Su(var) genes regulate the balance between euchromatin and heterochromatin in Drosophila

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Histone lysine methylation is an epigenetic mark to index chromosomal subdomains. In Drosophila, H3-K9 di- and trimethylation is mainly controlled by the heterochromatic Su(VAR)3-9 HMTase, a major regulator of position-effect variegation (PEV). In contrast, H3-K27 methylation states are independently mediated by the Pc-group enzyme E(Z). Isolation of 19 point mutants demonstrates that the silencing potential of Su(var)3-9 increases with its associated HMTase activity. A hyperactive Su(var)3-9 mutant, pitkinB, displays extensive H3-K9 di- and trimethylation within but also outside pericentric heterochromatin. Notably, mutations in a novel Su(var) gene, Su(var)3-1, severely restrict Su(var)3-9-mediated gene silencing. Su(var)3-1 was identified as “antimorphic” mutants of the euchromatic H3-S10 kinase JIL-1. JIL-1Su(var)3-1 mutants maintain kinase activity and do not detectably impair histone lysine methylation marks. However, analyses with seven different PEV rearrangements demonstrate a general role of JIL-1Su(var)3-1 in controlling heterochromatin compaction and expansion. Our data provide evidence for a dynamic balance between heterochromatin and euchromatin, and define two distinct mechanisms for Su(var) gene function. Whereas the majority of Su(var)s encode inherent components of heterochromatin that can establish repressive chromatin structures [intrinsic Su(var)s], Su(var)3-1 reflects gain-of-function mutants of a euchromatic component that antagonize the expansion of heterochromatic subdomains [acquired Su(var)s].

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HMTases for H3-K9 trimethylation at pericentric heterochromatin are Suv39h enzymes (Peters et al. 2003). Ezh2 is the major H3-K27 HMTase to index the inactive X-chromosome [X] and Pc-dependent gene silencing (Plath et al. 2003, Silva et al. 2003); however, it is not known whether Ezh2 contributes to pericentric H3-K27 monomethylation in mammals.

In Drosophila, H3-K9 dimethylation was found to be enriched at heterochromatin (Schotta et al. 2002). The development of highly specific antibodies that can discriminate mono-, di-, and trimethylation of H3-K9 versus H3-K27 (Peters et al. 2003) now allowed us to characterize all H3-K9 and H3-K27 methylation states in Drosophila. Our data reveal that Su(var)3-9 mainly controls H3-K9 di- and trimethylation at pericentric heterochromatin, whereas all H3-K27 methylation is mediated by Enhancer of zeste [E(z)]. The identification and characterization of novel point mutants in Su(var)3-9 that show differential HMTase activities demonstrate that the silencing potential of Su(var)3-9 is mainly determined by the kinetic properties of the HMTase reaction. Importantly, we identified the PEV modifier Su(var)3-3 as a key antagonist for Su(var)3-9-dependent gene silencing. Su(var)3-1 mutants are novel alleles of the euchromatic H3-S10 kinase JIL-1 (Wang et al. 2001). In these mutants, H3-S10 phosphorylation is not altered, indicating that the kinase function of JIL-1Su(var)3-1 is intact. However, condensation of the male X-chromosome and expansion of heterochromatic subdomains are impaired, suggesting that JIL-1-Su(var)3-1 may regulate the balance between euchromatin and heterochromatin. Although JIL-1 is not an intrinsic component of heterochromatin, JIL-1Su(var)3-1 mutants display the strongest PEV modifier effect and are dominant over Su(var)3-9. Our data define Su(var)3-1 as a novel class of Su(var) genes, in which gain-of-function mutants of a euchromatic component antagonize the expansion of heterochromatic subdomains.

Results

H3-K9 and H3-K27 methylation states in Drosophila heterochromatin

To investigate the distribution of distinct H3-K9 and H3-K27 methylation states in Drosophila heterochromatin, we used highly specific antibodies that can discriminate mono-, di-, and trimethylation of H3-K9 versus H3-K27 (Peters et al. 2003) in immunofluorescence analyses. Polytenic chromosomes allow the mapping of these marks at a very high resolution because they have a clearly discernible chromocenter where all chromosomes are joined. Attached to this chromocenter is the largely heterochromatic fourth chromosome.

In wild-type chromosomes, the prominent mark of the chromocenter and the fourth chromosome is H3-K9 dimethylation (Fig. 1A). In addition, H3-K9 dimethylation is associated with some euchromatic bands and telomeres (Supplementary Fig. S1). H3-K9 monomethylation is also found in the chromocenter (Fig. 1A) and is associated with many euchromatic bands (Supplementary Fig. S1). H3-K9 trimethylation shows a very distinct heterochromatic distribution with a strong enrichment in the chromocenter core (Fig. 1A, arrowhead). The chromocenter core region is also marked by CID, a centromere-specific histone variant in Drosophila [Henikoff et al. 2000, data not shown], and likely represents centromeric heterochromatin.

In Su(var)3-9-null mutants, H3-K9 monomethylation is not significantly altered. In contrast, H3-K9 di- and trimethylation are strongly reduced in the chromocenter but not in the fourth chromosome (Fig. 1A). Notably, the chromocenter core that is enriched for H3-K9 trimethylation in wild-type chromosomes is prominently marked with H3-K9 dimethylation in Su(var)3-9 mutants (Fig. 1A, arrowhead).

HP1 is an interaction partner of SU(VAR)3-9 and important to retain SU(VAR)3-9 at pericentric heterochromatin (Schotta et al. 2002). To test whether HP1 is involved in the control of Su(var)3-9-dependent H3-K9 di- and trimethylation, we analyzed these methyl-marks in Su(var)2-5 [HP1] mutants. In the chromocenter, H3-K9 mono-, di-, and trimethylation appear unaltered (Fig. 1A); however, there is a dramatic increase in H3-K9 mono- and dimethylation along euchromatic arms (Fig. 1B). Additionally, H3-K27 mono- and dimethylation can be detected at almost all bands in euchromatin, whereas only ~100 bands are marked by H3-K27 trimethylation (Supplementary Fig. S2). H3-K27 methylation states are not significantly altered in Su(var)3-9-null mutants (Fig. 1B); however, no H3-K27 methylation can be detected in E(z)-null mutants (Fig. 1B).

Immunofluorescence analyses and the different abundance of H3-K9 and H3-K27 methylation states were confirmed by mass spectrometric analyses of bulk nuclear extracts from wild-type and mutant larvae (Fig. 1C). Consistent with previous studies [Schotta et al. 2002] demonstrating dispersed localization of SU(VAR)3-9, H3-K9 methylation levels are significantly increased in the HP1-null background.

Together, the data demonstrate that pericentric heterochromatin in Drosophila is largely characterized by H3-K9 di- and H3-K27 mono- and dimethylation. Only the chromocenter core displays a focal enrichment for the trimethylated state [H3-K9 and H3-K27 trimethylation]. We provide evidence for the existence of Su(var)3-9-independent H3-K9 monomethylation at the chromocenter and of additional H3-K9 HMTases that target the fourth chromosome. In contrast, E(z) mediates all three H3-K27 methylation states but does not appear to affect H3-K9 methylation. Although E(Z) has been detected at a limited number of bands (Carrington and Jones 1996), the high abundance of H3-K27 methylation indicates that E(Z) complexes might not reside at all sites after accomplishing the methylation.
The silencing potential of Su(var)3-9 mutants correlates with their associated H3-K9 HMTase activity

To identify novel regulatory mechanisms for Su(var)3-9-dependent gene silencing, we performed EMS mutagenesis experiments and isolated 56 novel suppressor mutants together with 19 Su(var)3-9 alleles. Sequence analyses of these novel Su(var)3-9 alleles revealed that most mutants cluster around the SET domain (Fig. 2A; Supplementary Fig. S3). Although the screen was rather saturated, we could not detect mutants in the SU(VAR)3-9 chromo domain.

In the course of these studies we also identified the exceptionally strong PEV enhancer pitkinD [Kuhfittig et al. 2001] as a novel Su(var)3-9 allele. The pitkinD mutation causes extensive chromatin compaction in early embryos and induces ectopic recruitment of SU(VAR)3-9 with many euchromatic sites [Kuhfittig et al. 2001]. P-element-induced male recombination [cf. Materials and Methods] indicated that the pitkin locus maps to region 88E, near Su(var)3-9. Sequence analysis revealed a point mutation [R529S] within the SU(VAR)3-9 SET domain. To verify that this mutation is, indeed, pitkinD, we analyzed transgenic flies expressing SU(VAR)3-9R529S. After remobilization, four independent lines were selected that display a very strong PEV enhancer effect that was equivalent to three additional Su(var)3-9 gene copies (data not shown). Since these data classify the pitkinD mutation as a hypermorphic Su(var)3-9 allele, we propose to rename this mutant Su(var)3-9ptn.

Based on their ability to induce white gene silencing in the PEV rearrangement In(1)wm4, the other Su(var)3-9 mutants were categorized into null and hypomorphic alleles. One wild-type copy of Su(var)3-9 fails to induce
white gene repression (the eyes remain red), whereas two wild-type copies partially silence the white gene and display the characteristic white mottled phenotype [Fig. 2B]. Next, we crossed these two wild-type copies with our novel Su(var)3-9mut mutants. In this combination, some mutant alleles could moderately enhance white gene silencing and were regarded as hypomorphic mutants [Fig. 2B]. In contrast, mutant alleles that did not alter the In(1)wm4 phenotype were regarded as null alleles [Fig. 2B].

Next, we tested whether the reduced silencing potential of hypomorphic Su(var)3-9 mutants possibly correlated with their ability to induce H3-K9 di- and trimethylation at pericentric heterochromatin. In the homozygous Su[var]3-9mut-null mutant, pericentric H3-K9 dimethylation is nearly absent and H3-K9 trimethylation is entirely lost from the chromocenter [Fig. 2C]. All mutants that were classified as null alleles show a similar loss of H3-K9 methylation. In contrast, hypomorphic alleles show slightly elevated H3-K9 di- and trimethylation at the chromocenter [Fig. 2C]. Importantly, hypermorphic Su[var]3-9ptn induces strong H3-K9 di- and trimethylation at pericentric heterochromatin [Fig. 2C] and, additionally, very prominent signals at many euchromatic sites [Supplementary Fig. S4A]. Mass spectrometric analyses of Su[var]3-9ptn nuclear extracts further demonstrate that the overall levels of H3-K9 di- and trimethylation are significantly increased [Supplementary Fig. S4B].

To examine the mutant proteins in a more direct manner, we performed in vitro HMTase assays with 16 different point mutants. All loss-of-function mutants are catalytically inactive, whereas mutant proteins of hypomorphic alleles are less active than wild-type protein [Fig. 2D]. Su[VAR]3-9ptn displayed a higher HMTase activity [Fig. 2D].

Finally, we characterized reaction kinetics of the wild-type HMTase versus the Su[VAR]3-9ptn mutant. In the starting phase of the reaction, Su[VAR]3-9ptn methylates approximately twice as many H3 peptides as the wild-type HMTase and reaches a plateau within ~15 min [Fig. 2E]. These data characterize Su[VAR]3-9ptn as a hyperactive H3-K9 HMTase and strongly suggest that its hypermorphic phenotype (chromatin compaction and enhanced gene silencing) is caused by enhanced enzymatic activity.

Identification of JIL-1 mutants as novel regulators of PEV-based gene silencing

In our mutant screens to isolate novel Su(var)3-9 alleles, we also identified other suppressor mutants that impair Su(var)3-9-dependent silencing. The strongest of these mutants has been identified as an allele of the previously

![Figure 2](image-url)

**Figure 2.** SU(VAR)3-9 HMTase activity correlates with its ability to induce gene silencing. (A) Protein structure of SU(VAR)3-9 and positions of the molecularly characterized mutations. Point mutations resulting in amino acid exchanges are in red. (B) Genetic classification of Su(var)3-9 mutants. Su(var)3-9mut alleles were crossed to two extra Su(var)3-9 copies. Null mutants show the characteristic white mottled phenotype, whereas hypomorphic alleles partially enhance white repression. Su(var)3-9ptn is a hypermorphic mutant resulting in complete white and partial silencing of the roughest gene. (C) H3-K9 di- and trimethylation in chromocenter regions of heterozygous Su(var)3-9mut/Su(var)3-9mut mutants. Null mutants [exemplified by Su(var)3-9ptn] show the same loss of H3-K9 di- and trimethylation as the Su(var)3-9mut null mutant. H3-K9 methylation levels are elevated in hypomorphic mutants [exemplified by Su(var)3-9ptn]. Very high levels of H3-K9 di- and trimethylation are found in hypermorphic Su[var]3-9ptn. Arrowheads indicate the chromocenter core. (D) In vitro HMTase activity of recombinant SU(VAR)3-9 mutant proteins. The amount of incorporated label was measured by scintillation counting. (E) Time-course analysis of HMTase reactions with wild-type (wt) and SU(VAR)3-9ptn proteins. Incorporation of the radioactive label was measured after different timepoints by scintillation counting.
described Su(var)3-1 gene [Wustmann et al. 1989]. Su(var)3-1 mutants strongly suppress gene silencing in the In(1)wm4 PEV rearrangement [Fig. 3A, left panel] and even counteract gene repression that is caused by overexpression of prominent regulators of heterochromatin formation, such as Su(var)3-9, Su(var)2-5 [HP1], and Su(var)3-7 [Fig. 3A, right panels]. Using male recombination, we mapped the Su(var)3-1 locus to a small region comprising four genes [Mocs1, CG7839, CG6302, and JIL-1] between the two P-element insertions P[EP]EP3688 and P[EP]EP3657. Sequencing of candidate open reading frames (ORFs) in all six Su(var)3-1 alleles revealed nonsense mutations in the C-terminal part of JIL-1 that would generate truncated proteins [Fig. 3B]. We therefore propose naming these mutants JIL-1\textsuperscript{Su(var)3-1}.

JIL-1 is a kinase that controls H3-S10 phosphorylation (pH3S10) in interphase nuclei [Wang et al. 2001]. In JIL-1-null mutants, euchromatic pH3S10 is impaired, whereas mitotic pH3S10 is not altered. We analyzed pH3S10 in polytene chromosomes of JIL-1\textsuperscript{Su(var)3-1} mutants by immunofluorescence using a pH3S10-specific antibody. In wild-type chromosomes, pH3S10 is associated with many interbands along chromosomal arms. In JIL-1\textsuperscript{Su(var)3-1} mutants, we did not observe a significant change of this pattern [Fig. 3C], indicating that the kinase function of the truncated JIL-1\textsuperscript{Su(var)3-1} protein is intact.

JIL-1-null mutants are characterized by condensation of polytene chromosomes, an effect that is most apparent for the male X-chromosome [Wang et al. 2001]. To test for changes in the polytene chromosome structure of JIL-1\textsuperscript{Su(var)3-1} mutants, we performed IF analyses of polytene chromosomes from wild-type versus mutant larvae. In homozygous JIL-1\textsuperscript{Su(var)3-1} mutants, no obvious differences in the patterns of H3-K9 and H3-K27 methylation states or of H4-K20 trimethylation have been detected (Supplementary Fig. S5), which was confirmed by mass spectrometric analyses of nuclear extracts [data not shown]. One possibility to explain the strong derepression of the white gene in In(1)wm4 by JIL-1\textsuperscript{Su(var)3-1} could be a shift in the expansion of the heterochromatic structure. We analyzed the effect of JIL-1\textsuperscript{Su(var)3-1} mutants on heterochromatin expansion in the PEV rearrangement T(1;4)wm258-21, in which heterochromatization of a large chromosomal domain.

**Figure 3.** Su(var)3-1 mutants are antimorphic alleles of the JIL-1 kinase. (A) Genomic extra copies of Su(var)3-9, Su(var)2-5, and Su(var)3-7 display enhanced silencing of the white gene in In(1)wm4 (upper row), which is impaired in the presence of the strong PEV suppressor mutant Su(var)3-1 [lower row]. (B) Protein structure of JIL-1. All mutational lesions in Su(var)3-1 alleles result in a C-terminally truncated JIL-1 protein. (S/T) Serine/threonine kinase domain; (S/T/Y) Serine/threonine/tyrosine kinase domain. (C) Polytene chromosomes of wild-type and Su(var)3-1 mutant larvae were stained with pH3S10-specific antibodies. DNA was stained with propidium iodide. (D) X-chromosome decondensation phenotype of Su(var)3-1 mutants. In male homozygous mutant larvae, the X-chromosome is largely decondensed and partially loses its banding structure. The arrowhead points to the X-chromosome. The affected chromosome shows association with MOF, a specific marker for the male X-chromosome. DNA was stained with propidium iodide. Bar, 10 µm.
A euchromatic region can be monitored. T(1;4)wm258-21 is a complex rearrangement in which a part of the X-chromosome [cytological region 3C–3E] comprising ∼1 Mb of DNA is brought near to pericentric heterochromatin [Fig. 4A]. Because of a translocation of the fourth chromosome, this region can specifically be detected in polytene chromosome spreads. It contains several genes, of which inactivation of Notch [clipped wings] and white [white eyes] can be monitored phenotypically. In the presence of two extra Su(var)3-9 copies, the whole region becomes highly compacted and prominently marked by H3-K9 dimethylation [Fig. 4B, bracket, left panel]. The repressed state of this region can also be detected by inactivation of Notch and, to a lesser extent, of the more distant white gene [Fig. 4B, left panel]. Notably, heterochromatin does not expand along the fourth chromosome, which is modified by different HMTase complexes that might be critical to the spreading effect. Next, we crossed the two extra copies of Su(var)3-9 with JIL-1Su(var)3-1 and examined the polytene structure at T(1;4)wm258-21. Surprisingly, the whole region that was inactivated in the presence of the two extra Su(var)3-9 copies is not compacted and shows a normal banding pattern [Fig. 4B, right panel]. Derepression of this region is further supported by reactivation of the Notch and white marker genes [Fig. 4B, right panel]. These data illustrate that Su(var)3-9-mediated condensation of this chromosomal region is accompanied by strong H3-K9 dimethylation, however, we could not detect significant H3-K9 trimethylation [Fig. 4B, bracket, right panel]. These data demonstrate that in Drosophila, H3-K9 dimethylation is the major mark to induce chromatin condensation and expansion of heterochromatic structures. H3-K9 trimethylation plays a minor role for heterochromatin formation, and the strong enrichment for this mark in the chromocenter core rather supports the argument for a function of H3-K9 trimethylation in organizing chromatin structure at centromeres.

**Figure 4.** Su(var)3-1 mutants impair expansion of heterochromatin. (A) Translocation T(1;4)wm258-21 contains the X-chromosomal region 3C–3E comprising ∼1 Mb of DNA, which can be detected because of contacts to the fourth chromosome. Heterochromatin expansion inactivates the two marker genes Notch [clipped wings] and white [white eyes]. (B) Immunocytological analysis of H3-K9 di- and trimethylation at T(1;4)wm258-21. [Left panel] In the presence of two extra Su(var)3-9 copies, region 3C–3E becomes compacted [bracket] and marked with H3-K9 dimethylation but not H3-K9 trimethylation. Transcriptional repression is indicated by a large number of Notch wings [51%] and white mottled sectored eyes [41%]. [Right panel] The presence of Su(var)3-1 mutations impairs expansion of heterochromatin [bracket], resulting in a normal banding pattern, loss of H3-K9 dimethylation, and restored transcriptional activity as indicated by wild-type [Notch+] wings [91%] and red [white+ ] eyes [95%].
Control of heterochromatic gene silencing in Drosophila

Table 1. JIL-1Su(var)3-1 generally antagonizes Su(var)3-9 mediated heterochromatin expansion

| PEV epialleles | Marker gene(s)   | Chr. | Phenotype when marker is fully expressed | % wild-type expression of marker gene in P[3-9]/+ | Su(var)3-1/+ | Su(var)3-1/P[3-9]/+
|----------------|-----------------|------|------------------------------------------|-----------------------------------------------|-------------|----------------|
| Class I        |                 |      |                                          |                                               |             |                |
| In(1)w^ax4    | white           | X    | Red eyes                                 | 0%                                           | 100%        | 95%            |
| Dp(1;f)238    | yellow          | X    | Black abdominal pigmentation             | 6%                                           | 70%         | 45%            |
| T(2;3)Sb^2    | stable          | 3    | Short bristles                           | 2%                                           | 64%         | n.d.           |
| T(1;4)w^258-2t| Notch           | X    | Clipped wings                            | 49%                                          | 100%        | 91%            |
| white         |                 |      | Red eyes                                 | 41%                                          | 100%        | 95%            |
| Class II       |                 |      |                                          |                                               |             |                |
| In(3L)BL1     | lacZ-transgene  | 3    | lacZ expression                          | 26%                                          | 73%         | 60%            |
| w^r           |                 |      | Red eyes                                 | 33%                                          | 86%         | 58%            |
| Tp(3;Y)BL2    | lacZ-transgene  | Y    | lacZ expression                          | 26%                                          | 68%         | 53%            |
| w^r           |                 |      | Red eyes                                 | 25%                                          | 98%         | 68%            |
| T(2;3)V21^+   | tandem repeats  | 3    | Red eyes                                 | 22%                                          | 85%         | n.d.           |
| PlacW(92E)    |                 |      |                                          |                                               |             |                |

Expression of marker genes in different PEV epialleles was monitored in the presence of two Su(var)3-9 extra copies [P[3-9]/+], JIL-1Su(var)3-1 mutants [Su(var)3-1/+], and the combination of two Su(var)3-9 extra copies with JIL-1Su(var)3-1 mutants [Su(var)3-1/P[3-9]/+]. Expression of the respective marker genes was evaluated according to Materials and Methods. Representative phenotypes are depicted in Supplementary Figure 6.

In (In(1)w^ax4), yellow abdomen with few black spots [Dp(1;f)238], or long thoracic bristles due to inactivation of the dominant Sb^r mutant [T(2;3)Sb^r], respectively. JIL-1Su(var)3-1 can release silencing of these marker genes and even antagonizes Su(var)3-9-mediated gene silencing (Table 1; Supplementary Fig. 6).

In the second class of PEV rearrangements, transgene insertions containing lacZ and white marker genes are brought near to heterochromatic regions (Table 1). In these rearrangements we have analyzed expression of marker genes in different developmental stages, lacZ expression in larval salivary gland tissues, and white expression in adult flies. Both lacZ and white are silenced by overexpression of Su(var)3-9, but strongly derepressed in the presence of JIL-1Su(var)3-1 mutants (Table 1; Supplementary Fig. 5). Also in these rearrangements, Su(var)3-9-mediated expansion of heterochromatin is restricted by JIL-1Su(var)3-1, which is indicated by reactivation of both marker genes in flies containing genomic extra copies of Su(var)3-9 together with JIL-1Su(var)3-1 mutants.

These data demonstrate that JIL-1Su(var)3-1 antagonizes Su(var)3-9-mediated heterochromatin expansion at different genomic regions and developmental stages. We therefore conclude that JIL-1Su(var)3-1 is a general regulator for the balance between euchromatin and heterochromatin.

Discussion

Su(var) genes as inherent components of heterochromatin

The identification of Su(var) genes in Drosophila revealed many important proteins regulating higher-order chromatin structure. SU(VAR)3-9, HP1, and SU(VAR)3-7 are inherent components of heterochromatin that can establish and maintain a heterochromatic chromatin structure. By genetic means, Su(var)3-9 is the dominant component over Su(var)2-5 [HP1] and Su(var)3-7 (Schotta et al. 2002), indicating a major function of the associated HMTase in the formation of repressive chromatin regions.

In Drosophila, heterochromatic gene silencing is mainly determined by H3-K9 dimethylation. In agreement with its in vitro HMTase activity [Eskeland et al. 2004], Su(var)3-9 regulates H3-K9 di- and trimethylation at pericentric heterochromatin. In addition, other H3-K9-specific HMTases must exist that mediate H3-K9 dimethylation at the fourth chromosome and in the chromosome core of Su(var)3-9 mutants. HP1 is another important control factor for H3-K9 methylation because in Su(var)2-5 mutants, H3-K9 mono- and dimethylation are dramatically increased along chromosomal arms. Although SU(VAR)3-9 shows extensive association with heterochromatic and euchromatic regions in HP1-null cells, the shift in these methylation patterns could also be mediated by other H3-K9-specific HMTases. At pericentric heterochromatin, H3-K9 methylation states appear unaltered; however, a slight reduction of H3-K9 dimethylation that is not detectable by immunofluorescence might be the cause of the HP1 Su(var) effect. Alternatively, impairment of H4-K20 trimethylation at pericentric heterochromatin [Schotta et al. 2004] could cause the loss of silencing in HP1 mutants.

The analysis of Su(var)3-9 mutants revealed novel hypomorphic and null alleles that show differential effects on H3-K9 methylation states. A direct correlation between their HMTase activities and the amount of gene silencing demonstrates that establishment of heterochromatic structures is to a large extent determined by the kinetic properties of the SU(VAR)3-9 HMTase reaction. Consistently, hyperactive SU(VAR)3-9med-
duces enhanced H3-K9 di- and trimethylation, concomitant with an expansion of heterochromatin. This results in severe phenotypes such as dominant female sterility caused by overcompaction of the whole chromatin in early embryos [Kuhfittig et al. 2001]. In this study we show that Su(var)3-9"mutants display around 100–200 additional bands with enhanced H3-K9 di- and trimethylation in the arms of polytene chromosomes. These bands likely correspond to endogenous SU(VAR)3-9-binding sites [Greil et al. 2003] and demonstrate that hyperactive SU(VAR)3-9 can mediate the expansion of repressive structures also at chromosomal arms.

A loss-of-function Su(var) mutant reduces gene silencing by removing one or several intrinsic components of heterochromatin. Su(var)3-9" induces enhanced gene silencing, and it was therefore surprising to encode a mutant of a “classical” Su(var) gene. Based on our functional characterization, we identified this mutant as the strongest Su(var) gene to date. Su(var)3-1 can restrict heterochromatin formation and generally dominate over the strong silencing effect of Su(var)3-9 extra copies. Su(var)3-1 carries C-terminal truncations of the H3-S10 kinase JIL-1 that do not affect its ability to phosphorylate H3-S10 but selectively impair the expansion of heterochromatin.

Intrinsic Su(var) genes impair or weaken the heterochromatic structure. Loss-of-function Su(var)3-9 alleles or mutations in HDAC1 reduce H3-K9 dimethylation [Czernin et al. 2001], whereas HP1 mutants impair H4-K20 trimethylation [Schotta et al. 2004] at pericentric heterochromatin. JIL-1 is not an intrinsic component of heterochromatin [Wang et al. 2001] and does not affect any of the known repressive histone lysine methylation marks at pericentric heterochromatin. Nevertheless, JIL-1Su(var)3-1 alleles display a strong Su(var) effect. We therefore categorize JIL-1Su(var)3-1 as a novel type of mutants with acquired Su(var) function. In contrast to intrinsic Su(var)s that encode components directly involved in heterochromatin formation, acquired Su(var) mutants would antagonize heterochromatin formation by stabilizing the euchromatic state or preventing heterochromatin expansion.

Our data assign a novel function to the C terminus of the JIL-1 kinase that is independent of its H3-S10 kinase activity. Lack of the C terminus could prevent phosphorylation or interaction with an as-yet unknown target protein. Alternatively, the C terminus could comprise control functions that prevent excessive phosphorylation of this target. There are many possible target proteins that could be involved in higher-order chromatin formation. For example, the striking X-chromosome decondensation phenotype of JIL-1Su(var)3-1 mutants has also been described for iswi and nurf301, subunits of the NURF complex [Deuring et al. 2000], Badenhorst et al. 2002]. Particularly, nurf301 mutants display a dominant Su(var) effect, indicating that chromatin remodeling processes are involved in the regulation of heterochromatin. Another possible target of the JIL-1Su(var)3-1 kinase might be the histone variant H2A.Z. In budding yeast, H2A.Z is required to prevent spreading of heterochromatin into euchromatin [Meneghini et al. 2003]. This protective function might be modulated by JIL-1Su(var)3-1-mediated phosphorylation of H2A.Z. Finally, some intrinsic Su(var)s such as SU(VAR)3-9 and HP1 are phosphoproteins [Eisenberg et al. 1994], Aagaard et al. 2000] and JIL-1Su(var)3-1-mediated phosphorylation of those proteins might affect their function in heterochromatin expansion.

The balance between euchromatin and heterochromatin

Based on our data we propose a model for a dynamic balance between euchromatin and heterochromatin [Fig. 5]. In PEV rearrangements, the boundary between these two states is determined by the antagonistic functions of euchromatic regulators (e.g., JIL-1) and the SU(VAR)3-9 HMTase that mediates H3-K9 dimethylation, the major mark of heterochromatin [Fig. 5A]. The boundary between euchromatin and heterochromatin is not static and depends on the activity and abundance of inherent components of heterochromatin, such as, for example, hyperactive SU(VAR)3-9" or overexpression of Su(var)3-9 [Fig. 5B] or overexpression of Su(var)3-9 [Fig. 5C]. In contrast, hypoaactive or null mutants of Su(var)3-9 weaken heterochromatin formation and favor the propagation of the euchromatic state. In JIL-1Su(var)3-1 mutants, heterochromatin expansion is severely repressed, even under conditions of elevated Su(var)3-9 function, and is most likely antagonized by a currently unknown proteins that are potential targets of the gain-of-function JIL-1Su(var)3-1 kinase. Dependent on the phosphorylation state of these targets, either heterochromatin expansion can be blocked or the propagation of the euchromatic state is highly stabilized [Fig. 5D].

In this work we have significantly extended mechanistic insights into the formation of heterochromatin and discovered Su(var)3-1 as a novel class of PEV modifiers that control the balance between euchromatic and heterochromatin subdomains. Because of the dominant role of JIL-1Su(var)3-1 over major components of heterochromatin [intrinsic Su(var)s], the putative JIL-1 mutant targets are predicted to have important functions in gene silencing and the higher-order structuring of chromatin. The ongo-
enhancer effect and dominant female sterility by crosses to w^me^4 flies. The full-length Su(var)3-9 cDNA containing the ptn^1^ mutation was introduced into p[GSy^ry^, hsEGFP] [Schotta and Reuter 2000]. Independent p[GSy^ry^, hs cDNA Su(var)3-9^gen^ EGFP] transgenes were mobilized in crosses with TM3, ry^KK^ Sb e [P[y^ry^ -2]-3][99B] and derivative lines with ptn^1^-specific mutant effects established.

HP1-null Df[2L]TE128X11/Su(var)3-9^0^ larvae survive up to third instar and were identified as GFP-negative larvae in the progeny of CyO GFP/Df[2L]TE128X11 x CyO GFP/Su(var)2-5^0^. E(z)-null larvae were generated by a cross of y; mwh E(z)^2^ red e/TM3, y; Sb Ser x E(z)^15 red/TM3, Sb Ser. Transheterozygous mwh E(z)^2^ red e/E(z)^15 red larvae were identified by their red mutant Malpighian tube phenotype.

In vitro histone methyltransferase assays and mass spectrometry analysis
HM Tase activities of recombinant GST-tagged proteins were analyzed according to Czermin et al. (2001). For the methyltransferase assays 1 µg of recombinant protein, 1 µg of histone H3 peptide (amino acids 1-20, Upstate Biotech), and 0.5 µL of SAM [1 µCi/ml, Amersham] were incubated for various times at 30°C in 25 µL of HIM buffer [Czermin et al. 2001]. Mass spectrometry analysis of reaction products was performed according to Peters et al. (2003).

Nuclear extracts were prepared from wild-type, Su(var)3-9 null [Su(var)3-9^null]/Su(var)3-9^null], HP1-null [Df[2L]TE128X11/Su(var)2-5^0^], E(z)-null [E(z)^2^/E(z)^2^], Su(var)3-9^gen^ [Su(var)3-9^gen^/+ and Su(var)3-9^gen^/Su(var)3-9^gen^] third instar larvae and separated by 18% SDS-PAGE. Following Coomassie staining, histone H3 bands were excised and processed according to Peters et al. (2003).

Immunostaining of polytene chromosomes
Preparation and staining of polytene chromosomes was performed as described [Schotta et al. 2002]. Chromosomes were incubated with rabbit polyclonal a-mono-, a-di-, and a-trimethyl H3-K9 or H3-K27, respectively (1:50) or 100/5% dry milk), a-phospho H3-S10 (Upstate; 1:50/5% dry milk) antibodies at 4°C overnight, followed by incubation with Alexa Fluor 488 conjugated [Molecular Probes] secondary antibody for 2 h at 37°C (1:50/5% dry milk). Preparations were examined with confocal laser scanning microscopy [LSM 510, Zeiss].

Molecular cloning of Su(var)3-1 and analysis of heterochromatic silencing
Su(var)3-1 was mapped to the genomic region of IL-1 at 86A by P-transposase-induced male recombination using a series of EP(3) P-element insertions. After a cross of homozygous se ss females with +/y; CyO, H[w^wt^/mc^ = P2-3]Hope2.1/+; ru ptn^1^ e/EP(3) males were crossed to +/+; ru e/ru e females. Recombinant + e/ru e and ru + ru e females were tested for the presence of ptn^1^ [PEV

Materials and methods
Drosophila cultures, stocks, and genetic analysis
Flies were reared on standard medium at 25°C. Chromosomes and mutations noted here are described in FlyBase (http://flybase.net). Su(var)3-9 and Su(var)3-1 mutations were isolated by their dominant suppressor effect on white variegation of In(1)w^wt^ in the background of El(var) mutations or transgenic copies of Su(var)3-9 after EMS [2.5 mM] mutagenesis [Reuter et al. 1986; Tschiersch et al. 1994]. The effects of Su(var)3-9 mutations on white gene silencing in w^wt^ were quantified in transheterozygotes with an additional P[ry^+^] Su(var)3-9 11.5kb]100E genomic copy [Tschiersch et al. 1994]. For molecular analysis, genomic DNA of Su(var)3-9^gen^/Su(var)3-9^gen^ heterozygotes was used to amplify the coding region of the Su(var)3-9 locus. Due to the DNA fragment inserted into Su(var)3-9^gen^ (Schotta et al. 2002), only PCR products of Su(var)3-9^gen^ are generated. Sequence information for all available alleles is available at GenBank [AJ290956].

Pitkirt^3^, originally assigned to region 67C due to a modifier of dominant female sterility was finally mapped by P-transposase-induced male recombination near Su(var)3-9 with the help of EP elements. w/+/CyO, H[w^wt^/mc^ = P2-3]Hope2.1/+; ru ptn^1^ e/EP(3) males were crossed to +/+; ru e/ru e females. Recombinant + e/ru e and ru + ru e females were tested for the presence of ptn^1^ [PEV

Figure 5. Su(var) genes regulate the balance between euchromatin and heterochromatin. The antagonistic functions of acquired and intrinsic Su(var) genes establish a boundary between euchromatin and heterochromatin. Hyperactive SU[VAR]3-9^gen^ (B) or overexpression of Su(var)3-9 (C) can expand heterochromatin, whereas JIL-1^Su(var)3-1^ stabilizes the euchromatic state by acting on an unknown target protein X (D). Please see main text for details.
males. $P_{\text{[rp7]}}$ Su(var)3-9 11.5kb $P_{\text{[rp7]}}$ Su(var)3-9 11.5kb 100E contains two additional genomic Su(var)3-9 copies inserted in 96A and 100E. $T[1;4]w^{y26-} F[1;4]w^{y26-}$ and $T[1;4]w^{y26-}F[1;4]w^{y26-}$ heterochromatin levels of marker genes were determined by classifying the phe-
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