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Dengue virus NS1 protein interacts with the ribosomal protein RPL18: This interaction is required for viral translation and replication in Huh-7 cells

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ABSTRACT

Given dengue virus (DENV) genome austerity, it uses cellular molecules and structures for virion entry, translation and replication of the genome. NS1 is a multifunctional protein key to viral replication and pathogenesis. Identification of cellular proteins that interact with NS1 may help in further understanding the functions of NS1. In this paper we isolated a total of 64 proteins from DENV infected human hepatic cells (Huh-7) that interact with NS1 by affinity chromatography and immunoprecipitation assays. The subcellular location and expression levels during infection of the ribosomal proteins RPS3a, RPL7, RPL18, RPL18a plus GAPDH were determined. None of these proteins changed their expression levels during infection; however, RPL-18 was redistributed to the perinuclear region after 48 hpi. Silencing of the RPL-18 does not affect cell translation efficiency or viability, but it reduces significantly viral translation, replication and viral yield, suggesting that the RPL-18 is required during DENV replicative cycle.

Introduction

The persisting epidemics of dengue disease in the world as well as the increasing number of cases, especially of the severe forms of the infection, along with the growing number of countries where dengue outbreaks are reported, make this disease a severe public health problem (Gubler and Meltzer, 1999). Despite multiple studies conducted by several research groups to produce a vaccine or an effective antiviral treatment, so far neither is available (Chokephaibulkit and Perng, 2013). Dengue virus (DENV), a member of the *Flaviviridae* family and *Flavivirus* genus is a single-stranded RNA virus with positive polarity, which encodes for ten viral proteins, three structural, capsid (C), membrane (M) and envelope (E) proteins and seven non-structural NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5.

Because of the austerity of the viral genomes, viruses require during their replicative cycle the participation of different molecules and organelles to enter into the host cell, to translate and replicate their genome and to exit from the cell. The use of various strategies to identify molecules required during DENV infection has allowed to identify proteins involved in viral particle or E protein binding to the cell surface (Chen et al., 1997; Jindadamrongwech et al., 2004; Navarro-Sanchez et al., 2003; Reyes-Del Valle et al., 2005; Sakoonwatanyoo et al., 2006; Tassaneetrithep et al., 2003; Vega-Almeida et al., 2013), proteins that bind to the 5′ and 3′ untranslated regions of the viral genome (De Nova-Ocampo et al., 2002; Yocupicio-Monroy et al., 2007; Yocupicio-Monroy et al., 2003), as well as molecules that modify their location or expression levels during infection. Interestingly, although DENV replicative cycle occurs within the cytoplasm and specifically in the endoplasmic reticulum (ER), several of the proteins that bind to the viral RNA are located in the nucleus (Agis-Juarez et al., 2009; Blaney et al., 2005; De Nova-Ocampo et al., 2002). Thus, it is clear that there are major changes in the cellular physiology during infection. One of the tools used to identify and characterize molecules involved in viral replicative cycles is to identify cellular proteins which interact with viral proteins. One of the less known DENV viral protein is NS1. This is a protein of approximately 46–50 kDa with two glycosylation sites (N130 and N207) (Smith and Wright, 1985) and one of the most conserved proteins among the four DENV serotypes and even among flaviviruses (Deubel et al., 1988; Mackow et al., 1987). The precise function of NS1 in DENV replication is unclear, but NS1 is known to co-localizes with double-stranded RNA in infected cells

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and to be a necessary cofactor for viral RNA replication (Lindenbach and Rice, 1997, 1999; Mackenzie et al., 1996). In addition to the cytoplasmic localization of NS1, this protein has the particularity to be associated with the outer face of the plasma membrane, via a GPI link, and to be secreted during infection (Gutsche et al., 2011; Winkler et al., 1988). In its membrane-associated form, NS1 behaves as a dimer, whereas in its soluble form it behaves as a hexamer (Gutsche et al., 2011; Winkler et al., 1988). In patients, NS1 circulates at high levels in serum during the first 5–7 days after infection (de la Cruz-Hernandez et al., 2013). High levels of NS1 in patients' sera correlate with disease severity and with the risk of developing Dengue Hemorrhagic fever (DHF) (Avirutnan et al., 2006; de la Cruz-Hernandez et al., 2013). Thus, it has been postulated that NS1 protein is directly involved in the pathogenesis of DHF. Finally, it has been reported that anti-NS1 antibodies protected mice from infection with DENV (Falgout et al., 1990; Henchal et al., 1988).

It is likely that NS1 interacts with cellular proteins that contribute to its multifunctionality. The identification of host cell proteins that interact with NS1 may help to better understand the function of this protein during DENV replicative cycle. Thus, in this paper we used two different strategies to isolate cellular proteins that interact with NS1. First, a His-tag recombinant NS1 protein produced in bacteria was used as bait to separate cellular proteins with affinity to NS1. Second, a polyclonal anti-NS1 antibody was used in immunoprecipitation assays with DENV infected cell extracts to isolate cellular proteins with affinity to NS1. The cell proteins bound to NS1 were eluted with salt and identified by MALDI ToF, MS/MS. A total of 64 proteins were identified that belong to different functional groups such as ribosomal and histone proteins as well as cytoskeleton proteins. The subcellular distribution of some of the proteins identified such as GAPDH and RPL18 was altered in Huh-7 infected cells. Since RPL18 was redistributed to the perinuclear region during DENV infection, its participation in DENV replicative cycle was analyzed by siRNA techniques. RPL18 silencing reduces significantly viral translation and replication as well as viral yield suggesting that the RPL18 is required during DENV replicative cycle.

Results

Purification of recombinant NS1

To identify cellular proteins that interact with the nonstructural protein NS1 from DENV, the first step was to express and purify recombinant DENV2 NS1 protein (rNS1) in *E. coli*. The expression of the rNS1 was induced by IPTG and the recombinant protein was purified by metal affinity resin as described in the Materials and methods section. The full sequence of the cloned NS1 protein and the Western blot carried out to corroborate the nature of the expressed protein are shown in Fig. 1 of Supplemental material. After washing with different concentrations of imidazole, the rNS1 protein, with a molecular weight of approximately 55 kDa, was coupled to the resin (lane R, Fig. 2A, Supplemental material). The presence and purity of the rNS1 in the resin was confirmed by Western blot assay using an anti-NS1 polyclonal antibody (lane R, Fig. 2A and B, Supplemental material). Moreover, the rNS1 migrates as a single band of 55 kDa in a native gel, suggesting that the monomer is the isoform present in the preparation (lanes 1 and 2 Fig. 2C Supplemental material). No specific bands were observed after the analysis of lysates from induced bacteria transformed with the same plasmid without the NS1 sequence used as controls (Fig. 3A and B, Supplemental material).

Table 1
Functional classification of the NS1-interacting proteins identified of mass spectrometry. To investigate the biological process and signaling pathway, we used DAVID Bio-informatics Resources (http://david.abcc.ncifcrf.gov/) and the proteins were also searched through REACTOME pathway database (http://www.reactome.org).

Biological process	Proteins
Microtubule based process	8
Translation	33
Nucleosome assembly	4
Glycolysis	4
Stress response	3
Transport	2
Other process	12

Cellular proteins that interact with DENV NS1 in infected human liver cells

To isolate cellular proteins that interact with NS1 two different strategies were used: affinity chromatography using the rNS1 protein as bait and immunoprecipitation assays using polyclonal anti-NS1 antibodies.

For the affinity chromatography assay, the rNS1 protein, coupled to the cobalt column, and the control resin were incubated with cell extracts from uninfected Huh-7 cells, and after six washes with different concentrations of NaCl, (lanes 1–6, Fig. 3, Supplemental material), two elutions, at 1000 and 1500 mM of NaCl were performed. The majority of the proteins were eluted at 1000 mM NaCl (Fig. 4A Supplemental material). Most of the proteins obtained in this faction interact specifically with rNS1 because none of them were observed in the elution fraction of the negative controls (Fig. 4B, Supplementary material). Mass spectrometry (Maldi ToF) analysis of the eluted proteins allowed the identification of 80, 70 and 72 proteins in each of the three independent experiments from Huh-7 cells. These proteins were able to interact directly or indirectly with rNS1 and were absent in the proteins identified in the control resin.

The immunoprecipitation assays were carried out with anti-NS1 polyclonal antibody or rabbit IgG used as negative control, cross linked to protein-G agarose and infected Huh-7 cells cytoplasmic extracts. The immunoprecipitated proteins were washed five times (lanes 1–5, Fig. 5A and B, Supplemental material) and eluted as described in the Material and methods section. The proteins obtained in the elution fraction interact specifically with NS1 because none of them were immunoprecipitated with the anti-rabbit IgG used as negative control (lanes E, Fig. 5A and B, Supplemental material). Mass spectrometry (Maldi ToF) analysis of the immunoprecipitated proteins allowed to identify 70, 86 and 85 proteins from 3 independent experiments. A total of 64 proteins that were identified by the 2 methods and in the 3 independent experiments were considered as true NS1-interacting proteins (Table 2).

Functional characterization of NS1 interacting proteins

In an attempt to further characterize the cell proteins identified, the functional categorization and subcellular localization was determined based on a Swiss-Prot TrEMBL database search. The 64 proteins identified are involved in six different biological processes: 33 out of the 64 proteins identified are involved in translation; 8 out of 64 are involved in microtubule based process; 4 out of the 64 are involved in nucleosome assembly and glycolysis, 3 and out of the 64 are involved in stress response and 2 in transport (Table 1). Most proteins identified are cytoplasmic proteins (23.2%) and are ribosomal proteins (48.2%). Interestingly, 8.9% of the cell proteins identified are nuclear proteins, while only 1.7% of the proteins identified are from mitochondrion, 3.5% from membrane and 1.7% are secreted (Fig. 1).

Table 2
Summary of the NS1-interacting proteins identified of mass spectrometry in Huh-7 cells.

Protein identified	Mascot score	No. of peptide sequences indentified	String accesion no.
26S protease regulatory subunit 4	39	3	PSCM1
28S ribosomal protein S15, mitochondrial	37	1	MRPS15
40S ribosomal protein S13	27	1	RPS13
40S ribosomal protein S14	81	1	RPS14
40S ribosomal protein S15	39	1	RPS15
40S ribosomal protein S15a	38	1	RPS16
40S ribosomal protein S16	63	1	RPS16
40S ribosomal protein S18	56	5	RPS18
40S ribosomal protein S2	68	3	RPS2
40S ribosomal protein S23	115	1	RPS23
40S ribosomal protein S24	75	3	RPS24
40S ribosomal protein S25	82	1	RPS25
40S ribosomal protein S3a	150	5	RPS3A
40S ribosomal protein S4, X isoform	108	5	RPS4
40S ribosomal protein S5	30	3	RPS5
40S ribosomal protein S6	31	1	RPS6
40S ribosomal protein S8	87	2	RPS8
60S ribosomal protein L11	39	4	RPL11
60S ribosomal protein L13	71	3	RPL13
60S ribosomal protein L14	94	2	RPL14
60S ribosomal protein L17	40	1	RPL17
60S ribosomal protein L18	104	2	RPL18
60S ribosomal protein L18a	63	3	RPL18A
60S ribosomal protein L19	41	3	RPL19
60S ribosomal protein L21	50	2	RPL21
60S ribosomal protein L23a	27	1	RPL23A
60S ribosomal protein L24	108	2	RPL24
60S ribosomal protein L26	31	1	RPL26
60S ribosomal protein L27a	80	1	RPL27A
60S ribosomal protein L31	39	1	RPL31
60S ribosomal protein L36	60	2	RPL36
60S ribosomal protein L7	68	1	RPL7
Ankyrin repeat and KH domain-containing protein 1	61	2	ANKHD1
Cofilin-1	31	1	CFL1
Cytoplasmic dynein 1 heavy chain 1	47	5	DYNC1H1
Elongation factor 1 alpha 1	93	3	EEF1A1
Glucose-6-phosphate isomerase	37	1	GPI
Glyceraldehyde-3-phosphate dehydrogenase	59	1	GAPDH
GTP-binding protein Di-Ras2	47	1	DIRAS2
Heat shock cognate 71 kDa protein	73	2	HSPA8
Heat shock protein beta-1	114	1	HSPB1
Heat shock protein HSP 90-beta	55	1	HSP90AB1
Heterogeneous nuclear ribonucleoprotein F	43	1	HNRNPF
Histone H2B type 1-A	38	2	HIST1H2BA
Histone H2B type 1-B	61	4	HIST1H2BB
Histone H3.3C	38	2	H3F3C
Histone H4	50	2	HIST1H4H
Insulin-like growth factor 2 mRNA-binding protein 2	53	2	IGF2BP2
Oxidoreductase NAD-binding domain-containing protein 1	38	2	OXNAD1
Peroxiredoxin-1	34	2	PRDX1
Plectin	86	13	PLEC
Protein FAM132A	34	1	FAM132A
Pyruvate kinase PKM	38	3	PKM2
Serine/threonine-protein kinase Chk1	30	1	CHEK1
Serine/threonine-protein kinase SIK3	40	1	SIK3
Tetratricopeptide repeat protein 21B	39	1	TTC21B
Tubulin alpha-1A chain	239	6	TUBA1A
Tubulin alpha-4A chain	42	5	TUBB6
Tubulin beta chain	297	7	ENSG0000
Tubulin beta-2A chain	81	5	TUBB2A
Tubulin beta-3 chain	224	6	TUBB2C
Tubulin beta-4B chain	247	8	TUBA4A
Tubulin beta-6 chain	149	7	TUBB6
Vimentin	1179	38	VIM

Additionally, a protein network analysis of protein–protein interaction was performed to identify functionally linked proteins. We upload the 64 identified proteins along with their names into STRING. Even though all the proteins do not show interactions, 30 proteins were found to be strongly linked either directly or indirectly through one or more interacting proteins suggesting the existence of functional linkages. A merge network is shown in Fig. 2. Forty-three out of the 64 identified proteins were interconnected while 12 proteins did not show any link at the chosen confidence level (STRING score = 0.4).

DENV NS1 interacts with RPL18a, RPL18 and GAPDH in Huh-7 cells

Interestingly approximately 50% of the 64 proteins identified were ribosomal proteins. It is possible that NS1 interacts with many ribosomal proteins, or even with the entire ribosome. The

main function of the ribosomal proteins is mRNA translation; however, it has been described that several ribosomal proteins have extra-ribosomal functions. Among the proteins identify to interact with NS1, extra-ribosomal functions have been reported for RPL18a, RPL18, RPS3a, and RPL7 (Bhavsar et al., 2010). The extra ribosomal functions described for these proteins include, ribosomal binding to endoplasmic reticulum, cell cycle, apoptosis, etc (Bhavsar et al., 2010). Moreover, hits for ribosomal proteins RPL18 and RPL7 have been found in siRNA screening performed with yellow fever and West Nile virus (Le Sommer et al., 2012) suggesting that these ribosomal proteins are required for viral infection.

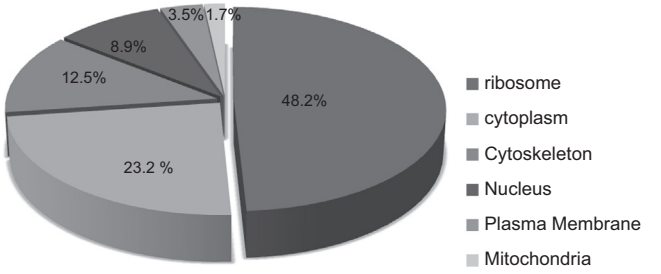

Fig. 1. Cellular localization of NS1-interacting proteins identified by mass spectrometry. The subcellular localization of the NS1-interacting proteins was analyzed using the information from Swiss-Prot/TrEMBL database.

To validate that proteins isolated and identified by affinity chromatography and immunoprecipitation assays are proteins that interact directly or indirectly with DENV NS1 protein, two ribosomal proteins RPL18 and RPL18a and the metabolic enzyme GAPDH were further analyzed by immunoprecipitation assays.

Cytoplasmic extracts from infected Huh-7 cells were immunoprecipitated with the anti-NS1 antibody and the immunoprecipitated fraction was analyzed by Western blot using specific anti-NS1, anti-GAPDH, anti-RPL18a and anti-RPL18 (Fig. 3). Extracts were also immunoprecipitated with anti-GAPDH, anti-RPL18a and anti-RPL18 and Western blot revealed with anti-NS1 antibodies. In the immunoprecipitation assays, the presence of RPL18a (18 kDa), GAPDH (38 kDa) and RPL18 (22 kDa) among the proteins immnoprecipitated with the anti-NS1 (55 kDa) antibody, as well as the detection of NS1 (55 kDa) in the fractions immunoprecipitated with anti-GAPDH, anti-RPL18a and anti-RPL18 fraction was observed (Fig. 3A and C respectively). Similar results were obtained when cellular extract was pretreated with RNAses (Fig. 6 Supplemental material) suggesting that DENV RNA does not mediate the interaction of NS1 with the ribosomal proteins

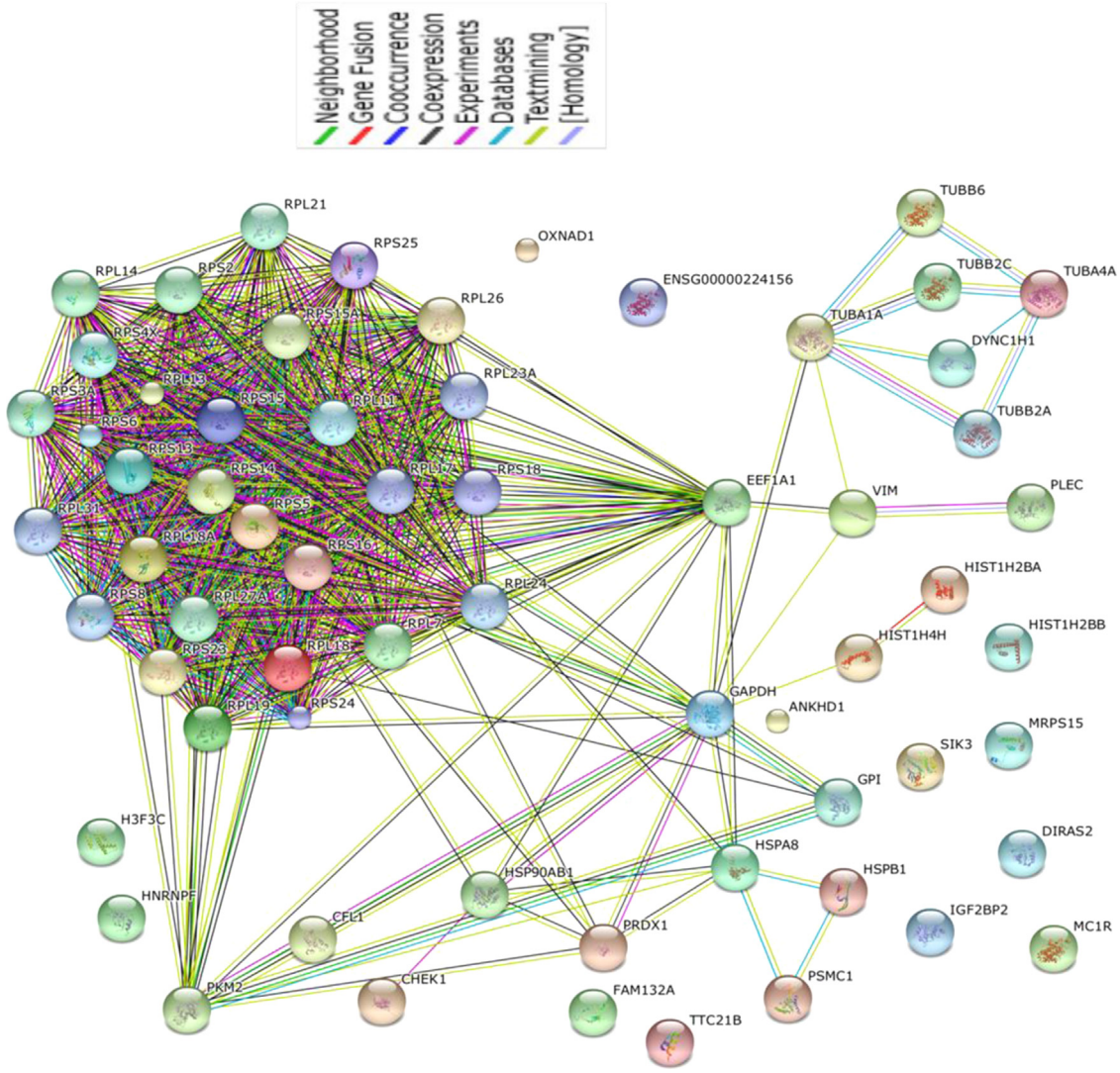

Fig. 2. Protein interaction network analysis of the NS1-interacting proteins. The network containing 64 identified proteins was mapped using STRING version 9.05. Line color indicates the type of supporting evidence.

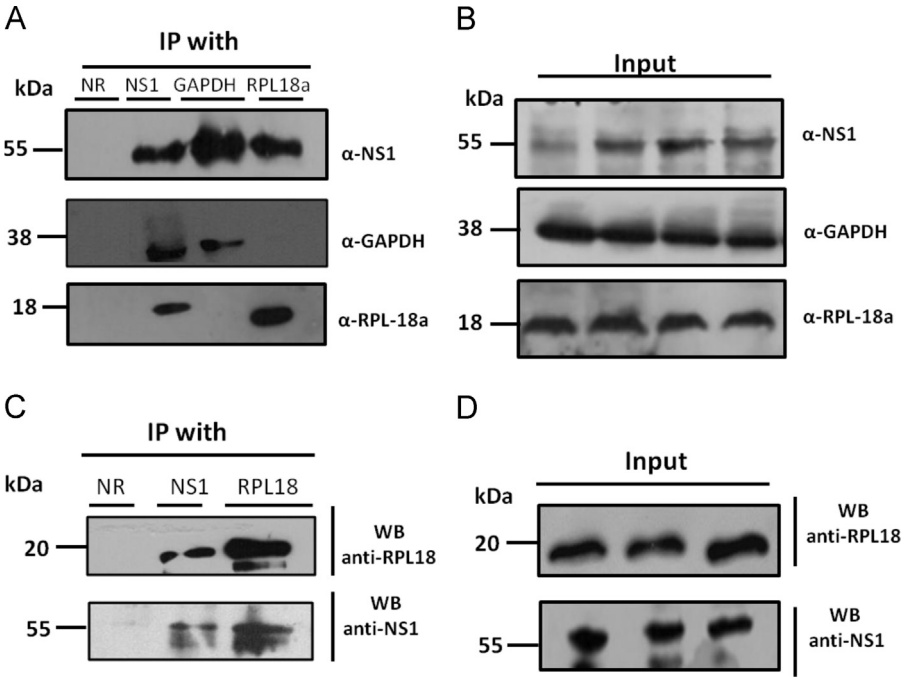

Fig. 3. NS1 interacts with GAPDH, RPL18a and RPL18 proteins. Infected cells extracts from Huh-7 cells were immunoprecipitated with anti-NS1 (panels A and C), anti-GAPDH and anti-RPL18a (panel A) or anti-RPL18 antibodies (panel C). The immunoprecipitated proteins (panels A and C) or the total proteins from cytoplasmic extract (panels B and D) were separated by SDS-PAGE and analyzed by Western blot assay using anti-NS1 (panels A and C), anti-GAPDH and anti-RPL18a (panel A) or anti-RPL18 antibodies (panel B).

and GADPH. The presence of the GAPDH, RPL18a, RPL18 and NS1 proteins, in the extract used for the immunopreciptation assay was also confirmed (Fig. 3B and D). These results confirm the interaction between the NS1 and GAPDH, RPL18a and RPL18 proteins and validate the results obtained by affinity chromatography and immunoprecipitation assays followed by mass spectroscopy identification.

The expression of GAPDH, RPL18a, RPL18, RPL7 and RPS3a proteins is not altered during DENV infection

To evaluate the expression levels and the localization of RPL18a, RPL18, RPL7, RPS3a and GADPH proteins during infection, Western-blot analysis and immunofluoresce assays were performed with Huh-7 cells infected at a MOI of 3 at 6, 24 and 48 hpi. Total cell extracts from mock infected Huh-7 cells (M), infected with UV inactivated virus (IV) or infected with DENV (INF) were used. The expression levels of GAPDH (Fig. 4A and B), RPL18a (Fig. 4A and C), RPL18 (Fig. 4A and D), RPL7 (Fig. 4E and F) and RPS3a (Fig. 4E and G) was not altered during infection with DENV or with UV inactivated DENV. To confirm this observation, quantitative analysis of the five proteins in three independent experiments with respect to actin level was performed. No statistically significant differences in the amount of the five proteins were detected in the infected cells al any time in relation to the protein level in mock infected cells. (Fig. 4B–D and F–G) [GAPDH ($p=0.090$), RPL18A ($p=0.158$) RPL18 ($p=0.588$), RPL7 ($p=0.126$) AND RPS3a ($p=0.560$)]. Thus, although RPL18a, RPL18, RPL7, RPS3a and GADPH interact with DENV NS1 protein they do not alter their expression levels during infection.

To analyze the subcellular localization of GAPDH, RPL18, RPL7 and RPS3a, Huh-7 cells were infected with DENV at a MOI of 3 for 48 h, incubated with specific antibodies against each protein and analyzed by confocal microscopy. Although we could no detect any difference in the expression of GAPDH and RPL18 and RPL7 by Western-blot using total cell extracts, a different distribution of these proteins in infected cells was detected. While GAPDH protein shows a punctuate distribution in uninfected cells, it was observed dispersedly distributed in infected cells (Fig. 5A). For GAPDH, it was not possible to detect a colocalization with NS1 by confocal microscopy. Protein RPL18, which shows a disperse distribution in the cytoplasm of uninfected cells; was found relocated to the perinuclear region in infected cells (Fig. 5B). Moreover, colocalization between RPL18 and NS1 was observed (Fig. 5B merge). Similarly, the RPL7 shows a disperse distribution in the cytoplasm of uninfected cells and relocation to the perinuclear region in infected cells (Fig. 6A).

Neither the distribution nor the expression of the ribosomal protein and RPS3a was altered during DENV infection, confirming the results obtained by Western-blot (Fig. 6B).

RPL18 is required during DENV infection

As was shown previously, RPL18 is one of the proteins identified as NS1-interacting protein and although this protein does not alter its expression during DENV infection, it changes its distribution in infected cells. Since it has been described that the RPL18 may have extra ribosomal functions (Bhavsar et al., 2010), the role of this protein in DENV infection was evaluated.

Given the abundance of the ribosomal proteins, the first step was to standardize silencing of the protein. To achieve siRNA silencing of RPL18, Huh-7 cells were transfected in suspension with two different concentrations of RPL18 siRNA (100 and 150 nM) or with 150 nM of an unrelated siRNA. Twenty-four hours later, the Huh-7 cells, grown in monolayer, were retransfected with the same concentrations of the two different siRNAs and levels of RPL18 protein were measured 24 h after the second transfection. Under these conditions, a 61% and 84% reduction in the expression of the RPL18 was detected with 100 and 150 nM of the RPL18 siRNA respectively, compared with the expression of the protein in cells transfected with the unrelated siRNA (Fig. 7A and B).

It is well known that ribosomal proteins are essential for cellular translation; however, some of them can be silenced

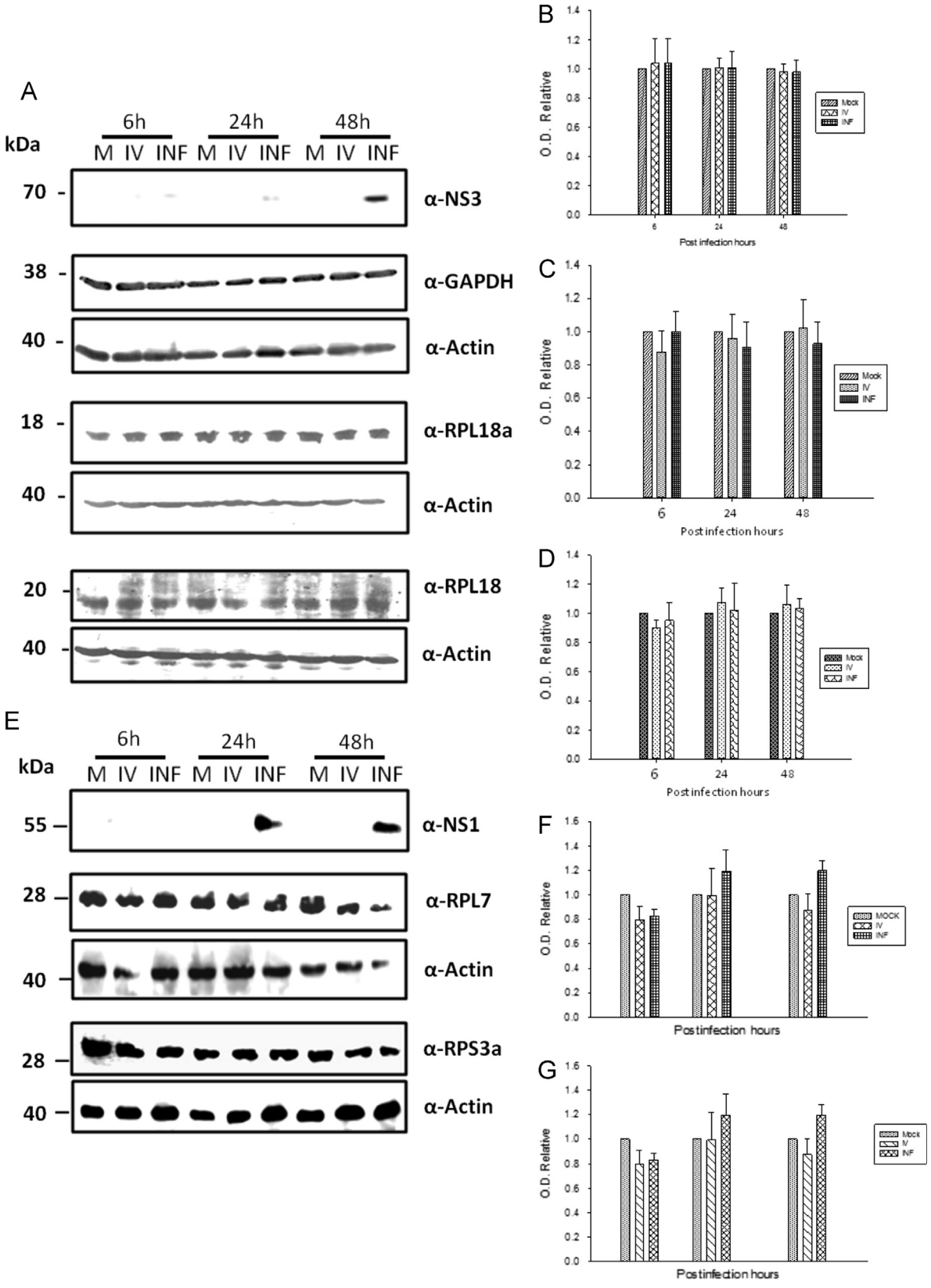

Fig. 4. Expression levels of GAPDH, RPL18a, RPL18, RPS3a and RPL7 proteins in DENV infected cells. Total cell extracts from Huh-7 cells Mock (M) infected or infected with UV-inactivated DENV (IV) or with DENV (INF) at three different times post-infection (6, 24 and 48 h), were analyzed by Western-blot assay using anti-GAPDH, anti-RPL18a, RPL18, (A) or anti-RPL7 and RPS3a antibodies (E). Anti-NS3 (A) and anti-NS1antibodies (E) were used to demonstrate DENV infection. Anti-actin antibody was used as a loading control. A representative Western-blot assay is shown in (A) and (B). Densitometric analysis from three independent experiments for GAPDH ($p=0.090$) (B), RPL18a ($p=0.158$) (C), RPL18 ($p=0.588$) (D), RPS3a ($p=0.560$) (F) and RPL7 ($p=0.126$) (G) is shown.

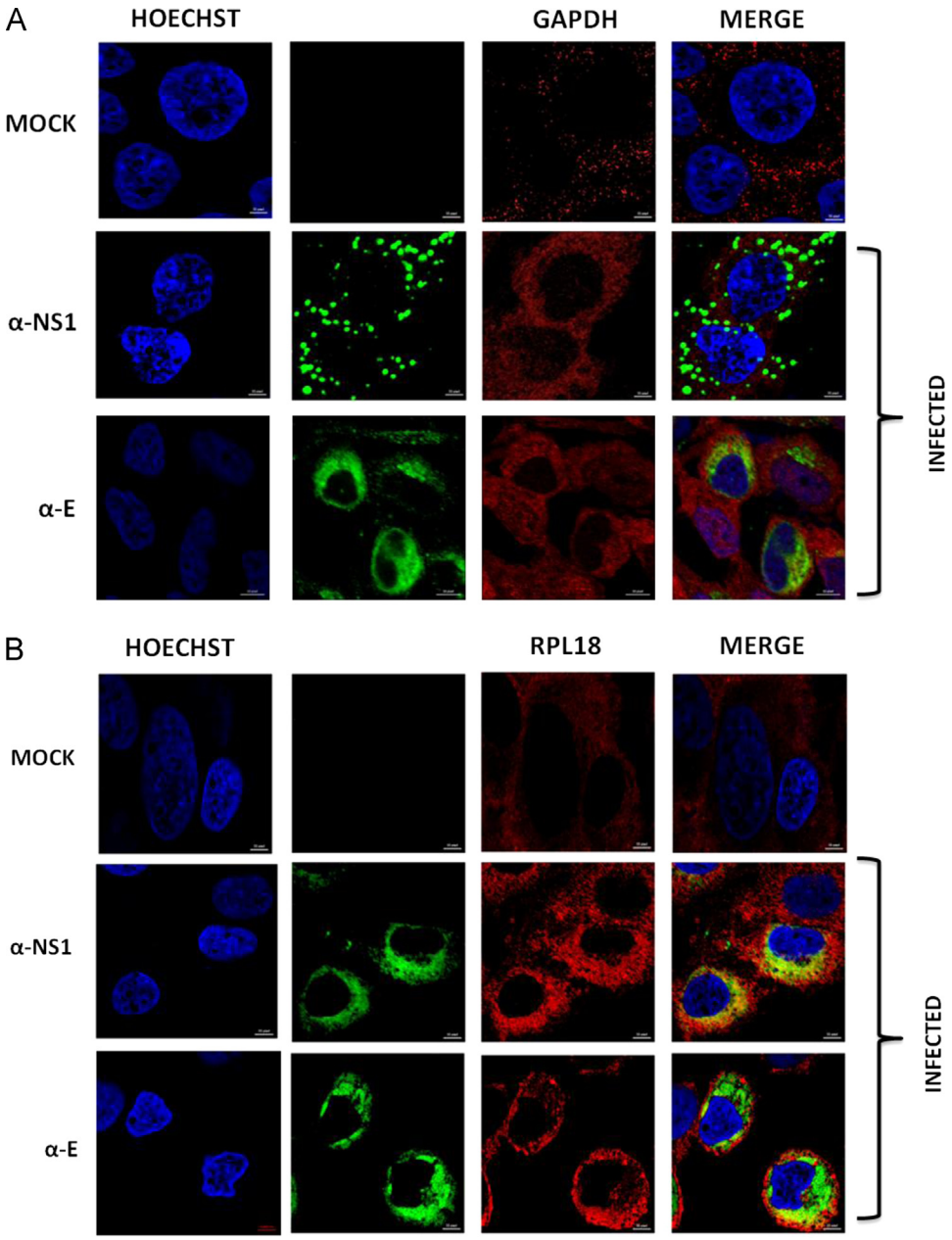

Fig. 5. Subcellular localization of GAPDH, and RPL18 proteins in DENV infected cells. Huh-7 cells infected with DENV2 for 48 h were fixed and stained with anti-E, anti-NS1 (green) and anti-GAPDH (A) and anti-RPL18 (B) (red) antibodies. Nuclei were counterstained with **Hoechst (blue).** Proteins location was analyzed by confocal microscopy. The images are representatives of three independent experiments.

without any effect in cell viability and in cellular translation. To evaluate if the expression of RPL18 could be silenced without a deleterious effect in the cell, cell viability and cellular translation efficiency were evaluated. Neither the RPL18 siRNA, nor the unrelated siRNA reduce cell viability of the transfected cells, indicating that the silencing of the RPL18 does not have deleterious effect on cell viability at 48 or 72 h post transfection (Fig. 7C). Moreover, translation of cellular mRNAs, assayed by the presence of S^{35}-methionine labeled cellular proteins (Fig. 7D), protein staining with Coomasie-blue biosafe and with silver (Fig. 7 Supplemental material) was not modified by knockdown of the expression of RPL18 at 48 or 72 h post transfection. Thus, silencing of RPL18 neither reduce cell viability nor inhibit translation efficiency.

Once the silencing of RPL18 was established, transfected cells were infected with DENV. To determine the effect of RPL18 silencing in viral infection, four different parameters were evaluated: viral yield, NS1 secretion, viral protein expression and levels of viral RNA.

One and 1.5 log reduction in viral yield was detected in the supernatant of cells collected at 48 hpi and transfected with 100 and 150 nM of the RPL18 siRNA respectively, compared to the viral yield obtained with the cells transfected with 150 nM of an unrelated siRNA, suggesting that RPL18 is involved in DENV replicative cycle (Fig. 8A). This suggestion was supported by the reduction in secreted NS1 protein levels detected by ELISA in the supernatants of cells transfected with 150 nM of the RPL18 siRNA and collected at 48 hpi compared with the amount of NS1 secreted by cells transfected with an unrelated siRNA (Fig. 8B).

Knowing that reduced levels of RPL18 induced a reduction in viral yield and in NS1 secretion, the expression levels of viral proteins and viral RNA were analyzed in RPL18 silenced cells. A

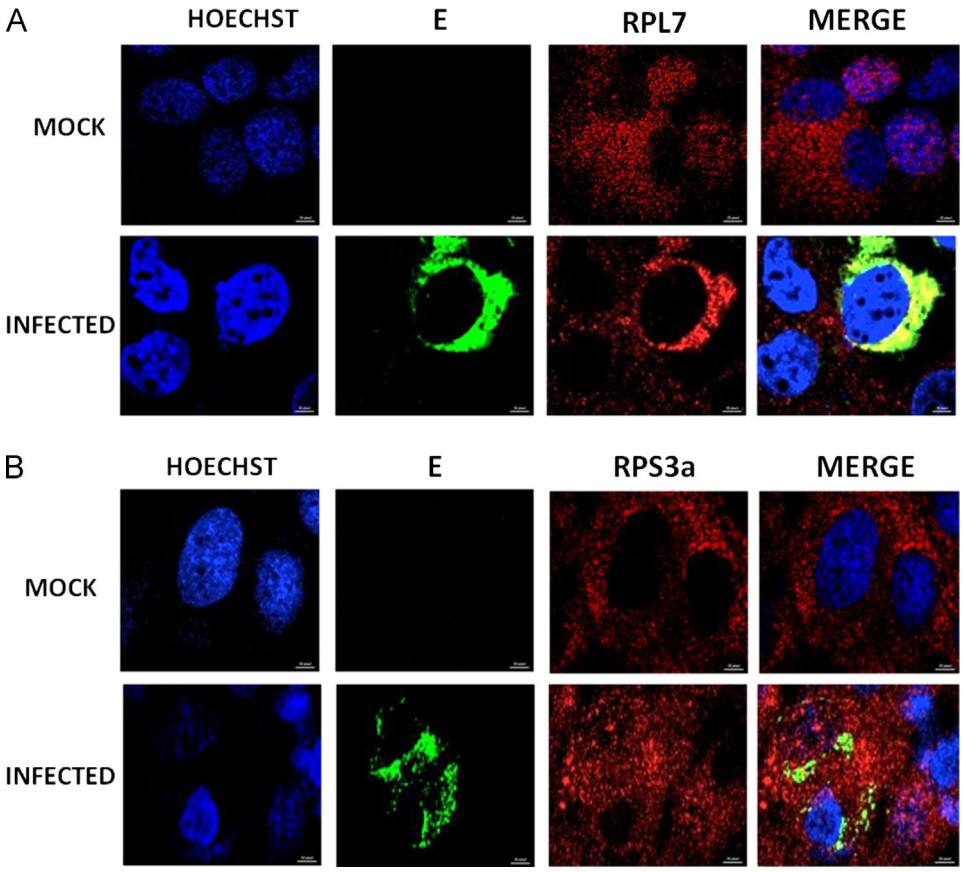

Fig. 6. Subcellular localization of RPL7, and RPS3a proteins in DENV infected cells. Huh-7 cells infected with DENV2 for 48 h were fixed and stained with anti-E (green) and anti-RPL7 (A) and anti-RPS3a (B) (red) antibodies. Nuclei were counterstained with Hoescht (blue). Proteins location was analyzed by confocal microscopy. The images are representatives of three independent experiments.

significant reduction in the presence of the viral proteins E (green) and NS1 (orange) was observed by confocal microscopy in cells transfected with RPL18 siRNA and analyzed at 24 hpi in comparison with cells transfected with the unrelated siRNA (Fig. 8C). This reduction was confirmed by Western-blot when a reduction in the expression of NS3 and NS5 was detected in RPL18 knockdown cells compared with cells transfected with the unrelated siRNA (Fig. 8D).

In addition to the reduction in viral protein expression after RPL18 silencing, one log reduction in the levels of viral RNA was also distinguished by qRT-PCR in cells transfected with 150 nM of RPL18 protein siRNA and harvested at 48 hpi compared with the amount of viral RNA present in cells transfected with the unrelated siRNA (Fig. 8E), confirming that the RPL18 is involved in DENV replicative cycle.

To further corroborate the role of RPL18, in DENV translation/replication a Vero-derived cell line, stably transfected with DENV4 encoding luciferase reporter replicon (kindly donated by Dr Padmanabhan, Georgetown University Washington DC), was transfected with 100 and 150 nM of RPL18 siRNA and with 150 nM of the unrelated siRNA. At 24 and 48 h post transfection luciferase activity was measured. At 24 h, a concentration dependent decrease, from 0.5 to 1 log, in the luciferase activity was observed in the cells transfected with 100 and 150 nM respectively of the RPL18 siRNA, compared with cells transfected with 150 nM of the unrelated siRNA (Fig. 9B). At 48 h post transfection a more dramatic effect was observed because more than 1.0 log reduction in the luciferase activity was observed in the cells transfected at both concentrations of the RPL18 siRNA compared with cells transfected with the unrelated siRNA (Fig. 9B). None of the concentrations of siRNA cause a reduction in the Vero-derived cell viability (Fig. 9A), confirming that the RPL18 is involved in viral translation/replication of DENV.

Discussion

NS1 protein of DENV is a multifunctional nonstructural protein that can be found in infected cells as a monomer, dimer and hexamer located in the cytoplasm, on the cell membrane and secreted (Gutsche et al., 2011; Lindenbach and Rice, 1999; Mackenzie et al., 1996; Winkler et al., 1988). Although the presence of NS1 in the replicative complexes in the endoplasmic reticulum point to that this protein plays an important role in viral replication (Lindenbach and Rice, 1999; Mackenzie et al., 1996), it has also involved the pathogenesis of DENV (Avirutnan et al., 2006; Cheng et al., 2009; de la Cruz-Hernandez et al., 2013). Moreover, the presence of NS1 in patients´ sera provides a rapid diagnostic marker for DENV infection, correlating the levels of this protein in the sera with the risk to develop DHF (de la Cruz-Hernandez et al., 2013).

Given the importance of NS1 in DENV replicative cycle and pathogenesis, several groups have tried to identify cell proteins that interact with this protein in the patients' sera as well as in infected cell.

To isolate cellular proteins that interact with viral proteins different methodological strategies have been used. Affinity chromatography allowed the identification of clusterin (Clu) that interacts with the recombinant NS1 from DENV. Clu is an inhibitor of the complement system and it is possible that the interaction

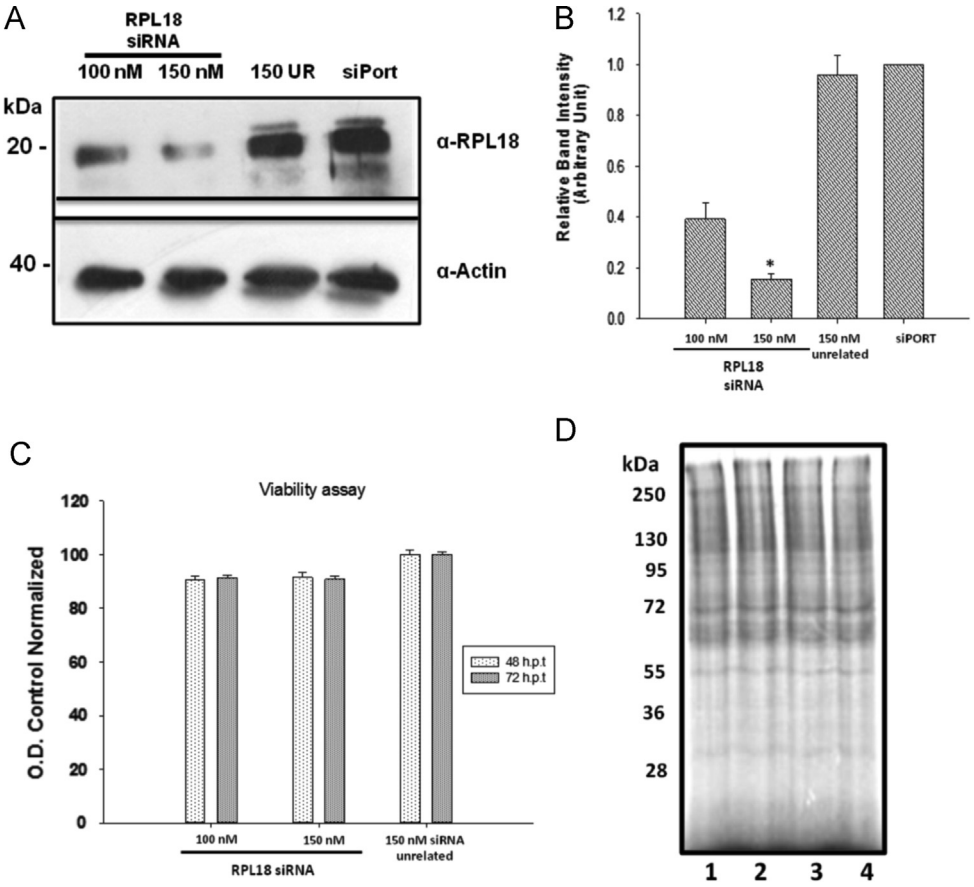

Fig. 7. Use of siRNA to knock down the expresion of RPL18 in Huh-7 cells. Huh-7 cells were transfected with 100 or 150 nM of RPL18 siRNA, with 150 nM of an unrelated siRNA and with the transfection reactive siPORT were analyzed. At 48 h post transfection, total cell extracts from transfected cells were prepared and analyzed by Western-blot using anti-RPL18 antibodies. Anti-actin antibodies were used as loading control. Densitometric analysis from three independent Western-blot assays was performed (B). $^{*}p < 0.05$. Cell viability assay (C) and Methionine S^{35} incorporation into cellular proteins were performed after 48 (lanes 1 and 2) and 72 h (lane 3 and 4) post transfection with unrelated (Lane 2 and 4) or RPL-18 siRNA (lanes 1 and 3) (D). Results are representatives of three independent experiments.

between NS1 and Clu may be involved in plasma leakage (Kurosu et al., 2007).

In this work, 64 proteins that interact directly or indirectly with NS1 were identified using two different strategies, affinity chromatography and immunoprecipitation assays. Among the proteins identified, we found interactions with proteins that were previously identified as molecules that interact with NS1, such as the translation elongation factor 1-alpha-1 (eEF1A1) that was identified by double hybrid system (Silva et al., 2013). This protein was also found bound to the 3′UTR of DENV4 (De Nova-Ocampo et al., 2002). However, the functional role of eEF1A1 in the replication cycle of DENV is unknown. We also identify the 60S ribosomal protein L24 (RPL24) and the Heat shock protein HSP 90-beta (HSP90) that were previously described as NS3 interacting proteins by double hybrid (Le Breton et al., 2011; Silva et al., 2013). The NS3 protein has two functional domains, protease and helicase and it is an important component of the replicative complex of DENV. Thus, since NS1 and NS3 are both interacting in DENV replicative complex it is likely that the proteins identified in our study may be interacting with both viral proteins or that they were isolated because they interact directly with NS3 and indirectly with NS1. The HSP90 was also previously isolated by its affinity to the E protein of DENV and it was described as DENV receptor in the monocytic cell line U937 and in neuroblastoma cells (Reyes-Del Valle et al., 2005).

Interestingly, we also identified 3 proteins previously reported as NS5 interacting protein. One of them was the 26S protease regulatory subunit 4 (PSMC1) (Mairiang et al., 2013) and the other two are vimentin and the heterogeneous nuclear ribonucleoprotein F (hnRPF) protein (Le Breton et al., 2011). Vimentin, which has the highest Mascot score in our proteomic analysis, has also been shown to interact with NS1 and NS4A (Kanlaya et al., 2010; Teo and Chu, 2014). Furthermore, DENV replication seems to modulate vimentin phosphorylation in a specific way by altering the phosphorylation levels, which regulate vimentin reorganization (Teo and Chu, 2014). Interestingly, vimentin silencing induced a significant alteration in the distribution of RCs in DENV-infected cells (Teo and Chu, 2014) suggesting that this protein plays a crucial role in localizing and concentrating DENV RCs at the perinuclear site.

In the case of hnRPs, it has been reported that NS1 also interacts with two members of the heterogeneous nuclear ribonucleoprotein family, hnRNP C1/C2 and hnRNP K (Kanlaya et al., 2010). The hnRNP C1 and C2 proteins (41 and 43 kDa) are involved in mRNA biogenesis and contain important conserved motifs essential for RNA binding, protein–protein interaction and nuclear localization. A partial co-localization of the hnRNP C1/C2 and NS1 was observed in perinuclear regions of DENV infected cells (Lindenbach and Rice, 1997) and the presence of DENV RNA in the immunoprecipitated complex containing hnRNP C1/C2 proteins has been demonstrated, supporting the idea that hnRNP C1/C2 is involved in DENV replication (Dechtawewat et al., 2015).

On the other hand, it has been described that DENV-2 infection promotes the cytoplasmic translocation of hnRNP K to favor viral multiplication, suggesting that this protein is a host factor required for DENV replication (Brunetti et al., 2015). Recently, it has been

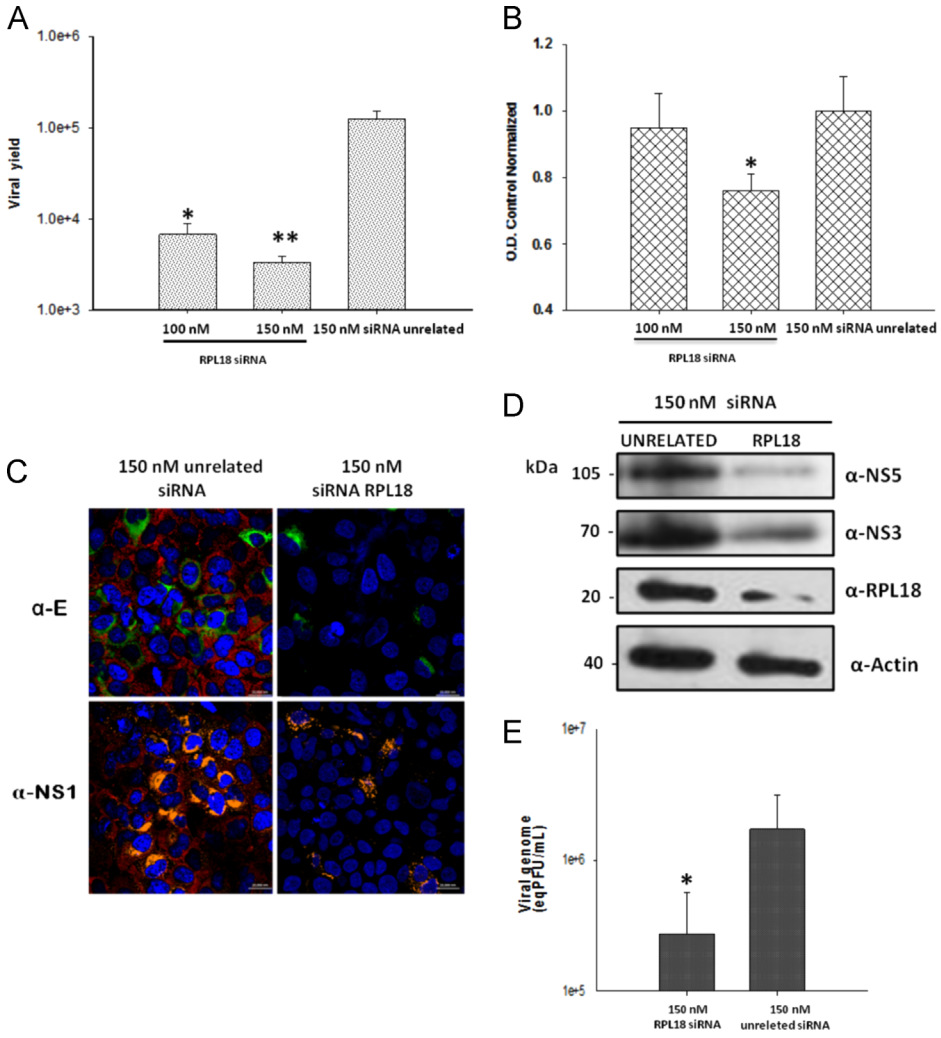

Fig. 8. RPL18 silencing inhibits DENV infection. At 48 h post transfection of Huh-7 cells with 100 or 150 nM of RPL18 siRNA or with 150 nM of an unrelated siRNA were infected with DENV2 at a MOI of 3. Supernatants of Huh-7 cells transfected and infected were analyzed for viral yield (A) by plaque assay and NS1, secretion (B) by ELISA. Viral yield is expressed as mean ± SEM of PFU/mL of three independent experiments. The NS1 secretion is expressed as mean of normalized absorbance ± SEM. $^{*}p < 0.05$. The presence of the two viral proteins NS1 (orange) and E (Green) and the cellular protein RPL18 (red) from transfected and infected cells was analyzed by confocal microscopy (C). The amount of viral proteins NS3 and NS5 and the cellular protein RPL18 was assayed by Western blot (24 and 48 h of treatment) (D). Viral genome was quantified by qRT-PVR and it is expressed as mean ± SEM of eqPFU/mL of three independent experiments. $^{*}p < 0.05$.

demonstrated that hnRNPC1/C2 and K interact with vimentin and with NS1, and that this complex plays a crucial role in replication and release of DENV from the cell (Kanlaya et al., 2010).

Additionally, we found as NS1 interacting proteins the histones H2B and H4. These histones were found interacting with the C protein of DENV in the nucleus of infected cells (Colpitts et al., 2011). The presence of C and NS1 in nucleus has been reported (Bhuvanakantham et al., 2009; Colpitts et al., 2011; Poungsawai et al., 2011). In any case, the role of the interaction between viral proteins with histones is uncertain.

It is possible that during DENV replicative cycle, NS1 interacts with host cellular proteins to facilitate its proper folding, trafficking and/or to promote favorable environment for virus production in the host cell. One of these proteins is STAT3β. It has been described that NS1 is able to interact with the N-terminal region of the STAT3β protein during DENV 2 infection. This interaction may modulate the activity of this protein (Chua et al., 2005).

Since an important group of NS1 interacting proteins identified in this study, were ribosomal proteins, we decide to analyze if DENV infection could be modifying the expression or location of some NS1 interacting ribosomal proteins as well as the GAPDH protein. All of these proteins are abundant cell proteins. The expression of the RPL18, RPL18a, PRL7, RPS3a and GAPDH was not altered during DENV infection. However, redistribution of GAPDH and relocation to the perinuclear region of the RPL18 and RPL7 proteins was observed in infected cells. The interaction between NS1 with two of the ribosomal protein RPL18 and RPL18a as well as with the cellular enzyme GADPH was confirmed by co-immunoprecipitation. Thus, although we found that the expression of 5 of the NS1-interacting proteins was not altered during DENV infection, 3 of them, GAPDH, RPL18 and RPL7 were relocated in infected cells. Additionally, we found that they interact directly or indirectly with NS1 because they were identified with specific antibodies in the fraction immunoprecipitated with anti-NS1 antibodies.

GAPDH is an important metabolic enzyme that binds to the 3′ end of the negative and positive strands of the Japanese Encephalitis virus (JEV) RNA (Yang et al., 2009). This protein colocalized with NS5 in the replicative complexes (Yang et al., 2009). Moreover, it has been described that GAPDH is incorporated into HIV-1 virions (HIV-1) downregulating viral infection in lymphocytes and macrophages. Additionally GAPDH interacts directly with Pr55gag

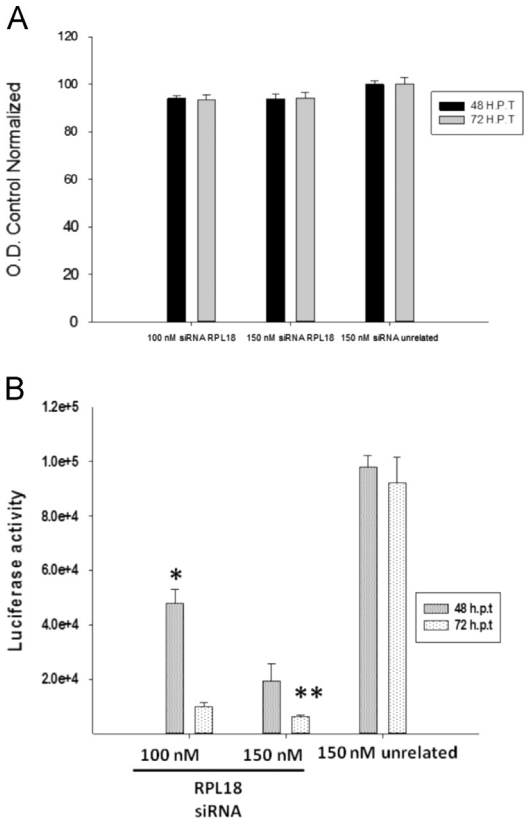

Fig. 9. LPL18 silencing inhibits DENV translation/replication. Vero cells, expressing DENV 4 replicon encoding luciferase gene, were transfected with100 or 150 nM of RPL18 siRNA or with 150 nM of an unrelated siRNA. At 48 and 72 post transfection, (A) Cell viability assay (B) Vero cells were lysed and luciferase activity was measured. Luciferase activity is expressed as relative luciferase units (RLU) and the data is mean ± SEM of three independent experiments. $^{*}p < 0.05$.

and p160gag-pol, however, the biological significance of this interaction is unclear (Kishimoto et al., 2012). Further studies directed to analyze the specific role of GAPDH during DENV infection are currently performed in our laboratory.

The ribosomal proteins RPL18, RPL18a and RPL7 are part of the large ribosomal subunit. It has been described that the three ribosomal proteins have extraribosomal functions. For example, RPL18 plays an important role in translation of some viruses such as the cauliflower mosaic virus (Leh et al., 2000), while the RPL18A protein interacts with the internal ribosomal entry site (IRES) of Hepatitis C virus (Dhar et al., 2006), a member of the *Flavividdae* family. Additionally, the RPL7 has been involved in the anchoring of the ribosome to the endoplasmic reticulum membranes (Wu et al., 2007). Additionally, since RPL7 contains multifaceted basic amino acid clusters present in the $NH_{(2)}$-region of the protein it can interacts with importin β3 which is essential for the nuclear import of RPL7 (Chou et al., 2010).

Since the RPL18 was clearly relocated to the perinuclear region, we decided to further analyze the role of this protein during DENV replicative cycle. Given the abundance of the ribosomal proteins, to silence the expression of the RPL18 protein, two transfections in tandem with RPL18 siRNA were performed in Huh-7 cells. Twenty-four hours after the second transfection, cells were infected with DENV and four different parameters were evaluated: viral yield, NS1 secretion, viral protein synthesis and amount of viral RNA. Silencing of RPL18 induced a significant reduction in viral yield, viral protein expression and RNA synthesis in infected cells. The direct effect of the silencing of RPL18 in DENV translation/replication was confirmed by the reduction in the luciferase activity in a stably transfected cell line with DENV4 encoding luciferase reporter replicon. Thus, the RPL18 may play a role in viral infection and it may be required for viral translation/replication. Specifically, it has been described that RPL18 plays an important role in translation of some viruses such as the cauliflower mosaic virus (Leh et al., 2000). Thus, it is possible that DENV could use a group of ribosomal proteins to favor its own translation as has been described for other mRNAs. For example it has been described that VSV mRNA translation depends specifically on the 60S ribosomal protein, RPL40. Lee et al. (2013) demonstrate that rpL40 is not essential for bulk cellular or cap-independent translation but is necessary for replication of VSV and other viruses within the order *Mononegavirales*. Additionally, the RPS25 directly interacts with the Dicistroviridae IRES to facilitate its translation (Nishiyama et al., 2007).

Another possibility to explain the participation of ribosomal protein in DENV translation/replication could be in anchoring the ribosomes that translate DENV mRNA to the endoplasmic reticulum such as the RPL7 (Wu et al., 2007). Since many of the DENV proteins are reticulum transmembrane proteins, viral genome has to be translated in the endoplasmic reticulum. In this process participates ribosomal proteins as well as trans-acting factors, such as the mRNA receptor p180, and cis-acting elements, such as transmembrane domain coding regions, that are responsible for this alternative mRNA localization process (Jagannathan et al., 2014). Thus, NS1 maybe a key scaffold protein necessary to bring together cellular proteins required for efficient DENV replication. Another option is that NS1 and other transmembrane viral proteins interact with ribosomal proteins during translocation through translocon after their translation in the endoplasmic reticulum. Further studies directed to understand the role of certain ribosomal proteins in anchoring and translocation of viral proteins are required.

Material and methods

Cell culture and virus propagation

Huh-7 cells, a differentiated hepatocyte derived cellular carcinoma cell line, (a gift from Dr. Ana Maria Rivas, Autonomous University of Nuevo León, México), were grown in advanced DMEM supplemented with 2 mM glutamine, penicillin (5×10^4 U/mL)-streptomycin (50 μg/mL), 5% fetal calf serum (FCS), 1 mL/L of amphotericin B (Fungizone) at 37 °C and a 5% CO_2 atmosphere. Propagation of DENV serotype 2 16681 strain, was carried out in CD1 suckling mice brains and titers were determined by plaque assays in BHK-21 cells as was previously described (Mosso et al., 2008). CD1 suckling mice brains lysates from mock-infected mice were used as control. Huh-7 cells were infected with DENV 2 at a multiplicity of infection of 3 for 24 or 48 h.

NS1 purification

The recombinant plasmid p-ProEx-NS1, containing the complete nucleotide sequence of the NS1 protein (2422–3477 nt) was used to transform competent *Escherichia coli* (BL21* strain) and positive clones were selected in an Luria broth (LB)-agar medium containing 100 μg/mL ampicillin at 37 °C overnight. Selected colonies were grown in 10 mL of LB medium containing 100 μg/mL ampicillin at 37 °C overnight. The culture was diluted to 1:10 in 1 L of fresh LB-ampicillin media and grown again until an optical density ($O.D._{600\ nm}$) of 0.6 to 0.8 was reached. Then, isopropyl-thiogalactopyranoside (IPTG, Invitrogen) was added to a final concentration of 0.5 mM for 18 h to induce protein expression. Cells were harvested by centrifugation at 8500 rpm for 20 min at 4 °C. To prepare a total-cell lysate the pellet was frozen (−20 °C) for 24 h and then resuspended in 5 mL of lysis buffer (50 mM TRIS

pH 8.0, 300 mM NaCl, 10% glycerol, 0.1% Triton X-100, 1 mM PMSF and lysozyme), and incubated for 1 h at 4 °C. The total-cell lysate was sonicated for 3 min using 10 s in on/10 s in off at 80% amplitude. The mix was centrifuged for 30 min at 6000 rpm and the supernatant saved. The purification of the His-tag NS1 protein was carried out by affinity chromatography. Briefly, 500 μL of resin (TALON Metal Affinity Resin, Clontech) were equilibrated with 5 volumes of a lysis buffer. Afterwards, resin was mixed by gently shaking with 3 mg/mL of preabsobed lysate of induced bacteria, and incubated overnight at 4 °C. The resin was pelleted by centrifugation for 5 min at 800 rpm and then washed four times with buffer A (50 mM TRIS pH 8.0, 500 mM NaCl, 0.1% Triton X-100, 10% of glycerol and 10% of ethanol) and five times with buffer B (imidazole 100–350 mM in Buffer A). All fractions were collected, analyzed by 10% SDS-PAGE and subjected to Western blot analysis. As a negative control, the same volume of resin was incubated with extracts from bacteria transfected with pProEx plasmid without NS1 insert.

Preparation of cytoplasmic protein extracts

Huh-7 cells grown on petri dish (7×10^6 cells/plate) were infected with DENV-2 at MOI of 3 for 24 h at 37 °C. Then, cells were washed twice with 2 mL of PBS and 1 mL of detaching buffer (40 mM TRIS HCl pH 7.5, 2 mM EDTA, 150 mM NaCl) was added per plate. Cells were harvested, centrifuged at 700 rpm for 10 min at 4 °C and the pellet was washed twice with 1 mL of buffer C (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl) and resuspended in 1 volume (100 or 200 μL) of buffer C in the presence of 20 μL of free protease inhibitor cocktail containing EDTA, EGTA and DTT. Cells were lysed in a homogenizer at 4 °C. Nuclei and debris were removed by centrifugation at 10,000 rpm for 30 min at 4 °C. Finally, cell extract was untreated or treated with 300 units of micrococcal nuclease (per 100 μL of total extract) in the presence of 5 mM of $CaCl_2$ at room temperature for 15 min.

Affinity chromatography with NS1

A total of 3 mg/mL of cytoplasmic cell extracts were preabsorbed by incubation with 200 μL of TALON resin (in the absence of NS1 recombinant protein) previously equilibrated with lysis buffer for 3 h in gentle shaking. Later, preabsorbed extracts were recovered by centrifugation at 3000 rpm for 3 min at 4 °C. Next, 500 μL of the resin coupled with recombinant NS1 protein or 500 μL of the control resin (described before), were incubated with preabsorbed cell extracts diluted in 5 volumes of interaction buffer (20 mM Tris base, NaCl 200 mM and 20 mM imidazole), overnight at 4 °C with gentle shaking. The resins were recovered by centrifugation and subjected to 8 washes with 5 volumes of washing buffer containing increasing concentrations of imidazole and NaCl in buffer A (6 mM imidazole and 100 mM NaCl; 7.5 mM imidazole and 150 mM NaCl; 7.5 mM imidazole and 200 mM; NaCl 7.5 mM imidazole 250 mM NaCl; 7.5 mM imidazole and 300 mM NaCl). Finally, the proteins bound to recombinant NS1 or the control resin were eluted with 500 mM and 1 M of NaCl and precipitated overnight with acetone at −20 °C. The pellets were resuspended in PBS pH 7.4 and the proteins were separated by SDS-PAGE for 20 min at 80 volts. The gels were washed 3 times for 5 min with milliQ water and fixed with F solution (40% methanol and 10% acetic acid) for 30 min. Proteins were stained with 50 mL Coomassie blue (blue R-250, Bio-Rad) for 30 min at room temperature, cut and sent to be analyzed by MALDI-TOF in the Protein Core Lab Facility at Columbia University in New York, NY.

Immunoprecipitation assay

Huh-7 cells were infected with DENV 2 at a MOI of 3 and harvested 48 h post infection. A total of 2 mg/mL of cytoplasmic extract were preabsorbed with 50 μL of protein G agarose (Roche) for 4 h at 4 °C. The lysate was recovered by centrifugation at 1000 rpm for 3 min. Anti-NS1 and a rabbit control IgG antibody were coupled to 100 μL of beads by crosslinking with DCG (disuccinimidyl glutarate, Thermo Scientific). The protein G agarose coupled with antibody was blocked in 200 μL of blocking buffer (1 mg/mL BSA in PBS), for 60 min at 4 °C. Then, beads were incubated with the preabsorbed cell lysate. The interaction was permitted overnight at 4 °C gentle shaking. The immunocomplexes were recovered by centrifugation at 1000 rpm for 3 min and the beads were washed five times with 400 μL of IP buffer with proteases inhibitor (50 mM TRIS pH 8.0, 1% NP40, 150 mM NaCl, 10 mM EDTA). Finally, proteins were eluted at 1 M of NaCl and precipitated with acetone overnight at −20 °C. The pellet was resuspended in 50 mM of Tris pH 7.4, analyzed by electrophoresis in SDS-PAGE and stained with Coomassie brilliant blue R-250.

The immunoprecipitated proteins were cut and analyzed by MALDI-TOF in the Protein Core Lab Facility at Columbia University in New York, NY.

Classification and protein network analysis

To investigate the biological process, subcellular localization and signaling pathway, associated with each identified protein, information from Swiss-Prot/TrEMBL database and DAVID Bioinformatics Resources (http://david.abcc.ncifcrf.gov/) were used. The proteins were also searched through REACTOME pathway database (http://www.reactome.org).

STRING network analysis of protein–protein interactions was performed to identify functionally linked proteins. The network is presented under confidence view, whereby stronger associations are represented by thicker lines or edges and vice versa, whereas proteins are represented as nodes.

Western blot assay

Huh-7 cells were infected with DENV 2 at a MOI of 3 and harvested at 6, 24 and 48 hpi. Cell extracts were prepared in a lysis buffer in the presence of protease inhibitor cocktail (Roche). A total of 35 μg/mL of cytoplasmic extract or inmunoprecipitated proteins which had been heated or not at 95 °C for 10 min in the presence of 3% β-mercaptoethanol, were separated by electrophoresis in 10% SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad). Membranes were blocked with 10% of non-fat milk in 0.5% PBS-Triton X-100. Cell proteins were detected by using an anti-GAPDH rabbit monoclonal antibody (1:10,000, Cell signaling), and rabbit polyclonal antibodies directed against RPL18a (1:4000, GeneTex), RPL18 (1:5000, AbCam), RPL7 and RPS3a (1:12,500 and 1:10,000, AbCam). The DENV NS1 and NS3 proteins were detected by using rabbit polyclonal antibodies (1:5000 and 1:1000, GeneTex) respectively. An anti-rabbit HRP antibody (1:10,000, Cell Signaling) was used as secondary antibody in all cases. The proteins were visualized by Super Signal West Femto Chemiluminescent Substrate (Thermo scientific).

Immunofluorescence analysis

Huh-7 cells grown on slides were transfected with the different siRNAs or/and infected with DENV-2 at a MOI of 3 and fixed at 24 or 48 hpi with 3% paraformaldehyde (PFA) for 20 min at room temperature. Later, cells were treated with permeabilizing solution (serum 1%, saponin 2 mg/mL in PBS) for 20 min at room

temperature. The cells were probed with 1 μg/mL of mouse anti-E protein antibody (4G2), or with mouse anti-NS1 and rabbit anti-NS3 antibodies and with rabbit antibodies directed against the cellular proteins GAPDH, RPL18, RPL7 and RPS3a in permeabilizing solution for 2 h at room temperature. Next, cells were incubated with 2 μg/mL of AlexaFluor 488-conjugated donkey anti-mouse IgG and AlexaFluor 555-conjugated goat anti-rabbit IgG. Nuclei were stained with Hoechst (Santa Cruz). Slides were observed in a Zeiss LSM700 laser confocal microscope.

siRNA transfection of Huh-7 cells

To knock down the expression of the RPL18 protein, Huh-7 and Vero R4 cells were transfected with two different concentrations (100 and 150 nM) of a specific RPL18 siRNAs (SC-97249 Santa Cruz). An unrelated siRNA was used as a control (SC-37007 Santa Cruz). Briefly, Huh-7 cells were grown in advanced DMEM supplemented with 10% fetal bovine serum (FBS), antibiotic free at 37 °C and a 5% CO_2 atmosphere until 70% confluence. Then, two mixtures were prepared: (a) 100 or 150 nM of siRNA diluted in 100 μL of transfection medium (opti-MEM, Life Technologies) and (b) 1.5 μL of siPORT (Applied Biosystems) in 100 μL of transfection medium. Mixtures were incubated 10 min at 37 °C. Later, the two mixtures were mixed by pipetting and incubated for 10 min more. Next 200 μL of siRNA transfection medium was added to each tube and total volume was added to the cells. Cells were incubated 8 h at 37 °C in a CO_2 incubator. Then, 200 μL of free medium containing 15% of fetal bovine serum was added. At 24 h post-transfection, cells were retransfected under the same conditions and 24 h after the second transfection, cells were infected with DENV-2 at a MOI of 3 for 24 and 48 h.

Viral yield and levels of secreted NS1 protein were determined from supernatants of transfected and infected cells by plaque assay (Mosso et al., 2008) and ELISA (Bio-Rad, Platelia) (Ludert et al., 2008), respectively.

Cell viability assay and viral yields

Cell viability of Huh-7 cells and Vero cells was evaluated by the MTS reduction used according with the manufacturer´s protocol (Cell Titer 96 Aqueous One Solution Cell Proliferation Assay, Promega). Supernatants of transfected and/or infected cells were analyzed for viral yield by plaque assay as previously described (Mosso et al., 2008).

Metabolic labeling with [^{35}S]-methionine

Huh-7 cells grown at confluence of 70% (6×10^5 cells) and transfected with RPL18 siRNA and with an unrelated siRNA were washed twice with 10 mL of pulse-labeling medium. Later, cells were incubated with 500 μL of prewarmed pulse-labeling medium for 25 min at 37 °C, to deplete intracellular pools of methionine. Next, the pulse-labeling medium was removed and replaced by 500 μL of methionine-free medium containing 7 μCi/mL of [^{35}S]-methionine (PerkinElmer) for 30 min at 37 °C. Finally, the medium was removed and the cells were washed once with ice-cold PBS. Total cell proteins were analyzed by an 8% SDS-PAGE, stained with Coomassie blue (Bio-Safe, Bio-Rad) and visualized by autoradiography.

Silver staining

Ten μg of protein extract from cells transfected with unrelated or with RPL18 siRNAs were separated by SDS-PAGE, washed once with milliQ water for 2 min, fixed with 30 mL of 50% methanol and 5% acetic acid for 30 min, and washed once with 20 mL of 50% methanol for 15 min. Next, gel was washed with milliQ water for 1 min twice, sensitized with 0.01% $Na_2S_sO_3$ for 1 min and was washed two times with milliQ water. Then, gel was stained with 30 mL of 0.1% $AgNO_3$ for 20 min at 4 °C with gently agitation. Next, gel was washed twice with milliQ water for 1 min, revealed with 2% sodium carbonate in 0.04% formaline and fixed with 5% acetic acid.

Luciferase activity and real time PCR analysis

Luciferase activity of transfected Vero 4R cells expressing a DENV 4 replicon with RPL18 and unrelated siRNA was measured as indicated in the kit protocol (Renilla luciferase assay system, Promega). To determine viral genome levels, total RNA was obtained using the kit Zymo Research Quick-RNA (MicroPrep). The qRT-PCR was conducted by the SYBR green method using the primers of NS5 reported by Chien et al. (2006) in the ECO ILLUMINA system. The amount of viral RNA transcripts was calculated by generating a standard curve from 10-fold dilutions of RNA isolated from a DENV-4 and DENV-2 preparation titrated in BHK-21 cells and expressed as plaque forming units equivalents per milliliter (PFU equivalents/mL) (Callahan et al., 2001; Chien et al., 2006; Johnson et al., 2005).

Statistical analysis

Differences between the diverse treatments and control groups were evaluated using the statistical program Sigma-Plot 11 in all cases. One tail analysis of variance (ANOVA) was used. For all tests used a $p \leq 0.05$ was considered statistically significant.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2015.05.017.

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