates imprinting in *Drosophila*, we generated *CTCF* mutant alleles and assayed gene expression from the imprinted *Dp(1;f)LJ9* mini-X chromosome in the presence of reduced *CTCF* expression. We observed disruption of the maternal imprint when *CTCF* levels were reduced, while no affect was observed on the paternal imprint. The effect was restricted to maintenance of the imprint and was specific for the *Dp(1;f)LJ9* mini-X chromosome.

Conclusions: CTCF in *Drosophila* functions in maintaining parent-specific expression from an imprinted domain, as it does in mammals. We propose that *Drosophila* CTCF maintains an insulator boundary on the maternal X chromosome, shielding genes from the imprint-induced silencing that occurs on the paternally inherited X chromosome.

3.2 Background

The correct establishment and propagation of epigenetic states are essential for normal development, and disruption of these processes leads to disease. Genomic imprinting is a striking example of the affect of epigenetics on gene regulation. In genomic imprinting, a mark, the imprint, is imposed on the two parental genomes during gametogenesis. In the zygote, the imprint is maintained through each mitotic division and results in the parental alleles of a gene, or entire homologous chromosomes, adopting different epigenetic states. As a result of these different epigenetic states, one parental

allele can be silenced, while the allele from the other parent, although identical in DNA sequence, is active.

The CCCTC-binding factor, CTCF, is a key player in maintaining epigenetically distinct chromatin domains. CTCF is an evolutionarily conserved zinc-finger-containing DNA-binding protein that can function both directly in gene regulation as a transcription factor and also indirectly by mediating long-range chromatin interactions. In this latter role, CTCF acts as a chromatin insulator by isolating enhancer and promoter regulatory units and as a barrier to the spread of heterochromatin (Filippova 2008). CTCF binds at multiple sites throughout the genome (Mukhopadhyay *et al.* 2004; Barski *et al.* 2007; Dion *et al.* 2007), indicating a widespread role in generating chromatin domains. Epigenetic isolation is necessary for correct maintenance of genomic imprints as imprinted domains are often interspersed among non-imprinted domains (Verona *et al.* 2003; Wan and Bartolomei 2008), necessitating their isolation from flanking regulatory regions. Additionally, the two homologous alleles must be isolated since differential gene expression patterns, chromatin conformations, and replication timing have all been associated with imprinted alleles (Pardo-Manuel de Villena *et al.* 2000).

CTCF binding has been reported at multiple mammalian imprinted domains (Wan and Bartolomei 2008), illustrating the importance of insulator function in maintaining parent-specific expression. The role of CTCF in imprinting has been best characterized for the mammalian *Igf2/H19* genes where only the maternal *H19* allele and paternal *Igf2* alleles are expressed (Bell and Felsenfeld 2000; Hark *et al.* 2000; Lewis and Murrell 2004). On the maternal chromosome, CTCF binds to a differentially methylated domain (DMD) located between the *Igf2* and *H19* genes, preventing interaction of downstream enhancer sequences with the promoter of *Igf2* and effectively silencing the gene. Methylation of the paternal DMD effectively blocks CTCF binding, allowing activation of *Igf2* expression while also initiating the silencing the *H19* gene. Binding of CTCF is necessary to maintain the epigenetic state of the imprinted alleles. Consequently, if the CTCF binding site in the *Igf2/H19* DMD is mutated, the monoallelic expression arising from the imprint is lost (Schoenherr *et al.* 2003; Szabo *et al.* 2004). An additional facet of

CTCF binding appears to be the facilitation of higher-order chromatin structures through DNA looping, a property which fortifies the silencing of *Igf2* and the activation of *H19* on the maternal chromosome (Yoon *et al.* 2007; Li *et al.* 2008). The details of CTCF binding and its consequences are less well studied at other imprinted loci; however, its insulator function and role in establishing higher-order chromatin function appear to be shared features of other mammalian imprinted loci which bind CTCF (Takada *et al.* 2002; Yoon *et al.* 2005; Wan and Bartolomei 2008; Shiura *et al.* 2009). The KvDMR1 imprinted domain, which contains two CTCF binding sites, regulates the tissue-specific expression of the gene *Cdkn1c*. It has been suggested that the tissue-specific imprinting of *Cdkn1c* is due to tissue-specific binding of CTCF to the KvDMR1 imprint domain (Fitzpatrick *et al.* 2007; Shin *et al.* 2008). The imprinted domain *Wsb1/Nf1* also requires CTCF mediated interchromosomal association with the *Igf2/H19* imprinted domain for proper parent-specific expression (Ling *et al.* 2006).

While CTCF appears to be the major insulator protein in vertebrates, the more compact *Drosophila* genome uses a variety of insulator proteins, among which is the *Drosophila* CTCF homolog dCTCF (Capelson and Corces 2004; Moon *et al.* 2005; Mohan *et al.* 2007). The insulator activity of dCTCF has been well characterized in the bithorax complex where it demarcates the chromatin domains that define separate regulatory regions (Holohan *et al.* 2007; Mohan *et al.* 2007; Kyrchanova *et al.* 2008) and, as in mammals, dCTCF is widely used as both an insulator throughout the *Drosophila* genome and also acts directly as a transcription factor (Bartkuhn *et al.* 2009; Cuddapah *et al.* 2009; Smith *et al.* 2009). Though the role of CTCF in the formation of distinct chromatin domains is conserved from *Drosophila* to mammals, the role of CTCF in epigenetic processes such as genomic imprinting have been assumed to differ (Filippova 2008). To assess the effect of dCTCF on *Drosophila* imprinting we used a well-characterized imprinting assay system, the *Dp(1;f)LJ9* mini-X chromosome, in which a readily visible eye color gene, *garnet* (*g*), is juxtaposed to an imprint control region and so becomes a marker for imprinting (Lloyd *et al.* 1999). Regulation of the *Dp(1;f)LJ9* imprint in *Drosophila* shares properties of mammalian imprinting, including transcriptional silencing of gene clusters and differential chromatin states between

homologues (Joanis and Lloyd 2002; Haigh and Lloyd 2006; Anaka *et al.* 2009). Here we present the first demonstration that *dCTCF* has a role in the regulation of genomic imprinting in *Drosophila*. As is the case in mammalian imprinting, *dCTCF* in *Drosophila* is involved in the regulation of the maternal imprint by maintaining parent-specific expression from the maternally inherited X chromosome.

3.3 Results

3.3.1 Characterization Of *CTCF* Alleles

The $CTCF^{EY15833}$ allele (FBrf0132177) was produced by insertion of the *P{EPgy2}* element into the +26 position relative to the transcription start site of the *dCTCF* gene by the Berkeley *Drosophila* Genome Project (BDGP) Gene Disruption Project (Bellen *et al.* 2004). Homozygous $CTCF^{EY15833}$ adults appear healthy and are reasonably fertile. $CTCF^{30}$, created by a partial deletion of the *P{EPgy2}* element, is homozygous lethal. $CTCF^{30}$ lacks the entire 5' end of the *P{EPgy2}* element but retains 4860 bp of the 3' end. Flanking *dCTCF* sequences and the *quemao* (*qm*) gene remain intact in $CTCF^{30}$. The reduced severity of $CTCF^{EY15833}$, with an intact *P{EPgy2}* element, suggests that a promoter in the 5' end of *P{EPgy2}* may partially rescue *dCTCF* expression. To test this idea we measured the *dCTCF* transcript levels by quantitative real time PCR (qRT PCR) of third instar larvae. Expression in homozygous $CTCF^{EY15833}$ larvae is 20% ± 4% of that in wild-type (*y w*) controls. This is consistent with previous studies showing that $CTCF^{EY15833}$ homozygotes produces approximately 50% of wild-type dCTCF protein levels (Mohan *et al.* 2007). Homozygous $CTCF^{30}$ larvae cannot be recovered in sufficient numbers for qRT PCR, but heterozygous $CTCF^{30}$/+ larvae display 68 ±9% of wild-type transcript levels, consistent with a severe reduction in expression by this mutation. Taken together, the phenotypic and expression analysis of *dCTCF* alleles indicates that both $CTCF^{EY15833}$ and $CTCF^{30}$ alleles have reduced *dCTCF* expression and that the 5' end of *P{EPgy2}* may drive sufficient expression to allow recovery of $CTCF^{EY15833}$ adults.

3.3.2 *Drosophila CTCF* Maintains The Maternal Imprint Of The *garnet* Gene On The *Dp(1;f)LJ9* Mini-X Chromosome

To test the effect of the *dCTCF* alleles on *Drosophila* imprinting we used the *Dp(1;f)LJ9* mini-X chromosome. Maternal inheritance of the *Dp(1;f)LJ9* mini-X chromosomes ($Dp(1;f)LJ9^{MAT}$) generate full expression of the marker gene *garnet*, while paternal inheritance ($Dp(1;f)LJ9^{PAT}$) generates variegated *garnet* expression (Figure 2.1 A and B, control). The variegated *garnet* gene phenotype arising from paternal transmission is mitotically stable and so results in distinct clonal regions exhibiting *garnet* expression in an eye devoid of *garnet* expression. Expression of *garnet* affects both red (pteridine) and brown (ommochrome) eye pigments, which makes this mini-X chromosome an easily assayed system in which to assess the effect of *dCTCF* alleles on imprinting in *Drosophila*.

Transmission of the *Dp(1;f)LJ9* mini-X chromosome through the female results in $y^1 z^a g^{53d}/Dp(1;f)LJ9$; +/+ mini-X chromosome-bearing male progeny with essentially wild-type expression of the *garnet* imprint marker gene. Eyes are phenotypically wild-type, with 85.3±1.4% wild-type red pigment levels and 85.7±3.4% wild-type brown pigment levels (Figure 3.1A, control). To determine the effects of *dCTCF* on the maternal maintenance of imprinted *garnet* expression, the $CTCF^X$ alleles (X represents $CTCF^{30}$ or $CTCF^{EY15833}$) were crossed to females with the *Dp(1;f)LJ9* mini-X chromosome: $y^1 z^a g^{53d}/Y$; $CTCF^X/TM3$, *Sb Ser* males x X^X/*Dp(1;f)LJ9* females. This cross generated progeny with a mutant *dCTCF* allele and a maternally imprinted mini-X chromosome ($y^1 z^a g^{53d}/Dp(1;f)LJ9^{MAT}$; $CTCF^X$/+) which were compared to progeny similarly carrying a maternally imprinted chromosome, but wild-type for *dCTCF*.

For each *dCTCF* allele tested, the mutant allele substantially reduced expression of the maternally transmitted imprint marker gene (Figure 3.1A). Progeny with a maternally inherited mini-X chromosome ($Dp(1;f)LJ9^{MAT}$) with $CTCF^{EY15833}$ reduced pigment levels to 67.2±3.1% ($p<0.001$) and 68.4±4.2% ($p<0.001$) for red and brown pigments, respectively. $Dp(1;f)LJ9^{MAT}$ progeny coupled with $CTCF^{30}$ resulted in an even greater reduction of pigment levels; 58.9±3.1% ($p<0.001$) and 56.9±4.7% ($p<0.001$) for

red and brown pigments, respectively. No variegated *garnet* expression was observed in flies with *Dp(1;f)LJ9*^{MAT} and wild-type for *dCTCF* (Figure 3.2A and B). However, when *Dp(1;f)LJ9*^{MAT} was inherited along with mutant *dCTCF* alleles, variegated *garnet* expression was observed (Figure 3.2A and B). These results demonstrate that the maintenance of the maternal imprint is highly sensitive to dCTCF dosage.

This cross also produced sibling progeny that have a maternally inherited *Dp(1;f)LJ9*^{MAT}, but with the balancer chromosome, and so wild-type for *dCTCF* (described in the methods, genotype: $y^1z^ag^{53d}$/*Dp(1;f)LJ9*^{MAT}; *TM3, Sb Ser* /+). These internal control flies are genotypically identical to the external controls but have the male parent mutant for $CTCF^X$ and so would allow detection of any paternal effects. None were detected (Figure 3.3A and B).

3.3.3 *Drosophila CTCF* Does Not Regulate The Paternal Imprint Of The *Dp(1;f)LJ9* Mini-X Chromosome

Variegated silencing of *garnet* from the paternally inherited *Dp(1;f)LJ9*^{PAT} is a consequence of the spreading of heterochromatin from the imprinted region (Lloyd *et al.* 1999; Joanis and Lloyd 2002). In contrast to the effects observed when the *Dp(1;f)LJ9* is maternally imprinted, *dCTCF* mutants had no affect on the paternal expression of the *garnet* imprint marker gene. Paternal inheritance of the mini-X chromosome (X^Y/*Dp(1;f)LJ9* males crossed to $y^1z^ag^{53d}/y^1z^ag^{53d}$; *TM3, Sb Ser*/+ females) results in $y^1z^ag^{53d}$/*Dp(1;f)LJ9*^{PAT}; +/+ progeny with variegated *garnet* expression and a marked reduction in eye pigment levels (35.5±1.5% red and 52.5±0.2% brown wild-type pigment levels, Figure 3.1B, control). The introduction of *dCTCF* mutant alleles ($y^1z^ag^{53d}/y^1z^ag^{53d}$; $CTCF^X$/*TM3, Sb Ser* females to X^Y/*Dp(1;f)LJ9 males*) to generate progeny with either mutant $CTCF^{EY15833}$ or $CTCF^{30}$ alleles and a paternally imprinted *Dp(1;f)LJ9*^{PAT} mini-X chromosome ($y^1z^ag^{53d}$/*Dp(1;f)LJ9*; $CTCF^X$/+), yielded no significant change in either red or brown eye pigment levels or phenotype (Figures 3.1B and 3.2C).

Sibling progeny with a paternally inherited *Dp(1;f)LJ9*^{PAT} along with the balancer chromosome, are wild-type for *dCTCF* (described in the methods, genotype:

$y^1 z^a g^{53d}$/*Dp(1;f)LJ9*; *TM3, Sb Ser*/+), and can be used to determine if there if a maternal effect from mothers mutant for $CTCF^X$. No maternal effect was detected; progeny wild-type for *dCTCF* from mothers with either $CTCF^{EY15833}$ or $CTCF^{30}$ showed no significant change in *garnet* expression levels (Figure 3.3C and D).

3.3.4 *Drosophila CTCF* Does Not Regulate The Establishment Of The Maternal Or Paternal Imprint Of The *Dp(1;f)LJ9* Mini-X Chromosome

To determine if the effect of *dCTCF* was on the somatic maintenance of the imprint or its establishment in the germ line of the parents, we examined the phenotype of progeny from male or female parents with both the *Dp(1;f)LJ9* mini-X chromosome and a mutant $CTCF^{30}$ allele. If *dCTCF* affects the establishment of the imprint, the imprint should be disrupted in the progeny of mutant $CTCF^{30}$ parents, but not wild-type ($CTCF^+$) parents. When we compared the phenotype of progeny wild-type for *dCTCF* but differing in their parental genotype, no significant alternation in *garnet* expression levels resulted between $Dp(1;f)LJ9^{MAT}$ progeny from mothers carrying $CTCF^{30}$ (Figure 3.4A; Mat–Est. $CTCF^{30}$) and either of the external or internal controls wild-type for *dCTCF* (Figure 3.4A; Ex. Control and Mat–Est. $CTCF^+$, respectively). This was reflected in the unchanged phenotype of progeny from mothers mutant or wild-type for *dCTCF* (Figure 3.4B; Mat–Est. $CTCF^{30}$ and Mat–Est. $CTCF^+$, respectively). Likewise, mutant *dCTCF* did not affect the establishment of the paternal imprint. $Dp(1;f)LJ9^{PAT}$ progeny from fathers carrying *Dp(1;f)LJ9* and $CTCF^{30}$ (Figure 3.4C; Pat–Est. $CTCF^{30}$) had no significant change in *garnet* expression compared to either the external on internal controls (Figure 3.4C; Ex. Control and Pat–Est. $CTCF^+$, respectively). Again, these $Dp(1;f)LJ9^{PAT}$ progeny also had no observable change in phenotype between fathers mutant or wild-type for *dCTCF* (Figure 3.4D; Pat–Est. $CTCF^{30}$ and Pat–Est. $CTCF^+$, respectively). These findings distinguish the function of dCTCF in the maintenance versus the establishment of the imprint on the *Dp(1;f)LJ9* mini-X chromosome. dCTCF is involved in the maintenance of the imprint in the soma of progeny as its reduction disrupts the maternal imprint. However, dCTCF is not involved in the establishment of the imprint, as the presence of mutant $CTCF^{30}$ in either the maternal or paternal germline, during establishment of the imprint, does not affect regulation of the imprint.

3.3.5 *Drosophila CTCF* Is Not A General Modifier Of Position-Effect Variegation

To determine if the effect of CTCF mutant alleles on the *Dp(1;f)LJ9*^{MAT} imprint, we tested the effect of *CTCF*^{30} on *In(1)w*^{m4}, a classical variegating rearrangement (Muller 1930) and two 4th chromosome transgenic constructs (Sun *et al*. 2004) in which the *white* (*w*) gene is variegated. Like the *Dp(1;f)LJ9* mini-X chromosome, the variegated silencing in *In(1)w*^{m4}is induced by the centric heterochromatin of the X chromosome (Talbert and Henikoff 2000) and the 4th chromosome has been proposed to be evolutionary related to the X chromosome (Larsson *et al.* 2001). We found that the *CTCF*^{30} allele decreased silencing of *white* in *In(1)w*^{m4};*CTCF*^{30}/+ females, while having no significant affect on *white* expression levels in *In(1)w*^{m4};*CTCF*^{30}/+ males, compared to sibling *In(1)w*^{m4};*Tb*/+ controls (Figure 3.5A). Similarly, the *6-M193* strain responded to *CTCF*^{30} with a modest decrease in *white* reporter silencing in females only (Figure 3.5B), while the *39C-33* strain showed no significant change in *white* reporter silencing from *CTCF*^{30} (Figure 3.5C). These results demonstrate that *CTCF*^{30} is not ubiquitous modifier of variegated heterochromatic silencing in *Drosophila*, consistent with the absence of an affect on silencing of the paternally inherited *Dp(1;f)LJ9* mini-X chromosome. Further, the decreased silencing of the non-imprinted variegators is opposite to the effect of *CTCF*^{30} on *Dp(1;f)LJ9*^{MAT} silencing. Thus, the role of *dCTCF* in the maintenance of the maternal *Dp(1;f)LJ9* imprint represents a distinct parent-specific function for *dCTCF* on the imprinted *Dp(1;f)LJ9* mini-X chromosome.

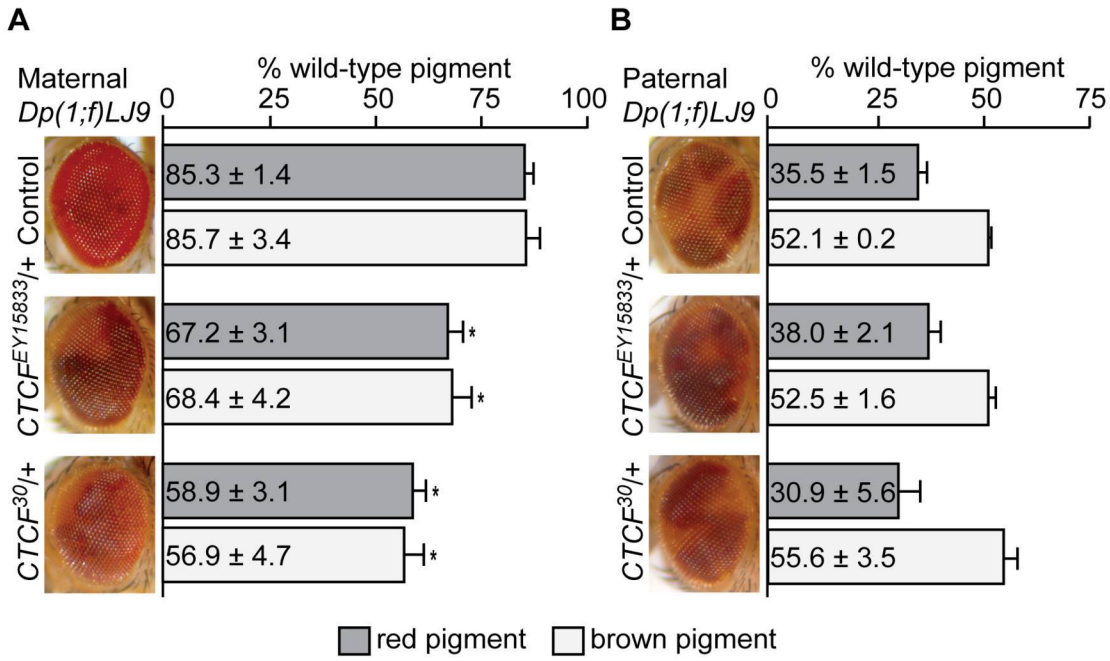

Figure 3.1 - Effect Of *dCTCF* Alleles On The Imprinted *Dp(1;f)LJ9 garnet* (*g*) Gene Expression.

A) Maternally transmitted *Dp(1;f)LJ9* mini-X chromosome; the control ($y^1z^ag^{53d}$/*Dp(1;f)LJ9*) displays full *garnet* expression with no variegation observed. Both *dCTCF* mutant alleles tested ($y^1z^ag^{53d}$/*Dp(1;f)LJ9*; $CTCF^{EY15833}$/+ and $y^1z^ag^{53d}$/ *Dp(1;f)LJ9*; $CTCF^{30}$/+) disrupt maintenance of the maternal imprint causing variegated *garnet* gene expression. Significant reduction in both red and brown pigment levels is observed in the presence of $CTCF^{EY15833}$or $CTCF^{30}$ alleles.

B) Paternally transmitted *Dp(1;f)LJ9* mini-X chromosome; the control ($y^1z^ag^{53d}$/*Dp(1;f)LJ9*) exhibits variegated *garnet* gene expression, while the introduction of $CTCF^{EY15833}$and $CTCF^{30}$ alleles had no significant affect on *garnet* gene variegation. Pigment assay values are expressed as a percentage of wild-type pigment levels ± standard deviation. Values that are significantly different from the controls are marked with an asterisk signifying p=<0.001.

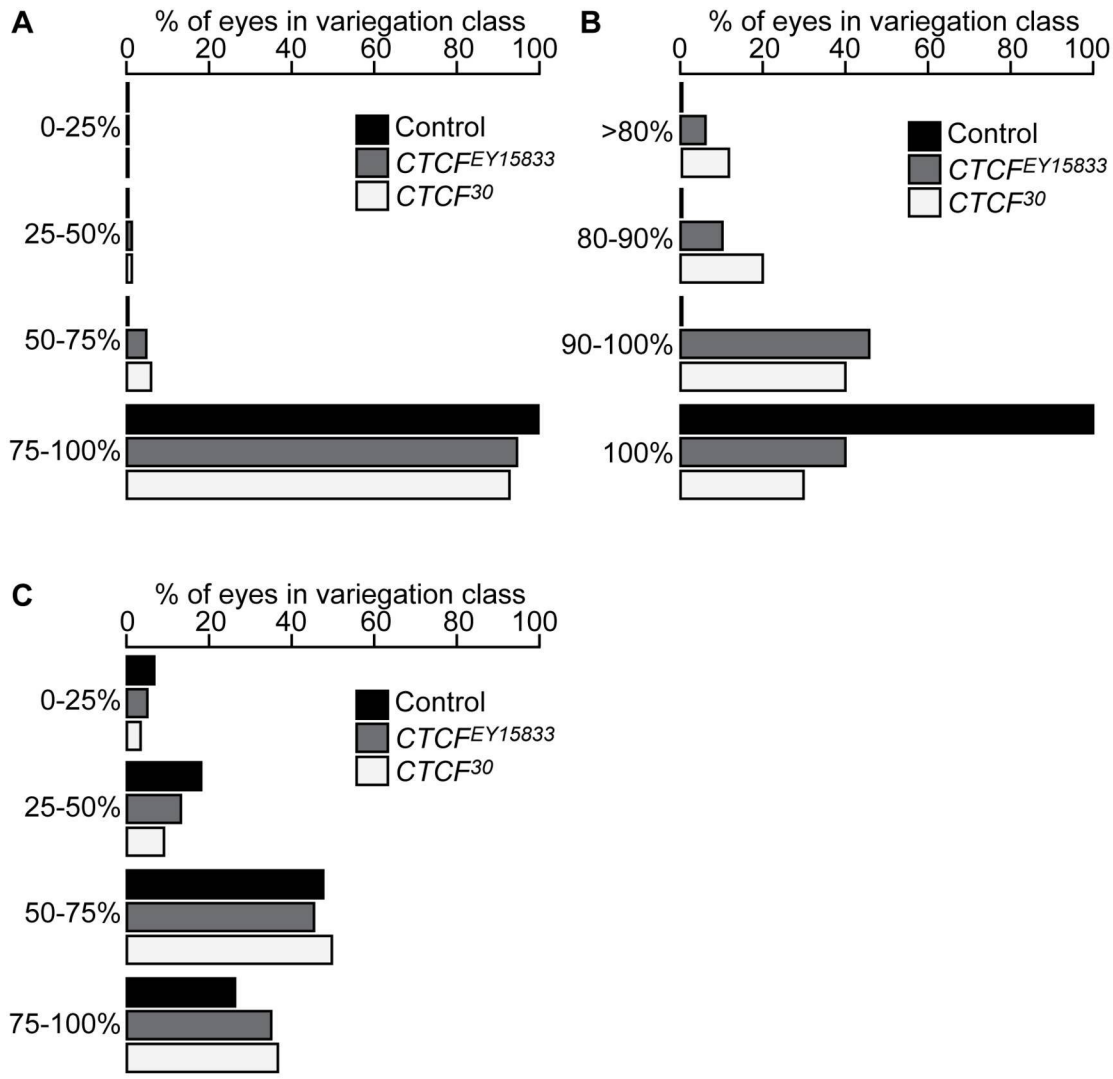

Figure 3.2 - Eye Phenotype Of *Dp(1;f)LJ9 Drosophila* With *dCTCF* Alleles.
A) Phenotypes of maternally inherited *Dp(1;f)LJ9* mini-X chromosome ranging from 0% to 100% pigmentation; control *n*= 300, $CTCF^{EY15833}$ *n*= 300, $CTCF^{30}$ *n*= 300.
B) Phenotypes of maternally inherited *Dp(1;f)LJ9* mini-X chromosome ranging from >80% to 100% pigmentation; control *n*= 150, $CTCF^{EY15833}$ *n*= 132 (Kolmogorov-Smirnov test $p<0.001$) and $CTCF^{30}$ *n*= 122 (Kolmogorov-Smirnov test $p<0.001$).
C) Phenotypes of paternally inherited *Dp(1;f)LJ9* mini-X chromosome ranging from 0% to 100% pigmentation; control *n*= 300, $CTCF^{EY15833}$ *n*= 300, $CTCF^{30}$ *n*= 300. Each eye was scored depending on its phenotypic class, and the prevalence of each phenotypic class is expressed as a percentage versus the total number of eyes scored (*n*).

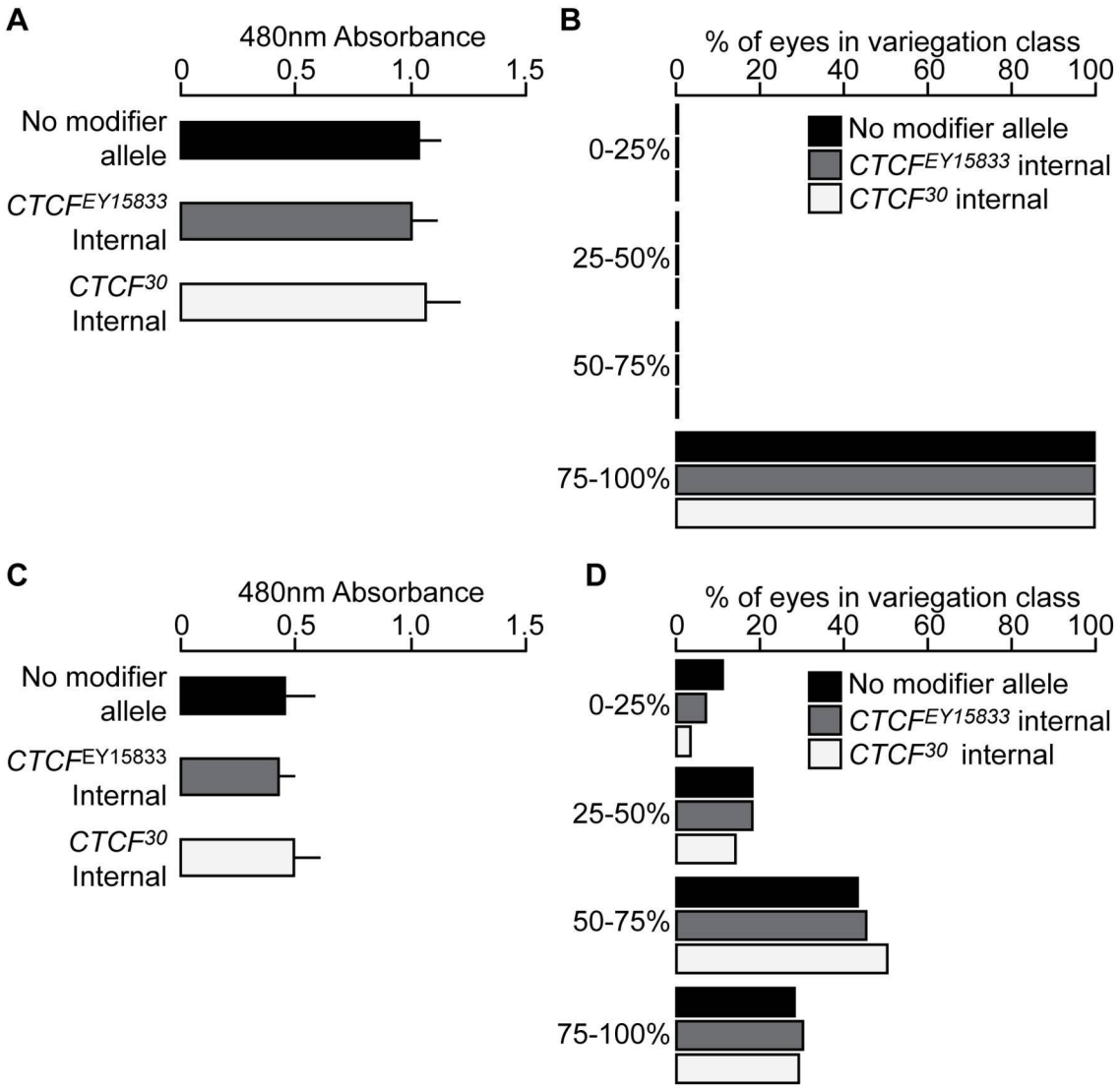

Figure 3.3 - Absence Of Maternal Or Paternal Effects From Mutant *dCTCF* On *Dp(1;f)LJ9 garnet* Expression.

External control progeny (No modifier allele) have the same genotype as internal control progeny ($y^1z^ag^{53d}$/*Dp(1;f)LJ9*; *TM3, Sb Ser*/+), but are generated from a separate cross with parents that have never encountered a mutant $CTCF^X$ allele.

A) Maternally inherited *Dp(1;f)LJ9 CTCF* internal control eye pigment levels; no significant difference in pigment levels was observed between external control progeny (No modifier allele) and internal control progeny from fathers carrying $CTCF^X$ ($CTCF^X$ internal), demonstrating that no paternal effect occurs

B) Phenotypes of maternally inherited *Dp(1;f)LJ9* ranging from 0% to 100% pigmentation; No modifier allele *n*= 300, $CTCF^{EY15833}$ internal *n*= 300, $CTCF^{30}$ internal *n*= 300. No *garnet* variegation was detected from the internal controls.

C) Paternally inherited *Dp(1;f)LJ9 CTCF* internal control eye pigment levels; no significant difference in pigment levels was observed between the external control progeny (No modifier allele) and internal control progeny from mothers carrying $CTCF^X$ ($CTCF^X$ internal), demonstrating that no maternal effect occurs.

(Figure 3.3 continued)

D) Phenotypes of paternally inherited *Dp(1;f)LJ9* ranging from 0% to 100% pigmentation; No modifier allele *n*= 300, $CTCF^{EY15833}$ internal *n*= 300, $CTCF^{30}$ internal *n*= 300. No significant change in *garnet* variegation was detected from the internal controls. Red eye pigment levels are measured by absorbance at 480nm and pigment quantification mean values for each group are based on: *n* = 5 samples (40 heads total), error bars represent standard deviation.

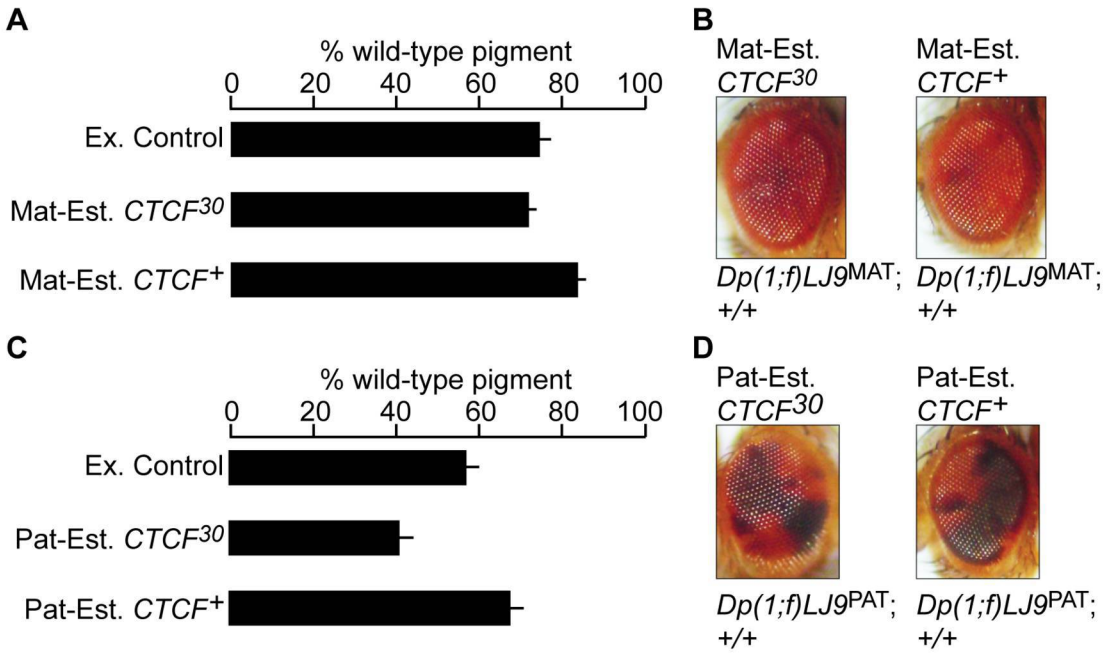

Figure 3.4 - *dCTCF* Does Not Effect Establishment Of The *Dp(1;f)LJ9* Imprint.
All genotypes tested are $y^1z^ag^{53d}$/*Dp(1;f)LJ9*; +/+, but differ in parental genotype. External control progeny (Ex. Control) are generated from parents carrying *Dp(1;f)LJ9* that have never been exposed to a mutant *dCTCF* allele. Progeny generated from a parent carrying both the imprinted *Dp(1;f)LJ9* mini-X chromosome and a mutant *dCTCF* allele test for effects on imprint establishment (Mat or Pat–Est. $CTCF^{30}$), while parental siblings carrying *Dp(1;f)LJ9* and wild-type for *CTCF* serve as an internal control (Mat or Pat–Est. $CTCF^{+}$).
A) Maternal establishment of the *Dp(1;f)LJ9* imprint is not affected by $CTCF^{30}$. No significant change in red pigment levels were detected between external control progeny (Ex. Control; n=23), mothers carrying $Dp(1;f)LJ9^{MAT}$ and $CTCF^{30}$ (Mat–Est. $CTCF^{30}$; n=10), and mothers carrying $Dp(1;f)LJ9^{MAT}$ and $CTCF^{+}$(Mat–Est. $CTCF^{+}$; n=10).
B) No phenotypic difference is present between progeny generated from *Dp(1;f)LJ9* carrying mothers, either mutant (Mat–Est. $CTCF^{30}$) or wild-type (Mat–Est. $CTCF^{+}$) for *CTCF*.
C) Paternal establishment of the *Dp(1;f)LJ9* imprint is not affected by $CTCF^{30}$. No significant change in red pigment levels were detected between external control (Ex. Control; n=44), fathers carrying $Dp(1;f)LJ9^{PAT}$ and $CTCF^{30}$ (Pat–Est. $CTCF^{30}$; n=18), and fathers carrying $Dp(1;f)LJ9^{PAT}$ and $CTCF^{+}$(Pat–Est. $CTCF^{+}$; n=29).
D) No phenotypic difference is present between progeny generated from *Dp(1;f)LJ9* carrying fathers, either mutant (Pat–Est. $CTCF^{30}$) or wild-type (Pat–Est. $CTCF^{+}$) for *dCTCF*.

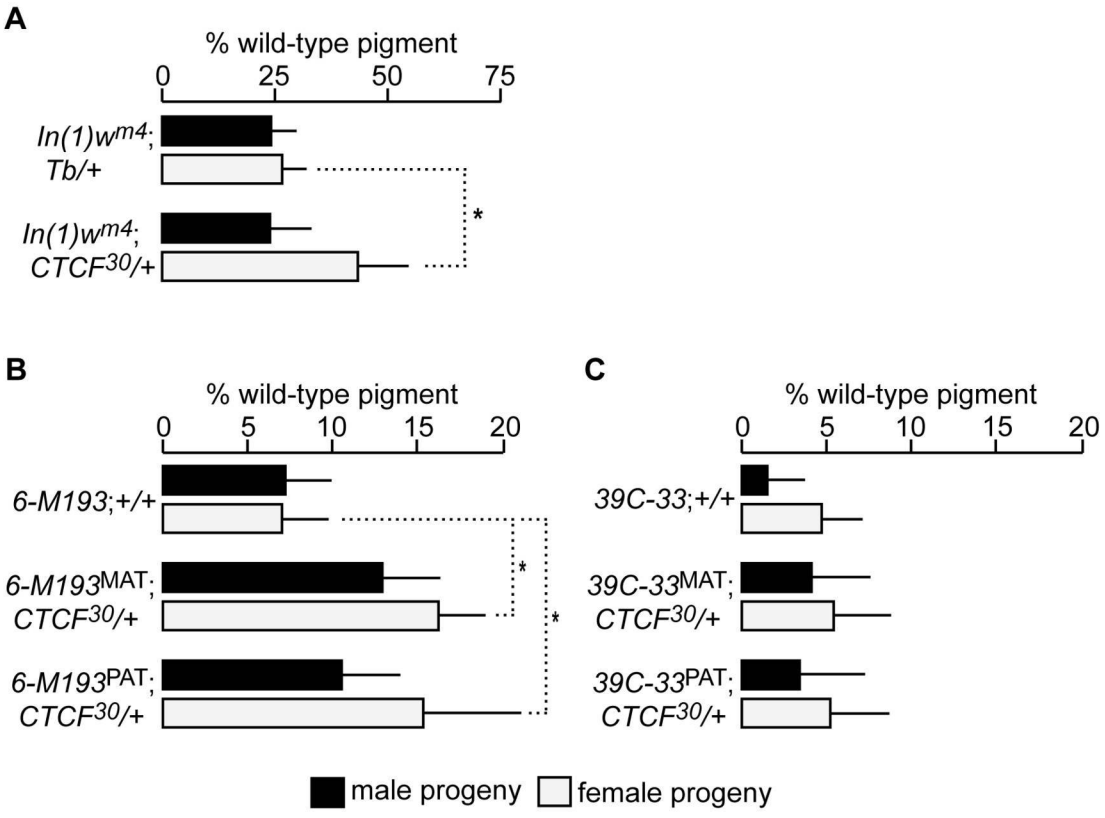

Figure 3.5 - The Effect Of *CTCF*^{30} On *White* Variegation In *In(1)w*^{m4} And The 4th Chromosome Variegating Strains 6-M193 And 39C-33

A) Pigment levels were measured for *In(1)w*^{m4}*/w*^{1118}*;CTCF*^{30}*/+* and *In(1)w*^{m4}*/Y;CTCF*^{30}*/+* genotypes compared to the corresponding sibling *In(1)w*^{m4}*;Tb/+* controls. *In(1)w*^{m4} heterozygous for *CTCF*^{30} results in an increase in *white* expression; however, this increase is only significant in female progeny (white bars). Red pigment quantification mean values for each group are based on: n = 10 (50 heads total). Error bars represent standard deviation, and values significantly different from the controls are marked with an asterisk (p=<0.001).

B) Pigment levels were independently measured for both the maternally (*6-M193*^{MAT}) and paternally (*6-M193*^{PAT}) inherited 4th chromosome variegator *6-M193*. *6-M193* heterozygous for *CTCF*^{30}, result in an increase in *white* expression when either maternally or paternally inherited; however, this increase is only significant in female progeny (white bars). Red pigment quantification mean values for each group are based on: n = 9 (45 heads total) for *6-M193*^{MAT} male and female progeny; n = 10 (50 heads total) for *6-M193*^{PAT} male and female progeny; *6-M193*;+/+ control male progeny, and n = 12 (60 heads total) for *6-M193*;+/+ control female progeny. Error bars represent standard deviation, and values significantly different from the controls are marked with an asterisk (p=<0.001).

(Figure 3.5 continued)

C) Pigment levels were independently measured for both the maternally (*39C-33*^{MAT}) and paternally (*39C-33*^{PAT}) inherited 4th chromosome variegator *39C-33*. *39C-33* heterozygous for *CTCF*^{30}, result in no significant change in *white* expression when either maternally or paternally inherited. Red pigment quantification mean values for each group are based on: n = 10 (50 heads total) for *39C-33*^{MAT} and *39C-33*^{PAT} male and female progeny, and n = 20 (100 heads total) for *39C-33*;+/+ control male and female progeny. Error bars represent standard deviation.

3.4 Discussion

CTCF is essential for insulator function in vertebrates where it plays an active role in regulating imprinted gene expression. In *Drosophila*, dCTCF has likewise been shown to be involved in the insulator function of boundary elements (Gerasimova *et al.* 2007; Smith *et al.* 2009). Our results show that parent-specific expression from an imprinted domain in *Drosophila* is dependent on dCTCF function. Maintenance of expression from maternally inherited *Dp(1;f)LJ9* mini-X chromosome is highly sensitive to dCTCF; even a modest decrease in *dCTCF* mRNA alters the maternal imprint so that it resembles the paternal imprint.

The effect of dCTCF on maternal-specific expression is limited to the maintenance of imprint. The presence of mutant *dCTCF* in either the maternal or paternal parents, when the imprint is being established, does not affect the imprint in the progeny. These results are strikingly similar to the role of CTCF in mammalian imprinting, where CTCF assists in the post-fertilization formation of an imprinted region, but is dispensable for the establishment of an imprint (Schoenherr *et al.* 2003; Szabo *et al.* 2004; Matsuzaki *et al.* 2009).

Further, the requirement for dCTCF for maintenance of the maternal *Dp(1;f)LJ9* imprint is specific, and does not represent a ubiquitous role for dCTCF in regulating heterochromatic silencing. Not only is the paternal *Dp(1;f)LJ9* imprint unaffected by mutant *dCTCF*, but other variegating *Drosophila* reporter genes respond differently to mutant *dCTCF*. Thus, the association of *dCTCF* expression with the maintenance of the maternal *Dp(1;f)LJ9* imprint boundary demonstrates a distinct function for *dCTCF* in imprinted gene expression.

In mammals, maternally imprinted regions that bind CTCF rely critically on this binding to insulate the imprinted loci and establish distinct chromatin domains. Our results show that a reduction in *dCTCF* levels disrupts the maternal imprint boundary on the *Drosophila Dp(1;f)LJ9* mini-X chromosome and consequently, the marker gene, *garnet*, is silenced. Variegated silencing of *garnet* from $Dp(1;f)LJ9^{PAT}$ inheritance is a

consequence of heterochromatin formation, nucleated from the paternal imprint control region, spreading in *cis* (Lloyd *et al.* 1999; Joanis and Lloyd 2002). The absence of an effect upon the introduction of *dCTCF* mutant alleles to *Dp(1;f)LJ9*^{PAT} suggests that dCTCF binding and boundary function occurs only on the maternal chromosome. Thus, it is conceivable that a reduction in *dCTCF* levels enables the spreading of heterochromatin on the maternal *Dp(1;f)LJ9*^{MAT} in a manner similar to that of the paternal *Dp(1;f)LJ9*^{PAT}. This would suggest that dCTCF defines the boundary of a distinct maternal-specific imprinted chromatin domain required to maintain maternal-specific gene expression on the X chromosome.

The model organism ENCyclopedia Of DNA Elements (modENCODE) project provides detailed mapping of regulatory elements throughout the *Drosophila* genome (Celniker *et al.* 2009). Large-scale profiling of dCTCF insulator sites from early embryo modENCODE data reveals several candidate dCTCF insulator sites present proximal to the predicted heterochromatic breakpoint of the *Dp(1;f)LJ9* mini-X chromosome. These dCTCF insulator sites, located between the centric heterochromatic imprinting center and the imprint marker gene *garnet*, could account for the sensitivity of the maternal imprint to dCTCF expression. If dCTCF were bound only when the X chromosome is transmitted maternally, mutations in *dCTCF* would disrupt insulator function and lead to maternal silencing of the imprint marker gene. While such binding remains to be tested, it is similar to the function of CTCF at mammalian imprinted regions.

That the structure of CTCF and its role as an insulator, barrier, and transcriptional regulator, is conserved between mammals and insects have been well established (Moon *et al.* 2005; Cuddapah *et al.* 2009; Smith *et al.* 2009). However, the finding that CTCF maintains its function in regulating the imprinting of diverse genes in such phylogenetically distinct organisms is remarkable. CTCF is a versatile DNA binding factor; subsets of its zinc fingers are adept at binding diverse DNA sequences, while the rest of the protein is able to maintain common regulator interactions and insulator function (Ohlsson *et al.* 2001). This feature may explain how CTCF can regulate

imprinting in organisms as diverse as insects and mammals, in which the imprinted target sequences are different.

Previously, the evolutionary origin of imprinting has been extrapolated from the conservation of imprinting among specific genes. Such studies have led to the proposal that mammalian imprinting is of relatively recent origin and restricted to eutherian mammals (Hurst and McVean 1998; Nolan *et al.* 2001). However, studies showing that the molecular mechanism of imprinting is highly conserved have suggested a much more ancient origin (Greally *et al.* 1999; Pardo-Manuel de Villena *et al.* 2000; Anaka *et al.* 2009). Mammalian imprint control elements inserted into transgenic *Drosophila* act as discrete silencing elements (Lyko *et al.* 1997; Lyko *et al.* 1998) and can retain post-transcriptional silencing mechanisms involving non-coding RNA (Schoenfelder *et al.* 2007). While these transgenic imprinting elements lose their parent-specific functions, the retention of epigenetic silencing mechanisms suggests an ancient and conserved origin of imprinting mechanisms. Our finding that CTCF has a role in the maintenance of maternal imprints in insects, as it does in mammals, supports the possibility of evolutionary conservation for both CTCF function and the mechanisms of genomic imprinting.

3.5 Conclusions

CTCF is a multifunctional protein with a conserved role as a chromosomal insulator in both mammals and *Drosophila*. To determine if dCTCF is involved in imprinted regulation in *Drosophila*, as it is in mammals, we generated a *dCTCF* mutant allele with severe reduction in *dCTCF* expression and tested its effects on the expression of the imprint marker gene, *garnet,* on the *Dp(1;f)LJ9* mini-X chromosome. Full *garnet* gene expression, which occurs when the *Dp(1;f)LJ9* mini-X chromosome is maternally inherited, was disrupted when *dCTCF* expression levels were reduced. No affect of reduced *dCTCF* expression was observed on the *Dp(1;f)LJ9* mini-X chromosome when it was inherited paternally. The affect of *dCTCF* mutations is on the maintenance rather than the establishment of the imprint, and is specific to the *Dp(1;f)LJ9* mini-X chromosome. These results demonstrate that dCTCF is involved in maintaining parent-

specific expression from the maternally inherited X chromosome in *Drosophila*, a role paralleling its involvement in mammalian imprinting.

3.6 Methods

3.6.1 *Drosophila* Culture

All crosses were maintained at 22°C and cultured on standard cornmeal-molasses *Drosophila* media with methylbenzoate (0.15%) as a mould inhibitor. Each set of crosses was performed in 55 ml shell vials and contained 10-15 virgin females and 10-15 males. Each of the crosses was subcultured three or four times at three-day intervals before the parents were discarded. Each cross was replicated four to six times and the progeny pooled. All stocks were obtained from the Bloomington *Drosophila* stock center with the exception of the $CTCF^{30}$ allele and the variegating 4th chromosome transgene strains. The $CTCF^{EY15833}$ allele was created by insertion of P{EPgy2} 27 bp downstream of the *dCTCF* transcription start site. The homozygous lethal $CTCF^{30}$ allele was generated by imprecise excision of $CTCF^{EY15833}$. The $CTCF^{30}$ deletion was characterized by amplification across the break and sequencing of the PCR product. $CTCF^{30}$ is deleted for all 5' transposon sequences but retains 4860 bp at the 3' end. No genomic sequence was removed by the $CTCF^{30}$ deletion. The 4th chromosome variegating strains were generated using the transposable *P*-element *P[hsp26-pt, hsp70-w]*, which contains the a *hsp70*-driven *white* gene that is susceptible to silencing caused by heterochromatin formation(Sun *et al.* 2004). The *6-M193* strain has the construct inserted within a *1360* transposon and inside the *Syt7* gene (4th chromosome coordinate: 323400, FlyBase release 3), while the *39C-33* strain is generated from the construct being inserted into gene of the RNA binding protein *gawky* (4th chromosome coordinate: 680211, FlyBase release 3) which is in close proximity to a *1360* transposon (Sun *et al.* 2004).

To determine the effect of mutant *dCTCF* on imprint maintenance, the $CTCF^{30}$ and $CTCF^{EY15833}$ alleles were crossed into $y^1z^ag^{53d}$ background to yield stable stocks of $y^1z^ag^{53d}/y^1z^ag^{53d}$; $CTCF^X/TM3, Sb\ Ser$ (where $CTCF^X$ is the $CTCF^{30}$ or $CTCF^{EY15833}$ allele). To test the effect of a *dCTCF* allele on the paternal imprinting of *garnet*,

Dp(1;f)LJ9, y^+g^+/X^Y males were crossed to $y^1z^ag^{53d}/y^1z^ag^{53d}$; $CTCF^X$/*TM3, Sb Ser* females, the reciprocal cross with *Dp(1;f)LJ9, y^+g^+/X^X* virgin females was performed to test the effect on the maternal imprinting of *garnet* (Figure 3.6). $y^1z^ag^{53d}$/*Dp(1;f)LJ9*; $CTCF^X$/+ male progeny were collected based on wild-type *yellow* (y^+) body color, which independently confirms the presence of the *Dp(1;f)LJ9* chromosome, while the *zeste* allele (z^a) reduces background eye color of the *g* allele (g^{53d}). The $y^1z^ag^{53d}$/*Dp(1;f)LJ9*; *TM3, Sb Ser*/+ sibling males were used as internal controls (Figure 3.6). The 'no modifier' control test cross for paternal *garnet* imprinting consisted of *Dp(1;f)LJ9, y^+g^+/X^Y* males crossed to $y^1z^ag^{53d}/y^1z^ag^{53d}$; *TM3, Sb Ser/+* females, with the reciprocal cross serving as the maternal control: $y^1z^ag^{53d}$/*Dp(1;f)LJ9*; +/+ and $y^1z^ag^{53d}$/*Dp(1;f)LJ9*; *TM3, Sb Ser*/+ male progeny were collected as controls.

To test for the effects of *CTCF* on germline imprint establishment, mutant *CTCF* must be present in parents carrying the *Dp(1;f)LJ9* mini-X chromosome. To detect the effect of $CTCF^{30}$ on the establishment of the imprint, *Dp(1;f)LJ9; e/e* flies were balanced over *X^X; $CTCF^{30}$/e* for maternal establishment (Figure 3.7), or *X^Y; $CTCF^{30}$/e* for paternal establishment (Figure 3.8). *X^X/Dp(1;f)LJ9; $CTCF^{30}$/e* females were crossed to $y^1z^ag^{53d}/Y$ males to test maternal imprint establishment (Mat Est. $CTCF^{30}$), and the reciprocal cross tested for paternal imprint establishment (Pat Est. $CTCF^{30}$). Maternal establishment controls (Mat-Est. $CTCF^+$) consisted of *X^X/Dp(1;f)LJ9; e/e* females crossed to $y^1z^ag^{53d}/Y$ males, and paternal establishment controls (Pat-Est. $CTCF^+$) were *X^Y/Dp(1;f)LJ9; e/e* males crossed to $y^1z^ag^{53d}/y^1z^ag^{53d}$ females. External controls were also produced by crossing F1 generation *X^X/Dp(1;f)LJ9; e/e* females to $y^1z^ag^{53d}/Y$ males for maternal establishment, and the reciprocal cross for paternal establishment.

To assess the effect of $CTCF^{30}$ on other variegating strains, $CTCF^{30}$ / *TM6, Tb* flies were crossed to $In(1)w^{m4}$ and two variegating 4th chromosome (*6-M193* or *39C-33*) strains. For the $In(1)w^{m4}$crosses, $In(1)w^{m4}$ females were crossed to w^{1118}/Y; $CTCF^{30}$ males and the red pigment levels of the $In(1)w^{m4}$/Y; $CTCF^{30}$/+and $In(1)w^{m4}/w^{1118}$; $CTCF^{30}$/+ progeny compared with that of their $In(1)w^{m4}$; *TM6, Tb* siblings. Reciprocal crosses were performed with the variegating 4th chromosome strains to control for both the maternal

and paternal inheritance of the variegating transgene. The maternal cross consisted of *w-/w-; +/+; +/+; var/var* females crossed to *y/w-; CTCF^{30} / TM6, Tb; +/+; +/+* males, and paternal inheritance used *y/w-; +/+; +/+; var/var* males crossed to *w-/w-; CTCF^{30}/+; +/+; +/+* females, where *var* represents the variegating 4th chromosome transgene. The resulting progeny were separated by sex (*y/w-; CTCF^{30}; +/+; var/var* males and *w-/w-; CTCF^{30}/+; +/+; var/var* females) and compared to the balancer controls (*y/w-; TM6, Tb/+; +/+; var/var* males and *w-/w-; TM6, Tb/+; +/+; var/var* females).

3.6.2 Measurement Of *dCTCF* Expression

Quantitative real time PCR (qRT PCR) was used to measure *dCTCF* expression. Total RNA was prepared from 3 groups of 50 larvae for each genotype. One μg of total RNA was reverse transcribed using random hexamers and ImProm-II reverse transcriptase (Promega). Quantitative PCR was performed as previously described (Deng *et al.* 2005). *dCTCF* primers (CTCF F2400, ACGAGGAGGTGTTGGTCAAG and CTCF R2485, ATCATCGTCGTCCTCGAAC) were used at 300 nM. Two technical replicates from each sample were amplified. Expression was normalized to *Dmn*, a gene that has proved reliable for this purpose (Deng *et al.* 2005).

3.6.3 Quantification Of Eye Pigment Levels

Expression of the imprint marker gene*, garnet,* was quantified both visually and through the use a spectrophotometeric assay of extracted eye pigments. The visual assay assigns each eye a score in relation to its variegation class as described by Joanis and Lloyd (2002); 0-25% pigmentation, 25-50% pigmentation, 50-75% pigmentation, and 75-100% pigmentation. The prevalence of each variegation class is expressed as a percentage of all eyes assayed. As the variegated phenotype of maternally inherited *Dp(1;f)LJ9* in the presence of mutant *dCTCF* is skewed towards fully pigmented eyes, a second assay with the following variegation classes was preformed; >80% pigmentation, 80-90% pigmentation, 90-100% pigmentation, and 100% pigmentation.

The spectrophotometric assay was adapted from Real *et al* (1985). For red (pteridine) pigment, flies of each test genotype were aged for 4 days then placed in 1.5 ml Eppendorf microtubes and stored at -30 °C. For each sample set, 8 heads were placed into a 0.6 ml microtube containing 400 μl of acidified ethanol (30% EtOH, acidified to pH 2 with HCl). Pigment was extracted on an orbital shaker at 150 rpm, in the dark, for 48 hours. Absorbance of the extracted pigments was measured at 480 nm. Each 400 μl sample of extracted pigment was split into two, 200 μl volumes, independently measured, and the values averaged. 5 tubes were run per sample set, with the values averaged and expressed as a percentage of wild-type pigment levels± standard deviation. For brown (ommochrome) pigment, flies of each test genotype were aged for 4 days then placed in 1.5 ml Eppendorf microtubes and stored at -80 °C. 10 heads were placed in a 1.5 ml Eppendorf tube and homogenized with 150 μL of 2M HCl and 0.66% sodium metabisulfite (w/v). 200 μl of 1-butanol was added and the mixture was placed on an orbital shaker 150 rpm for 30 min before centrifugation at 9000 *g* for 5 min. The organic layer was removed, washed with 150 μl of 0.66% sodium metabisulfite in dH2O and placed back on the orbital shaker for a further 30 min, this step was repeated for a second wash. The organic layer was removed and measured for absorbance at 492 nm. 5 tubes were run per sample set, with the values averaged and expressed as a percentage of wild-type (O. R) pigment levels ± standard deviation. Absorbance was determined with a Pharmacia Biotech Ultrospec 2000 spectrophotometer. Representative eye pictures were photographed with a Zeiss AxioCam MRc5 mounted on a Zeiss Stemi 2000-C dissecting microscope.

Spectrophotometeric assay for quantifying the expression of the *white* transgene on the 4th chromosome variegating strains and *In(1)w^{m4}* followed the same procedure for fly aging and head collection. Heads were split into groups of 5 and placed into 150 μL of 30% EtOH, acidified to pH 2 with HCl. Pigment extraction consisted of sonicating samples for 5 seconds at 50 MHz (Sonic 300 Dismembrator Sonicator) prior to soaking samples at room temperature for 24 hours in the dark. For each sample, 90 μL of extracted pigment was loaded into 96 well microtitre trays and quantified with a Microplate Reader (Benchmark Bio-Rad) at a wavelength of 480 nm. The results from

each sample group were pooled for a final mean pigment value. Pigment levels for imprint establishment were quantified using the same spectrophotometeric assay used for quantifying the *white* variegating strains, except one head was used per sample set.

3.6.4 Statistical Analysis

Kolmogorov-Smirnov two-sample tests were used to determine the statistical significance between the visual eye scores from control and *dCTCF* mutant data sets. Statistical significance for both red and brown pigments was determined by an ANOVA followed by Student's t-test with Bonferroni-corrected p values between the mean of the experimental *dCTCF* mutant data sets and the mean of the results from the appropriate control cross.

3.6.5 CTCF modENCODE Data

The modENCODE data for dCTCF insulator sites from 0-12 hour embryos were obtained from the White Lab project on the modENCODE website (http://www.modencode.org).

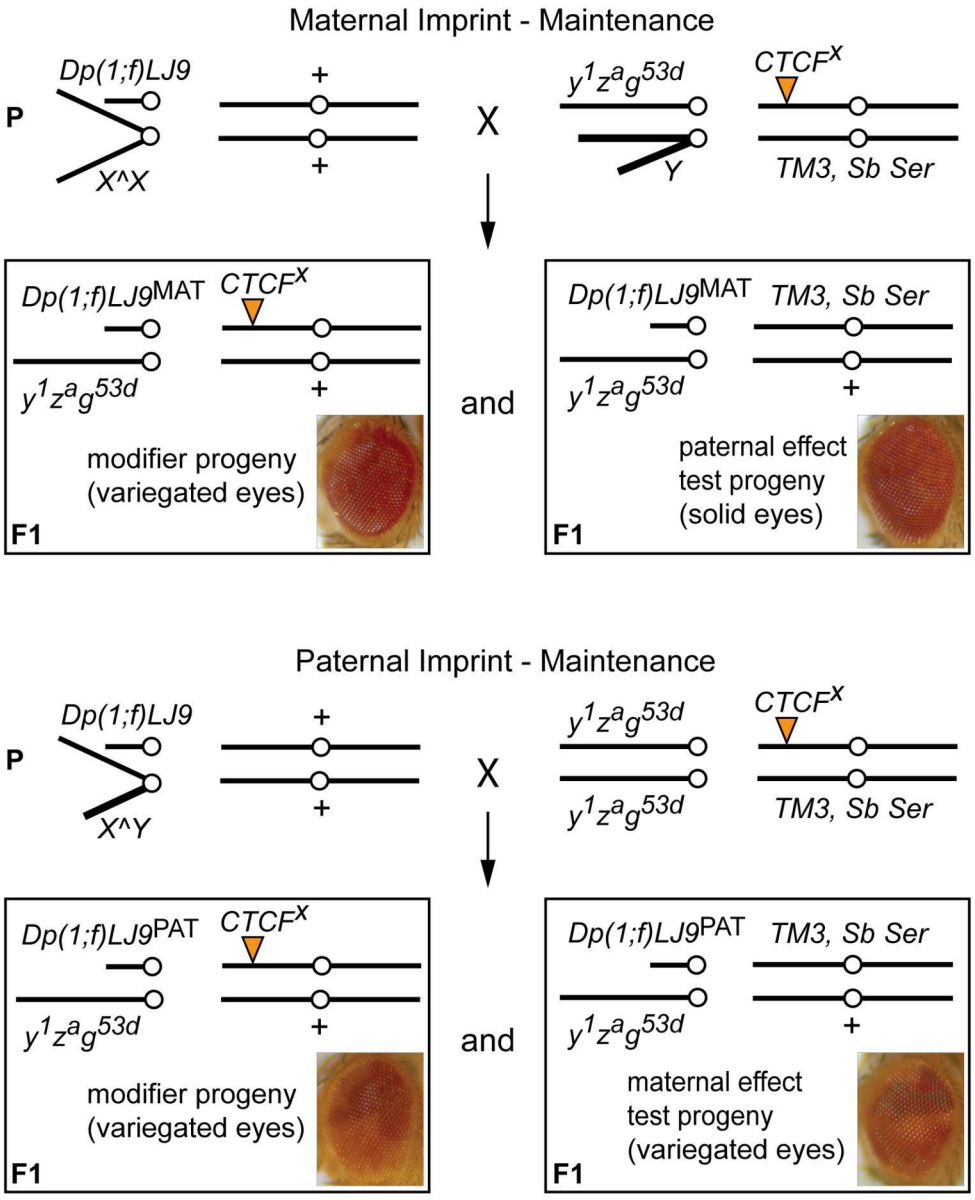

Figure 3.6 - Mating Schematic For Testing The Effect Of *dCTCF* On The Maintenance Of The *Dp(1;f)LJ9* Imprint.

Two sets of progeny are generated from this cross: progeny that have independently inherited the *Dp(1;f)LJ9* mini-X chromosome and a $CTCF^{X}$ mutant allele (modifier progeny), and progeny that have inherited *Dp(1;f)LJ9* and the *TM3, Sb Ser* balancer, but had a parent carrying a $CTCF^{X}$ mutant allele (maternal and paternal effect test progeny).

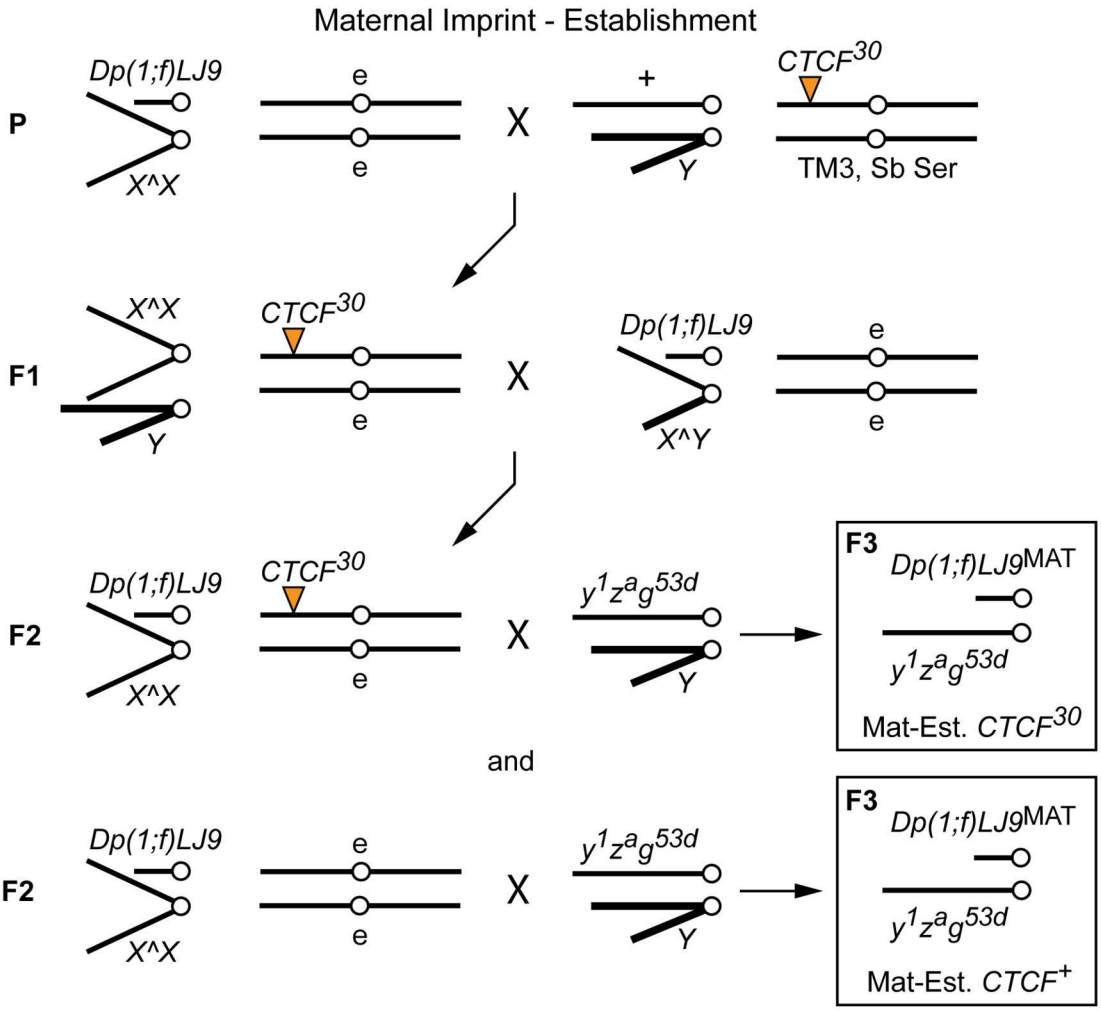

Figure 3.7 - Mating Schematic For Testing *dCTCF* For An Effect On Maternal Establishment Of The *Dp(1;f)LJ9* Imprint.

Two primary sets of progeny, and an external control, were generated from this cross: progeny with a maternally-inherited *Dp(1;f)LJ9* mini-X chromosome from mothers with the $CTCF^{30}$ mutation (Mat Est. $CTCF^{30}$), and progeny that also have a maternally imprinted *Dp(1;f)LJ9* chromosome but from mothers wild-type for *CTCF* (Mat Est. $CTCF^{+}$). External control crosses were produced by crossing F1 generation *X^X/Dp(1;f)LJ9; e/e* females to $y^1z^ag^{53d}/Y$ males (not depicted).

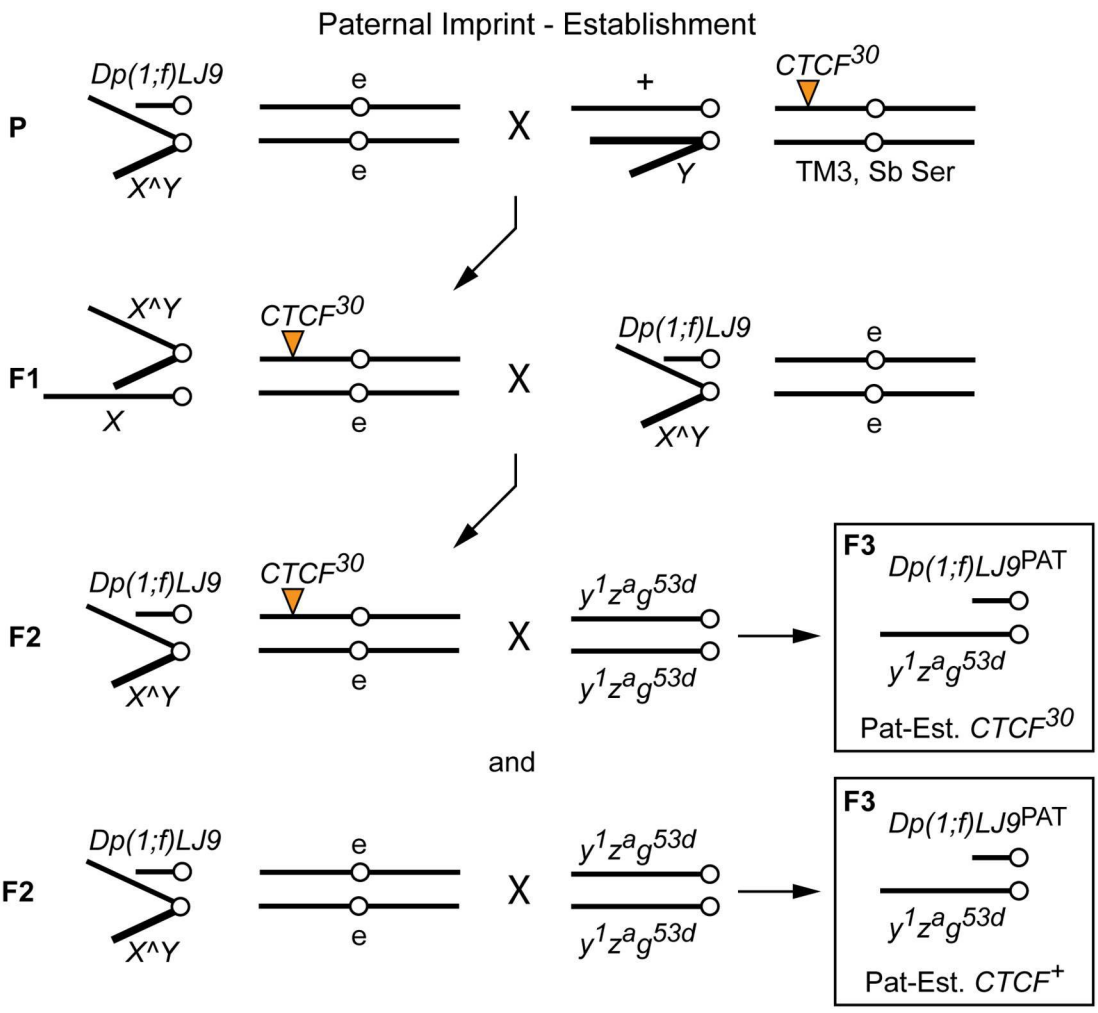

Figure 3.8 - Mating Schematic For Testing *dCTCF* For An Effect On The Paternal Establishment Of The *Dp(1;f)LJ9* Imprint.

Two primary sets of progeny, and an external control, were generated from this cross: progeny with a paternally-transmitted *Dp(1;f)LJ9* mini-X chromosome from fathers with the $CTCF^{30}$ mutation (Pat Est. $CTCF^{30}$), and progeny that also have a paternally imprinted *Dp(1;f)LJ9* mini-X chromosome but from fathers wild-type for *CTCF* (Pat Est. $CTCF^{+}$). External control crosses were produced by crossing F1 generation *X^Y/Dp(1;f)LJ9; e/e* males to $y^1z^ag^{53d}/y^1z^ag^{53d}$ females (not depicted).

3.7 Author's Contributions

WAM performed the imprinting experiments, provided analysis, and wrote the manuscript. DM characterized the CTCF alleles, NB performed the variegating 4th chromosome strain crosses and assays, GES performed the imprint establishment crosses and assays and VR produced the *CTCF*^{30} allele. VKL and VM participated in the conceptualization and design of the experiments, performed analysis, and participated in writing the manuscript. All authors read and approved the final manuscript.

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3.9 References

Anaka M, Lynn A, Mcginn P, Lloyd VK. (2009) Genomic imprinting in *Drosophila* has properties of both mammalian and insect imprinting. Dev Genes Evol. **219**:59-66.

Barski A, Cuddapah S, Cui K, Roh T-Y, Schones DE, Wang Z, Wei G, Chepelev I, Zhao K. (2007) High-resolution profiling of histone methylations in the human genome. Cell. **129**:823-837.

Bartkuhn M, Straub T, Herold M, Herrmann M, Rathke C, Saumweber H, Gilfillan GD, Becker PB, Renkawitz R. (2009) Active promoters and insulators are marked by the centrosomal protein 190. EMBO J. **28**:877-888.

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

Bellen HJ, Levis RW, Liao G, He Y, Carlson JW, Tsang G, Evans-Holm M, Hiesinger PR, Schulze KL, Rubin GM and others. (2004) The BDGP gene disruption project: single transposon insertions associated with 40% of *Drosophila genes*. Genetics. **167**:761-781.

Capelson M, Corces VG. (2004) Boundary elements and nuclear organization. Biol Cell. **96**:617-629.

Celniker SE, Dillon LAL, Gerstein MB, Gunsalus KC, Henikoff S, Karpen GH, Kellis M, Lai EC, Lieb JD, Macalpine DM and others. (2009) Unlocking the secrets of the genome. Nature. **459**:927-930.

Cuddapah S, Jothi R, Schones D, Roh T, Cui K, Zhao K. (2009) Global analysis of the insulator binding protein CTCF in chromatin barrier regions reveals demarcation of active and repressive domains. Genome Res. **19**:24-32.

Deng X, Rattner BP, Souter S, Meller VH. (2005) The severity of *roX1* mutations is predicted by MSL localization on the X chromosome. Mech Dev. **122**:1094-1105.

Filippova G. (2008) Genetics and Epigenetics of the Multifunctional Protein CTCF. Curr Top in Dev Biol. **80**:337-360.

Fitzpatrick GV, Pugacheva EM, Shin J-Y, Abdullaev Z, Yang Y, Khatod K, Lobanenkov VV, Higgins MJ. (2007) Allele-specific binding of CTCF to the multipartite imprinting control region *KvDMR1*. Mol Cell Biol. **27**:2636-2647.

Gerasimova T, Lei E, Bushey A, Corces V. (2007) Coordinated control of dCTCF and *gypsy* chromatin insulators in *Drosophila*. Mol Cell. **28**:761-772.

Greally JM, Gray TA, Gabriel JM, Song L, Zemel S, Nicholls RD. (1999) Conserved characteristics of heterochromatin-forming DNA at the *15q11-q13* imprinting center. PNAS. **96**:14430-14435.

Haigh A, Lloyd V. (2006) Loss of genomic imprinting in *Drosophila* clones. Genome. **49**:1043-1046.

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

Holohan E, Kwong C, Adryan B, Bartkuhn M, Herold M, Renkawitz R, Russell S, White R. (2007) CTCF Genomic Binding Sites in *Drosophila* and the Organisation of the *Bithorax* Complex. PLoS Genet. **3**:e112.

Hurst LD, McVean GT. (1998) Do we understand the evolution of genomic imprinting? Curr Opin Genet Dev. **8**:701-708.

Joanis V, Lloyd V. (2002) Genomic imprinting in *Drosophila* is maintained by the products of *Suppressor of variegation* and *trithorax* group, but not *Polycomb* group, genes. Mol Genet Genomics. **268**:103-112.

Kim TH, Abdullaev ZK, Smith AD, Ching KA, Loukinov DI, Green RD, Zhang MQ, Lobanenkov VV, Ren B. (2007) Analysis of the vertebrate insulator protein CTCF-binding sites in the human genome. Cell. **128**:1231-1245.

Kyrchanova O, Toshchakov S, Podstreshnaya Y, Parshikov A, Georgiev P. (2008) Functional Interaction between the Fab-7 and Fab-8 Boundaries and the Upstream Promoter Region in the *Drosophila Abd-B* Gene. Mol Cell Biol. **28**:4188-4195.

Larsson J, Chen JD, Rasheva V, Rasmuson-Lestander A, Pirrotta V. (2001) Painting of fourth, a chromosome-specific protein in *Drosophila*. PNAS. **98**:6273-6278.

Lewis A, Murrell A. (2004) Genomic Imprinting: CTCF Protects the Boundaries. Curr Biol. **14**:R284-R286.

Li T, Hu J-F, Qiu X, Ling J, Chen H, Wang S, Hou A, Vu TH, Hoffman AR. (2008) CTCF regulates allelic expression of *Igf2* by orchestrating a promoter-polycomb repressive complex 2 intrachromosomal loop. Mol Cell Biol. **28**:6473-6482.

Ling J, Li T, Hu J, Vu T, Chen H, Qiu X, Cherry A, Hoffman A. (2006) CTCF mediates interchromosomal colocalization between *Igf2/H19* and *Wsb1/Nf1*. Science. **312**:269-272.

Lloyd V, Sinclair D, Grigliatti T. (1999) Genomic imprinting and position-effect variegation in *Drosophila melanogaster*. Genetics. **151**:1503-1516.

Lyko F, Brenton J, Surani M, Paro R. (1997) An imprinting element from the mouse *H19* locus functions as a silencer in *Drosophila*. Nat Genet. **16**:171-173.

Lyko F, Buiting K, Horsthemke B, Paro R. (1998) Identification of a silencing element in the human *15q11-q13* imprinting center by using transgenic *Drosophila*. PNAS. **95**:1698-1702.

Matsuzaki H, Okamura E, Shimotsuma M, Fukamizu A, Tanimoto K. (2009) A randomly integrated transgenic *H19* imprinting control region acquires methylation imprinting independently of its establishment in germ cells. Mol Cell Biol. **29**:4595-4603.

Mohan M, Bartkuhn M, Herold M, Philippen A, Heinl N, Bardenhagen I, Leers J, White R, Renkawitz-Pohl R, Saumweber H and others. (2007) The *Drosophila* insulator proteins CTCF and CP190 link enhancer blocking to body patterning. EMBO J. **26**:4203-4214.

Moon H, Filippova G, Loukinov D, Pugacheva E, Chen Q, Smith S, Munhall A, Grewe B, Bartkuhn M, Arnold R and others. (2005) CTCF is conserved from *Drosophila* to humans and confers enhancer blocking of the *Fab-8* insulator. EMBO Rep. **6**:165-170.

Mukhopadhyay R, Yu W, Whitehead J, Xu J, Lezcano M, Pack S, Kanduri C, Kanduri M, Ginjala V, Vostrov A and others. (2004) The binding sites for the chromatin insulator protein CTCF map to DNA methylation-free domains genome-wide. Genome Res. **14**:1594-1602.

Muller H. (1930) Types of visible variations induced by X-rays in *Drosophila*. J Genet. **22**:299-334.

Nolan C, Killian J, Petitte J, Jirtle R. (2001) Imprint status of *M6P/IGF2R* and *IGF2* in chickens. Dev Genes Evol. **211**:179-183.

Ohlsson R, Renkawitz R, Lobanenkov V. (2001) CTCF is a uniquely versatile transcription regulator linked to epigenetics and disease. Trends Genet. **17**:520-527.

Pardo-Manuel de Villena F, de la Casa-Esperón E, Sapienza C. (2000) Natural selection and the function of genome imprinting: beyond the silenced minority. Trends Genet. **16**:573-579.

Real, MD, Ferre, J, Mensua, JL. (1985) Methods for the quantitative estimation of the red and brown pigments of *D. melanogaster.* Dros Inf Serv. **61**:198-200.

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

Schoenherr CJ, Levorse JM, Tilghman SM. (2003) CTCF maintains differential methylation at the *Igf2/H19* locus. Nat Genet. **33**:66-69.

Shin J-Y, Fitzpatrick GV, Higgins MJ. (2008) Two distinct mechanisms of silencing by the KvDMR1 imprinting control region. EMBO J. **27**:168-178.

Shiura H, Nakamura K, Hikichi T, Hino T, Oda K, Suzuki-Migishima R, Kohda T, Kaneko-ishino T, Ishino F. (2009) Paternal deletion of *Meg1/Grb10* DMR causes maternalization of the *Meg1/Grb10* cluster in mouse proximal Chromosome 11 leading to severe pre- and postnatal growth retardation. Hum Mol Genet. **18**:1424-1438.

Smith ST, Wickramasinghe P, Olson A, Loukinov D, Lin L, Deng J, Xiong Y, Rux J, Sachidanandam R, Sun H, Lobanenkov V, Zhou J. (2009) Genome wide ChIP-chip analyses reveal important roles for CTCF in *Drosophila* genome organization. Dev Biol. **328**:518-528.

Sun F-L, Haynes K, Simpson CL, Lee SD, Collins L, Wuller J, Eissenberg JC, Elgin SCR. (2004) *cis*-Acting determinants of heterochromatin formation on *Drosophila melanogaster* chromosome four. Mol Cell Biol. **24**:8210-8220.

Szabo P, Tang S, Silva F, Tsark W, Mann J. (2004) Role of CTCF binding sites in the *Igf2/H19* imprinting control region. Mol Cell Biol. **24**:4791-4800.

Takada S, Paulsen M, Tevendale M, Tsai C-E, Kelsey G, Cattanach BM, Ferguson-Smith AC. (2002) Epigenetic analysis of the *Dlk1-Gtl2* imprinted domain on mouse chromosome 12: implications for imprinting control from comparison with *Igf2-H19*. Hum Mol Genet. **11**:77-86.

Talbert PB, Henikoff S. (2000) A reexamination of spreading of position-effect variegation in the *white-roughest* region of *Drosophila melanogaster*. Genetics. **154**:259-272.

Verona RI, Mann MRW, Bartolomei MS. (2003) Genomic imprinting: intricacies of epigenetic regulation in clusters. Annu. Rev. Cell. Dev. Biol. **19**:237-259.

Wan L-B, Bartolomei MS. (2008) Regulation of imprinting in clusters: noncoding RNAs versus insulators. Adv Genet. **61**:207-223.

Yoon B, Herman H, Hu B, Park Y, Lindroth A, Bell A, West A, Chang Y, Stablewski A, Piel J, Loukinov D, Lobanenkov V, Soloway P. (2005) *Rasgrf1* imprinting is regulated by a CTCF-dependent methylation-sensitive enhancer blocker. Mol Cell Biol. **25**:11184-11190.

Yoon YS, Jeong S, Rong Q, Park K-Y, Chung JH, Pfeifer K. (2007) Analysis of the *H19* ICR insulator. Mol Cell Biol. **27**:3499-3510.

3.10 Transition To Chapter 4

The following chapter describes the involvement of DNA methylation in regulating the paternal *Dp(1;f)LJ9* imprint. This research involved treating embryos with 5-azacytidine, an inhibitor of DNA methyltransferase, in addition to crosses involving a null mutation in the methyl-DNA binding protein, MBD2/3. The results presented in this chapter show that DNA methylation acts within a defined period early in development to regulate paternal silencing. Subsequent MBD2/3 binding reinforces this silencing. These findings demonstrate that imprint regulation by DNA methylation, a feature of imprinting in both mammals and plants, is also found in *Drosophila*.

Chapter 4 5-azacytidine Treatment And Mutant *MBD2/3* Methyl-DNA Binding Protein Disrupt Genomic Imprinting In *Drosophila melanogaster*

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4.1 Abstract

DNA methylation is a major contributor to the epigenetic regulation of imprinted genes in mammals and plants. In *Drosophila melanogaster* DNA methylation and the methyl-DNA binding protein MBD2/3 regulate gene expression primarily during early embryonic development. We used the *Dp(1;f)LJ9* mini-X chromosome, which places a marker gene in proximity to a pericentric imprint control region, to determine if DNA methylation is involved in the epigenetic regulation of imprinting in *D. melanogaster*. We found that treatment with 5-azacytidine during the early stages of embryonic development, coinciding with peak DNA methylation levels, relaxed parent-specific silencing from the paternally inherited *Dp(1;f)LJ9* mini-X chromosome. A similar relaxation of paternal-specific silencing resulted from a *MBD2/3* null mutation. No disruption of marker gene expression was observed when the *Dp(1;f)LJ9* mini-X chromosome was maternally inherited, indicating that sensitivity to 5-azacytidine and *MBD2/3* expression levels are specific to the paternal imprint. Additionally, treatment with 5-azacytidine affected only the maintenance of the imprint in the early embryo, it did not affect the establishment of the imprint in either the paternal or maternal germline. These results implicate DNA methylation and recruitment of MBD2/3 in stabilizing silencing from an imprinted domain during the early stages of embryonic development in *D. melanogaster*.

4.2 Introduction

Genomic imprinting is an epigenetic process that can produce monoallelic expression of a gene or gene cluster dependent upon the sex of the parent. Imprint control regions (ICRs) regulate expression of imprinted genes and are the target sites of several epigenetic processes, including DNA methylation, histone modifications, and non-coding RNAs. The establishment and maintenance of many mammalian imprints are linked to parent-specific DNA methylation within ICRs. Examples include the well-characterized *IGF2*/*H19* imprinted genes in mammals (Bartolomei 2009). During gametogenesis, *de novo* DNA methyltransferases Dnmt3a and Dnmt3b, as well as cofactor Dnmt3L, establish differentially methylated regions (DMRs) by mono-allelic CpG dinucleotide

methylation in the germline (Hata *et al.* 2002; Kaneda *et al.* 2004). Once DMRs are established within ICRs, the methyltransferase Dnmt1 maintains parent-specific DNA methylation patterns (Hirasawa *et al.* 2008). If the parent-specific DNA methylation patterns are lost, Dnmt1 cannot re-set them, and only passage through the germline can correctly produce an imprint (Tucker *et al.* 1996). Germline re-programming and early embryonic propagation of mono-allelic expression comprise a critical period during which an imprint must be established and maintained to ensure correct expression at imprinted loci.

Although not as well characterized as the other methyltransferases, Dnmt2 is highly conserved and involved in both DNA and tRNA methylation (Goll *et al.* 2006; Schaefer and Lyko 2010). Both human and mouse Dnmt2 have only weak DNA methyltransferases activity (Liu *et al.* 2003; Hermann *et al.* 2004), and Dnmt2 knock-out mice are viable with only minor developmental defects (Hermann *et al.* 2003), suggesting a limited and possibly specialized role for Dnmt2 in mammalian DNA methylation. Dmnt2 is the only methyltransferase present in *D. melanogaster* (Kunert *et al.* 2003), where it directs methylation of CpT and CpA dinucleotides rather than the CpG dinucleotide as is typical in mammals (Lyko *et al.* 2000). Dnmt2 is localized to both the nucleus and cytoplasm, with its nuclear concentration increasing in early embryos when DNA methylation is at its peak (Lyko *et al.* 2000; Schaefer *et al.* 2008). The low levels of non-CpG methylation present in *D. melanogaster* are not essential for viability (Lyko *et al.* 2000). However, Dnmt2 is actively involved in genome regulation, as Dnmt2 stabilizes the silencing of retrotransposons and repetitive elements during early embryogenesis and contributes to the recruitment of histone methyltransferases that continue to maintain silencing in somatic cells (Phalke *et al.* 2009).

DNA methylation at CpT and CpA dinucleotides is also present in mammalian embryonic stem cells (Ramsahoye *et al.* 2000); a recent genome-wide mapping of human embryonic stem cell methylation found non-CpG methylation comprised a quarter of all methylated DNA in undifferentiated cells (Lister *et al.* 2009). Thus, it appears that the window of non-CpG methylation activity in the mammalian genome parallels that of *D.*

melanogaster being primarily restricted to early embryogenesis. The role of non-CpG methylation at mammalian ICRs is not yet fully understood. The imprinted *Peg1/Mest* gene in mice is marked by non-CpG methylation in addition to CpG methylation within DMRs at the oocyte and early blastocyst stages; however, only CpG methylation persists in somatic cells (Imamura *et al.* 2005). It remains to be determined if the brief period of non-CpG methylation stabilizes the *Peg1/Mest* imprint early in development.

Imprinted domains in *D. melanogaster* occur within heterochromatic regions of the genome (Lloyd 2000; Anaka *et al.* 2009). Distinct imprinted domains have been found in the centric heterochromatin of the X chromosome and autosomes (Lloyd *et al.* 1999a; Lloyd 2000), and the majority of the Y chromosome exhibits imprinted regulation (Maggert and Golic 2002; Menon and Meller 2009). Insight into structure and function of ICRs has progressed in part from the generation of transgenic *D. melanogaster* containing elements of mammalian ICRs. The upstream ICR of the imprinted gene *H19* acts as a silencing element in transgenic *D. melanogaster*, and this activity requires the presence of a discrete DMR within the *H19* ICR (Lyko *et al.* 1997; Brenton *et al.* 1999). Additionally, it has been shown that non-coding RNA transcripts are transcribed from the *H19* ICR in transgenic *Drosophila*, and that the nuclear localization of these non-coding transcripts are required for reporter gene silencing (Schoenfelder *et al.* 2007). The human 15q11-q13 imprinted domain, responsible for both Prader-Willi syndrome (PWS) and Angelman syndrome, also acts as a silencing element in transgenic *D. melanogaster* (Lyko *et al.* 1998). The induction of discrete silencing domains in *D. melanogaster* by mammalian ICRs suggests at least some conserved function in the molecular structure of an imprint.

The methyl-DNA binding protein MBD2/3, which shares homologies with both mammalian MBD2 and MBD3 (Tweedie *et al.* 1999; Hendrich and Tweedie 2003), is known to bind CpT and CpA methylated DNA in *D. melanogaster* (Marhold *et al.* 2004). While MBD2/3 is not directly associated with heterochromatin (Marhold *et al.* 2002), it does promote heterochromatic stability and is associated with chromatin remodelers (Marhold *et al.* 2004). Chromosomal association of MBD2/3 coincides with zygotic gene

activation in the early embryo and dissipates in the 1st instar larval stage, overlapping the active period of DNA methylation in the *D. melanogaster* embryo (Marhold *et al.* 2002). Together, these findings demonstrate that the DNA methylation and MBD2/3 silencing pathway may regulate targeted regions during the initial stages of *D. melanogaster* development.

To address the role of DNA methylation in imprinting in *D. melanogaster*, we used the well-characterized *Dp(1;f)LJ9* mini-X chromosome (mini-X chromosome) imprinting assay system. The mini-X chromosome is the product of an inversion and deletion, placing the eye color gene *garnet* (*g*) under the regulatory influence of an endogenous ICR in pericentric heterochromatin where it acts as a phenotypic reporter for imprinting. Paternal mini-X chromosome transmission results in variegated *garnet* expression (Figure 4.1A), while maternal transmission results in full *garnet* expression (Figure 4.1B) (Lloyd *et al.* 1999a). Maintenance of paternally inherited mini-X chromosome silencing is dependent upon histone H3 lysine 9 methylation (H3K9) and heterochromatin formation (Joanis and Lloyd 2002). The epigenetic mechanisms involved in the establishment of the *Dp(1;f)LJ9* imprint are unknown; however, paternally induced silencing is lost in cloned organisms, suggesting that passage through gametogenesis is critical for imprint establishment (Haigh and Lloyd 2006).

5-azacytidine has been shown to disrupt the DNA methyltransferase activity of Dnmt2 in *D. melanogaster* (Kunert *et al.* 2003). Here we report that treatment of embryos with 5-azacytidine relaxes imprinted silencing from the paternally inherited mini-X chromosome. The disruptive affect of 5-azacytidine on the paternally inherited mini-X chromosome silencing occurs in early embryogenesis, coinciding with peak DNA methylation levels. Furthermore, MBD2/3 null heterozygotes loose silencing from the paternally inherited mini-X chromosome, implying DNA methylation and MBD2/3 stabilize the paternal imprint in the developing early embryo. No significant affect from either the treatment of embryos with 5-azacytidine or the introduction of the MBD2/3 null mutation was observed on *garnet* expression from maternally inherited mini-X chromosomes. Additionally, 5-azacytidine disruption of DNA methylation did not affect

the establishment of the either the maternal or paternal imprint. These findings suggest that DNA methylation and MBD2/3 recruitment are involved in the initial propagation of imprinted silencing from the paternal *Dp(1;f)LJ9* mini-X chromosome ICR.

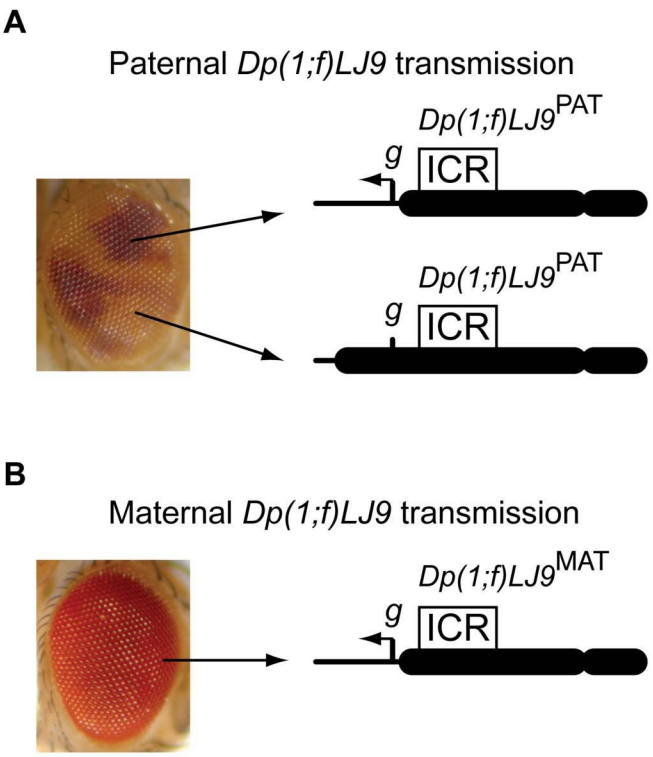

Figure 4.1 - The *D. melanogaster Dp(1;f)LJ9* Mini-X Chromosome.
X-ray deletion of a majority of the X chromosome euchromatin (thin line) and the distal tip of heterochromatin (thick line) places the *garnet* gene (*g*) next to the heterochromatic break, where it falls under control of an imprint control region (ICR). Cell-specific spreading of heterochromatin when the *Dp(1;f)LJ9* mini-X chromosome is paternally inherited (*Dp(1;f)LJ9*^{PAT}) results in variegated *garnet* expression (A), while maternal inheritance (*Dp(1;f)LJ9*^{MAT}) retains stable, full *garnet* expression (B).

4.3 Results

4.3.1 The Post-embryonic Imprinted State Of The Mini-X Chromosome Is Stably Maintained

The ICR of the mini-X chromosome induces distinct expression patterns on adjacent genes dependent upon parental inheritance. The easiest of these genes to monitor is the *garnet* eye color gene. Paternal inheritance of the mini-X chromosome (X^Y/*Dp(1;f)LJ9* males crossed to $y^1z^ag^{53d}/y^1z^ag^{53d}$ females) results in progeny ($y^1z^ag^{53d}/Dp(1;f)LJ9^{PAT}$; +/+), with reduced eye pigment levels due to mosaically silenced *garnet* expression (Figure 4.1A). Maternal inheritance of the mini-X chromosome (X^X/*Dp(1;f)LJ9* females crossed to $y^1z^ag^{53d}/y^1z^ag^{53d}$ males) results in progeny with the same genotype ($y^1z^ag^{53d}/Dp(1;f)LJ9^{MAT}$; +/+), yet phenotypically distinct with fully pigmented eyes as a result of full *garnet* expression (Figure 4.1B). Because these eye color phenotypes are only visible in the adult fly, to determine if the imprint is maintained through earlier stages of development, we used whole mount tissue *in situ* hybridization with RNA-specific probes to visualize *garnet* expression in the 2nd and 3rd instar larval stages. While the *garnet* gene is involved in directing pigment deposition in the adult eye, it is also ubiquitously transcribed throughout development, including in the larval precursors of the adult eye, the eye imaginal discs (Lloyd *et al.* 1999b). The paternally inherited mini-X chromosome results in mosaically silenced *garnet* expression in 3rd instar eye imaginal discs (Figure 4.2A), as well as their precursors, the 2nd instar eye imaginal discs (Figure 4.2B). This pattern of *garnet* expression is consistent with the variegated eye pigmentation observed in adults with a paternally inherited mini-X chromosome. Eye imaginal discs from 3rd and 2nd instar larvae with a maternally inherited mini-X chromosome (Figure 4.2D and Figure 4.2E, respectively) both displayed complete *garnet* expression, matching the expression pattern observed in adults. The stable propagation of both the paternal and maternal imprinted states though larval development and into adulthood suggests that nucleation of the chromatin state from the mini-X chromosome ICR occurs early in development.

4.3.2 5-azacytidine Relaxes Paternal Silencing From The Mini-X Chromosome ICR

To determine if DNA methylation might play a role in the regulation of the mini-X chromosome imprint, we treated embryos with the methyltransferase-inhibiting drug 5-azacytidine. 5-azacytidine treatments were conducted on embryos at specific times after egg lay (AEL) to assess if the mini-X chromosome ICR is sensitive to presence of methylated DNA at different stages of embryonic development (Figure 4.3A). We found that exposure to 5-azacytidine early in development disrupted imprinted silencing from the paternally inherited ICR; however, treatment towards the end of embryogenesis did not have any significant affect. Compared to the control, mock treated embryos (31 $\pm$ 7% pigment), disrupted silencing of the marker gene *garnet* was seen in 5-azacytidine treated embryos from the 0-1 hour (51±3% pigment), 3-4 hours (44±4% pigment), and 7-8 hours AEL (50±2% pigment) treatments. Of these treatment groups, disruption of paternally imprinted *garnet* silencing was greatest at 0-1 hour AEL and 7-8 hour AEL (Figure 4.3B). 5-azacytidine treated embryos aged 9-10 hours AEL also had elevated levels of pigment (43±7% pigment); however, the increase was not significantly different from the control mock treatment group. As 5-azacytidine exposure spanned two hours, it is possible that this treatment group represents the tail end of regulatory sensitivity towards DNA methylation, with only some embryos responding to treatment. By 12-13 hours AEL sensitivity to 5-azacytidine appeared to be lost (pigment levels 32±7%). Eye variegation index assays visually confirmed that treatment by 5-azacytidine within the first 8 hours of embryogenesis significantly reduced the mosaic silencing of the paternally imprinted mini-X chromosome (Supplemental Table 4.1).

Embryos with a maternally inherited mini-X chromosome were also subjected to the same developmentally-staged 5-azacytidine treatments. Disruption of DNA methylation by 5-azacytidine exposure did not affect the expression of *garnet* from the maternally transmitted mini-X chromosome (Figure 4.3C). Pigment levels, and thus *garnet* expression, were similar in embryos with maternally mini-X chromosomes at 0-1 hour AEL (90±6% pigment), 3-4 hours AEL (91±7% pigment), 7-8 hours AEL (98±3% pigment), 9-10 hours AEL (93±9% pigment), and 12-13 hours AEL (92±5% pigment)

and those mock treated with *D. melanogaster* Ringer's (92 + 5% pigment). Results from the eye variegation index assays supported the conclusion that the 5-azacytidine treatments did not affect *garnet* expression from maternally inherited mini-X chromosomes (Supplemental Table 4.1).

4.3.3 The *MBD2/3* Null Allele Dominantly Relaxes Silencing From The Paternal Mini-X Chromosome

Disruption of DNA methylation by 5-azacytidine causes the mislocalization of the methyl-binding domain protein MBD2/3 in *Drosophila* embryos, resulting in destabilization of heterochromatin (Marhold *et al.* 2004). We tested a null mutation of *MBD2/3* (*MBD^{1}*) to examine the effect of a reduction in the amount the MBD2/3 protein on the imprinted silencing of *garnet* on the mini-X chromosome. *MBD^{1}* heterozygotous progeny with a paternally inherited mini-X chromosome (*Dp(1;f)LJ9*^{PAT}; *MBD*^{1}/+) had elevated pigment levels (61±3%), indicating a significant decrease in *garnet* silencing compared to the control (*Dp(1;f)LJ9*^{PAT}; +/+ with 37±3% pigment; Figure 4.4A). The reduction in *garnet* variegation was clearly visible phenotypically (Figure 4.4B), resulting in a significant difference between control (*Dp(1;f)LJ9*^{PAT}; +/+) and *MBD^{1}* (*Dp(1;f)LJ9*^{PAT}; *MBD*^{1}/+) genotypes in the eye variegation index assay (Supplemental Table 4.2). As was found for 5-azacytidine, the effect of the *MBD^{1}* allele is also restricted to the paternally imprinted mini-X chromosome; no significant difference resulted from the presence of *MBD^{1}* with a maternally imprinted mini-X chromosome, (88±6% pigment, compared to the control with 97±6% pigment, Figure 4.4C). The eye variegation index assay likewise detected no significant affect of *MBD^{1}* on *garnet* expression from the maternal mini-X chromosome (*Dp(1;f)LJ9*^{MAT}; *MBD*^{1}/+, compared to the control, *Dp(1;f)LJ9*^{MAT}; +/+, Supplemental Table 4.2); phenotypically, both genotypes produced fully red eyes (Figure 4.4D).

4.3.4 Establishment Of The Imprint Is Not Affected By 5-azacytidine Treatment Of The Parental Generation

Soon after fertilization, a select set of cells at the posterior end of a *D. melanogaster* embryo, pole cells, are mitotically arrested and sequestered as primordial

germ cells which will give rise to the germline (Williamson and Lehmann 1996). To assess if disruption of DNA methylation during primordial germ cell formation compromises the establishment of the *Dp(1;f)LJ9* mini-X chromosome imprint, we treated *Dp(1;f)LJ9* mini-X chromosome-bearing embryos (0-1 AEL), a time when these cells are being determined. When treated *Dp(1;f)LJ9* mini-X chromosome males (*Dp(1;f)LJ9, y^+g^+/X^Y*) were mated there was no significant change to the expression of imprint the marker gene *garnet* in the next generation. The progeny produced from this cross (Figure 4.5A: Male Parent 5-azaC) averaged 38±10% pigment levels, which was not significantly different from 29±7% pigment levels of the *D. melanogaster* Ringer's treatment control embryos (Figure 4.5A: Male Parent Control). Eye variegation index assays likewise revealed no significant difference between the two groups (Figure 4.5B; Supplemental Table 4.3).

Mini-X chromosome females, both those treated as embryos with 5-azacytidine and controls, were similarly mated to determine if the establishment of the maternal *Dp(1;f)LJ9* mini-X chromosome imprint is regulated by DNA methylation. As was the case for the establishment of the paternal imprint, establishment of the maternal imprint was unaffected by 5-azacytidine treatment. Progeny from treated females (*Dp(1;f)LJ9, y^+ g^+/X^X*) averaged 89±4% pigment levels (Figure 4.5C: Female Parent 5-azaC), not significantly different from the control average of 87±2% pigment levels (Figure 4.5C: Female Parent Control). Additionally, no significant difference between the progeny of 5-azacytidine-treated female embryos and control embryos was determined by the eye variegation index assay (Figure 4.5D; Supplemental Table 4.3).

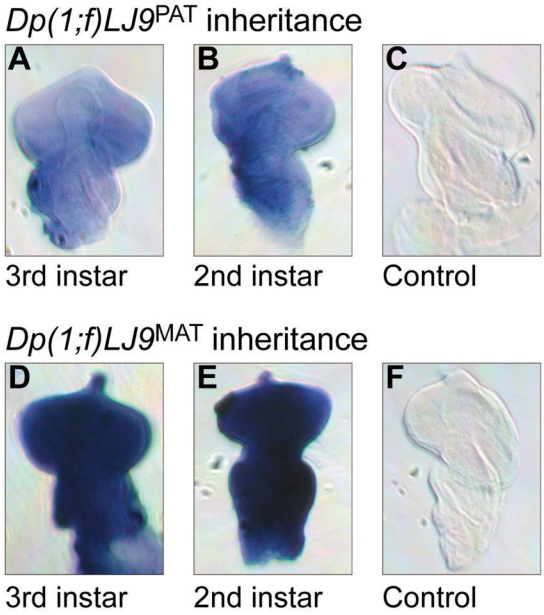

Figure 4.2 - Maintenance Of The Imprinted *garnet* Expression From The Mini-X Chromosome.

Expression of *garnet* in 3rd and 2nd instar larvae eye imaginal discs labeled with antisense probes specific for *garnet* RNA by whole mount tissue *in situ* hybridization. Paternal inheritance of mini-X chromosome (*Dp(1;f)LJ9*^{PAT}) results in a variegated eye phenotype. Expression patterns of *garnet* RNA are also variegated in the eye precursor imaginal discs, at both the 3rd (A) and 2nd (B) instar larval development stages. Maternal inheritance of mini-X chromosome (*Dp(1;f)LJ9*^{MAT}) results in a fully pigmented eye. Labeling of *garnet* RNA by *in situ* hybridization of the eye imaginal discs in the 3rd (D) and 2nd (E) instar larvae shows uniform *garnet* expression at earlier development stages. *In situ* hybridization using control sense probes reveals no non-specific hybridization (C and F).

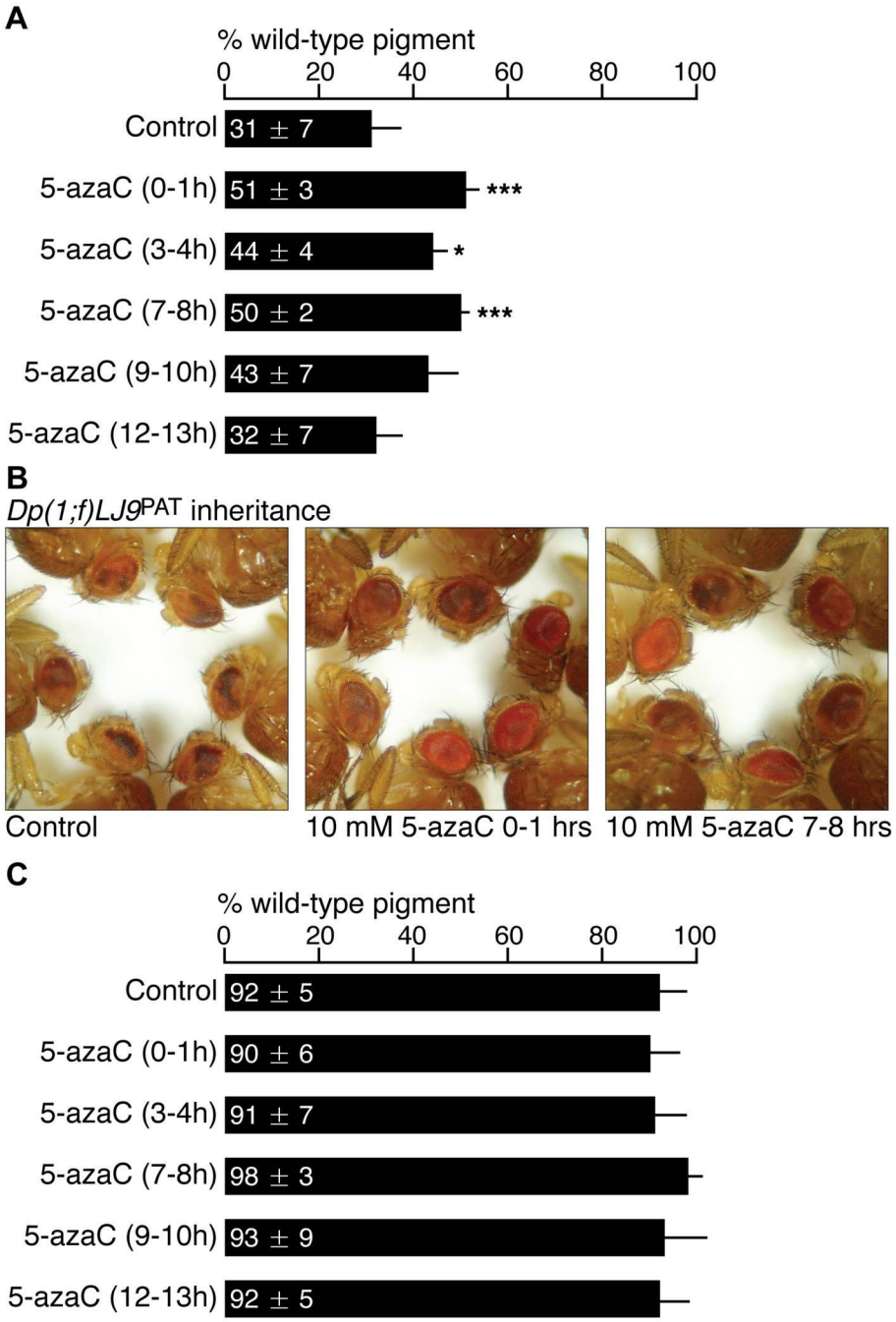

Figure 4.3 - The Effects Of 5-azacytidine (5-azaC) On The Expression Of *garnet* From The Imprinted *Dp(1;f) LJ9* Mini-X Chromosome.

Embryos at the ages shown were exposed to 10 mM 5-azacytidine for a total of 2 hours, while control embryos were treated with *D. melanogaster* Ringer's for 2 hours. Pigment levels are represented as a percentage of full, wild-type *garnet* expression levels. Error bars represent standard deviation. Mean values for each group are based on: $n = 6$ samples (48 heads total). Values that are significantly different from the control are marked with Bonferroni-corrected p values; *(p=0.0055-0.0011), **(p=0.0011-0.00011), ***(p<0.00011).

(Figure 4.3 continued)

A) Treatment with 5-azaC suppresses variegated silencing of *garnet* early in embryonic development, increasing pigment levels, after which, its affect is gradually reduced in older embryo treatment groups. Complete representative eye pictures from all treatment groups presented in Supplemental Figure 4.6
B) Representative examples of the variegated eye phenotype resulting from the paternal inheritance of the mini-X chromosome (Control), and 5-azacytidine suppression of variegation at both 0-1 hour and 7-8 hours AEL.
C) Pigment levels from the maternally derived mini-X chromosome; full *garnet* expression results in close to wild-type levels of pigment. Treatment with 5-azacytidine did not significantly affect expression from the maternal mini-X chromosome at any embryonic stage. Additional eye pictures of maternal and paternal treatment groups are presented in Supplemental Figure 4.7.

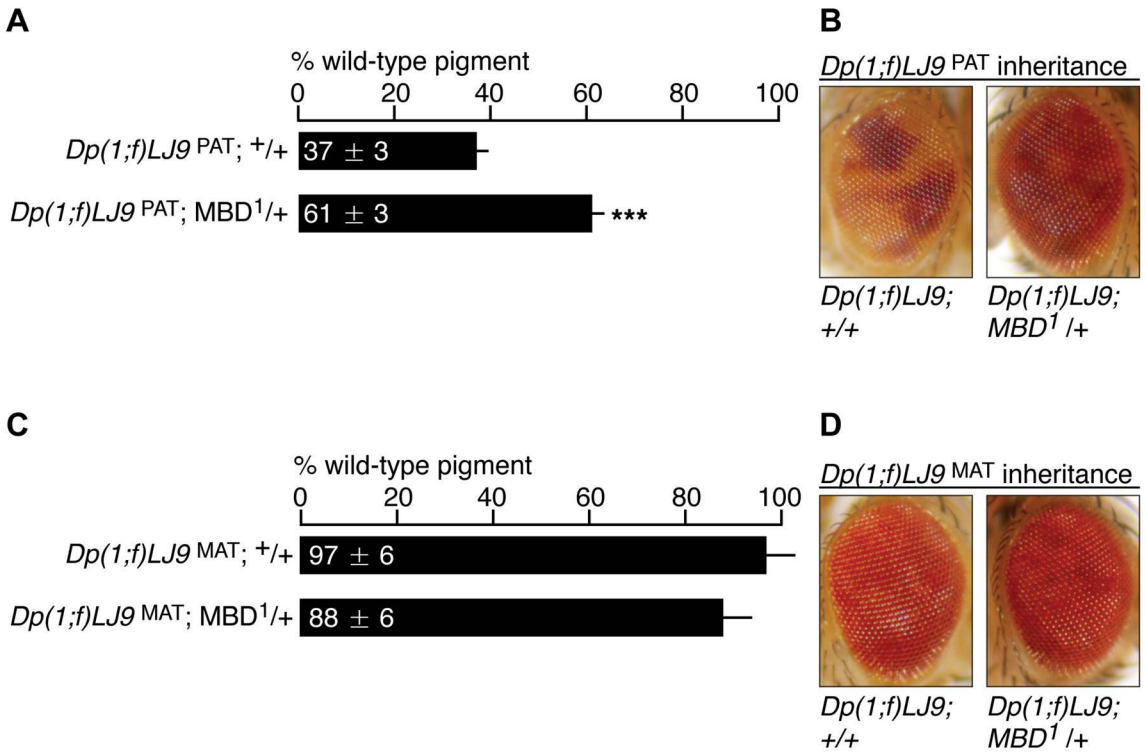

Figure 4.4 - *MBD2/3* Reduces The Silencing Of *garnet* On The Paternally Imprinted *Dp(1;f)LJ9* Mini-X Chromosome.

Pigment levels are represented as a percentage of full, wild-type *garnet* expression levels. Mean values for each group are based on n = 5 samples (40 heads total), error bars represent standard deviation.

A) Pigment levels from the paternally derived mini-X chromosome. MBD^1 ($Dp(1;f)LJ9^{PAT}$; MBD^1/+) significantly relaxed *garnet* silencing *** ($p < 0.001$) compared to the control ($Dp(1;f)LJ9^{PAT}$; +/+).

B) Representative eye phenotypes illustrate variegation of *garnet* is evident in flies with a paternally inherited mini-X chromosome, which is suppressed by the MBD^1 allele.

C) Pigment levels from the maternally derived mini-X chromosome. The MBD^1 allele ($Dp(1;f)LJ9^{MAT}$; MBD^1/+) yields no significant change in *garnet* expression compared to the control ($Dp(1;f)LJ9^{MAT}$; +/+).

D) Representative eye phenotypes of $Dp(1;f)LJ9^{MAT}$; MBD^1/+ and the control $Dp(1;f)LJ9^{MAT}$; +/+ are similar. Internal control data is provided in Supplemental Figure 4.8 and Supplemental Table 4.4.

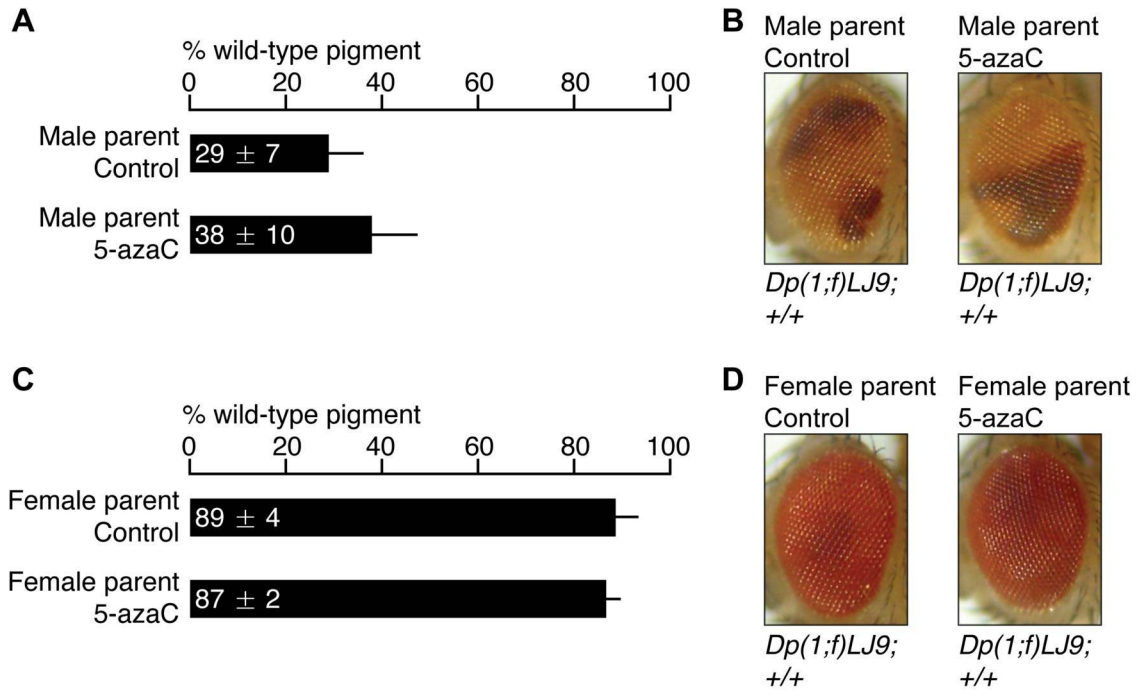

Figure 4.5 - The Effects Of 5-azacytidine (5-azaC) On The Establishment Of The Mini-X Chromosome Imprint.

Male and female *Dp(1;f)LJ9* mini-X chromosome-bearing embryos were treated with 5-azacytidine 0-1 hours AEL. These embryos were allowed to develop and their progeny were assessed for any alteration of the imprint.

A) Treatment of male parents (*Dp(1;f)LJ9, y^+g^+/X^Y*) with 5-azacytidine did not have any significant affect on *garnet* expression levels compared to male parent controls treated with *D. melanogaster* Ringer's.

B) No visible change in phenotype was observed from male parents treated with 5-azacytidine compared to male parent controls.

C) Treatment of female parents (*Dp(1;f)LJ9, y^+ g^+/X^X*) with 5-azacytidine did not have any significant affect on *garnet* expression levels compared to controls treated with *D. melanogaster* Ringer's.

D) No visible change in phenotype was observed from female parents treated with 5-azacytidine compared to female parent controls. Pigment levels are represented as a percentage of full, wild-type *garnet* expression levels. Mean values for each group are based on $n = 7$ samples (56 heads total), error bars represent standard deviation.

4.4 Discussion

4.4.1 Parent-specific Expression From The Mini-X Chromosome ICR Is Initiated Early In Development

When maternally inherited, the imprinted domain of the mini-X chromosome ICR is restricted by a stable boundary that protects neighboring genes, including the *garnet* reporter gene, from silencing. In contrast, paternal inheritance of the mini-X chromosome results in the spread of heterochromatin-induced silencing, from the ICR to adjacent genes, variably silencing the *garnet* reporter gene (Lloyd 2000; Joanis and Lloyd 2002). We found that this parent-specific expression pattern is initiated early in development and subsequently maintained throughout the life cycle, consistent with the formation of a developmentally stable heterochromatic domain, well characterized in *D. melanogaster* as position-effect variegation (Baker 1967; Spofford 1976; Ahmad and Henikoff 2001). Maintenance of the parent-specific imprinted state through the later stages of development demonstrates that the initial distinction between paternal and maternal ICRs occurs during embryonic development. Consistent with this distinction occurring early in development, sensitivity to 5-azacytidine treatment peaked between 0-1 hour AEL embryos and 7-8 hours AEL embryos. Remodeling of the paternal pronucleus by the maternal cytoplasm occurs immediately after fertilization and this stage is critical for maintenance of the imprint (Fitch *et al.* 1998). Shortly thereafter, heterochromatin becomes cytologically visible at nuclear division 14, approximately 2-3 hours after fertilization (Vlassova *et al.* 1991), and by 7-8 hours AEL the initial signaling of precursor tissues that develop in the eye imaginal disc and other organs, has commenced (Friedrich 2006). There is a gradual reduction in the effectiveness of 5-azacytidine treatments beyond 8 hours, corresponding to decreased levels of non-CpG methylation and *Dnmt2* expression reported in the late stages of embryonic development (Lyko *et al.* 2000; Phalke *et al.* 2009), to the point where no reduction of paternal mini-X chromosome silencing is observed in 12-13 AEL late embryos.

The developmental window where DNA methylation regulates paternal imprinting corresponds to the heightened levels of genome DNA methylation and Dnmt2 expression in early *D. melanogaster* embryogenesis (Lyko *et al.* 2000; Kunert *et al.* 2003;

Phalke *et al.* 2009). Similar losses of imprint-directed silencing have been reported in mammalian cell lines where 5-azacytidine has been used to disrupt DNA methylation (Eversole-Cire *et al.* 1993; Baqir and Smith 2006). 5-azacytidine has wide-ranging effects on histone modifications and heterochromatin formation in embryonic and differentiated mammalian cell lines (Komashko and Farnham 2010), demonstrating the importance of Dnmts in maintaining chromatin structure through the recruitment of additional epigenetic mechanisms. These results suggest that non-CpG DNA methylation early in *D. melanogaster* embryogenesis assists in maintaining the distinction between parental alleles until additional regulatory elements are able to generate a stable imprinted chromatin domain.

4.4.2 Treatment With 5-azacytidine Does Not Affect Establishment Of The Imprint On The Mini-X Chromosome

While DNA methylation appears to be required for the somatic maintenance of imprinted silencing from the mini-X chromosome ICR, we did not observe a role for DNA methylation in the gametic establishment of the imprint. *D. melanogaster* pole cells have detectable levels of methylated DNA at the blastoderm stage when the pole cells are mitotically arrested (Kunert *et al.* 2003). However, the treatment of blastoderm embryos with 5-azacytidine did not have any affect on mini-X chromosome imprinting in the subsequent generation. While it is possible that the 2 hour treatment period did not include the time at which DNA methylation could influence the establishment of the imprint, this result is consistent with previous findings that imprint maintenance and establishment in *D. melanogaster* employ different epigenetic mechanisms (Joanis and Lloyd 2002), and the suggestion that DNA methylation performs different roles in the soma and germline. Despite reports of cytosine methylation in the arrested germ cells of embryos (Kunert *et al.* 2003), cytosine methylation has not been conclusively associated with chromosomal DNA in spermatids and spermatocytes (Weyrich *et al.* 2008). Further, although transgenic *D. melanogaster* over-expressing mammalian Dnmts are not viable (Lyko *et al.* 1999), driving mammalian Dnmts exclusively in the germline does not have a significant affect on either viability or fertility (Weyrich *et al.* 2008). While this finding does not address the endogenous function of DNA methylation by *D. melanogaster*

Dnmt2, it does suggest a regulatory pathway responsive to DNA methylation is present in somatic cells but not in germ cells. Finally, the recent findings of Phalke *et al*. that in *D. melanogaster* DNA methylation is required to silence retrotransposons in somatic cells, but not germ cells, reinforces the conclusion that distinct regulatory pathways operate in these cell types (Phalke *et al.* 2009). Our finding that establishment of the mini-X chromosome imprint is unaffected by the disruption of DNA methylation in primordial germ cells could reflect the lack of active DNA methylation within *D. melanogaster* germ cells, and suggests a separate epigenetic pathway is responsible for establishing the mini-X chromosome imprint.

It is not uncommon for mammalian imprinted regions to be established by epigenetic mechanisms other than germline DNA methylation. Epigenetic histone modifications have been characterized as establishing epigenetic marks at some mammalian imprinted domains, where DNA methylation is recruited to maintain parent-specific silencing later in embryonic development (Lander *et al.* 2001; McEwen and Ferguson-Smith 2009; McEwen and Ferguson-Smith 2010). Additionally, there is a reciprocal-recruitment and self-reinforcing relationship between DNA methylation and histone modifications at differentially regulated domains, with DNA methylation being linked to directing histone methylation, and vice versa (Henckel *et al.* 2009). It has been proposed that in mammalian tissue-specific imprinting, the differential epigenetic modification of histones represent an ancestral mechanism of imprinting, lacking the extra genomic stability provided by DNA methylation (Lewis *et al.* 2004). An example of epigenetic histone modifications establishing an imprint in insects is provided by the mealybug, *Planococcus citri*, where paternal gametes are imprinted by histone H3K9 and H4K20 methylation marks that result in alterations in DNA methylation and paternal chromosome-wide silencing by heterochromatinization (Bongiorni *et al.* 2009). Accordingly, *D. melanogaster* may utilize DNA methylation to stabilize silencing within imprinted domains prior to, and immediately preceding, zygotic activation of the embryo.

4.4.3 MBD2/3 Maintains Silencing From Paternally Inherited Mini-X Chromosome ICR

We found the null allele *MBD*^{1} relaxes silencing originating from the paternally imprinted mini-X chromosome ICR. The *D. melanogaster* methyl-DNA binding protein MBD2/3 binds non-CpG methylated DNA after cellularization of the blastoderm, coinciding with zygotic activation of the embryo and the formation of cytologically visible heterochromatin (Marhold *et al.* 2002; Marhold *et al.* 2004). MBD2/3 mRNA is contributed maternally to the egg and is transcribed during the early stages of embryogenesis, but transcription ends after the 1st instar larval stage (Ballestar *et al.* 2001). Thus, it appears that the DNA methylation-MBD2/3 silencing pathway is only required to stabilize silencing of targeted domains during a short window in early development. Non-CpG DNA methylation, followed by MBD2/3 recruitment to the paternal mini-X chromosome ICR, may provide a stable mark that could be reliably propagated during rapid division early in embryogenesis.

Previously, MBD2/3 has been shown to be a transcriptional silencer in *D. melanogaster*, one that associates with chromatin remodelers and promotes heterochromatic stability (Roder *et al.* 2000; Marhold *et al.* 2004). While MBD2/3 shares homologies with both mammalian MBD2 and MBD3 (Tweedie *et al.* 1999; Hendrich and Tweedie 2003), its ability to directly bind methylated DNA has suggested it is a functional homologue of MBD2 (Hendrich *et al.* 2001; Marhold *et al.* 2004). However, MBD2 does not appear to have a functional association with imprinted domains in mice (Hendrich *et al.* 2001; Chen *et al.* 2008). In contrast, MBD3 is preferentially active at the imprinted *H19* DMR in mice, contributing to the silencing of the paternal allele and maintaining methylation within the DMR (Reese *et al.* 2007). MBD3 is also recruited to the silenced maternal allele of the imprinted *U2af1-rs1* gene as part of a chromatin remodeling complex that blocks active histone H3K4 methylation and promotes repressive histone H3K9 methylation (Fournier *et al.* 2002). Silencing of the maternal *U2af1-rs1* allele is maintained by this H3K9 methylation, which spreads across a large domain beyond the *U2af1-rs1* gene (Fournier *et al.* 2002). A similar removal of H3K4 methylation and spreading of H3K9 methylation propagates silencing from the paternal

mini-X chromosome ICR (Joanis and Lloyd 2002). It is not yet understood if MBD2/3 assists in the recruitment of H3K9 methyltransferases, but H3K9 methylation and DNA methylation are linked in *D. melanogaster*, as reduced levels of DNA methylation were observed in H3K9 methyltransferase mutants (Kunert *et al.* 2003). Our finding that MBD2/3 contributes to the allele-specific silencing generated from an imprinted domain suggests that MBD2/3 is also functionally similar to mammalian MBD3.

4.4.4 Paternal-specific Silencing Of The Mini-X Chromosome ICR Is Stabilized By A DNA Methylation-MBD2/3 Silencing Pathway

DNA methylation is a critical epigenetic component of mammalian genomic imprinting, involved in both the initial establishment of an imprint as well as the subsequent maintenance of imprinted state after fertilization (Lees-Murdock and Walsh 2008; Bartolomei 2009). In *D. melanogaster*, DNA methylation is proposed to have a role in heterochromatic organization via the recruitment of chromatin modifiers, such as MBD2/3, or histone methyltransferases (Marhold *et al.* 2004; Phalke *et al.* 2009), however its roel in genomic imprinting had not previously been examined. We find that 5-azacytidine treatment early in embryogenesis, or a null MBD2/3 allele, relaxed silencing initiated from the imprinted mini-X chromosome when paternally inherited. Stable expression of the imprint marker gene directed by the maternally inherited mini-X chromosome ICR remained unaltered by 5-azacytidine treatments or the null MBD2/3 allele. This demonstrates that regulation by DNA methylation and MBD2/3 is specific to silencing the paternal imprint. However, 5-azacytidine treatment was only effective in early developmental stages. Thus, the involvement of DNA methylation in paternal-specific silencing is transient and only required to stabilize the paternal imprint during early embryonic development. Following this stage the imprint is stabilized by parent-specific heterochromatin formation. Our findings suggest that active DNA methylation and, subsequent MBD2/3 recruitment, in the early embryo of *D. melanogaster* may be sufficient to stabilize the established imprint during stages of rapid cellular division. In this sense, *D. melanogaster* represents an organism with a conserved role for DNA methylation in stabilizing imprinted domains, despite a limited role for DNA methylation in the general regulation of the genome.

4.5 Materials And Methods

4.5.1 *D. melanogaster* Stocks

All stocks were provided by the Bloomington *Drosophila* Stock Center and were maintained at 22°C on standard cornmeal-molasses medium (Sullivan *et al.* 2000), supplemented with 0.15% methylbenzoate as a mold inhibitor (Sigma). Crosses were conducted in 12 dram shell vials with 15-20 paired adults and subcultured at three-day intervals. The MBD^{1} allele ($P\{EPgy2\}MBD\text{-}like^{EY04582}$) was created by the Berkeley *Drosophila* Genome Project (Bellen *et al.* 2004), and has been shown to be a null mutation (Marhold *et al.* 2004).

4.5.2 *D. melanogaster* Crosses

Flies with the *Dp(1;f)LJ9* mini-X chromosome were balanced over an attached X^X chromosome for maternal transmission or an attached an X^Y chromosome for paternal transmission. To visualize the imprinting of the *garnet* gene, *Dp(1;f)LJ9* mini-X chromosome bearing flies are crossed to $y^{1}z^{a}g^{53d}$ flies of the opposite sex: paternal cross (*Dp(1;f)LJ9,* $y^{+}g^{+}/X\text{^}Y$ males x $y^{1}z^{a}g^{53d}/y^{1}z^{a}g^{53d}$ females), and the reciprocal maternal cross ($y^{1}z^{a}g^{53d}/y^{1}z^{a}g^{53d}$ males x *Dp(1;f)LJ9,* $y^{+}g^{+}/X\text{^}X$ females) (Lloyd *et al.* 1999a). Each cross produces progeny of the genotype $y^{1}z^{a}g^{53d}/Dp(1;f)LJ9^{PAT}$; +/+ or $Dp(1;f)LJ9^{MAT}$; +/+ , which differ only in the parental origin of the mini-X chromosome, paternal or maternal, respectively (Cross schematic available in Supplemental Figure 4.9A).

MBD^{1} was crossed into a $y^{1}z^{a}g^{53d}$ background and balanced over *TM3, Sb* to yield a stock of $y^{1}z^{a}g^{53d}$; $MBD^{1}/TM3,\ Sb$. The effect of MBD^{1} on the paternal imprinting of *garnet* was determined by crossing *Dp(1;f)LJ9* males to $y^{1}z^{a}g^{53d}/y^{1}z^{a}g^{53d}$; $MBD^{1}/TM3,\ Sb$ females. The reciprocal cross determined the effect of MBD^{1} on the maternal imprinting of *garnet*. $y^{1}z^{a}g^{53d}/Dp(1;f)LJ9$; MBD^{1}/+ progeny were collected from both crosses to determine the effect of MBD^{1}, while $y^{1}z^{a}g^{53d}/Dp(1;f)LJ9$; *TM3, Sb*/+ progeny were collected as a internal control (Cross schematic available in Supplemental Figure 4.9B). The control cross for paternal *garnet* imprinting consisted of *Dp(1;f)LJ9* males crossed to

$y^1z^ag^{53d}/y^1z^ag^{53d}$; *TM3, Sb*/+ females, the reciprocal cross produces the maternal control; *Dp(1;f)LJ9*; +/+ and *Dp(1;f)LJ9*; *TM3, Sb*/+ progeny were collected as control genotypes.

4.5.3 5-azacytidine Treatment

5-azacytidine-induced demethylation of embryos was adapted from the procedure used in Kunert *et al* (Kunert *et al.* 2003). 5-azacytidine (Sigma) was dissolved in *D. melanogaster* Ringer's (Sullivan *et al.* 2000), and stored at -80°C until required. Adult flies were acclimated for 2 hours in egg collection chambers comprised of a glass vial over a grape juice agar plate (0.1% agar/ juice), before progressive 1 hr embryo collections were made on fresh grape juice agar plates smeared with a small amount of yeast paste (dry active yeast mixed with 1:1 ddH_2O/ 5% acetic acid). Embryos were dechorionated with a 50% bleach solution for 1 min, rinsed in distilled water for 3 min, then washed in *D. melanogaster* Ringer's for 1 min. Prepared embryos were split into two groups; the experimental group that received 5-azacytidine and the mock treatment control group that received *D. melanogaster* Ringer's. Experimental embryos were nested between Whatman filter paper soaked with freshly thawed 5-azacytidine, and placed in a sterile BD Falcon 6-well cell culture plate to maintain humidity. Embryos were treated with 5-azacytidine for a total of 2 hours, with a second dose of freshly thawed 5-azacytidine reapplied after 1 hour of exposure. Aged embryos were held on grape juice agar plates until the targeted hours after egg lay (AEL) treatment window was reached. After treatment, embryos were rinsed with *D. melanogaster* Ringer's and placed on standard *D. melanogaster* medium in 12 dram shell vials. Controls followed the same experimental procedures, except that embryos were treated with *D. melanogaster* Ringer's solution in place of 5-azacytidine. We tested dosages of 5 mM, 10 mM, and 20 mM 5-azacytidine on 0-1 hours after egg lay (AEL) embryos (Supplemental Figure 4.10). The similar results arising from the 10 mM and 20 mM treatment, suggest the 10 mM dosage of 5-azacytidine effectively disrupts DNA methylation in embryos, as has been reported previously (Kunert *et al.* 2003), and was the dosage used for all subsequent treatments.

The effect of 5-azacytidine on the maintenance of the mini-X chromosome imprint was determined by collecting embryos produced by the paternal (*Dp(1;f)LJ9* males x $y^1z^ag^{53d}$ females) or maternal ($y^1z^ag^{53d}$ males x *Dp(1;f)LJ9* females) crosses and treating them with 5-azacytidine as described above. The effect of 5-azacytidine demethylation on the establishment of the mini-X chromosome imprint was determined by collecting and treating *Dp(1;f)LJ9,* y^+ g^+*/X^Y* or *Dp(1;f)LJ9,* y^+ g^+*/X^X* embryos, which were raised to adults, and crossed to $y^1z^ag^{53d}$ flies as described above.

4.5.4 Quantification Of Eye Pigment

Eye pigment, as a measure of *garnet* gene expression, was assessed visually and quantified spectrophotometrically. The eye variegation index assay, described by Joanis and Lloyd (2002), consists of assigning a value to each eye representing its degree of variegation (0=0-25% pigmentation, 1=25-50%, 2=50-75%, 3=75-100%). The data for each sample set were pooled and expressed as the mean ± the standard error of the mean (SEM). Statistical significance was calculated by a Kolmogorov-Smirnov two-sample test.

The spectrophotometric assay was adapted from Real *et al* (1985). Randomly selected flies were aged for 3-4 days then placed in 1.5ml microtubes and stored at -30 °C. Heads were removed and divided into groups of eight and placed into 0.6ml microtubes containing 150ul of acidified ethanol (30% EtOH, acidified to pH 2 with HCl). Five to seven microtubes were prepared for each of the pigment assay trials. Pigment was extracted from the samples by staking at 150 rpm on an orbital shaker in the dark for 48 hours. Absorbance of the extracted pigments was measured at 480 nm with a Pharmacia Biotech Ultrospec 2000. Eye pigmentation levels are expressed as a percentage of O. R wild-type (full *garnet* expression) eye pigmentation levels. Statistical significance was determined by an ANOVA followed by a Student's t-test. Bonferroni corrected p values were used for multiple test comparisons between the mean of a sample set and the mean of the comparable control for the 5-azacytidine treatment results. Bonferroni correction produced significance levels of: p 0.05 = 0.0055, p 0.01 = 0.0011,

and p 0.001 = 0.00011. Eyes were photographed with a Zeiss AxioCam MRc5 or a Sony DSC-S70 mounted on a Zeiss Stemi 2000-C dissecting microscope.

4.5.5 Whole Mount *in situ* Hybridization

The in *situ* hybridization protocol was adapted from the manufacturer's instructions provided by Roche. Riboprobes were produced from a *garnet* cDNA fragment (Lloyd 1999b) inserted into a pBlueScript II SK (+) vector (Appendix B); digestion with either *Kpn*I or *Bam*H1 was followed by transcription using either T7 (antisense probe) or T3 (control probe) polymerase, respectively, in the presence of Digoxygenin (DIG)-conjugated UTP (Roche). Probes were shortened to a 1kb length via carbonate buffer hydrolysis (Roche). Eye discs were dissected and fixed in 4% paraformaldehyde, 1x PBS for 30 minutes. Fixed tissue was treated with 2µl/ ml proteinase K (Sigma) in PBT and post-fixed in 4% paraformaldehyde, 1x PBS for 20 minutes. Five successive washes with 1x PBS for 5 minutes was followed by pre-hybridization washes of 1:1 PBS/hybridization buffer (50% formamide, 5x SSC, 0.1% Tween-20, 1% Blocking Reagent (Roche), 50 µg/ ml Heparin) for 10 minutes and a 10 minute wash in hybridization buffer heated to 55°C. Hybridization was performed overnight at 55°C with the riboprobe concentration of 1µl/ 10µl total volume. Post-hybridization tissues were washed twice in hybridization buffer heated to 55°C for 30 minutes, followed by washes in successive dilutions (4/1, 3/2, 2/3, 1/4) of hybridization buffer/ 1x PBS for 10 minutes each wash. Tissues were then washed 3 times in 1x PBT with 10% BSA for 10 minutes per wash. Tissues were soaked in 1/5000 AP-conjugated anti-DIG antibody (Roche) in 1ml of 1x PBT overnight at 4°C, washed 3 times in 1x PBT for 10 minutes per wash and, developed with NBT/BCIP (Roche). Tissues were mounted in 90% glycerol in PBS. Eyes discs were photographed with a Sony DSC-S70 mounted on a Zeiss Axiovert 25 microscope.

4.5.6 Acknowledgements

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4.6 Supplemental

Table 4.1 - Eye Variegation Index For 5-azacytidine Treatment At Selected Developmental Stages

Paternal *Dp(1:f)LJ9* mini-X Inheritance						
		Eye Variegation Index				
Hours AEL	**n**	**0**	**1**	**2**	**3**	**Average**
Control	128	17	27	51	33	1.78 ± 0.09
0-1	112	6	18	29	59	2.26 ± 0.09*
3-4	112	6	17	32	57	2.25 ± 0.09*
7-8	112	6	14	24	68	2.38 ± 0.09*
10-11	96	9	15	40	32	1.99 ± 0.1
12-13	96	12	23	31	30	1.82 ± 0.1
Maternal *Dp(1:f)LJ9* mini-X Inheritance						
		Eye Variegation Index				
Hours AEL	**n**	**0**	**1**	**2**	**3**	**Average**
Control	96	0	0	2	94	2.98 ± 0.02
0-1	112	0	0	3	109	2.97 ± 0.02
3-4	96	0	0	0	96	3.00 ± 0.00
7-8	96	0	0	0	96	3.00 ± 0.00
10-11	96	0	0	0	96	3.00 ± 0.00
12-13	96	0	0	0	96	3.00 ± 0.00

Eye variegation index values are expressed as an average ± SEM based on a scale of visible red eye pigment levels; 0 (0-25%), 1 (25-50%,) 2 (50-75%), 3 (75-100%). AEL = after egg lay, the *n* value represents the number of individual eyes assayed. A concentration of 10 mM 5-azacytidine (5-azaC) was used in each treatment group, *D. melanogaster* Ringer's was used a control. Values that are significantly different from the control are marked with one asterisk ($p =< 0.01$).

Table 4.2 - Eye Variegation Index For The Effects of *MBD*^{1} On *Dp(1;f)LJ9 garnet* Expression

		Eye Variegation Index				
Genotype	**n**	**0**	**1**	**2**	**3**	**Average**
Dp(1;f)LJ9^{PAT}; +/+	200	17	31	101	51	1.93 ± 0.06
Dp(1;f)LJ9^{PAT}; *MBD*^{1}/+	200	5	10	84	101	2.41 ± 0.05*
Dp(1;f)LJ9^{MAT}; +/+	200	0	0	0	200	3.00 ± 0.00
Dp(1;f)LJ9^{MAT}; *MBD*^{1}/+	200	0	0	0	200	3.00 ± 0.00

Eye variegation index values are expressed as an average ± SEM based on a scale of visible red eye pigment levels; 0 (0-25%), 1 (25-50%,) 2 (50-75%), 3 (75-100%). The *n* value represents the number of individual eyes assayed. PAT and MAT denote the parental origin of the *Dp(1;f)LJ9* mini-X chromosome. Values that are significantly different from the control are marked with one asterisk ($p < 0.001$).

Table 4.3 - Eye Variegation Index For The Effects Of 5-azacytidine On The Establishment Of The *Dp(1;f)LJ9* Imprint

		Eye Variegation Index				
Parental Treatment	**n**	**0**	**1**	**2**	**3**	**Average**
Male Parent Control	200	26	34	72	68	1.91 ± 0.07
Male Parent 5-azaC	200	22	26	67	85	2.08 ± 0.07
Female Parent Control	200	0	0	0	200	3.00 ± 0.00
Female Parent 5-azaC	200	0	0	0	200	3.00 ± 0.00

Eye variegation index values are expressed as an average ± SEM based on a scale of visible red eye pigment levels; 0 (0-25%), 1 (25-50%,) 2 (50-75%), 3 (75-100%). The *n* value represents the number of individual eyes assayed. All embryos were treated 0-1 hours after egg lay for a total of 2 hours, a concentration of 10 mM 5-azacytidine (5-azaC) was used in each treatment group and *D. melanogaster* Ringer's was used a control.

Table 4.4 - Eye Variegation Index For The *MBD*[1] Internal Controls

		Eye Variegation Index				
Genotype	**n**	**0**	**1**	**2**	**3**	**Average**
Pat Control	200	20	34	98	48	1.87 ± 0.06
Pat *MBD* Internal	200	14	27	110	49	1.97 ± 0.06
Mat Control	200	0	0	0	200	3.00 ± 0.00
Mat *MBD* Internal	200	0	0	0	200	3.00 ± 0.00

Eye variegation index of MBD^{1} internal control progeny support the results from the pigment assay; there is no significant phenotypic difference between control progeny (Pat Control and Mat Control) and internal control progeny with parents carrying MBD^{1} allele (Pat *MBD* Internal and Mat *MBD* Internal). Eye variegation index values are expressed as an average ± SEM based on a scale of visible red eye pigment levels; 0 (0-25%), 1 (25-50%,) 2 (50-75%), 3 (75-100%).

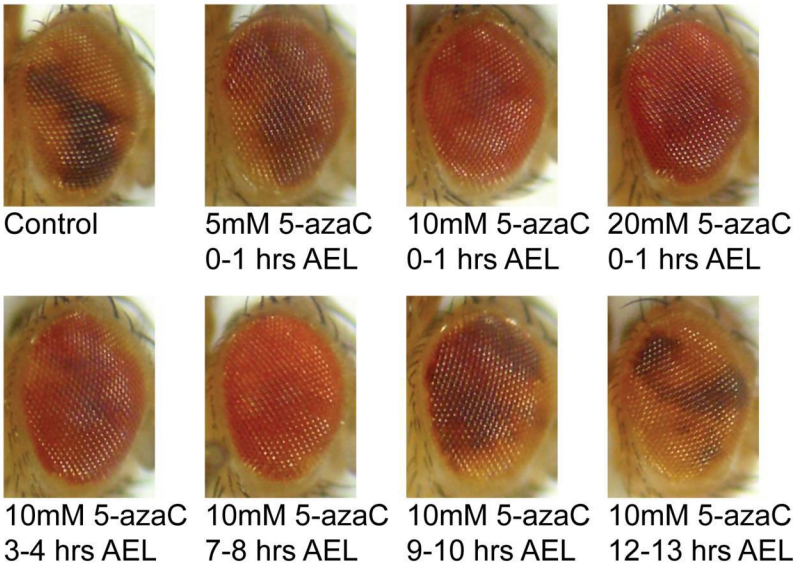

Figure 4.6 – Images Of Representative Eyes From The Paternally Inherited Mini-X Chromosome 5-azacytidine (5-azaC) Treatment Groups.
Relaxed silencing of the reporter gene *garnet* was observed at all dosages of 5-azacytidine tested and at each treatment time, except 9-10 hrs and 12-13 hrs AEL. For each sample, the 5-azacytidine dosage and time of treatment are listed below the picture. *D. melanogaster* Ringer's was used to treat the control group.

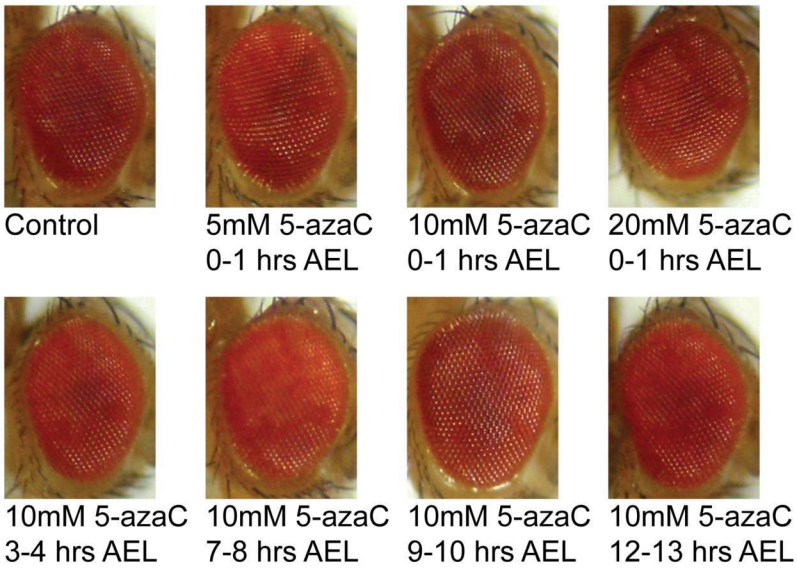

Figure 4.7 - Images Of Representative Eyes From The Maternally Inherited Mini-X Chromosome 5-azacytidine (5-azaC) Treatment Groups.
No affect was observed on expression of the reporter gene *garnet* when embryos with the maternally inherited mini-X chromosome were treated with 5-azacytidine; For each sample, the 5-azacytidine dosage and time of treatment are listed below the picture. *D. melanogaster* Ringer's was used to treat the control group.

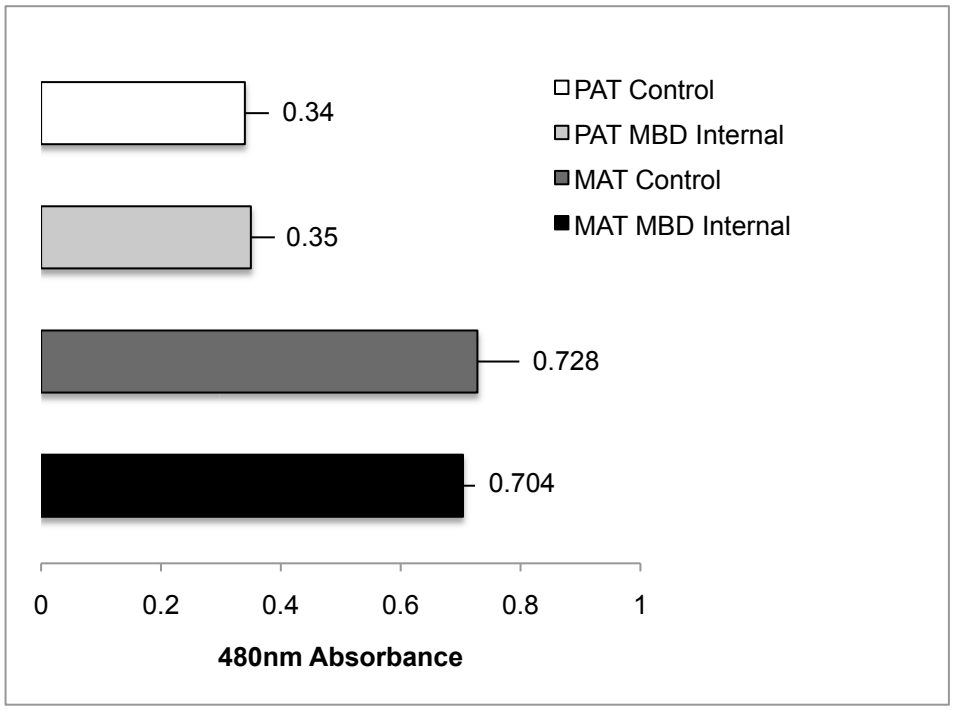

Figure 4.8 - *MBD*^{1} Internal Control Eye Pigment Levels.
Internal control progeny were compared to progeny of the same genotype ($y^{1}z^{a}g^{53d}$/*Dp(1;f)LJ9*; *TM3, Sb*/+) from a control cross using unrelated parents wild-type for *MBD*^{1}, as outlined in the Materials and Methods. Internal control progeny can be used to determine if there is a maternal effect in embryos produced from mothers carrying the *MBD*^{1} allele, as early embryonic development is dependent upon maternally contributed transcripts until zygotic activation of the embryo (Fitch *et al.* 1998). Paternal inheritance of the mini-X chromosome with mothers bearing *MBD*^{1} (PAT MBD Internal) did not yield any significant alteration to *garnet* expression levels compared to mothers with wild-type levels of *MBD2/3* expression (PAT Control). Thus, *MBD*^{1} has no maternal effect, which is consistent with MBD2/3 first associating with DNA during zygotic activation of the embryo (Marhold *et al.* 2002). No significant difference in *garnet* expression levels was observed in the maternal inheritance of the mini-X chromosome with fathers bearing *MBD*^{1} (MAT MBD Internal) compared to fathers with wild-type levels of *MBD2/3* expression (MAT Control). Pigment levels are measured by absorbance at 480nm, as outlined in the Material and Methods; pigment quantification mean values for each group are based on $n = 5$ samples (40 heads total), error bars represent standard deviation.

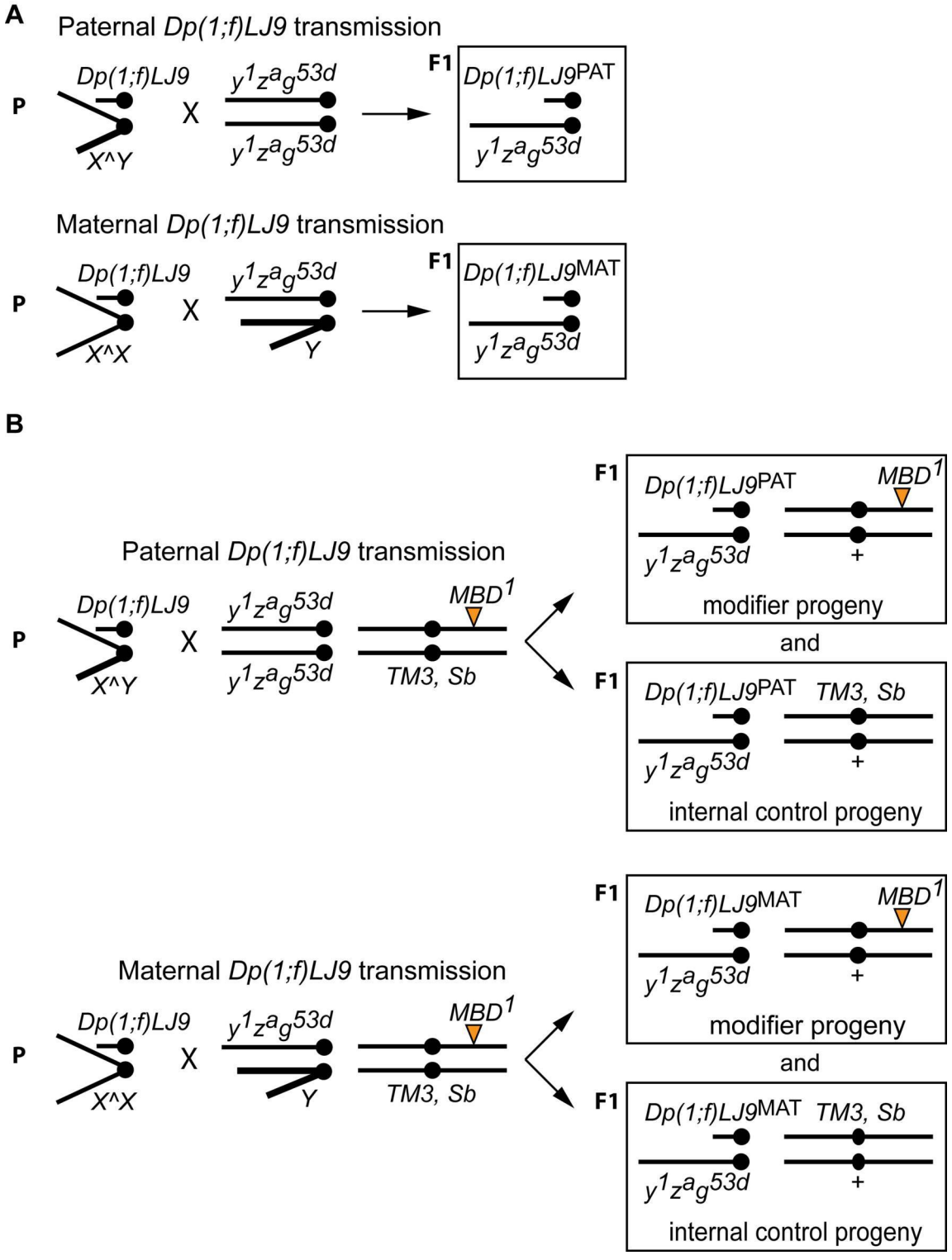

Figure 4.9 - Cross Schematic For *Dp(1;f)LJ9* Transmission.
A) Paternal and maternal transmission of *Dp(1;f)LJ9*.
B) Both paternal and maternal crosses involving MBD^{1} produce $y^{1}z^{a}g^{53d}$/*Dp(1;f)LJ9*; MBD^{1}/+ progeny, and sibling $y^{1}z^{a}g^{53d}$/*Dp(1;f)LJ9*; *TM3, Sb*/+ internal control progeny.

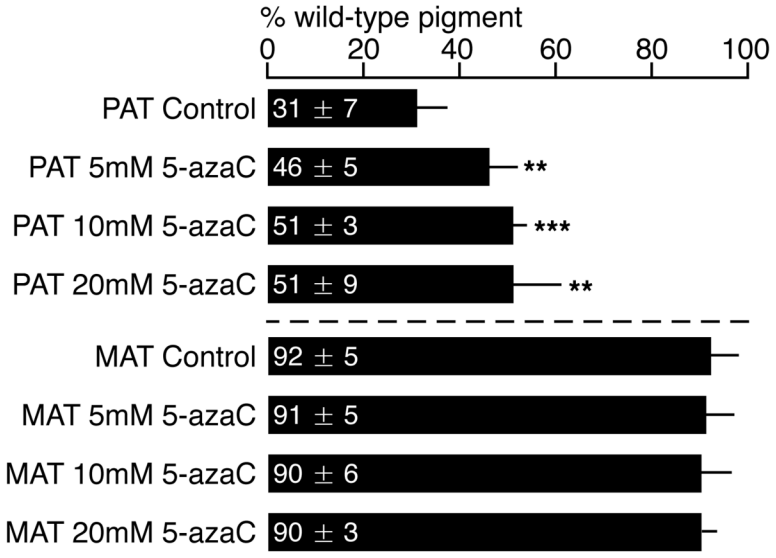

Figure 4.10 - The Effects Of 5-azacytidine (5-azaC) Dosages On *Dp(1;f)LJ9 garnet* Expression.

Each dosage of 5-azacytidine tested significantly increased *garnet* expression from paternally inherited mini-X chromosome (PAT), while no significant change occurred from maternally inherited mini-X chromosome (MAT). All embryos were treated 0-1 hours AEL for a total of 2 hours; control embryos were treated with *D. melanogaster* Ringer's. Eye pigment levels are expressed as a percentage of full, wild-type gene expression. Error bars represent standard deviation. Mean values for each group are based on: n = 6 samples (48 heads total). Values that are significantly different from the control are marked with Bonferroni-corrected p values: **(p=0.0011-0.00011), ***(p<0.00011). Representative eye pictures presented in Supplemental Figure 4.6 and S2.

4.7 Addendum: *Drosophila* Nuclear Organization By Lamin May Be Required For Proper DNA Methylation Of The Paternal *Dp(1;f)LJ9* Imprint

The preliminary results presented in this addendum contribute to the interpretation of the DNA methylation results.

Nuclear lamins are filamentous proteins that contribute to the structure of the nuclear matrix, and are involved in the nuclear localization of chromosomes and mitotic organization (Gruenbaum *et al.* 2003). The *Drosophila* B-type lamins form the majority of the nuclear envelope, and B-type lamin Dm_0 is able to directly bind chromatin through interaction with histones H2A and H2B (Goldberg *et al.* 1999; Mattout *et al.* 2007). During early embryogenesis, Dnmt2 associates with lamin and the nuclear matrix during mitosis, suggesting DNA modification by Dnmt2 may occur at this stage in the cell cycle (Schaefer *et al.* 2008). Thus, a disruption of nuclear organization by *lamin* mutations would be expected to affect Dnmt2 localization and interfere with the application of DNA methyl groups to target areas of the genome. I tested two *lamin* Dm_0 (*lam*) alleles for their effects on *Dp(1;f)LJ9 garnet* expression; the lam^{04634} loss-of-function allele and the hypomorphic lam^{sz18} allele (Osouda *et al.* 2005). Both alleles significantly relaxed *garnet* silencing from paternally inherited $Dp(1;f)LJ9^{PAT}$ (Figure 4.11A), causing a visible increase in red pigment levels (Table 4.5 and Figures 4.11A and 4.11B). Additionally, the internal controls for both *lam* alleles also significantly relaxed *garnet* silencing, demonstrating *lam* alleles have a maternal effect on $Dp(1;f)LJ9^{PAT}$ regulation prior to the zygotic activation of the embryo (Table 4.5 and Figures 4.11A and 4.11B). Neither *lam* allele had any significant affect on *garnet* expression from $Dp(1;f)LJ9^{MAT}$, and no paternal effects were detected (Table 4.5 and Figures 4.11C and 4.11D).

These results demonstrate the importance of nuclear organization for the stabilization of the *Dp(1;f)LJ9* imprint during early embryogenesis. Strict nuclear localization of imprinted loci in mice contributes to the asynchronous replication of these regions late in the cell cycle (Gribnau *et al.* 2003). The partial loss-of-function lam^{Ari3} allele has been reported to enhance heterochromatic silencing of the *white* gene in the

In(1)w^{m4} variegating strain, in both homo-allelic lam^{Ari3}/lam^{Ari3} and hetero-allelic lam^{Ari3}/lam^{04634} combinations, but no maternal effects were reported (Bao *et al.* 2006). These findings reinforce the importance of nuclear organization for the formation of higher order chromatin structures. However, the distinct requirement of lamin early in embryogenesis to form the paternal *Dp(1;f)LJ9* imprint, and its requirement for silencing rather than activation, distinguishes the regulation of imprinted regions from other regions of the *Drosophila* genome.

Developmentally regulated localization of Dnmts is thought to contribute to the regulation of imprinted regions in preimplantation mouse embryos (Carlson *et al.* 1992). Disruption of DNA methylation by 5-azacytidine affected maintenances of the imprint during the earliest stages of embryogenesis and prior to the zygotic activation of the embryo, consistent with the maternal effect of the *lam* mutations. Thus, it remains to be determined if lamins interact directly with Dnmt2 or act indirectly by enforcing heterochromatic nuclear compartments where Dnmt2 could be recruited. The nature of the interaction between Dnmt2 and lamin could be determined by examining the localization of Dnmt2, and the levels of in DNA methylation, in *lamin* mutants.

Table 4.5 - Eye Variegation Index For The Effects Of Lamin Mutants On *Dp(1;f)LJ9 garnet* Expression

Paternal *Dp(1:f)LJ9* mini-X Inheritance						
		Eye Variegation Index				
Genotype	**n**	**0**	**1**	**2**	**3**	**Average**
Dp(1;f)LJ9^{PAT}; +/+	300	17	57	138	88	1.99 ± 0.07
Dp(1;f)LJ9^{PAT}; *lam*^{04634}/+	300	10	24	97	169	2.42 ± 0.05*
Dp(1;f)LJ9^{PAT}; *lam*^{sz18}/+	300	13	24	89	164	2.35 ± 0.05*
(Mat +/+) *Dp(1;f)LJ9*^{PAT}; *CyO*/+	300	22	55	132	91	1.97 ± 0.05
(Mat *lam*^{04634}) *Dp(1;f)LJ9*^{PAT}; *CyO*/+	300	17	19	114	150	2.32 ± 0.05*
(Mat *lam*^{sz18}) *Dp(1;f)LJ9*^{PAT}; *CyO*/+	300	12	29	118	141	2.29 ± 0.05*
Maternal *Dp(1:f)LJ9* mini-X Inheritance						
		Eye Variegation Index				
Genotype	**n**	**0**	**1**	**2**	**3**	**Average**
Dp(1;f)LJ9^{MAT}; +/+	300	0	0	0	300	3.00 ± 0.0
Dp(1;f)LJ9^{MAT}; *lam*^{04634}/+	300	0	0	0	300	3.00 ± 0.0
Dp(1;f)LJ9^{MAT}; *lam*^{sz18}/+	300	0	0	0	300	3.00 ± 0.0
(Pat +/+) *Dp(1;f)LJ9*^{MAT}; *CyO*/+	300	0	0	0	300	3.00 ± 0.0
(Pat *lam*^{04634}) *Dp(1;f)LJ9*^{MAT}; *CyO*/+	300	0	0	0	300	3.00 ± 0.0
(Pat *lam*^{sz18}) *Dp(1;f)LJ9*^{MAT}; *CyO*/+	300	0	0	0	300	3.00 ± 0.0

Eye variegation index values are expressed as an average ± SEM based on a scale of visible red eye pigment levels; 0 (0-25%), 1 (25-50%,) 2 (50-75%), 3 (75-100%). The *n* value represents the number of individual eyes assayed. PAT and MAT denote the parental origin of the *Dp(1;f)LJ9* mini-X chromosome. Internal control progeny are balanced over *CyO* and the parental *lam* allele is listed in parentheses. Values that are significantly different from the control are marked with one asterisk ($p < 0.001$).

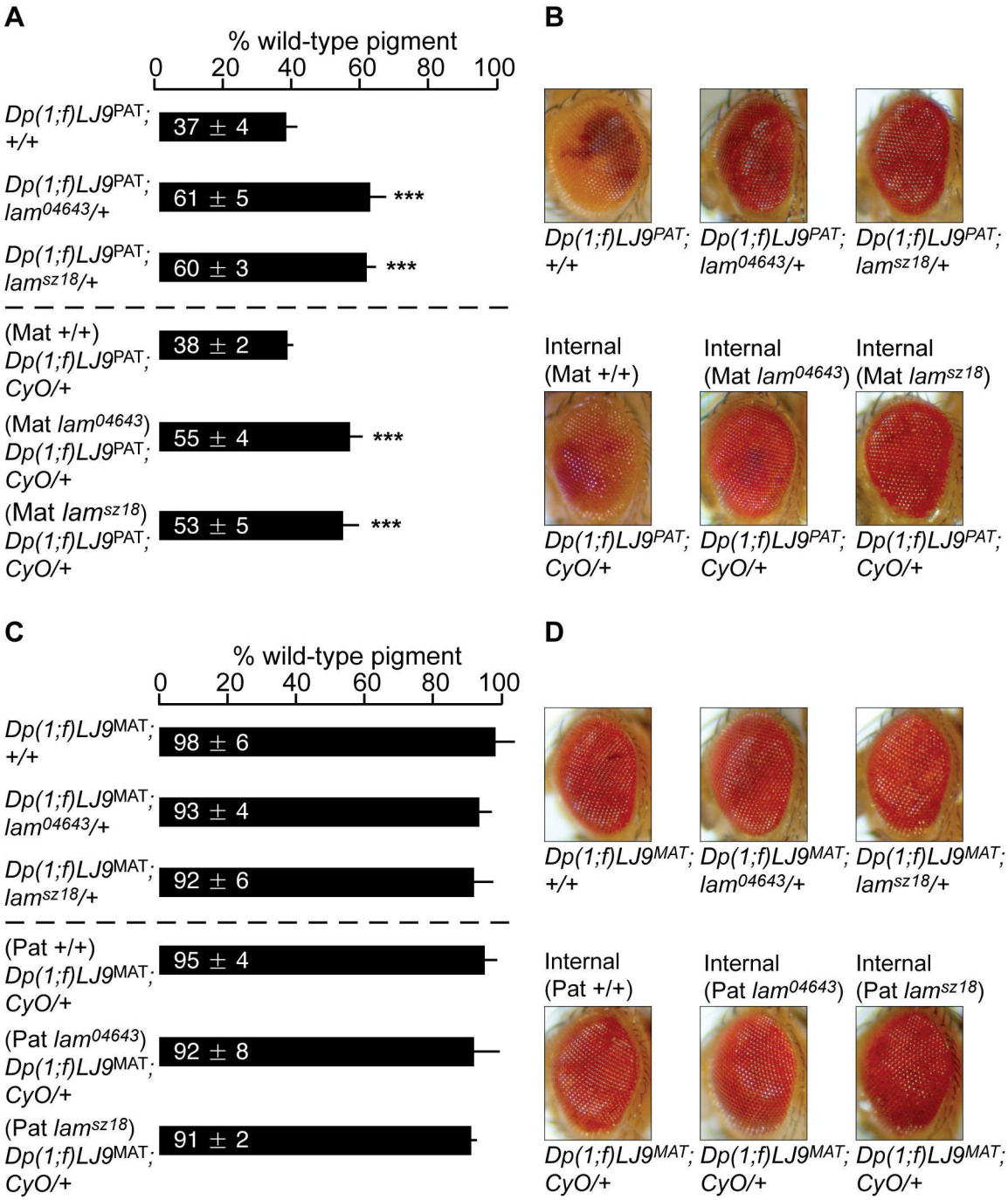

Figure 4.11 - Mutant *lamin* Alleles Relax Silencing Of *garnet* From The Paternal Mini-X Chromosome.

A) Pigment assays for paternally inherited mini-X chromosomes ($Dp(1;f)LJ9^{PAT}$), both *lam* alleles significantly relaxed *garnet* silencing *** ($p < 0.001$) compared to the control ($Dp(1;f)LJ9^{PAT}$; +/+). Internal control progeny are all the same genotype ($y^{1}z^{a}g^{53d}/Dp(1;f)LJ9^{PAT}$; *CyO*/+), but differ in the maternal presence of *lam* alleles (listed within parentheses). Both *lam* alleles when present maternally significantly relaxed *garnet* silencing *** ($p < 0.001$) compared to the control ((Mat +/+) $Dp(1;f)LJ9^{PAT}$; *CyO*/+).

(Figure 4.11 continued)

B) Representative eye pictures for paternally inherited mini-X chromosomes illustrate increased red pigment levels due to relaxed *garnet* silencing from *lam* mutations.
C) Pigment assays for maternally inherited mini-X chromosomes (*Dp(1;f)LJ9*^{MAT}). Internal control progeny are all the same genotype (*y*^{1}*z*^{a}*g*^{53d}/*Dp(1;f)LJ9*^{MAT}; *CyO*/+), but differ in the paternal presence of *lam* alleles (listed within parentheses).
D) Representative eye pictures for maternally inherited mini-X chromosomes illustrate no change red pigment levels from *lam* mutations.
Pigment levels are measured by absorbance at 480nm, as outlined in the Material and Methods; pigment quantification mean values for each group are based on $n = 5$ samples (40 heads total), error bars represent standard deviation.

4.8 References

Ahmad K, Henikoff S. (2001) Modulation of a transcription factor counteracts heterochromatic gene silencing in *Drosophila*. Cell. **104**:839-847.

Anaka M, Lynn A, Mcginn P, Lloyd VK. (2009) Genomic imprinting in *Drosophila* has properties of both mammalian and insect imprinting. Dev Genes Evol. **219**:59-66.

Baker WK. (1967) A clonal system of differential gene activity in *Drosophila*. Dev Biol. **16**:1-17.

Ballestar E, Pile L, Wassarman D, Wolffe A, Wade P. (2001) A *Drosophila* MBD family member is a transcriptional corepressor associated with specific genes. Eur J Biochem. **268**:5397-5406.

Bao X, Girton J, Johansen J, Johansen K. (2006) The lamin *Dm(0)* allele Ari3 acts as an enhancer of position effect variegation of the *wm4* allele in *Drosophila*. Genetica. **129**:339-342.

Baqir S, Smith LC. (2006) Inhibitors of histone deacetylases and DNA methyltransferases alter imprinted gene regulation in embryonic stem cells. Cloning Stem Cells. **8**:200-213.

Bartolomei MS. (2009) Genomic imprinting: employing and avoiding epigenetic processes. Genes Dev. **23**:2124-2133.

Bellen HJ, Levis RW, Liao G, He Y, Carlson JW, Tsang G, Evans-Holm M, Hiesinger PR, Schulze KL, Rubin GM and others. (2004) The BDGP gene disruption project: single transposon insertions associated with 40% of *Drosophila genes*. Genetics. **167**:761-781.

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

Brenton J, Drewell R, Viville S, Hilton K, Barton S, Ainscough J, Surani M. (1999) A silencer element identified in *Drosophila* is required for imprinting of *H19* reporter transgenes in mice. PNAS. **96**:9242-9247.

Carlson LL, Page AW, Bestor TH. (1992) Properties and localization of DNA methyltransferase in preimplantation mouse embryos: implications for genomic imprinting. Genes Dev. **6**:2536-2541.

Chen Y, Dhupelia A, Schoenherr C. (2008) The *Igf2/H19* imprinting control region exhibits sequence-specific and cell-type-dependent DNA methylation-mediated repression. Nucleic Acids Res. **37**:793-803.

Eversole-Cire P, Ferguson-Smith AC, Sasaki H, Brown KD, Cattanach BM, Gonzales FA, Surani MA, Jones PA. (1993) Activation of an imprinted *Igf2* gene in mouse somatic cell cultures. Mol Cell Biol. **13**:4928-4938.

Fitch K, Yasuda G, Owens K, Wakimoto B. (1998) Paternal effects in *Drosophila*: implications for mechanisms of early development. Curr Top Dev Biol. **38**:1-34.

Fournier C, Goto Y, Ballestar E, Delaval K, Hever A, Esteller M, Feil R. (2002) Allele-specific histone lysine methylation marks regulatory regions at imprinted mouse genes. EMBO J. **21**:6560-6570.

Friedrich M. (2006) Ancient mechanisms of visual sense organ development based on comparison of the gene networks controlling larval eye, ocellus, and compound eye specification in *Drosophila*. Arthropod Struct Dev. **35**:357-378.

Goldberg M, Harel A, Brandeis M, Rechsteiner T, Richmond TJ, Weiss AM, Gruenbaum Y. (1999) The tail domain of lamin Dm0 binds histones H2A and H2B. PNAS. **96**:2852-2857.

Goll M, Kirpekar F, Maggert K, Yoder J, Hsieh C, Zhang X, Golic K, Jacobsen S, Bestor T. (2006) Methylation of tRNAAsp by the DNA methyltransferase homolog Dnmt2. Science. **311**:395-398.

Gribnau J, Hochedlinger K, Hata K, Li E, Jaenisch R. (2003) Asynchronous replication timing of imprinted loci is independent of DNA methylation, but consistent with differential subnuclear localization. Genes Dev. **17**:759-773.

Gruenbaum Y, Goldman RD, Meyuhas R, Mills E, Margalit A, Fridkin A, Dayani Y, Prokocimer M, Enosh A. (2003) The nuclear lamina and its functions in the nucleus. Int Rev Cytol. **226**:1-62.

Haigh A, Lloyd V. (2006) Loss of genomic imprinting in *Drosophila* clones. Genome. **49**:1043-1046.

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

Henckel A, Nakabayashi K, Sanz LA, Feil R, Hata K, Arnaud P. (2009) Histone methylation is mechanistically linked to DNA methylation at imprinting control regions in mammals. Hum Mol Genet. **18**:3375-3383.

Hendrich B, Guy J, Ramsahoye B, Wilson VA, Bird A. (2001) Closely related proteins MBD2 and MBD3 play distinctive but interacting roles in mouse development. Genes Dev. **15**:710-723.

Hendrich B, Tweedie S. (2003) The methyl-CpG binding domain and the evolving role of DNA methylation in animals. Trends Genet. **19**:269-277.

Hermann A, Gowher H, Jeltsch A. (2004) Biochemistry and biology of mammalian DNA methyltransferases. Cell Mol Life Sci. **61**:2571-2587.

Hermann A, Schmitt S, Jeltsch A. (2003) The human Dnmt2 has residual DNA-(cytosine-C5) methyltransferase activity. J Biol Chem. **278**:31717-31721.

Hirasawa R, Chiba H, Kaneda M, Tajima S, Li E, Jaenisch R, Sasaki H. (2008) Maternal and zygotic Dnmt1 are necessary and sufficient for the maintenance of DNA methylation imprints during preimplantation development. Genes Dev. **22**:1607-1616.

Imamura T, Kerjean A, Heams T, Kupiec J-J, Thenevin C, Pàldi A. (2005) Dynamic CpG and non-CpG methylation of the *Peg1/Mest* gene in the mouse oocyte and preimplantation embryo. J Biol Chem. **280**:20171-20175.

Joanis V, Lloyd V. (2002) Genomic imprinting in *Drosophila* is maintained by the products of *Suppressor of variegation* and *trithorax* group, but not *Polycomb* group, genes. Mol Genet Genomics. **268**:103-112.

Kaneda M, Okano M, Hata K, Sado T, Tsujimoto N, Li E, Sasaki H. (2004) Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. Nature. **429**:900-903.

Komashko VM, Farnham PJ. (2010) 5-azacytidine treatment reorganizes genomic histone modification patterns. Epigenetics. **5**:229-240.

Kunert N, Marhold J, Stanke J, Stach D, Lyko F. (2003) A Dnmt2-like protein mediates DNA methylation in *Drosophila*. Development. **130**:5083-5090.

Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, FitzHugh W and others. (2001) Initial sequencing and analysis of the human genome. Nature. **409**:860-921.

Lees-Murdock DJ, Walsh CP. (2008) DNA methylation reprogramming in the germ line. Epigenetics. **3**:5-13.

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

Lister R, Pelizzola M, Dowen RH, Hawkins RD, Hon G, Tonti-Filippini J, Nery JR, Lee L, Ye Z, Ngo Q-M and others. (2009) Human DNA methylomes at base resolution show widespread epigenomic differences. Nature. **462**:315-322.

Liu K, Wang YF, Cantemir C, Muller MT. (2003) Endogenous assays of DNA methyltransferases: Evidence for differential activities of DNMT1, DNMT2, and DNMT3 in mammalian cells in vivo. Mol Cell Biol. **23**:2709-2719.

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

Lloyd V, Sinclair D, Grigliatti T. (1999a) Genomic imprinting and position-effect variegation in *Drosophila melanogaster*. Genetics. **151**:1503-1516.

Lloyd V, Sinclair D, Wennberg R, Warner T, Honda B, Grigliatti T. (1999b) A genetic and molecular characterization of the *garnet* gene of *Drosophila melanogaster*. Genome. **42**:1183-1193.

Lyko F, Brenton J, Surani M, Paro R. (1997) An imprinting element from the mouse *H19* locus functions as a silencer in *Drosophila*. Nat Genet. **16**:171-173.

Lyko F, Buiting K, Horsthemke B, Paro R. (1998) Identification of a silencing element in the human *15q11-q13* imprinting center by using transgenic *Drosophila*. PNAS. **95**:1698-1702.

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

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

Maggert K, Golic K. (2002) The Y chromosome of *Drosophila melanogaster* exhibits chromosome-wide imprinting. Genetics. **162**:1245-1258.

Marhold J, Kramer K, Kremmer E, Lyko F. (2004) The *Drosophila* MBD2/3 protein mediates interactions between the MI-2 chromatin complex and CpT/A-methylated DNA. Development. **131**:6033-6039.

Marhold J, Zbylut M, Lankenau D-H, Li M, Gerlich D, Ballestar E, Mechler BM, Lyko F. (2002) Stage-specific chromosomal association of *Drosophila* dMBD2/3 during genome activation. Chromosoma. **111**:13-21.

Mattout A, Goldberg M, Tzur Y, Margalit A, Gruenbaum Y. (2007) Specific and conserved sequences in *D. melanogaster* and *C. elegans* lamins and histone H2A mediate the attachment of lamins to chromosomes. J Cell Sci. **120**:77-85.

McEwen K, Ferguson-Smith A. (2009) Genomic Imprinting–A Model for Roles of Histone Modifications in Epigenetic Control. In: Epigenomics.235-258.

McEwen KR, Ferguson-Smith AC. (2010) Distinguishing epigenetic marks of developmental and imprinting regulation. Epigenet Chrom. **3**:2.

Menon DU, Meller VH. (2009) Imprinting of the Y chromosome influences dosage compensation in *roX1 roX2 Drosophila melanogaster*. Genetics. **183**:811-820.

Osouda S, Nakamura Y, de Saint Phalle B, McConnell M, Horigome T, Sugiyama S, Fisher PA, Furukawa K. (2005) *Null* mutants of *Drosophila* B-type *lamin Dm0* show aberrant tissue differentiation rather than obvious nuclear shape distortion or specific defects during cell proliferation. Dev Biol. **284**:219-232.

Phalke S, Nickel O, Walluscheck D, Hortig F, Onorati MC, Reuter G. (2009) Retrotransposon silencing and telomere integrity in somatic cells of *Drosophila* depends on the cytosine-5 methyltransferase DNMT2. Nat Genet. **41**:696-702.

Ramsahoye BH, Biniszkiewicz D, Lyko F, Clark V, Bird AP, Jaenisch R. (2000) Non-CpG methylation is prevalent in embryonic stem cells and may be mediated by DNA methyltransferase 3a. PNAS. **97**:5237-5242.

Real, MD, Ferre, J, Mensua, JL. (1985) Methods for the quantitative estimation of the red and brown pigments of *D. melanogaster.* Dros Inf Serv. **61**:198-200.

Reese KJ, Lin S, Verona RI, Schultz RM, Bartolomei MS. (2007) Maintenance of paternal methylation and repression of the imprinted *H19* gene requires MBD3. PLoS Genet. **3**:e137.

Roder K, Hung MS, Lee TL, Lin TY, Xiao H, Isobe KI, Juang JL, Shen CJ. (2000) Transcriptional repression by *Drosophila* methyl-CpG-binding proteins. Mol Cell Biol. **20**:7401-7409.

Schaefer M, Lyko F. (2010) Solving the Dnmt2 enigma. Chromosoma. **119**:35-40.

Schaefer M, Steringer JP, Lyko F. (2008) The *Drosophila* cytosine-5 methyltransferase Dnmt2 is associated with the nuclear matrix and can access DNA during mitosis. PLoS ONE. **3**:e1414.

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

Spofford JB. (1976) Position-effect variegation in *Drosophila*. In: The Genetics and Biology of *Drosophila* Edited by M. Ashburner and E. Novitski. Academic Press, London, U.K. **1c**:955-1018.

Sullivan W, Ashburner M, Scott Hawley R. (2000) *Drosophila* protocols. W. Sullivan, M. Ashburner, and R. S. Hawley (eds.) Appendix 3 Reagents & Solutions. Cold Spring Harbor Laboratory Press.655.

Tucker KL, Beard C, Dausmann J, Jackson-Grusby L, Laird PW, Lei H, Li E, Jaenisch R. (1996) Germ-line passage is required for establishment of methylation and expression patterns of imprinted but not of nonimprinted genes. Genes Dev. **10**:1008-1020.

Tweedie S, Ng HH, Barlow AL, Turner BM, Hendrich B, Bird A. (1999) Vestiges of a DNA methylation system in *Drosophila melanogaster*? Nat Genet. **23**:389-390.

Vlassova IE, Graphodatsky AS, Belyaeva ES, Zhimulev IF. (1991) Constitutive heterochromatin in early embryogenesis of *Drosophila melanogaster*. Mol Gen Genet. **229**:316-318.

Weyrich A, Tang X, Xu G, Schrattenholz A, Hunzinger C, Hennig W. (2008) Mammalian DNMTs in the male germ line DNA of *Drosophila*. Biochem Cell Biol. **86**:380-385.

Williamson A, Lehmann R. (1996) Germ cell development in *Drosophila*. Annu Rev Cell Dev Biol. **12**:365-391.

4.9 Transition To Chapter 5

The following chapter examines the role that histone modifications and histone variants have on the regulation of the *Dp(1;f)LJ9* imprint. A number of mutations in histone genes, histone variants, and histone modifiers were tested. The results show that differential histone modifications distinguish the maternal and paternal imprint domains and that heterochromatin nucleated from the *Dp(1;f)LJ9* imprint control center is distinct from general constitutive centric heterochromatin.

Chapter 5 The Histone Variant H2Av And Other Histone Modifications Maintain Genomic Imprinting In *Drosophila melanogaster*

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5.1 Abstract

The formation of higher-order chromatin structures is directed in part by histone modifications and histone replacements that designate the initiation of either euchromatic or heterochromatic structures. In chromosomal regions marked by a genomic imprint, distinct chromatin configurations are generated on maternally and paternally inherited chromosomes, resulting in the parent-specific expression of imprinted genes. The *Drosophila* imprinted *Dp(1:f)LJ9* mini-X chromosome exhibits distinct phenotypes dependent on parental inheritance; paternal inheritance results in variegated silencing of a euchromatic marker gene, while maternal inheritance yields full expression of the marker gene. Position-effect variegation (PEV) has long been used as a tool to assess chromatin structure. We tested mutant alleles of core histones, histone variants, and histone modifiers, all of which modify PEV, to assess the aspects of chromatin structure required to propagate the distinct parent-specific phenotypes from the imprinted domain on the mini-X chromosome. A null allele of the histone variant H2Av, which suppresses conventional PEV, acts as a dominant enhancer of variegation, increasing silencing from both the maternally and paternally imprinted domain. However, acetylation of H2Av is a modification that only affects the paternal imprint. Mutations affecting histone acetylation, deacetylation, and phosphorylation also interfered with regulation of the imprinted domain. Together, these results show variant H2Av is a critical component of the imprinted domain that is regulated in a parent-specific manner.

5.2 Introduction

Higher-order chromatin structure is a critical component of eukaryotic chromosome organization and essential for correct gene regulation. A dramatic example is provided by genomic imprinting, in which the two parental alleles are expressed differently. While the details of the imprinting mechanism vary by loci, one common feature is that the same region of DNA adopts different chromatin structures depending on whether it has been transmitted by a male or female (Pardo-Manuel de Villena *et al.* 2000). In *Drosophila,* imprinted domains have been found only in heterochromatic regions (Haller and Woodruff 2000; Lloyd 2000; Maggert and Golic 2002; Menon and

Meller 2009). Mammalian imprinted domains are similarly dependent on parent-specific histone modifications and chromatin formation (Latham 2005; Wen *et al.* 2008), and these domains of differential chromatin conformation are delimited by strict structural boundaries (Shirohzu *et al.* 2004; Regha *et al.* 2007). Thus, genomic imprinting in both *Drosophila* and mammals is initiated by the remodeling of chromatin on maternal and paternal chromosomes into differentially distinct domains.

The effect of chromatin on gene expression has long been studied using *Drosophila* position-effect variegation (PEV). Early experiments by Muller described a red and white mottled eye color resulting from placement of the *white* gene into the "inert" centric heterochromatin of the $In(1)w^{m4}$ chromosome (Muller 1930). It was determined that the degree of *white* silencing could be altered by mutations in genes influencing heterochromatic structure (Gowen and Gay 1934; Schultz 1936; Baker 1967; Spofford 1967). Histone genes in *Drosophila* are clustered in tandem repeats, and deficiencies for this region suppresses PEV (Moore *et al.* 1983; Doheny *et al.* 2008). Additionally, other genes, which either suppress, *Su(var)s*, or enhance, *E(var)s*, variegated silencing when mutant, allowed identification of key structural components of euchromatic and heterochromatic regions, prominent among them being histone modifying enzymes. Mutations in the histone H3 lysine 9 (H3K9) methyltransferase (*Su(var)3-9*) and histone H4 lysine 20 (H4K20) methyltransferase (*Su(var)4-20*) genes suppress PEV (Schotta *et al.* 2002; Schotta *et al.* 2004). Additional non-histone proteins are recruited during the formation of heterochromatin; heterochromatic protein HP1 (*Su(var)2-5*), and the HP1 associated DNA-binding protein (*Su(var)3-7*) both encode known components of heterochromatic regions (Eissenberg and Elgin 2000; Jaquet *et al.* 2006).

The prevailing model for heterochromatin formation in *Drosophila* is that replacement of the core histone H2A with H2Av is an early step towards the recruitment of H3K9 methyltransferase and HP1, leading to heterochromatinization. Swaminathan *et al*. (2005) showed that the H2Av null allele, $His2Av^{810}$ (van Daal and Elgin 1992), suppressed variegation of $In(1)w^{m4}$, disrupted localized H4K12 acetylation, and reduced

H3K9 methylation, leading to a disruption in heterochromatin formation (Swaminathan *et al.* 2005). However, H2Av localizes to both active and inactive sites in the genome (Leach *et al.* 2000) and, recently, *His2Av*^{810} has been reported to act as either a suppressor, enhancer, or have no affect, depending on the chromosomal insertion site of a variegating construct (Haynes *et al.* 2007). Additionally, acetylation of the N-terminus of H2Av has been linked to the activation of transiently silenced genes within heterochromatin (Tanabe *et al.* 2008). These findings suggest that H2Av may have a more complex role in the nucleation of chromatin domains than initially proposed; H2Av may be used to prime nucleosomes, which are then directed to form active or repressive chromatin conformations in a domain specific manner. *Drosophila* H2Av shares homologies with yeast, plant, and mammalian H2A.X and H2A.Z histone variants. The N-terminus of H2Av is similar to H2A.Z and accepts acetylation, while the C-terminus shares identity with H2A.X and is phosphorylated in response to DNA damage (Madigan *et al.* 2002; Tanabe *et al.* 2008). This dual identity of H2Av likely contributes towards its dual regulatory properties, as H2A.Z is commonly associated with active chromatin and H2A.X with repressive chromatin.

The study of PEV has revealed that heterochromatin is not a monolithic entity but rather a dynamic region that can be modulated by the availability of heterochromatic components to regulate gene expression. As genomic imprints are dependent on the formation of different chromatin domains at the two genetically identical parental alleles, we assessed the role of histone modifications and histone variants on heterochromatin formation at a *Drosophila* imprinted domain. We used the established *Dp(1;f)LJ9* mini-X (mini-X) chromosome imprinting assay system, in which the eye pigmentation gene, *garnet*, is juxtaposed to an endogenously imprinted domain. A paternally inherited mini-X (mini-X^{PAT}) results in the variegated silencing of *garnet* caused by the spreading of heterochromatic chromatin from the imprint control region, while a maternally inherited mini-X (mini-X^{MAT}) results in full *garnet* expression and no variegated silencing, as a stable boundary is maintained between the imprint control region and the *garnet* marker gene (Lloyd *et al.* 1999a; MacDonald *et al.* 2010), protecting the marker gene from silencing.

We found that mutations in core histone genes relax silencing of *garnet* variegation from mini-X^{PAT}, while having no affect on the stable *garnet* expression from mini-X^{MAT}, consistent with their established role in suppressing PEV. We also found that the *His2Av*^{810} allele acts as a strong, dominant, enhancer of variegation from mini-X^{PAT} and induces variegated silencing from mini-X^{MAT}, demonstrating that H2Av is a critical component in regulating expression from the imprinted domain. However, disrupting acetylation of H2Av affects only mini-X^{PAT}, causing dominant enhancement of *garnet* variegation, showing that H2Av is modified in a parent-specific manner. Together, these results support a role for H2Av as a dynamic component of higher-order chromatin structure, involved in the modulation of both active and silent transcriptional domains.

5.3 Materials And Methods

5.3.1 *Drosophila* Stocks

Stocks were maintained at 22°C and cultured on standard cornmeal, yeast, molasses and agar media with methylbenzoate (0.15%, Sigma). Crosses were conducted in 12 dram shell vials with 15-20 virgin females mated to 10-15 selected males. Each cross was subcultured at three-day intervals, up to four times before the parents were discarded. The progeny from all subcultures were collected and results pooled. All mutant stocks were provided by the Bloomington *Drosophila* Stock Center.

5.3.2 Experimental Crosses

The *Dp(1;f)LJ9* mini-X chromosome has the *garnet* (*g*) gene placed next to imprinted heterochromatin, and is derived from the *In(1)sc*^{29} inversion followed by X-ray induced deletion of the majority of X chromosome euchromatin and the distal end of pericentric heterochromatin (Hardy *et al.* 1984). *Dp(1;f)LJ9*, g^+, y^+ flies are balanced over attached *X^X* or *X^Y* chromosomes for maternal (mini-X^{MAT}) and paternal (mini-X^{PAT}) transmission, respectively. To visualize the imprinting of the *garnet* gene (*g*), *Dp(1;f)LJ9/X^X* or *Dp(1;f)LJ9/X^Y* flies were crossed to $y^1 z^a g^{53d}$ hemizygotes or homozygotes, respectively, and *Dp(1;f)LJ9/* y^1 z^a g^{53d} male progeny selected. Selecting

yellow^{+} (y^{+}) progeny confirms the presence of the *Dp(1;f)LJ9* mini-X chromosome, while the *zeste* allele (z^{a}) reduces background eye color of the eye-specific g^{53d} allele (Lloyd *et al.* 1999b). The control crosses for paternal or maternal imprinting consisted of *Dp(1;f)LJ9,* $y^{+}g^{+}$*/X^Y* or *Dp(1;f)LJ9,* $y^{+}g^{+}$*/X^X* flies crossed to homozygous or hemizygous $y^{1}z^{a}g^{53d}$; *CyO*/+ or $y^{1}z^{a}g^{53d}$; *TM3, Sb*/+ flies, carrying the relevant balancer for the modifier being tested (*CyO* or *TM3, Sb*). All mutant alleles being tested were crossed into a y^{1} z^{a} g^{53d} background to yield stable stocks of $y^{1}z^{a}g^{53d}/y^{1}z^{a}g^{53d}$; *allele*/*CyO* for second chromosome mutations and $y^{1}z^{a}g^{53d}/y^{1}z^{a}g^{53d}$; *allele*/*TM3, Sb* for third chromosome mutations. Experimental crosses mirrored the control crosses, with the addition of mutant alleles. Two male progeny genotypes were collected from the experimental crosses; $y^{1}z^{a}g^{53d}$*/Dp(1;f)LJ9; allele/*+ and the internal control $y^{1}z^{a}g^{53d}$*/ Dp(1;f)LJ9;* CyO/+ or *TM3, Sb*/+ (Figure 5.1). The internal controls allow detection of any parental effects caused by the mutant allele being present in one of the parental flies; the progeny carrying the balancers are wild-type for the tested alleles, yet their mothers (mini-X^{PAT} cross) or fathers (mini-X^{MAT} cross) were mutant for the tested allele. No such effects were noted (Supplementary Tables 5.2 to 5.5). *Oregon-R* was used as wild-type.

5.3.3 Quantification Of Eye Pigment Levels

Drosophila eye pigment levels were quantified following the methods of Joanis and Lloyd (2002). Flies were randomly selected and each eye was given a variegation index value in relation to its degree of variegation (0=0-25% pigmentation, 1=25-50%, 2=50-75%, 3=75-100%). Results from each sample set were pooled and expressed as the mean ± the standard error of the mean. Statistical significance was calculated by a Kolmogorov-Smirnov two-sample test between the test group and the designated control group. A subset of these flies was then randomly selected for a spectrophotometric pigment assay adapted from Real *et al.* (1985). Flies were aged for 3-4 days then placed in 1.5ml microtubes and stored at -80 °C. Heads were removed by vigorous shaking and placed into 0.6ml microtubes containing 150ul of acidified ethanol (30% EtOH, acidified to pH 2 with HCL). Pigment was extracted from up to 20 samples of 5 heads each on an orbital shaker at 150 rpm in the dark for 48 ± 4 hours. Absorbance of the extracted pigments was measured at 480 nm with a Pharmacia Biotech Ultrospec 2000. Eye

pigment levels are expressed as a mean percentage of wild-type pigment levels (full *garnet* expression) ± standard deviation. Statistical significance was calculated by an ANOVA, followed by Bonferroni-corrected Student's t-tests to determine the p values for groups with significant difference.

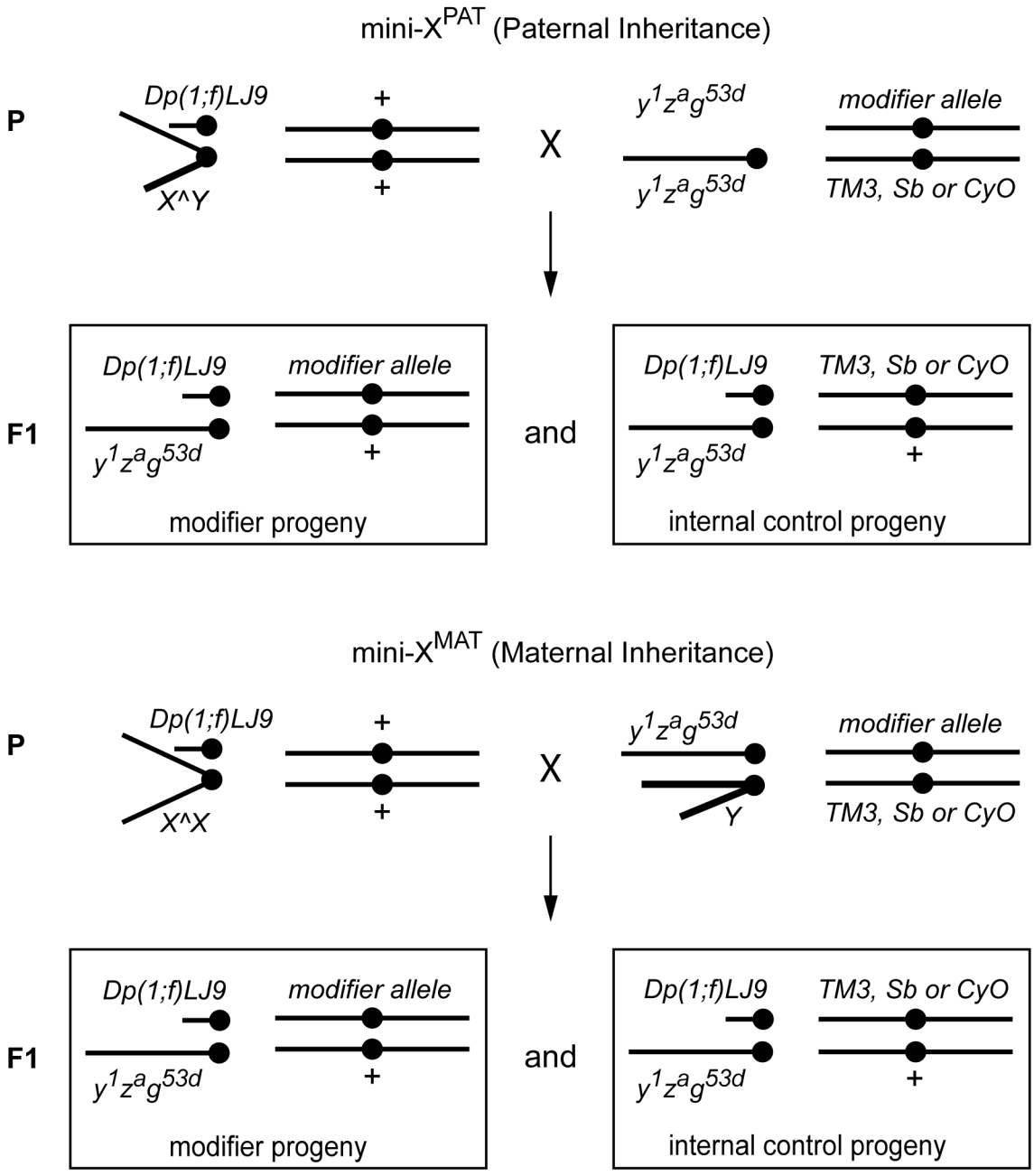

Figure 5.1 - Crosses For Maternally Or Paternally Inherited Mini-X *Dp(1;f)LJ9*. Two genotypes are created; test progeny with a modifier allele present, and internal control progeny containing a balancer. The internal control genotype is identical to the external control genotype (y^1 z^a g^{53d} */ Dp(1;f)LJ9;* CyO/+ or *TM3, Sb*/+); however, the modifier allele was present in one of the parental flies (mothers carry the mutation in the paternal cross; mini-X^{PAT}, fathers in the maternal cross; mini-X^{MAT}). Thus, assaying the internal control progeny will detect any pre-fertilization maternal effects for a mutation carried by mothers in the mini-X^{PAT} cross or sperm-associated effects from mutant fathers in the mini-X^{MAT} cross.

5.4 Results

5.4.1 H2Av Is Involved In Maintaining Expression Of *garnet* From The Mini-X Imprinted Domain

H2A replacement with H2Av is a proposed early step in the formation of heterochromatin (Swaminathan *et al.* 2005), so we tested the $His2Av^{810}$ null mutation for its effects on paternally-inherited (mini-X^{PAT}) and maternally-inherited (mini-X^{MAT}) *garnet* expression. Inheritance of mini-X^{PAT} along with $His2Av^{810}$ results in significant enhancement of *garnet* variegation (Table 5.1 and Figure 5.2A), resulting in pigment levels dropping to 16±4% wild-type from the control level of 37±7%. Inheritance of mini-X^{MAT} along with $His2Av^{810}$ also results in significant levels of *garnet* variegation observed in the eyes (Table 5.1 and Figure 5.2B), resulting in pigment levels dropping to 58±6% from the control level of 90±5%. Dominant enhancement of PEV in mini-X-bearing flies that are heterozygote for $His2Av^{810}$ suggests that H2Av is involved in the maintenance of paternal or maternal expression from the imprinted domain.

The dTip60 acetyltransferase complex is a chromatin-remodeler that utilizes the Domino ATPase to acetylate H2Av, promoting H2Av exchange (Kusch *et al.* 2004). To determine if Domino-assisted H2Av modification occurs at the mini-X imprinted domain, we tested two Domino hypomorphic alleles, dom^3 and dom^9. Both *dom* alleles significantly enhanced *garnet* variegation for mini-X^{PAT}, reducing pigment to 19±5% and 29±3% for dom^3 and dom^9 respectively (Table 5.1 and Figure 5.2A). However, unlike the variegation-inducing effect of $His2Av^{810}$ on mini-X^{PAT} *garnet* expression, neither dom^3 nor dom^9 had a significant affect on mini-X^{MAT} *garnet* expression, producing 89±6% and 92±6% pigment levels, respectively (Table 5.1 and Figure 5.2B). Thus, the Domino-assisted acetylation of H2Av is specific to only the paternally inherited mini-X imprinted domain.

To further assess the role of H2Av at the mini-X imprinted domain, we tested two mutant alleles of the *Drosophila* Remodeling and Spacing Factor (dRSF). dRSF is a chromatin remodeling factor that is also associated with dTip60 and involved in H2Av

replacement (Hanai *et al.* 2008). Both the *dRsf-1*^{KG00766} and *dRsf-1*^{KG02636} alleles had no affect on mini-X^{PAT} *garnet* expression, resulting in 34±8% and 40±5% pigment levels, respectively (Table 5.1 and Figure 5.2A). Similarly, mini-X^{MAT} flies heterozygous for *dRsf-1*^{KG00766} and *dRsf-1*^{KG02636} showed no significant response with pigment levels of 90±6% and 87±4% respectively (Table 5.1 and Figure 5.2B). The lack of dRSF involvement at the *Dp(1:f)LJ9* imprinted domain suggests the dTip60-Domino complex can modulate H2Av exchange independently of dRSF.

5.4.2 Mutant Alleles Of Core Histones *His3, His4,* And Replacement Histone *His4r* Relax *garnet* Silencing From Mini-X^{PAT}

Deficiencies for core histones are known to suppress PEV (Moore *et al.* 1983; Doheny *et al.* 2008), so we tested various mutant histone alleles for their effect on mini-X *garnet* expression. The histone H2A allele *His2A*^{KG00275} did not have any significant effect on either mini-X^{PAT} or mini-X^{MAT} *garnet* expression (Table 5.1 and Figure 5.3A, Figure 5.3B). However, we found that the H3 allele *His3*^{KG00688} and the H4 allele *His4*^{Scim} both significantly relaxed silencing of *garnet* from mini-X^{PAT}, increasing pigment levels to 45±6% and 61±5%, respectively (Table 5.1 and Figure 5.3A). Neither *His3*^{KG00688} nor *His4*^{Scim} had a significant affect on mini-X^{MAT} *garnet* expression (Table 5.1 and Figure 5.3B).

The histone replacement gene *His4r* encodes a histone H4 that is predominantly placed within a nucleosome in a replication-independent manner and is not located within the histone gene cluster (Akhmanova *et al.* 1996). The *His4r*^{EY06726} allele also caused a significant reduction in the level of variegated *garnet* silencing from mini-X^{PAT}, increasing pigment levels to 54±5% (Table 5.1 and Figure 5.3A), but did not have any significant affect on mini-X^{MAT} *garnet* expression (Table 5.1 and Figure 5.3B). Histone H3.3 is another histone variant that is deposited within a nucleosome in a replication-independent manner and is commonly associated with active genes (Ahmad and Henikoff 2002). The *His3.3A*^{KG02455} allele had no significant affect on *garnet* expression from either mini-X^{PAT} or mini-X^{MAT} (Table 5.1 and Figure 5.3A, Figure 5.3B), consistent with a previous report that *His3.3* mutations do not affect PEV (Doheny *et al.* 2008).

5.4.3 Disruption Of Histone Modifications Affects Imprinted *garnet* Gene Expression From Both Mini-X^{PAT} And Mini-X^{MAT}

Modification of histones is an important feature directing either active or repressive chromatin formation. Histone deacetylases (HDACs) are highly conserved subunits of multi-protein complexes that are responsible for removing acetyl groups from histones (Yang and Seto 2008). HDAC1 (Rpd3) is one of five identified HDACs in *Drosophila* that specifically targets histones and is involved in transcriptional regulation (Foglietti *et al.* 2006). Mutations in HDAC1 are generally regarded as suppressors of variegation (Mottus *et al.* 2000), due in part to the association of HDAC1 with the Su(var)3-9 histone methyl transferase, where the deacetylation of histone H3 is required prior to the methylation of lysine 9 on histone H3 (Czermin *et al.* 2001). However, opposite, variegation enhancing, effects been also reported for HDAC1 mutants (De Rubertis *et al.* 1996). These may be the result of allele-specific effects or the redundant activity of other deacetylases within the multi-protein deacetylating complexes (Mottus *et al.* 2000). $Rpd3^{04556}$ is a strong hypomorphic HDAC1 allele that has no dominant suppressing affect on $In(1)w^{m4}$ variegation, but does act to enhance suppression of variegation when combined with other HDAC1 mutant alleles (Chen *et al.* 1999; Mottus *et al.* 2000). $Rpd3^{04556}$ acted as a dominant enhancer of *garnet* variegation for both mini-X^{PAT} and mini-X^{MAT}, reducing pigment levels to 30±6% and 77±6% wild-type, respectively (Table 5.1 and Figures 5.4A, 5.4B).

Histone acetyltransferases (HATs) are enzymes that transfer acetyl groups to histones, and are involved in both transcriptional activation and repression (Carrozza *et al.* 2003). The HAT Chameau (Chm) is a member of the highly conserved MYST family of HATs that shows an affinity for H4, and is a dominant suppressor of *garnet* variegation that functions within centric heterochromatin (Grienenberger *et al.* 2002; Miotto *et al.* 2006). We tested the $chm^{BG02254}$ allele and found it to be a dominant suppressor of variegation for mini-X^{PAT}, increasing pigment levels to 49±5% (Table 5.1 and Figure 5.4A). No significant affect was observed from the $chm^{BG02254}$ allele on *garnet* expression from mini-X^{MAT} (Table 5.1 and Figure 5.4B).

Phosphorylation of histone H3 serine 10 by the kinase JIL-1 is a feature of active chromatin regions and has been shown to counteract heterochromatinization (Ebert *et al.* 2004; Bao *et al.* 2007). The gain-of-function *JIL-1*^{3} allele causes ectopic H3S10 phosphorylation, reinforcing euchromatic structure, and is one of the strongest characterized suppressors of PEV (Ebert *et al.* 2004). The *JIL-1*^{3} allele also acts as a dominant suppressor of mini-X^{PAT} *garnet* variegation, producing the strongest suppression of silencing amongst of all the mutations tested. Pigment levels increased to 70±4% (Figure 5.4A) and variegated silencing typical of paternal mini-X transmission was almost completely lost (Table 5.1). The *JIL-1*^{3} allele had the opposite effect on mini-X^{MAT}, producing mild variegated expression of *garnet* in some progeny and reducing pigment levels to 79±9% (Table 5.1 and Figure 5.4B).

Table 5.1 - The Effects Of Mutant Histone And Histone Modifying Alleles On The *Dp(1:f)LJ9* Mini-X Imprint.

Allele	*n*	Eye Variegation Index 0	1	2	3	Score
Paternal *Dp(1:f)LJ9* Inheritance (mini-X^{PAT})						
Control	300	17	57	138	88	1.99 ± 0.07
His2A^{KG00275} /+	280	19	36	118	107	2.12 ± 0.05
His2Av^{810} /+	300	98	86	79	37	1.18 ± 0.06***
His3^{KG00688} /+	300	17	53	103	127	2.14 ± 0.05**
His3.3A^{KG02455} /+	300	19	46	112	123	2.12 ± 0.05
His4^{Scim} /+	200	7	19	70	104	2.36 ± 0.06***
His4r^{EY06726} /+	200	7	23	74	96	2.30 ± 0.06***
chm^{BG02254} /+	300	17	53	103	127	2.14 ± 0.05**
dom^{3} /+	300	101	95	76	28	1.10 ± 0.06***
dom^{9} /+	280	55	77	96	52	1.52 ± 0.06***
JIL-1^{3} /+	250	0	0	11	239	2.96 ± 0.02***
Rpd3^{04556} /+	280	55	76	88	81	1.65 ± 0.06***
dRsf-1^{KG00766} /+	300	45	61	102	92	1.80 ± 0.06
dRsf-1^{KG02636} /+	300	29	64	108	99	1.92 ± 0.06
Maternal *Dp(1:f)LJ9* Inheritance (mini-X^{MAT})						
Control	300	0	0	0	300	3.00 ± 0.00
His2A^{KG00275} /+	300	0	0	0	300	3.00 ± 0.00
His2Av^{810} /+	300	0	9	25	266	2.86 ± 0.04*
His3^{KG00688} /+	300	0	0	0	300	3.00 ± 0.00
His3.3A^{KG02455} /+	300	0	0	0	300	3.00 ± 0.00
His4^{Scim} /+	300	0	0	0	300	3.00 ± 0.00
His4r^{EY06726} /+	200	0	0	0	300	3.00 ± 0.00
chm^{BG02254} /+	300	0	0	0	300	3.00 ± 0.00
dom^{3} /+	300	0	0	0	300	3.00 ± 0.00
dom^{9} /+	200	0	0	0	300	3.00 ± 0.00
JIL-1^{3} /+	300	0	0	3	297	2.99 ± 0.01
Rpd3^{04556} /+	200	0	0	8	192	2.96 ± 0.02
dRsf-1^{KG00766} /+	300	0	0	0	300	3.00 ± 0.00
dRsf-1^{KG02636} /+	250	0	0	0	300	3.00 ± 0.00

Eye variegation index values are based on a scale of visible red eye pigment levels; 0 (0-25%), 1 (25-50%,) 2 (50-75%), 3 (75-100%). The *n* value represents the number of individual eyes assayed, average score ± Standard Error of the Mean. Values significantly different from the control are marked with one asterisk ($p=<0.05$-0.01), two ($p=<0.01$-$p<0.001$) or three ($p<0.001$). Internal control values presented in Supplementary Tables 5.2 to 5.5.

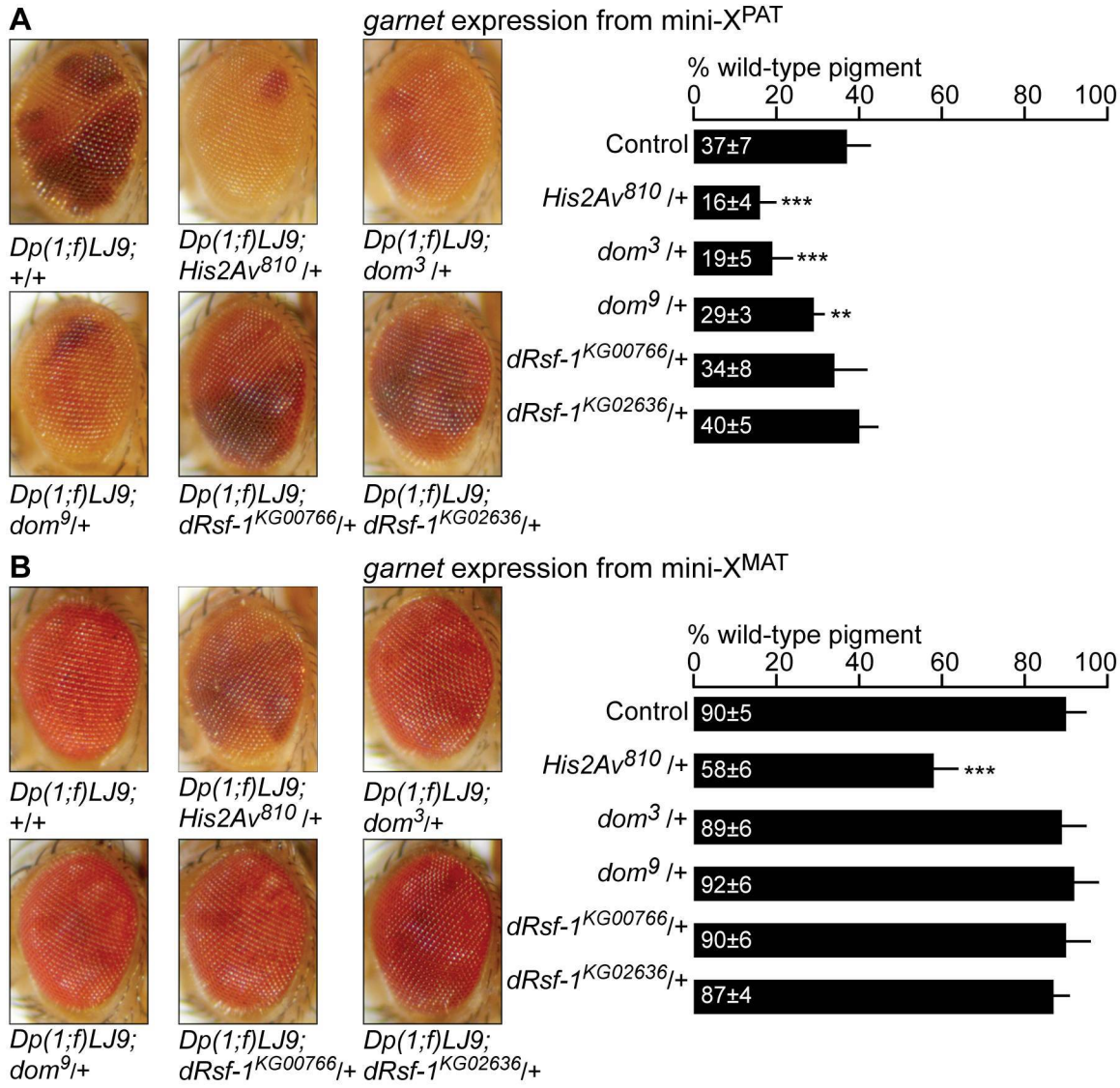

Figure 5.2 - The Effects Of *His2Av*^{810} And H2Av Modifiers On Mini-X^{PAT} And Mini-X^{MAT} *garnet* Expression.

A) *His2Av*^{810}, *dom*^{3} and *dom*^{9} dominantly enhance variegation of mini-X^{PAT} *garnet* compared to the control *Dp(1:f)LJ9*; +/+ genotype. Mutant alleles *dRsf-1*^{KG00766} and *dRsf-1*^{KG02636} did not significantly affect variegated mini-X^{PAT} *garnet* expression.

B) *His2Av*^{810} induces variegated silencing of *garnet* from mini-X^{MAT} whereas no significant affect on mini-X^{MAT} *garnet* expression was observed the *dom*^{3}, *dom*^{9}, *dRsf-1*^{KG00766} and *dRsf-1*^{KG02636} mutant alleles. Mean % wild-type pigment levels are shown with error bars representing standard deviation. Values significantly different from the control are marked with one asterisk ($p=<0.05$-0.01), two ($p=<0.01$- $p<0.001$) or three ($p<0.001$).

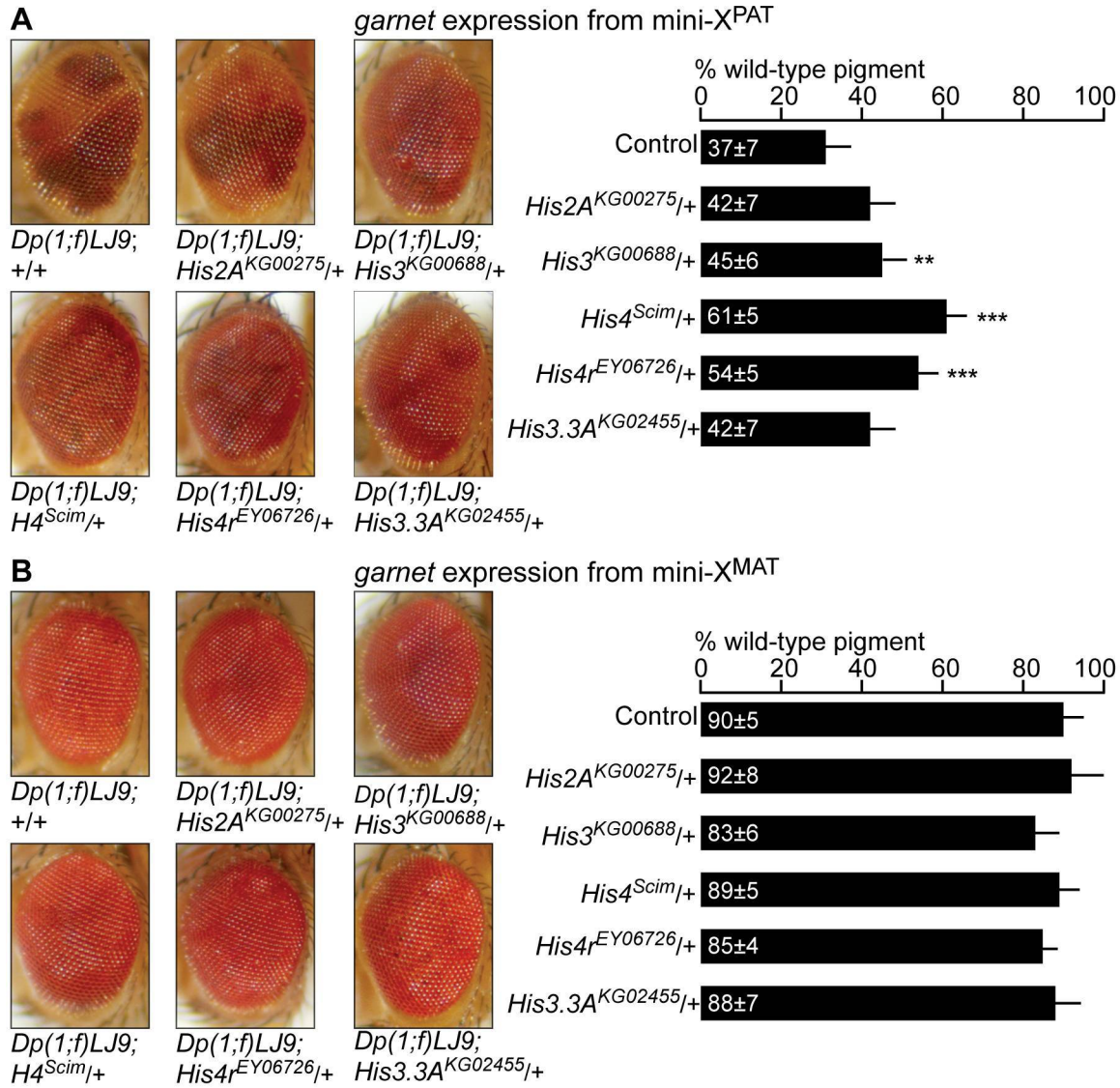

Figure 5.3 - The Effects Of Core Histone And Histone Variant Mutant Alleles On Mini-X^{PAT} And Mini-X^{MAT} *garnet* Expression.

A) Dominant suppression of variegation from mini-X^{PAT} was observed in *His3*^{KG00688}, *His4*^{Scim}, and *His4r*^{EY06726} heterozygote's, while the alleles *His2A*^{KG00275} and *His3.3A*^{KG02455} had no significant on mini-X^{PAT} *garnet* variegation.

B) No significant affect on mini-X^{MAT} *garnet* expression was observed for any of the histone or histone variant mutant alleles tested. Mean % wild-type pigment levels are shown with error bars representing standard deviation. Values significantly different from the control are marked with one asterisk (p=<0.05-0.01), two (p=<0.01- p<0.001) or three (p<0.001).

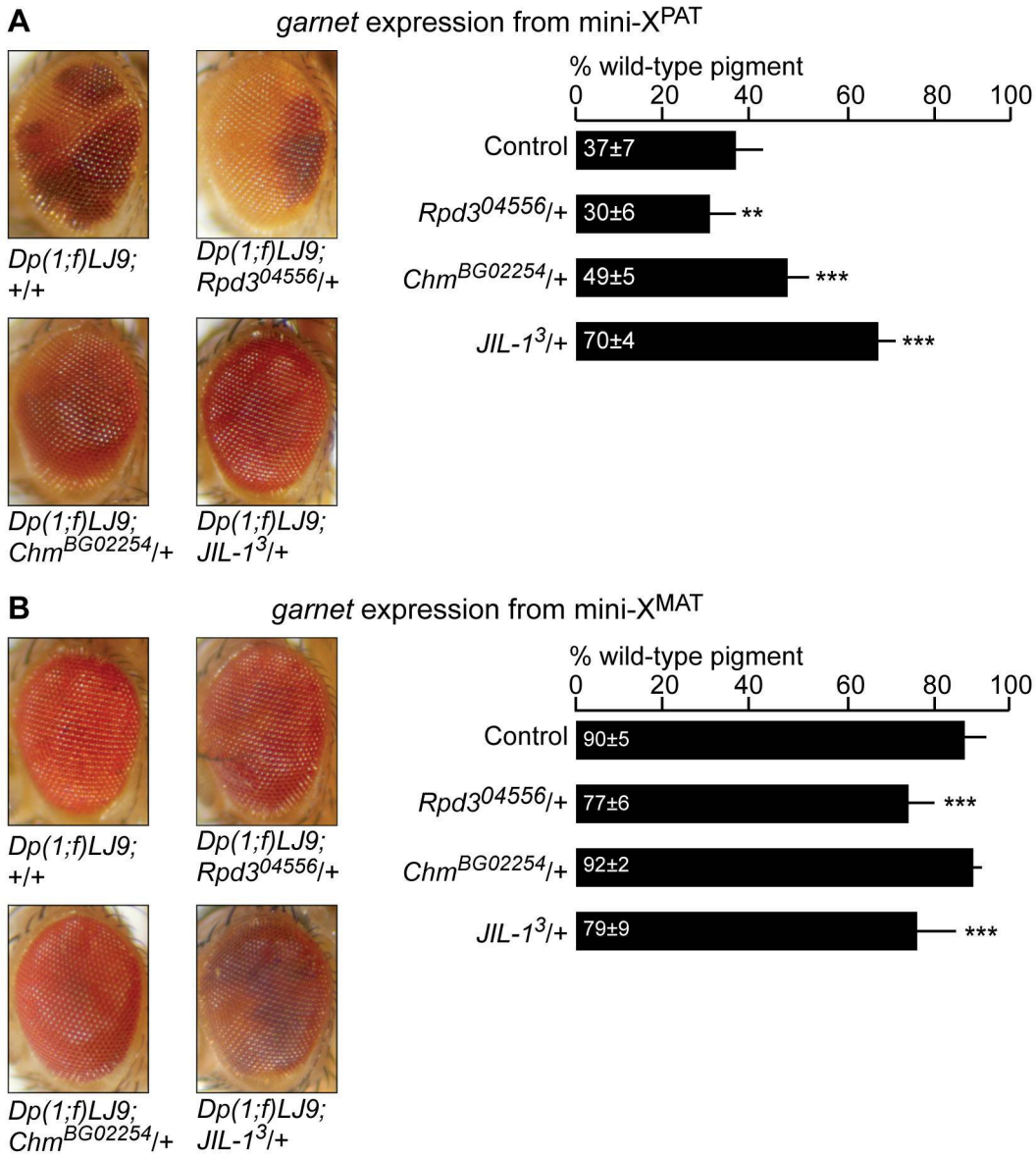

Figure 5.4 - The Effects Of Histone Modifier Mutant Alleles On Mini-X^{PAT} And Mini-X^{MAT} *garnet* Expression.

A) Dominant enhancement of mini-X^{PAT} *garnet* variegation is observed from the *Rpd3^{04556}* allele, while dominant suppression of variegation is observed from the *chm^{BG02254}* and *JIL-1^{3}* alleles.

B) mini-X^{MAT} *garnet* variegation is observed with both the *Rpd3^{04556}* and *JIL-1^{3}* alleles, while no significant affect is observed from the *chm^{BG02254}* allele. Mean % wild-type pigment levels are shown with error bars representing standard deviation. Values significantly different from the control are marked with one asterisk (p=<0.05-0.01), two (p=<0.01- p<0.001) or three (p<0.001).

5.5 Discussion

The imprinted domain exposed in the pericentric heterochromatin of the *Dp(1:f)LJ9* mini-X chromosome produces variegated silencing of the marker gene *garnet* when paternally inherited but initiates full *garnet* expression maternally inherited. Such parent-specific regulation requires differential construction of chromatin on maternally and paternally inherited chromosomes. Testing the effects mutant alleles of histones, histone variants and histone modification enzymes have on the imprinted mini-X chromosome provides the opportunity to assess which histone components maintain either repressive or active domains within imprint directed heterochromatin.

5.5.1 Mutant Alleles Of Core Histones And Histone Variants Disrupt Imprinted Silencing From Only The Paternally Imprinted Mini-X^{PAT}

Within a nucleosome, histone occupancy and histone modifications direct chromatin structure. The use of variegating constructs in *Drosophila* has shown that mutant alleles of histones and histone modifiers act as general suppressors of PEV (Moore *et al*. 1983; Ebert *et al*. 2006). Mutations in dominant suppressors of PEV, such as *Su(var)3-9* (histone H3K9 methylation) and *Su(var)2-5* (HP1), suppress variegation of g*arnet* from mini-X^{PAT}, thus disrupting silencing from the imprinted domain (Joanis and Lloyd 2002). However, these mutations do not impact the maintenance of *garnet* expression from mini-X^{MAT} (Joanis and Lloyd 2002). Similarly, we found that mutant alleles for core histones *His3*, *His4* and the histone variant *His4r* were all suppressors of variegation for mini-X^{PAT} while having no affect on the stable expression of *garnet* from mini-X^{MAT}. These genes acted exclusively zygotically on the somatic maintenance of the imprint, as no maternal effects were observed from mutant mothers.

In *Drosophila*, multiple copies of core histone genes are present in large cluster on the second chromosome (Moore *et al.* 1983), thus single histone gene mutations would not be expected to have a significant influence on chromatin formation. This could account for the lack of effect seen from $His2A^{KG00275}$. The $His3^{KG00688}$ allele remains uncharacterized, thus it is possible the moderate increase in eye pigmentation we observed could be due to other characteristics of this stock. The mutant alleles of $His4^{Scim}$

and *His4r* produced a pronounced relaxation mini-X^{PAT} *garnet* variegated silencing. The histone H4 allele $His4^{Scim}$ mutates a histone gene at the edge of the cluster that has been speculated to be a differentially expressed form of H4 (Dobie *et al.* 2001). Differential expression of $His4^{Scim}$ may account for the pronounced affect we observed from a mutation single mutation to a histone gene. While histone H4 methylation (H4K20) and acetylation (H4K12) are known to contribute to heterochromatin formation, little is known about any specific role histone H4 variants in the generation of silent heterochromatic domains. *His4r* is deposited within a nucleosome in a replication-independent manner (Akhmanova *et al.* 1996), suggesting it might be actively recruited to distinguish the mini-X^{PAT} imprinted domain from mini-X^{MAT}.

5.5.2 The Effects Of Histone Acetylation On The Mini-X Chromosome

The acetylation of histones H3 and H4 is generally associated with active euchromatic chromatin (Schübeler *et al.* 2004). A notable exception is H4K12 acetylation. While not ubiquitously required for heterochromatin formation (Ciurciu *et al.* 2008), H4K12 acetylation is associated with repressive pericentric heterochromatic regions (Turner *et al.* 1992). Treatment of *Drosophila* embryos with the histone deacetylase inhibitor sodium butyrate typically relaxes PEV, indicating a hypoacetylated histone state is generally associated with heterochromatic silencing (Mottus *et al.* 1980; Reuter *et al.* 1982). However, treatment of *Dp(1;f)LJ9* flies with sodium butyrate enhanced variegated silencing in mini-X^{PAT} flies and induced weak variegated silencing in mini-X^{MAT} flies (Lloyd *et al.* 1999a). Here we tested the histone deacetylase mutant allele $Rpd3^{04556}$ for its effect on mini-X expression and found a similar enhancement of *garnet* variegation from mini-X^{PAT}, and induction of weak variegated silencing of *garnet* from mini-X^{MAT}. Together, these results may indicate that the *Dp(1;f)LJ9* imprinted domain is highly sensitive to histone acetylation. The sensitivity of the imprint to acetylation is emphasized by the fact that the $Rpd3^{04556}$ allele has previously been shown not to affect PEV unless coupled with other HDAC mutants (Chen *et al.* 1999; Mottus *et al.* 2000). A role for Rpd3 demarcating telomeric and heterochromatic boundaries has been described in yeast where mutations in the Rpd3 gene cause an increase in H4K12 acetylation resulting in increased silencing spreading from the disrupted boundary

(Rundlett *et al.* 1996; Zhou *et al.* 2009). A similar involvement in Rpd3 boundary integrity has been proposed for *Drosophila* (De Rubertis *et al.* 1996). This role for Rpd3 would be consistent with induction of weak variegation of the mini-X^{MAT}, although no direct link has yet been demonstrated between Rpd3 and H4K12 deacetylation in *Drosophila*.

As differential H4 acetylation of imprinted alleles is a common feature of mammalian imprinted genes (Hu *et al.* 2000; Grandjean *et al.* 2001; Gregory *et al.* 2001), we tested the Chameau HAT, which has an affinity for histone H4 and is known to have an antagonistic relationship with Rpd3 (Miotto *et al.* 2006). Indeed, we found that the *chameau* allele dominantly relaxed silencing of *garnet* from mini-X^{PAT}. The opposite effects of *Rpd3* and *chm* mutant alleles, coupled with the strong suppression of mini-X^{PAT} silencing caused by *His4* and *His4r* mutations may point to histone H4 having a key role in heterochromatic spreading from the imprinted domain. Acetylation of H4K12 has been proposed to follow H2Av replacement as a step in the nucleation of heterochromatin, necessary for H3K9 methylation and subsequent recruitment of HP1 (Swaminathan *et al.* 2005). Our results demonstrate that H2Av recruitment is not necessarily required for histone H4 acetylation and heterochromatin formation at the *Dp(1;f)LJ9* imprinted domain; decreasing H2Av levels enhanced *garnet* silencing, while decreasing H4 acetyltransferase levels suppressed mini-X^{PAT} *garnet* silencing.

5.5.3 Histone H2Av Is Required For Both Maternal And Paternal Imprinting Of The Mini-X

The histone variant H2Av is widely distributed throughout the genome in both euchromatic and heterochromatic chromosomal regions and is essential for viability (van Daal and Elgin 1992; Leach *et al.* 2000). As distribution of H2Av is not specifically linked to the transcriptional state of a gene, it is likely that H2Av is not strictly utilized as a replacement histone during transcription, but instead H2Av incorporation is directed by pre-existing histone chromatin states (Leach *et al.* 2000). Additionally, loci-specific functions have been reported for H2Av; a variegating reporter construct inserted into the pericentric heterochromatin of the 2nd chromosome had enhanced PEV from *His2Av*^{810},

while insertions into the 4th chromosome either had suppressed variegation or no response to *His2Av*^{810} depending on the site of insertion (Haynes *et al.* 2007). Our results demonstrate that H2Av is also a critical component of an imprinted domain in *Drosophila*. We found that *His2Av*^{810} acts as a dominant enhancer of silencing on mini-X^{PAT}, dramatically reducing expression of the marker gene *garnet*. *His2Av*^{810} also disrupts the mini-X^{MAT} boundary, allowing the encroachment of heterochromatin and the initiation of *garnet* silencing. Together, these results demonstrate that H2Av is a critical component of both the maternally and paternally imprinted domains.

Consistent with a critical role for H2Av in gene regulation is the characterization of *H2Av* as a Polycomb group gene involved in maintaining homeotic gene silencing (Swaminathan *et al.* 2005). While H2Av has been characterized as a Polycomb group gene, it doesn't direct histone H3 lysine 27 methylation, a mark of homeotic gene silencing in euchromatin (Swaminathan *et al.* 2005). This suggests a differential role for H2Av in the silencing of euchromatic genes and heterochromatic regions (Swaminathan *et al.* 2005). Previously, mutations in Polycomb group genes were shown not to affect PEV from mini-X^{PAT} or disrupt the mini-X^{MAT} boundary (Joanis and Lloyd 2002), supporting evidence that constitutive heterochromatin and Polycomb-dependent chromatin are distinct (de Wit *et al.* 2007). The involvement of H2Av within diverse chromatin domains, as well as its ability to modulate differential chromatin conformations, distinguishes H2Av as a versatile member of the Polycomb group gene family. Furthermore, enhanced heterochromatic silencing resulting from the *His2Av*^{810} allele suggests that H2Av is not a ubiquitous component of heterochromatin formation, but is rather a dynamic component of domain-specific regulation.

5.5.4 Paternal-Specific Involvement Of Domino At The Mini-X Chromosome Imprinted Domain

The Domino ATPase is a necessary component of the dTip60 acetyltransferase chromatin-remodeler that acetylates phosphorylated H2Av before exchanging it for unmodified H2Av (Kusch *et al.* 2004). Phosphorylated H2Av is associated with double-strand breaks and DNA damage repair, as well as under-replicated heterochromatic

regions of *Drosophila* chromosomes (Kusch *et al.* 2004; Andreyeva *et al.* 2008). The yeast homologue of Domino, Swr1, is part of the SNF2/SWI2 family of ATPase chromatin remodelers involved in replacing the core histone H2A with H2A.Z (Htz1) (Kobor *et al.* 2004; Mizuguchi *et al.* 2004). Currently, it is unknown if in *Drosophila* Domino is also active in the process of replacing the core histone H2A with the histone variant H2Av in addition to its involvement in replacing phosphorylated H2Av with unmodified H2Av. Like H2Av, Domino has previously been associated with chromatin silencing, is linked to Polycomb group silencing, and *dom* mutant alleles act as suppressors of variegation (Ruhf *et al.* 2001). The mutant dom^{3} and dom^{9} alleles enhanced *garnet* variegation from mini-X^{PAT} to levels similar to those observed from the $His2Av^{810}$ allele. However, unlike the response to the $His2Av^{810}$ allele, mini-X^{MAT} was unaffected. These results show that H2Av is required for the maintenance of the boundary separating the maternally imprinted region from the *garnet* marker gene, but Domino is not. Thus acetylation of H2Av is a paternal-specific modification regulating expression from the imprinted domain. Domino could be specifically recruited to the mini-X^{PAT} domain to direct H2Av acetylation and facilitate H2Av exchange. This finding distinguishes H2Av as a target of distinct epigenetic modification in the maternal and paternal mini-X imprinted domain.

Domino involvement at transcriptionally active chromatin sites has previously been reported, where it shared characteristics similar to another ATPase chromatin remodeling factor, Brahma (Eissenberg *et al.* 2005). Brahma regulates homeotic gene expression and, unlike Domino, is commonly associated with transcriptional activation (Tamkun *et al.* 1992). Mutations in *brahma* had a similar effect on imprinted mini-X *garnet* expression as *dom* mutations (Joanis and Lloyd 2002); however, *brahma* mutations also had a maternal-effect on mini-X^{PAT}. None of the mutant alleles we tested produced a maternal-effect on mini-X^{PAT} *garnet* expression (Supplemental Tables 5.2 and 5.3), suggesting divergent roles for Brahma and Domino function, and that the recruitment of Domino to the paternally imprinted domain likely occurs after or during zygotic transcriptional activation of the *Drosophila* embryo.

To further investigate the role of H2Av modifiers, we tested mutations of the chromatin remodeling factor dRSF, which physically interacts with H2Av while associating with dTip60 and Domino (Hanai *et al.* 2008). Previously, mutations of *dRsf-1* have been found to be dominant suppressors of variegation, mechanistically linked to H2Av, as an enhanced phenotype is observed when coupled with $His2Av^{810}$ (Hanai *et al.* 2008). We found no evidence for involvement of dRSF at either the mini-X^{PAT} or mini-X^{MAT} imprinted domain. This result might be attributed to the available alleles; the $dRsf\text{-}1^{KG02636}$ allele still produces a transcript and is likely a weak allele (Hanai *et al.* 2008), while $dRsf\text{-}1^{KG00766}$ is uncharacterized. However, dRSF is not localized to centric heterochromatin, and is not always associated with Domino (Hanai *et al.* 2008), suggesting that these chromatin-remodeling components can function independently. The mini-X^{PAT} domain, which resides in centric heterochromatin, may recruit Domino-dependent chromatin-remodelers without the involvement of dRSF.

5.5.5 Ectopic JIL-1 Phosphorylation Of Histone H3K10 Is A Strong Suppressor Of Paternally Imprinted Mini-X Chromosome Expression

One of the primary histone modifications maintaining transcriptionally active euchromatin is the phosphorylation of histone H3K10 by the essential JIL-1 kinase. Phosphorylation of histone H3K10 by JIL-1 is a euchromatin-distinguishing modification that is both necessary and sufficient to antagonize or dissociate heterochromatic components and modifiers by antagonizing histone H3K9 methylation (Zhang *et al*. 2006; Deng *et al*. 2008). The gain-of-function $JIL\text{-}1^{3}$ allele retains functional kinase activity but has a corrupted localization domain, resulting in ectopic JIL-1 binding that counteracts heterochromatin formation (Ebert *et al.* 2004; Zhang *et al.* 2006). The $JIL\text{-}1^{3}$ allele is among the strongest characterized suppressors of variegation (Ebert *et al*. 2004). Consistent with this, we found the $JIL\text{-}1^{3}$ allele dramatically suppressed mini-X^{PAT} *garnet* variegation to the degree that the majority of eyes appeared wild-type. This nearly complete elimination of the mini-X^{PAT} imprint by the $JIL\text{-}1^{3}$ allele resembles the similar elimination of mini-X^{PAT} imprint silencing in *Su(var)3-9* and *Su(var)2-5* mutants, reinforcing the importance of histone H3K9 methylation and HP1 recruitment to the paternal imprinted domain.

In contrast to the effect on mini-X^{PAT}, inheritance of mini-X^{MAT} along with the *JIL-1*^{3} allele resulted in modest silencing observed in the form of variegated *garnet* expression and a reduction in eye pigment levels. This is notable in that this was the only mutation, out of all the genes tested, to produce an opposite effect on the mini-X^{PAT} and mini-X^{MAT} imprinted domain. This antipodal effect might be explained by the fact that the *JIL-1*^{3} allele is functional but mislocalized; if JIL-1 is directly recruited to the mini-X^{MAT} domain to assist in counteracting heterochromatinization, disruption of its localization domain would diminish its abundance in this domain and so weaken the boundary. Disruption of JIL-1 function can result in the redistribution of heterochromatic components from pericentric regions to other areas of the chromosome (Lerach 2006; Zhang *et al*. 2006), suggesting the regulation of chromatin domains within pericentric regions is particularly sensitive to JIL-1 function.

5.5.6 Distinct Histone Regulation Distinguishes Expression From The Paternally And Maternally Imprinted Domain

The mini-X^{MAT} and mini-X^{PAT} imprinted domains adopt very distinct chromatin conformations depending on inheritance. This causes silencing when the mini-X is paternally transmitted but allows full expression when it is maternally transmitted. In a recent *Drosophila* genome-wide profile of chromatin, nucleosomes replaced independent of replication were enriched with H2Av and located upstream of active promoters, suggesting H2Av incorporation could be a transitional stage of chromatin remodeling (Henikoff *et al*. 2009). Nucleosomal destabilization by H2Av deposition could conceivably prime a chromosomal region to respond to chromatin remodeling, which may explain our finding that H2Av is involved in the maintenance of both the paternally and maternally imprinted domains.

Silencing of *garnet* from the imprinted domain was disrupted by mutant alleles of both histones H3 and H4. The particular sensitivity of mini-X^{PAT} silencing to *His4*^{Scim} and *His4r* is significant, as both alleles are thought to be alternate forms of histone H4 (Akhmanova *et al.* 1996; Dobie *et al.* 2001). Loss of mini-X^{PAT} *garnet* silencing from a mutant *chameau* allele supports the requirement for proper H4 regulation to maintain

paternal-specific expression. Replacement of histone H4 on mini-X^{PAT} could assist in defining the paternally imprinted domain. It has been suggested that turnover of histone variants can assist in isolating chromatin domains by either removing or resisting histone modifications, and the resulting disruption of nucleosomes provides access for chromatin remodeling factors or transcription factors to dynamically direct expression (Dion *et al.* 2007; Mito *et al.* 2007). The generation of such a dynamic nucleosomal state could also account for how the chromatin remodeler Domino is able to access and modify H2Av from mini-X^{PAT}, yet is uninvolved with H2Av from mini-X^{MAT}.

The stable chromatin boundary maintained between the mini-X^{MAT} imprinted domain and the marker gene *garnet* is generated in part thought the recruitment of the CCCTC-binding factor (CTCF) (MacDonald *et al.* 2010). In mammals, H2A.Z has been linked to regions that bind CTCF, with nucleosomes flanking CTCF binding sites being enriched for H2A.Z, suggesting a role for H2Av in insulator function (Barski *et al.* 2007; Fu *et al.* 2008). Furthermore, the H2Av homologue in yeast displays a boundary function, protecting euchromatic regions from the encroachment of telomeric heterochromatin (Meneghini *et al.* 2003). Thus, the boundary function for H2Av reported here might be associated with the maternal-specific recruitment of CTCF to the imprinted domain. A higher threshold against heterochromatic silencing is likely an additional contributing factor to the stable expression of *garnet* from mini-X^{MAT}. Variegated silencing of the marker gene on mini-X^{MAT} can be induced, indicating that the boundary can be disrupted; however, the levels of variegation that result are low in comparison to mini-X^{PAT}, suggesting a higher threshold for heterochromatic silencing remains. Maintenance of an active transcriptional domain, featuring polymerase occupancy and the recruitment of JIL-1 as well as other euchromatic components, likely creates this higher threshold for silencing.

In PEV, context-specific differences such as chromosomal location, regional histone patterns, and the variegating gene's threshold for silencing, can determine effectiveness of heterochromatic silencing (Ahmad and Henikoff 2001; Haynes *et al.* 2007; Vogel *et al.* 2009). Our results dramatically demonstrate the dynamic range of

chromatin configurations that a specific domain can adopt; the imprinted domain of the *Dp(1:f)LJ9* mini-X chromosome produces distinct phenotypes as a result of the recruitment of distinct chromatin remodelers to the same domain in response to parent-specific epigenetic marks. When inherited from the father, heterochromatin originating in the imprinted domain spreads to the marker gene. In contrast, stable expression of *garnet* is maintained when the same domain, inherited from the mother, forms a boundary that is able to actively counteract heterochromatin formation and generates a higher threshold for gene silencing. The specific placement of histone variants and histone modifications thus distinguish the chromatin structure of the *Dp(1:f)LJ9* mini-X chromosome imprinted domain following maternal or paternal inheritance. These distinct structures demonstrate how the initial chromatin conformation of a specific region, shapes the response of that region to subsequent alterations in histones, histone variants and histone modifications.

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5.7 Supplemental

Table 5.2 - Internal Controls For Mini-X^{PAT}; 2^{nd} Chromosome Mutations Balanced Over *CyO*

	Eye Variegation Index						Pigment Assay	
Maternally present allele // balancer	***n***	**0**	**1**	**2**	**3**	**Score**	***n***	**% wild-type**
No modifier // CyO	300	22	55	132	91	1.97 ± 0.88	20	36±6
$His2A^{KG00275}$ // CyO	280	28	43	113	96	1.99 ± 0.95	20	42±7
$His3^{KG00688}$ // CyO	300	34	47	123	96	1.94 ± 0.96	20	39±6
$His3.3A^{KG02455}$ // CyO	300	27	26	136	95	1.99 ± 0.91	20	39±7
$His4^{Scim}$ // CyO	190	19	37	78	56	1.90 ± 0.94	20	41±6
$chm^{BG02254\ PAT}$ // CyO	300	27	61	119	93	1.93 ± 0.93	20	39±6
dom^{3} // CyO	300	33	63	123	81	1.84 ± 0.95	20	31±5
dom^{9} // CyO	280	28	46	110	96	1.98 ± 0.95	20	40±4
$dRsf\text{-}1^{KG00766}$ // CyO	300	26	59	112	103	1.97 ± 0.94	20	39±9
$dRsf\text{-}1^{KG02636}$ // CyO	300	30	64	112	94	1.90 ± 0.96	10	40±6

Eye variegation index values are based on a scale of visible red eye pigment levels; 0 (0-25%), 1 (25-50%,) 2 (50-75%), 3 (75-100%). The *n* value represents the number of individual eyes assayed, average score ± Standard Error of the Mean. Pigment assay values are presented as a mean percentage of wild-type levels ± standard deviation; the *n* value represents the number of independent measurements. An ANOVA followed by Bonferroni-corrected Student's t-tests determined no significant difference is present between the internal control progeny and the no modifier control.

Table 5.3 - Internal Controls For Mini-X^{PAT}; 3rd Chromosome Mutations Balanced Over *TM3, Sb*

	Eye Variegation Index						Pigment Assay	
Maternally present allele // balancer	***n***	**0**	**1**	**2**	**3**	**Score**	***n***	**% wild-type**
No modifier // TM3, Sb	300	34	54	128	84	1.87 ± 0.94	19	22±5
$His2Av^{810}$ // TM3, Sb	300	29	64	110	97	1.92 ± 0.96	20	19±5
$His4r^{EY06726}$ // TM3, Sb	200	15	34	97	54	1.95 ± 0.86	20	26±5
$JIL\text{-}1^{3}$ // TM3, Sb	250	21	55	93	81	1.94 ± 0.94	20	25±5
$Rpd3^{04556}$ // TM3, Sb	300	27	47	117	109	2.03 ± 0.94	20	27±6

Eye variegation index values are based on a scale of visible red eye pigment levels; 0 (0-25%), 1 (25-50%,) 2 (50-75%), 3 (75-100%). The *n* value represents the number of individual eyes assayed, average score ± Standard Error of the Mean. Pigment assay values are presented as a mean percentage of wild-type levels ± standard deviation; the *n* value represents the number of independent measurements. Males carrying the *TM3, Sb* balancer are physically smaller, which results in a lower pigment assay score despite no significant change in the variegation phenotype. There is no significant difference between *Dp(1;f)*LJ9; TM3*, Sb* balanced internal controls and the 'no modifier' *Dp(1:f)LJ9; TM3, Sb* controls. An ANOVA followed by Bonferroni-corrected Student's t-tests determined no significant difference is present between the internal control progeny and the no modifier control.

Table 5.4 - Internal Controls For Mini-X^{MAT}; 2nd Chromosome Mutations Balanced Over *CyO*

	Eye Variegation Index						Pigment Assay	
Paternally present allele // balancer	***n***	**0**	**1**	**2**	**3**	**Score**	***n***	**% wild-type**
No modifier // CyO	300	0	0	0	300	300± 0.0	20	89±6
His2A^{KG00275} // CyO	300	0	0	0	300	300± 0.0	20	91±5
His3^{KG00688} // CyO	300	0	0	0	300	300± 0.0	20	83±6
His3.3A^{KG02455} // CyO	300	0	0	0	300	300± 0.0	20	87±8
His4^{Scim} // CyO	300	0	0	0	300	300± 0.0	20	90±6
chm^{BG02254} // CyO	300	0	0	0	300	300± 0.0	20	92±4
dom^{3} // CyO	300	0	0	0	300	300± 0.0	20	90±6
dom^{9} // CyO	300	0	0	0	300	300± 0.0	20	92±3
dRsf-1^{KG00766} // CyO	300	0	0	0	300	300± 0.0	20	91±5
dRsf-1^{KG02636} // CyO	150	0	0	0	150	300± 0.0	10	87±4

Eye variegation index values are based on a scale of visible red eye pigment levels; 0 (0-25%), 1 (25-50%,) 2 (50-75%), 3 (75-100%). The *n* value represents the number of individual eyes assayed, average score ± Standard Error of the Mean. Pigment assay values are presented as a mean percentage of wild-type levels ± standard deviation; the *n* value represents the number of independent measurements. An ANOVA followed by Bonferroni-corrected Student's t-tests determined no significant difference is present between the internal control progeny and the no modifier control.

Table 5.5 - Internal Controls For Mini-X^{MAT}; 3rd Chromosome Mutations Balanced Over *TM3, Sb*

	Eye Variegation Index						Pigment Assay	
Paternally present allele // balancer	***n***	**0**	**1**	**2**	**3**	**Score**	***n***	**% wild-type**
No modifier // TM3, Sb	300	0	0	0	300	300± 0.0	19	55±4
His2Av^{810}// TM3, Sb	300	0	0	0	300	300± 0.0	20	52±6
His4r^{EY06726} // TM3, Sb	300	0	0	0	300	300± 0.0	20	53±3
JIL-1^{3} // TM3, Sb	300	0	0	0	300	300± 0.0	20	57±4
Rpd3^{04556} // TM3, Sb	300	0	0	0	300	300± 0.0	20	58±4

Eye variegation index values are based on a scale of visible red eye pigment levels; 0 (0-25%), 1 (25-50%,) 2 (50-75%), 3 (75-100%). The *n* value represents the number of individual eyes assayed, average score ± Standard Error of the Mean. Pigment assay values are presented as a mean percentage of wild-type levels ± standard deviation; the *n* value represents the number of independent measurements. Males carrying the *TM3, Sb* balancer are physically smaller, which results in a lower pigment assay score despite no significant change in the variegation phenotype. There is no significant difference between *Dp(1;f)*LJ9; TM3*, Sb* balanced internal controls and the 'no modifier' *Dp(1:f)LJ9; TM3, Sb* controls. An ANOVA followed by Bonferroni-corrected Student's t-tests determined no significant difference is present between the internal control progeny and the no modifier control.

5.8 References

Ahmad K, Henikoff S. (2001) Modulation of a transcription factor counteracts heterochromatic gene silencing in *Drosophila*. Cell. **104**:839-847.

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

Akhmanova A, Miedema K, Hennig W. (1996) Identification and characterization of the *Drosophila* histone H4 replacement gene. FEBS Lett. **388**:219-222.

Andreyeva EN, Kolesnikova TD, Belyaeva ES, Glaser RL, Zhimulev IF. (2008) Local DNA underreplication correlates with accumulation of phosphorylated H2Av in the *Drosophila melanogaster* polytene chromosomes. Chromosome Res. **16**:851-862.

Baker WK. (1967) A clonal system of differential gene activity in *Drosophila*. Dev Biol. **16**:1-17.

Bao X, Deng H, Johansen J, Girton J, Johansen KM. (2007) Loss-of-function alleles of the JIL-1 histone H3S10 kinase enhance position-effect variegation at pericentric sites in *Drosophila* heterochromatin. Genetics. **176**:1355-1358.

Barski A, Cuddapah S, Cui K, Roh T-Y, Schones DE, Wang Z, Wei G, Chepelev I, Zhao K. (2007) High-resolution profiling of histone methylations in the human genome. Cell. **129**:823-837.

Carrozza MJ, Utley RT, Workman JL, Côté J. (2003) The diverse functions of histone acetyltransferase complexes. Trends Genet. **19**:321-329.

Chen G, Fernandez J, Mische S, Courey AJ. (1999) A functional interaction between the histone deacetylase Rpd3 and the corepressor Groucho in *Drosophila* development. Genes Dev. **13**:2218-2230.

Ciurciu A, Komonyi O, Boros IM. (2008) Loss of ATAC-specific acetylation of histone H4 at Lys12 reduces binding of JIL-1 to chromatin and phosphorylation of histone H3 at Ser10. J Cell Sci. **121**:3366-3372.

Czermin B, Schotta G, Hülsmann BB, Brehm A, Becker PB, Reuter G, Imhof A. (2001) Physical and functional association of SU(VAR)3-9 and HDAC1 in *Drosophila*. EMBO Rep. **2**:915-919.

De Rubertis F, Kadosh D, Henchoz S, Pauli D, Reuter G, Struhl K, Spierer P. (1996) The histone deacetylase RPD3 counteracts genomic silencing in *Drosophila* and yeast. Nature. **384**:589-591.

de Wit E, Greil F, van Steensel B. (2007) High-resolution mapping reveals links of HP1 with active and inactive chromatin components. PLoS Genet. **3**:e38.

Deng H, Bao X, Cai W, Blacketer MJ, Belmont AS, Girton J, Johansen J, Johansen KM. (2008) Ectopic histone H3S10 phosphorylation causes chromatin structure remodeling in *Drosophila*. Development. **135**:699-705.

Deng H, Bao X, Zhang W, Girton J, Johansen J, Johansen KM. (2007) Reduced levels of Su(var)3-9 but not Su(var)2-5 (HP1) counteract the effects on chromatin structure and viability in loss-of-function mutants of the JIL-1 histone H3S10 kinase. Genetics. **177**:79-87.

Dion MF, Kaplan T, Kim M, Buratowski S, Friedman N, Rando OJ. (2007) Dynamics of replication-independent histone turnover in budding yeast. Science. **315**:1405-1408.

Dobie KW, Kennedy CD, Velasco VM, McGrath TL, Weko J, Patterson RW, Karpen GH. (2001) Identification of chromosome inheritance modifiers in *Drosophila melanogaster*. Genetics. **157**:1623-1637.

Doheny JG, Mottus R, Grigliatti TA, Bielinsky A-K. (2008) Telomeric Position Effect—A Third Silencing Mechanism in Eukaryotes. PLoS ONE. **3**:e3864.

Ebert A, Lein S, Schotta G, Reuter G. (2006) Histone modification and the control of heterochromatic gene silencing in *Drosophila*. Chromosome Res. **14**:377-392.

Ebert A, Schotta G, Lein S, Kubicek S, Krauss V, Jenuwein T, Reuter G. (2004) *Su(var)* genes regulate the balance between euchromatin and heterochromatin in *Drosophila*. Genes Dev. **18**:2973-2983.

Eissenberg J, Elgin S. (2000) The HP1 protein family: getting a grip on chromatin. Curr Opin Genet Dev. **10**:204-210.

Eissenberg J, Wong M, Chrivia J. (2005) Human *SRCAP* and *Drosophila melanogaster DOM* are homologs that function in the notch signaling pathway. Mol Cell Biol. **25**:6559-6569.

Foglietti C, Filocamo G, Cundari E, De Rinaldis E, Lahm A, Cortese R, Steinkühler C. (2006) Dissecting the biological functions of *Drosophila* histone deacetylases by RNA interference and transcriptional profiling. J Biol Chem. **281**:17968-17976.

Fu Y, Sinha M, Peterson CL, Weng Z. (2008) The insulator binding protein CTCF positions 20 nucleosomes around its binding sites across the human genome. PLoS Genet. **4**:e1000138.

Gowen J, Gay E. (1934) Chromosome constitution and behavior in eversporting and mottling in *Drosophila melanogaster*. Genetics. **19**:189-208.

Grandjean V, O'Neill L, Sado T, Turner B, Ferguson-Smith A. (2001) Relationship between DNA methylation, histone H4 acetylation and gene expression in the mouse imprinted Igf2-H19 domain. FEBS Letters. **488**:165-169.

Gregory RI, Randall TE, Johnson CA, Khosla S, Hatada I, O'Neill LP, Turner BM, Feil R. (2001) DNA methylation is linked to deacetylation of histone H3, but not H4, on the imprinted genes *Snrpn* and *U2af1-rs1*. Mol Cell Biol. **21**:5426-5436.

Grienenberger A, Miotto B, Sagnier T, Cavalli G, Schramke V, Geli V, Mariol MC, Berenger H, Graba Y, Pradel J. (2002) The MYST domain acetyltransferase Chameau functions in epigenetic mechanisms of transcriptional repression. Curr Biol. **12**:762-766.

Haller BS, Woodruff RC. (2000) Varied expression of a Y-linked *P[w+]* insert due to imprinting in *Drosophila melanogaster*. Genome. **43**:285-292.

Hanai K, Furuhashi H, Yamamoto T, Akasaka K, Hirose S. (2008) RSF governs silent chromatin formation via histone H2Av replacement. PLoS Genet. **4**:e1000011.

Hardy R, Lindsley D, Livak K, Lewis B, Siversten A, Joslyn G, Edwards J, Bonaccorsi S. (1984) Cytogenetic analysis of a segment of the Y chromosome of *Drosophila melanogaster*. Genetics. **107**:591-610.

Haynes KA, Gracheva E, Elgin SCR. (2007) A Distinct type of heterochromatin within *Drosophila melanogaster* chromosome 4. Genetics. **175**:1539-1542.

Henikoff S, Henikoff JG, Sakai A, Loeb GB, Ahmad K. (2009) Genome-wide profiling of salt fractions maps physical properties of chromatin. Genome Res. **19**:460-469.

Hu JF, Pham J, Dey I, Li T, Vu TH, Hoffman AR. (2000) Allele-specific histone acetylation accompanies genomic imprinting of the insulin-like growth factor II receptor gene. Endocrinology. **141**:4428-4435.

Jaquet Y, Delattre M, Montoya-Burgos J, Spierer A, Spierer P. (2006) Conserved domains control heterochromatin localization and silencing properties of SU(VAR)3-7. Chromosoma. **115**:139-150.

Joanis V, Lloyd V. (2002) Genomic imprinting in *Drosophila* is maintained by the products of *Suppressor of variegation* and *trithorax* group, but not *Polycomb* group, genes. Mol Genet Genomics. **268**:103-112.

Kobor M, Venkatasubrahmanyam S, Meneghini M, Gin J, Jennings J, Link A, Madhani H, Rine J. (2004) A protein complex containing the conserved Swi2/Snf2-related ATPase Swr1p deposits histone variant H2A.Z into euchromatin. PLoS Biol. **2**:E131.

Kusch T, Florens L, Macdonald WH, Swanson SK, Glaser RL, Yates JR, Abmayr SM, Washburn MP, Workman JL. (2004) Acetylation by Tip60 is required for selective histone variant exchange at DNA lesions. Science. **306**:2084-2087.

Latham K. (2005) X chromosome imprinting and inactivation in preimplantation mammalian embryos. Trends Genet. **21**:120-127.

Leach TJ, Mazzeo M, Chotkowski HL, Madigan JP, Wotring MG, Glaser RL. (2000) Histone H2A.Z is widely but nonrandomly distributed in chromosomes of *Drosophila melanogaster*. J Biol Chem. **275**:23267-23272.

Lerach S. (2006) Loss-Of-Function Alleles of the JIL-1 Kinase Are Strong Suppressors of Position Effect Variegation of the *wm4* Allele in *Drosophila*. Genetics. **173**:2403-2406.

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

Lloyd V, Sinclair D, Grigliatti T. (1999a) Genomic imprinting and position-effect variegation in *Drosophila melanogaster*. Genetics. **151**:1503-1516.

Lloyd V, Sinclair D, Wennberg R, Warner T, Honda B, Grigliatti T. (1999b) A genetic and molecular characterization of the *garnet* gene of *Drosophila melanogaster*. Genome. **42**:1183-1193.

MacDonald WA, Menon D, Bartlett NJ, Sperry GE, Rasheva V, Meller V, Lloyd VK. (2010) The *Drosophila* homolog of the mammalian imprint regulator, CTCF, maintains the maternal genomic imprint in *Drosophila melanogaster*. BMC Biology. **8**:105.

Madigan JP, Chotkowski HL, Glaser RL. (2002) DNA double-strand break-induced phosphorylation of *Drosophila* histone variant H2Av helps prevent radiation-induced apoptosis. Nucleic Acids Res. **30**:3698-3705.

Maggert K, Golic K. (2002) The Y chromosome of *Drosophila melanogaster* exhibits chromosome-wide imprinting. Genetics. **162**:1245-1258.

Meneghini MD, Wu M, Madhani HD. (2003) Conserved histone variant H2A.Z protects euchromatin from the ectopic spread of silent heterochromatin. Cell. **112**:725-736.

Menon DU, Meller VH. (2009) Imprinting of the Y chromosome influences dosage compensation in *roX1 roX2 Drosophila melanogaster*. Genetics. **183**:811-820.

Miotto B, Sagnier T, Berenger H, Bohmann D, Pradel J, Graba Y. (2006) Chameau HAT and DRpd3 HDAC function as antagonistic cofactors of JNK/AP-1-dependent transcription during *Drosophila* metamorphosis. Genes Dev. **20**:101-112.

Mito Y, Henikoff JG, Henikoff S. (2007) Histone replacement marks the boundaries of *cis*-regulatory domains. Science. **315**:1408-1411.

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

Moore G, Sinclair D, Grigliatti T. (1983) Histone Gene Multiplicity and Position Effect Variegation in *Drosophila Melanogaster*. Genetics. **105**:327-344.

Mottus R, Reeves R, Grigliatti TA. (1980) Butyrate suppression of position-effect variegation in *Drosophila melanogaster*. Mol Gen Genet. **178**:465-469.

Mottus R, Sobel RE, Grigliatti TA. (2000) Mutational analysis of a histone deacetylase in *Drosophila melanogaster*: missense mutations suppress gene silencing associated with position effect variegation. Genetics. **154**:657-668.

Muller H. (1930) Types of visible variations induced by X-rays in *Drosophila*. J Genet. **22**:299-334.

Pardo-Manuel de Villena F, de la Casa-Esperón E, Sapienza C. (2000) Natural selection and the function of genome imprinting: beyond the silenced minority. Trends Genet. **16**:573-579.

Real, MD, Ferre, J, Mensua, JL. (1985) Methods for the quantitative estimation of the red and brown pigments of *D. melanogaster.* Dros Inf Serv. **61**:198-200.

Regha K, Sloane MA, Huang R, Pauler FM, Warczok KE, Melikant B, Radolf M, Martens JHA, Schotta G, Jenuwein T and others. (2007) Active and repressive chromatin are interspersed without spreading in an imprinted gene cluster in the mammalian genome. Mol Cell. **27**:353-366.

Reuter G, Dorn R, Hoffmann H. (1982) Butyrate sensitive suppressor of position-effect variegation mutations in *Drosophila melanogaster*. Mol Genet Genomics. **188**:480-485.

Ruhf M, Braun A, Papoulas O, Tamkun J, Randsholt N, Meister M. (2001) The *domino* gene of *Drosophila* encodes novel members of the SWI2/SNF2 family of DNA-dependent ATPases, which contribute to the silencing of homeotic genes. Development. **128**:1429-1441.

Rundlett SE, Carmen AA, Kobayashi R, Bavykin S, Turner BM, Grunstein M. (1996) HDA1 and RPD3 are members of distinct yeast histone deacetylase complexes that regulate silencing and transcription. PNAS. **93**:14503-14508.

Schotta G, Ebert A, Krauss V, Fischer A, Hoffmann J, Rea S, Jenuwein T, Dorn R, 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.

Schotta G, Lachner M, Sarma K, Ebert A, Sengupta R, Reuter G, Reinberg D, Jenuwein T. (2004) A silencing pathway to induce H3-K9 and H4-K20 trimethylation at constitutive heterochromatin. Genes Dev. **18**:1251-1262.

Schübeler D, Macalpine DM, Scalzo D, Wirbelauer C, Kooperberg C, Van Leeuwen F, Gottschling DE, O'Neill LP, Turner BM, Delrow J and others. (2004) The histone modification pattern of active genes revealed through genome-wide chromatin analysis of a higher eukaryote. Genes Dev. **18**:1263-1271.

Schultz J. (1936) Variegation in *Drosophila* and the Inert Chromosome Regions. PNAS. **22**:27-33.

Shirohzu H, Yokomine T, Sato C, Kato R, Toyoda A, Purbowasito W, Suda C, Mukai T, Hattori M, Okumura K and others. (2004) A 210-kb segment of tandem repeats and retroelements located between imprinted subdomains of mouse distal chromosome 7. DNA Res. **11**:325-334.

Spofford J. (1967) Single-locus modification of position-effect variegation in *Drosophila melanogaster*. I. *white* variegation. Genetics. **57**:751-766.

Swaminathan J, Baxter E, Corces V. (2005) The role of histone H2Av variant replacement and histone H4 acetylation in the establishment of *Drosophila* heterochromatin. Genes Dev. **19**:65-76.

Tamkun JW, Deuring R, Scott MP, Kissinger M, Pattatucci AM, Kaufman TC, Kennison JA. (1992) brahma: a regulator of *Drosophila* homeotic genes structurally related to the yeast transcriptional activator *SNF2/SWI2*. Cell. **68**:561-572.

Tanabe M, Kouzmenko A, Ito S, Sawatsubashi S, Suzuki E, Fujiyama S, Yamagata K, Zhao Y, Kimura S, Ueda T and others. (2008) Activation of facultatively silenced *Drosophila* loci associates with increased acetylation of histone H2AvD. Genes Cells. **13**:1279-1288.

Turner BM, Birley AJ, Lavender J. (1992) Histone H4 isoforms acetylated at specific lysine residues define individual chromosomes and chromatin domains in *Drosophila* polytene nuclei. Cell. **69**:375-384.

van Daal A, Elgin SC. (1992) A histone variant, H2AvD, is essential in *Drosophila melanogaster*. Mol Biol Cell. **3**:593-602.

Vogel MJ, Pagie L, Talhout W, Nieuwland M, Kerkhoven RM, van Steensel B. (2009) High-resolution mapping of heterochromatin redistribution in a *Drosophila* position-effect variegation model. Epigenet. Chrom. **2**:1-18.

Wen B, Wu H, Bjornsson H, Green RD, Irizarry R, Feinberg AP. (2008) Overlapping euchromatin/heterochromatin- associated marks are enriched in imprinted gene regions and predict allele-specific modification. Genome Res. **18**:1806-1813.

Yang X-J, Seto E. (2008) The Rpd3/Hda1 family of lysine deacetylases: from bacteria and yeast to mice and men. Nat Rev Mol Cell Biol. **9**:206-218.

Zhang W, Deng H, Bao X, Lerach S, Girton J, Johansen J, Johansen KM. (2006) The JIL-1 histone H3S10 kinase regulates dimethyl H3K9 modifications and heterochromatic spreading in *Drosophila*. Development. **133**:229-235.

Zhou J, Zhou B, Lenzmeier B, Zhou J. (2009) Histone deacetylase *Rpd3* antagonizes Sir2-dependent silent chromatin propagation. Nucleic Acids Res. **37**:3699-3713.

5.9 Transition To Chapter 6

The following chapter examines the role of the RNA interference pathways in the regulation of the *Dp(1;f)LJ9* imprint. The findings presented in this chapter show distinct functions for the siRNA and piRNA silencing pathways in regulating the *Dp(1;f)LJ9* imprint. Mutations in siRNA pathway components disrupt paternal silencing and mutations affecting piRNA pathway components enhance both maternal and paternal silencing. The involvement of the siRNA pathway in paternal silencing correlates with the presence of antisense *garnet* transcripts. Additionally, the germline-specific piRNA pathway protein, Aubergine, was found to be involved in the establishment of the maternal imprint. This is the first report of a role for non-coding RNA, a feature of many mammalian imprinted genes, in *Drosophila* imprinting, and the first report of the RNAi pathway being involved in the establishment of an imprint in any organism. Because of the implications of these findings that RNAi is involved in both the establishment and maintenance of the imprint, additional RT-PCR experiments are required to better characterize the extent of the antisense RNA transcripts generated from the *Dp(1;f)LJ9* imprinted domain and further testing of RNAi pathway component alleles will also be necessary.

Chapter 6 The Role Of RNAi In Regulating Genomic Imprinting In *Drosophila melanogaster*: siRNA And piRNA Pathways Have Distinct Functions

6.1 Abstract

Background: Genomic imprinting involves multiple epigenetic mechanisms working in concert to establish and maintain parent-specific expression of a gene or gene cluster. DNA methylation, histone modification, and heterochromatin formation all act to maintain parent-specific transcriptional silencing; however, non-coding RNAs, which can engage RNA interference (RNAi) silencing pathways are being recognized as common features of imprinted domains. I utilized the *Drosophila* imprinted *Dp(1;f)LJ9* mini-X chromosome model to determine if RNAi is involved regulating the imprint.

Results: The findings presented in this paper demonstrate the involvement of the Dicer 2-dependent endogenous siRNA pathway in the silencing associated with paternal *Dp(1;f)LJ9* inheritance. Non-coding antisense RNA transcripts were detected preferentially from paternally inherited *Dp(1;f)LJ9*, providing a source for the production of siRNAs. Mutations in piRNA pathway components demonstrated that Piwi acts independently of the characterized piRNA pathway, contributing to *Dp(1;f)LJ9* paternal-specific silencing. Mutations in *aubergine*, *armitage*, *spindle-E*, *squash* and *zucchini*, enhanced silencing from both the maternally and paternally inherited *Dp(1;f)LJ9*. Aubergine, but not Piwi, was also found to function in the germline establishment of the maternal *Dp(1;f)LJ9* imprint, linking the piRNA pathway to the initiation of an imprint.

Conclusions: My results show that RNAi function is critical in both the establishment and maintenance of the Drosophila *Dp(1;f)LJ9* genomic imprint. The siRNA and piRNA pathways have distinct functions. The germline specific piRNA pathway contributed to the initial maternal establishment of the imprint, and may also function in regulating both the maternal and paternal imprint in the early stages of embryonic development. The siRNA pathway functions only in the somatic maintenance

of paternal-specific silencing, possibly interacting with Piwi to guide heterochromatin recruitment.

6.2 Introduction

Genomic imprinting is an epigenetic process that can result in the parent-specific expression of alleles. This is achieved first by the germline establishment of parent-specific epigenetic marks, followed by a subsequent somatic response to these epigenetic marks recruiting differential transcriptional regulators to the maternal and paternal alleles. The end result of an imprint can be the monoallelic expression of a gene absent of any direct alteration of the gene sequence. Imprinted regulation depends on multiple epigenetic mechanisms, including DNA methylation, histone modification and higher-order chromatin formation. Non-coding RNAs (ncRNAs) have been detected in many imprinted regions in both mammals and plants, yet their role in regulating imprinted expression remains to be fully elucidated (Royo and Cavaillé 2008; Latos and Barlow 2009; Verdel *et al.* 2009).

In mice, the *Air* and the *Kcnq1ot1* ncRNAs are well-characterized antisense transcripts involved in regulating parent-specific expression of the *Igf2r* gene cluster and *Kcnq1* gene cluster, respectively (Pauler *et al.* 2007). *Air* is transcribed from only the paternal chromosome, and it is able to maintain silencing of the paternal *Igf2r* gene cluster even when DNA methylation is lost (Barlow *et al.* 1991). Preventing transcription of *Air*, or truncation the *Air* transcript, results in biallelic expression of *Igf2r* and the neighboring gene clusters (Wutz *et al.* 1997; Sleutels *et al.* 2002). Similarly, *Kcnq1ot1* is also paternally transcribed and necessary for paternal silencing of the *Kcnq1* gene cluster (Mancini-Dinardo *et al.* 2006). The reciprocally imprinted genes *H19* and *Igf2* are primarily regulated in mice by parent-specific DNA methylation and maternal specific binding of the CTCF insulator within the *H19* imprint control region (ICR) (Latos and Barlow 2009). However, ncRNA produced from bidirectional sense and antisense transcription within the *H19* ICR contributes to paternal *H19* silencing and, furthermore, is a conserved silencing feature of the transgenic *H19* ICR element when inserted into *Drosophila* (Schoenfelder *et al.* 2007). The presence of ncRNA may be a common

feature of mammalian imprinted domains. Widespread transcription of ncRNAs has recently been reported at multiple imprinted loci in mammal and, in some cases, transcription of the ncRNAs extended beyond the previously established boundaries for the imprinted loci (Katayama *et al.* 2005; Babak *et al.* 2008).

The presence of antisense ncRNA transcripts at imprinted domains suggests the possible involvement of RNA interference (RNAi), a conserved posttranscriptional silencing mechanism that uses double stranded RNA (dsRNA) to target the destruction of complementary RNA transcripts (Zakharova *et al.* 2009). In addition to this posttranscriptional silencing, members of the RNAi silencing pathway are also able to direct transcriptional silencing through the recruitment of chromatin remodeling factors (Djupedal and Ekwall 2009). At some imprinted loci in mice, transcriptional silencing mediated by the methylation of histone 3 lysine 9 (H3K9) and histone 4 lysine 20 (H4K20) has been linked to the presence of antisense RNA (asRNA) (McEwen and Ferguson-Smith 2009). RNAi has also been implicated in the function of both *Air* and *Kcnq1ot1* in mammalian imprinting (Pauler *et al.* 2007). Additionally, treating human cell lines with RNAi transcripts matching a targeted gene sequence was able to initiate both DNA methylation and histone methylation of the targeted gene (Kawasaki and Taira 2004). Similarly, in *Arabidopsis,* the imprinted gene FWA generates dsRNA from tandem repeats located within the paternal gene promoter, which guides DNA methylation and heterochromatin formation that silence the paternal allele (Lippman and Martienssen 2004). The RNAi pathway is involved in both *de novo* and maintenance DNA methylation of the paternal FWA allele (Lippman *et al.* 2003; Chan *et al.* 2004), and mutations in genes involved in the *Arabidopsis* RNAi silencing pathway cause a loss of paternal FWA methylation (Chan *et al.* 2004). Recently, parent-specific generation of asRNAs has been detected in the developing endosperm of *Arabidopsis*, leading to the identification of new imprinted loci (Mosher *et al.* 2009). Thus, while the specific function of asRNA and RNAi silencing at imprinted domains is not fully understood, RNAi machinery has the ability to recruit epigenetic components commonly associated with imprint regulation in both mammals and plants.

RNAi silencing pathways use components of three general types: 1) RNAse III nucleases, such as Dicer (Dcr) and Drosha, which cleave long dsRNA strands into small RNAs 21 to 23 nucleotides in length. 2) dsRNA binding proteins, such as R2D2, Loquacious (Loqs), and Pasha, which pair with RNAse III nucleases to facilitate small RNA production. 3) The Argonaute (Ago) family of PAZ domain proteins, which includes Ago1 and Ago2, form an active part of the RNAi Silencing Complex (RISC) required to target and 'slice' the mRNA transcript complementing the small RNA guide (Matranga and Zamore 2007; Huisinga and Elgin 2009).

The silencing induced by RNAi can be separated into three broad pathways, determined by the origin of the dsRNA transcripts and the RNAi members that participate in the biogenesis of small silencing RNAs: 1) the micro RNA (miRNA) pathway (Figure 6.1), is triggered by the formation of hairpin dsRNA from the transcription of a single strand of RNA, 2) the short-interfering RNA (siRNA) pathway (Figure 6.1), is triggered by the production of dsRNA from non-coding antisense transcripts, 3) the piwi RNA (piRNA) RNAi pathway (Figure 6.2), is Dicer-independent and is triggered by transcripts from discrete clusters such as transposons and repetitive elements.

The link between RNAi mediated posttranscriptional silencing and transcriptional gene silencing in *Drosophila* was first demonstrated with piRNA components (Pal-Bhadra *et al.* 2002; Pal-Bhadra *et al.* 2004). Mutations in *aub*, *piwi*, and *spn-E* lead to a reduction in H3K9 methylation, a dispersal of heterochromatic protein 1 (HP1), and a general reduction of heterochromatic spreading (Pal-Bhadra *et al.* 2004). The precise role of piRNA components in formation of somatic heterochromatin remains to be elucidated. However, as expression of *aub* and *ago3* are limited to the early embryo (Williams and Rubin 2002; Gunawardane *et al.* 2007), thus, the function of the piRNA pathway is likely limited to the initial propagation of heterochromatin. Piwi stands out amongst members of the piRNA pathway due to its expression in both somatic and germline cells, as well as its presence the nucleus in addition to the cytoplasm, where the other piRNA components are localized (Cox *et al.* 2000; Nishida *et al.* 2007). Growing evidence suggests that in

somatic cells, Piwi may function outside of the conventional piRNA pathway to mediated heterochromatin formation in collaboration with HP1 (Brower-Toland *et al.* 2007) but independently of Ago3 (Li *et al.* 2009). Recently, a 'piwi-piRNA guidance hypothesis' has been proposed, which identifies Piwi as directing heterochromatin formation in somatic cells, either by direct piRNA-DNA binding, or piRNA targeting of specific ncRNAs from genomic sequences (Lin and Yin 2009).

The siRNA pathway has been linked more directly to the formation of somatic heterochromatin in *Drosophila*. In somatic cells, endogenous siRNAs (endo-siRNAs) act as RNAi guides to silence transcription from transposable elements in a Dcr2 and Ago2 dependent pathway (Ghildiyal *et al.* 2008; Kawamura *et al.* 2008); mutations in *dcr2*, *r2d2*, and *ago2* all result in reductions of histone H3K9 methylation and HP1 recruitment to transposable elements (Fagegaltier *et al.* 2009). Additionally, direct interactions between Dcr2 and RNA polymerase II have been associated with endo-siRNA pathway directed heterochromatin formation in *Drosophila* (Kavi and Birchler 2009).

To determine if RNAi is involved regulating imprinted regions in *Drosophila,* I tested members of the miRNA, siRNA and piRNA pathways for their effects on the establishment and maintenance of the *Dp(1;f)LJ9* imprint. The *Drosophila Dp(1;f)LJ9* mini-X chromosome is the result of a chromosomal inversion and deletion, which places the eye color gene *garnet* (*g*) under the regulatory influence of an endogenous imprinting center in pericentric heterochromatin (Lloyd *et al.* 1999a). Paternal inheritance of *Dp(1;f)LJ9* (*Dp(1;f)LJ9*^{PAT}) produces variegated *garnet* expression, while maternal inheritance (*Dp(1;f)LJ9*^{MAT}) produces full *garnet* expression. Paternal-specific silencing of *garnet* is dependent upon heterochromatin formation (Joanis and Lloyd 2002; Anaka *et al*. 2009). Maternal-specific full *garnet* expression is achieved by the generation of active chromatin supported by the formation of a CTCF-associated boundary that counteracts heterochromatin formation (MacDonald *et al*. 2010).

My results show distinct patterns of regulation by different RNAi pathways. The presence of asRNA transcripts in *Dp(1;f)LJ9*^{PAT} flies could constitute source transcripts

for siRNA directed heterochromatin formation. Consistent with the presence of these transcripts, mutations in endo-siRNA components *loqs*, *r2d2*, *dcr2*, and *ago2* all resulted in a relaxation of paternally imprinted *garnet* silencing, while having no affect on maternally imprinted *garnet* expression. In contrast, I found that dedicated miRNA component mutations had no affect on either maternal or paternal imprint regulation. Regulation by the piRNA pathway was more complex. My results show a role for Piwi in maintaining somatic silencing from the paternally inherited *Dp(1;f)LJ9*, similar to the action of the siRNA genes. This response distinguishes Piwi from all other piRNA pathway mutations tested, including *aub*, *armi*, *spn-E*, *squ*, and *zuc* which enhanced silencing of *garnet* from both paternally and maternally inherited *Dp(1;f)LJ9*. Furthermore, I found Aub is additionally involved the establishment of the maternal *Dp(1;f)LJ9* imprint, consistent with its documented role in maternal germline regulation. These findings demonstrate that both siRNAs and piRNAs, but not miRNAs, influence the somatic regulation of imprinted regions in *Drosophila*.

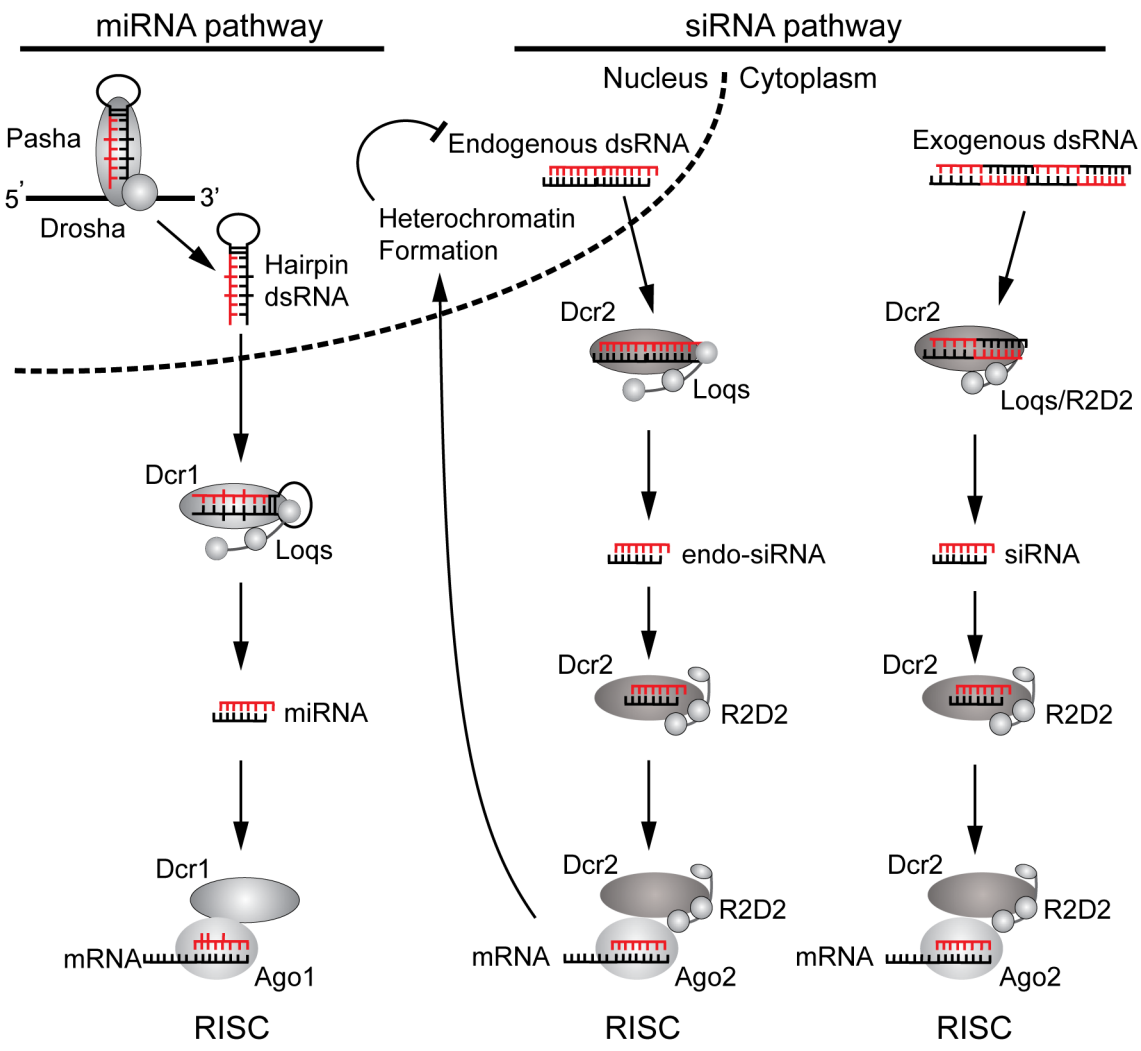

Figure 6.1 - The *Drosophila* miRNA And siRNA Silencing Pathways.
The origin of double-stranded RNA (dsRNA) determines the RNAi pathway that will be employed to process it: hairpin RNA, which result from short RNA sequences that fold onto themselves to form dsRNA with sense (black) and antisense (red) regions, is processed by the miRNA pathway, while dsRNA that is generated from endogenous repetitive elements or exogenous viruses is processed by the siRNA pathway. The miRNA and siRNA pathways in *Drosophila* are distinguished by the involvement of discrete dsRNA 'dicers' and mRNA 'slicers', as Dcr1 and Ago1 are involved in miRNA processing and Dcr2 and Ago2 are involved in siRNA processing. Loqs is primarily associated with Dcr1, where it is required for miRNA biogenesis, but is not involved in loading miRNA guides into Ago1-RISC (Liu *et al.* 2007). R2D2 is linked to the siRNA pathway, where it is required to load siRNA guides into Ago2-RISC (Liu *et al.* 2003b). Recently, Loqs has also been found to act upstream of R2D2 in the biogenesis of siRNAs, which are than loaded into the Ago2-RISC by R2D2 (Hartig *et al.* 2009; Marques *et al.* 2010). The Ago2-RISC is also involved in guiding heterochromatin formation in regions that generate endogenous siRNAs (endo-siRNA) (Fagegaltier *et al.* 2009). Adapted from (Hartig *et al.* 2009; Marques *et al.* 2010).

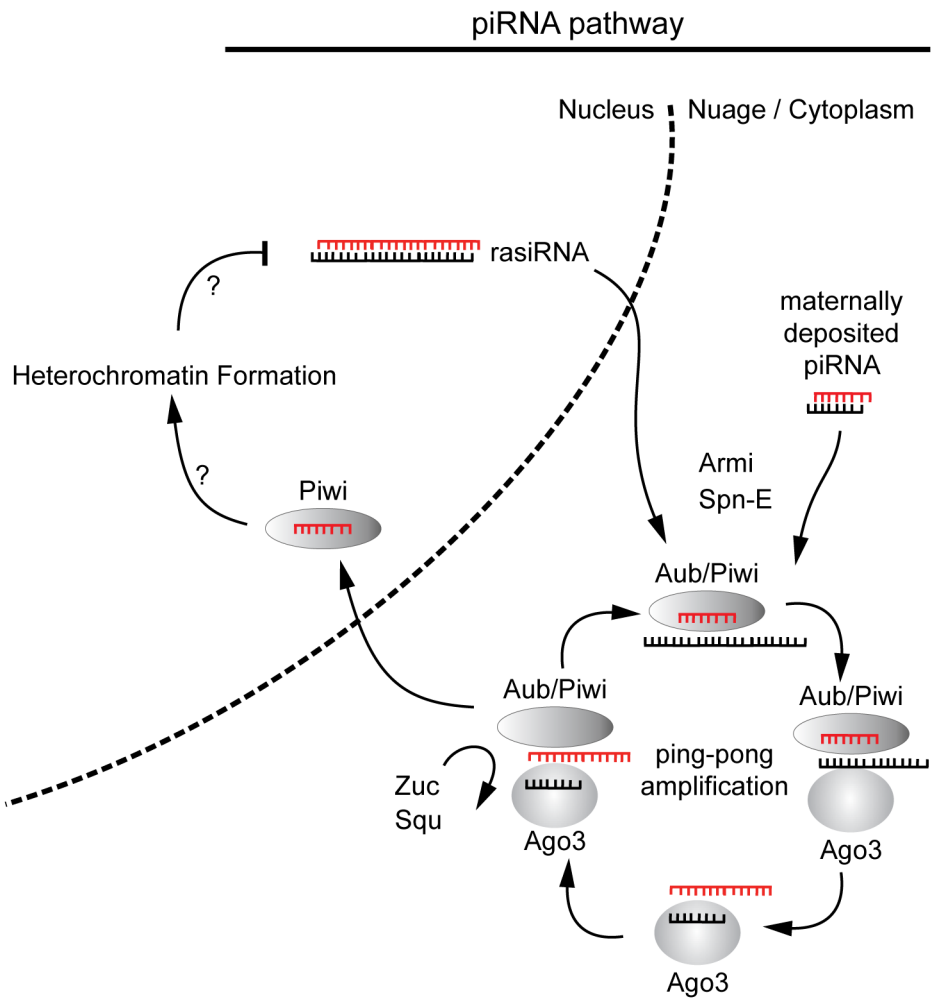

Figure 6.2 - The *Drosophila* piRNA Silencing Pathway.
The piRNAs, and repeat-associated small interfering RNAs (rasiRNAs), function primarily in the silencing of transposable elements and non-coding tandem repeats in the germline of *Drosophila*. The piRNA pathway does not use Dicer nucleases to cleave RNAs, instead the Argonaute proteins Ago3, Aubergine (Aub), and Piwi, function to cleave target RNAs (Klattenhoff and Theurkauf 2008). Within the germline, Aub and Ago3 mediate cytoplasmic and nuage (a germline-specific perinuclear structure) piRNA functions, while Piwi operates primarily in nucleus of cells, but may be loaded with piRNAs in the nuage (Klattenhoff and Theurkauf 2008). A unique feature of piRNA pathway is the so called ping-pong amplification cycle for generating piRNAs to silence transposon transcription; primary piRNAs, either produced by repetitive elements or maternally contributed to the germ cells, act as guides for sense-binding Ago3 and antisense-binding Aub/Piwi, that in turn amplify piRNA production and degradation of both sense (black) and antisense (red) transposon transcripts (Brennecke *et al.* 2007; Gunawardane *et al.* 2007). The RNA helicases Armitage (armi) and Spindle-E (Spn-E) assist in the processing of piRNAs (Vagin *et al.* 2006), while the RNA nucleases Squash (Squ) and Zucchini (Zuc) are thought to contribute to the production of mature piRNAs guides (Pane *et al.* 2007). Adapted from (Klattenhoff and Theurkauf 2008).

6.3 Results and Discussion

6.3.1 The siRNA Pathway Maintains *garnet* Silencing From *Dp(1;f)LJ9*^{PAT}

I tested mutations in members of both the miRNA and siRNA pathways for their effect on *garnet* expression on either paternally or maternally inherited *Dp(1;f)LJ9*. A relaxation of *garnet* silencing was observed from mutations in each of the established members of the siRNA pathway when *Dp(1;f)LJ9* was paternally inherited (Table 6.1).

Dcr2 function is specific to the siRNA pathway and is required for the biogenesis of siRNA guides. The *dcr2*$^{L811\ fsx}$ loss-of-function allele (Lee *et al.* 2004) caused a strong relaxation of *garnet* silencing from *Dp(1;f)LJ9*^{PAT} resulting in increased eye pigmentation (Figure 6.3A). R2D2 binds double-stranded RNA and is necessary for loading siRNA guides into Ago2 (Figure 6.1). I tested three *r2d2* alleles, of which *r2d2*^{1} and *r2d2*$^{S165\ fsx}$ are null mutations (Marques *et al.* 2010) and *r2d2*^{EY00028} is an uncharacterized P-element mutation. Each of the *r2d2* alleles relaxed silencing of *garnet* from *Dp(1;f)LJ9*^{PAT}, increasing pigmentation to levels similar to those resulting from the *dcr2*$^{L811\ fsx}$ allele (Figure 6.3A). Both the *ago2*^{EY04479} and *ago2*^{dop1} alleles tested have previously been shown to disrupt somatic RNAi function (Meyer *et al.* 2006; Zambon *et al.* 2006) and, as for other siRNA members tested, caused the relaxation of silencing from *Dp(1;f)LJ9*^{PAT} (Figure 6.3A). Collectively, these results demonstrate that the siRNA pathway is involved in maintaining paternal-specific silencing from the *Dp(1;f)LJ9* imprinting center.

Mutations in members of the miRNA pathway demonstrated no conclusive involvement in *Dp(1;f)LJ9*^{PAT} directed *garnet* silencing (Table 6.2). The biogenesis of miRNA guides depends on Pasha and Drosha to bind and process hairpin antisense RNAs into miRNAs (Matranga and Zamore 2007). The uncharacterized P-element mutation *pasha*^{EY01325} had no significant affect on *garnet* expression *Dp(1;f)LJ9*^{PAT} (Figure 6.4A). *Drosophila* Rm62 is an RNA helicase that is an ortholog of the Drosha complex in mammals (Buszczak 2006; Cziko *et al.* 2009) and which has been associated with RNAi and insulator function in *Drosophila* (Lei and Corces 2006; Zambon *et al.* 2006). As was the case with *pasha*^{EY01325}, the hypomorphic *rm62*^{01086} allele (Buszczak 2006) had no

significant affect on *garnet* expression *Dp(1;f)LJ9*^{PAT} (Figure 6.4A). The lack of involvement of Pasha and Drosha may indicate that the miRNAs are not involved in regulating paternal-silencing from *Dp(1;f)LJ9*. Loqs is now known to participate in processing of specific siRNAs, such as the endo-siRNAs (Marques *et al.* 2010), in addition to its originally defined function of pairing with Dcr1 for miRNA biogenesis (Liu *et al.* 2007). My finding that the hypomorphic *loqs*^{f00791} allele (Förstemann *et al.* 2005) also repressed *Dp(1;f)LJ9*^{PAT} *garnet* silencing (Table 6.2 and Figure 6.4A), supports the possibility that long, endogenous, antisense transcripts from *Dp(1;f)LJ9*^{PAT} are a target of the endo-siRNA pathway.

Ago1 has not been definitively linked to siRNA function; however, Ago1 and Dcr2 have been found to interact directly with RNA polymerase II during the formation of heterochromatin (Kavi and Birchler 2009). Additionally, Ago1 and Ago2 may have overlapping functions for degrading target transcripts (Williams and Rubin 2002). I tested three alleles of *ago1* that were generated through P-element insertion; the strong loss-of-function *ago1*^{k08121} and *ago1*^{k00208} alleles, and the hypomorphic *ago1*^{04845} allele (Kataoka *et al.* 2001; Williams and Rubin 2002; Jin *et al.* 2004). Both the *ago1*^{k08121} and *ago1*^{04845} alleles relaxed silencing of *garnet* from *Dp(1;f)LJ9*^{PAT}, while the *ago1*^{k00208} allele had no significant affect of paternally-inherited *garnet* expression (Table 6.2 and Figure 6.4A).

The *ago2*^{dop1} allele specifically impairs the ability of Ago2 to interact with Ago1 (Meyer *et al.* 2006), thus the relaxation of *Dp(1;f)LJ9*^{PAT} *garnet* silencing from *ago2*^{dop1} and two of the tested *ago1* alleles is consistent with the possibility of overlapping function for Ago1 and Ago2 in *Dp(1;f)LJ9* imprinted silencing. Both *ago1* and *ago2* are ubiquitously expressed and have transcripts maternally deposited into the egg during gametogenesis, (Williams and Rubin 2002); however, no maternal effects were detected for either *ago1* or *ago2* mutant alleles (Table 6.7 and 6.8), suggesting siRNA involvement in paternal-specific *garnet* silencing occurs at or after zygotic gene activation in the embryo. In agreement with this result is the finding that the *ago2*^{dop1} allele specifically disrupts developmental processes at the mid-blastula transition of the *Drosophila* embryo (Meyer *et al.* 2006).

The involvement of the siRNA pathway in regulating the *Dp(1;f)LJ9* imprint is limited to the paternally inherited *Dp(1;f)LJ9*. Mutations in either siRNA or miRNA pathway members had no affect on *garnet* expression in maternally inherited *Dp(1;f)LJ9*^{MAT} flies (Table 6.1 and Figure 6.3B), and the internal controls did not show any paternal effects when siRNA or miRNA mutations were present in fathers (Table 6.9 and 6.10).

6.3.2 The Involvement Of asRNA In Targeting *Dp(1;f)LJ9*^{PAT} Silencing

The siRNA pathway is involved in the recruitment of heterochromatin to target areas in somatic cells. Endogenous siRNAs generated from transposons and repetitive elements have been linked to both the degradation of transcripts and the transcriptional silencing of these regions by the recruitment of heterochromatic components (Kawamura *et al.* 2008; Obbard and Finnegan 2008). Mutations in *ago2*, *dcr2*, and *r2d2* have all been associated with disruption of histone H3K9 methylation and the disassociation of HP1 from heterochromatic regions of the chromosome (Peng and Karpen 2007; Fagegaltier *et al.* 2009; Peng and Karpen 2009). Additionally, paternal-specific silencing from the *Dp(1;f)LJ9* imprinted region is dependent upon histone H3K9 methylation and heterochromatin formation (Joanis and Lloyd 2002).

The finding that Ago2, Dcr2, Loqs, and R2D2 are all required to maintain paternal-specific *Dp(1;f)LJ9* silencing, suggests that paternal-specific siRNAs generated from endogenous dsRNAs may be involved in guiding heterochromatin formation. The production of non-coding asRNA transcripts could extend from the imprint control region through the *garnet* coding region to potentially engage siRNA-guided heterochromatin recruitment. To test this possibility, I produced digoxigenin-labeled riboprobes to detect both sense and antisense *garnet* transcripts. Probes were produced to detect a 3' coding region of the *garnet* gene and a 5' untranscribed region of the *garnet* promoter. These probes serve the dual function of determining if antisense transcription extends over the entire length of the *garnet* gene and is acting as a control for the sense detection of *garnet* transcripts. *In situ* hybridizations with the riboprobes were performed on *Drosophila* head

sections from both *Dp(1;f)LJ9*^{PAT} and *Dp(1;f)LJ9*^{MAT} newly eclosed adults. Sense *garnet* transcripts were detected in *Dp(1;f)LJ9*^{PAT} eyes (Figure 6.5A). Antisense *garnet* transcripts were also detected from both the 3' coding region and a 5' untranscribed region in *Dp(1;f)LJ9*^{PAT} eyes, while the control probes for sense 5' untranscribed region showed no specific staining (Figure 6.5A). *Dp(1;f)LJ9*^{MAT} eyes had strong staining for *garnet* sense transcripts, weak staining for *garnet* antisense transcripts, and no staining for either the sense or antisense *garnet* 5' untranscribed region (Figure 6.5B).

The detection of antisense transcripts spanning the *garnet* gene in *Dp(1;f)LJ9*^{PAT} eyes supports the possibility that non-coding RNAs, antisense to *garnet*, are generating siRNAs that assist in the paternal-specific formation of heterochromatin. The detection of 3' antisense *garnet* transcripts from *Dp(1;f)LJ9*^{MAT} eyes was weak and just above background levels, suggesting antisense transcripts are also present when *Dp(1;f)LJ9* is maternally inherited, but at much reduced levels. This is consistent with the infrequent weak variegation that can be induced from *Dp(1;f)LJ9*^{MAT}. The lack of detectable antisense *garnet* transcripts from the 5' untranscribed region in *Dp(1;f)LJ9*^{MAT} flies indicates a less expansive generation of antisense transcripts overall.

To confirm the presence of antisense *garnet* transcripts in *Dp(1;f)LJ9*^{PAT} flies, I designed sense and antisense specific primers for use in RT-PCR experiments on RNA extracted from adult heads. The use of complimentary primers detected antisense *garnet* transcripts from both *Dp(1;f)LJ9*^{PAT} and *Dp(1;f)LJ9*^{MAT} flies; however, the faint detection of *Dp(1;f)LJ9*^{MAT} antisense *garnet* transcripts was similar to the amplification seen in the no-primer RT control (Figure 6.6A). To eliminate self-primed transcripts present in the no-primer RT control, I attached an adaptor sequence to each RT primer as described by (Shpiz *et al.* 2009). Using adaptor primers, antisense *garnet* transcripts remain detectable from RNA extracted from *Dp(1;f)LJ9*^{PAT} heads, but amplification of transcripts is no longer seen in the no-primer RT control (Figure 6.6B). Faint detection of antisense *garnet* transcripts still occurs in RNA from *Dp(1;f)LJ9*^{MAT} flies using adaptor primers (Figure 6.6B), indicating that the low levels of antisense *garnet* transcripts detected in *Dp(1;f)LJ9*^{MAT} heads by *in situ* hybridization is indicative of scarce but detectable

antisense *garnet* transcripts. I also tested RNA extracted from *Dp(1;f)LJ9*^{PAT}; dcr2$^{L811\ fsx}$/+ flies to determine if a disruption in the siRNA pathway impacted the detection of antisense *garnet* transcripts. I found that in both the use of standard primers (Figure 6.6A), and adaptor primers (Figure 6.6B), stronger detection of antisense transcripts was observed. Increased detection of antisense *garnet* transcripts may be accounted for by the reduced degradation of transcripts caused by the dcr2$^{L811\ fsx}$ allele. Further quantitative or semi-quantitative RT-PCR would be needed to fully elucidate the abundance of antisense *garnet* transcripts in both *Dp(1;f)LJ9*^{PAT} and *Dp(1;f)LJ9*^{MAT} flies; however, this finding identifies the presence of non-coding antisense transcripts in flies bearing the *Dp(1;f)LJ9* mini-X chromosome.

6.3.3 piRNA Pathway Mutant Alleles Cause Enhanced Silencing Of *garnet* In Both *Dp(1;f)LJ9*^{PAT} *And Dp(1;f)LJ9*^{MAT}

The piRNA pathway is involved in the silencing of transposable elements and repetitive DNA regions in *Drosophila* through the Dcr-independent production of repeat-associated small interfering RNAs (rasiRNAs) (Aravin *et al.* 2003; Saito *et al.* 2006). Mutant alleles of piRNA pathway components, including *aub*, *armi*, *piwi*, *squ*, *spn-E*, or *zuc* alleles, relax the variegated silencing of reporter genes placed next to either repetitive sequence or transposable elements (Pal-Bhadra *et al.* 2004; Klenov *et al.* 2007; Pane *et al.* 2007). In contrast, I found that most piRNA mutant alleles enhanced silencing of the *garnet* reporter gene, from both maternally and paternally inherited *Dp(1;f)LJ9* (Table 6.3).

Aub, a member of the Argonaute protein family, is key a component of the piRNA pathway that functions in the germline biogenesis of piRNAs through the ping-pong amplification cycle and the degradation target transposon sense transcripts (Figure 6.2). I tested three *aub* alleles that have previously been shown to disrupt RNAi function (Kennerdell *et al.* 2002; Zambon *et al.* 2006); the *aub*^{HN} and *aub*^{QC42} alleles are strong hypomorphic mutations (Wilson *et al.* 1996; Harris and Macdonald 2001) that enhanced *garnet* silencing in *Dp(1;f)LJ9*^{PAT} flies, while the weaker *aub*^{KG05389} allele, generated from the P-element BDGP Gene Disruption Project (Bellen *et al.* 2004), did not have any

significant affect on *Dp(1;f)LJ9*^{PAT} *garnet* silencing (Table 6.3 and Figure 6.7A). All three of the *aub* alleles tested disrupted *garnet* expression in *Dp(1;f)LJ9*^{MAT} flies, causing variegated *garnet* expression (Table 6.3 and Figure 6.7B). The finding that all the tested *aub* alleles enhanced *garnet* silencing in *Dp(1;f)LJ9*^{MAT} flies, but only the stronger *aub*^{HN} and *aub*^{QC42} alleles enhanced *garnet* silencing in *Dp(1;f)LJ9*^{PAT} flies, suggests regulation of the maternal *Dp(1;f)LJ9* imprint is more sensitive to *aub* dosage.

Spn-E is an RNA helicase that is a component of the piRNA pathway in the germline that is primarily associated with the ping-pong amplification loop (Vagin *et al.* 2006; Malone *et al.* 2009). I tested two *spn-E* alleles for their effects on the *Dp(1;f)LJ9* imprint: *spn-E*^{1} , which has a point mutation within the helicase domain (Klenov *et al.* 2007), and the uncharacterized P-element mutation *spn-E*^{c00786}. Both *spn-E* alleles caused enhanced *garnet* silencing from *Dp(1;f)LJ9*^{PAT} resulting in a decrease in eye pigmentation (Table 6.3 and Figure 6.7A). However, unlike the Armi RNA helicase, mutations in *spn-E* did not display a maternal effect (Table 6.8). Both *spn-E* alleles tested also generated enhanced *garnet* silencing in *Dp(1;f)LJ9*^{MAT} flies, evidenced by the appearance of a variegated eye phenotype (Table 6.3 and Figure 6.7B). No paternal effects of *spn-E* alleles were found (Table 6.10).

Armi is an RNA helicase that functions in the processing of piRNAs and is involved in the silencing of transposons in both the germline and female follicle cells (Vagin *et al.* 2006; Klattenhoff *et al.* 2007). The hypomorphic *armi*$^{72.1}$ allele (Tomari *et al.* 2004) dramatically increased *garnet* silencing in *Dp(1;f)LJ9*^{PAT} flies, reducing the expression of *garnet* more drastically than any other tested mutation (Table 6.3 and Figure 6.7A). Furthermore, *armi*$^{72.1}$ showed a maternal effect, increasing *garnet* silencing in *Dp(1;f)LJ9*^{PAT}; +/+ flies that had mothers bearing the *armi*$^{72.1}$ allele (Table 6.8). *Armi* has previously been reported to act as a maternal effect gene and, when mutant in mothers, can affect the development of embryos prior to zygotic activation (Cook *et al.* 2004; Tomari *et al.* 2004). My finding that the *armi*$^{72.1}$ allele disrupts regulation of the paternal *Dp(1;f)LJ9* imprint prior to the zygotic activation of the embryo suggests that the influence of the piRNA pathway occurs during the initial formation of the imprinted

domain. In *Dp(1;f)LJ9*^{MAT} flies, the *armi*$^{72.1}$ allele also enhanced *garnet* silencing, causing visible variegation of eye pigments (Figure 6.7B). The *armi*$^{72.1}$ allele did not cause any paternal effects from being present in fathers (Table 6.10); yet similar to the effect on *Dp(1;f)LJ9*^{PAT}, the *armi*$^{72.1}$ allele showed the strongest enhancement of *garnet* silencing in *Dp(1;f)LJ9*^{MAT} flies amongst all the mutations tested (Table 6.3 and Figure 6.7B).

Piwi is an Argonaute protein localized primarily in the nucleus that is found in both somatic and germline cells (Klattenhoff and Theurkauf 2008). Like Aub, Piwi can cleave targeted RNA transcripts when loaded with piRNA guides (Gunawardane *et al.* 2007) and, *piwi* mutations result in an increase in transposon transcript abundance (Kalmykova *et al.* 2005; Vagin *et al.* 2006). I tested the *piwi*^{3} allele, which is the product of a single P-element insertion (Cox *et al.* 1998), for its effect on *Dp(1;f)LJ9 garnet* expression. Unlike the enhanced of silencing from both *Dp(1;f)LJ9*^{PAT} and *Dp(1;f)LJ9*^{MAT} induced by *aub* mutations, the *piwi*^{3} allele relaxed *garnet* silencing in *Dp(1;f)LJ9*^{PAT} flies (Table 6.3 and Figure 6.7A), while having no affect on *Dp(1;f)LJ9*^{MAT} flies (Table 6.3 and Figure 6.7B). No maternal or paternal effects were evident from the *piwi*^{3} allele being present in either parent (Tables 6.7 and 6.9, respectively). The distinct function of Aub and Piwi in the regulation of the *Dp(1;f)LJ9* imprint may be attributed to the distinct expression patterns and function of Aub and Piwi; Aub is cytoplasmic and primarily targets retrotransposons without long terminal repeats, while Piwi is cytoplasmic and nuclear and primarily targets retrotransposons with long terminal repeats (Huisinga and Elgin 2009). Thus, it is likely that Aub and Piwi functions do not overlap and that the piRNA pathway is compartmentalized into nuclear and cytoplasmic functions (Klattenhoff and Theurkauf 2008).

Squ and Zuc are cytoplasmic nucleases that interact with Aub and are involved in the processing of piRNAs (Pane *et al.* 2007). Mutations in *squ* and *zuc* result in increased levels of transposon transcripts due to defects in the biogenesis of germline piRNAs (Pane *et al.* 2007; Saito *et al.* 2009). The *zuc*^{RS49} allele is a mis-sense hypomorphic mutation, and the *squ*^{PP32} allele is a nonsense mutation with a stop codon at residue 111

(Pane *et al.* 2007). Both zuc^{RS49} and squ^{PP32} alleles moderately enhance silencing of *garnet* in both *Dp(1;f)LJ9*^{PAT} and *Dp(1;f)LJ9*^{MAT} flies (Table 6.3 and Figures 6.7A and 6.7B, respectively). No maternal or paternal effect was occurred for either allele (Tables 6.7 and 6.9).

The effect of mutations in *aub*, *armi*, *spn-E*, *squ*, and *zuc* to enhance the silencing of the *garnet* imprint reporter gene in both *Dp(1;f)LJ9*^{PAT} and *Dp(1;f)LJ9*^{MAT} flies suggests that the piRNA pathway is either directly, or indirectly, involved in regulating the *Dp(1;f)LJ9* imprint. The expression of piRNA pathway components occurs primarily in the early embryo and, in adults, is restricted to the gonads and germline cells. This means that any role for the piRNA pathway in regulating heterochromatin formation in somatic cells would be limited to the early embryo (Klenov *et al.* 2007).

6.3.4 Vasa Mutations Disrupt *Dp(1;f)LJ9*^{PAT} Silencing

Vasa is an RNA helicase specific to the female germline that is involved in oogenesis and has a strong maternal effect, causing embryonic lethality in the progeny of *vas*$^{-}$ mothers (Schüpbach and Wieschaus 1991). Mutations in *vasa* (*vas*) result in increased transcription of transposons in the maternal germline (Vagin *et al.* 2004), due in part to the disruption of the germline localization of piRNA pathway components Aub, Ago3, and Piwi (Malone *et al.* 2009). I tested two hypomorphic *vas* alleles, vas^{1} and vas^{RJ36}, for their effects on *Dp(1;f)LJ9 garnet* expression. The vas^{1} allele strongly relaxed *garnet* silencing in *Dp(1;f)LJ9*^{PAT} flies, producing eyes with only few and small visible patches of variegated silencing (Table 6.4 and Figure 6.8A). Furthermore, the vas^{1} allele also displayed a maternal effect, relaxing *garnet* silencing in *vasa*+ progeny when mothers were vas^{1}/+ (Table 6.7). The vas^{RJ36} allele caused a moderate relaxation of *garnet* silencing in *Dp(1;f)LJ9*^{PAT} flies (Table 6.4 and Figure 6.8A); however, no maternal effect was observed from mothers bearing the vas^{RJ36} allele (Table 6.7). Both of the tested vas^{1} and vas^{RJ36} alleles had no affect on *garnet* expression in *Dp(1;f)LJ9*^{MAT} flies (Table 6.4 and Figure 6.8B), additionally, no paternal effects were found for either *vas* allele (Table 6.9).

Within a *vasa* intron lies the *vasa intronic gene* (*vig*), which is associated with the Ago2-RISC complex and RNAi function (Caudy *et al.* 2002) in addition to the formation of heterochromatin (Gracheva *et al.* 2009). I tested two *vig* alleles, vig^{C274} and $vig^{EY07816}$, for their effects on *Dp(1;f)LJ9 garnet* expression. In $Dp(1;f)LJ9^{PAT}$ flies the mutant *vig* alleles resulted in increased *garnet* expression; however, when pigment levels were quantified the result was statistically insignificant (Table 6.4 and Figure 6.8A). No maternal effect was observed in $Dp(1;f)LJ9^{PAT}$ progeny from *vig* mothers (Table 6.7). Neither of the available *vig* alleles are well characterized; however, the $vig^{EY07816}$ allele has previously been shown to disrupt RNAi function (Zambon *et al.* 2006). It is possible that further testing with strong *vig* alleles will help to clarify if Vig is involved in silencing from $Dp(1;f)LJ9^{PAT}$. The expression of *vig* increases later in *Drosophila* embryonic development, and has been proposed to stabilize heterochromatin formation in these later stages of development (Gracheva *et al.* 2009). As discussed in Chapter 4, heterochromatin formation from the *Dp(1;f)LJ9* imprinting center is initiated early in development, and may be sufficiently stabilized prior to the period of peak *vig* expression, accounting for the weak affect of the *vig* alleles on $Dp(1;f)LJ9^{PAT}$ *garnet* silencing. None of the *vasa* or *vig* alleles had an affect on *garnet* expression from $Dp(1;f)LJ9^{MAT}$ flies (Table 6.4 and Figure 6.8B). Additionally, no paternal effect was observed for *vasa* or *vig* mutations (Table 6.9).

6.3.5 The Involvement Of *aubergine* In The Establishment Of The Imprint In The Maternal Germline

I tested a subset of RNAi pathway mutations for their effects on the establishment of the *Dp(1;f)LJ9* imprint. *Drosophila* bearing the *Dp(1;f)LJ9* mini-X chromosome in the presence of either mutant *aub*, *ago1*, or *piwi* alleles were used to determine the effect of the tested mutation on the germline establishment of the imprint. To test for an effect on the paternal establishment of the *Dp(1;f)LJ9* imprint, the mutant RNAi alleles were combined with the *Dp(1;f)LJ9* mini-X chromosome in the father. These crosses all resulted in embryonic lethality, probably due to the combination of sub-viable mutant alleles. However, females bearing both the *Dp(1;f)LJ9* mini-X chromosome and RNAi mutant alleles were viable and fertile. Those combined with the aub^{HN} allele produced

progeny with variegated *garnet* silencing, resembling paternally imprinted *Dp(1;f)LJ9* and distinct from their respective controls (Table 6.5 and Figure 6.9A and 6.9B, respectively). These results implicate Aub in establishing the maternal *Dp(1;f)LJ9* imprint in the germline. The other tested RNAi mutations, $piwi^{3}$, $ago1^{k08121}$, $ago1^{k00208}$, and $ago1^{04845}$ had no affect on *garnet* expression in the progeny compared to their respective controls (Table 6.5 and Figures 6.9A and 6.9B, respectively), and thus are not involved in establishing the maternal *Dp(1;f)LJ9* imprint.

Aub is critical in maintaining female germline integrity and homozygous *aub* mutant females for are sterile (Klattenhoff and Theurkauf 2008; Siomi *et al.* 2010). The involvement of Aub, but not Piwi, in the establishment of the maternal imprint suggests that there is not a general involvement of RNAi components, but rather a specific RNAi pathway is required to achieve establishment of the imprint. Further RNAi mutants will need to be tested to formulate a model of RNAi involvement in the establishment of the *Dp(1;f)LJ9* imprint, but it is tempting to speculate that Aub and the piRNA pathway may be involved in directing histone H1 to the maternal *Dp(1;f)LJ9* imprinting center. Recently, the linker histone H1 has been determined to be a factor in the maternal establishment of the *Dp(1;f)LJ9* imprint (Kent 2008). Histone H1 is able to form complexes with double-stranded RNAs (Haberland *et al.* 2009), and histone H1 is also associated with the DNA damage response (Yamanaka *et al.* 2002; Hashimoto *et al.* 2007; Rosidi *et al.* 2008). Thus, an Aub-dependent piRNA pathway may be initiated from the imprinted region to assist in establishing the epigenetic imprint mark that in embryos distinguishes maternally inherited *Dp(1;f)LJ9* and promote the maintenance of a transcriptionally competent domain.

6.3.6 The Role Of The piRNA Pathway In The Regulation Of The *Dp(1;f)LJ9* Imprint

Since the piRNA pathway is involved in the degradation of RNA transcripts and silencing of gene expression, my finding that piRNA pathway mutants cause increased silencing from the *Dp(1;f)LJ9* imprinted domain appears contradictory. Recently, similar results were reported for siRNA and piRNA pathway mutants; mutant alleles of *ago2*,

aub and *piwi* all increased silencing of a *white* reporter gene placed next to piRNA-generating clusters of transposable elements (Moshkovich and Lei 2010). Furthermore, the distribution of HP1 is also disrupted in in *ago2* and *piwi* mutations, suggesting a redistribution of heterochromatic factors can result from siRNA and piRNA pathway mutations (Moshkovich and Lei 2010). Together, these results suggest that heterochromatin formation is not directly dependent on RNAi function and, within some genomic regions, RNAi pathways may actively counteract heterochromatin formation.

In contrast to the findings reported by Moshkovich and Lei (2010), only piRNA pathway mutations caused an increase in *Dp(1;f)LJ9 garnet* silencing, while Piwi and siRNA pathway mutations relaxed silencing *Dp(1;f)LJ9*^{PAT} *garnet*. It is possible that redistribution of heterochromatic components is from a specific genomic region and not the result of a general mislocalization. Located close to the heterochromatic breakpoint of the *Dp(1;f)LJ9* mini-X chromosome are the *Stellate* repeats (Hardy *et al.* 1984), that are silenced by the piRNA pathway guided by transcripts from *Suppressor of Stellate* (*Su*(*Ste*)) locus on the Y chromosome (Vagin *et al.* 2006). These repeats constitute a possible candidate region for such a source of heterochromatic factors to account for the increase in silencing from piRNA mutations. Disruption of *Stellate* silencing can result in *trans*-silencing effects and the redistribution of heterochromatic factors similar to the effects described by Moshkovich and Lei (Gvozdev *et al.* 2007). Mutations in *aub*, *armi*, *spn-E*, *zuc*, and *squ* all result in a relaxation of *Stellate* silencing (Vagin *et al.* 2006; Nishida *et al.* 2007; Pane *et al.* 2007), whereas they result in increased *Dp(1;f)LJ9 garnet* silencing. Additionally, mutations in *piwi*, and the siRNA components *ago2*, *dcr2*, and *r2d2* that do not affect *Stellate* silencing (Förstemann *et al.* 2005; Vagin *et al.* 2006) result in decreased *Dp(1;f)LJ9*^{PAT} *garnet* silencing. Collectively, these findings suggest the possibility that a redistribution of heterochromatic factors from the *Stellate* repeats to the *Dp(1;f)LJ9* imprint center contributes to the increase in *garnet* silencing observed in piRNA mutants. However, there are a few discrepancies between silencing from the *Stellate* sequences and the *Dp(1;f)LJ9* imprint center that suggest such a redistribution of heterochromatic factors may not be the primary cause of the increased silencing of *garnet* from piRNA pathway mutations. First, Loqs is also involved in silencing *Stellate* repeats

(Förstemann *et al.* 2005) yet the *loqs* mutant allele did not enhance *garnet* silencing, rather it relaxed silencing. Second, histone H3K9 methylation and HP1 are required components of heterochromatin formation from the *Dp(1;f)LJ9* imprint center (Joanis and Lloyd 2002), but are not major contributors to the silencing of the *Stellate* repeats (Ner *et al.* 2002; Gvozdev *et al.* 2003), which occurs primarily through the posttranscriptional degradation of mRNA transcripts (Kotelnikov *et al.* 2009). Third, while piRNA pathway mutations do create a redistribution of heterochromatic proteins, they do not generally produce changes to heterochromatic structure sufficient enough to alter gene expression in protein coding genes (Klattenhoff *et al.* 2009).

Another possible explanation for piRNA pathway mutations' enhancement of *garnet* silencing from both paternally and maternally inherited *Dp(1;f)LJ9* is the involvement of piRNA pathway components in the DNA damage response. Meiotic recombination necessitates the formation and repair of DNA double-strand breaks, which is associated with the exchange of phosphorylated histone variant H2Av, and the activation of the ATR/Chk2 kinase DNA-repair pathway (Klattenhoff and Theurkauf 2008). Mutations in *aub*, *armi*, and *spn-E* all result in the accumulation of phosphorylated H2Av at distinct loci in the germline, indicating the formation and failed repair of DNA damage (Klattenhoff *et al.* 2007). The ATR/Chk2 DNA-repair pathway is not responsive to the loci of DNA damage in *aub*, *armi*, and *spn-E* mutants, suggesting the piRNA associated DNA-repair pathway and the meiotic ATR/Chk2 DNA-repair pathway are independent and respond to distinct sources of DNA damage (Klattenhoff *et al.* 2007; Klattenhoff and Theurkauf 2008). The source of the DNA damage in piRNA pathway mutants is unknown, but candidates include transposon mobilization or a general role for the piRNA pathway in maintaining the structural integrity of specific chromatin domains in the germline (Klattenhoff and Theurkauf 2008).

Mutations in *aub*, *armi*, and *spn-E*, which cause DNA damage and the accumulation of phosphorylated H2Av, result in enhanced *garnet* silencing in both *Dp(1;f)LJ9*^{PAT} and *Dp(1;f)LJ9*^{MAT} flies. This finding corresponds to the effect of H2Av mutations (Chapter 5). Furthermore, mutations in Domino, which is involved in the

exchange of H2Av at DNA double-strand breaks (Kusch *et al.* 2004), also results in enhanced *garnet* silencing in *Dp(1;f)LJ9*^{PAT} flies (Chapter 5). Together, these results present the intriguing possibility that the piRNA pathway is involved in maintaining the structural integrity of the *Dp(1;f)LJ9* imprinting center during the early stages of embryogenesis. To determine if disruption of the *Dp(1;f)LJ9* imprint is specific to the piRNA pathway DNA damage response, I tested a mutation in the DNA-repair gene *spn-D* for its effects on *Dp(1;f)LJ9 garnet* expression. Like *spn-E* mutations, mutations in *spn-D* resulted in the accumulation of phosphorylated H2Av (Abdu *et al.* 2003). However, unlike Spn-E, Spn-D function involves the ATR/Chk2 DNA-repair pathway and responds to meiotic double-strand breaks (Klattenhoff *et al.* 2007). I found that the *spn-D*^{2} null allele (Abdu *et al.* 2003) had no significant affect on *garnet* expression in either *Dp(1;f)LJ9*^{PAT} or *Dp(1;f)LJ9*^{MAT} flies (Table 6.6 and Figures 6.10A and 6.10B, respectively). These results reinforce the possibility that the piRNA pathway, independent of the DNA-repair pathway, is involved in regulating chromatin structure and integrity within the *Dp(1;f)LJ9* imprinting center.

6.3.7 Piwi Function Is Distinct From Other piRNA Pathway Components In The Regulation Of *Dp(1;f)LJ9*^{PAT} Silencing

The mutant *piwi* allele affected paternal-specific silencing from the *Dp(1;f)LJ9* imprinting center, distinguishing its function from all the other piRNA pathway mutations that enhanced silencing from both paternally and maternally inherited *Dp(1;f)LJ9*. Piwi also stands out from other piRNA components for it nuclear localization and general function in somatic cells as well as germline cells (Klattenhoff and Theurkauf 2008). While the details of the nuclear function of Piwi are still speculative, Piwi is presumed to have a role in chromatin formation (Klattenhoff and Theurkauf 2008). Piwi associates with chromatin in an RNA-dependent manner and directly interacts with HP1a (Brower-Toland *et al.* 2007), yet the recruitment of HP1 by Piwi may be region specific, as *piwi* mutations have been associated with both the decrease (Brower-Toland *et al.* 2007) and increase (Yin and Lin 2007; Moshkovich and Lei 2010) in the recruitment of heterochromatin. The finding that Piwi function is distinct from the other members of the piRNA pathway is interesting, and possible support for the

'Piwi-piRNA guidance' model of heterochromatin formation. The Piwi-piRNA guidance hypothesis proposes that the recruitment of HP1 is initiated by Piwi, and guided by piRNAs that target specific genomic regions for heterochromatin formation in somatic cells (Lin and Yin 2009). The generation of siRNAs from *Dp(1;f)LJ9*^{PAT} could act as guides for Piwi-directed HP1 recruitment, assisting in the initial paternal-specific formation of heterochromatin (Figure 6.11A).

Links between Piwi and the siRNA pathway have previously been described in *C. elegans* (Das *et al.* 2008; Han *et al.* 2009), but no such links between Piwi and siRNAs have been made definitively in *Drosophila*. It is known that Piwi does not interact with Dcr2 in cytoplasm (Megosh *et al.* 2006); however, Piwi function in the nucleus requires loading of target RNA guides from the cytoplasm (Saito *et al.* 2009), suggesting that guide siRNAs could direct Piwi nuclear targeting. Furthermore, heterochromatic silencing in somatic cells involves a Dcr2 and RNA polymerase II silencing complex that is associated with Piwi, suggesting that siRNA guided transcriptional silencing can involve Piwi (Kavi and Birchler 2009). The involvement of Piwi in heterochromatin formation could also account for the enhanced silencing observed from mutations in the other piRNA components. Piwi competes for piRNAs guides from multiple sources (Li *et al.* 2009); thus, a decrease in the available pool of piRNAs early in development, caused by piRNA mutations, may increase Piwi availability for targeting of the *Dp(1;f)LJ9* imprinted domain and silencing would be enhanced (Figure 6.11B). Further insight into Piwi function and heterochromatin formation comes from mammals, where Piwi directs DNA methylation of targeted genomic regions leading to heterochromatin formation (Aravin *et al.* 2007; Aravin and Hannon 2008; Ooi *et al.* 2009). In Chapter 4, I proposed that DNA methylation demarcates and stabilizes the paternal *Dp(1;f)LJ9* imprint, contributing to the formation of heterochromatin and *garnet* silencing in *Dp(1;f)LJ9*^{PAT} flies. It remains to be determined in Piwi is involved in directing paternal-specific DNA methylation on the *Dp(1;f)LJ9* mini-X chromosome.

Table 6.1 - The Effects Of siRNA Mutations On *Dp(1:f)LJ9 garnet* Expression

	Eye Variegation Index						Summary Effect
	n	**0**	**1**	**2**	**3**	**Score**	
***Dp(1;f)LJ9*^{PAT}**							
Control	300	21	57	143	79	1.93 ± 0.05	
ago2^{dop1} /+	300	9	24	71	196	2.51 ± 0.04*	↑↑↑
ago2^{EY04479} /+	300	7	25	65	203	2.55 ± 0.04*	↑↑↑
dcr2$^{L811\ fsx}$ /+	200	7	21	56	116	2.41 ± 0.06*	↑↑↑
r2d2^{1} /+	300	17	36	104	143	2.24 ± 0.05*	↑↑↑
r2d2$^{S165\ fsx}$ /+	200	7	20	40	133	2.50 ± 0.06*	↑↑↑
r2d2^{EY00028} /+	300	4	15	51	130	2.54 ± 0.05*	↑↑↑
***Dp(1;f)LJ9*^{MAT}**							
Control	300	0	0	0	300	3.00 ± 0.0	
ago2^{dop1} /+	300	0	0	0	300	3.00 ± 0.0	/
ago2^{EY04479} /+	300	0	0	0	300	3.00 ± 0.0	/
dcr2$^{L811\ fsx}$ /+	200	0	0	0	300	3.00 ± 0.0	/
r2d2^{1} /+	300	0	0	0	300	3.00 ± 0.0	/
r2d2$^{S165\ fsx}$ /+	200	0	0	0	300	3.00 ± 0.0	/
r2d2^{EY00028} /+	200	0	0	0	300	3.00 ± 0.0	/

Variegation index values are based on a scale of visible red eye pigment levels; 0 (0-25%), 1 (25-50%,) 2 (50-75%), 3 (75-100%). The *n* value represents the number of individual eyes assayed, average score ± SEM. The summary effect is determined by combined pigment assays (Figure 6.3) and eye variegation assays and represented as either: a strong (↑↑↑), moderate (↑↑), or weak (↑) increase in expression; a strong (↓↓↓), moderate (↓↓), or weak (↓) increase in silencing; or no affect (/).

Table 6.2 - The Effects Of miRNA Mutations On *Dp(1:f)LJ9 garnet* Expression

	Eye Variegation Index						Summary Effect
	n	**0**	**1**	**2**	**3**	**Score**	
***Dp(1;f)LJ9*^{PAT}**							
Control	300	21	57	143	79	1.93 ± 0.05	
ago1^{k08121} /+	300	8	43	64	185	2.42 ± 0.05*	↑↑↑
ago1^{04845} /+	300	2	34	80	184	2.49 ± 0.04*	↑↑↑
ago1^{k00208} /+	300	6	91	110	93	1.97 ± 0.05	/
loqs^{f00791} /+	300	4	15	105	176	2.51 ± 0.04*	↑↑↑
pasha^{EY01325} /+	300	22	61	106	111	2.02 ± 0.05	/
rm62^{01086} /+	200	29	40	66	65	1.84 ± 0.07	/
***Dp(1;f)LJ9*^{MAT}**							
Control	300	0	0	0	300	3.00 ± 0.0	
ago1^{k08121} /+	300	0	0	0	300	3.00 ± 0.0	/
ago1^{04845} /+	300	0	0	0	300	3.00 ± 0.0	/
ago1^{k00208} /+	300	0	0	0	300	3.00 ± 0.0	/
loqs^{f00791} /+	300	0	0	0	300	3.00 ± 0.0	/
pasha^{EY01325} /+	300	0	0	0	300	3.00 ± 0.0	/
rm62^{01086} /+	200	0	0	0	200	3.00 ± 0.00	/

Variegation index values are based on a scale of visible red eye pigment levels; 0 (0-25%), 1 (25-50%,) 2 (50-75%), 3 (75-100%). The *n* value represents the number of individual eyes assayed, average score ± SEM. The summary effect is determined by combined pigment assays (Figure 6.4) and eye variegation assays and represented as either: a strong (↑↑↑), moderate (↑↑), or weak (↑) increase in expression; a strong (↓↓↓), moderate (↓↓), or weak (↓) increase in silencing; or no affect (/).

Table 6.3 - The Effects Of piRNA Mutations On *Dp(1:f)LJ9 garnet* Expression

		Eye Variegation Index					Summary Effect
	n	**0**	**1**	**2**	**3**	**Score**	
***Dp(1;f)LJ9*^{PAT}**							
Control	300	21	57	143	79	1.93 ± 0.05	
aub^{HN} /+	300	48	67	120	65	1.67 ± 0.06*	↓↓
aub^{QC42} /+	300	34	77	113	76	1.77 ± 0.06*	↓↓
aub^{KG05389} /+	200	22	43	79	56	1.85 ± 0.07	/
armi$^{72.1}$ /+	300	76	78	97	49	1.39 ± 0.06*	↓↓↓[a]
piwi^{3} /+	300	7	18	87	188	2.52 ± 0.04*	↑↑↑
spn-E^{1} /+	300	83	76	88	53	1.37 ± 0.06*	↓↓↓
spn-E^{c00786} /+	300	63	81	124	32	1.42 ± 0.04*	↓↓↓
squ^{PP32} /+	300	39	73	105	83	1.77 ± 0.06*	↓↓
zuc^{RS49} /+	300	43	70	108	79	1.74 ± 0.06*	↓↓
***Dp(1;f)LJ9*^{MAT}**							
Control	300	0	0	0	300	3.00 ± 0.00	
aub^{HN} /+	300	0	0	7	293	2.98 ± 0.01	↓↓↓
aub^{QC42} /+	300	0	0	4	296	2.99 ± 0.01	↓↓
aub^{KG05389} /+	300	0	0	4	296	2.99 ± 0.01	↓↓
armi$^{72.1}$ /+	300	0	5	9	286	2.93 ± 0.02	↓↓↓
piwi^{3} /+	300	0	0	0	300	3.00 ± 0.00	/
spn-E^{1} /+	200	0	1	8	191	2.95 ± 0.01	↓↓↓
spn-E^{c00786} /+	300	0	0	5	295	2.98 ± 0.01	↓↓↓
squ^{PP32} /+	300	0	0	2	298	2.99 ± 0.01	↓↓
zuc^{RS49} /+	300	0	0	4	296	2.99 ± 0.01	↓

Variegation index values are based on a scale of visible red eye pigment levels; 0 (0-25%), 1 (25-50%,) 2 (50-75%), 3 (75-100%). The *n* value represents the number of individual eyes assayed, average score ± SEM. The summary effect is determined by combined pigment assays (Figure 6.7) and eye variegation assays and represented as either: a strong (↑↑↑), moderate (↑↑), or weak (↑) increase in expression; a strong (↓↓↓), moderate (↓↓), or weak (↓) increase in silencing; or no affect (/).

[a]pre-zygotic activation maternal effect caused by the allele being present in mothers

Table 6.4 - The Effects Of *vasa* Mutations On *Dp(1:f)LJ9 garnet* Expression

		Eye Variegation Index					Summary Effect
	n	**0**	**1**	**2**	**3**	**Score**	
***Dp(1;f)LJ9*^{PAT}**							
Control	300	21	57	143	79	1.93 ± 0.05	
vasa^{1} /+	300	14	25	109	152	2.33 ± 0.05*	↓↓↓[a]
vasa^{RJ36} /+	300	18	22	107	153	2.32 ± 0.05*	↓↓
vig^{C274} /+	300	17	51	112	120	2.12 ± 0.05*	↓
vig^{EY07816} /+	300	22	41	113	124	2.13 ± 0.05*	↓
***Dp(1;f)LJ9*^{MAT}**							
Control	300	0	0	0	300	3.00 ± 0.0	
vasa^{1} /+	300	0	0	0	300	3.00 ± 0.0	/
vasa^{RJ36} /+	300	0	0	0	300	3.00 ± 0.0	/
vig^{C274} /+	200	0	0	0	200	3.00 ± 0.0	/
vig^{EY07816} /+	300	0	0	0	300	3.00 ± 0.0	/

Variegation index values are based on a scale of visible red eye pigment levels; 0 (0-25%), 1 (25-50%,) 2 (50-75%), 3 (75-100%). The *n* value represents the number of individual eyes assayed, average score ± SEM. The summary effect is determined by combined pigment assays (Figure 6.8) and eye variegation assays and represented as either: a strong (↑↑↑), moderate (↑↑), or weak (↑) increase in expression; a strong (↓↓↓), moderate (↓↓), or weak (↓) increase in silencing; or no affect (/).

[a]pre-zygotic activation maternal effect caused by the allele being present in mothers

Table 6.5 - The Effects Of RNAi Mutations On The Establishment Of The Maternal *Dp(1:f)LJ9* Imprint

	Eye Variegation Index						Summary Effect
Maternally present allele	***n***	**0**	**1**	**2**	**3**	**Score**	
Control	100	0	0	0	100	3.00 ± 0.0	
aub^{HN} /+	100	0	3	21	76	2.73 ± 0.05*	↓↓↓
aub^{HN} Control	100	0	0	0	100	3.00 ± 0.0	/
ago1^{k08121} /+	100	0	0	0	100	3.00 ± 0.0	/
ago1^{k08121} Control	100	0	0	0	100	3.00 ± 0.0	/
ago1^{04845} /+	100	0	0	0	100	3.00 ± 0.0	/
ago1^{04845} Control	100	0	0	0	100	3.00 ± 0.0	/
ago1^{k00208} /+	100	0	0	0	100	3.00 ± 0.0	/
ago1^{k00208} Control	100	0	0	0	100	3.00 ± 0.0	/
piwi^{3} /+	100	0	0	0	100	3.00 ± 0.0	/
piwi^{3} Control	100	0	0	0	100	3.00 ± 0.0	/

Variegation index values are based on a scale of visible red eye pigment levels; 0 (0-25%), 1 (25-50%,) 2 (50-75%), 3 (75-100%). The *n* value represents the number of individual eyes assayed, average score ± SEM. The summary effect is determined by combined pigment assays (Figure 6.9) and eye variegation assays and represented as either: a strong (↑↑↑), moderate (↑↑), or weak (↑) increase in expression; a strong (↓↓↓), moderate (↓↓), or weak (↓) increase in silencing; or no affect (/).

Table 6.6 - The Effects Of *spn-D* Mutations On *Dp(1:f)LJ9 garnet* Expression

	Eye Variegation Index						Summary Effect
	n	**0**	**1**	**2**	**3**	**Score**	
***Dp(1;f)LJ9*^{PAT}**							
Control	300	21	57	143	79	1.93 ± 0.05	/
spn-D^2 /+	80	6	13	38	23	1.98 ± 0.1	/
***Dp(1;f)LJ9*^{MAT}**							
Control	300	0	0	0	300	3.00 ± 0.00	/
spn-D^2 /+	200	0	0	0	200	3.00 ± 0.00	/

Variegation index values are based on a scale of visible red eye pigment levels; 0 (0-25%), 1 (25-50%,) 2 (50-75%), 3 (75-100%). The *n* value represents the number of individual eyes assayed, average score ± SEM. The summary effect is determined by combined pigment assays (Figure 6.10) and eye variegation assays and represented as either: a strong (↑↑↑), moderate (↑↑), or weak (↑) increase in expression; a strong (↓↓↓), moderate (↓↓), or weak (↓) increase in silencing; or no affect (/).

Table 6.7 - Internal Controls For *Dp(1:f)LJ9*^{PAT}; 2nd Chromosome Mutations Balanced Over *CyO*

	Variegation Assay						Pigment Assay	
	Eye Variegation Index							
Maternally present allele // balancer	***n***	**0**	**1**	**2**	**3**	**Score**	***n***	**% wild-type**
No modifier // CyO	300	22	55	132	91	1.97 ± 0.05	20	39±5
ago1^{k08121} *// CyO*	300	12	56	122	110	2.1 ± 0.05	20	43±7
ago1^{04845} *// CyO*	300	13	43	121	123	2.18 ± 0.05	20	42±5
ago1^{k00208} *// CyO*	300	21	80	114	85	1.88 ± 0.05	20	36±4
aub^{HN} *// CyO*	300	27	39	127	107	2.05 ± 0.05	20	39±9
aub^{QC42} *// CyO*	300	25	51	146	78	1.92 ± 0.05	20	40±6
aub^{KG05389} */ CyO*	200	13	25	95	67	2.08 ± 0.05	20	41±6
dcr2$^{L811\ fsx}$ *// CyO*	200	18	48	77	57	1.87 ± 0.07	20	41±3
loqs^{f00791} *// CyO*	300	35	66	121	78	1.81 ± 0.06	20	38±6
piwi^{3} *// CyO*	300	20	57	138	85	1.95 ± 0.05	20	37±5
r2d2^{1} *// CyO*	300	21	41	124	114	2.10 ± 0.05	20	34±7
r2d2$^{S165\ fsx}$ *// CyO*	200	16	41	72	71	1.99 ± 0.07	20	40±6
r2d2^{EY00028} *// CyO*	300	23	33	85	59	1.90 ± 0.07	20	40±7
squ^{PP32} *// CyO*	300	26	55	120	99	1.97 ± 0.05	20	41±5
vasa^{1} *// CyO*	300	16	40	117	127	2.18 ± 0.05	20	49±5***
vasa^{RJ36} *// CyO*	300	22	52	103	123	2.09 ± 0.05	20	42±6
vig^{C274} *// CyO*	300	34	53	104	109	1.96 ± 0.06	20	33±4
vig^{EY07816} *// CyO*	300	24	51	101	84	1.94 ± 0.06	20	41±4
zuc^{RS49} *// CyO*	300	24	53	122	101	2.00 ± 0.05	20	39±5

Eye variegation index values are based on a scale of visible red eye pigment levels; 0 (0-25%), 1 (25-50%,) 2 (50-75%), 3 (75-100%). The *n* value represents the number of individual eyes assayed, average score ± SEM. Pigment assay values are presented as a mean percentage of wild-type levels ± SD, the *n* value represents the number of independent measurements, an ANOVA followed by Bonferroni-corrected Student's t-tests determined significance. Both the eye variegation assay and pigment assays determined that *vasa*^{1} is the only 2nd chromosome mutation tested that produces a maternal effect on *garnet* expression from *Dp(1;f)*LJ9^{PAT}, causing an increase in *garnet* pigment levels.

Table 6.8 - Internal Controls For *Dp(1:f)LJ9*^{PAT}; 3rd Chromosome Mutations Balanced Over *TM3, Sb*

	Variegation Assay						Pigment Assay	
	Eye Variegation Index							
Maternally present allele // balancer	***n***	**0**	**1**	**2**	**3**	**Score**	***n***	**% wild-type**
No modifier // TM3, Sb	300	34	54	128	84	1.87 ± 0.05	20	24±6
ago2^{dop1} *// TM3, Sb*	300	28	57	122	93	1.93 ± 0.05	20	26±6
ago2^{EY04479} *// TM3, Sb*	300	26	67	103	104	1.95 ± 0.05	20	27±6
armi$^{72.1}$ *// TM3, Sb*	200	45	43	57	55	1.61 ± 0.08*	20	18±3**
pasha^{EY01325} *// TM3, Sb*	200	10	33	87	70	2.09 ± 0.06	20	26±5
rm62^{01086} *// TM3, Sb*	100	11	22	34	33	1.89 ± 0.1	20	22±6
spn-D^{2} *// TM3, Sb*	80	7	10	44	19	1.94 ± 0.09	20	21±3
spn-E^{1} *// TM3, Sb*	200	24	41	58	77	1.94 ± 0.07	20	20±4
spn-E^{c00786} *// TM3, Sb*	200	21	39	79	61	1.90 ± 0.07	20	20±5

Eye variegation index values are based on a scale of visible red eye pigment levels; 0 (0-25%), 1 (25-50%,) 2 (50-75%), 3 (75-100%). The *n* value represents the number of individual eyes assayed, average score ± SEM. Pigment assay values are presented as a mean percentage of wild-type levels ± SD, the *n* value represents the number of independent measurements, an ANOVA followed by Bonferroni-corrected Student's t-tests determined significance. Males carrying the *TM3, Sb* balancers are physically smaller, which results in a lower pigment assay score despite no significant change in the levels of variegation present. There is no significant difference between *Dp(1;f)*LJ9; TM3*, Sb* balanced internal controls and the 'no modifier' *Dp(1:f)LJ9; TM3, Sb* controls, with the exception of the *armi*$^{72.1}$ allele. Both the eye variegation assay and pigment assays determined that *armi*$^{72.1}$ is the only 3rd chromosome allele tested that produces a maternal effect on *garnet* expression from *Dp(1;f)*LJ9^{PAT}, causing a reduction in *garnet* pigment levels.

Table 6.9 - Internal Controls For *Dp(1:f)LJ9*^{MAT}; 2nd Chromosome Mutations Balanced Over *CyO*

	Variegation Assay						Pigment Assay	
	Eye Variegation Index							
Paternally present allele // balancer	***n***	**0**	**1**	**2**	**3**	**Score**	***n***	**% wild-type**
No modifier // CyO	300	0	0	0	300	3.00 ± 0.0	20	80±4
ago1^{k08121} *// CyO*	280	0	0	0	300	3.00 ± 0.0	20	77±8
ago1^{04845} *// CyO*	300	0	0	0	300	3.00 ± 0.0	20	81±7
ago1^{k00208} *// CyO*	300	0	0	0	300	3.00 ± 0.0	20	82±6
aub^{HN} *// CyO*	190	0	0	0	300	3.00 ± 0.0	20	81±8
aub^{QC42} *// CyO*	300	0	0	1	299	3.00 ± 0.01	20	78±7
aub^{KG05389} */ CyO*	300	0	0	0	300	3.00 ± 0.0	20	82±7
dcr2$^{L811\ fsx}$ *// CyO*	200	0	0	0	200	3.00 ± 0.0	20	77±5
loqs^{f00791} *// CyO*	300	0	0	0	300	3.00 ± 0.0	20	83±6
piwi^{3} *// CyO*	300	0	0	0	300	3.00 ± 0.0	20	83±5
r2d2^{1} *// CyO*	300	0	0	0	300	3.00 ± 0.0	20	83±5
r2d2$^{S165\ fsx}$ *// CyO*	200	0	0	0	200	3.00 ± 0.0	20	76±7
r2d2^{EY00028} *// CyO*	200	0	0	0	200	3.00 ± 0.0	20	84±7
squ^{PP32} *// CyO*	300	0	0	0	300	3.00 ± 0.0	20	80±3
vasa^{1} *// CyO*	300	0	0	0	300	3.00 ± 0.0	20	81±5
vasa^{RJ36} *// CyO*	300	0	0	0	300	3.00 ± 0.0	20	77±7
vig^{C274} *// CyO*	300	0	0	0	300	3.00 ± 0.0	20	81±6
vig^{EY07816} *// CyO*	300	0	0	0	300	3.00 ± 0.0	20	79±7
zuc^{RS49} *// CyO*	300	0	0	0	300	3.00 ± 0.0	20	80±4

Eye variegation index values are based on a scale of visible red eye pigment levels; 0 (0-25%), 1 (25-50%,) 2 (50-75%), 3 (75-100%). The *n* value represents the number of individual eyes assayed, average score ± SEM. Pigment assay values are presented as a mean percentage of wild-type levels ± SD, the *n* value represents the number of independent measurements. An ANOVA followed by Bonferroni-corrected Student's t-tests determined no significant difference is present between the internal control progeny and the no modifier control for these mutations.

Table 6.10 - Internal Controls For *Dp(1:f)LJ9*^{MAT}; 3rd Chromosome Mutations Balanced Over *TM3, Sb*

	Variegation Assay						Pigment Assay	
	Eye Variegation Index							
Paternally present allele // balancer	***n***	**0**	**1**	**2**	**3**	**Score**	***n***	**% wild-type**
No modifier // TM3, Sb	300	0	0	0	300	3.00 ± 0.0	20	52±5
ago2^{dop1} // TM3, Sb	300	0	0	0	300	3.00 ± 0.0	20	50±6
ago2^{EY04479} // TM3, Sb	200	0	0	0	300	3.00 ± 0.0	20	51±5
armi$^{72.1}$ // TM3, Sb	300	0	0	1	299	3.00 ± 0.01	20	51±4
pasha^{EY01325} // TM3, Sb	300	0	0	0	300	3.00 ± 0.0	20	50±5
rm62^{01086} // TM3, Sb	300	0	0	0	300	3.00 ± 0.0	20	54±3
spn-D^{2} // TM3, Sb	200	0	0	0	200	3.00 ± 0.0	20	54±4
spn-E^{1} // TM3, Sb	300	0	0	0	300	3.00 ± 0.0	20	50±3
spn-E^{c00786} // TM3, Sb	300	0	0	1	299	3.00 ± 0.01	20	49±5

Eye variegation index values are based on a scale of visible red eye pigment levels; 0 (0-25%), 1 (25-50%,) 2 (50-75%), 3 (75-100%). The *n* value represents the number of individual eyes assayed, average score ± SEM. Pigment assay values are presented as a mean percentage of wild-type levels ± SD, the *n* value represents the number of independent measurements. Males carrying the *TM3, Sb* balancers are physically smaller, which results in a lower pigment assay score despite no significant change in the variegation phenotype. There is no significant difference between *Dp(1;f)*LJ9; TM3*, Sb* balanced internal controls and the 'no modifier' *Dp(1:f)LJ9; TM3, Sb* controls. An ANOVA followed by Bonferroni-corrected Student's t-tests determined no significant difference is present between the internal control progeny and the no modifier control for these mutations.

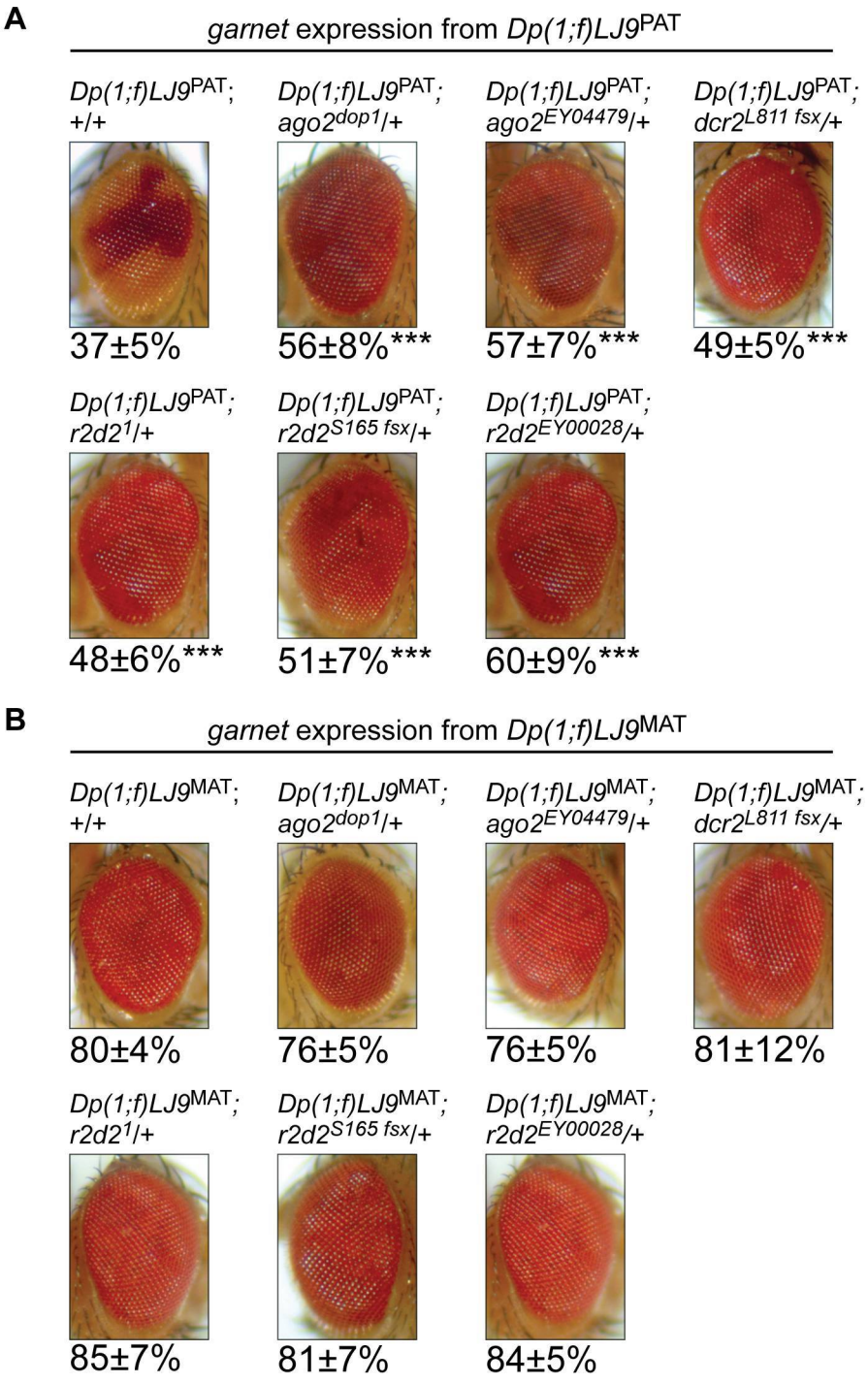

Figure 6.3 - The Effects Of siRNA Pathway Alleles On The Maintenance Of *Dp(1;f)LJ9 garnet* Expression.

Pigment assay values are presented below the representative eye picture as a mean percentage of wild-type levels ± standard deviation, n = 20 for all genotypes. Values significantly different from the no modifier controls are marked with one asterisk (p=<0.05-0.01), two (p=<0.01- p<0.001) or three (p<0.001).

(Figure 6.3 continued)

A) Representative eye pictures of *garnet* expression from paternally inherited *Dp(1;f)LJ9*^{PAT} illustrates relaxed silencing caused by all siRNA component alleles compared to the *Dp(1;f)LJ9; +/+* control, where variegated silencing of *garnet* is observed.
B**)** Maternally inherited *Dp(1;f)LJ9*^{MAT} produces full *garnet* expression (*Dp(1;f)LJ9; +/+*) , and no affect on *garnet* expression was observed from siRNA mutant alleles.

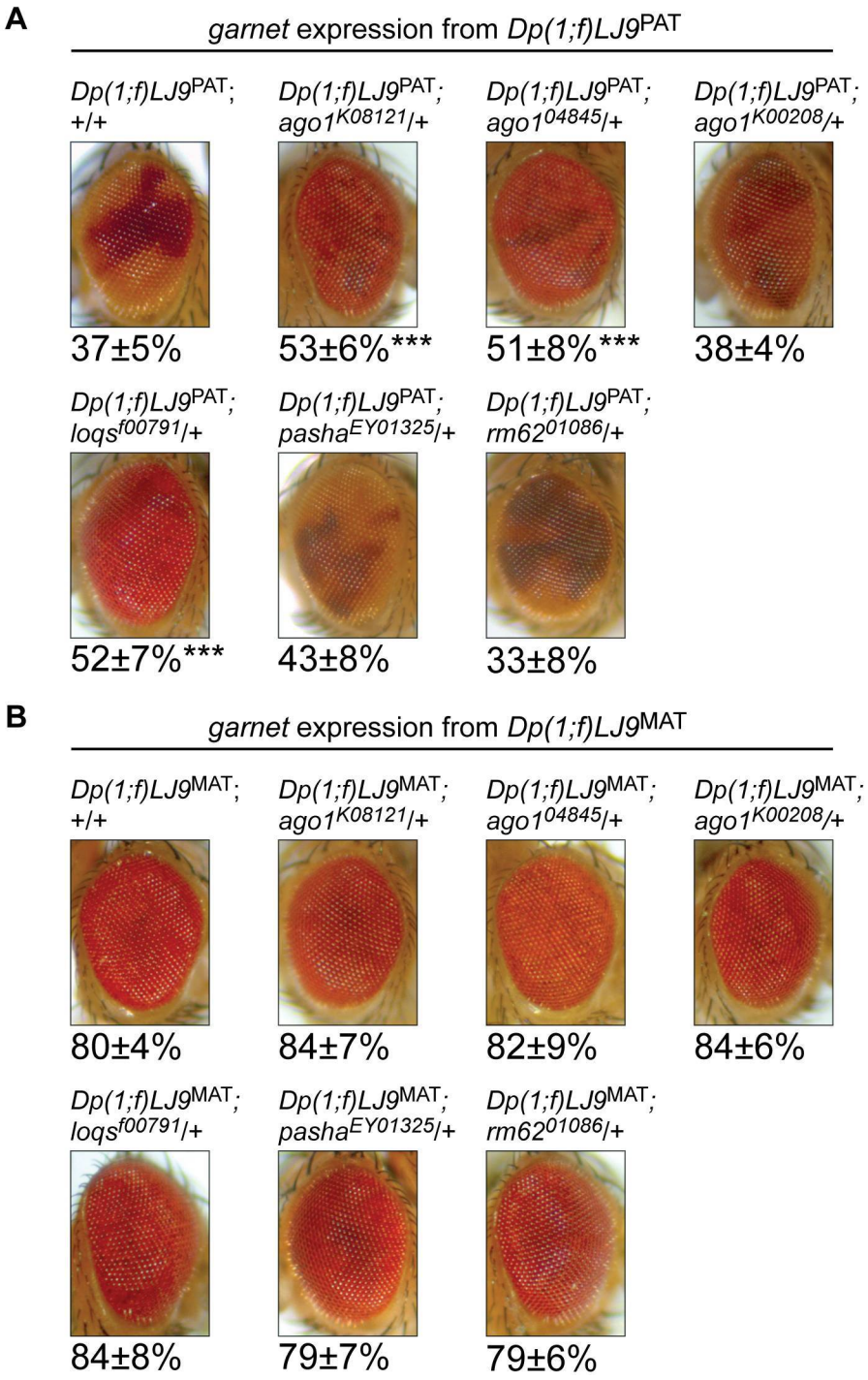

Figure 6.4 - The Effects Of miRNA Pathway Alleles On The Maintenance Of *Dp(1;f)LJ9 garnet* Expression.

Pigment assay values are presented below the representative eye picture as a mean percentage of wild-type levels ± standard deviation, n = 20 for all genotypes. Values significantly different from the no modifier controls are marked with one asterisk ($p=<0.05$-0.01), two ($p=<0.01$- $p<0.001$) or three ($p<0.001$).

(Figure 6.4 continued)

A) Representative eye pictures of *garnet* expression from paternally inherited *Dp(1;f)LJ9*^{PAT} illustrates relaxed silencing caused by the ,*ago1*^{k08121}, *ago1*^{k00208}, and *loqs*^{f00791} alleles compared to the *Dp(1;f)LJ9; +/+* control, where variegated silencing of *garnet* is observed.
B**)** Maternally inherited *Dp(1;f)LJ9*^{MAT} produces full *garnet* expression (*Dp(1;f)LJ9; +/+*) , and no affect on *garnet* expression was observed from miRNA mutant alleles.

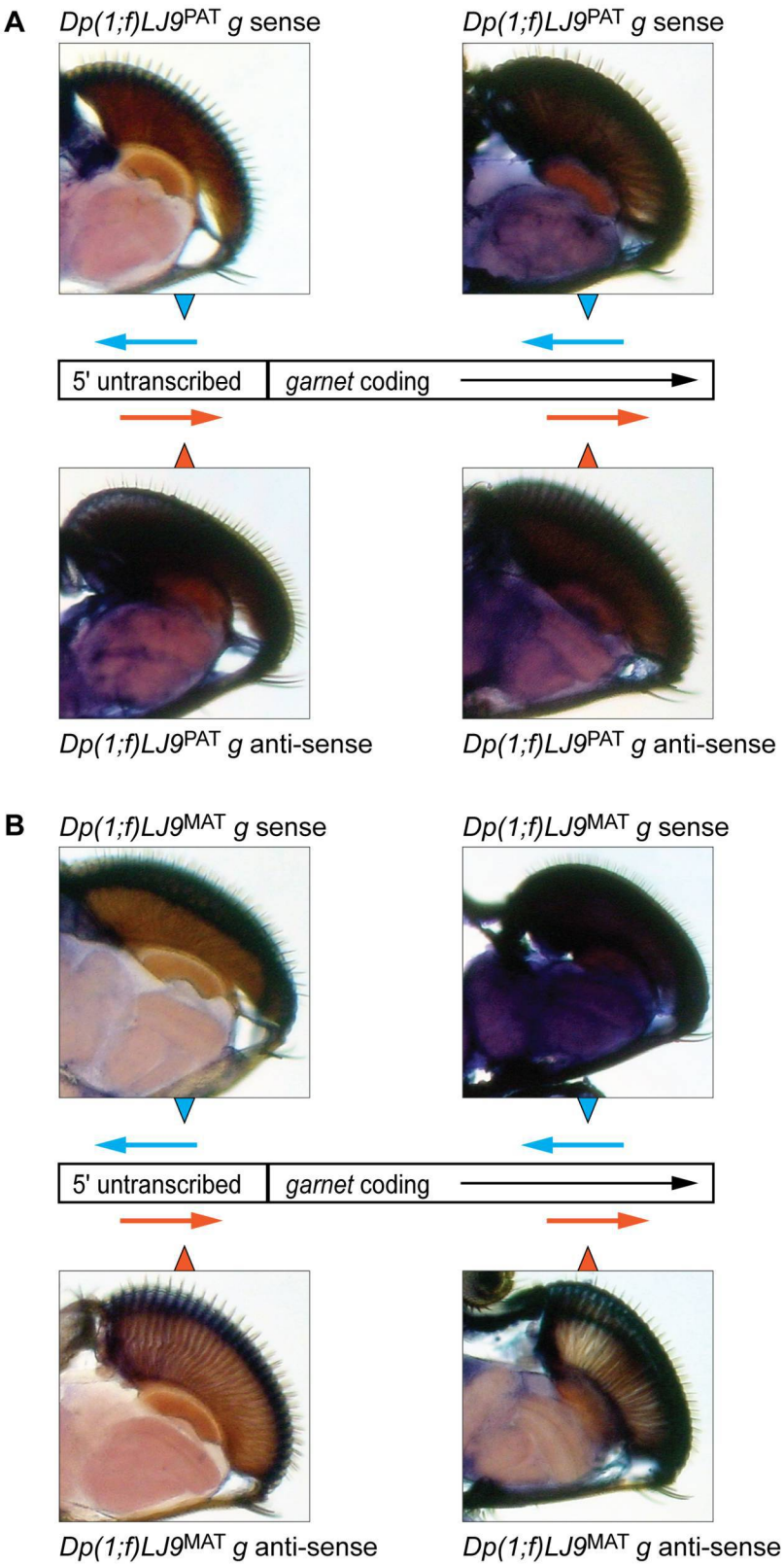

Figure 6.5 - Detection Of *garnet* Coding And Non-Coding Antisense Transcripts From *Dp(1;f)LJ9; +/+* Head Sections.

(Figure 6.5 continued)

In situ hybridization performed on sectioned *Drosophila* heads using probes to detect coding transcripts (blue) or non-coding antisense transcripts (orange). These probes detected either a 5' untranscribed region of the *garnet* promoter (left column) or a transcribed region of exon 9 of the *garnet* gene (right column).
A) *Dp(1;f)LJ9*^{PAT} inheritance results in detectable antisense, non-coding transcripts for both the coding and 5' untranscribed regions of *garnet*. As expected, sense strand detection of *garnet* occurs only from the coding region.
B) *Dp(1;f)LJ9*^{MAT} inheritance results in strong detection of sense *garnet*, corresponding to higher levels of *garnet* transcription. Low levels of antisense, non-coding *garnet* transcripts were detected from the coding region, while no transcripts were detected from the 5' untranscribed region by either sense or antisense probes.

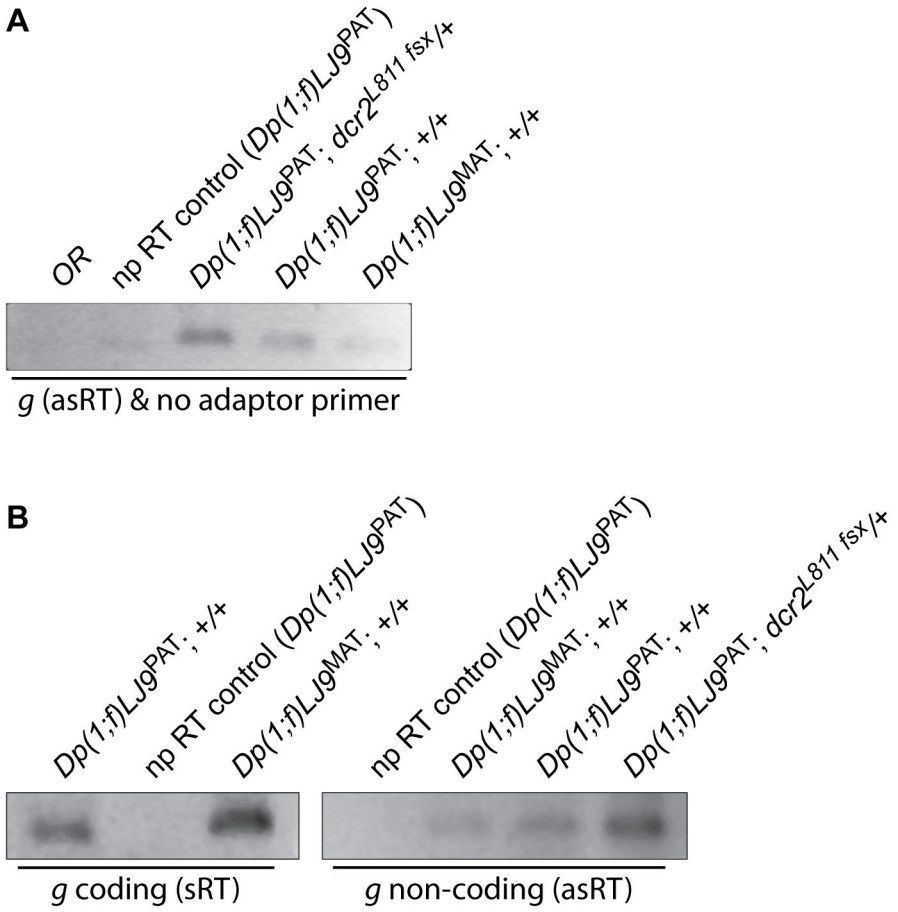

Figure 6.6 - RT-PCR Detection Of *garnet* Non-Coding Antisense Transcripts From RNA Extracted From *Dp(1;f)LJ9*^{PAT} And *Dp(1;f)LJ9*^{MAT} Heads.

A) No *garnet* antisense transcripts were detected in the heads of wild-type OR flies, while antisense *garnet* transcripts are detected in *Dp(1;f)LJ9*^{PAT}; +/+, *Dp(1;f)LJ9*^{PAT}; dcr2$^{L811\ fsx}$/+, and *Dp(1;f)LJ9*^{MAT}; +/+ heads. Self-primed transcripts were detected when standard primers are used; faint antisense transcripts were present in the no-primer (np) RT control, resembling the antisense *garnet* detection found in *Dp(1;f)LJ9*^{MAT}; +/+ flies.

B) Sense transcripts of *garnet* were detected in both *Dp(1;f)LJ9*^{PAT}; +/+ and *Dp(1;f)LJ9*^{MAT}; +/+ heads, and no self-primed transcripts were detected when the adaptor primer was used. Antisense *garnet* transcripts are detected in *Dp(1;f)LJ9*^{PAT}; +/+ and *Dp(1;f)LJ9*^{PAT}; dcr2$^{L811\ fsx}$/+ heads, while faint detection of antisense *garnet* transcripts were also found in *Dp(1;f)LJ9*^{MAT}; +/+ heads. No self-primed transcripts were detected in the antisense no-primer (np) RT control. No RT controls were also performed and yielded no amplification (data not shown).

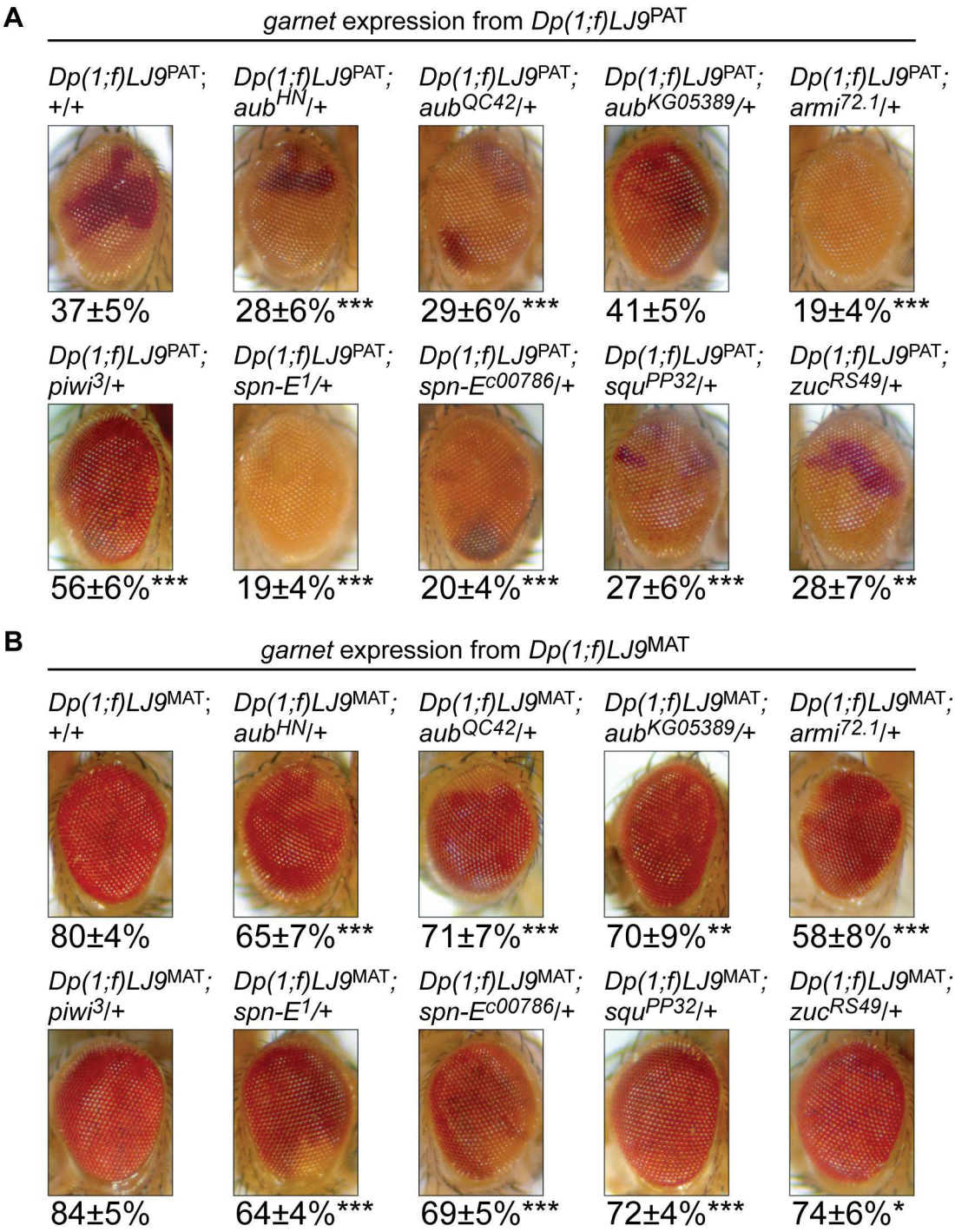

Figure 6.7 - The Effects Of piRNA Pathway Alleles On The Maintenance Of *Dp(1;f)LJ9 garnet* Expression.

Pigment assay values are presented below the representative eye picture as a mean percentage of wild-type levels ± standard deviation, n = 20 for all genotypes. Values significantly different from the no modifier controls are marked with one asterisk (p=<0.05-0.01), two (p=<0.01- p<0.001) or three (p<0.001).

(Figure 6.7 continued)

A) Representative eye pictures of *garnet* expression from paternally inherited *Dp(1;f)LJ9*^{PAT} illustrates enhanced silencing is caused by piRNA pathway mutations compared to the *Dp(1;f)LJ9; +/+* control. Only the *piwi*^{3} allele caused a reduction in the silencing of *garnet*.
B**)** Maternally inherited *Dp(1;f)LJ9*^{MAT} produces full *garnet* expression (*Dp(1;f)LJ9; +/+*), which was disrupted by all the tested piRNA mutant alleles except the *piwi*^{3} allele that had no affect on *garnet* expression.

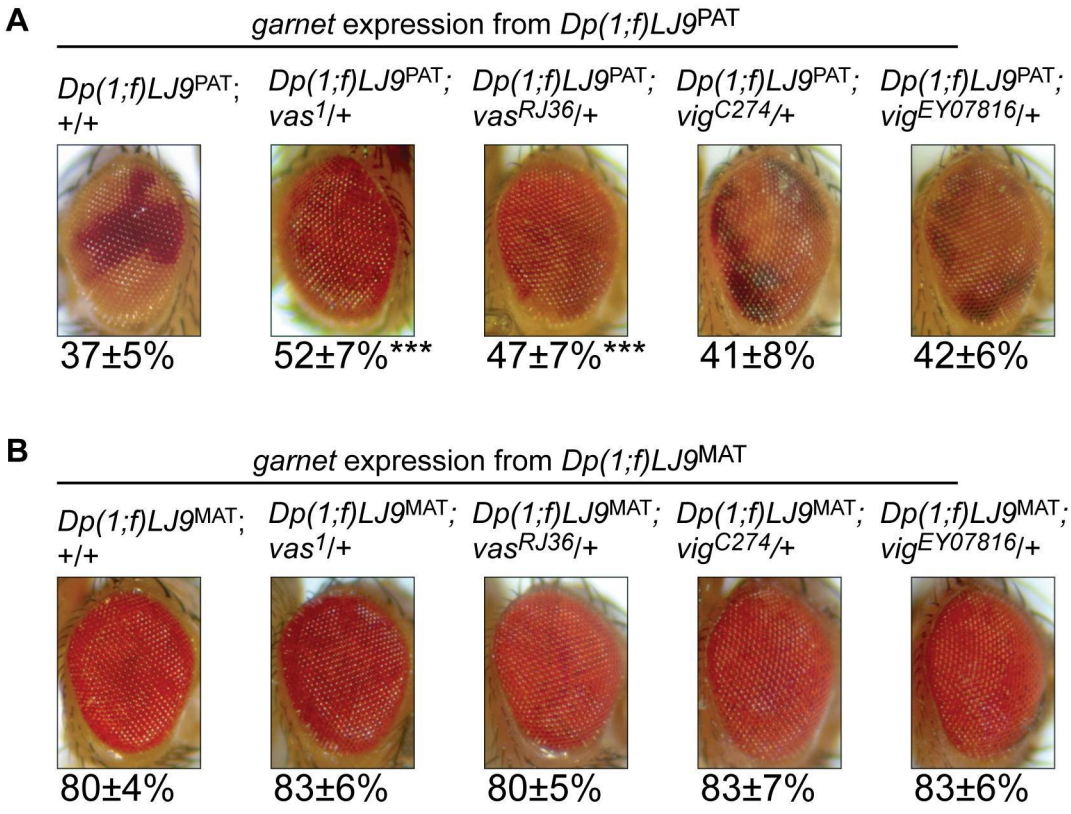

Figure 6.8 - The Effects Of *vas* And *vig* Alleles On The Maintenance Of *Dp(1;f)LJ9 garnet* Expression.

Pigment assay values are presented below the representative eye picture as a mean percentage of wild-type levels ± standard deviation, n = 20 for all genotypes except $Dp(1;f)LJ9^{PAT}$;vig^{C274}/+ (n=18) and $Dp(1;f)LJ9^{MAT}$;vig^{C274}/+ (n=15) . Values significantly different from the no modifier controls are marked with one asterisk (p=<0.05-0.01), two (p=<0.01- p<0.001) or three (p<0.001).

A) The tested *vas* alleles relax *garnet* silencing from paternally inherited $Dp(1;f)LJ9^{PAT}$, while the *vig* alleles show no affect on $Dp(1;f)LJ9^{PAT}$.

B**)** No affect was observed from either *vas* or *vig* alleles on maternally inherited $Dp(1;f)LJ9^{MAT}$ *garnet* expression.

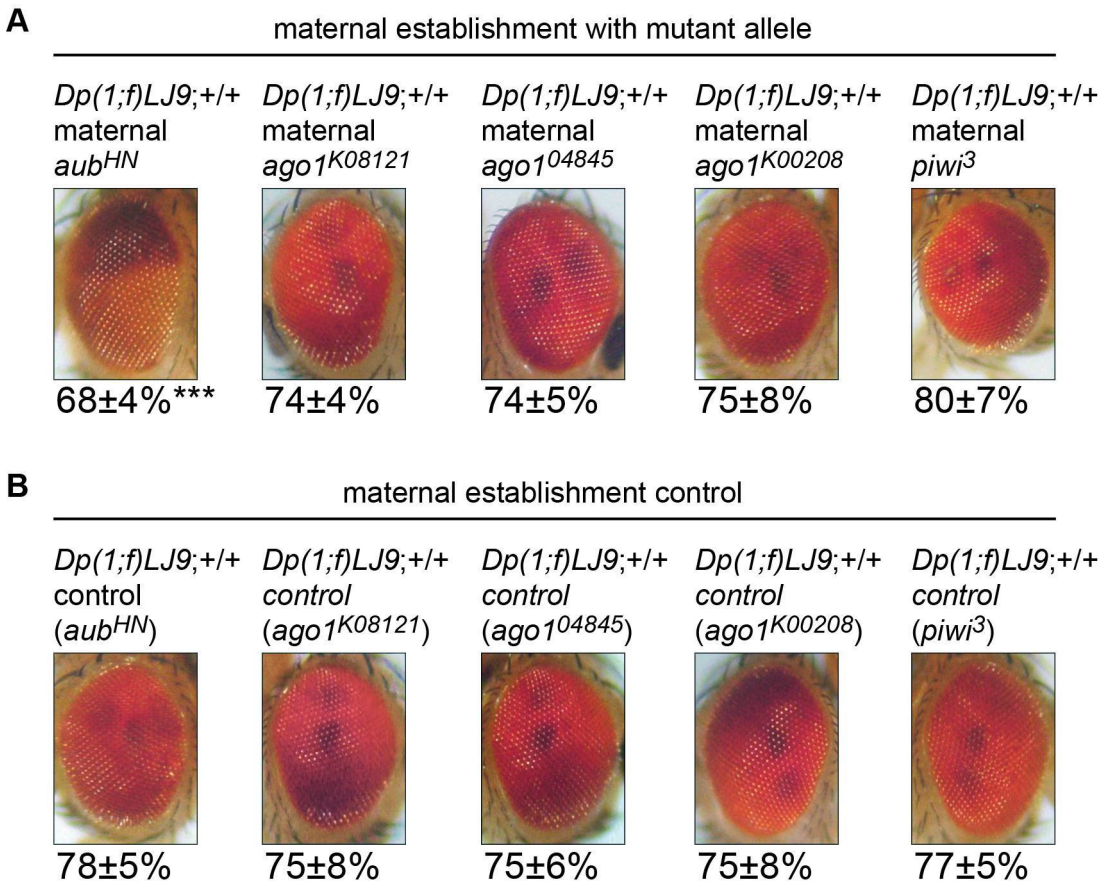

Figure 6.9 - *aub* Is Involved In The Establishment Of The *Dp(1;f)LJ9* Maternal Imprint.

Pigment assay values are presented below the representative eye picture as a mean percentage of wild-type levels ± standard deviation, n = 20 for all genotypes. Values significantly different from the no modifier controls are marked with one asterisk (p=<0.05-0.01), two (p=<0.01- p<0.001) or three (p<0.001).

A) The effect of maternally present alleles are shown; aub^{HN} was the only allele tested that disrupted the establishment of the $Dp(1;f)LJ9^{MAT}$ imprint, causing increased *garnet* silencing.

B) Representative controls for the effect of each maternally present allele, where the balancer *CyO* was maternally present instead of the modifier allele.

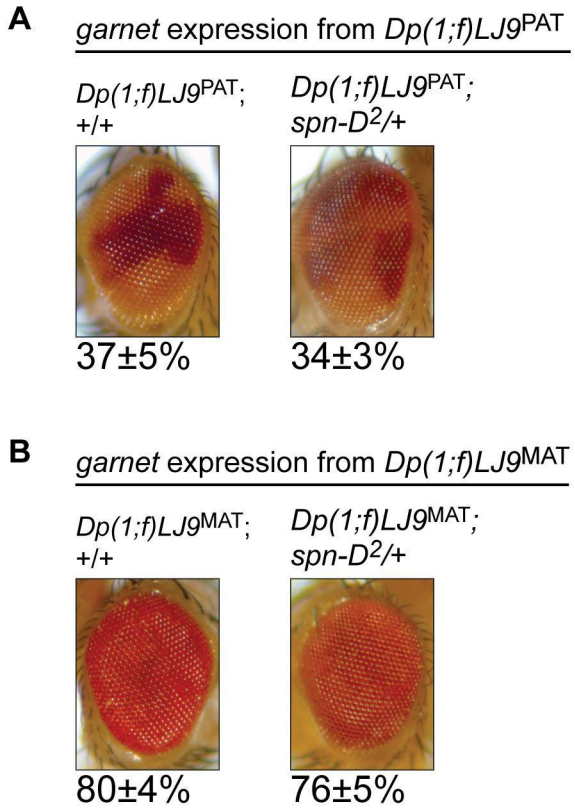

Figure 6.10 - The Effects Of The *spn-D*^{2} Allele On The Maintenance Of *Dp(1;f)LJ9 garnet* Expression.

Pigment assay values are presented below the representative eye picture as a mean percentage of wild-type levels ± standard deviation, n = 20 for all genotypes except *Dp(1;f)LJ9*^{PAT};*spn-D*^{2}/+ (n=10).

A) No affect was observed from the *spn-D*^{2} allele on paternally inherited *Dp(1;f)LJ9*^{PAT} *garnet* expression.

B**)** No affect was observed from the *spn-D*^{2} allele on maternally inherited *Dp(1;f)LJ9*^{MAT} *garnet* expression.

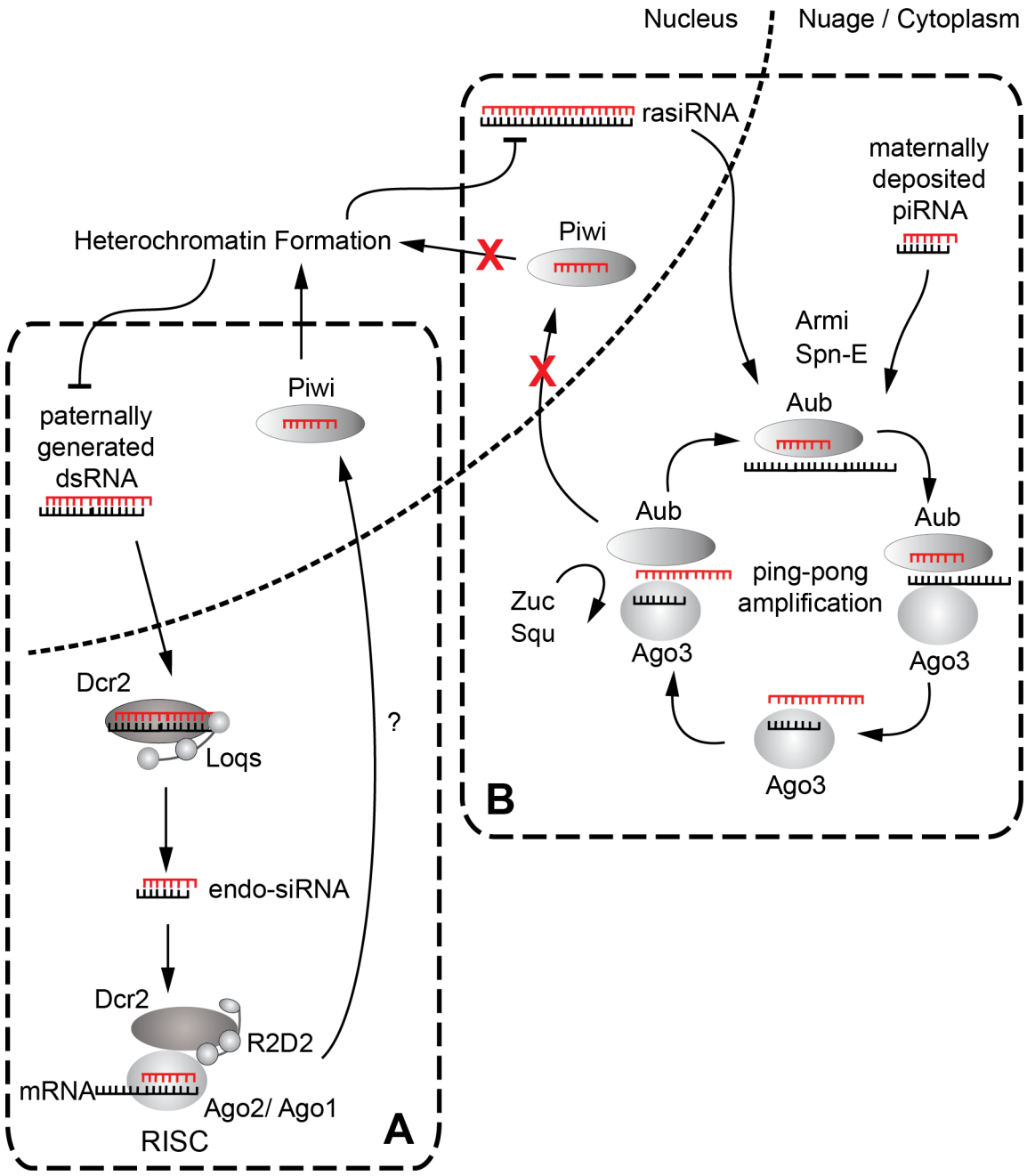

Figure 6.11 - Model For The Involvement Of Piwi And siRNAs In The Paternal-Specific Silencing Of The *Dp(1;f)LJ9* Imprint.

A) Antisense transcripts generated from the paternal *Dp(1;f)LJ9* imprinting center produce double-strand RNA (dsRNA) that is processed into endogenous siRNA guides. These siRNA guides direct posttranscriptional degradation of *garnet* transcripts, and the siRNA silencing complex may interact with Piwi to direct paternal-specific heterochromatin formation resulting in transcription silencing.

B) Disruption on the piRNA pathway prevents the loading of guide piRNAs into nuclear Piwi, which decreases competition for Piwi guided heterochromatin recruitment, resulting in an increase in Piwi guided silencing from the *Dp(1;f)LJ9* imprint center.

6.4 Conclusions

The establishment and maintenance of an imprinted domain require the function of multiple epigenetic mechanisms. Antisense transcripts are associated with a number of imprinted domains in both mammals and plants (Babak *et al.* 2008; Mosher *et al.* 2009), indicating that RNAi and posttranscriptional silencing may play a significant role in the regulation of genomic imprints. The mechanisms by which antisense transcripts and RNAi pathways participate in regulating imprinting remain to be determined for most imprinted domains.

I found that the siRNA pathway is involved in maintaining the paternal-specific silencing from the *Dp(1;f)LJ9* imprint center. Mutations in *ago2*, *dcr2*, *loqs*, and *r2d2* all resulted in a relaxation of $Dp(1;f)LJ9^{PAT}$ *garnet* silencing. Antisense transcripts, predominantly expressed from the paternally inherited *Dp(1;f)LJ9*, likely supply the siRNA pathway with guides to silence paternal-specific transcripts and recruit heterochromatin. Interestingly, my results functionally group the Dcr2 dependent siRNA pathway with both Ago1 and Piwi, which are components of the miRNA and piRNA pathways, respectively. Ago1 has overlapping function with Ago2 (Meyer *et al.* 2006) but, recently, Ago1 was found to form a heterochromatin recruitment complex with Dcr2 and RNA polymerase II, which was also associated with Piwi (Kavi and Birchler 2009). While the function of RNA polymerase II on $Dp(1;f)LJ9^{PAT}$ *garnet* silencing remains to be determined, my results suggest the involvement of the Dcr2-Ago1-Piwi silencing complex in regulating expression from paternally inherited *Dp(1;f)LJ9*. These results add to growing evidence that Piwi functions within the siRNA pathway in somatic cells, assisting in directing heterochromatin recruitment.

Significantly, my results showed distinct differences between the siRNA and piRNA pathways with regard to imprint regulation. Mutations in piRNA pathway components, with the exception of Piwi, caused enhanced silencing from both maternally and paternally inherited *Dp(1;f)LJ9*. This is the first reported example in *Drosophila* of Piwi functioning in a distinct and opposite manner to Aub and other members of the piRNA pathway. This irregularity might be a consequence of the regulatory requirements

of the imprint to both establish and maintain parent-specific expression that occurs in the germline and soma, respectively. While *piwi* is expressed in somatic cells, the piRNA genes *aub*, *armi*, and *spn-E* are only expressed in the germline and during early in embryonic development (Gauhar *et al.* 2008). The general disruption of both the maternal and paternal imprint from piRNA pathway mutations underscores the sensitivity of the imprint during the early stages of development, and suggests that the piRNA pathway may continue to maintain genomic integrity, as it does in the germline, during the early stages of embryonic development.

I also found that Aub is active in the establishment of the maternal imprint. Further experiments will need to be performed to determine the extent of piRNA involvement in establishing the imprint in the germline; nevertheless, this finding is significant, as it the first evidence for a link between piRNA function and imprint establishment. The specific function of Piwi in the somatic maintenance of the imprint but not germline establishment of the imprint clearly distinguishes Piwi function from Aub function. These findings may point to stratification of RNAi involvement in the regulation of an imprint, transitioning from germline establishment to somatic maintenance.

6.5 Materials And Methods

6.5.1 *Drosophila* Stocks

Stocks were maintained at 22°C and cultured on standard cornmeal, yeast, molasses and agar media with methylbenzoate (0.15%, Sigma). Crosses were held in shell vials with 15-20 virgin females mated to 10-15 selected males, and subcultured on three-day intervals (up to four times) before the parental cross was discarded. Crosses that produced low yields of desired progeny were replicated and the results were pooled. All mutant stocks were provided by the Bloomington *Drosophila* Stock Center except for the $dcr2^{L811\ fsx}$ and $R2D2^{S165\ fsx}$ alleles, which were kindly provided by R. Carthew (Northwestern University).

6.5.2 Experimental Crosses

Dp(1;f)LJ9 flies were balanced over an attached *X^X* or *X^Y* chromosome for maternal and paternal transmission. To visualize the imprinting of the *garnet* gene (*g*), *Dp(1;f)LJ9* flies are crossed to $y^1z^ag^{53d}$ homozygotes; selecting progeny with wild-type bodies (y^+) confirms the presence *Dp(1;f)LJ9*, while the zeste allele (z^a) reduces background eye color for the eye specific *garnet* allele (g^{53d}). To detect the effect of an allele on the maintenance of the imprint, all mutant alleles were crossed into a $y^1z^ag^{53d}$ background to yield stable stocks of: $y^1z^ag^{53d}/y^1z^ag^{53d}$; *allele*/*CyO* for second chromosome mutations, and $y^1z^ag^{53d}/y^1z^ag^{53d}$; *allele*/*TM3, Sb* for third chromosome mutations. The control cross for paternal imprint maintenance consisted of *Dp(1;f)LJ9*, y^+g^+/X^Y males crossed to $y^1z^ag^{53d}/y^1z^ag^{53d}$; CyO/+ or $y^1z^ag^{53d}/\ y^1z^ag^{53d}$; TM3, Sb/+ females. The reciprocal cross served as the control for maternal imprint maintenance. The experimental crosses mirrored the control crosses, except *Dp(1;f)LJ9*, y^+g^+/X^Y males were crossed to $y^1z^ag^{53d}/y^1z^ag^{53d}$; *allele*/*CyO* or $y^1z^ag^{53d}/y^1z^ag^{53d}$; *allele*/*TM3, Sb* females for paternal imprint maintenance, and the reciprocal cross tested for maternal imprint maintenance (Figure 6.12). Two male genotypes were collected from the maintenance experimental crosses; $y^1z^ag^{53d}$/*Dp(1;f)LJ9*; *allele*/+ progeny that determine the effect of the allele on imprint maintenance, and the internal control $y^1z^ag^{53d}$/*Dp(1;f)LJ9*; *CyO*/+ or *TM3, Sb*/+ progeny. The internal control genotype is notable for having the tested modifier allele present in the non-*Dp(1;f)LJ9* parent (mothers carry the mutation in the paternal cross; $Dp(1;f)LJ9^{PAT}$, fathers in the maternal cross; $Dp(1;f)LJ9^{MAT}$). These internal control progeny allow for the assessment of any pre-fertilization maternal or paternal effects for a mutation carried by mothers in the $Dp(1;f)LJ9^{PAT}$ cross and fathers in the $Dp(1;f)LJ9^{MAT}$ cross.

The imprint establishment crosses consisted of generating *Dp(1;f)LJ9* flies bearing mutant RNAi alleles (Figures 6.13 and 6.14): For paternal establishment, *X^Y/ Dp(1;f)LJ9* males were crossed to *modifier allele/CyO*, to yield *X^Y/ Dp(1;f)LJ9; modifier allele/*+ and control *X^Y/ Dp(1;f)LJ9; CyO/*+ flies. For maternal establishment, *X^X/ Dp(1;f)LJ9* females were crossed to *modifier allele/CyO*, to yield *X^X/ Dp(1;f)LJ9; modifier allele/*+ and control *X^X/ Dp(1;f)LJ9; CyO/*+ flies. To detect the effect of an allele on the establishment of the imprint, flies with the mutant allele being tested, *X^Y/*

Dp(1;f)LJ9; modifier allele/+ (paternal establishment) or *X^X/ Dp(1;f)LJ9; modifier allele/+* (maternal establishment) were crossed to opposite sex $y^1z^ag^{53d}$ flies, yielding $y^1z^ag^{53d}$*/Dp(1;f)LJ9* progeny from parents bearing RNAi mutant alleles. The corresponding control parental genotypes, with *CyO* instead of the mutant allele were used to generate $y^1z^ag^{53d}$*/Dp(1;f)LJ9* progeny wild-type for RNAi components. Eye pigment levels were compared between progeny from *modifier allele/+* parents and *CyO/+* parents. Oregon-R (OR) flies were used as wild-type.

6.5.3 Quantification Of Eye Pigment Levels

Drosophila eye pigment levels were quantified both visually and through the use a spectrophotometer to determine *garnet* expression levels. The visual assay consists of scoring each eye in relation to its degree of visible pigmentation (0=0-25% pigmentation, 1=25-50%, 2=50-75%, 3=75-100%), as described by Joanis and Lloyd (2002). Data from each sample set were pooled and expressed as the mean ± the standard error of the mean. Statistical significance was determined by a Kolmogorov-Smirnov two-sample test between the experimental sample group and the designated control sample group. The spectrophotometric assay was adapted from Real *et al.* (1985). Collected flies were aged for 3-4 days then placed in 1.5ml microtubes and stored at -30 °C. Heads were removed by chilling samples to -80 °C, then vigorously shaking until heads were detached. For each sample set, at least 100 heads were divided into groups of five and placed into 0.6ml microtubes containing 150ul of acidified ethanol (30% EtOH, pH 2 with HCL). Pigment was extracted from the samples by shaking at 150RPM in the dark for 36 ± 4 hours. Absorbance of the extracted pigments was measured at 480 nm with a Pharmacia Biotech Ultrospec 2000. Statistical significance was calculated by an ANOVA, followed by Bonferroni-corrected Student's t-tests to determine the p values for groups with significant difference between the mean of an experimental sample set and the mean of the comparative control group. Eyes were photographed with a Zeiss AxioCam MRc5, or a Sony DSC-S70, mounted on a Zeiss Stemi 2000-C dissecting microscope.

6.5.4 *In situ* Hybridization

Riboprobes were produced from *garnet* sequences inserted into a pBlueScript II SK (+) vector (Appendix B). Probes for the *garnet* coding region were created from a 0.7 kb *garnet* cDNA fragment (Lloyd 1999b) containing exon 9. Digestion with either *Xho*I or *Not*I was followed by transcription using either T7 (antisense probe; sense detecting) or T3 (sense probe; antisense detecting) polymerase, respectively, in the presence of Digoxygenin (DIG)-conjugated UTP (Roche). Probes for the *garnet* non-coding region were created from a 0.5 kb *garnet* sequence containing part of the untranscribed promoter region of *garnet* created from PCR amplification of genomic DNA with the primers 5'-GCTCTAGACTCCGTGGCGTTACTTTGAT-3' and 5'-GCGGGCCCAGCGAGTTATCGACAGTCCAC-3'. The generated product was inserted into a pBlueScript II SK (+) vector using the generated *Xba*I and *Apa*I sites. Digestion with either *Xba*I or *Apa*I was followed by transcription using either T7 (antisense probe; sense detecting) or T3 (sense probe; antisense detecting) polymerase, respectively, in the presence of Digoxygenin (DIG)-conjugated UTP (Roche). For increased specificity all probes were hydrolyzed to 300 bp in length with carbonate buffer following manufacturer's instructions (Roche).

Newly eclosed adult heads were dissected, with the mouthparts, removed and fixed in 4% paraformaldehyde for 2 x 20 min. Fixed heads were then embedded in 7% Type VIIA low geling Agar (Sigma) within silicone embedding molds (Polysciences, Inc.), with care taken to orient the heads correctly for sectioning. Set agar blocks were removed from the mold and glued to a cutting block with Loctite 404 instant adhesive. Heads were cut into 100 μm thick horizontal sections using a Vibratome and fixed again for 20 min in 4% paraformaldehyde. The *in situ* hybridization protocol was followed as described by Braissant and Wahli (1998), using the AP-conjugated anti-DIG antibody (Roche) and staining samples with NBT/BCIP (Roche). Stained head sections were mounted in 90% glycerol in PBS. Head sections were photographed with a Sony DSC-S70 mounted on a Zeiss Axiovert 25 microscope.

6.5.5 RT-PCR Analysis

The balanced X chromosome contains the g^{53d} allele, which affects eye specific *garnet* expression (Lloyd *et al*. 1999b); thus, to minimize background expression of *garnet*, I dissected newly eclosed adult heads from $y^1 z^a g^{53d}$/*Dp(1;f)LJ9* flies for source RNA. Mouthparts removed from the dissected heads prior to them being placed in RNAlater (Qiagen) on ice. Once 100 heads were dissected, RNA was extracted and purified using a Qiagen RNAeasy Kit, following manufacturer's instructions. cDNA was synthesized from head-extracted RNA using *garnet* gene-specific primers. RT reactions with no primers (np RT control) yielded amplification due to non-specific priming, a universal adapter sequence (**GCCTGCCCCAACCTCC**) attached to each gene-specific RT primer was used, as described by Shpiz *et al.* (2009). The universal adapter functions as a PCR primer site, avoiding non-specific priming artifacts when paired with a gene-specific primer.

Adaptor primers:

garnet sRT	(5'-**GCCTGCCCCAACCTCC**GATTCCCAGTGGCTGTTC-3')
garnet sPCR	(5'-GGTGTTCACAATGTGCAAGG-3')
garnet asRT	(5'-**GCCTGCCCCAACCTCC**GACGCTCAACATCCTGCT-3')
garnet asPCR	(5'-GCTGAAGCTTGTCCAACACA-3')
adapter PCR	(5'-GGAGGTTGGGGCAGGC-3')

Intron-spanning non-adaptor primers were used to distinguish self-primed transcripts arising from the RT reaction.

Intron-spanning non-adaptor primers:

garnet PCR1	(5'-GATGTCGTAGCCCAGCATTT-3')
garnet PCR2	(5'-CATATCGACGTGCATTGAGG-3')

5µg of total RNA from each sample was treated with DNAse I (Promega) prior to the RT reaction using SuperScript III Reverse Transcriptase Kit (Invitrogen), following manufacturer's instructions. 2µl of RT cDNA was used from each sample for a PCR

reaction using Tsg DNA Polymerase (BioBasic). PCR amplification was conducted using a Stratagene Robocycler and a touchdown protocol; 95°C for 1 min, followed by an amplification cycle of 95°C for 30 sec, 65-60°Cfor 1 min, and 72°C for 1min (annealing temperature decreased by 2°C per until 60°C was reached and used for the remaining 22 amplification cycles), with a final extension of 72°C for 7 min. Amplified PCR products were resolved on 1% agarose gels stained with SYBR Green (Invitrogen).

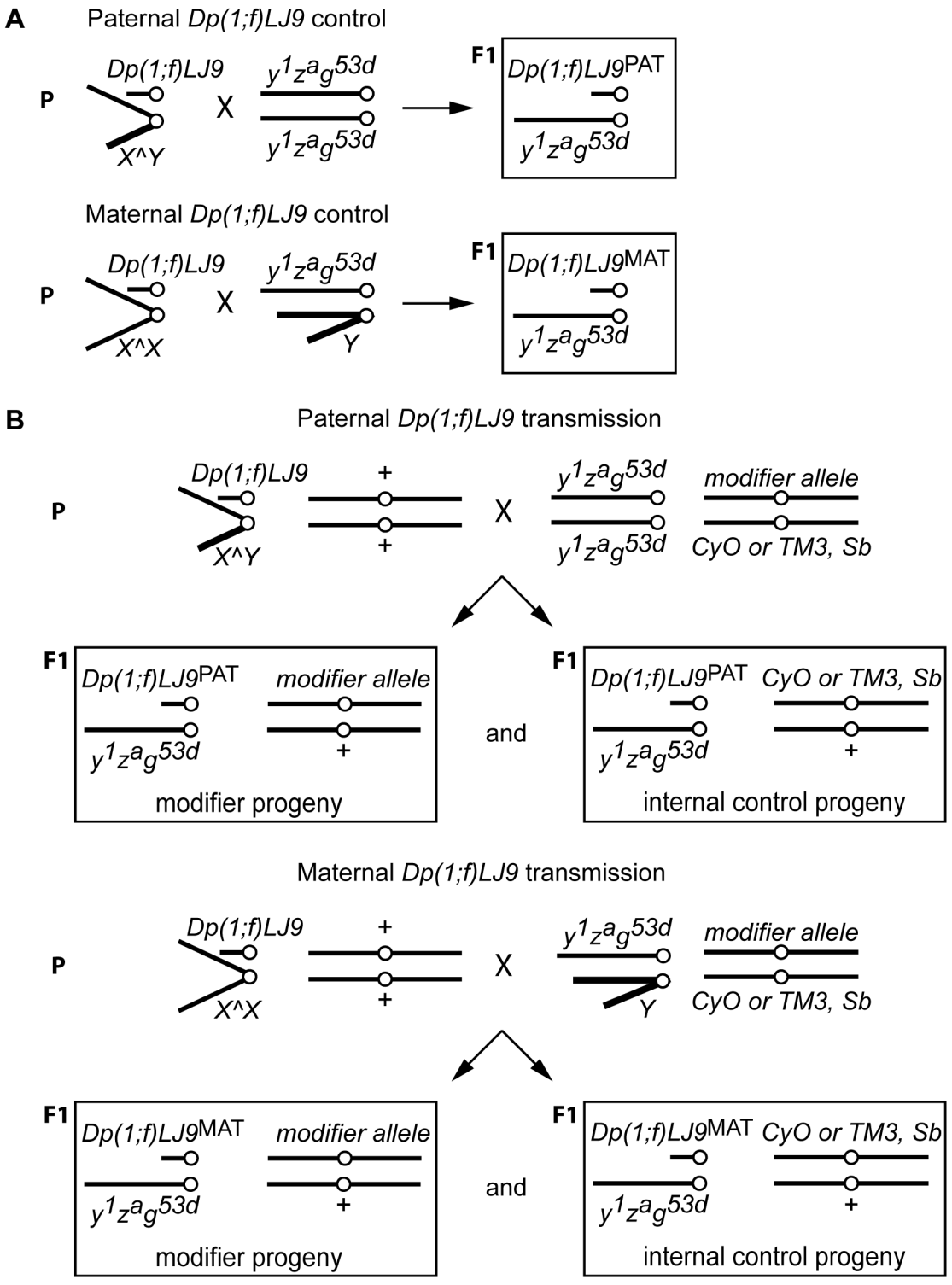

Figure 6.12 - Cross Schematic For *Dp(1;f)LJ9* Transmission.
A) Paternal and maternal transmission of *Dp(1;f)LJ9* control crosses.
B) Paternal and maternal crosses involving modifier alleles produce $y^1z^ag^{53d}$/*Dp(1;f)LJ9*; *modifier allele*/+, and sibling $y^1z^ag^{53d}$/*Dp(1;f)LJ9*; CyO or *TM3, Sb*/+ internal control progeny.

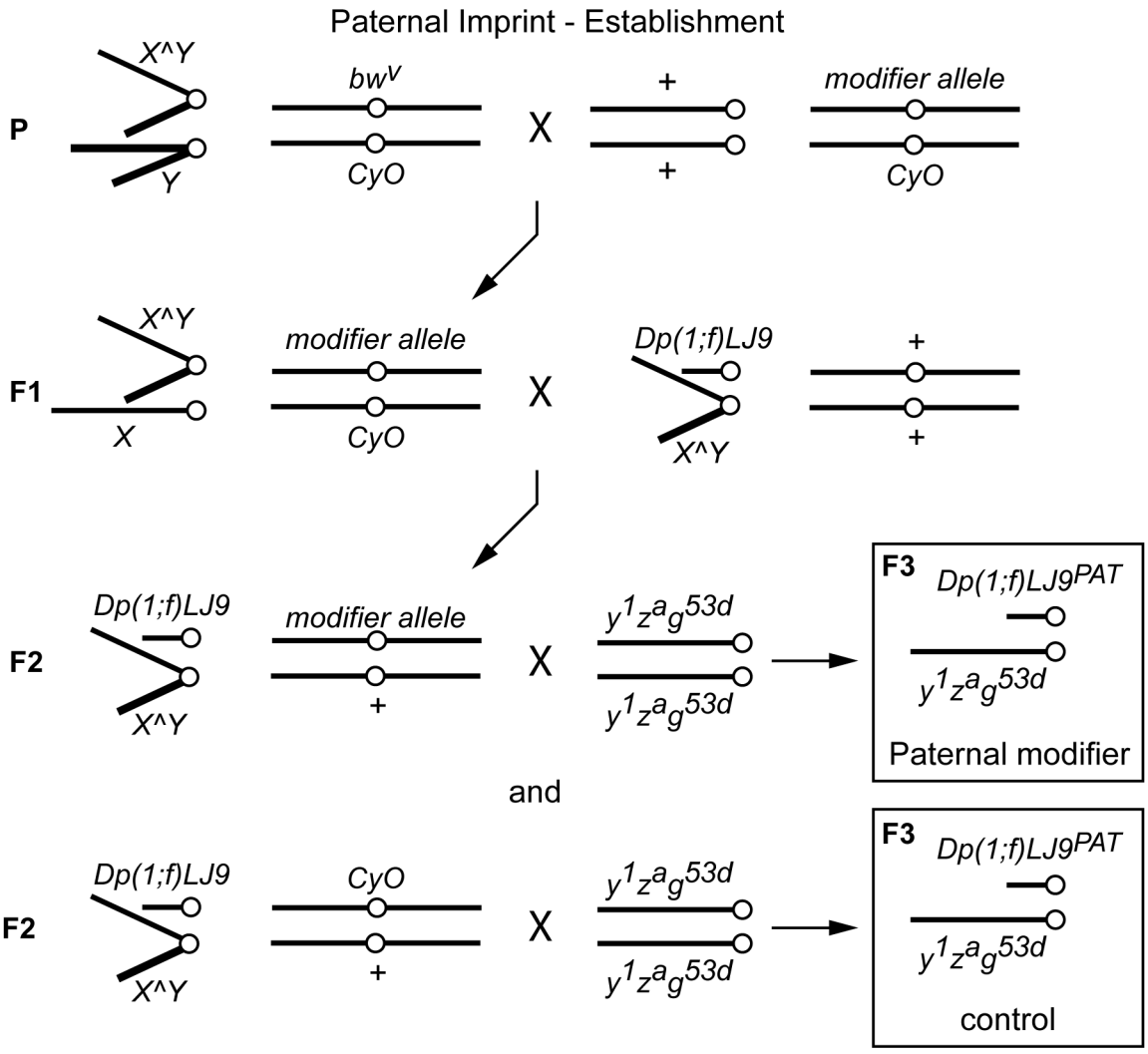

Figure 6.13 - Mating Schematic For Testing RNAi Component Mutations For An Effect On The Paternal Establishment Of The *Dp(1;f)LJ9* Imprint.

Two sets of progeny are generated from this cross: progeny with paternally-transmitted *Dp(1;f)LJ9* from fathers with the a modifier allele (maternal modifier), and progeny that also have paternally-transmitted *Dp(1;f)LJ9* from fathers wild-type for the tested allele (control).

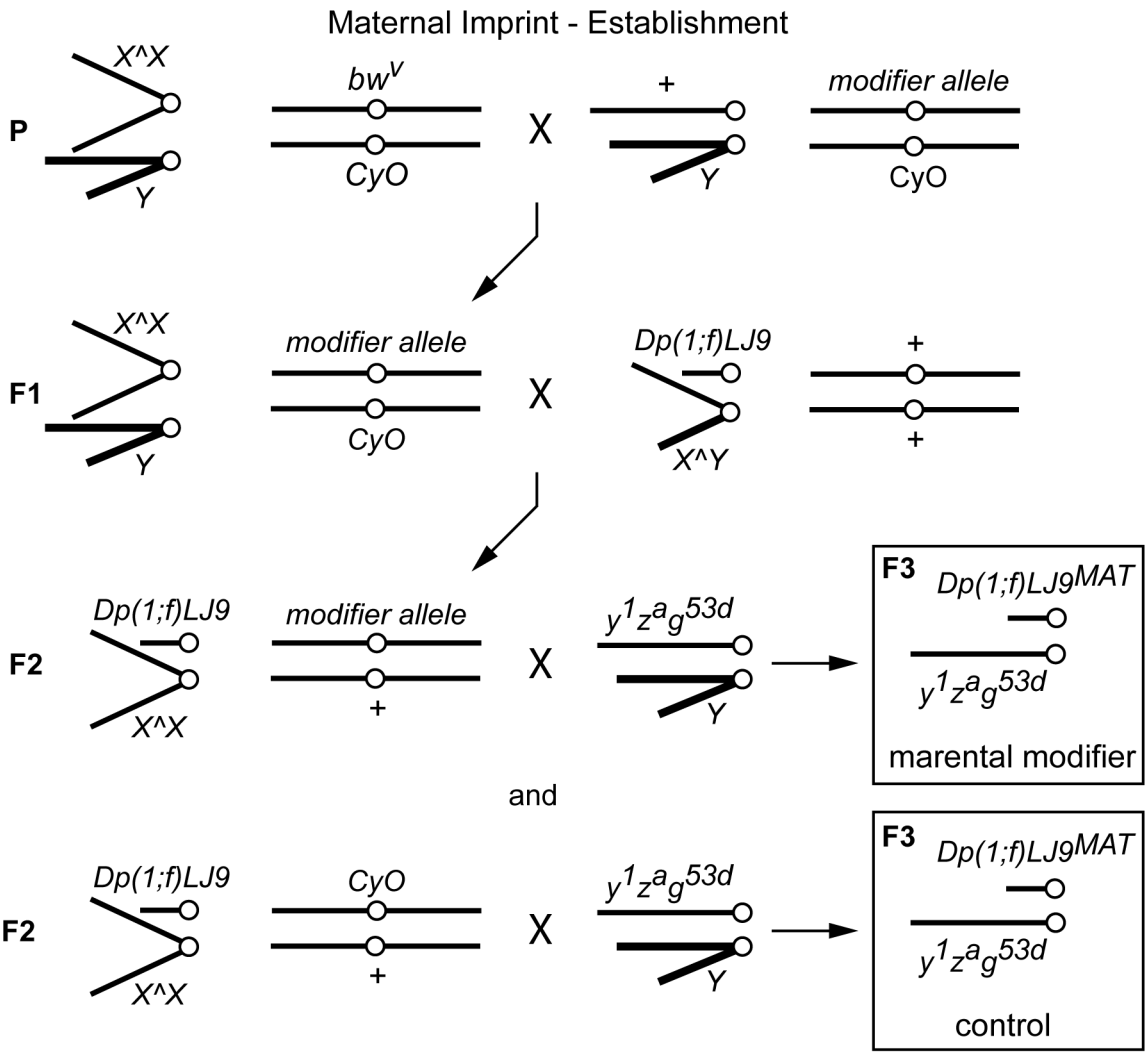

Figure 6.14 - Mating Schematic For Testing RNAi Component Mutations For An Effect On The Maternal Establishment Of The *Dp(1;f)LJ9* Imprint.
Two sets of progeny are generated from this cross: progeny with maternally-transmitted *Dp(1;f)LJ9* from mothers with the a modifier allele (maternal modifier), and progeny that also have maternally-transmitted *Dp(1;f)LJ9* from mothers wild-type for the tested allele (control).

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6.7 References

Abdu U, Gonzalez-Reyes A, Ghabrial A, Schupbach T. (2003) The *Drosophila spn-D* gene encodes a RAD51C-like protein that is required exclusively during meiosis. Genetics. **165**:197-204.

Anaka M, Lynn A, Mcginn P, Lloyd VK. (2009) Genomic imprinting in *Drosophila* has properties of both mammalian and insect imprinting. Dev Genes Evol. **219**:59-66.

Aravin A, Hannon G. (2008) Small RNA Silencing Pathways in Germ and Stem Cells. Cold Spring Harb Symp Quant Biol. **73**:283-290.

Aravin A, Lagos-Quintana M, Yalcin A, Zavolan M, Marks D, Snyder B, Gaasterland T, Meyer J, Tuschl T. (2003) The small RNA profile during *Drosophila melanogaster* development. Dev Cell. **5**:337-350.

Aravin AA, Sachidanandam R, Girard A, Fejes-Toth K, Hannon GJ. (2007) Developmentally regulated piRNA clusters implicate MILI in transposon control. Science. **316**:744-747.

Babak T, Deveale B, Armour C, Raymond C, Cleary MA, van der Kooy D, Johnson JM, Lim LP. (2008) Global survey of genomic imprinting by transcriptome sequencing. Curr Biol. **18**:1735-1741.

Barlow DP, Stoger R, Herrmann BG, Saito K, 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.

Bellen HJ, Levis RW, Liao G, He Y, Carlson JW, Tsang G, Evans-Holm M, Hiesinger PR, Schulze KL, Rubin GM and others. (2004) The BDGP gene disruption project: single transposon insertions associated with 40% of *Drosophila genes*. Genetics. **167**:761-781.

Braissant O, Wahli W. (1998) A simplified *in situ* hybridization protocol using non-radioactively labeled probes to detect abundant and rare mRNAs on tissue sections. Biochemica. **1**:10-16.

Brennecke J, Aravin A, Stark A, Dus M, Kellis M, Sachidanandam R, Hannon G. (2007) Discrete small RNA-generating loci as master regulators of transposon activity in *Drosophila*. Cell. **128**:1089-1103.

Brower-Toland B, Findley S, Jiang L, Liu L, Yin H, Dus M, Zhou P, Elgin S, Lin H. (2007) *Drosophila* PIWI associates with chromatin and interacts directly with HP1a. Genes Dev. **21**:2300-2311.

Buszczak M. (2006) The *Drosophila* P68 RNA helicase regulates transcriptional deactivation by promoting RNA release from chromatin. Genes Dev. **20**:977-989.

Caudy AA, Myers M, Hannon GJ, Hammond SM. (2002) Fragile X-related protein and VIG associate with the RNA interference machinery. Genes Dev. **16**:2491-2496.

Celniker SE, Dillon LAL, Gerstein MB, Gunsalus KC, Henikoff S, Karpen GH, Kellis M, Lai EC, Lieb JD, Macalpine DM and others. (2009) Unlocking the secrets of the genome. Nature. **459**:927-930.

Chan S, Zilberman D, Xie Z, Johansen L, Carrington J, Jacobsen S. (2004) RNA silencing genes control *de novo* DNA methylation. Science. **303**:1336.

Cook H, Koppetsch B, Wu J, Theurkauf W. (2004) The *Drosophila SDE3* homolog armitage is required for oskar mRNA silencing and embryonic axis specification. Cell. **116**:817-829.

Cox DN, Chao A, Baker J, Chang L, Qiao D, Lin H. (1998) A novel class of evolutionarily conserved genes defined by *piwi* are essential for stem cell self-renewal. Genes Dev. **12**:3715-3727.

Cox DN, Chao A, Lin H. (2000) *piwi* encodes a nucleoplasmic factor whose activity modulates the number and division rate of germline stem cells. Development. **127**:503-514.

Cziko A-MJ, McCann CT, Howlett IC, Barbee SA, Duncan RP, Luedemann R, Zarnescu D, Zinsmaier KE, Parker RR, Ramaswami M. (2009) Genetic modifiers of *dFMR1* encode RNA granule components in *Drosophila*. Genetics. **182**:1051-1060.

Das PP, Bagijn MP, Goldstein LD, Woolford JR, Lehrbach NJ, Sapetschnig A, Buhecha HR, Gilchrist MJ, Howe KL, Stark R and others. (2008) Piwi and piRNAs act upstream of an endogenous siRNA pathway to suppress *Tc3* transposon mobility in the *Caenorhabditis elegans* germline. Mol Cell. **31**:79-90.

Djupedal I, Ekwall K. (2009) Epigenetics: heterochromatin meets RNAi. Cell Res. **19**:282-295.

Fagegaltier D, Bougé A-L, Berry B, Poisot E, Sismeiro O, Coppée J-Y, Théodore L, Voinnet O, Antoniewski C. (2009) The endogenous siRNA pathway is involved in heterochromatin formation in *Drosophila*. PNAS. **106**:21258-21263.

Förstemann K, Tomari Y, Du T, Vagin V, Denli A, Bratu D, Klattenhoff C, Theurkauf W, Zamore P. (2005) Normal microRNA maturation and germ-line stem cell maintenance requires Loquacious, a double-stranded RNA-binding domain protein. PLoS Biol. **3**:e236.

Gauhar Z, Ghanim M, Herreman T, Lambert JD, Li TR, Mason C, Rifkin S, Sun L, White KP, Costello JC and others. (2008) *Drosophila melanogaster* life-cycle gene expression dataset and microarray normalisation protocols. FLYbase.

Ghildiyal M, Seitz H, Horwich MD, Li C, Du T, Lee S, Xu J, Kittler ELW, Zapp ML, Weng Z and others. (2008) Endogenous siRNAs Derived from Transposons and mRNAs in *Drosophila* Somatic Cells. Science. **320**:1077-1081.

Gracheva E, Dus M, Elgin SCR. (2009) *Drosophila* RISC Component VIG and Its Homolog Vig2 Impact Heterochromatin Formation. PLoS ONE. **4**:e6182.

Gunawardane L, Saito K, Nishida K, Miyoshi K, Kawamura Y, Nagami T, Siomi H, Siomi M. (2007) A slicer-mediated mechanism for repeat-associated siRNA 5' end formation in *Drosophila*. Science. **315**:1587-1590.

Gvozdev V, Aravin A, Abramov Y, Klenov M, Kogan G, Lavrov S, Naumova N, Olenkina O, Tulin A, Vagin V. (2003) *Stellate* repeats: targets of silencing and modules causing *cis*-inactivation and *trans*-activation. Genetica. **117**:239-245.

Gvozdev VA, Abramov IA, Kogan GL, Lavrov SA. (2007) Distorted heterochromatin replication in *Drosophila melanogaster* polytene chromosomes as a result of euchromatin-heterochromatin rearrangements. Genetika. **43**:18-26.

Haberland A, Zaitsev S, Waldöfner N, Erdmann B, Böttger M, Henke W. (2009) Structural appearance of linker histone H1/siRNA complexes. Mol Biol Rep. **36**:1083-1093.

Han T, Manoharan AP, Harkins TT, Bouffard P, Fitzpatrick C, Chu DS, Thierry-Mieg D, Thierry-Mieg J, Kim JK. (2009) 26G endo-siRNAs regulate spermatogenic and zygotic gene expression in *Caenorhabditis elegans*. PNAS. **106**:18674-18679.

Hardy R, Lindsley D, Livak K, Lewis B, Siversten A, Joslyn G, Edwards J, Bonaccorsi S. (1984) Cytogenetic analysis of a segment of the Y chromosome of *Drosophila melanogaster*. Genetics. **107**:591-610.

Harris AN, Macdonald PM. (2001) *Aubergine* encodes a *Drosophila* polar granule component required for pole cell formation and related to eIF2C. Development. **128**:2823-2832.

Hartig JV, Esslinger S, Böttcher R, Saito K, Förstemann K. (2009) Endo-siRNAs depend on a new isoform of loquacious and target artificially introduced, high-copy sequences. EMBO J. **28**:2932-2944.

Hashimoto H, Sonoda E, Takami Y, Kimura H, Nakayama T, Tachibana M, Takeda S, Shinkai Y. (2007) Histone H1 variant, H1R is involved in DNA damage response. DNA Repair (Amst). **6**:1584-1595.

Huisinga KL, Elgin SCR. (2009) Small RNA-directed heterochromatin formation in the context of development: what flies might learn from fission yeast. Biochim Biophys Acta. **1789**:3-16.

Jin P, Zarnescu D, Ceman S, Nakamoto M, Mowrey J, Jongens T, Nelson D, Moses K, Warren S. (2004) Biochemical and genetic interaction between the fragile X mental retardation protein and the microRNA pathway. Nat Neurosci. **7**:113-117.

Joanis V, Lloyd V. (2002) Genomic imprinting in *Drosophila* is maintained by the products of *Suppressor of variegation* and *trithorax* group, but not *Polycomb* group, genes. Mol Genet Genomics. **268**:103-112.

Kalmykova A, Klenov M, Gvozdev V. (2005) Argonaute protein PIWI controls mobilization of retrotransposons in the *Drosophila* male germline. Nucleic Acids Res. **33**:2052-2059.

Kataoka Y, Takeichi M, Uemura T. (2001) Developmental roles and molecular characterization of a *Drosophila* homologue of *Arabidopsis* Argonaute1, the founder of a novel gene superfamily. Genes Cells. **6**:313-325.

Katayama S, Tomaru Y, Kasukawa T, Waki K, Nakanishi M, Nakamura M, Nishida H, Yap CC, Suzuki M, Kawai J and others. (2005) Antisense transcription in the mammalian transcriptome. Science. **309**:1564-1566.

Kavi HH, Birchler JA. (2009) Interaction of RNA polymerase II and the small RNA machinery affects heterochromatic silencing in *Drosophila*. Epigenet Chrom. **2**:15.

Kawamura Y, Saito K, Kin T, Ono Y, Asai K, Sunohara T, Okada TN, Siomi MC, Siomi H. (2008) *Drosophila* endogenous small RNAs bind to Argonaute 2 in somatic cells. Nature. **453**:793-797.

Kawasaki H, Taira K. (2004) Induction of DNA methylation and gene silencing by short interfering RNAs in human cells. Nature. **431**:211-217.

Kennerdell J, Yamaguchi S, Carthew R. (2002) RNAi is activated during Drosophila oocyte maturation in a manner dependent on aubergine and spindle-E. Genes Dev. **16**:1884-1889.

Kent D. (2008) The role of histone H1 in imprint establishment in *Drosophila melanogaster*. MSc Thesis, Mount Allison Uiversity, Biology Department.

Klattenhoff C, Bratu D, McGinnis-Schultz N, Koppetsch B, Cook H, Theurkauf W. (2007) *Drosophila* rasiRNA pathway mutations disrupt embryonic axis specification through activation of an ATR/Chk2 DNA damage response. Dev Cell. **12**:45-55.

Klattenhoff C, Theurkauf W. (2008) Biogenesis and germline functions of piRNAs. Development. **135**:3-9.

Klattenhoff C, Xi H, Li C, Lee S, Xu J, Khurana JS, Zhang F, Schultz N, Koppetsch BS, Nowosielska A and others. (2009) The *Drosophila* HP1 homolog Rhino is required for transposon silencing and piRNA production by dual-strand clusters. Cell. **138**:1137-1149.

Klenov M, Lavrov S, Stolyarenko A, Ryazansky S, Aravin A, Tuschl T, Gvozdev V. (2007) Repeat-associated siRNAs cause chromatin silencing of retrotransposons in the *Drosophila melanogaster* germline. Nucleic Acids Res. **35**:5430-5438.

Kotelnikov R, Klenov M, Rozovsky Y, Olenina L, Kibanov M, Gvozdev V. (2009) Peculiarities of piRNA-mediated post-transcriptional silencing of *Stellate* repeats in testes of *Drosophila melanogaster*. Nucleic Acids Res. **37**:3254-3263.

Kusch T, Florens L, Macdonald WH, Swanson SK, Glaser RL, Yates JR, Abmayr SM, Washburn MP, Workman JL. (2004) Acetylation by Tip60 is required for selective histone variant exchange at DNA lesions. Science. **306**:2084-2087.

Latos PA, Barlow DP. (2009) Regulation of imprinted expression by macro non-coding RNAs. RNA Biol. **6**:100-106.

Lee Y, Nakahara K, Pham J, Kim K, He Z, Sontheimer E, Carthew R. (2004) Distinct roles for *Drosophila* Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. Cell. **117**:69-81.

Lei EP, Corces VG. (2006) RNA interference machinery influences the nuclear organization of a chromatin insulator. Nat Genet. **38**:936-941.

Li C, Vagin VV, Lee S, Xu J, Ma S, Xi H, Seitz H, Horwich MD, Syrzycka M, Honda BM and others. (2009) Collapse of germline piRNAs in the absence of Argonaute3 reveals somatic piRNAs in flies. Cell. **137**:509-521.

Lin H, Yin H. (2009) A Novel Epigenetic Mechanism in *Drosophila* Somatic Cells Mediated by PIWI and piRNAs. Cold Spring Harb Symp Quant Biol. **73**:273-281.

Lippman Z, Martienssen R. (2004) The role of RNA interference in heterochromatic silencing. Nature. **431**:364-370.

Lippman Z, May B, Yordan C, Singer T, Martienssen R. (2003) Distinct mechanisms determine transposon inheritance and methylation via small interfering RNA and histone modification. PLoS Biol. **1**:E67.

Liu Q, Rand TA, Kalidas S, Du F, Kim H-E, Smith DP, Wang X. (2003b) R2D2, a bridge between the initiation and effector steps of the *Drosophila* RNAi pathway. Science. **301**:1921-1925.

Liu X, Park J, Jiang F, Liu Y, McKearin D, Liu Q. (2007) Dicer-1, but not Loquacious, is critical for assembly of miRNA-induced silencing complexes. RNA. **13**:2324-2329.

Lloyd V, Sinclair D, Grigliatti T. (1999a) Genomic imprinting and position-effect variegation in *Drosophila melanogaster*. Genetics. **151**:1503-1516.

MacDonald W, Menon D, Bartlett NJ, Sperry GE, Rasheva V, Meller V, Lloyd VK. (2010) The *Drosophila* homolog of the mammalian imprint regulator, CTCF, maintains the maternal genomic imprint in *Drosophila melanogaster*. BMC Biology. **8**:105.

Malone CD, Brennecke J, Dus M, Stark A, Mccombie WR, Sachidanandam R, Hannon GJ. (2009) Specialized piRNA pathways act in germline and somatic tissues of the *Drosophila* ovary. Cell. **137**:522-535.

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

Marques JT, Kim K, Wu P-H, Alleyne TM, Jafari N, Carthew RW. (2010) Loqs and R2D2 act sequentially in the siRNA pathway in *Drosophila*. Nat Struct Mol Biol. **17**:24-30.

Matranga C, Zamore PD. (2007) Small silencing RNAs. Curr Biol. **17**:R789-793.

McEwen K, Ferguson-Smith A. (2009) Genomic Imprinting–A Model for Roles of Histone Modifications in Epigenetic Control. In: Epigenomics.235-258.

Megosh H, Cox D, Campbell C, Lin H. (2006) The role of PIWI and the miRNA machinery in *Drosophila* germline determination. Curr Biol. **16**:1884-1894.

Meyer W, Schreiber S, Guo Y, Volkmann T, Welte M, Muller H. (2006) Overlapping functions of argonaute proteins in patterning and morphogenesis of *Drosophila* embryos. PLoS Genet. **2**:e134.

Mosher RA, Melnyk CW, Kelly KA, Dunn RM, Studholme DJ, Baulcombe DC. (2009) Uniparental expression of PolIV-dependent siRNAs in developing endosperm of *Arabidopsis*. Nature. **460**:283-286.

Moshkovich N, Lei EP. (2010) HP1 recruitment in the absence of argonaute proteins in *Drosophila*. PLoS Genet. **6**:e1000880.

Ner SS, Harrington MJ, Grigliatti TA. (2002) A role for the *Drosophila* SU(VAR)3-9 protein in chromatin organization at the histone gene cluster and in suppression of position-effect variegation. Genetics. **162**:1763-1774.

Nishida K, Saito K, Mori T, Kawamura Y, Nagami-Okada T, Inagaki S, Siomi H, Siomi M. (2007) Gene silencing mechanisms mediated by Aubergine piRNA complexes in *Drosophila* male gonad. RNA. **13**:1911-1922.

Obbard DJ, Finnegan DJ. (2008) RNA interference: endogenous siRNAs derived from transposable elements. Curr Biol. **18**:R561-563.

Ooi SKT, O'Donnell AH, Bestor TH. (2009) Mammalian cytosine methylation at a glance. J Cell Sci. **122**:2787-2791.

Pal-Bhadra M, Bhadra U, Birchler JA. (2002) RNAi related mechanisms affect both transcriptional and posttranscriptional transgene silencing in *Drosophila*. Mol Cell. **9**:315-327.

Pal-Bhadra M, Leibovitch B, Gandhi S, Rao M, Bhadra U, Birchler J, Elgin S. (2004) Heterochromatic silencing and HP1 localization in *Drosophila* are dependent on the RNAi machinery. Science. **303**:669-672.

Pane A, Wehr K, Schüpbach T. (2007) *zucchini* and *squash* encode two putative nucleases required for rasiRNA production in the *Drosophila* germline. Dev Cell. **12**:851-862.

Pauler FM, Koerner MV, Barlow DP. (2007) Silencing by imprinted noncoding RNAs: is transcription the answer? Trends Genet. **23**:284-292.

Peng J, Karpen G. (2007) H3K9 methylation and RNA interference regulate nucleolar organization and repeated DNA stability. Nat Cell Biol. **9**:25-35.

Peng JC, Karpen GH. (2009) Heterochromatic genome stability requires regulators of histone H3 K9 methylation. PLoS Genet. **5**:e1000435.

Real, MD, Ferre, J, Mensua, JL. (1985) Methods for the quantitative estimation of the red and brown pigments of *D. melanogaster.* Dros Inf Serv. **61**:198-200.

Rosidi B, Wang M, Wu W, Sharma A, Wang H, Iliakis G. (2008) Histone H1 functions as a stimulatory factor in backup pathways of NHEJ. Nucleic Acids Res. **36**:1610-1623.

Royo H, Cavaillé J. (2008) Non-coding RNAs in imprinted gene clusters. Biol Cell. **100**:149-166.

Saito K, Inagaki S, Mituyama T, Kawamura Y, Ono Y, Sakota E, Kotani H, Asai K, Siomi H, Siomi MC. (2009) A regulatory circuit for piwi by the large Maf gene *traffic jam* in *Drosophila*. Nature. **461**:1296-1299.

Saito K, Nishida K, Mori T, Kawamura Y, Miyoshi K, Nagami T, Siomi H, Siomi M. (2006) Specific association of Piwi with rasiRNAs derived from retrotransposon and heterochromatic regions in the Drosophila genome. Genes Dev. **20**:2214-2222.

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

Schüpbach T, Wieschaus E. (1991) Female sterile mutations on the second chromosome of Drosophila melanogaster. II. Mutations blocking oogenesis or altering egg morphology. Genetics. **129**:1119-1136.

Shpiz S, Kwon D, Rozovsky Y, Kalmykova A. (2009) rasiRNA pathway controls antisense expression of *Drosophila* telomeric retrotransposons in the nucleus. Nucleic Acids Res. **37**:268-278.

Siomi MC, Miyoshi T, Siomi H. (2010) piRNA-mediated silencing in *Drosophila* germlines. Semin Cell Dev Biol. [Epub]

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

Tomari Y, Du T, Haley B, Schwarz D, Bennett R, Cook H, Koppetsch B, Theurkauf W, Zamore P. (2004) RISC assembly defects in the *Drosophila* RNAi mutant *armitage*. Cell. **116**:831-841.

Vagin V, Klenov M, Kalmykova A, Stolyarenko A, Kotelnikov R, Gvozdev V. (2004) The RNA interference proteins and vasa locus are involved in the silencing of retrotransposons in the female germline of *Drosophila melanogaster*. RNA Biology. **1**:54-58.

Vagin V, Sigova A, Li C, Seitz H, Gvozdev V, Zamore P. (2006) A distinct small RNA pathway silences selfish genetic elements in the germline. Science. **313**:320-324.

Verdel A, Vavasseur A, Le Gorrec M, Touat-Todeschini L. (2009) Common themes in siRNA-mediated epigenetic silencing pathways. Int. J. Dev. Biol. **53**:245-257.

Williams RW, Rubin GM. (2002) ARGONAUTE1 is required for efficient RNA interference in *Drosophila* embryos. PNAS. **99**:6889-6894.

Wilson JE, Connell JE, Macdonald PM. (1996) *aubergine* enhances *oskar* translation in the *Drosophila* ovary. Development. **122**:1631-1639.

Wutz A, Smrzka O, Schweifer N, Schellander K, Wagner E, Barlow D. (1997) Imprinted expression of the *Igf2r* gene depends on an intronic CpG island. Nature. **389**:745-749.

Yamanaka S, Katayama E, Yoshioka K-i, Nagaki S, Yoshida M, Teraoka H. (2002) Nucleosome linker proteins HMGB1 and histone H1 differentially enhance DNA ligation reactions. Biochem Biophys Res Commun. **292**:268-273.

Yin H, Lin H. (2007) An epigenetic activation role of Piwi and a Piwi-associated piRNA in *Drosophila melanogaster*. Nature. **450**:304-308.

Zakharova IS, Shevchenko AI, Zakian SM. (2009) Monoallelic gene expression in mammals. Chromosoma. **118**:279-290.

Zambon RA, Vakharia VN, Wu LP. (2006) RNAi is an antiviral immune response against a dsRNA virus in *Drosophila melanogaster*. Cell Microbiol. **8**:880-889.

6.8 Transition To Chapter 7

The following chapter is a summary of the research presented in this thesis. I present models for the epigenetic mechanisms involved in regulating both the paternal and maternal *Dp(1;f)LJ9* imprints, and the developmental progression of genomic imprint regulation in *Drosophila*. I also discuss the significance of the results presented in this thesis in context of the Host Defense hypothesis for the evolution of genomic imprinting.

Chapter 7 Conclusion

7.1 Epigenetic Regulation Of The *Dp(1;f)LJ9* Imprinted Domain

The chromosomal rearrangement that produces the *Dp(1;f)LJ9* mini-X chromosome exposes an imprinting center within heterochromatin that is assayed by expression of the euchromatic *garnet* gene. Typically, the placement of euchromatic genes next to heterochromatin in *Drosophila* results in variegated expression of these genes due to heterochromatic silencing (Schulze and Wallrath 2007). The findings presented in this thesis demonstrate that epigenetic regulation at both the maternal and paternal imprints are distinct from regulation at non-imprinted regions; aspects of paternal silencing and the presence of a stable maternal boundary are not typical of other variegating chromosomal arrangements. This suggests that both the paternal and maternal *Dp(1;f)LJ9* imprint centers are discrete regulatory domains and that differential epigenetic regulation is directed from the imprint center. For example, silencing from the paternal inheritance of *Dp(1;f)LJ9* is not simply the consequence of the absence of a heterochromatin boundary, but rather is the result of epigenetic components being actively recruited to silence this domain. Maternal inheritance of *Dp(1;f)LJ9* leads to the active formation of a robust heterochromatic boundary to protect gene expression.

7.1.1 The Paternal *Dp(1;f)LJ9* Imprint

A model for the epigenetic mechanisms involved in maintaining paternal silencing is presented in Figure 7.1A. Paternal inheritance of *Dp(1;f)LJ9* results in variegated silencing of the marker gene g*arnet*. Disruption of DNA methylation caused a relaxation of paternal-specific silencing, suggesting that Dnmt2 and DNA methylation are involved in regulation of the paternal imprint. Additionally, the methyl-DNA binding protein MBD2/3 is also involved maintaining paternal silencing. *Drosophila* DNA is not abundantly methylated; however, DNA methylation has been shown to assist in silencing targeted regions of the genome, such as transposable elements (Phalke *et al.* 2009). In mammals, DNA methylation is extensively associated with genomic imprinting, involved in both the establishment and maintenance of many imprinted loci (Delaval and Feil

2004). In both mammals and plants, DNA methylation contributes directly to the silencing of imprinted genes by blocking promoters and preventing transcription (Feil and Berger 2007). The involvement of DNA methylation in regulating the paternal imprint is restricted to the maintenance of silencing during the early stages of embryonic development. Considering the limited extent to which the *Drosophila* DNA is methylated, it is reasonable to speculate that the functional role of Dnmt2 and MBD2/3 is to target specific areas of the genome for localization into repressive nuclear compartments during development. Both Dnmt2 and MBD2/3 are associated with the nuclear matrix (Marhold *et al.* 2002; Kunert *et al.* 2003), and DNA methylation may be sufficient to mark chromosomal regions for nuclear compartments in which MBD2/3 is subsequently available. The finding that mutations in *lamin* significantly disrupt *Dp(1;f)LJ9* paternal-specific silencing reinforces the importance of nuclear chromosome organization in maintaining imprint regulation.

The histone variant H2Av is a dynamic component of chromatin associated with transient chromatin states (Henikoff *et al.* 2009). H2Av is involved in the formation of both the maternal and paternal imprinted domains. However, Domino, which is involved in H2Av exchange (Kusch *et al.* 2004), acts only on the paternal imprint, suggesting H2Av is differentially regulated to prime chromatin formation at the maternal and paternal imprinted domains. Another possible function for H2Av is counteracting DNA methylation; the homologous histone variant H2A.Z antagonizes DNA methylation in *Arabidopsis* (Zilberman *et al.* 2008). While such a function has yet to be determined for H2Av in *Drosophila*, it could explain the enhanced silencing of paternally inherited *Dp(1;f)LJ9* observed in *H2Av* mutants. Histone H4 is also important for the paternal imprint; mutations in H4 variants and H4 associated acetyltransferase Chameau caused strong relaxation of silencing. Acetylation of H4 may be required to initiate heterochromatin formation prior to H3K9 methylation and HP1 recruitment. The full extent of H2Av and H4 variant association with the paternal imprint remains to be determined; however, both histone variants are found within mature spermatozoa (Dorus *et al.* 2006; Miller *et al.* 2009), and thus could also be involved in establishing the paternal imprint.

Antisense *garnet* transcripts, predominantly from the paternally inherited *Dp(1;f)LJ9*, can be detected. Consistent with this finding, mutations in components of the endogenous siRNA pathway disrupted *garnet* silencing from the paternal imprint. This suggests posttranscriptional transcript degradation and RNAi-guided heterochromatin formation likely contributes to paternal silencing. The presence of antisense transcripts at imprinted domains is a regulatory feature of many imprinted loci in both mammals and plants (Royo and Cavaillé 2008; Zhang and Qu 2009) and, in some cases, the generation of antisense transcripts extends the regulatory influence of imprinted domain beyond their previously established boundaries (Babak *et al.* 2008). Disruption of the imprinted domain by the creation of the *Dp(1;f)LJ9* mini-X chromosome may account for the extension of antisense transcription beyond the original imprint control center.

7.1.2 The Maternal *Dp(1;f)LJ9* Imprint

A model for the epigenetic mechanisms involved in maintaining maternal expression is presented in Figure 7.1B. The *Dp(1;f)LJ9* imprinted domain resides within centric heterochromatin, and thus the domain must be insulated to protect the expression of flanking genes. A majority of the mutant alleles tested had no effect on the stable expression promoted by maternal inheritance of *Dp(1;f)LJ9*, thereby demonstrating the intrinsic stability of this domain. The insulator protein CTCF associates with maternally inherited *Dp(1;f)LJ9*, and is necessary to prevent the encroachment of silencing elements into the maternal imprinted domain. This finding is significant as CTCF is also associated with insulating imprinted domains in mammals (Filippova 2008). CTCF is only involved in maintaining the maternal imprint, as mutations in CTCF had no impact on the establishment of either the maternal or paternal imprints, paralleling the lack of CTCF involvement in establishing mammalian imprints (Schoenherr *et al.* 2003; Szabo *et al.* 2004).

Mutations in *H2Av* were found to affect regulation of both the maternal and paternal *Dp(1;f)LJ9* imprints; however, it is likely that H2Av functions independently at the maternal and paternal imprint domains. *Domino* mutations only affect the paternal imprint, suggesting that at the maternally imprinted domain the Tip60 complex does not

actively exchange H2Av. The H2Av homologue, H2A.Z, is associated with CTCF boundaries in mammals (Yusufzai *et al.* 2004; Barski *et al.* 2007), and thus H2Av may support the initial formation of the maternal boundary. Such function would also correlate with H2Av antagonizing DNA methylation (Zilberman *et al.* 2008). Active expression from the maternally imprinted domain is reinforced by the recruitment of Jil-1, which phosphorylates H3S10 and antagonizes H3K9 methylation and HP1 recruitment (Bao *et al.* 2007).

7.1.3 Developmental Progression Of *Dp(1;f)LJ9* Imprint Regulation

Achieving a genomic imprint requires the action of epigenetic processes to establish the initial imprint in the parental germline and to maintain that imprint in the somatic cells of the progeny for the span of their life. The critical events for the regulation of imprinted domains occur during the period leading to fertilization, immediately after fertilization, and during the zygotic activation of the embryo. Mouse genomes experience extensive DNA demethylation after fertilization; imprinted domains are protected from such demethylation, which is necessary to maintain the imprint (Bartolomei 2009). Sheep do not undergo the same post-fertilization DNA demethylation and detection of parental-specific imprinted expression does not occur until after the blastocyst stage during zygotic activation of the embryo, reflecting the importance of histone modifications in regulating sheep genomic imprints (Thurston *et al.* 2008; Colosimo *et al.* 2009). In plants, parent-specific DNA methylation occurs prior to fertilization, after which the imprinted domains are maintained in the endosperm (Garnier *et al.* 2008).

A model for the developmental progression of *Dp(1;f)LJ9* imprint regulation is presented in Figure 7.2. Regulation of genomic imprinting is generally divided into two stages: the establishment of the imprint in the germline, and the subsequent maintenance of the imprint in the soma (Reik and Walter 2001). I am proposing that a transitional stage between germline establishment and somatic maintenance exists in *Drosophila*, which I am defining as the initialization stage. The initialization stage encompasses regulatory activity in the gametes prior to fertilization through to the zygotic activation of

the embryo. This stage is a critical period for epigenetic imprint regulation, evident in the fact that some mutations affect the imprint through maternal effects, prior to zygotic activation of the embryo. The initialization of the imprint also involves epigenetic mechanisms that are specific to the early embryo and are not part of the general maintenance of the imprint later in development.

Of the mutations tested for their effects on imprint establishment, only mutations in *aubergine* disrupted the maternal imprint establishment. This finding is important in that it implicates the piRNA pathway in the germline establishment of the imprint. Disruption of the piRNA pathway can affect the formation of heterochromatin in developing embryos (Pal-Bhadra *et al.* 2004), which may suggest that the role of piRNA extends into the early stages of embryonic development (Klenov *et al.* 2007) coinciding with the initialization stage of imprint regulation. Indeed, *aubergine* mutations have similar effects on the establishment and maintenance of the imprint. In contrast, siRNA is involved in somatic heterochromatin formation (Fagegaltier *et al.* 2009) and was found to only be involved in maintaining paternal silencing. Interestingly, the piRNA pathway has been linked to regulating translation of maternally deposited RNA in oocytes (Kennerdell *et al.* 2002), which could contribute the disruption of imprint initialization in piRNA mutants.

CTCF RNA transcripts are maternally deposited into oocytes and their expression levels peak early in embryogenesis before they decline in larvae and adults (Moon *et al.* 2005). CTCF is critical for maintaining expression from the maternal imprint and may also function at the initialization stage to isolate the maternal imprint during fertilization and zygotic activation of the embryo. The *Drosophila* DNA methyltransferase, Dnmt2, is expressed in embryos and transcript levels begin to decrease shortly after zygotic activation of the embryo until it becomes undetectable in the larval stages (Kunert *et al.* 2003). Similarly, MBD2/3 is expressed only in embryos and associates with DNA after zygotic activation of the embryo (Marhold *et al.* 2002). I found that disruption of DNA methylation only affected the paternal imprint during the first 8 hours of development. This demonstrates DNA methylation has a central role in the initialization of the paternal

imprint, a role reinforced by MBD2/3. After fertilization, the sperm pronucleus is remodeled and paternal DNA is repackaged with maternally contributed nucleosomes and chromatin components (Fitch *et al.* 1998). During this stage, asynchronous replication between maternal and paternal DNA would allow targeted DNA methylation of paternal chromosomes, while maternal chromosomes may be protected within the female pronucleus by binding CTCF. The transition from the imprint initialization stage to the maintenance stage occurs with the formation of a stable chromatin domain. This would involve Jil-1 reinforcement of the maternal domain, and H3K9 methylation along with HP1 recruitment at the paternal domain to form heterochromatin.

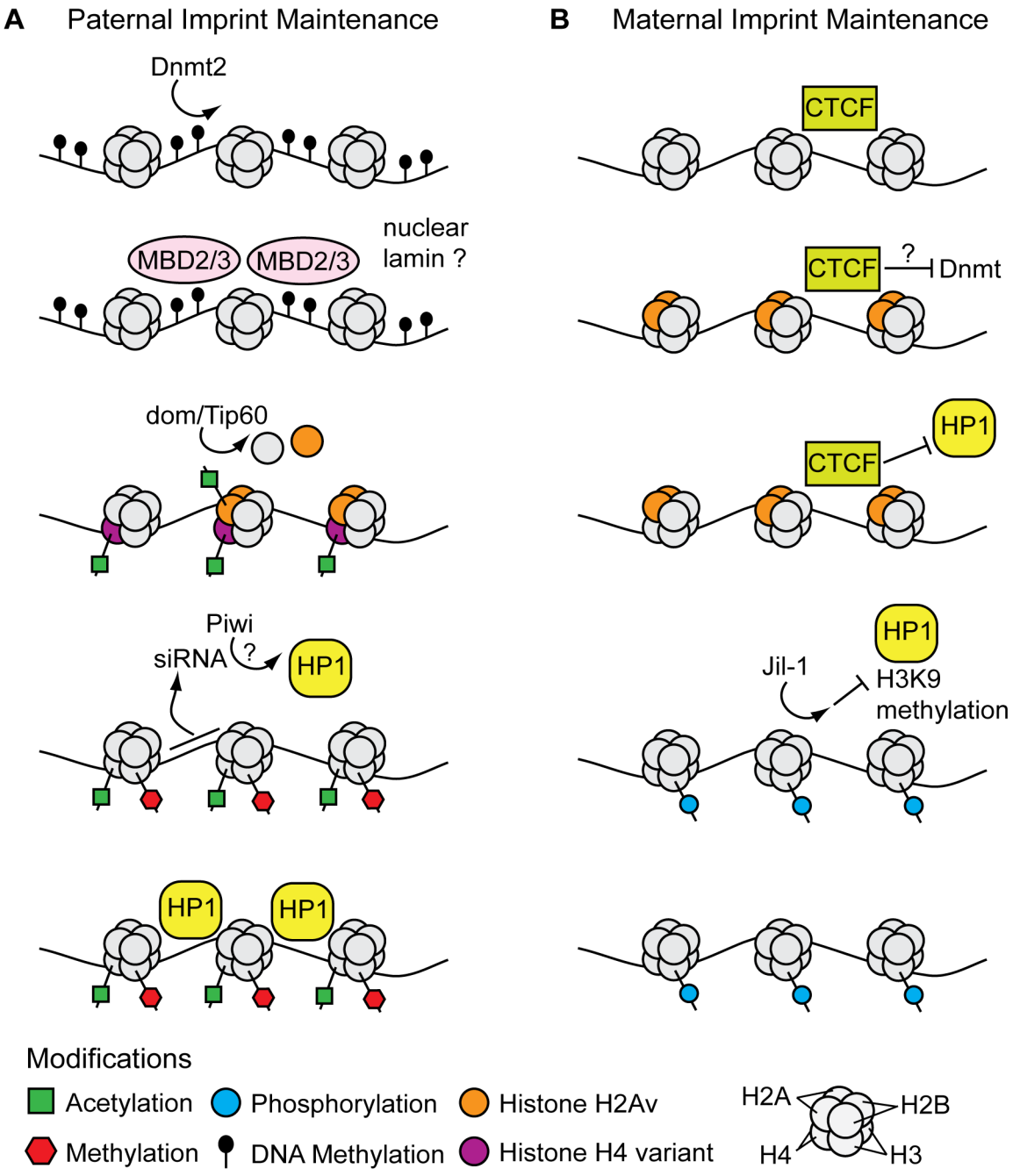


Figure 7.1 - Models For Paternal And Maternal *Dp(1;f)LJ9* Imprint Maintenance
A) Paternal *Dp(1;f)LJ9* imprint maintenance. DNA methylation by Dnmt2 and MBD2/3 binding stabilize the paternal imprint and guide nuclear localization though associations with nuclear lamin. The histone variant H2Av is exchanged for H2A after acetylation by Domino (dom) and the Tip60 complex. Histone H4 and the variant H4r assist silencing, and may help the initiation of heterochromatin formation when acetylated. Antisense transcripts generated from paternally inherited *Dp(1;f)LJ9* are processed by the siRNA pathway to reinforce silencing.

(Figure 7.1 continued)

Processed siRNAs may also actively recruit heterochromatin components through a possible association with Piwi. The silent paternal imprint domain is maintained by H3K9 methylation and the recruitment of HP1 to form heterochromatin.
B) Maternal *Dp(1;f)LJ9* imprint maintenance. CTCF associates with the maternal imprinted domain, insulating it from silencing factors. H2Av also associates with the maternal imprint, but is not responsive to modification by the dom/Tip60 complex. It is possible that H2Av assists CTCF in forming a stable boundary that can counteract DNA methylation and heterochromatin formation. Recruitment of Jil-1 leads to phosphorylation of H3S10, reinforcing the active maternal domain by counteracting H3K9 methylation and HP1 recruitment.

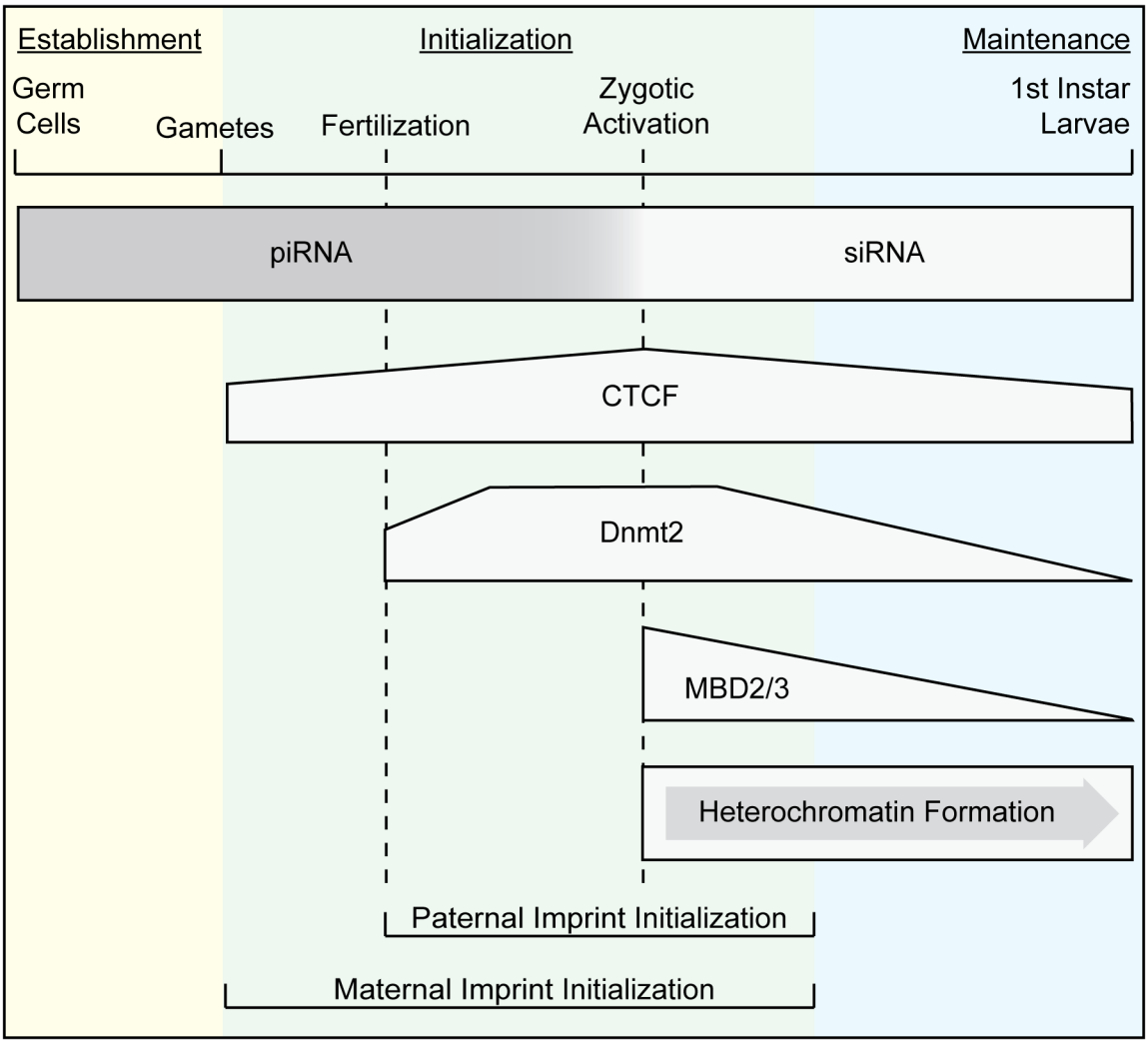


Figure 7.2 - Developmental Progression Of *Dp(1;f)LJ9* Imprint Regulation
Boxes represent the expression profiles of specific genes and the activity of epigenetic processes during embryonic development. Establishment of an imprint occurs in the germline. Initialization of an imprint could begin in the gametes and is responsible for the transition in imprint regulation from fertilization to zygotic gene activation in the embryo. Maintenance of an imprint begins after zygotic activation to maintain stable somatic inheritance and expression of the imprint. The piRNA pathway is involved in establishing the imprint and may also influence initialization of the imprint in mature oocytes and the early embryo prior to zygotic activation. CTCF RNA is maternally deposited and may assist in isolating the maternal imprint during fertilization, and thus would be involved in the initialization and maintenance of the imprint. DNA methylation by Dnmt2 peaks between fertilization and the zygotic activation of the embryo, when MBD2/3 association commences. Dnmt2 and MBD2/3 are only active during early embryogenesis, and thus are exclusive to the initialization of the paternal imprint. Maintenance of the paternal imprint involves the siRNA pathway and heterochromatin formation, which is initiated after zygotic activation of the embryo.

7.2 Regulation Of The *Dp(1;f)LJ9* Imprint And Host Defense Hypothesis

The host defense hypothesis proposes that the epigenetic regulation of genomic imprinting is evolutionarily linked to the control of transposable elements (Barlow 1993; McDonald *et al.* 2005). The foundation of this hypothesis rests on the dependence on DNA methylation for regulating both mammalian imprinting and transposable elements, as well as the similar mechanisms used to isolate imprinted regions and transposable elements in transcriptionally restrictive regions of chromosomes (Barlow 1993; McDonald *et al.* 2005).

7.2.1 DNA Methylation In *Drosophila*

DNA methylation in *Drosophila* is not as prevalent as it is in mammals and plants, yet, despite its limited activity, DNA methylation does participate in silencing transposable elements (Phalke *et al.* 2009). DNA methylation in *Drosophila* is not ubiquitously involved in heterochromatin formation (Phalke *et al.* 2009), suggesting that the involvement of DNA methylation in regulating both transposable elements and imprinted regions represents the creation of genomic domains distinct from the bulk of constitutive heterochromatin.

As in *Drosophila*, Dnmt2 is also the lone DNA methyltransferase found in the slime mold *Dictyostelium*. In *Dictyostelium*, Dnmt2 in which it also has a conserved role in silencing transposable elements via DNA methylation (Kuhlmann *et al.* 2005; Katoh *et al.* 2006). Thus, genome regulation by Dnmt2 may represent an ancient silencing pathway targeting viruses and retroelements. While MBD proteins are not present in all eukaryotes (Harony and Ankri 2008), MBD proteins are involved in the regulation of transposable elements in mammals (Steinhoff and Schulz 2003). Imprinting of *H19* in mice is considered to be the most ancient imprinted regulatory region identified in vertebrates (Smits *et al.* 2008), and binding MBD3 is required to stabilize this imprint early in development (Reese *et al.* 2007). This denotes an ancient functional significance for MBD proteins in regulating imprinting. The finding that DNA methylation and

MBD2/3 have a restricted, yet significant, role in *Drosophila* imprint regulation supports an epigenetic link between imprinting and transposable element regulation.

7.2.2 RNAi And The Host Defense Hypothesis

The host defense hypothesis was originally proposed by Barlow prior to the discovery of RNAi regulation by Fire *et. al.* in 1998 (Barlow 1993; Fire *et al.* 1998). Given the significance of RNAi in the host defense response of many organisms (Obbard *et al.* 2009), this regulatory pathway must also be considered when contemplating overlapping functions in regulating transposable elements and imprinted regions. I have shown that the piRNA pathway is involved in imprint establishment and the siRNA pathway is involved in imprint maintenance (Chapter 5). Both of these roles parallel their function in the germline and somatic repression of transposable elements (Brennecke *et al.* 2007; Marques *et al.* 2010). Piwi homologues, MILI and MIWI, in mammals are found to regulate germline expression of transposable elements (Aravin *et al.* 2007; Aravin *et al.* 2008), as well as to direct DNA methylation and heterochromatin formation (Ooi *et al.* 2009). While conclusive links between RNAi and imprint regulation have yet to be made in mammals, MIWI2 gonadal expression does coincide with *de novo* establishment of DNA methylation on imprinted regions and transposable elements (Aravin *et al.* 2008).

7.2.3 CTCF And The Host Defense Hypothesis

It has been suggested that the origin of imprinting at some loci could be attributed to the protection and activation of an otherwise silenced region (Brenton *et al.* 1998; Edwards and Ferguson-Smith 2007). Natural selection for silenced regions is explained by the host defense hypothesis; transposable elements are typically targeted for silencing and the same epigenetic mechanisms responsible for silencing transposable elements could also be engaged in other regions of the host genome with similar repetitive structures, resulting in genomic imprinting (McDonald *et al.* 2005). However, the formation of isolated active domains can also be addressed in the context of the host defense hypothesis. The host response to DNA damage and transposable elements has

long been linked to genomic activation (McClintock 1984). Transposable elements have recently been shown to be able to provide novel binding sites for transcription factors in mammalian genomes due select transposable elements harboring binding motifs for transcription factors, including CTCF (Bourque *et al.* 2008). In *Drosophila*, CTCF is necessary for the function of *gypsy* insulators (Gerasimova *et al.* 2007), insulators derived from *gypsy* retrotransposons involved in regulating gene expression in the early embryo (Ramos *et al.* 2006). Collectively, these findings show that transposable elements can both recruit gene silencing mechanisms and potentially counter silencing by providing binding sites for transcription factors and insulator proteins such as CTCF.

CTCF is a highly conserved multi-functional protein that can regulate the genome as a chromosomal insulator in addition to functioning as a transcriptional activator (Moon *et al.* 2005; Filippova 2008). The dynamic properties of CTCF, and its association with imprinting in mammals, make it a strong candidate for generating an insulated imprinted domain dependent on binding motifs within transposable elements. Select transposable elements contain binding motifs for different transcription factors, but only binding profiles for CTCF are conserved across cell types and species (Kunarso *et al.* 2010), suggesting that CTCF binding to transposable element motifs is more robust than that of other transcription factors. Additionally, CTCF is associated with maintaining active domains in repressed, gene poor regions of the genome (Gombert and Krumm 2009), which emphasizes the requirement of CTCF insulator function for regulating these domains. This dual activity of CTCF has been shown to influence gene activation in embryos and has shaped transcriptional regulatory networks over evolutionary time (Filippova 2008). Such dual functions could also provide the machinery underpinning genomic imprinting. CTCF is a maternal effect gene in mice that is critical for oocyte and preimplantation embryo development. Likewise, in *Drosophila*, CTCF RNA is also maternally deposited into the egg and is required for early development (Moon *et al.* 2005; Wan *et al.* 2008). The exclusive availability of CTCF to the maternal genome in the oocyte and early embryo provides a window of opportunity that could contribute to the advent of an imprinted domain. Such a process would certainly correlate with the known association of CTCF with maintaining maternal-specific expression at many

mammalian imprinted loci (Filippova 2008). The possibility that maternal-specific protection could account for the evolutionary advent of an imprinted domain is made even more appealing by the finding that CTCF has a conserved function for protecting the maternal imprint in *Drosophila* (MacDonald *et al.* 2010).

7.3 Summary

The understanding of epigenetic mechanisms governing genomic imprinting has progressed significantly since the first description of parent-of-origin chromosome silencing in insects (Crouse 1960) and parent-of-origin dependent DNA methylation in mammals (Sasaki *et al.* 1992; Bartolomei *et al.* 1993; Stöger *et al.* 1993). Currently, regulation of imprinted domains is known to require both concurrent and stepwise epigenetic processes to establish and maintain parent-specific imprinted expression. DNA methylation, histone modification, chromatin remodelers/regulators, and non-coding RNA processes are all associated with regulating imprinted domains. Comparisons of imprinted domains, both within species and between species, provide valuable insight into the common mechanisms necessary to construct an imprinted domain. Regulation of *Drosophila* imprinting is conducted in distinct stages in early development, utilizing epigenetic mechanisms unique to the early stages of embryogenesis. *Drosophila* imprinting therefore relies on waves of epigenetic regulation to transition from imprint establishment in the germline to imprint maintenance in somatic cells.

Considering that *Drosophila* has a rich history in genetic research, providing the foundation of our knowledge of the genetics of chromatin structure and function, characterizing genomic imprinting in this organism is both appropriate and significant. The research presented in this thesis makes a substantial contribution towards characterizing an imprinted domain, and broadens our understanding of epigenetic regulation in *Drosophila*.

References

Aagaard L, Laible G, Selenko P, Schmid M, Dorn R, Schotta G, Kuhfittig S, Wolf A, Lebersorger A, Singh P and others. (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.

Abdu U, Gonzalez-Reyes A, Ghabrial A, Schupbach T. (2003) The *Drosophila spn-D* gene encodes a RAD51C-like protein that is required exclusively during meiosis. Genetics. **165**:197-204.

Ahmad K, Henikoff S. (2001a) Centromeres are specialized replication domains in heterochromatin. J Cell Biol. **153**:101-110.

Ahmad K, Henikoff S. (2001b) Modulation of a transcription factor counteracts heterochromatic gene silencing in *Drosophila*. Cell. **104**:839-847.

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

Akhmanova A, Miedema K, Hennig W. (1996) Identification and characterization of the *Drosophila* histone H4 replacement gene. FEBS Lett. **388**:219-222.

Alleman M, Doctor J. (2000) Genomic imprinting in plants: observations and evolutionary implications. Plant Mol Biol. **43**:147-161.

Anaka M, Lynn A, Mcginn P, Lloyd VK. (2009) Genomic imprinting in *Drosophila* has properties of both mammalian and insect imprinting. Dev Genes Evol. **219**:59-66.

Andersen A, Panning B. (2003) Epigenetic gene regulation by noncoding RNAs. Curr Opin Cell Biol. **15**:281-289.

Andreyeva EN, Kolesnikova TD, Belyaeva ES, Glaser RL, Zhimulev IF. (2008) Local DNA underreplication correlates with accumulation of phosphorylated H2Av in the *Drosophila melanogaster* polytene chromosomes. Chromosome Res. **16**:851-862.

Aravin A, Hannon G. (2008) Small RNA Silencing Pathways in Germ and Stem Cells. Cold Spring Harb Symp Quant Biol. **73**:283-290.

Aravin A, Lagos-Quintana M, Yalcin A, Zavolan M, Marks D, Snyder B, Gaasterland T, Meyer J, Tuschl T. (2003) The small RNA profile during *Drosophila melanogaster* development. Dev Cell. **5**:337-350.

Aravin AA, Sachidanandam R, Bourc'his D, Schaefer C, Pezic D, Toth KF, Bestor T, Hannon GJ. (2008) A piRNA pathway primed by individual transposons is linked to *de novo* DNA methylation in mice. Mol Cell. **31**:785-799.

Aravin AA, Sachidanandam R, Girard A, Fejes-Toth K, Hannon GJ. (2007) Developmentally regulated piRNA clusters implicate MILI in transposon control. Science. **316**:744-747.

Arney K. (2003) *H19* and *Igf2*-enhancing the confusion? Trends Genet. **19**:17-23.

Arney KL, Bae E, Olsen C, Drewell RA. (2006) The human and mouse *H19* imprinting control regions harbor an evolutionarily conserved silencer element that functions on transgenes in *Drosophila*. Dev Genes Evol. **216**:811-819.

Babak T, Deveale B, Armour C, Raymond C, Cleary MA, van der Kooy D, Johnson JM, Lim LP. (2008) Global survey of genomic imprinting by transcriptome sequencing. Curr Biol. **18**:1735-1741.

Baker WK. (1967) A clonal system of differential gene activity in *Drosophila*. Dev Biol. **16**:1-17.

Ballestar E, Pile L, Wassarman D, Wolffe A, Wade P. (2001) A *Drosophila* MBD family member is a transcriptional corepressor associated with specific genes. Eur J Biochem. **268**:5397-5406.

Bao X, Deng H, Johansen J, Girton J, Johansen KM. (2007) Loss-of-function alleles of the JIL-1 histone H3S10 kinase enhance position-effect variegation at pericentric sites in *Drosophila* heterochromatin. Genetics. **176**:1355-1358.

Bao X, Girton J, Johansen J, Johansen K. (2006) The lamin *Dm(0)* allele Ari3 acts as an enhancer of position effect variegation of the *wm4* allele in *Drosophila*. Genetica. **129**:339-342.

Baqir S, Smith LC. (2006) Inhibitors of histone deacetylases and DNA methyltransferases alter imprinted gene regulation in embryonic stem cells. Cloning Stem Cells. **8**:200-213.

Barlow DP. (1993) Methylation and imprinting: from host defense to gene regulation? Science. **260**:309-310.

Barlow DP, Stoger R, Herrmann BG, Saito K, 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.

Barski A, Cuddapah S, Cui K, Roh T-Y, Schones DE, Wang Z, Wei G, Chepelev I, Zhao K. (2007) High-resolution profiling of histone methylations in the human genome. Cell. **129**:823-837.

Bartkuhn M, Straub T, Herold M, Herrmann M, Rathke C, Saumweber H, Gilfillan GD, Becker PB, Renkawitz R. (2009) Active promoters and insulators are marked by the centrosomal protein 190. EMBO J. **28**:877-888.

Bartolomei M, Webber A, Brunkow M, Tilghman S. (1993) Epigenetic mechanisms underlying the imprinting of the mouse *H19* gene. Genes Dev. **7**:1663-1673.

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

Bartolomei MS. (2009) Genomic imprinting: employing and avoiding epigenetic processes. Genes Dev. **23**:2124-2133.

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

Beaujean N, Hartshorne G, Cavilla J, Taylor J, Gardner J, Wilmut I, Meehan R, Young L. (2004) Non-conservation of mammalian preimplantation methylation dynamics. Curr Biol. **14**:R266-267.

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

Bellen HJ, Levis RW, Liao G, He Y, Carlson JW, Tsang G, Evans-Holm M, Hiesinger PR, Schulze KL, Rubin GM and others. (2004) The BDGP gene disruption project: single transposon insertions associated with 40% of *Drosophila genes*. Genetics. **167**:761-781.

Berger S. (2002) Histone modifications in transcriptional regulation. Curr Opin Genet Dev. **12**:142-148.

Bergman CM, Quesneville H, Anxolabéhère D, Ashburner M. (2006) Recurrent insertion and duplication generate networks of transposable element sequences in the *Drosophila melanogaster* genome. Genome Biol. **7**:R112.

Bernstein E, Caudy A, Hammond S, Hannon G. (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature. **409**:363-366.

Birchler J, Kavi H, Fernandez H. (2004) Heterochromatin: RNA points the way. Curr Biol. **14**:R759-761.

Blaydes S, Elmore M, Yang T, Brannan C. (1999) Analysis of murine *Snrpn* and human *SNRPN* gene imprinting in transgenic mice. Mamm Genome. **10**:549-555.

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

Bongiorni S, Pasqualini B, Taranta M, Singh PB, Prantera G. (2007) Epigenetic regulation of facultative heterochromatinisation in *Planococcus citri* via the Me(3)K9H3-HP1-Me(3)K20H4 pathway. J Cell Sci. **120**:1072-1080.

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

Bourque G, Leong B, Vega VB, Chen X, Lee YL, Srinivasan KG, Chew J-L, Ruan Y, Wei C-L, Ng HH and others. (2008) Evolution of the mammalian transcription factor binding repertoire via transposable elements. Genome Res. **18**:1752-1762.

Braissant O, Wahli W. (1998) A simplified *in situ* hybridization protocol using non-radioactively labeled probes to detect abundant and rare mRNAs on tissue sections. Biochemica. **1**:10-16.

Brennecke J, Aravin A, Stark A, Dus M, Kellis M, Sachidanandam R, Hannon G. (2007) Discrete small RNA-generating loci as master regulators of transposon activity in *Drosophila*. Cell. **128**:1089-1103.

Brenton J, Drewell R, Viville S, Hilton K, Barton S, Ainscough J, Surani M. (1999) A silencer element identified in *Drosophila* is required for imprinting of *H19* reporter transgenes in mice. PNAS. **96**:9242-9247.

Brenton JD, Ainscough JF-X, Lyko F, Paro R, Surani MA. (1998) Imprinting and Gene Silencing in Mice and *Drosophila*. Novartis Found Symp. **214**:233-244.

Brower-Toland B, Findley S, Jiang L, Liu L, Yin H, Dus M, Zhou P, Elgin S, Lin H. (2007) *Drosophila* PIWI associates with chromatin and interacts directly with HP1a. Genes Dev. **21**:2300-2311.

Bühler M. (2009) RNA turnover and chromatin-dependent gene silencing. Chromosoma. **118**:141-151.

Buszczak M. (2006) The *Drosophila* P68 RNA helicase regulates transcriptional deactivation by promoting RNA release from chromatin. Genes Dev. **20**:977-989.

Byrne K, Colgrave ML, Vuocolo T, Pearson R, Bidwell CA, Cockett NE, Lynn DJ, Fleming-Waddell JN, Tellam RL. (2010) The imprinted retrotransposon-like gene *PEG11* (*RTL1*) is expressed as a full-length protein in skeletal muscle from Callipyge sheep. PLoS ONE. **5**:e8638.

Capelson M, Corces VG. (2004) Boundary elements and nuclear organization. Biol Cell. **96**:617-629.

Carlson LL, Page AW, Bestor TH. (1992) Properties and localization of DNA methyltransferase in preimplantation mouse embryos: implications for genomic imprinting. Genes Dev. **6**:2536-2541.

Carrozza MJ, Utley RT, Workman JL, Côté J. (2003) The diverse functions of histone acetyltransferase complexes. Trends Genet. **19**:321-329.

Caudy AA, Myers M, Hannon GJ, Hammond SM. (2002) Fragile X-related protein and VIG associate with the RNA interference machinery. Genes Dev. **16**:2491-2496.

Cedar H, Bergman Y. (2009) Linking DNA methylation and histone modification: patterns and paradigms. Nat Rev Genet. **10**:295-304.

Celniker SE, Dillon LAL, Gerstein MB, Gunsalus KC, Henikoff S, Karpen GH, Kellis M, Lai EC, Lieb JD, Macalpine DM and others. (2009) Unlocking the secrets of the genome. Nature. **459**:927-930.

Cerrato F, Dean W, Davies K, Kagotani K, Mitsuya K, Okumura K, Riccio A, Reik W. (2003) Paternal imprints can be established on the maternal *Igf2-H19* locus without altering replication timing of DNA. Hum Mol Genet. **12**:3123-3132.

Chan S, Zilberman D, Xie Z, Johansen L, Carrington J, Jacobsen S. (2004) RNA silencing genes control *de novo* DNA methylation. Science. **303**:1336.

Charlier C, Segers K, Wagenaar D, Karim L, Berghmans S, Jaillon O, Shay T, Weissenbach J, Cockett N, Gyapay G and others. (2001) Human-ovine comparative sequencing of a 250-kb imprinted domain encompassing the callipyge (clpg) locus and identification of six imprinted transcripts: *DLK1, DAT, GTL2, PEG11, antiPEG11*, and *MEG8*. Genome Res. **11**:850-862.

Chen G, Fernandez J, Mische S, Courey AJ. (1999) A functional interaction between the histone deacetylase Rpd3 and the corepressor Groucho in *Drosophila* development. Genes Dev. **13**:2218-2230.

Chen Y, Dhupelia A, Schoenherr C. (2008) The *Igf2/H19* imprinting control region exhibits sequence-specific and cell-type-dependent DNA methylation-mediated repression. Nucleic Acids Res. **37**:793-803.

Cheung P, Lau P. (2005) Epigenetic regulation by histone methylation and histone variants. Mol Endocrinol. **19**:563-573.

Choi Y, Gehring M, Johnson L, Hannon M, Harada J, Goldberg R, Jacobsen S, Fischer R. (2002) DEMETER, a DNA glycosylase domain protein, is required for endosperm gene imprinting and seed viability in *Arabidopsis*. Cell. **110**:33-42.

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

Ciurciu A, Komonyi O, Boros IM. (2008) Loss of ATAC-specific acetylation of histone H4 at Lys12 reduces binding of JIL-1 to chromatin and phosphorylation of histone H3 at Ser10. J Cell Sci. **121**:3366-3372.

Cohen J. (1962) Position-effect variegation at several closely linked loci in *Drosophila melanogaster*. Genetics. **47**:647-659.

Colosimo A, Di Rocco G, Curini V, Russo V, Capacchietti G, Berardinelli P, Mattioli M, Barboni B. (2009) Characterization of the methylation status of five imprinted genes in sheep gametes. Animal Genet. **40**:900-908.

Constancia M, Dean W, Lopes S, Moore T, Kelsey G, Reik W. (2000) Deletion of a silencer element in *Igf2* results in loss of imprinting independent of *H19*. Nat Genet. **26**:203-206.

Cook H, Koppetsch B, Wu J, Theurkauf W. (2004) The *Drosophila SDE3* homolog armitage is required for oskar mRNA silencing and embryonic axis specification. Cell. **116**:817-829.

Cox DN, Chao A, Baker J, Chang L, Qiao D, Lin H. (1998) A novel class of evolutionarily conserved genes defined by *piwi* are essential for stem cell self-renewal. Genes Dev. **12**:3715-3727.

Cox DN, Chao A, Lin H. (2000) *piwi* encodes a nucleoplasmic factor whose activity modulates the number and division rate of germline stem cells. Development. **127**:503-514.

Crouse HV. (1960) The Controlling Element in Sex Chromosome Behavior in *Sciara*. Genetics. **45**:1429-1443.

Cuddapah S, Jothi R, Schones D, Roh T, Cui K, Zhao K. (2009) Global analysis of the insulator binding protein CTCF in chromatin barrier regions reveals demarcation of active and repressive domains. Genome Res. **19**:24-32.

Czermin B, Schotta G, Hülsmann BB, Brehm A, Becker PB, Reuter G, Imhof A. (2001) Physical and functional association of SU(VAR)3-9 and HDAC1 in *Drosophila*. EMBO Rep. **2**:915-919.

Cziko A-MJ, McCann CT, Howlett IC, Barbee SA, Duncan RP, Luedemann R, Zarnescu D, Zinsmaier KE, Parker RR, Ramaswami M. (2009) Genetic modifiers of *dFMR1* encode RNA granule components in *Drosophila*. Genetics. **182**:1051-1060.

Das PP, Bagijn MP, Goldstein LD, Woolford JR, Lehrbach NJ, Sapetschnig A, Buhecha HR, Gilchrist MJ, Howe KL, Stark R and others. (2008) Piwi and piRNAs act upstream of an endogenous siRNA pathway to suppress *Tc3* transposon mobility in the *Caenorhabditis elegans* germline. Mol Cell. **31**:79-90.

Davis E, Caiment F, Tordoir X, Cavaille J, Ferguson-Smith A, Cockett N, Georges M, Charlier C. (2005) RNAi-Mediated Allelic trans-Interaction at the Imprinted *Rtl1/Peg11* Locus. Curr Biol. **15**:743-749.

de la Casa-Esperón E, Sapienza C. (2003) Natural selection and the evolution of genome imprinting. Annu Rev Genet. **37**:349-370.

De Rubertis F, Kadosh D, Henchoz S, Pauli D, Reuter G, Struhl K, Spierer P. (1996) The histone deacetylase RPD3 counteracts genomic silencing in *Drosophila* and yeast. Nature. **384**:589-591.

de Wit E, Greil F, van Steensel B. (2007) High-resolution mapping reveals links of HP1 with active and inactive chromatin components. PLoS Genet. **3**:e38.

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

Delaval K, Feil R. (2004) Epigenetic regulation of mammalian genomic imprinting. Curr Opin Genet Dev. **14**:188-195.

Deng H, Bao X, Cai W, Blacketer MJ, Belmont AS, Girton J, Johansen J, Johansen KM. (2008) Ectopic histone H3S10 phosphorylation causes chromatin structure remodeling in *Drosophila*. Development. **135**:699-705.

Deng H, Bao X, Zhang W, Girton J, Johansen J, Johansen KM. (2007) Reduced levels of Su(var)3-9 but not Su(var)2-5 (HP1) counteract the effects on chromatin structure and viability in loss-of-function mutants of the JIL-1 histone H3S10 kinase. Genetics. **177**:79-87.

Deng X, Meller VH. (2006a) Non-coding RNA in fly dosage compensation. Trends Biochem Sci. **31**:526-532.

Deng X, Meller VH. (2006b) *roX* RNAs are required for increased expression of X-linked genes in *Drosophila melanogaster* males. Genetics. **174**:1859-1866.

Deng X, Rattner BP, Souter S, Meller VH. (2005) The severity of *roX1* mutations is predicted by MSL localization on the X chromosome. Mech Dev. **122**:1094-1105.

Dialynas GK, Vitalini MW, Wallrath LL. (2008) Linking Heterochromatin Protein 1 (HP1) to cancer progression. Mutat Res. **647**:13-20.

Dimitri P, Caizzi R, Giordano E, Carmela Accardo M, Lattanzi G, Biamonti G. (2009) Constitutive heterochromatin: a surprising variety of expressed sequences. Chromosoma. **118**:419-435.

Dimitri P, Corradini N, Rossi F, Verni F. (2005) The paradox of functional heterochromatin. Bioessays. **27**:29-41.

Dindot S, Kent K, Evers B, Loskutoff N, Womack J, Piedrahita J. (2004) Conservation of genomic imprinting at the XIST, IGF2, and GTL2 loci in the bovine. Mamm Genome. **15**:966-974.

Dion MF, Kaplan T, Kim M, Buratowski S, Friedman N, Rando OJ. (2007) Dynamics of replication-independent histone turnover in budding yeast. Science. **315**:1405-1408.

Djupedal I, Ekwall K. (2009) Epigenetics: heterochromatin meets RNAi. Cell Res. **19**:282-295.

Dobie K, Mehtali M, McClenaghan M, Lathe R. (1997) Variegated gene expression in mice. Trends Genet. **13**:127-130.

Dobie KW, Kennedy CD, Velasco VM, McGrath TL, Weko J, Patterson RW, Karpen GH. (2001) Identification of chromosome inheritance modifiers in *Drosophila melanogaster*. Genetics. **157**:1623-1637.

Doheny JG, Mottus R, Grigliatti TA, Bielinsky A-K. (2008) Telomeric Position Effect—A Third Silencing Mechanism in Eukaryotes. PLoS ONE. **3**:e3864.

Dorus S, Busby SA, Gerike U, Shabanowitz J, Hunt DF, Karr TL. (2006) Genomic and functional evolution of the *Drosophila melanogaster* sperm proteome. Nat Genet. **38**:1440-1445.

Drewell RA, Brenton JD, Ainscough JF, Barton SC, Hilton KJ, Arney KL, Dandolo L, Surani MA. (2000) Deletion of a silencer element disrupts *H19* imprinting independently of a DNA methylation epigenetic switch. Development. **127**:3419-3428.

Ebert A, Lein S, Schotta G, Reuter G. (2006) Histone modification and the control of heterochromatic gene silencing in *Drosophila*. Chromosome Res. **14**:377-392.

Ebert A, Schotta G, Lein S, Kubicek S, Krauss V, Jenuwein T, Reuter G. (2004) *Su(var)* genes regulate the balance between euchromatin and heterochromatin in *Drosophila*. Genes Dev. **18**:2973-2983.

Edwards CA, Ferguson-Smith AC. (2007) Mechanisms regulating imprinted genes in clusters. Curr Opin Cell Biol. **19**:281-289.

Edwards CA, Mungall AJ, Matthews L, Ryder E, Gray DJ, Pask AJ, Shaw G, Graves JAM, Rogers J, consortium tS and others. (2008) The evolution of the *DLK1-DIO3* imprinted domain in mammals. PLoS Biol. **6**:e135.

Eissenberg J, Elgin S. (2000) The HP1 protein family: getting a grip on chromatin. Curr Opin Genet Dev. **10**:204-210.

Eissenberg J, Wong M, Chrivia J. (2005) Human *SRCAP* and *Drosophila melanogaster DOM* are homologs that function in the notch signaling pathway. Mol Cell Biol. **25**:6559-6569.

Eissenberg JC, James TC, Foster-Hartnett DM, Hartnett T, Ngan V, Elgin SC. (1990) Mutation in a heterochromatin-specific chromosomal protein is associated with suppression of position-effect variegation in *Drosophila melanogaster*. PNAS. **87**:9923-9927.

Erhardt S, Lyko F, Ainscough J, Surani M, Paro R. (2003) Polycomb-group proteins are involved in silencing processes caused by a transgenic element from the murine imprinted *H19/Igf2* region in *Drosophila*. Dev Genes Evol. **213**:336-344.

Eversole-Cire P, Ferguson-Smith AC, Sasaki H, Brown KD, Cattanach BM, Gonzales FA, Surani MA, Jones PA. (1993) Activation of an imprinted *Igf2* gene in mouse somatic cell cultures. Mol Cell Biol. **13**:4928-4938.

Fagegaltier D, Bougé A-L, Berry B, Poisot E, Sismeiro O, Coppée J-Y, Théodore L, Voinnet O, Antoniewski C. (2009) The endogenous siRNA pathway is involved in heterochromatin formation in *Drosophila*. PNAS. **106**:21258-21263.

Fanti L, Perrini B, Piacentini L, Berloco M, Marchetti E, Palumbo G, Pimpinelli S. (2008) The trithorax group and Pc group proteins are differentially involved in heterochromatin formation in *Drosophila*. Chromosoma. **117**:25-39.

Fedoriw A, Stein P, Svoboda P, Schultz R, Bartolomei M. (2004) Transgenic RNAi reveals essential function for CTCF in *H19* gene imprinting. Science. **303**:238-240.

Feil R, Berger F. (2007) Convergent evolution of genomic imprinting in plants and mammals. Trends Genet. **23**:192-199.

Filippova G. (2008) Genetics and Epigenetics of the Multifunctional Protein CTCF. Curr Top in Dev Biol. **80**:337-360.

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature. **391**:806-811.

Fitch K, Yasuda G, Owens K, Wakimoto B. (1998) Paternal effects in *Drosophila*: implications for mechanisms of early development. Curr Top Dev Biol. **38**:1-34.

Fitzpatrick GV, Pugacheva EM, Shin J-Y, Abdullaev Z, Yang Y, Khatod K, Lobanenkov VV, Higgins MJ. (2007) Allele-specific binding of CTCF to the multipartite imprinting control region *KvDMR1*. Mol Cell Biol. **27**:2636-2647.

Foglietti C, Filocamo G, Cundari E, De Rinaldis E, Lahm A, Cortese R, Steinkühler C. (2006) Dissecting the biological functions of *Drosophila* histone deacetylases by RNA interference and transcriptional profiling. J Biol Chem. **281**:17968-17976.

Förstemann K, Tomari Y, Du T, Vagin V, Denli A, Bratu D, Klattenhoff C, Theurkauf W, Zamore P. (2005) Normal microRNA maturation and germ-line stem cell maintenance requires Loquacious, a double-stranded RNA-binding domain protein. PLoS Biol. **3**:e236.

Fournier C, Goto Y, Ballestar E, Delaval K, Hever A, Esteller M, Feil R. (2002) Allele-specific histone lysine methylation marks regulatory regions at imprinted mouse genes. EMBO J. **21**:6560-6570.

Friedrich M. (2006) Ancient mechanisms of visual sense organ development based on comparison of the gene networks controlling larval eye, ocellus, and compound eye specification in *Drosophila*. Arthropod Struct Dev. **35**:357-378.

Fu Y, Sinha M, Peterson CL, Weng Z. (2008) The insulator binding protein CTCF positions 20 nucleosomes around its binding sites across the human genome. PLoS Genet. **4**:e1000138.

Garnier O, Laoueillé-Duprat S, Spillane C. (2008) Genomic imprinting in plants. Epigenetics. **3**:14-20.

Gauhar Z, Ghanim M, Herreman T, Lambert JD, Li TR, Mason C, Rifkin S, Sun L, White KP, Costello JC and others. (2008) *Drosophila melanogaster* life-cycle gene expression dataset and microarray normalisation protocols. FLybase.

Gehring M, Bubb KL, Henikoff S. (2009a) Extensive demethylation of repetitive elements during seed development underlies gene imprinting. Science. **324**:1447-1451.

Gehring M, Reik W, Henikoff S. (2009b) DNA demethylation by DNA repair. Trends Genet. **25**:82-90.

Gerasimova T, Lei E, Bushey A, Corces V. (2007) Coordinated control of dCTCF and *gypsy* chromatin insulators in *Drosophila*. Mol Cell. **28**:761-772.

Gerasimova TI, Corces VG. (1998) Polycomb and trithorax group proteins mediate the function of a chromatin insulator. Cell. **92**:511-521.

Ghildiyal M, Seitz H, Horwich MD, Li C, Du T, Lee S, Xu J, Kittler ELW, Zapp ML, Weng Z and others. (2008) Endogenous siRNAs Derived from Transposons and mRNAs in *Drosophila* Somatic Cells. Science. **320**:1077-1081.

Giannoukakis N, Deal C, Paquette J, Goodyer C, Polychronakos C. (1993) Parental genomic imprinting of the human *IGF2* gene. Nat Genet. **4**:98-101.

Goldberg M, Harel A, Brandeis M, Rechsteiner T, Richmond TJ, Weiss AM, Gruenbaum Y. (1999) The tail domain of lamin Dm0 binds histones H2A and H2B. PNAS. **96**:2852-2857.

Goll M, Kirpekar F, Maggert K, Yoder J, Hsieh C, Zhang X, Golic K, Jacobsen S, Bestor T. (2006) Methylation of tRNAAsp by the DNA methyltransferase homolog Dnmt2. Science. **311**:395-398.

Gombert WM, Krumm A. (2009) Targeted deletion of multiple CTCF-binding elements in the human C-MYC gene reveals a requirement for CTCF in C-MYC expression. PLoS ONE. **4**:e6109.

Gowen J, Gay E. (1934) Chromosome constitution and behavior in eversporting and mottling in *Drosophila melanogaster*. Genetics. **19**:189-208.

Gracheva E, Dus M, Elgin SCR. (2009) *Drosophila* RISC Component VIG and Its Homolog Vig2 Impact Heterochromatin Formation. PLoS ONE. **4**:e6182.

Grandjean V, O'Neill L, Sado T, Turner B, Ferguson-Smith A. (2001) Relationship between DNA methylation, histone H4 acetylation and gene expression in the mouse imprinted Igf2-H19 domain. FEBS Letters. **488**:165-169.

Greally JM, Gray TA, Gabriel JM, Song L, Zemel S, Nicholls RD. (1999) Conserved characteristics of heterochromatin-forming DNA at the *15q11-q13* imprinting center. PNAS. **96**:14430-14435.

Gregory RI, Randall TE, Johnson CA, Khosla S, Hatada I, O'Neill LP, Turner BM, Feil R. (2001) DNA methylation is linked to deacetylation of histone H3, but not H4, on the imprinted genes *Snrpn* and *U2af1-rs1*. Mol Cell Biol. **21**:5426-5436.

Grewal S, Elgin S. (2002) Heterochromatin: new possibilities for the inheritance of structure. Curr Opin Genet Dev. **12**:178-187.

Gribnau J, Hochedlinger K, Hata K, Li E, Jaenisch R. (2003) Asynchronous replication timing of imprinted loci is independent of DNA methylation, but consistent with differential subnuclear localization. Genes Dev. **17**:759-773.

Grienenberger A, Miotto B, Sagnier T, Cavalli G, Schramke V, Geli V, Mariol MC, Berenger H, Graba Y, Pradel J. (2002) The MYST domain acetyltransferase Chameau functions in epigenetic mechanisms of transcriptional repression. Curr Biol. **12**:762-766.

Gruenbaum Y, Goldman RD, Meyuhas R, Mills E, Margalit A, Fridkin A, Dayani Y, Prokocimer M, Enosh A. (2003) The nuclear lamina and its functions in the nucleus. Int Rev Cytol. **226**:1-62.

Grunstein M. (1997) Molecular model for telomeric heterochromatin in yeast. Curr Opin Cell Biol. **9**:383-387.

Gunawardane L, Saito K, Nishida K, Miyoshi K, Kawamura Y, Nagami T, Siomi H, Siomi M. (2007) A slicer-mediated mechanism for repeat-associated siRNA 5' end formation in *Drosophila*. Science. **315**:1587-1590.

Gvozdev V, Aravin A, Abramov Y, Klenov M, Kogan G, Lavrov S, Naumova N, Olenkina O, Tulin A, Vagin V. (2003) *Stellate* repeats: targets of silencing and modules causing *cis*-inactivation and *trans*-activation. Genetica. **117**:239-245.

Gvozdev VA, Abramov IA, Kogan GL, Lavrov SA. (2007) Distorted heterochromatin replication in *Drosophila melanogaster* polytene chromosomes as a result of euchromatin-heterochromatin rearrangements. Genetika. **43**:18-26.

Haberland A, Zaitsev S, Waldöfner N, Erdmann B, Böttger M, Henke W. (2009) Structural appearance of linker histone H1/siRNA complexes. Mol Biol Rep. **36**:1083-1093.

Haigh A, Lloyd V. (2006) Loss of genomic imprinting in *Drosophila* clones. Genome. **49**:1043-1046.

Haller BS, Woodruff RC. (2000) Varied expression of a Y-linked *P[w+]* insert due to imprinting in *Drosophila melanogaster*. Genome. **43**:285-292.

Han T, Manoharan AP, Harkins TT, Bouffard P, Fitzpatrick C, Chu DS, Thierry-Mieg D, Thierry-Mieg J, Kim JK. (2009) 26G endo-siRNAs regulate spermatogenic and zygotic gene expression in *Caenorhabditis elegans*. PNAS. **106**:18674-18679.

Hanai K, Furuhashi H, Yamamoto T, Akasaka K, Hirose S. (2008) RSF governs silent chromatin formation via histone H2Av replacement. PLoS Genet. **4**:e1000011.

Hardy R, Lindsley D, Livak K, Lewis B, Siversten A, Joslyn G, Edwards J, Bonaccorsi S. (1984) Cytogenetic analysis of a segment of the Y chromosome of *Drosophila melanogaster*. Genetics. **107**:591-610.

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

Harony H, Ankri S. (2008) What do unicellular organisms teach us about DNA methylation? Trends Parasitol. **24**:205-209.

Harris AN, Macdonald PM. (2001) *Aubergine* encodes a *Drosophila* polar granule component required for pole cell formation and related to eIF2C. Development. **128**:2823-2832.

Hartig JV, Esslinger S, Böttcher R, Saito K, Förstemann K. (2009) Endo-siRNAs depend on a new isoform of loquacious and target artificially introduced, high-copy sequences. EMBO J. **28**:2932-2944.

Hashimoto H, Sonoda E, Takami Y, Kimura H, Nakayama T, Tachibana M, Takeda S, Shinkai Y. (2007) Histone H1 variant, H1R is involved in DNA damage response. DNA Repair (Amst). **6**:1584-1595.

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

Haynes KA, Gracheva E, Elgin SCR. (2007) A Distinct type of heterochromatin within *Drosophila melanogaster* chromosome 4. Genetics. **175**:1539-1542.

Henckel A, Nakabayashi K, Sanz LA, Feil R, Hata K, Arnaud P. (2009) Histone methylation is mechanistically linked to DNA methylation at imprinting control regions in mammals. Hum Mol Genet. **18**:3375-3383.

Hendrich B, Guy J, Ramsahoye B, Wilson VA, Bird A. (2001) Closely related proteins MBD2 and MBD3 play distinctive but interacting roles in mouse development. Genes Dev. **15**:710-723.

Hendrich B, Tweedie S. (2003) The methyl-CpG binding domain and the evolving role of DNA methylation in animals. Trends Genet. **19**:269-277.

Henikoff S, Henikoff JG, Sakai A, Loeb GB, Ahmad K. (2009) Genome-wide profiling of salt fractions maps physical properties of chromatin. Genome Res. **19**:460-469.

Hermann A, Gowher H, Jeltsch A. (2004) Biochemistry and biology of mammalian DNA methyltransferases. Cell Mol Life Sci. **61**:2571-2587.

Hermann A, Schmitt S, Jeltsch A. (2003) The human Dnmt2 has residual DNA-(cytosine-C5) methyltransferase activity. J Biol Chem. **278**:31717-31721.

Hiragami-Hamada K, Xie SQ, Saveliev A, Uribe-Lewis S, Pombo A, Festenstein R. (2009) The molecular basis for stability of heterochromatin-mediated silencing in mammals. Epigenet. Chrom. **2**:14.

Hirasawa R, Chiba H, Kaneda M, Tajima S, Li E, Jaenisch R, Sasaki H. (2008) Maternal and zygotic Dnmt1 are necessary and sufficient for the maintenance of DNA methylation imprints during preimplantation development. Genes Dev. **22**:1607-1616.

Holohan E, Kwong C, Adryan B, Bartkuhn M, Herold M, Renkawitz R, Russell S, White R. (2007) CTCF Genomic Binding Sites in *Drosophila* and the Organisation of the *Bithorax* Complex. PLoS Genet. **3**:e112.

Hou J, Liu L, Zhang J, Cui X-H, Yan F-X, Guan H, Chen Y-F, An X-R. (2008) Epigenetic modification of histone 3 at lysine 9 in sheep zygotes and its relationship with DNA methylation. BMC Dev Biol. **8**:60.

Hsieh T-F, Ibarra CA, Silva P, Zemach A, Eshed-Williams L, Fischer RL, Zilberman D. (2009) Genome-wide demethylation of *Arabidopsis* endosperm. Science. **324**:1451-1454.

Hu JF, Pham J, Dey I, Li T, Vu TH, Hoffman AR. (2000) Allele-specific histone acetylation accompanies genomic imprinting of the insulin-like growth factor II receptor gene. Endocrinology. **141**:4428-4435.

Huisinga KL, Elgin SCR. (2009) Small RNA-directed heterochromatin formation in the context of development: what flies might learn from fission yeast. Biochim Biophys Acta. **1789**:3-16.

Hurst LD, McVean GT. (1998) Do we understand the evolution of genomic imprinting? Curr Opin Genet Dev. **8**:701-708.

Hutter B, Helms V, Paulsen M. (2006) Tandem repeats in the CpG islands of imprinted genes. Genomics. **88**:323-332.

Imamura T, Kerjean A, Heams T, Kupiec J-J, Thenevin C, Pàldi A. (2005) Dynamic CpG and non-CpG methylation of the *Peg1/Mest* gene in the mouse oocyte and preimplantation embryo. J Biol Chem. **280**:20171-20175.

Jahnke S, Scholten S. (2009) Epigenetic resetting of a gene imprinted in plant embryos. Curr Biol. **19**:1677-1681.

James T, Elgin S. (1986) Identification of a nonhistone chromosomal protein associated with heterochromatin in *Drosophila melanogaster* and its gene. Mol Cell Biol. **6**:3862-3872.

Jaquet Y, Delattre M, Montoya-Burgos J, Spierer A, Spierer P. (2006) Conserved domains control heterochromatin localization and silencing properties of SU(VAR)3-7. Chromosoma. **115**:139-150.

Jelinic P, Shaw P. (2007) Loss of imprinting and cancer. J Pathol. **211**:261-268.

Jin P, Zarnescu D, Ceman S, Nakamoto M, Mowrey J, Jongens T, Nelson D, Moses K, Warren S. (2004) Biochemical and genetic interaction between the fragile X mental retardation protein and the microRNA pathway. Nat Neurosci. **7**:113-117.

Joanis V, Lloyd V. (2002) Genomic imprinting in *Drosophila* is maintained by the products of *Suppressor of variegation* and *trithorax* group, but not *Polycomb* group, genes. Mol Genet Genomics. **268**:103-112.

Jones B, Levorse J, Tilghman S. (2002) A human *H19* transgene exhibits impaired paternal-specific imprint acquisition and maintenance in mice. Hum Mol Genet. **11**:411-418.

Josefsson C, Dilkes B, Comai L. (2006) Parent-dependent loss of gene silencing during interspecies hybridization. Curr Biol. **16**:1322-1328.

Jullien P, Katz A, Oliva M, Ohad N, Berger F. (2006a) Polycomb group complexes self-regulate imprinting of the Polycomb group gene MEDEA in *Arabidopsis*. Curr Biol. **16**:486-492.

Jullien P, Kinoshita T, Ohad N, Berger F. (2006b) Maintenance of DNA Methylation during the *Arabidopsis* Life Cycle Is Essential for Parental Imprinting. Plant Cell. **18**:1360-1372.

Kacem S, Feil R. (2009) Chromatin mechanisms in genomic imprinting. Mamm Genome. **20**:544-556.

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

Kalmykova A, Klenov M, Gvozdev V. (2005) Argonaute protein PIWI controls mobilization of retrotransposons in the *Drosophila* male germline. Nucleic Acids Res. **33**:2052-2059.

Kamakaka R. (2003) Heterochromatin: proteins in flux lead to stable repression. Curr Biol. **13**:R317-319.

Kaneda M, Okano M, Hata K, Sado T, Tsujimoto N, Li E, Sasaki H. (2004) Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. Nature. **429**:900-903.

Kataoka Y, Takeichi M, Uemura T. (2001) Developmental roles and molecular characterization of a *Drosophila* homologue of *Arabidopsis* Argonaute1, the founder of a novel gene superfamily. Genes Cells. **6**:313-325.

Katayama S, Tomaru Y, Kasukawa T, Waki K, Nakanishi M, Nakamura M, Nishida H, Yap CC, Suzuki M, Kawai J and others. (2005) Antisense transcription in the mammalian transcriptome. Science. **309**:1564-1566.

Kato Y, Kaneda M, Hata K, Kumaki K, Hisano M, Kohara Y, Okano M, Li E, Nozaki M, Sasaki H. (2007) Role of the Dnmt3 family in de novo methylation of imprinted and repetitive sequences during male germ cell development in the mouse. Hum. Mol. Genet. **16**:2272-2280.

Katoh M, Curk T, Xu Q, Zupan B, Kuspa A, Shaulsky G. (2006) Developmentally regulated DNA methylation in *Dictyostelium discoideum*. Eukaryotic Cell. **5**:18-25.

Kavi HH, Birchler JA. (2009) Interaction of RNA polymerase II and the small RNA machinery affects heterochromatic silencing in *Drosophila*. Epigenet Chrom. **2**:15.

Kawahara M, Wu Q, Takahashi N, Morita S, Yamada K, Ito M, Ferguson-Smith AC, Kono T. (2007) High-frequency generation of viable mice from engineered bi-maternal embryos. Nat Biotechnol. **25**:1045-1050.

Kawamura Y, Saito K, Kin T, Ono Y, Asai K, Sunohara T, Okada TN, Siomi MC, Siomi H. (2008) *Drosophila* endogenous small RNAs bind to Argonaute 2 in somatic cells. Nature. **453**:793-797.

Kawasaki H, Taira K. (2004) Induction of DNA methylation and gene silencing by short interfering RNAs in human cells. Nature. **431**:211-217.

Kelley RL, Lee O-K, Shim Y-K. (2008) Transcription rate of noncoding *roX1* RNA controls local spreading of the *Drosophila* MSL chromatin remodeling complex. Mech. Dev. **125**:1009-1019.

Kennerdell J, Yamaguchi S, Carthew R. (2002) RNAi is activated during Drosophila oocyte maturation in a manner dependent on aubergine and spindle-E. Genes Dev. **16**:1884-1889.

Kent D. (2008) The role of histone H1 in imprint establishment in *Drosophila melanogaster*. MSc Thesis, Mount Allison Uiversity, Biology Department.

Kermicle JL. (1970) Dependenct of the R-Mottled Aleurone Phenotype in Maize on Mode of Sexual Transmission. Genetics. **66**:69-85.

Kim TH, Abdullaev ZK, Smith AD, Ching KA, Loukinov DI, Green RD, Zhang MQ, Lobanenkov VV, Ren B. (2007) Analysis of the vertebrate insulator protein CTCF-binding sites in the human genome. Cell. **128**:1231-1245.

Kinoshita T, Miura A, Choi Y, Kinoshita Y, Cao X, Jacobsen S, Fischer R, Kakutani T. (2004) One-way control of *FWA* imprinting in *Arabidopsis* endosperm by DNA methylation. Science. **303**:521-523.

Klattenhoff C, Bratu D, McGinnis-Schultz N, Koppetsch B, Cook H, Theurkauf W. (2007) *Drosophila* rasiRNA pathway mutations disrupt embryonic axis specification through activation of an ATR/Chk2 DNA damage response. Dev Cell. **12**:45-55.

Klattenhoff C, Theurkauf W. (2008) Biogenesis and germline functions of piRNAs. Development. **135**:3-9.

Klattenhoff C, Xi H, Li C, Lee S, Xu J, Khurana JS, Zhang F, Schultz N, Koppetsch BS, Nowosielska A and others. (2009) The *Drosophila* HP1 homolog Rhino is required for transposon silencing and piRNA production by dual-strand clusters. Cell. **138**:1137-1149.

Klenov M, Lavrov S, Stolyarenko A, Ryazansky S, Aravin A, Tuschl T, Gvozdev V. (2007) Repeat-associated siRNAs cause chromatin silencing of retrotransposons in the *Drosophila melanogaster* germline. Nucleic Acids Res. **35**:5430-5438.

Kobor M, Venkatasubrahmanyam S, Meneghini M, Gin J, Jennings J, Link A, Madhani H, Rine J. (2004) A protein complex containing the conserved Swi2/Snf2-related ATPase Swr1p deposits histone variant H2A.Z into euchromatin. PLoS Biol. **2**:E131.

Kohler C, Hennig L, Spillane C, Pien S, Gruissem W, Grossniklaus U. (2003) The Polycomb-group protein MEDEA regulates seed development by controlling expression of the MADS-box gene PHERES1. Genes Dev. **17**:1540-1553.

Kohler C, Page D, Gagliardini V, Grossniklaus U. (2005) The *Arabidopsis* thaliana MEDEA Polycomb group protein controls expression of PHERES1 by parental imprinting. Nat Genet. **37**:28-30.

Komashko VM, Farnham PJ. (2010) 5-azacytidine treatment reorganizes genomic histone modification patterns. Epigenetics. **5**:229-240.

Kotelnikov R, Klenov M, Rozovsky Y, Olenina L, Kibanov M, Gvozdev V. (2009) Peculiarities of piRNA-mediated post-transcriptional silencing of *Stellate* repeats in testes of *Drosophila melanogaster*. Nucleic Acids Res. **37**:3254-3263.

Kuhlmann M, Borisova BE, Kaller M, Larsson P, Stach D, Na J, Eichinger L, Lyko F, Ambros V, Söderbom F and others. (2005) Silencing of retrotransposons in Dictyostelium by DNA methylation and RNAi. Nucleic Acids Research. **33**:6405-6417.

Kunarso G, Chia N-Y, Jeyakani J, Hwang C, Lu X, Chan Y-S, Ng H-H, Bourque G. (2010) Transposable elements have rewired the core regulatory network of human embryonic stem cells. Nat Genet. **42**:631-634.

Kunert N, Marhold J, Stanke J, Stach D, Lyko F. (2003) A Dnmt2-like protein mediates DNA methylation in *Drosophila*. Development. **130**:5083-5090.

Kurukuti S, Tiwari VK, Tavoosidana G, Pugacheva E, Murrell A, Zhao Z, Lobanenkov V, Reik W, Ohlsson R. (2006) CTCF binding at the *H19* imprinting control region mediates maternally inherited higher-order chromatin conformation to restrict enhancer access to Igf2. PNAS. **103**:10684-10689.

Kusch T, Florens L, Macdonald WH, Swanson SK, Glaser RL, Yates JR, Abmayr SM, Washburn MP, Workman JL. (2004) Acetylation by Tip60 is required for selective histone variant exchange at DNA lesions. Science. **306**:2084-2087.

Kyrchanova O, Toshchakov S, Podstreshnaya Y, Parshikov A, Georgiev P. (2008) Functional Interaction between the Fab-7 and Fab-8 Boundaries and the Upstream Promoter Region in the *Drosophila Abd-B* Gene. Mol Cell Biol. **28**:4188-4195.

Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, FitzHugh W and others. (2001) Initial sequencing and analysis of the human genome. Nature. **409**:860-921.

Larsson J, Chen JD, Rasheva V, Rasmuson-Lestander A, Pirrotta V. (2001) Painting of fourth, a chromosome-specific protein in *Drosophila*. PNAS. **98**:6273-6278.

Latham K. (2005) X chromosome imprinting and inactivation in preimplantation mammalian embryos. Trends Genet. **21**:120-127.

Latos PA, Barlow DP. (2009) Regulation of imprinted expression by macro non-coding RNAs. RNA Biol. **6**:100-106.

Lawton B, Sevigny L, Obergfell C, Reznick D, O'Neill R J, O'Neill M J. (2005) Allelic expression of IGF2 in live-bearing, matrotrophic fishes. Dev Genes Evol. **215**:207-212.

Leach TJ, Mazzeo M, Chotkowski HL, Madigan JP, Wotring MG, Glaser RL. (2000) Histone H2A.Z is widely but nonrandomly distributed in chromosomes of *Drosophila melanogaster*. J Biol Chem. **275**:23267-23272.

Lee Y, Nakahara K, Pham J, Kim K, He Z, Sontheimer E, Carthew R. (2004) Distinct roles for *Drosophila* Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. Cell. **117**:69-81.

Lees-Murdock DJ, Walsh CP. (2008) DNA methylation reprogramming in the germ line. Epigenetics. **3**:5-13.

Lei EP, Corces VG. (2006) RNA interference machinery influences the nuclear organization of a chromatin insulator. Nat Genet. **38**:936-941.

Lerach S. (2006) Loss-Of-Function Alleles of the JIL-1 Kinase Are Strong Suppressors of Position Effect Variegation of the *wm4* Allele in *Drosophila*. Genetics. **173**:2403-2406.

Lewis A, Mitsuya K, Umlauf D, Smith P, Dean W, Walter J, Higgins M, Feil R, 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 A, Murrell A. (2004) Genomic Imprinting: CTCF Protects the Boundaries. Curr Biol. **14**:R284-R286.

Li C, Vagin VV, Lee S, Xu J, Ma S, Xi H, Seitz H, Horwich MD, Syrzycka M, Honda BM and others. (2009) Collapse of germline piRNAs in the absence of Argonaute3 reveals somatic piRNAs in flies. Cell. **137**:509-521.

Li T, Hu J-F, Qiu X, Ling J, Chen H, Wang S, Hou A, Vu TH, Hoffman AR. (2008) CTCF regulates allelic expression of *Igf2* by orchestrating a promoter-polycomb repressive complex 2 intrachromosomal loop. Mol Cell Biol. **28**:6473-6482.

Lin H, Yin H. (2009) A Novel Epigenetic Mechanism in *Drosophila* Somatic Cells Mediated by PIWI and piRNAs. Cold Spring Harb Symp Quant Biol. **73**:273-281.

Lin S, Youngson N, Takada S, Seitz H, Reik W, Paulsen M, Cavaille J, Ferguson-Smith A. (2003) Asymmetric regulation of imprinting on the maternal and paternal chromosomes at the *Dlk1-Gtl2* imprinted cluster on mouse chromosome 12. Nat Genet. **35**:97-102.

Ling J, Li T, Hu J, Vu T, Chen H, Qiu X, Cherry A, Hoffman A. (2006) CTCF mediates interchromosomal colocalization between *Igf2/H19* and *Wsb1/Nf1*. Science. **312**:269-272.

Lippman Z, Martienssen R. (2004) The role of RNA interference in heterochromatic silencing. Nature. **431**:364-370.

Lippman Z, May B, Yordan C, Singer T, Martienssen R. (2003) Distinct mechanisms determine transposon inheritance and methylation via small interfering RNA and histone modification. PLoS Biol. **1**:E67.

Lister R, Pelizzola M, Dowen RH, Hawkins RD, Hon G, Tonti-Filippini J, Nery JR, Lee L, Ye Z, Ngo Q-M and others. (2009) Human DNA methylomes at base resolution show widespread epigenomic differences. Nature. **462**:315-322.

Liu K, Wang YF, Cantemir C, Muller MT. (2003a) Endogenous assays of DNA methyltransferases: Evidence for differential activities of DNMT1, DNMT2, and DNMT3 in mammalian cells in vivo. Mol Cell Biol. **23**:2709-2719.

Liu Q, Rand TA, Kalidas S, Du F, Kim H-E, Smith DP, Wang X. (2003b) R2D2, a bridge between the initiation and effector steps of the *Drosophila* RNAi pathway. Science. **301**:1921-1925.

Liu X, Park J, Jiang F, Liu Y, McKearin D, Liu Q. (2007) Dicer-1, but not Loquacious, is critical for assembly of miRNA-induced silencing complexes. RNA. **13**:2324-2329.

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

Lloyd V, Sinclair D, Grigliatti T. (1999a) Genomic imprinting and position-effect variegation in *Drosophila melanogaster*. Genetics. **151**:1503-1516.

Lloyd V, Sinclair D, Wennberg R, Warner T, Honda B, Grigliatti T. (1999b) A genetic and molecular characterization of the *garnet* gene of *Drosophila melanogaster*. Genome. **42**:1183-1193.

Lopes S, Lewis A, Hajkova P, Dean W, Oswald J, Forne T, Murrell A, Constancia M, Bartolomei M, Walter J and others. (2003) Epigenetic modifications in an imprinting cluster are controlled by a hierarchy of DMRs suggesting long-range chromatin interactions. Hum Mol Genet. **12**:295-305.

Luo M, Bilodeau P, Dennis E, Peacock W, Chaudhury A. (2000) Expression and parent-of-origin effects for *FIS2*, *MEA*, and *FIE* in the endosperm and embryo of developing *Arabidopsis* seeds. PNAS. **97**:10637-10642.

Lyko F, Brenton J, Surani M, Paro R. (1997) An imprinting element from the mouse *H19* locus functions as a silencer in *Drosophila*. Nat Genet. **16**:171-173.

Lyko F, Buiting K, Horsthemke B, Paro R. (1998) Identification of a silencing element in the human *15q11-q13* imprinting center by using transgenic *Drosophila*. PNAS. **95**:1698-1702.

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

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

MacDonald WA, Menon D, Bartlett NJ, Sperry GE, Rasheva V, Meller V, Lloyd VK. (2010) The *Drosophila* homolog of the mammalian imprint regulator, CTCF, maintains the maternal genomic imprint in *Drosophila melanogaster*. BMC Biology. **8**:105.

Madigan JP, Chotkowski HL, Glaser RL. (2002) DNA double-strand break-induced phosphorylation of *Drosophila* histone variant H2Av helps prevent radiation-induced apoptosis. Nucleic Acids Res. **30**:3698-3705.

Maggert K, Golic K. (2002) The Y chromosome of *Drosophila melanogaster* exhibits chromosome-wide imprinting. Genetics. **162**:1245-1258.

Malone CD, Brennecke J, Dus M, Stark A, Mccombie WR, Sachidanandam R, Hannon GJ. (2009) Specialized piRNA pathways act in germline and somatic tissues of the *Drosophila* ovary. Cell. **137**:522-535.

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

Marhold J, Kramer K, Kremmer E, Lyko F. (2004a) The *Drosophila* MBD2/3 protein mediates interactions between the MI-2 chromatin complex and CpT/A-methylated DNA. Development. **131**:6033-6039.

Marhold J, Rothe N, Pauli A, Mund C, Kuehle K, Brueckner B, Lyko F. (2004b) Conservation of DNA methylation in dipteran insects. Insect Mol Biol. **13**:117-123.

Marhold J, Zbylut M, Lankenau D-H, Li M, Gerlich D, Ballestar E, Mechler BM, Lyko F. (2002) Stage-specific chromosomal association of *Drosophila* dMBD2/3 during genome activation. Chromosoma. **111**:13-21.

Marques JT, Kim K, Wu P-H, Alleyne TM, Jafari N, Carthew RW. (2010) Loqs and R2D2 act sequentially in the siRNA pathway in *Drosophila*. Nat Struct Mol Biol. **17**:24-30.

Matranga C, Zamore PD. (2007) Small silencing RNAs. Curr Biol. **17**:R789-793.

Matsuzaki H, Okamura E, Shimotsuma M, Fukamizu A, Tanimoto K. (2009) A randomly integrated transgenic *H19* imprinting control region acquires methylation imprinting independently of its establishment in germ cells. Mol Cell Biol. **29**:4595-4603.

Mattout A, Goldberg M, Tzur Y, Margalit A, Gruenbaum Y. (2007) Specific and conserved sequences in *D. melanogaster* and *C. elegans* lamins and histone H2A mediate the attachment of lamins to chromosomes. J Cell Sci. **120**:77-85.

Matzke AJ, Matzke MA. (1998) Position effects and epigenetic silencing of plant transgenes. Curr Opin Plant Biol. **1**:142-148.

McClintock B. (1984) The significance of responses of the genome to challenge. Science. **226**:792-801.

McDonald JF, Matzke MA, Matzke AJ. (2005) Host defenses to transposable elements and the evolution of genomic imprinting. Cytogenet Genome Res. **110**:242-249.

McEwen K, Ferguson-Smith A. (2009) Genomic Imprinting–A Model for Roles of Histone Modifications in Epigenetic Control. In: Epigenomics. 235-258.

McEwen KR, Ferguson-Smith AC. (2010) Distinguishing epigenetic marks of developmental and imprinting regulation. Epigenet Chrom. **3**:2.

McGowan R, Martin C. (1997) DNA methylation and genome imprinting in the zebrafish, *Danio rerio*: some evolutionary ramifications. Biochem Cell Biol. **75**:499-506.

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

Megosh H, Cox D, Campbell C, Lin H. (2006) The role of PIWI and the miRNA machinery in *Drosophila* germline determination. Curr Biol. **16**:1884-1894.

Meneghini MD, Wu M, Madhani HD. (2003) Conserved histone variant H2A.Z protects euchromatin from the ectopic spread of silent heterochromatin. Cell. **112**:725-736.

Menon DU, Meller VH. (2009) Imprinting of the Y chromosome influences dosage compensation in *roX1 roX2 Drosophila melanogaster*. Genetics. **183**:811-820.

Metz C. (1925) Chromosomes and sex in *Sciara*. Science. **61**:212-214.

Meyer W, Schreiber S, Guo Y, Volkmann T, Welte M, Muller H. (2006) Overlapping functions of argonaute proteins in patterning and morphogenesis of *Drosophila* embryos. PLoS Genet. **2**:e134.

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

Miller D, Brinkworth M, Iles D. (2009) Paternal DNA packaging in spermatozoa: more than the sum of its parts? Reproduction. **139**:287-301.

Miotto B, Sagnier T, Berenger H, Bohmann D, Pradel J, Graba Y. (2006) Chameau HAT and DRpd3 HDAC function as antagonistic cofactors of JNK/AP-1-dependent transcription during *Drosophila* metamorphosis. Genes Dev. **20**:101-112.

Mito Y, Henikoff JG, Henikoff S. (2007) Histone replacement marks the boundaries of *cis*-regulatory domains. Science. **315**:1408-1411.

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

Mohan M, Bartkuhn M, Herold M, Philippen A, Heinl N, Bardenhagen I, Leers J, White R, Renkawitz-Pohl R, Saumweber H and others. (2007) The *Drosophila* insulator proteins CTCF and CP190 link enhancer blocking to body patterning. EMBO J. **26**:4203-4214.

Moon H, Filippova G, Loukinov D, Pugacheva E, Chen Q, Smith S, Munhall A, Grewe B, Bartkuhn M, Arnold R and others. (2005) CTCF is conserved from *Drosophila* to humans and confers enhancer blocking of the *Fab-8* insulator. EMBO Rep. **6**:165-170.

Moore G, Sinclair D, Grigliatti T. (1983) Histone Gene Multiplicity and Position Effect Variegation in *Drosophila Melanogaster*. Genetics. **105**:327-344.

Moore T, Haig D. (1991) Genomic imprinting in mammalian development: a parental tug-of-war. Trends Genet. **7**:45-49.

Mosher RA, Melnyk CW, Kelly KA, Dunn RM, Studholme DJ, Baulcombe DC. (2009) Uniparental expression of PolIV-dependent siRNAs in developing endosperm of *Arabidopsis*. Nature. **460**:283-286.

Moshkovich N, Lei EP. (2010) HP1 recruitment in the absence of argonaute proteins in *Drosophila*. PLoS Genet. **6**:e1000880.

Mottus R, Reeves R, Grigliatti TA. (1980) Butyrate suppression of position-effect variegation in *Drosophila melanogaster*. Mol Gen Genet. **178**:465-469.

Mottus R, Sobel RE, Grigliatti TA. (2000) Mutational analysis of a histone deacetylase in *Drosophila melanogaster*: missense mutations suppress gene silencing associated with position effect variegation. Genetics. **154**:657-668.

Muchardt C, Guilleme M, Seeler J, Trouche D, Dejean A, Yaniv M. (2002) Coordinated methyl and RNA binding is required for heterochromatin localization of mammalian HP1alpha. EMBO Rep. **3**:975-981.

Mukhopadhyay R, Yu W, Whitehead J, Xu J, Lezcano M, Pack S, Kanduri C, Kanduri M, Ginjala V, Vostrov A and others. (2004) The binding sites for the chromatin insulator protein CTCF map to DNA methylation-free domains genome-wide. Genome Res. **14**:1594-1602.

Muller H. (1930) Types of visible variations induced by X-rays in *Drosophila*. J Genet. **22**:299-334.

Murrell A, Heeson S, Bowden L, Constancia M, Dean W, Kelsey G, Reik W. (2001) An intragenic methylated region in the imprinted *Igf2* gene augments transcription. EMBO Rep. **2**:1101-1106.

Murrell A, Heeson S, Reik W. (2004) Interaction between differentially methylated regions partitions the imprinted genes *Igf2* and *H19* into parent-specific chromatin loops. Nat Genet. **36**:889-893.

Ner SS, Harrington MJ, Grigliatti TA. (2002) A role for the *Drosophila* SU(VAR)3-9 protein in chromatin organization at the histone gene cluster and in suppression of position-effect variegation. Genetics. **162**:1763-1774.

Nicholls RD, Knoll JH, Butler MG, Karam S, Lalande M. (1989) Genetic imprinting suggested by maternal heterodisomy in nondeletion Prader-Willi syndrome. Nature. **342**:281-285.

Nishida K, Saito K, Mori T, Kawamura Y, Nagami-Okada T, Inagaki S, Siomi H, Siomi M. (2007) Gene silencing mechanisms mediated by Aubergine piRNA complexes in *Drosophila* male gonad. RNA. **13**:1911-1922.

Nolan C, Killian J, Petitte J, Jirtle R. (2001) Imprint status of *M6P/IGF2R* and *IGF2* in chickens. Dev Genes Evol. **211**:179-183.

Normark BB. (2003) The evolution of alternative genetic systems in insects. Annu Rev Entomol. **48**:397-423.

Nur U. (1990) Heterochromatization and euchromatization of whole genomes in scale insects (*Coccoidea: Homoptera*). Dev Suppl.29-34.

Obbard DJ, Finnegan DJ. (2008) RNA interference: endogenous siRNAs derived from transposable elements. Curr Biol. **18**:R561-563.

Obbard DJ, Gordon KHJ, Buck AH, Jiggins FM. (2009) The evolution of RNAi as a defence against viruses and transposable elements. Philos Trans R Soc Lond, B, Biol Sci. **364**:99-115.

Ogawa Y, Sun BK, Lee JT. (2008) Intersection of the RNA interference and X-inactivation pathways. Science. **320**:1336-1341.

Ohlsson R, Renkawitz R, Lobanenkov V. (2001) CTCF is a uniquely versatile transcription regulator linked to epigenetics and disease. Trends Genet. **17**:520-527.

Ooi SKT, O'Donnell AH, Bestor TH. (2009) Mammalian cytosine methylation at a glance. J Cell Sci. **122**:2787-2791.

Orlando V. (2003) Polycomb, epigenomes, and control of cell identity. Cell. **112**:599-606.

Osouda S, Nakamura Y, de Saint Phalle B, McConnell M, Horigome T, Sugiyama S, Fisher PA, Furukawa K. (2005) *Null* mutants of *Drosophila* B-type *lamin Dm0* show aberrant tissue differentiation rather than obvious nuclear shape distortion or specific defects during cell proliferation. Dev Biol. **284**:219-232.

Pal-Bhadra M, Bhadra U, Birchler JA. (2002) RNAi related mechanisms affect both transcriptional and posttranscriptional transgene silencing in *Drosophila*. Mol Cell. **9**:315-327.

Pal-Bhadra M, Leibovitch B, Gandhi S, Rao M, Bhadra U, Birchler J, Elgin S. (2004) Heterochromatic silencing and HP1 localization in *Drosophila* are dependent on the RNAi machinery. Science. **303**:669-672.

Pane A, Wehr K, Schüpbach T. (2007) *zucchini* and *squash* encode two putative nucleases required for rasiRNA production in the *Drosophila* germline. Dev Cell. **12**:851-862.

Pardo-Manuel de Villena F, de la Casa-Esperón E, Sapienza C. (2000) Natural selection and the function of genome imprinting: beyond the silenced minority. Trends Genet. **16**:573-579.

Pask A, Papenfuss A, Ager E, McColl K, Speed T, Renfree M. (2009) Analysis of the platypus genome suggests a transposon origin for mammalian imprinting. Genome Biol. **10**:R1.

Pauler FM, Koerner MV, Barlow DP. (2007) Silencing by imprinted noncoding RNAs: is transcription the answer? Trends Genet. **23**:284-292.

Pearsall R, Plass C, Romano M, Garrick M, Shibata H, Hayashizaki Y, Held W. (1999) A direct repeat sequence at the *Rasgrf1* locus and imprinted expression. Genomics. **55**:194-201.

Peng J, Karpen G. (2007) H3K9 methylation and RNA interference regulate nucleolar organization and repeated DNA stability. Nat Cell Biol. **9**:25-35.

Peng JC, Karpen GH. (2009) Heterochromatic genome stability requires regulators of histone H3 K9 methylation. PLoS Genet. **5**:e1000435.

Peters J, Beechey C. (2004) Identification and characterisation of imprinted genes in the mouse. Brief Funct Genomic Proteomic. **2**:320-333.

Phalke S, Nickel O, Walluscheck D, Hortig F, Onorati MC, Reuter G. (2009) Retrotransposon silencing and telomere integrity in somatic cells of *Drosophila* depends on the cytosine-5 methyltransferase DNMT2. Nat Genet. **41**:696-702.

Pimpinelli S, Berloco M, Fanti L, Dimitri P, Bonaccorsi S, Marchetti E, Caizzi R, Caggese C, Gatti M. (1995) Transposable elements are stable structural components of Drosophila melanogaster heterochromatin. PNAS. **92**:3804-3808.

Ramos E, Ghosh D, Baxter E, Corces VG. (2006) Genomic organization of *gypsy* chromatin insulators in *Drosophila melanogaster*. Genetics. **172**:2337-2349.

Ramsahoye BH, Biniszkiewicz D, Lyko F, Clark V, Bird AP, Jaenisch R. (2000) Non-CpG methylation is prevalent in embryonic stem cells and may be mediated by DNA methyltransferase 3a. PNAS. **97**:5237-5242.

Real, MD, Ferre, J, Mensua, JL. (1985) Methods for the quantitative estimation of the red and brown pigments of *D. melanogaster.* Dros Inf Serv. **61**:198-200.

Reese KJ, Lin S, Verona RI, Schultz RM, Bartolomei MS. (2007) Maintenance of paternal methylation and repression of the imprinted *H19* gene requires MBD3. PLoS Genet. **3**:e137.

Regha K, Sloane MA, Huang R, Pauler FM, Warczok KE, Melikant B, Radolf M, Martens JHA, Schotta G, Jenuwein T and others. (2007) Active and repressive chromatin are interspersed without spreading in an imprinted gene cluster in the mammalian genome. Mol Cell. **27**:353-366.

Reik W, Collick A, Norris M, Barton S, Surani M. (1987) Genomic imprinting determines methylation of parental alleles in transgenic mice. Nature. **328**:248-251.

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

Renfree MB, Hore TA, Shaw G, GRAVES JAM, Pask AJ. (2009) Evolution of genomic imprinting: insights from marsupials and monotremes. Annual review of genomics and human genetics. **10**:241-262.

Reuter G, Dorn R, Hoffmann H. (1982) Butyrate sensitive suppressor of position-effect variegation mutations in *Drosophila melanogaster*. Mol Genet Genomics. **188**:480-485.

Riclet R, Chendeb M, Vonesch J-L, Koczan D, Thiesen H-J, Losson R, Cammas F. (2009) Disruption of the interaction between transcriptional intermediary factor 1*β* and heterochromatin protein 1 leads to a switch from DNA hyper- to hypomethylation and H3K9 to H3K27 trimethylation on the *MEST* promoter correlating with gene reactivation. Mol Biol cell. **20**:296-305.

Roder K, Hung MS, Lee TL, Lin TY, Xiao H, Isobe KI, Juang JL, Shen CJ. (2000) Transcriptional repression by *Drosophila* methyl-CpG-binding proteins. Mol Cell Biol. **20**:7401-7409.

Rosidi B, Wang M, Wu W, Sharma A, Wang H, Iliakis G. (2008) Histone H1 functions as a stimulatory factor in backup pathways of NHEJ. Nucleic Acids Res. **36**:1610-1623.

Royo H, Cavaillé J. (2008) Non-coding RNAs in imprinted gene clusters. Biol Cell. **100**:149-166.

Rudolph T, Yonezawa M, Lein S, Heidrich K, Kubicek S, Schafer C, Phalke S, Walther M, Schmidt A, Jenuwein T and others. (2007) Heterochromatin formation in *Drosophila* is initiated through active removal of H3K4 methylation by the *LSD1* homolog *SU(VAR)3-3*. Mol Cell. **26**:103-115.

Ruhf M, Braun A, Papoulas O, Tamkun J, Randsholt N, Meister M. (2001) The *domino* gene of *Drosophila* encodes novel members of the SWI2/SNF2 family of DNA-dependent ATPases, which contribute to the silencing of homeotic genes. Development. **128**:1429-1441.

Rundlett SE, Carmen AA, Kobayashi R, Bavykin S, Turner BM, Grunstein M. (1996) HDA1 and RPD3 are members of distinct yeast histone deacetylase complexes that regulate silencing and transcription. PNAS. **93**:14503-14508.

Saito K, Inagaki S, Mituyama T, Kawamura Y, Ono Y, Sakota E, Kotani H, Asai K, Siomi H, Siomi MC. (2009) A regulatory circuit for piwi by the large Maf gene *traffic jam* in *Drosophila*. Nature. **461**:1296-1299.

Saito K, Nishida K, Mori T, Kawamura Y, Miyoshi K, Nagami T, Siomi H, Siomi M. (2006) Specific association of Piwi with rasiRNAs derived from retrotransposon and heterochromatic regions in the Drosophila genome. Genes Dev. **20**:2214-2222.

Sapienza C, Peterson A, Rossant J, Balling R. (1987) Degree of methylation of transgenes is dependent on gamete of origin. Nature. **328**:251-254.

Sasaki H, Jones PA, Chaillet JR, Ferguson-Smith AC, Barton SC, Reik W, Surani MA. (1992) Parental imprinting: potentially active chromatin of the repressed maternal allele of the mouse insulin-like growth factor II (Igf2) gene. Genes & Development. **6**:1843-1856.

Scarano MI, Strazzullo M, Matarazzo MR, D'Esposito M. (2005) DNA methylation 40 years later: Its role in human health and disease. J. Cell. Physiol. **204**:21-35.

Schaefer M, Lyko F. (2010) Solving the Dnmt2 enigma. Chromosoma. **119**:35-40.

Schaefer M, Steringer JP, Lyko F. (2008) The *Drosophila* cytosine-5 methyltransferase Dnmt2 is associated with the nuclear matrix and can access DNA during mitosis. PLoS ONE. **3**:e1414.

Schoborg TA, Labrador M. (2010) The phylogenetic distribution of non-CTCF insulator proteins is limited to insects and reveals that BEAF-32 is *Drosophila* lineage specific. J Mol Evol. **70**:74-84.

Schoenfelder S, Paro R. (2004) *Drosophila* Su(Hw) regulates an evolutionarily conserved silencer from the mouse *H19* imprinting control region. Cold Spring Harb Symp Quant Biol. **69**:47-54.

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

Schoenherr CJ, Levorse JM, Tilghman SM. (2003) CTCF maintains differential methylation at the *Igf2/H19* locus. Nat Genet. **33**:66-69.

Schotta G, Ebert A, Dorn R, Reuter G. (2003) Position-effect variegation and the genetic dissection of chromatin regulation in *Drosophila*. Semin Cell Dev Biol. **14**:67-75.

Schotta G, Ebert A, Krauss V, Fischer A, Hoffmann J, Rea S, Jenuwein T, Dorn R, 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.

Schotta G, Lachner M, Sarma K, Ebert A, Sengupta R, Reuter G, Reinberg D, Jenuwein T. (2004) A silencing pathway to induce H3-K9 and H4-K20 trimethylation at constitutive heterochromatin. Genes Dev. **18**:1251-1262.

Schrader F. (1921) The chromosomes of *Pseudococcus nipae*. Biol. bull. **40**:259-270

Schübeler D, Macalpine DM, Scalzo D, Wirbelauer C, Kooperberg C, Van Leeuwen F, Gottschling DE, O'Neill LP, Turner BM, Delrow J and others. (2004) The histone modification pattern of active genes revealed through genome-wide chromatin analysis of a higher eukaryote. Genes Dev. **18**:1263-1271.

Schultz J. (1936) Variegation in *Drosophila* and the Inert Chromosome Regions. PNAS. **22**:27-33.

Schulze SR, Wallrath LL. (2007) Gene regulation by chromatin structure: paradigms established in *Drosophila melanogaster*. Annu Rev Entomol. **52**:171-192.

Schüpbach T, Wieschaus E. (1991) Female sterile mutations on the second chromosome of Drosophila melanogaster. II. Mutations blocking oogenesis or altering egg morphology. Genetics. **129**:1119-1136.

Seitz H, Youngson N, Lin S, Dalbert S, Paulsen M, Bachellerie J, Ferguson-Smith A, Cavaille J. (2003) Imprinted microRNA genes transcribed antisense to a reciprocally imprinted retrotransposon-like gene. Nat Genet. **34**:261-262.

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

Shin J-Y, Fitzpatrick GV, Higgins MJ. (2008) Two distinct mechanisms of silencing by the KvDMR1 imprinting control region. EMBO J. **27**:168-178.

Shirohzu H, Yokomine T, Sato C, Kato R, Toyoda A, Purbowasito W, Suda C, Mukai T, Hattori M, Okumura K and others. (2004) A 210-kb segment of tandem repeats and retroelements located between imprinted subdomains of mouse distal chromosome 7. DNA Res. **11**:325-334.

Shiura H, Nakamura K, Hikichi T, Hino T, Oda K, Suzuki-Migishima R, Kohda T, Kaneko-ishino T, Ishino F. (2009) Paternal deletion of *Meg1/Grb10* DMR causes maternalization of the *Meg1/Grb10* cluster in mouse proximal Chromosome 11 leading to severe pre- and postnatal growth retardation. Hum Mol Genet. **18**:1424-1438.

Shpiz S, Kwon D, Rozovsky Y, Kalmykova A. (2009) rasiRNA pathway controls antisense expression of *Drosophila* telomeric retrotransposons in the nucleus. Nucleic Acids Res. **37**:268-278.

Siomi MC, Miyoshi T, Siomi H. (2010) piRNA-mediated silencing in *Drosophila* germlines. Semin Cell Dev Biol. [Epub]

Sleutels F, Tjon G, Ludwig T, Barlow D. (2003) Imprinted silencing of *Slc22a2* and *Slc22a3* does not need transcriptional overlap between *Igf2r* and *Air*. EMBO J. **22**:3696-3704.

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

Smith ST, Wickramasinghe P, Olson A, Loukinov D, Lin L, Deng J, Xiong Y, Rux J, Sachidanandam R, Sun H, Lobanenkov V, Zhou J. (2009) Genome wide ChIP-chip analyses reveal important roles for CTCF in *Drosophila* genome organization. Dev Biol. **328**:518-528.

Smits G, Mungall A, Griffiths-Jones S, Smith P, Beury D, Matthews L, Rogers J, Pask A, Shaw G, Vandeberg J and others. (2008) Conservation of the H19 noncoding RNA and H19-IGF2 imprinting mechanism in therians. Nat Genet. **40**:971-976

Spada F, Haemmer A, Kuch D, Rothbauer U, Schermelleh L, Kremmer E, Carell T, Längst G, Leonhardt H. (2007) DNMT1 but not its interaction with the replication machinery is required for maintenance of DNA methylation in human cells. J. Cell Biol. **176**:565-571.

Spofford J. (1967) Single-locus modification of position-effect variegation in *Drosophila melanogaster*. I. *white* variegation. Genetics. **57**:751-766.

Spofford JB. (1976) Position-effect variegation in *Drosophila*. In: The Genetics and Biology of *Drosophila* Edited by M. Ashburner and E. Novitski. Academic Press, London, U.K. **1c**:955-1018.

Steinemann S, Steinemann M. (2005) Y chromosomes: born to be destroyed. Bioessays. **27**:1076-1083.

Steinhoff C, Schulz WA. (2003) Transcriptional regulation of the human LINE-1 retrotransposon L1.2B. Mol Genet Genomics. **270**:394-402.

Stöger R, Kubicka P, Liu CG, Kafri T, Razin A, Cedar H, Barlow DP. (1993) Maternal-specific methylation of the imprinted mouse Igf2r locus identifies the expressed locus as carrying the imprinting signal. Cell. **73**:61-71.

Suetake I, Shinozaki F, Miyagawa J, Takeshima H, Tajima S. (2004) DNMT3L stimulates the DNA methylation activity of Dnmt3a and Dnmt3b through a direct interaction. J Biol Chem. **279**:27816-27823.

Sullivan W, Ashburner M, Scott Hawley R. (2000) *Drosophila* protocols. W. Sullivan, M. Ashburner, and R. S. Hawley (eds.) Appendix 3 Reagents & Solutions. Cold Spring Harbor Laboratory Press. 655.

Sun F-L, Haynes K, Simpson CL, Lee SD, Collins L, Wuller J, Eissenberg JC, Elgin SCR. (2004) *cis*-Acting determinants of heterochromatin formation on *Drosophila melanogaster* chromosome four. Mol Cell Biol. **24**:8210-8220.

Surani MA, Barton SC, Norris ML. (1984) Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. Nature. **308**:548-550.

Suzuki S, Ono R, Narita T, Pask AJ, Shaw G, Wang C, Kohda T, Alsop AE, Marshall Graves JA, Kohara Y and others. (2007) Retrotransposon silencing by DNA methylation can drive mammalian genomic imprinting. PLoS Genet. **3**:e55.

Suzuki S, Renfree M, Pask A, Shaw G, Kobayashi S, Kohda T, Kaneko-Ishino T, Ishino F. (2005) Genomic imprinting of IGF2, p57(KIP2) and PEG1/MEST in a marsupial, the tammar wallaby. Mech Dev. **122**:213-222.

Swaminathan J, Baxter E, Corces V. (2005) The role of histone H2Av variant replacement and histone H4 acetylation in the establishment of *Drosophila* heterochromatin. Genes Dev. **19**:65-76.

Szabo P, Tang S, Silva F, Tsark W, Mann J. (2004) Role of CTCF binding sites in the *Igf2/H19* imprinting control region. Mol Cell Biol. **24**:4791-4800.

Takada S, Paulsen M, Tevendale M, Tsai C-E, Kelsey G, Cattanach BM, Ferguson-Smith AC. (2002) Epigenetic analysis of the *Dlk1-Gtl2* imprinted domain on mouse chromosome 12: implications for imprinting control from comparison with *Igf2-H19*. Hum Mol Genet. **11**:77-86.

Takeda S, Paszkowski J. (2005) DNA methylation and epigenetic inheritance during plant gametogenesis. Chromosoma. **115**:27-35.

Talbert PB, Henikoff S. (2000) A reexamination of spreading of position-effect variegation in the *white-roughest* region of *Drosophila melanogaster*. Genetics. **154**:259-272.

Tamkun JW, Deuring R, Scott MP, Kissinger M, Pattatucci AM, Kaufman TC, Kennison JA. (1992) brahma: a regulator of *Drosophila* homeotic genes structurally related to the yeast transcriptional activator *SNF2/SWI2*. Cell. **68**:561-572.

Tanabe M, Kouzmenko A, Ito S, Sawatsubashi S, Suzuki E, Fujiyama S, Yamagata K, Zhao Y, Kimura S, Ueda T and others. (2008) Activation of facultatively silenced *Drosophila* loci associates with increased acetylation of histone H2AvD. Genes Cells. **13**:1279-1288.

Tariq M, Paszkowski J. (2004) DNA and histone methylation in plants. Trends Genet. **20**:244-251.

Thurston A, Taylor J, Gardner J, Sinclair KD, Young LE. (2008) Monoallelic expression of nine imprinted genes in the sheep embryo occurs after the blastocyst stage. Reproduction. **135**:29-40.

Tomari Y, Du T, Haley B, Schwarz D, Bennett R, Cook H, Koppetsch B, Theurkauf W, Zamore P. (2004) RISC assembly defects in the *Drosophila* RNAi mutant *armitage*. Cell. **116**:831-841.

Tucker KL, Beard C, Dausmann J, Jackson-Grusby L, Laird PW, Lei H, Li E, Jaenisch R. (1996) Germ-line passage is required for establishment of methylation and expression patterns of imprinted but not of nonimprinted genes. Genes Dev. **10**:1008-1020.

Tuna M, Knuutila S, Mills GB. (2009) Uniparental disomy in cancer. Trends Mol Med. **15**:120-128.

Turner BM, Birley AJ, Lavender J. (1992) Histone H4 isoforms acetylated at specific lysine residues define individual chromosomes and chromatin domains in *Drosophila* polytene nuclei. Cell. **69**:375-384.

Tweedie S, Ng HH, Barlow AL, Turner BM, Hendrich B, Bird A. (1999) Vestiges of a DNA methylation system in *Drosophila melanogaster*? Nat Genet. **23**:389-390.

Vagin V, Klenov M, Kalmykova A, Stolyarenko A, Kotelnikov R, Gvozdev V. (2004) The RNA interference proteins and vasa locus are involved in the silencing of retrotransposons in the female germline of *Drosophila melanogaster*. RNA Biology. **1**:54-58.

Vagin V, Sigova A, Li C, Seitz H, Gvozdev V, Zamore P. (2006) A distinct small RNA pathway silences selfish genetic elements in the germline. Science. **313**:320-324.

van Daal A, Elgin SC. (1992) A histone variant, H2AvD, is essential in *Drosophila melanogaster*. Mol Biol Cell. **3**:593-602.

Verdel A, Vavasseur A, Le Gorrec M, Touat-Todeschini L. (2009) Common themes in siRNA-mediated epigenetic silencing pathways. Int. J. Dev. Biol. **53**:245-257.

Verona RI, Mann MRW, Bartolomei MS. (2003) Genomic imprinting: intricacies of epigenetic regulation in clusters. Annu. Rev. Cell. Dev. Biol. **19**:237-259.

Vlassova IE, Graphodatsky AS, Belyaeva ES, Zhimulev IF. (1991) Constitutive heterochromatin in early embryogenesis of *Drosophila melanogaster*. Mol Gen Genet. **229**:316-318.

Vogel MJ, Pagie L, Talhout W, Nieuwland M, Kerkhoven RM, van Steensel B. (2009) High-resolution mapping of heterochromatin redistribution in a *Drosophila* position-effect variegation model. Epigenet. Chrom. **2**:1-18.

Volpe TA, Kidner C, Hall IM, Teng G, Grewal SIS, Martienssen RA. (2002) Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. Science. **297**:1833-1837.

Vrana PB, Fossella JA, Matteson P, del Rio T, O'Neill MJ, Tilghman SM. (2000) Genetic and epigenetic incompatibilities underlie hybrid dysgenesis in Peromyscus. Nat Genet. **25**:120-124.

Vu T, Li T, Hoffman A. (2004) Promoter-restricted histone code, not the differentially methylated DNA regions or antisense transcripts, marks the imprinting status of *IGF2R* in human and mouse. Hum Mol Genet. **13**:2233-2245.

Wan L, Pan H, Hannenhalli S, Cheng Y, Ma J, Fedoriw A, Lobanenkov V, Latham K, Schultz R, Bartolomei M. (2008) Maternal depletion of CTCF reveals multiple functions during oocyte and preimplantation embryo development. Development. **135**:2729-2738

Wan L-B, Bartolomei MS. (2008) Regulation of imprinting in clusters: noncoding RNAs versus insulators. Adv Genet. **61**:207-223.

Wang M, Wesley S, Finnegan E, Smith N, Waterhouse P. (2001) Replicating satellite RNA induces sequence-specific DNA methylation and truncated transcripts in plants. RNA. **7**:16-28.

Weidman J, Murphy S, Nolan C, Dietrich F, Jirtle R. (2004) Phylogenetic footprint analysis of *IGF2* in extant mammals. Genome Res. **14**:1726-1732.

Wen B, Wu H, Bjornsson H, Green RD, Irizarry R, Feinberg AP. (2008) Overlapping euchromatin/heterochromatin- associated marks are enriched in imprinted gene regions and predict allele-specific modification. Genome Res. **18**:1806-1813.

Weyrich A, Tang X, Xu G, Schrattenholz A, Hunzinger C, Hennig W. (2008) Mammalian DNMTs in the male germ line DNA of *Drosophila*. Biochem Cell Biol. **86**:380-385.

Wilkins J, Haig D. (2003) What good is genomic imprinting: the function of parent-specific gene expression. Nat Rev Genet. **4**:359-368.

Williams RW, Rubin GM. (2002) ARGONAUTE1 is required for efficient RNA interference in *Drosophila* embryos. PNAS. **99**:6889-6894.

Williamson A, Lehmann R. (1996) Germ cell development in *Drosophila*. Annu Rev Cell Dev Biol. **12**:365-391.

Wilson JE, Connell JE, Macdonald PM. (1996) *aubergine* enhances *oskar* translation in the *Drosophila* ovary. Development. **122**:1631-1639.

Wines DR, Henikoff S. (1992) Somatic instability of a *Drosophila* chromosome. Genetics. **131**:683-691.

Wutz A, Smrzka O, Schweifer N, Schellander K, Wagner E, Barlow D. (1997) Imprinted expression of the *Igf2r* gene depends on an intronic CpG island. Nature. **389**:745-749.

Xiao W, Custard KD, Brown RC, Lemmon BE, Harada JJ, Goldberg RB, Fischer RL. (2006) DNA methylation is critical for *Arabidopsis* embryogenesis and seed viability. Plant Cell. **18**:805-814.

Xiao W, Gehring M, Choi Y, Margossian L, Pu H, Harada J, Goldberg R, Pennell R, Fischer R. (2003) Imprinting of the MEA Polycomb gene is controlled by antagonism between MET1 methyltransferase and DME glycosylase. Dev Cell. **5**:891-901.

Yamamoto K, Sonoda M, Inokuchi J, Shirasawa S, Sasazuki T. (2004) Polycomb group Suppressor of Zeste 12 links Heterochromatin Protein 1alpha and Enhancer of Zeste 2. J Biol Chem. **279**:401-406.

Yamanaka S, Katayama E, Yoshioka K-i, Nagaki S, Yoshida M, Teraoka H. (2002) Nucleosome linker proteins HMGB1 and histone H1 differentially enhance DNA ligation reactions. Biochem Biophys Res Commun. **292**:268-273.

Yang X-J, Seto E. (2008) The Rpd3/Hda1 family of lysine deacetylases: from bacteria and yeast to mice and men. Nat Rev Mol Cell Biol. **9**:206-218.

Yin H, Lin H. (2007) An epigenetic activation role of Piwi and a Piwi-associated piRNA in *Drosophila melanogaster*. Nature. **450**:304-308.

Yoon B, Herman H, Hu B, Park Y, Lindroth A, Bell A, West A, Chang Y, Stablewski A, Piel J, Loukinov D, Lobanenkov V, Soloway P. (2005) *Rasgrf1* imprinting is regulated by a CTCF-dependent methylation-sensitive enhancer blocker. Mol Cell Biol. **25**:11184-11190.

Yoon YS, Jeong S, Rong Q, Park K-Y, Chung JH, Pfeifer K. (2007) Analysis of the *H19* ICR insulator. Mol Cell Biol. **27**:3499-3510.

Yusufzai T, Tagami H, Nakatani Y, Felsenfeld G. (2004) CTCF tethers an insulator to subnuclear sites, suggesting shared insulator mechanisms across species. Mol Cell. **13**:291-298.

Zakharova IS, Shevchenko AI, Zakian SM. (2009) Monoallelic gene expression in mammals. Chromosoma. **118**:279-290.

Zambon RA, Vakharia VN, Wu LP. (2006) RNAi is an antiviral immune response against a dsRNA virus in *Drosophila melanogaster*. Cell Microbiol. **8**:880-889.

Zeng Y, Yi R, Cullen B. (2003) MicroRNAs and small interfering RNAs can inhibit mRNA expression by similar mechanisms. PNAS. **100**:9779-9784.

Zhang W, Deng H, Bao X, Lerach S, Girton J, Johansen J, Johansen KM. (2006) The JIL-1 histone H3S10 kinase regulates dimethyl H3K9 modifications and heterochromatic spreading in *Drosophila*. Development. **133**:229-235.

Zhang X, Clarenz O, Cokus S, Bernatavichute YV, Pellegrini M, Goodrich J, Jacobsen SE. (2007) Whole-genome analysis of histone H3 lysine 27 trimethylation in *Arabidopsis*. PLoS Biol. **5**:e129.

Zhang Y, Qu L. (2009) Non-coding RNAs and the acquisition of genomic imprinting in mammals. Sci China, C, Life Sci. **52**:195-204.

Zhang Y, Tycko B. (1992) Monoallelic expression of the human *H19* gene. Nat Genet. **1**:40-44.

Zhao XD, Han X, Chew JL, Liu J, Chiu KP, Choo A, Orlov YL, Sung W-K, Shahab A, Kuznetsov VA, Bourque G, Oh S, Ruan Y, Ng H-H, Wei C-L. (2007) Whole-genome mapping of histone H3 Lys4 and 27 trimethylations reveals distinct genomic compartments in human embryonic stem cells. Cell Stem Cell. **1**:286-298.

Zhou J, Zhou B, Lenzmeier B, Zhou J. (2009) Histone deacetylase *Rpd3* antagonizes Sir2-dependent silent chromatin propagation. Nucleic Acids Res. **37**:3699-3713.

Zilberman D, Coleman-Derr D, Ballinger T, Henikoff S. (2008) Histone H2A.Z and DNA methylation are mutually antagonistic chromatin marks. Nature. **456**:125-129.

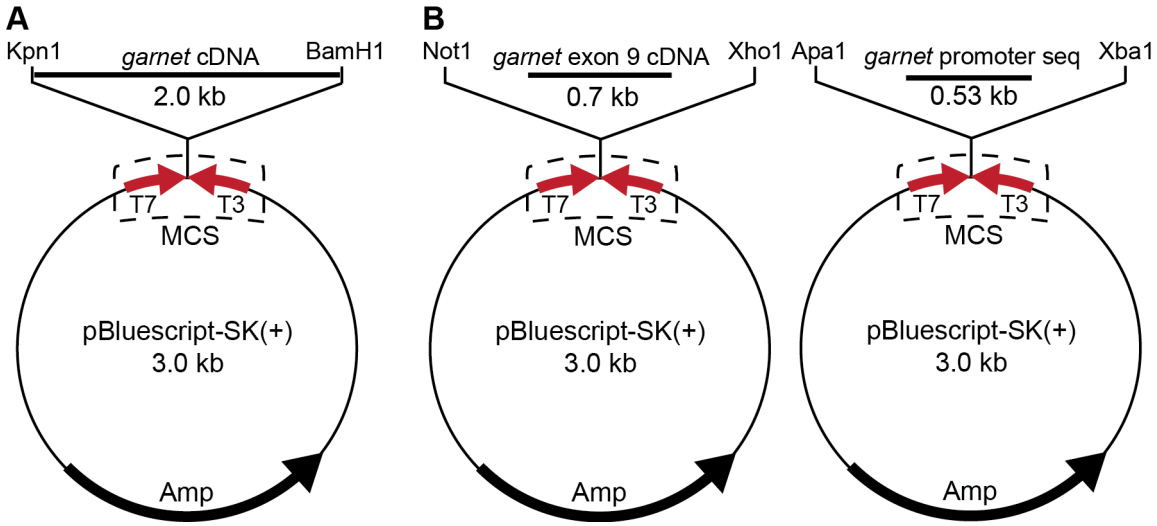
Appendix A *Drosophila* Stock List

Allele	Stock #	Function	Mutagen
ago1^{04845}	11388	RNAi miRNA pathway	P{PZ}
ago1^{k00208}	10470	RNAi miRNA pathway	P{lacW}
ago1^{k08121}	10772	RNAi miRNA pathway	P{lacW}
ago2^{dop1}	5242	RNAi siRNA pathway	EMS
ago2^{EY04479}	16608	RNAi siRNA pathway	P{EPgy2}
armi$^{72.1}$	8544	RNAi piRNA pathway	Δ2-3
aub^{HN}	8517	RNAi piRNA pathway	EMS
aub^{KG05389}	1400	RNAi piRNA pathway	P{SUPor-P}
aub^{QC42}	4968	RNAi piRNA pathway	EMS
chm^{BG02254}	12666	Histone acetyltransferase	P{GT1}
CTCF^{30}	V. Meller	Insulator Protein	P{EPgy2}
CTCF^{EY15833}	12262	Insulator Protein	P{EPgy2}
dcr2$^{L811\ fsx}$	R. Carthew	RNAi siRNA pathway	EMS
dom^{3}	9260	Histone Replacement	Δ2-3
dom^{9}	9261	Histone Replacement	Δ2-3
Dp(1;f)LJ9	3219	mini-X chromosome	X-ray
H2A^{KG00275}	14346	Histone	P{SUPor-P}
H2Av^{810}	9264	Histone variant	EMS
H3^{KG00688}	13689	Histone	P{SUPor-P}
H3.3A^{KG02455}	13315	Histone variant	P{SUPor-P}
H4^{Scim}	4979	Histone	Δ2-3
His4r^{EY06726}	15970	Histone variant	P{EPgy2}
In(1)w^{m4}	807	Chromosomal Inversion	X-ray
JIL-1^{3}	6347	Histone serine kinase	EMS
lam^{04643}	11384	Nuclear lamin	P{PZ}
lam^{sz18}	6392	Nuclear lamin	EMS
loqs^{f00791}	8371	RNAi miRNA	PBac{WH}
MBD-Like^{EY04582}	15753	DNA Methyl binding	P{EPgy2}

Allele	Stock #	Function	Mutagen
pasha^{EY01325}	15509	RNAi miRNA pathway	P{EPgy2}
piwi^{06843}	12225	RNAi piRNA pathway	P{PZ}
r2d2^{1}	8518	RNAi siRNA pathway	EMS
r2d2^{EY00028}	14997	RNAi siRNA pathway	P{EPgy2}
r2d2$^{S165\ fsx}$	R. Carthew	RNAi siRNA pathway	EMS
rm62^{01086}	11520	RNA Helicase	P{PZ}
rpd3^{04556}	11633	Histone Deacetylase	EMS
rsf-1^{KG00766}	12955	H2Av Replacement	P{SUPor-P}
rsf-1^{KG02636}	13325	H2Av Replacement	P{SUPor-P}
Spn-D^{2}	3326	DSB repair	EMS
Spn-E^{1}	3327	RNAi piRNA pathway	EMS
Spn-E^{c00786}	10274	RNAi piRNA pathway	PBac
squ^{PP32}	5114	RNAi piRNA pathway	EMS
vasa^{1}	284	RNA Helicase	EMS
vasa^{RJ36}	5011	RNA Helicase	EMS
vig^{EY07816}	17408	mRNA binding	P{EPgy2}
vig$^{\{3HPy+\}C274}$	16323	mRNA binding	PBac
X^X/X^Y; bw^{v}*/CyO*	1216	attached X	X-ray
zuc^{RS49}	5120	RNAi piRNA pathway	EMS
39C-33	S. Elgin	4th chromosome insert	*P{hsp26-pt,hsp70-w}*
6-M193	S. Elgin	4th chromosome insert	*P{hsp26-pt,hsp70-w}*

Stock number refers to the Bloomington *Drosophila* Stock Center reference number.
Stocks marked R. Carthew were created by and donated from the Dr. R. Carthew Lab.
Stocks marked S. Elgin were created by and donated from the Dr. S. Elgin Lab.
Stocks marked R. Meller were created by and donated from the Dr. V. Meller Lab.
EMS = ethyl methane sulfonate
P{*}, PBac{*}, Δ2-3 = P-element/ transposable element insertion or removal
X-ray = X-ray irradiation

Appendix B *In situ* Probe Constructs



A) Schematic of the *in situ* hybridization probe construct used in Chapter 4.

B) Schematic of the *in situ* hybridization probe constructs used in Chapter 6.

Probe constructs were generated by digesting DNA and pBlueScript-SK(+) (Fermentas) vector with the appropriate restriction enzymes, followed by purification with GFX (Fischer) columns and resuspension in 20uL RNAse free water. Ligation of DNA into the vector was accomplished with T4 ligase (Invitrogen) overnight at 18˚C. 10 µl of ligation sample was added to 50 µl of MAX Efficiency DH5α competent cells (Invitrogen) on ice of 30 minutes, heat shocked for 90 seconds at 37˚C, and placed on ice for 2 minutes. The competent cells were added to 500 µl LB media (Sigma-Aldrich) and incubated at 37˚C for up to 4 hours before being placed on agar plates with Ampicillin (Sigma-Aldrich) overnight at 37˚C. Single isolated colonies were selected for minipreparation of plasmid DNA, and successful transformations were determined by restriction digest and Gel electrophoresis.