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Physical and functional association of SU(VAR)3-9 and HDAC1 in *Drosophila*

Birgit Czermin, Gunnar Schotta[1], Bastian B. Hülsmann, Alexander Brehm, Peter B. Becker, Gunter Reuter[1] & Axel Imhof+

Adolf-Butenandt Institut, Molekularbiologie, Ludwig-Maximilians Universität, Schillerstrasse 44, D-80336 München and [1]Institut für Genetik, Martin Luther Universität Halle Biologicum, Weinbergweg 10, D-06120 Halle, Germany

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Modification of histones can have a dramatic impact on chromatin structure and function. Acetylation of lysines within the N-terminal tail of the histone octamer marks transcriptionally active regions of the genome whereas deacetylation seems to play a role in transcriptional silencing. Recently, the methylation of the histone tails has also been shown to be important for transcriptional regulation and chromosome structure. Here we show by immunoaffinity purification that two activities important for chromatin-mediated gene silencing, the histone methyltransferase SU(VAR)3-9 and the histone deacetylase HDAC1, associate *in vivo*. The two activities cooperate to methylate pre-acetylated histones. Both enzymes are modifiers of position effect variegation and interact genetically in flies. We suggest a model in which the concerted histone deacetylation and methylation by a SU(VAR)3-9/HDAC1-containing complex leads to a permanent silencing of transcription in particular areas of the genome.

INTRODUCTION

The packaging of DNA into chromatin plays a very important role during establishment and maintenance of stable gene expression patterns. Post-translational modifications of the nucleosome have a major regulatory function during this process by generating an epigenetic code (Grant *et al.*, 1998b; Strahl and Allis, 2000; Imhof and Becker, 2001). Acetylation of lysine residues within the N-terminal histone tails is associated with transcriptionally active regions within the genome and plays a causal role during the activation process (Wade *et al.*, 1997; Kuo and Allis, 1998; Mizzen and Allis, 1998). Conversely the removal of acetyl groups from the histones has a negative effect on gene expression in multiple model systems (Zhang *et al.*, 1997, 1999; Brehm *et al.*, 1998; Jones *et al.*, 1998; Wade *et al.*, 1999). The recent identification of the first lysine-specific histone methyltransferase (HIM) as the heterochromatin-associated protein SUV39H1 (Rea *et al.*, 2000) led to the hypothesis that histone methylation might also play a role during gene silencing. SUV39H1 selectively methylates lysine 9 within the H3 N-terminus, which can then serve as a docking site for another well characterized heterochromatin-associated protein, HP1 (Bannister *et al.*, 2001; Lachner *et al.*, 2001), connecting lysine 9 methylation with transcriptional repression.

A way of studying transcriptional silencing *in vivo* is the analysis of position effect variegation (PEV) in flies (Spofford, 1967; Henikoff, 1990; Reuter and Spierer, 1992). In extensive screens for modifiers of this phenomenon mutant alleles of the methyltransferase *Su(var)3-9* as well as the deacetylase *HDAC1* have been isolated as potent suppressors of PEV (Tschiersch *et al.*, 1994; Mottus *et al.*, 2000). This suggests a functional role for these histone-modifying enzymes during the establishment and/or maintenance of functional chromatin domains.

Here we report the isolation of SU(VAR)3-9 from *Drosophila* embryo extracts. We show that SU(VAR)3-9 isolated from embryos is associated with HIM as well as histone deacetylase (HDAC) activity. We show that this deacetylase activity is due to the association of SU(VAR)3-9 with HDAC1. The deacetylase activity is essential for methylation of a pre-acetylated peptide. This functional synergism between HDAC1 and SU(VAR)3-9 is also observed *in vivo* as a dominant-negative *HDAC1* mutant effectively represses a triplo-enhancer effect of *Su(var)3-9* on PEV. Recently, a genetic interaction between the methyltransferase *clr4* and the deacetylase *clr3* in *Schizosaccharomyces pombe* has been reported (Nakayama *et al.*, 2001). It thus seems likely that a comparable interaction between a HIM and a HDAC

+Corresponding author. Tel: +49 89 5996 435; Fax: +49 89 5996 425; E-mail: axel.imhof@mol-bio.med.uni-muenchen.de

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also exists in *S. pombe* and that an evolutionarily conserved deacetylation/methylation reaction serves to establish a specific epigenetic mark for heterochromatin formation.

RESULTS

Recombinant SUV39H1 has intrinsic HIM activity (Rea *et al.*, 2000). However, little is known about SU(VAR)3-9 *in vivo* and whether it acts as a single molecule or within a macromolecular assembly analogous to the acetyltransferase complexes SAGA and NuA4 (Grant *et al.*, 1998a; Allard *et al.*, 1999). In order to study this we fractionated nuclear extracts from *Drosophila* embryos (0–12 h after egg laying) (Figure 1A) prepared from a fly strain expressing a myc-tagged version of SU(VAR)3-9 (Aagaard *et al.*, 1999). In this strain the *Su(var)3-9* gene is expressed under the control of a heat shock promoter and can be switched on by a simple heat shock pulse (Aagaard *et al.*, 1999). Myc-tagged SU(VAR)3-9 binds to a resource Q column and can be eluted at a salt concentration between 150 and 250 mM KCl (Figure 1A and B). The anti-myc antibody immunoprecipitates HIM activity from a nuclear extract made from the tagged fly strain but not from wild-type flies (Figure 1C) and from active column fractions containing myc-SU(VAR)3-9 (Figure 1C).

To analyse the lysine specificity of immunoprecipitated SU(VAR)3-9 we used peptide substrates resembling the N-terminus of histone H3 that were either unmodified or premodified at specific amino acid residues (Figure 2A). SU(VAR)3-9 isolated from embryonic extracts could methylate a peptide premethylated at lysine 4 but was unable to methylate a peptide methylated at lysine 9, which matches the lysine specificity observed with recombinant SUV39H1 (Rea *et al.*, 2000) (Figure 2A). However, a peptide acetylated on lysine 9 could be methylated by immunoprecipitated SU(VAR)3-9, but not by recombinant SUV39H1 (Figure 2A). In order to find out whether this effect was due to a deacetylase activity associated with SU(VAR)3-9 we repeated the assay in the presence of a specific HDAC inhibitor, Trichostatin A (TSA). These experiments showed that TSA significantly reduces the observed HIM activity of SU(VAR)3-9 on a peptide acetylated at lysine 9 (Figure 2B). This inhibition by TSA suggests that the deacetylation of lysine 9 is indeed a prerequisite for a subsequent methylation of lysine 9 by SU(VAR)3-9.

Based on these observations we analysed the immunoprecipitated SU(VAR)3-9 for HDAC activity (Figure 3A). With the anti-myc antibody, we could indeed recover HDAC activity from extracts and partially purified SU(VAR)3-9-containing fractions prepared from the tagged fly strain but not from wild-type flies (Figure 3A). This HDAC activity is probably due to the presence of HDAC1 in these fractions as we could detect HDAC1 in the immunopurified material but not HDAC3 (Figure 3B). We cannot eliminate, however, the possibility that other HDACs different from HDAC1 and 3 contribute to the observed HDAC activity, but as we have identified HDAC1 as being associated with SU(VAR)3-9 we decided to further investigate this association. Neither recombinant HDAC1 nor SUV39H1 has a dual activity in our standard methyltransferase and deacetylase assays (Figure 3C), therefore we could exclude the possibility that a single polypeptide is responsible for the observed effects.

To further confirm the interaction between HDAC1 and SU(VAR)3-9, we used an anti-HDAC1 antibody to immunoprecipitate HIM activity from the partially purified SU(VAR)3-9 fractions mentioned above (Figure 3D). Independent evidence for an interaction of HDAC and HIM activities was obtained by using a cell line that was stably transfected with flag-HDAC1 and expresses it to high levels. Immunoprecipitation with anti-FLAG antibodies precipitated HDAC1 and, in addition to a strong HDAC activity, a pronounced HIM activity (Figure 3E). Although these experiments suggest a physical interaction between SU(VAR)3-9 and HDAC1 we were unable to show a direct interaction using GST pull-down assays (data not shown). Therefore, we suggest the presence of at least one additional factor bridging between SU(VAR)3-9 and HDAC1. Interestingly we have partially purified a very similar activity from extracts prepared from wild-type flies, which further confirms the observed interaction and argues against the possibility that the association we see is artificial due to an overexpression of SU(VAR)3-9 (B. Czermin *et al.*, manuscript in preparation).

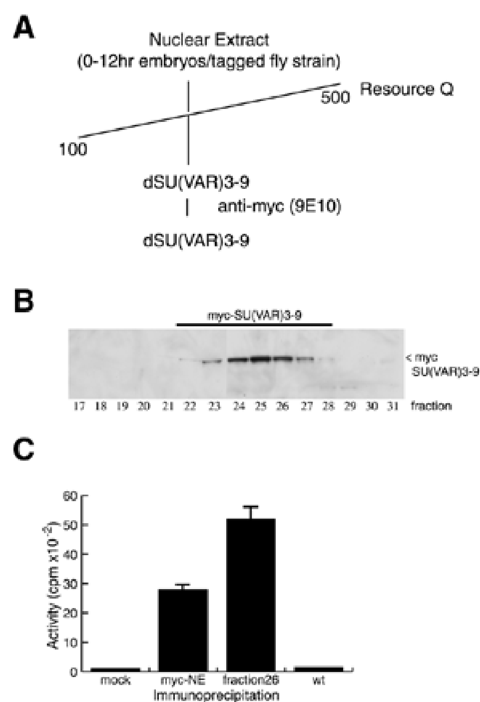

Fig. 1. Purification of a HIM from *Drosophila* embryo extracts. (**A**) Purification scheme for the purification of myc-tagged SU(VAR)3-9 from *Drosophila* embryonal extracts. (**B**) Purification of myc-SU(VAR)3-9 from nuclear extracts prepared from flies expressing a myc-tagged SU(VAR)3-9 (Aagaard, 1999) on a resource Q column. Western blot of fractions eluted from the resource Q column probed with an anti-c-myc antibody (9E10). (**C**) HIM activity assay with immunoprecipitates of SU(VAR)3-9 from an extract (myc-NE) or from partially purified myc-SU(VAR)3-9 fractions (fraction 26) prepared from flies expressing triple-myc-tagged SU(VAR)3-9 using an anti-myc antibody (9E10). As controls immunoprecipitations were also performed without the addition of antibody (mock) or from an extract prepared from wild-type embryos (wt).

To study the functional interaction of HDAC1 and SU(VAR)3-9 *in vivo* we made use of the well characterized PEV tester strain *In(1)w^m4h^* (Reuter *et al.*, 1986), which contains an inversion placing the white marker gene adjacent to pericentric X heterochromatin. In this experimental setting extra copies of *Su(var)3-9*

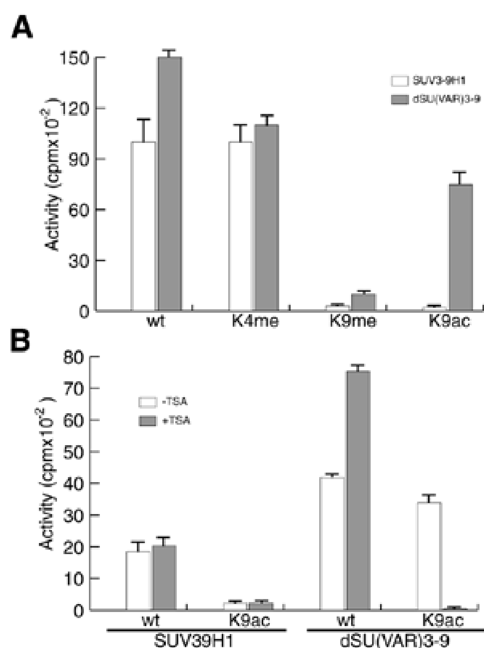

Fig. 2. Lysine specificity of purified SU(VAR)3-9. (**A**) Specificity assay of recombinant SUV3-9H1 (white bars) and SU(VAR)3-9 prepared as described in Figure 1A (dark bars) on various modified peptides. Modifications used were unmodified (wt), methylated on lysine 4 (K4me), methylated on lysine 9 (K9me) and acetylated on lysine 9 (K9ac). (**B**) Reactions were performed as in (A) with the exception that reactions contained either 50 nM Trichostatin A (+TSA) dissolved in ethanol or only ethanol (–TSA).

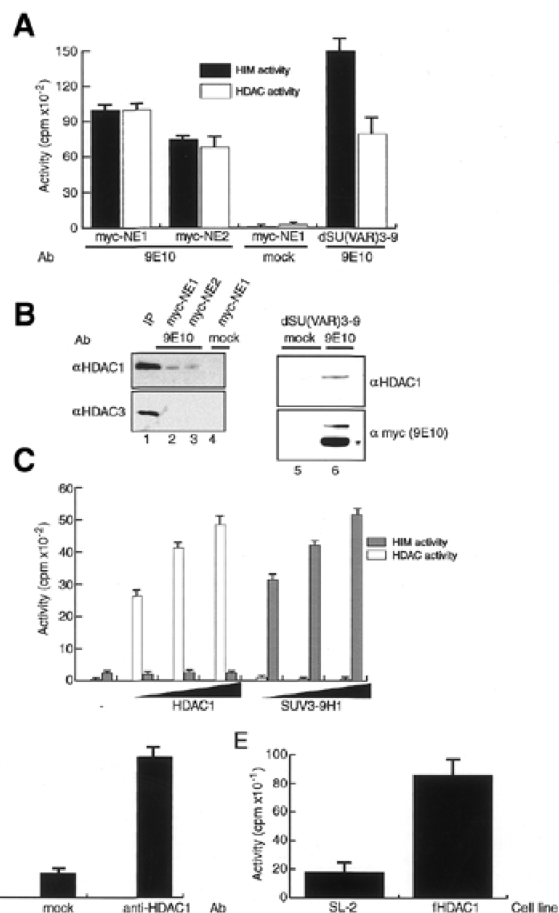

Fig. 3. Association of SU(VAR)3-9 with HDAC1. (**A**) HDAC activity assay using immunoprecipitated material from two independently prepared nuclear extracts (myc-NE1 and myc-NE2) or a partially purified fraction (fraction 26) from the tagged fly strain precipitated with an anti-c-myc antibody (9E10) or no antibody (mock). (**B**) Western blot of immunoprecipitated material from (A) probed either with anti-HDAC1 (top panel), anti-HDAC3 antibodies (bottom panel, lanes 1–3) or anti-c-myc antibody (9E10) (bottom panel, lanes 5–6); the asterisk indicates the light chain of the antibody used for immunoprecipitation. The input lane (lane 1) contains ~30% of the material used for immunoprecipitations. (**C**) HIM and HDAC activity assays of recombinantly expressed HDAC1 (left panel) and SUV3-91 (right panel). (**D**) HIM activity with immunoprecipitates using an anti-HDAC1 antibody from a partially purified fraction (Figure 1B, fraction 26). (**E**) HIM activity assay using material immunoprecipitated with an anti-flag antibody. Immunoprecipitates were purified from *Drosophila* control cells (SL-2) or cells stably expressing flag-tagged HDAC1 (fHDAC1).

significantly enhance silencing of the white gene (Tschiersch *et al.*, 1994) (Figure 4, top panel). This so-called triplo-enhancer effect of *Su(var)3-9* displays a dominant effect over most other known *Su(var)*s. However, when the strain expressing an extra copy of *Su(var)3-9* is crossed with a strain carrying the $HDAC1^{326}$ mutation (Mottus *et al.*, 2000) we still observe a strong suppression of PEV leading to a red eye phenotype (Figure 4, bottom panel). Because *Drosophila* has four different HDACs with sequence similarities to *HDAC1* (Adams *et al.*, 2000) we used a point mutation of *HDAC1*, which is still expressed (Mottus *et al.*, 2000) and will get incorporated into multiprotein complexes where it exerts dominant-negative functions. We concluded from these experiments that *HDAC1* lies upstream of *Su(var)3-9* in the regulatory cascade leading to the formation of pericentric heterochromatin.

DISCUSSION

Here we report the association of the HIM SU(VAR)3-9 and the deacetylase HDAC1 within *Drosophila* embryo extracts. We show that this interaction plays an essential role in SU(VAR)3-9's ability to methylate acetylated histone tails, which is probably important during the 'invasion' of euchromatic regions of the genome by heterochromatin that is observed when a gene is placed closed to heterochromatin (Spofford, 1967).

In addition to their biochemical interaction, we also see a strong genetic interaction between *Su(var)3-9* and *HDAC1*. A point mutation within the *HDAC1* gene, which has a strong Su(var) phenotype, very efficiently dominates the 'triplo-enhancer effect' usually seen in flies carrying additional copies of *Su(var)3-9*. The effect of different *HDAC1* mutations on PEV has been described as enhancing, suppressing or neutral (De Rubertis *et al.*, 1996; Mannervik and Levine, 1999; Mottus *et al.*, 2000) depending on the experimental setup. These controversial results are probably due to the different nature of the mutants used in the different laboratories. A functional redundancy of HDAC1 with other known HDACs could, for example, obscure the contribution of *HDAC1* in a hypomorphic strain. We postulate that in the $HDAC1^{326}$ strain a mutant protein is made that is able to interact with SU(VAR)3-9 but fails to

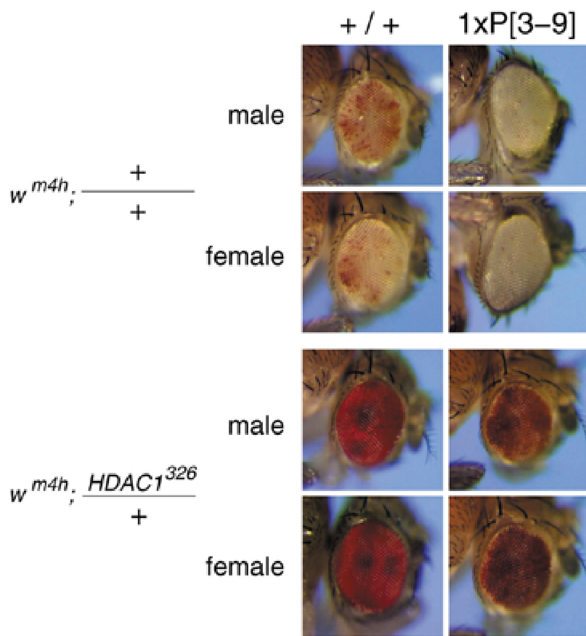

Fig. 4. The *HDAC*^{326} mutation dominates the PEV enhancer effect of an additional genomic copy of *Su(var)3-9*. The P[3–9] carries one additional genomic copy of *Su(var)3-9* introduced by P[(ry^{+}) 11kb *SU(VAR)3-9*] at 100E.

deacetylate the histone substrate and therefore acts as a dominant-negative suppressor.

Based on these findings we propose a model in which deacetylation precedes methylation of lysine 9 in the N-terminus of histone H3. Methylated H3 would then serve as a docking site for HP1 (Bannister *et al.*, 2001; Lachner *et al.*, 2001), which in turn could help in assembling a specialized higher order chromatin structure. As the turnover of methylated histones is slow in comparison to acetylation (Waterborg, 1993), we suggest that histone methylation serves as a permanent epigenetic mark that freezes a particular chromatin conformation.

The concerted action of deacetylation and methylation of lysine 9 in histone H3 could allow the generation of a permanently repressed chromatin structure within otherwise more accessible, acetylated chromatin of the early embryo. The interaction between SU(VAR)3-9 and HDAC1 is probably especially important at boundaries between eu- and heterochromatin and under circumstances when heterochromatin has been shown to 'invade' euchromatic regions like the *white* gene in the *In(1)w*^{m4h} strain (Reuter *et al.*, 1986).

The existence of a complex containing a HDAC and a HIM provides a molecular mechanism for the close connection between histone deacetylation and histone methylation. This link between deacetylation and methylation is also evident in *S. pombe*, where inhibition of deacetylases by TSA as well as mutations of the *Su(var)3-9* orthologue *clr4* leads to defects in centromere function (Ekwall *et al.*, 1997; Ivanova *et al.*, 1998). It will be very interesting to see whether the coupling of histone deacetylation and histone methylation is a mechanism commonly used to stably repress gene expression not only around the centromere but also in euchromatic regions of the genome.

METHODS

Histone methylation. Peptide HIM assays were carried out in 25 µl of methyltransferase buffer [50 mM Tris–HCl pH 8.0, 0.5 mM DTT] containing 1 µg of peptide (ARTKQTARKSTGG-KAPRKQL, synthesised by Peptide Speciality Laboratories, Heidelberg) as substrate and 500 nCi *S*-adenosyl-[methyl-^{3}H]-l-methionine (25 µCi/ml) (Amersham) as methyl donor. Reactions were stopped by spotting 20 µl on P81 filter paper. Filter papers were then washed three times for 10 min in 50 mM carbonate buffer pH 9.2, dried and ^{3}H-incorporation was measured by scintillation counting. All methylation assays were repeated at least three times. Error bars on the graphs represent the SEM values derived from at least three experiments.

Histone deacetylation. For HDAC activity 20 µl aliquots of column fractions were incubated at 30°C for 90 min with 1 µg of ^{3}H-labelled core histones (25 000 c.p.m./µg). Then 230 µl of IPH buffer (50 mM Tris–HCl pH 7.5, 300 mM NaCl, 0.5% NP-40, 0.2 mM PMSF) and 65 µl of 1 M HCl/0.16 M acetic acid were added and the released acetate was extracted with 700 µl of ethylacetate. A 500 µl aliquot of the upper ethylacetate phase was used for scintillation counting. All deacetylation assays were repeated at least three times. Error bars on the graphs represent the SEM values derived from at least three experiments.

Immunoprecipitation. To immunoprecipitate SU(VAR)3-9-associated proteins, 200 µl of nuclear extract (NE) from myc-SU(VAR)3-9 flies were incubated with 10 µl of 9E10 antibody crosslinked to protein G–agarose beads. After incubation for 3 h at 4°C, the beads were washed three times in 1 ml of IPH 300 buffer and resuspended in 45 µl of buffer A (16 mM NaCl, 20 mM Tris–HCl pH 8.0, 1 mM DTT). For activity assays 20 µl of this material were used.

Fly culture. *Drosophila* cultures and stocks were reared on standard medium at 25°C. Except where noted chromosomes and mutations are described in FLYBASE. Heterochromatin-induced gene silencing has been studied in flies carrying the white variegating rearrangement *In(1)w*^{m4h} (Wade *et al.*, 1997). The *HDAC*^{326} mutation was isolated as a dominant-negative suppressor of PEV (Nakayama *et al.*, 2001). The mutation is caused by a P204S amino acid exchange. Extra genomic copies of *Su(var)3-9* were introduced by P[(ry^{+}) 11kb *Su(var)3-9*] (Mottus *et al.*, 2000). A cross of w^{m4h}; P[(ry^{+}) 11kb *Su(var)3-9*] (100E)/TM3, *Sb Ser* females to w^{m4h}/Y; *HDAC1*^{326}/TM3, *Sb* males produced w^{m4h}; P[(ry^{+}) 11kb *Su(var)3-9*] (100E)/*HDAC1*^{326} flies with one additional genomic copy of *Su(var)3-9*. In the reciprocal crosses identical results were obtained.

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