Flavivirus infection uncouples translation suppression from cellular stress responses

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Running Title: Control of host cell translation by flaviviruses

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Summary

As obligate parasites, viruses strictly depend on host cell translation for the production of new progeny, yet infected cells also synthesize antiviral proteins to limit virus infection. Modulation of host cell translation therefore represents a frequent strategy by which viruses optimize their replication and spread. Here we sought to define how host cell translation is regulated during infection of human cells with Dengue Virus (DENV) and Zika Virus (ZIKV), two positive-strand RNA flaviviruses. Polysome profiling and analysis of de novo protein synthesis revealed that flavivirus infection causes potent repression of host cell translation while synthesis of viral proteins remains efficient. Selective repression of host cell translation was mediated by the DENV polyprotein at the level of translation initiation. In addition, DENV and ZIKV infection suppressed host cell stress responses such as the formation of stress granules and phosphorylation of the translation initiation factor eIF2α. Mechanistic analyses revealed that translation repression was uncoupled from the disruption of stress granule formation and eIF2α signaling. Rather, DENV infection induced p38-Mnk1 signaling that resulted in the phosphorylation of the eukaryotic translation initiation factor eIF4E and was essential for the efficient production of virus particles. Together, these results identify the uncoupling of translation suppression from the cellular stress responses as a conserved strategy by which flaviviruses ensure efficient replication in human cells.

Importance

For efficient production of new progeny, viruses need to balance their dependency on the host cell translation machinery with potentially adverse effects of antiviral proteins produced by the infected cell. To achieve this, many viruses evolved mechanisms to manipulate
host cell translation. Here we find that infection of human cells with two major human
pathogens Dengue Virus (DENV) and Zika Virus (ZIKV) leads to the potent repression of host cell
translation initiation while the synthesis of viral protein remains unaffected. Unlike other RNA
viruses, these flaviviruses concomitantly suppress host cell stress responses, thereby uncoupling
translation suppression from stress granule formation. We identified that the p38-Mnk1
cascade regulating phosphorylation of eIF4E is a target of DENV infection and plays an
important role in virus production. Our results define several molecular interfaces by which
flaviviruses hijack host cell translation and interfere with stress responses to optimize the
production of new virus particles.

Introduction

During infection by viruses, the translation stimulation of specific host mRNAs encoding
innate response effector proteins can limit viral replication and spread (1). Therefore,
interference with host mRNA translation represents a frequent evasion strategy evolved by
viruses to subvert nearly every step of the host cell translation process (2). Translational arrest
can be triggered by the phosphorylation of the eukaryotic translation initiation factor (eIF) 2
subunit alpha (eIF2α), which interferes with formation of the eIF2-GTP-trNAiMet ternary
complex and causes stalling of translation initiation and polysome disassembly (3). Among the
four eIF2α-kinases, protein kinase R (PKR) is activated by viral double-stranded (ds) RNA in the
cytoplasm and mediates translation suppression upon replication of many RNA viruses (4).
Inhibition of protein synthesis is tightly linked to the assembly of stress granules (SGs), which
are cytosolic aggregates of stalled translation pre-initiation complexes (5-7). As they require an
intact translation machinery to translate their viral genome, several viruses antagonize SG
formation during infection, although some may also exploit SG responses for their replication (8, 9).

Viruses can also interfere with host cell translation by targeting eIF4E availability or activity to limit the initial cap-binding step in the translation process (10). The activity of eIF4E is regulated by the eIF4E-binding proteins (4E-BPs), which can sequester eIF4E when hypophosphorylated while hyperphosphorylation of 4E-BP by mechanistic target of rapamycin (mTOR) frees eIF4E (11-13). Moreover, the phosphorylation of eIF4E at serine residue 209 by the mitogen activated protein (MAP) kinase interacting kinases Mnk1/2 can lead to translational activation of mRNAs encoding proteins involved with cell proliferation, inflammation and interferon production (14-17). Therefore, several viruses manipulate mTOR or MAP kinase signaling pathways to exert translational control on the host (reviewed in (2, 10)).

DENV infection is considered the most important arboviral disease (18) and causes an estimated 390 million cases annually worldwide (19). DENV infection leads to a wide spectrum of clinical manifestations ranging from asymptomatic or self-limited Dengue Fever to more severe symptoms such as Dengue Hemorrhagic Fever, Dengue Shock Syndrome and eventually death that occur in a small proportion of patients and often result from secondary infections with heterologous serotypes (20-22). DENV is a member of the Flavivirus genus of the Flaviviridae family that also includes West Nile virus (WNV), Japanese encephalitis virus, Yellow Fever virus, and Zika virus (ZIKV). In addition to clinical manifestations similar to the febrile illness caused by DENV infection, ZIKV has recently been associated with severe neurological disease in newborns (23-25). DENV is a positive-strand RNA virus with a genome length of approximately 10.7 kilobases that harbors a type I cap structure at the 5’ end but lacks a polyadenylated tail at the 3’ end (26, 27). The DENV RNA genome encodes a single polyprotein
which is proteolytically processed into three structural proteins (capsid, prM and envelope) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) required for viral RNA replication (28). Viral RNA is replicated through dsRNA intermediates likely shielded in virus-induced rearrangements of the endoplasmic reticulum membranes called vesicle packets (29, 30).

DENV infection interferes with cellular processes such as autophagy (31, 32) and host innate immune responses (33-35), however the regulation of host cell translation during DENV infection is not well characterized. Paradoxically, DENV induces host stress responses but seems to repress them almost simultaneously (36-39) supposedly to avoid repression of host cell translation which could also affect translation of the viral genome. Interestingly, DENV can switch from cap-dependent to cap-independent RNA translation when host cell translation is inhibited experimentally (40).

Herein, we aimed at defining the complex interaction of flaviviruses such as DENV and ZIKV with the host cell translation machinery in human cells. We show that flaviviruses suppress host cell translation early post infection while translation of their RNA genome is maintained. Translation suppression is uncoupled from the activation of the eIF2α-dependent stress response, and is part of a multilayered strategy that manipulates several steps of host cell translation.

**Results**

**DENV induces a strong repression of host cell translation early post infection.**
To characterize the effect of DENV infection on global host cell translation we compared polysome profiles from naïve and DENV-infected human hepatoma Huh7 cells. DENV infections were carried out with the serotype 2 strain New Guinea C (NGC) at a high multiplicity of infection (MOI) of 10 TCID<sub>50</sub> per cell, unless otherwise stated, to synchronize the infection kinetic and reduce effects resulting from viral spread. Cell extracts were analyzed by sucrose density gradient centrifugation and absorbance at 254 nm was continuously recorded. This allowed separating actively translated, polysomal mRNAs associated with multiple ribosomes from sub-polysomal mRNAs that are not or poorly translated (Fig. 1A). As illustrated by the increase in 80S peak and concomitant decrease of the polysomal peaks, DENV-infected cells showed a progressive loss of polysomal mRNAs (Fig. 1A). This effect was already measurable 18 h post infection (p.i.) and became even more pronounced at later times (Fig. 1A). The rate of translation for each condition was assessed by measuring the proportion of ribosomes associated with polysomes (Fig. 1B). While naïve Huh7 cells had on average more than 55% polysomal ribosomes (Fig. 1B), the proportion of polysomal ribosomes strongly decreased in DENV-infected cells to 12.5% at 36 h p.i..

DENV genome translation is able to switch from a cap-dependent to a cap-independent mechanism when infected cells are treated with a translation inhibitor (40). Since the DENV positive strand (+) RNA genome directly binds to ribosomes for translation, we were able to investigate its association with actively translating ribosomes in the same time course experiments (Fig. 1C). Polysome profiles were recorded and fractions collected based on the elution time (Fig. S1A). Distributions of DENV (+) RNA genome and GAPDH mRNA, a housekeeping gene whose translation is stalled upon translation shutoff, were quantified in each fraction by qRT-PCR (Fig. S1B). As expected, the association of GAPDH mRNA with polysomal
ribosomes decreased upon infection from 85 to 40% (Fig. 1C). In contrast, a constant fraction of
approximately 40% of the DENV (+) RNA genome remained associated with polysomal
ribosomes throughout the experiment, during which DENV RNA genome replication increased
gradually up to 36 h p.i. (Fig. S1C). Similar translation repression of host mRNAs upon DENV
infection was observed in human lung epithelial A549 cells, which are immune-competent in
contrast to Huh7 cells (41, 42) (Fig. S1D), arguing that the observed phenotype does not depend
on interferon.

Repression of global protein synthesis in DENV-infected cells was also observed by
measuring incorporation of puromycin (43), a structural tRNA analog that is covalently coupled
to the carboxyl terminus of nascent polypeptides and causes their premature release from
ribosomes (44). Puromycin incorporation was strongly reduced after 36 h p.i. comparable with
the effect of cycloheximide (CHX), a potent translation elongation inhibitor (45) (Fig. S1E).
Interestingly, viral protein levels as detected by immunostaining of DENV NS4B increased over
time (Fig. S1E).

This block in cellular protein synthesis was also apparent at the single cell level (46, 47).
Huh7 cells were infected at a lower MOI (0.5 TCID$_{50}$ per cell) to allow for parallel visualization of
infected and uninfected cells and harvested at 24, 36 and 48 h p.i.. Puromycylated native
peptide chains were detected using an anti-puromycin antibody as a measure of protein
synthesis (Fig. 1D). Quantification of the puromycin signal in individual cells showed a
progressive reduction of protein synthesis over the 48 h period of infection (Fig. 1E). As a
positive control, cells were treated with arsenite, a potent inducer of eIF2α phosphorylation
that inhibits global protein translation (48, 49). Similar protein synthesis repression was found in
cells electroporated with DENV subgenomic RNA (also called replicon, DENV$_{rep}$) (50), a DENV
RNA genome lacking the structural proteins sequences but still capable of autonomous amplification (Fig. S2A). In this system, protein suppression was stronger at later time points. This delay accounted for a slower RNA replication kinetics of the replicon (starting at 48 h post electroporation, Fig. S2C) compared to the replication kinetics of viral particles (Fig. S2D). We further tested the role of DENV proteins, independently of viral replication. Importantly, transient transfection of a plasmid encoding for DENV non-structural proteins NS1 to NS5 (DENV$_{NS1-5}$) was sufficient to induce translational repression, demonstrating that viral replication is dispensable for this process (Fig. S2E and S2F). As expected (51), no effect on cellular translation was observed in Huh7 cells transiently expressing the hepatitis A virus replicon (HAV$_{rep}$) or the HAV polyprotein 2ABC-3ABCD (Fig. S2B, S2E and S2F). Together, these results establish that the DENV polyprotein potently represses host cell translation at early times p.i., irrespective of the immune competence of host cells, while RNA genome translation is maintained throughout the course of viral infection.

Host cell translation repression is a hallmark of flavivirus infection.

To test whether early repression of host cell translation is a general feature of flavivirus infection, we analyzed other DENV serotypes (DENV1, DENV3, DENV4), ZIKV Uganda strain MR766 and Polynesian strain H/PF/2013, as well as WNV strain New-York 99 (NY). Infection kinetics were defined based on virus replication and cytopathogenicity in Huh7 cells. Of note, cell confluency was maintained at a maximum of 95% even at late time points after infection to avoid translation repression artifacts due to growth restriction. As shown by polysome profile analyses (Fig. S3A-F) and estimation of translation rates (Fig. 1F), all flaviviruses analyzed repressed host cell translation, albeit to different extents. DENV4 and WNV NY infection
induced a repression comparable to that observed with DENV2 NGC infection. Infection with DENV1, DENV3 and ZIKV H/PF/2013 induced a slower and slightly less potent translation repression, which correlated with a reduced cytopathic effect in Huh7 cells compared to DENV2 NGC. Finally, infection with ZIKV strain MR766 induced a very strong reduction of translation rates as early as 18 h p.i.. The suppression of a global translation accompanied by the disassembly of heavy polysomes is therefore a general feature of flavivirus infection.

Host cell translation is impaired at the initiation stage.

Global protein synthesis shut off can result from reducing the rate of translation, initiation or elongation (52). Our previous observation that sub-polysomal mRNAs are markedly increased in DENV-infected cells (Fig. 1B) suggested repression of translation at the initiation stage. To test if elongation was also affected, we analyzed translation elongation rates by ribosome run-off experiments (53). Naïve Huh7 cells (Fig. 2A) or Huh7 cells infected with DENV for 24 h (Fig. 2B) were treated with harringtonine, an alkaloid that inhibits translation initiation only by a block following 60S subunit joining (54). Polysome profile analysis revealed a similar decrease of polysomes due to ribosome runoff over the 4.5 min treatment in naïve and DENV-infected cells (Fig. 2C), indicating similar rates of translation. Consistently, phosphorylation levels of eukaryotic elongation factor 2 (eEF2), reflecting altered binding to the ribosome and impaired elongation (55), remained unaltered during the course of infection (Fig. 2D). We conclude that DENV infection impairs host cell translation at the initiation step but does not alter translation elongation.
DENV-induced host cell translation initiation repression is uncoupled from host cell stress responses.

Stalling of translation initiation is tightly linked to SG assembly (5). To overcome host translation shutoff, several RNA viruses evolved different strategies including mechanisms that interfere with SG formation (8, 9). It has been reported that DENV inhibits SG formation in baby hamster kidney (BHK-21) cells by sequestering the SG-initiating proteins T cell internal antigen-1 (TIA-1) and TIA-1-related protein R (TIAR) on the 3’ untranslated region (UTR) of its RNA genome (36), although we found previously that infection of Huh7 cells with DENV induced few sporadically oscillating SGs (56). To test if DENV-induced translation initiation repression was linked to the activation of a (sporadic) stress response in Huh7 cells, we analyzed SG formation in detail (Fig. 3A). The eukaryotic initiation factor eIF3B, which binds the 40S subunit, was used as *bona fide* SG marker. No SGs were detectable in DENV-infected Huh7 cells (Fig. 3A) and, as expected (36), in WNV NY-infected cells (Fig. S4A). Next, we assessed the response to arsenite-induced oxidative stress, which causes translation suppression and SG formation through eIF2α phosphorylation. When treated with arsenite, DENV-infected cells showed an attenuation of SG formation affecting both the number (Fig. 3B and 3C) and size (Fig. S4C) of SGs. Moreover, inhibition of arsenite-induced SG formation in DENV-infected cells correlated negatively with the viral expression level as measured by the accumulation of NS5 signal intensity in the nucleus (Fig. S4D). In agreement with earlier reports (36, 57), these results demonstrate that DENV and WNV NY (Fig. 3D) repress eIF2α phosphorylation-dependent SG formation in Huh7 cells.

Surprisingly, although infection with ZIKV strain MR766 was also capable to repress arsenite-induced SG formation to levels similar to DENV infection (Fig. 3D and S4B), small eIF3B positive-foci, which co-localized with the SG markers Hu protein R (HuR), poly(rC)-binding
protein 2 (PCBP2) and TIAR, were detected in around 55% of naïve Huh7 cells infected with ZIKV (Fig. S4A and S4E).

SG formation can be triggered in an eIF2α phosphorylation-independent manner by treatment with hippuristanol (58), an inhibitor of eIF4A RNA-binding (59). Huh7 cells infected with DENV were treated with hippuristanol and compared to DMSO-treated cells (Fig. 3E). Remarkably, DENV infection also impaired hippuristanol-induced SG formation (Fig. 3F) in an eIF2α phosphorylation-independent manner (Fig. S4E). Altogether, these results suggest that DENV infection inhibits SG formation induced by both eIF2α-dependent and -independent pathways.

DENV-induced repression of host cell translation is independent of PKR and eIF2α phosphorylation.

Cells respond to various stressors including viral infection by inducing phosphorylation of eIF2α, which results in translational stalling. Since inconsistent results have been observed in different experimental systems regarding the phosphorylation status of eIF2α in the course of DENV infection (37, 38), we tested whether the PKR-eIF2α signaling pathway is activated by DENV infection in Huh7 cells despite the absence of SGs. Remarkably, and in agreement with previous reports (35), basal PKR levels were dramatically reduced in the course of DENV infection. However, PKR phosphorylation was markedly increased at 24 h p.i., a time at which SG were not detected (Fig. 4A). In stark contrast, eIF2α phosphorylation remained at basal levels in DENV-infected cells at all time points examined (Fig. 4B). Similarly, the analysis of global eIF2α phosphorylation by Phos-tag acrylamide gel electrophoresis (60), a mobility shift detection assay of phosphorylated proteins, confirmed the absence of eIF2α phosphorylation
(Fig. 4B, lower panel). Of note, impairment of eIF2α phosphorylation in response to kinases other than PKR was previously reported (36, 37). Consistently, DENV and ZIKV infection blocked eIF2α phosphorylation induced by treatment with arsenite that activates both heme-regulated eIF2α kinase (HRI) and general control nonderepressible 2 (GCN2), thapsigargin that activates the PKR-like endoplasmic reticulum kinase (PERK), and FCCP that activates HRI (61) (Fig. S5A-F).

While the above experiments excluded a role for PKR in DENV-induced eIF2α phosphorylation, they did not address a potential role of PKR in translation repression. To test this, we verified by siRNA-mediated gene silencing that transient PKR suppression did not affect DENV replication (Fig. S6A and S6B) and then established Huh7-derived PKR knock-out (ko) cell clones. Three ko cell clones (2#2, 2#3 and 3#1) were selected that lacked basal levels of PKR and failed to produce detectable amounts of PKR upon induction with interferon α (62) (Fig. S6C). Polysome profiles of Huh7 PKR ko cell clone 2#3, in which DENV replication levels were similar to those of parental cells (Fig. S6D), showed a similar reduction of polysomal RNAs throughout the course of infection (Fig. 4C), as observed for the parental Huh7 cells (Fig. 1B). Similar results were obtained with Huh7 PKR ko cell clones 2#2 and 3#1. This suggests that PKR activation during DENV infection is not required for translation suppression in human Huh7 cells.

Growth arrest and DNA-damage-inducible 34 (GADD34), a regulatory subunit protein of phosphatase PP1, is induced in response to eIF2α phosphorylation. GADD34 promotes dephosphorylation of eIF2α and thereby serves as negative feedback mechanism to trigger recovery from the translation arrest (63-65). GADD34 mRNA levels were upregulated in the course of late DENV infection (Fig. S7A), consistent with our earlier report (56). However, at 18 h p.i. when DENV-induced translational repression starts, levels were only moderately upregulated. We therefore hypothesized that early p.i. GADD34 levels are insufficient to
antagonize the virus-induced host translation repression. To address this possibility, we used Huh7 cell pools that stably overexpress GADD34 (56) (Fig. S7B). We tested their ability to antagonize arsenite-induced translation inhibition by polysome profile analysis. While Huh7 control (Ctrl) cells responded to increasing arsenite concentrations by a strong translational repression (6.5 % of polysomal ribosomes at 500 µM, Fig. S7C), Huh7 GADD34 cells efficiently antagonized the translation block (42.6 % of polysomal ribosomes at 500 µM, Fig. S7D). In contrast, polysomes were similarly reduced in DENV-infected Huh7 Ctrl and GADD34 cells (Fig. 4D). For control, GADD34 overexpression was found to reduce DENV replication only slightly (Fig. S7E). Polysome profiles and immunofluorescence analyses of Huh7 PKR ko cells (Fig. S6E and S6F) as well as Huh7 GADD34 cells (Fig. S7F and S7G) confirmed that both SG-like foci formation and translation repression upon ZIKV infection do not require of the PKR-eIF2α signaling pathway. Hence, flavivirus suppress translation through a pathway that is independent of the canonical PKR-eIF2α cascade.

DENV infection does not impair assembly of the cap eIF4F complex.

Since translation suppression in DENV-infected cells did not result from eIF2α inactivation, we next explored whether DENV infection impairs alternative regulators of translation initiation, e.g. the ability to assemble the cap eIF4F complex (66). To address this question, cap-binding proteins were isolated by immunoprecipitation from naïve and DENV-infected Huh7 cells using immobilized m7GTP (Fig. 5A). As a control for cap disassembly, Huh7 cells were treated with Torin1, a mTOR inhibitor that blocks 4E-BP1 phosphorylation and thereby lowers the availability of eIF4E for translation (67). As expected, treatment of Huh7 cells with Torin1 resulted in hypophosphorylation and increased association of 4E-BP1 with m7GTP
whereas binding of eIF4A, eIF4G, eIF3B, eIF3E and PABP to the cap was decreased (Fig. 5A and 5B). However, DENV infection did not alter 4E-BP1 phosphorylation or cause a decrease of initiation factor binding to the cap (Fig. 5A and Fig. 5B). Thus, DENV infection does not suppress translation initiation by the disassembly of the eIF4E cap-binding complex.

DENV requires activation of the p38-Mnk1 kinase pathway for virus production.

Association of eIF4E with eIF4G within the cap-binding complex is important for cap recognition and for the regulation of eIF4E phosphorylation (68). In response to activation of the MAP kinases p38 and ERK, the MAP kinase-interacting kinases Mnk1/2, when bound to the eIF4E-eIF4G complex, phosphorylate eIF4E on serine residue 209 (17, 69). Several viruses are known to hijack this pathway or interfere with eIF4E dephosphorylation (10). Thus, we investigated the phosphorylation status of eIF4E during DENV infection. eIF4E phosphorylation showed a transient increase at 24 h p.i. and returned to basal levels at 48 h p.i. (Fig. 6A).

Induction of eIF4E phosphorylation upon DENV infection was also observed using Phos-tag acrylamide gel electrophoresis (Fig. 6A, lower panel). eIF4E is phosphorylated by Mnk1/2 downstream of MAP kinases p38 and ERK (70, 71). We therefore tested whether DENV infection activated the MAPK signaling pathway using a human phospho-MAPK array and examined ERK1/2 and p38 phosphorylation levels at 24 and 43 h p.i. (Fig. 6B). While inoculation with UV-inactivated virus did not trigger MAPK activation, the p38 main isoform, p38α, was strongly phosphorylated at 24 h and 43 h post DENV infection. In contrast, phosphorylation of the other isoforms p38β, p38δ and p38γ, as well as of ERK1 and ERK2, remained unchanged (Fig. 6B). p38α phosphorylation in DENV-infected cells was confirmed by Western blot analysis and observed as early as 12 h p.i. (Fig. 6C). Upon activation by p38, Mnk1 is activated to
phosphorylate eIF4E (17). Consistent with the activation of p38α, the phosphorylation of the eIF4E upstream kinase Mnk1 was increased at 24 h.p.i. (Fig. 6D). However, this phosphorylation was weaker than that previously shown to be induced during murine norovirus (MNV) infection (72).

To test whether phosphorylation of eIF4E was involved in DENV-induced repression of host cell translation, we established Huh7 cell pools overexpressing wild-type HA-tagged eIF4E, phospho-ablative HA-eIF4E-S209A and phosphomimetic HA-eIF4E-S209D mutants (71). The characterization of these cells pools showed that ectopic expression of HA-tagged eIF4E variants resulted in reduced expression levels of endogenous eIF4E compared to control cells (Fig. 6E), reflecting the tight regulation control of eIF4E expression levels (73). Importantly, addition of a HA tag at the N-terminus of eIF4E variants did not impair eIF4E association with polysomes (Fig. S8A). Finally, ectopic expression of eIF4E variants did not affect rates of translation of Huh7 cell pools compared to those of parental cells (Fig. S8A and S8B). Stable overexpression of the phosphomimetic HA-eIF4E S209D mutant in naïve Huh7 cells did not reduce translation rates (Fig. S8B) providing first evidence that eIF4E phosphorylation itself does not induce host translation repression. Comparison of polysome profiles of DENV-infected Huh7 HA-eIF4E wildtype and Huh7 HA-eIF4E-S209A cells, in which DENV replication levels were similar to those of control cells (Fig. S8C), revealed that overexpression of the phospho-ablative HA-eIF4E-S209A mutant failed to rescue the host translation repression (Fig. 6F). These results confirm that eIF4E phosphorylation is dispensable for DENV-induced translation repression. Furthermore, inhibition of Mnk1 function using a specific chemical inhibitor of Mnk1 activity CGP57380 (74, 75) at a not cytotoxic concentration in Huh7 cells (Fig. S8D) prevented eIF4E phosphorylation in naïve and DENV-infected Huh7 cells (Fig. S8E) but failed to rescue DENV-induced translation
repression (Fig. 6G). Altogether, these results indicate that eIF4E phosphorylation during DENV infection is dispensable for the translation suppression and support the current model in which eIF4E phosphorylation would rather favor the translation of selective mRNAs than impair translation initiation on a more global level (15).

Our results indicated that eIF4E phosphorylation did not influence DENV replication (Fig. S8C). To investigate the importance of the p38-Mnk1 pathway during DENV infection, we analyzed DENV particle production in presence of the p38 inhibitor, SB203580 (76) and of the Mnk1 inhibitor, CGP57380. At a concentration of 50 µM, both SB203580 and CGP57380 did not affect Huh7 cell viability (Fig. S8D and S8F) and reduced eIF4E phosphorylation levels in DENV-infected Huh7 cells (Fig. S8E and S8G). Inhibition of p38 by SB203580 treatment resulted in a moderate but significant reduction of DENV infectious titers compared to the control DMSO-treated cells (Fig. 6H). Consistently, inhibition of Mnk1 by CGP57380 treatment strongly impaired DENV particle production of approximately 100-fold (Fig 6I). These results suggest that during DENV infection the activation of the p38-Mnk1 signaling pathway plays a major role for virus production that is independent of eIF4E phosphorylation.

Discussion

Suppression of host cell translation is a strategy that several viruses, including RNA viruses such as poliovirus, vesicular stomatitis virus, encephalomyocarditis virus and Influenza virus, have developed to favor the translation of their viral genome (reviewed in (2, 77)). Here, we report an unexpected repression of global cellular protein synthesis by cap-dependent flaviviruses (Fig. 7). Our results support the model of a possible switch in DENV genome translation from cap-dependent to cap-independent under conditions of translation
suppression (40). Importantly, our results indicate that this switch occurs in the context of a natural infection, as reflected by the subsistence of viral RNA association with actively translating ribosomes. Consistently, viral protein levels are not affected by the host translation repression while global protein synthesis is strongly reduced.

Flaviviruses were proposed to prevent rather than to induce host translation shutoff (36, 37, 40). Several technical aspects might explain this difference to our findings, including the use of non-human cells, different DENV serotype 2 strains, and time points p.i. chosen for analysis. Of note, polysome profiles of Huh7 cells infected with the DENV serotype 2 Bangkok strain 16681 (40) revealed in our experiments a similar translation repression to that induced by DENV1 infection (see Fig. 1F for reference), which also correlated with reduced cytopathic effect in Huh7 cells (data not shown). Analyses of human cells early p.i. by polysome profiling identified host cell translation repression as a new feature of flavivirus infection.

Flaviviruses actively block eIF2α-mediated stress response at different levels, supposedly to avoid the associated host translation suppression. First, WNV and DENV inhibit SG formation in non-human cells (36). Consistently, we observed that DENV, WNV as well as ZIKV block SG formation in human Huh7 cells (Fig. 7). Second, infection with DENV was reported to trigger the activation of integrated stress response, phosphorylation of eIF2α through PERK activation and presumably eIF2α dephosphorylation by the GADD34-PP1 complex (37, 38). However, discrepancies exist about eIF2α phosphorylation and the timing of its regulation. Our results support an absence of eIF2α phosphorylation at late times p.i. as well as the activation of a stress response as reflected by the upregulation of GADD34 mRNA levels in DENV-infected cells. Importantly, our results demonstrate that activation of eIF2α-dependent stress response is uncoupled from the host translation repression during DENV infection. Strikingly, our results
indicate that DENV infection also represses eIF2α-independent SGs assembly since SG formation induced with hippuristanol, an inhibitor of eIF4A RNA-binding (59), was repressed. Since flaviviruses actively block both eIF2α-dependent and -independent SG formation but override the associated translation suppression, it is tempting to speculate that they thereby avoid the sequestration of their RNA genome into SG or its degradation in SG associated processing bodies (78, 79).

None of the canonical pathways leading to translation initiation alteration that are usually hijacked by viruses to favor their genome translation (2) was involved in the suppression of host translation upon DENV infection. Neither eIF2α nor the eiF4F cap-binding complex was altered as translation was repressed by DENV infection. Nevertheless, our study showed that DENV infection activates of the p38-Mnk1 signaling pathway, which regulates eIF4E phosphorylation and contributed to DENV particle production. While we ruled out that eIF4E phosphorylation accounted for DENV-induced translation repression, using stable overexpression of the phospho-ablative mutant, eIF4E-S209A, or the inhibition of Mnk-1 activity, our results imply that eIF4E phosphorylation may rather play a role in the translational remodeling and control of specific mRNAs encoding proteins associated with cell proliferation, inflammation and interferon production (14-16), similar to that previously proposed during norovirus infection (72). The p38-Mnk1 signaling pathway activation is known to phosphorylate a number of downstream targets other than eIF4E including the heterogeneous ribonucleoprotein hnRNPA1 (80). Phosphorylation of hnRNPA1 by Mnk1 results in its disassociation from the tumor necrosis factor-α (TNF-α) 3’ UTR and promotes the translation of the TNF-α mRNA (81). Interestingly, hnRNPA1 phosphorylation also leads to its recruitment in SG (80) and was recently shown to play an essential role in SG aggregation (82). Whether Mnk1-
mediated hnRNPA1 phosphorylation could be involved in the absence of SG formation upon DENV infection remains to be investigated.

While our analysis did not reveal the mechanism by which flaviviruses block host cell translation, several scenarios, not mutually exclusive, might explain this repression. First, DENV genome translation could be favored by an optimized usage of codons with low prevalence in the host cell (83-85) and thereby translation of host mRNAs be attenuated. Second, DENV infection might limit the availability of the translation machinery components such as ribosomal subunits. Affinity purification and mass spectrometry analyses of DENV-infected cells revealed the interaction of NS1 with over 30 ribosomal proteins such as RPL18 which is required for both viral replication and translation (86). Third, our results indicate that viral replication is dispensable for the induction of translational repression, as illustrated by the absence of puromycin incorporation in single cell expressing DENV polyprotein. Interestingly, expression of the DENV polyprotein in Huh7 cells induces ER membrane rearrangements as observed in DENV-infected cells (M.C. and R.B., unpublished results). Although we cannot exclude the role of single DENV proteins, it is tempting to speculate that host translational repression might also be a consequence of the ER membrane rearrangements which are essential for virus replication. Altogether, relevance of the host translation repression for virus production is underscored by the simultaneous targeting of multiple pathways that regulate host translation and by its conservation among all flaviviruses. These findings provide a novel perspective on the role of cap-independent translation as a crucial step of flavivirus life cycle and highlight the importance of unraveling underlying mechanisms. Further analyses of these complex virus-host interactions in human immature dendritic cells, target cells of DENV at the first site of infection (87), will be required.
**Material and Methods**

**Cell Culture**

Information about the cell lines is provided as supplementary information.

**Plasmids**

A description of the plasmids used in this study is provided as supplementary information.

**Production of DENV NGC and Titration**

Ten µg of pDVWSK601 *in vitro* transcript were electroporated in BHK-21 cells. Virus supernatants were collected from day 3 to day 5 post electroporation. Infectious titers of virus stocks were determined by limiting dilution assay which protocol was adapted from (88). Detailed information about *in vitro* transcription protocols, virus production and titration is provided as supplementary information.

**Production of Flaviviruses and Titration by Plaque Assay**

DENV serotype 1 (strain Hawaii), DENV serotype 3 (strain H87), DENV serotype 4 (strain H241) were kindly provided by Progen Biotechnik (Heidelberg, Germany). ZIKV strains MR766 and H/PF/2013 were obtained from the European Virus Archive (EVA, France). WNV (strain New-York99) was a kind gift of Jonas Schmidt-Chanasit (Hamburg, Germany). All viruses were passaged once on C6/36 cells and stocks were prepared by virus amplification in VeroE6 cells.
Virus stock titers were determined by plaque assay. Detailed information is provided as supplementary information.

**Polysome Profile Analysis**

Cells were seeded to reach a maximum of 90 percent confluence on the day of analysis (1x10^6 cells for 24 h infection). Polysome profile analysis was performed as previously described (89). Prior to lysis, cells were treated with 100 µg/ml cycloheximide (CHX, Sigma-Aldrich) for 10 min and washed with ice-cold PBS containing 100 µg/ml CHX. Cells were lysed by scraping with 200 µl polysome lysis buffer and cleared by centrifugation at 10,000 rpm for 10 min at 4°C. Lysates were loaded onto a linear gradient of 17.5 to 50% and subjected to ultracentrifugation at 35,000 rpm at 4°C using a SW60 rotor (Beckman) for 2.5 h. Fractions were eluted from the top using a Telodyne ISCO gradient elution system. Polysome profiles were obtained by measuring the absorbance at 254 nm. Detailed information about the calculation of translation rates and polysome fractionation procedure is provided as supplementary information.

**Ribopuromycylation Assay and Quantification of Fluorescence Intensities**

De novo synthesized proteins were quantified by measuring the incorporation of puromycin on native peptide chains as described previously (46, 47). Detailed information is provided as supplementary information.

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References


Figure legends

Figure 1. Induction of host translation repression by flavivirus infection. (A) Exemplary polysome profiles of naïve Huh7 cells (Mock) and DENV-infected cells at 18, 24 and 36 h p.i.. Cell extracts of naïve Huh7 cells or cells infected with DENV (MOI = 10) for the indicated time periods were loaded on sucrose gradient and separated by ultra-centrifugation. Sucrose gradients were eluted from the top using a fractionator and absorption at 254 nm was continuously recorded. Shown in the Mock panel is the separation between actively translated, polysomal mRNAs associated with multiple ribosomes and not or poorly translated sub-polysomal mRNAs (40S and 60S, single ribosomal subunits; 80S, monosome). DENV-infected cells show an increase of the monosomal 80S peak throughout the infection. (B) Representative polysome profile analysis (lower panel). The percentage of polysomal ribosomes (actively
translating mRNAs) is assessed by measuring the area below the polysomal part of the curve and the area of sub-polysomal and polysomal parts of the curve. Histogram bars shown in the upper panel represent the mean percentages of polysomal ribosomes ± SEM; n, number of profiles analyzed. (C) Abundance of specific mRNAs in gradient fractions. Polysome profiles of naïve (Mock) and DENV-infected Huh7 cells were recorded at the indicated time points p.i.. Total RNA was extracted from all fractions and relative abundance of specific mRNAs in each fraction was quantified by qRT-PCR. Histogram bars represent mean percentages of GAPDH mRNA and DENV (+) RNA genome associated with the polysomal fractions ± SD (n=3). (D) Reduction of protein synthesis in DENV-infected cells. Naïve Huh7 cells (Mock) and cells infected with DENV for the indicated time period were treated with puromycin to induce a premature release of nascent polypeptidic chains. Arsenite-treated Huh7 cells were used as control. Puromycylated chains are visualized using an anti-puromycin antibody (red) and infection by immunostaining of DENV NS3 (green). Exemplary fields of view are shown. Yellow squares represent the cropped section shown in the merge panel. Scale bar, 50 µm. (E) Scatter plot of de novo protein synthesis measured by fluorescence intensity of the puromycin signal (mean fluorescence intensities ± SD; n=3; a.u., arbitrary units). Statistical significance and the number of analyzed cells (n) are given at the top. ***, p < 0.001; **, p < 0.01. (F) Host cell translation repression is a general feature of flavivirus infection. Polysomal profiles of Huh7 cells infected with DENV serotype 1, 3 or 4, WNV strain NY, or ZIKV MR766 or H/PF/2013 (MOI = 10) for the indicated time periods were recorded (see Fig. S2). Shown are mean percentages of polysomal ribosomes ± SEM; n, number of profiles analyzed.
**Figure 2. Host cell translation is impaired at the initiation step by DENV infection. (A, B and C)**

DENV infection does not affect RNA translation elongation. Naïve Huh7 cells (Mock, A) and cells infected with DENV (MOI = 10) for 24h (B) were treated with harringtonine for 1.5, 3 and 4.5 min to allow ribosome run-off. Treatment with DMSO for 4.5 min was used as control. Shown are exemplary polysome profile analyses (lower panel) and mean percentages of polysomal ribosomes ± SEM (upper panel; n, number of profiles analyzed). (C) Mean percentages ± SD (n=3) of total mRNAs associated with polysomes in Mock and DENV-infected cells upon harringtonine treatment (corresponding to panels A and B) were normalized to the values of DMSO-treated cells, respectively. **(D)** Phosphorylation levels of the eEF2 are not affected by DENV infection. Representative Western blot analysis (n=2) of Huh7 cells infected with DENV (MOI = 10) for 12, 24, 36 and 48h (lanes 5 to 8). Naïve Huh7 cells (Mock) cultured in parallel for the same time periods were used as reference (lanes 1 to 4).

**Figure 3. Flavivirus infection inhibits eIF2α-dependent and -independent SG formation in Huh7 cells. (A, B and C)** Huh7 cells were infected with DENV (MOI = 0.5) for the indicated time period and left untreated (A) or treated with arsenite (B) before fixation. DENV infection was visualized by immunostaining of NS5 (green) and SGs by immunostaining of eIF3B (red). Naïve cells (Mock) served as control. Exemplary fields of view are shown. Yellow squares represent the cropped section shown in the merge panel. Scale bar, 50 µm. **(C)** Scatter plot displaying the number of SGs in cells treated with arsenite. Shown are mean values ± SD of a representative experiment (n=3); n, number of cells analyzed; ***, p < 0.001; n.s., not significant. **(D)** ZIKV and WNV inhibit arsenite-induced SG assembly. Similar to panel (C), Huh7 cells were infected with ZIKV MR766 or WNV NY (MOI = 0.5) for 24 h and treated with arsenite before fixation. **(E and F)**
Huh7 cells were infected with DENV (MOI = 0.5) for 30 h and treated with hippuristanol for 8 h before fixation. (E) DENV infection was visualized by immunostaining of NS5 (green) and SGs by immunostaining of elf3B (red). Naïve cells (Mock) served as control. Exemplary fields of view are shown. Yellow squares represent the cropped section shown in the merge panel. Scale bar, 50 µm. (F) Scatter plot displaying the number of SGs in cells treated with hippuristanol. Shown are mean values ± SD of a representative experiment (n=3); n, number of cells analyzed; ***, p < 0.001.

Figure 4. DENV-induced host cell translation repression is independent of the PKR-elf2α signaling pathway. (A and B) Activation of PKR by DENV does not result in elf2α phosphorylation. Cells were infected with DENV (MOI = 10) for 12, 24, 36 and 48 h (lanes 5 to 8). Naïve Huh7 cells (Mock) cultured in parallel for the same time periods were used as reference (lanes 1 to 4). Shown are representative Western-blot analyses (n=4). (A) Analysis of PKR and phospho-PKR (p-PKR) abundance. Cells transfected with the synthetic dsRNA poly(I:C) (lane 9) were used as positive control. (B) Analysis of elf2α and phospho-elf2α (p-elf2α) abundance. Cells treated with arsenite (lane 9) were used as positive control. Phosphorylation of elf2α was analyzed by Phos-tag acrylamide gel (lower panel). (C) DENV-induced translational repression is PKR-independent. Polysome profiles of Huh7 PKR ko cells (clone 2#3) left untreated (Mock) or infected with DENV (MOI = 10) were recorded at the indicated times. Shown are exemplary polysome profile analyses (lower panel) and mean percentages of polysomal ribosomes ± SEM (upper panel; n, number of profiles analyzed). (D) DENV-induced translational repression is elf2α-independent. Polysome profiles of Huh7 control cells (Ctrl) and Huh7 cells expressing stably GADD34 infected with DENV (MOI = 10) were recorded 24 h p.i.. Naïve cells (Mock) were
used as control. Shown are exemplary polysome profile analyses (lower panel) and mean percentages of polysomal ribosomes ± SEM (upper panel; n, number of profiles analyzed).

**Figure 5. Cap-binding complex assembly is not affected by DENV infection.** (A) m\(^7\)GTP immunoprecipitation from naïve Huh7 cells or cells infected with DENV (MOI = 10) for 24 h. Cells treated with Torin1 for 16 h were used as control of cap-binding complex disassembly. Representative Western blot analysis of cap-binding proteins co-immunoprecipitated with m\(^7\)GTP immobilized agarose beads. Shown are input cell extracts (1% of total, left panel) and immunoprecipitated proteins (25% of eluate, right panel). (B) Quantification of cap-binding proteins associated to m\(^7\)GTP. Shown are mean ± SEM of fold changes (n=3). *, p < 0.05; n.s., not significant.

**Figure 6. Activation of the p38-Mnk1 signaling pathway is required for virus production.** (A) eIF4E phosphorylation levels increase in DENV-infected cells. Representative Western blot analysis (n=3) of phospho-eIF4E (p-eIF4E) abundance in naïve and DENV-infected Huh7 cells at 12, 24, 36 and 48 h p.i.. Phosphorylation of eIF4E was analyzed by Phos-tag acrylamide gel (lower panel) (B) Analysis of MAPK phosphorylation levels in Huh7 cells inoculated with UV-inactivated DENV (Ctrl) or infected with DENV for 24 and 43 h by using the Proteome Profiler Human Phospho-MAPK Array. Shown in the lower panel are mean relative pixel densities normalized to the control of two independent experiments with two measurements each. Upper panel: representative Western blot analysis (n=2) of Huh7 cell extracts used for analysis of MAPK phosphorylations. Naïve Huh7 cells, Huh7 cells infected with DENV for 24 and 43 h, and Huh7 cells inoculated with UV-inactivated DENV for 43 h were analyzed. (C) Representative
Western blot analysis of phospho-p38α (p-p38α) abundance in naïve and DENV-infected Huh7 cells (n=3). (D) Representative Western blot analysis of phospho-Mnk1 (p-Mnk1) abundance in naïve and DENV-infected Huh7 cells (n=2). Lysates of naïve and murine norovirus (MNV)-infected mouse leukemic monocyte-macrophage (RAW264.7) served a positive control (72). (E, F and G) **Phosphorylation of eIF4E is dispensable for DENV-induced repression of translation.**

(E) Ectopic expression of HA-tagged eIF4E in Huh7 cells. Representative Western blot analysis (n=3) of endogenous eIF4E and HA-eIF4E abundance in Huh7 HA-eIF4E, Huh7 HA-eIF4E S209A and Huh7 HA-eIF4E S209D. Huh7 control (Ctrl) cells served as control. Upper panel: immunobloting using an anti-eIF4E antibody; lower panel: immunoblotting using an anti-HA antibody. (F and G) DENV-induced translational repression is phospho-eIF4E-independent. (F) Polysome profiles of Huh7 cells stably expressing HA-eIF4E (WT) and the mutant HA-eIF4E S209A (S209A) infected with DENV (MOI = 10) were recorded 24 h p.i.. Naïve cells (Mock) were used as control. Shown are exemplary polysome profile analyses (lower panel) and mean percentages of polysomal ribosomes ± SEM (upper panel; n, number of profiles analyzed). (G) Naïve Huh7 cells (Mock) and cells infected with DENV (MOI = 10) for 8h (DENV) were treated with CGP57380, an inhibitor of Mnk1 phosphorylation, for 16 h. Treatment with DMSO was used as control. Shown are exemplary polysome profile analyses (lower panel) and mean percentages of polysomal ribosomes ± SEM (upper panel; n, number of profiles analyzed). (H) Inhibition of p38α reduces DENV virus production. Huh7 cells were infected with DENV (MOI = 0.1) and co-treated with DMSO or 50 µM SB203580, an inhibitor of p38α activity. Virus titers were determined by limiting dilution assay. Shown is the mean ± SD (n=3). *, p < 0.05. (I) Inhibition of Mnk1 phosphorylation severely diminishes DENV virus production. Huh7 cells were infected with DENV (MOI = 0.1) for 8 h and subsequently treated with DMSO or 50 µM
Figure 7. Modifications of host cell translation and stress response by DENV infection.

Early in DENV infection, host cell translation is repressed. While assembly of the pre-initiation complex and translation elongation remain unaffected, translation initiation is stalled after association of the 60S ribosomal subunit with cellular mRNA (monosomal RNA) (red blind-ended arrow). Interestingly, DENV infection activates the p38α-Mnk1 pathway resulting in the phosphorylation of eIF4E (green arrows). While eIF4E phosphorylation does not account for DENV-induced translation repression, it might regulate the selective translation of specific mRNA subsets (green dashed arrow). DENV genome replication occurs through dsRNA intermediates that are sensed by PKR leading to PKR autophosphorylation and activation (green arrow). However, the downstream phosphorylation of eIF2α, a direct target of the activated PKR, is inhibited as well as the assembly of both eIF2α-dependent and -independent SGs (red blind-ended arrows). Altogether, during DENV infection translation suppression is uncoupled from the activation of the stress response.

Supplemental Figure Legends

Figure S1. DENV infection suppresses global protein synthesis. (A) Exemplary polysome profile of Huh7 cells infected with DENV (MOI = 10) for 24h. After separation by ultra-centrifugation, sucrose gradients were eluted from the top using a fractionator and absorption at 254 nm was continuously recorded (upper panel). Fractions were collected (1 to 13), total RNA was...
extracted and analyzed by formaldehyde gel electrophoresis (lower panel) to confirm the presence of 18S and 28S ribosomal RNA and distinguish precisely sub-polysomal from polysomal fractions. (B) Analysis of mRNA abundance in polysome gradient fractions by qRT-PCR. After gradient fractionation, a non-human in vitro transcript (eGFP transcript) is spiked in each fraction before purification to measure the loss of total RNA upon RNA extraction. eGFP transcripts are quantified in each fraction by qRT-PCR and used to normalize values of specific mRNAs. Abundance of GAPDH mRNAs in naïve and DENV-infected Huh7 cells is shown as example. Histogram bars represent the percentage of GAPDH transcripts in each fraction relative to total amount of GAPDH transcripts in the gradient. (C) Quantification of DENV (+) RNA genome levels by qRT-PCR in total cell extract before separation by ultra-centrifugation. All values were normalized to GAPDH mRNA levels. Shown are means ± SD of triplicate measurements of a representative experiment. (D) DENV infection induces a translational repression in human A549 cells. Shown are exemplary polysome profile analyses (lower panel) and mean percentages of polysomal ribosomes ± SEM (upper panel). The number of profiles analyzed (n) is given at the top. (E) DENV induces a reduction of global protein synthesis. Representative Western blot analysis (n=2) of puromycin incorporation in Huh7 cells infected with DENV for 12, 24, 36 and 48 h. Naïve cells served as control. Extracts of cells treated for 2 h with cycloheximide (CHX) were used as control. DENV antigens were stained using DENV NS4B antiserum. GAPDH served as loading control.

**Figure S2. Ribopuromycylation assay. (A and B)** Analysis of de novo protein synthesis in Huh7 cells expressing transiently DENV replicon. Huh7 cells were electroporated with wildtype DENV Firefly luciferase replicon (DENVrep) and HAV Firefly luciferase replicon (HAVrep) as control.
Cells were treated with puromycin at the indicated time points. After fixation, puromycylated polypeptidic chains were visualized using an anti-puromycin antibody. Viral antigens were immunostained with DENV NS3 antiserum and HAV proteinase 3C antiserum. Shown are scatter plots of puromycin mean fluorescence intensities (a.u.) ± SD of a representative experiment. Statistical significance and the number of analyzed cells (n) are given at the top. ***, p < 0.001; n.s., not significant. (A) Huh7 cells expressing DENVrep (n=3). (B) Huh7 cells expressing HAVrep (n=2). (C and D) Analysis of DENV Firefly luciferase replicons and DENV serotype 2 strain NGC replication kinetics. (C) DENV replicon system expresses a Firefly Luciferase reporter gene that allows for the measurement of luciferase activity as a surrogate of RNA replication. In vitro transcripts of wildtype DENV Firefly luciferase replicon (DENVrep) and replication-defective DENV Firefly luciferase replicon (DENVrep GND) were electroporated in Huh7 cells and harvested at 4, 24, 48, and 72 h post electroporation. To assess DENVrep RNA replication cells were lysed at time points specified and Firefly luciferase activities were determined (Relative Light Units, RLU). Values were normalized to the 4 h (input RNA) value. Shown are mean RLU values ± SD of three independent experiments. (D) Huh7 cells (1x10^5) were infected at an MOI of 0.1 TCID_{50} per cell for 2 h. Twenty-four, 48, 72 and 96 h post infection, cells were harvested and infectious titers were determined by limiting dilution assay (TCID_{50}/ml). Shown are mean values ± SD of three independent experiments. (E and F) DENV polyprotein is sufficient for translational repression. Expression of DENV polyprotein NS1-NS5 and HAV polyprotein in Huh7 Lunet T7 cells. Forty-eight hours post transfection, cells were treated with puromycin and fixed. (E) Exemplary fields of view are shown. Yellow squares represent the cropped section shown in the merge panel. Scale bar, 50 µm. (F) Scatter plots of puromycin mean fluorescence intensities ±
Figure S3. Polysome profiles of Huh7 cells infected with flaviviruses. Huh7 cells were infected (MOI = 10) with (A) DENV serotype 1 strain Hawaii (DENV1), (B) DENV serotype 3 strain H87 (DENV3), (C) DENV serotype 4 strain H241 (DENV4), (D) WNV strain New-York 99 (WNV NY), (E) ZIKV strain MR766 or (F) ZIKV strain H/PF/2013. Shown are exemplary polysome profile analyses (lower panels) and mean percentages of polysomal ribosomes ± SEM (upper panels). The number of profiles analyzed (n) is given at the top.

Figure S4. Inhibition of arsenite-induced SG formation by DENV and ZIKV infection. (A and B) ZIKV and WNV inhibit SG assembly. Naïve Huh7 cells and Huh7 cells infected with ZIKV MR766 and WNV NY (MOI = 0.5) for 24 h were left untreated (A) or treated with arsenite for 45 min (B) before fixation. ZIKV and WNV infection was visualized by immunostaining of dsRNA (green) and SGs by immunostaining of eIF3B (red). Naïve cells (Mock) served as control. Exemplary fields of view are shown. Yellow squares represent a cropped section showed in the merge panel. Scale bar, 50 µm. (C and D) Inhibition of arsenite-induced SGs by DENV infection. Huh7 cells were infected with DENV (MOI = 0.5) for the indicated time periods, left untreated or treated with 500 µM arsenite for 45 min before fixation. DENV infection was visualized by immunostaining of NS5 and SGs by immunostaining of eIF3B (see Fig. 3). (C) Scatter plot of SG size (µm²) in cells treated with arsenite. Shown are mean values ± SD of a representative experiment (n=3). Statistical significance and the number of analyzed cells (n) are given at the top. ***, p < 0.001; **, p < 0.01. (D) Scatter plot of correlation between DENV NS5 mean fluorescence intensity...
(reflecting the level of DENV replication) and number of arsenite-induced SGs in DENV-infected
cells at 24, 36 and 48 h post infection. n, number of analyzed cells; PC, Pearson’s correlation; R²,
coefficient of determination. (E) SG-like foci in ZIKV-infected cells. Huh7 cells infected with ZIKV
MR766 for 24 h. ZIKV infection was visualized by immunostaining of dsRNA (green) or NS3 (far
red) and SGs by immunostaining of eIF3B (red), HuR and PCBP2 (green) and TIAR (red) and
analyzed by confocal microscopy. Exemplary fields of view are shown. Yellow squares represent
the cropped section shown in the merge panel. Scale bar, 50 µm. (F) Treatment with
hippuristanol does not induce eIF2α phosphorylation. Naïve Huh7 cells infected with DENV (MOI
= 10) for 30 h were left untreated or treated with arsenite, an eIF2α-kinase inducing stressor,
and hippuristanol, an eIF2α-kinase independent stressor. Representative Western blot analysis
of phospho-eIF2α (p-eIF2α) abundance (n=2).

**Figure S5. Flaviviruses impair eIF2α phosphorylation by other eIF2α kinases. (A, B and C)**
Naïve
Huh7 cells and Huh7 cells infected with DENV (MOI = 10) for 30 h were left untreated or treated
with different eIF2α-kinase inducing stressors arsenite, FCCP and Thapsigargin. (A)
Representative Western blot analysis of phospho-eIF2α (p-eIF2α) abundance (n=3). (B)
Quantification of p-eIF2α density intensities. Values were normalized to the loading control β-
actin density values and shown relative to untreated cells (n=3). (C) Stress induction was also
dcored by immunofluorescence analysis of SG formation. SGs were visualized by
immunostaining of eIF3B (red) and DENV infection by immunostaining of NS1 (green).
Exemplary fields of view are shown. Yellow squares represent the cropped section shown in the
merge panel. Scale bar, 50 µm. (D, E and F) Similar analyses with naïve Huh7 cells and Huh7 cells
infected with MR766 (MOI = 10) for 30 h were left untreated or treated with different eIF2α-
kinase inducing stressors arsenite, FCCP and thapsigargin. (D) Western blot analysis of phospho-eIF2α (p-eIF2α) abundance (n=3). (E) Quantification of p-eIF2α density intensities (n=3). (F) Control of stress induction by immunofluorescence. ZIKV infection was visualized by immunostaining of dsRNA (green) and SGs by immunostaining of eIF3B (red).

**Figure S6. Impact of PKR on DENV replication.** (A and B) Transient PKR silencing does not alter DENV replication. *In vitro* transcript of DENV *Firefly* luciferase reporter virus (50) was co-expressed with nontargeting siRNA (siNT) or PKR-specific siRNA in Huh7 cells. Cells were harvested at 4, 24, 48, and 72 h post electroporation and PKR mRNA levels were quantified at 72 h by qRT-PCR (A). All values were normalized to GAPDH mRNA levels. Results represent fold induction relative to cells transfected with siNT. Shown are means of triplicate measurements ± SD of a representative experiment (n=3). (B) To assess DENV RNA replication cells were lysed at time points specified and *Firefly* luciferase activities were determined (Relative Light Units, RLU). Values were normalized to the 4 h (input RNA) value. Shown are mean RLU values ± SD of three independent experiments. (C) Representative Western blot analysis (n=3) of PKR abundance in Huh7 PKR ko cell clones upon stimulation with IFN-α. Three different clones of Huh7 PKR ko cells (2#2, 2#3 and 3#1) were left untreated or treated with 1000 IU/ml IFN-α for 24 h. Huh7 parental cells served as control. (D) RNA replication of DENV *Firefly* luciferase reporter virus in Huh7 PKR ko cell clones (2#2, 2#3 and 3#1) was analyzed as described in panel (B). Shown are mean RLU values ± SD of three independent experiments. (E) ZIKV MR766-induced translational repression is PKR-independent. Polysome profiles of naïve Huh7 PKR ko cell clone 2#3 (Mock) and Huh7 PKR ko cell clone 2#3 infected with ZIKV MR766 (MOI = 10) were recorded at the indicated times. Shown are exemplary polysome profile analyses (lower
Figure S7. Impact of GADD34 ectopic expression on DENV infection.

(A) DENV infection induces GADD34 mRNA levels. Quantification of GADD34 mRNA levels by qRT-PCR in Huh7 cells infected with DENV for 18, 24 and 36 h. All values were normalized to GAPDH mRNA levels. Results represent fold induction relative to naïve cells. Shown are means of triplicate measurements ± SD of a representative experiment. (B, C, D and E) Characterization of GADD34-overexpressing cells. (B) Quantification of GADD34 mRNA in parental Huh7 (Ctrl) and GADD34 cells normalized to GAPDH mRNA levels. Results represent fold induction relative to Ctrl cells. Shown are means of triplicate measurements ± SD of a representative experiment. (C and D) Ectopic expression of GADD34 antagonizes arsenite-induced translation repression. Huh7 Ctrl (C) and GADD34 (D) cells were treated with increasing concentrations of arsenite (50, 125, 250 and 500 µM) for 45 min. Shown are exemplary polysome profile analyses (lower panels) and mean percentages of polysomal ribosomes ± SEM (upper panels). The number of profiles analyzed (n) is given at the top. (E) Ectopic expression of GADD34 reduces DENV replication. Huh7 Ctrl and GADD34 cells were infected with DENV (MOI of 0.01) and supernatants harvested at 24, 48, 72 and 96 h post infection. Virus titers were determined by plaque assay (pfu/ml). (F) ZIKV MR766-induced host translational repression is eIF2α-
independent. Polysome profiles of Huh7 control cells (Ctrl) and Huh7 cells expressing stably
GADD34 (GADD34) infected with ZIKV MR766 (MOI = 10) were recorded 24 h post infection.
Naïve cells (Mock) were used as control. Shown are exemplary polysome profile analyses (lower
panel) and mean percentages of polysomal ribosomes ± SEM (upper panel). The number of
profiles analyzed (n) is given at the top. (G) ZIKV-induced SG-like foci are phospho-eIF2α-
independent. Huh7 cells overexpressing GADD34 and Huh7 Ctrl cells were infected with ZIKV
(MOI = 0.5) for 24 h. ZIKV infection was visualized by immunostaining of dsRNA (green) and SGs
by immunostaining of eIF3B (red) or TIAR (red). Exemplary fields of view are shown. Yellow
squares represent the cropped section shown in the merge panel. Scale bar, 50 µm.

Figure S8. Activation of the p38-Mnk1 pathway. (A and B). Characterization of Huh7 cells stably
overexpressing HA-eIF4E wildtype, the phospho-ablative mutant HA-eIF4E S209A and the
phosphomimetic mutant HA-eIF4E S209D. (A) Exemplary polysome profiles of Huh7 cells stably
over-expressing HA-tagged eIF4E wild-type, S209A and S209D mutants. After separation by
ultra-centrifugation, sucrose gradients were eluted from the top using a fractionator and
absorption at 254 nm was continuously recorded (upper panel). Fractions were collected (1 to
14), and proteins purified and analyzed by Western blot analysis (lower panel) to confirm the
presence of HA-tagged eIF4E in polysomal fractions. (B) Shown are mean percentages of
polysomal ribosomes ± SEM (upper panels). The number of profiles analyzed (n) is given at the
top. (C) Stable over-expression of HA-tagged eIF4E and mutants does not alter DENV virus
production. Huh7 control (Ctrl), HA-eIF4E wildtype (WT), HA-eIF4E S209A (S209A) and HA-eIF4E
S209D (S209D) cells were infected at an MOI of 0.1 TCID$_{50}$ per cell for 2 h. Twenty-four, 48, 72
and 96 h post infection, cells were harvested and infectious titers were determined by limiting
dilution assay (TCID_{50}/ml). Shown are mean values ± SD of three independent experiments. (D)

Cytotoxicity of CGP57380 treatment. Huh7 cells were treated with increasing concentrations of CGP57380 (2.5, 5, 10, 25, 50, 75, and 100 µM) for 16 h. Cell viability was determined by measuring release ATP using the CellTiter-Glo assay. DMSO was used as control. Values were normalized to untreated cells and are represented as percentage of cell survival (n=3). (E)

Representative Western blot analysis (n=3) of phospho-eIF4E (p-eIF4E) abundance in Huh7 cells treated with the Mnk1 inhibitor CGP57380. Huh7 cells were infected for 8 h and subsequently treated with 50 µM CGP57380 (CGP) for 16 h, with DMSO or left untreated (Untr.). Naïve cells were used as control. (F) Cytotoxicity of SB203580 treatment. Huh7 cells were treated with increasing concentrations of SB203580 for 16 h (2.5, 5, 10, 25, 50, 75, and 100 µM). Cell viability was determined as described in panel (B) (n=3). (G) Representative Western blot analysis (n=2) of phospho-eIF4E (p-eIF4E) abundance in Huh7 cells treated with p38 inhibitor. Huh7 cells were infected and co-treated with 50µM SB203580 (SB) for 16 h, with DMSO or left untreated (Untr.). Naïve cells were used as control.
Figure 4
The diagram illustrates the interaction between the translational machinery and viral infection. It shows the phosphorylation of p38α and MNK1, which leads to the phosphorylation of elf4E and elf4E-m^G^G, affecting the translation of capped mRNA. The assembly of the pre-initiation complex on the mRNA depends on elf4E and elf2α, which are involved in the 40S and 60S ribosome formation. The monosomal mRNA is involved in stress granule assembly, which is induced by DENV. The polysomal mRNA is shown at the end, indicating the translation process.