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Acetylations of Ftz-F1 and histone H4K5 are required for the fine-tuning of ecdysone biosynthesis during Drosophila metamorphosis

Barbara N. Borsos [a], Tibor Pankotai [a,b,*], Dávid Kovács [a], Christina Popescu [a], Zoltán Páhi [a], Imre M. Boros [a,b]

[a] Department of Biochemistry and Molecular Biology, University of Szeged, Szeged, Hungary
[b] Institute of Biochemistry, Biological Research Center, Szeged, Hungary

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ABSTRACT

The molting during Drosophila development is tightly regulated by the ecdysone hormone. Several steps of the ecdysone biosynthesis have been already identified but the regulation of the entire process has not been clarified yet. We have previously reported that dATAC histone acetyltransferase complex is necessary for the steroid hormone biosynthesis process. To reveal possible mechanisms controlled by dATAC we made assumptions that either dATAC may influence directly the transcription of *Halloween* genes involved in steroid hormone biosynthesis or it may exert an indirect effect on it by acetylating the Ftz-F1 transcription factor which regulates the transcription of steroid converting genes.

Here we show that the lack of dATAC complex results in increased mRNA level and decreased protein level of Ftz-F1. In this context, decreased mRNA and increased protein levels of Ftz-F1 were detected upon treatment of Drosophila S2 cells with histone deacetylase inhibitor trichostatin A. We showed that Ftz-F1, the transcriptional activator of *Halloween* genes, is acetylated in S2 cells. In addition, we found that ecdysone biosynthetic *Halloween* genes are transcribed in S2 cells and their expression can be influenced by deacetylase inhibitors. Furthermore, we could detect H4K5 acetylation at the regulatory regions of *disembodied* and *shade Halloween* genes, while H3K9 acetylation is absent on these genes.

Based on our findings we conclude that the dATAC HAT complex might play a dual regulatory role in *Drosophila* steroid hormone biosynthesis through the acetylation of Ftz-F1 protein and the regulation of the H4K5 acetylation at the promoters of *Halloween* genes.

Introduction

In Drosophila, molting transitions are hormonally regulated by the steroid hormone ecdysone. Steroidogenesis takes place in the ring gland resulting in the conversion of cholesterol into 20-hydroxyecdysone (20E). The ecdysone synthesis steps are catalyzed by cytochrome P450 enzymes encoded by *Halloween* genes (*spook/Cyp 307A1 (spo), spookier/Cyp 307A2 (spok), phantom/Cyp 306A1 (phm), disembodied/Cyp 302 A1 (dib)* and *shadow/Cyp315A1 (sad)*) that are expressed in the prothoracic cells of the ring gland. The final step of the synthesis is catalyzed by the product of *shade/Cyp314A1 (shd)* (Gilbert, 2004; Warren et al., 2002), which is present mostly in the midgut (Huang et al., 2008).

The transcriptional regulator βFtz-F1 is required for larval molting and puparium formation during *Drosophila* metamorphosis (Broadus et al., 1999; Woodard et al., 1994). At late larval stages, the ecdysone level falls and βFtz-F1 protein level is increased inducing ecdysone production. βFtz-F1 was shown to bind to ecdysone-regulated puffs in late prepupal stage and enhance the ecdysone-induction of early ecdysone responsive genes like BR-C, E74A, E75A and E93 (Lavorgna et al., 1993; Woodard et al., 1994). Mutations of *βftz-f1* result in defects of adult head eversion, leg elongation and salivary gland cell death, which are hallmarks of the prepupal–pupal transition. In addition, βFtz-F1 enhances the ecdysone-induction of early ecdysone-response genes, but it has no direct effect on them (Woodard et al., 1994). Furthermore, Ftz-F1 interacts with nuclear receptor (NR) coactivators such as CBP/p300 (Monte et al., 1998), SRC-1 (Ito et al., 1998), RIP140 (Sugawara et al., 2001), TIF2 (Borud et al., 2002) and components of the general transcription machinery such as TFIIB

* Corresponding author at: Department of Biochemistry and Molecular Biology, University of Szeged, Szeged, Hungary.
E-mail address: pankotai@bio.u-szeged.hu (T. Pankotai).

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(Li et al., 1999). It also interacts with nuclear receptors by allowing the recruitment of histone modifying enzymes such as HDACs (Wang et al., 2008).

Reversible histone modifications by acetyl groups are carried out by specific histone acetyltranferase (HAT) and histone deacetylase (HDAC) enzymes (Cheung et al., 2000; Nagy and Tora, 2007; Narlikar et al., 2002). In *Drosophila,* one of the major HAT is the GCN5 (KAT2a) (general control none-derepressible 5) which has been identified in two related HAT complexes: dSAGA (*S*pt-*A*da-*G*cn5-*A*cetyltransferase) is the metazoan counterpart of the canonical related yeast complex, while dATAC (*A*DA-*T*wo-*A*-*C*ontaining) is a HAT complex characteristic only for metazoa. dATAC is involved in the acetylation of H4 lysine 5 (H4K5ac) and lysine 12 (H4K12ac), while dSAGA takes part in the acetylation of H3K9 and H3K14. While the lack of dSAGA has only mild effect on steroid biosynthesis (Pankotai et al., 2010), dATAC complex is essential for steroid biosynthesis (Pankotai et al., 2010). The mechanism by which dATAC affects steroid synthesis, however, has not been clarified yet. The two most probable assumptions could be that dATAC influences the transcription of genes involved in steroid hormones biosynthesis directly by histone acetylation at their regulatory regions or indirectly by acetylation of Ftz-F1. Here we report that histone acetylation has an important role in the transcription of *Halloween* genes. dATAC HAT complex is indispensable for the regulation of expression and probably for the stabilization of Ftz-F1, the main transcription regulator of *Halloween* genes.

Materials and methods

Fly strains

Fly stocks were raised and crosses were performed at 25 °C on standard medium containing propionic acid. The genotype of the Ada2a mutant fly stock is the following: P[Dtl^{+} Rpb4^{+}] Ada2a^{d189} as it was described (Pankotai et al., 2005).

Cell lines, media, culture conditions

S2 *Drosophila* embryonic cell line was maintained at 25 °C in Schneider medium (Lonza) supplemented with 10% fetal calf serum (Lonza).

Immunostaining

For immunostaining of polytene chromosome spreads obtained from salivary glands of wandering larvae were treated in phosphate-buffered saline (PBS) containing 3.7% paraformaldehyde and 45% acetic acid. Slides were blocked in PBST (PBS+0.1% Tween20) supplemented with 5% fetal calf serum for 1 h at 25 °C and incubated overnight at 4 °C in the mixture of Ftz-F1 (Lavorgna et al., 1993; Yamada et al., 2000) and RNAPII 7G5 (Pankotai et al., 2005) antibodies. 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, covered with Vecta-Shield mounting medium containing DAPI and examined with an OLYMPUS BX51 microscope. Photos were taken with an Olympus DP70 camera using identical settings for mutant and control samples.

S2 cells were fixed with 4% paraformaldehyde (Sigma) for 25 min, then permeabilized with 0.3% Triton-X-100 (Fluka) in PBS for 20 min. Nonspecific staining was blocked with 5% BSA in PBST (0.1% Tween 20 (Molar Chemicals) in PBS) for 20 min. To detect the acetylated lysine residues, anti-acetyl lysine primary antibody (Abcam) was used in 1:150 dilution. After washing steps, goat anti-rabbit Alexa-Fluor-555 (Molecular Probes) secondary antibody was used in 1:300 dilution. Both the primary and secondary antibodies were applied in 1% BSA in PBST. To detect DNA, DAPI fluorescence dye was used in 1:1000 dilution following incubation with secondary antibody. Cells were rinsed with PBS between each step. Samples were visualized with Nikon eclipse 80i fluorescence microscope. At image capturing the same exposition time was used.

RNA extraction, reverse transcription, RT-PCR and qRT-PCR

Total RNA was isolated from S2 cells using RNeasy mini kit (Qiagen) according to the intsructions of the manufacturer. 1 μg RNA from each sample was transcribed to cDNA using TaqMan Reverse Transcription Reagent (ABI) according to the instructions of the manufacturer. cDNA amounts were measured after 35 cycles PCR. PCR products were separated in 2% agarose gel. We quantified the previous data with ImageJ software ($n=14$). Statistical analysis was done using Sigma Plot 12.0. Statistical significance was determined using the Mann–Whitney *U* nonparametric test of significance with a $p<0.05$ considered statistically significant.

Real-time PCR experiments were performed in ABI 7500 real time PCR system (Applied Biosystems) using SYBR Green (Fermentas), under the following conditions: 1 cycle of 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 45 s. 18S RNA was used as internal control. C_t values of each sample were normalized to the internal control and the changes in mRNA levels were calculated by the $\Delta\Delta C_t$ method (Johnson et al., 2000). Data were obtained from three independent experiments (Table 1).

Western blot

For protein analysis on immunoblots, total protein samples from animals of the indicated genotypes and developmental stages were separated on SDS-PAGE and transferred to nitrocellulose membrane (GE Healthcare). Membranes were blocked for 1 h in 5% non-fat 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 TBST. For the detection of Ftz-F1, polyclonal antibody (a generous gift from Prof. Ueda) was used in 1: 20,000 dilution. 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.

S2 cells were harvested in sonication buffer (50 mM Tris–HCl pH 8.0, 2 mM EDTA, 0.5 mM DTT, 50 mM NaCl) (Sigma-Aldrich), incubated in ice for an hour then centrifugated (13,000 rpm, at 4 °C for 5 min). The supernatant lysates were mixed with the same amount of 2 × SDS loading buffer containing 5% β-mercaptoethanol (Sigma-Aldrich), boiled for 5 min. The lysates were separated in 8% SDS-PAGE, transferred to Amersham Hybond ECL-membrane (GE Healthcare) and incubated with the following primary and secondary antibodies: anti-Ftz-F1 (Santa-Cruz) 1:2500, anti-α-Actin 1:5000, donkey-anti-goat IgG-HRP (Santa Cruz) 1:7500, goat-anti-rabbit IgG-HRP (Dako) 1:10,000. Chemiluminescent detection was

Table 1
Primers used in RT-PCR experiments.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
dib	TGCCCTCAATCCCTATCTGGTC	ACAGGGTCTTCACACCCATCTC
phm	GGATTTCTTTCGGCGCGATGTG	TGCCTCAGTATCGAAAAGCCGT
sad	CCGCATTCAGCAGTCAGTGG	ACCTGCCGTGTACAAGGAGAG
shd	CGGGCTACTCGCTTAATGCAG	AGCAGCACCACCTCCATTTC
spok	TATCTCTTGGGCACACTCGCTG	GCCGAGCTAAATTTCTCCGCTT
ftz-f1	TTGTTCAGTTGCGTCTCGTC	CTTCGAGCTGATGTGCAAAG
rpL17a	GTGATGAACTGTGCCGACAA	CCTTCATTTCGCCCTTGTTG
18S RNA	GCCAGCTAGCAATTGGGTGTA	CCGGAGCCCAAAAAGCTT

done using Immobilon Western Chemiluminescent HRP substrate (Millipore).

TSA treatment, transfection and luciferase activity measurement

S2 cells were seeded in six-well plates at 3×10^6 cells/well in supplemented Schneider medium (Lonza) 24 h before transfection or TSA treatment.

TSA was used in 25, 50, 75 and 100 ng/ml concentration. When cells were both transfected and treated with TSA, the treatment was applied 24 h after transfection. To measure *dib* promoter activity we used pGL3-basic luciferase vector which contains 963 bp of the *dib* promoter region.

Plasmids were transfected into the cells using HilyMax (Dojindo) transfection reagent according to the manufacturer's instructions. In each experiment a total of 5 μg DNA were transfected into the cells. After transfection, cells were incubated for 24 h at 25 °C. After 24 h incubation, cells were washed twice with 1 × PBS and then harvested in 1 × lysis buffer (Promega, Cell Culture Lysis Reagent 5 ×) and incubated in ice for 1 h. The supernatants were collected by centrifugation (13,000 rpm, 4 °C, 5 min) and used to perform luciferase enzyme assay. Luciferase activities were determined using a Promega luciferase assay kit and Orion L Microplate Luminometer (Berthold Detection System, Simplicity 4.2 software). Transfections were performed in at least three repeats. Luciferase activity values were normalized to a reference sample indicated in Fig. 4. Statistical analysis was done using Sigma Plot 12.0. Statistical significance was determined using the Mann–Whitney *U* nonparametric test of significance with a $p < 0.05$ considered statistically significant.

In vivo acetylation assay

S2 cells were seeded in 10 cm plates at 1.38×10^7 cells/plate in supplemented Schneider medium (Lonza) 24 h before 75 ng/ml TSA treatment.

Cells were lysed in NP-40 buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris–HCl pH 8.0) containing 1 × PIC (Protease Inhibitor Cocktail) on ice for an hour. After lysation, cell debris was pelleted by 2000 rpm, 5 min at 4 °C and then discarded. 300 μg of the lysates were precleared for 2 h with blocked Protein A-Sepharose beads (Sigma-Aldrich). Polyclonal acetyl-lysine antibody (Abcam) was used for the immunoprecipitation of the acetylated protein pool. Acetylated protein-antibody complexes were collected with 30 μl of blocked Protein A Sepharose beads (Sigma-Aldrich). Then beads were washed four times with NP-40 lysis buffer supplemented with 1 × PIC. After the washing steps, beads were boiled in 2 × SDS loading buffer for 5 min and centrifugated at 13,000 rpm for 5 min at 4 °C. For the detection of the acetylated form of the Ftz-F1 protein, polyclonal Ftz-F1 antibody was used in 1:2000 or 1:20,000 dilution in case of the immunoprecipitated and input samples, respectively. Anti-Tubulin antibody (Sigma) was used to show the equal loading of the samples.

Chromatin immunoprecipitation

Chromatin samples were prepared from S2 cells. Chromatin samples were cross-linked with 1% formaldehyde (Sigma-Aldrich) for 10 min then incubated with 125 mM glycine (Sigma-Aldrich) for 5 min to stop the fixation. Cells were collected with centrifugation (2000 rpm, 5 min, 4 °C). Cell pellets were suspended in Cell lysis buffer [5 mM PIPES (Sigma-Aldrich) pH 8.0, 85 mM KCl (Sigma-Aldrich), 0.5% NP-40 (IGEPAL) (Sigma-Aldrich), 1 × PIC (Calbiochem)] and incubated in ice for 10 min. Cells were collected with centrifugation (2000 rpm, 5 min, 4 °C). Pellets were resuspended in Nuclear lysis buffer [50 mM Tris–HCl pH 8.0 (Sigma-Aldrich), 10 mM EDTA pH 8.0 (Sigma-Aldrich), 0.8% SDS (Sigma-Aldrich), 1 × PIC (Calbiochem)] and incubated in ice for 1 h. Chromatin samples were fragmented by sonication in a Bioruptor (Diagenode). Chromatin samples were diluted four-fold by using dilution buffer [10 mM Tris–HCl pH 8.0 (Sigma-Aldrich), 0.5 mM EGTA pH 8.0 (Sigma-Aldrich), 1% Triton-X-100 (Sigma-Aldrich), 140 mM NaCl (Sigma-Aldrich), 1 × PIC (Calbiochem)]. 25 μg Chromatin was used for immunoprecipitation after preclearing with BSA (Sigma-Aldrich) and salmon sperm DNA (Sigma-Aldrich) blocked Protein A-Sepharose beads (Sigma-Aldrich). Immunoprecipitations were incubated overnight at 4 °C with antibodies listed below, then chromatin-antibody complexes were collected with blocked Protein A-Sepharose beads (Sigma-Aldrich) at 4 °C for 3 h. The supernatant of the mock control was used as total input chromatin (TIC) control. After washing steps the samples were reverse crosslinked and the amount of extracted DNA was determined by Q-PCR using SYBR Green PCR Master Mix (Fermentas). Sequences of primers used in the Q-PCR are listed at Table 2. Samples were quantitied using a TIC standard curve, the amount of DNA specifically precipitated by the given antibody was calculated by deducting the amount of DNA in the no antibody control (NAC). The immunoprecipitates were normalized to histone H3 (Supplement 4A). The following antibodies were used in the ChIP experiments: anti-H3 ab1791 (Abcam), anti-H3K9ac ab4441 (Abcam), anti-H4K5ac ab61236 (Abcam).

Table 2
Primers used in chromatin immunoprecipitations (Papp and Muller, 2006).

Gene region	Forward primer (5′-3′)	Reverse primer (5′-3′)
Dib-1000 bp	GCCCAACGTGTAATGGAAC	GTGGTTCGGCGATAAGTTGT
Dib TSS	CCAGTGTGCGTTTAATGCC	TCCTCGTGGTCCTGGTAT
Dib 5′ gene body	GCTAAGATTGCACCAAGCCG	TATTGAGCCAGTGCCAGGTG
Dib middle gene body	GGCGATTTCTGGAGACACCT	CCTCCTCGTGCAGTTTTTGC
Dib 3′ UTR	GTCGCCAGAGCATTAAGACT	ACCCACAGCCTTTCAATCAC
Shd-1000 bp	CTGCCGTAATGTGTTGCA	GGACTGTATTGATAGCGCC
Shd TSS	TCGTGCGCTGGTGTGTGTGT	CCGCAACCGAATCCCAGATC
Shd 5′ gene body	ATTAGCCCCTCAGCTCTCCA	TCAACGAGAAACTCCACCAGT
Shd middle gene body	GAGTGAGCCGTAGAATGGGG	GAGGCGAGCACTTCCTTCTT
Shd 3′ UTR	AACAGTGCCTACCCTCAG	GCTGGCTCATCAGGGATAAA
Rpl 32 promoter	TTTCACACCACCAGCTTTTTC	CACGGACTAACGCAGTTCAA
Rpl 32 gene body	CGGTTACGGATCGAACAAGC	CTCGACAATCTCCTTGCGCT
Intergenic	CAGTTGATGGGATGAATTTGG	TGCCTGTGGTTCTATCCAAAC

Results

dATAC HAT complex has a role in the regulation of Drosophila steroid hormone biosynthesis

Failures in the synthesis of the molting hormone ecdysone result in complete abolishment of the larval–pupal transition in the Drosophila development. We have previously shown that dATAC HAT complex plays a role in the expression of the cytochrome P450 *Halloween* genes that are essential for the ecdysone hormone synthesis (Pankotai et al., 2010). In dATAC mutants *Halloween* genes expressed in the prothoracic gland (*spookier/Cyp307A2*, *phantom/Cyp306A1*, *disembodied/Cyp302A1* and *shadow/Cyp315A1)* are downregulated while the peripherial *shade/Cyp314A1* is upregulated.

Since dADA2a is a specific subunit of dATAC complex, we used *dAda2a* null alleles to further investigate the role of dATAC in the Drosophila ecdysone synthesis. First we checked whether Ftz-F1, the master transcriptional activator of steroid hormone biosynthesis has any alterations in *dAda2a* mutants. By measuring the expression of *ftz-f1* in Drosophila third instar larvae, we detected an increased

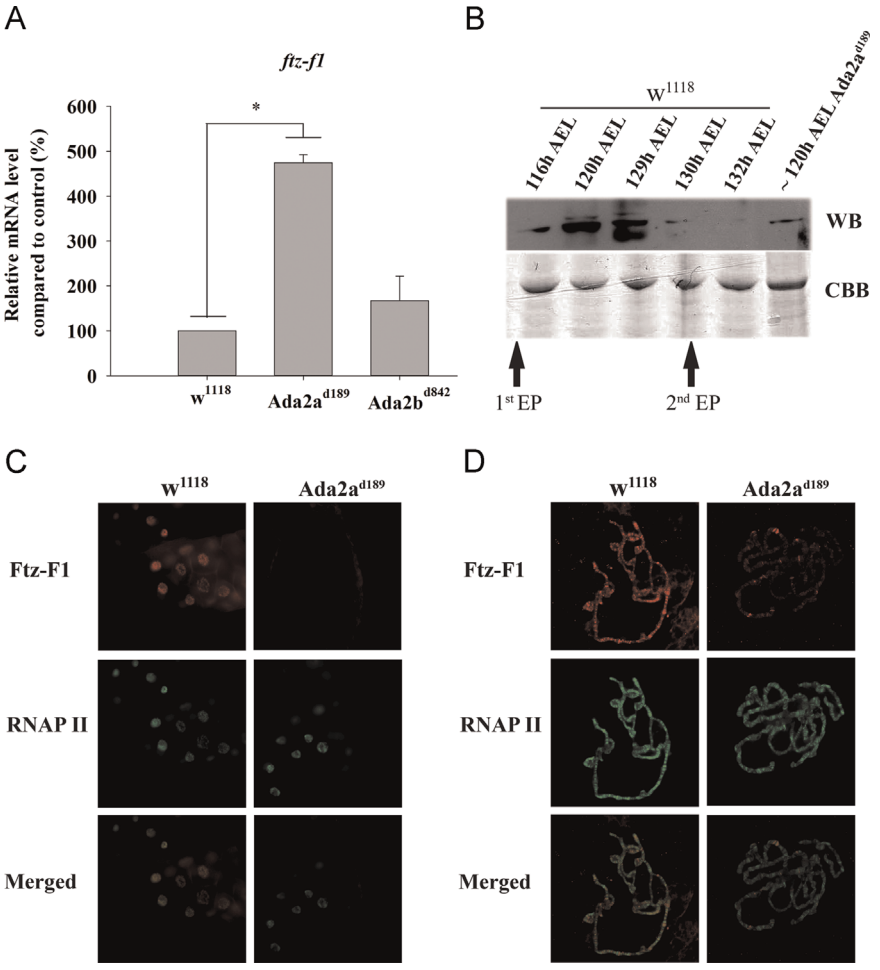

Fig. 1. *Ftz-f1* mRNA level is increased, while its protein level is decreased in *Ada2a* mutant Drosophila L3 larvae. (A) *ftz-f1* mRNA levels were compared by qRT-PCR in w^{1118}, $Ada2a^{d189}$ and $Ada2b^{d842}$ L3 larvae. Data were derived from three independent experiments and were normalized to 18S RNA (*$p < 0.05$, Mann–Whitney test). (B) Ftz-F1 protein level was detected in different stages of wild type (w^{1118}) and in $Ada2a^{d189}$ mutant Drosophila. $Ada2a^{d189}$ mutant flies were syncronized and collected at the time of spiracle eversion corresponding to the control animals. Coomassie Brilliant Blue (CBB) staining was used to show the equal loading. AEL: after egg laying. 1st and 2nd EP indicate the ecdysone peaks, respectively. (C) Ftz-F1 and RNAPII protein levels were detected using immunofluorescent staining in prothoracic cells of wild type and $Ada2a^{d189}$ mutant L3 larvae. (D) Ftz-F1 and RNAPII detection in polytene chromosomes of wild type and $Ada2a^{d189}$ mutant L3 larvae by immunostainings.

mRNA level of *ftz-f1* in *dAda2a* mutants compared to wild type (wt) control (Fig. 1A). In order to investigate whether the increased mRNA production also resulted in the accumulation of the Ftz-F1 protein, we compared the Ftz-F1 protein levels in wt and *dAda2a* mutant flies by Western blot. We found that during metamorphosis 120 h after egg laying when the ecdysone titer reached its maximum, the level of Ftz-F1 protein decreased in the control animals. Additionally, during molting when the ecdysone titer was high, we detected a lower level of Ftz-F1 protein in *dAda2a* mutant flies, while in the control animals the protein level was high (Fig. 1B). To test whether the reduction in Ftz-F1 protein level in *dAda2a* mutant animals affected the localization of Ftz-F1 onto the chromatin, we performed immunostainings on ring glands (Fig. 1C) and also on salivary gland polytene chromosomes of wt (w^{1118}) and $Ada2a^{d189}$ third instar larvae (Fig. 1D). We detected reduced Ftz-F1 level in the examined Drosophila tissues, while the RNA polymerase II levels were comparable in w^{1118} and $Ada2a^{d189}$ animals (Fig. 1C and D). Based on these observations we concluded that Ftz-F1 protein level is reduced in the absence of the dADA2a dATAC subunit that indicates additional function of dATAC in the regulation of ecdysone synthesis. Thus, the dATAC complex might have an important role in Drosophila steroid biosynthesis by regulating the Ftz-F1 protein level in the cells.

Acetylation plays a role in the expression of Drosophila cytochrome genes

Our results indicate that the dATAC HAT complex influences Drosophila steroid hormone synthesis through the Ftz-F1 protein and the lack of dATAC results in transcriptional down-regulation of the *Halloween* genes. In order to investigate whether the dATAC complex regulates Drosophila steroid hormone biosynthetic genes through histone acetylation, we determined the mRNA level of *Halloween* genes upon inhibition of histone deacetylases. Since the PT glands of L3 animals consist of only a small number of cells and therefore they are hardly accessible for DNA manipulation methods, we used insect Schneider2 (S2) cell line for further analyses to compare *Halloween* genes expression levels.

Using gene specific primers by RT-PCR (Table 1), we detected the expressions of *phantom* (*phm*), *disembodied* (*dib*), *shadow* (*sad*) and *shade* (*shd*), while *spookier* (*spok*) expression was not detectable in S2 cells (Supplement 1). For further studies we chose *disembodied* (*dib*) and *shade* (*shd*) because these showed the highest expression level. In order to determine whether histone deacetylase inhibitor trichostatin A (TSA) affected the global acetylation level we performed immunostainings with acetyl-lysine antibody, which recognizes all the acetylated lysine residues in the cell (Supplement 2). We found that treatment with 75 ng/ml TSA, significantly increased the global acetylation level in S2 cells. Then we measured

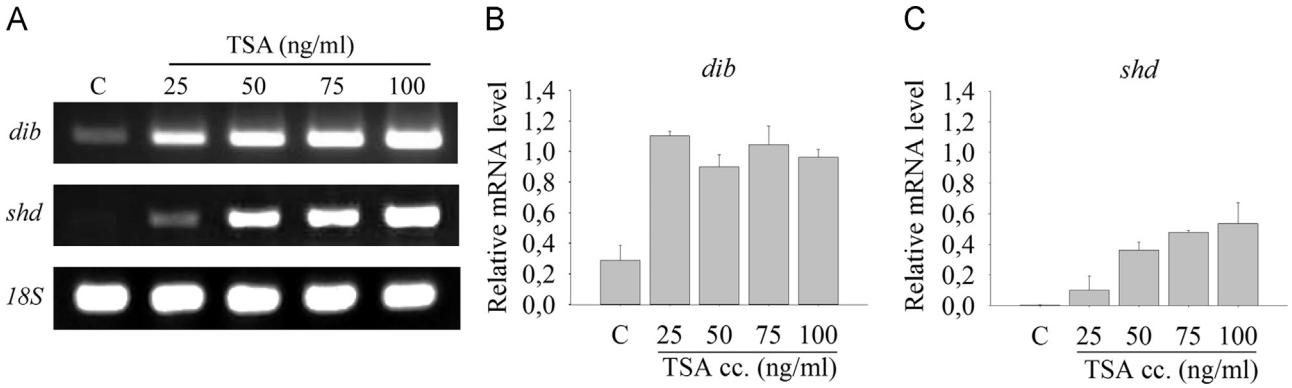

Fig. 2. Acetylation increases *disembodied (dib)* and *shade (shd)* gene expression in S2 cells. (A) *Dib* and *shd* gene expressions were analyzed by RT-PCR upon treatment with 25, 50, 75 and 100 ng/ml TSA. 18S RNA was used as internal control. (B, C) Band intensities were quantified and normalized to 18S RNA with ImageJ software.

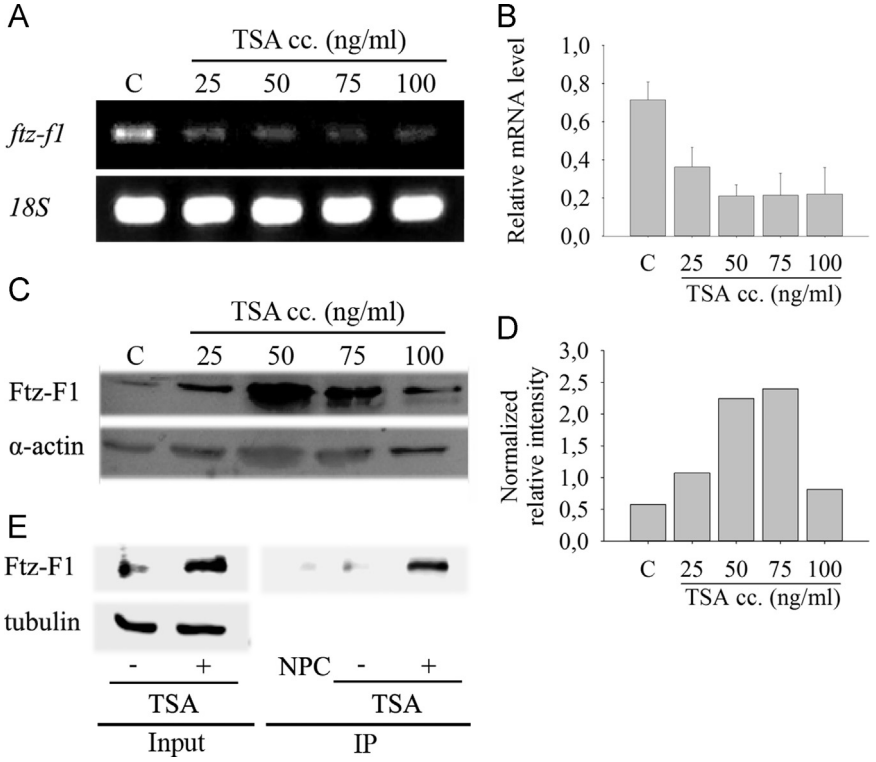

Fig. 3. Acetylation decreases Drosophila *ftz-f1* mRNA level, while it increases its protein level. Ftz-F1 is acetylated *in vivo*. S2 cells were treated with elevating levels of TSA (ng/ml). (A) *ftz-f1* mRNA levels were compared by RT-PCR upon TSA treatment. 18S RNA was used as internal control. (B) Band intensities of RT-PCR were quantified and normalized to 18S RNA with Image J software. (C) Western blot detection of Ftz-F1 protein level upon TSA treatment. α-actin was used to show the equal loading. (D) Band intensities of Western blot were quantified and normalized to actin with Image J software. (E) Western blot detection of the immunoprecipitated acetylated Ftz-F1 protein. Tubulin was used to show the equal loading. The samples with or without TSA treatment and the NPC is no protein control as the negative control of the immunoprecipitation are shown.

whether the alteration in the acetylation state affected the expression of *dib* and *shd*. We found that the mRNA levels corresponding to *dib* and *shd* were significantly increased upon TSA treatment (Fig. 2A–C, Supplement 3).

In order to determine whether alterations in the global acetylation level influenced *ftz-f1* expression as well, we measured *ftz-f1* mRNA level upon TSA treatment (Fig. 3A and B). We found that the mRNA level of *ftz-f1* decreased in TSA treated cells. This observation is surprising since we detected elevated level of Ftz-F1 protein under the same conditions (Fig. 3C and D). These results suggest that dATAC complex may regulate the Ftz-F1 protein level by a stabilizing acetylation step.

Thus, our results demonstrate that acetylation could regulate the *ftz-f1* expression as well as stabilize the Ftz-F1 protein. Furthermore, these data also suggest that dATAC histone acetyltransferase complex could control the activation of the *Halloween* genes probably directly by acetylation.

Ftz-F1 protein is acetylated in vivo

To further investigate whether the dATAC complex has a direct role in Drosophila steroidogenesis through the acetylation of Ftz-F1, we performed immunoprecipitation experiments to determine the acetylation state of Ftz-F1. We compared Ftz-F1 protein acetylation state in control and TSA treated S2 cells using acetyl-lysine antibody. We were able to detect the acetylated form of Ftz-F1 protein both in untreated and TSA treated S2 cells. As we expected the inhibition of deacetylation markedly increased the acetylation

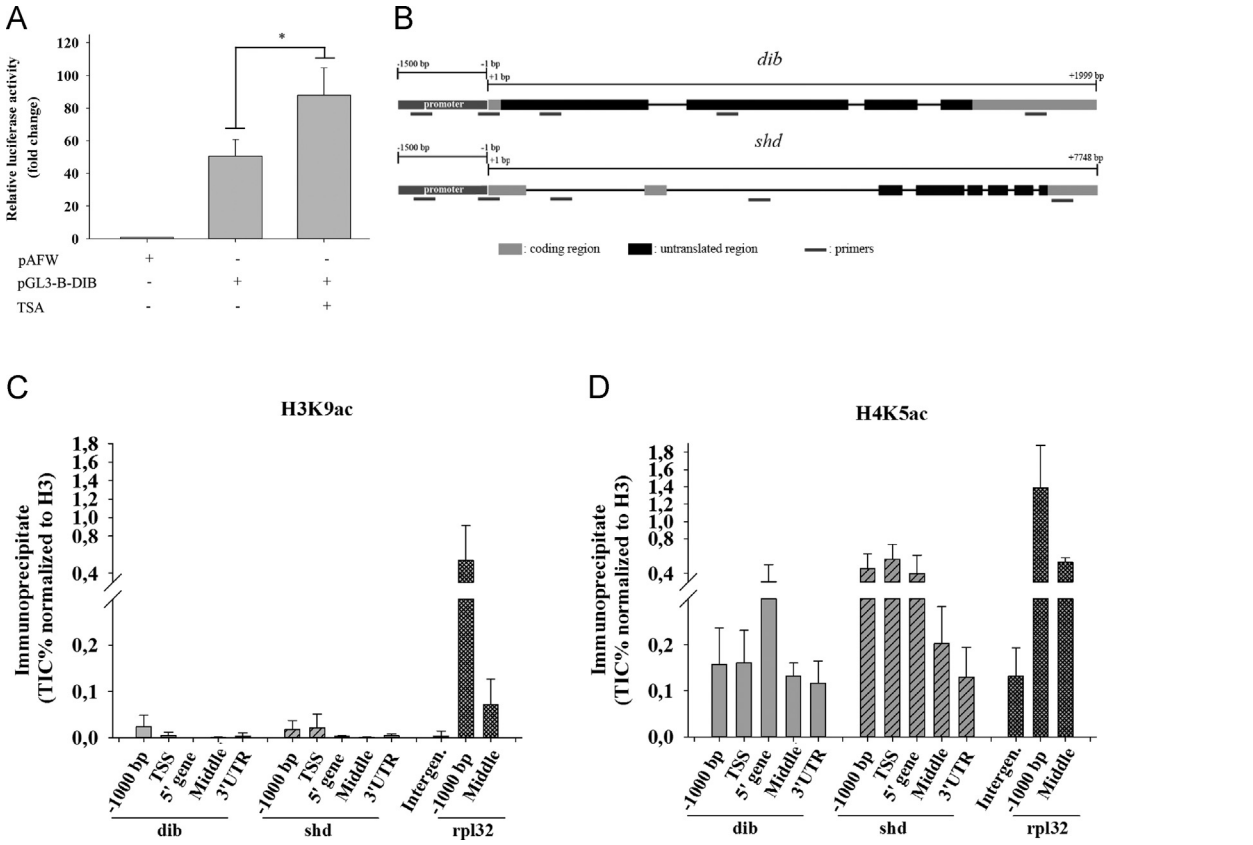

Fig. 4. dATAC HAT complex plays a role in the regulation of ecdysone synthesis by specific histone modification. (A) The promoter activity of *disembodied* (*dib*) gene is significantly increased upon trichostatin A (TSA) treatment. S2 cells were treated with 75 ng/ml TSA. Luciferase activities were measured 48 h after transfection. pAFW empty vector was used as mock control and data were normalized to its activity. Data are derived from three independent experiments ($^*p < 0.05$, Mann–Whitney test). (B) The schematic structures of *dib* and *shd* genes. Gray rectangles indicate the location of the products amplified with qRT-PCR in ChIP experiments at the different gene regions (-1000 bp, TSS regions, 5′ and middle parts of the gene body and 3′ UTR). (C) Levels of histone H3 lysine 9 acetylation (H3K9ac) of Drosophila *dib* and *shd* gene regions. Data were normalized to H3 and represented in TIC%. (D) Levels of histone H4 lysine 5 acetylation (H4K5ac) at Drosophila *dib* and *shd* gene regions. Data were normalized to H3 and represented in TIC%.

of Ftz-F1 protein (Fig. 3E), suggesting that the Ftz-F1 can be acetylated *in vivo* and the acetylation is necessary for the Ftz-F1 stabilization.

ATAC influences the steroid biosynthesis through histone acetylation

To further investigate the dATAC role in the transcription of *Halloween* genes, we measured whether acetylation takes part in the activation of *dib* promoter. To carry out this, we generated a *dib* promoter containing luciferase reporter gene construct. To analyze the effect of acetylation on that promoter we transfected S2 cells with the reporter construct, applied TSA treatment and measured the activity of the luciferase reporter gene (Fig. 4A). We detected approximately two-fold increase in the promoter activity compared to the untreated control which confirmed the positive effect of acetylation on the expression of the *dib* gene.

Our next aim was to reveal whether dATAC is the histone acetyltransferase complex which influences the steroid biosynthesis through histone acetylation. In order to obtain information on this, we analyzed the levels of dATAC-specific histone H4K5ac and dSAGA specific histone H3K9 acetylation by chromatin immunoprecipitation at the -1000 bp region, transcriptional start site (TSS) (approximately -100 to $+100$ bp region), throughout the gene body and at the 3′ untranslated regions (3′ UTR) of *dib* and *shd* genes (Fig. 4B). We used specific primers for intergenic region and for the ribosomal *rpl32* promoter and gene body as a negative and as a positive control, respectively. Although we found that dATAC specific H4K5 acetylation can be observed at the -1000 bp region, at the transcription start site (TSS) and also at the gene body of *dib* and *shd* (Fig. 4D), considerable levels of acetylated H3K9 were not detected (Fig. 4C). Previously we showed that the transcriptional level of *ftz-f1* was down-regulated after TSA treatment. To investigate whether TSA has any effects on H4K5ac levels at the regulatory regions of *Halloween* genes, we also performed ChIP experiments on TSA treated cells. We found that treatment with TSA influenced neither the H3K9ac nor the H4K5ac levels at *dib* or *shd* gene (Supplement 4B and C). These findings indicate that the H4K5 acetylation could play a role in the regulation of the transcriptional rate of *dib* and *shd* genes which is in accord with that dATAC complex has a role in the transcriptional activation steps of steroidogenesis.

Discussion

Our previous data highlighted the fact that dATAC HAT complex is involved in the ecdysone biosynthesis. The *Halloween* genes (*spookier, phantom, disembodied, shadow*) which are expressed in the ring gland are downregulated while *shade*, which is mainly expressed in the larval gut and fat body, is overrepresented in dATAC mutants. dATAC mutants show failures in the larval–pupal transition due to the lack of ecdysone (Pankotai et al., 2010). It was previously shown that *Halloween* genes were regulated by Ftz-F1 transcription factor (Warren et al., 2002). These results suggest that Ftz-F1 protein level might be affected in dATAC mutant Drosophila larvae that can reveal another function of dATAC complex in the regulation of Drosophila steroid hormone synthesis. Thus,

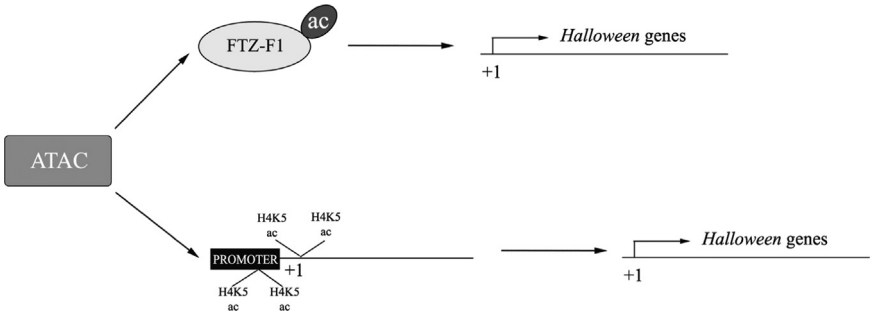

Fig. 5. dATAC HAT complex is able to regulate transcription of *Halloween* genes by different mechanisms. We hypothetized that dATAC affects the regulation of cytochrome P450 genes through two ways: it may acetylate the main regulator of steroidogenesis, Ftz-F1 and it controls the transcription of CYP genes through histone acetylation.

our hypothesis is that dATAC complex has a regulatory role in Drosophila steroidogenesis through the tight regulation of Ftz-F1.

In previous studies the regulation of chromatin structure and transcription activation by HAT complexes, such as ATAC and SAGA, have been extensively investigated (Lee and Workman, 2007; Nagy and Tora, 2007). It has been shown that GCN5 HAT, the common catalytic subunit of the dATAC and dSAGA multisubunit HAT complexes could acetylate both histone and non-histone proteins (Carre et al., 2005). The latter function of ATAC has been less known, although recent studies have shown that GCN5-containing ATAC complex localizes to the mitotic spindles and mediate acetylation of Cyclin A at specific lysine residues which triggers its degradation (Mateo et al., 2009; Orpinell et al., 2010). This indicates that ATAC can acetylate non-histone proteins which reveals another, still less known function of ATAC. In the light of these data, we hypothetized two possible scenarios by which dATAC HAT complex can regulate ecdysone biosynthesis: it might play a role in steroidogenesis through histone acetylation and it could acetylate the master regulator of the *Halloween* gene transcription, Ftz-F1 that is able to promote the molting hormone synthesis (Fig. 5). In order to validate the existence of these roles of ATAC, first we investigated the role of acetylation on *Halloween* genes and *ftz-f1* gene expression with histone deacetylase inhibitor (TSA) treatment and we measured the acetylation state of *dib* promoter region. In agreement with our expectations, acetylation has a role in the regulation of *dib* and *shd* *Halloween* genes. In contrary, *ftz-f1* mRNA level was decreased while its protein level was increased upon TSA treatment. One possible explanation of the detected increased mRNA level and decreased protein level of Ftz-F1 could be that the lack of the protein provokes an autocompensatory effect through its transcriptional upregulation (Broadus et al., 1999; Yamada et al., 2000). This would imply that the dATAC complex regulates the Ftz-F1 protein level by a stabilizing acetylation step. ATAC might also have a role in the Ftz-F1 regulated transcriptional activation step by regulating the histone acetylation state of specific genes controlled by Ftz-F1 transcription factor. Accordingly, we hypothesize that lysine acetylation has a dual role in *Halloween* gene expression: in the Ftz-F1 protein stabilization and in histone modification.

In order to further study the effect of histone acetylation on *Halloween* genes we performed experiments to reveal whether dATAC regulates Drosophila steroidogenesis through histone acetylation. We have already shown that H4K5 and K12 acetylation are mostly depended on dATAC complex function (Ciurciu et al., 2006; Pankotai et al., 2005). These acetylations are mainly present at the promoter and coding region of genes indicating that H4 acetylations are markers of actively transcribed genes (Park et al., 2013). In addition, H4K5 acetylation can be also observed at intergenic regions where non-coding RNAs and microRNAs are transcribed (Park et al., 2013). In contrast, H3K9 acetylation – a SAGA-specific mark – shows enrichment mainly at the 5′ end regions of genes where it is necessary for the recruitment of TFIID to the promoters (Agalioti et al., 2002; Guenther et al., 2007). Our results demonstrate that H4K5ac is present in an increased level at the regulatory regions of *dib* and *shd* genes. However, we cannot exclude the possibility that an increased level of K12 acetylated H4, which is also depends on dATAC function is also required for *Halloween* gene transcription. On the contrary, we could not detect significant change in H3K9 acetylation, suggesting that the dSAGA mediated H3 acetylation is not critical for the transcriptional regulation of *dib* and *shd*. These data suggest that dATAC-dependent H4 acetylation is required for the transcriptional regulation of *dib* and *shd*.

In summary, our results suggest that the dATAC HAT complex regulates Drosophila steroid hormone biosynthesis by acetylation of both specific histone residues and non histone regulatory protein as well. The dual role that ATAC seems to exert on steroid conversion through mediating histone H4 acetylation level and Ftz-F1 mRNA and protein levels might be important for the tight regulation of ecdysone hormone synthesis at critical developmental stages. Based on our results, it seems that during ecdysone biosynthesis ATAC might act at some steps directly, while contributes to others indirectly. Further studies are required to elucidate the fine details of its dual role.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2015.04.020.

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