Fatty Acid Synthase Promotes the Palmitoylation of Chikungunya Virus nsP1

Na Zhang,[a] Hongjian Zhao,[a] (ID) Leiliang Zhang[a,b]

[a]MOH Key Laboratory of Systems Biology of Pathogens, Institute of Pathogen Biology, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, China
[b]Key Laboratory of Medicine and Biotechnology of Qingdao, Department of Microbiology, Medical College of Qingdao University, Qingdao, China

ABSTRACT Chikungunya virus (CHIKV) is transmitted to people by mosquitoes, and CHIKV infection causes fever and joint pain. Fatty acid synthase (FASN) has been identified as a proviral factor for CHIKV. How FASN participates in CHIKV replication remains to be elucidated. In this study, we demonstrated that palmitic acid (PA) can restore the suppression of CHIKV replication by FASN inhibitors. The palmitoylation and plasma membrane localization of CHIKV nsP1 were reduced by FASN inhibitors. Triple mutation of Cys417, Cys418, and Cys419 in nsP1 blocked its palmitoylation and severely disrupted CHIKV replication. Furthermore, two zinc finger DHHC domain-containing palmitoyltransferases (ZDHHCs), ZDHHC2 and ZDHHC19, promoted nsP1 palmitoylation and CHIKV replication. Our results not only identified the key enzymes for the palmitoylation of nsP1 but also provided mechanistic insights into the roles of FASN in CHIKV replication.

IMPORTANCE S-palmitoylation is an important form of lipid posttranslational modification, which affects the function of proteins by regulating their transport, stability, and localization. Previous studies have shown that FASN is critical for CHIKV replication; however, the mechanism for this function of FASN remains unknown. The key zinc finger DHHC domain-containing palmitoyltransferases involved in the palmitoylation of nsP1 are not clear. We demonstrated that FASN promoted CHIKV replication through nsP1 palmitoylation. ZDHHC2 and ZDHHC19 were identified as the major enzymes for nsP1 palmitoylation. Since nsP1 proteins are conserved in alphaviruses, our results highlight the mechanisms by which alphavirus nsP1 is palmitoylated.

KEYWORDS CHIKV, FASN, ZDHHC, nsP1, palmitoylation

Chikungunya virus (CHIKV) is transmitted to people by *Aedes aegypti* and *Aedes albopictus* mosquitoes (1). The most common symptoms of CHIKV infection include severe joint pain, fever, headache, muscle pain, and rash (1, 2). CHIKV is responsible for a reemerging epidemic in countries in the Indian Ocean region and has been identified in Europe and the United States. Currently, no vaccine exists to prevent CHIKV infection.

CHIKV is a member of the genus *Alphavirus* in the family *Togaviridae* (3). The genome of CHIKV consists of two open reading frames (ORFs). One ORF encodes a nonstructural polyprotein that is posttranslationally processed to generate four nonstructural proteins (nsP1, nsP2, nsP3, and nsP4) that are involved in genome replication. The other ORF encodes a structural polyprotein that is also processed and forms the viral particles, which are composed of the capsid, E3, E2, 6K, and E1 proteins (3).

CHIKV genomic viral RNA and the 26S subgenomic RNA require a 5′ cap structure to direct efficient translation of the viral polyprotein and prevent degradation of the viral RNA genome by host exonucleases. Capping of the CHIKV RNA is orchestrated by nsP1 protein, which binds GTP and confers the N-7 methyltransferase and guanylyltransferase activities that are necessary for cap formation in a noncanonical manner (4).

Addition of palmitic acid (PA), a 16-carbon saturated fatty acid, to the thiol group of a cycteine residue of substrate is defined as S-palmitoylation, which was generalized as "palmitoylation" (5). Palmitoylation is catalyzed by the zinc finger Asp-His-His-Cys (DHHC) domain-containing (ZDHHC) palmitoyl acyltransferase (PAT) family (6). ZDHHC proteins were paired with their specific substrates and were closely involved in the substrates' function. As a posttranslational lipid modification, palmitoylation increases protein hydrophobicity and facilitates protein trafficking to cellular membranes. The RNA capping enzyme nsP1 from Semliki Forest virus (SFV) and Sindbis virus (SINV) anchors to the cellular membrane through an amphipathic peptide and a palmitoylated cysteine in nsP1 (7, 8). Mutations that prevent nsP1 palmitoylation reduce the replication of SFV and SINV (9). Whether CHIKV nsP1 is also palmitoylated is unknown. Moreover, which ZDHHC enzymes contribute nsP1 palmitoylation is not clear.

Fatty acid synthase (FASN) catalyzes the *de novo* synthesis of palmitic acid, a fatty acid that is utilized for the posttranslational palmitoylation of host and viral proteins. FASN has been demonstrated to be critical for the replication of many viruses (2, 8, 10, 11). How FASN participates in CHIKV replication remains to be elucidated.

In this study, we found that FASN inhibitor reduced the palmitoylation of nsP1. ZDHHC2 and ZDHHC19 associated with nsP1 and mediated its palmitoylation. Silencing of ZDHHC2 and ZDHHC19 reduced CHIKV replication. Taken together, our findings identified the key enzymes for the palmitoylation of nsP1 and revealed the mechanism by which FASN contributes to CHIKV replication.

RESULTS

FASN inhibitors reduced CHIKV replication. To determine whether the inhibition of FASN enzyme activity disrupted CHIKV replication, HeLa cells were treated with the compounds C75 and cerulenin, two known FASN inhibitors (12), and then infected with CHIKV strain 181/25. Consistent with the results from a previous study (13), C75 inhibited CHIKV replication, as assessed by the protein level of CHIKV nsP1 and the CHIKV RNA level (Fig. 1A and B). C75 did not reduce the cell viability as measured by the intracellular ATP level (Fig. 1C). Similarly, cerulenin reduced the CHIKV viral nsP1 protein expression and viral RNA level (Fig. 1D and E) without reducing the cell viability (Fig. 1F).

Palmitic acid restored the suppression of CHIKV replication caused by FASN inhibitors or a palmitoylation inhibitor. Since FASN inhibitors suppressed CHIKV replication, we hypothesized that FASN might promote CHIKV replication through its product palmitic acid. To test this hypothesis, we treated HeLa cells with palmitic acid (PA) or left them untreated in the presence of a FASN inhibitor and infected the cells with CHIKV strain 181/25. As shown in Fig. 2A and B, palmitic acid rescued the suppression of CHIKV replication by the FASN inhibitors C75 without reducing the cell viability. Similarly, palmitic acid rescued the suppression of CHIKV replication by cerulenin without reducing the cell viability (Fig. 2C and D). The palmitate analog 2-bromopalmitate (2-BP) is commonly used to inhibit palmitoylation (14). We treated the HeLa cells with 2-BP and then infected the cells with CHIKV strain 181/25. The results indicated that 2-BP inhibited CHIKV replication in a dose-dependent manner without reducing the cell viability (Fig. 2E and F). PA partially rescued the suppression of CHIKV replication by 2-BP (Fig. 2G and H). However, stearic acid (SA), a downstream lipid from palmitic acid, failed to rescue the suppression of CHIKV replication by C75 (Fig. 2I and J), cerulenin (Fig. 2K and L), or 2-BP (Fig. 2M and N), excluding the downstream lipid role of palmitic acid complementation. Taken together, these data suggested that palmitic acid could restore the suppression of CHIKV replication by FASN inhibitors and a palmitoylation inhibitor.

Palmitoylation of nsP1 was reduced by a FASN inhibitor. The palmitoylation of nsP1 from other alphaviruses, such as SINV, is critical for viral replication (9); thus, we hypothesized that the palmitoylation of nsP1 from CHIKV is dependent on the synthesis of palmitic acid by FASN. To investigate whether the palmitoylation of endogenous nsP1 existed, Huh7.5.1 cells were infected with CHIKV strain 181/25 (multiplicity of
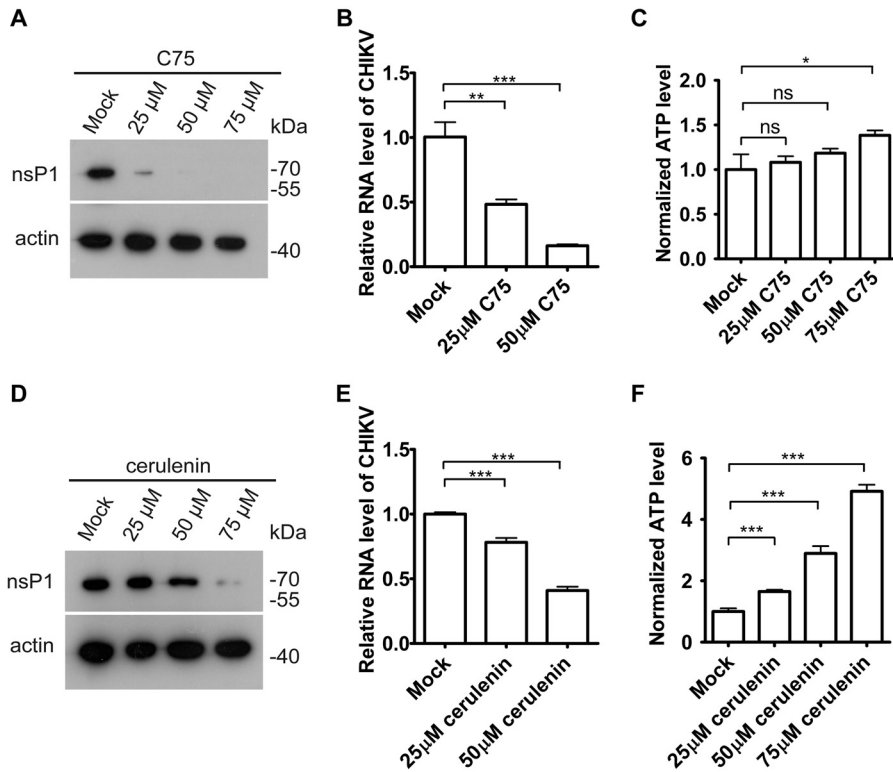
FIG 1 FASN activity was required for CHIKV replication. (A) HeLa cells were treated with difference dosages of C75 for 30 min and then infected with CHIKV strain 181/25 (multiplicity of infection [MOI] = 1) for 8 h. The lysates were immunoblotted for nsP1 and actin. (B) HeLa cells were treated with difference dosages of C75 for 30 min and then infected with CHIKV strain 181/25 (MOI = 1) for 5 h. RNA was assessed by qPCR. (C) HeLa cells were treated with difference dosages of C75 for 30 min and then infected with CHIKV strain 181/25 (MOI = 1) for 5 h. ATP levels were measured by CellTiter-Glo luminescent cell viability assay. (D) HeLa cells were treated with difference dosages of cerulenin for 30 min and then infected with CHIKV strain 181/25 (MOI = 1) for 5 h. The lysates were immunoblotted for nsP1 and actin. (E) HeLa cells were treated with difference dosages of cerulenin for 30 min and then infected with CHIKV strain 181/25 (MOI = 1) for 5 h. RNA was assessed by qPCR. (F) HeLa cells were treated with difference dosages of cerulenin for 30 min and then infected with CHIKV strain 181/25 (MOI = 1) for 5 h. ATP levels were measured by CellTiter-Glo luminescent cell viability assay.

infection [MOI] = 1) for 8 h and the palmitoylation of nsP1 was assayed by acyl-biotin exchange (ABE) assay. The ABE assay contains three biochemical steps: blocking the unmodified cystein thiol groups by *N*-ethylmaleimide (NEM), cleaving and unmasking the palmitoylated cysteine's thiol group by hydroxylamine (HAM), and labeling the palmitoylated cysteine by a thiol-reactive biotinylation reagent, 1-biotinamido-4-[4'-(maleimidomethyl)cyclohexanecarboxamido]butane (biotin-BMCC). CHIKV nsP1 was confirmed to be palmitoylated as measured by ABE assay (Fig. 3A). To investigate whether the inhibition of FASN reduces the palmitoylation of nsP1, 293T cells were transfected with FLAG-nsP1 for 24 h and treated with the FASN inhibitor C75 or subjected to mock treatment for 12 h. As shown in Fig. 3B, the palmitoylation of CHIKV nsP1 was reduced by the FASN inhibitor.

Palmitoylated alphavirus nsP1 is located in the plasma membrane and filopodial extensions, while nonpalmitoylated nsP1 is not observed in filopodial extensions (7). Indeed, immunofluorescence experiments showed that the filopodial localization of nsP1 was reduced by the FASN inhibitor (Fig. 3C). The plasma membrane localization of nsP1 was also reduced by the FASN inhibitor (Fig. 3D). We quantified the blots and found that FASN inhibitor reduced the nsP1 protein level in the total lysate. The nsP1 without proper localization is degraded eventually, leading the reduction of the plasma membrane level of nsP1. Taken together, these data suggested that FASN is important for the palmitoylation and proper localization of nsP1.
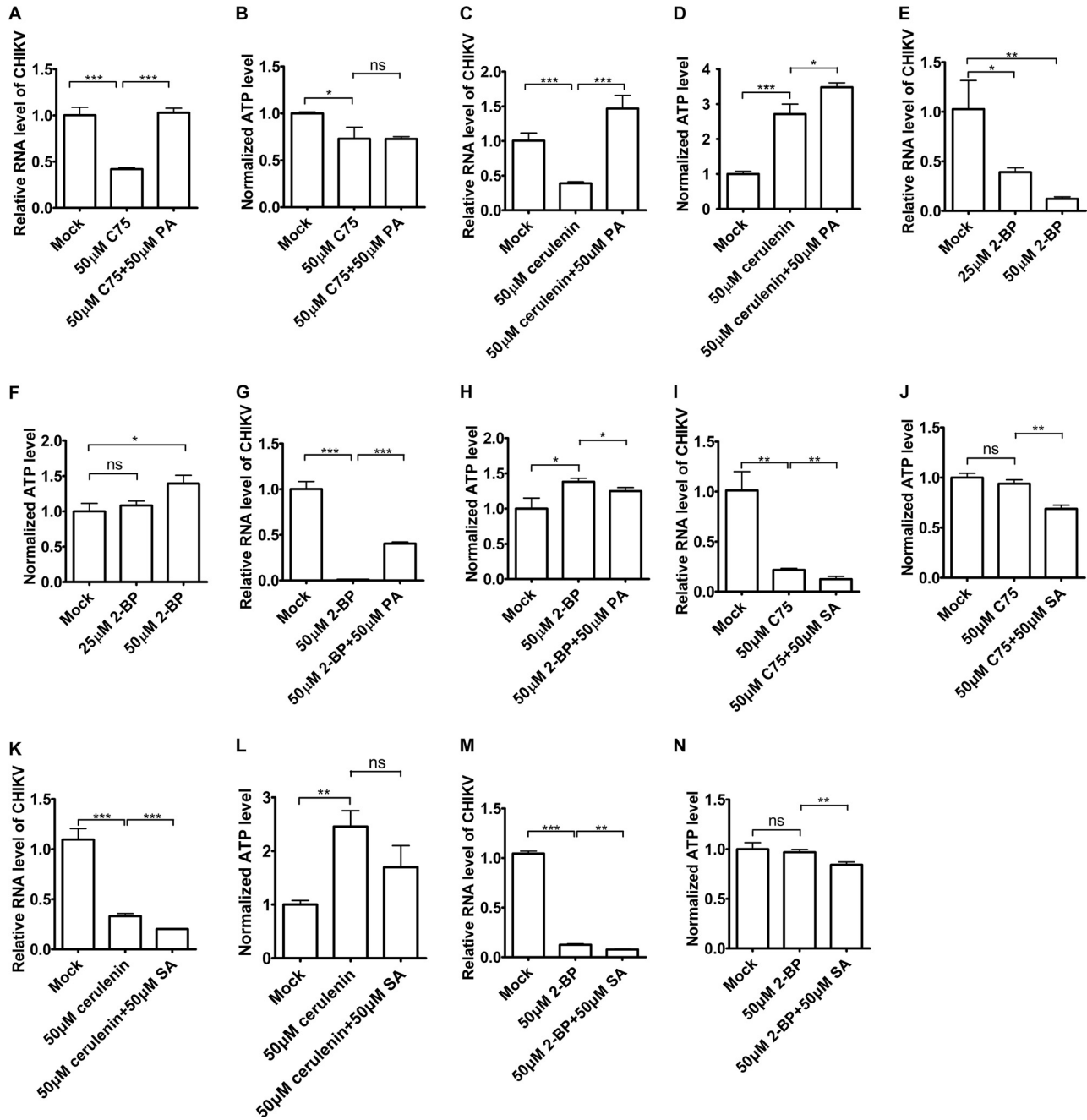
FIG 2 Palmitic acid could rescue the inhibitory effects of 2-BP and FASN inhibitors on CHIKV replication. (A and B) HeLa cells were preinfected with CHIKV vaccine strain 181/25 (MOI = 1) for 1 h and then treated with C75 or C75-palmitic acid for 5 h in the presence of CHIKV. Total RNA was isolated and reverse transcribed, and qPCR was then performed to measure CHIKV RNA levels (A). ATP levels were measured by CellTiter-Glo luminescent cell viability assay (B). (C and D) HeLa cells were preinfected with CHIKV vaccine strain 181/25 (MOI = 1) for 1 h and then treated with cerulenin or cerulenin-palmitic acid for 5 h in the presence of CHIKV. Total RNA was isolated and reverse transcribed, and qPCR was then performed to measure CHIKV RNA levels (C). ATP levels were measured by CellTiter-Glo luminescent cell viability assay (D). (E and F) HeLa cells were preinfected with CHIKV vaccine strain 181/25 (MOI = 1) for 1 h and then treated with 2-BP for 3 h in the presence of CHIKV. Total RNA was isolated and reverse transcribed, and qPCR was then performed to measure CHIKV RNA levels (E). ATP levels were measured by CellTiter-Glo luminescent cell viability assay (F). (G and H) HeLa cells were preinfected with CHIKV vaccine strain 181/25 (MOI = 1) for 1 h and then treated with 2-BP or 2-BP–palmitic acid for 3 h in the presence of CHIKV. Total RNA was isolated and reverse transcribed, and qPCR was then performed to measure CHIKV RNA levels (G). ATP levels were measured by CellTiter-Glo luminescent cell viability assay (H). (I and J) HeLa cells were preinfected with CHIKV vaccine strain 181/25 (MOI = 1) for 1 h and then treated with C75 or C75-SA for 5 h in the presence of CHIKV. Total RNA was isolated and reverse transcribed, and qPCR was then performed to measure CHIKV RNA levels (I). ATP levels were measured by CellTiter-Glo luminescent cell viability assay (J). (K and L) HeLa cells were preinfected with CHIKV vaccine strain 181/25 (MOI = 1) for 1 h and then treated with cerulenin or cerulenin-SA for 5 h in the presence of CHIKV. Total RNA was isolated and reverse transcribed, and qPCR was then performed to measure CHIKV RNA levels (K). ATP levels were measured by CellTiter-Glo luminescent cell viability assay (L). (M and N) HeLa cells were preinfected with CHIKV vaccine strain 181/25 (MOI = 1) for 1 h and then treated with 2-BP or 2-BP–palmitic acid for 3 h in the presence of CHIKV. Total RNA was isolated and reverse transcribed, and qPCR was then performed to measure CHIKV RNA levels (M). ATP levels were measured by CellTiter-Glo luminescent cell viability assay (N).
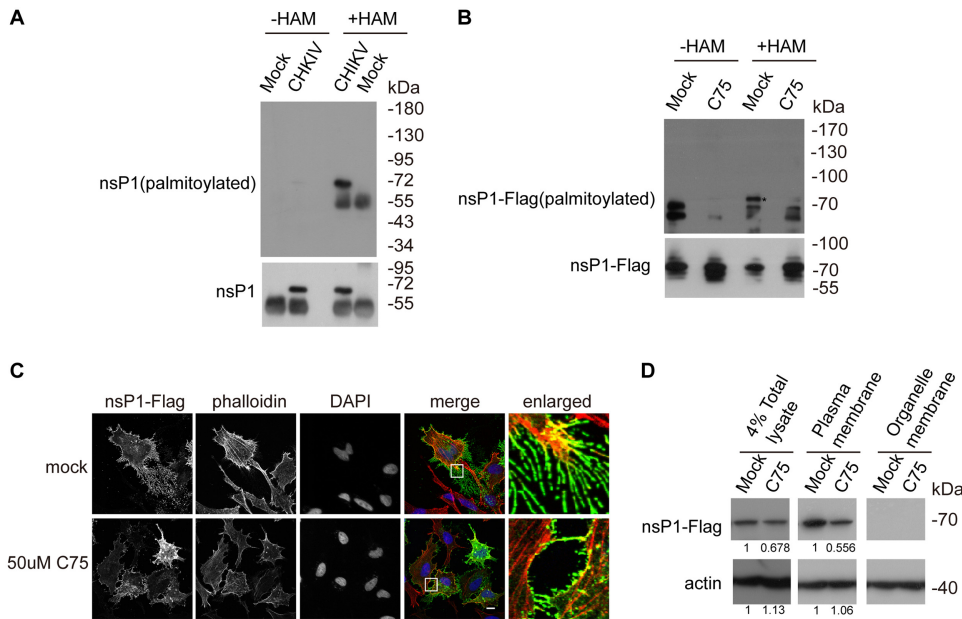
FIG 3 Palmitoylation of nsP1 was reduced by a FASN inhibitor. (A) Huh7.5.1 cells were infected with CHIKV strain 181/25 (MOI = 1) for 8 h. The palmitoylation level of nsP1 was measured by ABE assay, and then cells were blotted with streptavidin-HRP and nsP1 antibody. −HAM, without HAM; +HAM, with HAM. (B) 293T cells were transfected with nsP1-FLAG plasmid overnight and treated with 50 μM C75 for 20 h. The palmitoylation level of nsP1 was measured by ABE assay, and then cells were blotted with streptavidin-HRP and FLAG antibody. The asterisk indicates the palmitoylated nsP1-FLAG. (C) HeLa cells were transfected with nsP1-FLAG plasmid overnight and then treated with 50 μM C75 for 8 h. The cells were fixed and immunolabeled with FLAG antibody (green). Phalloidin marks the actin (red). DAPI marks the nucleus. Scale bar: 10 μm. (D) HeLa cells were transfected with nsP1-FLAG plasmid overnight and then treated with 50 μM C75 for 8 h. The plasma membrane and other fractions of the cells were separated by a plasma membrane protein isolation and cell fractionation kit and then blotted with FLAG and actin antibodies. The blots were quantified by ImageJ.

The cysteines at sites 417 to 419 of CHIKV nsP1 are the key residues for nsP1 palmitoylation. Based on predictions (15), the cysteines at sites 417 and 419 of CHIKV nsP1 were found to be potential key residues for nsP1 palmitoylation (Fig. 4A). We performed homology analysis to compare the sequence of CHIKV nsP1 with those of SFV and SINV and found that C419 is the most conserved amino acid among C417 to C419. We generated C417A, C418A, C419A, and C417/8/9A mutants of nsP1 and found that the cysteines at sites 417 to 419 are the key residues for nsP1 palmitoylation (Fig. 4B). These cysteines are also critical for the plasma membrane targeting of nsP1 (Fig. 4C). The C419A and C417/8/9A mutants of nsP1 showed reduced localization to filopodial extensions (Fig. 4D). Thus, we concluded that the cysteines at sites 417 to 419 of nsP1 are critical for the palmitoylation and proper localization of nsP1.

The cysteines at sites 417 to 419 of CHIKV nsP1 are critical for CHIKV replication. To investigate whether the palmitoylation of nsP1 could affect CHIKV replication, we generated the nsP1 mutant CHIKV-nsP1C/A, which is deficient in the palmitoylation of nsP1, by mutating C417 to C419 to A417 to A419 in the context of CHIKV 181/25 (Fig. 5A). The infectivity of CHIKV-C3A-expressing virus was 4 orders of magnitude lower than that of wild-type (WT) CHIKV, as measured by quantitative PCR (qPCR) (Fig. 5B), and this finding is consistent with the result obtained with the nsP1 palmitoylation-deficient mutant of SFV (9).

ZDHHC2 and ZDHHC19 promote the palmitoylation of nsP1. Protein palmitoylation is mediated mainly by ZDHHCs (16). Next, we screened for ZDHHCs associated with nsP1. 293T cells were transfected with 23 FLAG-tagged ZDHHCs and green fluorescent protein (GFP)-tagged nsP1, and the lysates were incubated with an anti-FLAG antibody to perform immunoprecipitation (IP) experiments. ZDHHC2 and ZDHHC19 were identified as binding partners for nsP1 (Fig. 6A). Interestingly, nsP1C/A
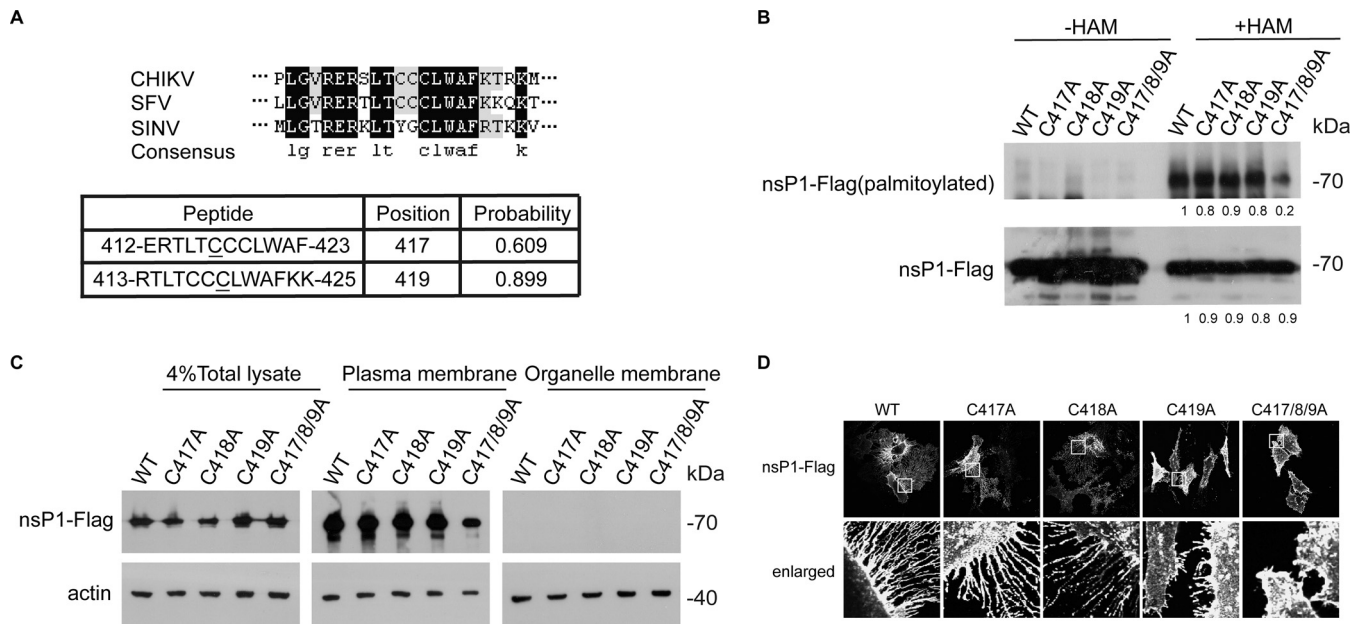
FIG 4 The crucial sites of nsP1 were the three cysteines located at positions 417 to 419. (A) nsP1 from CHIKV nsP1 was compared with those from SFV and SINV. Protein palmitoylation software was used to predict the nsP1 palmitoylation sites. (B) 293T cells were transfected with the WT nsP1-FLAG plasmid and mutant plasmids for 24 h. The palmitoylation level of nsP1 was measured by an ABE assay, and the cells were then immunoblotted with streptavidin-HRP and an anti-FLAG antibody. The blots were quantified by ImageJ. (C) HeLa cells were transfected with a WT nsP1-FLAG plasmid and mutant plasmids for 24 h. Next the plasma membrane and other cell fractions were separated by a plasma membrane protein isolation and cell fractionation kit and then immunoblotted with antibodies against FLAG and actin. (D) HeLa cells were transfected with a WT nsP1-FLAG plasmid and mutant plasmids for 24 h and then immunolabeled with an anti-FLAG antibody. Scale bar: 10 μm.

mutants no longer interacted with ZDHHC2 or ZDHHC19 (Fig. 6B and C), further confirming the specific association of ZDHHC2 and ZDHHC19 with nsP1.

We hypothesized that ZDHHC2 and ZDHHC19 participated in the palmitoylation of nsP1. To test this hypothesis, ZDHHC2 or ZDHHC19 was knocked down in 293T cells by small interfering RNAs (siRNAs) for 48 h, and the cells were then transfected with nsP1-FLAG for 24 h. ABE assays showed that the palmitoylation of nsP1 was reduced after silencing ZDHHC2 (Fig. 6D), ZDHHC19 (Fig. 6E) or ZDHHC2/ZDHHC19 (Fig. 6F). These results suggested key roles for ZDHHC2 and ZDHHC19 in the palmitoylation of nsP1.

ZDHHC2 and ZDHHC19 promote CHIKV replication. To assess whether ZDHHC2 and ZDHHC19 are crucial for CHIKV replication, we knocked down ZDHHC2 and ZDHHC19 with siRNAs. Silencing ZDHHC2 inhibited CHIKV replication, as measured by
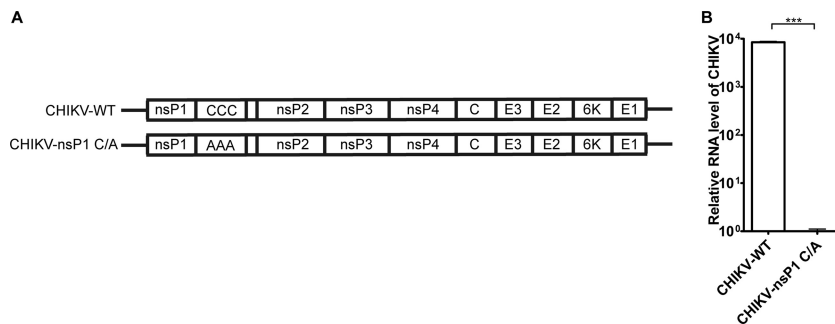
FIG 5 Mutation of nsP1 palmitoylation sites inhibited CHIKV replication. (A) Diagram of CHIKV-WT and CHIKV-nsP1 C/A. (B) Plasmids containing the CHIKV WT genome and the CHIKV-nsP1 C/A genome were linearized by NotI digestion and transcribed to generate viral RNA. In addition, Huh7.5.1 cells were transfected with viral RNA by electroporation, and the cells were cultured in 24-well plates. Sixteen hours later, the intracellular RNA was isolated and reverse transcribed. qPCR was performed to measure CHIKV RNA levels.
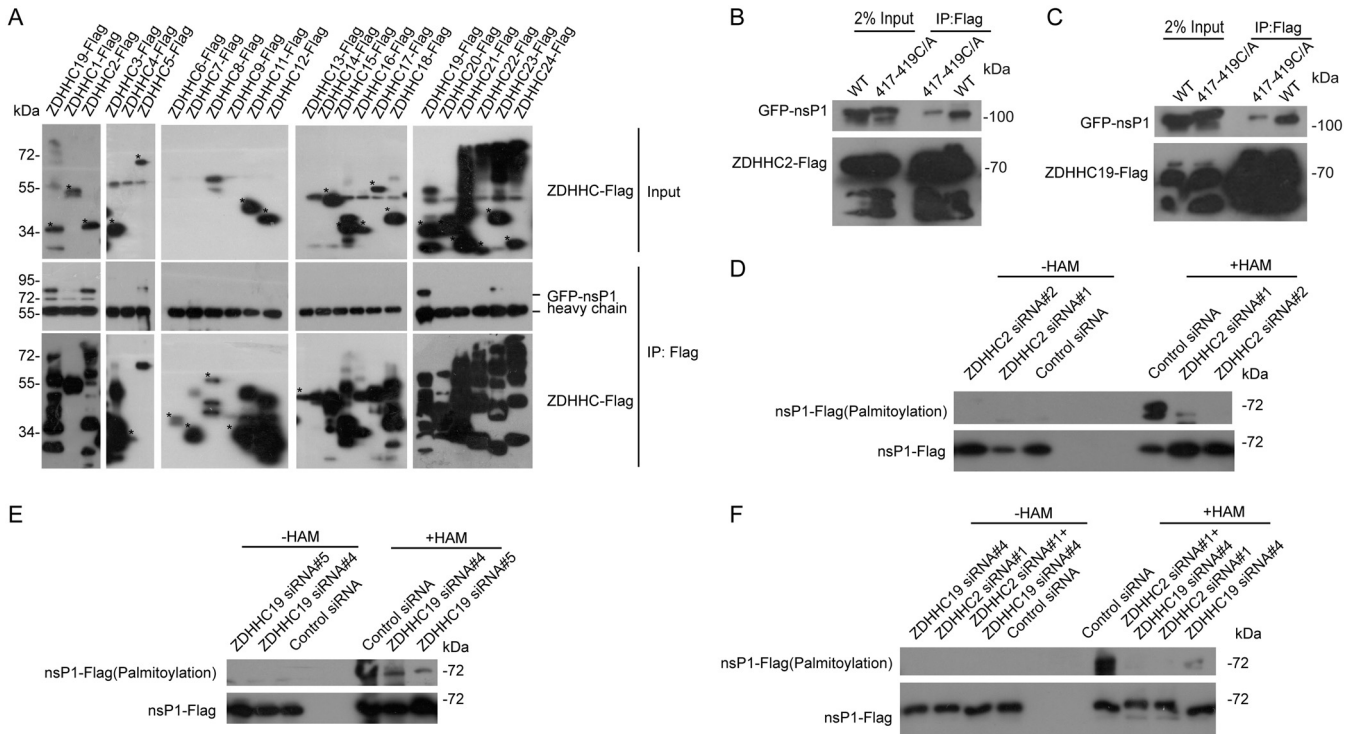
FIG 6 ZDHHC2 and ZDHHC19 interacted with nsP1 and mediate nsP1 palmitoylation. (A) Lysates from 293T cells transfected with 23 ZDHHCs fused to FLAG and GFP-nsP1 of CHIKV were immunoprecipitated with an anti-FLAG antibody and then immunoblotted with antibodies against GFP and FLAG to screen for ZDHHCs that could interact with nsP1. Asterisks indicate the ZDHHCs. (B) Lysates from 293T cells transfected with ZDHHC2-FLAG and GFP-nsP1 or GFP-nsP1417-419C/A were immunoprecipitated with an anti-FLAG antibody and then immunoblotted with antibodies against GFP and FLAG. (C) Lysates from 293T cells transfected with ZDHHC2-FLAG and GFP-nsP1 or GFP-nsP1417-419C/A were immunoprecipitated with an anti-FLAG antibody and then immunoblotted with antibodies against GFP and FLAG. (D) 293T cells were treated with siRNAs targeting ZDHHC2 or a control siRNA for 48 h and then transfected with an nsP1-FLAG plasmid for 24 h. The lysates were collected, and an ABE assay was performed to detect the nsP1 palmitoylation level. (E) 293T cells were treated with siRNAs targeting ZDHHC19 or a control siRNA for 48 h and then transfected with an nsP1-FLAG plasmid for 24 h. The lysates were collected, and an ABE assay was performed to detect the nsP1 palmitoylation level. (F) 293T cells were treated with siRNAs targeting ZDHHC2, ZDHHC19, or ZDHHC2/ZDHHC19 or a control siRNA for 48 h and then transfected with an nsP1-FLAG plasmid for 24 h. The lysates were collected, and an ABE assay was performed to detect the nsP1 palmitoylation level.

the levels of viral RNA and the viral protein nsP1, without reducing the cell viability (Fig. 7A to C). Similarly, knockdown of ZDHHC19 reduced the levels of CHIKV RNA replication and the viral protein nsP1 (Fig. 7D to F). Combination of ZDHHC2 siRNA and ZDHHC19 siRNA treatment also reduced the CHIKV replication (Fig. 7G to I). Taken together, these data indicate that both ZDHHC2 and ZDHHC19 are involved in CHIKV replication.

DISCUSSION

In this study, we demonstrated that FASN contributed to CHIKV replication by producing palmitic acid for the palmitoylation of nsP1 (Fig. 8). Inhibitors of FASN and ZDHHCs reduced the palmitoylation of nsP1 and suppressed CHIKV replication (Fig. 8). The key residues in nsP1 for palmitoylation are C417 to C419. ZDHHC2 and ZDHHC19 interacted with nsP1, promoted nsP1 palmitoylation, and enhanced viral replication.

FASN catalyzes the synthesis of palmitic acid, which can be utilized for the post-translational palmitoylation of proteins, including viral proteins. Alphavirus nsP1 proteins from SFV and SINV were reported to be palmitoylated (9, 17, 18). Our current report raises the possibility that FASN is involved in viral replication through the palmitoylation of CHIKV nsP1. Indeed, inhibition of FASN reduced CHIKV replication, which was consistent with the findings of a previous study (13). Although we established that FASN played a crucial role in CHIKV nsP1 palmitoyaltion, our data did not rule out the possibility that FASN-mediated palmitoylation of other CHIKV proteins and host proteins involved in replication. A previous study found that transframe (TF) protein from SINV was palmitoylated (19). Thus, it is conceivable that inhibition of FASN would also reduce the palmitoylation of CHIKV TF protein.
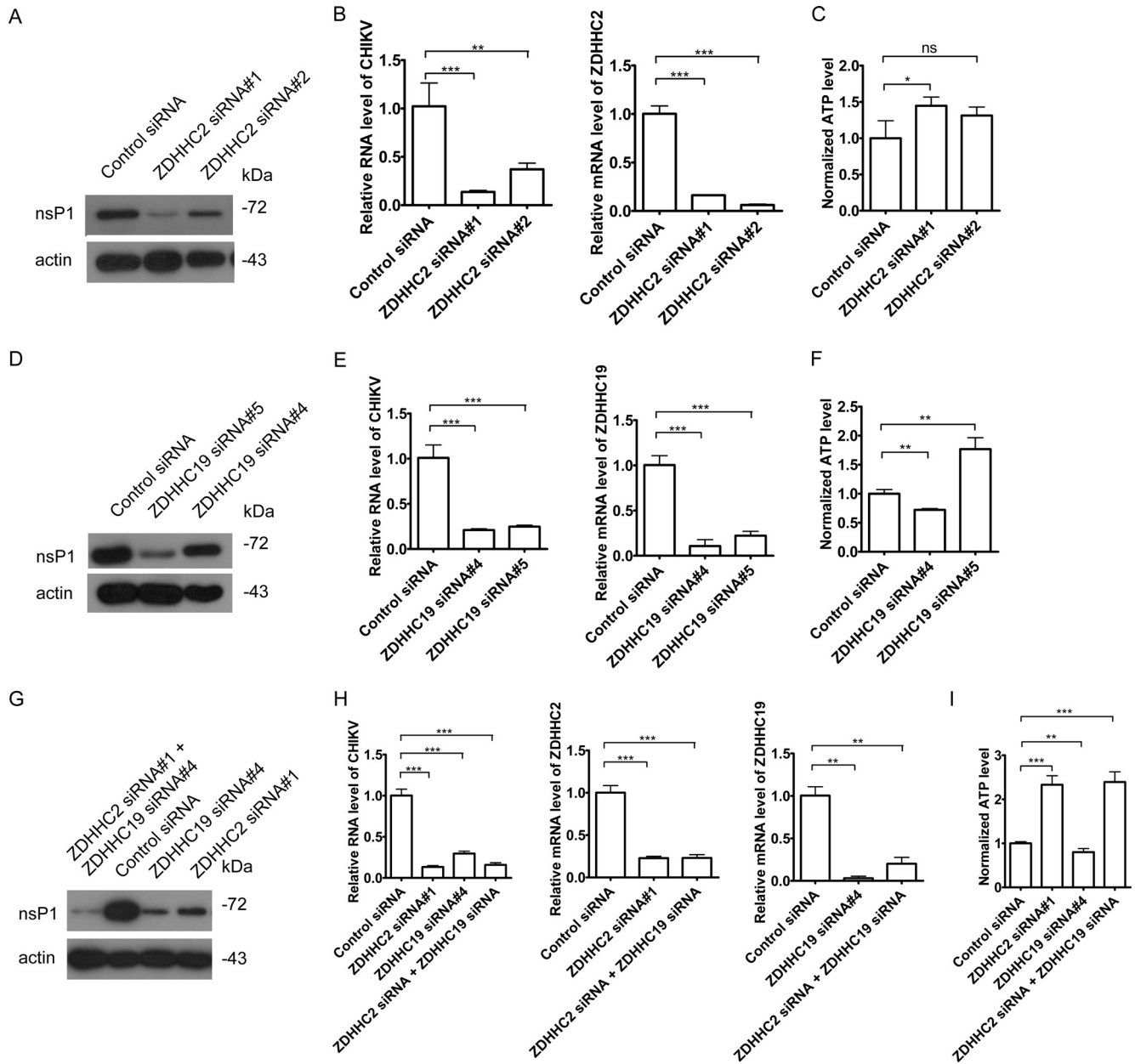
FIG 7 ZDHHC2 and ZDHHC19 were important for CHIKV replication. (A to C) HeLa cells were treated with siRNAs targeting ZDHHC2 or a control siRNA for 60 h and then infected with CHIKV vaccine strain 181/25 (MOI = 1) for 6 h. The samples were analyzed by Western blotting (A). Total RNA was isolated and reverse transcribed. Subsequently, qPCR was performed (B). ATP levels were measured by CellTiter-Glo luminescent cell viability assay (C). (D to F) HeLa cells were treated with siRNAs targeting ZDHHC19 or a control siRNA for 60 h and then infected with CHIKV vaccine strain 181/25 (MOI = 1) for 6 h. The samples were analyzed by Western blotting (D). Total RNA was isolated and reverse transcribed. Subsequently, qPCR was performed (E). ATP levels were measured by CellTiter-Glo luminescent cell viability assay (F). (G to I) HeLa cells were treated with siRNAs targeting ZDHHC2, ZDHHC19, or ZDHHC2/ZDHHC19 or a control siRNA for 60 h and then infected with CHIKV vaccine strain 181/25 (MOI = 1) for 6 h. The samples were analyzed by Western blotting (G). Total RNA was isolated and reverse transcribed. Subsequently, qPCR was performed (H). ATP levels were measured by CellTiter-Glo luminescent cell viability assay (I).

Protein palmitoylation plays a key role in regulating the localization and function of many proteins (20). Palmitoylated nsP1 from CHIKV localized in the plasma membrane and filopodial extensions. CHIKV nsP1 was not observed in the plasma membrane or filopodial extensions of cells treated with a FASN inhibitor (Fig. 3C), confirming that FASN contributed to the proper distribution of nsP1 by producing palmitic acid.

The palmitoylation of nsP1 has been shown to be critical for alphavirus replication (9). Since nsP1 proteins are conserved across alphaviruses, it is possible that the palmitoylation of nsP1 from alphaviruses other than CHIKV is also reduced by FASN
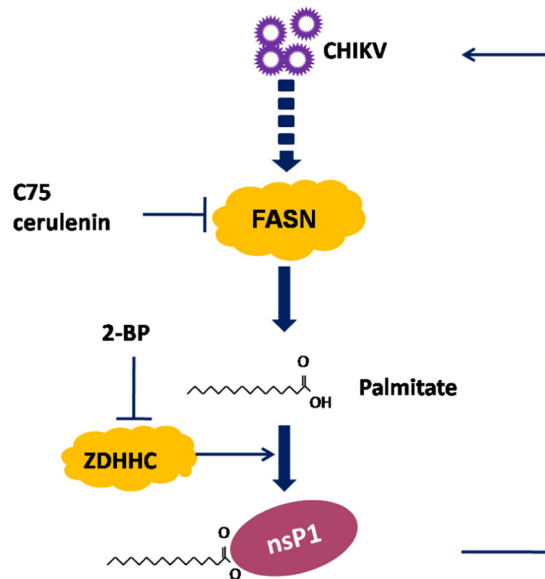
FIG 8 Schematic diagram of the proposed model depicting the mechanism of nsP1 palmitoylation. Palmitic acid synthesized by FASN was covalently attached to nsP1, and nsP1 palmitoylation was critical for CHIKV replication. Pharmacological inhibition of FASN by C75 and cerulenin inhibited nsP1 palmitoylation and restricted CHIKV replication. The ZDHHC inhibitor 2-BP reduced nsP1 palmitoylation and suppressed CHIKV replication.

inhibitors, reflecting a general mechanism for the role of FASN in alphavirus replication via the production of palmitic acid.

By prediction and mutagenesis experiments, we demonstrated that the cysteines at sites 417 to 419 of CHIKV nsP1 are the key residues for nsP1 palmitoylation. In Fig. 4B, mutations in individual cysteine residue showed no impact on nsp1 palmitoylation, whereas mutations of three cysteine residues reduced nsp1 palmitoylation by about 80%. Mutation of the key cysteine for the palmitoylation could generate another new palmitoylation site which did not exist in the endogenous protein. That is why we saw the reduction of nsP1 palmitoylation only with the triple mutation. C417 to C419 of CHIKV nsP1 affected more than just nsP1 palmitoylation. For instance, those residues might affect other processes, such as nsP1 disulfide bond formation. Consistent with its critical role in palmitoylation, CHIKV-nsP1 C/A triple mutation reduced viral replication greatly. We did not isolate CHIKV-nsP1 C/A virus or a reversion mutant.

Protein palmitoylation is mediated by PATs and protein thioesterases (16). In our study, ZDHHC2 and ZDHHC19 are the major PATs for nsP1. By associating with nsP1, ZDHHC2 and ZDHHC19 promoted the palmitoylation of nsP1. ZDHHC2 has been involved in the palmitoylation of postsynaptic density protein of 95 kDa (PSD-95) (21), protein A kinase-anchoring protein 79 (AKAP79) (22), CD9 (23), and CD151 (23). For instance, upon blocking synaptic activity, ZDHHC2 rapidly translocated to the postsynaptic plasma membrane to induce the palmitoylation of PSD-95, which is beneficial for synaptic recruitment of AMPA receptors (21). ZDHHC2 localized in plasma membrane in some cells (24) and thus is a credible candidate to palmitoylate nsP1. ZDHHC19 is able to promote the palmitoylation of R-Ras (25). R-Ras palmitoylation resulted in an increased association with membranes enriched in cholesterol and sphingomyelin (25). Given that nsP1 proteins are conserved across alphaviruses, ZDHHC2 and ZDHHC19 might also promote the palmitoylation of nsP1 from other alphaviruses. Since ZDHHC2 and ZDHHC19 palmitoylated more than one substrate, it is possible that silencing ZDHHC2 or ZDHHC19 would inhibit CHIKV replication indirectly by affecting the palmitoylation of other host proteins or CHIKV TF protein. Individual silencing of ZDHHC2 or ZDHHC19 strongly inhibits nsP1 palmitoylation and viral replication. We have a theory that ZDHHC2 and ZDHHC19 might participate in the palmitoylation of

nsP1 in different steps of the life cycle. More work must be conducted to further dissect the detailed mechanisms.

FASN is a host factor for several other viruses, such as hepatitis C virus (11, 26), HIV (27), respiratory syncytial virus (RSV) (8), rotavirus (28), and dengue virus (10). Thus, it is possible that FASN contributes to the replication of other viruses through the palmitoylation of some host and viral proteins.

How does nsP1 palmitoylation facilitate CHIKV replication? We think that nsP1 palmitoylation is not only crucial for CHIKV genome replication but also important for postreplication steps. The nsP1 palmitoylation-deficient mutation reduced viral RNA replication greatly, which suggested that nsP1 palmitoylation is critical for genome replication. In order to fulfill the genome replication role, nsP1 required the palmitoylation modification. The palmitoylation of nsP1 might affect the life cycle of CHIKV during a process other than genome replication. The protrusion of nsP1 also required palmitoylation (Fig. 4D). Previously, nsP1 was reported to reverse the anti-CHIKV role of bone marrow stromal cell antigen 2 (BST2) to produce more viruses (29). It is possible that the palmitoylation of nsP1 is important for this role.

In conclusion, we propose that FASN functions in CHIKV replication through nsP1 palmitoylation. ZDHHC2 and ZDHHC19 are the major PATs for nsP1. Our results provide mechanistic insights into the role of FASN in CHIKV replication.

MATERIALS AND METHODS

Cells, virus, and reagents. HeLa, 293T, and Huh7.5.1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS), glutamine, and gentamicin. CHIKV strain 181/25 plasmid pSinRep5-181/25ic was provided by Terence Dermody through Addgene (pSinRep5-181/25ic, plasmid 60078) (30). Plasmid pSinRep5-181/25ic was linearized by NotI digestion. RNA was synthesized from these cDNAs *in vitro* using the mMESSAGE mMACHINE SP6 transcription kit (Thermo Fisher Scientific). The cells were transfected with RNA by electroporation with a Bio-Rad Green Pulser II unit, as follows. The cells were resuspended in Opti-MEM and electroporated with one pulse at 270 V and 960 μF. Electroporations were performed in 4-mm cuvettes (Thermo Fisher Scientific). Cerulenin, C75, palmitic acid (PA), stearic acid (SA), and 2-bromopalmitate (2-BP) were purchased from Sigma-Aldrich.

Antibodies. The mouse antibodies used in this study have been described previously and included anti-FLAG (Sigma-Aldrich; A2220) and anti-actin (Sigma-Aldrich; A1978) antibodies. The rabbit antibodies used in this study included anti-nsP1 (provided by Andres Merits) and anti-GFP (XHY, XHY026L) antibodies. Horseradish peroxidase (HRP)-conjugated donkey antibodies against mouse IgG, rabbit IgG, and goat IgG were also used (Jackson ImmunoResearch, West Grove, PA).

Plasmids. Plasmid transfection into 293T cells was performed using FuGENE HD (Promega, Madison, WI) according to the manufacturer's instructions. A mammalian expression construct for nsP1 was generated by using the HindIII and EcoRI sites of 3×FLAG–CMV-14. The forward and reverse primers for PCR were as follows: 5'-CCCAAGCTTATGGATCCTGTGTA-3' and 5'-CGGAATTCGCTGCGCCCGCTCT-3'. Constructs expressing nsP1C417A, nsP1C418A, nsP1C419A, and nsP1C417/8/9A were generated by a QuikChange site-directed mutagenesis kit (Thermo Fisher Scientific, Waltham, MA). CHIKV-nsP1C/A was generated by mutating C417 to C419 to A417 to A419. The plasmids expressing FLAG-tagged ZDHHC1 (MG5A3618-CF), ZDHHC2 (MG5A1611-CF), ZDHHC3 (MG5A0430-CF), ZDHHC4 (HG16190-NF), ZDHHC5 (MG5A2307-CF), ZDHHC6 (MG53691-NF), ZDHHC7 (HG19166-CF), ZDHHC8 (MG5A0792-CF), ZDHHC9 (HG21413-NF), ZDHHC11 (MG55410-CF), ZDHHC12 (MG5A7057-CF), ZDHHC13 (MG5A1809-CF), ZDHHC14 (MG54007-CF), ZDHHC15 (MG5A4866-CF), ZDHHC16 (MG5A5172-CF), ZDHHC17 (MG5A0647-CF), ZDHHC18 (MG5A2533-CF), ZDHHC19 (MG54666-CF), ZDHHC20 (MG5A6992-CF), ZDHHC21 (MG5A1530-CF), ZDHHC22 (MG54364-CF), ZDHHC23 (MG5A6405-CF), and ZDHHC24 (MG5A6597-CF) were from Sino Biological (Beijing, China). All ZDHHC plasmids contained a cytomegalovirus (CMV) promoter and a kanamycin resistance cassette.

Immunoprecipitation. Cells were washed three times with ice-cold phosphate-buffered saline (PBS) and lysed in lysis buffer (20 mM Tris [pH 7.4], 2 mM EDTA, 150 mM NaCl, and 1% Triton X-100) supplemented with a protease inhibitor cocktail. The cell lysates were incubated with protein A/G agarose beads slurry at 4°C for 30 min and centrifuged at 12,000 × *g* at 4°C for 15 min. Then the cell lysate was incubated with protein A/G agarose beads bound to the antibody for 1.5 h and washed three times with PBS at 4°C at 500 × *g*. The proteins bound to the beads were boiled in 4× loading buffer and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Prediction of palmitoylation sites. Palmitoylation sites of nsP1 from CHIKV were predicted in NBA-Palm (nbapalm.biocuckoo.org) (15).

ABE assay. The acyl-biotin exchange (ABE) assay contains three biochemical steps: blocking the unmodified cysteine thiol groups by *N*-ethylmaleimide (NEM), cleaving and unmasking the palmitoylated cysteine's thiol group by hydroxylamine (HAM), and labeling the palmitoylated cysteine by a thiol-reactive biotinylation reagent, 1-biotinamido-4-[4'-(maleimidomethyl)cyclohexanecarboxamido]butane

(biotin-BMCC). Detection of nsP1 palmitoylation by an ABE assay was performed based on a previous study (31).

Plasma membrane isolation. The plasma membrane was isolated using a Minute plasma membrane protein isolation and cell fractionation kit (Invent Biotechnologies; SM-005), and the assay was performed according to the manufacturer's protocol.

Western blot analysis. The protein samples were separated by SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane, and probed with appropriate primary and secondary antibodies.

Immunofluorescence microscopy. Cells seeded onto glass coverslips were washed with PBS three times and fixed with 4% formaldehyde in PBS for 10 min at room temperature. The fixed cells were incubated with blocking solution (PBS containing 10% normal donkey serum) for 5 min at room temperature. For intracellular staining, the coverslips were incubated with primary antibodies diluted in permeabilization buffer (0.1% Triton X-100 in blocking solution) for 1 h. The coverslips were then washed three times with blocking solution, followed by incubation with an Alexa Fluor 488-conjugated secondary antibody, Alexa Fluor 555 phalloidin (Molecular Probes; A34055), and 4′,6-diamidino-2-phenylindole (DAPI) for 1 h at room temperature. The cells were washed three times with blocking solution and one time with PBS. The cells were imaged using a 40× oil immersion lens with a Leica TCS microscope (Germany).

qPCR. Total cellular and viral RNA was isolated using RNeasy minicolumns (Qiagen) 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 quantitative PCR (qPCR) using a DyNAmo HS SYBR green qPCR kit (Finnzyme, Espoo, Finland). The sequences of the primers used in qPCR were as follows. The forward and reverse primers for CHIKV were 5′-CATCTGCACCCAAGTGTACCA-3′ and 5′-GCGCATTT TGCCTTCGTAATG-3′, the forward and reverse primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5′-GGAGCGAGATCCCTCCAAAAT-3′ and 5′-GGCTGTTGTCATACTTCTCATGG-3′, the forward and reverse primers for ZDHHC2 were 5′-TCTTAGGCGAGCAGCCAAGGAT-3′ and 5′-CAGTGATGGACGCG ATCTGGTT-3′, and the forward and reverse primers for ZDHHC19 were 5′-TTGCTGCCTTCAATGCGGTG-3′ and 5′-CGGAGCCTTGATGTAAGATGC-3′.

siRNA and transfection. siRNAs were transfected into cells using Lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA). The siRNAs were from GenePharma (Shanghai, China) and had the following sequences: GCUACUGCUAGAAGUCUUAUU (ZDHHC2#1), CCAAGGAUCUUCCCAUCUAUU (ZDHHC2#2), GCAUCGGUCACCGCAACUUTT (ZDHHC19#4), GCACAACCCACCUGCCCUUTT (ZDHHC19#5), and UUCUCCGAACGUGUCACGUTT (negative control).

Cell viability assays. Cell viability was assessed using a CellTiter-Glo luminescent cell viability assay kit (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, the CellTiter-Glo reagent was added to the cells in 96-well plates at room temperature. The plate was shaken for 2 min and incubated for 10 min. Luminescence was detected using an EnVision multilabel plate reader (Perkin Elmer).

Statistics. Statistically significant differences were assessed using a paired Student's t test in GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA). Data represent the averages from at least three independent experiments $\pm$ the standard deviations (SD), unless stated otherwise. In figures, significant differences are represented as follows: *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$; and ns, no significant difference.

REFERENCES

1. Burt FJ, Rolph MS, Rulli NE, Mahalingam S, Heise MT. 2012. Chikungunya: a re-emerging virus. Lancet 379:662–671. https://doi.org/10.1016/S0140 -6736(11)60281-X.
2. Couderc T, Lecuit M. 2015. Chikungunya virus pathogenesis: from bedside to bench. Antiviral Res 121:120–131. https://doi.org/10.1016/j .antiviral.2015.07.002.
3. Schwartz O, Albert ML. 2010. Biology and pathogenesis of chikungunya virus. Nat Rev Microbiol 8:491–500. https://doi.org/10.1038/nrmicro2368.
4. Delang L, Li C, Tas A, Querat G, Albulescu IC, De Burghgraeve T, Guerrero NA, Gigante A, Piorkowski G, Decroly E, Jochmans D, Canard B, Snijder EJ, Perez-Perez MJ, van Hemert MJ, Coutard B, Leyssen P, Neyts J. 2016. The viral capping enzyme nsP1: a novel target for the inhibition of chikungunya virus infection. Sci Rep 6:31819. https://doi.org/10.1038/srep31819.
5. Salaun C, Greaves J, Chamberlain LH. 2010. The intracellular dynamic of

protein palmitoylation. J Cell Biol 191:1229–1238. https://doi.org/10 .1083/jcb.201008160.
6. Gottlieb CD, Linder ME. 2017. Structure and function of DHHC protein S-acyltransferases. Biochem Soc Trans 45:923–928. https://doi.org/10 .1042/BST20160304.
7. Laakkonen P, Ahola T, Kaariainen L. 1996. The effects of palmitoylation on membrane association of Semliki forest virus RNA capping enzyme. J Biol Chem 271:28567–28571. https://doi.org/10.1074/jbc.271.45.28567.
8. Ohol YM, Wang Z, Kemble G, Duke G. 2015. Direct inhibition of cellular fatty acid synthase impairs replication of respiratory syncytial virus and other respiratory viruses. PLoS One 10:e0144648. https://doi.org/10 .1371/journal.pone.0144648.
9. Ahola T, Kujala P, Tuittila M, Blom T, Laakkonen P, Hinkkanen A, Auvinen P. 2000. Effects of palmitoylation of replicase protein nsP1 on alphavirus

infection. J Virol 74:6725–6733. https://doi.org/10.1128/JVI.74.15.6725
-6733.2000.

10. Heaton NS, Perera R, Berger KL, Khadka S, Lacount DJ, Kuhn RJ, Randall
    G. 2010. Dengue virus nonstructural protein 3 redistributes fatty acid
    synthase to sites of viral replication and increases cellular fatty acid
    synthesis. Proc Natl Acad Sci U S A 107:17345–17350. https://doi.org/10
    .1073/pnas.1010811107.

11. Yang W, Hood BL, Chadwick SL, Liu S, Watkins SC, Luo G, Conrads TP,
    Wang T. 2008. Fatty acid synthase is up-regulated during hepatitis C
    virus infection and regulates hepatitis C virus entry and production.
    Hepatology 48:1396–1403. https://doi.org/10.1002/hep.22508.

12. Flavin R, Peluso S, Nguyen PL, Loda M. 2010. Fatty acid synthase as a
    potential therapeutic target in cancer. Future Oncol 6:551–562. https://
    doi.org/10.2217/fon.10.11.

13. Karlas A, Berre S, Couderc T, Varjak M, Braun P, Meyer M, Gangneux N,
    Karo-Astover L, Weege F, Raftery M, Schonrich G, Klemm U, Wurzlbauer
    A, Bracher F, Merits A, Meyer TF, Lecuit M. 2016. A human genome-wide
    loss-of-function screen identifies effective chikungunya antiviral drugs.
    Nat Commun 7:11320. https://doi.org/10.1038/ncomms11320.

14. Webb Y, Hermida-Matsumoto L, Resh MD. 2000. Inhibition of protein pal-
    mitoylation, raft localization, and T cell signaling by 2-bromopalmitate and
    polyunsaturated fatty acids. J Biol Chem 275:261–270. https://doi.org/10
    .1074/jbc.275.1.261.

15. Xue Y, Chen H, Jin C, Sun Z, Yao X. 2006. NBA-Palm: prediction of
    palmitoylation site implemented in Naive Bayes algorithm. BMC Bioin-
    formatics 7:458. https://doi.org/10.1186/1471-2105-7-458.

16. Lemonidis K, Werno MW, Greaves J, Diez-Ardanuy C, Sanchez-Perez MC,
    Salaun C, Thomson DM, Chamberlain LH. 2015. The zDHHC family of
    S-acyltransferases. Biochem Soc Trans 43:217–221. https://doi.org/10
    .1042/BST20140270.

17. Kiiver K, Tagen I, Zusinaite E, Tamberg N, Fazakerley JK, Merits A. 2008.
    Properties of non-structural protein 1 of Semliki Forest virus and its
    interference with virus replication. J Gen Virol 89:1457–1466. https://doi
    .org/10.1099/vir.0.2008/000299-0.

18. Zusinaite E, Tints K, Kiiver K, Spuul P, Karo-Astover L, Merits A, Sarand I.
    2007. Mutations at the palmitoylation site of non-structural protein nsP1
    of Semliki Forest virus attenuate virus replication and cause accumula-
    tion of compensatory mutations. J Gen Virol 88:1977–1985. https://doi
    .org/10.1099/vir.0.82865-0.

19. Ramsey J, Renzi EC, Arnold RJ, Trinidad JC, Mukhopadhyay S. 2017.
    Palmitoylation of Sindbis virus TF protein regulates its plasma mem-
    brane localization and subsequent incorporation into virions. J Virol
    91:e02000-16. https://doi.org/10.1128/JVI.02000-16.

20. Daniotti JL, Pedro MP, Valdez Taubas J. 2017. The role of S-acylation in
    protein trafficking. Traffic 18:699–710. https://doi.org/10.1111/tra.12510.

21. Noritake J, Fukata Y, Iwanaga T, Hosomi N, Tsutsumi R, Matsuda N, Tani
    H, Iwanari H, Mochizuki Y, Kodama T, Matsuura Y, Bredt DS, Hamakubo
    T, Fukata M. 2009. Mobile DHHC palmitoylating enzyme mediates
    activity-sensitive synaptic targeting of PSD-95. J Cell Biol 186:147–160.
    https://doi.org/10.1083/jcb.200903101.

22. Woolfrey KM, Sanderson JL, Dell'Acqua ML. 2015. The palmitoyl acyl-
    transferase DHHC2 regulates recycling endosome exocytosis and syn-
    aptic potentiation through palmitoylation of AKAP79/150. J Neurosci
    35:442–456. https://doi.org/10.1523/JNEUROSCI.2243-14.2015.

23. Sharma C, Yang XH, Hemler ME. 2008. DHHC2 affects palmitoylation,
    stability, and functions of tetraspanins CD9 and CD151. Mol Biol Cell
    19:3415–3425. https://doi.org/10.1091/mbc.e07-11-1164.

24. Greaves J, Carmichael JA, Chamberlain LH. 2011. The palmitoyl trans-
    ferase DHHC2 targets a dynamic membrane cycling pathway: regulation
    by a C-terminal domain. Mol Biol Cell 22:1887–1895. https://doi.org/10
    .1091/mbc.E10-11-0924.

25. Baumgart F, Corral-Escariz M, Pérez-Gil J, Rodríguez-Crespo I. 2010.
    Palmitoylation of R-Ras by human DHHC19, a palmitoyl transferase with
    a CaaX box. Biochim Biophys Acta 1798:592–604. https://doi.org/10
    .1016/j.bbamem.2010.01.002.

26. Huang JT, Tseng CP, Liao MH, Lu SC, Yeh WZ, Sakamoto N, Chen CM,
    Cheng JC. 2013. Hepatitis C virus replication is modulated by the inter-
    action of nonstructural protein NS5B and fatty acid synthase. J Virol
    87:4994–5004. https://doi.org/10.1128/JVI.02526-12.

27. Kulkarni MM, Ratcliff AN, Bhat M, Alwarawrah Y, Hughes P, Arcos J,
    Loiselle D, Torrelles JB, Funderburg NT, Haystead TA, Kwiek JJ. 2017.
    Cellular fatty acid synthase is required for late stages of HIV-1 replication.
    Retrovirology 14:45. https://doi.org/10.1186/s12977-017-0368-z.

28. Gaunt ER, Cheung W, Richards JE, Lever A, Desselberger U. 2013. Inhi-
    bition of rotavirus replication by downregulation of fatty acid synthesis.
    J Gen Virol 94:1310–1317. https://doi.org/10.1099/vir.0.050146-0.

29. Jones PH, Maric M, Madison MN, Maury W, Roller RJ, Okeoma CM. 2013.
    BST-2/tetherin-mediated restriction of chikungunya (CHIKV) VLP bud-
    ding is counteracted by CHIKV non-structural protein 1 (nsP1). Virology
    438:37–49. https://doi.org/10.1016/j.virol.2013.01.010.

30. Mainou BA, Zamora PF, Ashbrook AW, Dorset DC, Kim KS, Dermody TS.
    2013. Reovirus cell entry requires functional microtubules. mBio
    4:e00405-13. https://doi.org/10.1128/mBio.00405-13.

31. Brigidi GS, Bamji SX. 2013. Detection of protein palmitoylation in cul-
    tured hippocampal neurons by immunoprecipitation and acyl-biotin
    exchange (ABE). J Vis Exp 2013(72):50031. https://doi.org/10.3791/
    50031.