CHAPTER ONE

Cellular Mechanism for Targeting Heterochromatin Formation in *Drosophila*

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Contents

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Abstract

Near the end of their 1990 historical perspective article "60 Years of Mystery," Spradling and Karpen (1990) observe: "Recent progress in understanding variegation at the molecular level has encouraged some workers to conclude that the heterochromatization model is essentially correct and that position-effect variegation can now join the mainstream of molecular biology." In the 18 years since those words were written, heterochromatin and its associated position effects have indeed joined the mainstream of molecular biology. Here, we review the findings that led to our current understanding of heterochromatin formation in *Drosophila* and the mechanistic insights into heterochromatin structural and functional properties gained through molecular genetics and cytology.

Key Words: *Drosophila*, Heterochromatin, Position-effect variegation, Chromatin.

1. Introduction

The term "heterochromatin" was coined by Heitz (1928) as the material he observed in liverwort nuclei that failed to disappear after telophase in the mitotic cell cycle. In subsequent studies (Zacharias, 1995), Heitz showed that *Drosophila* somatic nuclei contained heterochromatic material resembling what he had seen in plants. He noted the heterochromatic Y chromosome of *Drosophila* and initially characterized the heterochromatin as "genicly passive." Since that time, heterochromatin has been the subject of considerable research and conjecture. The extraordinary cytology afforded by the giant polytene chromosomes of *Drosophila* third-instar-larval salivary glands, combined with the rapid accumulation of cytological aberrations and genetic mutations, made *Drosophila* the organism of choice for the elucidation of heterochromatin properties. Although both euchromatin and heterochromatin are composed of DNA and are packaged into nucleosomes, the sequence composition in heterochromatin and the structural modifications of histones in heterochromatin are distinctive, respectively. Heterochromatin-associated nonhistone proteins have been identified through genetic and biochemical approaches. Much of the success in the molecular dissection of heterochromatin has been the result of genetic screens that identified modifiers of heterochromatin silencing activity.

2. Heterochromatin Domains in the *Drosophila* Genome

2.1. Cytological heterochromatin in *Drosophila*

2.1.1. Pericentric heterochromatin

2.1.1.1. Pycnotic appearance In *Drosophila*, as in all higher eukaryotes, certain regions of each chromosome do not cyclically change their degree of condensation between interphase and metaphase but remain condensed throughout most of the cell cycle. These regions comprise the heterochromatin of the genome and include substantial amounts of the chromatin surrounding each centromere (pericentric heterochromatin). Regions that show an allocyclic behavior are collectively called euchromatin. During interphase, euchromatin is differentially packed too, and in polytene giant chromosomes of certain larval tissues, this differential condensation of interphase euchromatin is visible as transverse bands of condensed chromatin. This higher-order structure of chromatin and the mechanism of its assembly are by no means understood. The non-DNA moiety of chromatin represents not only a structural component, but is implicated in fundamental regulatory processes. During development, the determined programs of gene activity are stably inherited over mitoses; this cell memory information stored in the chromatin has been called ''epigenetic information.'' The mechanisms of establishment and maintenance of epigenetic information are now being dissected and the biochemical and genetic tools to detect and manipulate epigenetic marks are becoming available (see the following sections).

2.1.1.2. Under-replication in polytene chromosomes The giant polytene chromosomes of *Drosophila* larval salivary glands (and various other larval tissues as well) are the result of multiple rounds of chromosome replication in the absence of mitosis. The replication products in euchromatin remain paired, giving rise to large bundles of chromatin fibers (Zhimulev *et al.*, 2004). In wild-type polytene chromosomes, both homologs pair. The alternating intervals of chromatin condensation and decondensation along the axis of each chromosome arm result, in the paired polytene chromosome bundles, in the appearance of transverse bands of condensed chromatin alternating with interbands of decondensed chromatin.

Relative to the euchromatin, most of the pericentric heterochromatin in polytene tissues is under-replicated (Gall *et al.*, 1971), giving rise to the amorphous, attenuated appearance of this material. Remarkably, certain loci that reside in pericentric heterochromatin are nonetheless fully polytenized, even as flanking repetitious DNA sequences are severely under-replicated (Berghella and Dimitri, 1996; Zhang and Spradling, 1995).

In addition, the pericentric regions of all the polytene chromosomes coalesce into a single structure called the chromocenter.

Using a combination of mutations in genes encoding the heterochromatin-associated proteins SU(UR) and SU(VAR)3–9 (discussed later in this chapter), Zhimulev and colleagues were able to force the polytenization of pericentric heterochromatin (Andreyeva *et al.*, 2007). Under these conditions, the polytenized heterochromatin forms banded material that resembles euchromatin, permitting *in situ* hybridization mapping of specific genes in formerly heterochromatic regions. Immunolocalization on polytenized heterochromatin showed a number of bands that stained for HP1 and for histone H3 dimethylated at lysine 9 (H3K9me2). Presumably, these sites represent the vestigial staining sites previously observed within the heterochromatic chromocenter in *Su(var)3–9* homozygous mutant chromosomes (Schotta *et al.*, 2002). Interestingly, the remaining HP1 and H3K9me2 show relatively little overlap, suggesting that HP1 is targeted by a distinct mechanism from that used elsewhere in the genome (discussed later in this chapter). While the polytenization of heterochromatin by doubly inactivating SU(UR) and SU(VAR)3–9 creates a high-resolution picture of the DNA organization in this elusive region, caution should certainly be exercised in interpreting the distributions of proteins and chromatin modifications, considering that both SU(UR) and SU(VAR)3–9 are themselves being chromatin modifying proteins. Their loss could have secondary effects on gene expression and chromatin structure.

Heterochromatin protein distribution has mainly been studied in larval salivary gland chromosomes. Some mutations (e.g., *otu*) cause formation of polytene chromosomes in female ovarian nurse cells with morphology similar to salivary gland chromosomes (Heino, 1989; King *et al.*, 1981). Although similar in banding pattern, most of the cytological manifestations of heterochromatin are significantly less pronounced in nurse cell polytene chromosomes (Mal'ceva and Zhimulev, 1993). Interestingly, the heterochromatin proteins HP1, SUUR, and SU(VAR)3–9 are more abundant (Koryakov *et al.*, 2006). For the chromosomal distribution of SU(VAR)3–9, besides chromocentic heterochromatin, more than 200 additional binding sites along the euchromatic arms are detected. In these chromosomes, SU (VAR)3–9 binding only depends on SUUR in autosomes but not in the X chromosomes. Since ovarian nurse cells represent germ line cells, these findings suggest differential organization of heterochromatin in somatic and germ line chromosomes.

2.1.2. Intercalary heterochromatin

The term ''intercalary heterochromatin'' was first coined to describe certain sites along the euchromatic polytene X chromosome that showed an elevated frequency of breakage in squash preparations, similar to that seen at the heterochromatic base of the X (Kaufmann, 1939). This property has

been generalized to sites on the major autosomal arms as well, and the definition of intercalary heterochromatin has been expanded to include sites that are relatively under-replicated in polytene chromosomes and that undergo a thread-like physical association with the chromocenter termed ''ectopic pairing'' (Zhimulev, 1998; Zhimulev *et al.*, 2003a). The definition of intercalary heterochromatin, like the definition of heterochromatin itself, is a cytological one. As the genetic dissection of heterochromatin has progressed, and molecular probes for chromosomal proteins have been developed, similarities and distinctions between intercalary and pericentric heterochromatin have emerged (Zhimulev and Belyaeva, 2003).

The SUUR protein controls under-replication of intercalary heterochromatin as well as pericentric heterochromatin in polytene tissue (Belyaeva *et al.*, 1998). Many of these regions contain unique genes (e.g., BX-C and ANT-C) and about 60% of sites of intercalary heterochromatin show binding of PC-G proteins, suggesting that intercalary heterochromatin reflects silenced genes (Belyakin *et al.*, 2005).

2.1.3. Telomeric heterochromatin

The sequence organization of telomeric DNA in *Drosophila* is unusual among eukaryotes. Rather than being composed of monotonous polymers of short repeats synthesized by telomerase, *Drosophila* telomeres are built and maintained by the saltatory addition of copies of the HeT-A and TART non-LTR retrotransposable elements (Biessmann *et al.*, 1990; Cenci *et al.*, 2003a; Danilevskaya *et al.*, 1994; Levis *et al.*, 1993; Young *et al.*, 1983).

The tendency of telomeres to undergo ectopic pairing in polytene chromosomes (Hinton and Atwood, 1941; Kaufmann and Gay, 1969) gives them the impression of being heterochromatic. They are also sites of binding for the heterochromatin-associated protein HP1 (Fanti *et al.*, 1998; James *et al.*, 1989; discussed later in the following sections), which seems to have a role in telomere capping (Fanti *et al.*, 1998) and telomere length regulation (Savitsky *et al.*, 2002). Like the pericentric and intercalary heterochromatin, telomeres are under-replicated in polytene salivary gland chromosomes (George *et al.*, 2006).

As will be discussed further, transgene insertions into subtelomeric regions in *Drosophila* show variegated silencing, but the genetic basis for this silencing appears to be distinct from that of pericentric heterochromatin.

2.1.4. Y chromosome

The Y chromosome of *Drosophila* is entirely heterochromatic in somatic tissue. Like pericentric heterochromatin, the Y is under-replicated in polytene tissue (Gall *et al.*, 1971). Chromosome translocations with breakpoints anywhere in the Y chromosome can cause heterochromatic position-effect silencing, and transposon insertions into the Y also experience heterochromatic silencing (Zhang and Spradling, 1994).

A supernumerary Y chromosome can suppress heterochromatic position-effect silencing (discussed later in this chapter) that occurs in chromosome rearrangements (Gowen and Gay, 1934). This suppression has since been confirmed for all rearrangements that variegate for euchromatic loci and has been generalized into a rule by Morgan and Schultz (1942). Noujdin (1936, 1944) first reported the suppression of PEV by additional heterochromatin of either of the Y chromosome arms. Schultz (1936) extended the analysis of the effect of the Y chromosome aneuploidy on PEV and first described the strong enhancement due to a loss of Y chromosome in XO males. He was also the first to observe that susceptibility of variegation to Y chromosome aneuploidy represents a diagnostic feature for PEV. Since then, the modifier effect of Y chromosome aneuploidy has been used to discriminate between PEV and other cases of variegated gene expression (Gans, 1953; Becker, 1957).

The suppressive effect of additional heterochromatic material can best be understood if the heterochromatic regions in the nucleus are considered to be a sink for limiting quantities of silencing factors. For euchromatic genes experiencing silencing by neighboring heterochromatin, the silencing complexes propagated across the heterochromatic breakpoint would be recruited by additional heterochromatic material in *trans*, depleting them from the variegation-inducing site and thereby relieving silencing. The sequestering effect of additional heterochromatin may account for the curious observation of Cooper (1955) that, in XXYY females and XYYY males having an otherwise wild type chromosomal constitution, the adult eye becomes variegated, with large patches of bleached pigmentation. This effect could be explained if the consequence of such a large amount of additional heterochromatin was the misregulation of normally heterochromatic genes (such as *light*) even within their normal chromosomal context.

Taken together, the genetics and cytology of the Y chromosome in *Drosophila* argue that it is an example of heterochromatin. Since the Y chromosome is required in males for fertility and is extensively decondensed and transcribed in primary spermatocytes (Bonaccorsi *et al.*, 1988), it is probably best regarded as facultative heterochromatin: heterochromatic in somatic tissue but euchromatic in the germ line.

2.2. Genetic properties of heterochromatin in *Drosophila*

2.2.1. Gene silencing: Heterochromatic position-effect variegation

Beginning with Muller (1930), chromosome rearrangements that break within pericentric heterochromatin, the Y chromosome or the fourth chromosome have been associated with variegated gene silencing, a phenomenon called ''position-effect variegation'' (Spofford, 1976).

The term ''position effect'' was first used by Sturtevant (1925) and Dobzhansky (1932, 1936) to designate effects on gene action that were clearly dependent on a new position in the chromosome complement.

In this early period, the experimental work of geneticists on position effects was aimed at learning about chromosome organization and regulation of gene action. Lewis (1950) first classified the different phenomena of position effects into the relatively rare cases of a stable type and the more frequently observed variegated type position effects. For variegated type position effects, it was first shown by Schultz (1936) that the new position of a gene adjacent to heterochromatic regions is the cause for the variegated expression. Such rearrangements were first described by Muller (1930) in his classical experiments demonstrating the mutagenic action of X-rays, and referred to by him as ''eversporting displacements,'' ''sport'' being an early term referring to mutants. Between 1930 and 1940, all of the defining characteristics of PEV were described. Morgan and Schultz (1942) first defined a series of general rules which hold true for the cases of PEV he had studied. In the three classical reviews on PEV (Baker, 1968; Lewis, 1950; Spofford, 1976), these main rules have been further refined on the basis of a large amount of data then available.

2.2.2. Silencing requires the placement of the silenced locus in *cis* to silencing heterochromatin

This rule, according to Baker (1968), consists of demonstrating a wild-type function of the variegating gene after its restoration from the rearrangement back to a normal chromosome via crossover. This was shown by Dubinin and Sidorov (1935), Panshin (1935), and Judd (1955) in *Drosophila melanogaster*. Additional proof for the *cis* dominance came from revertant analysis (Griffen and Stone, 1940; Grüneberg, 1937; Kaufmann, 1942; Panshin, 1938). Reversion of the variegated phenotype was found to be accompanied by reversion of the rearrangement or by relocation of the affected locus into a new position.

An apparent exception to this rule is the dominant variegation seen at the *brown* locus (discussed later in this chapter). In this case, a wild-type *brown* allele on the homologous chromosome is inactivated by a variegating rearrangement. It appears, however, that the underlying basis for the dominant variegation is the conjunction of two mechanisms: (1) the establishment of a heterochromatic domain as a result of chromosome rearrangement and (2) an unusual sensitivity of the *brown* regulatory machinery to pairing interactions which, in the case of a paired homolog associated with a heterochromatic breakpoint, results in *trans*-silencing. The mechanistic basis for pairing-dependent inactivation is unknown, but since inactivation is coupled to a mechanism which does fulfill the rules of PEV, the exceptional nature of this phenomenon is more apparent than real.

2.2.3. Additional dosage of heterochromatin titrates silencing

As discussed above, increasing the dose of the Y chromosome, X chromosome heterochromatin or the fourth chromosome all have the effect of suppressing position-effect variegation.

Interdependence between the amount of heterochromatin and the dosage of factors controlling heterochromatin formation is supported by identification of mutations displaying heterochromatin-sensitive lethality. Altogether, mutations for three genes (*Su(var)2–1*, *Su(var)3–3* and *bonus*) have been identified which display recessive lethal interaction with additional heterochromatin, such as an extra copy of the Y chromosome. The mutations are homozygous viable in XX females and XO males but semilethal in XY males and almost completely lethal if an additional Y chromosome (XXY females or XYY males) is present (Beckstead *et al.*, 2005; Reuter *et al.*, 1982b). The *Su(var)3–3* genes encodes the *Drosophila* homolog of the mammalian histone H3 lysine 4 demethylase LSD1 and was shown to control the balance between euchromatin and heterochromatin in early *Drosophila* embryogenesis (Rudolph *et al.*, 2007).

2.2.4. The silencing effect of heterochromatin diminishes with distance from nearby heterochromatin

The polar effect of inactivation for PEV was discovered as a consequence of extensive cytogenetic analyses by Demerec and coworkers (Demerec, 1940, 1941; Demerec and Slizynska, 1937) using different *white*- and *Notch*-variegating rearrangements. In the inversion $In(1)N^{264-52}$, variegation was observed for five linked genes located within a ca. 50-band interval adjacent to the heterochromatic breakpoint. Heterochromatin appears to propagate for a variable distance into euchromatin and cause the variegated expression of genes in PEV (Demerec, 1941; Prokofieva-Belgovskaya, 1939, 1941, 1947). Prokofieva-Belgovskaya (1947) summarized her results of a thorough cytological analysis of several different position-effect rearrangements (sc^8, w^{m5}, rst^3, and w^{m4}), finding that gene inactivation in all these rearrangements could be correlated with a visible heterochromatinization of the euchromatic regions immediately adjacent to the rearrangement breakpoint. She also studied the frequency of heterochromatinization under the influence of different modifiers of PEV like the presence of an additional Y chromosome, temperature of development, parental origin of the rearrangement, and age of parents. Most of these studies were performed with $In(1)sc^8$, a rearrangement with visible variegation for the *yellow*, *achaete*, and *scute* genes, which had been already extensively used for a study of different PEV modifier effects by Noujdin (1944).

Using a series of secondary rearrangements derived from X-irradiation of *In(1LR)pn2a*, Gvozdev and colleagues (Tolchkov *et al.*, 2000) showed that the amount of heterochromatin at the breakpoint has a quantitative effect on variegation. Importantly, though, the presence of a centromere within the heterochromatic block had a stronger effect than a much larger block of paracentric heterochromatin lacking the centromere. This suggests that sequence composition of heterochromatin plays an important role in the induction and/or propagation of silencing heterochromatin. In addition

to the rules described earlier, there are a number of frequently (though not universally) observed properties associated with heterochromatic silencing.

2.2.5. The clonal nature of PEV

The clonal nature of gene silencing in PEV was suggested by mitotic twin spot analysis and comparisons to pattern formation during development of the *Drosophila* eye anlage. These studies were performed by Becker (1957) and Baker (1967) for the eye anlage of *D. melanogaster* and *D. virilis*, respectively. Comparison of clonal patches marked by X-ray-induced mitotic crossing-over induced at different developmental stages with the variegation pattern of different *white*-variegating rearrangements suggested that inactivation of the *white* gene is based on cell lineage and determined by the end of first larval instar, when about 20 presumptive eye cells are present (Baker, 1967; Becker, 1961, 1966). Janning (1970, 1971) induced twin spots in the eye anlage at different times during development and analyzed their overlap with PEV induced mutant white spots in a $Dp(1;3)w^{m264-58}$ background. Because clones induced at the end of the first larval instar overlap with the white PEV spots, the time of *white* inactivation by heterochromatin was inferred to be clonally decided at this time during development of the eye anlage.

An outstanding question in chromatin biology is whether levels of a histone modifier must be maintained continuously to set levels of gene expression or whether modifier levels initiate an epigenetic mark that is stable to changes in modifier levels later in development. Reuter and colleagues tested whether reduced SU(VAR)3–9 is required continuously in the *Drosophila* eye to set PEV levels. Clonal analysis with *Su(var)3–9* null mutations suggests that SU(VAR)3–9 controls heterochromatin formation by dimethylation of histone H3 at lysine 9 (H3K9me2) during early embryonic development (Rudolph *et al.*, 2007; Fig. 1.1). *Su+/Su+* clones

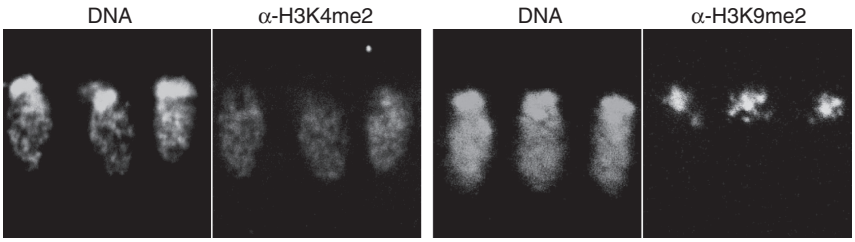

Figure 1.1 **Establishment of the euchromatin-associated (H3K4me2) and heterochromatin (H3K9me2) histone methylation marks first occurs during early embryogenesis at the syncytial blastoderm stage when nuclei show an apico-basal polarity ("Rabl configuration"). Euchromatin, identified by diffuse H3K4me2 staining, is located towards the lower basal side of the nuclei. DAPI ("DNA"; red) intensely stains the pericentric heterochromatin at the upper apical pole, which is enriched in histone H3K9me2 methylation mark. Photo provided byThomas Rudolph. (See Color Insert.)**

were induced in *Su(var)3–9/+* heterozygotes by the flipase/FRT mitotic recombination system. Although two wild type copies of the *Su(var)3–9* gene are present in the *Su+/Su+* clones, *white* gene silencing in the w^{m4} PEV rearrangement is only restored in large clones induced early in development. Reversion of the suppressor effect in late-induced clones does not occur with additional cell divisions, suggesting that after early establishment of heterochromatin, the chromatin state established by reduced *Su(var)3–9* dosage is stably maintained throughout consecutive development. This contrasts with another chromatin modifier, the histone H3 lysine 4 trimethylase TRITHORAX, which is required continuously to maintain HOX gene activity in *Drosophila* (Ingham, 1985). Thus, SU(VAR)3–9 functions in heterochromatin as part of a mechanism of epigenetic memory.

2.2.6. Paternal and parental effects

Although a basic analysis of paternal and maternal effects in PEV had been reported by Noujdin (1944), the analysis of such effects was mainly extended within a period (1950–1970) when PEV was only studied by few scientists. Genetic analysis of most basic characteristics of PEV was already advanced and experimental work was more focused on modifying factors. A maternal suppressor effect of an extra Y was first reported by Noujdin (1944) for the $In(1)sc^8$ rearrangement. The sc^8 homozygous female offspring showed a ca. eightfold reduction in variegation for *yellow* and *acheate* if additional Y chromosome material was present in the mother. Spofford (1976) reported a similar maternal suppressor effect of an additional Y chromosome for $Dp(1;3)N^{264-58}$and a similar effect was reported for the *white*-variegating rearrangement $Dp(1;3)w^{vco}$ (Khesin and Bashkirov, 1978). Noujdin (1944) also reported a paternal suppressor effect of additional Y chromosome material for $In(1)y^{3P}$ variegating for *yellow*. In other variegating rearrangements of *D. melanogaster*, no significant maternal or paternal effects of additional Y chromosome material have been reported. Schneider (1962) found a maternal suppressor effect of an additional Y chromosome in one of the six *peach*-variegating rearrangement of *D. virilis* studied.

Parental source of the rearrangement can also affect significantly the extent of variegation in the offspring. Such effects were described for $Dp(1;3)N^{264-58}$ by Spofford (1959), and for $In(1)sc^8$ by Prokofieva-Belgovskaya (1947). In the case of $Dp(1;3)N^{264-58}$, variegation was more enhanced if the rearrangement was maternal in origin, whereas in sc^8, heterochromatinization was enhanced with a paternal origin of the rearrangement.

A stable paternal effect was observed in crosses with several *Enhancer of variegation* [*E(var)*] mutations (Dorn *et al.*, 1993). In a cross of $w^{m4}/w^{m4}+/+$ females to w^{m4}/Y *E(var)*/+ males the w^{m4}/Y+/+ offspring males show enhanced *white* variegation, although the enhancer mutation is not present. In a series of crosses, it was demonstrated that the Y chromosome has

acquired a stable ability to enhance *white* variegation in w^{m4} independent of the genetic background. Once acquired, the enhancer effect of the Y chromosome is maintained through consecutive generations. The molecular basis for this Y chromosome imprinting effect is unknown.

2.2.7. Temperature sensitivity of PEV

As a rule, in *Drosophila*, low temperature of development enhances variegation of euchromatic genes subject to PEV, whereas a higher temperature shows a suppressor effect. This was first described for *white* variegating rearrangements by Gowen and Gay (1934). The effect of temperature on PEV extended to the polar effect of genetic inactivation as well as cytological condensation (Hartmann-Goldstein, 1967; Rudkin, 1965). The temperature effect can be understood in the general context of thermal effects on protein folding if one imagines silencing as mediated by a proteinaceous complex: high temperature would weaken such a complex, while lower temperatures would stabilize it. There are consistent differences in the strength of the modifying effect of temperature on PEV when different rearrangements are compared. This might be due to differences in the genetic and molecular nature of the heterochromatin inducing PEV. Temperature-sensitive periods were mapped in order to determine the developmental stage(s) when gene inactivation in PEV takes place. In the rearrangement studied, the major temperature sensitive period was found during the first two days of puparium formation (Becker, 1961; Chen, 1948; Hartmann-Goldstein, 1967; Schultz, 1956). Possible tissue-specific differences were indicated by results of a study of temperature-sensitive periods for PEV in salivary glands and Malpighian tubules (Hartmann-Goldstein, 1967).

By inspection of the giant polytene chromosomes of *Drosophila* larval salivary glands taken from PEV lines, the cytological site of the variegating locus loses its banded, fully polytenized euchromatic appearance in some nuclei, and to take on the disorganized, densely staining, attenuated appearance of heterochromatin (Caspersson and Schultz, 1938; Henikoff, 1981; Kornher and Kauffman, 1986; Prokofyeva-Belgovskaya, 1939). Moreover, this structural dimorphism is sensitive to temperature (Belyaeva and Zhimulev, 1991; Prokofyeva-Belgovskaya 1947; Schultz, 1941; Zhimulev *et al.* 1988) and to genetic modifiers of PEV (Belyaeva and Zhimulev, 1991; Reuter *et al.*, 1982a; Zhimulev *et al.*, 1988). In at least one case, the morphologically heterochromatic variegating locus recruits the normally heterochromatin-associated protein HP1 (Belyaeva *et al.*, 1993). Thus, the cytological compaction of a variegating locus is taken to be a morphological manifestation of the silencing mechanism.

The chromosome structural changes that correlate with genetic silencing imply that the mechanism operates at the level of transcription. Indeed, a reduction in transcription of a variegating locus has been demonstrated by

in vivo pulse-labeling (Henikoff, 1981) and by quantitative mRNA blot hybridization (Henikoff and Dreesen, 1989; Kornher and Kauffman, 1986; Rushlow *et al.*, 1984; Wallrath and Elgin, 1995). While position-dependent changes in RNA turnover rates cannot rigorously be excluded in any of these cases, the simplest interpretation is that the silencing mechanism acts at the level of transcript synthesis. Central to the notion that epigenetic changes underlie the phenomenon of PEV is that no irreversible genetic change explains the silencing effect (although covalent changes in DNA structure may be a concomitant of the silencing mechanism in some cases). Thus, demonstrating that silencing can be efficiently reversed represents strong evidence that the mechanistic basis of silencing does not require the mutation or deletion of variegating genes.

Two recent studies bear on this point. Lu *et al.* (1996) used a ubiquitously inducible reporter for PEV to investigate the developmental progression of PEV silencing (see further for details on this system). They found that silencing of an *HSP70-lacZ* reporter was extensive in the third instar precursors of the adult eye (the eye imaginal discs) but was dramatically relaxed in the adult eye. This extensive relaxation of silencing during differentiation is difficult to explain as an efficient reversal of mutational inactivation. In a separate study, Ahmad and Golic (1996) used a *white* reporter flanked by the FRT recombination sites, subject to PEV silencing. When they mobilized somatic DNA excision with a heat shock-inducible FLP "flippase" activity, they found red-pigmented facets among the white sectors of the adult eye. This result suggests that when the *white* reporter escapes the chromosomal context by FLP-catalyzed excision and circularization as an episome, it recovers full function. Here too, such efficient recovery of function is best explained by a model of chromosome-dependent epigenetic silencing.

Interest in the phenomenon of heterochromatic PEV has intensified in the last 20 years because it has proven to be such a useful tool for the structural and functional dissection of heterochromatin and euchromatin (Eissenberg, 1989; Eissenberg and Wallrath, 2003; Grigliatti, 1991; Reuter and Spierer, 1992). As modifiers of PEV have been cloned and characterized, the vast majority have proven to encode chromosomal proteins or their modifiers. Many of these are evolutionarily conserved, arguing for a conserved mechanism for heterochromatin assembly and maintenance.

2.2.8. Centromere activity

In *Drosophila*, the centromeres of chromosomes are diffuse structures. The *Drosophila* X centromeric region, the best characterized of the *Drosophila* centromeres, consists of interspersed blocks of unique sequence and repetitious DNA (Le *et al.*, 1995; Murphy and Karpen, 1995). Using a series of deletions that remove blocks of pericentric X chromosome heterochromatin, Karpen *et al.* (1996) found evidence that centric heterochromatin

contains multiple pairing elements that are required for the proper alignment of achiasmate chromosomes in meiosis I in *Drosophila* females. Genetic screens for factors influencing the fidelity of meiotic chromosome transmission identified a number of genes previously implicated in heterochromatic position-effect variegation (Hari *et al.*, 2001; Le *et al.*, 2004).

Surprisingly, the pattern of histone modifications in the putative centromeric chromatin is distinct from those associated with pericentric heterochromatin (Sullivan and Karpen, 2004). Presumably, such differences reflect the distinctive role of centromeric chromatin in the formation of the kinetochore and the recruitment of microtubules during mitosis.

2.2.9. Suppression of meiotic recombination

The frequency of meiotic recombination is very low within the pericentric heterochromatin of *Drosophila* (Brown, 1940; Muller and Painter, 1932). Furthermore, the frequency of recombination relative to physical distance is reduced in the euchromatin near the pericentric heterochromatin and the telomeres of the major chromosome arms (Ashburner, 1989). A mechanistic link between the inhibition of meiotic recombination in heterochromatin and the position-effect silencing of gene expression observed at heterochromatic breakpoints is suggested by the observation that several mutations that cause dominant suppression of heterochromatic position-effect variegation (discussed later in this chapter) enhance recombination in pericentric heterochromatin (Westphal and Reuter, 2002). Thus, the mechanism that silences euchromatic gene as a result of position effects also interferes with normal meiotic recombination.

2.2.10. Suppressor of under-replication

The *Suppressor of Underreplication* [*Su(UR)*] locus was discovered serendipitously in a stock carrying an X-ray induced X chromosome rearrangement (Belyaeva *et al.*, 1998). The original *Su(UR)* allele is a semidominant, maternal-effect enhancer of polytenization of pericentric and intercalary heterochromatin in larval salivary gland and ovarian polytene chromosomes. Flies homozygous for *Su(UR)* mutation are viable, and the enhancement of polytenization of heterochromatin is more extreme. The protein encoded by the *Su(UR)* locus contains an AT hook domain and homology to the ATPase domain of SWI2/SNF2 family chromatin remodeling proteins (Makunin *et al.*, 2002). Outside of these motifs, though, there are no obvious homologs of SU(UR) in other organisms. In salivary gland polytene chromosomes, the SU(UR) protein is concentrated in the chromocenter and at sites of intercalary heterochromatin on the euchromatic arms.

Overexpression of SU(UR) protein in transgenic larvae results in a striking, temperature-sensitive distension, or swelling of the chromocenter and intercalary heterochromatin sites (Zhimulev *et al.*, 2003a). These swellings, unlike classical polytene chromosome puffs, are not a consequence of

transcription, since they do not accumulate tritiated uridine. EM micrographs reveal lacunae within these swellings, and at the light microscope level, the swellings are largely devoid of SU(UR) protein while still staining strongly for DNA.

In flies that are doubly mutant for *Su(UR)* and *Su(var)3–9* (which encodes a histone H3 lysine 9 methyltransferase; discussed later in this chapter), a further polytenization of the pericentric heterochromatin of the third chromosome occurs beyond that seen in *Su(UR)* mutants alone (Andreyeva *et al.*, 2007). Surprisingly, double mutation for *Su(UR)* and the gene encoding HP1 (discussed below) does not have the same effect, suggesting that the phenotype of the *Su(UR)-Su(var)3–9* double mutant is not simply due to the loss of HP1 binding to the methyl mark normally created by the *Su(var)3–9* gene product.

2.3. Biochemical properties of heterochromatin

Several studies have tested the accessibility of DNA at variegating loci to nucleolytic attack or enzymatic modification; most yielded results suggesting little or no structural difference between a silenced locus and its euchromatic counterpart (Hayashi *et al.*, 1990; Locke and McDermid, 1993; Schloßherr *et al.*, 1994; Wines *et al.*, 1996). However, the relatively low resolution of the measurements in these cases, together with a lack of detailed structural information concerning the euchromatic structure of the variegating locus argue for caution in the interpretation of these experiments. When the conditions of high resolution measurements and prior knowledge of the gene involved were met; however, a close correlation between silencing and DNA packaging was observed (Wallrath and Elgin, 1995).

Wallrath and Elgin (1995) employed a transposon bearing the *hsp26* promoter to analyze the structural consequences of heterochromatic inactivation. The choice of the *hsp26* promoter was especially apposite in this case, since the chromatin structure of this promoter at its normal chromosomal position at position 67B has been characterized in detail (Cartwright and Elgin, 1986; Thomas and Elgin, 1988) and since the *in vivo* structural requirements for its chromosomal architecture and activity have been extensively investigated (Lu *et al.*, 1992, 1993). Transgene insertions showing variegation of a linked *Hsp70*-mini-*white* reporter were selected and characterized as to their insertion sites: 4 were insertions in pericentric heterochromatin, 9 were insertions at telomeric sites, and 18 were found at various positions throughout the fourth chromosome. Only for the pericentric and fourth chromosome inserts did inclusion of the dominant suppressor *Su(var)2–1*^{01} or reduced HP1 levels result in suppression of *white* variegation; none of the inserts at the telomeres of the second or third chromosome responded to these modifiers. For selected lines, suppression of *hsp26*-driven transcription was confirmed by quantitative

Nothern blotting. It was possible to distinguish transgene transcription from the endogenous *hsp26* gene transcription because the transgene promoter directs transcription of a barley cDNA fragment not found in flies. For all pericentric insertion lines tested, transcripts of the barley sequence were reduced, and this reduction was reversed for all but one line when HP1 dose was reduced. Again, though, variegating transgene insertions in the telomeres showed levels of barley transcripts comparable to euchromatic controls, and this expression was either not affected or slightly reduced by reduced HP1 dosage.

Nuclei were prepared from non-heat-shocked transgenic third instar larvae and the sensitivity to digestion with *Xba* 1, which cleaves within each of two DNase I hypersensitive sites in the *hsp26* promoter, was measured by Southern blot hybridization using a transgene-specific probe. With the digestion efficiency obtained using a euchromatic insertion arbitrarily set as 100% accessibility, it was found that all variegating inserts showed significantly reduced accessibility, even a telomeric insert on the second chromosome which showed no effect on *hsp26*-mediated transcript levels. In this and a more recent study (Sun *et al.*, 2001), micrococcal nuclease digests revealed a more regular nucleosomal ladder at the silenced loci compared to their euchromatic counterparts, suggesting a more ordered chromatin structure underlies the nuclease resistance.

One reservation concerning the interpretation of nuclease sensitivity studies is that, of necessity, suspensions of free nuclei must be used, since nucleases cannot penetrate the plasma membrane. Thus, one cannot be certain that no rearrangement of chromatin structure occurs during the preparation of nuclei. To circumvent this objection, the accessibility of the DNA in silenced loci to modification by *E. coli* Dam methyltransferase in transformed flies has been tested (Boivin and Dura, 1998). Since methylation is occurring in intact cells, this differential accessibility is the *in vivo* state. Adenine methylation is tolerated well in flies (Boivin and Dura, 1998; Wines *et al.*, 1996); a maximum of 50% methylation overall is observed. Boivin and Dura (1998) used transgenic flies expressing Dam methyltransferase under an *Hsp70* promoter, and assayed for methylation based on sensitivity of the purified DNA to digestion with a methylation-sensitive restriction endonuclease. Under these conditions, the efficiency of methylation is the same for a euchromatic locus independent of its transcriptional state. In contrast, *white* DNA sequences in a classical PEV reporter as well as in transgene reporters subject to heterochromatic PEV showed reduced methylation compared to euchromatic DNA controls. Methylation in 5′ and 3′ sequences was similar at a given locus, suggesting that the chromatin differences were not restricted to the promoter.

The pycnotic appearance of heterochromatin, the relative resistance of heterochromatic DNA to exogenous nuclease digestion (Wallrath and Elgin, 1995) and endogenously expressed DNA methylase (Boivin and

Dura, 1998), and the relatively ordered nucleosome arrays that package heterochromatic DNA (Sun *et al.*, 2001) together suggest a model in which heterochromatin silencing is imposed by occlusion of DNA binding sites for transcription activators and/or RNA polymerase. Chromatin footprinting analysis supports this model (Cryderman *et al.*, 1999a).

In some cases, though, transcription factors with high affinity for their DNA target sites can compete successfully with heterochromatin to prevent the establishment of silencing (Ahmad and Henikoff, 2001; Eissenberg, 2001a). Furthermore, there are a number of genes in *Drosophila* that reside in pericentric heterochromatin and require a heterochromatin context in order to function (Eissenberg and Hilliker, 2000; Rossi *et al.*, 2007; Yasuhara and Wakimoto, 2006). Thus, heterochromatin does not present an impenetrable barrier to DNA binding proteins, but would seem to shift the binding equilibrium away from the bound state.

3. DNA Sequences that Target Heterochromatin

Unlike mammals and plants, *Drosophila* has little DNA methylation, and whatever cytosine methylation exists has no discernible role in gene regulation. In respect to the DNA sequence composition of heterochromatin of *Drosophila*, however, there are characteristics that distinguish it from the sequence composition of euchromatin.

3.1. Pericentric DNA

*Drosophila*is the first organism for which a large part of the heterochromatic sequence was successfully mapped, assembled, and finished (Hoskin *et al.*, 2007; Smith *et al.*, 2007). The studies revealed that the constitutive heterochromatin of *Drosophila*, like constitutive heterochromatin in other animals and plants, is highly enriched in middle repetitive and satellite DNA. Within complex repeats, though, islands of highly conserved genes are found. Altogether, more than 230 protein-coding genes were detected which are also found in other *Drosophila* species. Altogether, 77% of the heterochromatic sequences are repetitive or transposable element (TE) sequences. Frequently, nests of TE elements that are fragmented, interdigitated, and transposed into one another are found. Almost 900 such repeat nests could be defined. Specific TE elements have been implicated in gene silencing by heterochromatin (discussed later in this chapter).

There is no conserved sequence feature shared by eukaryotic heterochromatin. Between different species and even different strains of *Drosophila*, the amount of heterochromatin and satellite compositions vary widely (Bosco *et al.*, 2007; Gall *et al.*, 1971; Halfer, 1981; Kuhn and Sene, 2005;

Lohe and Brutlag, 1986; Schweber, 1974). These studies suggest that variation in heterochromatic satellite DNA contributes significantly to genome size evolution. Such evolutionary diversity may reflect competition between centromeres for success in the egg, as has been proposed for the centromeric satellite DNA (Henikoff *et al.*, 2001; Malik and Henikoff, 2002). But regardless of the evolutionary basis, the extraordinary diversity of DNA sequences that underlie pericentric heterochromatin suggests that the mechanism of heterochromatin formation requires factors besides an underlying consensus DNA sequence.

The heterochromatin-associated protein HP1 binds DNA *in vitro* (Perrini *et al.*, 2004; Zhao *et al.*, 2000). Crosslinking studies suggest that direct binding of HP1 to telomeric DNA could target HP1 to these chromosomal sites (Perrini *et al.*, 2004). In an attempt to infer a preferred DNA sequence target site for HP1 binding, Greil *et al.* (2003) compared the nonrepetitive sequences targeted by HP1 in Kc cultured cells. They found that AT-rich motifs, consisting of stretches of adenosines or stretches of thymidines were enriched at these sites. Previous *in vitro* experiments showed that there is no strong DNA sequence preference for purified recombinant HP1 (Zhao *et al.*, 2000). However, *Drosophila* HP1 is multiply phosphorylated (Eissenberg *et al.*, 1994; Zhao and Eissenberg, 1999; Zhao *et al.*, 2001), so either posttranslational modification or association with other factors could confer sequence-preferential binding *in vivo*.

3.2. The fourth chromosome

The fourth chromosome in *D. melanogster* is by far the shortest autosome, representing ca. 3.5% of the genome and estimated to be ca. 5 Mb in length (Locke and McDermid, 1993). The fourth chromosome shares some attributes of heterochromatin, notably that it undergoes no detectable meiotic recombination (Bridges, 1935; Hochman, 1976), replicates late in the cell cycle (Barigozzi *et al.*, 1966), is relatively enriched in middle repetitive DNA (Locke *et al.*, 1999; Miklos *et al.*, 1988), is associated with rearrangements that induce heterochromatic position effects, and is enriched in the heterochromatin-asociated protein HP1 (discussed below) and histone H3 dimethylated at lysine 9 (de Wit *et al.*, 2007; Greil *et al.*, 2003; James *et al.*, 1989; Schotta *et al.*, 2002). Interestingly, however, the enzyme that methylates lysine 9 of histone H3 in pericentric heterochromatin, and that is required for silencing of genes mislocalized to pericentric heterochromatin (see below) is not required for methylation of lysine 9 of histone H3 (H3K9) on the fourth chromosome (Schotta *et al.*, 2002). Instead, the *Drosophila* SETDB1 protein is required for H3K9 dimethylation on chromosome four and for silencing of transgene reporters on the fourth (Seum *et al.*, 2007).

Genetic analysis suggests that one or more TE families mediate heterochromatin-like transgene silencing on the *Drosophila* fourth chromosome.

Using the expression of *white*-marked transposons as reporters and a series of nested deletions, Sun *et al.* (2004) implicated nearby copies of the 1360/*hoppel* transposon as sources for transgene silencing on the fourth chromosome. The silencing depended on the dosage of the heterochromatin-associated protein HP1. At other chromosomal sites, members of the 1360/*hoppel* transposon family recruit high levels of HP1, although the levels appear to depend on whether the transposon is in chromosome regions enriched for repetitious DNA (de Wit *et al.*, 2005, 2007). Short RNAs corresponding to both sense and antisense strands of 1360/*hoppel* elements are detectable in Kc cultured cells (Haynes *et al.*, 2006), suggesting that RNA interference (RNAi) is part of the mechanism of 1360/*hoppel*-dependent silencing. Indeed, mutations in genes encoding subunits of the *Drosophila* RNAi machinery partially relieve silencing associated with 1360/*hoppel* elements (Pal-Bhadra *et al.*, 2004). In a direct test of the ability of 1360/*hoppel* elements to impose position-effect silencing, Haynes *et al.* (2006) cloned a copy of 1360/*hoppel* adjacent to a mini-*white* transposon reporter. Of 22 independent insertions spanning both major autosomes, only one insert showed variegated silencing. This insert is located near the base of the left arm of the second chromosome at a transposon-rich site, and the silencing is sensitive to HP1 dosage and mutation in *Su(var)3–9,* mutation in genes encoding components of the *Drosophila* RNAi mechanism. While these results clearly show a requirement for chromosomal positioning near natural heterochromatin in the silencing mechanism, the authors show that deletion of the 1360/*hoppel* sequence at this insertion site partially relieves silencing. Thus, 1360/*hoppel* elements appear unable to independently target heterochromatin formation to ectopic sites, but can cooperate with nearby repeat elements to enhance or spread heterochromatin. This model for cooperative interactions to target heterochromatin assembly fits well with tethering experiments showing that tethering HP1 to a transgene reporter can target heterochromatic silencing only when the transgene insertion is in a repeat-rich region (Seum *et al.*, 2001).

3.3. Transposon arrays and ectopic heterochromatin

In *Drosophila*, the introduction of multiple transgenes carrying the same marker generally results in dosage effect for the expression of the marker. In certain cases, however, the multiplication of transgenes leads to silencing. The first report of this paradoxical phenomenon involved transposons marked with mini-*white*; local transposition of the transposon results in transposon arrays, some of which result in variegated *white* expression (Dorer and Henikoff, 1994, 1997). HP1 is found at the site of transgene arrays in polytene chromosomes (Fanti *et al.*, 1998). Interestingly, lower amount of HP1 are present at arrays at which no silencing is detectable, suggesting that HP1 binding alone is not sufficient for the silencing.

X-irradiation of stocks carrying variegating mini-*white* transposon arrays resulted in lines showing enhanced or suppressed variegation (Dorer and Henikoff, 1997). Lines showing enhanced variegation had chromosome rearrangements that placed the transgene closer to pericentric heterochromatin, and recombining the transgene back to its original position suppressed the variegation. Transgene array silencing is not limited to mini-*white* transposons. Arrays composed of transposons marked with *brown* also show variegation (Sabl and Henikoff, 1996), but in this case, only when the array is located very close to pericentric heterochromatin. Thus, mini-*white* transposons seem more prone to array-induced heterochromatin formation than are transposons marked with *brown*.

On the other hand, a transposon carrying a *Prat-brown* fusion transgene appears refractory to array induced silencing. Arrays of up to 320 kb at a euchromatic insertion site show no evidence of silencing (Clark *et al.*, 1998). Thus, like other transposons in the *Drosophila* genome, P-elements can promote heterochromatin assembly, but not all P-elements do so. These differences could be explained by the differences in protein complexes assembled on different transgenes, differences in promoter strength of the transgenes, the presence of elements that inhibit heterochromatin assembly, or some combination of factors.

3.4. Spreading of heterochromatin at rearrangement breakpoints

In 1988, Tartof and colleagues proposed a mass-action model for the assembly and propagation of heterochromatin based on the genetics of heterochromatin position-effect silencing in *Drosophila* (Locke *et al.*, 1988). This model was designed to explain the genetic observation that several genes each appeared to have dosage-dependent effects on heterochromatic silencing, implying that multiple rate-limiting factors for heterochromatin exist simultaneously. The model also incorporated the idea that spreading of heterochromatic silencing appears to occur from a heterochromatic breakpoint. There are three general features of this model: (1) that heterochromatin consists of nucleoprotein complexes whose integrity depends on a multiplicity of interactions, and that multiple complex subunits can each be limiting for the *cis*-spreading of the complex; (2) that such complexes normally are targeted to one or more ''initiation sites'' in the DNA of pericentric heterochromatin and spread in *cis* from these sites in a cooperative fashion; and (3) that spreading is normally contained by termination sites and that position-effects arise when chromosome rearrangements permit spreading into a normally euchromatic region. Considerable molecular and genetic data support all three elements of this model.

Spreading of heterochromatin has been demonstrated directly by chromatin immunoprecipitation analysis in the w^{m4} PEV rearrangement

(Rudolph *et al.*, 2007). Here, spreading of the heterochromatic dimethylated H3K9 (H3K9me2) histone methylation mark, a histone modification normally enriched in pericentric heterochromatin (discussed later in this chapter) into the euchromatic *white* gene region has been demonstrated. Spreading of H3K9me2 clearly depends on dosage of the histone H3K9 methyltransferase SU(VAR)3–9 (discussed later in this chapter) and the function of the histone H3 lysine 4 (H3K4) demethylase SU(VAR)3–3 (DmLSD1).

The mechanism that normally constrains heterochromatin spreading in higher eukaryotes is poorly understood. One contributor could be the abundant DNA binding protein GAGA factor. GAGA factor was shown to interact with the FACT complex, which facilitates nucleosome remodeling essential for maintenance of Hox gene expression (Shimojima *et al.*, 2003). The GAGA factor–FACT complex is also involved in control of heterochromatin spreading in PEV by facilitating replacement of H3K9-methylated histones by the unmethylated histone variant H3.3 (Fig. 1.2). This function counteracts heterochromatin spreading and could explain at the molecular level why GAGA factor mutations are enhancers of PEV (Nakayama *et al.*, 2008).

An overly literal reading of the model would infer that heterochromatin complexes can only be initiated within canonical pericentric heterochromatin and must spread continuously from initiation sites within these regions in a crystallization-like process without interruption to silence genes in nearby euchromatin. However, the mass-action model is also completely consistent with the idea that abnormal proximity of euchromatin to heterochromatin enforced by rearrangements results in the stochastic

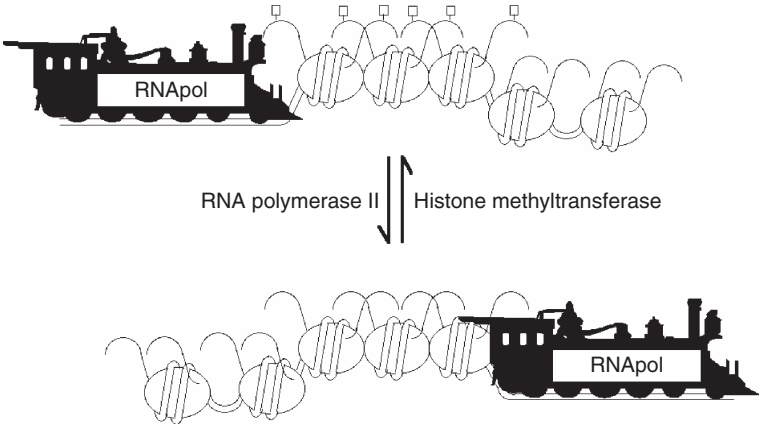

Figure 1.2 **Remodeling of methylated histone H3 by RNA Polymerase II passage. As RNA Polymerase II traverses a chromatin domain containing methylated histone H3 (□, the nucleosomes in its path are evicted from the DNA). As chromatin is reassembled in the wake of the Polymerase, unmethylated histone H3.3 is used, leaving a domain of unmarked chromatin. Redrawn from Eissenberg and Elgin (2005).**

colonization of euchromatin by heterochromatin at latent, cryptic ''initiation'' sites in euchromatin such as TE or repetitive sequences. Since key factors that assemble heterochromatin by mass action are diffusible, they could spread in *cis* or in *trans*, either continuously or discontinuously.

4. Histone Modifications and Heterochromatin Targeting

4.1. Heterochromatin-associated chromatin marks

The idea that covalent histone modifications could regulate transcription was first advanced by Vincent Allfrey and colleagues (Allfrey *et al.*, 1964; Pogo *et al.*, 1966). In the past 15 years, this inference has gained robust support, as the tools for the genetic dissection and cytological characterization of chromatin modifications have been developed. The current model driving experiments on histone modifications is the ''histone code'' hypothesis, which posits that combinations of covalent histone modifications partition chromosomes into distinct functional domains (Jenuwein and Allis, 2001; Strahl and Allis, 2000; Turner, 1993, 2002). In many cases, the key observations that have led to our current understanding of histone modifications in heterochromatin were first made in *Drosophila*.

The different histone H4 isoforms acetylated at lysines 5, 8, 12, or 16 were shown to have distinct genomic distributions in *Drosophila* polytene chromosomes (Turner *et al.*, 1992). H4 isoforms acetylated at lysines 5 or 8 are found widely dispersed throughout the euchromatic arms, but only in low amounts in the chromocenter heterochromatin. In contrast, the isoform acetylated at lysine 12 is significantly enriched in the chromocenter and in bands along the polytene fourth chromosome, a pattern highly reminiscent of HP1 distribution. Subsequent genome-wide analysis using chromatin immunoprecipitation and cDNA microarrays confirmed that hyperactylated histone H3 and H4 isoforms are enriched in the transcription units of active genes in *Drosophila* (Schübeler *et al.*, 2004).

H3K9me2 is found at high concentrations throughout the pericentric heterochromatin, along the fourth chromosome and at dispersed euchromatic sites in *Drosophila* polytene chromosomes, while H3K9me3 has a more restricted distribution within pericentric heterochromatin (Cowell *et al.*, 2002; Ebert *et al.*, 2004; Jacobs *et al.*, 2001; Schotta *et al.*, 2002; Fig. 1.3). The SU(VAR)3–9 protein is required for most of the H3K9me2 in pericentric heterochromatin (Ebert *et al.*, 2004; Schotta *et al.*, 2002), while the SETDB1 protein is required for most of the H3K9me2 on the fourth chromosome (Seum *et al.*, 2007). Histone H4 trimethylated at lysine 20 (H4K20me3) is also found at high concentrations in the pericentric heterochromatin in *Drosophila*, although it is also

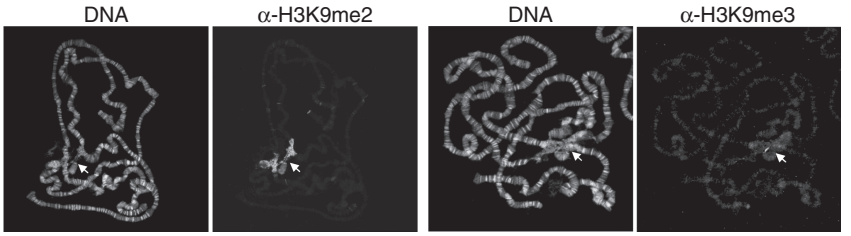

Figure 1.3 In salivary gland nuclei, all pericentric heterochromatin coalesces into a structure called the chromocenter (arrow) that stains strongly for H3K9me2 (left), a characteristic histone methylation mark of heterochromatin in *Drosophila*. In *Drosophila*, only a low amount of H3K9me3 within the core of chromocenter heterochromatin is detected in polytene chromosomes (right). Photo provided by Anja Ebert. (See Color Insert.)

widespread in euchromatin (Schotta *et al.*, 2004). In larvae lacking SU (VAR)3–9, the H3K9 dimethylase (see below), the heterochromatic concentration of H4K20me3 is markedly reduced. Strikingly, loss of HP1, a heterochromatin-associate protein that binds the H3K9me2 methyl mark (see below) results in loss of the H4K20me3 mark from both heterochromatin and euchromatin. A mutation in the gene encoding the H4K20 trimethylase, *Suv4–20*, is a dominant suppressor of heterochromatic position-effect variegation (Schotta *et al.*, 2004; but see also Sakaguchi *et al.*, 2008), suggesting that the H4K20me3 methyl mark plays a role in the establishment or maintenance of heterochromatin.

4.2. Proteins that bind heterochromatin-associated marks

Methylated H3K9 is specifically recognized by HP1-family proteins in mammal and *Drosophila* (Bannister *et al.*, 2001; Jacobs and Khorasanizadeh, 2002; Lachner *et al.*, 2001; Nielsen *et al.*, 2002; Peters *et al.*, 2002). In a genome-location analysis, dimetH3K9 was associated with loci that were also associated with hypoacetylated histones in human cells (Miao and Natarajan, 2005). HP1 colocalizes with H3K9me2 in *Drosophila* and fission yeast (Jacobs *et al.*, 2001; Noma *et al.*, 2001), and loss of methylation causes a dramatic reduction of HP1 in heterochromatin in mammalian, yeast, and *Drosophila* cells (Ebert *et al.*, 2004; Lachner *et al.*, 2001; Nakayama *et al.*, 2001; Schotta *et al.*, 2002). Targeted H3K9 methylase represses a mammalian gene *in vivo* (Snowden *et al.*, 2002), although it is not clear that the repression is by the same mechanism as that operating in heterochromatin. An HP1 chromo domain mutation that ablates PEV silencing in *Drosophila* (Platero *et al.*, 1995) also interferes with the ability of HP1 to bind H3K9me2 *in vitro* (Jacobs *et al.*, 2001), strengthening the mechanistic connection between HP1–nucleosome interactions and HP1-mediated repression. However, evidence suggests that other HP1–histone

and HP1–DNA interactions could contribute to HP1 targeting in chromosomes (Eskeland *et al.*, 2007; Perrini *et al*, 2004; Zhao *et al.*, 2000).

5. Nonhistone Proteins and Heterochromatin Targeting

5.1. Heterochromatin protein 1

One of the first genes encoding a heterochromatin-associated protein to be cloned was the *Drosophila* Heterochromatin Protein 1 (HP1; James and Elgin, 1986). It was identified as a band on SDS-PAGE among proteins from embryo nuclei that remained complexed with DNA in 0.25 M potassisum thiocyanate, but were solubilized by 1 M potassium thiocynate. This band was used to immunize mice, and a monoclonal antibody generated from these mice immunolocalized the antigen primarily to the heterochromatic chromocenter of fixed salivary gland polytene chromosomes (James and Elgin, 1986; James *et al.*, 1989). The antibody was used to screen a λgt11 expression library, resulting in the cloning of HP1 cDNA (James and Elgin, 1986).

The *in situ* hybridization of this cDNA clone to cytological region 29A on the second chromosome coincided with the map location of a genetic suppressor of heterochromatic position-effect silencing termed *Su(var)205* (Sinclair *et al.*, 1983), and molecular characterization of alleles from multiple independent screens established HP1 as a dose-dependent modifier of heterochromatin silencing (Eissenberg and Hartnett, 1993; Eissenberg *et al.*, 1990, 1992). Subsequent work shows that HP1 family proteins are found in fission yeast, animals and plants, though not in bacteria or budding yeast.

In all higher eukaryotes, at least one HP1 family protein shows a preferential localization to pericentric heterochromatin. In cases where they have been tested, these HP1 family proteins have been implicated in silencing in certain assays; all three mammalian HP1 homologs target heterochromatin when expressed in *Drosophila*, and one isoform promotes heterochromatin silencing in flies (Ma *et al.*, 2001). In other contexts, though, HP1 is required for normal transcription. It has long been known that several genes map to pericentric heterochromatin in *Drosophila*. In some cases, the normal expression of these genes has been shown to require HP1 (Lu *et al.*, 2000) and the chromatin containing such genes is enriched in HP1 (de Wit *et al.*, 2005, 2007; Greil *et al.*, 2003).

Evidence from immunolocalization of *Drosophila* HP1 on polytene chromosomes (Fanti *et al.*, 2003; James *et al.*, 1989) and from DamID genomic localization in cultured Kc cells (de Wit *et al.*, 2005, 2007; Greil

et al., 2003) shows that HP1 is enriched at certain euchromatic loci. Prominent among these in polytene chromosomes is the cytological interval 31 in the middle of the left arm of the second chromosome, a region subsequently shown to contain genes that are HP1-repressed in third instar larvae (Hwang *et al.*, 2001). At least one polytene chromosome puff site also shows significant staining, suggesting that transcriptionally active chromatin could accumulate significant amounts of HP1. This observation was extended by Piacentini *et al.* (2003), who found HP1 associated with heat shock puffs as well. HP1 is probably not targeted by directly binding to nascent RNA, since HP1 binding in polytene chromosomes is not significantly affected by RNase digestion (Piacentini *et al.*, 2003) although some studies suggest RNA contributes to HP1 binding (Muchardt *et al.*, 2002).

The first immunolocalization of HP1 in polytene chromosomes also revealed significant concentrations of HP1 at telomeres (James *et al.*, 1989). Although telomeres are associated with transgene silencing in *Drosophila* (Cryderman *et al.*, 1999b; Golubovsky *et al.*, 2001; Hazelrigg *et al.*, 1984; Karpen and Spradling, 1992; Levis *et al.*, 1993; Marin *et al.*, 2000; Wallrath and Elgin, 1995), the telomeric silencing mechanism in *Drosophila* is, for the most part, genetically distinct from centromeric silencing (Donaldson *et al.*, 2002; Mason *et al.*, 2004). Mutations in *tefu*, the *Drosophila* ATM homologue, reduce the amount of HP1 at telomeres and cause a recessive suppression of telomeric silencing (Oikemus *et al.*, 2004), suggesting an indirect mechanistic link between HP1 binding at telomeres and telomeric silencing.

Mutations in the *Drosophila* HP1-encoding gene *Su(var)2–5* are recessive lethal. The lethal period was mapped to the third larval instar by temperature shift experiments using a *Hsp70*-HP1 transgene (Eissenberg and Hartnett, 1993) and by using larval cuticle markers (Lu *et al.*, 2000). Loss of heterochromatic silencing and reduced expression of heterochromatic genes were noted in larvae approaching the lethal period (Lu *et al.*, 2000), suggesting a failure of HP1-dependent gene regulation could contribute to lethality. However, adult flies rescued by HP1 expression in midlarval development show eye and wing defects suggesting defects in imaginal disc cell proliferation and behavioral defects consistent with CNS defects (Eissenberg and Hartnett, 1993). Detailed examination of neuroblasts in HP1 mutant larvae revealed extensive telomeric fusions (Fanti *et al.*, 1998; Perrini *et al.*, 2004). These fusions could result in proliferation defects in the dividing cells of the imaginal disks and CNS, and suggest that HP1 also plays an essential role in telomere capping in *Drosophila*.

In biochemical fractionation, HP1 is associated with the telomeric HOAP protein (Badugu *et al.*, 2003; Shareef *et al.*, 2003). HOAP is encoded by the *caravaggio* locus, and mutations in *caravaggio* cause telomere fusions in larval neuroblasts that resemble those seen in HP1 mutant neuroblasts (Cenci *et al.*, 2003b). Thus, HOAP and HP1 are thought to be essential

components of the *Drosophila* telomere capping complex, although it is unknown whether HP1 is targeted to telomeres by HOAP itself or through a distinct mechanism (Cenci *et al.*, 2003b). HOAP is also enriched in the pericentric heterochromatin, and *caravaggio* mutations dominantly suppress heterochromatic silencing (Badugu *et al.*, 2003).

In situ measurements of HP1 family proteins in mammalian cells using fluorescence recovery after photobleaching (FRAP) indicate that much of nuclear HP1 is remarkably dynamic (Cheutin *et al.*, 2003; Festenstein *et al.*, 2003). In heterochromatic regions, most HP1 turns over within 60–200 s. This observation is consistent with the mass action model of Locke *et al.* (1988), which invokes a dynamic equilibrium between dissociated and assembled heterochromatic subunits. However, the original protocol used to identify HP1 in *Drosophila* demonstrates that a fraction of HP1 is sufficiently tightly bound to chromatin in embryos to resist extraction by moderate concentrations of chaotropic agents (James and Elgin, 1986). FRAP studies in transgenic *ex vivo* T cells indicate that ca. 30% of heterochromatic HP1 is immobile (Festenstein *et al.*, 2003), while in Hep-2 cells this represents ca. 5% of HP1 in heterochromatin (Schmiedeberg *et al.*, 2004). Perhaps this relatively immobile fraction corresponds to the tightly bound HP1 in *Drosophila*, and could account for the stability of heterochromatic silencing.

Drosophila HP1 was the first characterized protein with a chromo domain motif (Eissenberg, 2001b; Eissenberg and Khorasanizadeh, 2005). The HP1 chromo domain is located in the N-terminal half of all HP1 family proteins (Eissenberg and Elgin, 2000). Subsequent sequence comparisons identified a second chromo domain motif in the C-terminal half of HP1 family proteins, called the ''chromo shadow domain'' (Aasland and Stewart, 1995; Epstein *et al.*, 1992; Koonin *et al.*, 1995). The HP1 chromo domain is sufficient to target heterochromatin *in vivo*, and a point mutation in the *Drosophila* HP1 chromo domain inactivates the ability of the protein to contribute to heterochromatin silencing (Platero *et al.*, 1995). Structural studies revealed that the chromo domain is a high-affinity binding site di- and tri-methylated lysine 9 of histone H3 (Jacobs and Khorasanizadeh, 2002; Nielsen *et al.*, 2002). The chromo shadow domain is also capable of targeting heterochromatin *in vivo* (Powers and Eissenberg, 1993). Structural studies show that the HP1 family chromo shadow domain is a self-association motif (Brasher *et al.*, 2000; Cowieson *et al.*, 2000). Self-association through the chromo shadow domain could explain the heterochromatin-targeting ability of this domain in a nucleus containing endogenous HP1. Deletions that remove part or all of the *Drosophila* HP1 chromo shadow domain also abolish silencing activity (Eissenberg and Hartnett, 1993; Eissenberg *et al.*, 1992).

Multiple mechanisms could target HP1 to distinct chromosomal sites directly or by recruiting one of the four known histone H3K9

methylase orthologs in *Drosophila* [SU(VAR)3–9, G9A, SETDB1, and EU-HMTase 1], resulting in HP1 binding. Loss of SU(VAR)3–9 leads to a dramatic reduction in HP1 levels in pericentric heterochromatin (Schotta *et al.*, 2002), while loss of SETDB1 leads to a dramatic reduction of HP1 at nontelomeric sites in euchromatin and along the euchromatic fourth chromosome (Seum *et al.*, 2007). However, *in vitro* studies indicate that HP1 can bind to nucleosomes lacking tails, and that nonhistone proteins may contribute to HP1 binding to methylated chromatin (Eskeland *et al.*, 2007; Zhao *et al.*, 2000).

An RNAi mechanism is required for HP1-mediated silencing in fission yeast, and one study suggests that this mechanism may also occur in flies (Pal-Bhadra *et al.*, 2004; Verdel *et al.*, 2004; Volpe *et al.*, 2002). Since transposon transcripts are an important biological target for RNAi, transposons could mediate heterochromatin formation through an RNAi mechanism. In *Drosophila*, a study mapping HP1 binding sites in >3 Mb of DNA using the DamID method found 17 regions of significant HP1 binding, all but one of which were TE or other repeated elements (Sun *et al.*, 2003). This is consistent with a role for 1360/*hoppel* transposons in mediating HP1-dependent silencing on the fourth chromosome (see above). Using an array containing over 6200 cDNA fragments, Greil *et al.* (2003) found significant HP1 concentrations in pericentric and subtelomeric sequences in chromatin from the Kc cell line. Thus, repetitious DNA, rather than any particular DNA sequence, may be an important sequence determinant in heterochromatin formation.

5.2. Su(var)3–7

SU(VAR)3–7 was the second heterochromatin-associated protein to be cloned in *Drosophila* (Reuter *et al.*, 1990). Its gene product, SU(VAR)3–7, is a seven zinc finger protein that binds DNA *in vitro* (Cléard and Spierer, 2001; Cléard *et al.*, 1995), binds HP1 and is enriched in pericentric heterochromatin (Cléard *et al.*, 1997; Delattre *et al.*, 2000). Like HP1, it is essential (Seum *et al.*, 2002) and it is a dosage dependent modifier of heterochromatic position-effect variegation (Reuter *et al.*, 1990). Importantly, however, HP1 binding to the chromocenter does not require SU(VAR)3–7 (Spierer *et al.*, 2005).

When SU(VAR)3–7 is overexpressed from a heat shock inducible transgene, the protein binds extensively to all the euchromatic arms in polytene chromosomes (Delattre *et al.*, 2004). Ectopic SU(VAR)3–7 binding is accompanied by increased euchromatic levels of dimetH3K9 and this increased dimetH3K9 depends on the heterochromatic H3K9 methylase SU(VAR)3–9 (see below). Additionally, SU(VAR)3–7 overexpression results in increased euchromatic accumulation of HP1, which also depends on SU(VAR)3–9. These findings point to model in which SU(VAR)3–7

recruits the H3K9 methylase SU(VAR)3–9, which dimethylates H3K9, resulting in ectopic HP1 binding (Delattre *et al.*, 2004).

Unlike HP1, however, SU(VAR)3–7 does not belong to an evolutionarily conserved family of heterochromatin-associated proteins. Either the SU(VAR)3–7:HP1 interaction and the role of SU(VAR)3–7 in H3K9 dimethylation is a specialized adaptation of *Drosophila* or the homologous zinc finger protein that fulfills this role in other organisms has diverged so much as to be unrecognizable.

5.3. Su(var)3–9

Su(var)3–9 was first identified in *Drosophila* as a gene whose mutations cause strong dominant suppression of heterochromatic silencing (Tschiersch *et al.*, 1994). The protein product of this gene contains a chromo domain motif—a binding motif for methylated lysine—and a SET domain, the catalytic domain of histone methylases.

Biochemical and yeast two-hybrid protein analysis demonstrate that the heterochromatin-associated protein SU(VAR)3–9 interacts directly with HP1 (Aagaard *et al.*, 1999; Schotta *et al.*, 2002). This interaction is consistent with the colocalization of HP1 and SU(VAR)3–9 proteins in the *Drosophila* pericentric heterochromatin (Fig. 1.4). Studies in *Drosophila*, fission yeast, and mammals demonstrate that SU(VAR)3–9 family proteins are H3K9 methylating enzymes (Czermin *et al.*, 2001; Ebert *et al.*, 2004; Eskeland *et al.*, 2004; Nakayama *et al.*, 2001; Rea *et al.*, 2000; Schotta *et al.*, 2002) and that HP1 family proteins bind specifically to the H3K9me2 methyl mark (Bannister *et al.*, 2001; Jacobs and Khorasanizadeh, 2002; Jacobs *et al.*, 2001; Lachner *et al.*, 2001; Nielsen *et al.*, 2002). These findings have coalesced around an attractive cascade model for HP1 family protein recruitment and the assembly and spreading of HP1-dependent heterochromatin: (1) SU(VAR)3–9 proteins are recruited to specific chromosomal sites (by an unknown mechanism) and through its HP1-binding activity, recruits HP1; (2) SU(VAR)3–9 methylates H3K9 residues on nearby nucleosomes; (3) additional HP1 binds to the newly methylated nucleosome; (4) more SU

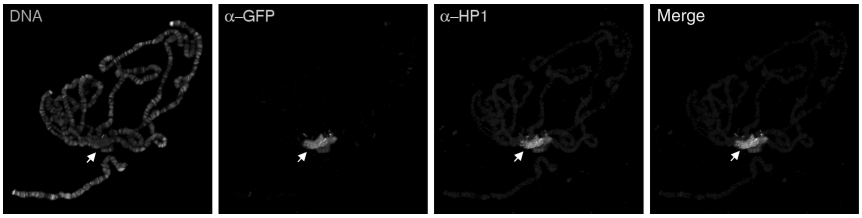

Figure 1.4 The SU(VAR)3–9 and HP1 proteins colocalize in chromocenter heterochromatin. Photo provided by Anja Ebert. (See Color Insert.)

(VAR)3–9 protein is recruited by the additional HP1; and (5) the additional SU(VAR)3–9 protein methylates adjacent nucleosomes, which creates new HP1 binding sites, etc. Loss of HP1 results in increased H3K9 dimethylation in the euchromatin of polytene chromosomes, consistent with the model that SU(VAR)3–9:HP1 interaction constrains SU(VAR)3–9 histone methylating activity to heterochromatin (Ebert *et al.*, 2004).

A mutation in the SU(VAR)3–9 SET domain, *pitkin*^{D}, is a strong dominant enhancer of heterochromatic position effect (Ebert *et al.*, 2004; Kuhfittig *et al.*, 2001). *pitkin*^{D} behaves as a hypermorphic allele of *Su(var)3–9*, and results in increased euchromatic H3K9me2 (Ebert *et al.*, 2004). Gene silencing in heterochromatic rearrangements is suppressed by *Su(var)3–9*, *Su(var)2–5* (encodes HP1), *Suv4–20*, and *Su(var)3–1* (encodes the JIL-1 protein kinase) mutations, reflecting a silencing pathway initiated by SU(VAR)3–9-dependent H3K9 dimethylation, a mark recognized by HP1 which anchors SU(VAR)3–9 to heterochromatin and recruits the H4K20me3-catalyzing SUV4–20 histone methyltransferase (Schotta *et al.*, 2002, 2004).

Spreading of heterochromatic H3K9 methylation into euchromatin is normally inhibited by the JIL-1 kinase (Ebert *et al.*, 2006; Lerach *et al.*, 2006; Zhang *et al.*, 2006), which phosphorylates serine 10 of histone H3 (H3S10) in euchromatin. Surprisingly, loss-of-function mutations in JIL-1 suppress PEV of *white* in the classical variegating rearrangement w^{m4}, but enhance PEV of *white* when the gene is inserted within pericentric heterochromatin on a P-element transposon (Bao *et al.*, 2007). The suppression of w^{m4} could be explained by ectopic relocalization of SU(VAR)3–9 and HP1 proteins to euchromatin from pericentric heterochromatin, reducing the ability of the remaining heterochromatic complexes to spread across the w^{m4} breakpoint and silence *white* at a distance. The enhancement of *white* transposon silencing could reflect the local loss of H3S10 phosphorylation at the *white* and the local shift in the euchromatin–heterochromatin balance in favor of heterochromatin. The mechanism by which H3S10 phosphorylation antagonizes heterochromatin appears to involve competition with SU(VAR)3–9, since the lethality associated with loss of JIL-1 can be partially rescued by loss of SU(VAR)3–9 (Deng *et al.*, 2007). Taken together, these results point to a role of JIL-1 and/or H3K10 phosphorylation in the spatial restriction of heterochromatin in the nucleus.

5.4. Origin recognition complex

Studies in budding yeast have implicated components of replication origins in the establishment and maintenance of silencing at the silent mating type cassettes (Laurenson and Rine, 1992). Since some of the key proteins involved in yeast silencing have no clear homologs in *Drosophila*, and since budding yeast lacks both cytologically visible heterochromatin and homologs of some of the key players implicated in *Drosophila*

heterochromatin (e.g., HP1, SU(VAR)3–9, methylation of H3K9, the RNAi mechanism, etc.), it is not clear that replication origins in *Drosophila* play an analogous role in heterochromatic silencing. Antibody to the *Drosophila* origin recognition complex subunit Orc2 shows significant concentration in apical regions of interphase nuclei and in pericentromeric regions of metaphase chromosomes at all stages of syncytial embryos, irrespective of whether cytologically visible heterochromatin is present (Pak *et al.*, 1997). Upon cellularization, embryonic Orc2 is primarily detectable in extrachromosomal material at the metaphase plate in anaphase cells, but shows preferential localization to pericentric heterochromatin in metaphase chromosomes of SL2 cultured cells (Pak *et al.*, 1997). In the giant polytene chromosomes of larval salivary glands, little or no selective association with pericentric heterochromatin by immunostaining of polytene chromosomes is evident (Pak *et al.*, 1997), suggesting that interaction with HP1 in the heterochromatin of these nuclei is limited at best. Interestingly, orc-containing protein complexes copurify with HP1 and HOAP, both of which are found at telomeres and act to prevent telomere fusions in mitotic cells (discussed above). Thus, although origin recognition complexes appear to be enriched in the pericentric regions of mitotically cycling chromosomes, there is no compelling evidence that replication origins are mechanistically involved in heterochromatin assembly in *Drosophila*.

5.5. Cohesins and heterochromatin

In fission yeast, interaction between cohesin and the HP1 family protein Swi6 is important for the establishment of sister chromatid cohesion in centromeric heterochromatin (Bernard *et al.*, 2001; Nonaka *et al.*, 2002). However, cohesions and HP1 show little or no colocalization in *Drosophila* polytene chromosomes, particularly in the heterochromatic chromocenter (Gause *et al.*, 2008). Moreover, mitotic chromosomes from larvae lacking HP1 show no significant loss of sister chromatid cohesion or defects in polytene chromosome organization (Fanti *et al.*, 1998), while loss of cohesin function leads to cohesion defects in mitotic cells (Gause *et al.*, 2008) and altered polytene chromosome morphology (Dorsett *et al.*, 2005). Thus, the evidence from *Drosophila* suggests that interactions between cohesin and HP1-family proteins in heterochromatin are not an evolutionarily conserved feature of sister chromatid cohesion.

5.6. Artificial targeting proteins and ectopic heterochromatin

Studies in *Drosophila* have demonstrated that heterochromatin-like properties can be targeted to euchromatic sites by tethering HP1 protein. Seum *et al.* (2001) tethered HP1, expressed as a Gal4-HP1 fusion protein, to transgene reporters inserted at euchromatic sites. One of six transgene

insertion sites showed GAL4-HP1-dependent variegated silencing; the silencing required the GAL4 binding site and was suppressed by reducing dosage of *Su(var)3–7*, a gene that encodes a heterochromatin-associated protein and silencing modifier (discussed earlier). It was also associated with ectopic pairing to sites of intercalary heterochromatin in polytene chromosomes, another hallmark of heterochromatin. To explain the exceptional behavior of the transposon that did show silencing, the authors note that this transgene was inserted into a copy of the micropia retrotransposon, and near a cluster of middle repetitive elements that are mostly found in centric heterochromatin. Thus, like the case of the 1360/*hoppel* element and fourth chromosome position effects,★★★ ectopic heterochromatin formation with tethered HP1 appears to require a chromosomal context containing nearby transposons and/or middle repetitive DNA. Importantly, transgenes that do not show silencing still bind the GAL4-HP1 fusion protein, as was seen previously for *white* transgene arrays (Fanti *et al.*, 1998). This is consistent with a heterochromatin mass action model in which subthreshold amounts of heterochromatic factors assemble at non-silenced arrays, and a conversion to silencing results from juxtaposition with other chromosomal regions that attain sufficient concentrations of factors to establish stable silencing.

Somewhat different results are obtained when HP1 is tethered as a HP1–lacI fusion protein to tandem arrays of 4–256 lacI binding sites (Li *et al.*, 2003). For 25/26 euchromatic transgene insertion sites, tethering of HP1 resulted in reduced expression of the mini-*white* transgene marker and a large fraction of polytene chromosomes showed ectopic fibers linking the transgene sites to other chromosomal loci, as is seen with intercalary heterochromatin. However, in no case was variegated silencing typical of heterochromatic position effect observed. Also in contrast to classical heterochromatic position effect, no significant accumulation of H3K9me2 was observed even though SU(VAR)3–9 is recruited to the loci where HP1 is tethered. Furthermore, the repression imposed by tethered HP1 was insensitive to loss of SU(VAR)3–9 protein, suggesting that the mechanism of HP1-mediated repression in these tethered HP1 transgenes differs in significant respects from heterochromatin silencing.

In follow-up studies, the effects of tethering HP1 to 256-copy lac I arrays on transgene chromatin structure was investigated (Danzer and Wallrath, 2004). Consistent with previous reports on the effects of classical heterochromatin silencing on transgene chromatin structure (Sun *et al.*, 2001; Wallrath and Elgin, 1995), the repression associated with HP1–lac I arrays is associated with ordered nucleosome arrays and increased resistance to restriction endonuclease cleavage. Surprisingly, the repression of an Hsp 26 heat shock promoter located on one side of the lac I array was independent of SU(VAR)3–9, while repression of an Hsp70 heat shock promoter on the other side of the array is completely relieved in the absence

of SU(VAR)3–9. Taken together, the results of HP1 tethering experiments suggest that HP1 targeting alone is insufficient to nucleate heterochromatin formation, but that HP1 can impose transcriptional repression and modify chromatin by multiple mechanisms.

6. Nuclear Associations and Heterochromatin in *Drosophila*

6.1. *Trans*-inactivation

Most examples of heterochromatic position-effect silencing are recessive; the function of an unrearranged allele masks the silencing of the rearranged allele. A notable exception to this is dominant variegation for the *brown* [*bw*] locus. Rearrangements variegating for the *brown* [*bw*] eye pigment gene cause dominant inactivation of the wild type *bw* allele in *trans* to heterochromatin (Slatis, 1955), a phenomenon termed ''*trans*-inactivation'' (Henikoff and Dreesen, 1989).

In the case of $brown^{Dominant}$ (bw^{D}), a 1–2 Mb block of heterochromatic satellite sequence has been inserted into the *brown* (*bw*) locus. However, in contrast to null alleles of *brown*, which are recessive, the bw^{D} allele causes variegation of a wild type *bw* allele in *trans*. The silenced *bw* allele acquires neither the condensed cytological appearance of heterochromatin nor recruits detectable amounts of HP1 in polytene chromosomes (Belyaeva *et al.*, 1997). This suggests that dominant silencing of *bw* allele in *trans* by bw^{D} occurs by a distinct mechanism from other forms of heterochromatic silencing. Detailed confocal microscopic analysis, combined with immuno-FISH, showed that silencing by bw^{D} is correlated with association with pericentric heterochromatin (Csink and Henikoff, 1996). The idea that dragging a euchromatic locus into proximity with pericentric heterochromatin could silence that locus is consistent with a mass action model of chromatin assembly. However, it seems unlikely that physical proximity alone would be sufficient, since the nearby essential genes *chrw* (ca. 6 kb downstream of *bw*) and *wmd* (ca. 6 kb upstream of *bw*) would otherwise be inactivated. Indeed, a later study demonstrated that heterochromatic associations are compatible with an active *brown* allele (Sass and Henikoff, 1999). Thus, proximity to a domain of heterochromatin alone is not sufficient to confer heterochromatic silencing; such nuclear associations may be a concomitant of, but not causative of, heterochromatic silencing.

Trans-inactivation is not limited to the *brown* locus in *Drosophila*. Martin-Morris *et al.* (1997) and Dorer and Henikoff (1997) described *white* transgenes that variegate due to *cis*-inactivation by pericentric heterochromatin and that can impose heterochromatic silencing on a transgene located at a homologous position in *trans*. Moreover, they present evidence suggesting

that a neighboring essential gene is silenced in *trans* due to spreading of heterochomatic silencing from the *trans*-inactivated allele. These remarkable findings provide particularly clear and dramatic examples of position effects, reflecting the ability of local concentrations of heterochromatic factors to drive the establishment of ectopic heterochromatin domains on nearby loci, either in *cis* or in *trans*.

6.2. Heterochromatin associations

Several studies in yeast strongly implicate regions around the nuclear membrane in promoting transcriptional silencing (Andrulis *et al.*, 1998; Gartenberg *et al.*, 2004; Gasser *et al.*, 1998; Hediger *et al.*, 2002; Taddei *et al.*, 2004). The relationship between silencing and proximity to the nuclear membrane is not a simple one; however, the nuclear pore complex, imbedded in the nuclear membrane, has been implicated in transcriptional activity (Casolari *et al.*, 2004; Schmid *et al.*, 2006). Live-cell measurements have also shown that genes move to the nuclear envelope upon transcriptional activation (Cabal *et al.*, 2006; Drubin *et al.*, 2006; Taddei *et al.*, 2006).

In a rigorous study comparing nuclear associations and heterochromatic silencing on a cell-by-cell basis for three different variegating loci, Harmon and Sedat (2005) found that in the nuclei of cells in which the variegating gene was silenced, the association of the silenced locus with heterochromatin was significantly more intimate than in neighboring cells of the same tissue in which the locus escaped silencing. Oddly, however, they found that in mitotically cycling, undifferentiated cells where silencing of one of the rearrangements occurs in all cells, there was no difference in the extent of associations between these cells and differentiated cells in the same tissue, where variegated activation occurs. This suggests that the associations have no necessary functional relationship to silencing.

In mammals, correlations have also been noted between transcriptionally silent loci and blocks of heterochromatin (Brown *et al.*, 1997). The timing of such associations, however, suggests that they are not an obligatory part of the silencing mechanism (Brown *et al.*, 1999). The inactive X in mammalian females is associated with the nucleolus in mid-to-late S phase (Zhang *et al.*, 2007). An ectopic X inactivation center can target an autosome to the nucleolus, and deletion of the gene encoding the Xist noncoding RNA results in loss of nucleolar targeting (Zhang *et al.*, 2007). Such interactions suggest a physical affinity of certain types of chromatin, but the consequences for gene expression of these interactions may depend on additional factors besides physical proximity.

Taken together, the data concerning gene silencing and nuclear positioning are most consistent with a model in which associations between specific silent loci and certain nuclear regions are correlative but not causative. A simple way to think about this is that certain protein complexes that

promote silencing are recruited to silenced genes, that these complexes are shared with other chromosomal regions (including, in some cases, heterochromatin), and that aggregation between distant chromosomal regions may be facilitated by the affinities of shared complex components for one another. By this model, the association of a variegating gene with heterochromatin is a byproduct of gene silencing. Though a reinforcing or stabilizing role for heterochromatic associations is not excluded by this model, the targeting of heterochromatin to a silenced locus does not appear to require heterochromatic associations.

7. Summary and Perspectives

What has *Drosophila* taught us about heterochromatin? Heterochromatic position-effect silencing was first described in *Drosophila* (Muller, 1930) and genetic screens for modifiers of such position effects uncovered key factors in heterochromatin assembly (Dorn *et al.*, 1986; Sinclair *et al.*, 1983; Wustmann *et al.*, 1989). Many of these factors are structurally and functionally conserved.

The findings in *Drosophila* have pointed to a mechanism of heterochromatin assembly in which multiple factors contribute to silencing and in which cooperative protein complex assembly plays an important role. Transgene studies in *Drosophila* have emphasized the role both of specific sequences and proteins, and the cooperativity of interactions that are required to target heterochromatin assembly. As neatly summarized by Yasuhara and Wakimoto (2008): "... it is not repetitive sequences *per se* that specifies heterochromatin but the physical proximity of multiple repetitive sequences of a certain type... [suggesting] versatility in the activities of different types of heterochromatin-enriched repetitive DNA sequences and modified histones and... the importance of chromosomal context."

Cloning and characterization of heterochromatic DNA and genetic modifiers of PEV in *Drosophila* allowed the study of heterochromatin to join the mainstream of molecular biology. Versatile genetics and extraordinary cytology account for the important role *Drosophila* has played in our understanding of heterochromatin and its formation. With the advent of genome sequencing, chromatin immunoprecipitation and microarray analysis, the advantage of a high-resolution genome-wide map afforded by polytene chromosome cytology has been eclipsed. The genetic toolkit of *Schizosaccharomyces pombe* has also come to complement the genetics of *Drosophila* in this field. To the extent that *Drosophila* will continue to offer unique insights into heterochromatin assembly and function, this will be primarily as a model metazoan. The role, if any, of heterochromatin in development, differentiation, cell signaling and aging is unclear.

The genetic dissection of each of these processes is advancing rapidly using *Drosophila*, and numerous examples of control at the level of chromatin have already been uncovered, including HP1 and heterochromatic histone marks. Many of the mechanisms used to establish heterochromatin in *Drosophila* are likely to underlie mechanisms of growth, development, and aging in *Drosophila* and other animals.

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