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Activities of proteasome and m-calpain are essential for Chikungunya virus replication

Yogesh A. Karpe[1] · **Kunal D. Pingale**[1] · **Gayatri D. Kanade**[1]

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Abstract Replication of many viruses is dependent on the ubiquitin proteasome system. The present study demonstrates that Chikungunya virus replication increases proteasome activity and induces unfolded protein response (UPR) in cultured cells. Further, it was seen that the virus replication was dependent on the activities of proteasomes and m-calpain. Proteasome inhibition induced accumulation of polyubiquitinated proteins and earlier visualization of UPR.

Keywords Alphaviruses · Chikungunya virus · Ubiquitin proteasome system (UPS) · Calpain · m-Calpain

Chikungunya virus (CHIKV) is a medically important mosquito-borne pathogen. It has an approximately 12 kb positive-stranded RNA genome and belongs to the genus *Alphavirus* and family *Togaviridae* [1]. CHIKV is endemic in parts of Africa and many Asian countries [2, 3]. CHIKV outbreaks have been reported to be spread in the European as well as North American continent through CHIKV-infected travelers [4, 5]. In spite of its medical importance, CHIKV has been poorly studied. There are no licensed vaccine and effective therapeutic treatments available for human use against CHIKV [6].

In this study, we investigated the role of ubiquitin proteasome system (UPS) and m-calpain on CHIKV replication. UPS is the major pathway for protein degradation in eukaryotic cells [7]. It is composed of ubiquitin conjugation and substrate degradation machinery. The replication of many positive-stranded RNA viruses is dependent on UPS [8–13]. UPS maintains a proper stoichiometric ratio of viral proteins through its specific degradation. The post-translational modification of viral proteins by UPS with ubiquitin/ubiquitin-like modifications is the major way of regulating protein functions [13, 14]. Previous reports have shown that the function of Sindbis virus (*Alphavirus*) polymerase is regulated by ubiquitin-dependent proteolysis by selective degradation in the early stages of the life cycle [15]. Sindbis virus polymerase is also reported to be short-lived in the reticulocyte lysate system due to the ubiquitin-dependent proteolysis [16, 17]. Previously, it was indicated that the alphavirus nonstructural protein-2 directs RNA polymerase subunit Rpb1 to the ubiquitin-dependent degradation pathway. Rpb1 degradation induces the transcriptional shut off and subsequently inhibits the cellular antiviral response [18].

Calpains are a family of ubiquitously expressed, calcium-dependent non lysosomal cysteine proteases. The exact role of calpain in cells is unknown; however, they may be involved in the regulation of vesicular trafficking and cytoskeletal remodeling [19–22]. μ-calpain is localized in cytosolic fractions, whereas m-calpain is known to associate with cellular membranes and subcellular organelles [23–25]. Replication of Echovirus and SARS coronavirus has been previously shown to be dependent on the activity of m-calpain [19–25].

To study the importance of UPS in the life cycle of CHIKV, proteasomal activity was monitored after the virus infection. For all experiments, CHIKV strain No.-065173 isolated during the 2006 outbreak in India [Genbank-EF027134.1] was used. The experiments were carried out using Vero-E6 (ATCC) and GripTite-293 MSR cell lines.

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✉ Yogesh A. Karpe
yakarpe@aripune.org

[1] Nanobioscience Group, Agharkar Research Institute, G. G. Agarkar Road, Pune 411004, India

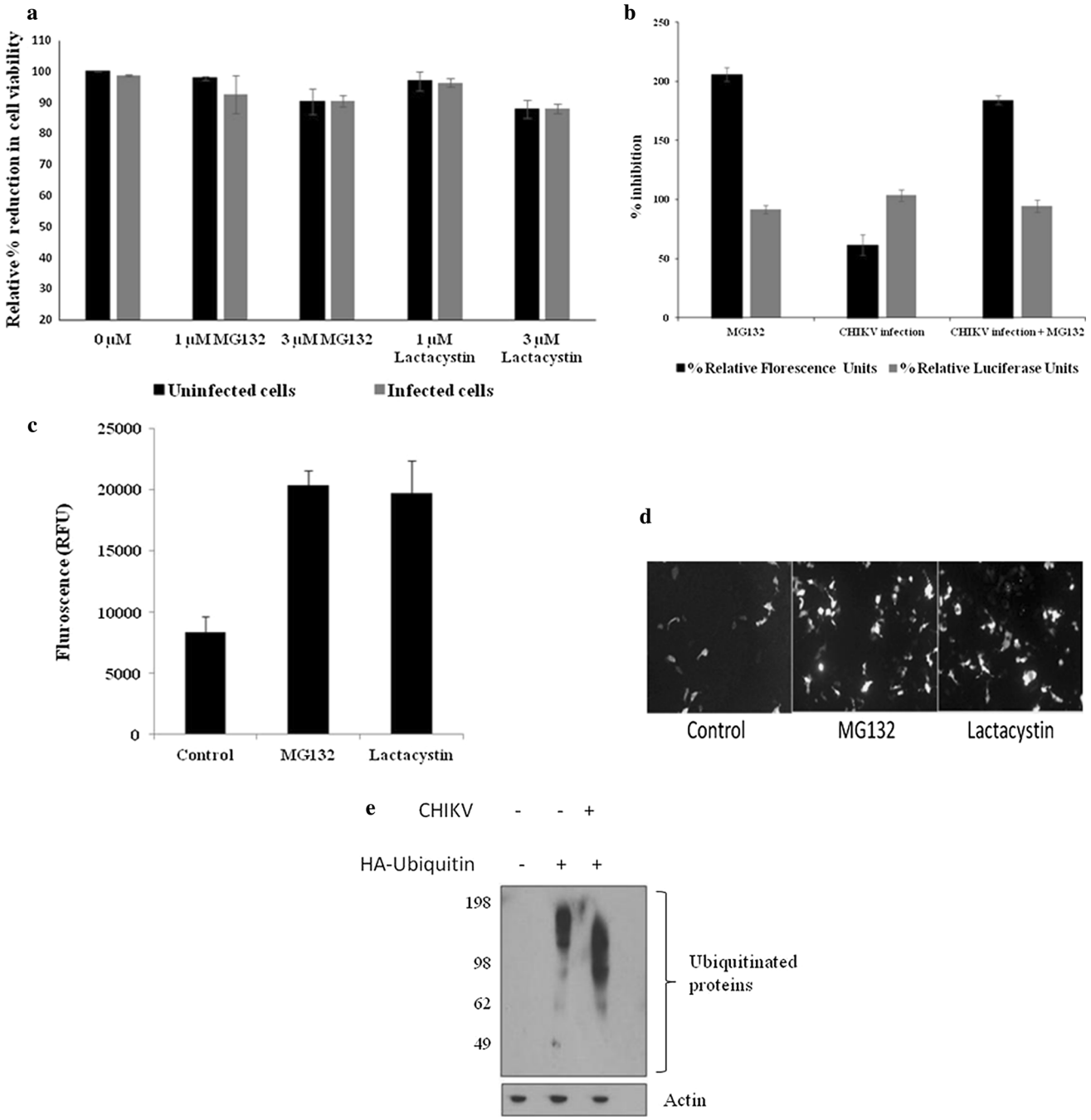

Fig. 1 Activity of proteasomes during the course of CHIKV infection to cells. **a** GripTite-293 MSR cells were infected with CHIKV (1-MOI) for 1 h and treated with inhibitors for 12 h (0–12 hpi). The results shown are from an alamarBlue assay for cytotoxicity. The assay was performed at 12 hpi. Mean values from three independent experiments are plotted. **b** Plasmid pZsProSensor-1 and pGL-4.1 (Luciferase vector) were co-transfected into GripTite-293 MSR cells in 6-well plate and the cells were infected with CHIKV (1-MOI) 24-h post-transfection. Degradation of ZsGreen-MODC-d410 protein was monitored by fluorometer at 12 hpi. The values of Relative Fluorescence Unit (RFU) and Relative Luciferase Unit (RLU) from two independent experiments are plotted. All the values have been normalized to control cells treated with DMSO. **c** Inhibition of proteasomes by MG132 and lactacystin. Plasmid pZsProSensor-1 was transfected into GripTite-293 MSR cells in 6-well plate and 24-h post-transfection, cells were treated with 1-µM MG132 and lactacystin independently for 12 h. Degradation of ZsGreen-MODC-d410 protein was monitored with fluorometer; Relative Fluorescence Unit (RFU) values from three independent experiments are plotted. **d** Plasmid pZsProSensor-1 was transfected into GripTite-293 MSR cells in 6-well plate and 24-h post-transfection, cells were treated with 1-µM MG132 and lactacystin independently for 12 h. Degradation of ZsGreen-MODC-d410 protein was monitored with microscope at 12-h post-transfection. **e** Plasmid pCDNA-3.1-HA-UbiC was transfected into Vero-E6 Cells and 3-h post-transfection cells were infected CHIKV (1-MOI) for 1 h and the cells were harvested after 6 h. Cell lysates were analyzed by immunoblot with anti-HA tag antibody

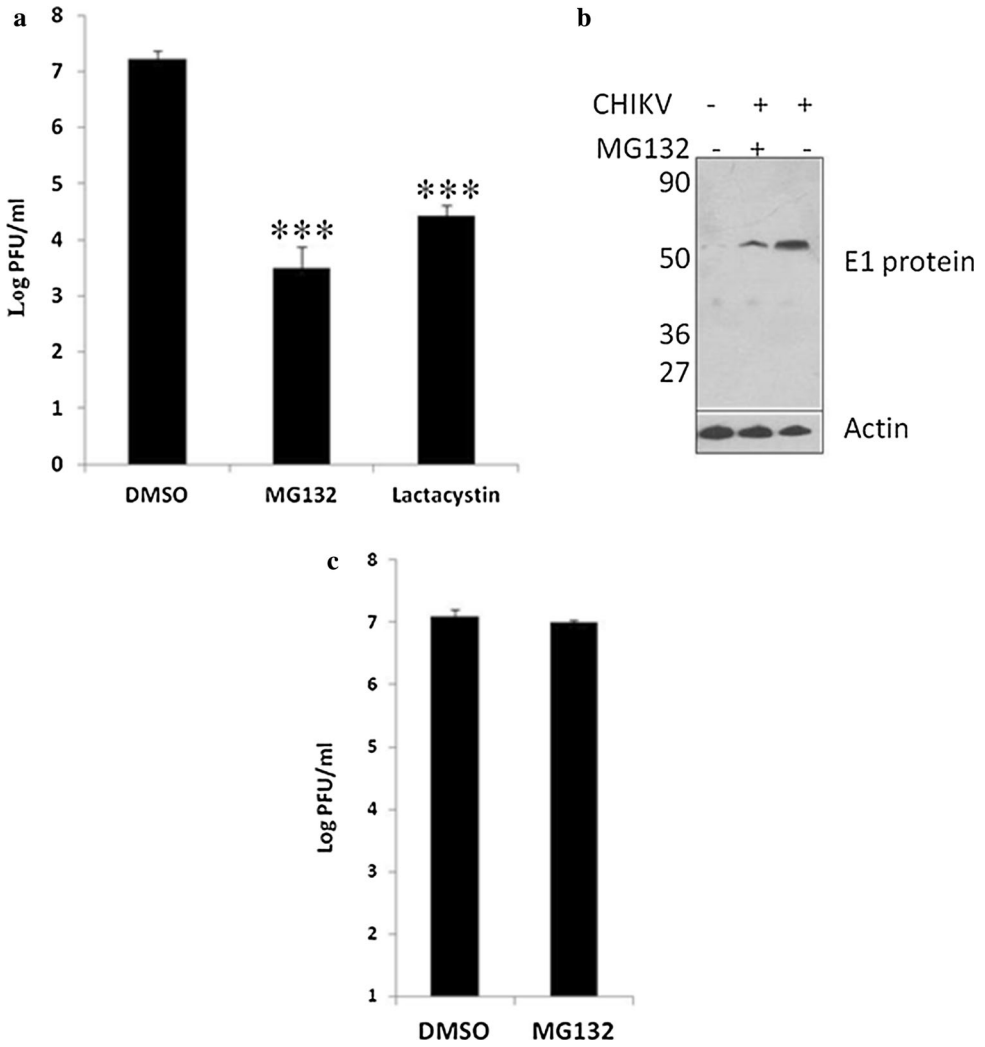

Fig. 2 The proteasome inhibitors significantly reduced the levels of CHIKV replication. **a** Inhibition of CHIKV replication by MG132 and Lactacystin. GripTite-293 MSR cells were infected with CHIKV (1-MOI) for 1 h and treated with proteasome inhibitors 1 μM-MG132 and 1 μM-Lactacystin independently for 12 h (0–12 hpi). At 72 hpi, cell supernatant was analyzed by plaque assay to determine virus titer. Mean values from two independent experiments, performed in duplicates, are plotted. **b** Inhibition of viral protein translation by inhibition of proteasome. GripTite-293 MSR cells were infected with CHIKV (1-MOI) for 1 h and treated with proteasome inhibitors 1 μM-MG132 for 12 h (0–12 hpi). At 24 hpi, cells were harvested and subjected to immunoblot analysis with polyclonal antibodies of mice raised against E1-protein. *Lane 1* uninfected cells, *Lane 2* infected cells with MG132 treatment, *Lane 3* infected cells without MG132 treatment. Actin was used as a loading control. **c** MG132 does not inhibit virus internalization. GripTite-293 MSR cells were treated with 2.5-μM MG132 (1 h before infection to 1 hpi). Cells were infected with CHIKV (1-MOI) for 1 h. After 72 hpi, cell supernatant was analyzed by plaque assay to determine the virus titer. Mean values from two independent experiments, performed in duplicates, are plotted

Proteasome sensor vector (pZsProSensor-1) (Clontech) was used to monitor proteasomal activity in cells. In vector pZsProSensor-1, the mouse ornithine decarboxylase degradation domain (MODC-d410) was fused with the C-terminus of the ZsGreen. The amount of ZsGreen-MODC-d410 protein in infected cells was the indicator of proteasomal activity and was monitored using fluorometer and microscope.

The inhibition of proteasomes by proteasome inhibitor MG132 and lactacystin was monitored in cells transfected with vector pZsProSensor-1. Both the inhibitors effectively repressed the proteasomal activity (Fig. 1b–d). The inhibitors used in this study were not cytotoxic at the applied concentrations as checked with the alamarBlue assay (Invitrogen) (Fig. 1a).

Plasmid pZsProSensor-1 vector was transfected into GripTite-293 MSR cells. The 24-h post-transfected cells were infected with CHIKV [1-MOI], and the activity of ZsGreen was monitored. The increase in proteasomal activity was observed at 12 hpi (hours post infection)

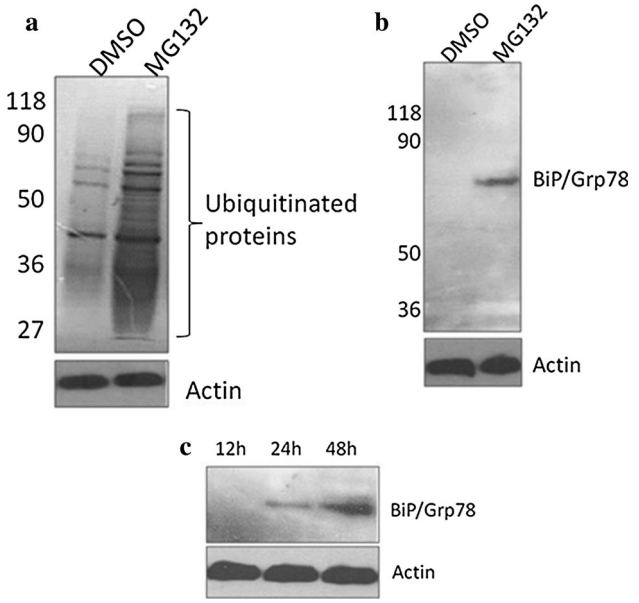

Fig. 3 Accumulation of polyubiquitinated proteins and induction of UPR may be responsible for inhibition of CHIKV replication. **a** GripTite-293 MSR cells were infected with CHIKV (1-MOI) for 1 h and treated with proteasome inhibitors 1-μM MG132 for 12 h (0–12 hpi). At 12 hpi, cells were harvested and subjected to immunoblot analysis with Rabbit anti-ubiquitin antibody. Actin was used as loading control. **b** GripTite-293 MSR cells were infected with CHIKV (1- MOI) for 1 h and treated with proteasome inhibitors 1 μM-MG132 for 12 h (0–12 hpi). At 12 hpi, cells were harvested and subjected to immunoblot analysis with Rabbit anti-BiP/Grp78 antibody. Actin was used as a loading control. **c** GripTite-293 MSR cells were infected with CHIKV (1-MOI) for 1 h. Cells were harvested at indicated time points and subjected to immunoblot analysis with Rabbit anti-BiP/Grp78 antibody. Actin was used as a loading control

compared to the uninfected control cells, suggesting that the proteasomal activity was elevated after the CHIKV infection. This experiment was performed to study the activity of proteasome system in context of CHIKV replication. Irrespective of whether protein degradation is ubiquitin-dependent or ubiquitin-independent, proteasomes are the central component of protein degradation in eukaryotic cells. Further to study the effect of virus replication on overall cellular ubiquitination, ubiquitin-C gene was amplified from human embryonic kidney cells and cloned in vector pCDNA3.1. The following primers were used for the same: UbC_F_ EcoRI:5′-GTTAGAATTCATGGCATATCCGTATGACGTGCCCGA CTATGCCATGCAGATCTTCGTGAAGACT-3′ and UbC_R_BamHI: 5′-CTCGGAT CCTCACCCACCTCTGAGACGGAGTAC-3′. These primers were designed to arrange fusion of hemagglutinin tag to C-terminus of ubiquitin-C protein. This construct was designated as pCDNA-3.1-HA-UbiC. Construct pCDNA-3.1-HA-UbiC was transfected into Vero-E6 Cells and 3-h post-transfection cells were infected with CHIKV (1-MOI) for 1 h and the cells were harvested after 6 hpi. Cell lysates were analyzed by immunoblot to monitor the ubiquitinated proteins with anti-HA tag antibody (Sigma). We observed increase in protein ubiquitination in the virus-infected cells when compared to the control cells (Fig. 1e).

To study the role of UPS in CHIKV life cycle, GripTite-293 MSR cells were infected with CHIKV (1-MOI) for 1 h and treated with 1-μM proteasome inhibitors MG132/lactacystin (Sigma) for 12 h (0–12 hpi). After 72 hpi, virus titers were determined by plaque assay and cell lysates were analyzed by immunoblot to monitor the expression of CHIKV E1-protein. Both the inhibitors were shown to reduce the viral replication; however, inhibition with MG132 was more efficient (Fig. 2a) and concentration-dependent (data not shown). Immunoblot analysis indicated that the inhibition of proteasomes led to reduced expression of CHIKV E1-protein (Fig. 2b). Pretreatment of cells with 2.5-μM MG132 (1 h before infection to 1 hpi) did not inhibit virus replication, which suggested that inhibition of proteasome did not affect CHIKV entry into the cells (Fig. 2c).

Inhibition of proteasome is known to induce accumulation of polyubiquitinated proteins [10]. It also affects protein secretory pathways due to the protein accumulation in the endoplasmic reticulum (ER), leading to ER stress and eventually unfolded protein response (UPR) [26]. To analyze proteasome dysfunction, GripTite-293 MSR cells were treated with MG132 as mentioned above and were harvested at 12 hpi. Immunoblot assay was performed with anti-ubiquitin antibody (Sigma) and anti-BiP/Grp78 antibody (Sigma). BiP/Grp78 is a chaperone protein induced by UPR, and is a marker for proteasome dysfunction. Immunoblot analysis clearly indicated the accumulation of polyubiquitinated proteins and UPR (Fig. 3a, b). Depletion of free ubiquitin in cells may be responsible for the inhibition of CHIKV replication. Accumulation of polyubiquitinated proteins in cells is known to deplete the cellular pool of free ubiquitin [10].

Robust replication of the virus in cultured cells can induce ER stress. Time point analysis of expression of BiP/Grp78 in virus-infected GripTite-293 MSR cells (without proteasome inhibition) was performed by the immunoblot technique. BiP/Grp78 started expressing at 24 hpi but not at 12 hpi (Fig. 3c). However, in the presence of a proteasome inhibitor, UPR was induced at 12 hpi (Fig. 3b). It can be speculated that infected culture cells showed early induction of UPR due to inhibition of proteasome.

There has been a previous report on the inhibition of SARS coronavirus replication by MG132 as a result of proteasome-independent inhibition of m-calpain. MG132 can impair the activity of m-calpain [19]. The possible role of calpain in CHIKV replication was investigated by studying CHIKV replication in the presence of calpain inhibitors MDL28170 (Calpain inhibitor III) (Sigma) and

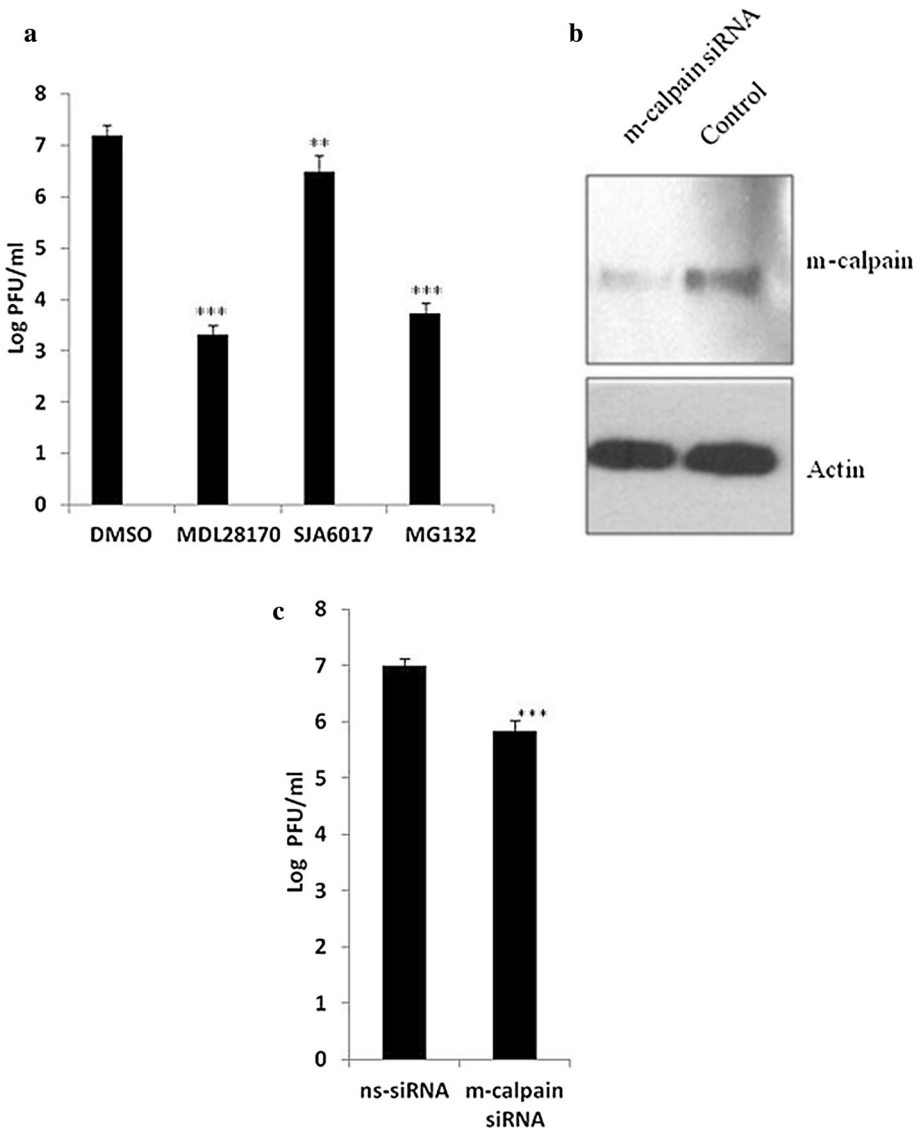

Fig. 4 m-Calpain activity is essential for efficient CHIKV replication. **a** Inhibition of CHIKV replication by MDL28170. GripTite-293 MSR cells were infected with CHIKV (1-MOI) for 1 h and treated with 1 μM-MDL28170, 1 μM-SJA6017, 1 μM-MG132 independently for 12 h (0–12 hpi). At 72 hpi, cell supernatant was analyzed by plaque assay to determine the virus titer. Mean values from two independent experiments, performed in duplicates, are plotted. **b** GripTite-293 MSR cells were transfected with 250 pmol siRNA against m-calpain, in 25-cM^2 tissue culture flask. 48-h post-transfection cells were harvested and subjected to immunoblot analysis with anti-m-calpain antibody. Actin was used as a loading control. **c** GripTite-293 MSR cells were transfected with siRNA against m-calpain. After 48-h post-transfection, cells were infected with CHIKV (1-MOI) for 1 h. After 72 hpi, cell supernatant was analyzed by plaque assay to determine virus titer. Mean values from two independent experiments, performed in duplicates, are plotted

SJA6017 (Calpain inhibitor VI) (Merck). GripTite 293-MSR cells were infected with CHIKV (1-MOI) for 1 h and treated with 1-μM Calpain inhibitor III and VI independently for 12 h (0–12 hpi). After 72 hpi, the cell supernatant was analyzed by plaque assay to monitor virus replication. Only calpain inhibitor III was found to inhibit CHIKV replication significantly (3–4 log reduction in virus titer) (Fig. 4a). Calpain inhibitor IV targets μ-calpain, whereas calpain inhibitor III is known to inhibit both m-calpain and μ-calpain [19]. This suggested that CHIKV replication may require active m-calpain. Small molecule inhibitors can have nonspecific or off-target effects. Thus, to rule out nonspecific effects of MDL28170 and to confirm the role of m-calpain in virus replication, siRNA-mediated knockdown experiment was performed. GripTite-293 MSR cells were transfected with siRNA to knock down m-calpain expression (Silencer Validated siRNA, Ambion). Virus titers were determined by plaque assay, and immunoblot analysis was performed using Rabbit anti-calpain antibody (Cell signaling) to confirm knockdown of m-calpain. Knockdown of synthesis of m-calpain by siRNA-reduced virus replication (Fig. 4b, c), suggesting the important role of m-calpain during CHIKV replication. To investigate whether activity of calpain increases as a consequence of viral replication, total calpain enzyme activity was checked in Vero-E6 cells using calpain-glo protease assay kit (Promega). Cells infected with virus did not show significant increase in the activity of calpain when compared to the noninfected control cells (data not shown).

MG132 has been known to inhibit activities of both proteasome and calpain, whereas proteasome inhibitor lactacystin did not inhibit calpain. SARS coronavirus replication was shown to be significantly inhibited by only

MG132 and not by lactacystin [20], while the inhibition of CHIKV replication was carried out by both MG132 and lactacystin. This suggested that CHIKV replication is dependent on the proteasomal activity. Further, MG132 efficiently inhibited CHIKV replication than lactacystin, probably due to its synergistic effect on proteasome and m-calpain.

Echovirus (*Picornaviridae*) utilizes cellular membranes for the formation of replication complex (RC), which is dependent on the m-calpain activity [25]. SARS coronavirus replication is also found to be dependent on the activity of m-calpain [19]. Replication of many positive-stranded RNA viruses requires the formation of RC in cellular membranes/subcellular organelles. Thus, it is possible that m-calpain may be essential for the early events in the life cycle of positive-stranded RNA viruses.

In summary, the results of this study suggest that CHIKV replication increases proteasomal activity in cells, and virus replication is dependent on the activity of proteasomes and m-calpain. Inhibition of proteasomes leads to UPR and accumulation of polyubiquitinated proteins. m-calpain may be essential for the formation of RC of CHIKV. Both proteasome system and m-calpain could be potential therapeutic targets against CHIKV infections.

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Author's Contribution YK: conceived of the study, carried out the experiments, and drafted the manuscript. KP, GK carried out the experiments and wrote the manuscript. All authors read, revised, and approved the final manuscript.

Compliance with ethical standards

Conflicts of interest This work is funded by INSPIRE Faculty research grant of Dept. of Science and Technology, Govt. of India. The authors have declared that they have no conflict of interest. This article does not contain any studies with human participants or animals performed by any of the authors. The authors declare that they have no competing interests.

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