INTRODUCTION

It is now well established that the hijacking and manipulation of host cell biosynthetic pathways by human enveloped viruses are shared molecular events essential for the viral lifecycle [1–3]. For example, lipid metabolism and associated host-cell pathways are manipulated by Flaviviridae members such as hepatitis C virus (HCV) and dengue virus (DENV) to enhance viral production [1, 4]. In addition, mounting evidence implicates the manipulation by HCV and DENV of cellular functions associated with the host secretory pathway in the viral entry and virus maturation steps of the viral lifecycle [5, 6].

An important step towards understanding Flaviviridae biology and host–virus interactions is to further characterize how Flaviviridae members HCV and DENV are able to successfully exploit specific pathway components and associated proteins in these and other host pathways. Moreover, uncovering the molecular players involved during viral hijacking may unearth a common Achilles’ heel in the Flaviviridae lifecycle, leading to the identification of potential broad-spectrum therapeutic targets [6].

HCV, a hepacivirus member of the Flaviviridae family, is a single-stranded, positive-sense RNA virus [7]. The positive-strand RNA genome of HCV is directly translated by the host machinery into a single viral polyprotein, which is cleaved by host- and virus-encoded proteases to release the individual structural (core, E1 and E2) proteins and non-structural (NS) proteins (p7, NS2, NS3, NS4A, NS4B, NS5A...
and NS5B) [7]. HCV is a globally important human pathogen afflicting more than 170 million people worldwide [8].

The four DENV serotypes (DENV-1–4) are members of the *Flavivirus* genus of the *Flaviviridae* family, with single-stranded positive-sense RNA genomes encoding three structural [capsid (C), precursor membrane (prM), and envelope (E)] proteins and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) proteins [9]. DENV represents an important number of non-glycolytic functions such as intracellular membrane trafficking [12], endocytosis [13], receptor-mediated cell signalling [14, 15] and translational control of gene expression [16–18], all important biological processes for HCV and DENV lifecycles. Interestingly, GAPDH has been shown to interact with the genomes of several viruses, suggesting that GAPDH could be involved in the regulation of viral replication [19–22]. For HCV, GAPDH binds to the untranslated region of the 3′ end of viral RNA, but its function is uncertain [23]. Together, these results underline the ‘moonlighting’ nature of GAPDH (i.e. an enzyme with additional functional activities [24, 25]), but the contributions of these moonlighting activities to host–*Flavivirusidae* interactions remain poorly understood.

In this study, we investigated whether the multifunctional GAPDH is a host factor that regulates the lifecycle steps of HCV and DENV in human hepatoma Huh-7.5.1 cells. First, we demonstrated that siRNA-mediated GAPDH silencing in naïve Huh-7.5.1 cells not only inhibits primary HCV infection, but also inhibits the production and/or release of infectious HCV particles in HCV-infected Huh-7.5.1 cell supernatants. Second, we demonstrated that GAPDH also plays an important role during the post-replication steps of the HCV lifecycle because siRNA-mediated GAPDH silencing in HCV-infected Huh-7.5.1 cells results in a significant decrease in infectivity of the HCV-infected cell supernatants. Importantly, siRNA-mediated GAPDH suppression in HCV replicon-harbouring cells has no effect on HCV RNA replication. Third, we demonstrated that exogenous expression of V5-tagged human GAPDH pre- and post-infection increases the viral infectivity of HCV-infected Huh-7.5.1 cell supernatants, suggesting a predominant role of GAPDH during the post-replication steps of the HCV lifecycle.

Finally, we validated the important role of GAPDH in the HCV lifecycle by generating two Huh-7-derived stable GAPDH-knockdown (kd) clones (67D2 and 67B3), which show a robust reduction in intracellular GAPDH mRNA levels and GAPDH protein abundance, with a concomitant reduction in GAPDH enzymatic activity. Analysis of the GAPDH-kd clone’s susceptibility to HCV (JFH-1) infection showed a reduction in intracellular HCV core abundance and a significant decrease in viral infectivity of the HCV-infected GAPDH-kd clone supernatants. Interestingly, our preliminary results with DENV have shown that siRNA-mediated GAPDH suppression in naïve human Huh-7.5.1 cells robustly inhibits primary DENV-2 infection.

Collectively, the results of our studies demonstrate that the moonlighting enzyme, human GAPDH, is an important host factor for HCV infection in human Huh-7.5.1 cells, and they support its potential role in the DENV-2 lifecycle.

**RESULTS AND DISCUSSION**

**siRNA-mediated GAPDH mRNA silencing in naïve human Huh-7.5.1 cells dramatically decreases GAPDH protein abundance**

We first optimized the experimental conditions needed to obtain a robust transient knockdown of GAPDH by RNA interference. Cultured Huh-7.5.1 cells were initially transfected with an siRNA pool targeting human GAPDH (siGAPDH) or a non-targeting siRNA pool (siCTRL) with 15 nM of each respective siRNA pool. Seventy-two hours post-transfection, cell lysates were prepared for Western blot analysis (Fig. 1, upper panel). A 14.3-fold decrease in GAPDH abundance was observed for cells treated with 15 nM of siGAPDH relative to control cells (siCTRL) (Fig. 1, lower panel). These results confirmed that a robust transient knockdown of GAPDH is achieved by using 15 nM of the GAPDH siRNA pool in Huh-7.5.1 cells for up to 72 h, the duration of the HCV infection examined in this study. Importantly, no significant effect on cell density was detected in siRNA-treated Huh-7.5.1 cells using the siGAPDH compared to siCTRL at a concentration of 15 nM and up to 5 days post-transfection (p.t.; Fig. S1, available in the online Supplementary Material).

**siRNA-mediated GAPDH knockdown in naïve human Huh-7.5.1 cells decreases host-cell susceptibility to primary and secondary HCV infections**

First, to examine the effect of siRNA-mediated GAPDH suppression on primary HCV infection of naïve Huh-7.5.1 cells, cells were transfected with siGAPDH or siCTRL for 24 h. Forty-eight hours p.t., treated cells were infected with HCV at an m.o.i. of 0.1. Seventy-two hours post-viral-infection (p.v.i.), HCV-infected Huh-7.5.1 cells were fixed for in-cell Western (ICW) analysis (Fig. 2a; lower panel). Thus, siRNA-mediated GAPDH suppression in naïve human Huh-7.5.1 cells robustly inhibits primary HCV infection.

Next, to examine the effect of siRNA-mediated GAPDH suppression on HCV infectious virus particle production and its spread to naïve cells, we performed an ICW assay involving HCV secondary infection. Briefly, Huh-7.5.1 cells...
were treated as described for the primary infection with the exception that at 72 h p.i., the HCV-infected Huh-7.5.1 cell supernatants were collected and used to perform a secondary infection on naïve Huh-7.5.1 cells. After the secondary infection had proceeded for 72 h, the HCV-infected Huh-7.5.1 cells were fixed for ICW assays. A 3.9-fold reduction of HCV core abundance was measured for siGAPDH-treated cells during the secondary infection compared to siCTRL (Fig. 2b; lower panel). Thus, siRNA-mediated GAPDH silencing in naïve Huh-7.5.1 cells not only inhibits primary HCV infection, but also seems to inhibit the production and/or release of infectious HCV particles in HCV-infected Huh-7.5.1 cell supernatants.

At least in part, the robust decrease of HCV core abundance observed during the primary infection in siGAPDH-treated cells could be attributed to host cells compromised for viral entry and/or replication, as the measurement of intracellular HCV core abundance is not sufficient to discriminate between these possibilities. Notably, GAPDH may play important roles in the early steps of the HCV lifecycle. GAPDH is associated with tetraspanin-enriched microdomains in the host plasma membrane, and it has been shown to directly interact with putative HCV receptor CD81 in this context [26]; roles for GAPDH in endocytosis as well as intracellular membrane fusion have also been identified [12, 13]. Thus, HCV attachment, entry and fusion could all be affected by siRNA-mediated GAPDH silencing.

Interestingly, the secondary infection of naïve Huh-7.5.1 cells with the HCV-infected cell supernatants collected from the primary infection resulted in an even further decrease in intracellular HCV core abundance. These results are consistent with a compromised host-cell susceptibility to HCV infection observed during the primary infection of siGAPDH-treated cells, but they also imply that key maturation and trafficking steps leading to the production of infectious virus particles and release are also compromised. Because of the multifunctional roles of GAPDH in intracellular membrane trafficking [25], an important molecular event for the biogenesis of organelles in the secretory pathway, siRNA-mediated suppression of GAPDH may impact essential cellular biological processes that HCV requires for maturation in the host secretory pathway, including the formation of lipoviroparticles and their release from the infected cells [4].

**GAPDH knockdown in HCV-infected Huh-7.5.1 cells leads to a decrease in viral infectivity of the HCV-infected cell supernatants**

To test the effect of siRNA-mediated GAPDH silencing in established infections, Huh-7.5.1 cells were first infected with HCV for 24 h to allow uninterrupted HCV replication and establishment of infection. At the end of this time, Huh-7.5.1 cells were transfected with siGAPDH or siCTRL and incubated for an additional 72 h (Fig. 2c, d). The HCV-infected Huh-7.5.1 cell supernatants were collected to perform a secondary infection on naïve Huh-7.5.1 cells (Fig. 2d) and cells were fixed for ICW analysis (Fig. 2c).

We found that GAPDH suppression in Huh-7.5.1 cells when HCV infection has already been established does not have an effect on intracellular HCV core abundance during the primary infection (Fig. 2c). However, the relative abundance of HCV core associated with the secondary infection is reduced 2.5-fold compared to siCTRL-treated cells (Fig. 2d). This suggests that GAPDH also plays an important role during the post-replication steps of the virus lifecycle because siRNA-mediated GAPDH silencing in HCV-infected Huh-7.5.1 cells caused a significant decrease in viral infectivity of the HCV-infected cell supernatants.

In addition to its potential roles early in the HCV lifecycle, GAPDH is an important component of the Rab2-Src-αPKCɛ–GAPDH microtubule-bound complex, which is vital to endoplasmic reticulum–Golgi trafficking in the secretory pathway [12]. Since HCV hijacks this pathway to promote the formation and egress of lipoviroparticles, it seems that the assembly and budding of infectious virions could be inhibited as a result of siRNA-mediated GAPDH silencing. Thus, silencing GAPDH may significantly impact the pre- and post-replication steps of the HCV lifecycle, underlining
Fig. 2. siRNA-mediated GAPDH knockdown in human Huh-7.5.1 cells leads to decreased infectivity of HCV-infected cell supernatants. (a, b) siRNA-mediated GAPDH suppression in naïve human Huh-7.5.1 cells decreases host-cell susceptibility to primary and secondary HCV infections. (a) Primary infection. Huh-7.5.1 cells were transfected with an siRNA pool targeting human GAPDH (siGAPDH, 15 nM) or with a non-targeting siRNA pool (siCTRL, 15 nM) for 24 h. 48 h p.t., treated cells were infected with HCV (m.o.i. of 0.1); 24 h p.i., cell supernatants were replaced with fresh medium for secondary infection. 72 h p.i., HCV-infected supernatants were collected for a secondary infection in naïve Huh-7.5.1 cells (b), and HCV-infected Huh-7.5.1 cells were fixed for ICW analysis (a) as described in Methods. (b) Secondary infection. HCV-infected Huh-7.5.1 cell supernatants collected in (a) were used to infect naïve Huh-7.5.1 cells for 72 h, then fixed for ICW assays (b). (c, d) siRNA-mediated GAPDH suppression in HCV-infected Huh-7.5.1 cells leads to decreased viral infectivity of the HCV-infected cell supernatants. (c) Primary infection. Huh-7.5.1 cells were infected with HCV (m.o.i. of 0.1) for 24 h. HCV-infected Huh-7.5.1 cells were then transfected with an siRNA pool targeting GAPDH (siGAPDH, 15 nM) or with a non-targeting siRNA pool (siCTRL, 15 nM). 24 h p.t., cell supernatants were replaced with fresh medium for secondary infection. 72 h p.t., cell supernatants...
the varied and numerous functions performed by this moonlighting enzyme [24, 27, 28].

GAPDH knockdown in HCV replicon-harbouring cells has no effect on HCV replication

To determine if the host-cell susceptibility to primary and secondary HCV infections observed in siGAPDH-treated cells is due to reduced viral RNA replication, we examined the impact of siRNA-mediated GAPDH suppression on viral RNA replication using HCV subgenomic replicons [29, 30].

Human hepatoma cells harbouring stable HCV replicons encoding wild-type NS5A (Huh.8 cells: Fig. 3a) or NS5A with an adaptive mutation (Huh.2 cells: Fig. 3b) were transfected for 72 h with siGAPDH or siCTRL. Total RNA was harvested and the levels of HCV and GAPDH RNA were quantified using qRT-PCR analysis (Fig. 3a, b). Although a robust knockdown of GAPDH was observed in Huh.8 cells (16.7-fold decrease) and Huh.2 cells (25-fold decrease), no significant differences in HCV RNA levels were observed between siGAPDH and siCTRL (Fig. 3a, b). In contrast, Huh.8 cells treated with the positive control, BILN2061, a viral protease inhibitor that inhibits HCV replication [31], resulted in a 33.3-fold decrease in HCV RNA compared to DMSO (Fig. 3a (right panel)). These findings strongly suggest that the robust reduction in HCV core abundance observed during primary and secondary HCV infections in siGAPDH-treated cells (Fig. 2a, b) is not due to a defect in HCV RNA replication. In addition, since we have not observed any significant differences in HCV RNA replication between siGAPDH (>95 % kd) and siCTRL in both Huh.8 and Huh.2 replicon-harbouring cells, our results support the apparent lack of cytotoxic effects associated with GADH-kd in human hepatoma cells under our experimental conditions.

Interestingly, GAPDH has been shown to interact directly with 3’-UTRs, in the context of cellular mRNAs including angiotensin II type 1 receptor and endothelin-1 [16, 17] as well as the genomes of several RNA viruses, including hepatitis A virus and Japanese encephalitis virus [19, 22]. These interactions generally have the effect of destabilizing the mRNA or preventing translation by other means. Despite this, it seems

Fig. 3. siRNA-mediated GAPDH knockdown in HCV replicon-harbouring cells has no effect on HCV RNA replication. (a) Huh.8 replicon-harbouring cells. Left panel: Huh.8 cells were transfected with an siRNA pool targeting human GAPDH (siGAPDH, 15 nM) or with a non-targeting siRNA pool (siCTRL, 15 nM). Right panel: Huh.8 cells were treated with 1 µM of HCV NS3/4A protease inhibitor (BILN2061 or DMSO (control)). 72 h.p.t., total RNA was harvested from Huh.8 cells. (b) Huh.2 replicon-harbouring cells. Huh.2 cells were transfected with an siRNA pool targeting human GAPDH (siGAPDH, 15 nM) or with a non-targeting siRNA pool (siCTRL, 15 nM). 72 h.p.t., total RNA was harvested from the Huh.2 cells. (a, b) GAPDH mRNA levels and HCV RNA levels, normalized to β-actin transcript levels, were relatively quantified in cell extracts using RT-PCR. All values are expressed as relative GAPDH and HCV levels in treated cells (siGAPDH or BILN2061) compared to control-treated cells (siCTRL or DMSO). Results: mean±SEM of three independent experiments in triplicate. Significance is based on Student’s t-test (****, P<0.0001).
that any ability GAPDH may have to interact with HCV RNA [23] does not confer an inhibitory or stimulatory effect on HCV replication. Thus, it appears that the role of GAPDH in the HCV lifecycle is limited to pre- and post-replication steps while RNA replication itself is GAPDH-independent.

**Exogenous expression of V5-tagged human GAPDH increases the viral infectivity of HCV-infected Huh-7.5.1 cell supernatants**

Next, we wanted to test whether over-expression of human GAPDH in Huh-7.5.1 cells would increase host-cell susceptibility to primary and secondary HCV infections. This was accomplished by first producing a plasmid encoding the V5-tagged full-length human GAPDH (pcDNA3.1-GAPDH-V5, abbreviated GAPDH-V5) (Fig. 4a). This construct was then transfected in Huh-7.5.1 cells for 72 h. WB analysis of GAPDH-V5-transfected cellular lysates using anti-GAPDH antibody (Fig. 4b, left side) and anti-V5 antibody (Fig. 4b, right side) demonstrated the successful over-expression of the GAPDH-V5 recombinant protein, associated with a 1.9-fold increase in GAPDH intracellular enzymatic activity in GAPDH-V5-transfected cells versus control cells (CTRL) (Fig. 4c).

To determine whether exogenous expression of GAPDH-V5 can increase primary and secondary HCV infection in Huh-7.5.1 cells, we examined the effect of over-expressing GAPDH-V5 on HCV core abundance pre- (Fig. 5a, b) and post-establishment (Fig. 5c, d) of viral infection in Huh-7.5.1 cells. First, Huh-7.5.1 cells were transfected with pcDNA3.1-GAPDH-V5 (GAPDH-V5) or pcDNA3.1 (CTRL) for 24 h. Treated cells were infected with HCV (m.o.i. of 0.1). Seventy-two hours p.i., the HCV-infected Huh-7.5.1 cell supernatants were collected to perform a secondary infection on naïve Huh-7.5.1 cells (Fig. 5b) and cells were fixed for ICW analysis (Fig. 5a). Results showed that transfection of Huh-7.5.1 naïve cells with GAPDH-V5 prior to infection with HCV does not have an effect on the level of intracellular HCV core protein in primary infected cells (Fig. 5a), but it increases the level of HCV core protein 1.7-fold in secondary infected cells (Fig. 5b). Since HCV core abundance is an indicator of viral load in the host cells, this shows that the over-expression of GAPDH and the concomitant boost in GAPDH enzymatic activity pre-establishment of HCV infection lead to an increase in the viral infectivity of HCV-infected Huh-7.5.1 cell supernatants while cell susceptibility remains unaffected.
Fig. 5. Over-expression of human GAPDH-V5 increases the viral infectivity of HCV-infected Huh-7.5.1 cell supernatants. (a, b) Effect of GAPDH-V5 on host-cell susceptibility to HCV infection when expressed before establishing viral infection in Huh-7.5.1 cells. (a) Primary infection. Huh-7.5.1 cells were transfected with pcDNA3.1-GAPDH-V5 (GAPDH-V5) or pcDNA3.1 (CTRL) for 24 h. Treated cells were infected with HCV (m.o.i. of 0.1). 24 h p.i., cell supernatants were replaced with fresh medium for secondary infection. 72 h p.i., the HCV-infected Huh-7.5.1 cell supernatants were collected for secondary infection in naïve Huh-7.5.1 cells (b) and fixed for ICW analysis (a). (b) Secondary infection. HCV-infected Huh-7.5.1 cell supernatants from (a) were used to infect naïve Huh-7.5.1 cells for 72 h, then
To test the effect of GAPDH-V5 over-expression in established infections, Huh-7.5.1 cells were first infected with HCV for 24 h. HCV-infected Huh-7.5.1 cells were transfected with pcDNA3.1-GAPDH-V5 (GAPDH-V5) or pcDNA3.1 (CTRL); 72 h.p.t., the HCV-infected Huh-7.5.1 cell supernatants were collected for a re-infection experiment. (c) Effect of GAPDH-V5 on host-cell susceptibility to HCV infection when expressed after established viral infection in Huh-7.5.1 cells. (c) Primary infection. Huh-7.5.1 cells were infected with HCV (m.o.i. of 0.1) for 24 h. HCV-infected Huh-7.5.1 cells were transfected with pcDNA3.1-GAPDH-V5 (GAPDH-V5) or pcDNA3.1 (CTRL). 24 h.p.t., cell supernatants were replaced with fresh medium for secondary infection. 72 h.p.t., HCV-infected Huh-7.5.1 cell supernatants were collected for a secondary infection in naïve Huh-7.5.1 cells (d) and fixed for ICW analysis (c). (d) Secondary infection. HCV-infected Huh-7.5.1 cell supernatants from (c) were used to infect naïve Huh-7.5.1 cells for 72 h, then fixed for ICW assays (d). Upper panel (a–d): representative ICW wells, probed with HCV anti-core antibody (green) and anti-V5 antibody (green) (a, c) or anti-core alone (green) (b, d) and stained with two dyes for cell density (CD) normalization (red). Lower panel (a–d): HCV core protein abundance was quantified, averaged across triplicate wells, and expressed relative to core protein abundance in control cells (CTRL). Results: means±SD of one representative experiment in triplicate.
Fig. 6. The Huh-7-derived stable GAPDH-kd clones 67D2 and 67B3 show a robust reduction in intracellular GAPDH protein abundance, GAPDH enzymatic activity, and GAPDH mRNA levels. (a) GAPDH protein abundance determined by WB for Huh-7-derived GAPDH-kd clones 67D2 and 67B3. Cell lysates were harvested from parental Huh-7 cells (Parental), control Huh-7 cells (CTRL), and Huh-7-derived stable GAPDH-kd clones (67D2 and 67B3) for WB analysis and probed with human GAPDH antibody (green) and β-tubulin antibody (red) (see Methods). Upper panel: a representative WB. Lower panel: protein abundance was quantified as described in Methods, averaged across WB bands and expressed relative to protein abundance in CTRL. Results: mean±SEM of three independent WB analyses. Significance is based on Student’s t-test (***, P<0.0001). (b) GAPDH protein abundance determined by ICW for Huh-7-derived stable GAPDH-kd
clones 67D2 and 67B3. Equal numbers of parental, CTRL and 67D2 and 67B3 cells were seeded in a 96-well plate. 24 h post-seeding, cells were fixed for ICW analysis (b) as described in Methods. Upper panel: representative ICW wells probed with anti-GAPDH antibody (green) and stained with two dyes to determine CD (red) for normalization. Lower panel: protein abundance was quantified as described in Methods, averaged across triplicate wells and expressed relative to GAPDH protein abundance in CTRL. Results: mean±SEM of four independent ICW experiments, each in triplicate. Significance is based on Student’s t-test (***, P<0.0001). (c) GAPDH enzymatic activity determined for Huh-7-derived stable GAPDH-kd clones 67D2 and 67B3. Parental, CTRL, and 67D2 and 67B3 cells were seeded in a 96-well plate. 24 h post-seeding, cell lysates were prepared to detect intracellular GAPDH enzymatic activity as described in Methods. Values are plotted relative to GAPDH enzymatic activity detected in CTRL. Results: mean±SEM of four independent experiments, each in triplicate. Significance is based on Student’s t-test (***, P<0.0001). (d) GAPDH mRNA levels determined for 67D2 and 67B3. Total RNA was harvested from parental, CTRL, 67D2 and 67B3 clones. GAPDH mRNA levels, normalized to β-actin mRNA levels, were relatively quantified by QPCR as described in Methods. Values are plotted as relative GAPDH mRNA levels in comparison to CTRL. Results: mean±SEM of two independent experiments in triplicate. Significance is based on Student’s t-test (***, P<0.0001).

CD normalization (red). HCV core was quantified and expressed relative to protein abundance in control Huh-7 cells (CTRL) (Fig. 7a; lower panel). A significant reduction in HCV core abundance was measured for GAPDH-kd clones [4-fold decrease (67D2) and 8.3-fold decrease (67B3)] during the primary infection compared to CTRL (Fig. 7a; lower panel).

Next, supernatants from the HCV-infected GAPDH-kd clones were collected and used to perform a secondary infection in naïve Huh-7.5.1 cells. After the secondary infection had proceeded for 72 h, these supernatant-infected Huh-7.5.1 cells were fixed for ICW assay. Interestingly, during the secondary infection (Fig. 7b), a more robust reduction in intracellular HCV core abundance (~threefold compared to primary infected cells) was measured in the naïve Huh-7.5.1 cells infected with GAPDH-kd clone supernatants [12.5-fold decrease (67D2) and 25-fold decrease (67B3)] with control Huh-7 (CTRL)]. Thus, shRNA-mediated knockdown of GAPDH in GAPDH-kd clones inhibits primary HCV infection (Fig. 7a), which appears to compromise the production and/or release of infectious HCV virus particles in HCV-infected GAPDH-kd clone supernatants (Fig. 7b). The combined results of primary infection (Fig. 7a) and secondary infection (Fig. 7b) suggest a reduction in extracellular infectious virus due to compromised primary HCV infection – as exemplified by the reduction in intracellular HCV core abundance – in GAPDH-kd clones.

Our results obtained using the Huh-7-derived stable GAPDH-kd clones 67D2 and 67B3 (Fig. 7a, b) are consistent with our results obtained using siRNA-mediated GAPDH silencing in naïve Huh-7.5.1 cells, where we showed that siRNA-mediated GAPDH silencing in naïve Huh-7.5.1 cells robustly inhibits primary HCV infection (Fig. 2a) and leads to a decrease in viral infectivity of the HCV-infected cell supernatants (Fig. 2b). Our results are also consistent with the work done by Randall et al. [32], who identified human GAPDH as one of the 26 genes that modulate HCV replication by performing a systematic RNAi screen in Huh-7.5 cells infected with a chimeric HCV genotype 2a virus, J6/JFH-1. Importantly, and in agreement with our findings, there was no correlation between the effect of GAPDH siRNA on HCV replication and cell viability, suggesting that the phenotype reported was not the result of changes in cellular physiology [32].

**GAPDH knockdown in naïve human Huh-7.5.1 cells decreases host-cell susceptibility to primary DENV-2 infection**

Finally, to investigate whether GAPDH is an important host factor for other *Flaviviridae* members, we determined the effect of siRNA-mediated GAPDH silencing in naïve human Huh-7.5.1 cells on DENV-2 infection. Huh-7.5.1 cells were transfected with siGAPDH or siCTRL. Forty-eight hours post-transfection, treated cells were infected with DENV-2 (m.o.i. of 0.005); 72 h.p.i., the DENV-2-infected Huh-7.5.1 cells were fixed for ICW analysis (Fig. 8). A 1.7-fold reduction of intracellular DENV-2 E protein abundance was measured for the siGAPDH-treated cells during the primary infection compared to the siCTRL-treated cells (Fig. 8; lower panel). Thus, siRNA-mediated GAPDH suppression in naïve human Huh-7.5.1 cells significantly inhibits DENV-2 infection, although the specific lifecycle steps compromised in these GAPDH-kd cells remain to be determined. Interestingly, Alonso et al. [33] recently identified GAPDH as a binding partner interacting with intracellular DENV NS1. Both DENV infection and DENV NS1 over-expression increased GAPDH glycolytic activity. Those authors hypothesized that DENV NS1 is responsible for modulating the host metabolism by increasing GAPDH glycolytic activity during the early steps of infection. Together, these results suggest that GAPDH is a host factor required to enhance glycolysis and, consequently, energy production during the DENV lifecycle [33].

**Conclusion**

Uncovering the molecular players involved during viral hijacking may unearth a common Achilles’ heel in the lifecycle of important human pathogens such as HCV and DENV, thus leading to a better understanding of host–virus interactions and to identifying potential broad-spectrum therapeutic targets [34]. The results of our studies underlined the important contributions of GAPDH’s ‘moonlighting’ activities to host–*Flaviviridae* interactions, and they uncovered novel complex molecular interplays between human GAPDH and the *Flaviviridae* lifecycle steps. Importantly, in this study, we established unique Huh-7-derived stable GAPDH-kd clonal cell lines that are valuable tools not only for further investigations of GAPDH’s function in DENV infection but also for unravelling the functional role of GAPDH in other important human pathogens.
of GAPDH during viral infection of other emerging flaviviruses such as Zika virus [35].

METHODS

Cell culture and reagents

Human hepatoma Huh-7.5.1 cells were kindly provided by Dr Francis Chisari (Scripps Research Institute) [36, 37]. Huh-7 cells, Huh.8 (Con1/SG-Neo) cells supporting the HCV genotype 1b subgenomic replicon, and Huh.2 (Con1/SG-Neo: S2197P) cells supporting the same replicon with an NS5A adaptive mutation were provided by Dr Charles Rice (The Rockefeller University and Apath, LLC, St Louis, USA) [29, 30]. BILN2061 was kindly provided by Dr Peter W. White (Boehringer Ingelheim, Canada). Vero cells (ATCC CCL-81) were obtained from ATCC [38]. Cells were cultured and maintained as described previously [6, 39, 40].

Antibodies and dyes

Two anti-GAPDH antibodies were employed for detecting human GAPDH in cultured cells: a mouse anti-GAPDH monoclonal antibody (1:1000 for WB) and a rabbit anti-GAPDH monoclonal antibody (1:1000 for WB or ICW assay). A mouse anti-core monoclonal antibody (1:1000,
Viral stock production and titre determination

The plasmid pUCvJFH-1 (a generous gift from Dr Takaji Wakita, National Institute of Infectious Diseases, Japan) was used for generating HCV RNA and infectious HCV stocks as described previously [37, 41]. HCV stocks were produced and titres determined as described previously [6, 40]. DENV serotype-2 (DENV-2; New Guinea C) was kindly provided by Dr Mike Drebot from the National Microbiology Laboratory (Canada). DENV-2 stocks were generated and titres determined as described elsewhere [42].

Viral infections

Human hepatoma cells were either infected with HCV (m.o. i. of 0.1 or 0.5) or DENV-2 (m.o.i of 0.005). Twenty-four hours p.i., medium was removed and fresh complete medium added. Thereafter, the cells were incubated for the indicated number of days p.i. (see Figs 2, 5, 7 and 8) and cells fixed for ICW assay. Alternatively, cell supernatants were collected at various time-points p.i. for performing secondary infection of naïve Huh-7.5.1 cells and determining viral protein abundance (HCV core; DENV-2 E-glycoprotein) using ICW assay.

RNA isolation

Total RNA was isolated using a miniRNAeasy kit (Qiagen) according to the manufacturer’s instructions. The concentration and purity of RNA were determined by a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific) as described previously [6, 43, 44].

Quantitative real-time (qRT) PCR

Purified total RNA (15 ng) was reverse transcribed to cDNA using the qScript cDNA synthesis kit (Quanta Biosciences) according to the manufacturer’s instructions. qRT-PCR was carried out using PerfeCTa Multiplex qPCR SuperMix (Quanta Biosciences) according to the manufacturer’s instructions on an Mx3005P QPCR system (Stratagene) as described previously [43, 44].

PrimerQuest [Integrated DNA Technologies (IDT)] was used to design qRT-PCR assays for detecting (i) HCV genomic abundance [probe: (5'-CAGTACACAAAGGCTTTTCGC AACC-3'), forward primer (5'-CAAGAICTCAGCAGGAG T-3'), reverse primer (5'-ACTCGCAA GGCCTCTATC-3')]; (ii) GAPDH RNA levels [probe: (5'-GCTCCAGAGATGG TGATGGG-3'), forward primer (5'-AGAACGGGAAGCTTG TCATC-3'), reverse primer (5'-CATCCGCCCAACTTGA TTTTG-3')]; and (iii) human β-actin (ACTB) RNA levels [probe: (5'-ACTCCATGCCAGGAAGAGGCG-3'), forward primer (5'-GCCCAGGGCAGACTTCC-3'), reverse primer (5'-GGATCTCCACGTCACACTTC-3')].

HCV RNA levels were relatively quantified across samples and normalized to β-actin RNA levels (5’ Cy5 fluorophore and 3’ Iowa Black RQ quencher) using 300 nM primers and 150 nM double-quenched probes (5’ FAM fluorophore and
TransIT-LT1 (Mirus) according to the manufacturer.

GAPDH-V5 construct using the transfection reagent, Huh-7.5.1 cells were transfected with the pcDNA3.1-

Transfection of Huh-7 cells with the hGAPDH-V5

hGAPDH-V5 construct (pcDNA3.1-GAPDH-V5). The final

hGAPDH construct entails the introduction of KpnI and

V5-tagged human GAPDH construct

Total RNA isolated from human Huh-7 cells (PureLink RNA Mini Kit, Ambion) was reverse transcribed to cDNA (TaqMan Reverse Transcription Reagents, Applied Biosystems) using manufacturer-supplied oligo d(T) 16 primers. KOD Hot Start DNA Polymerase (Toyobo, EMD Biosciences) was used to amplify the cDNA corresponding to the coding region of human GAPDH (hGAPDH) using reverse transcription mix with primer sets 5'-ATGGGGAAGG TGAAGGTCGG-3' and 5'-TTACTCCTTTGAGGCCCATG TGGGC-3'. The purified hGAPDH PCR fragment (PCR purification kit, Invitrogen) was cloned into a pGEM-t vector (Promega). Briefly, the cloning method of the V5-tagged hGAPDH construct entails the introduction of KpnI and EcoRI restriction cloning sites on the 5' end and the 3' end of the hGAPDH PCR fragment, respectively, along with the elimination of a stop codon using primer sets 5'-GCTTGG TACCATGGGGAAGG TTACGTAGAATCGAGACCGAG-3' (forward) and 5'- TAGGCCTCGAGTTACTCCTTTGAGGCCCATG TGGGC-3' (reverse) (IDT). The double-digested PCR fragment was then cloned into the mammalian expression vector pcDNA3.1+ (Invitrogen). A duplex sequence of V5-tag was designed with restriction sites EcoRI at the 5' end and XhoI at the 3' end (5'-TAGGCCTCGAGTTACTCCTTTGAGGCCCATG TGGGC-3' IDT)). The duplex was double-digested with EcoRI (NEB) and XhoI (NEB), and it was introduced into a GAPDH-containing pcDNA3.1+ vector to generate the hGAPDH-V5 construct (pcDNA3.1-GAPDH-V5). The final construct was verified by sequencing.

Transfection of Huh-7 cells with the hGAPDH-V5 construct

Huh-7.5.1 cells were transfected with the pcDNA3.1-

ICW assays and WBs

ICW assays were performed using black flat-bottom 96-well plates (BD Biosciences). Cells were seeded onto 96-well plates for 24 h, and were then transfected and/or infected as described in the figure legends. Cells were fixed in 4% v/v formaldehyde in PBS for 45 min, then permeabilized and blocked in PBS containing 0.1% Triton-X 100 (permeabilization buffer) and PBS diluted 1:1 with Odyssey blocking buffer for 1.5 h. Primary and secondary antibodies were diluted in Odyssey blocking buffer containing 0.02% Tween 20; proteins were detected by probing with the appropriate primary antibodies overnight at 4°C followed by the addition of the secondary antibodies for 1 h. For normalization, the fixed cells were stained with two dyes [Sapphire 700 (LI-COR Biosciences) and a DNA interactive stain, DRAQ5 (BioStatus)], for the purpose of determining CD. The fixed cells were scanned at a wavelength of 700 nm for detecting IRDye 680 labelled antibodies and at a wavelength of 800 nm for detecting IRDye 800CW conjugated antibodies. Signal intensities were quantified by means of Odyssey software version 2.0. Western blots were performed on whole cell lysate as described previously and intracellular β-tubulin was used as a loading internal control for normalization of the GAPDH abundance in each sample [6].

GAPDH enzymatic assays

GAPDH enzymatic activity was measured using the KDalert GAPDH assay kit according to the manufacturer’s instructions (Invitrogen Life Technologies). The fluorescence signal was detected using a Gemini EM fluorescence microplate reader ( Molecular Devices).

Huh-7-derived GAPDH-kd clones (67D2 and 67B3)

The human GAPDH MISSION short-hairpin RNA (hGAPDH-shRNA) sequence [Seq: CCGGGTGGATATTG TGGCCCATCAATCTCGAGATTGATGGCAACAATATCCA TGGGC-3'] was custom-designed using GAPDH RefSeq: NM_002046.2. The hGAPDH-shRNA targeted the 5' region of the hGAPDH mRNA (see Fig. S2a, b). MISSION pLKO.1-puro control (SHC001V-lentiviral vector empty of shRNA) was used as control (CTRL). Huh-7 cells (5 × 10^4 cells) were infected with hGAPDH-shRNA encoding lentiviral transduction particles (Sigma Aldrich) at an m.o.i. of 1 in the presence of 2.5 µM hexadimethrine bromide (Sigma Aldrich) for 24 h. Seventy-two hours p.i., transduced cells were selected and maintained in medium containing 7.35 µM puromycin and supplemented with 10 mM sodium pyruvate to compensate for GAPDH knockdown. Over the next 5–10 days p.i., selection medium was replaced when excessive cell death was observed. Upon repopulation (10–20 days) of transduced cells, single-cell Huh-7-derived cell clones 67D2 and 67B3 were isolated by serially diluting transduced cells in a 96-well plate. Thereafter, these Huh-7-derived cell clones were characterized for stable GAPDH knockdown.

**Funding information**

This work was supported by the Canadian Institutes of Health Research (CIHR) (MOP-84462). M. Raj acknowledges generous support from a Michael Smith Foundation for Health Research (MSFHR) Junior Trainee Award.
Acknowledgements
We would like to thank the following people for kindly providing materials used in this work: Dr Francis Chisari (Scripps Research Institute, La Jolla, CA, USA) for Huh-7.5.1 cells, Apath LLC (St Louis, MO, USA) for Huh.2 and Huh.8 cells, Dr Takaji Wakita (National Institute of Infectious Diseases, Tokyo, Japan) for JFH-1 HCV cDNA, Dr Peter W. White (Boehringer Ingelhein (Canada) Ltd) for BILN2061. The authors would like to thank Dr Jill Kelly for proofreading the manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest.

References

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