Ecdysone Induced Gene Expression Is Associated with Acetylation of Histone H3 Lysine 23 in Drosophila melanogaster

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Abstract

Posttranslational modification of histones regulates transcription but the exact role that acetylation of specific lysine residues plays in biological processes in vivo is still not clearly understood. To assess the contribution of different histone modifications to transcriptional activation in vivo, we determined the acetylation patterns on the ecdysone induced Eip74EF and Eip75B genes in Drosophila melanogaster larvae by chromatin immunoprecipitation. We found that acetylation of histone H3 lysine 23 is localized to promoters and correlates with endogenous ecdysone induced gene activation. In contrast, acetylation of lysines 8, 12 and 16 of histone H4 and lysine 9 of histone H3 showed minor differences in their distribution on the regulatory and transcribed regions tested, and had limited or no correlation with ecdysone induced transcriptional activity. We found that dCBP, which is encoded by the nejire gene, acetylates H3 lysine 23 in vivo, and silencing of nejire leads to reduced expression of the Eip74EF and Eip75B genes. Our results suggest that acetylation of specific lysine residues of histones contribute specifically to the dynamic regulation of transcription. Furthermore, along with previous studies identify CBP dependent H3 lysine 23 acetylation as an evolutionarily conserved chromatin modification involved in steroid induced gene activation.

Introduction

The hereditary material of eukaryotes can be found in a complex structure called chromatin, which beside DNA also contains protein and RNA molecules. The basic building blocks of chromatin are the nucleosomes that consist of a protein core, containing two of each histone proteins H2A, H2B, H3 and H4, wrapped around twice with 146 bp of DNA [1]. The organization of nucleosomes and posttranslational modifications (PTMs) of histone proteins play a pivotal role in the regulation of DNA dependent nuclear processes by modulating the accessibility of the chromatin template [2]. Histones can be covalently modified by a variety of chemical appendages ranging from small functional groups to whole proteins, like ubiquitin or SUMO [3]. These modifications may alter the interaction of histones with DNA, neighboring nucleosomes and chromatin binding proteins. The observation that a high number of histone PTMs affect the unstructured N-terminal histone tails not involved in the formation of the nucleosome core particle, and the identification of protein domains responsible for the binding of modified residues of histones favors the idea that PTMs act primarily by providing binding surfaces to chromatin associated proteins i.e. rendering the chromatin more or less accessible to these factors [4]. This notion forms the basis of the histone code hypothesis that proposes that combinations of histone PTMs recruit specific binding factors thereby leading to specific functional outcomes [5].

Acetylation of lysine residues, one of the firstly described histone PTMs [6], affects the lysine rich N-terminal tails of all four core histones [4]. As acetylation neutralizes the positive charge of lysine residues first it was proposed that it loosens chromatin by weakening the association of the negatively charged DNA with the protein core of the nucleosome. Later characterization of the acetyl-lysine binding bromodomain [7], which can be found in several chromatin binding proteins, proved that this PTM can also exert its effect by recruiting chromatin binding proteins. The spatial and temporal pattern of histone acetylation is established by the opposing action of two enzyme groups, the histone acetyltransferases (HATs) and the histone deacetylases (HDACs), both consisting of several conserved protein families [8,9]. Histone acetylation is dynamically regulated, with a usual half-life of 2–3 minutes that rarely exceeds 30–40 minutes [10]. The high turnover rate strongly suggests that instead of influencing epigenetic
memory acetylation participates in the regulation of dynamic processes on the chromatin template.

Although a large body of knowledge had been accumulated about the characteristics and biological functions of histone acetylation, information about its pattern and role during dynamic transcriptional changes in vivo is scarce. Therefore, we designed experiments to reveal histone acetylation patterns associated with gene activation in *Drosophila* in vivo. Loss of several HAT genes in *flies* (for example *en3* [11], *endik mushroom* [12] or *chameau* [13]) causes lethality during the larva - pupa transition or during metamorphosis, suggesting that histone acetylation might play an essential role in the 20-hydroxyecdysone (20E) induced transcription response. 20E is the steroid molting hormone responsible for the timing of transitions between developmental stages [14]. In its target cells 20E binds to and activates its receptor, a heterodimer of the Ecdysone Receptor and Ultraspircacle proteins [15], then the activated hormone-receptor complex acts as a transcription factor to induce the expression of its target genes [16]. The primary targets of the activated receptor, the so called early genes, are themselves transcriptional factors that activate the expression of late (effector) genes [17]. Thus, release of the hormone leads to a well regulated gene activation cascade. We investigated histone acetylation patterns on different functional regions of the *Eip74EF* [18] and *Eip75B* [19] ecdysone induced early genes during their activation in late third instar larvae. The acetylation of lysine 23 of histone H3 was associated with the promoters of activated ecdysone induced genes, while other residues (H3K9, H4K8, H4K12 and H4K16) showed little or no change in their level of acetylation in response to 20E. We found, that H3K23 acetylation is catalyzed by dCBP, the product of the *nejire* gene, in vivo and that normal cCBP function is required for the proper activation of the *Eip74EF* and *Eip75B* genes.

**Results**

*Eip74EF* and *Eip75B* Promoters are Induced in Late L3 Larvae

Mutations of several histone acetyltransferase genes cause lethality in late L3 larvae or in early pupae when pulses of the molting hormone ecdysone trigger metamorphosis by activating the transcription of ecdysone responsive genes. Therefore, we hypothesized that acetylation of nucleosomal histones plays a significant role in the regulation of the ecdysone response and by analyzing the pattern of acetylation on ecdysone induced genes during this period we can gain insight into the role of this PTM in gene regulation in vivo.

Our aim was to characterize inducible promoters that are activated in late third larval instar (L3) when the level of 20-hydroxyecdysone is elevated. Therefore, we determined the transcriptional activity profile of the *Eip74EF-RA*, *Eip74EF-RB* and *Eip75B-RC* promoters during the L3 stage in four hours resolution. The *Eip74EF* and *Eip75B* ecdysone induced genes are complex transcription units that express several transcript variants from alternative promoters (Fig. 1). Promoter activity was determined by Q-PCR using primers specific for intronic regions 1–2 kb downstream of the selected promoters that amplify only cDNAs that are derived from nascent RNAs but not those from accumulated mRNAs. The *Eip74EF-RA* promoter is silent during the first 40 hours of the L3 stage and is strongly activated 8 hours before pupariation during the wandering L3 (w-L3) stage (Fig. 2A). The *Eip74EF-RB* promoter is active throughout the L3 stage. Its activity is moderately increased during the second day of L3, reaches its highest level 4–8 hours before pupariation then it is downregulated (Fig. 2B). The *Eip75B-RC* promoter shows low transcriptional activity during the first 40 hours of the L3 stage; then it is activated in wandering larvae and reaches its peak level in the last four hours before pupariation (Fig. 2C). Thus, we found that all of the three selected promoters are activated during the late third larval ecdysone pulse and they show a somewhat different transcriptional profile.

**Histone Modification Patterns on the *Eip74EF* and *Eip75B* Genes**

To determine the pattern of histone modifications during the activation of the *Eip74EF* and *Eip75B* genes, we collected *w*1118 (wild-type) larvae for chromatin immunoprecipitation (ChIP) analysis at three time points representing different stages of ecdysone response. We collected synchronized larvae in the middle of the L3 stage (mid-L3), which has low ecdysone level; wandering L3 larvae (w-L3) in which the ecdysone level is elevated; and from larvae with everted anterior spiracles (spev-L3), which are immediately before pupariation and have the highest ecdysone level. The occurrence of nucleosomes and acetylated histones on functionally distinct gene regions was determined by ChIP of larval chromatin samples with antibodies raised against histone H3 or against specific acetylated histones, followed by Q-PCR quantitation (Fig. 3). The PCR primers used amplified sequences of promoters, 5’ introns and 3’ exons of the *Eip74EF-RB*, *Eip74-RB* and *Eip75B-RC* transcriptional units (Fig. 1), and also two control regions: one at the promoter of the highly expressed Rpl32 ribosomal protein gene, and another in an euchromatic intergenic region.

By measuring the levels of histone H3 we found that all regions in question were occupied by nucleosomes at all time points (Fig. 3A). The level of histone H3 did not change significantly at any of the regions tested during gene induction. However, significant differences were detected between the investigated gene regions during the w-L3 and spev-L3 stage that could be mainly contributed to the low nucleosome occupancy of the *Eip74EF-RB* and *RB* promoters. It is important to note that there was no significant difference in H3 level between the intergenic control region and the transcribed regions of the *Eip74EF* and *Eip75B* genes; or between the four investigated promoters, i.e. the three ecdysone inducible ones and the constitutively highly active Rpl32 promoter.

In order to identify chromatin marks present at the *Eip74EF* and *Eip75B* genes we tested a panel of antibodies recognizing histone H3 or H4 proteins acetylated or methylated at specific lysine residues in a pilot ChIP experiment, and found that acetylated forms of the H3K9 (lysine 9 of histone H3), H3K23, H4K8, H4K12 and H4K16 residues could be reliably detected. Thus, we determined the pattern of these PTMs during the late L3 stage ecdysone response.

The H3K9 residue was highly acetylated on the Rpl32 control gene, while all other regions exhibited more than ten times lower level of acetylation, indicating that this modification is primarily associated with highly active gene expression (Fig. 3B). We did not find significant differences between the *Eip74EF* and *Eip75B* gene regions and the intergenic control region at any time point, nor significant changes in H3K9 acetylation at any region between the three time points. These results suggest that the level of H3K9 acetylation we observed at the *Eip74EF* and *Eip75B* genes is not directly linked to ecdysone induced transcription.

Acetyl-H3K23 could be primarily detected at regulatory regions of the *Eip74EF* and *Eip75B* genes, the level of this modification was significantly higher on the *Eip74EF-RB* and *RB* promoters in the w-L3 and spev-L3 stages than on other regions (Fig. 3C). Furthermore, the level of H3K23 acetylation significantly
increased during the ecdysone response on the Eip74-RA and -RB promoters. In contrast to the ecdysone induced genes, acetyl-H3K23 levels were low on the Rpl32 promoter. Thus, the dynamics of H3K23 acetylation showed characteristics that implicate this PTM in ecdysone dependent gene regulation.

Although the level of H4K8 acetylation shows a seemingly even distribution, several gene sequences (Eip74EF-RA promoter and intron 5, Eip75B intron 1 and exon 8, Rpl32) is enriched in this modification compared to the intergenic control region in the mid-L3 sample (Fig. 3D). Furthermore, a statistically significant general decrease of acetyl-H4K8 levels was detected in the spev-L3 samples compared to the mid-L3 or w-L3 samples.

The level of H4K12 and H4K16 acetylation (Fig. 3 E and F, respectively) showed a pattern resembling to H3K9 acetylation i.e. they were enriched on the highly expressed Rpl32 gene, but showed minor regional or temporal changes on the Eip74EF and Eip75B genes. However, similarly to H4K8 acetylation, a general decline in acetyl-H4K16 levels was observed in the spev-L3 stage.

**Drosophila** CBP has H3K23 Specific Acetyltransferase Activity

Among the H3 and H4 specific histone modifications tested acetylation of H3K23 was the only one that showed strong specificity to the promoters of Eip74EF and Eip75B and correlated with their activation suggesting that this modification is involved in the regulatory steps leading to gene induction. To be able to modulate the level of this PTM we aimed to identify the HAT enzyme specific for it in flies. Previous studies have implicated the acetyltransferases GCN5 in yeast [20,21] and CBP in mammals [22,23] in the acetylation of the H3K23 residue, thus, we considered the *Drosophila* orthologs of these factors as candidates.

*Drosophila* GCN5 is the catalytic subunit of the ATAC and SAGA acetyltransferase complexes, which can be inactivated by mutating the complex specific ADA2a or ADA2b subunits, respectively. Null mutations of the *gen5*, *Ada2a* or *Ada2b* genes cause lethality at the end of the third larval instar or in early pupae [11,24], therefore we analyzed the level of acetyl-H3K23 in late L3 larvae in which GCN5 activity supposed to reach critically low levels. We tested samples from homozygous *gen5* flyes, *Ada2a* and *Ada2b* mutants next to homozygous *cha14* and *moF* (two *H4K16 specific HATs [25,26]) and w*1118* (wild-type) controls by western analysis and found that loss of neither of these factors led to decreased H3K23 acetylation (Fig. 4A).

To rule out the possibility, that although GCN5 is not required for the maintenance of global H3K23 acetylation levels but it is required specifically for the acetylation of the same residue on the *Eip74EF* and *Eip75B* genes, we performed ChIP experiments on chromatin samples derived from *gen5* homozygous and heterozygous (control) wandering L3 larvae (Fig. 4C). We found that acetyl-H3K23 can be detected in *gen5* mutants, although its level is slightly diminished at several tested regions. As previously we have found, that at this developmental stage *gen5* larvae are protein null [27], and the acetylation of main GCN5 targets, such as H3K9 and H3K14 nearly completely vanishes [28], we concluded that GCN5 is not, or at least not solely responsible for H3K23 acetylation.

Next we asked whether dCBP/nejire, the single *Drosophila* ortholog of the mammalian CBP and p300 proteins is responsible for the acetylation of the H3K23 residue. As loss of dCBP activity in *nej* null mutants results in embryonic lethality [29], we investigated the consequences of loss of dCBP function in embryos. By comparing the intensity of anti-acetyl-H3K23 immunostaining of whole mount wild-type and hemizygous *nej* embryos we found that the level of acetyl-H3K23 immunoreactivity was significantly lower (P<0.01) in *nej* mutants (Fig. 4B). The residual acetyl-H3K23 immunoreactivity in *nej* mutants can be attributed to maternal effects as it was reported previously that dCBP mRNA and protein is present in *nej* mutant embryos at a...
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Discussion

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Acetylation of nucleosomal histones is generally thought to be associated with active transcription, although the exact role of specific acetyl-lysines is often not clearly elucidated. The understanding of the functional consequences of histone acetylation is made difficult not only by the variety of molecular interactions (histone - DNA, histone – chromatin binding factor, and intra- and internucleosomal histone - histone) it can alter but also by the often contradictory findings different experimental approaches provide. To resolve these discrepancies a two-step model had been proposed by Anamika et al. according to which each segment as is visualized by anti-GFP immunostaining (Fig. 4D and 4E). Anti-acetyl-H3K23 immunostaining reveals similar pattern in embryos carrying UAS-dCBP, i.e. strong anti-acetyl-H3K23 immunoreactivity can be observed in the posterior part of every segment, where dCBP is overexpressed, while only weak staining is detected in the anterior parts of the segments where the UAS-dCBP transgene is silent (Fig. 4F). In contrast, in embryos overexpressing the UAS-dCBP-FLAD transgene increased anti-acetyl-H3K23 immunoreactivity cannot be observed (Fig. 4G). We observed similar staining pattern by detecting the acetylation level of H3K27, a previously characterized substrate [32] of dCBP (Fig. S1). Taken together these findings indicate that dCBP participates in the acetylation of the H3K23 residue in vivo.

dCBP is Required for Larval development and Proper Eip74EF and Eip75B Transcription

The early lethality of nejire mutants hinders the assessment of dCBP functions in larval development, therefore we turned to the use of inducible RNAi lines. To be able to determine whether dCBP is essential in specific developmental stages we generated flies that carried UAS-nejire-RNAi constructs along with a heat-shock inducible hs-GAL4 driver, and activated the expression of the nejire-RNAi construct by a single heat-shock on specific days of development from day 1 to day 7 after egg laying. As Figure 5A shows, without induction the viability of hs-GAL4/+ UAS-nejire-RNAiKK105115 flies was not significantly lower than that of UAS-nejire-RNAiKK105115 control siblings. However, one-off heat-shock seriously compromised the viability of hs-GAL4/+ UAS-nejire-RNAiKK105115 flies. Especially strong reduction in viability was observed if the heat-shock was between days 3 to 6, i.e. during the L2 or L3 instars or in the prepupal stage, while much weaker or no effect could be seen if the heat-shock was in day 1, 2 or 7 after egg laying, corresponding to the embryonic, L1 larval or pupal stages (Fig. 5A). This suggests that dCBP is required for larval development and the prepupal-pupal transition, but is not essential for pupal development or residual dCBP could be sufficient to promote development at this stage. The resistance of embryos and L1 larvae to the effects of nejire-RNAi could be attributed to maternally deposited dCBP.

After we established that dCBP is required during larval development, we sought to determine whether expression of nejire-RNAi influences the expression of the Eip74EF and Eip75B genes. Therefore, we crossed UAS-nejire-RNAiKK105115 females with hs-GAL4/TM6 Tb males and compared the expression of the Eip74EF and Eip75B genes in nejire-RNAi hs-GAL4 versus nejire-RNAi/TM6 Tb control larvae after heat-shock (Fig. 5B). We observed dramatic reduction in the activity of the Eip74EF-R A and -RB and the Eip75B-R C promoters. The amount of accumulated Eip74EF mRNA decreased to half, while a small reduction could be detected in the level of the Eip75B mRNA.

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Conserved Epigenetic Patterns on Steroid Induced Genes

Steroid hormones regulate the development and cellular and physiological functions of vertebrate and invertebrate species and also play an important role in human health and disease. They exert their effects primarily through inducing transcriptional changes with the aid of their receptors, members of the conserved nuclear hormone receptor family, which act as transcription factors. Besides ligand binding, the activity of these receptors is also influenced by posttranslational modifications, interaction with co-factors and the chromatin environment. Protein acetylation is involved in the regulation of steroid hormone action in at least two levels: acetylation of the receptor and acetylation of nucleosomal histones. Homologs of nejire/dCBP participate in both processes. For example, the human p300 protein acetylates the androgen receptor [38] and the estrogen receptor α (ERα) [39] while CBP acetylates the thyroid hormone receptor α [40], the modifications resulting in enhanced ligand dependent transactivating activities and modulated DNA or ligand binding affinities of the receptors. At the chromatin level, p300 is recruited to the promoters of thyroid hormone responsive genes and required for their proper expression and thyroid induced metamorphosis in Xenopus tadpoles [41]. Human CBP is recruited to both promoter and enhancer regions of androgen receptor target genes in a ligand dependent manner where it acetylates core histones [42]. CBP is also recruited to estrogen induced gene promoters upon estrogen induction resulting in sequential acetylation of the H3K18 and H3K23 lysines, which leads to the methylation of H3R17 by the arginine methyltransferase CARM1 [22], demonstrating that specific acetyl-lysines can attract chromatin modifying factors as predicted by the histone code model. Importantly, Trim24 (Tripartite motif-containing 24), a co-activator of ERα, recognizes histone tails having concurrent non-methylated H3K4 and acetylated H3K23 residues [43]. Upon estrogen treatment of MCF7 breast cancer cells, Trim24 is recruited along with ERα to estrogen responsive genes that are also became enriched in acetyl-H3K23 [43]. In Drosophila S2 cells, dCBP associates with the Ecr-B1 receptor isoform upon 20E addition, and acetylates the H3K27 lysine on the 5′ region of the Sox14 ecdysone induced early gene, a key regulator of dendrite pruning during metamorphosis [44]. Accordingly, dCBP is required for the proper expression of Sox14 in ddaC neurons during the white pupal stage and its silencing causes pruning defects 18 hours after pupariation [44]. We have shown that dCBP acetylates the H3K23 residue in vivo, and that this chromatin mark is enriched on the promoters of the Eip74EF and Eip75B ecdysone induced genes during the late L3 stage. Although a direct causal relationship between acetylation and gene induction was not demonstrated, the role of dCBP in the regulation of the Eip74EF and Eip75B genes is supported by the finding that both genes are downregulated in nej-RXl h larvae and also by data from the modENCODE project [45] showing extensive binding of dCBP on both gene regions. Previous attempts to detect H3K23 specific acetyltransferase activity of dCBP gave contradicting results: while increased acetylation of H3K23 was not detected in S2 cells expressing transiently transfected dCBP; recombinant dCBP acetylated H3K23 in an in vitro acetylation assay [32]. Furthermore, the level of H3K23 acetylation was unchanged in ddaC neurons after RNAi silencing of dCBP [44] suggesting that a HAT other than CBP might also possess this activity.

Our findings along with those of others indicate that CBP dependent histone acetylation at the regulatory region of steroid induced genes might be a conserved chromatin modification in response to the hormone. Furthermore, CARMER, the Drosophila homologue of CARM1, associates with the Ecdysone Receptor and is required for ecdysone induced upregulation of apoptotic genes [46], suggesting that it may play a role similar to that of its human counterpart in the regulation of steroid response genes [22]. Together, these results imply the existence of an evolutionarily conserved, multistep chromatin modification mechanism in
Figure 4. The nej/dCBP protein is responsible for H3K23 specific acetylation in Drosophila. (A) The level of acetylated H3K23 is unchanged in L3 larvae homo- or hemizygous for loss of function mutant alleles of the gcn5, mol or chn histone acetyltransferase genes, and also in homozygous Ada2a or Ada2b mutants specific for the GCNS containing ATAC and SAGA complexes, respectively. (B) In nej7 mutant embryos the level of acetyl-H3K23 is significantly reduced compared to the wild-type as quantitated by immunostaining of whole mount embryos. Mean pixel intensity ± s.e.m. are shown, P<0.01. (C) ChIP analysis of chromatin samples from gcn5mof2 homozygous null mutant (gcn5) and heterozygous (cont) wandering L3 larvae using acetyl-H3K23 specific antibody detects the presence of the acetyl-H3K23 mark in gcn5 mutants. In engrailed-GAL4 UAS-GFP embryos also carrying either an UAS-dCBP (D) or an UAS-dCBP-FLAD (E) transgene, the UAS transgenes are expressed in the posterior part of every segment, as visualized by GFP fluorescence. Immunostaining with anti-acetyl-H3K23 specific antibody reveals that the level of acetyl-H3K23 is dramatically increased in embryos overexpressing UAS-dCBP (F), while it is unchanged in embryos overexpressing the UAS-dCBP-FLAD construct (G), which is mutated in the acetyltransferase domain of CBP. doi:10.1371/journal.pone.0040565.g004

the transcriptional regulation of nuclear hormone receptor activated genes.

Materials and Methods

Drosophila Stocks

Fly strains were maintained and crossed out on standard cornmeal – yeast – sucrose Drosophila medium at 25°C unless otherwise noted. The mutant strains gcn5mof2, enak7 and nej7 were obtained from the Bloomington Drosophila Stock Center (Bloomington, IN, USA). The nejire RNAi lines KK105115 and 15319R-2 were obtained from the VDRC (Vienna, Austria) or NIG-FLY (Mishima, Japan) stock centers, respectively. The UAS-dCBP and UAS-dCBP-FLAD transgenic lines were provided by Justin P. Kumar (Indiana University, Bloomington, IN, USA); The chn14 and mof mutant lines were provided by Jacques Pradel (Developmental Biology Institute of Marseille Luminy, Marseille, France) and John Lucchesi (Emory University, Atlanta, GA, USA), respectively.

Transcript Analysis

To measure the transcriptional activity of edcysone induced genes during the third larval instar RNA was prepared from synchronized larvae. w1118 eggs were collected on agar – raspberry juice plates for eight hours and then transferred to standard Drosophila medium. Synchronization was carried out three days later by collecting larvae that molted from second to third instar during a four-hour interval. Synchronized L3 larvae were transferred to a new plate from which larvae were collected every four hours until pupariation. Third independent replicates of the synchronized developmental series were collected.

To determine the expression level of the Eip74EF and Eip75B genes at reduced nejire levels, we crossed homozygous UAS-nejire RNAi KK105115 females with hs-GALA/TME Tb males at 18°C and heat-shocked the L3 larvae four times for 1 hour at 37°C. UAS-nejire RNAi KK105115/hs-GALA and UAS-nejire RNAi KK105115/TM6 Tb control larvae were collected four hours after the last heat shock based on the Tb phenotype.

Larvae were homogenized in Trizol Reagent (Invitrogen, Carlsbad, CA, USA) and RNA was prepared according to the manufacturer’s recommendation. The RNA samples were quantitated by a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), and treated with RNAase free DNAseI (Fermentas, Vilnius, Lithuania) to remove genomic DNA contamination. First strand cDNA was generated from 1 µg RNA samples using Taqman Reverse Transcription Reagent (Applied Biosystems, Foster City, CA, USA) with random hexamer primers. Transcript levels were determined by quantitative real-time PCR (Q-PCR) using gene specific primers with Power SYBR Green PCR Master Mix (Applied Biosystems) in an ABI 7500 Real-Time PCR System (Applied Biosystems). Relative cDNA quantities were calculated by setting Ct values against template calibration curves, and normalizing to the level of the housekeep-
mobilon Western Chemiluminescent HRP substrate (Millipore, Billerica, MA, USA).

Immunohistochemistry

Immunostaining of Drosophila embryos was performed as described previously [49] with modifications. In short, embryos were dechorionated in bleach, rinsed in water and fixed in 1:1 heptane : PBS-formaldehyde (4%) for 30 minutes. After devitellinization in 1:1 methanol : heptane for 20 seconds, embryos were treated with methanol and then with PBS - 0.3% Triton X-100. Embryos were incubated with blocking solution (PBS, 5% NGS, 0.3% Triton X-100) for 30 minutes at room temperature, with primary antibody overnight at 4°C, then with secondary antibody for 1 hour at room temperature. The antibodies were diluted in blocking solution. The primary and secondary antibodies used for immunohistochemistry were the following: mouse anti-β-galactosidase G4644 (Sigma-Aldrich), goat anti-mouse IgG (H+L)-FITC 115-095-166 (Jackson Immunoresearch, West Grove, PA, USA), chicken anti-GFP ab13970 (Abcam), donkey anti-chicken IgG (H+L)-FITC 703-095-155 (Jackson Immunoresearch), anti-H3K23ac ab46982 (Abcam), anti-H3K27ac ab4729 (Abcam), donkey anti-rabbit IgG (H+L)-Cy3 711-165-152 (Jackson Immunoresearch).

To determine acetyl-H3K23 levels in nej mutant and control embryos, hemizygous nej3/Y embryos were stained together with FM7c, P{ftz/lacC}YH1/Y and FM7c, P{ftz/lacC}YH1/nej3 control siblings, then separated based on anti-β-galactosidase immunostaining. The strength of anti-H3K23ac immunoreactivity was quantitated by calculating the mean pixel intensity of stained embryos using the ImageJ software [50]. To determine acetyl-H3K23 levels upon dCBP overexpression, engerailed-GAL4 UAS-EGFP/UAS-dCBP FLAD embryos were stained with anti-H3K23ac and anti-GFP antibodies. Micrographs were taken with a Leica SP5 confocal microscope.

Supporting Information

Figure S1 dCBP acetylates the H3K27 residue in vivo. In engerailed-GAL4 UAS-EGFP UAS-dCBP (A) and engerailed-GAL4 UAS-EGFP UAS-dCBP-FLAD (B) transgene carrying embryos the expression pattern of the UAS transgenes are visualized by GFP fluorescence. Immunostaining using anti-acetyl-H3K27 specific antibody shows that the level of acetyl-H3K27 is increased in embryos overexpressing UAS-dCBP (C), while it is unchanged in embryos overexpressing the UAS-dCBP-FLAD enzymatically dead construct (D).

Table S1 Oligonucleotides used as PCR primers in transcriptional analyzes and quantitation of ChIP assays. a Chromosomal location data correspond to BDGP/FlyBaseGenBank assembly Release 5.44. b The distance of the 5’ end of the oligonucleotide primer relative to the transcriptional start site of the transcript in parentheses.

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Author Contributions

Conceived and designed the experiments: LB NZ OK. Performed the experiments: LB NZ RG IK. Analyzed the data: LB NZ RG IK OK IMB. Contributed reagents/materials/analysis tools: LB OK IMB. Wrote the paper: LB. Revised the manuscript: NZ RG IK OK IMB.
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