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Understanding the Biological Context of NS5A–Host Interactions in HCV Infection: A Network-Based Approach

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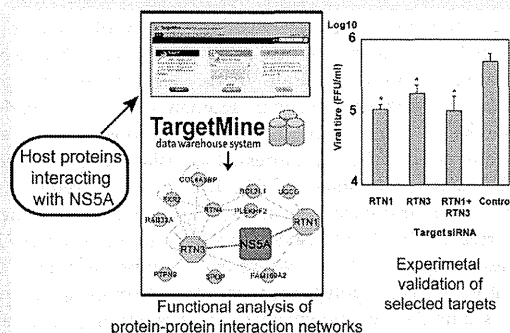
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Supporting Information

ABSTRACT: Hepatitis C virus (HCV) is a major cause of chronic liver disease. HCV NS5A protein plays an important role in HCV infection through its interactions with other HCV proteins and host factors. In an attempt to further our understanding of the biological context of protein interactions between NS5A and host factors in HCV pathogenesis, we generated an extensive physical interaction map between NS5A and cellular factors. By combining a yeast two-hybrid assay with comprehensive literature mining, we built the NS5A interactome composed of 132 human proteins that interact with NS5A. These interactions were integrated into a high-confidence human protein interactome (HPI) with the help of the TargetMine data warehouse system to infer an overall protein interaction map linking NS5A with the components of the host cellular networks. The NS5A–host interactions that were integrated with the HPI were shown to participate in compact and well-connected cellular networks. Functional analysis of the NS5A “infection” network using TargetMine highlighted cellular pathways associated with immune system, cellular signaling, cell adhesion, cellular growth and death among others, which were significantly targeted by NS5A–host interactions. In addition, cellular assays with *in vitro* HCV cell culture systems identified two ER-localized host proteins RTN1 and RTN3 as novel regulators of HCV propagation. Our analysis builds upon the present understanding of the role of NS5A protein in HCV pathogenesis and provides potential targets for more effective anti-HCV therapeutic intervention.

KEYWORDS: HCV, NS5A, host–pathogen protein–protein interactions, biological network analysis, literature mining, pathway enrichment analysis, siRNA knockdown, target discovery, TargetMine, yeast two-hybrid



INTRODUCTION

Hepatitis C virus (HCV) causes chronic liver disease including liver steatosis, cirrhosis and hepatocellular carcinoma (HCC) and infects nearly 3% of the world population. HCV possesses a single-stranded RNA genome encoding a 3000 amino acid polyprotein, which is processed by host and viral proteases to yield 10 viral proteins, Core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B.^{1–5} HCV variants are classified into seven genotypes that display phylogenetic heterogeneity, differences in infectivity and interferon sensitivity.^{6,7} However, despite considerable research, a precise understanding of the molecular mechanisms underlying HCV pathology remains elusive.

HCV NS5A protein (hereafter referred to as NS5A) is a RNA binding phosphoprotein, which consists of three domains; domain I includes a zinc-finger motif necessary for HCV replication and an N-terminal membrane anchor region, and the unstructured domains II and III facilitate protein–protein

interactions. NS5A plays a critical role in regulating viral replication, production of infectious viral particles, interferon resistance and modulation of apoptosis in HCV pathogenesis via interactions with other HCV proteins and host factors.^{8–12} Furthermore, BMS-790052, a small molecule inhibitor of NS5A, is the most potent inhibitor of HCV infection known so far.¹³ Consequently, NS5A has emerged as a unique, attractive and promising target for anti-HCV therapy.^{14–19} In particular, impairing interactions between NS5A and host factors has been shown to impede HCV infection, which may offer novel anti-HCV therapeutic approaches.^{12,20} However, the overall structure and precise functions of NS5A in HCV pathogenesis are poorly understood.

Pathogens such as viruses infect their hosts by interacting with the components of the host cellular networks and

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exploiting the cellular machinery for their survival and propagation. Therefore, elucidating host–pathogen interactions is crucial for a better understanding of pathogenesis.^{21–26} Here, we report the host biological processes likely to be influenced by NSSA by virtue of an inferred protein–protein interaction (PPI) network. We describe our integrated approach that combines an experimental yeast two-hybrid (Y2H) assay using NSSA as bait to screen a library of human cDNAs with comprehensive literature mining. The analysis of the NSSA infection network illustrates the functional pathways likely to be influenced by NSSA–host interactions in HCV pathogenesis, thus providing novel insights into the NSSA function in HCV pathogenesis. Furthermore, RTN1 and RTN3, which are endoplasmic reticulum (ER)-localized proteins involved in regulating ER integrity, will be demonstrated as novel regulators of HCV propagation and thus attractive targets for anti-HCV therapy.

MATERIALS AND METHODS

Yeast Two-Hybrid Protein Assay

Screening for the genes encoding host proteins that interact with NSSA was performed using the Matchmaker two-hybrid system (Clontech, Palo Alto, CA, USA) as per the manufacturers' specifications. Human adult liver libraries were purchased from Clontech and were cloned into the pAct2 vector (Clontech) and expressed as fusion proteins fused to the Gal4-activation domain (Gal4-AD). Since Y2H requires the bait protein to translocate to the nucleus, the cDNA of the region corresponding to the NSSA encoding amino acids 1973–2419 (excluding the NSSA N-terminal membrane anchor region) within the HCV polyprotein from the J1 strain (genotype 1b)²⁷ was amplified by polymerase chain reaction (PCR) and was cloned into the pGBKT7 vector (Clontech)²⁸ and expressed as Gal4-DNA binding domain (Gal4-DB) fusion in the AH109 yeast strain. The human liver libraries were subsequently screened by Y2H using NSSA as bait. A total of 4×10^6 transformants were screened in this manner, and the positive clones (see Supporting Information) were isolated and sequenced to identify the genes coding for the NSSA interacting host factors (Supporting Information, Table S1).

Literature Mining for Pairwise NSSA–Human Interactions

Literature information describing pairwise interactions between NSSA and cellular proteins were extracted from Medline using the PubMed interface and two other information retrieval and extraction tools, EBIMed²⁹ and Protein Corral. These tools employ an automatic text-mining approach, but we supplemented them with a follow-up manual inspection. All abstracts related to “NSSA” and “HCV NSSA” keywords and interaction verbs (including “interact”, “bind”, “attach”, “associate”)³⁰ were gathered and manually examined to retrieve direct pairwise NSSA–human protein interactions (see Supporting Information, Tables S2, S3, S4, S5a).

Construction of Extended Protein–Protein Interaction Networks

Physical and direct binary interactions between all human proteins were retrieved from BioGRID 3.1.93³¹ and iRefindex 9.0³² databases using TargetMine.³³ TargetMine is an integrated data warehouse that combines different types of biological data and employs an objective protocol to prioritise candidate genes for further experimental investigation.³³ The interactions were filtered for redundancy, potential false

positives and isolated components to infer a representative undirected and singly connected high-confidence human protein interactome (HPI) comprising 22 532 nonredundant binary physical interactions between 7277 proteins (see Supporting Information, Figure S2, Table S5b). The inferred HPI was used to identify biologically relevant trends, the significance of which was assessed by using randomized networks (see below). Secondary interactors of the NSSA interacting proteins were retrieved from the HPI and were appended to the NSSA–host interactions to construct a representative NSSA infection network (Supporting Information, Table S5a).

Topological Analysis

Network components were visualized using Cytoscape,^{34,35} while network properties such as *node degree distribution*, *average shortest path* and *betweenness* measures were computed using Cytoscape NetworkAnalyzer plugin³⁶ as described earlier.²⁴ For comparison, degree preserved randomized PPI networks were generated by edge rewiring using the Cytoscape RandomNetworks plugin and were used as control networks to assess the statistical significance of the topological trends observed in the inferred PPI networks (see Supporting Information).

Functional Analysis by Characterization of Enriched Biological Associations

Protein structural domain assignments were retrieved from the Gene3D database,³⁷ Gene ontology associations from the GO consortium,³⁸ and biological pathway data from KEGG³⁹ were used to assign functional annotations to the genes in the NSSA infection network. The enrichment of specific biological associations within the NSSA infection network was estimated by performing the hypergeometric test within TargetMine. The inferred *p*-values were further adjusted for multiple test correction to control the false discovery rate using the Benjamini and Hochberg procedure,^{40,41} and the annotations/pathways were considered significant if the adjusted *p* ≤ 0.005 .

RNAi and Transfection

A mixture of four siRNA targets each to RTN1 and RTN3 (SMARTpool:siGENOME RTN1 siRNA and SMARTpool:siGENOME RTN3 siRNA, respectively) were purchased from Thermo Scientific (Thermo Scientific, Waltham, MA, USA). siGENOME Non-Targeting siRNA Pool #1 (Thermo Scientific) was used as a control siRNA. Thermo Scientific ID numbers of siRNA mixtures of RTN1 and RTN3 and the control were M-014138-00, M-020088-00 and D-001206-13-05, respectively. Each siRNA mixture was introduced into the cell lines by using lipofectamine RNAiMax (Invitrogen, Carlsbad, CA, USA). The replicon cell line, as will be described below, was transfected with each siRNA at a final concentration of 20 nM as per the manufacturer's protocol and then seeded at 2.5×10^4 cells per well of a 24-well plate. The transfected cells were harvested at 72 h post-transfection. The Huh7OK1 cell line, as will be described below, was transfected with each siRNA at a final concentration of 20 nM as per the manufacturer's protocol and then seeded at 2.5×10^4 cells per well of a 24-well plate. The transfected cells were infected with JFH1 at an MOI of 0.05 at 24 h post-transfection. The resulting cells were harvested at the indicated time.

Table 1. List of 132 Human Proteins Interacting with the HCV NSSA Protein

gene ID	symbol	description	refs
47	ACLY	ATP citrate lyase	22
60	ACTB	actin, beta	101
79026	AHNAK	AHNAK nucleoprotein	22
10598	AHSA1	AHA1, activator of heat shock 90 kDa protein ATPase homologue 1 (yeast)	102
207	AKT1	v-akt murine thymoma viral oncogene homologue 1	22
302	ANXA2	annexin A2	103
335	APOA1	apolipoprotein A-I	22
348	APOE	apolipoprotein E	22
116985	ARAP1	ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 1	22
27236	ARFIP1	ADP-ribosylation factor interacting protein 1	22
23204	ARL6IP1	ADP-ribosylation factor-like 6 interacting protein 1	this study
4508	ATP6	ATP synthase F0 subunit 6	this study
8312	AXIN1	axin 1	22
581	BAX	BCL2-associated X protein	22
222389	BEND7	BEN domain containing 7	22
274	BIN1	bridging integrator 1	this study; ^{22,47}
89927	C16orf45	chromosome 16 open reading frame 45	this study
8618	CADPS	Ca ⁺⁺ -dependent secretion activator	22
93664	CADPS2	Ca ⁺⁺ -dependent secretion activator 2	22
79080	CCDC86	coiled-coil domain containing 86	22
983	CDK1	cyclin-dependent kinase 1	22
1021	CDK6	cyclin-dependent kinase 6	22
1060	CENPC1	centromere protein C 1	22
153241	CEP120	centrosomal protein 120 kDa	22
11190	CEP250	centrosomal protein 250 kDa	22
9702	CEP57	centrosomal protein 57 kDa	22
80254	CEP63	centrosomal protein 63 kDa	22
1381	CRABP1	cellular retinoic acid binding protein 1	22
1445	CSK	c-src tyrosine kinase	22
1452	CSNK1A1	casein kinase 1, alpha 1	104
1457	CSNK2A1	casein kinase 2, alpha 1 polypeptide	63,105
1499	CTNNB1	catenin (cadherin-associated protein), beta 1, 88 kDa	84,106
9093	DNAJA3	Dnaj (Hsp40) homologue, subfamily A, member 3	22
2202	EFEMP1	EGF containing fibulin-like extracellular matrix protein 1	22
5610	EIF2AK2	eukaryotic translation initiation factor 2-alpha kinase 2	22
2051	EPHB6	EPH receptor B6	this study
54942	FAM206A	family with sequence similarity 206, member A	22
25827	FBXL2	F-box and leucine-rich repeat protein 2	22
2274	FHL2	four and a half LIM domains 2	22
23770	FKBP8	FK506 binding protein 8, 38 kDa	this study; ^{43,45}
2316	FLNA	filamin A, alpha	12
2495	FBP1	ferritin, heavy polypeptide 1	22
8880	FUBP1	far upstream element (FUSE) binding protein 1	107
2534	FYN	FYN oncogene related to SRC, FGR, YES	22
11345	GABARAPL2	GABA(A) receptor-associated protein-like 2	this study
54826	GIN1	gypsy retrotransposon integrase 1	22
2801	GOLGA2	golgin A2	22
2874	GPS2	G protein pathway suppressor 2	22
2885	GRB2	growth factor receptor-bound protein 2	22
2931	GSK3A	glycogen synthase kinase 3 alpha	22
2932	GSK3B	glycogen synthase kinase 3 beta	22
3055	HCK	hemopoietic cell kinase	22
3320	HSP90AA1	heat shock protein 90 kDa alpha (cytosolic), class A member 1	22
3303	HSPA1A	heat shock 70 kDa protein 1A	108
3315	HSPB1	heat shock 27 kDa protein 1	109
3537	IGLC1	immunoglobulin lambda constant 1 (Mcg marker)	22
79711	IPO4	importin 4	22
3843	IPO5	importin 5	22
3683	ITGAL	integrin, alpha L (antigen CD11A (p180), lymphocyte function-associated antigen 1; alpha polypeptide)	22
6453	ITSN1	intersectin 1	this study
3716	JAK1	Janus kinase 1	22

Table 1. continued

gene ID	symbol	description	refs
3932	LCK	lymphocyte-specific protein tyrosine kinase	22
55679	LIMS2	LIM and senescent cell antigen-like domains 2	22
4067	LYN	v-yes-1 Yamaguchi sarcoma viral related oncogene homologue	22
9448	MAP4K4	mitogen-activated protein kinase kinase kinase kinase 4	this study
6300	MAPK12	mitogen-activated protein kinase 12	22
4155	MBP	myelin basic protein	22
4256	MGP	matrix Gla protein	110
55233	MOB1A	MOB kinase activator 1A	22
4673	NAP1L1	nucleosome assembly protein 1-like 1	22
4674	NAP1L2	nucleosome assembly protein 1-like 2	22
10397	NDRG1	N-myc downstream regulated 1	22
4778	NFE2	nuclear factor (erythroid-derived 2), 45 kDa	22
11188	NISCH	nischarin	this study
4924	NUCB1	nucleobindin 1	22
4938	OAS1	2'-5'-oligoadenylate synthetase 1, 40/46 kDa	22
5007	OSBP	oxysterol binding protein	111
64098	PARVG	parvin, gamma	22
5170	PDPK1	3-phosphoinositide dependent protein kinase-1	22
5297	PI4KA	phosphatidylinositol 4-kinase, catalytic, alpha	22
5291	PIK3CB	phosphoinositide-3-kinase, catalytic, beta polypeptide	22
5295	PIK3R1	phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	55,84,106
5300	PIN1	peptidylprolyl cis/trans isomerase, NIMA-interacting 1	112
5307	PITX1	paired-like homeodomain 1	22
5347	PLK1	polo-like kinase 1	113
10654	PMVK	phosphomevalonate kinase	22
5478	PPIA	peptidylprolyl isomerase A (cyclophilin A)	114,115
10848	PPP1R13L	protein phosphatase 1, regulatory subunit 13 like	22
5515	PPP2CA	protein phosphatase 2, catalytic subunit, alpha isozyme	116
5518	PPP2R1A	protein phosphatase 2, regulatory subunit A, alpha	116
5698	PSMB9	proteasome (prosome, macropain) subunit, beta type, 9 (large multifunctional peptidase 2)	22
5757	PTMA	prothymosin, alpha	22
5894	RAF1	v-raf-1 murine leukemia viral oncogene homologue 1	22
6142	RPL18A	ribosomal protein L18a	22
6167	RPL37	ribosomal protein L37	this study
6238	RRBP1	ribosome binding protein 1 homologue 180 kDa (dog)	22
91543	RSAD2	radical S-adenosyl methionine domain containing 2	117
6252	RTN1	reticulon 1	this study
10313	RTN3	reticulon 3	this study
6424	SFRP4	secreted frizzled-related protein 4	22
81858	SHARPIN	SHANK-associated RH domain interactor	22
64754	SMYD3	SET and MYND domain containing 3	22
8470	SORBS2	sorbin and SH3 domain containing 2	22
10174	SORBS3	sorbin and SH3 domain containing 3	22
6714	SRC	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homologue (avian)	22
10847	SRCAP	Snf2-related CREBBP activator protein	22
6741	SSB	Sjogren syndrome antigen B (autoantigen La)	22
284297	SSCS	scavenger receptor cysteine rich domain containing (5 domains)	110
6772	STAT1	signal transducer and activator of transcription 1	118
25777	SUN2	Sad1 and UNC84 domain containing 2	this study
6850	SYK	spleen tyrosine kinase	119
4070	TACSTD2	tumor-associated calcium signal transducer 2	22
6880	TAF9	TAF9 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 32 kDa	22
6908	TBP	TATA box binding protein	22
7046	TGFBR1	transforming growth factor, beta receptor 1	22
7057	THBS1	thrombospondin 1	22
374395	TMEM179B	transmembrane protein 179B	22
7110	TMF1	TATA element modulatory factor 1	22
7157	TP53	tumor protein p53	22
7159	TP53BP2	tumor protein p53 binding protein, 2	22
7186	TRAF2	TNF receptor-associated factor 2	22
11078	TRIOBP	TRIO and F-actin binding protein	22

Table 1. continued

gene ID	symbol	description	refs
51061	TXNDC11	thioredoxin domain containing 11	22
53347	UBASH3A	ubiquitin associated and SH3 domain containing A	22
10869	USP19	ubiquitin specific peptidase 19	22
9218	VAPA	VAMP (vesicle-associated membrane protein)-associated protein A, 33 kDa	22
9217	VAPB	VAMP (vesicle-associated membrane protein)-associated protein B and C	this study; ^{22,28,46}
10493	VAT1	vesicle amine transport protein 1 homologue (<i>T. californica</i>)	this study
55737	VPS35	vacuolar protein sorting 35 homologue (<i>S. cerevisiae</i>)	22
6293	VPS52	vacuolar protein sorting 52 homologue (<i>S. cerevisiae</i>)	22
140612	ZFP28	zinc finger protein 28 homologue (mouse)	this study
9726	ZNF646	zinc finger protein 646	22

Quantitative Reverse-Transcription PCR (qRT-PCR)

Total RNA was prepared from the cell and culture supernatant using the RNeasy mini kit (QIAGEN, Hilden, Germany) and QIAamp Viral RNA Mini Kit (QIAGEN), respectively. First-strand cDNA was synthesized using high capacity cDNA reverse transcription kit (Applied biosystems, Carlsbad, CA, USA) with random primers. Each cDNA was estimated by Platinum SYBR Green qPCR Super Mix UDG (Invitrogen) as per the manufacturer's protocol. Fluorescent signals of SYBR Green were analyzed with ABI PRISM 7000 (Applied Biosystems). The HCV internal ribosomal entry site (IRES) region and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene were amplified with the primer pairs 5'-GAGTGTCTGTCGAGCCCTCCA-3' and 5'-CACTCGCAAGCACCTATCA-3', and 5'-GAAGGTCGGAGTCAACGGATT-3' and 5'-GATGACAAGCTTCCCCTTCTC-3', respectively.⁴² The quantities of the HCV genome and the other host mRNAs were normalized with that of GAPDH mRNA. RTN1 and RTN3 genes were amplified using the primer pairs purchased from QIAGEN.

Cell Lines and Virus Infection

Cells from the Huh7OK1 cell line are highly permissive to HCV JFH1 strain (genotype 2a) infection compared to Huh 7.5.1 and exhibit the highest propagation efficiency for JFH1.⁴³ These cells were maintained at 37 °C in a humidified atmosphere and 5% CO₂ in the Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with nonessential amino acids (NEAA) and 10% fetal calf serum (FCS). The viral RNA of JFH1 was introduced into Huh7OK1 as described by Wakita et al.⁴⁴ The viral RNA of JFH1 derived from the plasmid pJFH1 was prepared as described by Wakita et al.⁴⁴

Statistical Analysis

Experiments for RNAi transfection and qRT-PCR were performed two times. The estimated values were represented as the mean \pm standard deviation ($n = 2$). The significance of differences in the means was determined by the Student's *t*-test.

RESULTS AND DISCUSSION

Identifying Host Proteins That Interact with HCV NSSA Protein

We employed an integrated approach that combined an experimental Y2H assay and comprehensive literature mining to identify human host proteins interacting with NSSA.

First, we performed a Y2H screening to characterize the interactions between NSSA and host proteins. The analysis of positive colonies revealed 17 host factors as interacting partners

of NSSA (Tables 1, S1, Supporting Information), 14 of which are novel. The other three interactions have been characterized previously; vesicle-associated membrane protein (VAMP)-associated protein B (VAPB), a membrane trafficking factor, and FK506-binding protein 8 (FKBP8), an immunoregulation protein, independently regulate HCV replication via interactions with NSSA,^{28,43,45,46} Bridging integrator 1 (BIN1), a tumor suppressor protein, interacts with NSSA and significantly contributes to HCC.⁴⁷ Among the newly discovered interactors, MAP4K4 is overexpressed in HCC, and knock-down of MAP4K4 expression inhibits HCC progression;⁴⁸ RTN1 and VAT1 were previously observed to be elevated in HCV infected cells,⁴⁹ and ARL6IP1, EPHB6, GABARAPL2, ITSN1 and NISCH were differentially expressed in HCV infection *in vitro*.⁵⁰ Furthermore, five (ARL6IP1, FKBP8, RTN1, RTN3, VAPB) of the 17 interactors (29.4%) localize to the endoplasmic reticulum (ER; GO:0005783; $p = 0.0028$), which is consistent with the role of NSSA as a crucial constituent of the HCV replication complex associated with the ER.⁵¹ These results suggest that the PPIs detected by our Y2H assay may closely reflect NSSA interactions *in vivo*.

We next scanned the biomedical literature to expand the repertoire of NSSA–host interactions. Because of an ever increasing volume of biomedical literature describing the pathogenesis of infectious diseases, the identification of specific host–pathogen interactions and their roles in pathogenicity is a nontrivial task, and therefore, recent years have witnessed a rapid development of computational tools for biomedical literature mining. We performed extensive literature mining using computational tools that facilitate the retrieval and extraction of relevant information from the biomedical literature (Pubmed, EBIMed, Protein Coral) and followed it up with a careful manual inspection to identify additional host factors, which directly interact with NSSA and which were not present in the Y2H data set. One hundred and fifteen pairwise interactions between NSSA and human proteins (consisting of 93 catalogued by a high throughput study of binary HCV–host interactions²² and 22 from assorted reports; see Supporting Information, Table S2) were extracted from the literature in this manner and were added to the existing interactors. The resulting NSSA–human interactome thus comprised 132 human host proteins directly interacting with NSSA (Table 1), all of which are expressed in the liver (see Supporting Information, Table S3).

Network Topological Analysis of the NSSA–host Interactions: NSSA Preferentially Targets Hubs and Bottlenecks in the Host Protein Interactome

To further understand the biological significance of the NSSA–host interactions, we retrieved PPIs for the nodes targeted by

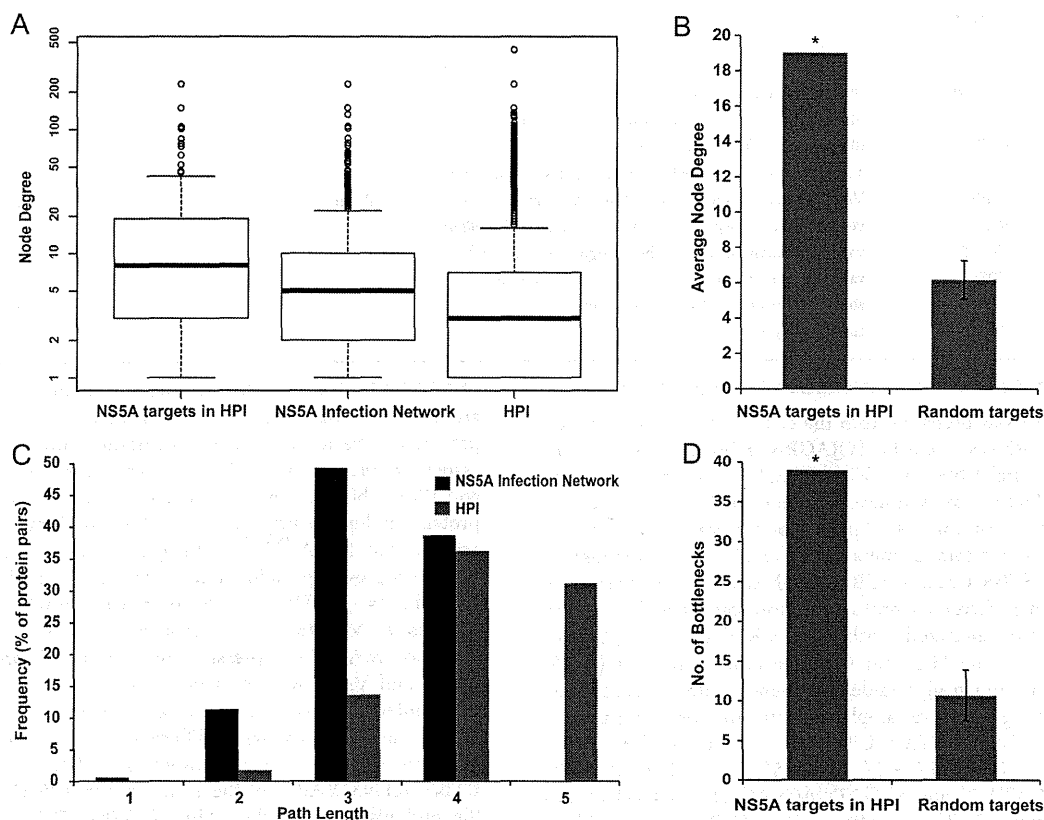


Figure 1. Topological analysis of the NSSA infection network. (A) The node degree distributions of the NSSA interactors in the HPI, NSSA infection network, and HPI are represented as box plots. The average degree of the NSSA interactors in HPI (19.02) was higher than those of the NSSA infection network (8.24) and HPI (5.96). Median node degrees (indicated by thick horizontal lines) of the NSSA interactors in HPI, NSSA infection network, and HPI are 8, 5, and 3, respectively. (B) The average degree of the nodes targeted by NSSA in HPI was much higher than mean average degree of 1000 sets of the randomly selected 108 nodes in HPI. (C) The shortest path length distributions of the NSSA infection network and HPI. The path length is represented on the x-axis while the y-axis describes the frequency, i.e., the percentage of node (protein) pairs within the PPI network with a given shortest path length. For simplicity, only the node frequencies for path lengths 1–5 in the HPI are displayed. (D) The number of bottlenecks among the nodes targeted by NSSA in HPI was much higher than mean of the number of bottlenecks among 1000 sets of the randomly selected 108 nodes in HPI. *: $p < 0.001$.

NSSA in the HPI and incorporated them with the initial interactions to infer an extended NSSA infection network. PPIs for 108 of 132 NSSA interactors were retrieved in this manner; 24 of 132 NSSA interactors had no PPIs in the HPI (Supporting Information, Tables S4, S5a, S5b). For the NSSA infection network and the HPI, we computed the node degree distribution and the characteristic/average path length measures to capture the topologies of the two networks. The degree of a protein, which corresponds to the number of its interacting partners, may often reflect its biological relevance since a better connected protein is likely to have a higher ability to influence biological networks via PPIs. Average path lengths provide an approximate measure of the relative ease and speed of dissemination of information between the proteins in a network.

The NSSA infection network consisted of 1442 entities (nearly all of which are expressed in the liver; see Supporting Information) with 6263 interactions between them (Supporting Information, Tables S4, S5a). The average degree (defined as the number of interactions for a given protein) of the NSSA infection network (8.24) was notably higher than the degree inferred for the HPI (5.96) (Figure 1A). Furthermore, the

average degree of the nodes targeted by NSSA in the HPI (19.02) was even higher; this number is significantly greater than the average degree obtained from a sample of randomly selected nodes (6.17 ± 1.08 with $p < 0.001$; Figure 1B; see Supporting Information). Also the degrees inferred for the majority of the NSSA interactors in the HPI (65 of 108; 60.18%) were higher than the mean degree of the HPI (5.96) (Figure 1A). Our observations therefore suggest that NSSA preferentially targets several highly connected cellular proteins (hubs) with an ability to influence a large number of host factors in HCV infection. The average (shortest) path length of the NSSA infection network (3.26) was significantly shorter than the HPI (4.54), and also the distribution of shortest path lengths was shifted toward the left (Figure 1C), thereby suggesting that the NSSA influenced cellular network is more compact and inclined toward faster communication between the constituents relative to the host cellular network.

Next, we examined the betweenness measures of the NSSA interactors in the HPI to assess their significance in the HPI and the NSSA infection network. The betweenness of a node, determined by the number of shortest paths passing through it, reflects the importance of that node in the network; the nodes

with the highest betweenness prominently regulate the flow of signaling information and are therefore “bottlenecks”, representing central points for communication in an interaction network.⁵² Previously, proteins with high betweenness have been implicated in crucial roles in HCV infection and pathogenesis.^{53,54} To investigate if NSSA preferentially targets bottlenecks (defined as the top 10% of the nodes in the HPI ranked by betweenness), we estimated the fraction of NSSA interactors that were bottlenecks in the HPI. A significant proportion (39 of 108; 36.1%) of the NSSA interactors were identified as bottlenecks in the HPI (Supporting Information, Table S6); this number is significantly higher than the number of bottlenecks among randomly selected nodes (10.72 ± 3.17 with $p < 0.001$; Figure 1D; see Supporting Information). These include growth factor receptor-binding protein 2 (GRB2), which plays an important role in the subversion of host signaling pathways by NSSA,⁵⁵ tumor protein 53 (TP53), a key mediator of the oncogenic effect of NSSA in HCV-induced HCC;⁵⁶ and tyrosine kinase SRC, which regulates the formation of NSSA-containing HCV replication complex.⁵⁷ Among the NSSA interacting proteins identified by our Y2H screening, ITSN1, an endocytic traffic associated protein, and GABARAPL2, an autophagy associated protein, were identified as network bottlenecks.

Our observations therefore suggest that NSSA preferentially interacts with highly central proteins in the host protein interactome; these interactions may help the virus to regulate efficiently the flow of the infection-related information in the host cellular network and manipulate the host metabolic machinery for its own survival and pathogenesis. Our observations are consistent with studies that suggested that viral pathogens tend to interact with well-connected host proteins that are central to the host cellular networks, thus enabling them to appropriate essential cellular functions.^{21,22,26,58,59}

Functional Analysis of NSSA Interaction Network

Next, we investigated the NSSA infection network for the enrichment of specific biological associations (KEGG pathways, CATH structural domains; GO terms and Reactome Pathways; Supporting Information, Tables S7a, S7b, S7c and S7d). Notably, a significant proportion of the proteins in the NSSA infection network were mapped to the CATH Phosphorylase Kinase; domain 1, domain (CATH:3.30.200.20; 138 out of 1442, $p = 2.61 \times 10^{-45}$) including 23 of the 132 NSSA interacting host proteins ($p = 3.38 \times 10^{-14}$) (13 of which are bottlenecks in the HPI), based on the Gene3D protein domain assignments (Supporting Information, Table S7b). These include two novel interactions between EPHB6 (a kinase deficient receptor) and MAP4K4 and NSSA, identified by our Y2H assay (Table 1). The significant representation of cellular kinases in the NSSA infection network is consistent with the key roles played by reversible phosphorylation of NSSA in modulating various NSSA functions in HCV pathogenesis. Impairing NSSA hyperphosphorylation has been shown to inhibit HCV replication, and thus, the cellular kinases that regulate NSSA phosphorylation are important targets for anti-HCV therapy.^{9,60–63}

The analysis of NSSA infection network revealed an enrichment of 79 KEGG pathways (Supporting Information, Table S7a). Furthermore, 31 of the 39 NSSA interacting bottlenecks (hereafter referred to as bottlenecks) were mapped to 75 of the 79 enriched KEGG pathways (Supporting

Information, Table S5). Among the 75 bottleneck-associated enriched KEGG pathways, the highest numbers were associated with various cancers and infectious diseases (31 enriched KEGG pathways; 27 bottlenecks), followed by immune system, signal transduction and endocrine system (23 enriched KEGG pathways; 27 bottlenecks), cell growth and death (4 enriched KEGG pathways; 9 bottlenecks), nervous system (4 enriched KEGG pathways; 8 bottlenecks) and cellular communication (3 enriched KEGG pathways; 14 bottlenecks) among others (Tables 2, S8a, Supporting Information). Below we describe our observations on the most prominent enriched biological themes of interest that were associated with the NSSA infection network, with a specific focus on the bottlenecks.

Cancers and Infectious Diseases

The analysis of the NSSA interaction network revealed that NSSA specifically targets host factors that participate in various complex human diseases. Thirty-four NSSA interactors including 24 bottlenecks were mapped to one or more of the 17 enriched KEGG pathways associated with different infectious diseases (Supporting Information, Tables S7a, S8a). Among the most prominent associations, 12 bottlenecks were mapped to “Epstein–Barr virus infection” ($p = 1.36 \times 10^{-27}$); 10 to “Hepatitis C” ($p = 3.47 \times 10^{-24}$); 10 to “HTLV-I infection” ($p = 1.39 \times 10^{-20}$); 9 to “Hepatitis B” ($p = 3.33 \times 10^{-26}$); 8 to “Measles” ($p = 5.69 \times 10^{-17}$); 7 bottlenecks were mapped to “Influenza A” ($p = 5.01 \times 10^{-12}$); 7 to “Herpes simplex infection” ($p = 1.47 \times 10^{-13}$) and 6 to “Tuberculosis” ($p = 3.02 \times 10^{-6}$) (Supporting Information, Tables S7a, S8a). These associations include infectious diseases induced by various bacterial and viral pathogens thereby suggesting that HCV and other pathogens may systematically target specific host factors, the perturbation of which may contribute to the onset of various human diseases.

Also, 19 bottlenecks were mapped to one or more of the 16 enriched KEGG pathways associated with various cancers. Among the most prominent associations, 10 bottlenecks were mapped to “Viral carcinogenesis” ($p = 1.3 \times 10^{-30}$); 8 each were mapped to “Prostate cancer” ($p = 4.27 \times 10^{-25}$), “Endometrial cancer” ($p = 5.52 \times 10^{-21}$) and “Colorectal cancer” ($p = 4.22 \times 10^{-18}$); 7 to “Pancreatic cancer” ($p = 1.94 \times 10^{-18}$); 6 to “Chronic myeloid leukemia” ($p = 1.61 \times 10^{-30}$) and 5 each to “Non-small cell lung cancer” ($p = 8.66 \times 10^{-15}$) and “Glioma” ($p = 2.38 \times 10^{-14}$) (Supporting Information, Tables S7a, S8a). The significant association of HCV with host factors central to various cancer pathways (including tumor suppressors such as TP53) is consistent with previous observations that viral pathogens significantly targeted host proteins associated with cancer pathways,^{59,64,65} which likely plays major roles in tumorigenesis.

Immune System and Signal Transduction

HCV infection induces various active and passive host immune responses including the recognition of viral RNA by host cell receptors. These events lead to the production of Type I interferons (IFN- α/β) and inflammatory cytokines in the infected hepatocytes, initiating the antiviral response. HCV persistence in the host is determined by the virus’s ability to impair host immune responses.^{66–69}

The analysis of the NSSA interaction network revealed that 21 of the 132 NSSA interacting proteins, including 16 bottlenecks and their interacting partners, were mapped to one or more enriched KEGG pathways associated with the immune system (Supporting Information, Tables S7a, S8a).

Table 2. KEGG Pathway Functional Categories (Subclasses) Sorted by the Number of Enriched Pathways (≥ 3) Associated with One or More NSSA Interacting Bottlenecks

category	no. of enriched pathways	no. of bottlenecks	associated bottlenecks	KEGG pathways in the given category associated with most number of bottlenecks
infectious diseases	16	24	ACTB, AKT1, CDK1, CSNK2A1, CTNNB1, FLNA, FYN, GPC2, GRB2, GSK3B, HSPB1, JAK1, LCK, LYN, PIK3R1, PPP2CA, RAF1, SRC, STAT1, SYK, TBP, TGFBRI1, TP53, TRAF2	"Epstein-Barr virus infection"; "HTLV-1 infection"; "Hepatitis C"; "Hepatitis B"; "Measles"; "Influenza A"; "Herpes simplex infection"; "Tuberculosis"; "Toxoplasmosis"; "Chagas disease (American trypanosomiasis)"; "Bacterial invasion of epithelial cells"
cancers	16	19	AKT1, AXIN1, CDK1, CTNNB1, GRB2, GSK3B, HSP90AA1, JAK1, LYN, RAF1, SRC, STAT1, SYK, TBP, TGFBRI1, THBS1, TP53, TRAF2	"Pathways in cancer"; "Viral carcinogenesis"; "Prostate cancer"; "Endometrial cancer"; "Colorectal cancer"; "Pancreatic cancer"; "Chronic myeloid leukemia"; "Non-small cell lung cancer"; "Glioma"; "Small cell lung cancer"; "Renal cell carcinoma"; "Melanoma"; "Acute myeloid leukemia"
immune system	10	16	ACTB, AKT1, CTNNB1, FYN, GRB2, GSK3B, HSP90AA1, LCK, LYN, PIK3R1, PIN1, RAF1, SRC, STAT1, SYK, TRAF	"Chemokine signaling pathway"; "T cell receptor signaling pathway"; "Fc epsilon RI signaling pathway"; "B cell receptor signaling pathway"; "Natural killer cell mediated cytotoxicity"; "Fc gamma R-mediated phagocytosis"
signal transduction	9	22	AKT1, AXIN1, CSKN1A1, CTNNB1, FLN, GRB2, GSK3B, HSP90AA1, HSPB1, JAK1, LCK, LYN, PIK3R1, PPP2CA, RAF1, SRC, STAT1, SYK, TGFBRI1, THBS1, TP53, TRAF2	"PI3K-Akt signaling pathway"; "MAPK signaling pathway"; "Wnt signaling pathway"; "ErbB signaling pathway"; "VEGF signaling pathway"; "NF-kappa B signaling pathway"; "Jak-STAT signaling pathway"
nervous system	5	8	AKT1, GRB2, GSK3B, LYN, PIK3R1, PPP2CA, RAF1, TP53	"Neurotrophin signaling pathway"; "Long-term depression"; "Dopaminergic synapse"; "Long-term potentiation"
endocrine system	4	10	AKT1, CDK1, GRB2, GSK3B, HSP90AA1, PIK3R1, PLK1, RAF1, SRC, TRAF2	"Progesterone-mediated oocyte maturation"; "Insulin signaling pathway"; "GnRH signaling pathway"; "Adipocytokine signaling pathway"
cell growth and death	4	9	AKT1, CDK1, GSK3B, PIK3R1, PLK1, PPP2CA, THBS1, TP53, TRAF2	"Cell cycle"; "Apoptosis"; "p53 signaling pathway"; "Oocyte meiosis"
cell communication	3	14	ACTB, AKT1, CSNK2A1, CTNNB1, FLNA, FYN, GRB2, GSK3B, PIK3R1, PPP2CA, RAF1, SRC, TGFBRI1, THBS1	"Focal adhesion"; "Tight junction"; "Adherens junction"
development	3	12	AKT1, FHL2, FYN, GRB2, GSK3B, JAK1, LCK, PIK3R1, STAT1, SYK, TGFBRI1, THBS1	"Osteoclast differentiation"; "Axon guidance"; "Dorso-ventral axis formation"

Eight bottlenecks were mapped to the enriched KEGG pathway "Chemokine signaling pathway" ($p = 2.27 \times 10^{-10}$), which is consistent with the modulation of host interferon signaling by NSSA in HCV infection.⁷⁰ In addition, 7 bottlenecks each were mapped to "T cell receptor signaling pathway" ($p = 4.6 \times 10^{-24}$), "Fc epsilon RI signaling pathway" ($p = 2.86 \times 10^{-14}$) and "B cell receptor signaling pathway" ($p = 1.8 \times 10^{-14}$) and 6 bottlenecks were mapped to "Natural killer cell mediated cytotoxicity" ($p = 1.92 \times 10^{-12}$). Three bottlenecks (AKT1, PIK3R1 and STAT1) were also mapped to the enriched KEGG pathway "Toll-like receptor signaling pathway" ($p = 3.23 \times 10^{-7}$; Supporting Information, Tables S7a, S8a). Toll-like receptor 3 mediated chemokine and cytokine signaling plays an important role in the host immune response in HCV infection.⁷¹ Therefore, NSSA interaction with bottlenecks, which function in various aspects of the host immune response, may significantly contribute to the perturbation of the host immune system in HCV pathogenesis.

Additionally, 32 of 132 NSSA interacting proteins examined in the present study, including 24 bottlenecks, were mapped to various pathways associated with the signal transduction and the endocrine system (Supporting Information, Tables S7a, S8a), many of which are implicated in HCV infection and HCC progression and are targets for molecular therapy in HCC.^{22,72-74}

Eleven bottlenecks were mapped to the enriched KEGG pathway "PI3K-Akt signaling pathway" ($p = 2.2 \times 10^{-24}$; Supporting Information, Tables S7a, S8a), which is consistent with a previous study that NSSA stimulates the activation of PI3K-Akt pathway, which contributes to HCC in HCV infection.⁷⁵ Eight bottlenecks were mapped to the enriched KEGG pathway "MAPK signaling pathway" ($p = 2.4 \times 10^{-19}$; Supporting Information, Tables S7a, S8a). Elements of the MAPK signaling cascades are directly involved in the progression of HCV infection, particularly in association with HCV Core and E2 proteins,^{22,24,76,77} thereby suggesting that NSSA interactions with the key facilitators of MAPK signaling in the host interactome may play an important role in regulating the reversible phosphorylation of NSSA and may contribute to the progression of HCV pathogenesis.

Bottlenecks AKT1, GRB2, GSK3B, PIK3R1 and RAF1 and many of their interactors were mapped to the enriched KEGG pathway "Insulin signaling pathway" ($p = 2.42 \times 10^{-13}$; Supporting Information, Tables S7a, S8a); these proteins are highlighted in Figure 2. Insulin signaling plays an important role in regulating glucose and lipid metabolism, and the disruption of this process may contribute to insulin resistance (IR). IR is linked with steatosis, fibrosis progression and poor interferon- α response in HCV infection.⁷⁸⁻⁸⁰ Suppression of AKT1 and GSK3B activity in HCV infection disrupts glucose metabolism and contributes to IR.^{81,82} Furthermore, PIK3R1 and NSSA interactor PIK3CB (Figure 2) are subunits of phosphatidylinositol 3-kinase (PI3K), which controls insulin secretion;⁸³ PI3K also facilitates the activation of the proto-oncogene beta-catenin (CTNNB1) by NSSA, which contributes to the development of HCC in HCV pathogenesis.⁸⁴ Previously, HCV Core protein has been directly implicated in the induction of IR in HCV infection,⁸⁵ while there is little evidence suggesting definitive links between NSSA and IR. Our observations, however, suggest that NSSA directly interacts with key regulators of insulin metabolism and may, therefore, play a major role in modulating HCV-induced IR and eventually HCC.

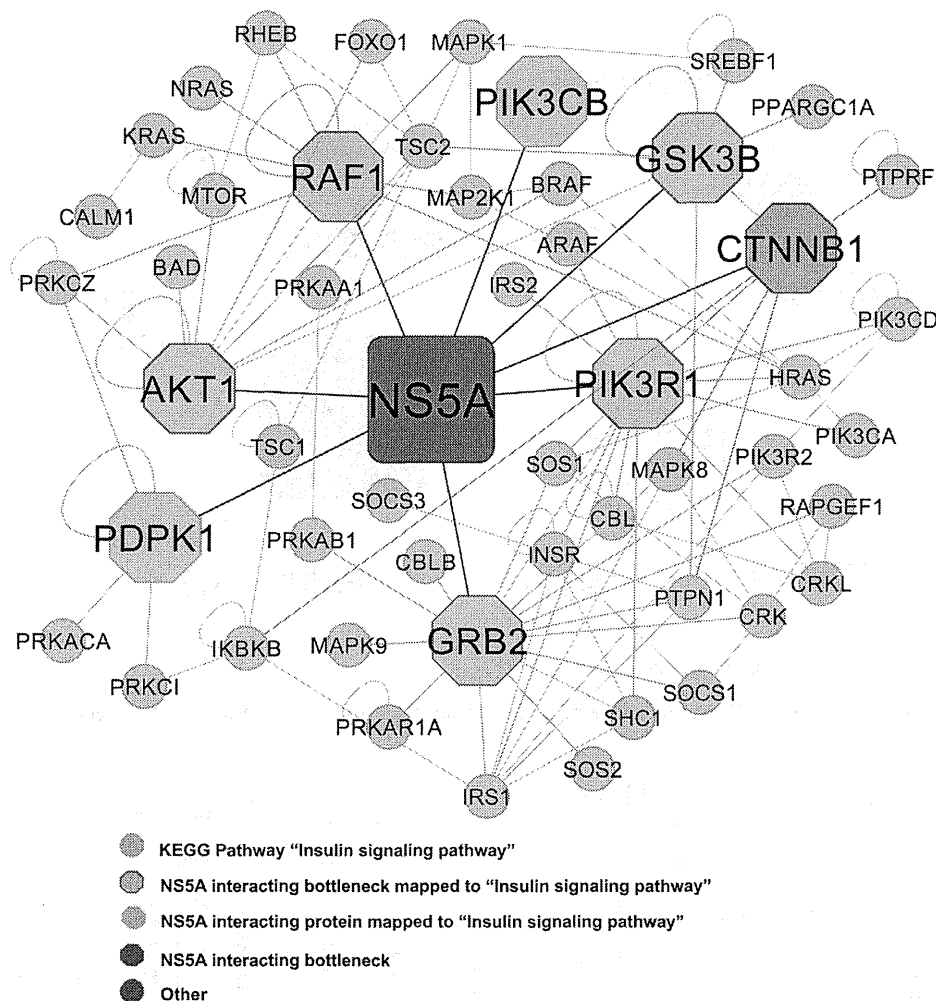


Figure 2. NS5A interacting bottlenecks and their interacting partners associated with the enriched KEGG pathway hsa04910: "Insulin signaling pathway".

Cell Adhesion and Communication

The perturbation of adherens and tight junction associated proteins has been implicated in HCV entry, cell–cell transmission and hepatoma migration in HCV infection.^{86–88} In the NS5A infection network, eight bottlenecks (ACTB, AKT1, CSNK2A1, CTNNB1, FYN, PPP2CA, SRC and TGFBR1) were mapped to either or both of the enriched KEGG pathways "Adherens Junction" ($p = 1.03 \times 10^{-15}$) and "Tight junction" ($p = 1.19 \times 10^{-5}$), which are associated with cell adhesion junctions and cellular communication (Supporting Information, Tables S7a, S8a). CSNK2A1 is the catalytic (alpha) subunit of Casein Kinase II (CK2), which phosphorylates NS5A and regulates the production of infectious viral particles.⁶³ CTNNB1, a key component of cell-adhesion complexes, is positively regulated by CK2.⁸⁹ Furthermore, the activation of CTNNB1 by NS5A significantly contributes to HCC.⁸⁴ Taken together, our observations suggest that NS5A interactions with bottlenecks, which regulate cell–cell adhesion (CSNK2A1, CTNNB1) and cytoskeletal organization (ACTB), may significantly contribute to the progression of HCV life cycle and tumorigenesis in HCV pathogenesis.

Eleven bottlenecks were mapped to the enriched KEGG pathway "Focal Adhesion" ($p = 1.02 \times 10^{-17}$; Supporting Information, Tables S7a, S8a), thereby reiterating that focal adhesion is a major target of NS5A.²² Focal adhesion regulates cell migration and adhesion, and some of its components were directly implicated in the regulation of HCV replication and propagation in our earlier study.²⁴ Our observations thus suggest that NS5A interactions with key components of the focal adhesion machinery may play important roles in the HCV lifecycle. For instance, NS5A interacts with bottleneck THBS1 (Thrombospondin-1), a glycoprotein, which was mapped to the KEGG "Focal Adhesion" pathway. THBS1 plays a key role in NS5A-mediated activation of the cytokine TGF- β 1, which facilitates HCV replication and progressive liver fibrosis in HCV infection.⁹⁰ Our observations suggest that direct NS5A interactions with the bottlenecks THBS1 and TGFBR1 (TGF- β receptor 1; KEGG Pathway "Adherens Junction"), a key facilitator of TGF- β downstream signaling, may be crucial in facilitating HCV replication and tumorigenesis in HCV pathogenesis.

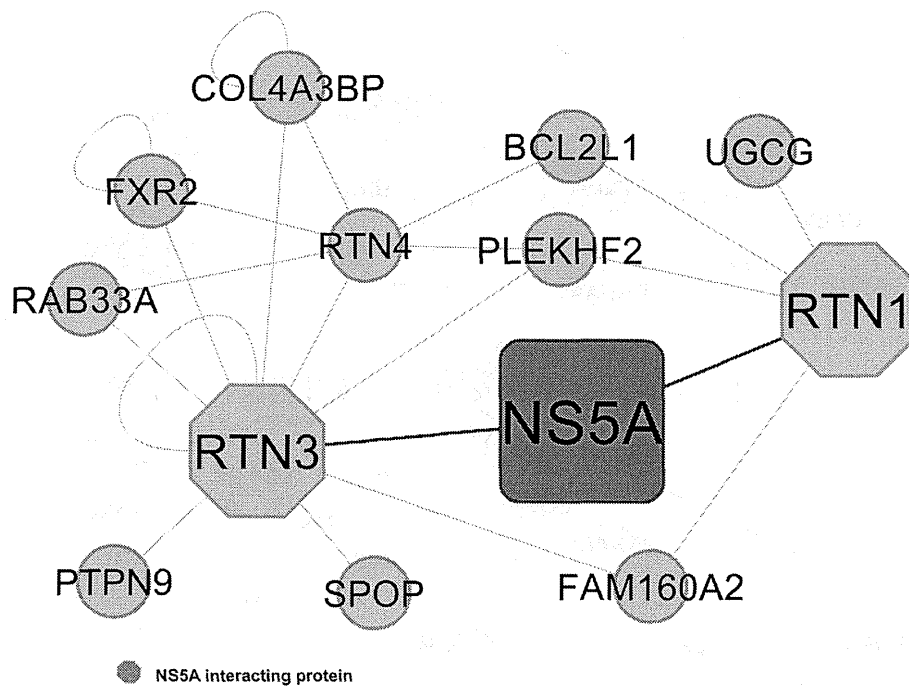


Figure 3. ER-localized host factors RTN1 and RTN3 were found to interact (blue edges) with NS5A in an Y2H screening of human liver cDNA library using NS5A as bait.

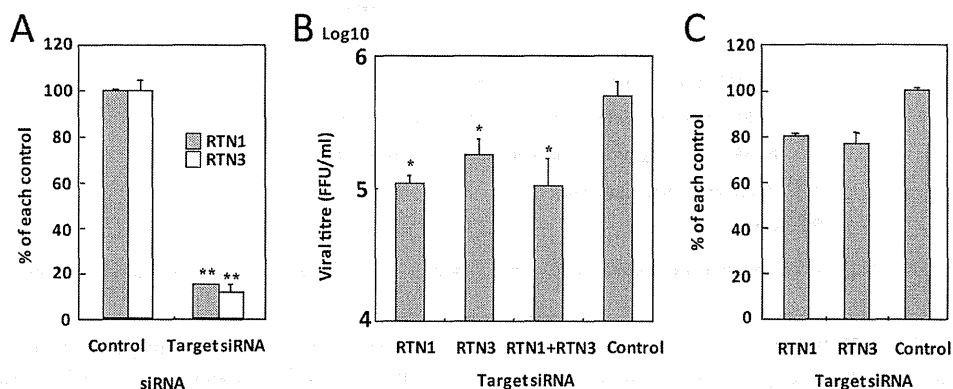


Figure 4. Effects of knockdown of RTN1 and RTN3 on HCV propagation and replication. Host factors RTN1 and RTN3 were suppressed by RNAi (A) in Huh7OK1 cells infected with HCV JFH1 strain (genotype 2a). The amounts of viral titer (B) and intracellular viral RNA (C) were estimated. Each value was represented as percentage of the cells transfected with the control siRNA. FFU: Focus-forming units; *: $p < 0.05$, **: $p < 0.01$.

Cellular Transport

Cellular factors associated with endocytic trafficking are key facilitators of the HCV life cycle, particularly HCV entry into the hepatic cells.^{91–93} Endocytosis of the extracellular growth factor receptor (EGFR) in association with the cell surface glycoprotein CD81 plays a crucial role in HCV internalization and entry and is, therefore, an attractive target of anti-HCV strategies.⁹⁴ In the NSSA infection network, NSSA interactors ARAP1 and HSPA1A together with two bottlenecks (SRC, TGFBR1) were mapped to the enriched KEGG pathway “Endocytosis” ($p = 2.97 \times 10^{-8}$; Supporting Information, Tables S7a, S8a). ARAP1, a Golgi associated protein, negatively regulates EGFR trafficking, and decreased ARAP1 expression contributes to enhanced EGFR endocytosis.⁹⁵ Therefore, NSSA

interaction with ARAP1 may facilitate EGFR internalization and thus viral entry in HCV infection.

NSSA Interacting Host Proteins RTN1 and RTN3 Function in HCV Propagation but Not Replication

Traditionally, viral and host proteins associated with the HCV lifecycle (internalization, replication, assembly and release) have been preferred targets in the anti-HCV studies. During infection, HCV localizes to the detergent-resistant membrane fraction (DRM) derived from the ER, where the viral replication and assembly take place.⁴ Thus, of the novel interactions identified in our Y2H assay, we focused on two ER-localized host factors RTN1 and RTN3 (Figure 3). RTN1 and RTN3 belong to a group of proteins named Reticulons, which are integral to maintaining the shape and organization of the

ER and have been implicated in facilitating the replication of various positive-strand RNA viruses.^{96–98} Furthermore, both RTN1 and RTN3 have been specifically detected in the very low density lipoprotein (VLDL) transport vesicle (VTV);⁹⁹ VTV is a key component of the VLDL secretory pathway, which plays an essential role in the production and the release of the infectious HCV particles.¹⁰⁰ Therefore, NSSA interactions with RTN1 and RTN3 suggested novel and potentially crucial roles of the two host proteins in the replication and/or release stages of the HCV lifecycle.

We performed cellular assays to assess the impact of RTN1 and RTN3 siRNA knockdowns on HCV replication and release. Since the HCV-production systems using the HCV JFH1 infectious strain (genotype 2a) isolates alone are capable of both efficient replication and the production of the infectious HCV particles, JFH1 was used to infect the Huh7OK1 cell line 24h after transfection with each siRNA (see Materials and Methods). The infected cells were harvested after 72 h postinfection, and the expression of each host protein was assessed by qRT-PCR (Figure 4A). The viral titer was significantly decreased by individual and double knockdowns of RTN1 and RTN3 (Figure 4B). However, RTN1 and RTN3 knockdowns had no effect on the intracellular viral RNA levels in the HCV infected cells (Figure 4C), suggesting that RTN1 and RTN3 regulate HCV propagation but not HCV replication.

CONCLUSIONS

We describe here our observations of PPIs between HCV NSSA and host proteins. By employing a multifold approach involving an experimental Y2H assay and literature mining, we derived a comprehensive set of experimentally determined binary interactions between NSSA and host proteins. We proceeded to map the combined NSSA–host interactions onto an overall interaction network, which comprised a repertoire of connections, which potentially enable NSSA to link up with and modulate the components of the host cellular networks. We then employed a network-based approach to understand the biological context of these connections in HCV pathogenesis with the help of the TargetMine data warehouse.

A functional analysis of the PPI networks highlighted NSSA interactions with several well connected host factors (hubs) and centrally located “bottlenecks” in the host cellular networks that function in cellular pathways associated with immune system and cell signaling, cellular adhesion and cell transport, cell growth and cell death and ER homeostasis among others. The “bottlenecks” include several proteins that were previously implicated in HCV pathogenesis, thereby suggesting that NSSA interactions with centrally connected host factors may enable the virus to influence strongly the host cellular processes in HCV infection. Notably, many bottlenecks were mapped to pathways associated with the infectious diseases induced by diverse bacterial and viral pathogens of the human host. These observations thus suggest the presence of some common themes underlying the onset of various human diseases associated with pathogenic infection in humans, a better understanding of which may be helpful in optimizing broad spectrum approaches to counteracting a wide range of pathogenic infections.

Cellular assays based on siRNA knockdowns in the HCV infected and replicon cells demonstrated RTN1 and RTN3, ER-localized NSSA interacting proteins, to be novel regulators of HCV propagation, but not replication, and thus promising novel candidates for anti-HCV therapy.

Our analysis therefore provides further insights into the role of NSSA–host interactions in HCV infection, a deeper understanding of which may aid in the identification of new clinically relevant targets for optimizing the therapeutic strategies to manipulate HCV–host interactions and thus more effectively combating HCV infection. Our analysis also emphasizes the importance of elaborate network-based computational approaches that integrate diverse biological data types in investigating host–pathogen interactions.

ASSOCIATED CONTENT

Supporting Information

Supporting methods, figures, and tables. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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Japanese Encephalitis Virus Core Protein Inhibits Stress Granule Formation through an Interaction with Caprin-1 and Facilitates Viral Propagation

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Stress granules (SGs) are cytoplasmic foci composed of stalled translation preinitiation complexes induced by environmental stress stimuli, including viral infection. Since viral propagation completely depends on the host translational machinery, many viruses have evolved to circumvent the induction of SGs or co-opt SG components. In this study, we found that expression of Japanese encephalitis virus (JEV) core protein inhibits SG formation. Caprin-1 was identified as a binding partner of the core protein by an affinity capture mass spectrometry analysis. Alanine scanning mutagenesis revealed that Lys⁹⁷ and Arg⁹⁸ in the α -helix of the JEV core protein play a crucial role in the interaction with Caprin-1. In cells infected with a mutant JEV in which Lys⁹⁷ and Arg⁹⁸ were replaced with alanines in the core protein, the inhibition of SG formation was abrogated, and viral propagation was impaired. Furthermore, the mutant JEV exhibited attenuated virulence in mice. These results suggest that the JEV core protein circumvents translational shutoff by inhibiting SG formation through an interaction with Caprin-1 and facilitates viral propagation *in vitro* and *in vivo*.

In eukaryotic cells, environmental stresses such as heat shock, oxidative stress, UV irradiation, and viral infection trigger a sudden translational arrest, leading to stress granule (SG) formation (1). SGs are cytoplasmic foci composed of stalled translation preinitiation complexes and are postulated to play a critical role in regulating mRNA metabolism during stress via so-called “mRNA triage” (2). The initiation of SG formation results from phosphorylation of eukaryotic translation initiation factor 2 α (eIF2 α) at Ser⁵¹ by various kinases, including protein kinase R (PKR), PKR-like endoplasmic reticulum kinase (PERK), general control non-repressed 2 (GCN2), and heme-regulated translation inhibitor (HRI), which are commonly activated by double-stranded RNA (dsRNA), endoplasmic reticulum (ER) stress, nutrient starvation, and oxidative stress, respectively. Phosphorylation of eIF2 α reduces the amount of eIF2-GTP-tRNA complex and inhibits translation initiation, leading to runoff of elongating ribosomes from mRNA transcripts and the accumulation of stalled translation preinitiation complexes. Thus, SGs are defined by the presence of components of translation initiation machinery, including 40S ribosome subunits, poly(A)-binding protein (PABP), eIF2, eIF3, eIF4A, eIF4E, eIF4G, and eIF5. Then, primary aggregation occurs through several RNA-binding proteins (RBPs), including T-cell intracellular antigen-1 (TIA-1), TIA-1-related protein 1 (TIAR), and Ras-Gap-SH3 domain-binding protein (G3BP). These RBPs are independently self-oligomerized with the stalled initiation factors and with other RBPs, such as USP10, hnRNP Q, cytoplasmic activation/proliferation-associated protein-1 (Caprin-1), and Staufen and with nucleated mRNA-protein complex (mRNP) aggregations (3, 4). SG assembly begins with the simultaneous formation of numerous small mRNP granules which then progressively fuse into larger and fewer structures, a process known as secondary aggregation (5). The aggregation of TIA-1 or TIAR is regulated by molecular chaperones, such as heat shock protein 70 (Hsp70) (3), whereas that of G3BP is controlled by its phosphor-

ylation at Ser¹⁴⁹ (4). SG formation and disassembly in response to cellular stresses are strictly regulated by multiple factors.

Viral infection can certainly be viewed as a stressor for cells, and SGs have been reported in some virus-infected cells. Since the propagation of viruses is completely reliant on the host translational machinery, stress-induced translational arrest plays an important role in host antiviral defense. To antagonize this host defense, most viruses have evolved to circumvent SG formation during infection. For example, poliovirus (PV) proteinase 3C cleaves G3BP, leading to effective SG dispersion and virus propagation (6). Influenza A virus nonstructural protein 1 (NS1) has been shown to inactivate PKR and prevent SG formation (7). In the case of human immunodeficiency virus 1 (HIV-1) infection, Staufen1 is recruited in ribonucleoproteins for encapsidation through interaction with the Gag protein to prevent SG formation (8). In contrast, some viruses employ alternative mechanisms of translation initiation and promote SG formation to limit cap-dependent translation of host mRNA (9, 10). In addition, vaccinia virus induces cytoplasmic “factories” in which viral translation, replication, and assembly take place. These factories include G3BP and Caprin-1 to promote transcription of viral mRNA (11).

Japanese encephalitis virus (JEV) belongs to the genus *Flavivirus* within the family *Flaviviridae*, which includes other mosquito-borne human pathogens, such as dengue virus (DENV), West Nile virus (WNV), and yellow fever virus, that frequently cause significant morbidity and mortality in mammals and birds (12). JEV has

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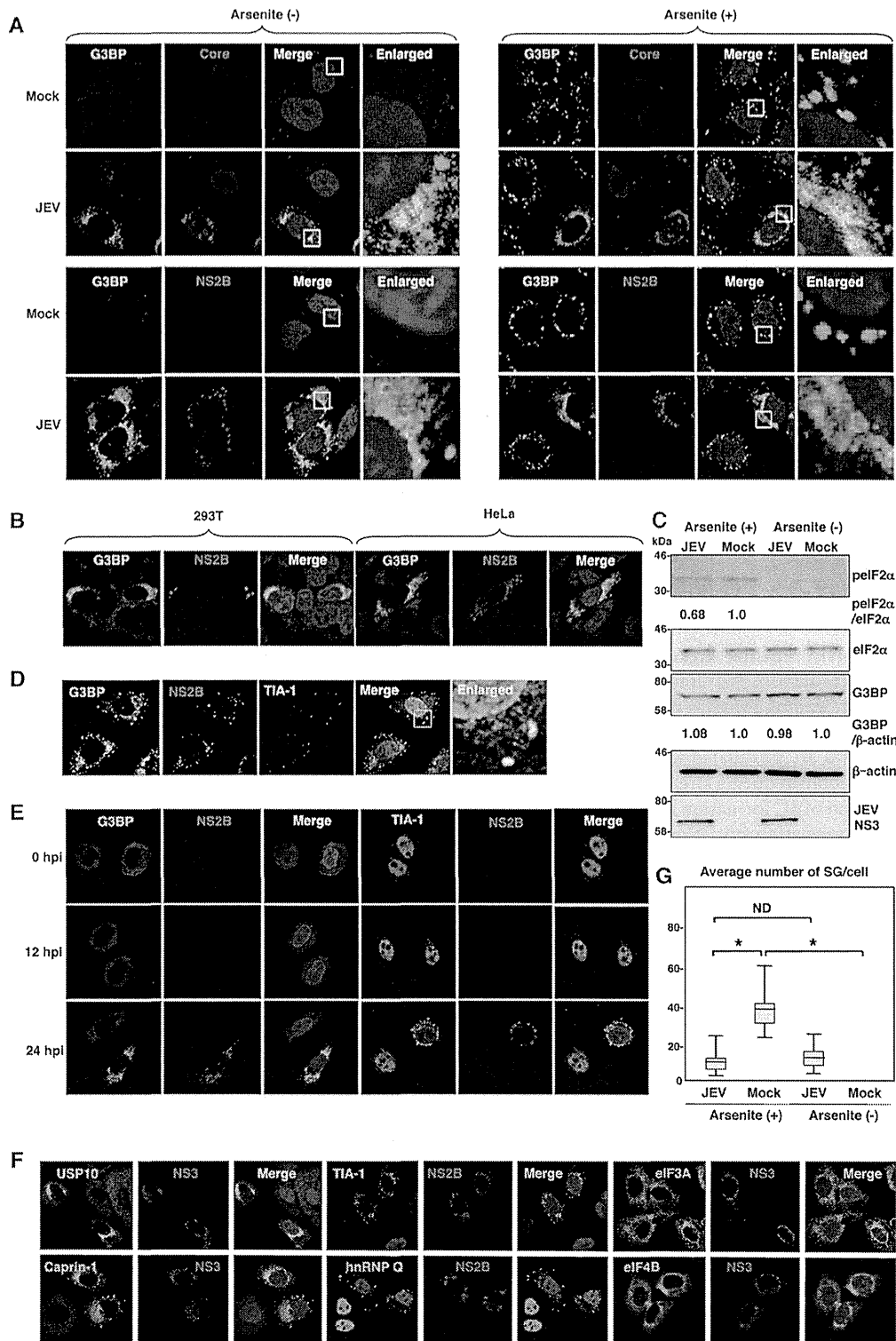


FIG 1 Dynamics of SG-associated factors during JEV infection. (A) Huh7 cells infected with JEV at an MOI of 0.5 were treated with or without 1.0 mM sodium arsenite for 30 min at 37°C, and the levels of expression of G3BP and JEV core protein/NS2B were determined at 24 h postinfection by immunofluorescence analysis with mouse anti-G3BP MAb and rabbit anti-core protein or anti-NS2B PAb, followed by AF488-conjugated anti-mouse IgG (Invitrogen) and AF594-conjugated anti-rabbit IgG, respectively. Cell nuclei were stained with DAPI (blue). (B) Cellular localizations of G3BP and JEV NS2B in 293T and HeLa cells infected with JEV were determined at 24 h postinfection by immunofluorescence analysis with mouse anti-G3BP MAb and rabbit anti-NS2B PAb, followed by AF488-conjugated anti-mouse IgG and AF594-conjugated anti-rabbit IgG, respectively. Cell nuclei were stained with DAPI (blue). (C) Phosphorylation of eIF2α in cells prepared as described in panel A was determined by immunoblotting using the indicated antibodies. The band intensities were quantified by ImageJ

a single-stranded positive-sense RNA genome of approximately 11 kb. The genomic RNA carries a single large open reading frame, and a polyprotein translated from the genome is cleaved co- and posttranslationally by host and viral proteases to yield three structural proteins, the core, precursor membrane (PrM), and envelope (E) proteins, and seven nonstructural (NS) proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (13). PrM is further cleaved by the multibasic protease, furin, and matured to membrane (M) protein. The core, M, and E proteins are components of extracellular mature virus particles. NS proteins are not incorporated into particles and are thought to be involved in viral replication, which occurs in close association with ER-derived membranes (14). Previous reports have shown that WNV and DENV inhibit SG formation by sequestering TIA-1 and TIAR through specific interaction with viral RNA (15, 16). In addition, the membrane structure induced by WNV infection was suggested to prevent PKR activation and avoid induction of SG formation (17). In this study, we show that JEV core protein plays an important role in inhibition of SG formation. JEV core protein recruited several SG-associated proteins, including G3BP and USP10, through an interaction with Caprin-1 and suppressed SG formation. Furthermore, a mutant JEV carrying a core protein incapable of binding to Caprin-1 exhibited lower propagation *in vitro* and lower pathogenicity in mice than the wild-type (WT) JEV, suggesting that inhibition of SG formation by the core protein is crucial to antagonize host defense. These results reveal a novel strategy of JEV to inhibit SG formation through an interaction with Caprin-1 and facilitate viral propagation.

MATERIALS AND METHODS

Plasmids. Plasmids encoding FLAG-tagged JEV core protein (pCAGPM-FLAG-Core) and hemagglutinin (HA)-tagged JEV proteins (pCAGPM-HA-JEV proteins) were generated as previously described (18, 19). The cDNA of the core protein of JEV AT31 (amino acid residues 2 to 105) was amplified from the pCAGPM-FLAG-Core plasmid by PCR and cloned into pET21b (Novagen-Merck, Darmstadt, Germany) for expression in bacteria as a His-tagged protein and in pCAG-MCS2-FOS for expression in mammalian cells as a FLAG-One-STrEP (FOS)-tagged protein. The resulting plasmids were designated pET21b-Core-His and pCAG-Core-FOS, respectively. The cDNA of the core protein of DENV2 (amino acid residues 2 to 100) was amplified from the pCAG/FLAG-DEN2C-HA plasmid (19) by PCR and cloned into pCAGPM-N-FLAG. The cDNA of human Caprin-1 was amplified from 293T cells by reverse transcription-PCR (RT-PCR) and cloned into pCAGPM-N-HA (20) and pGEX 6P-1 (GE Healthcare, Buckinghamshire, United Kingdom) for expression in bacteria as a glutathione S-transferase (GST) fusion protein and designated pCAGPM-HA-Caprin-1 and pGEX-GST-Caprin-1, respectively. The cDNAs of human G3BP1 and USP10 were also amplified from 293T cells by RT-PCR and cloned into pCAGPM-N-HA. The nucleotide residues of the adenine at 384, adenine at 385, cytosine at 387, and guanine at

388 of the JEV genome in pMWATG1 were replaced with guanine, cytosine, guanine, and cytosine, respectively, by PCR-based mutagenesis to change Lys⁹⁷ and Arg⁹⁸ of the core protein to Ala, yielding pMWAT/KR9798A. The cDNA of the mutant core protein was also cloned into pCAGPM-N-FLAG and pET21b. To generate stable cell lines expressing *Aequorea coerulescens* green fluorescent protein (AcGFP)-fused Caprin-1, the cDNA of human Caprin-1 was amplified by RT-PCR and cloned into pAcGFP N1 (Clontech, Mountain View, CA), and the Caprin-1-AcGFP gene was subcloned into the lentiviral vector pCSII-EF-RFA (21) and designated pCSII-EF-Caprin-1-AcGFP. All plasmids were confirmed by sequencing with an ABI Prism 3130 genetic analyzer (Applied Biosystems, Tokyo, Japan).

Cells and stress treatment. Mammalian cell lines, Vero (African green monkey kidney), 293T (human kidney), Huh7 (human hepatocellular carcinoma), and HeLa (human cervical carcinoma), were maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM) (Sigma, St. Louis, MO) supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, nonessential amino acids (Sigma), and 10% fetal bovine serum (FBS). The mosquito cell line C6/36 (*Aedes albopictus*) was grown in Leibovitz's L-15 medium with 10% FBS. Huh7 cells were transduced with a lentiviral vector expressing Caprin-1-AcGFP and AcGFP and designated Huh7/Caprin-1-AcGFP and Huh7/AcGFP, respectively. For induction of SGs, cells were treated with sodium arsenite at a final concentration of 1.0 mM in the culture medium for 30 min prior to fixation or lysis of the cells. SG formation was defined morphologically by immunostaining using anti-SG-related factor antibodies described below. Cell viability was determined by using CellTiter-Glo (Promega, Madison, WI) according to the manufacturer's instruction.

Viruses. The wild-type and 9798A mutant of the JEV AT31 strain were generated by the transfection of pMWATG1 and pMWAT/KR9798A, respectively, as described previously (22). Viral infectivity was determined by an immunostaining focus assay as described previously (20), and the results are expressed in focus-forming units (FFU). JEV and DENV serotype 2 New Guinea C strain were amplified in C6/36 cells.

Antibodies. Anti-JEV core rabbit polyclonal antibody (PAb) and anti-JEV NS3 mouse monoclonal antibody (MAb) were prepared as described previously (20, 23). Anti-JEV NS2B rabbit PAb was generated with synthetic peptides of JEV NS2B at Scrum, Inc. (Tokyo, Japan). Anti-DENV core protein rabbit PAb was prepared by using a GST-fused recombinant protein containing amino acid residues 2 to 100 of the DENV core protein. Anti-FLAG mouse MAb (M2) and rabbit PAb and anti- β -actin mouse MAb were purchased from Sigma. Anti-hnRNP Q mouse MAb (ab10687), anti-USP10 rabbit PAb (ab70895), and anti-eIF4B rabbit PAb (ab78916) were purchased from Abcam (Cambridge, United Kingdom). Anti-eIF2 α , anti-phospho-eIF2 α , and anti-eIF3A rabbit PABs were purchased from Cell Signaling Technology (Danvers, MA). Anti-HA mouse MAb (HA11), anti-HA rat MAb (3F10), anti-His mouse MAb, anti-GFP mouse MAb (JL-8), anti-JEV envelope protein mouse MAb (6B4A-10), anti-G3BP mouse MAb, anti-TIA-1 goat PAb, anti-Caprin-1 rabbit PAb, and anti-dsRNA mouse MAb were purchased from Covance (Richmond, CA), Roche (Mannheim, Germany), R&D Systems (Minneapolis, MN), Clontech, Chemicon (Temecula, CA), BD Biosciences (Franklin Lakes, NJ), Santa Cruz (Santa Cruz, CA), Proteintech (Chicago, IL), and Bio-

software (NIH, Bethesda, MD), and the relative levels for the indicated proteins are shown based on the level of the mock-infected cells. (D) Cellular localizations of G3BP, NS2B, and TIA-1 in Huh7 cells infected with JEV were determined at 24 h postinfection by immunofluorescence analysis with mouse anti-G3BP MAb, rabbit anti-NS2B PAb, and goat anti-TIA-1 PAb, followed by AF488-conjugated anti-mouse IgG, AF594-conjugated anti-rabbit IgG, and AF633-conjugated anti-goat IgG, respectively. Cell nuclei were stained with DAPI (gray). (E) Dynamics of G3BP and TIA-1 during JEV infection. Huh7 cells infected with JEV were immunostained at 0, 12, and 24 h postinfection (hpi) with mouse anti-G3BP MAb or goat anti-TIA-1 PAb and rabbit anti-NS2B PAb, followed by AF488-conjugated anti-mouse IgG or AF488-conjugated anti-goat IgG and AF594-conjugated anti-rabbit IgG, respectively. Cell nuclei were stained with DAPI (blue). (F) Cellular localization of SG-associated proteins (USP10, Caprin-1, TIA-1, hnRNP Q, eIF3A, and eIF4B) (green, AF488-conjugated secondary antibody) and JEV NS2B/NS3 (red, AF-594-conjugate secondary antibody) in Huh7 cells infected with JEV was determined by immunoblotting at 24 h postinfection. Cell nuclei were stained with DAPI (blue). (G) Numbers of G3BP-positive foci in 30 cells prepared as described in panel A were counted for each experimental condition. Lines, boxes, and error bars indicate the means, 25th to 75th percentiles, and 95th percentiles, respectively. The significance of differences between the means was determined by a Student's *t* test. *, *P* < 0.01; ND, no significant difference.

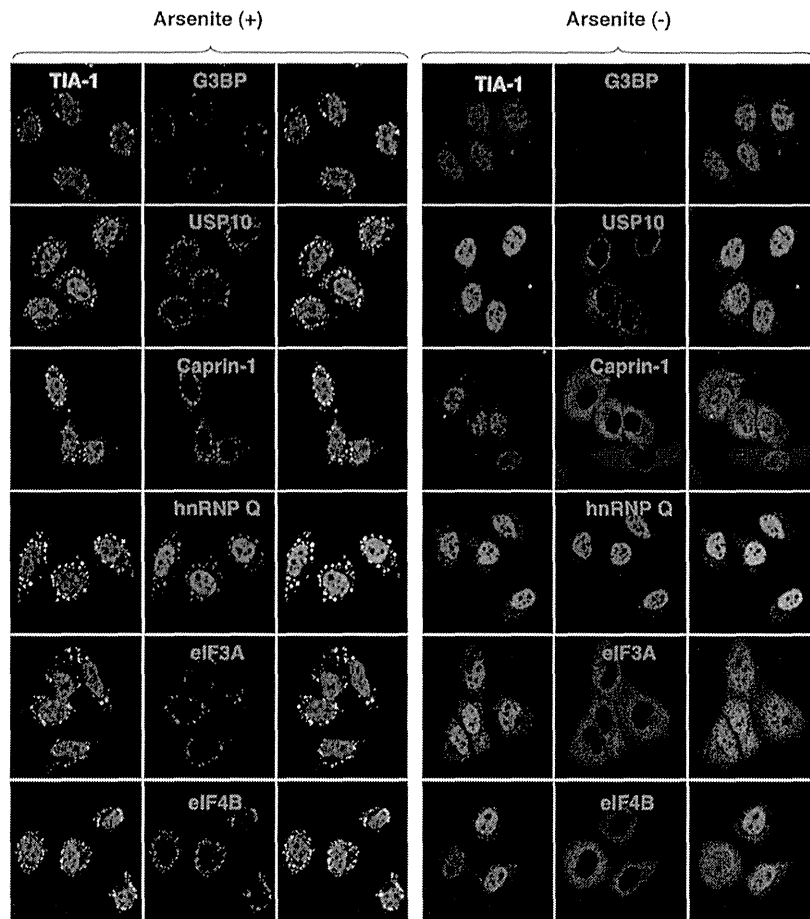


FIG 2 Each SG-associated factor forms SGs under oxidative stress. After treatment with 1.0 mM sodium arsenite for 30 min at 37°C, Huh7 cells were subjected to immunofluorescence analysis with the indicated primary antibodies, followed by AF488-conjugated anti-goat IgG and AF594-conjugated anti-mouse or rabbit IgG. Cell nuclei were stained with DAPI (blue).

center (Szirak, Hungary), respectively. Alexa Fluor (AF)-conjugated secondary antibodies were purchased from Invitrogen (Carlsbad, CA).

Immunofluorescence microscopy. Huh7 cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature. After cells were quenched for 10 min with PBS containing 50 mM ammonium chloride (NH_4Cl), they were permeabilized with 0.2% Triton X-100 in PBS for 10 min and blocked with PBS containing 2% bovine serum albumin (BSA) for 30 min at room temperature. The cells were then incubated with the antibodies indicated in the figure legends. Nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI). The samples were examined by a Fluoview FV1000 laser scanning confocal microscope (Olympus, Tokyo, Japan).

Transfection, immunoprecipitation, and immunoblotting. Plasmids were transfected into 293T or Huh7 cells by use of TransIT LTI (Mirus, Madison, WI), and cells collected at 24 h posttransfection were subjected to immunostaining, immunoprecipitation, and/or immunoblotting as described previously (24). The immunoprecipitates were boiled in sodium dodecyl sulfate (SDS) sample buffer and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and incubated with the appropriate antibodies. The immune complexes were visualized with SuperSignal West Femto substrate (Thermo Scientific, Rockford, IL) and detected by use of an LAS-3000 image analyzer system (Fujifilm, Tokyo, Japan).

FOS-tagged purification and mass spectrometry. pCAG-Core-FOS or empty vector was transfected into 293T cells, harvested at 24 h posttransfection, washed with cold PBS, suspended in cell lysis buffer (20 mM Tris-HCl, pH 7.4, 135 mM NaCl, 1% Triton X-100, and protease inhibitor cocktail [Complete; Roche]), and centrifuged at $14,000 \times g$ for 20 min at 4°C. The supernatant was pulled down using 50 μl of STreP-Tactin Sepharose (IBA, Gottingen, Germany) equilibrated with cell lysis buffer for 2 h at 4°C. The affinity beads were washed three times with cell lysis buffer and suspended in $2 \times$ SDS-PAGE sample buffer. The proteins were subjected to SDS-PAGE, followed by Coomassie brilliant blue (CBB) staining using CBB Stain One (Nakalai Tesque, Kyoto, Japan). The gels were divided into 10 pieces, and each fraction was trypsinized and subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis to identify coimmunoprecipitated proteins. All of the proteins in gels were identified comprehensively, and the proteins detected in cells transfected with pCAG-Core-FOS but not in those with empty vector were regarded as candidates for binding partners of JEV core.

Gene silencing. A commercially available small interfering RNA (siRNA) pool targeting Caprin-1 (siGENOME SMARTpool, human Caprin1) and control nontargeting siRNA were purchased from Dharmacon (Buckinghamshire, United Kingdom) and transfected into 293T cells using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol.