DENGUE VIRUS CONTROLS ACTIVATION OF CELLULAR STRESS RESPONSES THROUGH INHIBITION OF eIF2α PHOSPHORYLATION INDEPENDENTLY OF ATF4

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Work in progress
Abstract

During virus infection, an integrated cellular stress response (ISR) is activated to limit cell damage. The phosphorylation status of the eukaryotic initiator factor 2 alpha (eIF2α) is key in stress regulation. eIF2α phosphorylation leads to a global translational arrest and results in the formation of stress granules. At these conditions, activating transcription factor 4 (ATF4) is selectively expressed and serves as a gatekeeper by inducing expression of genes aimed at the recovery of homeostasis or otherwise, cell death. For example, DNA damage inducible 34 (GADD34) dephosphorylates eIF2α thereby reestablishing the cellular translational rates. An overall decrease in protein translation is not beneficial for virus reproduction; therefore viruses have evolved to hijack the ISR. Intriguingly, we recently described an increase in ATF4 protein levels upon dengue virus (DENV) infection and others have described the nuclear redistribution of ATF4 during DENV infection. DENV might therefore use ATF4 to maintain protein translation. Here, we explored the activation and importance of the eIF2α/ATF4 pathway in DENV-infected Huh7 cells in more detail. The results show that under normal infection conditions, the ISR is not activated. Subsequent experiments revealed that DENV actively inhibits eIF2α phosphorylation and SG formation in Huh7 cells. Thus, the increased expression of ATF4 is regulated independently of the ISR. Furthermore, increased mRNA levels were detected. Despite high levels of ATF4 in infected cells, this transcription factor was not involved in the relief of cellular stress during DENV infection of Huh7 cells.
1. Introduction

Viruses, as obligatory parasites, are dependent on the host cell for genome replication and protein translation. Abundant replication and translation of viral proteins in infected cells, however, induces a cellular stress response that leads to a global decrease in protein translation. Therefore, many viruses have evolved to manipulate the antiviral stress response thereby allowing efficient virus multiplication.

The integrated stress response (ISR) is activated upon sensing of extrinsic and intrinsic stress signals by four distinct kinases. Protein kinase R (PKR) recognizes double stranded RNA (dsRNA); PKR-like endoplasmic reticulum kinase (PERK) senses endoplasmatic reticulum (ER) stress; heme-regulated initiation factor 2α kinase (HRI) is activated upon oxidative stress and nutrient deprivation triggers general control nonderepressible 2 (GCN2). Activation of these kinases leads to the phosphorylation of the alpha subunit of the eukaryotic translation initiation factor 2 (eIF2α). eIF2α phosphorylation on serine 51 (pS51-eIF2α) impairs the exchange of eIF2-GDP to eIF2-GTP which is needed for the delivery of Met-tRNAi to the 40S ribosome and the recognition of the start codon. The stalled translational complexes subsequently aggregate to form cytoplasmic stress granules. Thus, SGs mainly consist of mRNA transcripts, small ribosomal subunits and several eukaryotic initiation factors such as eIF3, eIF4F and eIF4B. Other RNA-binding proteins, which are reliable SG markers, but are not universal to all SGs, are TIA-1 (T cell internal antigen-1), TIAR (TIA-1-related) and G3BP1 (Ras-GTPase-activating-protein binding protein 1).

During the ISR translation of most mRNA transcripts is inhibited; however, selective translation of ATF4 is observed. ATF4 functions as a gatekeeper and induces a tailored gene expression program aimed at adaptation to new environmental conditions or alternatively, cell death. Preferential translation of ATF4 is regulated through two upstream open reading frames (uORFs). Upon translation of uORF1, the presence of pS51-eIF2α delays the ribosomal re-initiation at the inhibitory uORF2 allowing the translation of the ATF4 coding region. This translational control is the main mechanism by which ATF4 protein levels are regulated during the ISR. Recently, however, it was reported that the activation of the mechanistic/mammalian target of rapamycin complex 1 (mTORC1), a key regulator of cap-dependent translation, also controls the expression of ATF4 at the translational level independently of the activation of the ISR. Upon activation, ATF4 regulates the expression of DNA damage inducible 34 (GADD34), the regulatory subunit of the protein phosphatase 1 (PP1) which mediates dephosphorylation of eIF2α. Hence, expression of GADD34 is an important negative feedback loop that allows termination of the ISR.

Antagonizing eIF2α phosphorylation is a common strategy deployed by viruses to avoid shut off of protein synthesis. For example, influenza virus and vesicular
stomatitis virus recruit the host chaperone p58IPK, a PKR and PERK inhibitor, to prevent eIF2α phosphorylation. Moreover, human papilloma virus and herpes simplex virus encode viral proteins that increase the activity of the GADD34/PP1 phosphatase through which eIF2α is dephosphorylated and translation is re-initiated. Indeed, the presence of pS51-eIF2α had a detrimental effect on virus production. Other viruses actually induce eIF2α phosphorylation and hijack other cellular processes to ensure protein translation. For instance, phosphorylation of eIF2α during cytomegalovirus infection promotes ATF4 expression thereby alleviating ER stress and prolonging cell survival.

In case of dengue virus (DENV), an early report described inhibition of eIF2α phosphorylation during infection. However, other studies described a time and serotype dependent regulation of the levels of pS51-eIF2α. Additionally, it has been reported that DENV induces nuclear accumulation of ATF4, and we recently observed an increase in the protein levels of ATF4 upon DENV infection (chapter 6). Therefore, we here explored the eIF2α/ATF4 pathway in more detail. We observed that DENV actively inhibits eIF2α phosphorylation and SG formation in Huh7 cells. The increased expression of ATF4 was found independent of the ISR and mTORC1. Finally, ATF4 is not required to relief the cellular stress during DENV infection of Huh7 cells.

2. Materials and methods

Cell culture. The human hepatic Huh7 cells (JCRB0403) were a kind gift from Tonya Colpitts (University of South Carolina) and were cultured in Dulbecco’s minimal essential medium (DMEM)/Glutamax (Gibco, the Netherlands) supplemented with 10% of fetal bovine serum (FBS) (Lonza, USA), 100U/mL penicillin and 100mg/mL streptomycin (PAA, Switzerland). The lung epithelial A549 cells (ATCC CCL-185) were cultured in DMEM (Gibco) supplemented with 10% of FBS, 100U/mL penicillin and 100mg/mL streptomycin, 2mM L-glutamine (Gibco) and 1X of non-essential amino acids (Gibco). Baby hamster kidney cells clone 21-15 (not commercially available) were a kind gift from Richard Kuhn (Purdue University) and were grown in DMEM (Gibco) supplemented with 10% FBS, 100U/mL penicillin and 100mg/mL streptomycin, 1X of non-essential amino acids (Gibco) and 10mM of hepes (Gibco). All cell lines were cultured at 37°C and 5% CO2.

Virus stocks, infection and stress induction. DENV serotype 2, strain 16681 was propagated on mosquito cell line C6/36, as described previously. The number of infectious virus particles was determined by plaque assay on BHK-15 cells, as described before. Huh7 cells were infected with DENV at a multiplicity of infection.
DENV inhibits eIF2α phosphorylation independently of ATF4

(MOI) of 5 or 10. At 2 hours post-infection (hpi), cells were extensively washed and incubation was continued in DMEM/Glutamax supplemented with 10% FBS. When indicated, cells were treated with sodium arsenite (NaAs Sigma-Aldrich, USA) at a final concentration of 0.5 mM. NaAs was added 45 minutes (min) prior to the time point of harvesting. Salubrinal (Sigma-Aldrich, USA) was added at a final concentration of 5uM at 2 hpi and was present for the duration of the experiment.

Transfection of siRNAs. Huh7 cells were seeded in 24-well plates at a cell density of 7,0*10⁴ cells per well. Twenty-four hours post-seeding cells were transfected with 1.5µl of lipofectamine RNAi/Max (Invitrogen) and 10nM of siRNAs (Dharmacon, ATF4: L-005125-00-0005 and siRNA negative control (siNC): D-001810-01-05 5). When indicated, cells were infected 48 hours post transfection (hpt).

Real-time PCR. Huh7 cells were mock-infected or infected with DENV at MOI 10. At 18, 24 and 30 hpi, cell pellets were lysed in RNA later (Ambion, USA). Total RNA was extracted using Trizol (Ambion) following manufacturer’s instructions. cDNA synthesis was performed with the QuantiTec Reverse transcription (Qiagen, Germany) kit using 100ng of RNA. Real-time PCR was performed with QuantiNova SYBR (Qiagen) using the following primers: ATF4-Fwd: 5’-TTGGTCAGTCCCTCCAACAAC-3’, ATF4-Rev: 5’-CAACAGGGCATCCAAGTGCAAA-3’, GAPDH-Fwd: AATGAAGGGGTCATTGATGG, GAPDH-Rev: AAGGTTGAAGGTCGGAGTCAA. The relative expression of ATF4 was determined using the 2(-delta delta Ct) method taking GAPDH as a reference gene.

Western blot. The proteins were extracted from cells using the RIPA Lysis Buffer System (Santa Cruz Biotechnology). Subsequently, the protein concentration was determined via a Bradford assay (Expedeon, UK). Samples (50-90 µg protein) were mixed with 5x Laemmli buffer and heated for 5 min at 95°C. Thereafter, the proteins were fractionated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Germany). The membranes were subsequently incubated in 5% bovine serum albumin (BSA; GE Healthcare) for 10 min. Then, primary antibodies were added and incubation was continued overnight at 4°C. The antibodies against eIF2α, phospho-S51-eIF2α, ATF4, S6K, phospho-T389-S6K, 4EBP1 and phospho-T37/46-4EBP1 (all from Cell Signalling, The Netherlands) were diluted 1:1000, the antibody for GADD34 (Santa Cruz Biotechnologies) was diluted 1:1000 and the antibody for GAPDH (Abcam, UK) was diluted 1:10000. After extensive washing, membranes were incubated with secondary HRP-conjugated antibodies, goat anti-mouse or goat anti-rabbit (Thermo Fisher Scientific), diluted 1:4000. All dilutions were prepared in TBST at 5% of BSA and 0.1% of sodium azide. Pierce ECL western blotting substrate (Thermo Fisher Scientific) or Super Signal West FEMTO (Thermo Fisher Scientific) was used for detection by means of chemiluminescence using a LAS-4000 mini camera system (GE Healthcare, Little Chalfont, UK). Image
analysis was performed using the Image QuantTL software (GE Healthcare). The band intensity of each protein was normalized to that of GAPDH and expressed as the fold-change over non-treated/mock-infected cells.

**Immunofluorescence.** Huh-7 cells were grown on 12-mm coverslips to 50% confluency. Cells were infected and treated as described above. At the end of an experiment, cells were fixed with 4% paraformaldehyde (PFA) and permeabilized with 0.1% SDS (Merck Millipore). Then, primary antibodies were added and incubation was continued for 1 h at RT in a humid chamber. The antibodies against TIA, G3BP1 and eIF3ε were from Santa Cruz Biotechnologies and diluted 1:50, 1:100 and 1:100, respectively. The J2 antibody against dsRNA (English and Scientific Consulting, Hungary) was diluted 1:200. Upon extensive washing, secondary antibody was added and incubation was continued for 1 h at RT in the dark. All secondary antibodies were from Life technologies and diluted as follows: rabbit α mouse AF647 (1:2000), donkey α goat AF488 (1:500), goat α mouse AF647 and goat α rabbit AF488 (1:1000). All antibodies were diluted in 0.5% milk powder (Nutricia, The Netherlands) prepared in PBS. Nuclei were stained with DAPI (BioLegend, USA). Coverslips were mounted with DABCO (Sigma-Aldrich) and scanned with a fluorescent microscope (Leica, Germany) using a 100x oil-immersion objective. The images were analyzed with ImageJ. An in house-macro was designed to automatically select the SGs. Counting (SGs per cell) was done manually in at least 100 cells per experimental condition.

**Statistical analysis.** All data were visualized using GraphPad Prism software and presented as mean ± SEM. The tests used to evaluate statistical differences between treatments are specified in each figure and a p value <0.05 was considered significant in all cases.

### 3. Results

In chapter 6, we described that DENV infection increases ATF4 protein expression in a time-dependent manner in Huh7 cells. Earlier studies showed that the expression of ATF4 is mainly driven by eIF2α phosphorylation. Therefore, we first evaluated the phosphorylation status of eIF2α in DENV-infected Huh7 cells at 3, 6, 12, 18, 24 and 30 hpi. As shown in Chapter 6, the replicative cycle of DENV in Huh7 cells takes around 18 h, as initial virus particle production is seen at this time point post-infection. Abundant virus particle production is seen from 24 to 30 hpi (Chapter 6, Fig. S1). No difference was found in the levels of pS51- eIF2α expression between mock-infected cells and DENV-infected cells at all evaluated time points (Fig. 1A – 1F; compare first and second lane of each figure and the respective quantitation). Huh7 mock-infected cells treated with the stressor NaAsO2 did show an increase in pS51- eIF2α levels,
indicating that eIF2α phosphorylation can be induced in these cells (Fig. 1A – 1F; compare first and third lane of each figure). NaAs induces oxidative stress which leads to eIF2α phosphorylation by the activation of the kinase HRI. Interestingly, DENV was found to significantly reduce NaAs-mediated eIF2α phosphorylation at 30 hpi (Fig. 1F). This suggests that DENV has the capacity to actively control the levels of eIF2α phosphorylation in cells. Furthermore, the above data suggest that ATF4 induction in DENV-infected cells, which was shown in Chapter 6, is independent of eIF2α phosphorylation.

Upon eIF2α phosphorylation SGs are assembled in the cytoplasm of stressed cells. In order to confirm our previous finding, we next analyzed the presence of SGs in DENV-infected Huh7 cells. In line with the literature in mock-infected non-stressed cells, G3BP1 distributes homogeneously in the cytoplasm and TIA mainly localizes to the nucleus (Fig. 2A). Furthermore, when mock-infected cells were treated with 0.5 mM NaAs for 45 min, G3BP1- and TIA-positive SGs were visible (Fig. 2A). At these conditions, more than 95% of the cells had SGs. In line with the results in Fig. 1, no SG formation was detected in DENV-infected cells at 3, 6, 12, 18, 24 and 30 hpi (Fig. 2B). In these experiments, cells were co-stained for dsRNA molecules to visualize infected cells, which can be clearly detected at 18 hpi and at latter time points (Fig. 2B). We next investigated whether DENV infection actively inhibits SG formation. To this end, mock-infected and DENV-infected cells were treated with NaAs (0.5 mM, for 45 min) at 18, 24 and 30 hpi (Fig. 3). Representative images for 24 hpi are depicted in Fig. 3A whereas Fig. 3B and 3C show the quantification plots at each time point investigated. The results indicate, and in line with the results presented in Fig. 1, that the number of NaAs-induced SGs per cell is significantly lower in DENV-infected cells when compared to time-matched mock-infected controls (Fig. 3A and 3B). We did not observe a decrease in the percentage of SG-positive cells (Fig. 3C), suggesting that DENV reduces SG formation although cells remain susceptible to stress induction.
FIGURE 1 | DENV infection does not induce eIF2α phosphorylation. Huh7 cells were mock-infected (white bars) or infected with DENV at MOI 10 (black bars). When indicated, 0.5 mM NaAs was added to the cells 45 min prior to the end of the experiment. NT stands for non-treated. In each figure the upper panel shows representative western blot images of eIF2α, pS51-eIF2α and GAPDH, whereas the lower panel shows the pS51-eIF2α/eIF2α ratio normalized to GAPDH and relative to mock-infected non-treated cells. (A) 3 hpi, (B) 6 hpi, (C) 12 hpi, (D) 18 hpi, (E) 24 hpi, (F) 30 hpi. Values represent mean ± SEM of four independent experiments. Mann-Whitney U was used as a statistical test. *=p≤0.05.
FIGURE 2 | DENV infection does not induce the formation of stress granules. Immunofluorescence of Huh7 stained for the SG markers G3BP1 and TIA (green), for dsRNA (red) and with DAPI. (A) Mock-infected Huh7 cells left untreated (NT) or treated with 0.5 mM NaAs for 45 min. Cells were processed simultaneously with DENV-infected cells cultured for 30h. (B) Huh7 cells were infected with DENV at MOI 5. At the indicated time points post-infection, cells were fixed and processed for immunofluorescence.
However, is important to note that inconsistent results have been reported regarding the phosphorylation of eIF2α and the assembly of SGs during DENV infection\textsuperscript{17–19,24}. For example, in A549 lung epithelial cells, DENV was found to increase eIF2α phosphorylation and induce G3BP1-positive SGs\textsuperscript{24}. To investigate whether these differences are related to the cell type used, we also investigated the assembly of SGs in A549 cells. In line with the published report\textsuperscript{24}, G3BP1-positive SGs were observed in DENV-infected A549 cells at 24 hpi (Supplementary Fig. S1). We noticed both an increase in the number of G3BP1-positive SGs per cell and an increase in the percentage of cells with G3BP1-positive SGs. This confirms that the regulation of the stress response upon DENV infection is cell type specific. Of note, hepatic cells are natural targets of DENV, whereas lung epithelial cells are not targeted during the course of human DENV infection\textsuperscript{25,26}.
Collectively, our data suggests that in Huh7 cells, DENV actively controls the cellular stress response by inhibiting eIF2α phosphorylation/SG formation. This also implies that the canonical pathway involved in the induction of ATF4 expression is not activated by DENV in Huh7 cells. Recently, the activation of mTORC1 was also associated with ATF4 expression. Therefore, we next evaluated whether DENV induces mTORC1 activation. The phosphorylation status of 4E-BP1 and S6K were used as markers for mTORC1 activation. mTORC1 promotes protein synthesis by 4E-BP1 phosphorylation to form cap-recognition complexes and S6K phosphorylation to increase the biogenesis of ribosomes. We determined the levels of total S6K and total 4E-BP1 as well as the phosphorylated forms of both proteins at 18, 24 and 30 hpi. Because mTORC1 activation is dependent on the metabolic status of the cells, we again performed comparisons with time-matched controls. No differences were found between mock-infected and DENV-infected cells regarding the expression of total S6K at any investigated time point. Similarly, the levels of phosphorylated S6K (p-T389-S6K) in DENV-infected cells were not significantly different to that of mock-infected cells; although, a slight non-significant increase was observed at 18 hpi. Moreover, at 18 hpi, the expression of total 4E-BP1 and the phosphorylated form (p-T37/46-4EBP1) were increased in DENV-infected cells, yet the differences were not statistically significant. Given the results, it is not very likely that mTORC1 is activated upon DENV infection.

Although the expression of ATF4 is typically regulated at the translational level, the aforementioned results indicate that this is not the case in DENV-infected Huh7 cells. Therefore, we next investigated through real time PCR whether DENV altered the mRNA levels of ATF4. As shown in Fig. 5, Huh7 cells infected with DENV indeed have increased levels of ATF4 mRNA, which in accordance with the protein levels (Chapter 6, Fig. 5A), is also time-dependent. Altogether the data suggest that the regulation of ATF4 expression in DENV-infected cells is not controlled at the translational level.
FIGURE 4 | DENV does not induce phosphorylation of S6K or 4EBP1. Huh7 cells were mock-infected or infected with DENV at MOI 10. (A) Representative western blot images of total S6K, pT389-S6K, total 4EBP1, pT37/46-4EBP1 and GAPDH expression at 18, 24 and 30 hpi. (B – E) Quantitation of protein levels at 18, 24 and 30 hpi, normalized to GAPDH and relative to the time-matched mock-infected cells. (B) Total S6K. (C) pT389-S6K. (D) Total 4E-BP1. (E) pT37/46-4EBP1. White bars represent mock-infected cells and black bars depict DENV-infected cells. Values represent mean ± SEM of four independent experiments.
The mechanism by which DENV inhibits eIF2α phosphorylation late in infection has not yet been elucidated. We hypothesized that DENV-induced ATF4 expression might dephosphorylate eIF2α through the expression of GADD34. Therefore, we examined the protein levels of GADD34 upon DENV infection. However, no differences were found in the expression of GADD34 between mock-infected and DENV-infected cells at 30 hpi (Fig. 6A). To further evaluate the role of GADD34 in DENV infection, we next tried to inhibit its phosphatase activity by the use of guanabenz and salubrinal. Active inhibition of GADD34 phosphatase activity has previously been shown to increase pS51-eIF2α levels in Huh7 cells when treated with 75 µM guanabenz for 4h\textsuperscript{29}. However, we did not observe any change in pS51-eIF2α levels neither at this condition, nor at other guanabenz concentrations and incubations times (Supplementary Fig. S2). Therefore, we next attempted to use salubrinal (Fig. 6B). We again tested multiple concentrations and incubation times (data not shown) and at best we found a 1.5-fold increase in pS51-eIF2α levels in mock-infected Huh7 cells treated with 5 µM of salubrinal for 28 h when compared to non-treated cells (Fig. 6B; compare lane one with five). However, no increase in eIF2α phosphorylation was seen in DENV-infected cells treated with salubrinal when compared to control cells (Fig. 6B; compare lane two and six). Importantly, treatment with salubrinal and NaAs has no additive effect on pS51-eIF2α levels (Fig. 6B; compare lane three and seven). Furthermore, salubrinal had no effect on eIF2α phosphorylation levels in DENV-infected cells stressed with NaAs (Fig. 6B; compare lane four and eight). Although salubrinal shows minimal effect in our Huh7 cells, these results do suggest that it is not very likely that GADD34 is involved in controlling the levels of pS51-eIF2α in DENV-infected cells.
FIGURE 6 | ATF4 does not control eIF2α phosphorylation levels in DENV-infected cells. Huh7 cells were infected and treated as described in the legend to Figure 1. (A) The upper panel shows a representative western blot image of GADD34 and GAPDH expression at 30 hpi. Lower panel shows the quantitation of the GADD34 levels normalized to GAPDH and relative to mock-infected cells. (B) Expression levels of total eIF2α, pS51-eIF2α and GAPDH. Salubrinal (Sal) was added at 2 hpi and left for 28 h. Upper panel shows a representative western blot image of the indicated proteins. Lower panel shows the quantitation of the pS51-eIF2α/eIF2α ratio normalized to GAPDH and relative to mock-non-treated (NT) cells. (C) Upper panel shows a representative western blot image visualizing the expression level of total eIF2α, pS51-eIF2α and GAPDH in DENV-infected cells prior transfected with 10 nM of negative control siRNA (siNC) or siRNA targeting ATF4 (siATF4). Lower panel shows the quantitation of the pS51-eIF2α/eIF2α ratio normalized to GAPDH and relative to mock-NT-siNC-transfected cells. Values represent mean ± SEM of at least three independent experiments (A and C) and two independent experiments (B). Statistical differences were assessed with Mann-Whitney U test. *=p≤0.05, **=p≤0.01.
Although GADD34 is a well-described target of ATF4, ATF4 also controls the expression of other genes involved in the restoration of cell homeostasis upon stress. In order to determine whether ATF4 plays a role in controlling the stress responses during DENV infection, ATF4 expression was silenced by the means of siRNAs and eIF2α phosphorylation was monitored. To this end, a negative control (NC) siRNA and a pool of 4 siRNAs targeting ATF4 (siATF4) were used. The average efficiency of knockdown was 70% (Fig. 6C, last four lanes). Again, as also shown in Figure 1, no difference in eIF2α phosphorylation was seen between non-treated mock-infected and DENV-infected cells (Fig. 6C; compare lane one and two). Also, DENV was found to actively inhibit eIF2α phosphorylation in NaAs stressed cells (Fig. 6C; compare lane three and four). Furthermore, comparable levels of pS51-eIF2α were seen in NC- and siATF4-transfected mock-infected cells stressed with NaAs (Fig. 6C; compare lane three and seven). Unexpectedly, however, silencing of ATF4 did not increase the levels of pS51-eIF2α in DENV-infected cells neither in the absence nor in the presence of NaAs (Fig 6C; compare lane two with six and lane four and eight, respectively). Collectively, our results therefore suggest that the increased expression of ATF4 during DENV infection does not contribute to active inhibition of eIF2α phosphorylation.

4. Discussion

During DENV replication, the ER undergoes drastic membrane rearrangements, dsRNA intermediates are formed and there is an accumulation of reactive oxygen species\textsuperscript{30,31}. These phenomena are known to trigger the ISR yet we did not observe eIF2α phosphorylation and SG formation. Several hypotheses have been postulated to explain the lack of eIF2α phosphorylation/SG formation upon DENV infection. For example, as DENV replication occurs in compartmentalized membranes, the dsRNA intermediate might not be recognized by PKR\textsuperscript{32}. However, during the course of our study, Roth and co-workers\textsuperscript{33} reported an increase in the phosphorylation status of PKR upon DENV infection thereby disproving the above hypothesis. Furthermore, and in line with our data, the authors show that eIF2α is not phosphorylated during DENV infection and describe reduced levels of pS51-eIF2α in DENV-infected cells treated with activators of HRI, GCN2 and PERK kinases\textsuperscript{33}. Collectively, this suggests that DENV actively inhibits the ISR by inhibiting eIF2α phosphorylation.

Other studies argue that DENV controls the ISR at the level of SG formation. Indeed, in BHK-21 cells DENV was shown to recruit TIA and TIAR to the replication sites thereby reducing the cellular pools of these proteins. The authors reasoned that the recruitment of these proteins decreased SG assembly in DENV-infected cells\textsuperscript{17}. However, more recently, SG formation was observed in DENV-infected A549 cells.
Despite recruitment of TIA to the genome replication sites. Although the composition of SGs is known to vary between cell types, and particularly in infected cells, the above results do suggest that single factors such as TIA cannot prevent the formation of SGs. Furthermore, in both studies, SG formation was dependent on the phosphorylation status of eIF2α. In DENV-infected BHK-21 cells there was no eIF2α phosphorylation and no SG assembly whereas in DENV-infected A549 cells both eIF2α phosphorylation and SG assembly were observed. This strongly suggests that DENV controls the activation of the ISR upstream of SG assembly by a cell type specific mechanism. Therefore, it is clear that studies regarding the cellular stress response to DENV should be conducted in relevant cell types. Preferentially, future studies should be conducted in primary cells as, compared to cell lines, these better reflect the natural targets of infection.

The absence of eIF2α phosphorylation in Huh7 cells also implies that the observed increased expression of ATF4 in DENV-infected cells is independent of the ISR. A recent study linked ATF4 expression to mTORC1 activation. Yet, here we observed that neither 4E-BP1 nor S6K, two well-known targets of mTORC1, were phosphorylated during DENV infection. Likewise, Roth and coworkers did not find changes in 4E-BP1 phosphorylation levels upon DENV infection. Nevertheless, mTOR targets many more cellular proteins; thus, we cannot rule out that other mTOR substrates regulate ATF4 expression. The absence of 4E-BP1 phosphorylation is also intriguing from another point of view as it suggests that DENV uses a cap-independent mechanism for viral translation. Indeed, it has been reported that DENV translation occurs in a cap-independent manner when cap-dependent translation is inhibited, and more recently Roth et al postulated that cap-independent translation represents a crucial step in the replication cycle of flaviviruses. This concept challenges the traditional view on flavivirus translation and warrants future research as it might open new avenues for therapeutic interventions.

The results show that ATF4 is either regulated at the transcriptional level or its mRNA turnover is reduced in infected cells. Although ATF4 is predominantly regulated at the translational level, several transcription factors have been shown to induce transcription of ATF4. For example, nuclear factor erythroid 2-related factor (NRF2) induces ATF4 transcription in response to oxidative stress, and transcription factors EB and E3 under ER stress and starvation. Interestingly, activation of NRF2-mediated antioxidant pathways was described to be part of the stress response in DENV-infected dendritic cells. Another study reported DENV-induced activation and nuclear localization of NRF2 in mononuclear phagocytes. NRF2 activation is triggered by the viral proteins NS2B/NS3 and contributes to TNF-α production during DENV infection. The authors did not study ATF4 expression, but the activation of NRF2 due to the oxidative stress caused by DENV infection might be
DENV inhibits eIF2α phosphorylation independently of ATF4

a plausible explanation for the observed increase in ATF4 levels. On the other hand, DENV-infected cells may have slower rates of mRNA turnover. This as the activity of processing bodies (P-bodies), which is tightly linked to the assembly of SGs, has been shown to be reduced in DENV-infected cells. Reduced P-body activity may result in enhanced stability of certain mRNAs. Further studies are being conducted in our group to determine the pathway leading to ATF4 mRNA transcription/stability and the viral protein(s) involved in these processes.

Activation of ATF4 leads to the transcription of various genes involved in the recovery from stress. Surprisingly, though, we did not find differences in GADD34 expression between mock-infected and DENV-infected cells. This result is puzzling, as GADD34 is a well-known target of ATF4 and a recent study did report an increase in mRNA levels of GADD34 in DENV-infected Huh7 cells. It is possible that transcription but not translation of GADD34 is induced upon DENV infection. On the other hand, GADD34 inhibitors have been shown to have potent antiviral activity in mouse embryonic fibroblasts, A549 and MDCK cells, which does suggest a role for this protein during infection. In our model, Huh7 cells were rather resistant to the chemical inhibition of GADD34 activity. Therefore, it is difficult to conclude whether GADD34 is involved in the control of eIF2α phosphorylation in these cells. Nevertheless, because the overall silencing of ATF4 did not have an effect on pS51-eIF2α levels in DENV-infected cells stressed with NaAs, it is likely that the ATF4/GADD34 axis of the ISR is not involved in the DENV-mediated control of eIF2α phosphorylation.

The importance of DENV-induced ATF4 expression remains an open question. We hypothesize that ATF4 is involved in controlling other traits related to infection. For example, ATF4 contributes to the production of several pro-inflammatory cytokines and prolonged cell survival due to induction of autophagy. Future studies, with several cell types and other DENV serotypes, are needed to clarify the role that ATF4 plays during infection.
**Supplementary material**

A. 

**Supplementary Figure S1.** DENV induces G3BP1-positive SGs in A549 cells. A549 cells mock infected (white bars) or infected with DENV at MOI 10 (black bars). (A) At 24 hpi, cells were fixed and stained for the SG marker G3BP1 (green), viral dsRNA (red) and with DAPI. (B) Average number of G3BP1-positive SGs per cell. (C) Percentage of G3BP1-SG-positive cells. Two independent experiments were performed and for each experimental condition at least 35 cells were counted. Values are presented as mean ± SEM. Statistical differences were assessed with Mann-Whitney U test. ***=p≤0.001.

**Supplementary Figure S2.** Guanabenz treatment does not increase pS51-elf2α levels in Huh7 cells. Huh7 cells were treated with several concentrations of Guanabenz for 4, 3, 18, 24 or 30 h as indicated. As control of elf2α phosphorylation, cells were treated with 0.5 mM NaAs for 45 min. Upper panel shows the western blot images of elf2α, pS51-elf2α and GAPDH and the lower panel shows the pS51-elf2α/elf2α ratio normalized to GAPDH and relative to non-treated (NT) cells.
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