Antiviral Research 124 (2015) 11–19

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Requirement of the eukaryotic translation initiation factor 4F complex in hepatitis E virus replication

Xinying Zhou, Lei Xu, Yijin Wang, Wenshi Wang, Dave Sprengers, Herold J. Metselaar, Maikel P. Peppelenbosch, Qiuwei Pan*

Department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center, Rotterdam, The Netherlands

ARTICLE INFO

Article history:
Received 11 August 2015
Received in revised form
15 October 2015
Accepted 19 October 2015
Available online 23 October 2015

Keywords:
Hepatitis E virus
Eukaryotic translation initiation factor 4F complex
eIF4E-binding protein 1
Ribavirin
IFN-α

ABSTRACT

Hepatitis E virus (HEV) infection, one of the foremost causes of acute hepatitis, is becoming a health problem of increasing magnitude. As other viruses, HEV exploits elements from host cell biochemistry, but we understand little as to which components of the human hepatocellular machinery are perverted for HEV multiplication. It is, however, known that the eukaryotic translation initiation factors 4F (eIF4F) complex, the key regulator of the mRNA-ribosome recruitment phase of translation initiation, serves as an important component for the translation and replication of many viruses. Here we aim to investigate the role of three subunits of the eIF4F complex: eukaryotic translation initiation factor 4A (eIF4A), eukaryotic translation initiation factor 4G (eIF4G) and eukaryotic translation initiation factor 4E (eIF4E) in HEV replication. We found that efficient replication of HEV requires eIF4A, eIF4G and eIF4E. Consistently, the negative regulatory factors of this complex: programmed cell death 4 (PDCD4) and eIF4E-binding protein 1 (4E-BP1) exert anti-HEV activities, which further illustrates the requirement for eIF4A and eIF4E in supporting HEV replication. Notably, phosphorylation of eIF4E induced by MNK1/2 activation is not involved in HEV replication. Although ribavirin and interferon-α (IFN-α), the most often-used off-label drugs for treating hepatitis E, interact with this complex, their antiviral activities are independent of eIF4E. In contrast, eIF4E silencing provokes enhanced anti-HEV activity of these compounds. Thus, HEV replication requires eIF4F complex and targeting essential elements of this complex provides important clues for the development of novel antiviral therapy against HEV.

1. Introduction

Hepatitis E virus (HEV), a single-strand-positive RNA virus classified within the genus *Hepevirus* in the family *Hepeviridae*, represents the most common cause of acute viral hepatitis (Kamar et al., 2014a). Like all viruses, HEV is completely dependent on the translational machinery of host cells to synthesize the viral proteins essential for its productive infection (Walsh and Mohr, 2004). The host protein synthesis machinery commandeered by viruses has major impact on viral protein synthesis and genome replication (Montero et al., 2015), but little is known regarding how HEV uses host translational machinery for its life-cycle.

As a heterotrimeric protein complex, eukaryotic translation initiation factor 4F (eIF4F) mediates recruitment of ribosomes to mRNA and is the rate-limiting step for cap-dependent translation in viruses and cells under most circumstances (Gingras et al., 1999). Functions of the constituent proteins of eIF4F include delivery of an RNA helicase eukaryotic initiation translation factor 4A (eIF4A) to the 5′ region, bridging mRNA and ribosome by eukaryotic initiation translation factor 4G (eIF4G) scaffolding protein and recognition of the mRNA 5′ cap structure by eukaryotic initiation translation factor 4E (eIF4E) cap-binding protein (Gingras et al., 1999). Not surprisingly, all these translation initiation factors are required for

Abbreviations: HEV, hepatitis E virus; eIF4F, eukaryotic translation initiation factor 4F; eIF4A, eukaryotic initiation translation factor 4A; eIF4G, eukaryotic initiation translation factor 4G; eIF4B, eukaryotic initiation translation factor 4B; PDCD4, programmed cell death 4; eIF4E, eukaryotic initiation translation factor 4E; 4E-BP1, eIF4E-binding protein 1; MEFs, mouse embryonic fibroblasts; IFN, interferon; JAK1, Janus kinase 1; STAT1, signal transducers and activators of transcription 1; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle medium; qRT-PCR, quantitative real-time polymerase chain reaction; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

* Corresponding author. Department of Gastroenterology and Hepatology, Erasmus MC, Room Na-617, 's-Gravendijkwal 230, NL-3015 CE Rotterdam, The Netherlands.
E-mail address: q.pan@erasmusmc.nl (Q. Pan).

http://dx.doi.org/10.1016/j.antiviral.2015.10.016
0166-3542/

various types of viruses during their translation and replication (de Breyne et al., 2012; George et al., 2012; Ventoso et al., 2001; Walsh and Mohr, 2004). In addition, eIF4E phosphorylation is induced by the eIF4G-associated kinase MNK1 to facilitate eIF4F assembly (Walsh et al., 2013). This process of translational control has been reported to be critical for the efficient viral infection (Royall et al., 2015; Walsh and Mohr, 2004; Walsh et al., 2005). Furthermore, other cellular regulatory proteins of eIF4F complex such as eukaryotic translation initiation factor 4B (eIF4B) (Harms et al., 2014), programmed cell death 4 (PDCD4) (Dennis et al., 2012) and eIF4E-binding protein 1 (4E-BP1) have been reported vital for viral protein synthesis (Damania et al., 2014; Kaur et al., 2007; Wang et al., 2014a). HEV, however, has not been investigated in this context and it is currently unknown whether the virus requires eIF4F complex for efficient replication.

Interestingly, the eIF4F complex can interact with antiviral regimens, such as ribavirin or interferon-α (IFN-α), which are the classical standard therapy of chronic hepatitis C but also as off-label drugs for treating individual HEV cases or small case series (Kamar et al., 2014b; Zhou et al., 2013). Ribavirin can directly bind to eIF4E and compete for 5′ cap mRNA binding (De la Cruz-Hernandez et al., 2015; Kentsis et al., 2004), whereas some regulatory factors of eIF4F complex are involved in interferon mediated antiviral immune response (Burke et al., 2011; Nehdi et al., 2014). In absence, however, of information as to requirement of HEV for elements of the host translational machinery it is impossible to make statements whether ribavirin exerts its anti-HEV action through inhibition of the eIF4F complex or whether alternative mechanisms are involved.

The lack of knowledge as to the requirements made by HEV on the hepatocellular host cell machinery with respect to translation of viral gene products represents a major gap in our understanding of the biology of this virus and hampers design of rational treatment. Therefore, this study has investigated the role of the eIF4F complex and its regulatory factors in HEV replication, as well as their potential involvements in the anti-HEV actions of ribavirin and IFN-α.

2. Materials and methods

2.1. Reagents

Compound GCP57380 as Mnk1 inhibitor (>98% purity) was purchased from Abcam Biochemicals (UK). Ribavirin was purchased from Sigma-Aldrich (St Louis, MO). Human IFN-α (Thermo Scientific, the Neterlands) was dissolved in PBS. Doxycycline hyclate (≥98% TLC) was purchased from Sigma-Aldrich (St Louis, MO). Stocks of Jak inhibitor I (Santa Cruz Biotech, Santa Cruz, CA) was dissolved in DMSO (Sigma-Aldrich, St Louis, MO) with a final concentration of 5 mg/ml. Antibodies including total-eIF4E, phosphor-eIF4E, total-4E-BP1 (Cell Signalling Technology, Netherlands) and β-actin (Santa Cruz Biotech, Santa Cruz, CA); anti-rabbit or anti-mouse IRDye-conjugated secondary antibodies (Stressgen, Glandford Ave, Victoria, BC, Canada) were also used.

2.2. Cell culture and cell models

Naïve or vector transduced HuH7 cells was established from a hepatocellular carcinoma, immortalized mouse embryonic fibroblasts (MEFs) derived from wild-type and 4E-BP1 knock-out (4E-BP1−/−) mice (kind gifts from E.N. Fish's lab), eIF4E−S209A MEFs containing an eIF4E mutation in which eIF4E cannot be phosphorylated (kind gift from Dr Sonenberg's lab, McGill University) were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen-Gibco, Breda, The Netherlands) complemented with 10% v/v fetal calf serum (Hyclone, Lonan, Utah), 100 IU/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine (Invitrogen-Gibco). Authentication of cell line was performed at the Department of Pathology, Erasmus MC and regular testing for mycoplasma contamination was performed at the Laboratory of Gastroenterology and Hepatology, Erasmus MC.

HEV genomic RNA was generated from a plasmid construct containing the full-length HEV genome (Kernow-C1 p6 clone, GenBank Accession Number JQ679013) or a construct containing subgenomic HEV sequence coupled with a Gaussia luciferase reporter gene (p6-luc), using the Ambion MESSAGE MACHINE in vitro RNA transcription Kit (Life Technologies Corporation) (Shukla et al., 2012, 2011). The human hepatoma 7 (HuH7) cells were collected and centrifuged for 5 min, 1500 rpm, 4 °C. Supernatant was removed and washed with 4 ml Opti-MEM by centrifuging for 5 min, 1500 rpm, 4 °C. The cell pellet was re-suspended in 100 μl Opti-MEM and mixed with p6 full-length HEV RNA or p6-Luc subgenomic RNA. Electroporation was performed with the Bio-Rad's electroporation systems using the protocol of a designed program (240 V, pulse length 0.5, number 1 and cuvette 4 mm) (Shukla et al., 2012). All cells were grown at 37 °C, 5% CO_2, and 100% humidity.

2.3. Gene knockdown and overexpression by lentiviral vector

Lentiviral vectors of shRNA (Sigma–Aldrich) targeting eIF4A, eIF4G, eIF4B, PDCD4, eIF4E, 4E-BP1 and controls were obtained from the Erasmus Center for Biomics and produced in HEK 293 T cells as previously described (Pan et al., 2009). Three types of control vectors have been tested on HEV replication (CTR1: Control that will not activate the RNAi pathway because the vector does not contain an shRNA insert, CTR2: Control that will activate RISC and the RNAi pathway, but does not target any human or mouse genes. The short hairpin sequence contains 5 bp mismatches and scrambled sequences to any known human or mouse gene, CTR3: Control contains shRNA sequence that targets GFP reporter that is not expressed in our cell lines. Since no off-target effect was observed (Fig. S1), the most advanced shRNA control vector targeting GFP (GFP is not expressed in our cell lines) was used in this study as control (shCTR). After a pilot study, the shRNA vectors exerting optimal gene knockdown were selected. These shRNA sequences were described in Supplementary Table 1. To generate gene knockdown cells, HuH7 cells were transduced with lentiviral vectors. Since the knockdown vectors also express a puromycin resistance gene, transduced cells were subsequently selected by adding 3 μg/ml puromycin to the cell culture medium. Overexpression of 4E-BP1 lentivector (Addgene) was a kind gift from Dr Sonenberg's lab, McGill University. To generate overexpression cells, HuH7 cells were transduced with lentiviral vectors and doxycycline was used to add in the 4E-BP1 overexpression cell lines as the stimulation factor.

2.4. Quantitative real-time polymerase chain reaction

RNA was isolated with a Machery-Nucleo Spin RNA II kit (Bioke, Leiden, The Netherlands) and quantified using a Nanodrop ND-1000 (Wilmington, DE, USA). cDNA was prepared from total RNA using a cDNA Synthesis Kit (TAKARA BIO INC). The cDNA was quantified with a SYBR Green-based real-time PCR (MJ Research Opticon, Hercules, CA, USA) according to the manufacturer's instructions. GAPDH or β-actin was considered as reference gene to normalize gene expression. The HEV primer sequences were 5′-ATTGGCCAGAAGTTGGTTTTCAC-3’ (sense) and 5′-CCGTGGCTATAATTGTGGTCT-3′ (antisense). Other human and mouse primer sequences were included in Supplementary Table 2.

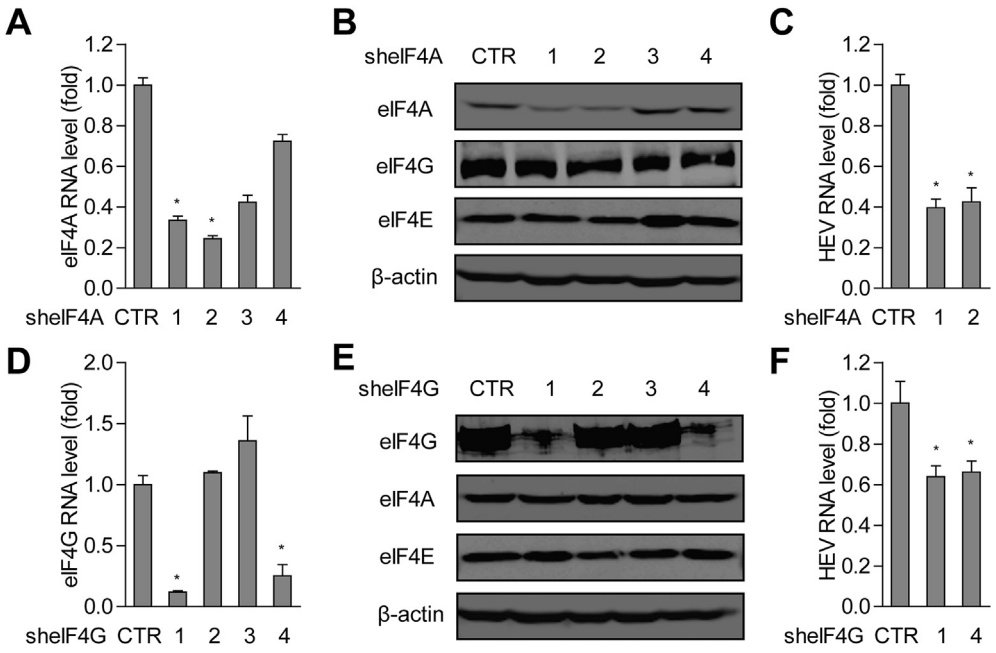

Fig. 1. Requirements of eIF4A and eIF4G for HEV replication. Knockdown of eIF4A and eIF4G by lentiviral shRNA vectors were performed in Huh7 cells. Compared with the control vector transduced cells, the sheIF4A clone 1 and 2 (A and B) or the sheIF4G clone 1 and 4 (D and E) showed potent gene silencing at mRNA level and protein level. Correspondingly, knockdown of eIF4A (C) and eIF4G (F) resulted in significant increase of cellular HEV RNA level (Mean ± SEM, n = 4). $^*P < 0.05$.

2.5. Western blot assay

Proteins in cell lysates were heated 5 min at 95 °C followed by loading onto a 10-15% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) and separated by electrophoresis. After 90 min running at 100 V, proteins were electrophoretically transferred onto a polyvinylidenedifluoride (PVDF) membrane (Invitrogen) for 1.5 h with an electric current of 250 mA. Subsequently, the membrane was blocked with blocking buffer. It was followed by incubation with rabbit t-eIF4E, p-eIF4E, t-4E-BP1 (1:1000) antibodies overnight at

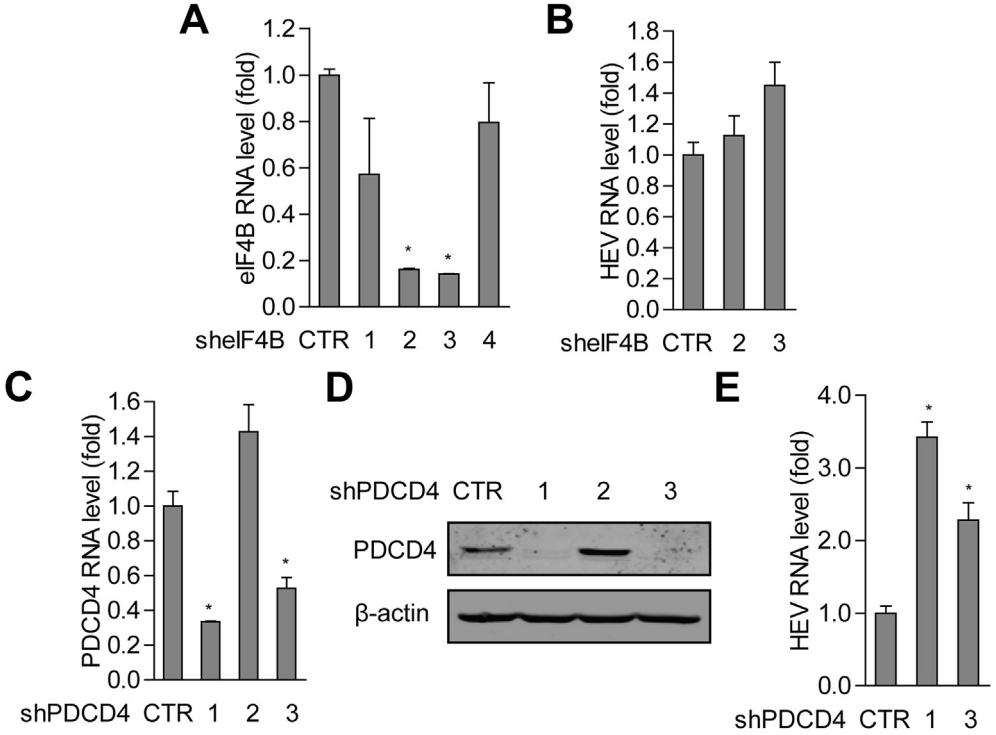

Fig. 2. The regulatory factors of eIF4A, PDCD4 but not eIF4B, restricted HEV replication. (A) Clone 2 and 3 Knockdown of eIF4B by lentiviral shRNA vectors exerted significant downregulation of eIF4B at mRNA level. (B) Silencing of eIF4B did not influence HEV replication during 72 h inoculation of viral particles. mRNA level (C) and protein level (D) of PDCD4 were significantly reduced in clone 1 and 3. (E) HEV RNA level were dramatically increased when silencing PDCD4 in clone 1 and 3. (Mean ± SEM, n = 4). $^*P < 0.05$.

4 °C. The membrane was washed 3 times followed by incubation for 1 h with anti-rabbit or anti-mouse IRDye-conjugated secondary antibodies (LI-COR Biosciences, Lincoln, USA) (1:5000) at room temperature. Blots were assayed for β-actin content as standardization of sample loading, scanned, and quantified by Odyssey infrared imaging (Li-COR Biosciences, Lincoln, NE, USA). Results were visualized and quantitated with Odyssey 3.0 software.

2.6. Measurement of luciferase activity

For Gaussia luciferase, the activity of secreted luciferase in the cell culture medium was measured by BioLux® Gaussia Luciferase Flex Assay Kit (New England Biolabs). For firefly luciferase, luciferin potassium salt (100 mM; Sigma) was added to cells and incubated for 30 min at 37 °C. Both gaussia and firefly Luciferase activity was quantified with a LumiStar Optima luminescence counter (BMG LabTech, Offenburg, Germany).

2.7. Statistical analysis

All results were presented as mean ± SEM. Comparisons between groups were performed with Mann–Whitney test. Differences were considered significant at a p value less than 0.05 *or 0.01 **.

3. Results

3.1. Requirements of eIF4A and eIF4G for efficient HEV replication

Most of cellular and viral mRNAs rely on cap-dependent mRNA translation. The canonical mechanism of initiation commences with recognition of 5′ end m^7GpppN cap structure by the eIF4F complex formed by the DEAD-box helicase eIF4A, the scaffolding protein eIF4G and the cap recognition factor eIF4E (Sonenberg and Hinnebusch, 2009). Among these initiation factors, eIF4A is a subunit in charge of unwinding of secondary structure within the leader sequence of mRNA, while large scaffolding subunit eIF4G is associated with many other translation initiation factors (de Breyne et al., 2012). Because of the important roles of both eIF4A and eIF4G subunits reported in translation and replication of many viruses, we investigated their roles in HEV replication.

Firstly, we evaluated the role of the DEAD-box RNA helicase eIF4A by using RNAi-based gene loss-of-function approach. Two out of four (sheIF4A-1 and sheIF4A-2) shRNAs targeting eIF4A showed significant reduction of its mRNA level in HuH7 cells, compared with a control shRNA targeting GFP (shCTR) (Fig. 1A). Consistently, their protein levels were also downregulated without affecting the expression of eIF4G and eIF4E, which suggested a successful knockdown of eIF4A (Fig. 1B). No cytotoxicity has been observed in these cells as measured by MTT assay (Fig. S2A). Silencing of eIF4A resulted in significant decrease of cellular HEV RNA level by 63.1% ± 8.6% and 57.6% ± 13.8% (mean ± SEM, n = 4, $P < 0.05$) after three days inoculation of HEV particles, respectively (Fig. 1C). Next, knockdown of scaffold protein eIF4G by four shRNAs were also performed in HuH7 cells. Two clones (sheIF4G-1 and sheIF4G-4) showed efficient down-regulation of eIF4G at both mRNA level and protein level, but did not influence the protein level of eIF4A or eIF4E (Fig. 1D and E). No cytotoxicity was observed in these knockdown cells (Fig. S2B). Correspondingly, HEV RNA levels was significantly reduced by 36.1% ± 10.6% and 33.8% ± 11.0% (mean ± SEM, n = 4, $P < 0.05$) in both eIF4G knockdown cells, respectively, compared to shCTR cells (Fig. 1F). These results demonstrate that both eIF4A and eIF4G are required for efficient HEV replication.

3.2. PDCD4, the negative regulatory factor of eIF4A, restricts HEV replication

Given the fact that the function of eIF4A is regulated by multiple cellular factors, we first investigated the effect of its activator eIF4B that can increase the helicase activity of eIF4A. Knockdown of eIF4B by two out of four shRNA clones (sheIF4B-2 and sheIF4B-3) resulted in significant down-regulation of eIF4B expression (Fig. 2A), but has no significant influence on cellular HEV RNA level (Fig. 2B).

We next examined a negative regulatory factor of eIF4A, PDCD4, which prevents the binding of eIF4A to eIF4G and thereby inhibits

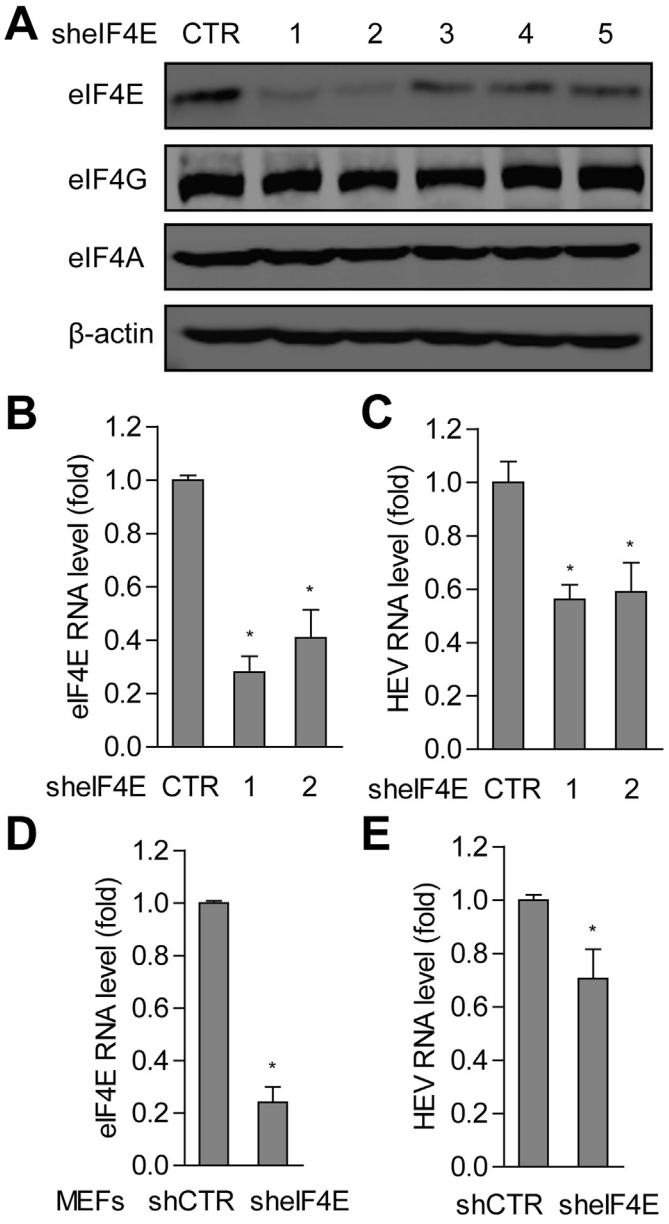

Fig. 3. eIF4E supports HEV replication. Knockdown of human eIF4E by five lentiviral shRNA vectors were performed in HuH7 cells. Clone 1 and 2 exhibited a potent down-regulation of eIF4E at protein (A) and mRNA (B) levels. β-actin served as an internal reference. (C) Correspondingly, these two clones of eIF4E knockdown inhibited HEV replication. (D) Knockdown of mouse eIF4E by lentiviral shRNA vector was applied in MEFs showed a significant decrease at eIF4E mRNA level. (E) HEV RNA level was significantly reduced with eIF4E silencing in MEFs. (Mean ± SEM, n = 4). *$P < 0.05$.

the initiation of translation (Pelletier et al., 2015), To assess the effect of PDCD4 on HEV replication, lentiviral shRNA vectors were used to stably knockdown its expression in HuH7 cells. Potent down-regulation of PDCD4 mRNA and protein expression of two clones (shPDCD4-1 and shPDCD4-3) (Fig. 2C and D) resulted in a significant increase of HEV RNA after inoculation of HEV particles for three days (Fig. 2E). No cytotoxicity was observed in knockdown cells (Fig. S2C). These results are consistent with the finding that eIF4A supports HEV replication and inhibiting the function of eIF4A by PDCD4 in turn suppresses HEV replication.

3.3. eIF4E is also required for efficient HEV replication

eIF4E, the least abundant component of the eIF4F complex, is a rate-limiting factor for translation (Mohr and Sonenberg, 2012), To investigate the role of eIF4E in HEV replication, its expression was silenced by RNAi. Two out of five shRNAs targeting eIF4E exerted potent knockdown at both protein (Fig. 3A) and mRNA levels (Fig. 3B). No off-target effect was observed on protein expression of eIF4G or eIF4A (Fig. 3A), or on genes such as CyA, CyB, 4E-BP1 and mTOR, which are known to affect HEV replication as previously shown (Wang et al., 2014b; Zhou et al., 2014) (Fig. S3). MTT assay showed no cytotoxicity of eIF4E silencing in cells (Fig. S2D). Accordingly, inoculation of HEV led to reduction by 44% ± 12% and 41% ± 25% (mean ± SEM, n = 5, $P < 0.05$) in viral RNA level in these two knockdown cells compared to shCTR cells (Fig. 3C). We observed similar effect in MEFs (Fig. 3D and E), further confirming that eIF4E plays an important role in HEV replication.

3.4. Phosphorylation is not required for eIF4E to support HEV replication

Ser209 phosphorylation has been shown to be required for the oncogenic potential of eIF4E (Panda et al., 2014). To examine whether this is also important in the context of HEV infection, HuH7 cells harboring the HEV subgenomic replicon or the full-length genome were treated with 10–100 μM CGP57380, a well-characterized inhibitor of MNK. MNK is the only known physiologic kinase that phosphorylates eIF4E (Ser209 site) (Kyriakis, 2014). This compound potently inhibited eIF4E–S209 phosphorylation without effect on total eIF4E protein level (Fig. 4A). However, CGP57380 had no effect on HEV replication in both luciferase replicon model (Fig. 4B) and infectious model (Fig. 4C). To further confirm the function of phosphorylation of eIF4E, MEFs cultured from mice with S209A mutation were used. This mutation targeting the conserved phosphorylation site for MNK1/2 kinase with serine-to-alanine completely abolished phosphorylation of eIF4E at Ser209 without effect on total eIF4E (Fig. 4D). Consistently, no significant effect was observed on HEV replication between mutated and wild type MEFs (Fig. 4E). These data suggest that phosphorylation of eIF4E is dispensable for HEV replication.

3.5. HEV replication is inhibited by the eIF4E suppressor, 4E-BP1

eIF4E can be released by 4E-BP1 hyperphosphorylation with elimination of translational repression (Nehdi et al., 2014). For a more detailed characterization of the role of eIF4E suppressor 4E-BP1 in HEV infection, we employed both loss- and gain-of-function approaches in HuH7 cells. Using lentiviral RNAi technique, 4E-BP1 was dramatically down-regulated at both protein and mRNA levels (Fig. 5A). Accordingly, 48 h inoculation of HEV resulted in 2.6 ± 1.2 fold (mean ± SEM, n = 4, $P < 0.05$) increase of viral RNA in 4E-BP1 knockdown HuH7 cells, compared with the mock knockdown cells (Fig. 5A). In contrast, using an inducible over-expression lentiviral vector, 4E-BP1 expression was drastically up-regulated at both protein and mRNA levels with treatment of dose dependent doxycycline, which resulted in significant reduction of HEV RNA by 59% ± 17% (mean ± SEM, n = 4, $P < 0.05$) (Fig. 5B).

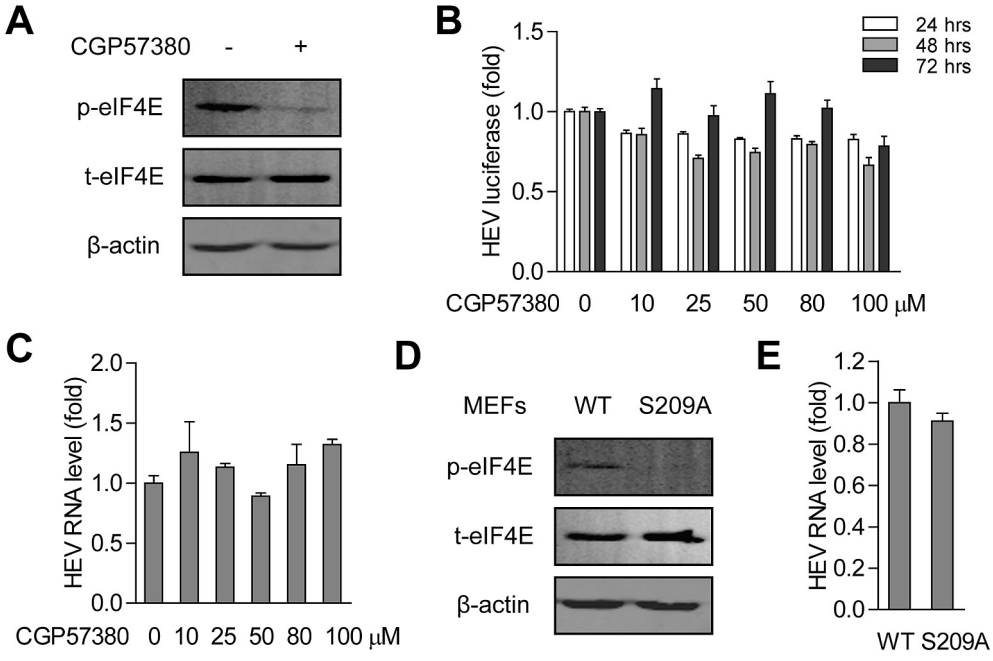

Fig. 4. eIF4E phosphorylation did not significantly affect HEV replication. (A) Treatment with MNK1/2 inhibitor CGP57380 of 100 μM dramatically decreased the phosphorylation of eIF4E, but not total eIF4E protein shown by Western blot assay. β-actin served as an internal reference. 10–100 μM CGP57380 did not significantly affected viral replication-related luciferase activity during the three days (B) and viral RNA level in HuH7-p6 infectious model for 48 h (C). (D) MEFs of S209A mutation abolished phosphorylation of eIF4E at Ser209 shown by Western blot assay. β-actin served as an internal reference. (E) Inhibition of phosphorylation in S209A MEFs did not significantly influence HEV replication. (Mean ± SEM, n = 4).

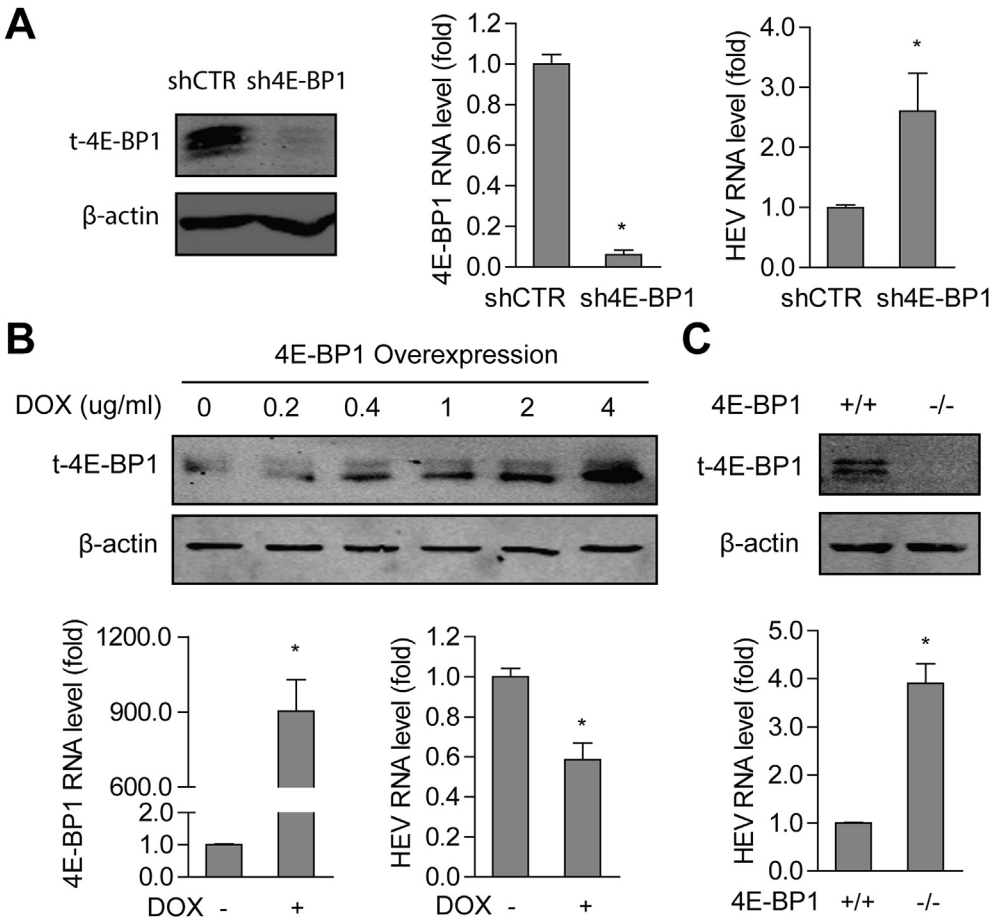

Fig. 5. The eIF4E suppressor, 4E-BP1, limited HEV replication. (A) Efficient silencing of 4E-BP1 at protein level was detected by Western blot assay. β-actin served as an internal reference. Similarly, 4E-BP1 was significantly down-regulated at mRNA level (Mean ± SEM, n = 7). Correspondingly, inoculation of HEV resulted in significant increase of viral RNA in 4E-BP1 knockdown Huh7 cells, compared with the mock knockdown cells (Mean ± SEM, n = 4). (B) 4E-BP1 was drastically up-regulated at protein level when the over-expression cell lines were treated with different concentrations of doxycycline for 24 h β-actin served as an internal reference. Similarly, mRNA levels of 4E-BP1 was dramatically increased after treatment of 4 μg/ml doxycycline for 24 h (Mean ± SEM, n = 5). Correspondingly, 4E-BP1 over-expression cell line with treatment of 4 μg/ml doxycycline for 24 h resulted in significant reduction of HEV RNA level (Mean ± SEM, n = 4). (C). MEFs derived from 4E-BP1 knockout (4E-BP1−/−) mice presented an efficient silencing of 4E-BP1 at protein level compared to WT MEFs, leading to significant increase of HEV RNA level (Mean ± SEM, n = 4). *$P < 0.05$.

To further validate 4E-BP1 function in HEV infection, MEFs cultured from 4E-BP1 knockout mice were studied. Western blot assay showed a complete knockout of 4E-BP1 at protein level (Fig. 5C). Accordingly, 48 h inoculation of HEV led to 3.9 ± 0.8 fold (mean ± SEM, n = 4, $P < 0.05$) increase of cellular HEV RNA level in 4E-BP1 knockout MEFs (4E-BP1−/−) compared to wild type MEFs (4E-BP1+/+) (Fig. 5C). Hence, these data supported a role of 4E-BP1 in constraining HEV replication.

3.6. The anti-HEV activities of ribavirin and IFN-α are independent of eIF4E

Ribavirin is a well-known inhibitor of eIF4E (Kentsis et al., 2004). To evaluate whether the anti-HEV activity of ribavirin is mediated by the inhibition of eIF4E, both shCTR and sheIF4E-1 cells with HEV inoculation were used for the treatments of 6.25, 12.5, 25, 50, 100 μM ribavirin for 48 h. Comparable IC50 in shCTR and sheIF4E cells were found with the range between 12.5 and 25 μM (Fig. 6A).

The antiviral activity of IFN-α has also been associated to the regulation of translation initiation factors in particular circumstances (Burke et al., 2011; Nehdi et al., 2014). To further assess whether eIF4E could mediate the anti-HEV activity of IFN-α, treatments of 1, 10, 100, 1000, 10,000 IU/ml IFN-α on HEV in both shCTR and sheIF4E-1 cells for 48 h has been performed. Comparable IC50 of IFN-α were observed with the range between 10 and 50 IU/ml in shCTR and sheIF4E cells (Fig. 6B). Furthermore, as expected, HEV replication were significantly inhibited with treatment of 25 μM ribavirin and 1000 IU/ml IFN-α for 48 h. However, the anti-HEV effects of ribavirin and IFN-α were further enhanced by eIF4E knockdown (Fig. 6C). In addition, no clear cytotoxicity was observed in both shCTR and sheIF4E cells (Fig. S4A and 4B). These results indicated that the antiviral effects of ribavirin and IFN-α are independent of eIF4E, although silencing of eIF4E could already inhibit HEV replication.

4. Discussion

Most of the viruses can only encode restricted numbers of proteins and therefore they heavily rely on the host cellular machinery and their ingredients to accomplish the virus life-cycles (Montero et al., 2015). Recent studies show that translation initiation mechanisms especially eIF4F complex is employed by many viruses as a primary target for cap-dependent translational control to confer advantages to generate progeny (Bushell and Sarnow, 2002). Three proteins: RNA helicase eIF4A, scaffolding protein eIF4G and cap binding protein eIF4E, which are components of the eIF4F complex, are related to the efficient translation and replication of various viruses (de Breyne et al., 2012; George et al., 2012;

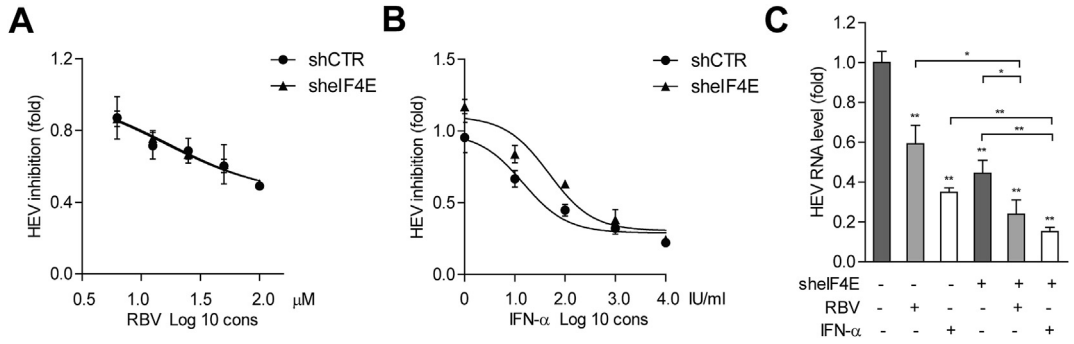

Fig. 6. The anti-HEV activities of ribavirin and IFN-α is independent of eIF4E. (A) IC50 of RBV was with the range between 12.5 and 25 μM: 15.67 μM in shCTR cells and 17.24 μM in sheIF4E cells and (B) IFN-α was with the range between 10 - 50 IU/ml: 14.55 IU/ml in shCTR cells and 47.75 IU/ml in sheIF4E cells). (C) 25 μM ribavirin and 1000 IU/ml IFN-α were treated in shCTR and sheIF4E cells. HEV RNA level was detected by qRT-PCR after 72 h inoculation of viral particle. (Mean ± SEM, n = 4-8). $^{*}P < 0.05$. $^{**}P < 0.01$.

Ventoso et al., 2001; Walsh and Mohr, 2004). It is, however, unknown to what extent HEV requires elements from the translation initiation complex. Our study was aimed to provide more insight in this area of HEV biology, also with the explicit goal to provide directions for the development of rational treatment of HEV-related disease. Our study demonstrated a requirement of the eIF4F complex for efficient HEV replication (Figs. 1 and 3).

Among all three subunits of the eIF4F complex, eIF4E is the main regulatory nexus involved in the complex formation and has impact on many types of viral infections (Montero et al., 2015), including on HEV as we showed in this study. One of the mechanisms by which eIF4E takes control of complex formation and translation initiation process is via phosphorylation on serine 209 carried out by MNK1/2 (Montero et al., 2015). Stimulation of eIF4E phosphorylation is correlated with facilitated translation and replication of some viruses (Panda et al., 2014; Royall et al., 2015; Walsh and Mohr, 2004). In contrast, we found that S209 phosphorylation is not required for eIF4E to support HEV replication (Fig. 4). Another regulatory mechanism of eIF4E is exerted via 4E-BP1, a small-molecular-weight repressor of 5′ capped mRNA translation, which has also been implicated in host defense against viral infection (Kaur et al., 2007). In apparent agreement, we show that 4E-BP1 can inhibit HEV replication (Fig. 5). 4E-BP1 is a phosphoprotein that binds to eIF4E depending on its phosphorylation status. 4E-BP1 hyperphosphorylation results in releasing eIF4E to form the functional eIF4F complex. Conversely, 4E-BP1 hypophosphorylation allows binding of this protein to eIF4E and counteracts the formation of eIF4F complex (Montero et al., 2015). Therefore, without this hijacking of eIF4E in a 4E-BP1-deficient context, eIF4E can still exert its pro-HEV activity (as we have shown in Fig. S5). Similarly, the eIF4A suppressor PDCD4 can also restrict HEV replication (Fig. 2). PDCD4 sequesters eIF4A from the eIF4E-eIF4G complex, resulting in repressed translation of mRNAs (Dennis et al., 2012) and thus modulates replication of various viruses (Damania et al., 2014; Wang et al., 2014a). Apparently this notion also holds true in the biology of HEV infection.

Despite the absence of proven medications for treating HEV, ribavirin, IFN-α, or the combination have been use as off-label antiviral drugs to treat individual HEV cases or small case series (Zhou et al., 2013). The antiviral effect of interferons and their signaling pathways have been attributed to effects in the 4E-BP1 cascade (Burke et al., 2011; Kaur et al., 2007). However, loss- or gain-function of 4E-BP1 had no significant effects on the expression of IFN-α and -β (Fig. S6A) and no effect on phosphorylation of STAT1, the key element of interferon signalling transduction (Fig. S6B). Furthermore the effect of 4E-BP1 on HEV is independent of JAK-STAT cascades (Fig. S6C). Conversely, the anti-HEV effect of IFN-α is also independent of 4E-BP1 (Fig. S6D). Although ribavirin directly binds to eIF4E and competes for 5′ cap mRNA binding (De la Cruz-Hernandez et al., 2015; Kentsis et al., 2004), the anti-HEV

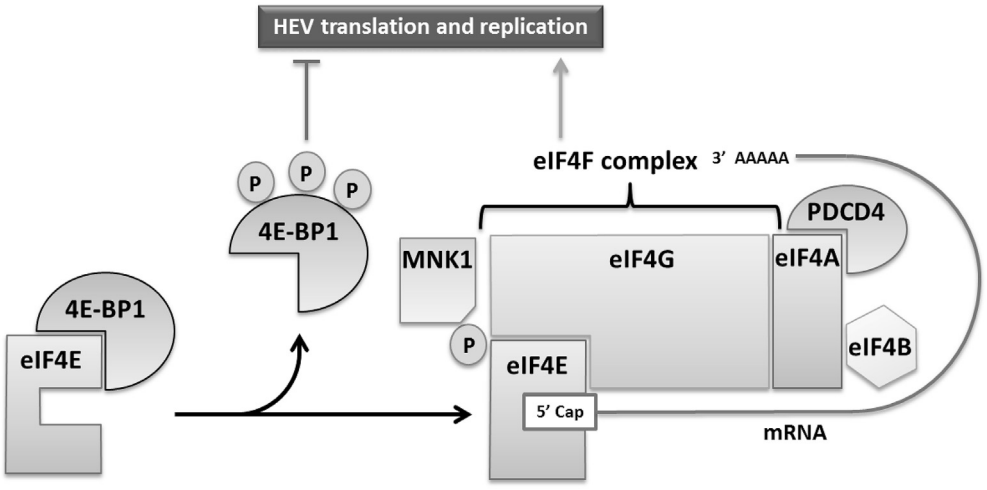

Fig. 7. Schematic illustration of the involvement of the eIF4F complex in HEV replication. Three subunits of eIF4F complex: eIF4A, eIF4G and eIF4E play important roles in efficient HEV replication. Furthermore, HEV replication is limited by the cap dependent translational suppressors, PDCD4 and 4E-BP1, but is not influenced by eIF4E phosphorylation induced by MNK1/2 kinase activation.

activity of ribavirin is also independent of eIF4E in our experimental system (Fig. 6). Instead, loss of eIF4E exerts additive anti-HEV effect of IFN-α or ribavirin and suggests that treatments aimed at targeting the translation initiation complex in conjunction with IFN-α or ribavirin have significant promise.

In conclusion, we revealed that cap dependent translation machinery plays a critical role in help with efficient HEV replication. The translational suppressors PDCD4 and 4E-BP1 are important antiviral factors in restraining HEV infection (Fig. 7). Thus, these results have shed new light on virus-host interactions and provided new avenue for potential antiviral drug development against HEV infection.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

X.Z. contributed to study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript; statistical analysis; L. X., Y. W., and W. W., contributed to acquisition of data and critical revision of the manuscript; D. S., and H. J. M contributed to study concept and critical revision of the manuscript; M. P. P. contributed to study concept, study supervision and critical revision of the manuscript; Q. P. contributed to study concept and design, obtained funding, study supervision and critical revision of the manuscript.

Acknowledgments

The authors would like to thank Dr. Suzanne U. Emerson (National Institute of Allergy and Infectious Diseases, NIH, USA) for generously providing the plasmids to generate subgenomic and full-length HEV genomic RNA. The authors also thank E.N. Fish's lab (University Health Network) for kind gift of 4E-BP1 knock-out MEFs from 4E-BP1−/− mice, Dr Sonenberg's lab (McGill University) for kind gift of lenti-vector of 4E-BP1 overexpression and eIF4E-S209A MEFs containing an eIF4E mutation in which eIF4E cannot be phosphorylated. The authors also thank the support by the Netherlands Organization for Scientific Research (NWO/ZonMw) for a VENI grant (No. 916-13-032) (to Q. Pan), the Dutch Digestive Foundation (MLDS) for a career development grant (No. CDG 1304) (to Q. Pan), the European Association for the Study of the Liver (EASL) for a Sheila Sherlock Fellowship (to Q. Pan), the Daniel den Hoed Foundation for a Centennial Award fellowship (to Q. Pan) and the China Scholarship Council for funding PhD fellowships to X. Zhou (No. 201206150075), L. Xu (201306300027), Y. Wang (201207720007) and W. Wang (201303250056).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.antiviral.2015.10.016.

References

Burke, J.D., Sonenberg, N., Platanias, L.C., Fish, E.N., 2011. Antiviral effects of interferon-beta are enhanced in the absence of the translational suppressor 4E-BP1 in myocarditis induced by Coxsackievirus B3. Antivir. Ther. 16, 577–584.
Bushell, M., Sarnow, P., 2002. Hijacking the translation apparatus by RNA viruses. J. Cell Biol. 158, 395–399.
Damania, P., Sen, B., Dar, S.B., Kumar, S., Kumari, A., Gupta, E., Sarin, S.K., Venugopal, S.K., 2014. Hepatitis B virus induces cell proliferation via HBx-induced microRNA-21 in hepatocellular carcinoma by targeting programmed cell death protein4 (PDCD4) and phosphatase and tensin homologue (PTEN). PLoS One 9, e91745.
de Breyne, S., Chamond, N., Decimo, D., Trabaud, M.A., Andre, P., Sargueil, B., Ohlmann, T., 2012. In vitro studies reveal that different modes of initiation on HIV-1 mRNA have different levels of requirement for eukaryotic initiation factor 4F. FEBS J. 279, 3098–3111.
De la Cruz-Hernandez, E., Medina-Franco, J.L., Trujillo, J., Chavez-Blanco, A., Dominguez-Gomez, G., Perez-Cardenas, E., Gonzalez-Fierro, A., Taja-Chayeb, L., Duenas-Gonzalez, A., 2015. Ribavirin as a tri-targeted antitumor repositioned drug. Oncol. Rep. 33, 2384–2392.
Dennis, M.D., Jefferson, L.S., Kimball, S.R., 2012. Role of p70S6K1-mediated phosphorylation of eIF4B and PDCD4 proteins in the regulation of protein synthesis. J. Biol. Chem. 287, 42890–42899.
George, A., Panda, S., Kudmulwar, D., Chhatbar, S.P., Nayak, S.C., Krishnan, H.H., 2012. Hepatitis C virus NS5A binds to the mRNA cap-binding eukaryotic translation initiation 4F (eIF4F) complex and up-regulates host translation initiation machinery through eIF4E-binding protein 1 inactivation. J. Biol. Chem. 287, 5042–5058.
Gingras, A.C., Raught, B., Sonenberg, N., 1999. eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. Annu. Rev. Biochem. 68, 913–963.
Harms, U., Andreou, A.Z., Gubaev, A., Klostermeier, D., 2014. eIF4B, eIF4G and RNA regulate eIF4A activity in translation initiation by modulating the eIF4A conformational cycle. Nucleic Acids Res. 42, 7911–7922.
Kamar, N., Dalton, H.R., Abravanel, F., Izopet, J., 2014a. Hepatitis E virus infection. Clin. Microbiol. Rev. 27, 116–138.
Kamar, N., Izopet, J., Tripon, S., Bismuth, M., Hillaire, S., Dumortier, J., Radenne, S., Coilly, A., Garrigue, V., D'Alteroche, L., Buchler, M., Couzi, L., Lebray, P., Dharancy, S., Minello, A., Hourmant, M., Roque-Afonso, A.M., Abravanel, F., Pol, S., Rostaing, L., Mallet, V., 2014b. Ribavirin for chronic hepatitis E virus infection in transplant recipients. N. Engl. J. Med. 370, 1111–1120.
Kaur, S., Lal, L., Sassano, A., Majchrzak-Kita, B., Srikanth, M., Baker, D.P., Petroulakis, E., Hay, N., Sonenberg, N., Fish, E.N., Platanias, L.C., 2007. Regulatory effects of mammalian target of rapamycin-activated pathways in type I and II interferon signaling. J. Biol. Chem. 282, 1757–1768.
Kentsis, A., Topisirovic, I., Culjkovic, B., Shao, L., Borden, K.L., 2004. Ribavirin suppresses eIF4E-mediated oncogenic transformation by physical mimicry of the 7-methyl guanosine mRNA cap. Proc. Natl. Acad. Sci. U. S. A. 101, 18105–18110.
Kyriakis, J.M., 2014. In the beginning, there was protein phosphorylation. J. Biol. Chem. 289, 9460–9462.
Mohr, I., Sonenberg, N., 2012. Host translation at the nexus of infection and immunity. Cell Host Microbe 12, 470–483.
Montero, H., Garcia-Roman, R., Mora, S.I., 2015. eIF4E as a control target for viruses. Viruses 7, 739–750.
Nehdi, A., Sean, P., Linares, I., Colina, R., Jaramillo, M., Alain, T., 2014. Deficiency in either 4E-BP1 or 4E-BP2 augments innate antiviral immune responses. PLoS One 9, e114854.
Pan, Q., Henry, S.D., Metselaar, H.J., Scholte, B., Kwekkeboom, J., Tilanus, H.W., Janssen, H.L., van der Laan, L.J., 2009. Combined antiviral activity of interferon-alpha and RNA interference directed against hepatitis C without affecting vector delivery and gene silencing. J. Mol. Med. Berl. 87, 713–722.
Panda, S., Vedagiri, D., Viveka, T.S., Harshan, K.H., 2014. A unique phosphorylation-dependent eIF4E assembly on 40S ribosomes co-ordinated by hepatitis C virus protein NS5A that activates internal ribosome entry site translation. Biochem. J. 462, 291–302.
Pelletier, J., Graff, J., Ruggero, D., Sonenberg, N., 2015. Targeting the eIF4F translation initiation complex: a critical nexus for cancer development. Cancer Res. 75, 250–263.
Royall, E., Doyle, N., Abdul-Wahab, A., Emmott, E., Morley, S.J., Goodfellow, I., Roberts, L.O., Locker, N., 2015. Murine norovirus 1 (MNV1) replication induces translational control of the host by regulating eIF4E activity during infection. J. Biol. Chem. 290, 4748–4758.
Shukla, P., Nguyen, H.T., Faulk, K., Mather, K., Torian, U., Engle, R.E., Emerson, S.U., 2012. Adaptation of a genotype 3 hepatitis E virus to efficient growth in cell culture depends on an inserted human gene segment acquired by recombination. J. Virol. 86, 5697–5707.
Shukla, P., Nguyen, H.T., Torian, U., Engle, R.E., Faulk, K., Dalton, H.R., Bendall, R.P., Keane, F.E., Purcell, R.H., Emerson, S.U., 2011. Cross-species infections of cultured cells by hepatitis E virus and discovery of an infectious virus-host recombinant. Proc. Natl. Acad. Sci. U. S. A. 108, 2438–2443.
Sonenberg, N., Hinnebusch, A.G., 2009. Regulation of translation initiation in eukaryotes: mechanisms and biological targets. Cell 136, 731–745.
Ventoso, I., Blanco, R., Perales, C., Carrasco, L., 2001. HIV-1 protease cleaves eukaryotic initiation factor 4G and inhibits cap-dependent translation. Proc. Natl. Acad. Sci. U. S. A. 98, 12966–12971.
Walsh, D., Mathews, M.B., Mohr, I., 2013. Tinkering with translation: protein synthesis in virus-infected cells. Cold Spring Harb. Perspect. Biol. 5, a012351.
Walsh, D., Mohr, I., 2004. Phosphorylation of eIF4E by Mnk-1 enhances HSV-1 translation and replication in quiescent cells. Genes Dev. 18, 660–672.
Walsh, D., Perez, C., Notary, J., Mohr, I., 2005. Regulation of the translation initiation factor eIF4F by multiple mechanisms in human cytomegalovirus-infected cells. J. Virol. 79, 8057–8064.
Wang, S., Chi, X., Wei, H., Chen, Y., Chen, Z., Huang, S., Chen, J.L., 2014a. Influenza A virus-induced degradation of eukaryotic translation initiation factor 4B contributes to viral replication by suppressing IFITM3 protein expression. J. Virol. 88, 8375–8385.
Wang, Y., Zhou, X., Debing, Y., Chen, K., Van Der Laan, L.J., Neyts, J., Janssen, H.L.,

Metselaar, H.J., Peppelenbosch, M.P., Pan, Q., 2014b. Calcineurin inhibitors stimulate and mycophenolic acid inhibits replication of hepatitis E virus. Gastroenterology 146, 1775–1783.
Zhou, X., de Man, R.A., de Knegt, R.J., Metselaar, H.J., Peppelenbosch, M.P., Pan, Q., 2013. Epidemiology and management of chronic hepatitis E infection in solid organ transplantation: a comprehensive literature review. Rev. Med. Virol. 23, 295–304.
Zhou, X., Wang, Y., Metselaar, H.J., Janssen, H.L., Peppelenbosch, M.P., Pan, Q., 2014. Rapamycin and everolimus facilitate hepatitis E virus replication: revealing a basal defense mechanism of PI3K-PKB-mTOR pathway. J. Hepatol. 61, 746–754.