

CD2-Associated Protein Contributes to Hepatitis C, Virus Propagation and Steatosis by Disrupting Insulin Signaling

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Chronic hepatitis C virus (HCV) infection can result in steatosis, a condition displaying aberrant accumulation of neutral lipid vesicles, the component of lipid droplets (LDs), which are essential for HCV assembly. However, the interplay between HCV infection and steatosis remains unclear. Here, we show that HCV-infected cells have higher levels of CD2-associated protein (CD2AP), which plays two distinct, yet tightly linked, roles in HCV pathogenesis: Elevated CD2AP binds to non-structural protein 5A (NS5A) and participates in the transport of NS5A to LDs to facilitate viral assembly; Up-regulated CD2AP also interacts with casitas B-lineage lymphoma (b) (Cbl/Cbl-b) E3 ligases to degrade insulin receptor substrate 1 (IRS1), which, in turn, disrupts insulin signaling and increases LD accumulation through the IRS1/protein kinase B (Akt)/adenosine monophosphate-activated protein kinase (AMPK)/hormone-sensitive lipase (HSL) signaling axis to accommodate viral assembly. In the HCV-infected mouse model, CD2AP expression is up-regulated during the chronic infection stage and this up-regulation correlates well with liver steatosis. Importantly, CD2AP up-regulation was also detected in HCV-infected human liver biopsies showing steatosis compared to non-HCV-infected controls. **Conclusion:** CD2AP is indicated as a protein up-regulated by HCV infection, which, in turn, stimulates HCV propagation and steatosis by disrupting insulin signaling; targeting CD2AP may offer an opportunity for alleviating HCV infection and its associated liver pathology. (HEPATOLOGY 2018;68:1710-1725).

Hepatitis C virus (HCV) infects approximately 180 million people worldwide, causing serious chronic liver diseases such as steatosis, liver cirrhosis, and, eventually, hepatocellular carcinoma (HCC).⁽¹⁾ Although a myriad of host factors have been reported to regulate viral propagation from entry to release of infectious particles,⁽²⁻⁴⁾ it is not fully understood how chronic HCV infection causes steatosis.

Lipid droplets (LDs), an organelle composed of a single phosphor-lipid layer,⁽⁵⁾ participate in many biological processes, such as energy storage and lipid metabolism.⁽⁶⁾ HCV uses LDs as hubs for assembly.^(7,8) HCV proteins, especially nonstructural protein 5A (NS5A) and HCV core protein, are in close proximity to LDs in HCV-infected cells.^(9,10) Transport of core and NS5A proteins to LDs depends on interactions

Abbreviations: ACC1/2, acetyl-CoA carboxylases 1 and 2; Akt, protein kinase B; AMPK, adenosine monophosphate kinase; BioID, proximity-dependent biotinylation method; BirA, BirA (R118G)-HA; Cbl/Cbl-b, casitas B-lineage lymphoma (b); CD2AP, CD2-associated protein; Erk, extracellular signal-regulated kinase; HA, hemagglutinin; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HSL, hormone-sensitive lipase; Ig, immunoglobulin; IHC, immunohistochemistry; IRS1, insulin receptor substrate 1; JFH1, Japanese fulminant hepatitis type 1; LDs, lipid droplets; LS, liver steatosis; NS5A, nonstructural protein 5A; OA, oleic acid; p, phosphorylated; SH3, Src homology 3.*

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between viral proteins, such as NS5A, and cytoskeletal filaments, such as actin and microtubules.^(11,12)

The goal of our studies was to better understand how HCV controls LD accumulation and contributes to liver pathology. We applied the proximity-dependent biotinylation (BioID) method to find NS5A interacting proteins *in situ*.⁽¹³⁾ One of the identified proteins was CD2-associated protein (CD2AP), an adaptor protein that is known to bind capping protein and regulate actin polymerization.⁽¹⁴⁾ We further show that CD2AP facilitates HCV propagation by two mechanistically linked pathways: participating in transporting NS5A to LDs and disrupting insulin signaling to boost more hubs for HCV assembly. In transgenic ICR mice infected with HCV, CD2AP up-regulation is detected in hepatocytes during the chronic stage of infection, concurrent with the appearance of steatosis. Importantly, levels of CD2AP are also up-regulated in liver biopsies from HCV-infected patients showing steatosis. Thus, our study indicates a link between CD2AP, HCV infection, and steatosis.

Materials and Methods

MATERIALS

Cell cultures, viruses, plasmids, and reagents are described in the Supporting Information.

ANIMAL MODEL

Eight- to 12-week-old age- and sex-matched littermates of human CD81 and occludin transgenic ICR

mice were used for *in vivo* study and infected with HCV as described.⁽¹⁵⁾ Mice were tail-vein injected with HCV J399EM (tissue culture infective dose, $50 = 1 \times 10^8$ /mL; 1 mL in 1–2 minutes to avoid liver injury). Mouse blood (0.1 mL) and liver tissues (0.1 g) were collected to quantify HCV genomic RNA at the indicated time. Five mice at each time point were infected with HCV. Two to three HCV infection-confirmed mice were used for further analysis. One of the noninfected mice at each time point was used as negative control. Data collection and data analysis were performed by different persons in a blinded manner. Use of animals was approved by the Institutional Review Board of Wuhan Institute of Virology, Chinese Academy of Sciences (Wuhan, China).⁽¹⁵⁾

HUMAN SUBJECTS

Seventy-two serologically confirmed HCV-infected human liver biopsies were obtained from resected liver tissues containing HCC, hemangioma, or cholangiocarcinoma from patients at the Tongji Hospital (Wuhan, China) and Eastern Hepatobiliary Surgery Hospital (Shanghai, China; patients' information in Supporting Table S1). No biopsies were obtained from executed prisoners or other institutionalized persons. Liver samples from HCV/HBV (hepatitis B virus) coinfection were excluded. Twelve non-HCV- and non-HBV-infected control specimens were obtained from normal regions of liver adjacent to resected hemangioma (patients' information in Supporting Table S1). Biopsies were scored for steatosis, cirrhosis, and HCC by two pathologists, Changshu Ke (M.D., Ph.D.) and Yu Hu (M.D.,

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Ph.D.; Department of Pathology, Tongji Hospital). Among the 72 biopsies, 53 also had HCC, 7 cases did not show steatosis and cirrhosis, 17 cases showed only steatosis, 20 cases showed steatosis and cirrhosis, and 4 cases showed only cirrhosis. Some cases could not be determined and thus were excluded. Informed consent was obtained from all subjects. Use of liver sections was approved by the Institutional Review Board of Wuhan Institute of Virology, Chinese Academy of Sciences (Approval Number: WIVH28201601).

METHODS OF *IN VITRO* ASSAYS, STATISTICAL ANALYSIS

In vitro assays and statistical analysis are described in the Supporting Information.

Results

HCV NS5A BINDS CD2AP

HCV NS5A plays an important role in HCV propagation. To identify proteins participating in HCV propagation, Huh7 cells with the NS5A-BirA*-HA construct (Fig. 1A) were cultured with or without biotin; more biotin-labeled proteins were detected in cells cultured with biotin (Fig. 1A). Several bands presented only in samples with biotin were sequenced and identified (Supporting Table S2). Among those proteins, CD2AP is an adaptor protein with three Src homology 3 (SH3) domains. Conversely, NS5A has several proline-rich motifs that are reported to bind the SH3 domain.⁽¹⁶⁾ Structural information thus suggests that CD2AP might interact with NS5A. Furthermore, the association of HCV replication complexes with actin has been shown depending on NS3 and NS5A interaction,⁽¹⁷⁾ and CD2AP can regulate actin organization.⁽¹⁸⁾ We thus focused on studying interactions between CD2AP and NS5A.

We found that CD2AP indeed interacted with NS5A (Supporting Fig. S1A), but not core protein (Supporting Fig. S1B) *in vitro*. In HCV Japanese fulminant hepatitis type 1 (JFH1)-infected cells, anti-CD2AP antibody pulled down NS5A and CD2AP (Fig. 1B). Conversely, anti-NS5A antibody also pulled down CD2AP and NS5A (Fig. 1B). Copurification was not observed with an isotype control mouse immunoglobulin (Ig)G2a (Fig. 1C). In addition, we observed colocalization of CD2AP and NS5A in cells infected with HCV-J399EM (Fig. 1D), the HCV 2a strain in which NS5A is enhanced green fluorescent protein

tagged. Thus, in HCV-infected cells, CD2AP interacts with NS5A. More important, levels of CD2AP were consistently up-regulated in infected cells compared to mock-infected cells (Fig. 1B).

DOMAIN III OF NS5A INTERACTS WITH SH3 DOMAIN OF CD2AP

To investigate which domain bound NS5A, a panel of FLAG-tagged CD2AP mutants was generated (Fig. 1E) and coexpressed with hemagglutinin (HA)-tagged NS5A in 293T cells. By co-immunoprecipitation (Co-IP), CD2AP containing at least one SH3 domain was able to bind NS5A, but CD2AP mutant lacking all three SH3 domains did not (Fig. 1E). NS5A contains an N-terminal amphipathic helix, three domains (I, II, and III), separated by two low-complexity sequences.⁽¹⁹⁾ We generated a series of NS5A mutants and found that when domain III of NS5A was deleted, it failed to bind CD2AP (Fig. 1F). Therefore, domain III of NS5A can bind the SH3 domains of CD2AP.

CD2AP PARTICIPATES IN THE TRANSPORT OF NS5A TO LD

To investigate the function of the binding between CD2AP and NS5A, we expressed an mCherry-tagged full-length CD2AP or CD2AP lacking all SH3 domains in Huh7.5.1 cells. After infecting these cells with HCV-J399EM, we observed that full-length CD2AP, but not truncated CD2AP, colocalized with NS5A (Fig. 2A). We then performed live cell tracking of the colocalized NS5A/CD2AP complex. The trajectory and velocity of the complex supported that CD2AP and NS5A indeed comigrated (Fig. 2B; Supporting Movie S1).

HCV particles travel along actin filaments.⁽²⁰⁾ Motility of CD2AP in podocytes depends on actin polymerization.⁽²¹⁾ Others have reported that transport of the HCV NS5A to LDs depend on microtubules.^(12,17,22) To address whether cotransport of NS5A and CD2AP was actin or microtubule dependent, we cultured HCV-infected cells with either cytochalasin B (an actin polymerization inhibitor) or colchicine (a microtubulin polymerization inhibitor). Live cell tracking of NS5A and CD2AP showed that cytochalasin B reduced the colocalization of NS5A and CD2AP (Fig. 2C). However, colchicine did not reduce the colocalization, but blocked their co-movement (Fig. 2D; Supporting Movie S2). Four hours after removing cytochalasin B, colocalization of CD2AP and NS5A

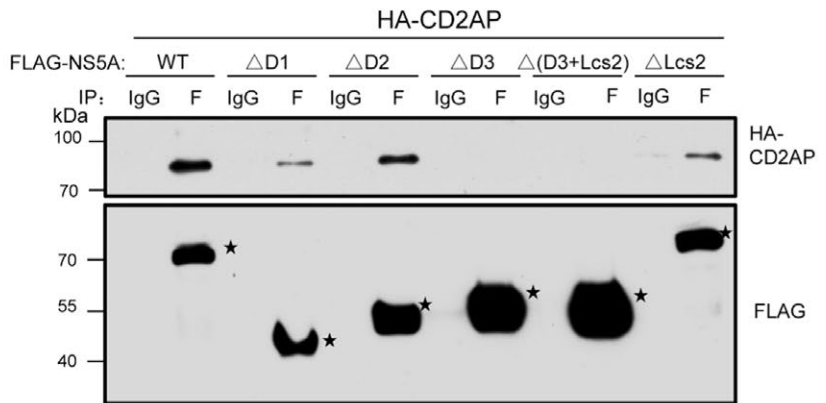
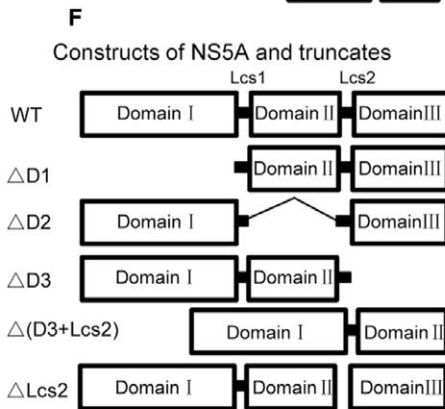
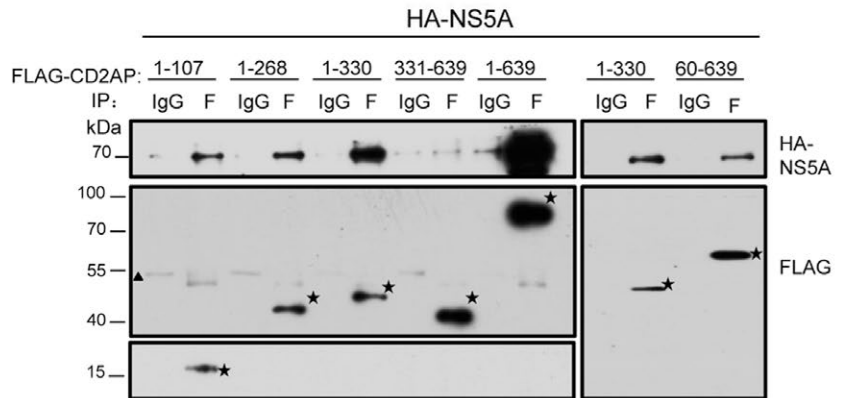
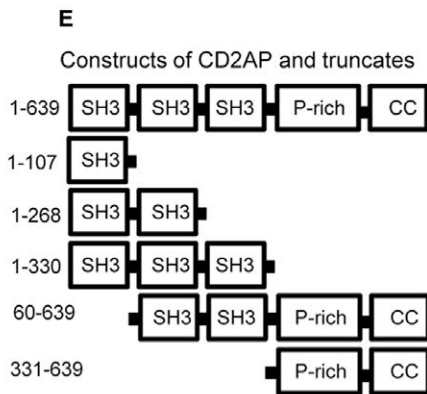
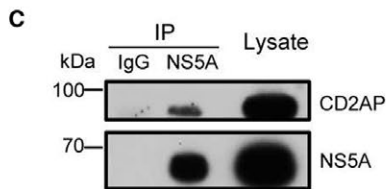
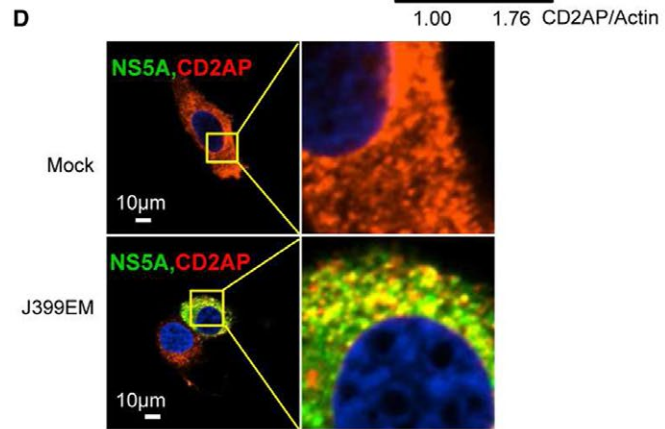
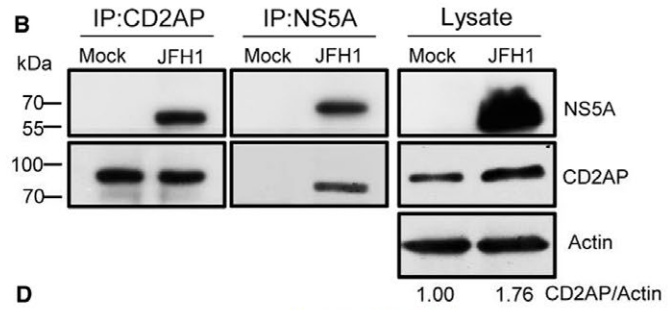
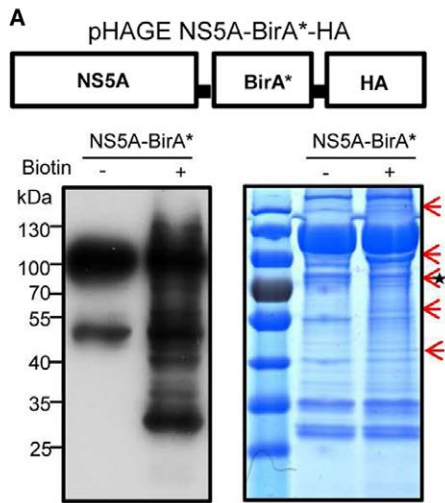


FIG. 1. HCV NS5A binds host protein CD2AP. (A) A diagram of the HCV NS5A- BirA* construct. Huh7 cells expressing NS5A BirA*-HA were incubated for 24 hours in complete media supplemented with or without 50 μ M of biotin. Lysates were purified by streptavidin-agarose beads and analyzed by mass spectrometry. CD2AP band was labeled with the asterisk. (B) Analysis of interactions between CD2AP and NS5A in Huh7.5.1 cells under HCV infection. Huh7.5.1 cells were infected with HCV JFH1 or left uninfected for 72 hours. Cell lysates were co-immunoprecipitated with anti-CD2AP antibody or anti-NS5A antibody. The immunoprecipitation complex was analyzed by immunoblotting with anti-NS5A antibody and anti-CD2AP antibody. (C) HCV-infected cell lysates were co-immunoprecipitated with anti-NS5A antibody or control mouse IgG2a. (D) Colocation of CD2AP and NS5A in HCV J399EM-infected Huh7.5.1 cells. (E) A schematic diagram of full-length and truncated CD2AP. Analysis of the interactions between CD2AP truncates with NS5A in 293T cells. FLAG-tagged CD2AP truncates are labeled with the asterisk. Nonspecific bands with molecular weight between 20 and 35 kDa are not shown. (F) A schematic representation of full-length and truncated NS5A. Analysis of the interactions between NS5A truncates with CD2AP in 293T cells. FLAG-tagged NS5A truncates are denoted with the asterisk. Abbreviations: IP, immunoprecipitation.

was restored (Fig. 2C). However, removal of colchicine did not restore NS5A/CD2AP comigration (movie not shown). These results suggest that NS5A and CD2AP colocalization is actin dependent and cytochalasin B treatment is reversible. Co-movement of the NS5A/CD2AP complex is microtubule dependent and colchicine treatment is irreversible.

Next, we investigated whether CD2AP participated in transport of NS5A to LD. We established two CD2AP-silenced Con1 cell lines (Supporting Fig. S2, c4# and c6#). Con1 cells support the subgenomic replication of HCV RNA, but do not support HCV assembly and production of virus particles.⁽²³⁾ Silencing CD2AP did not affect the total level of NS5A, but reduced the association of NS5A with fractionated LDs (Fig. 2E). Purity of LDs was confirmed by the absence of calnexin, a marker for endoplasmic reticulum (ER), and the presence of adipose differentiation-related protein, a marker for LDs (Fig. 2E). Thus, these results suggest that binding between NS5A and CD2AP is actin polymerization dependent and NS5A/CD2AP complex may be transported to LDs in a microtubule-dependent manner.

SILENCING CD2AP REDUCES THE LEVEL OF LDS

Because down-regulation of CD2AP diminished NS5A association with LDs in Con1 cells, we next investigated whether the phenomenon could be observed in Huh7.5.1 cells infected with HCV by generating two stably CD2AP-silenced cell lines (Supporting Fig. S3A, 4# and 6#). Immunofluorescent staining showed that less NS5A was found on LDs in CD2AP-silenced cells after infection with HCV (Fig. 3A). Surprisingly, levels of LDs were also reduced after CD2AP silencing (Fig. 3A). To investigate whether LD levels are enhanced by CD2AP expression or simply attributed to HCV infection, we stained LDs in those

cells without HCV infection. We found that the level of LDs was reduced in CD2AP-silenced cells treated with or without oleic acid (OA), which can stimulate LD formation by activating the long-chain fatty acid receptor, free fatty acid receptor 4 (Fig. 3B).⁽²⁴⁾ To prove there is a functional interplay between CD2AP and LD accumulation, we reintroduced HA-tagged CD2AP back into CD2AP-silenced cells (Supporting Fig. S3B). CD2AP-rescued cells (6#-res) had more LDs than control (6#-NC; Fig. 3C). Therefore, in addition to targeting HCV components to LDs, CD2AP also plays a role in boosting LD level.

CD2AP INCREASES INSULIN RECEPTOR SUBSTRATE 1 UBIQUITINATION BY ENGAGING CASITAS B-LINEAGE LYMPHOMA (B) E3 UBIQUITIN LIGASES

We then investigated the mechanism of CD2AP regulating LD accumulation. Level of LDs is modulated by insulin signaling.^(25,26) To study whether CD2AP boosted LD level through insulin signaling, we detected the expression of insulin receptor and insulin receptor substrate 1 (IRS1) in CD2AP-silenced cells. We found that levels of total IRS1, but not total insulin receptor, were obviously up-regulated (Fig. 4A). However, there was no difference for IRS1 mRNA levels between control and CD2AP-silenced cells (Supporting Fig. S4A). Hence, expression of IRS1 is likely regulated at the protein level. We found that after 2 hours of treatment with proteasome degradation inhibitor MG132, but not other inhibitors, such as NH_4Cl /3-methyladenine/chloroquine, level of IRS1 was increased (Supporting Fig. S4B). These results suggest that IRS1 stability may be regulated by protein ubiquitination. Furthermore, MG132 treatment increased much more IRS1 in wild-type cells

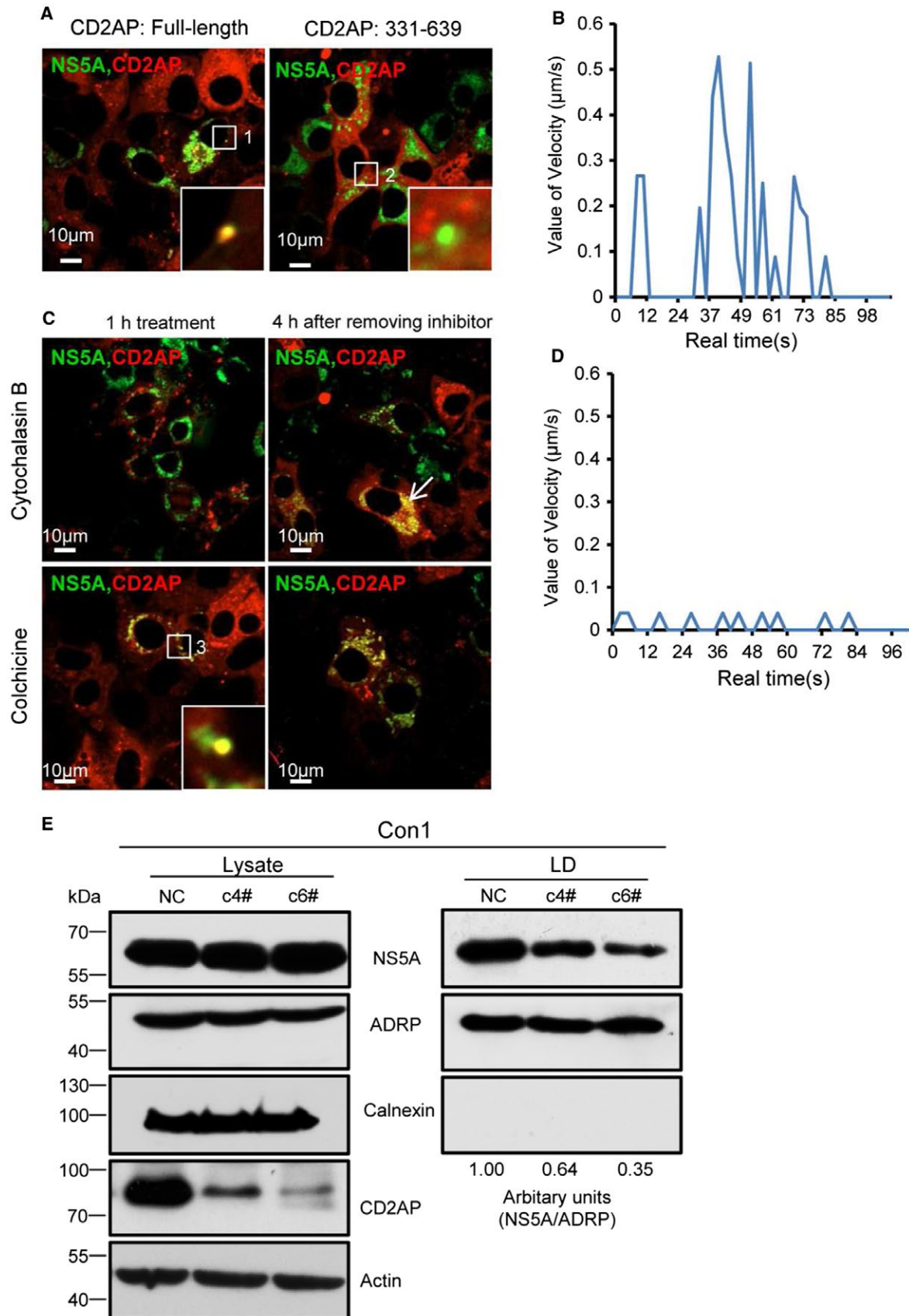


FIG. 2. CD2AP participates in the transport of NS5A to LD. (A) Huh7.5.1 cells expressing mCherry-tagged full-length CD2AP or SH3 domains deleted CD2AP (CD2AP:331-639) were infected with HCV-J399EM. At 48 hours, live cell tracking of CD2AP (red) and NS5A (green) were performed using confocal microscopy. (B) Velocity analysis of the colocalized NS5A/CD2AP complex shown in (A) (square 1). (C) Huh7.5.1 cells expressing mCherry-tagged CD2AP were infected with HCV-J399EM for 48 hours and then treated with 1 μ g/mL of cytochalasin B or 10 μ M of colchicine for 1 hours; or after 1 hour of drug treatment, culture media were replaced with fresh media without drugs for an additional 4 hours and cells were subjected to confocal observation. (D) Velocity analysis of the colocalized NS5A/CD2AP dot shown in (C) (square 3) after colchicine treatment. (E) Effect of CD2AP silencing on the association of NS5A with LDs in Con1 cells. Subgenomic replicon Con1 cells were transiently transduced with CD2AP sh-RNA (c4#, c6#) or control sh-RNA (NC). LD fractions were isolated as described in the Materials and Methods. Quantification of NS5A level was analyzed by Image J software (NIH, Bethesda, MD). ADRP and calnexin served as markers for LD and ER, respectively. Abbreviations: ADRP, adipose differentiation-related protein; shRNA, short hairpin RNA.

than in CD2AP-silenced cells (Fig. 4B). These results implicate that CD2AP down-regulation may diminish IRS1 ubiquitination. To prove this hypothesis, we purified a similar amount of IRS1 from CD2AP-silenced and control cells, and found that CD2AP down-regulated cells indeed showed less ubiquitination (Fig. 4C). Thus, CD2AP affects IRS1 level by modulating its ubiquitination. However, we could not exclude the possibility that some IRS1 might be degraded in a ubiquitin-independent manner.

We then investigated how CD2AP regulated IRS1 ubiquitination. In Huh7.5.1 cells, IRS1 copurified and colocalized with CD2AP (Fig. 4D). Because IRS1 is a client of several E3 ubiquitin ligases^(27,28) and CD2AP is known to interact with Cbl family E3 ligases,^(29,30) we then investigated whether IRS1 interacted with casitas B-lineage lymphoma (b) (Cbl/Cbl-b) E3 ligases. We found that Cbl/Cbl-b copurified and colocalized with IRS1 (Fig. 4D). To address whether Cbl/Cbl-b ubiquitinated IRS1, we silenced Cbl/Cbl-b in Huh7.5.1 cells. Accordingly, levels of IRS1 were up-regulated (Fig. 4E). Thus, CD2AP promotes IRS1 ubiquitination and degradation by engaging Cbl/Cbl-b E3 ligases.

CD2AP DISRUPTS IRS1 SIGNALING TO BOOST LD LEVEL

Because CD2AP promotes IRS1 degradation, we then investigated whether CD2AP influenced LD formation by the IRS1 signaling pathway, which affects lipid metabolism through the IRS1/phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) axis.^(31,32) In CD2AP-silenced cells, the level of phosphorylated (p)-Akt (S473), but not p-Erk (extracellular signal-regulated kinase) was increased (Fig. 5A). Akt is a negative regulator of adenosine monophosphate kinase (AMPK).⁽³³⁾ Then, we blotted for AMPK and found that level of p-AMPK (T172) was reduced

in CD2AP-silenced cells (Fig. 5A). Activation of AMPK regulates many enzymes in lipid metabolism, such as acetyl-CoA carboxylases 1 and 2 (ACC1/2), adipose triglyceride lipase (ATGL), sterol regulatory element-binding protein 1 (SREBP-1), and hormone-sensitive lipase (HSL).^(34,35) Binding of HSL to p-perilipin A on the surface of LD causes hydrolysis of LDs.⁽³⁶⁾ AMPK phosphorylates S554, inactivating the lipase activity of HSL.⁽³⁷⁾ We found that levels of p-HSL (S554), a marker of HSL inactivation, were reduced in CD2AP-silenced cells (Fig. 5A). Furthermore, silencing CD2AP did not affect other proteins involved in lipid metabolism, such as p-ACC1/2, ACC1/2, SREBP1, and ATGL (Supporting Fig. S5). To confirm that HSL (S554) is phosphorylated by AMPK, we treated cells with dorsomorphin, an AMPK inhibitor. Dorsomorphin reduced levels of p-AMPK (T172) and p-HSL (S554; Fig. 5B). To further prove that CD2AP disrupts IRS1 signaling by the PI3K-Akt pathway, we reintroduced CD2AP into CD2AP-silenced cells; the level of p-Akt (S473) was reduced (Fig. 5C, compare lane 3 to lane 4). Considering that CD2AP functionally rescued p-Akt (Fig. 5C) and LD levels (Fig. 3C) when reintroduced into CD2AP-silenced cells, these results provided strong evidences that enhanced CD2AP affects lipid metabolism by the IRS1-Akt-AMPK-HSL axis, causing inactivation of HSL to increase LD accumulation.

CD2AP SILENCING INHIBITS HCV PROPAGATION INDEPENDENT OF VIRUS ENTRY, SUBGENOMIC REPLICATION, AND INTERNAL RIBOSOMAL ENTRY SITE-DEPENDENT TRANSLATION

Because silencing CD2AP affects LD accumulation and NS5A transport to LDs, we posited that CD2AP

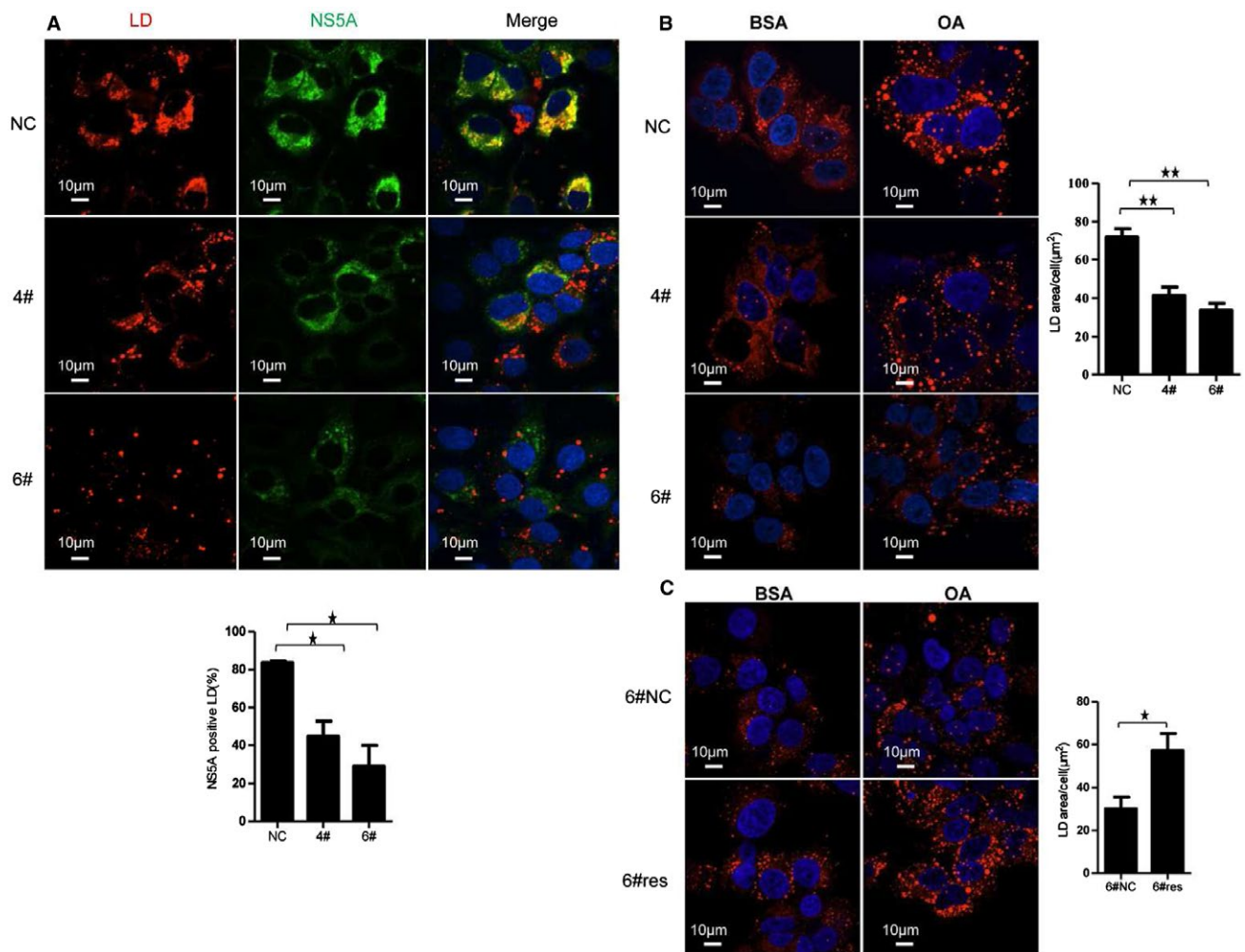


FIG. 3. CD2AP down-regulation affects LD accumulation. (A) Effects of CD2AP silencing on the association of NS5A with LDs under HCV JFH-1 infection. CD2AP down-regulated (4#, 6#) and control (NC) Huh7.5.1 cells were infected with JFH-1 for 72 hours and stained for LDs using HCS LipidTOX Deep Red neutral lipid stains. Nuclei were counterstained with DAPI. Quantification of NS5A-positive LDs is shown in diagram. (B,C) Effect of CD2AP silencing (B) and CD2AP rescuing (C) on LD accumulation. CD2AP silenced-cells (6#) were rescued with HA-tagged CD2AP (6#res) or empty vector (6#NC). Silenced (6#) and rescued (6#res) cells were treated with BSA alone or with 0.5 mM of OA-BSA complexes for 16 hours and then stained for LD. Quantification of LDs area in single cell after OA stimulation was analyzed by Volocity software and is shown in diagram. Experiments were repeated three times. A two-tailed Student *t* test was used for statistical analysis. **P* < 0.05; ***P* < 0.01. Abbreviations: BSA, bovine serum albumin; DAPI, 4',6-diamidino-2-phenylindole.

down-regulation should affect HCV propagation. First, we infected CD2AP-silenced Huh7.5.1 cells with HCV-JFH1 and found that CD2AP down-regulation did not affect cell viability (Supporting Fig. S6A), but reduced intracellular total HCV-RNA level, protein level, and supernatant RNA copy numbers (Fig. 6A), and the reduction seems to be correlated with level of CD2AP reduction. In addition, silencing CD2AP also decreased the JFH1-Luc-5AGFP reporter virus activity (Fig. 6B) and CD2AP-rescued cells (6#-res) could recover HCV-RNA and protein level (Fig. 6C). Thus, CD2AP indeed regulates HCV propagation.

We then tried to identify the step(s) affecting HCV replication after CD2AP silencing. We found that silencing CD2AP did not affect HCV entry (by pseudovirus assay; Supporting Fig. S6B), subgenomic replication in Con1 cells, which do not support HCV assembly (Supporting Fig. S6C and Fig. 2E), and HCV internal ribosomal entry site-dependent translation (Supporting Fig. S6D). However, intracellular and supernatant HCV titers (Fig. 6D) were reduced in CD2AP-silenced cells infected with JFH1. This suggested that CD2AP silencing impaired production of infectious virions in steps other than virus entry,

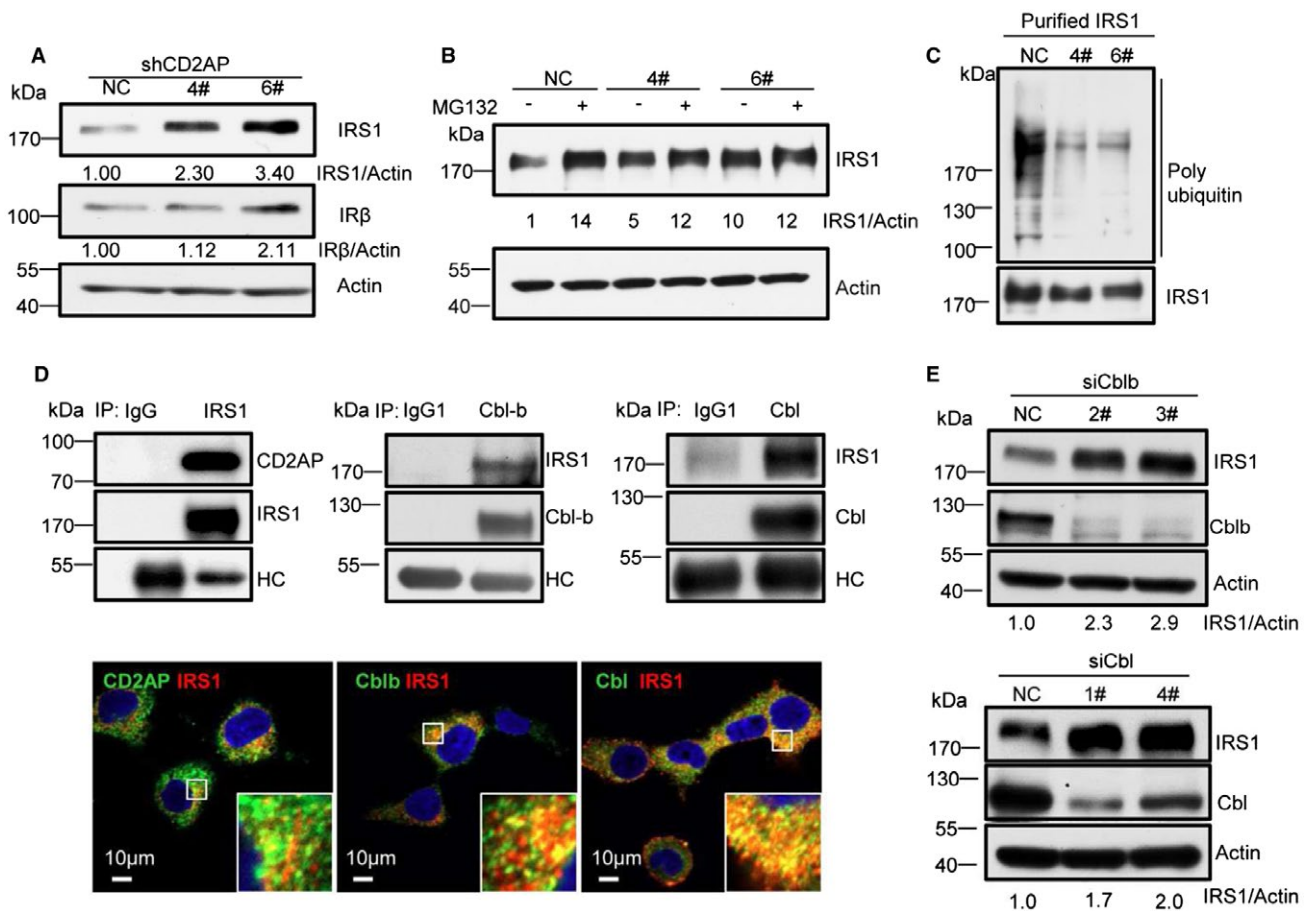


FIG. 4. CD2AP increases IRS ubiquitination by engaging Cbl/Cbl-b E3 ubiquitin ligases. (A) Effects of CD2AP silencing on IRS1 after HCV JFH-1 infection for 72 hours. (B) Effect of MG132 treatment (10 μ M, 2 hours) on IRS1 in CD2AP-silenced cells. (C) Effect of CD2AP silencing on ubiquitination of IRS1. Cell lysates were purified with anti-IRS1 antibody. Purified IRS1 was adjusted to the similar amount and then immunoblotted with antibodies against polyubiquitin or IRS1. (D) Co-IP and colocalization of IRS1 with CD2AP, Cbl-b, or Cbl. Huh7.5.1 cell lysates were purified with anti-IRS1, anti-Cbl-b, or anti-Cbl antibodies. The immunoprecipitation complex was immunoblotted with specific antibodies. Heavy chain (HC) served as the loading control. (E) Effects of Cbl-b or Cbl silencing on IRS1 expression. Huh7.5.1 cells were transfected with 30 nM of siRNA specific to Cbl-b or Cbl or control siRNA for 48 hours. Quantification was analyzed by ImageJ software (NIH, Bethesda, MD). Abbreviation: siRNA, small interfering RNA.

subgenomic replication, and RNA translation. We further performed one single-cycle assay to determine which step of HCV infection was affected by CD2AP silencing. We found that HCV could complete one single cycle between 12 and 18 hours postinfection (Supporting Fig. S7A) and CD2AP silencing significantly reduced intracellular HCV RNA 18 hours, but not 12 hours, postinfection (Fig. 6E). However, we did not detect NS5A protein expression until 24 hours postinfection (Supporting Fig. S7B), probably attributed to low sensitivity of immunoblotting. These results, together with the observation that less LD accumulation and less NS5A was found on LDs after CD2AP silencing (Fig. 3), supported that HCV

assembly might be impaired in CD2AP-silenced cells. However, it is also possible that steps other than HCV assembly are also impacted by CD2AP down-regulation. Thus, HCV infection increased CD2AP expression to enhance HCV propagation.

CD2AP UP-REGULATION CORRELATES WITH STEATOSIS IN AN ANIMAL MODEL AND IN HUMAN BIOPSIES

HCV is known to cause steatosis, and CD2AP is up-regulated in HCV-infected cells and enhanced CD2AP could stimulate LD accumulation, a

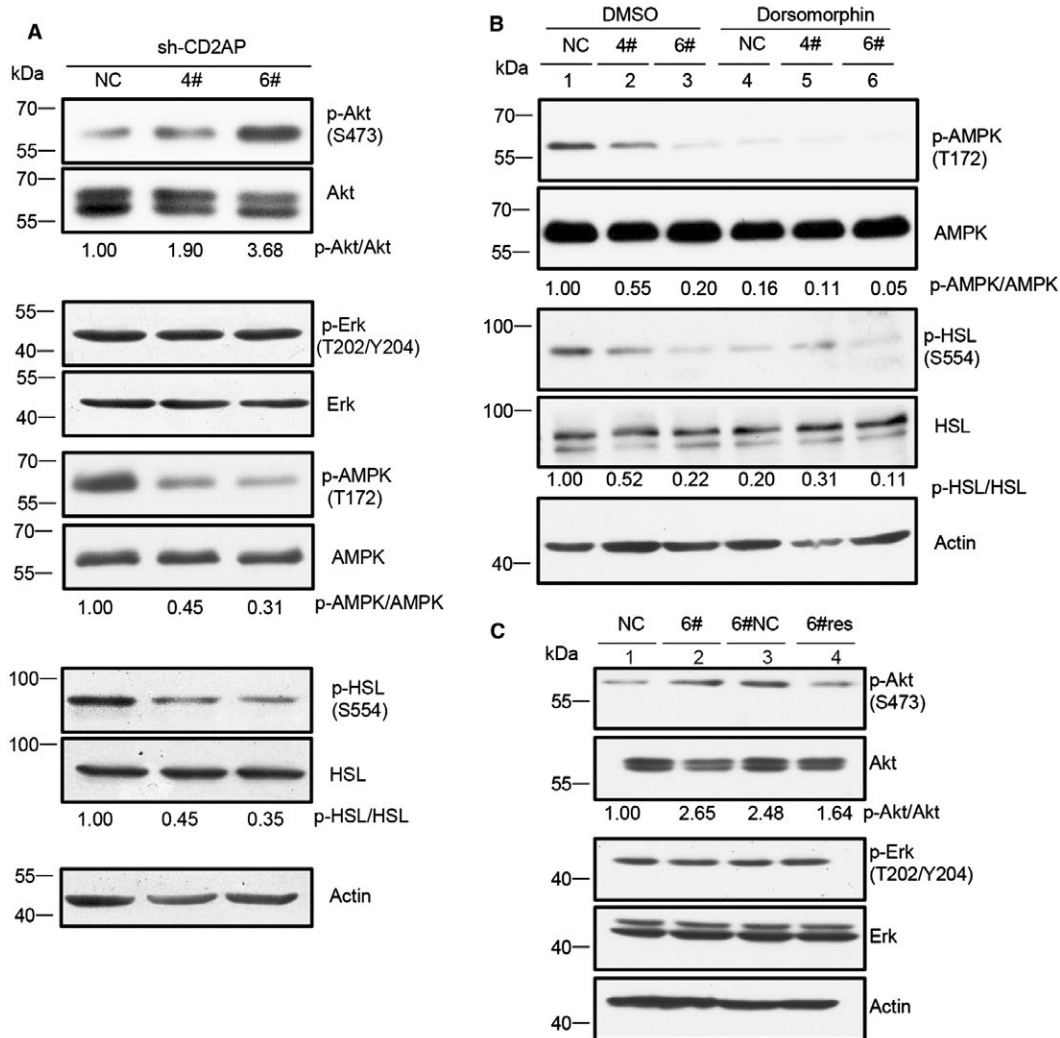


FIG. 5. CD2AP regulates LD accumulation by the IRS1-Akt-AMPK-HSL axis. (A) Effects of CD2AP silencing on Akt-AMPK-HSL axis. (B) Effect of dorsomorphin (an inhibitor for AMPK) treatment on p-AMPK (T172) and p-HSL (S554) levels. CD2AP down-regulated (4# & 6#) and control (NC) cells were cultured in complete medium for 48 hours and then treated with DMSO or dorsomorphin (5 μ M) for 4 hours. (C) Effect of CD2AP rescuing on p-Akt expression in CD2AP-rescuing cells. Abbreviation: DMSO, dimethyl sulfoxide.

requirement for steatosis. We then investigated whether steatosis and CD2AP up-regulation could be observed in a human CD81 and occludin transgenic ICR mouse model.⁽¹⁵⁾ Based on liver and blood HCV-RNA copy number, the first week after HCV infection represented the acute infection stage (Fig. 7A). At this time, there was little CD2AP staining in the liver (Fig. 7B). In contrast, CD2AP expression became highly noticeable from the first to fourth month during the chronic infection stage, when lipids began to accumulate and steatosis was evident (Fig. 7B, arrows indicate steatosis). Thus, up-regulation of CD2AP correlates with manifestation of steatosis.

Finally, we stained 72 liver biopsies from HCV-infected patients and 12 liver biopsies from non-HCV-infected controls for CD2AP. We found CD2AP immunohistochemistry (IHC) staining was faint or undetectable in most of the non-HCV-infected controls, whereas most of the 72 HCV-infected biopsies showed positive CD2AP staining (Fig. 7C,D). Most important, HCV-infected biopsies showing only steatosis or HCC also showed stronger CD2AP staining than non-HCV-infected hepatocytes (Fig. 7D). Thus, HCV infection activated CD2AP expression in the mouse model and human subjects, and the up-regulation correlates well with liver steatosis (LS).

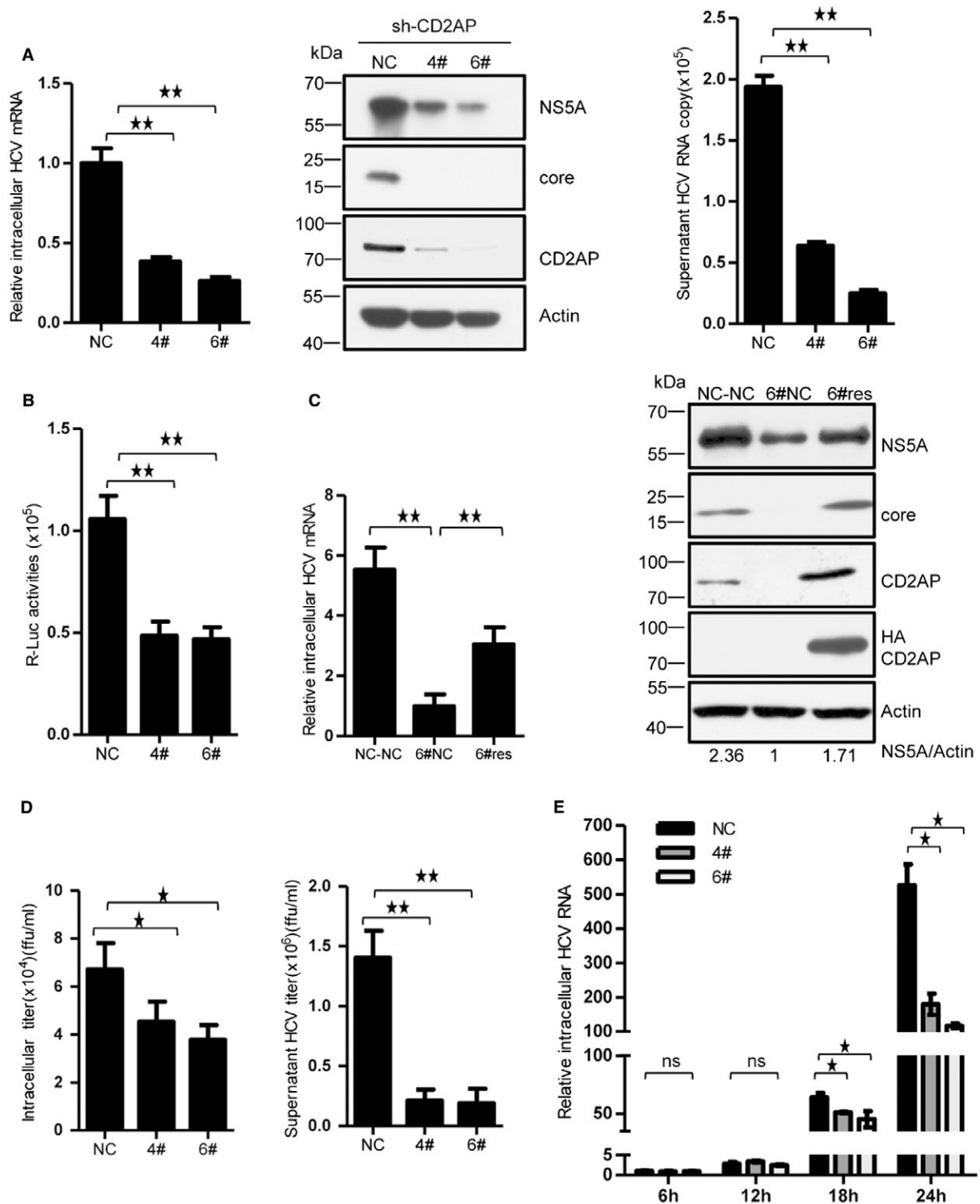


FIG. 6. Silencing CD2AP inhibits HCV propagation. (A) Effects of CD2AP silencing on intracellular HCV-RNA level, NS5A and core protein level, and supernatant HCV-RNA copy number. CD2AP down-regulated (4#, 6#) and control (NC) Huh 7.5.1 cells were infected with HCV JFH1 for 72 hours. Total intracellular HCV RNAs were extracted and subjected for quantitative PCR. Total cell lysates were immunoblotted for CD2AP, HCV NS5A, and core. Supernatant HCV RNA was extracted as described in the Materials and Methods and detected by quantitative PCR. (B) Effect of CD2AP down-regulation on HCV reporter virus JFH1-Luc-5AGFP. CD2AP down-regulated (4#, 6#) and control (NC) cells were infected with a reporter virus JFH1-Luc-5AGFP with a Renilla luciferase gene. Luciferase activities were measured 72 hours postinfection. (C) Effect of CD2AP rescuing on intracellular HCV RNA and protein level. CD2AP-rescued cells (6#res) and control cells (6#NC) were infected with HCV JFH1. Intracellular HCV RNA levels were detected by quantitative PCR, and cell lysates were immunoblotted with CD2AP, core, and NS5A antibodies 72 hours postinfection. (D) Effect of CD2AP silencing on intracellular and supernatant HCV titers. CD2AP down-regulated (4#, 6#) and control (NC) cells were infected with J399EM (MOI=1) for 72 hours. Cell pellet and culture supernatant were quantified for intracellular and extracellular virus titers as described in the Materials and Methods. (E) CD2AP down-regulated (4#, 6#) and control (NC) Huh 7.5.1 cells were infected with HCV J399EM for indicated time points (6, 12, 18, and 24 hours). Total intracellular HCV RNAs were quantified. Normalized intracellular HCV-RNA level in control cells at 6 hours postinfection was defined as 1. Experiments were repeated three times. A two-tailed Student *t* test was used for statistical analysis. Data were mean \pm SEM. **P* < 0.05; ***P* < 0.01. Abbreviations: MOI, multiplicity of infection; h, hours.

Discussion

Liver pathologies, such as steatosis, are affected by many factors.⁽³⁸⁻⁴⁰⁾ Here, we provide insights into the propagation of HCV and steatosis caused by chronic HCV infection. We identify a host protein, CD2AP, as being stimulated by HCV infection and pivotal in HCV propagation and lipid metabolism. CD2AP participates in cytoskeleton reorganization and trafficking of cellular organelles and vesicles.^(18,21) In HCV-infected cells, the SH3 domain of CD2AP binds domain III of NS5A (Fig. 1). Binding of CD2AP to NS5A delivers NS5A toward LDs for HCV assembly. We posit that during HCV assembly, trafficking of the CD2AP/NS5A complex to LDs is a two-step process. First, CD2AP meets NS5A in an actin-filament-dependent manner. Subsequently, NS5A is transported to LDs through the microtubule (Fig. 2). At this time, the molecular mechanism mediating these two steps is unknown. Given that CD2AP can bind small G-coupled proteins that are important in vesicle formation and migration,⁽⁴¹⁾ it is likely that additional host proteins, such as motor proteins and small GTPases, are involved in the transport process.

Unexpectedly, CD2AP also regulates the levels of LDs. In HCV-infected cells, CD2AP modulates the activity of IRS1 by securing Cbl/Cbl-b E3 ligases, enhancing IRS1 ubiquitination, and augmenting IRS1 degradation by the proteasome (Fig. 4), which then regulates the Akt-AMPK-HSL signaling axis. Accordingly, levels of LDs, IRS1, and p-Akt are correlated with CD2AP (Figs. 3-6).

When CD2AP is silenced, production of infectious HCV particles is also decreased (Fig. 6D). We

postulate that reduction of infectious HCV particles is attributed to diminished transport of NS5A to LDs and reduced levels of LDs for virus assembly (Figs. 2 and 3). This hypothesis is supported by the observation that when CD2AP was rescued, production of HCV was also restored (Fig. 6C).

In an HCV infection transgenic ICR mouse model, levels of HCV RNA were detected in liver and serum of infected mice 2 days postinfection (Fig. 7A). However, hepatocyte CD2AP staining in these mice did not appear until much later, at around 1 month postinfection, a time when steatosis began to appear (Fig. 7B). Thus, CD2AP up-regulation may contribute to steatosis. In cells, up-regulation of CD2AP occurs within days, but in infected animals, this up-regulation can only be observed at the chronic stage of infection. The reason for this difference is not known. One possibility is that in the cell model, the hepatoma cells already express high levels of CD2AP, whereas the normal hepatocyte has no detectable CD2AP. Titers of the HCV used for *in vitro* infection and *in vivo* infection may also contribute to the difference. Furthermore, immunoblotting is more sensitive than IHC staining; a slight increase in CD2AP level may escape detection by immunostaining.

In human liver biopsies, only 2 of the 12 noninfected control tissues showed discernable CD2AP expression (Fig. 7D), whereas most of the HCV-infected peritumor biopsies showed positive CD2AP staining. The reason why some of the biopsies from HCV-infected individuals are CD2AP negative is not clear at this time. These patients may express CD2AP that is beyond the detection limit of IHC, or they are at an earlier stage of infection. Most important, CD2AP

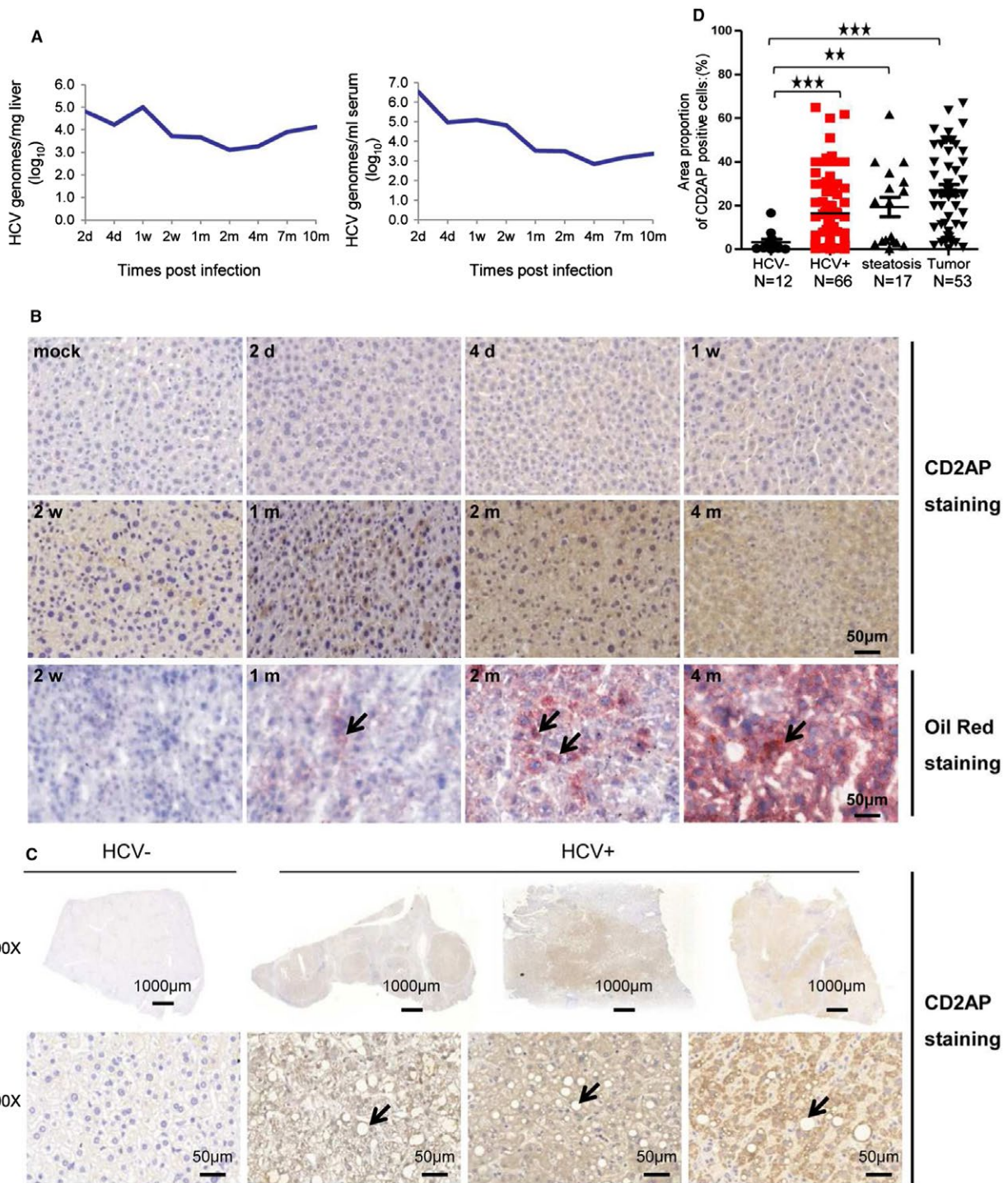


FIG. 7. CD2AP up-regulation correlates with steatosis in an animal model and in human biopsies. Human CD81 and occludin transgenic ICR mice were infected with HCV J399EM. HCV-RNA copy number in liver and serum (A) and IHC staining of CD2AP in liver sections (B) were determined at the indicated time points postinfection. Steatosis is shown by Oil Red O staining (arrows indicate steatosis at 1, 2, and 4 months postinfection). Scale bar, 50 μ m. (C) Representative IHC staining of CD2AP in human liver biopsies without HCV infection (HCV⁻) or with HCV infection (HCV⁺; arrows indicate steatosis). (D) CD2AP expression in peritumor of human liver biopsies without HCV infection (n = 12 cases), or with HCV infection (n = 66 cases); or with HCV infection showing steatosis (n = 17 cases), or CD2AP expression in tumor of HCV-positive human liver biopsies (n = 53 cases). Quantification was analyzed by Panoramic viewer software and is shown in scatter plot. A two-tailed Student *t* test was used for statistical analysis. Data are mean \pm SEM. **P* < 0.05; ***P* < 0.01; ****P* < 0.001. Abbreviations: d, day; w, week; m, month.

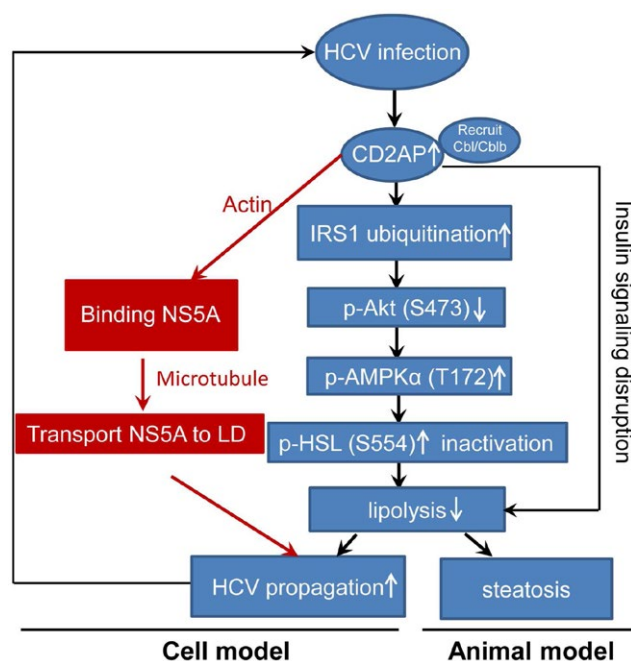


FIG. 8. Proposed model for CD2AP regulating HCV propagation and steatosis. HCV infection up-regulates CD2AP level, which binds NS5A in an actin-dependent manner and then NS5A is transported to LDs by the microtubule. Moreover, CD2AP recruits Cbl/Cbl-b E3 ligases to promote ubiquitination and degradation of IRS-1 to damp insulin signaling and thus preventing LDs hydrolysis to accommodate viral propagation. In the HCV-infected mouse model and human subjects, CD2AP up-regulation correlates well with LS.

expression is also significantly higher in liver biopsies with steatosis than in HCV-negative liver biopsies, further proving that CD2AP may contribute to steatosis (Fig. 7D).

In summary, we propose a model in which CD2AP regulates HCV propagation and steatosis (Fig. 8). HCV infection up-regulates CD2AP to facilitate HCV propagation by distinct methods: On one hand, CD2AP binds NS5A by actin to form a complex that is then transported to LDs in a microtubule-dependent manner. On the other hand, CD2AP recruits Cbl/Cbl-b E3 ligases to promote the ubiquitination and degradation of IRS-1, which causes a decrease in p-Akt, a negative regulator of AMPK. When p-Akt is reduced, the level of p-AMPK is increased. An increase in p-AMPK promotes phosphorylation and inactivation of HSL. Inactivation of HSL reduces LD hydrolysis, boosting LD levels, providing more hubs for HCV assembly. At present, we cannot exclude other possible ways in which CD2AP might affect HCV

propagation. In the HCV-infected mouse model and human subjects, CD2AP expression is up-regulated, and this up-regulation correlates well with LS. Thus, HCV infection-induced CD2AP expression results in a vicious cycle for HCV infection. However, how the vicious cycle is blocked by host responses remains to be investigated. Finally, the interplays between HCV infection, steatosis, and CD2AP expression are complicated. Elucidating these interplays will require studying a much larger cohort of patients as well as an additional animal model.

Although our study has added details into the biology of the HCV life cycle and liver pathology, many important questions remain to be explored. For example, how is CD2AP up-regulated by HCV infection? Does the detection of CD2AP have any prognostic values in HCV-infected patients? Furthermore, systematic studies of other already identified proteins will impart additional details to our understanding of HCV biology. Likewise, using a different HCV-encoded protein as the “bait” in the BioID approach, we may identify additional host proteins that are important in the HCV life cycle. Finally, a myriad of studies suggested that lowering of IRS1 level contributed to insulin resistance in type II diabetes.⁽⁴²⁾ Our finding that CD2AP regulates IRS-1 turnover may provide a potential therapeutic target to treat type 2 diabetes, in addition to curtailing HCV-associated pathology.

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