Epigenetic modifications acetylation and deacetylation play important roles in juvenile hormone action

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Abstract

Background: Epigenetic modifications including DNA methylation and post-translational modifications of histones are known to regulate gene expression. Antagonistic activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs) mediate transcriptional reprogramming during insect development as shown in Drosophila melanogaster and other insects. Juvenile hormones (JH) play vital roles in the regulation of growth, development, metamorphosis, reproduction and other physiological processes. However, our current understanding of epigenetic regulation of JH action is still limited. Hence, we studied the role of CREB binding protein (CBP, contains HAT domain) and Trichostatin A (TSA, HDAC inhibitor) on JH action.

Results: Exposure of Tribolium castaneum cells (TcA cells) to JH or TSA caused an increase in expression of Kr-h1 (a known JH-response gene) and 31 or 698 other genes respectively. Knockdown of the gene coding for CBP caused a decrease in the expression of 456 genes including Kr-h1. Interestingly, the expression of several genes coding for transcription factors, nuclear receptors, P450 and fatty acid synthase family members that are known to mediate JH action were affected by CBP knockdown or TSA treatment.

Conclusions: These data suggest that acetylation and deacetylation mediated by HATs and HDACs play an important role in JH action.

Keywords: HAT, HDAC, Kr-h1, FOXO Tribolium and TcA cells

Introduction

Immature juvenile forms of animals transform to become reproductive adults during their life cycle. In insects, two types of such transformation are generally observed; incomplete metamorphosis where nymphs turn into adults in hemimetabolous insects (e.g. true bugs, locusts & cockroaches) and complete metamorphosis which comprises dramatic morphological changes that result in the transformation from larva to the intermediate form, pupa and then to reproductive adults as observed in holometabolous insects (e.g. beetles and butterflies) [1]. Despite the differences in the strategies used by the insects, the genetic switch between different forms is tightly regulated by changes in the levels of two major hormones, ecdysteroids (20-hydroxyecdysone, 20E is the most active form) and juvenile hormones (JH) [2, 3]. 20-hydroxyecdysone works through its receptor complex, ecdysone receptor (EcR) and ultraspiracle (USP) that binds to ecdysone response elements (EcRE) present in the promoter regions of ecdysone-response genes including ecdysone-induced transcription factors (“early genes”) and others [4, 5]. Periodic pulses of 20E ensure developmental transitions during molting and metamorphosis. The presence of JH prevents metamorphosis, and the absence of JH promotes metamorphosis. Juvenile hormones function through its receptor, Methoprene-tolerant (Met) and steroid receptor coactivator (SRC, also known as FISC or Taiman). The JH/MET/SRC complex binds to JH response elements (JHRE) present in the promoter regions of JH-response genes including Krüppel homolog 1 (Kr-h1) and plays key roles in “status quo” action of JH [6, 7]. In Bombyx mori, Kr-h1 functions as a direct transcriptional repressor of E93, the adult specifier gene, through binding to response elements present in the promoter region of this gene [8].
In addition to signaling through nuclear receptors and transcription factors, JH may also function through membrane receptors and second messengers [9, 10]. Our previous work in *T. castaneum* has documented the dopamine D2-like receptor [G-protein-coupled-receptor (GPCR) family member] playing critical roles in regulating JH-mediated Vg uptake [11]. Cai et al., [12] reported the involvement of GPCRs during the 20E signaling suggesting that membrane receptors may be involved in this hormone signaling. Furthermore, it has been proposed that JH could regulate Ca\(^{2+}\) homeostasis within the cell during metamorphosis by interacting with membrane receptors or ion channels. Previously, farnesol (a precursor of JH) was shown to block Ca\(^{2+}\) channels [13]. It has been suggested that the JH maintains the “status quo effect” by reducing the cellular Ca\(^{2+}\) levels and when JH titers decline, such regulation is released, and the cellular Ca\(^{2+}\) levels are elevated to induce apoptosis, a process that occurs during metamorphosis [13].

In addition to metamorphosis, JH also regulates other crucial aspects of insect life including reproduction, diapause and caste differentiation [14]. In *D. melanogaster* females, JH controls yolk protein synthesis in the fat body and ovaries [15]. In males, JH regulates courtship behavior and promotes synthesis of accessory gland proteins [16–18]. The regulatory mechanisms underlying orchestrated synthesis of JH and expression of its receptors and target genes including transcription factors, co-activators for regulation of various physiological processes such as molting and metamorphosis are not yet precise.

Recent studies documented the role of CBP (or Nejire), a protein with acetyltransferase activity (HAT), as a transcriptional co-regulator in various developmental processes in *D. melanogaster* [19, 20]. In addition, the circadian rhythms in *D. melanogaster* also rely on the presence of Nejire/CBP [21]. Intriguingly, with more than 400 documented interacting partners mostly belonging to transcription factor and growth regulator families, CBP acts as a hub for transcriptional network involved in regulation of hormone signaling pathways and other fundamental physiological processes in insect life [19, 22, 23]. Recently, the involvement of CBP in the metamorphosis of *Blatella germanica* was reported [24]. The HAT activity of CBP increases acetylation of histones resulting in neutralization of lysine residues, which in turn increases accessibility of the promoters to cellular transcriptional machinery resulting in higher gene expression.

Histone deacetylases (HDACs) remove acetyl groups, increasing DNA and histone interactions resulting in repression of target gene expression through diminishing the accessibility of the promoters to transcription factors. Hence, stringent regulation of DNA packaging by HATs and HDACs can control gene expression. In insects, HDAC inhibitor activity might facilitate caste specification behavior in honey bees [25]. Epigenetic control of neural plasticity in honey bee has been documented recently [26]. Furthermore, the size and their nutrition-dependent hyper-variability of mandibles in the broad-horned flour beetle, *Gnatocerus cornutus*, are regulated by HDACs. HDAC1 RNAi in the beetle larvae caused shortening of mandibles in the adults, whereas HDAC3 RNAi resulted in hypertrophy [27]. In the case of ants, the caste-specific foraging and scouting behaviors are also regulated epigenetically by the equilibrium between CBP-mediated acetylation and HDAC-mediated deacetylation of histones in the brain [28].

However, only limited information on the contribution of HATs or HDACs to JH action is available. In the current study, we elucidated the effect of CBP and an HDAC inhibitor TSA on gene expression using *TcA* cells as a model system because it has already served as a robust research platform for molecular analysis of JH signaling pathway [29, 30]. The expression of CBP was knocked down by exposing *TcA* cells to *dsCBP*. Exposure of CBP knockdown cells to DMSO (control), JH or TSA followed by sequencing of RNA isolated from the treated cells revealed the influence of acetylation status of histones on the expression of genes involved in JH action.

**Materials and methods**

**Cell culture**

The *Tribolium castaneum* cells (BCIRL-TcA-CLG1 are gift from Dr. Cynthia Goodman from Biological control of insects research laboratory, USDA-ARS, 1503 S. Providence, Columbia, MO, USA) were grown at 28 °C in Ex-cell 420 (Sigma) supplemented with 10% Fetal Bovine Serum (Life Technologies) following the protocol described recently [31].

**Knockdown of the CBP gene**

Double-stranded RNA targeting CBP gene (*dsCBP*) was synthesized using the cDNA template and MEGAscript RNAi kit (Ambion”). For knockdown experiments, *TcA* cells were seeded in 12-well plates. After overnight culture in the medium containing 10% FBS, the cells were treated with either 1 μg of *dsCBP* or *dsmaLE* (as control dsRNA targeting a fragment of *E. coli maltase* gene) for 72 h. At the end of the incubation with dsRNA, the cells were treated with JH III or TSA dissolved in DMSO. DMSO was added to the control cells. After 6 h of hormone treatment, the *TcA* cells were washed with 1X PBS, harvested and processed for isolation of RNA.

Total RNA was isolated using the TRI reagent (Molecular Research Center Inc., Cincinnati, OH). The DNA was eliminated from the total RNA using DNase I (Ambion Inc., Austin, TX). RNA quantity was measured using a NanoDrop 2000 spectrophotometer (Thermo
Fisher Scientific), and quality was determined by running on 1% agarose gel. Two micrograms of total RNA from each sample was used for cDNA synthesis. cDNA was used to amplify a fragment of the target gene, and the PCR product was used for dsRNA synthesis. Primers used for dsRNA synthesis and reverse-transcriptase quantitative real-time PCR (RT-qPCR) are listed in Additional file 1: Table S1 or reported in our previous publication [33]. The MEGAscript RNAi Kit (Ambion Inc., Austin, TX) was employed for dsRNA synthesis. After total RNA extraction, cDNA synthesis and RT-qPCR were performed following the protocol described previously [33].

**RNA-seq study design, library preparation, and sequencing**

Total RNA was extracted from *dsma*E (M) treated cells exposed to DMSO (*dsma*E+ DMSO or MD), JH III (*dsma*E+ JHIII or M) or TSA (*dsma*E+ TSA or MT) and dsCBP (C) treated cells exposed to DMSO (*dsCBP*+ DMSO or CD), JH III (*dsCBP*+ JHIII or C) or TSA (*dsCBP*+ TSA or CT). Three biological replicates were used for each treatment. Design of RNA seq experiment is presented in Table 1. The protocol used for RNA seq library preparation include oligo-dT based total RNA separation, RNA fragmentation, two-step cDNA preparation and library barcoding, as described previously [33, 34]. Briefly, mRNA was purified and enriched from 3 μg of high-quality RNA from each biological replicate, fragmented and used for cDNA preparation. First strand cDNA synthesis was performed using SMARTScrbe reverse transcriptase enzyme (Clontech Laboratories, Inc) and reverse transcriptase primers (Integrated DNA technologies) with a sequencing adaptor followed by a random hexamer for Illumina sequencing and unique barcode for multiplexing. Second strand cDNA was synthesized using SMART 7.5 primer (Clontech Laboratories, Inc). The cDNA fragments were size selected (~300 bp) using HighPrep TM PCR beads (MAGBIO) and finally amplified using PCR. Libraries are quantified by Nanodrop and verified by running them on 1.2% agarose gels. Finally, the libraries were multiplexed and sent for sequencing (single-end) using the HiSeq4000 sequencer at the Genomics Technologies Center of Duke University, NC, USA. Raw sequencing data statistics are presented in Table 2.

### RNA-seq data analysis

Raw reads after quality control (demultiplexing, trimming and adaptor removal) were mapped to the reference genome (GA-2 strain of *T. castaneum*) [35]. The parameters used for the mapping are mismatch cost = 2, insertion cost = 3, deletion cost = 3, length fraction = 0.8, similarity fraction = 0.8, unique exon read mapping. The read counts were Log10 transformed followed by normalization using the scaling method where the sets of the transformed expression values for the samples were multiplied by a constant so that the sets of normalized values for the samples had the same ‘target’ value. The RNA-seq tool from CLC workbench (Version 9.5.9) was used to quantify gene expression employing standard pre-optimized settings and parameters such as mapping to exon regions only [36]. Further, biases in the sequence datasets and different transcript sizes were corrected using the RPKM algorithm to obtain correct estimates for relative expression levels. Finally, EDGE (Empirical analysis of differential gene expression) analysis was performed through “Advanced RNA-Seq plug-in” in CLC workbench using the recommended parameters such as estimated count filter cut off 5, estimate tag-wise dispersion [33]. For differential gene expression, we set a p-value cut-off of < 0.01 and fold change cut-off of ±2-fold as a threshold value for being significant. Differentially expressed genes were functionally annotated using “cloud blast” feature within the “Blas to2GO plug In” in CLC Genomic Workbench (Version 9.5.9). Nucleotide blast was performed against arthropod database with an E-value cut off 1.0E-3. Both, annex and GO slim were used to improve the GO term identification further by crossing the three GO categories (biological process, molecular function, and cellular component) to search for name similarities, GO term and enzyme relationships within KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways.

### RT-qPCR validation

RT-qPCR was conducted to validate the RNA seq results with a subset of 15 genes induced after JH treatment. Primers were designed using IDT’s primer design software (www.idtdna.com) with parameters including length 18–25 nt, melting temperature 55–65 °C, GC content 50–60% and product size 100–150 bp (Additional file 1: Table S1). Primers were tested by PCR followed by gel electrophoresis for correct product amplification. cDNA for RT-qPCR were synthesized using RNA from a

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**Table 1** RNA-seq study design

| No | Comparisons                              | Abbreviation |
|----|------------------------------------------|--------------|
| 1  | DMSO and JH treated cells exposed to *dsma*E | MD vs MJ     |
| 2  | DMSO treated cells exposed to *dsma*E or dsCBP | MD vs CD     |
| 3  | DMSO or JH treated cells exposed to *dsma*E or dsCBP | MD vs CJ     |
| 4  | DMSO or TSA treated cells exposed to *dsma*E or dsCBP | MD vs CT     |
| 5  | DMSO or TSA treated cell exposed to *dsma*E | MD vs MT     |

The table shows a list of comparisons made after RNA sequencing. All the treatments are compared with the control (MD)
new set of dsmaE, and dsCBP treated samples. cDNA was prepared from 3 μg RNA per sample using M-MLV reverse transcriptase kit (Invitrogen TM) following the manufacturer protocol. Resulting cDNA samples were diluted (4 times), and RT-qPCR was performed using SYBR Green Supermix (Bio-rad) following existing lab protocol [33]. Melt curves were generated to ensure single product amplification. The target genes expression levels were calculated using the 2-ΔCt method with RP49 (a ribosomal protein) as a reference gene, which had already been validated as a housekeeping gene in Tribolium castaneum [37–39].

**Results and discussion**

Ecdysteroids and JH regulate insect metamorphosis by performing antagonistic roles [40]. 20E facilitates metamorphosis whereas the presence of JH prevents it until the appropriate time. The role of JH in preventing precocious metamorphosis is documented in some hemimetabolous and holometabolous insects [6, 41–43]. We studied the contribution of CBP (HAT, acetylation) and TSA (HDAC inhibitor) to JH action. TcA cell line, originated from the non-embryonic adult insect tissues, was used in the study. The cells were treated with dsmaE (dsRNA targeting E. coli maltase gene as control) or dsCBP followed by exposure to JH III, TSA and treated with JH or TSA indicating the requirement of CBP for the cellular response to JH and TSA treatments (Fig. 1). These results suggest a possible role for CBP and TSA in JH induction of Kr-h1 gene.

**RNA-seq and gene expression analysis**

To identify the impact of CBP on gene expression after JH III or TSA treatment, we performed sequencing of RNA isolated from TcA cells exposed to dsmaE or dsCBP followed by treatment with JH or TSA. Illumina sequencing resulted in 90.28, 64.05, 86.01, 98.4, 100.32 and 94.04 million reads for MD (dsmaE+ DMSO), MJ (dsmaE+ JHIII), MT (dsmaE+ TSA), CD (dsCBP+ DMSO), CJ (dsCBP+ JHIII) and CT (dsCBP+ TSA) treatments respectively. After quality control measures, approximately 83–93% of reads from all treatments were mapped back to the exon regions of the T. castaneum genome (Table 2). Further details of RNA-seq statistics per sample are given in Additional file 1: Table S2.

| Samples         | dsmaE         | dsCBP         |
|-----------------|---------------|---------------|
| MD (dsmaE+ DMSO)| MJ (dsmaE+ JHIII) | MT (dsmaE+ TSA) | CD (dsCBP+ DMSO) | CJ (dsCBP+ JHIII) | CT (dsCBP+ TSA) |
| No. of high-quality reads | 90,280,120 | 86,015,875 | 98,406,728 | 100,327,204 | 94,045,683 |
| Total no. of reads | 90,280,120 | 86,015,875 | 98,406,728 | 100,327,204 | 94,045,683 |
| Uniquely mapped reads [b] | 70,309,618 | 45,547,725 | 75,753,713 | 68,994,581 | 67,063,490 |

### Table 2 RNA-seq statistics

[a] Exon regions of Tribolium castaneum genome
[b] Reads considered for downstream gene expression analysis

The table is showing a summary of read statistics after Illumina Hi-seq 4000 sequencing. Total read counts for three biological replicates from each treatment varies within 64,000,000 to 101,000,000. Number of high quality reads indicates the number of reads left after read trimming and filtering (Quality control step). Percentage of high quality reads unambiguously mapped to reference Tribolium genome represented in the second last row. Uniquely mapped reads were considered for differential gene expression analysis. Further details of RNA-seq statistics per sample are given in Additional file 1: Table S2.

After statistical analysis, 32 and 699 genes were upregulated after JH (MD vs. MJ) or TSA (MD vs. MT) treatment of control cells exposed to dsmaE. Similarly, 181 and 602 genes were significantly upregulated in TcA cells exposed to dsCBP followed by treatment with JH (MD vs. CJ) or TSA (MD vs. CT). Also, CBP knockout
alone caused down-regulation of 456 genes in DMSO treated cells compared to control (MD vs. CD). Details on Blast2GO hits in the NR databases are included in Additional files 2, 3, 4, 5 and 6: Excel Files S1-S5. The expression of 15 selected genes from all the treatments was verified by RT-qPCR (Fig. 3). Comparison of the gene expression levels between RT-qPCR and RNA-seq study showed a correlation coefficient of 0.74 (Additional file 1: Figure S1) indicating a good agreement of gene expression data obtained by both methods.

**JH response genes in TcA cells**

JH III treatment of TcA cells (dsmaE+ JHIII or MJ) caused an increase in expression of known JH response genes including *Kr-h1* (Fig. 4a), a mediator of anti-metamorphic action of JH [44–47] when compared to control cells treated with DMSO (MD or dsmaE+ DMSO). A decrease in JH titers at the end of final instar larval stage reduces the *Kr-h1* mRNA levels that relax the inhibitory influence on metamorphic changes and leads to the onset of metamorphosis through an increase in expression of genes such as *Broad* and *E93* required for pupal and adult development respectively [8, 48]. In *T. castaneum*, RNAi-mediated depletion of *Kr-h1* expression led to precocious pupation [45]. Besides *Kr-h1*, there were 31 other genes whose expression increased after JH treatment of TcA cells. Among them, eight genes including *ankyrin 3*, *Vitellogenin* (*Vg*), *matrix metalloproteinase-3* (*Mmp-3*), *neurexin-1* and *alpha-2C adrenergic* receptor showed expression pattern similar to *Kr-h1* in different comparisons (Fig. 4b). Ankyrin or ankyrin repeat containing proteins are involved in signal transduction processes in *D. melanogaster* and play a crucial role in the development or initiation of the immune response in eukaryotic cells [49–51]. Induction of ankyrin-3 gene by JH suggests its function in the regulation of gene expression in response to the immune challenge as reported previously [52]. Induction of Vg (a protein made by female insects to produce yolk) by JH in TcA cells support our previous finding on the regulation of *Vg* gene in *T. castaneum* by JH through insulin-like peptide signaling pathway [53]. In other insects including *B. germanica*, ectopic application of JH caused re-expression of Vg in JH-deficient female adults [54]. Similarly, in *Locusta migratoria*, induction of Vg was observed after methoprene (JH analog) injection [55]. Furthermore, JH also induced *Mmp-3*, and it showed a similar expression profile as *Kr-h1* after various treatments. In *T. castaneum*, three Mmps, i.e., *Mmp1*, *Mmp2*, and *Mmp3* were identified [56, 57]. RNAi mediated knockdown of *Mmp1* caused an arrest in the initial stage of pupation.
resulting in the altered development of wings, legs, antennae, compound eye, etc. Whereas, Mmp2 knockdown resulted in abnormal gut development during beetle embryogenesis [58]. However, RNAi mediated knockdown of Mmp3 did not show any abnormality although the expression of Mmp3 was highly upregulated during metamorphosis. Hence, it was postulated that both Mmp2 and Mmp3 had overlapping functions. Moreover, JH also induced an \( \alpha 2C \) adrenergic receptor, a G-protein coupled receptor (GPCR) in TcA cells. Interaction of GPCR with JH had been reported in other insects [9, 59]. Our previous studies in \( T. castaneum \) screened multiple candidate GPCRs that may be involved in Vg uptake into oocytes and identified promising candidate membrane receptor mediating JH regulation of patency [11]. However, the contribution of intracellular and membrane receptors in JH action need to be investigated further.

**TSA-response genes**

Trichostatin A (TSA) is a class I and II histone deacetylase (HDACs) inhibitor. It is known that HDACs mediated epigenetic mechanisms regulate various physiological processes [60]. In the present study, TSA application resulted in up-regulation of approximately 699 genes in dsmaE treated cells (MT or dsmaE + TSA) when compared to control cells treated with DMSO (MD or dsmaE + DMSO) (Fig. 5a, Additional file 3: Excel File S2). 22% of these genes contained “epifactor” domains (Fig. 5b) that are detected most frequently in proteins with capabilities of modifying histones and thus involved in chromatin remodeling [61]. Epifactor domains, observed predominantly within the gene set induced by TSA are shown in Fig. 5c. Some of these domains are well known due to their involvement in various cellular processes. For example, the SET domain proteins are involved in DNA methylation [62], and the Jmj (Jumonji) domain proteins are involved in histone demethylation [63]. It is known that ‘eggless’ family of SET domains fused to the TAM/MBD domain play a distinct role in epigenetic regulation through DNA and histone methylation in \( Acyrthosiphon pisum \) [64]. In MD vs. MT comparison, we identified nine SET domain-containing genes that are induced by TSA.

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**Fig. 2** Gene expression comparisons in TcA cells exposed to dsmaE or dsCBP and treated with JH III and TSA using the empirical analysis of DGE (EDGE) algorithm in CLC Genomics Workbench (Version: 9.5.9). (a-e) Heatmaps showing the overall comparison between DMSO and JH III treated cells exposed to dsmaE (MD vs. MJ), DMSO treated cells exposed to dsmaE or dsCBP (MC vs. CD), DMSO or JH III treated cells exposed to dsmaE or dsCBP (MD vs. CJ), DMSO or TSA treated cells exposed to dsmaE or dsCBP (MD vs. CT), and DMSO or TSA treated cells exposed to dsmaE (MD vs. MT) (Table 1). The dsmaE plus DMSO treated cells act as a control. The color spectrum, stretching from yellow to blue, represents TMM (trimmed mean of M values) normalized expression values obtained after EDGE analysis. (f-j) Volcano plots of expression data after EDGE analysis. The red dots indicate the number of significantly up- and down-regulated genes using \( p < 0.01 \) and ±2-fold change as the cut-off threshold corresponding to each comparison.
Moreover, genes coding for polycomb group protein Psc and trithorax that maintain active or repressed gene expression states in a cell by regulating chromatin architecture at multiple levels ranging from local structural modifications to the three-dimensional organization within the genome were also induced by TSA treatment. Indeed, most prominent epigenetic regulatory systems use these well-conserved groups of proteins to function antagonistically for orchestrating the expression of key genes during development [65].

Transcription factors are also important regulators of gene expression. In *D. melanogaster*, transcription factors containing helix-loop-helix, C2H2 Zn-finger or forkhead DNA-binding domain play essential roles in developmental
processes such as body plan determination and cell fate specification [66]. Interestingly, many genes coding for DNA-binding domain containing transcription factors were induced by TSA (Fig. 5c, Additional file 3: Excel File S2). A list of TSA response genes after various treatments is shown in Additional file 1: Figure S2. Interestingly, the expression levels of these genes decreased after CBP knockdown suggesting that acetylation levels maintained by HATs (CBP) and HDAC inhibitors (TSA) regulate the expression. Gene coding for Forkhead box protein O (FoxO), a transcription factor that plays important roles in the regulation of metabolism, development, and organogenesis through either activation or repression of the target gene expression, was induced by TSA (Additional file 3: Excel File S2). FoxO regulates lipolysis in fat body cells by regulating the expression of lipases (i.e., brummer, acid lipase-1) during molting and pupation in Bombyx mori [67]. Recently, Zeng et al., (2017) [68] documented the role of FoxO transcription factor in regulating JH degradation in Bombyx mori. However, the function of FoxO could vary among different insect orders. In D. melanogaster (Diptera), delayed metamorphosis was caused by overexpression of FoxO [69] whereas, RNAi of FoxO caused delayed pupation in T. castaneum (Coleoptera) [70]. FoxO also regulates the timing of pupation through regulating ecdysone biosynthesis in T. castaneum, [70]. Our previous studies also documented the regulation of Vg gene expression by FoxO and JH in T. castaneum [71].

Genes coding for nuclear receptors (NRs) including Ftz transcription factor 1 (Ftz-f1), Hormone receptor 51 (HR51), Seven-up (SVP) and Hormone receptor 38 (HR38) were induced by TSA. The NRs play a fundamental role in embryonic development and metamorphosis [72–75]. For example, expression of NRs, such as E75, HR3, and Ftz-f1 is crucial for the development of D. melanogaster embryos [76]. The NRs also serve as the early response genes in the 20E regulation of molting and metamorphosis in D. melanogaster [77]. During postembryonic development, the Ftz-f1 expression is required for successful molting and metamorphosis [78, 79]. Specifically, βFtz-f1 acts as a competence factor for stage-specific responses to 20E pulse that initiates the pupal molt [80]. Similarly, D. melanogaster HR38 regulates metamorphosis [81]. Although HR38 expression was not directly under the control of 20E, it participates in the 20E signaling pathway as an alternative partner of ultraspiracle (USP) [82]. Our previous RNAi-based studies demonstrated the role of TcFtz-f1 and TcHR51 during larval-pupal metamorphosis. Precisely, 100% mortality before pupation was observed when dsTcFtz-f1 and dsTcHR51 were injected into one-day old final instar T. castaneum larvae [83]. Likewise, TcSVP and TcHR38 play crucial roles in both larval-pupal and pupal-adult metamorphosis as TcSVP, and TcHR38 RNAi larvae died either during larval-pupal transition or pupal-adult transition [83]. A similar RNAi-based study from our lab further disclosed the
importance of TcSVP in reproduction, as indicated by the observation that newly eclosed *T. castaneum* females injected with dsTcSVP caused a decrease in TcVg transcript levels and egg production [84]. Recently, the contribution of SVP and Ftz-f1 in JH biosynthesis was also documented in *B. germanica* [85].

Interestingly, JH signal transduction pathway genes including SRC and Kr-h1 were also induced by TSA. However, SRC induction by TSA was not confirmed by RT-qPCR (Fig. 3). Kr-h1, a mediator of JH action during metamorphosis in most of the insects, was induced TSA. Remarkably, knockdown of CBP suppressed TSA induction of Kr-h1 (Additional file 3: Excel File S2). The expression of Met gene was unchanged by TSA suggesting some degree of selectivity (Fig. 3). The present study also revealed that TSA induces fatty acid synthase (FAS) expression. The contribution of FAS to lipid accumulation during diapause preparation in insects has been reported. Recently, Tan et al., [86] found that fatty acid synthase 2 contributed explicitly to lipid accumulation and stress tolerance gene expression in the beetle, *Colaphellus bowringii*. Furthermore, the induction of calcium channel flower (LOC658279), calcium-transporting ATPase type 2C member 1 (LOC658547), calpain-C (LOC662931), etc. after TSA application suggested the possibility of regulation of gene expression through altering cellular Ca²⁺ homeostasis. However, such possibilities need to be confirmed.

The widespread changes in gene expression after TSA treatment was also shown in the pathway enrichment analysis of 699 genes induced by TSA. Some metabolic pathways including MAPK, Hippo, FoxO, and Wnt signaling and fatty acid metabolism, Longevity regulating pathways and TGFβ signaling pathway were also enriched after TSA treatment (Additional file 1: Figure S3A). Among them, the TGF-β signaling pathway and FoxO signaling were shown to regulate insect metamorphosis [70, 87]. Thus TSA (alternatively HDACs) may indirectly involve in hormonal regulation during metamorphosis in *T. castaneum*.  

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**Fig. 5** Effect of TSA application on gene expression. (a) Heatmap illustrating the TMM normalized expression values of 699 up-regulated genes (p < 0.01, ≥ 2-fold↑) after TSA treatment in TcA cells. (b) Figure showing the percent containing epifactor domains within 699 genes. (c) Epifactor domains present in TSA induced genes. [MD: dsmaE treated cells exposed to DMSO; MT: dsmaE treated cells exposed to TSA]
CBP regulates genes involved in the development and hormone action

CBP is a transcriptional co-activator with HAT activity regulating the activity of hundreds of transcription factors. During postembryonic development, Nejire (a homolog of CBP), contributes to the ecdysone signaling pathway. Nejire regulates the expression of sox1A, an ecdysone-response gene through acetylation of H3K27 [88]. Our previous in vivo studies in T. castaneum larvae indicated the role of CBP in insect postembryonic development [89]. However, due to interference from endogenous hormones, we did not obtain a satisfactory picture of the impact of CBP RNAi on epigenetic regulation of JH action. In the current study, we aimed to get a more comprehensive picture using JH and TSA treatments after CBP RNAi (Table 1). CBP knockdown (CD or dsCBP + DMSO) caused down-regulation of 456 genes in TcA cells compared to control cells exposed to dsmaLE (MD or dsmaLE + DMSO) (Fig. 6a, Additional file 4: Excel S3). Nearly one third (approximately 147) of the 456 genes were also found to be down-regulated in the CBP dsRNA injected T. castaneum larvae [89] (Fig. 6b, Additional file 7: Excel S6). Among these 147 genes, many of them such as FoxO, fatty acid synthase, hairy (H), insulin receptor (InR), Laminin subunit alpha (Lana), adenosine deaminase, bambi, eyegone, etc. are known to be involved in hormonal regulation of development and metamorphosis. The contributions of FoxO and fatty acid synthase in hormone biosynthesis (20E) in the regulation of metamorphosis, reproduction, and lipid biosynthesis during diapause preparation were already discussed in the previous section. Among the other genes, JH-response gene hairy (H) acts downstream to the Met in the JH action cascade in Aedes aegypti [90].

Insulin-like growth factor (IGF) signaling plays a vital role in insect growth, development and metabolism by controlling glucose uptake and protein translation. In T. castaneum, the two InRs play distinct roles during development and reproduction. Thus, down-regulation of InR by CBP knockdown could affect those processes, but such an assumption needs to be experimentally confirmed. LanaA was another essential gene whose expression was affected by depletion of CBP both in larvae and in TcA cells. Loss of function mutations of lanaA gene in D. melanogaster caused embryonic lethality, whereas individuals with holomorphic alleles in specific combination gave rise to escaper adults with reduced viability and tissue defects demonstrating the contribution of Lana during D. melanogaster development [91]. Adenosine deaminase CECR1-A (Cat Eye Critical Region 1) expression was also affected by CBP knockdown. In D. melanogaster, expression of adenosine deaminases increases during late larval stage suggesting that it may function during metamorphosis. The enzyme, adenosine deaminase may protect the tissues from toxic nucleosides released from cellular apoptosis during metamorphosis by transforming them into their respective inosine nucleosides and thus helping in tissue reconstruction during metamorphosis [92]. Reduced expression of these physiologically important genes by CBP RNAi could induce mortality during metamorphosis as reported recently [89].

Transcription factors such as mad2 and bambi were also down-regulated in cells exposed to dsCBP. Bambi, a Bone Morphogenetic Protein (BMP) inhibitor present in T. castaneum andApis genotypes but not in Drosophila, was reported to be expressed in regions with high BMP signaling indicating its role in modulating TGFβ signaling [93]. In the dipteran model insect, D. melanogaster, TGF-β signaling induced JH production through up-regulation of JH acid methyltransferase (JHAMT) gene transcription through transcription factor Mothers Against Dpp (MAD) [94]. These data suggest that CBP could modulate the TGFβ signaling and JH biosynthesis by controlling the expression of bambi and JHAMT (via mad2). Similarly, the expression of Pax transcription factor gene eyegone (eyg) was also affected by depletion of CBP. In T. castaneum, eyegone is essential for normal development of hind wings, antennae, and eyes [95].

Some other key functional genes are affected in TcA cells after CBP RNAi treatment. For instance, a nuclear receptor, HR38, was down-regulated after CBP RNAi in these cells. Besides H38, the expression of other essential genes such as hexokinase 2 (HK2), Takeout, H17 and different cytochromes (i.e., Cyt18A1, Cyt303A1, Cyp4q6, Cyp4C1, etc.) that are known to be involved in various physiological function in insects, were affected by CBP RNAi. HK2 was one of the key components in insect chitin biosynthesis pathway that contributes to insect growth and development [96, 97]. In T. castaneum, RNAi of trehalose-6-phosphate synthase (TcTPS) significantly reduced the expression of HK2, and the larvae could not complete the transition from larvae to pupae due to a lower level of chitin and resulted in abnormal phenotypes [98]. Hence, down-regulation of HK2 could also lead to abnormal molting phenotype by negatively influencing chitin synthesis in T. castaneum as observed in our recent study where CBP was knocked down in T. castaneum larvae [89]. Similarly, the expression of take-out gene, reported to be regulated by JH [99], was also affected by CBP RNAi. Interestingly, in N. lugens, the expression of the takeout gene increased after knockdown of Met and Kr-h1 genes and decreased by RNAi of Met interacting partners such as NItTai and NIβ-Ftz-f1 [99]. Although the depletion of CBP has no direct effect on Met expression pattern, the indirect involvement of CBP via takeout in JH signaling pathway mediated by Met and other Met interacting partners is possible but needs to be experimentally validated.
Two genes, H17 and farnesol dehydrogenase, which play antagonistic roles in JH biosynthesis, were also affected by CBP RNAi. H17, a mimic of the allatostatin A, inhibits JH biosynthesis in *B. germanica* and thus acts as an insect growth regulator (IGR) [100]. The influence of allatostatin A on vitellogenesis was also documented in *B. germanica* [101]. CBP RNAi also affected farnesol dehydrogenase expression. Oxidation of NADP-dependent farnesol dehydrogenase is a rate-limiting step in JH biosynthesis pathway in adult mosquitoes [102]. The decrease in farnesol dehydrogenase expression could lead to reduced endogenous JH synthesis. Hence, CBP may also influence JH production in insects. Another gene family, Cytochrome P450s (CYPs), perform vital physiological functions during all life stages of an insect through their involvement in the biosynthesis of 20E and JH [103, 104]. The expression of different cytochrome P450 family members was downregulated by CBP RNAi. For example, the expression of CYP18A1 that is essential for *D. melanogaster* growth and development was reduced after CBP RNAi [105]. Some of the genes from a CYP4 family that is known to be involved in the metabolism of JH III were also downregulated by CBP knockdown [106]. Furthermore, RNAi of CBP decreased the expression of transcripts such as calcium-activated chloride channel regulator 4 (LOC662351), sodium/potassium-transporting ATPase subunit alpha (LOC663833) and calcium release-activated calcium channel protein 1 (LOC657890).
possibly suggesting a role in the maintenance of cellular Ca^{2+} homeostasis. However, additional experiments are needed to test this hypothesis.

Finally, Pathway enrichment analysis of the 456 downregulated genes revealed the signaling pathways regulated by CBP (Additional file 1: Figure S3 B). There were many pathways, related to insect growth, immunity and development, affected by CBP RNAi. For instance, FoxO signaling, fatty acid metabolism, Insect hormone biosynthesis, Toll and IMD pathway, TGF-β, and Hedgehog signaling pathways were among the pathways enriched >1.5 times in CBP RNAi samples. Functionally, FoxO signaling, insect hormone biosynthesis, and TGF-β signaling are involved in insect hormone biosynthesis; reproduction and metamorphosis, Toll and IMD pathway, TGF-β, and Hedgehog signaling pathways were among the pathways involved in adult appendages patterning [87, 107–111].

Functional correlation between CBP RNAi and TSA treatment
Both CBP and TSA work as epigenetic modulators of gene expression through their different roles as writer and eraser of acetylation. The expression of some genes was regulated by both CBP and TSA. We identified 35 genes that were regulated by both CBP and TSA (Fig. 6b, Additional file 7: Excel S6). The application of TSA after CBP knockdown could not rescue the expression of these genes. Importantly, FoxO and HR38 that are known to mediate JH and 20E signaling respectively were affected by both CBP and TSA. These data suggest that acetylation (by HATs, e.g., CBP) and deacetylation (HDACs, TSA studies) could regulate expression of key players involved in JH and 20E action thus adding an extra layer of epigenetic regulation to hormone action (Fig. 6c, Additional file 7: Excel File S6). Pathway enrichment analysis has also revealed the regulation of common pathways by both CBP and TSA. Pathways such as TGF-β, FoxO, and Wnt signaling, ECM receptor interaction and fatty acid biosynthesis were regulated by both CBP and TSA in TcA cells. Interestingly, comparing the CBP RNAi in T. castaneum in larvae [89] and TSA treatment of TcA cells, we found 148 genes that were affected by both these treatments. Among them, Kr-h1, svp, Fz2, polycomb, lanA, FoxO and BMP receptor type-1B were shown to regulate insect development and metamorphosis (Additional file 7: Excel S6). Moreover, ~22 genes including FoxO and lanA responded to CBP RNAi in larvae and TcA cells as well as TSA treatment in TcA cells (Fig. 6b and c; Additional file 7: excel S6). The expression levels of FoxO was further validated by RT-qPCR (Additional file 1: Figure S4). The function of FoxO in T. castaneum growth, reproduction and metamorphosis has been reported previously, but the report on epigenetic regulation of FoxO through CBP and HDACs is one of the major contributions of this paper. However, the effect of epigenetic regulation of FoxO on hormone action requires further studies.

Conclusions
JH and 20E play vital roles in insect life by orchestrating the molting and metamorphosis processes. Synthesis of these hormones and their spatiotemporal action are indispensable for successful molting and metamorphosis. Such level of precision in the synthesis of these hormones and expression of their downstream response genes during different life stages of insects indeed demands a higher degree of transcriptional as well as translational regulation. The present study shows the function of CBP and HDAC in regulating JH hormone action and corroborates our previous findings on T. castaneum larvae with only CBP RNAi treatment. The impact of dsCBP and TSA on the expression of the JH response gene, Kr-h1, was confirmed. The expression of Vg gene was also affected by dsCBP to such an extent that the expression level did not recover by further JH treatment. The expression of other key developmental genes such as bambi, eyegone, FoxO, lanA, svp, polycomb, Fz-2, HR38 and hairy was also influenced by the CBP RNAi and/or HDAC inhibition. Many key physiological pathways involved in insect growth and development such as TGF-β and FoxO signaling were modulated by both treatments. Thus, CBP and HDAC may contribute significantly to the hormonal regulation of T. castaneum metamorphosis. Interestingly, FoxO gene expression was significantly altered after CBP RNAi or TSA treatment confirming the epigenetic regulation of this important transcription factor. Further studies based on chip sequencing and functional proteomics will help to elucidate the precise mechanisms involved in epigenetic regulation of JH action in insects.

Additional files

Additional file 1: Table S1. List of primers. Table S2. Detailed RNA seq statistics. Table is showing a summary of read statistics after Illumina Hi-seq 4000 sequencing for individual biological replicates in different treatments. Figure S1. Correlation of gene expression levels of selected genes (15) by comparing both RT-qPCR and RNA-seq data. Individual log fold changes obtained by RT-qPCR and RNA-seq for each gene in the sample group. Figure S2. Relative expression of the epi-factor domain-containing genes in MD vs. MT, MD vs. CD and MD vs. CT comparisons. Heatmaps are illustrating the relative expression of genes with the epi-factor domain, in all three treatments (MT, CD, and CT). Figure S3. Pathway enrichment analysis. A) Pathways enriched after TSA induction. B) Pathway affected due to CBP RNAi. Here, the negative impact is equivalent to the enrichment. Figure S4. Relative expression of FoxO transcription factor after different treatments in TcA cells and CBP RNAi in larvae. The data shown are the mean ± S.E. (n = 4). (Letters represent significance at 95% CI). (PDF 9301 kb)

Additional file 2: Excel S1. Excel sheet-containing details of 32 genes induced by JH III (MD vs. MJ) and their relative expression in all other treatments compared to control. Genes behaving like Kr-h1 showed separately in a second excel sheet. (XLSX 23 kb)
Additional file 3: Excel S2. Excel sheet containing details of 699 genes induced by TSA (MD vs. MT) and their relative expression in other treatments of interest such as MD vs. CD and MD vs. CT. Genes with epifactor domains showed separately in a second excel sheet. Expression of Met and SRC in all three treatments showed in another excel sheet. (XLSX 133 kb)

Additional file 4: Excel S3. Excel sheet containing details of 456 genes suppressed by CBP RNAi (MD vs. CD) and their relative expression in all other comparisons such as MD vs. CT, MD vs. MJ, MD vs. CJ and MD vs. MT. (XLS 89 kb)

Additional file 5: Excel S4. Excel sheet containing details of 181 genes responded after CBP RNAi and subsequent TSA treatment. (XLS 132 kb)

Additional file 6: Excel S5. Excel sheet containing details of 602 genes responded after CBP RNAi and subsequent TSA treatment. (XLS 132 kb)

Additional file 7: Excel S6. Excel sheet containing details of gene clusters obtained after Venn diagram analysis shown in main Fig. 6b. (XLSX 43 kb)

Abbreviations
20E: 20-hydroxyecdysone; CD: dsCBP treated cells exposed to DMSO; CJ: dsCBP treated cells exposed to JH III; CT: dsCBP treated cells exposed to TSA; dscBP: dsRNA targeting a fragment of CREB-binding protein; dsmalE: dsRNA targeting a fragment of E. coli maltase gene; EcR: Ecdysone receptor; FOXO: Forkhead transcription factor; GPCR: G protein-coupled receptor; HATs: Histone acetyltransferases; HDACs: Histone deacetylases; JH: Juvenile Hormone; Kr-h1: Krüppel homolog 1; MD: dsmalE treated cells exposed to DMSO; Met: Methoprene-tolerant; MJ: dsmalE treated cells exposed to JH III; Mmp-3: Matrix metalloproteinase-3; MT: DSMAL treated cells exposed to TSA; nACHr9α: Nicotinic acetylcholine receptor alpha9 subunit; SRC: Steroid receptor coactivator; TCA cells: Tribolium castaneum cells; TSA: Trichostatin A

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Availability of data and materials
We have deposited the short read (Illumina HiSeq4000) data with the following accession number: PRJNA392465 [EBI short read archive (SRA)], SRA Ids - SRR5787268 to SRR5787285. The complete study can also be accessed directly at the following URL: http://www.ebi.ac.uk/ena/data/view/PRJNA392465.

Authors' contributions
AR and SRP designed research, AR conducted the experiments, AR and SRP wrote the manuscript, and both authors approved the manuscript.

Ethics approval and consent to participate
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Not applicable.

Competing interests
The authors declare that they have no competing interests.

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