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The spliceosome factor SART1 exerts its anti-HCV action through mRNA splicing

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Abstract

Background/Aims—The broadly antiviral cytokine interferon- α (IFN α)'s mechanisms of action against HCV infection are not well understood. We previously identified SART1, a host

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List of author contribution:

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Notes: More supporting Materials and Methods are available in the online supplementary.

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protein involved in RNA splicing and pre-mRNA processing, as a regulator of IFN's antiviral effects. We hypothesized that SART1 regulates antiviral effector genes (IEGs) through mRNA processing and splicing.

Methods—We performed siRNA knockdown in Huh7.5.1 cells and mRNA-seq with or without IFN treatment. Selected gene mRNA variants and their proteins, together with HCV replication, were monitored by qRT-PCR and Western blot in HCV OR6 replicon cells and the JFH1 HCV infectious model.

Results—we identified 419 genes with greater than 2-fold expression difference between Neg siRNA and SART1 siRNA treated cells in the presence or absence of IFN. Bioinformatic analysis identified at least 10 functional pathways. SART1 knockdown reduced classical ISG mRNA transcription including MX1 and OAS3. However, SART1 did not affect Jak-STAT pathway gene mRNA expression and IFN stimulated response element (ISRE) signaling. We identified alternative mRNA splicing events for several genes, including EIF4G3, GORASP2, ZFAND6, and RAB6A that contribute to their antiviral effects. EIF4G3 and GORASP2 were also confirmed to have anti-HCV effect.

Conclusions—The spliceosome factor SART1 is not IFN-inducible but is an IFN effector gene. SART1 exerts its anti-HCV action through direct transcriptional regulation for some ISGs and alternative splicing for others, including EIF4G3, GORASP2. SART1 does not have effects on IFN receptor or canonical signal transduction components. Thus, SART1 regulates ISGs using a novel, non-classical mechanism.

Keywords

hepatitis C virus (HCV); janus kinase-signal transducers and activators of transcription (JAK-STAT) pathway; squamous cell carcinoma antigen recognized by T cells (SART1); RNA Sequencing (RNA-Seq); RNA splicing

Introduction

Hepatitis C virus (HCV) infects over 180 million people worldwide and is the leading cause of chronic viral liver disease [1]. Until now, type I interferon α (IFN α) has been the backbone of therapy for HCV. Unfortunately, there is still a sizable population of HCV-infected persons nonresponsive to current therapy. The precise mechanisms for HCV's persistence are still incompletely understood. We previously performed a high throughput whole-genome siRNA library screen and identified 22 genes that mediate IFN's antiviral effects. Many of the identified genes are involved in RNA splicing and pre-mRNA processing [2]. Silencing of one of the identified splicing genes, SART1 (squamous cell carcinoma antigen recognized by T cells) abrogates IFN's suppressive effects against HCV. SART1 is a component of the spliceosome [3]. We hypothesized that SART1 regulates downstream ISGs and IFN effector genes (IEGs) through mRNA splicing. The majority of human genes express multiple isoforms and more than 80 percent are alternatively spliced, so incorporating exon-level analysis would allow us to study this transcriptional variation [4, 5]. Alternative splicing and isoform variations may shape the host response to HCV. Characterization of gene variants that are differentially expressed in the presence of IFN stimulation will allow us to specifically identify hundreds of thousands of proteins that arise

from the relatively low number of protein-coding interferon effector genes (IEGs) [4]. mRNA-seq using next-generation DNA sequencing (NGS) technologies provides more precise measurement of mRNA transcript levels and their isoforms than does existing microarray technology [6]. We reasoned that the application of RNA-Seq technology could significantly increase the likelihood of identifying key processes that drive HCV persistence and discovering possible new host pathways and target proteins that regulate JAK-STAT, IEGs, and virus. In this report, we found that SART1 regulated HCV replication through differential expression of interferon stimulated genes (ISGs) and alternative splicing of IEGs. Our findings imply that SART1 regulates both ISGs and IEGs in a non-classical manner to contribute to the IFN-induced antiviral state.

Materials and Methods

Cell cultures and infectious viruses

The Huh7.5.1 was grown in DMEM with 10% FBS. The Huh7.5.1 cells were infected with genotype 2a JFH1 HCV virus (0.2 moi) (JFH1 cells) as previously described [7–9].

RNA-Sequencing

The four Huh7.5.1 cell RNA sample (2 µg each) sets included #1. Neg siRNA/Neg IFN α , #2. Neg siRNA/ IFN α (30 IU/ml), #3. SART1 siRNA/Neg IFN α , and #4. SART1 siRNA/ IFN α (30 IU/ml). Non-strand specific RNA sequencing performed at the Broad Institute (Cambridge, MA) uses a large-scale, automated variant of the Illumina Tru Seq™ RNA Sample Preparation protocol.

Statistical analysis

Data analysis was performed using a 2-tailed Student's t-test. Data are expressed as mean $\pm$ SD of at least three sample replicates, unless stated otherwise. *P < 0.05, ** P < 0.01, and #P < 0.001 for comparison of indicated treatments.

Results

Differential gene expression analysis using mRNA-seq

We obtained sequence reads for 26,090 genes from the mRNA-Seq data (Supplemental Table 1 (STab1)). There are 419 genes that demonstrated greater than 2-fold difference between negative control siRNA and SART1 siRNA treated cells in the presence or absence of IFN (STab 2). Ingenuity Pathway Analysis (IPA) on this set of selected genes uncovered SART1 regulated functional pathways. We identified at least 10 pathway categories with significant enrichment using Gene Ontology biological pathway terms (p<0.05) (Supplemental Figure 1 (SFig1)). Genes in these enriched pathway include classical interferon and virus response genes such as Mx1, OAS3, IFIH1 (MDA5), and DDX58 (RIG-I). These findings suggest that SART1 regulates multiple functional pathways, including interferon and virus response, molecular transport and metabolism.

SART1 regulates classical ISGs through differential expression

We performed qPCR to compare the mRNA expression of two selected groups of genes (classical ISGs and Jak-STAT1 pathway) in Huh7.5.1 cells to verify differential expression by RNA-Seq bioinformatics analysis (STab 3). The qPCR data confirmed the consistency between RNA-Seq and qPCR analysis (STab 3). We confirmed that knock-down of SART1 reduced classical ISG mRNA expression including MX1, OAS3 [2], IFIH1, and DDX58 (STab 3-1). These findings indicate that SART1 regulates classical ISG differential expression. We found that siRNA to SART1 did not significantly affect IFNAR1, STAT1, JAK1, IRF1, or IRF9 mRNA expression (STab 3-2). Western blot confirmed that SART1 knock down did not affect protein expression levels for IFNAR1, JAK1, and STAT1 (Fig 2F). These findings indicate that SART1 regulation of classical ISGs did not act through direct regulation of the Jak-STAT pathway.

SART1 knockdown reduces classical ISG mRNA expression

We found that HCV replication reduced MX1, OAS3, DDX58, and IFIH1 mRNA expression in JFH1 cells. RNAi mediated knock down of SART1 reduced MX1, OAS3, RIG-I, and IFIH1 mRNA expression in both Huh7.5.1 and JFH1 cells (Fig 1A–D). These findings indicate that MX1, OAS3, RIG-I, and IFIH1 are under SART1 regulation. We also performed siRNA mediated knockdown of MX1 and OAS3 to test their effects on HCV replication in OR6 replicon cells and in JFH1 cells. The OR6 cell line is an HCV replicon that stably harbors full-length genotype 1b HCV RNA and co-expresses *Renilla* luciferase [10, 11]. siRNA to MX1 or OAS3 rescued IFN α inhibition of HCV replication in OR6 (STab 4, SFig 2) and JFH1 cells (Fig 1E–G). It has been reported that OAS3 is constitutively expressed in Huh7 cells, and is significantly induced following IFN treatment. OAS3 may exert its anti-HCV replication through the activation of RNase L in the early stage of antiviral response [12]. The MX1(A) protein has also been shown to be involved in HCV induced stress granules formation that regulate HCV replication through virus assembly and egress [13]. We also demonstrated that knockdown of MX1 or OAS3 each blocked IFN-mediated HCV inhibition in JFH1 replication model. We found that overexpression of SART1 rescued HCV-mediated inhibition of MX1 and OAS3 mRNA expression (Fig 1H–I). In addition, we found that SART1 siRNA influenced the frequency of IFN-induced MX1 and OAS3 variant mRNA expression (SFig 3–4). Further characterization of these variants' effects on HCV replication are warranted. These data suggest that SART1 regulates HCV replication through differential expression of the classical ISGs such as MX1 and OAS3.

SART1 regulation of classical ISGs and HCV is not mediated by the Jak-STAT pathway

HCV replication reduced IFNAR1, JAK1, and IRF9 mRNA expression in JFH1 cells. siRNA to SART1 did not affect IFNAR1, JAK1, and IRF9 mRNA and protein expressions compared to negative control siRNA, indicating that JAK-STAT1 pathway genes are not under SART1 regulation (Fig 2A–C, 2F, STab 3-2).

SART1 effects on HCV do not act through the ISRE signaling pathway

We confirmed that SART1 siRNA significantly increased HCV and partially rescued HCV RNA from IFN α inhibition [2] (Fig 2D). IFN treatment did not induce SART1 mRNA expression in Huh7.5.1 or JFH1 cells. HCV infection significantly reduced SART1 mRNA and protein expression in JFH1 cells (Fig 2E–F). HCV infection inhibited IFN-stimulated ISRE promoter signaling in JFH1 cells (Fig 2G). However, SART1 siRNA did not affect ISRE promoter signaling in either Huh7.5.1 or JFH1 cells, further demonstrating that SART1 suppression of HCV replication did not act through classical JAK-STAT or ISRE – directed signaling pathways (Fig 2G). In previous reports, we demonstrated that HCV NS5A or core bound to STAT1 to block STAT1 phosphorylation and IFN stimulated Jak-STAT signaling pathway [9, 14, 15]. In this study, we found that HCV replication also significantly reduced SART1 mRNA expression (Fig 2E), indicating that HCV may also contribute to its persistence partially through non-classical means, such as SART1 suppression.

Alternative splicing analysis

One of the advantages of RNA-Seq over microarray is that it can provide more precise assessment of transcript levels and their isoforms. From the mRNA-seq data, we identified a total of 2744 exons with altered inclusion status (1589 exons with decreased inclusion and 1155 exons with increased inclusion) by SART1 or IFN α . We then selected the top 26 altered exons for RT-PCR validation (STab 5). We have verified that several genes' mRNA alternative splicing variants are regulated by SART1 and IFN treatment. These confirmed genes included eukaryotic translation initiation factor 4 gamma 3 (EIF4G3), Golgi reassembly stacking protein 2 (GORASP2), zinc finger AN1-type domain 6 (ZFAND6), and RAB6A member RAS oncogene family (RAB6A). We found little overlap between differentially expressed genes and alternatively spliced genes, which is consistent with previous reports [16, 17].

EIF4G3, GORASP2, and ZFAND6 regulate HCV replication

We found that siRNA to EIF4G3, GORASP2 or ZFAND6 significantly increased HCV replication. In contrast, siRNA to RAB6A had no effect on HCV replication (SFig 5A). Cell Titer Glo assay showed that siRNA to EIF4G3, GORASP2, RAB6A or ZFAND6 did not affect cell viability (SFig 5B). These findings indicated that EIF4G3, GORASP2 and ZFAND6 participate in regulation of HCV replication. Moreover, we found that knockdown of EIF4G3 or GORASP2 did not affect MX1 or OAS3 mRNA expression (SFig 6), indicating that EIF4G3 or GORASP2 do not regulate these genes. We therefore selected EIF4G3 and GORASP2 to further characterize their mRNA isoforms' expression and their effects on HCV replication.

SART1 knock down selectively reduces EIF4G3 variant 2 expression

EIF4G3 has two isoforms based on exon splicing bioinformatics analysis (STab 5). EIF4G3 V2 includes exon 4 while V1 does not. We designed several pairs of isoform-specific primers to monitor expression of EIF4G3 mRNA variants (STab 6, Figure 3A). The RT-PCR images and qPCR results confirmed the RNA-Seq alternative splicing bioinformatics analysis for EIF4G3 (Figure 3A–B, STab 5). We found that siRNA to SART1 significantly

inhibited EIF4G3 total and V2 mRNA expression with or without IFN α treatment in Huh7.5.1 cells. In contrast, siRNA to SART1 did not significantly affect EIF4G3 V1 levels (Figure 3B–C). These findings indicate that SART1 regulates EIF4G3 specifically through modulation of EIF4G3 V2 expression.

EIF4G3 is antiviral in the JFH1 HCV replication model

Knock-down of EIF4G3 increased HCV RNA (Figure 4A) and HCV core protein levels (Figure 4B). IFN α did not affect EIF4G3 mRNA or protein expression (Fig 4A–B). These findings suggested that EIF4G3 regulation of HCV replication is independent of direct IFN regulation. We also found that knockdown of EIF4G3 increased HCV internal ribosome entry site (IRES)-directed translation (Fig 4C), indicating that EIF4G3 regulates HCV replication through IRES-mediated translation. To further analyze EIF4G3 variants' effects on HCV replication, we overexpressed EIF4G3 V1 and V2 in JFH1 cells. We found that both EIF4G3 V1 and V2 overexpression reduced HCV RNA and HCV core protein levels in JFH1 cells (Figure 4D–E), further confirming EIF4G3's anti-HCV function. These findings suggested that SART1 regulates HCV replication partially through EIF4G3 V2 expression.

SART1 knockdown decreases GORASP2 mRNA and protein expression

The GORASP2 has two isoforms based on exon splicing bioinformatics analysis (STab 5). GORASP2 V2 includes exon #2 while V1 does not. We applied a similar strategy to monitor total and GORASP2 variants (STab 6, Fig 3A). We confirmed the RNA-Seq alternative splicing bioinformatics analysis for GORASP2 gene (Fig 3A–B, STab 5). We found that siRNA to SART1 significantly inhibited GORASP2 V1, V2, and total mRNA expression with or without IFN α treatment in Huh7.5.1 cells (Fig 3D).

GORASP2 V1 inhibits HCV replication in JFH1 cells

We found that siRNA knockdown of GORASP2 increased HCV RNA and protein levels (Fig 4F–G). GORASP2 mRNA and protein expression were not affected by IFN treatment (Fig 3D, 4G), indicating that GORASP2 expression is independent of direct IFN regulation. We found that GORASP2 V1 overexpression reduced HCV RNA and protein levels in JFH1 cells (Fig 4H–I). In contrast, overexpression of GORASP2 V2 had no significant effect on HCV RNA or protein levels (Fig 4H–I) in JFH1 cells. By Western blot, we confirmed that endogenous GORASP2 V1 expressed a 55 kDa protein. GORASP2 V1 –Myc-DDK-tagged overexpression produced a 60 kDa protein. In contrast, as expected, GORASP2 V2 –Myc-DDK-tagged with exon #2 insertion created a premature stop, and the resultant overexpressed protein degraded (Fig 4I). These findings imply that SART1 knockdown rescued HCV replication partially through splicing that led to reduced GORASP2 protein expression. GORASP2 was not in the list of genes with greater than two fold differential expression by SART1 siRNA. The RNA-Seq alternative splicing analysis provided much more detailed measurement in this novel identification of GORASP2 splicing variants. To further explore the possible mechanisms by which GORASP2 regulates HCV replication, we monitored HCV RNA in JFH1-infected Huh7.5.1 cells and supernatant treated with Neg siRNA, GORASP2 siRNA, or GOLPH3 siRNA (Table 1). We confirmed that GOLPH3 knockdown reduced HCV virion secretion in JFH1-infected Huh7.5.1 culture supernatant, but not HCV RNA replication, consistent with a previous report [18]. Moreover, we found

that GORASP2 knockdown increased both intracellular HCV RNA replication and virion trafficking and release to the supernatant (Table 1), suggesting that GORASP2 protein regulates HCV replication through HCV RNA replication and virion protein trafficking.

Discussion

The antiviral ISGs are thought to be the primary effectors of the IFN response [19]. Moreover, both the type I and III IFN families have been shown to exert their anti-HCV effects through utilization of the intracellular JAK-STAT and ISG pathway in hepatoma cells, primary hepatocytes, and *in vivo* [14, 15, 20, 21]. However, there is growing evidence that host factors regulate HCV replication independent of JAK-STAT signaling. For example, it has been reported that a ligand of a member of the tumor necrosis factor receptor family CD40L (CD154), inhibits HCV. The antiviral effect of CD40L requires c-Jun N terminal kinase (JNK)1/2 activation, but not induction of the JAK/STAT pathway [22].

In this report, we showed that SART1 siRNA reduced MX1 and OAS3 mRNA expression, which in turn rescued IFN inhibition of HCV replication in the JFH1 infectious replication model. We found that SART1 regulates HCV directly through classical ISGs such as MX1 and OAS3, which are differentially expressed, independent of JAK-STAT activation. We found that SART1 is antiviral for HCV [2], and that HCV infection reduces SART1 expression, strongly suggesting that HCV promotes its persistence through suppression of multiple levels of host innate immunity, including not only the classical IFN- JAK-STAT pathway but also host components of the spliceosome. Changes in exon usage and alternative splicing are increasingly being shown to affect important pathways and phenotypes [23, 24]. RNA Seq reveals significantly more details of the transcripts and novel splicing variants, thereby greatly increasing our understanding of the molecular mechanisms of disease [24, 25]. Our RNA-Seq approach provides the first assessment of the relationship between SART1 and the anti-HCV IEG pathway. We identified several genes, including EIF4G3, GORASP2, and ZFAND6, that are alternatively regulated by SART1, and we were able to functionally confirm their anti-HCV effect in the fully infectious JFH1 HCV system.

Our findings indicate that SART1 can regulate HCV replication through ISGs and antiviral IEGs through differential expression and alternative splicing. However, one of the limitations of our study is that we only had one replicate of each of our RNA-Seq samples. We were able to confirm the RNA-Seq bioinformatics differential analysis by qPCR in the most selected genes in both classical ISGs and Jak-STAT group genes, suggesting the accuracy and consistency of RNA-Seq differential analysis. However, we only confirmed 4 splicing exons of the 26 selected exons by RT-PCR. It is bioinformatics consensus that more replicates for each RNA-Seq sample could increase the confirmation rate for exon splicing analysis [24, 26–28]. Thus, our initial analysis is likely to provide only a *minimum* estimate of the number of altered exon splicing events. Our study therefore underscores both the power and shortcomings of RNA-Seq in revealing host-pathogen interactions [29].

Among those confirmed splicing variants (isoforms), we found that SART1 regulated HCV replication partially through reducing EIF4G3 V2 protein expression. The EIF4G3 (also known as eIF4GII) protein contains mRNA binding domains and is a critical component in

the initiation of protein translation [30-32]. It has been reported that host and viral protein translation were regulated through EIF4G3 by several viruses [33, 34]. For example, foot-and-mouth disease virus (FMDV) and human rhinovirus inhibit host cell protein synthesis within infected cells through cleavage of eIF4GII. Vaccinia virus (VV) can not translate its RNA or replicate without EIF4G3 [35, 36]. However, HCV viral internal ribosome entry site (IRES) does not need to compete for limiting EIF4 proteins to hijack host ribosomes in infected cells [30]. The HCV IRES can bind directly to 40S and eIF3 and either eIF2/eIF5 to pair tRNAi with the IRES start codon to produce HCV proteins. HCV selectively inhibits cap-dependent translation of cellular mRNAs through EIF4G3, while viral mRNA translation is unaffected because of its utilization of IRESs [30, 37]. We therefore speculate that SART1 siRNA increases HCV replication through reduction of EIF4G3 V2 and subsequent antiviral protein translation to short circuit the host innate immune response.

We also found that SART1 knockdown increased HCV replication partially through reducing GORASP2 (also known as GRASP55) expression. Golgi apparatus matrix proteins include two homologues, the Golgi reassembly stacking protein of 65 kDa (GRASP65, or GORASP1) and the 55 kDa GRASP55 (or GORASP2) [38-40]. GORASP proteins are involved in establishing the Golgi apparatus structure, which plays an important role in the processing and sorting of proteins exported from the endoplasmic reticulum [41, 42]. It has been demonstrated that GRASP proteins do not affect cell apoptosis or ER stress. However, GRASP2 protein knockdown has been shown to increase vesicular stomatitis virus glycoprotein (VSV-G) traffic through the Golgi in cells infected with VSV-G ts045-GFP adenovirus [42]. We therefore speculate that GORASP2 protein affects HCV replication partially through regulation of HCV protein trafficking and virus assembly. Further mechanistic studies on EIF4G3 and GORASP2 regulation of HCV are warranted. It is likely that ongoing analysis of our exon-level experimental data may further provide us even more examples of transcriptional events underlying the host antiviral response to HCV.

We finally conclude that SART1 is not IFN-inducible but is an IFN effector gene. SART1 exerts its anti-HCV action through direct transcriptional regulation for some ISGs (e.g., MX1, OAS3) independently of the JAK-STAT pathway, and alternative splicing for others, including EIF4G3, GORASP2, and ZFAND6. SART1 does not have effects on IFN receptor or signal transduction components, but regulates HCV replication partially through EIF4G3 V2 and GORASP2 V1 alternative exon splicing. We therefore propose a SART1 regulation pathway model, in which SART1 regulates HCV replication through differential splicing of classical ISGs and IEGs, independent of the JAK-STAT and ISRE pathway. We found that SART1 also directly regulates antiviral IEGs by alternative exon splicing (SFig 7). Taken together, these data imply that the spliceosome factor SART1 regulates ISGs and antiviral IEGs in a non-classical manner. This previously unrecognized endogenous antiviral mechanism has implications for development of host-directed strategies to interrupt the viral lifecycle and prevent establishment of persistent infection for HCV and, potentially, other viral infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. SART1 knockdown reduced anti-HCV ISGs MX1, OAS3, RIG-I, and IFIH1 mRNA expression

SART1 siRNA inhibited IFN α activated MX1, OAS3, DDX58, and IFIH1 mRNA expression in Huh7.5.1 and JFH1 cells. Each selected gene mRNA levels were normalized to GAPDH mRNA yielding arbitrary units (fold). *P < 0.05, **P < 0.01, and #P < 0.001 for comparison of indicated SART1 siRNA treatment and Neg siRNA without or with IFN respectively.

(A) MX1 mRNA.

(B) OAS3 mRNA.

- (C) DDX58 mRNA.
- (D) IFIH1 mRNA.
- (E) Knockdown MX1 or OAS3 increased HCV replication in JFH1 cells.
- (F) MX1 mRNA knockdown by MX1 siRNA.
- (G) OAS3 mRNA knockdown by OAS3 siRNA.

Figure 2. SART1 siRNA does not affect JAK-STAT component mRNA expression and IFN-stimulated ISRE signaling

Results are expressed as mean $\pm$ STDs from three experiments.

(A) SART1 siRNA does not affect IFNAR1 mRNA.

(B) SART1 siRNA does not affect JAK1 mRNA.

(C) SART1 siRNA does not affect IRF9 mRNA.

(D) SART1 knockdown significantly increased HCV replication and partially rescued IFN suppression of HCV replication. JFH1 HCV RNA levels were normalized to GAPDH mRNA yielding arbitrary units (fold).

(E) SART1 is not IFN-inducible. IFN α treatment did not induce SART1 mRNA expression. qPCR confirmed SART1 mRNA knocked down in Huh7.5.1 and JFH1 cells. HCV infection significantly reduced SART1 mRNA levels in JFH1 cells.

(F) SART1 siRNA increased HCV core expression.

(G) SART1 siRNA does not affect IFN α -stimulated ISRE signaling. Neg siRNA or SART1 siRNA were transfected in Huh7.5.1 or JFH1 cells 24 hours before pISRE-luc (expressing firefly luciferase) and pRL-TK (expressing *renilla* luciferase) co-transfection. PEG-IFN α -2b (30 IU/mL) was added 48 hours later. IFN α -stimulated ISRE luciferase levels were normalized to RL-TK luciferase levels yielding the relative luciferase unit (RLU).

Figure 3. SART1 knocked down selectively reduced EIF4G3 and GORASP2 variants mRNA levels

(A) EIF4G3 and GORASP2 variant 1 and 2 mRNA structure, and the primer strategy for PCR to monitor each variant. V2 has included extra exon while V1 without extra exon.

(B) RT-PCR images. To monitor both EIF4G3 V1 and V2 in the same gel image, we used primer sequences F1 and R1, which are located in exon #3 and #5, respectively, for RT-PCR. The gel images showed that siRNA SART1 knocked down SART1 mRNA, and reduced V2 mRNA expression. GAPDH demonstrated relatively equal levels of mRNA for each treatment.

(C) SART1 siRNA reduced EIF4G3 variant 2 and total mRNA levels. To assess EIF4G3 V2 quantification, we used primer sequence F1 (located in exon #3) and R2 (located in exon #4) for qPCR. To monitor V1, we used primer sequence F2 (located across exon#3 and #5) and R3 (located in exon #5). To assess total amount of EIF4G3 mRNA level, we used primers F3 and R3 (both located in exon #5). We found that siRNA SART1 significantly reduced EIF4G3 V2 and total mRNA levels with or without IFN α in Huh7.5.1 cells. In contrast, siRNA to SART1 did not significantly reduce EIF4G3 V1 levels. *P < 0.05, ** P < 0.01, and #P < 0.001 for comparison of indicated treatment and Neg siRNA/Neg IFN.

(D) siRNA SART1 significantly reduced GORASP2 V2, V1, and total mRNA levels. To assess GORASP2 V2 quantification, we used primer sequence F2 (located in exon #2) and R3 (located in exon #3) for qPCR. To monitor V1, we used primer sequence F4 (located across exon#1 and #3) and R3 (located in exon #3). To assess total amount of GORASP2 mRNA level, we used primers F3 and R3 (both located in exon #3).

Figure 4. EIF4G3 or GORASP2 siRNA increased HCV replication and Overexpression EIF4G3 or GORASP2 variants reduced HCV in JFH1 cells

* $P < 0.05$, ** $P < 0.01$, and # $P < 0.001$ for comparison of indicated treatment and pCMV-Empty vector/Neg IFN.

(A) EIF4G3 siRNA increased HCV RNA level and partially rescued IFN α inhibited HCV RNA level.

(B) EIF4G3 siRNA increased HCV core protein levels in JFH1 cells. Western blot confirmed EIF4G3 protein knocked down by EIF4G3 siRNA, EIF4G3 siRNA increased HCV core protein expression in JFH1 cells.

(C) EIF4G3 siRNA increased HCV IRES-directed translation. Huh7.5.1 cells were transfected with Neg siRNA, EIF4G3 siRNA, or GORASP2 siRNA for 24 hr followed by

transfection of pHCV-CLX-CMV RNA (HCV IRES harboring a firefly luciferase reporter) for 24 hr as previously described [43]. We found that knockdown of EIF4G3 increased IRES-directed luciferase activity compared to Neg siRNA or GORASP2 siRNA.

(D) EIF4G3 V1 or V2 overexpression reduced HCV RNA levels in JFH1 cells. pCMV-Empty, pEIF4G3 V1 and V2 plasmids were transfected to Huh7.51 and JFH1 cells. Overexpression EIF4G3V1 or V2 reduced HCV RNA level. Overexpression EIF4G3 V2 exerts stronger inhibition on HCV RNA level compared to V1. IFN treatment inhibited HCV RNA level. V1 or V2 plus IFN further inhibited HCV RNA levels.

(E) Overexpression EIF4G3 V1 or V2 reduced HCV core protein levels in JFH1 cells. Western blot confirmed the over expression of EIF4G3 V1 (1591 aa, 175 KDa) or V2 (1628 aa, 179 KDa) in Huh7.5.1 and JFH1 cells. EIF4G3 V1 or V2 over expressions reduced HCV core protein level in JFH1 cells, further confirming EIF4G3 anti-HCV function. EIF4G3 V2 overexpression had stronger reduction of HCV core level compared to EIF4G3 V1. #1. Huh7.5.1 / pCMV Empty / IFN α -, #2-7. JFH1 cells.

(F) GORASP2 siRNA increased HCV RNA level and partially rescued IFN α inhibited HCV RNA level.

(G) GORASP2 siRNA increased HCV core protein level in JFH1 cells. Western blot confirmed GORASP2 protein knocked down by GORASP2 siRNA, GORASP2 siRNA increased HCV core protein expression in JFH1 cells.

(H) GORASP2 V1 overexpression reduced HCV RNA levels in JFH1 cells. pCMV-Empty, pGORASP2 V1 and V2 plasmids were transfected to Huh7.51 and JFH1 cells. The qPCR showed overexpression GORASP2 V1 HCV RNA level. In contrast, overexpression of GORASP2 V2 had not significantly effect on HCV RNA level in JFH1 cells.

(I) Overexpression of GORASP2 V1 decreased HCV core protein levels in JFH1 cells. Western blot confirmed the endogenous GORASP2 V1 expressed a 55 kDa protein (lower band). GORASP2 V1 –Myc-DDK-tagged overexpression produced a 60 kDa protein (upper band). In contrast, as expected, GORASP2 V2 –Myc-DDK-tagged with exon #2 insertion created premature stop, the overexpressed protein degraded.

Table 1

Time course of JFH1 HCV RNA in culture cell and supernatant

Huh7.5.1 cells were transfected with Neg siRNA, GORASP2 siRNA, or GOLPH3 siRNA respectively for 48 hrs. The cells were inoculated with JFH1 virus for 2 hr and then washed with PBS twice. We found that GORASP2 siRNA increased HCV RNA levels in cells and supernatant started from 12 hr to 48 hr post infection compared to Neg siRNA, suggesting that GORASP2 regulates both intracellular HCV RNA replication and virion trafficking and release to the supernatant. In contrast, GOLPH3 siRNA did not affect intracellular HCV RNA levels at 6, 12, 24, and 48 hr compared to Neg siRNA, but reduced HCV RNA level in supernatant beginning from 12 hr, 24, and 48 hr, suggesting that GOLPH3 affects HCV virion trafficking and assembly. JFH1/GAPDH arbitrary unit is expressed as means and STDs from three sample replicates. P value reflects comparison of Neg siRNA with the indicated treatment in each time point.

Time	Cell	Treatment	JFH1/GAPDH Arbitrary Unit in cell			JFH1/GAPDH Arbitrary Unit in supernatant		
			Ave	STD	P-Value	Ave	STD	P-Value
6 hr	Huh7.5.1	Neg siRNA	1.01	0.20	n/a	1.01	0.17	n/a
6 hr	JFH1	Neg siRNA	514.55	46.95	n/a	1.63	0.26	n/a
6 hr	JFH1	GORASP2 siRNA	538.64	94.32	0.712	1.66	0.25	0.905
6 hr	JFH1	GOLPH3 siRNA	534.42	101.53	0.774	1.69	0.23	0.768
12 hr	JFH1	Neg siRNA	704.29	110.08	n/a	1.96	0.36	n/a
12 hr	JFH1	GORASP2 siRNA	932.66	141.63	0.092	3.03	0.56	0.049
12 hr	JFH1	GOLPH3 siRNA	708.68	101.66	0.962	1.62	0.17	0.213
24 hr	JFH1	Neg siRNA	18746.07	2850.13	n/a	4.50	0.46	n/a
24 hr	JFH1	GORASP2 siRNA	29852.92	5079.85	0.030	8.29	1.33	0.006
24 hr	JFH1	GOLPH3 siRNA	17436.15	2142.24	0.559	3.21	0.35	0.017
48 hr	JFH1	Neg siRNA	378882.28	58884.71	n/a	553.30	89.08	n/a
48 hr	JFH1	GORASP2 siRNA	631023.17	119519.00	0.031	855.32	92.94	0.015
48 hr	JFH1	GOLPH3 siRNA	350105.49	57632.00	0.578	387.83	38.67	0.042