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

Nóra Zsindely\textsuperscript{1}, Tibor Pankotai\textsuperscript{1}, Zsuzsanna Újfaludi\textsuperscript{1}, Dániel Lakatos\textsuperscript{1}, Orbán Komonyi\textsuperscript{1}, László Bodai\textsuperscript{1}, László Tora\textsuperscript{2} and Imre M. Boros\textsuperscript{1,3,*}

\textsuperscript{1}Chromatin Research Group of HAS, Department of Biochemistry and Molecular Biology, University of Szeged, Közép fasor 52, H-6726 Szeged, Hungary, \textsuperscript{2}Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), UMR 7104, CNRS, ULP, INSERM U 596, Parc d’Innovation, 1, rue Laurent Fries, BP 10142–67404 ILLKIRCH Cedex, CU de Strasbourg, France and \textsuperscript{3}Institute of Biochemistry, Biological Research Center, Temesvári krt. 62, H-6726 Szeged, Hungary

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

ABSTRACT

In \textit{Drosophila}, the \textit{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 \textit{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 \textit{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 \textit{dAda2b} mutants, particularly high numbers are those which play roles in antimicrobial defense mechanisms. Results of chromatin immunoprecipitation experiments indicate that in \textit{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 \textit{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 \textit{Saccharomyces cerevisiae} for example, Ada2p is present in the Spt-Ada-Gen5-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 \textit{Drosophila}, two related ADA2-type factors (\textit{dADA2a} and b) have been identified (10,11). Several lines of evidence indicate that the two \textit{dADA2} proteins are specific components of different GCN5 HAT complexes. \textit{dADA2a} is present in the 0.6 MDa ATAC (\textit{Ada2a}-containing) complex, which acetylates histone H4 at lysine K5 and K12 (12,13). \textit{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 \textit{dAda2b} mutations result in a significant decrease in the level of K14 and K9 acetylated histone H3.
(14,15). A decrease in histone H3K9ac and H3K14ac levels in \textit{dAda2b} mutants is detectable all along with the polytene chromosomes including highly compacted bands, suggesting that global histone H3 acetylation is dependent on \textit{dADA2b} (14,15). Interestingly, despite the dramatic decrease in the levels of H3K9ac and H3K14ac by the later developmental stages, \textit{dAda2b} null animals reach almost full development and die only as pupae. The ability of flies to nearly complete development in the absence of \textit{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 \textit{S. cerevisiae}, deletion of \textit{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 \textit{dADA2b}-dependent histone H3 acetylation in a multicellular organism. We took advantage of \textit{dADA2b} being a specific component of dSAGA and performed whole genome ribonucleic acid (RNA) profiling of \textit{dAda2b} null mutants to reveal the function of dSAGA in the late stages of \textit{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 \textit{Drosophila} medium. The null allele \textit{dAda2b} used in this work has been described (14). As a control, in some of these experiments we used \textit{w1118}, 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 \textit{dAda2bL}-enhanced green fluorescent protein (EGFP) transgene (short hereafter referred as \textit{Ada2bEGFP}), the 5’ half of the \textit{dAda2b} gene was amplified using primers \textit{Ada2bRI} and \textit{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 \textit{Ada2bL3BamHI} and \textit{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 \textit{dAda2b} gene by BamHI and NotI restriction endonucleases. The \textit{dAda2b} promoter was amplified using primers \textit{Ada2bgene} and \textit{Ada2bgeneL}, and assembled with the coding region in pKS using BglII and SalI. Finally, the fragment corresponding to the \textit{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: \textit{w/w; +/+; P\{Ada2bEGFP\}} \textit{dAda2bL}\textit{d842}, and \textit{w/w; +/+; P\{Ada2bEGFP\}} \textit{dAda2bL} \textit{d842}.

The \textit{attacin A} and \textit{defensin} promoter fused GFP-containing att-GFP and def-GFP transgene carrier stocks were kindly provided by Dr J.L. Imler and Dr B. Lemaître (23). For the detection of GFP expression under the control of \textit{att} and \textit{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 \textit{dAda2b} and \textit{w1118} 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 \textit{Ada2b}, polyclonal antibodies raised in rabbits against a \textit{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 (DAKO), 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 \textit{dAda2b} and \textit{w1118} 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     | GCCAGTGGACAAATTGGGTTGTA                              |
| 18S rev     | CGGAGGCCAAAGAATCTTAA                                 |
| Lcp1 fwd    | TTTCGGAATCCGAGATGTTT                                |
| Lcp1 rev    | GGTCGCCCTATCCTAGTTT                                  |
| Lcp4 fwd    | TCAAAGATCCCTGCTGCTGTC                                |
| Lcp4 rev    | CACCTGCAAAATCTCCGAGTTT                               |
| Eig71E fwd  | CTCGGTGGAAATGTTCCCTGTC                                |
| Eig71E rev  | ACCGGATGGGCTGCTACCAT                                  |
| Eig71EEd fwd| ATG2GAAACCCCTGTGAGGAA                                 |
| Eig71EEd rev| CGACGAGTCATGCACATTG                                    |
| Eig71Eg fwd | CCACTATGCCATGGCGGG                                         |
| Eig71Eh fwd | CCTAGCTGCTGCTTCCTGTC                                   |
| Eig71Eh rev | CTCGAGTTGGGAGTTCAC                                     |
| Ada2BHII    | CGATGGATCCCCGACAGCTATCCAA                             |
| Ada2Bgene   | TTATTCTTGCAAGCACGCT                                    |
| Ada2BgeneL  | CAGGTTGGGTCCATAGTGTGTC                                 |
| Ada2L3BamHI | GGA TCC OTG GCT CAG CCA GCC GCA                        |
| sug prom fwd (+34) | CGCATATTACCAGAACCTCT                          |
| sug prom rev (+66) | GTTGCTTGTGGTGGCTTCT                              |
| sug 3' fwd (+1525) | TTCGCTAAAACCAACAAGG                                 |
| sug 3' rev (+1638) | GTGACTCCACGCTCCATTCT                                 |
| Fst prom fwd (+59) | GCCATGTAATGAAATGTTG                                  |
| Fst prom rev (+62) | CAAAGCAGATGAAAGGATAA                                  |
| Fst 3' fwd (+864) | ATACTGACGTTCCTACCGGCTCT                               |
| Fst 3' rev (+964) | GTTAATCAGGAACCCCAAGAT                                 |
| cnn prom fwd (+80) | AACCAGCAGAAAACCAAAACCT                                |
| cnn prom rev (+116) | GTGATGAGTAAAGACGAG                                   |
| cnn 3' fwd (+33 437) | TGGAACTAGTGAGCCAGGA                                 |
| cnn 3' rev (+33 545) | TGTATAGTCCAGGGGAAAAG                                 |
| CycB prom fwd (+39) | TGCAGTAAAAAGAGGCAAAT                                 |
| CycB prom rev (+101) | TGATCGAACCGTTTGACAGCA                                 |
| RpS23 prom fwd (+8)  | GCCCTCATGACTGAAACACAT                                  |
| RpS23 prom rev (+140) | TTTCCCTATTCCACCAAACAA                                 |
| Rpl32 prom fwd (+19) | TTTTACACCCACGTTCTTTC                                  |
| Rpl32 prom rev (+120) | CACCCGACTACCGCTTCAAAC                                 |
| Hus1-like prom fwd (+62) | TGCTGATGCTGCTGATCTGTC                                 |
| Hus1-like prom rev (+96) | GCAAGGCGGATGCTATTACCT                                 |
| Hus1-like 3' fwd (+3527) | GGGCTCTTGGAGGACCTGT                                  |
| Hus1-like 3' rev (+3628) | CCAATCCTGCTGCTCAGAG                                  |
| AttD prom fwd (+62) | AGTGGGAGTACTTCTGGCAAC                                 |
| AttD prom rev (+130) | TCTACAGGAGCCAGCTTACC                                  |

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 (w1118 and dAda2b) at both time-points were obtained, permitting nine pair-wise comparisons of w1118 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 w1118 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 1μg 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. CT values were set against a calibration curve. The ΔΔCT 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: z-H3 (1 μg, Abcam ab1791), z-H3K9ac (4 μg, Abcam ab4441), z-H3K14ac (4 μg, Upstate 07-353), z-dADA2b (5 μl, (10), z-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-cleaned using BSA and salmon sperm DNA blocked Protein A—Sepharose CL-4B beads (Sigma). Pre-cleaned 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 (dAda2bΔ642) 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 w1118 and dAda2b larvae to activate heat shock genes, and stained polytene chromosomes 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 w1118 and dAda2b null mutants at two stages: 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 w1118 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
dAda2b mutants in any of the stages tested. A comparison of the mRNA profiles of dAda2b mutant and control (w1118) 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 w1118 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 w1118 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 w1118 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 w1118 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 w1118 and dAda2b animals. This might show gene expression alterations resulting from genetic differences unrelated to dADA2b isoforms are indicated. Note that in Ada2bEGFP 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 mutants, w1118 control, and Ada2bEGFP transgene carrier Ada2b animals. The labels are P: pupa, L: larvae Ada2bmrec. Ada2bEGFP transgene carrier Ada2b animals and as indicated. The Mw of the two

Figure 1. (A) Western blot of total protein samples of Ada2b mutant, w1118 control, and Ada2bEGFP transgene carrier Ada2b animals. The labels are P: pupa, L: larvae Ada2bmrec. Ada2bEGFP transgene carrier Ada2b animals and as indicated. The Mw of the two

Nucleic Acids Research, 2009, Vol. 37, No. 20 | 6669
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 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 \(Ada2b^{d842}\) homozygotes are shown.
follows that of those genes identified in the dAda2b versus w^{118} comparison (Figure 2C). We believe therefore that the two estimates i.e. dAda2b versus Ada2bEGFP carrier, and dAda2b versus w^{118} 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^{118} 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^{118} 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^{118} animals, the levels of both mRNAs decrease dramatically during the transition from L3 to pupa stage (Figure 3). In dAda2b mutants, the
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.

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

| PART A |
| --- | |
| **Gene** | **Lex** | **Pupa** | **Total** | **P(lex)** | **P(pupa)** |
| **Ada2b** | **Ada2b**<sup>rescue</sup> | **Ada2b**<sup>rescue</sup> | **Ada2b**<sup>rescue</sup> | | |
| C3 | 22 550 22 | 1.17 0.86 | 1.32 0.59 | 1.56 0.01 | |
| C5 | 19 559 19 | 1.63 3.71 | 1.52 0.91 | 1.62 0.01 | |
| C6 | 10 225 10 | 3.33 0.22 | 3.54 0.21 | 1.12 0.31 | |
| C7 | 52 655 52 | 3.26 0.09 | 3.26 0.09 | 7.06 0.02 | |
| **Dipterine (dpg) family** | **2 members** | | | | |
| dpg | 68 219 15 | 0.68 0.04 | 1.40 0.01 | | |
| dpgB | 62 219 15 | 0.68 0.04 | 1.40 0.01 | | |
| **Cecropin (cec) family** | **4 members** | | | | |
| cecA1 | 19 285 19 | 2.97 0.02 | 2.97 0.02 | | |
| cecA2 | 93 562 93 | 2.59 0.02 | 2.59 0.02 | | |
| cecC | 8 49 8 | 8.22 0.00 | 8.22 0.00 | | |
| cecB | 6 73 6 | 0.71 0.00 | 0.71 0.00 | | |
| **Drosomycin (drs) family** | **6 members** | | | | |
| drr1 | 1195 3103 13 | 1.08 0.00 | 7.06 0.00 | | |
| drr2 | 20 52 20 | 1.52 0.00 | 1.52 0.00 | | |
| drr3 | 12 27 12 | 0.76 0.00 | 1.28 0.00 | | |
| drr4 | 1389 4520 15 | 3.37 0.00 | 3.37 0.00 | | |
| drr5 | 5265 1052 5 | 2.70 0.00 | 2.70 0.00 | | |
| **Other Amps** | | | | | |
| amphiA | 44 795 44 | 0.11 0.00 | 0.11 0.00 | | |
| hmyain | 329 701 33 | 0.20 0.00 | 0.20 0.00 | | |
| defensin | 30 102 67 | 1.78 0.00 | 1.78 0.00 | | |
| mpd | 29 63 29 | 0.52 0.00 | 0.52 0.00 | | |
| **Peptidoglycan recognition protein (PGRP) family** | | | | | |
| PGRP-Sc2 | 167 301 278 | 0.71 0.00 | 1.83 0.00 | | |
| PGRP-Sclb | 41 239 910 10 | 2.53 1.67 | 1.45 0.00 | | |
| PGRP-Sclt | 26 54 26 | 1.06 0.00 | 1.06 0.00 | | |
| PGRP-Spl | 263 426 26 | 2.46 0.00 | 2.46 0.00 | | |
| PGRP-Spl2 | 3445 170 2269 46 | 0.55 0.00 | 0.55 0.00 | | |
| PGRP-D | 72 47 2 | 1.88 0.00 | 1.88 0.00 | | |
| PGRP-P | 34 12 34 | | | | |
| **Lysozyme (lya) family** | | | | | |
| lyaX | 2487 1157 1067 2095 495 | 2.10 2.08 | 1.47 0.00 | | |
| lya | 2549 7164 25 | 1.58 | 1.46 | | |
| lyaB | 236 135 | 236 | -0.81 | -0.14 | | |
| lyaC | 1179 5221 1179 | 1.58 | 1.47 | | |
| Nf | 14 33 14 | 0.57 0.00 | 0.57 0.00 | | |
| CG1798 | 22 6 6 | 0.26 | 0.26 | | |
| CG1849 | 98 30 98 | 1.20 | 0.78 | | |
| CG1794 | 33 27 33 | -1.84 | -0.92 | | |
| **Induced serine proteases** | | | | | |
| CG1853 | 43 27 43 | -0.65 | -0.65 | | |
| CG1854 | 48 20 48 | 2.09 | 2.09 | | |
| CG1856 | 14 29 14 | 0.60 | 0.60 | | |
| CG1857 | 65 21 65 | 0.62 | 0.62 | | |
| CG1873 | 16 57 16 | -0.80 | -0.80 | | |
| CG1861 | 856 213 856 | 0.08 | 0.08 | | |
| **Induced small peptides (40-134aa)** | | | | | |
| CG1809 | 56 129 56 | 1.19 | 4.02 | | |
| CG1810 | 183 389 183 | 1.31 | 3.05 | | |
| M1 | 34 4 | 34 4 | 0.90 | 0.90 | 1.15 | |
| M2 | 15 4 | 15 4 | 0.90 | 0.90 | 1.15 | |
| M3 | 3 5 | 3 5 | 0.65 | 0.65 | 0.65 | |
| M4 | 13 39 30 | 1.65 | 0.96 | 1.65 | |
| CG1845 | 150 582 150 | 2.46 | 0.12 | 2.46 | |
| CG1847 | 953 2241 2222 | 1.33 | 2.14 | |
| M5 | 3 9 | 3 9 | 0.55 | 0.55 | 2.12 | |
| CG13324 | 22 48 22 | 0.59 | 0.59 | 1.01 | |
| CG15114 | 38 16 | 38 16 | 2.52 | | |
| CG1848 | 9 9 9 | 2.99 | 2.99 | 2.99 | |
| CG1942 | 32 48 42 | 0.15 | 0.15 | 1.38 | |
| CG17167 | 18 57 25 | 3.65 | 5.24 | | |
| CG1870 | 508 985 1237 | 0.38 | 2.58 | | |
| CG1773 | 129 48 | 129 48 | 0.48 | 0.48 | 2.32 | |
| CG1816 | 183 362 183 | 1.33 | 2.12 | |
| CG1818 | 19 44 | 19 44 | 1.04 | 1.04 | | |
| CG1819 | 35 233 | 35 233 | 1.04 | 1.04 | | |
| CG1820 | 25 233 | 25 233 | 1.04 | 1.04 | | |
| CG1821 | 150 310 150 | 2.38 | 0.12 | 2.38 | |
| CG1822 | 90 18 | 90 18 | 0.71 | 0.71 | | |
| CG1823 | 65 33 | 65 33 | 0.71 | 0.71 | | |
| CG1824 | 30 48 | 30 48 | 0.71 | 0.71 | | |

The average hybridization signal determined on three microarrays in control (w<sub>1118</sub>, dAda2b<sup>d842</sup>, and dAda2b<sup>d842</sup> Ada2bEGFP transgene carrier larvae (Ada2b<sup>rescue</sup>) 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<sup>rescue</sup> 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).

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 mutants. 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, Husl), 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 \( dAda2b^{null} \) 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), dADAAb- (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 dC\(_t\) 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.
For the detection of dADA2b binding to chromatin, preimmune serum control (Preimm) was used instead of no antibody control (NAC).

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 synchronized wild-type and dAda2b mutant third instar larvae

| Region          | w^{1118}   | dAda2b   |
|-----------------|------------|----------|
|                 | Ct         | dCt (Ct_{NAC} - Ct_{H3K9ac}) | Ct         | dCt (Ct_{NAC} - Ct_{H3K9ac}) |
|                 | NAC H3K9ac | ADA2b    | NAC H3K9ac | 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 region 1 | 29.4 | 28.3 | 1.1 | 28.1 | 27.5 | 0.4 |
| Intergenic region 2 | 30.0 | 29.2 | 0.8 | 27.3 | 27.2 | 0.1 |
| Intergenic region 3 | 32.0 | 31.6 | 0.4 | 28.6 | 28.5 | 0.2 |
| Intergenic region 4 | 29.6 | 28.8 | 0.9 | 27.1 | 27.0 | 0.3 |

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^{842} mutant we used can also contribute to this, although as rescue of the dAda2b^{842} 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 gen5 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 analysis of Arabidopsis (At) Ada2b and Gen5 mutants, however, provided opposing results, in that in both AtAda2b and AtGen5 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 TFIIID- 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 Lep genes in dAda2b mutants at the larva-pupa transition. The minor alterations in the timing of the expression change of the Lep 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 dAda2b0842 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 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 acetylation 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 SAGA-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 yeasts 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 Génapoles (N° 260), European Community (EUTRACC LHSG-CT-2007-037445) grants to L.T.; European Community (HPRN-CT-2004-504228: LHSG-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.

REFERENCES
1. Carrozza,M.J., Utey,R.T., Workman,J.L. and Cote,J. (2003) The diverse functions of histone acetyltransferase complexes. Trends Genet., 19, 321–329.
2. Nagy,Z. and Tora,L. (2007) Distinct GCN5/PCAF-containing complexes function as co-activators and are involved in transcription factor and global histone acetylation. Oncogene, 26, 5341–5357.
3. Wu,P.Y., Ruhlmann,C., Winston,F. and Schultz,P. (2004) Molecular architecture of the S. cerevisiae SAGA complex. Mol. Cell, 15, 199–208.
4. Daniel,J.A. and Grant,P.A. (2007) Multi-tasking on chromatin with the SAGA coactivator complexes. Mutat. Res., 618, 135-148.
5. Grant,P.A., Duggan,L., Cote,J., Roberts,S.M., Brownell,J.E., Candau,R., Ohba,R., Owen-Hughes,T., Allis,C.D., Winston,F. et al. (1997) Yeast Gcn5 functions in two multisubunit complexes to acetylate nucleosomal histones: characterization of an Ada complex and the SAGA (Spt/Ada) complex. Genes Dev., 11, 1640–1650.
6. Pray-Grant,M.G., Schiltz,D., McMahon,S.J., Wood,J.M., Kennedy,E.L., Cook,R.G., Workman,J.L., Yates,J. R. 3rd. and Grant,P.A. (2002) The novel SLIK histone acetyltransferase
complex functions in the yeast retrograde response pathway.

7. Berger, S.L., Pina, B., Silverman, N., Marcus, G.A., Agapite, J., Regier, J.L., Triebenj, S.J. and Guarente, L. (1999) Genetic isolation of ADA2: a potential transcriptional adaptor required for function of certain acidic activation domains. Cell, 70, 251–265.

8. Pina, B., Berger, S., Marcus, G.A., Silverman, N., Agapite, J. and Guarente, L. (1999) ADA3: a gene, identified by resistance to GAL-VP16, with properties similar to and different from those of ADA2. Mol. Cell. Biol., 13, 5981–5989.

9. Baker, S.P. and Grant, P.A. (2007) The SAGA complexes expands: the cellular role of a transcriptional co-activator complex. Oncogene, 26, 5329–5340.

10. Muratoglu, S., Georgieva, S., Papai, G., Scheer, E., Enunlu, I., Komonyi, O., Csorpan, I., Lebedeva, L., Nabirolchikina, E., Udvardy, A. et al (2007) Two different Drosophila ADA2 homologues are present in distinct GCN5 histone acetyltransferase-containing complexes. Mol. Cell. Biol., 23, 306–321.

11. Kusch, T., Guelman, S., Abmayr, S.M. and Workman, J.L. (2003) Two Drosophila Ada2 homologues function in different multiprotein complexes. Mol. Cell. Biol., 23, 3305–3319.

12. Ciurciuc, A., Komonyi, O., Pankotai, T. and Boros, I.M. (2006) The Drosophila histone acetyltransferase Gcn5 and transcriptional adaptor Ada2a are involved in nucleosomal histone H4 acetylation. Mol. Cell. Biol., 26, 9413–9423.

13. Guelman, S., Saganuma, T., Florens, L., Swanson, S.K., Kieseker, C.L., Kusch, T., Anderson, S., Yates, J.R. 3rd, Washburn, M.P., Abmayr, S.M. et al (2006) Host cell factor and an uncharacterized SANT domain protein are stable components of ATAC, a novel dAda2a/dGcn5-containing histone acetyltransferase complex in Drosophila. Mol. Cell. Biol., 26, 871–882.

14. Pankotai, T., Komonyi, O., Bodai, L., Ujfaludi, Z., Muratoglu, S., Ciurciuc, A., Tora, L., Szabad, J. and Boros, I. (2005) The homologous Drosophila transcriptional adaptors ADA2a and ADA2b are both required for normal development but have different functions. Mol. Cell. Biol., 25, 8215–8227.

15. Qi, D., Larsson, J. and Mannervik, M. (2004) Drosophila Ada2b is required for viability and normal histone H3 acetylation. Mol. Cell. Biol., 24, 8080–8089.

16. Pokholok, D.K., Harbison, C.T., Levine, S., Cole, M., Hannett, N.M., Lee, T.I., Bell, G.W., Walker, K., Rolfe, P.A., Herbolsheimer, E. et al (2005) Genome-wide map of nucleosome acetylation and methylation in yeast. Cell, 122, 517–527.

17. Chanas, G., Lavrov, S., Iral, F., Cavalli, G. and Maschat, F. (2004) Engrailed and polyhomeotic maintain posterior cell identity through cubsitus-interruptus regulation. Dev. Biol., 272, 522–535.

18. Besse, S., Vignon, M., Pichard, E. and Puvion-Dutilleul, F. (1995) Synthesis and maturation of viral transcripts in herpes simplex virus type 1 infected HeLa cells: the role of interchromatin granules. Gene Expr., 4, 143–161.

19. Winer, J., Jung, C.K., Shackle, I. and Williams, P.M. (1999) Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. Anal Biochem., 270, 41–49.

20. Janaki, R., Papai, G., Scheer, E., Enunlu, I., Komonyi, O., Csorpan, I., Lebedeva, L., Nabirolchikina, E., Udvardy, A. et al (2007) Two different Drosophila ADA2 homologues are present in distinct GCN5 histone acetyltransferase-containing complexes. Mol. Cell. Biol., 23, 306–321.

21. Yan, C. and Boyd, D.D. (2006) Histone H3 acetylation and H3 K4 methylation define distinct chromatin regions permissive for transgene expression. Mol. Cell. Biol., 26, 6357–6371.

22. Ryder, E., Blows, F., Ashburner, M., Bautista-Llacer, R., Coulson, D., Drummond, J., Webster, J., Gubb, D., Gunton, N., Johnson, G. et al (2004) The DrosDel collection: a set of P-element insertions for generating custom chromosomal aberrations in Drosophila melanogaster. Genetics, 167, 797–813.

23. Tzou, P., Ohresser, S., Ferrandon, D., Capovilla, M., Reichhart, J.M., Lemaire, B., Hoffmann, J.A. and Imler, J.L. (2000) Tissue-specific inducible expression of antimicrobial peptide genes in Drosophila surface epithelia. Immunity, 13, 737–748.

24. Besse, S., Vignon, M., Pichard, E. and Puvion-Dutilleul, F. (1995) Synthesis and maturation of viral transcripts in herpes simplex virus type 1 infected HeLa cells: the role of interchromatin granules. Gene Expr., 4, 143–161.

25. Winer, J., Jung, C.K., Shackle, I. and Williams, P.M. (1999) Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. Anal Biochem., 270, 41–49.

26. Kim, S., Lavois, S., Iral, F., Cavalli, G., and Maschat, F. (2004) Engrailed and polyhomeotic maintain posterior cell identity through cubsitus-interruptus regulation. Dev. Biol., 272, 522–535.

27. Besse, S., Vignon, M., Pichard, E. and Puvion-Dutilleul, F. (1995) Synthesis and maturation of viral transcripts in herpes simplex virus type 1 infected HeLa cells: the role of interchromatin granules. Gene Expr., 4, 143–161.