An E3 Ubiquitin Ligase Scaffolding Protein Is Proviral during Chikungunya Virus Infection in Aedes aegypti

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ABSTRACT Chikungunya virus (CHIKV) is a reemerging alphavirus causing chikungunya disease (CHIKD) and is transmitted to humans by Aedes mosquitoes. The virus establishes an intricate balance of cellular interactions that ultimately helps in its replication and dodges cellular immune response. In an attempt to identify cellular host factors required during CHIKV replication in Aag2 cells, we performed global transcriptomics of CHIKV-infected Aag2 cells, and further, we compared this library with the Drosophila RNAi Screening Center (DRSC) database and identified transcripts that were regulated in Aedes aegypti during CHIKV infection. These analyses revealed specific pathways, such as ubiquitin-related pathways, proteolysis pathways, protein catabolic processes, protein modification, and cellular protein metabolic processes, involved during replication of the virus. Loss-of-function assays of selected candidates revealed their proviral or antiviral characteristics upon CHIKV infection in A. aegypti-derived Aag2 cells. Further validations identified that the ubiquitin proteasomal pathway is required for CHIKV infection in A. aegypti and that an important member of this family of proteins, namely, AeCullin-3 (Aedes ortholog of human cullin-3), is a proviral host factor of CHIKV replication in Aag2 cells.

IMPORTANCE Arboviruses cause several diseases in humans and livestock. Vector control is the main strategy for controlling diseases transmitted by mosquitoes. In this context, it becomes paramount to understand how the viruses replicate in the vector for designing better transmission blocking strategies. We obtained the global transcriptome signature of A. aegypti cells during CHIKV infection, and in order to obtain the maximum information from these data sets, we further utilized the well-characterized Drosophila system and arrived upon a set of transcripts and their pathways that affect A. aegypti cells during CHIKV infection. These analyses and further validations reveal that important pathways related to protein degradation are actively involved during CHIKV infection in A. aegypti and are mainly proviral. Targeting these molecules may provide novel approaches for blocking CHIKV replication in A. aegypti.

KEYWORDS Chikungunya virus, Aedes aegypti, vector–virus interactions, ubiquitin proteasomal pathway

Chikungunya virus (CHIKV) is one of the reemerging zoonotic viruses endemic mainly to tropical and sub-Saharan regions. The virus is transmitted by mosquitoes belonging to the genus Aedes, with Aedes aegypti serving as the favored vector in the tropics and Aedes albopictus in temperate regions (1). Increased travel and trade have led to reemergence and large-scale epidemics in India, Africa, and Europe (1–3). Infection with CHIKV has been associated with extensive morbidity causing severe and self-limiting arthralgia in affected individuals that is sometimes chronic (4). Several approaches are under way for the development of an effective treatment against the infection. Despite the efforts, no effective therapeutics are developed that could limit pathogen spread and infectivity. Disease control strategies pertain mostly to demolishing vector breeding grounds and usage of insecticides (5, 6).
Newer approaches involve developing genetically modified mosquitoes that could block viral transmission (7–9). Therefore, increased understanding of vector-virus interactome will help design newer and more effective strategies to restrict viral spread.

Host proteins play a significant role during viral replication that can be categorized as either proviral or antiviral depending on whether they facilitate or counter the establishment of virus in the host/vector. A number of elaborated studies in the last decade that were focused on CHIKV and host/vector interactions recognized a plethora of cellular processes and pathways that were of significance during infection (10–14). These included components of stress granules (15), mitochondria (13), and endoplasmic reticulum (16). Thus, by virtue of modulating these essential cellular proteins, the virus becomes capable of hijacking cellular responses enabling its efficient replication.

High-throughput screening and computational analyses are some of the tactics that are routinely employed to identify cellular factors. These studies provide a global perspective for the role of the host genes in viral infection. The present study is one such study where we identified Aedes aegypti host factors involved in CHIKV replication. We first performed global transcriptomic analysis of an A. aegypti cell line post CHIKV infection and identified transcripts that were regulated during CHIKV replication. In addition, we selected known genes from the Drosophila RNAi Screening Center (DRSC) database and performed a hybrid analysis to identify transcripts that may be involved in arboviral replication in the vector. The identified transcripts were evaluated for their role in CHIKV replication through a double-stranded RNA (dsRNA)-mediated RNAi screen. Finally, A. aegypti ortholog of human cullin-3 protein, referred to here as AeCullin-3, was found to be proviral during CHIKV replication.

RESULTS

Identification of Aedes aegypti host factors involved during infection with CHIKV.

The pipeline used for the identification of A. aegypti host factors is depicted in Fig. 1. RNA-Seq data acquired by processing of the uninfected and infected libraries (done in triplicates) were processed for quality trimming and removal of adapter sequences before proceeding with mapping to A. aegypti genome. The mapping percentage for all samples was found to be above 80%, and the total number of transcripts identified in each individual library varied between 12,000 to 15,000 (Table 1, Fig. 2a).

To further explore the vector-virus interactome within the host cell, we utilized the extensive information available on the model insect, namely, Drosophila, to better annotate the Aedes genes. From the early 1900s to the present, Drosophila melanogaster has been central to groundbreaking research in diverse organisms ranging from humans to insects (17–19). Previous studies have performed hybrid high-throughput screens of Drosophila genes, including RNAi screens, to understand host-pathogen interactions in their organisms of interest and have established different protocols to perform the same (17, 20). In the present study, using a similar approach, we compared our transcriptome libraries with Drosophila RNAi Screening Center (DRSC) database. Comparison of the Drosophila data sets with our transcriptome data presented a total of 8,608 transcripts that were found to be common and were taken for further analysis. Pathway analysis revealed that ubiquitin-related pathways, proteolysis pathways, protein catabolic processes, protein modification, and cellular protein metabolic processes were significantly affected (Fig. 2b and c). Differential expression analysis revealed 346 genes to be differentially expressed, of which 34 genes were found to be significant (P value of <0.05 and/or fold change difference of ±1.5) (Data set S1). This set of genes was further reviewed for genes’ pathways, and 7 genes were taken for downstream validation.

dsRNA-based screening to elucidate the proviral or antiviral role of identified cellular factors. In addition to the above 7 genes, we performed a literature search for putative host factors in insects that played a role in infection and cellular physiology and selected 23 additional genes for a dsRNA-based screening to evaluate their role in CHIKV infection in A. aegypti cells (Table 2). The final set of 30 transcripts for dsRNA screening were involved in cellular processes, including metabolism, biosynthesis, and oxidative pathways (AAEL001895 [21], AAEL013361 [22, 23], AAEL014840 [25], AAEL001292 [26], AAEL012868 [27]), protein modification and proteolysis (AAEL011287, AAEL007187, AAEL019450, AAEL006797 [28], AAEL017567 [29], AAEL003104 [30], AAEL010641,
AAEL001112, AAEL012337), transcriptional/translational regulatory and nucleic acid binding proteins (AAEL019736 [31], AAEL019431, AAEL008073, AAEL007945, AAEL001612), and ion channels, receptors, cell adhesion proteins, and transmembrane proteins (AAEL002922, AAEL009229, AAEL012421, AAEL005681 [32], AAEL005133, AAEL008641, AAEL006685). One transcript was found to be involved in endocytosis (AAEL007041 [32]). Apart from these, one uncharacterized transcript was also included in the study (AAEL024345).

The selected transcripts were silenced using dsRNA after estimating their silencing efficiencies (14). dsRNAs showing a silencing efficiency of >50% were taken further for the loss-of-function studies (data not shown). A known host factor, V-type proton ATPase catalytic subunit A, that was previously reported to be involved in arboviral infections was also taken and served as the positive control (AAEL008787) (33), and dsRNA against GFP served as the negative control. Five genes (AAEL012337, AAEL006685, AAEL008641, AAEL010641, AAEL001112) could not be optimized for silencing and were therefore not included further. Screening of the selected transcripts during CHIKV infection using dsRNA-mediated silencing

TABLE 1 RNA-Seq data processing: reads obtained after trimming and mapping percentage and number of identified transcripts

| Sample name               | No. of reads after trimming (bp) | Mapping percentage (%) | No. of identified transcripts |
|---------------------------|----------------------------------|------------------------|------------------------------|
| Aag2_infected_1           | 32,897,025                       | 84.39%                 | 14,179                       |
| Aag2_uninfected_1         | 67,948,278                       | 84.15%                 | 14,893                       |
| Aag2_infected_2           | 20,229,369                       | 85.33%                 | 12,716                       |
| Aag2_uninfected_2         | 24,229,683                       | 84.10%                 | 12,987                       |
| Aag2_infected_3           | 22,723,606                       | 87.73%                 | 12,687                       |
| Aag2_uninfected_3         | 20,948,346                       | 85.31%                 | 12,704                       |

FIG 1 Pipeline used for the identification of *Aedes aegypti* host factors during infection with CHIKV.
in A. aegypti-derived Aag2 cells revealed that CHIKV viral RNA levels differed significantly among cells with silenced transcripts compared to those in the negative control (GFP dsRNA-transfected cells) upon CHIKV infection. The relative CHIKV genomic RNA level for AAEL011287, AAEL014840, and AAEL001895 were found to be higher than that of the negative control. Among these factors, AAEL001895 (4-alpha-galactosyltransferase) was the most significant one exhibiting antiviral activity. Viral genomic RNA levels showed no significant change for six genes relative to negative control, namely, AAEL011287, AAEL014840, AAEL017567, AAEL024345, AAEL001292, and AAEL003104. Genes AAEL019736, AAEL007187, AAEL019431, AAEL002922, AAEL019450, AAEL008073, AAEL013603, AAEL013361, AAEL007041, AAEL009229, AAEL007945, AAEL007945, AAEL001612, AAEL012868, and AAEL005133 all showed reduction in relative CHIKV genomic RNA level, suggesting potential proviral activity. Among these factors, AAEL007187 (AeCullin-3) was the most significant factor showing the proviral activity for CHIKV (Fig. 3).

Proteasomal pathway is required for CHIKV replication in Aag2 cells post entry. Previous studies have established a functional role for ubiquitin proteasomal machinery during viral replication in host cells (as reviewed in references 34 and 35). Studies done on alphaviruses have employed proteasome inhibitors to decipher the function of ubiquitin proteosome pathway (UPP) machinery (36–38). Global proteomic analysis of CHIKV-infected host cells has
also revealed several components of the proteasome to be differentially regulated (39). In our initial analysis, we found the protein modification pathway to be evidently involved during the process of CHIKV replication. Transcripts for proteins such as proteases, ubiquitin ligases, and deubiquitinases were found to be significantly regulated in Aag2 cells upon infection. E3 ubiquitin ligase belonging to the TRIM family (AAEL003104) showed no significant impact on CHIKV replication upon being silenced. The F-box and leucine-rich repeat protein 7 (which functions as an adaptor for SCF E3 ligase; AAEL006797) along with cullin-3 (now referred to as AeCullin-3) were found to be proviral for CHIKV replication.

To elucidate if there was any role of proteasomal pathway in CHIKV replication, we used a well-known proteasome inhibitor MG132 in pre and posttreatment assays. MG132 has also previously been employed as a proteasome inhibitor in Aag2 cells (40, 41). Prior to the assay, cell viability upon MG132 treatment was tested in Aag2 cells, and it was found that the cell viability remained above 80% at the three drug concentrations tested, namely, 0.1 μM, 1 μM, and 10 μM (Fig. 4a). Based on the results, 0.1 μM and 1 μM were used for the

**FIG 2** (Continued).
inhibitor assays. Pretreatment assay determined whether proteasomal pathway had a significant involvement during early stages of viral entry and internalization. A significant reduction of approximately 40% (P < 0.0001) was observed in PFU at both 0.1 mM and 1 mM concentrations of MG132 (Fig. 4b). These results were also reproduced using quantitative real-time-PCR (qRT-PCR), and a 2-fold reduction (P < 0.01) was observed at both these concentrations (Fig. 4c). A posttreatment assay, on the other hand, determined the possible involvement at a postentry stage of viral life cycle within the cells. It was observed that during posttreatment of the drug, viral inhibition was dose dependent. While at 0.1 mM concentration a 30% reduction was obtained in plaque forming units (PFU/mL; P < 0.05), we observed a 50% reduction at 1 mM concentration (P < 0.01). These results were also corroborated by the posttreatment assay.

**TABLE 2** Detailed information on the putative host factors selected for dsRNA-based screening; the description shown is acquired from UniProt (83) and Vectorbase (72)

| Gene ID         | Gene name                                    | Gene ontology                                      |
|-----------------|----------------------------------------------|---------------------------------------------------|
| AAEL011287      | Ubiquitin specific protease 1                | Protein deubiquitination                           |
| AAEL014840      | Short change dehydrogenase                   | Oxidoreductase activity                            |
| AAEL001895      | Alpha-1,4-galactosyltransferase              | Glycan biosynthesis and metabolism                 |
| AAEL001112      | Ubiquitin carboxyl-terminal hydrolase        | Protein deubiquitination                           |
| AAEL019736      | HTH CENPB-type domain-containing protein     | DNA binding                                        |
| AAEL007187      | Cullin ubiquitin ligase                      | E3 ubiquitin ligase                                |
| AAEL019431      | Ec dysone receptor 8a                        | DNA-binding transcription factor activity          |
| AAEL002922      | Ionotropie receptor 8a                       | Ion channel                                        |
| AAEL019450      | Peptidase_58 domain-containing protein       | Protease                                           |
| AAEL008641      | GTP-binding protein (o) alpha subunit, gnao  | G protein coupled receptor                         |
| AAEL008073      | Putative mRNA binding protein                | RNA binding                                        |
| AAEL013603      | Short-chain dehydrogenase                    | Oxidoreductase activity                            |
| AAEL013361      | Lipase                                        | Hydrolase activity, lipid catabolic process        |
| AAEL009229      | Mitochondrial citrate transport protein, putative | Transmembrane transport                           |
| AAEL003977      | Very-long-chain 3-oxoacyl-CoA synthase       | Fatty acid biosynthesis                            |
| AAEL012421      | Cadherin                                      | Cell-cell adhesions                                |
| AAEL010641      | SUMO-activating enzyme subunit                | Protein sumoylation                                |
| AAEL006797      | F-box and leucine-rich repeat protein 7       | Ubiquitination                                     |
| AAEL005681      | GPRHIS                                        | G protein coupled receptor                         |
| AAEL007945      | Eukaryotic translation initiation factor 3 subunit H | Translational initiation factor activity          |
| AAEL001612      | Dicer-1                                       | Posttranscriptional gene silencing by RNA          |
| AAEL017567      | Metalloprotease                               | Uncharacterized protein                            |
| AAEL024345      | Uncharacterized protein                       | Uncharacterized protein                            |
| AAEL012337      | Goliath E3 ubiquitin ligase                  | E3 ubiquitin ligase                                |
| AAEL012868      | Cmp-n-acetylneuraminic acid synthase          | Metabolism                                         |
| AAEL001292      | Cytochrome P450                               | Oxidoreductase activity                            |
| AAEL003104      | Tripartite motif protein trim 2,3             | Metal ion binding, ubiquitination                  |
| AAEL005133      | Tetra tricopeptide repeat protein, tpr        | Nonmotile cilium assembly                          |
| AAEL007041      | Low-density lipoprotein receptor (ldl)        | Calcium ion binding, endocytosis                   |
| AAEL006685      | Guanine nucleotide-binding protein subunit gamma | G protein coupled receptor                        |

**FIG 3** RNAi screening for *A. aegypti* host factors in CHIKV replication. Quantitative real-time PCR (qRT-PCR) analysis of CHIKV genomic RNA levels upon dsRNA-mediated silencing followed by CHIKV infection. Rps17 was used as an endogenous control and data are analyzed using 2^−ΔΔCT. Data are expressed as mean ± SD; ****, P < 0.0001 versus control group; ***, P < 0.001 versus control group; **, P < 0.01 versus control; *, P < 0.1 versus control.
reduction in viral genomic RNA levels at 1 μM concentration of MG132 (P < 0.001) (Fig. 4d). Similar results were also obtained in qRT-PCR, showing a 2-fold reduction at 0.1 μM (P < 0.05) and 4-fold reduction at 1 μM concentration of MG132 (P value < 0.05) (Fig. 4e).

**Role of AeCullin-3 during CHIKV infection in A. aegypti.** In order to further validate the relevance of AeCullin-3 in CHIKV infection, we performed a dsRNA-based silencing in *A. aegypti* mosquitoes. First, expression levels of AeCullin-3 were determined using qRT-PCR in normal blood-fed and CHIKV-spiked blood-fed female *A. aegypti* individuals. AeCullin-3 expression levels increased to up to 10-fold at 48 h and starting at 72 h postinfection gradually returned to normalcy (Fig. 5a).

We then went on to further characterize the role of AeCullin-3 by performing a loss-of-function assay on the mosquitoes (Fig. 5b). Four- to six-day-old female *A. aegypti* individuals were injected with dsRNA against green fluorescent protein (GFP; control) or AeCullin-3. As seen in Fig. 5c, a 2-fold reduction in AeCullin-3 expression levels was obtained in all the time points tested (from 24 h to 120 h post nanoinjection). Based on this, 24-h post nanoinjection of both GFP and AeCullin-3, the mosquitoes were subjected to CHIKV infectious blood meal (10^6 PFU/mL). In dsAeCullin-3-injected mosquitoes, a reduction of more than 3-fold in CHIKV genomic RNA levels (P < 0.0001) was obtained compared to those in the GFP control group (Fig. 5d), thereby confirming that AeCullin-3 acts as a proviral CHIKV host factor in *A. aegypti* mosquitoes.

**DISCUSSION**

While much research has been executed to identify cellular factors of the mammalian host that play important roles in CHIKV replication (42–45), limited literature is available...
with respect to host factors regulated upon CHIKV infection in Aedes (13, 46–48). A deficit AeCullin-3 gene expression was quantified in the two groups by qRT-PCR. The experiments were performed a minimum of three times, with each experiment set in triplicates. Fold change in expression in CHIKV-spiked blood-fed mosquitoes is compared to that in uninfected blood-fed mosquitoes. (b) Experimental plan for dsRNA-mediated knockdown of AeCullin-3 in A. aegypti mosquitoes. (c) Mosquitoes were intrathoracically injected with either dsRNA against GFP (dsGFP, control group) or dsRNA against AeCullin-3 (dsCUL3 group), and the mosquitoes (n = 5) from both groups were then collected and AeCullin-3 gene expression was estimated in each individual mosquito using qRT-PCR. (d) dsGFP- and dsCUL3-injected mosquitoes were subjected to CHIKV-spiked blood feeding 24 h post nano-injection. Mosquitoes (n = 5) were then collected and CHIKV genomic RNA levels were analyzed in each individual mosquito. (a to d) Rps17 served as endogenous control, and qRT-PCR analysis for experiments indicated in this figure was done using 2−ΔΔCT method. Fold change in expression in dsCUL3-injected mosquitoes is compared to that in dsGFP-injected mosquitoes. Data are expressed as mean ± SD. ***, P < 0.001; **, P < 0.01; *, P < 0.1 (versus control).

FIG 5 Role of AeCullin-3 during CHIKV infection in A. aegypti. (a) A. aegypti mosquitoes were fed with either uninfected blood (control group) or CHIKV-spiked blood (10⁶ PFU/mL), a pool of n = 5 mosquitoes was collected at indicated time points in TRIzol for RNA extractions. AeCullin-3 gene expression was quantified in the two groups by qRT-PCR. The experiments were performed a minimum of three times, with each experiment set in triplicates. Fold change in expression in CHIKV-spiked blood-fed mosquitoes is compared to that in uninfected blood-fed mosquitoes.

Researchers have also resorted to computational analyses and better studied insect systems such as Drosophila to identify orthologs and Aedes factors that could affect alphavirus infection (19). Combining the knowledge available on the Drosophila system with lab-generated A. aegypti transcriptome data, and with further validations, our study unravels the role of the protein modification pathways such as ubiquitin-proteasome pathway (UPP) during CHIKV infection in Aag2 cells. Other than UPP, protein catabolic processes as well as cellular protein metabolism were found to be significantly affected during CHIKV infection, as has been emphasized by previous reports (11, 36, 39). In our analysis, we found that a major proportion of genes were part of the UPP, the pathway that regulates cellular protein turnover by an intricate interplay of three diverse enzymes, E1 ubiquitin activating enzyme, E2 ubiquitin conjugating enzyme, and E3 ubiquitin ligase. Briefly, the E1 enzyme activates ubiquitin by ATP to a high-energy intermediate followed by transfer of ubiquitin from E1 to substrate which is mediated by the E2 and E3 enzymes. Cellular proteins with covalently attached ubiquitin are targeted for degradation in the 26S proteasome (53). Ubiquitin proteasomal pathway is considered to be one of the most important regulatory machineries in the cellular system. The machinery governs biological processes such as cell cycle regulation, apoptosis, transcription, translation, and cell signaling and therefore serves as
an attractive target for viral manipulation (54). Viruses are often found to subvert ubiquitin proteasomal machinery by redirecting the E3 ubiquitin ligases to degrade host proteins in order to ensure survival and promote viral replication and dissemination (as reviewed in references 55 and 56). Using both proteasomal inhibitors and performing loss-of-function studies, our present study corroborates with these earlier studies and identified AeCullin-3 to be an important proviral host factor during CHIKV infection in both Aag2 cells and A. aegypti mosquitoes.

AeCullin-3 is known to serve as a scaffolding protein for the cullin-RING E3 ubiquitin ligase (CRUL). CRULs are the largest family of E3 ubiquitin ligases and are distinguished by the presence of a structural motif called RING domain that forms the interface for recruiting E2-conjugating enzymes (57). There are eight mammalian homologues for cullin, each pertaining to different substrate specificities. Viral proteins are often found to interact with CRULs (cullinRING ubiquitin ligases) to subvert the machinery to newer cellular targets or to protect existing targets thus modulating protein turnover (55). Elaborate studies in the past two decades have established a number of downstream effects of CRULs subversion that include (i) degradation of proteins of host defense mechanism, (ii) degradation of cellular proteins to promote oncogenic transformation, and (iii) degradation of cell cycle regulatory proteins.

Viruses are known to encode proteins that interact with CRULs, as seen in Ectromelia virus and Vaccinia virus, where they encode proteins that contain domains structurally similar to the one present in cullin-3 adapters, and by virtue of the domain (BTB Kelch), viral proteins are able to interact with this sophisticated machinery and bring about changes in cellular physiology (58, 59). Similarly, viral proteins are known to participate in the subversions as reported in paramyxoviruses (60–63). All these studies, irrespective of the cullin homolog involved in the process, seem to target the STAT proteins. STAT dimerization is an important messenger for downstream activation of interferon (IFN) response, which might explain the subversion of different cullin homologues during virus replication. In the case of arboviruses, a study suggests that West Nile virus, when present in Culex mosquitoes, also adopts this subversion mechanism of CRULs for STAT degradation (64). However, unlike in the case of flaviviruses, immunity during an alphavirus infection in A. aegypti is imparted mainly by RNAi defenses, and other pathways such as JAK STAT and Toll/Imd have limited roles to play (65). Based on these findings, it would be interesting to identify whether subversion of A. aegypti cullin (AeCullin 3)-based E3 ubiquitin ligases is responsible for the nominal involvement of JAK STAT pathway during alphavirus infections. Apart from STAT proteins, viruses also divert CRULs to target other proteins of host defense, as well, such as in the case of HBV, where HBx protein mediates the degradation of antiviral protein through cullin-4-based CRULs (66). During retroviral infection, as well, HIV vif protein hijacks cullin 1 machinery to degrade APOBEC3G and promote viral replication (67). Oncogenic viruses also utilize cullin homologues to induce transformations; a well-known example includes SV40 LT antigen, which inhibits cullin-7 and increases downstream signaling pathway, ultimately leading to tumorigenic transformation (68). Thus, based on their requirements, viruses of different families often interact with CRULs in order to facilitate the degradation of host defense proteins or, in the case of retroviruses, promote oncogenic transformation. In the present study, we observed that within the A. aegypti-derived Aag2 cells, presence of AeCullin-3 promoted viral replication. We do hypothesize that CHIKV might be subverting CRUL-3 for degradation of host defense proteins; however, further studies are required to decipher the exact mechanism of action.

**Conclusion.** In summary, based on integrative analysis using global transcriptomics of A. aegypti-derived Aag2 cells upon CHIKV infection and *Drosophila* database screening, AeCullin-3 was identified and validated as one of the major and important host factors differentially regulated upon CHIKV infection in A. aegypti. Our study also revealed that the novel host factor has a proviral role during the postentry stage of viral replication.

**MATERIALS AND METHODS**

**Cell culture and virus maintenance.** A. aegypti cell line (Aag2) was a kind gift from Alain Kohl (University of Glasgow, Scotland, UK). The cells were maintained at 28°C in Leibovitz medium (L-15; cat no. AL011S, HiMedia) supplemented with glutamine, tryptose phosphate broth, penicillin/streptomycin, and 10% fetal bovine serum (FBS). Vero cells (ATCC-CCL-81) and C6/36 cells were used for chikungunya...
Expression levels were calculated using the 2 control. Experiments were conducted a minimum of three times, with each experiment set up in triplicates. (Qiagen, Germany). To estimate CHIKV genomic RNA, E1 gene was used and Rps17 served as endogenous polymerase were used for dsRNA preparation. pGEMT-easy vector was used for cloning genes for generating

Sample preparation and RNA sequencing. Briefly, Aag2 cells were seeded in two 6-well plates. Cells from one plate were incubated with CHIKV at a multiplicity of infection (MOI) of 1, and cells from the other were used as uninfected control. Twenty-four hours postinfection, cells were collected in TRizol reagent (Invitrogen). RNA isolation was subsequently performed using TRizol method (70). For RNA-Seq, three samples from uninfected control cells and three samples from CHIKV-infected cells were used. RNA-Seq was performed on total RNA using Illumina HiSeq 4000 at NGB Diagnostics Pvt. Ltd. (Noida, UP, India).

Identification of putative host factors from analysis of public databases. The RNA-Seq data were supplemented with publicly available data from the Drosophila DRSC (76) to identify well-annotated host factors of A. aegypti upon CHIKV infection. (Fig. 1b). The gene/transcript IDs of A. aegypti genome (AaegL5.3) downloaded from VectorBase [https://vectorbase.org/] were used for further analyses like count identification and differential expression analysis studies. The annotations of identified transcripts were also fetched from the VectorBase database using BioMart tool.

Double-stranded RNA preparation and transfection. For in vitro transcription (IVT), T7 and SP6 polymerase were used for dsRNA preparation. pGEMT-easy vector was used for cloning genes for generating dsRNA. After IVT, the dsRNAs were purified using the TRizol method. Purified dsRNAs were then used for transfections in Aag2 cell lines using transfection reagent Attractene (Qiagen). For transfections, 2 × 10^5 to 8 × 10^5 cells were seeded into 24-well plates. In each well, 400 ng of each dsRNA was transfected when 70% confluency was reached, along with negative control. After 4 h of incubation, the transfection medium was changed and fresh medium was added to the wells. Post 24 h, cells from one set of plates were collected to check silencing of the factors. The other plates were infected with CHIKV at an MOI of 1 and further processed for primary and secondary screening. dsRNAs were designed using snapdragon (80) primer details in Data set S2.

RNAi screening of putative host factors using qRT-PCR. Silencing efficiencies of dsRNA were estimated before setting up the experiments by transfecting cells with dsRNA and analyzing gene expression of the target transcript using qRT-PCR. Cells were transfected with 400 ng dsRNA 24 h prior to CHIKV infection and collected at 24 h postinfection. Further, RNA was isolated using TRizol reagent (Invitrogen) per manufacturer’s instructions and was used for qRT-PCR analysis using QuantiTect SYBR green PCR kit (Qiagen, Germany). To estimate CHIKV genomic RNA, E1 gene was used and Rps17 served as endogenous control. Experiments were conducted a minimum of three times, with each experiment set up in triplicates. Expression levels were calculated using the 2^−ACT method (81).

Cell viability using MTT assay. Aag2 cells were seeded in a 96-well format at a density of 20,000 cells per well and incubated at 28°C overnight. MG132 (Sigma; dissolved in dimethyl sulfoxide [DMSO]) was used at 0.1, 1, and 10 μM (final concentration of DMSO, 1%) in serum-free L-15 medium and incubated at 28°C for 24 h. Cells were washed with phosphate-buffered saline (PBS), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reagent (0.5 mg/mL) was added to each well, after which the cells were incubated for 4 h at 37°C. Cells were again washed, 100 μL DMSO was added to each well to dissolve MTT crystals, and cells were incubated at 37°C for 30 min. Absorbance was recorded at 570 nm and was used to calculate percent cell viability.

Pretreatment and posttreatment assays with proteasomal inhibitor. MG132 (Sigma) proteasome inhibitor was used for suppression of proteasomal machinery. For pretreatment assay, cells were initially treated with 0.1 μM and 1 μM drug concentrations with 1% DMSO as a vehicle control and incubated at 28°C for 24 h in 2% FBS-containing L-15 medium, which was followed by washing and CHIKV infection (MOI = 1) for 2 h. Cells and supernatants were collected at 24 h for qRT-PCR (viral genome quantification using primers for CHIKV E1 gene) and plaque assay (determination of viral titer), respectively.

For posttreatment assay, cells were infected with CHIKV (MOI = 1) for 2 h, washed, and then treated with MG132 at 0.1 μM and 1 μM concentrations again using 1% DMSO as vehicle. After a 24-h incubation, cells and supernatant were collected for qRT-PCR and plaque assays, respectively.

Plaque assay. Vero cells were plated in a 96-well format in DMEM containing 10% FBS and were incubated overnight such that the cells formed a monolayer the following day. The virus was used starting at a dilution of 1:200 and was subsequently double diluted. Cells were incubated with the virus particles for 2 h at 37°C for virus adsorption in serum-free medium. The medium was then removed and a final volume of 200 μL containing equal parts 2% carboxymethyl cellulose (CMC) and FBS-containing medium was used to overlay. Cells were further incubated for 72 h for the plaques to develop. Five percent formaldehyde was used as a fixative for 1 h and 0.25% crystal violet (in 30% methanol) was used for staining cells. The plates were washed

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three times with 1× PBS, and finally, the plaques obtained were calculated as follows to determine viral titer: PFU/mL = no. of plaques/dilution factor × volume of diluted virus per well.

Mosquito maintenance, blood feeding, and nanoinjections. *A. aegypti* mosquitoes were maintained as described previously (82). Blood feeding was performed by initially starving female mosquitoes for 4 h. Uninfected rabbit blood or blood spiked with CHIKV (10^6 PFU/mL) was then fed to mosquitoes for a period of 1 h using membrane feeders. Fully fed mosquitoes were distinguished by presence of engorged midgut and separated.

dsRNA injections were performed by injecting 800 ng of total dsRNA into female mosquitoes. For experiments with subsequent blood feeding, injected mosquitoes were fed with CHIKV-infected blood 24 h after nanoinjection. Fed mosquitoes were separated from unfed mosquitoes. A total of 5 individual mosquitoes were then collected in TRizol and analyzed for culin-3 and CHIKV genomic RNA levels in qRT-PCR using primers against E1 gene.

qRT-PCR. A total of 2 × 10^6 cells were collected in TRizol reagent (Invitrogen) for total RNA isolation. Gene expression levels were determined using specific primers with qRT-PCR using QuantiTect SYBR green PCR kit (QiaGen) following manufacturer’s instructions.

For mosquito experiments, 4- to 5-day-old female *A. aegypti* mosquitoes were fed with either CHIKV-spiked blood (10^6 PFU/mL) or uninfected blood. Fully engorged mosquitoes were separated. A pool of 5 mosquitoes were then collected in TRizol every 24 h for 5 days. RNA was extracted from the pool of mosquitoes subjected to qRT-PCR using QuantiTect SYBR green PCR kit (QiaGen) with AeCullin-3-specific primers. Rps17 was used as endogenous control. The experiment was performed three times with three technical replicates. Data were analyzed by 2^ΔΔCt method and 2^−ΔΔCt and expressed as mean ± standard deviation (SD).

Primer details for qRT-PCR have been listed in Data set S2.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 6. All experiments were performed a minimum of three times with three technical replicates. Data are expressed as mean ± SD. The statistical significance of primary screening. MG132 treatment assays, and expression profile for culin in infected *Aedes aegypti* mosquitoes was done using one-way analysis of variance (ANOVA) with Dunnett’s multiple-comparison test. Statistical significance of expression profile for viral genomic RNA levels in AeCullin-3-silenced mosquitoes was performed using unpaired Student’s t tests. P values of <0.05 were considered significant and are represented with an asterisk in the figures.

Data availability. Transcriptomics data sets from the current study have been submitted to National Center for Biotechnology Information accession number GSE195852.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.04 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.01 MB.

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The authors declare no competing interests.

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