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Establishment of a versatile cell line for juvenile hormone signaling analysis in *Tribolium castaneum*

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The red flour beetle, *Tribolium castaneum*, has been widely used as a laboratory model for analyzing gene function. In this study, we established a novel cell line (Tc81) from *T. castaneum* embryos and validated the utility of this cell line by analyzing the juvenile hormone (JH) signaling pathway. In Tc81 cells, the *Krüppel homolog 1* gene (*Kr-h1*), which is a JH-dependent repressor of insect metamorphosis, was rapidly induced by subnanomolar levels of JHs. Bioinformatics analysis and reporter assays identified 2 JH response elements (*k*JHREs) located in the region upstream of the transcription start site and in the first intron of *Kr-h1*. Furthermore, *methoprene tolerant* (*Met*) and *steroid receptor co-activator* (*SRC*) RNAi reduced JH-dependent induction of *Kr-h1* transcripts and *k*JHRE-reporter activities. Thus, this novel Tc81 cell line is useful for the elucidation of JH signaling and is a promising tool for the functional analysis of genes by RNAi and reporter assays.

The red flour beetle *Tribolium castaneum* is a serious pest often found in stored agricultural products and is widely used as a laboratory model[1,2]. *T. castaneum* has many advantages as an experimental organism. For example, it has a relatively short life cycle (about 4 weeks from embryo to adult)[3] and is easy to maintain. The elucidation of complete annotated genomic sequences[4], transposon-based genetic transformation[5], and systemic RNA interference (RNAi)[3] has facilitated the analysis of gene function in this model. In addition, unlike the fruit fly *Drosophila melanogaster*, which has a highly derived developmental pattern, *T. castaneum* development follows a typical process that can be considered representative of primitive holometabolous insects[3,4]. These features have made this species an indispensable model insect for research in evolutional and developmental biology, as well as the study of pest management.

One of a few disadvantages of this experimental insect is the shortage of cell lines derived from the organism. Although over 500 cell lines have been established from various tissue sources of many insect species, cell lines of *T. castaneum* have not yet been established, except for one such cell line reported very recently by Goodman et al.[6]. Some of these insect cell lines have been used as research tools to elucidate functions and regulatory mechanisms of genes involved in various biological phenomena[7]. In particular, they are useful for the functional analysis of genes involved in complex signaling pathways, where the functions of individual genes would be too difficult to determine using whole organisms. In addition, these cell lines are valuable for the development of efficient screening systems to discover new drugs, including insecticides[7]. Therefore, the establishment of new *T. castaneum* cell lines will undoubtedly enhance the value of this model insect.

Juvenile hormones (JHs) comprise a group of sesquiterpenoids that regulate a wide array of developmental and physiological events in insects, such as metamorphosis, reproduction, diapause, and polyphenism[8,9]. JH is known as a "status quo" hormone and is necessary for maintaining larval nature during molting and for repressing metamorphosis[10]. Although the molecular mechanisms facilitating the antimetamorphic action of JH have long been a mystery[11], recent breakthroughs in the study of *T. castaneum* have largely expanded our knowledge of these processes[12].

Methoprene-tolerant (*Met*) and *Krüppel homolog 1* (*Kr-h1*) have critical roles in the JH signaling cascade during metamorphosis[12]. Met is a basic helix-loop-helix Per-ARNT-Sim (bHLH–PAS) transcription factor that was initially identified from a *D. melanogaster* mutant as a resistance gene to the JH agonist methoprene[13,14]. Kr-h1 is a C_2H_2 zinc-finger-type transcription factor that was initially identified as a modulator of the prepupal ecdysone response in *D. melanogaster*[15]; it was later found to be induced by JH[16]. RNAi analysis of *Met* (*TcMet*) in the larvae and pupae of *T. castaneum* revealed that JH carries out its antimetamorphic action via TcMet[17,18]. Additional RNAi analyses in *T. castaneum* revealed that JH induces *Kr-h1* (*TcKr-h1*), a metamorphosis inhibitor, via TcMet,

thereby mediating the antimetamorphic activity of JH[19]. Importantly, this JH-Met-Kr-h1 cascade is conserved in the larval-pupal transition in holometabolous insects and the nymphal-adult transition in hemimetabolous insects[19–21].

Very recently, Met was found to form a functional JH receptor with another bHLH-PAS transcription factor, steroid receptor coactivator (SRC, also named FISC and Taiman), based on studies in *D. melanogaster*, *Aedes aegyptii*, and *T. castaneum*[12]. Met proteins bound to JH with high affinity[22,23], heterodimerized with SRC in a JH-dependent manner, and stimulated the transcription of JH-inducible genes, such as *Kr-h1* and *early trypsin* (*ET*)[24,25]. Furthermore, using a cell line derived from *A. aegyptii*, Li et al.[24] identified a JH response element (JHRE) that interacts with Met and SRC in the promoter region of the *ET* gene.

Using a cell line derived from *Bombyx mori*, we previously identified a different JHRE (*k*JHRE) in the promoter of the *Kr-h1* (*BmKr-h1*) gene and revealed that the JH/Met/SRC complex uses *k*JHRE to activate *BmKr-h1* transcription[26]. Intriguingly, sequences similar to the *k*JHRE are also present in the promoter of *Kr-h1*s in *T. castaneum* and other insect species[26], suggesting their conserved and relevant roles in JH signaling. However, the function of these *k*JHRE-like sequences in *T. castaneum* and other insects remains to be characterized.

In this study, we established a novel cell line (Tc81) from *T. castaneum* embryos and used this cell line for molecular analysis of the JH signaling pathway. Using a combination of RNAi and reporter gene assays in Tc81 cells, we analyzed the functions of *Met*, *SRC*, and *k*JHREs in the JH-dependent induction of *Kr-h1* in *T. castaneum*. This is the first report to describe the establishment of a *T. castaneum*-derived cell line that is applicable for the RNAi-based functional analysis of various genes.

Results

Characterization of Tc81 cells. Cells derived from *T. castaneum* embryos (Tc81) were suspended throughout the majority of the culture medium, with vesicles forming and cells occasionally adhering to the bottom of the culture flask (Fig. 1A). The origin of the Tc81 cells was confirmed by the sequences of 3 representative genes in the genomic DNA of the cells, which perfectly matched with the previously published sequences of the respective *T. castaneum* genes[6] (Supplementary Fig. 1A online). DAPI staining of the nuclei of Tc81 cells suggested that each lattice in the Fig. 1A inset represented a cell (Fig. 1B). The majority of Tc81 cells contained 20 chromosomes/cell, which was double the standard number of chromosomes for in vivo haploid *T. cataneum* cells (10 chromosomes)[6], indicating that Tc81 cells are mainly diploid (Supplementary Fig. 1B online). The size and shape of individual Tc81 cells were uniform, measuring about 10 μm in diameter, while the shape of vesicles was variable, with sizes ranging from about 30 to 300 μm in length (Fig. 1A). The vesicles were collected by centrifugation and dispersed into fresh medium by gentle pipetting (Fig. 1C). After this manipulation, most vesicles temporarily withered, but supple vesicles were regenerated within 2 days. After transfer to fresh medium, cell numbers decreased slightly, but started to increase again after day 2 (Fig. 1D).

Efficiency of soaking Tc81 cells in RNAi. To evaluate the efficiency of RNAi in Tc81 cells, we selected the JH receptor *TcMet* as a target gene and *MalE* of *Escherichia coli* as a control. Cells were soaked with medium containing one of these dsRNAs, and the expression of *TcMet* was analyzed by qPCR. In cells treated with *TcMet* dsRNA, *TcMet* transcripts declined rapidly to about half of baseline levels by day 1 and to about one-third of baseline levels by day 2, maintaining

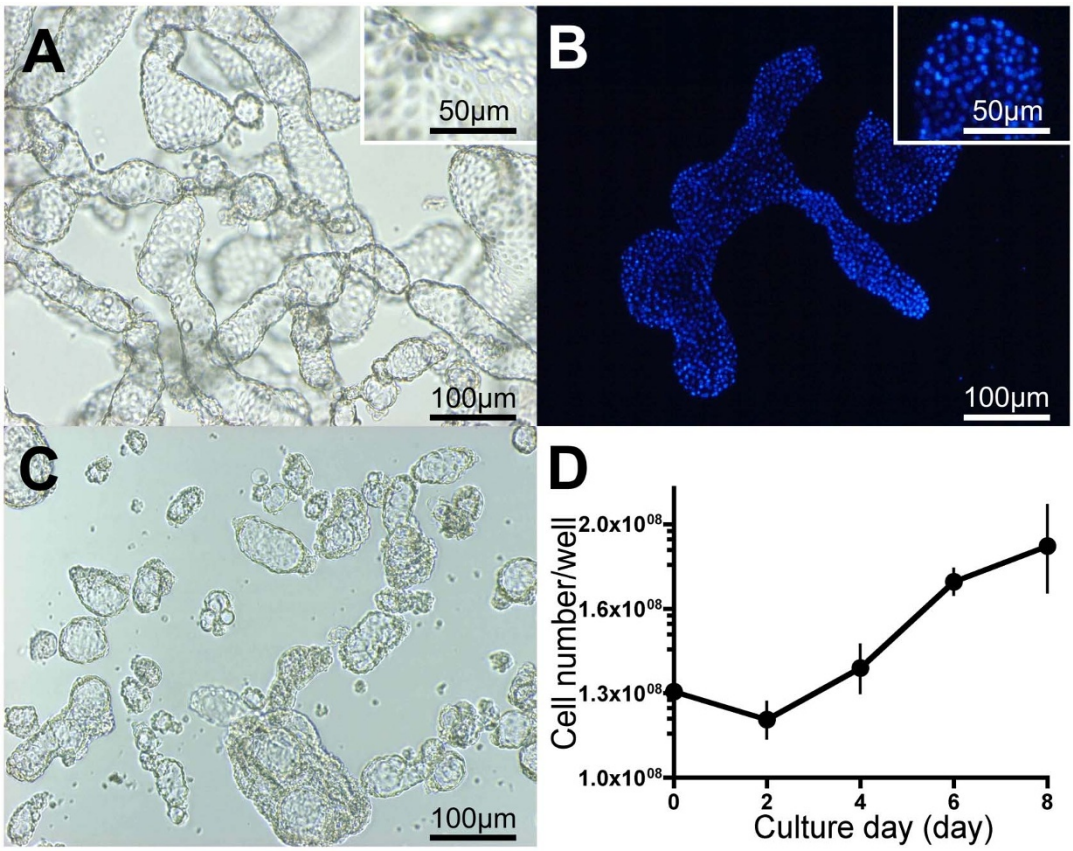

Figure 1 | Morphology and growth capacity of Tc81 cells. (A) Phase contrast micrograph of Tc81 cells forming vesicles. (B) Fluorescence micrograph of Tc81 cells stained with DAPI. (C) Phase contrast micrograph of vesicles disrupted by centrifugation and pipetting. (D) Growth curves of Tc81 cells after 120 passages. The cultures were maintained at 25°C. Mean ± SD (n = 4).

this reduced expression until day 8 (Fig. 2A). In contrast, in cells soaked with *MalE* dsRNA, *TcMet* transcript expression was similar to that in untreated cells and showed little decline throughout the experiment (Fig. 2A). These results suggested that the decline in *TcMet* transcript expression observed in the cells soaked with *TcMet* dsRNA was a target-specific effect. No further suppression of *TcMet* transcripts was achieved by additional dsRNA treatment, indicating that repeated treatment was not necessary to enhance RNAi efficiency, at least until day 8. Fig. 2B shows the relationship between the concentration of dsRNA and the suppression of target transcripts. Expression of *TcMet* transcripts was decreased by treatment with *TcMet* dsRNA in a concentration-dependent manner (0.0005–0.5 ng/μL). Virtually no further suppression was observed at higher concentrations (0.5–500 ng/μL). A near maximal RNAi effect was obtained at a concentration of 0.05 ng/μL dsRNA (Fig. 2B). Treatment with *dsMalE* dsRNA showed no significant effects on *TcMet* transcript expression at any concentration tested (0.0005–500 ng/μL; Fig. 2B).

Effects of JH and its analog on the expression of *TcKr-h1* in Tc81 cells. The effects of JH and its analog (methoprene; JHA) on the expression of *TcKr-h1* in Tc81 cells were analyzed by qPCR. *TcKr-h1* transcript expression was marginal before JHA treatment, but increased significantly within 0.5 h after treatment, reaching a peak (1.5×10^2-fold increase in expression compared to baseline levels) at 1 h after treatment (Fig. 3A). The expression decreased to about half of the maximum level by 2 h and was maintained at this level until 24 h after treatment (Fig. 3A). JH I, JH III, and JHA induced *TcKr-h1* transcript expression dose-dependently in Tc81 cells; the 50% effective concentrations (EC_{50}s) were 4.7×10^{-11}, 2.7×10^{-11}, and 1.8×10^{-9} M, respectively (Fig. 3B). Farnesoic acid (FA) stimulated weak induction of *TcKr-h1* transcripts, but only at a high concentration (1 μM; Fig. 3B).

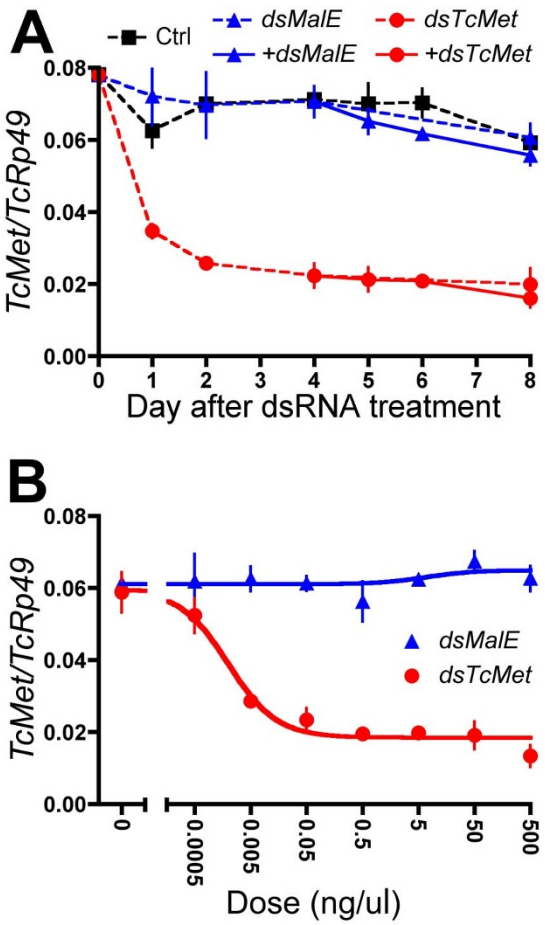

Figure 2 | Effects of soaking Tc81 cells in *TcMet* dsRNA. *TcMet* transcript levels were determined by qPCR (mean ± SD, n = 3). (A) Cells were soaked with *TcMet* (*dsMet*) or *MalE* (*dsMalE*, control) dsRNA (50 ng/μL), and temporal changes in *TcMet* transcript levels were measured (*dsMalE* and *dsTcMet*). Some cells were retreated with dsRNA (50 ng/μL) 4 days after the first treatment (+*dsMalE* and +*dsTcMet*). (B) Cells were treated with different concentrations of dsRNA for *MalE* or *TcMet*, and the expression levels of *TcMet* were determined 60 h after treatment.

Effects of *TcMet* and *TcSRC* RNAi on the JH-dependent induction of *TcKr-h1* in Tc81 cells. Next, we analyzed the functions of *TcMet* and *SRC* (*TcSRC*) in *T. castaneum* in the JH-dependent induction of *TcKr-h1* by RNAi in our newly established Tc81 cell line. Since only a partial sequence for *TcSRC* has been reported in public databases and the literature[23,25], we cloned the full-length cDNA of *TcSRC* (AB762694) by RACE. The open reading frame (ORF) encoded a predicted protein with 1,344 amino acid residues. Four domains, i.e., bHLH, PASA, LXXLL, and PolyQ, of TcSRC showed high homology with the SRCs of other insect species (Supplementary Fig. 2 online).

In Tc81 cells soaked with dsRNA for *TcMet* or *TcSRC*, the transcript levels of the respective target genes were significantly reduced compared to untreated cells or cells treated with *MalE* dsRNA (Fig. 4A and B). The application of JHA did not significantly affect the expression of *TcMet* or *TcSRC* in either control or test cells (Fig. 4A and B). In contrast, *TcKr-h1* transcript expression in Tc81

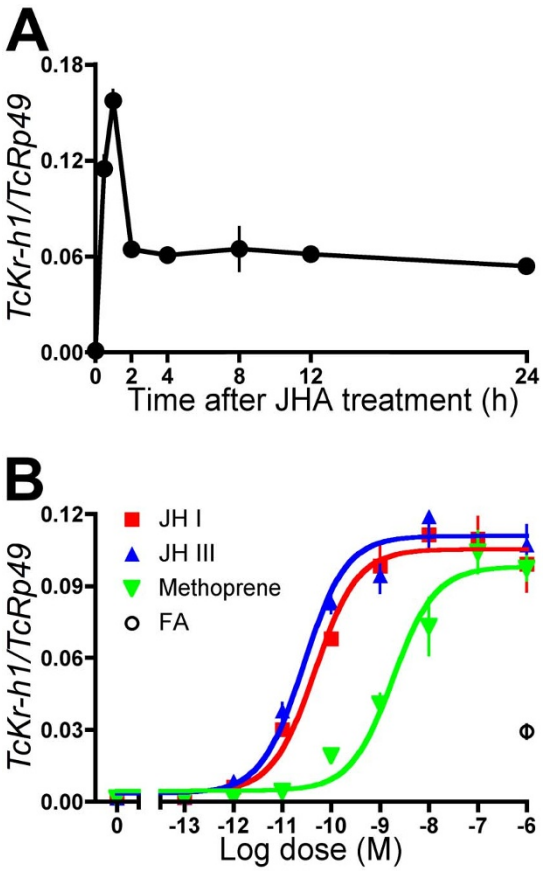

Figure 3 | Induction of *TcKr-h1* transcripts by JH in Tc81 cells. Transcript levels of *TcKr-h1* were determined by qPCR (mean ± SD, n = 3). (A) Cells were treated with 10 μM methoprene (JHA), and temporal changes in *TcKr-h1* expression were monitored. (B) Cells were treated with different concentrations of JH I, JH III, JHA, or farnesoic acid (FA), and the expression levels of *TcKr-h1* were determined 2 h after treatment.

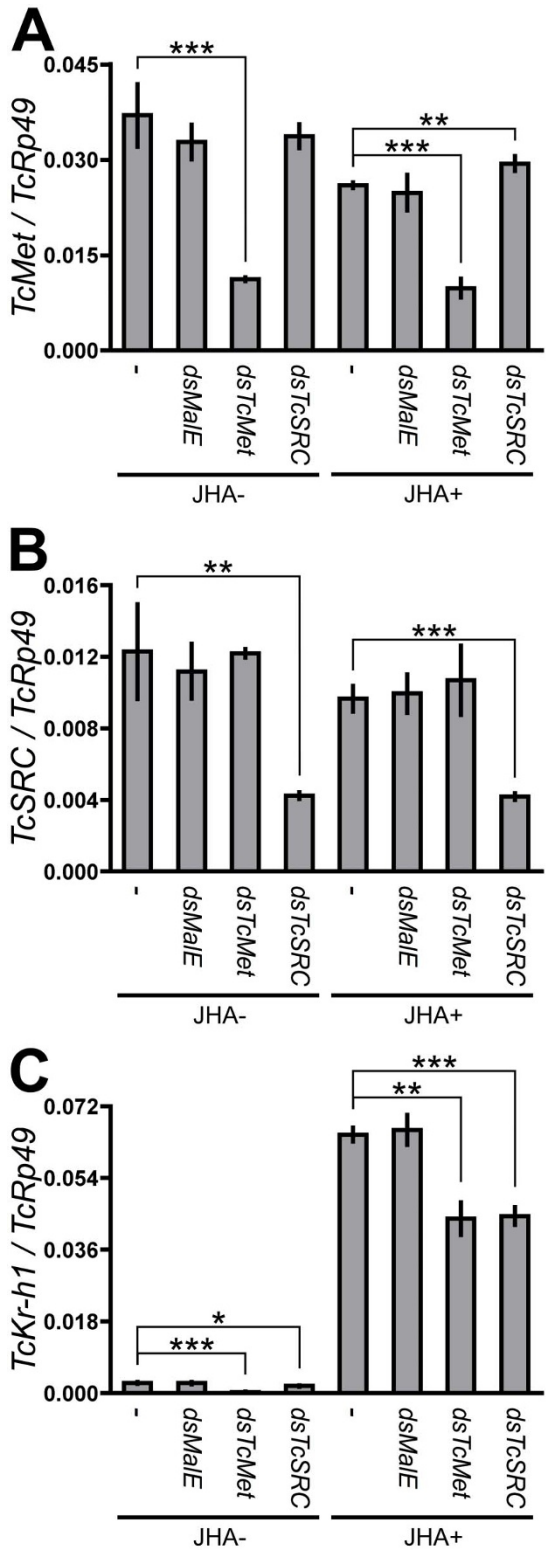

Figure 4 | Effects of RNAi-mediated knockdown of *TcMet* and *TcSRC* on the JH-dependent induction of *TcKr-h1* in Tc81 cells. Cells were soaked in 50 ng/μL dsRNA for *MalE*, *TcMet*, or *TcSRC* for 60 h. Control cells (−) were not treated with dsRNA. Cells were then incubated in media containing 10 μM JHA (JHA+) or JHA-free media (JHA-) for 2 h, and *TcMet* (A), *TcSRC* (B), and *TcKr-h1* (C) transcript levels were determined by qPCR (mean ± SD, n = 3). Data were analyzed using Student's *t*-tests (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; not indicated, $P > 0.05$).

cells was low before JHA treatment, but dramatically increased following JHA treatment. However, this induction by JHA was significantly suppressed in cells treated with *TcMet* or *TcSRC* dsRNAs (Fig. 4C). These results confirmed that both *TcMet* and *TcSRC* were involved in the signaling pathway mediating JH-induced *TcKr-h1* expression.

Identification and functional characterization of *k*JHREc in the *TcKr-h1* gene. Next, we searched for *k*JHREs in *Kr-h1* genes from 4 insect species. This search yielded candidate sequences in the first intron in addition to ones previously identified upstream of the first exon (Fig. 5A). These 11-bp sequences (GCCTCCACGTG) found in the first intron of *Kr-h1* in *B. mori* (2 sequences), *T. castaneum* (1 sequence), and *A. mellifera* (1 sequence) matched the original *k*JHREc identified upstream of *BmKr-h1* perfectly (Fig. 5B, top). Two putative *k*JHREcs, each having 1- or 2-nucleotide substitutes in the 5′ region, were also found in the first intron of the *Kr-h1* gene in *D. melanogaster* (Fig. 5A, B). All of these sequences contained an identical canonical E-box sequence (CACGTG).

To characterize the function of the putative *k*JHREcs found in the *TcKr-h1* gene in the context of JH stimulation, we carried out reporter assays in Tc81 cells. We tested reporter constructs carrying the upstream and first intron region (−477 to +1883), the upstream region alone (−477 to +106), and the first intron region alone (+106 to +1883). All 3 constructs showed 8- to 12-fold increases in luciferase reporter activity in the presence of JHA (Fig. 5C). When a mutation (CGCCTCCACGTG to TTTAAATTTAAA) was introduced in one or both of the *k*JHREcs in the −477 to +1883 region, reporter activity in the presence of JHA decreased by 51%–63% with single mutations and by 73% with double mutations, as compared to the wild-type reporter (Fig. 5C). When *k*JHREcs were mutated in the reporter harboring the upstream region alone (−477 to +106) or the first intron region alone (+106 to +1883), JHA-dependent stimulation was completely abolished (Fig. 5C). These results indicated that the 2 *k*JHREcs located upstream of the transcription start site and first intron of *TcKr-h1* contributed independently and additively to the JH-dependent induction of *TcKr-h1*.

Effects of *TcMet* and *TcSRC* RNAi on *k*JHRE reporter activity in Tc81 cells. To examine the interactions of *TcMet*, *TcSRC*, and *k*JHRE in the JH-dependent induction of *TcKr-h1*, we further performed reporter assays in Tc81 cells using a *k*JHRE reporter vector (−477 to +1883) in combination with *TcMet* or *TcSRC* RNAi. The reporter activities induced by JHA treatment were decreased by about 75% in cells treated with *TcMet* or *TcSRC* dsRNA, as compared to untreated control cells or cells treated with *MalE* dsRNA (Fig. 6). This result was consistent with the effects of *TcMet* and *TcSRC* RNAi on the JH-dependent induction of *TcKr-h1* transcripts (Fig. 4C), indicating that TcMet and TcSRC induced the transcription of the *TcKr-h1* gene via interaction with the *k*JHRE in the presence of JH.

Analysis of the interactions between *TcMet*, *TcSRC*, and *k*JHRE in mammalian cells. Next, we examined the JH-dependent interaction of *TcMet* and *TcSRC* using full ORFs of these proteins in mammalian 2-hybrid reporter assays. As expected, no JHA-dependent increase in UAS reporter activity was observed when HEK293 cells were transfected with Gal4DBD-TcMet, Gal4DBD-TcSRC, VP16AD-TcMet, or VP16AD-TcSRC (Fig. 7A). In contrast, when cells were cotransfected with Gal4DBD-TcMet:VP16AD-TcSRC or Gal4DBD-TcSRC:VP16AD-TcMet, UAS reporter activity was significantly increased in the presence of JHA (Fig. 7A). These results suggested that the full ORFs of *TcMet* and *TcSRC* proteins interacted with each other in a JH-dependent manner in HEK293 cells.

Finally, we examined whether the *k*JHRE reporter (−477 to +1883) containing the 2 *k*JHREc sequences could be induced by JH in HEK293 cells and *Drosophila* S2 cells in which full-length *TcMet* and *TcSRC* proteins were heterologously expressed.

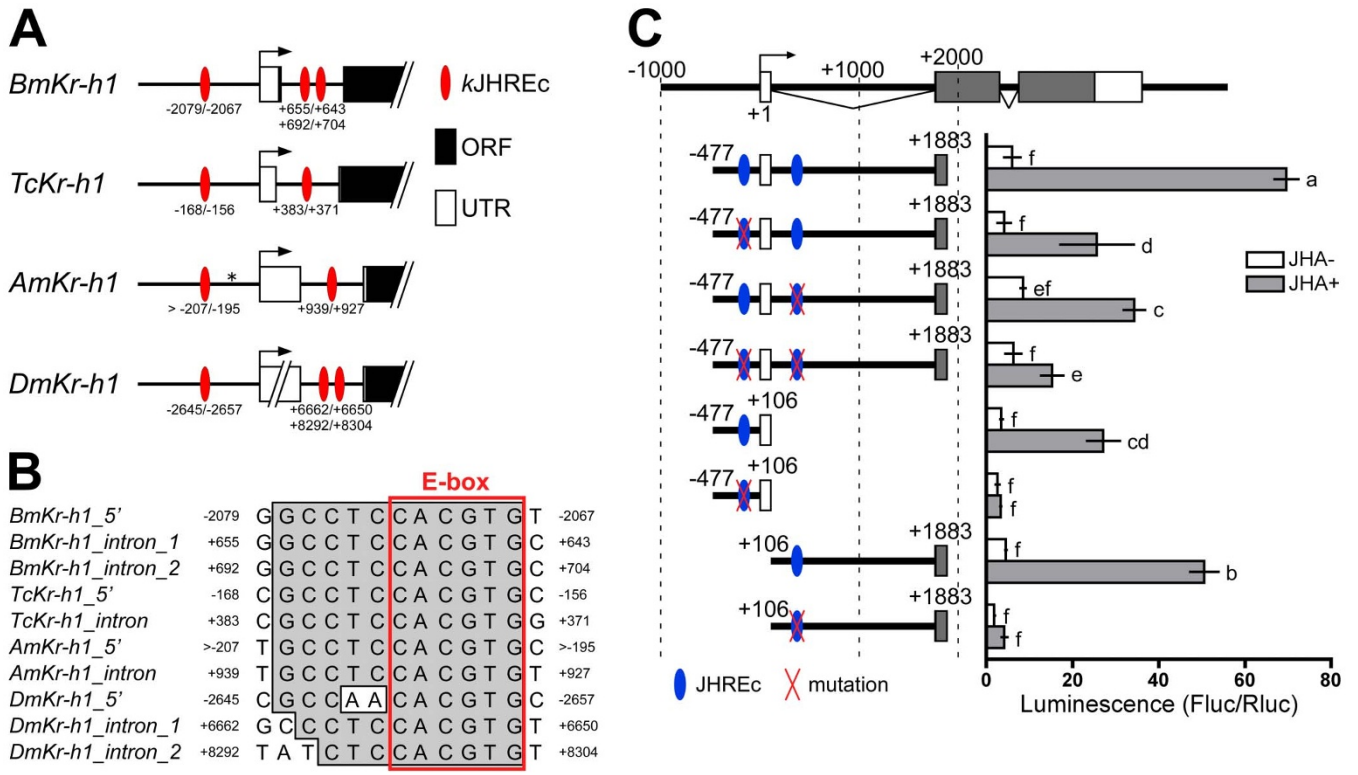

Figure 5 | Prediction and functional determination of JHREs of *TcKr-h1*. (A) Schematic representation of core JHRE (*k*JHREc)-like sequences present upstream and within the first intron of *Kr-h1* genes from 4 insect species. *Tc*, *T. castaneum*; *Bm*, *B. mori*; *Am*, *A. mellifera*; *Dm*, *D. melanogaster*. White boxes, black boxes, and arrows represent the 5′-UTRs, ORFs, and transcription start sites, respectively. The numbers indicate distances from the transcription start site. Red ellipses represent the authentic *k*JHREc (-2079/-2068) in *B. mori*[26] and its homologs. The asterisk shows a gap in the genomic sequence. (B) Alignment of the putative *k*JHREc sequences is shown in (A). (C) Functional characterization of putative *k*JHREcs of *TcKr-h1*. Tc81 cells were cotransfected with reporter plasmids that express *firefly luciferase* under the regulation of indicated regions and a reference reporter plasmid carrying *Renilla luciferase*. Red Xs indicate mutations in putative *k*JHREc sequences (see Materials and Methods for details). Cells were treated with 10 μM JHA for 24 h, and reporter activity was measured using a dual-luciferase reporter assay system. The activity of the firefly luciferase reporter was normalized against that of the *Renilla* luciferase reporter in the same samples. Data represent means ± SD (n = 3). Means with the same letter are not significantly different (Tukey–Kramer test, $P < 0.05$).

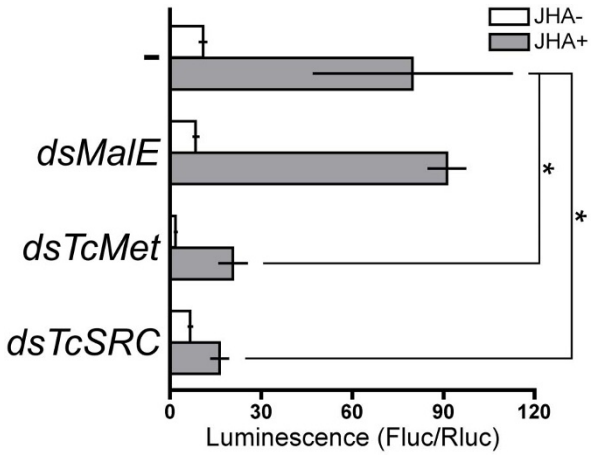

Figure 6 | Effects of *TcMet* and *TcSRC* RNAi and JHA treatment on reporter activity in Tc81 cells. Tc81 cells were cotransfected with a reporter plasmid carrying a *k*JHRE region (−477 to +1883, pGL4.14) conjugated to *firefly luciferase*, a reference reporter plasmid carrying *Renilla luciferase*, and the indicated dsRNAs. The cells were treated with 10 μM methoprene (JHA) for 24 h, and reporter activities were measured using a dual-luciferase reporter assay system. The activity of the firefly luciferase reporter was normalized to that of the *Renilla* luciferase reporter in the same samples. Data represent means ± SD (n = 3). Data were analyzed using Student's *t*-tests (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; not indicated, $P > 0.05$).

The wild-type reporter showed no JH-dependent induction in HEK293 cells in which neither *TcMet* nor *TcSRC* were expressed (Fig. 7B). Similarly, when only one construct (i.e., *TcMet* or *TcSCR*) was expressed, the wild-type reporter again exhibited no JH-dependent induction. In contrast, weak but significant JH-dependent induction was observed in cells in which both *TcMet* and *TcSRC* were expressed. Similar levels of JH-dependent induction were observed when only the upstream *k*JHRE was mutated in the reporter vector; this effect was abolished when the *k*JHRE in the first intron was mutated or when both *k*JHREs were mutated (Fig. 7B). The insertion of the mammalian minimal promoter (minP), which is suitable for mammalian cells, into the downstream region of the *k*JHRE reporter (−477 to +1883), did not enhance JH-dependent induction (Supplementary Fig. 3A online). In *Drosophila* S2 cells, the *k*JHRE reporter was significantly activated by JH only when the both *TcMet* and *TcSRC* were expressed, and this induction was abolished by the use of a mutated *k*JHRE reporter (Supplementary Fig. 3B online).

Discussion

In the current study, we established a novel cell line (Tc81) from *T. castaneum* embryos. This highly sought after cell line was highly efficient in RNAi and could be used for the analysis of JH-dependent signaling pathways.

In *T. castaneum*, RNAi-mediated knockdown of a target gene by direct injection of dsRNA into individuals has been shown to provide systemic and highly efficient knockdown throughout the embryonic and postembryonic stages[27]. Therefore, we hypothesized that a cell

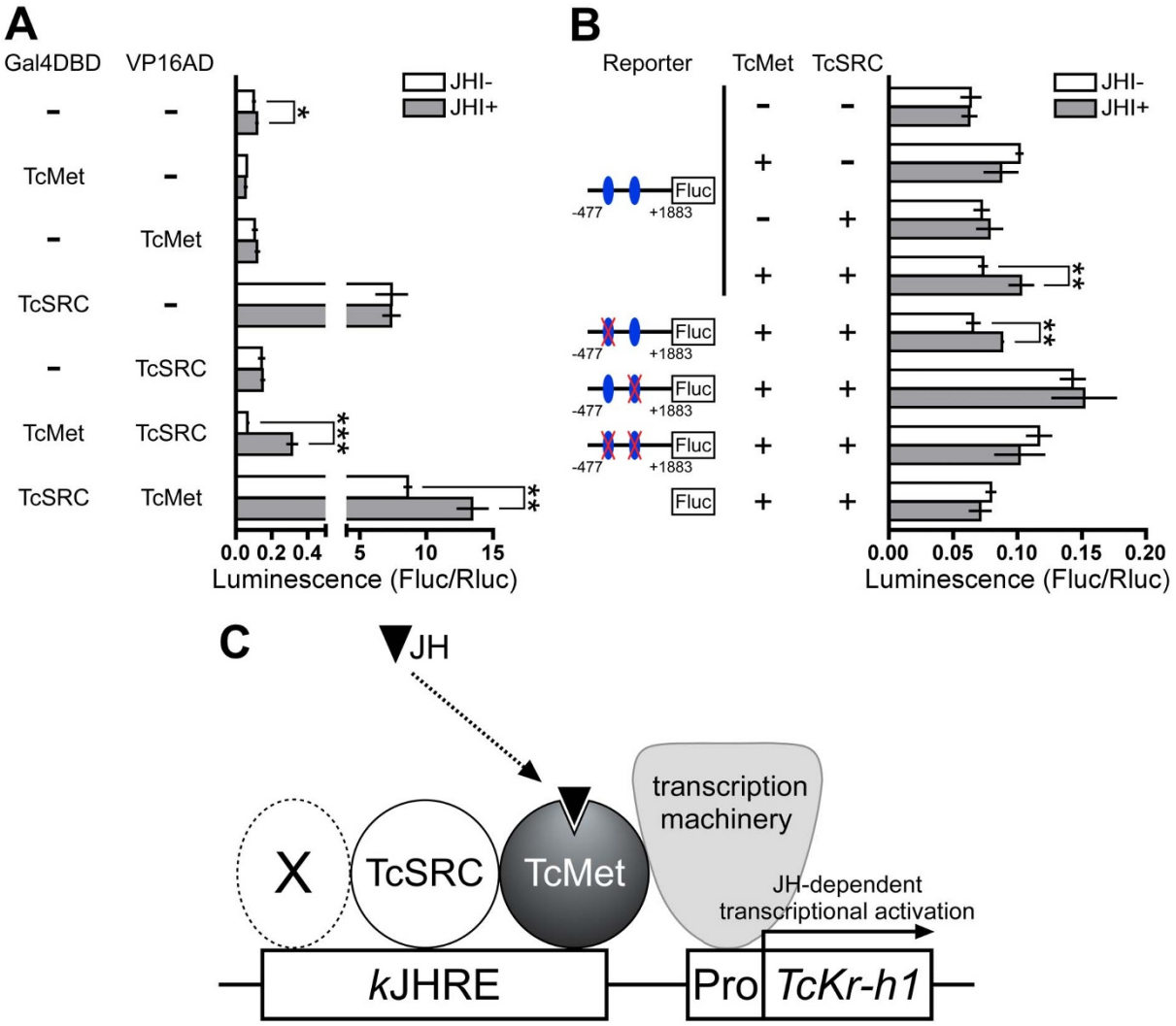

Figure 7 | Functional analysis of *TcMet*, *TcSRC*, and *k*JHRE in mammalian HEK293 cells. (A) Cells were cotransfected with a UAS reporter plasmid carrying *firefly luciferase*, a reference reporter plasmid (pRL-TK) carrying *Renilla luciferase*, and an expression plasmid carrying *GAL4DBD* or *VP16AD* fused with *TcMet* or *TcSRC*. Cells were then treated with 0.1 μM JH III for 24 h. (B) Cells were cotransfected with a *k*JHRE-reporter vector (−477 to +1883, pGL4.14) and a plasmid expressing the full ORF of *TcMet* or *TcSRC*. Cells were then treated with 0.1 μM JH III for 24 h. Xs indicate mutations in the *k*JHREc. (A, B) Reporter activity was examined using a dual-luciferase reporter assay system. Data represent means ± SD (n = 3). Data were analyzed using Student's *t*-tests (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; not indicated, $P > 0.05$). (C) Schematic representation of the interactions of *k*JHRE, TcMet, and TcSRC in the JH-dependent transcription of *TcKr-h1*. X represents an additional cofactor.

line generated from *T. castaneum* tissues would also maintain the characteristic of high RNAi efficiency. Although one *T. castaneum* cell line was established very recently from adult and pupal tissues[6], the efficiency of RNAi in these cells has not yet been determined. In this study, we generated a new *T. castaneum* cell line (Tc81) with a different origin (embryos) and demonstrated that this cell line maintained high RNAi efficiency, as expected. The cells exhibited a rapid decline in *Met* transcripts in a gene-specific manner following a single soaking with low concentrations of *Met* dsRNA. Repeated dsRNA treatment was not required to obtain highly efficient knockdown through day 8 after dsRNA treatment. Thus, Tc81 cells appear to be valuable for RNAi-based functional gene analysis.

Tc81 cells were shown to be highly sensitive to JH in terms of the induction of the *Kr-h1* gene. The *Kr-h1* gene has been reported to be induced by JH in a wide variety of insect species[16,19–21,26,28–30] and also in several insect cell lines[25,26]. Therefore, *Kr-h1* is recognized as an indicator of the JH sensitivity of individual insects and insect cells. In Tc81 cells, *TcKr-h1* transcripts were rapidly induced by JHA (within 30 min) and by subnanomolar concentrations of natural JHs. This high JH sensitivity implied that Tc81 cells may be valuable for the analysis of the JH signaling pathway at the cellular level.

Moreover, the dose-response and temporal JH-induction profiles of *TcKr-h1* suggested that Tc81 cells maintained some characteristic features of JH responsiveness observed in *T. castaneum* individuals. In terms of *TcKr-h1* induction, Tc81 cells were more sensitive to JH III (EC_{50}, 2.7×10^{-11} M) than to JH I (EC_{50}, 4.7×10^{-11} M). In contrast, NIAS-Bm-aff3 cells derived from *B. mori* fat bodies are more sensitive to JH I (EC_{50}, 1.2×10^{-10} M) than to JH III (EC_{50}, 2.6×10^{-10} M)[26]. Since JH I and JH III are the major JHs in Lepidoptera and Coleoptera, respectively[31], Tc81 and NIAS-Bm-aff3 cells reflect the characteristic response to JH species in their respective organisms. In addition, the temporal JH-induction profile of the *Kr-h1* gene in Tc81 was similar to that observed in *T. castaneum* pupae[19]. *TcKr-h1* reached its peak at 1 h and decreased soon after in *T. castaneum* pupa that were topically treated with JHA[19]. Likewise, *TcKr-h1* was induced in 1 h and then declined rapidly by 2 h in Tc81 cells. In contrast, in *B. mori* NIAS-Bm-aff3 cells, *Kr-h1* transcripts reached a peak at 2 h after JHA treatment and were maintained at high levels until 12 h after treatment[26]. Therefore,

the early decline in *TcKr-h1* after JH induction observed both in pupae and cells may be regulated by a common mechanism at the cellular level.

Taking advantage of the high RNAi efficiency and JH sensitivity of Tc81 cells, we established the function of TcMet and TcSRC in JH signaling. Previously, using *in vivo* RNAi in the larvae and pupae of *T. castaneum* individuals, we found that *TcKr-h1* is induced by JH via Met[19]. However, since systemic RNAi was employed in *T. castaneum* individuals, it was unclear whether the effects of RNAi were tissue autonomous or occurred via humoral and/or neuronal regulation from other tissues in which the target gene was knocked down. In addition, *in vivo* RNAi is limited in that it cannot be used for the functional analysis of vital genes whose knockdown results in a lethal phenotype. For example, *SRC* RNAi in *T. castaneum* larvae caused a lethal phenotype[32], which hampered the elucidation of its function in JH signaling. Thus, the use of RNAi in Tc81 cells could circumvent these disadvantages of *in vivo* RNAi. Indeed, our study revealed that both *TcMet* and *TcSRC* mediated JH signaling to induce *TcKr-h1* in a cell-autonomous manner, demonstrating the usefulness of this novel cell line.

In a previous study, we reported the presence of a *k*JHREc-like sequence upstream of *TcKr-h1*[26]. In the current study, we identified an additional putative *k*JHREc in the first intron of *TcKr-h1*. By reporter assays in Tc81 cells, we demonstrated that both *k*JHREcs were critical for the JH-dependent induction of the *TcKr-h1* gene. Intriguingly, additional *k*JHREc-like sequences were also found in the first intron of *Kr-h1* genes in *B. mori*, *A. mellifera*, *and D. melanogaster*. Recently, Shin et al. reported an E-box-like sequence (CACGCG), which interacts with the protein complex of Met and Cycle, upstream of the *Kr-h1* (*AaKr-h1*) gene in *A. aegyptii*[33]. This E-box-like sequence was also found in the JHRE of the *early trypsin* gene[24], but was not found in the promoter or first intron region of the *TcKr-h1* gene examined in this study (Supplementary Fig. 4 online). On the other hand, a *k*JHREc-like sequence (GCCTCCACGTG) was found in the vicinity of the E-box-like sequence in *AaKr-h1*[33]. Since *Kr-h1* is commonly induced by JH in a wide variety of insect species as described above, it is reasonable to hypothesize that the critical role of *k*JHREc for JH-dependent transcriptional induction is highly conserved in insects.

The presence of multiple *Kr-h1* transcript isoforms that differ in their 5′ regions were reported in *D. melanogaster*, *B. mori*, and *Frankliniella occidentalis*[15,26,30]. In *D. melanogaster* and *B. mori*, the α-isoform is transcribed from a promoter located upstream of the first exon, and the β-isoform is transcribed from an alternative promoter located in the first intron[15,26]. Therefore, the *k*JHRE upstream of the first exon and in the first intron may regulate the α- and β-isoforms, respectively, in these insects. Alternatively, *k*JHREc in the first intron may work together with the upstream *k*JHREc to induce *Kr-h1* transcripts from the upstream promoter. We have attempted to isolate the second isoform of *TcKr-h1*, for which transcription starts downstream of *k*JHREc in the first intron, but our attempts using RACE have not yet been successful. Further analyses of the role of individual *k*JHREcs in the isoform-specific regulation of *Kr-h1* are required in *T. castaneum* and other insect species.

In combination with reporter assays and RNAi in Tc81 cells, we clearly demonstrated that TcMet and TcSRC were involved in the JH-dependent induction of *TcKr-h1* via interactions with the *k*JHRE. We have previously shown that BmMet2 and BmSRC induce *BmKr-h1* via interaction with the *k*JHRE using reporter assays in HEK293 cells heterologously expressing BmMet2 and BmSRC[26]. However, since RNAi has not been shown to be effective in *B. mori* individuals and cells, we failed to demonstrate direct interactions between *k*JHRE, BmMet2, and BmSRC in *B. mori* cells[26]. Thus, in the current study, we used a combination of reporter assays and RNAi in Tc81 cells to facilitate the analysis of JH signaling. This strategy in Tc81 cells may be more generally applicable to the analysis of interactions between transcription factors and *cis*-regulatory elements.

Finally, we attempted to reconstitute the JH induction system using *k*JHRE, TcMet, and TcSRC in mammalian HEK293 cells lacking an intrinsic JH signaling pathway. Using partial recombinant proteins containing PAS domains, previous studies have shown that TcMet and TcSRC physically interact with each other depending on the presence of JH[23,25]. The 2-hybrid assay system in HEK293 cells using full-length TcMet and TcSRC proteins in the current study was able to confirm the JH-dependent interaction of these proteins, indicating that both proteins were functional in mammalian cells. However, contrary to our expectations, the heterologous expression of full-length TcMet and TcSRC proteins in HEK293 cells could only marginally activate a reporter carrying 2 *k*JHREc sequences, unlike the case of BmMet2 and BmSRC proteins[26]. In contrast, in *Drosophila* S2 cells, JH-dependent induction was observed only in the context of TcMet and TcSRC expression. These results suggested that additional factors intrinsic to insect cells are vital for the JH-dependent activation of the *TcKr-h1* gene via *k*JHREc (Fig. 7C). Using Tc81 cells, RNAi-based screening of the missing factors guided by *k*JHRE reporter activity is currently underway.

In conclusion, we have succeeded in establishing a new *T. castaneum* cell line and elucidated a portion of the JH signaling pathway in *T. castaneum* using this novel cell line. Since reverse genetic analysis using injection of dsRNA *in vivo* is highly effective in *T. castaneum*, *T. castaneum* is frequently used as a model organism to elucidate the molecular mechanisms of various physiological phenomena, such as hormonal regulation, immunity, apoptosis, and RNAi[7]. We anticipate that our novel Tc81 cell line will become a vital tool applicable to a wide range of research fields in *T. castaneum*.

Methods

Establishment of a *T. castaneum* cell line (Tc81). A wild-type strain of *T. castaneum*, which was obtained from the National Food Research Institute (Japan), was reared in whole wheat flour at 30°C. Twenty eggs were sterilized within 24 h of being laid by immersion in 1% Kitchen Haiter solution (Kao) for 30 min and 70% ethanol for 5 min. The sterilized eggs were rinsed twice with Carlson's solution[34] and once with MGM-464 medium supplemented with 20% fetal bovine serum (FBS)[35]. The rinsed eggs were gently homogenized with a pestle by hand, and the homogenized cells were cultured at 25°C with gentle shaking in 600 μL MGM-464 medium containing 20% FBS, 1 mg/mL reduced-form glutathione, and 1% antibiotic-antimycotic (Gibco, Invitrogen) for 5 days. Fresh medium (500 μL) was then added to the culture, and the shaking culture was changed to a static culture. The medium was partially replaced (30%) with fresh medium once a week. After the fifth generation of culture, the medium was changed to MGM-464 medium containing 10% FBS (glutathione- and antibiotic-free), and this process of adding and replacing medium was continued for 15 months. Unless stated otherwise, MGM-464 medium supplemented with 10% FBS was used for the following experiments. The average sizes of vesicles and cells were calculated by measuring the lengths of 18 vesicles in Fig. 1 and 5 cells in the inset in Fig. 1, respectively. The nuclei were stained with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories). For dsRNA treatment and reporter assays, healthy vesicles were collected by centrifugation at 300 × *g* for 3 min, and pellets were disrupted by gentle pipetting 5 times. To obtain growth curves for Tc81 cells, the cells were seeded at the 120th passage at a density of 1.27×10^8 cells/well in 100 μL medium in 96-well plates (Iwaki) and were collected every other day for 8 days. The collected cells were centrifuged at 500 × *g* for 3 min and were lysed with cell lysis buffer from the ProteoJET Cytoplasmic and Nuclear Protein Extraction Kit (Fermentas). The lysed cells were stained with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories), and DAPI-stained nuclei were counted under a fluorescence microscope; the number of nuclei was assumed to represent the number of cells.

Chemicals. JH I was purchased from SciTech, JH III was purchased from Sigma-Aldrich, and FA was purchased from Echelon Research Laboratories. Methoprene (SDS Biotech) was a kind gift from Dr. Syo Sakurai (Kanazawa University). Appropriate amounts of compounds dissolved in methanol were transferred into glass vials coated with polyethylene glycol 20,000 (PEG20,000; Wako). The methanol was evaporated under a stream of nitrogen, appropriate amounts of medium were added to the vial, and the compounds were dissolved by sonication using a Biorupter (Cosmo Bio).

JH treatment in Tc81 cells. To examine temporal changes in the expression of *TcKr-h1* after methoprene (JHA) treatment, Tc81 cells were seeded at a density of 1.0×10^3 vesicles with 100 μL medium in a well of 96-well plate and incubated for 1 day before

JHA treatment. Fresh medium (100 μL) containing 20 μM JHA was added to precultured cells (final concentration of JHA, 10 μM), and the cells were cultured for 30 min to 24 h at 25°C and collected for RNA extraction.

To examine the concentration-response relationship, cells were seeded at 1.0×10^3 vesicles/well in 100 μL medium in 96-well plates coated with PEG20,000 and incubated for 1 day before JH treatment. Fresh medium containing JH I, JH III, JHA, or FA was added to precultured cells, and the cells were incubated for 2 h at 25°C and collected for RNA extraction.

cDNA cloning of *TcSRC*. Primers used for the cloning of *TcSRC* cDNA were designed based on the sequence of the *T. castaneum* genome (Supplementary Table 1 online). A *TcSRC* cDNA fragment was amplified by PCR with first-strand cDNA prepared from the whole bodies of *T. castaneum* larvae and RTPCR_FW and RTPCR_RV primers (Supplementary Table 1 online). The 5′- and 3′-end sequences were obtained by 5′ RACE and 3′ RACE with 5′ RACE and 3′ RACE primers, respectively (Supplementary Table 1 online) using a GeneRacer kit (Invitrogen). The full-length cDNA sequence was obtained by combining the sequence data obtained from RT-PCR and RACE analyses. The full ORF of *TcSRC* was amplified from the cDNAs by PCR using ORF_F and ORF_R primers (Supplementary Table 1 online), subcloned into the pCR4Blunt-TOPO vector (Invitrogen), and sequenced.

RNAi experiments. Template DNA fragments of *TcMet*, *TcSRC*, and *TcKr-h1* used for the synthesis of double-stranded RNA (dsRNA) were amplified by PCR with primers and templates listed in Supplementary Table 2 online and purified using a Wizard SV Gel and PCR Clean-Up System (Promega). dsRNAs were synthesized from the amplified DNA using RiboMAX SP6 and T7 Large Scale RNA Production Systems according to the manufacturer's instructions (Promega).

To examine the temporal effect of RNAi by soaking cells in dsRNAs, Tc81 cells were seeded at a density of 1.0×10^3 vesicles in 100 μL medium in a well of a 96-well plate, and dsRNA for *MalE* or *TcMet* (1 μL, 5 μg/μL) was added to the medium (final concentration, 50 ng/μL). The cells were cultured for 1–8 days after the first dsRNA treatment. In some experiments, dsRNA for *MalE* or *TcMet* (1 μL, 5 μg/μL) was further added to the cells at day 4 after the first dsRNA treatment (final concentration, 100 ng/μL) and incubated for an additional 1–4 days.

To examine concentration-response relationships, cells were seeded at a density of 1.0×10^3 vesicles/well in 100 μL medium containing various concentrations of dsRNAs for *MalE* or *TcMet* (0.0005–500 ng/μL) in 96-well plates. Cells were then incubated at 25°C for 60 h and collected for RNA extraction.

Quantitative RT-PCR (qPCR). qPCR analysis was performed essentially as described previously[26]. The primers used for qPCR are listed in Supplementary Table 2 online.

Identification of the *k*JHREc in *Kr-h1* genes of several insect species. We searched for putative *k*JHRE core sequences (*k*JHREcs) by homology using the sequence 5′-CTCCACGTG or its complementary sequence 5′-CACGTGGAG as queries in the first intron of *Kr-h1* genes of 4 insect species (*T. castaneum*, *B. mori*, *Apis mellifera*, and *D. melanogaster*).

Construction of reporter and expression vectors. Genomic DNA was extracted from *T. castaneum* larvae by conventional methods[36]. A region containing the 5′-flanking and first intron (−477 to +1833) of the *TcKr-h1* gene (Supplementary Fig. 4 online) was amplified from genomic DNA by PCR using the KOD FX DNA polymerase (Toyobo, Japan) and gene-specific primers tagged with attB1 or attB2 (listed in Supplementary Table 3 online). The amplified DNA was inserted into the pGL4.14 luciferase reporter vector (Promega) modified for the Gateway system (Invitrogen)[26] to construct pGL4.14_−477/+1883. Deletion and mutation vectors were constructed from pGL4.14_−477/+1883 by inverse PCR (iPCR) using the KOD -Plus- Mutagenesis Kit (Toyobo) with TcKrh1_ProiPCR primers (Supplementary Table 3 online).

Vectors for expressing TcMet or TcSRC proteins fused at the N terminus with the GAL4 DNA-binding domain (GAL4DBD) or VP16 activation domain (VP16AD) were constructed using the CheckMate Mammalian Two-Hybrid System (Promega). Full ORFs of TcMet and TcSRC cDNAs were amplified by PCR using the templates and primers listed in Supplementary Table 1 online. The amplified DNAs were ligated into *Not*I- and *Kpn*I-digested pBIND and pACT plasmids (Promega). To create vectors that expressed native TcMet and TcSRC proteins, GAL4DBD was removed from pBIND_TcMet and pBIND_TcSRC plasmids by iPCR with the pBINDiPCR primers listed in Supplementary Table 1 online.

Transfection and reporter assays. Tc81 cells were seeded at a density of 1.0×10^3 vesicles/well in 96-well plates and transfected with 40 μL medium (IPL-41, Gibco, Invitrogen) containing a mixture of plasmid DNAs (a reporter vector and a reference vector carrying *Renilla* luciferase, pIZT_RLuc[37], 0.2 μg each) and a transfection reagent (0.6 μL; Fugene HD, Promega). Fresh medium (100 μL MGM-464 medium containing 10% FBS) was added to the wells 1 h after the transfection. In reporter assays with RNAi, dsRNA (500 ng), plasmid DNAs (0.2 μg each), and transfection reagent (0.6 μL) were mixed with 40 μL IPL-41 medium and used for transfection. HEK293 cells were seeded at a density of 0.2×10^5 cells/well in 96-well plates and cultured for 2 days before transfection. HEK293 cells were then transfected with 40 μL medium (MEM, Sigma-Aldrich) containing a mixture of plasmid DNAs (a reporter vector and a reference vector carrying *Renilla* luciferase, pRL-TK [Promega], 0.2 μg each) and Lipofectamine 2000 (0.6 μL; Invitrogen). The medium was replaced with MEM supplemented with 10% FBS and nonessential amino acids (Gibco, Invitrogen) 6 h after transfection. The transfected cells were then incubated for 60 h and treated with JHs for 1 day. Reporter activities were measured using a Dual-Luciferase Reporter Assay System (Promega) and a luminometer (ARVO; PerkinElmer) according to the manufacturers' instructions.

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Author contributions

T.K., K.T. and T.S. designed the research, T.K. and K.T. performed the experiments, T.K., K.T. and T.S. analyzed data, and T.K. and T.S. wrote the paper.

Additional information

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