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BRIEF REPORT

SUMO1 depletion prevents lipid droplet accumulation and HCV replication

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Abstract Infection by hepatitis C virus (HCV) is a major public-health problem. Chronic infection often leads to cirrhosis, steatosis, and hepatocellular carcinoma. The life cycle of HCV depends on the host cell machinery and involves intimate interaction between viral and host proteins. However, the role of host proteins in the life cycle of HCV remains poorly understood. Here, we identify the small ubiquitin-related modifier (SUMO1) as a key host factor required for HCV replication. We performed a series of cell biology and biochemistry experiments using the HCV JFH-1 (Japanese fulminate hepatitis 1) genotype 2a strain, which produces infectious particles and recapitulates all the steps of the HCV life cycle. We observed that SUMO1 is upregulated in Huh7.5 infected cells. Reciprocally, SUMO1 was found to regulate the expression of viral core protein. Moreover, knockdown of SUMO1 using specific siRNA influenced the accumulation of lipid droplets and reduced HCV replication as measured by qRT-PCR. Thus, we identify SUMO1 as a key host factor required for HCV replication. To our knowledge, this is the first report showing that SUMO1 regulates lipid droplets in the context of viral infection. Our report provides a meaningful insight into how HCV replicates and interacts with host proteins and is of significant importance for the field of HCV and RNA viruses.

Keywords HCV · SUMO1 · Lipid droplets

Introduction

Hepatitis C virus (HCV) has long been considered a public-health problem, with an estimated 170 million infected individuals worldwide. It is a major human pathogen responsible for development of serious liver diseases, including steatosis, fibrosis, cirrhosis, and eventually, hepatocellular carcinoma [1]. HCV is a small, enveloped, positive-sense, single-stranded RNA virus of the family *Flaviviridae* [2]. The HCV genome is transported to the endoplasmic reticulum, where it is translated into a large polyprotein by the host translation machinery and processed by viral and cellular proteases into the mature structural (core, E1, E2) and non-structural proteins (p7, NS2, NS3, NS4A-B, NS5A-B) [2–4]. HCV replication occurs in replication complexes (RCs) in a membranous web (MW) consisting of lipid droplets (LDs) [4, 5].

There is not yet a vaccine to protect against HCV infection. However, a major improvement regarding treatment of HCV infection has been recently achieved. Two direct-acting antivirals (DAAs), the protease inhibitors telaprevir and boceprevir, were approved for the treatment of HCV infection in combination with pegylated interferon alfa/ribavirin (peg-IFN/RBV) [6, 7]. The addition of DAAs targeting different viral proteins was found to increase the sustained virologic response (SVR) [8], defined as the lack

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of detection of HCV RNA in blood several months after a complete course of treatment, and therefore to improve treatment success [9–11]. Unfortunately, resistance to DAAs has been reported recently, due to the high genetic variability of the HCV RNA genome [12–14]. Therapies that target host cell pathways may overcome or limit the occurrence of viral resistance and thus improve treatment responses. In this therapeutic context, there is an increased need to define the pathways that are hijacked by HCV for its life cycle. Moreover, HCV infection is tightly associated with alterations in lipid metabolism, and lipids have been shown to play important roles during the viral life cycle [15, 16]. The HCV core induces the redistribution of LDs through the clustering of these organelles in the perinuclear area, providing a platform for virus replication, assembly and production [17].

LDs have long been considered simple reservoirs for lipid storage, but they are now considered dynamic organelles that are involved in many biological processes, and several reports have highlighted their movement by attachment to microtubules [15–19]. Nevertheless, the molecular details of the mechanisms driving LDs biogenesis, growth and intracellular movement remain largely unknown. Modification of proteins involved in lipogenesis, such as the sterol regulatory element-binding proteins (SREBPs) and liver receptor homolog 1 (LRH-1) by SUMO has been demonstrated [20, 21]. Additionally, Talamillo and coworkers have demonstrated that silencing the drosophila SUMO homologue smt3 in the prothoracic gland leads to reduced lipid content [23]. These recent advances suggest that SUMO might have critical roles in lipogenesis [20, 21, 23].

The SUMO protein was first identified in mammals, where it was found to be covalently linked to the GTPase-activating protein RanGAP1 [24]. Consequently, SUMO is expressed by all eukaryotes but is absent from prokaryotes/archaea. Lower eukaryotes have a single SUMO gene, whereas plants and vertebrates express several SUMO paralogues [25]. In mammals, the SUMO family consists of four different isoforms, termed SUMO1 to 4. The SUMOs are post-translationally conjugated to other proteins, thereby regulating diverse cellular processes, including transcription, DNA repair, chromosome dynamics, subcellular localization of proteins, and protein-protein interactions via a mechanism essentially analogous to that of ubiquitination [26, 27].

In addition to its critical roles in a variety of cellular signalling pathways, SUMO family members play important roles in the life cycle of various viruses, such as KSHV (Kaposi's sarcoma-associated virus) [28] and influenza virus [29]. In this study, we performed different experiments using HCVcc-JFH1 and investigated the importance of SUMO1 for HCV replication.

Materials and methods

Antibodies and reagents

Anti-mouse actin and oleic acid were obtained from Sigma, and anti-mouse core and anti-rabbit SUMO1 were from Abcam. Anti-mouse Alexa Fluor 488 and 546 were obtained from Invitrogen. Nuclei were stained with TO-PRO-3 Iodide or Hoechst from Invitrogen.

Cell culture

Huh7.5 cells were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen) containing 4.5 g of glucose per liter, supplemented with 10 % heat-inactivated fetal bovine serum, 1 % nonessential amino acids (Invitrogen) and 1 % penicillin/streptomycin (Invitrogen).

Small interfering RNA and cell transfection

Endogenous expression of SUMO1 was silenced by transfection with a specific siRNA (sc-29498, Santa Cruz). Huh7.5 cells were grown in 12-well plates or on glass coverslips and infected with HCVcc. At 24 hours post-infection, cells were transiently transfected with 100 pmol of a siRNA oligonucleotide against SUMO1 using a Lipofectamine RNAiMAX Kit (Invitrogen) according to the manufacturer's protocol and incubated for another 48 hours. Control non-targeting siRNA was used as a reference (Genecust). At 48 hours post-transfection, cells were harvested for extraction of proteins and isolation of total RNA or fixed with a 3.7 % paraformaldehyde. The samples were then analyzed via western blot, qRT-PCR and immunofluorescence.

Immunoblot

Cells were collected on ice, washed and lysed in 20 mM Tris-HCl, 100 mM NaCl, 1 % Triton X-100 and 10 mM EDTA, pH 7.4, containing protease and phosphatase inhibitor cocktail (Roche diagnostics). The proteins were separated by SDS-PAGE, blotted onto a PVDF membrane, and visualized using a chemiluminescence reagent (Amersham).

Immunofluorescence staining

Huh7.5 cells were grown on glass coverslips in 12-well plates, treated according to the experiments, fixed with 3.7 % paraformaldehyde, permeabilized, and saturated with PBS supplemented with 0.7 % fish gelatin and 0.025 % saponin. Primary antibodies were diluted with permeabilization solution and incubated with cells. The

cells were washed and stained with fluorescent secondary antibodies, phalloidin and TO-PRO 3 or Hoechst. Samples were observed with a Zeiss 510 LSM confocal microscope or a Leica TCS SP5 (Leica Microsystems). The lipophilic fluorescence dye LD 540 was used to stain lipid droplets.

JFH1/HCVcc production and inoculation

The JFH1/HCVcc stock was prepared as described [30]. Cells were infected with HCV at 37 °C for 16 h, and unbound viruses were removed by aspiration and by washing cells with PBS and DMEM. The infected cells were harvested for extraction of proteins and isolation of total RNA or fixed with a 3.7 % paraformaldehyde. The samples were then analyzed via western blot, qRT-PCR and immunofluorescence.

RNA extraction and qRT-PCR analysis

Total RNA was isolated using RNAble solution (Eurobio), and intracellular levels of positive-strand HCV RNA were quantified by a strand-specific qRT-PCR technique described previously [36] using a Light Cycler Fast Start DNA MasterPlus SYBR Green (Roche) on a Real-Time PCR System (Roche). The triplicate mean values were calculated according to the Ct quantification method (Pfaffl 2001) using GAPDH gene transcription as reference for normalization. The average levels of HCV RNA relative to the positive control (100 %) from triplicate experiments, are shown.

Statistical analysis

Comparison of mean values was conducted using an unpaired Student's t-test. Statistical significance was determined at $*P < 0.05$, $**P < 0.001$, and $***P < 0.0001$.

Results

The abundance of SUMO1 is altered in infected Huh7.5 cells

We examined the expression of SUMO1 in uninfected Huh7.5 cells (Mock) and infected Huh7.5 cells (HCV) (Fig. 1). Based on the specific bands size, immunoblot analysis indicated an increase in the amount of SUMO1 (Fig. 1B and Fig. 1C). In addition to investigating protein level of SUMO1, we examined the localization of SUMO-1 in uninfected and infected cells. SUMO-1 appeared in the nucleus and cytoplasm of uninfected and infected cells (Fig. 1A).

SUMO1 regulates HCV genomic RNA replication and core expression

We assessed the requirement for SUMO1 in HCV viral protein expression and RNA replication. First, Huh7.5 cells were infected with HCV-JFH1 for 24 hours and then treated with SUMO1 siRNA for another 48 hours and analyzed by immunoblot, immunofluorescence staining and qRT-PCR. A reduction in the SUMO1 signal was observed, and this was associated with a decrease in the core protein level (Fig. 1E). Next, the cells were analyzed by confocal microscopy. Treatment of cells with SUMO1 siRNA reduced cellular expression of the core protein, as shown by immunoblotting and immunofluorescence (Fig. 1D). After that, we tested the effect of SUMO1 on HCV replication using qRT-PCR. The intracellular level of HCV RNA was quantified, and a significant drop in the level of HCV genomic RNA was observed after depletion of SUMO1 (Fig. 1F), indicating a critical role for this protein in HCV replication. The efficiency of SUMO1 siRNA was evaluated by immunoblotting and immunofluorescence staining (Fig. 1E and D).

Depletion of SUMO1 affects colocalization of HCV core protein and LDs

Attachment of the core protein to LDs is crucial for HCV replication [15]. Recent advances suggest that SUMO might have critical roles in lipogenesis [20–22], silencing the drosophila SUMO in the prothoracic gland has been shown to result in reduced lipid content [23]. Taking these data into account, we analysed the behaviour of LDs in SUMO1-siRNA-treated infected Huh7.5 cells. As presented in Fig. 2A, in uninfected cells, LDs were present at low levels and dispersed in the cytoplasm, whereas in infected cells, there was an increase and redistribution of LDs as clusters that colocalized with core protein around the nucleus (Fig. 2B and SI Fig. 1A). Treatment of cells with SUMO1 siRNA strongly reduced accumulation of LDs and cluster formation (Fig. 2C). Confocal images were analysed using Image J software, and the results are presented as graph under to each panel with a cartoon representing the LD content of the cell separated in three zones from the nucleus (Z1, Z2, and Z3). The LD signal was mainly present around the nucleus (Z2), and the intensity of the LD signal strongly increased with HCV infection. However, treatment of cells with SUMO1 siRNA led to a decrease in the intensity of the LD signal and a shift from the centre to the cell periphery. Fifty cells were analysed from three experiments, and the intensity of the LD signal was evaluated using Image J software. The data are presented as histograms in Fig. 2C and D. The number of LDs and the percentage of cells expressing LDs

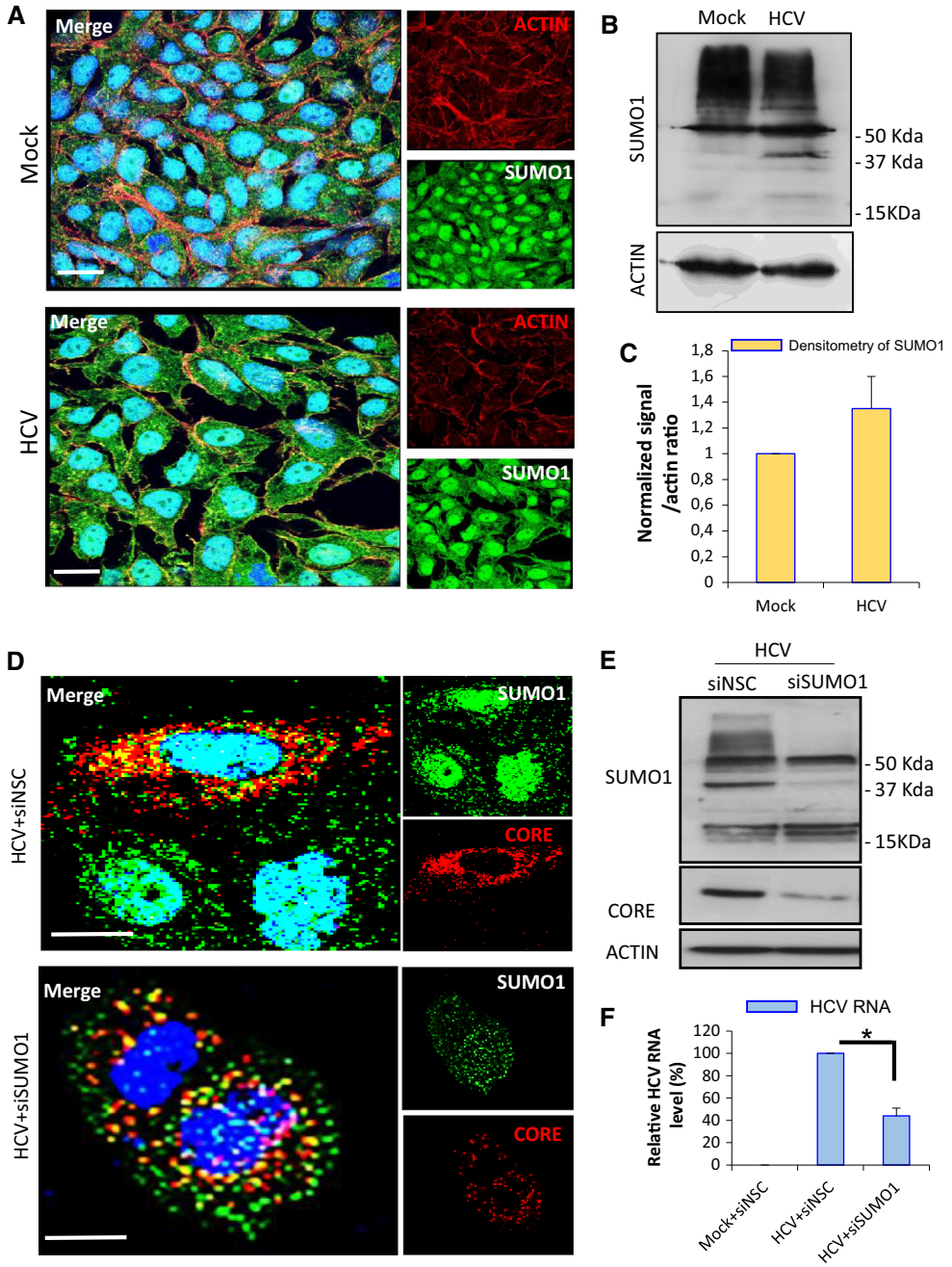

Fig. 1 SUMO1 regulates expression of the HCV core protein and genomic RNA expression. (A) Immunofluorescence analysis of SUMO1 (green), actin (red) and nuclei (blue) in uninfected (Mock) and infected (HCV) Huh7.5 cells. Scale bars represent 10 µm). (B) Immunoblot analysis of SUMO1 and HCV core in lysates of uninfected and infected Huh7.5 cells. Actin was used as a loading control. (C) Data analysis of the immunoblot staining in B, recapitulated as a histogram. (D) Immunofluorescence analysis of SUMO1 (green), core (red) and nuclei (blue) in Huh7.5 cells infected with HCV for 24 hours and then transfected with control siRNA (siNSC) or with SUMO1 siRNA (100 pmoles/assay) at 37 °C. At 48 hours post-transfection, cells were fixed with 3.7 % paraformaldehyde and stained as described in Materials and methods. Scale bars, 10 µm. (E) Immunoblot analysis of HCV core and SUMO1 in Huh7.5 cells infected with HCV for 24 hours and then transfected with control siRNA (siNSC) or with SUMO1 siRNAs (100 pmoles/assay) at 37 °C. At 48 hours post-transfection, cells were harvested for protein extraction. Actin was used as loading control. (F) Inhibition of HCV replication in Huh7.5 cells by siRNA specific for SUMO1. Huh7.5 cells in 12-well plates were infected with HCV. At 24 hours postinfection, cells were transiently transfected with control siRNA (siNSC) or with SUMO1 siRNA and incubated for another 48 hours at 37 °C. Total RNAs in the uninfected and infected Huh7.5 cells were isolated with RNABLE reagent (Eurobio). The levels of positive-strand HCV RNA were determined by qRT-PCR as described in Materials and methods. The average levels of HCV RNA relative to the control (100 %), from triplicate experiments, are shown (color figure online)

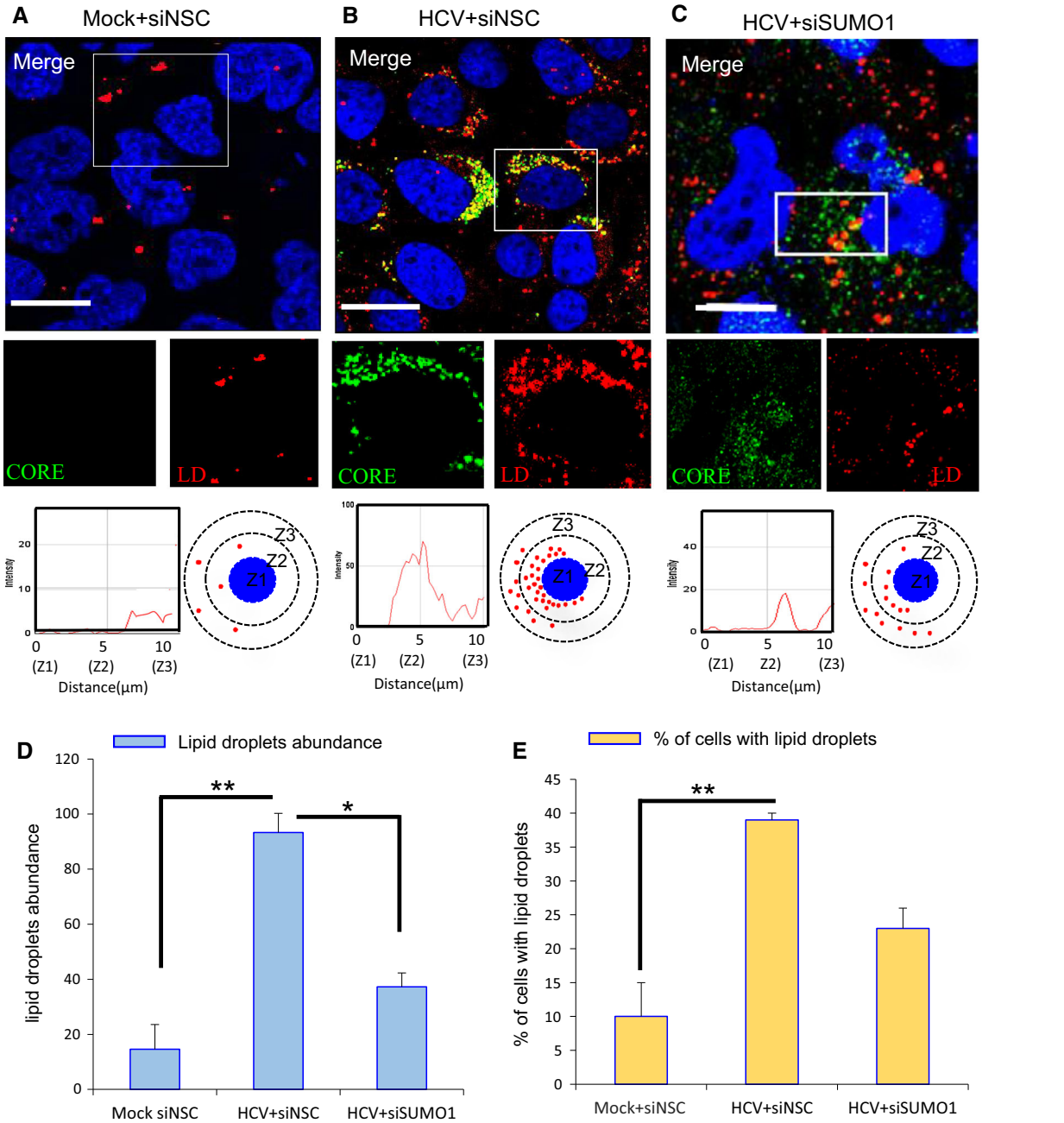

Fig. 2 Effect of SUMO1 on LD formation and on their association with the HCV core protein. (A) Immunofluorescence labeling of lipid droplets (red), core (green) and nuclei (blue) in uninfected Huh7.5 cells (Mock) treated with control siRNA. Scale bar, 10 µm. (B) Immunofluorescence labeling of lipid droplets (red), core (green) and nuclei (blue) in infected Huh7.5 cells treated with control siRNA as in Fig. 1D. Scale bar, 10 µm. (C) Immunofluorescence labeling of lipid droplets (red), core (green) and nuclei (blue) in infected Huh7.5 cells treated with SUMO1 siRNA as in Fig. 1D. Scale bar, 10 µm. (D) The average number of lipid droplets according to the experiments in panels A, B and C (lipid droplet count). (E) The effect of SUMO1 on the percentage of cells that expressed lipid droplets (color figure online)

increased in the presence of HCV infection (Fig. 2D). However, the silencing of SUMO1 expression led to a statistically significant decrease in the number of LDs (Fig. 2D) and also caused a reduction in the percentage of cells expressing LDs (Fig. 2E), indicating a role of SUMO1 in HCV induced- accumulation of LDs.

Depletion of SUMO1 alters the cellular level and distribution of LDs

Because of the important regulatory function of SUMO1 on LD accumulation, we decided to analyse the effect of SUMO1 on LD accumulation induced by oleic acid

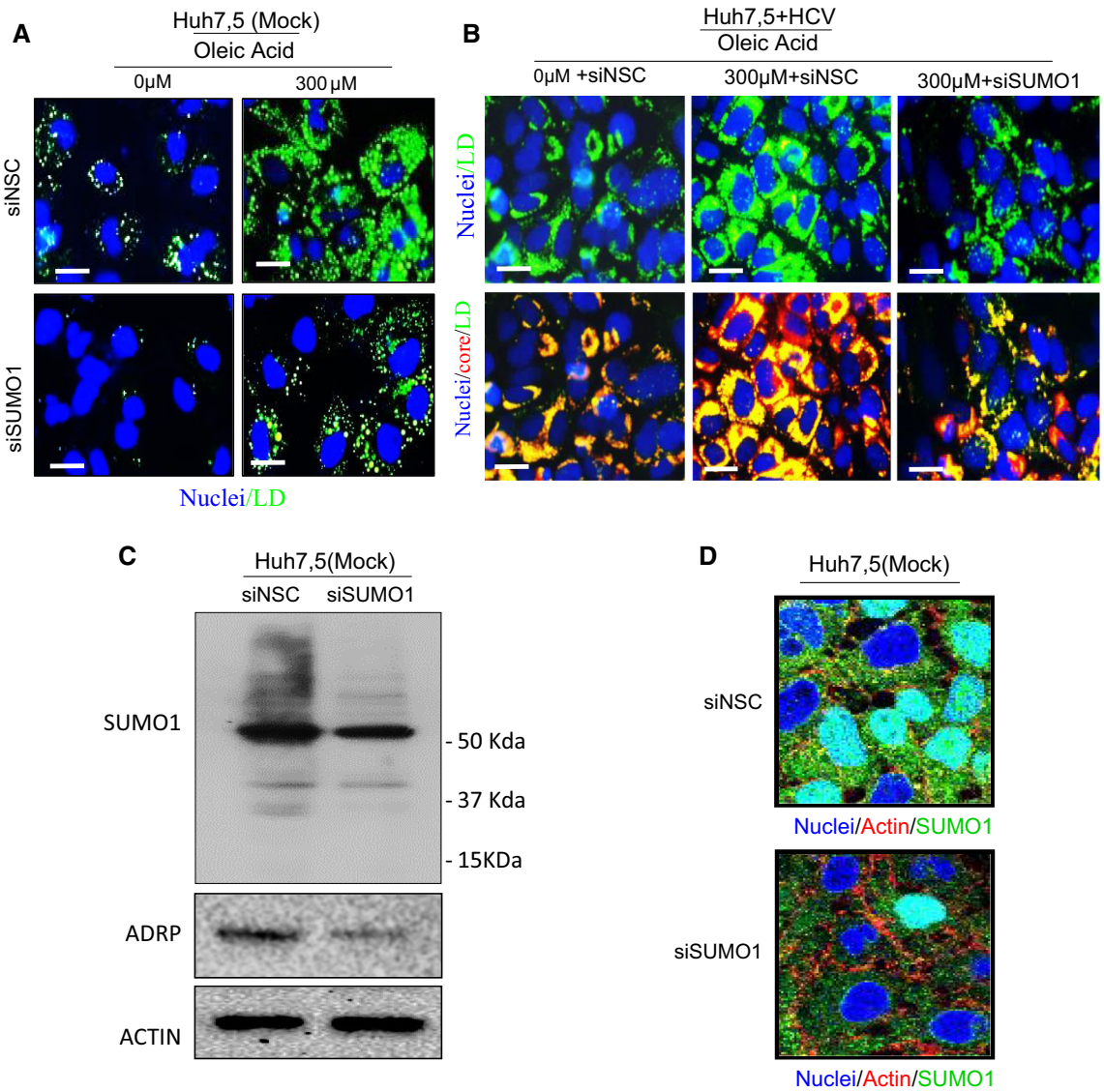

Fig. 3 SUMO1 alters cellular accumulation of LDs. (A) Immunofluorescence analysis of LD (green) and nuclei (blue) in uninfected cells treated with 300 μM oleic acid for 24 hours and then transfected with control siRNA or SUMO1 siRNA (100 pmoles/assay) for another 48 hours. Scale bar, 10 μm. (B) Immunofluorescence analysis of LD (green) and nuclei (blue) and core (red) in infected cells treated with 300 μM of oleic acid for 24 hours, transfected with control siRNA or SUMO1 siRNA (100 pmoles/assay). Scale bar, 10 μm. (D) Immunoblot analysis of SUMO1 and ADRP in lysates of uninfected Huh7.5 cells treated with control or with SUMO1 siRNAs for 48 hours. (E) Immunofluorescence analysis of SUMO1 and actin in uninfected Huh7.5 cells treated as in Fig. 3D. Scale bar, 10 μm (color figure online)

(Fig. 3A and B). Interestingly, treatment of cells with siRNA against SUMO1 suppressed LD formation induced by oleic acid in uninfected (Fig. 3A) and in infected cells (Fig. 3B), indicating that SUMO1 is required for formation of lipid droplets. Moreover, depletion of SUMO1 affected the expression of adipose differentiation-related protein (ADRP), an important protein associated with lipid droplets (Fig. 3C), confirming that knockdown of SUMO1 leads to reduced lipid content. The efficiency of SUMO1 siRNA was evaluated by immunoblot and immunofluorescence staining (Fig. 3C and D).

Discussion

This study provides another example of SUMO being needed for viral infection. We showed that HCV infection increases SUMO1 expression, which is necessary for its replication. This work provided significant insights into the host cellular machineries exploited by HCV for its replication, as well as new fundamental data on the functional features of SUMO1.

Different studies have suggested that LDs play a crucial role in the life cycle of HCV. Interactions between HCV

proteins, especially the core protein and LDs, are required for the morphogenesis and production of infectious HCV [31]. Our study revealed that SUMO1 modulated the accumulation of LDs and their association with HCV core and thus contributed to the establishment of the lipid environment required for virus replication.

SUMO proteins are important regulators of cell signalling and are covalently linked to other proteins in order to alter their structure, localization or function. To date, descriptions of SUMO conjugated pathogen proteins are limited to viruses, for which protein SUMOylation facilitates viral entry, nuclear translocation, changes in gene expression, and vesicular trafficking [34]. Studies conducted by Dunphy and coworkers have shown that *Ehrlichia chaffeensis* exploits SUMOylation pathways to promote its intracellular survival [33]. Moreover, previous work has shown that global cellular SUMOylation increases during influenza virus infection, and silencing the key enzymes required for SUMO conjugation prevents virus replication [32]. Our own work indicated also that negative changes in SUMO1 levels disturb HCV replication. This raised the possibility that this virus may manipulate SUMO and/or the fate of SUMO-modified proteins to facilitate its replication. In this report, SUMO1 was found mainly within the nucleus in both uninfected and infected cells. However, there appeared to be an increase in SUMOylation during HCV infection. This change in SUMO1 might be an important step for determining the abundance of lipid droplets and for their recruitment, a process that is necessary for HCV replication. It is difficult to provide a simple hypothesis that attempts to explain these results, because the biological effects of SUMOylation are quite diverse, and the mechanisms and signal pathways involved in most of them remain unclear. Recent advances suggest that SUMO might have critical roles in lipogenesis [20–22]. Moreover, silencing of SUMO in the prothoracic gland of drosophila led to reduced lipid content [23]. More experiments will be needed to identify the factors involved in the connection of SUMO1 and lipid droplets. This study provided information about the role of SUMO1 in production and redistribution of LDs, which is timely, because there is increasing evidence in the literature that lipid droplets play a crucial role in the replication of different pathogens [35]. Our study suggested that HCV may alter the localization and abundance of SUMO1, and this, in turn, might help to recruit and organize the LDs required for HCV assembly and replication. Thus, one explanation for the implication of SUMO1 in LDs redistribution could be connected to the SUMO E3 ligase function.

To conclude, this study illuminates an important function for SUMO1 in HCV replication. These findings also represent a step forward in our understanding of the relationship between HCV and its host and may open new therapeutic directions. A future key aim may be to correlate changes to the SUMO sub-proteome with the function of lipid droplet biogenesis, thereby establishing the mechanistic role of these modifications during HCV replication. Understanding the basic mechanisms underlying SUMOylation in HCV infection will provide new insights into the fundamental biology of this important pathogen. Future studies focused on the key enzymes required for general SUMOylation could also lead to identification of key cellular pathways that can be exploited by novel therapeutic targets.

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