Comparative RNA-sequencing analysis of ER-based *HSP90* functions and signal pathways in *Tribolium castaneum*

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Abstract *Tribolium castaneum*, the red flour beetle, is a major agriculture pest that damages stored grains and cereal products. Heat-shock protein 90 of *T. castaneum* (*Tchsp90*) has been reported to play pivotal roles in heat stress response, development, reproduction, and life span. However, the signaling pathway of *Tchsp90* remains unclear. Thus, the global transcriptome profiles between RNA interference (RNAi)-treated insects (ds-*Tchsp90*) and control insects of *T. castaneum* were investigated and compared by RNA sequencing. In all, we obtained 14,145,451 sequence reads, which assembled into 13,243 genes. Among these genes, 461 differentially expressed genes (DEGs) were identified between the ds-*Tchsp90* and control samples. These DEGs were classified into 44 gene ontology (GO) functional groups, including the cellular process, the response to stimulus, the immune system process, the development process, and reproduction. Interestingly, knocking down the expression of *Tchsp90* suppressed both the DNA replication and cell division signaling pathways, which most likely modulated the effects of

Tchsp90 on development, reproduction, and life span. Moreover, the DEGs encoding *AnnexinB9*, *frizzled-4*, *sno*, *Fem1B*, *TSL*, and *CSW* might be related to the regulation of the development and reproduction of ds-*Tchsp90* insects. The DEGs including *TLR6*, *PGRP2*, *defensin1*, and *defensin2* were involved in heat stress and immune response simultaneously, which suggested that cross talk might exist between immunity and stress response. Additionally, RNAi of *Tchsp90* altered large-scale *serine protease* (*sp*) gene expression patterns and amplified the SP signaling pathway to regulate the development and reproduction as well as the stress response and innate immunity in *T. castaneum*. All these results shed new light onto the regulatory mechanism of *Tchsp90* involved in insect physiology and could further facilitate research into appropriate and sustainable pest control management.

Keywords *HSP90* · RNA interference · RNA sequencing · DNA replication · Cell division · *Tribolium castaneum*

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Introduction

Heat shock proteins (HSPs) are a gene family that code for abundant molecular chaperone proteins typically expressed in response to environmental stress factors and which were originally identified in *Drosophila melanogaster* by Ritossa (Ritossa and Vonborstel 1964). The highly conserved heat shock protein 90 (HSP90), one of the most significant members of the HSP family, was discovered in all species except for *Archaea* (Large, Goldberg et al. 2009). It acted as a global cellular regulator that bound to a wide array of downstream signaling proteins and then stabilized their conformation or facilitated their conformational remodeling during normal growth conditions (Caplan, Mandal et al. 2007). This effect was further intensified and magnified when HSP90 levels were induced by different environmental stress factors (McClellan, Xia et al. 2007).

Basically, four different types of HSP90 were shown to exist in higher eukaryotes: chloroplast HSP90, mitochondrial HSP90, cytosolic HSP90, and endoplasmic reticulum-based HSP90 (ER-based HSP90) (Johnson 2012). These diverse HSP90 homologs appear to cooperate with other chaperone molecules, such as HSP70 and HSP40, to mediate protein folding and/or stability, transport, and/or assembly into multi-protein complexes (Johnson and Brown 2009; Marzec, Eletto et al. 2012). Among them, mitochondrial HSP90, cytosolic HSP90, and ER-based HSP90 were widely identified across the metazoan, whereas chloroplast HSP90 was found only in plants (Chen, Zhong et al. 2006). Mitochondrial HSP90s were specifically located in mitochondria and were evolutionarily and structurally distinct from the cytosolic HSP90 and ER-based HSP90 (Chen, Zhong et al. 2006; Johnson 2012). However, several studies have indicated that most of the HSP90s were cytosolic HSP90 and ER-based HSP90 in the insects (Chen, Zhong et al. 2006; Johnson and Brown 2009; Johnson 2012), and that they possessed the same origin and evolved separately after species differentiation (Knorr and Vilcinskas 2011; Zhang, Gu et al. 2014). The physiological roles and signaling pathways for cytosolic HSP90 have been well studied over the past decade, while relatively little is known about ER-based HSP90.

ER-based HSP90 is an endoplasmic reticulum stress protein with an ER retention signal (Johnson 2012), and acts as a ubiquitously expressed chaperone with especially high levels in secreted tissues, indicating that it is essential for the functional expression of secretory and/or membrane proteins that enable the integration of cells into tissues (Eletto, Dersh et al. 2010; Maynard, Pham et al. 2010). Usually, upregulation of ER-based HSP90 is considered a hallmark in responses to ER stress and plays a vital role in ER quality control pathways (Eletto, Dersh et al. 2010). Recent studies have suggested that ER-based HSP90 is a master chaperone for cell-surface toll-like receptors, and therefore displays the unique specialized

function to modulate the immune system by influencing both native and adaptive immune responses (Yang, Liu et al. 2007; Tramentozzi, Zamarchi et al. 2011). ER-based HSP90 is also involved in mesoderm induction and muscle development through the regulation of insulin-like growth factor secretion (Wanderling, Simen et al. 2007). Moreover, ER-based *hsp90* is required for early development in *Caenorhabditis elegans*, *Drosophila*, *Arabidopsis thaliana*, and *mouse* (Maynard, Pham et al. 2010; Marzec, Eletto et al. 2012). A lack of ER-based *hsp90* caused a development arrest at the early larval stage, without maturation of adult nematodes (Marzec, Eletto et al. 2012). The loss of function of ER-based *hsp90* also resulted in delayed development and mutant larvae have pronounced defects in the midgut epithelium of *Drosophila* (Maynard, Pham et al. 2010). Also, silencing of ER-based *hsp90* led to defects in meristem development and mesoderm formation in *Arabidopsis* and *mouse*, respectively (Marzec, Eletto et al. 2012). Therefore, ER-based *hsp90* is not only essential for support traffic of secreted or plasma membrane proteins but has many physiological functions such as ER stress, innate immunity response, and early development.

Most recently, ER-based HSP90 has also been discovered in *T. castaneum*, the red flour beetle, which is a worldwide coleopteran pest of stored grain and cereal products (Zhang, Gu et al. 2014; Perkin, Elpidina et al. 2017). The cytosolic HSP90 and ER-based HSP90 of *T. castaneum* were renamed as HSP83 and HSP90 based on their molecular weight, respectively (Knorr and Vilcinskas 2011; Zhang, Gu et al. 2014). Larval RNA interference (RNAi) of *Tchsp90* resulted in morphological deficiency with a significant lethality at the larval and pupal stages (Zhang, Gu et al. 2014), which seriously hindered development of *T. castaneum*, and this phenotype was also observed in GP93 (the orthologous of ER-based HSP90) mutated *Drosophila* (Maynard, Pham et al. 2010). Moreover, knocking down of *Tchsp90* at the pupal stage dramatically decreased the fertility of *T. castaneum* (Zhang, Gu et al. 2014). In addition, *Tchsp90* showed a positive effect on the resistance to starvation and high temperature as well as on life span regulation (Zhang, Gu et al. 2014). However, it is still unknown how *Tchsp90* exerts these functions in insect model. In order to clarify the regulatory mechanism of *Tchsp90* involved in insect physiology, RNAi and RNA-sequencing technology were jointly used in this study, and our study also sheds new lights into signaling modulated systems of relative genes in insect species.

Materials and methods

Insect strains

The *T. castaneum* GA-1 line insects were reared at 40% relative humidity in whole wheat flour with 5% yeast powder at

30 °C under standard conditions (Zhang, Gu et al. 2014; Sang, Li et al. 2016).

RNA isolation and complementary DNA synthesis

Total RNAs were isolated separately from *T. castaneum* larvae using RNAiso™ Plus (TaKaRa, Dalian, China), following the manufacturer's protocol. The ratio of absorbance (Abs) at 260 to 280 nm (A260/A280 ratio) was performed to assess the yield and purity of RNAs. The RNAs were only used when the Abs260 nm/Abs280 nm ratio was >1.8 and the RNA integrity was further evaluated by 1% agarose gel electrophoresis with an Agilent 2100 Bioanalyzer. Then, HiScript II Reverse Transcriptase (Vazyme, Nanjing, China) was employed for the first-strand complementary DNA (cDNA) synthesis with 1 µg RNAs.

Double-strand RNA synthesis and injection

For double-strand RNA (dsRNA) synthesis, primers containing gene-specific sequences (sense primer 5'-TTAA AAGAACTGCGCGACAAAGC-3', antisense primer 5'-CACAATGGAAAAGCTTGACGAATC-3') and the T7 polymerase promoter (TAATACGACTCACTATAGGG) at the 5'-end of both the sense primer and antisense primer were used to amplify double-strand DNAs (dsDNAs) of *Tchsp90*. These dsDNAs were employed for *Tchsp90* dsRNA synthesis with the TranscriptAid™ T7 High Yield Transcription Kit (Fermentas, Vilnius, Lithuania) and for the larval RNAi experiments which were performed as described previously (Li, Zhang et al. 2014). Larvae injected with *Tchsp90* dsRNAs were called ds-*Tchsp90* group insects, while larvae injected with an equal volume of physiological buffer were used as a control group.

Illumina sequencing

Total RNAs were extracted from six larvae of the ds-*Tchsp90* and control groups on the fifth day after injection as in the above description. Then, the poly(A) messenger RNA (mRNA) was enriched by using oligo(dT) magnetic beads and the mRNA was disrupted into short fragments (approximately 200 bp). Subsequently, the cDNA was synthesized using the Illumina Tru-Seq™ RNA sample preparation kit according to the manufacturer's instructions. The double-strand cDNA was purified with a QIAquick PCR Extraction Kit (Qiagen, Germany) and washed with EB buffer for end repair and single nucleotide A (adenine) addition. Ultimately, sequencing adaptors were assembled to the fragment. The required fragments were purified by agarose gel electrophoresis and enriched by PCR amplification. The library products were then sequenced via Illumina HiSeq™ 2000 platform. Raw sequence data were deposited to the NCBI Short Read Archive

(SRA) database under the accession numbers SRR3087513 (control) and SRR4454083 (ds-*Tchsp90*). Received raw image sequencing data were transformed by base calling into sequence data, which were defined as raw reads. To reduce the noise caused by sequencing errors, we filtered all the sequences to remove the adaptor sequences and low-quality sequences before data analysis.

Mapping reads to a reference genome and annotated genes

After filtering out the dirty raw reads, the clean reads were then aligned to the reference genome of *T. castaneum* (<http://www.beetlebase.org/>) using SOAPaligner/soap2 (Li, Yu et al. 2009), allowing up to two base mismatches. Failed mapped reads were progressively trimmed off, one base at a time, from the 3'-end and mapped to the genome again until a match was found (unless the read had been trimmed by <27 bases). For single-end reads, the insert between paired reads was set at 1 base–5 kb, allowing them to span introns of various sizes in the genome. The same method was used to align single-end reads to the non-redundant gene, except that the insert was changed to 1 base–1 kb.

Normalized expression levels of genes from RNA-sequencing and gene ontology analysis

ERANGE software (version 4.0) (<http://woldlab.caltech.edu/gitweb/>) was applied to calculate the normalized gene locus expression level by assigning reads to their origin sites and counting them. The expression level of a gene from the RNA sequencing was normalized by RPKM method (Reads Per Kilobase of exon model per Million mapped reads) (Wang, Wu et al. 2009), a normalized measure of read density that allows transcriptional levels to be compared both within and between samples. The cutoff value for determining the gene transcriptional activity was determined based on a 95% confidence interval for all the RPKM values for each gene.

We obtained the gene ontology (GO) terms for each *T. castaneum* gene by using Blast2GO (version 2.3.5) (<http://www.blast2go.org/>) (Conesa, Gotz et al. 2005). The GO classification was carried out using Web Gene Ontology Annotation Plot (WEGO; <http://wego.genomics.org.cn/cgi-bin/wego/index.pl>) to classify these GO terms by performing Fisher's exact test with an false discovery rate (FDR) correction in order to obtain an adjusted *P* value between certain test gene groups and the whole annotation genome (Ye, Fang et al. 2006).

Identification and annotation of differentially expressed genes between the ds-*Tchsp90* and control groups

We applied the R package, DEGseq, to identify the differentially expressed genes (DEGs) using a random sampling

model based on the read count for each gene in the ds-*Tchsp90* and control groups. We used the “robust $FDR \leq 0.001$ and the absolute value of $\log_2$ ratio ≥ 1 ” as the threshold to judge the significance of each gene expression difference. GO enrichment analysis provided all GO terms that significantly enriched in DEGs in comparison to the genome background, and filtered the DEGs that corresponded to biological functions. This method mapped all DEGs to GO terms in the database (<http://www.geneontology.org/>), calculating gene numbers for every term, and using a hypergeometric test to find significantly enriched GO terms in DEGs compared to the genome background. The calculated P value goes through Bonferroni correction, taking corrected P value ≤ 0.05 as a threshold. GO terms fulfilling this condition are defined as significantly enriched GO terms in DEGs (Ye, Fang et al. 2006).

KEGG pathway analyses of the DEGs were used by Cytoscape software (version 2.6.2) (<http://www.cytoscape.org/>) with the ClueGO plug-in (<http://www.ici.upmc.fr/cluego/cluegoDownload.shtml>). Pathway enrichment analysis identifies significantly enriched metabolic pathways or signal transduction pathways in DEGs compared with the whole-genome background. Pathways with Q value ≤ 0.05 are significantly enriched ones with DEGs compared with the whole-genome background.

Quantitative real-time PCR

The quantitative real-time PCR (qRT-PCR) was performed to validate the DEGs that were randomly selected from the RNA sequencing for three biological samples using Power SYBR Green PCR Master Mix (Roche) with StepOnePlus Real-Time PCR System (ABI, Applied Biosystems, Life Technologies) following the manufacturer's recommendations. Data expressed here as relative mRNA levels were standardized to a control gene, *T. castaneum* ribosomal protein S3 (*rps3*, GenBank accession number CB335975) (Begum, Li et al. 2009), and gene expression relative-fold was calculated with the $\Delta\Delta CT$ method (Livak and Schmittgen 2001). Primers for these genes are listed in supplementary Table S10. All data were analyzed by Student's t test for statistically significant differences ($P < 0.05$) using ANOVA program of SPSS version 13.0 and were presented as the mean $\pm$ standard error.

Results

Illumina sequencing and alignment to the reference genome

Knockdown efficiency of *Tchsp90* gene by RNAi was measured and validated before RNA sequencing in our present study. The result confirms that *Tchsp90* was depleted in the

test samples when compared to control insects (data not shown). Illumina sequencing yielded 14,145,451 sequence reads, including 7,315,113 clean reads and 6830,338 clean reads from the control and ds-*Tchsp90* of *T. castaneum* insects, respectively (Fig. S1 and Table 1). While sequence saturation analysis indicated that when the number of reads reached 1.5 million, it was sufficient for the quantitative analysis of gene expression (Fig. S2). Then, the clean reads were aligned to the reference genome database of *T. castaneum* by using SOAPaligner/soap2 software. Of the total control reads, 65.97% mapped either to a unique (63.03%) or to multiple (2.94%) genomic locations, while 62.36% of the ds-*Tchsp90* reads showed either a unique match (59.56%) or a multiple-position match (2.80%). The remaining 34.03% and 37.64% from the above two samples reads were unmapped (Table 1) because only reads aligning entirely inside exonic regions were matched.

Global analysis of gene expression

There are 12,761 and 12,494 genes that were detected in the control and ds-*Tchsp90* groups, respectively, which ranged from 81 to 63,354 bp (Fig. 1a). Based on their lengths, these genes were divided into seven categories. Approximately half of the genes were 600 to 1800 bp, an average of 31.92% of the genes ranged from 600 to 1200 bp, while 21.80% were distributed between 1200 and 1800 bp (Fig. 1a and Table S1). There were nearly equal proportions of genes in each category between the control and ds-*Tchsp90* groups. In all, these two cDNA libraries produced 13,243 genes (Table S1), which provided abundant information for the analysis of the *Tchsp90* signaling system.

In addition, RPKM values were used to normalize and quantify the gene expression. The highest number of genes' RPKM values were from 10 to 50 for both the control (5263 genes, 41.24%) and ds-*Tchsp90* (4877 genes, 39.03%) samples, while the lowest number of genes were detected when the RPKM values were more than 1000, suggesting that there is only small proportion of genes at high-expression levels (Fig. 1b and Table S2). No significant differences were observed from the analysis of the RPKM values between the control and ds-*Tchsp90* groups. For mRNA expression, heterogeneity and redundancy are two significant characteristics.

Table 1 Summary of read numbers based on the RNA-sequencing data

	Control	ds- <i>Tchsp90</i>
Total reads	7,315,113 (100%)	6,830,338 (100%)
Total mapped reads	4,825,890 (65.97%)	4,259,212 (62.36%)
Unique match	4,610,640 (63.03%)	4,068,089 (59.56%)
Multi-position match	215,250 (2.94%)	191,123 (2.80%)
Total unmapped reads	2,489,223 (34.03%)	2,571,126 (37.64%)

Fig. 1 Length distribution of the gene detected by RNA sequencing (a). The x axis and y axis indicate the length of the genes and the number of genes, respectively. RPKM statistic of the control and ds-*Tchsp90* (b).

While the majority of mRNA is expressed at low levels, a small proportion of mRNA is highly expressed. Therefore, the distribution of tag expression was performed to evaluate the normality of our RNA-sequencing data. Meanwhile, the distribution of distinct tags over different tag abundance categories showed similar patterns between the two RNA-sequencing libraries (Fig. 2). The distribution of similarity had a comparable pattern, with more than half of detected genes having a similarity of >70%, while approximately 45% of the hits had a similar range (Table S3).

Annotation of all detected genes by GO classification and KEGG analysis

Of a total of 13,243 detected genes, 6830 could be divided into 49 functional groups based on sequence homology. In the three main categories (biological process, cellular component, and molecular function), according to GO classification, there were 25, 14, and 10 functional groups, respectively (Fig. 3 and Table S4). Cellular process (GO 0009987) with 3689 genes (54.01%) was dominant in the main categories of biological

The x axis and y axis indicate the RPKM value of the genes and the number of genes, respectively

process. Cell part (GO 0044464) and binding (GO 0005488) consisting of 3183 (46.60%) and 3389 genes (49.62%) were dominant in the main category of cellular component and molecular function, respectively (Fig. 3 and Table S4). All detected beetle genes were queried against the KEGG pathway database, and 73.31% of the 13,243 genes were assigned to 181 pathways (Table S5). Those pathways with the greatest representation by unique genes were metabolic pathways (1537 genes, ko01100), neuroactive ligand-receptor interaction (416 genes, ko04080), pathways in cancer (293 genes, ko05200), ubiquitin-mediated proteolysis (289 genes, ko04120), purine metabolism (284 genes, ko00230), and regulation of actin cytoskeleton (270 genes, ko04810) (Table S5).

Changes in gene expression profiles between the ds-*Tchsp90* and control groups

To confirm the significant differences in gene expression between the ds-*Tchsp90* and control groups, a likelihood ratio test was used to compare the RPKM-derived read count. A reliable statistical analysis was applied to the genes with an

Fig. 2 Distribution of genes' coverage (control vs ds-*Tchsp90*). Percent of coverage representing the percentage of genes that expressed in each of the two libraries mapped in the red flour beetle genome. The gene

coverage is the percentage of a gene covered by the reads. This value is equal to the ratio of the base number in a gene covered by unique mapping reads to the total base number of that gene

Fig. 3 Histogram presentation of gene ontology (GO) classifications. The results are summarized in three main categories: biological process, molecular function, and cellular component. Differentially expressed

genes (DEGs) are indicated in green, and all detected genes are indicated in wine color. The y axis indicates the number of genes in a category

RPKM value ≥ 2 in at least one of the two samples to minimize false positives and negatives. It should be noted that these statistical significances were based on expected sampling distributions. To determine which of the 13,243 genes were expressed significantly and differentially between the two samples, we required a two fold or greater change in expression and an FDR of 10^{-3} or less, which resulted in a set of 461 significantly DEGs. Compared with the control, 223 genes were upregulated and 238 genes were downregulated in the *ds-Tchsp90* sample, and the number of upregulated was almost as many as those of downregulated of DEGs (Fig. 4a). In order to validate the quality of RNA-sequencing results, 12 DEGs were randomly selected for qRT-PCR. Expression

trends of these selected genes were similar between qRT-PCR and RNA sequencing (Fig. 4b), which further validated that our RNA-sequencing data could be used to identify and quantify gene expression patterns.

Functional analysis of DEGs based on the RNA-sequencing data

The GO analyses were used to classify the functions of the DEGs between the two samples. Of the DEGs (P value ≤ 0.05), 244 were annotated to three main GO functional categories and were then divided into 44 sub-categories. There were 24 sub-categories for biological process, 11 for cellular

Fig. 4 Changes in the gene expression profile between the *ds-Tchsp90* and control (a) and a comparison of the expression profiles of the selected genes as determined by RNA sequencing and qRT-PCR (b)

component, and 9 for molecular function (Table S4). Of these groups, cellular process (GO 0009987), developmental process (GO 0032502), reproduction (GO 0000003), response to stimulus (GO 0050896), and immune system process (GO 0002376) were closely related to *Tchsp90* functions. The cellular process (GO 0009987) contains 94 DEGs, which included 6 *MCM* genes and 3 *DNA polymerase* genes associated with DNA replication (Table 2) and encoding *CDC16*, *NDC80*, *CENPE*, *KIF4A*, *KIF18A*, *KRP-130*, and *Anillin* involved in cell division (Table 3). *PCNA* and *RCC1* contributed to DNA replication and cell division pathways simultaneously in insects (Tables 2 and 3). We also found that there were 34 DEGs connected with developmental process (GO 0032502) (Table 4), which included *Annexin B9*, *frizzled-4*, *Torso-like (TSL)*, and *corkscrew (CSW)* that acted as significant regulators of 20E, Wnt, and Torso signaling pathway, respectively. The reproduction (GO 0000003) contains 12 DEGs (Table S6), which included *strawberry notch (sno)* and *feminization factor 1B (Fem1B)* related to oogenesis and spermatogenesis, respectively. *Toll-like receptor 6 (TLR6)*, *peptidoglycan recognition protein 2 (PGRP2)*, *defensin1*, and *defensin2* were annotated on the response to stimulus (GO 0050896) (Table S7), which were associated with heat stress and also acted as bacteria defense agents in the NF- κ B pathway that confers immune challenge. Seven DEGs were classified into the immune system process (GO 0002376) (Table S8), and six of them were also included in the group of response to stimulus (GO 0050896) (Table S7). Moreover, a high percentage of DEGs were observed in the metabolic process (GO 0008152) with 117 DEGs, cell part (GO 0044464) with 72 DEGs, and catalytic activity (GO 0003824) consisting of 127 DEGs (Table S4).

Additionally, KEGG analysis showed that 295 DEGs were enriched with 181 biological pathways (Table S5). And there were 11 pathways significantly enriched in DEGs compared with the whole transcriptome background with Q value ≤ 0.05 , including DNA replication (ko03030), pancreatic secretion (ko04972), and protein digestion and absorption (ko04974) (Fig. 5 and Table S5). Eleven DEGs were distributed in the DNA replication pathway (ko03030) (Table 2). Twenty-four DEGs and 19 DEGs participated in the pancreatic secretion (ko04972) (Table 5) and protein digestion and absorption (ko04974) (Table S9) pathways, respectively. Among them, 18 DEGs are the same, among which 17 DEGs are members of the *serine protease (sp)* family, which likely contributed to development and reproduction, stress response, and innate immunity in *T. castaneum*. These indicated that there are considerable differences in the physiological processes between the ds-*Tchsp90* and control samples.

Discussion

Our previous study illustrated that *Tchsp90* was associated inextricably with the heat stress, development, reproduction, and life span of *T. castaneum* (Zhang, Gu et al. 2014). Therefore, we used a high-throughput RNA-sequencing technology to further identify the regulatory mechanisms and/or signaling pathways of *Tchsp90* in *T. castaneum*. Between the ds-*Tchsp90* and control samples, 461 DEGs (223 DEGs up-regulated and 238 DEGs downregulated) were screened out (Fig. 4a). Moreover, 244 DEGs were classified into 44

Table 2 DEGs related to DNA replication (significant at P value ≤ 0.05)

Gene ID	Log ₂ ratio (T/C)	Regulation (T/C)	FDR	Protein	Phenotype after RNAi of DEGs in <i>T. castaneum</i> ^a
TC003862	-3.33	Down	4.10E-04	RCC1	90% ^b
TC013712	-2.03	Down	4.28E-07	Pol α	/
TC009146	-1.89	Down	3.75E-11	MCM5	/
TC011437	-1.81	Down	5.65E-07	MCM2	/
TC002088	-1.57	Down	2.75E-09	MCM3	40% ^b . 25% animals showed mature egg deposition blocked and 25% animals reduced egg chamber number ^c
TC011200	-1.46	Down	6.42E-07	MCM6	90% ^b
TC009571	-1.32	Down	5.97E-09	MCM4	/
TC013713	-1.26	Down	2.76E-07	MCM7	/
TC008874	-1.24	Down	5.28E-09	PCNA	100% ^b
TC004992	-1.13	Down	2.31E-05	Pol δ	10% ^b
TC009600	-1.10	Down	2.53E-05	Pol ϵ	/

C control, T ds-*Tchsp90*, FDR false discovery rate, *RCC1* regulator of chromosome condensation 1, *Pol* DNA polymerase, *MCM* minichromosome maintenance, *PCNA* proliferating cell nuclear antigen

^a Metamorphosis and survival after RNAi were analyzed (<http://ibeetle-base.uni-goettingen.de>)

^b Lethalities 11 days after larval injection (includes death as larva, prepupa, pupa)

^c 13 days after female pupal injection

Table 3 DEGs involved in cell division (significant at P value ≤ 0.05)

Gene ID	Log ₂ ratio (T/C)	Regulation (T/C)	FDR	Protein	Phenotype after RNAi of DEGs in <i>T. castaneum</i> ^a
TC003862	-3.33	Down	4.10E-04	RCC1	90% ^b
TC011249	-1.61	Down	6.42E-04	NDC80	/
TC003759	-1.61	Down	2.57E-06	KRP-130	100% ^b
TC002763	-1.52	Down	2.88E-04	KIF18A	50–80% embryo/egg not fertilized ^c
TC015937	-1.39	Down	6.55E-10	Anillin	/
TC008874	-1.24	Down	5.28E-09	PCNA	100% ^b
TC015915	-1.19	Down	6.10E-06	KIF4A	More than 80% embryonic tissue, no cuticle ^c
TC014111	-1.03	Down	5.04E-14	CENPE	60% ^b
TC030737	-1.01	Down	2.10E-04	CDC16	/

C control, *T* ds-*Tchsp90*, FDR false discovery rate, *RCC1* regulator of chromosome condensation 1, *NDC80* nuclear division cycle 80, *KRP-130* bipolar kinesin 130, *KIF* kinesin, *PCNA* proliferating cell nuclear antigen, *CENPE* centromere protein E, *CDC16* cell cycle protein 16

^a Metamorphosis and survival after RNAi were analyzed (<http://ibeetle-base.uni-goettingen.de>)

^b Lethalities 11 days after larval injection (includes death as larva, prepupa, pupa)

^c 14 days after female pupal injection

functional groups by GO analyses and 295 DEGs were enriched with 181 biological pathways through KEGG analysis based on gene functional annotation (Fig. 3 and Table S5). Among these functional groups and biological pathways, the DEGs involved in DNA replication process, cell division control, stress response, development, reproduction, and life span were discussed in detail as they were most likely related to *Tchsp90* functions.

Treatment of the cytosolic HSP90 inhibitor, geldanamycin, resulted in the inactivation of the DNA replication signaling pathway (Crevel, Bates et al. 2001), and knockdown of cytosolic *hsp90* also caused cell division stagnation in *Drosophila* (McClellan, Xia et al. 2007; Bandura, Jiang et al. 2013). But little is known about whether the abnormal expression of ER-based *hsp90* in insects is involved in DNA replication and cell division. Interestingly, based on the GO annotations, KEGG pathway analyses, and references, 11 DEGs and 9 DEGs related to DNA replication and cell division signaling pathways were identified between ds-*Tchsp90* and control groups in *T. castaneum*, respectively (Tables 2 and 3). This implied that ER-based HSP90, which may be similar to cytosolic HSP90, also plays a crucial role in the pathway of DNA replication and cell division in insects.

There are two steps, initiation and elongation, responsible for DNA replication as described previously (Yamaguchi, Hirose et al. 1995; Ritzi and Knippers 2000). The initiation of DNA replication is an ordered biochemical pathway that is activated by the *MCM* gene family (*MCM2*–*MCM7*), which provides essential helicase functions from two DNA replication forks through the unwinding of the DNA double strands (Ritzi and Knippers 2000; Ilves, Petojevic et al. 2010). In *Drosophila*, the mutation of each member of these six *MCMs* reduced the DNA helicase activity although the extent of defect varied significantly among different members (Ilves,

Petojevic et al. 2010). Subsequently, the elongation step of DNA replication is triggered by three major *DNA polymerases* (*Polα*, *Polδ*, and *Polε*) (Yamaguchi, Hirose et al. 1995). *Polα* plays a crucial role in the synthesis of RNA primers for replicative chain elongation, and HeLa cell extracts depleted of *Polα* by immunoaffinity column chromatography do not support cell-free replication of SV40 DNA, which can be restored specifically by the addition of an appropriate (human or monkey but not murine) *Polα* (Miyazawa, Izumi et al. 1993). In *Schizosaccharomyces pombe*, strains encoding a *Polδ* and *Polε* mutator allele caused a moderate block in elongation of lagging strand and leading strand, respectively (Miyabe, Kunkel et al. 2011). Thus, a critical step of this DNA replication pathway is the engagement of *MCM* proteins that unwinds the two strands of genomic DNA to allow the *DNA polymerases* to use these strands as templates for the synthesis of new daughter DNA molecules (Enemark and Joshua-Tor 2008). Here, we offer the first evidence to demonstrate that RNAi of *Tchsp90* led to the downexpression of *MCM2*, 3, 4, 5, 6, 7, *Polα*, *Polδ*, and *Polε* in *T. castaneum* (Table 2) and suggesting that *Tchsp90* also participated in the DNA replication pathways through cross talk with *MCMs* and *DNA polymerases*.

Moreover, *CDC16*, *CENPE*, *NDC80*, *KIF4A*, *KIF18A*, *KRP-130*, and *Anillin* were downregulated through the RNAi of *Tchsp90* in *T. castaneum* (Table 3). Reducing the expression of *CDC16* in fly tissue culture cells caused a transient mitotic arrest (Huang and Raff 2002), and RNAi-mediated depletion of *CDC16* resulted in delayed or blocked cell division in a premetaphase-metaphase-like state with over-condensed chromosomes in *Drosophila* (Pal, Nagy et al. 2007). It indicated that non-presence of *CDC16* likely perturbed the cell division pathway. Furthermore, there are two kinetochore proteins, *CENPE* and *NDC80*, which play a

Table 4 DEGs associated with development process (significant at *P* value ≤ 0.05)

Gene ID	Log ₂ ratio (T/C)	Regulation (T/C)	FDR	Protein	Phenotype after RNAi of DEGs in <i>T. castaneum</i> ^a
TC014501	2.81	Up	8.52E-18	L(3)mbn	/
TC006736	2.17	Up	8.35E-66	SKRP	/
TC010564	2.17	Up	3.15E-39	GTP cyclohydrolase I isoform A	/
TC003136	1.65	Up	6.95E-05	Flocculation protein FLO11-like	/
TC012910	1.44	Up	1.98E-68	CSW	/
TC013304	1.30	Up	1.29E-04	Spaetzle	/
TC002550	1.22	Up	4.01E-27	Nuclear hormone receptor	/
TC000175	1.13	Up	9.07E-13	Peroxidase	/
TC010234	-4.13	Down	3.78E-04	Zinc finger protein	/
TC010547	-2.07	Down	1.81E-16	Myosin-I heavy chain	/
TC011793	-1.93	Down	4.96E-07	Annexin B9	50% ^b
TC013067	-1.79	Down	3.92E-07	unc-5	/
TC006527	-1.63	Down	3.11E-04	Frizzled-4	/
TC003254	-1.55	Down	2.98E-04	ITP122H	/
TC002893	-1.46	Down	1.37E-08	Integrin beta-PS-like	/
TC008808	-1.44	Down	2.17E-19	Fem1B	/
TC012185	-1.44	Down	4.03E-76	Endoplasmin	/
TC014023	-1.41	Down	3.46E-04	Serine/threonine-protein kinase	/
TC015937	-1.39	Down	6.55E-10	Anillin	/
TC007877	-1.36	Down	1.07E-10	Fat protein	/
TC012521	-1.34	Down	1.66E-20	PWPP	/
TC013099	-1.32	Down	5.63E-05	Protein abrupt isoform X2	/
TC008110	-1.23	Down	5.89E-05	Parathyroid hormone	/
TC003821	-1.17	Down	1.56E-05	WDR43	/
TC002775	-1.15	Down	6.74E-04	Roundabout-like protein	/
TC009906	-1.11	Down	1.93E-06	TSL	20% ^b . More than 80% embryonic and larval musculature patterns become irregular ^c
TC015177	-1.10	Down	7.40E-08	sno	/
TC007878	-1.10	Down	1.31E-20	Cadherin-like protein	/
TC007760	-1.08	Down	9.07E-06	Protein abrupt isoform X1	/
TC003891	-1.07	Down	1.21E-23	Zn finger homeodomain 2	/
TC006775	-1.04	Down	7.57E-08	rbbp4	/
TC012545	-1.02	Down	1.07E-13	Starry night	/
TC003728	-1.01	Down	7.57E-04	Pangolin	/
TC001720	-1.01	Down	4.02E-05	RNA-binding protein 24B	/

C control, *T* ds-*Tchsp90*, *FDR* false discovery rate, *L(3)mbn* lethal (3) malignant blood neoplasm, *SKRP* serine/threonine kinase receptor-associated protein, *CSW* corkscrew, *ITP122H* intraflagellar transport protein 122 homolog, *Fem1B* feminization factor 1B, *PWPP* protocadherin-like wing polarity protein, *WDR43* WD repeat-containing protein 43, *TSL* receptor tyrosine kinase Torso-like protein, *sno* strawberry notch, *rbbp4* retinoblastoma-binding protein 4

^a Metamorphosis and survival after RNAi were analyzed (<http://ibeetle-base.uni-goettingen.de>)

^b Lethalities 11 days after larval injection (includes death as larva, prepupa, pupa)

^c 14 days after female pupal injection

key role in the movement of chromosomes toward the equatorial plate during mitosis metaphase and is required for kinetochore integrity and chromosome segregation during mitosis anaphase, respectively (Przewloka, Zhang et al. 2007; Williams, Leung et al. 2007; Radford, Hoang et al. 2015). Mitotic cells lacking *NDC80* had persistent lateral

kinetochore-microtubule attachments, while the loss of *CENPE* resulted in chromosomes becoming unaligned in the *Drosophila* cell cycle (Radford, Hoang et al. 2015). Ablation of *KIF18A* and *KIF4A* led to a significant delay of mitosis due to chromosome congression defects and caused microtubule dynamic instability, respectively (Nunes Bastos, Gandhi et al.

Fig. 5 Summary of KEGG pathway enrichment with Q value ≤ 0.05 by RNA sequencing. Differentially expressed genes (DEGs) are indicated in green, and all detected genes are indicated in wine color. The y axis indicates the number of genes in a category

2013; Yang, Chen et al. 2015). *KRP-130*, a bipolar kinesin purified from *fly*, is essential for spindle body separation and pushing apart the associated spindle poles during spindle assembly (Kashina, Baskin et al. 1996). Ultimately, cytokinesis, the final step of cell division, is controlled by *Anillin* that is required for the structural integrity of the cleavage furrow and for completion of cleavage furrow ingression. Depletion of *Anillin* gave rise to binucleate cells and fewer intercellular bridges in *Drosophila* (Hickson and O'Farrell 2008; Haglund, Nezis et al. 2010). Thus, RNAi of *Tchsp90* probably led to dramatic defects in cell division accompanied by improper assembly of the kinetochore and spindle, then chromosome failed to congression or segregation and incomplete cytokinesis in insects.

The coordination and synchronization of DNA replication and cell division are crucial for proper cell development (Klusza and Deng 2011; Zhou and Luo 2013). Mutation of *RCC1* failed to detect small amounts of unreplicated DNA, so that it could not produce a signal that inhibits the cell move into the G_2 phase of the cell cycle and prevent the activation of mitotic regulators (Dasso 1993), suggesting that *RCC1* was necessary for the control of the DNA replication to mitosis transition in the whole cell cycle process. *PCNA* plays a significant role during DNA replication through loading to the *Pol α* and is considered a polymerase processivity factor for DNA *Pol δ* and *Pol ϵ* , and participates in multiple DNA repair processes (Wang, Shi et al. 2013). Apart from its functions in DNA replication and repair, it is also required for cell division control (Warbrick, Heatherington et al. 1998). The *PCNA* mutants in *flies* have abnormal mitotic spindles which are either anastral or have poorly focused poles, and then are arrested in a defective metaphase with shorter and thicker chromosomes that fail to align at the metaphase plate within a poorly

assembled mitotic spindle (Pflumm and Botchan 2001). These results suggested that DNA replication defects led to the mitotic arrest; *RCC1* and *PCNA* were coupled with DNA replication and mitosis. Strikingly, *RCC1* and *PCNA* were both downregulated in *T. castaneum* by RNAi of *Tchsp90* (Tables 2 and 3), confirming that *Tchsp90* is involved in the coordination between DNA replication and mitosis, which cooperated with *RCC1* and *PCNA*.

DNA replication and cell division have also been shown to be associated with development, reproduction, and life span in many studies (Schwed, May et al. 2002; Franke, Gehlen et al. 2008; Wang, Shi et al. 2013). Mutations of three *MCM* genes in the *fly*, *MCM2*, *MCM4*, and *MCM6*, resulted in lethality before adulthood, and *MCM6* mutant larvae had no identifiable imaginal disks and brain lobes which were reduced to 50% of the diameter of wild type (Schwed, May et al. 2002). Injection of antibodies against *MCMs* disrupted early embryonic divisions, and in *Drosophila* *MCM2* and *MCM4* mutants, defectives in DNA replication were evident from mid-embryogenesis through larval development (Su, Yakubovich et al. 1997). Beyond the functions in development and reproduction, *MCMs* have a pivotal role in determining longevity. *MCM* proteins are less abundant with age in eukaryotes, and lower levels of *MCM7* correlate with decreased cell proliferation, lower autophagic capacity, and higher intracellular protein expression, which severely reduced adult life span (Dumit, Kuttner et al. 2014). Moreover, *Pol ϵ* mutant resulted in less developed imaginal disks and a pupal lethal mutation which hindered the development of the larvae and pupae in *Drosophila* (Sahashi, Matsuda et al. 2013). *Pol ϵ* and *Pol α* were maternally stored in unfertilized eggs and maintained at high levels during embryogenesis, suggesting that these gene products were required in early development and essential for reproduction (Yamaguchi, Hirose et al. 1995). Meanwhile, reduction of *Pol α* was associated with the loss of telomerase activity and resulted in telomere shortening which caused abnormal cell senescence, cell death, and eventually led to a significantly shorter life (Franke, Gehlen et al. 2008). Additionally, mutation of *fly* *PCNA* exhibited a complex pleiotropy of temperature sensitive lethality and female sterility (Henderson, Wiegand et al. 2000; Wang, Shi et al. 2013). More importantly, silencing of *T. castaneum* larvae *MCM3*, *MCM6*, *Pol δ* , *CENPE*, *KRP-130*, *RCC1*, and *PCNA* caused development arrest (including larvae and pupae) although the extent of lethality varied significantly from differential genes (Tables 2 and 3). The lack of *MCM3*, *KIF18A*, and *KIF4A* at *T. castaneum* pupal stage induced a certain degree of embryonic defects (Tables 2 and 3) (Donitz, Schmitt-Engel et al. 2015). Consistent with our findings, RNAi of larvae *Tchsp90* led to development arrest before eclosion and the loss of *Tchsp90* at pupal stage decreased egg production and resulted in a significantly reduction in life span in *T. castaneum*.

Table 5 DEGs related to pancreatic secretion pathway (significant at Q value ≤ 0.05)

Gene ID	Log ₂ ratio (T/C)	Regulation (T/C)	FDR	Protein	Phenotype after RNAi of DEGs in <i>T. castaneum</i> ^a
TC005503	7.29	Up	4.50E-85	PTL	/
TC003081	2.32	Up	8.57E-10	Serine protease H42	/
TC013416	2.03	Up	6.57E-09	Serine protease P142	/
TC013419	1.86	Up	2.70E-04	Phospholipase A2C	/
TC006246	1.66	Up	4.97E-20	Serine protease H70	/
TC005908	1.60	Up	1.96E-05	Serine protease H64	/
TC008659	1.57	Up	1.25E-06	Serine protease P87	/
TC000249	1.57	Up	5.51E-59	Serine protease H4	/
TC010930	1.37	Up	2.38E-120	Serine protease H112	/
TC009602	1.14	Up	5.91E-14	Serine protease P96	/
TC011014	-12.98	Down	2.39E-06	Serine protease P124	/
TC014083	-5.93	Down	9.93E-68	CTLP5B	30% embryonic tissue, no cuticle ^c
TC004635	-3.95	Down	3.35E-114	Serine protease-like protein	/
TC002785	-3.38	Down	2.22E-20	Serine protease P40	/
TC000547	-2.97	Down	1.11E-21	Serine protease P13	/
TC004656	-2.70	Down	5.44E-91	Phospholipase A1	10% ^b
TC030074	-2.45	Down	2.24E-26	Serine protease 9-like protein	/
TC010909	-1.90	Down	5.09E-21	Serine protease H107	/
TC010934	-1.62	Down	2.30E-15	Serine protease H115	60% ^b
TC009724	-1.59	Down	7.24E-146	Inactive pancreatic lipase	/
TC010935	-1.44	Down	3.64E-04	Serine protease H116	/
TC004657	-1.24	Down	1.04E-18	Lipase	10% ^b
TC012528	-1.13	Down	2.40E-22	PLRP1	10% ^b . 25% mature egg deposition blocked and 100% yolk structure irregular ^d
TC002767	-1.12	Down	1.32E-05	Serine protease P38	/

C control, *T* ds-*Tchsp90*, FDR false discovery rate, PTL pancreatic triacylglycerol lipase, CTLP5B chymotrypsin-like proteinase 5B, PLRP1 pancreatic lipase-related protein 1

^a Metamorphosis and survival after RNAi were analyzed (<http://ibeetle-base.uni-goettingen.de>)

^b Lethalities 11 days after larval injection (includes death as larva, prepupa, pupa)

^c 14 days after female pupal injection

^d 13 days after female pupal injection

(Zhang, Gu et al. 2014). Thus, the effect of ds- *Tchsp90* on the development, reproduction, and life span of *T. castaneum* might be the consequence of the decline in DNA replication and cell division.

Furthermore, 34 DEGs and 12 DEGs were associated with developmental process and reproduction GO categories, respectively (Tables 4 and S6). Meanwhile, the DEGs encoding *AnnexinB9* and *TSL* were specifically involved in the developmental process and 9 DEGs such as *frizzled-4*, *sno*, *Fem1B*, and *CSW* existed in both GO terms (Tables 4 and S6). Specially, *CSW* was significantly upregulated, and the other five genes were significantly downregulated by RNAi of *Tchsp90*. During the pupation, 20E triggers programmed cell death (PCD) to remove larval-specific tissues and upregulates *AnnexinB9* expression in *Bombyx mori* (Tsuzuki, Iwami et al. 2001). And low levels of *AnnexinB9* mRNA were detected during the prepupal stage in tissues that are destined to die

but not in tissues that survive pupal metamorphosis, providing evidence that *AnnexinB9* plays a key role in 20E regulation of metamorphosis in the way of PCD (Kaneko, Takaki et al. 2006). Indeed, RNAi of *AnnexinB9* at the larval stage resulted in 50% lethality before adulthood in *T. castaneum* (Table 4) (Donitz, Schmitt-Engel et al. 2015). The Wnt signaling pathway is essential for short germ embryos, which elongate their primary body axis by consecutively adding segments from a posteriorly located growth zone in *T. castaneum*, and this is regulated by the binding of the Wnt ligand to transmembrane receptors encoded by the *frizzled* gene family (Beermann, Pruhs et al. 2011). *Frizzled-4*, apart from the function in control axis elongation in a short germ insect, has a significant role in the process of promoting hindgut development at the larval stage. When *frizzled-4* was inhibited in *T. castaneum*, the elongation of the gut tube was impaired and subsequently died at early pupa (Beermann, Pruhs et al. 2011). Together

with RNAi phenotypes of *Tchsp90* analysis, we show that silencing of *Tchsp90* expression reduced the levels of *AnnexinB9* and *frizzled-4* expressions and further resulted in development arrest before adulthood. Moreover, the lack of *sno* gene caused females to be sterile and also led to severe cuticle and nervous system defects in the early stage of embryonic development and then led to death at later stages in *Drosophila* (Coyle-Thompson and Banerjee 1993). *Fem1B*, an orthologous of the *C. elegans Fem1*, was identified as being necessary in the signaling pathway for male determination, and null mutation of the *Fem1* prevented male development especially in the process of spermatogenesis (Oyhenart, Benichou et al. 2005; Subauste, Sansom et al. 2010). Knocking down of *Fem1* gene expression inhibited *Tnfrsf6*, which acts as a strong candidate gene for elimination of defective cells by playing an apoptosis inducer during the spermatogenic process, subsequently causing the continuous accumulation of defective cells and a further decline in sperm quality (Oyhenart, Benichou et al. 2005; Subauste, Sansom et al. 2010). *Fem1B* was significantly expressed in the rodent testis and its mRNA presented at high levels during spermatogenesis, and similar results were described for the human *Fem1B* (Oyhenart, Benichou et al. 2005). This maybe the reason why ds-*Tchsp90* females were completely sterile, and the males had low reproduction rates in *T. castaneum*. Additionally, the Torso (Tor) pathway is responsible for the specification of the most anterior and posterior regions of the fly embryo and is triggered by secretion of *Trunk* (Grillo, Furriols et al. 2012). But the levels of extracellular *Trunk* are greatly reduced in *TSL* null mutants (Johnson, Henstridge et al. 2015), demonstrating that *TSL* functions to mediated secretion of *Trunk* and then regulated Tor pathway. It was directly observed that *TSL* female mutant suppressed the formation of abdomen in *Drosophila* embryo (Martin, Raibaud et al. 1994). Also, more than 80% of embryonic and larval musculature patterns became irregular after female pupal injection of ds-*TcTSL* in *T. castaneum* (Table 4) (Donitz, Schmitt-Engel et al. 2015). The lack of *CSW* would contain high levels of *Tor* than wild type in fly embryos, further resulting in the activation of the Tor pathway, suggesting that the over-expression of *CSW* probably inhibited the Tor signaling pathway (Cleghon, Feldmann et al. 1998). These results indicated that *Tchsp90* likely participates in the Tor pathway to regulate insect embryogenesis cooperating with *TSL* and *CSW*.

Septic injury results in the induced expression of potential immune-effector genes and stress-responsive genes in *T. castaneum*, suggesting that there might be cross talk between immune and stress responses (Altincicek, Knorr et al. 2008). In *Drosophila*, GP96 was the master immune chaperone for both cell surface and intracellular *TLRs* (Yang, Liu et al. 2007), while *Tchsp90* expression level was increased significantly after heat stress and reached a peak after 2 hour

of heat treatment at 45 °C in *T. castaneum* (Zhang, Gu et al. 2014), indicating that ER-based HSP90 could play a pivotal role in both the response to immunity and heat stress. There are two distinct NF- κ B transcription factor-dependent immune signaling pathways, toll and the immune deficiency (IMD) pathways, responsible for antimicrobial peptide (AMP) induction in fly (Li, Wu et al. 2014). The toll signaling pathway was inactivated for *TLR6* knockout in mice, which further reduced the expression of AMP genes (Zhang 2016). And it has been reported that *TLR6* mutation mice were more susceptible to *Brucella abortus* than the wild-type mice at 1–6 weeks after infection (de Almeida, Macedo et al. 2013). As we expected, our RNA-sequencing results revealed that *TLR6* was downregulated in *T. castaneum* by RNAi of *Tchsp90* (Table S7), illustrating that ds-*Tchsp90* probably led to a decline in the activity of toll pathway. Moreover, *PGRP2*, *defensin1*, and *defensin2* were upregulated by RNAi of *Tchsp90* in *T. castaneum* (Tables S7 and S8), while silencing fly *PGRP-LB* resulted in the activation of the IMD signaling pathway and downregulation of *PGRP-SC* causing a significantly increased expression of dipterin and attacin in housefly (Wang, Wu et al. 2009; Gao, Tang et al. 2015), suggesting that the presence of *PGRP2* probably decreased the synthesis of AMPs and further reduced the resistance to gram-negative bacteria infection although the detail mechanism still needs further study. Furthermore, *defensin1* was strongly upregulated by wounding and bacterial challenge in *Bombus terrestris* and *defensin2* was also upregulated following Cry3Ba spore-crystal intoxication and induced upon Cry3Aa spore-crystal treatment in *T. castaneum* (Erler, Popp et al. 2011; Contreras, Benito-Jardon et al. 2015). The over-expressed *defensin1* and *defensin2* probably are compensated for the loss of *Tchsp90* as well as that of *TLR6*, which maintained the homeostasis of microorganisms in insects. Interestingly, *PGRP2*, *defensin1*, and *defensin2* were upregulated in fourth-instar larvae of *Aedes aegypti* exposed to temperature stress, further confirming the relationship between immunity and the heat stress response (Muturi, Kim et al. 2011). According to the GO analysis, seven DEGs were associated with the immune system process (Table S8), among which six DEGs were also found to be related to response to stimulus (Table S7), suggesting that cross talk might exist between immunity and stress response and the interaction is mediated through the *Tchsp90* in *T. castaneum*.

More importantly, combining the pathways of pancreatic secretion (Table 5) and protein digestion and absorption (Table S9), the nine members of the *sp* family were downregulated, but eight members were upregulated in response to RNAi of *Tchsp90* treatment (*Q* value ≤ 0.05). Beyond their digestive function, *SPs* have important and vital roles in various physiological processes including development and reproduction (Zou, Lopez et al. 2006; Wang, Zhang et al. 2016), and the stress and immune response such as hemolymph

coagulation and AMP synthesis (Gorman, Andreeva et al. 2000). Silencing of larval *SPH155* caused development arrest with 60% lethality (including larvae and pupae) in *T. castaneum* (Table 5) (Donitz, Schmitt-Engel et al. 2015) and the lack of *SPP2c* in silkworm embryos significantly reduced the degradation rate of residual yolk proteins on embryonic day 10, and further led to the suspension of embryogenesis (Wang, Zhang et al. 2016). Also, *SP3*, *SPH42*, and *SP49* transcripts were elevated after the saline or *Escherichia coli* injection and the pathogen challenge gave rise to a much stronger induction of *SP1*, *SP2*, *SP6*, and *SP41* gene transcription in *Apis mellifera* (Zou, Lopez et al. 2006). These results indicated that the SP signaling pathway, in parallel with the DNA replication and cell division pathway, was activated and amplified by *Tchsp90*, which, in turn, affected development and reproduction, and was even involved in the stress response and innate immunity of the red flour beetle. Interestingly, the loss of these *SPs* activity caused by reduction of *Tchsp90* in *T. castaneum* larvae is also likely compensated by increasing the expression of the other major *SPs* (Tables 5 and S9), at least partially. Actually, this result was similar to depletion of *cysteine peptidase* (*CP*) genes or fed *SP* inhibitors, in which the expressions of some *CPs* and *SPs* were decreased whereas other vital *CPs* and *SPs* were increased, as part of an integrated compensation response in *T. castaneum* (Oppert, Elpidina et al. 2010; Perkin, Elpidina et al. 2017). Taken together, these data suggested that *T. castaneum* larvae also altered large-scale *SP* gene expression patterns and amplified the SP signaling pathways after RNAi of *Tchsp90* in addition to reducing the activities of DNA replication and cell division, further regulating the beetle's diverse physiological functions.

Conclusions

In total, 461 DEGs were identified between the ds-*Tchsp90* and control groups. Among these genes, *MCMs* and *DNA polymerases* were downregulated in the ds-*Tchsp90* larvae, which would reduce the activity of the DNA replication signaling pathway; *CENPE*, *NDC80*, and other cell division associated proteins were also downregulated in the ds-*Tchsp90* larvae, which would suppress the activation of the cell division signaling pathway in the insects. These results indicated that *Tchsp90* was involved in the regulation of DNA replication and cell division and then further participated in the insect development, reproduction, and life span through these two pathways. Moreover, the DEGs encoding *AnnexinB9*, *frizzled-4*, *sno*, *Fem1B*, *TSL*, and *CSW* may be related to the development and reproduction of ds-*Tchsp90* insects. And cross talk between stress response and immunity is regulated by *Tchsp90*. Additionally, the serine protease signaling pathway, paralleling with DNA replication and cell division

pathways, was activated and amplified by *Tchsp90* to participate in *T. castaneum* larval development and reproduction as well as in the heat stress and innate immunity response. Taken together, these DEGs were closely related to the functions of *Tchsp90* in *T. castaneum* and this study will promote the study of the *Tchsp90* signaling pathway in the insects.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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