Table 1. Summary of PowerBlot Detected Protein Expression Levels in Protein Samples

CoreTGvsWT					
protein	gene ID	symbol	confidence level[a]	(−) under, (+) over[b]	fold change[c]
P31749	207	AKT1	3	−	2.68
P07355	302	ANXA2	3	+	2.92
O43747	164	AP1G1	3	+	5.72
P63010	163	AP2B1	3	+	2.40
Q96CW1	1173	AP2M1	2	+	1.93
Q9Y2T2	26985	AP3M1	2	+	1.89
P05089	383	ARG1	2	+	2.08
P52566	397	ARHGDIB	3	+	2.02
O15145	10094	ARPC3	2	−	2.25
P49407	408	ARRB1	3	−	2.33
Q07812	581	BAX	3	+	2.03
P55212	839	CASP6	3	−	1.95
Q14790	841	CASP8	2	+	2.18
Q03135	857	CAV1	2	+	2.07
P12830	999	CDH1	3	−	2.33
P19022	1000	CDH2	3	+	4.57
Q53SH4	1134	CHRNA1	3	−	3.11
P21964	1312	COMT	2	+	2.96
P00450	1356	CP	3	+	2.36
P21291	1465	CSRP1	3	+	2.23
P49711	10664	CTCF	3	+	6.13
P25685	3337	DNAJB1	3	−	2.16
P63241	1984	EIF5A	3	+	1.94
P42566	2060	EPS15	3	+	4.28
Q92889	2072	ERCC4	3	+	5.43
O75899	9568	GABBR2	3	+	3.39
O43719	27336	HTATSF1	3	+	5.76
P06756	3685	ITGAV	3	+	6.32
Q14974	3837	KPNB1	3	−	1.86
Q16539	1432	MAPK14	3	−	2.81
Q9UPY8	22924	MAPRE3	3	+	2.46
P49736	4171	MCM2	3	+	1.87
P62166	23413	NCS1	3	−	2.24
Q8IZ57	140767	NRSN1	3	+	1.89
Q16620	4915	NTRK2	3	+	2.40
Q14980	4926	NUMA1	3	−	1.94
P07237	5034	P4HB	3	+	2.27
Q92878	10111	RAD50	3	+	4.93
Q99638	5883	RAD9A	2	−	3.10
P20936	5921	RASA1	3	+	1.86
Q96SB4	6732	SRPK1	3	+	3.11
Q92186	8128	ST8SIA2	3	+	5.11
P42224	6772	STAT1	3	+	2.00
P40763	6774	STAT3	3	+	2.30
Q9UNK0	9482	STX8	3	+	1.88
Q12800	7024	TFCP2	3	+	5.04
Q92752	7143	TNR	3	+	5.36
Q13263	10155	TRIM28	3	+	4.70
O43396	9352	TXNL1	3	−	4.82
P50552	7408	VASP	2	−	2.61
Q96AJ9	143187	VTI1A	3	+	3.25
Q14191	7486	WRN	3	+	17.12

altered protein levels in $PA28\gamma^{-}/^{-}$CoreTGvsWT					
protein	gene ID	symbol	confidence level[a]	(−) under, (+) over[b]	fold change[c]
O15145	10094	ARPC3	2	−	1.96
P49407	408	ARRB1	3	−	2.17
P55212	839	CASP6	3	−	2.04
O60508	51362	CDC40	2	+	1.99
P54105	1207	CLNS1A	3	+	2.41
P21964	1312	COMT	2	+	2.71
P67870	1460	CSNK2B	3	+	1.90
P78352	1742	DLG4	2	+	4.08
Q95GK7	1837	DTNA	3	−	2.42
P55010	1983	EIF5	3	+	2.19
Q08495	2039	EPB49	3	+	2.66
P37268	2222	FDFT1	2	+	5.68
P09038	2247	FGF2	2	+	2.69
P62962	2280	FKBP1A	2	+	1.89
O75146	9026	HIP1R	2	−	2.08
Q9NZL4	23640	HSPBP1	3	+	3.46
P05412	3725	JUN	2	+	2.15
P52292	3838	KPNA2	3	−	7.28
P36507	5605	MAP2K2	3	−	2.35
Q16539	1432	MAPK14	3	−	3.29
P22033	4594	MUT	3	+	2.46
P54920	8775	NAPA	2	−	1.97
Q8IZ57	140767	NRSN1	3	+	1.93
Q16620	4915	NTRK2	3	+	2.50
P07237	5034	P4HB	3	+	2.04
Q08209	5530	PPP3CA	3	+	7.55
Q06124	5781	PTPN11	2	+	2.33
Q99638	5883	RAD9A	2	−	1.97
P43487	5902	RANBP1	3	+	2.29
Q9UPX8	22941	SHANK2	3	−	1.94
P29353	6464	SHC1	3	+	3.27
Q92186	8128	ST8SIA2	3	+	4.06
P31948	10963	STIP1	3	−	1.99
O75558	8676	STX11	2	+	2.04
P23193	6917	TCEA1	3	−	2.17
P07101	7054	TH	2	+	2.78
P13693	7178	TPT1	3	−	1.93
Q15628	8717	TRADD	3	−	2.00
P50607	7275	TUB	3	+	1.91

altered protein levels in $PA28\gamma^{-}/^{-}$CoreTGvsCoreTG					
protein	gene ID	symbol	confidence level[a]	(−) under, (+) over[b]	fold change[c]
P07355	302	ANXA2	3	−	2.96
O43747	164	AP1G1	3	−	4.20
P63010	163	AP2B1	3	−	3.01
Q96CW1	1173	AP2M1	2	−	1.88
Q9Y2T2	26985	AP3M1	3	+	2.38
O00499	274	BIN1	2	−	1.88
Q9UQM7	815	CAM2KA	3	+	2.06
Q8N5S9	84254	CAMKK1	2	+	5.78
P19022	1000	CDH2	3	−	3.85
P25108	1134	CHRNA1	3	+	2.55
P49674	1454	CSNK1E	3	+	1.97
P67870	1460	CSNK2B	3	+	1.88
P21291	1465	CSRP1	3	+	1.87
P49711	10664	CTCF	3	−	5.43
Q8WTW3	9382	COG1	3	−	7.02
P00450	1356	CP	3	−	3.55
Q13618	8452	CUL3	3	+	1.91
P78352	1742	DLG4	2	+	2.13
Q9Y4J8	1837	DTNA	3	−	2.94

Table 1. continued

altered protein levels in PA28$\gamma^{-/-}$CoreTGvsCoreTG					
protein	gene ID	symbol	confidence level[a]	(−) under, (+) over[b]	fold change[c]
Q08495	2039	EBP49	3	+	2.30
O14682	8507	ENC1	3	+	2.88
P42566	2060	EPS15	3	−	2.11
Q92889	2072	ERCC4	3	−	3.49
P09038	2247	FGF2	2	−	2.18
P62962	2280	FKBP1A	2	+	2.59
P49356	2342	FNTB	2	+	1.95
O75899	9568	GABBR2	3	−	2.53
O75146	9026	HIP1R	3	−	1.97
Q9NZL4	23640	HSPBP1	3	+	3.60
P61604	3336	HSPE1	3	−	2.17
Q99730	27336	HTATSF1	3	−	9.24
Q9Y6K9	8517	IKBKG	2	+	1.97
P52292	3838	KPNA2	3	−	3.94
P36507	5605	MAP2K2	3	−	2.66
Q13505	4580	MTX1	3	−	1.90
P62166	23413	NCS1	3	+	2.56
Q14980	4926	NUMA1	2	+	1.87
P41236	5504	PPP1R2	3	−	2.25
Q08209	5530	PPP3CA	3	+	12.94
P13861	5576	PRKAR2A	3	−	1.88
Q15276	9135	RABEP1	2	−	2.74
Q92878	10111	RAD50	3	−	5.19
P20936	5921	RASA1	3	+	2.50
P06400	5925	RB1	3	+	2.50
Q92854	10507	SEMA4D	2	+	2.00
Q92529	53358	SHC3	3	−	1.90
P63208	6500	SKP1	3	−	2.45
P43004	6506	SLC1A2	2	+	2.27
Q4U2R8	9356	SLC22A6	3	−	2.23
P42224	6772	STAT1	3	−	1.90
P31948	10963	STIP1	3	−	1.98
O75558	8676	STX11	2	+	3.52
Q8IZU3	50511	SYCP3	3	+	1.88
P07101	7054	TH	2	+	2.62
Q92752	7143	TNR	3	−	4.62
O43396	9352	TXNL1	2	+	3.05
P13693	7178	TPT1	3	−	2.85
Q13263	10155	TRIM28	3	−	3.53
Q15628	8717	TRADD	3	−	2.98
P50607	7275	TUB	3	+	1.96
P41542	8615	USO1	3	−	2.05
Q14191	7486	WRN	3	−	3.44

[a]Defined as follows: Level 3 = changes greater than 2-fold from good quality signals that also pass a visual inspection. Level 2 = changes greater than 2-fold from good quality signals that do not pass a visual inspection. [b]+ indicates an increase in protein level in the experimental sample relative to control. − indicates a decrease in protein level in the experimental sample relative to control. [c]A semiquantitative value that represents the general trend of protein changes for the experimental sample relative to control.

by performing the hypergeometric test within TargetMine.[16] The inferred p-values were further adjusted for multiple test correction to control the false discovery rate using the Benjamini and Hochberg procedure,[26,27] and the annotations/pathways were considered significant if $p \leq 0.05$.

Transcription Factor-Target Associations

Transcription factor (TF)-target associations for the differentially expressed proteins were retrieved from the TF-target repository compiled from Amadeus[28] and ORegAnno[29] in TargetMine[16] and are discussed in the Supporting Information.

RNAi and Transfection

The siRNA pair targets to VTI1A, STX8, and COMT were purchased from Ambion (Ambion, Austin, TX, USA). Stealth RNAi Negative Control Low GC Duplex (Invitrogen, Carlsbad, CA, USA) was used as a control siRNA. Each siRNA duplex was introduced into the cell lines by using lipofectamine RNAiMax (Invitrogen). Ambion ID numbers of siRNA duplex of VTI1A and STX8 were S225671 and S18183, respectively. 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.

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 a 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′-CACTCGCAAGCACCCTATCA-3′, and 5′-GAAGGTCGGAGTCAACGGATT-3′ and 5′-TGATGACAAGCTTCCCGTTCTC-3′, respectively.[30] The quantities of the HCV genome and the other host mRNAs were normalized with that of GAPDH mRNA. VTI1A and STX8 genes were amplified using the primer pairs 5′-TGACAGGGATGTTGCGAAGA-3′ and 5′-CAACCCACATGCAAACAGGA-3′, and 5′-TTGAAGGGGACCGAAGACAGAACCTC-3′, and 5′-TCAAAACCCAAGCCTCTGGTCTCCT-3′, respectively.

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.[30] These cells were maintained at 37 °C in a humidified atmosphere and 5% CO_2, in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with nonessential amino acids (NEAA), sodium pyruvate, and

10% fetal calf serum (FCS). The human hepatoma cell line Huh7, harboring the full genome of the HCV Con1 strain (genotype 1b), was prepared as described by Pietschmann et al.[31] We also established an Huh7 cell line harboring the subgenome of the JFH1 strain by the transfection of the plasmid pSGR-JFH1.[32] The Huh7-derived cell lines harboring a full length HCV replicon were maintained in DMEM containing 10% FCS, nonessential amino acids, sodium pyruvate, and 1 mg/mL G418 (Nakarai Tesque, Tokyo, Japan). The viral RNA of JFH1 was introduced into Huh7OK1 as described by Wakita et al.[33] The viral RNA of JFH1 derived from the plasmid pJFH1 was prepared as described by Wakita et al.[33]

Statistical Analysis

Experiments for RNAi transfection and qRT-PCR were performed three times. The estimated values were represented as the mean ± standard deviation (n = 3). The significance of differences in the means was determined by the Student's t test.

RESULTS AND DISCUSSION

Core Expression and PA28γ Knockout Induce Substantial Changes in the Expression Levels of Host Proteins Associated with HCV Infection in the Liver

The PowerBlot immunoblots showed proteins with increased or decreased levels (defined as those that displayed >1.8-fold change in abundance) in the transgenic samples relative to the WT samples and also relative to each other. In all, we identified 37 proteins with increased levels and 15 proteins with decreased levels in CoreTGvsWT, 24 proteins with increased levels and 15 proteins with decreased levels in PA28$\gamma^{-/-}$-CoreTGvsWT, and 26 proteins with increased levels and 36 proteins with decreased levels in PA28$\gamma^{-/-}$CoreTGvsCoreTG. While most proteins with altered abundance display changes between 1.8-fold and 6-fold, some proteins displayed much higher fold changes. For instance, WRN protein levels increased 17-fold in CoreTGvsWT (Table 1).

Our analysis detected changes in the abundance of proteins, known to be associated with HCV pathogenesis, in the liver samples from CoreTG compared with WT. These include Arginase I (ARG1; +2.08-fold), a liver enzyme associated with the polyamine metabolism, which is known to be overexpressed in HCV-mediated hepatocarcinogenesis;[34] STAT3 (+2.30-fold), which is directly activated by the Core and HCV-mediated oxidative stress facilitating tumorigenesis and is also essential for HCV replication;[35−37] STAT1 (+2-fold), which interacts with Core and facilitates the HCV-mediated attenuation of the host interferon signaling;[38] and MAPK14 (p38 MAPK; −2.81-fold), which is cooperatively activated by Core and ethanol in HCV infection[39] (Table 1). These results are in line with the previous observations that Core expression can induce HCV pathogenesis and hepatocarcinogenesis in transgenic mice.[2] Among other examples, BIN1, which interacts with the HCV NS5A protein and contributes to the pathogenesis of HCC,[40] was suppressed 1.88-fold in PA28$\gamma^{-/-}$CoreTGvsCoreTG; this is consistent with the lack of HCC pathogenesis in PA28$\gamma^{-/-}$CoreTG mice. Similar studies have aimed to characterize the global changes in the host transcriptome and proteome in response to HCV infection.[41−44] These studies, however, have not provided specific insights into PA28γ's roles in HCV pathogenesis. Our observations suggest that the PowerBlot assay was able to capture successfully some of the molecular signatures associated with the Core-PA28γ interplay in HCV pathogenesis.

Topological Analysis of the Extended Protein Interaction Networks

To further understand the biological significance of the differential protein levels, we retrieved PPIs for the proteins with increased and decreased levels in CoreTGvsWT, PA28$\gamma^{-/-}$CoreTGvsWT, and PA28$\gamma^{-/-}$CoreTGvsCoreTG and inferred the corresponding extended protein interaction networks for each data set using TargetMine (see Materials and Methods). First, we computed the *node degree distribution* and *characteristic/average path length* measures to capture the topology of the extended PPI networks as described earlier.[22] 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 may have a higher ability of influencing biological networks via PPIs. Average path lengths provide an approximate measure of the relative ease and speed of transfer of information between the proteins in a network. The CoreTGvsWT extended network was made up of 1373 entities (proteins) with 12535 interactions, the PA28$\gamma^{-/-}$CoreTGvsWT extended network of 1057 entities with 8988 interactions, and the PA28$\gamma^{-/-}$CoreTGvsCoreTG of 1476 entities with 12871 interactions between them, respectively (Tables S3, S4). For comparison, we also derived an extended PPI network for all the non-genetic PPIs in the human genome as compiled in BioGRID and iRefindex repositories (data not shown). The average degree (defined as the number of interactions for a given protein) of the CoreTGvsWT (17.31), PA28$\gamma^{-/-}$CoreTGvsWT (16.1), and PA28$\gamma^{-/-}$CoreTGvsCoreTG (16.57) extended networks was higher than the degree inferred for the human interactome (10.17). This observation suggests that HCV infection targets several highly connected cellular proteins with an ability to influence a large number of host factors in HCV pathogenesis. The average (shortest) path lengths of the three extended networks (2.93, 2.9, and 2.97, respectively) were significantly shorter than that inferred for the human interactome (3.88), suggesting that the Core and PA28γ influenced cellular networks are more compact and inclined toward faster communication between the constituents relative to the human interactome (Figure 1). Our observations are consistent with previous studies on the protein interaction networks associated with HCV infection.[22,45]

The compactness of the HCV-influenced protein networks coupled with the ability to influence a wide array of factors in the host cellular networks may facilitate a rapid propagation of the signaling information and allow the virus to respond rapidly to the host mobilization against HCV infection.

Functional Analysis of the Extended Protein Interaction Networks

Next, we investigated the extended networks for the enrichment of specific biological associations (KEGG pathways, GO terms, and OMIM phenotypes, Tables S5, S6, and S7). The analysis of the CoreTGvsWT, PA28$\gamma^{-/-}$CoreTGvsWT, and PA28$\gamma^{-/-}$CoreTGvsCoreTG extended networks revealed an enrichment ($p \leq 0.05$) of 116, 104, and 118 KEGG pathways, respectively (Table S5). Below we describe our observations on the selected enriched biological themes of interest, chiefly associated with the PA28$\gamma^{-/-}$CoreTGvsCoreTG network. Functional associations for the host factors previously known to be associated with HCV pathogenesis and HCC are

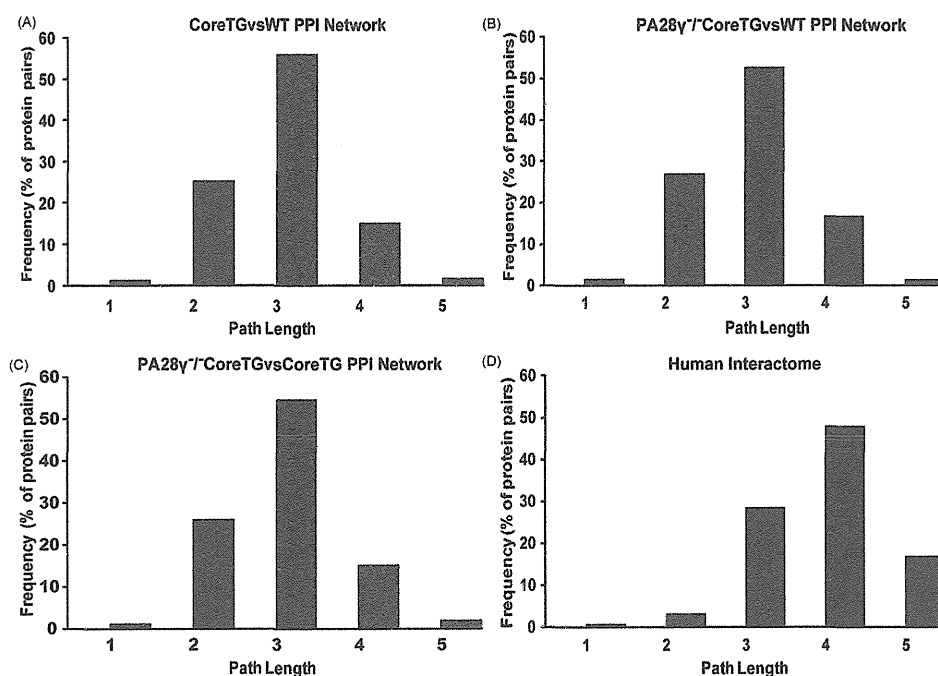

Figure 1. Graphical representation of the shortest path length distribution for (A) CoreTGvsWT extended network, (B) PA28γ^{-}/$^{-}$CoreTGvsWT extended network, (C) PA28γ^{-}/$^{-}$CoreTGvsCoreTG extended network, and (D) human protein interactome. The path length is represented on the x-axis, while 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 are displayed.

summarized in Table 2. Specific functional associations for the CoreTGvsWT and PA28$\gamma^{-/-}$CoreTGvsWT networks, except when discussed below, are detailed in the Supporting Information. It will highlight the biological significance of the differentially expressed proteins, their interactions, and their probable roles in HCV infection and help identify potentially novel regulators of and biomarkers for HCV pathogenesis.

Vesicular Transport

HCV infection involves the formation of the HCV replication complex in the detergent-resistant membrane (DRM) fraction or lipid rafts. These subcellular membrane fractions are utilized by some pathogens including viruses to facilitate viral entry and assembly.[46−49] HCV infection induces modifications in the host lipid raft proteome, which directly impacts HCV replication in the infected cells.[50] Core targeting to the early and late endosomes and the viral particle production requires the components of the endosome-based secretory pathways.[51,52]

CoreTGvsWT Extended Network. The PowerBlot analysis revealed the two endosomal proteins VTI1A and STX8 (KEGG Pathway "SNARE interactions in vesicular transport"; $p = 0.023$; Table S5) that were upregulated 3.25- and 1.88-fold, respectively, in CoreTGvsWT (Table 1). SNAREs are membrane-anchored proteins involved in membrane trafficking.[53] Some SNAREs may function in HCV egress by possibly facilitating the fusion of the late endosomes that carry HCV particles with the plasma membrane resulting in their release into the extracellular environment.[52] VTI1A is a SNARE involved in the vesicular transport from the late endosomes to the trans-Golgi network and forms a SNARE complex with STX16 and VAMP4 (Table S4).[54,55] STX8 is involved in the protein trafficking from the early to the late endosomes and exocytosis and forms a SNARE complex with STX7, VAMP8, and VTI1B.[55,56] A reduction in the expression of STX7, which interacts with both VTI1A and STX8 (Figure 2; Table S4), decreases HCV replication.[50] Taken together, the increased abundances of VTI1A and STX8 in CoreTGvsWT, but not PA28$\gamma^{-/-}$CoreTGvsWT, suggest potentially crucial roles of the two proteins in the HCV life cycle.

PA28$\gamma^{-/-}$CoreTGvsCoreTG Extended Network. Syntaxin 11 (STX11), a SNARE, was upregulated 3.52-fold in PA28$\gamma^{-/-}$CoreTGvsCoreTG (Table 1) and was mapped to the enriched KEGG Pathway "SNARE interactions in vesicular transport" ($p = 0.003$; Table S5). STX11 associates with the late endosomes and functions in the essential trafficking pathways (such as cytokine secretion) in the immune cells, with enhanced STX11 expression contributing to increased NK-cell mediated cytotoxicity.[57−61] STX11 binds with the SNARE VTI1B (Figure 3, Table S4) and regulates its participation the Q-SNARE complexes and, thus, the endocytic and exocytic trafficking in the macrophages. Overexpression of STX11 alters the VTI1B binding to STX6 and STX8 and likely reduces the endosomal transport to the cell surface.[57]

USO1, a Golgi-associated peripheral membrane protein, was decreased 2.05-fold in PA28$\gamma^{-/-}$CoreTGvsCoreTG (Table 1) and was identified as a significant linking component of the PA28$\gamma^{-/-}$CoreTGvsCoreTG SNARE network (Figure 3). USO1 plays an important role in ER to Golgi trafficking and its knockdown leads to the disintegration of the Golgi complex.[62,63] Decreased USO1 levels in PA28$\gamma^{-/-}$CoreTGvsCoreTG may, therefore, significantly impact the endosomal pathways associated with HCV release.

PA28γ knockdown impairs the production of the infectious HCV particles (but not replication) in the JFH1 (HCV genotype 2a) infected cells, largely due to the deregulation of the E6AP-dependent Core degradation, which contributes to an antiviral response.[14] Our analysis suggests a potentially novel

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Table 2. Functional Analysis of the Extended Protein Interaction Networks[a]

proteins	data set[b]	KEGG pathways	prior involvement with HCV pathogenesis and HCC	probable associations with Core/PA28γ functions in HCV pathogenesis
AKT1	I (−)	Insulin signaling pathway ($p = 5.72 \times 10^{-12}$)	reduced levels associated with insulin resistance (IR) in rats;[105] Akt phosphorylation is suppressed in the CoreTG mice and contributes to IR.[12]	Core-induced suppression of AKT1 levels may contribute to IR in HCV pathogenesis.
BAX	I (+)	Apoptosis ($p = 1.58 \times 10^{-18}$)	interacts with NS5A; disrupts mitochondrial homeostasis leading to abnormal cytochrome *c* release and apoptosis in HCV infection.[45,90,92,93]	Core-mediated BAX upregulation may induce host cell apoptosis.
CASP8	I (+)	Apoptosis ($p = 1.58 \times 10^{-18}$)	activated in HCV infection.[90]	Core-mediated CASP8 upregulation may induce host cell apoptosis.
CDH1	I (+)	Adherens junction ($p = 1.45 \times 10^{-20}$)	downregulated in the rat liver during hepatocarcinogenesis[106]	Core-mediated decrease in CDH1 levels may contribute to HCC
COMT	I (+); II (+)	Steroid hormone biosynthesis ($p = 0.002$)	upregulated in the lipid rafts in HCV infection;[50] COMT siRNA knockdown decreases HCV replication[103]	Core-induced activation of COMT may play an important role in HCV entry and replication
CSNK2B	II (+); III (+)	Adherens junction ($p = 1.4 \times 10^{-13}$); tight junction ($p = 4.46 \times 10^{-7}$)	interacts with the HCV NS3 protein;[45] regulates NS5A phosphorylation and hence infectious HCV particle production.[87]	loss of PA28γ activity may perturb CK2-mediated NS5A phosphorylation leading to decreased viral propagation.
EPS15	I (+); III (−)	Endocytosis ($p = 2.08 \times 10^{-22}$)	elevated in human and mouse HCC;[107] mediates human enterovirus 71 entry via clathrin-mediated endocytosis,[108] which also mediates HCV internalization.[109]	elevated EPS15 levels may facilitate HCC; decreased EPS15 levels may contribute to the lack of HCC in the PA28γ$^{-/-}$CoreTG. May facilitate HCV entry via clathrin-mediated endocytosis.
MCM2	I (+)	Cell cycle ($p = 1.82 \times 10^{-25}$)	increased hepatocyte MCM2 expression linked with fibrosis progression in HCV infection.[110]	Core-mediated enhanced MCM2 activity may contribute to fibrosis in HCV pathogenesis.
PTPN11	II (+)	Natural killer cell mediated cytotoxicity ($p = 3.64 \times 10^{-10}$); Jak-STAT signaling pathway ($p = 5.74 \times 10^{-5}$)	functions as a tumor suppressor in HCC and negatively regulates hepatic insulin action.[111,112]	increased PTPN11 levels may be associated with the absence of HCC progression in PA28γ$^{-/-}$CoreTG.
RABEP1	III (−)	Endocytosis ($p = 2.08 \times 10^{-22}$)	interacts with NS3;[45] functions in early endocytic events and regulates mast cell activation.[113,114]	may possibly function in HCV propagation.
RB1	III (+)	Cell cycle ($p = 4.04 \times 10^{-20}$)	tumour suppressor, downregulated in HCC.[115,116]	increased RB1 levels in III consistent with the lack of HCC progression in the PA28γ$^{-/-}$CoreTG mice.
TRADD	III (−)	Apoptosis ($p = 1.84 \times 10^{-15}$)	forms a complex with Core and TNFR1, implicated in HCV-induced chronic liver disease.[117]	decreased TRADD levels may contribute to the lack of IR and liver disease in PA28γ^{-}/$^{-}$CoreTG

[a]Host factors that were previously known to be associated with HCV pathogenesis and HCC and were mapped to various enriched KEGG pathways associated with the CoreTGvsWT, PA28γ$^{-/-}$CoreTGvsWT, and PA28γ$^{-/-}$CoreTGvsCoreTG PPI networks. [b]Data set I: CoreTGvsWT; Data set II: PA28γ$^{-/-}$/CoreTGvsWT; Data set III: PA28γ$^{-/-}$/CoreTGvsCoreTG; +: upregulated; −: downregulated.

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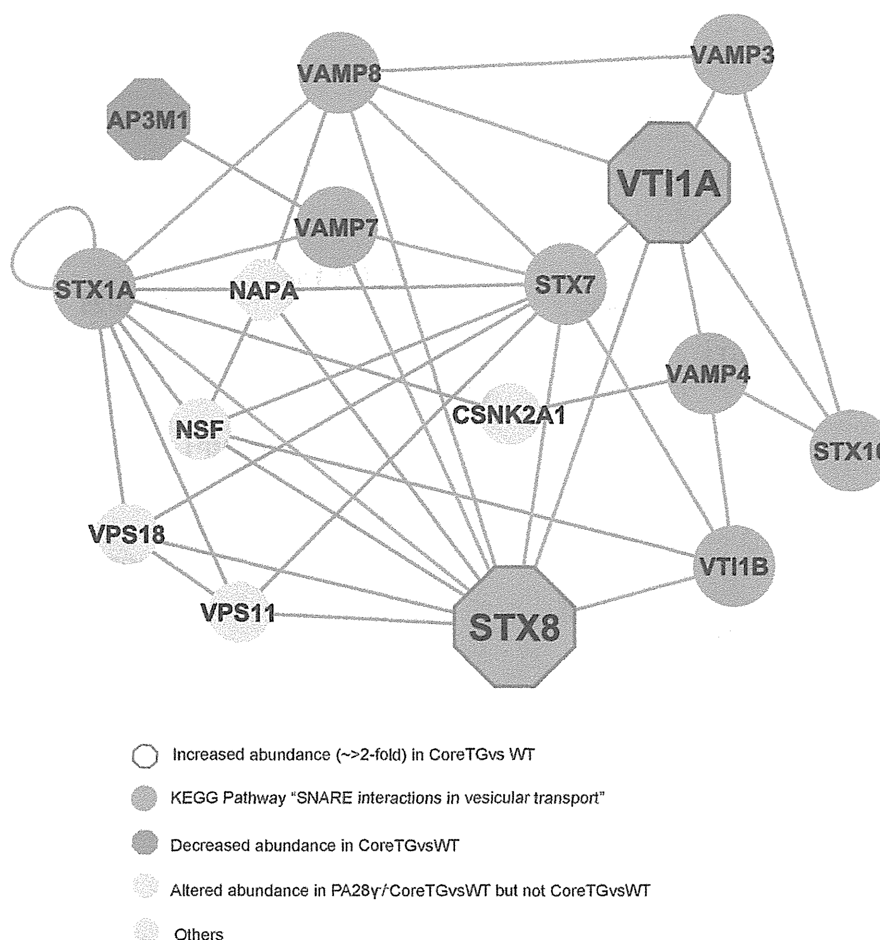

Figure 2. CoreTGvsWT SNARE network. Network illustration of the interactions between the PowerBlot identified differentially expressed proteins in CoreTGvsWT and human proteins mapped to the enriched KEGG pathway "SNARE interactions in vesicular transport". The node sizes differ for better clarity and do not reflect any topological attributes.

mechanism for the involvement of PA28γ in HCV propagation. Potentially, the suppression of PA28γ activity in PA28$\gamma^{-/-}$CoreTG mice may contribute to the overexpression of STX11 (and downregulation of USO1), thereby impairing the trafficking to the cell surface and consequently the release of the infectious HCV particles.

HCV has also been detected in the macrophages of certain infected patients,[64] suggesting that HCV may possibly infect the macrophages *in vivo* and regulate the STX11 (and USO1) expression to modulate the viral release and cytokine secretion.

EPS15 and RABEP1 (KEGG pathway "Endocytosis", $p = 2.08 \times 10^{-22}$) were decreased 2.11- and 2.74-fold, respectively, in PA28$\gamma^{-/-}$CoreTGvsCoreTG (Table 1, Table S5). EPS15 is an adaptor protein associated with the epidermal growth factor (EGF) signaling; it is localized to the clathrin-coated pits and functions in receptor-mediated endocytosis[65,66] and may play an important role in HCV pathogenesis (Table 2).

Immune System and Signal Transduction

HCV infection induces varied active and passive host immune responses such as the recognition of the infecting HCV RNA and proteins by the macrophages and the dendritic cells expressing Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs). These events trigger the production of Type I interferons (IFN-α/β) and inflammatory cytokines in the infected hepatocytes, thereby initiating viral clearance. The ability to impair host immune responses contributes to the HCV persistence in the host.[67−72]

The PowerBlot analysis showed differentially expressed host proteins (IKBKG, MAP2K2, PPP3CA, SHC3, STAT1, TRADD) in PA28$\gamma^{-/-}$CoreTGvsCoreTG and their interacting partners that were mapped to one or more enriched KEGG pathways associated with the immune system (Table S5). IKBKG (IKK Gamma) is an antiapoptotic protein that is essential for NFKB activation and modulates TNF-mediated apoptosis.[73] IKBKG mutations are associated with immune deficiency phenotype (Table S7) and IKBKG may contribute to the activity of the hepatic carcinoma associated protein MAFIP in suppressing the proliferation of the cancer cells.[74] Additionally, specific deletion of IKBKG in the hepatocytes promotes NK-cell dependent liver damage.[75] Taken together, the elevated IKBKG abundance as observed in PA28$\gamma^{-/-}$CoreTGvsCoreTG (1.97-fold; Table 1) may contribute to the lack of HCC progression and reduced liver damage in the PA28$\gamma^{-/-}$CoreTG mice.

PPP3CA, MAP2K2, and SHC3 were mapped to the KEGG pathway "Natural killer cell mediated cytotoxicity" ($p = 1.67 \times 10^{-5}$; Table S5), the components of which function in the host immune response against the cancer cells and cells beset with pathogen infection.[76] PPP3CA levels were increased 12.94-fold,

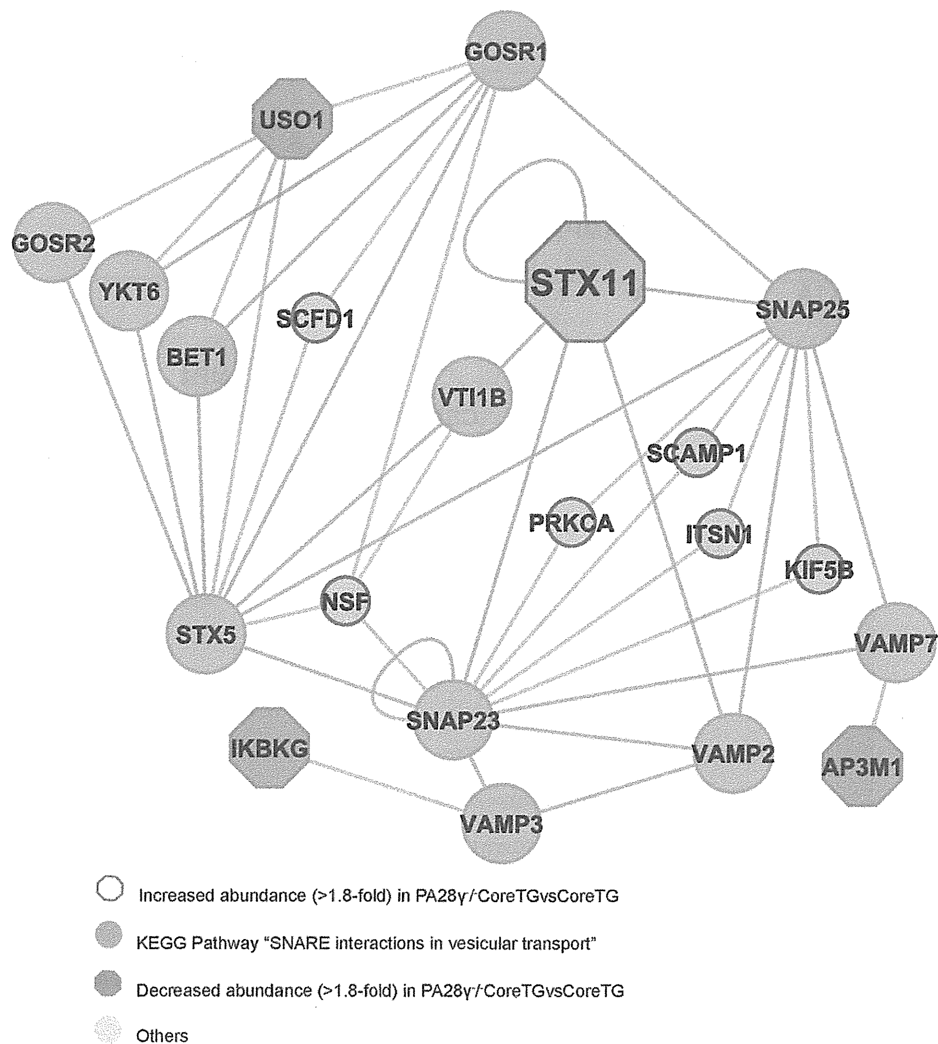

Figure 3. PA28γ^{-}/$^{-}$CoreTGvsCoreTG SNARE network. Network illustration of the interactions between the PowerBlot identified differentially expressed proteins in PA28γ^{-}/$^{-}$CoreTGvsCoreTG and human proteins mapped to the enriched KEGG pathway "SNARE interactions in vesicular transport". The node sizes differ for better clarity and do not reflect any topological attributes.

while MAP2K2 and SHC3 levels were suppressed 2.35-fold and 1.9-fold, respectively, in PA28$\gamma^{-/-}$CoreTGvsCoreTG (Table 1). These observations together with the increased STX11 abundance are consistent with the enhanced NK-cell mediated cytotoxicity that accompanies the STX11 overexpression.

PPP3CA is a tumor suppressor that negatively modulates the vascular endothelial growth factor (VEGF)-stimulated cell proliferation[77] and is downregulated in some cancerous cells.[78,79] PPP3CA was also mapped to the enriched KEGG pathways "VEGF signaling pathway" (p = 2.59 × 10^{-5}), "MAPK signaling pathway" (p = 2.37 × 10^{-16}) and "Wnt signaling pathway" (p = 1.048 × 10^{-10}; Table S5), which are implicated in the HCV infection and HCC. The 7.55-fold increase in PPP3CA abundance in PA28$\gamma^{-/-}$CoreTGvsCoreTG (Table 1) suggests that increased PPP3CA activity may significantly contribute to the lack of tumorigenesis and HCC progression in PA28$\gamma^{-/-}$CoreTG mice.

MAP2PK2 is a dual specificity MAP kinase that plays a critical role in the mitogen growth factor signal transduction. It is a key regulator of the TNF-α signaling and plays an important role in the tumor progression in certain cancers.[80] Reduced MAP2K2 levels in the hepatocytes are implicated in enhanced apoptosis.[81] Therefore, the 2.66-fold decrease in MAP2K2 protein levels in PA28$\gamma^{-/-}$CoreTGvsCoreTG (Table 1) suggests that decreased MAP2K2 activity may contribute to the lack of HCC progression in PA28$\gamma^{-/-}$CoreTG mice. Our analysis thus identified potentially significant PA28γ-dependent roles of MAP2K2 and PPP3CA in a probable STX11-mediated regulation of NK cell activity in HCV pathogenesis.

MAP2K2, PPP3CA and SHC3 were also associated with the "Insulin signaling pathway" (p = 5.88 × 10^{-9}; Table S5), the disruption of which may contribute to insulin resistance (IR). IR is linked with steatosis, fibrosis progression and poor interferon-α response in HCV infection.[82,83] PA28γ contributes to hyperinsulinemia and IR in the CoreTG mice by impairing the insulin-signaling pathway through the suppression of Insulin receptor substrate 1 (IRS1) phosphorylation and increased tumor necrosis factor alpha (TNF-α) secretion.[12,84] The Powerblot analysis revealed that TRADD, which regulates TNF-α signaling as an antiapoptotic factor[85,86] and possibly functions in HCV pathogenesis (Table 2), was suppressed 2-fold in PA28$\gamma^{-/-}$CoreTGvsWT (Table 1).

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Table 3. Summary of Proteins and Pathways Prioritized with TargetMine and Adjusted with the Help of Knowledge-Based Inputs for Experimental Investigation and/or Biomarker Discovery

(a) HCV replication and release					
KEGG pathways	*p*-value	proteins[a]	data set[b]	knowledge-based evidence	refs
SNARE interactions in vesicular transport	0.023	VTI1A (+), STX8 (+)	I	regulation of the endosome-based membrane trafficking pathway implicated in HCV release	50, 52, 55
Steroid hormone biosynthesis	0.002	COMT (+)	I	COMT siRNA impairs HCV genotype 1b replication; functions in genotype 2a replication not known	50, 103
SNARE interactions in vesicular transport	0.003	STX11 (+)	III	modulation of specific components of the endosome-based membrane trafficking pathway implicated in HCV release; modulation of cytokine secretion in immune cells	50, 55, 57
Endocytosis	1.03×10^{-18}	CAV1 (+)	I	close homologue CAV2 associated with HCV replication complex; possible role in HCV replication	103
(b) steatosis, fibrosis, and hepatcarcinogenesis in HCV infection					
KEGG pathways	*p*-value	proteins[a]	data set[b]	knowledge-based evidence	refs
Insulin signaling pathway	5.72×10^{-12}	AKT1 (−)	I	reduced AKT1 levels and phosophorylation associated with insulin resistance, which contributes to steatosis, fibrosis and HCC	118
Natural killer cell mediated cytotoxicity	1.67×10^{-5}	PPP3CA (+), MAP2K2 (−)	III	PPP3CA is a tumor suppressor with decreased levels in some cancers; PTPN1 is a tumor suppressor and regulates hepatic insulin signaling; decreased MAP2K2 levels in hepatocytes associated with enhanced apoptosis	78, 79, 81
Adherens junction	1.45×10^{-20}	CDH1 (−)	I	decreased CDH1 abundance associated with hepatocarcinogenesis and various cancers	106
Focal adhesion	5.57×10^{-12}	SHC1 (+)	II	loss of SHC1 function associated with tumor metastasis	119
Apoptosis	1.58×10^{-18}	BAX (+), CASP8 (+)	I	BAX interacts with NS5A and contributes to abnormal cytochrome *c* release in HCV infection; CASP8 activated in HCV infection	45, 90, 92, 93
Apoptosis	1.84×10^{-15}	TRADD (−)	III	antiapoptotic factor that forms a ternary complex containing Core, with likely functions in HCV-induced chronic liver disease	117
Chemokine signaling pathway	1.18×10^{-13}	ARRB1 (−)	I	interacts with PKM2, a key enzyme in glycolytic metabolism and cell growth and death in tumor cells	120

[a]+: upregulated. −: downregulated. [b]Data set I: CoreTGvsWT; Data set II: PA28$\gamma^{-/-}$/CoreTGvsWT; Data set III: PA28$\gamma^{-/-}$/CoreTGvsCoreTG.

Cell Adhesion

The Powerblot analysis revealed that host protein CSNK2B, the regulatory (beta) subunit of Casein Kinase II (CK2), was increased 1.88-fold in PA28$\gamma^{-/-}$CoreTGvsCoreTG (1.9-fold in PA28$\gamma^{-/-}$CoreTGvsWT). CK2 phosphorylates NS5A and regulates the production of infectious viral particles[87] and thus HCV pathogenesis (Table 2). CSNK2B was mapped to the enriched KEGG pathways "Adherens junction" ($p = 1.4 \times 10^{-13}$) and "Tight junction" ($p = 4.46 \times 10^{-7}$), some components of which are implicated in HCV entry and infection.[88]

SHC3 and TNR were decreased 1.9- and 4.62-fold, respectively, in PA28$\gamma^{-/-}$CoreTGvsCoreTG and were mapped to the enriched KEGG pathway "Focal adhesion" ($p = 3.56 \times 10^{-12}$; Table 1; Table S5), which regulates cell migration and adhesion to the extracellular matrix. Its deregulation is linked with tumor progression and possibly HCV propagation.[45] Previously, a Core interacting protein ENO1, associated with the focal adhesion, was identified as a novel regulator of HCV replication and release,[22] suggesting that SHC3 and TNR may play important roles in HCV pathogenesis.

Cell Growth and Death

Host cell apoptosis plays a critical role in HCV pathogenesis. The induction of apoptosis in the hepatocytes contributes to cell damage and fibrosis, whereas the induction of apoptosis in the peripheral blood mononuclear cells (PMBC), such as the T-cells, contributes significantly to the impaired immune response and HCV persistence in the host.[89−94] PA28γ is implicated in the cell cycle regulation, cell proliferation, and apoptosis[95−98] and likely plays a critical role in the manipulation of the cell cycle and apoptosis in HCV pathogenesis.

PPP3CA, TRADD, PRKAR2A, and IKBKG, with increased or decreased abundances in PA28$\gamma^{-/-}$CoreTGvsCoreTG, were mapped to KEGG pathway "Apoptosis" ($p = 1.84 \times 10^{-15}$; Table S5). PPP3CA was also mapped to "Oocyte meiosis" ($p = 9.84 \times 10^{-10}$; Table S5), associated with cell division. PPP3CA levels were highly elevated (12.94-fold) in PA28$\gamma^{-/-}$CoreTGvsCoreTG (Table 1), which may contribute to the accelerated cell death and the lack of tumor progression in PA28$\gamma^{-/-}$CoreTG mice.

PRKAR2A levels were decreased 1.88-fold in PA28$\gamma^{-/-}$CoreTGvsCoreTG (Table 1). PRKAR2A codes for a regulatory subunit of the cAMP dependent protein kinase (PKA), an important mediator of the cAMP signal transduction and elevated PRKAR2A expression is associated with an increased proliferation of the rat alveolar cells.[99] The suppression of PRKAR2A activity may therefore contribute to the lack of tumor proliferation in the PA28$\gamma^{-/-}$CoreTG mice.

Prioritization and Validation of the Novel Candidates for Their Role in HCV Replication and Release

Target prioritiszation using TargetMine is a simple process that involves uploading an initial list of candidates (in this instance the proteins in the CoreTGvsWT, PA28$\gamma^{-/-}$CoreTGvsWT, and PA28$\gamma^{-/-}$CoreTGvsCoreTG extended PPI networks) and estimating enriched biological themes associated with the input list.[16] Knowledge-based inputs may then be employed to further screen the proteins mapped to the top ranking significant associations to infer a manageable set of candidates. With the help of TargetMine, we previously investigated the significance of interactions between HCV Core and NS4B proteins and host factors in HCV infection and identified three novel regulators of HCV replication and propagation.[22]

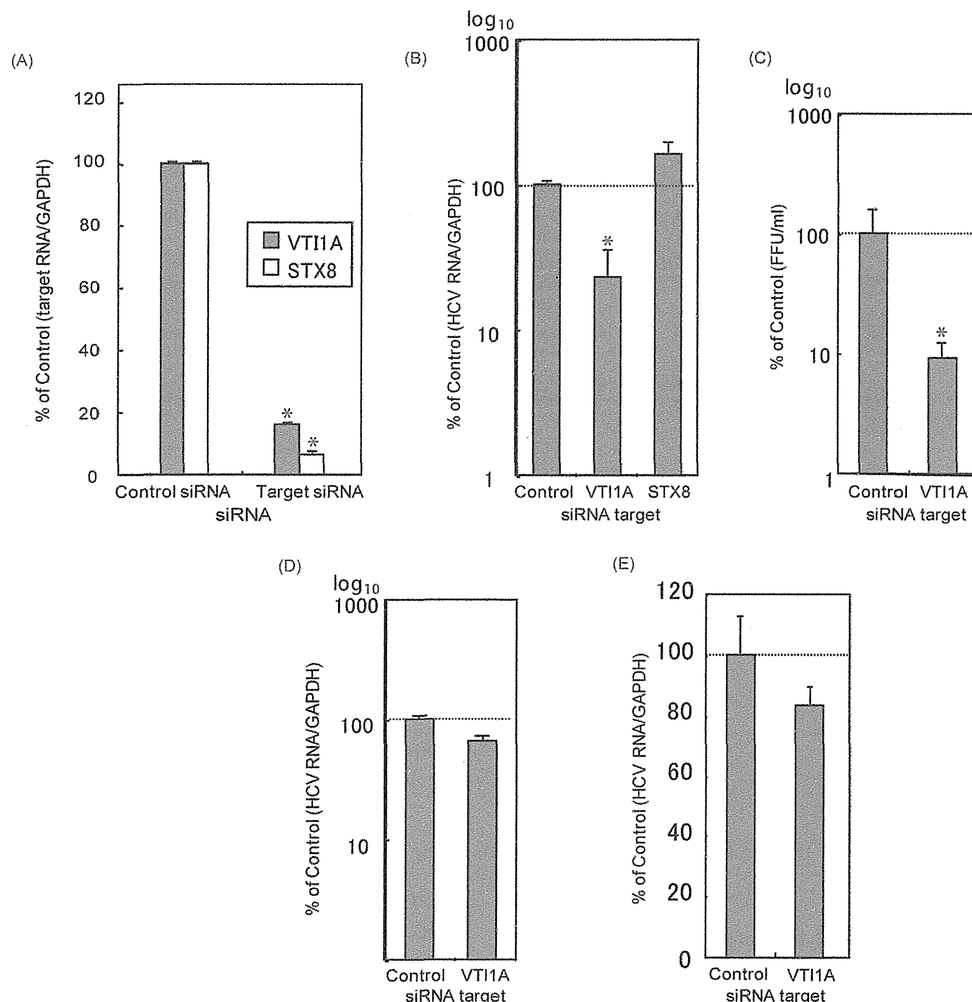

Figure 4. Effects of knockdown of candidate proteins on HCV propagation and replication. Host proteins VTI1A and STX8 were suppressed by RNAi (A) in Huh7OK1 cells infected with HCV JFH1 strain (genotype 2a; B, C, D) and in cells including subgenomic JFH1 replicon (E). The amounts of mRNA of the intracellular host proteins (A) and the supernatant viral RNA (B), viral titer (C), and intracellular viral RNA were estimated (D). The amount of the subgenomic viral RNA was also estimated (E). Each value was represented as percentage of the cells transfected with control siRNA; FFU: focus-forming units; * $p < 0.01$.

Table 3 summarizes the prioritized candidates and pathways, all of which have been discussed above. Traditionally, viral and host proteins associated with the HCV lifecycle (internalization, replication, assembly, and release) have been preferred targets in the anti-HCV studies. The prioritized candidates and pathways in Table 3a fall within this category. In particular, our analysis suggested novel and potentially crucial roles of the host proteins VTI1A and STX8, which were elevated in CoreTG but not in PA28$\gamma^{-/-}$CoreTG, in the replication and/or the release stages of the HCV lifecycle, therefore making these host proteins attractive targets for further investigation.

Because of the lack of a suitable model system for HCV infection, cell-culture-based systems for HCV RNA replication and infectious viral particle production have been extensively exploited to identify potential anti-HCV drug targets.[5,7−9] To further explore the roles of selected candidates in the HCV life cycle, we performed cellular assays to assess the impact of VTI1A and STX8 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,[33,100] JFH1 was used to infect the Huh7OK1 cell line 24 h after transfection with each siRNA (see Materials and Methods). The infected cells were harvested after 72 h post-infection and the expression of each host protein was assessed by qRT-PCR (Figure 4A). Supernatant viral RNA and the viral titer were significantly decreased by the knockdown of VTI1A but were unaffected by the STX8 knockdown (Figure 4B,C). However, VTI1A and STX8A knockdowns had no effect on the intracellular viral RNA levels in the HCV infected cells (Figure 4D) or replicon cells derived from JFH1 strain (Figure 4E) or replicon cells derived from the Con1 (genotype 1b) strain (data not shown). These observations suggest that VTI1A regulates HCV propagation but not HCV replication.

The standard therapy of PEGylated interferon-α plus rebavirin treatment often results in severe side effects such as depression, flu-like symptoms, anemia, and fatigue that force the treatment to discontinue in affected patients, thus necessitating improved and combinatorial treatment strategies.[101,102] The genetic variability of HCV has led to increasing drug resistance. Thus, antivirals that target host proteins critical to viral pathogenesis, with a lower rate of mutation and

preferably with minimal adverse side effects, may provide attractive alternatives to HCV protein targets. VTI1A-deficient (knockout) mice are viable and fertile,[55] suggesting that the suppression of VTI1A activity may not have significantly undesirable side effects.

Inhibition of COMT (which was increased 2.71- and 2.96-fold in CoreTGvsWT and PA28$\gamma^{-/-}$CoreTG, respectively; Table 1) activity via siRNA knockdown was previously shown to result in a decreased HCV replication in cells infected with the Con1 strain.[103] To investigate other possible aspects (such as genotype specificity) of COMT function in the HCV life cycle, we assessed the impact of the COMT siRNA knockdown on HCV replication and release. COMT knockdown, however, had no effect on HCV propagation or replication in the cells including full length or subgenomic replicons derived from JFH1 or Con1 strains (data not shown). The discrepancy between our observations and those of Chan et al.[103] may be explained by the differences in the methodologies. We employed a transient transfection method to knockdown the selected targets to assess their roles in HCV replication and release, whereas Chan et al. employed a lentiviral expressing system for their experiments. Lentiviral mediated siRNA delivery is known to result in a persistent knockdown of gene expression,[104] and a persistent knockdown of COMT expression may be necessary to inhibit HCV replication *in vitro*.

That we were able to experimentally validate one of the three genes selected for experimental characterization reinforces the strengths of the elaborate PPI network-based approach to identify and prioritize suitable targets for experimental and therapeutic investigation.

CONCLUSIONS

By analyzing high-throughput proteomics data from transgenic mice expressing HCV Core protein in the liver (an *in vivo* model of HCV pathogenesis) with or without the knockout of the proteasome activator PA28γ, we highlighted the cellular responses to HCV infection *in vivo* and obtained further insights into the role of PA28γ in HCV infection.

We investigated the network context of the changes in the protein abundances by mapping them onto the human interactome with the help of the TargetMine data warehouse. The differentially expressed proteins that were integrated with the human interactome were observed to participate in compact and well connected cellular networks reflecting the ability of HCV to rapidly and efficiently react to the host responses to HCV infection. A functional analysis of the PPI networks highlighted the cellular pathways associated with vesicular transport, immune system, cellular adhesion, cell growth, and cell death among others that were most prominently influenced by Core and PA28γ in HCV infection. We also confirmed the previous observations that host factors such as AKT1, BAX, CASP8 CDH1, COMT, MCM2, PTPN11, and RB1 showed increased or decreased abundances in HCV infection. However, to the best of our knowledge, the precise molecular mechanisms of these factors' involvement in HCV pathogenesis and HCC were unknown, and our analysis suggests novel contributions of Core and PA28γ to the functions of these proteins.

Our observations were then used to prioritize potential candidates for the follow-up experimental investigations. Cellular assays based on siRNA knockdowns of selected candidates in the HCV infected and replicon cells validated VTI1A, a SNARE protein associated with vesicular transport, which was upregulated in CoreTG but not in PA28$\gamma^{-/-}$CoreTG, as a novel regulator of HCV propagation but not replication. VTIA-deficient mice are largely indistinguishable from the normal mice except for minor growth retardation in a few instances; therefore, VTI1A is a promising novel candidate for anti-HCV therapy.

Our analysis not only builds on the present understanding of the Core-PA28γ interplay in HCV infection but also provides novel insights that would facilitate the clinical evaluation of proteomic changes associated with HCV pathogenesis. Our analysis also provides a generic framework for investigating large scale proteomic data. Such investigation may help identify common themes associated with different physiological conditions, especially pathogen (such as viral) infection and disease, and help develop effective broad spectrum strategies aimed at ameliorating pathogen infection and diseases.

ASSOCIATED CONTENT

Supporting Information

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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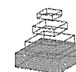

Exploitation of lipid components by viral and host proteins for hepatitis C virus infection

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Hepatitis C virus (HCV), which is a major causative agent of blood-borne hepatitis, has chronically infected about 170 million individuals worldwide and leads to chronic infection, resulting in development of steatosis, cirrhosis, and eventually hepatocellular carcinoma. Hepatocellular carcinoma associated with HCV infection is not only caused by chronic inflammation, but also by the biological activity of HCV proteins. HCV core protein is known as a main component of the viral nucleocapsid. It cooperates with host factors and possesses biological activity causing lipid alteration, oxidative stress, and progression of cell growth, while other viral proteins also interact with host proteins including molecular chaperones, membrane-anchoring proteins, and enzymes associated with lipid metabolism to maintain the efficiency of viral replication and production. HCV core protein is localized on the surface of lipid droplets in infected cells. However, the role of lipid droplets in HCV infection has not yet been elucidated. Several groups recently reported that other viral proteins also support viral infection by regulation of lipid droplets and core localization in infected cells. Furthermore, lipid components are required for modification of host factors and the intracellular membrane to maintain or up-regulate viral replication. In this review, we summarize the current status of knowledge regarding the exploitation of lipid components by viral and host proteins in HCV infection.

Keywords: HCV, hepatitis, lipid droplets, host factor

INTRODUCTION

Hepatitis C virus (HCV) is a major causative agent of chronic liver disease including steatosis, cirrhosis, and hepatocellular carcinoma. Epidemiological studies indicate that HCV is also associated with extrahepatic manifestations including type 2 diabetes mellitus, B-cell non-Hodgkin lymphoma, mixed cryoglobulinemia, and Sjögren's syndrome (Jacobson et al., 2010). It has been estimated that there are 170 million patients worldwide, of whom most are infected with HCV. Combination therapy with pegylated interferon (PEG-IFN) and ribavirin has been the standard treatment but it fails to cure ~50% of treated patients (Soriano et al., 2009).

Hepatitis C virus belongs to the genus *Hepacivirus* of the family Flaviviridae. The viral genome of HCV is characterized by a single positive strand RNA with a nucleotide length of 9.6 kb and it encodes a single polypeptide (**Figure 1**). This polyprotein is cleaved by host and viral proteases into structural and non-structural proteins (Harada et al., 1991; Hijikata et al., 1991; Grakoui et al., 1993a,b). Structural proteins, including the core protein and two envelope proteins, and the viroporin p7 are located within one-third of the N-terminal, while the remaining viral proteins are classified as non-structural proteins which form a replication complex with host factors (Grakoui et al., 1993c). HCV core protein is cleaved by signal peptide cleavage and then released from E1 (Santolini et al., 1994). After cleavage by signal peptidase (SP), the C-terminal transmembrane region of the core protein is further cleaved by signal peptide peptidase (SPP; Hussy et al., 1996; McLauchlan et al., 2002). The nucleocapsid, composed of matured core proteins and the viral genome, is surrounded by an envelope composed of host lipids and viral envelope proteins (Wakita et al., 2005). The life cycle of HCV is shown in **Figure 2**. The viral envelope proteins play a role in the binding to host receptors and membrane fusion for uncoating. Recently, several groups reported that the viral particle binds to a very low-density lipoprotein (VLDL), including apolipoprotein E (apoE), which is required for the binding step (Andre et al., 2002; Nielsen et al., 2006; Chang et al., 2007; Benga et al., 2010) as described below. The virus infects hepatocytes via entry factors known as receptors and co-receptors. The viral particle complex composed of the enveloped nucleocapsid and VLDL including apoE (Merz et al., 2011), is reported to bind to heparin sulfate (HS; Barth et al., 2003) and the low-density lipoprotein (LDL) receptor (LDLR; Agnello et al., 1999), although Albecka et al. (2012) recently reported that LDLR is required for optimal replication of the HCV genome rather than entry of the infectious viral particle. Other host factors may be involved in apoE-mediated entry. The HCV viral particle is transferred to the scavenger receptor class B type I (SR-BI; Scarselli et al., 2002; Bartosch et al., 2003) and CD81 (Pileri et al., 1998) through E2 binding and then enters cells with claudin-1 (CLDN1; Evans et al., 2007) and occludin (OCLN; Ploss et al., 2009) by endocytosis. The Niemann–Pick C1-like 1 cholesterol absorption receptor has recently been reported to be an HCV cell entry factor that is involved in the entry step between post-binding and

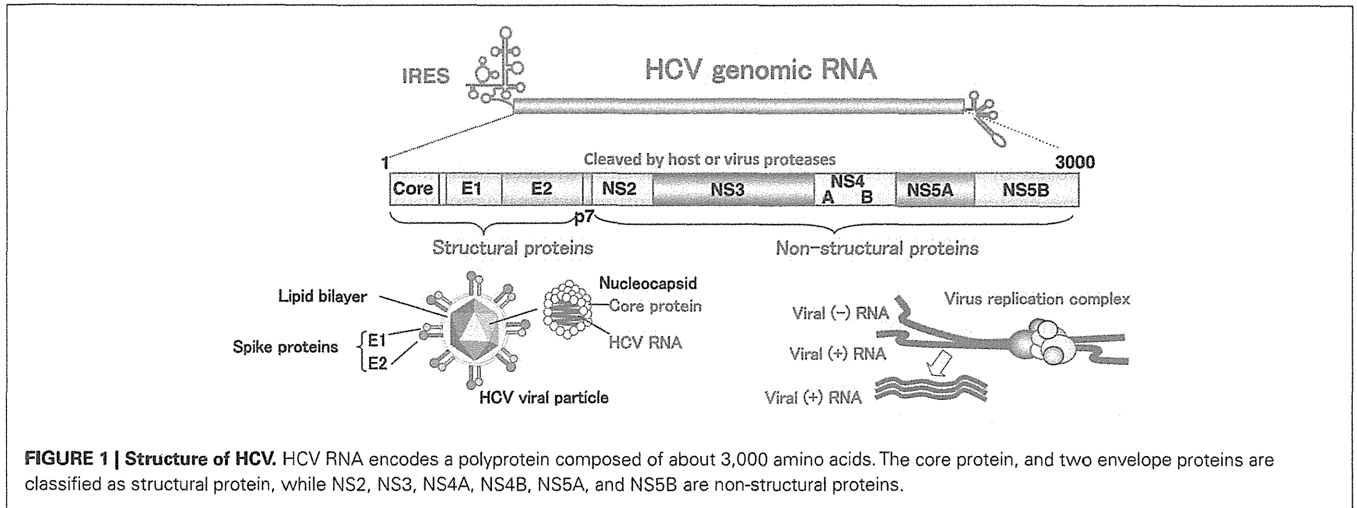

FIGURE 1 | Structure of HCV. HCV RNA encodes a polyprotein composed of about 3,000 amino acids. The core protein, and two envelope proteins are classified as structural protein, while NS2, NS3, NS4A, NS4B, NS5A, and NS5B are non-structural proteins.

FIGURE 2 | The infection cycle of HCV. The HCV complex with VLDL binds to entry factors on the surface of hepatocytes and then enters cells by endocytosis. After uncoating, viral replication is carried out in the convoluted membrane structure called the membranous web. The viral nucleocapsid egresses into the lumen side of the ER and binds to VLDL. The HCV complex with VLDL is released from the infected cells.

pre-fusion (Sainz et al., 2012). The viral envelope fuses with the host plasma membrane in an endosome under a low pH condition (Takikawa et al., 2000; Hsu et al., 2003; Blanchard et al., 2006; Codran et al., 2006; Meertens et al., 2006; Tscherne et al., 2006). The capsid protein and viral genome are expected to be released into the cytoplasm of infected cells. The viral replication, assembly, and budding are summarized in **Figure 3** on the basis of current information. The viral genome is translated dependent on own internal ribosome entry site (Tsukiyama-Kohara et al., 1992) and transcribed by the translated and processed NS3 to NS5B (Lohmann et al., 1999). The viral protein NS4B induces a convoluted membrane structure (termed a membranous web) with host lipid components and proteins, in which the viral replication is carried out (Egger et al., 2002; Gosert et al., 2005; Ferraris et al., 2010). The newly synthesized viral positive stranded RNA genome is released from the membranous web and passes to the

core protein via NS5A (Masaki et al., 2008). The core protein is translocated on the surface of the lipid droplet or endoplasmic reticulum (ER) membrane for efficient formation of viral particles, and then encloses the synthesized viral genome to form a capsid near the membranous web (Miyanari et al., 2007; Boson et al., 2011). The capsids are enclosed by an endoplasmic membrane containing the viral envelope proteins E1 and E2 and are then released into ER lumen side, since intracellular envelope proteins are categorized as high-mannose type glycoproteins and the viral particle composed of core proteins and envelope proteins egresses into the lumen side of the intracellular compartment associated with lipid droplets (Miyanari et al., 2007; Vieyres et al., 2010). The viral particle is secreted through a host secretion pathway, although the mechanism by which HCV particles are secreted in infected cells remains poorly understood.

Although no effective vaccine for HCV has been developed, antiviral drugs targeting to the viral and host factors have been reported recently. The HCV replicon system was reported for a screening system based on cultured cells (Lohmann et al., 1999) and has been improved by modification of cell lines and marker genes and introduction of adaptive mutations in the region of the viral RNA genome for high efficiency of viral replication (Blight et al., 2000; Krieger et al., 2001; Lohmann et al., 2001; Ikeda et al., 2002; Pietschmann et al., 2002). The complete infectious cycle of HCV in cultured cells was established in a highly permissive cell line by using the genotype 2a strain JFH1 or its chimeric recombinant virus (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). A system based on the cultured cell line has been an exclusive drug-screening system for finding antiviral compounds that interfere with the process of viral RNA replication under intracellular conditions. NS3 forms a complex with cofactor NS4A (Failla et al., 1994, 1995; Koch et al., 1996). This interaction stabilizes NS3 and retains it on the ER where it acquires the ability of a protease against viral polyprotein (Wolk et al., 2000) and host protein IPS-1/MAVS (Foy et al., 2005; Loo et al., 2006), which is a molecule downstream of the RIG-I sensor molecule (Sumpter et al., 2005; Loo et al., 2008). NS3 serine protease activity is a target of the direct acting HCV antiviral drugs known as telaprevir and boceprevir, which are available clinically by combination therapy with PEG-INF and ribavirin (Hofmann and Zeuzem, 2011). The RNA helicase activity of NS3 and NS5B RNA-dependent RNA polymerase are also used for drug-screening in particular (Hicham Alaoui-Ismaili et al., 2000; Dhanak et al., 2002; Borowski et al., 2003; De Francesco et al., 2003; Boguszewska-Chachulska et al., 2004; Maga et al., 2005; Najda-Bernatowicz et al., 2010). Combination therapy using several compounds targeting host and viral factors may be able to completely eradicate the virus and suppress the pathogenicity induced by HCV infection.

Liver steatosis, which is characterized by accumulation of lipid droplets in hepatocytes, is significantly associated with the incidence of hepatocellular carcinoma in HCV-infected patients (Ohata et al., 2003). Severe liver steatosis has been frequently found in patients infected with the genotype 3a virus (Rubbia-Brandt et al., 2000; Adinolfi et al., 2001). Successful clearance of HCV reduces steatosis in genotype 3a patients, suggesting an association between genotype 3a and severe steatosis. Furthermore, HCV core protein derived from genotype 1 also induced liver steatosis in mouse and cultured cells (Barba et al., 1997; Moriya et al., 1997; Hope and McLauchlan, 2000). Lipid droplets containing triglycerides and cholesteryl ester are increased in cells expressing core protein and are surrounded by the core protein (Hope and McLauchlan, 2000). Non-structural proteins associate with the lipid droplets surrounded by HCV core proteins to supply the synthesized viral genome for viral assembly (Miyanari et al., 2007). Other lipid components are reported to be involved in formation of viral particles and the viral RNA replication as described below. This review mainly summarizes the viral and host factors that are associated with lipid metabolism with regard to HCV replication and pathogenicity.

THE ROLE OF VLDL IN HCV INFECTION

Hepatitis C virus replicates in a convoluted membrane structure as a membranous web (Egger et al., 2002; Gosert et al., 2005; Ferraris et al., 2010) and assembles in the area of the ER membrane-associated with lipid droplets surrounded by the core protein (Miyanari et al., 2007). The LDLR has also been proposed to function as one of entry factors described above for HCV entry, in which interaction between LDLR and HCV particles is facilitated though interaction of the virus with host lipoprotein components (Monazahian et al., 1999; Chang et al., 2007; Huang et al., 2007; Miyanari et al., 2007; Gastaminza et al., 2008). HCV RNA containing particles derived from infected human serum were fractionated in densities with a value of 1.03–1.25 g/ml (Thomssen et al., 1992, 1993). The HCV RNA particles of the fraction with a density of lower than 1.06 g/ml possessed infectivity against chimpanzees, while HCV RNA derived from fractions with a higher density showed poor infectious ability (Bradley et al., 1991; Hijikata et al., 1993). The infectious HCV particles form a LDL–virus complex in the sera of human patients (Andre et al., 2002). An LDL–virus complex was found in the fractions with very low to low buoyant densities (1.03–1.25 g/ml), which varied with the stage of infection (Pumeechockchai et al., 2002; Carabaich et al., 2005). HCV particles prepared from infected human serum forms a complex with lipoproteins designated as lipo-viro-particles (LVP; **Figure 3**; Andre et al., 2002; Nielsen et al., 2006). LVP includes triglycerides, HCV RNA, core protein, and apolipoproteins B and E (Andre et al., 2002), which are components of VLDLs and LDL (Brodsky et al., 2004).

Very low-density lipoprotein is formed with a hydrophobic particle composed of triglycerides and cholesteryl ester that is surrounded by a surface coat containing phospholipid, free cholesterol, and two dominant lipoproteins, apoB and apoE (review to see Havel, 2000). Both apoB and apoE were found in a low-density fraction of HCV RNA particles (Andre et al., 2002; Chang et al., 2007). HCV virions could also be precipitated with antibodies against apoB or apoE (Andre et al., 2002; Chang et al., 2007). ApoB and microsomal triglyceride transfer protein (MTP) are required for HCV assembly and production, since knockdown of apoB or a specific antibody to MTP could inhibit HCV production (Huang et al., 2007; Gastaminza et al., 2008). However, another report suggests that knockdown of apoB or antibodies to apoB exhibited no significant effect on HCV infectivity and production (Jiang and Luo, 2009). The monoclonal antibodies against apoE neutralized HCV infection in cultured cells (Chang et al., 2007; Jiang and Luo,

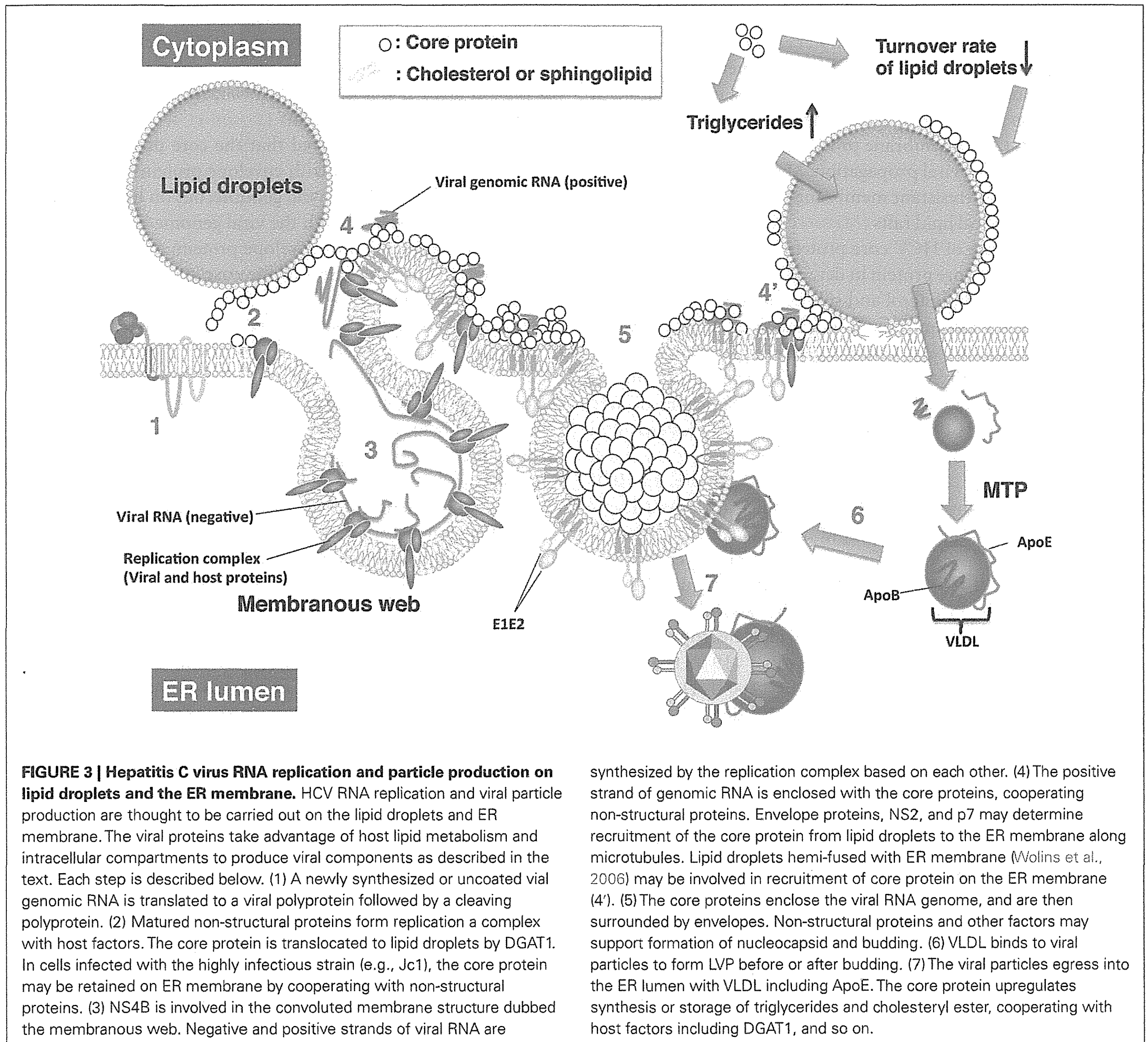

FIGURE 3 | Hepatitis C virus RNA replication and particle production on lipid droplets and the ER membrane. HCV RNA replication and viral particle production are thought to be carried out on the lipid droplets and ER membrane. The viral proteins take advantage of host lipid metabolism and intracellular compartments to produce viral components as described in the text. Each step is described below. (1) A newly synthesized or uncoated vial genomic RNA is translated to a viral polyprotein followed by a cleaving polyprotein. (2) Matured non-structural proteins form replication a complex with host factors. The core protein is translocated to lipid droplets by DGAT1. In cells infected with the highly infectious strain (e.g., Jc1), the core protein may be retained on ER membrane by cooperating with non-structural proteins. (3) NS4B is involved in the convoluted membrane structure dubbed the membranous web. Negative and positive strands of viral RNA are synthesized by the replication complex based on each other. (4) The positive strand of genomic RNA is enclosed with the core proteins, cooperating non-structural proteins. Envelope proteins, NS2, and p7 may determine recruitment of the core protein from lipid droplets to the ER membrane along microtubules. Lipid droplets hemi-fused with ER membrane (Wolins et al., 2006) may be involved in recruitment of core protein on the ER membrane (4'). (5) The core proteins enclose the viral RNA genome, and are then surrounded by envelopes. Non-structural proteins and other factors may support formation of nucleocapsid and budding. (6) VLDL binds to viral particles to form LVP before or after budding. (7) The viral particles egress into the ER lumen with VLDL including ApoE. The core protein upregulates synthesis or storage of triglycerides and cholesteryl ester, cooperating with host factors including DGAT1, and so on.

2009), while knockdown of apoE markedly reduced HCV infectivity and infectious viral production without affecting viral entry and replication (Chang et al., 2007; Berger et al., 2009; Jiang and Luo, 2009). Hishiki et al. (2010) suggested that the isoforms 3 and 4, but not 2, of apoE are critical for HCV infectivity dependent of affinity to LDLR. Furthermore, NS5A could interact with apoE in infected cells and colocalization of both proteins supports the notion of intracellular interaction in infected cells (Benga et al., 2010). The C-terminal alpha-helix region spanning from residue 205 to 280 was critical for NS5A–apoE interaction and viral production (Cun et al., 2010). ApoE included in LVP may directly bind to LDLR or LDLR-related proteins in hepatocytes (**Figure 2**), since apoE is a ligand for all members of the LDLR gene family (see review described by Herz et al., 2009). These results suggest that apoE is an essential host factor for HCV entry.

LOCALIZATION OF THE CORE PROTEIN ON BOTH ER AND LIPID DROPLETS IN INFECTED CELLS

Hepatitis C virus core protein is located at the N-terminus of the HCV polyprotein (**Figure 1**). The HCV core protein is cleaved from a precursor polyprotein by a SP, releasing it from an envelope E1 protein. Then, the C-terminal transmembrane region of the core protein is further processed by a SPP (McLauchlan et al., 2002). The intramembrane processing of the HCV core protein by SPP is critical for the production of infectious viral particles (Okamoto et al., 2008). The C-terminal end of the mature HCV core protein expressed in insect and human cell lines was determined to be Phe^{177} (Ogino et al., 2004; Okamoto et al., 2008). Randall et al. (2007) reported that the introduction of an siRNA targeted to SPP (called HM13) reduced the production of infectious HCV particles, suggesting that SPP is required for HCV

particle production. Our previous report (Okamoto et al., 2008) showed that the production of HCV in cells persistently infected with the JFH1 strain was impaired by treatment with an SPP inhibitor and that JFH1 viruses bearing SPP-resistant mutations in the core protein failed to propagate in a permissive cell line. These data suggest that intramembrane processing of HCV core protein by SPP is required for viral propagation. Matured core protein was found in a detergent-resistant membrane fraction, which was distinct from the classical lipid rafts (Matto et al., 2004). Our data also suggest that cleavage of HCV core protein by SPP is required for localization of HCV core protein in detergent-resistant membrane fractions including cholesterol and sphingolipid (**Figure 3**, step 4 and 5). Detergent-resistant membrane fractions may be derived from the membranous web where the viral replication complex synthesizes the viral RNA genome, since the replication complex is fractionated in lipid raft fractions including Vesicle-associated membrane protein-associated protein (VAP)-A, cholesterol, and sphingolipid (**Figure 3**, step 3; Shi et al., 2003; Aizaki et al., 2004; Gao et al., 2004; Sakamoto et al., 2005). Furthermore, an HCV core protein mutation resistant to SPP results in delayed localization of HCV core protein on lipid droplets and reduction of virus production (Targett-Adams et al., 2008). These reports suggest that cleavage of HCV core protein by SPP is required for its suitable intracellular localization for the viral assembly. Sequence analysis of the core protein suggests that high hydrophobicity is found in the region from amino acid residues 119 to 174, which is called domain 2 (Hope and McLauchlan, 2000). Domain 2 is critical for localization of the core protein on lipid droplets and shares common features with the core protein of GBV-B, but not of other viruses belonging to the Flaviviridae family (Hope et al., 2002). When three hydrophobic amino acids, Leu139, Val140, and Leu144, in the most hydrophobic peak in domain 2 were replaced with Ala to reduce hydrophobicity, the triple mutations in the core protein led to resistance to SPP cleavage, dislocation of the detergent-resistant membrane, and a reduction in virus production (Okamoto et al., 2004, 2008). Furthermore, comparative analysis between JFH1 and Jc1 suggest that binding strength of domain 2 of core for lipid droplets determines efficiency of virus assembly (Shavinskaya et al., 2007). These results suggest that hydrophobicity of domain 2 in the core protein is required for lipid droplet localization, SPP cleavage, and virus production.

Host lipid biogenesis is responsible for replication and assembly. HCV core protein contributes to the accumulation and production of host lipid components and is detected on the surface of lipid droplets (Hope and McLauchlan, 2000). The core protein is translocated into the lipid droplets near the replication complex and encloses newly synthesized viral RNA to form the nucleocapsid (**Figure 3**, step 2–4 or 4′), egresses into the lumen side of the ER, then is surrounded with host lipid components and viral envelope proteins (**Figure 3**, step 5; Miyanari et al., 2007). HCV core protein interacts with diacylglycerol acyl transferase 1 (DGAT1), which is required for the trafficking of core protein to lipid droplets (**Figure 3**, step 2; Herker et al., 2010). However, the translocation of the core protein to lipid droplets may not be required for efficient production of viral particles. The recombinant virus Jc1 exhibits a higher virus titer than the JFH1 strain (Lindenbach et al., 2006; Pietschmann et al., 2006). The core protein of the Jc1 strain is hardly detected on lipid droplets in infected cells and is mainly localized on ER membranes, together with envelope protein E2 (Miyanari et al., 2007; Shavinskaya et al., 2007; Boson et al., 2011). Expression of p7 increases the ER localization of core protein in the absence of envelope proteins (Boson et al., 2011). However, Miyanari et al. (2007) reported that the core protein of the Jc1 strain was mainly localized with envelope proteins on ER in cells transfected with a complete viral genome, but on lipid droplets in cells that were transfected with the viral genome lacking envelope protein genes. Expression of envelope proteins and p7 may determine intracellular localization of the core protein with regard to viral assembly (**Figure 3**, step 2 and 4 or 4′).

NS2 has been reported to be involved in the assembly process of HCV particles (Jones et al., 2007; Jirasko et al., 2008; Dentzer et al., 2009). NS2, which is composed of three transmembrane regions and a cytoplasmic domain in order after p7 (Lorenz et al., 2006), is known as the autoprotease of which C-terminal cytoplasmic domain is involved in *cis* cleavage at the NS2–NS3 junction (Santolini et al., 1995; Yamaga and Ou, 2002; Lorenz et al., 2006). Genetic interaction was implied between the N-terminal region of NS2 and the upstream structural proteins, since the first transmembrane of NS2 was identified as a genetic determinant for infectivity by construction of chimeric HCV with various genotypes (Pietschmann et al., 2006). Analyses by co-immunoprecipitation and imaging microscopy for interaction between NS2 and other viral proteins in cultured cells suggest that NS2 interacts with p7 and E2 on the ER-derived dotted structure closed to lipid droplets that are surrounded by HCV core protein (Popescu et al., 2011). NS2 also interacts with NS3/4A to recruit the core protein from lipid droplets to the cytoplasmic motile puncta along microtubules (Counihan et al., 2011). HCV p7 is a short hydrophobic protein composed of 63 amino acids and is encoded between the structural and non-structural proteins (Carrere-Kremer et al., 2002). The cytoplasmic loop of p7 is located between the N-terminal and C-terminal transmembrane regions (Carrere-Kremer et al., 2002). HCV p7 is known as a viroprotein that forms homooligomerize to be a ion channel, which is then involved in assembly and release of virus particle in infected cells by modulating pH equilibration in intracellular vesicles (Carrere-Kremer et al., 2002; Jones et al., 2007; Steinmann et al., 2007; Wozniak et al., 2010). Mutations of conserved amino acids required for ion channel activity impaired the production of infectious virus (Jones et al., 2007). However, recruitment of HCV core protein from lipid droplets to the ER assembly site was independent of the ion channel activity of HCV p7 (Boson et al., 2011). HCV p7 enhanced ER localization of the core protein without other viral proteins regardless of viral genotype, although compatibilities between two transmembrane regions of p7 and the first transmembrane domain of NS2 are responsible for ER localization of core protein and infection (Boson et al., 2011). The second transmembrane region of p7, rather than the first, is critical for compatibilities with NS2 regarding recruitment of core protein to the ER assembly site, although both transmembrane regions of p7 are important to sustain infectivity (Boson et al., 2011). These reports speculate that localization of the core protein on lipid droplets may contribute to suppression of virus production and maintenance of persistent HCV infection, while localization of the

core protein on ER may positively support virus production under the fulminant condition.

REGULATION OF HOST LIPID METABOLISM BY THE CORE PROTEIN

The mechanisms by which the core protein can induce liver diseases and extrahepatic manifestations are unknown. Liver steatosis, which is one of the characteristics associated with persistent HCV infection, develops by accumulation of triglyceride-rich lipids in hepatocytes. However, the precise functions of HCV proteins in the development of fatty liver remain unknown due to the lack of an adequate system to investigate the pathogenesis of HCV. HCV core protein expression has been shown to induce lipid droplets in cell lines and hepatic steatosis and hepatocellular carcinoma in transgenic mice (Barba et al., 1997; Moriya et al., 1997; Hope and McLauchlan, 2000). The lipid composition of the core-transgenic mouse is similar to that of a hepatitis C patient (Koike et al., 2010; Miyoshi et al., 2011). These reports suggest that HCV core protein plays an important role in the development of various types of liver failure, including steatosis and hepatocellular carcinoma. Biosynthesis of triglycerides is mainly regulated by the sterol regulatory element-binding protein (SREBP)-1c. It has been reported that many genes regulated by SREBPs were induced during the early stage of HCV infection in the livers of chimpanzees (Bigger et al., 2004). Our study has demonstrated that the core protein enhances the binding activity of the LXRα–RXRα complex to the *srebp-1c* promoter in a PA28γ-dependent manner, resulting in upregulation of SREBP-1c and its regulating genes (Moriishi et al., 2007). The activation may be mediated by the direct interaction between the core protein and RXRα (Tsutsumi et al., 2002). Another mechanism is thought to be suppression of lipid secretion. Reduced serum levels of cholesterol and apolipoprotein B have been reported in patients with severe hepatitis C and core-transgenic mice (Perlemuter et al., 2002). The MTP regulates the assembly and secretion of VLDLs consisting of apolipoprotein E, cholesterol, and triglycerides. In core-transgenic mice, MTP-specific activity is significantly decreased (Perlemuter et al., 2002). In addition, DGAT1, which plays an important role in trafficking core protein from lipid droplets to the ER membrane (Herker et al., 2010), was reported to delay the turnover of lipid droplets that are coated by the core protein (Harris et al., 2011; **Figure 3**). Furthermore, increases in saturated and monounsaturated fatty acids enhance HCV RNA replication (Kapadia and Chisari, 2005). The core protein can enhance the production of reactive oxygen species (ROS) by induction of induced nitric oxide synthetase (iNOS) or by damage to the mitochondrial electron transport system, contributing to the emergence of hepatocellular carcinoma (Moriya et al., 2001; Okuda et al., 2002; Nunez et al., 2004), suggesting that accumulation of lipids hastens the occurrence of hepatocellular carcinoma by enhancing ROS production. The core protein is reported to be degraded by PA28gamma-dependent, but ubiquitin-independent, proteasome activity, and directly binds to PA28gamma (Moriishi et al., 2003; Suzuki et al., 2009). PA28gamma knockdown diminished liver steatosis, hepatocellular carcinoma, and insulin resistance induced by HCV core protein in the mouse liver (Moriishi et al., 2007). After our reports, several groups found that PA28gamma plays an important role in cell cycling by degradation of SRC-3, p16, p19, and p53 (Li et al., 2006; Chen et al., 2007; Zhang and Zhang, 2008). Furthermore, HCV propagation in a cell culture system is potently suppressed by PA28gamma knockdown, regardless of cell growth (Moriishi et al., 2010). One possibility is that E6AP-dependent ubiquitination of the core protein in cytoplasm is competitively suppressed by peptide fragments deduced from nuclear core protein. However, there is still the possibility of an indirect effect of PA28gamma, since potent reduction of PA28gamma, but not intermediate reduction, can induce nuclear accumulation of HCV core protein in cultured cells and the mouse liver, but both potent and intermediate reductions could suppress viral production (Moriishi et al., 2007, 2010; Cerutti et al., 2011). Further study will be required to clarify the mechanism by which PA28gamma regulates core-induced liver diseases and the HCV life cycle.

NS3/4A AND LIPID DROPLETS

The NS3 also cleaves the host adaptor proteins IPS-1/MAVS and TRIF to modulate TLR and RIG-I signaling, resulting in inhibition of type I interferon production (Ferreon et al., 2005; Li et al., 2005a,b; Cheng et al., 2006; Loo et al., 2006). It is speculated that NS3 suppresses the activation of host innate immunity induced by HCV RNA and then contributes to persistent infection with HCV. NS3/4A may be responsible for not only the replication, but also the virus assembly and production by interaction with viral and host proteins on a region close to lipid droplets/ER assembly site. NS3/4A interacts with NS2 cooperating with p7 and E2 to recruit the core protein from lipid droplets to the cytoplasmic motile puncta along microtubules (Boson et al., 2011; Counihan et al., 2011; Popescu et al., 2011). HCV NS3/4A also interacts with host protein Y-box-binding protein-1 (YB-1) and influences the equilibrium between viral replication and infectious particle production (Chatel-Chaix et al., 2011). Knockdown of YB-1 impaired HCV RNA replication, regardless of the viral genotype, but did not affect NS3/4A autoprocessing and MAVS cleavage (Chatel-Chaix et al., 2011). JFH1 infection allowed YB-1 to translocate to lipid droplets containing core protein and NS3 (Chatel-Chaix et al., 2011), although knockdown of YB-1 enhanced the production of viral infectious particles (Chatel-Chaix et al., 2011). YB-1 may cooperate with NS3/4A to negatively regulate the steps after replication and to positively regulate viral replication.

NS5A AND CYCLOPHILINS

The peptide bond *cis*/*trans* isomerase converts between *cis* and *trans* peptide bonds leading to correct folding of the protein substrate. Peptidyl prolyl *cis*/*trans* isomerase (PPIase) includes the families of cyclophilin (Fischer et al., 1989), FK506-binding proteins (FKBP; Siekierka et al., 1989a,b) and parvulins (Rahfeld et al., 1994), and the secondary amide peptide bond *cis*/*trans* isomerase (Schiene-Fischer et al., 2002). Cyclophilin and FKBP are categorized as immunophilins, which are targeted by the immunosuppressants cyclosporin and FK506, respectively (Liu et al., 1991). Some cyclophilins and FKBP8 were shown to interact with NS5B and/or NS5A and to regulate HCV replication (Watashi et al., 2005; Okamoto et al., 2006), suggesting that immunophilins could lead to promising therapies for chronic hepatitis C, as discussed below.