# Increased expression of *Drosophila* Su(var)3-7 triggers Su(var)3-9-dependent heterochromatin formation

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## Summary

The Su(var)3-7 protein is essential for fly viability, and several lines of evidence support its key importance in heterochromatin formation: it binds to pericentric heterochromatin, it potently suppresses variegation and it interacts with HP1. However, the mode of action of Su(var)3-7 is poorly understood. Here we investigate in vivo the consequences of increased Su(var)3-7 expression on fly viability and chromatin structure. A large excess of Su(var)3-7 induces lethality, whereas lower doses permit survival and cause spectacular changes in the morphology of polytene chromosomes in males, and to a lesser extent in females. The male X is always the most affected chromosome: it becomes highly condensed and shortened, and its characteristic banding pattern is modified. In addition, Su(var)3-7 was found over the complete length of all chromosomes. This event coincides with the appearance of heterochromatin markers such as histone H3K9 dimethylation and HP1 at many sites on autosomes and, more strikingly, on the male X chromosome. These two features are strictly dependent on the histone-methyltransferase Su(var)3-9, whereas the generalised localisation of Su(var)3-7 is not. These data provide evidence for a dose-dependent regulatory role of Su(var)3-7 in chromosome morphology and heterochromatin formation. Moreover they show that Su(var)3-7 expression is sufficient to induce Su(var)3-9-dependent ectopic heterochromatinisation and suggest a functional link between Su(var)3-7 and the histone-methyltransferase Su(var)3-9.

Key words: *Drosophila*, Su(var)3-7, Heterochromatin, Position-effect variegation

## Introduction

In eukaryotes, a large proportion of the genome is represented by heterochromatin, the chromosomal regions characterised by a dense cytological appearance throughout the cell cycle. Heterochromatic regions are late-replicating, predominantly located near centromeres and telomeres, and consist mainly of repeated DNA sequences with a low density of unique genes. In contrast, euchromatin replicates relatively early in the cell cycle and contains most genes. Euchromatin and heterochromatin are also distinguished by specific histone tail modifications (Turner et al., 1992; Jacobs et al., 2001; Li et al., 2002; Richards and Elgin, 2002). Heterochromatin can silence the expression of euchromatic genes when translocated in its vicinity, a phenomenon known as position-effect variegation (PEV) (reviewed by Weiler and Wakimoto, 1995; Wallrath, 1998; Henikoff, 2000). Repression typically occurs only in a subset of cells and is heritable, leading to mosaic patterns of gene expression. Genetic analysis in *Drosophila* has identified mutations that enhance or suppress PEV (reviewed by Reuter and Spierer, 1992; Schotta et al., 2003). Some loci exhibit both a haplo-suppressor and a triplo-enhancer effect on PEV, making them good candidates as structural components of constitutive heterochromatin. Among them are HP1, Su(var)3-9 and Su(var)3-7 (Eissenberg et al., 1990; Tschiersch et al., 1994; Reuter et al., 1990). The well-known HP1 is encoded by the *Su(var)2-5* gene of *Drosophila melanogaster* (Eissenberg et al., 1990). On polytene chromosomes, HP1 is mainly associated with the chromocentre, telomeres and a few euchromatic sites (James et al., 1989). HP1 contains two domains, the N-terminal chromodomain and a C-terminal chromoshadow domain, conserved from yeast to human (Eissenberg and Elgin, 2000). HP1 was shown to interact with a number of other chromosomal proteins, including several involved in nuclear assembly, replication and gene regulation (Eissenberg and Elgin, 2000; Li et al., 2002). The mechanism of HP1 association with heterochromatin remained a mystery until the discovery that its chromodomain binds to methylated lysine 9 of histone 3 (Bannister et al., 2001; Lachner et al., 2001). This modified histone is a mark of heterochromatin. Immunofluorescence staining of *Drosophila* polytene chromosomes shows that the bulk of dimethyl-lysine 9 histone 3 (H3-diMeK9) is in pericentric heterochromatin, and in a banded pattern on the fourth chromosome (Jacobs et al., 2001). Moreover, methylation of H3-K9 has also been associated with the silencing of euchromatic genes (Nielsen et al., 2001; Ayyanathan et al., 2003). In *Drosophila*, most of the dimethylation of H3K9 is catalysed by the product of the modifier of PEV *Su(var)3-9* (Tschiersch et al., 1994; Schotta et al., 2002; Czermin et al., 2001; Eskeland et al., 2004). Su(var)3-9 and HP1 physically interact and their localisation

in heterochromatin is mutually interdependent (Schotta et al., 2002). The Su(var)3-9 protein contains a SET domain, carrying the histone methyl transferase activity, adjacent to a cysteine-rich region (Rea et al., 2000). In mammals, in addition to Su(var)3-9 orthologues, at least three additional proteins (G9A, ESET/SETDB1 and EU-HMTase 1) have been reported to possess H3-K9-specific HMTase activity (Tachibana et al., 2002; Schultz et al., 2002; Ogawa et al., 2002). These proteins are also present in *Drosophila*, and their specificity remains to be defined.

Su(var)3-7 is a third modifier of PEV, which appears to be a structural component of heterochromatin. *Su(var)3-7* encodes a protein mainly associated with pericentromeric heterochromatin and telomeres (Cléard et al., 1997; Delattre et al., 2000). It encodes a large protein of 1169 amino acids that contains seven atypical and widely spaced zinc finger motifs, which were shown to bind DNA in vitro (Reuter et al., 1990; Cléard et al., 1995; Cléard and Spierer, 2001). Specific binding to pericentric heterochromatin is conferred to Su(var)3-7 by its C-terminal region (Jaquet et al., 2002). Extra doses of Su(var)3-7 enhance silencing of variegating genes, whereas a decreased level of Su(var)3-7 reduces the silencing (Reuter et al., 1990). Loss of the Su(var)3-7 protein results in lethality, making it an essential protein for the fly. Interestingly, males are more sensitive to the lack of Su(var)3-7 than females, but the cause of lethality is unknown (Seum et al., 2002). Although the *Su(var)3-7* gene was identified more than ten years ago as a modifier of PEV, the exact role of its protein in heterochromatin structure is still poorly understood.

There are links between Su(var)3-7 and HP1. First the two genes show strong genetic interaction (Cléard et al., 1997). Second, the two proteins colocalise in the *Drosophila* embryos and on polytene chromosomes. Third, physical interaction between HP1 and Su(var)3-7 has been demonstrated by coimmunoprecipitation and yeast two-hybrid analysis (Cléard et al., 1997; Delattre et al., 2000). Su(var)3-7 was also shown to interact by two-hybrid interaction trapping in yeast with the histone methyltransferase Su(var)3-9 (Schotta et al., 2002), but this interaction has not been confirmed in vivo until now. Despite these indications, the mode of action of Su(var)3-7 remains elusive. To address the role of Su(var)3-7, we analysed in vivo the consequences of increased Su(var)3-7 expression on fly viability and chromatin structure.

Here, we provide evidence for a dose-dependent regulatory role of Su(var)3-7 in chromosome morphology and heterochromatin formation and demonstrate in vivo a functional link between Su(var)3-7 and the histone methyl-transferase Su(var)3-9.

## Materials and Methods

### Induction of Su(var)3-7

Heat-shocks were carried out on a *Drosophila melanogaster* line containing the inducible *Su(var)3-7* transgene *HA:FL* (lines *HA:FL1A* and *HA:FL4D*) (Jaquet et al., 2002) and on the $yw^{67}$ control line. Flies were allowed to lay eggs at 25°C for 24 hours, and embryos were incubated at 30°C for 2 days. Three heat-shocks of 15 minutes at 35°C were then performed each day from the fourth day of development until adulthood. For stronger induction, daily heat-shocks were applied from the third day of development. Other conditions of induction are noted in the text.

### Immunostaining of polytene chromosomes

Procedures for immunostaining were as described (Platero et al., 1995). Briefly, salivary glands were dissected in Cohen's buffer, fixed for 2 minutes in 2% formaldehyde, 2% Triton X-100 and then squashed in 2% formaldehyde, 45% acetic acid. These fixation conditions were used for all immunostaining except in one case, as indicated in the text, where milder fixation consisted of squashing directly in 2% formaldehyde, 45% acetic acid. Primary antibodies were used at the following dilutions: 1:10 for anti-Su(var)3-7 (Cléard et al., 1997), 1:20 for anti-H3-diMeK9 from Upstate, 1:400 for anti-HP1 (a gift of Lori Wallrath), 1:200 for anti-GAGA (a gift of Vincenzo Pirrotta) and 1:100 for anti-H3-diMeK27 (a gift of Thomas Jenuwein).

### Orcein staining of polytene chromosomes

Larvae were dissected, and salivary glands transferred and squashed in 45% acetic acid. Slides were dehydrated for at least 20 minutes in 100% ethanol and air-dried. A drop of staining solution (1% orcein in a 1:1 mix of 60% acetic acid and lactic acid) was deposited on a coverslip and applied on the polytene chromosomes. Excess staining solution was removed and the coverslip sealed with nail varnish.

## Results

### Overexpression of Su(var)3-7 protein induces lethality

To investigate the role of the chromosome-associated protein Su(var)3-7 in vivo, we examined the consequences of an increase of its expression. In order to induce high levels of Su(var)3-7, we used the *HA:FL* transgenic lines expressing the full-length Su(var)3-7 cDNA tagged with an haemagglutinin epitope (HA) (Jaquet et al., 2002). Expression of the transgene is controlled by the heat-shock *hsp70* inducible promoter, as demonstrated by western blot analysis (Jaquet et al., 2002). This transgene rescues the lethality of *Su(var)3-7* mutations (Seum et al., 2002). After heat-shock, the *HA:FL1A* or *HA:FL4D* lines overproduce a Su(var)3-7-tagged protein with the same chromatin binding properties as the heat-shock inducible non-tagged protein (Jaquet et al., 2002). We first looked at the consequences of high levels of Su(var)3-7 on the flies. At 25°C, the flies are viable and fertile. However, after three daily heat inductions of Su(var)3-7 the flies die, whereas the control flies (the $yw^{67}$ line) survive. Lethality was observed with independent *HA:FL* lines harbouring the transgene at different locations, showing that it is not due to a position effect. The stage of lethality depends on the time at which the heat-shocks start to be applied. When the young larvae are submitted to three heat-shocks per day at 35°C starting 24 hours after egg laying, individuals stop developing at the second instar larval stage, and eventually die after several days. If heat-shocks are applied at the second instar larval stage, the flies die as late third instar larvae or as pupae. Finally, if heat-shocks are performed later (at beginning of the pupal stage), some adults survive but display non-specific developmental defects like loss of macrochaetes on the thorax (Carole Seum, personal communication). In summary, induction of large amount of the heterochromatic protein Su(var)3-7 throughout development kills the flies concomitantly with perturbations of development.

### Chromosome morphology, particularly that of the male X, is profoundly affected by the dose of Su(var)3-7
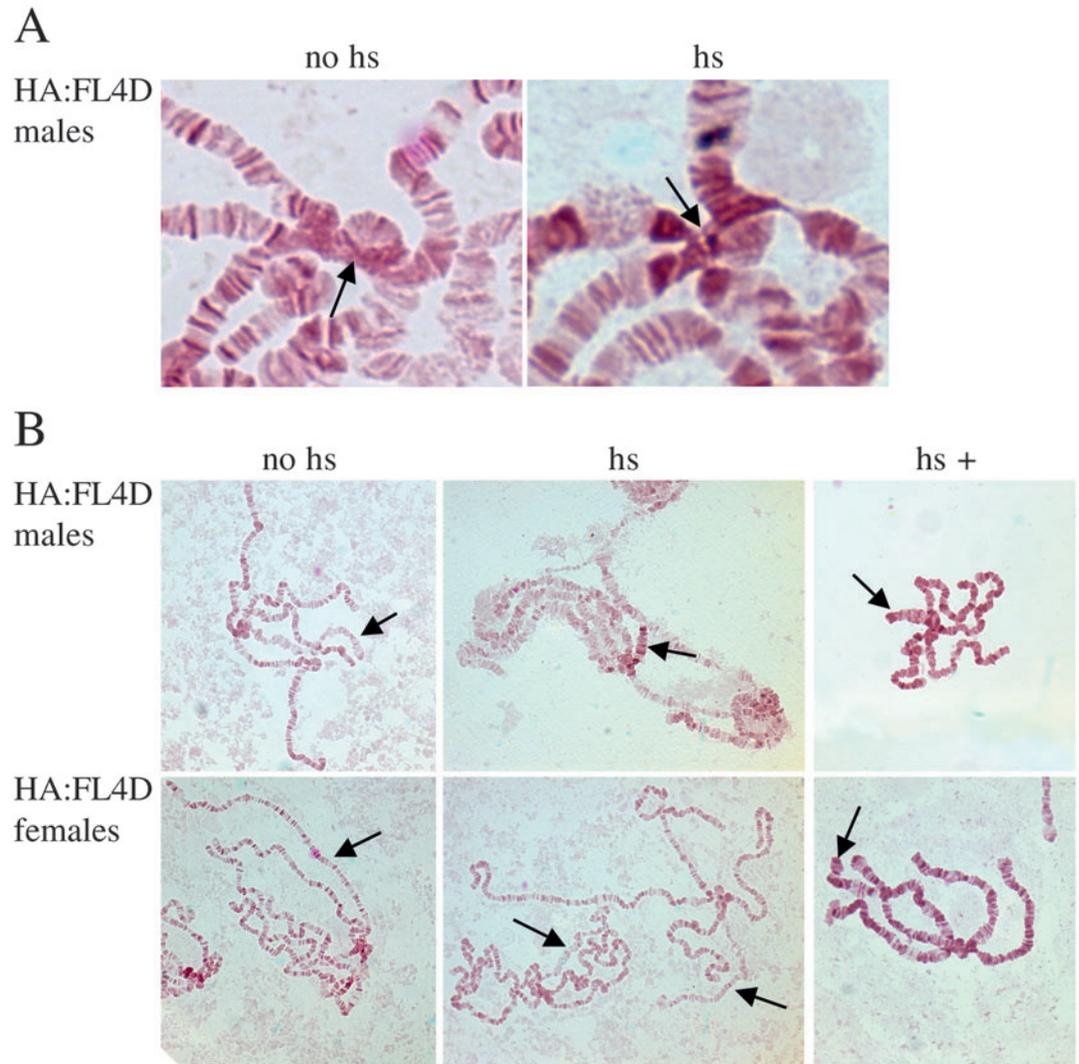
As Su(var)3-7 is a chromosome-associated protein, we

**Fig. 1.** Orcein staining of polytene chromosomes from third instar larvae. (A) Detail of the chromocentre (arrow) from *HA:FL4D* larvae after daily heat-shocks (hs) or without heat-shocks. (B) Polytene chromosomes from males and females containing a heat-inducible *Su(var)3-7* transgene. Homozygous *HA:FL4D* males and females kept at 18°C (no hs) or submitted to daily heat-shock at 35°C from the second larval instar stage (hs) or three daily heat-shocks from the first instar larval stage (hs+). Arrows indicate the X chromosome.

examined chromosome morphology in larvae expressing high level of the protein. Polytene chromosomes from *HA:FL4D* third instar larvae submitted to daily heat-shocks from the second instar larval stage were squashed and stained with orcein. High levels of Su(var)3-7 induce dramatic changes in chromosome morphology. First, in males as in females the chromocentre reproducibly appears denser when compared to non heat-shocked controls (Fig. 1A). Second, the morphology of the chromosome arms is affected: the banding pattern is altered, and more strikingly the male X chromosome becomes very small and compact (Fig. 1B). In this very spectacular phenotype, the length of the male X chromosome can be reduced by more than tenfold by increasing the expression of just one protein. Third, with stronger heat-shocks, starting from the first instar larval stage, extreme phenotypes appear (Fig. 1B): in males all the chromosomes are reduced in size, with the X always the most affected, and in females chromosomes also start to condense. These defects are never observed on non heat-shocked chromosomes, and are never observed on the control *yw⁶⁷* line submitted to the same treatment. These phenotypes are not specific to the *HA:FL4D* line: the *HA:FL1A* line, which contains the same full-length Su(var)3-7 transgene

but inserted at a different location, shows the same effects although to a lesser extent, probably due to a lower level of induction (not shown). Fourth, the male X chromosome exhibits a reproducible novel array of bands and interbands. Compaction of the chromosome is not uniform and we surmise that some regions are specifically condensed while others are not. Taken together these observations provide evidence for a dose-dependent regulatory role of Su(var)3-7 in chromosome morphology.

### Su(var)3-7 invades chromosome euchromatic arms, particularly the male X, and induces methylation of H3-K9

We next assessed the consequences of excess of Su(var)3-7 on its association with chromosomes. Immunostaining of wild-type polytene chromosomes with anti-Su(var)3-7 antibodies shows staining restricted to the chromocentre and some telomeres (Delattre et al., 2000) (Fig. 2A). Staining for Su(var)3-7 in *HA:FL4D* male and female larvae submitted to daily heat-shocks reveals however that the protein invades all the chromosomes extensively (Delattre et al., 2000) (Fig. 2A).
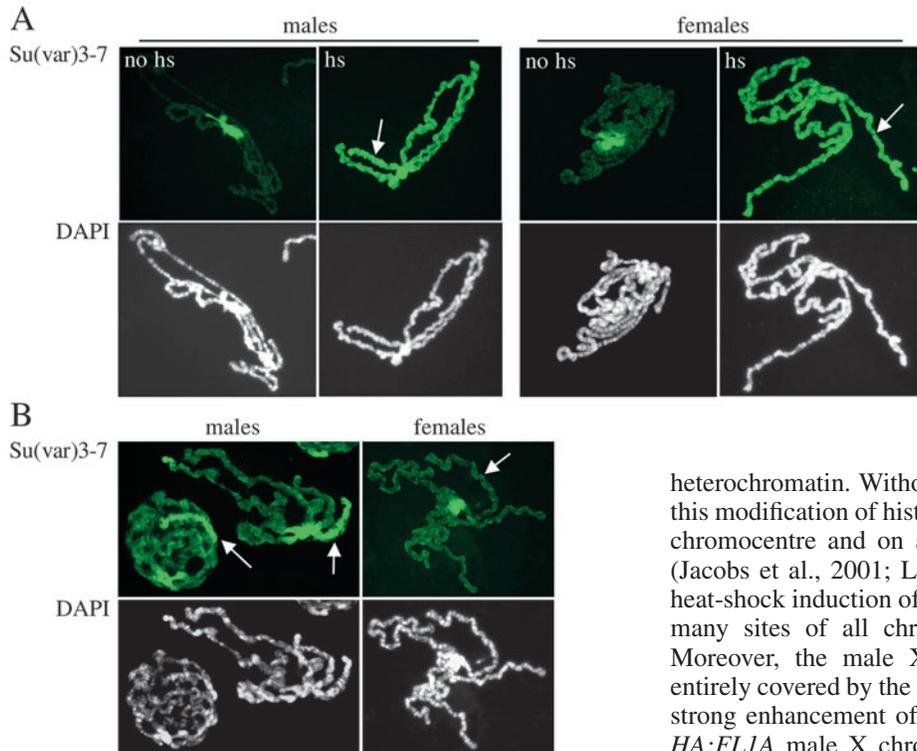
**Fig. 2.** Immunodetection of Su(var)3-7 on polytene chromosomes from male and female larvae containing the heat-inducible Su(var)3-7 transgene.
(A) Chromosomes after standard fixation. Green, anti-Su(var)3-7; white, DAPI staining. Larvae were kept at 18°C (no hs) or submitted to daily heat-shocks at 35°C (hs).
(B) Chromosomes after the same heat-shock conditions, but a milder fixation regime (see Materials and Methods). Arrows indicate the X chromosome.

Interestingly, in milder conditions of fixation, Su(var)3-7 is seen only on the chromocentre and on the compacted male X chromosome, probably revealing a stronger binding or a better affinity to these regions (Fig. 2B).

We propose that the reduction in size and the alteration of the banding pattern of the chromosomes, especially of the male X chromosome, is due to heterochromatinisation. To test this hypothesis, we stained the *HA:FL4D* chromosomes with an antibody raised against H3-diMeK9, a marker of heterochromatin. Without heat-shock induction of Su(var)3-7, this modification of histone H3 is indeed detected mainly at the chromocentre and on a few telomeres, as already described (Jacobs et al., 2001; Li et al., 2002) (Fig. 3). However, after heat-shock induction of Su(var)3-7, H3-diMeK9 is detected on many sites of all chromosomes in males and in females. Moreover, the male X chromosome is specifically almost entirely covered by the H3-diMeK9 staining (Fig. 3). The same strong enhancement of H3-diMeK9 staining is visible on the *HA:FL1A* male X chromosome even if the X is not greatly reduced in size in this line (Fig. 3).

We investigated this particular sensitivity of the male X chromosome to overproduction of Su(var)3-7. First, we verified that this effect was not specific to the X chromosome of the $yw^{67}$ fly stock containing the *HA:FL* construct by exchanging this X chromosome with the X from Canton S or $w^{1118}$ stocks. Hypermethylation of H3-diMeK9 after overexpression of *Su(var)3-7* was also observed on these two X chromosomes (not shown). We also verified that the increase of the H3-diMeK9 staining was not due to the change in the X
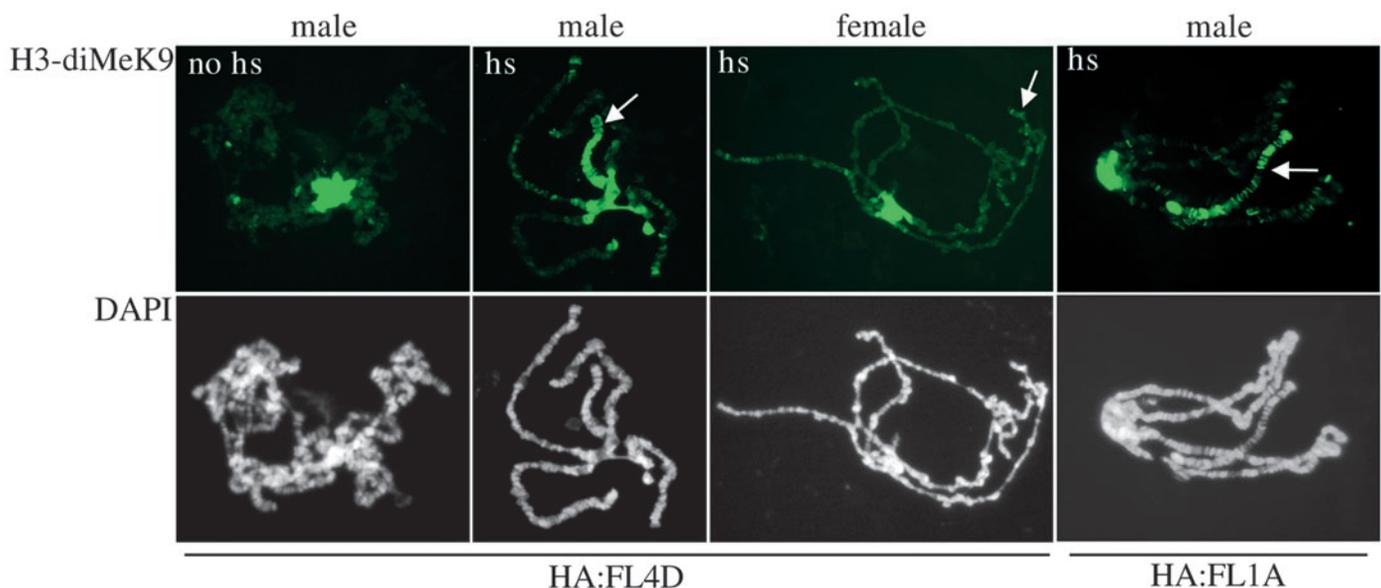


**Fig. 3.** Immunodetection of H3-diMeK9 on polytene chromosomes from larvae containing a heat-inducible *Su(var)3-7* transgene. Green, anti-dimethyl-H3K9; white, DAPI staining. Homozygous *HA:FL4D* or *HA:FL1A* male or female larvae raised at 18°C (no hs) or submitted to daily heat-shocks at 35°C (hs). Arrows indicate the X chromosome.
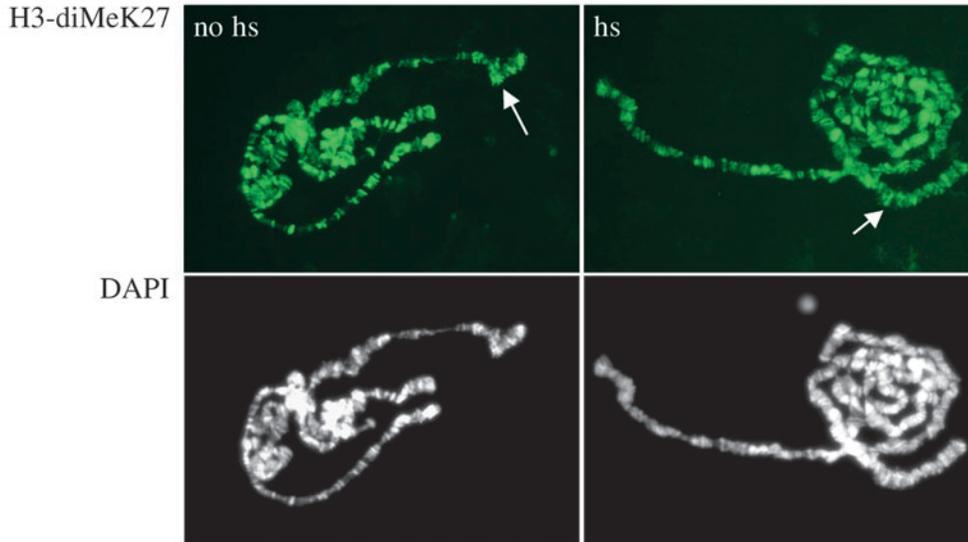
**Fig. 4.** Immunodetection of H3-diMeK27 on polytene chromosomes of homozygous *HA:FL4D* males raised at 18°C (no hs) or submitted to daily heat shocks at 35°C (hs). Arrows indicate the X chromosome.

morphology (as the chromosome becomes more compact, the staining of discrete bands scattered on the chromosome could appear continuous). We stained the chromosomes of the *HA:FL4D* line with antibodies against another modified histone, H3-diMeK27 (dimethylated on lysine 27) and with antibodies against the chromatin protein GAGA. Excess Su(var)3-7 did not increase the pattern of these antibodies specifically on the compacted X chromosome, although the general aspect of the staining is slightly modified by the change of morphology of all the chromosomes (Fig. 4 and data not shown). In conclusion, an increase in dose of Su(var)3-7 induces a heterochromatin-specific modification of histone H3

all over the chromosomes and most dramatically on the male X chromosome.

## Accumulation of Su(var)3-7 on male X chromosome induces recruitment of the HP1 protein

Knowing first that HP1 and Su(var)3-7 interact (Cléard et al., 1997; Delattre et al., 2000), second that HP1 specifically binds H3-diMeK9 (Bannister et al., 2001; Lachner et al., 2001) and third that HP1 plays a crucial role in heterochromatin formation and gene silencing (Ayyanathan et al., 2003; Li et al., 2003), we tested whether excess Su(var)3-7 modifies the
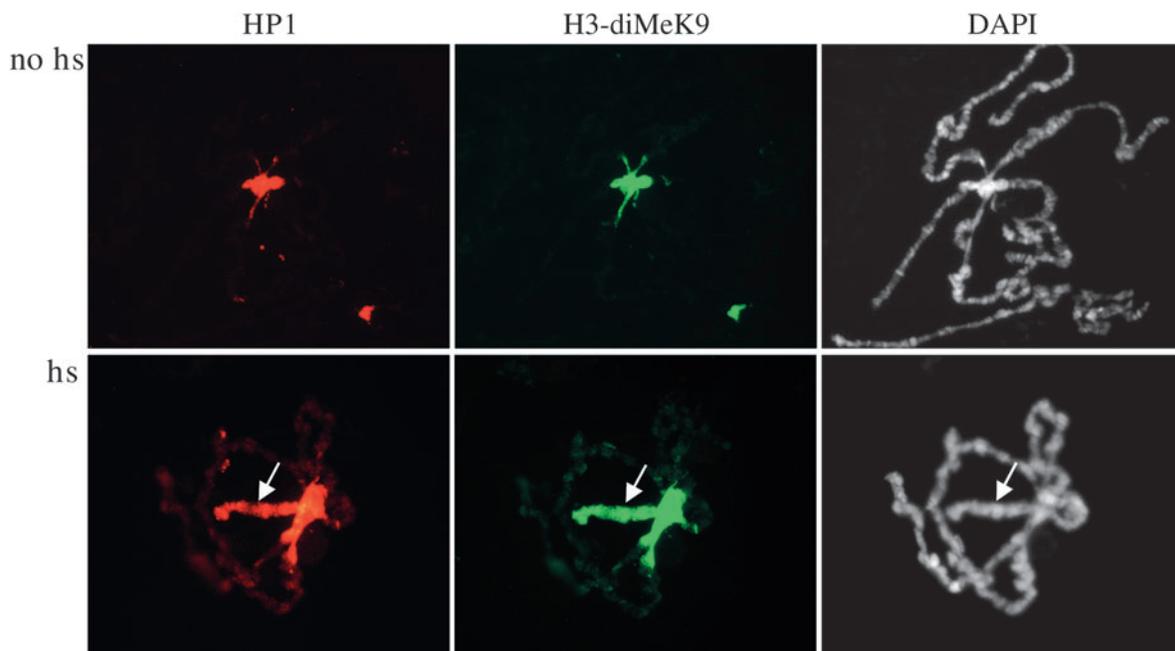


**Fig. 5.** Double-immunostaining of homozygous *HA:FL4D* male chromosomes. Antibodies against HP1 (red) and H3-diMeK9 (green) were used and corresponding DAPI staining is shown. Larvae were raised at 18°C (no hs) or submitted to daily heat-shocks at 35°C (hs). Arrows indicate the X chromosome.

localisation of HP1. Polytene chromosomes of *HA:FL4D* larvae were stained with the anti-HP1 antibody (Fig. 5). Without heat-shock induction of Su(var)3-7, HP1 associates preferentially with pericentric heterochromatin, where it colocalises with H3-diMeK9 (Fig. 5). However, with an excess of Su(var)3-7, HP1 strongly decorates the entire male X chromosome in addition to the chromocentre (Fig. 5). On autosomes, overproduction of Su(var)3-7 increases the number of sites bound by HP1 (not shown). The recruitment of HP1 on the entire male X chromosome, together with the condensation and the increase of amount of H3-diMeK9, constitutes the third mark of heterochromatin formation induced by high levels of Su(var)3-7.

### Increased methylation of H3-K9 depends on Su(var)3-9

At least four different histone methyl-transferases selectively methylate H3 at lysine 9 (Rea et al., 2000; Tachibana et al., 2002; Schultz et al., 2002; Ogawa et al., 2002). In contrast with the three others, Su(var)3-9 and its orthologues function primarily in heterochromatin. Moreover, Su(var)3-9 interacts in vivo with HP1, and interacts in the yeast two-hybrid system with Su(var)3-7 (Schotta et al., 2002). We therefore tested the requirement for Su(var)3-9 on the increased methylation of H3-K9 induced by Su(var)3-7. We constructed a line harbouring the *HA:FL1A* transgene and the *Su(var)3-9^{06}* null mutation (Tschiersch et al., 1994), both of which are homozygous. We submitted larvae from this genotype to daily heat-shocks and examined the resulting chromosome morphology and H3-diMeK9 pattern. As a control, we analysed separate *HA:FL1A* and *Su(var)3-9^{06}* genotypes. First, we observed that the morphology of the male X chromosome of *HA:FL1A;Su(var)3-9^{06}* larvae is not altered by a high dose of Su(var)3-7 whereas that of *HA:FL1A* is affected (Fig. 6 and orcein staining not shown). Second, the pattern of H3-diMeK9 in *HA:FL1A;Su(var)3-9^{06}* larvae is very similar to the *Su(var)3-9^{06}* control but dramatically different from the *HA:FL1A* larvae (Fig. 6A). H3-diMeK9 staining does not accumulate on the X chromosome and is strongly reduced on euchromatic arms and on the chromocentre, except for a reproducible bright point (Fig. 6A) (Schotta et al., 2002). In the same conditions *HA:FL1A* control chromosomes show strong accumulation of staining on the male X chromosome and over autosomes (not shown). This suggests that Su(var)3-9 is responsible not only for the methylation of H3-K9 at the chromocentre as previously reported (Schotta et al., 2002), but also for the hypermethylation of the male X chromosome and autosomes triggered by increasing expression of Su(var)3-7. Interestingly, HP1 binding is also dramatically different in *HA:FL1A; Su(var)3-9^{06}* larvae when compared with *HA:FL1A* larvae. HP1 staining is indeed strongly reduced everywhere, except for a reproducible bright point at the chromocentre (Fig. 6B). We conclude that the recruitment of HP1 by overproduced Su(var)3-7 requires functional Su(var)3-9.

The loss of histone modification observed in a *Su(var)3-9^{06}* mutant background could nevertheless result from a loss of expansion of Su(var)3-7 on these chromosomes. To test this possibility, we examined whether Su(var)3-7 binding on chromosomes was altered by the loss of Su(var)3-9. Immunostaining clearly showed that it is not the case: Su(var)3-7 is still bound to all chromosomes, in euchromatin as in heterochromatin, without the influence of *Su(var)3-9* (Fig. 6B). The ability of Su(var)3-7 to spread on chromosomes does not depend on Su(var)3-9. Interestingly, we observed that the combination of *HA:FL1A* with *Su(var)3-9^{06}* partially rescues the lethality induced by the overexpression of Su(var)3-7. Whereas all *HA:FL1A* individuals die after heat-shock, about 10% of *HA:FL1A;Su(var)3-9^{06}* flies survive to adulthood without obvious phenotype (not shown). In conclusion, these results demonstrate that Su(var)3-7 and Su(var)3-9 are partners in vivo and that effects induced by overproduced Su(var)3-7 require wild-type amounts of Su(var)3-9.

## Discussion

### Su(var)3-7 and chromosome morphology

Increased amounts of Su(var)3-7 induce striking changes in polytene chromosome morphology, underlining a role for Su(var)3-7 in the modulation of higher order chromatin structure. The compaction of the chromocentre that we observed with excess Su(var)3-7, is consistent with the preferential binding of the protein to heterochromatin. We also observed a reduction of chromosomes size and a modification of their banding pattern. Compaction of chromosomes is not
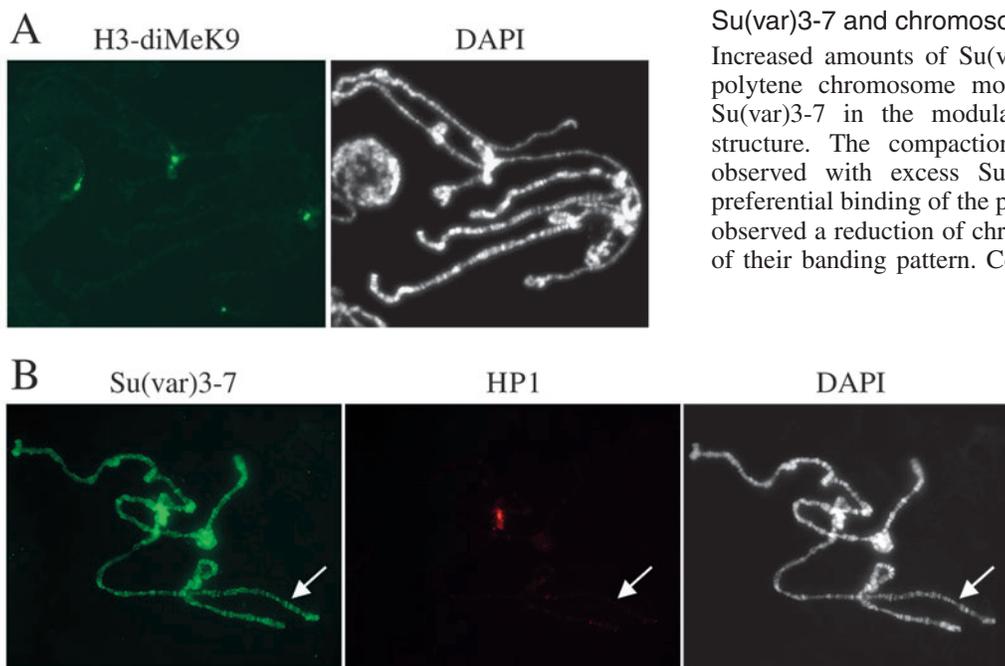


**Fig. 6.** Immunodetection of H3-diMeK9, HP1 and Su(var)3-7 in *HA:FL1A*; *Su(var)3-9^{06}* homozygous null mutant chromosomes submitted to heat-shock. (A) H3-diMeK9 staining (green) and corresponding DAPI staining. (B) Double staining with Su(var)3-7 antibodies (green) and HP1 antibodies (red) and the corresponding DAPI staining.

uniform and we surmise that some regions are specifically condensed whereas others are not. We noticed that the male X chromosome is the most affected: it is dramatically reduced in size, altered in its banding pattern, covered by H3-diMeK9 and to a lesser extent by HP1. These facts led us to the conclusion that increased amounts of Su(var)3-7 trigger progressive heterochromatinisation of the chromosomes. These new data show that the level of expression of Su(var)3-7 is critical for chromosome morphology, probably through control of the compaction level, and that the male X chromosome is especially sensitive to the dose of Su(var)3-7.

Given that an increase in the dose of Su(var)3-7 provokes ectopic heterochromatinisation, we would expect an opposite phenotype in the absence of Su(var)3-7. This is indeed the case: an extensive analysis of previously described Su(var)3-7 mutations (Seum et al., 2002) revealed that loss of the protein results in decondensation of the male X chromosome (A.S., M.D., C.S. and P.S., in preparation). In addition, we have noticed that males suffer more than females from the lack of Su(var)3-7: the rescue of the lethality of Su(var)3-7 mutations by Su(var)3-7 transgenes was better in females than in males (Seum et al., 2002). This observation is consistent with a particular susceptibility of the male X chromosome to the lack of Su(var)3-7. Decreased and increased levels of Su(var)3-7 have opposite phenotypes on the male X chromosome, showing that the effects described here are specific and that Su(var)3-7 plays a particular role, directly or indirectly, on the morphology of the male X chromosome.

The reason for the particular susceptibility of the male X chromosome in response to change of Su(var)3-7 dose is not yet clear. The main difference between the male X and other chromosomes is its interaction with the dosage compensation complex. In *Drosophila*, equalisation of X-linked gene expression between males and females is accomplished by a twofold hyper-transcription of most genes on the male X chromosome (reviewed by Akhtar et al., 2003). A complex made of male-specific proteins and RNAs binds to the male X and modifies histone tails. This unique chromatin environment may render the male X chromosome more accessible to Su(var)3-7, or increase the affinity of the protein to it. We are currently exploring the possibility of an interaction between Su(var)3-7 and the dosage compensation complex.

## Spreading of Su(var)3-7 on euchromatin depends on its expression level

The increase of Su(var)3-7 levels reveals a new dimension to the previously described specificity of Su(var)3-7 binding. The protein is detected not only at the chromocentre but also on euchromatic arms in males as in females. These data suggest that in wild-type conditions, the heterochromatin restricted binding of Su(var)3-7 needs a precise control of the level of its expression. The dose-dependent capacity of Su(var)3-7 to spread all over chromosomes is reminiscent of the dose-dependent effect of Su(var)3-7 on PEV: the loss of a gene results in a strong suppression of variegation, whereas extra doses of the gene progressively enhance silencing of genes in the vicinity of heterochromatin (Reuter et al., 1990). The capacity of Su(var)3-7 to invade euchromatin might result from its general affinity for DNA (Cléard and Spierer, 2001). When in excess, and without excesses of its heterochromatic partners like HP1, Su(var)3-7 could escape the heterochromatic complex and invade the rest of the chromosome only because of its affinity for DNA. This invasion property is not purely random as Su(var)3-7 shows preferential association with the male X chromosome. The non-monotonous compaction of euchromatin also reveals that Su(var)3-7 finds sites of higher affinity on euchromatic arms, and we suggest that it could expand from these sites as it does in position-effect variegation.

## Su(var)3-7 and Su(var)3-9 interact in vivo

We first observed that an excess of Su(var)3-7 increases the number of sites on the chromosomes containing the H3-diMeK9, especially on the male X chromosome. Then we provided genetic evidence that the histone methyl-transferase leading to this increase is Su(var)3-9. In addition, we determined that the lethality induced by high levels of Su(var)3-7 is partially dependent on wild-type dose of Su(var)3-9. This is consistent with the fact that the suppressor effect of *Su(var)3-9* loss of function is epistatic to the triplo-enhancer effect of *Su(var)3-7* on PEV (Schotta et al., 2002). These pieces of evidence suggest that the function of Su(var)3-7 in silencing requires the wild-type activity of *Su(var)3-9*. Su(var)3-7 is able to expand the site of action of the histone H3 methyl-transferase, normally mainly restricted to the chromocentre. Conversely, expansion of Su(var)3-7 binding is not dependent on Su(var)3-9. Our study shows for the first time, evidence that Su(var)3-7 and Su(var)3-9 truly interact in vivo. This validates the interaction observed between the two proteins in the yeast two-hybrid system (Schotta et al., 2002). Although Su(var)3-7 produced in excess is able to cover all chromosomes, methylation of H3-K9 occurs only at a number of loci on autosomes and almost uniformly on the male X chromosome. We conclude that ectopic methylation of H3K9 by increased level of Su(var)3-7 is dependent on Su(var)3-9 but appears only in some particular chromatin context.

## Excess Su(var)3-7 recruits HP1 specifically to the male X chromosome

We know that Su(var)3-7 interacts with HP1 (Cléard et al., 1997; Delattre et al., 2000), that Su(var)3-9 also interacts with HP1 (Aargard et al., 1999; Schotta et al., 2002) and that Su(var)3-7 interacts with Su(var)3-9 according to two-hybrid data (Schotta et al., 2002). The next question is how does Su(var)3-7 recruit the two others? We have determined that in the absence of Su(var)3-9 methyl-transferase, HP1 cannot be recruited on the X chromosome by overproduction of Su(var)3-7. This implies that H3-K9 methylation is a prerequisite for HP1 recruitment. Hence, we propose that Su(var)3-7 recruits first Su(var)3-9 on the male X chromosome and then HP1. Accumulation of H3-diMeK9 and HP1 on the male X chromosome is consistent with the finding that methylation of H3-K9 provides binding sites for HP1 (Bannister et al., 2001; Lachner et al., 2001; Jacobs et al., 2001). It is interesting to note that new sites of H3-diMeK9 on autosomes and on the female X chromosomes do not efficiently recruit HP1 (within the limits of detection allowed by the technique). There might not be enough endogenous HP1 for recruitment at all new H3-diMeK9 sites. Alternatively, a certain amount of H3-diMeK9 may be necessary to efficiently recruit HP1, or the presence of

H3-diMeK9 sites is not sufficient to recruit and maintain HP1 on chromosomes. A special chromatin environment existing on the male X chromosome seems critical for efficient HP1 targeting. Interestingly, Greil et al. (Greil et al., 2003) have shown that HP1 and Su(var)3-9 can form different complexes and bind independently of each other at distinct sets of genes. Hence, access of HP1 to H3-diMeK9 may be blocked at some loci, owing to the chromatin environment or competition with other H3-MeK9-binding proteins. Our work reveals the existence of two types of heterochromatic complexes established on euchromatin: a complex made of Su(var)3-7, HP1 and H3-diMeK9 on the male X chromosome and a complex made of only Su(var)3-7 and H3-diMeK9 at some euchromatic loci on autosomes.

In summary, we have shown that the level of expression of Su(var)3-7 is critical for fly viability and integrity of chromosome morphology, that increased expression of Su(var)3-7 alone is sufficient to trigger heterochromatin formation by recruitment of its heterochromatic partners and that the male X chromosome is particularly sensitive to the dose of the protein. It will be interesting to determine which chromatin environment is prerequisite for efficient recruitment of Su(var)3-9 and HP1 by Su(var)3-7. The male X chromosome is of special interest given its particular chromatin environment and we are currently examining a potential link between the dosage compensation complex and Su(var)3-7.

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