Downregulation of a cell polarity protein potentiates Chikungunya Virus infection in host cells

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Abstract:

Downregulation of the host cell pro-apoptotic pathways confers a selective advantage to viral pathogens, and many viruses interfere with such regulatory mechanisms in order to enhance their propagation in infected cells. The Scribble cell polarity complex, which is composed of multiple adapter proteins including human Scribble, Dlg and MAGI, controls a variety of host cell functionalities including apoptosis, morphology, polarity, signaling and migration. In the recent years, components from several viral pathogens, including oncogenic viruses, have been found to associate with and modulate the activities of this complex, particularly that of the pro-apoptotic protein Scribble. Here, we establish a critical role for the downregulation of Scribble expression for the potentiation of Chikungunya Virus (CHIKV) infection. CHIKV is a well-known mosquito-borne alphavirus, which has caused outbreaks in more than 100 countries and constitutes a global health hazard. While the majority of the CHIKV proteome is well characterized, the role of a small structural protein 6K, and its transframe variant, TF, which is generated from the 6K sequence by ribosomal slippage and frameshifting, is not established as yet. We show that TF triggers the punctation, ubiquitination and degradation of Scribble during CHIKV infection, and that the downregulation of Scribble during CHIKV infection. We show that the association of TF with the PDZ domains of Scribble is mediated through a PDZ-domain binding motif at the C-terminus of TF. Thus, our work establishes a role for the downregulation of Scribble in alphavirus infections for the first time, while allocating a novel role in host modulation to the mysterious 6K/TF component of alphaviruses.

Introduction

Chikungunya Virus (CHIKV), a member of the alphavirus genus in the *Togaviridae* family, is a mosquito-borne human pathogen that causes ~ 3,000, 000 infections per year and affects almost 100 countries [1]. In spite of the significant morbidities caused by this virus globally, there are no targeted therapeutics or preventive strategies at this point and the treatment is primarily symptomatic [2], [3]. While the symptoms caused by CHIKV are primarily self-limiting, there are occasional complications which involve persistence of arthritis-like symptoms for months, and infections in the very young and elderly populations can be fatal. As such, a clearer understanding of the functionalities of the virally encoded proteins and identification of their host interaction partners is crucial for the development of targeted therapeutics [4].

The CHIKV capsid, like that of all alphaviruses, is composed of a 60-70 nm shell assembled in accordance with T=4 icosahedral geometry [5]–[7]. The outer layer is composed of 240 copies each of the heterodimers of envelope glycoproteins E1 and E2, embedded in a host derived lipid envelope. While E2 is responsible for the initial association with the host cellular receptor, E1 contains the fusion peptide that orchestrates the fusion of the viral and host lipid membranes. The inner layer consists of 240 copies of a multifunctional capsid protein (C) which encapsulates the genome [8]. Another envelope protein E3, encoded by the viral genome, promotes the association between E1 and E2 during virus assembly.

While the functionalities of the structural proteins of CHIKV – namely E1, E2, E3 and C are well investigated and established; a fifth protein – a small structural peptide named “6K”, remains relatively uncharacterized [9]. While 6K is categorized as a structural protein, its presence in the CHIKV capsid is as yet unconfirmed and its role in the virus life cycle remains mysterious. Our laboratory, as well as others, have shown that recombinantly generated alphavirus 6K is an ion channel forming protein [10], [11]. The 6K component from Ross River Virus (RRV) and Barmah Forest Virus (BFV) have been shown to form cation specific channels in bilayer membranes in electrophysiology experiments. The expression of CHIKV 6K in bacterial cells have been shown to be detrimental to the growth of the cells, which is consistent with 6K possessing membrane disrupting activity [11], [12]. We have shown that bacterially produced CHIKV 6K, in conjunction with the expression partner GST (Glutathione-S-transferase) forms heterogenous pores in membrane *in vitro* [10]. Further, *in vitro* liposome

disruption experiments as well as *ex vivo* cellular overexpression models have shown that CHIKV 6K tends to have a strong affinity towards Endoplasmic Reticulum (ER) membranes and tends to congregate in the ER. CHIKV 6K, as well as Sindbis Virus (SV) 6K have been shown to co-localize with the glycoprotein E2, and co-localize with E2 to cytopathic vesicles on the way to the plasma membrane, which is the budding site for alphaviruses [10], [13], [14]. It has been conjectured that the ion channel activity of 6K is somehow related to its ability to perhaps promoting association or allowing correct transport of viral protein and genomic complexes during assembly, although there is no clearcut connection between these two functionalities of 6K at present.

The role of 6K in the alphavirus life cycle has been further complicated by the discovery of a transframe variant "TF" of 6K, which is generated in ~10-18 % frequency during viral protein translation [15], [16]. A ribosomal slippage site in 6K results in the production of TF, which shares the majority of the primary amino acid sequence with 6K but varies in the C-terminal region. The production of TF also results in premature termination of the translation of the structural polyprotein of CHIKV; thus E1 is not generated along with TF (Figure 1A, schematic of CHIKV structural polyprotein production). TF is palmitoylated in alphaviruses such as Sindbis Virus (SV) and the palmitoylation is necessary for the accumulation of this component to the plasma membrane [17]. It has also been shown that alphavirus capsids may contain TF as a structural component, albeit in substoichiometric quantities [15], [18]. The exact functionality of TF during alphavirus infections is not established; thus it remains a mysterious entity like 6K. It is to be noted that the functionalities attributed to 6K in many previous work may have been, to some limited extent, influenced by the coexistence of sections of TF due to the non-removal of the ribosomal slippage site within the 6K genomic sequence in recombinant expression systems.

Upon a close examination of the CHIKV TF amino acid sequence, we noted that the unique region corresponding to the C-terminal end of TF contains a strong PDZ binding motif. These motifs associate with PDZ domains which are protein-protein interaction domains found in practically all life forms. The acronym PDZ originates from the first three proteins discovered to contain this domain – the post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (ZO-1) [19], [20]. *In silico* docking and simulation studies suggested that the PDZ domain binding motif (PBM) at the C-

terminus of TF is expected to interact with Scribble, a human cell polarity protein which has pro-apoptotic activity [21]. Previous studies have shown the targeting of Scribble by non-structural proteins from viruses such as Influenza, Human T lymphotrophic virus-1 and hepatitis C virus; however, the role of Scribble in cellular infections by CHIKV or any other alphavirus is not yet reported. In this study, we report that CHIKV infection results in more than 90 % abrogation in the expression of Scribble, and that Scribble protein is ubiquitinated and marked for degradation within 24 hours of CHIKV infection. We show that CHIKV TF colocalizes with Scribble during viral infection and that overexpression of TF results in the sequestration of Scribble into puncta. The co-localization of TF with Scribble appears to be mediated by the PBM of TF. Further, we show that knockdown of Scribble results in a significant enhancement in CHIKV multiplication in cells. Our data suggests that TF eliminates the pro-apoptotic activity of Scribble, thus accelerating CHIKV propagation, and therefore attributes an essential host response modulation functionality to poorly characterized TF component of alphaviruses.

Materials and methods

Docking and Molecular Dynamics (MD) simulation: Since there is no experimental structure available for CHIKV TF, a structure predicted using the I-TASSER web server [22], [23] was utilized for docking. The model with the highest c-score was selected, and further refined using the GalaxyRefine web server [24]–[26]. The X-ray crystallographic structures of Scribble PDZ domains (1 and 4, referred to as ScPDZ1 and ScPDZ4) and mammalian HTRA1 PDZ (HtPDZ) domain were downloaded from the Protein data bank (PDB IDs 5VWC, 1UJU and 2JOA respectively). All structures were energy minimized prior to docking and simulation studies.

Protein-protein docking between TF and the PDZ domains of Scribble and HTRA1 was carried out using the ClusPro web server [27]–[30]. Binding interface and affinity of each docked complex was analyzed using LigPlot [31]. All-atom MD simulation was carried out for each complex in triplicates as described. Briefly, GROMACS [32] was used to set up and execute the simulations. Each complex was solvated separately using the spc water model [33] and neutralized using counter-ions. Periodic boundary condition was applied to each system. Energy minimization was executed using the steepest descent algorithm [34]. Position-restrained NVT-NPT ensemble equilibrations were carried out for 500 ps and 1 ns respectively.

Finally, the systems were subjected to unrestrained dynamics for 100 ns. Nose-Hoover [35] and Parrinello-Rehmann [36] were used for maintaining the temperature (300K) and pressure (1 bar) during simulations. Electrostatics was maintained using Reaction-Field [37] and LINCS [38] was used as the constraints algorithm. A time-step of 2 femtoseconds and the verlet cutoff-scheme was applied. All simulations were carried out using the Gromos 54a7 force field. Trajectory analysis was carried out using in-built gromacs tools. VMD [39] and UCSF Chimera [40] were used as the visualization tools.

Molecular Mechanics Poisson-Boltzmann Surface Area (MM-PBSA) analysis: MM-PBSA was utilized to determine the Gibb's free energy (GFE) of protein-protein interactions. The script g_mmpbsa [41] was used for evaluating GFE of TF and PDZ domains from simulation trajectories. The following equations were used for the analysis

$\Delta G\ bind = \Delta G\ complex - (\Delta G\ protein1 + \Delta G\ protein2)$

$\Delta GX = \Delta EMM + \Delta G\ solv - T\Delta SMM$

$\Delta EMM = \Delta E\ bonded + \Delta E\ nonbonded = \Delta E\ bonded + (\Delta E\ elec + \Delta E\ vdw)$

$\Delta G\ solv = \Delta G\ polar + \Delta G\ nonpolar$

where $\Delta G\ complex$ = total free energy of protein-protein complex

$\Delta G\ protein1$, $\Delta G\ protein2$ = free energies of proteins in solvent

ΔGX = free energy for each entity

$\Delta E\ MM$ = vacuum potential energy (combined bonded and nonbonded interactions)

$\Delta G\ solv$ = solvation free energy [polar ($\Delta G\ polar$), nonpolar ($\Delta G\ nonpolar$)]

$T\Delta S$ = entropic contribution of free energy in vacuum. T is temperature and ΔS is entropy respectively.

$T\Delta S$ MM was not included in this study as g_mmpbsa does not calculate entropy (S). Hence, $\Delta G\ bind$ was calculated as follows,

$\Delta G\ bind = (\Delta E\ elec + \Delta E\ vdw) + (\Delta G\ polar + \Delta G\ SASA)$

where, $\Delta G\ SASA = \Delta G\ nonpolar$ since nonpolar solvation energy was calculated based on the SASA model in this study.

Cells and plasmids: Human embryonic kidney (HEK) 293T and Vero cells were grown in Dulbecco's modified Eagle medium (DMEM) (Gibco) supplemented with 10 % fetal bovine serum (FBS) (Gibco) and 1 % antibiotic antimycotic solution (Himedia). The cells were maintained at 37 °C with 5 % CO_2. The cDNA sequences of CHIKV 6K and TF

(Supplementary Figure S1) were cloned in the pEGFPC1 vector (Clontech) to generate fusion proteins tagged with the Enhanced Green Fluorescent Protein (EGFP) at the N-terminus. The ribosomal slippery site UUUUUUA in the 6K cDNA sequence was silently mutated to UUUCCTG to disrupt the consensus ribosomal frameshifting sequence and produce only 6K without affecting its primary structure [15]. Alanine substitution mutations were carried out to disrupt the PDZ domain binding motif within amino acids 71-74 of TF. Two mutants – a) G73A S74A and b) H71A G72A G73A S74A, were generated using sequential site-directed mutagenesis (Life Technologies) and were confirmed by DNA sequencing (Supplementary Table S1).

CHIKV infection: For all CHIKV infections, HEK293T cells were seeded 24 h prior to being infected by CHIKV (strain 119067) [42] at a MOI of 0.1. CHIKV was added to the cells post phosphate-buffered saline (PBS) washing for adsorption. After 90 min, cells were again washed with PBS, and complete media was added. Post-infection, cells were incubated for 24 - 48 h in normal cell growth conditions.

Western blotting: HEK293T cells infected with CHIKV were washed with PBS and lysed with RIPA lysis buffer supplemented with a protease inhibitor cocktail (ThermoFisher Scientific) as per the manufacturer's protocol. Total protein was quantified using the bicinchoninic acid (BCA) assay, followed by SDS-PAGE and Western blotting with a primary antibody against Scribble (1:500, Santa Cruz sc55543), beta-actin (1:1000, Cell signaling Technology 4967), beta-tubulin (1:200, Santa Cruz sc55529), CHIKV E2 (1:3000, The Native antigen company MAB12130-200), and EGFP (1:3000, Bio Bharati BB-AB0065). Densitometric analysis was performed to quantify the protein levels in western blots using the Fiji software as described elsewhere [43], [44].

TF peptide production and generation of specific antibodies: A 15-amino acid peptide corresponding to the C-terminal region of TF was chemically synthesized and coupled to a carrier protein, Keyhole Limpet Hemocyanin (KLH), via the N-terminus (ThermoFisher Scientific). The KLH-TF peptide was dissolved in DMSO and stored at –20 ^{o}C until use.

Polyclonal sera against TF were raised by immunizing two New Zealand white rabbits, aged 3-4 months, with the KLH-TF peptide emulsified in complete/incomplete Freund's adjuvant (CFA/IFA) as per the following protocol. On Day 0 (priming), 1 mg of KLH-TF peptide was emulsified with CFA and injected subcutaneously into the rabbits. Subsequently, four boosts

(on Days 21, 42, 63, 84) were given with 300 µg of the peptide emulsified in IFA intramuscularly. Sera were collected two weeks post-immunization from boost 2 onwards and checked for anti-TF antibodies using immunoblotting.

Immunoprecipitation: HEK 293T cells were infected with CHIKV at an MOI of 0.1. Cell extracts were prepared 24 hours post-infection (hpi). A polyclonal antibody against Scribble (Invitrogen PA5-54821) was added overnight to precleared cell extracts at 4 °C on a rocker, followed by incubation with protein A/G agarose beads (Santa Cruz sc-2003) for 1 hour at 4 °C with gentle mixing. The beads were washed thrice with lysis buffer (Tris HCL – 20 mM, KCL – 120 mM, EDTA – 0.2 mM, DTT – 1 mM, and protease inhibitor cocktail). Post-washing, the beads were resuspended in SDS-PAGE buffer, and the samples were analyzed in immunoblots with anti-Scribble and anti-TF antibodies.

MBP-tagged protein production in bacteria: The cDNA corresponding to the TF and 6K regions of CHIKV were cloned into the pMALc6T vector (New England Biolabs), in conjunction with N-terminal Histidine and Maltose binding protein (MBP) tags, and a TEV protease cleavage site. Recombinant expression of MBP-tagged TF and 6K proteins was induced by the addition of IPTG in *E. coli* Rosetta pLysS cells at 16 °C at an OD 600 of 0.6. The expressed proteins were 2-step purified by Ni-NTA affinity chromatography followed by Size Exclusion Chromatography (SEC) in a buffer containing 25 mM HEPES, 100 mM NaCl and the detergent n-Dodecyl β-D maltoside (DDM).

Confocal microscopy: HEK293T cells were cultured on glass coverslips and infected with CHIKV at an MOI of 0.1. Twenty-four hours post infection, cells were washed with chilled PBS and fixed with 4 % paraformaldehyde (PFA) for 10 min, followed by permeabilization with 0.1 % Triton X-100 in PBS for 5 min and blocking with 5 % bovine serum albumin for 60 min. Immunostaining was performed at 4 °C for Scribble and CHIKV proteins using anti-Scrib (1:500, Invitrogen PA5-54821), anti-E2 (1:500, MAB12130-200), and anti-capsid (1:200, MAB12131-200) antibodies. Post-primary antibody incubation, Alexa fluor-labeled appropriate secondary antibodies were added for 60 min. All washes were carried out using chilled PBS. Nuclei were labeled with DAPI (Invitrogen D1306) for 10 min, and cells were mounted using Prolong Glass Antifade Mountant (Invitrogen P36982). The slides were dried for a few minutes and sealed with nail paint. Imaging was performed on a confocal microscope (Leica TCS SP8) at a magnification of 63X using the oil immersion objective.

For transfection of pEGFPC1 constructs, HEK293T cells were seeded and grown at 37 °C for 24 h, followed by addition of plasmid DNA in conjunction with lipofectamine2000 (Invitrogen) according to the manufacturer's protocol. Briefly, DNA and lipofectamine (1:3 w/v) were diluted in Opti-MEM (Gibco) and incubated at room temperature (RT) for 20 min. The DNA-lipofectamine complexes were used to replace the cellular growth medium for 5 h. Twenty-four hours post-transfection, cells were washed thrice with PBS, fixed with 4 % PFA, and processed as above.

Electron Microscopy: HEK293T cells were seeded and incubated for 24 h at 37 °C, 5 % CO_2, prior to infection with CHIKV at an MOI of 1.0. After a post-infection period of 48 h, the media was discarded, and chilled phosphate buffer (pH 7.4) with 0.1 M sucrose was immediately added. After 10 min of incubation at RT, the supernatant was discarded, and fresh buffer was added to the cells. This step was repeated thrice, followed by the addition of Karnovsky's fixative. After 5-6 h of incubation, the cells were scraped and centrifuged at 2000 rpm for 3 min. The used fixative was discarded, and fresh fixative (1-2 ml) was added to the cell pellet. The pellet was resuspended slowly in the fixative and stored at 4 °C. Immunostaining for Scribble was performed using an anti-Scrib primary antibody (1:20, Invitrogen PA5-54821) and 18 nm gold-labeled secondary antibody.

shRNA mediated knockdown of Scribble: HEK293T cells were seeded and grown at 37 °C for 24 h, and then the Scribble short hairpin RNA (shRNA) containing plasmid DNAs were transfected with lipofectamine 2000 as described earlier. Four different shRNAs were used in different conditions, either alone or in combination, to optimize Scribble knockdown. The effect of the knockdown was analyzed 36 hours post-transfection using western blot analysis and the results were quantified via densitometry. Two conditions, shRNA.C1: AAACCACAAAATAGAGTCT, and shRNA.C2: a combination of AAACCACAAAATAGAGTCT and TGTACAAACATCACTAGTT, produced maximum and consistent knockdown of ~50 %, and were utilized for further studies.

MTT assay: Assays with the tetrazolium dye 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) were performed as described in the product's manual (Himedia) with slight modifications. Briefly, 10 µl MTT (5 mg/ml, Himedia) was added to cells, and the formazan crystals were dissolved in dimethyl sulfoxide after 30 min. The absorbance measurements were recorded at 570 and 690 nm.

Real-Time PCR: CHIKV infected HEK293T cells were washed with PBS, harvested in Trizol (Takara), and stored at -80 °C. RNA was isolated from the samples as per the manufacturer's protocol, quantitated using nanodrop (Thermo Scientific), and 5 µg RNA was reverse transcribed into cDNA using a cDNA synthesis kit (Takara Bio) as per the manufacturer's instructions. The cDNA was used to analyze human *scribble* and CHIKV E1 expression levels by real-time PCR using gene-specific primers (Supplementary Table S1). PCR reactions were conducted on an ABI 7500 FAST mastercycler, and the relative quantification of gene expression was carried out using the ΔΔCt method with *gapdh* as the housekeeping gene.

Plaque assay: Vero cells were seeded in a six well plate in DMEM with 10 % FBS (Gibco) and antibiotics (Corning) and incubated for 24 h at 37 °C, 5 % CO_2. The cell growth media was then replaced with serum-free media, and cells were incubated for 4-6 h. Subsequently, 10-fold dilutions (10^{-5} to 10^{-7}) of the virus-containing culture supernatants of CHIKV-infected HEK293T cells, in serum-free media, were used to replace the cellular growth media. Virus particles in the diluted culture supernatant were allowed to infect Vero cells for 1 h with shaking every 15 min. Post 1 h, the cells were washed with PBS, and 3 ml of an overlay mix of 2 % low melting agarose in 2X DMEM was added to each well. The agarose was allowed to solidify at 4 °C for 15 min, and cells were then incubated at 37 °C for 48 h. For plaque visualization, cells were fixed with 10 % formaldehyde for 4-5 h at room temperature and stained with crystal violet for 5 min, followed by washing with tap water. The plate was allowed to dry for a few minutes, and the plaques were counted. The plaque-forming units (PFU) were calculated using the following formula: PFU/ml = (number of plaques x dilution factor)/(inoculum volume).

Results

The unique C-terminus of CHIKV TF contains a PDZ domain binding motif capable of association with Scribble: The first 49 amino acids of 6K and TF are identical, highly hydrophobic, and capable of lipid membrane interaction as shown before [10], [15]. A ribosomal -1 frameshift at a slippery sequence corresponding to residues 48-49 within the 6K gene results in the production of TF with a 27 residue, largely hydrophilic tail (Figure 1A). A motif search of this region unique to TF, indicated the presence of a putative PDZ domain binding region or PBM at the extreme C-terminus (Figure 1B). The Eukaryotic Linear Motif

(ELM) resource [45] predicted that TF residues 71-76, with the sequence HGGSTV, constitutes a class I PBM. This was further confirmed by PDZPepInt, a PDZ predicting software [46], [47], which predicted that residues 71-75 of TF (HGGST) is a potential interaction partner for the host protein Scribble. Mammalian Scribble, a cell polarity component with four PDZ domains, is targeted by multiple viral proteins through PDZ-PBM interactions [48]. We attempted to check the association of CHIKV TF with the PDZ binding domains of Scribble using *in silico* methods. A predicted structure of full-length TF was docked against PDZ domains 1 and 4 of Scribble (designated ScPDZ1 and ScPDZ4) using the ClusPro web server. Both these domains have been previously reported to be high affinity binders [49]. The best scoring models were selected for further analysis and the binding interface was analyzed using Ligplot (Figure 1C). Several residues at the C-terminus of TF were found to be involved in mediating hydrogen bonds and hydrophobic interactions with residues of the PDZ domains (Table 1). In order to further investigate the interaction, MD simulations of TF in conjunction with ScPDZ1 and ScPDZ4 were carried out. To determine whether the interaction of TF with Scribble PDZ domains is specific, a PDZ domain from the mammalian High temperature requirement A serine protease (HtrA1) [50] was also included in the study. An RMSF plot analysis of the simulation trajectory showed that TF was stabilized by the Scribble PDZ domains (Figure 1D, Supplementary Figure S2). In particular, local fluctuations in the C-terminal tail of TF were significantly reduced upon interaction with ScPDZ1 (Figure 1D). Local fluctuations in the C-terminal tail were most pronounced in simulations of TF alone. However, HtrA PDZ interaction had minimal effect on the C-tail fluctuations of TF (Figure 1D). Higher binding energies were observed for TF interaction with the PDZ domains of Scribble in contrast to the PDZ domain of HtrA1 (ScPDZ1>ScPDZ4>>HtPDZ) (Supplementary Table S2). Specifically, TF-HtPDZ interaction was observed to be ~3.8 times weaker than that with ScPDZ4 and ~5.8 times weaker as compared to TF-ScPDZ1. The binding energies in all cases were primarily mediated by electrostatic and van der Waals interactions. Thus, *in silico* analysis indicates that CHIKV TF preferentially binds to the PDZ domains 1 and 4 of Scribble, as compared to PDZ domains from other mammalian proteins. Further analysis of the TF interface residues with ScPDZ1 (Figure 1E) revealed that several C-terminal residues of TF, which are part of the putative PDZ binding motif, contributed favorably towards binding with ScPDZ1.

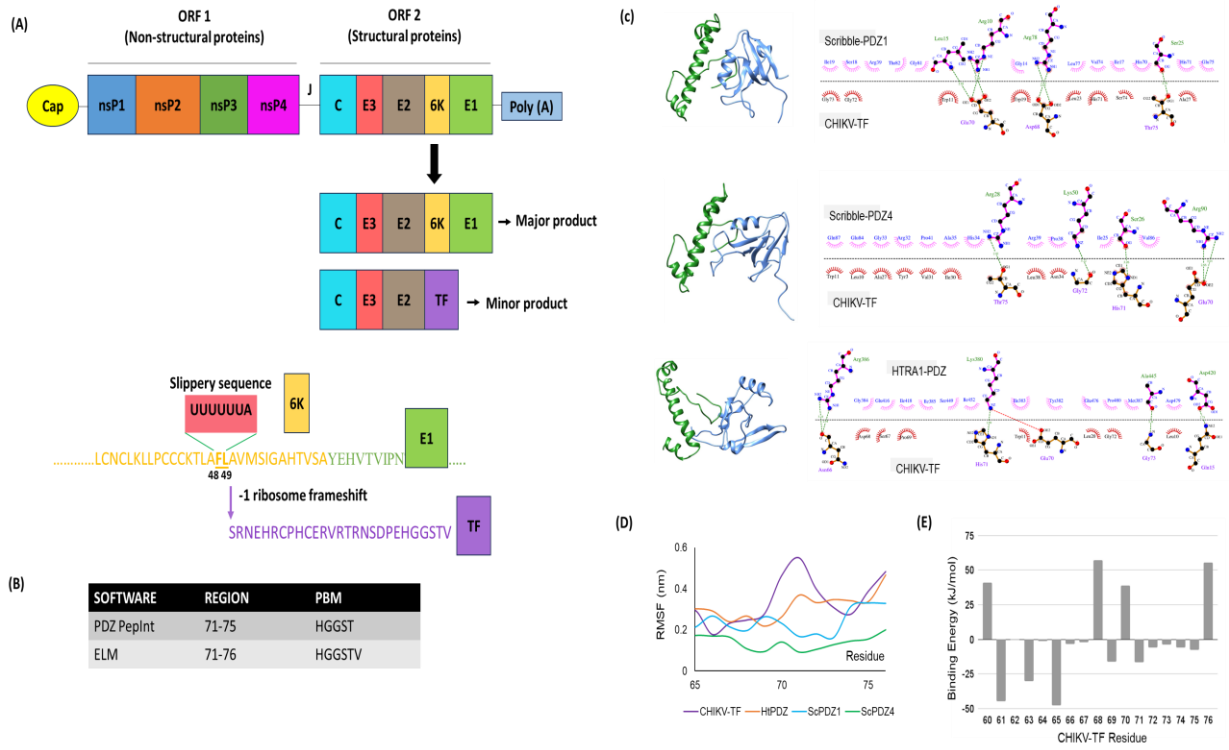

Figure 1. CHIKV TF may be a binding partner for human Scribble. The CHIKV RNA genome has two open reading frames (ORFs) separated by a junctional region (J). The 5' ORF (ORF 1) codes for four non-structural proteins (nsP1-4). The 3' ORF (ORF 2) codes for 5 structural proteins, namely capsid (C), E3, E2, 6K, and E1. The 6K has a ribosomal slippery site which leads to the production of a transframe (TF) protein (Firth et al., 2008) (upper panel, A). Schematic of 6K and TF sequences and the ribosome slip site (F at 48th and L at 49th position) (lower panel, A). Potential PDZ binding motifs (PBMs) present in TF as predicted by different softwares (B). Predicted interactions between TF and Scribble PDZ1, Scribble PDZ4, and HTRA1 (C) . RMSF plot for TF interactions with Scribble and HTRA1 (D). Binding of TF C-terminal residues with ScPDZ1.

Table 1. Binding interface of docked complexes

System	CHIKV-TF (Hydrogen bonds)	CHIKV-TF (Hydrophobic interactions)
TF-ScPDZ1	Asp68, Glu70, Thr75	Trp11, Trp19, Leu23, Ala27, His71, Gly72, Gly73, Ser74
TF-ScPDZ4	Glu70, His71, Gly72, Thr75	Tyr3, Leu10, Trp11, Ala27, Ile30, Val31, Asn34, Leu38
TF-HtPDZ	Gln15, Asn66, Glu70, His71, Gly73	Leu10, Trp11, Leu20, Ser67, Asp68, Pro69, Gly72

Overexpressed CHIKV TF co-localizes with Scribble: In order to check the interaction of TF with Scribble the TF cDNA, fused to EGFP at the C-terminus, was transfected in HEK293T cells. To check for the specificity of the putative interaction, CHIKV 6K, which shares the N-terminal 49 residues with TF, similarly fused with EGFP, was also transfected. The ribosomal slippage site was mutated in the 6K construct to prevent the production of TF. Twenty-four hours post-transfection, cells were fixed, and immunostaining was performed to identify the localizations of TF, 6K and Scribble (Figure 2A). In mock-transfected cells, Scribble was found to majorly localize to the cell periphery near the plasma membrane. Similarly, Scribble localized to the plasma membrane in cells transfected with an empty vector (pEGFPC1), and no co-localization with EGFP was noted (Figure 2A). However, Scribble formed clusters in the cytoplasm in cells transfected with EGFP-TF. TF was also found to form distinct puncta in the cytoplasm and to co-localize with Scribble (Figure 2A). This interaction was specific to TF as neither Scribble clustering nor co-localization was observed with EGFP-6K or EGFP alone (Figure 2A).

Since Scribble is known to interact with its target proteins via its PDZ domains, the putative PBM at the unique C-terminus of TF (HGGSTV) was mutated to contain either two or four alanine substitutions (HGAATV/AAAATV). The corresponding plasmids containing the mutated EGFP-TF sequence were individually transfected in HEK293T cells, and the effect of these mutants on Scribble localization was analyzed using confocal microscopy.

No puncta formation was observed in cells transfected with mutated TF constructs (Figure 2B). In contrast to the distribution of wildtype TF, the fluorescence associated with mutated TF was detected throughout the cytoplasm, similar to the expression of EGFP only. No co-localization was detected between Scribble and mutated TF constructs (Figure 2B). The expression levels of TF PBM mutants, along with the wildtype construct, were also investigated (Figure 2C). A slight reduction in the protein expression level of TF with the PBM mutated to HGAATV (TF Mut1) compared to wildtype was observed, while the TF construct with a quadruple alanine mutation (TF Mut2) displayed further reduced expression. However, the alteration in the cellular expression pattern of the mutated constructs and the lack of co-localization with Scribble clearly indicated that TF associates with Scribble through its C-terminal region, and the putative PDZ binding motif HGGSTV is critical for this interaction (Figure 2A-C).

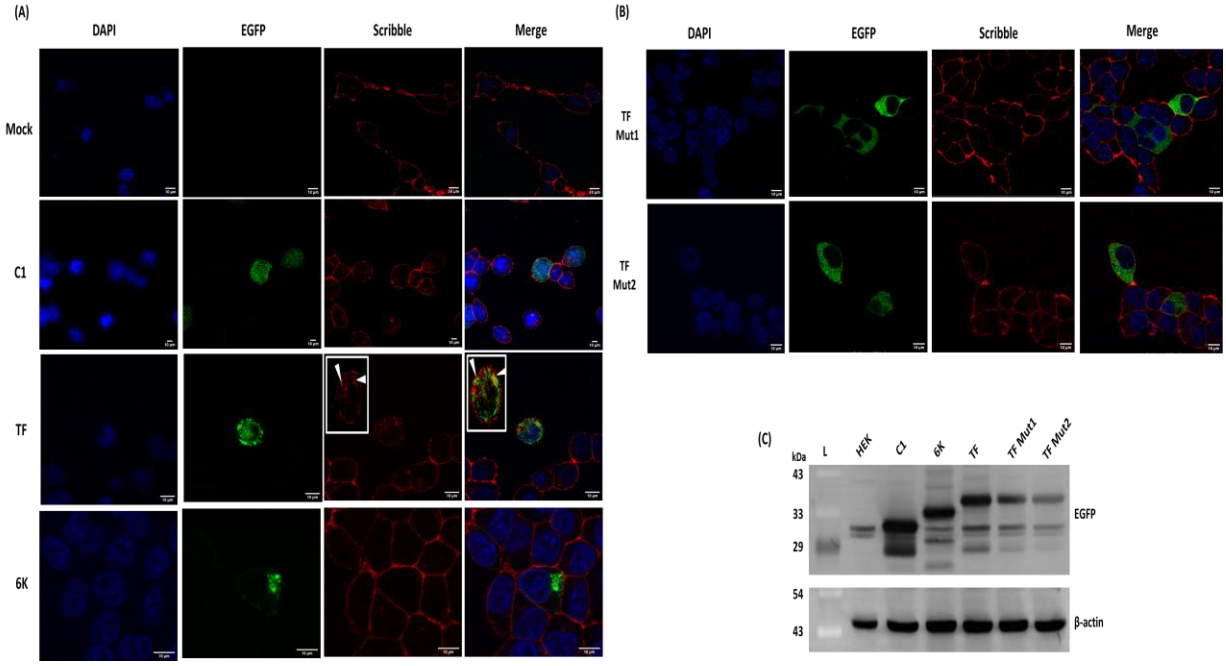

Figure 2. Indirect assessment of TF and Scribble association through TF PDZ binding motif. HEK293T cells were transfected with pEGFPC1 vector or pEGFPC1 plasmids carrying TF or 6K. Twenty-four hours post-transfection, cells were fixed, and immunostaining was performed for Scribble (red). Inset shows zoomed images of an EGFP-TF expressing cell. Arrowheads indicate Scribble puncta and co-localization with TF (A). Mutational analysis of TF and its effect on Scribble localization (B). Expression data of TF and TF mutants in HEK293T cells. Twenty-four hours post-transfection, cells were lysed, and 6K and TF expression were analyzed using western blot with anti-GFP antibody (C). L: protein ladder, C1: pEGFPC1 empty vector, TF Mut1: TF-HGAATV, TF Mut2: TF-AAAATV.

TF associates with Scribble during CHIKV infections: In order to determine if the association of TF with Scribble has any functional relevance, the interaction was investigated during CHIKV infection of HEK293T cells. An antibody was raised against the unique region of CHIKV TF, by utilizing the C-terminal 27 amino acids coupled with Keyhole limpet hemocyanin (KLH) as an antigen. The antibody, generated in rabbit, was tested for its ability to recognize TF in Western blots as well as in immunofluorescence assays (Supplementary Figure S3). The association of the antibody with TF was found to be quite specific as it was able to recognize bacterially produced and purified TF (conjugated with the Maltose Binding Protein,

MBP) and EGFP-TF from a HEK293T cell lysate but was unable to bind to bacterially produced MBP-6K or overexpressed EGFP-6K.

The antibody against TF was utilized to visualize the localization and association of TF during CHIKV infection. Confocal microscopy studies showed substantial production of TF during CHIKV infection of HEK293T cells, and co-localization of TF with Scribble in punctate forms (Figure 3A). A polyclonal anti-Scribble antibody was utilized for co-immunoprecipitation of binding partners from CHIKV infected HEK293T cell lysates. Western blotting of the co-IP fractions with TF unique-region specific antibody clearly indicated the presence of TF (Figure 3B), thus suggesting direct interaction of TF with Scribble during CHIKV infection.

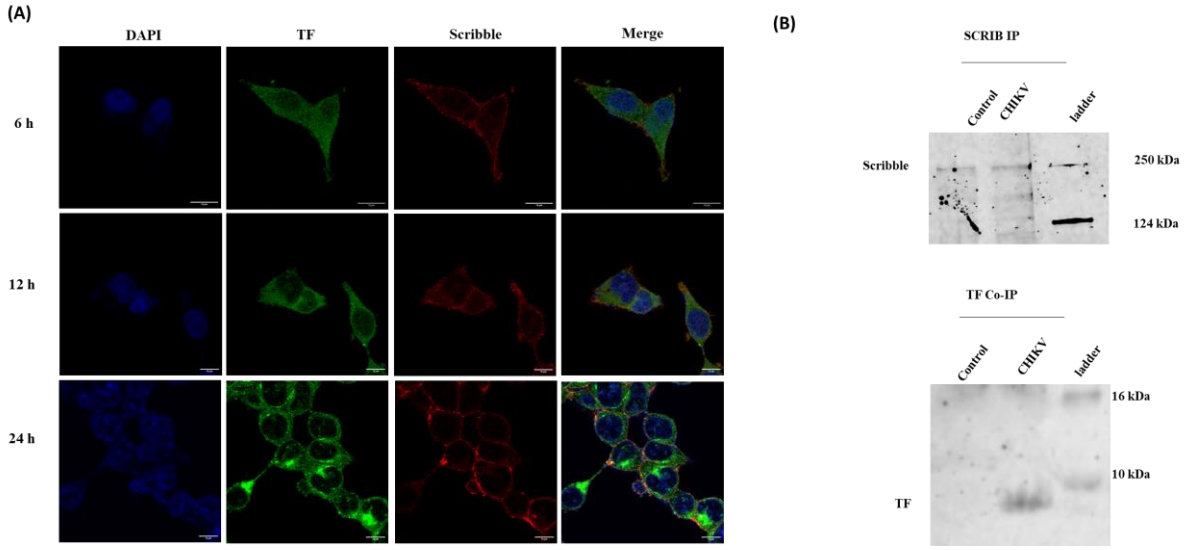

Figure 3. Assessment of TF –Scribble association in infected cells. Co-localization of TF-Scribble using TF specific antibody during infection at different time points (6, 12 and 24 hours) (A). Immunoprecipitation (IP) of scribble using scribble specific antibody (i) and Co-IP of TF-Scribble in infected cells (ii) (B).

CHIKV infection, or TF expression, results in punctation and degradation of Scribble: Multiple viruses have been shown to target Scribble for degradation in order to manipulate host immune responses, cell junction integrity and apoptosis pathways [48]. In order to analyze the effect of CHIKV infection on the level of Scribble, the levels of Scribble protein were analyzed by Western blotting in HEK293T cells 24 and 48 h post infection (Figure 4A). A significant decrease in Scribble protein level was noted upon CHIKV infection in a time-dependent

manner. At 24 hours post-infection (hpi), Scribble levels were reduced to nearly 50 % compared to uninfected cells. The protein levels reduced to greater than 95 % at 48 hpi (Figure 4A), while that in uninfected cells remained at the same level. Thus, CHIKV infection appeared to cause drastic reduction in the expression of Scribble protein in cells.

To investigate if CHIKV infection affects Scribble localization, CHIKV infected HEK293T cells were immunostained with a Scribble antibody 24 hpi. CHIKV capsid protein and glycoprotein E2 were also immunostained using specific antibodies (Figure 4B). While Scribble primarily localized to the plasma membrane in case of mock-infected cells, cytoplasmic sequestration was noted in CHIKV infected cells, with prominent puncta as previously noted in TF transfected cells (Figure 2A). Together with the decrease in Scribble protein levels, this data suggests that CHIKV infection leads to mislocalization and eventual degradation of Scribble, which is likely executed by TF.

To confirm this, the effect of wildtype TF, and that of TF with a mutated PBM, on Scribble protein expression level was also evaluated (Figure 4C). Since the maximum CHIKV-induced reduction in Scribble protein levels was observed at 48 h post infection, the effect of TF, upon transfection, on Scribble expression was also studied at 48 h. Transfection of HEK293T cells with the EGFP-TF plasmid, containing the wildtype sequence of TF, was found to reduce the Scribble protein levels by ~20 %. This reduction was absent in cells transfected with EGFP-6K, which further indicates lack of association between 6K and Scribble. The reduction in Scribble protein levels was found to be restored to slightly more than normal levels (increased by up to 14 %) in cells transfected with EGFP-TF, containing double or quadruple alanine mutations in the C-terminal PBM. Taken together, our results suggest that the CHIKV TF mediates Scribble punctation and degradation using its C-terminal PBM HGGSTV. The relatively higher increase in the levels of Scribble degradation in CHIKV-infected versus TF-transfected cells is likely due to the significantly higher efficiency of TF expression, as well as differences in the time period of maximum expression, in the infection model compared to the transfection model.

In order to morphologically characterize the puncta formed by Scribble at a relatively higher resolution, CHIKV infected HEK293T cells were fixed, sectioned, and immunostained with an anti-Scribble primary antibody and a nanogold labeled secondary antibody and subjected to electron microscopy (Figure 4D). Consistent with the results of confocal microscopy studies, clustering of Scribble was observed in the CHIKV-infected cells.

The mode of eventual degradation of Scribble was investigated by co-staining CHIKV infected cells with anti-Scribble and anti-Ubiquitin primary antibodies and appropriate secondary antibodies (Figure 4E). We found strong co-localization of Scribble and ubiquitin specific antibodies in CHIKV infected HEK293T cells 24 hpi, which intensified after 48 h of infection. Particularly, Scribble was found to be present prominently in punctate form 48 hpi, and was strongly associated with Ubiquitin, indicating that CHIKV-mediated degradation of Scribble is probably mediated by the ubiquitin-proteasome system (UPS).

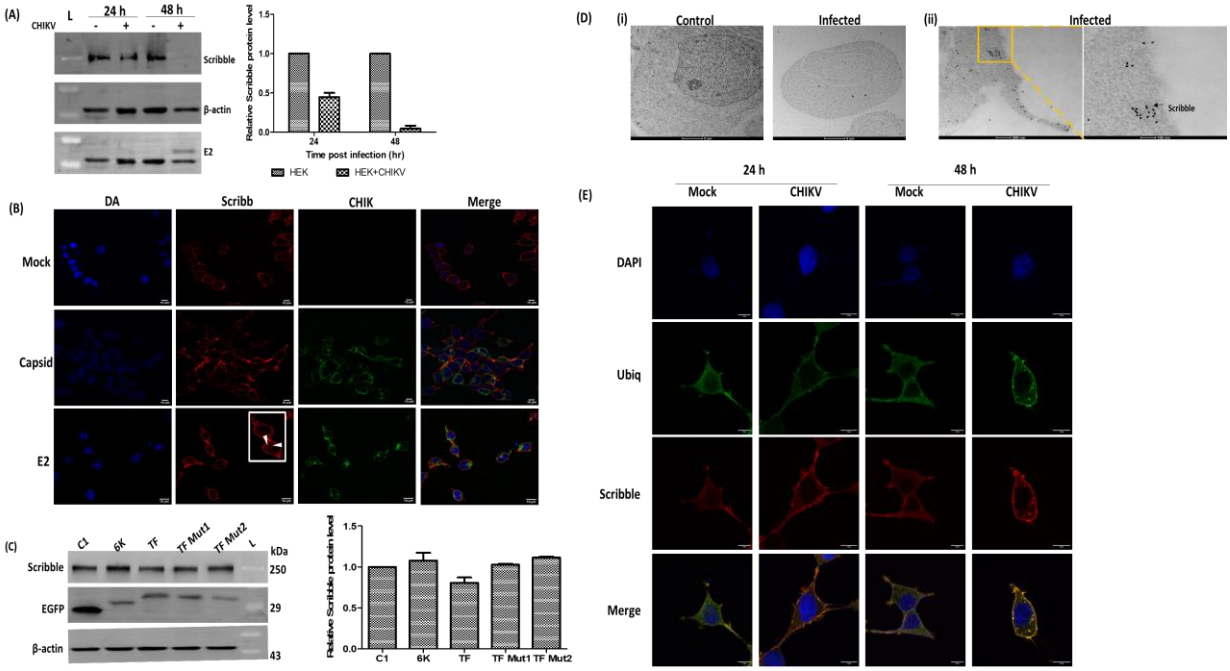

Figure 4. Effect of CHIKV infection on Scribble localization and expression. HEK293T cells were infected with CHIKV at 0.1 MOI. Scribble levels were analyzed at 24 and 48 hpi using western blot and quantitated using densitometry (A). Twenty-four hours post-infection, the cells were fixed, and immunostaining was performed for Scribble and CHIKV capsid and E2 proteins. Inset shows zoomed images of an E2 expressing cell. Arrowheads indicate Scribble puncta (B). Effect of TF on Scribble expression. HEK293T cells were transfected with pEGFPC1-TF and its mutants. Only vector and pEGFPC1-6K were also transfected, and Scribble levels were analyzed 48 h post-transfection using western blot and quantitated using densitometry (C). Electron microscopy (EM) imaging of CHIKV-infected HEK293T cells. HEK293T cells were infected with CHIKV at 1.0 MOI and Scribble was visualized using a gold-labeled secondary antibody (D-i). EM images of an infected cell at higher magnification

(D-ii). Immunostaining for ubiquitin and Scribble in CHIKV infected HEK293T cells (E). Data represents mean ± SEM of two biological replicates.

The effect of Scribble knockdown on CHIKV infection

In order to investigate the significance of Scribble downregulation during CHIKV infection, the level of CHIKV propagation in Scribble knockdown cells was compared to that in untreated cells. Two short hairpin RNAs (shRNAs) were used to knock down Scribble protein expression in HEK293T cells according to standard protocols. The effect of the shRNAs were analyzed 36 hours post-transfection using western blot analysis. Densitometric analysis, to quantitate the relative percentage of gene knockdown for each sample, indicated a relatively efficient Scribble protein knockdown of approximately 50 % with both shRNA conditions (Figure 5A). The degree of knockdown at the RNA level was also confirmed using real-time PCR (Figure 5B).

To ensure that any alteration observed in CHIKV infectivity upon Scribble knockdown was indeed because of reduction in the quantity of Scribble, and not due to a difference in the number of viable cells as an effect of knockdown, cell viability was assessed using an MTT assay (Figure 5C). No significant change in cell viability was observed with Scribble knockdown.

The Scribble knockdown cells were then infected with 0.1 MOI of CHIKV. Twenty-four hours post-infection, the culture supernatants containing new virus were harvested and plaque assay was performed using standard methodologies in Vero cells. CHIKV infectivity was calculated by counting the number of plaques formed 48 h post-virus addition. We found that the virus titer, represented as the plaque forming unit per ml (pfu/ml), was significantly increased upon Scribble knockdown (Figure 5D). We also tested the levels of CHIKV RNA in infected HEK293T cells with Scribble knockdown by RT PCR and found that the levels of CHIKV RNA were also markedly upregulated in these cells, compared to regular infected cells (Figure 5E). The level of increase in CHIKV pfu/ml and genomic RNA was approximately 2-3 times higher upon Scribble knockdown. Taken together, our data strongly suggests that punctation and degradation of Scribble is a viral countermeasure against the host pro-apoptotic response against CHIKV infection and is mediated by the transframe protein of CHIKV 6K component.

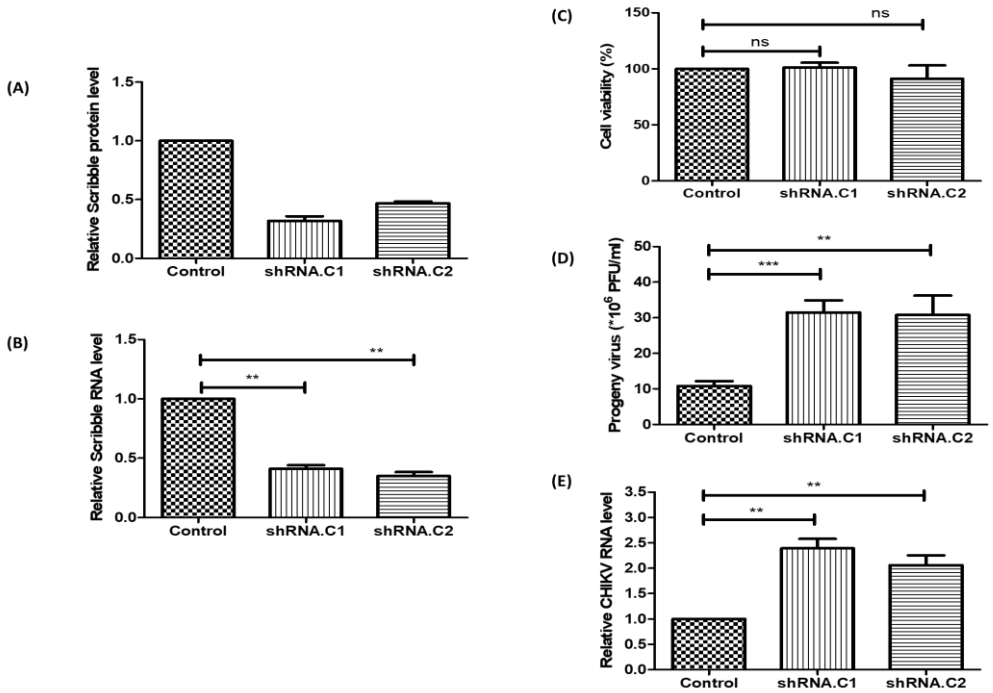

Figure 5. Effect of Scribble knockdown on CHIKV infection. Scrib shRNAs were transfected into HEK293T cells, and knockdown was confirmed 36 hours post-transfection using western blot and quantitated using densitometry (A). Scribble knockdown was also confirmed at RNA level using real-time PCR (B). The effect of Scribble knockdown on cell viability was assessed using MTT assay (C). Thirty-six hours post-transfection, cells were infected with CHIKV at MOI of 0.1 for 24 hours. The effect of Scribble knockdown on CHIKV progeny virus was determined using plaque assay (D) and CHIKV RNA levels using real-time PCR (E). Data represents mean ± SEM. **, $p \leq 0.01$; ***, $p \leq 0.001$, ns: non-significant (Student's t-test).

Discussion

Host-virus interactions play a crucial role in defining the trajectory of pathogenesis [51]. Key host molecules and pathways are consistently hijacked by viruses in order to ensure propagation of infection. Some widely targeted host cellular cofactors include nuclear pore/envelope, COPI vesicles, ribosomes and the translational pathway, kinases in the signaling pathway etc. [52]. Increasing evidence suggest that the Scribble cell polarity complex could be one of the central host factors targeted by multiple virus families [48]. Human Scribble is part of a large module of adapters, including several Dlg and Lgl proteins, which has a central role

in regulating cell migration, adhesion, polarity, signaling, apoptosis, morphogenesis, and development [53]–[55]. The members of this complex associate with a wide range of cellular factors such as kinases and other signaling proteins to modulate cellular activities. Scribble is a member of the LAP (leucine-rich repeats and PDZ domain) family and contains numerous protein-protein interaction domains including 16 Leucine-Rich Repeat domains (LRRs), 2 LAP specific domains and 4 PDZ domains (PDZ1-4). Loss of Scribble in cells causes disruption of cellular adhesion and polarity, resulting in hyperproliferation [53]. As a determinant of cellular growth, apoptosis and proliferation, Scribble has been a target of tumorigenic viruses such as Human Papillomavirus (HPV) and Human T-lymphotropic virus 1 (HTLV-1) [56], [57]. Recently, effectors from several other viruses such as the NS1 protein from Influenza A Virus (IAV), NS5 from Tick-borne encephalitis virus (TBEV), and the core protein from Hepatitis C Virus (HCV) have been shown to associate with the PDZ domains of Scribble through PBMs, resulting in mistargeting of Scribble and increase in viral proliferation [48]. However, there have been no reports of Scribble modulation by alphaviruses so far. In this manuscript, we establish Scribble as a host target of Chikungunya Virus (CHIKV) and identify the viral factor which induces the mislocalization and degradation of Scribble to ensure increased viral proliferation.

We found that CHIKV infections downregulate Scribble expression by as much as 95 % in a time-dependent manner. Immunofluorescence analysis of CHIKV-infected cells showed clustering of Scribble into cytoplasmic puncta, and ubiquitin-mediated degradation by the proteasome. The cellular localization of Scribble is important for its functioning [21], [48], [55], and mislocalization of Scribble and its sequestration in discrete puncta likely affects its regulatory role in cellular proliferation [56], [58]. To confirm the role of Scribble in CHIKV infection, shRNA-based downregulation of Scribble expression was carried out. A ~ 50 % downregulation in Scribble levels resulted in 2-3 times increase in the virus titer and genomic RNA levels. It is possible that this effect is due to the elimination of the pro-apoptotic activity of Scribble, resulting in prolonged cell survival and consequent enhancement in viral replication [58]. Scribble is a cell polarity protein that is important for maintaining the integrity of cell junctions, and disruption of these junctions has been reported to promote the egress and dissemination of some effect [48], [53], [59]. Thus, the beneficial effect of Scribble knockdown on CHIKV replication might thus also be due to the alterations in cell-cell barriers, which

enhances viral spread. A more detailed analysis of the timeline of CHIKV-mediated Scribble downregulation and consequent spatiotemporal effects on cellular morphology, adhesion and signaling may be essential for understanding the functional basis for viral countermeasures to host response.

The small structural protein 6K, and its transframe variant TF, appear to have outsized importance in regulation of CHIKV morphology and virus-host interactions [60]. We have shown previously that CHIKV 6K, like its homologues from other alphaviruses, is an ion-channel forming protein [10]. We also showed that inhibition of 6K may affect CHIKV assembly, resulting in the formation of morphologically unusual particles. Recent work has shown that 6K co-localizes with alphavirus glycoproteins in cytopathic vesicles (CPV-II), which may support its role in promoting viral assembly [14]. TF, in contrast, localizes at the plasma membrane and is packaged in virions [15], [17]. Our data attributes an essential role to TF in the modulation of host response and in the enhancement of viral proliferation. Expression of TF in HEK293T cells by transfection also led to the sequestration of Scribble into distinct cytoplasmic puncta, similar to that noted in CHIKV infected cells. This sequestration was absent in cells transfected with the cDNA for EGFP-6K, suggesting that TF, and not 6K, is the regulator of the human Scribble protein. TF was found to co-localize with the Scribble, unlike 6K, which has a distinctly separate localization pattern. Since TF and 6K share approximately two-thirds of their amino acid sequence, this functional difference is likely to originate from the unique C-terminal region of TF.

Other viral proteins such as IAV NS1, TBEV NS5, HTLV tax, HCV NS4B and HPV-16 E6 are known to associate with the PDZ domains of Scribble through their PBMs [48]. *In silico* analysis of the unique C-terminus of TF indicated the presence of a PBM which could interact with the PDZ domains of Scribble. Mutational analysis of the predicted PBM confirmed its involvement in regulating Scribble localization. The TF constructs with the PBM mutated were mis-localized, as they were distributed throughout the cytoplasm, and were unable to co-localize with or trigger the sequestration of Scribble. Mutations in TF PBM also resulted in somewhat decreased protein levels, particularly in the quadruple alanine mutant, which could be due to the drastic nature of the mutation. However, the expressed protein clearly showed an altered localization pattern.

Although the expression of wildtype TF by transfection in HEK293T cells caused mislocalization of Scribble, the protein quantity was only marginally reduced compared to the drastic decrease in Scribble protein content during CHIKV infection. This could be because of temporal alterations in TF expression in the transfection versus the infection model, or due to the differences in the quantity of TF production in these cases. The onset of protein detection is usually delayed in transfected compared to infected cells, and it is possible that in HEK293T cells expressing TF, mislocalization and punctation of Scribble precedes protein degradation, which occurs at later time points. Also, we found that in spite of the high transfection efficiency of HEK293T cells, as seen with empty vector producing EGFP, the expression efficiency of EGFP-TF or EGFP-6K by transfection is fairly low, which could be due to the membrane permeabilization capability of these components. It has been shown earlier that expression of 6K in bacterial cells impedes the growth curve significantly, as the membrane activity is likely toxic to the cells [11], [12]. Thus, reduced levels of TF expression in transfected HEK293T cells may be a possible reason for decreased levels of Scribble degradation, compared to that observed in CHIKV infected cells. However, this slight decline in Scribble protein levels triggered by transfection of EGFP-TF was absent in case of PBM-mutated TF constructs. Instead, a slight increase in Scribble levels was noted in these cells indicating the role of TF PBM in PDZ-domain dependent association with Scribble.

The PDZ domain-PBM interaction is one of the most abundant modes of protein-protein interaction. The homologues of CHIKV TF from several other alphaviruses also have PBMs in their unique C-terminal tails that can potentially associate with host partners (Supplementary Figure S4). For example, the predicted TF sequences from O'nyong nyong Virus and Igbo-Ora Virus have Class I PBMs at the C-termini that are almost identical to the PBM in CHIKV TF; while TF sequences from Ross River Virus, Mayaro Virus and Semliki Forest Virus are predicted to contain Class II PBMs (Table 2)**.** Two prominent PBMs, in addition to the one at the C-terminus of TF, are predicted in the CHIKV proteome (Table 3). Residues 418-423 in CHIKV glycoprotein E2 are expected to constitute a Class I PBM, while the C-terminus of 6K is predicted to have a Class II PBM. However, we did not note any co-localization of transiently expressed 6K with Scribble, therefore it is possible that this PBM may mediate interactions with other host or viral proteins.

Table 2. Prediction of PBM in alphavirus TF using the ELM software [45]

Alphavirus	Region	PBM	Class
SINV	65-70	CATDTV	Class_3
ONNV	71-76	HGGSTV	Class_1
UNAV	69-74	HGGISV	Class_2
RRV	70-75	CGGVPV	Class_2
IGBO-ORBA	71-76	HGGSTV	Class_1
MAYV	70-75	PSGIPV	Class_2
SFV	70-75	RGGVPV	Class_2
EEEV	NA		
VEEV	NA		
MIDV	NA		

Table 3. Prediction of PBM in CHIKV proteins using the ELM software [45]

Protein	Region	PBM	Class
nsP1	NA		
nsP2	NA		
nsP3	NA		
nsP4	NA		
Capsid	NA		
E3	NA		
E2	418-423	VRTTKA	Class_1
6K	56-61	AHTVSA	Class_2
TF	71-76	HGGSTV	Class_1
E1	NA		

Human Scribble has four PDZ domains, and their association with several viral proteins has been monitored using biophysical methods such as Isothermal Titration Calorimetry (ITC) and X-ray Crystallography [61], [62]. *In vitro*, the IAV NS1 proteins appear to bind to all four PDZ domains of Scribble, with PDZ4 being the tightest binder and PDZ1 being the weakest [62]. The TBEV NS5 protein appears to show strong binding to both PDZ4 and PDZ3 of Scribble [61], [63]. Interestingly, the NS4B protein of HCV requires interaction with multiple PDZ

domains of Scribble for strong binding [64]. A detailed biophysical analysis of the CHIKV TF protein with full-length Scribble, as well as the isolated PDZ domains, is essential in order to understand the binding modalities, including cooperativity between different PDZ domains. It is to be noted that other host proteins that are part of the Scribble complex, such as Dlg-1, also contain PDZ domains. The potential association of alphavirus TF with other components in the Scribble cell polarity module requires further study. This will lead to a clearer understanding of host responses and viral countermeasures during infection.

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Author contributions:

Conceptualization: RK, DD, MB; Methodology: RK, DD, MB; Investigation: RK, PT, DD, YR, SC, SYM, KS; Resources: SB, MB; Writing-original draft: RK, PT, DD, MB; Writing-review & editing: RK, PT, MB; Visualization: RK, PT, MB; Supervision: SB, MB. Project administration: MB; Funding acquisition: MB.

Conflict of interest:

The authors declare no conflict of interest.

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