Developmental Biology

Receptor tyrosine kinases CAD96CA and FGFR1 function as the cell membrane receptors of insect juvenile hormone

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Abstract

Juvenile hormone (JH) is important to maintain insect larval status; however, its cell membrane receptor has not been identified. Using the lepidopteran insect *Helicoverpa armigera* (cotton bollworm), a serious agricultural pest, as a model, we determined that receptor tyrosine kinases (RTKs) cadherin 96ca (CAD96CA) and fibroblast growth factor receptor homologue (FGFR1) function as JH cell membrane receptors by their roles in JH-regulated gene expression, larval status maintaining, rapid intracellular calcium increase, phosphorylation of JH intracellular receptor MET1 and cofactor Taiman, and high affinity to JH III. Gene knockout of *Cad96ca* and *Fgfr1* by CRISPR/Cas9 in embryo and knockdown in various insect cells, and overexpression of CAD96CA and FGFR1 in mammalian HEK-293T cells all supported CAD96CA and FGFR1 transmitting JH signal as JH cell membrane receptors.

eLife Assessment

In this **valuable** study, Li and others identified cell membrane receptors for juvenile hormone (JH), a terpenoid hormone in insects that regulates their development and reproduction. While intracellular receptors for JH have been well characterized, membrane receptors for JH remained elusive for many years. Although the authors provide **solid** evidence to indicate that the receptor tyrosine kinases they identified bind to JH in vitro and induce non-genomic responses in cultured cells, their loss-of-function phenotypes are not consistent with known JH functions, so additional work is required to define physiological roles of these receptors.

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Introduction

Juvenile hormone (JH) plays a vital role in insect development and maintaining insect larval status. JH is an acyclic sesquiterpenoid known to enter cells freely via diffusion because of its lipid-soluble character (Riddiford, 2020). JH binds its intracellular receptor methoprene-tolerant

protein (MET), a basic helix-loop-helix/Per-ARNT-SIM (bHLH-PAS) family protein (Charles et al., 2011; Jindra et al., 2021). MET forms a transcription complex with the transcription factor Taiman (TAI, also known as FISC, p160/SRC, and is a steroid receptor coactivator) to initiate gene transcription (Charles et al., 2011; Zhu et al., 2003). An important gene in the JH pathway is Krüppel homologue 1 (Kr-h1), which encodes the zinc-finger transcription factor Kr-h1 (Minakuchi et al., 2008; Pecasse et al., 2000; Wu et al., 2021). Kr-h1 acts downstream of MET and is induced rapidly by JH to regulate larval growth and development (Minakuchi et al., 2009). Other genes, for example, the early trypsin gene of *Aedes aegypti* (*AaEt*) (Li et al., 2011; Noriega et al., 2003), JH-inducible 21 kDa protein (*Jhp21*) (Zhang et al., 1996), JH esterase (*Jhe*) (Feng et al., 1999; Wroblewski et al., 1990), vitellogenin (*Vg*) (Comas et al., 1999; Xu et al., 2014), *Drosophila* JH-inducible gene 1(*Jhi-1*), and JH-inducible gene 26 (*Jhi-26*) (Dubrovsky et al., 2000) are regulated by JH.

However, some studies suggest that cell membrane receptors also play essential roles in JH signaling (Davey, 2000; Jindra et al., 2021). For example, in *A. aegypti*, receptor tyrosine kinases (RTKs) are involved in JH-induced rapid increases in inositol 1,4,5-trisphosphate, diacylglycerol, and intracellular calcium, leading to activation of calcium/calmodulin-dependent protein kinase II (CaMKII) to phosphorylate MET and TAI, resulting in *Kr-h1* gene transcription in response to JH (Liu et al., 2015). JH III, also via RTKs, leads to rapid calcium release and influx in *Helicoverpa armigera* epidermal cells (HaEpi cells) (Wang et al., 2016). JH induces MET phosphorylation to increase MET interacting with TAI, which enhances Kr-h1 transcription in *H. armigera* (Li et al., 2021). In *Drosophila melanogaster*, JH through RTK and PKC protein kinase C (PKC) induces phosphorylation of ultraspiracle (USP) (Gao et al., 2022). The phenomenon that RTK transmits JH signal has long been predicted (Liu et al., 2015; Ojani et al., 2016); however, the RTKs critical for JH signaling have yet to be identified from numerous RTKs *in vivo*.

RTKs constitute a class of cell surface transmembrane proteins that play important roles in mediating extracellular to intracellular signaling. Humans carry approximately 60 RTKs (Manning et al., 2002), the *Drosophila* genome encodes 21 RTK genes (Sopko and Perrimon, 2013), *Bombyx mori* has 20 RTKs (Alexandratos et al., 2016), and the *German cockroach* genome identifies 16 RTKs (Li et al., 2022). *H. armigera* has 20 RTK candidates with gene codes in the *H. armigera* genome by our analysis. The cotton bollworm, is a well-known and worldwide distributing agricultural pest in Lepidoptera, which threatens cotton and many other vegetable crops by rapidly producing resistance to various chemical insecticides and Bt-transgenic cotton. Using *H. armigera* as a model, we focus on identifying the RTKs functioning as the JH receptors and demonstrating the mechanism. We screened the RTKs sequentially, including examining the roles of 20 RTKs identified in the *H. armigera* genome in JH regulated-gene expression to obtain primary candidates, followed by screening of the candidates by their roles in maintaining larval status, JH induced-rapid increase of intracellular calcium levels, JH induced-phosphorylation of MET and TAI, and affinity to JH. The cadherin 96ca (CAD96CA) and fibroblast growth factor receptor 1 (FGFR1) were finally determined as JH cell membrane receptors by their roles in JH regulated-gene expression, maintaining larval status, JH induced-rapid increase of intracellular calcium levels, JH induced-phosphorylation of MET and TAI, and their JH-binding affinity. Their roles as JH cell membrane receptors were further determined by knockdown and knockout of them *in vivo* and cell lines, and overexpression of them in mammal HEK-293T heterogeneously. These data not only improve our knowledge of JH signaling and open the door to studying insect development, but also present new targets to explore the new growth regulators to control the pest.

Results

The screen of the RTKs involved in JH signaling

To explore which RTKs may be involved in JH signaling, the total of RTKs were identified in the *H. armigera* genome. We found 20 RTK-like proteins encoded in the *H. armigera* genome and named the RTKs according to the nomenclature typically used in the genome or according to their homologues in *B. mori* or *D. melanogaster* (*Supplementary file 1*). Phylogenetic analysis showed that the 20 RTK candidates in *H. armigera* were conserved in *B. mori* and *D. melanogaster* (**Figure 1**—*figure supplement 1*). All the analyzed RTKs were grouped according to the basis of their structural characteristics and homology to the structure of 20 subfamilies of human (Honegger et al., 1989; Lemmon and Schlessinger, 2010; Sparrow et al., 1997; Yarden and Ullrich, 1988); the cell wall integrity and stress response component kinase (WSCK), tyrosine-protein kinase receptor torso like (TORSO) and serine/threonine-protein kinase STE20-like (STE 20-like) were not classed (**Figure 1**—*figure supplement 2*).

To identify the RTKs involved in JH III signaling, 20 RTKs of *H. armigera* were knocked down by RNA interference (RNAi) in HaEpi cells using JH III-induced *Kr-h1*, *Vg*, *Jhi-1*, and *Jhi-26* gene expression as readouts. When *Cad96ca*, *Drl* (encoding derailed), *Fgfr1*, *Nrk* (encoding neurotropic receptor kinase), *Vegfr1* (encoding vascular endothelial growth factor receptor 1), and *Wsck* were knocked down, respectively, JH III-upregulated expression of *Kr-h1* was decreased. However, knocking down other *Rtks* did not decrease the *Kr-h1* transcription level. When *Cad96ca*, *Drl*, *Fgfr1*, *Nrk*, *Vegfr1*, *Wsck*, and *Inr* (encoding insulin-like receptor) were knocked down, JH III-upregulated expression of *Vg* was decreased. RNAi of *Rtks* did not affect JH-induced *Jhi-1* expression. When *Cad96ca*, *Fgfr1*, *Nrk*, and *Vegfr1* were knocked down, JH III-upregulated expression of *Jhi-26* was decreased (**Figure 1A**). *Rtks* were confirmed to be knocked down significantly in HaEpi cells (**Figure 1**—*figure supplement 3A*). The off-target effects of their knockdown were excluded from the genes we detected. Off–target genes were selected based on the identity rate of nucleotide sequences (**Figure 1**—*figure supplement 3B*). By the primary screening of RNAi, six RTKs, CAD96CA, DRL, FGFR1, NRK, VEGFR1, and WSCK were chosen for further screening.

The tissue–specific and developmental expression profiles of the six selected RTKs were determined using qRT−PCR to identify their possible roles in tissues at different developmental stages. The mRNA levels of *Vegfr1*, *Drl*, *Cad96ca*, and *Nrk* showed no tissue specificity in the epidermis, midgut and fat body. Their transcript levels were high at the sixth instar feeding stage (6th–6 h to 6th–48 h) compared with those at the metamorphic molting stage (6th–72 h to 6th–120 h) and pupal stages (P–0 d to P–8 d). *Fgfr1* was highly expressed in the midgut at these feeding stages. *Wsck* was highly expressed from the 6th–48 h to the pupal stage and showed no tissue specificity (**Figure 1**—*figure supplement 4A*). These data suggested that the RTKs are differentially distributed in tissues and highly expressed during larval feeding stages.

We further examined the roles played by these six RTKs in JH III-delayed pupation by injecting double-stranded RNA (dsRNA) into the fifth instar 20 h larval hemocoel. The interference of these six RTK genes in larvae led to a significant decrease in the expression of *Kr-h1*, in which, *Cad96ca*, *Nrk*, *Fgfr1*, and *Wsck* knockdown resulted in the expression of *Br-z7* (encoding broad isoform Z7) increase (**Figure 1**—*figure supplement 4B*). The pupation time was approximately 162 h in 93% of the larvae in the dimethyl sulfoxide (DMSO) control group. After injection of JH III, the pupation time was approximately 187 h in 76% of the larvae, which was 25 h later than that of the DMSO control group, suggesting that JH III delayed pupation. In the *dsGFP*+JH III-injected control, 70% larvae pupated approximately the same time as larvae after JH III treatment. In the *dsVegfr1*+JH III and *dsDrl*+JH III treatment groups, 60–63% larvae exhibited delayed pupation; only 9–10% of the larvae did not show delayed pupation, and 28–30% died at the larval or pupal stage. However, 66–68% of the larvae did not show delayed pupation after *dsCad96ca*+JH III, *dsNrk*+JH III, *dsFgfr1*+JH III or *dsWsck*+JH III injection (**Figure 1B**, *C and* **Figure 1**—*figure supplement 4C*). These results indicated that CAD96CA, NRK, FGFR1, and WSCK are involved in JH III-induced delayed pupation.

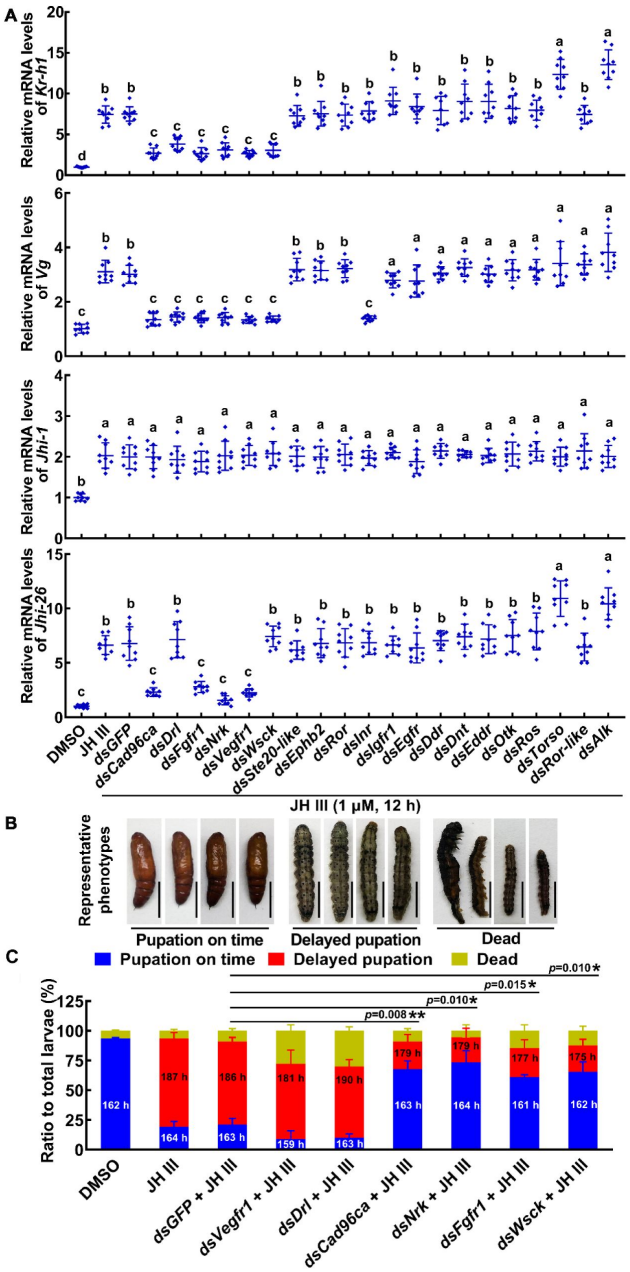

Figure 1.

RTKs were screened to determine their involvement in the JH signal pathway in HaEpi cells and larvae.

(**A**) The roles of RTKs in JH III-induced *Kr-h1*, *Vg*, *Jhi-1*, and *Jhi-26* expression were determined by RNAi of *Rtk* genes (1 μg/mL dsRNA, 48 h, 1 μM JH III for 12 h). DMSO as solvent control. The relative mRNA levels were calculated via the $2^{-\Delta\Delta CT}$ method and the bars indicate the mean ± SD. n = 3. Multiple sets of data were compared by analysis of variance (ANOVA). The different lowercase letters show significant differences. (**B**) The examples of phenotype after *Vegfr1*, *Drl*, *Cad96ca*, *Nrk*, *Fgfr1*, and *Wsck* knockdown in larvae. Scale = 1 cm. (**C**) Phenotype percentage and pupation time after *Vegfr1*, *Drl*, *Cad96ca*, *Nrk*, *Fgfr1*, and *Wsck* knockdown in larvae. The time was recorded from the bursting of the head shell of the 5th instar to pupal development. Images were collected after more than 80% of the larvae had pupated in the DMSO control group. Two-group significant differences were calculated using Student's *t* test (**p*<0.05, ***p*<0.01 indicate the significant difference between the percentages of the delayed pupation in *dsGFP* + JH III control group and gene knockdown) based on three replicates, n = 30 × 3 larvae.

To address the mechanism involved in the RTK effects on JH signaling, we examined the roles played by the selected RTKs in JH III-induced cellular responses by knocking down RTK gene expression in HaEpi cells (Wang et al., 2016). JH III-induced rapid calcium mobilization was repressed after knockdown of *Vegfr1*, *Drl*, *Cad96ca*, *Nrk*, *Fgfr1*, or *Wsck* compared with that after *dsGFP* knockdown (**Figure 2A**). The efficacy of RNAi was confirmed (**Figure 2B**). However, only *Cad96ca*, *Nrk*, and *Fgfr1* knocking down decreased the JH III-induced phosphorylation of MET1 and TAI (**Figure 2C**). The results suggested that these RTKs are involved in JH III-induced rapid cellular calcium increase but are differentially involved in JH III-induced MET1 and TAI phosphorylation.

CAD96CA and FGFR1 had high affinity to JH III

Since Cad96CA, FGFR1, and NRK were not only involved in JH-regulated *Kr-h1* expression, JH III-induced delayed pupation, and calcium levels increase, but also involved in MET and TAI phosphorylation, we further analyzed their binding affinity to JH III. OTK did not respond to JH III, so we used it as a control protein on the cell membrane to exclude the possibility of nonspecific binding. The affinity of CAD96CA, FGFR1, NRK, and OTK for JH III was determined using saturable specific–binding curve analysis via microscale thermophoresis (MST). The experiment used full–length sequences of CAD96CA, FGFR1, NRK, and OTK. CAD96CA-CopGFP-His (CopGFP is a 26 kDa green fluorescent protein cloned from copepod *Pontellina plumate*), FGFR1-CopGFP-His, NRK-CopGFP-His, and OTK-CopGFP-His were overexpressed in the Sf9 cell line (Sf9 cells expressed the proteins at a higher level than HaEpi cells) and then, the proteins were isolated separately to determine the JH III-binding strength of each. Immunocytochemistry showed that CAD96CA-CopGFP-His, FGFR1-CopGFP-His, NRK-CopGFP-His, and OTK-CopGFP-His located in the plasma membrane (**Figure 3A**). The purity of the proteins was assessed and confirmed using sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) with Coomassie brilliant blue staining (**Figure 3B**). CAD96CA-CopGFP-His binding to JH III exhibited a dissociation constant (Kd) = 11.96 ± 1.61 nM. Similarly, the saturable specific binding of FGFR1-CopGFP-His to JH III exhibited a Kd = 23.61 ± 0.90 nM, and NRK-CopGFP-His and OTK-CopGFP-His showed no obvious binding (**Figure 3C**). These results suggested that CAD96CA and FGFR1 bind JH III.

The JH intracellular receptor MET has been reported to bind to JH in *Tribolium* (Charles et al., 2011); therefore, the JH intracellular receptor MET1 in *H. armigera* was used as the positive control in analyses to assess the applicability of the MST method. MET1-CopGFP-His and CopGFP-His were overexpressed in the Sf9 cell line and then isolated to determine their binding strength to JH III. Immunocytochemistry showed the nuclear location of MET1 (**Figure 3**—*figure supplement 1A*). The purities of the isolated CopGFP-His and MET1-CopGFP-His proteins were examined and confirmed using SDS−PAGE with coomassie brilliant blue staining (**Figure 3**—*figure supplement 1B*). The saturable specific binding of MET1-CopGFP-His to JH III exhibited a Kd = 6.38 ± 1.41 nM. CopGFP-His showed weaker binding to JH III (**Figure 3**—*figure supplement 1C*). In comparison with the Kd of *Tribolium* MET to JH III of 2.94 ± 0.68 nM as detected by [^{3}H]JH III (Charles et al., 2011), the MST method was a valid approach to detect the JH III binding to a protein.

To further validate CAD96CA and FGFR1 binding JH III, saturation assays were performed using the analogs of JH, the farnesol, methoprene, and farnesoate (MF). Results showed that CAD96CA-CopGFP-His bound farnesol with a Kd of 1039.2 ± 0.68 nM. CAD96CA-CopGFP-His bound methoprene with a Kd of 553.94 ± 1.11 nM. CAD96CA-CopGFP-His bound methyl farnesoate (MF) with a Kd of 446.55 ± 0.80 nM. CAD96CA-CopGFP-His bound JH III with a Kd of 12.10 ± 1.4 nM (**Figure 3D**). The results confirmed that CAD96CA has the highest affinity to JH III. Because methoprene is known as an effective juvenoid (Konopova and Jindra, 2007) and competes with JH III in binding to MET (Charles et al., 2011), the compete experiment was performed to confirm CAD96CA bound JH III. CAD96CA-CopGFP-His bound to methoprene plus JH III with a Kd

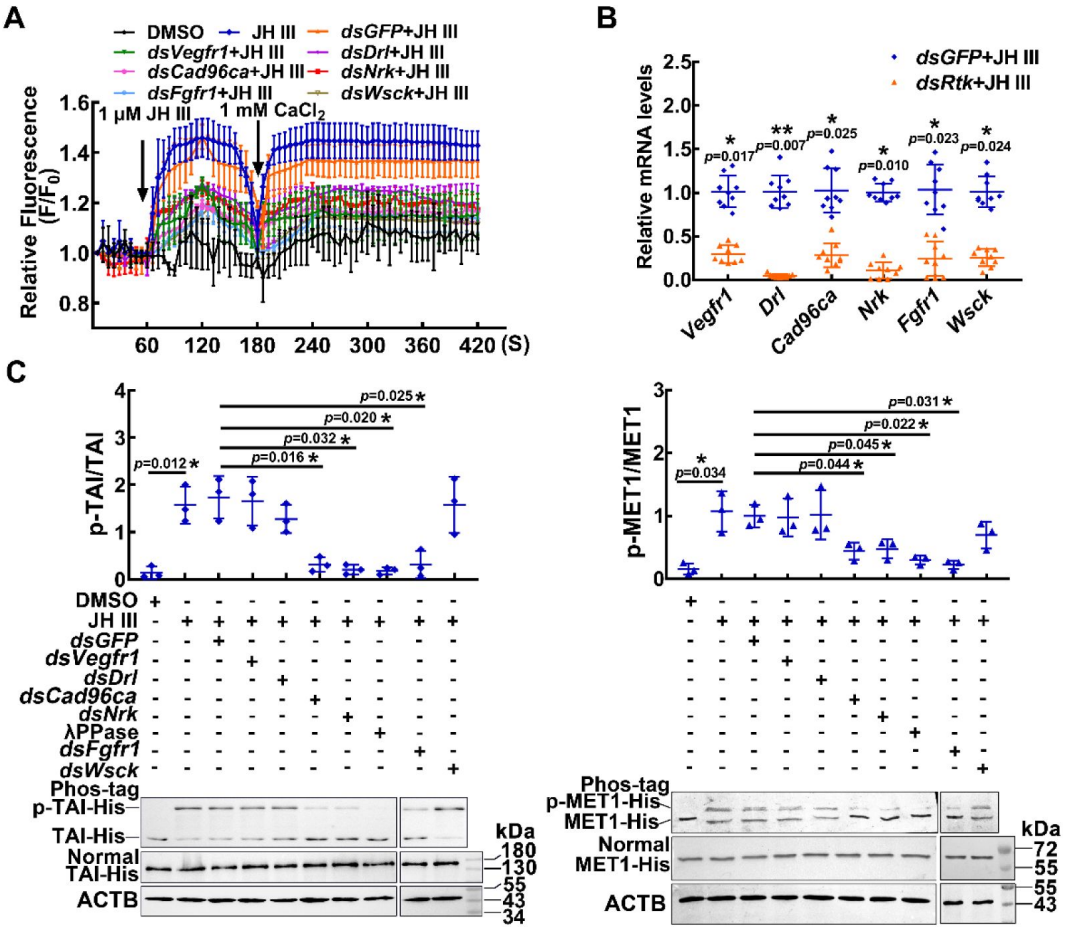

Figure 2.

RTKs involved in JH III-regulated Ca^{2+} increase and protein phosphorylation.

(**A**) The level of Ca^{2+} after *Vegfr1*, *Drl*, *Cad96ca*, *Nrk*, *Fgfr1*, and *Wsck* knockdown in HaEpi cells. The cells were incubated with dsRNA (the final concentration was 1 μg/mL for 48 h) and AM ester calcium crimson dye (3 μM, 30 min). F_0: the fluorescence intensity of HaEpi cells without treatment. F: the fluorescence intensity of HaEpi cells after different treatments. DMSO as solvent control. (**B**) The interference efficiency of dsRNA in HaEpi cells. (**C**) Western blotting was performed to analyze TAI-His and MET1-His phosphorylation after treatment with dsRNA and JH III (1 μM, 3 h). Phos-tag: phosphate affinity SDS–PAGE gel, Normal: normal SDS–PAGE gel, which was a 7.5 or 10% SDS–PAGE gel. The results of three independent repeated western blots were statistically analyzed by ImageJ software. The *p* value was calculated by Student's *t* test based on three independent replicate experiments. The error bar indicates the mean ± SD.

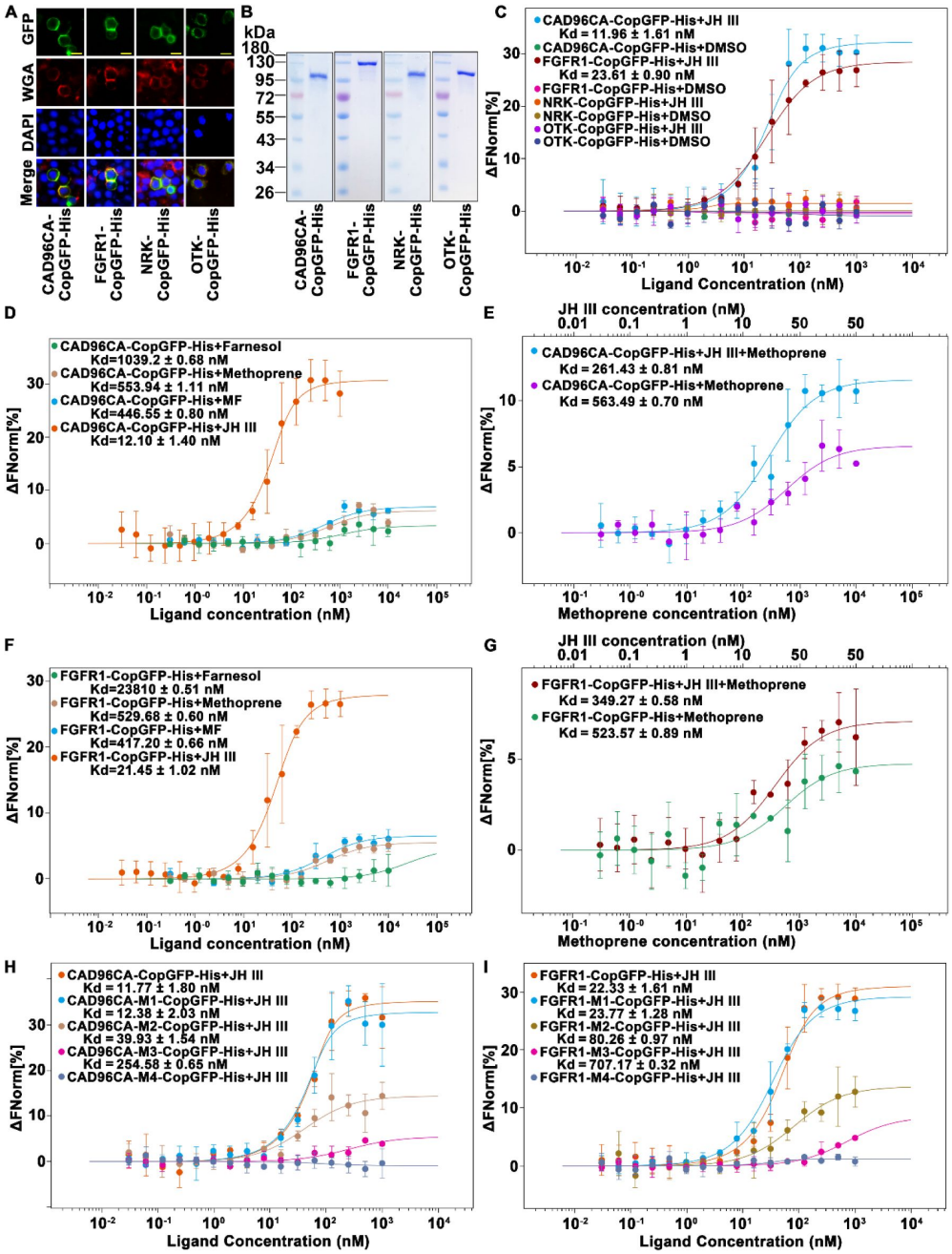

Figure 3.

CAD96CA and FGFR1 could bind JH III.

(**A**) Cell membrane localization of the overexpressed CAD96CA-CopGFP-His, FGFR1-CopGFP-His, NRK-CopGFP-His and OTK-CopGFP-His. GFP: green fluorescence of RTKs fused with a green fluorescent protein. WGA: red fluorescence, the cell membrane was labeled with wheat germ agglutinin. DAPI: nuclear staining. Merge: the pictures of different fluorescent-labeled cells were combined. The cells were observed with a fluorescence microscope. Scale bar = 20 μm. (**B**) Coomassie brilliant blue staining of the SDS−PAGE gel showed the purity of the separated CAD96CA-CopGFP-His, FGFR1-CopGFP-His, NRK-CopGFP-His, and OTK-CopGFP-His proteins. (**C**) Saturation binding curves of CAD96CA-CopGFP-His, FGFR1-CopGFP-His, NRK-CopGFP-His and OTK-CopGFP-His. (**D**) Saturation binding curves of CAD96CA-CopGFP-His were incubated with the indicated compounds. (**E**) The binding and competition curves of CAD96CA and methoprene. (**F**) Saturation binding curves of FGFR1-CopGFP-His were incubated with the indicated compounds. (**G**) The binding and competition curves of FGFR1 and methoprene. (**H**) The binding curves of CAD96CA mutants and JH III. (**I**) The binding curves of FGFR1 mutants with JH III. Data are mean ± SE of three replicates.

value of 261.43 ± 0.81 nM, whereas CAD96CA-CopGFP-His bound to methoprene with a Kd value of 563.49 ± 0.7 (**Figure 3E**). These suggested that CAD96CA-CopGFP-His has the highest affinity to JH III compared with the analogs.

Similarly, the saturable specific binding of FGFR1-CopGFP-His bound farnesol with a Kd = 23810 ± 0.51 nM; FGFR1-CopGFP-His bound methoprene with a Kd = 529.68 ± 0.60 nM; FGFR1-CopGFP-His to MF exhibited a Kd = 417.20 ± 0.66 nM; and FGFR1-CopGFP-His to JH III exhibited a Kd = 21.45 ± 1.02 (**Figure 3F**), suggesting FGFR1 had the highest affinity to JH III. The compete binding of FGFR1-CopGFP-His to methoprene plus JH III with a Kd value = 349.27 ± 0.58 nM, whereas FGFR1-CopGFP-His to methoprene with a Kd value = 523.57 ± 0.89 (**Figure 3G**). These suggested that FGFR1 has the highest affinity to JH III compared with the analogs.

Various mutants of CAD96CA and FGFR1 were further constructed to identify the key motifs in CAD96CA and FGFR1 critical for JH binding. Truncated mutations were performed on extracellular regions of CAD96CA and FGFR1, including CAD96CA-M1 (51-615 AA, amino acid), CAD96CA-M2 (101-615 AA), CAD96CA-M3 (151-615 AA), CAD96CA-M4 (201-615 AA), FGFR1-M1 (101-615 AA), FGFR1-M2 (201-615 AA), FGFR1-M3 (301-615 AA) and FGFR1-M4 (401-615 AA). Mutants were overexpressed, and the encoded mutants located in the plasma membrane, as confirmed via immunocytochemistry, and the purity of the proteins was confirmed using SDS− PAGE with Coomassie brilliant blue staining (**Figure 3**—*figure supplement 1D-I*). Compared with the wild-type counterparts, CAD96CA-M2, CAD96CA-M3, and CAD96CA-M4 mutants' affinity to JH III was significantly reduced (**Figure 3H**). Similarly, FGFR1-M2, FGFR1-M3, and FGFR1-M4 mutants for the binding affinity to JH III were significantly reduced (**Figure 3I**). These results suggested that the extracellular domain 51-151 AA in CAD96CA and the extracellular domain 101-301 AA in FGFR1 play a vital role in JH binding.

The affinity of CAD96CA, FGFR1, NRK, and OTK for JH III was further determined using saturable specific–binding curve analysis via isothermal titration calorimetry (ITC). ITC as an alternative method to further examine the affinity of CAD96CA and FGFR1 to JH III. CAD96CA-CopGFP-His bound JH III with a Kd value of 79.6 ± 27.5 nM. Similarly, the saturable specific binding of FGFR1-CopGFP-His to JH III with a Kd value of 88.5 ± 19.4 nM, and NRK-CopGFP-His and OTK-CopGFP-His showed no remarkable binding (**Figure 3**—*figure supplement 2*). These results also suggested that CAD96CA and FGFR1 bind JH III.

Gene knockout of *Cad96ca* or *Fgfr1* by CRISPR/Cas9 caused early pupation and a decrease of JH signaling

To verify the roles of CAD96CA and FGFR1 in JH signaling *in vivo*, we mutated *Cad96ca* or *Fgfr1* by CRISPR/Cas9 technology. We synthesized two gRNAs targeting different sites in the *Cad96ca* and *Fgfr1* coding regions with a low probability of causing off–target effects. Two gRNAs (referred to as *Cad96ca*-gRNAs) are located at the third exon of the *Cad96ca* gene (**Figure 4A**), and two gRNAs (referred to as *Fgfr1*-gRNAs) located at the second exon of the *Fgfr1* gene (**Figure 4B**) were used for the experiment.

When the Cas9-gRNA injected eggs (105 eggs were injected each for three injections, a total of 315 experimental eggs) had developed into second instar larvae, the survival rates were determined. The survival rate of the Cas9-gRNA-injected eggs (19.4~20.6%) did not significantly differ from that of the control eggs injected with Dulbecco's phosphate-buffered saline (DPBS) (a survival rate of 22.6%), suggesting that the mixture of gRNA and Cas9 protein was nontoxic to the *H. armigera* eggs. In 61 survivors of Cas9 protein plus *Cad96ca*-gRNA injection, 30 mutants were sequenced, and a mutation efficiency was 49.2%. Similarly, in the 65 survivors of Cas9 protein plus *Fgfr1*-gRNA injection, 35 mutants were sequenced, and a mutation efficiency was 53.8% (**Figure 4C**). The DNA sequences, deduced amino acids and off–target were analyzed (**Figure 4**—*figure supplement 1*). Most wild-type larvae showed a phenotype of pupation on time. However, in the

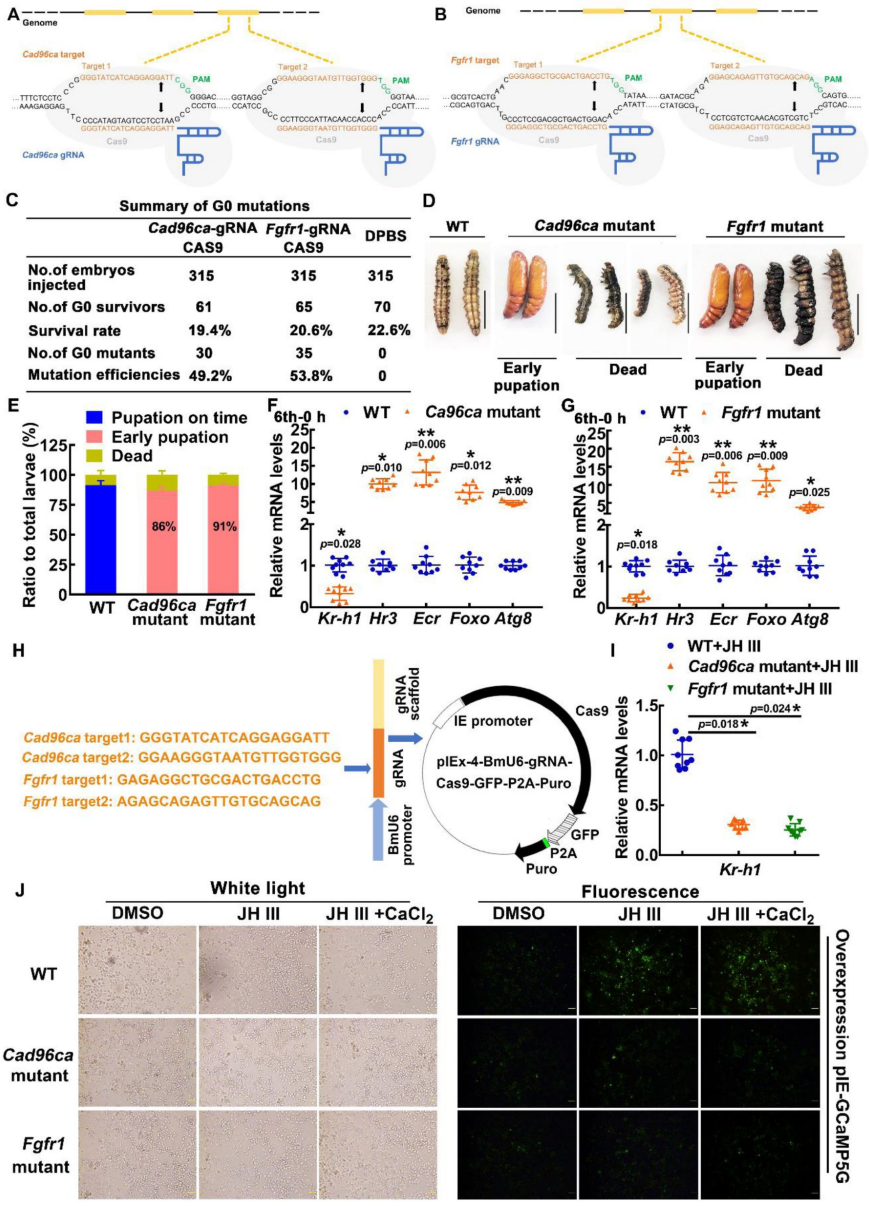

Figure 4.

The roles of CAD96CA and FGFR1 in larval development were determined by CRISPR/Cas9 system-mediated mutants.

(**A** and **B**) Schematic showing the injection mixture of the CRISPR/Cas9 system. The black line refers to the genome of *H. armigera*; the yellow blocks correspond to exons. The Cas9 nuclease (in grey) was targeted to genomic DNA by *Cad96ca*-gRNA or *Fgfr1*-gRNA with an ~20-nt guide sequence (orange) and a scaffold (blue). The guide sequence pairs with the DNA target (orange sequence on the top strand), which requires the upstream sequence of the 5′-CGG-3′ adjacent motif (PAM; green). Cas9 induces a double-strand break (DSB) ~3 bp upstream of the PAM (black triangle). (**C**) Summary of G0 mutations. (**D**) Images showing WT and mutant *H. armigera* phenotypes. The scale represents 1 cm. (**E**) Morphology and statistical analysis of WT and mutant *H. armigera*. Both *Cad96ca* and *Fgfr1* mutant larvae showed earlier pupation than WT controls. (**F** and **G**) qRT−PCR showing the mRNA levels of the JH/20E response genes in WT and mutant *H. armigera*. (**H**) Schematic showing the CRISPR/Cas9 editing in HaEpi cells by pIEx-4-BmU6-*Cad96ca*-gRNA-Cas9-GFP-P2A-Puro and pIEx-4-BmU6-*Fgfr1*-gRNA-Cas9-GFP-P2A-Puro recombination vectors. (**I**) qRT−PCR showing the mRNA levels of *Kr-h1* in WT and mutant HaEpi cells. (**J**) pIEx-GCaMP5G was overexpressed in WT and mutant HaEpi cells, and calcium mobilization was detected. Green fluorescence shows the calcium signal. The concentration of JH III was 1 μM, and that of $CaCl_2$ was 1 mM. The scale bar represents 100 μm.

Cad96ca mutant, 86% of the larvae (an editing efficiency of 67% by TA clone analysis) had a shortened feeding stage in the sixth instar and entered the metamorphic molting stage earlier, showing early pupation, with the pupation time being 24 h earlier. In the *Fgfr1* mutant, 91% of the larvae (an editing efficiency of 61%) had a shortened feeding stage in the sixth instar and entered the metamorphic molting stage earlier, showing early pupation, with the pupation time being 23 h earlier (**Figure 4D** and *E*). The data suggested that CAD96CA and FGFR1 support larval growth and prevent pupation *in vivo*.

To address the mechanism of early pupation caused by knockout of *Cad96ca* or *Fgfr1*, we compared the expression of the genes in the JH and 20E pathways between mutant and wild-type *H. armigera*. Both the mutants *Cad96ca* or *Fgfr1* led to a significant decrease in *Kr-h1* expression and an increase in 20E pathway gene expression compared with the wild-type *H. armigera*, respectively (**Figure 4F** and *G*), indicating that CAD96CA and FGFR1 prevented pupation by increasing *Kr-h1* expression and repressing 20E pathway gene expression.

To confirm the roles played by CAD96CA and FGFR1 in JH signaling, we further examined the response of HaEpi cells to JH III induction after editing of *Cad96ca* and *Fgfr1* by CRISPR/Cas9 in HaEpi cells using the gRNAs inserted in the pIEx-4-BmU6-gRNA-Cas9-GFP-P2A-Puro plasmid (**Figure 4H**). The mutation of *Cad96ca* and *Fgfr1* in HaEpi cells was confirmed by sequencing the mutants and deduced amino acids (**Figure 4***—figure supplement 2A-D*). *Cad96ca* or *Fgfr1* mutation repressed the JH III-induced expression of *Kr-h1* in HaEpi cells compared with wild-type cells (**Figure 4I**), and repressed the JH III-induced rapid calcium mobilization in cells (**Figure 4J** *and* **Figure 4***—figure supplement 2E*), suggesting that CAD96CA and FGFR1 were involved in JH III-induced expression of *Kr-h1* and rapid calcium mobilization. These results supported the hypothesized roles played by CAD96CA and FGFR1 in JH signaling.

CAD96CA and FGFR1 transmitted JH signal in different insect cells and HEK-293T cells

To demonstrate the universality of CAD96CA and FGFR1 in JH signaling in different insect cells, we investigated JH-triggered calcium ion mobilization and *Kr-h1* expression in Sf9 cells developed from *S. frugiperda* and S2 cells developed from *D. melanogaster*. Knockdown of *Cad96ca* and *Fgfr1* (named *Htl* or *Btl* in *D. melanogaster*), respectively, significantly decreased JH III-induced intracellular Ca^{2+} release and extracellular Ca^{2+} influx, and *Kr-h1* expression (**Figure 5A**, *B*, **Figure 5***—figure supplement 1A and B*). The efficacy of RNAi of *Cad96ca* and *Fgfr1* was confirmed in the cells (**Figure 5***—figure supplement 1C and D*), suggesting that CAD96CA and FGFR1 had a general function to transmit JH signal in *S. frugiperda* and *D. melanogaster*.

To confirm the roles of CAD96CA and FGFR1 transmitting JH signal, CAD96CA and FGFR1 of *H. armigera* were overexpressed heterogeneously in mammalian HEK-293T cells to exclude the unknown endogenous effect in insect cells. Immunocytochemistry showed that CAD96CA-GFP, FGFR1-GFP, and NRK-GFP locate in the plasma membrane. The proteins were confirmed using western blotting (**Figure 5***—figure supplement 2A*). HEK-293T cells had no significant changes at calcium ion levels under JH III induction (**Figure 5C**), indicating that HEK-239T cells did not respond to JH III induction. However, when HEK-293T cells were overexpressed CAD96CA and FGFR1, respectively, JH III triggered rapid cytosolic Ca^{2+} increase, by comparison with the DMSO condition, His tag, and other RTK NRK-His controls (**Figure 5D**). These results further confirmed that CAD96CA and FGFR1 transmit JH III signal.

CAD96CA and FGFR1 mutants were used to further confirm their roles in transmitting the JH signal in HEK-293T. Mutants were overexpressed, and the encoded mutants located in the plasma membrane, as confirmed via immunocytochemistry, and the proteins were confirmed using western blotting (**Figure 5***—figure supplement 2B*). Results showed that Ca^{2+} increase was not detected in CAD96CA-M3 and CAD96CA-M4 under JH III-induced (**Figure 5E**), JH III-induced Ca^{2+}

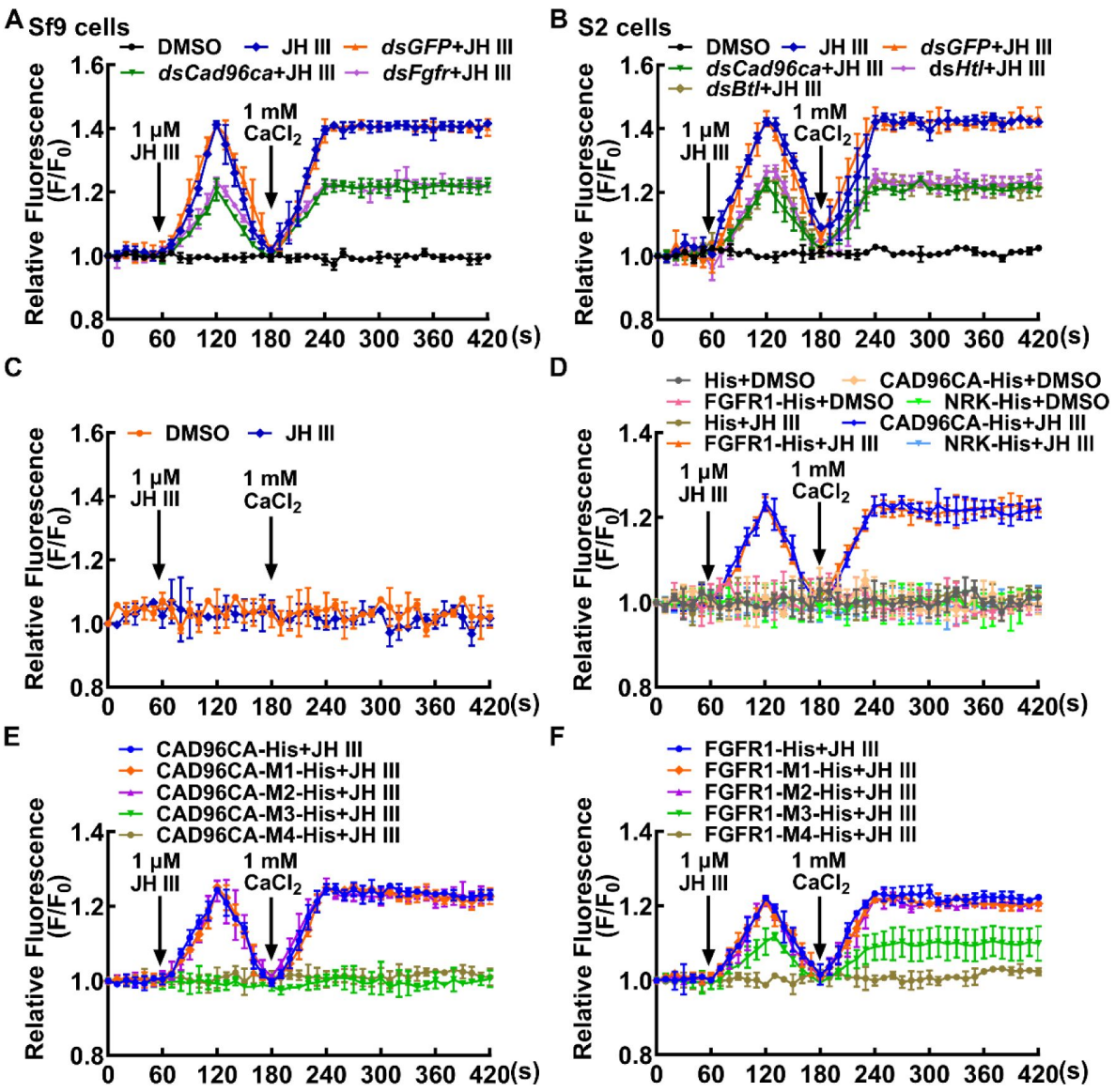

Figure 5.

CAD96CA and FGFR1 participated in JH-induced calcium ion mobilization.

(**A**) The level of Ca^{2+} after *Cad96ca* and *Fgfr* knockdown in Sf9 cells. The cells were incubated with dsRNA (the final concentration was 1 μg/mL for 48 h). F_0: the fluorescence intensity of Sf9 cells without treatment. F: the fluorescence intensity of Sf9 cells after different treatments. DMSO as solvent control. (**B**) Effect of JH III on calcium ion levels in S2 cells after *Cad96ca* and *Htl* knockdown. (**C**) The response of calcium ion levels to JH III in HEK-293T cells. (**D**) The analysis of calcium ion flow after HEK-293T cells overexpressed RTK. DMSO as solvent control. His as tag control. (**E** and **F**) The calcium was quantitated after HEK-293T cells overexpressed CAD96CA-His, FGFR1-His, and mutants. The error bar indicates the mean ± SD.

mobilization was slightly detected in FGFR1-M3, and JH III-induced Ca^{2+} mobilization was not detected in FGFR1-M4 (**Figure 5F**). These results confirmed that CAD96CA and FGFR1 play roles in transmitting JH III signal.

Discussion

JH regulates insect development through intracellular and membrane signaling; however, the cell membrane receptors and the mechanism are unclear. In this study, CAD96CA and FGFR1 were screened out from the total 20 RTKs in the *H. armiger* genome and identified as JH III cell membrane receptors, which transmit JH signal for gene expression and have a high affinity to JH III.

CAD96CA and FGFR1 transmit JH signal

JH induces a set of gene expression, such as *Kr-h1* (Truman, 2019), *Vg* (Roy et al., 2018; Song et al., 2014), *Jhi-1*, and *Jhi-26* (Dubrovsky et al., 2000), a rapid intracellular calcium increase, phosphorylation of MET and Tai (Liu et al., 2015), and prevents pupation. We found several RTKs are involved in JH III-induced gene expression and calcium increase; however, only *Cad96ca*, *Nrk*, *Fgfr1*, and *Wsck* are involved in the JH III-induced pupation delay, in which, only CAD96CA, NRK, and FGFR1 are involved in the JH-induced phosphorylation of MET1 and TAI, and only CAD96CA and FGFR1 can bind JH III. Therefore, CAD96CA and FGFR1 are finally determined as JH III receptors.

CAD96CA (also known as Stitcher, Ret-like receptor tyrosine kinase) activates upon epidermal wounding in *Drosophila* embryos (Tsarouhas et al., 2014) and promotes growth and suppresses autophagy in the *Drosophila* epithelial imaginal wing discs (O'Farrell et al., 2013). There is a CAD96CA in the genome of the *H. armigera*, which is without function study. Here, we reported that CAD96CA prevents pupation by transmitting JH signal as a JH cell membrane receptor. We also showed that CAD96CA of other insects has a universal function of transmitting JH signal to trigger Ca^{2+} mobilization, as demonstrated by the study in Sf9 cell lines of *S. frugiperda* and S2 cell lines of *D. melanogaster*.

FGFRs control cell migration and differentiation in the developing embryo of *D. melanogaster* (Muha and Muller, 2013). The ligand of FGFR is FGF in *D. melanogaster* (Du et al., 2018). FGF binds FGFR and triggers cell proliferation, differentiation, migration, and survival (Beenken and Mohammadi, 2009; Lemmon and Schlessinger, 2010). Three FGF ligands and two FGF receptors (FGFRs) are identified in *Drosophila* (Huang and Stern, 2005). The *Drosophila* FGF-FGFR interaction is specific. Different ligands have different functions. The activation of FGFRs by specific ligands can affect specific biological processes (Kadam et al., 2009). The FGFR in the membrane of Sf9 cells can bind to Vip3Aa (Jiang et al., 2018). One FGF and one FGFR are in the *H. armigera* genome, which has yet to be studied functionally. The study found that FGFR prevents insect pupation by transmitting JH signal as a JH cell membrane receptor. Exploring the molecular mechanism and output by which multiple ligands transmit signals through the same receptor is exciting and challenging.

CAD96CA and FGFR1 have similar functions in JH signaling, including transmitting JH signal for *Kr-h1* expression, larval status maintaining, rapid intracellular calcium increase, phosphorylation of transcription factors MET1 and TAI, and high affinity to JH III. CAD96CA and FGFR1 are essential in the JH signal pathway, and loss-of-function for each is sufficient to trigger strong effects on pupation. The difference is that CAD96CA expression has no tissue specificity, and the *Fgfr1* gene is highly expressed in the midgut; possibly, it plays a significant role in the midgut.

Other possibility is that they play roles by forming heterodimer with each other or other RTKs, which needs to be addressed in future study. CAD96CA and FGFR1 transmit JH III signals in three different insect cell lines, suggesting their conserved roles in other insects.

Homozygous *Cad96ca* null *Drosophila* die at late pupal stages (Wang et al., 2009). However, we found that 86% of the larvae of the *Cad96ca* mutant successfully pupated in G0 generation, although earlier than the control. Similarly, null mutation of *Fgfr1* or *Fgfr2* in mouse is embryonic lethal (Arman et al., 1998; Deng et al., 1994; Yamaguchi et al., 1994). In *D. melanogaster*, homozygous *Htl* (*Fgfr*) mutant embryos die during late embryogenesis, too (Beati et al., 2020; Beiman et al., 1996; Gisselbrecht et al., 1996). However, in *H. armigera*, 91% of larvae successfully pupated in G0 generation after *Fgfr1* knockout. The low death rate after *Cad96ca* and *Fgfr1* knockout might be because of following reasons, including the editing efficiency (67% and 61% for *Cad96ca* mutant and *Fgfr1* mutant, respectively), the chimera of the gene knockout at the G0 generation, and the redundant RTKs that play similar roles in JH signaling, similar to the redundant roles of MET and Germ-cell expressed bHLH-PAS (GCE) in JH signaling (Liu et al., 2009), which needs to obtain alive G1 homozygote mutants and double knockout of these two receptors in future study. We indeed observed that the eggs did not hatch successfully after mixed-mating of G0 *Cad96ca* mutant or *Fgfr1* mutant, respectively, but the reason was not addressed further due to the embryonic death. By the similar reasons, most of the *Cad96ca* and *Fgfr1* mutants showed a slight acceleration of pupation (about one day) without the typical precocious metamorphosis (at least one instar earlier) phenotype caused by JH signaling defects (Daimon et al., 2012; Fukuda, 1944; Riddiford et al., 2010) and JH pathway gene deletions (Abdou et al., 2011; Liu et al., 2009). On other side, JH can regulate gene transcription by diffusing into cells and binding to the intracellular receptor MET to conduct JH signal, which might affect the results of gene knockdown and knockout.

It is generally believed that the primary role of JH is to antagonize 20E during larval molting (Riddiford, 2008). The knockdown of *Cad96ca*, *Nrk*, *Fgfr1*, and *Wsck* showed phenotypes resistant to JH III induction and the decrease of *Kr-h1* and increase of *Br-z7* expression, but knockdown of *Vegfr* and *Drl* only decrease *Kr-h1*, without increase of *Br-z7*. *Br-z7* is involved in 20E-induced metamorphosis in *H. armigera* (Cai et al., 2014), whereas, Kr-h1 is a JH early response gene that mediates JH action (Minakuchi et al., 2009) and represses *Br* expression (Riddiford et al., 2010). The high expression of *Br-z7* is possible due to the down-regulation of *Kr-h1* in *Cad96ca*, *Nrk*, *Fgfr1* and *Wsck* knockdown larvae. The different expression profiles of *Br-z7* in *Vegfr* and *Drl* knockdown larvae suggest other roles of *Vegfr* and *Drl* in JH signaling, which need further study.

This study found six RTKs that respond to JH induction by participating in JH induced-gene expression and intracellular calcium increase, however; they exert different functions in JH signaling, and finally CAD96CA and FGFR1 are determined as JH cell membrane receptors by their roles in JH induced-phosphorylation of MET and TAI and binding to JH III. We screen the RTKs transmitting JH signal primarily by examining some of JH induced-gene expression. By examining other genes or by other strategies to screen the RTKs might find new RTKs functioning as JH cell membrane receptors; however, the key evaluation indicators, such as the binding affinity of the RTKs to JH and the function in transmitting JH signal to maintain larval status are essential. In addition, GPCRs also play a role in JH signaling. JH triggers GPCR, RTK, PLC, IP3R, and PKC to phosphorylate Na^+/K^+-ATPase-subunit, consequently activating Na^+/K^+-ATPase for the induction of patency in *L. migratoria* vitellogenin follicular epithelium (Jing et al., 2018); JH activates a signaling cascade including GPCR, PLC, extracellular Ca^{2+}, and PKC, which induces vitellogenin receptor (VgR) phosphorylation and promotes vitellogenin (Vg) endocytosis in *Locusta migratoria* (Jing et al., 2021). JH activates a signaling cascade including GPCR, Cdc42, Par6, and aPKC, leading to an enlarged opening of patency for Vg transport (Zheng et al., 2022). In *Tribolium castaneum*, the dopamine D2-like receptor-mediated JH signaling promotes the accumulation of vitellogenin and increases the level of cAMP in oocytes (Bai and Palli, 2016). In *H. armigera*,

GPCRs are involved in JH III-induced broad isoform 7 (BRZ7) phosphorylation (Cai et al., 2014). In summary, these published results indicate that RTKs and GPCRs contribute to JH signaling on the cell membrane, however, the GPCR functions as JH receptor needs to be addressed in the future study. The RNAi of RTKs does not affect JH-induced *Jhi-1* expression, which implies other receptors exist, presenting a target for future study of the new JH III receptor.

The affinity of CAD96CA and FGFR1 to JH III

RTKs are high–affinity cell surface receptors for many cytokines, polypeptide growth factors, and peptide hormones (Trenker and Jura, 2020). Up to date, there is no report that RTK binds lipid hormone. We determined that CAD96CA and FGFR1 have a high affinity to JH III by MST and ITC methods after they are isolated from the cell membrane.

The [^{3}H]JH III detection method is used to determine *Drosophila* MET *in vitro* translation product binding JH III (Kd = 5.3 nM) (Miura et al., 2005), and *Tribolium* MET binding JH III (Kd = 2.94 nM) (Charles et al., 2011). However, the commercial production of [^{3}H]JH III has ceased, whereas the microscale thermophoresis (MST) method is a widely used method to detect protein binding of small molecules (Welsch et al., 2017). Therefore, MST was used in our study as the alternative method to measure the binding strengths of RTKs with JH III. Using the MST method, we determined that the saturable specific binding of *Helicoverpa* MET1 to JH III is Kd of 6.38 nM, which is comparable to that report for *Drosophila* MET and *Tribolium* MET determined using [^{3}H]JH III, confirming MST method can be used to detect protein binding JH III. The CAD96CA exhibited saturable specific binding to JH III with a Kd of 11.96 nM, and FGFR1 showed a Kd of 23.61 nM, which is higher than that of MET1 for JH III, suggesting lower binding affinity of RTKs than the intracellular receptor MET1 for JH III. A similar phenomenon is reported in another study, the binding affinities of steroid membrane receptors are orders of magnitude lower than those of nuclear receptors (Falkenstein et al., 2000). NRK did not bind JH III. One possible explanation is that NRK has a low affinity to JH III and thus transmits JH signal without binding JH, or NRK requires association with other proteins to play roles. Our study provides new evidence for the binding of lipid hormones by RTK and a new method to study the binding of ligands to receptors.

We also verified the affinity of CAD96CA and FGFR1 with JH III through the ITC method, determining their respective Kd values as 79.6 and 88.5 nanomolar. ITC is a versatile analytical method for the character of molecular interactions (Johnson, 2021). ITC is applied in the membrane protein family, containing GPCRs, ion channels, and transporters (Draczkowski et al., 2014). The ITC method requires relatively high ligand and receptor concentrations for better saturation curves (Rajarathnam and Rösgen, 2014). However, when we prepared a protein solution of 1000 nM, protein aggregation occurred, thus we used a protein solution with a concentration of 700 nM. The Kd value detected by ITC is slightly higher than the result of the MST method; the results are sufficient to confirm the high affinity of CAD96CA and FGFR1 binding to JH III.

Although JH I and JH II are natural hormones for lepidopteran larvae (Furuta et al., 2013; Schooley et al., 1984), *H. armigera* (Liu et al., 2013) and *B. mori* (Deng et al., 2011; Kayukawa et al., 2012) also respond to JH III. In *B. mori* Bm-aff3 cells, the effective concentrations (EC50) of JHs (JH I, JH II, JH III, JHA, or methyl farnesoate) to induce *Kr-h1* transcription are 1.6×10^{-10}, 1.2×10^{-10}, 2.6×10^{-10}, 6.0×10^{-8}, and 1.1×10^{-7} M, respectively (Kayukawa et al., 2012). In cultures of wing imaginal discs from *B. mori*, 1–2 μM JH III promotes cuticle protein 4 gene expression (Deng et al., 2011). The effective concentration of JH III to induce rapid calcium increase in HaEpi cells is ≥ 1 μM (Wang et al., 2016) and 500 ng of 6th instar larva (Cai et al., 2014). JH III is a commercially available reagent; therefore, we used JH III to carry out the experiments in this study, and the results hypothesize the possibility of CAD96CA and FGFR1 binding other JHs in addition to JH III, which should be addressed in future study.

Relationship of cell membrane receptor and intracellular receptor

MET is determined as JH's intracellular receptor by its characters binding to JH and regulating *Kr-h1* expression (Charles et al., 2011; Jindra et al., 2021). In our study, cell membrane receptors CAD96CA and FGFR1 are also able to bind JH III and transmit JH III signal to regulate a set of JH III-induced gene expression including *Kr-h1*. Obviously, both intracellular receptor MET and cell membrane receptor CAD96CA and FGFR1 are involved in JH III signaling as receptors. JH III transmits signal by cell membrane receptor and intracellular receptor at different stages, with cell membrane receptor CAD96CA and FGFR1 inducing rapid Ca^{2+} signaling, which regulates the phosphorylation of MET and TAI to enhance the function of MET for gene transcription, and the intracellular receptor MET regulates gene transcription by diffusion into cells based its lipid characteristics.

The study in human cell line HEK293 shows that overexpression of *B. mori* JH intracellular receptor MET2 and its cofactor SRC together in HEK293 cells may induce JH-dependent luciferase reporter expression through a plasmid that contains the JH specific kJHRE (JH response element containing the E-box core sequence for JH binding) (Kayukawa et al., 2012), suggesting JH can diffuse into cells to initiate a gene expression when the insect MET2 and SRC and kJHRE exist. Our study showed that overexpressed CAD96CA or FGFR1 in HEK-293T cells elicits Ca^{2+} elevation under JH III induction, suggesting CAD96CA or FGFR1 transmit a rapid signal of JH III in HEK-293T cells, which might trigger further cellular responses of HEK-293T to JH III. These data suggest that both cell membrane receptors CAD96CA and FGFR1 and intracellular receptor MET of JH can respond to JH. These proteins might be used as switches to induce a gene expression or regulate cell fate in heterogeneous cells by JH induction when the side effects are determined.

Conclusions

CAD96CA and FGFR1 were involved in JH III signaling, including larval status maintaining, JH III-induced rapid intracellular calcium increase, gene expression, and phosphorylation of MET and TAI. CAD96CA and FGFR1 had high affinity to JH III and were possible cell membrane receptors of JH III and other JHs. CAD96CA and FGFR1 had a general role in transmitting the JH III signal for gene expression in various insect cells, suggesting their conserved roles in other insects. JH III transmits signal by either cell membrane receptor and intracellular receptor at different stages in the signaling, with JH III transmitting the signal by cell membrane receptor CAD96CA and FGFR1 to induce rapid Ca^{2+} signaling, which regulates the phosphorylation of MET and TAI to enhance the function of MET for gene transcription, and intracellular receptor MET regulates gene transcription by diffusion into cells based its lipid characteristics (**Figure 6**). This study presents a platform to identify the agonist or inhibitor of JH cell membrane receptors to develop an environmental friend insect growth regulator.

Materials and methods

Experimental insects

Cotton bollworms (*H. armigera*) were raised on an artificial diet comprising wheat germ and soybean powder with various vitamins and inorganic salts. The insects were kept in an insectarium at 26 ± 1 °C with 60 to 70% relative humidity and under a 14 h light:10 h dark cycle.

Cell culture

Our laboratory established the *H. armigera* epidermal cell line (HaEpi) (Shao et al., 2008). The cells were cultured as a loosely attached monolayer and maintained at 27 °C in cell culture flasks. The cell culture flasks had an area of 25 cm^2 with 4 mL of Grace's medium supplemented with 10%

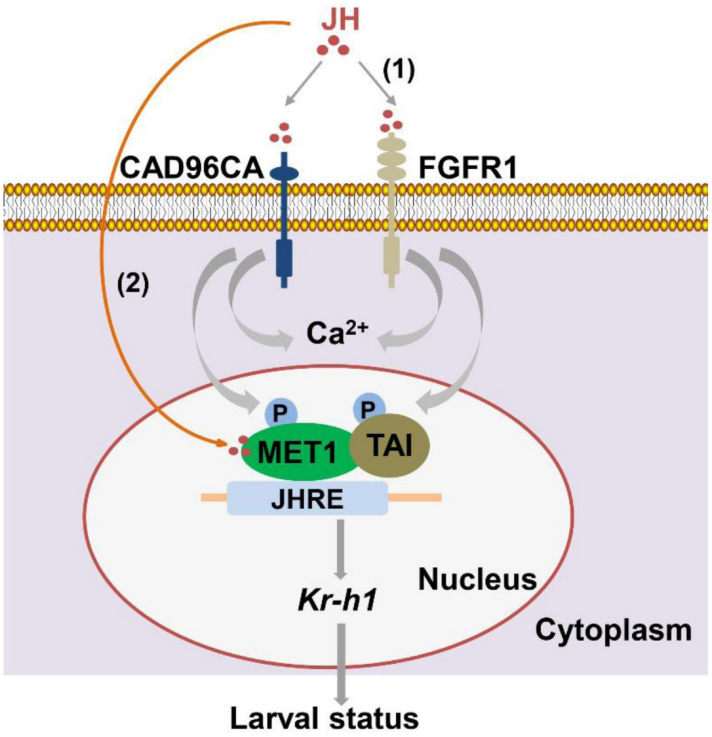

Figure 6.

A diagram illustrating CAD96CA and FGFR1 transmitting juvenile hormone signal for gene expression.

(1) JH binds to cell membrane receptors CAD96CA and FGFR1 to increase intracellular calcium, phosphorylation of MET1 and TAI to enhance their function in gene expression to maintain larval status. (2) On the other hand, JH enters cells freely via diffusion to bind its intracellular receptor MET. MET interacts with TAI and then binds to the JH response element (JHRE, containing the E-box core sequence, in the *Kr-h1* promoter region) to promote gene expression to keep larval status. Therefore, JH III transmits signal by either cell membrane receptor and intracellular receptor at different stages in the signaling.

fetal bovine serum (Biological Industries, Cromwell, CT, USA). The Sf9 cell line (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was cultured in ESF921 medium at 27 °C. The S2 cell line was cultured in Schneider's *Drosophila* medium (Gibco, California, USA) with 10% FBS (Sigma, San Francisco, CA, USA) at 27 °C. The cells were subcultured when cells covered 80% of the culture flasks. The HEK-293T cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, California, USA) with 10% FBS (Sigma, St. Louis, Missouri, USA) at 37 °C with 5% carbon dioxide.

Bioinformatic analyses

Identification of RTKs by looking for the name of RTK in the genome of *H. armigera* using bioinformatics. Then, blast analysis was used to search for more RTKs. These RTKs were compared with previously reported RTK species in *B. mori*, *D. melanogaster*, and *H. sapiens* to confirm the amount of RTK in *H. armigera*. The phylogenetic trees were constructed from amino acid sequences using the Neighbor Joining (NJ) method in MEGA 5.0. The structure domains of the proteins were predicted using SMART (*http://smart.embl-heidelberg.de/*). Although the SMART tool did not predict that the TORSO has a transmembrane structure, the TORSO of *H. armigera* is 79% identity to that of TORSO of RTK members in *B. mori*. We believe that the TORSO of *H. armigera* belongs to the RTK family, but SMART failed to predict its structure successfully. Although the SMART tool did not predict the complete structure of STE20-like, it was clustered with the RTK of *D. melanogaster* Tie-like in evolutionary tree clustering analysis. In addition, in sequence alignment, the named flocculation protein FLO11-like in *Hyposmocoma kahamanoa* was 85% identity to it, and FLO11-like protein showed transmembrane structure in domain prediction, so the STE20-like of *H. armigera* was classified as a member of the RTK family.

Double-stranded RNA synthesis

RNA interference (RNAi) has been used widely in moths of 10 families (Xu et al., 2016). Long double-stranded RNA (dsRNA) can be processed into smaller fragments, with a length of 21–23 nucleotides (Zamore et al., 2000), to restrain transcription of the target gene (Fire et al., 1998). dsRNA transcription was performed as follows: 2 μg of DNA template, 20 μL of 5 × transcription buffer, 3 μL of T7 RNA polymerase (20 U/μL), 2.4 μL of A/U/C/GTP (10 mM) each, 3 μL of RNase inhibitor (40 U/μL, Thermo Fisher Scientific, Waltham, USA), and RNase-free water were mixed to a volume of 50 μL. After incubation at 37 °C for 4–6 h, 10 μL RNase-free DNase I (1 U/μL, Thermo Fisher Scientific), 10 μL of DNase I Buffer, and 30 μL RNase-free water were added to the solution, which was incubated at 37 °C for 1 h. The solution was extracted with phenol/chloroform and precipitated with ethanol; the precipitate was resuspended with 50 μL RNase-free water. The purity and integrity of the dsRNA was determined using agarose gel electrophoresis. A MicroSpectrophotometer (GeneQuant; Amersham Biosciences, Little Chalfont, UK) was used to quantify the dsRNAs.

RNA interference in HaEpi cells

When the HaEpi cell density reached 70 to 80% in six-well culture plates, the cells were transfected with dsRNA (1 μg/mL) and Quick Shuttle Enhanced transfection reagent (8 μL) (Biodragon Immunotechnologies, Beijing, China) diluted in sterilized saline medium (200 μL), and incubated with Grace's medium. The cells were cultivated for 48 h at 27 °C. After that, the medium was replaced with a fresh Grace's medium with JH III at a final concentration of 1 μM for 12 h. An equivalent volume of DMSO was a control. The total mRNA was then extracted for qRT-PCR.

RNA interference in larvae

The DNA fragments of *Rtks* were amplified as a template for dsRNA synthesis using the primers RTK-RNAiF and RTK-RNAiR (*Supplementary file 2*). The dsRNAs (*dsRtk*, *dsGFP*) were injected using a micro-syringe into the larval hemocoel of the fifth instar 20 h at 500 ng/larva, using three injections at 36 h intervals. At 12 h after the last injection, 500 ng of JH III (Santa Cruz

Biotechnology, Santa Cruz, CA, USA) was injected into each larva. Dimethyl sulfoxide (DMSO) was used as a control. The phenotypes and developmental rates of the larvae were recorded. The mRNA was isolated from the larvae at 12 h after JH III injection.

Protein overexpression

The nucleotide sequence of the genes involved in this study was cloned into the pIEx-4-His, pIEx-4-GFP-His, pIEx-4-CopGFP-His, pcDNA3.1-GFP-His or pcDNA3.1-His vector. The cells were cultured to 80% confluence at 27 °C or 37 °C in the medium. For transfection, approximately 5 μg of plasmids, 200 μL of sterilized saline water medium or Opti-MEM medium, and 8 μL of transfection reagent (Biodragon, Beijing, China) were mixed with the cells in the medium for 24–48 h.

Quantitative real–time reverse transcription PCR (qRT–PCR)

Total RNA was extracted from HaEpi cells and larvae using the Trizol reagent (TransGen Biotech, Beijing, China). According to the manufacturer's instructions, first-strand cDNA was synthesized using a 5 × All-In-One RT Master Mix (Abm, Vancouver, Canada). qRT–PCR was then performed using the CFX96 real–time system (Bio-Rad, Hercules, CA, USA). The relative expression levels of the genes were quantified using *Actb* (β-actin) expression as the internal control. The primers are listed in *Supplementary file 2*. The experiments were conducted in triplicate with independent experimental samples. The relative expression data from qRT–PCR were calculated using the formula: $R= 2^{-\Delta\Delta CT}$ ($\Delta\Delta Ct = \Delta Ct_{sample}-\Delta Ct_{control}$, $\Delta Ct = Ct_{gene}-Ct_{\beta\text{-}actin}$) (Livak and Schmittgen, 2001).

Detection of the cellular levels of calcium ions

The cells were cultured to a density of 70–80%. The cells were incubated with Dulbecco's phosphate-buffered saline (DPBS) (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8 mM Na_2HPO_4) including 3 μM acetoxymethyl (AM) ester calcium crimson^{TM} dye (Invitrogen, Carlsbad, CA, USA) for 30 min at 27 °C. The cells were washed with fresh DPBS three times. The cells were then exposed to 1 μM JH III to detect the intracellular calcium release. After that, cells were treated with Calcium chloride (final concentration 1 mM) and JH III (final concentration 1 μM), and put into a microscope dish. Fluorescence was detected at 555 nm, and the cells were photographed automatically once every 6 s for 420 s using a Carl Zeiss LSM 700 laser scanning confocal microscope (Thornwood, NY, USA). The fluorescence intensity of each image was analyzed using Image Pro-Plus software (Media Cybernetics, Rockville, MD, USA).

Western blotting

Epidermis, midgut, and fat body tissues were homogenized in 500 μL Tris-HCl buffer (40 mM, pH 7.5) on ice with 5 μL phenylmethylsulfonyl fluoride (PMSF, 17.4 mg/mL in isopropyl alcohol), respectively. The homogenate was centrifuged for 15 min at 4 °C at 12,000 × g, then supernatant was collected. The protein concentration in the supernatant was measured using the Bradford protein assay. Proteins (20 μg per sample) sample was subjected to 7.5% or 12.5% SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was incubated in blocking buffer (Tris-buffered saline, 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, with 3–5% fat-free powdered milk) for 1 h at room temperature. The primary antibody was diluted in blocking buffer, then incubated with the membrane at 4 °C overnight. The membrane was washed three times wash with TBST (0.02% tween in TBS) for 10 min each. Subsequently, the membrane was incubated with secondary antibodies, 1:10,000 diluted, alkaline phosphatase-conjugated (AP) or horseradish peroxidase-conjugated (HRP) AffiniPure Goat Anti-Rabbit/-Mouse IgG (ZSGB-BIO, Beijing, China). The membrane was washed twice with TBST and once with TBS. The immunoreactive protein bands marked by AP were observed after incubating in 10 mL of TBS solution combined with 45 μL of P-nitro-blue tetrazolium chloride (NBT, 75 μg/μL) and 30 μL of 5-bromo-4-chloro-3 indolyl phosphate (BCIP, 50 μg/μL) in the dark for 10–30 min. The reactions were stopped by washing the membrane with deionized water and images by the scanner. The proteins marked by HRP were detected

using a High-Sig ECL Western Blotting Substrate and exposed to a Chemiluminescence imaging system (Tanon, Shanghai, China), according to the manufacturer's instructions. The immunoreactive protein band density was calculated using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The data were analyzed using GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA).

Lambda protein phosphatase (λPPase) treatment

The protein suspension (40 μL, 0.1 mg/mL) was incubated with λPPase (0.5 μL), buffer (5 μL), and $MnCl_2$ (5 μL) at 30 °C for 30 min, according to the manufacturer's specifications (New England Biolabs, Beijing LTD, Beijing, China). Total proteins were subjected to SDS-PAGE and then electrophoretically transferred onto a nitrocellulose membrane for western blotting.

Phos-tag SDS-PAGE

Phos-tag Acrylamide (20 μM; Fujiflm Wako Pure Chemical Corporation, Osaka, Japan) and $MnCl_2$ (80 μM) were mixed into a normal SDS-PAGE gel. The phosphates of the phosphorylated protein can bind to Mn^{2+}, which reduces the mobility of the phosphorylated protein in the gel. The protein sample was treated with 20% trichloroacetic acid (TCA) to remove the chelating agent. The gel was shaken and incubated three times in 10 mmol/L EDTA transfer buffer solution for Phos-tag SDS-PAGE for 10 min each time. Mn^{2+} was removed, and then the proteins were electrophoretically transferred to a nitrocellulose membrane and analyzed using western blotting.

Immunocytochemistry

The cells were grown on coverslips, treated with hormones, washed three times with DPBS, and fixed using 4% paraformaldehyde in PBS for 10 min in the dark. The fixed cells were incubated with 0.2% Triton-X 100 diluted in PBS for 10 min. The cells were washed with DPBS five times for 3 min each, and the plasma membrane was stained with Alexa Fluor 594-conjugated wheat germ agglutinin (WGA) (1:2,000 in PBS) (Invitrogen, Carlsbad, CA, USA) for 8 min. The cells were washed with DPBS five times for 3 min each, and stained with 4', 6-diamidino-2-phenylindole (DAPI, 1 μg/mL in PBS) (Sigma, San Francisco, CA, USA) in the dark at room temperature for 8 min. The fluorescence signal was detected using an Olympus BX51 fluorescence microscope (Olympus, Tokyo, Japan). Scale bar = 20 μm.

Mutations of CAD96CA and FGFR1

The structures of CAD96CA and FGFR1 were predicted online with SMART. According to the location of the predicted domain, the target fragment was amplified with mutated primers (*Supplementary file 2*) and cloned into the pIEx-4-CopGFP-His, pcDNA3.1-GFP-His or pcDNA3.1-His vector. The CAD96CA mutants were constructed to CAD96CA-M1-CopGFP-His (AA: 51-615), CAD96CA-M2-CopGFP-His (AA: 101-615), CAD96CA-M3-CopGFP-His (AA: 151-615), and CAD96CA-M4-CopGFP-His (AA: 201-615). FGFR1 mutants were constructed to FGFR1-M1-CopGFP-His (AA: 101-852), FGFR1-M2-CopGFP-His (AA: 201-852), FGFR1-M3-CopGFP-His (AA: 301-852), and FGFR1-M4-CopGFP-His (AA: 401-852).

Detection of RTK binding JH III by microscale thermophoresis

RTKs and MET1 were recombined in plasmid pIEx-4-CopGFP-His, which was overexpressed in Sf9 cells. After 48 h, total plasma membrane RTKs were extracted using a cell transmembrane protein extraction kit (BestBio, Shanghai, China). MET1-CopGFP-His and CopGFP-His were extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (20 mM Tris-HCl, pH 7.5; 150 mM NaCl; and 1% Triton X-100) without ethylenediaminetetraacetic acid (EDTA) (Beyotime, Shanghai, China). A 100 μL of slurry of chelating Sepharose with Ni^{2+} was washed three times with binding buffer (500 mM NaCl; 20 mM Tris-HCl, pH 7.9; and 5 mM imidazole) for 5 min. The overexpressed proteins were bound to the washed Ni^{2+}-chelating Sepharose (GE Healthcare, Pittsburgh, PA, USA). The

suspension was mixed on a three-dimensional rotating mixer for 40 min at 4 °C. Then, the resin was washed three times for 5 min each time with wash buffer (0.5 M NaCl; 20 mM Tris-HCl, pH 7.9; and 20 mM imidazole). After centrifugation at 500 × *g* for 3 min at 4 °C, the RTKs were washed three times with wash buffer for 5 min each time. The RTKs were eluted using 100 μL of elution buffer (0.5 M NaCl; 20 mM Tris-HCl, pH 7.9; 100 mM imidazole; and 0.5% Triton X-100) and then diafiltration was carried out three times with PBST (PBS, 0.05% Tween, and 0.5% Triton X-100) buffer using Amicon Ultra 0.5 (Merck Millipore, Temecula, CA, USA) to reduce the concentration of imidazole in preparation for the subsequent experiment. The concentration of the isolated RTK was detected using a BCA protein assay kit (Beyotime, Shanghai, China). JH III bound by 50 nM RTK was detected using the microscale thermophoresis (MST) method (Huang and Zhang, 2021; Welsch et al., 2017). Firstly, the fluorescence intensity and the homogeneity of the protein solution were detected. We confirmed that the fluorescence intensity of the protein samples was within the range of the instrument, and there was no aggregation of the protein samples. Then, we carried out experiments. 16 microtubes were prepared, and the ligand was diluted for use at the initial concentration of 1 μM JH III. Specifically, 5 μL of the ligand buffer was added to prepared microtubes No. 2-16. After, 10 μL of the ligand was added to tube No. 1, 5 μL of the ligand solution in tube No. 1 was pipetted out of tube No. 1, added to tube No. 2, and mixed well. Then 5 μL of solution was pipetted from tube No. 2 and added to tube No. 3. Finally, 5 μL of mixed liquid was removed from tube No. 16 and discarded. (The original concentration of JH III was dissolved in DMSO, and therefore, DMSO needed to be added to the ligand dilution buffer to ensure an equal amount of DMSO in each tube). Then, 5 μL of the fluorescence molecule (target protein) was added to each tube and mixed well. With each tube holding a 10 μL volume in total, the tubes were incubated at 4 °C for 30 to 60 minutes. Finally, samples were removed with a capillary tube and tested with an MST Monolith NT.115 (NanoTempers, Munich, Germany).

Detection of RTK binding JH III by isothermal titration calorimetry

The protein purification method was described in the MST experiment. The isothermal titration calorimetry (ITC) assay was performed using MicroCal PEAQ-ITC (Malvern Panalytical, Malvern, U.K.). JH III was dissolved in ethanol, JH III stock solution to a final concentration of 10 μM with PBST buffer. The protein solution with same concentration ethanol, make sure the buffer identity. According to the manufacturer's instructions, JH III (10 μM) was loaded in a syringe, and the protein solution (700 nM) was injected into the ITC cell. Injection of 3 μl of JH III solution over a period of 150 s at a stirring speed of 750 rpm was performed. For the control test, JH III solution was pumped into syringe, and the buffer was injected into the ITC cell. For the data, the experimental data were subtracted with that from the control test by analysis software.

Methyl farnesoate, farnesol, methoprene binding assays, and competition assays

Methyl farnesoate (Echelon Biosciences, Utah, USA), farnesol (Sigma, San Francisco, CA, USA), and methoprene (Sigma, San Francisco, CA, USA) were dissolved in DMSO, respectively, diluted to the corresponding concentration, and the experimental method as described by the MST method for detection of binding. The competitive binding by MST requires fluorescent labeling of ligands (JH III). Currently, there is no suitable method to label JH III, and we only have fluorescently labeled receptors (target protein). The binding curve of adding both JH III and methoprene, but the maximum concentration of JH used in the experiment was 50 nM, while the concentration of methoprene was increasing. The Kd value is generated automatically by the software of the instrument.

Generation of *Cad96ca* or *Fgfr1* edited *H. armigera* using the CRISPR/Cas9 system

The gRNAs were designed using the CRISPRscan tool (*https://www.crisprscan.org/?page=sequence*) (Zhang et al., 2021) and each consisted of an ~20-nucleotide (nt) region in complementary reverse to one strand of the target DNA (protospacer) with an NGG motif at the 3' end (PAM) of the target site and a GGN at position (5' end) of the T7 promoter. The sgRNA primer and universal primer were used as corresponding templates to obtain amplification products. Product transcription was carried out with a T7 Transcription Kit (Thermo Fisher Scientific, Waltham, USA) following the manufacturer's instructions.

Freshly laid eggs on gauze (within 2 h) were collected from gauze using 0.1% (v/v) 84 solution and rinsed with distilled water. The eggs were affixed onto microscope slides using double-sided adhesive tape (Zuo et al., 2017; Zuo et al., 2018). A mixture of 100 ng/μL Cas9 protein (GenScript, New Jersey, USA) and 300 ng/μL gRNA for the injection into the eggs (per egg 2 nL was injected) within 4 h of oviposition using a Pico-litre Microinjector (Warner Instruments, Holliston, USA) (Hou et al., 2021). The injected eggs were incubated at 26 ± 1 °C with 60 to 70% relative humidity for 3– 4 days until they hatched. To detect the mutagenesis of *H. armigera* induced by CRISPR/Cas9, we used PCR to amplify the targeted genomic region obtained from fresh epidermis samples of larvae moulted from G0 individuals and used primers at approximately 50-200 base pairs upstream and downstream from the expected double strand break site by HiFi DNA Polymerase (Transgen, Beijing, China). The corresponding PCR products were sequenced, and the PCR fragments from the mutant animals were ligated into a pMD19-T vector (TaKaRa, Osaka, Japan) in preparation for sequencing. The mutated sites were identified by comparison with the wild-type sequence. To detect off-target activity of the CRISPR/Cas9 system-created *Cad96ca* and *Fgfr1* mutants, we searched the *H. armigera* genome for homologues of the target sequences of *Cad96ca* and *Fgfr1* and found that the genes possibly included similar target sequences. PCR amplification and sequencing were performed with these genes.

Generation of *Cad96ca*- or *Fgfr1*-mutant HaEpi cells using the CRISPR/Cas9 system

The target sites were selected according to the CRISPRscan tool (*Supplementary file 2*). Then, two complementary oligonucleotides were synthesized according to the target sequences, and the annealed fragments were cloned into a pUCm-T-U6-gRNA plasmid after forming double chains. Primers gRNAwf-F and gRNAwf-R were used for PCR amplification with the pUCm-T-U6-gRNA plasmid carrying with target gRNA sequence as a template. The obtained fragment was cloned into a pIEx-Cas9-GFP-P2A-Puro plasmid, and pIEx-4-BmU6-gRNA-Cas9-GFP-P2A-Puro was successfully constructed. The pIEx-4-BmU6-Cad96ca-gRNA-Cas9-GFP-P2A-Puro or pIEx-4-BmU6-*Fgfr1*-gRNA-Cas9-GFP-P2A-Puro recombinant vectors were transfected into HaEpi cells with transfection reagent (Roche, Basel, Switzerland). After 48 h of vector transfection (cells can be observed to express green fluorescent protein), fresh medium containing puromycin (Solarbio, Beijing, China) (15 μg/mL) was added to the cells, the medium containing puromycin was replaced every two days until the green fluorescence was gone (about five days), and the medium was replaced. The puromycin-screened cells were used for subsequent experiments. Messy peak figures reporting the results of DNA sequencing showed mutations induced by CRISPR/Cas9 in the HaEpi cells.

Detection of the cellular levels of calcium ions as indicated by protein calcium-sensing GCaMPs

GCaMPs are the most widely used protein calcium sensors (Dana et al., 2019). The CMV promoter of pCMV-GCaMP5G was replaced with an IE promoter and transformed into pIE-GCaMP5G, which can be expressed in HaEpi cells. pIE-GCaMP5G was transfected into normal

HaEpi cells, *Cad96ca*- and *Fgfr1*-mutant HaEpi cells for 48 h and incubated with JH III (1 μM) or JH III (1 μM) plus $CaCl_2$ (1 mM) for 60 s. First, the cells were photographed in white light and then imaged with a fluorescence microscope.

Calcium levels were detected by Flow-8 AM fluorescence probe

Intracellular calcium levels in Sf9 cells, S2 cells, and HEK-293T cells were determined using the fluorescent probe Fluo-8 AM (MKBio, Shanghai, China). Cells were seeded overnight at 50,000 cells per 100 μL per well in a 96-well black wall/clear bottomed plate. The Fluo-8 dye was diluted to 2 μM with DPBS, while the 20% PluronicF-127 solution was added for a final concentration of 0.02%. Add 100 μl Fluo-8 dye solution to each well. Then the plate was incubated at room temperature for 30 min. The cells were washed with DPBS three times. Then, JH III (1 μM) was added to the cells to detect the intracellular calcium release. After that, cells were treated with Calcium chloride (final concentration 1 mM) and JH III (final concentration 1 μM) to detect the extracellular calcium influx. The fluorescence intensities were measured using an ENSPIE plate reader (PE, New York, USA) with a filter set of Ex/Em = 490/514 nm.

Antibodies

The following antibodies were used in the study: anti-His monoclonal antibody (1:3000, AE003, RRID: AB_2728734, ABclonal, Wuhan, China), anti-GFP monoclonal antibody (1:3000, AE012, RRID: AB_2770402, ABclonal, Wuhan, China), anti-ACTB polyclonal antibodies (1:3000, AC026, RRID: AB_2768234, ABclonal, Wuhan, China).

Statistical analysis

All data were from at least three biologically independent experiments. The western blotting results were quantified using ImageJ software (NIH, Bethesda, MA, USA). The fluorescence intensity of each image of calcium detection was analyzed using Image Pro-Plus software (Media Cybernetics, Rockville, MD, USA). GraphPad Prism 7 was used for data analysis and producing figures (GraphPad Software Inc., La Jolla, CA, USA). Multiple sets of data were compared by analysis of variance (ANOVA). The different lowercase letters show significant differences. Two group datasets were analyzed using a two-tailed Student's *t* test. Asterisks indicate significant differences between the groups (*$p < 0.05$, **$p < 0.01$). Error bars indicate the standard deviation (SD) or standard error (SE) of three independent experiments.

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Data and materials availability

All data generated or analyzed in this work are included in the manuscript and the supporting source data files.

Additional information

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Author Contributions

Yan-Xue Li, Conceptualization, Data curation, Investigation, Visualization, Methodology, Writing - original draft; Xin-Le Kang, Software operation, Investigation; Yan-Li Li, Investigation; Xiao-Pei Wang, Investigation; Qiao Yan, Investigation; Jin-Xing Wang, Conceptualization, Writing - review and editing; Xiao-Fan Zhao, Conceptualization, Funding acquisition, Writing - original draft, Writing - review and editing.

Competing Interest Statement

The following authors have previously disclosed a patent application that is relevant to this manuscript: Xiao-Fan Zhao, Yan-Xue Li, and Jin-Xing Wang. The remaining authors declare no competing interests.

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Editors

Reviewer #1 (Public review):

Summary:

Juvenile Hormone (JH) plays a key role in insect development and physiology. Although the intracellular receptor for JH was identified long ago, a number of studies have shown that part of JH functions should be fulfilled through binding to an unknown membrane receptor, which was proposed to belong to the RTK family. In this study, the authors screened all RTKs from the H. armigera genome for their ability to mediate responses to JH III treatment both in cultured cells and in developping animals. They also present convincing evidence that CAD96CA and FGFR1 directly bind JH III, and that their role might be conserved in other insect species.

Strengths:

Altogether, the experimental approach is very complete and elegant, providing evidence for the role of CAD96CA and FGFR1 in JH signalling using different techniques and in different contexts. I believe that this work will open new perspectives to study the role of JH and better understand what is the contribution of signalling through membrane receptors for JH-dependent developmental processes.

Weaknesses:

Unfortunately, the revised manuscript does not show significant improvement. While the identification of the receptors is highly convincing, important issues about the biological relevance remain unaddressed.

First, the main point I raised about the first version of this article is that the redundancy and/or specificity of the two receptors should be clarified, even though I understand that it cannot be deeply investigated here. I believe that this point, shared by all reviewers, is highly relevant for the scope of this work. In this revised version, it is still unclear how to reconcile gain and loss-of-function experiments and the different expression profiles of the receptors.

Second, the newly added explanations and pieces of discussion provided about the mild in vivo phenotypes of early pupation upon Cad96ca or Fgfr1 knock-out do not clarify the issue but instead put emphasis on methodological issues. Indeed, it is not clear whether the mild phenotypes reflect the biological role of Cad96ca and Fgfr1, or the redundancy of these two RTKs (and/or others), or some issue with the knock-out strategy (partial efficiency, mosaicism...).

Finally, parts of the updated discussion and the modifications to the figures are confusing.

https://doi.org/10.7554/eLife.97189.2.sa3

Reviewer #2 (Public review):

Summary:

Juvenile hormone (JH) is a pleiotropic terpenoid hormone in insects that mainly regulates their development and reproduction. In particular, its developmental functions are described as the "status quo" action, as its presence in the hemolymph (the insect blood) prevents metamorphosis-initiating effects of ecdysone, another important hormone in insect development, and maintains the juvenile status of insects.

While such canonical functions of JH are known to be mediated by its intracellular receptor complex composed of Met and Tai, there have been multiple reports suggesting the presence of cell membrane receptor(s) for JH, which mediate non-genomic effects of this terpenoid hormone. In particular, the presence of receptor tyrosine kinase(s) that phosphorylate Met/Tai in response to JH and thus indirectly affect the canonical JH signaling pathway has been strongly suggested. Given the importance of JH in insect physiology and the fact that the JH signaling pathway is a major target of insect growth regulators, elucidating the identify and functions of putative JH membrane receptors is of great significance from both basic and applied perspectives.

In the present study, the authors identified candidate receptors for such cell membrane JH receptors, CAD96CA and FGFR1, in the cotton bollworm Helicoverpa armigera.

Strengths:

Their in vitro analyses are conducted thoroughly using multiple methods, which overall supports their claim that these receptors can bind to JH and mediate their non-genomic effects.

Weaknesses:

Results of their in vivo experiments, particularly those of their loss-of-function analyses using CRISPR mutants are still preliminary, and the results rather indicate that these membrane receptors do not have any physiologically significant roles in vivo. More specifically, previous studies in lepidopteran species have clearly and repeatedly shown that precocious metamorphosis is the hallmark phenotype for all JH signaling-deficient larvae. In contrast, the present study showed that Cad96ca and Fgfr1 G0 mutants only showed slight acceleration in their pupation timing, which is not a typical phenotype one would expect from JH signaling deficiency. This is inconsistent with their working model provided in Figure 6, which indicates that these cell membrane JH receptors promote the canonical JH signaling by phosphorylating Met/Tai.

If the authors argue that this slight acceleration of pupation is indeed a major JH signaling-deficient phenotype in Helicoverpa, they need to provide more data to support their claim by analyzing CRISPR mutants of other genes involved in JH signaling, such as Jhamt and Met. An alternative explanation is that there is functional redundancy between CAD96CA and FGFR1 in mediating phosphorylation of Met/Tai. This possibility can be tested by analyzing double knockouts of these two receptors.

Currently, the validity of their calcium imaging analysis in Figure 5 is also questionable. When performing calcium imaging in cultured cells, it is critically important to treat all the cells at the end of each experiment with a hormone or other chemical reagents that universally induce calcium increase in each particular cell line. Without such positive

control, the validity of calcium imaging data remains unknown, and readers cannot properly evaluate their results.

https://doi.org/10.7554/eLife.97189.2.sa2

Reviewer #3 (Public review):

Summary:

In this study, Li et al. identified CAD96CA and FGF1 among 20 receptor tyrosine kinase receptors as mediators of JH signaling. By performing a screen in HaEpi cells with overactivated JH signaling, the authors pinpointed two main RTKs that contribute to the transduction of JH. Using the CRISPR/Cas9 system to generate mutants, the authors confirmed that these RTKs are required for normal JH activation, as precocious pupariation was observed in their absence. Additionally, the authors demonstrated that both CAD96CA and FGF1 exhibit a high affinity for JH, and their activation is necessary for the proper phosphorylation of Tai and Met, transcription factors that promote the transcriptional response. Finally, the authors provided evidence suggesting that the function of CAD96CA and FGF1 as JH receptors is conserved across insects.

Strengths:

The data provided by the authors are convincing and support the main conclusions of the study, providing ample evidence to demonstrate that phosphorylation of the transducers Met and Tai mainly depends on the activity of two RTKs. Additionally, the binding assays conducted by the authors support the function of CAD96CA and FGF1 as membrane receptors of JH. The study's results validate, at least in H. amigera, the predicted existence of membrane receptors for JH.

Weaknesses:

The authors have provided evidences that the Cad96Ca and FGF1 RTK receptors contribute to JH signaling through CRISPR/Cas9, inducing precocious metamorphosis, although not to the same extent as absence of JH. Therefore, it still remains unclear whether these RTKs are completely required for pathway activation or only necessary for high activation levels during the last larval stage.

While the authors have included some additional data, the mechanism by which different RTKs function in transducing JH signaling in a tissue specific manner is still unclear. As the authors note in the discussion, it is possible that other RTKs may also play a role in facilitating the transduction of JH signaling.

Lastly, the study does not yet explain how RTKs with known ligands could also bind JH and contribute to JH signaling activation. Although receptor promiscuity has been suggested as a possible mechanism, future studies could explore whether activation of RTK pathways by their known ligands induces certain levels of JH transducer phosphorylation, which, in the presence of JH, could contribute to full pathway activation without the need for direct JH-RTK binding.

https://doi.org/10.7554/eLife.97189.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *Juvenile Hormone (JH) plays a key role in insect development and physiology. Although the intracellular receptor for JH was identified long ago, a number of studies have shown that part of JH functions should be fulfilled through binding to an unknown membrane receptor, which was proposed to belong to the RTK family. In this study, the authors screened all RTKs from the H. armigera genome for their ability to mediate responses to JH III treatment both in cultured cells and in developing animals. They also present convincing evidence that CAD96CA and FGFR1 directly bind JH III, and that their role might be conserved in other insect species.*
>
> *Strengths:*
>
> *Altogether, the experimental approach is very complete and elegant, providing evidence for the role of CAD96CA and FGFR1 in JH signalling using different techniques and in different contexts. I believe that this work will open new perspectives to study the role of JH and better understand what is the contribution of signalling through membrane receptors for JH-dependent developmental processes.*
>
> *Weaknesses:*
>
> *I don't see major weaknesses in this study. However, I think that the manuscript would benefit from further information or discussion regarding the relationship between the two newly identified receptors. Experiments (especially in HEK-293T cells) suggest that CAD96CA and FGFR1 are sufficient on their own to transduce JH signalling. However, they are also necessary since loss-of-function conditions for each of them are sufficient to trigger strong effects (while the other is supposed to be still present).*

Thank you for the suggestion. We have added the discussion in the text: "CAD96CA and FGFR1 have similar functions in JH signaling, including transmitting JH signal for *Kr-h1* expression, larval status maintaining, rapid intracellular calcium increase, phosphorylation of transcription factors MET1 and TAI, and high affinity to JH III. CAD96CA and FGFR1 are essential in the JH signal pathway, and loss-of-function for each is sufficient to trigger strong effects on pupation. The difference is that CAD96CA expression has no tissue specificity, and the *Fgfr1* gene is highly expressed in the midgut; possibly, it plays a significant role in the midgut. Other possibility is that they play roles by forming heterodimer with each other or other RTKs, which needs to be addressed in future study. CAD96CA and FGFR1 transmit JH III signals in three different insect cell lines, suggesting their conserved roles in other insects.".

> *In addition, despite showing different expression patterns, the two receptors seem to display similar developmental functions according to loss-of-function phenotypes. It is therefore unclear how to draw a model for membrane receptor-mediated JH signalling that includes both CAD96CA and FGFR1.*

Thank you for your question. We have modified the figure and the legends to make the conception clear.

> ***Reviewer #2 (Public Review):***
>
> *Summary:*
>
> *Juvenile hormone (JH) is a pleiotropic terpenoid hormone in insects that mainly regulates their development and reproduction. In particular, its developmental functions are described as the "status quo" action, as its presence in the hemolymph (the insect blood)*

> *prevents metamorphosis-initiating effects of ecdysone, another important hormone in insect development, and maintains the juvenile status of insects. While such canonical functions of JH are known to be mediated by its intracellular receptor complex composed of Met and Tai, there have been multiple reports suggesting the presence of cell membrane receptor(s) for JH, which mediate non-genomic effects of this terpenoid hormone. In particular, the presence of receptor tyrosine kinase(s) that phosphorylate Met/Tai in response to JH and thus indirectly affect the canonical JH signaling pathway has been strongly suggested. Given the importance of JH in insect physiology and the fact that the JH signaling pathway is a major target of insect growth regulators, elucidating the identification and functions of putative JH membrane receptors is of great significance from both basic and applied perspectives. In the present study, the authors identified candidate receptors for such cell membrane JH receptors, CAD96CA and FGFR1, in the cotton bollworm* Helicoverpa armigera*.*
>
> *Strengths:*
>
> *Their in vitro analyses are conducted thoroughly using multiple methods, which overall supports their claim that these receptors can bind to JH and mediate their non-genomic effects.*
>
> *Weaknesses:*
>
> *Results of their in vivo experiments, particularly those of their loss-of-function analyses using CRISPR mutants are still preliminary, and the results rather indicate that these membrane receptors do not have any physiologically significant roles in vivo. More specifically, previous studies in lepidopteran species have clearly and repeatedly shown that precocious metamorphosis is the hallmark phenotype for all JH signaling-deficient larvae. In contrast, the present study showed that Cad96ca and Fgfr1 G0 mutants only showed a slight acceleration in their pupation timing, which is not a typical phenotype one would expect from JH signaling deficiency. This is inconsistent with their working model provided in Figure 6, which indicates that these cell membrane JH receptors promote the canonical JH signaling by phosphorylating Met/Tai.*
>
> *If the authors argue that this slight acceleration of pupation is indeed a major JH signaling-deficient phenotype in Helicoverpa, they need to provide more data to support their claim by analyzing CRISPR mutants of other genes involved in JH signaling, such as Jhamt and Met. An alternative explanation is that there is functional redundancy between CAD96CA and FGFR1 in mediating phosphorylation of Met/Tai. This possibility can be tested by analyzing double knockouts of these two receptors.*

Thank you for your question and suggestion. The cadherin 96ca (CAD96CA) and fibroblast growth factor receptor 1 (FGFR1) were finally determined as JH cell membrane receptors by their roles in JH regulated-gene expression, maintaining larval status, JH induced-rapid increase of intracellular calcium levels, JH induced-phosphorylation of MET and TAI, and their JH-binding affinity. Their roles as JH cell membrane receptors were further determined by knockdown and knockout of them *in vivo* and in cell lines, and overexpression of them in mammal HEK-293T heterogeneously. Figure 6 is drafted by these solidate evidences.

Cad96ca and *Fgfr1* G0 mutants caused slight acceleration of pupation is one of the types of evidence of JH signaling-deficient. Othe evidences include a set of gene expression and the block of JH induced-rapid intracellular calcium increase.

Kr-h1 is a typical indicator gene at the downstream of Jhamt and in JH signaling, so we used it as an indicator to examine JH signaling. *Jhamt* and *Met* or other genes might be affected in *Cad96ca* and *Fgfr1* G0 mutants, which can be examined in future study.

We have discussed the question that *Cad96ca* and *Fgfr1* G0 mutants only showed a slight acceleration in their pupation timing: "Homozygous *Cad96ca* null *Drosophila* die at late pupal stages (*Wang et al., 2009*). However, we found that 86% of the larvae of the *Cad96ca* mutant successfully pupated in G0 generation, although earlier than the control. Similarly, null mutation of *Fgfr1* or *Fgfr2* in mouse is embryonic lethal (*Arman et al., 1998; Deng et al., 1994; Yamaguchi et al., 1994*). In *D. melanogaster*, homozygous *Htl* (*Fgfr*) mutant embryos die during late embryogenesis, too (*Beati et al., 2020; Beiman et al., 1996; Gisselbrecht et al., 1996*). However, in *H. armigera*, 91% of larvae successfully pupated in G0 generation after *Fgfr1* knockout. The low death rate after *Cad96ca* and *Fgfr1* knockout might be because of following reasons, including the editing efficiency (67% and 61% for *Cad96ca* mutant and *Fgfr1* mutant, respectively), the chimera of the gene knockout at the G0 generation, and the redundant RTKs that play similar roles in JH signaling, similar to the redundant roles of MET and Germ-cell expressed bHLH-PAS (GCE) in JH signaling *(Liu et al., 2009)*, which needs to obtain alive G1 homozygote mutants and double knockout of these two receptors in future study. We indeed observed that the eggs did not hatch successfully after mixed-mating of G0 *Cad96ca* mutant or *Fgfr1* mutant, respectively, but the reason was not addressed further due to the embryonic death. By the similar reasons, most of the *Cad96ca* and *Fgfr1* mutants showed a slight acceleration of pupation (about one day) without the typical precocious metamorphosis (at least one instar earlier) phenotype caused by JH signaling defects (*Daimon et al., 2012; Fukuda, 1944; Riddiford et al., 2010*) and JH pathway gene deletions (*Abdou et al., 2011; Liu et al., 2009*). On other side, JH can regulate gene transcription by diffusing into cells and binding to the intracellular receptor MET to conduct JH signal, which might affect the results of gene knockdown and knockout.".

> *Currently, the validity of their calcium imaging analysis in Figure 5 is also questionable. When performing calcium imaging in cultured cells, it is critically important to treat all the cells at the end of each experiment with a hormone or other chemical reagents that universally induce calcium increase in each particular cell line. Without such positive control, the validity of calcium imaging data remains unknown, and readers cannot properly evaluate their results.*

Thank you for your question. For Figure 5, our goal was to demonstrate that JH can induce calcium mobilization through CAD96CA and FGFR1. Controls have been established between different experimental groups within the same cell, as well as between different cells. Increasing the positive experimental group would make the results more complex.

> ***Reviewer #3 (Public Review):***
>
> *Summary:*
>
> *In this study, Li et al. identified CAD96CA and FGF1 among 20 receptor tyrosine kinase receptors as mediators of JH signaling. By performing a screen in HaEpi cells with overactivated JH signaling, the authors pinpointed two main RTKs that contribute to the transduction of JH. Using the CRISPR/Cas9 system to generate mutants, the authors confirmed that these RTKs are required for normal JH activation, as precocious pupariation was observed in their absence. Additionally, the authors demonstrated that both CAD96CA and FGF1 exhibit a high affinity for JH, and their activation is necessary for the proper phosphorylation of Tai and Met, transcription factors that promote the transcriptional response. Finally, the authors provided evidence suggesting that the function of CAD96CA and FGF1 as JH receptors is conserved across insects.*
>
> *Strengths:*
>
> *The data provided by the authors are convincing and support the main conclusions of the study, providing ample evidence to demonstrate that phosphorylation of the*

> *transducers Met and Tai mainly depends on the activity of two RTKs. Additionally, the binding assays conducted by the authors support the function of CAD96CA and FGF1 as membrane receptors of JH. The study's results validate, at least in H. amigera, the predicted existence of membrane receptors for JH.*
>
> *Weaknesses:*
>
> *The study has several weaknesses that need to be addressed. Firstly, it is not clear what criteria were used by the authors to discard several other RTKs that were identified as repressors of JH signaling. For example, while NRK and Wsck may not fulfill all the requirements to become JH receptors, other evidence, such as depletion analysis and target gene expression, suggests they are involved in proper JH signaling activation.*

Thank you for your question. We screened the RTKs sequentially, including examining the roles of 20 RTKs identified in the *H. armigera* genome in JH regulated-gene expression to obtain primary candidates, followed by screening of the candidates by their roles in maintaining larval status, JH induced-rapid increase of intracellular calcium levels, JH induced-phosphorylation of MET and TAI, and affinity to JH. WSCK was not involved in the phosphorylation of MET and TAI and was discarded during subsequent screening. NRK did not bind to JH III, did not meet the screening strategy, and was discarded.

We increased the information in the Introduction: "We screened the RTKs sequentially, including examining the roles of 20 RTKs identified in the *H. armigera* genome in JH regulated-gene expression to obtain primary candidates, followed by screening of the candidates by their roles in maintaining larval status, JH induced-rapid increase of intracellular calcium levels, JH induced-phosphorylation of MET and TAI, and affinity to JH. The cadherin 96ca (CAD96CA) and fibroblast growth factor receptor 1 (FGFR1) were finally determined as JH cell membrane receptors by their roles in JH regulated-gene expression, maintaining larval status, JH induced-rapid increase of intracellular calcium levels, JH induced-phosphorylation of MET and TAI, and their JH-binding affinity. Their roles as JH cell membrane receptors were further determined by knockdown and knockout of them *in vivo* and cell lines, and overexpression of them in mammal HEK-293T heterogeneously.".

We increased discussion: "This study found six RTKs that respond to JH induction by participating in JH induced-gene expression and intracellular calcium increase, however; they exert different functions in JH signaling, and finally CAD96CA and FGFR1 are determined as JH cell membrane receptors by their roles in JH induced-phosphorylation of MET and TAI and binding to JH III. We screen the RTKs transmitting JH signal primarily by examining some of JH induced-gene expression. By examining other genes or by other strategies to screen the RTKs might find new RTKs functioning as JH cell membrane receptors; however, the key evaluation indicators, such as the binding affinity of the RTKs to JH and the function in transmitting JH signal to maintain larval status are essential.".

> *Secondly, the expression of the six RTKs, which, when knocked down, were able to revert JH signaling activation, was mainly detected in the last larval stage of H. amigera. However, since JH signaling is active throughout larval development, it is unclear whether these RTKs are completely required for pathway activation or only needed for high activation levels at the last larval stage.*

Thank you for the question. We knocked down the genes at last larval stage to observe pupation, which is a relatively simple and easily to be observed target to examine the role of the gene in JH-maintained larval status. The results from CRISPR/Cas9 experiments showed: "Most wild-type larvae showed a phenotype of pupation on time. However, in the *Cad96ca* mutant, 86% of the larvae (an editing efficiency of 67% by TA clone analysis) had a shortened feeding stage in the sixth instar and entered the metamorphic molting stage earlier, showing

early pupation, with the pupation time being 24 h earlier. In the *Fgfr1* mutant, 91% of the larvae (an editing efficiency of 61%) had a shortened feeding stage in the sixth instar and entered the metamorphic molting stage earlier, showing early pupation, with the pupation time being 23 h earlier (*Figure 4D and E*). The data suggested that CAD96CA and FGFR1 support larval growth and prevent pupation *in vivo*.".

> *Additionally, the mechanism by which different RTKs exert their functions in a specific manner is not clear. According to the expression profile of the different RTKs, one might expect some redundant role of those receptors. In fact the no reversion of phosphorilation of tai and met upon depletion of Wsck in cells with overactivated JH signalling seems to support this idea.*
>
> *Nevertheless, and despite the overlapping expression of the different receptors, all RTKs seem to be required for proper pathway activation, even in the case of FGF1 which seems to be only expressed in the midgut. This is an intriguing point unresolved in the study.*

Thank you for your comments. Yes, from our study, different RTKs exert their functions in a specific manner. We have increased discussion: "This study found six RTKs that respond to JH induction by participating in JH induced-gene expression and intracellular calcium increase, however; they exert different functions in JH signaling, and finally CAD96CA and FGFR1 are determined as JH cell membrane receptors by their roles in JH induced-phosphorylation of MET and TAI and binding to JH III. We screen the RTKs transmitting JH signal primarily by examining some of JH induced-gene expression. By examining other genes or by other strategies to screen the RTKs might find new RTKs functioning as JH cell membrane receptors; however, the key evaluation indicators, such as the binding affinity of the RTKs to JH and the function in transmitting JH signal to maintain larval status are essential.".

> *Finally, the study does not explain how RTKs with known ligands could also bind JH and contribute to JH signaling activation. in Drosophila, FGF1 is activated by pyramus and thisbe for mesoderm development, while CAD96CA is activated by collagen during wound healing. Now the authors claim that in addition to these ligands, the receptors also bind to JH. However, it is unclear whether these RTKs are activated by JH independently of their known ligands, suggesting a specific binding site for JH, or if they are only induced by JH activation when those ligands are present in a synergistic manner. Alternatively, another explanation could be that the RTK pathways by their known ligands activation may induce certain levels of JH transducer phosphorylation, which, in the presence of JH, contributes to the full pathway activation without JH-RTK binding being necessary.*

Thank you for your professional questions. It is an exciting and challenging to explore the molecular mechanism by which multiple ligands transmit signals through the same receptor. It requires a long-term research plan and in-depth studies. We added discussion in the text: "CAD96CA (also known as Stitcher, Ret-like receptor tyrosine kinase) activates upon epidermal wounding in *Drosophila* embryos (*Tsarouhas et al., 2014*) and promotes growth and suppresses autophagy in the *Drosophila* epithelial imaginal wing discs (*O'Farrell et al., 2013*). There is a CAD96CA in the genome of the *H. armigera*, which is without function study. Here, we reported that CAD96CA prevents pupation by transmitting JH signal as a JH cell membrane receptor. We also showed that CAD96CA of other insects has a universal function of transmitting JH signal to trigger Ca2+ mobilization, as demonstrated by the study in Sf9 cell lines of *S. frugiperda* and S2 cell lines of *D. melanogaster*.

FGFRs control cell migration and differentiation in the developing embryo of *D. melanogaster* (*Muha and Muller, 2013*). The ligand of FGFR is FGF in _D. melanogaste_r *(Du et al., 2018*). FGF binds FGFR and triggers cell proliferation, differentiation, migration, and survival (*Beenken and Mohammadi, 2009; Lemmon and Schlessinger, 2010*). Three FGF ligands and two FGF receptors (FGFRs) are identified in *Drosophila* (*Huang and Stern, 2005*). The *Drosophila* FGF-

FGFR interaction is specific. Different ligands have different functions. The activation of FGFRs by specific ligands can affect specific biological processes (*Kadam et al., 2009*). The FGFR in the membrane of Sf9 cells can bind to Vip3Aa (*Jiang et al., 2018*). One FGF and one FGFR are in the *H. armigera* genome, which has yet to be studied functionally. The study found that FGFR prevents insect pupation by transmitting JH signal as a JH cell membrane receptor. Exploring the molecular mechanism and output by which multiple ligands transmit signals through the same receptor is exciting and challenging.".

> ***Reviewer #1 (Recommendations For The Authors):***
>
> *As an experimental suggestion, I will only propose that authors test the double knock-down/knock-out or overexpression of CAD96CA and FGFR1 to give some hints into how redundant/independent the two receptors are.*

Thank you very much for your professional advice. We agree with your point of view that double knockout of CAD96CA and FGFR1 is very important to resolve the redundant/independent of the two receptors, which can make our research more complete. Unfortunately, due to experimental difficulty and time constraints, we did not provide supplementary experiments. In this study, we aim to screen the cell membrane receptors of JH. Therefore, we focused on which RTKs can function as receptors. This article is a preliminary study to identify the cell membrane receptors of JH. To further understand the relationship between the two membrane receptors, we will conduct in-depth research in future work.

> *Apart from that, here are some minor points about the manuscript:*
>
> *Figure 2A: changing the scale on the y-axis would help to better see the different genotypes (similar to the way it is presented in Figure 5).*

Thanks for your reminding, we have changed the scale in Figure 2A.

> *Figure 4J: image settings could be improved to better highlight the green fluorescence.*

Thank you for your advice, we have improved the imaged in Figure 4J.

> *In general, the manuscript would benefit from some proofreading since a number of sentences are incorrect.*

Thanks for your reminding, we have carefully revised the manuscript.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *(1) Although the authors note that there are 21 RTK genes in Drosophila (line 55), I can only see 16 Drosophila RTKs in Figure 1 - Figure Supplement 1. Some important Drosophila RTKs such as breathless are missing. The authors need to redraw the phylogenetic tree.*

Thanks for your reminding, we have presented the new phylogenetic tree in Figure 1-figure supplement 1.

> *(2) The accelerated pupation phenotype in Cad96ca and Fgfr1 G0 mutants needs to be better described. In particular, it is critical to examine which developmental stage(s) are shortened in these mutant larvae. Refer to a similar study on a JH biosynthetic enzyme in Bombyx (PMID: 22412378) regarding how to describe the developmental timing phenotype.*

Thank you for your advice. We have re-shown Figure 4E and added the explanation in the text: "In 61 survivors of Cas9 protein plus *Cad96ca*-gRNA injection, 30 mutants were sequenced, and a mutation efficiency was 49.2%. Similarly, in the 65 survivors of Cas9 protein plus *Fgfr1*-gRNA injection, 35 mutants were sequenced, and a mutation efficiency was 53.8% (*Figure 4C*). The DNA sequences, deduced amino acids and off–target were analyzed (*Figure 4—figure supplement 1*). Most wild-type larvae showed a phenotype of pupation on time. However, in the *Cad96ca* mutant, 86% of the larvae (an editing efficiency of 67% by TA clone analysis) had a shortened feeding stage in the sixth instar and entered the metamorphic molting stage earlier, showing early pupation, with the pupation time being 24 h earlier. In the *Fgfr1* mutant, 91% of the larvae (an editing efficiency of 61%) had a shortened feeding stage in the sixth instar and entered the metamorphic molting stage earlier, showing early pupation, with the pupation time being 23 h earlier (*Figure 4D and E*). The data suggested that CAD96CA and FGFR1 support larval growth and prevent pupation *in vivo*.".

> *(3) The editing efficiency described in lines 211-213 is obscure. Does this indicate the percentage of animals with noisy sequencing spectra or the percentage of mutation rates analyzed by TA cloning?*

Thanks for your reminder. We have revised the description in the text: "In 61 survivors of Cas9 protein plus *Cad96ca*-gRNA injection, 30 mutants were sequenced, and a mutation efficiency was 49.2%. Similarly, in the 65 survivors of Cas9 protein plus *Fgfr1*-gRNA injection, 35 mutants were sequenced, and a mutation efficiency was 53.8% (*Figure 4C*). The DNA sequences, deduced amino acids and off–target were analyzed (*Figure 4—figure supplement 1*). Most wild-type larvae showed a phenotype of pupation on time. However, in the *Cad96ca* mutant, 86% of the larvae (an editing efficiency of 67% by TA clone analysis) had a shortened feeding stage in the sixth instar and entered the metamorphic molting stage earlier, showing early pupation, with the pupation time being 24 h earlier. In the *Fgfr1* mutant, 91% of the larvae (an editing efficiency of 61%) had a shortened feeding stage in the sixth instar and entered the metamorphic molting stage earlier, showing early pupation, with the pupation time being 23 h earlier (*Figure 4D and E*). The data suggested that CAD96CA and FGFR1 support larval growth and prevent pupation *in vivo*.".

> *(4) In Figures 4F and G, the authors examined expression levels of some JH/ecdysone responsive genes only at 0 hr-old 6th instar larvae. This single developmental stage is not enough for this analysis. In particular, the expression level of Fgfr1 only goes up in the mid-6th instar according to their own data (Figure 1-Figure Supplement 4), so it is critical to examine expression levels of these genes at least throughout the 6th larval instar.*

Thank you for your advice. Indeed, it is essential to detect the expression levels of JH/ecdysone response genes in the whole sixth instar larvae. Because we observed that the mutation has a shorter feeding stage at the sixth instar, we examined the expression level of the JH/ecdysone response gene at the early sixth instar. Due to the number of mutants obtained in the experiment was small and non-destructive sampling could not be performed in sixth instar period, there were no enough samples to test. In the future, we will generate *Cad96ca Fgfr1* double mutations to carry out studies and detect the expression level of JH/ecdysone response genes in the whole sixth instar.

> *(5) As mentioned above, some important Drosophila RTKs such as breathless are missing in their analyses. As breathless is a close paralog of heartless (Htl), I am sure that Drosophila breathless is also orthologous to Helicoverpa FGFR1. The authors therefore need to analyze breathless in Figure 5B in addition to Htl.*

Thank you for your advice. We added experiments and the results are shown in Figure 5B and Figure 5—figure supplement 1.

> *(6) More discussion about the reason why dsNrk and dsWsck can provide resistance to JHIII in Figure 1 is required.*

Thank you for your advice. We added explanation in the discussion: "It is generally believed that the primary role of JH is to antagonize 20E during larval molting (*Riddiford, 2008*). The knockdown of *Cad96ca*, *Nrk*, *Fgfr1*, and *Wsck* showed phenotypes resistant to JH III induction and the decrease of *Kr-h1* and increase of *Br-z7* expression, but knockdown of *Vegfr* and *Drl* only decrease *Kr-h1*, without increase of *Br-z7*. *Br-z7* is involved in 20E-induced metamorphosis in *H. armigera (Cai et al., 2014)*, whereas, Kr-h1 is a JH early response gene that mediates JH action (Minakuchi et al., 2009) and represses *Br* expression (*Riddiford et al., 2010*). The high expression of *Br-z7* is possible due to the down-regulation of *Kr-h1* in *Cad96ca*, *Nrk*, *Fgfr1* and *Wsck* knockdown larvae. The different expression profiles of *Br-z7* in *Vegfr* and *Drl* knockdown larvae suggest other roles of *Vegfr* and *Drl* in JH signaling, which need further study."

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *(1) The authors should consider optimizing their experimental approach by depleting the six candidate RTKs in an early larval stage rather than using a sensitized background with JH application in the last larval stage.*

Thank you for your precious suggestion. We knocked down the genes at last larval stage to observe pupation, which is a relatively simple and easily to be observed target to examine the role of the gene in JH-maintained larval status. The results from CRISPR/Cas9 experiments showed: "Most wild-type larvae showed a phenotype of pupation on time. However, in the *Cad96ca* mutant, 86% of the larvae (an editing efficiency of 67% by TA clone analysis) had a shortened feeding stage in the sixth instar and entered the metamorphic molting stage earlier, showing early pupation, with the pupation time being 24 h earlier. In the *Fgfr1* mutant, 91% of the larvae (an editing efficiency of 61%) had a shortened feeding stage in the sixth instar and entered the metamorphic molting stage earlier, showing early pupation, with the pupation time being 23 h earlier (*Figure 4D and E*). The data suggested that CAD96CA and FGFR1 support larval growth and prevent pupation *in vivo*.". To know the roles of other RTKs in the whole larval development needs future work since a lot of experiments are needed.

> *(2) Including a positive control for JH signaling, such as met or tai, would strengthen the assays and provide a benchmark for evaluating the downregulation of target genes and phenotype reversion upon JH application. This addition, especially in Figure 1, would enhance the interpretability of the results.*

Thank you for your suggestion. We agree with your point of view that adding the detection of *Met* or *Tai* as a positive control. Our laboratory has reported in previous studies that knockdown of *Met* leads to decreased expression of genes in the JH signaling pathway and precocious pupation (PMID: 24872508), so we did not repeat this related experiment in this study. In the future, when performg *Cad96ca* and *Fgfr1* double mutant experiments, *Met* mutant can be generated as a control to provide more references for the interpretation of the results.

> *(3) I recommend revising the manuscript to improve readability, particularly in the Results section, where descriptions of the binding part are particularly dense.*

Thank you for your advice. We have carefully revised the manuscript.

> *(4) In line 122, please add the reference Wang et al., 2016.*

Thank you for your reminding, we have added the reference in line 125 of the new manuscript.

> *(5) The authors should clarify why they chose to test the possible binding to JH of only Cad96CA, FGFR1, and NRK after conducting various assays while including OTK in the study as a negative control. This explanation should be included in the text.*

Thank you for the suggestion. We added the explanation, as described in the text: "We screened the RTKs sequentially, including examining the roles of 20 RTKs identified in the *H. armigera* genome in JH regulated-gene expression to obtain primary candidates, followed by screening of the candidates by their roles in maintaining larval status, JH induced-rapid increase of intracellular calcium levels, JH induced-phosphorylation of MET and TAI, and affinity to JH. The cadherin 96ca (CAD96CA) and fibroblast growth factor receptor 1 (FGFR1) were finally determined as JH cell membrane receptors by their roles in JH regulated-gene expression, maintaining larval status, JH induced-rapid increase of intracellular calcium levels, JH induced-phosphorylation of MET and TAI, and their JH-binding affinity. Their roles as JH cell membrane receptors were further determined by knockdown and knockout of them *in vivo* and cell lines, and overexpression of them in mammal HEK-293T heterogeneously.".

"Since Cad96CA, FGFR1, and NRK were not only involved in JH-regulated *Kr-h1* expression, JH III-induced delayed pupation, and calcium levels increase, but also involved in MET and TAI phosphorylation, we further analyzed their binding affinity to JH III. OTK did not respond to JH III, so we used it as a control protein on the cell membrane to exclude the possibility of nonspecific binding.".

> *(6) The observed embryonic lethality of cad96ca and FGF1 mutants in Drosophila contrasts with the ability of the respective mutants in H. armigera to reach the pupal stage. The authors should discuss this significant difference.*

Thank you for the suggestion. We added the explanation in the discussion, as described in the text: "Homozygous *Cad96ca* null *Drosophila* die at late pupal stages (*Wang et al., 2009*). However, we found that 86% of the larvae of the *Cad96ca* mutant successfully pupated in G0 generation, although earlier than the control. Similarly, null mutation of *Fgfr1* or *Fgfr2* in mouse is embryonic lethal (*Arman et al., 1998; Deng et al., 1994; Yamaguchi et al., 1994*). In *D. melanogaster*, homozygous *Htl* (*Fgfr*) mutant embryos die during late embryogenesis, too (*Beati et al., 2020; Beiman et al., 1996; Gisselbrecht et al., 1996*). However, in *H. armigera*, 91% of larvae successfully pupated in G0 generation after *Fgfr1* knockout. The low death rate after *Cad96ca* and *Fgfr1* knockout might be because of following reasons, including the editing efficiency (67% and 61% for *Cad96ca* mutant and *Fgfr1* mutant, respectively), the chimera of the gene knockout at the G0 generation, and the redundant RTKs that play similar roles in JH signaling, similar to the redundant roles of MET and Germ-cell expressed bHLH-PAS (GCE) in JH signaling *(Liu et al., 2009)*, which needs to obtain alive G1 homozygote mutants and double knockout of these two receptors in future study. We indeed observed that the eggs did not hatch successfully after mixed-mating of G0 *Cad96ca* mutant or *Fgfr1* mutant, respectively, but the reason was not addressed further due to the embryonic death. By the similar reasons, most of the *Cad96ca* and *Fgfr1* mutants showed a slight acceleration of pupation (about one day) without the typical precocious metamorphosis (at least one instar earlier) phenotype caused by JH signaling defects (*Daimon et al., 2012; Fukuda, 1944; Riddiford et al., 2010*) and JH pathway gene deletions (*Abdou et al., 2011; Liu et al., 2009*). On other side, JH can regulate

gene transcription by diffusing into cells and binding to the intracellular receptor MET to conduct JH signal, which might affect the results of gene knockdown and knockout.".

(7) Building upon the previous point, it is noteworthy that the cad96ca and FGF1 mutants exhibit only a 24-hour early pupation phenotype, contrasting with the 48-hour early pupation induced by Kr-h1 depletion. This discrepancy suggests that while the function of these RTKs is necessary, it may not be sufficient to fully activate JH signaling. The expression profile of these receptors, primarily observed in the last larval stage, supports this hypothesis.

Thank you for your suggestion. We added the explanation in the discussion, as described in the text: "Homozygous *Cad96ca* null *Drosophila* die at late pupal stages (*Wang et al., 2009*). However, we found that 86% of the larvae of the *Cad96ca* mutant successfully pupated in G0 generation, although earlier than the control. Similarly, null mutation of *Fgfr1* or *Fgfr2* in mouse is embryonic lethal (*Arman et al., 1998; Deng et al., 1994; Yamaguchi et al., 1994*). In *D. melanogaster*, homozygous *Htl* (*Fgfr*) mutant embryos die during late embryogenesis, too (*Beati et al., 2020; Beiman et al., 1996; Gisselbrecht et al., 1996*). However, in *H. armigera*, 91% of larvae successfully pupated in G0 generation after *Fgfr1* knockout. The low death rate after *Cad96ca* and *Fgfr1* knockout might be because of following reasons, including the editing efficiency (67% and 61% for *Cad96ca* mutant and *Fgfr1* mutant, respectively), the chimera of the gene knockout at the G0 generation, and the redundant RTKs that play similar roles in JH signaling, similar to the redundant roles of MET and Germ-cell expressed bHLH-PAS (GCE) in JH signaling *(Liu et al., 2009)*, which needs to obtain alive G1 homozygote mutants and double knockout of these two receptors in future study. We indeed observed that the eggs did not hatch successfully after mixed-mating of G0 *Cad96ca* mutant or *Fgfr1* mutant, respectively, but the reason was not addressed further due to the embryonic death. By the similar reasons, most of the *Cad96ca* and *Fgfr1* mutants showed a slight acceleration of pupation (about one day) without the typical precocious metamorphosis (at least one instar earlier) phenotype caused by JH signaling defects (*Daimon et al., 2012; Fukuda, 1944; Riddiford et al., 2010*) and JH pathway gene deletions (*Abdou et al., 2011; Liu et al., 2009*). On other side, JH can regulate gene transcription by diffusing into cells and binding to the intracellular receptor MET to conduct JH signal, which might affect the results of gene knockdown and knockout.".

> *(8) The expression profile of the RTK hits described in Supplementary Figure 4A appears to be limited to the last larval stage until pupation. The authors should clarify whether these receptors are expressed earlier, and the meaning of the letters in the plot should be described in the figure legend.*

Thank you for the suggestion. We added the explanation in the Figure 1—figure supplement 4 legend, as described in the text: "The expression profiles of *Vegfr1*, *Drl*, *Cad96ca*, *Nrk*, *Fgfr1*, and *Wsck* during development. 5F: fifth instar feeding larvae; 5M: fifth instar molting larvae; 6th-6 h to 6th-120 h: sixth instar at 6 h to sixth instar 120 h larvae; P0 d to P8 d: pupal stage at 0-day to pupal stage at 8-day F: feeding stage; M: molting stage; MM: metamorphic molting stage; P: pupae.".

We are very sorry, but due to time limitations, we will investigate the expression profile of RTK throughout the larval stage in future work.

> *(9) In Figure 4, panels F and G, the levels of Kr-h1 are shown in cad96ca and FGF1 mutants in the last larval stage. The authors should indicate whether Kr-h1 levels are also low in earlier larval stages or only detected in the last larval stage, as this would imply that these RTKs are only required at this stage.*

Thank you for your suggestion. In this study, the *Cad96ca* and *Fgfr1* mutants' feeding stage was shortened in the sixth instar, and they entered the metamorphic molting stage earlier. So, we detected the expression of *Kr-h1* in the sixth instar. It is an excellent idea to detect the

expression of *Kr-h1* at various larvae stages to analyze the stages in which CAD96CA and FGFR1 play a role and to study the relationship between CAD96CA and FGFR1 in future.

> *(10) While Figure 5 demonstrates JH-triggered calcium ion mobilization in Sf9 cells and S2 cells, the authors should also include data on JH signaling target genes, such as Kr-h1, for a more comprehensive analysis.*

Thank you for your advice. We added experiments, as described in the text: "To demonstrate the universality of CAD96CA and FGFR1 in JH signaling in different insect cells, we investigated JH-triggered calcium ion mobilization and *Kr-h1* expression in Sf9 cells developed from *S. frugiperda* and S2 cells developed from *D. melanogaster*. Knockdown of *Cad96ca* and *Fgfr1* (named *Htl* or *Btl* in *D. melanogaster*), respectively, significantly decreased JH III-induced intracellular Ca2+ release and extracellular Ca2+ influx, and *Kr-h1* expression (*Figure 5A, B*, *Figure 5—figure supplement 1A and B*). The efficacy of RNAi of *Cad96ca* and *Fgfr1* was confirmed in the cells (*Figure 5—figure supplement 1C and D*), suggesting that CAD96CA and FGFR1 had a general function to transmit JH signal in *S. frugiperda* and *D. melanogaster*.".

> *(11) The authors should consider improving the quality of images and some plots, particularly enlarging panels showing larval and pupal phenotypes, such as Figure 1B and Supplementary Figure C. Additionally, adding a plot showing the statistical analysis of the phenotype in Supplementary Figure C would enhance clarity. Some plots are overly busy and difficult to read due to small size, such as Figure 1C, Figure 2A, and all the plots in Figure 3. Figure 4E also requires improvement for better readability.*

Thank you for your suggestion. We have adjusted Figure 1B, Figure 1C, Figure 1—figure supplement 1C, Figure 2A and Figure 4E. However, for Figure 3, we have not found a better way to arrange and adapt them, considering the overall arrangement of the results and the page space, so we keep them in their original state.

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