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Sequence Determinants for Heterochromatin Targeting in *Drosophila melanogaster*

by

Monica F. Sentmanat

A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
partial fulfillment of the
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ABSTRACT OF THE DISSERTATION

Sequence Determinants for Heterochromatin Targeting in *Drosophila melanogaster*

by

Monica F. Sentmanat

Doctor of Philosophy in Molecular Genetics and Genomics

Washington University in St. Louis, 2012

Professor Sarah C.R. Elgin, Chairperson

Heterochromatin is classically defined as densely staining regions of the genome; these domains are typically late replicating and show little recombination. Correct assembly of heterochromatin is critical for chromosome stability. Assembly begins with histone deacetylation and H3 lysine 9 di- and trimethylation (H3K9me2/3); the methylated H3 is typically bound by Heterochromatin Protein 1a (HP1a). Heterochromatin predominates at pericentric and telomeric domains —regions abundant in transposable elements (TEs) and satellite repeats. Transcription of these TEs has been found to generate a platform for assembly of heterochromatin through RNAi in *S. pombe* and *A. thaliana*, and may play a critical role in *Drosophila melanogaster*. However, the precise role of RNAi in heterochromatin assembly for a metazoan system such as flies remains unclear. However, *1360,* a DNA transposable element in *D. melanogaster*, has been found to be sufficient to promote heterochromatin assembly in a repeat-rich region, as shown by a variegating phenotype of a *hsp70-white* reporter. RNAi components and heterochromatin factors such as HP1a were both implicated in this *1360*-sensitive variegation, a form of position effect variegation (PEV).

Here, I sought to determine the extent and mechanism of TE-sensitive PEV. A collection of *1360*-sensitive landing pad insertion lines containing the *hsp70-w* reporter was generated. This tool allows for the repeated sampling of altered *1360* constructs in a variety of chromatin contexts, a useful a platform to study the attributes of *1360*-sensitive variegation as well as PEV generally. We found *1360*-sensitive PEV to extend to sites outside of annotated heterochromatin,

although most sensitive sites lie within or proximal to heterochromatic masses. I used biochemical approaches to show that *1360*-sensitive PEV corresponds to HP1a accumulation over the *hsp70-w* promoter region, confirming that the silencing is due to heterochromatin assembly. The deletion of sites within the *1360* element with homology to the PIWI-interacting RNAs (piRNAs) in *1360* suppressed PEV, as did dominant mutations in PIWI domain proteins. Similar results were obtained using *Invader4,* a retrotransposon, in the same landing pad site. The results support a mechanism that uses piRNAs for transposon-sensitive HP1a-silencing, likely early in development, with persistent effects observed in the adult somatic tissue of the eye.

To determine if the sequence determinants required for *1360*-sensitive silencing in a euchromatic region (as seen above) also operate in a repetitious sequence environment, where interspersed signals may operate cooperatively, I investigated a *1360*-sensitive site in the piRNA generating locus 42AB. We find that mutations in *piwi,* along with many prototypical *Su(var)* mutations, result in weak suppression of variegation at this site, while an *ago2* mutation enhances variegation. Tests of various fragments of the TEs do not reveal a strong dependency on piRNA matching sequences, contrary to the euchromatic site driven to a heterochromatic form by the added TE. These findings indicate that suppression of PEV by mutations in the genes for RNAi components occurs in a limited number of heterochromatic domains, predominantly those near gene clusters – sites typically found at the border between euchromatin and heterochromatin. Thus chromosomal context appears to be an important determinant for RNAi-dependent *1360*-sensitive PEV. This finding helps to reconcile reports of inconsistent PEV effects from mutations in RNAi components that have been carried out using reporters in different domains. Collectively, these results indicate the TEs can act as sequence determinants of heterochromatin assembly at a subset of genomic sites using an RNAi-mediated targeting mechanism.

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I applied to graduate school six and half years ago in the hopes of becoming a scientist. I understood this to be an individual who can independently carry out experiments to contribute to ongoing scientific discourse in their field. It seemed easy enough, and possibly one of the most exciting pursuits I have ever taken on. I had never wanted something more intensely than this. As it turned out, it wasn't that easy. This journey has had some excruciating moments that the love and support of my friends and family have seen me through. I am especially thankful to my mother, Eulalia Sentmanat for her encouragement and confidence in me. I am also greatly thankful to my best friend and partner William Gass who has been a beacon of support and strength.

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CHAPTER 1

INTRODUCTION

PREAMBLE

Transposable elements populate a large fraction of most eukaryotic genomes, contributing to roughly half of the human genome and 22% to the *Drosophila melanogaster* genome. Genome restructuring by TEs has played a pivotal part in composing lineage specific regulatory networks, and thus are regarded as potent drivers of evolution (Feschotte, 2008). However, mechanisms have evolved to create an equilibrium between genome stability and plasticity, wherein quelling transposon activity typically takes precedence. From plants to mammals, a well conserved mechanism for TE silencing involves small RNA-mediated post-transcriptional (RNA degradation) and transcriptional silencing (chromatin-mediated) by PIWI domain proteins. The PIWI domain proteins primarily reside in germ line tissue and the early embryo, suggesting the extent of their observable influences may be limited, particularly at the chromatin level (Brennecke et al., 2007). Ovaries depleted of germ line Piwi exhibit the upregulation of TE transcripts, which is correlated to a loss of chromatin silencing marks over a subset of reactivated TE promoters (Klenov et al., 2011; Wang and Elgin, 2011). Such work draws into question whether chromatin silencing marks at some TEs are directly deposited by Piwi and, if so, if such activity is solely restricted to the germ line.

The review presented herein (Chapter 1) provides a comprehensive survey of the literature regarding the role of PIWI domain proteins in chromatin-based silencing, particularly as it relates to their primary targets - transposable elements. This topic is one of active investigation for many in the *Drosophila* community interested in chromatin biology, as it adds a rapidly adaptable and sequence-specific mechanism to the current palette of heterochromatin assembly pathways. The review nicely converges evidence of *cis*-acting targets that promote HP1a recruitment and *trans*-acting PIWI effectors that support the phenomenon. The evidence presented highlights the layers of complexity associated with chromatin biology. For one, there are multiple mechanisms operating in tandem at any given time and, two, this likely contributes to context-dependent effects observed between reporter inserts. To directly address the question at

large, the role of Piwi at the chromatin level, these layers of complexity will have to be experimentally addressed.

The work I present provides a new platform with which to address context specific silencing effects (Chapter 2). The use of phiC31 landing pad reporter inserts in a wide variety of silent chromatin environments (e.g. telomere proximal, fourth chromosome) can help resolve issues relating to genetic context so that multiple, putative *cis*-acting targets of heterochromatin assembly may be sampled at a single insertion site. Proof of concept is presented for *cis*-acting TE targets of HP1a-dependent heterochromatin assembly. The targets included the DNA transposon *1360* and the retrotransposon *Invader4,* both shown to produce HP1a-dependent silencing of the *hsp70-w* reporter*.* The genetic dissection of these two unrelated TEs at a euchromatic landing pad site, near a block of heterochromatin revealed that the deletion of sites with sequence homology to PIWI-interacting RNAs (piRNAs) impact HP1a-dependent silencing. Thus, a useful resource has been developed to explore the mechanisms employed for *cis*-targets, in a variety of chromatin contexts. Importantly, the results suggest that sequences with homology to piRNAs may be specifically targeted for silencing, presumably by Piwi. Indeed, read-through transcription of the landing pad construct is observed in 0-10 hr embryos – generating a possible target for complementary piRNAs. The combined observations lay important groundwork for understanding the mechanisms of small RNA mediated silencing.

The euchromatic reporter insert was dominantly suppressed by mutations in PIWI domain proteins Piwi and Aub. Interestingly, context appears to be an important determinant of the mechanism behind TE-induced silencing (Chapter 3). Although most reporter inserts silenced by a single copy of *1360* are also dominantly suppressed by *piwi*, not all are. The unifying feature of those that are is that they are at the base of 2L, suggesting the chromatin status of this domain may be particularly susceptible to the effects of additional copies of TEs. This domain may be under the surveillance of the piRNA pathway – targeting heterochromatin to sites of new TE insertions. Also of interest were sites more densely populated with TE remnants, particularly at the primary piRNA generating cluster 42AB. This site is reportedly enriched in HP1a in *piwi*

mutants, suggesting that this domain might be alternatively regulated (Moshkovich and Lei, 2010). Sampling of partial *1360* fragments at a landing pad reporter insert in 42AB revealed that *1360*-sensitive silencing is refractory to the loss of sites with homology to piRNA reads at this site. These observations suggest that in this repetitious environment, additional targets or mechanisms may act redundantly.

Together, these observations support a context specific model, whereby euchromatic sites interspersed with repetitious sequence (or vice versa) are particularly susceptible to the epigenetic effects of new TE insertions, which may be mediated by Piwi. These observations invite exciting new experimental work (Chapter 4) to address the implications of such a model – particularly methods to directly measure the effect of Piwi on chromatin structure.

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CONTRIBUTIONS TO MANUSCRIPT

This manuscript has been prepared as an invited review for the journal *Biochemistry* (Moscow). I wrote the section on cis-acting elements, most of the introduction and contributed to a portion (alternative targeting mechanisms) of the concluding remarks. Sidney Wang wrote the section on trans-acting machineries and most of the concluding remarks. I have also assisted in revising the manuscript together with the other co-authors (SW and SCRE).

Targeting heterochromatin formation to transposable elements in Drosophila: potential roles of the piRNA system

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ABSTRACT

Heterochromatin formation is critical for genome stability in eukaryotes. Inappropriate heterochromatin assembly can have deletrious effects on gene expression, and consequently the orgranism. Here we focus on heterochromatin assembly mechanisms in *Drosophila melanogaster*. In particular, we review the potential role of transposable elements as genetic determinants of the chromatin state, and examine how small RNA pathways may participate in the process of targeted heterochromatin formation.

INTRODUCTION

Cytological staining of interphase chromatin reveals two apparent states of compaction – a euchromatic state which is lightly stained, diffuse in appearance; and a heterochromatic state which is densely stained, appearing compact (Zacharias, 1995). Thus heterochromatin is classically defined as densely packaged throughout cell cycle, peripherally localized nuclear material. The repetitious sequence content of eukaryotic genomes was initially recognized by quantitative DNA reassociation analysis (or Cot curves) using principles pioneered by Roy Britten and colleagues (Britten and Kohne, 1968). These studies revealed the abundance and arrangement of repetitive DNA, and ultimately led to the understanding that heterochromatin is enriched in satellite and transposable element sequences of varying copy numbers. Although understanding genome organization within the euchromatic, more complex, gene-rich compartment took precedence for many years, heterochromatin has more recently received attention with the development of improved sequencing technologies and bioinformatics strategies. These tools have enabled improved assemblies and annotation of repeats present in heterochromatin.

In a complex organism consisting of differentiated cells, “constitutve heterochromatin“ is that found at the same place in all cell types, while “facultative heterochromatin“ (important for developmentally controlled genes) occurs in some cells but not others (Beisel and Paro, 2011). Along a chromosome, constitutive heterochromatin is usually found at pericentric repeats and telomeres, while facultative heterochromatin can be interspersed along the chromosome arms. Heterochromatin is generally characterized by a signature of histone modifications that includes

H3K9me2/3; in plants, mammals, and some other organisms it is also associated with DNA methylation at CpG or CpNpG repeats.

In the fruit fly, *Drosophila melanogaster*, heterochromatin becomes visible during nuclear cycle 11-14 of embryogenesis (3-4 hrs), establishing post-translational histone modifications that persist throughout development (Hathaway et al.). Most heterochromatic sites are enriched for H3K9me2/3, the chromo domain protein HP1a and the histone methyltransferase (HMT) SU(VAR)3-9, whose catalytic SET domain delivers the H3K9me2/3 mark. Two other SET domain proteins have been identified, SETDB1 (encoded by *egg*) and G9a; both are also H3K9 histone methyltransferases, although Su(var)3-9 and Egg appear to have the dominant role (Brower-Toland et al., 2009).

Functional studies that deplete SU(VAR)3-9 homologues in mammals or in yeast have shown that the protein is important for kinetochore assembly and chromosome segregation (Aagaard et al., 1999; Ekwall et al., 1996), while a loss of HP1a in Drosophila results in telomere fusions (Fanti et al., 1998). Another form of instability from the loss of heterochromatin (HP1a in particular) is the activation of transposable elements (Wang and Elgin, 2011), which could lead to double strand breaks as well as the obvious mutagenizing effects of TE insertions within protein-coding DNA. Gain-of-function mutations in *Su(var)3-9* cause heterochromatin expansion and female sterility in Drosophila (Kuhfittig et al., 2001). Alternatively, facultative heterochromatin proteins play an important role in cell identity. Examples include X-inactivation in mammals and developmentally controlled silencing progams associated with Polycomb group (PcG) proteins, which accomplish targeted gene silencing using an H3K27me3-based mechanism. This review will primarily focus on mechansims associated with HP1a targeting. Our discussion of “heterochromatin“ will be in reference to constitutive hetrochromatin unless otherwise specified. In Drosophila, the constitutive heterochromatin domains include the pericentric heterochromatin, regions in the telomeres, and the bulk of the small fourth chromosome (Muller F element) (Kharchenko et al., 2011).

A classic and commonly used assay to dissect the cis- and trans-acting factors involved

in heterochromatic silencing in Drosophila (among other systems) involves position-effect variegation (PEV) – first observed in Drosophila by Herman Muller in the 1930s. Following X-ray mutagenesis, Muller recovered fly lines (termed w^m, *white mottled*) that had a variegating, red-interspersed-with-white pattern across the fly eye, rather than its normally solid-red (or completely white, if mutant) appearance (Muller, 1930). The phenotype is caused by a DNA rearrangement that places the euchromatic *white* gene, which has a transport function required cell-autonomously for red eye pigmentation, proximal to repeat-rich pericentric heterochromatin. This results in the stochastic "spreading" of heterochromatin components along the now proximally located euchromatic mass that includes *white* (**Fig. 1A**). Dominant loss-of-function mutations in heterochromatin components such as *Su(var)3-9* or the HP1a gene *Su(var)205* suppress the PEV phenotype such that the expression of *white* is restored in a greater fraction of cells, whereas over-expression can have the opposite effect. At the chromatin level, PEV is characterized as resulting in a relatively regular nucleosome array (Sun et al., 2001; Wallrath and Elgin, 1995), indicative of heterochromatic packaging. Biochemical analysis across the inverted breakpoint of one strain from the w^m collection, w^{m4}, shows variable enrichment of heterochromatin proteins along a 30 kb stretch, suggesting some sequence determinants might be more susceptible than others to ectopic heterochromatin assembly (Vogel et al., 2009). Together, these observations suggest that heterochromatin assembly can spread in *cis-* provided a permissible sequence context and sufficient trans-acting molecules. These properties have made PEV a widely used model with which to dissect the cis- and trans-acting factors responsible for heterochromatin assembly.

Localized distribution of heterochromatin in the genome implies an underlying sequence determinant for its targeted formation. The immediate question following this observation asks for a mechanistic explanation for the targeting process. In recent years, work from plants and the fission yeast *S. pombe* have established that many of the heterochromatin components in these systems are associated with RNA-directed transcriptional silencing (Slotkin and Martienssen, 2007). In these systems, RNA transcribed from repetitive, heterochromatic loci is processed into small RNAs that ultimately become the targeting signal for heterochromatin assembly. Such a

targeting mechanism, in which the targeting signal is generated from heterochromatin (the target) itself, allows plasticity. This is necessary to accommodate imprecision during DNA replication or new TE invasions that change the system's DNA composition, while ensuring functional precision (faithful heterochromatin assembly).

S. pombe, a system for which RNA-directed transcriptional silencing is well described, serves as an excellent model of how cis-sequence determinants work with trans-acting factors to assemble heterochromatin at repeats, generally remnants of transposable elements (TEs). Targeting of the HP1 family protein Swi6 and the H3K9 HMT Clr4 depends on the processing of RNA Pol II transcripts generated from heterochromatic loci. The RNAi-induced transcriptional silencing complex (RITS) contains the chromo domain protein Chp1, as well as the RNAi component Ago1, which binds small RNAs generated from target sites (e.g. *dg/dh* repeats, *cis*-acting signals) located in pericentric heterochromatin (Kloc and Martienssen, 2008) (**Fig. 2**). Mutations in the slicer activity of Ago1 result in a loss of silencing for reporters located at heterochromatic sites (Irvine et al., 2006), indicating that Ago1 is an essential trans-acting factor for heterochromatin assembly in *S. pombe,* and that processing the long RNA cis-acting signal from *dg/dh* repeats into smaller fragments is required. The small RNAs generated by Ago1 provide a primer for RNA-dependent RNA polymerase, which generates additional dsRNA products to be processed by Dicer1. The amplified small RNA is used to achieve additional RITS targeting. However, whether such a mechanism also operates in metazoan systems remains an open question.

It is important to distinguish between RNA-based silencing systems (here referred to as RNA interference or RNAi), which are associated with post-transcriptional mRNA silencing, and those implicated in chromatin-based silencing (**Fig. 3**). In Drosophila, RNAi primarily involves two families of proteins: Argonaute proteins, AGO1, AGO2, AGO3, Piwi and Aub, and RNase III helicases, DICER-1 and DICER-2. The Argonaute family comprises two clades, the more ubiquitous AGO clade (AGO1 and AGO2) and the primarily germ line PIWI clade (AGO3, Aub and Piwi). AGO1 and DICER-1 generate microRNAs, derived from imperfect stem-loop

transcripts, that participate in translational repression or degradation of mRNA target transcripts. Short-interfering RNA (siRNA) is derived from exogenous or endogenous (endoRNAs) dsRNA processed by AGO2 and DICER-2 (Ghildiyal et al., 2008; Kawamura et al., 2008). Although siRNA is generally considered to function through a post-transcriptional silencing mechanism in the cytoplasm, both AGO2 and DICER-2 have recently been documented to associate with chromatin in somatic nuclei, suggesting a role in nuclear silencing (**Fig. 3**) (Cernilogar et al., 2011; Moshkovich et al., 2011). PIWI-interacting RNAs, piRNAs, are derived from master clusters enriched in transposon sequences (Brennecke et al., 2007; Gunawardane et al., 2007). Both transcriptional and post-transcriptional silencing mechanisms have been reported for transposon silencing by piRNA (**Fig. 3**) (Brennecke et al., 2007; Gunawardane et al., 2007; Klenov et al., 2011; Wang and Elgin, 2011). .

In spite of their hazardous potential, transposons are among the genome's most important tools, providing the host new material for cis-acting regulatory features and protein-coding capacity (Feschotte, 2008). The paradox between a necessity to maintain genome integrity, while also achieving diversity within a population has been empirically linked to RNAi-mediated transposon regulation (Gangaraju et al., 2010). Indeed, such mechanisms have been speculated to participate in generating new variants in a changing environment, with profound consequences over the evolutionary trajectory of the population. Thus, RNAi systems in Drosophila, particularly the piRNA pathway, can be thought of as a master regulatory switchboard, with the primary task of TE repression. Whether these effects occur at the chromatin level is the topic of this review.

We recognize that a multiplicity of targeting mechanisms for sites with similar chromatin marks has been observed in systems that possess well-documented RNAi-mediated transcriptional silencing, such as *S. pombe* and *N. crassa*. This should come as no surprise; in light of the complex chromatin environments present in a genome (Kharchenko et al., 2011), equally complex targeting systems have been developed. In *S. pombe*, all of the major heterochromatic domains are targeted for silencing by proteins recognizing specific DNA

sequences (i.e. CENP-B recruitment of histone deacetylases to silence Tf2 retrotransposons; (Cam et al., 2008), in addition to the RNAi-based mechanisms (Slotkin and Martienssen, 2007). Conversely, TE elements have been used for different recognition events as well. For example, transposase-derived chromatin modifiers have been documented in Drosophila as well as in mammals. For example, BEAF-32, derived from the *hAT* transposase, is a chromatin insulator protein that binds the *scs* chromatin boundary element (Aravind, 2000).

Similar mechanisms may have evolved in Drosophila to specifically target heterochromatin factors to TEs. One possible candidate (but with no known transposase-derived domains) is Bonus, a Tif1 homolog that derives from a family of proteins identified to interact with HP1 and recruit HDACs to mediate transcriptional repression (Nielsen et al., 1999). In Drosophila, Bonus can suppress or enhance PEV, depending on the reporter insert, and binds repetitive sequence elements in euchromatin (Beckstead et al., 2005). This suggests that Bonus has a role in chromatin organization, but precisely what that is remains an open question.

Additional mechanisms of silencing include the AT-hook, DNA binding protein D1, which has been found to localize to centromeric heterochromatin and suppress PEV (Aulner et al., 2002). Genome-wide mapping analysis has revealed that D1 overlaps with several combinatorial categories of chromatin marks that can be generally ascribed to silent chromatin, in particular, HP1a-dependent heterochromatin and PcG-associated silencing (Filion et al., 2010). Indeed, D1 overexpression induces pairing among its targets in polytene chromosomes suggesting a role in higher order chromatin organization (Smith and Weiler, 2010).

In this review we strictly aim to synthesize the evidence for RNAi-induced heterochromatin targeting in Drosophila. In particular, we focus on repetitious elements acting as cis-acting signals. We begin by discussing established examples of cis-acting silencing signals, which serve as precedents for sequence-specific targeting of chromatin modifying enzymes. Although many empirical examples exist that involve transcriptional activation (Feschotte, 2008), we explore the potential of TE remnants to act as silencing signals to be used by RNAi pathway component effectors.

CIS-ACTING ELEMENTS

Cis-acting factors that are targets for complexes that bind, rearrange, and/or modify histones have profound effects on nucleosome organization and higher-order interactions. A classic example are polycomb response elements (PREs), cis-acting DNA sequence targets. PREs are targets for the developmentally controlled Polycomb group (PcG) repressor complexes PRC1, PRC2, and PhoRC (Muller and Kassis, 2006), responsible for one form of facultative silencing. Although these complexes contain histone binding and modifying subunits, it is the cis-acting sequence content present in PREs that is required for appropriate targeting. Indeed, PREs have been found to be nucleosome-free assembly platforms (Muller and Kassis, 2006), supporting a sequence-specific targeting event (as opposed to a modified histone-protein interaction). Reporter assays using upstream putative regulatory regions of the animal polarity-determining Hox genes identified PREs as necessary sequence components for targeted gene silencing (Chan et al., 1994; Simon et al., 1993). In flies, genome-wide analysis of the sequence composition of PREs has revealed low conservation (Hauenschild et al., 2008), with individual PREs possessing inherently different propensities for silencing (Okulski et al.). The low sequence conservation has been suggested to impart a certain degree of plasticity to these sites which enables them to evolve rapidly (Moazed, 2009).

TEs are an abundant resource for potential cis-regulatory elements. Transposable elements have the ability to retool their host's gene regulatory programs, and so to contribute to networks involved in cell identity during tissue specialization, much like PREs. The capacity of TEs to establish novel gene regulatory networks, particularly species-specific programs that contribute to new evolutionary trajectories, is supported by much empirical evidence (Feschotte, 2008). Although such new networks are fortuitous for the system, particularly under times of environmental stress, it is generally in the best interest of genome integrity for TE expression and mobilization to remain suppressed. Transposable elements and their remnants comprise 22% of the Drosophila genome (Kapitonov and Jurka, 2003) and roughly half of the human genome

(Lander et al., 2001); they reside primarily in repressive, heterochromatic regions. The non-random distribution and evolutionary conservation of heterochromatic TE clusters suggests that their residence is functionally required. As previously discussed, TEs inherently possess regulatory signals or may acquire them *de novo*; this, combined with their capacity for insertional mutagenesis, more often than not results in a substantial blow to the system during mobilization events. Thus, repression of these elements takes precedence under most circumstances. Indeed, the flux of TEs in the genome requires a rapidly adaptive targeted silencing system for survival. Deep sequencing of small RNA libraries has shown that TEs are expressed, and become targets for small RNA-mediated silencing in flies (Brennecke et al., 2007; Ghildiyal et al., 2008). Although small RNA pathways are better known for their function in a post-transcriptional capacity, evidence for chromatin-based silencing in Drosophila has been reported (Huisinga and Elgin, 2009). Both piRNA and chromatin structural proteins (and/or their mRNAs) are present in the early embryo (0-6 hr) (Aravin et al., 2003) during the early stages of heterochromatin formation (Rudolph et al., 2007). Thus, piRNA sequence elements could help define some heterochromatic domains, particularly for a subset of repeats represented in the piRNA repretoire.

Chromosome organization *per se* suggests that TEs could be targets for silencing, as many Drosophila PEV reporters showing the variegating phenotype typical of heterochromatic domains map to repeat-rich regions of the genome. Studies aimed at mapping heterochromatic domains on the repeat-rich 4th chromosome of *Drosophila melanogaster* using an *hsp70-white* reporter have shown that 20-60 kb deletions or duplications of flanking DNA can be sufficient to shift a red phenotype to vareigating (and vice versa), indicating local variation in chromatin packaging at that scale (Sun et al., 2004) (**Fig. 1B**). Genomic analysis of these variegating lines found a correlation between the presence of the DNA transposable element *1360* and silencing. Follow-up experiments using FLP-mediated excision of a *1360* remnant upstream of an *hsp70-white* reporter revealed that *1360* is indeed capable of supporting heterochromatin formation predominantly in repeat-rich areas of the genome (~30% repeats) (Haynes et al., 2006). Interestingly, *1360* is sufficient to induce ectopic, HP1a-dependent heterochromatin assembly in a domain of annotated euchromatin that is close to a heterochromatic mass (Sentmanat and Elgin,

2012). Variegation in both contexts, repeat-rich and euchromatic, is suppressed in *Su(var)205* and *piwi* mutants, suggesting that RNAi components may facilitate the HP1a targeting event. RNAi-based heterochromatin targeting in both *S. pombe* and plants is thought to act through RNA-RNA recognition events. A mechanistic connection between such transcriptional silencing and *1360*-induced heterochromatin assembly was observed when read-through transcripts of the P element insert containing *1360* were found to be present in 0-10 hr embryos, suggesting a plausible RNA targeting signal. Further, deletion of sites within the *1360* element with homology to piRNA sequences abundantly found in Drosophila compromised *1360*-induced PEV. These results directly implicate the piRNA pathway in *1360*-induced silencing (Sentmanat and Elgin, 2012).

Given that the piRNA pathway generates the most complex small RNA population in the fly – needed to target hundreds of TEs - it is likely that alternative TEs should behave similarly at a *1360*-sensitive site. This was confirmed using the retroelement *Invader4*, which recapitulated *1360*-sensitive PEV. Deletion of sites complementary to piRNA sequence elements again compromised the effect (Sentmanat and Elgin, 2012). The combined results support a model in which a small RNA targeting event utilizing read-through transcripts participates in the HP1a-dependent assembly of heterochromatin at this site.

Sites sensitive to *1360* appear to be limited to sites proximal to pericentric repeats, or in some cases within mapped pericentric regions. As noted above, the presence of a single copy of *1360* within the euchromatic arms (which have a low repeat density, <10%) is insufficient to trigger a variegating phenotype. A survey of *1360*-sensitive and –independent (no change in PEV +/- *1360*) lines revealed that PEV reporters close to the base of the 2L euchromatic arm are consistently suppressed by *piwi* mutations and almost all are *1360*-sensitive. Many PEV reporters that show no change in variegation in *piwi* mutant backgrounds are *1360*-insensitive and reside in regions associated with polycomb group proteins (TAS sequences). These observations suggest that piRNA pathway target sites are likely HP1a-target sites (as *1360*-sensitive silencing is an HP1a-dependent phenomenon), but limited to a subset of domains. The need for a reporter

insertion site that results in read-through transcription of the *1360* element could also limit the set of reporter loci demonstrating this form of targeted silencing.

The repertoire of possible cis targets for the piRNA system is wide, but few elements have been associated with chromatin-based changes in *piwi* mutants. Knockdown of germline Piwi has been shown to compromise HP1a deposition at promoters of HeT-A, Blood, Bari1 and Invader1, among a small set of TEs tested in Drosophila ovaries (Klenov et al., 2011; Wang and Elgin, 2011). The lack of sufficient polymorphisms among repetitive element types makes it difficult to identify the precise location of HP1a loss. Thus, the high copy number and lack of complete genome assembly in heterochromatic regions has hampered efforts to identify additional targets. Genomic context at a larger scale (at least over 10 kb, and perhaps much more) may prove to be an important factor in identifying additional *cis*-acting determinants of heterochromatin formation.

TRANS-ACTING MACHINERIES: SMALL RNA TARGETING

A small RNA-mediated targeting model (Huisinga and Elgin, 2009), representing a mechanism of remarkable simplicity and adaptability, uses sequence information encoded in small RNAs to achieve highly specific target site recognition. The coding capacity of a 20-30 nucleotide long RNA allows a wide range of potential target sequences to be identified. Recently, both endo-siRNA and piRNA have been implicated in heterochromatin targeting (Fagegaltier et al., 2009; Wang and Elgin, 2011). In both cases, however, many critical questions remain to be clarified; in particular, whether changes observed at the chromatin level in endo-siRNA and piRNA pathway mutants are a result of direct or indirect effects. The potential redundancy and/or cross talk between the two pathways further confounds our ability to interpret results from genetic perturbation experiments.

In flies, endo-siRNAs were first observed by sequencing small RNAs associated with AGO2 and small RNAs bearing 2’O-methylation at their 3’ terminus from somatic cells (Ghildiyal et al., 2008; Kawamura et al., 2008). It was found that these small RNAs are enriched in

transposon and intergenic sequences, and that their production is strongly impacted by mutations disrupting the siRNA pathway. Interestingly, the involvement of these small RNAs in heterochromatin targeting had been implicated even before their identification. It had already been shown, mostly by cytological assays, that mutations in *ago2* result in defects in centromeric heterochromatin formation (Deshpande et al., 2005). Given the well-established role of AGO2 in a small RNA-based silencing mechanism, and a potential parallel mechanism in *S. pombe* (describing small RNA targeting of heterochromatin formation), these observations pointed to the enticing possibility of siRNA targeting for heterochromatin formation. The model is particularly attractive when taken together with the observed enrichment of transposon sequences in endo-siRNAs.

A test of this model, looking at perturbation of heterochromatin formation and targeting under conditions where endo-siRNA production is disrupted, provides encouraging support. It has been shown that both viral protein sequestering of endo-siRNA, and mutations impacting endo-siRNA production, have a dominant suppression effect on a stubble PEV reporter, Sb^{V} (a translocation of *Sb* to the 2R pericentric region) (Fagegaltier et al., 2009). It has also been shown that trans-heterozygous mutations in components needed for endo-siRNA production, such as AGO2 and DCR2, also show strong suppression of w^{m4} PEV. In addition, in the same study Fagegaltier and colleagues further demonstrated that endo-siRNA component mutations have an impact on localization of HP1a and H3K9me2/3 using immuno-fluorescent staining of polytene chromosomes. While for a good percentage of samples examined, a clear impact on heterochromatin distribution is observed, it should be noted that pericentric heterochromatin remains visibly stained in all cases. These results argue that while the endo-siRNA pathway is critical in determining the localization pattern of heterochromatin, the specific targeting of heterochromatin formation at the pericentric region is either independent of the endo-siRNA pathway or (more likely) the role of endo-siRNA in this process is redundant with other mechanisms. It is interesting to note that while dominant mutations of these same genes have little to no impact on PEV at some reporter sites (Haynes et al., 2006), inserts of reporter transgenes in other regions of the genome show significant suppression. It appears that

involvement of endo-siRNA in targeting heterochromatin formation could be context dependent.

One conundrum of the endo-siRNA targeting model for heterochromatin formation is the fact that the siRNA pathway is better known for its function in post-transcriptional silencing in the cytoplasm. It is therefore difficult to draw a direct mechanistic link to a nuclear targeting process for heterochromatin. However, two recent studies have independently demonstrated chromatin-bound AGO2 protein (Cernilogar et al., 2011; Moshkovich et al., 2011) albeit in larval or adult tissues. Although a direct mechanistic link is still missing (i.e. it remains unclear what is recruited by AGO2 to initiate heterochromatinization), the endo-siRNA pathway is clearly involved in the process of heterochromatin formation, at least in certain regions of the heterochromatic genome.

Amongst the five Argonaute proteins in the fly genome, the one conspicuously localized in the nucleus is Piwi, of the PIWI family proteins (Brennecke et al., 2007). Piwi has therefore been regarded as the primary candidate Argonaute protein for heterochromatin targeting in Drosophila. The PIWI proteins associate with piRNAs, 26-30 nt small RNAs that are enriched for TE sequences. Piwi and Aub primarily bind antisense piRNAs derived from “piRNA loci“, postulated to be discrete regulatory loci that can be several kilobases long, proposed to generate a transposon defense system. In 2007, two groups independently proposed that a ‘ping-pong’ amplification loop is responsible for piRNA biogenesis (Brennecke et al., 2007; Gunawardane et al., 2007). piRNA master regulatory loci and endo-siRNA clusters predominantly map to the edges of pericentric and telomeric regions—which are highly enriched in repeats and transposable elements. Work from Pal-Bhadra and colleagues have demonstrated that mutations in PIWI family proteins impact two types of PEV at multiple genomic loci (Pal-Bhadra et al., 2004). In a study of *Spn-E*, a putative helicase involved in the piRNA pathway (**Fig. 3**), Gvozdev and colleagues demonstrated a loss of heterochromatic structure at transposon sites due to this perturbation of the piRNA pathway (Klenov et al., 2007).

Further evidence supporting the piRNA-targeting model comes from biochemical experiments showing a direct interaction between Piwi and HP1a (Brower-Toland et al., 2007). Additionally, it has been demonstrated that the direct interaction between Piwi and HP1a is

dependent on the PXVXL motif at the Piwi N-terminus. A point mutation in this domain disrupts the interaction between Piwi and HP1a in a yeast two-hybrid setting and *in vitro* (Mendez et al., 2011). This observation connects the targeting model directly to the well-established HP1a-centric model for the spread of heterochromatin (Girton and Johansen, 2008), and provides a theoretical framework for understanding the heterochromatin formation process in flies.

Piwi was first described to be involved in the maintenance of germline stem cells (Cox et al., 1998). This function was shown to be required in the stem cell niche of ovarian soma. Deep sequencing of piRNA initially positioned Piwi alongside Aub in the Ping-Pong amplification cycle for generating secondary piRNAs (Brennecke et al., 2007). This model was later modified in response to results from sequencing piRNA in *ago3* mutant ovaries (Li et al., 2009) that showed Piwi could silence a subset of transposons in the absence of Ago3, and the role of Piwi in generating piRNAs became obscure. A functional test of the piRNA targeting model for heterochromatin formation in the female germline demonstrated a function for Piwi downstream of piRNA production in deposition of HP1a at the putative promoter region for most of the transposons tested (Wang and Elgin, 2011). This interpretation is supported by an independent study using an N-terminal truncation mutant of Piwi, which fails to localize in the nucleus, to demonstrate the critical function of Piwi nuclear localization in transposon silencing and enrichment of heterochromatic markers at a subset of transposon sites (Klenov et al., 2011). Taken together, results from these two studies and a previous observation from Saito and colleagues, on the dependency of Piwi nuclear localization on piRNA binding (Saito et al., 2009), make a compelling case that piRNA targeting of Piwi plays a role in transcriptional silencing of transposons.

Evidence supporting the transcriptional silencing model for Piwi-dependent transposon suppression also arises from an independent report showing an increase in HeT-A transcription using nuclear run-on assays performed in ovaries depleted for Piwi (Shpiz et al., 2011). It should be noted that an earlier report from Zamore and colleagues found a lack of impact on the transcription rate of transposons (e.g. *mst40*) in *armitage* mutants, suggesting a post-

transcriptional silencing mechanism for piRNA in transposon silencing (Sigova et al., 2006) (Vagin et al., 2006). Consistent with this observation, silencing of the transposon Jockey is not impacted by HP1a depletion, indicating that it is not regulated by a chromatin-based mechanism, even though it is dependent on Piwi (Wang and Elgin, 2011). Thus, a post-transcriptional component is clearly part of the piRNA silencing mechanism, and maybe particularly relevant to a subset of TEs. However, given the predominant nuclear localization pattern of Piwi, and the concordance between TE over-expression and depletion of HP1a at these TEs, we argue that a transcriptional silencing mechanism mediated through a piRNA-directed heterochromatin targeting process is a major mechanism for transposon silencing by piRNA.

The physical interaction between Piwi and HP1a that connects the targeting model with the spreading model of heterochromatin formation is a substantive link. However, an attempt to verify the importance of this direct interaction in transposon silencing *in vivo* led to the discovery of unexpected complexities. By substituting the wild type Piwi in the germline with a single residue mutant form (V30A) that fails to interact with HP1a in a yeast two-hybrid experiment had no obvious impact on transposon silencing (Wang and Elgin, 2011). It was hypothesized that additional proteins bridge the Piwi and HP1a interaction, perhaps in a way similar to Tas3 in the *S. pombe* RITS complex, and that this creates a more robust system. Further biochemical work will likely be needed to yield insights into these interactions. Alternatively, other chromosomal proteins than HP1a might be initially targeted to the TEs. A tudor-domain containing histone methyl-transferase, EGG, appears to be a promising alternative candidate for Piwi targeting of heterochromatin formation; this key protein is prominently associated with piRNA loci, and necessary to maintain their heterochromatic status(Rangan et al., 2011).

In future studies, experiments using constructs bypassing the need for small RNA targeting of Piwi to induce heterochromatin formation could be informative in deciphering how Piwi recruits relevant downstream factors, if indeed it does. Ectopic tethering of a wild type or PAZ domain mutant form of Piwi is being tested in an attempt to induce ectopic heterochromatin formation. Tethering of HP1a to euchromatic reporters has been reported to induce ectopic

heterochromatin over the reporter and sufficient to induce new chromosomal interactions with other endogenous heterochromatic sites (Li et al., 2003; Seum et al., 2001). A strong claim could potentially be made from this type of sufficiency, but the results from these experiments may be difficult to interpret due to the context-dependent nature of heterochromatin silencing. Given the discussion above, a context-dependent impact of tethering is the likely outcome.

One critical question concerning the piRNA targeting model for heterochromatin formation stems from the fact that piRNA is thought to be restricted to the reproductive system and the early zygote (Brennecke et al., 2007). However, heterochromatin is critical for maintaining genome stability and adequate chromosome segregation during mitosis throughout the lifetime of the individual; thus, the lack of a heterochromatin targeting/assembly mechanism in most tissue types does not seem plausible. While the endo-siRNA pathway could potentially be an alternative targeting mechanism in the soma, many of the studies cited above show an impact of piRNA component mutations in the larval and adult tissues normally scored in PEV assays. It remains unclear how mutations in genes that are not known to be expressed could impact chromatin structure in those tissues. One intriguing possibility is the epigenetic inheritance of chromatin structure through mitosis. Heterochromatin formation is first observed during embryonic stage four (nuclear cycle 11-14) and is thought to maintain complete silencing until the relaxation phase during the late third instar larval stage (Lu et al., 1998). It is conceivable that the impact of Piwi depletion in the early zygote could be maintained epigenetically through mitosis and lead to the observed phenotype in later developmental stages of the zygote. In fact, a recent study has shown such an impact upon the conditional depletion of Piwi in the early zygote by RNAi knockdown; a strong impact on suppression of PEV is visible in adults (Tingting Gu and SCR Elgin, personal communication). While significant depletion of HP1a is apparent at the reporter site, the impact on heterochromatin as a whole appears to be minimal, suggesting how these animals might display a visible phenotype while maintaining the minimum required heterochromatin for the progression of the developmental program. We note that while the TEs are an important component of heterochromatin, satellite DNA sequences are also a significant part of the whole, and might be targeted by other mechanisms. Studies of mitotic inheritance

upon ectopic heterochromatin formation induced by conditional (temporal) tethering of Piwi could provide a strong argument for the epigenetic inheritance model described here.

CONCLUDING REMARKS AND FUTURE PERSPECTIVE

Here, we focused our discussion on the targeting aspect of heterochromatin formation. We reviewed the tremendous progress in the past decade on this issue using the fruit fly as a model organism. Clearly, small RNAs are instrumental in the targeting process required to silence transposons. However, a reoccurring theme throughout the review is that most of the reported experimental observations are dependent on genome context (proximity to heterochromatic masses, etc), thus making the derivation of a general rule difficult. For example, the impacts of mutations in the genes for RNAi pathway components show a differential response when tested on PEV reporter inserts present in different genomic loci. This no doubt reflects the mosaic nature of heterochromatin, and could also relate to the special features of the piRNA loci, which are certainly packaged as heterochromatin in somatic cells (Kharchenko et al 2011). The effectiveness of *1360* to enhance or drive HP1a-dependent silencing also varies depending on the site tested (see discussion above). It is apparent to us that complex interactions between multiple mechanisms must be in place, preventing us from deriving simple rules from our observations. From an evolutionary point of view, the involvement of transposons in this process almost guarantees a convoluted mechanism like the one we observed. There is no doubt an ”arms race” between the host species and the invading transposable elements through the evolutionary time scale, similar to that reported for viral defense systems. Whichever strategy succeeds in helping the host cope with the invasive new transposon will result in a further (potentially redundant) mechanism built into the system.

The idea of heterochromatin targeting originated from a vision in which only two types of chromatin exist in the genome. In this scenario, while the majority of the genome is composed of euchromatin, the formation of the densely localized heterochromatic regions must be specifically

targeted. The dichotomous classification of chromatin structure, while a good starting point and still useful in many cases, is insufficient to describe observations made from recent experiments. Domains and subtypes of heterochromatin have therefore been reported to describe the differences between pericentric and telomeric heterochromatin (Cryderman et al., 1999; Doheny et al., 2008). More recently, results from genome-wide chromatin immunoprecipitation mapping of chromosomal proteins and histone modifications has suggested other informative ways of classifying chromatin structure across the genome. For example the nine-state model can be used to adequately identify enhancer regions, transcription start sites and polycomb-regulated regions in addition to classic heterochromatin (Kharchenko et al., 2011). These new additions to our knowledge have in many ways made the euchromatin/heterochromatin more nuanced. As our resolution of chromatin states continues to improves our definitions of these states will likely require modifications.

While there is no doubt that certain targeting events are needed to ensure proper heterochromatin silencing, as supported by ample evidence reviewed in this introduction, the pursuit of a single unifying mechanism in heterochromatin targeting is likely to be futile. We propose, instead, that multiple mechanisms function in a complex network to ensure proper chromatin structure formation in the genome. This complex interactive network forms the basis of the context-dependent effects that we so often see in genetic dissections of chromatin biology. Towards a better understanding of chromatin based gene regulation, perhaps the reductionist approach, seeking simple explanations for targeting mechanisms should be replaced. To gain predictive power on the outcomes from simple perturbation experiments, we will have to embrace the inherent complexity of the system and utilize the wealth of genomic information derived from high throughput technologies. Where possible, this philosophy has been applied in the studies that follow.

FIGURES

1.Position-effect variegation in *Drosophila melanogaster.* Schematic depiction of the chromosomal inversion generating the white-mottled four line (*In(1)w*^{m4}) by Muller (Muller, 1930),

that places the euchromatic *white* gene (coding for a transporter protein required for red eye pigment) adjacent to pericentric heterochromatin. The light red bar represents heterochromatin while the light green bar represents a euchromatic chromatin state. The chromosomal inversion results in silencing for some cells (white, due to heterchromatin spreading over the *w* gene) and expression in others (red).

2. RNAi-transcriptional silencing in *S. pombe*. Transcripts of *dg/dh* pericentric repeats are targeted by the RNA-induced transcriptional silencing complex (RITS). RITS consists of the chromo domain protein Chp1, Tas3 and the small RNA associated protein Ago1. A second complex, the RNA-directed RNA polymerase complex (RDRC) consists of the RNA-directed RNA polymerase 1 (Rdp1), a putative polyA polymerase Cid12 and helicase Hrr1. RDRC is recruited to *dh/dg* repeats by a physical interaction with RITS to synthesize double stranded RNA, which are targeted by Dicer to make additional siRNAs to reinforce RITS recruitment.

3. Small RNA-mediated silencing in *D. melanogaster*. Only siRNA and piRNA pathways are illustrated. Note that while the piRNA pathway is more restricted to the reproductive system, the siRNA pathway has a broader distribution. Both pathways have been implicated in a small RNA mediated heterochromatin targeting process. In the siRNA pathway, small RNA generated by Dcr2 is loaded to AGO2 RISC. The AGO2 complex can suppress expression via either slicing target mRNA in the cytoplasm through a well-characterized post-transcriptional gene silencing (PTGS) mechanism or through a yet to be characterized chromatin-based transcriptional silencing mechanism (TGS) in the nucleus. In the piRNA pathway, primary piRNA generated by a process involving Zuc is fed into the Ping-Pong cycle involving Aub and AGO3 to generate secondary piRNA. This step is proposed to function simultaneously in amplifying antisense secondary piRNA and suppress transposon expression via slicing. *Spn-E* is required for secondary piRNA production although the detailed mechanism is unclear. Secondary piRNAs loaded onto Piwi, likely by Armitage, allows nuclear localization of Piwi and downstream recruitment of HP1a to induce heterochromatin silencing of transpososns.

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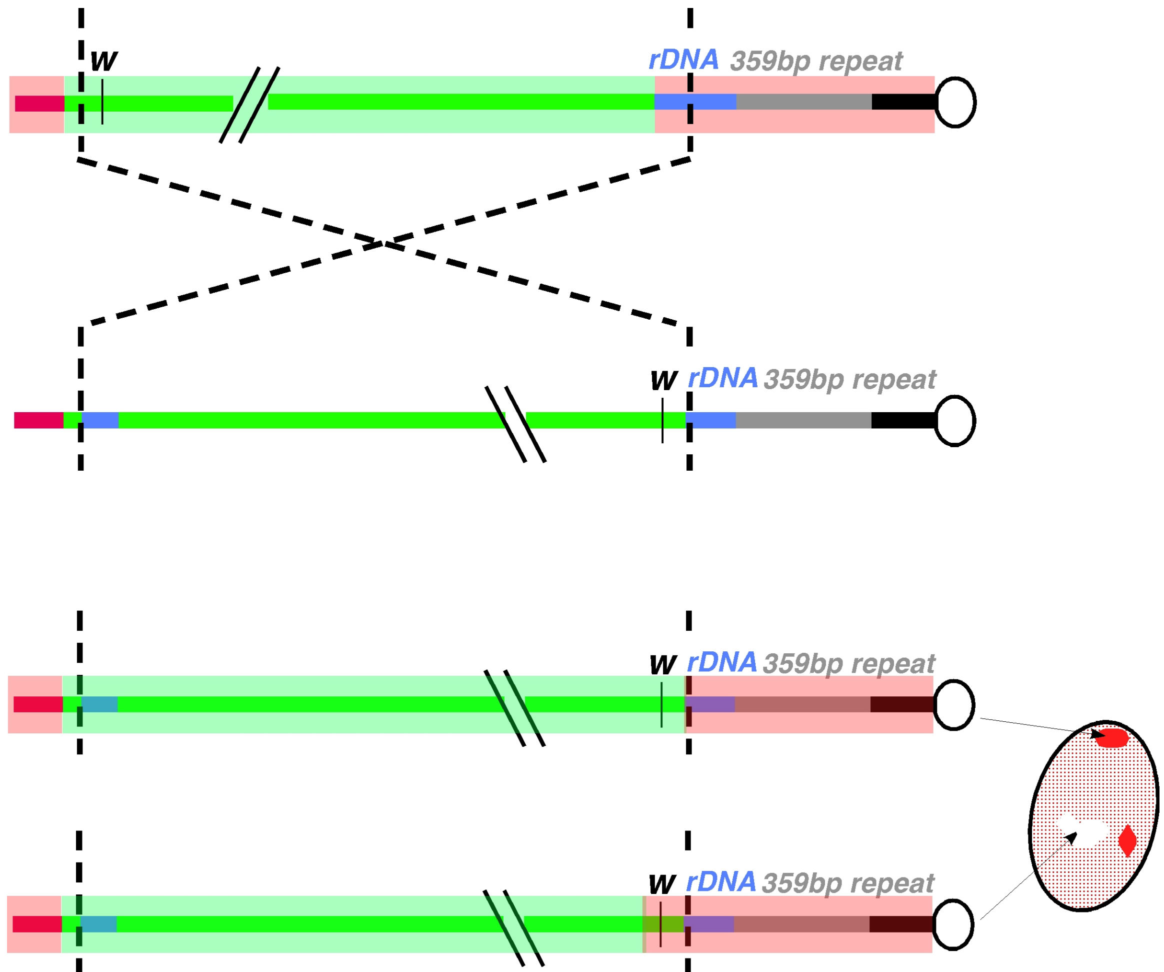
Chapter 1, Figure 1



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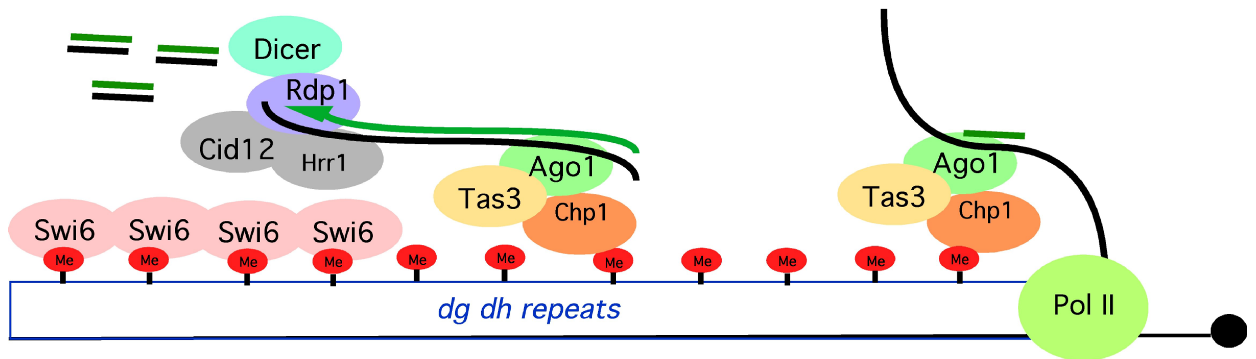
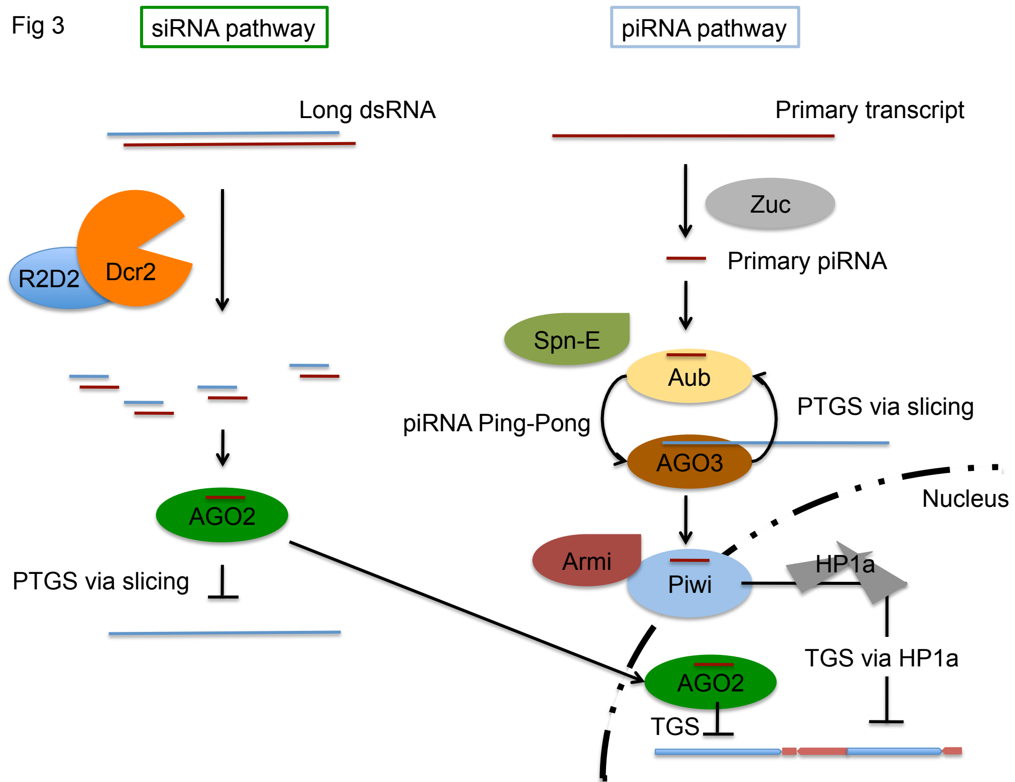


Chapter 1, Figure 3



CHAPTER 2

Ectopic assembly of heterochromatin in *Drosophila melanogaster* triggered by transposable elements

Ectopic assembly of heterochromatin in *Drosophila melanogaster* triggered by transposable elements

Classification: Biological Sciences, Genetics

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ABSTRACT

A persistent question in biology is how *cis*-acting sequence elements influence *trans*-acting factors and the local chromatin environment to modulate gene expression. We previously reported that the DNA transposon *1360* can enhance silencing of a reporter in a heterochromatic domain of *Drosophila melanogaster*. We have now generated a collection of variegating phiC31 landing pad insertion lines containing *1360* and an *hsp70*-driven *white* reporter to explore the mechanism of *1360*-sensitive silencing. Many *1360*-sensitive sites were identified, some in apparently euchromatic domains, although all are close to heterochromatic masses. One such site (line 1198; insertion near the base of chromosome arm 2L) has been investigated in detail. ChIP analysis shows *1360*-dependent HP1a accumulation at this otherwise euchromatic site. The phiC31 landing pad system allows different *1360* constructs to be swapped with the full-length element at the same genomic site to identify the sequences that mediate *1360*-sensitive silencing. Short deletions over sites with homology to piRNA reads are sufficient to compromise *1360*-sensitive silencing. Similar results were obtained on replacing *1360* with *Invader4* (a retroviral-derived element), suggesting that this phenomenon likely applies to a broader set of transposable elements. Our results suggest a model in which piRNA sequence elements behave as *cis*-acting targets for heterochromatin assembly, likely in the early embryo where piRNA pathway components are abundant, with the heterochromatic state subsequently propagated by chromatin modifiers present in somatic tissue.

INTRODUCTION

Transposable elements (TEs) are major structural features of nearly all eukaryotic genomes, and can play a role as *cis*-acting regulatory features with profound influence over the gene regulatory networks of their host (Feschotte, 2008). However, if left unchecked, the deleterious effects of TE mobilization can become insurmountable for the system, damaging genome integrity. Thus silencing mechanisms that prevent TE mobilization are fundamental to the faithful propagation of genetic information. Position-effect variegation (PEV), a mosaic expression pattern resulting from silencing in some cells that normally express a gene, has frequently been

used as an indicator of heterochromatic environments (Girton and Johansen, 2008; Wallrath and Elgin, 1995). We previously reported that the DNA transposon *1360* can enhance reporter silencing in repeat-rich heterochromatic regions (Haynes et al., 2006). To gain insight into the mechanisms involved, we sought to identify sequence elements present in *1360* that contribute to *1360*-sensitive PEV, using an adjacent *hsp70*-driven *white* gene as the reporter.

We generated a *D.melanogaster* strain that allows the incorporation of different transgenes, each possessing the same reporter but a different *1360* sequence element, into a single *1360*-sensitive genomic site. We used the phiC31 landing pad system, where phiC31-integrase mediates recombination between a landing pad (inserted in the genome) containing phage attachment (attP) sites, and a donor construct containing bacterial attachment (attB) sites (Bateman et al., 2006). This system allows us to maintain a constant genomic context for multiple transgenic constructs. A genomic test site identified at the base of chromosome arm 2L (site 1198) indeed exhibits *1360*-dependent silencing, which we find is dependent on components of the heterochromatin system, and reflects accumulation of HP1a.

Testing various features of *1360*, we found that *1360*-sensitive PEV at site 1198 does not require the inverted repeats at the ends of the element, nor internal transcription start sites, but is impacted by small deletions over sites homologous to piRNAs, a small RNA population initially generated in germ-line tissue and loaded into the zygote. This observation was extended to the retrotransposon *Invader4*, suggesting that the mechanism is broadly applicable. Thus sites with homology to piRNAs may be *cis*-acting targets for heterochromatin assembly, likely in the early embryo – a stage when piRNA pathway components are abundant - with this decision subsequently propagated in somatic tissue by chromatin modifying factors.

RESULTS

***1360*-sensitive silencing is observed in a subset of euchromatic domains.** We generated a collection of landing pad lines by mobilizing a *P* element construct containing attP sites flanking a FLP-excisable *1360*, cloned upstream of an *hsp70-white* reporter (**Fig. 1A**). Mobilization led to the recovery of 38 variegating landing pad lines (~5% recovery) with inserts

mapping to unique genomic sites (**Fig. 1B**). Many (34%) mapped to the telomere-associated repeats (TAS) present on 2R and 3R. The subtelomeric repeats are known targets for *P* element insertion (Karpen and Spradling, 1992). These genomic regions are enriched for polycomb group proteins E(Z), PSC and PC, and the associated histone mark H3K27me3 (Andreyeva et al., 2005; Kharchenko et al., 2011). In 60% of the variegating lines (including those in TAS) the construct mapped to regions with a high repeat density (>30%), a property of canonical heterochromatin (Smith et al., 2007). (See **Dataset S1** for a list of all lines.). In most of these cases the construct insertion sites lie in the pericentric heterochromatin of 2L and 2R, in the fourth or the Y chromosomes. A subset of lines have insertion sites in regions of low repeat density (<10%) that are enriched for chromatin marks associated with transcriptional activity, such as H3K4me2 and H3K9ac, in both S2 and BG3 cells (**Dataset S1**) (Kharchenko et al., 2011). The distribution of variegating insertions within transcriptionally active domains is not random; all lie within two divisions (or numerical cytological positions) of pericentric or telomeric heterochromatin. These lines represent a unique resource with which to study position effects.

To determine which variegating landing pad reporters are *1360*-sensitive, eye pigment assays were performed comparing 3-5 day old adults with the *1360* element removed by FLP-mediated excision with their sibs with the *1360* element present, i.e. +/-*1360*. Fourteen lines exhibited pigment levels that were increased in the absence of *1360*, a suppression of PEV (**Fig. 1B**; see also **Dataset S1** and **Fig. S1**). Some of these lines had a reporter inserted within or close to genes, in repeat-poor regions enriched for euchromatic marks in S2 and BG3 cells (Kharchenko et al., 2011). Thus *1360* can support heterochromatin formation at a wider variety of sites than previously recognized (Haynes et al., 2006; Sun et al., 2004). We chose the *1360*-sensitive landing pad line 1198, with an insert at the base of 2L, distal to a block of heterochromatin (cytological position 38B6, 2L:20094149) for further investigation. This line exhibits >2-fold change +/-*1360* in eye pigment, a range sufficient to detect partial suppression of *1360*-sensitive silencing (**Fig. 1D)**.

The construct is inserted within a gene (*nesd*). A survey of the chromatin environment at the insertion site (in the absence of the *P* element insert) shows that the region is enriched for marks associated with transcription (H3K4me2 and H3K9ac) in S2 and BG3 cells, as well as in 3rd instar larvae (Riddle et al., 2011; Roy et al., 2010) (**Fig. 1C**). The 20 kb region surrounding the reporter has a repeat density of 2%, consistent with the notion that the reporter is not in canonical heterochromatin. Notably, the eye phenotype in the absence of *1360* corresponds with that observed on insertion of the reporter into a euchromatic environment, displaying a solid red eye; variegation is only observed in the presence of the *1360*. However, in the presence of the *1360* element pigment levels become comparable to some lines present in annotated heterochromatin (e.g. line 1310; compare line 1198 with *1360* to line 1310 without *1360*; see **Fig. S1**), indicating that in the presence of a *1360* the site may be more representative of heterochromatin. The degree of silencing is greater in the female than the male, as is typical of PEV (**Fig. 1D**). This line gives us an opportunity to study *1360*-sensitive ectopic silencing of a reporter.

***1360* supports ectopic heterochromatin assembly.** We hypothesized that the PEV observed in the presence of *1360* for line 1198 is due to a change in the local chromatin environment, from a native euchromatic domain to a *1360*-dependent heterochromatic state, which is reversed on loss of *1360*. Heterochromatin assembly is associated with the presence of H3K9me2/3, deposited by a histone methyltransferase (HMT), typically (but not always) SU(VAR)3-9, and the chromo domain protein HP1a, encoded by *Su(var)205* in flies. We assessed eye pigment levels for the 1198 reporter +/-*1360* when mutant for either *Su(var)3-9* or *Su(var)205* to test whether these components are required for *1360*-senseitive PEV. In the presence of *1360*, a >2-fold increase in pigment is detected with each mutant in both sexes, while no significant change is observed in the absence of *1360* in males (a minor quantitative change is seen in females), corroborating that *1360*-sensitive PEV is dependent on heterochromatin components (**Fig. 2A**). Importantly, this also indicates that in the absence of *1360,* HP1a-dependent silencing is not supported at this site.

To determine if *1360*-sensitive PEV is a consequence of a change in the chromatin landscape, we performed ChIP-qPCR using antibodies for HP1a and H3K9me2, assaying the promoter region of *hsp70-white* (as marked in **Fig. 1A**). This demonstrated that the local chromatin environment is substantially enriched for HP1a and H3K9me2 in the presence of *1360*, while in the absence of *1360* levels were low (**Fig. 2B**). No significant difference in the euchromatic mark H3K4me2 was detectable +/-*1360*. Thus the *1360*-sensitive PEV exhibited by line 1198 reflects HP1a-dependent heterochromatin assembly at a normally euchromatic site in the genome.

A *cis*-acting sequence element in the right half of *1360* is necessary and sufficient for *1360*-sensitive PEV. Several features present in *1360* could be used as a signal for heterochromatin assembly. Possibilities include the terminally inverted repeats; putative transcription start sites (TSSs) found within the right half of the element; and regions with similarity to piRNA reads. Terminally inverted repeats can contribute secondary structure or serve as binding sites for transposon-derived proteins, both mechanisms utilized in plants (Ebbs et al., 2005; Huettel et al., 2006). Present within the right half of *1360* are three putative TSSs, with similarity to the *1360* transcription initiation sites suggested to produce antisense *Suppressor of stellate* transcripts in the *D. melanogaster* male germ-line (Aravin et al., 2001). If the integrated copy of *1360* is a target for the transcription machinery, this might cause inappropriate local transcript production, targeting the site for silencing by a similar mechanism. The right half also contains sites which map to piRNA reads with an antisense bias; these sites could be targets for antisense piRNAs bound to Piwi, possibly targeting sense transcripts of the integrated *1360* (Fig 3A).

To identify which sequence features in *1360* contribute to *1360*-sensitive PEV, we tested *1360* fragments lacking the terminal inverted repeats (*1360*ΔIR), left half (*1360*ΔL), or right half (*1360*ΔR) for induction of silencing as measured by pigment assays (**Fig. 3A**). Deletion of the right half of *1360* has the most pronounced effect on *1360*-sensitive variegation, resulting in a 2-fold increase in eye pigment (**Fig. 3B**; compare red bars). Lines lacking the inverted repeats or

the left half of *1360* retained *1360*-sensitive silencing, indicating that these sequence elements are dispensable for *1360*-sensitive PEV.

Since each *1360* element is flanked by FRT sites (**Fig. 1A**), we can test whether silencing for each line is dependent on the presence of the *1360*Δ construct. FLP-mediated excision of *1360*ΔL and *1360*ΔIR leads to a loss of silencing comparable to the loss of the full-length *1360* donor, while excision of *1360*ΔR produced no change in pigment levels (**Fig. 3B**). Thus, deletion of the left half or inverted repeats of *1360* does not impact *1360*-sensitive PEV, while deletion of the right half does, suggesting that sequence feature(s) within the right half of the element, independent of the terminally inverted repeat, are necessary and sufficient for *1360*-sensitive PEV.

Putative transcription start sites present in *1360* are not required for *1360*-sensitive PEV. Although there are no universal core promoter elements in *D. melanogaster*, there are some canonical elements, spanning approximately 80 bp, that are common targets for the RNA Pol II transcription machinery, including the TATA box and the Initiator (Inr) motif (Juven-Gershon et al., 2008). The *1360* element in our construct contains one TATA box with a downstream Inr motif close to one of the mapped *Su(ste)* promoter sites, while the other two sites have nearby Inr motifs. To test whether these putative promoters are required, we made three 80 bp deletions in *1360*ΔL, each spanning one of these sequence elements, to make *1360*ΔTSS (**Fig. 3A**). The resulting construct maintains *1360*-sensitive PEV, indicating that the putative TSSs are dispensable for silencing (**Fig. 3B**). FLP-mediated excision of *1360*ΔTSS led to a ~2-fold increase in pigment levels, confirming that the sequences in *1360*ΔTSS are sufficient to impart silencing (**Fig. 3B**). Thus an alternative sequence element(s) must be responsible.

The disruption of sites matching antisense piRNA reads compromises TE-dependent variegation. The piRNA pathway, previously implicated in *1360*-sensitive PEV (Haynes et al., 2006), suppresses TEs in the germ-line through post-transcriptional targeting of sense and antisense transcripts by the slicer-mediated activities of Aubergine (AUB) and Argonaute 3 (AGO3), using a ping-pong mechanism (Brennecke et al., 2007). The repertoire of

defective and active TE copies present in the genome can skew the piRNA population to bias against active TEs, necessary to prevent further TE colonization of the genome (Brennecke et al., 2008; Khurana et al., 2011). A third component, PIWI, is a nuclear protein that binds mostly antisense piRNAs, a subset of which are generated by ping-pong cycle dependent piRNA biogenesis (Brennecke et al., 2007; Li et al., 2009). PIWI interacts with HP1a, and has been implicated in a transcriptional silencing role in the germline (Klenov et al., 2011; Mendez et al., 2011; Wang and Elgin, 2011). These piRNA components are loaded into the oocyte (Aravin et al., 2003; Brennecke et al., 2007); their mode of TE repression in the early embryo has been relatively unexplored, but could include a role in establishing heterochromatin domains. Heterochromatin formation, and the boundary between heterochromatic and euchromatic domains, is established in the syncytium, at nuclear cycle 10-14, and must be propagated by chromatin modifying mechanisms present in somatic tissue (Rudolph et al., 2007).

Large-scale sequencing of piRNA populations has revealed that individual TE families have distinct patterns and ratios of sense and antisense piRNAs (Klattenhoff et al., 2009; Li et al., 2009). To determine if the right half of the *1360* element has a distinguishing piRNA distribution that could indicate key sites for silencing, we mapped the piRNA read density from wt ovary piRNA data generated by Li et al., 2009 (Li et al., 2009), against the *1360* copy present in our construct, *1360{}1503*. Both halves of the element exhibit matches to piRNA reads (**Fig. 3A**), but antisense peaks (one with a 10-nt overlapping sense and antisense peaks indicative of ping-pong cycle biogenesis) were present at two positions only, in the right half of the element (**Fig. 3A**). We tested whether these regions contributed to *1360*-sensitive PEV by removing the signature ping-pong overlapping peaks (36 bp deletion) and the neighboring shorter antisense peak (10 bp deletion) of *1360*ΔL to make *1360*ΔpiRNA. The new junctions formed by the deletions did not result in new piRNA matches. Indeed, these relatively small deletions, 46 bp in total, significantly compromised the *1360* effect, although a complete loss of *1360*-sensitive silencing was not observed (**Fig. 3B**).

Whether the observed loss of silencing was directly associated with the *1360*ΔpiRNA construct was tested by assaying PEV after FLP excision of this *1360* fragment. Indeed, a 1.3-fold change was observed, indicating that much of the capacity to silence had been lost, and that the residual silencing was due to the *1360* remnant (**Fig. 3B**). The results indicate that this 46 bp sequence component contributes significantly to *1360*-dependent silencing, but is not the sole sequence responsible for the effect. To verify that the PEV assay data was truly a reflection of a change in silencing (from less capacity for transcription +*1360* to more capacity for transcription -*1360*), we performed RT-qPCR for *hsp70*-driven *white* transcripts in 3-5 day old non-heat shocked (NHS) and heat shocked (HS) adults, comparing the full-length and *1360*ΔpiRNA recombinant lines. Transcript levels for *hsp70-w* at 25°C are sufficient to generate the eye phenotype. Heat shock increases the level of the appropriate transcription factor, and provides a more robust test of the accessibility of the promoter region, producing levels of transcript suitable for a quantitative assay (Haynes et al., 2006; Wallrath and Elgin, 1995). Indeed, the presence of *1360* significantly decreases the capacity for expression under heat shock; this capacity is restored on loss of *1360*, and partially restored with the *1360*ΔpiRNA construct (**Fig. 3C**). These findings indicate that piRNA sequence elements present in *1360* contribute to *1360*-sensitive heterochromatin assembly.

To explore whether piRNA pathway components could impact *1360*-sensitive PEV at this site, we tested reporter line 1198 both +/- *1360* in the presence of *piwi* and *aub* mutant alleles. Mutations in both genes dominantly suppressed *1360*-sensitive PEV (**Fig. S2**). This finding implicates an RNAi mechanism in establishing this *1360*-sensitive silencing.

To determine whether this phenomenon is unique to element *1360* we swapped *1360* with the long terminal repeat (LTR) element *Invader4* (**Fig. 4A)**. *Invader4* was chosen for two reasons. First, many variegating reporters generated for this study (**Fig. 1B**) are located within or in close proximity to an *Invader4* element. (Similar criteria were used initially to select *1360* as a potential target for heterochromatin assembly (Sun et al., 2004).) Second, *Invader4* is regulated similarly to *1360* by piRNA pathway components in that transcription from both elements is up-

regulated in ovaries in *aub*, *ago3* and *piwi* mutant backgrounds (Brennecke et al., 2007; Li et al., 2009). When *Invader4* replaces *1360* in the test construct, we found an equivalent level of variegation (**Fig. 4B**); this loss of expression was also suppressed by mutations in heterochromatin and piRNA components (**Fig. S3**). Deletion of sites with homology to piRNA reads (*Invader4*ΔLTR; **Fig. 4A**) resulted in an increase in pigment levels - similar to the loss of full-length *Invader4* [compare silencing by the full length element (left) with that by the truncated element (right), **Fig 4B**]. This evidence supports the suggestion that piRNA sequence elements can promote silencing; importantly, the phenomenon is not exclusive to *1360*, and can be observed using either a DNA transposon- or a retrotransposon-derived element.

Work from plants and fungi argues that such a recognition event would likely be based on RNA-RNA base pairing (Slotkin and Martienssen, 2007), suggesting a requirement for transcription over the transposable element to generate a target template. While the putative TSSs in *1360* are dispensable for *1360*-sensitive PEV at this insertion site, read-through transcription could occur from a nearby promoter. In this case, the P element is inserted downstream of the transcription start site for *nesd*. This gene is expressed at low levels in the female ovary, and at moderately high levels in the early embryo (Graveley et al.). To determine if read-through transcription of the P element is occurring, we looked for transcripts across the P element junction by RT-PCR in 0-10 hr embryos; such transcripts are detected (**Fig. 4C**). The combined results support a model in which a small RNA targeting event utilizing this transcript participates in the HP1a-dependent assembly of heterochromatin at this site in the early embryo, with the consequences of that event being evident in the tissues that go on to form the eye (see model **Fig. S4**).

DISCUSSION

Our screen to identify a *1360*-sensitive phiC31 landing pad site generated many landing pad lines with inserts in unique chromatin domains, including several on the fourth chromosome and in repetitious elements such as the telomere-associated sequences on chromosomes 2R and 3R (**Fig. 1**). This study adds useful resources to the collection of phiC31 lines currently available

(Venken et al., 2011). We find that reporters in a wide-range of chromatin domains are sensitive to the presence of *1360*;, thus, the influence of *1360* is not limited to repeat-rich, heterochromatic sites. However, proximity to repeat-rich regions appears to be important, as most *1360*-sensitive reporters are close to the base of the euchromatic arms or are telomere proximal (**Fig 1B**). Genetic analysis and ChIP experiments show that *1360*-sensitive PEV is representative of HP1a-dependent heterochromatin assembly (**Fig. 2**). The use of phiC31 recombineering technology allowed us to show that a unique sequence element within the right half of *1360*, independent of the inverted repeat terminal sequences and of putative transcription start sites, is required for optimal *1360* impact in line 1198 (**Fig. 3**). Importantly, an alternative, unrelated transposable element (*Invader4*) has the same effect as *1360* – implicating a mechanism that can be broadly applied to other TEs (**Fig 4A, 4B**). In both cases the deletion of sites with homology to antisense-oriented piRNAs compromised silencing at this ectopic site. This suggests that a small RNA directed, RNA-RNA targeting event is contributing to transposon-sensitive heterochromatin assembly, potentially using a mechanism similar to that documented in *S. pombe* and plants (**Fig. S4**) (Chen et al., 2008; Kloc et al., 2008; Mette et al., 2000; Noma et al., 2004).

The ability to trigger ectopic HP1a assembly appears to be limited to genomic sites close to heterochromatic masses, as the distribution of *1360*-sensitive sites is limited (**Fig. 1B**). Indeed, half of *1360*-sensitive sites are in annotated heterochromatin (chromatin states 7/8, BG3 cells), while most sites inducing variegation but lacking *1360* sensitivity (without a change in pigment levels +/-*1360*) are in Polycomb enriched regions, frequently in telomere-associated repeats (**Dataset S1**). Thus *1360*-sensitive PEV appears to be an HP1a-dependent phenomenon. Prior reports as well as this study find that single repetitious elements within the euchromatic arms do not trigger detectable reporter silencing (Haynes et al., 2006). The high density of repetitious elements in heterochromatic domains is a fundamental characteristic of these regions, suggesting that these repeats may cooperatively participate in stabilizing heterochromatin factors. Cooperative function of interspersed signals has been reported for other domain-wide chromatin structures, for example that developed to provide dosage compensation (Straub and Becker, 2008). Many of the *1360*-sensitive sites identified here are surrounded by repeats of different

types; thus at different insertion sites, additional mechanisms could be at work. In the future it will be of interest to determine if our observations extend to other *1360*-sensitive sites, both those adjacent to and within annotated heterochromatin. It is probably no coincidence that our experimentally recovered test site (**Fig 1C**) resembles the classical rearrangements that result in PEV, having a reporter in a normally euchromatic domain close to a heterochromatic mass.

Evidence for piRNA-mediated effects on chromatin assembly in flies is mixed, with data suggesting both silencing and activating roles for RNAi system components, depending on reporter location (Haynes et al., 2006; Moshkovich and Lei, 2010; Yin and Lin, 2007). Mutations in piRNA pathway components are weak suppressors of PEV for a reporter in a repeat-rich environment, with and without *1360* present, suggesting that both local heterochromatin and *1360*-sensitive silencing are impacted (Haynes et al., 2006). Other TEs with piRNA signals include *HeT-A*, *I* element and *copia* (Li et al., 2009). All three transposon families have been found to be silenced by HP1a and H3K9me2/3 assembly over their promoter elements in the female germ line by a mechanism dependent on RNAi components (Klenov et al., 2007; Wang and Elgin, 2011). piRNA biogenesis is most active at this stage, and it has been argued that its effects may be limited to germ cells. For example, mutations in the RNA helicase Spn-E, required for piRNA biogenesis, exhibit TE de-repression in the female germline but not in mature somatic tissues (no-ovary carcasses)(Klenov et al., 2007). Mutations in many of the RNAi components and chromosomal proteins result in female sterility, making the issue difficult to study.

Both piRNA and chromatin structural proteins (and/or their mRNAs) are synthesized during oogenesis, loaded into the ovary, and present in the early embryo (0-6 hr)(Aravin et al., 2003). Their presence overlaps the early stages of heterochromatin formation (cycles 11-14) and of zygotic transcription (nuclear cycle 14) (Rudolph et al., 2007). The latter coincides with the appearance of the histone H3K4 demethylase SU(VAR)3-3, without which HP1a and H3K9me2 levels in the heterochromatin become substantially reduced while levels for the activating H3K4me2 mark become high (immunofluorescent staining of nuclei in cycle 14 embryos), resulting in suppression of PEV. Generation of wild type clones from heterozygous *Su(var)3-3*

lines suggests that the chromatin patterns established in the late embryo persist during differentiation (Rudolph et al., 2007). Thus, piRNA components could play a role in initiation of heterochromatin formation in the early embryo at select sites (e.g. a subset of TEs) in the genome, with the structure maintained during development by chromosomal protein interactions. However, whether or not depletion of these products in the female germline/early embryo alone is sufficient to see an impact in the larvae/adult remains to be tested.

While the impact on silencing of deleting the piRNA sites in *1360* and *Invader4* is striking, full suppression of the PEV induced by *1360* was not observed (**Fig. 3B, 4B**), which suggests that additional sites may be critical. Such sequences could be missing from the small RNA library used for our analysis. Additional piRNA libraries (Brennecke et al., 2007) examined did not identify additional sites within the right half of the *1360* element. The piRNA libraries reported to date do not achieve saturation, so it is possible that additional piRNA targets remain.

Alternatively, a different mode of targeting could also be in effect, unrelated to a piRNA-mediated event. Such dual mechanisms have been documented for heterochromatin formation in *S. pombe*, a system in which RNAi-mediated heterochromatin targeting is well established (Cam et al., 2008; Kloc and Martienssen, 2008). In many eukaryotes, transposases have been found to be sources of DNA binding domains, co-opted by their host to achieve new transcriptional regulatory networks, in many cases targeting their cognate transposon sequences (Feschotte, 2008). For example, *Drosophila* BEAF-32, derived from the *hAT* transposase, is a chromatin insulator that binds the *scs* chromatin boundary element (Aravind, 2000). Similar mechanisms may have evolved in Drosophila to specifically target heterochromatin factors to TEs. However, no candidate proteins have emerged to date.

Evidence for an RNAi-based mechanism that contributes to heterochromatin assembly has been found in several model systems (worms, plants, fission yeast). In *S. pombe*, targeting of the HP1a homolog Swi6 and the H3K9 HMT Clr4 depends on the processing of RNA Pol II transcripts (generated from heterochromatic loci) by RNAi components. The RNAi-induced transcriptional silencing complex (RITS) contains the chromo domain-containing protein Chp1 as

well as the RNAi component Ago1, which binds small RNAs with homology to target sites (e.g. *dg/dh* repeats) in pericentric heterochromatin (Kloc and Martienssen, 2008). In the system described here, the read-through transcripts of the *nesd* gene detected in early embryo samples (**Fig 4C**) are plausible targets for Piwi associated with *1360* or *Invader4* piRNAs, utilizing a base-pairing interaction. Analysis of EST libraries from Drosophila embryos indicates that most TE families are transcribed (Deloger et al., 2009). However determining the origin of these transcripts is a challenging task given the high sequence similarity among TE family members. The current experiments have not allowed us to determine whether the *1360*-sensitive silencing observed is transcription dependent, as would be required for an RNA-RNA recognition event for targeted silencing; such a test will be critical for future work to establish the mechanism involved. The present results support a mechanism that utilizes piRNAs, possibly ping-pong cycle derived (as are *1360* and *Invader4* piRNAs (Li et al., 2009)), for transposon sensitive targeting of HP1a, most likely early in development, with persistent effects observed using reporters in adult tissues.

MATERIALS AND METHODS

Fly Stocks: All *Drosophila* stocks and crosses were maintained at 25°C on cornmeal sucrose-based media (Shaffer et al., 1994). Fly stocks were from the Bloomington *Drosophila* Stock Center unless otherwise indicated. Transgenesis of the landing pad construct into the starting stock yw^{67c12} was carried out by Genetic Services (Cambridge MA). Stocks and constructs used, plus the mobilization and mapping procedures are described in detail in ***SI Materials and Methods***.

Chromatin Immunoprecipitation: Chromatin isolation and immunoprecipitation from 3rd instar larvae were carried out as previously described (Riddle et al., 2011). The antibodies used were HP1a W191, Abcam2012 H3K9me2, and Millipore 07-030 H3K4me2. Antibodies were validated by us and by others: see the Antibody Validation Database (http://compbio.med.harvard.edu/antibodies/about) (Egelhofer et al., 2011). Quantitaive PCR (see **Table S2** for the list of primers) was performed using BioRad 2x Master Mix SYBR Green in a

Cepheid SmartCycler. Two biological replicates, each consisting of two technical replicates, were assayed for each immunoprecipitation assay.

Mapping piRNA reads and assessing RNA products: Direct sequence mapping was carried out using small RNA sequence reads derived from wild-type Oregon R ovaries (Li et al., 2009) retrieved from the NCBI trace archives with accession number SRP000458 (Figs 3A, 4A). For RT-PCR, RNA was isolated from 0-10 hr embryos (**Fig. 4B**) or 3-5 day adult flies, +/- heat shock (**Fig. 3C**), DNAse I treated and reverse transcribed using random hexamer primers (**Fig. 4B**) or oligo dT (**Fig. 3C**). See ***SI Materials and Methods*** for details.

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FIGURE LEGENDS

Figure 1. *1360*-senstive PEV can occur at euchromatic sites. (A) Schematic of phiC31-mediated cassette exchange between the donor construct and a landing pad, resulting in the recombinant product at the same genomic site. The *yellow* gene, used as a marker to recover recombinants, was removed by *loxP* excision prior to all assays. The bar below *hsp70-white* denotes the region assayed by ChIP-qPCR in **Fig. 2**. (B) Map of the landing pad insertions exhibiting variegation. Those showing suppressed variegation on excision of *1360* are marked by green triangles; those with no change, red triangles (see also **Dataset S1**). (Black triangles indicate that the line was not assayed.). (C) Chromatin state of the 1198 insertion site based on modENCODE ChIP-array data from 3rd instar larvae (active marks H3K9ac, H3K4me2, and silent marks H3K9me2 and HP1a shown). A vertical line denotes the *P* element insertion site within *nesd*. (D) Pigment assays +/- *1360* for females (red) and males (blue), with representative eye pictures (see also **Fig. S1**). Bars

represent the mean from two biological replicates, +/- SEM. Excision of the *1360* element results in a loss of silencing.

Figure 2. *1360* promotes heterochromatin assembly. (A) Eye phenotypes (top) of line 1198 +/- *1360*, comparing the starting line (wt, left) with those mutant for *Su(var)205* (HP1a) or the HMT *Su(var)3-9*. Below: corresponding pigment assay readings (OD 480). Bars give the mean from two biological replicas, +/-SEM. **(**B) ChIP assessment of levels of HP1a, H3K9me2 and H3K4me2 at the *hsp70-white* promoter for line 1198 +*1360* (black bars) and -*1360* (grey bars). qPCR primers bracket the *hsp70-white* promoter (see **Fig. 1A**). All data was normalized to input; fold enrichment over the *alpha actinin* locus is shown, +/- SEM.

Figure 3. piRNA sequence elements in the right half of *1360* support *1360*-sensitive silencing. (A) *1360* variants used in donor constructs for phiC31-mediated cassette exchange. Bent arrows indicate putative transcription start sites. Frequencies of sense (red line) and antisense (blue line) piRNA reads (Li et al., 2009) that map to *1360{}1503* are shown. The bars below illustrate the deletions for the *1360*DTSS and *1360*ΔpiRNA constructs. (B) Representative eye pictures for each *1360*Δ recombinant line are shown under the pigment assay data (from male flies) using OD 480, +/-SEM. Asterisks indicate a significant change +*1360* (red) vs -*1360* (blue) with a *P*-value of <.05 (Student’s *t*-test); numbers indicate fold change. Loss of the right half of *1360{}1503* or loss of the piRNA hotspots results in a significant loss of silencing (no or reduced change on excision of the *1360* remnant). (C) RT-qPCR of *hsp70-w* transcript levels in non-heat shocked (NHS) and heat shocked (HS) *wt* adult flies heterozygous for the *P* element. Shown is fold expression (normalized to *RpL32*) relative to full-length *1360* recombinant lines, +/-SEM.

Figure 4. *Invader4* piRNA sequence elements support PEV. (A) *Invader4* variants used in donor constructs for phiC31-mediated cassette exchange are shown. Frequencies of sense (red line) and antisense (blue line) piRNA reads mapping to *Invader4{}1541* are shown. The bar below denotes regions of *Invader4* deleted in *Invader4ΔLTR*. (B) Pigment assay data using OD480, +/- SEM is shown for +/- *Invader4* test constructs. Loss of regions with homology to piRNA reads (*Invader4*ΔLTR) results in a loss of *Invader4*-sensitive PEV. (C) Diagram of the *P* element

insertion site for line 1198 in the 5' end of *nesd*. Black bars represent primers used for RT-PCR (top). Read-through transcripts spanning the 3'P region of the *P* element (3'P) and control *RpL32* products are shown (bottom). Lines used were wt control (no *P* element insert) and 1198, where +/-RT indicates the addition or omission of reverse transcriptase.

SUPPORTING INFORMATION

Fly Stocks: FLP-mediated excision of inserts was performed using *hs-FLP Cy/noc Sco* (6876) as described previously (Haynes et al., 2006). The yw^{67c23} strain was used to outcross reporter lines for pigment assays (for +/-*1360* assays and WT controls for mutant analysis) and ChIP-qPCR. Alleles used for PEV assays are $yw; Su(var)205^{02}/CyO$, $yw; Su(var)205^{05}/CyO$, $w^{118};Su(var)3\text{-}9^{06}$, $w^{118}; aub^{QC42}/CyO$, $w^{118}; aub^{\Delta P\text{-}3a}/CyO$ (Pal-Bhadra et al., 2004), $w^{118}; piwi^{1}/CyO$ (Cox et al., 1998), $w^{118}; piwi^{2}/CyO$ (Cox et al., 1998).

Constructs: The P element landing pad construct was derived from P[T1] (Haynes et al., 2006). Phage attachment sites attP1 and attP2 were PCR amplified (using primers attP1F, attP1R, attP2F, and attP2R) from pUASTP2 (Bateman et al., 2006), and cloned into *Stu*I and *Cla*I sites of P[T1], respectively. The Clontech In-Fusion PCR Cloning System was used; primers were designed according to manufacturer's specifications (Zhu et al., 2007).

Donor constructs were derived from pCiB-yin (Bateman et al., 2006) by PCR amplifying *yellow* (primers y F, y R) to clone into the pCR2.1 TOPO vector to make pCR2.1-y (Bateman et al., 2006). The attB1 and attB2 sites were cloned from pCiB-GFP (Bateman et al., 2006) and the loxP sites from pP[wlo+GS]. The attB2 site was inserted into *Apa*I of pCR2.1-y to generate pCR2.1-y-attB2. A loxP site was cloned into a new pCR2.1 TOPO vector and attB1 was inserted upstream at *Kpn*I to make pCR2.1-attB1-loxP. PCR amplification of attB1-loxP and insertion into the *Sac*I site of pCR2.1-y-attB2 was carried out to produce pCR2.1-attB1-loxP-y-attB2. A second loxP site from pP[wlo+GS] and a frt site from P[T1] were cloned into a new pCR2.1 TOPO vector to make pCR2.1-loxP-frt. Each deletion construct (**Fig. 3A**) was derived from the *1360{}1503* copy present in P[T1]. The full-length *Invader4* construct (**Fig. 4A**) was derived from *Invader4{}1541* (sub-cloned from the fourth chromosome into pCR2.1 TOPO-TA vector using

primers Invader4F/R) and cloned into the *XhoI* site of pCR2.1-loxP-frt using primers XhoIInvader4F/R. The list of primers used to amplify deletion constructs can be found in Table S2. Each amplified deletion construct was cloned into the *Xho*I site of pCR2.1-loxP-frt, to generate pCR2.1-loxP-frt-*1360*Δ. Primers XhoI8-24F and XhoI8-24R were used to amplify loxP-frt-*1360*Δ to clone into the *Xho*I site of pCR2.1-attB-loxP-y-attB to make pCR2.1-attB-loxP-y-loxP-frt-*1360*Δ-attB. Preparing the *1360*ΔTSS and *1360*ΔpiRNA constructs required consecutive inverse PCR steps; primers are listed in **Table S2**. Donor constructs were injected by Genetic Services (Cambridge MA).

Mobilization: Mobilization was from the X chromosome (Line 5, X:3589639). Females homozygous for the P-element insertion were crossed to *w/Y, Sb Δ2-3/TM6* males. The male progeny carrying the *Sb Δ2-3* chromosome and the landing pad construct were crossed to *yw*^{67c23}*; net; sbd*; *spa*^{pol} (MMR- multiply marked recessive) females. Male progeny which carried the landing pad construct but not the *Sb Δ2-3* chromosome were backcrossed to *yw*^{67c23}*; net; sbd; spa*^{pol} females, facilitating genetic mapping as indicated by the absence of one of the recessive phenotypes, *net, sbd* or *spa*^{pol}*.* Landing pad lines generated from the screen were maintained over appropriate 2nd or 3rd chromosome balancers (*CyO* or *TM3Sb*), or for the 4th, a chromosome marked by a dominant mutation (*ci*^{D}).

Mapping insertion sites in landing pad lines: Insertion sites in landing pad lines were mapped by inverse PCR from the 5'P end to the transposon as previously described (Sun et al., 2004). The genomic position of the landing pad P element in line 1198 was confirmed by amplifying and sequencing the 3' end of the construct (primer 3'w v.2) and predicted flanking genomic region (primer 1198 F).

PhiC31 cassette exchange: To screen for putative recombinants we crossed adults to *yw* and screened F1 males for *y*+. PCR was used to confirm that cassette exchange had occurred in the desired orientation, by screening for the loss of attP sites and gain of attL and attR (**Fig. 1A**). We crossed each recombinant to *yw* P{*y*[+*mDint2*]=*Crey*}1b for *Cre* recombinase-

mediated excision of the *yellow* marker prior to analysis by pigment assay; this was necessary, as enhancers present in the *yellow* gene interfered with our reporter readout.

Eye pigment analysis: Quantitative eye pigment analysis was performed on 3-5 day-old adults. All mutant lines analyzed were heterozygous for the reporter and for the mutant allele. Flies were homogenized in 250 µL of 0.01 M HCl in ethanol, incubated for 10 min at 50°C and the extract clarified by centrifugation. A final volume of 150 µL was used to measure optical density at 480 nm (adapted from (Khesin and Leibovitch, 1978)).

Assessment of RNA products by RT-PCR: RNA was isolated from 0-10 hr embryos (**Fig. 4B**) or 3-5 day adult flies (**Fig. 3C**) using Trizol (Invitrogen) according to the manufacture's protocol. For quantitative analysis of *hsp70-w* (primers *white* exon6 F/R) from +/-*1360* and *1360*ΔpiRNA (**Fig. 3C**) flies were non-heat shocked or heat shocked at 37°C for 55 min and allowed to recover for 2 hours prior to RNA extraction. RNA was DNAse I treated (Promega RQ1 RNase-Free DNase) and reverse transcribed (Invitrogen SuperScript II) using random hexamer primers (**Fig. 4B**) or oligo dT (**Fig. 3C**). qPCR of the 3'P end of the P element (**Fig. 4B**) was performed using primers 1198 F and 3'P A412 R.

Supplemental Figure Legends

Figure S1. Pigment assay (OD 480; a measure of expression of the *hsp70-white* gene) for females and males carrying the reporter element (Fig 1) at different insertion sites, with (+) or without (-) the *1360* element. The left half of the graph is a compilation of all *1360*-sensitive landing pad lines, while the right half displays data from those that showed no change in pigment levels +/-*1360*. Error bars derived from two biological replicate experiments (four technical replicates per experiment), +/- SD.

Figure S2. Representative eye pictures for 1198 lines +/-*1360* (top) in the presence of *piwi* and *aub* mutant alleles. Pigment assay data (OD 480, below) shows the mean of two biological replicate experiments, +/-SEM. When *1360* is present, silencing is sensitive to mutations in *piwi* and *aub*, which code for components of the RNAi system.

Figure S3. Representative eye pictures and pigment assays (OD 480) (+/- SEM) for line 1198 females and males carrying the reporter construct with an *Invader4* element, demonstrating the impact of mutations in *Su(var)205, piwi,* or *aub*. Silencing is sensitive both to mutations in the heterochromatin system and the piRNA system.

Figure S4. A model for piRNA targeting of *1360*. piRNAs loaded onto Piwi could bind to the read-through transcript from the *nesd* promoter. Our results suggest that these RNA products help target HP1a to the *1360* element upstream of our reporter.

Supplemental Tables

Table S1. (Dataset S1) Landing pad lines generated from our screen, giving the eye phenotype, location in the genome (chromosome, band, and sequence position of the insertion), orientation (strand), gene and repeat densities (or number of bases annotated as genes (Flybase) or repeats (repeat masker) reported as percentages measured 10 kb on either side of the insertion site for a total of a 20 kb window), with the corresponding chromatin state of the native insertion site in BG3 and S2 cells (Kharchenko et al., 2011), *1360*-dependent silencing is indicated as Y = yes, N = no, and U = unknown (not tested). Note that sites where no chromatin state information is available are marked “NA” in those columns.

Table S2. Oligonucleotides

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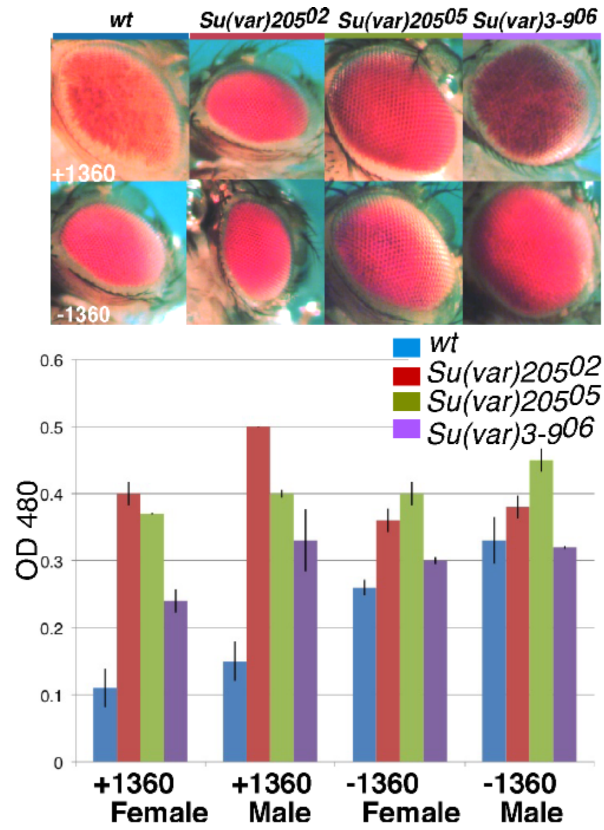
Chapter 2, Figure 1

Chapter 2, Figure 2

A



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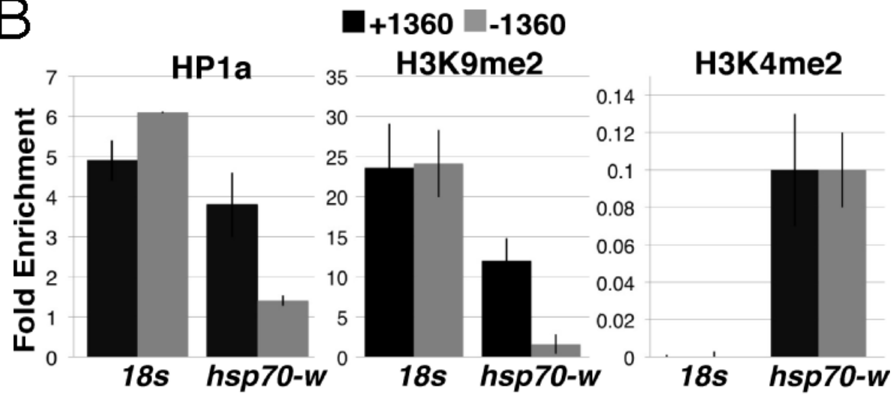
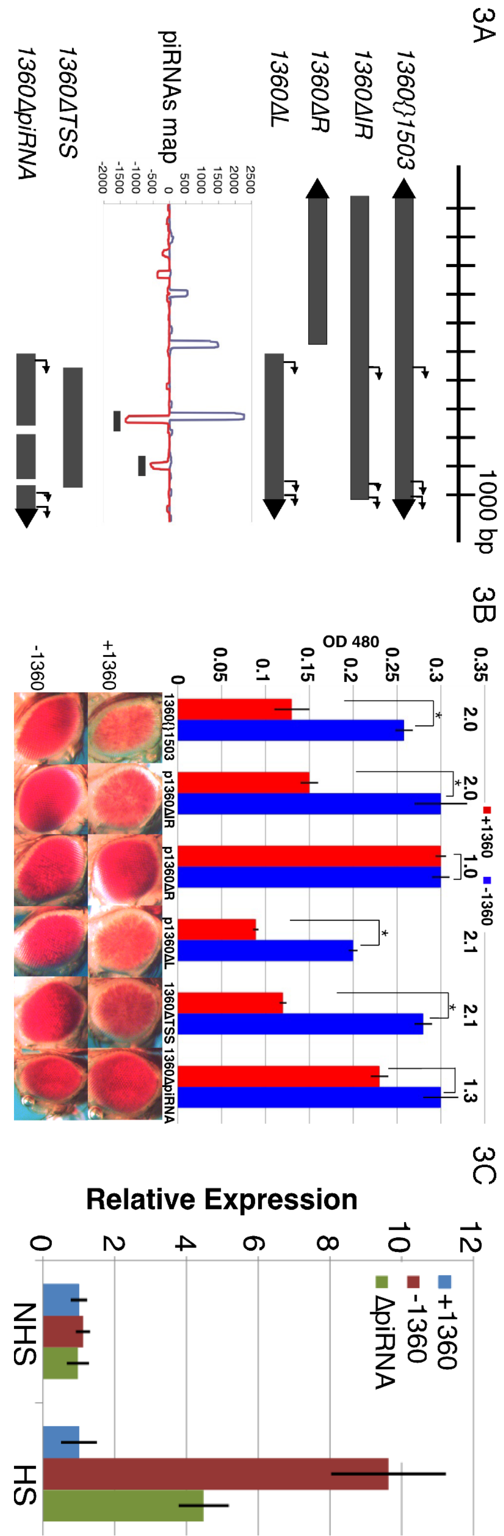
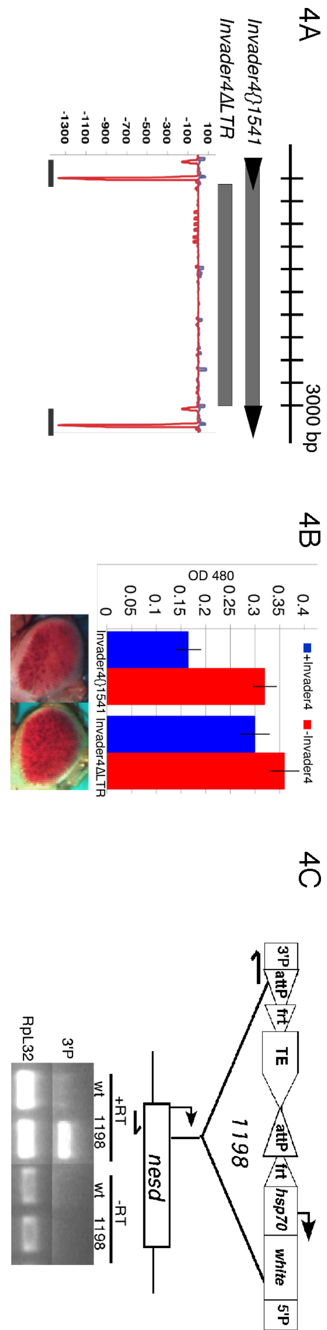


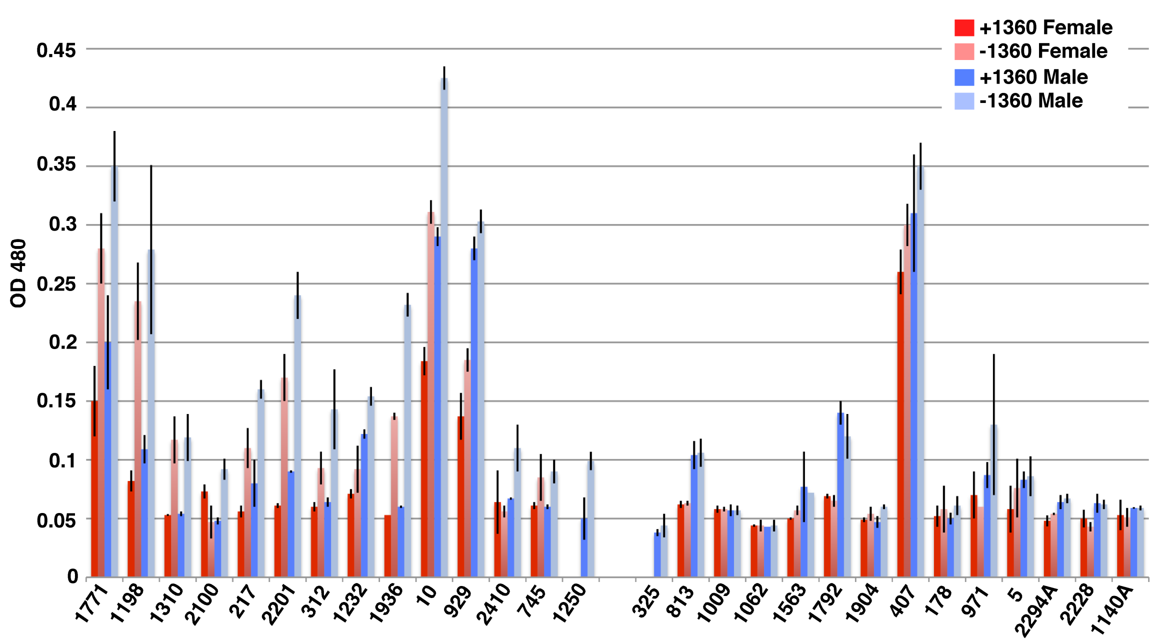
Chapter 2, Figure 3



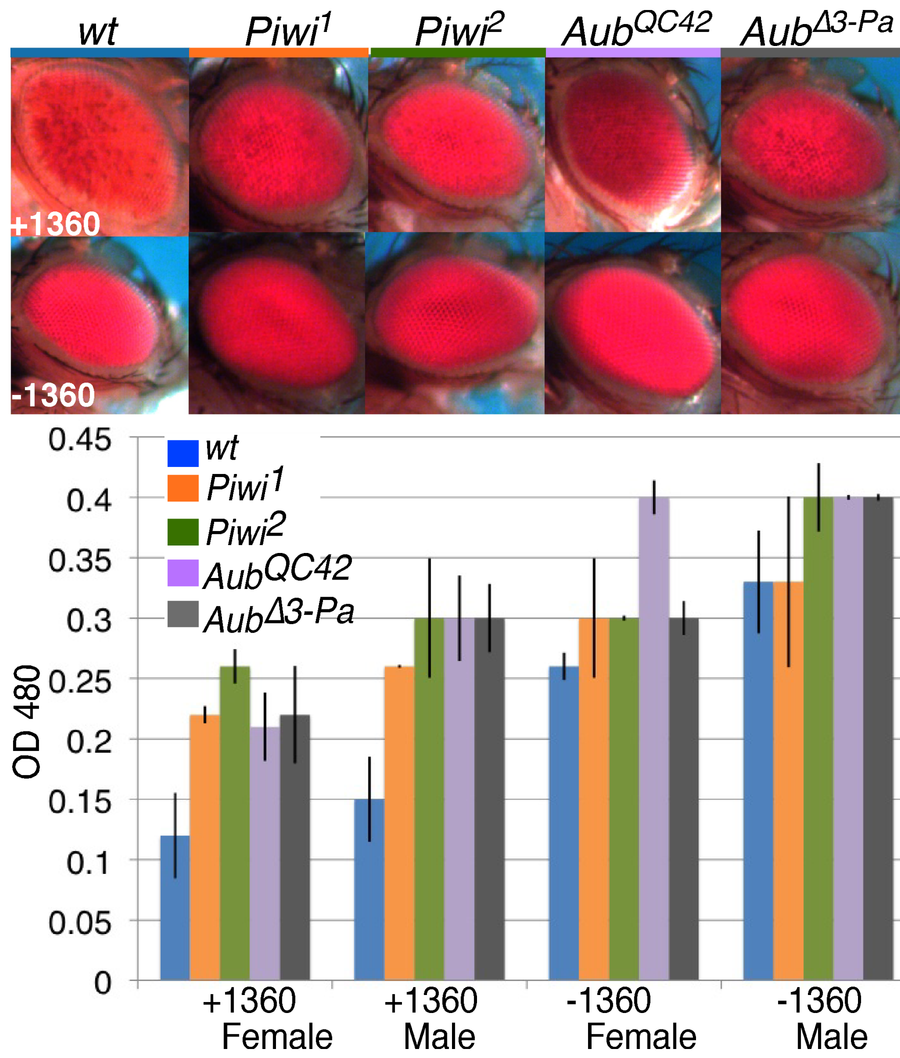
Chapter 2, Figure 4



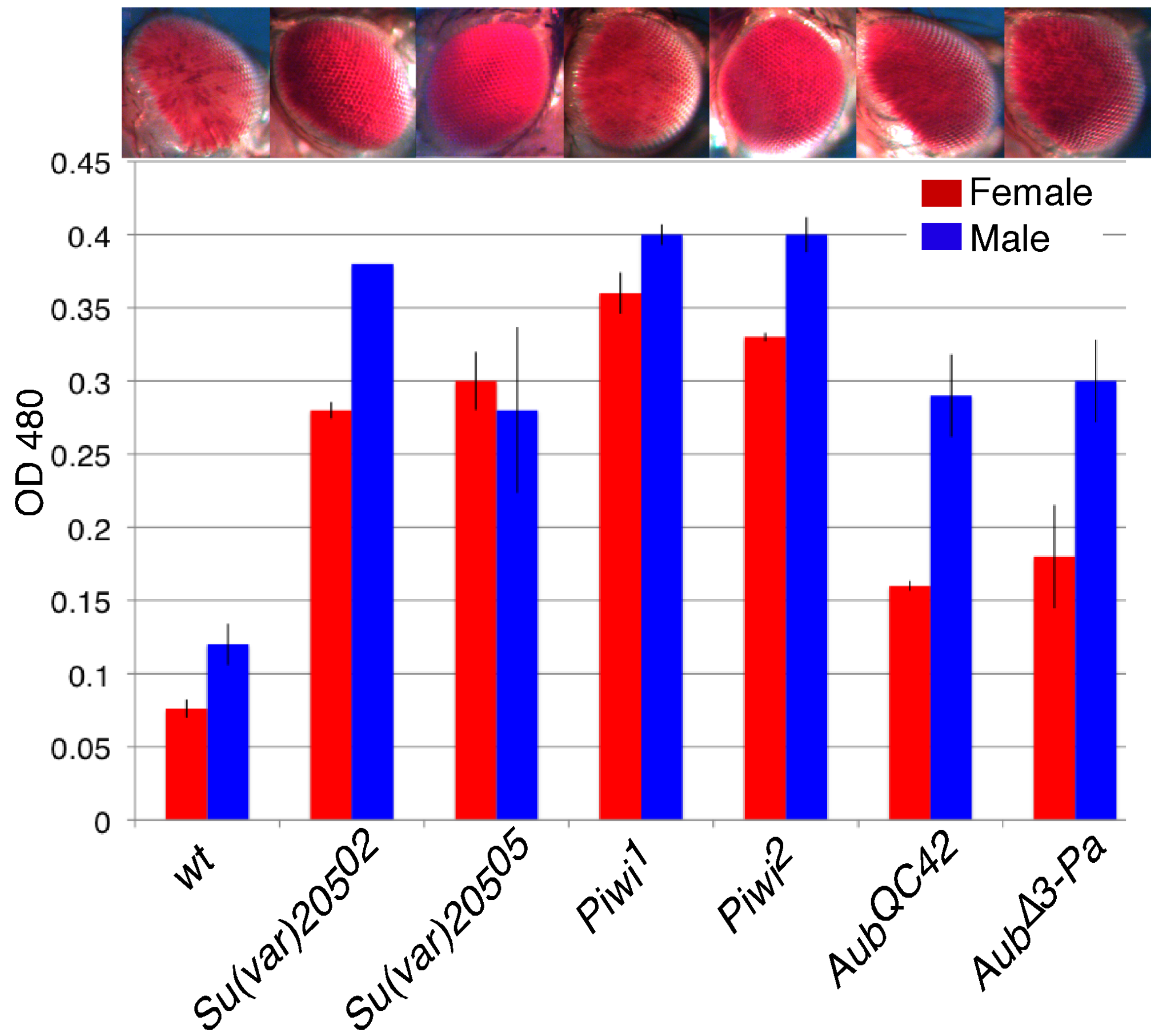
Chapter 2, Figure S1



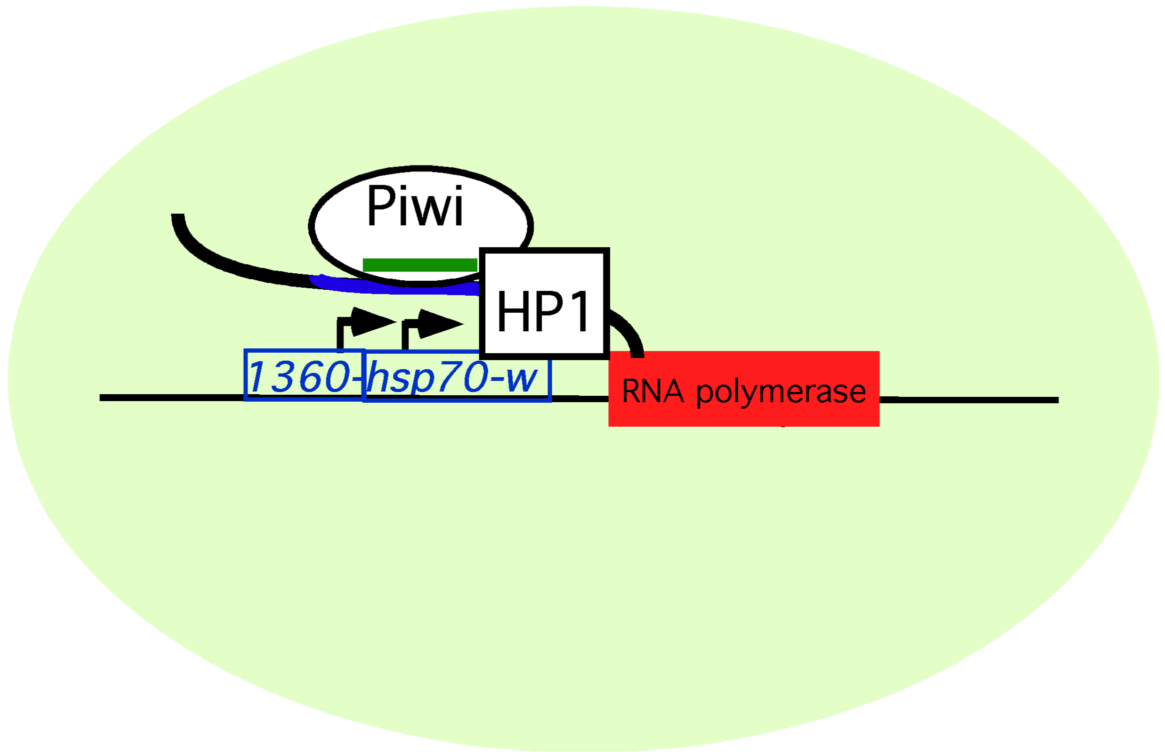
Chapter 2, Figure S2



Chapter 2, Figure S3



Chapter 2, Figure S4



Chapter 2, Table S1 'Dataset S1'

Line	Phenotype	Chr	Band	Position	Strand	*1360*-sensitive	Gene density	Repeat density	BG3	S2
line_1771	moderate PEV, red on white	X	3E5	3589639	plus	Y	65.86	6.25	3	3
line_1198	weak PEV, red on brick red	2L	38B6	20094149	plus	Y	76.69	2.26	1	1
line_1310	strong PEV, red on yellow	2L	39E2	21572961	plus	Y	41.45	61.38	7	NA
line_2100	moderate PEV, orange on white	2L	39D3	21419980	minus	Y	36.25	36.29	7	NA
line_217	moderate PEV, red on white	2L	39E1	21540808	plus	Y	27.53	43.83	7	NA
line_2201	moderate PEV, red on orange	2L	39F1	21708198	plus	Y	100	34.37	7	7
line_312	weak PEV, red on orange	2L	38C2	20148548	minus	Y	0	45.14	8	7
line_1232	weak PEV, red on brick red	2R	44A4	3948956	plus	Y	81.2	5.59	3	3
line_1936	moderate PEV, orange on yellow	2R	42A16	2301664	minus	Y	0	47.52	7	7
line_10	weak PEV, red on orange/red	3L	64D14	3579448	minus	Y	100	1.38	4	4
line_929	weak PEV, orange on red	3L	61F1	1101501	minus	Y	54.23	2.96	6	6
line_2410	moderate PEV, red on orange	3R	100A4	26591628	minus	Y	49.9	2.98	3	3
line_745	strong PEV, orange on yellow	4	102B5	380116	plus	Y	93.61	18.46	7	7
line_1250	moderate PEV, white on red	Y	U	7542035	minus	Y	0	92.49	NA	NA
line_325	moderate PEV, red on white	Y	U	9095030	minus	N	0	83.81	NA	NA
line_813	weakPEV, red on orange	2L	22A7	1668526	minus	N	100	2.86	6	3
line_1009	moderate PEV, red on orange	2R	43C3	3344853	minus	N	74.51	4.32	1	1
line_1062	solid light orange	2R	57A7	16490478	plus	N	99.87	2.86	3	2
line_1563	solid orange	2R	54B1	13152997	plus	N	49.7	1.81	3	3
line_1792	weak PEV, orange on white	2R	58D2	18130495	plus	N	45.14	1.34	6	6
line_1904	strong PEV, orange on yellow	2R	42A16	2287667	minus	N	0	45.28	7	7
line_407	weak PEV, red on brick red	3R	95A7	19505875	minus	N	51.23	3.8	1	1
line_178	strong PEV, orange on white/yellow	3R	100E3	27899585	plus	N	29.59	50.6	6	6
line_971	strong PEV, peach on white	3R	100E3	27899499	plus	N	29.98	50.32	6	6
line_5	weak PEV on orange	3R	100E3	27899517	plus	N	29.9	50.38	6	6
line_2294A	strong PEV, red on orange	3R	100E3	27899570	plus	N	29.66	50.56	6	6

Chapter 2, Table S2

PRIMER NAME	SEQUENCE
Cloning	
attP1 F	aggtcgacctcgaggcctttgagatgcagtacctgacggacac
attP1 R	aacgttactcgaggccttgacctgttcggagtgattagcgt
attP2 F	tcatcaagcttatcggtcacaccacagaagtaaggttcc
attP2 R	tcctcgacggtatcgtagggaattgggaattcggcttcg
y F	aagccacctgattacccgaacact
y R	tcaagcgaccaggcgatctcaaat
attB1 F	attacgccaagcttggtaccgactcactatagggcgaattgg
attB1 R	gtggatccgagctcggtaccatcaagcttatcgataccgtcg
attB2 F	agcatgcatctagagggcccgtggatccactagttctagagc
attB2 R	ctatagggcgaattgggcccgatgtagctcggtctcgaagc
loxP F	tgtggacagagaaggaggcaaaca
loxP R	agcgacactcccagttgttcttca
XhoI8-24F	ctggcggccgctcgacaggaaacagctatgaccatga
XhoI8-24R	tagatgcatgctcgactatagggcgaattgggccctc
1360{}1503F	cttgcggccgctcgagaaaggaatacggtattaccaagacac
1360{}1503R	tagatgcatgctcgacatcggttgatgatcaataaatttc
p1360R F	cttgcggccgctcgatgtaacaataacattaaaagtgtt
p1360R R	tagatgcatgctcgacatcggttgatgatcaataaatttc
p1360L F	cttgcggccgctcgagaaaggaatacggtattaccaagacac
p1360L R	tagatgcatgctcgatcatattaagtcaaatgatttaat
p1360-IR F	cttgcggccgctcgagccatacattggtttgcccaaag
p1360-IR R	tagatgcatgctcgagccatacattggtttggcactatg
TSSΔ1	tcaaatgatttaataaatatactaaataat
TSSΔ2	attagaattaaacataaatataaatgtgtaaac
TSSΔ3	ttttcgggccgaaatcaattctgatc
TSSΔ4	tagatttcttacgctctcagcggg
TSSΔ5	attactctctttccgctcactcc
TSSΔ6	ggttaaactaaagtatttttaaag
piRNA IΔF	aacaaacttaaaaagctttaa
piRNA IΔR	catttatatttatgtttaattct
piRNA IIΔF	ccatcgatggacacgcacacttatac
piRNA IIΔR	ccatcgatggcacgattttcgggcc
Invader4 F	gtatgtgtcgaagagtcatcaggatg
Invader4 R	ggtagaagaaagcccttaaaggtatg
XhoI Invader4 F	cttgcggccgctcgagatctgcgac
XhoI Invader4 R	tagatgcatgctcgacatctgggcat
Invader4ΔLTR F	cttgcggccgctcgacggccttcct
Invader4ΔLTR R	tagatgcatgctcgatcagaagtggg
Mapping	
5'P v.2	cttcggctatcgacgggaccacctta
3'w v.2	gacgaaatgaaccactcggaacc
1198 F	gcattgaatgagcattgtaatcgatactt
Cassette exchange	
1X1360 3'P inner F	attaacccttagcatgtccgtggg
AttB4/09 R	atcaagcttatcgataccgtcgacc
attB2F	gtggatccactagttctagagc
InFusion Primer Bind	tagcgaattgggaattcggcttcg
1X1360 3'P R	gtatcatcagtgggaagggcgaa

CHAPTER 3

A variegating reporter in the 42AB piRNA locus of *Drosophila melanogaster* is not sensitive to mutations in the piRNA system.

A variegating reporter in the 42AB piRNA locus of *Drosophila melanogaster* is not sensitive to mutations in the piRNA system.

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ABSTRACT

Position-effect variegation (PEV), the stochastic silencing of a gene in some of the cells in which it is normally expressed, can be observed when a typical euchromatic gene is placed adjacent to or within a heterochromatic environment. Variegating reporters have been widely used to study the *trans*-acting factors responsible for silencing, helping to uncover the assembly mechanisms necessary for a heterochromatin state. We have previously reported that the DNA transposon remnant *1360,* acting in *cis,* and RNAi components (specifically PIWI domain proteins), acting in *trans*, impact variegating reporters proximal to repeat-rich genomic regions near the base of chromosome 2L in *Drosophila melanogaster*. The requirement for PIWI domain proteins suggests that these components may be involved in establishing or maintaining *1360*-sensitive heterochromatin formation. Here we investigate whether this is general, restricted to specific domains, or a peculiarity of *1360*-induced PEV. We find that *1360*-sensitive variegating reporters, particularly at the base of 2L, exhibit suppression of variegation in *piwi* mutant backgrounds at a greater frequency than do variegating reporters which are not sensitive to an added *1360* element. We investigated in detail a TE-sensitive site in the piRNA generating locus 42AB, which is considered heterochromatic by multiple criteria. The reporter shows enhanced silencing when associated with either a copy of *1360* or a copy of *Invader4*, with enhanced HP1a accumulation at its promoter. However, mutations in *piwi,* along with many prototypical Su(var) mutations, result in only weak suppression of variegation at this site, while an *ago2* mutation enhances variegation. In addition, tests of various fragments of the TEs do not reveal the strong dependence on piRNA matching sequences which is observed at a euchromatic site driven to a heterochromatic form by an added TE. Our findings indicate that suppression of PEV by mutations in the genes for RNAi components occurs in a limited number of heterochromatic domains, predominantly those near gene cluseters – sites resembling the border between euchromatin and heterochromatin, generated in rearrangement-induced PEV. Thus chromosomal context is an important determinant of sensitivity to Su(var) mutations. This finding helps to explain the inconsistent PEV effects obtained using different reporters to test the impact of mutations in RNAi components.

INTRODUCTION

Position-effect variegation (PEV) is defined as the stochastic silencing of a gene in some of the cells in which it is normally expressed due to a repressive chromatin environment. This phenomenon results in a “mottled” expression pattern as observed for many prototypical examples of PEV (Girton and Johansen, 2008). The first documented instance of PEV in Drosophila was seen in w^{m4} (*white mottled four*), the product of a chromosomal inversion that positions a gene required for eye pigment transport adjacent to pericentric heterochromatin (Muller, 1930). Such rearrangements result in the splotched appearance of red across the eyes, interspersed with white or lighter shades of red (yellow, orange). Variegating lines are also recovered when a transposon carrying an appropriate reporter gene inserts into a heterochromatic domain (Wallrath and Elgin, 1995). Large-scale screens for modifiers of the PEV phenotype have revealed ~150 genes that suppress [Su(var)] and more that enhance [E(var)] variegation (Girton and Johansen, 2008). The study of variegation has led to the genetic and biochemical characterization of many core chromatin-modifying components responsible for expressed and silenced states of gene regulation. Many of the genes required to support PEV are involved in heterochromatin assembly, generating the relatively inaccessible, compact chromatin state associated with transcriptional silencing. Components required for heterochromatin assembly include histone methyltransferases (HMTs) such as SU(VAR)3-9 and dSETDB1, as well as the chromo domain protein Heterochromatin Protein 1 (HP1a) (Eissenberg and Reuter, 2009). Cytological studies have revealed that these components are present at high concentrations in the pericentric and telomeric domains, as well as the small fourth chromosome (the Muller F element) of *D. melanogaster.* Not surprisingly, almost all variegating reporters lie within or proximal to such sites, providing a means to study these chromosomal domains (Wallrath and Elgin, 1995).

The availability of reporter inserts in a wide variety of chromosomal domains provides access to the unique regulatory features associated with each site. Thus the *trans*-acting factors that influence heterochromatin assembly have been shown to differ among the heterochromatic

domains (pericentric heterochromatin, telomeres, other sites). A notable example from Drosophila is the 4th chromosome, where the most prominent HMT along the arm is dSETDB1; accordingly mutations in this HMT result in the strongest suppression of variegation for many 4th chromosome PEV reporters (Brower-Toland et al., 2009). However, the extent of differential targeting of heterochromatin components to the various heterochromatic domains in Drosophila is relatively unexplored. Given that heterochromatin formation is required for many different complex chromatin assemblies, with different structural and transcriptional roles, the means of finding the appropriate targets is likely equally complex.

In *S. pombe*, RNAi proteins are required for the appropriate assembly of heterochromatin at the centromeric repeats (Grewal, 2010). In Drosophila, the RNAi effector Argonaute family proteins PIWI and AGO2 have been linked to heterochromatin assembly (Fagegaltier et al., 2009; Klenov et al., 2011; Wang and Elgin, 2011). Both proteins have been found to associate with chromatin and to bind small RNAs with homology to repetitive elements that are packaged into heterochromatin. Three lines of evidence support a possible role for Piwi in heterochromatin assembly. First, Piwi has a predominantly nuclear localization (Brennecke et al., 2007). Second, the loss of Piwi leads to increased expression of many transposable elements, sequences with homology to piRNA products (24-30 nt small RNAs) generated by PIWI proteins (Brennecke et al., 2007; Gunawardane et al., 2007); in some cases increased expression has been correlated with a loss in HP1a over the TE promoters (Klenov et al., 2011; Wang and Elgin, 2011). Third, there is *in vitro* evidence of a physical associated between HP1a and Piwi (Brower-Toland et al., 2007; Mendez et al., 2011).

However, Piwi is reportedly dispensable for HP1a targeting to variegating reporters present in piRNA generating sites of the genome (Moshkovich and Lei, 2010; Phalke et al., 2009). Surprisingly, in *piwi* mutants, an increased accumulation of HP1a over reporters inserted at these sites is observed by ChIP-qPCR (Moshkovich and Lei, 2010; Yin and Lin, 2007), indicating that alternative mechanisms may support heterochromatin assembly at such sites. Previous studies from our lab show that a *1360* remnant cloned upstream of an *hsp70-w* reporter

is sufficient to support HP1a-dependent heterochromatin formation at sites near the base of chromosome 2L (Haynes et al., 2006; Sentmanat and Elgin, 2012). Variegation of these *1360*-associated reporters is strongly suppressed (>2-fold increase in pigment levels) in many lines when mutant for *Su(var)205* (the gene encoding HP1a) or *piwi* or *aub* (encoding PIWI domain proteins) (Haynes et al., 2006). Enrichment of HP1a and H3K9me2 was observed in the presence of *1360,* corroborating the notion that *1360*-sensitive PEV is the result of an HP1a targeting event. In a test at one such site, the deletion of sites within *1360* with homology to piRNAs resulted in a loss of silencing (Sentmanat and Elgin, 2012). These findings suggest that piRNA pathway components contribute to HP1a-dependent, *1360*-sensitive PEV.

While variegating reporters at many sites in the genome are sensitive to the presence of a copy of *1360* (referred to as *1360*-sensitive), others are not (*1360*-insensitive). To investigate whether suppression of variegation in *piwi* mutant backgrounds is a general feature of *1360*-sensitive sites, we surveyed 27 *1360*-sensitive and *1360*–insensitive variegating reporter lines recovered in an earlier screen (Sentmanat and Elgin, 2012). We find that many, but not all, *1360*-sensitive variegating reporters respond similarly to mutations in *piwi* with a loss of silencing. Here we investigate in detail a *1360*-sensitive reporter in the piRNA generating locus 42AB, which consists of a string of repetitious elements. Second site mutations in *piwi* as well as many prototypical *Su(var)* loci are only weak suppressors of variegation at this site, while *ago2* enhances PEV. Genetic dissection of the *1360*-element inserted at this site shows that while the right half of *1360* contributes most significantly to *1360*-sensitive PEV, deletion of sites with homology to piRNA sequence elements has no significant impact. Our findings indicate that while our reporter in the 42AB locus is sensitive to the presence of an additional copy of *1360*, elimination of either *cis* or *trans* components of a potential piRNA system has little impact. These results suggest that there are a limited number of heterochromatic reporter sites that support *1360*-sensitive silencing that is suppressed by RNAi components. Surprisingly, the piRNA locus tested is not among these sites. Clearly chromosomal context is an important determinant of the silencing mechanisms at work. This finding is significant in that it helps to reconcile inconsistent

PEV effects between different reporters in RNAi mutant lines, and provides a starting point for analyzing the mechanisms involved.

RESULTS

Variegating reporters sensitive to *piwi* mutations tend to reside at *1360*-sensitive sites

An *hsp70*-driven *white* reporter gene, cloned downstream of a *1360* remnant in a *P* element landing pad construct (see Figure 2a), was previously mobilized to determine the extent of *1360*-sensitive PEV (Sentmanat and Elgin, 2012). Twenty-seven variegating lines were recovered (~5% of total mobilizations); of these, eleven of the lines are *1360*-sensitive, determined by a statistically significant difference in pigment levels +/-*1360* (p<.05, **Table 1**), while the variegation phenotype of the remaining 16 lines are unaffected by removal of *1360* (Sentmanat and Elgin, 2012). To determine if PIWI domain proteins generally contribute to the variegating status of reporters in *1360*-sensitive sites, we determined the impact of *piwi* mutations on the level of PEV, looking for a dominant phenotype. The chromatin status of each insert was also determined using the chromatin profile associated with the site in BG3 cells or S2 cells when BG3 data was not available (**Table 1**) (Kharchenko et al., 2011). We hypothesized that if *1360*-sensitive PEV requires PIWI domain proteins to target silencing complexes to the TE, and so to the site of the reporter, then on depletion of Piwi, pigment levels should equal or exceed the levels observed in the absence of *1360* – a suppression of *1360*-sensitive PEV.

On average, variegated expression from *1360*-sensitive inserts is suppressed by *piwi* mutations more frequently than is the case for *1360*-independent reporters. This difference is significant and reproducible in many cases using the $piwi^1$ *or* $piwi^2$ alleles, although the effect is stonger in the $piwi^1$ background. A commonality between *1360*-sensitive reporters suppressed by *piwi* mutations is that, like the previously published *1360*-sensitive lines, they tend to cluster at the base of 2L. Indeed, all variegating inserts at the base of 2L tested by our lab have been suppressed by mutations in $piwi^1$, $piwi^2$, or both. This suggests that inserts in this genomic

domain are particularly sensitive to mutations in piRNA components, in contrast to others, indicating that genomic location is an important factor.

A survey of the chromatin environment surrounding reporter inserts (20 kb window) suppressed by *piwi* suggests that they tend to reside in annotated heterochromatin, although most are close to euchromatic masses (see **Table 1**, gene density). Many *1360*-insensitive inserts lie within or in close proximity to telomeric associated sequences (TAS), particularly at 2R and 3R. These domains are enriched for polycomb marks such as H3K27me3 (Kharchenko et al., 2011; Sentmanat and Elgin, 2012). These observations support the hypothesis that suppression of variegation by Piwi components, like *1360*-sensitive variegation, is an HP1a-dependent phenomenon.

Thus, suppression of variegation by *piwi* mutations is not observed for the majority of this set of variegating reporters, in agreement with previous reports (Moshkovich and Lei, 2010; Yin and Lin, 2007), but is limited to inserts that lie in domains resembling euchromatin-heterochromatin borders (particularly at a subset of genomic sites at the base of 2L). These genomic sites tend to be *1360*-sensitive, suggesting a chromatin environment that can be altered by addition of sequence components (TEs) that have been implicated as silencing targets for the RNAi system.

Several PEV reporters in our collection are present in piRNA generating loci, sites previously reported to be enriched in HP1a in the absence of PIWI (Moshkovich and Lei, 2010; Yin and Lin, 2007). It has been suggested that Piwi may act as a transcriptional activator for such sites. Indeed, transcription of repeats present in these genomic regions is necessary to produce piRNA molecules, present in the germ line and early embryo. However, our study found some variegating inserts in piRNA generating clusters that are suppressed by mutations in *piwi* (e.g. line 1310 and 153), suggesting that variegating reporters in piRNA generating sites are not uniformly enhanced by *piwi* mutations. This suggests domain-specific effects, but the presence of the *1360* remnant may be a critical difference between the reporters used in these experiments.

1360-sensitive PEV at the 42AB piRNA cluster

To explore the nature of silencing at a site reportedly refractory to PIWI-domain mutants we chose to study *1360*-sensitive silencing of line 1936, which has a reporter inserted in the primary piRNA cluster 42AB (**Fig. 1**). This region has been previously shown to be enriched in HP1a in *piwi* mutants (Moshkovich and Lei, 2010). In line 1936 the presence of *1360* has a marked effect on silencing of our adjacent reporter (>3-fold increase in pigment levels in the absence of *1360*); this silencing is suppressed by mutations in *piwi,* and thus linked to RNAi-dependent silencing (**Table 1**). Consequently, this reporter insert can be used to investigate how *1360*-sensitive PEV operates in a region reported to be generally unaffected by RNAi components.

Detailed mapping of the site revealed that the reporter is inserted in the non-LTR retroelement *Juan*, surrounded by a dense array of repeats near the base of 2R (**Fig. 1A**). The insertion site is annotated as enriched for heterochromatin marks HP1a and H3K9me2 in 3rd instar larvae, while depleted for activating H3K4me2 (Riddle et al., 2011). The presence of the *1360* element enhances variegation by >3-fold (**Fig. 1B**), suggesting HP1a enrichment is occurring over the reporter in the presence of *1360* (Sentmanat and Elgin, 2012). This was verified at the chromatin level using chromatin immunoprecipitation (ChIP)-qPCR of *hsp70-w* in +/-*1360* 3rd instar larvae. Indeed, the presence of *1360* increased the accumulation of HP1a at *hsp70-w* by ~3-fold (**Fig. 2A**).

PEV is typically affected by the dose of heterochromatin components, displaying less variegation when the availability of silencing effectors is reduced. We tested the impact of mutations in a subset of heterochromatin assembly compnents – the H3K9 histone methyl transferases (HMTs) SETDB1, Su(var)3-9 and G9a, in addition to the chromo-domain heterochromatin protein 1 (HP1a). Dominant effects (while small) were most strongly observed for mutant alleles of HP1a and SETDB1 (encoded by the *egg* gene) (**Fig. 2B**). An enhancement of variegation was observed in the *Su(var)3-9*^{06}/+ mutant background, visible in eye pictures and reproducible without the *1360* present – suggesting Su(var)3-9 is not required for heterochromatin assembly at the

reporter. Notably, however, the Su(var) effects were not particularly strong (<2-fold pigment level changes) for any of the mutant alleles sampled, which may be indicative of redundant targeting mechanisms operating at this site, given the complex array of repeats surrounding the reporter insert.

A quintessential characteristic of an HP1a targeting event is the spreading of silencing in *cis*. The physical interactions between HP1a and methylated histone H3K9 facilitate this process, which is compromised when the HP1a domains required for such interactions are mutated (Hines et al., 2009). To determine if a spreading effect could be detected along the P element insert we assayed HP1a enrichment +/- *1360* at either end of the reporter insert (**Fig. 2A**, see red bars). Indeed, spreading was detected up to 8 kb downstream (**Fig. 2A**, see 5'P bars) of the *1360* element. The combined results suggest *1360*-sensitive silencing is an HP1a-dependent event at this site, corroborated by HP1a enrichment over the reporter with concomitant spreading over the length of the P element insert.

Enhancement of*1360*-sensitive PEV at 42AB by mutations in *ago2*

The low impact of mutations in *piwi* led us to explore the impact of mutations in another PIWI domain protein, AGO2. Interestingly, $ago2^{414}$ mutants displayed an dominant enhancement of variegation, but only in the case without the *1360* element (**Fig 3A**). A similar result was previously found using a reporter carrying *mini-white* (and no additional repetitious elements) inserted into this 42AB region (Moshkovich and Lei, 2010). Eye pictures suggest a slight enhancement in $ago2^{414}$ when *1360* is present (**Fig 3B**), but this is not sufficient for quantitative detection in pigment assays. Mutations in *piwi* had weak suppression effects in the presence of *1360,* but no impact in its absence (**Fig 3A, B**), indicating that upon the insertion of a new target element (+*1360*) Piwi may facilitate silencing at this otherwise refractory site. Thus most reporters exhibiting *1360*-dependent PEV appear to be dominantly suppressed by mutations in *piwi*– even when in locations reportedly refractory to such effects, namely 42AB. This sensitivity is particularly evident in regions where euchromatin and heterochromatin are closely interspersed, for example near the base of 2L (**Table 1**). This genomic region seems to be

particularly amendable, suggesting genomic context is an important determinant of suppression by RNAi components.

Dissection of the *1360* element at 42AB

To determine the sequence elements required for *1360*-sensitivity at a site reportedly refractory to piRNA components we swapped the full-length element with partial *1360* fragments (*1360*∆, **Fig. 4A;** Sentmanat & Elgin, 2012) using phiC31-mediated recombination. The deletion of sequences of the *1360* remnant required to sustain *1360*-sensitive PEV should lead to suppression of variegation. Removal of the left half (*1360*∆L) or of the terminal inverted repeats (*1360*∆IR) did not compromise *1360*-sensitive silencing (**Fig. 4B**). The deletion of the right half of *1360* (*1360*∆R) has a weak suppression effect, suggesting that sequence components present within the right half of the element promote *1360*-sensitive silencing. These results (while less dramatic) agree with data previously gathered for a *1360*-sensitive reporter inserted into 2L (Sentmanat and Elgin, 2012). Sites with homology to piRNA sequences present in *1360*∆L were deleted to determine if these sites contribute to the *1360*-sensitive effect at this site. No significant suppression of variegation is observed. These results indicate that additional sequence elements present in *1360*∆piRNA are sufficient to sustain *1360*-sensitive PEV. Interestingly, of all the constructs tested, none achieved pigment levels originally observed for the -*1360* landing pad line (**Fig. 1B**). The combined results support a model where a combinatorial set of sequence elements within *1360* facilitate the *1360*-sensitive effect at this site, again suggesting that more than one mechanism may be at work.

***Invader4* induces PEV at the *1360*-sensitive reporter site in piRNA cluster 42AB**

We previously reported that replacing *1360* with the LTR retroelement *Invader4* at a *1360*-sensitive insertion site in euchromatin, recapitulated the full-length *1360* effect. In the euchromatic site, deletion of sequences with homology to piRNA sequence elements (LTRs in this case, see **Fig. 5A** for map), resulted in a loss of *Invader4*-dependent silencing. To determine if a reporter at a different site, in a different chromatin environment, would respond similarly, we swapped *1360*

for *Invader4* in line 1936. We find that the *Invader4* element displays a similar impact on silencing as observed with *1360*. A suppression of PEV is observed on loss of *Invader4*, again suggesting that a sequence determinant present in the element contributes to the effect. Deletion of the left- or right-half of Invader4 did not compromise the effects seen with the full-length element (**Fig. 5B**). Loss of the LTR sequence elements, which have homology to piRNA sequence reads, resulted in a weak suppression of PEV, but did not achieve levels comparable to deletion of the full-length element. Thus, at this site both *1360* and *Invader4* appear to be robust targets for silencing, but that cannot be attributed to their piRNA sites. Although the silencing effects were compromised upon deletion of the right half of *1360* or the LTRs of *Invader4*, neither deletion was sufficient to eliminate silencing entirely (pigment levels without the element). Note that in both cases, variegation of the reporter is still evident in the absence of the TE (*1360* or *Invader4*), no doubt a reflection of the heterochromatic nature of this site (piRNA cluster 42AB) in larvae.

DISCUSSION

We have recovered a series of *D melanogaster* lines carrying a *1360+, hsp70-white* reporter construct that exhibits a variegating phenotype when inserted in a subset of chromosomal domains, including some euchromatic domains that shift toward heterochromatin (Sentmanat and Elgin, 2012), and some otherwise heterochromatic domains. Our results suggest that *1360*-sensitive sites are more often than not impacted by dominant mutations in *piwi*. However, *piwi*-mutant sensitivity is not a defining feature of all sites Impacted by an additional copy of a TE. We find that all *1360*-sensitive sites present at the base of 2L tested to date are sensitive to *piwi* mutations, suggesting that this chromatin domain may be under the regulatory control of this pathway. Interestingly, we find that a *1360*-sensitive insert at the primary piRNA generating locus 42AB responds rather differently: although weakly suppressed by *piwi* in the presence of *1360*, the insert shows striking enhancement of variegation on *ago2* mutation when *1360* is absent. However, no enhancement was detected in the presence of *1360*. These results argue that multiple silencing mechanisms are in operation at this site. Together, these results support the

notion that chromosomal context is an important determinant of how dominant mutations in RNAi components can impact variegation.

Work from *N. crassa* and *S. pombe* suggest multiple repressive complexes target heterochromatin to distinct repeat regions. In *N. crassa*, different HP1-associated complexes regulate accessibility at centromeric and non-centromeric repeats (Honda et al., 2012). Similar observations have been made in *S. pombe*, where the RNA induced transcriptional silencing complex (RITS) containing the PIWI domain argonuate protein Ago1 and the chromo domain protein Chp1, targets heterochromatin to *dg/dh* centromeric repeats and to the mating type locus but not to other repeat regions. Similarly, *w* reporter inserts in distinct chromosomal domains reported respond differently to dominant modifiers of PEV. Five unique silencing mechanisms were identified, which were dependent on the local repetitious sequence content (Phalke et al., 2009). The possibility that distinct repeats are targeted for silencing by different complexes has received some support. In particular, knockdown of *piwi* in the ovaries results in marked depression for only a subset of transposons (e.g. *HeT-A*, *Bari)* with concomitant decrease in HP1a over their promoters (Klenov et al., 2011; Wang and Elgin, 2011). Why some repeats are targeted for repression by this Piwi-based mechanism and not others has remained elusive.

The collection of variegating reporters used here is limited, and some genomic regions were overrepresented. For example, most *1360*-independent variegating reporters recovered lie in the telomeric associated repeats (TAS) in 2R and 3R (Sentmanat, M and Elgin, SCR 2012). This bias may be a result of *P* element targeting, possibly due to the presence of *1360* as has been previously observed (Husinga et al., 2012). This targeting bias has likely limited our ability to survey other repetitious environments that may also be refractory to *1360*. Thus, there may be additional *1360*-independent variegating environments not represented in this study.Most *1360*-insensitive reporters were not suppressed by mutations in RNAi components, corroborating previous reports that these components do not impact all silent domains using the present reporter assays.

While many *1360*-sensitive reporter sites exhibit some suppression of variegation in the presence of *piwi* mutations, there is a range of responses. The reporter studied here, inserted into a normally heterochromatic piRNA locus, shows relatively little response, while a euchromatic site driven to heterochromatin formation by the *1360* shows a strong response; further, in the latter case, much of the *1360 s*ensitivity can be associated with piRNA elements within the TE. The results suggest suggests a distinct subset of genomic regions shifts chromatin state on addition of an additional TE, and that of these, only a subset appear to be silenced through a piRNA-directed mechanism.

METHODS

Fly stocks and husbandry: Crosses used for this analysis were performed and maintained in bottles at 25 C, 70% humidity on sucrose-corn meal media (Schaffer, 1994). Fly stocks were obtained from the Bloomington *Drosophila* Stock Center unless otherwise indicated. The reporter construct and methods for replacing the *1360* element with a partial *1360* element or the *Invader4* element are reported in detail in Sentmanat and Elgin (2012). The y^1w^{67c23} strain was used to outcross reporter lines for pigment assays (*+/-1360* assays; WT controls for mutant analysis). Lines used for PEV assays are *yw; Su(var)205*^{02}*/CyO*, *yw*; Su*(var)205*^{05}*/CyO*, w^{118}; *aub*^{QC42}/*CyO*, w^{118}; *aub*$^{\Delta P\text{-}3a}$/*CyO*; w^{118}; *piwi*^{1}/*CyO*, w^{118}; *piwi*^{2}/*CyO.*

Pigment assay: Quantitative eye pigment analysis was performed on 3-5 day-old adults. All mutant lines analyzed were heterozygous for the reporter and for the mutant allele. Flies were homogenized in 250 µL of 0.01 M HCl in ethanol, incubated for 10 min at 50°C and the extract clarified by centrifugation. A final volume of 150 µL was used to measure optical density at 480 nm (adapted from (Khesin and Leibovitch, 1978)).

Chromatin immuonprecipitation (ChIP): Chromatin isolation and immunoprecipitation from 3rd instar larvae were carried out as previously described (Riddle et al., 2011). The antibodies used were HP1a W191, Abcam2012 H3K9me2, and Millipore 07-030 H3K4me2. Antibodies were validated by us and by others (Egelhofer et al., 2011). Quantitative PCR was

performed using Bio-Rad 2x iQ SYBR Green Supermix in a Cepheid SmartCycler. Primers used to assay *alpha actinin*, *18s* and *hsp70-w* can be found in Sentmanat and Elgin, 2012. Two biological replicates, each consisting of two technical replicates, were assayed for each immunoprecipitation assay.

FIGURE LEGENDS

Table 1. Reporter lines used for this study. Shown are the fold change +/- *1360* (table split between *1360*-sensitive lines, top; and *1360*-independent, bottom); the fold change in response to $piwi^1$ and $piwi^2$ mutations, assessed in the presence of *1360*; the chromatin state (Bg3 cells); and whether the reporter lies in a piRNA cluster. Chromatin states reported (Kharchenko et al., 2011) are 8 - heterochromatin-like, 7 - heterochromatin, 6 - polycomb, 4-1-chromatin associated with transcriptionally active regions. Insertion sites are annotated as unique piRNA generating loci based on the report by Brennecke et al (Brennecke et al., 2007). Bold values indicate significant changes in pigment values (*p*=<.05, Student's t-test). NA-Not available, * S2 cell chromatin data.

Figure 1 *1360*-sensitive PEV is observed in piRNA cluster 42AB. A. Map showing the landing pad insertion in piRNA cluster 42AB. The region is enriched for HP1a and H3K9me2 in 3rd instar larvae (modENCODE). Below is a schematic representation of the landing pad construct. Black bars indicate fragments amplified for ChIP-qPCR. B. Quantitative eye pigment assay +/-*1360* and accompanying eye pictures.

Figure 2 HP1a-dependent silencing over the *1360*-sensitive insert in piRNA cluster 42AB A. HP1a enrichment along the length of the P element insert. Samples were normalized to input and fold-enrichment over *alpha actinin* is reported, +/- SEM for two biological replicates. The region is enriched for HP1a in the presence of *1360*. B. Impact of mutations in prototypical suppressors of variegation. Pigment assays for the reporter with *1360* present are reported, +/- SEM.

Figure 3 Mutations in the genes encoding RNAi components have little impact on silencing of the reporter in this chromatin domain. A. Pigment assays of the *1936* reporter insert (+/- *1360*) in

RNAi mutant backgrounds. B. Corresponding eye pictures. The *ago2* mutation results in enhanced silencing at this locus, most apparent in the absence of *1360.*

Figure 4 Partial fragments of *1360* are sufficient to generate the added silencing. A. *1360* fragments swapped for the full length *1360* remnant. The map of sense (blue) and antisense (red) piRNA reads from wild-type *Drosophila* ovaries that guided design of *1360*DpiRNA is shown. B. Pigment assays for each *1360* test fragment. The asterisk indicates a statistically significant difference between full-length *1360* and a test construct (*p*-value <.05).

Figure 5 *Invader4* constructs swapped for the full-length *1360* remnant. A. A map of the sense (blue) and antisense (red) piRNA reads from wild-type *Drosophila* ovaries is shown. B. Pigment assayed for each *Invader4* construct sampled. A single asterisk indicates a statistically significant difference at a *p*-value <.05, and a double asterisk at *p*-vaule of <.005.

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Chapter 3, Table 1

1360-sensitive lines

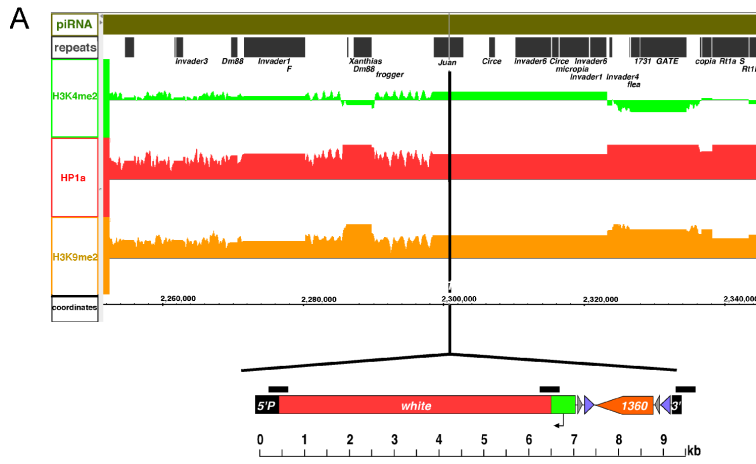
Line	Cytological	Coordinate	+/-*1360*[1]	*Piwi*[1]	*Piwi*[2]	State	piRNA cluster
1936	42A16	2R:2301664	**3.83**	**1.67**	**1.5**	7	piRNACluster_1
2201	39F1	2L:21708198	**2.67**	**5.11**	**1.89**	7	
1310	39E2	2L:21572961	**2.4**	**2.66**	**2.24**	7	piRNACluster_141
312	38C2	2L:20148548	**2.23**	**5.31**	**6.09**	8	piRNACluster_5
217	39E1	2L:21540808	**2.0**	**2.88**	**3.38**	7	
2100	39D3	2L:21419980	**1.84**	**1.60**	1.08	7	
2410	100A4	3R:26591628	**1.64**	**1.34**	1.19	3	
745	102B5	4:380116	**1.5**	1.17	1.17	7	
10	64D14	3L:3579448	**1.43**	1.2	1.37	4	

1360-independent lines

Line	Cytological	Coordinate	+/-*1360*[1]	*Piwi*[1]	*Piwi*[2]	State	piRNA cluster
971	42A16	3R:27899499	1.44	**1.67**	1.11	6	
1904	39F1	2R:2287667	1.27	**1.63**	1.45	7	piRNACluster_1
1232	44A4	2R:3948956	1.25	**1.83**	1.5	3	
178	39E2	3R:27899585	1.22	1.0	1.0	6	
153	60F5	2R:21146267	1.18	1.55	**2.05**	6	piRNACluster_97
407	38C2	3R:19505875	1.13	**1.48**	NA	1	
813	39E1	2L:1668526	1.1	**1.8**	**1.8**	6	
325	39D3	U:9095030	1.1	**1.75**	0.85	NA	
5	100A4	3R:27899517	1.08	1.05	1.29	6	piRNACluster_11
929	102B5	3L:1101501	1.03	**1.21**	1.0	6	
2228	64D14	3R:27899409	1.03	0.81	0.76	6	piRNACluster_11
1062	44A4	2R:16490478	1.02	1.2	1.23	3	
1009	60F5	2R:3344853	1.0	1.05	1.14	1	
837	67E6	3L:10667411	1.0	1.24	0.94	3	
1140	100E3	3R:27899311	1.0	1.0	0.68	6	piRNACluster_11
2294	100E3	3R:27899570	0.96	.97	0.79	6	
1563	54B1	2R:13152997	0.94	1.01	1.25	3	
1792	58D2	2R:18130495	0.86	1.07	NA	6	

Chapter 3, Figure 1

A



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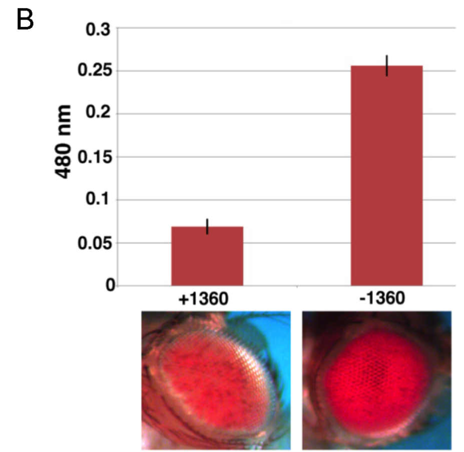
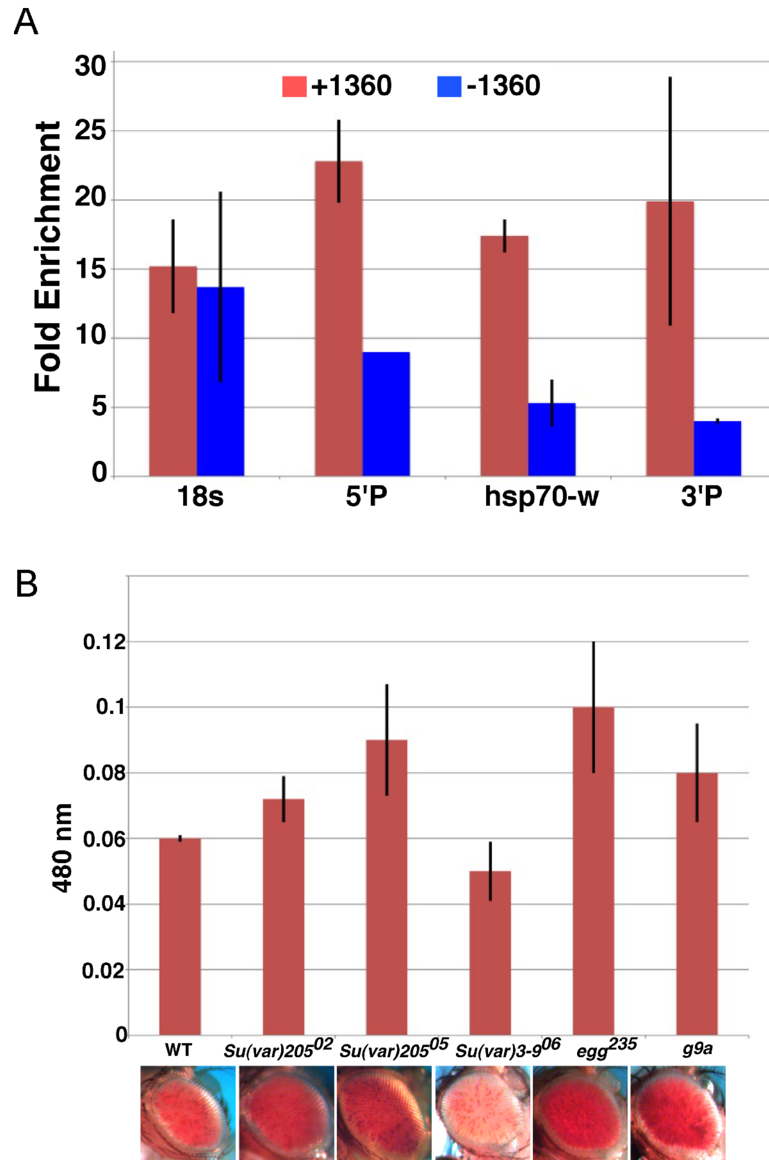
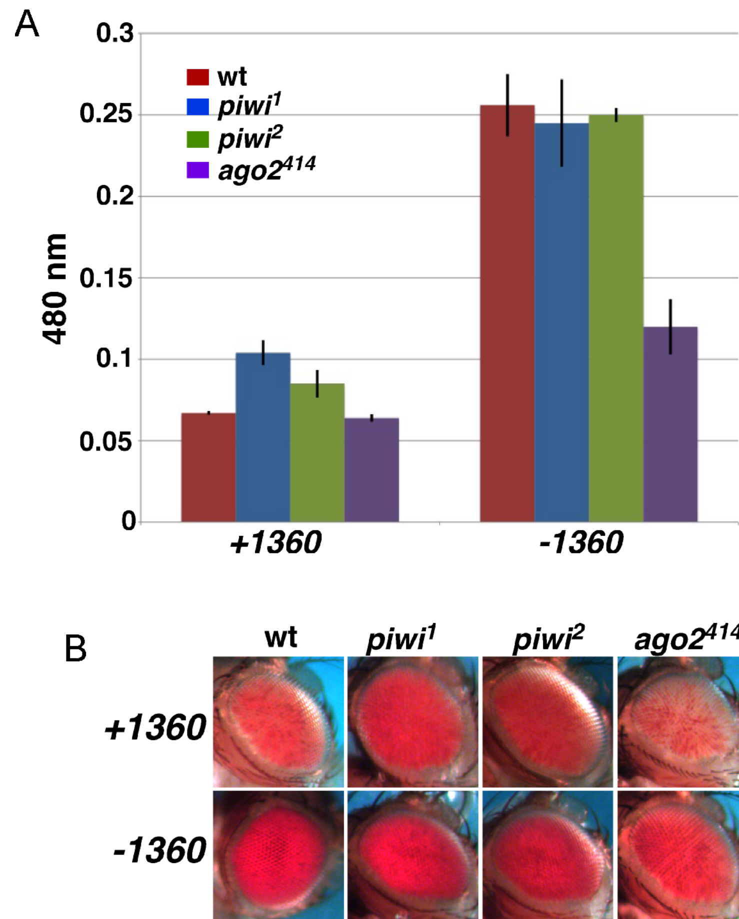


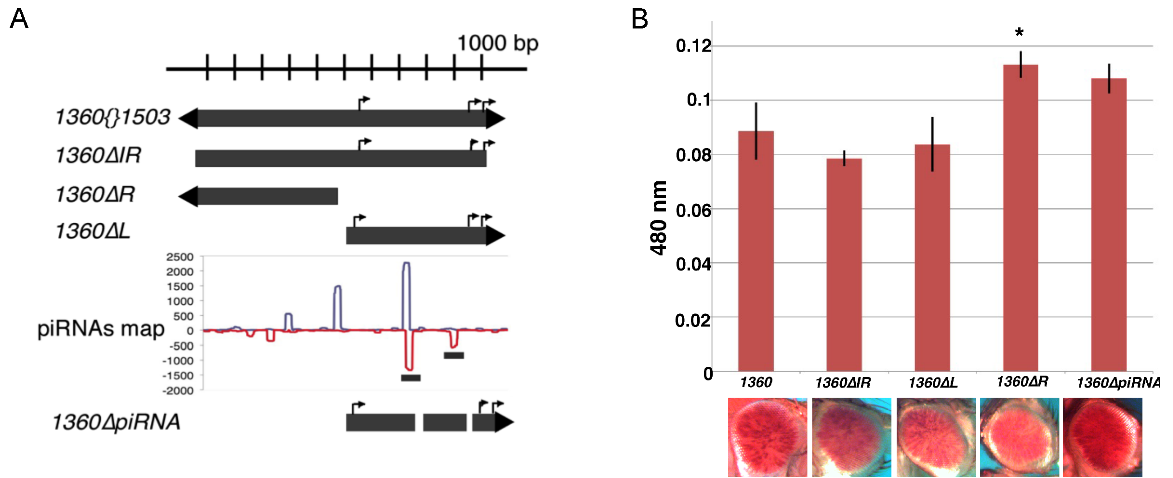
Chapter 3, Figure 2



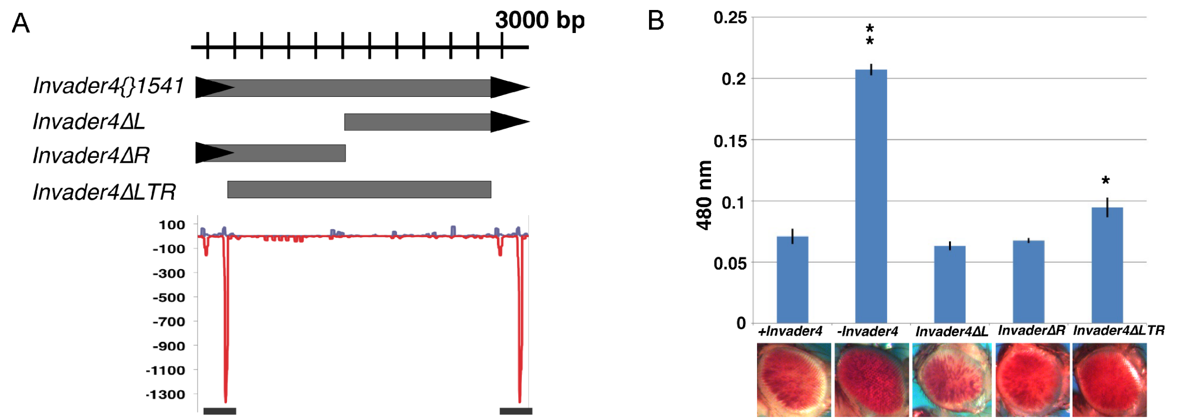
Chapter 3, Figure 3



Chapter 3, Figure 4



Chapter 3, Figure 5



CHAPTER 4

CONCLUSION AND FUTURE DIRECTIONS

CONCLUSION AND FUTURE DIRECTIONS

A model for RNAi-based heterochromatin assembly. The vast majority of transposable element (TE) remnants reside in heterochromatic environments, which suggests TEs may be an underlying sequence determinant for heterochromatin assembly. An exploration of this possibility, implementing reverse genetic approaches to determine the cis-acting sequence requirements to target a TE for silencing has provided important groundwork in support of RNAi-based transcriptional silencing in a metazoan system. The use of site-specific recombination by phiC31-integrase facilitated the sampling of multiple constructs while maintaining a constant genomic context, providing proof of concept that such a system could be applied to study the sequence determinants of silencing. The elimination of sites with homology to PIWI-interacting RNAs (piRNAs) from either a DNA transposable element or a retrotransposon compromised TE-sensitive silencing. Further, read-through transcripts detected in 0-10 embryo samples of the *P* element insert at the base of 2L (2L:20094149, cytological position 38B6) provide a plausible target for complementary, small RNA recognition and subsequent silencing by HP1a (chapter two of this thesis).

Heterochromatin first becomes cytologically visible in syncytial blastoderm embryos (130 min after fertilization) with the appearance of the chromocenter and dispersed heterochromatic regions (Mahowald and Hardy, 1985). The presence of heterochromatin on DNA leads to reduced accessibility to transcriptional activators. The consequences of this state become apparent during gastrulation (190 min after ferilization) as noted in studies analyzing the onset of position-effect variegation (Lu et al., 1998). Once established, it is faithfully propagated and becomes increasingly pronounced (as observed by PEV analysis) in undifferentiated imaginal precursor cells of *D. melanogaster* (Lu et al., 1998). Differentiation of precursor cells during late 3rd instar triggers a release of silencing, which leads to the variegating pattern observed in terminally differentiated adult tissues (Lu et al., 1996; Lu et al., 1998). This is supported by evidence that ecdysone, a hormone required for differentiation, is sufficient to trigger PEV of cultured primordial cells *in vitro* (Lu et al., 1998). Maternally loaded Piwi and associated piRNAs are present during

the first 240 min after fertilization – within the critical period of heterochromatin establishment during gastrulation.

Our model suggests that the read-through transcripts of the *P* element produced from the *nesd* gene promoter occur during this stage and are targeted due to the presence of sites with homology to piRNA reads present within *1360*. We believe silencing must be established early over the *hsp70-w* reporter in most, if not all, eye primordial cells. As differentiation begins, reporter silencing is lost in some cells during progressive mitotic divisions. The speckled eye phenotype is the result of the relative loss of silencing in some cells and not others during differentiation. The eye phenotype associated with *1360*-sensitive silencing at the base of 2L is clearly not an all or nothing event for each cell. Variegation is relatively weak and presents as interspersed dark and light shades of red. If a release of silencing is coincident with activation during differentiation the weak PEV observed suggests a relatively strong activation signal assists in countering the initial silencing event. Indeed, *nesd* is reported to not only be highly expressed in early embryos (0-10 hrs) but also begins mounting transcriptional activity during late 3rd instar larval development, peaking at the prepupae stage (Graveley et al., 2011). Thus, the reactivation of *nesd* during late 3rd instar development may reduce the local HP1a density and facilitate *hsp70-w* accessability – explaining the relatively weak PEV observed at this site.

Validating predictions associated with RNAi-mediated heterochromatin assembly. The conventional RNAi-mediated transcriptional targeting mechanisms, found in yeast and plants, require target transcript production. If a similar mechanism exists at the tested TE-sensitive site (described above), the read-through *P* element transcripts should be required for Piwi-mediated silencing. To test this possibility, one would need to interrupt read-through transcription. It is likely that the transcripts emerge from the promoter of the *nesd* gene, as the *P* element insertion lies within the *nesd* gene body. Peak transcript production necessary for silencing occurs during early embryogenesis (2-10 hours). To interrupt read-through transcription, a transcription termination signal could be integrated into the *P* element using site-specific recombination mediated by phiC31-integrase. For example, the transcription terminator signal from SV40 is routinely used in

P element constructs to signal transcription termination. If read-through transcription is required for *1360*-sensitive silencing at this site, this strategy should suppress PEV of the *hsp70-w* reporter.

The impact of deleting short sequence elements with homology to piRNAs was sufficient to compromise silencing, which suggests these elements are required. It would be interesting to determine if they are sufficient using site-specific recombination at *1360*-sensitive inserts. If so, this could be a means to ectopically target Piwi, as well as associated co-factors. Members of Haifan Lin's laboratory (Yale University) have presented data (Huang and Lin, Poster Abstracts – Chromatin and Epigenetics, Drosophila Research Conference, Chicago, IL; The Genetics Society of America Conferences, 2012; Abstract 370A.) that suggests a tandem array of piRNA sequence elements with homology to piRNA reads are sufficient to silence a reporter, through a Piwi-dependent mechanism. The *in vitro* physical association between HP1a and Piwi at the PxVxL interface suggests that Piwi could directly recruit HP1a to chromatin (Brower-Toland et al., 2007; Mendez et al., 2011).

However, whether this occurs *in vivo* remains to be demonstrated. A mutant form of Piwi with an alanine substitution at the valine in the proposed PxVxL interaction interface does not bind HP1a *in vitro,* but is still able to rescue *piwi* null germ cells, repressing the typical, TE over-expression phenotype (Wang and Elgin, 2011). Thus it appears that the PxVxL interface is not required for an *in vivo* Piwi - HP1a interaction, or that the interaction does not exist *in vivo* and the reported effects thus far have been the result of indirect consequences. (For example, TE mobilization will lead to increased DNA damage, which can impact HP1a distribution, and thus, TE silencing.) To delineate among the various possibilities, directly tethering of LacI-Piwi to LacO repeats may provide the means to gather substantive evidence (**Figure 1**).

Tethering experiments have previously been performed to explore the impact of localized HP1a. Using a LacI-HP1a fusion protein binding to LacO repeats cloned upstream of an *hsp26-tag, hsp70-w* reporter at euchromatic locations, (Danzer and Wallrath, 2004) found that tethered HP1a caused a more compact chromatin state to emerge, and that this state is sensitive to

Su(var)3-9 mutations. This finding supports the model that an interaction between HP1a and SU(VAR)3-9 is needed for heterochromatin spreading (Bannister et al., 2001; Lachner et al., 2001). Thus, using these same reporters, proven to form a more compact chromatin state in the presence of HP1a, one could begin to address how Piwi might impact chromatin structure. One would predict that the N-terminal PxVxL domain of PIWI is the most likely domain for HP1a-interaction. Thus, if recruitment of HP1a is observed following LacI-Piwi binding, testing for the requirement of this domain would help dissect the role of PIWI (e.g. transcriptional or post-transcriptional silencing). These efforts have begun, and lines containing LacI-Piwi and LacI-PiwiΔN have been generated. The impact of the expression and binding of these fusion proteins on silencing of a reporter adjacent to a lacO binding site will be tested using reporter lines with a lacO cluster (cytological locations 45D, 54F1 and 87C1) close to a heterochromatic mass. These experiments will be completed by collaboration with my colleague Tingting Gu.

Mobilization of the landing pad construct containing*1360* allowed us to determine that *1360*-sensitive PEV is largely restricted to heterochromatic domains but can be induced in annotated euchromatin that is close to repetitive DNA clusters. Many *1360*-sensitive reporters dominantly suppressed by *piwi* mutant background are present in heterochromatic domains, but almost all were close to interspersed euchromatic domains. There were some inserts in heterochromatin refractory to *piwi*, particularly at the primary piRNA generating cluster 42AB. This observation suggests that while not all heterochromatic domains are equally susceptible to the effects of *piwi* mutations, those that are are most likely to be inappropriately expressed as a consequence of a nearby promoter.

An important extension of this work is to determine whether in the absence of a reporter, *piwi* susceptible *1360*-sensitive domains are indeed under the regulatory control of the piRNA pathway for heterochromatin targeting. It would be interesting to determine if endogenous repeats at these sites (e.g. base of chromosome 2L) experience HP1a-directed targeting by Piwi as reporter inserts appear to. This can be carried out with the use of ChIP-Seq technology to determine if, in the absence of a reporter, such domains are indeed depleted of HP1a in *piwi* mutants. Given the confounding issues that arise due to maternal loading of Piwi, the best

approach would be to examine embryos or larvae depleted of the maternal Piwi load – accomplished using the appropriate Gal4 drivers (NGT40;NGT(A), GAL4 expressed in the female germ line for loading into the oocyte) coupled with a UAS-Piwi hairpin. Depletion of Piwi in the early embryo has been successfully accomplished using this technique (personal communication, Tingting Gu, Washington University in St Louis).

Transposons as sequence determinants of heterochromatin. There is an extraordinary positive correlation between heterochromatin and transposon sequence content (Riddle et al., 2011; Yasuhara and Wakimoto, 2008). There are two predominant explanations for this observation: (1) heterochromatic regions are gene-poor and undergo little to no meiotic recombination, thus, these sites are less deleterious, or (2) transposons are sequence determinants of heterochromatin assembly. These explanations are not mutually exclusive. There are mounting examples of transposable elements that are co-opted by their host to serve as sequence-specific modules to regulate gene expression (Feschotte, 2008). Thus, it is plausible that while there is some initial selective advantage upon a new TE invasion to insert within a heterochromatic environment, over time these sequences may be co-opted for a regulatory role. This predicts that at least some TEs would have become fixed within the population due to their functional property in the host genome. This notion is supported by evidence that the transposon sequence composition of the TE-rich *flamenco* locus on the X chromosome are well conserved between closely related species of *D. melanogaster* (e.g. *D. yakuba* and *D. erecta*) and are necessary in *D. melanogaster* to repress active TE copies (Malone et al., 2009). The regulatory role of these TEs may require a heterochromatic environment to properly function as has been observed for heterochromatic genes such as *light* and *rolled* (Lu et al., 2000). In the absence of the histone methyltransferase *egg* the regulatory role of a similar TE-rich cluster on the second chromosome is lost (Rangan et al., 2011).

The silencing signals (in the form of small RNAs) produced from these heterochromatic TE-rich clusters are most notably required for post-transcriptional degradation of active TEs (Brennecke et al., 2007). However, as discussed in the chapter 1 of this thesis, a transcriptional role is possible. Experiments to investigate this possibility are outlined in this chapter. If a

transcriptional role is indeed in effect, this would strongly support that sequence-specific targeting occurs at sites with sequences (likely TEs) that represent those targeted by the transcriptional silencing component of the pathway.

The contribution of transposable elements to genetic variability. A delicate balance exists in the genome between the efforts to maintain genome integrity and the ability to respond to new environmental pressures. As is often advisable in unfavorable circumstances, adaptability is one's best recourse. My work has shown that TEs can impart an epigenetic switch at sites in their immediate proximity, enhancing the variability of expression (on/off state from cell to cell) for a reporter at some genomic sites. Such phenotypic variability could have a selective advantage in a changing environment. However, whether the sources of epigenetic modulation that result in changes in gene expression are mostly the consequences of environmental perturbations is an area of active investigation in the field. Feinberg and Irizarry argue that the source of phenotypic variability is not necessarily Lamarckian (or the environment influencing heritable phenotypes), as has been previously proposed, but genetic (Feinberg and Irizarry, 2010). Many tissue-specific differentially methylated regions that differ between species (e.g. mouse and human) are associated with developmentally important genes and, in some cases there is a gain or loss of proximal CpG islands targeted for methylation (Feinberg and Irizarry, 2010). This observation links underlying genetic determinants to epigenetic regulation of potentially species-specific developmental programs. Could TEs be a source of similar phenotypic variation, helping to reshape developmental networks?

Transposable elements are sources of empirically determined species-specific *cis*-regulatory features (Feschotte, 2008). A quarter of the human promoters have been documented to contain TE-derived sequences (Jordan et al., 2003). Notably, >30% of binding sites for the tumor suppressor protein p53 in humans, determined by chromatin immunoprecipitation (ChIP), are enriched for long terminal repeats (LTRs) of the LTR class I endogenous retrovirus (ERV) (Wang et al., 2007). The ERV families found at these sites are primate-specific, suggesting a species-specific *cis*-regulatory network. Such events require mobilization(s) that distribute TEs into sites that facilitate a gene regulatory role.

The extent to which TEs may influence the epigenetic landscape is unknown, but some intriguing examples have emerged. A classic example of heritable epigenetic changes resulting from a TE comes from the agouti locus in the mouse, where variable DNA methylation of a retrotransposon leads to changes in expressivity between individuals. The retrotransposon insertion transforms the agouti locus to a metastable epiallele, a locus variably expressed between genetically identical individuals. Environmental factors, particularly potent during early embryogenesis, can influence the DNA methylation levels at epialleles leading to altered adult phenotypes. Gestational exposure to bisphenol A (BPA), used in plastics, lowers methylation levels at the agouti epiallele (among other loci), while dietary supplementation with methyl donors such as folic acid can counteract the effect (Dolinoy et al., 2007). Thus, genetic determinants within TEs can introduce a target for effectors of epigenetic regulation at some genomic sites, while environmental factors may influence the activity of those effectors. Collectively, the data presented here suggest that piRNA sequence elements present in TEs are targets for silencing at some sites of the genome.

Figure 1. Tethering LacI-Piwi to a reporter locus. Two experimental constructs containing wild type Piwi and Piwi∆N (first 35 amino acids deleted, red) have been cloned separately downstream of a LacI tag. The control, LacI-GFP, will also be targeted to the reporter locus to determine if a change in the nucleosome density around the reporter is observed between experimental constructs and this control. All constructs have an upstream activation sequence (UAS) at which Gal4 protein (driver) binds to activate transcription. The Gal4 driver here will be under the control of the *nos* promoter, which is expressed in germ line cells (top right corner) of the embryo and adult where Piwi is known to be active. The LacI tag specifically localizes to LacO repeats (256 array), here cloned upstream of a reporter. Proteins that associate with Piwi should also localize to the LacO repeats with LacI-Piwi, if those associations depend on the N-terminal PxVxV sequence (as postulated for HP1a), those associations should be lost in LacI-Piwi∆N lines.

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Chapter 4, Figure 1