SAP18 Promotes Krüppel-dependent Transcriptional Repression by Enhancer-specific Histone Deacetylation*

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Body pattern formation during early embryogenesis of Drosofila melanogaster relies on a zygotic cascade of spatially restricted transcription factor activities. The gap gene Krüppel ranks at the top level of this cascade. It encodes a C2H2 zinc finger protein that interacts directly with cis-acting stripe enhancer elements of pair rule genes, such as even skipped and hairy, at the next level of the gene hierarchy. Krüppel mediating their transcriptional repression by direct association with the corepressor Drosophila C terminus-binding protein (dCtBP). However, for some Krüppel target genes, deletion of the dCtBP-binding sites does not abolish repression, implying a dCtBP-independent mode of repression. We identified Krüppel-binding proteins by mass spectrometry and found that SAP18 can both associate with Krüppel and support Krüppel-dependent repression. Genetic interaction studies combined with pharmacological and biochemical approaches suggest a site-specific mechanism of Krüppel-dependent gene silencing. The results suggest that Krüppel tethers the SAP18-bound histone deacetylase complex 1 at distinct enhancer elements, which causes repression via histone H3 deacetylation.

Kr (Krüppel) is an essential gene of the segmentation gene hierarchy in Drosophila melanogaster. It encodes a C2H2 zinc finger protein necessary for the formation of thoracic and abdominal body parts of the embryo (1–3). Kr is expressed in the central region of the embryo where it controls transcription of pair rule genes, such as eve (even skipped) and h (hairy) (4, 5). The Kr protein (Kr) binds to multiple specific DNA-binding sites (6, 7) within distinct cis-acting stripe elements of pair rule genes and provides repression in a concentration-dependent manner. In addition, Kr functions later in embryogenesis by acting as a key regulator of multiple developmental genes necessary for the formation of various organs (8–11). Furthermore, ectopic expression of Kr in imaginal eye discs interferes with their normal development by causing an Irregular facet gain-of-function phenotype (12, 13). The available evidence suggests that Kr acts mainly as a transcriptional repressor (14–18) and is likely to control several hundred target genes (14, 19). However, the mechanism of Kr-dependent transcriptional repression is still not fully understood.

Initial in vitro studies showed that Kr is capable of forming concentration-dependent homodimers that interact with the basal transcriptional machinery and prevent transcription (20). Although this mechanism would explain a concentration-dependent action of Kr, its relevance for in vivo action of Kr has not yet been demonstrated. A second mechanism of Kr-dependent repression rests on multiple and overlapping DNA-binding sites for activators within the enhancer region of target genes. In this context, binding of Kr can compete for binding of activators and thereby prevent gene activation (21, 22). Finally, the C terminus of Kr contains functional binding sites for the corepressor dCtBP (22, 23) and acts as a repressor via distinct cis-acting enhancers such as the eve stripe 2. In the absence of the dCtBP-binding sites, eve stripe 2 expression cannot be repressed by Kr, and thus, the expression domain extends into the Kr expression domain. Conversely, overexpression of Kr throughout the blastoderm embryo causes repression of eve stripe 2 expression, whereas overexpression of mutant Kr lacking the dCtBP-binding sites has no such effect. Although the dCtBP-dependent repression by Kr is firmly established, the mechanism of this type of repression is not yet elucidated.

In contrast to the eve stripe 2 enhancer, dCtBP does not seem to be necessary for Kr-dependent regulation of the h stripe 7 enhancer. Overexpression of Kr in maternal dCtBP mutant embryos still represses h stripe 7 expression (24), suggesting that there must be an additional and dCtBP-independent repressor function of Kr. To seek a factor that confers this missing repressor function on Kr and to elucidate the mechanism of this type of repressor action, we performed a protein-protein interaction screen to identify proteins that associate with Kr. We found dSAP18, a protein that is known to associate with components of the histone deacetylase complex 1 (dHDAC1) (25). The results suggest that Kr participates in the site-specific deacetylation of histone 3 at the h stripe 7 element but not at the

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2 The abbreviations used are: HDAC, histone deacetylase; CtBP, C terminus-binding protein; ESI-MS/MS, electrospray ionization tandem mass spectrometry; HPLC, high performance liquid chromatography; HRP, horseradish peroxidase; PepSpot, peptide spot; TSA, trichostatin A; ChIP, chromatin immunoprecipitation; xChIP, cross-linked ChIP; GST, glutathione S-transferase; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

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eve stripe 2 enhancer. We propose a model in which DNA-bound Kr associates with dSAP18 and thereby tethers dHDAC1 at the respective loci to silence target genes by local histone deacetylation.

**EXPERIMENTAL PROCEDURES**

**Fly Genetics**—The following fly stocks were used: b pr cn wx K(y)h23041 (Tübingen stock collection), the balancers y,w; Sco/Cyo, P(hb-lacZ) and y,w; Ly/TM3, Sb, and hs-Kr/Cyo, P(hb-lacZ); h7-lacZ (for details see Ref. 24). The +/Cyo, P(hb-lacZ); h7-lacZ fly stock was generated from hs-Kr/Cyo, P(hb-lacZ); h7-lacZ and y,w; Ly/TM3, Sb flies to obtain the genotype +/Cyo, P(hb-lacZ); h7-lacZ. The P element insertion G13322 (dsap18 w+,EP) was obtained from the GenExel fly stock center. sap18R7–18/TM3, ftz-lacZ and sap18-14/TM3, ftz-lacZ and the dsap18 rescue stock y,w; P(y+ dsap18) on the II-chr.; sap18R7–18 were described earlier (26). Germ line clones (27, 28) homozygous for sap18-14 were generated as described (26) involving sap18-14/ovoD1 virgins that were mated to sap18-14/TM3, ftz-lacZ or hs-Kr/Cyo, P(hb-lacZ); h7-lacZ males.

**Liquid Chromatography-coupled ESI-MS/MS**—Proteins were excised from SDS-PAGE, digested with trypsin (29), and subjected to analysis by HPLC-coupled ESI-MS/MS. HPLC-ESI-MS/MS was carried out under standard conditions on a hybrid triple quadruple/linear ion trap mass spectrometer (4000 Qtrap; Applied Biosystems) coupled to an Agilent 1100 nano-chromatography system (30). Peptide fragment spectra were searched in the NCBInr data base using Mascot as search engine. Mass accuracy was 1.4 Da and 400 milli-mass units, respectively, for samples analyzed on the linear ion trap. The peptides were constrained to be tryptic with a maximum of one missed cleavage site. Carbamidomethylation of cysteines and oxidation of methionine residues were considered as variable modifications. The highest scoring peptide from each protein as well as single hit peptides were manually inspected to eliminate the false positives in the data set.

**PepSpot**—Cellulose-bound peptide arrays (31) were prepared by JPT Peptide Technologies GmbH (Berlin, Germany). One peptide array (PepSpot39) contained 39 spots of 14-mer peptides representing the Krüppel protein (amino acids 301–502; see Fig. 1A) with seven amino acid overlaps. A second peptide array (PepSpot54) contained 54 species of partially overlapping 16-mer peptides representing the full-length Krüppel sequence with an overlap length of four amino acids (supplemental Table S1). To prevent oxidation of SH groups, cysteine residues of the PepSpot54 array were replaced by serines.

**In Vitro Translation**—[35S]Methionine-labeled full-length dsAP18 was produced from the pETWZ1 plasmid (32) using the Tnt system (Promega; L1170) supplemented with Redivue® PRO-MIX [35S] label (65 MBeq, 1.75 mCi of methionine; Amersham). Prior to incubation with membranes, the [35S]-labeled dsAP18 (80 μl) was diluted five times in TBS buffer (10 mM of Tris-HCl, pH 8.0, 100 mM NaCl) and filtered through a Durapore® membrane (Ultrafree®-MC, 0.22 micron; Millipore). Unincorporated [35S]methionine and low molecular weight solutes were removed by ultrafiltration using the regenerated cellulose filter (Microcon YM-10; 10,000 molecular weight cut-off) at 4,000 rpm. The PepSpot membranes were blocked with the blocking buffer (Sigma) to minimize nonspecific binding of dsAP18. Autoradiographs were obtained after exposure for 40 h, followed by scanning with the Typhoon™ 8600 laser-scanner (Molecular Dynamics). Signal quantification was done with a QuantityOne™ software tool (Bio-Rad).

As a control, dsAP18 was tagged with an N-terminal His6 epitope and expressed in a coupled TnT system as described above (except that nonradioactive methionine was added) using the pETWZ2 plasmid as a template. The peptide spots bound by the His6-dSAP18 were visualized by mouse monoclonal anti-His6 primary antibodies (DIA900; Dianova) followed by goat anti-mouse HRP-conjugated secondary antibody detection (Pierce). Enhanced luminescence was recorded by CCD-based Lumi-Imager™ (Roche Applied Science).

**Heat Shock and TSA Treatments**—Embryos were collected (100 min; 23 °C) from flies bearing a single Kr cDNA transgene under control of the heat-inducible hsp70 promoter balanced over a CyO balancer chromosome carrying a hb-lacZ transgene (second chromosome) and two h7-lacZ transgenes (third chromosome). The embryos were aged (20 or 60 min; 23 °C), dechorionated, and permeabilized (20–30 s) with octane (Fluka) (33) and incubated in a 30 μm TSA (Biomol; dissolved in dimethyl sulfoxide) solution of 1× phosphate-buffered saline solution. Control embryos were treated in parallel but without TSA. The embryos were either exposed to 37 °C (30–60 min) or kept at room temperature (23 °C). After recovery (20 min; 23 °C), the embryos were fixed (in situ hybridization) or snap-frozen (liquid nitrogen, RNA isolation, Western blot analysis). For heat shock-induced Kr expression in maternal dsAP18 mutant embryos, the embryos were collected from a cross of females with homozygous dsAP18 mutant germ lines and males bearing the hs-Kr and h7-lacZ transgenes.

**Whole Mount in Situ Hybridization and Antibody Staining**—In vitro translation—lacZ and eve expression were examined by in situ hybridization to whole mount preparations using antisense RNA probes as described (14). Kr expression was monitored by staining embryos with rabbit-raised anti-Kr antiserum followed by staining with goat anti-rabbit Cy2-conjugated fluorescent antibody (Dianova).

For chromatin immunoprecipitation, affinity-purified anti-acetyl K9,14-histone 3 rabbit polyclonal antibodies (Millipore-Upstate) were used. Western blots and PepSpot39 binding assays were performed with anti-His6 mouse monoclonal antibody (Dianova). Affinity-purified anti-histone H2A serum, anti-acetylK9, and anti-acetylK14 histone 3 rabbit polyclonal antibodies (Millipore-Upstate) were used for quantitative Western blot experiments. Goat anti-mouse HRP-conjugated antibody and goat anti-rabbit HRP-conjugated antibody (Pierce) were employed as secondary antibodies. For in situ detection of protein and digoxigenin-labeled RNA probes, we used anti-Kr rabbit polyclonal antiserum, goat anti-rabbit Cy2-conjugated antibodies (Dianova), alkaline phosphatase-conjugated anti-digoxigenin antibodies (Roche Applied Science), rabbit anti-digoxigenin antibodies (Roche Applied Science), sheep anti-rabbit IgG antibodies, and donkey anti-sheep IgG biotinylated antibodies.
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*In Silico Analysis and Statistical Methods—In silico prediction of nucleosome patterns at the h stripe 7 and eve stripe 2 elements (including the respective 10-kb flanking regions) were carried out using the available nucleosome/DNA interaction models (34) and the Genomica software tool. To test the significance of differences between medians representing relative enrichment factors as revealed by xChIP analysis via semi-quantitative PCR, the Kruskal–Wallis nonparametric statistical test was used (Analyze-it®, standard edition, v.2.03, Analyze-it Software, Ltd.).*

*In Vitro Pulldown Experiments—GST-(N)Kr2–502 aa fusion protein was produced as described (14). Constructs expressing different fragments of Kr fused N-terminally to the GST moiety were produced using primer pairs containing either EcoRI (forward primers) or XhoI (reverse primers) sites (supplemental Table S2) for cloning into the pGEX-4T-3 (Amersham Biosciences). Kr sequences were fused N-terminally to GST via the thrombin sensitive linker. The constructs were expressed in Escherichia coli BL21 bacterial cells (see Ref. 14) to produce the respective GST-Kr fusions. The proteins were purified by binding to GST-Sepharose 4B affinity resin (Pharmacia) and washed under moderate stringency conditions (20 mM HEPES, pH 8.0, 300 mM NaCl, 0.1 mM ZnSO4, 0.1% Triton X-100, 4% glycerol). The amount of GST-Sepharose for binding was adjusted to yield resins containing GST-Kr (concentration of 1 mg/ml). Fusion protein was covalently fixed to the affinity resin (homobifunctional imidoester cross-linker, dimethyl adipimide; Pierce) and processed according to the instructions of the manufacturer.

*Drosophila* S2 Schneider cells were grown to confluency (0.5–1 × 10^6 cells), harvested into a 50-ml Falcon tube, washed twice with phosphate-buffered saline, and cross-linked with 10 mM of dimethyl adipimide (room temperature). The cells were disrupted in 1× phosphate-buffered saline (containing protease inhibitors (Roche Applied Science), 5% glycerol, and 1 mM of dithiothreitol) by sonication with ultrasound (five bursts; 30 s each). Triton X-100 was added to 0.05%. Cell debris was removed by centrifugation. Lysate was passed through the pGEX-4T-3 (Amersham Biosciences). Kr sequences were disrupted in 1 ml of binding buffer (20 mM HEPES, pH 8.0, 300 mM NaCl, 0.1 mM ZnSO4, 0.1% Triton X-100, 4% glycerol). The amount of GST-Sepharose for binding was adjusted to yield resins containing GST-Kr (concentration of 1 mg/ml). Fusion protein was covalently fixed to the affinity resin (homobifunctional imidoester cross-linker, dimethyl adipimide; Pierce) and processed according to the instructions of the manufacturer.

**Chromatin Immunoprecipitation and Semi-quantitative PCR—**Chromatin was prepared from ~500 µl of 110–230-min-old embryos. Immunoprecipitations of cross-linked chromatin (14) were carried out with affinity-purified anti-acetyl h9.14-histone 3 rabbit polyclonal antibodies (Millipore-Upstate; about 3000 ng/ml). 100 µl of Affi-Prep® protein A support beads (Bio-Rad) were used to adsorb the antibody-chromatin complexes from 10 ml of immunoprecipitation solution, resulting in between 300 and 500 ng of immunoprecipitated DNA/500 µl of embryos.

Primers to amplify h stripe 7, eve stripe 2 or dSec23 sequences are listed in supplemental Table S2. To estimate enrichment of full-length sequences of the eve2 (772 bp) and h7 (799 bp) enhancers, as well as the quantification of the small genomic fragments (sizes around 200 bp; see Fig. 5, C and D), semi-quantitative PCR was performed as described (14). The relative enrichment (vertical axis values in the Fig. 5, D and G) was calculated as (w/x)/(y/z), where w is the ChIP target fragment, x is the ChIP reference fragment, y is the input target fragment, z is the input reference, and all values are the sums of pixel intensities for each band (local background was subtracted). The numbers reflect the relative immunoprecipitation efficiency; values less than 1 are not unexpected. The increased acetylation in response to TSA (Fig. 5E, grey bars) was calculated as [(w/x)/(y/z)] with TSA/[(w/x)/(y/z)] without TSA. The relative sensitivity to sonication (Fig. 5E, black bars) was calculated as (y/z) without TSA/(y/z) with TSA. To increase the range of the exponential amplification phase, PCR was paused after 18 cycles of the following conditions: 40 s at 95 °C, 40 s at 54 °C, and 90 s (600–900-bp fragments) or 30 s (200-bp fragments) at 72 °C. The reaction mix was diluted twice with freshly activated 1× PCR Mastermix (total volume, 50 µl) prior to adding 12 cycles (conditions as described above). Each reaction was done in triplicate. PCR fragments were size fractionated on polyacrylamide gels and stained with EtBr followed by imaging and signal quantification (Lumi-Imager™, Roche Applied Science). PCR products were controlled by sequencing.

**Quantitative Western Blots—**Embryos (50-µl aliquots) were thawed in 300 µl of Laemmli buffer, ground, and heated (95 °C, 10 min). Aliquots of the supernatant with equal amount of protein were separated by 15% SDS-PAGE and transferred to a nitrocellulose membrane (Millipore). The membranes were stained (0.1% Ponceau S; Sigma; in 5% acetic acid) to estimate...
the protein content and probed with either anti acetylK9H3 or anti acetylK14H3 antibody (Millipore). ECL was quantified with LAS-1000 (Fujifilm). As controls, the antibodies were removed details see text.

FIGURE 1. dSAP18 is a Kr binding protein in vitro. A, schematic representation of the Kr protein showing the N- and C-terminal transrepressor domains (regions 1–93 and 400–502; orange), the transactivator and coactivator domains (region 93–168; green), the five zinc finger motifs (ovals; blue), the dCtBP-binding sites (positions 414–420 and 464–469; triangles), and the five dSAP18 in vitro binding peptides (details in E; arrows plus numbers). B, SDS-PAGE showing the proteins bound to GST-fused full-length Kr (GST-Kr) and GST control protein, respectively. GST-Kr-specific proteins were submitted to MALDI-TOF-MS analysis, and the previously known Kr-binding protein dCtBP and dSAP18 (see C) were identified. Apparent molecular masses (kDa) are indicated. C, amino acid sequence of dSAP18 showing Kr-associated peptides as revealed by combined mass spectrometry/Mascot data base analysis. D, Western blots of proteins from pull down experiments. Staining with His tag antibodies showing that recombinant His-tagged dSAP18 is bound to Kr. Lane 1, Kr region 168–399. Lane 2, Kr region 400–502. Lane 3, control experiment; dSAP18 does not bind to His-GST. Lane 4, control experiment lacking the His6-SAP18 input. Lane 5, His-tagged dSAP18 input. The asterisk indicates the position of His-tagged dSAP18; kDa of marker proteins are indicated. E, autoradiograph of peptide spots covering the full-length Kr sequence; spots 1–54 for sequences; supplement Table S1) after incubation with [35S]methionine-labeled dSAP18. Position of dSAP18-binding peptides 25, 30–32, and 46 within Kr is shown in A. For details see text.

RESULTS

Krüppel Binds dSAP18 in Vitro—To identify proteins that interact with the transcription factor Kr (Fig. 1A), we isolated proteins from crude extracts of Drosophila S2 Schneider cells that bind to a GST-Kr fusion protein. The GST-Kr-associated proteins were identified by liquid chromatography coupled ESI-MS/MS subsequent to SDS-PAGE (Fig. 1B). The Kr-bound proteins included dCtBP (35) and dSAP18 (also called dBin1 (Bicoid-interacting protein 1)), a transcriptional co-repressor known to be active during early Drosophila embryogenesis (26). Because Kr was shown to require an unknown co-repressor in addition to dCtBP (24), we asked whether dSAP18 could serve this function.

Previous results had shown that such a factor must bind to a region N-terminal to the dCtBP-binding domain of Kr (24). To determine the SAP18-binding region of Kr, we used pulldown experiments involving a set of GST fusions that contain various parts of Kr. We confirmed that dCtBP binds to the C-terminal portion (amino acids 400–502) of Kr (22, 23), whereas dSAP18 binds to the amino acid region 168–399, which includes the C2H2 zinc finger domain (Fig. 1A). MALDI-TOF-MS control experiments identified N-terminal peptides of dSAP18 that were associated with full-length Kr (Fig. 1C) but not with GST fusions that lack the Kr168–399 region, and pulldown experiments with bacterially expressed His-tagged dSAP18 protein confirmed strong in vitro binding of dSAP18 to the central region of Kr (amino acid region 168–399) as well as weak binding to the C-terminal part (Fig. 1D). We also expressed GST fusions containing the N-terminal region of Kr, but they were not soluble under the conditions used for the other GST fusions of Kr. Thus, we cannot exclude the possibility that dSAP18 is capable of binding also to the N-terminal portion of Kr.

To determine the dSAP18-binding sites of Kr, we performed peptide array analysis employing tiled 16-mer peptides (see supplemental material), which cover the entire Kr sequence (peptides 1–54; Fig. 1E). In vitro translated [35S]methionine-labeled dSAP18 bound strongly to three overlapping peptides (peptides 30–32; Fig. 1E) that contain the region 342KFRRR346 of the fifth zinc finger motif of Kr. Two additional sites were found in the third zinc finger motif 299LRRHLRVH309, peptide 25 and the C-terminal domain (amino acid interval 431NIARROAK438, peptide 46), respectively (Fig. 1, A and E). A complementary immunological approach with His-tagged dSAP18 confirmed the identified binding sites (data not shown). To exclude the possibility that dSAP18 binds to a short stretch of basic amino acids in an unspecified manner only, we also tested a total of 14 arbitrary peptides of comparable basicity. No binding was observed except when the consensus sequence RR(K/R)H(H/K) was present in target peptides. This motif was found in a number of Drosophila transcription factors including Bicoid, which was previously shown to functionally interact with dSAP18 (26). A detailed analysis of the dSAP18-binding sites in Drosophila transcription factors and their functional requirement will be presented else-
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FIGURE 2. Loss of maternal dSap18 activity generates transformations of eye structures in the dominant Kr mutant (If) and suggests an epigenetic maternal effect of dSap18 on Kr function. A, light micrograph of an adult wild type eye. B, dominant Kr mutant (If/1-/+ ) eye. Note that If causes a slit-like rough eye phenotype. C–F, flies from matings of If-1/If-1 males and either sap18R7–18 h/h11001 (C–E) or sap18R7–18 h/ h11001/TM3.Sb1 (F) females. C and D, eyes of If-1/+ ; sap18R7–18 h/+ (C) and If-1/+ ; +/TM3.Sb1 (D) individuals showing that sap18R7–18 h or balancer chromosomes used in the crossings do not alter the If phenotype. E and F, transformations of eye structures observed in about 2% of If-1/+ ; sap18R7–18 h/+ (E) and If-1/+ ; sap18R7–18 h/+ (F) individuals (not seen in more than 500 If-1/+ ; +/+ or +/+ ; sap18R7–18 h mutant flies. E and F: enlargements of areas marked by dotted lines in E and F.

where.3 We also note that the binding of Kr sequences and dSAP18 withstands salt concentrations up to at least 300 mmol (see “Experimental Procedures”). Taken together, our results indicate that dSAP18 can bind Kr in vitro and that the binding involves distinct binding sites.

Genetic Interactions Suggest That SAP18 and Kr Interact in Vivo—To establish a functional basis for an interaction between Kr and dSAP18, we performed genetic studies using a loss-of-function mutant allele, sap18R7–18 h/h11001 (26) and the dominant Kr gain-of-function mutation If (Irregular facets) (13). If causes ectopic Kr activity in the developing eye imaginal discs, resulting in a dosage-dependent slit-like eye phenotype (13). The If mutant phenotype is modified in response to reduced activity of zygotically expressed genes that act in the same developmental pathway (12, 13), and it responds to reduced maternally expressed gene activities, resulting in an outgrowth of transformed adult eye tissue (36). Such transformations are observed in low frequency, providing evidence for an epigenetic “inherited maternal effect” caused by histone acetylation/deacetylation events (36). sap18R7–18 h mutants alone have no scorable effects on eyes.

Crosses between the If-1 homozygous males and heterozygous sap18R7–18 h or sap18R7–18 h females did not alter the If mutant phenotype (Fig. 2A–D), but resulted in transformations in about 2% of the offspring (Fig. 2, E and F). No transformation was observed in more than 500 individuals resulting from reciprocal crosses between heterozygous sap18 males and If-1 females. Thus, the modifier effect was neither allele-specific nor dependent on the genetic background of the female flies, and it was not due to reduced zygotic dSap18 activity. It can be attributed to an earlier reported maternal effect, likely to involve epigenetic events such as histone acetylation/deacetylation events (36). These results highlight that SAP18 not only interacts with Kr in vitro but could also play a role in one or several Kr-dependent gene regulation processes in vivo.

dSAP18 and Kr-dependent Regulation of h Stripe 7 but Not eve Stripe 2—During the blastoderm stage, Kr binds to and represses via the eve stripe 2 element (18, 37) in a dCtBP-dependent manner (22). It also binds to the h stripe 7 element and represses h stripe 7 expression in a dCtBP-independent manner (24), indicating that Kr-dependent repression involves more than one mechanism (24).

Previous studies of Singh et al. (26) suggested that the absence of dSAP18 activity did not interfere with the spatial pattern of embryonic eve or h expression. However, this finding does not rule out the possibility that dSAP18 participates in Kr-dependent repression. It could be that dCtBP and/or the activity of other corepressors are sufficient to mediate Kr-dependent repression in the absence of dSAP18 and that the corepressors may act, at least in part, redundantly. To test this possibility and to explore whether dSAP18 functions as a corepressor on Kr target genes, we examined eve expression in wild type and dSAP18-deficient embryos after heat shock-induced Kr expression during blastoderm formation. In wild type embryos (Fig. 3, A–C), ubiquitous Kr overexpression caused full repression of eve stripes 1–5, whereas in some embryos weak eve stripe 6 and 7 expression remained (Fig. 3C’). Thus, anterior eve stripes are more sensitive to Kr-dependent repression than posterior stripes. In the absence of maternal dSAP18 activity, no effect could be observed on Kr-dependent eve stripe 2 and 5 repression, but eve stripes 1, 3, and 4 were derepressed (Fig. 3, D–F). The lacZ reporter transgenes (as described below and in Fig. 3 legend) used to identify embryos of particular genotypes had no effect on the eve expression. These results show that dSAP18 is not required for Kr-dependent repression of eve stripes 2 and 5 but to various degrees for the repression of the other eve stripes.

We next asked whether dSAP18 contributes to Kr-dependent hairy stripe 7 repression, which does not require dCtBP (24). We used a transgene that consists of a lacZ reporter under the control of the h stripe 7 minimal enhancer (24) and mediates either full repression in response to overexpressed full-length Kr or partial repression in response to truncated Kr (region 1–351 in Kr mutant), which lacks the two dCtBP-binding sites (24) as well as one of the three in vitro identified dSAP18 sites. In the absence of maternal dSAP18, overexpression of Kr in response to 30 min of heat shock induction was unable to repress the h-lacZ reporter gene (Fig. 4, A–F). However, prolonged expression of Kr (60 min) caused repression of the h-lacZ reporter in dSAP18 mutant embryos (Fig. 3, F and F’). These observations indicate that dSAP18 can indeed participate in the Kr-dependent repression of the h-lacZ gene. In the presence of high levels of Kr, as obtained after its prolonged expression for 60 min, however, Kr-dependent repression can also occur by a mechanism that does not involve dSAP18. In contrast, the eve stripes 1, 3, and 4 remain unsuppressed in response to the prolonged expression of Kr as shown in Fig. 3 (C and C’).

3 A. Matyash and H. Jäckle, manuscript in preparation.
SAP18-dependent Repression by Kr Involves Histone Deacetylation—SAP18 interacts with SIN3A, a component of the dSin3A/dRpd3 dHDAC1 (25). RNA interference experiments with Drosophila cell culture and transcriptional profiling indicates that the transcriptional effects of the HDAC-inhibitor TSA are due to inhibited dHDAC1 activity that results in increased acetylation of histone 3 (H3) on lysine 9 and 14 (38, 39). To test the possible link between Kr-dependent repression and SAP18-mediated H3 deacetylation, we examined the effect of TSA on Kr-overexpressing embryos. In the presence of TSA, Kr-overexpressing embryos did not repress expression of h7-lacZ (Fig. 4H and supplemental Fig. S2), suggesting that h stripe 7 repression by Kr involves H3 deacetylation. This idea was further studied using acetylK9H3- and acetylK14H3-specific antibodies. TSA treatment caused a strong increase of K9,14H3 acetylation in wild type embryos, whereas in Kr-overexpressing embryos, this effect was strongly reduced (supplemental Fig. S1). In addition, in the absence of TSA, the level of acetylK9H3 in embryos overexpressing Kr was lower than in wild type embryos (supplemental Fig. S1).

Kr-dependent Deacetylation at the h Stripe 7 but Not the eve Stripe 2 Element—We next asked whether a lack of Kr-dependent repression of h7-lacZ correlates with an increased K9,14H3 acetylation at the h stripe 7 element. We performed immunoprecipitation experiments with anti-acetylK9,14H3 antibodies and chemically cross-linked chromatin from Kr-overexpressing Drosophila embryos that contain a h7-lacZ transgene either in the presence or the absence of TSA. Immunoprecipitated chromatin was examined by PCR for enrichment of h7-lacZ sequences (Fig. 5C; for a 23 °C control see Fig. 5F). We attribute
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**FIGURE 5.** Acetylation of histone H3 at h7 and eve stripe 2 enhancer elements in response to Kr activity, inhibition of dHDAC1, and reduced maternal dSAP18 activity. A and B, schematic representation of the eve stripe 2 (A) and h stripe 7 (B) enhancers. Binding sites for Kr (red; (15, 24)), Giant (blue; (17, 18, 37)), Bicoid (green; (18, 21)) and Caudal (brown; (21)) are shown. Primers used to amplify enhancer regions (C and F) are listed in supplemental Table S2. Predicted positions of stable nucleosomes (NS; stability probability > 0.2; (34)) are marked (green, chicken model, NS0–4; c; brown, yeast model, NS0–3); regions designated according to both models are framed (dotted lines). Note that the h stripe 7 element contains a denser array of nucleosomes than the eve stripe 2 element (for details see text and “Experimental Procedures”). Black bars, amplified regions of the eve stripe 2 and h7 enhancers in C–E, C–E, xChIP analysis showing K\(^9,14\)H3 acetylation of histone H3 in the eve stripe 2 and h7 enhancers (black lines in A and B) in response to overexpressed Kr and the HDAC1-inhibitor TSA (30 μM), respectively. C, chromatin isolated after immunoprecipitation with anti-acetylK\(^9,14\)H3 antibodies (+) from TSA treated (+) or untreated (−) embryos. DNA of the eve stripe 2 and h7 enhancers were amplified by PCR; control PCRs were carried out with dSec23 DNA, which lacks Kr-binding sites. D–E, quantification of the xChIP results shown in C (see “Experimental Procedures” for details). Note that K\(^9,14\)H3 acetylation of histone H3 is increased at the h7 after TSA treatment and heat shock-induced Kr activity but not at the eve stripe 2 enhancer (D and E). The effect correlates with increased sensitivity to ultrasound treatment (E; black bars). K\(^9,14\)H3 acetylation at the three subregions of the h7 element is strongly reduced in response to Kr, and this effect is reverted by TSA treatment. F and G, ChIP analysis (F) and quantification of the K\(^9,14\)H3 acetylation (G) at the eve2 and h7 enhancers from heterozygous dSAP18 females (sap18-14, Mut) or from females that contain two copies of the dSap18 transgene (Rescue, Res) at the 772-bp eve stripe 2 enhancer (black line in A), the 799-bp h stripe 7 enhancer (black line in B), and the Sec23 control sequences, respectively. Note that K\(^9,14\) acetylation at the h7 stripe 7 enhancer is about twice as high as at the eve stripe 2 element (p < 0.0001) and that H3 deacetylation in response to two dSap18 transgenes (Res) is enhanced accordingly. The 95% notched box plot shows the calculated median value (horizontal line within the box) for the relative enrichments and the corresponding 95% CI of the median (notched part); the error bars show the limits of the data spread. A nonparametric Kruskal-Wallis test was used to assess the significance of the differences in the group of four medians, all pair-wise (Bonferroni protected). The details are outlined under “Experimental Procedures.”

This effect to a lower sensitivity of h7 element sequences to sonication as compared with eve stripe 2 and the control sequences. These findings suggest that h7 element sequences are better protected against sonication than eve stripe 2 sequences, perhaps as a result of chromatin compaction that might occur on the h7 element as a result of Kr-dependent deacetylation in response to HDAC activity (40). Indeed, ChIP experiments (Fig. 5, C–E) showed that TSA treatment of Kr-overexpressing embryos caused an increase of K\(^9,14\)H3 acetylation at the h7 element. In contrast, TSA treatment of Kr-overexpressing embryos did not increase the K\(^9,14\)H3 acetylation at the eve stripe 2 element as compared with the Sec23 control gene. Furthermore, TSA did not lead to any change in sensitivity to sonication. Additional xChIP experiments performed with primer sets directing amplification of the full-length h7- and eve2 element data (not shown) confirmed the above mentioned results. Collectively, these findings suggest that Kr-dependent repression via the h stripe 7 involves deacetylation of H3, whereas repression via the eve stripe 2 element does not.

Maternal dSAP18 Participates in H3 Acetylation at Kr-regulated Enhancers—No effect on h and eve stripe expression patterns is seen in blastoderm stage embryos lacking maternal dSAP18 activity (26). Thus, dCtBP and other possible corepressors may support repression by Kr in the absence of dSAP18. However, h7-lacZ expression was only weakly repressed when Kr was overexpressed in embryos lacking the maternal complement of SAP18 (Fig. 4D). We therefore asked whether the loss of maternal SAP18 activity in embryos affects K\(^9,14\)H3 acetylation at the h stripe 7, but not at the eve stripe 2 enhancer sequences. Fig. 5 (F and G) shows the results of ChIP assays with anti-acetyl K\(^9,14\)H3 antibodies, indicating that K\(^9,14\)H3 acetylation in embryo collections obtained from crosses of sap18-14 heterozygous...
parents was higher at the h stripe 7 enhancer as compared with the SAP18-insensitive eve stripe 2 enhancer or the sec23 control gene. This result is consistent with the in silico prediction of nucleosome packing (Fig. 5, A and B), suggesting a more dense array of nucleosomes at the h stripe 7 enhancer as compared with the eve stripe 2 enhancer. In conclusion, both biochemical and functional data suggest that Kr recruits dSAP18-mediated dHDAC1 activity in an enhancer-specific manner.

**DISCUSSION**

We provide evidence that Kr exerts transcriptional repression not only by association with the corepressor dCtBP but also by site-specific deacetylation of histones, a mechanism that involves an interaction between Kr and dSAP18. The dual mode of Kr-dependent repression might explain earlier studies showing that Kr represses eve stripe 2 expression, but not h stripe 7 expression, in a dCtBP-dependent manner (24). Consistent with these observations, a mutant Kr protein that lacks dCtBP-binding sites still associates with dSAP18, which in turn interacts with the Sin3A-HDAC1 repressor complex (26, 41). dSAP18 was also shown to bind the homeodomain transcription factor Bicoid, causing repression of anterior gap genes such as hunchback in the late Drosophila blastoderm embryo. SAP18-dependent repression involves histone deacetylase both in flies (26, 42) and mammals (41), and SAP18 that links the HDAC1 complex with sequence-specific transcriptional repressors bound to chromatin is also found in plants (43). The results shown here are consistent with such a SAP18-dependent mode of Kr-dependent repression that provides target gene-specific repression. Because both dCtBP and SAP18 are uniformly distributed in the embryo, it will be important to learn how the eve stripe 2 and the h stripe 7 enhancer distinguish between the dCtBP- or SAP18-dependent modes of repression. One possibility is that differential packing of the enhancer DNA into nucleosomes might account for the difference in susceptibility to the SAP18/HDAC1-mediated repression.

dSAP18 binds to three distinct regions of Kr (Fig. 1A), including the 42-amino acid-long repressor region (21), which is conserved in Kr homologs of all Drosophila species. However, as observed for dCtBP, dSAP18 alone cannot account for Kr-dependent repression of h7-lacZ, because prolonged expression of Kr is able to overcome the lack of dSAP18 activity as observed for the h7 element in dSAP18 mutants. Therefore, it is likely that the full spectrum of Kr-dependent repression is mediated redundantly, employing at least two different corepressors that involve different modes of repression.

In vitro, dSAP18 binds to the sequence motif 344RRRHHL349 of Kr and to a similar motif (14RRRRHHK149) of Bicoid (this study, data not shown); the latter is consistent with the results reported by Zhu et al. (42). In both proteins, the dSAP18-binding sites are localized in the C-terminal portion of their DNA-binding domains. Thus, when acting from weak binding sites in vivo, transcription factors might be able to form strong complexes with dSAP18. In fact, Bicoid-dependent repression of hunchback, which depends on both SAP18 and HDAC1 (26), occurs only at the very anterior tip of blastoderm embryos where the Bicoid concentration is highest and the target gene enhancers contain multiple weak Bicoid-binding sites.

dSAP18 also interacts with the histone-specific H3K27 methyl-transferase E(z) (Enhancer of zeste) (44), a component of the polycomb group protein complex (45, 46), and with the GAGA factor (47), a transcription factor of the trxG (trithorax group) protein complex (48). Thus, dSAP18 is capable of interacting with two regulatory protein complexes that have antagonistic functions in gene regulation. Whereas the polycomb group complex acts as a repressor of homeotic genes in ectopic locations (see reviews in Refs. 49–51), the trxG complex is required for activation and maintenance of their transcription (52). However, this clear-cut distinction between polycomb group and trxG functions has been questioned, because polycomb group and trxG group members were shown to act both as context-dependent repressors and activators of transcription (53), and factors with such dual functions include both the E(z) and GAGA factor proteins (54, 55). In fact, interactions between dSAP18 and GAGA factor at the iab-6 element of the bithorax complex, for example, were shown to cause transcriptional activation and not repression (47, 56).

Our study suggests that Kr mediates repression through at least two pathways involving either dCtBP or SAP18. dCtBP-dependent and -independent repression of the transcription factors Knirps and Hairless exert quantitative effects (57, 58), whereas Kr distinguishes dCtBP and dSAP18 recruitment at different enhancers. We observed, however, that the loss of SAP18 activity does not affect the pattern of eve stripe expression and that prolonged Kr can suppress h7-lacZ expression in the absence of dSAP18. Thus, although both dSAP18 and dCtBP act independently from each other, the two corepressors, or other yet unknown corepressors, can functionally substitute for each other under forced conditions. However, their mode of repression appears to involve different mechanisms. One mechanism is exemplified by the dCtBP-dependent repression of eve-stripe 2 (22, 23) and not yet established at the molecular level. dCtBP-dependent repression does not act via unleashing local heterochromatization (59), does not require HDAC1 activity (60), and is insensitive to the HDAC inhibitor TSA (61). Consistently, communoprecipitation studies failed to detect HDAC activity in the dCtBP immunoprecipitates (62), histone H3 remained acetylated in dCtBP-deficient embryos, and transcription was not repressed (63, 64). Other studies, however, implied an association of dCtBP with HDACs (65, 66). Thus, the mechanism of the dCtBP mode of repression is not yet fully understood.

Our results showing a lack of H3 deacetylation at the eve stripe 2 enhancer in response to Kr repression are consistent with the argument that eve stripe 2-mediated repression involves the corepressor CtBP. The second, dCtBP-independent mode of Kr-dependent repression, as exemplified by the h stripe 7 element (and possibly also eve stripes 1, 3, and 4) does require both dSAP18 and HDAC1 activities. In support of this mode of repression, we observed in Kr-overexpressing embryos (i) a dSAP18-dependent loss of K9,14H3 acetylation on the h stripe 7 element, (ii) an increased resistance of the h7 enhancer DNA to sonication, and (iii) SAP18-dependent repression of the h7 reporter gene in response to Kr activity. These Kr-dependent effects were dependent on HDAC1 enzymatic activity as revealed by experiments using the HDAC1 inhibitor, TSA.
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Our results therefore suggest that dSAP18-dependent repression by Kr involves structural changes of chromatin, such as compaction or condensation, likely to be caused by site-specific heterochromatization in response to enhancer-specific HDAC1 activity.

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