Published online 8 September 2009 *Nucleic Acids Research, 2009, Vol. 37, No. 20* ***6665–6680***
doi:10.1093/nar/gkp722

The loss of histone H3 lysine 9 acetylation due to dSAGA-specific *dAda2b* mutation influences the expression of only a small subset of genes

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Received March 22, 2009; Revised July 23, 2009; Accepted August 16, 2009

ABSTRACT

In *Drosophila,* the *dADA2b*-containing dSAGA complex is involved in histone H3 lysine 9 and 14 acetylation. Curiously, although the lysine 9- and 14-acetylated histone H3 levels are drastically reduced in *dAda2b* mutants, these animals survive until a late developmental stage. To study the molecular consequences of the loss of histone H3 lysine 9 and 14 acetylation, we compared the total messenger ribonucleic acid (mRNA) profiles of wild type and *dAda2b* mutant animals at two developmental stages. Global gene expression profiling indicates that the loss of dSAGA-specific H3 lysine 9 and 14 acetylation results in the expression change (up- or down-regulation) of a rather small subset of genes and does not cause a general transcription de-regulation. Among the genes up-regulated in *dAda2b* mutants, particularly high numbers are those which play roles in antimicrobial defense mechanisms. Results of chromatin immunoprecipitation experiments indicate that in *dAda2b* mutants, the lysine 9-acetylated histone H3 levels are decreased both at dSAGA up- and down-regulated genes. In contrast to that, in the promoters of dSAGA-independent ribosomal protein genes a high level of histone H3K9ac is maintained in *dAda2b* mutants. Our data suggest that by acetylating H3 at lysine 9, dSAGA modifies Pol II accessibility to specific promoters differently.

INTRODUCTION

Histone acetyltransferase (HAT) complexes play a role in chromatin structure modifications which might lead to changes in the gene expression (1). The GCN5 (general control nonderepressed 5) protein is the catalytic component of several multiprotein HAT complexes, which modifies chromatin structure by acetylating specific lysine residues at the N-terminal tails of histone H3 and H4. Many of the GCN5-containing HAT complexes also contain ADA-type adaptor proteins, which play roles in modulating HAT activity and specificity (2,3). In *Saccharomyces cerevisiae* for example, Ada2p is present in the Spt-Ada-Gcn5-acetyltransferase (SAGA), SAGA-like (SLIK), alteration/deficiency in activation (ADA), and HAT-A2 GCN5-HAT complexes (4–6). These complexes are involved in transcription activation, and in accord with that, Ada2p was originally discovered as it was necessary for transcription activation by acidic activators such as Gcn4 and VP16 (7,8). For some of these complexes, however, further roles in additional processes have been recognized recently (9).

In *Drosophila,* two related ADA2-type factors (dADA2a and b) have been identified (10,11). Several lines of evidence indicate that the two dADA2 proteins are specific components of different GCN5 HAT complexes. dADA2a is present in the 0.6 MDa ATAC (Ada2a-containing) complex, which acetylates histone H4 at lysine K5 and K12 (12,13). *dADA2b* is present in the 1.8 MDa dSAGA complex. dSAGA is involved in the post-translational modification of nucleosomal histone H3 at K9 and K14 (14,15). Recently, we and others demonstrated that *dAda2b* mutations result in a significant decrease in the level of K14 and K9 acetylated histone H3

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(14,15). A decrease in histone H3K9ac and H3K14ac levels in *dAda2b* mutants is detectable all along with the polytene chromosomes including highly compacted bands, suggesting that global histone H3 acetylation is dependent on *dADA2b* (14,15). Interestingly, despite the dramatic decrease in the levels of H3K9ac and H3K14ac by the later developmental stages, *dAda2b* null animals reach almost full development and die only as pupae. The ability of flies to nearly complete development in the absence of *dADA2b* is surprising as H3K9 and H3K14 acetylation generally correlates with transcription activation; in yeast, genome-wide mappings indicated the presence of Gcn5p, and high levels of H3K9ac and H3K14ac at promoters of actively transcribed genes (16,17). Gcn5p, however, is present in several yeast HAT complexes which have overlapping patterns of acetylation with complexes containing the Sas3p acetyltransferase (18).

In *S. cerevisiae*, deletion of *Ada2* inhibits the global transcriptional response to glucose (19). This might be interpreted as indication of a role for SAGA in the coordinated expression of functionally-related genes. In accord with this, acetylation clusters were proposed to define groups of genes with related expression patterns (20). In mammalian cells, high levels of histone H3K9ac and H3K14ac together with H3K4me were found to define chromatin regions permissive for transgene expression. In contrast with that, reduced H3K9ac, H3K14ac and H3K4me levels were accompanied by the progressive silencing of transgenes (21). Thus, the role of histone H3K9/K14 acetylation in transcription regulation is demonstrated; however, the question, whether the SAGA-deposited histone marks affect primarily global or promoter specific transcription in a multicellular eukaryote remains to be elucidated.

Here we report the effects of the loss of *dADA2b*-dependent histone H3 acetylation in a multicellular organism. We took advantage of *dADA2b* being a specific component of dSAGA and performed whole genome ribonucleic acid (RNA) profiling of *dAda2b* null mutants to reveal the function of dSAGA in the late stages of *Drosophila* development. Our aims were to uncover whether the altered expression of specific genes in the absence of dSAGA is in correlation with the (i) localization; (ii) expression level and/or; (iii) biological function of the affected genes.

MATERIALS AND METHODS

Drosophila melanogaster strains

Fly stocks were raised at 25°C on standard *Drosophila* medium. The null allele $dAda2b^{d842}$ used in this work has been described (14). As a control, in some of these experiments we used w^{1118}, which is an isogenized strain constructed in the DROSDEL project (22). The mutant chromosomes were kept over TM6c, Tb, Sb balancer. For the construction of dAda2bL-enhanced green fluorescent protein (EGFP) transgene (for short hereafter referred as Ada2bEGFP), the 5′ half of the *dAda2b* gene was amplified using primers Ada2bRI and Ada2bBHI (Table 1 for primer sequences), and were inserted into pBluescriptKS (pKS) (Fermentas). The 3′ part of the gene was amplified without translational stop codons using primers Ada2bL3′BamHI and Ada2bNco, and the obtained fragment was combined with the 5′ region in pKS using NcoI and BamHI. A fragment encoding the EGFP tag was isolated from pEGFPN3 (Clontech) and joined to the 3′-end of *dAda2b* gene by BamHI and NotI restriction endonucleases. The *dAda2b* promoter was amplified using primers Ada2bgene and Ada2bgeneL, and assembled with the coding region in pKS using BglII and SalI. Finally, the fragment corresponding to the dAda2b cDNA with its regulatory region (up to −400) with an EGFP coding region attached to the 3′-end was inserted into pUASP. With this plasmid transgenic lines were established using the standard embryo injection protocol. For rescue the following genotypes were used: *w/w; +/+; P{Ada2bEGFP}* $dAda2b^{d842}$/$dAda2b^{d842}$ and *w/w; +/+; P{Ada2bEGFP}* $dAda2b^{d842}$/*P{Ada2bEGFP}* $dAda2b^{d842}$.

The *attacin A* and *defensin* promoter fused GFP-containing att-GFP and def-GFP transgene carrier stocks were kindly provided by Dr J.L. Imler and Dr B. Lemaitre (23). For the detection of GFP expression under the control of *att* and *def* promoters, the transgenes were transferred to the desired genetic background as indicated in the Figure 5 by genetic crosses, and GFP expression in the resulting offspring was examined with an OLYMPUS SZX-12 microscope using GFP band-pass filter. Photos were taken with an OLYMPUS C7070WZ camera using identical settings for mutants and controls.

Western blot

For protein analysis by immunoblot total protein samples of *dAda2b* and w^{1118} control animals at developmental stages as indicated in figure legends were separated on SDS–PAGE and transferred by electroblotting to nitrocellulose membrane. The membranes were blocked for 1 h in 5% nonfat dry milk in TBST (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) and incubated overnight with primary antibody diluted in 2% BSA (Sigma) TBST. For the detection of dADA2b, polyclonal antibodies raised in rabbits against a dADA2b-specific peptide (10), and for the detection of histone H3K9ac (Abcam) and H3K14ac (Upstate), commercially available polyclonal antibodies were used as recommended by the supplier. Membranes were washed, incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (DACO), washed again extensively, and developed using the ECL (Millipore) kit following the manufacturer's recommendations.

Immunostaining

Polytene chromosome spreads obtained from the salivary glands of wandering *dAda2b* and w^{1118} larvae were fixed in 3.7% formaldehyde dissolved in phosphate-buffered saline (PBS) followed by incubation in 45% acetic acid. Slides were blocked in PBS supplemented with 5% fetal calf serum and 0.1% Tween-20 for 1 h at 25°C and incubated

Table 1. Oligonucleotides used as PCR primers for plasmid constructions, for the determination of RNA levels by RT-PCR, and in ChIP experiments. The numbers in parenthesis at the names of primers used in ChIP experiments indicate the position of the primer relative to the transcription start site of the gene

Primer	Sequence 5′–3′
18S fwd	GCCAGCTAGCAATTGGGTGTA
18S rev	CCGGAGCCCAAAAAGCTT
Lcp1 fwd	TTTCCCAATCCGATGATGTT
Lcp1 rev	GGCTGGTATCCGTTCTCATT
Lcp4 fwd	TCAAGATCCTGCTTGTCTGC
Lcp4 rev	CACTCGAAAACTCCGTCGAT
Eig71Ec fwd	CTCGGTGCGAATTGTCTCTG
Eig71Ec rev	ACGGGTAGTTGGGGTCCTAC
Eig71Ed fwd	ATGTGAACGCTGTGTGGAAA
Eig71Ed rev	GCCAGCGAGTTCAGCAATA
Eig71Eg fwd	TGGCTTTCTGCTGCATATTG
Eig71Eg rev	CCAGCTCACAACGGGTTAAT
Eig71Eh fwd	TGACTGTCTGCTTCCTGGTG
Eig71Eh rev	CCTGGAGTTTGGAGTCAC
Ada2bRI	GCATGAATTCATGACCACAATCGCGGATTT
Ada2bBHI	CGATGGATCCCCGACAGCTATCCAA
Ada2bNco	CCATATGGCCATGGCAAG
Ada2bgene	TTTAATCCTGACCACCGCT
Ada2bgeneL	CAGGGTGGGTCGATTATGTTG
Ada2bL3′BamHI	GGA TCC GTG GCT CAG CCA GCC GCA
sug prom fwd (−34)	CGCATATTACCCGAACCTCT
sug prom rev (+66)	GTTGTCTGTGGTGGGTGCT
sug 3′ fwd (+1525)	CTCGCTAAAACCCAAACAGG
sug 3′ rev (+1638)	GGTGACTCCACGTCCATCTT
Fst prom fwd (−59)	GGCAGTGAATGGAAGTGGTT
Fst prom rev (+62)	CCAAGGCAGTGAAGAGGATAA
Fst 3′ fwd (+864)	ACTATCGATTCTTCAGCGGTCTA
Fst 3′ rev (+964)	GTTACTCGGAAACGCCAAAT
cnc prom fwd (−80)	AACCGCAAAAGCACAAAACT
cnc prom rev (+116)	GTGGTGAGCTTGAAAACGTG
cnc 3′ fwd (+33 437)	TGGAATCAGTGAGCCAGGA
cnc 3′ rev (+33 545)	TGTATAGTCGCCGGAAAAGG
CycB prom fwd (−39)	TGCGGCTTAAAAGGGAACTA
CycB prom rev (+101)	TGATCGAGTTTTTGCACACG
RpS23 prom fwd (−8)	GCGGTCACACTGAAAACATC
RpS23 prom rev (+140)	TTCGCTTAATTCGCACAAAA
RpL32 prom fwd (−19)	TTTCACACCACCAGCTTTTTC
RpL32 prom rev (+120)	CACGGACTAACGCAGTTCAA
Hus1-like prom fwd (−62)	TCGTTATCGGTTTTCGATGTC
Hus1-like prom rev (+96)	GCAGCAGTCGCACTTACCTT
Hus1-like 3′ fwd (+3527)	GGCCTTCTTTGGAGCACTT
Hus1-like 3′ rev (+3628)	CCACATCCTGTCGTACATCG
AttD prom fwd (−62)	AGTTGCGTACTTTTGCGACA
AttD prom rev (+130)	TCATCACCGACCCTTACTCC

overnight at 4°C in a mixture of anti-modified histone H3 polyclonal and anti-RNA polymerase II monoclonal antibodies. H3K9ac-specific antibody was from Abcam (dilution 1 : 200), H3K14ac-specific antibody was from Upstate (07-353, dilution 1 : 200), Pol II specific antibodies were 7G5 (24) or H14 (Covance Research Products) (dilution 1 : 500) as indicated. Samples were washed in PBST and incubated with a mixture of secondary antibodies (Alexa Fluor 555-conjugated anti-rabbit-, and Alexa Fluor 488-conjugated anti-mouse IgGs, Molecular Probes) for 1 h at 25°C. The slides were washed again and covered with VectaShield mounting medium containing 4′-6-diamidino-2-phenylindole (DAPI). For immunostaining of larval tissue samples, animals were dissected in PBS and fixed in 4% formaldehyde solution. Treatment with anti-H3K9ac primary antibody (1 : 200, Abcam) at 4°C was followed by Alexa Fluor 555-conjugated anti-rabbit secondary antibody (Molecular Probes). Stained samples were examined with a NIKON eclipse 80i microscope and photos were taken with a Retiga 4000R camera using identical settings for mutant and control samples.

Microarray analysis and quantitative RT-PCR

Total RNA was isolated from groups of 10 larvae or pupae using RNeasy Mini Kit (Qiagen). RNA labeling, hybridization to Affymetrix DrosGenome1 (pupae) and Drosophila 2 (larvae) GeneChips and scanning were performed at the IGBMC DNA CHIP Facility following the recommended standard Affymetrix protocols. Three biological replicates for each genotype (w^{1118} and *dAda2b*) at both time-points were obtained, permitting nine pair-wise comparisons of w^{1118} and mutant expression patterns. Only those genes which were indicated as ''present'' in at least two out of three samples of a given type/time-point were included in data analysis.

For the quantitative determination of larval cuticle protein (*Lcp*) and *Eig71E* mRNAs, total RNAs were isolated from w^{1118} and *dAda2b* larvae and pupae at the indicated developmental stages with RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 μg RNA using TaqMan Reverse Transcription Reagent (ABI). Quantitative real-time polymerase chain reaction (Q-RT-PCR) was performed (ABI, 7500 RT-PCR System) using primers specific for the respective cDNAs and 18S rRNA as internal control, following the incorporation of SYBRGreen. C_T values were set against a calibration curve. The $\Delta\Delta C_T$ method was used for the calculation of the relative abundances (25). The sequence of primers is given in Table 1.

Chromatin immunoprecipitation

Chromatin samples were prepared from L3 larvae with everted anterior spiracles based on the protocol described (26) with modifications. All steps were done in the presence of a protease inhibitor cocktail (Calbiochem). Samples (1 g L3 ea.) were ground in liquid nitrogen in a mortar and resuspended in 7 ml Buffer A (60 mM KCl, 0.5906 in NaCl, 15 mM Hepes–KOH pH 7.6, 13 mM EDTA, 0.1 mM EGTA, 10 mM Na-butyrate, 0.15 mM spermine, 0.5 mM spermidine, 0.5% NP-40, 0.5 mM DTT). The suspension was homogenized in a dounce homogenizer with pestle B and filtered through two layers of Miracloth (Calbiochem) filter. Homogenate was transferred over 2 ml Buffer AS (60 mM KCl, 15 mM NaCl, 15 mM Hepes–KOH pH 7.6, 1 mM EDTA, 0.1 mM EGTA, 10 mM Na-butyrate, 0.15 mM spermine, 0.5 mM spermidine, 0.5 mM DTT and 10% sucrose) and nuclei were pelleted with centrifugation (3000 r.p.m., 5 min, 4°C). The pellet was resuspended in 3 ml Buffer A, further homogenized in a dounce homogenizer, transferred over 1 ml Buffer AS and nuclei collected by centrifugation. The nuclear pellet was

resuspended in wash buffer (60 mM KCl, 15 mM NaCl, 15 mM Hepes–KOH pH 7.6, 1 mM EDTA, 0.1 mM EGTA, 10 mM Na-butyrate, 0.1% NP-40), and cross-linked with 1% formaldehyde for 10 min at room temperature. Crosslinking was stopped by the addition of 300 µl 1 M glycine. The nuclei were pelleted and washed two times with 10 ml wash buffer. After washing, nuclei were resuspended in 1.5 ml nuclei lysis buffer (50 mM Tris–HCl pH 8.0, 1% SDS, 10 mM EDTA, 10 mM Na-butyrate) and sonicated for 4 × 20 s on high setting in a Diogenode Bioruptor. Debris was removed by centrifugation at 14 000 r.p.m. for 10 min at 4°C, and the concentration of chromatin was determined by spectrophotometer.

Immunoprecipitations were performed as described in (27) using 25 µg chromatin samples with the following antibodies: α-H3 (1 µg, Abcam ab1791), α-H3K9ac (4 µg, Abcam ab4441), α-H3K14ac (4 µg, Upstate 07-353), α-dADA2b (5 µl, (10), α-Pol. II (2.5 µg, clone 7G5, (24). The specificity of modified histone-specific antibodies used here has been tested and verified by their suppliers, the specificity of the dADA2b Ab has been shown earlier (10) and here as well, though this Ab has not been used for chromatin immunoprecipitation (ChIP) experiments previously. The Pol II-specific anti-C-terminal domain 7G5 Ab has been used in ChIP experiments and its specificity has been demonstrated (28). Chromatin was pre-cleared using BSA and salmon sperm DNA blocked Protein A—Sepharose CL-4B beads (Sigma). Pre-cleared chromatin lysates were incubated with antibodies at 4°C overnight, then chromatin-antibody complexes were collected with blocked Protein A—Sepharose beads at 4°C for 4 h. The supernatant of the mock control was used as total input chromatin (TIC) control. After several washing steps with RIPA, LiCl (0.25 M LiCl, 0.5% NP-40, 0.5% Na-deoxycholate, 1 mM EDTA, 10 mM Tris–HCl pH 8.0) and TE buffers, the beads were resuspended in 100 µl TE buffer, the cross-links were reversed and the precipitated DNA was extracted with phenol/chloroform. The amount of precipitated DNA was determined with quantitative RT PCR using Power SYBR Green PCR master mix (Applied Biosystems) in an ABI 7500 Real-Time PCR system. In quantitative PCR analysis, reactions were done in duplicates next to a TIC standard curve, and the quantity of DNA bound by specific antibodies was calculated by deducting the amount of DNA bound by the mock controls. Primers used for PCR are given in Table 1. The primers for the eu- and hetero-chromatic intergenic regions were as described (29).

RESULTS

dAda2b mutation results in a drastic decrease in histone H3K9ac and H3K14ac levels

Recently, we reported the isolation of a *dAda2b* null allele (*dAda2b*^{d842}) and showed that the loss of *dAda2b* function results in lethality in later developmental stages, and a decrease of histone H3K9ac and H3K14ac levels on polytene chromosomes (14). Similar data were reported using independently-isolated *dAda2b* alleles (15). In *dAda2b* mutant flies, neither of the two dADA2b protein isoforms (14) are detectable by immunoblot in L3 or later stages of development (Figure 1A), and a decreased level of H3K9ac is observed by immunoblots developed with H3K9ac-specific antibodies (Figure 1B). In agreement, decreased levels of H3K9ac and H3K14ac are observed by staining of either larval tissues or polytene chromosomes of *Ada2b* null mutants (Figure 1C and data not shown). Interestingly, despite the severely reduced H3K9 and K14 acetylation, homozygous *dAda2b* animals follow a seemingly normal development until P5 stage, except that they complete the larval and the first stages of pupal development slightly slower than their heterozygous siblings (Figure 2A). *dAda2b* mutants do not show morphological abnormalities until P5, at this stage the reduced development of the legs and head becomes obvious and 85% of the animals die. A small fraction of mutants even develop further, occasionally reaching pharate adult stage. The normal development until pupa stage in the absence of dADA2b wondered us whether H3K9/K14 acetylation is required for transcription activation and whether the pattern of gene expression is changed in the lack of H3K9/K14 acetylation. To answer these questions, first we heat stressed third instar w^{1118} and *dAda2b* larvae to activate heat shock genes, and stained polytene chromosome squashes with antibodies raised against the Ser5-phosphorylated C-terminal domain of the largest subunit of RNA polymerase II, and acetylated H3K9. This experiment revealed that Pol II recruited the puffs containing heat shock genes; while the H3K9ac signal in the same regions remained low (Figure 1D). Based on these observations we concluded that a significant change in H3K9 acetylation is not prerequisite for the strong transcription activation, and decided to study the effects of the loss of dSAGA-specific acetylation on the total RNA profile of *dAda2b* mutants.

dAda2b mutation affects the expression of only a small subset of genes and results in both down- and up-regulation of gene expression

To determine the effect of *dAda2b* on gene expression, we compared the total mRNA profiles of w^{1118} and *dAda2b* null mutants at two stages: in late L3 and in P4 (Figure 2A). The narrow time windows of spiracle eversion and white pupa stages provided a convenient means of collecting synchronized samples for the comparison of the RNA content of mutant and control animals. We prepared poly(A)$^+$ RNA samples from w^{1118} and *dAda2b* mutant animals, labeled them with fluorescent dye, and hybridized to *Drosophila* whole genome cDNA microarrays (Affymetrix). For the validation of hybridization data we compared the level of selected mRNAs by Q-RT-PCR. For this, RNA samples were obtained from animals in developmental stages identical to those used for hybridization sample preparation, and as well as from additional two time points corresponding to mid L3 (12–16 h before puparium formation) and white pupa stage (1 h after puparium formation) (Figure 2A). As expected, no *dAda2b* specific message was detected in

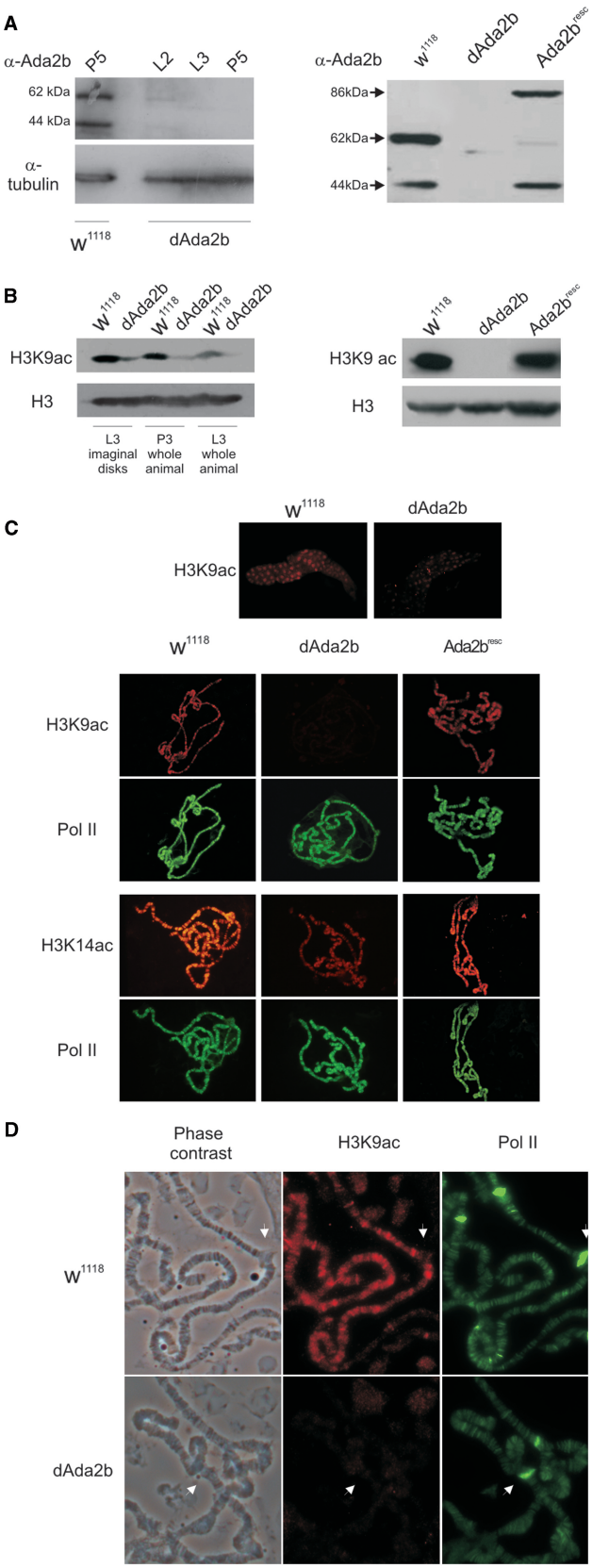

Figure 1. (A) Western blot of total protein samples of *Ada2b*^{d842} mutant, w^{1118} control, and Ada2bEGFP transgene carrier *Ada2b*^{d842} animals. The labels are P: pupa, L: larvae Ada2b^{resc}: Ada2bEGFP transgene carrier *Ada2b*^{d842} and as indicated. The Mw of the two dADA2b isoforms are indicated. Note that in Ada2b^{resc} the EGFP tag attached to the C-terminus of the larger dADA2b isoform increases its size. At the bottom on the left panel the same filter developed with alpha-tubulin-specific Ab as loading control is shown. (**B**) Western blot of total protein samples of *Ada2b*^{d842} mutants, w^{1118} control and Ada2bEGFP transgene carrier *Ada2b*^{d842} animals developed with histone H3K9ac-specific antibody. Labels, genotypes and developmental stages are as indicate, and as in (A). On the bottom: the same filters developed with anti-histone H3 antibody. (**C**) Immunostaining of salivary glands (top) and of polytene chromosomes (bottom) of late third instar *Ada2b*^{d842}, control (w^{1118}) and Ada2bEGFP transgene carrier *Ada2b*^{d842} larvae with H3K9ac- and H3K14ac-specific Abs. Pol II-specific staining (Ab: 7G5) of the same polytene chromosomes is shown as staining controls. (**D**) Accumulation of H3K9ac is not detectable in wild type (w^{1118}) heat-shock puff (top). Puff is formed in the absence of H3K9ac in *Ada2b*^{d842} mutant (bottom). Puffs formed at the 93D cytological region are indicated by arrow as an example. Red: H3K9ac-specific, green: Pol II-specific Ab (H14) staining.

dAda2b mutants in any of the stages tested. A comparison of the mRNA profiles of *dAda2b* mutant and control (w^{1118}) animals revealed a relatively small number of mRNAs present at a significantly lower or higher level in the mutants than in the corresponding control samples at both time points analyzed. (We considered only those mRNAs which gave a 'present' score in at least two out of three hybridizations at each time point). In *dAda2b* mutants, the level of 239 and 437 mRNAs were less than 50% of that detected in w^{1118} samples in larva and pupa stages, respectively (Figure 2B). For approximately one-third of these, the difference between the mRNA levels in the mutant and control were more than three-fold. Surprisingly, a higher number of mRNAs, 334 in larvae and 466 in pupae, were detected to be present at more than 2-fold increased levels in the mutants compared to w^{1118} samples (Figure 2B). Again, for ~35% of these, the levels in mutants were more than three-fold higher as the levels detected in the control samples. By comparing the RNA profiles corresponding to larva and pupa stages, we observed a relatively small overlap between the two stages in both mutant and w^{1118} samples. Only ~10% of the mRNAs affected by *dAda2b* mutation in larva stage was also affected in pupae. We believe this reflects the shift that takes place in the expression profile of the *Drosophila* genome at the time of larva to pupa transition. These data together indicate that: (i) *dAda2b* affects the expression level of a relatively small fraction of genes in both stages tested; (ii) a direct or indirect involvement of *dAda2b* both in down- and up-regulation of gene expression can be assumed since in *dAda2b* mutants some mRNAs can be detected at lower, while others at higher levels than in w^{1118} samples, and finally; (iii) the significant shift in the gene expression profile required for transition from larva to pupa can take place in the absence of dADA2b.

Although the microarray comparisons described above indicated a rather small number of genes affected by *dAda2b* mutation, even this number might be an overestimate, since—in order to facilitate further comparisons with other dSAGA and ATAC mutations—in these experiments we compared the RNA profiles of w^{1118} and *dAda2b* animals. This might show gene expression alterations resulting from genetic differences unrelated to

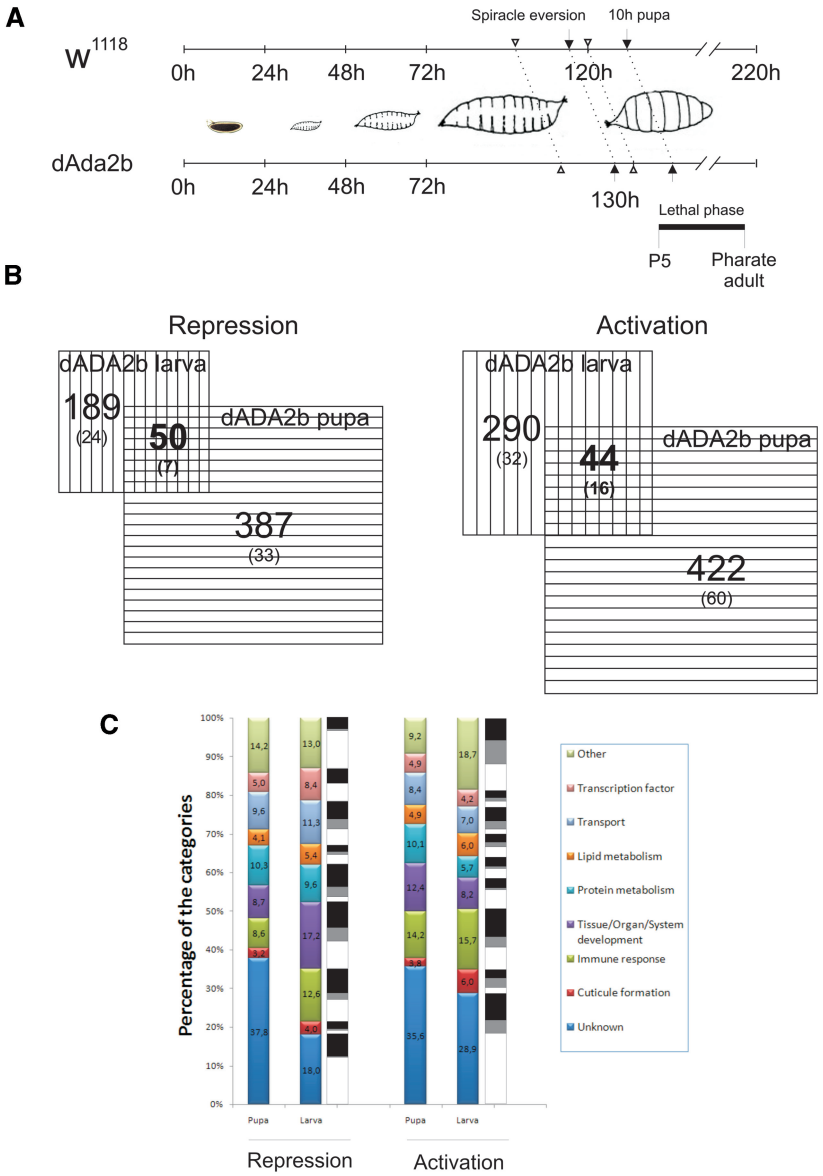

Figure 2. (**A**) Time scale showing the development of wild type (w^{1118}) and *dAda2b* animals. The lethal phase of *dAda2b* null mutants and the time points at which samples were collected for microarray (filled arrows) and Q-RT-PCR analysis (filled and open arrows) are indicated. (**B**) VENN-diagrams showing the numbers of up- and down-regulated genes in *dAda2b* larvae and pupae. The numbers of affected defense-related genes are shown in parentheses. (**C**) The distribution of genes represented with a more than two-fold altered RNA level in *dAda2b* mutants according to gene ontology categories. In the Larva columns the fractions of the genes in each category which were rescued (black), rescued to some extent (grey), and not rescued (white), by the Ada2bEGFP transgene in $dAda2b^{d842}$ homozygotes are shown.

the *dAda2b* status as well. Therefore, to obtain a further control, we performed additional sets of microarray in which we compared the total RNA profiles of *dAda2b* null mutants with that of an Ada2bEGFP transgene carrier *dAda2b* null. The expression of the Ada2bEGFP transgene, used for this, can be regulated by either the cognate *dAda2b*, or by a GAL4-inducible promoter. When expressed under the control of the *dAda2b* promoter, the expression level of dADA2b proteins in the transgene carriers is comparable to wild type controls (Figure 1A) and the transgene results in an 80% phenotypic rescue of *dAda2b* null mutants, with a detectable restoration of H3K9ac and H3K14ac levels (Figure 1B and C). With the help of a strong GAL4 driver, such as Act5C-GAL4, even a higher level of dADA2b expression can be achieved from the Ada2bEGFP transgene, however, this does not result in a more effective rescue. We assume, therefore, that the incomplete rescue is not due to a limiting level of dADA2b proteins; rather the EGFP tag at the C-terminus might interfere with dADA2b function. In Ada2bEGFP carrier $Ada2b^{d842}$ larvae, the mRNA levels of 186 (56%) and 123 (52%) out of those genes which are activated or repressed in w^{1118} versus *dAda2b* null mutants are partially restored. The distribution of these genes among the different gene ontology categories

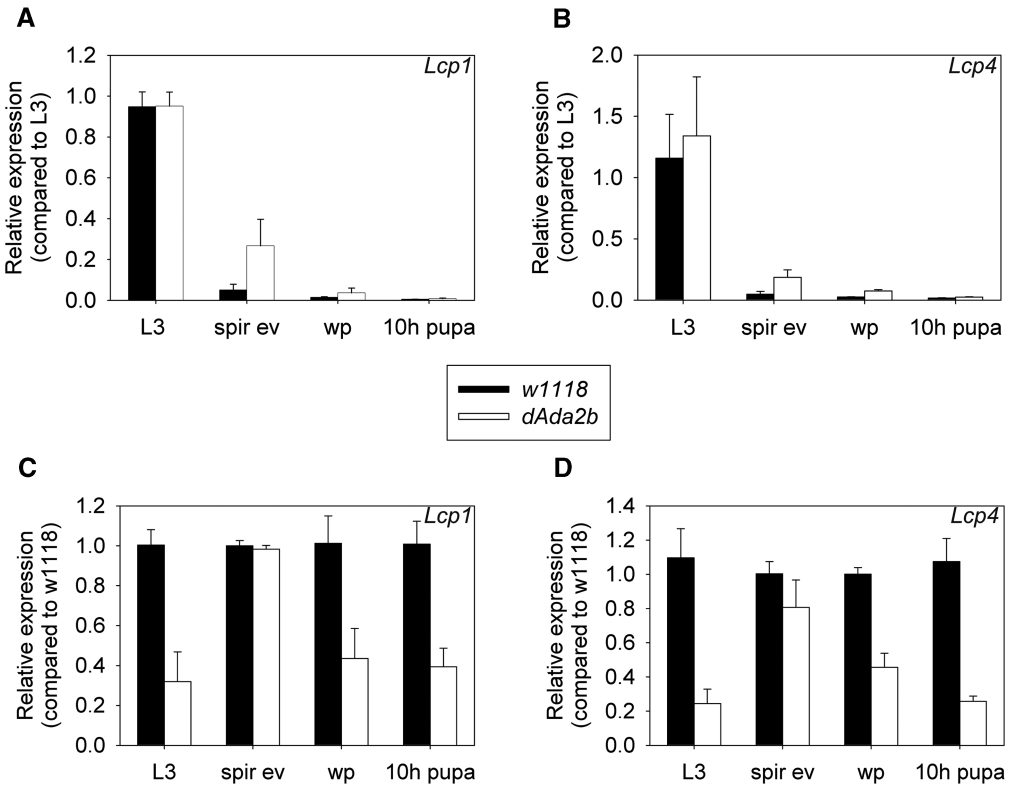

Figure 3. RNA levels of larval cuticle protein (*Lcp*) genes at the 44C-D cytological region as determined by Q-RT-PCR in *dAda2b* mutants at four time points in L3 and pupa stages (spir ev: spiracle eversion, wp: white pupa, 10 h pupa: 10 h after pupariation). The RNA levels of *Lcp1* (**A** and **C**), and *Lcp4* (**B** and **D**) at the indicated stages are shown in comparison with the level observed in the L3 stage of the particular genotype (A and B), and in comparison with the levels observed in the control samples (w^{1118}) in the given time point (C and D).

follows that of those genes identified in the *dAda2b* versus w^{1118} comparison (Figure 2C). We believe therefore that the two estimates i.e. *dAda2b* versus Ada2bEGFP carrier, and *dAda2b* versus w^{1118} represent a low and a high approximation of the number of genes affected by *dAda2b* mutation.

No linkage among dADA2b-regulated genes can be observed based on their topology or expression levels

Since dADA2b is a component of the dSAGA HAT complex, which might have both global and locus specific effects on transcription, we next analyzed the gene expression profiling data asking whether mRNAs detected at either increased or decreased levels in *dAda2b* mutant represent genes localized in close proximity in the genome (i.e. are there islands of activated/inactivated genes). The other question we asked was whether genes expressed at a high level in wild type animals are regulated by dADA2b. Our data analysis revealed that the answers to both questions were negative. Genes represented in mutants by either higher or lower mRNA levels than in w^{1118} samples were distributed evenly among the four chromosomes. In a few instances groups of adjacent three to five genes each up- or down-regulated at a particular stage can be identified, the number of these co-localizations, however, is not significant. Similarly, no pattern among the affected genes based on the level of their expression can be recognized. Among the most highly expressed genes at both larva and pupa stages are those coding for ribosomal proteins, ecdysone-induced genes, genes encoding proteins involved in cuticle synthesis and immune functions. Out of these, the level of none of the ribosomal protein messages change significantly in *dAda2b* mutants compared to w^{1118} control in either larva or pupa stage. In summary, based on these observations we concluded that a coordinated regulation of topologically linked or highly expressed housekeeping genes by dADA2b does not exist. It is worth to note that despite the small overlap between genes affected in larvae and pupae a functional grouping of the genes with altered mRNA levels in either larva or pupa stage revealed similar distribution of both the up- and down-regulated genes (Figure 2C).

Next, we considered the change of expression of mRNAs corresponding to functionally-related genes. In *dAda2b* mutants, we detected marked changes in the levels of several, but not all, ecdysone-induced mRNAs, in a number of immune function-related mRNAs, and some cuticle protein genes. In order to validate the hybridization data, we quantified the expression changes of two *Lcp* genes by Q-RT-PCR, at four time points (Figure 2A). *Lcp1* and *Lcp4* are the proximal and distal genes of an *Lcp* gene group at 44C6-D1. In w^{1118} animals, the levels of both mRNAs decrease dramatically during the transition from L3 to pupa stage (Figure 3). In *dAda2b* mutants, the

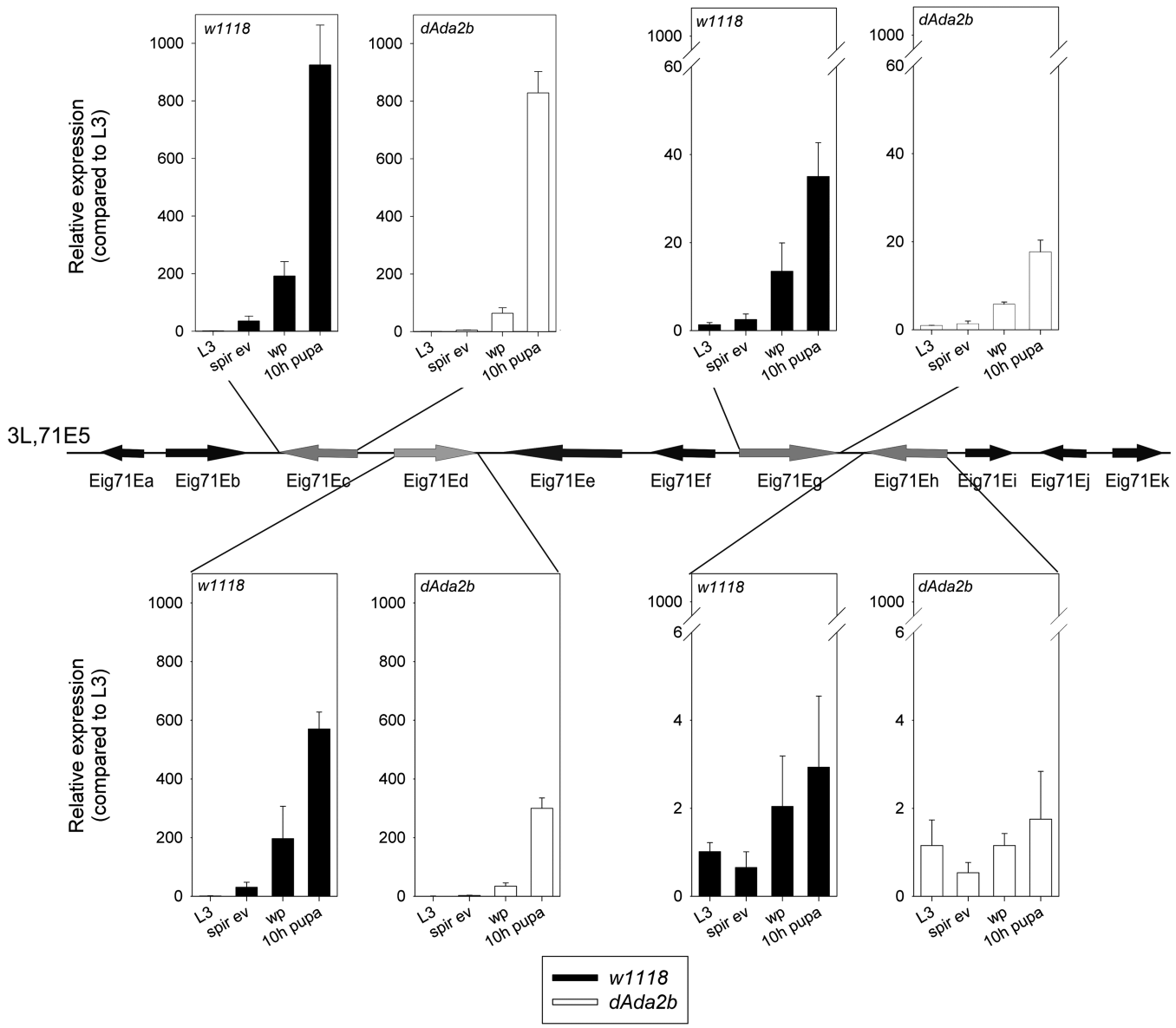

Figure 4. The change of RNA levels of ecdysone-induced genes (*Eig71E*) located at the 71E5 cytological region. The organization of the *Eig71E* gene cluster is shown in the center, with the direction of transcription indicated by arrows. The graphs show the relative level of *Eig71Ec*, *d*, *g* and *h* in control (w^{1118}) and *dAda2b* mutants compared to the levels of RNAs found in L3 stage. Note that the scales of the graphs are different, in order to show the dramatically different changes in expression among the four genes studied. Abbreviations are as in Figure 3.

change of *Lcp1* and *Lcp4* expression follows a slightly different pattern in that both the *Lcp1* and the *Lcp4* mRNA level drops during an extended time period. Thus, the sharp down-regulation, characteristic for both *Lcp1* and *Lcp4* expression from L3 to spiracle eversion is observable in the absence of dADA2b, but the kinetics of the expression change is altered.

We also observed alterations in the expression levels of ecdysone-regulated genes in *dAda2b* mutants. A cluster of late responding ecdysone-induced genes is located at the 71E cytological region (30). The cluster extends to an approximately 13 Kb region, and consists of five pairs of head-to-head oriented genes, each encoding a short cysteine-rich peptide. These genes are believed to be functionally related, and originated from duplication of a single copy gene. Therefore, we found interesting to compare their expression in *dAda2b* mutants and w^{1118} controls. Members of the *Eig71E* gene family are expressed at a low level in L3 stage, but some of them are among the most highly expressed genes in pupae. In *dAda2b* mutants they show a similar change, with small differences in expression kinetics (Figure 4). Significantly, despite their similar structure, some genes of the cluster show very high, while others only moderate activation in pupae both in w^{1118} and mutant animals (Figure 4, compare *Eig71Ec* and *Eig71Eh*). At specific stages the relative expression of different members of the cluster is modified similarly

Table 2. Expression changes of selected defense-related genes in *dAda2b* mutants

PART A

name	larva w1118	larva dAda2b	larva Ada2b^{resc}	pupa w1118	pupa dAda2b	log2 dAda2b/ w1118 larva	log2 dAda2b/ w1118 pupa	P(larva)	P(pupa)
Attacins: 4 members									
att A	77	167	183	22	550	1.12	4.63	7.3E-02	1.5E-01
att B1	65	202	225	43	559	1.63	3.71	1.2E-01	1.6E-01
att C	-	-	-	10	331	-	5.12	-	1.9E-01
att D	42	312	43	72	655	2.88	3.19	6.8E-02	7.0E-02
Diptericin (dpt) family: 2 members									
dpt	-	-	-	68	800	-	3.56	-	1.4E-01
dpt B	15	65	19	62	499	2.09	3.00	1.9E-01	1.8E-01
Cecropin (cec) family: 4 members									
cecA2	-	-	-	19	285	-	3.91	-	2.0E-01
cecA1	-	-	-	93	562	-	2.59	-	2.2E-01
cecC	-	-	-	8	49	-	2.62	-	2.2E-01
cecB	-	-	-	6	73	-	3.71	-	2.0E-01
Drosomycin (drs) family: 6 members									
drs	-	-	-	1195	3103	-	1.38	-	7.0E-05
dro2	-	-	-	20	52	-	1.40	-	5.7E-02
dro3	12	20	16	-	-	0.70	-	1.2E-01	-
dro4	27	140	66	-	-	2.38	-	7.6E-05	-
dro5	-	-	-	1389	4520	-	1.70	-	4.9E-05
dro6	-	-	-	5266	1052	-	-2.32	-	2.2E-04
other AMPs									
drosocin	-	-	-	44	795	-	4.17	-	8.1E-02
metchnikowin	-	-	-	329	701	-	1.09	-	2.1E-01
defensin	30	102	57	6	171	1.78	4.72	1.9E-01	1.7E-01
andropin	-	-	-	39	63	-	0.70	-	5.1E-02
Peptidoglycan recognition protein (PGRP) family									
PGRP-SC2	187	301	278	91	223	0.69	1.29	2.8E-03	2.9E-02
PRGP-SC1b	41	239	910	6	19	2.53	1.67	1.6E-03	1.4E-01
PRGP-SC1a	-	-	-	26	54	-	1.06	-	2.5E-01
PGRP-SB1	263	46	26	5	28	-2.52	2.59	3.7E-04	1.8E-01
PGRP-SB2	3445	170	2269	46	29	-4.34	-0.66	1.4E-04	9.1E-02
PGRP-SD	-	-	-	72	47	-	-0.63	-	1.8E-01
PGRP-LA	-	-	-	34	12	-	-1.55	-	1.2E-01
Lysozyme (lys) family									
lysX	2487	1157	1067	2095	495	-1.10	-2.08	1.4E-03	5.9E-04
lysB	-	-	-	2549	7164	-	1.49	-	1.4E-04
lysS	236	135	136	-	-	-0.81	-0.14	2.2E-01	-
lysE	-	-	-	1179	5321	-	2.17	-	2.9E-05
lysP	4	130	231	-	-	5.01	0.57	7.4E-04	-
CG7798	22	6	6	72	40	-1.95	-0.86	2.4E-03	2.1E-02
CG8492	-	-	-	98	30	1.20	-1.70	-	4.7E-03
CG11159	-	-	-	29	77	-1.64	1.40	-	4.2E-03
Induced serine proteases									
CG6639	-	-	-	439	1026	-	1.23	-	4.8E-03
CG18563	680	115	166	183	70	-2.56	-1.39	9.1E-05	3.0E-05
CG9631	9	34	35	25	13	2.00	-1.02	6.0E-05	1.8E-02
CG15046	43	27	34	-	-	-0.65	-	2.2E-02	-
Jon65Aiv	-	-	-	48	206	-	2.09	-	3.6E-02
CG9645	-	-	-	14	29	-	1.04	-	6.0E-02
Ser7	-	-	-	65	21	-	-1.64	-	2.6E-03
CG9733	16	57	56	-	-	1.80	0.91	3.3E-04	-
CG3505	-	-	-	856	1430	-	0.74	-	1.3E-03
Induced small peptides (40-134aa)									
CG30080	56	128	66	-	-	1.19	-	4.2E-02	-
CG9080	183	398	357	-	-	1.12	-	3.5E-03	-
IM1	3	34	4	5	67	3.50	3.65	5.0E-02	1.1E-01
CG16978	-	-	-	14	6	-	-1.15	-	1.8E-02
IM23	-	-	-	3	35	-	3.66	-	6.6E-02
CG6429	13	39	30	981	213	1.65	-2.20	1.9E-03	9.7E-03
CG4250	150	1052	3297	220	1193	2.81	2.44	6.4E-04	9.7E-04
CG14957	953	2241	2222	-	-	1.23	-	2.1E-05	-
IM2	3	50	3	6	51	3.91	3.02	6.6E-02	5.3E-02
CG13324	-	-	-	25	74	-	1.58	-	8.7E-03
CG15126	-	-	-	38	16	-	-1.29	-	2.5E-02
CG16836	3	39	2	9	29	3.56	1.68	9.2E-02	1.2E-01
CG15829	32	48	42	21	41	0.59	0.95	1.1E-01	7.8E-02
CG17107	8	18	57	25	38	1.19	0.59	5.1E-02	2.4E-01
CG5773	501	986	1237	-	-	0.98	-	1.4E-02	-
CG7738	-	-	-	35	70	-	1.00	-	9.9E-02
CG15065	-	-	-	35	233	-	2.75	-	1.4E-01
CG13323	-	-	-	25	239	-	3.23	-	2.0E-03
CG8157	-	-	-	154	301	-	0.97	-	4.8E-02
MtnC	-	-	-	65	23	-	-1.50	-	1.1E-02
CG14599	-	-	-	30	48	-	0.68	-	5.3E-02

name	larva w1118	larva dAda2b	larva Ada2b^{resc}	pupa w1118	pupa dAda2b	log2 dAda2b/ w1118 larva	log2 dAda2b/ w1118 pupa	P(larva)	P(pupa)
Induced unknown proteins									
Fst	1177	2728	2771	30	341	1.21	3.53	1.7E-04	8.2E-04
CG13905	224	999	414	59	392	2.16	2.74	2.1E-03	6.5E-04
CG14567	269	111	130	307	85	-1.27	-1.86	5.2E-05	3.7E-03
CG15043	382	977	318	-	-	1.36	-	5.9E-02	-
CG16772	-	-	-	201	97	-	-1.05	-	3.0E-02
CG18279/IM10	4	56	19	37	151	3.70	2.04	1.7E-02	2.1E-02
CG9989	1821	1112	1016	112	28	-0.71	-1.99	1.0E-02	9.1E-03
CG16718	-	-	-	15	37	-	1.27	-	6.9E-03
CG2217	24	48	96	18	37	1.01	1.06	7.6E-02	1.8E-01
CG10420	66	28	44	-	-	-1.23	-	5.1E-05	-
CG18067	-	-	-	406	204	-	-0.99	-	2.0E-02
CG10912	-	-	-	34	72	-	1.10	-	1.1E-01
CG15678	86	156	127	13	34	0.87	1.43	2.5E-03	2.8E-04
Vago	190	118	168	-	-	-0.69	-	6.4E-02	-
CG12505	-	-	-	243	2075	-	3.10	-	4.6E-04
CG13618	-	-	-	271	102	-	-1.41	-	2.2E-02
CG13641	63	121	140	-	-	0.93	-	6.1E-03	-
CG18348	11	20	18	9	28	0.78	1.67	1.5E-02	4.0E-02
CG6426	-	-	-	4520	2562	-	-0.82	-	4.0E-03
CG11413	-	-	-	84	49	-	-0.77	-	4.6E-02
CG16887	49	33	62	-	-	-0.61	-	4.5E-02	-
CG14762	-	-	-	11	18	-	0.62	-	2.5E-01
CG16743	119	58	60	42	27	-1.04	-0.61	8.2E-03	7.8E-02
CG6357	418	217	233	-	-	-0.95	-	1.5E-02	-
CG15784	142	473	833	391	2960	1.74	2.92	1.6E-02	7.8E-04
BG:DS07721.3	-	-	-	32	62	-	0.96	-	3.0E-02
C7695	-	-	-	19	35	-	0.92	-	4.0E-02
CG10910	83	155	134	16	64	0.89	2.04	4.7E-02	7.7E-02
CG14661	-	-	-	99	30	-	-1.72	-	1.7E-03
CG14907	-	-	-	21	32	-	0.60	-	3.7E-02
CG5765	-	-	-	39	14	-	-1.47	-	2.0E-02
CG2875	-	-	-	33	22	-	-0.62	-	1.3E-01
DNApol-i	-	-	-	14	29	-	1.07	-	1.3E-01
CG6073	13	26	26	-	-	1.00	-	8.5E-03	-

PART B

name	larva w1118	larva dAda2b	larva Ada2b^{resc}	pupa w1118	pupa dAda2b	log2 dAda2b/ w1118 larva	log2 dAda2b/ w1118 pupa	P(larva)	P(pupa)
Genes encoding canonic components of the Toll signaling pathway									
spatzle	117	116	141	58	65	-0.02	0.16	2.4E-01	1.9E-01
Toll	2	6	5	49	59	1.26	0.25	4.5E-02	2.0E-01
DmMyD88	233	211	199	487	251	-0.14	-0.95	2.7E-01	4.3E-03
tube	214	222	224	91	74	0.06	-0.31	1.4E-01	9.5E-02
pelle	125	113	90	119	129	-0.15	0.12	3.4E-01	2.8E-01
cactus	136	107	120	274	303	-0.34	0.15	3.8E-02	1.1E-01
DIF	21	20	17	12	19	-0.08	0.68	3.1E-01	6.9E-02
dorsal	38	18	20	11	20	-1.10	0.94	1.2E-03	1.4E-02
Serine Proteases/Serpins acting upstream of the Toll pathway									
Necrotic	103	43	55	663	390	-1.28	-0.76	6.3E-03	1.4E-02
SPE	14	5	5	214	85	-1.58	-1.33	2.0E-02	5.0E-02
persephone	31	39	37	84	150	0.34	0.83	2.6E-02	2.7E-02
Spirit	213	226	284	34	58	0.09	0.77	1.4E-01	1.7E-01
Grass	190	202	192	495	114	0.09	-2.12	9.7E-02	2.2E-04
spheroide	8	10	6	8	17	0.47	1.04	7.6E-02	1.0E-01
sphinx1/2	4	2	3	59	75	-1.45	0.33	1.0E-01	3.6E-02
Genes encoding canonic components of the Imd pathway									
imd	71	69	65	8	7	-0.06	-0.28	4.9E-01	3.6E-01
DmTak1	180	248	166	57	70	0.46	0.29	1.4E-02	1.8E-01
Tab2	180	220	166	123	106	0.29	-0.21	6.2E-02	1.2E-01
Iap2	208	247	253	135	155	0.25	0.20	1.3E-01	1.5E-01
dFADD/BG4	72	76	63	-	-	0.07	-	4.9E-01	-
dredd	106	113	130	94	120	0.08	0.36	2.0E-01	5.8E-02
kenny	360	332	319	76	89	-0.12	0.24	1.2E-01	8.7E-02
Dmikkb/ird5	111	112	111	-	-	0.01	-	4.7E-01	-
relish	404	427	474	191	270	0.08	0.50	2.0E-01	9.4E-03
Genes encoding proteins that affect the Toll and/or the Imd pathway(s)									
skpA	1635	1699	1474	522	505	0.06	-0.05	2.3E-01	2.7E-01
UEV1a	1223	1127	984	300	264	-0.12	-0.19	2.0E-01	2.5E-01
Helicase89B	132	138	171	-	-	0.07	-	3.3E-01	-
POSH	66	61	69	69	65	-0.10	-0.09	2.6E-01	3.3E-01
Caspar	551	575	485	288	338	0.06	0.23	2.6E-01	5.8E-03
Defense rep 1	78	97	102	6	53	0.31	3.06	7.2E-02	3.8E-02

The average hybridization signal determined on three microarrays in control (w^{1118}), *dAda2b*^{d842} (dAda2b), and *dAda2b*^{d842} Ada2bEGFP transgene carrier larvae (Ada2b^{resc}) and pupae, and the relative changes in mutants as compared to the control samples are shown. Colors indicate: red: larger than 100% increase, orange: 50–100% increase, dark blue: larger than 100% decrease, light blue 50–100% decrease in signal intensity compared to the control. *P*-values are calculated by using Student's *t*-test (one tailed distribution, two sample equal variance, homoscedastic, calculation methods). Shaded boxes in the Ada2b^{resc} column indicate genes for which rescue was detected. Immune-related genes are grouped (31).

in *dAda2b* mutants and controls. These data thus indicate that a robust induction of transcription by the metamorphic regulatory hormone ecdysone can take place in the absence of dADA2b. Furthermore, although these related genes are within a short region in a cluster, during the transition from L to P stage, individual members of the cluster are up-regulated at very different extent. In w^{1118} and *dAda2b* mutants these expression changes are similar, both in quantitative and qualitative term.

A third group of functionally-related genes, which shows dramatic changes in expression levels in *dAda2b* mutants involved in defense mechanisms. In fact, among the genes displaying altered expression in *dAda2b* mutants, genes induced by microbial infection are represented in the highest number. These include genes encoding peptidoglycan recognition proteins, lysozymes and serine proteases, genes encoding antimicrobial peptides and other small peptides identified by other studies as immune-induced factors. Several other genes as well, with unknown functions belong to this group based on that they have been found to be induced by immune challenges (Table 2) (31). Most of the RNAs corresponding to genes in this group are present in dramatically increased levels in *dAda2b* mutant samples, some of them displaying a 20-40-fold induction. The presence of a large number of immune function related mRNAs in increased levels in *dAda2b* mutants is particularly striking in the *dAda2b* versus w^{1118} comparison at pupa stage. A smaller, still significant number of immune function related genes are up-regulated in *dAda2b* mutants in larva stage. In Ada2bEGFP-containing *dAda2b* null samples, the levels of many of the mRNAs corresponding to these genes are changed towards the values seen in the w^{1118} control sample, suggesting that the expression of these genes indeed depends on the function of *dAda2b* (Table 2).

Particularly, striking is the high induction level of genes encoding antimicrobial peptides (AMP). These include all the known seven classes of AMPs that play roles in defense against gram-negative and gram-positive bacteria and fungi. In order to obtain an independent verification that the expression of these genes is indeed up-regulated in *dAda2b* mutants, we compared the expression of AMP-promoter-GFP transgenes in wild type and *dAda2b* mutant animals. As it is shown in Figure 5, both the *attacin* (*att*) and *defensin* (*def*) promoter–driven GFP transgenes were expressed in higher levels in *dAda2b* null animals than in wild type, or in heterozygous siblings pupariated in the same vials.

While RNAs corresponding to AMPs are present exclusively at higher levels in *dAda2b* mutants than in w^{1118} samples, very few other components of the immune response show either an increased or decreased RNA level. Surprisingly, the RNA levels of members of the Toll and Imd pathways, the two major branches of signaling pathways involved in *Drosophila* innate immune responses, are only moderately, if at all, affected by *dAda2b* mutations, and only a few genes belonging to these pathways show significantly altered RNA levels in *dAda2b* mutants (Table 2).

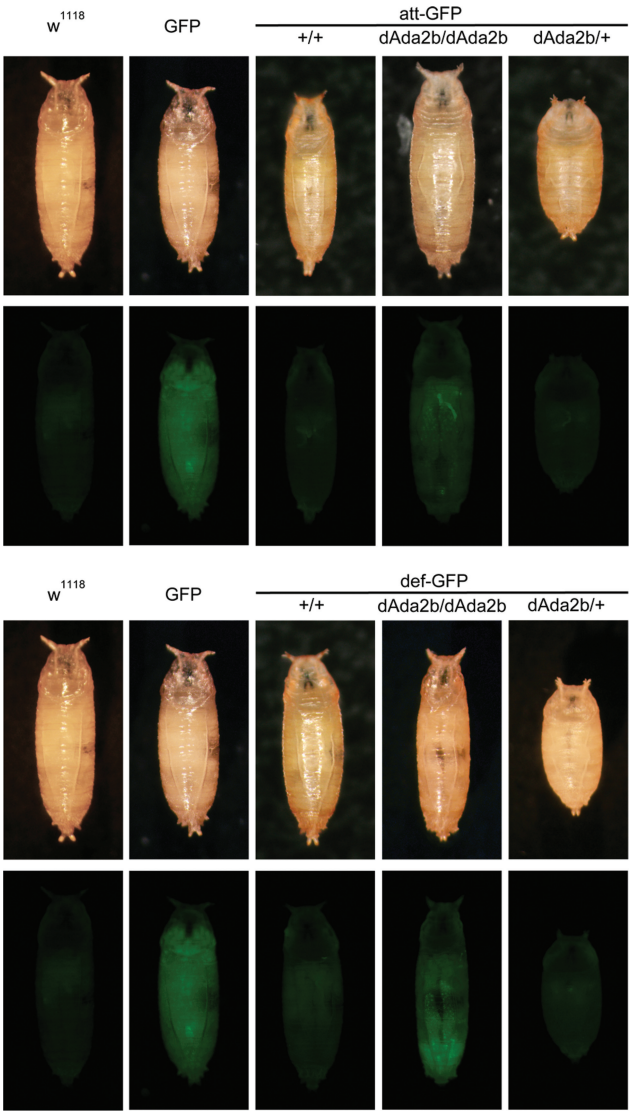

Figure 5. AMP promoter-GFP reporter gene expression in *dAda2b* mutant pupae. The expression of GFP from *defensin* promoter- (def-GFP) and *attacin* promoter-GFP (att-GFP) transgenes in different *dAda2b* background are shown. An Act5C-GFP transgene carrier is shown (GFP) for comparison. The different AMP promoter-GFP transgene carrier animals are siblings obtained from the same crosses and cultured in the same vials.

Localization of dADA2b, SAGA-specific histone mark and Pol II at promoters affected differently by *dAda2b* mutations

The gene expression changes observed in *dAda2b* mutants wondered us whether a direct role of the dADA2b-containing HAT complex can be demonstrated in the transcription of genes, which are affected either positively or negatively in mutants. Searching for an answer to this question, we performed ChIPs to detect the presence of SAGA-specific histone H3 acetylation marks (H3K9ac and H3K14ac) and also dADA2b and Pol II localization in the promoter regions of selected genes. In the ChIP analysis we included genes which were found to be up- [Frost (*Fst*), Hus1-like (*Hus1*), Attacin-D (*AttD*)] or

down-regulated [sugarbabe (*sug*), cap and collar (*cnc*), cyclin B (*CycB*)] in *dAda2b* mutants, and as well two genes [(ribosomal protein L32 (*RpL32*), ribosomal protein S23 (*RpS23*)], which were unaffected by *dAda2b* mutations (Figure 6A). For further control intergenic regions of the genome (29) were included in some of the ChIP analysis. Some of the genes chosen for the analysis have been shown to play roles in processes believed to be regulated by SAGA in *Drosophila* or another organism: *Frost* (up-regulated in *dAda2b* mutants), for example, encodes a protein involved in cold hardening response in *Arabidopsis*, and is up-regulated during recovery after cold shock in *Drosophila* (32,33). On the other hand, the gene of the zinc finger transcription factor, *sugarbabe* (*sug*), down-regulated in *dAda2b* mutants, is the highest and earliest activated gene upon sugar ingestion in *Drosophila* (34). We performed ChIP experiments on samples obtained from wild type control and *dAda2b* mutant larvae. First we used dADA2b-specific antibodies to test the association of dADA2b with the selected genes. Fragments corresponding to promoter and 3′ regions of the selected genes were detected in immunoprecipitated samples by Q-PCR, using specific primers (Table 1). ChIPs performed with dADA2b-specific Abs resulted in weak signals both from promoter and 3′ regions. We obtained similarly weak signals irrespective whether the specific sequence amplified from a precipitated sample corresponded to promoter or 3′ region of an up- or down-regulated or dSAGA-independent gene (Figure 6D and Table 3). *dAda2b* mutant chromatin samples resulted even weaker signals corresponding to 50% or less of that obtained from wild type samples, and again no differences between different genes or regions were observable. On the transcriptionally silent intergenic regions, we detected dADA2b localization neither in wild-type nor in mutant samples. ChIPs performed with H3K9ac-specific Abs gave different results revealing differences in H3K9ac levels between dSAGA-affected and unaffected genes in wild type and *dAda2b* samples (Figure 6B and C). Specifically, in dSAGA-regulated genes the H3K9ac levels were decreased in *dAda2b* mutants compared to wild type samples. On the other hand, in the two ribosomal protein genes, which were not affected by dSAGA, the amount of H3K9ac-specific Abs precipitated chromatin were equally high in both wild type and *dAda2b* samples (Figure 6C and Table 3). In contrast with these, on the intergenic regions we detected very low levels of K9-acetylated H3 (Figure 6C and Table 3). We detected H3K14ac, the other dSAGA-specific histone modification, in much lower level than H3K9ac in each gene we tested. Therefore, on this form of dSAGA-modified histone we can conclude only that no strong differences in its levels can be observed in these genes, despite that they are affected differently by *dAda2b* mutations. The small differences in the H3K9ac levels in the promoters of the RpL32 and RpS23 genes in wild type versus *dAda2b* mutants might result from low nucleosome occupancy of these regions. To assess this possibility, we performed ChIP experiments using H3-specific Abs. The amounts of amplified probes did not indicate that the levels of H3 at the ribosomal gene promoter were significantly different from that at the other promoters, studied: H3 specific Abs precipitated similar fractions, ~3% of input chromatin from both the dSAGA-independent ribosomal protein and the dSAGA-dependent other genes. Finally, we performed ChIPs using Pol II large subunit-specific antibodies. Comparisons of the Pol II occupancy in regions of SAGA-independent and dSAGA-dependent genes in wild type and *dAda2b* mutants are shown in Figure 6E. The amounts of chromatin immunoprecipitated with Pol II-specific Abs from wild type and *dAda2b* samples indicate very little differences in the Pol II levels on the dSAGA-independent RpL and RpS promoters. On the promoter regions of genes up-regulated in *dAda2b* mutants (*Fst, Hus1*), Pol II is present in higher level in *dAda2b* samples, while on the promoters of genes down-regulated in *dAda2b* mutants (*sug, cnc*), Pol II is present at a lower level in the mutant samples than in wild type ones.

DISCUSSION

dADA2b is a complex specific constituent of the dSAGA histone modifying complex. Since dGCN5, the HAT component of dSAGA plays a role in at least one other histone modifying complex, ATAC, *dGcn5* mutants cannot be used to study dSAGA-regulated genes. However, as recent data have indicated that the loss of *dAda2b* function interferes with dSAGA histone modifying activity (14,15), we reasoned that by employing *dAda2b* mutants we will uncover dSAGA-specific functions. Therefore, we used the *dAda2b* mutants to learn new information on the function of the dSAGA complex. Recently, dSAGA has shown to play a role in histone H2A and H2B deubiquitination as well (35,36). This function of dSAGA is believed to be associated with a module which is not or only partly affected by *dAda2b* mutations. Our data, thus, are related mainly to the dGCN5 HAT function of the dSAGA complex.

We assessed the effect of the loss of *dAda2b* zygotic function. Results of earlier studies showed that *dAda2b* is essential in the germ line (14,15). During early embryogenesis *dAda2b* might play specific roles, the effect of which are not observed in these experiments. We assume that at the developmental stages we studied, very small fraction, if any, of the maternal dADA2b is remaining. Nonetheless, we cannot exclude a long lasting maternal effect. Neither can we exclude the possibility that, although *dAda2b* mutations hamper the H3K9 and H3K14 specific HAT activity of dSAGA, they do not eliminate it completely. Thus, despite that we studied *dAda2b* null mutants; an activity remaining in H3 acetyltransferase of dSAGA in these animals might play a role.

In order to tackle questions concerning the role of dADA2b (and dSAGA) in determining gene expression changes during the late course of fly development, we choose stages for mutant and control sample comparisons in which a significant decrease in the levels of H3K9ac and H3K14ac in *dAda2b* null mutants is unquestionable. Thus, if dADA2b plays a role in transcription regulation,

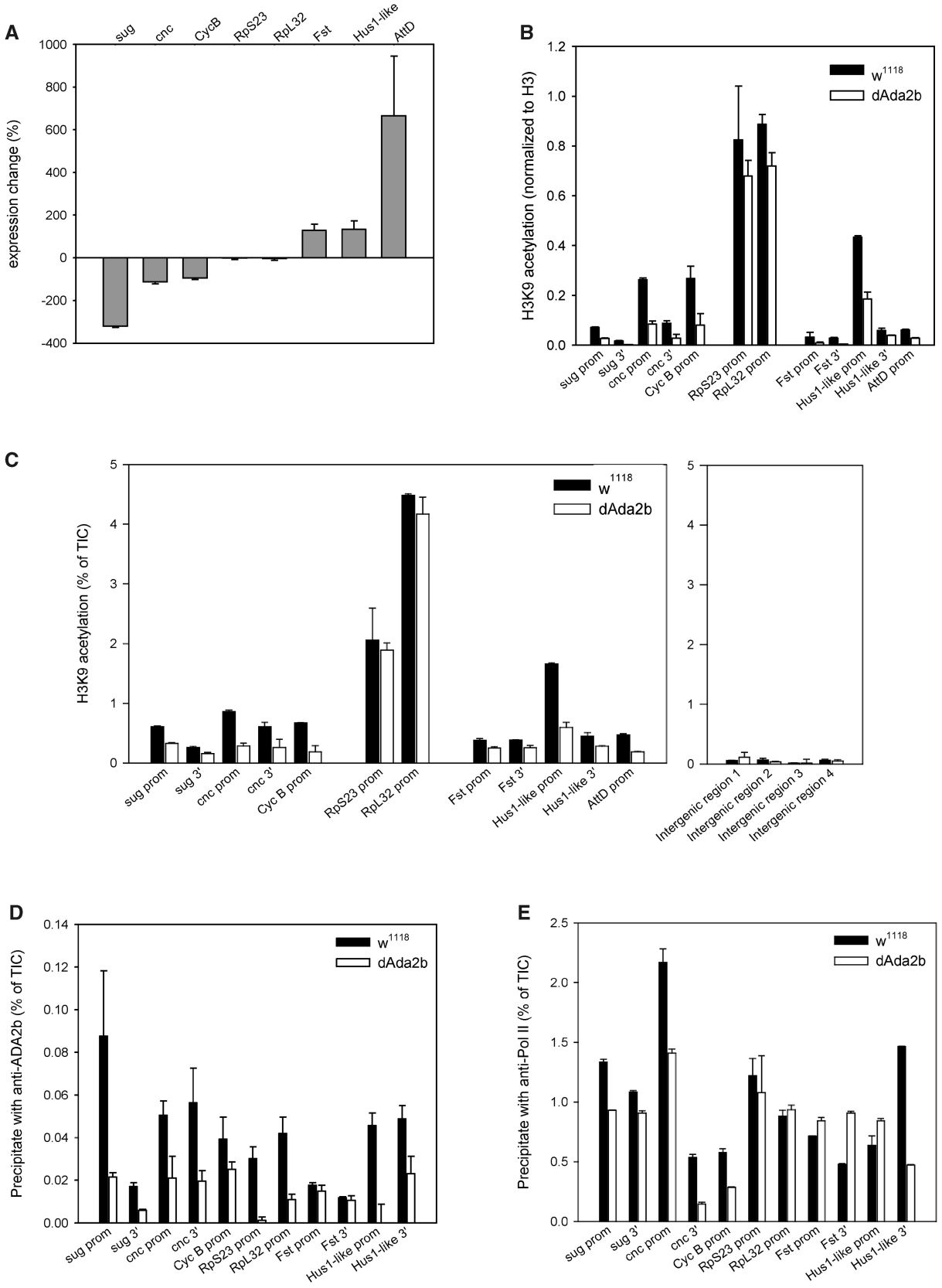

Figure 6. (**A**) Changes in the levels of specific mRNAs in *dAda2b*^{d842} mutants compared to wild type (w^{1118}) samples. Average changes in mRNA levels determined by microarray hybridizations in three biological samples are shown. (**B–E)** Q-PCR detection of specific fragments of selected genes in chromatin immunoprecipitated samples. The primers used for Q-PCR are listed in Table 1. H3K9ac-specific (B and C), dADAb- (D) and Pol II-specific (E) antibody-precipitated chromatin from wild type (w^{1118}) and *dAda2b* samples obtained from sycronised third instar larvae. C_t and dCt values of representative experiments of those shown here are given in Table 3. Note that ChIP experiments to detect H3K9ac in the intergenic regions (C right) were done in separate experiments from those shown on B left.

Table 3. Ct and dCt values of Q-PCR experiments performed to detect K9-acetylated histone H3 levels and the presence of dADA2b at specific gene and intergenic regions in chromatin samples obtained from syncronized wild-type and *dAda2b* mutant third instar larvae

	w^{1118}					dAda2b				
	Ct					*Ct*				
	NAC	H3K9ac	ADA2b	$dCt\ (Ct_{NAC}\text{-}Ct_{H3K9ac})$	$dCt\ (Ct_{NAC}\text{-}Ct_{ADA2b})$	NAC	H3K9ac	ADA2b	$dCt\ (Ct_{NAC}\text{-}Ct_{H3K9ac})$	$dCt\ (Ct_{NAC}\text{-}Ct_{ADA2b})$
sug prom	28.8	24.8	27.5	3.9	1.2	29.4	25.9	28.8	3.4	0.5
sug 3′	31.9	27.5	30.1	4.4	1.9	30.3	29.2	29.9	1.1	0.5
cnc prom	31.1	24.9	29.3	6.2	1.7	31.2	26.9	30.3	4.2	0.9
cnc 3′	28.8	24.5	27.7	4.3	1.1	29.3	26.0	28.7	3.2	0.6
CycB prom	32.1	26.5	30.8	5.6	1.3	33.0	29.1	31.8	3.8	1.1
RpS23 prom	33.3	24.6	32.0	8.6	1.3	34.5	25.1	33.9	9.4	0.6
RpL32 prom	28.7	20.7	28.1	8.0	0.7	29.7	21.0	29.7	8.7	0.1
Fst prom	32.2	26.8	30.0	5.4	2.2	30.2	28.0	29.3	2.2	0.9
Fst 3′	30.2	25.4	28.8	4.8	1.5	28.9	27.3	28.1	1.6	0.8
Hus1-like prom	29.1	22.6	28.1	6.5	1.0	33.3	28.3	33.8	5.0	−0.4
Hus1-like 3′	28.6	25.4	27.9	3.2	0.7	29.4	26.3	28.9	3.1	0.5
AttD prom	28.3	25.1	28.0	3.2	0.2	28.8	26.4	28.7	2.4	0.1
Intergenic region1	29.4	28.3		1.1		28.1	27.5		0.4	
Intergenic region2	30.0	29.2		0.8		27.3	27.2		0.1	
Intergenic region3	32.0	31.6		0.4		28.6	28.5		0.1	
Intergenic region4	29.6	28.8		0.9		27.1	27.0		0.3	
	Preimm		ADA2b		$dCt\ (Ct_{Preimm}\text{-}Ct_{ADA2b})$	Preimm		ADA2b		$dCt\ (Ct_{Preimm}\text{-}Ct_{ADA2b})$
Intergenic region1	26.3		26.6		−0.3	27.0		26.6		0.4
Intergenic region2	27.3		27.5		−0.2	26.0		26.1		−0.1
Intergenic region3	28.5		28.8		−0.4	26.6		26.3		0.2
Intergenic region4	27.0		27.0		−0.1	24.8		25.1		−0.1

For the detection of dADA2b binding to chromatin, preimmune serum control (Preimm) was used instead of no antibody control (NAC).

then comparisons of the total RNA profiles of mutant and w^{1118} flies at these stages are expected to unravel this role. Surprisingly, the number of genes displaying an altered expression in *dAda2b* mutant compared to control is rather small. The *dAda2b* versus w^{1118} and *dAda2b* versus *dAda2b* Ada2bEGFP comparisons we made gave most likely a low and a high estimate of the number of genes affected by *dAda2b* function. The main reason that these numbers differ considerably might be that the Ada2bEGFP transgene does not provide a complete *dAda2b* function. Differences in the genetic background of the w^{1118} and the $dAda2b^{d842}$ mutant we used can also contribute to this, although as rescue of $dAda2b^{d842}$ homozygotes by a genomic transgene results in fertile adults (14), we do not think that this can be a major factor. The similar gene ontology distribution of affected genes of the two samples does not indicate a significant effect arising from different genetic backgrounds either.

Out of those genes affected by *dAda2b* mutation, more show increased than decreased transcript levels in mutants relative to w^{1118} control either in larva or in pupa stage. This might seem surprising as subunits of dSAGA were originally identified based on their role in transcription activation. Indeed, in *gcn5* mutant yeast cells, most of the affected genes show decreased expression relative to wild type cells, as expected for a coactivator protein (19). Gene expression profile analysis of *Arabidopsis* (At) Ada2b and Gcn5 mutants, however, provided opposing results, in that in both *AtAda2b* and *AtGcn5* mutants, most of the affected genes had increased transcript levels, similarly to our observations in *Drosophila* (37). Based on these data, we assume that the dSAGA complex plays dual roles, acting both in repression and activation of target genes. The number of genes that we found to be affected by *dAda2b* mutations is very close to the numbers reported by Weake et al. recently (35). Noteworthy, they also found that the number of those genes which are represented by an elevated mRNA level in *dAda2b* mutants (186) is slightly larger than that of those which have a reduced RNA level (158). Since the comparisons in the Workman's lab and in our laboratory were performed in animals at different developmental stages, we did not attempt a systematical comparison of the two datasets.

Our data did not indicate that the absence of *dAda2b* had a synchronous effect on physically linked clusters of genes, or that dADA2b was essential for the transcription of highly expressed housekeeping genes. Genome-wide profiling of yeast gene expression also suggested a housekeeping role more to TFIID- than SAGA-dominated genes (38). During the time period we tested, the expression changes of ecdysteroid-regulated genes play a crucial role in larva-pupa transition. The lack of *dAda2b* does not abolish ecdysone-mediated gene regulation, thus it seems that activation of transcription by *Drosophila* nuclear receptors can take place in the absence of dADA2b. This observation is somewhat unexpected

because several studies documented that SAGA and TFTC/STAGA-type complexes function as cofactors in the activation process of nuclear receptors (36,39,40). We must assume that in *dAda2b* mutant flies, either redundant activities are present at these stages of *Drosophila* development, or partial dSAGA complexes can form and function as co-activators.

We also observed that down-regulation of gene expression also takes place without a significant defect in *dAda2b* mutants. An example for this is the sharp drop in the expression of some *Lcp* genes in *dAda2b* mutants at the larva-pupa transition. The minor alterations in the timing of the expression change of the *Lcp* genes might indicate a role for *dAda2b* in modulating the kinetics of the transcriptional response. A similar contribution was suggested for yeast ADA2 in the transcription response to glucose (19). This result is thus consistent with the idea that HATs and/or HAT-containing complexes can contribute to chromatin restructuring and by this modify transcriptional activation. The response of *dAda2b* mutants to heat stress is also in accord with this view: in *dAda2b* mutants we observed a slower development of heat shock puffs as compared to controls.

The genes affected by dADA2b depletion most dramatically and in the highest number belong to the group of genes involved in *Drosophila* immune response and specifically those that play a role in defense against microorganisms. The increased level of some of the immune-function related messages is particularly striking in pupa comparisons. While our hybridization data show very high induction of some of the immune effector genes involved in defense against pathogens, we also noted a high level of fluctuation in the level of expression of some of these genes as compared among the triplicates. Nonetheless, we detected the induction of immune-related genes repeatedly in RNA samples obtained independently over a time period more than a year. Since the expression of many of the affected immune function related genes is readjusted at least partially in transgene carrier *dAda2b* null mutants (Table 2), a causal link between these gene expression changes and the loss of *dAda2b* function is highly probable. The activation of AMP-promoter linked GFP reporter genes in *dAda2b* mutants gives a further support this conclusion.

In contrast to the large number of AMP genes affected, only a limited number belonging to the Toll and Imd pathways are misregulated in *dAda2b* mutants. This might indicate that the lack of *dAda2b* affects mainly the downstream part of the immune response. Our data do not indicate whether the role of dADA2b in immune-related gene induction is direct or indirect. We did not observe that sensitivity towards experimental infections by bacteria was higher in *dAda2b* mutants than in their controls. We favor the idea that in the absence of dADA2b, a functional defect induces immune response genes. Among numerous possibilities, this defect could be in cuticle formation, or activation of an autoimmune mechanism. We believe a direct role of dADA2b in stress response, including immune response gene regulation can be assumed. In this respect the induction of *Frost* expression in *dAda2b* mutants is noteworthy as *AtAda2b* has also been found to be involved in cold response (37). Moreover, *Fst* also has been found among immune-induced genes in *Drosophila* (31).

Earlier, physical interaction has been demonstrated between Dmp53 and dADA2b (11). We also reported that *dAda2b* mutations interfered with Dmp53-mediated functions, and that X-ray irradiation induced apoptosis in a smaller number of cells in *dAda2b* mutants than in wild type controls (14). In contrast with that, another group found increased level of Dmp53-dependent apoptosis in response to X-ray radiation in *dAda2b* mutants (15). These authors concluded that *dAda2b* is acting upstream of *reaper* induction in response to irradiation. A more recent report has demonstrated that in mammalian system, among others, the dADA2b subunit of STAGA (the human homologue of dSAGA) makes contacts with p53, and plays a role in p53-dependent gene activation (41). These partially contradicting data on Dmp53 and *dAda2b* functional interaction made us interested to see whether the mRNA levels of pro- and anti-apoptotic genes were affected in *dAda2b* mutants. We found repeatedly a decreased number of cells in apoptosis following X-ray irradiation in $dAda2b^{d842}$ larvae (data not shown). In the microarrays only a small number of those genes implicated in apoptosis showed altered mRNA levels in *dAda2b* mutants. Among them, however, the level of *reaper* mRNA was decreased to 50% of the wild-type level, and importantly, in the presence of the rescue transgene the normal level was restored.

The results of ChIP experiments indicated dADA2b in low levels at promoter and 3′ regions of several genes we tested. Despite that some of the genes we studied by ChIP are up- or down-regulated in *dAda2b* mutants, while others are unaffected, we found with none of them the dADA2 protein associated in a significantly higher level than with any other. At first this seems to be an unexpected finding which might result from technical problems. On the other hand, it is in accord with the observation that SAGA is involved in global histone acetylation and in its absence a reduced level of histone acetylation is seen along the polytene chromosomes. The observation that the dSAGA-modified H3K9ac level is lower in all genes we tested is also in accord with this observation. The cause of failure in detecting specific and selective dADA2b-chromatin interaction by ChIP could be that dADA2b is in such a position within the dSAGA complex that it is not accessible to the Abs or is not crosslinked effectively. Alternatively, the contact between dSAGA and the nucleosomes required to deposit the modification could be a ''hit-and-run'' type interaction, which is not detectable by the ChIPs we performed. Interestingly, *dAda2b* mutation affects the H3K9ac level by a different extent in dSAGA-dependent and independent genes. In the promoters of the ribosomal protein genes, which are expressed in a high level both in *dAda2b* and wild type cells, we detected H3K9ac in only 10–20% lower levels in *dAda2b* mutant than in wild type samples. In the promoters (and also in the 3′ regions) of SAGA-regulated genes the H3K9ac levels were much lower in *dAda2b* mutant than wild type samples.

Comparisons of the H3 and H3K9ac levels at the promoters reveal a further interesting fact; in the promoters of the highly expressed ribosomal genes the level of K9 acetylated H3 is high even in *dAda2b* mutants (Figure 6C). The ratio of total and K9 acetylated H3 cannot be determined directly by these ChIP experiments because of the different efficiency of immunoprecipitations by the different Abs, nonetheless under the same conditions a much higher fraction of H3 can be precipitated in K9 acetylated form with the RpL32 and RpS23 promoters than with the dSAGA-dependent ones (Figure 6B). In other words, the promoters of ribosomal protein genes are associated with high levels of H3K9ac even in the absence of dSAGA. This observation might point to two important facts: first, that at different regions H3K9 can be acetylated by enzyme(s) other than dSAGA, and second, that the H3K9ac marks might have an important role in ensuring the high level expression of the RpL32 and RpS23 (and probably also other highly expressed dSAGA-independent) genes. On the other hand, the H3K9ac levels in dSAGA-regulated genes depend more on dADA2b. On these genes the activity of dSAGA plays a role in determining the transcription intensity. On genes down-regulated in *dAda2b* mutants the decreased acetylation is paralleled by a decreased Pol II occupancy on the promoters. On the up-regulated genes the situation is the opposite; a decreased H3K9ac level is detectable together with an increased Pol II occupancy.

The observation that *dAda2b* mutation results in a drastic decrease in global histone H3 acetylation, yet in *dAda2b* mutants the expression of only a relatively small numbers of genes are affected, poses an interesting question: does dSAGA play a role in global and gene-specific transcription regulation by the same or different mechanisms? In light of the data presented here we propose that the two effects are only seemingly different. We envision that in the landscape of modified histones established in the genome by enzymes in various protein complexes, the loss of *dAda2b* function (and by this the loss of those acetyltransferase functions of dSAGA which are affected by *dAda2b*) results in a global decrease in H3 acetylation. Depending on other type of histone modifications and the availability and activity of regulators this can lead to an increase or a decrease in the transcription level of selected genes. In the case of most of the genes, this is observable as a delay in the change in expression in the lack of dADA2b. At other regulatory regions, the combinations of histone modifications which exist in wild type animals are perturbed by the loss of dSAGA-specific histone H3 acetylation more drastically. These genes respond to *dAda2b* mutations as dSAGA-specific targets. It is worth to point out here that genes are defined as dSAGA-specific on an arbitrary criteria. The genome-wide response to *dAda2b* mutation is a continuous spectrum of changes in both directions. This interpretation is in accord with the observation that H3 acetylation by dSAGA is deposited all along the polytene chromosomes, and also with the suggested global role of H3 acetylation on transcription in other systems. The phenotype of *dAda2b* mutants i.e. that they develop late and have an extended lethal phase, is also in accord with this interpretation. We note, however, that since we studied the effects of *dAda2b* mutation using RNA samples from whole animals, which were in the later stages of their development, and cultured under normal conditions, a more direct promoter-specific *dAda2b*-dependent regulation of selected genes or under specific conditions cannot be excluded.

This study shows that *Drosophila dAda2b* mutants can serve as a valuable model for the dissection of metazoan SAGA functions. We expect that further studies will uncover details in dSAGA function, among others, will give an answer whether the up-regulation of immune-response related genes we observed in *Drosophila dAda2b* mutants, is a new function of metazoan SAGA which evolved from the general stress protection role SAGA plays in yeast cells.

ACKNOWLEDGEMENTS

The authors thank Adrienn Bakota for technical assistance. They also thank C. Thibault (IGBMC, Illkirch, France) for the microarray analyses, Dr J.L. Imler (CNTR-UPR9022, Strasbourg, France) and Dr B. Lemaitre (EPFL-SV-GHI, Lausanne), for providing us AMP-promoter-GFP transgene stocks.

FUNDING

Hungarian Science Fund (OTKA K77443 to I.B., PD72491 to L.B.); Hungarian Ministry of Health (ETT 078/2003 to I.B.); INSERM, CNRS, Réseau National des Génopoles (N° 260), European Community (EUTRACC LSHG-CT-2007-037445) grants to L.T.; European Community (HPRN-CT-2004-504228: LSHG-CT-2004-502950) to L.T. and I.B.). Funding for open access charge: Hungarian Science Fund OTKA K77443.

Conflict of interest statement. None declared.

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