FOR GENES THAT ENCODE ONE COMPONENT OF A MULTIMERIC PROTEIN COMPLEX, MEASURING ONLY ONE PHENOTYPE OFTEN GIVES A BIASED VIEW OF FUNCTION: SU(VAR)3-9 AND CHROMATIN ARCHITECTURE AS AN EXAMPLE.

by

Pamela Kalas
ABSTRACT

Eukaryotic genomes are organized into chromatin, a highly dynamic complex of DNA and proteins, which plays a critical role in the regulation of genes expression. This thesis focuses on the study of a non-histone chromatin protein, the SET domain-containing H3K9 methyltransferase (HMTase) SU(VAR)3-9, and its role in the packaging and regulation of a euchromatic locus, the histone genes cluster (HIS-C). SU(VAR)3-9 was discovered in Drosophila melanogaster, but it is highly conserved from yeast to mammals. It has two conserved domains, the chromo- and the SET domains, and both are required for its function in gene silencing. The SET domain is responsible for the catalytic activity of SU(VAR)3-9, while the exact function of the chromo domain is still unclear.

To gain an insight on the role(s) of SU(VAR)3-9 in the regulation of gene silencing, we first characterized a collection of Su(var)3-9 EMS-induced mutants that had been isolated in a genetic screen for strong, dominant suppressors of position-effect variegation (PEV). These mutants were characterized at the molecular, enzymatic, and cellular level, and their effect on gene silencing was also examined. We found that all mutants have single amino acid substitutions in the conserved preSET/SET/postSET domain, and that they all display a dramatic or complete loss of HMTase activity, strongly suggesting that suppression of PEV is linked to SU(VAR)3-9’s ability to methylate H3K9.

The HIS-C is a natural, euchromatic target of SU(VAR)3-9, and mutations in Su(var)3-9 can alter its chromatin structure. To investigate the exact role(s) of SU(VAR)3-9 in the regulation of this locus, we analyzed the effects of a series of
Su(var)3-9 missense mutants on the chromatin architecture of the HIS-C and on the expression of the histone genes. We detected a drastic reduction in the levels of H3K9me2 and HP1 associated with the his genes in all Su(var)3-9 missense mutants, although the mutant SU(VAR)3-9 still associate with the HIS-C. In addition, these mutants have elevated amounts of histone H2A and histone H3 RNA, suggesting that the enzyme function of SU(VAR)3-9 is critical for the regulation of the histone genes.
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<th>Definition</th>
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<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced-green fluorescent protein</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione-S-transferase</td>
</tr>
<tr>
<td>H3K4</td>
<td>Lysine 4 of histone H3</td>
</tr>
<tr>
<td>H3K9</td>
<td>Lysine 9 of histone H3</td>
</tr>
<tr>
<td>H3K27</td>
<td>Lysine 27 of histone H3</td>
</tr>
<tr>
<td>H4K20</td>
<td>Lysine 20 of histone H4</td>
</tr>
<tr>
<td>HDAC1</td>
<td>Histone deacetylase 1</td>
</tr>
<tr>
<td>HIS-C</td>
<td><em>Histone Gene</em> cluster</td>
</tr>
<tr>
<td>HP1</td>
<td>Heterochromatin Protein 1</td>
</tr>
<tr>
<td>MNase</td>
<td>Micrococcal nuclease</td>
</tr>
<tr>
<td>MOF</td>
<td>Males absent On the First</td>
</tr>
<tr>
<td>PEV</td>
<td>Position-effect variegation</td>
</tr>
<tr>
<td>PC</td>
<td>Polycomb</td>
</tr>
<tr>
<td>RTase</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription-PCR</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Su(var)</td>
<td>Suppressor of position-effect variegation</td>
</tr>
</tbody>
</table>
CO-AUTHORSHIP STATEMENT

A version of chapters 2 and 3 of this thesis will be submitted for publication.

I (P. Kalas) performed all the experiments, and the data analysis, presented in
this thesis. Dr T.A. Grigliatti supervised the project and provided assistance with
experimental design and editing of this manuscript.
1. INTRODUCTION


In metazoans, each somatic cell contains the same genetic information, but the fate and specificity of the cells comprising various tissue types is determined by and dependent upon the expression of different subsets of genes. While some genes go through "on" (expressed) and "off" (not expressed) states throughout the whole life of the organism, others remain permanently "off" in certain cell types, or after a certain stage of development. So, for example, nerve-specific genes are not expressed in muscle cells, and mitosis-specific genes are not expressed in cells that are not dividing anymore. Failure to express, or conversely, failure to silence a particular set of genes at the appropriate time in a specific cell type can have dramatic consequences such as congenital malformations, cancer, or death (reviewed for example by Ausio et al., 2003; Oligny, 2003; Jaffe, 2003; Moss and Wallrath, 2007; Nelson et al., 2007). Indeed, tight, accurate regulation of gene expression is absolutely crucial for the survival of any organism.

Gene expression is regulated at many levels. At the transcriptional level, protein complexes assembled on regulatory sequences such as enhancers interact with the transcriptional machinery assembled on a gene’s promoter(s), stimulating gene transcription. However, in order for this to happen, the gene in question must first be "transcriptionally competent", or in other words, in a form that makes it accessible to transcription factors. In prokaryotes, the entire genome is transcriptionally competent, that is, the DNA is readily accessible to regulatory proteins. However, this is not the case in eukaryotes.
The eukaryotic genome is subdivided into chromosomes and organized and compacted into chromatin, a complex of DNA and proteins (see below, section 1.2.). Chromatin exists in different forms, depending on its level of compaction, the presence of specific proteins, and particular post-translational modifications of some of its components (recently reviewed by Ebert et al., 2006; Razin et al., 2007; Kouzarides, 2007). Some forms, often referred to as "open chromatin" or euchromatin, are more accessible to the transcriptional machinery, while "silent chromatin", or heterochromatin, in contrast, is typically refractory to transcription. Chromatin is extremely dynamic, having the ability to convert, under the appropriate circumstances, from an open to a silent conformation, and vice-versa. By modulating the chromatin architecture of certain regions, cells are able to make genes, and entire chromosome domains, transcriptionally competent or silenced (for a review see for example Struhl, 1999; Talbert and henikoff, 2006). Hence, in order to fully understand the biological process of gene regulation, it is necessary to uncover the mechanisms underlying chromatin biology and chromatin architecture. This thesis focuses on the study of SU(VAR)3-9, a key non-histone chromatin protein with a histone methyltransferase activity, and on its roles in modulating the packaging and thus the regulation of a euchromatic locus, the histone gene cluster.

1.2. The components of chromatin.

Chromatin is a dynamic, highly organized complex of DNA and proteins. Histones are the most abundant chromatin proteins, but there are also numerous non-
histone chromatin proteins (NHCPs) that have crucial roles in regulating and maintaining chromatin architecture.

1.2.1. Histones

The basic unit of chromatin is the nucleosome, 146 bp of DNA wrapped around an octamer consisting of two copies of each of the core histones, H2A, H2B, H3 and H4 (Finch et al., 1977; Klug et al., 1980; Luger et al., 1997), and chromatin at its simplest can be described as an array of nucleosomes (Kornberg, 1977). Although devoid of enzymatic activity, histones are far from being inert structural components of chromatin. Post-translational modifications of their N-terminal tails play an active role in recruiting and/or stabilizing the binding of non-histone chromatin proteins (NHCPs) to the chromatin fibre. To date, about a dozen histone modifications have been described, including methylation, acetylation, ubiquitylation and SUMOylation of lysines, phosphorylation of serines and threonines, methylation of arginines, and ADP-ribosylation (for a systematic review of the current nomenclature, see Turner, 2005). Table 1.1. summarizes the histone modifications thus far described in Drosophila, and the enzymes catalyzing them.

The availability of antibodies specific for most histone modifications, in combination with high throughput analyses in yeast, Drosophila and mammals have been providing high resolution, genome-wide maps of histone post-translational modifications (reviewed by Schones and Zhao, 2008). This has allowed the systematic study of the correlations between the chromatin structure and the transcriptional state of a locus (Crawford et al., 2006), as well as the
presence of particular histone modifications in that region. In all organisms analyzed, including yeast, flies and mammals, acetylated histone H3 (H3ac) and methylated lysines 4 and 36 of histone H3 (H3K4me, H3K36me) are enriched in regions corresponding to active promoters and transcribed genes (Roh et al., 2005; Schubeler et al., 2004; Liu et al., 2005; Bernsterin et al., 2005; Kim et al., 2005; Pokholok et al., 2005; Barski et al., 2007). In animals, the presence of phosphorylated histone H3 serine 10 (H3S10ph) is also characteristic of transcriptionally competent regions of the genome (Wang et al., 2001; Ebert et al., 2004; 2006). On the other hand, di- and tri-methylated histone H3 lysines 9 and 27, and methylated histone H4 lysine 20 (H3K9me2,3, H3K27me2,3, H4K20me3) are typically associated with large heterochromatic regions (Schotta et al., 2002; 2004; Peters et al., 2002; Ebert et al., 2004; 2006).

Two main mechanisms have been proposed to be responsible for the "translation" of a given set of histone modifications into a particular transcriptional state of the region in question. One of them postulates that the addition of a charged group (such as an acetyl or a phosphate group) to the histone tail can cause a localized decondensation of the chromatin fibre, making it accessible to the transcriptional machinery (Turner, 2000). The other, more widely applicable, proposes that specific histone modifications, or combinations thereof, serve as a binding platforms for specific chromatin proteins (for example Lachner and Jenuwein, 2002; de la Cruz et al., 2005). Evidence exists in support of both models, suggesting that both mechanisms probably play a role. For instance, it has been demonstrated that in vitro reconstituted chromatin arrays can form compact
fibres, but this compaction is prevented when H4K16 is acetylated, suggesting that the acetyl group could directly affect chromatin architecture (Shogren-Knaak et al., 2006; Chodaparambil et al., 2007).

However, histone modifications have also been widely shown to affect chromatin structure and gene regulation via the action of NHCPs. The chromodomain of HP1 has been shown to recognize and bind to H3K9me2,3 (Lachner et al., 2001; Jacobs et al., 2001; Fischle et al., 2003), while the chromodomain of Polycomb specifically recognizes H3K27me2,3 (Cao et al., 2002) and those of CHD1 and CHD3 proteins bind to H3K4me and H3K36me, respectively (reviewed by Mellor, 2006). In contrast, bromodomains typically recognize acetylated histone residues (reviewed by Yang, 2004; Mujtaba et al., 2007).

The next section will focus on the roles of lysine methylation, since histone lysines are the target of SU(VAR)3-9’s methyltransferase activity, and have important functions in the regulation of gene silencing as well as transcriptional competence.
Table 1.1. Post-translational modifications of canonical histones that have been formally detected in *Drosophila*.

<table>
<thead>
<tr>
<th>MODIFICATION</th>
<th>PREDOMINANT LOCATIONS</th>
<th>ENZYME(S) RESPONSIBLE</th>
<th>SELECTED REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3K9me</td>
<td>IV chromosome</td>
<td>DmSetDB1</td>
<td>Seum <em>et al.</em>, 2007; Tzeng <em>et al.</em>, 2007.</td>
</tr>
<tr>
<td>H3K9me1,2</td>
<td>Heterochromatin</td>
<td>SU(VAR)3-9, dG9a</td>
<td>Schotta <em>et al.</em>, 2002; 2003; Eskeland <em>et al.</em>, 2004; Mis <em>et al.</em>, 2006; Stabell <em>et al.</em>, 2006.</td>
</tr>
<tr>
<td>H3K9me</td>
<td>Euchromatic sites</td>
<td>dG9a, SU(VAR)3-9</td>
<td>Ner <em>et al.</em>, 2002; Schotta <em>et al.</em>, 2002; Stabell <em>et al.</em>, 2006.</td>
</tr>
<tr>
<td>H3K9me3</td>
<td>Heterochromatin</td>
<td>SU(VAR)3-9</td>
<td>Schotta <em>et al.</em>, 2002; 2003; Eskeland <em>et al.</em>, 2004.</td>
</tr>
<tr>
<td>H3K9ac</td>
<td>Euchromatin, bands</td>
<td>dADA2b-containing complex</td>
<td>Pankotai <em>et al.</em>, 2005; Ebert <em>et al.</em>, 2006.</td>
</tr>
<tr>
<td>H3K27me</td>
<td>Heterochromatin</td>
<td>E(Z)/ESC/SU(Z)12 complex</td>
<td>Muller <em>et al.</em>, 2002; Ebert <em>et al.</em>, 2006.</td>
</tr>
<tr>
<td>H3K36me</td>
<td>Euchromatin/interbands</td>
<td>dSet2</td>
<td>Stabell <em>et al.</em>, 2007.</td>
</tr>
<tr>
<td>H3K79me</td>
<td>Euchromatin (puffs, interbands, some bands)</td>
<td>GRAPPA</td>
<td>Shanower <em>et al.</em>, 2005.</td>
</tr>
<tr>
<td>H4K20me</td>
<td>Euchromatin, bands and interbands</td>
<td>PR-Set7, ASH1</td>
<td>Karachentsev <em>et al.</em>, 2005; Beisel <em>et al.</em>, 2002.</td>
</tr>
<tr>
<td>H4K20me3</td>
<td>Heterochromatin</td>
<td>SUV4-20</td>
<td>Schotta <em>et al.</em>, 2004.</td>
</tr>
</tbody>
</table>
Figure 1.1. A schematic of the nucleosome core particle.
Figure 1.1. A schematic of the nucleosome core particle. The histone proteins are represented as solid circles. Their unstructured N-terminal tails, which comprise many of the residues targeted by histone-modifying enzymes, are shown as «squiggles» protruding from the core particle. Histones H2A and H2B are represented in light and dark green, respectively, H3 is represented in yellow and H4 in red (due to the perspective, only one H4 molecule is visible). The DNA double helix is represented as a plain black line.
1.2.1.1. DISTRIBUTION AND FUNCTIONS OF METHYLATED HISTONE LYSINES

Over a dozen lysine residues have been shown to be susceptible to methylation (reviewed by Kouzarides, 2007). These lysines reside mainly within the H3 and H4 histone proteins, but also on H2A and H2B. Lysines can be mono-, di-, or trimethylated (me1, me2, me3, respectively), giving rise to a large number of possible methylation states for each nucleosome. Distinct methylation states are observed not only in different regions of the genome (i.e. transcriptionally active vs. inactive), but also within different portions of a given gene. In addition, methylation of certain lysines seems to be incompatible with that of others, suggesting that the co-ordinated action of histone methyltransferases (HMTases) and demethylases (DMTases) is a critical factor in the regulation of gene expression.

Methylated H3K4 (H3K4me) is generally associated with transcriptionally active genes. It appears that H3K4me3 tends to be enriched within promoters and the 5’-most region of transcriptionally competent genes, while H3K4me2 is more abundant within the middle portion of active genes, and H3K4me1 is localized towards the 3’-most region (reviewed by Schones and Zhang, 2008). H3K36me3 is also associated with transcriptionally active chromatin (Barski et al., 2007). However, unlike the H3K4me "marks", it seems to be less localized, and spread over the entire length of the transcribed regions and peaking at their 3’ end (Bannister et al., 2005; Barski et al., 2007). Also, while H3K4me3 is detected at the start of genes that are transcriptionally competent, but not necessarily transcribed, the presence of H3K36me3 seems to be restricted, at least in mammals, to genes that are actively transcribed (Mikkelsen et al., 2007).
Trimethylated H3K9 and H4K20, on the other hand, are generally considered hallmarks of heterochromatin. In mammals, H3K9me3 and, to a lesser degree, H3K9me2, are associated with heterochromatin and also with silent regions of the genome, while H3K9me1 and H4K20me1 appear to be associated with active genes (Barski et al., 2007). In Drosophila, H3K9me1,2, H3K27me1,2,3 and H4K20me3 are all highly enriched in pericentric heterochromatin, but they are also present in other regions of the genome (Schotta et al., 2004; Ebert et al., 2004; 2006). For instance, H3K9me1,2,3 are associated with a number of euchromatic sites and with telomeres and, in polytene chromosomes, H3K27me1,2 are associated with virtually all bands, which represent regions of the genome that are somewhat condensed (Ebert et al., 2006). H3K9me3, on the other hand, does not appear to be very abundant, and is only detected within the core of the chromocentre and at a few other sites (Ebert et al., 2004; 2006).

It is becoming increasingly apparent that these "methyl marks" may be involved in combinatorial, as well as in step-by-step mechanisms that regulate chromatin condensation/decondensation. In Drosophila, mutations in the H3K4 demethylase SU(VAR)3-3/dLSD1 are epistatic to the presence of additional copies of the H3K9 methyltransferase SU(VAR)3-9, indicating that demethylation of H3K4 must precede methylation of H3K9 (Rudolph et al., 2007). However, the fact that no "methyl mark" is completely restricted to transcriptionally active or inactive chromatin, suggests that none of these modifications is sufficient, by itself, to determine the transcriptional state of a chromatin region.
The addition of methyl groups to lysine residues is catalyzed by histone methyltransferase enzymes (HMTases). The vast majority of HMTases characterized so far are non-histone chromatin proteins (NHCPs) containing the signature SET domain (see below). Differences within key residues of their catalytic region confer SET domain-containing HMTases their substrate specificity. A wealth of information is available about SET domain-containing proteins, and it will be discussed in section 1.2.2.1. Much less is known about histone demethylases, as their discovery is much more recent (Wang et al., 2004; Cuthberg et al., 2004; Shi et al., 2004; Tsukada et al., 2006). So far, four histone lysine demethylases have been identified in Drosophila: SU(VAR)3-3/dLsd1, which demethylates H3K4me1,2, Lid, which appears to be specific for H3K4me3, and the JMJD2 homologs, dJMJD2(1) and (2) that demethylate H3K9me3 and H3K36me3, respectively (Rudolph et al., 2007; Di Stefano et al., 2007; Eissenberg et al., 2007; Lee et al., 2007; Secombe et al., 2007; Lloret-Llinares et al., 2008).
Histone H3

**Hs** 1 artkqtarkstggkaprkqlatkaarksapatgvkkphyrprgtvalreirrykstellirklpfqrlnveiaq
d
**Dm** 1 artkqtarkstggkaprkqlatkaarksapatgvkkphyrprgtvalreirrykstellirklpfqrlnveiaq
d

**Hs** 78 fktdlrfqssavmalqeasealvglfednlaiahakrvtimkdpdgllarrirgera

**Dm** 78 fktdlrfqssavmalqeasealvglfednlaiahakritimkdpdgllarrirgera

Histone H4

**Hs** 1 sgrkgkkglkglgakhrkvlrdnigtkpairrrarrggvkrigyleetrvgvklvflenmdvtyehak

**Dm** 1 tgrkgkkglkglgakhrkvlrdnigtkpairrrarrggvkrigyleetrvgvklvflenmdvtyehak

**Hs** 78 rktvamdvyalkrggtrlygfg

**Dm** 78 rktvamdvyalkrggtrlygfg
Figure 1.2. Histone H3 and H4 post-translational modifications. Amino acid sequence of human (H.s.) and Drosophila (D.m.) histones H3 and H4. The sequences corresponding to H3 and H4’s N-terminal tails are underlined in light blue, while yellow underlining denotes the sequences comprising the histone folds (according to Luger et al., 1997). Formally identified post-translational modifications are represented as blue squares (methylation), green circles (phosphorylation) and red asterisks (acetylation) above the residues concerned. More details about the modifications identified in Drosophila, including all relevant references, are listed in Table 1.4. The diagrams relative to human H3 and H4 modifications are based on the Abcam/Millipore histone modification map (http://www.histone.com).
1.2.2. **Non-histone chromatin proteins**

Non-histone chromatin proteins (NHCPs) are generally defined as either "structural" components of chromatin or chromatin-modifying enzymes. The former include all the proteins that are physically associated with chromatin, but that do not appear to have catalytic functions, such as HP1, *Drosophila*’s SU(VAR)3-7 and *S. pombe*’s Rik1. Chromatin-modifying enzymes, in contrast, include histone acetyl-transferases and deacetylases (HATs and HDACs), HMTases and DMTases, histone kinases and phosphatases, and so forth. In many cases, these modifying enzymes also physically associate with chromatin and play a structural role in chromatin architecture. The next section will focus on HMTases and, in particular, SET domain-containing HMTases, since SU(VAR)3-9 belongs to this family of enzymes.

### 1.2.2.1. SET DOMAIN-CONTAINING METHYLTRANSFERASES

The SET domain was originally identified as a ~140 amino acid region present and highly conserved in the gene products of *Su(var)3-9, Enhancer of zeste* and *trithorax* (Jones and Gelbart, 1993; Tschiersch et al., 1994). Dozens of SET-containing proteins have since been described in eukaryotes, and many of them appear to have HMTase or, more generally, protein lysine MTase activities (reviewed by Qian and Zhou, 2006). Table 1.2. summarizes the known *Drosophila* SET domain-containing HMTases and their substrate specificities.

SET domain-containing proteins can be classified into families based on phylogenetic analyses, on the presence of other protein domains (such as a
chromodomain, ankyrin repeats, zinc fingers, and so forth), and/or based on their substrate specificity. Most SET domain-containing HMTases can only catalyze the addition of methyl groups to one or two histone residues and, while some HMTases are strictly mono-methylases, others are able to catalyze the addition of multiple methyl groups (Eskeland et al., 2004; Xiao et al., 2005; Chin et al., 2006; Qian et al., 2006; Guo et al., 2007). The crystal structures of a dozen SET domains have been resolved (reviewed by Qian and Zhou, 2006), providing an explanation for their respective substrate specificities, and allowing investigators to rationalize the specificity of yet un-crystallized SET-containing HMTases.

In most cases, the SET domain is surrounded by a preSET and a postSET regions, which contain a number of conserved cysteines. These residues are not involved in the catalytic process, but they coordinate a set of zinc ions and are necessary to stabilize the structure of the SET domain (Min et al., 2002; reviewed in Qian and Zhou, 2006). The substrate (histone tail) and the methyl group/methyl donor complex (S-adenosyl-methyl-methionine) bind to two distinct clefts located at opposite sides of the SET domain. These two clefts are connected by a hydrophobic channel, and the substrate specificity of SET domains is likely determined by the side chains of the residues forming this channel (Qian and Zhou, 2006). The combination of side chains is thought to recognize particular residues that flank the target lysine in the substrate, which would explain why several HMTases also display lysine MTase activity on non-histone proteins (Kouskouti et al., 2004; Couture et al., 2006; Chin et al., 2007; Sampath et al., 2007). In addition, the nature and size of the side chains in the hydrophobic
channel can determine the degree of methylation that can be achieved by a given SET domain. For example, the presence of a large, bulky side chain will not permit an already methylated histone tail access to the channel, thus precluding the transfer of multiple methyl groups on the target lysine.

SET-containing HMTases also have an additional layer of specificity, as it is becoming apparent that different HMTases act within different chromatin regions. In *Drosophila*, for example, SU(VAR)3-9 is responsible for H3K9me2,3 within centric and pericentric heterochromatin, as well as some euchromatic sites. In contrast, DmSetDB1 mono- and dimethylates H3K9 almost exclusively on the mainly heterochromatic fourth chromosome (Schotta *et al.*, 2002; Eskeland *et al.*, 2004; Mis *et al.*, 2006; Stabell *et al.*, 2006; Seum *et al.*, 2007; Tzeng *et al.*, 2007; Ebert *et al.*, 2006, and references therein).

When this thesis was begun, the catalytic activity of one SET domain, that of SUV39H and CLR4 (the human and yeast homologs of SU(VAR)3-9, respectively) had just been discovered (Rea *et al.*, 2000). Prior to that, SET domains were thought to have protein-protein interaction roles (for example Aagard *et al.*, 1999; Cui *et al.*, 1998), and no structure/function analyses had ever been performed.
Figure 1.3.
**Figure 1.3. Structure of a SET domain, in this case SET7/9.** The substrate (histone H3) binds to the shallow groove adjacent to the channel, on the side of the protein that is facing the viewer in this figure. The methyl donor (AdoMet) binds to a pocket on the opposite side of the protein (hidden in this figure). The narrow channel indicated with an arrow allows the H3 tail to come in contact with the AdoMet moiety. The blue shading indicates different levels of residue conservation relative to other SET domain proteins. This figure was modified from Kwon *et al.*: Mechanism of histone lysine methyl transfer revealed by the structure of SET7/9-AdoMet. EMBO J 22, 292-303 (2003).
Table 1.2. A list of characterized *Drosophila* SET domain-containing proteins. Note that predicted/hypothetical proteins are not included.

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Activity and specificity reported</th>
<th>Selected references</th>
</tr>
</thead>
<tbody>
<tr>
<td>E(Z)</td>
<td>H3K27me (as part of the E(Z)/ESC/SU(Z)12 complex)</td>
<td>Muller <em>et al.</em>, 2002; Ebert <em>et al.</em>, 2006.</td>
</tr>
<tr>
<td>TRITHORAX</td>
<td>H3K4me3</td>
<td>Smith <em>et al.</em>, 2004</td>
</tr>
<tr>
<td>TRR</td>
<td>H3K4me3</td>
<td>Sedkov <em>et al.</em>, 2003</td>
</tr>
<tr>
<td>ASH1</td>
<td>H3K4me3, H3K9me3, H4K20me3</td>
<td>Beisel <em>et al.</em>, 2002; Byrd and Shearn, 2003.</td>
</tr>
<tr>
<td>ASH2</td>
<td>H3K4me3</td>
<td>Beltran <em>et al.</em>, 2007</td>
</tr>
<tr>
<td>SUV4-20</td>
<td>H4K20me3</td>
<td>Schotta <em>et al.</em>, 2004</td>
</tr>
<tr>
<td>dG9a</td>
<td>H3K9me1,2, H3K27me, H4K20me (<em>in vitro</em>)</td>
<td>Mis <em>et al.</em>, 2006; Stabell <em>et al.</em>, 2006.</td>
</tr>
<tr>
<td>DmSetDB1</td>
<td>H3K9me1,2</td>
<td>Seum <em>et al.</em>, 2007; Tzeng <em>et al.</em>, 2007.</td>
</tr>
<tr>
<td>dSet2</td>
<td>H3K36me2</td>
<td>Stabell <em>et al.</em>, 2007</td>
</tr>
</tbody>
</table>
1.2.2.2. SU(VAR)3-9

In *Drosophila*, SU(VAR)3-9, is one of the most prominent HMTases. It appears to be very conserved, with homologs in virtually every eukaryote from the fission yeast to mammals and plants (Krauss *et al.*, 2006). SU(VAR)3-9, as well as most of its homologs, has two conserved domains: a SET domain (see above) and a chromodomain, which is also found in other chromatin proteins such as HP1 and Polycomb (Paro and Hogness, 1991). Chromodomains recognize and bind methylated histone residues, and each chromodomain seems to be very specific for one particular methyl-residue. For example, the chromodomain of HP1 binds to H3K9me (Bannister *et al.*, 2001; Lachner *et al.* 2001; Eskeland *et al.* 2007), that of PC binds to H3K27me, and it was recently shown that the chromodomain of CLR4, the yeast homolog of SU(VAR)3-9, binds to H3K9me (Zhang *et al.*, 2008).

*Drosophila*’s SU(VAR)3-9 is 635 amino acids long, which is larger than its known homologs, and it is unique in that it contains an N-terminal domain responsible for dimerization (Eskeland *et al.*, 2004). The N-terminal moiety of SU(VAR)3-9 also comprises motifs that are required for interaction with two other chromatin proteins, HP1 and SU(VAR)3-7 (Schotta *et al.*, 2002).

In yeast, *clr4* mutants are viable, but show impaired silencing at centromeres and at the *mating type* locus (Ivanova *et al.*, 1998). In mouse, homozygosity for knockout mutations in either *Suv39h1* or *Suv39h2* doesn’t lead to any noticeable phenotypes (Peters *et al.*, 2001). However, a double knockout for both *Su(var)3-9* paralogs, *Suv39h1* and *Suv39h2*, is semi-lethal, and those few homozygous double mutant mice that survive display growth retardation, a high incidence of B cell lymphomas and chromosomal instability (Peters *et al.*, 2001).
In *Drosophila*, homozygous *Su(var)*3-9 mutant adults are viable and fertile, but the chance of *Su(var)*3-9 null embryos reaching adulthood is only about 50% of that of their wild-type counterparts (Mis *et al.*, 2006). These observations suggest that SU(VAR)3-9 is not absolutely essential for development, possibly because other HMTases are able to mimic some of SU(VAR)3-9's functions at least to some degree. On polytene chromosomes, SU(VAR)3-9 is detected at the chromocentre, as well as at some euchromatic sites on the chromosomal arms (Schotta *et al.*, 2002), including the HIS-C (Ner *et al.*, 2002). In addition to controlling methylation of H3K9 in heterochromatin, it interacts physically and functionally with a number of other chromatin proteins, including HP1 and the histone deacetylase HDAC1, which is responsible for deacetylating H3K9 (Schotta *et al.*, 2002; Ebert *et al.*, 2004; 2006; Czermin *et al.*, 2001). These and additional pieces of evidence obtained in yeast, have given rise to the current models for SU(VAR)3-9’s role in chromatin-based gene silencing (see below, 1.3.1).
1.2.2.3. OTHER NON-HISTONE CHROMATIN PROTEINS IN DROSOPHILA

Lysine HMTases, and SU(VAR)3-9 in particular, are thought to work in conjunction with other NHCPs to form and maintain chromatin architecture. In *Drosophila*, the genes encoding many of these proteins were originally identified in genetic screens for dominant suppressors of position-effect variegation (PEV, reviewed by Spofford, 1976; Weiler and Wakimoto, 1995). Table 1.3 summarizes the genes originally identified through screens for dominant suppressors of PEV and the characteristics of their products, most of which are NHCPs. These include SU(VAR)3-7 and HDAC1, both of which are known to interact with SU(VAR)3-9 (Reuter *et al*., 1990; Cléard *et al*., 1997; Mottus *et al*., 2000; Czermin *et al*., 2001; Schotta *et al*., 2002).

SU(VAR)3-7 is a large, *Drosophila*-specific, zinc-finger protein that binds to repetitive DNA and physically interacts with both SU(VAR)3-9 and HP1 (Cléard and Spierer, 2001; Schotta *et al*., 2002; Jaquet *et al*., 2006). It is preferentially associated with heterochromatin and appears to be sufficient to induce SU(VAR)3-9-dependent heterochromatisation (Reuter *et al*., 1990; Cléard *et al*., 1995; Delattre *et al*., 2004). In contrast, localization of the histone deacetylase HDAC1, the homolog of *S. cerevisiae* Rpd3, does not appear to be heterochromatin-specific, as this protein is detected at hundreds of sites on polytene chromosomes (Chang *et al*., 2001; Tie *et al*., 2003). HDAC1 is an H3K9 deacetylase that has been shown to co-immunoprecipitate with SU(VAR)3-9, suggesting that the two proteins may be part of the same complex (Czermin *et al*., 2001; Rudolph *et al*., 2007). In addition, the deacetylation activity of HDAC1 is necessary for H3K9 methylation (Nakayama *et al*., 2001; Czermin *et al*., 2001; Vaute *et al*., 2002).
One NHCP, HP1, was originally identified as the specific target of a monoclonal antibody that associated preferentially with heterochromatin in polytene chromosomes (James and Elgin, 1986; James et al., 1989) and Su(var)2-5, the gene encoding HP1, was identified as a strong dominant suppressor of PEV (Eissenberg et al., 1990; 1992). HP1 has homologs in almost all eukaryotes from the fission yeast (Swi6) to mammals and plants; it is comprised of two conserved domains, the chromodomain and the chromo-shadow domain, linked by what is known as the "hinge" or "linker" region (reviewed by Lomberk et al., 2006). The chromodomain is responsible for specifically recognizing and binding H3K9me, while the chromo-shadow domain is required for protein-protein interactions and for dimerisation (Bannister et al., 2001; Lachner et al., 2001; Jacobs et al., 2001; Brasher et al., 2000; Jones et al., 2000; Nielsen et al., 2001; Cowieson et al., 2000). Both the chromo-shadow and the "hinge" domains are necessary for interaction with SU(VAR)3-9 and SU(VAR)3-7 (Schotta et al., 2002; Jaquet et al., 2002).

With the advent of genome-wide sequencing efforts, genomic databases and bioinformatics tools, a number of genes encoding NHCPs were cloned and characterized based on sequence homologies and in silico searches. These include two HMTase-encoding genes, dG9a and Su(var)4-20, as well as several DMTases (Schotta et al., 2004; Mis et al., 2006; Stabell et al., 2006; Schotta et al., 2004; Secombe et al., 2007; Lloret-Llinares et al., 2008). Several NHCPs identified through an in silico approach have also been shown to suppress PEV, indicating that they are functionally involved in epigenetic silencing (Table 1.4).
Table 1.3. Drosophila proteins identified through screens for strong, dominant suppressors of PEV. «Location» refers to the cytological location to which the gene in question has been mapped. Note that, under «homologs», hypothetical/predicted proteins are not included.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Location</th>
<th>Gene product</th>
<th>Attributed functions</th>
<th>Homologs</th>
<th>Selected references</th>
</tr>
</thead>
<tbody>
<tr>
<td>Su(var)2-5</td>
<td>28F2-F3</td>
<td>HP1</td>
<td>Chromatin silencing and assembly, telomere maintenance, transcriptional regulation, chromosome segregation, binding to H3K9me, RNA-binding.</td>
<td>Orthologs: HP1a (mammals), SWI6 (S. pombe), LHP1 (Arabidopsis), *M31 (mouse)</td>
<td>James and Elgin, 1986; Essenberg et al., 1992; Kellum and Alberts, 1995; Nielsen et al., 2001; Grell et al., 2003.</td>
</tr>
<tr>
<td>Su(var)2-10</td>
<td>45A8-A9</td>
<td>dPIAS*</td>
<td>DNA binding, chromatin organization, transcriptional regulation, regulation of the JAK-STAT cascade.</td>
<td>PIAS** (mammals)</td>
<td>Mohr and Boswell, 1999; Evans et al., 2003; Hari et al., 2001; Muller et al., 2005.</td>
</tr>
<tr>
<td>(Note that Su(var)3-1 is a hypermorphic allele of JIL-1)</td>
<td>68A5-A6</td>
<td>JIL-1</td>
<td>Histone kinase (specific for H3S10), regulation of H3K9methylation, chromatin architecture, transcriptional regulation.</td>
<td>Undetermined</td>
<td>Jin et al., 1999; Wang et al., 2004; Ebert et al., 2004; Bao et al., 2005; Zhang et al., 2006.</td>
</tr>
<tr>
<td>Su(var)3-1</td>
<td>77A3</td>
<td>SU(VAR)3-3</td>
<td>Histone demethylase (specific for H3K4me2), transcriptional regulation.</td>
<td>LSD1 (mammals), SPR-8 (C. elegans), LSD1 (S. pombe).</td>
<td>Di Stefano et al., 2007; Rudolph et al., 2007.</td>
</tr>
<tr>
<td>Su(var)3-3</td>
<td></td>
<td></td>
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</table>

* Synonym: HP1a, C1A9 nuclear antigen

** multiple isomorphs present in mammals.
<table>
<thead>
<tr>
<th>Gene name</th>
<th>Location</th>
<th>Gene product</th>
<th>Attributed functions</th>
<th>Homologs</th>
<th>Selected references</th>
</tr>
</thead>
<tbody>
<tr>
<td>Su(var)3-6</td>
<td>87B9-B10</td>
<td>PP1-87B</td>
<td>Ser/Thr phosphatase, cell cycle regulation/mitosis, glycogen metabolism, gametogenesis, wing, eye, nervous system and muscle development.</td>
<td>PP1 (vertebrates), TOPP2 (Arabidopsis), Ppz1p (S. cerevisiae).</td>
<td>Dombradi et al., 1990; Baksa et al., 1993; Bennett et al., 2003; Babu et al., 2005.</td>
</tr>
<tr>
<td>Su(var)3-7</td>
<td>87E3</td>
<td>SU(VAR)3-7</td>
<td>DNA-binding (preference for satellite sequences), interaction with HP1 and SU(VAR)3-9, chromatin compaction, architecture and silencing.</td>
<td>To date, no homologs identified outside the genus Drosophila.</td>
<td>Reuter et al., 1990; Cléard and Spierer, 2001; Jaquet et al., 2002; Delattre et al., 2004; Demakova et al., 2007.</td>
</tr>
<tr>
<td>Su(var)3-9</td>
<td>88E6-E8</td>
<td>SU(VAR)3-9</td>
<td>HMTase (specific for H3K9), gene regulation, chromatin architecture and silencing, interaction with HP1, SU(VAR)3-7, SU(VAR)3-3, HDAC1.</td>
<td>Orthologs: SUV39H1 (human), Suv39h1 (mouse), CLR4/Clr4p (S. pombe); DIM-5 (N. crassa).</td>
<td>Tschiersch et al., 1994; Ner et al., 2002; Schotta et al., 2002; Eskeland et al., 2004; Swaminathan et al., 2005.</td>
</tr>
<tr>
<td>(Note: pitkin, a very strong E(var), is a hypermorphic allele of Su(var)3-9)</td>
<td></td>
<td></td>
<td></td>
<td>Paralogs: SUV39H2 (human), Suv39h2 (mouse).</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>SU VH3.4.5 (Arabidopsis).</td>
<td></td>
</tr>
<tr>
<td>Su(var)326/Rpd3</td>
<td>64B12</td>
<td>HDAC1/RPD3</td>
<td>Histone deacetylase (preference for H3K9ac), interaction with SU(VAR)3-9, gene regulation, chromatin architecture, silencing, cell cycle regulation.</td>
<td>HDAC2/1 (mammals), Rpd3 (S. cerevisiae).</td>
<td>Maixner et al., 1998; Mottus et al., 2000; Huang and Kadonaga, 2001; Czermin et al., 2001.</td>
</tr>
</tbody>
</table>
Table 1.4. Additional Su(var)s. Mutations in the following *Drosophila* genes were also found to act as dominant suppressors of PEV on at least two different variegating rearrangements. Note that the Su(var) phenotype associated *puc* and *dSas4* was determined in our lab (Toub, unpublished), and that, since *dG9a* and *Suv4-20* are located on the X chromosome, their Su(var) phenotypes have so far only been tested on one variegating rearrangement, *SbV*.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Location</th>
<th>Gene product</th>
<th>Major attributed functions</th>
<th>Homologs</th>
<th>Selected references</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>chm</em></td>
<td>27F3-4</td>
<td>CHAMEAU (Synonym: HAT1)</td>
<td>HAT (preference for H4K16), chromatin architecture, silencing, regulation of the JNK cascade, transcriptional regulation.</td>
<td>HBO1 (human), Sas2 (<em>S. cerevisiae</em>).</td>
<td>Grienenberger <em>et al.</em>, 2002; Miotto <em>et al.</em>, 2006;</td>
</tr>
<tr>
<td>Gene name</td>
<td>Location</td>
<td>Gene product</td>
<td>Major attributed functions</td>
<td>Homologs</td>
<td>Selected references</td>
</tr>
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</tr>
<tr>
<td><strong>dSas-4</strong> (Previously known as S2214)</td>
<td>84C6-7</td>
<td>dSAS-4</td>
<td>Centriole replication, biogenesis of centrosomes, flagella and cilia.</td>
<td>SAS-4* (*C. elegans). (*Spindle assembly 4)</td>
<td>Basto et al., 2006.</td>
</tr>
<tr>
<td><strong>puc</strong></td>
<td>84E12-13</td>
<td>PUCKERED</td>
<td>Ser/Thr and Tyr phosphatase, regulation of JNK cascade, dorsal closure and metamorphosis.</td>
<td>Pyst1 (human); CEL-F081 (*C. elegans).</td>
<td>Glise and Noselli, 1997; Martin-Blanco et al., 1998; Agnès et al., 1999.</td>
</tr>
</tbody>
</table>
1.3. SU(VAR)3-9 and heterochromatin assembly

Heterochromatin is a transcriptionally inert form of chromatin that remains highly compacted throughout the cell cycle. Heterochromatin is characterized by the presence of repetitive DNA, transposable elements, highly ordered nucleosomal arrays (Wallrath and Elgin, 1995), specific subsets of histone modifications (e.g. H3K9me2,3, H4K20me3) and enrichment for particular NHCPs, such as SU(VAR)3-9, HP1, SU(VAR)3-7 (Eissenberg and Elgin, 2000; Schotta et al., 2002; Reuter et al., 1990; for a comprehensive review see Ebert et al., 2006).

Heterochromatic regions of the genome tend to be replicated late during S phase and appear underreplicated in polytene chromosomes. Chromosomes’ centromeres and telomeres, the inactive X chromosome in female mammals and most of Drosophila’s fourth chromosome are heterochromatic.

However, chromatin is highly dynamic, and its packaging status, as well as the histone modifications and NHCPs present at a given locus, are not fixed; instead they change throughout the cell cycle, and during development, as the cell responds to cellular and extracellular signals (de Wit et al., 2005; Ebert et al., 2004; Dormann et al., 2006). Results obtained from genetic and biochemical analyses in yeast, and from the study of polytene chromosomes in flies suggest that heterochromatin formation is a multi-step process. The model presented in section 1.3.1 focuses on the roles of SU(VAR)3-9, based on what is known from Drosophila and yeast.
1.3.1. Postulated mechanism for heterochromatin formation

Demethylation of H3K4me by SU(VAR)3-3/dLSD1 is thought to be one of the first steps in heterochromatisation of a region, since a loss of function mutation in Su(var)3-3 is epistatic to the presence of extra copies of Su(var)3-9, and SU(VAR)3-9’s activity is absolutely necessary for the formation of heterochromatin (Rudolph et al., 2007; Schotta et al., 2002; 2003). Deacetylation of H3K9ac by HDAC1 also needs to occur prior to SU(VAR)3-9-dependent methylation, as H3K9 can’t be simultaneously acetylated and methylated. In addition, loss of function mutations in the gene encoding HDAC1 are also epistatic to the presence of extra copies of Su(var)3-9 (Czermin et al., 2001).

Once H3K4 is demethylated and H3K9 is deacetylated, SU(VAR)3-9 can di- and trimethylate H3K9. It should however be noted that, since SU(VAR)3-9 seems to preferentially di- and trimethylate H3K9me1, its action is probably preceded by that of an H3K9 monomethylase, possibly dG9a (Mis et al., 2006). How SU(VAR)3-9 is recruited to its target sites is not completely understood, but we know that its recruitment is at least partially dependent on SU(VAR)3-7, and that the presence of HP1 is required to prevent the binding of SU(VAR)3-9 to ectopic sites (Delattre et al., 2004; Schotta et al., 2002; 2003). Since SU(VAR)3-9 is able to physically interact with HDAC1, SU(VAR)3-7 and HP1, it is possible that these chromatin proteins form a complex (Delattre et al., 2000; Czermin et al., 2001, Schotta et al., 2002; 2003).

The H3K9me2,3 probably acts as a binding platform for the chromodomain of HP1 (Bannister et al. 2001; Jacobs et al., 2001). There is also some evidence
showing that, at least in yeast, H3K9me2,3 helps recruit and stabilize the binding of Clr4, the yeast homolog of SU(VAR)3-9, to chromatin (Zhang et al., 2008). If the same is true in Drosophila, the presence of H3K9me2,3 may stabilize the association of SU(VAR)3-9 with chromatin, in addition to facilitating, with the help of auxiliary factors, the binding of HP1. This, in turn, is thought to recruit SU(VAR)4-20, an HMTase that trimethylates H4K20 (Ebert et al., 2006; Eskeland et al., 2007; Schotta et al., 2004).

SU(VAR)3-9 has also been associated with euchromatic gene regulation (Vandel et al., 2001; Nielsen et al., 2001; Ner et al., 2002; Greil et al., 2003). Because of its role in heterochromatin formation, the presence of SU(VAR)3-9 at euchromatic loci is hypothesized to be associated with silencing of the genes within these loci. Whether this is always the case, or not, has not been well documented. In addition, it is presently unclear whether SU(VAR)3-9 acts through similar or completely distinct mechanisms at heterochromatic and euchromatic sites. Investigating its role in the regulation of the histone genes, which are located in euchromatin should provide useful information in this regard, and is the subject of this thesis.
Figure 1.4.
Figure 1.4. A simplified diagram showing the assembly of heterochromatin.

The nucleosome core is represented as a cylinder, with the dotted line representing the DNA wrapped around it. For simplicity, only 4 N-terminal histone tails are shown instead of 8; the histone H3 tail in yellow, H4 in red, H2A in light green and H2B in dark green. The blue squares indicate methyl groups, the red asterisks represent acetyl groups. The enzymes known to catalyze the addition and removal of these groups are shown. Proteins known to be associated with the HIS-C are highlighted in yellow.
1.4. The *histone* gene cluster (HIS-C)

One of the targets of SU(VAR)3-9 in polytene chromosomes is the HIS-C, and some evidence exists that the regulation of the *histone* genes is, at least partially, dependent on SU(VAR)3-9 (Ner et al., 2002; see below), thus making the HIS-C a very good model system to start dissecting the SU(VAR)3-9’s mechanism of action at a euchromatic locus.

1.4.1. Structure of the HIS-C and characteristics of the *histone* genes

*Drosophila*’s *histone* genes are organized into a “*histone unit*” consisting of one copy of each of the five *histone* genes (h2a, h2b, h3, h4 and h1). About 110 tandemly repeated copies of this histone unit form the *Histone Gene cluster* (HIS-C). The order of the *histone* genes and their direction of transcription are shown in Figure 1.2. Located on the left arm of the second chromosome, *Drosophila*’s HIS-C spans over 500 kb (Saigo et al., 1981) and has some peculiar characteristics. In spite of being a euchromatic locus, it displays some features that are usually associated with heterochromatin. It replicates slightly later in S-phase than other euchromatic loci, it is somewhat underreplicated in polytene chromosomes, nuclease sensitivity assays suggest that it is packaged as a higher order chromatin structure, and, at DNA level, it is a reiterated locus (Zhimulev and Belyaeva, 2003; Samal et al., 1981).

In metazoans, *histone* genes differ from most genes in that they are intronless and, more importantly, their mRNAs are, for the most part, not polyadenylated in spite of being transcribed by RNA polymerase II. Instead, the
processing of *histone* pre-mRNAs depends on two elements located at their 3’ end: a highly conserved, 16 nucleotide stem-loop sequence, and a purine-rich sequence, known as HDE (histone downstream element). The processing also requires several trans-acting factors: the stem-loop binding protein (SLBP), which binds to the stem-loop structure, a U7 snRNP, which binds the HDE, plus some additional (poorly characterized) factors (for a review, see for example Dominski and Marzluff, 1999; 2007). Relatively little is known about the precise functions of SLBP, and much of the data available comes from studies in vertebrates. We know that SLBP remains associated with mature *histone* mRNAs as they are transported into the cytoplasm, where it is thought to play a role in the stability and translation of the transcripts (reviewed by Dominski and Marzluff, 1999; 2007). More importantly, SLBP is only present in large amounts during S-phase, possibly explaining why non-polyadenylated *histone* mRNAs only accumulate at this stage of the cell cycle. One of its main roles may be to stabilize and protect the *histone* mRNA from degradation (reviewed by Dominski and Marzluff, 1999; 2007).
Figure 1.5.
Figure 1.5. A schematic of Drosophila’s histone unit. The BglII fragment defining the histone unit is comprised of one copy of each histone gene. The histone gene cluster (HIS-C) consists of a tandem array of about 110 copies of the histone unit. The coding regions of the histone genes are represented in blue (H2A and H2B), red (H3 and H4), and yellow (H1). The arrowheads indicate the direction of transcription. Selected restriction sites, and their relative distances and positions along the histone unit, are indicated.
1.4.2. H3K9me and SU(VAR)3-9 distribution across the HIS-C

Several pieces of evidence suggest that SU(VAR)3-9 must have a role in the regulation of the histone gene cluster. Based on MNase and DNaseI sensitivity assays, it appears that the HIS-C has an altered chromatin structure in Su(var)3-9 mutants, and at least three Su(var)3-9 mutants have increased levels of histone h1 and histone h4 transcripts (Ner et al., 2002).

ChIP experiments have shown that SU(VAR)3-9 is associated with transcribed and non-transcribed/intergenic regions of the histone unit in staged embryos (Ner et al., 2002; Ner et al., in preparation), indicating that it probably plays a direct role in the regulation of the locus. The association of SU(VAR)3-9 with the HIS-C was confirmed in adults using a SU(VAR)3-9::DAM fusion protein (Ner et al., in preparation). In addition, localization of SU(VAR)3-9 at the HIS-C was also reported in Drosophila nurse cells (Koryakov et al., 2006). H3K9me2 follows the same distribution pattern as SU(VAR)3-9 along the histone unit, suggesting that SU(VAR)3-9 is not only present at the locus, but it is also enzymatically functional (Ner et al., 2002). Other NHCPs known to interact with SU(VAR)3-9, such as HP1 and HDAC1, have also been detected at the HIS-C (Greil et al., 2003; Koryakov et al., 2006; Ner et al., in preparation).

1.4.3. Possible function of SU(VAR)3-9 in the regulation of the histone genes

What could be the function of SU(VAR)3-9 in the regulation of histone gene expression? First of all, a role at the transcriptional level seems most compatible
with its nature as a chromatin-associated H3K9 HMTase. Assuming that this is the case, SU(VAR)3-9 could be involved in modulating the chromatin structure, and therefore transcriptional competency, of the HIS-C. It could do so in at least three ways. The first possibility is that it acts on individual histone units, determining which/how many of the ~110 of them are available for transcription. This possibility implies that the five histone genes comprising a given unit are all either accessible, or inaccessible to the transcriptional machinery. Alternatively, SU(VAR)3-9 could work as a transcriptional regulator that modulates the expression level of each histone gene independently. In this case, genes belonging to the same histone unit may be transcribed at different rates.

Finally, it is also possible that SU(VAR)3-9’s function is somehow involved in the coupling of histone genes expression with the cell cycle. Chapter 3 of this thesis will address some of these issues.

1.5. Subject(s) of this study

Chromatin structure and dynamics play crucial roles in gene regulation. Thus, the importance of identifying chromatin components and understanding their functions and interaction is obvious. However, chromatin’s size and complexity make it necessary to study its components one, or a few, at a time. The focus of this study is a non-histone chromatin protein, the H3K9-specific HMTase SU(VAR)3-9, and its role in the packaging and regulation of a euchromatic locus, the histone genes cluster.
Highly conserved from yeast to mammals (Krauss et al., 2006), SU(VAR)3-9 is very well characterized. However, when this study was begun, the effects of single amino acid substitutions on the protein’s HMTase activity were not known. Subsequently, the enzymatic activity of a dozen missense mutants has been reported, but only with respect to a single synthetic substrate (a histone tail peptide), which does not allow for a detailed characterization of the catalytic characteristics of each mutant (Ebert et al., 2004). Also, the mutants’ phenotypes at the enzymatic and cellular level, and their effect on chromatin-based gene silencing, have never been systematically related to the position of the mutated residue within the three dimensional structure of SU(VAR)3-9. Part of the reason for this lack of detailed structure-function analyses of SU(VAR)3-9 may be that its crystal structure has not yet been resolved, so one needs to "extrapolate" from structural information relative to similar HMTases.

Finally, this thesis represents the first attempt to determine whether the effect(s) of specific Su(var)3-9 mutations on one phenotype (for example, catalytic activity on a given substrate) are good predictors of how these mutations will affect other phenotypes (for example, overexpression of the histone genes).

The first section of my thesis (chapter 2) is concerned with the characterization of a series of Su(var)3-9 missense alleles isolated in our laboratory as strong, dominant suppressors of position-effect variegation (PEV, see below, section 1.2.3.1.) at a number of different phenotypic levels, including: the molecular, enzymatic, and cellular levels, as well as at their morphological phenotype, suppression of PEV, the phenotype against which the mutants were
identified and isolated. Taking advantage of the crystal structure of CLR4 (Min et al., 2002), the yeast homolog of SU(VAR)3-9, and of the high level of identity between the primary sequences of the two proteins, the results are then discussed in terms of structure-function. This analysis allows us to propose roles for the various mutated amino acids in the HMTase function of SU(VAR)3-9. In addition, it highlights the functional biases of the original genetic screen that was used to identify Su(var)3-9 as well as the interpretive biases that result from characterizing mutants against a single molecular or cellular phenotype. Based on this molecular, biochemical and functional analyses we can postulate a possible mechanism by which SU(VAR)3-9 acts as a regulator of chromatin-based gene silencing (see "Discussion").

The second part of this thesis (chapter 3) deals with the role of SU(VAR)3-9 as a regulator of euchromatic gene expression. Although it was first identified as a chromatin protein, and found to be associated mainly with heterochromatic regions of the genome (Schotta et al., 2002; 2003; Ebert et al., 2004), there is reason to believe that SU(VAR)3-9 also plays an important role in the regulation of many euchromatic genes (Nielsen et al., 2001; Greil et al., 2003). Among the pieces of evidence supporting this idea is the relationship between Su(var)3-9 function and regulation of the HIS-C transcription. The HIS-C appears to be one of the major euchromatic targets of SU(VAR)3-9, and at least two Su(var)3-9 mutants show an altered chromatin structure at the HIS-C locus and elevated levels of histone transcripts (Ner et al., 2002).
Assuming that SU(VAR)3-9 does indeed play a direct role in the regulation of histone genes expression, its mechanism of action is not known. In order to gain some insights on the function(s) of SU(VAR)3-9 at the HIS-C, its distribution along this locus is studied in wild-type and particular Su(var)3-9 mutants. The distributions of dimethyl-H3K9 (H3K9me2), the histone modification elicited by SU(VAR)3-9, and that of HP1, another chromatin protein thought to bind to H3K9me2, are also analyzed. This study gives us a general picture of the HIS-C "landscape" in terms of SU(VAR)3-9, HP1 and H3K9me2, and allows us to determine the interdependence (or lack thereof) among these three factors. Finally, the relative amounts of histone transcripts are accurately quantified in the different Su(var)3-9 mutants and, by relating this information to the distribution of SU(VAR)3-9, HP1 and H3K9me2 at the HIS-C in each mutant, we can propose a mechanism for SU(VAR)3-9's function at the HIS-C (chapter 3).
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2. ALTERED HISTONE H3 METHYLTRANSFERASE ACTIVITY OF Su(VAR)3-9 IMPAIRS GENE SILENCING¹.

2.1. Introduction

The chromatin of eukaryotic organisms is a dynamic complex of DNA and proteins. The nucleosome, its basic structural unit, is comprised of 146 bp of DNA wrapped around an octamer of the core histones H3, H4, H2A and H2B (Finch et al. 1977; Klug et al., 1980, Luger et al., 1997). Higher order assemblies of nucleosomes package the genome into structured regions that typically have different functional properties. For example, regions of the genome surrounding the centromere have nucleosomes organized into highly condensed and tightly packaged chromatin, are transcriptionally inert, late replicating and are collectively termed heterochromatin (Gatti and Pimpinelli, 1992; Lohe and Hilliker, 1995). Regions that are less densely packaged, accessible to the transcriptional machinery, and which replicate earlier in S-phase, are collectively known as euchromatin (often also referred to as “open” chromatin).

However, even euchromatin is a mosaic of silenced (repressed) and transcriptionally competent (open) domains. Numerous non-histone proteins and a range of covalent histone modifications influence nucleosome-nucleosome interactions and higher order packaging and thus control the transitions between transcriptionally competent (open) and transcriptionally repressed states of the euchromatic portion of the genome. The histone modifications, particularly at the N-terminal tails, include acetylation,

¹ A version of this chapter will be submitted for publication. Kalas, P. and Grigliatti, T.A. Altered histone H3 methyltransferase activity of SU(VAR)3-9 impairs gene silencing.
Specific patterns of histone modifications correlate with gene activity and chromatin structure - the “histone code” hypothesis (Jenuwein and Allis, 2001). For example, acetylation of lysine 9 of histone H3 (H3K9ac) is typically associated with gene expression, while methylation of H3K9 (H3K9me) and H4K20me are usually associated with gene repression or silenced regions of the genome (Litt et al., 2001; Schotta et al., 2004; reviewed in Rice and Allis, 2001; Berger, 2002; Ebert et al., 2006).

In *Drosophila*, methylation of H3K9 is catalyzed by Su(var)3-9, the archetypal SET domain-containing histone methyltransferase (HMTase) that is highly conserved in eukaryotes (Aagaard et al., 1999; Rea et al., 2000; Ivanova et al., 1998). Mutations in Su(var)3-9 were recovered in genetic screens (Reuter and Wolff, 1981; Sinclair et al., 1983; Donaldson et al., 2002) as strong dominant suppressors of the heterochromatin-associated gene silencing phenomenon, position-effect variegation (PEV) (Muller, 1930; Grigliatti, 1991; Lewis, 1950; Weiler and Wakimoto, 1995). All Su(var)3-9 mutants known to date are strong, dominant suppressors of w<sup>m4</sup>, Sb<sup>v</sup> and bw<sup>v</sup> variegation (Sinclair et al., 1983; Reuter and Wolff, 1981). Su(var)3-9 has now been extensively characterized. It associates with heterochromatic regions (Schotta et al., 2002; 2003) and numerous euchromatic loci (Greil et al., 2003), including the tandemly reiterated histone gene cluster (HIS-C) (Ner et al., 2002), where it epigenetically modulates the expression of the histone genes by altering the chromatin structure of the locus via methylation of H3K9 and recruitment of HP1 (Ner et al., 2002; Ner et al., in preparation).

The original EMS mutagenesis screen isolated over 2 dozen mutants that mapped near the Su(var)3-9 locus (Sinclair et al. 1983). This chapter focuses on a subset of these mutants. We demonstrate that these mutations are indeed Su(var)3-9
alleles, and examine their phenotypes at four different levels: molecular, cellular, biochemical and morphological (eye colour/PEV). At the molecular level, we found that all alleles have missense mutations resulting in single amino acid substitutions in the catalytic region of the protein, which consists of the evolutionarily conserved preSET, SET and postSET domains. In contrast, no mutations were recovered in the other highly conserved region of Su(var)3-9, the chromodomain, or in the Drosophila-specific N-terminus, suggesting alterations in these regions don’t influence the epigenetic silencing resulting from PEV. At the cellular level, the Su(var)3-9 mutants had reduced levels of H3K9me2 and HP1 at both the chromocentre and a euchromatic locus, the HIS-C. Biochemically, we showed that all amino acid substitutions either abolish or dramatically reduce the HMTase activity of Su(var)3-9 in vitro. Substitutions of conserved cysteine residues in the preSET domain directly correlated with a complete loss-of-HMTase function in vitro, while changes in the SET or postSET regions resulted in partial loss-of-function. Finally, at the level of morphological phenotypes, eye colour/PEV, all Su(var)3-9 mutants were able to suppress wmt4 variegation in a strain that is hyperploid for Su(var)3-9, but we observed differences in their strength of suppression.

2.2. Results

2.2.1. Recombination mapping and DNA Sequence analysis

In the early 1980s our lab recovered about 50 third chromosome EMS-induced mutants that were strong, dominant suppressors of PEV (Sinclair et al., 1983). Over twenty of these mutants are homozygous viable, clustered on the right arm of the third chromosome and circumstantial evidence strongly indicated that a large subset of these
were Su(var)3-9 alleles (Harrington, 2001). The mutations are homozygous viable, which obviated the use of complementation analyses to define allelism. Thus, to determine which of the mutations were Su(var)3-9 alleles, we performed recombination analysis using a recessive lethal P-element insert, Su(var)3-9\textsuperscript{P25} (Harrington, 2001). For each of the 20 putative Su(var)3-9 alleles, we generated heterozygous Su(var)3-9\textsuperscript{P25}/Su(var)3-9\textsuperscript{putative} females and crossed them to wild-type males, and examined the progeny. For 13 mutants we observed no wild-type recombinants (after screening > 2000 flies for each cross), indicating that the mutations they carry are very closely linked to Su(var)3-9\textsuperscript{P25} (<0.1 cM; Table 1). These 13 mutants were then characterized by DNA sequence analysis to determine the precise nature and position of the mutation. Nine out of the 12 were missense mutants in Su(var)3-9 (Table 1). Accordingly, we renamed these mutants as Su(var)3-9\textsuperscript{allele number} (Table 1). Three mutants, Su(var)3-9\textsuperscript{317}, Su(var)3-9\textsuperscript{327}, and Su(var)3-9\textsuperscript{329}, did not contain mutations in the coding region of Su(var)3-9. Since the estimated distance between the mutations in each of these three mutants and Su(var)3-9\textsuperscript{P25} is <0.1 cM, which represents about 50kb or less in the region surrounding the Su(var)3-9 locus (http://flybase.bio.indiana.edu), these mutants may still be alleles of Su(var)3-9 and represent alterations in the regulatory region of Su(var)3-9 or cause defects in mRNA processing. Alternatively, their Su(var) phenotype may be the result of mutations in one or more genes that are closely linked to Su(var)3-9, such as Set or Oscp. Indeed, Su(var) mutations are rather commonly found in clusters, closely mapping, but not contiguous (Sinclair et al., 1983).

The sequence analysis also revealed single base-pair changes leading to ALA304PHE and/or ILE375LEU in Su(var)305, Su(var)3-9\textsuperscript{324}, Su(var)3-9\textsuperscript{329} and Su(var)331
(Table 2.1). Since Su(var)305, which carries both substitutions, and Su(var)331, which carries the I375L substitution, are not allelic to Su(var)3-9 (as determined by recombination mapping with Su(var)3-9P25, see Materials and Methods), we concluded that these substitutions are not the cause of the Su(var) phenotype in Su(var)3-9324 or Su(var)3-9329. In Su(var)3-9324, the C428Y is most likely the cause of the Su(var) effect, while for Su(var)3-9329 the mutation responsible for this phenotype may be located in one of the cis-regulatory elements of the gene, which are not known and were therefore not sequenced.
Table 2.1. Putative *Su(var)3-9* alleles that were mapped and sequenced in this study. Allelism to *Su(var)3-9* was determined by recombination mapping with the P-element induced *Su(var)3-9*<sup>P25</sup> mutant (Ner et al., 2002). Mutants that failed to yield wild-type recombinants were further analyzed by sequencing and, if allelism to *Su(var)3-9* was confirmed, they were renamed accordingly.

<table>
<thead>
<tr>
<th>MUTANT</th>
<th><em>Su(var)3-9</em> allele (genetically)?</th>
<th>Allelism confirmed by sequencing?</th>
<th>Mutation</th>
<th>New name</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Su(var)301</em></td>
<td>NO</td>
<td>N/A (not tested)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td><em>Su(var)305</em></td>
<td>NO</td>
<td>No</td>
<td>A304F&lt;sup&gt;b&lt;/sup&gt;, I375L&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><em>Su(var)306</em></td>
<td>NO&lt;sup&gt;a&lt;/sup&gt;</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td><em>Su(var)309</em></td>
<td>YES</td>
<td>Yes</td>
<td>C462Y</td>
<td><em>Su(var)3-9</em>&lt;sup&gt;309&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Su(var)311</em></td>
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<td>Yes</td>
<td>G521D</td>
<td><em>Su(var)3-9</em>&lt;sup&gt;311&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Su(var)312</em></td>
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<td>Yes</td>
<td>C462Y</td>
<td><em>Su(var)3-9</em>&lt;sup&gt;312&lt;/sup&gt;</td>
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<tr>
<td><em>Su(var)314</em></td>
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<td>N/A</td>
<td>N/A</td>
<td></td>
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<tr>
<td><em>Su(var)315</em></td>
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<td>N/A</td>
<td>N/A</td>
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<tr>
<td><em>Su(var)317</em></td>
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<td>No</td>
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<tr>
<td><em>Su(var)318</em></td>
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<td>Yes</td>
<td>S616L</td>
<td><em>Su(var)3-9</em>&lt;sup&gt;318&lt;/sup&gt;</td>
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<tr>
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<td>Yes</td>
<td>S616L</td>
<td><em>Su(var)3-9</em>&lt;sup&gt;319&lt;/sup&gt;</td>
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<tr>
<td><em>Su(var)320</em></td>
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<td>N/A</td>
<td>N/A</td>
<td></td>
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<tr>
<td><em>Su(var)324</em></td>
<td>YES</td>
<td>Yes</td>
<td>C428Y, I375L&lt;sup&gt;b&lt;/sup&gt;</td>
<td><em>Su(var)3-9</em>&lt;sup&gt;324&lt;/sup&gt;</td>
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<tr>
<td><em>Su(var)325</em></td>
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<td>Yes</td>
<td>P582Q</td>
<td><em>Su(var)3-9</em>&lt;sup&gt;325&lt;/sup&gt;</td>
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<td><em>Su(var)327</em></td>
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<td></td>
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<tr>
<td><em>Su(var)329</em></td>
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<td>No</td>
<td>A304F&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td><em>Su(var)C76</em></td>
<td>YES</td>
<td>Yes</td>
<td>C421S</td>
<td><em>Su(var)3-9</em>&lt;sup&gt;376&lt;/sup&gt;</td>
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<tr>
<td><em>Su(var)330</em></td>
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<td>Yes</td>
<td>D536N</td>
<td><em>Su(var)3-9</em>&lt;sup&gt;330&lt;/sup&gt;</td>
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<tr>
<td><em>Su(var)331</em></td>
<td>NO</td>
<td>N/A</td>
<td>I375L&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Harrington, 2001.
<sup>b</sup> These amino acid substitutions likely represent naturally occurring polymorphisms, and are not responsible for the *Su(var)* phenotype.
2.2.2. Amino acid substitutions in Su(var)3-9 target the preSET/SET/postSET domain.

Su(var)3-9 has two distinct, and highly conserved domains: a chromodomain, and a SET domain, which includes the flanking pre- and a postSET regions (Figure 2.1). The chromodomain of Su(var)3-9 is about 40 amino acids long and its function is not entirely clear, but it is speculated to have a role in protein-protein interactions that may control H3K9 methylation (Schotta et al., 2003). The preSET/SET/postSET region is ~250 amino acids long, represents less than 40% of the entire length of the protein, and constitutes the catalytic region of the Su(var)3-9 HMTase activity (Tschiersch et al., 1994; Rea et al., 2000; Schotta et al., 2002; Ner et al., 2002). Both domains are also found in other chromatin proteins, where they play critical roles in silencing (Ivanova et al., 1998; Nakayama et al., 2001; Schotta et al., 2002; Platero et al., 1995: Messmer et al., 1992; Ma et al., 2001; Akhtar et al., 2000; Jacobs et al., 2001; Bannister et al., 2001; Lachner et al., 2001; Bouazoune et al., 2002; Fischle et al., 2003; Min et al., 2003; Pray-Grant et al., 2005). The Drosophila Su(var)3-9 has a longer N-terminus than its mammalian and yeast counterparts. This novel region of the protein appears to be required for interactions with chromatin proteins Su(var)3-7 and HP1, and for Su(var)3-9 dimerization (Schotta et al., 2002; 2003; Eskeland et al., 2004). Theoretically, mutations in the Drosophila specific N-terminus, the chromodomain, or the preSET/SET/postSET regions could lead to a loss of the protein’s silencing function, and to a Su(var) phenotype. Mutations in the N-terminus may do so by preventing Su(var)3-9 dimerization and/or its interaction with other chromatin proteins, such as HP1 and Su(var)3-7. Mutations in the chromodomain could affect the targeting of Su(var)3-9 and/or its interaction with other proteins. Finally, alterations in the
preSET/SET/postSET may impair its catalytic activity, with or without affecting the protein’s ability to participate in protein-protein interactions. In addition, mutations in any region could potentially affect the protein’s folding, thus rendering it inactive.

All Su(var)3-9 mutations were recovered in an EMS screen. EMS causes mainly base-pair substitutions, principally G:C to A:T transitions and thus, effectively causes random mutations. Accordingly, we reasoned that 1) if the Su(var)3-9 mutations isolated in the original screen preferentially target certain regions of the protein, then these regions must be crucial for PEV-type gene silencing, and 2) if some of the EMS-induced lesions represent missense mutations, the affected residues are likely crucial for the silencing function of Su(var)3-9. Strikingly, we found that all 9 mutants are located solely in the preSET/SET/postSET domain. Not surprisingly, all are single base pair substitutions resulting in missense mutations. Since all the mutations cluster within the catalytic (preSET/SET/postSET) domain of Su(var)3-9, our results suggest the catalytic function of Su(var)3-9 is essential for the silencing associated with PEV, and perhaps, amino acid substitutions in the remainder of the protein do not influence the repression caused by PEV.

The nature of the amino acid substitutions in the various Su(var)3-9 alleles was examined further. Su(var)3-9$^{309}$ and Su(var)3-9$^{312}$ arose independently, but have the same A-to-G transition, giving rise to a CYS to TYR substitution at position 462. CYS462 is part of the preSET domain, and is conserved in all homologues of Su(var)3-9, including SUV39H1, Suv39h1 and Clr4p. It corresponds to one of nine cysteine residues that coordinate three zinc ions (Min et al., 2002). Su(var)3-9$^{324}$ (CYS428TYR) and Su(var)3-9$^{376}$ (CYS421SER) also cause substitutions in this highly conserved group...
of cysteines (Fig. 2.1). Three alleles, \textit{Su(var)3-9}^{325}, \textit{Su(var)3-9}^{330} and \textit{Su(var)3-9}^{311}, cause amino acid substitutions in the SET domain. They correspond to \textit{PRO582GLU} in \textit{Su(var)3-9}^{325}, \textit{GLY521ASP} in \textit{Su(var)3-9}^{311}, and \textit{ASP536ASN} in \textit{Su(var)3-9}^{330}. Both \textit{PRO582} and \textit{GLY521} are conserved in the mammalian homologues of \textit{Su(VAR)3-9}, but not in the yeast homologue, \textit{Clr4p}. \textit{ASP536} is conserved in all H3K9-specific HMTases and plays a key role in the interaction with the N-terminal tail of H3 (Zhang \textit{et al.}, 2003). \textit{Su(var)3-9}^{318} and \textit{Su(var)3-9}^{319} represent a pair of independently induced mutations and each causes a \textit{SER616LEU} substitution. This residue is part of the post-SET, but it is not conserved in \textit{SUV39H1}, \textit{Suv39h1} or \textit{Clr4p}. A similar screen performed by Reuter and colleagues (Reuter and Wolff, 1981; Ebert \textit{et al.}, 2004) also recovered multiple alleles of \textit{Su(var)3-9}, but none of them correspond to the nine mutations we recovered (see Discussion).

In summary, the screen yielded nine different \textit{Su(var)3-9} mutants of which 4 carry mutations in the preSET domain, 3 in the SET domain, and 2 in the postSET domain. In two cases (\textit{Su(var)3-9}^{309} and \textit{Su(var)3-9}^{312}, and \textit{Su(var)3-9}^{318} and \textit{Su(var)3-9}^{319}) the same residue was mutated independently, so the 9 different mutants represent 7 different, new alleles/mutations.
Figure 2.1.
Figure 2.1. *Su(var)3-9* mutants are clustered in the preSET, SET and postSET domains. a) A schematic of the domain structure of SU(VAR)3-9 (635 amino acids). The relative positions of the chromodomain (red box), the pre- and postSET domains (green boxes), the SET domain (blue box), and the region that SU(VAR)3-9 shares with eIF2γ (eIF2γ) are shown. The region N-terminal to the chromodomain is longer in the Drosophila protein than the mammalian or yeast homologues and is involved in protein-protein interactions with HP1, SU(VAR)3-7 and SU(VAR)3-9 (Schotta et al., 2003, Eskeland et al., 2004). The vertical bars mark the positions of amino acids substitutions in 9 *Su(var)3-9* mutants identified by sequence analysis. b) Amino acid alignment of the catalytic domain of SU(VAR)3-9, SUV39H1 (human) and CLR4P (fission yeast) showing major conserved residues (red), conserved preSET cysteines that coordinate 3 zinc ions in CLR4P (yellow) (Min et al. 2002) and the conserved aspartate that directly interacts with the substrate (green) (Zhang et al., 2003). The amino acid changes observed in each EMS-induced mutant are shown below the alignment. Allele pairs *Su(var)3-9*^{309} and *Su(var)3-9*^{312}, and *Su(var)3-9*^{318} and *Su(var)3-9*^{319}, are independently generated mutants resulting in the same amino acid substitutions.
2.2.3. The missense alleles express (mutant) Su(var)3-9 gene products.

At the morphological level (suppression of PEV in the w\textsuperscript{m4} strain), the phenotype of the EMS-induced Su(var)3-9 missense mutants is virtually indistinguishable from that of the "protein null", Su(var)3-9\textsuperscript{06} (Reuter and Wolff, 1981; Sinclair \textit{et al.}, 1983; Tschiersch \textit{et al.}, 1994; Schotta \textit{et al.}, 2002). Su(var)3-9\textsuperscript{06} is an X ray-induced, null allele that results in no RNA or protein products (Figures 2.2a and 2.2b, and Tschiersch \textit{et al.}, 1994; Schotta \textit{et al.}, 2002). Western blots were performed on each homozygous mutant strain to ensure that the EMS-induced Su(var)3-9 alleles are not effectively "protein nulls", as a consequence of, for example, an instability of their mutant gene products. All strains tested (Su(var)3-9\textsuperscript{309}, Su(var)3-9\textsuperscript{330}, Su(var)3-9\textsuperscript{318}, Su(var)3-9\textsuperscript{319} and Su(var)3-9\textsuperscript{311}) showed approximately the same level of Su(var)3-9 as the wild-type, confirming that the protein is still present in the mutant strains, in spite of single base-pair substitutions. A representative blot is shown in Figure 2.2b.
Figure 2.2

a.

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<th>cDNA amplified strain</th>
<th>RP49</th>
<th>Su(var)3-9</th>
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<tr>
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<td>-</td>
</tr>
<tr>
<td>Su(var)3-g06</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RTase</td>
<td>-</td>
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b.

<table>
<thead>
<tr>
<th>rec. GST-SU(VAR)3-9</th>
<th>wild type</th>
<th>Su(var)3-g06</th>
<th>Su(var)3-g09</th>
<th>Su(var)3-g50</th>
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Figure 2.2. Detection of Su(var)3-9 gene products in the EMS-induced Su(var)3-9 missense mutants, and in the null allele Su(var)3-906.

a) RT-PCR reactions using total RNA extracted from wild-type (lanes 1, 2, 5 and 6) or Su(var)3-906 female flies as starting material. RNA samples were reverse-transcribed with Su(var)3-9- and RP49-specific primers simultaneously, and the cDNA mixture obtained was PCR amplified separately using primers specific for RP49 (lanes 1-4) or Su(var)3-9 (lanes 5-8). The odd-numbered lanes represent PCR amplification of mock RT reactions (no reverse transcriptase). Identical results were obtained using total RNA from males. Note that primer 3906rt hybridizes to the first 17 nucleotides of Su(var)3-9’s third exon. b) Western blot analysis of wild-type and mutant embryo extracts. Lane 1: GST-SU(VAR)3-9[residues 310-635] recombinant protein (1µg), lane 2: wild-type embryo extract (150µg), lanes 3-5: extracts from homozygous Su(var)3-9 mutant embryos (150µg).
2.2.4. *Su(var)3-9* mutants display a reduction in the levels of H3K9me and HP1 associated with the chromocentre and with the HIS-C.

Since di- and trimethylated H3K9 (H3K9me2/H3K9me3) are the predominant products of Su(var)3-9 catalytic activity (Eskeland *et al*., 2004), we asked whether our EMS-induced *Su(var)3-9* mutations had an effect on the level of H3K9me2 *in vivo*. First, a set of western blots was performed with an anti-H3K9me2 antibody, to compare the levels of H3K9me2 present in embryo extracts from the *Su(var)3-9* homozygous mutant strains to the levels in wild-type and *Su(var)3-9*06 ("protein null") extracts. The western blots were also probed with anti-tubulin antibody to establish equal loading of protein extracts, and the total histone H3 present in each extract was detected on parallel blots to ensure that any difference we observed in H3K9me2 levels between the extracts were meaningful (Figure 3a). The *Su(var)3-9* mutant extracts had significantly reduced levels of H3K9me2 relative to the wild-type extract (Figure 3a, compare lane 2 with lanes 3-6). However, all *Su(var)3-9* missense mutant extracts showed a residual level of H3K9me2. We also detected residual H3K9me2 in the protein null strain, *Su(var)3-9*06. This was not unexpected since there are several other HMTases, including dG9a and DmSETDB1, that can methylate H3K9 (Ayyanathan *et al*., 2003; Mis *et al*., 2006; Seum *et al*., 2007).

H3K9me2 is generally considered a mark of heterochromatin, but is also detected at many euchromatic regions (Nielsen *et al*., 2001; Schotta *et al*., 2002; Ner *et al*., 2002; Greil *et al*., 2003; Ebert *et al*., 2006). Thus, we next asked whether the reduction in the level of methylated H3K9 observed in the *Su(var)3-9* mutants affects heterochromatin exclusively, or whether it can also be observed in some euchromatic...
regions. For this purpose, we examined the distribution of H3K9me2 at a the chromocentre (heterochromatin) and at the histone genes cluster (HIS-C), a euchromatic target of Su(var)3-9 (Ner et al., 2002), in wild-type and Su(var)3-9 mutants. As expected, the chromocenter of wild-type nuclei was richly stained for H3K9me2 (Figure 2.3c, panels A-C). In contrast, the amount of H3K9me2 detected at the chromocentre was substantially reduced in the Su(var)3-9 mutants (Figure 2.3c, compare panel A with panels D, G, J and M). This reduction was particularly dramatic in Su(var)3-906, confirming previous observations (Schotta et al., 2002; Ebert et al., 2006). Note however that H3K9me2, despite its low level, was still detected at the chromocenter. The presence of H3K9me2 at the HIS-C was examined by chromatin immunoprecipitation (ChIP) of cross-linked extracts with α-H3K9me2. Extracts from 12-16 hours old embryos of wild-type, Su(var)3-906 and the various Su(var)3-9 missense mutant strains were tested, and HIS-C DNA sequences were detected in all extracts (Figure 2.3b, lane 3). However, the proportion of HIS-C DNA pulled down by the antibody was lower in the Su(var)3-9 mutants than it was in the wild-type strain (Figure 2.3b, compare lanes 1 and 3 in the wild-type extracts (WT) with lanes 1 and 3 in the Su(var)3-9 mutants). We conclude that the missense Su(var)3-9 mutants, as well as the null mutant Su(var)3-906, are associated with reduced levels of H3K9me2 at both the highly compacted pericentric heterochromatin and at the largely silenced HIS-C, two natural targets of Su(var)3-9.

Several pieces of evidence suggest that H3K9me2 serves as a substrate for HP1 in the formation of centric heterochromatin (Bannister et al. 2001; Lachner et al. 2001; Nakayama et al. 2001; Ebert et al., 2006), and HP1 has been shown to colocalize with
H3K9me2 in heterochromatin (Cryderman et al., 2005; Ebert et al., 2006). Co-localization of H3K9me2 and HP1 within euchromatic regions is much less frequent, but has been observed for a few loci, such as cdc2, the HIS-C and a few others (Cryderman et al., 2005; Greil et al., 2003; Ner et al., in preparation). Accordingly, we asked whether the drastic reductions in the levels of H3K9me2 at the chromocentre and at the HIS-C observed in the Su(var)3-9 mutants correlated with a disruption of HP1 targeting to these two regions. Consistent with previous results, we found that HP1 is still present at the chromocentre of Su(var)3-9 mutants, but in much lower amounts (Figure 2.3b, panels E, H, K, N). Similarly, ChIP analyses demonstrated that although HP1 is present at the HIS-C locus in both wild-type and Su(var)3-9 mutant embryos, it is considerably less abundant in the Su(var)3-9 mutant extracts (Figure 3b, compare lanes 4 and 6 in the wild-type to lanes 4 and 6 in the mutants). We conclude that, like in the case of H3K9me, Su(var)3-9 mutants display reduced levels of HP1 associated with pericentric heterochromatin and with the HIS-C.
Figure 2.3.
Figure 2.3. Detection of H3K9me2 and HP1 in Su(var)3-9 mutants. a) Western blot analysis of wild-type and mutant embryo extracts. Lane 1: bulk core histones (control), lanes 2: wild-type embryo extracts, lanes 3-6: extracts from homozygous Su(var)3-9 mutant embryos. The allele numbers are indicated above the panel. The antibodies used in each case are indicated to the left of the panels. Each lane was loaded with the following: core histones (2 µg), extracts (15 µg for the H3 blot and 150 µg for the H3K9me2/tubulin blots). The H3K9me2 and tubulin panels represent the same western blot probed first with anti-H3K9me2 and subsequently with anti-tubulin. b) Chromatin immunoprecipitation of wild-type and Su(var)3-9 mutant embryo extracts. In each case, the recovered DNA was PCR amplified using primers specific for the coding region of the histone H3 gene (H3F and H3R, see Suppl.table 2). Lane 1: input DNA at a 1:10 dilution (IN (1:10)), lanes 2 and 5: mock IP, no antibody (NO Ab), lane 3: IP with anti-H3K9me2 (IP (K9me2)), lane 4: input DNA at a 1: 100 dilution (IN 1:100), lane 6: IP with anti-HP1 (IP (HP1)). c) Immunostaining of polytene nuclei from salivary glands of wild-type and Su(var)3-9 mutant larvae with anti-H3K9me2 and anti-HP1 (HP1). The genotypes of the larvae are indicated to the left of each set of panels. The arrowheads point to the chromocenter region.
2.2.5. The HMTase activity of the Su(var)3-9 variants is impaired.

Since all Su(var)3-9 missense alleles tested displayed a reduction in the levels of H3K9me2, and carried mutations in the catalytic region of Su(var)3-9, we next asked how much, if any, HMTase activity is retained by each of the mutant proteins, and whether the levels of residual activity correlate with the positions of the different amino acid substitutions. To address this, we tested the in vitro enzyme activity of five of our seven Su(var)3-9 mutant proteins. We chose two mutants with substitutions in the preSET (Su(var)3-9\textsuperscript{376} and Su(var)3-9\textsuperscript{309}), two in the SET domain (Su(var)3-9\textsuperscript{311} and Su(var)3-9\textsuperscript{330}), and one in the postSET domain (Su(var)3-9\textsuperscript{318}). Su(var)3-9\textsuperscript{330} and Su(var)3-9\textsuperscript{311} were selected because they represent substitutions of a highly conserved (ASP536), and relatively un-conserved (GLY521) residues, respectively.

We produced various GST-Su(var)3-9 recombinant proteins (referred to as Su(var)3-9\textsuperscript{allele number for simplicity}) and tested their HMTase activity. As an additional control, we generated a GST-Su(var)3-9 fusion protein that carries the 2 polymorphisms (ALA304PHE, ILE375LEU) detected in some of our strains (Table 2.1). We named this recombinant protein Su(var)3-9\textsuperscript{pol}, and expected it to retain wild-type levels of enzyme activity since these polymorphisms don’t result in suppression of PEV. HMTase assays were first performed on bulk histones. Although the natural substrate for Su(var)3-9 is histone H3, we used all the histones to rule out the possibility that the mutants had acquired activity towards the other histones. As expected, Su(var)3-9\textsuperscript{WT} (Fig. 2.4b, lane 2) and Su(var)3-9\textsuperscript{pol} (data not shown) efficiently catalyzed the transfer of the methyl moiety onto H3. All mutant Su(var)3-9 proteins showed either no activity or a dramatically reduced HMTase function compared to Su(var)3-9\textsuperscript{WT}. Su(var)3-9\textsuperscript{309} (Fig.
2.4b, lane 4) and SU(VAR)3-9^{376} (data not shown) had no detectable HMTase activity. SU(VAR)3-9^{330}, SU(VAR)3-9^{318} and SU(VAR)3-9^{311} showed partial loss of function and they varied in the level of residual enzyme activity (Fig 2.4b, lanes 5-7). Finally, none of the mutants showed any obvious activity on histones other than H3.

Since bulk histones are prepared from nuclei, they contain extensively modified histones, which could have skewed our HMTase measurements. Therefore, we re-tested the mutants using unmodified recombinant histone H3. The results of this analysis were similar to those obtained for the bulk histones (Fig 2.4c and 2.4e). SU(VAR)3-9^{309} and SU(VAR)3-9^{376} showed no detectable HMTase function (Fig. 2.4d, lanes 11 and 15), while SU(VAR)3-9^{330}, SU(VAR)3-9^{318} and SU(VAR)3-9^{311} had reduced levels of activity (Fig. 2.4d, compare lanes 12-14 with lane 9). The relative activities of the different mutants are shown in Figure 2.4e and Table 2.2. Finally, as expected, SU(VAR)3-9^{pol} methylated H3 as efficiently as SU(VAR)3-9^{WT} (99.0% +/-1.55%, data not shown).

In summary, the in vitro HMTase data indicate that all 5 variant SU(VAR)3-9 proteins have dramatically reduced enzymatic activity. While preSET mutants displayed a complete loss-of-function, SET and postSET domain mutants were hypomorphs that retained a small fraction of the wild-type enzyme activity.
Figure 2.4

a. GST-3-9 fusion (r3-9)

b. BULK HISTONES

c. Specific activity on core histones

    Relative activity (%)

    100  80  60  40  20  0

    wild type  GST  309  330  318  311

    24.3  14.3  12.8 <1

d. RECOMBINANT H3

    Specific activity on recombinant H3

    Relative activity (%)

    100  80  60  40  20  0

    wild type  GST  309  330  318  311

    31.3  24.7  22.7 <1
Figure 2.4. The HMTase activity of variant SU(VAR)3-9 proteins. a) Each SU(VAR)3-9 variant protein was expressed and purified as a GST fusion polypeptide (r3-9). The position of the chromodomain (red box), the pre- and postSET (green box) and the SET domain (blue box) are indicated in the stick diagram. The vertical black bars indicate the positions of the amino acid substitutions in 5 Su(var)3-9 alleles that were selected for HMTase analysis. The numbers above each bar indicate the corresponding allele. b) and d) HMTase assay on bulk histones and recombinant H3 respectively. The enzyme activity of the variant proteins was tested using labeled Ado-Met as the methyl donor. The fluorogram (top panel) and the corresponding Coomassie stained gel (bottom panel) are shown. Lanes 1 and 8: molecular weight marker, lanes 2 and 9: wild-type GST-SU(VAR)3-9, lanes 3 and 10: recombinant GST (negative control), lanes 4-7 and 11-15: GST-SU(VAR)3-9 mutant proteins. c) and e) Relative HMTase activity of the various mutants on bulk histones and recombinant H3, respectively. Presented as a bar graph. Following each assay, the methyl H3 radioactive signals and the Coomassie-stained bands corresponding to the appropriate GST-SU(VAR)3-9 proteins were quantified using the ImageQuant and NIH Image softwares respectively (see materials and methods). The bars represent the average of 3 independent assays and the error bars span two S.E.M. Both Su(var)3-9<sup>309</sup> and Su(var)3-9<sup>376</sup> displayed no detectable activity, but for simplicity only Su(var)3-9<sup>309</sup> is shown on the graph.
2.2.6. A mutation in the postSET prevents the addition of a third methyl group to H3K9me2.

There are a wide variety of SET domain-containing HMTases that differ in the lysine residues that they target and/or the number of methyl groups they transfer (Eskeland et al., 2004; Mis et al., 2006). Some methyltransferases can exclusively mono-methylate H3K9 while others, like SU(VAR)3-9, can (mono-), di- or trimethylate (Eskeland et al., 2004). X-ray crystal structural analyses have revealed the key amino acid residues in the preSET, SET and postSET domains that contribute to these activities. For example, the presence of VAL569 in the active site and PHE602 in the enzyme channel allows addition of multiple methyl groups to H3K9 by creating sufficient space to accommodate tri-methylated H3K9 (Xiao et al., 2003; Collins et al., 2005; Zhang et al., 2003).

Since some of the mutations in our Su(var)3-9 alleles affect amino acids located in close proximity to residues in the active site, we asked if the variant SU(VAR)3-9 proteins, which show a partial loss of function, have an altered ability to mono/di- or trimethylate. The bulk histones and recombinant H3 assays did not allow us to distinguish between these three levels of K9 methylation. So, we addressed this issue using H3 tail peptides. We took advantage of an unmodified peptide corresponding to residues 1-20 of H3, and a peptide comprising amino acids 1-21 of H3 in which K9 is dimethylated. Both H3 peptides are very efficient substrates for SU(VAR)3-9WT and are readily methylated (Fig 2.5a and 2.5c, lanes 1 and 7), clearly showing that SU(VAR)3-9 is able to catalyze the addition of a third methyl group to H3K9me2.
The mutant protein Su(Var)3-9\textsuperscript{309} showed no enzyme function on either the unmodified or the dimethylated peptides (Fig. 2.5a, lane 3, and 2.5c, lane 9). Su(Var)3-9\textsuperscript{311} was a hypomorph, and it retained a fraction of the wild-type activity (Fig. 5a, lane 6, and 2.5c, lane 12, respectively) on both peptides. These results are similar to those obtained when full length (unmodified) H3 was used as a substrate. Thus the activity of the Su(Var)3-9\textsuperscript{309} and Su(Var)3-9\textsuperscript{311} mutant forms of the enzyme was consistent on all substrates tested.

In contrast, Su(Var)3-9\textsuperscript{318} behaved differently with the different substrates. While it had reduced activity towards the unmodified peptide and the full-length histone H3, it was completely inactive toward the H3K9me2 peptide (Fig. 2.5a and 2.5c, lanes 5 and 11). Thus, not only did Su(Var)3-9\textsuperscript{318} have a reduced overall catalytic activity, but it also appeared to be unable to tri-methylate K9. Su(Var)3-9\textsuperscript{318} has a SER616LEU substitution in the postSET domain.

Su(Var)3-9\textsuperscript{330} also showed a partial loss-of-enzyme function when either bulk histones or recombinant H3 were used as the substrate. However, rather surprisingly, this variant was unable to methylate the H3 peptides. It retained less than 1% and 4% activity towards the unmodified and H3K9me2 peptides, respectively (Fig. 2.5a and 2.5c, lanes 4 and 10).

In summary, the Su(Var)3-9 mutants displayed an array of severely reduced enzyme activities, ranging from complete abolition to ~25% of the wild-type activity. In addition, some of the mutants showed distinct HMTase characteristics (summarized in Table 2.2) depending on the substrate used.
Figure 2.5

H3 TAIL (aa 1-20)

- Relative activity (%)
  - 100
  - 80
  - 60
  - 40
  - 20
  - 0

H3 K9me2 TAIL (aa 1-21)

- Relative activity (%)
  - 100
  - 80
  - 60
  - 40
  - 20
  - 0

HMTase activity on H3 tail peptide

- Wild type GST 309 330 318 311
  - Relative activity (%)
    - 0
    - <1
    - 1.3
    - 18.3
    - 14.3

HMTase activity on H3K9me2 peptide

- Wild type GST 309 330 318 311
  - Relative activity (%)
    - 0
    - <1
    - 4
    - <1
    - 11.5
Figure 2.5. HMTase activity of Su(var)3-9 alleles using unmodified and dimethylated H3 tail peptides. a) and c) Fluorograms of an HMTase assay on H3 peptide (H3 tail, aa 1-20) and dimethylK9H3 peptide (H3K9me2 tail, aa 1-21) respectively. Lanes 1 and 7: wild-type GST-SU(VAR)3-9, lanes 2 and 8: GST (negative control), lanes 3-6 and lanes 9-12: GST-SU(VAR)3-9 mutants. The allele numbers are indicated above the corresponding lanes. b) and d) Relative in vitro activity of the indicated Su(var)3-9 alleles on unmethylated (b) and dimethylK9 (d), H3K9me2 H3 tail peptides. The numbers used in the bar graphs were obtained as described in the materials and methods.
2.2.7. **Effect of Su(var)3-9 missense and null alleles on PEV in the presence of one mutant and 2 wild type copies of Su(var)3-9.**

All Su(var)3-9 mutants were originally identified on the basis of a morphological phenotype, dominant suppression of PEV. Here, we examine the morphological phenotype of the Su(var)3-9 mutants in more detail. We specifically asked whether the mutant alleles differ in their ability to suppress heterochromatic gene silencing that results from PEV and, if so, whether these are correlated to the differences observed at the biochemical level (*in vitro* enzyme function). Direct examination of the Su(var)3-9 alleles with the “classical” variegating strain, w\(^{m4}\), is inadequate for this study since all the Su(var)3-9 mutants almost completely suppress w\(^{+}\) gene silencing. So, we used a variation on the w\(^{m4}\) assay system and examined the suppression effect in the presence of an extra copy of wild-type Su(var)3-9, that is, in flies with w\(^{m4}\); Su(var)3-9\(^{mutation}\)/Su(var)3-9\(^{+}\), Su(var)3-9\(^{+}\) genotypes. We took advantage of a Su(var)3-9::eGFP transgene inserted in the third chromosome (*pP{GS[ry\(^+\), (10kb Su(var)3-9)EGFP]}`, (Schotta and Reuter, 2000), here referred to as *P[3-9egfp]* for simplicity). Crosses were set up, in triplicate, between males homozygous for this transgene and for the wild-type, endogenous Su(var)3-9 allele (*w^{m4}\!/Y; Su(var)3-9\(^+\), P[3-9egfp]/Su(var)3-9\(^{+}\), P[3-9egfp]) and females homozygous for each of the Su(var)3-9 alleles (see material and methods). The progeny of these crosses carry one maternally inherited mutant copy (or, in the case of the control cross, one wild-type copy) and two paternally derived wild-type copies of Su(var)3-9 (the endogenous Su(var)3-9\(^{+}\) and the ectopically inserted *P[3-9egfp]*). For each cross, the eyes of the male progeny were visually scored for the amount of eye pigment and thus the level of suppression of PEV.
Over 98% of the flies derived from the control cross, which have 3 copies of the 
$Su(var)3-9^+$ allele ($w^m4/Y; Su(var)3-9^+/Su(var)3-9^+,P[3-9egfp]$), displayed a strong $E(var)$ phenotype, confirming that the $Su(var)3-9::eGFP$ transgene is functional and over-production of $Su(var)3-9$ enhances the gene silencing that results from PEV (Fig. 2.6). On the other hand, in the progeny bearing a mutant allele, a wild-type (endogenous) allele and an extra (transgenic) copy of $Su(var)3-9^+$, we observed a broad range of variegating eye phenotypes. None of the individuals displayed the almost completely white eye phenotype of their control counterparts. However, we did note a significant proportion of flies with bilaterally unequal eye pigmentation, that is, one eye strongly suppressed and the other unsuppressed. For this reason, instead of performing standard pigment assays, we scored each eye visually and assigned it to one of three categories: strongly suppressed (~75-100% pigment), mildly suppressed (~20-75%), or unsuppressed (~5-20%) (Fig. 2.6b, panels A, B & C respectively). The proportion of strongly suppressed, mildly suppressed and unsuppressed eyes varied with the genotype (Fig. 2.6b). For the sake of simplicity, and since all these individuals have the identical wild type and transgenic alleles, and only differ in their mutant allele of $Su(var)3-9$, the description of their genotypes is limited to the identity of the mutant allele they carry.

Over half of the individuals carrying the $Su(var)3-9^{06}$ allele had strongly suppressed eyes. Since $Su(var)3-9^{06}$ is a null allele for which no mRNA or protein products are detected (Fig. 2.2 and Tschiersch et al., 1994; Schotta et al., 2002), these individuals should be phenotypically equivalent to $w^m4, Su(var)3-9^+/Su(var)3-9^+$ flies, which typically have variegating, unsuppressed eyes (Tartof et al., 1984; Grigliatti, 1991;
Reuter and Spierer, 1992, and references therein). Thus, the presence of a large proportion of strongly suppressed eyes in \( w^{nd}/\gamma; Su(var)3-9^{06}/Su(var)3-9^{+}, P[3-9egfp] \) individuals suggests that the ectopically inserted \( Su(var)3-9 \) transgene, \( P[3-9egfp] \), may not be functionally equivalent to the endogenous gene. The EGFP-tagged \( Su(var)3-9 \) may produce less protein product, and/or its product may function less efficiently than the wild-type \( Su(var)3-9 \).

The highest proportion of strongly suppressed eyes was observed in flies carrying the \( Su(var)3-9^{311} \) allele, which suggests \( Su(var)3-9^{311} \) is the strongest suppressor of PEV in this genetic test. Individuals bearing the \( Su(var)3-9^{318} \) and \( Su(var)3-9^{330} \) alleles displayed approximately the same fraction of strongly suppressed eyes, indicating that \( Su(var)3-9^{318} \) and \( Su(var)3-9^{330} \) suppress PEV roughly to the same degree. Curiously, in this assay, \( Su(var)3-9^{309} \) resulted in the lowest proportion of strongly suppressed, and the highest proportion of unsuppressed eyes, suggesting \( Su(var)3-9^{309} \) is a weaker suppressor of PEV than \( Su(var)3-9^{330}, Su(var)3-9^{318} \) or \( Su(var)3-9^{311} \).

In summary, differences in the strength of PEV suppression were detected among the four \( Su(var)3-9 \) missense alleles. Using this particular genetic assay of function, \( Su(var)3-9^{311} \) was the strongest suppressor, while \( Su(var)3-9^{309} \) was the weakest, and also the only one to suppress PEV less efficiently than the «protein null» allele, \( Su(var)3-9^{06} \). With the exception of \( Su(var)3-9^{309} \), these results correlate well with the levels of remaining HMTase activity of each mutant (see Discussion).
Figure 2.6.
Figure 2.6. Effect of several $Su(var)3$-$9$ alleles on PEV (here, $w^{md}$ variegation) in $w^{md}/Y; Su(var)3$-$9^+, P[3-9egfp]/Su(var)3$-$9^{mutant}$ individuals. a) A schematic of the genetic configuration of the flies examined in this experiment. The EGFP-tagged $Su(var)3$-$9$ (also indicated as $P[3-9egfp]$ for simplicity) represents the ectopic $Su(var)3$-$9$ insertion $pP\{GS[ry^+, (10kb Su(var)3$-$9)EGFP]\}$ (Schotta and Reuter, 2000). This construct is inserted on the third chromosome, thus all flies harboring this insert also carry a wild-type copy of the endogenous $Su(var)3$-$9$ gene.

b) Frequencies (%) of strongly suppressed, mildly suppressed, unsuppressed, and enhanced eyes in $w^{md}/Y; Su(var)3$-$9^+, P[3-9egfp]/Su(var)3$-$9^{mutant}$ individuals carrying different $Su(var)3$-$9$ alleles. Pictures A-D show examples of strongly suppressed, mildly suppressed, unsuppressed and enhanced $w^{md}$ variegation, respectively. Genotypes are shown on the left, n=number of crosses analyzed, tot=total number of eyes scored. The bar graphs below each eye picture represent the fraction (%) of eyes displaying the depicted phenotype amongst the individuals of the genotype indicated (average of 3 independent crosses +/- S.E.M., rounded to the closest second decimal). Panels A and C show eyes of $w^{md}/Y; Su(var)3$-$9^{309}/Su(var)3$-$9^+, P[3-9egfp]$ flies. The eye in panel B is from a $w^{md}/Y; Su(var)3$-$9^{06}/Su(var)3$-$9^+, P[3-9egfp]$ fly and the eye in D is from a $w^{md}/Y; Su(var)3$-$9^+/Su(var)3$-$9^+, P[3-9egfp]$ individual.
2.3. Discussion

2.3.1. The $Su(var)3-9$ mutations are single base-pair substitutions clustered in the catalytic region.

$Su(var)3-9$ has two highly conserved and functionally distinct regions: a chromodomain and a SET domain; the latter, together with the flanking pre- and postSET sequences, constitutes the HMTase activity of the protein. Both regions are present in several chromatin associated proteins and are highly conserved, and therefore it is reasonable to assume that the chromo and SET domains each have important roles in the function of $Su(var)3-9$. Other domains of the protein, as yet unidentified, may also be required for the function of $Su(var)3-9$. Indeed, we expected that the dominant suppressors of PEV would comprise mutations in the chromo and SET domains, and perhaps identify other regions of $Su(var)3-9$ that are required for the epigenetic gene silencing observed in PEV. Interestingly, the 9 confirmed EMS-induced $Su(var)3-9$ mutations altered 7 residues, with 2 residues hit twice, all of which are located in the catalytic region of $Su(var)3-9$; none of the $Su(var)3-9$ mutations occurred in or near the chromodomain of the protein. The concentration of missense mutations within the preSET/SET/postSET region of the protein suggests that the HMTase activity of $Su(var)3-9$ plays a crucial role in suppression of PEV. In contrast, single amino acid substitutions in the chromodomain are probably insufficient to cause a dominant suppression of PEV and thus affect the gene silencing function of $Su(var)3-9$. Alternatively, the absence of chromodomain mutants among the dominant $Su(var)$s recovered in the original screen may be due to the fact that the EMS mutagenesis failed to induce mutations in or around the chromodomain of $Su(var)3-9$. This latter hypothesis
seems unlikely, because Reuter and colleagues also failed to recover mutations in the chromodomain of Su(var)3-9 by screening for strong, dominant suppressors of PEV (Reuter and Wolff, 1981; Ebert et al., 2004). Mutations in the N-terminal region of the protein were indeed isolated in several screens using P element transposition or gamma rays as the mutagenic agent, but they represent insertions and deletions that produce either truncated proteins, or no protein at all (Tschiersch et al., 1994; Harrington, 2001; Schotta et al., 2002; Ebert et al., 2004, 2006). These mutants act as dominant Su(var)s because the Su(var)3-9 locus is dosage sensitive, that is hemizygosity for Su(var)3-9 suppresses PEV (Grigliatti, 1991; Reuter and Spierer, 1992; and references therein). Nevertheless, the observation that mobile genetic elements can insert into or near the chromodomain suggests that this region should be amenable to mutagenesis via EMS.

Thus, the function of the chromodomain in Su(var)3-9 remains elusive, although it is more defined in other proteins, such as HP1, Pc and MOF. In these proteins, the chromodomain appears to be required for chromatin binding (Platero et al., 1995; Messmer et al., 1992; Ma et al., 2001; Akhtar et al., 2000; Jacobs et al., 2001; Bannister et al., 2001; Lachner et al., 2001; Bouazoune et al., 2002; Fischle et al., 2003; Min et al., 2003; Pray-Grant et al., 2005). By analogy, a similar role was suggested for the Su(var)3-9 chromodomain (Schotta et al., 2002; 2003), but, to date, direct evidence is lacking. If the Su(var)3-9 chromodomain is involved in chromatin binding, then one would expect mutations within the chromodomain to cause mis-targeting, which should have a strong dominant Su(var) phenotype. The absence of chromodomain mutations among the respective collections of dominant Su(var)3-9 mutants (this paper and Ebert
et al., 2004; Donaldson et al., 2002), suggests that substitution of any of the ~40 amino acids that comprise the chromodomain is not enough to cause significant mis-targeting.

2.3.2. Reduction of H3K9me2 and HP1 in Su(var)3-9 mutants.

At the cellular level, we found that all Su(var)3-9 missense mutants display a dramatic reduction in the levels of both H3K9me2 and HP1 associated with centric heterochromatin and with the HIS-C. Firstly, these in vivo observations corroborate the results obtained from the in vitro enzyme assay, namely that the amino acid substitutions present in the mutant alleles of Su(var)3-9 impair the protein’s HMTase function. Missense mutations in Su(var)3-9 also altered the abundance of HP1 binding, as measured by in situ immunofluorescence and ChIP analysis, which is consistent with the hypothesis that H3K9me2 constitutes a binding platform for HP1 (Bannister et al., 2001; Lachner et al., 2001; Nakayama et al., 2001; Ebert et al., 2006). Secondly, the observation that Su(var)3-9 mutants cause the same set of effects at the chromocentre and at the HIS-C, a euchromatic locus and natural target of Su(var)3-9, suggests that Su(var)3-9 may function as part of a silencing mechanism that affects numerous loci in the euchromatic as well as heterochromatic regions of the genome.
2.3.3. The SET mutants are hypomorphs

Based on the crystal structures of the Su(var)3-9 homologues Clr4p and DIM-5 (Min et al., 2002; Zhang et al., 2003), both the HMTase active site, and the substrate-binding cleft, are part of the SET domain. The mutant alleles Su(var)3-9^{311}, Su(var)3-9^{325} and Su(var)3-9^{330} cause single amino acid substitutions in the SET domain. Each of the affected residues is predicted to lie within, or very near, the active site of the enzyme (Figure 2.7). Su(var)3-9^{330}, which affects ASP536, is the most interesting of these SET mutants. ASP536 is conserved in all H3K9 and in some H3K4 methyltransferases (Aagard et al., 1999; Zhang et al., 2002; Mis et al., 2006). It is located in the portion of the cleft that is involved in stabilizing the enzyme-substrate complex; its side chain forms a hydrogen bond with the hydroxyl oxygen of SER10 of H3 (H3S10) (Min et al., 2002; Zhang et al., 2003). The HMTase activity of Su(var)3-9^{330}, on full-length, unmodified histone H3, is reduced to about 31% of the wild-type. This partial loss of function is probably the result of an unstable enzyme-substrate interaction due to the replacement of ASP536 with ASN, which could disrupt the hydrogen bond formed between H3S10 and ASP536. Interestingly, the H3 peptides are much poorer substrates for Su(var)3-9^{330} compared with full length H3. The enzyme activity on unmodified, and H3K9me2 peptides is less than 5% of wild-type. Their smaller size, and the absence of backbone residues, which are present in full length H3, reduce the stability of binding of the peptides compared to the full length H3. Thus, the substitution of ASP536 probably has a more drastic effect on the peptides than it does on the full-length histones. Alternatively, the ASP536ASN substitution in Su(var)3-9^{330} may lead to an altered substrate specificity from H3K9 to another H3 lysine that is not present in the H3 tail.
peptides, for example K27. Since K27 is absent in these peptides, the only activity detected would correspond to weak, residual methylation of K9. Since no appropriately modified peptides are available that include residues 1-27 of H3, this hypothesis cannot be tested without using mass spectrometry.

2.3.4. The preSET mutants are enzymatically inactive.

There are nine key cysteine residues in the preSET region of Su(var)3-9. These residues are highly conserved (Aagaard et al., 1999; Min et al., 2002) and crystal structures of the Clr4p and DIM-5 proteins reveal that these cysteines coordinate 3 zinc ions that form a “zinc cluster”. This “cluster” has an important structural role as it holds together two random coils that form the bottom surface of the catalytic region of the protein (Min et al., 2002; Zhang et al., 2003). Although the preSET domain is not part of the enzyme active site, or the regions binding the substrate or cofactor per se, it is required for efficient H3 methylation (Rea et al., 2000).

Four of our Su(var)3-9 missense alleles have mutations in preSET cysteine residues. We tested two of them, Su(var)3-9376 (CYS428TYR) and Su(var)3-9309/312 (CYS462TYR), for their in vitro HMTase activity, and in both cases the variant proteins were completely inactive. Given the role of these residues in protein structure, the complete loss of enzyme function is likely due to misfolding of the protein, which dramatically alters many aspects of substrate binding and enzyme function.
2.3.4. The postSET mutant lacks the ability to add a third methyl group to H3K9me2.

Su(var)3-9^{318} and Su(var)3-9^{319} are two independently isolated mutations with the same SER616LEU mutation in the postSET region. Although this amino acid is not conserved in Clr4p, Suv39h1 and SUV39H1 (fig 1b), our results suggest that it is crucial for the addition of the third methyl group to H3K9. In addition, the fact that the corresponding residues are a GLN in Clr4p and an ASP in SUV39H1 and Suv39h1 (Fig.1b), and that the SER616LEU substitution in SU(VAR)3-9 causes a strong, dominant Su(var) phenotype, suggest that the presence of a polar residue at this position may be critical for SU(VAR)3-9 function.

In the Clr4p structure the flexible postSET region is positioned near the active site where it acts as a “lid” and creates a solvent-secluded space for optimal methyl transfer (Min et al., 2002). The postSET domain of DIM-5 works in a similar manner and in the presence of H3 substrate it interacts directly with the enzyme active site, with the substrate, and with the cofactor AdoMet (Zhang et al., 2003). Hence, mutations in the postSET region could disrupt methyl transfer by altering the local architecture and exposing the active site to the solvent. Alternatively, the altered residue in the postSET may interfere with the normal positioning of the substrate or the cofactor in their respective binding pockets. As expected, Su(var)3-9^{318} has reduced HMTase function on unmodified substrates such as bulk histones, recombinant H3 and H3 tail peptide (1-20). However, Su(var)3-9^{318} fails to add a third methyl group to an H3 tail peptide already dimethylated at K9, suggesting that the mutation either interferes with the stability of the dimethylated peptide or the AdoMet in the catalytic cleft, or creates sufficient steric hindrance to impair methyl transfer.
Figure 2.7.
Figure 2.7. Probable tertiary structure of SU(VAR)3-9 and relative positions of the mutated residues. a) Amino acid sequence alignment of the catalytic domain of Clr4p and SU(VAR)3-9. Identical residues are highlighted in black, similar residues are highlighted in grey. Red dots indicate the residues that are mutated in the different Su(var)3-9 alleles. Above the alignment is represented the secondary structure of Clr4p (aa 220-490), according to Min et al. (2002). The N-terminal region of the catalytic domain is shown in black, the pre- and postSET domains in green, and the SET domain in blue. The high level of homology between the two proteins at the primary sequence level suggests that their secondary and tertiary structures could also be very similar. b) A ribbon diagram representation of the Clr4 structure (residues 220-490). Under the assumption that the tertiary structure of SU(VAR)3-9 is very similar to Clr4’s, the red dots show the approximate positions of the amino acids that are mutated in SU(VAR)3-9309, SU(VAR)3-9330, SU(VAR)3-9318 and SU(VAR)3-9311. The diagram was generated partially using Raster3D (Kraulis, 1991) and MolScript (Merritt and Murphy, 1994).
2.3.5. **Effect of Su(var)3-9 missense and null alleles on PEV in a strain that is hyperploid for Su(var)3-9.**

In our structure-function analysis of Su(var)3-9 we wanted to examine the morphological phenotype of PEV and attempt to correlate the morphological end point (eye colour pattern) with the cytological and molecular effects of the different mutations in Su(var)3-9. However, all Su(var)3-9 missense mutants suppress PEV very strongly. Therefore, we employed an assay for PEV, which examined the relative strength of the Su(var)3-9 mutations in individuals that carried three copies of Su(var)3-9. The three alleles were one wild-type, endogenous allele (Su(var)3-9+), one Su(var)3-9+−EGFP transgene, and one mutant Su(var)3-9 allele.

At the morphological level (suppression of PEV) we found that all Su(var)3-9 mutant alleles were able to suppress PEV in at least a substantial fraction of w^m4/Y; Su(var)3-9mutant/Su(var)3-9+, P[3-9egfp] individuals, but they differed in their strength of suppression. These differences may be due to differences in the residual HMTase activity of the mutant SU(VAR)3-9 proteins. However, it should be kept in mind that, unlike the HMTase assay, which provides a direct measure of enzyme activity under given conditions, or the immunohistochemical analyses, which identify the distribution patterns and can delineate the relative abundance of given proteins, PEV suppression is a tertiary phenotype involving numerous unknown variables, and may therefore not be directly indicative of SU(VAR)3-9 HMTase function.

Flies carrying the Su(var)3-9^{06} mutant allele (w^m4/Y; Su(var)3-9^{06}/Su(var)3-9^+, P[3-9egfp]) served as "baseline", as Su(var)3-9^{06} does not produce any Su(var)3-9 mRNA or protein, and the products of the endogenous Su(var)3-9^+ and the transgenic
\textit{Su(var)3-9^*-EGFP} must therefore account for the \textit{Su(var)3-9} function present in this strain. Individuals of this genotype showed strongly suppressed eyes at a frequency of about 53\%. Curiously, the presence of missense \textit{Su(var)3-9} allele (\textit{w^m4/Y; Su(var)3-9^{\text{missense}}/Su(var)3-9^*}, \textit{P[3-9egfp]}) resulted in either stronger (\textit{Su(var)3-9^{330}}, \textit{Su(var)3-9^{318}}, \textit{Su(var)3-9^{311}}) or weaker (\textit{Su(var)3-9^{309}}) suppression of PEV than with the \textit{Su(var)3-9^{06}} null allele (Fig. 6b). \textit{Su(var)3-9} alleles resulting in stronger suppression of PEV than \textit{Su(var)3-9^{06}} are likely antimorphs, while alleles that are weaker suppressors than \textit{Su(var)3-9^{06}}, are probably hypomorphs. This logic suggests that \textit{Su(var)3-9^{311}}, \textit{Su(var)3-9^{318}} and \textit{Su(var)3-9^{330}} are antimorphs, and \textit{Su(var)3-9^{309}} is a hypomorph.

At a mechanistic level, the three antimorphic mutants possibly act as dominant negatives. The mutant \textit{Su(var)3-9} products may be incorporated into protein complexes like their wild-type counterparts, and these complexes would be correctly targeted, but would fail to efficiently methylate H3K9, interfering with the function of the wild-type \textit{Su(var)3-9}. Indeed, the mutations present in \textit{Su(var)3-9^{311}}, \textit{Su(var)3-9^{318}} and \textit{Su(var)3-9^{330}} are located in the catalytic region of the protein, and do not affect its N-terminal protein-protein interaction domains. However, the possibility that amino acid substitutions in the SET domain may play a role in the assembly, stability, or targeting of \textit{Su(var)3-9}-containing complexes cannot be excluded.

In this hyperploid genotype, \textit{Su(var)3-9^{311}} is a stronger suppressor of PEV than \textit{Su(var)3-9^{330}}. This may be due to the fact that \textit{Su(var)3-9^{311}} retains significantly less HMTase activity than \textit{Su(var)3-9^{330}}, as the \textit{in vitro} enzyme assays indicate. In this genetic assay, \textit{Su(var)3-9^{311}} is also a morphologically stronger PEV suppressor than \textit{Su(var)3-9^{318}}, but the \textit{in vitro} enzyme activities of \textit{Su(var)3-9^{311}} and \textit{Su(var)3-9^{318}} do
not differ significantly. This may be explained by the peculiar biochemical phenotype of SU(VAR)3-9^{318}. If, as suggested by the in vitro data (Figure 5), this mutant protein is an inefficient HMTase that is also completely unable to trimethylate histone H3, then its observed residual activity on unmodified substrates probably represents mono/dimethylation exclusively. Therefore, comparing the HMTase activity of SU(VAR)3-9^{318} and SU(VAR)3-9^{330} or SU(VAR)3-9^{311} based on the relative amount of radiolabeled methyl transferred onto unmodified H3 substrate, could be misleading. With respect to mono/dimethylation, SU(VAR)3-9^{318} may well retain as much activity as SU(VAR)3-9^{330} (i.e. significantly more than SU(VAR)3-9^{311}) and, since H3K9me2 is sufficient for heterochromatic silencing in Drosophila (Fischle et al., 2003; Swaminathan et al., 2005), it would not be surprising that Su(var)3-9^{318} and Su(var)3-9^{330} may be equivalent in their ability to suppress PEV, and that neither is as strong as Su(var)3-9^{311}. However, we cannot exclude the possibilities that 1) the in vitro HMTase activity of the mutant SU(VAR)3-9 proteins is not always a good indicator of their in vivo activity, and/or 2) the Su(var)3-9 missense mutations affect more than just the protein’s enzyme activity, and the enzyme activity of the different mutant SU(VAR)3-9 proteins is not the only factor determining the strength of the Su(var) phenotype.

Interestingly Su(var)3-9^{309}, which behaves as a hypomorph in the PEV assay employed here, results in a protein product with no in vitro HMTase activity at all. We propose that the amino acid substitution present in SU(VAR)3-9^{309} (CYS462TYR) causes the catalytic region of the protein to be misfolded since it affects a residue that likely plays an important structural role (Min et al., 2002). Thus, in vitro, the complete loss of enzyme function associated with this mutation is due to severe misfolding of the
polypeptide. Furthermore, we suggest that the catalytic domain of Su(VAR)3-9\textsuperscript{309} is misfolded \textit{in vivo}. This misfolding not only renders it inactive, but also prevents it from being incorporated into Su(VAR)3-9-containing complexes, therefore not interfering with the function of the wild-type protein. Hence, Su(var)3-9\textsuperscript{309} is not an antimorph. We are left with the observation that Su(var)3-9\textsuperscript{309} is a weaker suppressor of PEV than the null allele, Su(var)3-9\textsuperscript{06}, indicating that Su(var)3-9\textsuperscript{309} is a hypomorph. One possibility is that, occasionally (\textit{i.e.} at a low frequency, \textit{e.g.} 10\% of the time), Su(VAR)3-9\textsuperscript{309} is still incorporated into the Su(VAR)3-9-containing complex, and its incorporation into the complex stabilizes its tertiary structure. In such cases, Su(VAR)3-9\textsuperscript{309} is able to methylate histone H3 like its wild-type counterpart, since its active site is intact.
Table 2.2. Summary of the molecular, biochemical, and Su(var) phenotypes of five Su(var)3-9 mutant alleles examined in this study. The stick diagram of SU(VAR)3-9 shows the positions of the point mutations in Su(var)3-9<sup>309</sup>, Su(var)3-9<sup>311</sup>, Su(var)3-9<sup>330</sup> and Su(var)3-9<sup>318</sup> (vertical black bars). The chromodomain (red box), pre- and postSET domains (green boxes) and SET domain (blue box), and the N-terminal region that is in common with eIF2g are also indicated. For each allele, the result of the mutation (amino acid change), the relative amount of residual HMTase activity with 3 different substrates, and the percentage of strongly suppressed eyes in the PEV assay are shown.

<table>
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<th>Su(var)3-9 allele</th>
<th>H3K9me2 in vivo</th>
<th>H3HMTase in vitro (% of wild-type)</th>
<th>% strongly suppressed eyes</th>
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<tr>
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</tr>
<tr>
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<td>S616L</td>
<td>Reduced</td>
<td>24.7 +/- 1.7</td>
</tr>
</tbody>
</table>
2.4. Materials and Methods

2.4.1. *Drosophila* strains

Unless otherwise specified, all fly strains were grown under standard conditions on glucose/yeast/cornmeal medium, with Tegosept (methyl-p-hydroxybenzoate) as a mold inhibitor.

2.4.2. Recombination mapping

Allelism to *Su(var)*3-9 was determined by recombination analysis. Each putative EMS-induced *Su(var)*3-9 mutant (*w*\(^{m4}\);*Su(var)*X/*Su(var)*X) was first crossed to *w*\(^{m4}\);*Su(var)*3-9\(^{P25}\), which harbours a P-element insert in the first intron of the dual *Su(var)*3-9/elF2g transcription units (Harrington, 2001; Ner et al., 2002). F1 females (*w*\(^{m4}\)/*w*\(^{m4}\); *Su(var)*3-9\(^{P25}\)/*Su(var)*X) were then crossed to *w*\(^{m4}\)/Y;+/- males, and in each case >2000 offspring were scored with respect to PEV suppression. Mutants that did not yield any *w*\(^{m4}\);+/- recombinants, indicating that the distance between *Su(var)*3-9 and the *Su(var)*X mutation they carried was less than 0.1 cM, were further characterized by DNA sequence analysis. The maximum distance between *Su(var)*X and *Su(var)*3-9 was calculated as if the next fly to eclose would have been a wild-type recombinant; max distance = 2 [since the reciprocal event yields a double mutant, indistinguishable from the parentals]/(1+total number of flies scored).
2.4.3. Suppression of PEV in the presence of 2 wild-type copies of \( Su(var)3-9 \)

Homozygous \( w^{m4}; \) \( Su(var)3-9 \) females were crossed to \( w^{m4}/Y; \) \( pP[GS[ry+,(10kb \ Su(var)3-9)EGFP]] \) homozygous males (Schotta and Reuter, 2000) and the eye colors of the offspring were scored. Only males were scored in order to avoid effects due to the presence of two copies of the \textit{white} \ gene. All crosses were conducted in triplicate at 18 °C. For each cross, a total of 356 to 580 eyes were examined. The phenotype of each eye was classified as “strongly suppressed”, “mildly suppressed”, “unsuppressed” (\( w^{m4} \)-like), or enhanced. The reciprocal crosses gave similar results, but the number of offspring was much lower due to the low fecundity of \( w^{m4}/w^{m4}; \) \( pP[GS[ry+,(10kb \ Su(var)3-9)EGFP]] \) homozygous females at 18 °C. Crosses were set up in triplicates, and strength of PEV suppression was assessed based on the percentage of strongly suppressed eyes in the male offspring of each cross (average +/- S.E.M.). Student’s T-tests (p=0.05) were employed to determine whether differences between alleles were statistically significant.

2.4.4. Sequence analysis

Genomic DNA was extracted from the following stocks: Oregon-R, \( w^{m4} \), \( w^{m4};Su(var)3-9^{309}/Su(var)3-9^{309} \), \( w^{m4};Su(var)3-9^{312}/Su(var)3-9^{312} \), \( w^{m4};Su(var)3-9^{311}/Su(var)3-9^{311} \), \( w^{m4};Su(var)3-9^{317}/TM3,Sb,Ser \), \( w^{m4};Su(var)3-9^{318}/Su(var)3-9^{318} \), \( w^{m4};Su(var)3-9^{324}/TM3,Sb,Ser \), \( w^{m4};Su(var)3-9^{325}/TM3,Sb,Ser \), \( w^{m4};Su(var)3-9^{327}/TM3,Sb,Ser \), \( Su(var)3-9^{319}/TM3,Sb,Ser \), and \( w^{m4};Su(var)3-9^{330}/Su(var)3-9^{330} \).

Segments of the \( Su(var)3-9 \) gene were amplified by PCR using the following primer
pairs: 39KYLE and 3SET, 3-95’ and 39-1, 39-2 and 3-95’, 5RI and 3RI, and 5SET and
3SET (all primer sequences are listed in supplementary table 2). The amplification
conditions were: 94°C, 5 min; (94°C 45 sec, 57-60°C, 30 sec, 72°C, 1.5 min) for 30
cycles, 72°C, 10 min. Each PCR reaction was performed three times and both strands
of each product were sequenced twice.

2.4.5. RT-PCR

Total RNA was prepared from wild-type and Su(var)3-906 homozygous flies by
TRIzol® extraction as recommended by the manufacturer (Invitrogen). 50 adults were
used for each extraction. 2 µg of each RNA sample were reverse-transcribed with
primers RP49rt (control) and 3906rt simultaneously, following standard procedures.
Mock reactions (no reverse transcriptase) were carried out to ensure that no
contaminating genomic DNA was present. cDNA (first strand) samples and mock
reactions were amplified using primer pairs RP495-RP493, and 3906rt-3906pcr,
separately. The amplification conditions were: 94°C, 5 min; (94°C, 30 sec; 52°C, 30 sec;
72°C, 30 sec) for 30 cycles; 72°C, 5 min.

2.4.6. Western blots

Western blot analyses were performed according to standard procedure (Lacey
et al., 1994). Embryo extracts were prepared from wild-type and mutant 12-16 hour old
embryos as described below under (ChIP analysis). About 150 µg of each extract were
used for the Su(var)3-9 and H3K9me analyses. The blots were probed with a polyclonal
anti- Su\textsuperscript{VAR}3-9 antibody (a-3-9\textsuperscript{chr}) (Ner \textit{et al.}, 2002) at 1:2000 dilution and subsequently the blots were probed with an anti-tubulin monoclonal antibody at 1:1000. To detect the methylation status of K9H3 we used commercial anti-H3K9me2 (Upstate Biotech #07-212) at 1:1000 and to detect total histone H3 the blots were probed with an anti-H3 monoclonal antibody at 1:30,000 dilution (Sauvé \textit{et al.}, 1999).

2.4.7. Immunostaining of polytene nuclei

Salivary glands of wild-type and Su\textit{(var)}3-9 mutant 3rd instar larvae were dissected in PBS, fixed in PBS + 2% formaldehyde for 15 min at room temperature, washed 3 times in PBS\textsuperscript{2+}, and blocked in PBS\textsuperscript{2+} with 1% BSA for 60 min at room temperature (Cryderman \textit{et al.}, 1999). Protease inhibitors were added as required. The anti-H3K9me2 (Upstate Biotech #07-212) and anti-HP1 (C1A9) (James \textit{et al.}, 1989) were added at a final dilution of 1:250 each. The secondary antibodies were anti-rabbit Alexa488 and anti-mouse Alexa568 (Molecular Probes) at 1:1000 each.

2.4.8. ChIP analysis

12-16 hours old embryos were dechorionated in 50% bleach for 2 min and washed extensively with PBS+0.01% Triton-X 100. Cross-linking was achieved by incubation in 2% formaldehyde, 50mM Hepes pH 8.0, 100mM NaCl, 1mM EDTA, 0.5mM EGTA for 10 min at room temperature and then for 20 min at 4°C, and terminated by adding glycine at a final concentration of 250mM. The embryos were then washed twice with 10mM Tris-HCl, pH 8.0, 1mM EDTA, 0.5 mM EGTA and subjected to
sonication (9X15 sec at 30% output). Protease inhibitors were added as required. The soluble fraction of the lysate was adjusted to a final concentration of 3M urea and incubated on ice for 10 min. Nucleoprotein complexes were purified using a polyclonal anti-H3K9me2 (UPSTATE #02-441) or a polyclonal anti-HP1 (a-HP1 (Ner et al., in preparation)) antibodies. Mock reactions (no antibody) were included in each set of experiments. Immunoprecipitated DNA was purified as described by Nelson et al., (2006). Genomic sequences of interest (HIS-C) were detected by PCR using primer pair H3F/H3R. The amplification conditions were: 94°C, 5min; (94°C, 45 sec; 58°C, 30 sec; 72°C, 40 sec) for 26 cycles; 72°C, 5 min.

2.4.9. Expression and purification of active GST-SU(VAR)3-9 fusion proteins

DNA fragments encoding amino acids 182 to 635 of wild-type SU(VAR)3-9, SU(VAR)3-9\(^{311}\), SU(VAR)3-9\(^{309}\), SU(VAR)3-9\(^{330}\), SU(VAR)3- SU(VAR)3-9\(^{318}\) and SU(VAR)3-9\(^{376}\), were amplified from genomic DNA of the corresponding strains using primers 39KYLE and 3SET. Each purified amplification product was cloned into the EcoRV site of pBluescript KS- and sequenced. An EcoRI/NotI fragment from the pBluescript Su(var)3-9 constructs was then cloned into pGEX 4T-1 resulting in an in-frame construct that produces GST-SU(VAR)3-9 polypeptides. The junctions and specific Su(var)3-9 mutation of each clone were verified by sequencing.

The pGEX-Su(var)3-9 constructs were transformed into BL21(DE3)pLysS. For each construct, 500 ml of LB containing 100 µg/ml of ampicillin were inoculated with a single colony and incubated overnight in a 37°C shaker. The culture was induced by adding 150 ml of LB supplemented with 600 µg of ampicillin and 650 µl of 1M IPTG,
incubated for 5 hours at 37º C and then processed as described by Frangioni and Neel (1993) with the following modifications. The bacterial pellet was repeatedly frozen and thawed (four times) in liquid nitrogen and a 25º C water bath. The cells were resuspended in 36 ml of STE+ (10mM Tris, pH 8.0, 300 mM NaCl, 1mM EDTA) and lysozyme added to a final concentration of 1 mg/ml. After 20 minutes at room temperature the cells were adjusted to 5 mM DTT and 1.4% N-lauryl-sarcosine. The lysate was placed on ice for 5 min and then sonicated (Sonic 300 dismembrator, 6x45 sec at 40% power). Triton X-100 was added to a final concentration of 3.4%. The sonicate was then incubated for 5 min. at room temperature and spun for 6 min at 12000 rpm (bench top centrifuge) at 4º C. The supernatant was collected and the GST-Su(VAR)3-9 fusions were bound to glutathione-coupled matrix (Pharmacia Biotech) as recommended by the manufacturer. After binding, the matrix was washed 4 times with PBS, 0.1% NP40, 6 times with PBS, 500mM NaCl, 0.1% NP40, and twice with PBS alone. The matrix-bound fusion protein was stored at –80°C in 50% glycerol in PBS.

2.4.10. HMTase assays

The recombinant variant GST- Su(VAR)3-9 polypeptides (1-10 µg) including the wild-type were incubated for 2 hours at room temperature in HMTase buffer (50mM Tris, pH 8.1, 20mM KCl, 10mM MgCl2, 10mM 2-mercaptoethanol, 250mM sucrose (Rea et al., 2000)) with 20 mg of bulk histones (Roche), or 2 µg of recombinant H3 (Upstate Biotech #14-411), or 2 µg of H3 tail peptide (Upstate Biotech #12-357, Upstate Biotech #12-430), and 0.125-0.25 µCi of S-adenosyl-methyl-methionine. The reactions were carried out in a final volume of 50 ml and stopped by adding 10 µl of 6X SDS loading
buffer. 30µl of each reaction was separated on a 13 or 15% polyacrylamide gel and stained with Coomassie. After drying, the gels were exposed and radioactive signal detected using a Phosphor Imager. The data were processed using the Image Quant software. The NIH Image software was used to quantify the amount of the various GST-Su(var)3-9 proteins in each Coomassie-stained band.

The specific activity of each mutant was calculated as the ratio of the radioactive signal corresponding to the (methylated) substrate and relative amount of recombinant GST-Su(var)3-9 used as determined by the intensity of the Coomassie stained band. The relative activity of each mutant, expressed as a percentage, is the ratio between its specific activity and the specific activity of the wild-type recombinant protein (run on the same gel). Each experiment was performed three times using recombinant proteins from independent preparations. The results are expressed as the average of three independent trials +/- the S.E.M. Student’s T-tests (p=0.05) were used to determine whether differences between mutants were statistically significant.
Suppl. table 2.1. Enzymatic activity of selected SU(VAR)3-9 mutants on 4 different substrates (see Materials & Methods for details).

<table>
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<tr>
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<th>Percent activity (average +/- SEM) on the indicated substrates</th>
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<tr>
<td></td>
<td>Bulk histones</td>
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<tr>
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<td>SU(VAR)3-9^WT</td>
<td>100</td>
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<tr>
<td>SU(VAR)3-9309/312</td>
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<tr>
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<td>SU(VAR)3-9318</td>
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<tr>
<td>SU(VAR)3-9330</td>
<td>24.3 +/- 1.9</td>
</tr>
<tr>
<td>SU(VAR)3-9376</td>
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^1 Values below 1% are listed as 0
**Suppl. table 2.2.** Sequences of the primers used in this study.

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<tr>
<td>5SET</td>
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</tr>
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<td>3-95’</td>
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<td>H3R</td>
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</table>
2.5. References


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


3. THE ROLE OF SU(VAR)3-9 IN THE REGULATION OF DROSOPHILA'S HISTONE GENE CLUSTER (HIS-C)^2.

3.1. Introduction

In Drosophila, methylation of lysine 9 of histone H3 (H3K9me) is a hallmark of heterochromatin (Rea et al., 2000; Nakayama et al., 2001; Peters et al., 2001; Schotta et al., 2002; 2004; Ebert et al., 2004; 2006), and SU(VAR)3-9 is one of the major methyltransferases responsible for this modification (Rea et al., 2000; Schotta et al., 2002; 2003). SU(VAR)3-9 itself is associated with heterochromatic regions of the genome, and particularly with centromeric and pericentric heterochromatin, which in polytene chromosomes form the chromocentre. The formation of heterochromatin is thought to involve several steps, and a relatively detailed model has emerged, which describes the sequence of events and the role of SU(VAR)3-9 in this process (Nakayama et al., 2001; Czermin et al., 2001; Schotta et al., 2003; Swaminathan et al., 2005; Rudolph et al., 2007). In this model, chromatin compaction is initiated by the demethylation of H3K4 by the demethylase SU(VAR)3-3/dLSD1, followed by the deacetylation of H3K9 by the histone deacetylase HDAC1/RPD3 (Czermin et al., 2001; Rudolph et al., 2007). HP1 and SU(VAR)3-7, two other NHCPs, are then responsible for targeting and restricting SU(VAR)3-9 to the chromocentre, where it methylates H3K9, thus creating a binding site for the chromodomain of HP1 (Jaquet et al., 2002; Schotta et al., 2002; Delattre et al., 2004; Ebert et al., 2006; Jacobs et al., 2001;

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^2 A version of this chapter will be submitted for publication. Kalas, P. and Grigliatti, T.A. The role of SU(VAR)3-9 in the regulation of Drosophila's Histone Gene cluster.
help of auxiliary factors, HP1 then binds to H3K9me, allowing the recruitment of more SU(VAR)3-9 and other factors, such as the H4K20 HMTase SUV4-20 (Schotta et al., 2004; Eskeland et al., 2007).

However, SU(VAR)3-9 is also detected at a number of euchromatic loci, where it contributes to the regulation of gene expression (Nielsen et al., 2001; Ner et al., 2002; Greil et al., 2003; Koryakov et al., 2006). One of these euchromatic sites is the histone genes cluster (HIS-C), where SU(VAR)3-9 appears to affect gene expression by altering the chromatin structure of the locus (Ner et al., 2002). Several pieces of evidence seem to connect the function of SU(VAR)3-9 in euchromatin with silencing or down regulation of the target genes, but its mechanism of action is largely unknown (Nielsen et al., 2001; Ner et al., 2002; Macaluso et al., 2003). Moreover, it is becoming increasingly apparent that SU(VAR)3-9’s role and function may vary in a context-dependent manner. For instance, genome-wide localization studies showed that SU(VAR)3-9 is present at a large number of euchromatic loci, and it colocalizes with HP1 only at a subset of these loci; unlike in heterochromatin, where SU(VAR)3-9 and HP1 appear to overlap very broadly (Greil et al., 2003; Schotta et al., 2002). This suggests that SU(VAR)3-9 may have slightly different functions at different loci, and/or that it may elicit its function(s) through different mechanisms (e.g. in collaboration with HP1 or with some other non-histone chromatin protein).

Particular attention has been devoted to the study of SU(VAR)3-9’s catalytic core, the preSET/SET/postSET domain (here referred to as "the SET domain" for simplicity). It has been demonstrated that the integrity of this domain is
necessary not only for the protein to carry out its enzymatic activity, which is believed to play a key role in heterochromatin formation, but also for its association with centric and pericentric heterochromatin (Schotta et al., 2002). FRAP-based studies in mammalian cell lines have also shown that the SET domain of SUV39H1 (the human ortholog of SU(VAR)3-9) contributes to its stable association to (hetero)chromatin, and that this function seems independent from its catalytic activity (Krouwels et al., 2005). Again, it is possible that the relative importance of each of SU(VAR)3-9's multiple functions are context-dependent.

Here, we use the HIS-C as a "model system" for investigating the function(s) of SU(VAR)3-9, and specifically those associated with its SET/preSET/postSET domain, in the regulation of a euchromatic locus. There are several reasons why the HIS-C was chosen. First, SU(VAR)3-9 has been shown to physically associate with the HIS-C, indicating that it probably plays a direct role in the chromatin architecture of this locus. Second, we know that the expression of at least two of the histone genes (H1 and H4) is altered in at least three Su(var)3-9 mutants, suggesting that SU(VAR)3-9 must play a role in their regulation (Ner et al., 2002). In addition, its mechanism of action seems to be chromatin-mediated, since the nucleosome spacing at the HIS-C appears altered in several Su(var)3-9 mutants (Ner et al., 2002).

The simplest initial working model is that SU(VAR)3-9 acts at the HIS-C through the same mechanism as it does in heterochromatin. In particular, we hypothesize that methylation of H3K9 across the HIS-C is mainly dependent on SU(VAR)3-9,
and that the presence of SU(VAR)3-9 and that of H3K9me2 are necessary for proper localization of HP1 at this locus. In turn, the presence of HP1 at the HIS-C would be necessary for proper regulation of the histone genes expression. In order to test this hypothesis, we first need to obtain a reasonably detailed picture of the HIS-C "landscape" in terms of the distribution of SU(VAR)3-9, H3K9me2 and HP1 across the locus in a wild-type strain. Then, we take advantage of a set of well-characterized Su(var)3-9 mutants to investigate the functional relationships among SU(VAR)3-9, H3K9me2 and HP1 in the context of histone gene regulation. Specifically, we ask whether the distribution of SU(VAR)3-9, H3K9me2 and HP1 across the HIS-C, and the level of histone transcripts are altered in Su(var)3-9 missense mutants. We show that, in the three missense mutants analyzed, the Su(var)3-9 gene product is still present across the HIS-C. The levels of H3K9me2 and HP1 associated with the HIS-C are significantly reduced in all Su(var)3-9 mutants tested. These mutants also display an increase in the amount of histone H3 and histone H2A transcript levels, supporting the hypothesis that the enzymatic function of SU(VAR)3-9 is critical for regulation of the histone genes. Interestingly, in one Su(var)3-9 missense allele (Su(var)3-9\textsuperscript{330}) the relative increase in the level of H3 transcript is much more pronounced than those of H2A and H2B, suggesting that the stoechiometry of these core nucleosome proteins may be disrupted in this particular strain. Finally, we show that the elevated levels of histone transcripts detected in Su(var)3-9 mutants are not due to an accumulation of abnormally high levels of histone mRNA synthesized outside S-phase. Hence, we conclude that the increased amount of
*h2a* and *h3* transcripts is most likely a consequence of an increase in the number of templates transcribed/unit time, or an increase in the rate of transcription from each of an invariant number of templates, or a combination of these two factors.
Figure 3.1.
**Figure 3.1. A schematic of the histone unit.** The histone genes cluster (HIS-C) is comprised of ~110 tandemly repeated histone units; the diagram shows the *BglII* genomic fragment representing the histone unit. The coding regions of histones H1 (yellow), H3 and H4 (red) and H2A and H2B (blue) are shown as plain boxes with an arrowhead pointing in the direction of transcription. The region delimited by the brackets represents the approximate extent and location of the deletion present in about 25% of the histone units. The blue lines labelled "H1/H3 intergenic", "H3/H4 intergenic", H2A/H2B intergenic", "H3 coding" and "H2A coding" indicate the sizes and positions of the fragments analyzed in the ChIP experiments. The dark red arrows show the positions and directions of the primers used to reverse-transcribe the histone RNAs, and the dark red lines labelled "H3", "H2A" and "H2B" indicate the extents of the amplified cDNAs produced. Below, the relative positions of four unique restriction sites are shown in relation to the two *BglII* sites defining the unit.
3.2. Results

3.2.1. SU(VAR)3-9 is associated with the HIS-C in wild-type and Su(var)3-9 missense mutants

The Histone Gene Cluster (HIS-C) region of the second chromosome is comprised of about 100 tandemly reiterated copies of the histone unit (his unit). The his unit contains one copy of each of the five histone genes, with the core histone genes arranged into two gene pairs, the h3/h4 and the h2a/h2b couplets (Figure 3.1). The two members of each couplet share a regulatory region. There are two versions of the his unit: one, representing about 75% of the his units present in the HIS-C, is 5kb, and the other, less represented, is about 4.75kb in length (Lifton et al., 1977). The 250 bp difference is due to an indel located in the H1/H3 intergenic region (see parentheses in Figure 3.1.).

SU(VAR)3-9 is associated with the HIS-C, where it is detected across the whole locus (Ner et al., 2002; Koryakov et al., 2006; Ner et al., in preparation). In addition, several tested Su(var)3-9 mutants, including the missense mutant Su(var)3-9330 display an alteration in the chromatin structure of the HIS-C and elevated levels of histone gene transcripts (Harrington, 2001; Ner et al., 2002). However, whether the physical presence of SU(VAR)3-9 is necessary and/or sufficient for proper regulation of the histone genes, and whether the single amino acid substitutions in its catalytic region affect its localization at the HIS-C, is unclear.

In order to address these issues, we first used chromatin immunoprecipitation (ChIP) to determine the distribution of SU(VAR)3-9 at the HIS-C in wild-type and
Su(var)3-9 missense mutant embryos. Homozygous Su(var)3-906 individuals, which represent complete nulls producing no Su(var)3-9 mRNA or protein (Tschiersch et al., 1994; Schotta et al., 2002; this work, chapter 2) were also included in the analysis to serve as an internal standard/negative control. ChIP analysis of cross-linked extracts prepared from 12-16 hour old staged embryos was performed using an antibody raised against the N-terminal half of SU(VAR)3-9 (α-SU(VAR)3-9chr (Ner et al., 2002)), and the immunoprecipitated DNA was tested for the presence of 3 sequences belonging to the HIS-C: the H3 coding region ("H3 coding"), a fragment including the intergenic/regulatory region between the H2A and H2B genes ("H2A/H2B intergenic") and a section of the intergenic region between the H1 and H3 genes ("H1/H3 intergenic") (see also Figure 3.1). This last fragment spans a region of the HIS-C that, in ~25% of the 110 or so copies of the his unit, contains a ~250bp indel (Lifton et al., 1977).

Thus, amplification with H1/H3 intergenic-specific primers gives rise to two different fragments: one representing the "longer" version of the his unit (5 kb) and one corresponding to the "shorter" one (4.75 kb). The sizes of these two fragments are roughly 600 bp and 350bp, respectively (Lifton et al., 1977; Samal et al., 1981; Worcel et al., 1983).

As expected, the cross-linked material pulled down by the α-SU(VAR)3-9chr antibody from Su(var)3-9+/ Su(var)3-9+ extracts contains each of the three HIS-C sequences ("H3 coding", "H2 intergenic" and "H3/H1 intergenic"). In contrast, these fragments were not enriched in the material pulled down from Su(var)3-906 extracts (Figure 3.2.b), allowing us to conclude that the enrichment observed in
the wild-type strain is indeed due to the association of SU(VAR)3-9 with the HIS-C. In all the Su(var)3-9 missense mutants, fragments corresponding to the "H3 coding", "H2A/H2B intergenic" and "H1/H3 intergenic" regions were detected among the immunoprecipitated material (Figure 3.2). Accurate quantifications of one of the his unit fragments, "H3 coding", revealed that the relative enrichment obtained with α-SU(VAR)3-9chr is relatively small, although significant (Figure 3.2.c and appendix 2). Still, the data obtained allow us to conclude that that wild-type SU(VAR)3-9 protein (SU(VAR)3-9WT) is associated with the his unit. These data, together with those of Ner and colleagues suggest that SU(VAR)3-9WT is distributed throughout the his unit (Ner et al., 2002; Ner et al., in preparation).

A similar set of ChIP analyses, performed on Su(var)3-9309, Su(var)3-9330 and Su(var)3-9318 12-16 hours old embryos, demonstrated that the SU(VAR)3-9309, SU(VAR)3-9330 and SU(VAR)3-9318 mutant proteins also associate with the his unit. The enrichment for the "H3 coding" fragment detected in the three missense mutants Su(var)3-9309, Su(var)3-9330 and Su(var)3-9318 was not significantly different from that of the wild-type strain (Figure 3.2.c and appendix 2). Hence, our results also suggest that the single amino acid substitutions present in the SU(VAR)3-9309, SU(VAR)3-9330 and SU(VAR)3-9318 variants do not prevent their association with the HIS-C.
Figure 3.2
Figure 3.2. Relative levels of SU(VAR)3-9 associated with three regions of the histone unit in wild type and Su(var)3-9 mutant embryos. a) Schematic of the BglII fragment defining the histone unit. The five histone genes (H1, H3, H4, H2A and H2B) and the three regions analysed (H3 coding, H2A/H2B intergenic and H1/H3 intergenic) are shown. b) Chromatin immunoprecipitation of wild-type and Su(var)3-9 mutant embryo extracts. The recovered DNA was PCR amplified using primers specific for the “H3 coding” region (lanes 1-3), the “H2A/H2B intergenic region” (lanes 4-6) or the “H1/H3 intergenic” region (lanes 7-9). The template used in each PCR reaction is indicated above the corresponding lane; IN (1:100): input material diluted 100X, NO Ab: mock (no antibody) reaction, IP (α-3-9chr): immunoprecipitated material. The antibody used was raised against the N-terminal region (including the chromodomain) of SU(VAR)3-9 and has been previously described (Ner et al., 2002). c) Relative amounts of “H3 coding” fragment in the precipitated material as determined by real-time PCR (see materials and methods for details). The bar graphs represent the average of 3 independent experiments and the error bars span two standard deviations. d) Average amounts of immunoprecipitated “H3 coding” fragment (minus the average for the respective “mock” reaction) expressed as fractions of the wild type.
3.2.2. The level of H3K9me2 associated with the HIS-C is significantly reduced in 
\textit{Su(var)3-9} missense mutants

H3K9me2 is enriched at the HIS-C (Figure 3.3 and Ner \textit{et al.}, 2002) and, since \textit{Su(var)3-9}\textsuperscript{309}, \textit{Su(var)3-9}\textsuperscript{330} and \textit{Su(var)3-9}\textsuperscript{318} have all been shown to produce SU(VAR)3-9 proteins with an impaired HMTase activity (chapter 2), we asked whether the levels and/or distribution of H3K9me2 across the HIS-C was altered in these \textit{Su(var)3-9} mutants.

ChIP analyses of cross-linked embryo extracts from homozygous \textit{Su(var)3-9}\textsuperscript{+}, \textit{Su(var)3-9}\textsuperscript{06}, and the three \textit{Su(var)3-9} missense mutant strains were performed using an antibody that specifically recognizes H3K9me2 (see material and methods for details). As shown previously (Ner \textit{et al.}, 2002), all three HIS-C fragments, "H3 coding", "H2A/H2B intergenic" and "H1/H3 intergenic" were detected in the material immunoprecipitated from the wild-type strain (Figure 3.3.a). Their enrichment was about 100-fold higher than that observed using non-specific IgG (Supplementary Figure 1 and appendices 1 and 3), allowing us to conclude that H3K9me2 is indeed present at the HIS-C in the wild-type strain. As expected, the three HIS-C fragments analyzed were significantly less abundant in the material immunoprecipitated from \textit{Su(var)3-9}\textsuperscript{06} mutant extracts (Figure 3.3). The \textit{Su(var)3-9}\textsuperscript{06} mutant completely lacks the SU(VAR)3-9 protein. However, it is not surprising that a small amount of H3K9me2 associated with its HIS-C in this strain since other HMTases, capable of methylating H3K9, are present in the nucleus (see discussion). The target \textit{his} unit fragments were also detected in the material immunoprecipitated from each of the three \textit{Su(var)3-9}
missense mutants, but, as expected, their relative abundance was significantly lower than in the wild-type strain (Figure 3.3).

The relative enrichment for one particular fragment, "H3 coding", was accurately quantified by real-time PCR. With regard to this fragment, Su(var)3-9\textsuperscript{06} and the missense mutant Su(var)3-9\textsuperscript{330} displayed the lowest level of enrichment, less than 5% of the wild-type (Figures 3.3.c and d). Su(var)3-9\textsuperscript{309} and Su(var)3-9\textsuperscript{318} showed a slightly higher enrichment for this fragment, corresponding to about 10% and 13% of the wild-type, respectively (Figure 3.3 and appendix 3). We conclude that the levels of H3K9me2 associated with the three regions of the HIS-C, "H3 coding", "H2A/H2B intergenic" and "H3/H1 intergenic" are significantly lower in all Su(var)3-9 mutants tested than they are in the wild-type strain.
Figure 3.3
Figure 3.3. Relative levels of H3K9me2 associated with three regions of the histone unit in wild type and Su(var)3-9 mutant embryos. a) Schematic of the *BglII* fragment defining the histone unit. The five histone genes (H1, H3, H4, H2A and H2B) and the three regions analysed (H3 coding, H2A/H2B intergenic and H1/H3 intergenic) are shown. b) Chromatin immunoprecipitation of wild-type and Su(var)3-9 mutant embryo extracts. The recovered DNA was PCR amplified using primers specific for the “H3 coding” region (lanes 1-3), the “H2A/H2B intergenic region” (lanes 4-6) or the “H1/H3 intergenic” region (lanes 7-9). The template used in each PCR reaction is indicated above the corresponding lane; IN (1:10): input material diluted 10X, NO Ab: mock (no antibody) reaction, IP (K9me2): immunoprecipitated material. The antibody used was an anti-H3K9me2 from UPSTATE (#07-441). c) Relative amounts of “H3 coding” fragment in the precipitated material as determined by real-time PCR (see materials and methods for details). The bar graphs represent the average of 3 independent experiments and the error bars span two standard deviations. d) Average amounts of immunoprecipitated “H3 coding” fragment (minus the average for the respective “mock” reaction) expressed as fractions of the wild type.
3.2.3. Association of HP1 with the HIS-C

In heterochromatin, H3K9me is thought to represent a binding platform for HP1 (Lachner et al., 2001; Jacobs et al., 2002; Bannister et al., 2001; Nielsen et al., 2002). In addition, SU(VAR)3-9 is known to interact with HP1 physically and genetically (Schotta et al., 2002; 2003; our lab, unpublished data). Thus, not surprisingly, most models postulate an interaction among SU(VAR)3-9, HP1 and H3K9me as a central step in the formation of heterochromatin (Nakayama et al., 2001; Schotta et al., 2002; 2003; Ebert et al., 2006; Rudolph et al., 2007), and SU(VAR)3-9 and HP1 have been suggested to work together in the regulation of a subset of genes (Nielsen et al., 2001; Greil et al., 2003). Since HP1 has been detected at the HIS-C (Greil et al., 2003; Koryakov et al., 2006; chapter 2 of this work), we hypothesized that it may play a role in regulating the expression of the histone genes, as part of a SU(VAR)3-9-dependent mechanism. To test this hypothesis, we performed another set of ChIP analyses, in this case, with an anti-HP1 antibody (α−HP1 (Ner et al., in preparation)).

The material immunoprecipitated with α−HP1 from wild-type embryo extracts contained significant, although not copious amounts, of his unit fragments (Figure 3.4 and appendix 4). This enrichment for his unit fragments was significantly higher than that obtained with pre-immune serum (Supplementary Figure 3.1 and Appendix 1), allowing us to conclude that HP1 is associated with the HIS-C in wild-type strains. In contrast, the material immunoprecipitated with α−HP1 from Su(var)3-9 mutant extracts displayed very low levels of enrichment for all HIS-C fragments analyzed (Figure 3.4.b). For the "H3 coding" and "H2A coding"
fragments, relative quantifications were performed by real-time PCR, and the 
Su(var)3-9 mutants showed a ~3 to 19-fold, and ~4 to 13-fold reduction in the 
enrichment for HP1, respectively, relative to the wild-type.
We conclude that overall, in Su(var)3-9 mutants, the levels of HP1 associated 
with the HIS-C are significantly lower than in wild-type individuals.
**Figure 3.4**

A. Schematic representation of the genomic regions analyzed.

- **H3 coding** regions
- **H2A/H2B intergenic** regions
- **H1/H3 intergenic** regions

B. Gel images showing the relative amounts of “H3 coding” and “H2A/H2B intergenic” fragments in the material immunoprecipitated from 12-16 hrs old embryos.

C. Graph showing the relative amounts of “H2A coding” fragment in the material immunoprecipitated from 12-16 hrs old embryos.

D. Graph showing the relative amounts of “H3 coding” fragment in the material immunoprecipitated from 12-16 hrs old embryos.
Figure 3.4. Relative levels of HP1 associated with four regions of the histone unit in wild-type and Su(var)3-9 mutant embryos. a) Schematic of the BglII fragment defining the histone unit. The five histone genes (H1, H3, H4, H2A and H2B) and the four regions analysed (H3 coding, H2A/H2B intergenic, H2A coding and H1/H3 intergenic) are shown. b) Chromatin immunoprecipitation of wild-type and Su(var)3-9 mutant embryo extracts. The recovered DNA was PCR amplified using primers specific for the “H3 coding” region (lanes 1-3), the “H2A/H2B intergenic region” (lanes 4-6) or the “H1/H3 intergenic” region (lanes 7-9). The material used as template is indicated above each lane; IN (1:100): input material diluted 100X, NO Ab: mock (no antibody) reaction, IP (HP1): immunoprecipitated material. See material and methods for details about the antibody. c) and d) Relative amounts of “H2A coding” and “H3 coding” fragments, respectively, in the precipitated material as determined by real-time PCR (see materials and methods for details). The bar graphs represent the average of 3 independent experiments and the error bars span two standard deviations.
3.2.4. The null, as well as the missense Su(var)3-9 mutants have elevated levels of H2A and H3 transcripts

It was previously reported that the levels of H1 and H4 transcripts are elevated in three Su(var)3-9 mutants, including the missense mutant Su(var)3-9\textsuperscript{330} (Ner et al., 2002). To determine whether this is a common feature of Su(var)3-9 mutants, and its relationship to the abundance of H3K9me2, SU(VAR)3-9 and HP1 associated with the HIS-C, respectively, we quantified the relative amounts of two histone transcripts present in wild-type and several Su(var)3-9 mutants. We used 12-16 hour old embryos as our source of RNA, for two reasons. Firstly, at this stage of embryogenesis a significant proportion of cells are still going through the cell cycle (reviewed by Lee and Orr-Weaver, 2003) and therefore synthesizing copious amounts of histone mRNAs. Thus, if SU(VAR)3-9 participates in the regulation of the histone genes, the effect of Su(var)3-9 mutations on the levels of histone should be more pronounced at this stage of development than in adults. Secondly, we wanted to be able to relate the alterations (or lack thereof) in the relative levels of histone mRNA to the results of the ChIP analyses, which were performed on 12-16 hour old embryo extracts. For each strain (Su(var)3-9\textsuperscript{+}, the various Su(var)3-9 missense mutants and Su(var)3-9\textsuperscript{06}), the relative levels of two histone genes transcripts, H2A and H3, were quantified by real time RT-PCR using rp49 as an internal standard (see materials and methods). Within the histone unit, H2A and H2B are transcribed in opposite directions and they share a promoter region, and the same is true for H3 and H4 (Figure 3.1). H2A and H3 were chosen for our analysis as
representative of each of the two "gene pairs", as we assumed that the two members of each pair would be co-regulated.

The relative amount of H3 transcript detected in all Su(var)3-9 mutants was significantly higher than in the Su(var)3-9+ strain (Figure 3.5.b and 3.5.d, and Appendix 5). Su(var)3-9<sup>06</sup>, Su(var)3-9<sup>330</sup>, and Su(var)3-9<sup>318</sup> showed, on average a 6.46, 6.07 and 4.72 fold increase over the wild-type (n=3). For Su(var)3-9<sup>309</sup> the increase was less substantial (1.56 fold relative to the wild-type), but still statistically significant (p=0.05).

For Su(var)3-9<sup>309</sup> and Su(var)3-9<sup>318</sup>, the relative increase in H2A was 1.86 and 5.60, respectively, which is comparable (not statistically different at p=0.05) to that observed for H3. In Su(var)3-9<sup>06</sup>, the increase in H2A was only about 70% of that observed for H3 (see Appendix 6), but still significantly higher than what was observed both in the wild-type and in Su(var)3-9<sup>309</sup> (Figure 3.5. and Appendix 6). Su(var)3-9<sup>330</sup> was an exception in that the relative increase (over the wild-type) in its level of H2A transcript was much lower (<30%) than that observed for its H3 transcript. In fact, the relative abundance of H2A detected in Su(var)3-9<sup>330</sup> was so low as not to be statistically different from that observed in the wild-type (Appendix 5).

Despite the exceptional case of the H2A transcript in Su(var)3-9<sup>330</sup>, we conclude that elevated levels of histone transcripts are probably a common feature of those Su(var)3-9 mutations that suppress PEV, although the magnitude of the increase seems to be, at least in part, allele-dependent (see section 3.2.5 and discussion). In addition, the data obtained with Su(var)3-9<sup>06</sup>, and especially with
Su(var)3-9 \(^{230}\), suggest that the regulation of the \(H2A/H2B\) and the \(H3/H4\) gene pairs may be uncoupled.
**Figure 3.5**

Relative amounts of total H2A transcript in 12-16 hr old embryos (normalized to rp49)

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Relative amounts of total H3 transcript in 12-16 hr old embryos (normalized to rp49)

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<th>330</th>
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* Statistically different from wild type (at p=0.05)
Figure 3.5. Relative quantifications of total histone H3 and histone H2A transcript in wild-type and Su(var)3-9 mutant embryos. a) and b) End point RT-PCR reactions on total RNA extracted from 12-16 hours old wild-type (lanes 1,2, 11 and 12) or Su(var)3-9 mutant embryos (lanes 3-10 and 13-20). The transcripts amplified were H2A and H3, respectively, as well as RP49 as an internal standard. In each case, the allele number is indicated above the corresponding lanes. "-" signs denote mock reactions (no reverse transcriptase). c) and d) Relative quantifications of H2A and H3 transcripts by real-time PCR. For each reaction, the ratio of H2A or H3 between mutants and wild-type (standardized for RP49) was reported. The histograms represent the average of three independent reactions and the error bars span two standard deviations. See material and methods for additional details.
3.2.5. *Histone* genes expression in mutant *Su(var)3-9*<sup>330</sup>

Since the four core histones are required in equal amounts, and *SU(VAR)3-9* is associated with both the *H2A/H2B* and the *H3/H4* gene pairs, one would expect each *Su(var)3-9* mutation to affect the expression of the *core histone* genes to the same degree. This was indeed the case for *Su(var)3-9*<sup>309</sup> and *Su(var)3-9*<sup>318</sup>, but it was not the case in the *Su(var)3-9*<sup>06</sup> and *Su(var)3-9*<sup>330</sup> strains. In the *Su(var)3-9*<sup>06</sup>, the "protein null" strain, there was, on average, a 4.65 and 6.46 fold increase in *H2A* and *H3*, respectively. Statistically (p=0.05), the difference between these two values is only marginally significant. The difference between the increase in *H2A* and *H3* was much more pronounced in the *Su(var)3-9*<sup>330</sup> strain (1.76 fold and 6.07 fold, respectively). Since in *Su(var)3-9*<sup>330</sup> there is such a dramatic difference in the increase of *H2A* versus *H3* mRNAs, we decided to focus on this strain, and to ask whether the regulation of the *histone* genes can be misregulated in such a way that the expression of each of the four core histones, or each of the two usually co-regulated pairs, *H2A/H2B* and *H3/H4* is decoupled. To examine this possibility, we measured the total *H2B* transcript present in embryo extracts from wild-type and *Su(var)3-9*<sup>330</sup>.

We found that, as it is the case for *H2A*, the relative levels of *H2B* detected in *Su(var)3-9*<sup>330</sup> embryo extracts are, on average, slightly less than 2 fold higher than in the wild-type (Figure 3.6). Similarly to what was observed for *H2A*, such difference is not statistically significant (at p=0.05), although it is probably significant biologically. We conclude that somehow, the amino acid substitution present in *SU(VAR)3-9*<sup>330</sup> affects the relative abundance of *H3* transcript, but not
that of H2A or H2B. This suggests that the H2A/H2B and the H3/H4 gene pairs may be independently regulated.

To examine this further, we next asked whether, in Su(var)3-9330, the intergenic regions between the H3/H4 and H2A/H2B pairs show any differences in the level of H3K9me2 versus the wild-type. If H3K9me2 is responsible for the regulation of the histone genes, we would expect to see a substantial difference between the wild-type and Su(var)3-9330, with respect to such modification, within the H3/H4 region, and a less dramatic difference within the H2A/H2B region. A ChIP analysis was performed, and relative quantifications of the fragments of interest were carried out by real-time PCR. The results showed that the material immunoprecipitated from both the wild-type and the mutant extracts is enriched for "H3/H4 intergenic" and "H2A/H2B intergenic" fragments (Figure 3.7).

However, as for all other regions tested, this enrichment is significantly higher in the wild-type than in Su(var)3-9330 (Figure 3.7. and Appendix 3). Intriguingly, it was the "H2A/H2B intergenic" region that showed the most substantial difference between Su(var)3-9330 and the wild-type (Figure 3.7. and Appendix 3), suggesting that there is no direct correlation between the levels of H2A, H2B and H3 transcripts and the amount of H3K9me2 associated with the genes' regulatory regions.
Figure 3.6

a. 

<table>
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<td>rp49</td>
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b. Relative amounts of total H2B transcript (standard=rp49)
Figure 3.6. Comparison of H2B transcript present in wild-type vs. Su(var)3-9330 mutant embryos. a) and b) Relative abundance of total H2B transcript in 12-16 hours embryos, quantified by real-time RT-PCR following the same procedure as in Figure 3.5. "-" signs denote mock reactions (no reverse transcriptase).
Figure 3.7
Figure 3.7. Relative levels of H3K9me2 associated with two intergenic regions of the histone unit in wild type and Su(var)3-9^{330} mutant embryos. a) Detection (end point PCR) and b) quantification (real-time PCR) of the "H2A/H2B intergenic" and "H3/H4 intergenic" fragments in the material immunoprecipitated with anti-H3K9me2 from wild-type and Su(var)3-9^{330} cross-linked embryonic extracts.

In all cases the histograms represent the average of three independent experiments and the error bars span two standard deviations. See material and methods for additional details.
3.2.6. The elevated levels of histone transcripts in Su(var)3-9 mutants are not due to an increase in polyadenylated transcripts.

The observed overproduction of histone H2A and histone H3 transcripts is an interesting phenotype, and its mechanism is unknown. There are three simple possibilities. First, in the Su(var)3-9 mutant strains there may be an increase in the number of histone templates transcribed at any point in time with respect to the wild-type, while the transcription rate remains constant. Second, Su(var)3-9 may result in an increase in the transcription rate of the histone genes, while the number of his templates that are transcribed within the HIS-C region remains constant. Finally, it is possible that neither the number of transcribed templates, nor the transcription rate are affected, but, in Su(var)3-9 mutants, expression of the histone genes may be uncoupled from the cell cycle (i.e. the histone genes may be transcribed outside, as well as during, S-phase).

The latter hypothesis is easily tested. Histone transcripts synthesized during S-phase are not polyadenylated, while those synthesized outside S-phase are (Akhmanova et al., 1997). Therefore, histone transcripts produced outside S-phase (decoupled from DNA synthesis) can easily be detected and quantified using an appropriate primer (oligodT) for the reverse-transcription (RT) step.

Taking this approach, we first measured the amount of polyadenylated H2A and H3 mRNAs in Su(var)3-9+/ Su(var)3-9+ embryos, in order to determine what proportion of the total histone transcripts are polyadenylated RNA. We found that the relative ratio of polyadenylated/total H2A and H3 was roughly 1/1000 (Figures 3.8.a and 3.8.b).
Hence, if the increase in the level of total H2A and H3 transcripts observed in the mutants is due, even only in part, to transcription outside S-phase, the Su(var)3-9 mutants should show a very prominent increase in the amount of polyadenylated H2A and H3 transcripts compared to the wild-type. This is not what we observed. Instead, we detected a small reduction in the levels of polyadenylated H2A and H3 in Su(var)3-9\textsuperscript{06}, Su(var)3-9\textsuperscript{309} and Su(var)3-9\textsuperscript{318}, while Su(var)3-9\textsuperscript{330} showed a small reduction in H3 and an increase in H2A (Figures 3.8.c and 3.8.d). The differences in the levels of polyadenylated H2A and H3 between the wild-type and each of the Su(var)3-9 mutants are statistically significant. However, since the polyadenylated histone RNAs represent less than 1% of the total, these differences certainly do not account for the increase in the levels of total histone transcripts. Thus, we conclude that increased expression outside S-phase is not the mechanism responsible for the observed elevation in total histone transcripts.
Figure 3.8.
Figure 3.8. Relative quantifications of polyadenylated (polyA) histone H3 and histone H2A transcript in wild-type and Su(var)3-9 mutant embryos.

a) Comparison between the relative amount of total and polyA H2A transcript in wild-type 12-16 hours old embryos. In lanes 1 and 2 the RNA was reverse-transcribed with primers H2AR and RP49rt, and in lanes 3 and 4 with an oligo(dT) primer. In both cases the reverse-transcribed material was then PCR amplified (separately) with primer pairs H2AFc/H2AR and RP495/RP493. Lanes 1 and 3 represent "mock" (no RTase) reactions. For the total H2A transcript sample, only 1/10 of the amplified product was loaded on the gel. b) Ratios of total H2A/total RP49, and polyadenylated H2A/polyadenylated RP49 transcripts, respectively, as determined by real-time PCR. The histograms represent the average of three independent experiments and the error bars span two standard deviations. c) RT-PCRs on total RNA extracted from 12-16 hours old wild-type and Su(var)3-9 mutant embryos. The extracts were reverse-transcribed with an oligo(dT) primer and the material obtained amplified with primer pairs H2AFc/H2AR, H3F/H3R and RP495/RP493, respectively. "Mock" (no RTase) reactions are indicated with a "-" sign above the corresponding lanes. d) Relative quantifications of polyadenylated H2A and H3 transcripts by real-time PCR. For each reaction, the ratio of H2A or H3 between mutants and wild-type (standardized for RP49) was reported. The histograms represent the average of three independent experiments and the error bars span two standard deviations.
3.3 Discussion

3.3.1. Association of SU(VAR)3-9 with the HIS-C in 3-9 mutants

The ChIP data demonstrate that SU(VAR)3-9 is present at the HIS-C in wild-type and in Su(var)3-9^{309}, Su(var)3-9^{330} and Su(var)3-9^{318} embryos. This confirms previous observations (Ner et al., 2002; Koryakov et al., 2006) and suggests that in all three cases, single amino acid substitutions in the catalytic region of SU(VAR)3-9 do not substantially affect the protein’s ability to be recruited to, or associate with, the HIS-C. Since the three missense mutants tested carry distinct amino acid substitutions, and all of them are still targeted to, and associated with the HIS-C, we propose that their 3-dimensional structure, as well as their ability to interact with the other chromatin components, are mainly unaffected. If this were not the case, and one or more of the mutant gene products were misfolded and/or unable to properly interact with the customary partners of SU(VAR)3-9, we would expect them to fail to associate with their targets. The fact that no SU(VAR)3-9 was detected in Su(var)3-9^{06} further validates this hypothesis.

All Su(var)3-9 mutants tested, regardless of whether they were "protein nulls" (Su(var)3-9^{06}) or missense alleles (Su(var)3-9^{309}, Su(var)3-9^{330} and Su(var)3-9^{318}) display elevated levels of histone transcripts, suggesting that the physical presence of SU(VAR)3-9 at the HIS-C is not sufficient for normal regulation of the histone genes. It is certainly possible, at least in theory, that SU(VAR)3-9 has an essential structural role at the HIS-C, and that each one of the single amino acid substitutions present in the mutants analyzed impairs this function. We do not favour this possibility because, as discussed above, the mutant proteins still
associate with their target (although in some cases less efficiently), which suggests that their ability to interact with other chromatin proteins does not differ significantly from that of the wild-type SU(VAR)3-9.

Although significant, the enrichment for HIS-C fragment detected in the material immunoprecipitated with $\alpha$-SU(VAR)3-9$^{chr}$ was very weak, which may reflect the fact that the association of SU(VAR)3-9 with this locus is very dynamic, and the amount of protein physically associated with the HIS-C chromatin at any given time is very small. This would fit with the observation that SUV39H1, the human homolog of SU(VAR)3-9, can be found stably associated with heterochromatin, but not with euchromatin (Krouwels et al., 2005). It would also argue in favour of the hypothesis that SU(VAR)3-9 does not play a major structural role at the HIS-C. Alternatively, it is possible that the antibody was not very efficient in its recognition of SU(VAR)3-9 (possibly because the epitope against which it was raised is partially hidden by other chromatin proteins interacting with SU(VAR)3-9).

3.3.2. Reduced levels of H3K9me2 in Su(var)3-9 mutants

Using ChIP analyses we were able to demonstrate conclusively that 1) at least three segments of the histone unit are enriched for H3K9me2, and 2) this enrichment is noticeably reduced in all four homozygous Su(var)3-9 mutants tested (and, for at least one fragment, "H3 coding" the reduction is as high as ~7 to 25-fold). The product of Su(var)3-9$^{309}$ (SU(VAR)3-9$^{309}$) is catalytically inactive in vitro, and those of Su(var)3-9$^{330}$ (SU(VAR)3-9$^{330}$) and Su(var)3-9$^{318}$
(SU(VAR)3-9318) have dramatically reduced enzymatic activity (chapter 2).

Nevertheless, all strains, including Su(var)3-906, display a statistically significant enrichment for H3K9me2 within at least one region of the his unit ("H3 coding", see also Appendix 3). Although weak cross-reactivity of the antibody with H3K9me1 or H3K27me2 cannot be completely excluded, we favour the hypothesis that the residual signal observed in Su(var)3-906 is due to the presence of other methyltransferases (MTases) that are able to methylate H3K9 at euchromatic loci, such as dG9a and DmSetDB1 (Mis et al., 2006; Stabell et al., 2006; Seum et al., 2007; Tzeng et al., 2007). This hypothesis could be tested by analysing cross-linked extracts from homozygous dG9a; Su(var)3-906 and DmSetdb1; Su(var)3-906 mutant embryos, respectively, and asking whether their level of H3K9me2 associated with the HIS-C is lower than that observed in their Su(var)3-906 counterparts. Unfortunately, this approach may not be simple. In the case of dSetdb1, homozygous dSetdb1− mutants survive until third instar larval stage (Seum et al., 2007), so dSetdb1−; Su(var)3-906 homozygous embryos could in principle be produced, although the viability of the double mutant strain is not known. In addition, it is likely that traces of H3K9me2, generated by the dG9a protein, would still be present in homozygous dSetdb−; Su(var)3-906 embryos. Since the levels of H3K9me2 associated with the HIS-C in Su(var)3-906 embryos are also very low, a comparison between dG9a−; Su(var)3-906 and Su(var)3-906 would likely involve working with trace amounts of material and trying to detect a very small difference, which would require extremely accurate and reliable quantification methods, as well as exceedingly specific antibodies. In a way, the
task could be simpler for \textit{dG9a}, since the \textit{dG9a}^{RG5} mutant line survives, as a homozygote, in combination with \textit{Su(var)3-9}^{06} (Seum \textit{et al.}, 2007). However, this particular \textit{dG9a} mutant line does not have any particular phenotype and fails to show reduced levels of H3K9me or H3K9me27 at larval stages, so there is no guarantee that a difference in the levels of such modifications could be detected in homozygous \textit{dG9a}^{RG5}; \textit{Su(var)3-9}^{06} vs. \textit{dG9a}^{+};\textit{Su(var)3-9}^{06} embryos.

All \textit{Su(var)3-9} missense mutants tested showed a significant reduction in the levels of H3K9me2 associated with the HIS-C. In principle, this could be due to either a reduction in the catalytic function of the mutant SU(VAR)3-9 products, or a reduction in the amount of SU(VAR)3-9 protein present at the HIS-C. Since we have shown that the level of (mutant) SU(VAR)3-9 associated with the HIS-C in the \textit{Su(var)3-9} missense mutants are comparable to those observed in wild-type embryos (Figure 3.2), we conclude that the reduced levels of H3K9me2 are due to the altered enzymatic activity of the mutant products (SU(VAR)3-9^{309}, SU(VAR)3-9^{330} and SU(VAR)3-9^{318}, respectively). This was expected, as the catalytic activity of these mutant proteins is significantly impaired, at least \textit{in vitro} (chapter 2).

Curiously, although SU(VAR)3-9^{309}, SU(VAR)3-9^{330} and SU(VAR)3-9^{318} differ in the strength of their catalytic phenotypes based on \textit{in vitro} tests (chapter 2), we detected no significant differences in the levels of H3K9me2 that is associated with the HIS-C among the corresponding mutants. There are a number of possible reasons for this apparent discrepancy. The simplest explanation is that the conditions employed for the HMTase assay reported in chapter 2 do not
wholly simulate the nuclear environment in which SU(VAR)3-9 normally functions. For instance, in the *in vitro* assay the reactions were allowed to proceed uninterrupted for several hours, which is probably not the case in an *in vivo* context. More importantly, the *in vitro* reactions were carried out on free histones, in the absence of all the NHCPs and additional factors normally present in a cell nucleus, and the results obtained may not be representative of the enzyme’s activity on a chromatin template in an *in vivo* context.

The dramatically reduced levels of HIS-C-associated H3K9me2 observed in the *Su(var)3*-9 mutants correlate with an overall upregulation of the histone genes, suggesting that dimethylation of H3K9 at the HIS-C is necessary to maintain normal levels of histone transcripts in the cell. However, this conclusion may be too simplistic since we don’t know what other functions SU(VAR)3-9 may have, and, if applicable, whether these other functions are affected in the *Su(var)3*-9 missense mutants. It has been suggested that the SET domain of SUV39H1, the human homolog of SU(VAR)3-9, may play an important structural role in the stable association of the protein with chromatin (Krouwels *et al.*, 2005). We know that the residues that are affected in the *Su(var)3*-9 mutants tested here are not necessary for this process, since the association of these mutant forms of SU(VAR)3-9 with the HIS-C is not significantly affected (Figure 3.2). However, as discussed above, we can’t exclude the possibility that such residues are required for proper interaction with other chromatin components and/or regulators of the histone genes, and that the elevated levels of histone transcripts are partially due to the inability of the mutant SU(VAR)3-9 proteins to carry out such interactions.
3.3.3. Recruitment and role of HP1 at the HIS-C

The ChIP results obtained show that HP1 is present at the HIS-C in wild-type embryos, at least within the three regions of the histone unit that we analyzed (Figure 3.4). This is in agreement with previous reports (Greil et al., 2003; Koryakov et al., 2006). We also detected HP1 association with the histone units in the Su(var)3-9 mutants, but its levels were drastically reduced in all mutants (Figure 3.4). We did not see a correlation between the magnitude of the reduction in the levels of H3K9me2 and the relative amount of HP1 associated with the different regions of the histone unit. This suggests that the presence of H3K9me2 is necessary for stable binding of HP1, but other factors, such as auxiliary proteins, are probably involved in the process, and these may influence the efficiency of HP1 binding to this chromatin domain.

Interestingly, the amount of HP1 associated with the histone units in the Su(var)3-9 missense mutants was not significantly different from that found at the same locations in the "protein null" Su(var)3-9<sup>06</sup>. Thus the presence of a SU(VAR)3-9 protein with a single amino acid substitution in its catalytic region is not sufficient to recruit and stabilize the association of HP1 with the HIS-C. There are at least two possible explanations for this. The first one is that each of the SU(VAR)3-9 residues that are mutated in the missense alleles is critical for the binding of HP1. The second one is that the physical presence of SU(VAR)3-9, whether wild-type or mutant, is not sufficient to recruit and/or to stabilize HP1 at the HIS-C.
Although *a priori* it is difficult to decide which hypothesis is most plausible, we tend to favour the second one, for the following reasons. Firstly, it has been shown that, at least *in vitro*, the presence of auxiliary factors is required for stable binding of HP1 to a chromatin template, even in the presence of H3K9me2,3 (Eskeland *et al.*, 2007). Secondly, we showed that all the mutant SU(VAR)3-9 proteins, SU(VAR)3-9309, SU(VAR)3-9330 and SU(VAR)3-9318, localize at the HIS-C like their wild-type counterpart (Figure 3.2), suggesting that *in vivo* their structure and folding are relatively unaffected. Thus, it is likely that their ability to interact with other chromatin proteins does not differ significantly from that of wild-type SU(VAR)3-9.

At this point, we propose that the most essential factor in the recruitment of HP1 at the HIS-C is the presence of a certain level of H3K9me2, and that this histone modification, in combination with the presence of NHCPs other than SU(VAR)3-9, are responsible for the stable association of HP1 with chromatin at this locus. Curiously, the relative enrichment for HP1 in Su(var)3-9330 is significantly lower than that observed in Su(var)3-906, suggesting that Su(var)3-9330 may be acting as an antimorph, effectively hindering the stable association of HP1 with the HIS-C. In chapter 2 we showed that, *in vitro*, the recombinant SU(VAR)3-9330 protein partially retains the ability to methylate histone H3, but can’t methylate a peptide representing the histone H3 tail alone. One of the hypotheses proposed to explain this result was that the amino acid substitution present in SU(VAR)3-9330 (D536N) might result in a change in specificity, causing SU(VAR)3-9330 to methylate a histone H3 residue other than K9. Several studies have shown that
chromodomains can show very high specificity with respect to modified histone residues; for example, the chromodomain of HP1 specifically binds H3K9me2,3, but not H3K27me, while the opposite is true for the chromodomain of POLYCOMB (Bannister et al., 2001; Nakayama et al., 2001; Muller et al., 2002; Jacobs and Khorasanizadeh, 2002; Nielsen et al., 2002; Cao et al., 2002; Min et al., 2003; Fischle et al., 2004; Pray-Grant et al., 2005; reviewed by Daniel et al., 2005). If the change in specificity hypothesized for SU(VAR)3-9330 occurs in vivo, then the methylation of this other H3 residue may create a binding platform that recruits or stabilizes the binding of a different chromodomain protein, and the binding of this inappropriate NHCP may preclude binding of HP1. Alternatively, it is conceivable that SU(VAR)3-9330 binds to histone H3, and may or may not methylate K9, but it remains tightly associated with the H3 tail, thus making it unavailable for the recruitment of HP1. We do not favour this possibility because, if SU(VAR)3-9330 remained tightly bound to the H3 tail, and thus to chromatin, we would expect to see higher levels of (mutant) SU(VAR)3-9 associated with the HIS-C in the ChIP experiments on Su(var)3-9330 extracts than in their wild-type counterparts. However, this is not the case (Figure 3.2).

3.3.4. Elevated levels of the histone transcripts in Su(var)3-9 mutants

In a previous study, members of our lab (Ner et al., 2002) showed that the levels of histone H1 and H4 mRNA present in two Su(var)3-9 mutants (one missense allele, Su(var)3-9330, and one P element-induced allele, Su(var)3-9330P225) is about two fold higher than in wild-type individuals. We confirmed and expanded this
observation. Our results show that the level of both the $H2A$ and the $H3$ histone transcripts are elevated in three $Su(var)3$-9 missense mutants and the complete null allele $Su(var)3$-9$^{06}$. We find the difference between the mutant and wild-type strains to range between ~1.8 and 6.5-fold.

The apparent discrepancies between these results and the data obtained in the 2002 study are likely due, at least in part, to differences in the experimental setup. In the former study the relative abundance of each histone transcript was quantified by northern blot analyses. In contrast, this study employed real-time/RT-PCR, a more accurate and reliable quantification system. Moreover, our mRNA samples are derived from staged embryos at a time when many of the cells are still undergoing mitosis (reviewed by Lee and Orr-Weaver, 2003). Since histone genes are expressed almost exclusively during S-phase, their misregulation would be more noticeable at this stage than in adult flies, which were used for the previous study (Ner et al., 2002).

In the present study, we were able to detect differences in the relative amounts of histone $h2a$ and $h3$ transcripts between some of the mutants. In particular, $Su(var)3$-9$^{06}$ and $Su(var)3$-9$^{318}$ have significantly higher levels of $h2a$ and $h3$ than $Su(var)3$-9$^{309}$. Since $Su(var)3$-9$^{06}$ is a "protein null" (no RNA or protein product detected, see chapter 2) we suggest that $Su(var)3$-9$^{318}$ acts as an amorph (at least functionally) and $Su(var)3$-9$^{309}$ behaves as a hypomorph, with respect to the regulation of $h2a$ and $h3$ gene expression.
Once again, the $Su(var)3-9^{330}$ mutant strain has a very curious phenotype. It shows dramatically elevated levels of $h3$ (~6 fold over the wild-type), but only a ~1.8 to 1.9 fold increase in the $h2a$ and $h2b$ transcripts. Thus, the $Su(var)3-9^{330}$ mutation is unique in that, unlike the other alleles studied, it seems to decouple the $h2a/h2b$ from the $h3$ expression levels. This mutant is therefore difficult to categorize, as it would be classified as a loss-of-function mutant (same phenotype as $Su(var)3-9^{06}$ and $Su(var)3-9^{318}$) based on its $h3$ expression level, but not with respect to $h2a$ and $h2b$.

3.3.5. Possible mechanism for the functions of SU(VAR)3-9 at the HIS-C

In general, the results obtained in this study are consistent with the notion that the function of SU(VAR)3-9 at the HIS-C, a euchromatic locus, is very similar to its function in (pericentric) heterochromatin. In both cases SU(VAR)3-9 is required for downregulation or silencing, and its ability to methylate H3K9 is required to allow stable association of HP1 with chromatin.

Our results suggest that the physical presence of SU(VAR)3-9 probably does not play a major role in the regulation of the histone transcripts levels. $Su(var)3-9^{318}$, a mutant that produces a SU(VAR)3-9 protein with a single amino acid substitution, and which localizes at the HIS-C like its wild-type counterpart, shows the same $h2a$ and $h3$ hyperexpression phenotype as $Su(var)3-9^{06}$, a mutant that does not produce any SU(VAR)3-9 protein at all (no statistical difference in a t test at $p=0.05$). In addition, the missense mutant $Su(var)3-9^{330}$ also displays the same hyperexpression phenotype as $Su(var)3-9^{06}$ with respect
to the histone h3 gene. If the physical presence of SU(VAR)3-9 played a major role in the regulation of the histone genes expression, one would expect most missense mutants to have a milder overexpression phenotype than the "protein null" Su(var)3-906.

All data presented point to the crucial role of SU(VAR)3-9's enzymatic activity in the regulation of the levels of histone transcripts; all mutants have an impaired HMTase activity (chapter 2), all of them display a drastic reduction in the abundance of H3K9me2 associated with the HIS-C, and all of them show elevated levels of h2a and h3 transcripts. However, the relative levels of H3K9me2 associated with the various regions of the histone units are virtually indistinguishable in all Su(var)3-9 mutants, while the abundance of h3 and h2a transcripts may differ. The simplest explanation for this apparent disparity is that differences in the levels of H3K9me2 do exist among the mutants, but the resolution of the ChIP technique is not sensitive enough to detect them. It is also possible that the mutants do not differ significantly for H3K9me2, but they do with respect to another NHCP, or another histone modification, which could have a role in the fine-tuning of the regulation of histone genes expression.

Interestingly, the levels of HP1 associated with the "H3 coding" region in the Su(var)3-906, Su(var)3-9318 and Su(var)3-9309 mutants correlates with their respective h3 transcript levels. For instance, the first two mutants show similar levels of HP1 associated with this region of the histone unit, and a similar increase in h3 transcript. The Su(var)3-9309 has a higher level of HP1 associated with the "H3 coding" region and a lower increase in its h3 transcript, which is
consistent with the view that the presence of HP1 is associated with silencing. As usual, \textit{Su(var)3-9}^{330} represents an exception. In this case, its levels of "H3 coding"-associated HP1 are significantly lower than those observed in the other mutants, yet the increase in its \textit{h3} transcript level is similar to that of \textit{Su(var)3-9}^{06} and of \textit{Su(var)3-9}^{318}.

In contrast, we did not detect a correlation between the level of HP1 associated with the "H2A coding" region and the relative increase in \textit{h2a} transcript produced in the various mutants. The levels of HP1 associated with the "H2A coding" region did not differ significantly in \textit{Su(var)3-9}^{06}, \textit{Su(var)3-9}^{318} and \textit{Su(var)3-9}^{309}, but \textit{Su(var)3-9}^{309} has a much weaker \textit{h2a} hyperexpression phenotype than the other two strains.

We have demonstrated that reduced levels of H3K9me2 and HP1 at the HIS-C correlate with an increase in the abundance of \textit{h2a} and \textit{h3} transcripts produced. We propose that there is a causal relationship between the two observations, and that the presence of appropriate amounts of H3K9me2 and HP1 at the HIS locus is absolutely necessary for the production of wild-type levels of \textit{histone} transcripts. Our data also suggest that high levels of H3K9me2 and/or of HP1 may not be sufficient, by themselves, to ensure a normal regulation of the \textit{histone} genes expression. We propose that additional factors are involved in the process of fine-tuning the production of \textit{histone} transcripts.

In order to gain a thorough understanding of the mechanisms involved in the regulation of the HIS-C at the chromatin level, it will be necessary to identify the other factors associated with this \textit{locus}, their respective functions and how they
interact with one another. So far, we know that the gene products of at least three other suppressors of PEV \((abo, Bonus\) and \(Su(var)326/HDAC1\)) localize to the HIS-C (Berloco et al., 2001; Beackstead et al., 2005; Ner et al., in preparation). Investigations of their respective roles and functions in the modulation of the histone gene expression will likely help our understanding of this complex system.

3.3.6. Regulation of the histone gene expression

Our data show that the amounts of \(h2a\) and \(h3\) transcripts are higher in \(Su(var)3-9\) mutants than in \(Su(var)3-9^+\) embryos and, as discussed previously, we think that this is most likely the result of overexpression of the histone genes. We have shown that the over production of histone transcripts is not due to decoupling of histone genes expression from the cell cycle (\(i.e.\) synthesis of histones outside S phase). Thus, we are left with two possibilities for the role of SU(VAR)3-9 in the regulation of the histone genes expression.

In the first scenario, SU(VAR)3-9 determines the number of histone templates that are actively transcribed in a nucleus. In this model, mutations in SU(VAR)3-9 would cause an increase in the number of transcribed templates without affecting transcription rates. In a wild-type situation the HMTase activity of SU(VAR)3-9 may be mainly responsible for keeping a certain number of histone units inaccessible to transcription factors. In \(Su(var)3-9\) mutant strains more units may be accessible and thus more histone transcripts are produced during S-phase while the transcription rate/template remains unchanged. This hypothesis is
supported by the fact that the chromatin structure of the HIS-C, as measured by
the pattern of DNaseI and MNase hypersensitive sites, is altered in at least a
subset of Su(var)3-9 mutants (Ner et al., 2002). The alternative model is that
SU(VAR)3-9 modulates the rate of transcription of each histone template or unit.
In this scenario, the same number of histone templates would be transcribed in
wild-type and Su(var)3-9 mutants, but the mutants would have higher
transcription rates.
Of course, these two hypotheses are not mutually exclusive, and SU(VAR)3-9
could have several functions regulating template accessibility as well as
modulating the transcription rate of each histone gene.
Finally, it should be noted that the peculiar phenotype of the Su(var)3-9330
mutant, which shows a drastic increase in its levels of h3, but only a moderate
elevation in h2a and h2b transcripts, suggests that the h2a/h2b and h3/h4 gene
pairs may be regulated somewhat independently. In a wild-type situation, the
regulation of these two gene pairs is usually co-ordinated. In two Su(var)3-9
mutants (Su(var)3-9309 and Su(var)3-9318) this co-ordinated regulation appears to
be maintained, as their increase in h2a transcript is virtually identical to their
increase in h3 transcript. Su(var)3-906 shows a slightly higher increase in h3 than
h2a, and for Su(var)3-9330 this decoupling of h2a/h2b couplet from h3 is very
dramatic. We do not have an explanation for these results, but it is possible that
SU(VAR)3-9 is involved, directly or indirectly in the co-regulation of the h2a/h2b
and h3/h4 pairs.
3.4. Materials and methods

3.4.1 Drosophila strains

All Drosophila strains used in this study are described in chapter 2, and were grown under standard conditions.

3.4.2 ChIP analysis

ChIP analysis of the HIS-C was performed as described in chapter 2. The antibody to H3K9me2 was purchased from Upstate (#07-441) and the anti-SU(VAR)3-9 antibody is described in chapter 2 and by Ner et al. (2002). The antibody to HP1 is a polyclonal and was raised in our lab (Ner et al., in preparation). The primers pairs used for amplification were H3F/H3R ("H3 coding" region), iH2AF/iH2BR ("H2A/H2B intergenic" region), H2AFc/H2BR ("H2A coding" region), iH3F/iH4R ("H3/H4 intergenic" region) and iH1Bf/iH3r ("H1/H3 intergenic" region) (see supplementary table 3.1 and Ner et al., 2002). The relative amounts of target DNA present in the immunoprecipitated material was quantified by real-time PCR. A dilution series (1:10-1:2000) of the input DNA was used to generate a standard curve, which was then used to estimate the amount of the fragment of interest present in the immunoprecipitated material (or in the mock reaction). In each case, the material pulled down in 3 independent experiments was individually analyzed for its enrichment in target (HIS-C) sequences, expressed as a % of the input for each IP (same for the mock IPs). The results obtained with each mutant strain were compared to those relative to the wild-type strain using Student’s T test. Differences were considered...
significant if the absolute t value was above 2.78 (critical value for p=0.05, 4 df, in a two-tailed T test). The same test was also employed to determine whether, in each strain, the enrichment observed was above the background.

3.4.3. Quantifications of histone mRNAs

Total RNA was isolated from 12-16 hours old embryos by TRIzol extraction as recommended by the manufacturer. Three independent extracts were prepared from each strain. For each extract, reverse-transcription reactions were performed as described in chapter 2. To reverse-transcribe total histone H2A, H3 and H2B mRNA, primers H2AR, H3R and dH2BR were used, each one in combination with RP49rt (internal control). A 17 nucleotide oligodT primer was employed to reverse-transcribe polyadenylated mRNAs. The relative amounts of histone cDNAs obtained from each RT reaction were quantified by real-time PCR amplification. For each sample, three reactions were run and, since the resulting Ct values were almost identical (<1% difference) among the three measurements, they were averaged, and referred to as "one measurement". The ratio between the histone and the rp49 measurements was calculated for three independent RNA extracts. In each case, the resulting value was then divided by the value obtained, by the same procedure, with the wild-type strain, giving rise to one "data point". For each strain, the results are presented as an average of three (independent) "data points" +/- the standard deviation. Differences among strains were considered significant if they yielded a |t value| >2.78 (critical value for p=0.05, 4df) in a two-tailed Student’s t-test.
Supplementary figure 3.1

a. Relative amounts of "H3coding" fragment immunoprecipitated from 12-16 hr old wild-type embryos

b. Relative amounts of "H2Acoding" fragment immunoprecipitated from 12-16 hr old wild-type embryos

c. Relative amounts of "H3coding" fragment immunoprecipitated from 12-16 hr old wild-type embryos

d. Relative amounts of "H2Acoding" fragment immunoprecipitated from 12-16 hr old wild-type embryos
Supplementary figure 3.1. Control ChIP reactions from wild-type 12-16 hours old embryo extracts using commercial, non-specific IgG and pre-immune serum, respectively. a) Representative gels showing the PCR products obtained using the primers that amplify the "H3 coding" (H3c), "H2A coding" (H2Ac) and "H2A/H2B intergenic" fragments, respectively. The template was a 1:10 dilution of the ChIP input DNA (lane 1), the DNA obtained from a mock IP (no antibody, lane 2), or the DNA IPed with commercial non-specific IgG (lane 3) or with the anti-H3K9me2 antibody (lane 4). b) Relative abundance of the "H3 coding" fragment in the material IPed with IgG and with anti-H3K9me2 determined by real-time PCR (see materials and methods for details). c) Representative gels showing the PCR products obtained using the primers that amplify the "H2A coding" (H2Ac) and "H2A/H2B intergenic" fragments, respectively. The template was a 1:100 dilution of the ChIP input DNA (lanes 5 and 8), the DNA obtained from a mock IP (no antibody, lanes 5 and 9), or the DNA IPed with the anti-HP1 antibody (lane 7) or with pre-immune serum (lane 10). d) Relative abundance of the "H2A coding" fragment in the material IPed with IgG and with anti-H3K9me2 determined by real-time PCR (see materials and methods for details).
### Supplementary table 3.1.
List of primers used in this study.

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<th>Primer name</th>
<th>Primer sequence (5'→3')</th>
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<tr>
<td>H2AFc</td>
<td>TCTGGACGTGAAAAAGGTGG</td>
</tr>
<tr>
<td>H3R</td>
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<tr>
<td>H3F</td>
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<td>iH4R</td>
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4. GENERAL SUMMARY AND DISCUSSION

4.1. Summary of results

In chapter 2 we characterized a subset of EMS-induced \( Su(var)3-9 \) missense alleles with respect to a variety of phenotypes, including the relative strength with which they suppress PEV, and the levels of residual HMTase activity of their respective gene products. Based on sequence information, and on the crystal structure of Clr4 (the \( S. pombe \) homolog of \( SU(VAR)3-9 \)) (Min et al., 2002) we were able to carry out a structure/function study of \( SU(VAR)3-9 \) and to rationalize the effects of each amino acid substitution on the various phenotypes analyzed. The nine mutants characterized were originally generated in a screen for strong, dominant modifiers of PEV (Sinclair et al., 1983) but, remarkably, all of them have single amino acid substitutions only in the catalytic region of the protein and show significantly impaired HMTase activity. This result strongly suggests that the enzymatic activity of \( SU(VAR)3-9 \) is crucial for its silencing function and, perhaps more interestingly, that single amino acid substitutions elsewhere in the protein have no discernable effect on silencing, at least in this particular assay.

The data presented in chapter 3 shows the effects of one "protein null", and three of the \( Su(var)3-9 \) missense mutants, on the regulation of a euchromatic locus, the HIS-C. Again, our results suggest that the HMTase activity of \( SU(VAR)3-9 \) is essential for the recruitment and/or binding of HP1 to the \( his \) units that comprise the HIS-C, and in the (down)regulation/silencing of the locus. Taken together, these two data sets strongly suggest that \( SU(VAR)3-9 \) likely acts in similar ways at the HIS-C and within the \( w^{m4} \) variegating
rearrangement and, by extension, heterochromatin. The results presented in chapter 3 strongly suggest that it is the catalytic activity of SU(VAR)3-9, rather than its physical association with the HIS-C locus, that plays a major role in the regulation of the histone gene transcription or in the association of HP1 with this locus.

Overall, we compared between 4 and 7 Su(var)3-9 mutants with the wild-type strain (and with each other) in a number of biochemical, molecular, cytological and morphological assays. When trying to integrate the data from the different types of assays, in most cases it was possible to detect some general trends and to rationalize the results obtained. However, no two assays gave results that were completely consistent with each other for every mutant (Table 4.1 and Figure 4.1).

I suggest that these slight variations may derive mostly from the nature and limitations of the assays employed. However, slight differences in the role(s) of SU(VAR)3-9 in the regulation of the HIS-C and in PEV cannot be ruled out. The presence of these apparent "inconsistencies" between data sets from different assays highlights a third, very important point, namely, the advantage of testing mutants for a variety of parameters, rather than solely focusing on one specific aspect of their phenotypes. In this specific case, such a multi-assay approach has allowed us to uncover some subtle, and yet informative, differences among the Su(var)3-9 mutants. Indeed, this thesis represents one of a few cases where the function of mutant alleles of a gene have been assayed in a wide variety of contexts. The results presented indicate that in many cases, the
effect(s) of a mutation on one particular phenotype do not accurately predict its effect(s) on a different phenotype. This may be true not only for Su(var)3-9, but also for other genes encoding chromatin and, most likely, other proteins. I suggest that this may be the case especially for those proteins that function as components of one or more multimeric complexes. Hence, it should be kept in mind that studies relying on only one or two phenotypic assays may give a biased, and possibly misleading idea of the function of the gene/gene product in question.

4.2. The function(s) of SU(VAR)3-9 in PEV and in the regulation of the HIS-C.

In this and the following sections I will try to briefly relate the role(s) and function(s) of SU(VAR)3-9 in the regulation of the HIS-C and in PEV, as well as some of the limitations of our experimental setup.

4.2.1. The white gene in the wm4 variegating rearrangement and the HIS-C.

In chapter 3 we set out to address the role of SU(VAR)3-9 in the regulation of one of its natural targets, the histone genes, and to compare it to what we know about its functions in heterochromatin, represented here by the wm4 variegating rearrangement. There is a valid rationale behind the choice of these two loci, but one should also keep in mind that the HIS-C and the white gene in the wm4 strain are only single examples of a euchromatic locus and a heterochromatin-induced variegating rearrangement, respectively, and as such may not be representative of what typically occurs in euchromatin and heterochromatin, respectively. The white locus in the wm4 variegating rearrangement has many features in common
with heterochromatin, for instance, it is enriched in heterochromatin marks such as H3K9me2, HP1 and HDAC1 (Rudolph et al., 2007; Mottus, personal communication). However, the very fact that it shows variegated expression suggests that, at least in some nuclei, it is transcriptionally competent, and therefore presumably has an "open" chromatin structure. Moreover, in PEV the variegating gene does not reside in heterochromatin per se, but rather in close proximity to a disrupted heterochromatic region, and this distance varies depending on the white^+ rearrangement in question (Tartof et al., 1989). Hence, one should not automatically extrapolate all that applies to the role of SU(VAR)3-9 in the context of the white gene in the w^md rearrangement to heterochromatin in general.

The case of the HIS-C is the opposite in that, while it is definitely a euchromatic locus, the HIS-C also has several characteristics that are perhaps more typical of heterochromatin: it is a reiterated locus, somewhat late-replicating, mildly underreplicated in polytene chromosomes, and it is organized into a higher order chromatin structure (Samal et al., 1981; Ner et al., 2002; Zhimulev and Belyaeva, 2003). For these reasons the HIS-C, albeit euchromatic, may not be representative of all euchromatic genes, especially single copy genes, and it would not be surprising if SU(VAR)3-9 acted through slightly different mechanisms and the recruitment of slightly distinct factors, in the regulation of other euchromatic loci.
4.2.2. Considerations regarding the assays used to assess the effect of *Su(var)3-9* mutants on gene expression.

For our purposes, the relative proportion of pigmented (red) vs. white pigment cells in flies carrying the *wm* variegating rearrangement can be considered as the readout of an assay for gene expression. In this sense, the PEV assay employed in chapter 2 is not substantially different from the RT-PCR approach used to determine the relative levels of *histone* transcripts in chapter 3. However, each of the two assays has its advantages and disadvantages. The RT-PCR approach is a direct assay of transcript accumulation, as it allows us to measure the relative amounts of transcripts of interest. Obviously, infer that an elevated transcript level reflects an increase in expression. However, we have not ruled out the possibility that the elevated transcript levels result from an increase in RNA stability, or a decrease in RNA turnover.

In contrast, the PEV assay is based on a tertiary phenotype, eye pigmentation, which is the result of the coordinated action of several factors, including events and proteins that are not related to the expression of the *white* gene *per se*. This assay has, however, one big advantage over the RT-PCR approach. Since the phenotype of each eye is individually recorded, it is possible to observe and document the phenotypic variability within each strain. This allows for a more accurate and, possibly, more relevant comparison among strains. For example, we were able to notice that the presence of the *Su(var)3-9*^06^ mutant allele in a strain that is hyperploid for *Su(var)3-9* (2 wild-type copies of *Su(var)3-9*, plus the mutant allele *Su(var)3-9*^06^) results in a surprisingly high
frequency of "mildly suppressed" eyes (chapter 2, Figure 2.6.b), and that the presence of \textit{Su(var)3-9}\textsuperscript{318}, instead of \textit{Su(var)3-9}\textsuperscript{06}, in the same hyperploid strain often causes the eyes to be "sectored" (large, well-defined regions of red and large, well-defined regions of white). Such observations would be impossible with the approach employed to assess the levels of \textit{histone} transcripts, since each RNA preparation used was derived from a population of embryos, which invariably contain cells that are in different stages of the cell cycle. Hence, it is formally possible, although probably highly unlikely, that embryos of a certain genotype could have a great variation in their levels of \textit{histone} transcripts, with some showing strong overexpression and others being indistinguishable from the wild type, while those of a different genotype could all be overexpressing the \textit{histone} genes. In both cases, the RT-PCR assay would show a moderate increase in the transcripts levels.

4.2.3. Correlations between the effects of the \textit{Su(var)3-9} mutants on PEV and on the levels of \textit{histone} transcripts.

Keeping in mind the respective limitations of the techniques used, if the function of SU(VAR)3-9 within the HIS-C is the same as in the \textit{w}\textsuperscript{m4} variegating rearrangement, and if it acts through the same mechanism at both loci, then any given mutation in \textit{Su(var)3-9} should affect the expression of the \textit{white} gene in \textit{w}\textsuperscript{m4}, and the \textit{histone} genes transcription similarly. Thus, some general predictions can be made. Firstly, a screen for dominant mutants that result in overexpression of the \textit{histone} genes should lead to the isolation of the same
Su(var)3-9 alleles that were recovered in the original screen for strong, dominant suppressors of PEV, as well as mutations in other loci. Secondly, all Su(var)3-9 alleles that strongly suppress PEV should also show elevated levels of histone transcripts. Finally, mutations in Su(var)3-9 that do not cause suppression of PEV should not result in elevated levels of histone transcripts.

An extension of this hypothesis predicts that, for each Su(var)3-9 mutation, there is a correlation between overproduction of histone transcripts and the strength of Su(var) phenotype. That is, an allele that shows a very high increase in the level of H2A and H3 transcripts should also be a very strong suppressor of PEV. Conversely, alleles that are weaker suppressors of PEV are expected to show a smaller elevation in the abundance of H2A and H3 RNAs. In the present case, Su(var)3-9309 was the weakest suppressor in our PEV assay (chapter 2), and it also showed the most limited increase in the levels of H2A and H3 transcripts (only ~1.6-1.8 fold higher than the wild-type, see chapter 3).

The H2A and H3 transcripts are significantly more abundant in Su(var)3-906 and Su(var)3-9318 embryos (~4.5 to 6-fold higher than wild-type, see chapter 3), and both Su(var)3-906 and Su(var)3-9318 displayed a stronger Su(var) phenotype than Su(var)3-9309. However, while no significant differences are detectable between Su(var)3-906 and Su(var)3-9318 at the level of histone transcripts, Su(var)3-9318 is a stronger Su(var) than Su(var)3-906 (chapters 2 and 3, respectively). This apparent discrepancy may be a result of the experimental systems used (discussed above), or may reflect a difference in the role of SU(VAR)3-9 in the regulation of the two loci, or in their respective sensitivity to
the presence of a malfunctioning SU(VAR)3-9 (as in Su(var)3-9^{318}) versus no SU(VAR)3-9 at all (as in Su(var)3-9^{06}).

4.2.4. The Su(var)3-9^{330} mutation appears to be a special case.

The Su(var)3-9^{330} mutant shows peculiar phenotypes in nearly every assay that we employed. In the in vitro enzyme assay this strain showed a unique characteristic. Its HMTase activity on full length histone H3 is higher than that of any other mutant SU(VAR)3-9 protein tested (>30% of wild-type), but it fails to function if a peptide representing the H3 N-terminal tail (20 amino acids) is used as the substrate. Su(var)3-9^{330} is also peculiar in that the levels of H3K9me2 associated with the histone unit, as determined by ChIP, are comparable to those observed in Su(var)3-9^{06}, yet the relative amount of HP1 associated with the same region is significantly lower than in Su(var)3-9^{06}. In fact, Su(var)3-9^{330} has the lowest level of his unit-associated HP1 among the mutants tested, suggesting that it may be acting as an antimorph. With respect to suppression of PEV, the strength of suppression of the Su(var)3-9^{330} mutant is similar to that of Su(var)3-9^{318} (although Su(var)3-9^{330} individuals do not show "sectored" eyes).

The most interesting feature of this mutant, however, is that the expression of its h2a/h2b and h3 histone genes appears to be dramatically decoupled, to the point where the relative abundance of the h3 transcript is 6 fold higher than in wild-type (one of the strongest hyperexpression phenotypes observed), while the levels of h2a and h2b are only about 1.7-1.8 fold above the wild-type (Table 4.1). This is a very unique, and intriguing feature. It may be
worth pointing out that there are presently no data formally demonstrating that the $H2A/H2B$, and the $H3/H4$ transcripts, are expressed in equal amounts in a wild type *Drosophila* (we are currently examining this). The data obtained with the $Su(var)3-9^{330}$ mutant strain suggest that there is some degree of independence between the regulation of $h2a/h2b$ and $h3$ expression, and that there may be a mechanism in place to ensure co-ordinated expression.
Figure 4.1. A semi-quantitative summary of the phenotypes of four Su(var)3-9 mutants analyzed.
Figure 4.1. A semi-quantitative summary of the phenotypes of four Su(var)3-9 mutants analyzed. The results presented in chapters 2 and 3 relative to in vitro HMTase activities of recombinant SU(VAR)3-9 proteins, association of H3K9me2 and HP1 with the HIS-C, suppression of PEV in Su(var)3-9 hyperploid strains and abundance of h3 and h2a transcripts were simplified and compiled in order to give a general "overview" of each mutant’s phenotype. For the in vitro enzyme assay, only the averages of 3 independent trials are indicated. The same is true for the ChIP data reported. Such data refer to the enrichment for "H3 coding" fragment in the material IPed with anti-H3K9me2 and anti-HP1, respectively. Note that no data regarding the relative enrichment for H3K9me2 or HP1 within the chromocentre is reported here. For the abundance of h3 and h2a transcripts, the wild-type strain was arbitrarily assigned the value of 10, and the mutants are expressed as proportions of the wild type. For the in vitro assays, the activity of the wild-type strain was always arbitrarily assigned a value of 100. The ChIP data is expressed in each case as % of the input, and the PEV value represent the % of fly eyes showing a strong Su(var) phenotype in the assay described in chapter 2.
4.2.5. **A possible mode of action for SU(VAR)3-9**

The genetic screens for dominant Su(var)s were originally designed as a way to identify non-histone chromatin proteins (NHCPs). The term NHCP generally describes proteins that are either relatively stable components of chromatin, or that act as chromatin-modifying enzymes. There are of course proteins that fit both descriptions: SU(VAR)3-9, for example, is both a chromatin-modifier and a chromatin-associated protein (Rea *et al*., 2000; Nakayama *et al*., 2001; Schotta *et al*., 2002; Ebert *et al*., 2004; 2006). Given these two functions, it is easy to hypothesize how SU(VAR)3-9 is involved in the variegated, epigenetic silencing of the *white* gene in the *wm4* rearrangement: it is recruited to centromeric and pericentric chromatin, where it methylates H3K9, thus allowing the recruitment and binding of HP1, a crucial step in the "heterochromatinisation" of the region. Therefore, the absence of functional SU(VAR)3-9 will result in a drastic reduction on H3K9me2 within pericentric chromatin; consequently low levels of HP1 are recruited and/or bound to the region (Rudolph *et al*., 2007; Mottus, personal communication). This will prevent or reduce the rate at which heterochromatin is assembled in most cells, leading to the Su(var) phenotype.

However, we now know that Su(var)3-9 mutations that suppress PEV cause increased levels of *histone* transcripts (chapter 3 and Ner *et al*., 2002). We also know that strains that are hemizygous for the HIS-C have a Su(var) phenotype (Moore *et al*., 1979; 1983) and surprisingly these strains also produce increased levels of *histone* transcripts (Ner *et al*., 2002). Therefore, it is possible that the Su(var) phenotype observed in *wm4*; Su(var)3-9 mutant individuals is due
to the combination of a lack of methylation of H3K9 within pericentric chromatin, where the variegating white gene is located, and an increase in the amount of histone transcripts produced.

It has previously been shown that individuals with elevated levels of histone gene transcripts also have an increased level of nucleus-associated histone proteins (Ner et al., 2002). We hypothesize that, since the euchromatic portion of the genome generally replicates earlier than the heterochromatic regions, the increased availability of histone proteins may give the euchromatic portion of the genome a competitive advantage over the later replicating heterochromatin. Therefore, in Su(var)3-9 mutants the formation of heterochromatin would be impeded in two ways. Firstly, by the failure to methylate H3K9 and secondly, by an excess of histone proteins that may allow the euchromatin to replicate slightly more rapidly or more completely, thus interfering with heterochromatin assembly.

It will be interesting to investigate whether other Su(var)s also have elevated levels of histone transcripts, and how their levels of histone transcripts correlate with the strength of their Su(var) phenotype, and with that of individuals that are hemizygous for the HIS-C. Our hypothesis would predict that 1) all Su(var) mutations that result in increased levels of histone transcripts should be stronger suppressors of PEV than the lack of one copy of the HIS-C, and 2) hemizygosity for the HIS-C and Su(var) mutations should have an additive effect on the Su(var) phenotype, particularly if the Su(var) mutation in question does not result in increased levels of histone gene products.
Table 4.1. Summary of the characteristics of the Su(var)3-9 mutants analyzed. All numerical data reported represent the average of 3 independent experiments (for details, see corresponding sections in chapters 2 and 3). The detection of H3K9me2 and HP1 associated with the chromocentre was carried out by immunofluorescence on polytene nuclei, which only provided qualitative data (expressed as "strong" vs. "reduced"). No HMTase assays were run for mutant Su(var)3-9\textsuperscript{06} because such allele results in no gene product (see chapter 2). Su(var)3-9\textsuperscript{311} was not chosen for the HIS-C and histone transcripts analysis (NT=not tested).

<table>
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<th>Allele</th>
<th>Result of mutation</th>
<th>HMTase function</th>
<th>HP1 recruitment/ association</th>
<th>Loss of silencing/ overexpression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>\textit{In vitro} (enzyme assays)</td>
<td>\textit{In vivo} (H3K9me2)</td>
<td>Chromo-centre</td>
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<td></td>
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<td>H3 tail</td>
<td>K9me tail</td>
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<td>100%</td>
<td>100%</td>
</tr>
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<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
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<td>0%</td>
<td>0%</td>
</tr>
<tr>
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<td>D536N</td>
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<td>1.2%</td>
<td>4.0%</td>
</tr>
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<td>S616L</td>
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<td>18.3%</td>
<td>0%</td>
</tr>
<tr>
<td>311</td>
<td>G521D</td>
<td>22.7%</td>
<td>14.3%</td>
<td>11.5%</td>
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</table>
4.3. *Drosophila* SU(VAR)3-9 has a unique N-terminal region

Like many Su(var) genes, Su(var)3-9 is highly conserved from yeast to mammals. Therefore, one might infer that the basic function of the protein and our findings in *Drosophila* are transferable to other organisms. However, *Drosophila*’s SU(VAR)3-9 is larger than its homologs, and it contains a "unique" N-terminal region. This region is comprised of the 82 amino acids that are in common with (eIF2γ common domain) and a region, sometimes called "region 2" (residues 83-219), that is required for the dimerization and full enzymatic function of SU(VAR)3-9, as well as for the interactions with SU(VAR)3-7 and HP1 (Krauss and Reuter, 2000; Schotta *et al*., 2002; Eskeland *et al*., 2004; Krauss *et al*., 2006).

The lack of these N-terminal domains in the yeast and vertebrate homologs of SU(VAR)3-9 suggest that they may function slightly differently from the *Drosophila* protein. Neither the vertebrate nor the yeast homologs have the regions required for dimerisation, implying that they may not need to dimerize in order to be fully functional. The fact that they also lack the region of interaction with SU(VAR)3-7 should not be surprising, since SU(VAR)3-7 does not exist outside the *Drosophila* genus (Jaquet *et al*., 2006), where it is required for proper localization of SU(VAR)3-9 (Delattre *et al*., 2004). In the fission yeast this function is carried out, at least in part, by an RNAi-based mechanism (Bühler *et al*., 2006; Zhang *et al*., 2008). In spite of its lack of region 2, the mammalian homolog of SU(VAR)3-9 can interact with HP1 (Aagaard *et al*., 1999; Melcher *et al*., 1999; Melcher *et al*., 2002; Schotta *et al*., 2002; Eskeland *et al*., 2004; Krauss *et al*., 2006).

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This interaction occurs through the first 44 amino acids of Suv39h1 (Melcher et al., 2000), which correspond to the last 44 residues of SU(VAR)3-9’s region 2. In addition, SUV39H1 (the human homolog) can partially rescue the dominant Su(var) phenotype of Su(var)3-9 mutants (Schotta et al., 2002). A physical interaction between S. pombe’s Clr4 and Swi6 (the homolog of HP1) has not been formally demonstrated, and in any case it would be unlikely to occur through the N-terminal region of Clr4, since this portion of the protein is only 7 amino acids long.

Curiously, although the N-terminal region of SU(VAR)3-9 has such important functions, no single amino acid substitutions within this portion of the protein have been isolated in the screens for dominant suppressors of PEV (Sinclair et al., 1983; Tschiersch et al., 1994; Ebert et al., 2004). As in the case of the chromodomain (see discussion in chapter 2), this suggests that mutating any single amino acid may not be sufficient to cause a dominant Su(var) phenotype. In order to dissect the function of region 2, it would be interesting to create transgenic Drosophila lines that express tagged SU(VAR)3-9 proteins with single and multiple amino acid substitutions within this region, and investigate the targeting and chromatin association of the mutant proteins, as well as their effects on the targeting of the endogenous (wild-type) SU(VAR)3-9 and some of its known partners, HP1 and SU(VAR)3-7. We would not expect the single amino acid substitutions to have any effects on the behaviour of the endogenous protein. The same experiment, carried out in a Su(var)3-9 null background, would allow us to determine whether any missense mutations in region 2 result in a
recessive suppression of PEV phenotype, and what their effect on \textit{in vivo} H3K9me2,3 is.
4.4. References


Appendix 1: ChIP with commercial non-specific IgG, and "SN1 pre-immune" (Ner et al., in preparation) serum.

(Critical value for a two-sided, unpaired T-test at p=0.05: 2.78)

Strain: WT
Region: H3c
Antibody: non-specific IgG (CALBIOCHEM NI01, control for H3K9me2 IPs)

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<th>SD</th>
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Strain: WT
Region: H2Ac
Antibody: "SN1 pre-immune serum" (control for HP1 IPs)

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Comparison between "control IPs" and corresponding "specific IPs"

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<th>Specific Ab-NO Ab (average, in % input)</th>
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Appendix 2: ChIP with anti-SU(VAR)3-9 (α-SU(VAR)3-9chr; Ner et al., 2002).

(|Critical value| for a two-sided, unpaired T-test at p=0.05: 2.78)

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Comparison of mutants to WT

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<th>Significantly different from WT?</th>
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<td>0.0406 (%)</td>
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Appendix 3: ChIP with anti-H3K9me2 (UPSTATE#07-441)
((Critical value) for a two-sided, unpaired T-test at p=0.05: 2.78)

Strain: WT
Region: H3c

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Strain: 06
Region: H3c

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Strain: 309
Region: H3c

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Strain: 330
Region: H3c

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Strain: 318
Region: H3c

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Comparison of mutants to WT

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<th>Significantly different from WT?</th>
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<tbody>
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<td>t=9.36; YES at p=0.05</td>
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<td>0.953 (%)</td>
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### Strain: WT
Region: H3/H4 intergenic

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Region: H3/H4 intergenic

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### Strain: WT
Region: H2A/H2B intergenic

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### Strain: 330
Region: H2A/H2B intergenic

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### Comparison of 330 to WT for H3/H4 intergenic region

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### Comparison of 330 to WT for H2A/H2B intergenic region

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### Appendix 4: ChIP with anti-HP1 (α-HP1; Ner et al., in preparation).

(Critical value for a two-sided, unpaired T-test at p=0.05: 2.78)

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### Comparison of mutants to WT

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<tr>
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### Strain: WT
#### Region: H3c

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### Strain: 309
#### Region: H3c

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<th>SD</th>
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<th>SD</th>
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#### Region: H3c

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<th>Avg</th>
<th>SD</th>
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### Strain: 318
#### Region: H3c

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<th>SD</th>
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<th>t</th>
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### Comparison of mutants to WT

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<th>Ratio to WT</th>
<th>Significantly different from WT?</th>
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<td>0.3595</td>
<td>t= 4.35; YES at p=0.05</td>
</tr>
<tr>
<td>330</td>
<td>0.0121</td>
<td>0.0531</td>
<td>t= 6.52; YES at p=0.05</td>
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<tr>
<td>318</td>
<td>0.0343</td>
<td>0.137</td>
<td>t= 18.0; YES at p=0.05</td>
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</table>
Appendix 5: relative quantifications of histone transcripts by RT-PCR. Comparison between the relative abundance of total h2a and h3 transcripts in wild-type vs. Su(var)3-9 mutant embryo extracts. (Critical value for a two-sided, unpaired T-test at p=0.05: 2.78)

<table>
<thead>
<tr>
<th>Exp #</th>
<th>Strain</th>
<th>h2a/rp49 in mut. H2a in WT</th>
<th>Avg</th>
<th>St. dev.</th>
<th>t* (mutant/WT)</th>
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<td>N/A</td>
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<tr>
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<td>0.53</td>
<td>-2.82 √</td>
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<table>
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<th>Avg</th>
<th>St. dev.</th>
<th>t*</th>
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</tr>
<tr>
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Appendix 6.
Comparison between the relative increase in $h2a$ and $h3$ transcripts detected in the $Su(var)3-9$ mutants. The data presented in appendix 5 were compared using a Student’s T test.
(|Critical value| for a two-sided, unpaired T-test at p=0.05: 2.78)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relative increase $h2a$</th>
<th>Relative increase $h3$</th>
<th>Statistically different?</th>
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<tbody>
<tr>
<td>06</td>
<td>4.56 +/- 0.51</td>
<td>6.46 +/- 0.53</td>
<td>YES at p=0.05; t=-4.46</td>
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<tr>
<td>309</td>
<td>1.86 +/- 0.53</td>
<td>1.56 +/- 0.15</td>
<td>NO at p=0.05; t=0.943</td>
</tr>
<tr>
<td>330</td>
<td>1.76 +/- 0.52</td>
<td>6.07 +/- 0.50</td>
<td>YES at p=0.05; t=-10.5</td>
</tr>
<tr>
<td>318</td>
<td>5.60 +/- 0.72</td>
<td>4.72 +/- 1.38</td>
<td>NO at p=0.05; t=0.994</td>
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</table>

Appendix 7.
Comparison between the relative abundance of total $h2b$ transcript in wild-type vs. $Su(var)3-9^{330}$ embryo extracts. The indicated $t^*$ refers to the result of an unpaired T-test in which the $h2b/rp49$ ratio of each mutant was compared to that of the wild-type strain.
(|Critical value| for a two-sided, unpaired T-test at p=0.05: 2.78)

<table>
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<tr>
<th>Exp #</th>
<th>Strain</th>
<th>$h2b/rp49$ in mut.</th>
<th>$h2b/rp49$ in WT</th>
<th>Avg</th>
<th>St. dev.</th>
<th>$t^*$</th>
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