



LETTER

Interplay between hepatitis C virus and ARF4

Dear Editor,

Hepatitis C virus (HCV) is a major cause of chronic liver diseases and hepatocellular carcinoma (HCC) with about 71 million people globally infected. HCV encodes only 10 viral proteins and its replication relies on host proteins. Many host factors including ADP-ribosylation factors (ARFs) have been characterized (Tai et al., 2009; Matto et al., 2011; Zhang et al., 2012; Zhang et al., 2016; Zhou and Zhang, 2016).

The ARFs are evolutionarily conserved and ubiquitously expressed guanosine triphosphatases (GTPase), which belong to the Ras superfamily of small G proteins (Gillingham and Munro, 2007). ARFs have several important functions, including the recruitment of coat proteins that promote sorting of cargo into vesicles, the recruitment and activation of enzymes such as the phosphatidylinositol kinases that alter membrane lipid composition, and interaction with cytoskeletal factors. ARF proteins that can be divided into three classes based on sequence homology: Class I (ARF1, ARF3), Class II (ARF4, ARF5) and Class III (ARF6) (Gillingham and Munro, 2007). Recent study found that ARF4 is induced by Brefeldin A (BFA) to maintain golgi structure (Reiling et al., 2013). The transcription factor for ARF4 is CREB3/LUMAN/LZIP, a cell stress related endoplasmic reticulum (ER)-bound cellular transcription factor (Jang et al., 2012). The roles of ARFs in viral replication including HCV have been reported (Matto et al., 2011; Zhang et al., 2012; Zhang et al., 2016). Individual silencing ARF4 or ARF5 did not reduce HCV infection though knocking down ARF4 and ARF5 simultaneously reduced HCV replication (Farhat et al., 2016). Thus, the current study is to investigate the interplay between HCV and ARF4.

To perform the cell culture experiments, Huh7.5.1 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum. Jc1FLAG2 (p7-nsGluc2A) was obtained from Dr. Charles Rice. The OR6 cell line, which harbors full-length genotype 1b HCV RNA and coexpresses *Renilla* luciferase (from Drs. Nobuyuki Kato and Masanori Ikeda), was grown in DMEM supplemented with 10% FBS and 400 µg/mL of G418 (Promega, Madison, WI, USA). OR6 cells were treated with IFN α (10 IU/mL) for 7 days

to generate cured OR6 cells. HCV replication in OR6 cells or Jc1FLAG2(p7-nsGluc2A)-infected Huh7.5.1 cells was determined by monitoring *Renilla* or *Gaussia* luciferase activity (Promega, Madison, WI). Cellular ATP level was assessed for cell viability using the Cell Titer-Glo Luminescent Cell Viability Assay Kit (Promega, Madison, WI) according to the manufacturer's protocol.

To detect the mRNA transcription of ARF4 and RNA level of Jc1, quantitative PCR (qPCR) was performed as previously described (Li et al., 2014). Sequences of primers used in qPCR were as follows: the forward and reverse primers for GAPDH were 5'-ACCTTCCCCATGGTGCTGA-3' and 5'-GCTCCTCCTGTTTCGACAGTCA-3'; the forward and reverse primers for ARF4 were 5'-TGGTGGTGACTATCTCCCT-3' and 5'-CCGACTATTTGGCAAGAAGC-3'; the forward and reverse primers for Jc1 were 5'-TCTGCGGAACCGGTGAGTA-3' and 5'-TCAGGCAGTACCACAAGGC-3'.

To study the location of CREB3 upon HCV infection, we performed the immunofluorescence microscopy assay. Cells seeded onto glass coverslips were washed with phosphate-buffered saline (PBS) and fixed with 4% formaldehyde in PBS buffer for 5 min at room temperature. Fixed cells were stained as previously described (Li et al., 2014). 4', 6-diamidino-2-phenylindole (DAPI) is from Invitrogen (Carlsbad, CA).

To investigate how ARF4 protein expression is regulated by HCV, we infected Huh7.5.1 cells with Jc1 virus and the expression of ARFs was assessed by western blot. As shown in [Figure 1A](#), Jc1 infection induced the protein expression of ARF4. To confirm that, we used the OR6 cells to estimate the expression level of ARF4 in contrast to cured OR6 cells. The protein expression of ARF4 was significantly increased in OR6 cells compared with that in cured OR6 cells ([Figure 1B](#)). Likewise, the RNA level of ARF4 assessed by PCR was apparently enhanced in OR6 cells compared to cured OR6 cells ([Figure 1C](#)). Taken together, these data strongly suggested that HCV up-regulated ARF4 expression.

Upon stimulation, CREB3 is relocated to nucleus from ER to activate the transcription of ARF4 (Raggio et al., 2002). We wondered whether CREB3 is activated in OR6 cells. As shown in [Figure 1D](#), the CREB3 signal was detected in nucleus in OR6 cells, while most of CREB3 signal in cured OR6 cells was distributed in

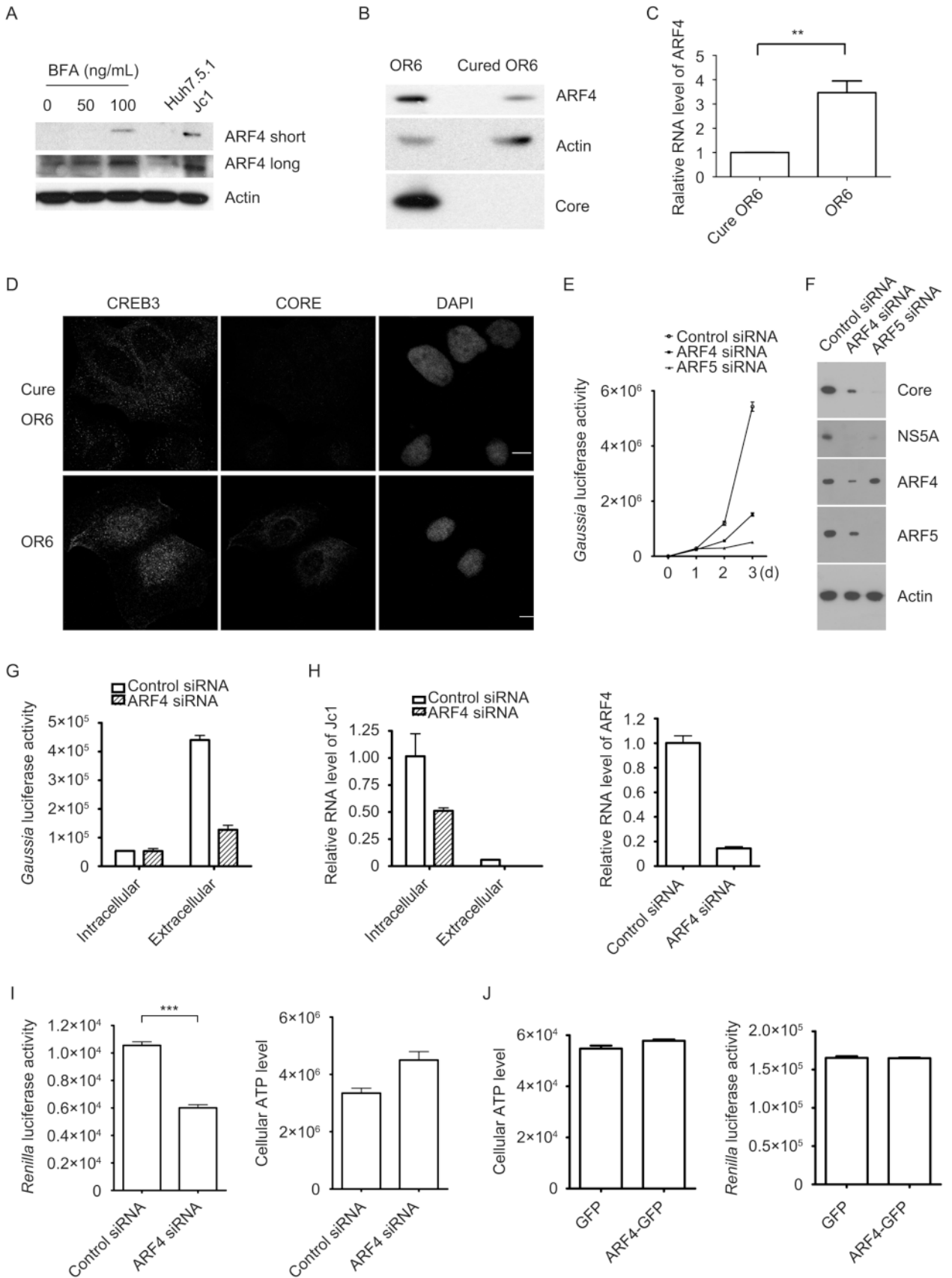


Figure 1. HCV upregulates ARF4, which is crucial for viral replication. (A) Cell lysates from Huh7.5.1 cells infected with Jc1FLAG2(p7-nsGluc2A) virus for 72 hours, or from Huh7.5.1 cells treated with different dosages of BFA for 16 hours were immunoblotted for ARF4 and actin as indicated. (B) Cell lysates from cured OR6 or OR6 cells were immunoblotted for ARF4, actin, and core as indicated. (C) Total RNA was isolated from OR6 or cured OR6 cells and reversely transcribed and then the quantitative PCR was performed. (D) OR6 or cured OR6 cells were fixed and immunolabeled with anti-CREB3 and anti-core. DAPI marks the nucleus. The scale bars represented 10 μ m. (E) Huh7.5.1 cells were treated with siRNA targeting ARF4 or control siRNA for 72 hours, and then infected with Jc1FLAG2 (p7-nsGluc2A) virus, followed by measuring the *Gaussia* luciferase activity in different days post infection. Data were represented the average \pm SD. (F) Cell lysates from Jc1-infected Huh7.5.1 cells treated with siRNA against ARF4 or control siRNA for 72 hours were immunoblotted for core, NS5A, ARF4, ARF5, and actin as indicated. (G) Huh7.5.1 cells were transfected with ARF4 siRNA or control siRNA for 72 h and then infected with Jc1 for 72 h. The cells were collected and tittered as intracellular titer. The supernatants were tittered as extracellular titer. (H) Huh7.5.1 cells were transfected with ARF4 siRNA or control siRNA for 72 h and then infected with Jc1 for 72 h. Total RNA from cells and supernatants was isolated and reverse transcribed and then the real time PCR was performed. (I) OR6 cells were treated with siRNA against ARF4 or control siRNA for 72 hours, followed by measuring the *Renilla* luciferase activity and cellular ATP levels. Data were represented the average \pm SD. (J) OR6 cells were transfected with constructs expressing ARF4-GFP or GFP for 48 hours followed by measuring the *Renilla* luciferase activity and cellular ATP levels. Data were represented the average \pm SD.

cytoplasm, indicating that HCV induces the nuclear localization of CREB3.

Previous study reported that individual silencing ARF4 or ARF5 resulted in moderate (30%–35%) inhibition of HCV infection (Farhat *et al.*, 2016). However, their conclusion was only based on qPCR analysis and they didn't validate the depletion of proteins of ARF4 or ARF5 in JFH1-CSN6A4 infected Huh7 cells. Therefore, we explored whether ARF4 and ARF5 are required for HCV infection in our system. Huh7.5.1 cells were treated with siRNA against ARF4 (AGACAACCAUUCUGUA UAA), ARF5 (CCACAAUCCUGUACAAACU) or control siRNA for 72 hours and then infected with Jc1FLAG2 (p7-nsGluc2A). As shown in [Figure 1E](#), HCV replication measured by *Gaussia* luciferase reporter assay was reduced by siRNA against ARF4 or ARF5. Moreover, protein levels of HCV NS5A and core were decreased by ARF4 or ARF5 siRNA ([Figure 1F](#)). These results suggested that individually silencing ARF4 or ARF5 reduced HCV replication, suggesting a key role for ARF4 and ARF5 in HCV replication.

To further investigate the potential role of ARF4 on HCV life cycle. We examined the intracellular and extracellular virus titers. Upon ARF4 siRNA treatment, the extracellular titer was reduced, while the intracellular titer remained the same ([Figure 1G](#)). We also quantified the intracellular and extracellular RNA by qPCR experiments. Both intracellular and extracellular viral RNA were reduced by ARF4 siRNA ([Figure 1H](#)). Silencing ARF4 also reduced HCV replication in OR6 cells measured by *Renilla* luciferase reporter assay without reducing cell viability measured by cellular ATP level ([Figure 1I](#)). However, overexpression of ARF4-GFP has no role in HCV replication in OR6 cells compared with overexpression of GFP ([Figure 1J](#)). Taken together we concluded that ARF4 played a positive role in HCV replication

and release.

It has been known that HCV core protein inactivates CREB3 function through subcellular sequestration (Jin *et al.*, 2000). However we found that HCV caused the relocation of CREB3 to nucleus. We speculate that HCV might induce Golgi stress to activate the transcription factor CREB3 to express ARF4. Considering the suppression of CREB3 function by HCV core, there must be a limited activation of CREB3 during HCV chronic infection, resulting in a balance between ARF4 and HCV.

In summary, we show here that HCV infection is associated with an upregulation of ARF4, which promotes HCV replication. Upon HCV infection, CREB3 was redistributed to nucleus and activated ARF4 transcription. Our studies demonstrate a host factor ARF4 upregulated in HCV replication, which may provide new therapeutic targets for antiviral therapy.

FOOTNOTES

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