Histone deacetylase 1 suppresses Krüppel homolog 1 gene expression and influences juvenile hormone action in *Tribolium castaneum*

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Posttranslational modifications, including acetylation and deacetylation of histones and other proteins, modulate hormone action. In *Tribolium castaneum* TcA cells, Trichostatin A, a histone deacetylase (HDAC) inhibitor, mimics juvenile hormone (JH) in inducing JH response genes (e.g., *Kr-h1*), suggesting that HDACs may be involved in JH action. To test this hypothesis, we identified genes coding for HDACs in *T. castaneum* and studied their function. Knockdown of 12 *HDAC* genes showed variable phenotypes; the most severe phenotype was detected in insects injected with double-stranded RNA targeting *HDAC1* (dsHDAC1). The dsHDAC1-injected insects showed arrested growth and development and eventually died. Application of JH analogs hydroprene to *T. castaneum* larvae and JH III to TcA cells suppressed *HDAC1* expression. Sequencing of RNA isolated from control and dsHDAC1-injected larvae identified 1,720 differentially expressed genes, of which 1,664 were up-regulated in dsHDAC1-treated insects. The acetylation levels of core histones were increased in TcA cells exposed to dsHDAC1 or JH III. ChIP assays performed using histone H2BK5ac antibodies showed an increase in acetylation in the *Kr-h1* promoter region of cells exposed to JH III or dsHDAC1. Overexpression or knockdown of *HDAC1*, *SIN3*, or both resulted in a decrease or increase in *Kr-h1* mRNA levels and its promoter activity, respectively. Overexpression of the JH receptor Methoprene tolerant (*Met*) was unable to induce *Kr-h1* in the presence of HDAC1 or SIN3. These data suggest that epigenetic modifications influence JH action by modulating acetylation levels of histones and by affecting the recruitment of proteins involved in the regulation of JH response genes.

epigenetics | *Kr-h1* | histone | hydroprene | SIN3

The major epigenetic changes, such as DNA and histone modifications and microRNA regulation, by themselves or in combination with other proteins regulate gene expression (1–3). Posttranslational modifications (PTMs) of histones, including acetylation, phosphorylation, methylation, ubiquitination, and sumoylation, play important roles in the epigenetic regulation of chromatin. One of the common PTMs of histones is acetylation by multiprotein complexes containing histone acetyltransferases (HATs) and histone deacetylases (HDACs) that add and remove acetyl groups, respectively (4). Modulation of the positive charge density of core histone by lysine acetylation is a reversible PTM that plays key roles in the formation and function of large macromolecular complexes involved in diverse cellular processes, such as chromatin remodeling, cell cycle, splicing, nuclear transport, and actin nucleation (5).

HDACs belong to a highly conserved family of proteins that regulate gene expression through histone modifications and formation of complexes with transcription activators and repressors (6). Along with their involvement in the acetylation and deacetylation of histones, HATs and HDACs interact with and/or modulate the acetylation levels of many receptors, transcription factors, coactivators, and corepressors and influence their function in the regulation of gene expression (7). Histone-modifying enzymes are also known to regulate nuclear receptor expression and activity; many nuclear receptors are subjected to acetylation that regulates their stability, ligand sensitivity, and transactivation (8, 9). In the fruit fly, *Drosophila melanogaster*, numerous acetylation sites have been identified in the proteome using high-resolution mass spectrometry (10). Research in *D. melanogaster* and other eukaryotes has shown that HDAC1 in complex with the corepressor SIN3 is often associated with sites of transcription repression (11). Knockdown of the *HDAC1* gene has been shown to increase acetylation levels of histone H3 and H4 (12) and to cause up-regulation of genes involved in multiple processes, including nucleotide and lipid metabolism, DNA replication, cell cycle regulation, and signal transduction (13).

The 2 major insect hormones, ecdysteroids (20-hydroxyecdysone, 20E, the most active form) and juvenile hormone (JH), regulate many developmental and physiological processes (14). Recent studies have identified Methoprene-tolerant (Met) and steroid receptor coactivator (SRC, also known as Taiman in *D. melanogaster* and FISC in *Aedes aegypti*), which act as a receptor protein and its binding partner, respectively, in JH signal transduction (15, 16). Many genes that are regulated by JH have been identified; one gene consistently identified as an important transcription factor in JH action is Krüppel homolog 1 (*Kr-h1*) (17). Recent studies reported *Kr-h1* repression of key genes, including Broad-Complex (*BR-C*), a pupal specifier, and *E93*, involved in adult

Significance

Juvenile hormone (JH) regulates many processes in insects, and JH mimics are used to control them. However, not much is known about the epigenetic regulation of JH action. Histone deacetylases (HDACs) are known to modulate hormone action. We identified 12 HDACs and analyzed their function in *Tribolium castaneum*. RNA interference-mediated knockdown of HDAC genes showed that HDAC1 plays critical roles in the regulation of growth and development by suppressing the expression of many genes, including those involved in JH action. Expression of the *HDAC1* gene is suppressed by JH, resulting in an increase in acetylation levels of histones, which promotes expression of JH response genes. SIN3:HDAC1 multiprotein complexes suppress the expression of JH response genes in the absence of JH.

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development and steroidogenic enzymes, in immature stages (18–22). Posttranslational modifications, including acetylation and phosphorylation, influence JH and 20E action (23, 24). The function of HDACs and HATs in epigenetic reprogramming during insect development, diapause, and polyphenism has been proposed (25–28). In *Blattella germanica* and *Tribolium castaneum*, CREB binding protein (CBP) has been shown to function in post-embryonic development and metamorphosis by regulating the expression of *Kr-h1*, *BR-C*, and *E93* (29–31). CBP is required for the acetylation of H3K18 and H3K27 in larvae of *T. castaneum* and in TcA cells (30, 31). We previously showed that Trichostatin A (TSA), an inhibitor of HDACs, mimics JH in the induction of JH response genes, including *Kr-h1*, in a dose- and time-dependent manner (31), suggesting that one or more HDACs may be involved in JH action. The function of HDACs in the action of JH is not known. Thus, the main goals of the present study were to identify HDACs involved in the expression of JH response genes and to investigate their mechanism of action in the regulation of growth and development using *T. castaneum* as a model insect.

Results

HDAC Enzymes Are Required for the Survival of Larvae, Pupae, and Adults. The genes coding for HDACs from *D. melanogaster* were used to search the *T. castaneum* genome, and 12 homologs were identified and classified into 4 classes (*SI Appendix*, Table S1). dsRNA targeting each *HDAC* gene was injected into newly molted last instar larvae, pupae, and adults. Control animals were injected with dsRNA targeting the gene encoding for maltose-binding protein from *Escherichia coli* (*malE*). The knockdown of *HDAC1* and *HDAC11* caused 100% larval mortality. In addition, larval mortality and significant pupal mortality were observed in animals injected with dsHDAC3. Knockdown of class III Sirtuins did not cause significant mortality (Fig. 1*A* and *SI Appendix*, Table S1). The control insects injected with dsmalE pupated by 5 to 6 d after injection and developed into normal adults (Fig. 1 *B*, *a*–*d*). In contrast, the larvae injected with dsHDAC1 showed developmental arrest during the final instar larval stage and died during the quiescent stage (Fig. 1 *B*, *e*). In addition, a larval-pupal intermediate phenotype with the dorsal split was observed when dsHDAC1 was injected into 72-h-old last instar larvae (Fig. 1 *B*, *f*). In these animals, removal of larval integument revealed a white-colored larval-pupal intermediate with dark eyes, sclerotized legs, and antennae (Fig. 1 *B*, *g* and *SI Appendix*, Fig. S1). Knockdown of *HDAC1* during the pupal stage arrested adult development, and the pupae eventually died (Fig. 1 *B*, *h*). Moreover, *HDAC1* knockdown caused 90% to 100% mortality in pupae and adults at approximately 5 d after dsRNA injection (Fig. 1*C*). Last instar larvae injected with dsHDAC11 arrested development before entering the quiescent stage; regions of midgut tissue became dark, and the larvae eventually died (*SI Appendix*, Fig. S2). In contrast, the last instar larvae injected with dsHDAC3 completed larval development and larval-pupal metamorphosis but had improperly folded wings and died during the pupal stage (*SI Appendix*, Fig. S2). Among the 12 *HDAC* genes knockdown, *HDAC1* knockdown caused the most severe effects; therefore, we focused our investigation on HDAC1.

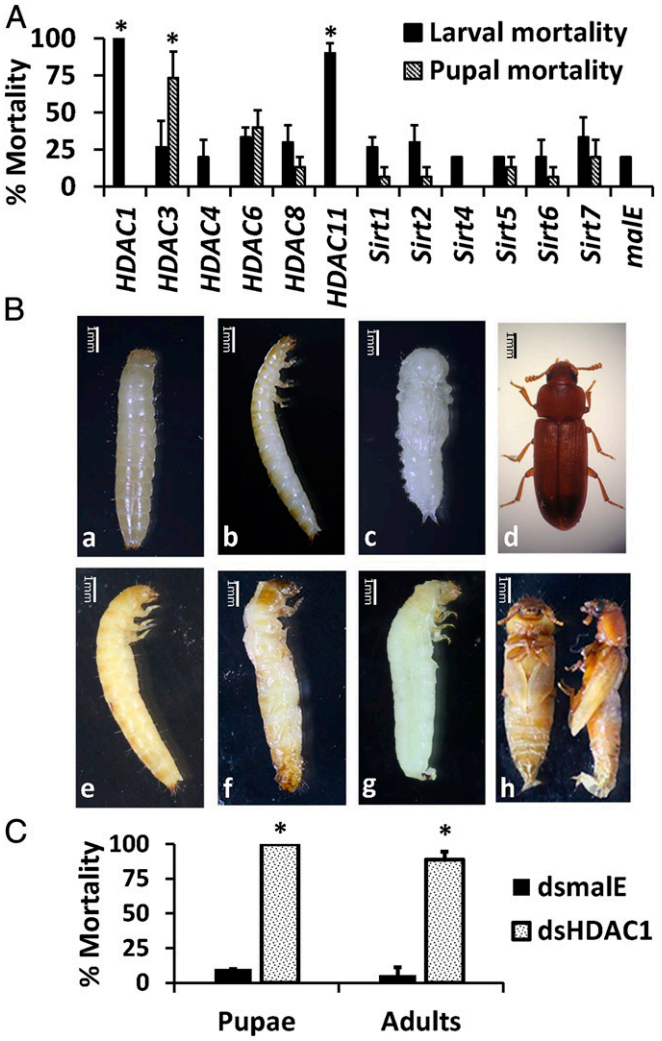

Fig. 1. Phenotypes and mortality caused by knockdown of *HDAC1* in *T. castaneum*. (*A*) dsRNA targeting 12 *HDAC* genes were injected into newly molted last instar larvae. Mortality and development defects were recorded every day until death or adult eclosion. Control insects were injected with dsRNA targeting the gene encoding for maltose-binding protein from *E. coli* (*malE*). Data are mean ± SE percent mortality; $n = 30$. In all panels, * indicates treatments that are significantly different from control; $P \leq 0.05$, *t* test. (*B*) dsHDACs were injected into newly molted last instar larvae or newly molted pupae. Phenotypes were photographed on the eighth day after injection. (*Top*) Normal newly molted last instar larva (*a*; untanned cuticle, white), quiescent stage nonfeeding larva (*b*; 96 h after the last molt), pupa (*c*), and adult (*d*). dsHDAC1-injected larva stuck in the quiescent stage (*e* and *f*), larval-pupal intermediate phenotypes caused by dsHDAC1 injection into 3-d-old last instar larvae (*g*), and pupal-adult intermediate phenotypes caused by dsHDAC1 injection during pupal stage (*h*) are shown. (*C*) 100% pupal and 90% adult mortality were observed after injection of dsHDAC1 into day 0 pupae and adults. Data are mean ± SE percent mortality; $n = 30$.

JH Suppresses Expression of *HDAC1*. Since HDAC1 is required for the survival and development of larvae and pupae, we determined the expression of this gene during larval and pupal stages. The mRNA levels were quantified by RT-qPCR using gene-specific primers (*SI Appendix*, Table S2). The maximum levels of *HDAC1* mRNA were detected at 24 h after entering the pupal stage (*SI Appendix*, Fig. S3). Treatment of last instar larvae with the JH analog hydroprene for 6 h caused a significant decrease in *HDAC1* mRNA levels (Fig. 2*A*). The expression of *Kr-h1* (a JH response gene), used as a positive control for JH response, was induced by hydroprene treatment. Similarly, exposure of *T. castaneum* TcA cells to JH III for 6 h caused a significant decrease in *HDAC1* and an increase in *Kr-h1* mRNA levels (Fig. 2*A*). These data suggest that JH suppresses the expression of *HDAC1*. To determine whether JH suppression of *HDAC1* expression is mediated by the JH receptor Met, dsMet or dsmalE was injected into the newly molted last instar larvae. At 48 h after injection of dsRNA, the larvae were treated with cyclohexane or hydroprene for 6 h. As shown in Fig. 2*B*, hydroprene treatment caused a significant decrease in *HDAC1* mRNA levels in larvae injected with dsmalE, but not in larvae injected with dsMet. The *HDAC1* mRNA levels in *Met* knockdown larvae were similar to

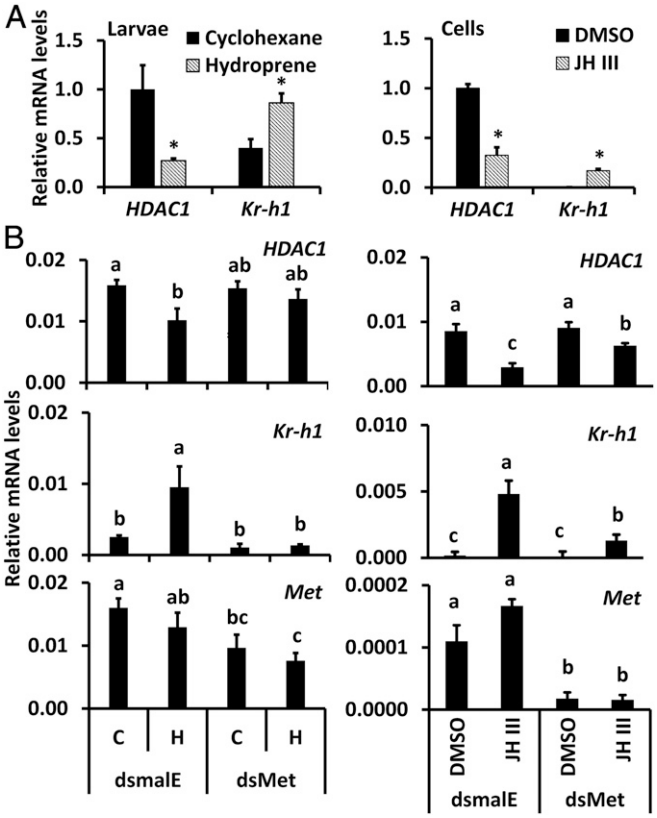

Fig. 2. JH suppresses the expression of *HDAC1* in *T. castaneum* larvae and TcA cells. (*A*) JH III and its analog hydroprene suppress the expression of *HDAC1* in TcA cells (*Right*) and *T. castaneum* larvae (*Left*), respectively. 0.5 μL of 2 μg/μL hydroprene in cyclohexane or cyclohexane alone was applied topically to 48-h-old final instar larvae. At 6 h after treatment, total RNA was isolated. TcA cells were exposed to 10 μm of JH III in DMSO or DMSO alone for 6 h, and the total RNA was isolated. The RNA was used to determine *HDAC1* and *Kr-h1* mRNA levels. Data are means ± SE; $n = 4$. * indicates treatments significantly different from control; $P \leq 0.05$, *t* test. (*B*) Met is required for suppression of *HDAC1* by hydroprene/JH III. Day 0 last instar larvae were injected with dsMet or dsmalE. At 48 h after injection of dsRNA, hydroprene was applied topically. TcA cells were exposed to dsMet or dsmalE. At 72 h after the addition of dsRNA, the cells were exposed to DMSO or JH III. Total RNA isolated from larvae (*Left*) and TcA cells (*Right*) was used to quantify *Kr-h1*, *HDAC1*, and *Met* mRNA levels. C, cyclohexane; H, hydroprene. Mean values with the same letter are not significantly different from each other.

those in control larvae treated with cyclohexane. Similar results were observed in TcA cells treated with dsMet or dsmalE followed by exposure to DMSO or JH III for 6 h. As expected, *HDAC1* mRNA levels decreased and *Kr-h1* mRNA levels increased after JH III treatment of control cells exposed to dsmalE. However, the suppression of *HDAC1* and induction of *Kr-h1* were reduced in the cells treated with dsMet (Fig. 2*B*). These data suggest that Met is required for JH III suppression of *HDAC1* and induction of *Kr-h1* expression.

HDAC1 Suppresses the Expression of Genes Involved in JH Action. Since JH suppresses the expression of *HDAC1*, we wanted to determine whether the expression of genes involved in JH action or those induced by JH are affected by *HDAC1* knockdown. A >50% reduction in *HDAC1* mRNA levels was detected at 12 h after injection of 1 μg of dsHDAC1 into each final instar larva (Fig. 3*A*). We also tested the injection of 0.5, 0.25, and 0.125 μg of dsHDAC1 per larva and found that at 12 h after dsRNA injection, there was no significant difference in *HDAC1* mRNA levels between larvae injected with 0.25 or 0.125 μg of dsHDAC1 per larva and the control larvae injected with the same doses of dsmalE (*SI Appendix*, Fig. S4). The extent of phenotypes detected at 15 d after injection of dsRNA decreased in treatments with 0.25 or 0.125 μg of dsHDAC1 per larva; therefore, we decided to use 1 μg per larva in subsequent studies. The mRNA levels of JH response genes *Kr-h1* and *4EBP* showed increase in *HDAC1* knockdown insects compared with their levels in control insects injected with dsmalE (Fig. 3*A*). Similarly, the mRNA levels of genes coding for SRC and CBP that are known to be involved in JH action were increased in *HDAC1* knockdown insects. The expression of *Met* increased by 1.1- to 1.7-fold in dsHDAC1-injected insects compared with dsmalE-injected insects in 3 independent experiments, but the differences were not statistically significant (Fig. 3*A*).

We also determined mRNA levels of *HSP90* (gene coding for heat shock protein) to check whether the *HDAC1* knockdown effect is universal. *HSP90* mRNA levels were not affected by *HDAC1* knockdown (Fig. 3*A*). These data suggest that HDAC1 may function in the regulation of JH response gene expression during the larval stage.

To identify other genes whose expression is affected by *HDAC1* knockdown, we sequenced RNA isolated from *HDAC1* knockdown and control insects. RNA samples isolated from larvae

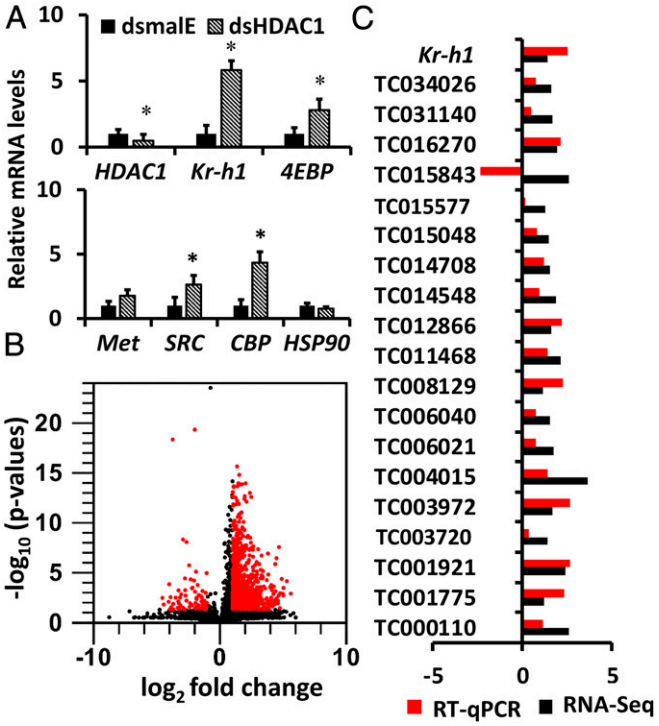

Fig. 3. *HDAC1* knockdown in last instar larvae of *T. castaneum* affects transcription of many genes involved in multiple pathways. (*A*) Knockdown of *HDAC1* causes an increase in expression of genes involved in JH action and JH response. Newly molted last instar larvae were injected with dsHDAC1 or dsmalE. Total RNA was extracted from larvae collected at 12 h after treatment and used to quantify mRNA levels of *Met*, *SRC*, *CBP*, and *HSP90*. * indicates treatments significantly different from control; $P \leq 0.05$, *t* test. (*B*) Volcano plot showing differentially expressed genes between larvae injected with dsHDAC1 or with dsmalE. The red dots show differentially expressed genes (≥2-fold difference and $P \leq 0.05$). (*C*) Comparison of the differential expression pattern of selected genes obtained by RNA-seq and RT-qPCR methods. The expression of 20 genes selected from the up-regulated group (based on RNA-seq data) was verified by RT-qPCR. Gene names are listed in *SI Appendix*, Table S5.

at 12 h after injection of dsHDAC1 were sequenced. Run summary and read count statistics are shown in *SI Appendix*, Table S3. Analysis of RNA-seq data identified 1,720 genes that are differentially expressed between dsHDAC1- and dsmalE-treated larvae with a ≥2-fold change and $P \leq 0.05$. Among 1,720 differentially expressed genes, 1,664 were up-regulated and the remainder were down-regulated (*SI Appendix*, Fig. S5 and Dataset S1). In Fig. 3*B*, the differential expression of genes is shown as a volcano plot with red dots indicating the genes showing statistically significant differences in expression between treatment and control. The expression of some of the genes known to function in 20E and JH action was affected by the *HDAC1* knockdown; these genes include *Kr-h1*, *SRC*, and *CBP* (*SI Appendix*, Table S4).

To confirm the gene expression differences revealed by the RNA-seq data, we performed RT-qPCR for 20 selected genes. The 20 up-regulated genes in dsHDAC1-treated larvae were selected based on the presence of DNA-binding domains with possible functions as transcription factors (*SI Appendix*, Table S5). The differential expression levels of selected genes obtained by the RNA-seq and RT-qPCR methods are compared in Fig. 3*C*. Nineteen out of 20 genes tested showed an increase in mRNA levels in dsHDAC1-treated larvae compared with control larvae treated with dsmalE (*SI Appendix*, Fig. S6). Although the fold differences in mRNA levels between treatment and control determined by RNA-seq and RT-qPCR methods are not the same due to differences in sensitivity between the 2 methods, 19 out of 20 genes tested showed increased expression in *HDAC1* knockdown samples analyzed by both methods.

To identify genes that are induced by JH and affected by *HDAC1* knockdown, we compared the RNA-seq data from our previous experiments on JH induction of gene expression in *T. castaneum* larvae and TcA cells (30) with our current RNA-seq data. Ten genes, including *Kr-h1*, Rho GTPase-activating protein 100F (LOC660562), lachesin (LOC659929), DNA damage-regulated autophagy modulator protein 2-like, 4 hydroxyphenyl pyruvate dioxygenase, hemicentin-1, and calpain-7, were induced by JH and showed increased expression levels in dsHDAC1-treated larvae (*SI Appendix*, Table S6). Gene ontology (GO) enrichment analysis of differentially expressed genes (dsHDAC1 vs. control) against the *Tribolium* reference transcriptome GOs revealed significant enrichment of the genes involved in the protein-containing complex, protein binding, response to stimuli, signal transduction, and transcription regulator activity (*SI Appendix*, Fig. S7).

***HDAC1* Knockdown Affects the Acetylation Status of Histones.** To identify targets of HDAC1 deacetylation, proteins were extracted from dsRNA-treated TcA cells and subjected to Western blot hybridization using an antibody that recognizes acetylated lysine. Increased acetylation of histones H2 and H3 was detected in cells exposed to dsHDAC1 compared with control cells treated with dsmalE and DMSO (Fig. 4*A* and *SI Appendix*, Fig. S8). Interestingly, cells treated with JH III also showed higher acetylation levels for all 3 histones (H2, H3, and H4) compared with their acetylation levels in DMSO-treated cells (Fig. 4*A*). Western blots were probed with antibodies targeting acetylated H3K9, H3K27, H2AK5, and H2BK5 to identify changes in the acetylation status of specific lysine residues in *HDAC1* knockdown or JH III-treated cells. These targets were selected based on information on HDAC1-deacetylated residues in humans and animals (6). Of the 4 antibodies tested, only the acetylation status of H2BK5 was increased significantly in JH III-treated and *HDAC1* knockdown cells compared with control cells treated with DMSO and dsmalE (Fig. 4*A* and *SI Appendix*, Fig. S8). These data suggest that both *HDAC1* knockdown and JH III treatment, which suppresses HDAC1 expression, increase the acetylation of histones.

To test whether enhanced acetylation of histones in cells exposed to JH III or dsHDAC1 is enriched at the promoter regions

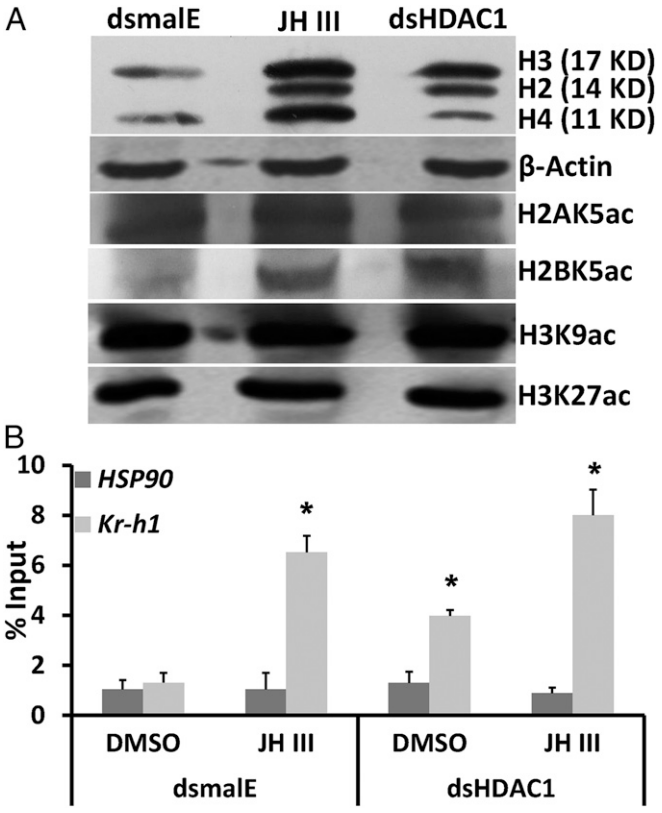

Fig. 4. Knockdown of *HDAC1* or treatment with JH III increases acetylation levels of histones. (*A*) Exposure of TcA cells to dsHDAC1 or JH III increased acetylation levels of core histones. TcA cells were treated with dsHDAC1 or dsmalE for 72 h, followed by DMSO or JH III for 24 h. The chromatin-bound proteins were extracted and analyzed by Western blot using antibodies that detect acetylated lysines in histones or specific acetylated lysine residues (H2AK5, H2BK5, H3K9, and H3K27) in H2 and H3. β-actin served as a loading control. (*B*) ChIP assay revealed that H2BK5 acetylation mark was enriched at the *Kr-h1* promoter. TcA cells were treated with dsmalE or dsHDAC1 for 72 h, followed by exposure to DMSO or JH III for 6 h. The chromatin was cross-linked and enriched using the H2BK5ac antibody. The enrichment levels of promoters of *Kr-h1* and *HSP90* (control) were determined by qPCR. Data were analyzed using 1-way ANOVA. * denotes the significant differences in enrichment levels of target gene promoter between the control (dsmalE + DMSO) and treatments at $P < 0.01$.

of JH-response genes such as *Kr-h1*, we performed a chromatin immunoprecipitation (ChIP) assay using TcA cells and H2BK5ac antibody. Compared with control cells exposed to DMSO and dsmalE, significantly enhanced acetylation at the *Kr-h1* promoter region was detected in the cells exposed to JH III or dsHDAC1 and precipitated with H2BK5ac antibody (Fig. 4*B*). No significant enrichment of acetylation at the promoter region of *HSP90* was detected in cells exposed to JH III or dsHDAC1 compared with DMSO- and dsmalE-treated cells.

HDAC1/SIN3 Protein Complexes Regulate Expression of *Kr-h1*. HDAC1 and SIN3 are components of multiprotein complexes conserved from yeast to humans (32). These complexes are known to regulate gene expression by deacetylation and/or acting as a scaffold to recruit transcription factors to the promoter region (33). Since HDAC1-mediated acetylated H2B proteins are enriched at the *Kr-h1* promoter, we tested the effects of HDAC1 and SIN3 on the expression of the *Kr-h1* gene and the activity of the *Kr-h1* promoter. Knockdown of *HDAC1*, *SIN3*, or both in TcA cells significantly increased *Kr-h1* mRNA levels compared with levels in control cells exposed to dsmalE (Fig. 5*A* and *SI Appendix*,

Fig. S9). JH III induced *Kr-h1* gene expression in TcA cells exposed to dsmalE. Interestingly, JH III induction of *Kr-h1* mRNA levels was further increased in cells exposed to dsRNAs targeting *HDAC1*, *SIN3*, or both. A similar effect of knockdown of *HDAC1*, *SIN3*, or both was observed on *Kr-h1* promoter activity in TcA cells. The luciferase gene regulated by the *Kr-h1* promoter was induced in cells exposed to dsHDAC1, dsSIN3, or both (Fig. 5*C*). JH III induced the luciferase in control cells exposed to dsmalE, and the JH III induction of the luciferase expression was further increased by 2-fold in cells exposed to dsHDAC1, dsSIN3, or both compared with control cells exposed to dsmalE and DMSO. Constructs expressing *HDAC1* and *SIN3* genes under the control of baculovirus IE1 promoter (which is active in TcA cells) were prepared and tested for their effect on *Kr-h1* gene expression. As shown in Fig. 5*C*, *Kr-h1* mRNA levels were low in DMSO- treated cells but increased in JH III-treated cells. Overexpression of *HDAC1*, *SIN3*, or both in TcA cells reduced JH III induction of *Kr-h1*. Unlike the effects on *Kr-h1* expression and *Kr-h1* promoter activity observed after knockdown of *SIN3* and *HDAC1* genes, the overexpression of *HDAC1* and *SIN3* caused a synergistic effect resulting in the maximum reduction in JH induction of *Kr-h1* mRNA levels and promoter activity when both *HDAC1* and *SIN3* were expressed together (Fig. 5*C*). Similarly, overexpression of *HDAC1* and *SIN3* also caused a significant decrease in JH III induction of the luciferase gene regulated by the *Kr-h1* promoter, and again the suppression was at the maximum levels when both *HDAC1* and *SIN3* were expressed together (Fig. 5*D*). Overexpression of *Met* in TcA cells increased *Kr-h1* mRNA levels and expression levels of the luciferase gene regulated by the *Kr-h1* promoter in DMSO, as well as JH III-treated cells (Fig. 5 *E* and *F*). Overexpression of *HDAC1* and *SIN3* prevented a Met-mediated increase in *Kr-h1* mRNA levels and luciferase activity in DMSO- and JH III-treated cells compared with control cells transfected with an empty vector construct. These data suggest that the SIN3 complex containing HDAC1 is involved in the suppression of *Kr-h1* gene expression.

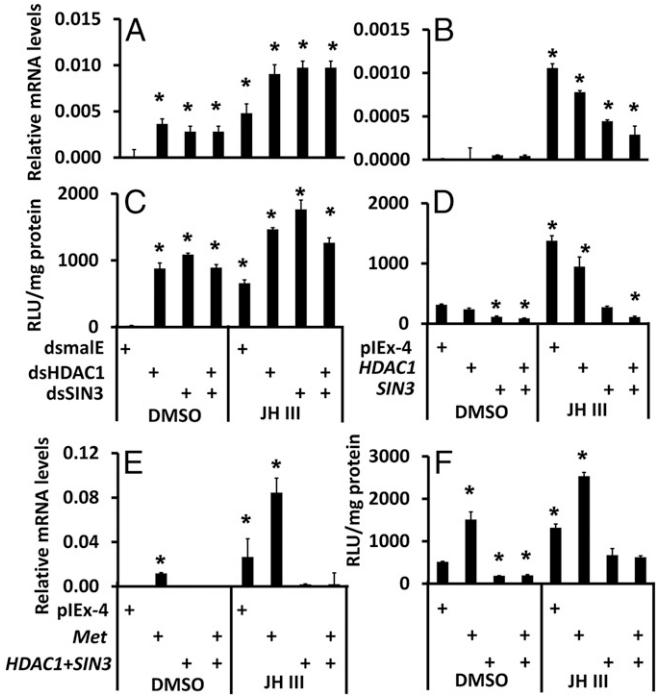

Fig. 5. Overexpression or knockdown of *HDAC1, SIN3*, or both affect the expression of *Kr-h1* gene and activity of its promoter. (*A*) TcA cells were exposed to dsmalE, dsHDAC1, dsSIN3, or both dsHDAC1 and dsSIN3 for 72 h, followed by treatment with 10 μM of JH III or DMSO for 6 h. Total RNA was extracted and used in RT-qPCR to quantify *Kr-h1* mRNA levels. Data were analyzed using 1-way ANOVA. * denotes the significant differences in *Kr-h1* mRNA levels between dsmalE/DMSO-treated cells compared with the other treatments at $P < 0.01$. Data are mean ± SE; $n = 3$. (*B*) *Kr-h1* mRNA levels were determined in cells transfected with vector alone (pIEX-4) or vector expressing *HDAC1, SIN3*, or both. (*C*) Luciferase activity was determined in TcA cells transfected with 500 ng of pGL3 basic vector containing the *Kr-h1* promoter using the X-tremeGENE HP DNA transfection reagent (Sigma-Aldrich). These cells were exposed to dsmalE, dsHDAC1, dsSIN3, or both dsHDAC1 and dsSIN3 for 48 h, followed by treatment with 10 μM JH III/DMSO for 24 h. Then the cells were lysed, and the luciferase activity was determined. Data are mean ± SE; $n = 4$. (*D*) Luciferase activity was determined in cells transfected with vector alone (pIEX-4) or vector expressing *HDAC1, SIN3*, or both. (*E* and *F*) *Kr-h1* mRNA levels (*E*) and luciferase activity (*F*) were determined in cells transfected with vector alone (pIEX-4) or vector expressing *Met* or *HDAC1* plus *SIN3*, or all 3 together.

Discussion

Based on the information on the function of HDACs reported in other organisms, we hypothesize that HDAC1 plays an important role in the regulation of basic cellular processes in *T. castaneum*, including DNA replication, cell cycle, metabolism, and signal transduction required for postembryonic development. Differential gene expression analysis of sequences from RNA isolated from dsHDAC1- or control dsmalE-injected larvae identified 1,664 genes suppressed by HDAC1. These data are consistent with the previous reports from *D. melanogaster* and other eukaryotes in which HDAC1 in complex with corepressors such as SIN3 is often associated with sites of transcription repression (11). The GO terms associated with HDAC1-suppressed genes are similar to those used to describe HDAC1 function in the FlyBase (http://flybase.org/reports/FBgn0015805), suggesting that the function of HDAC1 is conserved between *D. melanogaster* and *T. castaneum*. Individual HDACs modulate the transcription of distinct groups of genes in *D. melanogaster* (34). Similar gene groups are regulated by HDAC1 in both *T. castaneum* and *D. melanogaster* (*SI Appendix*, Tables S4–S6). Taken together, these data suggest that HDAC1 is involved in the regulation of multiple genes that contribute to basic processes, including cell cycle regulation, metabolism, and signal transduction—making HDAC1 an essential gene for the postembryonic development of *T. castaneum*.

Previous studies have shown that HDAC1 affects the acetylation status of all core histones with varying efficiency (35). Interestingly, in TcA cells, JH treatment suppresses the expression of *HDAC1* gene and increases the acetylation levels of core histones. Since acetylated histones are generally associated with activation of gene expression, it is likely that JH suppresses *HDAC1* expression and promote the acetylation of histones to ensure expression of genes such as *Kr-h1*. JH induces gene coding for Kr-h1, which in turn represses genes coding for BR-C, E93, and steroidogenic enzymes to prevent premature metamorphosis during immature stages (18, 21, 22). Knockdown of *CBP* results in decreased expression of *Kr-h1* (29–31). Trichostatin A induces *Kr-h1* gene expression in TcA cells (31). The data presented here show that knockdown of *HDAC1* results in increased *Kr-h1* expression in *T. castaneum* larvae. Taken together, these data point to *Kr-h1* as a central player in the regulation of gene activation and repression cascades during the postembryonic development of insects. After a commitment to undergo metamorphosis, JH titers decrease, allowing increased expression of *HDAC1*, resulting in deacetylation of histones, and decreased expression of *Kr-h1*, allowing expression of *BR-C* and *E93*, which promote metamorphosis. Thus, HDAC1 may play a key role in the expression of genes coding for proteins in MEKRE93 pathway that regulates insect metamorphosis (20).

How does HDAC1 influence expression of JH response genes such as *Kr-h1*? HDACs have been reported to be associated with large multimeric protein complexes, including SIN3A, nucleosome

remodeling and deacetylase (NuRD) and corepressor of REST transcription (CoREST), which are highly conserved in the animal kingdom (33). RNAi and RNA sequencing experiments in *T. castaneum* have identified regulation of cell cycle and metabolism as the major biological processes influenced by HDAC1, and these 2 processes were reported to be regulated by SIN3A/HDAC1 complexes in *D. melanogaster* (36). We hypothesized that HDAC1 might function in the SIN3 complex for regulating *Kr-h1* gene expression during postembryonic development in *T. castaneum*. SIN3 was first discovered in yeast and is conserved across eukaryotes. Mutations in SIN3 induce multiple defects in yeast, mammals, and flies (33). Knockdown of *SIN3*, *HDAC1*, or both together showed an increase in *Kr-h1* mRNA levels and the luciferase gene regulated by the *Kr-h1* promoter. While overexpression of these 2 genes by themselves or together showed a decrease in *Kr-h1* mRNA levels and the luciferase gene regulated by the *Kr-h1* promoter, these data suggest that HDAC1 functions in the SIN3 multiprotein complex and participates in the suppression of *Kr-h1* gene expression in the absence of JH. HDAC1 likely accomplishes this function by deacetylation of core histones as well as proteins in the SIN3 multiprotein complex localized to the *Kr-h1* promoter. How SIN3 is recruited to *Kr-h1* promoter remains unknown and requires further investigation. SIN3 has been shown to interact with the bHLH-ZIP transcription factor Mad and to form complexes with Mad-Max, and these complexes specifically recognize the Mad-Max E box-binding site and repress transcription of target genes (37). As proposed in the model (*SI Appendix*, Fig. S10), it is possible that SIN3 may interact with the JH receptor Met at the promoters of JH response genes, such as *Kr-h1*, and help recruit HDAC1 and other proteins. The HDAC1 assembled at the promoters may deacetylate transcription factors as well as core histones near the promoter region, resulting in suppression of gene expression and condensed chromatin. Work is in progress to test the proposed model.

Materials and Methods

Insect rearing, cell culture, hormone treatments, dsRNA synthesis, microinjection, cDNA synthesis, RT-qPCR, RNA-seq, data analysis and annotation, protein extraction and Western blot analysis, imaging and documentation, ChIP assays, and statistical analysis were performed as described previously (30). Methods described by Kalsi and Palli (38) were used to perform transfection of TcA cells and the luciferase assays. Details are provided in *SI Appendix*.

Data Accessibility. We have deposited the short-read (Illumina HiSeq 4000) sequence data in the NCBI SRA (accession no. PRJNA495026).

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