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# 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].

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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 *Blattella 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, *Gnathocerus 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 *dsmaIE* (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 [32]. 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 *dsmalE* (M) treated cells exposed to DMSO (*dsmalE*+ DMSO or MD), JH III (*dsmalE*+ JHIII or MJ) or TSA (*dsmalE*+ TSA or MT) and dsCBP (C) treated cells exposed to DMSO (*dsCBP*+ DMSO or CD), JH III (*dsCBP*+ JHIII or CJ) or TSA (*dsCBP*+ TSA or CT). Three biological replicates were used for each treatment. The 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 SMARTScribe 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™ PCR beads (MAGBIO) and finally amplified using PCR. Libraries

**Table 1** RNA-seq study design

No	Comparisons	Abbreviation
1	DMSO and JH treated cells exposed to <i>dsmalE</i>	MD vs MJ
2	DMSO treated cells exposed to <i>dsmalE</i> or <i>dsCBP</i>	MD vs CD
3	DMSO or JH treated cells exposed to <i>dsmalE</i> or <i>dsCBP</i>	MD vs CJ
4	DMSO or TSA treated cells exposed to <i>dsmalE</i> or <i>dsCBP</i>	MD vs CT
5	DMSO or TSA treated cell exposed to <i>dsmalE</i>	MD vs MT

The table shows a list of comparisons made after RNA sequencing. All the treatments are compared with the control (MD)

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 Log<sub>10</sub> 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 "Blasto2GO 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](http://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

**Table 2** RNA-seq statistics

Samples	dsmaIE			dsCBP		
	MD1-MD3	MJ1-MJ3	MT1-MT3	CD1-CD3	CJ1-CJ3	CT1-CT3
Total no. of reads	90,280,120	64,052,276	86,015,875	98,406,728	100,327,204	94,045,683
No. of high-quality reads	80,437,710	57,466,708	54,928,048	86,047,960	79,067,179	79,188,207
Mapped reads to Exon <sup>a</sup>	75,27,476	53,578,082	48,830,575	81,330,901	73,271,933	71,579,430
Percent reads mapped	83.59	93.29	87.89	93.36	92.97	90.37
Uniquely mapped reads <sup>b</sup>	70,309,618	49,895,642	45,547,725	75,753,713	68,994,581	67,063,490

<sup>a</sup>Exon regions of *Tribolium castaneum* genome

<sup>b</sup>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

new set of dsmaIE, 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- $\Delta$ Ct 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 dsmaIE (dsRNA targeting *E. coli maltase* gene as control) or dsCBP followed by exposure to JH III, TSA or DMSO (Table 1).

### TcA cell response to different treatments

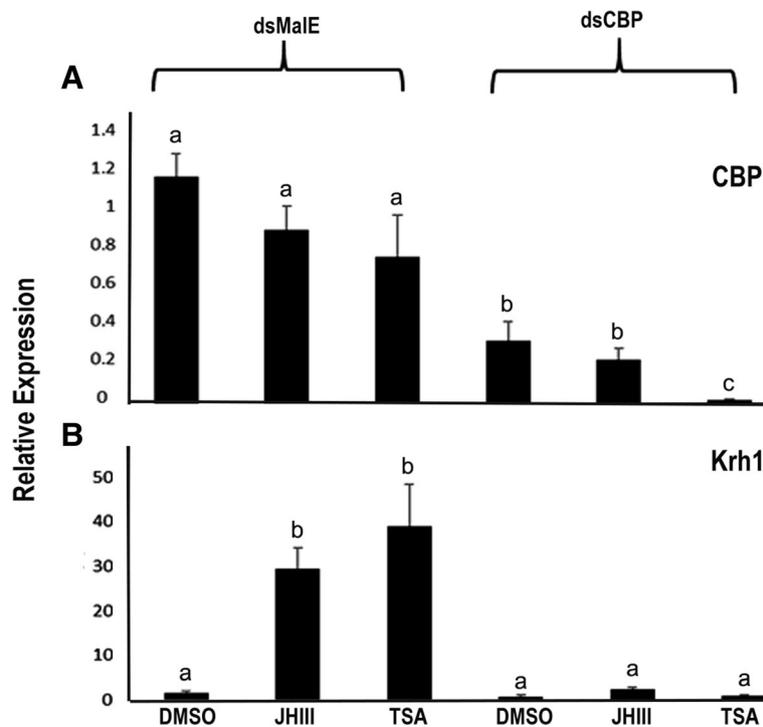
The response of TcA cells to different treatments [MD (dsmaIE+ DMSO), MJ (dsmaIE+ JHIII), MT (dsmaIE+ TSA), CD (dsCBP+ DMSO), CJ (dsCBP+ JHIII), and CT(dsCBP+ TSA)] was determined before the sequencing experiments by quantifying the expression of CBP and *Kr-h1* genes in all the treatments using RT-qPCR method [33]. Results showed more than 50% reduction of CBP mRNA levels after dsCBP treatment. The *Kr-h1* mRNA levels in control cells exposed to dsmaIE increased after JH or TSA treatment when compared to

their levels in DMSO treated cells. However, there was no increase in *Kr-h1* mRNA in cells exposed to dsCBP 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 dsmaIE 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 (dsmaIE+ DMSO), MJ (dsmaIE+ JHIII), MT (dsmaIE+ 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 for each biological replicate are included in Additional file 1: Table S2. After RPKM based gene expression normalization, differential gene expression (DGE) analysis was performed using EDGE analysis software package of CLC workbench, and the overall gene expression patterns for each treatment compared to the control (MD) are represented as heatmaps (Fig. 2a-e). Comparing with the control (MD), the gene expression levels in different samples was normalized in a way that made all treatments comparable to each other. Differentially expressed ( $p < 0.01$  and  $\pm 2$ -fold  $\uparrow\downarrow$ ) genes for each comparison (Table 1) are represented as red dots in the volcano plots (Fig. 2f-j).

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 dsmaIE. 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 knockdown



**Fig. 1** TcA cells respond to both juvenile hormone and TSA after dsMalE and dsCBP treatment. Total RNA was isolated from 100,000 cells that were cultured in the medium containing either dsMalE or dsCBP for 72 h. The cells were then exposed to DMSO (< 0.1%) or JH III (2  $\mu$ M) or TSA (5  $\mu$ M) for 6 h. Total RNA was isolated from the treated cells and used to quantify mRNA levels of CBP (a) and Krh1 (b). The data shown are the mean  $\pm$  S.E. ( $n = 4$ ). (Letters represent significance at 95% CI)

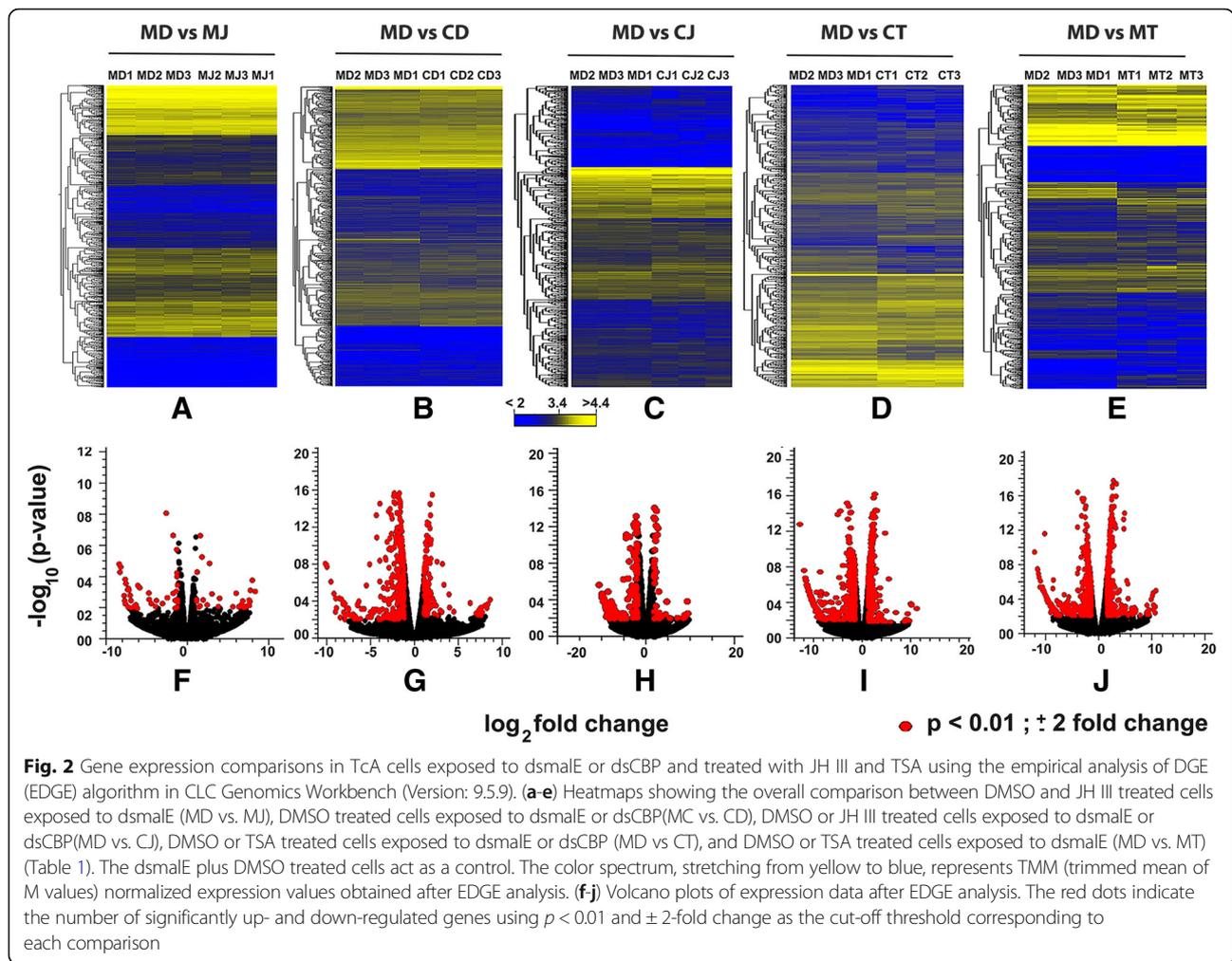
alone caused down-regulation of 456 genes in DMSO treated cells compared to control (MD vs. CD). Details on Blast2GO hits in the NR databases are included in Additional files 2, 3, 4, 5 and 6: Excel Files S1-S5. The expression of 15 selected genes from all the treatments was verified by RT-qPCR (Fig. 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 (*dsMalE*+ JHIII or MJ) caused an increase in expression of known JH response genes including *Krh1* (Fig. 4a), a mediator of anti-metamorphic action of JH [44–47] when compared to control cells treated with DMSO (MD or *dsMalE*+ DMSO). A decrease in JH titers at the end of final instar larval stage reduces the *Krh1* 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 *Krh1* expression led to precocious pupation [45]. Besides *Krh1*, 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 *Krh1* 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 *Krh1* 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

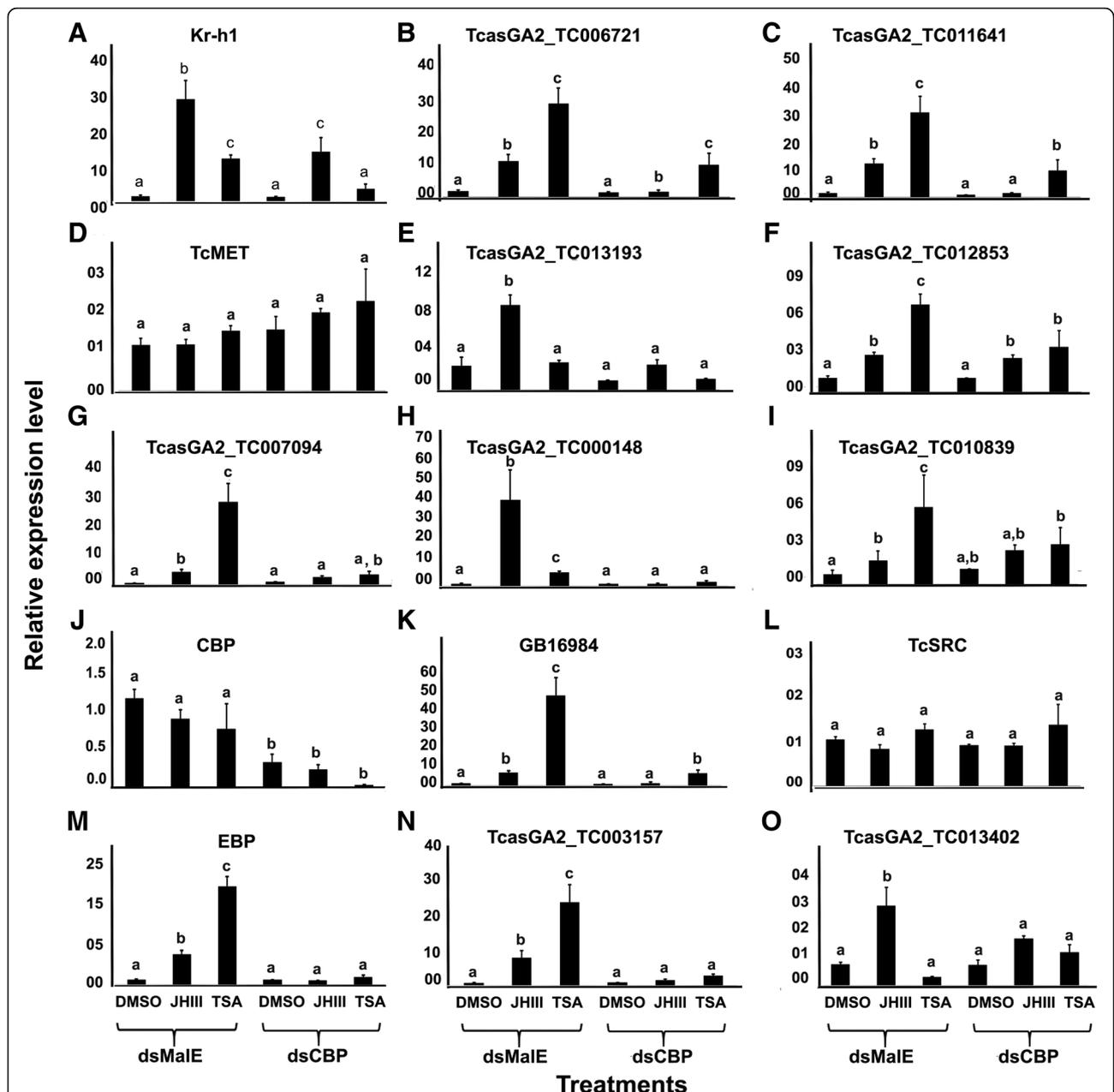


resulting in the altered development of wings, legs, antennae, compound eye, etc. Whereas, *Mmp2* knockdown resulted in abnormal gut development during beetle embryogenesis [58]. However, RNAi mediated knockdown of *Mmp3* did not show any abnormality although the expression of *Mmp3* was highly upregulated 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

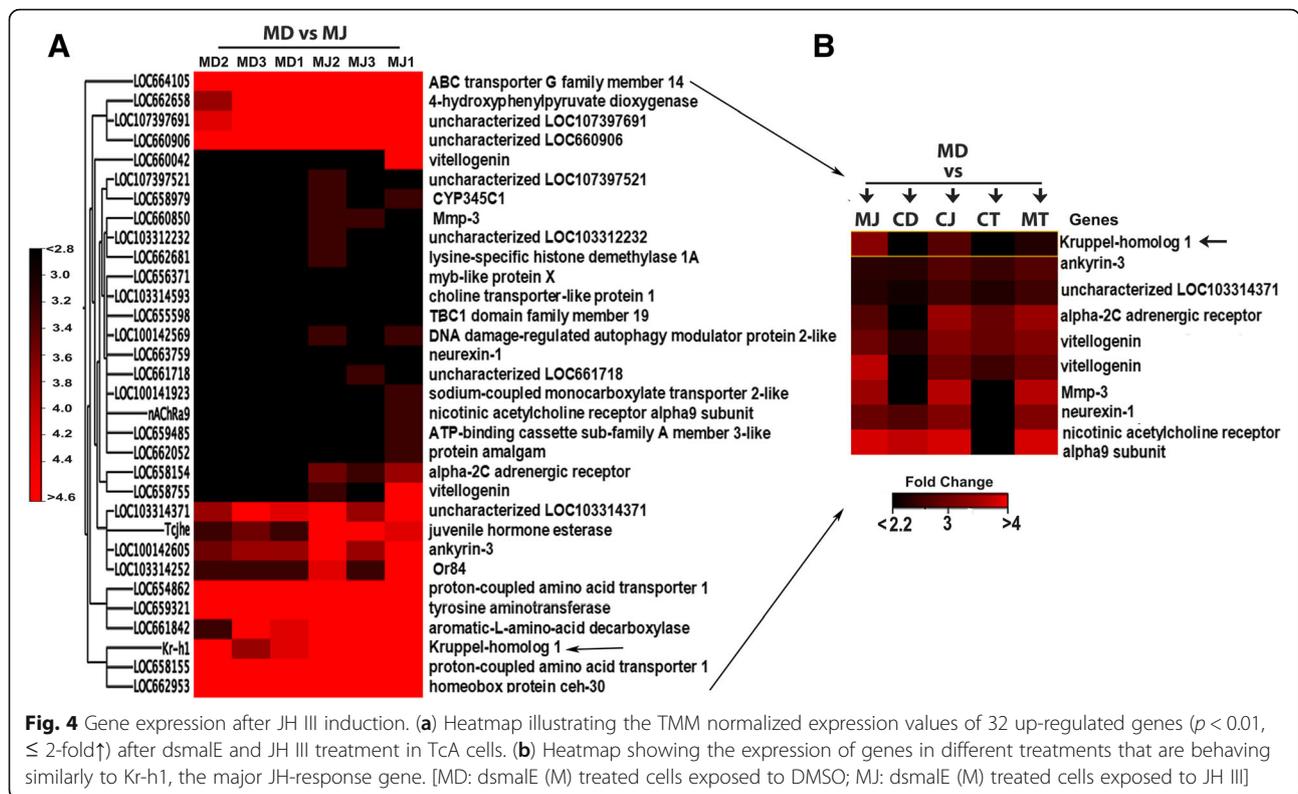
epigenetic mechanisms regulate various physiological processes [60]. In the present study, TSA application resulted in up-regulation of approximately 699 genes in *dsmaE* treated cells (MT or *dsmaE* + TSA) when compared to control cells treated with DMSO (MD or *dsmaE* + DMSO) (Fig. 5a, Additional file 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 *Acyrtosiphon pisum* [64]. In MD vs. MT comparison, we identified nine SET domain-containing genes that are induced by TSA



**Fig. 3** Relative mRNA levels of a subset of 15 genes selected from the genes that were upregulated after JH III application in different treatments. Relative mRNA levels of these genes were determined by RT-qPCR as described in Fig. 1. The data shown are the mean + S.E. (n = 4). (Letters represent significance at 95% CI). [TcasGA2\_TC006721: myb-like protein X; TcasGA2\_TC011641: alpha-2C adrenergic receptor; TcasGA2\_TC013193: juvenile hormone esterase; TcasGA2\_TC012853: Mmp-3; TcasGA2\_TC007094: TBC1 domain family member 19; TcasGA2\_TC000148: CYP345C1; TcasGA2\_TC010839: Vitellogenin; GB16984: nicotinic acetylcholine receptor alpha9 subunit (nAChRa9); TcasGA2\_TC003157: uncharacterized LOC103312232; TcasGA2\_TC013402: aromatic-L-amino-acid decarboxylase]

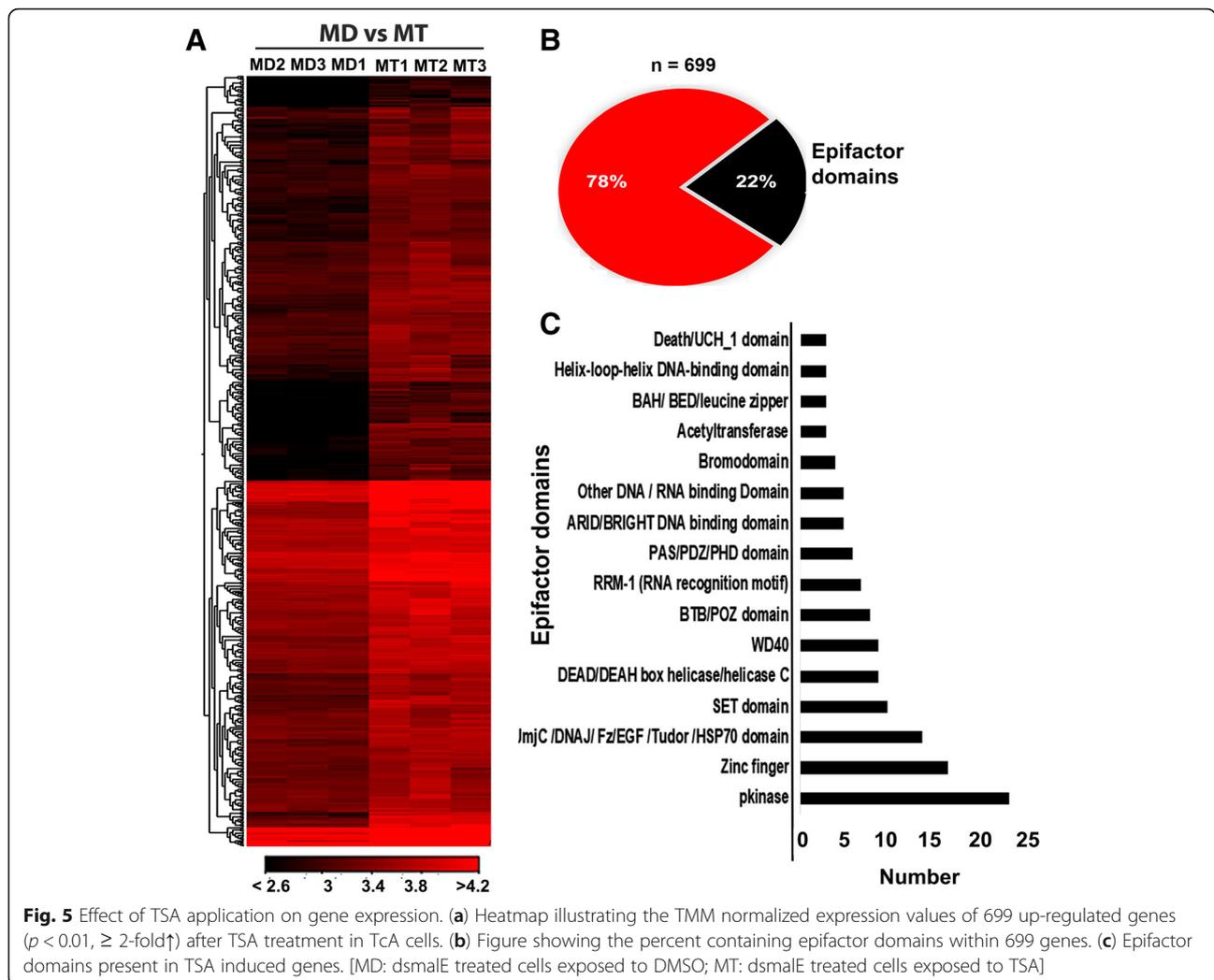
(Fig. 5c & Additional file 3: Excel File S2). Moreover, genes coding for polycomb group protein Psc and trithorax that maintain active or repressed gene expression states in a cell by regulating chromatin architecture at multiple levels ranging from local structural modifications to the three-dimensional 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, C<sub>2</sub>H<sub>2</sub> 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 over-expression of *FoxO* [69] whereas, RNAi of *FoxO* caused delayed pupation in *T. castaneum* (Coleoptera) [70]. *FoxO* also regulates the timing of pupation through regulating ecdysteroid 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,  $\beta$ *Ftz-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. bowringi*. Furthermore, the induction of *calcium channel flower (LOC6 58279)*, *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  $Ca^{2+}$  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 TGF $\beta$  signaling pathway were also enriched after TSA treatment (Additional file 1: Figure S3A). Among them, the TGF- $\beta$  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*.

### CBP regulates genes involved in the development and hormone action

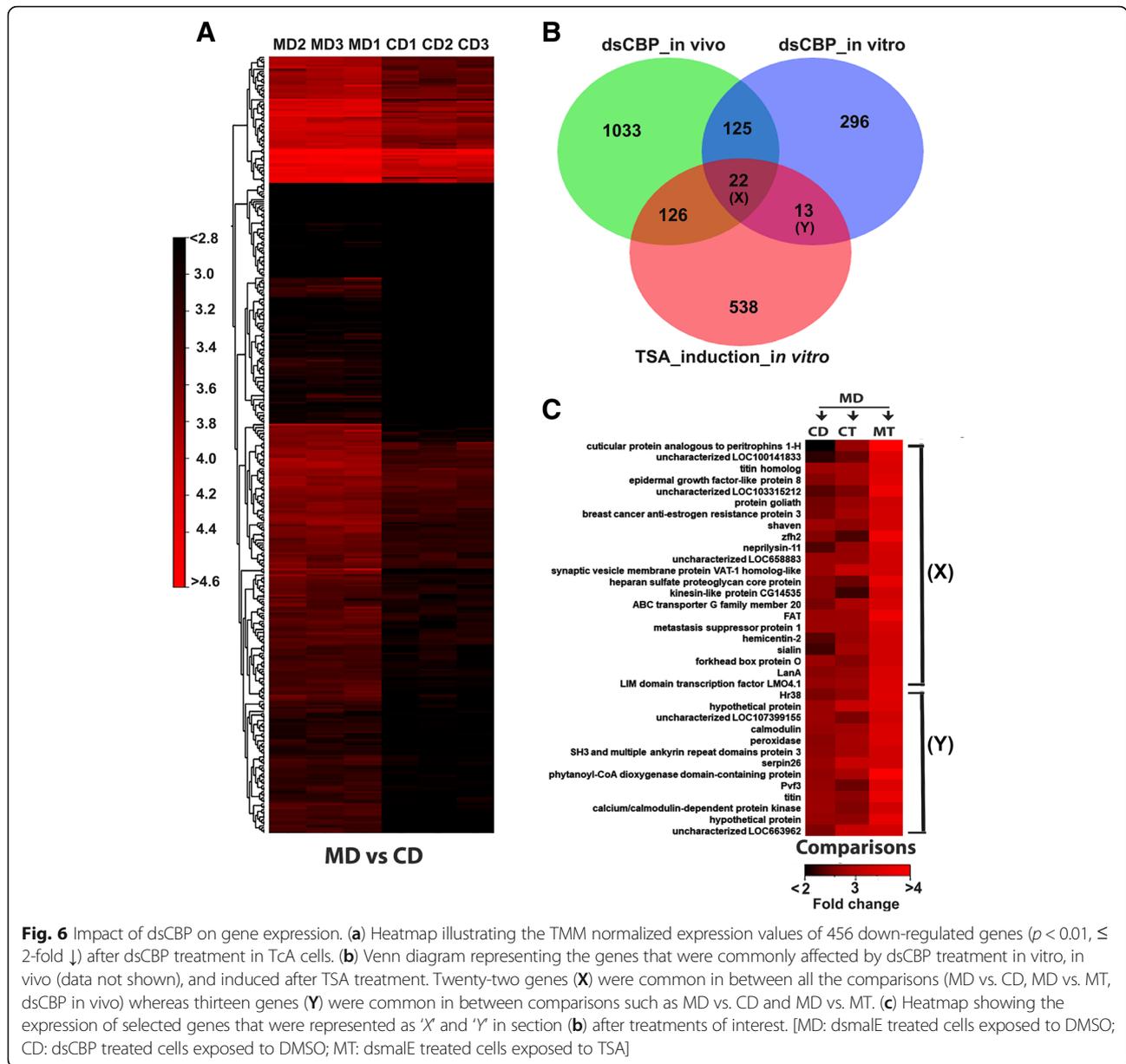
CBP is a transcriptional co-activator with HAT activity regulating the activity of hundreds of transcription factors. During postembryonic development, Nejire (a homolog of CBP), contributes to the ecdysone signaling pathway. Nejire regulates the expression of *sox14*, an ecdysone-response gene through 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. *LanA* was another essential gene whose expression was affected by depletion of *CBP* both in larvae and in TcA cells. Loss of function mutations of *lanA* 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 CECRI-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* and *Apis* genomes but not in *Drosophila*, was reported to be expressed in regions with high BMP signaling indicating its role in modulating TGF $\beta$  signaling [93]. In the dipteran model insect, *D. melanogaster*, TGF- $\beta$  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 TGF $\beta$  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 *takeout* 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 *NITai* and *NI $\beta$ -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.



**Fig. 6** Impact of dsCBP on gene expression. (a) Heatmap illustrating the TMM normalized expression values of 456 down-regulated genes ( $p < 0.01$ ,  $\leq 2$ -fold ↓) after dsCBP treatment in TcA cells. (b) Venn diagram representing the genes that were commonly affected by dsCBP treatment in vitro, in vivo (data not shown), and induced after TSA treatment. Twenty-two genes (X) were common in between all the comparisons (MD vs. CD, MD vs. MT, dsCBP in vivo) whereas thirteen genes (Y) were common in between comparisons such as MD vs. CD and MD vs. MT. (c) Heatmap showing the expression of selected genes that were represented as 'X' and 'Y' in section (b) after treatments of interest. [MD: dsmaIE treated cells exposed to DMSO; CD: dsCBP treated cells exposed to DMSO; MT: dsmaIE treated cells exposed to TSA]

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  $\text{Ca}^{2+}$  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- $\beta$ , and Hedgehog signaling pathways were among the pathways enriched > 1.5 times in *CBP* RNAi samples. Functionally, FoxO signaling, insect hormone biosynthesis, and TGF- $\beta$  signaling are involved in insect hormone biosynthesis; reproduction and metamorphosis, Toll and IMD pathway are involved in insect immunity development and hedgehog signaling pathway 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- $\beta$ , 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- $\beta$  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. (XLSX 89 kb)

**Additional file 5: Excel S4.** Excel sheet containing details of 181 genes responded after CBP RNAi and subsequent JH III treatment. (XLS 69 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; dsmaIE: 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; JHAMT: JH acid methyltransferase; JHIII: Juvenile Hormone III; Kr-h1: Krüppel homolog 1; MD: dsmaIE treated cells exposed to DMSO; Met: Methoprene-tolerant; MJ: dsmaIE treated cells exposed to JH III; Mmp-3: Matrix metalloproteinase-3; MT: dsmaIE treated cells exposed to TSA; nAChR $\alpha$ 9: 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

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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