Systematic identification of anti-interferon function on hepatitis C virus genome reveals p7 as an immune evasion protein

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Hepatitis C virus (HCV) encodes mechanisms to evade the multilayered antiviral actions of the host immune system. Great progress has been made in elucidating the strategies HCV employs to downregulate interferon (IFN) production, impede IFN signaling transduction, and impair IFN-stimulated gene (ISG) expression. However, there is a limited understanding of the mechanisms governing how viral proteins counteract the antiviral functions of downstream IFN effectors due to the lack of an efficient approach to identify such interactions systematically. To study the mechanisms by which HCV antagonizes the IFN responses, we have developed a high-throughput profiling platform that enables mapping of HCV sequences critical for anti-IFN function at high resolution. Genome-wide profiling performed with a 15-nt insertion mutant library of HCV showed that mutations in the p7 region conferred high levels of IFN sensitivity, which could be alleviated by the expression of WT p7 protein. This finding suggests that p7 protein of HCV has an immune evasion function. By screening a liver-specific ISG library, we identified that IFI6-16 significantly inhibits the replication of p7 mutant viruses without affecting WT virus replication. In contrast, knockout of IFI6-16 reversed the IFN hypersensitivity of p7 mutant virus. In addition, p7 was found to be coimmunoprecipitated with IFI6-16 and to counteract the function of IFI6-16 by depolarizing the mitochondria potential. Our data suggest that p7 is a critical immune evasion protein that suppresses the antiviral IFN function by counteracting the function of IFI6-16.

HCV | innate immune evasion mechanism | IF6-16 antiviral function | high-throughput mutagenesis | p7 ion channel protein

With an estimated 170 million people persistently infected worldwide, hepatitis C virus (HCV) has emerged as a major cause of human liver diseases, including chronic hepatitis, cirrhosis, and hepatocellular carcinoma (1, 2). Despite the recent breakthroughs in the development of HCV direct antiviral agents (DAAs) aiming to cure chronic HCV infection, emerging resistant mutations and drug-resistant polymorphisms at the baseline of treatments remain major challenges to eradicate HCV (3–8). In addition, the high cost of these DAAs limits their accessibility to the majority of patients worldwide. Therefore, HCV eradication is still heavily dependent on the development of an effective preventative vaccine (9). Understanding how the virus evades the immune system, which results in a poor immune response of the infected host against the virus, will provide important information for immune therapy and vaccine development.

HCV is an enveloped positive-strand RNA virus that encodes a polyprotein of around 3,000 amino acids. The genome is composed of two untranslated regions (5′UTR and 3′UTR), three structural proteins (core, E1, and E2), and seven nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (10). Due to the limited genome space, viral proteins have evolved multiple functions for viral survival within the host. For example, in addition to their roles in viral replication (11), core, E2, NS3/4A, and NS5A proteins encode immune evasion functions (12) to help the virus establish persistent infection in the host.

Virus–host interactions, such as the virus-IFN response, are very complex and involve a diverse range of mechanisms (13–15). Type I IFNs are critical components of the innate immune defense against viruses by controlling viral replication at multiple steps (15). Detection of viral infection triggers type I IFN expression, which then leads secreted IFNs to bind to their receptors on the targeted cell surface. The IFN-receptor binding results in the activation of the Jak/STAT pathway, where signal transducer and activator of transcription (STAT) proteins are phosphorylated, dimerized, and associated with interferon regulatory factor 9 (IRF-9). The complex translocates to the nucleus and binds to IFN-stimulated response elements (ISREs) within the promoter region of IFN-stimulated genes (ISGs), inducing many antiviral effectors. However, viruses have evolved to circumvent the IFN response via different strategies, which dampens the antiviral efficacy of IFN-α therapy (13). Previous studies have discovered several viral mechanisms (12), mainly through avoiding the induction of an IFN-mediated antiviral

Significance

Understanding how viruses interact with their hosts, especially the mechanisms that restrict virus replication, will provide a molecular basis for vaccine development. However, the search for restriction factors is oftentimes difficult if the virus has already evolved to counteract the restriction. Here, we describe a systematic approach to identify such restriction and counter-restriction mechanisms. We constructed a library of mutant hepatitis C viruses, where each mutant has a 15-nt stretch randomly inserted on the genome. We aimed to identify mutations that lose the anti-IFN function, but maintain replication capacity. We have identified p7 as an immune evasion protein and further characterize the antiviral function of IFI6-16 against hepatitus C virus (HCV) replication.

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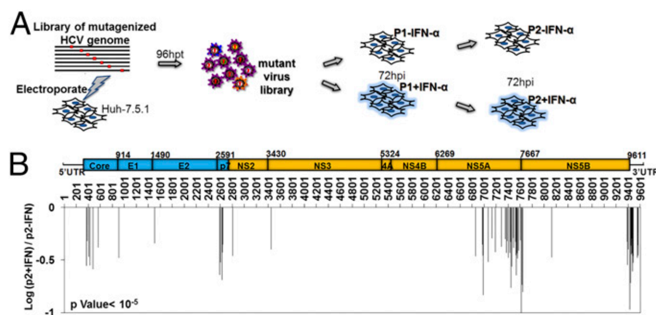

Fig. 1. Genomic screen of mutant virus library with IFN-α treatment. (*A*) Schematic diagram of selection to identify viral sequences critical for counteracting IFN-α responses. A 15-nt insertional mutant HCV library was subjected to infection with Huh-7.5.1 cells in the presence or absence of IFN-α treatment at the IC_{50} concentration (1 U/mL; Fig. S1) for two rounds, and the supernatant was collected (p2). (*B*) IFN-sensitive mutations are clustered at four regions on the virus genome: the N terminus of core, the N terminus of p7, NS5A domains II and III, and the 3′UTR. The *x* axis indicates the positions of the 15-nt insertion on the genome. The *y* axis shows the ratio of mutant frequency with IFN-α treatment to mutant frequency without IFN-α treatment. The schematic picture above the histogram shows the FNX-HCV virus genome composition. Blue is from the J6 strain, and yellow is from the JFH1 strain.

state (16–23). Several studies on the interactions between HCV and downstream IFN effectors have led to the identification of ISGs with inhibitory activity on HCV replication (12, 24–30). In a recent study, a comprehensive library of human ISGs was cloned and overexpressed individually to test their ability in controlling the replication of several human viruses (31). A subset of ISGs was found to inhibit HCV replication at different levels, but most ISGs were ineffective when overexpressed in virus-infected cells due to unknown mechanisms.

In vivo studies of experimentally infected chimpanzees have demonstrated that HCV infection strongly induces the expression of ISGs in the liver (32, 33). ISG induction has also been observed in patients upon viral infection (34). HCV persistence in the liver despite the apparent induction of an antiviral state raises the possibility that the virus encodes mechanisms to counteract the antiviral functions executed by ISGs. However, the cDNA ectopic expression screens are not optimal for identifying such interactions. To interrogate the anti-IFN functions of HCV systematically, we carried out genome-wide mutagenesis of HCV and determined the replication rate of each mutant in the presence and absence of IFN-α. We have identified p7 as an immune evasion viral protein. Measuring the impact of each liver-specific ISG (29, 30, 34, 35) on WT and p7 mutant virus replication revealed that IFI6-16 preferentially inhibits replication of p7 mutants, but does not affect the WT. Furthermore, we showed that p7 coimmunoprecipitates with IFI6-16 and that the overexpression of p7 causes depolarization of mitochondrial membrane potential, which inhibits the function of IFI6-16. In conclusion, these findings suggest that p7 antagonizes the antiviral responses of IFN by inhibiting the antiviral function of IFI6-16.

Results

High-Resolution Profiling of HCV Genome Revealed Four IFN-Hypersensitive Domains. To profile the HCV genome systematically in an unbiased manner, we constructed a mutant library by in vitro Mu transposon-mediated random insertional mutagenesis on a plasmid carrying the HCV genome (pFNX-HCV; a genome that we chemically synthesized based on the chimeric genotype 2a clone, J6/JFH1) (36) (Fig. 1*A*). We introduced seven silent mutations to distinguish the virus from the J6/JFH1 clone, which include a mutation to eliminate the endogenous NotI site. After digestion with NotI enzyme and ligation to remove the coding sequence in the transposon, a 15-nt insertion consisting of a NotI site and a 5-nt duplication from the targeted virus sequence remained, and was randomly distributed throughout the virus genome as described in our previous paper (37) (Table S1). After reconstitution of the virus library, we passaged it in Huh-7.5.1 cells for two rounds under IFN-α treatment at 1 unit (U)/mL (IC_{50}; Fig. 1*A* and Fig. S1). The frequency of each mutant in each round of selection was determined by next-generation sequencing (Fig. S2). After two passages, the effect of IFN-α on each mutant was evaluated by calculating the ratio of mutant virus abundance in IFN-α–treated library (p2 + IFN) to the control (p2 − IFN). By the binomial exact test, the *P* value was also determined for each mutant virus using a null hypothesis of 0.125. With a cutoff for IFN-α hypersensitivity of a ratio <0.5 and *P* value $<10^{-5}$, mutations conferring increased IFN sensitivity were found to be clustered in four regions in the genome: the N terminus of core protein, the N terminus of p7, domains II and III in NS5A protein, and the 3′UTR (Fig. 1*B*).

Validation of the Phenotype of Mutants Identified by IFN-α Screen. To verify the screen results, we constructed eight putative IFN-sensitive mutant viruses and one WT-like mutant virus (insertion at nucleotide position 7,351) by inserting 15 nt at the positions identified in the screen. The nucleotide/amino acid sequences inserted in the virus genome are shown in Table S2. Infectious virus production of the mutants at 48 and 96 h posttransfection indicated that viral replication was not significantly affected by these insertions (Fig. 2*A*). More importantly, consistent with our screen data, their replication was inhibited by IFN-α treatment quantitatively more than the WT-like control virus (Fig. 2*B*). The phenotype was also observed by assaying the replication of viral genome by quantitative PCR (Fig. S3). Furthermore, we found that insertions in NS5A domain II and domain III were located within regions previously identified as IFN sensitivity-determining regions (38, 39), protein kinase R-binding domain (21, 22), variable region 3, and IFN/ribavirin resistance-determining region (40–42) (Fig. S4). Collectively, the data demonstrate the reliability of the IFN screen results and, more generally, the utility of our profiling platform.

Mutations in p7 Confer Hypersensitivity of the Virus to IFN-α Treatment. Our screen reveals that p7 carries a previously uncharacterized immune evasion function. Disruption of this function causes significant

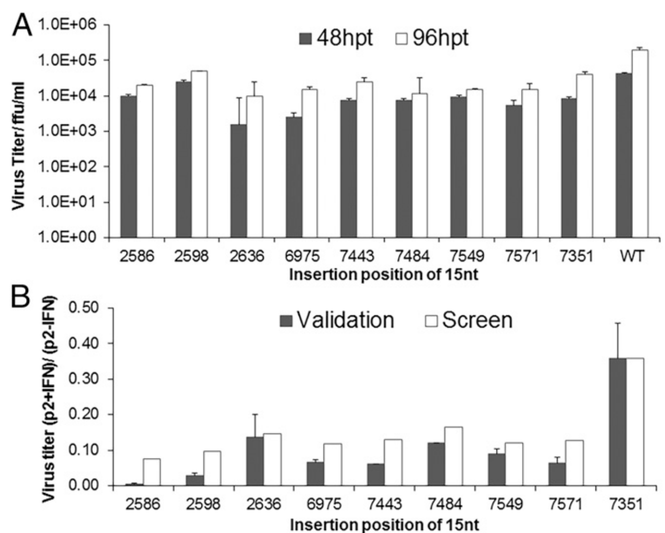

Fig. 2. Validation of the IFN screen with individual mutant viruses. (*A*) Eight putative IFN-sensitive mutants and one WT-like mutant (insertion at amino acid 7,351) were constructed individually to characterize their sensitivity to IFN-α. The infectious virus particle production was measured at 48 h posttransfection (hpt; gray) and 96 hpt (white). (*B*) Replication of the eight mutants in IFN-α treatment (gray), compared with the screen data (white). The *y* axis is the ratio of virus production in IFN-α treatment to control.

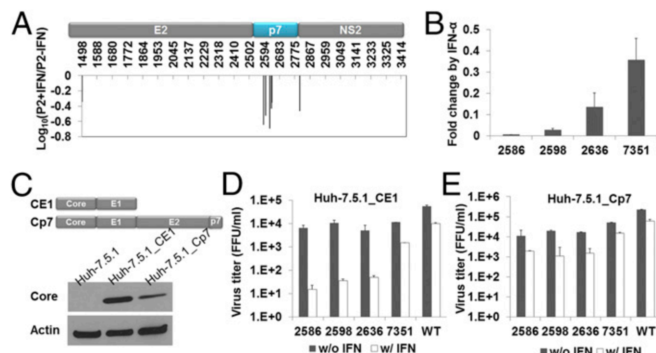

Fig. 3. Mutations in p7 confer hypersensitivity of the virus to IFN-α and are rescued by WT p7 protein. (*A*) Fifteen-nucleotide insertion mutagenesis profiling screen shows that mutations causing higher IFN sensitivity are clustered in the N terminus of p7. (*B*) Individual mutant viruses with 15-nt insertions in p7 confirmed the screen results. The three p7 mutant viruses are 2,586, 2,598, and 2,636. and 7,351 is a control mutant virus with a 15-nt insertion in NS5A. The Huh-7.5.1 cells were pretreated with 1 U/mL IFN-α for 18 h before being infected with the mutant viruses as indicated. At 72 h postinfection, the virus production in the supernatant was measured as the fold of change upon IFN-α treatment. (*C*) Two Huh-7.5.1 cell lines constitutively expressing core and E1 (CE1) or core, E1, E2, and p7 (Cp7) proteins. (*D* and *E*) Infectious virus particle production in the supernatant of the CE1 cell line or Cp7 cell line in the presence or absence of IFN-α treatment. FFU, focus forming units.

inhibition of viral replication by IFN treatment (Fig. 3*A*). This phenotype was validated with individually constructed p7 mutant viruses carrying 15-nt insertions at positions 2,586, 2,598, and 2,636 (Fig. 3*B*). To examine the specificity of the IFN-α inhibition on p7 mutants further, we tested whether overexpression of WT p7 protein could alleviate the inhibitory effect of IFN-α on p7 mutants. An Huh-7.5.1 cell line constitutively expressing p7 protein (Cp7) was thus established. A cell line harboring the proteins core and E1 (CE1) served as the control (Fig. 3*C*). We found that replication of p7 mutant viruses was inhibited by ∼2-log with IFN-α treatment (Fig. 3*D*), whereas the defective replication of p7 mutants in response to IFN treatment was significantly rescued in Cp7 cells (Fig. 3*E*). The rescuing effect was also observed on the viral genome replication in Cp7 cells, suggesting that p7 suppresses the antiviral effect activated by IFN-α treatment and mutations in p7 result in the loss of immune evasion function and hypersensitivity to IFN-α.

Identify Cellular Factor(s) Interacting with p7. After confirming the regulatory function of p7 on the IFN antiviral effects, we examined whether p7 protein expression affected the ISRE promoter activity. HEK293T cells were transfected with plasmids carrying WT or mutant p7, along with a luciferase reporter under the control of ISRE, which is responsive to IFN-α induction. The transfected cells were subsequently stimulated with 5 U/mL IFN-α to induce the activation of ISRE promoter. The luciferase activities were measured at 20 h after treatment, and induction was calculated in comparison to the untreated sample as the readout of ISRE activation. As shown in Fig. 4*A*, neither WT nor mutant p7 protein inhibited the activation of ISRE promoter induced by IFN-α, suggesting that p7 functions downstream of ISRE promoter activation.

To interrogate the molecular basis of p7 counteracting innate immune responses further, we searched for ISGs that preferentially inhibit replication of p7 mutant, but not the WT virus, by screening a cDNA expression library of ISGs that are expressed in liver. The hypothesis is that KO of immune evasion function encoded in p7 restores the antiviral effect of the ISG, which is otherwise suppressed by WT p7 protein. We analyzed the published microarray data from IFN-treated liver hepatoma cells or fetal liver cells (26), and compiled a list of 107 ISGs that are expressed in liver cells upon IFN induction. To give a clean background, we chose the Huh-7.5.1 cell for the screen, which carries a mutation in the RIG-I gene that results in impaired IFN signaling (43). Cotransfecting ISG constructs and a puromycin-resistant vector allowed for selecting ISG-delivered Huh-7.5.1 cells, which were then challenged with WT or p7 mutant virus carrying a monocistronic Renilla luciferase reporter (Fig. 4*B*). Viral replication in ISG-transfected cells was evaluated at 72 h postinfection by measuring Renilla luciferase activity. We compared the antiviral effect of each ISG on p7 mutant and WT viral replication, and the ratio was calculated (Fig. S5).

P7 Forms Complex with IFI6-16. Upon carrying out statistical analyses on the screen results, we found that 13 ISGs (Fig. S6) preferentially inhibited the replication of p7 mutant over WT. We then performed protein–protein interaction analysis through coimmunoprecipitation between p7 and the 13 ISGs to identify potential physical interactions, and found that IFI6-16 was the only one that formed a protein complex with p7 protein. P7 and IFI6-16 were constructed in mammalian expression vectors as fusions to the epitope tags HA or Flag. Flag-tagged IFI6-16 was cotransfected with the HA-tagged p7 construct into Huh-7.5.1 cells. Communoprecipitation results showed that IFI6-16 could form a complex with p7. The interaction can be detected both in

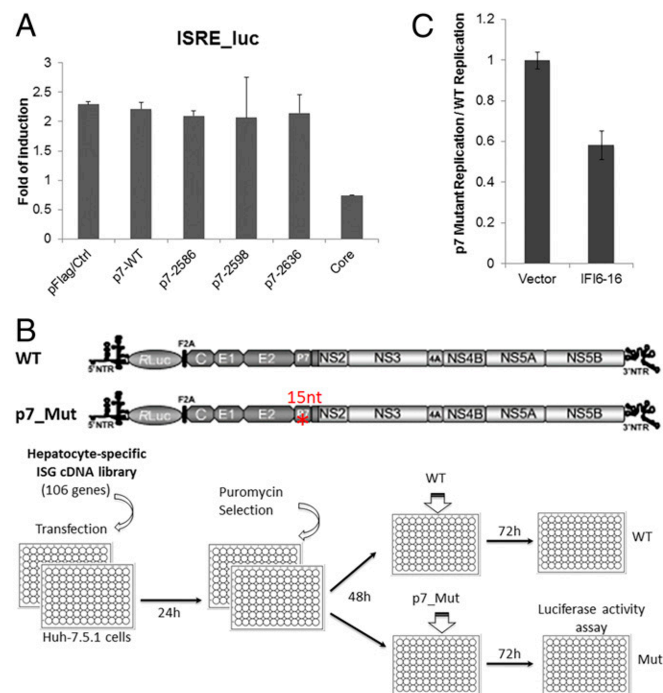

Fig. 4. Liver-specific ISG library screen to identify ISGs that inhibit p7 mutant replication. (*A*) Effect of WT or mutant p7 protein overexpression on ISRE promoter activity upon IFN-α stimuli. HEK293T were transfected with ISRE-driving luciferase reporter plasmids, along with the indicated HCV protein-expressing plasmids, respectively. The fold of activation was determined upon IFN treatment in comparison to untreated control. (*B*) Schematic of genome constructions of WT and p7 mutant (p7-2,598) Renilla reporter viruses and overall scheme of the liver-specific ISG library screen. Luciferase activities in the Huh-7.5.1 cells were measured as the readout of viral replication at 72 h postinfection. (*C*) Inhibition of IFI6-16 on p7 mutant virus replication was identified and validated. The replication of p7 mutant virus in ISG-expressed cells was normalized to control vector and compared with the normalized activity of WT. A relative activity equals to 1 suggests that the effect of ISG is the same on p7 mutant and WT. A larger than 1 relative activity implies a weaker inhibition of ISG on p7 mutant, whereas a smaller than 1 ratio means a stronger inhibition of ISG on p7 mutant.

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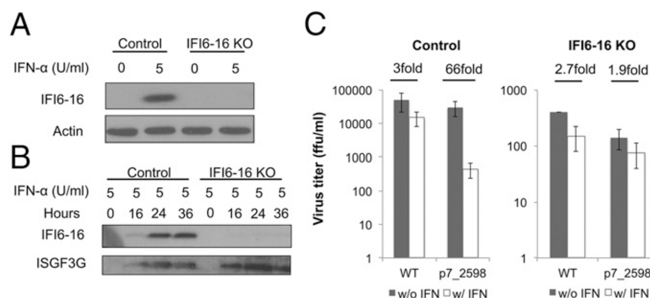

Fig. 5. KO of IFI6-16 in Huh-7.5.1 cells restores the IFN resistance of p7 mutant virus. (*A*) Huh-7.5.1 cells were used as parental cells to construct IFI6-16 KO cell lines with CRISPR technology. The induction of IFI6-16 expression upon IFN-α treatment is lost in the KO cell line. (*B*) IFN signaling pathway is intact in the IFI6-16 KO cells. ISGF3G is induced to a similar level of parental cells. (*C*) IFN sensitivity of p7 mutant virus replication was measured in a similar way as previously described for IFI6-16 KO cells and their parental cells. WT virus is used as control.

Flag-tagged IFI6-16 immunoprecipitated complex (Fig. S7*A*) and HA-tagged p7 immunoprecipitated complex (Fig. S7*B*).

KO of IFI6-16 Restores the IFN Resistance of p7 Mutant Virus. To verify the significance of IFI6-16 on the inhibition of p7 mutant virus replication, we constructed an IFI6-16 KO cell line with CRISPR/Cas9. The Western blot shows that IFI6-16 protein expression was undetectable in the KO cells (Fig. 5*A*). To determine whether the IFN signaling is affected in the KO cells, we examined the protein level of ISGF3G upon IFN-α treatment. Our data showed that the expression of ISGF3G is not affected (Fig. 5*B*), which suggested that the KO of IFI6-16 is specific and the IFN response signaling pathway is functional. The IFI6-16 KO cells were infected with WT or p7 mutant viruses and treated with or without IFN-α. Inhibition of p7 mutant virus replication upon IFN-α treatment was significantly alleviated when IFI6-16 is knocked out (Fig. 5*C*). This result suggests that IFI6-16 is a key antiviral regulator induced by IFN-α, and its function can be blocked by the viral protein p7.

P7 and IFI6-16 Counteract by Regulating the Mitochondrial Membrane Potential. From previous studies, IFI6-16 is known to be one of the most up-regulated ISGs induced upon IFN-α treatment and viral infection (24, 30, 31, 35, 44, 45). However, little was known about its function or the mechanism in blocking viral infection. Recent studies suggest that IFI6-16 plays a critical role in stabilizing cancer cells by inhibiting mitochondrial-mediated apoptosis (46, 47), and it also regulates apoptosis in Dengue virus (DENV)-infected cells (48). To examine whether p7 counteracts with IFI6-16 to regulate the mitochondrial function, we performed a mitochondrial membrane potential ($\Delta\psi$) assay using a well-characterized potentiometric fluorescent dye, tetramethylrhodamine methyl ester (TMRM). The lipophilic TMRM dye penetrates cells and mitochondrial lipid bilayer membranes. When the mitochondria are intact and the mitochondrial potential is maintained, positively charged TMRM dye is accumulated in the mitochondria and exhibits a dramatic increase in red fluorescence at 573 nm. Once the mitochondria are depolarized, the dye leaks out and disperses throughout the cytosol and yields minimal fluorescence upon excitation. P-triflouromethoxyphenylhydrazone (FCCP) transports protons across mitochondrial inner membranes and induces the depolarization of mitochondria potential (49). Consistent with previous studies (46–48), our results also suggest that IFI6-16 stabilizes the mitochondrial membrane potential, as shown by residual TMRM fluorescence signal even in cells treated with a low FCCP dose for 10 min (Fig. 6). By contrast, p7 appears to cause depolarization of mitochondria as the gradual increase of p7 protein reduces the TMRM signal (Fig. 6). This observation suggests that p7 may counteract IFI6-16 by regulating the mitochondria membrane potential to block the antiviral function of IFI6-16.

Discussion

The host IFN response has been described as the first line of defense against invading viral pathogens (50). However, it has also been recognized that viruses encode multiple mechanisms to evade these antiviral actions of the IFN response (13, 15, 51). Great progress has been made in elucidating the strategies that HCV uses to down-regulate IFN production, impede IFN signaling transduction, and impair ISG expression. However, understanding how viral proteins counteract the antiviral functions of downstream IFN effectors was hampered by the lack of an experimental approach to identify such interactions systematically. With a high-resolution mutagenesis profiling approach, we found that mutations in the p7 region increase IFN sensitivity and this phenotype can be rescued by overexpression of WT p7 protein in the infected cells, implicating p7 as an

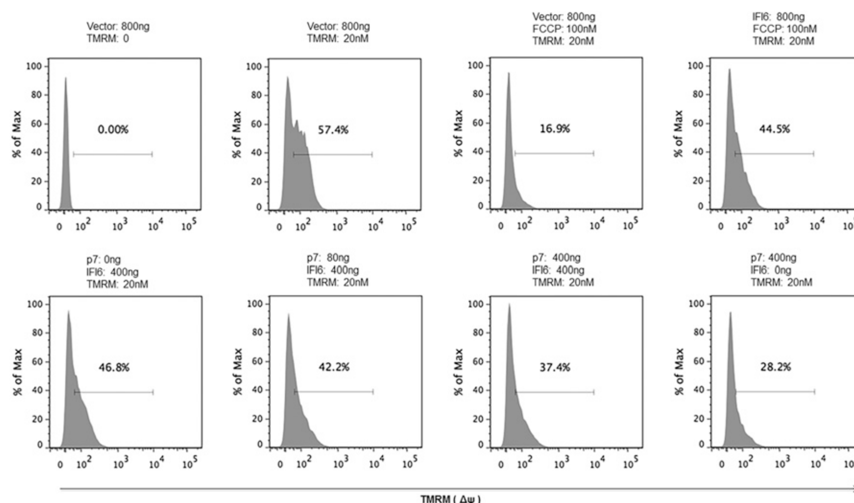

Fig. 6. P7 counteracts the function of IFI6-16 by depolarizing the mitochondrial potential. Cells were transfected with vector or p7 or IFI6-16 or a mixture of p7 and IFI6-16 at different ratios. The mitochondrial membrane potential was analyzed with flow cytometry under treatment with 20 nM TMRM for 30 min.

immune evasion protein of HCV. We found that p7 does not diminish the ISRE activity induced by IFN-α, which suggests that p7 might interfere with the antiviral functions downstream of IFN effectors. A liver-specific ISG library screen was then conducted, and it identified genetic and physical interaction between p7 and an ISG, IFI6-16.

Previous studies suggest that HCV proteins antagonize the innate immune response through inhibiting the production of type I IFN and suppression of JAK/STAT signal transduction to avoid the induction of an IFN-mediated antiviral state (52). However, in vivo studies of experimentally infected chimpanzees or human patient biopsy samples have demonstrated that HCV infection strongly induces the expression of ISGs in the liver (33, 53). Despite the apparent induction of an antiviral state, HCV persists in the liver, raising the possibility of the virus encoding mechanisms to counteract the antiviral functions executed by ISGs. All of these studies lead us to hypothesize that p7 could inhibit the function of ISG(s) to facilitate robust viral replication despite the induction of antiviral stage by IFN.

IFI6-16, also known as G1P3, was first identified as an ISG whose mRNA was highly inducible in multiple cell lines upon type I IFN stimulation (54–57). The expression of IFI6-16 is responsive to viral infections, including vesicular stomatitis virus, HCV, cytomegalovirus, and DENV (58, 59). It can also be induced by poly(I):poly(C) treatment and other immune regulators, namely, lipopolysaccharide and TNF-related apoptosis-induced ligand (58, 60). Despite the early identification of IFI6-16 as an ISG and implications that it mediates innate immunity, the antiviral mechanism of the protein still remains obscure and elusive. Early studies attempting to evaluate the antiviral function of IFI6-16 showed that introduction of IFI6-16 in a KO cell line (HT1080_IFI6$^{-/-}$) does not affect the replication of encephalomyocarditis virus, Semliki forest virus, or coccal virus, suggesting that IFI6-16 is not required to control these viral replications (61). In contrast, IFI6-16 was identified as a negative regulator that markedly inhibited the replication of yellow fever virus (31), DENV (62), and West Nile virus (63). The expression of the gene was also found to suppress respiratory syncytial virus replication and was down-regulated by the virus (64). The effect of IFI6-16 on HCV replication, however, seems a bit contradictory. In the replicon cells harboring HCV subgenomic RNA, overexpression of IFI6-16 inhibited HCV replication, and expression of viral proteins, whereas knockdown of IFI6-16 increased the level of RNA replication. Interestingly, IFI6-16 did not activate the IFN activation pathway, suggesting that it functions directly against viral replication without going through the IFN activity, which may amplify antiviral actions (24, 25). In contrast, a comprehensive ISG cDNA screen using an infection system demonstrated that IFI6-16 shows moderate or no significant suppression on HCV replication in either Huh-7 or Huh-7.5 cell lines (31). HCV persists in chimpanzee livers regardless of the up-regulation of IFI6-16, suggesting that either IFI6-16 does not regulate viral replication (64) or the virus has developed strategies to overcome the antiviral functions of IFI6-16, as proposed in this study.

Our data suggest that p7 functions as an immune evasion protein, most likely by counteracting the antiviral function of IFI6-16. On one hand, IFI6-16 is one of the earliest ISGs induced upon IFN treatment according to the previous studies. Studies showed that overexpression of IFI6-16 can delay the apoptosis of the cells through stabilizing the mitochondria, and therefore may extend the production of IFN in the infected cells, which may sustain and extend the antiviral effects of the IFN system (60). On the other hand, HCV replication has been known to induce mitochondrial dysfunction and mitophagy. This observation is very likely attributable to the ion channel function of p7 because the mitochondrial dysfunction can be blocked by amantadine, an ion channel inhibitor that interacts with p7. Therefore, a plausible explanation will be that p7 may break the balance that IFI6-16 confers on mitochondria through depolarizing mitochondria and induces mitochondrial dysfunction to interfere with the antiviral state of the infected cell. Our data explain the discrepant observations that expression of IFI6-16 protein presents a substantial level of antiviral effect in the HCV replicon system, but not in the infectious system (25, 31) (Fig. S8). This interaction could not have been identified without identification of the mutant viruses through the genome-wide mutagenesis study.

To determine the mechanism of p7 counteracting IFN signaling, we took two independent approaches to determine the cellular protein(s) functionally and physically interacting with p7. In the ISG screen, we have also noticed that there are several ISGs that inhibit p7 mutant virus replication over WT, but do not form a protein complex with p7 protein. Those ISGs may display an indirect effect on p7 mutant virus replication. We noticed that some of these ISGs are involved in the IFN signaling pathway, which may amplify the effect of IFN or IFI6-16 on HCV replication when p7 is mutated. For example, IFIT5 is an IFN-induced RNA-binding protein that recognizes single-stranded RNA and initiates IFN production upon recognizing single-stranded 5-triphosphate RNAs, which further reinforces the antiviral effect of the system.

Although we have identified IFI6-16 as a direct counteracting protein of p7, it does not rule out possibilities that other ISGs also have an impact on p7 mutant virus replication. It will be interesting to carry out a counterscreen as an orthogonal approach to eliminate errors from the high-throughput screen assay and to characterize the antiviral function of the ISG(s).

Because the HCV genome does not tolerate the 15-nt insertion very well, which leaves a large portion of the virus genome unexplored in the IFN screen, we anticipate that we would identify more immune evasion functions on the virus genome at a much higher resolution, and possibly novel antiviral ISGs with a complex single-amino acid mutant library.

Collectively, these multilayered systematic approaches offer comprehensive insights into HCV and host interactions, which will provide a basis for understanding innate immune evasion mechanisms. In addition, systematic screening of a viral genome to identify immune evasion functions, including anti-IFN functions, will enable the construction of recombinant viruses with desired biological properties. Multiple immune evasion functions can be knocked out to generate recombinant viruses that are replication-competent in immune-deficient hosts, such as IFN-deficient cells, but defective in healthy hosts. It can be expected that they will generate strong innate and adaptive immune responses and provide protection against WT virus challenge. Thus, our work also presents an approach for vaccine development based on rational design, enabled by systematic understanding of the viral genome.

Materials and Methods

The mutant plasmid library was linearized and transcribed into RNA in vitro, followed by electroporation into Huh-7.5.1 cells to reconstitute the mutant virus library. The virus library underwent two rounds of selection in Huh-7.5.1 cells. A detailed description of reagents and protocols used in this study can be found in *SI Materials and Methods*.

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