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dSAP18 and dHDAC1 contribute to the functional regulation of the *Drosophila Fab-7* element

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ABSTRACT

It was described earlier that the *Drosophila* GAGA factor [*Trithorax-like* (*Trl*)] interacts with dSAP18, which, in mammals, was reported to be a component of the Sin3–HDAC co-repressor complex. GAGA–dSAP18 interaction was proposed to contribute to the functional regulation of the bithorax complex (BX-C). Here, we show that mutant alleles of *Trl*, *dsap18* and *drpd3/hdac1* enhance A6-to-A5 transformation indicating a contribution to the regulation of *Abd-B* expression at A6. In A6, expression of *Abd-B* is driven by the *iab-6* enhancer, which is insulated from *iab-7* by the *Fab-7* element. Here, we report that GAGA, dSAP18 and dRPD3/HDAC1 co-localize to ectopic *Fab-7* sites in polytene chromosomes and that mutant *Trl*, *dsap18* and *drpd3/hdac1* alleles affect *Fab-7*-dependent silencing. Consistent with these findings, chromatin immunoprecipitation analysis shows that, in *Drosophila* embryos, the endogenous *Fab-7* element is hypoacetylated at histones H3 and H4. These results indicate a contribution of GAGA, dSAP18 and dRPD3/HDAC1 to the regulation of *Fab-7* function.

INTRODUCTION

The GAGA protein of *Drosophila* is a sequence-specific DNA-binding protein that plays essential roles during development (1–3). In particular, GAGA, which is encoded by the Trithorax-like (*Trl*) gene (4), contributes to the maintenance of homeotic gene expression and to silencing. Mutant *Trl* alleles show posterior-to-anterior transformations indicating a loss of *Ubx* and *Abd-B* function (4). GAGA was also found to bind *in vitro* and *in vivo* to several polycomb-response-elements (PREs) of the bithorax complex (BX-C), and to be required for their silencing activity (5–12). In this context, GAGA was found to co-immunoprecipitate with components of the polycomb repressive complex 1 (7,13), suggesting a contribution to its recruitment.

GAGA was also shown to interact with dSAP18 (14), a polypeptide that, in mammals, associates with the Sin3–HDAC co-repressor complex (15). The GAGA–dSAP18 interaction was proposed to contribute to the regulation of BX-C (14) as, in polytene chromosomes, GAGA and dSAP18 co-localize at BX-C and deficiencies uncovering *dsap18* enhance the homeotic A6-to-A5 transformation associated with some *Trl* mutations. In this study, the contribution of dSAP18 to the regulation of *Abd-B* expression is confirmed through the analysis of mutant *dsap18* alleles. Moreover, mutations in *drpd3*/*hdac1* were also found to enhance A6-to-A5 transformation. Expression of *Abd-B* in A6 is under the control of the *iab-6* enhancer that is insulated from the *iab-7* enhancer by the *Fab-7* element. *Fab-7* contains two functionally independent elements: a PRE, responsible for polycomb-dependent silencing of the *iab-7* enhancer, and a boundary element located 5′ of the PRE (6,16). Here, we show that GAGA, dSAP18 and dRPD3/HDAC1 co-localize to ectopic *Fab-7* elements and that mutant alleles of these genes affect silencing imposed by *Fab-7*. These results indicate that GAGA, dSAP18 and dRPD3/HDAC1 contribute to the regulation of *Fab-7* function.

MATERIALS AND METHODS

Drosophila stocks

Trl, d*rpd3*/*hdac1* and *taranis* alleles used in these experiments are described previously (4,17,18). *EP*(3)*3462*, a P-element

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insertion in the 5′-untranslated region (5′-UTR) of d*sap18*, and *Df(3R)sbd*^{45}, which uncovers d*sap18* (19), were obtained from the Bloomington Stock Center. The transgenic GCD6 and 5F24(25,2) lines are described previously (20,21). *dsap18*^{18} (this study) and *dsap18*^{117} (22) were generated as imprecise excisions from *EP*(3)*3462* by P-element mobilization. *dsap18*^{18} carries ~5.4 kb of the original P-element insertion and shows no alteration of the d*sap18* open reading frame (ORF) (data not shown). *dsap18*^{117} corresponds to a deficiency of 341 bp of the 5′ region of the *dsap18* ORF and carries ~1.7 kb of the original P-element insertion (22). *dsap18*^{117} is a null *dsap18* allele as judged by northern and western analyses of *dsap18*^{117}/*Df(3R)sbd*^{45} flies (data not shown). *dsap18*$^{R7-18}$ stock was obtained from the original *dsap18*^{117} line by meiotic recombination (22). All three *dsap18* mutations used here are lethal in homozygous or *trans*-heterozygous. For the rescue experiment, a transgenic line was generated carrying a pCaSpeR vector containing ~4 kb of the 5′ region of the *dsap18* ORF and the coding sequence of *dsap18* fused to a HA-tag. Details of the construct are available upon request. The transgene was mapped onto chromosome X. Expression of dSAP18-HA protein was characterized by western and immunofluorescence analyses (data not shown) using an α-HA mouse monoclonal antibody (Roche).

Immunofluorescence analysis

Immunostaining of polytene chromosomes with rat αGAGA (1:50), rabbit αdSAP18 (1:20) and rabbit αdRPD3 (1:100) was performed according to the method of James *et al*. (23). For *in situ* hybridization the 3.6 kb long *Fab-7* element was labeled with fluorescein and used as a probe. Images were recorded in a computer-controlled Zeiss Axioplan epifluorescence microscope equipped with a cooled CCD camera (Photometrics). The fluorescent signals, recorded separately as gray-scale digital images, were pseudocoloured and were merged using Adobe Photoshop.

Analysis of the effects on silencing

To analyze the effects of different mutations on *Fab-7*-dependent silencing of the mini-*white* gene in GCD6 flies, all stocks were crossed to a *w* background. GCD6 flies homozygous for the *Fab-7*-transgene were crossed with flies heterozygous for the indicated mutations and the eye phenotype of the progeny carrying the mutations compared with their wild-type siblings.

To analyze the effects on pairing-sensitive silencing of the *sd* gene, homozygous 5F24(25,2) fly stocks carrying the different mutations to be analyzed were generated by conventional crosses.

Chromatin immunoprecipitation (ChIP) analysis

Drosophila embryos 0–18 h old were dechorionated and resuspended in ENB buffer [10% sucrose, 10 mM Tris–HCl, pH 8.0, 1 mM $CaCl_2$ and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)]. Embryos were transferred to a 15 ml dounce homogenizer, disrupted with 20 strokes and filtered. Nuclei were pelleted at 2300 *g* for 5 min at 4°C, and resuspended in buffer I (15 mM Tris–HCl, pH 7.5, 60 mM KCl, 2 mM EDTA and 1 mM DTT). Cross-linking was carried out with 1% formaldehyde in buffer I for 30 min at 4°C. To stop the cross-linking reaction glycine was added to 0.125 M. After centrifugation, nuclei were resuspended in buffer I and sonicated in a Branson sonifier set at 30% output, 10 s for three times. The sonicate was spun at 14 000 *g* for 15 min at 4°C. For immunoprecipitation assays the extract was diluted 1/10 with IP buffer (1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1 mM PMSF, 2 μg/μl aprotinin and 1 μg/μl leupeptin). Preclearing was performed by adding 2 μg single-stranded salmon sperm DNA, 1 μg preimmune serum and 30 μl of equilibrated Protein A beads (Protein A–Sepharose CL-4B; Amersham Biosciences) in IP buffer and samples were rotated for 1 h at 4°C. Beads were removed by centrifugation and the appropriate antibody (2 μg of anti-acetyl-Histone H3 ref. 06-599 upstate and 2 μl anti-acetyl-Histone H4 ref. 06-866 upstate) was added and incubated overnight at 4°C with gentle mixing. Immunocomplexes were purified by adding 2 μg single-stranded salmon sperm DNA and 50 μl of equilibrated Protein A beads and were incubated for 3 h at 4°C. Beads were recovered by centrifugation for 2 min at 3000 *g* and washed sequentially with TSE I buffer (1% Triton X-100, 0.1% SDS, 2 mM EDTA, 20 mM Tris–HCl, pH 8.0 and 150 mM NaCl), TSE II buffer (1% Triton X-100, 0.1% SDS, 2 mM EDTA, 20 mM Tris–HCl, pH 8.0 and 500 mM NaCl), buffer III (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA and 10 mM Tris–HCl, pH 8.0) and twice with TE. Samples were extracted for three times with 100 μl of elution buffer (1% SDS and 0.1 M $NaHCO_3$) and were incubated for 6 h at 65°C to reverse formaldehyde cross-links. DNA was purified by GFX™ PCR Kit (Amersham Biosciences) and was resuspended in 50 μl. Input samples were obtained using 10% of the sonicated chromatin solution used for immunoprecipitation reactions. PCRs were performed by standard procedures with 2 μl of undiluted samples and 1/5 and 1/25 dilutions. The following primer pairs were used: boundary, 5′-TTGCGGTGGTGTGCGTGC-3′ and 5′-TCAAGCTGTGTGGCGGGG-3′; PRE, 5′-GTCGGCAATTCGGATTCCC-3′ and 5′-TTCGGTCGCTCACGTCGC-3′; Fab7-X, 5′-GTAGGTGCAAAAGGCGATG-3′ and 5′-TCAATCCACACGCACTGCC-3′; Trl, 5′-AGTGGGCAGTGATGGAGCAG-3′ and 5′-ATGATTGAAGGCTCGGCTGG-3′; and Sap18, 5′-GTGCGATAGGATTGCTGC-3′ and 5′-GTTGGGGGTACACGACAGC-3′. The amplified DNA was separated on 1% agarose gels and visualized by using ethidium bromide. Quantification of the results was carried out by determining for each genomic region the fold-enrichment obtained after immunoprecipitation with respect to the background precipitation obtained in the absence of any added antibody. Relative enrichment was determined by normalizing the fold-enrichment obtained for each genomic region for that corresponding to the *dsap18* promoter region. Results are the average of three independent experiments.

RESULTS

Mutations in *Trl*, *dsap18* and *drpd3*/*hdac1* enhance A6-to-A5 transformation

It was shown elsewhere (4) that males homozygous for the hypomorphic *Trl*^{13C} allele contain bristles on the sixth sternite, which in the wild-type is devoid of any bristles, indicating that cells of A6 have acquired an A5 identity. This homeotic

Table 1. Frequency of the homeotic A6-to-A5 transformation in different genetic backgrounds

Genotype	N^a	0 (%)	⩽2 (%)	>2 (%)
Effect of *Trl*				
$Trl^{67}/Df(3R)sbd^{45}$	209	44	28	28
$Trl^{67}/+$	135	94	6	0
$Df(3R)sbd^{45}/+$	190	82	13	5
Effect of *dsap18*				
$dsap18^{117}/Df(3R)sbd^{45}$	54	20	46	34
$dsap18^{117}/+$	92	96	4	0
$dsap18^{R7-18}/Df(3R)sbd^{45}$	59	14	51	35
$dsap18^{R7-18}/+$	48	93	7	0
$dsap18^{117}/Df(3R)sbd^{45}$; dsap18/+[b]	87	50	46	4
$dsap18^{18}/Df(3R)sbd^{45}$	134	33	55	12
$dsap18^{18}/+$	93	80	20	0
EP(3R)3462/$Df(3R)sbd^{45}$	31	38	54	8
EP(3R)3462/+	50	98	2	0
$Trl^{67}/dsap18^{117}$	124	99	1	0
Effect of *drpd3/hdac1*				
$HDAC^{def24}/Df(3R)sbd^{45}$	51	14	27	59
$HDAC^{def24}/+$	244	46	41	13
$HDAC^{303}/Df(3R)sbd^{45}$	116	4	43	53
$HDAC^{303}/+$	59	98	2	0
$HDAC^{313}/Df(3R)sbd^{45}$	393	40	38	22
$HDAC^{313}/+$	56	98	2	0

The percentage of males containing 0, ⩽2 and >2 bristles in the sixth sternite is presented as a function of the indicated genotypes.
[a]*N* indicates the number of males scored.
[b]Flies carry one copy of a transgene expressing wild-type *dsap18* under the control of its own promoter.

transformation results from a partial loss of *Abd-B* function at A6 and, therefore, indicates a contribution of GAGA to the regulation of *Abd-B*. This transformation is enhanced in flies heterozygous for the null Trl^{67} allele and hemizygous for a deficiency uncovering *dsap18* (14). Approximately 60% of $Trl^{67}/Df(3R)sbd^{45}$ males showed A6-to-A5 transformation, as judged by the presence of at least one bristle at the sixth sternite (Table 1, effect of *Trl* genotype). This transformation is infrequent in $Trl^{67}/+$ (6%) or $Df(3R)sbd^{45}/+$ (18%) males. Similar results were obtained with other mutant *Trl* alleles (data not shown). This genetic interaction might reflect a contribution of dSAP18 to the regulation of *Abd-B* expression. Confirming this hypothesis, a null $dsap18^{117}$ mutation (22) shows a strong A6-to-A5 transformation with 80% of $dsap18^{117}/Df(3R)sbd^{45}$ males containing at least one bristle in the sixth sternite (Table 1, effect of *dsap18* genotype). Similar results were obtained with $dsap18^{R7-18}$, a stock derived from $sap18^{117}$, where recessive background mutations were removed by meiotic recombination (22) (Table 1, effect of *dsap18* genotype). Other *dsap18* mutations, such as *EP(3R)3462* and $dsap18^{18}$, show a significantly weaker transformation (Table 1, effect of *dsap18* genotype). The transformation observed in $dsap18^{117}/Df(3R)sbd^{45}$ flies is significantly rescued by a transgene expressing dSAP18 under the control of its own promoter, as the number of males containing at least one bristle in the sixth sternite is reduced from 80%, in the absence of the transgene, to 50%, in flies carrying the transgene in the heterozygous condition. Moreover, the intensity of the transformation is strongly reduced as the frequency of flies showing >2 bristles highly diminishes in the presence of the transgene (from 34 to 4%) (Table 1, effect of *dsap18* genotype). Altogether, these results demonstrate the contribution of *dsap18* to the regulation of A6 identity.

In mammals, SAP18 was found to be associated with the Sin3–HDAC co-repressor complex (15). Therefore, we analyzed whether mutations in d*rpd3*/*hdac1* also enhance the A6-to-A5 transformation. Three different mutant alleles were analyzed: deficiency $HDAC1^{def24}$, a null mutation in which most of the 5′-UTR region into the second exon is deleted, and two specific missense mutations, $HDAC1^{303}$ and $HDAC1^{313}$, each carrying single amino acid substitutions in highly conserved protein regions (18). As shown in Table 1 (effect of *drpd3*/*hdac1* genotype) all three mutants show intense A6-to-A5 transformation in *trans*-heterozygous to $Df(3R)sbd^{45}$, as shown by the high frequency of flies containing >2 bristles in the sixth sternite, with some individuals having up to 10 bristles. These results indicate that similar to dSAP18, dRPD3/HDAC1 also participates in the regulation of *Abd-B* expression at A6 and are consistent with the association of dSAP18 with the Sin3–HDAC complex.

The homeotic A6-to-A5 transformation observed in $Trl^{67}/Df(3R)sbd^{45}$ or $HDAC1^{def24}/Df(3R)sbd^{45}$ flies cannot be attributed only to a loss of *dsap18* function since no transformation is detected in $Trl^{67}/dsap18^{117}$ (Table 1, effect of *dsap18* genotype) or $HDAC1^{def24}/dsap18^{117}$ flies (data not shown), indicating that additional elements contained within the genomic region uncovered by $Df(3R)sbd^{45}$ are also contributing to the observed effects. In particular, $Df(3R)sbd^{45}$ uncovers *taranis*, an essential trithorax gene (17), which could contribute to the observed homeotic transformation. Null *taranis* alleles, as $tara^{L4}$, are lethal in *trans*-heterozygous to $Df(3R)sbd^{45}$. However, no significant homeotic transformation is observed in flies *trans*-heterozygous for $tara^{L4}$ and Trl^{67}, $dsap18^{117}$, $HDAC^{def24}$ or $HDAC^{313}$ (data not shown), indicating that the homeotic transformation described above is not due only to a loss of *taranis* function either.

GAGA, dSAP18 and dRPD3/HDAC1 co-localize at ectopic *Fab-7* copies

At A6, the expression of *Abd-B* is regulated by the *iab-6* enhancer. Therefore, the homeotic transformation observed in the mutant conditions described above could reflect their contribution to the activation of *iab-6*. It is also possible that GAGA, dSAP18 and dRPD3/HDAC1 are required for the function of the *Fab-7* element, so that in their absence, *iab-6* is not insulated efficiently from the negative regulators that maintain silencing of *iab-7* in A6.

To test this hypothesis, we performed immunolocalization experiments to determine whether GAGA, dSAP18 and dRPD3/HDAC1 were present at the *Fab-7* element. For these experiments, we used the transgenic GCD-6 line, which carries, at position 61C9, a transgene containing two copies of the *Fab-7* element flanking a UAS-*lacZ* construct upstream of a mini-*white* gene (20). As shown in Figure 1A, in polytene chromosomes from GCD-6 flies, a strong αGAGA signal is observed at position 61C9 (indicated by the arrow), which overlaps with the *in situ* signal corresponding to the transgene, and is not detected in wild-type ORE-R flies. This additional αGAGA signal is located close to a significantly less intense endogenous αGAGA band that is observed both in

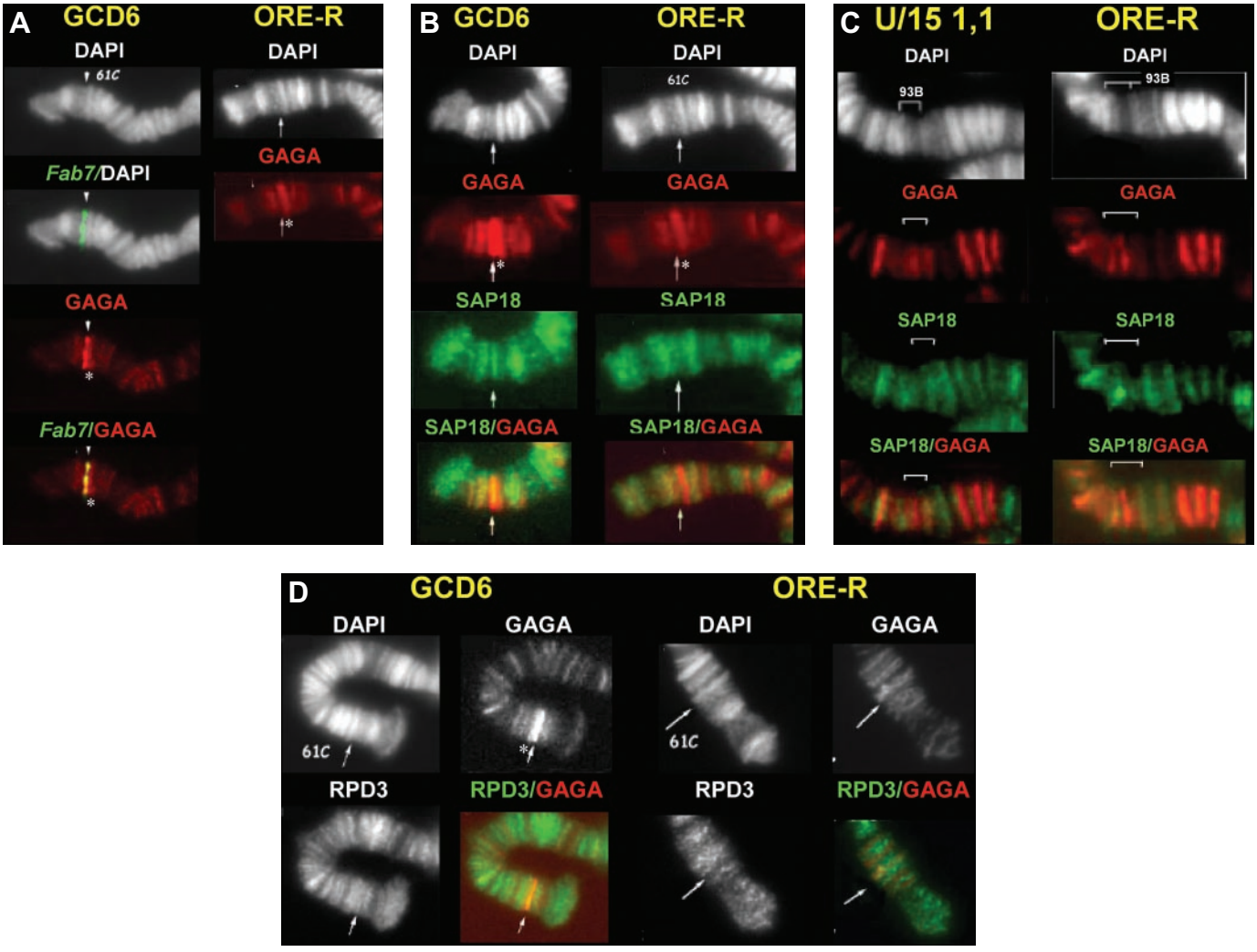

Figure 1. GAGA, dSAP18 and dRPD3/HDAC1 localize at ectopic *Fab-7* elements. (**A**) The immunolocalization pattern of GAGA (red) is presented for GCD6 (left) and ORE-R (right) polytene chromosomes. In GCD6, the position of insertion of the transgene (61C9) was determined by *in situ* hybridization (green) using the 3.6 kb long *Fab-7* element as probe. (**B**) Immunolocalization patterns of GAGA (red) and dSAP18 (green) in GCD6 (left) and ORE-R (right) chromosomes. (**C**) Immunolocalization patterns of GAGA (red) and dSAP18 (green) in U/15 1,1 (left) and ORE-R (right) chromosomes. The site of insertion of the transgene in U/15 1,1 chromosomes (93B) is indicated. (**D**) Immunolocalization patterns of GAGA and dRPD3/HDAC1 in GCD6 (left) and ORE-R (right) chromosomes. In the merge, GAGA and dRPD3/HDCA1 were pseudocoloured in red and green, respectively. Arrows indicate the position of the αGAGA signal associated with the presence of the transgene. Asterisks indicate the position of an endogenous αGAGA signal adjacent to the site of insertion of the transgene in GCD6 that is also present in ORE-R chromosomes.

wild-type and GCD-6 chromosomes (indicated by an asterisk in Figure 1).

As shown in Figure 1B, dSAP18 also localizes to the position of the transgene in GCD-6 chromosomes. A sharp αdSAP18 signal is detected at position 61C9 overlapping with the additional αGAGA signal that marks the position of the transgene but not with the endogenous αGAGA signal mentioned above. This αdSAP18 signal is not present in ORE-R chromosomes. Localization of GAGA and dSAP18 to the transgene is associated with the presence of the *Fab-7* element in the construct since no recruitment is observed in a control transgenic U/15 1,1 line that, at position 93B, carries a transgene similar to that in GCD-6, but missing the two *Fab-7* elements (20). The immunolocalization patterns of GAGA and dSAP18 at region 93B show no significant differences in polytene chromosomes from U/15 1,1 flies compared with ORE-R (Figure 1C). Recruitment of dRPD3/HDAC1 to the transgene was also tested (Figure 1D). In this case, the analysis was more difficult due to the large number of αdRPD3/HDAC1 bands detected at position 61C9. Actually, as described by others (24), the global immunolocalization pattern of dRPD3/HDAC1 in polytene chromosomes is much more complex than the patterns of GAGA and dSAP18. Nevertheless, in GCD-6 chromosomes, a faint αdRPD3/HDAC1 band could be detected that co-localizes with the additional αGAGA band associated with the presence of the transgene and which does not appear to be present in ORE-R chromosomes.

d*sap18* and d*rpd3*/*hdac1* mutations affect *Fab-7*-dependent silencing

Fab-7 is required to maintain silencing at *iab-7* and ectopic *Fab-7* constructs impose silencing on flanking reporter genes

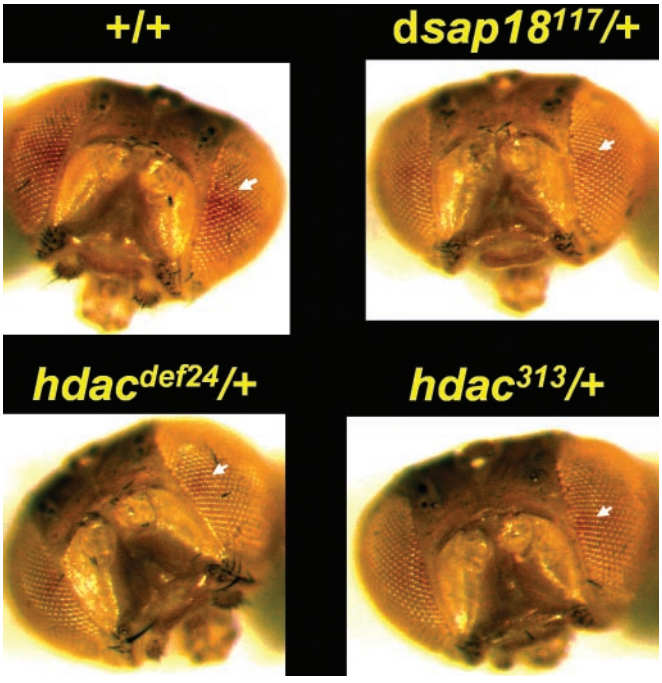

Figure 2. Mutations in *dsap18* and *drpd3*/*hdac1* enhance *Fab-7*-dependent silencing of the mini-*white* gene in GCD6 flies. The eye phenotypes of flies of the indicated genotypes carrying one copy of the *Fab-7*-transgene are presented. Arrows indicate the position of the red triangle characteristic of the eye phenotype of GCD6 flies.

(20,21,25,26). In the transgenic GCD-6 line, the expression of the mini-*white* gene is silenced *in cis* by *Fab-7* so that strong silencing is observed in flies carrying the transgene in a heterozygous state. Heterozygous GCD-6 flies show strongly patterned variegated eyes with a red triangle in a pale yellow background (Figure 2, +/+). Silencing imposed by *Fab-7* is known to depend on PcG (20,21). Consistent with these observations, in heterozygous GCD-6 flies, the mutant Pc^3 allele relieves silencing of the mini-*white* gene (data not shown). In contrast, *cis*-silencing by *Fab-7* is enhanced in GCD-6 flies heterozygous for the Trl^{13C} mutation (20). A similar enhancement is observed in the presence of other mutant *Trl* alleles, such as Trl^{67} (data not shown). Here, the effects of mutations in *dsap18* and *drpd3*/*hdac1* on *Fab-7*-dependent *cis*-silencing were also analyzed. In heterozygous GCD-6 flies, *Fab-7*-silencing is enhanced by the presence of the mutant $dsap18^{117}$ allele, where the red triangle characteristic of the eye phenotype of GCD6 flies is hardly detectable (Figure 2). Similarly, *drpd3*/*hdac1* mutations, such as $hdac^{def24}$ and $hdac^{313}$, also enhance *Fab-7*-dependent *cis*-silencing (Figure 2).

Fab-7 is also known to mediate silencing *in trans*, so that, in some transgenic lines, *Fab-7*-mediated silencing is pairing-sensitive being observed only when the transgene is in a homozygous state (6,21,26). In the GCD-6 line described above, silencing of the mini-*white* gene is not pairing-sensitive; heterozygous flies show strong silencing and a higher expression is observed in the homozygous condition. On the contrary, a different transgenic line, the 5F24(25,2), that carries a similar transgene as GCD-6 but missing one of the two *Fab-7* elements, shows strong silencing only in the homozygous condition (26). In the 5F24(25,2) line, two copies of the transgene

Table 2. Penetrance of the *sd* phenotype in different genetic backgrounds

Genotype	N^a	0 (%)	1 (%)	2 (%)
wt	64	12	16	72
$dsap18^{117}/Df(3R)sbd^{45}$	54	83	13	4
$dsap18^{117}/+$	59	25	39	36
$HDAC^{def24}/+$	61	44	26	30
$Trl^{67}/+$	94	17	32	51

The percentage of homozygous 5F24(25,2) females showing normal wings (0) and wing blade destruction in one (1) or both (2) wings is presented as a function of the indicated genotypes.
[a]*N* indicates the number of females scored.

are inserted in tandem, at position 13F on the X-chromosome, 9.6 kb upstream from the scalloped (*sd*) gene that is involved in wing development (27). It was shown earlier that, in the 5F24(25,2) line, the *Fab-7* insertion silences *sd* expression in a pairing-dependent manner (26). Reduced expression of the *sd* gene causes characteristic wing defects, from small lesions in the margin to complete destruction of the wing blade. As shown previously (26), homozygous 5F24(25,2) females manifest a strong *sd* phenotype with a high penetrance; destruction of the two wings is observed in 72% of the individuals and 16% showed destruction of at least one wing (Table 2). Heterozygous females or hemizygous males showed no *sd* phenotype at all (data not shown) (26). The *sd* phenotype of homozygous 5F24(25,2) females is strongly suppressed in *trans*-heterozygous $dsap18^{117}/Df(3R)sbd^{45}$ flies (Table 2), with only 4% of the individuals showing destruction of both wings. Significant suppression is also observed in heterozygous $dsap18^{117}$ flies (Table 2), which showed destruction of both wings only in 36% of the individuals. Mutations in *Trl* and *drpd3*/*hdac1* showed similar effects (Table 2). In the presence of the $HDAC^{def24}$ mutation a similar suppression of the *sd* phenotype is detected with only 30% of the flies showing destruction of the two wings (Table 2). On the other hand suppression by the Trl^{67} mutation was slightly weaker with destruction of both wings observed in up to 51% of the females (Table 2). A similar suppression of *Fab-7*-dependent pairing-sensitive silencing was reported earlier in the presence of a different Trl^{13C} mutant allele (6,10).

Histones at the endogenous *Fab-7* element of BX-C are hypoacetylated

The results reported above indicate a contribution of *drpd3*/*hdac1* to the regulation of *Fab-7* function suggesting that chromatin at the endogenous *Fab-7* element of BX-C is likely to be hypoacetylated. Indeed, as judged by ChIP-analysis, both the PRE and the boundary elements of *Fab-7* are significantly hypoacetylated (Figure 3). In these experiments, cross-linked chromatin from *Drosophila* embryos was subjected to immunoprecipitation with α-acetylH3 antibodies, recognizing histone H3 acetylated at residues K9 and K14, and with α-acetylH4 antibodies, recognizing histone H4 polyacetylated at residues K5, K8, K12 and K16. Immunoprecipated material was then analyzed by PCR for relative enrichment in specific regions of the *Fab-7* element in comparison with other genomic locations, namely the *dsap18* promoter. As shown in Figure 3B, both the PRE and

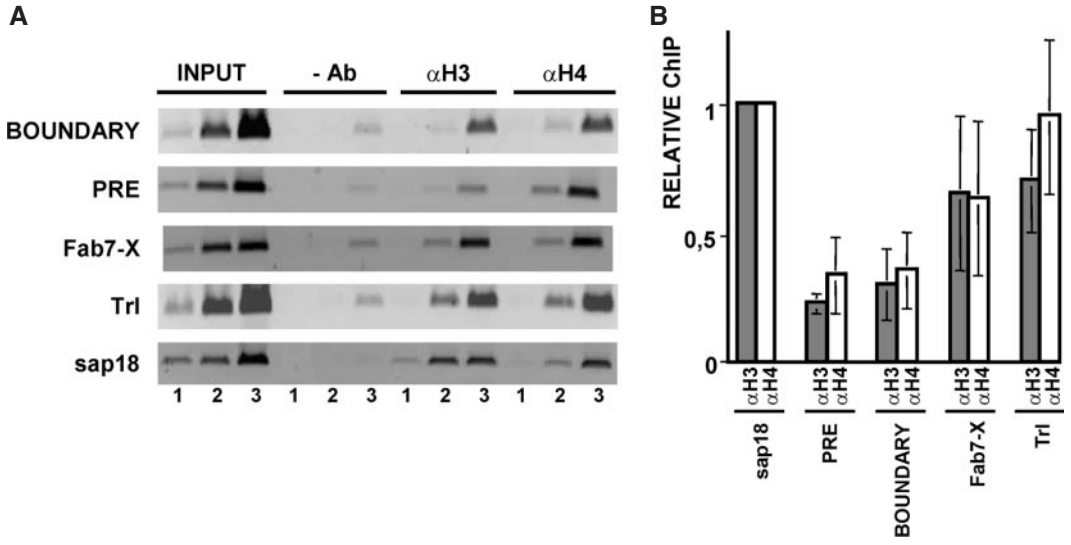

Figure 3. The endogenous *Fab-7* element of BX-C is hypoacetylated. (**A**) Cross-linked chromatin from *Drosophila* embryos was immunoprecipitated with αacetylH3 (lanes αH3), αacetylH4 (lanes αH4) or no antibodies (lanes –Ab). Immunoprecipitated material was analyzed by PCR using specific primers of different *Fab-7* regions: boundary, PRE and a proximal region located 1 kb from the boundary (Fab7-X), the *dsap18* promoter, and the *Trl* coding regions. PCRs were carried out with increasing amounts of immunoprecipitate: 0.08, 0.4 and 2 µl (lanes 1–3). Lanes INPUT correspond to PCR products obtained before immunoprecipitation from 10% of the material used for the immunoprecipitation. (**B**) Quantification of the results shown in (A). Relative enrichment with respect to the *dsap18* promoter region is presented for each genomic region analyzed.

the boundary regions of *Fab-7* are poorly represented in the immunoprecipitated material when compared with the *dsap18* promoter region (Figure 3B, columns PRE and boundary). In contrast, a proximal *Fab-7* region located ~1 kb from the boundary shows a higher relative enrichment (Figure 3B, columns Fab7-X) similar to that observed for the *Trl* coding region (Figure 3B, columns *Trl*). These results indicate that, in *Drosophila* embryos, the endogenous *Fab-7* of BX-C is hypoacetylated at the boundary and PRE regions. It was reported earlier that, in cultured S2 cells, the *Fab-7* PRE is methylated at histone H3 (28,29), which is in agreement with our results as deacetylation is a pre-requisite for histone methylation.

DISCUSSION

Here, we have shown results indicating that GAGA, dSAP18 and dRPD3/HDAC1 contribute to the function of the *Fab-7* element of BX-C. This conclusion is based on the following observations:

(i) the localization of GAGA, dSAP18 and dRPD3/HDAC1 at ectopic *Fab-7* elements.

(ii) the effects of *Trl*, *dsap18* and *drpd3*/*hdac1* mutations on *Fab-7*-dependent silencing. Ectopic *Fab-7* constructs are known to mediate silencing of flanking reporter genes (20,21,25,26) both *in cis*, as in heterozygous GCD6 flies (20,21), as well as *in trans*, as in 5F24(25,2) flies (26), where silencing is pairing-sensitive being observed only when the transgene is in a homozygous state (26). Here, we have shown that *Trl*, *dsap18* and *drpd3*/*hdac1* mutations affect both *cis*- and *trans*-silencing mediated by *Fab-7*.

(iii) the homeotic A6-to-A5 transformation observed in flies heterozygous for various *Trl*, *dsap18* and *drpd3*/*hdac1* mutant alleles and hemizygous for *Df(3R)sbd*^{45}, which uncovers *dsap18*. This homeotic transformation results from the ectopic repression of the *iab-6* enhancer at A6 that is insulated from the repressed *iab-7* enhancer by the *Fab-7* element. The fact that this homeotic transformation is very infrequent in hemizygous *Df(3R)sbd*^{45} flies, as well as in the heterozygous mutants, demonstrates that it is directly associated to the *Trl*, *dsap18* and *drpd3*/*hdac1* mutations. Moreover, a single copy of a transgene expressing *dsap18* significantly rescues this phenotype. Our results also indicate that an unidentified element(s) contained within *Df(3R)sbd*^{45} is also contributing to the establishment of the phenotype. In addition to *sap18*, *Df(3R)sbd*^{45} uncovers at least 11 other genes including the trithorax gene, *taranis* (17). However, the homeotic transformation described here does not appear to be associated to a loss of *taranis* function as no transformation is observed in flies *trans*-heterozygous for a null *taranis* allele and *Trl*, *dsap18* or *drpd3*/*hdac1* mutations.

Together, these results indicate a contribution of GAGA, dSAP18 and dRPD3/HDAC1 to the structural and functional properties of *Fab-7*. What could this contribution be? Several models might account for our results. *Fab-7* is known to contain two functional elements: a PRE, which is required for Pc-dependent silencing, and an adjacent boundary element that insulates *iab-6* from *iab-7* (6,16). The finding that, in heterozygous GCD6 flies, mutant *Trl*, *dsap18* and *drpd3*/*hdac1* alleles enhance *cis*-silencing imposed by *Fab-7* suggests that their functions might antagonize Pc-dependent silencing. Several observations, however, make this hypothesis unlikely. First, at some PREs, GAGA helps recruitment of PcG complexes and contributes to silencing (7,8). Second, dRPD3/HDAC1 was shown to be a component of several

PcG complexes (13,30), and genetic analysis indicates a contribution to homeotic silencing (31). Finally, in mammals, SAP18 acts as a repressor when targeted to an active promoter (15).

An alternative possibility is that GAGA, dSAP18 and dRPD3/HDAC1 contribute to the function of the *Fab-7* boundary element. In fact, the *Fab-7* boundary contains several GAGA-binding sites that are required for its enhancer blocking activity (32) and, as shown here, it is hypoacetylated at histones H3 and H4. In GCD-6 flies, the *Fab-7* boundary element is located proximal to the reporter mini-*white* gene with respect to the PRE (20) so that it might help to insulate the reporter gene from repression by the PRE. In this context, mutations that affect boundary function would result in a less efficient insulation and, therefore, would enhance silencing.

In contrast to the enhancer effect observed in heterozygous GCD6 flies, mutations in *Trl*, *dsap18* and *drpd3*/*hdac1* suppress pairing-dependent *trans*-silencing in 5F24(25,2) flies. A contribution to boundary-functions might also account for this effect. Pairing-sensitive *trans*-silencing results from long-distance chromosomal interactions that involve the association of the transgenes with each other and with the endogenous *Fab-7* element, even when located in different chromosomes (26). These long-distance interactions that require the contribution of PcG proteins might be facilitated by a functional boundary element as was described previously for the gypsy insulator (33,34).

The incomplete A6-to-A5 homeotic transformation observed in the presence of *Trl*, *dsap18* and *drpd3*/*hdac1* mutations might also reflect a contribution to the boundary function of *Fab-7* as, in the mutant conditions, it might not properly insulate the *iab-6* enhancer from the repressing activity of the *Fab-7* PRE, thereby becoming partially inactivated. Interestingly, mutations that delete the *Fab-7* boundary but not the PRE produce, in addition to strong A6-to-A7 transformation, incomplete A6-to-A5 transformation (16). Moreover, replacement of the *Fab-7* boundary by the gypsy or the scs insulator, which are not functional in the context of BX-C, results in complete A6-to-A5 transformation (35).

Our results indicate that GAGA, dSAP18 and dRPD3/HDAC1 have similar effects on the functional properties of *Fab-7* suggesting a functional link. A physical interaction between GAGA and dSAP18 was reported earlier (14). Moreover, in mammals, SAP18 was found to be associated with the Sin3–HDAC co-repressor complex (15) and, in *Drosophila*, dSAP18 modulates bicoid activity through the recruitment of dRPD3/HDAC1 (19) and it is required to suppress bicoid activity in the anterior tip of the embryo (22). In this context, it is tempting to speculate that GAGA helps in the recruitment of dSAP18 and dRPD3/HDAC1 to *Fab-7* resulting in a concerted contribution to its boundary function.

In mammals, SAP18 was also found to be associated with ASAP, a protein complex involved in RNA processing (36). In *Drosophila*, dSAP18 could also participate in RNA processing as, in cultured S2 cells, a large proportion of dSAP18 co-immunoprecipitates with factors that participate in RNA processing (M.L. Espinás *et al*., unpublished data). It is possible that, in response to cellular signals, the association of dSAP18 to different protein complexes would be regulated during development and/or cell cycle progression.

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