Knockout of juvenile hormone receptor, Methoprene-tolerant, induces black larval phenotype in the yellow fever mosquito, Aedes aegypti

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Juvenile hormones (JH) control almost every aspect of an insect’s life, and JH analogs are currently used to control mosquito larvae. Since RNA interference does not work efficiently during the larval stages of this insect, JH regulation of larval development and mode of action of JH analogs are not well studied. To overcome this limitation, we used a multiple single guide RNA-based CRISPR/Cas9 genome-editing method to knockout the methoprene-tolerant (Met) gene coding for a JH receptor. The Met knockout larvae exhibited a black larval phenotype during the L3 (third instar larvae) and L4 (fourth instar larvae) stages and died before pupation. However, Met knockout did not affect embryonic development or the L1 and L2 stages. Microscopy studies revealed the precocious synthesis of a dark pupal cuticle during the L3 and L4 stages. Gene expression analysis showed that Krüppel homolog 1, a key transcription factor in JH action, was down-regulated, but genes coding for proteins involved in melanization, pupal and adult cuticle synthesis, and blood meal digestion in adults were up-regulated in L3 Met mutants.

The function of Met in JH regulation of JH suppression of pupal/adult genes involved in the synthesis and melanization of the cuticle and blood meal digestion. These results help to advance our knowledge of JH regulation of larval development and the mode of action of JH analogs in Ae. aegypti. CRISPR/Cas9 | gene editing | sgRNA | cuticle | metamorphosis

Juvenile hormones (JH) and ecdysteroids [20-hydroxyecdysone (20E)] are the most active forms regulating molting and metamorphosis in insects (1, 2). While 20E initiates a cascade of gene expression and repression events during molting, JH preserves the juvenile status preventing metamorphosis (1, 2). Thus, during the larval stages, JH suppresses the expression of pupal and adult genes to ensure that the next molt is into a larva rather than metamorphosis to pupa or adult. At the end of the larval stage, JH levels decrease, allowing 20E to induce expression of pupal/adult genes resulting in metamorphosis to pupa or adult (1, 2). The methoprene-tolerant (Met) gene was discovered in the fruit fly, Drosophila melanogaster, based on the mutations that confer resistance to the JH analog, methoprene (3). However, Met was not confirmed as a JH receptor due to the lack of distinct effects of the Met mutation on D. melanogaster development. Studies in the red flour beetle, Tribolium castaneum, where RNA interference (RNAi)-mediated knockdown of Met induces precocious metamorphosis and premature development of pupal and adult structures, confirmed Met as a JH receptor (4). Later, it was shown that the D. melanogaster genome codes for 2 genes, Met and its paralog, germ cell-expressed (gce); mutation of Met and deletion of gce simultaneously induced lethality during metamorphosis similar to the mortality detected in allatomecas treated pupae (5). The Met protein binds to JH with a high affinity (6). In D. melanogaster, expression of Met/gce can rescue flies with Met mutations and gce deletion, establishing Met/gce as a JH receptor (7). Studies in multiple insects established Met, a member of the basic helix-loop-helix–Per-Arnt-Sim transcription factor family, as a JH receptor (2). Met functions through the recruitment of Taiman and induces the expression of JH-response genes, including the gene coding for a transcription factor, Krüppel homolog 1 (Kr-h1) (8, 9). During immature stages, Kr-h1 suppresses the expression of genes coding for the pupal specifier, Broad-Complex (BC) (10) and the adult specifier, E93 (11).

Overexpression of a gene coding for a JH-degrading enzyme, JH esterase (jhe), in Bombyx mori induced precocious metamorphosis from the third instar larval stage (12). Also, JH deficiency due to the null mutation of dimolting in B. mori induced metamorphosis in miniature pupae after the L3 or L4 stage (13). Furthermore, blocking the JH response by knocking out a gene coding for a JH biosynthesis enzyme, JH acid methyltransferase (jhamt), or Met induced precocious development of pupal structures in the L4 stage (14). These experiments showed that B. mori embryogenesis and early larval development do not require Met.

The yellow fever mosquito Aedes aegypti transmits many arboviruses that cause diseases such as yellow fever, dengue fever, Zika fever, and chikungunya. The JH analog, methoprene, is widely used for controlling Ae. aegypti and other mosquito larvae. The function of Met in JH regulation of Ae. aegypti female reproduction has been well studied (15). RNAi-mediated knockdown of Cycle (CYC) and Met showed that the Met/CYC heterodimer

**Significance**

Juvenile hormone (JH) analogs are used to control mosquitoes. However, both larval development and action of JH analogs are not well studied in these insects because RNA interference does not work well. A multiple single guide RNA-based CRISPR/Cas9 genome-editing method was used to knockout the methoprene-tolerant gene (Met, a JH receptor). The Met knockout larvae showed precocious development of pupal cuticle and expression of pupal/adult genes involved in the synthesis and melanization of cuticle and blood meal digestion. The methods developed here could help to overcome the major hurdle in functional genomics studies in Aedes aegypti and facilitate advances in understanding larval development and mode of action of JH analogs.

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mediates JH activation of *Kr-h1* and *Hairy* genes in female mosquitoes (16). However, JH action during larval stages and mode of action of JH analogs are not well studied because it is challenging to manipulate *Ae. aegypti* larvae that are growing in water and also due to problems associated with the delivery of double-stranded RNA and its stability; RNAi is inefficient in larval stages when compared to other stages of this insect.

Various genome-editing methods, including CRISPR/Cas9, have been used to knock out genes in *Ae. aegypti*. We injected multiple single guide RNAs (sgRNAs) targeting the gene coding for Met into embryos and detected a loss-of-function phenotype in G0. The Met knockout larvae exhibited a black larval phenotype during L3 and L4 and died during the late larval stage before pupation. Gene expression studies revealed that during larval stages Met suppresses pupal and adult genes, including those coding for proteins involved in pupal/adult cuticle synthesis, melanization, and blood meal digestion.

**Results and Discussion**

Multiple sgRNAs Improve Detection of the Loss-of-Function Phenotype in G0. Previous studies in *Ae. aegypti* reported CRISPR/Cas9-induced mutations of the gene coding for the enzyme kynurenine 3-monooxygenase (*kmo*), which is involved in eye pigmentation (17, 20). Methods for CRISPR/Cas9-mediated genome editing in *Ae. aegypti* were developed by multiple groups (17, 18, 24) and used to study sex determination (19), microRNA function (21, 23), and insulin signaling (22). In most cases, mutant phenotypes have been detected in the offspring developed from CRISPR/Cas9-injected embryos. However, in some cases, identification of a loss-of-function phenotype for studying the function of a target gene may require establishing a homozygous line. In the preliminary studies, injection of 1 sgRNA targeting the *kmo* gene into *Ae. aegypti* embryos showed a mosaic-eye phenotype in G0. We tested the injection of multiple sgRNAs to determine if the detection rate of the loss-of-function phenotype in G0 could be improved. Four sgRNAs were designed targeting exon 5 and introns on either side of this exon of the *kmo* gene (*SI Appendix, Fig. S1A*). Injection of 1 sgRNA/Cas9 (sgRNA-B) targeting exon 5 induced a mosaic-eye phenotype in 40% (36/90) of larvae and the pupae developed from injected eggs (*SI Appendix, Table S1*). Only 1 larva and the pupa with complete wild-type sequence (1/80, which is similar to the white-eye phenotype observed in the compound heterozygous G1 insects obtained after injection of sgRNA/Cas9 and self-crossing (*SI Appendix, Fig. S1B*). Injection of 2 sgRNAs (one targeting exon 5, sgRNA-B and the other targeting the intron, sgRNA-A) induced mosaic eyes in 50% (40/80) of larvae and pupae (*SI Appendix, Table S1*), but a complete white-eye phenotype was not detected in any of the G0 larvae. In contrast, injection of 3 sgRNAs targeting exon 5 and an intron (sgRNA-A, sgRNA-B, and sgRNA-C) induced a white-eye phenotype in 11% (21/190) of G0 larvae and pupae (*SI Appendix, Table S1*). Also, the injection of 4 sgRNAs (sgRNA-A, sgRNA-B, sgRNA-C, and sgRNA-D) induced a white-eye phenotype in 19–22% (32/165, 42/180) of G0 larvae and pupae (*SI Appendix, Table S1*). These data suggest that multiple sgRNAs could induce a high frequency of fragment deletions between different target sites, resulting in an increase in detection of loss of function in G0 (*SI Appendix, Fig. S1B*). To analyze the mutation frequency in white-eye phenotype insects, 12 white-eye pupae were randomly selected for isolation of genomic DNA and PCR amplification of the target gene. The mutation rate was quantified by the T7 Endonuclease I (T7EI) assay. As shown in *SI Appendix, Fig. S2A*, the PCR products revealed multiple bands suggesting that a high frequency of long fragment deletions was induced in the target gene. Compared to wild type (WT), the mutant DNA showed low-intensity WT gene fragments. The mutation efficiency of each line was quantified based on the band intensity in the gel. The average mutation efficiency was estimated to be 94% (*SI Appendix, Fig. S2B*). Because T7EI recognizes and cleaves imperfectly matched DNA, if the 2 strands of hybridized PCR products had the same mutation, the T7EI could not cleave the fragment at the mutation site. Therefore, the 94% mutation rate calculated could be an underestimate. The PCR products were sequenced, and the sequencing data showed mutations in DNA isolated from all mutants; some of the mutants showed the same deletions (*SI Appendix, Fig. S3*). Taken together, the T7EI and sequencing data suggest that injection of multiple sgRNAs induced a loss-of-function phenotype of the *kmo* gene, inducing the white-eye phenotype in the G0 generation.

Recently, Li et al. (20) produced transgenic *Ae. aegypti* expressing the Cas9 gene under the control of the AAE1010097 promoter (AAE1010097-Cas9 strain). Injection of sgRNA into eggs of this strain showed an improvement in sgRNA-induced mutagenesis when compared to injection of sgRNA/Cas9 into WT strain eggs. We tested *kmo* sgRNA combinations in the AAE1010097-Cas9 strain. Injection of each single sgRNA-A, sgRNA-B, sgRNA-C, and sgRNA-D induced a mosaic-eye phenotype in 0, 70 (67/97), 48 (31/65), and 0% of larvae and the white-eye phenotype in 0, 8 (8/97), 6 (4/65), and 0% of larvae, respectively (*SI Appendix, Table S1*). While injection of 1, 2, 3, or 4 sgRNAs induced a mosaic-eye phenotype in 46% (77/166), 59% (109/183), and 81% (104/127) and the white-eye phenotype in 4% (71/166), 25% (46/183), and 50% (65/127) of insects, respectively (*SI Appendix, Fig. S4A and Table S1*). Mutations in the target gene in white-eye insects were confirmed by sequencing (*SI Appendix, Fig. S4 B and C*). In each sgRNA combination tested, the loss-of-function phenotype frequency of the AAE1010097-Cas9 strain was higher than that observed in the Liverpool IB12 strain (*SI Appendix, Table S1*). These data demonstrate that the use of the AAE1010097-Cas9 strain could increase sgRNA/Cas9-induced mutation efficiency.

Detection of a Loss-of-Function Phenotype of the *Met* Gene in G0. To achieve a loss-of-function phenotype of the *Met* gene in G0, 4 sgRNAs were designed targeting exons 2 and 4 of the *Met* gene (*Fig. 1A*). The 4 sgRNAs were injected into AAE1010097-Cas9 strain eggs and 22 to 31% of the injected eggs hatched (*SI Appendix, Table S2*). Interestingly, 25 to 48% of the hatched larvae exhibited a black larval phenotype (*Fig. 1B and *SI Appendix, Table S2*) beginning in L3, and these larvae started dying during the L4 stage before entering the pupal stage. The black color of the skin was lighter and showed a mosaic pattern during L3 but was uniformly dark during L4. We also found that the abdomen of the Met loss-of-function phenotype 48-h-old L4 larvae is similar to that of the 24-h-old pupa (dark and shorter); the WT 48-h-old L4 larva did not show any dark pupa abdomen phenotype (*Fig. 1B*). The mutations in the *Met* gene in the black larvae were confirmed by sequencing (*Fig. 1C*). To test the mutation efficiency of each sgRNA, 4 sgRNAs were injected individually into eggs. Only larvae derived from Met-sgRNA-C and Met-sgRNA-D–injected eggs showed 14 and 9% black larval phenotype, respectively (*Fig. 1D* and *SI Appendix, Table S2*). The injection of 2 sgRNAs (*Met-sgRNA-C and Met-sgRNA-D*) induced 25% black larval phenotypes in G0 L3 and L4 (*Fig. 1D* and *SI Appendix, Table S2*). Mutations in each *Met* sgRNA target site were confirmed by sequencing (*SI Appendix, Fig. S5*). These data on Met mutations confirm data obtained with marker gene mutations and demonstrate that a multiple sgRNA-based method could be used to improve chances of identifying gene knockout phenotypes in G0. Recent studies in *D. melanogaster* reported improved improvement in the frequency of CRISPR/Cas9 editing of genes when 2 or more sgRNAs are used (25–27).

The off-target effects caused by single or multiple sgRNAs are a concern that needs to be addressed in gene-editing experiments (28). The sgRNAs were designed and evaluated for their potential off-target effects using the CRISPR optimal
target finder (http://targetfinder.flycrispr.neuro.brown.edu/). Forty-two potential off-target sites for 4 Met sgRNAs were identified. The alignment files of the sequences obtained using RNA isolated from Met knockout larvae were mapped to the Ae. aegypti reference genome; no insertions/deletions were detected in the RNA sequences at potential off-target sites. We also performed a variant analysis of the RNA sequences from WT and Met knockout larvae using samtools and bcftools (29, 30); the resulting alignment files were visualized by CLC genomics software. Again, no variants were detected at the potential off-target sites. Also, the black larval phenotype and mortality induced by Met knockout were not detected in any of the larvae developed from eggs injected with single or multiple sgRNAs targeting the kmo gene. The larvae that developed from eggs injected with single sgRNA or multiple sgRNAs targeting the Met gene showed black larval phenotype during only L3 and L4. The development of L1 and L2 stages appeared to be normal, and no abnormal phenotypes were detected in these larvae. Taken together, these data suggest that the black larval phenotype and mortality observed in larvae developed from the eggs injected with the sgRNA-targeting Met gene appear to be specific to knockout of the Met gene.

To further confirm the result that the black larval phenotype induced by knockout of the Met gene is due to a block in JH action, 3 sgRNAs targeting the jhamt (AAEL006280) gene were designed and synthesized (SI Appendix, Fig. S6). When these 3 sgRNAs were injected into eggs to achieve a loss-of-function phenotype of the jhamt gene, only 3.2% of injected eggs hatched and the DNA isolated from larvae developed from injected eggs did not contain any mutations in the jhamt gene. Attempts at improving the hatch rate by application of JH III (10 μM) or methoprene (50 ng) were not successful. In B. mori, after knockout of the jhamt gene, without JH, the neonate larvae were unable to break the eggshell and hatch (14). The application of JH III or methoprene helped with hatching of some of the jhamt mutants. We also tested single sgRNA by injecting jhamt-sgRNA-B into 510 eggs from which 107 larvae hatched. During the L3 stage, 9% (10/107) of larvae showed black color body (SI Appendix, Fig. S6B) as seen in the larvae developed from eggs injected with Met sgRNAs. The larvae that showed the black larval phenotype died at the end of L4. Genomic DNA isolated from larvae that showed black color was used as a template to amplify the sgRNA target region of the jhamt gene, and the PCR products were sequenced. Mutations were detected in 4 of the 12 clones sequenced (SI Appendix, Fig. S6C).

To determine if jhamt expression is required for completion of embryonic development and/or hatching in Ae. aegypti, we compared embryonic development and hatch rate of uninjected eggs and those injected with kmo sgRNA-ABC, jhamt sgRNA-ABC, or jhamt sgRNA-B. Under our rearing conditions, segmentation of embryos is visible 24 h after egg laying (SI Appendix, Fig. S7). About 90% of embryos from uninjected or kmo sgRNA-ABC-injected eggs and 76% from jhamt sgRNA-B–injected eggs showed segmentation (SI Appendix, Figs. S8 and S9). The un.injected eggs or those injected with kmo sgRNA-ABC or jhamt sgRNA-B showed 91, 59, and 56% hatch rates, respectively (SI Appendix, Fig. S9). In contrast, only 20% of embryos from jhamt sgRNA-ABC–injected eggs showed segmentation, and 13% of them hatched (SI Appendix, Fig. S9). These data showed that the effect on embryonic development is more pronounced in jhamt multiple sgRNA-injected eggs when compared to those injected with jhamt single sgRNA. This might be due to higher levels of loss of function of the jhamt gene in multiple sgRNA-injected eggs when compared to that in eggs injected with single sgRNA. These data suggest that Ae. aegypti embryonic development may require jhamt gene expression. However, further research is required to understand the role of JH and identify its receptor during the embryonic development of Ae. aegypti.

Identification of Loss-of-Function Phenotypes by Coinjecting Single-Marker Gene sgRNA and Multiple-Target Gene sgRNAs. As described above, multiple sgRNAs induced Met gene knockout in G0. However, the Met gene knockout phenotypes were seen only in the L3 and L4 stages. It would be useful if the target gene knockout could be identified before the onset of the target gene phenotype because, in some cases, target genes may not show distinct phenotypes. To determine if it is possible to improve identification of target gene phenotypes by coinjecting the sgRNA targeting marker gene and the target gene, we tested multiple sgRNAs targeting Met and 1 sgRNA targeting kmo. A mixture of 1 kmo sgRNA (sgRNA-B) and 4 Met sgRNAs (sgRNA-A, sgRNA-B, sgRNA-C, and sgRNA-D) was injected into AAEL010097-Cas9 eggs. From 462 eggs injected, 243 larvae hatched. In the L2 stage, 40% (88/223) mosaic-eye larvae were identified. Interestingly, 75% (66/88) of the mosaic-eye larvae showed a black larval phenotype during the L3 and L4 stages (Fig. 1D and SI Appendix, Table S3). The mutations in the kmo and Met genes were confirmed by sequencing (SI Appendix, Table S3).
Two additional kmo and Met sgRNA injections were performed to confirm the results from the first experiment, and in these 2 experiments, 90 and 69% of mosaic-eye larvae showed a black larval phenotype (SI Appendix, Table S3). In 3 trials, injection of multiple Met sgRNAs and 1 kmo sgRNA showed that 78% of the marker gene mutants identified during L2 also showed the black larval phenotype during L3 and L4. In contrast, only 33% of the larvae exhibited the black larval phenotype in larvae hatched from eggs injected with only Met sgRNAs (Fig. 1D). These data demonstrate that injection of multiple sgRNAs targeting a marker gene and the gene of interest could significantly improve identification of mutants for the gene of interest in G0. Recent studies in D. melanogaster employed simultaneous targeting of a marker gene and a target gene (26) and a negative coselection strategy using a dominant female sterile allele (27) to improve screening efficiency for identification of CRISPR/Cas9-induced genome-editing events. These improvements could save labor and time involved in screening for mutants and facilitate identification of mutants of genes the knockout of which does not induce distinct phenotypes. The multiple sgRNA-based CRISPR/Cas9 genome-editing method reported in this paper adds another improvement to the genome-editing toolkit available for use in Ae. aegypti functional genomics studies.

**Characterization of the Black Larval Phenotype Induced by Met Knockout.** The black larval body color detected in Met mutants is similar to the black larval mutants discovered as natural variants in populations of Manduca sexta and other lepidopteran insects. In M. sexta, JH deficiency induces black melanized cuticle in the last larval instar, and application of JH during molting to the last larval instar prevents the appearance of the black larval phenotype (31). Similar phenotypes have been reported in other lepidopteran insects, including Celerio euphorbiae, Papilio machaon, and Phalera bucephala (32). Studies in B. mori and D. melanogaster showed that ebony and yellow genes are associated with larval color mutants (33, 34). In this study, JH analog methoprene was applied to rescue the L3 black larvae. Sixteen black larvae were maintained in 100 ng/μL methoprene and provided sufficient diet. None of the larvae were rescued and all of these larvae died later in the L4 stage. To determine mechanisms responsible for the black body color detected in Met knockout Ae. aegypti larvae, these mutants were further characterized using scanning (SEM) and transmission electron microscopy (TEM), RNA sequencing, and RT-qPCR. The WT L4 cuticle is lighter in color when compared to the pupal cuticle. Observation of Met knockout mutants under a stereomicroscope revealed 2 layers of cuticle, a transparent outer cuticle and a dark inner cuticle (Fig. 2A). Observations under the scanning electron microscope identified the outer layer of the cuticle as the larval cuticle similar to that seen in L4 larvae (Fig. 2B). The inner layer of cuticle in the Met mutant is similar to the pupal cuticle seen in 24-h-old pupae (Fig. 2B). To analyze the ultrastructure of abdominal cuticles from 24-h-old L4 WT and Met mutant larvae, TEM was performed. The cuticles of L4 WT larvae

![Fig. 2](https://example.com/Fig2.png)

**Fig. 2.** Met mutants synthesize pupal cuticle during the larval stage. (A) Images of 48-h-old WT L4, 48-h-old Met knockout L4, and 24-h-old WT pupa are shown. (Scale bar, 1 mm.) (B) The SEM images of the cuticle from 48-h-old WT L4, 48-h-old Met knockout L4, and 24-h-old WT pupae. The staged larvae and pupae were fixed, critical-point-dried, coated, and photographed under a scanning electron microscope. To see the inner layer of WT and Met mutant cuticles, the outer layer of cuticle was peeled off using forceps. The Bottom panels show an enlarged view of marked areas. LC, larval cuticle; PC, pupal cuticle; EC, epidermal cell. (C) Ultrastructure of the larval body cuticle from WT and the Met mutant. Ultrastructure of the larval body cuticle from 24-h-old L4 WT, Met mutant, or WT pupa shortly after pupation was photographed under a transmission electron microscope. Arrows indicate the larval cuticle protuberances. (Bottom) An enlarged view of marked areas. LC, larval cuticle; EC, epidermal cell; ES, ecdysial space; and PC, pupal cuticle.
consist of a few less compact horizontal laminae and unique regular protuberances (arrows in Fig. 2C). However, there is no evidence of any pupal cuticle at this developmental stage (Fig. 2 C, Left). Unlike L4 WT larvae, there are 2 cuticles in the L4 Met mutant larvae (Fig. 2 C, Middle). The outer cuticle is similar to that of the WT L4 larval cuticle (Fig. 2 C, Left). In contrast, the inner cuticle is composed of a number of electron-dense compact horizontal laminae as seen in the pupal cuticle dissected from 0-h-old WT pupae (Fig. 2 C, Right). These data suggest that, unlike the WT strain, Met knockout larvae produced pupal cuticle precociously during the early stage of the last instar larvae.

Studies on variation in cuticle color in lepidopteran and other insects identified differential expression of genes coding for enzymes involved in melanization as one of the major contributors to the differences in cuticle color. To determine if any of the genes coding for enzymes involved in melanization are differentially expressed between WT and Met mutants, we sequenced RNA isolated from the AAEL010097-Cas9 strain (control) and black larval Met mutant larvae collected at 24 h after entering the L4 stage. Raw sequencing data statistics are presented in SI Appendix, Table S4. Differential gene expression between the WT and Met mutant was analyzed, and the overall gene expression differences are shown as the heatmap (Fig. S34) and the volcano plot (Fig. S3B). Gene Ontology (GO) enrichment analysis was performed using the WEGO tool by plotting the GO information of the up- and down-regulated genes (SI Appendix, Figs. S11 and S12). Compared to WT, 417 genes were up-regulated, and 224 genes were down-regulated (≥2-fold, *P ≤ 0.05) in the Met knockout larvae. In D. melanogaster, the Wnt (35) and TGF-β (36) signaling pathways modulate JH action and synthesis, respectively. In the Met knockout larvae, genes involved in Wnt and the TGF-β signaling pathway were up-regulated (SI Appendix, Fig. S13A). Genes involved in other pathways such as the phagosome, AGE-RAGE signaling pathway in diabetic complication, neuroactive ligand-receptor interaction, mitophagy, apoptosis, glycine, serine and threonine metabolism, MAPK, and hippo are also up-regulated in the Met knockout larvae. Interestingly, most of the genes down-regulated in the Met knockout larvae seem to be involved in metabolic pathways, such as fatty acid metabolism (SI Appendix, Fig. S13B). Expression of the JH-response gene and transcription regulator Kr-h1 (AAEL002590) was down-regulated (5.7-fold, but there seems to be a dominance of Hairy (AAEL005498), Hox response gene, and transcription regulator), BR-C (AAEL008426), and E93 (AAEL0004572) was not significantly different between WT and Met mutants. This may be due to the depth and range of RNA-seq data and the variability among the biological replicates. The mRNA levels of these 4 genes were quantified by RT-qPCR. As shown in Fig. 3C, Kr-h1 was down-regulated in L3 and L4 Met mutants, while Hairy, BR-C, and E93 were down-regulated only in L4 Met mutants. In Ae. aegypti, the expression of BR-C and E93 was lower during the pupal stage when compared to that in L4 (37). The Met mutant larvae proceeding to pupal stage seem to express lower levels of BR-C and E93 when compared to the WT larvae.

The Ae. aegypti genome contains 10 genes coding for prophenokidasises (PPO), while there are only 5 PPO genes in D. melanogaster (38). RNA-seq data showed up-regulation of 9 genes coding for enzymes, including PPO (PPO3 and PPO5), that are known to be involved in melanization. The RT-qPCR analysis showed that the PPO3 gene was up-regulated in Met knockout L3, and the PPO5 gene was up-regulated in Met knockout L4 larvae (Fig. 3C). The dopa decarboxylase (DDC) gene, which codes for another key in the melanization pathway was also up-regulated in Met knockout L3 larvae (Fig. 3C). The up-regulation of genes coding for enzymes involved in melanization in the Met mutant was confirmed by RT-qPCR (Fig. 3D). In Ae. aegypti, serine proteases and their inhibitors, serpins, carry out activation/inactivation of PPOs involved in melanization. Immune melanization proteases (IMP-1 and IMP-2) mediate PPO cleavage and immune response against Plasmodium (38), and RNA sequencing identified 11 genes coding for serine proteases that are up-regulated in Met knockout larvae (Fig. 4A). Determining whether or not any of these proteases are involved in the activation of PPOs requires further studies. These data suggest that JH suppresses the expression of genes coding for enzymes involved in the melanization pathway in Ae. aegypti. In Met knockout larvae, the JH response is disabled, resulting in up-regulation of genes coding for enzymes including PPO and DDC involved in melanization. It appears that the black larval phenotype seen in Met knockout larvae is due to a combined effect on the expression of multiple genes coding for proteins involved in melanization. These data are in line with findings in other insects on the changes in the expression of multiple genes coding for proteins involved in the melanization pathway (39, 40).

RNA-seq data showed up-regulation of 54 genes coding for cuticle proteins in the Met knockout larvae. Blast2go annotation identified them to be coding for pupal or adult cuticle proteins, which included 11 adult cuticle proteins (Fig. 4B and SI Appendix, Table SS) (41). In adults, JH participates in the regulation of reproduction, including previtellogenic development, vitellogenesis, and oogenesis (15). Early trypsin is one of the first JH-regulated genes identified in Ae. aegypti (42). Female Ae. aegypti adults synthesize multiple trypsins after a blood meal, where the early trypsin is expressed in the midgut within 1 h after ingestion of a blood meal for 6 to 8 h. Late trypsin begins to express 8 to 10 h after the ingestion of a blood meal (42). RNA-seq data showed up-regulation of 16 trypsin and chymotrypsin genes, including JH-regulated early and late trypsin genes identified in Ae. aegypti adults in the Met knockout larvae (Fig. 4C). This suggests that the genes coding for digestive enzymes, that are normally expressed in the adults, are suppressed by JH during the larval stage. We also
found 4 chitinase genes that were up-regulated and 1 chitinase gene that was down-regulated in Met mutant larvae (Fig. 4D). These data suggest that JH suppresses the expression of pupal and adult genes during larval stages.

Interestingly, knockout of the Met gene did not affect the development of embryos and early larval instars. The black larval phenotype appeared during L3 and L4 stages of the mutant larvae that died before pupation. Studies on the developmental expression of Ae. aegypti Met, jhamt, and Kr-h1 during larval stages detected these mRNAs throughout the larval stages (SI Appendix, Fig. S14). The jhamt mRNA levels showed 3 peaks during early L1, L2, and L3 stages, while Met and Kr-h1 mRNA levels showed a single major peak at the beginning of L4 (SI Appendix, Fig. S14). Although the general requirement of JH to prevent precocious metamorphosis is similar to that reported in other insects, the black larval phenotype induced by Met knockout in Ae. aegypti appears to be unique. Met knockout in B. mori induced the development of pupal cuticle patches but not a complete black pupal cuticle (14). Induction of the black larval phenotype was also not reported in D. melanogaster larvae that had simultaneous Met mutation and gce deletion (7). The black larval phenotype was also not reported after RNA-mediated knockdown of Met in insects such as T. castaneum (4). Whether or not induction of the black larval phenotype is unique to Ae. aegypti remains to be investigated. The multiple sgRNA-based CRISPR/Cas9 method reported here could help to overcome some of the hurdles in gene knockout in Ae. aegypti and other insects. Future studies using these methods should help in making advances toward understanding the function of genes in Ae. aegypti and other insects.

**Materials and Methods**

Mosquito rearing, egg collection, microinjection, and mutagenesis analysis were performed using the methods described previously (17, 24). RNA isolation, RNA sequencing, and RT-qPCR were performed as described previously (43). Electron microscopy analysis was conducted as previously described (44). The details on the materials and methods used are included in SI Appendix.

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