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Kinome siRNA screen identifies novel cell-type specific dengue host target genes

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ABSTRACT

Dengue is a global emerging infectious disease, with no specific treatment available. To identify novel human host cell targets important for dengue virus infection and replication, an image-based high-throughput siRNA assay screening of a human kinome siRNA library was conducted using human hepatocyte cell line Huh7 infected with a recent dengue serotype 2 virus isolate BR DEN2 01-01. In the primary siRNA screening of 779 kinase-related genes, knockdown of 22 genes showed a reduction in DENV-2 infection. Conversely, knockdown of 8 genes enhanced viral infection. To assess host cell specificity, the confirmed hits were tested in the DENV-infected monocytic cell line U937. While the expression of EIF2AK3, ETNK2 and SMAD7 was regulated in both cell lines after infection, most kinases were hepatocyte-specific. Monocytic cells represent initial targets of infection and an antiviral treatment targeting these cells is probably most effective to reduce initial viral load. In turn, infection of the liver could contribute to pathogenesis, and the novel hepatocyte-specific human targets identified here could be important for dengue infection and pathogenesis.

1. Introduction

Dengue virus (DENV) is a member of the flavivirus family and has 4 serotypes. It causes a wide spectrum of symptoms in infected humans. After the mosquito carrying virus feeds on a host, the infection is thought to start from dendritic cells in the skin, thereafter spreading to lymphatic tissue and infecting more dendritic cells, macrophages and monocytes (Clyde et al., 2006). Eventually, virus is released into the circulation and systemic infection can occur, including the liver (Seneviratne et al., 2006). Disease severity ranges from mild fever to more serious complications. Recent studies have estimated a large global burden of dengue, amounting to 390 million infections per year, of which 96 million are symptomatic (Bhatt et al., 2013). Currently, there are no available effective drugs and approved vaccines against this virus, and treatment remains supportive. Thus, there is a critical need for the development of anti-dengue treatments.

A number of high throughput siRNA screens to identify host factors involved in flavivirus infection have been reported. For example, genome-scale siRNA screens were used to discover host factors required for yellow fever virus propagation in Huh7 hepatocyte cell lines. Further validation of candidate genes in human hepatocyte cell lines and a murine fibroblast cell line identified G protein-coupled receptor kinase 2 as important for yellow fever virus and DENV replication (Le Sommer et al., 2012). A similar genome-wide siRNA-based screen silencing 21,121 human genes in Hela cells identified more than 300 host genes important in the infection

Abbreviation: DENV, dengue virus.

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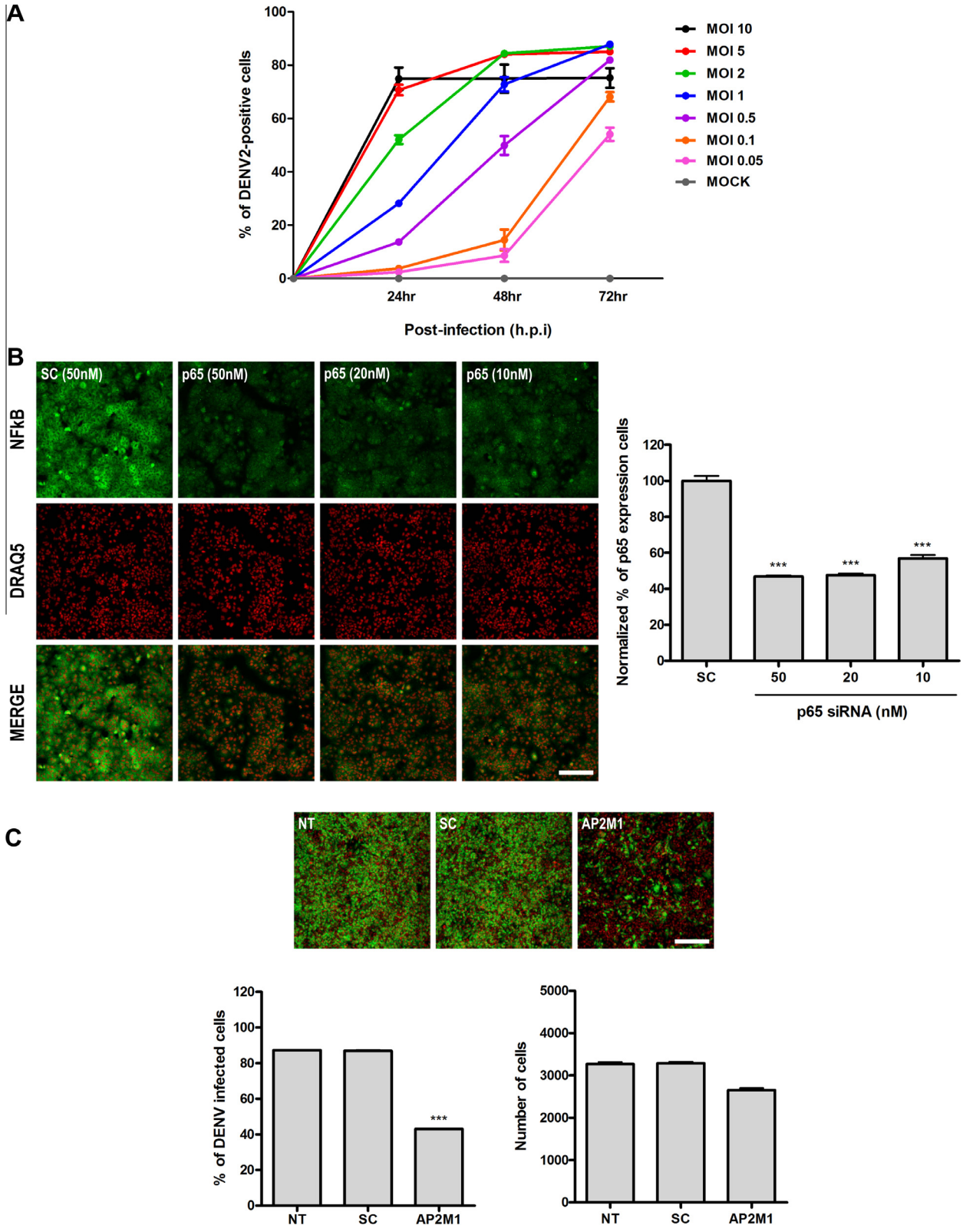

Fig. 1. Adaptation and validation of the image-based siRNA assay for high-throughput screening. Optimal conditions were established for DENV-2 infection and siRNA knockdown in Huh7 cells in 96-well plate format. (A) Infection kinetics of BR DEN2 01-01 at various MOIs. (B) Decrease in the expression of NFκB after transfection with different concentrations of p65 siRNA. (C)Transfection of Huh7 with AP2M1 siRNA reduced the percentage of DENV-2 infected cells. Bars show means ± SD from triplicates from 1 out of 4 independent experiments. Asterisks (∗) indicate statistical significance with a *p*-value of less than 0.05 using an unpaired *t* test (sc – scrambled, NT - non-transfected cells, scale bars = 250 μm).

with West Nile virus, another flavivirus (Krishnan et al., 2008). Host factors important in DENV infection have been discovered in *Drosophila melanogaster* cells, with 42 out of 116 hits later also being confirmed in hepatocyte cell lines (Sessions et al., 2009). A siRNA library targeted at genes regulating endocytosis pathways, polymerization of actin and cytoskeleton rearrangement and

vesicle/cargo trafficking identified human membrane trafficking genes that were necessary for the infectious entry of DENV in Huh7 cells (Ang et al., 2010).

The use of RNA interference is thus a powerful forward genetics approach to dissect virus-host cell interactions. Depending on the screening approach the hits do not necessarily overlap, and the host cell specificity of a hit in particular is an important parameter to assess its value as a drug target. Discovery of novel human factors important in immune cells could present attractive targets for the development of anti-dengue therapies.

We have carried out siRNA screening with a human kinase library in Huh7 cells. Eight candidate genes from the screen were subsequently followed up in U937 cells, a monocytic cell line. Most hits were specific for Huh7 cells, indicating that DENV uses different genes in different cell types for replication. This finding has implications for the interpretation of siRNA screens in general.

2. Materials and methods

2.1. Cells, viruses and antibodies

Huh7 cells (JCRB0403) were cultured in RPMI 1640 medium containing 25 mM HEPES (WelGene, Korea) and supplemented with 10% fetal bovine serum (FBS, Gibco Invitrogen, USA) and antibiotics (100 U/mL Penicillin and 100 μg/mL Streptomycin, Gibco Invitrogen, USA). U937-DC-SIGN (human monocytic cell line stably transfected with DC-SIGN) cells were maintained in RPMI 1640 (Gibco) containing 10% heat-inactivated FBS. DENV-2 BR DEN2 01-01 (GenBank JX073928) was a kind gift from Dr. Claudia N. Duarte dos Santos. BR DEN2 01-01 and DENV-2 strain TSV01 were propagated in C6/36 cells. Flavivirus group-specific anti-E 4G2 monoclonal antibody (mAb) from mouse hybridoma cell D1-4G2-4-15 (HB-112, American Type Culture Collection) (Henchal et al., 1982) was prepared as described previously (Cruz et al., 2013). The p65 monoclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA) while Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L) was from Invitrogen Molecular Probes (Carlsbad, CA).

2.2. Immunofluorescence detection of DENV-2 infected Huh7 cells

DENV-2 infected cells were detected and quantified using an immunofluorescence (IF) assay as described previously (Cruz et al., 2013), with some modifications. Briefly, cell monolayers were fixed with 4% (w/v) paraformaldehyde (PFA) for 30 min at room temperature. Wells were washed thrice with Dulbecco's Phosphate Buffered Saline (DPBS, WelGene, Korea) (Note: DPBS washing was performed following each subsequent step), then treated with 0.25% (v/v) Triton X-100 for 30 min at room temperature. The cells were probed with 1 μg/mL 4G2 mAb in blocking buffer (DPBS containing 5% FBS) for 30 min at 37 °C, followed by 2 μg/mL Alexa Fluor 488-conjugated goat anti-mouse IgG and 5 μM DRAQ5 (BioStatus, Shepshed, UK) in blocking buffer for 30 min at 37 °C. Confocal fluorescence images of 4 representative fields from each well (with each image consisting of approximately 2500–3000 cells) were digitally acquired using the high throughput cellular imaging system ImageXpress Ultra (Molecular Devices, USA) at 20× magnification. Nuclei were stained with DRAQ5 while DENV-2 infected cells were stained with anti-E 4G2 mAb complexed with Alexa Fluor 488. The total number of cells was quantified using the MetaXpress image-analysis software (Molecular Devices, USA). When computing the rate of DENV infection, an optimized imaging algorithm was used in order to exclude non-healthy cells and dead cells.

2.3. siGenome kinase library screening

Huh7 cells were prepared in ten 96-well black clear bottom plates (3×10^4 cells per well) and transfected with a previously defined concentration of siRNA from the Dharmacon siGenome kinase library (Catalogue number: G-003500-05), which contains 3116 siRNAs targeting 779 unique human genes (each targeted by 4 unique siRNA duplexes). p65, AP2M1 and scrambled siRNA were used as transfection, positive inhibition and infection controls, respectively, and siRNAs were transfected for 48 h before infection. All siRNAs and transfection reagent DharmaFECT 4 were purchased from ThermoFisher (West Lafayette, CO). Transfected cells were inoculated with BR DEN2 01-01 and incubated at

Table 1
Host factors which inhibited infection when knocked down in the primary screening.

Gene symbol	Description	Percentage of infection normalised to scrambled control (%)[a]
COPB2	Coatomer protein complex, subunit beta 2 (beta prime)	26.5
EIF2AK3	Eukaryotic translation initiation factor 2-alpha kinase 3	44
AP2M1	Adaptor-related protein complex 2, mu 1 subunit	50
PRKCD	Protein kinase C, delta	56.3
PKIB	Protein kinase (cAMP-dependent, catalytic) inhibitor beta	59.6
STYK1	Serine/threonine/tyrosine kinase 1	62.8
FES	Feline sarcoma oncogene	62.9
UHMK1	U2AF homology motif (UHM) kinase 1	63.2
TLR6	Toll-like receptor 6	63.9
PLK3	Polo-like kinase 3	65.6
SIK3	SIK family kinase 3	66.5
MAP4K5	Mitogen-activated protein kinase kinase kinase kinase 5	66.6
TESK2	Testis-specific kinase 2	67.5
LCP2	Lymphocyte cytosolic protein 2	68.7
SMAD7	SMAD family member 7	68.9
STK32B	Serine/threonine kinase 32B	69.9
ALPK3	ALPK3, alpha-kinase 3	70
MAK	Male germ cell-associated kinase	70.4
PIP5K2B	Phosphatidylinositol-4-phosphate 4-kinase, type II, beta	71.1
PKN3	Protein kinase N3	71.1
UGP2	UDP-glucose pyrophosphorylase 2	71.9
TTK	TTK protein kinase	74.5
EIF2AK1	Eukaryotic translation initiation factor 2-alpha kinase 1	75.6

[a] Percentages are means from two independent experiments.

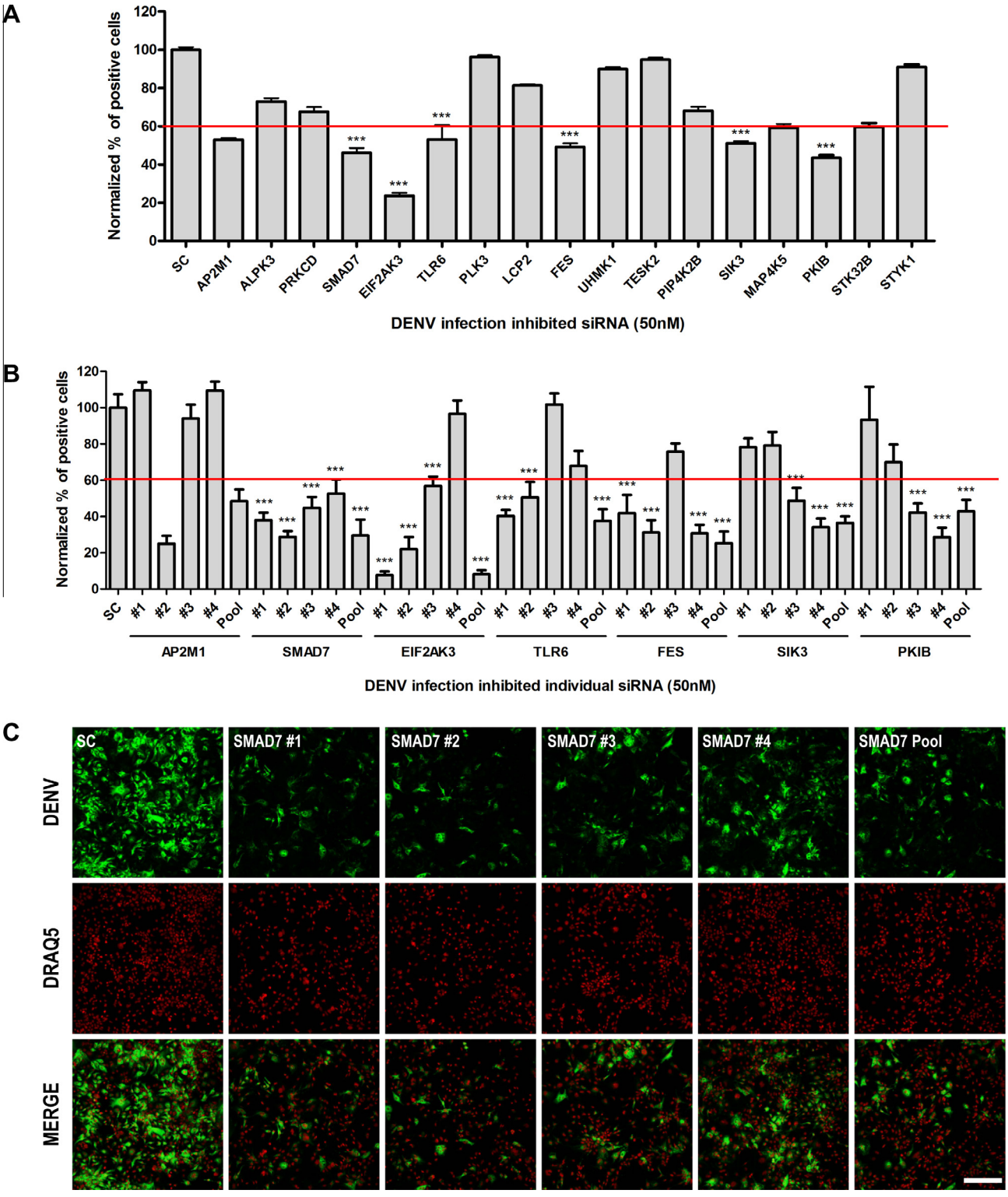

Fig. 2. Knock down of target host genes inhibit DENV-2 infection in Huh7 cells. (A) Among 16 siRNAs identified in the primary screening, 6 were confirmed to inhibit DENV-2 infection in Huh7 cells by more than 40%. (B) Target deconvolution using 4 separate siRNAs (50 nM) targeting the same host factor confirmed the specificity of the siRNA knockdown and the reduction in DENV-2 infection by BR DEN2 01-01. (C) Knockdown of SMAD7 by 4 separate siRNAs and SMAD7 siRNA pool showed a dramatic reduction in the number of DENV-2 positive Huh7 cells. Bars show means ± SD of triplicates from 1 out of 3 independent experiments. Asterisks (∗) indicate statistical significance with a *p*-value of less than 0.05 using unpaired *t*-test sc – scrambled, scale bars = 250 μm).

37 °C, 5% CO_2 for 48 h. The percentage of DENV-2 infected cells was determined by IF staining and confocal fluorescence imaging. The siRNA kinase library primary screening was conducted in two independent experiments. Scatter-plot and cross tabulation of the results were generated using TIBCO Spotfire 4.5.0 (TIBCO Software Inc. Somerville, MA). The mean and standard deviation for the percentage of DENV-2 infected cells was calculated. Primary hits for inhibitors and enhancers of DENV-2 infection were selected based

on outliers from the mean ± 2 standard deviations of the entire siRNA kinase library. A hit confirmation of selected hit genes was conducted (three independent experiments), using the siRNA SMART pools. Eventually, target deconvolution using four different siRNAs targeting the same gene was used to confirm the inhibition or enhancement of infection due to the knockdown of selected host genes (three independent experiments).

Transfection and infection of Huh7 cells with DENV-2 was conducted as described previously (Cruz et al., 2013) and analyzed by confocal fluorescence imaging. A reduction in the percentage of DENV-2 infected cells by more than 40% was defined as a positive inhibition resulting from gene knockdown by siRNA. Conversely, an increase of more than 40% infection was defined as a positive enhancement resulting from the gene knockdown.

2.4. Western blot

Huh7 cells were transfected with 50nM of siRNA with RNAi max (Life Technologies) for 48 h. The cells were lysed and total cell lysates were resolved by SDS–PAGE and transferred to PVDF membrane. EIF2AK3 was detected using a rabbit monoclonal antibody at 1:2500 dilution (Cell signaling Technology) followed by a HRP-conjugated anti-rabbit antibody (Promega). As a loading control, β-actin was detected using a mouse monoclonal antibody at 1:20,000 dilution (Millipore) followed by a HRP-conjugated anti-mouse antibody (Sigma).

2.5. Flow-cytometry readout of infection in Huh7 and U937-DC-SIGN cells

Infected cells were fixed with BD Cytofix–Cytoperm, followed by intracellular staining with 4G2 antibody. Cell viability was determined with a blue live/dead cell stain kit (Life Technologies). Data were collected on a BD FACS Canto (BD Bioscience, CA, San Jose).

2.6. RNA isolation, quantitative reverse transcription PCR (qRT-PCR) and determination of virus titre

For human gene expression analysis, Huh7 cells were infected with TSV01 at an MOI of 5 for 48 h and U937-DC-SIGN cell lines were infected similarly for 24 h. Total cellular RNA was extracted using Trizol (Life Technologies) and Qiagen RNeasy Mini Kit using on column DNase 1 treatment according to manufacturer's instructions (Qiagen, USA). Reverse transcription of RNA to cDNA was carried out with SuperScript III Reverse Transcriptase (Life Technologies). The quantitative PCR reactions were run on an ABI 7900 system (Applied Biosystems, USA). Data was analysed using the SDS2.2 software where baseline and threshold settings were automatically adjusted. Relative gene expression levels were obtained using the $2-\Delta\Delta CT$ method (Livak and Schmittgen, 2001) and normalised with respect to either beta-actin (for U937-DC-SIGN cells) or GAPDH (for Huh7 cells) housekeeping genes. Primers used for the qRT-PCR reactions to determine the relative gene expression levels of the various host genes are as described in Supplementary Table 1.

Viral RNA was quantified by real-time quantitative reverse transcription-PCR (qRT-PCR) using primers and methods reported previously (Gurukumar et al., 2009). Virus titers in cell culture supernatants were determined by standard plaque-forming assay using BHK-21 cells.

2.7. siRNA transfection of U937-DC-SIGN cells

To knock down the various target genes, U937-DC-SIGN cells were transfected with 750nM of siRNA (Dharmacon) using kit V from the Amaxa Nucleofector System (Lonza) with T20 program. 48 h after transfection, cells were infected with TSV01 for 24 h and then collected for flow cytometry and RNA analysis.

3. Results

3.1. Development and validation of an image-based siRNA assay against DENV infection

To screen the RNAi kinome library, the image-based siRNA assay established previously (Cruz et al., 2013) was adapted to 96-well plates. The infection of DENV-2 in Huh7 cells was determined by inoculating different MOIs of DENV-2 (BR DEN2 01-01) and measuring the percentage of infection by immunofluorescence 24, 48 and 72 h post infection (hpi). At MOI $\geqslant 2$, there was more than 80% infection at 48 hpi, while at MOI $\leqslant 0.1$, there was less than 15% of infection at 48 hpi (Fig. 1A). Therefore, MOIs of 5 and 0.5 were used to determine the inhibition or enhancement of the siRNAs on DENV-2 infection, respectively. The transfection efficiency of siRNA into Huh7 cells was established by transfecting different concentrations of p65 siRNA and measuring the down-regulation of p65 expression. 50 nM of the siRNA resulted in more than 50% reduction in p65 expression (Fig. 1B). AP2M1, the μ subunit of clathrin adaptor protein complex 2 (AP-2) (Owen et al., 2004) was used as a positive control for inhibition of infection. It has been reported previously to be essential for HCV assembly (Neveu et al., 2012). HCV and DENV both belong to the *Flaviviridae* family, and have been proposed to have a similar mechanism of assembly (Lindenbach and Rice, 2013). As shown in Fig. 1C, the knockdown of AP2M1 inhibited DENV-2 infection by more than 50% compared to non-treated and non-targeted (scrambled) siRNA controls. Fig. S1 illustrates the screening process. The Dharmacon siGenome kinase library was used to transfect Huh7 cells for 48 h, followed by infection with DENV-2 at an MOI of 5 (for inhibition of infection) or 0.5 (for enhancement of infection) for 48 h. DENV-2 infected cells were stained with 4G2 monoclonal antibody, visualized by confocal microscopy and analyzed by MetaXpress.

Table 2
Host factors which enhanced infection when knocked down in the primary screening.

Gene symbol	Description	Percentage of infection normalised to scrambled control (%)[a]
SHPK	Sedoheptulokinase	131.7
PICK1	Protein interacting with PRKCA 1	129.5
AVPR1B	Arginine vasopressin receptor 1B	129.4
CDKN3	Cyclin-dependent kinase inhibitor 3	127.8
PFKFB3	6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	125.2
ETNK2	Ethanolamine kinase 2	124.5
CSNK1D	Casein kinase 1, delta	123
NEK6	NIMA-related kinase 6	120

[a] Percentages are means from two independent experiments.

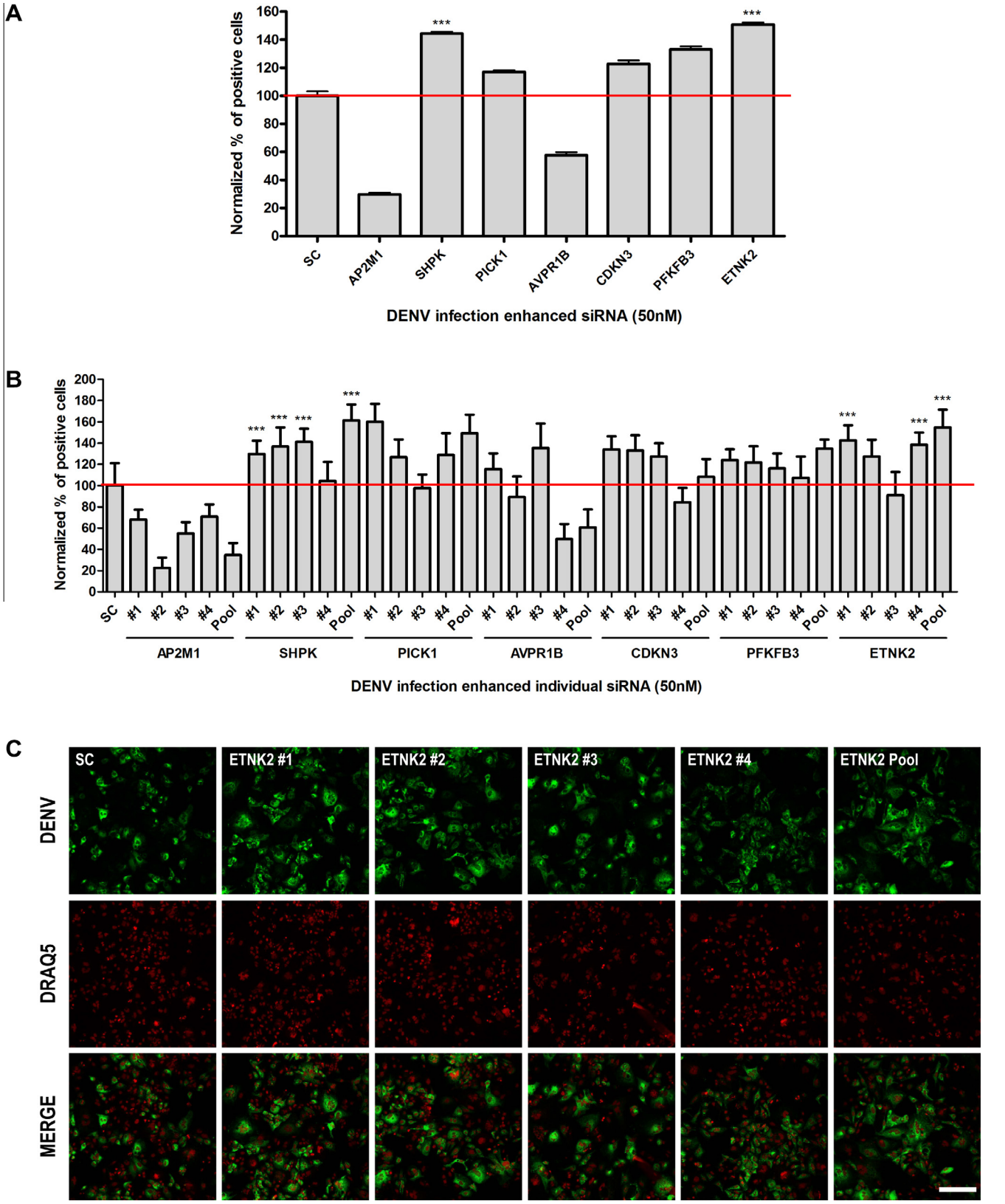

Fig. 3. Knock-down of target host genes enhance DENV-2 infection in Huh7 cells. (A) Among 6 siRNAs identified in the primary screening, 5 were confirmed to increase DENV-2 infection. (B) Target deconvolution with 4 separate siRNAs (50 nM) targeting the same host factor confirmed that the siRNA knockdown of two host factors (SHPK and ETNK2) were specific and resulted in an increase in DENV-2 infection. (C) Images showing BR DEN2 01-01 infection in Huh7 cells transfected with 4 separate siRNAs and siRNA pool of ETNK2. Bars are means ± SD from 1 out of 3 independent experiments. Asterisks (∗) indicate statistical significance with a *p*-value of less than 0.05 using unpaired *t* test (sc – scrambled, scale bars = 250 μm).

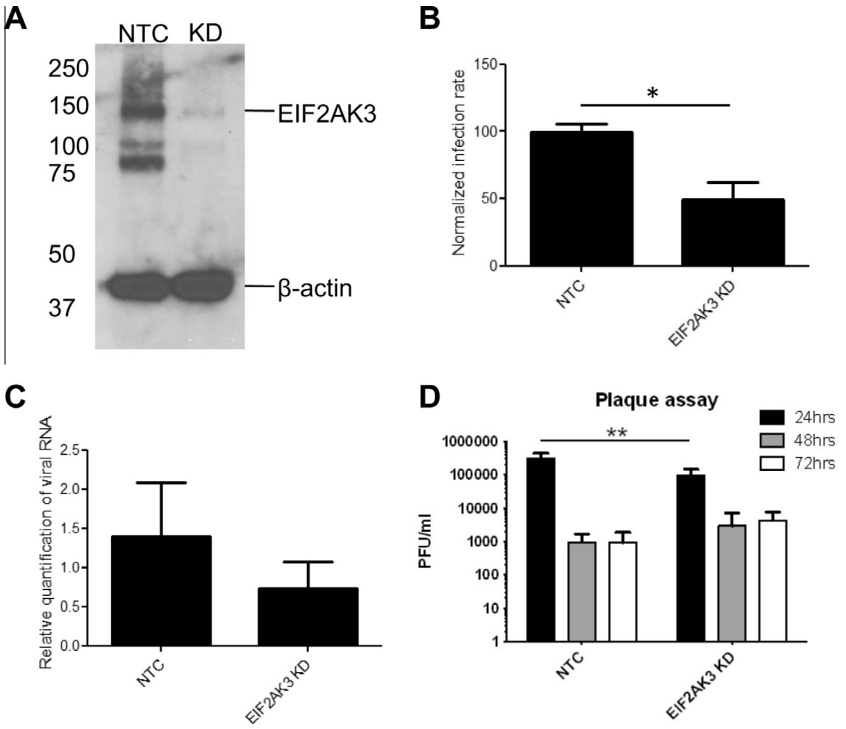

Fig. 4. EIF2AK3 knock-down reduces DENV infection in Huh7 cells. (A) Reduced expression of EIF2AK3 protein in Huh7 cells treated with EIF2AK3 siRNA was confirmed by Western blot using an EIF2AK3-specific antibody 48 h after siRNA transfection. This time point was used for infection. DENV infection in EIF2AK3 knock-down (KD) compared to non-targeting control (NTC)-treated cells was detected by flow cytometry 48 h after infection (B), by qRT-PCR of cell lysates to detect DENV RNA 48 h after infection (C) and by plaque assay 24 h, 48 h and 72 h after infection. Graphs show means ± SD from two independent experiments (B, C) or one experiment, $n = 3$ (D). Asterisks (∗) indicate statistical significance with a p-value of less than 0.05 using an unpaired t test (B, C) or a one-way Anova test (D).

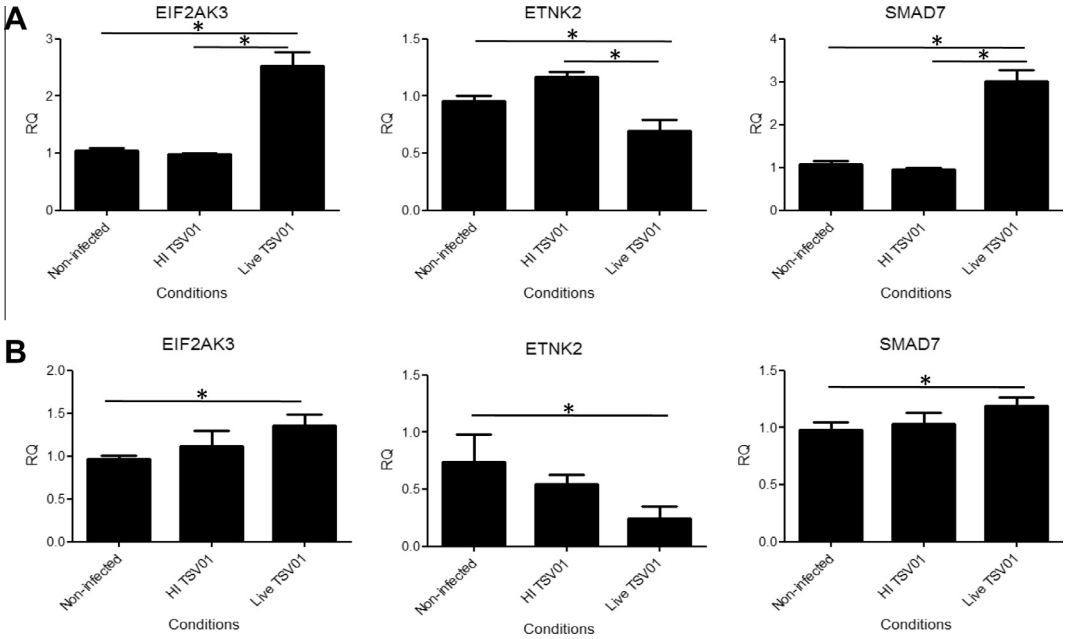

Fig. 5. Genes regulated upon dengue infection in both Huh7 and U937-DC-SIGN cells. The expression of EIF2AK3, ETNK2 and SMAD7 was affected when cells were infected with live but not heat-inactivated (HI) TSV01 in both Huh7 (48 h infection) (A) and U937-DC-SIGN cells (24 h infection) (B). Results are from triplicate wells and show mean RQ (Relative Quantification) ±SD from one experiment. Asterisks (∗) indicate statistical significance with a p-value of less than 0.05 using 1-way ANOVA test.

3.2. Potential host factors that support DENV infection

The siRNAs that reduced the percentage of DENV-2 infected cells more than 2 standard deviations below the mean infection percentage for the entire set of kinase siRNAs were identified as potential inhibitors of DENV-2 infection. Knock-down of 22 host factors resulted in a significant reduction of DENV-2 infection (Table 1). Based on their value as potential drug targets and on host genes identified in dengue patient microarrays (unpublished data), we selected 16 out of 22 host factors for hit confirmation (Fig. 2A).

Knock down of six (SMAD7, EIF2AK3, TLR6, FES, SIK3 and PKIB) out of the 16 host genes reduced DENV-2 infection by more than 40% (Fig. 2A). These six genes were selected for deconvolution, using four individual siRNAs instead of the SMART pools used for the initial screen. For all six hits tested at least two of the four individual siRNAs significantly inhibited the infection (Fig. 2B), while knock-down of SMAD7 reduced DENV-2 infection with all four individual siRNAs (Fig. 2C). The hit deconvolution confirmed the specificity of the hits identified with the siRNA pools and proved that SMAD7, EIF2AK3, TLR6, FES, SIK3 and PKIB support DENV replication in Huh7 cells.

3.3. Potential host factors that inhibit DENV infection

The siRNAs that most significantly increased the percentage of DENV-2 infected cells by 2 standard deviations above the mean infection percentage for the entire set of kinase siRNAs were identified as potential enhancers of DENV-2 infection. In the primary screening, siRNA knockdown of 8 host genes increased DENV-2 infection (Table 2). Based on their value as potential drug targets and on host genes identified in dengue patient microarrays (unpublished data), we selected SHPK, PICK1, AVPR1B, CDKN3, PFKFB3 and ETNK2 for hit confirmation (Fig. 3A). Knockdown of PICK1, CDKN3 and PFKFB3 increased DENV-2 infection, but only SHPK and ETNK2 significantly increased the infection by more than 40% (Fig. 3A). Knockdown of SHPK and ETNK2 with four individual siRNAs targeting different sequences of the same gene confirmed the enhancement of DENV-2 infection (Fig. 3B and C). Hence, the lack of SHPK (sedoheptulose kinase) and ETNK2 (ethanolamine kinase 2) directly or indirectly promoted DENV-2 infection in Huh7 cells.

3.4. Validation of EIF2AK3 and its impact on DENV infection upon knock down in Huh7 cells

As the knock down of EIF2AK3 showed the highest inhibition of DENV infection in the hit validation (Fig 2A), we further investigated the role of EIF2AK3 in Huh7 cells (Fig. 4). Knock-down of EIF2AK3 at the protein level at the time point of infection was confirmed by Western blot (Fig 4A). Cells were infected with DENV-2 and the infection rate was analyzed by flow cytometry 48 h after infection. The data confirmed that knock-down of EIF2AK3 reduced DENV infection significantly compared to treatment with non-targeting control (NTC) siRNA (Fig 4B). However, there was only a slight reduction in cellular viral RNA that was not significant when compared to the control, suggesting that EIF2AK3 impacts on viral protein levels in infected cells but not on viral RNA transcription (Fig 4C). We next tested whether virus particle production was affected in EIF2AK3 knock-down cells and therefore collected supernatants of siRNA-treated and infected cells. We detected significantly less virus output by EIF2AK3 knock-down cells 24 h after infection. However, while their E protein levels were still significantly reduced at 48 h, virus particle production by EIF2AK3 knock-down cells was similar to control cells at this time point (Fig 4D). In conclusion, EIF2AK3 knock-down reduced DENV infection by affecting viral protein production and virus particle output, at least at early time points after infection.

3.5. Expression of candidate genes in Huh7 and U937-DC-SIGN cell lines upon dengue infection

To determine if expression of the candidate genes from the siRNA screening in Huh7 cells was also regulated upon DENV infection, RNA was extracted from Huh7 cells infected with DENV-2 TSV01 and qRT-PCR was carried out for the eight genes described in Figs. 2B and 3B (EIF2AK3, ETNK2, FES, PKIB, SIK3, SHPK, SMAD7 and TLR6). To further compare the expression of these genes in a human monocytic cell line, we used U937 cells stably expressing DC-SIGN to enhance virus attachment and infection. As shown in Supplementary Fig. S2, approximately 50% of Huh7 cells and 40% of U937-DC-SIGN cells were infected after 48 h and 24 h, respectively, which were the time points used in the flow-cytometry based readout.

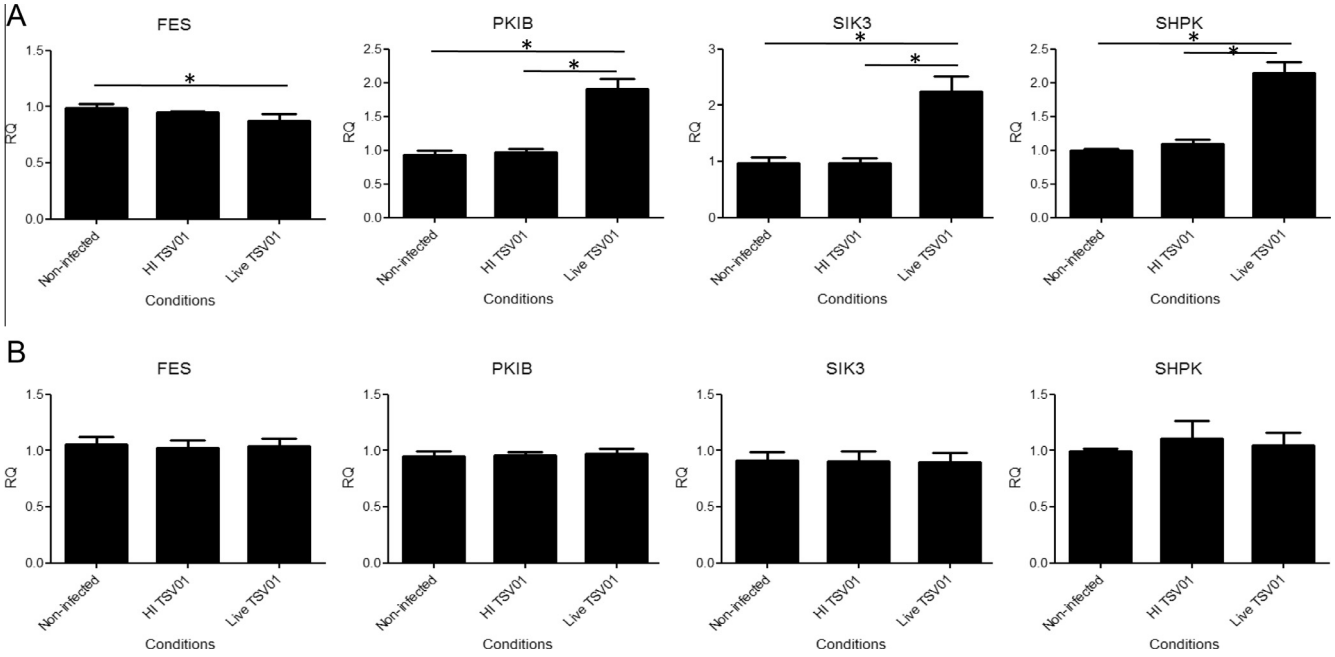

Fig. 6. Genes specifically regulated in infected Huh7 cells. (A) The expression of FES, PKIB, SIK3 and SHPK was affected in infected compared to non-infected or heat-inactivated (HI) DENV-2-treated Huh7 cells 48 h after infection. (B) In contrast, the same genes were not regulated upon infection in U937-DC-SIGN cells at 24 h. Results are from triplicate wells and show mean RQ (Relative Quantification) ±SD from one experiment. Asterisks (*) indicate statistical significance with a *p*-value of less than 0.05 using 1-way ANOVA test.

From our gene expression analysis, three genes (EIF2AK3, ETNK2 and SMAD7) were regulated upon infection in both Huh7 (Fig. 5A) and U937-DC-SIGN cells (Fig. 5B). The expression of ETNK2 was reduced while the expression of EIF2AK3 and SMAD7 was increased in response to infection compared to non-infected cells or cells treated with heat-inactivated (HI) virus, in line with the previous opposing effects of EIF2AK3 and SMAD7 versus ETNK2 (Figs. 2 and 3).

Contrary to EIF2AK3, SMAD7 and ETNK2, which were up- or down-regulated in both Huh7 and U937-DC-SIGN cells, mRNA levels of FES, PKIB, SIK3 and SHPK were affected in Huh7 cells after infection (Fig. 6A) but not in U937-DC-SIGN cells (Fig. 6B). The expression of FES was slightly reduced while the expression of PKIB, SIK3 and SHPK was up-regulated upon infection with DENV compared to non-infected controls. In contrast, the expression of TLR6 did not change upon DENV infection in both Huh7 and U937-DC-SIGN cells (data not shown).

3.6. siRNA knock down of target host genes did not significantly affect DENV infection in U937-DC-SIGN cells

While the increase or decrease in mRNA as detected by qRT-PCR is indicative of changes in protein expression levels after infection, a host protein may be important for viral infection without its expression level being affected. We therefore also knocked down Huh7 target genes in U937-DC-SIGN cells and tested whether DENV infection was affected in these cells. qRT-PCR and Western blot analysis confirmed the knock-down of EIF2AK3, SMAD7, FES

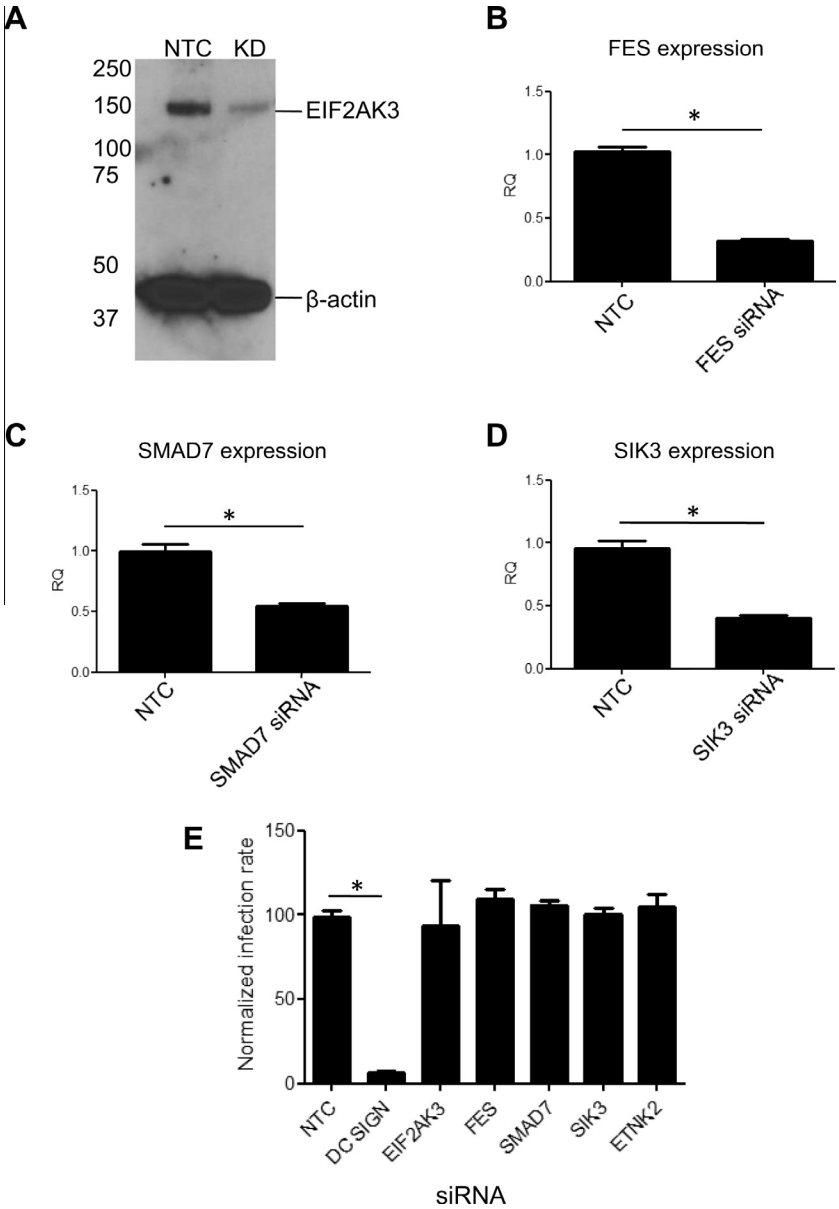

Fig. 7. siRNA knock down of selected target genes does not affect dengue infection in U937-DC-SIGN cells. U937-DC-SIGN cells were transfected individually with siRNAs targeting EIF2AK3, SMAD7, FES, SIK3, ETNK2 and DC-SIGN (control) for 48 h and were infected for 24 h before assessing infection by flow cytometry. (A) EIF2AK3 protein expression was reduced in U937-DC-SIGN cells 48 h post transfection as shown by Western blot. (B) FES, (C) SMAD7 and (D) SIK3 were successfully knocked down at the mRNA level 48 h post transfection. (B)–(D) Results are from triplicate wells and show mean RQ (Relative Quantification) ±SD from one representative experiment out of two. Asterisks (∗) indicate statistical significance with a p-value of less than 0.05 using unpaired t test. (E) Infection of cells transfected with DC-SIGN siRNA was significantly reduced compared to cells transfected with the non-targeting control (NTC), while there was no significant difference in infection in cells transfected with siRNA targeting EIF2AK3, SMAD7, FES, SIK3 and ETNK2. Results show means ± SD from two independent experiments. Asterisks (∗) indicate statistical significance with a p-value of less than 0.05 using 1-way ANOVA test.

and SIK3 (Fig. 7A–D), while there was no reduction of ETNK2 mRNA (data not shown). As shown in Fig. 7E, there was no significant difference in infection when EIF2AK3, SMAD7, FES, SIK3 and ETNK2 were knocked down in U937-DC-SIGN cells compared to the non-targeting control. As a positive control, DC-SIGN, when knocked down, reduced DENV infection by about 95%. Therefore, our data indicated that knockdown of EIF2AK3, SMAD7, FES and SIK3 significantly reduced DENV infection in Huh7 cells (Figs. 2 and 4), but had no effect on DENV infection in U937-DC-SIGN cells.

4. Discussion

In this study, we have developed a high throughput assay to identify novel host targets of DENV virus infection in Huh7 cells. From our primary screen 22 genes and 8 genes out of 779 genes assessed were found to inhibit or enhance viral infection, respectively, when knocked down. Comparison of our screen with that from (Le Sommer et al., 2012), who screened for genes important for yellow fever virus propagation in Huh7 hepatocyte cell lines, revealed PRKCD as a common gene found in both studies. This indicates that PRKCD, a member of the protein kinase C family involved in diverse cellular signaling pathways, could be essential for flavivirus replication in hepatocytes. The hits identified here did not overlap with the findings from (Sessions et al., 2009) who conducted a genome-wide RNA interference screen in *D. melanogaster* cells and subsequently tested human homologues of the *Drosophila* host target hits, which led to the identification of 42 human host targets of DENV in Huh7 cells. Several experiment variables may account for the different results, including viral strains, library types and different conditions for transfection and infection.

We selected hits (EIF2AK3, ETNK2, FES, PKIB, SIK3, SHPK, SMAD7 and TLR6) from our siRNA screen and analyzed their gene expression in DENV-infected Huh7 and U937-DC-SIGN monocytic cells. Expression of all genes except TLR6 was changed in DENV-infected Huh7 cells compared to non-infected cells.

It has been documented previously that DENV infection activates the unfolded protein response (UPR), which is partially controlled by EIF2AK3 (also known as PERK) (Pena and Harris, 2011). EIF2AK3 phosphorylates the inhibitory α unit of the eukaryotic initiation factor 2 (eIF2α), which results in the attenuation of global protein translation. Increased eIF2α phosphorylation has been shown in DENV-infected A549 cells (Umareddy et al., 2007) and in mouse embryonic fibroblasts (MEFs) (Pena and Harris, 2011). Furthermore, transcripts downstream of the eIF2α pathway including growth arrest DNA damage-inducible protein 34 (GADD34) and CCAAT/enhancer-binding protein-homologous protein (CHOP) were induced following DENV infection in Huh7 cells (Fraser et al., 2014), confirming the activation of the UPR after DENV infection. Consistent with these studies we found an up-regulation of EIF2AK3/PERK transcripts in both Huh7 and U937-DC-SIGN cells after DENV infection. However, while it has been shown that MEFs from EIF2AK3 knock-out (EIF2AK3−/−) mice produce more virus particles (Pena and Harris, 2011) compared to MEFs from wildtype mice, we found that EIF2AK3 siRNA-treated Huh7 cells produced less viral protein and less virus particles compared to scramble siRNA-treated Huh7 cells early after infection. The different results observed in MEFs and Huh7 cells could be due to cell type-specific differences. Also, a permanent and complete absence of EIF2AK3 in EIF2AK3−/− cells might have a different outcome on infection compared to a transient knock-down of EIF2AK3 with siRNA. Using the latter approach, our experiments may help to further understand and elucidate the role of EIF2AK3 during DENV infection.

While EIF2AK3 was described before to be involved in the unfolded protein response during dengue infection (Pena and Harris, 2011), the other genes are novel targets. SIK3 and ETNK2 are involved in lipid metabolism and may be associated with virus entry and cellular trafficking. SIK3 is a cytoplasmic serine/threonine protein kinase that modulates cholesterol metabolism (Uebi et al., 2012) while ETNK2 is a member of the choline/ethanolamine kinase family which catalyses the first step of phosphatidylethanolamine biosynthesis, a class of phospholipids in cellular membranes. It has been shown previously that the cholesterol and lipid metabolism is important for DENV uptake and replication, as dengue infection was affected when the cholesterol intake in infected cells was disrupted using a cholesterol transport inhibitor (Poh et al., 2012).

It is interesting to note that while knock-down of both SHPK (sedoheptulose kinase) and ETNK2 resulted in enhanced dengue infection (Fig. 3), mRNA levels of SHPK were increased upon DENV infection in Huh7 cells (Fig. 5) whereas mRNA levels of ETNK2 were decreased after infection of Huh7 and U937 cells (Fig. 4). While down-regulation of ETNK2 after infection is in line with an inhibitory function, the up-regulation of SHPK after infection may be indicative of a compensatory mechanism that eventually results in increased instead of decreased expression of SHPK after infection. Interestingly, SHPK was slightly down-regulated in a whole blood microarray of dengue patient samples collected <72 h after fever onset compared to the convalescent samples of the same patients (unpublished data). SHPK (also named CARKL) is a carbohydrate kinase of the pentose phosphate pathway that has recently been shown to be involved in macrophage polarization (Haschemi et al., 2012). In our experiments, however, the role of SHPK for DENV infection seemed to be Huh7-specific.

SMAD7 can induce tumorigenicity by inhibiting TGF-β induced growth inhibition and apoptosis, and thus perhaps prolongs cell survival and thus enhances virus replication and production in infected cells (Halder et al., 2005). Although the mRNA level of TLR6 did not change in DENV-infected Huh7 compared to non-infected controls, TLR6 still seems to play a role in dengue infection, as shown in our siRNA screen, where knocking it down reduced infection in Huh7 cells. It may be that dengue infection does not significantly change the expression of TLR6 in Huh7 cells because the basal level is already high.

EIF2AK3, ETNK2 and SMAD7 were regulated upon dengue infection in both Huh7 and U937-DC-SIGN cells. In contrast, regulation of expression of FES, PKIB, SIK3 and SHPK was only observed in dengue-infected Huh7 cells. Knocking down EIF2AK3, FES, SMAD7, SIK3 and SHPK in U937-DC-SIGN did not affect dengue infection. We were unable to efficiently knock down ETNK2 as this gene was expressed at very low levels in U937-DC-SIGN cells (data not shown), which made it more difficult to further reduce its expression.

The liver is an important target organ during dengue infection and has been shown to be involved in the pathogenesis of dengue in both adults and children (Roy et al., 2013; Seneviratne et al., 2006), with viral genome (Rosen et al., 1999), antigens (Couvelard et al., 1999) and virion particles (Rosen et al., 1989) detected in liver tissue and hepatocytes from dengue infected patients. A recent study assessed the effect of kinase inhibitors on DENV infection, and it found that small molecules such as AZD0530 and dasatinib inhibit DENV replication by their inhibiting action on Fyn kinase (de Wispelaere et al., 2013). However, the cell type-specific effect of these drugs and *in vivo* efficacy was not tested. In the kinase siRNA library used here Fyn was not targeted and we could therefore not confirm the role of Fyn in our screen. Unfortunately, no specific inhibitors for the kinases identified in our screen are commercially available and the hits could therefore not be validated with inhibitors.

In conclusion, we have identified several additional human kinases important for dengue infection, with relevance in hepatocytes. With the liver being an important target organ during

dengue infection, inhibition of viral infection in the liver could have an impact on pathogenesis in patients. It is not possible to predict the therapeutic value of liver or immune-cell-specific drugs with the current limited understanding of dengue pathogenesis. However, data gathered from mouse, monkey and human studies show that immune cells such as dendritic cells, monocyte-derived macrophages and tissue-resident macrophages are major targets for at least the initial steps of dengue infection. Knowledge about cell-type specific differences in host gene expression during infection is therefore critical for drug development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2014.07.006.

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