GENOMIC IMPRINTING IN *Drosophila melanogaster*: EPigenetic Regulation of the *Dp(1;f)LJ9* Imprinted Domain

by

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Dedicated to Hal & Myrtle Tincombe, Bill & Gina MacDonald
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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 further 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

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

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 interspecies 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. Prokofyev-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)wm*4, 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)wm*4 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 heterochromatins, 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)sc29*, 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 \textit{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 \textit{Drosophila} (Kunert \textit{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 \textit{garnet} from paternally-inherited \textit{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 \textit{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 \textit{et al.} 2005), yet an \textit{H2Av} null mutation enhanced silencing of \textit{garnet} from paternally-inherited \textit{Dp(1;f)LJ9} and disrupted the stable boundary of maternally-inherited \textit{Dp(1;f)LJ9}. This finding suggests that H2Av is a dynamic component of heterochromatin, and that the \textit{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 \textit{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 \textit{Dp(1;f)LJ9} imprint. Many mutations from the piRNA pathway enhanced silencing from both paternally and maternally inherited \textit{Dp(1;f)LJ9}. These results suggest different roles for the RNAi pathways in regulating the \textit{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 \textit{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 in 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 \textit{Igf2} and \textit{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 \textit{Igf2} and \textit{H19} imprinting. Within the \textit{H19} ICR there is a CCCTC domain that is only functional on the unmethylated maternal allele. When CTCF binds the maternally unmethylated \textit{H19} ICR, it acts as an insulator, blocking access of the \textit{Igf2} promoter to enhancers (Szabo et al. 2004). Paternal methylation of the \textit{H19} ICR inhibits CTCF binding, allowing enhancers access to the \textit{Igf2} promoter on the paternal chromosome (Bell and Felsenfeld 2000; Hark et al. 2000). Silencing of the \textit{Igf2} maternal allele is also facilitated by CTCF, which insulates maternal DMR1 and DMR2 from methylation when bound to the maternal \textit{H19} ICR (Lopes et al. 2003). A loss of CTCF function results in \textit{de novo} methylation of the maternal H19 ICR, which effectively erases imprinted expression of \textit{H19} and \textit{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 \textit{Igf2}. CTCF binding sites are conserved in humans, mice and, marsupials, which all have imprinted \textit{Igf2} and \textit{H19}, while they are lacking in monotremes that do not imprint \textit{Igf2} or \textit{H19} (Weidman et al. 2004). Furthermore, \textit{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 \textit{Igf2r}, but also to the silencing of the genes which are in the same region as \textit{Igf2r}, yet do not overlap the \textit{Air} transcript (Sleutels \textit{et al.} 2002).

Histone methylation patterns are critical components of the parent-specific expression of \textit{Igf2r} and \textit{Air} genes. In mice, the expressed maternal \textit{Igf2r} allele and paternal \textit{Air} allele are both marked by H3K4 di- and trimethylation marks, while the repressed paternal \textit{Igf2r} allele and maternal \textit{Air} allele are both marked by H3K9 trimethylation within the promoter region (Vu \textit{et al.} 2004). Indeed, histone methylation marks are more reflective of the imprinted state of \textit{Igf2r} than the presence of \textit{Air} transcripts or DNA methylation patterns. In the mouse brain, \textit{Igf2r} is biallelically expressed. This correlates with the presence of activating H3K4 methylation in both the paternal and maternal \textit{Igf2r} DMR1 promoter region, despite retention of paternal \textit{Air} transcription (Vu \textit{et al.} 2004). In humans, activating H3K4 methylation is present within both the maternal and paternal \textit{Igf2r} promoter regions (Figure 2.1D), yet is absent from the \textit{Air} promoter region, eliminating \textit{Air} expression while facilitating biallelic \textit{Igf2r} expression (Vu \textit{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 \textit{de novo} DNA methylation of DMRs (Ciccone \textit{et al.} 2009). H3K4 demethylation appeared critical for imprinted genes that undergo \textit{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.
A) The \textit{Igf2} and \textit{H19} genes are reciprocally imprinted, with \textit{H19} and \textit{Igf2} being expressed maternally and paternally, respectively. CTCF binds the maternal \textit{H19} ICR and acts as an insulator sequestering enhancers to initiate maternal \textit{H19} transcription while also protecting the \textit{H19} ICR from methylation. Methylation on the paternal \textit{H19} ICR prevents CTCF binding and silences paternal transcription. \textit{Igf2} is only expressed paternally as a lack of CTCF binding in the paternal \textit{H19} ICR allows enhancers to activate the \textit{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 \textit{Igf2} transcription and blocking maternal transcription.

B) CTCF mediate an intrachromosomal loop, which prevents DNA methylation of the \textit{H19} DMR and \textit{Igf2} DMRs, while facilitating \textit{H19} expression.

C) In mice, \textit{Igf2r} is maternally expressed while the overlapping \textit{Air} antisense transcript is paternally expressed. Histone H3K4 methylation in the maternal \textit{Igf2r} promoter (DRM1) initiates transcription, while DNA methylation and histone H3K9 methylation in the downstream \textit{Air} promoter region (DRM2) silences maternal \textit{Air} transcription. Activating H3K4 methylation at the paternal \textit{Air} promoter region initiates paternal transcription of the \textit{Air} transcript. The \textit{Air} transcript overlaps the \textit{Igf2r} promoter and contributes to the silencing of the paternal \textit{Igf2r} allele along with DNA methylation and histone H3K9 methylation.

D) In humans, \textit{Igf2r} is biallelically expressed. Activating H3K4 methylation is found in both the maternal and paternal promoter regions of \textit{Igf2r}. While maternal specific DNA methylation of DMR2 is maintained, there is no H3K4 methylation of paternal DRM2, preventing the transcription of the \textit{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.
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 (Grealy 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.

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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 \textit{Igf2} (paternally expressed) and \textit{Igf2r} (maternally expressed) are the primary example used to illustrate parental conflict, as \textit{Igf2} expression encourages fetal growth, while \textit{Igf2r} expression clears circulating \textit{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 to 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 \textit{Rasgrf}, a gene for
which imprinted expression has been attributed to the acquisition of repetitive elements (Pearsall et al. 1999).

The \textit{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 \textit{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 \textit{Peg10} in therian mammals has resulted in the imprinted expression of the \textit{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 \textit{Peg10} gene. This suggests that the presence of retrotransposon elements has implanted imprinted epigenetic marks to the \textit{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 \textit{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 \textit{D. melanogaster}. However, imprinted chromosomal regions identified in \textit{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

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^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*. 

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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 \textit{dCTCF} has a role in the regulation of genomic imprinting in \textit{Drosophila}. As is the case in mammalian imprinting, \textit{dCTCF} in \textit{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 \textit{CTCF}^{EY15833} allele (FBrf0132177) was produced by insertion of the \textit{P\{EPgy2\}} element into the +26 position relative to the transcription start site of the \textit{dCTCF} gene by the Berkeley \textit{Drosophila} Genome Project (BDGP) Gene Disruption Project (Bellen et al. 2004). Homozygous \textit{CTCF}^{EY15833} adults appear healthy and are reasonably fertile. \textit{CTCF}^{30}, created by a partial deletion of the \textit{P\{EPgy2\}} element, is homozygous lethal. \textit{CTCF}^{30} lacks the entire 5’ end of the \textit{P\{EPgy2\}} element but retains 4860 bp of the 3’ end. Flanking \textit{dCTCF} sequences and the \textit{quemao (qm)} gene remain intact in \textit{CTCF}^{30}. The reduced severity of \textit{CTCF}^{EY15833}, with an intact \textit{P\{EPgy2\}} element, suggests that a promoter in the 5’ end of \textit{P\{EPgy2\}} may partially rescue \textit{dCTCF} expression. To test this idea we measured the \textit{dCTCF} transcript levels by quantitative real time PCR (qRT PCR) of third instar larvae. Expression in homozygous \textit{CTCF}^{EY15833} larvae is 20% ± 4% of that in wild-type (\textit{y w}) controls. This is consistent with previous studies showing that \textit{CTCF}^{EY15833} homozygotes produces approximately 50% of wild-type \textit{dCTCF} protein levels (Mohan et al. 2007). Homozygous \textit{CTCF}^{30} larvae cannot be recovered in sufficient numbers for qRT PCR, but heterozygous \textit{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 \textit{dCTCF} alleles indicates that both \textit{CTCF}^{EY15833} and \textit{CTCF}^{30} alleles have reduced \textit{dCTCF} expression and that the 5’ end of \textit{P\{EPgy2\}} may drive sufficient expression to allow recovery of \textit{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\textsuperscript{MAT}*) generate full expression of the marker gene *garnet*, while paternal inheritance (*Dp(1;f)LJ9\textsuperscript{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 \( y^1z^a g^{53d}/Dp(1;f)LJ9; +/+ \) 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\textsuperscript{X}* alleles (X represents *CTCF\textsuperscript{30} or *CTCF\textsuperscript{EY15833}*) were crossed to females with the *Dp(1;f)LJ9* mini-X chromosome: \( y^1z^a g^{53d}/y; CTCF\textsuperscript{X}/TM3, Sb Ser \) males x X\( ^X/X\)/Dp(1;f)LJ9 females. This cross generated progeny with a mutant *dCTCF* allele and a maternally imprinted mini-X chromosome (\( y^1z^a g^{53d}/Dp(1;f)LJ9\textsuperscript{MAT}; CTCF\textsuperscript{XY1}+)\) 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\textsuperscript{MAT}*) with *CTCF\textsuperscript{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\textsuperscript{MAT} progeny coupled with *CTCF\textsuperscript{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 \( Dp(1;f)LJ9^{\text{MAT}} \) and wild-type for \( dCTCF \) (Figure 3.2A and B). However, when \( Dp(1;f)LJ9^{\text{MAT}} \) 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 \( Dp(1;f)LJ9^{\text{MAT}} \), but with the balancer chromosome, and so wild-type for \( dCTCF \) (described in the methods, genotype: \( y^{l}z^{a}g^{53d}/Dp(1;f)LJ9^{\text{MAT}}; \ TM3, \ Sb \ Ser/++ \)). 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 \( Dp(1;f)LJ9^{\text{PAT}} \) 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 (\( X^\wedge Y/Dp(1;f)LJ9 \) males crossed to \( y^{l}z^{a}g^{53d}/y^{l}z^{a}g^{53d}; \ TM3, \ Sb \ Ser/++ \) females) results in \( y^{l}z^{a}g^{53d}/Dp(1;f)LJ9^{\text{PAT}}, \ +/+ \) 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 (\( y^{l}z^{a}g^{53d}/y^{l}z^{a}g^{53d}; \ CTCF^{VY}; \ TM3, \ Sb \ Ser \) females to \( X^\wedge Y/Dp(1;f)LJ9 \) males) to generate progeny with either mutant \( CTCF^{Ey15833} \) or \( CTCF^{30} \) alleles and a paternally imprinted \( Dp(1;f)LJ9^{\text{PAT}} \) mini-X chromosome (\( y^{l}z^{a}g^{53d}/Dp(1;f)LJ9; \ CTCF^{VY}/\)\(+), 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 \( Dp(1;f)LJ9^{\text{PAT}} \) along with the balancer chromosome, are wild-type for \( dCTCF \) (described in the methods, genotype:
y¹z⁺g⁵d/Dp(1;f)LJ9; TM3, Sb Ser/+) is used to determine if there is a maternal effect from mothers mutant for CTCF<sup>X</sup>. No maternal effect was detected; progeny wild-type for dCTCF from mothers with either CTCF<sup>EY15833</sup> or CTCF<sup>30</sup> 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<sup>30</sup> allele. If dCTCF affects the establishment of the imprint, the imprint should be disrupted in the progeny of mutant CTCF<sup>30</sup> parents, but not wild-type (CTCF<sup>+</sup>) 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<sup>MAT</sup> progeny from mothers carrying CTCF<sup>30</sup> (Figure 3.4A; Mat–Est. CTCF<sup>30</sup>) and either of the external or internal controls wild-type for dCTCF (Figure 3.4A; Ex. Control and Mat–Est. CTCF<sup>+</sup>, respectively). This was reflected in the unchanged phenotype of progeny from mothers mutant or wild-type for dCTCF (Figure 3.4B; Mat–Est. CTCF<sup>30</sup> and Mat–Est. CTCF<sup>+</sup>, respectively). Likewise, mutant dCTCF did not affect the establishment of the paternal imprint. Dp(1;f)LJ9<sup>PAT</sup> progeny from fathers carrying Dp(1;f)LJ9 and CTCF<sup>30</sup> (Figure 3.4C; Pat–Est. CTCF<sup>30</sup>) had no significant change in garnet expression compared to either the external or internal controls (Figure 3.4C; Ex. Control and Pat–Est. CTCF<sup>+</sup>, respectively). Again, these Dp(1;f)LJ9<sup>PAT</sup> progeny also had no observable change in phenotype between fathers mutant or wild-type for dCTCF (Figure 3.4D; Pat–Est. CTCF<sup>30</sup> and Pat–Est. CTCF<sup>+</sup>, 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<sup>30</sup> 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* imprinted imprint, we tested the effect of *CTCF* on *In(1)w*⁴, 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*⁴ 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* allele decreased silencing of *white* in *In(1)w*⁴;*CTCF*/+ females, while having no significant affect on *white* expression levels in *In(1)w*⁴;*CTCF*/+ males, compared to sibling *In(1)w*⁴;Tb/+ controls (Figure 3.5A). Similarly, the 6-M193 strain responded to *CTCF* 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* (Figure 3.5C). These results demonstrate that *CTCF* 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* on *Dp(1;f)LJ9* 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^{l,a}g^{53d}/Dp(1;f)LJ9) displays full garnet expression with no variegation observed. Both dCTCF mutant alleles tested (y^{l,a}g^{53d}/Dp(1;f)LJ9; CTCFEY15833/+ and y^{l,a}g^{53d}/Dp(1;f)LJ9; CTCF30/+) 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 CTCFEY15833 or CTCF30 alleles.

B) Paternally transmitted Dp(1;f)LJ9 mini-X chromosome; the control (y^{l,a}g^{53d}/Dp(1;f)LJ9) exhibits variegated garnet gene expression, while the introduction of CTCFEY15833 and CTCF30 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\text{EY15833} n = 300, CTCF\text{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\text{EY15833} n = 132 (Kolmogorov-Smirnov test p<0.001) and CTCF\text{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\text{EY15833} n = 300, CTCF\text{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^l2,Da^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.
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 \( y^{d}z^{d}/y^{3d}/Dp(1;f)LJ9; +/- \), 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<sup>30</sup> On White Variegation In In(1)w<sup>md</sup> And The 4th Chromosome Variegating Strains 6-M193 And 39C-33

A) Pigment levels were measured for In(1)w<sup>md</sup>/w<sup>1118</sup>;CTCF<sup>30</sup>/+ and In(1)w<sup>md</sup>/Y;CTCF<sup>30</sup>/+ genotypes compared to the corresponding sibling In(1)w<sup>md</sup>;Tb/+ controls. In(1)w<sup>md</sup> heterozygous for CTCF<sup>30</sup> 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<sup>MAT</sup>) and paternally (6-M193<sup>PAT</sup>) inherited 4<sup>th</sup> chromosome variegator 6-M193. 6-M193 heterozygous for CTCF<sup>30</sup>, 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<sup>MAT</sup> male and female progeny; n = 10 (50 heads total) for 6-M193<sup>PAT</sup> 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).
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\textsuperscript{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)LJ9PAP 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)LJ9MAT in a manner similar to that of the paternal Dp(1;f)LJ9PAT. 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<sup>30</sup>* allele and the variegating 4<sup>th</sup> chromosome transgene strains. The *CTCF<sup>EY15833</sup>* allele was created by insertion of P{EPgy2} 27 bp downstream of the *dCTCF* transcription start site. The homozygous lethal *CTCF<sup>30</sup>* allele was generated by imprecise excision of *CTCF<sup>EY15833</sup>*. The *CTCF<sup>30</sup>* deletion was characterized by amplification across the break and sequencing of the PCR product. *CTCF<sup>30</sup>* is deleted for all 5′ transposon sequences but retains 4860 bp at the 3′ end. No genomic sequence was removed by the *CTCF<sup>30</sup>* deletion. The 4<sup>th</sup> 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 (4<sup>th</sup> 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* (4<sup>th</sup> 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<sup>30</sup>* and *CTCF<sup>EY15833</sup>* alleles were crossed into *y<sup>1</sup> z<sup>y</sup> g<sup>53d</sup>* background to yield stable stocks of *y<sup>1</sup> z<sup>y</sup> g<sup>53d</sup>/y<sup>1</sup> z<sup>y</sup> g<sup>53d</sup>, *CTCF<sup>x</sup>/TM3, Sb Ser* (where *CTCF<sup>x</sup>* is the *CTCF<sup>30</sup>* or *CTCF<sup>EY15833</sup>* 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^a g^53d /y^1z^a g^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^a g^53d /Dp(1;f)LJ9; CTCF^Y/+ 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^a g^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^a g^53d /y^1z^a g^53d; TM3, Sb Ser/+ females, with the reciprocal cross serving as the maternal control: y^1z^a g^53d /Dp(1;f)LJ9; +/+ and y^1z^a g^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^a g^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^a g^53d /Y males, and paternal establishment controls (Pat-Est. CTCF^+) were X^Y/Dp(1;f)LJ9; e/e males crossed to y^1z^a g^53d /y^1z^a g^53d females. External controls were also produced by crossing F1 generation X^X/Dp(1;f)LJ9; e/e females to y^1z^a g^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^md and two variegating 4th chromosome (6-M193 or 39C-33) strains. For the In(1)w^md crosses, In(1)w^md females were crossed to w^1118/Y; CTCF^30 males and the red pigment levels of the In(1)w^md/Y; CTCF^30/+ and In(1)w^md/w^1118; CTCF^30/+ progeny compared with that of their In(1)w^md; 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-; CTCF30 / TM6, Tb; +/+; +/+ males, and paternal inheritance used y/w-; +/+; +/+; var/var males crossed to w-/w-; CTCF30/+; +/+; +/+ females, where var represents the variegating 4th chromosome transgene. The resulting progeny were separated by sex (y/w-; CTCF30; +/+; var/var males and w-/w-; CTCF30/+; +/+; 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 spectrophotometric 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 (omnochrome) 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.

Spectrophotometric assay for quantifying the expression of the white transgene on the 4th chromosome variegating strains and In(1)wmd 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 spectrophotometric 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<sup><i>x</i></sup> 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<sup><i>x</i></sup> mutant allele (maternal and paternal effect test progeny).
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\textsuperscript{30} mutation (Mat Est. CTCF\textsuperscript{30}), and progeny that also have a maternally imprinted Dp(1;f)LJ9 chromosome but from mothers wild-type for CTCF (Mat Est. CTCF\textsuperscript{+}). External control crosses were produced by crossing F1 generation X\textsuperscript{c}X/Dp(1;f)LJ9; e/e females to \( y^1 z^a g^{53d}/Y \) 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 $X^\lambda Y/Dp(1;f)LJ9; e/e$ males to $y^1z^a g^{53d}/y^1z^a g^{53d}$ 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


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 \textit{MBD2/3} Methyl-DNA Binding Protein Disrupt Genomic Imprinting In \textit{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 Dmnt1 maintains parent-specific DNA methylation patterns (Hirasawa et al. 2008). If the parent-specific DNA methylation patterns are lost, Dmnt1 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, Dmnt2 is highly conserved and involved in both DNA and tRNA methylation (Goll et al. 2006; Schaefer and Lyko 2010). Both human and mouse Dmnt2 have only weak DNA methyltransferases activity (Liu et al. 2003; Hermann et al. 2004), and Dmnt2 knock-out mice are viable with only minor developmental defects (Hermann et al. 2003), suggesting a limited and possibly specialized role for Dmnt2 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). Dmnt2 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, Dmnt2 is actively involved in genome regulation, as Dmnt2 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^a,g^53d/y^a,g^53d females) results in progeny (y^a,g^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^a,g^53d/ y^a,g^53d males) results in progeny with the same genotype (y^a,g^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 ± 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* (*MBD1*) 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. *MBD1* heterozygous progeny with a paternally inherited mini-X chromosome (*Dp(1;f)LJ9\textsuperscript{PAT}; MBD1/+) had elevated pigment levels (61±3%), indicating a significant decrease in *garnet* silencing compared to the control (*Dp(1;f)LJ9\textsuperscript{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\textsuperscript{PAT}; +/+\) and *MBD1* (*Dp(1;f)LJ9\textsuperscript{PAT}; MBD1/+) genotypes in the eye variegation index assay (Supplemental Table 4.2). As was found for 5-azacytidine, the effect of the *MBD1* allele is also restricted to the paternally imprinted mini-X chromosome; no significant difference resulted from the presence of *MBD1* 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 *MBD1* on *garnet* expression from the maternal mini-X chromosome (*Dp(1;f)LJ9\textsuperscript{MAT}; MBD1/+, compared to the control, *Dp(1;f)LJ9\textsuperscript{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\text{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\text{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).
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 role 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* \(^I\) allele (\(P\{EPgy2\}MBD-like\textsuperscript{EY04582}\)) 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 \(X^Y\) chromosome for maternal transmission or an attached an \(X^X\) chromosome for paternal transmission. To visualize the imprinting of the *garnet* gene, *Dp(1;f)LJ9* mini-X chromosome bearing flies are crossed to \(y^Iz^a g^{53d}\) flies of the opposite sex: paternal cross (\(Dp(1;f)LJ9, y^Ig^+/X^Y\) males \(x\) \(y^Iz^a g^{53d}/y^Iz^a g^{53d}\) females), and the reciprocal maternal cross \((y^Iz^a g^{53d}/y^Iz^a g^{53d}\) males \(x\) *Dp(1;f)LJ9, y^Ig^+/X^X* females) (Lloyd et al. 1999a). Each cross produces progeny of the genotype \(y^Iz^a g^{53d}/Dp(1;f)LJ9\textsuperscript{PAT}; +/+\) or *Dp(1;f)LJ9\textsuperscript{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* \(^I\) was crossed into a *y^Iz^a g^{53d}* background and balanced over *TM3, Sb* to yield a stock of *y^Iz^a g^{53d}; MBD* \(^I\)/*TM3, Sb*. The effect of *MBD* \(^I\) on the paternal imprinting of *garnet* was determined by crossing *Dp(1;f)LJ9* males to *y^Iz^a g^{53d}/y^Iz^a g^{53d}; MBD* \(^I\)/*TM3, Sb* females. The reciprocal cross determined the effect of *MBD* \(^I\) on the maternal imprinting of *garnet*. *y^Iz^a g^{53d}/Dp(1;f)LJ9*; *MBD* \(^I\)/+ progeny were collected from both crosses to determine the effect of *MBD* \(^I\), while *y^Iz^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^{1}z^{a}g^{53d}; y^{1}z^{a}g^{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 ddH2O/ 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 \(\times y^{1}z^{a}g^{53d}\) females) or maternal (\(y^{1}z^{a}g^{53d}\) males \(\times Dp(1;f)LJ9\) 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 \(y^{1}z^{a}g^{53d}\) flies as described above.

4.5.4 Quantification Of Eye Pigment

Eye pigment, as a measure of \textit{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 \(\pm\) 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 \(^\circ\)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 \textit{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 KpnI or BamHI 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

We are grateful to Lori McEachern for discussion. We would like to thank the Bloomington Stock Centre for supplying mutant strains used in this study. The authors acknowledge the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Dalhousie Patrick Lett fund for support of this research.
4.6 Supplemental

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

<table>
<thead>
<tr>
<th>Paternal Dp(1:f)LJ9 mini-X Inheritance</th>
<th>Eye Variegation Index</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hours AEL</td>
<td>n 0 1 2 3</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>128 17 27 51 33</td>
<td>1.78 ± 0.09</td>
</tr>
<tr>
<td>0-1</td>
<td>112 6 18 29 59</td>
<td>2.26 ± 0.09*</td>
</tr>
<tr>
<td>3-4</td>
<td>112 6 17 32 57</td>
<td>2.25 ± 0.09*</td>
</tr>
<tr>
<td>7-8</td>
<td>112 6 14 24 68</td>
<td>2.38 ± 0.09*</td>
</tr>
<tr>
<td>10-11</td>
<td>96 9 15 40 32</td>
<td>1.99 ± 0.1</td>
</tr>
<tr>
<td>12-13</td>
<td>96 12 23 31 30</td>
<td>1.82 ± 0.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Maternal Dp(1:f)LJ9 mini-X Inheritance</th>
<th>Eye Variegation Index</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hours AEL</td>
<td>n 0 1 2 3</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>96 0 0 2 94</td>
<td>2.98 ± 0.02</td>
</tr>
<tr>
<td>0-1</td>
<td>112 0 0 3 109</td>
<td>2.97 ± 0.02</td>
</tr>
<tr>
<td>3-4</td>
<td>96 0 0 0 96</td>
<td>3.00 ± 0.00</td>
</tr>
<tr>
<td>7-8</td>
<td>96 0 0 0 96</td>
<td>3.00 ± 0.00</td>
</tr>
<tr>
<td>10-11</td>
<td>96 0 0 0 96</td>
<td>3.00 ± 0.00</td>
</tr>
<tr>
<td>12-13</td>
<td>96 0 0 0 96</td>
<td>3.00 ± 0.00</td>
</tr>
</tbody>
</table>

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 MBD1 On Dp(1;f)LJ9 garnet Expression

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dp(1;f)LJ9\textsuperscript{PAT}, +/+</td>
<td>200</td>
<td>17</td>
<td>31</td>
<td>101</td>
<td>51</td>
<td>1.93 ± 0.06</td>
</tr>
<tr>
<td>Dp(1;f)LJ9\textsuperscript{PAT}, MBD\textsuperscript{1/+}</td>
<td>200</td>
<td>5</td>
<td>10</td>
<td>84</td>
<td>101</td>
<td>2.41 ± 0.05*</td>
</tr>
<tr>
<td>Dp(1;f)LJ9\textsuperscript{MAT}, +/+</td>
<td>200</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>200</td>
<td>3.00 ± 0.00</td>
</tr>
<tr>
<td>Dp(1;f)LJ9\textsuperscript{MAT}, MBD\textsuperscript{1/+}</td>
<td>200</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>200</td>
<td>3.00 ± 0.00</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Parental Treatment</th>
<th>n</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male Parent Control</td>
<td>200</td>
<td>26</td>
<td>34</td>
<td>72</td>
<td>68</td>
<td>1.91 ± 0.07</td>
</tr>
<tr>
<td>Male Parent 5-azaC</td>
<td>200</td>
<td>22</td>
<td>26</td>
<td>67</td>
<td>85</td>
<td>2.08 ± 0.07</td>
</tr>
<tr>
<td>Female Parent Control</td>
<td>200</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>200</td>
<td>3.00 ± 0.00</td>
</tr>
<tr>
<td>Female Parent 5-azaC</td>
<td>200</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>200</td>
<td>3.00 ± 0.00</td>
</tr>
</tbody>
</table>

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<sup>1</sup> Internal Controls**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Average ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pat Control</td>
<td>200</td>
<td>20</td>
<td>34</td>
<td>98</td>
<td>48</td>
<td>1.87 ± 0.06</td>
</tr>
<tr>
<td>Pat MBD Internal</td>
<td>200</td>
<td>14</td>
<td>27</td>
<td>110</td>
<td>49</td>
<td>1.97 ± 0.06</td>
</tr>
<tr>
<td>Mat Control</td>
<td>200</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>200</td>
<td>3.00 ± 0.00</td>
</tr>
<tr>
<td>Mat MBD Internal</td>
<td>200</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>200</td>
<td>3.00 ± 0.00</td>
</tr>
</tbody>
</table>

Eye variegation index of MBD<sup>1</sup> 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<sup>1</sup> 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.
Internal control progeny were compared to progeny of the same genotype ($y^1 z^2 a^g 53d^{(1;)} Dp(1;f)LJ9; TM3, Sb/+)$ from a control cross using unrelated parents wild-type for $MBD^I$, 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 $MBD^I$ 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 $MBD^I$ (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, $MBD^I$ 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 $MBD^I$ (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 4.8 - $MBD^I$ Internal Control Eye Pigment Levels.**
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\(^1\)zug53d/Dp(1;f)LJ9; MBD\(^1\)/+ progeny, and sibling y\(^1\)zug53d/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 Dm0 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 Dm0 (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^no4 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 DNA methylation, in lamin mutants.
Table 4.5 - Eye Variegation Index For The Effects Of Lamin Mutants On
Dp(1;f)LJ9 garnet Expression

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Average</th>
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<tbody>
<tr>
<td><strong>Paternal Dp(1;f)LJ9 mini-X Inheritance</strong></td>
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<tr>
<td>Dp(1;f)LJ9&lt;sup&gt;PAT&lt;/sup&gt;; +/+</td>
<td>300</td>
<td>17</td>
<td>57</td>
<td>138</td>
<td>88</td>
<td>1.99 ± 0.07</td>
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<tr>
<td>Dp(1;f)LJ9&lt;sup&gt;PAT&lt;/sup&gt;; lam&lt;sup&gt;04634&lt;/sup&gt;/+</td>
<td>300</td>
<td>10</td>
<td>24</td>
<td>97</td>
<td>169</td>
<td>2.42 ± 0.05*</td>
</tr>
<tr>
<td>Dp(1;f)LJ9&lt;sup&gt;PAT&lt;/sup&gt;; lam&lt;sup&gt;sz18&lt;/sup&gt;/+ (Mat +/+)</td>
<td>300</td>
<td>13</td>
<td>24</td>
<td>89</td>
<td>164</td>
<td>2.35 ± 0.05*</td>
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<tr>
<td>Dp(1;f)LJ9&lt;sup&gt;PAT&lt;/sup&gt;; CyO/+ (Mat lam&lt;sup&gt;04634&lt;/sup&gt;)</td>
<td>300</td>
<td>22</td>
<td>55</td>
<td>132</td>
<td>91</td>
<td>1.97 ± 0.05</td>
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<tr>
<td>Dp(1;f)LJ9&lt;sup&gt;PAT&lt;/sup&gt;; CyO/+ (Mat lam&lt;sup&gt;sz18&lt;/sup&gt;)</td>
<td>300</td>
<td>17</td>
<td>19</td>
<td>114</td>
<td>150</td>
<td>2.32 ± 0.05*</td>
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<td><strong>Maternal Dp(1;f)LJ9 mini-X Inheritance</strong></td>
<td></td>
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<tr>
<td>Dp(1;f)LJ9&lt;sup&gt;MAT&lt;/sup&gt;; +/+</td>
<td>300</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>300</td>
<td>3.00 ± 0.0</td>
</tr>
<tr>
<td>Dp(1;f)LJ9&lt;sup&gt;MAT&lt;/sup&gt;; lam&lt;sup&gt;04634&lt;/sup&gt;/+</td>
<td>300</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>300</td>
<td>3.00 ± 0.0</td>
</tr>
<tr>
<td>Dp(1;f)LJ9&lt;sup&gt;MAT&lt;/sup&gt;; lam&lt;sup&gt;sz18&lt;/sup&gt;/+ (Pat +/+)</td>
<td>300</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>300</td>
<td>3.00 ± 0.0</td>
</tr>
<tr>
<td>Dp(1;f)LJ9&lt;sup&gt;MAT&lt;/sup&gt;; CyO/+ (Pat lam&lt;sup&gt;04634&lt;/sup&gt;)</td>
<td>300</td>
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<td>300</td>
<td>3.00 ± 0.0</td>
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<tr>
<td>Dp(1;f)LJ9&lt;sup&gt;MAT&lt;/sup&gt;; CyO/+ (Pat lam&lt;sup&gt;sz18&lt;/sup&gt;)</td>
<td>300</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>300</td>
<td>3.00 ± 0.0</td>
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</tbody>
</table>

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\textsuperscript{PAT}), both lam alleles significantly relaxed garnet silencing *** (p < 0.001) compared to the control (Dp(1;f)LJ9\textsuperscript{PAT}; +/+). Internal control progeny are all the same genotype (y\textsuperscript{1}zag\textsuperscript{53d}/Dp(1;f)LJ9\textsuperscript{PAT}; CyO/+), but differ in the paternal 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\textsuperscript{PAT}; CyO/+).
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 ($y^5z^{a53}/Dp(1;f)LJ9^{MAT}; CyO/+$), 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


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
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)wmd* 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, *His2Av810* (van Daal and Elgin 1992), suppressed variegation of *In(1)wmd*, 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\textsuperscript{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\textsuperscript{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\textsuperscript{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-XPAT, while having no affect on the stable garnet expression from mini-XMAT, consistent with their established role in suppressing PEV. We also found that the His2AvR10 allele acts as a strong, dominant, enhancer of variegation from mini-XPAT and induces variegated silencing from mini-XMAT, demonstrating that H2Av is a critical component in regulating expression from the imprinted domain. However, disrupting acetylation of H2Av affects only mini-XPAT, 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)sc29 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-XMAT) and paternal (mini-XPAT) 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 y¹z⁰g³d hemizygotes or homozygotes, respectively, and Dp(1;f)LJ9/ y¹z⁰g³d 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 \(y^1z^ag^{53d}; CyO/\) or \(y^1z^ag^{53d}; TM3, Sb/+\) flies, carrying the relevant balancer for the modifier being tested (CyO or TM3, Sb). All mutant alleles being tested were crossed into a \(y^1z^ag^{53d}\) 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. Experimental crosses mirrored the control crosses, with the addition of mutant alleles. Two male progeny genotypes were collected from the experimental crosses; \(y^1z^ag^{53d}/Dp(1;f)LJ9; allele/+\) and the internal control \(y^1z^ag^{53d}/Dp(1;f)LJ9; CyO/+\) 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

\textit{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^1z^2a^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<sup>810</sup> null mutation for its effects on paternally-inherited (mini-X<sup>PAT</sup>) and maternally-inherited (mini-X<sup>MAT</sup>) garnet expression. Inheritance of mini-X<sup>PAT</sup> along with His2Av<sup>810</sup> 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<sup>MAT</sup> along with His2Av<sup>810</sup> 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<sup>810</sup> 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, <i>dom</i><sup>3</sup> and <i>dom</i><sup>9</sup>. Both <i>dom</i> alleles significantly enhanced garnet variegation for mini-X<sup>PAT</sup>, reducing pigment to 19±5% and 29±3% for <i>dom</i><sup>3</sup> and <i>dom</i><sup>9</sup> respectively (Table 5.1 and Figure 5.2A). However, unlike the variegation-inducing effect of His2Av<sup>810</sup> on mini-X<sup>PAT</sup> garnet expression, neither <i>dom</i><sup>3</sup> nor <i>dom</i><sup>9</sup> had a significant affect on mini-X<sup>MAT</sup> 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 <i>Drosophila</i> 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\textsuperscript{KG00766} and dRsf-1\textsuperscript{KG02636} alleles had no affect on mini-X\textsuperscript{PAT} garnet expression, resulting in 34±8% and 40±5% pigment levels, respectively (Table 5.1 and Figure 5.2A). Similarly, mini-X\textsuperscript{MAT} flies heterozygous for dRsf-1\textsuperscript{KG00766} and dRsf-1\textsuperscript{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 His4\textsuperscript{r} Relax garnet Silencing From Mini-X\textsuperscript{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\textsuperscript{KG00275} did not have any significant effect on either mini-X\textsuperscript{PAT} or mini-X\textsuperscript{MAT} garnet expression (Table 5.1 and Figure 5.3A, Figure 5.3B). However, we found that the H3 allele His3\textsuperscript{KG00688} and the H4 allele His4\textsuperscript{Scim} both significantly relaxed silencing of garnet from mini-X\textsuperscript{PAT}, increasing pigment levels to 45±6% and 61±5%, respectively (Table 5.1 and Figure 5.3A). Neither His3\textsuperscript{KG00688} nor His4\textsuperscript{Scim} had a significant affect on mini-X\textsuperscript{MAT} garnet expression (Table 5.1 and Figure 5.3B).

The histone replacement gene His4\textsuperscript{r} 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 His4\textsuperscript{r}\textsuperscript{EY06726} allele also caused a significant reduction in the level of variegated garnet silencing from mini-X\textsuperscript{PAT}, increasing pigment levels to 54±5% (Table 5.1 and Figure 5.3A), but did not have any significant affect on mini-X\textsuperscript{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 His3.3\textsuperscript{A KG02455} allele had no significant affect on garnet expression from either mini-X\textsuperscript{PAT} or mini-X\textsuperscript{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^{md} 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<sup>3</sup> 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<sup>3</sup> allele also acts as a dominant suppressor of mini-X<sup>PAT</sup> 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<sup>3</sup> allele had the opposite effect on mini-X<sup>MAT</sup>, 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.

<table>
<thead>
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<th>Allele</th>
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<th>1</th>
<th>2</th>
<th>3</th>
<th>Score</th>
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<td>138</td>
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<td>1.99 ± 0.07</td>
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<tr>
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<tr>
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<td>79</td>
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<td>1.18 ± 0.06***</td>
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<td>127</td>
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</tr>
<tr>
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<td>74</td>
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<td>1.10 ± 0.06***</td>
</tr>
<tr>
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<td>Control</td>
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<td>3.00 ± 0.00</td>
</tr>
<tr>
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<td>3.00 ± 0.00</td>
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<tr>
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<tr>
<td>His4\textsuperscript{FY06726} /+</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>300</td>
<td>3.00 ± 0.00</td>
</tr>
<tr>
<td>chmBG02254 /+</td>
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<td>dom\textsuperscript{3} /+</td>
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<td>0</td>
<td>0</td>
<td>300</td>
<td>3.00 ± 0.00</td>
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</table>

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\textsuperscript{810} And H2Av Modifiers On Mini-X\textsuperscript{PAT} And Mini-X\textsuperscript{MAT} garnet Expression.

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

B) His2Av\textsuperscript{810} induces variegated silencing of garnet from mini-X\textsuperscript{MAT} whereas no significant effect on mini-X\textsuperscript{MAT} garnet expression was observed the dom\textsuperscript{3}, dom\textsuperscript{9}, dRsf-1\textsuperscript{KG00766} and dRsf-1\textsuperscript{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-XP\textsuperscript{AT} And Mini-X\textsuperscript{MAT} garnet Expression.

A) Dominant enhancement of mini-XP\textsuperscript{AT} 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\textsuperscript{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\textsuperscript{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 garnet from mini-X\textsuperscript{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\textsuperscript{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\textsuperscript{PAT} while having no affect on the stable expression of garnet from mini-X\textsuperscript{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\textsuperscript{KG00275}. The His3\textsuperscript{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\textsuperscript{Scim}
and His4r produced a pronounced relaxation mini-X\textsuperscript{PAT} garnet variegated silencing. The histone H4 allele His4\textsuperscript{Scim} mutates a histone gene at the edge of the cluster that has been speculated to be a differentially expressed form of H4 (Dobie \textit{et al.} 2001). Differential expression of His4\textsuperscript{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 \textit{et al.} 1996), suggesting it might be actively recruited to distinguish the mini-X\textsuperscript{PAT} imprinted domain from mini-X\textsuperscript{MAT}.

\subsection*{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 \textit{et al.} 2004). A notable exception is H4K12 acetylation. While not ubiquitously required for heterochromatin formation (Ciurciu \textit{et al.} 2008), H4K12 acetylation is associated with repressive pericentric heterochromatic regions (Turner \textit{et al.} 1992). Treatment of \textit{Drosophila} embryos with the histone deacetylase inhibitor sodium butyrate typically relaxes PEV, indicating a hypoacetylated histone state is generally associated with heterochromatic silencing (Mottus \textit{et al.} 1980; Reuter \textit{et al.} 1982). However, treatment of \textit{Dp(1;f)LJ9} flies with sodium butyrate enhanced variegated silencing in mini-X\textsuperscript{PAT} flies and induced weak variegated silencing in mini-X\textsuperscript{MAT} flies (Lloyd \textit{et al.} 1999a). Here we tested the histone deacetylase mutant allele Rpd3\textsuperscript{04556} for its effect on mini-X expression and found a similar enhancement of garnet variegation from mini-X\textsuperscript{PAT}, and induction of weak variegated silencing of garnet from mini-X\textsuperscript{MAT}. Together, these results may indicate that the \textit{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\textsuperscript{04556} allele has previously been shown not to affect PEV unless coupled with other HDAC mutants (Chen \textit{et al.} 1999; Mottus \textit{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\textsuperscript{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\textsuperscript{PAT}. The opposite effects of Rpd3 and chm mutant alleles, coupled with the strong suppression of mini-X\textsuperscript{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\textsuperscript{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 2\textsuperscript{nd} chromosome had enhanced PEV from His2Av\textsuperscript{810},

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while insertions into the 4th chromosome either had suppressed variegation or no response to \textit{His2Av}^{810} depending on the site of insertion (Haynes \textit{et al.} 2007). Our results demonstrate that H2Av is also a critical component of an imprinted domain in \textit{Drosophila}. We found that \textit{His2Av}^{810} acts as a dominant enhancer of silencing on mini-X^{\text{PAT}}, dramatically reducing expression of the marker gene \textit{garnet}. \textit{His2Av}^{810} also disrupts the mini-X^{\text{MAT}} boundary, allowing the encroachment of heterochromatin and the initiation of \textit{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 \textit{H2Av} as a Polycomb group gene involved in maintaining homeotic gene silencing (Swaminathan \textit{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 \textit{et al.} 2005). This suggests a differential role for H2Av in the silencing of euchromatic genes and heterochromatic regions (Swaminathan \textit{et al.} 2005). Previously, mutations in Polycomb group genes were shown not to affect PEV from mini-X^{\text{PAT}} or disrupt the mini-X^{\text{MAT}} boundary (Joanis and Lloyd 2002), supporting evidence that constitutive heterochromatin and Polycomb-dependent chromatin are distinct (de Wit \textit{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 \textit{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 \textit{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 dom³ and dom⁹ alleles enhanced garnet variegation from mini-XPAT to levels similar to those observed from the His2Av⁸₁₀ allele. However, unlike the response to the His2Av⁸₁₀ allele, mini-XMAT 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-XPAT 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-XPAT. None of the mutant alleles we tested produced a maternal-effect on mini-XPAT 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$^{\text{PAT}}$ or mini-X$^{\text{MAT}}$ imprinted domain. This result might be attributed to the available alleles; the dRsf-1$^{KG02636}$ allele still produces a transcript and is likely a weak allele (Hanai et al. 2008), while dRsf-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$^{\text{PAT}}$ 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-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-1$^3$ allele is among the strongest characterized suppressors of variegation (Ebert et al. 2004). Consistent with this, we found the JIL-1$^3$ allele dramatically suppressed mini-X$^{\text{PAT}}$ garnet variegation to the degree that the majority of eyes appeared wild-type. This nearly complete elimination of the mini-X$^{\text{PAT}}$ imprint by the JIL-1$^3$ allele resembles the similar elimination of mini-X$^{\text{PAT}}$ 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-XPAT, inheritance of mini-XMAT along with the \textit{JIL-1}\textsuperscript{3} allele resulted in modest silencing observed in the form of variegated \textit{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-XPAT and mini-XMAT imprinted domain. This antipodal effect might be explained by the fact that the \textit{JIL-1}\textsuperscript{3} allele is functional but mislocalized; if JIL-1 is directly recruited to the mini-XMAT 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 \textit{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-XMAT and mini-XPAT 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 \textit{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 \textit{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 \textit{garnet} from the imprinted domain was disrupted by mutant alleles of both histones H3 and H4. The particular sensitivity of mini-XPAT silencing to \textit{His4}\textsuperscript{Scim} and \textit{His4r} is significant, as both alleles are thought to be alternate forms of histone H4 (Akhmanova \textit{et al.} 1996; Dobie \textit{et al.} 2001). Loss of mini-XPAT \textit{garnet} silencing from a mutant \textit{chameau} allele supports the requirement for proper H4 regulation to maintain
paternal-specific expression. Replacement of histone H4 on mini-X$^{\text{PAT}}$ 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$^{\text{PAT}}$, yet is uninvolved with H2Av from mini-X$^{\text{MAT}}$.

The stable chromatin boundary maintained between the mini-X$^{\text{MAT}}$ 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$^{\text{MAT}}$. Variegated silencing of the marker gene on mini-X$^{\text{MAT}}$ can be induced, indicating that the boundary can be disrupted; however, the levels of variegation that result are low in comparison to mini-X$^{\text{PAT}}$, 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.

5.6 Acknowledgements

We would like to thank the Bloomington Stock Centre for supplying mutant strains. This research is supported by the Natural Sciences and Engineering Research Council of Canada (NSERC), the Nova Scotia Health Research Foundation (NSHRF) and the Dalhousie University Lett Fund.
### 5.7 Supplemental

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

<table>
<thead>
<tr>
<th>Maternally present allele // balancer</th>
<th>Eye Variegation Index</th>
<th>Pigment Assay</th>
<th>% wild-type</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td><strong>0</strong></td>
<td><strong>1</strong></td>
<td><strong>2</strong></td>
</tr>
<tr>
<td>No modifier // CyO</td>
<td>300</td>
<td>22</td>
<td>55</td>
</tr>
<tr>
<td>His2A_{KG00275} // CyO</td>
<td>280</td>
<td>28</td>
<td>43</td>
</tr>
<tr>
<td>His3_{KG00688} // CyO</td>
<td>300</td>
<td>34</td>
<td>47</td>
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<tr>
<td>His3.3A_{KG02455} // CyO</td>
<td>300</td>
<td>27</td>
<td>26</td>
</tr>
<tr>
<td>His4_{Scim} // CyO</td>
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<td>19</td>
<td>37</td>
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<tr>
<td>chm^{BG02254 PAT} // CyO</td>
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<td>61</td>
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<tr>
<td>dom^{3} // CyO</td>
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<td>63</td>
</tr>
<tr>
<td>dom^{9} // CyO</td>
<td>280</td>
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<td>46</td>
</tr>
<tr>
<td>dRsf-1_{KG00766} // CyO</td>
<td>300</td>
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<td>59</td>
</tr>
<tr>
<td>dRsf-1_{KG02636} // CyO</td>
<td>300</td>
<td>30</td>
<td>64</td>
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</table>

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$^{\text{PAT}}$; 3$^{\text{rd}}$ Chromosome Mutations Balanced Over $\text{TM3, Sb}$

<table>
<thead>
<tr>
<th>Maternally present allele // balancer</th>
<th>Eye Variegation Index</th>
<th>Pigment Assay</th>
<th>% wild-type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>No modifier // TM3, Sb</td>
<td>300</td>
<td>34</td>
<td>54</td>
</tr>
<tr>
<td>His2Av$^{810}$ // TM3, Sb</td>
<td>300</td>
<td>29</td>
<td>64</td>
</tr>
<tr>
<td>His4r$^{EY06726}$ // TM3, Sb</td>
<td>200</td>
<td>15</td>
<td>34</td>
</tr>
<tr>
<td>JIL-1$^{J}$ // TM3, Sb</td>
<td>250</td>
<td>21</td>
<td>55</td>
</tr>
<tr>
<td>Rpd3$^{34556}$ // TM3, Sb</td>
<td>300</td>
<td>27</td>
<td>47</td>
</tr>
</tbody>
</table>

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 $\pm$ Standard Error of the Mean. Pigment assay values are presented as a mean percentage of wild-type levels $\pm$ standard deviation; the $n$ value represents the number of independent measurements. Males carrying the $\text{TM3, Sb}$ 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 $\text{Dp(1;f)LJ9;}$ $\text{TM3, Sb}$ balanced internal controls and the ‘no modifier’ $\text{Dp(1;f)LJ9;}$ $\text{TM3, Sb}$ 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\textsuperscript{MAT}; 2\textsuperscript{nd} Chromosome Mutations Balanced Over CyO**

<table>
<thead>
<tr>
<th>Paternally present allele // balancer</th>
<th>Eye Variegation Index</th>
<th>Pigment Assay</th>
<th>% wild-type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>No modifier // CyO</td>
<td>300</td>
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<td>0</td>
</tr>
<tr>
<td>His2\textsuperscript{AKG00275} // CyO</td>
<td>300</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>His3\textsuperscript{AKG00688} // CyO</td>
<td>300</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>His3.3A\textsuperscript{AKG02455} // CyO</td>
<td>300</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>His4\textsuperscript{Scm} // CyO</td>
<td>300</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>chm\textsuperscript{BG02254} // CyO</td>
<td>300</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>dom\textsuperscript{3} // CyO</td>
<td>300</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>dom\textsuperscript{9} // CyO</td>
<td>300</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>dRsf-1\textsuperscript{KG00766} // CyO</td>
<td>300</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>dRsf-1\textsuperscript{KG02636} // CyO</td>
<td>150</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

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; 3\textsuperscript{rd} Chromosome Mutations Balanced Over TM3, Sb

<table>
<thead>
<tr>
<th>Paternally present allele // balancer</th>
<th>Eye Variegation Index</th>
<th>Pigment Assay</th>
<th>% wild-type</th>
</tr>
</thead>
<tbody>
<tr>
<td>No modifier // TM3, Sb</td>
<td>n=300</td>
<td>0 0 0 0 300</td>
<td>300±0.0 19</td>
</tr>
<tr>
<td>His2Av\textsuperscript{810} // TM3, Sb</td>
<td>300</td>
<td>0 0 0 0 300</td>
<td>300±0.0 20</td>
</tr>
<tr>
<td>His4r\textsuperscript{EY06726} // TM3, Sb</td>
<td>300</td>
<td>0 0 0 0 300</td>
<td>300±0.0 20</td>
</tr>
<tr>
<td>JIL-1\textsuperscript{3} // TM3, Sb</td>
<td>300</td>
<td>0 0 0 0 300</td>
<td>300±0.0 20</td>
</tr>
<tr>
<td>Rpd3\textsuperscript{304556} // TM3, Sb</td>
<td>300</td>
<td>0 0 0 0 300</td>
<td>300±0.0 20</td>
</tr>
</tbody>
</table>

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 \textit{Dp(1;f)LJ9; TM3, Sb} balanced internal controls and the ‘no modifier’ \textit{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


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


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 Der2 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 then 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*<sup>PAT</sup>

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<sup>L811 fsx</sup>* loss-of-function allele (Lee *et al.* 2004) caused a strong relaxation of *garnet* silencing from *Dp(1;f)LJ9*<sup>PAT</sup> 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<sup>1</sup>* and *r2d2<sup>S165 fsx</sup>* are null mutations (Marques *et al.* 2010) and *r2d2<sup>EY00028</sup>* is an uncharacterized P-element mutation. Each of the *r2d2* alleles relaxed silencing of *garnet* from *Dp(1;f)LJ9*<sup>PAT</sup>, increasing pigmentation to levels similar to those resulting from the *dcr2<sup>L811 fsx</sup>* allele (Figure 6.3A). Both the *ago2<sup>EY04479</sup>* and *ago2<sup>dop1</sup>* 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*<sup>PAT</sup> (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*<sup>PAT</sup> 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<sup>EY01325</sup>* had no significant affect on *garnet* expression *Dp(1;f)LJ9*<sup>PAT</sup> (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<sup>EY01325</sup>* , the hypomorphic *rm62<sup>01086</sup>* 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 hypomorph $loqs^{f00791}$ allele (Fürstemann 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 \textit{Dp(1;f)LJ9} imprint is limited to the paternally inherited \textit{Dp(1;f)LJ9}. Mutations in either siRNA or miRNA pathway members had no affect on \textit{garnet} expression in maternally inherited \textit{Dp(1;f)LJ9}\textsuperscript{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 \textit{Dp(1;f)LJ9}\textsuperscript{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 \textit{et al.} 2008; Obbard and Finnegan 2008). Mutations in \textit{ago2}, \textit{dcr2}, and \textit{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 \textit{et al.} 2009; Peng and Karpen 2009). Additionally, paternal-specific silencing from the \textit{Dp(1;f)LJ9} imprinted region is dependent upon histone H3K9 methylation and heterochromatin formation (Joanis and Lloyd 2002).

The finding that \textit{Ago2}, \textit{Dcr2}, \textit{Loqs}, and \textit{R2D2} are all required to maintain paternal-specific \textit{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 \textit{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 \textit{garnet} transcripts. Probes were produced to detect a 3’ coding region of the \textit{garnet} gene and a 5’ untranscribed region of the \textit{garnet} promoter. These probes serve the dual function of determining if antisense transcription extends over the entire length of the \textit{garnet} gene and is acting as a control for the sense detection of \textit{garnet} transcripts. \textit{In situ} hybridizations with the riboprobes were performed on \textit{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\(^{\text{PAT}}\); dcr\(^{L811\text{fxs}}\) 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 dcr\(^{L811\text{fxs}}\) 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\(^{\text{PAT}}\) and Dp(1;f)LJ9\(^{\text{MAT}}\) 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\(^{\text{PAT}}\)And Dp(1;f)LJ9\(^{\text{MAT}}\)

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\(^{OC42}\) alleles are strong hypomorph mutations (Wilson et al. 1996; Harris and Macdonald 2001) that enhanced garnet silencing in Dp(1;f)LJ9\(^{\text{PAT}}\) flies, while the weaker aub\(^{KGO5389}\) 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 $aub^{OC42}$ 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 \(Dp(1;f)LJ9\text{MAT}\) flies, the \(armi^{72.1}\) allele also enhanced \textit{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 \(Dp(1;f)LJ9\text{PAT}\), the \(armi^{72.1}\) allele showed the strongest enhancement of \textit{garnet} silencing in \(Dp(1;f)LJ9\text{MAT}\) 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 \textit{et al.} 2007) and, \textit{piwi} mutations result in an increase in transposon transcript abundance (Kalmykova \textit{et al.} 2005; Vagin \textit{et al.} 2006). I tested the \(piwi^{3}\) allele, which is the product of a single P-element insertion (Cox \textit{et al.} 1998), for its effect on \(Dp(1;f)LJ9\) \textit{garnet} expression. Unlike the enhanced of silencing from both \(Dp(1;f)LJ9\text{PAT}\) and \(Dp(1;f)LJ9\text{MAT}\) induced by \textit{aub} mutations, the \(piwi^{3}\) allele relaxed \textit{garnet} silencing in \(Dp(1;f)LJ9\text{PAT}\) flies (Table 6.3 and Figure 6.7A), while having no affect on \(Dp(1;f)LJ9\text{MAT}\) 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 \textit{et al.} 2007). Mutations in \textit{squ} and \textit{zuc} result in increased levels of transposon transcripts due to defects in the biogenesis of germline piRNAs (Pane \textit{et al.} 2007; Saito \textit{et al.} 2009). The \textit{zuc}^{B549} allele is a mis-sense hypomorphic mutation, and the \textit{squ}^{PP32} allele is a nonsense mutation with a stop codon at residue 111.
(Pane et al. 2007). Both \(\text{zuc}^{\text{RS}49}\) and \(\text{squ}^{\text{PP}32}\) alleles moderately enhance silencing of \text{garnet} in both \(\text{Dp}(1;f)\text{LJ9}^{\text{PAT}}\) and \(\text{Dp}(1;f)\text{LJ9}^{\text{MAT}}\) 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 \text{aub}, \text{armi}, \text{spn-E}, \text{squ}, and \text{zuc} to enhance the silencing of the \text{garnet} imprint reporter gene in both \(\text{Dp}(1;f)\text{LJ9}^{\text{PAT}}\) and \(\text{Dp}(1;f)\text{LJ9}^{\text{MAT}}\) flies suggests that the piRNA pathway is either directly, or indirectly, involved in regulating the \(\text{Dp}(1;f)\text{LJ9}\) 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 \(\text{Dp}(1;f)\text{LJ9}^{\text{PAT}}\) 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 \text{vas}^{-} mothers (Schüpbach and Wieschaus 1991). Mutations in \text{vasa} (\text{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 \text{Aub}, \text{Ago3}, and Piwi (Malone et al. 2009). I tested two hypomorphic \text{vas} alleles, \(\text{vas}^{l}\) and \(\text{vas}^{\text{RJ36}}\), for their effects on \(\text{Dp}(1;f)\text{LJ9}\) \text{garnet} expression. The \(\text{vas}^{l}\) allele strongly relaxed \text{garnet} silencing in \(\text{Dp}(1;f)\text{LJ9}^{\text{PAT}}\) flies, producing eyes with only few and small visible patches of variegated silencing (Table 6.4 and Figure 6.8A). Furthermore, the \(\text{vas}^{l}\) allele also displayed a maternal effect, relaxing \text{garnet} silencing in \text{vasa}^{+} progeny when mothers were \(\text{vas}^{l}+/\) (Table 6.7). The \(\text{vas}^{\text{RJ36}}\) allele caused a moderate relaxation of \text{garnet} silencing in \(\text{Dp}(1;f)\text{LJ9}^{\text{PAT}}\) flies (Table 6.4 and Figure 6.8A); however, no maternal effect was observed from mothers bearing the \(\text{vas}^{\text{RJ36}}\) allele (Table 6.7). Both of the tested \(\text{vas}^{l}\) and \(\text{vas}^{\text{RJ36}}\) alleles had no affect on \text{garnet} expression in \(\text{Dp}(1;f)\text{LJ9}^{\text{MAT}}\) flies (Table 6.4 and Figure 6.8B), additionally, no paternal effects were found for either \text{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 \textit{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 \textit{Dp(1;f)LJ9} imprint in the germline. The other tested RNAi mutations, \textit{piwi3}, \textit{ago1k08121}, \textit{ago1k00208}, and \textit{ago104845} 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 \textit{Dp(1;f)LJ9} imprint.

Aub is critical in maintaining female germline integrity and homozygous \textit{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 \textit{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 \textit{Dp(1;f)LJ9} imprinting center. Recently, the linker histone H1 has been determined to be a factor in the maternal establishment of the \textit{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 \textit{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 \textit{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 \textit{Dp(1;f)LJ9} imprinted domain appears contradictory. Recently, similar results were reported for siRNA and piRNA pathway mutants; mutant alleles of \textit{ago2},
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\textsuperscript{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\textsuperscript{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 heterochromatin 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\textsuperscript{PAT} and Dp(1;f)LJ9\textsuperscript{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 \textit{Dp(1;f)LJ9} 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 \textit{C. elegans} (Das \textit{et al.} 2008; Han \textit{et al.} 2009), but no such links between Piwi and siRNAs have been made definitively in \textit{Drosophila}. It is known that Piwi does not interact with Dcr2 in cytoplasm (Megosh \textit{et al.} 2006); however, Piwi function in the nucleus requires loading of target RNA guides from the cytoplasm (Saito \textit{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 \textit{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 \textit{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 \textit{et al.} 2007; Aravin and Hannon 2008; Ooi \textit{et al.} 2009). In Chapter 4, I proposed that DNA methylation demarcates and stabilizes the paternal \textit{Dp(1;f)LJ9} imprint, contributing to the formation of heterochromatin and garnet silencing in \textit{Dp(1;f)LJ9} flies. It remains to be determined if Piwi is involved in directing paternal-specific DNA methylation on the \textit{Dp(1;f)LJ9} mini-X chromosome.
Table 6.1 - The Effects Of siRNA Mutations On \textit{Dp(1;f)LJ9 garnet} Expression

<table>
<thead>
<tr>
<th></th>
<th>Eye Variegation Index</th>
<th>Summary Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Dp(1;f)LJ9}^{\text{PAT}}</td>
<td>n 0 1 2 3 Score</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>300 21 57 143 79 .93 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>ago2^{top1} /+</td>
<td>300 9 24 71 196 2.51 ± 0.04*</td>
<td>▲▲▲</td>
</tr>
<tr>
<td>ago2^{EY04479} /+</td>
<td>300 7 25 65 203 2.55 ± 0.04*</td>
<td>▲▲▲</td>
</tr>
<tr>
<td>dcr2^{L811 fsx} /+</td>
<td>200 7 21 56 116 2.41 ± 0.06*</td>
<td>▲▲▲</td>
</tr>
<tr>
<td>r2d2^{1} /+</td>
<td>300 17 36 104 143 2.24 ± 0.05*</td>
<td>▲▲▲</td>
</tr>
<tr>
<td>r2d2^{S165 fsx} /+</td>
<td>200 7 20 40 133 2.50 ± 0.06*</td>
<td>▲▲▲</td>
</tr>
<tr>
<td>r2d2^{EY00028} /+</td>
<td>300 4 15 51 130 2.54 ± 0.05*</td>
<td>▲▲▲</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Eye Variegation Index</th>
<th>Summary Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Dp(1;f)LJ9}^{\text{MAT}}</td>
<td>n 0 0 0 300 3.00 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>ago2^{top1} /+</td>
<td>300 0 0 0 300 3.00 ± 0.0</td>
<td>/</td>
</tr>
<tr>
<td>ago2^{EY04479} /+</td>
<td>300 0 0 0 300 3.00 ± 0.0</td>
<td>/</td>
</tr>
<tr>
<td>dcr2^{L811 fsx} /+</td>
<td>200 0 0 0 300 3.00 ± 0.0</td>
<td>/</td>
</tr>
<tr>
<td>r2d2^{1} /+</td>
<td>300 0 0 0 300 3.00 ± 0.0</td>
<td>/</td>
</tr>
<tr>
<td>r2d2^{S165 fsx} /+</td>
<td>200 0 0 0 300 3.00 ± 0.0</td>
<td>/</td>
</tr>
<tr>
<td>r2d2^{EY00028} /+</td>
<td>200 0 0 0 300 3.00 ± 0.0</td>
<td>/</td>
</tr>
</tbody>
</table>

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

<table>
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<tr>
<th></th>
<th>Eye Variegation Index</th>
<th>Summary Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>0</td>
</tr>
<tr>
<td><strong>Dp(1;f)LJ9</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PAT</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>300</td>
<td>21</td>
</tr>
<tr>
<td>ago1*08121 /+</td>
<td>300</td>
<td>8</td>
</tr>
<tr>
<td>ago1*04845 /+</td>
<td>300</td>
<td>2</td>
</tr>
<tr>
<td>ago1*00208 /+</td>
<td>300</td>
<td>6</td>
</tr>
<tr>
<td>loqs*60791 /+</td>
<td>300</td>
<td>4</td>
</tr>
<tr>
<td>pasha*EY01325/+</td>
<td>300</td>
<td>22</td>
</tr>
<tr>
<td>rm62*01086 /+</td>
<td>200</td>
<td>29</td>
</tr>
<tr>
<td><strong>MAT</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>300</td>
<td>0</td>
</tr>
<tr>
<td>ago1*08121 /+</td>
<td>300</td>
<td>0</td>
</tr>
<tr>
<td>ago1*04845 /+</td>
<td>300</td>
<td>0</td>
</tr>
<tr>
<td>ago1*00208 /+</td>
<td>300</td>
<td>0</td>
</tr>
<tr>
<td>loqs*60791 /+</td>
<td>300</td>
<td>0</td>
</tr>
<tr>
<td>pasha*EY01325/+</td>
<td>300</td>
<td>0</td>
</tr>
<tr>
<td>rm62*01086 /+</td>
<td>200</td>
<td>0</td>
</tr>
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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

<table>
<thead>
<tr>
<th></th>
<th>Eye Variegation Index</th>
<th>Summary Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dp(1;f)LJ9^PAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>300 21 57 143 79</td>
<td>1.93 ± 0.05</td>
</tr>
<tr>
<td>aub^HN /+</td>
<td>300 48 67 120 65</td>
<td>1.67 ± 0.06*</td>
</tr>
<tr>
<td>aub^QC42 /+</td>
<td>300 34 77 113 76</td>
<td>1.77 ± 0.06*</td>
</tr>
<tr>
<td>aub^KG05389 /+</td>
<td>200 22 43 79 56</td>
<td>1.85 ± 0.07</td>
</tr>
<tr>
<td>armi^72.1 /+</td>
<td>300 76 78 97 49</td>
<td>1.39 ± 0.06*</td>
</tr>
<tr>
<td>piwi^3 /+</td>
<td>300 7 18 87 188</td>
<td>2.52 ± 0.04*</td>
</tr>
<tr>
<td>spn-E^1 /+</td>
<td>300 83 76 88 53</td>
<td>1.37 ± 0.06*</td>
</tr>
<tr>
<td>spn-E^00786 /+</td>
<td>300 63 81 124 32</td>
<td>1.42 ± 0.04*</td>
</tr>
<tr>
<td>squ^PP32 /+</td>
<td>300 39 73 105 83</td>
<td>1.77 ± 0.06*</td>
</tr>
<tr>
<td>zuc^RS49 /+</td>
<td>300 43 70 108 79</td>
<td>1.74 ± 0.06*</td>
</tr>
<tr>
<td>Dp(1;f)LJ9^MAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>300 0 0 0 300</td>
<td>3.00 ± 0.00</td>
</tr>
<tr>
<td>aub^HN /+</td>
<td>300 0 0 7 293</td>
<td>2.98 ± 0.01</td>
</tr>
<tr>
<td>aub^QC42 /+</td>
<td>300 0 0 4 296</td>
<td>2.99 ± 0.01</td>
</tr>
<tr>
<td>aub^KG05389 /+</td>
<td>300 0 0 4 296</td>
<td>2.99 ± 0.01</td>
</tr>
<tr>
<td>armi^72.1 /+</td>
<td>300 0 5 9 286</td>
<td>2.93 ± 0.02</td>
</tr>
<tr>
<td>piwi^3 /+</td>
<td>300 0 0 0 300</td>
<td>3.00 ± 0.00</td>
</tr>
<tr>
<td>spn-E^1 /+</td>
<td>200 0 1 8 191</td>
<td>2.95 ± 0.01</td>
</tr>
<tr>
<td>spn-E^00786 /+</td>
<td>300 0 0 5 295</td>
<td>2.98 ± 0.01</td>
</tr>
<tr>
<td>squ^PP32 /+</td>
<td>300 0 0 2 298</td>
<td>2.99 ± 0.01</td>
</tr>
<tr>
<td>zuc^RS49 /+</td>
<td>300 0 0 4 296</td>
<td>2.99 ± 0.01</td>
</tr>
</tbody>
</table>

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 (/).

^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

<table>
<thead>
<tr>
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<th>Eye Variegation Index</th>
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<tbody>
<tr>
<td></td>
<td>n 0 1 2 3 Score</td>
<td></td>
</tr>
<tr>
<td><strong>Dp(1;f)LJ9</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Control              | 300 21 57 143 79 1.93±0.05 | ↓↓↓↓
| vasa¹/+              | 300 14 25 109 152 2.33±0.05* | ↓↓↓
| vasa²/RJ36/+         | 300 18 22 107 153 2.32±0.05* | ↓↓
| viga²/C274/+         | 300 17 51 112 120 2.12±0.05* | ↓
| viga²/EY07816/+      | 300 22 41 113 124 2.13±0.05* | ↓
| **Dp(1;f)LJ9**       |                       |                |
| Control              | 300 0 0 0 300 3.00±0.0 | /              |
| vasa¹/+              | 300 0 0 0 300 3.00±0.0 | /              |
| vasa²/RJ36/+         | 300 0 0 0 300 3.00±0.0 | /              |
| viga²/C274/+         | 200 0 0 0 200 3.00±0.0 | /              |
| viga²/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 (/).

⁠*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 \textit{Dp(1;f)LJ9} Imprint

<table>
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<th>Maternally present allele</th>
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<th>Summary Effect</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>\textit{n}</td>
<td>0 1 2 3  Score</td>
</tr>
<tr>
<td>Control</td>
<td>100 0 0 0 100 3.00 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>\textit{aub}^{HN} /+</td>
<td>100 0 3 21 76 2.73 ± 0.05$^*$</td>
<td></td>
</tr>
<tr>
<td>\textit{aub}^{HN} Control</td>
<td>100 0 0 0 100 3.00 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>\textit{ago}^{1K08121} /+</td>
<td>100 0 0 0 100 3.00 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>\textit{ago}^{1K08121} Control</td>
<td>100 0 0 0 100 3.00 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>\textit{ago}^{1K0845} /+</td>
<td>100 0 0 0 100 3.00 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>\textit{ago}^{1K0845} Control</td>
<td>100 0 0 0 100 3.00 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>\textit{ago}^{1K00208} /+</td>
<td>100 0 0 0 100 3.00 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>\textit{ago}^{1K00208} Control</td>
<td>100 0 0 0 100 3.00 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>\textit{piwi}^{3} /+</td>
<td>100 0 0 0 100 3.00 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>\textit{piwi}^{3} Control</td>
<td>100 0 0 0 100 3.00 ± 0.0</td>
<td></td>
</tr>
</tbody>
</table>

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 \textit{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 (\textit{↑↑↑}), moderate (\textit{↑↑}), or weak (\textit{↑}) increase in expression; a strong (\textit{↓↓↓}), moderate (\textit{↓↓}), or weak (\textit{↓}) increase in silencing; or no affect (/).
Table 6.6 - The Effects Of spn-D Mutations On Dp(1;f)LJ9 garnet Expression

<table>
<thead>
<tr>
<th></th>
<th>Eye Variegation Index</th>
<th>Summary Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n 0 1 2 3 Score</td>
<td></td>
</tr>
<tr>
<td><strong>Dp(1;f)LJ9</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>300 21 57 143 79 1.93 ± 0.05 /</td>
<td></td>
</tr>
<tr>
<td>spn-D(^2) /+</td>
<td>80 6 13 38 23 1.98 ± 0.1 /</td>
<td></td>
</tr>
<tr>
<td><strong>Dp(1;f)LJ9</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>300 0 0 0 300 3.00 ± 0.00 /</td>
<td></td>
</tr>
<tr>
<td>spn-D(^2) /+</td>
<td>200 0 0 0 200 3.00 ± 0.00 /</td>
<td></td>
</tr>
</tbody>
</table>

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 \(CyO\)

<table>
<thead>
<tr>
<th>Maternally present allele // balancer</th>
<th>Variegation Assay</th>
<th>Pigment Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Eye Variegation Index</td>
<td>% wild-type</td>
</tr>
<tr>
<td></td>
<td>(n)</td>
<td>0</td>
</tr>
<tr>
<td>No modifier // (CyO)</td>
<td>300</td>
<td>22</td>
</tr>
<tr>
<td>(ago1^{T08121}) // (CyO)</td>
<td>300</td>
<td>12</td>
</tr>
<tr>
<td>(ago1^{D4845}) // (CyO)</td>
<td>300</td>
<td>13</td>
</tr>
<tr>
<td>(ago1^{k00208}) // (CyO)</td>
<td>300</td>
<td>21</td>
</tr>
<tr>
<td>(aub^{HN}) // (CyO)</td>
<td>300</td>
<td>27</td>
</tr>
<tr>
<td>(aub^{QC42}) // (CyO)</td>
<td>300</td>
<td>25</td>
</tr>
<tr>
<td>(aub^{KG05389}) // (CyO)</td>
<td>300</td>
<td>13</td>
</tr>
<tr>
<td>(dcr^{L811fs}) // (CyO)</td>
<td>200</td>
<td>18</td>
</tr>
<tr>
<td>(loqs^{60791}) // (CyO)</td>
<td>300</td>
<td>35</td>
</tr>
<tr>
<td>(piwi^{3}) // (CyO)</td>
<td>300</td>
<td>20</td>
</tr>
<tr>
<td>(r2d2^{i}) // (CyO)</td>
<td>300</td>
<td>21</td>
</tr>
<tr>
<td>(r2d2^{S165fs}) // (CyO)</td>
<td>200</td>
<td>16</td>
</tr>
<tr>
<td>(r2d2^{Y00028}) // (CyO)</td>
<td>300</td>
<td>23</td>
</tr>
<tr>
<td>(squ^{PP32}) // (CyO)</td>
<td>300</td>
<td>26</td>
</tr>
<tr>
<td>(vasa^{1}) // (CyO)</td>
<td>300</td>
<td>16</td>
</tr>
<tr>
<td>(vasa^{RJ36}) // (CyO)</td>
<td>300</td>
<td>22</td>
</tr>
<tr>
<td>(vig^{C274}) // (CyO)</td>
<td>300</td>
<td>34</td>
</tr>
<tr>
<td>(vig^{EY07816}) // (CyO)</td>
<td>300</td>
<td>24</td>
</tr>
<tr>
<td>(zuc^{RS49}) // (CyO)</td>
<td>300</td>
<td>24</td>
</tr>
</tbody>
</table>

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 2\(^{nd}\) 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

<table>
<thead>
<tr>
<th>Maternally present allele // balancer</th>
<th>Variation Assay</th>
<th>Eye Variegation Index</th>
<th>Pigment Assay</th>
<th>% wild-type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$n$</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>No modifier // TM3, Sb</td>
<td>300</td>
<td>34</td>
<td>54</td>
<td>128</td>
</tr>
<tr>
<td>ago2$^{Dop1}$ // TM3, Sb</td>
<td>300</td>
<td>28</td>
<td>57</td>
<td>122</td>
</tr>
<tr>
<td>ago2$^{EYO4479}$ // TM3, Sb</td>
<td>300</td>
<td>26</td>
<td>67</td>
<td>103</td>
</tr>
<tr>
<td>armi$^{72.1}$ // TM3, Sb</td>
<td>200</td>
<td>45</td>
<td>43</td>
<td>57</td>
</tr>
<tr>
<td>pasha$^{EYO1325}$ // TM3, Sb</td>
<td>200</td>
<td>10</td>
<td>33</td>
<td>87</td>
</tr>
<tr>
<td>rm62$^{1086}$ // TM3, Sb</td>
<td>100</td>
<td>11</td>
<td>22</td>
<td>34</td>
</tr>
<tr>
<td>spn-D$^2$ // TM3, Sb</td>
<td>80</td>
<td>7</td>
<td>10</td>
<td>44</td>
</tr>
<tr>
<td>spn-E$^1$ // TM3, Sb</td>
<td>200</td>
<td>24</td>
<td>41</td>
<td>58</td>
</tr>
<tr>
<td>spn-E$^{100786}$ // TM3, Sb</td>
<td>200</td>
<td>21</td>
<td>39</td>
<td>79</td>
</tr>
</tbody>
</table>

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*<sup>MAT</sup>; 2nd Chromosome Mutations Balanced Over *CyO*

<table>
<thead>
<tr>
<th>Paternally present allele // balancer</th>
<th>Variegation Assay</th>
<th>Pigment Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Eye Variegation Index</td>
<td>% wild-type</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>0</td>
</tr>
<tr>
<td>No modifier // CyO</td>
<td>300</td>
<td>0</td>
</tr>
<tr>
<td>ago&lt;sup&gt;1&lt;/sup&gt; &lt;sub&gt;08121&lt;/sub&gt; // CyO</td>
<td>280</td>
<td>0</td>
</tr>
<tr>
<td>ago&lt;sup&gt;1&lt;/sup&gt; &lt;sub&gt;64845&lt;/sub&gt; // CyO</td>
<td>300</td>
<td>0</td>
</tr>
<tr>
<td>ago&lt;sup&gt;1&lt;/sup&gt; &lt;sub&gt;100208&lt;/sub&gt; // CyO</td>
<td>300</td>
<td>0</td>
</tr>
<tr>
<td>aub&lt;sup&gt;HN&lt;/sup&gt; // CyO</td>
<td>190</td>
<td>0</td>
</tr>
<tr>
<td>aub&lt;sup&gt;QC42&lt;/sup&gt; // CyO</td>
<td>300</td>
<td>0</td>
</tr>
<tr>
<td>aub&lt;sup&gt;KG05389&lt;/sup&gt; // CyO</td>
<td>300</td>
<td>0</td>
</tr>
<tr>
<td>dcr&lt;sup&gt;L811 fsx&lt;/sup&gt; // CyO</td>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td>loqs&lt;sup&gt;600791&lt;/sup&gt; // CyO</td>
<td>300</td>
<td>0</td>
</tr>
<tr>
<td>piwi&lt;sup&gt;3&lt;/sup&gt; // CyO</td>
<td>300</td>
<td>0</td>
</tr>
<tr>
<td>r2d2&lt;sup&gt;3&lt;/sup&gt; // CyO</td>
<td>300</td>
<td>0</td>
</tr>
<tr>
<td>r2d2&lt;sup&gt;S165 fsx&lt;/sup&gt; // CyO</td>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td>r2d2&lt;sup&gt;EY00028&lt;/sup&gt; // CyO</td>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td>squ&lt;sup&gt;PP32&lt;/sup&gt; // CyO</td>
<td>300</td>
<td>0</td>
</tr>
<tr>
<td>vasa&lt;sup&gt;3&lt;/sup&gt; // CyO</td>
<td>300</td>
<td>0</td>
</tr>
<tr>
<td>vasa&lt;sup&gt;RJ36&lt;/sup&gt; // CyO</td>
<td>300</td>
<td>0</td>
</tr>
<tr>
<td>vig&lt;sup&gt;C274&lt;/sup&gt; // CyO</td>
<td>300</td>
<td>0</td>
</tr>
<tr>
<td>vig&lt;sup&gt;EY07816&lt;/sup&gt; // CyO</td>
<td>300</td>
<td>0</td>
</tr>
<tr>
<td>zuc&lt;sup&gt;RS49&lt;/sup&gt; // CyO</td>
<td>300</td>
<td>0</td>
</tr>
</tbody>
</table>

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 $TM3, Sb$

<table>
<thead>
<tr>
<th>Paternally present allele // balancer</th>
<th>Variegation Assay</th>
<th>Pigment Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Eye Variegation Index</td>
<td>% wild-type</td>
</tr>
<tr>
<td></td>
<td>$n$ 0 1 2 3 Score</td>
<td>$n$</td>
</tr>
<tr>
<td>No modifier // $TM3, Sb$</td>
<td>300 0 0 0 300 3.00 ± 0.0</td>
<td>20 52±5</td>
</tr>
<tr>
<td>$ago2^{ago1}$ // $TM3, Sb$</td>
<td>300 0 0 0 300 3.00 ± 0.0</td>
<td>20 50±6</td>
</tr>
<tr>
<td>$ago2^{EY04479}$ // $TM3, Sb$</td>
<td>200 0 0 0 300 3.00 ± 0.0</td>
<td>20 51±5</td>
</tr>
<tr>
<td>$arm^{2.1}$ // $TM3, Sb$</td>
<td>300 0 0 1 299 3.00 ± 0.01</td>
<td>20 51±4</td>
</tr>
<tr>
<td>$pasha^{EY01325}$ // $TM3, Sb$</td>
<td>300 0 0 0 300 3.00 ± 0.0</td>
<td>20 50±5</td>
</tr>
<tr>
<td>$rm62^{E01086}$ // $TM3, Sb$</td>
<td>300 0 0 0 300 3.00 ± 0.0</td>
<td>20 54±3</td>
</tr>
<tr>
<td>$spn-D^2$ // $TM3, Sb$</td>
<td>200 0 0 0 200 3.00 ± 0.0</td>
<td>20 54±4</td>
</tr>
<tr>
<td>$spn-E^f$ // $TM3, Sb$</td>
<td>300 0 0 0 300 3.00 ± 0.0</td>
<td>20 50±3</td>
</tr>
<tr>
<td>$spn-E^{c00786}$ // $TM3, Sb$</td>
<td>300 0 0 1 299 3.00 ± 0.01</td>
<td>20 49±5</td>
</tr>
</tbody>
</table>

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)$$LJ9^{MAT}$; $TM3, Sb$ balanced internal controls and the ‘no modifier’ $Dp(1;f)$$LJ9^{MAT}$; $TM3, Sb$ 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 $Dp(1;f)LJ9; +/+ \text{ control}$, where variegated silencing of garnet is observed.

B) Maternally inherited $Dp(1;f)LJ9^{MAT}$ produces full garnet expression ($Dp(1;f)LJ9; +/+ \text{ )}$, 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).
A) Representative eye pictures of garnet expression from paternally inherited $Dp(1;f)LJ9^{\text{PAT}}$ illustrates relaxed silencing caused by the $ago1^{k00208}$, $ago1^{k008121}$, and $loqs^{f00791}$ alleles compared to the $Dp(1;f)LJ9; +/+ \text{ control}$, where variegated silencing of garnet is observed.

B) Maternally inherited $Dp(1;f)LJ9^{\text{MAT}}$ 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.
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\textsuperscript{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\textsuperscript{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\textsuperscript{PAT} And Dp(1;f)LJ9\textsuperscript{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\textsuperscript{PAT}; +/+, Dp(1;f)LJ9\textsuperscript{PAT}; dcr2\textsuperscript{L811 fsx}/+, and Dp(1;f)LJ9\textsuperscript{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\textsuperscript{MAT}; +/+ flies.

B) Sense transcripts of garnet were detected in both Dp(1;f)LJ9\textsuperscript{PAT}; +/+ and Dp(1;f)LJ9\textsuperscript{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\textsuperscript{PAT}; +/+, Dp(1;f)LJ9\textsuperscript{PAT}; dcr2\textsuperscript{L811 fsx}/+, heads, while faint detection of antisense garnet transcripts were also found in Dp(1;f)LJ9\textsuperscript{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=<0.01-0.001) 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\textsuperscript{PAT};vig\textsuperscript{C274/+} (n=18) and Dp(1;f)LJ9\textsuperscript{MAT};vig\textsuperscript{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\textsuperscript{PAT}, while the vig alleles show no affect on Dp(1;f)LJ9\textsuperscript{PAT}.

B) No affect was observed from either vas or vig alleles on maternally inherited Dp(1;f)LJ9\textsuperscript{MAT} garnet expression.
A) The effect of maternally present alleles are shown; aub<sup>HN</sup> was the only allele tested that disrupted the establishment of the Dp(1;f)LJ9<sup>MAT</sup> 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 6.9 - 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).
Figure 6.10 - The Effects Of The \textit{spn-D}^2 Allele On The Maintenance Of \textit{Dp(1;f)LJ9} \textit{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 \textit{Dp(1;f)LJ9}^\text{PAT}; \textit{spn-D}^2/+ (\( n = 10 \)).

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

B) No affect was observed from the \textit{spn-D}^2 allele on maternally inherited \textit{Dp(1;f)LJ9}^\text{MAT} \textit{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 \textit{Dp(1;f)LJ9} imprint center. Mutations in \textit{ago2}, \textit{dcr2}, \textit{loqs}, and \textit{r2d2} all resulted in a relaxation of \textit{Dp(1;f)LJ9}^{PAT} garnet silencing. Antisense transcripts, predominantly expressed from the paternally inherited \textit{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 \textit{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 \textit{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 \textit{Dp(1;f)LJ9}. This is the first reported example in \textit{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 dcr2L811 fsx and R2D2S165 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^l1 z^a g^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^l1 z^a g^53d* background to yield stable stocks of: *y^l1 z^a g^53d/ y^l1 z^a g^53d*; *allele/CyO* for second chromosome mutations, and *y^l1 z^a g^53d/ y^l1 z^a g^53d*; *allele/TM3, Sb* for third chromosome mutations. The control cross for paternal imprint maintenance consisted of *Dp(1;f)LJ9, y^+ g^+ /X^X Y* males crossed to *y^l1 zag53d/ y^l1 zag53d*; *CyO/+ or y^l1 zag53d/ y^l1 zag53d*; *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^X Y* males were crossed to *y^l1 zag53d/ y^l1 zag53d*; *allele/CyO* or *y^l1 zag53d/ y^l1 zag53d*; *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^l1 zag53d/Dp(1;f)LJ9; allele/+* progeny that determine the effect of the allele on imprint maintenance, and the internal control *y^l1 zag53d/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^a g^53d flies, yielding y^1z^a g^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^a g^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.
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 XhoI or NotI 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'-GCTCTAGACTCCGTGGCAGTTTAACTTTTGAT-3’ and 5'-GCGGGCCAGCGTTATCGACGATCCAC-3’. The generated product was inserted into a pBlueScript II SK (+) vector using the generated XbaI and ApaI sites. Digestion with either XbaI or ApaI 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 gelling 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 $y^{1}z^{9}g^{53d}/Dp(1;f) LJ9$ 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’-GCCTGCCCCAACCTCCGATTCCCAGTGGCTGTTC-3’)
- **garnet sPCR**: (5’-GGTGTTCACAATGTGCAAGG-3’)
- **garnet asRT**: (5’-GCCTGCCCCAACCTCCGACGCTCAACATCCTGCT-3’)
- **garnet asPCR**: (5’-GCTGAAGCTTGTCCAACACA-3’)
- **adapter PCR**: (5’-GGAGGTTGCGGCGAGGC-3’)

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

- **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°C for 1 min, and 72°C for 1 min (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 $y^1z^a g^{53d}/Dp(1;f)LJ9; modifier allele/+$, and sibling $y^1z^a g^{53d}/Dp(1;f)LJ9; CyO$ 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


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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 \textit{Dp(1;f)LJ9} Imprinted Domain

The chromosomal rearrangement that produces the \textit{Dp(1;f)LJ9} mini-X chromosome exposes an imprinting center within heterochromatin that is assayed by expression of the euchromatic \textit{garnet} gene. Typically, the placement of euchromatic genes next to heterochromatin in \textit{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 \textit{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 \textit{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 \textit{Dp(1;f)LJ9} leads to the active formation of a robust heterochromatic boundary to protect gene expression.

7.1.1  The Paternal \textit{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 \textit{Dp(1;f)LJ9} results in variegated silencing of the marker gene \textit{garnet}. 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. \textit{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
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 \textit{Dp(1;f)LJ9} Imprint Maintenance

A) Paternal \textit{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 \textit{Dp(1;f)LJ9} are processed by the siRNA pathway to reinforce silencing.

B) Maternal Imprint Maintenance

CTCF

nuclear
lamin?

Dnmt

HP1

Jil-1

H3K9
methylation

HP1
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 to 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


and non-CpG methylation of the Peg1/Mest gene in the mouse oocyte and


domains control heterochromatin localization and silencing properties of
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products of Suppressor of variegation and trithorax group, but not Polycomb

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18:1360-1372.

20:544-556.


### Appendix A  Drosophila Stock List

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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.