Histone deacetylase 3 is required for development and metamorphosis in the red flour beetle, *Tribolium castaneum*

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**Abstract**

**Background:** Hormones are chemical communication signaling molecules released into the body fluids to stimulate target cells of multicellular organisms. We recently showed that histone deacetylase 1 (HDAC1) plays an important role in juvenile hormone (JH) suppression of metamorphosis in the red flour beetle, *Tribolium castaneum*. Here, we investigated the function of another class I HDAC member, HDAC3, and show that it is required for the normal development of *T. castaneum*.

**Results:** RNA interference-mediated knockdown of the HDAC3 gene affected development resulting in abnormally folded wings in pupae and adults. JH analog, hydroprene, suppressed the expression of HDAC3 in *T. castaneum* larvae. The knockdown of HDAC3 during the final instar larval stage resulted in an increase in the expression of genes coding for proteins involved in JH action. Sequencing of RNA isolated from larvae injected with dsRNA targeting *malE* (*E. coli* gene, control) or HDAC3 followed by differential gene expression analysis identified 148 and 741 differentially expressed genes based on the *P*-value < 0.01 and four-fold difference, and the *P*-value < 0.05 and two-fold difference, respectively. Several genes, including those coding for myosin-I heavy chain (Myosin 22), Shaven, and nuclear receptor corepressor 1 were identified as differentially expressed genes in HDAC3 knockdown larvae. An increase in histone H3 acetylation, specifically H3K9, H3K18, and H3K27, was detected in HDAC3 knockdown insects.

**Conclusion:** Overall, these data suggest that HDAC3 affects the acetylation levels of histones and influences the expression of genes coding for proteins involved in the regulation of growth, development, and metamorphosis.

**Keywords:** HDAC3, Juvenile hormone, *Tribolium castaneum*, Acetylation, Histone H3

**Background**

Lysine acetylation is one of the major epigenetic modifications of proteins, which contributes to chromatin remodeling and expression of genes that regulate important biological processes [1]. In eukaryotes, the levels of acetylation of histones and other proteins are regulated by lysine acetyltransferases (KATs or Histone acetyltransferases HATs) and lysine deacetylases (KDACs or histone deacetylases HDACs), which catalyze the addition and removal of acetyl groups, respectively [2, 3]. Lysine acetylation targets large macromolecular complexes responsible for various nuclear and cytoplasmic cellular processes: such as splicing, cell cycle, chromatin remodeling, DNA replication, etc. [4]. HDAC enzymes depend on zinc ions for their catalytic activity, and human HDACs were grouped into four classes [5, 6]. Class I HDACs are localized in the nucleus, expressed universally, and play essential roles in cell proliferation, whereas class II and IV HDACs have a tissue-specific role [7, 8].
Recent studies using HDAC inhibitors have suggested multiple roles for HDACs in cell proliferation, cell cycle arrest, and apoptosis [9]. The knockdown of HDAC3 induced changes in gene expression, DNA damage, and caused cell cycle delay in mouse embryonic fibroblasts [10]. In Drosophila melanogaster, six HDACs (Rpd3, HDAC3, HDAC4, HDAC6-S, HDAC6-L, and Sir2) were characterized by studying temporal expression patterns and transcriptional profiling and the effect of HDAC inhibitors [11]. The D. melanogaster HDAC3 was cloned in 1998 and described as a metal-substituted enzyme [12]. RNA interference (RNAi)-mediated silencing of HDAC1 or HDAC3 in Drosophila S2 cells resulted in cell growth inhibition and deregulation of genes such as sox14, ecdysone-induced eip74ef, and nvy [13]. Chemical genomics studies revealed that HDAC1, 2 and 3 are essential for core regulatory transcription and cell proliferation in cancer models [14]. Deacetylation by HDAC3 plays a vital role in the suppression of apoptosis in D. melanogaster imaginal tissue [15]. Acetylation of specific lysine residues of histones contributes to the dynamic regulation of ecdysone induced genes in D. melanogaster [16]. However, the role of acetylation in the regulation of juvenile hormone (JH) action in insects is not well studied.

Juvenile hormones secreted by the corpora allata have multiple functions in an insect’s life cycle and regulate diverse biological processes, including larval development, molting, metabolism, polyphenism, diapause, reproduction, and metamorphosis [17–21]. The JH signals are transduced through JH receptor, Methoprene-tolerant (Met) [22, 23], Steroid receptor co-activator (SRC) [24], and CREB-binding protein (CBP) [25–27] (binding partners). JH represses the expression of genes involved in metamorphosis. Kr-h1 is an early JH response gene downstream of Met, and RNAi mediated knockdown of Met or Kr-h1 induces a precocious larval-pupal transition in the red flour beetle [28]. JH/Met-dependent Kr-h1 activity mediates the larval development. Lower JH titers result in lower levels of Kr-h1 expression in the last instar larvae allowing expression of pupal specifier, Broad complex and adult specifier, E93 and metamorphosis [29].

Recent research from our lab showed that the class I and II HDAC inhibitor Trichostatin A (TSA) mimics JH in the induction of JH response genes [27], suggesting a role for HDACs in JH action. We also demonstrated that HDAC1 influences JH action by regulating acetylation levels of histones, which promotes the expression of JH response genes [30]. In the present study, we focused on another member of the class I HDAC family, HDAC3 (TC006104). Knockdown of the HDAC3 gene during the final instar larval stage of the red flour beetle, Tribolium castaneum resulted in a pupa that showed abnormally folded wings and eventually died. RNA-seq analysis identified several genes including, Myo22, paired box protein Pax-5 (Shaven), and PDGF- and VEGF-related factor 3 (Pv3), whose expression is influenced by HDAC3.

Results

HDAC3 plays a key role in development and metamorphosis

HDAC3 is a member of the Arginase/deacetylase superfamily that belongs to class I and is structurally and functionally related to HDAC1 and HDAC8 (Additional file 1, Fig. S1. A). Orthologues of HDAC3 are present in insects, other arthropods, and vertebrates (Additional file 1, Fig. S1. B, Gregoretti, Lee, and Goodson 2004). Injection of one microgram of dsRNA into newly molted last instar larvae induced 30% larval mortality by eight days after dsRNA injection. The remaining larvae pupated but showed wing abnormalities, especially with wing folding, and could not complete development to the adult stage (Fig. 1Aa). Control larvae injected with dsmaE (dsRNA targeting maE gene from Escherichia coli) developed into normal pupae (Fig. 1Ab). Similarly, pupae with wing defects were observed when dsHDAC3 was injected into 72 h-old (day 3) last instar larvae (Fig. 1Ac). Also, adults developed from pupae injected with dsHDAC3 showed wing defects (Fig. 1Ad). The pupae that developed from dsHDAC3 treated larvae are smaller in size than the control larvae treated with dsmaE (Additional file 1, Fig. S2). Conversely, dsmaE injected pupae developed into normal adults (Fig. 1B). Injection of dsHDAC3 into larvae, pupae and adults induced 78, 61 and 89% of knockdown of target gene respectively in larva, pupae and adults (Fig. 1B) and resulted in 30, 41 and 54% mortality, respectively in larvae, pupae and adults (Fig. 1C).

Expression of HDAC3 in larval and pupal stages

Developmental expression of HDAC3 during the penultimate and last instar larval and pupal stages was determined using reverse transcription-quantitative PCR (RT-qPCR) and HDAC3-specific primers (Additional file 1, Table S1). The HDAC3 mRNA levels were low during the penultimate and last instar larval and early pupal stages but increased at 24 h after pupal ecdysis. (Fig. 2A). The HDAC3 mRNA levels then decreased again, and lower levels were maintained throughout the pupal stage. In general, the HDAC3 mRNA levels were higher during the pupal stage when compared to those during the penultimate and last instar larval stages.

JH analog hydronprene suppresses the expression of HDAC3 in T. castaneum larvae

The HDAC3 mRNA levels were significantly lower in hydronprene treated larvae when compared to those in
Fig. 1 (See legend on next page.)

A

Injected at last larval stage (0 h)

Control pupae (malE)

Injected after 3 days of last larval stage (72 h)

Injected at pupal stage (0 h)

Control adult (malE)

B

Relative mRNA levels

|          | TcHDAC3 |
|----------|---------|
| Larvae   | 78      |
| Pupae    | 61      |
| Adults   | 89      |

C

% Mortality

|          | Larvae | Pupae | Adults |
|----------|--------|-------|--------|
| 30       | a      | b     | b      |
| 41       |         |       | a      |
| 54       |         |       | a      |

Fig. 1 (See legend on next page.)
control larvae treated with solvent (Fig. 2B). As expected, the mRNA levels of JH response gene Kr-h1 increased in hydroprene treated larvae when compared to those in control larvae treated with cyclohexane (Fig. 2B). Also, the difference in expression levels of HDAC3 in larvae, pupae, and adults was detected (Additional file 1, Fig. S3). Higher HDAC3 mRNA levels were detected in wing discs when compared to the other tissues isolated from 72-h-old pupae developed from dsHDAC3 injected larvae. The CBP mRNA levels did not increase in pupae developed from dsHDAC3 injected larvae (Fig. 3B). The CBP mRNA levels did not increase in pupae developed from dsHDAC3 injected larvae. Also, the mRNA levels of the JH-response gene, G13402 did not increase in dsHDAC3 injected larvae (Fig. 3A) but increased in pupae developed from dsHDAC3 injected larvae (Fig. 3B).

To identify other target genes whose expression is affected by HDAC3 knockdown, we sequenced the RNA isolated from dsHDAC3 and dsmalE injected larvae. Run summary and read count statistics of sequencing output are shown in Additional file 1, Table S2. The overall pattern of normalized mean expression values of differentially expressed genes (DEGs) is represented as a heatmap (Fig. 4A). The DEGs are shown as a volcano plot with red dots indicating statistically significant genes after the EDGE test between treatment and control (Fig. 4B). After statistical analysis using Baggerley’s test to compare gene expression between dsHDAC3 and dsmalE treated insects, we identified 148 and 741 DGEs test to compare gene expression between dsHDAC3 and dsmalE regulated in both HDAC3 knockdown larvae (Additional file 1, Table S3). Web-based GO analysis of differently expressed genes showed enrichment of GO terms for binding, especially nucleic acid and ion binding, regulation of the cellular process, biological regulation, and transport (Additional file 1, Fig. S4).

Twenty genes (Additional file 1, Table S4) that are up-regulated in both HDAC3 and HDAC1 knockdown larvae [30] were selected for verification of RNA-seq data DEG predictions by RT-qPCR. The genes were selected based on the presence of a DNA-binding domain with possible functions as transcription factors, and RT-qPCR was used to determine their mRNA levels. Sixteen out of 20 genes tested showed an increase in their mRNA levels in HDAC3 knockdown larvae when compared to those in control dsmalE treated larvae (Fig. 4C). Comparison of up-regulated genes between JH III [31] and dsHDAC3

Knockdown of HDAC3 induces expression of genes involved in JH action and response in T. castaneum larvae and pupae

HDAC3 knockdown efficiency and its effect on the expression of JH response genes were tested using RT-qPCR. A significant knockdown of HDAC3 was detected in larvae collected at 12 h after dsHDAC3 injection (Fig. 3A). The Kr-h1, 4EFP, SRC, and CBP mRNA levels increased significantly in dsHDAC3 injected larvae when compared to those in dsmalE injected larvae. The expression of Met was not affected by HDAC3 knockdown. We also tested the housekeeping genes actin and heat shock protein (HSP90) to determine whether this effect is universal. Actin and HSP90 mRNA levels were not affected by HDAC3 knockdown (Fig. 3A). A similar pattern of HDAC3 knockdown and an increase in the expression of Kr-h1, 4EFP, and SRC were detected in 24 h-old pupae developed from dsHDAC3 injected larva (Fig. 3B). The CBP mRNA levels did not increase in pupae developed from dsHDAC3 injected larvae. Also, the mRNA levels of the JH-response gene, G13402 did not increase in dsHDAC3 injected larvae (Fig. 3A) but increased in pupae developed from dsHDAC3 injected larvae (Fig. 3B).
Fig. 2 (See legend on next page.)
treated larvae identified six common genes, including *Kr-h1* (Additional file 1, Table S5). Six genes that code for proteins containing zinc finger COG5048 domains found in *Kr-h1* were also up-regulated in HDAC3 knockdown larvae (Additional file 1, Table S6).

Identification of genes affected by both HDAC3 knockdown and TSA treatment

TSA selectively inhibits class I and II HDACs and was shown to alter gene expression by preventing the removal of acetyl groups from histones [32]. Previous studies from our lab identified TSA induced genes in *T. castaneum* TcA cells [31]. Comparison of TSA induced genes with up-regulated genes in HDAC3 knockdown insects identified multiple genes (5.3% of DGEs) that are common in both the treatments (Additional file 1, Fig. S5). The common genes identified from this analysis are listed in Additional file 4. To verify the results, we selected nine genes from this list (Additional file 1, Table S7) and determined their mRNA levels in dsHDAC3 treated *T. castaneum* pupae (Fig. 5A) and TcA cells (Fig. 5B).

**HDAC3 regulates acetylation levels of histone H3**

Total proteins were isolated from the dsHDAC3-treated last instar larval tissues and subjected to the western blot assay using acetyl-histone H3 antibody sampler kit #9927 (Cell Signaling, MA) to determine the targets of HDAC3 deacetylation. We evaluated the various lysine acetylation sites of histone H3 using Lys9, Lys14, Lys18, Lys27, and Lys56 specific antibodies. Increased acetylation of H3K9 and H3K27 was detected in dsHDAC1, and dsHDAC3 treated larvae compared to their levels in dsmalE treated larvae (Fig. 6A, B). These data suggest that H3 is one of the targets for HDAC1 and HDAC3.

**Discussion**

Recent research in our laboratory demonstrated that HDAC1 suppresses *Kr-h1* gene expression and regulate JH suppression of metamorphosis in *T. castaneum* [30]. In the current studies, we investigated the role of the other member of the HDAC class I, the HDAC3. Unlike HDAC1 knockdown, which causes complete lethality during the larval stage, some of the HDAC3 knockdown larvae undergo pupation, but the pupae exhibited defects, especially wing folding and the pupae that developed from dsHDAC3 treated larvae are smaller in size compared to the control larvae treated with dsmalE (Additional file 1, Fig. S2). Injection of dsHDAC1 into *T. castaneum* induced a block in growth and development and 100% mortality of larvae before pupation [30].

In contrast, HDAC3 knockdown is less severe, and some of the treated larvae completed larval development and died during the pupal stage. Some of the differences may be due to differences in the expression pattern of these two HDACs during the last larval stage. Further research is needed to uncover differences in the function of these two Class 1 HDACs. In *D. melanogaster*, mutations in *HDAC3* caused death during the late third instar larval and early pupal stages. Also, the imaginal discs are significantly reduced, and the pouch region of the wing disc was smaller in size compared to the wild-type [15]. RNAi-mediated HDAC3 knockdown in the beetle, *Gnatocerus cornutus*, caused a reduction in hind wing size [35].
One of the primary outcomes of this research is the discovery that HDAC3 is required for normal larval, pupal and adult development in *T. castaneum*. The knockdown of HDAC3 in newly molted last instar larvae caused an upregulation of genes involved in JH action (SRC, CBP) and JH response (*Kr-h1, 4EBP, G13402*). Newly molted last instar larvae were injected with dsHDAC3 or dsmalE. Total RNA was extracted at 12 h after treatment, and the mRNA levels of JH-response genes (*Kr-h1, 4EBP*) genes involved in JH action (Met, SRC, CBP), HSP90 and Actin were quantified. The mean ± SE (n = 4) are shown. The data were analyzed using analysis of variance, each pair student’s t-test. Mean values with the same letter are not significantly different from each other. B The knockdown of HDAC3 in pupae caused an upregulation of JH response genes (*Kr-h1, 4EBP, G13402*). 72 h-old last instar larvae were injected with dsHDAC3 or dsmalE. Total RNA was extracted on the fifth day after injection was used to determine relative mRNA levels of SRC, CBP, *Kr-h1, 4EBP, G13402, HSP90*, and Actin.

One of the primary outcomes of this research is the discovery that HDAC3 is required for normal larval, pupal and adult development in *T. castaneum*. The knockdown of HDAC3 in newly molted last instar larvae caused an upregulation of genes involved in JH action (SRC, CBP) and JH response (*Kr-h1 and 4EBP*). In *D. melanogaster*, HDAC3 plays a crucial role in development, consistent with their relatively high expression during the embryonic and adult stages [11]. Our developmental expression studies showed a significant upregulation of the HDAC3 gene expression in 24 h-old pupae (Fig. 2A). Previous studies reported that *T. castaneum*
Fig. 4 (See legend on next page.)
Fig. 4 HDAC3 knockdown in the last instar larvae of T. castaneum affects the transcription of genes involved in multiple pathways. A Heatmap of RNA-seq data. The heatmap of normalized mean expression values of 741 differentially expressed genes (≥ 2-fold and a $P < 0.05$) between dsma1E and dsHDAC3 treated insects. B Differentially expressed genes identified after HDAC3 knockdown represented as the volcano plot. The X and Y-axis represent the $-\log_{10} P$-values and $\log_{2}$ fold change of mean normalized values, respectively. The red dots indicate the genes that showed ≥2-fold difference in expression with a $P < 0.05$. C RT-qPCR verified the expression of 20 selected genes from the up-regulated group (RNA-seq data). Descriptions of genes are listed in (Additional file 1, Table S4).

Fig. 5 Confirmation of HDAC3 targets in T. castaneum pupae and TcA cells. A 72 h-old last instar larvae were injected with dsHDAC3 or dsma1E. Total RNA was extracted at five days after injection, and mRNA levels were quantified. The mean ± SE of four replications is shown. The data were analyzed using analysis of variance, each pair student’s t-test. Mean values with the same letter are not significantly different from each other. TcA cells were treated with dsHDAC3 or dsma1E. Total RNA was extracted 72 h after dsRNA treatment, and mRNA levels were quantified. The mean ± SE of four replications is shown. The data were analyzed using analysis of variance, each pair student’s t-test. Mean values with the same letter are not significantly different from each other.
HDAC3 is expressed during all developmental stages, and the highest mRNA levels were detected in one-day-old pupae [36]. We also demonstrated that the depletion of HDAC3 during the pre-pupal stage (72-h old) T. castaneum results in abnormal pupal development. Interestingly, transcription factor E93 (adult specifier) was down-regulated in dsHDAC3 knockdown pupal and cell samples (Fig. 5A, B). In T. castaneum and D. melanogaster, E93 promotes adult metamorphosis and represses the expression of antimetamorphic genes Kr-h1 and pupal specifier, Broad-complex [37]. In HDAC3 knockdown insects, we found up-regulation of Kr-h1, Br-C,
and downregulation of E93. The timing and expression levels of Kr-h1, Br-C, and E93 (metamorphic genetic network) facilitate the proper larval-pupal-adult transition in holometabolous insects [38]. Based on these data, we propose that the misregulation of critical hormone-related genes caused the abnormal pupal development in HDAC3 knockdown insects. In general, histone acetylation and deacetylation at the promoter region are associated with transcription activation and repression, respectively [39]. Interestingly, differential gene expression analysis of sequences of RNA isolated from dsHDAC3 and dsmaE treated larvae identified 563 (76%) up-regulated, and 178 (24%) down-regulated genes. The data suggest that the HDAC3 is involved in suppression of gene expression in T. castaneum larvae. The maintenance of equilibrium between acetylation and deacetylation of histones and non-histone proteins is essential for healthy cell growth. Loss of HDAC1 or HDAC3 leads to cell growth inhibition and overexpression of genes involved in lipid metabolism, DNA replication, cell cycle regulation, and signal transduction [13]. Histone acetyltransferases and deacetylases control cell proliferation and differentiation [40].

Myo22, a myosin heavy chain gene essential for muscle development, is a common gene up-regulated by HDAC3 knockdown larvae, and TSA treated cells. (Additional file 4, Fig. 5A, B). Functions of myosin heavy chain (MHC) family genes in T. castaneum were reported recently [41]. The TcMyo20 is essential for wing and leg morphogenesis in T. castaneum. MHC isoforms regulate muscle function and are critical for specialized functions such as flying and jumping in D. melanogaster [42]. The activity of HDACs (class I, II) promote swimming performance, but reduced slow and fast MHC content in cardiac and skeletal muscles in zebrafish [43].

Trichostatin A (TSA, Class I, II HDAC inhibitor) induces JH response genes in a dose-dependent manner in T. castaneum [27]. TSA works as an epigenetic modulator, and deacetylation by TSA regulates the expression of key players involved in JH and 20E action in Tca cells [31]. HDAC inhibition by TSA led to an increase in the concentration of MHC in both skeletal and cardiac muscle in zebrafish (Danio rerio) [43]. Histone acetyltransferases CBP/p300 binds to the muscle-specific promoter, and this leads to enhanced transcription of muscle-specific genes [40]. Our data suggest that HDAC3 could play an important role in muscle development and function. However, further studies are needed to identify the exact mechanism of HDAC3 suppression of Myo22 in T. castaneum.

Our results also demonstrated the role of HDAC3 in the regulation of ‘shaven,’ a transcription factor that is involved in the development of a variety of sensory organs [44]. Additionally, we confirmed the upregulation of Pvf3 in HDAC3 knockdown larvae. Pvf3 functions in embryonic plasmatocyte survival and migration in D. melanogaster [45]. Interestingly, LOC103313779 (nuclear receptor corepressor 1, TC006021; Drosophila orthologue for smrter (smr), corepressor of ec dysone receptor) was up-regulated in HDAC3 knockdown samples (Additional file 1, Table S3). Suppression of HDAC3 enhances apoptosis induced by paclitaxel in human maxillary cancer cells [46].

Histone deacetylase 3 is associated with the nuclear receptor corepressor complex containing N-CoR and SMRT (Silencing mediator for retinoid and thyroid hormone receptors) [47, 48]. HDAC3 is crucial for repression by multiple nuclear receptors, and the N-CoR-HDAC3 complex plays a unique role in thyroid hormone receptor-mediated gene repression in human 232 T cells [49]. Our previous studies demonstrated that the HDAC1/SIN3 multiprotein complex regulates the expression of Kr-h1 [30]. Further studies with the NCoR-HDAC3 complex is required to identify the mechanism of gene regulation by HDAC3.

In D. melanogaster, knockdown of RPD3 by RNAi affects global histone acetylation, especially K9/14 of histone H3 and K8/K12 of histone H4 [50]. Similarly, HDAC1 and RPD3 disruptions result in histone H4 and H3 hyperacetylation, especially at H3K9/18 and H4K5 and K12 in the Saccharomyces cerevisiae [51]. Our western blot results showed that RNAi-mediated knockdown of HDAC3 results in an increase in acetylation of Histone H3K9 and HDAC3 expression by small interfering RNA caused hyperacylation of Lys-9 in histone H3 near the growth-differentiation factor 11 (gdf11) promoter [53]. HDAC3 selectively represses CREB3-mediated transcription and migration of metastatic breast cancer cells [54]. The histone H3 acetylase dGcn5 is a notable player in D. melanogaster metamorphosis [55]. HDAC3 deacetylates myocyte enhancer factor 2, a transcription factor essential for controlling gene expression, muscle differentiation, apoptosis, and survival of different cell types [33]. These data suggest that HDAC3 suppression induces the acetylation status of histone H3. WEGO plot showed enrichment of terms involved in biological regulation, regulation of the cellular process, signal transduction, ion binding, and catalytic activity in HDAC3 knockdown insects.

Conclusions

HDAC3 knockdown interferes with the JH response gene Kr-h1, pupal specifier Br-C, and the adult specifier transcription factor E93. JH response gene Kr-h1 and Br-C was significantly up-regulated by HDAC3
knockdown; the ecdysone response gene E93 was significantly down-regulated. Six genes were commonly up-regulated in dsHDAC3- and TSA-treatment, and down-regulated in dsCBP treatment (Additional file 1, Table S8). We further confirmed our result with RT-qPCR and identified that TC003570 (Shaven) and TC008417 (pvf3), which are important for sensory and cell proliferation, respectively, are significantly up-regulated in HDAC3 knockdown pupae. A model for the HDAC3 function is shown in Additional file 1, Fig. S6. In conclusion, we identified that HDAC3 reduction in T. castaneum affects the genes responsible for muscle development and signal transduction, and thereby affecting the development and metamorphosis.

Methods
Insect rearing and cell culture
T. castaneum GA-1 strain [56] beetles were reared on organic wheat flour (Heartland Mill, Marienthal, KS) containing 10% dried baker yeast (MP biomedicals, Solon, OH) at 30 °C and 65 ± 5% relative humidity. The T. castaneum cells (BCIRL-TcA-CLG1, TcA) were grown in EX-CELL 420 (Sigma-Aldrich, St-Louis, MO) medium supplemented with 10% Fetal Bovine Serum (FBS, VWR-Seradigm, Radnor, PA) at 28 °C [57].

Hormone treatments
Insect growth regulator/JH analog, S-Hydroprene (Sigma-Aldrich, MO), was used to test the response of the HDAC3 gene to JH. Cyclohexane was used as a solvent control where hydroprene was dissolved to obtain the concentration of 2 μg/μl, and 1 μg (0.5 μl/larvae) hydroprene was topically applied to 48 h-old final instar larvae for in vivo hormone treatments. Six hours after treatment, the samples were collected and analyzed by RT-qPCR. The relative HDAC3 and Kr-h1 mRNA were determined.

Double-stranded RNA synthesis (dsRNA) treatment and differential gene expression analysis
RNA isolation, cDNA synthesis, quantitative reverse transcription PCR (RT-qPCR), Double-stranded RNA synthesis (dsRNA) and microinjection, RNA-sequencing (RNA-seq) and data analysis and annotations were performed as described our previous publications [26, 30, 31]. RT-qPCR was performed using gene-specific primers (Additional file 1, Table S1) and iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA) in Applied Biosystems StepOnePlus Real-time PCR instrument. The libraries for RNA sequencing were prepared, as described previously [26]. The pooled libraries were sequenced using the Illumina HiSeq 4000 sequencer at Duke University Sequencing and Genomic Technologies (NC, USA). The raw reads were demultiplexed, trimmed, and transcripts were mapped back to the T. castaneum reference genome (assembly Tcas5.2) using the CLC genomic workbench pipeline (Version 11.0.1).

Protein extraction and western blotting
The larvae were cleaned with ice-cold PBS and lysed in RIPA lysis buffer (Radioimmunoprecipitation assay buffer- 0.5 M Tris-HCl, pH 7.4, 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10 mM EDTA- Millipore Sigma, MA, USA) containing Halt protease inhibitor cocktail (ThermoFisher, Rockford, IL, USA). The lysate was clarified in a microfuge at 8000 rpm for 5 min. The proteins were then centrifuged in a microfuge at 12000 rpm for 20 min. The pellet was washed in ice-cold acetone twice to remove the TCA traces, air-dried, and dissolved in 10% SDS. The proteins quantified using Bio-Rad protein assay concentrate and standard. Samples were denatured (95–100 °C for 5 min) in 5X SDS loading buffer to denature and stored at −20 °C for future use. An equal amount of proteins were loaded and resolved on 12% SDS-polyacrylamide gels, along with the protein ladder. The proteins were transferred from the gel to the polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA) and blocked with 5% nonfat dried milk blocking buffer for one hour at room temperature. The membranes were washed and incubated with 1000x diluted primary lysine-acetylated antibody-Histone H3 antibody sampler kit #9927 (Cell Signaling Technology, Danvers, MA) overnight at 4 °C with gentle shaking. The membranes were washed three times in Tris-buffered saline with Tween 20 (TBST) and incubated with 1000x diluted HRP-conjugated secondary antibody (#7074- Cell Signaling) in blocking buffer at room temperature for one hour as described previously [58]. The signals were developed with Supersignal West Femto Maximum sensitivity Substrate (ThermoFisher, IL), following the manufacturer’s protocol. The images were acquired in a darkroom using the chemiluminescence technique. Western blot bands were quantified by Image-J software and normalized with β-Actin loading control protein.

Statistical analysis
JMP Pro 14.0 (SAS Institute Inc., Cary, NC) software was used for t-test, comparing means, P-value < 0.05.

Supplementary information
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