luciferase activity. To evaluate the interferon response, Huh7OK1 cells were seeded on a 48 well plate at a density of 2×10^4 cells per well, and transfected with pISRE-TA-Luc (Takara bio, Shiga, Japan) and phRG-TK (Promega). These transfected cells were incubated in the presence of compounds, IFN-α 2b, or DMSO, and then harvested at 48 h post-treatment. The firefly luciferase and *Renilla* luciferase activities were quantified by using Dual luciferase reporter assay system (Promega).

Prediction of Clog*P* for compounds

The *Clog*P value deduced from chemical structure roughly corresponds to a value of hydrophobicity. The *Clog*P values of compounds used in this study were calculated using the computer software Chem Bio Office Ultra 2008 (PerkinElmer, Cambridge, MA, USA).

Synergistic effect of caffeic acid n-octyl ester on anti-HCV activities of other drugs

The effects of drug-drug combinations were evaluated by using the Con1 LUN Sb #26 replicon cells, and were analyzed by using the computer software CalcuSyn (Biosoft, Cambridge, United Kingdom). Dose inhibition curves of two different drugs were plotted with each other. In each drug combination, EC_{90} values of several combinations of two different drugs were plotted as the fractional concentration (FC) of both drugs on the *x* and *y*-axes. Additivity indicates the line linked between 1.0 FC value points of both drugs in the absence of each other. Synergy and antagonism are indicated by values plotted under and above, respectively, an additivity line. The explanatory diagram of isobologram is shown in a right end of lower panels of Figure 5A. Combination indexes (CIs) were calculated at the EC_{50}, EC_{75}, and EC_{90} by using CalcuSyn. A CI value of less than 0.9 indicates synergy. A CI value ranging from 0.9 to 1.1 indicates additivity. A CI value of more than 1.1 indicates antagonism. The explanatory diagram was shown in a right end of lower panels of Figure 5B.

Supporting Information

Figure S1 Molecular structure of CAPE and commercial CAPE-related compounds. CAPE structure is divided into three parts: (I) the catechol moiety, (II) the alkenyl moiety on alpha, beta -unsaturated ester, and (III) the ester part. Molecular structures of CAPE and its commercial derivatives are shown.
(TIF)

Figure S2 The basic structure and side moieties of compounds shown in Table 2. Each compound structure is represented on the basis of the basic structure (top).
(TIF)

Figure S3 The molecular structures of compounds 7 and 12, which are shown in Table 3. Both compounds are different in alpha, beta-unsaturated or saturated part attached to ester.
(TIF)

Figure S4 The basic structure and side moieties of compounds shown in Table 4. Each compound structure is represented on the basis of the basic structure (top).
(TIF)

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Author Contributions

Conceived and designed the experiments: MT KM. Performed the experiments: H. Shen AY MN HY MS H. Shindo SM. Analyzed the data: HK TT NE. Contributed reagents/materials/analysis tools: YF MI NK NS. Wrote the paper: H. Shen AY MT KM.

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

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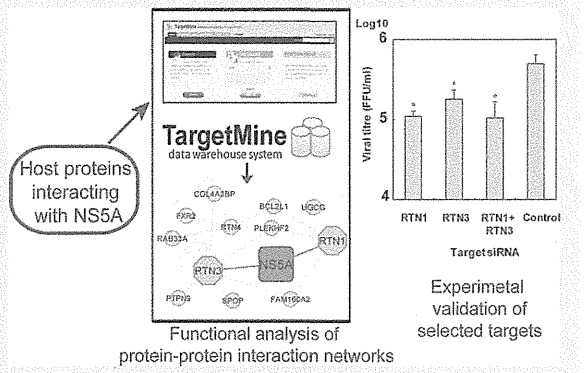
Supporting Information

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 NS5A 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 NS5A as bait to screen a library of human cDNAs with comprehensive literature mining. The analysis of the NS5A infection network illustrates the functional pathways likely to be influenced by NS5A–host interactions in HCV pathogenesis, thus providing novel insights into the NS5A 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 NS5A 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 NS5A encoding amino acids 1973–2419 (excluding the NS5A 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 NS5A 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 NS5A interacting host factors (Supporting Information, Table S1).

Literature Mining for Pairwise NS5A–Human Interactions

Literature information describing pairwise interactions between NS5A and cellular proteins were extracted from Medline using the PubMed interface and two other information retrieval and extraction tools, EBIMed[29] 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 "NS5A" and "HCV NS5A" keywords and interaction verbs (including "interact", "bind", "attach", "associate")[30] were gathered and manually examined to retrieve direct pairwise NS5A–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 3.1.93[31] and iRefindex 9.0[32] 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 NS5A interacting proteins were retrieved from the HPI and were appended to the NS5A–host interactions to construct a representative NS5A 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 plugin[36] 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 KEGG[39] were used to assign functional annotations to the genes in the NS5A infection network. The enrichment of specific biological associations within the NS5A 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 $p \leq 0.005$.

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 NS5A 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	104
1452	CSNK1A1	casein kinase 1, alpha 1	63,105
1457	CSNK2A1	casein kinase 2, alpha 1 polypeptide	84,106
1499	CTNNB1	catenin (cadherin-associated protein), beta 1, 88 kDa	22
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	this study
2051	EPHB6	EPH receptor B6	22
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	this study; 43,45
23770	FKBP8	FK506 binding protein 8, 38 kDa	12
2316	FLNA	filamin A, alpha	22
2495	FTH1	ferritin, heavy polypeptide 1	107
8880	FUBP1	far upstream element (FUSE) binding protein 1	22
2534	FYN	FYN oncogene related to SRC, FGR, YES	this study
11345	GABARAPL2	GABA(A) receptor-associated protein-like 2	22
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	108
3303	HSPA1A	heat shock 70 kDa protein 1A	109
3315	HSPB1	heat shock 27 kDa protein 1	22
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)	this study
6453	ITSN1	intersectin 1	22
3716	JAK1	Janus kinase 1	

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	SSC5D	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 (*T. californica*)	this study
55737	VPS35	vacuolar protein sorting 35 homologue (*S. cerevisiae*)	22
6293	VPS52	vacuolar protein sorting 52 homologue (*S. cerevisiae*)	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′-GAGTGTCGTGCAGCCTCCA-3′ and 5′-CACTCG-CAAGCACCCTATCA-3′, and 5′-GAAGGTCGGAGT-CAACGGATT-3′ and 5′-GATGACAAGCTTCCCGTTCTC-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% CO_2, 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 ± 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 NS5A Protein

We employed an integrated approach that combined an experimental Y2H assay and comprehensive literature mining to identify human host proteins interacting with NS5A.

First, we performed an Y2H screening to characterize the interactions between NS5A and host proteins. The analysis of positive colonies revealed 17 host factors as interacting partners of NS5A (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 NS5A;[28,43,45,46] Bridging integrator 1 (BIN1), a tumor suppressor protein, interacts with NS5A and significantly contributes to HCC.[47] Among the newly discovered interactors, MAP4K4 is overexpressed in HCC, and knockdown 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 NS5A 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 NS5A interactions in vivo.

We next scanned the biomedical literature to expand the repertoire of NS5A–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 NS5A and which were not present in the Y2H data set. One hundred and fifteen pairwise interactions between NS5A and human proteins (consisting of 93 catalogued by a high throughput study of binary HCV–host interactions[22] 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 NS5A–human interactome thus comprised 132 human host proteins directly interacting with NS5A (Table 1), all of which are expressed in the liver (see Supporting Information, Table S3).

Network Topological Analysis of the NS5A–host Interactions: NS5A Preferentially Targets Hubs and Bottlenecks in the Host Protein Interactome

To further understand the biological significance of the NS5A–host interactions, we retrieved PPIs for the nodes targeted by

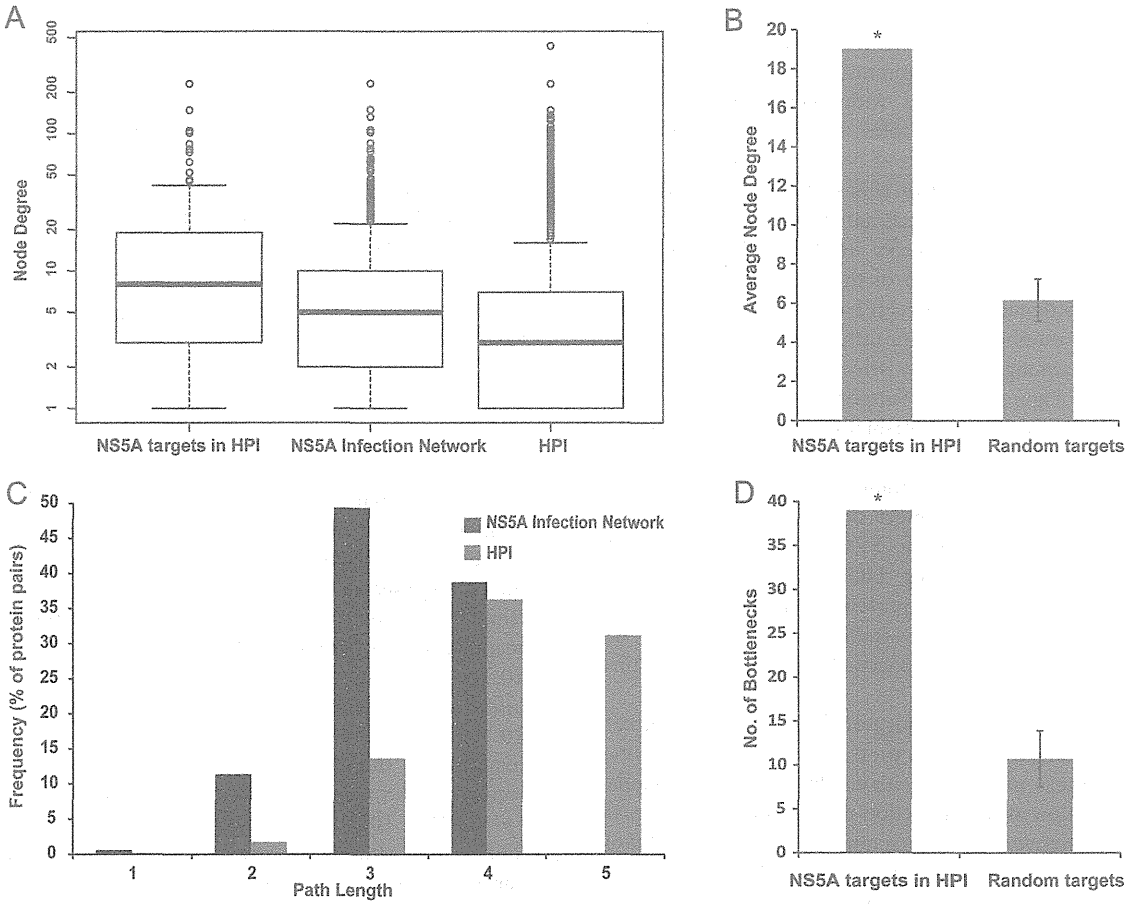

Figure 1. Topological analysis of the NS5A infection network. (A) The node degree distributions of the NS5A interatcors in the HPI, NS5A infection network, and HPI are represented as box plots. The average degree of the NS5A interactors in HPI (19.02) was higher than those of the NS5A infection network (8.24) and HPI (5.96). Median node degrees (indicated by thick horizontal lines) of the NS5A interactors in HPI, NS5A infection network, and HPI are 8, 5, and 3, respectively. (B) The average degree of the nodes targeted by NS5A 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 NS5A 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 NS5A in HPI was much higher than mean of the number of bottlenecks among 1000 sets of the randomly selected 108 nodes in HPI. *: $p < 0.001$.

NS5A in the HPI and incorporated them with the initial interactions to infer an extended NS5A infection network. PPIs for 108 of 132 NS5A interactors were retrieved in this manner; 24 of 132 NS5A interactors had no PPIs in the HPI (Supporting Information, Tables S4, S5a, S5b). For the NS5A 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 NS5A 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 NS5A 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 NS5A 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 NS5A 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 NS5A 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 NS5A 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 NS5A 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 NS5A interactors in the HPI to assess their significance in the HPI and the NS5A 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 NS5A preferentially targets bottlenecks (defined as the top 10% of the nodes in the HPI ranked by betweenness), we estimated the fraction of NS5A interactors that were bottlenecks in the HPI. A significant proportion (39 of 108; 36.1%) of the NS5A 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 NS5A;[55] tumor protein 53 (TP53), a key mediator of the oncogenic effect of NS5A in HCV-induced HCC;[56] and tyrosine kinase SRC, which regulates the formation of NS5A-containing HCV replication complex.[57] Among the NS5A 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 NS5A 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 NS5A Interaction Network

Next, we investigated the NS5A 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 NS5A 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 NS5A 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 NS5A, identified by our Y2H assay (Table 1). The significant representation of cellular kinases in the NS5A infection network is consistent with the key roles played by reversible phosphorylation of NS5A in modulating various NS5A functions in HCV pathogenesis. Impairing NS5A hyperphosphorylation has been shown to inhibit HCV replication, and thus, the cellular kinases that regulate NS5A phosphorylation are important targets for anti-HCV therapy.[9,60−63]

The analysis of NS5A infection network revealed an enrichment of 79 KEGG pathways (Supporting Information, Table S7a). Furthermore, 31 of the 39 NS5A 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 NS5A infection network, with a specific focus on the bottlenecks.

Cancers and Infectious Diseases

The analysis of the NS5A interaction network revealed that NS5A specifically targets host factors that participate in various complex human diseases. Thirty-four NS5A 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 "Prostrate 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 NS5A interaction network revealed that 21 of the 132 NS5A 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 NS5A 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, GPS2, GRB2, GSK3B, HSPB1, JAK1, LCK, LYN, PIK3R1, PPP2CA, RAF1, SRC, STAT1, SYK, TBP, TGFBR1, TP53, TRAF2	"Epstein–Barr virus infection"; "HTLV-I 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, TGFBR1, 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, TGFBR1, 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, TGFBR1, THBS1	"Focal adhesion"; "Tight junction"; "Adherens junction"
development	3	12	AKT1, FHL2, FYN, GRB2, GSK3B, JAK1, LCK, PIK3R1, STAT1, SYK, TGFBR1, 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 NS5A 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, NS5A 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 NS5A 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 NS5A 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 NS5A interactions with the key facilitators of MAPK signaling in the host interactome may play an important role in regulating the reversible phosphorylation of NS5A 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 NS5A 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 NS5A, 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 NS5A and IR. Our observations, however, suggest that NS5A directly interacts with key regulators of insulin metabolism and may, therefore, play a major role in modulating HCV-induced IR and eventually HCC.

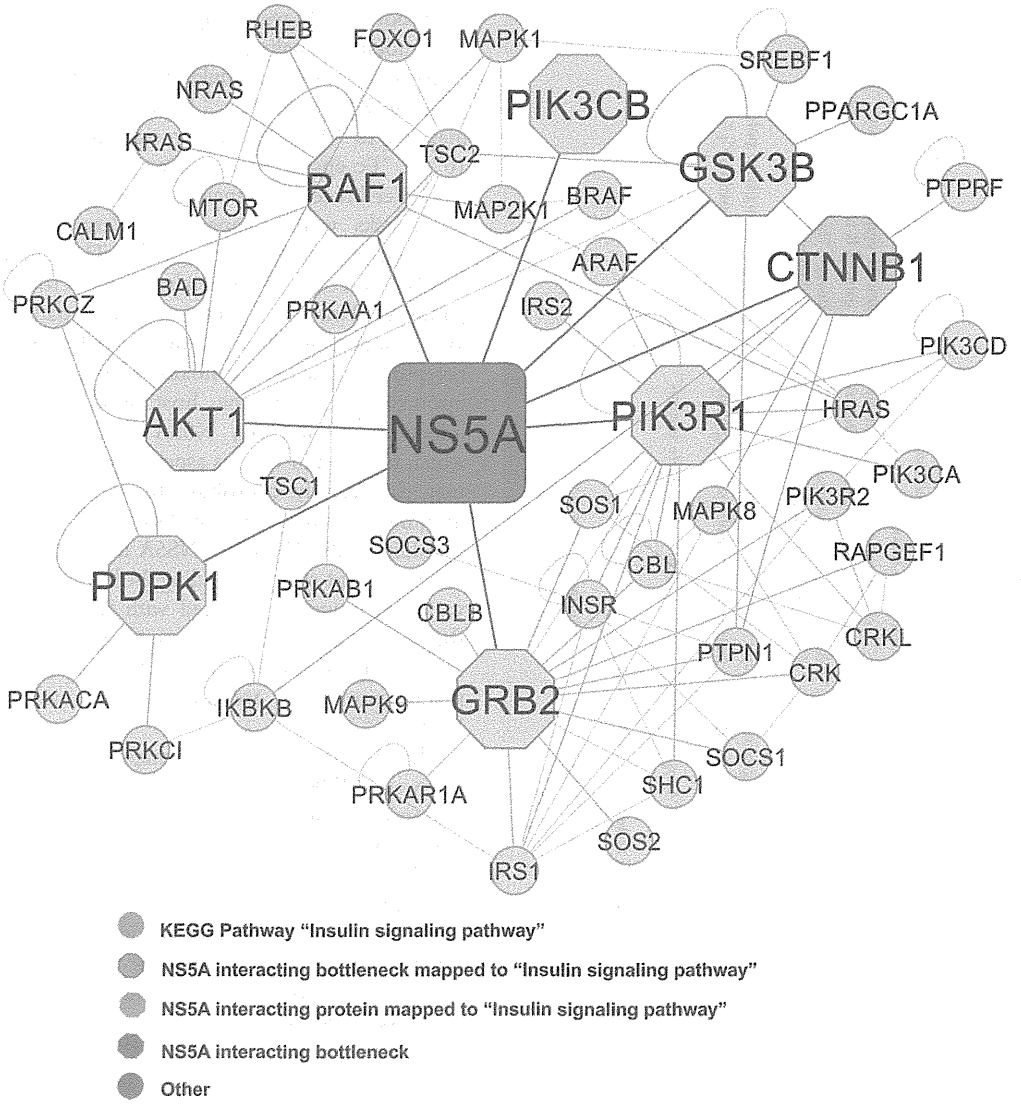

Figure 2. NS5A interacting bottlenecks and their interacting partners associated with the enriched KEGG pathway hsa04910: "Insulin signaling pathway".

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-β1, 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.

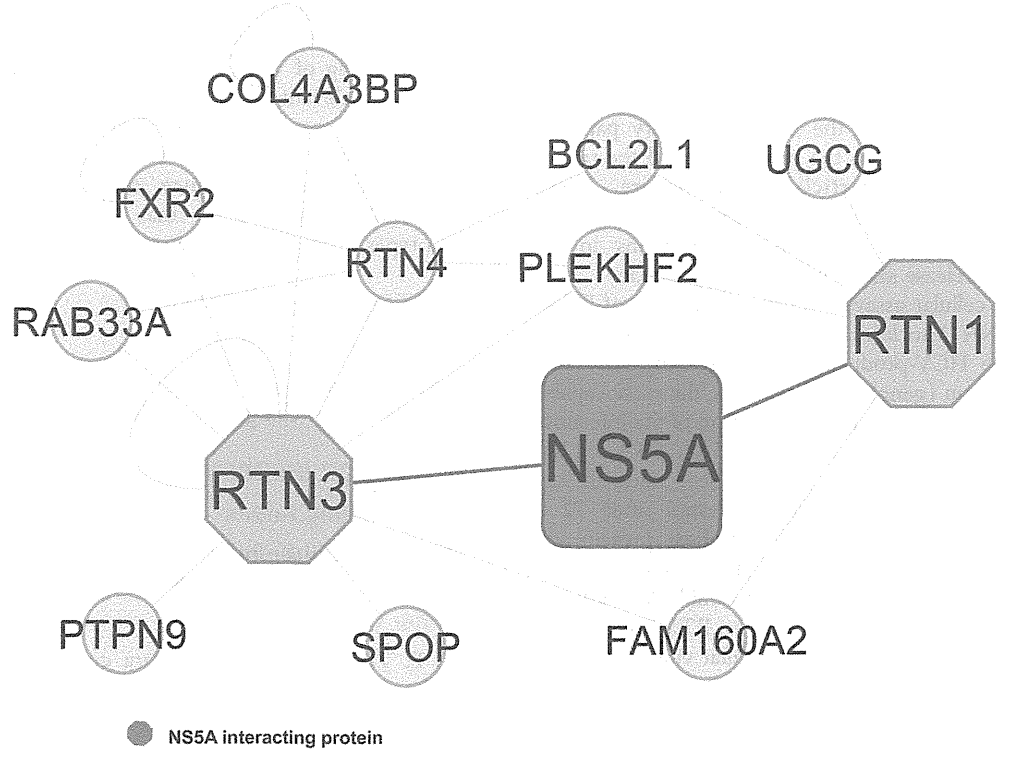

Figure 3. ER-localized host factors RTN1 and RTN3 were found to interact (blue edges) with NS5A in an Y2H screening of human liver cDNA library using NS5A as bait.

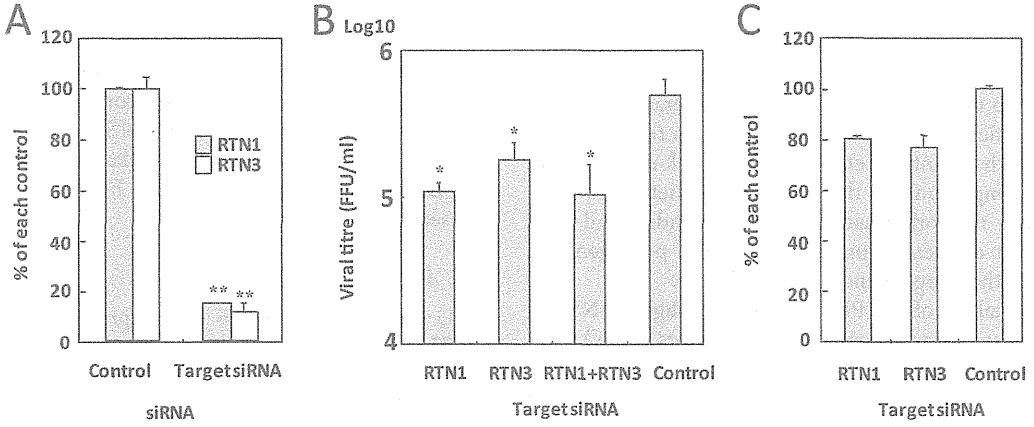

Figure 4. Effects of knockdown of RTN1 and RTN3 on HCV propagation and replication. Host factors RTN1 and RTN3 were suppressed by RNAi (A) in Huh7OK1 cells infected with HCV JFH1 strain (genotype 2a). The amounts of viral titer (B) and intracellular viral RNA (C) were estimated. Each value was represented 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 NS5A infection network, NS5A 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, NS5A interaction with ARAP1 may facilitate EGFR internalization and thus viral entry in HCV infection.

NS5A 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, NS5A 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 NS5A 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 NS5A and host proteins. We proceeded to map the combined NS5A−host interactions onto an overall interaction network, which comprised a repertoire of connections, which potentially enable NS5A 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 NS5A 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 NS5A 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 NS5A 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 NS5A−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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VIRAL HEPATITIS

Ca^{2+}/S100 proteins regulate HCV virus NS5A–FKBP8/FKBP38 interaction and HCV virus RNA replication

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Abstract

Background & Aim: FKBP8/FKBP38 is a unique FK506-binding protein with a C-terminal membrane anchor and localizes at the outer membranes of mitochondria and the endoplasmic reticulum. Similar to some immunophilins, such as FKBP51, FKBP52 and Cyclophilin 40, FKBP8/FKBP38 contain a putative Calmodulin-binding domain and a tetratricopeptide-repeat (TPR) domain for the binding of Hsp90. Both Hsp90 and the non-structural protein 5A (NS5A) of the hepatitis C virus (HCV) interact specifically with FKBP8/FKBP38 through its TPR domain, and the ternary complex formation plays a critical role in HCV RNA replication. The goal of this study is to evaluate that the host factor inhibits the ternary complex formation and the replication of HCV *in vitro* and *in vivo*. *Methods:* S100 proteins, FKBP38, FKBP8, HCV NS5A, Hsp90, and calmodulin were expressed in E.coli and purified. *In vitro* binding studies were performed by GST pull-down, S-tag pull-down and surface plasmon resonance analyses. The effect of S100 proteins on HCV replication was analysed by Western blotting using an HCV NS3 antibody following transfection of S100 proteins into the HCV replicon harbouring cell line (sO cells). *Results: In vitro* binding studies showed that S100A1, S100A2, S100A6, S100B and S100P directly interacted with FKBP8/FKBP38 in a Ca^{2+}-dependent manner and inhibited the FKBP8/FKBP38–Hsp90 and FKBP8/FKBP38–NS5A interactions. Furthermore, overexpression of S100A1, S100A2 and S100A6 in sO cells resulted in the efficient inhibition of HCV replication. *Conclusion:* The association of the S100 proteins with FKBP8/FKBP38 provides a novel Ca^{2+}-dependent regulatory role in HCV replication through the NS5A–host protein interaction.

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease, which frequently progresses to cirrhosis and hepatocellular carcinoma (1, 2). HCV represents a global public health problem, affecting approximately 170 million people worldwide, which is more than 3% of the world population (3, 4). HCV is a member of the positive strand RNA viruses, belongs to the family Flaviviridae, genus Hepacivirus, and contains a genome of approximately 9.6 kb in length, which encodes a large polyprotein precursor of approximately 3000 amino acids (5, 6). The polyprotein is cleaved by host and viral proteases to release the individual enzymes and proteins that mediate virus replication, assembly and release, producing viral structural proteins (Core, E1 and E2), a putative viropore protein (p7) and non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) (5, 6). Importantly, the NS5A of HCV plays a critical role in HCV replication and is an attractive target for antiviral therapy of HCV infection (7). NS5A is a multifunctional 56–58 kDa serine phosphoprotein and interacts with a number of cellular proteins thereby affecting numerous host functions, including the modulation of signal transduction pathways, suppression of apoptosis and modulation of transcription (8–10). Recently, FK506-binding protein 8 (FKBP8)/FK506-binding protein 38 (FKBP38) was shown to interact with NS5A and to regulate HCV replication (11, 12),

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suggesting that the immunophilins are promising therapies for chronic hepatitis C. FKBP8/FKBP38 binds to both NS5A and heat-shock protein 90 (Hsp90) through the tetratricopeptide-repeat (TPR) domain, and the ternary complex (FKBP8/FKBP38–Hsp90–NS5A) plays a critical role in HCV replication (11, 12). TPRs are loosely conserved 34 amino acid helix–turn–helix sequence motifs that mediate protein–protein interactions (13). This property enables TPR-containing proteins to function as scaffold proteins and allows them to be involved in a variety of cellular functions (14, 15).

The S100 protein family is composed of at least 25 members that share two EF-hand motifs, a 25–65% amino acid sequence homology and a molecular weight of 10–12 kDa (16–18).

Recently, we demonstrated that S100A2 and S100A6 interacted with the TPR domains of Hsp70/Hsp90-organizing protein (Hop), kinesin light chain (KLC) and Tom70 in a Ca^{2+}-dependent manner, leading to the dissociation of the Hsp90–Hop–Hsp70, KLC–JIP1 and Tom70–Hsps interactions *in vitro* and *in vivo* (19). Further studies have revealed an interaction of S100A1 and S100A2 bound to FK506-binding protein 52 (FKBP52) and cyclophilin 40 (Cyp40), which contain a TPR domain; in the presence of Ca^{2+}, this interaction led to the inhibition of the Cyp40–Hsp90 and FKBP52–Hsp90 interactions (20).

Because S100 proteins interact with TPR motifs (19, 20), we explored the potential role for S100 proteins in the regulation of the FKBP8/FKBP38–HSP90 and/or FKBP8/FKBP38–NS5A interactions and thereby the control of HCV replication. In this study, we demonstrate that Ca^{2+}/S100 proteins modulate replication of HCV via two different mechanisms. Firstly, S100A1, S100A2, S100A6, S100B and S100P interact with the TPR domain of FKBP8/FKBP38 and compete with Hsp90 binding to FKBP38. Secondly, these S100 proteins inhibit the interaction between NS5A and FKBP8/FKBP38. Using HCV replicon harbouring cells (sO cell: HCV O strain of genotype 1b) (21, 22), the overexpression of S100A1, S100A2 and S100A6 with A23187 treatment has shown a significant decrease in NS3 expression. These observations indicate that Ca^{2+}/S100 proteins could modulate HCV replication by inhibiting the interaction between FKBP8/FKBP38 and its binding partners.

Materials and methods

Materials

Phenyl–Sepharose and glutathione–Sepharose were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Nickel–nitrilotriacetic acid agarose was purchased from Qiagen. S-protein agarose was purchased from Merck. Antibodies were obtained as follows: anti-S100A1 (Novus Biologicals, Littleton, Colorado, USA), anti-S100A2 (ANOVA, Santa Clara, CA, USA), anti-S100A6 (Proteintech Group Inc., Chicago, IL, USA), Anti-HCVNS3 (Leica Microsystems, Wetzlar, Germany), anti-β-actin (BioVision, Milpitas, CA, USA) and horseradish peroxidase-conjugated antimouse or antirabbit IgG antibody (GE Healthcare). All other chemicals were obtained from standard commercial sources.

Plasmids

The following plasmids were previously described: pME18S-S100A1, -S100A2, -S100A6, pET-Calmodulin (pET-CaM), pET-CaM-Glutathione-S-transferase (pET-CaM-GST) and pET16b-Hsp90 (19, 20, 23, 24). Human FKBP38 and FKBP8 complementary DNAs (cDNAs) were purchased from Open Biosystems and subcloned into pET30a. For the expression of FKBP38 C-terminal deletion mutants, polymerase chain reaction (PCR) fragments encoding residues 1–166, −297, −315, −325 and −335 were also cloned into pET30a. To create a plasmid of the pET30a-FKBP38 carboxylate clamp mutant (K250E/R254E), the plasmid was generated using inverse PCR using pET30a-FKBP38 (1–335) as a template. pETUbHis-NS5A was kindly provided by Dr Craig E. Cameron (25). The sequence integrity of the all inserts was confirmed through automated sequence analysis (Applied Biosystems, Foster City, CA, USA).

Preparation of recombinant proteins

All recombinant proteins were produced in *Escherichia coli* strain BL21(DE3) or BL21(DE3) CodonPlus-RIL (Novagen, Darmstadt, Germany). S100 proteins (S100A1, S100A2, S100A4, S100A6, S100A10, S100A11, S100A12, S100B and S100P) were expressed and prepared as described previously (26, 27). Calmodulin (CaM) was prepared as described by Hayashi and colleagues (28). C-terminally GST-tagged CaM linked by a Gly_6 spacer (CaM-GST) was prepared as described (23, 24). A C-terminally His-tagged NS5A, lacking the N-terminal 32 amino acid residues of the membrane-anchoring region (NS5A-His), was expressed via the pET-ubiquitin expression system, in which the NS5A-His was fused with ubiquitin at the C-terminus and was cleaved by a ubiquitin-specific protease, Ubp1, in *E. coli* as described by Huang and colleagues (25). The His-tagged Hsp90 and NS5A were purified via the nickel–nitrilotriacetic acid (NTA)-agarose method according to the manufacturer's protocol. Protein expression of N-terminally His_6 and S-tagged FKBP38 (His_6-S-tag -FKBP38) and FKBP8 (His_6-S-tag-FKBP8) was induced with the addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 1 mM and further incubation at 16°C for 4 h. The His_6-S-tag-FKBP38 mutants (1–166, −297, −315, −325) and the His_6-S-tag-FKBP8 mutants (1–382) were purified using NTA agarose. The His_6-S-tag-FKBP38 (1–335), His_6-S-tag-FKBP8 (1–392) and His_6-S-tag-FKBP38-K250E/R254E mutants were purified with CaM-GST-coupled glutathione–Sepharose

columns as follows. The bacterial pellet from 200 ml of culture was resuspended in 10 ml of Buffer A (20 mM Tris-HCl, 200 mM NaCl and 5 mM dithiothreitol, pH 7.5), lysed using ultrasonic disruption and centrifuged at 35 000 *g* for 30 min at 4°C. The purified CaM-GST (3 mg) and $CaCl_2$ (2 mM final concentration) were mixed with the supernatant. The resulting mixture was applied to a glutathione – Sepharose column (1 ml bed volume; GE Healthcare) and then the column was washed with 5 ml of Buffer A supplemented with 0.2 mM $CaCl_2$. His_6-S-tag-FKBP38 or His_6-S-tag-FKBP8 was eluted using Buffer A supplemented with 0.5 mM ethylene glycol tetraacetic acid (EGTA). The concentration and purity of the isolated proteins were determined with a Bradford assay (Bio-Rad, Hercules, CA, USA) and Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE).

Pull-down assay

To assess the binding of S100 proteins, Hsp90 and NS5A to FKBP8/FKBP38, His_6-S-tag-FKBP8/FKBP38 and either S100 proteins, Hsp90 or NS5A, were mixed with S-protein-agarose beads (20 µl) in Buffer B (20 mM Tris-HCl, 150 mM NaCl and 0.02% Tween 20, pH 7.5) in the presence of 1 mM $CaCl_2$ or EGTA. The reaction mixtures (200 µl) were incubated for 60 min at 25°C. After the resin was washed three times with 1.0 ml of Buffer B, the resin was boiled in SDS-sample buffer (30 µl). Next, the samples were subjected to SDS-PAGE and visualized with Coomassie Blue staining. The details of the experimental conditions are described in the figure legends.

Surface Plasmon Resonance (SPR)

Binding kinetics were analysed using a SPR Biacore 2000 system (Biacore AB, Little Chalfont, Buckinghamshire, UK). CM5 research grade chips, N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Biacore amine coupling kit) were used for the amine coupling of the recombinant N-terminally His_6 and S-tagged FKBP8 (His_6-S-tag-FKBP8) (4540RU) to the dextran surface of the CM5 chip. Recombinant S100A1, S100A2, S100A6, S100B, S100P and CaM (1.25 µM, 625 nM, 313 nM, 156 nM and 78 nM respectively) were injected over the sensor surface at a flow rate of 20 µl/min in HBS-P buffer (10 mM Hepes, 150 mM NaCl and 0.005% Surfactant P20, pH 7.4) containing 1 mM $CaCl_2$. The S100 proteins were allowed to interact with the surface of the sensor chip for 2.5 min, after which HBS-P buffer was injected over the sensor surface to monitor the dissociation of the S100 protein. At intervals based on the sample injection, an FKBP8-coupled sensor chip was regenerated with HBS-P buffer supplemented with 2.5 mM EGTA and 0.75% n-Octyl-ß-D-glucopyranoside. The response curves were prepared for fit using a subtraction of the signal generated simultaneously on the control flow cell. Biacore sensorgram curves were evaluated in BIA-evaluation 4.1 using a 1:2 binding model for the S100 proteins. A 1:1 Langmuir model was used for the CaM binding.

Cell culture, transfection and preparation of cell lysates

HuH-7-derived cells harbouring HCV replicon harbouring cells (sO cells) were maintained in Dulbecco's Modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) containing 0.3 mg/ml G418 (Promega, Fitchburg, MA, USA) supplemented with 10% fetal bovine serum (Invitrogen) in a humidified 5% CO_2 incubator at 37°C as described (21). Transient transfections were performed using Fugene 6 (Roche, Mannheim, Germany) according to the manufacturer's instructions. The sO cells (10 cm dish) were transfected with pME18S-S100A1, pME18S-S100A2 or pME18S-S100A6 (each 7 µg). After 8 h of incubation with the transfection reagents, the medium was changed and cells were grown until 100% confluent. Next, the cells were treated with or without A23187 (5 µM) for 6, 12 and 24 h and were then lysed via the addition of 1 ml of the lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 0.5% Nonidet-P40, 10% glycerol, pH 7.5), briefly sonicated and centrifuged at 15 000 *g* for 30 min at 4°C. The supernatants were subjected to SDS-PAGE, followed by Western blot analysis.

Results

Interaction between the members of the S100 protein family and FKBP38

Previously, we demonstrated that S100A1, S100A2 and S100A6 interacted with the TPR domains of Hop, Tom70, CyP40, FKBP52 and KLC in a Ca^{2+}-dependent manner and led to the disruption of the TPR protein –client protein interactions *in vitro* and *in vivo* (9, 20). Because S100 proteins interact with TPR motifs, we explored the potential role of S100 proteins in regulating the functions of FKBP8/FKBP38. Firstly, we examined the interaction of the S100 proteins with FKBP8/FKBP38. FKBP8/FKBP38, a TPR-containing non-canonical member of the immunosuppressive drug FK506-binding protein (FKBP) family, consists of four structural and functional domains. The FK506-binding domain is located in the N-terminal half, followed by a TPR domain, a putative CaM-binding site and a transmembrane domain. Human FKBP8 is identical to FKBP38 except for the extra 58 amino acid residues at the N-terminus. The domain structure organization of FKBP8/FKBP38 is shown in Figure 1a. Because our preliminary binding analyses demonstrated that the last 10 residues immediately upstream of the transmembrane domain (^{326}AWSIPWKWLF^{335}) in FKBP38, but not the putative CaM-binding site, compose the actual