

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

CoreTGvsWT						altered protein levels in PA28γ ^{-/-} CoreTGvsWT					
protein	gene ID	symbol	confidence level ^a	(-) under, (+) over ^b	fold change ^c	protein	gene ID	symbol	confidence level ^a	(-) under, (+) over ^b	fold change ^c
P31749	207	AKT1	3	-	2.68	O60508	51362	CDC40	2	+	1.99
P07355	302	ANXA2	3	+	2.92	P54105	1207	CLNS1A	3	+	2.41
O43747	164	AP1G1	3	+	5.72	P21964	1312	COMT	2	+	2.71
P63010	163	AP2B1	3	+	2.40	P67870	1460	CSNK2B	3	+	1.90
Q96CW1	1173	AP2M1	2	+	1.93	P78352	1742	DLG4	2	+	4.08
Q9Y2T2	26985	AP3M1	2	+	1.89	Q95GK7	1837	DTNA	3	-	2.42
P05089	383	ARG1	2	+	2.08	P55010	1983	EIF5	3	+	2.19
P52566	397	ARHGDIB	3	+	2.02	Q08495	2039	EPB49	3	+	2.66
O15145	10094	ARPC3	2	-	2.25	P37268	2222	FDFT1	2	+	5.68
P49407	408	ARRB1	3	-	2.33	P09038	2247	FGF2	2	+	2.69
Q07812	581	BAX	3	+	2.03	P62962	2280	FKBP1A	2	+	1.89
P55212	839	CASP6	3	-	1.95	O75146	9026	HIP1R	2	-	2.08
Q14790	841	CASP8	2	+	2.18	Q9NZL4	23640	HSPBP1	3	+	3.46
Q03135	857	CAV1	2	+	2.07	P05412	3725	JUN	2	+	2.15
P12830	999	CDH1	3	-	2.33	P52292	3838	KPNA2	3	-	7.28
P19022	1000	CDH2	3	+	4.57	P36507	5605	MAP2K2	3	-	2.35
Q53SH4	1134	CHRNA1	3	-	3.11	Q16539	1432	MAPK14	3	-	3.29
P21964	1312	COMT	2	+	2.96	P22033	4594	MUT	3	+	2.46
P00450	1356	CP	3	+	2.36	P54920	8775	NAPA	2	-	1.97
P21291	1465	CSRP1	3	+	2.23	Q8IZ57	140767	NRSN1	3	+	1.93
P49711	10664	CTCF	3	+	6.13	Q16620	4915	NTRK2	3	+	2.50
P25685	3337	DNAJB1	3	-	2.16	P07237	5034	P4HB	3	+	2.04
P63241	1984	EIF5A	3	+	1.94	Q08209	5530	PPP3CA	3	+	7.55
P42566	2060	EPS15	3	+	4.28	Q06124	5781	PTPN11	2	+	2.33
Q92889	2072	ERCC4	3	+	5.43	Q99638	5883	RAD9A	2	-	1.97
O75899	9568	GABBR2	3	+	3.39	P43487	5902	RANBP1	3	+	2.29
O43719	27336	HTATSF1	3	+	5.76	Q9UPX8	22941	SHANK2	3	-	1.94
P06756	3685	ITGAV	3	+	6.32	P29353	6464	SHC1	3	+	3.27
Q14974	3837	KPNB1	3	-	1.86	Q92186	8128	ST8SIA2	3	+	4.06
Q16539	1432	MAPK14	3	-	2.81	P31948	10963	STIP1	3	-	1.99
Q9UPY8	22924	MAPRE3	3	+	2.46	O75558	8676	STX11	2	+	2.04
P49736	4171	MCM2	3	+	1.87	P23193	6917	TCEA1	3	-	2.17
P62166	23413	NCS1	3	-	2.24	P07101	7054	TH	2	+	2.78
Q8IZ57	140767	NRSN1	3	+	1.89	P13693	7178	TPT1	3	-	1.93
Q16620	4915	NTRK2	3	+	2.40	Q15628	8717	TRADD	3	-	2.00
Q14980	4926	NUMA1	3	-	1.94	P50607	7275	TUB	3	+	1.91
P07237	5034	P4HB	3	+	2.27	altered protein levels in PA28γ ^{-/-} CoreTGvsCoreTG					
Q92878	10111	RAD50	3	+	4.93	protein	gene ID	symbol	confidence level ^a	(-) under, (+) over ^b	fold change ^c
Q99638	5883	RAD9A	2	-	3.10	P07355	302	ANXA2	3	-	2.96
P20936	5921	RASA1	3	+	1.86	O43747	164	AP1G1	3	-	4.20
Q96SB4	6732	SRPK1	3	+	3.11	P63010	163	AP2B1	3	-	3.01
Q92186	8128	ST8SIA2	3	+	5.11	Q96CW1	1173	AP2M1	2	-	1.88
P42224	6772	STAT1	3	+	2.00	Q9Y2T2	26985	AP3M1	3	+	2.38
P40763	6774	STAT3	3	+	2.30	O00499	274	BIN1	2	-	1.88
Q9UNK0	9482	STX8	3	+	1.88	Q9UQM7	815	CAM2KA	3	+	2.06
Q12800	7024	TFCP2	3	+	5.04	Q8N5S9	84254	CAMKK1	2	+	5.78
Q92752	7143	TNR	3	+	5.36	P19022	1000	CDH2	3	-	3.85
Q13263	10155	TRIM28	3	+	4.70	P25108	1134	CHRNA1	3	+	2.55
O43396	9352	TXNL1	3	-	4.82	P49674	1454	CSNK1E	3	+	1.97
P50552	7408	VASP	2	-	2.61	P67870	1460	CSNK2B	3	+	1.88
Q96AJ9	143187	VTI1A	3	+	3.25	P21291	1465	CSRP1	3	+	1.87
Q14191	7486	WRN	3	+	17.12	P49711	10664	CTCF	3	-	5.43
altered protein levels in PA28γ ^{-/-} CoreTGvsWT						Q8WTW3	9382	COG1	3	-	7.02
protein	gene ID	symbol	confidence level ^a	(-) under, (+) over ^b	fold change ^c	P00450	1356	CP	3	-	3.55
O15145	10094	ARPC3	2	-	1.96	Q13618	8452	CUL3	3	+	1.91
P49407	408	ARRB1	3	-	2.17	P78352	1742	DLG4	2	+	2.13
P55212	839	CASP6	3	-	2.04	Q9Y4J8	1837	DTNA	3	-	2.94

Table 1. continued

altered protein levels in PA28γ ^{-/-} CoreTGvsCoreTG						altered protein levels in PA28γ ^{-/-} CoreTGvsCoreTG					
protein	gene ID	symbol	confidence level ^a	(-) under, (+) over ^b	fold change ^c	protein	gene ID	symbol	confidence level ^a	(-) under, (+) over ^b	fold change ^c
Q08495	2039	EBP49	3	+	2.30	Q92878	10111	RAD50	3	-	5.19
O14682	8507	ENC1	3	+	2.88	P20936	5921	RASA1	3	+	2.50
P42566	2060	EPS15	3	-	2.11	P06400	5925	RB1	3	+	2.50
Q92889	2072	ERCC4	3	-	3.49	Q92854	10507	SEMA4D	2	+	2.00
P09038	2247	FGF2	2	-	2.18	Q92529	53358	SHC3	3	-	1.90
P62962	2280	FKBP1A	2	+	2.59	P63208	6500	SKP1	3	-	2.45
P49356	2342	FNTB	2	+	1.95	P43004	6506	SLC1A2	2	+	2.27
O75899	9568	GABBR2	3	-	2.53	Q4U2R8	9356	SLC22A6	3	-	2.23
O75146	9026	HIP1R	3	-	1.97	P42224	6772	STAT1	3	-	1.90
Q9NZL4	23640	HSPBP1	3	+	3.60	P31948	10963	STIP1	3	-	1.98
P61604	3336	HSPE1	3	-	2.17	O75558	8676	STX11	2	+	3.52
Q99730	27336	HTATSF1	3	-	9.24	Q8IZU3	50511	SYCP3	3	+	1.88
Q9Y6K9	8517	IKBK9	2	+	1.97	P07101	7054	TH	2	+	2.62
P52292	3838	KPNA2	3	-	3.94	Q92752	7143	TNR	3	-	4.62
P36507	5605	MAP2K2	3	-	2.66	O43396	9352	TXNL1	2	+	3.05
Q13505	4580	MTX1	3	-	1.90	P13693	7178	TPT1	3	-	2.85
P62166	23413	NCS1	3	+	2.56	Q13263	10155	TRIM28	3	-	3.53
Q14980	4926	NUMA1	2	+	1.87	Q15628	8717	TRADD	3	-	2.98
P41236	5504	PPP1R2	3	-	2.25	P50607	7275	TUB	3	+	1.96
Q08209	5530	PPP3CA	3	+	12.94	P41542	8615	USO1	3	-	2.05
P13861	5576	PRKAR2A	3	-	1.88	Q14191	7486	WRN	3	-	3.44
Q15276	9135	RABEP1	2	-	2.74						

^aDefined 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. ^cA 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* ≤ 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 Amadeus²⁸ and ORegAnno²⁹ in TargetMine¹⁶ 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 × 10⁴ 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 × 10⁴ 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'-GAGTGTCTGTCAGCCTCCA-3' and 5'-CACTCGCAAG-CACCCATCA-3', and 5'-GAAGGTCGGAGTCAACG-GATT-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'-TTGAAGGG-GACCGAAGACAGAACCTC-3', and 5'-TCAAACCCAA-GCCTCTGGTCTCCT-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% CO₂ 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.³⁵

Statistical Analysis

Experiments for RNAi transfection and qRT-PCR were performed three times. The estimated values were represented as the mean \pm 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 PA28 γ 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 γ ^{-/-}-CoreTGvsWT, and 26 proteins with increased levels and 36 proteins with decreased levels in PA28 γ ^{-/-}-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 infection³⁹ (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 NSSA protein and contributes to the pathogenesis of HCC,⁴⁰ was suppressed 1.88-fold in PA28 γ ^{-/-}-CoreTGvsCoreTG; this is consistent with the lack of HCC pathogenesis in PA28 γ ^{-/-}-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 PA28 γ '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-PA28 γ 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 γ ^{-/-}-CoreTGvsWT, and PA28 γ ^{-/-}-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 γ ^{-/-}-CoreTGvsWT extended network of 1057 entities with 8988 interactions, and the PA28 γ ^{-/-}-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 γ ^{-/-}-CoreTGvsWT (16.1), and PA28 γ ^{-/-}-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 PA28 γ 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 γ ^{-/-}-CoreTGvsWT, and PA28 γ ^{-/-}-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 γ ^{-/-}-CoreTGvsCoreTG network. Functional associations for the host factors previously known to be associated with HCV pathogenesis and HCC are

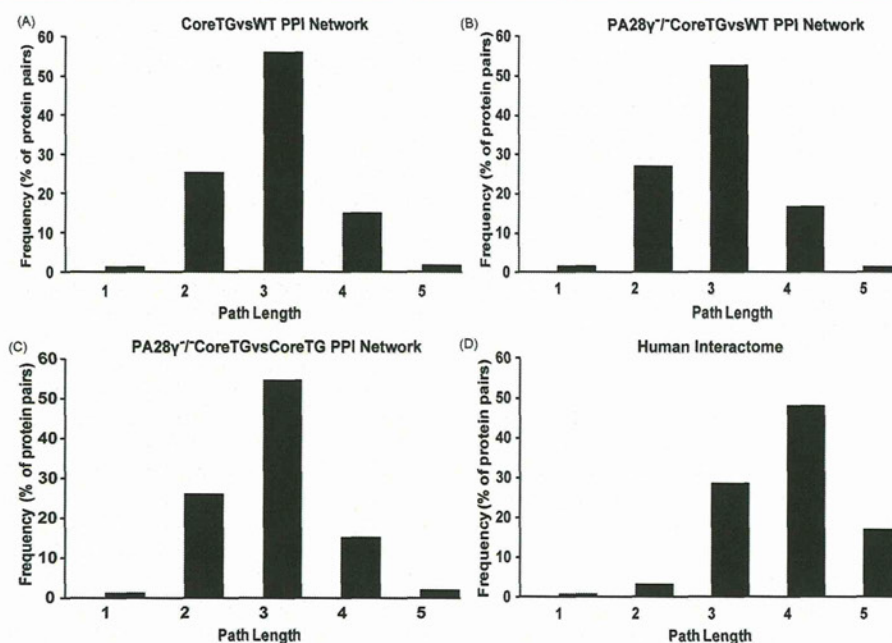


Figure 1. Graphical representation of the shortest path length distribution for (A) CoreTGvsWT extended network, (B) PA28 $\gamma^{-/-}$ CoreTGvsWT extended network, (C) PA28 $\gamma^{-/-}$ CoreTGvsCoreTG extended network, and (D) human protein interactome. The path length is represented on the x -axis, 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.

PA28 γ 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

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/PA28 γ 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 NSSA; disrupts mitochondrial homeostasis leading to abnormal cytochrome <i>c</i> 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 NSSA phosphorylation and hence infectious HCV particle production. ⁸⁷	loss of PA28 γ activity may perturb CK2-mediated NSSA 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 PA28 $\gamma^{-/-}$ 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 PA28 $\gamma^{-/-}$ 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 PA28 $\gamma^{-/-}$ 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 PA28 $\gamma^{-/-}$ CoreTG

^aHost 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, PA28 $\gamma^{-/-}$ CoreTGvsWT, and PA28 $\gamma^{-/-}$ CoreTGvsCoreTG PPI networks. ^bData set I: CoreTGvsWT; Data set II: PA28 $\gamma^{-/-}$ CoreTGvsWT; Data set III: PA28 $\gamma^{-/-}$ CoreTGvsCoreTG; +: upregulated; -: downregulated.

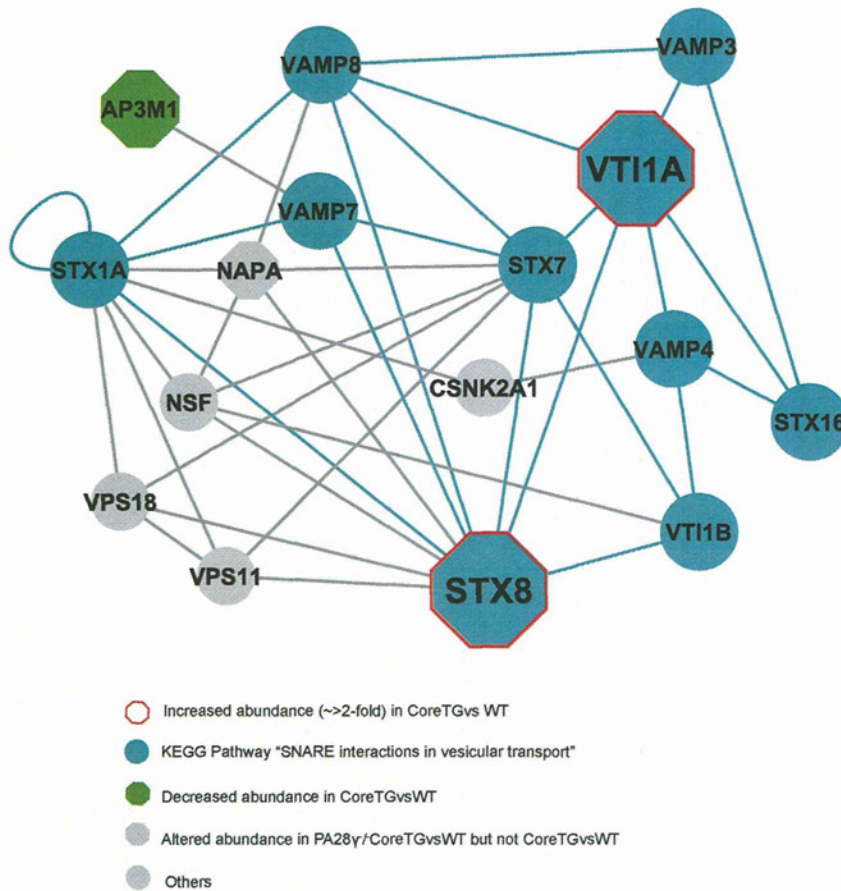


Figure 2. CoreTGvsWT SNARE network. Network illustration of the interactions between the PowerBlot identified differentially expressed proteins in CoreTGvsWT and human proteins mapped to the enriched KEGG pathway “SNARE interactions in vesicular transport”. The node sizes differ for better clarity and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

mechanism for the involvement of PA28 γ in HCV propagation. Potentially, the suppression of PA28 γ 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 NF κ B 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,

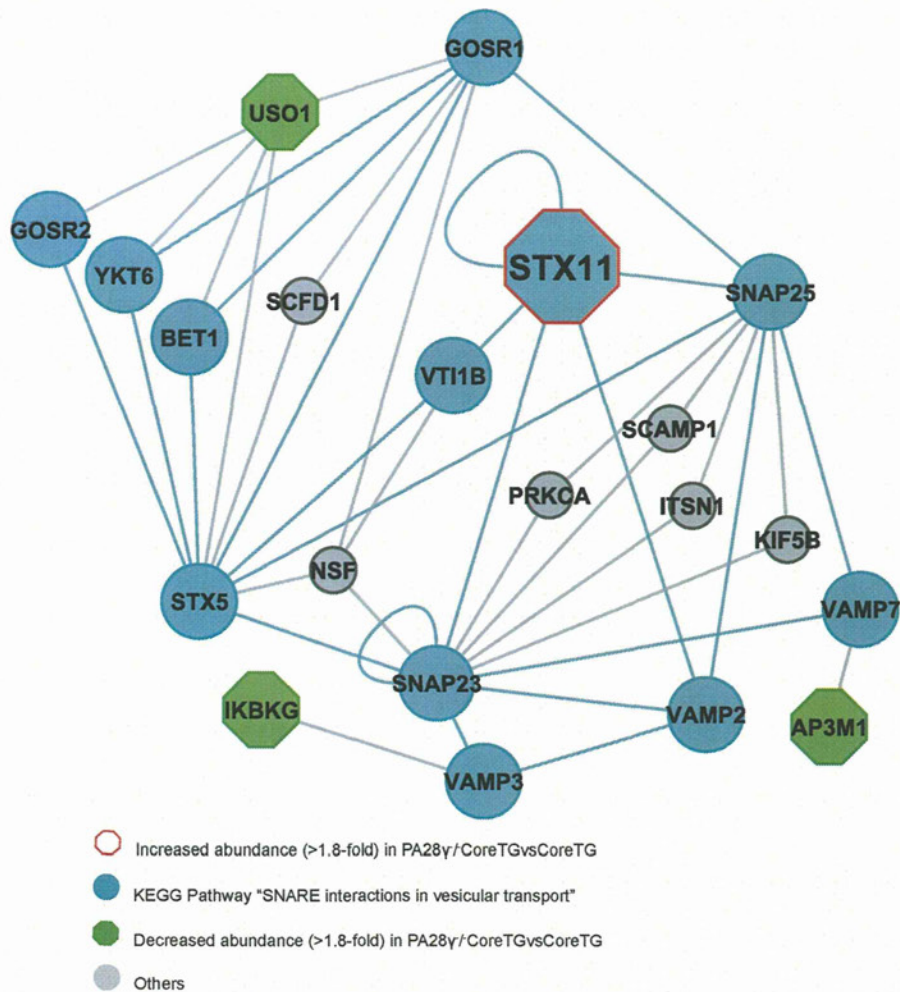


Figure 3. PA28 $\gamma^{-/-}$ CoreTGvsCoreTG SNARE network. Network illustration of the interactions between the PowerBlot identified differentially expressed proteins in PA28 $\gamma^{-/-}$ CoreTGvsCoreTG and human proteins mapped to the enriched KEGG pathway "SNARE interactions in vesicular transport". 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 proliferation⁷⁷ and is downregulated in some cancerous cells.^{78,79} PPP3CA was also mapped to the enriched KEGG pathways "VEGF signaling pathway" ($p = 2.59 \times 10^{-5}$), "MAPK signaling pathway" ($p = 2.37 \times 10^{-16}$) and "Wnt signaling pathway" ($p = 1.048 \times 10^{-10}$; 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.

MAP2K2 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 PA28 γ -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" ($p = 5.88 \times 10^{-9}$; 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} PA28 γ 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).

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	VTG1A (+), 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 hepatocarcinogenesis 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 phosphorylation 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 NSSA 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. ^bData 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 NSSA and regulates the production of infectious viral particles⁸⁷ 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 TNFR 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 TNFR 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 (PBMC), such as the T-cells, contributes significantly to the impaired immune response and HCV persistence in the host.⁸⁹⁻⁹⁴ PA28 γ is implicated in the cell cycle regulation, cell proliferation, and apoptosis⁹⁵⁻⁹⁸ 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 prioritization 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

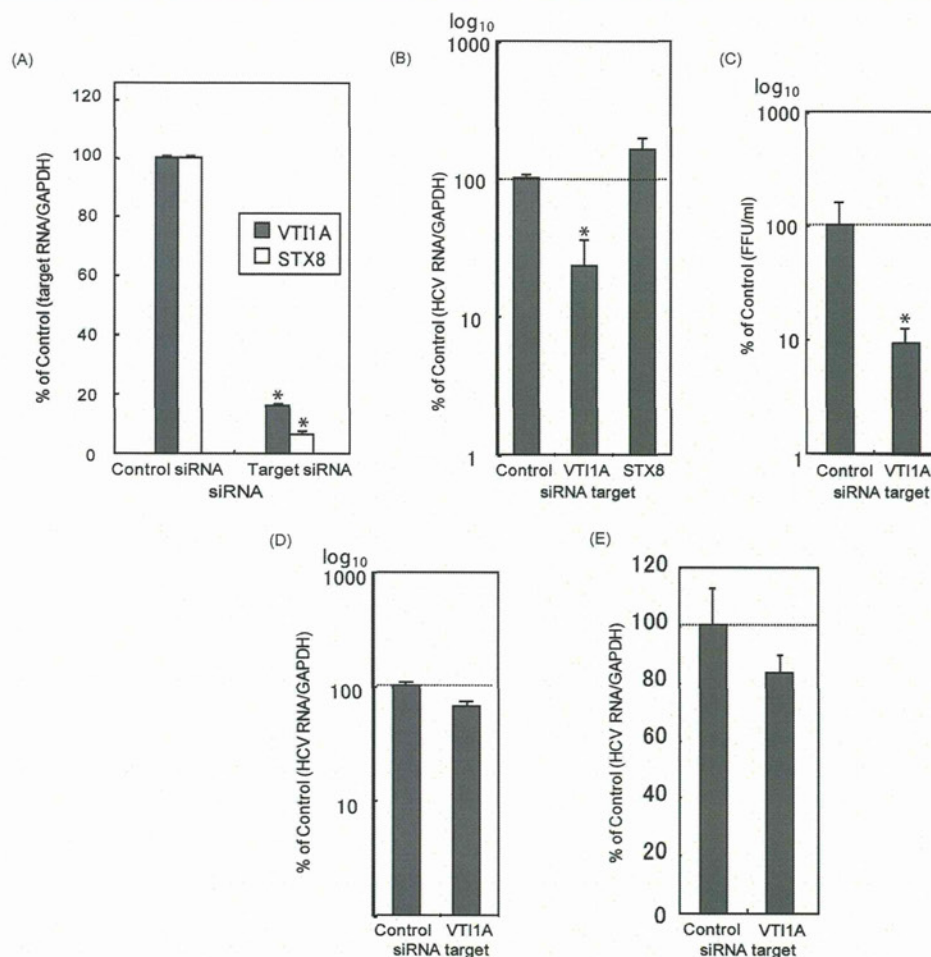


Figure 4. Effects of knockdown of candidate proteins on HCV propagation and replication. Host proteins VTI1A and STX8 were suppressed by RNAi (A) in Huh7OK1 cells infected with HCV JFH1 strain (genotype 2a; B, C, D) and in cells including subgenomic JFH1 replicon (E). The amounts of mRNA of the intracellular 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 γ ^{-/-}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 PA28 γ , we highlighted the cellular responses to HCV infection *in vivo* and obtained further insights into the role of PA28 γ 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 PA28 γ 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 PA28 γ 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 γ ^{-/-}CoreTG, as a novel regulator of HCV propagation but not replication. VTI1A-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-PA28 γ 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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