

Sumoylation of *Drosophila* SU(VAR)3-7 is required for its heterochromatic function

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ABSTRACT

In *Drosophila*, SU(VAR)3-7 is an essential heterochromatin component. It is required for proper chromatin condensation, and changing its dose modifies position-effect variegation. Sumoylation is a post-translational modification shown to play a role in diverse biological processes. Here, we demonstrate that sumoylation is essential for proper heterochromatin function in *Drosophila* through modification of SU(VAR)3-7. Indeed, SU(VAR)3-7 is sumoylated at lysine K839; this modification is required for localization of SU(VAR)3-7 at pericentric heterochromatin, chromosome 4, and telomeres. In addition, sumoylation of SU(VAR)3-7 is a prerequisite for its ability to enhance position-effect variegation. Thus, these results show that the heterochromatic function of SU(VAR)3-7 depends on its own sumoylation, and unveil a role for sumoylation in *Drosophila* heterochromatin.

INTRODUCTION

Heterochromatin represents the condensed part of chromosomes, characterized by a high content of repetitive elements and low gene density. In *Drosophila*, it is found mainly in pericentric regions, at telomeres and chromosome 4 (1). SU(VAR)3-7 is a key component of *Drosophila* heterochromatin essential for chromosome structure and heterochromatin formation (2–4). The *Su(var)3-7* gene was historically identified in genetic screens, together with *Su(var)3-9* and *Su(var)2-5* [encoding respectively SU(VAR)3-9 and heterochromatin protein 1 (HP1)], as haplo-suppressors and triplo-enhancers of position-effect variegation (PEV) [(5) and references in (6)]. PEV reflects the mosaic and stochastic heterochromatin-induced silencing of genes located in the vicinity of heterochromatin. Loss of one dose of *Su(var)3-7*, *Su(var)3-9*, or *Su(var)2-5*, suppresses the

genomic silencing of variegating genes, while a triple dose enhances it. *Su(var)3-7*, *Su(var)3-9* and *Su(var)2-5* were thereafter shown to encode key structural and enzymatic components of heterochromatin (6,7).

The product of the *Su(var)3-7* gene, SU(VAR)3-7, is composed of seven N-terminal zinc finger domains with high affinity for DNA, and of the C-terminal Bess- and BoxA-motifs involved in self-interaction and in specific heterochromatin association (5,8,9). SU(VAR)3-7 localizes mainly at pericentric heterochromatin, together with SU(VAR)3-9 and HP1 (1,10). SU(VAR)3-9 is the heterochromatic histone H3 lysine 9 (H3K9) methyltransferase (KMTase) (also referred to KMT1), that dimethylates and trimethylates H3K9 at pericentric heterochromatin and at the core of the chromocenter, respectively (11–13). In this way, it forms a docking site for the recruitment of HP1 through its chromodomain (14–16). SU(VAR)3-7 interacts genetically and physically with SU(VAR)3-9 and HP1 (2,10,13,17), and its presence at heterochromatin is HP1-dependent (4). Moreover, SU(VAR)3-7 and HP1 associate with telomeres, chromosome 4, and with a few euchromatic sites among which the well characterized region 31 of chromosome 2 (10,17–20). At these positions, H3K9 methylation is present, but deposited by other H3K9 KMTases, DmSETDB1 at chromosome 4 (21), and possibly G9a at euchromatic regions (22). Consistent with its role in heterochromatin formation, overexpression of SU(VAR)3-7 leads to further condensation, and absence of it to decondensation of chromatin, most strikingly at the chromocenter and the male X chromosome (2,4), the effect on the male X chromosome being due to an interaction between heterochromatin and dosage compensation (23).

Sumoylation is an essential post-translational modification, featuring a SUMO (small ubiquitin-related modifier) molecule (Smt3 in *Drosophila*) that is covalently conjugated to a protein substrate (for reviews on sumoylation, see for example (24,25)). A growing number of proteins were shown to be dependent on sumoylation for proper regulation, and sumoylation plays a role in

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several biological processes, such as for instance cell survival and proliferation, transcriptional regulation, intracellular trafficking, and maintenance of telomeric and nuclear organization (25–27). The sumoylation reaction cascade is similar to ubiquitination, involving the E1-activating enzyme (Aos1/Uba2 heterodimer in *Drosophila*), the E2-conjugating enzyme (Ubc9, encoded by *lwr* in *Drosophila*), and several E3-ligases mediating substrate specificity. The SUMO molecule is attached to the ϵ -NH₂ group of lysine (K) residues usually located within the Ψ KxE (where Ψ is a large hydrophobic amino acid) consensus target site (28). This modification is dynamic and reversible, since SUMO molecule can be cleaved by specific proteases (29). Sumoylation can have several functional outcomes; it can alter the activity of a protein, change its sub-cellular localization, or create a new platform for interaction with another protein (25,26). Indeed, the SUMO moiety is recognized by partners bearing a SUMO-interacting motif (SIM) (30). Cross-talk with other post-translational modifications, such as phosphorylation (31), adds an additional level of regulation to these modifications. In *Drosophila*, sumoylation has been involved in embryogenesis, wing morphogenesis, central nervous system development, immune system (32), Ras signaling, cell cycle control, and embryonic patterning (33). Several lines of evidence also implicate sumoylation in certain aspects of chromosome regulation, namely *lwr* (encoding the E2-conjugating Ubc9 enzyme) in the dissociation of heterochromatic regions of homologs at the end of the meiotic prophase (34), SUMO in the negative regulation of the activity of the *gypsy* chromatin insulator (35), and the SU(VAR)2-10 E3-ligase in the establishment and maintenance of chromosome organization in interphase (36). Given this diverse array of functions, it appears reasonable to assume that sumoylation could also be involved in additional, yet undiscovered biological processes in *Drosophila*.

Here, we show that sumoylation is required for heterochromatin function in *Drosophila*, through the modification of SU(VAR)3-7. We demonstrate that SU(VAR)3-7 is sumoylated, and identify the target lysine. We also show that sumoylation is necessary for proper heterochromatic localization of SU(VAR)3-7, and for its ability to enhance PEV. Thus, these results reveal a role for sumoylation in *Drosophila* heterochromatin, mediated by SU(VAR)3-7 modification.

MATERIALS AND METHODS

Drosophila lines and generation of transgenic lines

The *Su(var)3-7^{R2a8}* allele was described in ref. (4). The *lwr⁴⁻³* and *lwr⁵* alleles were described in (34) and were a gift from Soichi Tanda. *In(1)wm4h* and Heidi were described in (37) and (38). *daGal4.w-* (Bloomington stock #8641) was a gift from J.M. Dura. For the generation of the transgenic lines, *3HA-Su(var)3-7(WT)* and (*K839R*) variant coding sequences were cloned in the C4yellowUAST vector. Constructs were injected into *yw* embryos with the pUChs π delta-2-3 plasmid at a 3:1 ratio.

Transgenic flies were selected with the *yellow* marker. Expression was induced by the *daGal4* or *daGal4.w-* driver.

DNA constructs

For *Su(var)3-7*, the cDNA encoding amino acids 82–1250 was cloned downstream of EGFP, and the cDNA encoding amino acids 1–1250 was cloned downstream of the 3HA tag. Point mutations were generated by PCR. SUMO and ubiquitin cDNAs were cloned downstream of the 3HA. For expression in S2 cells, constructs were cloned in the pMT vector. pMT was generated from pMT/V5-HisB (Invitrogen), where the V5-His6 tag was removed by BstBI-PmeI double digestion, blunting and self-ligation. All constructs were verified by sequencing. Cloning details are available upon request.

Cell culture and transfection

S2 cells were cultivated in Schneider's *Drosophila* medium (Invitrogen) supplemented with 1 \times Penicillin–Streptomycin–Glutamin and 10% heat inactivated fetal calf serum (Invitrogen). For each transfection, 950 ng of linearized expression construct(s) (in the pMT vector) were cotransfected with 50 ng of linearized pCoblast (Invitrogen) using the FuGene6 reagent (Roche). Twenty four hours after transfection, integrative transfectants were selected with 25 μ g/ml blasticidin for at least 5 days. After selection, expression of the cDNA constructs was induced for 3 days with 50 μ M CuSO₄.

Immunoprecipitation

Proteins were extracted from fresh S2 cell pellets or from frozen canton S embryos with 150 mM NaCl, 50 mM Tris pH 7.4, 1 mM EDTA, 1% NP40, 0.25% sodium deoxycholate, 1 mM PMSF, 1 mM NEM, and anti proteases (complete, Roche). Rabbit antibodies directed against SU(VAR)3-7 [20 μ l Ab212 (10) or 20 μ l Ab1399] were preincubated with 25 μ l protein G agarose (Roche) in 150 mM NaCl, 50 mM Tris pH 7.4, 1 mM EDTA, 0.1% NP40, 1 mM PMSF, 1 mM NEM, anti proteases (complete, Roche). An amount of 500 μ g extract were incubated with the beads-antibody complex in 1 ml final 150 mM NaCl, 50 mM Tris pH 7.4, 1 mM EDTA, 0.1% NP40, 1 mM PMSF, 1 mM NEM, anti proteases (complete, Roche) for 2 h at 4°C on a wheel. Beads were washed three times with the same buffer, and the one-tenth of the immunoprecipitated material was analyzed by western blot.

Western blot analysis

Proteins from cultured cells were extracted from frozen pellets with 150 mM NaCl, 50 mM Tris pH 7.4, 1 mM EDTA, 1% NP40, 0.25% sodium deoxycholate, 1 mM PMSF, and anti proteases (complete, Roche). Protein extracts from brain, salivary glands and imaginal discs of third instar larvae were prepared as described (21). Western blot analyses were performed as described (21), with the following primary antibodies: α -HA (MMS-101R, Covance, 1/2000), α -GFP (11814460001,

Roche, 1/1000), α -alpha-Tubulin (T9026, Sigma, 1/5000), α -SU(VAR)3-7 Ab212 (10) (1/750) or Ab1399 (1/750), α -SUMO (1/2000) (a gift from A.J. Courey).

Eye pigment measurement

Each measure was done as a triplicate. Three days old adults were frozen. Ten heads were collected, resuspended in 50 μ l 30% ethanol pH 1.5, and squashed. An amount of 950 μ l 30% ethanol pH 1.5 were added and samples were incubated in 24-wells plates O/N at 37°C to extract pigments. Samples were centrifuged, the supernatants were collected, and the pigments values were measured with a spectrophotometer at OD 480 nm.

Immunostaining of polytene chromosomes and whole mount salivary glands

Polytene chromosomes immunostainings were performed as described in (21). Slides were hybridized with α -HA (MMS-101R, Covance, 1/200) and α -HP1 (a gift from L. Wallrath, 1/100) as primary antibodies. For immunostaining of whole mounts, salivary glands were dissected in PBS, fixed for 20 min in PBS 4% formaldehyde, washed two times with PBT (PBS, 0.5% Tween-20), washed six times with BBT (PBS, 0.5% TritonX-100, 1% BSA), incubated overnight at 4°C in BBT with the α -HA (MMS-101R, Covance), washed six times with BBT, incubated 2 h at RT with the secondary antibody (Alexa Fluor 555 goat anti-mouse IgG No A21422, Invitrogen), washed two times with BBT, three times with PBT, incubated 5' with DAPI in PBT, washed once with PBT, and mounted in Vectashield (Vector Laboratories). Confocal images of whole mount salivary glands were taken with a Leica TCS SP2 AOBS microscope.

RESULTS

SU(VAR)3-7 is modified by SUMO at lysine K839

The first indication that SU(VAR)3-7 is post-translationally modified comes from western blot analysis. Indeed, tagged versions of SU(VAR)3-7 expressed from a cDNA transfected in S2 cells or from a cDNA transgene appear as a major band at approximately 200 kDa, with an additional band of significant higher molecular weight (see e.g. Figures 1A and G). The upper band is not due to phosphorylation of SU(VAR)3-7, as it is resistant to phosphatase treatment (data not shown). Two other post-translational modifications were then considered, namely sumoylation and ubiquitination. To address this issue, a GFP-tagged version of amino acids 82–1250 of SU(VAR)3-7 (*GFP-Su(var)3-7*) was co-expressed in S2 cells with 3HA-tagged SUMO (3HA-SUMO) or with 3HA-tagged ubiquitin (3HA-ubi). These exploratory experiments were performed with a truncated yet functional protein (39). Indeed, when this work was initiated, the 81 amino acid N-terminal extension of SU(VAR)3-7 was not yet known. For this reason, the results were subsequently confirmed with the full-length protein (Figure 1F). If SU(VAR)3-7 were to be SUMO or ubiquitin modified, part of the upper band

should increase in size due to the 3HA-tag carried by the co-transfected 3HA-SUMO or 3HA-ubi. Figure 1A effectively shows that GFP-SU(VAR)3-7 displays an additional third band above the doublet when co-expressed with 3HA-SUMO, but not with 3HA-ubi, suggesting that SU(VAR)3-7 is subjected to sumoylation. To strengthen this further, the experiment was repeated in the presence of the SUMO isopeptidase Ulp1 (40). Upon co-expression of Ulp1, the SUMO and 3HA-SUMO modified forms of SU(VAR)3-7 disappear, further confirming that SU(VAR)3-7 is sumoylated (Figure 1B). We next addressed whether the endogenously expressed SU(VAR)3-7 was also sumoylated. Therefore, endogenous SU(VAR)3-7 protein was immunoprecipitated from S2 cells, and analyzed by western blot with antibodies specific for SU(VAR)3-7 or SUMO. Figure 1C shows that SU(VAR)3-7 displays one band corresponding to the non-modified form (square), and one band of higher molecular weight corresponding to the sumoylated SU(VAR)3-7 (circle). The identity of these two bands was confirmed by parallel analysis of an extract from cells overexpressing SU(VAR)3-7 (data not shown). In addition, two bands of even higher molecular weight are present (stars). The three upper bands, but not the one corresponding to the non-sumoylated SU(VAR)3-7, are recognized by the α -SUMO (Figure 1C). Similar results were obtained with another SU(VAR)3-7 antibody or with proteins extracted from embryos (Supplementary Figure S1). Thus, SU(VAR)3-7 can accommodate several SUMO molecules, probably when expressed at low level (see 'Discussion' section). In summary, three lines of evidence show that SU(VAR)3-7 is sumoylated: (i) the band corresponding to sumoylated SU(VAR)3-7 is shifted upon co-expression of 3HA-SUMO, (ii) the modified forms disappear in presence of the SUMO isopeptidase Ulp1, and (iii) the modified forms of SU(VAR)3-7 are recognized by an α -SUMO.

SUMO molecules are covalently attached to lysines, according to the Ψ KxE/D consensus (28). The SUMOplot™ Analysis Program (<http://www.abgent.com/tool/sumoplot>) predicts and scores sumoylation sites in any protein based on the presence of the SUMO consensus sequence. When the full-length SU(VAR)3-7 protein sequence (amino acids 1–1250) was analyzed with this program, the lysine 269 (K269) displayed the highest score. K269 was mutated into an arginine in *GFP-Su(var)3-7 (GFP-Su(var)3-7(K269R))* and this construct was transfected into S2 cells, with or without the 3HA-SUMO. Western blot analysis shows that the SUMO-modification is still present on GFP-SU(VAR)3-7(K269R) (Figure 1D). We then mutated the three next lysines displaying the highest scores in the SUMOplot™ analysis into arginines, namely K813, K1056 and K982. Mutations were done in a cumulative manner, because loss of a major sumoylation site could lead to sumoylation at secondary minor sites. Sumoylation still occurs on the protein carrying all four substitutions (data not shown). Four additional lysines were then cumulatively mutated (K134, K254, K508, K727), resulting into a molecule with eight lysines replaced by arginines (named 8KR), and again

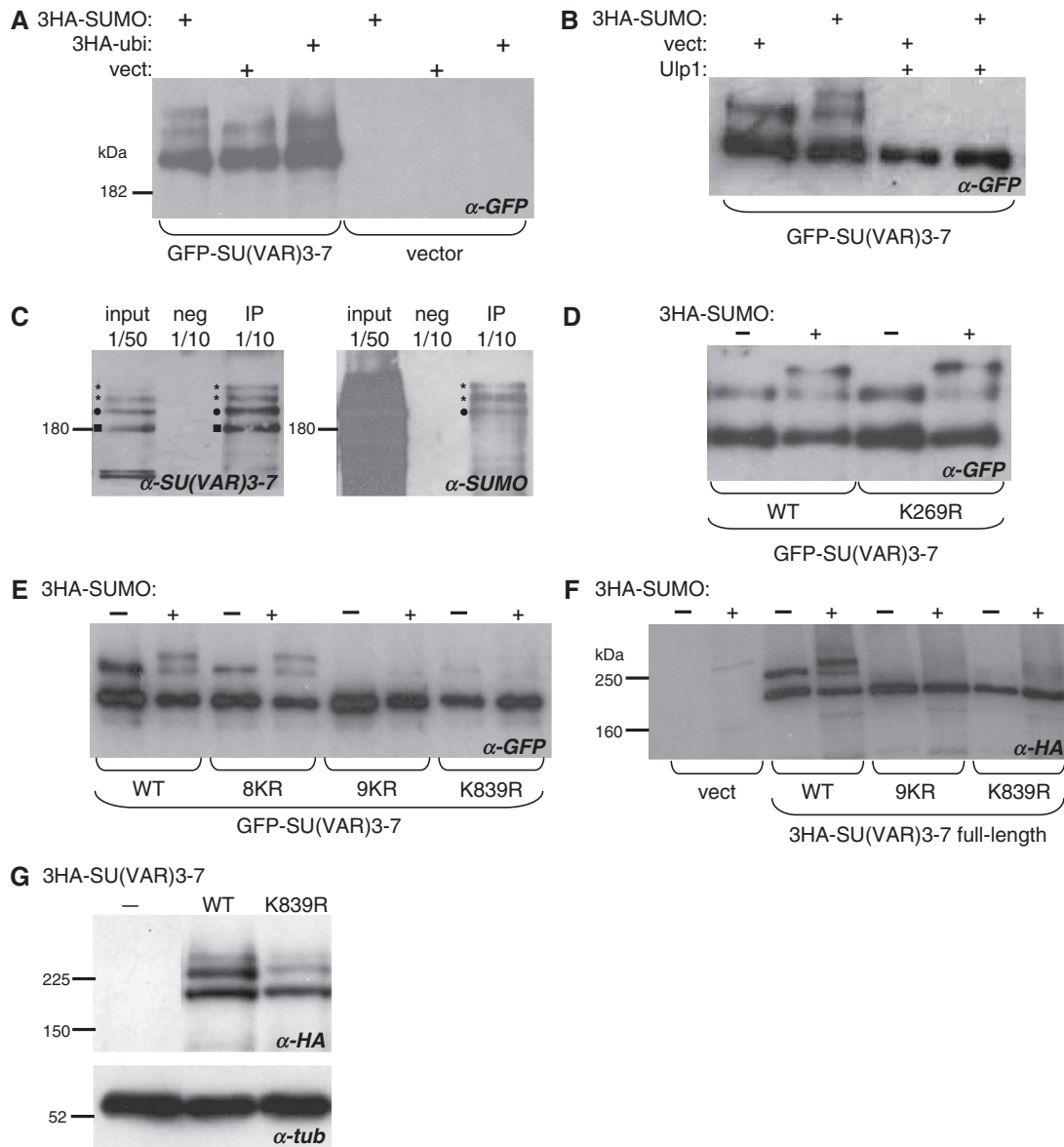


Figure 1. SU(VAR)3-7 is sumoylated at lysine K839. (A) Sumoylation of SU(VAR)3-7. A *GFP-Su(var)3-7* construct (amino acids 82–1250) or the empty vector (vect) were transfected in S2 cells together with the empty vector, 3HA-SUMO, or 3HA-ubi. Western blot analysis with an α -GFP shows the different forms of GFP-SU(VAR)3-7, namely a doublet when expressed alone, and an additional band when co-expressed with 3HA-SUMO. 3HA-SUMO and 3HA-ubi are both expressed (data not shown). (B) Profile of SU(VAR)3-7 upon co-expression of Ulp1. GFP-SU(VAR)3-7 was expressed with 3HA-SUMO or the empty vector, and with or without Ulp1. DNA amount was kept constant in transfections with empty vector. Proteins were analyzed as in A. (C) Sumoylation of the endogenous SU(VAR)3-7 protein. Total proteins were extracted from S2 cells and endogenous SU(VAR)3-7 was immunoprecipitated from 500 μ g total extract with α -SU(VAR)3-7 Ab1399. One-fiftieth of the total extract (input), one-tenth of the negative control IP without antibody (neg) and one-tenth of the IP (IP) were loaded twice and analyzed by western blot with α -SU(VAR)3-7 Ab1399 or α -SUMO. The identity of the bands was verified by running in parallel extract from cells overexpressing SU(VAR)3-7 (data not shown). Square: non-modified SU(VAR)3-7. Circle: sumoylated SU(VAR)3-7. Stars: SU(VAR)3-7 with possibly several molecules of SUMO or other additional post-translational modifications (see discussion). (D) Sumoylation of the K269R mutated form. GFP-SU(VAR)3-7(WT) or (K269R) expressed with the empty vector or 3HA-SUMO were analyzed as in A. (E) Lack of sumoylation in the SU(VAR)3-7(K839R) mutant. GFP-SU(VAR)3-7(WT), with eight or nine K to R substitutions (8KR or 9KR), or with the K839R substitution, were expressed with 3HA-SUMO or the empty vector. Proteins were analyzed as in A. (F) Absence of sumoylation in the 3HA-tagged full-length SU(VAR)3-7 bearing the K839R substitution. *3HA-Su(var)3-7* full-length (amino acids 1–1250) constructs, namely WT, 9KR and K839R mutant forms, or the empty vector, were expressed in S2 cells together with the empty vector or 3HA-SUMO. Proteins were analyzed as in A., but with α -HA. (G) Expression of 3HA-SU(VAR)3-7(WT) and (K839R) in transgenic lines. Western blot analysis on brain, salivary glands, and imaginal discs (dissected together) protein extracts of control, 3HA-SU(VAR)3-7(WT) or (K839R) expressing third instar larvae. Membranes were cut and probed with α -HA and α -tubulin. Genotypes are the following: lane 1: y^-w^- ; $P\{daGal4.w^-\}/+$. Lane 2: y^-w^- ; $P\{UAST-3HA-Su(var)3-7(WT); y^+\}/+$; $P\{daGal4.w^-\}/+$. Lane 3: y^-w^- ; $P\{UAST-3HA-Su(var)3-7(K839R); y^+\}/+$; $P\{daGal4.w^-\}/+$.

SU(VAR)3-7 retained sumoylation (Figure 1E). Mutation of a ninth lysine, K839, resulted into a molecule (9KR) that could not be sumoylated anymore (Figure 1E). The K839R substitution, when present in an otherwise wild-type background, recapitulates the same finding, since the resulting protein is not sumoylated (Figure 1E). Given that the single K839R change abolishes sumoylation, we conclude that K839 is the main lysine modified by SUMO in SU(VAR)3-7. As mentioned, the full-length protein is 1250 amino acids long [sequence can be found in GenBank (AAF54918) or (09)]. An initially non characterized in frame ATG is located 243-bp upstream of the second ATG (in the same exon), resulting into a protein with an 81 amino acid N-terminal extension. Thus, the results were confirmed with full-length 3HA-tagged SU(VAR)3-7 constructs. Similarly as the truncated form, full-length SU(VAR)3-7 is sumoylated at position K839 (Figure 1F). Full-length 3HA-*Su(var)3-7* constructs were used henceforth for functional *in vivo* studies.

Sumoylation of SU(VAR)3-7 is required for localization at heterochromatin

SU(VAR)3-7 is a heterochromatic protein associated with pericentric heterochromatin, chromosome 4, region 31 and telomeres (4,10,17). We therefore asked whether sumoylation is required for its proper localization. To this end, we generated transgenic lines with full length 3HA-*Su(var)3-7*(WT) or (K839R) placed downstream of the UAS element. The transgenes were expressed with the *daGal4* or the *daGal4.w⁻* driver. *In vivo*, the non modified 3HA-SU(VAR)3-7 wild-type and mutant proteins are expressed at similar levels (Figure 1G). The wild-type form displays a SUMO-modified upper band that is almost as intense as the non-modified form, as well as a faint band of higher molecular weight possibly corresponding to a protein carrying two SUMO molecules (Figure 1G). In a parallel experiment, the SU(VAR)3-7 protein expressed from the previously published *HA:FL* transgenic line (39) was reanalyzed; this construct also produces SU(VAR)3-7 modified forms (Supplementary Figure S2). The 3HA-SU(VAR)3-7(K839R) generates two upper bands, both of them much weaker than the wild-type. Thus, sumoylation of the 3HA-SU(VAR)3-7(K839R) is strongly reduced (Figure 1G); the residual signal could represent sumoylation at alternative sites in absence of the major site, or could reflect other post-translational modifications.

The transgenic lines were further used to localize 3HA-SU(VAR)3-7(WT) and (K839R) in whole mount salivary glands and on chromatin. These experiments were performed in the *Su(var)3-7^{R2a8}* deletion null mutant background to avoid potential homo-dimerization with the endogenous wild-type protein. Whole mount salivary glands of third instar larvae stained with an HA antibody show that both wild-type and mutant proteins are expressed and present in the nucleus (Figure 2A, first lane). The pictures were then taken at higher magnification with a confocal microscope. The 3HA-SU(VAR)3-7(WT) protein preferentially localizes

at chromatin dense regions and at some distinct bands, a pattern reminiscent of polytene chromosomes (see below). In contrast, 3HA-SU(VAR)3-7(K839R) shows no preferential chromatin association (Figure 2A, second lane). In conclusion, analysis of whole mount salivary glands shows that the non-sumoylable SU(VAR)3-7 variant, despite its nuclear localization, does not bind to chromatin dense regions similarly as the wild-type form.

On polytene chromosomes, 3HA-SU(VAR)3-7(WT) localizes at the chromocenter, telomeres, chromosome 4 (Figure 2B) and region 31 (data not shown), as described for the endogenous protein. Similarly, HP1 also localizes properly, namely at the chromocenter, telomeres, chromosome 4 and region 31 (Figure 2B). However, staining of polytene chromosome of larvae expressing 3HA-SU(VAR)3-7(K839R) revealed that the non-sumoylable protein does not co-localize with chromatin dense regions (Figure 2C). Co-staining of these polytene chromosomes with an HP1 antibody demonstrated a similar localization as in the wild-type (Figure 2C). The observed pattern for 3HA-SU(VAR)3-7(K839R) is identical in male and female larvae (Supplementary Figure S2). Taken together, these data show that SU(VAR)3-7 localization at pericentric heterochromatin, telomeres, chromosome 4 and region 31 requires post-translational modification by SUMO at lysine K839. On the other hand, sumoylation of SU(VAR)3-7 is not required for proper localization of HP1.

Sumoylation of SU(VAR)3-7 is required for enhancement of PEV

One of the main features of *Su(var)3-7* is its ability to influence PEV (6). We therefore asked next whether this property was dependent on its sumoylation. Two PEV reporter lines were used, the *In(1)w^{m4h}* line, where an inversion relocates the endogenous *white* gene next to centromeric heterochromatin, and the Heidi line (38), which has a *P* element containing the *white* gene relocated near centromeric heterochromatin of chromosome 2. Before testing the role of sumoylation of SU(VAR)3-7 in PEV, we first analyzed whether sumoylation *per se* was involved in this phenomenon. In other words, we tested whether loss of one dose of the ubiquitin-E2 ligase encoded in *Drosophila* by the *lesswright* (*lwr*) gene influences PEV. Two alleles of the *lwr* gene were used, *lwr⁴⁻³* and *lwr⁵*; both are recessive lethal and UAS-*lwr* transgene can rescue the lethality (34). In *In(1)w^{m4h}* and Heidi lines, the variegating *white* gene is more expressed in the *lwr* heterozygous mutant than in *lwr+* homozygous wild-type background (Figure 3A, left part and data not shown). This argues in favor of a role for sumoylation in the repression of these variegating genes. The same experiment was performed with the *In(1)w^{m4h}* line, in the presence of an additional copy of *Su(var)3-7* [with the T21A line (5)]. Due to this additional dose of *Su(var)3-7*, PEV is enhanced in the *lwr+* wild-type background. This is not the case in the *lwr* heterozygous mutant backgrounds (Figure 3A, right part), again underscoring that sumoylation plays a role in PEV.

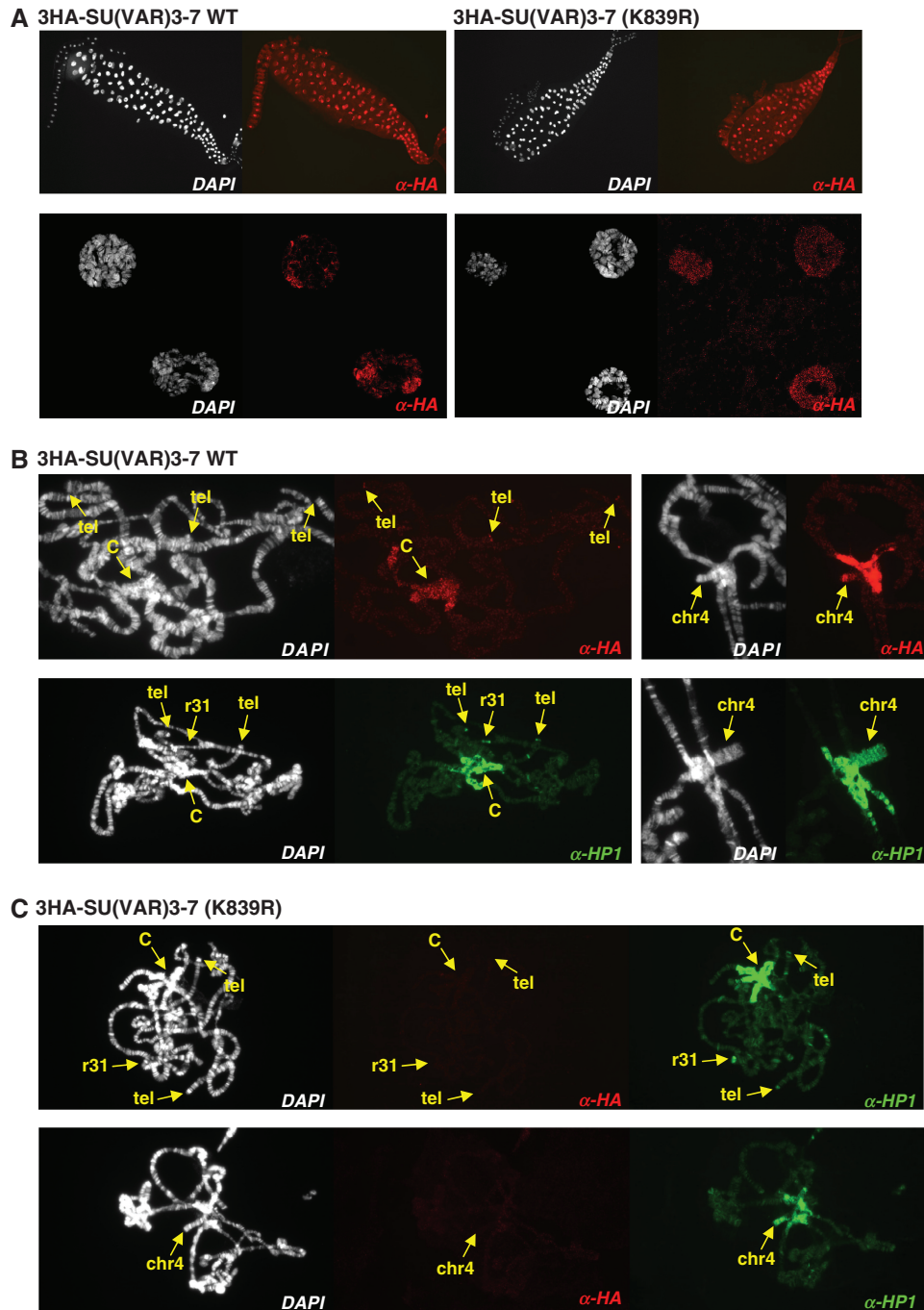


Figure 2. Sumoylation of SU(VAR)3-7 is required for localization at heterochromatin. (A) Expression and cellular localization of 3HA-SU(VAR)3-7(WT) and (K839R) proteins. Whole mount salivary glands of $y^{-}w^{-}; P\{UAST-3HA-Su(var)3-7(WT)or(K839R); y^{+}\}/P\{UAST-3HA-Su(var)3-7(WT)or(K839R); y^{+}\}; Su(var)3-7^{R2a8}/P\{daGal4; w^{+}\} Su(var)3-7^{R2a8}$ third instar larvae were stained with α -HA. First lane shows whole salivary glands, second lane enlargements of the nuclei (pictures taken on a confocal microscope). (B) Localization of 3HA-SU(VAR)3-7(WT) and HP1 on chromatin. Polytene chromosomes of larvae expressing 3HA-SU(VAR)3-7(WT) were stained with α -HA or α -HP1. DNA was stained with DAPI. Genotypes are the same as in Figure 2A. 'C' stands for chromocenter, 'tel' for telomere, 'r31' for region 31, and 'chr4' for Chromosome 4. (C) Localization of 3HA-SU(VAR)3-7(K839R) and HP1 on chromatin. Polytene chromosomes of larvae expressing 3HA-SU(VAR)3-7(K839R) were stained with α -HA and α -HP1 together. Genotypes and arrows codes are the same as in Figure 2A and B.

We next undertook to look specifically at the role of sumoylation of SU(VAR)3-7 itself in PEV. We compared the variegated phenotype of the Heidi line, in the presence of an excess of 3HA-SU(VAR)3-7(WT) or (K839R). Overexpression of the wild-type protein, but

not of the K839R non-sumoylable variant, enhances variegation in males and females (Figure 3B), demonstrating that the property of SU(VAR)3-7 to enhance variegation requires its own sumoylation. Taken together, these results show that the previously described

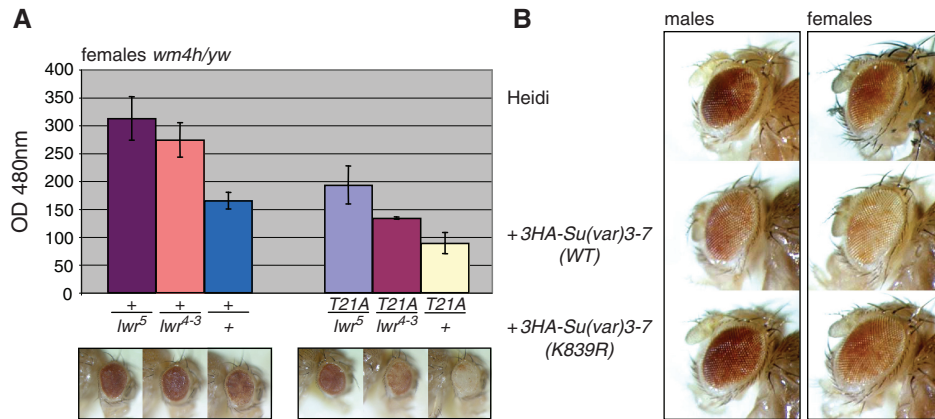


Figure 3. (A) Sumoylation is required for PEV. Repression of the variegating *white* gene in the $In(1)w^{m4h}$ line is relieved in the *lwr* heterozygous mutant background (left part). Data are shown for the females; the same trend is observed in males, as well as in the *Heidi* line (data not shown). The repression of the variegating *white* gene in the presence of an additional dose of *Su(var)3-7* (*T21A* transgene) is also relieved in the *lwr* heterozygous mutant background (right part). Pictures were taken and quantification of pigments was done 3 days after eclosion. The scale corresponds to the absorption at OD 480 nm. Genotypes, from left to right: 1) $In(1)w^{m4h} / y^{-w^{-}}; + / lwr^5 FRT40A$. 2) $In(1)w^{m4h} / y^{-w^{-}}; + / lwr^{4-3} FRT40A$. 3) $In(1)w^{m4h} / y^{-w^{-}}; + / +$. 4) $In(1)w^{m4h} / y^{-w^{-}}; T21A / lwr^5 FRT40A$. 5) $In(1)w^{m4h} / y^{-w^{-}}; T21A / lwr^{4-3} FRT40A$. 6) $In(1)w^{m4h} / y^{-w^{-}}; T21A / +$. (B) Sumoylation of SU(VAR)3-7 is required for PEV. The variegating *white* transgene in the *Heidi* line is further repressed upon expression of 3HA-Su(var)3-7(WT), but not upon expression of 3HA-Su(var)3-7(K839R). Pictures were taken 2 days after eclosion. The eye color phenotype was constant from fly to fly. Genotypes, from top: (i) $y^{-w^{-}}; Heidi / +; P\{daGal4.w^{-}\} / +$. (ii) $y^{-w^{-}}; Heidi / P\{UAST-3HA-Su(var)3-7(WT); y^{+}\}; P\{daGal4.w^{-}\} / +$. (iii) $y^{-w^{-}}; Heidi / P\{UAST-3HA-Su(var)3-7(K839R); y^{+}\}; P\{daGal4.w^{-}\} / +$.

functions and characteristics of SU(VAR)3-7, namely heterochromatic localization and ability to enhance PEV, rely on the post-translational modification of SUMO on lysine at position K839.

DISCUSSION

Our data show that sumoylation of SU(VAR)3-7 is required for its proper heterochromatic function in *Drosophila*. Indeed, sumoylation of SU(VAR)3-7 at lysine K839 is a prerequisite for heterochromatin localization, and for the ability of SU(VAR)3-7 to enhance PEV. To our knowledge, the specific requirement of sumoylation for the function of a heterochromatic protein in *Drosophila* had not been demonstrated before. Thus, in addition to its role in regulating multiple biological processes, sumoylation also plays a role in *Drosophila* heterochromatin.

SUMO modification of SU(VAR)3-7 was demonstrated by four approaches: (i) the shift of the modified form upon co-expression of 3HA-SUMO (Figure 1A), (ii) the disappearance of the sumoylated form upon overexpression of the SUMO isopeptidase Ulp1 (Figure 1B), (iii) the SUMO modification of the endogenously expressed SU(VAR)3-7 (Figure 1C), and iv) the identification of the lysine where the SUMO molecule is attached to (Figure 1E and F). The SU(VAR)3-7 proteins expressed from constructs transfected into S2 cells (*GFP-Su(var)3-7*, *3HA-Su(var)3-7*) always give the same pattern in western blot analysis, namely the SU(VAR)3-7 non-modified form, and one additional band corresponding to the sumoylated form (see, e.g. Figure 1A and F). A third (and fourth, depending on the experiment) band of higher molecular weight is detected in the *HA:FL* (Supplementary Figure S2) and *3HA-Su(var)3-7* (Figure

1G) transgenics lines, or with the endogenous protein in S2 cells (Figure 1C, Supplementary Figure S1A). We propose that these bands correspond to SU(VAR)3-7 modified by two (or three) SUMO molecules. This hypothesis is supported by immunoprecipitation experiments (Figure 1C). The band corresponding to SU(VAR)3-7 carrying one SUMO molecule shows higher intensity than the band with potentially two SUMO molecules when detected with the α -SU(VAR)3-7. These two bands show the same intensity when revealed by the α -SUMO, potentially due to two SUMO molecules. We surmise that those additional forms of SU(VAR)3-7 were not detected in transfectants, because the SU(VAR)3-7 protein is expressed at higher level, and only one SUMO molecule would be attached to a given SU(VAR)3-7 protein by its specific SUMO E3-ligase. Nevertheless, the major sumoylation site of SU(VAR)3-7 is the K839 position; its mutation prevents or strongly reduces the presence of all SUMO-modified SU(VAR)3-7 forms (Figures 1E–G), and impairs the function of the protein. It would be interesting—in a future study—to determine if those additional SUMO molecules are bound in chain to the main SUMO at position K839, or whether they are attached to other lysines that would be secondary minor sites.

The SU(VAR)3-7 sumoylation target sequence IK839VF is located in the middle of the seventh zinc finger of the protein. This particular sequence is conserved in *Drosophila pseudobscura* (*Dpse*), and highly similar (IKLF) in *Drosophila virilis* (*Dv*) *Su(var)3-7* orthologs (9). The seventh zinc finger of SU(VAR)3-7 is the most conserved part of the protein displaying 100% similarity (9), reinforcing the functional importance of this domain (see below) and of sumoylation at position K839. Sumoylated lysines are usually embedded in the Ψ KxEx/D consensus. The consensus surrounding lysine K839 is quite surprising, containing a phenylalanine (F) instead of

a glutamic (E) or an aspartic (D) acid at the fourth position. Such a variant was not found among several sumoylation targets listed (28). Based solely on the consensus sequence, the lysine K269 was a better candidate, as it lies in the IK269HE environment, it is also conserved in *Dpse*, and highly similar (VKHE) in *Dv* orthologs (9). Nevertheless, functional data show that K839 and not K269 is the main sumoylation target in SU(VAR)3-7 (Figures 1D and E). Recently, an early *Drosophila* embryonic ‘SUMO-ome’ (a catalog of sumoylated proteins) was established, and SU(VAR)3-7 was identified, among 144 proteins, as a sumoylation substrate (33). Interestingly, this study also revealed that ‘epigenetic regulation’ is one of the six enriched functional groups of sumoylation substrates.

Sumoylation of SU(VAR)3-7 is required for localization at the chromocenter, telomeres, chromosome 4, and region 31 (Figure 2A and B). It should be noted that in conditions where the polytene chromosomes are fixed more strongly, some non-specific staining appears on the chromosomes, for both the WT and the K839R forms, most likely due to the fact that the proteins are present in the nucleus, overexpressed, and contain several zinc fingers. The impairment in chromatin binding of the SU(VAR)3-7(K839R) protein is not due to a defect in nuclear import, as it is present in the nuclei of whole mount salivary glands. This experiment also shows that in intact tissue—in contrary to the wild-type form—the 3HA-SU(VAR)3-7(K839R) protein displays no preferential binding to the DAPI dense regions (Figure 2A). Since SU(VAR)3-7 can homodimerize (39), localization experiments were performed in a *Su(var)3-7* mutant background. However, localization of SU(VAR)3-7(K839R) was also assessed in *Su(var)3-7* wild-type larvae. In this context, the mutant form gives a signal at the chromocenter, although not as strong as the SU(VAR)3-7(WT) (Supplementary Figure S4). This argues in favor of a conserved capacity of the SU(VAR)3-7(K839R) protein to at least partially interact with the wild-type form. Some truncated forms of SU(VAR)3-7 were shown to have a dominant negative phenotype (39); the SU(VAR)3-7(K839R) form does not behave in such a way, as its overexpression does not suppress variegation (Figure 3B). The absence of a dominant negative phenotype of the 3HA-SU(VAR)3-7(K839R) protein could be due to a weaker association with the wild-type SU(VAR)3-7. In addition, although it can associate with the wild-type protein, the non-sumoylable SU(VAR)3-7 might not delocalize it from chromatin and therefore not prevent its function.

The N-terminal part of SU(VAR)3-7 is composed of seven widely spaced zinc fingers (8) which have affinity for DNA in a non-heterochromatin-specific manner (39,41). The C-terminal part contains BESS- and BoxA-motifs, involved respectively in self-interaction and in interaction with other heterochromatic partners (9,39). Interestingly, while the C-terminal part of SU(VAR)3-7 binds to heterochromatin only if endogenous SU(VAR)3-7 is present, probably through homodimerization, addition of the seventh zinc finger containing the K839 sumoylation site or of the sixth zinc

finger together with the seventh zinc finger mutated for the two cysteines but leaving the sumoylation site intact, suffices for proper localization at pericentric heterochromatin, even in the absence of the wild-type protein (9). Thus, together with the data presented here, we propose that the ability for SU(VAR)3-7 to be sumoylated in its seventh zinc finger is critical for its specific binding to heterochromatin. The fact that sumoylation of SU(VAR)3-7 is required for enhancement of PEV (Figure 3A and B) is coherent with the observation that the mutant protein does not co-localize with chromatin. Moreover, in the *lwr* mutant background we observe a suppression of variegation (Figure 3A), reinforcing our view on the role of sumoylation for PEV. SU(VAR)3-7 is a substrate for sumoylation, but we cannot rule out that other factors involved in PEV are modified by SUMO.

Taken together, these data decipher the importance of sumoylation for heterochromatin function in *Drosophila*. Interestingly, a similar mechanism was put forward in *Saccharomyces pombe*, where Ubc9 sumoylates Swi6 and Clr4 [the homologs of HP1 and SU(VAR)3-9]; SUMO modification of those two factors is required for the maintenance and silencing of heterochromatin (42). We assessed whether *Drosophila* HP1 or SU(VAR)3-9 were modified by SUMO, but could not detect even traces of SUMO-modified forms (data not shown). An independent recent study confirmed that HP1 is not a sumoylation substrate (33). Thus, sumoylation plays a role in heterochromatin function, and this key modification is carried by HP1 and SU(VAR)3-9 homologs in *Saccharomyces pombe*, and by SU(VAR)3-7 in *Drosophila*. As a model, we propose that the SUMO modification of SU(VAR)3-7 forms a new platform mediating interactions with for instance repetitive heterochromatic DNA-sequences, or with a heterochromatin-bound protein, for example HP1 or SU(VAR)3-9. In such a context, forming heterochromatin would recruit sumoylated SU(VAR)3-7 that would in turn contribute to the condensed state.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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