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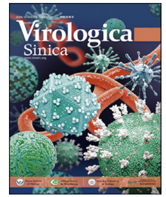

Research Article

Elucidating cellular interactome of chikungunya virus identifies host dependency factors

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ABSTRACT

Chikungunya virus (CHIKV) is a re-emerging mosquito-transmitted RNA virus causing joint and muscle pain. To better understand how CHIKV rewires the host cell and usurps host cell functions, we generated a systematic CHIKV-human protein-protein interaction map and revealed several novel connections that will inform further mechanistic studies. One of these novel interactions, between the viral protein E1 and STIP1 homology and U-box containing protein 1 (STUB1), was found to mediate ubiquitination of E1 and degrade E1 through the proteasome. Capsid associated with G3BP1, G3BP2 and AAA+ ATPase valosin-containing protein (VCP). Furthermore, VCP inhibitors blocked CHIKV infection, suggesting VCP could serve as a therapeutic target. Further work is required to fully understand the functional consequences of these interactions. Given that CHIKV proteins are conserved across alphaviruses, many virus-host protein-protein interactions identified in this study might also exist in other alphaviruses. Construction of interactome of CHIKV provides the basis for further studying the function of alphavirus biology.

1. Introduction

Chikungunya virus (CHIKV) is transmitted to humans by mosquitoes including *Aedes albopictus* and *Aedes aegypti* (Burt et al., 2012). The common symptoms of CHIKV infection are severe joint pain, fever, headache, muscle pain, and rash. Starting in 2004, CHIKV caused a pandemic in countries around the Indian Ocean, with millions of reported cases and a number of deaths (Benditt and Markowitz, 1986; Enserink, 2007). CHIKV was first reported in the Americas in 2013, and has rapidly expanded into more than 43 countries, causing more than a million cases (Morrison, 2014; Johansson, 2015; Shen et al., 2021). As the expanding geographic range of virus activity, increasing numbers of cases worldwide, and the severe and long-lasting arthralgic sequelae of the disease, the burden of disease caused by CHIKV is very high (Burt et al., 2012).

CHIKV, belonging to the genus *Alphavirus* in the family *Togaviridae*, contains a 12-kb positive-sense RNA genome including two open reading frames (ORFs) (Powers et al., 2001). One ORF encodes four nonstructural proteins (nsP1, nsP2, nsP3 and nsP4) that are involved in genome replication (Schwartz and Albert, 2010). The other ORF encodes structural proteins including capsid, E3, E2, 6K, TF and E1 (Schwartz and Albert, 2010). The CHIKV virion is highly organized and approximately 70 nm in diameter (Brown et al., 2018). Capsid packages the genome into the nucleocapsid core, which is surrounded by a lipid membrane containing 240 copies of the E2/E1 heterodimer. CHIKV entry is initiated by binding to cell surface receptors followed by clathrin-mediated uptake of the virus into the endosome compartment, where acidic pH triggers conformational change of the E1 fusion protein and virus membrane fusion. The nucleocapsid is then released into the cytoplasm where the genome replication and viral protein synthesis occur (Kielian et al., 2010; Kielian, 2014; Holmes et al., 2020). CHIKV structural proteins are synthesized as a polyprotein, and then the polyprotein is processed and transported to the plasma membrane, where the virus assembly and budding occur (Brown et al., 2018).

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Previously, we constructed the intraviral interactome of CHIKV, which will provide important insights into the function of CHIKV proteins (Yin et al., 2019). More importantly, viruses must hijack a considerable number of host proteins to rewire cellular pathways and carry out processes required for replication. This is often achieved through physical interactions between viral and host proteins. In light of the absence of effective vaccines to prevent against CHIKV infection, elucidating virus-host protein-protein interactions (PPIs) will be critical for developing antiviral therapies. Unbiased systematic studies play a powerful role in the effort to identify new virus-host PPIs by elucidating how viruses replicate, uncovering new functions of host machinery and revealing how dysfunction of these pathways can cause disease. Several systematic and unbiased approaches to identify host factors for individual CHIKV proteins have been conducted to date (Bourai et al., 2012; Meshram et al., 2018), but none have systematically identified CHIKV-host PPIs for all CHIKV proteins.

In the present study, we investigated the virus-host protein interactome of CHIKV. Using affinity purifications followed by high-throughput quantitative mass spectrometry, we identified host protein interactors of individual CHIKV proteins in human cells and provided a comprehensive protein-protein interaction map between the viral proteins of CHIKV and human proteins. Moreover, we found that STIP1 homology and U-box containing protein 1 (STUB1) interacted with viral protein E1 and mediated degradation of E1 through the proteasome, which could promote CHIKV replication. We also found that valosin-containing protein (VCP/p97) bound to capsid of the virus and VCP inhibitors blocked CHIKV infection.

2. Materials and methods

2.1. Cells and virus

293T and Huh7.5.1 cells were cultured at 37 °C in high glucose Dulbecco's modified Eagle's medium (DMEM, HyClone, Marlborough, MA, US) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 100 U penicillin/mL, and 100 μg streptomycin/mL. CHIKV strain 181/25 plasmid pSinRep5-181/25ic was a gift from Terence Dermody through Addgene (pSinRep5-181/25ic, plasmid 60078). Virus stocks of CHIKV were produced in Huh7.5.1 by electroporation of in vitro transcribed viral RNA, which was synthesized from linearized pSinRep5-181/25ic using the mMESSAGE mMACHINE SP6 transcription kit (Thermo Fisher Scientific, Waltham, MA, US).

2.2. Plasmids

Constructs encoding for nsP1, nsP2, nsP3, nsP4, E1, E2, E3, 6K, capsid and TF were generated by amplifying from pSinRep5-181/25ic and inserting into pEGFP-C1 expression plasmids. All constructs were confirmed by sequencing. The viral plasmids or empty vector were transfected using FuGENE HD (Promega, Madison, WI, US) according to the manufacturer instructions.

2.3. Antibodies and reagents

The mouse antibodies used in this study have been described previously and included anti-actin (A1978, Sigma-Aldrich, St. Louis, MO, US) and anti-GFP (XHY038L, Xuheyuan, Beijing, China) antibodies. The rabbit antibodies used in this study included anti-E2/E1 (Kielian et al., 1990), anti-nsP1 (provided by Dr. Andres Merits), anti-GFP (XHY026L, Xuheyuan, Beijing, China), anti-VCP (ab11433, Abcam, Cambridge, MA, US), anti-STUB1(12496-R034, Sino Biological, Beijing, China), anti-NAP1L1 antibody (ab33076, Abcam) and anti-G3BP1 antibody (ab181150, Abcam) antibodies. The secondary antibodies included donkey anti-mouse Alexa Fluor 488, donkey anti-rabbit Alexa Fluor 555, donkey anti-rabbit Alexa Fluor 488 and donkey anti-mouse Alexa Fluor 555 (all, Invitrogen, Carlsbad, CA), horseradish peroxidase (HRP)-conjugated enhanced chemiluminescent (ECL) goat anti-mouse IgG (A4416; Sigma-Aldrich), HRP-conjugated ECL goat anti-rabbit IgG (A6154; Sigma-Aldrich). DBeQ (S7199) and NMS-873 (S7285) were purchased from Selleckchem (Houston, TX, US).

2.4. GFP-Trap assays

293T Cells expressing CHIKV viral proteins were lysed with lysis buffer (50 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 0.5 mmol/L EDTA, 0.5% NP-40, protease inhibitor cocktail). Cell lysates were incubated with GFP-Trap_A beads (gta-20, Chromotech, Planegg-Martinsried, Germany) for 1 h at 4 °C and then the beads were washed three times with wash buffer (50 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 0.5 mmol/L EDTA). SDS-sample buffer was added to the beads. Binding proteins were eluted by boiling for 10 min at 95 °C and then analyzed by Western blotting or SDS-PAGE.

2.5. Mass spectrometry and data processing

To identify CHIKV viral protein interactors, the eluates from GFP-Trap were electrophoresed into about 1 cm 10-well SDS-PAGE. The gels were stained with Coomassie-blue, and then each lane was cut manually. The gel pieces were digested by trypsin and then analyzed by electrospray ionization tandem mass spectrometry on a Thermo LTQ Orbitrap instrument (Thermo Fisher Scientific). Individual ion scores are indicated in the form of a Mascot-derived confidence score [calculated from the posterior error probability (PEP) as $-10 \log (PEP)$]. The default significance threshold is a P value of <0.05. The unique peptides of each sample were used to score the hits. First, the hits of viral protein simples with less than three unique peptides were removed from list. Second, to compare the hits of viral protein samples with empty vector, the number of unique peptides of all the samples was added one and then the ratio of peptide number of viral proteins to empty vector was used to rank the hits. Third, ribosomal proteins were also removed from the list.

2.6. KEGG terms and protein domains analysis

The final lists of hits were used to analyze KEGG terms and protein domains using online STRING (Global Biodata Coalition and ELIXIR, string-db.org, version 11.0). False discovery rate is below 0.05. The CHIKV-protein interactome networks were created using Cytoscape_v3.7.2 (Cytoscape Team).

2.7. Knockdown by siRNA

The siRNAs were transfected into Huh7.5.1 cells using Lipofectamine RNAiMAX transfection reagent (Invitrogen). The siRNAs used for hits knockdown were obtained from GenePharma (Suzhou, China) and siRNA sequences were shown in Supplementary Table S1.

2.8. Quantitative PCR (qPCR)

Total cellular and viral RNA was isolated using RNeasy minicolumns (Qiagen, Hilden, Germany) and reverse transcribed by random priming with a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Then the cDNA was quantitated by qPCR using a Power SYBR Green PCR Master Mix (Thermo Fisher Scientific). Sequences of primers used in qPCR were listed in the Supplementary Table S2.

2.9. Immunofluorescence microscopy

Briefly, cells seeded onto glass coverslips were washed with PBS and fixed with 4% PFA in PBS buffer for 10 min at room temperature. Fixed cells were incubated with blocking solution (PBS containing 10% normal goat serum) for 5 min at room temperature. Next, the coverslips were incubated with primary antibodies in permeabilizing buffer (0.1% Triton

X-100 in PBS containing 10% normal goat serum) for 1 h. The coverslips were then washed three times with blocking solution, followed by incubation with Alexa Fluor 488- and Alexa Fluor 555-conjugated secondary antibodies for 1 h at room temperature. After being washed three times with blocking solution, the coverslips were mounted with mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI). The cells were imaged using a Leica TCS SP5 microscope (Germany) equipped with a 40× oil immersion lens.

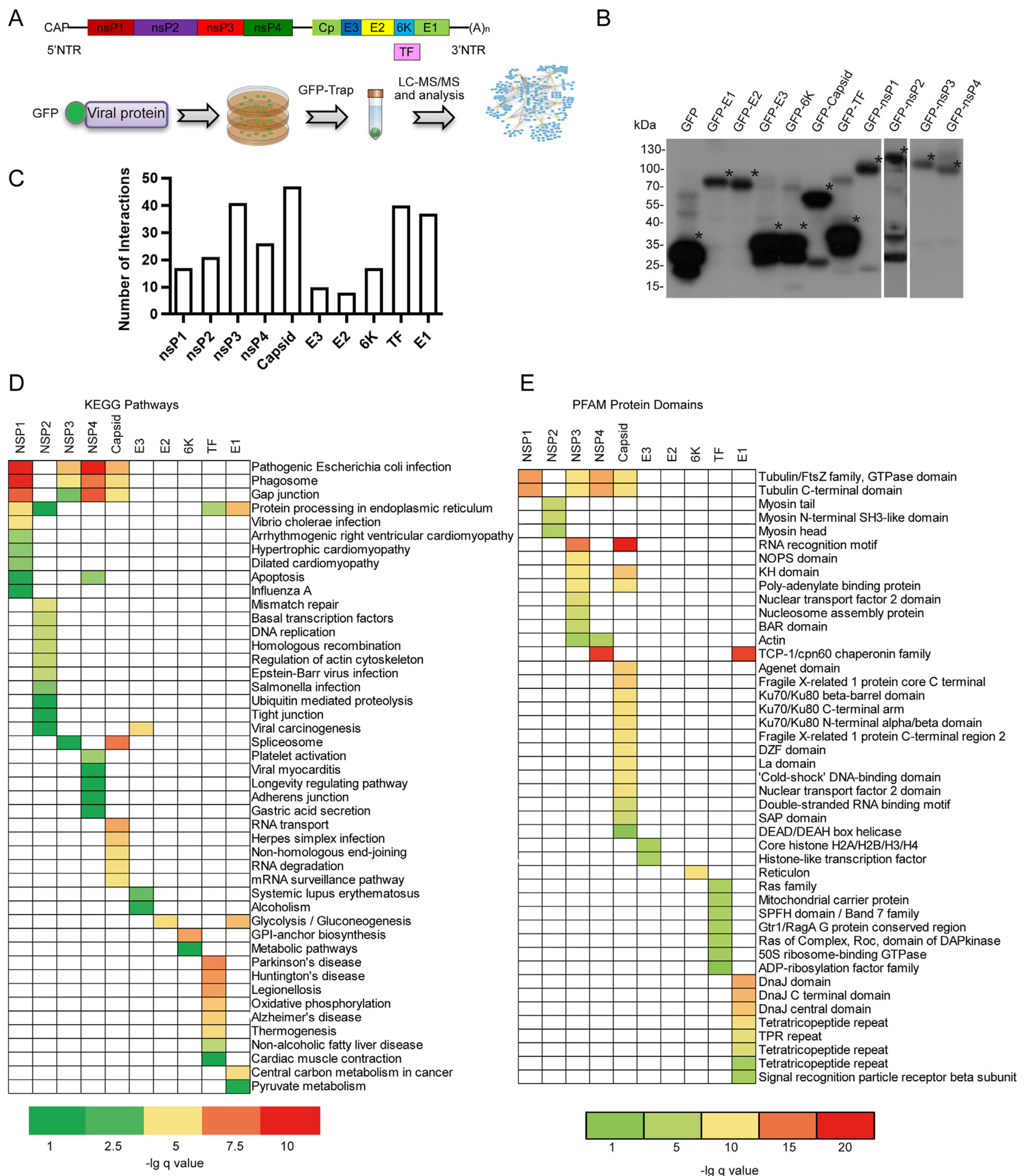

Fig. 1. Exploring the CHIKV-human protein-protein interactome. **A** Overall pipeline for the integrated AP-MS approach to define CHIKV-human protein-protein interactions in 293T cells. **B** Expression of GFP-tagged viral proteins. 293T cells in 6-well plate were transfected with 1 μg plasmids expressing GFP-tagged viral proteins or GFP control for 24 h and subjected to Western blotting for analysis. Aserisk indicates the expected protein. **C** Bar graph summarizing the number of human proteins identified as interacting with each of the indicated CHIKV baits. **D, E** Heat maps of KEGG terms (**D**) and protein domains (**E**) significantly enriched (false discovery rate value < 0.05) among the host factors that interact with CHIKV baits. Colors represent statistical significance (false discovery rate value) as indicated by the accompanying scale.

2.10. Cell viability assay

The cell viability was measured by the Cell Titer-Glo Luminescent Cell Viability Assay Kit (Promega) according to the manufacturer's protocol.

2.11. Statistical analysis

Statistically significant differences were assessed using an unpaired Student *t*-test in GraphPad Prism, version 5 (GraphPad Software, Inc., La Jolla, CA). Unless otherwise stated, the data represent the means from at least three independent experiments ± the standard deviations (SD). ns, not significant; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$.

3. Results

3.1. Systematic analysis of the host binding partners for CHIKV proteins

To gain further insight into the interplay between CHIKV and the host cell, we employed a systematic affinity purification mass spectrometry (AP-MS) approach to define the human proteins and complexes that interact with CHIKV proteins (Fig. 1A). The ten processed CHIKV proteins (nsP1, nsP2, nsP3, nsP4, capsid, E3, E2, 6K, E1 and TF) were cloned and individually expressed with N-terminal GFP tags in 293T cells (Fig. 1B). The ten tagged proteins were affinity purified using GFP-Trap_A beads and subjected to analysis by mass spectrometry to identify co-purifying proteins. To identify high-confidence protein-protein interactions (PPIs), we quantitatively scored PPIs by the peptide numbers in viral proteins divided by GFP control (Supplementary Table S3) and we identified a total of 264 PPIs across ten viral proteins. The numbers of PPIs for each CHIKV proteins were shown in Fig. 1C. To find pathways and processes that interact with specific CHKV proteins, an enrichment test for each viral protein was performed using KEGG pathway analysis (Fig. 1D) and PFAM protein domains analysis (Fig. 1E). These tests uncovered that CHIKV has strong ties with biological processes. A large number of host binding partners for CHIKV proteins play important roles in apoptosis, cell adhesion and pathogen infection, which fit the main functions of CHIKV proteins during virus infection.

3.2. Construction of CHIKV-human PPI network

We next constructed a network representing the 264 CHIKV-human interactors with viral bait proteins (Fig. 2). This network recapitulates several examples of known biology, such as the interaction between nsP3 and G3BPs as well as the connection between nsP3 and DHX9 (Götte et al., 2019; Matkovic et al., 2019), and reveals several novel connections between the CHIKV and human cellular machinery. Forty-six of the identified human proteins interact with at least two CHIKV proteins. The whole chaperonin containing TCP-1 ring complex (TRiC) was identified as interactors of nsP4 or E1. The TRiC/CCT complex, consisting of eight different subunits (CCT1–CCT8), is essential for protein folding in an ATP-dependent manner. Previous studies have shown that the TRiC/CCT complex participates in the life cycle of several viruses, including dengue virus (Hafirassou et al., 2017), hepatitis C virus (Inoue et al., 2011), and reovirus (Knowlton et al., 2018). Interestingly, we also found viral protein 6K interacts with glycosylphosphatidylinositol (GPI)-transamidase (GPI-TA) complex including PIGT, PIGS and PIGK, which transfers the GPI lipid to the newly made GPI protein in the endoplasmic reticulum, replacing the C-terminal GPI attachment signal peptide of a protein with the lipid (Gamage and Hendrickson, 2013).

3.3. RNAi screening identifies a set of CHIKV cofactors across all viral proteins

Next, we silenced the host hits by siRNA strategies and infected with CHIKV 181/25 strain to identify the host dependency factors or host restriction factors. Although we focused on proteins with robust

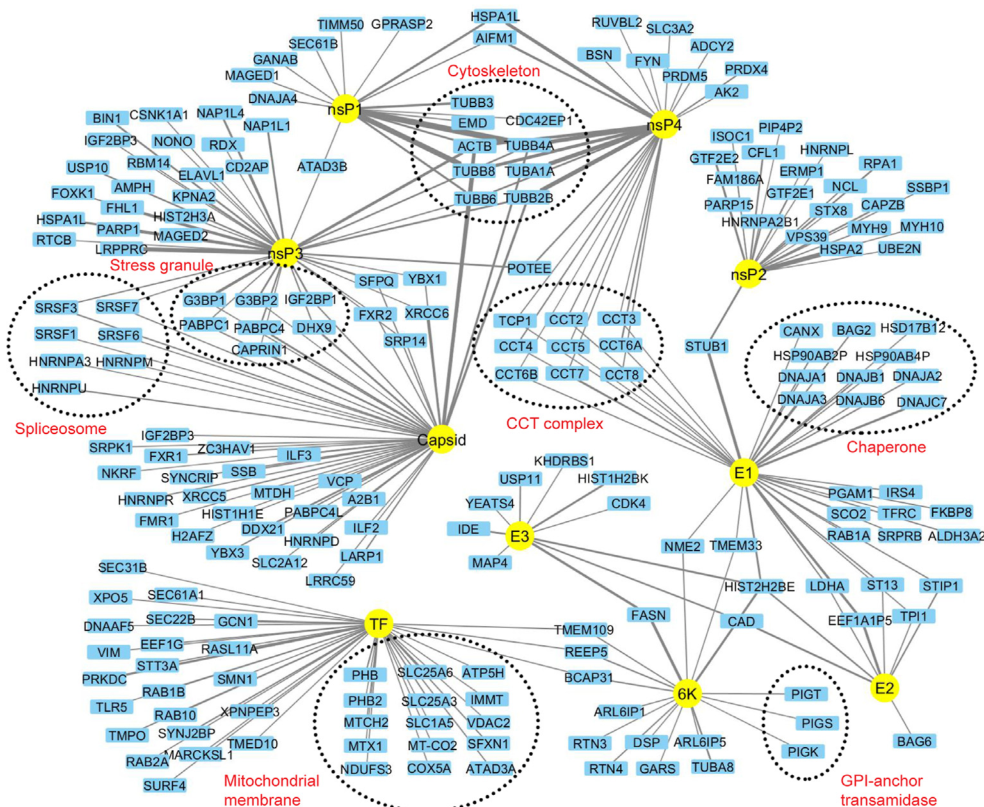

Fig. 2. Network of the CHIKV-host interactome in 293T cells using Cytoscape. There are ten CHIKV viral proteins (yellow circle) and interacting host proteins (blue rectangle). The black lines indicated interactions of viral proteins and host proteins.

specificity scores, we also selected hits that were enriched in a pathway. We included the known CHIKV host factors G3BP1 and G3BP2. Huh7.5.1 cells, a subclone of Huh7 cells that express CHIKV receptor, MXRA8, were transfected with siRNAs against hits or control siRNA. The knockdown efficiency of siRNAs was validated by quantitative RT-PCR (Fig. 3A). The reduction of target transcript expression >50% was considered as effective knockdown. All the siRNAs in Fig. 3A reduced the expression of host genes >50%. To determine whether a candidate affected the infection rate, Huh7.5.1 cells transfected with siRNA were challenged with CHIKV 181/25 strain. The viral RNA in cells was measured by quantitative RT-PCR. SiRNAs against the same target had to reduce the CHIKV RNA level. For example, treatment of CCT6A siRNA #1 and #3 decreased the CHIKV RNA level. However, CCT6A siRNA #2 increased the CHIKV RNA level. Thus we excluded CCT6A as a candidate. As shown in Fig. 3B, we found several host dependency factors had roles in CHIKV life cycle, such as STUB1 and VCP. Consistent with previous study, silencing G3BP2 but not G3BP1 reduced CHIKV replication (Scholte et al., 2015). Although the whole CCT complex was potential interactors of nsP4 or E1, the effect of two or more siRNAs against the same CCT complex subunit on CHIKV RNA level was distinct. We found the same phenotype in cells treated with siRNAs targeting translocon proteins.

3.4. STUB1 interacts with E1 and promotes its downregulation

STUB1, also known as cellular U-box E3 ubiquitin ligase protein (CHIP), is an ubiquitin E3 ligase that controls the stability of various cellular proteins by promoting their ubiquitination and degradation (van den Boom and Meyer, 2018). Our mass spectrometry results showed STUB1 interacted with E1. To validate the interaction between STUB1 and E1, we performed immunoprecipitation using GFP-Trap_A beads in 293T cells expressing GFP-E1 or GFP. We found STUB1 specifically binds with GFP-E1 but not GFP (Fig. 4A). To investigate whether E1 is ubiquitinated, 293T cells were co-transfected with E1-Flag and HA-ubiquitin-expressing plasmids followed by immunoprecipitation with an anti-Flag antibody. As shown in Fig. 4B, Western blot analysis

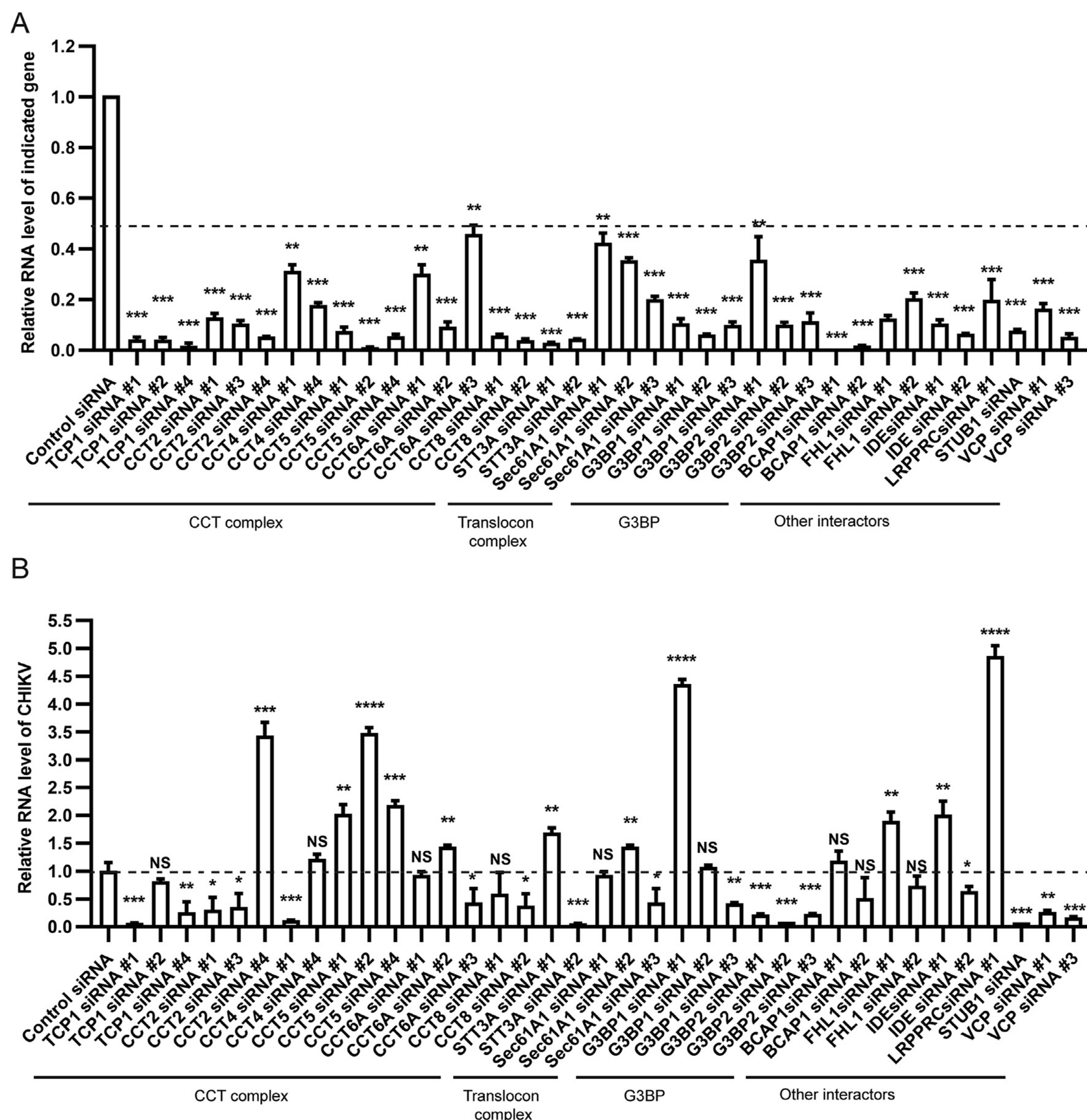

Fig. 3. SiRNA screen identifies CHIKV cofactors. **A** Knockdown efficiency of host genes confirmed by qPCR. Huh7.5.1 cells treated with different siRNAs or a control siRNA for 72 h. The mRNA level of host factors was quantitated by qPCR normalized by GAPDH. **B** Huh7.5.1 cells treated with host factors siRNA or a control siRNA for 72 h were infected with CHIKV for 8 h. Virus RNA was analyzed by qPCR. Data were represented the mean ± standard deviation from three independent experiments. Statistical analysis was performed by an unpaired Student *t*-test. NS, not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

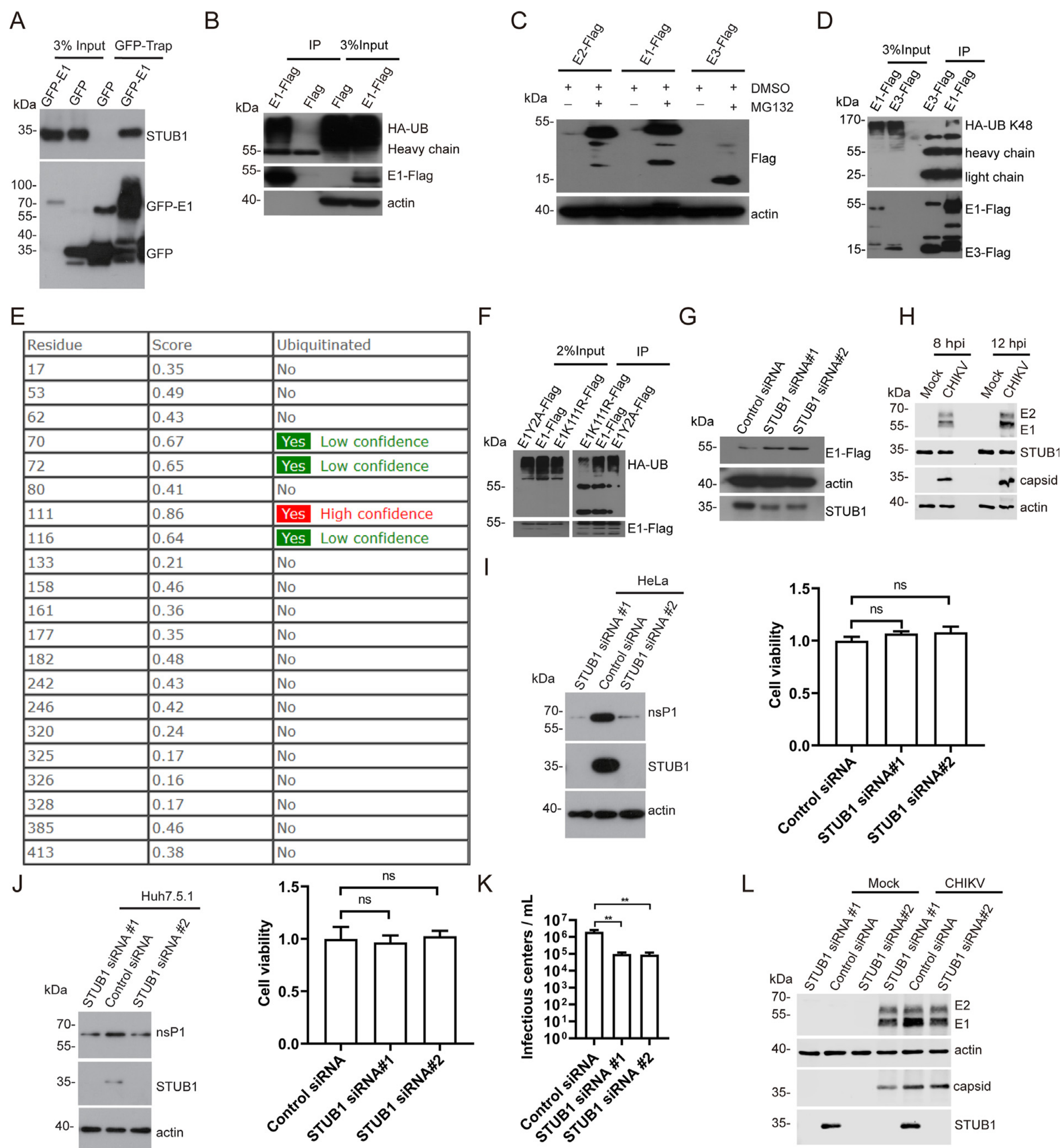

Fig. 4. STUB1 interacts with E1 and promotes its downregulation. **A** Immunoprecipitation using GFP-Trap analysis confirms STUB1 interacts with E1. 293T cells in 100 mm plates were transfected with 3 μg GFP-E1 or GFP empty vector for 48 h. GFP-Trap analysis was performed with GFP-Trap_A beads beads followed by Western blot. **B** E1 is modified by ubiquitin. 293T cells in 100 mm plate were co-transfected with 3 μg E1-Flag and HA-ubiquitin-expressing plasmids for 24 h followed by immunoprecipitation with an anti-Flag antibody and Western blot analysis using an anti-HA antibody. **C** MG132 inhibits degradation of E1. 293T cells in 6-well plate transfected with 1 μg plasmids expressing E2-Flag, E1-Flag or E3-Flag for 24 h were treated with 20 μmol/L MG132 or DMSO for 6 h. The expression of E1-Flag, E2-Flag or E3-Flag was analyzed by Western blot. **D** E1 is modified by K48-linked ubiquitination. 293T cells in 100 mm plates were co-transfected with 3 μg HA-ubiquitin-K48 and either E1-Flag or E3-Flag for 48 h. The cell lysates were subjected to immunoprecipitation with an anti-Flag antibody followed by Western blot analysis using an anti-HA or anti-Flag antibody. **E** The potential ubiquitination sites of E1 were predicted by BDM-PUB (http://bdmpub.biocuckoo.org/prediction.php). **F** 293T cells in 100 mm plate were transfected with 3 μg HA-ubiquitin together with E1-Flag, E1-K111R-Flag or E1-Y2A-Flag for 48 h followed by immunoprecipitation with an anti-Flag antibody. The ubiquitination of E1 was analyzed by Western blot analysis using an anti-HA antibody. **G** Knocking down of STUB1 increases expression of E1. 293T cells in 24-well plate were transfected with 20 μmol/L control or STUB1 specific siRNAs for 48 h followed by expression of E1-Flag for another 24 h. The protein expression of E1-Flag and knockdown efficiency of STUB1 by siRNA were measured by Western blot. **H** CHIKV infection didn't change the protein level of STUB1. Huh7.5.1 cells were infected with CHIKV (MOI = 1) for 8 h or 12 h. The cell lysates were analyzed by Western blot using indicated antibodies. **I, J** Silence of STUB1 inhibits CHIKV replication. HeLa (**I**) or Huh7.5.1 (**J**) cells were transfected with 20 μmol/L control or STUB1 specific siRNAs for 72 h, and were then infected with CHIKV (MOI = 1) for 8 h. The cell lysates were analyzed by Western blot analysis using antibodies against nsP1, STUB1 and actin. **K** HeLa cells were transfected with 20 μmol/L control or STUB1 specific siRNAs for 72 h, and were then infected with CHIKV (MOI = 1) for 12 h. The culture media were then harvested, and clarified by centrifugation. Virus titers were measured by infectious center assays in HeLa cells. **L** HeLa cells were transfected with 20 μmol/L control or STUB1 specific siRNAs for 72 h, and were then infected with CHIKV (MOI = 1) for 8 h. The cell lysates were analyzed by Western blot analysis using antibodies against E2/E1 and actin. For panels (**I, J** and **K**), data were represented the mean ± standard deviation from three independent experiments. Statistical analysis was performed by an unpaired Student *t*-test. ns, not significant; $^{**}P < 0.01$.

using an anti-HA antibody revealed that E1 was modified by ubiquitin. Thus, we hypothesized that STUB1 could mediate degradation of E1 by the proteasome. To test whether CHIKV envelope proteins are degraded by the proteasome, 293T cells expressing E1-Flag, E2-Flag or E3-Flag were treated with proteasome inhibitor, MG132. We found that significant accumulation of E1 and E2 in cells after treatment of MG132 (Fig. 4C). This result suggested that E1 and E2 were degraded by ubiquitination in cells. The polyubiquitin linked through Lys-48 are the primary targeting signals for proteasomal degradation (Glickman and Ciechanover, 2002). To confirm whether the accumulation of E1 protein in response to MG132 treatment was ubiquitin-linked through Lys-48, 293T cells were co-transfected with E1-Flag or E3-Flag and HA-ubiquitin Lys-48-expressing plasmids followed by immunoprecipitation with an anti-Flag antibody. As shown in Fig. 4D, E1 protein ubiquitination occurred by Lys-48-linked ubiquitin chains. Based on Prediction of Ubiquitination sites with Bayesian Discriminant Method (BDM-PUB) (http://bdmpub.biocuckoo.org/prediction.php), the Lys at site K111 was found to be the highly potential residue for ubiquitination (Fig. 4E). To confirm this, ubiquitination of E1 K111R mutant was investigated in 293T cells. We found that compared with E1 WT or unrelated mutant E1 Y2A, ubiquitination of E1 K111R was reduced (Fig. 4F). Together, these results suggest CHIKV viral protein E1 is ubiquitinated by STUB1.

To explore the potential role for STUB1 in degradation of E1, we measured the E1 protein level in 293T cells transfected with STUB1 siRNA. We found that silencing STUB1 increased the protein levels of E1, indicating that STUB1 promoted degradation of E1 (Fig. 4G). Next, we investigated the role of STUB1 in CHIKV replication. We measured the level of STUB1 protein upon CHIKV infection and found that CHIKV infection didn't change the protein level of STUB1 (Fig. 4H). We used two siRNAs to knockdown STUB1 in HeLa or Huh7.5.1 cells followed by infection with CHIKV. As shown in Fig. 4I and J, knockdown of STUB1 reduced nsP1 protein of CHIKV in HeLa and Huh7.5.1 cells, which suggested that STUB1 might promote CHIKV replication through degradation of E1. Cell viabilities were not changed by STUB1 siRNAs (Fig. 4I and J). We showed that knockdown of STUB1 decreased CHIKV RNA level (Fig. 3B), and protein level of nsP1 (Fig. 4I and J), which is key component of RNA replication factory. We measured the progeny CHIKV production in HeLa cells that were transfected with siRNAs against STUB1. We found that knockdown of STUB1 reduced CHIKV production, resulting in an about 1-log 10 decrease (Fig. 4K). We also determined the protein level of E1 in infected STUB1-knock down cells (Fig. 4L) and found that protein level of E1 did not change significantly. We are seeing two very different phenotypes, one with an artificial over-expression system (Fig. 4G), and a second in the context of a complete viral infection (Fig. 4L). Taken together, these results demonstrate that CHIKV viral protein E1 is degraded through the proteasome regulated by STUB1 in the E1-Flag overexpression system.

3.5. VCP interacts with capsid

To confirm the interaction between VCP and capsid, we performed immunoprecipitation using GFP-Trap_A beads in 293T cells expressing GFP-capsid, GFP-nsP3 or GFP-nsP1 (Fig. 5A). We found that VCP specifically binds with capsid but not nsP3 or nsP1. During CHIKV infection, nsP3 mainly locates at stress granules (Fros et al., 2015; Remenyi et al., 2018) while capsid distributed in stress granules and cytoplasm (Zheng and Kielian, 2013). Consistent with the previous finding (Götte et al., 2019), we found that nsP3 associated with many stress granules proteins (Fig. 2). We blotted the known nsP3 interactor NAP1L1, which has colocation with nsP3 in stress granules (Dominguez et al., 2021) and was identified as nsP3 binder in our mass spectrometry. We found that NAP1L1 only interacts with nsP3 but not capsid or nsP1 (Fig. 5A). Surprisingly, we uncovered the physical connections between capsid and stress granules proteins such as G3BP1 (Fig. 5A). We further identified residues 1–105 aa of capsid was critical for association with VCP and G3BP1 (Fig. 5B). N-terminus (residues 1–105 aa) of capsid is a disordered region. Disordered region of nsP3 was previously found to interact with G3BPs (Meshram et al., 2018). Thus, the disordered region of both capsid and nsP3 could interact with G3BPs and the disordered region of capsid is sufficient for interacting with VCP. To investigate the specificity of VCP antibody for immunofluorescence experiments, we applied an siRNA against VCP to knockdown VCP. As shown in Fig. 5C, VCP siRNA reduced the intensity of VCP measured by VCP antibody, confirming the specificity of VCP antibody. We found VCP localized with capsid in the cytoplasm (Fig. 5D). Moreover, the recombinant GFP-capsid with 1–105 aa showed the similar location in cytoplasm and co-localized with VCP (Fig. 5D). However, the recombinant GFP-capsid with 106–261 aa mainly located in the nucleus (Fig. 5D). Overall, our results indicate that VCP interacts with capsid in the cytoplasm.

3.6. VCP is a proviral host factor

We measured the level of VCP protein upon CHIKV infection in Huh7.5.1 cells and found that CHIKV infection didn't change the protein level of VCP (Fig. 6A). To test the effect of lowered VCP levels on CHIKV infection, we decreased VCP expression prior to CHIKV infection by transfecting Huh7.5.1 cells with either nontargeting control siRNA or VCP-specific siRNAs. We then infected the transfected cells with CHIKV and measured viral nsP1 protein levels, as well as viral RNA levels. VCP knockdown was successful in siRNA-treated cells, as confirmed by Western blot analysis (Fig. 6B). Cell viabilities were not changed in Huh7.5.1 cells by VCP siRNAs (Fig. 6B). The depletion of VCP in Huh7.5.1 cells prior to infection resulted in a significant decrease in both CHIKV RNA levels (Fig. 3B) and viral protein levels (Fig. 6B), indicating that VCP is important in CHIKV life cycle. We also measured the CHIKV production in cells that were transfected with siRNA against VCP. As shown in Fig. 6C, infectious virus particle production was reduced with >1 log 10 fold in VCP knocking down cells when compared to the control siRNA. Cell viabilities were not changed in HeLa cells by VCP siRNAs (Fig. 6C). This result is corresponding to decreased CHIKV RNA level (Fig. 3B) and nsP1 protein level in these cells (Fig. 6B). Consistent with our qPCR results (Fig. 3) and a previous study (Dominguez et al., 2021), silencing G3BP2 but not G3BP1 reduced CHIKV nsP1 protein expression (Fig. 6D). A recent study also identified VCP as a proviral host factor for CHIKV (Carissimo et al., 2019). To investigate whether VCP played a functional role in CHIKV infected cells, we used small inhibitors DBeQ and NMS-873 to block VCP activity. Both pre-treatment and post-treatment of inhibitors suppressed CHIKV replication in dose-dependent manners without changing the cell viabilities (Fig. 6E and F), further confirming the critical role for VCP in CHIKV replication.

4. Discussion

In this study, we present a systematic analysis of CHIKV-host PPIs. By using a complementary and integrative physical (AP-MS) and gene perturbation (RNAi) approach, a comprehensive CHIKV-human PPI network for all CHIKV proteins was generated. This platform revealed several new aspects about CHIKV replication and pathogenesis. Our data with VCP suggest that inhibition of VCP is a unique approach to impair virus replication. Additional detailed characterization of other PPIs revealed by our study is expected to reveal even more insights into CHIKV biology and to identify more potential targets for anti-CHIKV drug development.

We analyzed our results with other alphavirus-host interactomes to find pathways targeted by multiple viruses and those unique to CHIKV. According to our current study and the SFV-host interactome study (Contu et al., 2021), 37 host proteins have been identified as interactors for both SFV and CHIKV. These include the RNA binding proteins and stress granule proteins. Consistent with our study from CHIKV, both SFV capsid and SFV nsP3 interact with stress granule proteins, such as G3BP1 and G3BP2. Hypervariable domain (HVD) of CHIKV nsP3

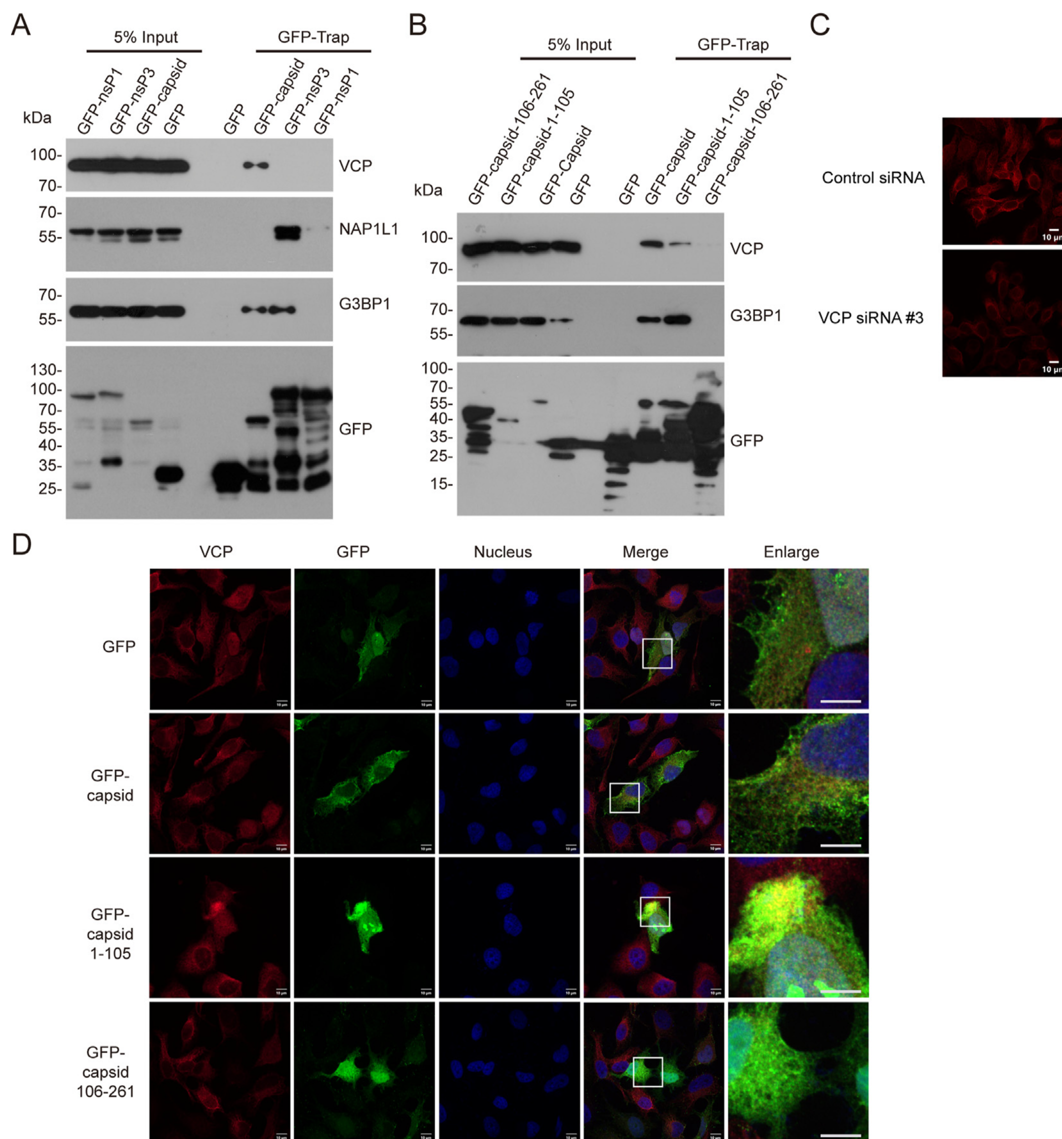

Fig. 5. Validation of the interactions of VCP-capsid, NAP1L1-nsP3, G3BP1-capsid and G3BP1-nsP3. **A** 293T cells in 100 mm plate were transfected with 3 μg GFP-capsid, GFP-nsP3, GFP-nsP1 or GFP empty vector for 48 h. GFP-Trap analysis was performed with GFP-Trap_A beads followed by Western blot. **B** N-terminal of capsid interacts with VCP and G3BP1. 293T cells in 100 mm plate were transfected with 3 μg GFP-capsid, GFP-capsid-1–105, GFP-capsid-106–261 or GFP-C1 empty vector for 48 h. Cell lysates were incubated with GFP-Trap_A beads, and co-IP proteins were subjected to Western blotting for analysis. **C** Validation of VCP antibody. HeLa cells treated with 20 μmol/L VCP siRNA#3 or control siRNA for 72 h were immunofluorescently labeled for VCP. **D** VCP co-localizes with capsid. HeLa cells in 6-well plate were transfected with 1 μg GFP-capsid, GFP-capsid-1–105, GFP-capsid-106–261 or GFP-C1 empty vector for 48 h. Cells fixed and immunofluorescently labeled for VCP. Scale bar, 10 μm.

protein is responsible for its interaction with G3BP proteins and these interactions are requirements for CHIKV replication. The host interactors of CHIKV nsP3 HVD are identified in the system expressing CHIKV nsP3 HVD from Venezuelan equine encephalitis virus (VEEV) replicon as a Flag-GFP-HVD fusion (Meshram et al., 2018). Nine host proteins are determined as the interactors of CHIKV nsP3 HVD including two members of the G3BP family, two members of the NAP1 family, and several members of the SH3 domain-containing protein family, while seven of them crossing three different families are identified in our nsP3-host interactome.

Studies have shown that alphavirus proteins nsP4, capsid and E2 can be degraded through ubiquitination (de Groot et al., 1991; Amaya et al., 2015; Zhang Y. et al., 2022). However, the detailed ubiquitination of alphavirus E1 is unknown. We have experimentally shown that the CHIKV envelope protein E1 can be degraded by ubiquitination in cells and E1 interacts with STUB1. By examining the effects of silencing STUB1 on the expression, protein stability and ubiquitination levels of E1, we further showed that silencing STUB1 induced the accumulation of E1 protein. Moreover, we found silencing STUB1 inhibited CHIKV replication. Protein homeostasis requires the degradation of misfolded proteins by protein quality control, which can deliver misfolded proteins and their aggregates to the ubiquitin-proteasome system (Chen et al., 2011; Ciechanover and Kwon, 2017). CHIKV viral protein quality control is critical to virus replication and treatment of proteasome inhibitor reduced CHIKV titers in CHIKV-infected cells (Kaur et al., 2020). We believe that misfolded E1 could induce endoplasmic reticulum (ER) stress in the infected cells, which may inhibit viral replication. STUB1 is a specialized ubiquitin ligase, which contains a Tetratricopeptide Repeat Domain (TPR domain), a charge domain and U-box domain (Zhang S. et al., 2020). The TRP domain of STUB1 is responsible for chaperone (HSP70 and HSP90) binding while U-box domain has an ubiquitin ligase activity (Ballinger et al., 1999; Jiang et al., 2001; Murata et al., 2001; Zhang M. et al., 2005). Since STUB1 has the dual

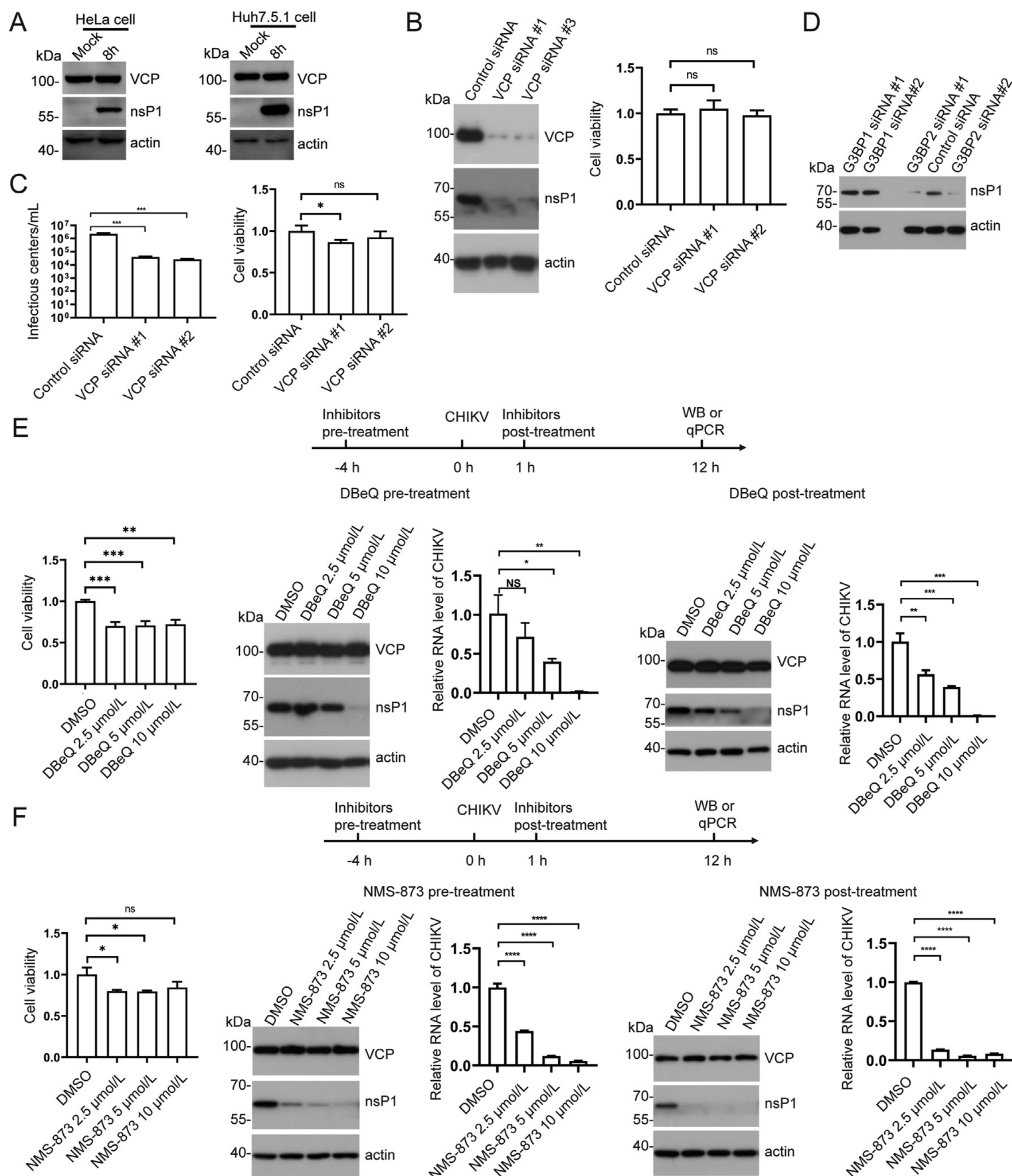

Fig. 6. VCP is required for CHIKV infection. **A** CHIKV infection did not change the protein level of VCP. HeLa or Huh7.5.1 cells were infected with CHIKV (MOI = 1) for 8 h and the lysates were subjected to Western blotting for analysis with indicated antibodies. **B** Huh7.5.1 cells treated with two distinct VCP siRNA or a control siRNA for 72 h were infected with CHIKV for 8 h and analyzed by Western blot for VCP, nsP1 and actin as a loading control. Cell viability was accessed with cellular ATP level. **C** HeLa cells were transfected with control or VCP specific siRNAs for 72 h then infected with CHIKV for 12 h. The culture media were then harvested, clarified by centrifugation. Virus titers were measured by infectious center assays in HeLa cells. Cell viability was accessed with cellular ATP level. **D** Knocking down G3BP2 attenuates CHIKV replication. Huh7.5.1 cells were transfected with siRNA against G3BP1 or G3BP2 for 72 h and then infected with CHIKV for 8 h. Cell lysates were analyzed by Western blot for nsP1 and actin. **E, F** VCP inhibitors impair CHIKV infection. For pre-treatment, Huh7.5.1 cells were pre-treated with different concentrations of DBeQ (**E**) or NMS-873 (**F**) for 4 h then infected with CHIKV at MOI = 1. After 1 h infection at 37 °C, the virus was removed, and the cells were washed by complete medium. For post-treatment, Huh7.5.1 cells were infected with CHIKV at MOI = 1 for 1 h at 37 °C. The cells were washed and incubated in complete medium containing different concentrations of DBeQ (**E**) or NMS-873 (**F**). For both treatments, cell lysates were harvested at 12 h post-infection to measure the protein level of VCP, nsP1 and actin by Western blot. The cellular virus RNA was measured by qPCR. Cell viability was accessed with cellular ATP level. For panels (**B**, **C**, **E** and **F**), data were represented the mean ± standard deviation from three independent experiments. Statistical analysis was performed by an unpaired Student *t*-test. ns, not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

functions of co-chaperone and ubiquitination ligase, it acts as a regulator of the protein control system in cells and involves cell differentiation, apoptosis and tumorigenesis (Rinaldi et al., 2019). In the future, we will examine whether HSP70 or HSP90 binds to E1, and determine the role of HSP70 or HSP90 in STUB1-regulated ubiquitination of E1.

We identified the specific interaction between VCP and CHIKV capsid. And these two proteins were colocalized in the cytoplasm.

Using VCP-specific siRNAs and VCP inhibitors, our results show that VCP is important for CHIKV infection. VCP is a highly conserved AAA+ ATPase that promotes replication of diverse RNA- and DNA-containing viruses (Das and Dudley, 2021). VCP is mainly localized in cell cytoplasm as a soluble protein, while a small fraction is on membranes of Golgi, endoplasmic reticulum, endosomes, mitochondria and nucleus (van den Boom and Meyer, 2018). Apart from the numerous cellular functions, VCP/p97 has been shown to participate in multiple stages of virus infection, including entry and uncoating, intracellular trafficking, nucleic acid replication, and egress (Das and Dudley, 2021). For example, VCP inhibitor blocks pre-replication events of yellow fever virus and shows a critical role of VCP in flavivirus uncoating (Ramanathan et al., 2020). In the case of alphaviruses, VCP also facilitates the entry of Sindbis virus (SINV) by regulating the surface levels of the SINV receptor NRAMP and some specific plasma membrane proteins, such as GLUT1 and intergrin (Panda et al., 2013). VCP has been identified as a proviral host factor that is required for CHIKV RNA replication but not for virus entry (Carissimo et al., 2019). However, 293T cells used in this study lacks of the expression of CHIKV receptor MXRA8, which makes this result less convincing. In our study, we confirmed that the proviral role of VCP in CHIKV RNA replication. We also found that VCP interacted with capsid. In the future, we will investigate the role of VCP in CHIKV uncoating and surface level of CHIKV receptor MXRA8.

5. Conclusions

In conclusion, our study provides a systematic CHIKV-human PPI map and investigates the role of two novel interactors, STUB1 and VCP in CHIKV life cycle. Ubiquitin E3 ligase, STUB1 mediates ubiquitination of CHIKV viral protein E1 at K111 and degrades E1 through the proteasome. Knockdown of STUB1 by siRNA reduces viral protein level, which suggests STUB1 may promote CHIKV replication through degradation of E1. VCP interacts with capsid and co-localizes with capsid in the cytoplasm. Blocking VCP activity by either siRNA or inhibitors decreased viral protein and RNA level, indicating that VCP is a proviral host factor.

Data availability

All data included in this study are available upon request by contact with the corresponding author.

Ethics statement

This study does not contain any studies with human or animal subjects performed by any of the authors.

Author contributions

Peiqi Yin: investigation, methodology, validation, data curation, writing-original draft. Xia Jian: investigation, methodology. Yihan Liu: investigation. Yuwen Liu: writing-reviewing and editing. Lu Lv: validation. Haoran Cui: data curation. Leiliang Zhang: conceptualization, project administration, writing-reviewing and editing.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2023.05.007.

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