the GFP gene and pseudotyped with the vesicular stomatitis virus G envelope (VSVG) protein) (generous gift from C. Aiken and D. Gabuzda). Forty-eight hours post-infection, intracellular GFP levels were quantified by FACS. HSV-1 infection: Cyp-knockdown Huh7 cells lines were infected with HSV-1-GFP (K26GFP virus encoding the GFP gene) (generous gift from P. Desai). Forty-eight hours post-infection, intracellular GFP levels were quantified by FACS. Dengue infection: Cyp-knockdown Huh7 cells lines were infected with Dengue-2 (Dengue-2 16681) (generous gift from R. Kinney). Dengue-2 infection was examined using an intracellular FACS staining assay (IFSA). Briefly, IFSA was conducted as described previously (27-28) with minor modifications. Three days post-infection, cells were washed, trypsinized, resuspended in PBS at 1×10^6/ml and fixed with 0.2% paraformaldehyde in PBS for 30 min on ice. Cells were washed, permeabilized in PBS containing 0.2% Tween for 15 min at 37°C, washed and resuspended in FACS buffer (PBS containing 2% FBS). For intracellular staining, cells (10^5) were incubated for 30 min at 4°C with 10 µg/ml of isotype controls, mouse monoclonal anti-Dengue capsid 9A7 IgG (TSRI, Antibody Core Facility). Cell permeabilization was confirmed by staining cells with mouse anti-tubulin IgG (Santa Cruz Biotechnologies). Cells were washed, incubated with secondary phycoerythrin (PE)-conjugated anti-mouse IgG (10 µg/ml) for 30 min at 4°C, washed again, resuspended in PBS, fixed in 2% paraformaldehyde and stored at 4°C until FACS analysis. HCV replication: Cyp-knockdown Huh7 cells lines were electroporated with 10 µg of *in vitro*–transcribed genomic Con1 RNA. Seven days post-transfection, HCV infection was quantified by measuring intracellular NS5B levels by IFSA using anti-NS5B IgG (Alexis Biochemicals).

CypA Binding to HIV-1 Gag in Hepatocytes. CypA-HIV-1 Gag interaction was studied by examining the incorporation of host CypA into budding HIV-1 particles by Western blot. Briefly, HIV-1 particles were transiently expressed in Huh7 cells by genejuice (EMD Biosciences) transfection with a mixture of 5 µg of proviral HIV-1 (NL4.3) DNA together with 5 µg of CypA plasmids (pcDNA3-resistant WT, H126Q and R55A). Viral supernatants, harvested 48 h post-transfection, were filtered through a 0.2-µm-pore-size filter to remove cellular debris, pelleted through a sucrose cushion, standardized for HIV-1 capsid content by p24 ELISA (Alliance, PerkinElmer), resuspended in 2x sodium dodecyl sulfate loading buffer, and subjected to Western blotting with antibodies directed against the HA tag.

Co-immunoprecipitations. Parental Huh7 cells (3 million) were co-transfected with NS5B-myc (5 µg DNA) and wild-type CypA-HA or H126Q CypA-HA (5 µg DNA) plasmids in the presence or absence of Debio 025 (2 µM). Three days post-transfection, cells were collected and lysed. Cell lysates (1 mL) were pre-cleared for 1 h with 20 µL of agarose beads. Co-immunoprecipation procedures were conducted according to the manufacturer's instructions (Pierce HA Tag IP/Co-IP Kit). Bound material was eluted and analyzed by Western blotting using anti-HA and anti-myc IgG (Santa Cruz Biotechnology).

RESULTS

Analysis of the Respective Contribution of Cyp Members to HCV Replication by Transient Small RNA Interference. Previous studies suggested that at least three members of the Cyp family – CypA, CypB and CypC – modulate HCV replication (9, 17-18). We thus examined if HCV exploits all these Cyp members or rather a unique Cyp member to efficiently replicate in human hepatocytes. To address this issue, we knocked down each of these Cyps by transient small inhibitory RNA (siRNA) interference and examined the effect of the Cyp knockdown on HCV replication. Specifically, Huh7 cells containing the subgenomic HCV Con1 replicon (genotype 1b) were transfected with siRNAs that target either luciferase (control siRNA), CypA (CypA siRNA), CypB (CypB siRNA), CypC (CypC siRNA) or CypD (CypD siRNA). To avoid siRNA toxicity, cells were washed 24 h post-transfection. Seven days post-transfection, cells were collected and lysed. To ensure that the

siRNA treatments did not non-specifically influence growth and viability of transfected hepatocytes, cells were counted and analyzed for trypan blue uptake 7 days post-transfection. We exclusively analyzed the lysates of cells, which gave numbers of viable cells comparable to those of control siRNA-treated cells. Cell lysates were standardized for protein content and analyzed for Cyp content by Western blot using antibodies directed against CypA, CypB, CypC or CypD.

The expression of each Cyp was profoundly reduced by siRNAs Cyp compared to siRNA control (Figure 1A), demonstrating the efficacy of the siRNA treatments. Moreover, siRNA Cyp treatments were specific since each siRNA Cyp treatment did not alter the expression of the other Cyps (Figure 1A). For example, the siRNA, which targeted CypA did not influence CypB, CypC or CypD expression (Figure 1A). Importantly, transient CypB, CypC or CypD knockdown did not significantly influence HCV protein expression. Indeed, NS5A and NS5B levels in siRNA CypB-, CypC- and CypD-treated hepatocytes were similar to those of control siRNA-treated cells (Figure 1B), suggesting that these Cyps play no or a minor role in HCV protein expression. In sharp contrast, NS5A and NS5B levels were profoundly diminished in hepatocytes treated with the siRNA CypA (Figure 1B), demonstrating that CypA rather than CypB, CypC and CypD, plays a major role in HCV protein expression.

We then asked whether CypA, but not other Cyp members, is also critical for HCV RNA replication. To address this issue, naïve Huh7 cells were electroporated with *in vitro*–transcribed genomic Con1 RNA. One day post-electroporation, cells were transfected with control or siRNA Cyp as described above. Viral RNA replication was monitored for 8 days by analyzing intracellular HCV RNA via reverse-transcription quantitative polymerase chain reaction as described previously (23). We verified that the expression of CypA, CypB, CypC and CypD was knocked down via the Cyp siRNA treatment 8 days post-electroporation (data not shown). We found that the HCV RNA replication in hepatocytes treated with siRNA CypC and CypD was comparable to that in siRNA control-treated cells, whereas the viral RNA replication in siRNA CypB was only slightly diminished (Figure 1C). In contrast, viral RNA replication was profoundly attenuated in the siRNA CypA-treated cells (Figure 1C). Altogether these data suggest that CypA, but not CypB, CypC and CypD, represents an essential host factor for both HCV RNA replication and HCV protein expression.

Demonstration of the Exclusive Contribution of CypA to HCV Replication by Stable Small RNA Interference. The introduction of siRNA into cultured cells provides a fast and efficient means of knocking down gene expression. However, siRNA has been shown to be effective for only short-term gene inhibition in certain mammalian cells. Supporting this notion, we found that although Cyp expression was dramatically reduced 5 to 7 days after siRNA transfection, Cyp expression recovered 9 to 10 days post-siRNA transfection (data now shown). Even a slight rebound in Cyp protein expression may interfere with the interpretation of the data. To avoid this issue, we conducted experiments similar to those described above, but using shRNA to stably silence Cyp gene expression. We constructed plasmids encoding shRNA that target CypA, CypB or CypC. As above, we constructed as control, a plasmid that encodes an shRNA that targets luciferase. Plasmids were packaged into HIV-1-based particles pseudotyped with the VSV-G envelope to permit entry into and infection of hepatocytes. The advantage of using the HIV-1 based vector is that the DNA encoding the shRNA will be stably integrated into the host chromosomes of the hepatocytes. Parental Huh7 cells were exposed to the HIV-1-based particles for 24 h, cultured under puromycin selection for 3-4 weeks and analyzed for Cyp content by Western blot. As expected, CypA, CypB and CypC levels in shRNA Cyp-transduced cells were severely reduced compared to control shRNA-treated cells (Figure 2A).

We then tested the Cyp-knockdown (KD) cell lines for their capacities to support HCV RNA replication as described above. Importantly, the HCV RNA replication was only profoundly reduced in the stable CypA-KD cell line (Figure 2B), further suggesting that CypA,

but not CypB, CypC and CypD, is essential for HCV replication. It is important to note that we did not observe differences in growth between the cell lines (data not shown), suggesting that these particular Cyps do not play a significant role in Huh7 cell division and multiplication.

To further demonstrate the specificity of the CypA requirement for HCV replication, CypA-, CypB-, CypC-knockdown and control cell lines were exposed to various viruses including the human immunodeficiency virus type-1 (HIV-1), the herpes simplex virus type-1 (HSV-1), the flavivirus Dengue and HCV. Infectivity was scored by measuring the intracellular levels of GFP for both HIV-1 and HSV-1, levels of capsid for Dengue and levels of NS5B for HCV. GFP levels were significantly reduced in HIV-1-exposed CypA-knockdown cells compared to control, CypB- and CypC-KD cells (Figure 2C, top left panel). This is in accordance with previous studies, which suggest that HIV-1 requires CypA to optimally infect human cells (29-30). All cell lines exposed to HSV-1 or Dengue expressed similar levels of GFP (HSV-1) and capsid (Dengue) (Figure 2C, top right and bottom left panel, respectively), suggesting that HSV-1 and Dengue do no require any of these Cyps to infect human cells. Importantly, NS5B levels were dramatically reduced in the CypA-knockdown cells compared to control, CypB- and CypC-knockdown cells (Figure 2C). This further suggests that HCV, like HIV-1, specifically exploits CypA to infect and replicate in human cells, more specifically in hepatocytes.

HCV Requires the Isomerase Activity of CypA to Replicate in Human Hepatocytes. The peptide bond generally exists in two relatively stable isomeric forms: *cis* and *trans* (31). The ribosome synthesizes peptide bonds in the lower energy-state *trans* peptide bond form, which is sterically favored, and whose side chains are 180 degrees opposite each other (32). However, bonds preceding each proline (peptidyl-prolyl bonds) also occur in the *cis* form in both unfolded and native proteins, with the side chains adjacent to each other (33). The isomerization to the *cis* form is required for both de novo protein folding, protein restructuring and refolding processes following cellular membrane traffic. Spontaneous cis/trans isomerization of peptidyl-prolyl bonds is a slow process at room temperature that does not require free energy. Thus, this isomerization represents a rate-limiting step in the refolding of chemically denatured proteins (34).

We thus asked if CypA promotes HCV replication via its isomerase activity. To address this issue, we created a CypA mutant deprived of its isomerase activity. Specifically, we replaced the histidine located at position 126 (H126) in the hydrophobic pocket of CypA by a glutamine, creating the H126Q CypA mutant. Importantly, this mutation diminishes CypA isomerase activity by more than 99% compare to wild-type CypA (25). To determine if HCV requires the isomerase activity of CypA to replicate, we had to transfect the H126Q CypA mutant into the CypA-KD cells and asked if HCV replication can be rescued. In order to introduce the H126Q CypA mutant into the CypA-KD cell line, we had to generate the H126Q mutation into the context of an shRNA-escape CypA plasmid. We thus generated one plasmid encoding the wild-type shRNA-escape CypA and another encoding the H126Q shRNA-escape CypA. CypA-KD cells were transfected with wild-type and H126Q shRNA-escape CypA plasmids and tested for their capacities to support HCV RNA replication as described above. Importantly, the introduction of the wild-type shRNA-escape CypA into the CypA-KD cells restored HCV RNA replication at levels similar to those observed in parental Huh7 cells (Figure 3A). This rescue not only demonstrates the specificity of the CypA knockdown, but it also further demonstrates the importance of CypA in HCV replication. More importantly, the introduction of the H126Q shRNA-escape CypA into the CypA-KD cells did not restore viral RNA replication (Figure 3A). Note that the cellular levels of H126Q CypA were similar to those of wild-type CypA (Figure 3B). Importantly, we obtained similar data for the R55A CypA mutant (data not shown), which is also deprived of isomerase activity (25). These findings strongly suggest that the isomerase activity of CypA is essential for HCV replication in human hepatocytes.

The hydrophobic pocket of CypA does not only contain the residues vital for the

isomerase activity of CypA, it also contains the residues responsible for the binding of CypA to its, to date, unique known viral ligand - the structural polyprotein HIV-1 Gag. One can thus envision that the H126Q mutation fails to rescue HCV activity in hepatocytes because the mutation, not only blocks the enzymatic activity of CypA, but it also precludes CypA binding to its still unidentified HCV ligand. Therefore, we asked if the H126Q mutation, which abolishes the enzymatic activity of CypA, also prevents the binding of CypA to HIV-1 Gag within human hepatocytes. CypA binding to HIV-1 Gag was never examined in hepatocytes. To address this issue, we measured amounts of CypA incorporated via Gag into HIV-1 particles released from human hepatocytes. As controls, hepatocytes were treated with CsA or Debio 025, which, by binding to the hydrophobic pocket of CypA, prevents CypA-Gag interaction (30, 35). Specifically, Huh7 cells were co-transfected with HIV-1 together with wild-type or H126Q CypA-HA in the presence or absence of CsA or Debio 025. Forty-eight post-transfection, both transfected cells and released virions were analyzed for CypA content by Western blotting using anti-HA antibodies.

In the absence of any treatment, HIV-1 particles released from hepatocytes contain significant amounts of wild-type CypA (Figure 3B, top panel), demonstrating that CypA-Gag interactions also occur in human hepatocytes. Virions released from hepatocytes treated with either CsA or Debio 025 contain minimal amounts of CypA (Figure 3B, top panel), suggesting that the two compounds, by binding to the hydrophobic pocket of CypA, interfere with CypA-Gag interactions in hepatocytes. Although the H126Q CypA mutant was efficiently expressed in transfected hepatocytes (Figure 3B, bottom panel), it was not incorporated into released virions (Figure 3B, top panel), suggesting that the H126Q CypA mutation in the hydrophobic pocket of CypA alters both the enzymatic activity of CypA and the binding capacity of CypA to its viral ligand in hepatocytes. Thus, the inability of the H126Q CypA mutant to support HCV replication may arise from either its inability to isomerizes peptidyl-prolyl bonds, its inability to bind to its viral or host ligand, or both. It is also important to note that the inability of the overexpressed isomerase-deficient H126Q CypA mutant to restore HCV replication argues against the possibility that CypA plays a more important role in HCV replication than other Cyp members simply due to its superabundance in a cell. Overexpression of CypB or CypC in the CypA-knockdown cells did not rescue HCV replication either, further supporting the notion that the CypA requirement for HCV is specific (data not shown).

HCV NS5B Polymerase Associates With CypA Via Its Enzymatic Pocket. A previous study presented data suggesting that CypA binds to NS5B (18). We thus asked if the isomerase pocket of CypA is critical for the interaction between host CypA and the HCV NS5B polymerase. To address this issue, hepatocytes were co-transfected with myc-tagged NS5B and HA-tagged wild-type or H126Q CypA plasmids in the presence or absence of the Cyp inhibitor Debio 025. Three days post-transfection, cells were collected and lysed. Cell lysates were then used for co-immunoprecipitation experiments. First, we confirmed that wild-type CypA associates with HCV NS5B (Figure 4). Importantly, the Cyp inhibitor prevents NS5B-CypA association (Figure 4). Most importantly, we found that the isomerase-deficient CypA, although well expressed in transfected cells, fails to associate with NS5B (Figure 4). This demonstrates for the first time that the enzymatic pocket of CypA is critical for the contact between host CypA and the viral NS5B polymerase.

DISCUSSION

It is well established that host proteins highly regulate the viral life cycles. Because cellular chaperones and enzymes control the correct folding of host proteins, one could assume that they also control the correct folding of viral proteins. This assumption is now apparently proven to be correct for two prime human pathogens: HIV-1 and HCV. In 1993, Luban et al. using the yeast two-hybrid system identified for the first time the interaction between CypA

and HIV-1 Gag (35). One year later, two independent studies elegantly demonstrated that CypA-Gag interactions are critical for HIV-1 replication in human cells (29). Specifically, Thali et al. showed that CsA, by preventing CypA-Gag interactions, inhibits HIV-1 infection (30). Moreover, Franke et al. showed that the introduction of mutations in the CypA-binding region of Gag also decreases HIV-1 infection of human cells (29). Further supporting the notion that HIV-1 requires CypA to replicate in human cells, several studies demonstrated that CypA-knockout or -knockdown human cells poorly support HIV-1 replication (24, 36-37). Similarly to HIV-1, we present here several lines of evidence that CypA is also required for HCV replication. We showed that both transient and stable small RNA interferences, which specifically target CypA, profoundly hamper HCV RNA replication as well as HCV protein expression. In contrast to previous studies (9, 17), we did not observe a significant contribution to HCV replication of other Cyp members including CypB, CypC and CypD. Although we do not have any clear explanation for these apparent divergent results, one cannot exclude the possibility that the use of different cells, HCV strains or replication systems somehow modulates the respective contribution of Cyp members to HCV replication. It is critical to emphasize that during the course of our study, two independent studies from the Tang (18) and the Bartenschlager (38) laboratories obtained similar results, which convincingly showed that CypA, but not CypB and CypC, is an essential factor for HCV infection. Thus, to date, the findings of three independent studies including ours, all using a stable shRNA knockdown approach, converge to the same conclusion, which is that CypA serves as a major host cofactor for HCV replication. A couple of observations may explain why HCV preferentially exploits CypA rather than CypB and CypC. One is that CypA is 10- and 100-fold more abundant in a cell than CypB and CypC, respectively (22, 39). Another is that CypA, which resides in the cytosol, is more appropriately located to interact with the HCV replication complex than CypB and CypC, which reside in the lumen of the endoplasmic reticulum (22).

Does CypA assist HCV replication as a peptidyl isomerase? To date, an auxiliary and an essential biochemical functions can be attributed to Cyps. The auxiliary function is characterized by polypeptide sequestration using extended catalytic subsites of the enzyme whereas catalysis essentially requires direct participation of active site residues (40). We demonstrated here that when we mutate residues that reside in the hydrophobic pocket of CypA (histidine or arginine in position 126 and R55, respectively) where the proline-containing peptide substrates bind, the resulting CypA mutants fail to restore HCV replication. The simplest hypothesis for the mechanism of action of CypA in the HCV life cycle is that it catalyses a *trans* to *cis* or a *cis* to *trans* isomerization of a peptidyl-prolyl bond in a viral or host protein critical for HCV replication. The observation that Cyp inhibitors (Debio 025, NIM811 and SCY-635), which neutralize PPIase activity, but which are not immunosuppressive, also block HCV replication is consistent with this hypothesis. In *Drosophila melanogaster*, the CypA homolog, called NinaA, forms a stable and specific complex with the Rh1 isoform of rhodopsin. The formation of this complex is essential for the transit of the visual pigment through the endoplasmic reticulum (41-42). Interestingly, an elegant study by Schmid and colleagues showed that a proline serves as a molecular timer in the infection of Escherichia coli by the filamentous phage fd (43). The phage infection is activated by the disassembly of two domains of its gene-3-protein, which is located at the phage tip. A proline (Pro213) located in the hinge between the two gene-3-protein domains, serves as a timer for the infective state. The timer is switched on by cis-to-trans and switched off by the unusually slow trans-to-cis isomerization of the Gln212-Pro213 peptide bond. Importantly, the switching rate and the phage infectivity are determined by the local sequence surrounding Pro213, and can be tuned by mutagenesis (43). Another hypothesis is that the PPIase activity and the auxiliary polypeptide sequestration function of CypA are both required for its function in HCV replication. Indeed, mutagenesis of NinaA failed to identify a mutant, which distinguishes the isomerase from

the polypeptide sequestration activity of CypA (41). Similarly, we showed here that mutations, which disrupt the isomerase activity of CypA, also disrupt the capacity of CypA to bind HIV-1 Gag in hepatocytes. This is in accordance with the work of Luban and colleagues, who showed that all mutations, which neutralize CypA enzymatic activity, also preclude CypA incorporation into HIV-1 (44).

The mechanisms of action of CypA in HCV replication remain to be unraveled. A current hypothesis is that Cyps by interacting with the nonstructural protein 5B (NS5B), increase the affinity of the polymerase to the viral RNA, and therefore enhances HCV replication (45). This hypothesis is supported by the fact that two studies showed that HCV resistance to CsA (39, 46), resulted in the emergence of mutations in NS5B. Moreover, Yang et al. presented convincing pulldown data showing that CypA binds NS5B (18). These data are in accordance with our co-immunoprecipitation data, which showed that the isomerase pocket of CypA serves as a binding site for the NS5B polymerase. This finding demonstrates for the first time that there is a direct correlation between NS5B binding to the isomerase pocket of CypA and HCV replication. These new findings are important because they suggest that CypA, by catalyzing a *trans* to *cis* or a *cis* to *trans* isomerization of a peptidyl-prolyl bond within NS5B, enhances HCV replication. Interestingly, recent resistance mapping studies suggest that Cyp inhibition may also act on the HCV NS5A protein (46-48). Thus, further work is required to determine if NS5A, NS5B, both or another viral protein represent(s) the true ligand(s) for CypA.

Although both HIV-1 and HCV exploit the abundant cytosolic CypA, it is likely that the immunophilin acts at distinct steps of their viral life cycles. HIV-1 requires CypA post-entry (early events) (49). It is currently thought that target cell CypA, by interacting with the HIV-1 core delivered into the cytosol of infected cells, protects these cores from an antiviral activity present in human cells (50-53). The identity of the anti-HIV-1 factor counteracted by CypA remains unknown. In contrast to HIV-1, HCV requires CypA before budding (late events). CypA likely does not act on the HCV core, like HIV-1, since HCV replicon (no core expressed) replication is also CypA-dependent. Nevertheless, further studies are required to determine if CypA acts at several steps of the HCV life cycle. Although CypA apparently acts at distinct steps of the HIV-1 and HCV life cycles, one cannot exclude the possibility that CypA via its hydrophobic pocket assists these two prime human pathogens by accomplishing the same task such as peptidyl-prolyl cis-trans isomerization or another unknown task. Given that the Cyp inhibitor Debio 025 exhibits a remarkable anti-HCV activity in patients (15-16), it is imperative to understand at a molecular level how this novel class of anti-HCV agents - Cyp inhibitors - block HCV replication.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1: Respective Contribution of Cyp Members to HCV Replication. A. Huh7 cells containing the subgenomic HCV Con1 replicon were transfected with an irrelevant control siRNA or siRNAs that target CypA (siRNA CypA), CypB (siRNA CypB), CypC (siRNA CypC) or CypD (siRNA CypD). Cells were washed 24 h post-transfection. Seven days post-transfection, cells were collected an dialysed. To ensure that the siRNA treatments did not non-specifically influence growth and viability of transfected

hepatocytes, cells were counted and analyzed for trypan blue uptake at the time of collection. Cell lysates were standardized for protein content and analyzed for Cyp content by Western blot using antibodies directed against CypA, CypB, CypC or CypD. **B.** Same as A. except that cell lysates were analyzed for HCV protein expression using antibodies directed against NS5A and NS5B. **C.** Naïve Huh7 cells were electroporated with 10 μg of in vitro-transcribed genomic Con1 RNA. Twenty-four hours post-HCV RNA eletroporation, cells were transfected with siRNA Cyp and then retransfected 24 hours later. At the indicated time points, intracellular HCV RNA was analyzed via reverse-transcription quantitative polymerase chain reaction and presented as genome equivalents (GE) per microgram total RNA.

Figure 2: HCV, Like HIV-1, Requires Host CypA to Fully Replicate in Human Cells. A. Naïve Huh7 cells were transduced with an VSVG-pseudotyped HIV-1-based vector containing an irrelevant control shRNA or shRNAs that target CypA, CypB or CypC. Transduced cells were selected for 7 weeks under puromycin (1 μg/mL). Stable cell lines were analyzed for CypA, CypB and CypC content by Western blotting. **B.** Stable Cyp-KD cell lines were electroporated with 10 μg of in vitro-transcribed genomic Con1 RNA. At the indicated time points, intracellular HCV RNA was analyzed via reverse-transcription quantitative polymerase chain reaction and presented as genome equivalents (GE) per microgram total RNA. **C.** Top panels. Cyp-KD cells lines were exposed to cell-free VSVG-HIV-1-GFP (left panel) or HSV-1-GFP (right panel) at a multiplicity of infection (MOI) of 0.1. Forty-eight hours post-infection, percentage of GFP-positive cells was quantified by FACS. Bottom left panel. Cells were exposed to cell-free Dengue-2 (MOI of 0.1). Three days post-infection, Dengue-2 infection was quantified by measuring the amounts of intracellular Dengue-2 capsid using an intracellular FACS staining assay (IFSA). Bottom right panel. Cyp-KD cells lines were electroporated with 10 μg of *in vitro*–transcribed genomic Con1 RNA. Seven days post-transfection, HCV infection was quantified by measuring the amounts of intracellular HCV NS5B. Data are expressed in % of infected cells (GFP-, capsid- or NS5B-positive cells) by fixing arbitrary infection of parental cells at 100. Results are representative of three independent experiments.

Figure 3: HCV Requires the Isomerase Activity of CypA to Replicate in Hepatocytes. A. Parental or CypA-knockdown Huh7 cell lines were electroporated with 10 μg of in vitro-transcribed genomic Con1 RNA. Twenty-four hours post-HCV RNA eletroporation, cells were transfected with shRNA-resistant wild-type or H126Q CypA-HA in the presence or absence of the Cyp inhibitors CsA (2.5 μM) or Debio 025 (2 μM). At the indicated time points, intracellular HCV RNA was analyzed via reverse-transcription quantitative polymerase chain reaction and presented as genome equivalents (GE) per microgram total RNA. **B.** The inability of the H126Q CypA mutant to bind to HIV-1 Gag in hepatocytes was examined by measuring amounts of CypA incorporated into released particles. Huh7 cells were co-transfected with HIV-1 together with wild-type or H126 CypA-HA in the presence or absence of Cyp inhibitors CsA (10 μM) or Debio 025 (2 μM). Forty-eight post-transfection, both transfected cells and released virions were analyzed for CypA content by Western blotting using anti-HA antibodies. Cell lysates were standardized for protein content, whereas virions were standardized for HIV-1 capsid content by p24 ELISA.

Figure 4: The HCV NS5B Polymerase Associates With CypA Via Its Enzymatic Pocket. Parental Huh7 cells (3 million) were co-transfected with NS5B-myc (5 μg DNA) and wild-type CypA-HA or H126Q CypA-HA (5 μg DNA) in the presence or absence of the Cyp inhibitor Debio 025 (2 μM). Three days post-transfection, cells were collected and lysed. Cell lysates were pre-cleared with agarose beads. CypA-NS5B association was assessed by co-immunoprecipation using the Pierce HA tag Co-IP kit. Bound material was eluted and analyzed by Western blotting using anti-HA and anti-myc antibodies. Results are representative of three independent transfections.

FIGURE 1

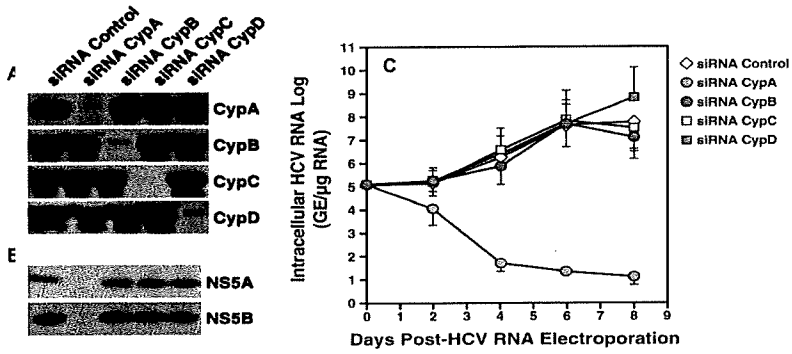


FIGURE 2

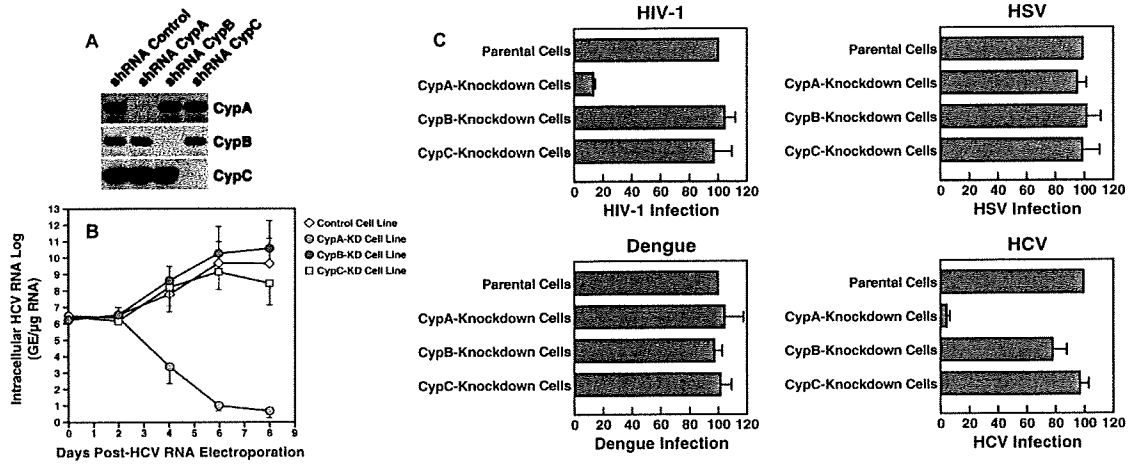


FIGURE 3

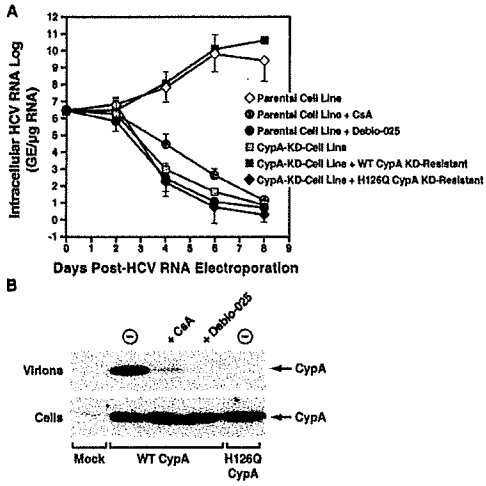
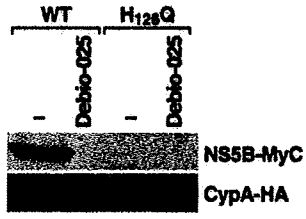


FIGURE 4



nature genetics

Genome-wide association of *IL28B* with response to pegylated interferon-α and ribavirin therapy for chronic hepatitis C

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The recommended treatment for patients with chronic hepatitis C, pegylated interferon-α (PEG-IFN-α) plus ribavirin (RBV), does not provide sustained virologic response (SVR) in all patients. We report a genome-wide association study (GWAS) to null virological response (NVR) in the treatment of patients with hepatitis C virus (HCV) genotype 1 within a Japanese population. We found two SNPs near the gene *IL28B* on chromosome 19 to be strongly associated with NVR (rs12980275, $P = 1.93 \times 10^{-13}$, and rs8099917, 3.11×10^{-15}). We replicated these associations in an independent cohort (combined *P* values, 2.84×10^{-27} (OR = 17.7; 95% CI = 10.0–31.3) and 2.68×10^{-32} (OR = 27.1; 95% CI = 14.6–50.3), respectively). Compared to NVR, these SNPs were also associated with SVR (rs12980275, $P = 3.99 \times 10^{-24}$, and rs8099917, $P = 1.11 \times 10^{-27}$). In further fine mapping of the region, seven SNPs (rs8105790, rs11881222, rs8103142, rs28416813, rs4803219, rs8099917 and rs7248668) located in the *IL28B* region showed the most significant associations ($P = 5.52 \times 10^{-28}$–2.68×10^{-32}; OR = 22.3–27.1). Real-time quantitative PCR assays in peripheral blood mononuclear cells showed lower *IL28B* expression levels in individuals carrying the minor alleles ($P = 0.015$).

Hepatitis C is a global health problem that affects a significant proportion of the world's population. The World Health Organization estimated that in 1999, there were 170 million HCV carriers worldwide, with 3–4 million new cases appearing each year. HCV infection affects more than 4 million people in the United States, where it represents the leading cause of cirrhosis and hepatocellular carcinoma as well as the leading cause of liver transplantation[1].The American Gastroenterological Association estimated that drugs are the largest direct costs of hepatitis C[1].

The most effective current standard of care in patients with chronic hepatitis C, a combination of PEG-IFN-α with ribavirin, does not produce SVR in all patients treated. Large-scale studies on 48-week-long PEG-IFN-α/RBV treatment in the United States and Europe showed that 42–52% of patients with HCV genotype 1 achieved SVR[2–4], and similar results were found in Japan. However, older patients (greater than 50 years of age) had a significantly lower rate of SVR due to poor adherence resulting from adverse events and laboratory-detectable abnormalities such as neutropenia and thrombocytopenia[5,6]. Specifically, various well-described side effects (such as a flu-like syndrome, hematologic abnormalities and adverse neuropsychiatric events) often necessitate dose reduction, and 10–14% of patients require premature withdrawal from interferon-based therapy[7]. To avoid these side effects in patients who will not be helped by the treatment, as well as to reduce the substantial cost of PEG-IFN-α/RBV treatment, it would be useful to be able to predict an individual's response before or early in treatment. Several viral factors, such as genotype 1, high baseline viral load, viral

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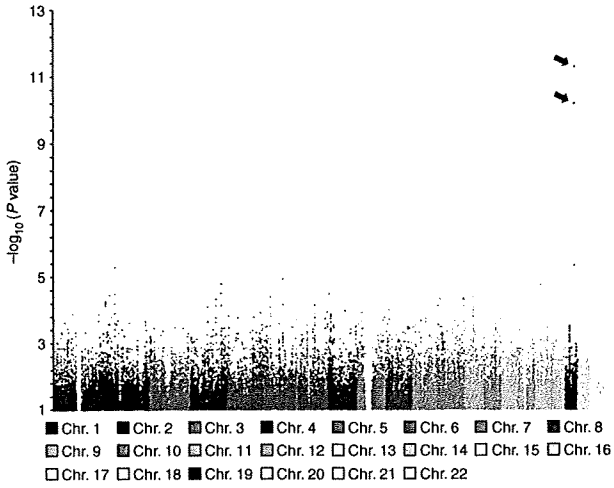

Figure 1 Genome-wide association results with PEG-IFN-α/RBV treatment in 142 Japanese patients with HCV (78 NVR and 64 VR samples). *P* values were calculated by using a χ^2 test for allele frequencies. The dots with arrows for chromosome 19 denote SNPs that showed significant genome-wide associations ($P < 8.05 \times 10^{-8}$) with response to PEG-IFN-α/RBV treatment.

kinetics during treatment, and amino acid pattern in the interferon sensitivity–determining region, have been reported to be significantly associated with the treatment outcome in a number of independent studies[8–10]. Studies have also provided strong evidence that ~20% of patients with HCV genotype 1 and 5% of patients with genotype 2 or 3 have a null response to PEG-IFN-α/RBV. No definite predictor of this resistance is currently available that make it possible to bypass the initial 12–24 weeks' treatment before deciding whether treatment should be continued. If a reliable predictor of non-response were identified for use in patients before treatment initiation, then an estimated 20%, including those who have little or no chance to achieve SVR, could be spared the side effects and cost of treatment.

Host factors, including age, sex, race, liver fibrosis and obesity, have also been reported to be associated with PEG-IFN-α/RBV therapy outcome[11,12]. However, little is known about the host genetic factors that might be associated with the response to therapy: thus far only a few candidate genes, including those encoding type I interferon receptor-1 (*IFNAR1*) and mitogen-activated protein kinase–activated protein kinase 3 (*MAPKAPK3*), have been reported to be associated with treatment response[13,14]. We describe here a GWAS for response to PEG-IFN-α/RBV treatment.

We conducted this GWAS to identify host genes associated with response to PEG-IFN-α/RBV treatment in 154 Japanese patients with HCV genotype 1 (82 with NVR and 72 with virologic response (VR), based on the selection criteria as described in Online Methods). We used the Affymetrix SNP 6.0 genome-wide SNP typing array for 900,000 SNPs. A total of 621,220 SNPs met the following criteria: (i) SNP call rate ≥95%, (ii) minor allele frequency (MAF) ≥1% and (iii) deviation from Hardy-Weinberg equilibrium (HWE) $P \geq 0.001$ in VR samples. After excluding 4 NVR and 8 VR samples that showed quality control (QC) call rates of <95%, 78 NVR and 64 VR samples were included in the association analysis. **Figure 1** shows a genome-wide view of the single-point association data based on allele frequencies. Two SNPs located close to *IL28B* on chromosome 19 showed strong associations, with a minor allele dominant model (rs12980275, $P = 1.93 \times 10^{-13}$, and rs8099917, $P = 3.11 \times 10^{-15}$, respectively), with NVR to PEG-IFN-α/RBV treatment (**Table 1**). The rs8099917 lies between *IL28B* and *IL28A*, ~8 kb downstream from *IL28B* and ~16 kb upstream from *IL28A*. These associations reached genome-wide levels of significance for both SNPs in this initial GWAS cohort (Bonferroni criterion $P < 8.05 \times 10^{-8}$ (0.05/621,220)). The frequencies of minor allele–positive patients were much higher in the NVR group than in the VR group for both SNPs (74.3% in NVR, 12.5% in VR for rs12980275; 75.6% in NVR, 9.4% in VR for rs8099917). Notably, individuals homozygous for the minor allele were observed only in the NVR group. The VR group, as compared to the NVR group, showed genotype frequencies closer to those in the healthy Japanese population[15], yet the minor allele frequencies were slightly higher in the transient virologic response (TVR) group (23.1%, 15.4%) than in the SVR group (9.8%, 7.8%) (**Table 1**). We applied the Cochrane-Armitage test on all the SNPs and found a genetic inflation factor, λ, of 1.029 for the GWAS stage (**Supplementary Fig. 1**). We also carried out principal component analysis in 142 samples for the GWAS stage together with the HapMap samples (CEU, YRI, CHB and JPT) (**Supplementary Fig. 2**); this suggested that the effect of population stratification was negligible.

Table 1 Significant association of two SNPs (rs12980275 and rs8099917) with response to PEG-IFN-α/RBV treatment

					Null responder (NVR[a], *n* = 128)			Responder (VR[a], *n* = 186)			Responder (SVR[a], *n* = 140)			NVR vs. VR		NVR vs. SVR	
dbSNP rsID	Nearest gene	MAF[b] (allele)	Allele (1/2)	Stage	11	12	22	11	12	22	11	12	22	OR (95% CI)[c]	*P* value[d]	OR (95% CI)[c]	*P* value[d]
rs12980275	*IL28B*	0.15 (G)	A/G	GWAS	20 (25.6)	54 (69.2)	4 (5.1)	56 (87.5)	8 (12.5)	0 (0.0)	46 (90.2)	5 (9.8)	0 (0.0)	20.3 (8.3–49.9)	1.93×10^{-13}	26.7 (9.3–76.5)	7.41×10^{-13}
				Replication	10 (20.0)	37 (74.0)	3 (6.0)	101 (82.8)	21 (17.2)	0 (0.0)	73 (82.0)	16 (18.0)	0 (0.0)	19.2 (8.3–44.4)	5.46×10^{-15}	18.3 (7.6–44.0)	8.37×10^{-13}
				Combined	30 (23.4)	91 (71.1)	7 (5.5)	157 (84.4)	29 (15.6)	0 (0.0)	119 (85.0)	21 (15.0)	0 (0.0)	17.7 (10.0–31.3)	2.84×10^{-27}	18.5 (10.0–34.4)	3.99×10^{-24}
rs8099917	*IL28B*	0.12 (G)	T/G	GWAS	19 (24.4)	56 (71.8)	3 (3.8)	58 (90.6)	6 (9.4)	0 (0.0)	47 (92.2)	4 (7.8)	0 (0.0)	30.0 (11.2–80.5)	3.11×10^{-15}	36.5 (11.6–114.6)	5.00×10^{-14}
				Replication	11 (22.0)	37 (74.0)	2 (4.0)	108 (88.5)	14 (11.5)	0 (0.0)	78 (87.6)	11 (12.4)	0 (0.0)	27.4 (11.5–65.3)	9.47×10^{-18}	25.1 (10.0–63.1)	1.00×10^{-14}
				Combined	30 (23.4)	93 (72.7)	5 (3.9)	166 (89.2)	20 (10.8)	0 (0.0)	125 (89.3)	15 (10.7)	0 (0.0)	27.1 (14.6–50.3)	2.68×10^{-32}	27.2 (13.9–53.4)	1.11×10^{-27}

[a]NVR, null virologic response; VR, virologic response; SVR, sustained virologic response. The 186 VRs consisted of 46 transient virologic response (TVRs) and 140 SVRs. [b]Minor allele frequency and minor allele in 184 healthy Japanese individuals[15]. The MAF of the SNPs in SVR is similar to that of TVR group, whereas that of NVR is much higher (76.6%). [c]Odds ratio for the minor allele in a dominant model. [d]*P* value by χ^2 test for the minor allele dominant model.

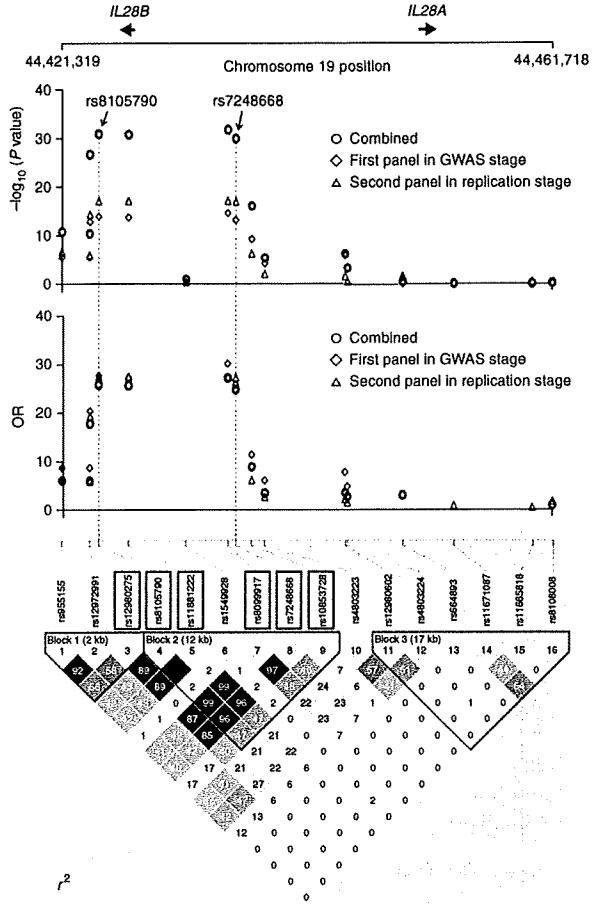

Figure 2 Genomic structure, *P* value and OR plots in association analysis and LD map around *IL28B* and *IL28A* (chr.19, nucleotide positions 44421319–44461718; build 35). *P* values by the χ^2 test for minor allele dominant effect model are shown for the first panel of 142 samples in the GWAS stage, the second panel of 172 samples in the replication stage, and the combined analysis. Below are estimates of pairwise r^2 for 16 SNPs selected in the replication study using a total of 314 Japanese patients with HCV treated with PEG-IFN-α/RBV. Boxes indicate the significantly associated SNPs with response to PEG-IFN-α/RBV treatment both in the GWAS stage and in the replication stage. Dotted lines indicate the region with the strongest associations from the positions of rs8105790 to rs7248668.

We analyzed the region of ~40 kb (chr. 19, nucleotide positions 44421319–44461718; build 35) containing the significantly associated SNPs (rs12980275 and rs8099917) using Haploview software for linkage disequilibrium (LD) and haplotype structure based on the HapMap data for individuals of Japanese ancestry. The LD blocks were analyzed using the four-gamete rule, and four blocks were observed (**Supplementary Fig. 3**). We selected 16 SNPs for both replication study and high-density association mapping, including tagging SNPs estimated on the basis of the haplotype blocks, one SNP located within *IL28B* (rs11881222) and the significantly associated SNPs from the GWAS stage (rs12980275 and rs8099917) (**Supplementary Table 1**).

To validate the results of the GWAS stage, 16 SNPs selected for the replication stage, including the original SNPs, were genotyped using the DigiTag2 assay in an independent set of 172 Japanese patients with HCV treated with PEG-IFN-α/RBV treatment (50 NVR and 122 VR samples), together with the first panel of 142 samples analyzed in the GWAS stage (**Supplementary Table 1**). The associations of the original SNPs were replicated in the replication cohort of 172 patients ($P = 5.46 \times 10^{-15}$, OR = 19.2 for rs12980275; $P = 9.47 \times 10^{-18}$, OR = 27.4 for rs8099917; **Table 1**). The combined *P* values for both stages reached 2.84×10^{-27} (OR = 17.7; 95% CI = 10.0–31.3) and 2.68×10^{-32} (OR = 27.1; 95% CI = 14.6–50.3), respectively (**Table 1**). Notably, when we compared the SVR ($n = 140$) with the NVR group ($n = 128$), the original two SNPs (rs12980275 and rs8099917) again showed strong associations: both *P* values and ORs were similar to those observed in the comparison between VR and NVR, and the combined *P* values for both stages reached 3.99×10^{-24} (OR = 18.5; 95% CI = 10.0–34.4) and 1.11×10^{-27} (OR = 27.2; 95% CI = 13.9–53.4), respectively (**Table 1**). Comparing SVR ($n = 140$) versus NVR plus TVR ($n = 174$), we again found that these SNPs were significantly associated ($P = 1.71 \times 10^{-16}$, OR = 8.8; 95% CI 5.1–15.4 for rs12980275; $P = 1.18 \times 10^{-18}$, OR = 12.1; 95% CI 6.5–22.4 for rs8099917, **Supplementary Table 2**), suggesting that these SNPs would predict NVR as well as SVR before PEG-IFN-α/RBV therapy.

Among the newly analyzed SNPs in the replication study, six (rs12980275, rs8105790, rs11881222, rs8099917, rs7248668 and rs10853728) showed significant associations both in the GWAS stage ($P < 8.05 \times 10^{-8}$) and in the replication stage ($P < 0.0031$ (0.05/16)) after Bonferroni correction. These SNPs are located within a 15.7-kb region that includes *IL28B* (**Fig. 2** and **Supplementary Table 1**). In particular, the strongest associations with NVR were observed for four SNPs, rs8105790, rs11881222, rs8099917 and rs7248668, that are located in the downstream flanking region, the third intron and the upstream flanking region of *IL28B*. The combined *P* values for these polymorphisms were 1.98×10^{-31} (OR = 25.7; 95% CI = 13.9–47.6), 2.84×10^{-31} (OR = 25.6; 95% CI = 13.8–47.3), 2.68×10^{-32} (OR = 27.1; 95% CI = 14.6–50.3) and 1.84×10^{-30} (OR = 24.7; 95% CI = 13.3–45.8), respectively (**Supplementary Table 1**). We then sequenced this region to identify further variants and found three SNPs (rs8103142, rs28416813 and rs4803219) located in the third exon, the first intron and the upstream flanking region of *IL28B*, and a few infrequent variations. These SNPs also showed strong associations in the combined dataset of 128 NVR and 186 VR samples ($P = 1.40 \times 10^{-29}$, OR = 26.6 for rs8103142; $P = 5.52 \times 10^{-28}$, OR = 22.3 for rs28416813; $P = 2.45 \times 10^{-29}$, OR = 23.3 for rs4803219; **Supplementary Table 3**). We also performed LD and haplotype analyses with seven SNPs. These SNPs were in strong LD, and the risk haplotype showed a level of association similar to those of individual SNPs ($P = 1.35 \times 10^{-25}$, OR = 11.1; 95% CI = 6.6–18.6) (**Table 2**). These results suggest that the association with NVR was primarily driven by one of these SNPs.

Table 2 Association analysis of response to treatment by *IL28B* haplotype

SNP							Frequencies			
rs8105790	rs11881222	rs8103142	rs28416813	rs4803219	rs8099917	rs7248668	NVR group	VR group	*P* value	OR (95% CI)
T	**A**	**T**	**C**	**C**	T	G	0.543	0.942	1.81×10^{-32}	0.1 (0.04–0.12)
C	**G**	**C**	**G**	**T**	G	A	0.387	0.054	1.35×10^{-25}	11.1 (6.6–18.6)

Association analysis of haplotypes consisting of seven SNPs with response to PEG-IFN-α/RBV treatment in 314 Japanese patients with HCV. Boldface letters: rs11881222 (third intron); rs8103142 (third exon).

Table 3 Factors associated with NVR by logistic regression model

Factors	Odds ratio	95% CI	*P* value
rs8099917 (G allele)	37.68	16.71–83.85	<0.0001
Age	1.02	0.98–1.07	0.292
Gender (Female)	3.32	1.49–7.39	0.003
Re-treatment[a]	1.12	0.55–2.33	0.750
Platelet count	0.93	0.87–1.01	0.080
Aminotransferase level	1.00	0.99–1.00	0.735
Fibrosis stage[20]	1.10	0.73–1.66	0.658
HCV-RNA level	1.01	0.99–1.02	0.139

[a]Re-treatment, non-response to previous treatment with interferon-α (plus RBV).

To examine the relative contribution of factors associated with NVR, we used a logistic regression model. One tagging SNP located within *IL28B* (minor allele of rs8099917) was the most significant factor for predicting NVR, followed by gender (**Table 3**). Clinically, viral factors such as HCV genotype and HCV RNA level are important for the outcome of PEG-IFN-α/RBV therapy. Indeed, mean HCV-RNA level was significantly lower in SVR (SVR versus TVR, $P = 0.002$; SVR versus NVR, $P = 0.016$; **Supplementary Table 4**). Mean platelet count and the proportion of mild fibrosis (F1–F2) were significantly higher in SVR than in NVR.

Real-time quantitative PCR assays in peripheral blood mononuclear cells revealed a significantly lower level of *IL28* mRNA expression in individuals with the minor alleles (**Fig. 3**), suggesting that variant(s) regulating *IL28* expression is associated with a response to PEG-IFN-α/RBV treatment. *IL28B* encodes a cytokine distantly related to type I (α and β) interferons and the interleukin (IL)-10 family. This gene and *IL28A* and *IL29* (encoding IL-28A and IL-29, respectively) are three closely related cytokine genes that encode proteins known as type III IFNs (IFN-λs) and that form a cytokine gene cluster at chromosomal region 19q13 (ref. 16). The three cytokines are induced by viral infection and have antiviral activity[16,17]. All three interact with a heterodimeric class II cytokine receptor that consists of IL-10 receptor beta (IL10Rβ) and IL-28 receptor alpha (IL28Rα, encoded by *IL28RA*)[16,17], and they may serve as an alternative to type I IFNs in providing immunity to viral infection.

Notably, a recent report showed that the strong antiviral activity evoked by treating mice with TLR3 or TLR9 agonists was significantly reduced in both *IL28RA*$^{-/-}$ and *IFNAR*$^{-/-}$ mice, indicating that IFN-λ is important in mediating antiviral protection by ligands for TLR3 and TRL9 (ref. 18). IFN-λ induced a steady increase in the expression of a subset of IFN-stimulated genes, whereas IFN-α induced the same genes with more rapid and transient kinetics[19]. Therefore, it is possible that IFN-λ induces a slower but more sustained response that is important for TLR-mediated antiviral protection. This might be one of the ways that a genetic variant regulating *IL28* expression influences the response to PEG-IFN-α/RBV treatment. Further research will be required to fully understand the specific mechanism by which a genotype might affect the response to treatment.

In conclusion, the strongest associations with NVR were observed for seven SNPs, rs8105790, rs11881222, rs8103142, rs28416813, rs4803219, rs8099917 and rs7248668, that are located in the downstream flanking region, the third intron, the third exon, the first intron and the upstream flanking region of *IL28B*. Further studies following our report of this robust genetic association to NVR may make it possible to develop a pre-treatment predictor of which individuals are likely to respond to PEG-IFN-α/RBV treatment. This would remove the need for the initial 12–24 weeks of treatment that is currently used as a basis for a clinical decision about whether treatment should be continued. That would allow better targeting of PEG-IFN-α/RBV treatment, avoiding the unpleasant side effects that commonly accompany the treatment where it is unlikely to be beneficial, and reduce overall treatment costs. Because of the small number of samples in this study, we plan to conduct a further prospective multicenter study to establish these SNPs as a clinically useful marker.

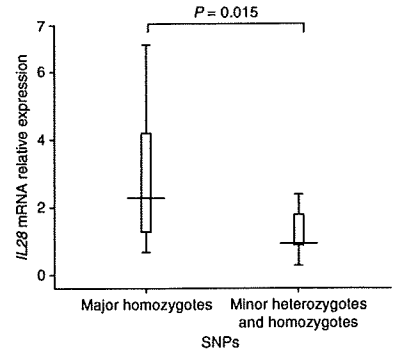

Figure 3 Quantification of *IL28* mRNA expression. The expression level of *IL28* genes was determined by real-time quantitative RT-PCR using RNA purified from peripheral blood mononuclear cells. Distribution of relative gene expression levels was compared between the individuals homozygous for major alleles ($n = 10$) and the heterozygous or homozygous individuals carrying minor alleles ($n = 10$) of rs8099917 by using the Mann-Whitney *U*-test. The bars indicate the median. All samples were obtained from HCV-infected patients before PEG-IFN-α/RBV therapy.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

Study design and discussion: Y.T., N.N., N.M., K.T., M.M.; sample collection: Y.T., M.K., K.M., N.S., M.N., M.K., K.H., S.H., Y.I., E.M., E.T., S.M., Y.M., M.H., A.S., Y.H., S.N., I.S., M.I., K.I., K.Y., F.S., N.I.; genotyping: N.N.; statistical analysis: N.N., A.K., K.I.; quantitative RT-PCR: M.S.; manuscript writing: Y.T., N.N., K.T., M.M.

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ONLINE METHODS

Study cohorts. From April 2007 to April 2009, samples were obtained from 314 patients with chronic HCV (genotype 1) infection who were treated at 15 multicenter hospitals (liver units with hepatologists) throughout Japan. Each patient was treated with PEG-IFN-α2b (1.5 μg per kg body weight (μg/kg) subcutaneously once a week) or PEG-IFN-α2a (180 μg/kg once a week) plus RBV (600–1,000 mg daily depending on body weight). As a reduction in the dose of PEG-IFN-α and RBV can contribute to a less sustained virological response[21], only patients with an adherence of >80% dose for both drugs during the first 12 weeks were included in this study. HBsAg-positive and/or anti-HIV–positive individuals were excluded from this study.

NVR (seen in ~20% of total treated patients) was defined as less than a 2-log-unit decline in the serum level of HCV RNA from the pre-treatment baseline value within the first 12 weeks and detectable viremia 24 weeks after treatment. VR was defined as the achievement of SVR or transient TVR in this study; SVR was defined as undetectable HCV RNA in serum 6 months after the end of treatment, whereas TVR was defined as a reappearance of HCV RNA in serum after treatment was discontinued in a patient who had undetectable HCV RNA during the therapy or on completion of the therapy. Of 878 patients with HCV genotype 1 treated by PEG-IFN-α/RBV at 14 hospitals, only 114 (13.0%) met the criteria for NVR in this study. For the GWAS stage of the study, a case-control study was conducted comparing individuals with NVR (82 individuals) and VR (72 individuals). For the replication stage, an independent cohort of samples from 172 Japanese patients with HCV genotype 1, including 50 with NVR and 122 with VR, was obtained from an independent cohort study at Tokyo Medical and Dental University Hospital (Ochanomizu Liver Conference Study Group) and Musashino Red Cross Hospital. Clinical data from the combined cohorts, with a total of 140 SVR, 46 TVR and 128 NVR patients, are shown in **Supplementary Table 4**.

Informed consent was obtained from each patient who participated in the study. The study protocol conforms to the relevant ethical guidelines as reflected in *a priori* approval by the ethics committees of all the participating universities and hospitals.

SNP genotyping and data cleaning. In the GWAS stage, we genotyped 154 Japanese patients with HCV receiving PEG-IFN-α/RBV treatment using the Affymetrix Genome-Wide Human SNP Array 6.0 according to the manufacturer's instructions. After exclusion of 4 NVR samples and 8 SVR samples with QC call rates <95%, the remaining 142 samples were recalled using the Birdseed version 3 software (Affymetrix). The average overall call rate of 78 NVR and 64 VR samples reached 99.46% and 99.46%, respectively. We then applied the following thresholds for QC in data cleaning: SNP call rate ≥95% for all samples, MAF ≥1% for all samples and HWE P value ≥0.001 for VR group[22,23]. A total of 621,220 SNPs on autosomal chromosomes passed the QC filters and were used for association analysis. All cluster plots for the SNPs showing $P < 0.001$ in association analyses by comparing allele frequencies in NVR and VR groups were checked by visual inspection. SNPs with ambiguous genotype calls were excluded. **Supplementary Table 5** shows SNPs that might be weakly associated with NVR ($P < 10^{-4}$).

Although the 12 samples noted above were excluded from the GWAS stage by data cleaning, their quality was good enough for the SNP typing in the replication study, and thus they were included in the replication stage. In the subsequent replication stage with high-density association mapping, SNP genotyping in the independent set of 172 patients was completed using the DigiTag2 assay[24] and direct sequencing using the Applied Biosystems 3730 DNA Analyzer (Applied Biosystems). In addition, strongly associated SNPs identified in the GWAS stage were also genotyped for the GWAS samples using the DigiTag2 assay, and the results were 100% concordant to those from the GWAS platform.

Screening for new polymorphisms. To determine possible genomic variants in the region of *IL28B* and its promoter, we sequenced the 3.3-kb region in a total of 48 Japanese patients with HCV (28 NVR and 20 VR). We selected 7 samples from NVR patients who were minor allele homozygotes for 2 SNPs (rs12980275 and rs8099917), 11 samples from NVR and 10 samples from VR heterozygotes, and 10 samples from NVR and 10 samples from VR major allele homozygotes. The sequencing primers were designed using the Visual OMP Nucleic Acid software (**Supplementary Table 6**). PCR was carried using TaKaRa LA *Taq* polymerase (Takara Biochemicals) under the following thermal cycler conditions: stage 1, 94 °C for 1 min; stage 2, 98 °C for 10 s, 68 °C for 15 min, for a total of 30 cycles; stage 3, 72 °C for 10 min. A 50-μl PCR analysis was performed using 2.5 U TaKaRa LA *Taq* with 1× LA PCR buffer II, 0.4 mM dNTP, 10 pmol of each primer and 10 ng of genomic DNA. For sequencing, 7.0 μl of the PCR products were incubated with 3 μl of Exonuclease I/Shrimp Alkali Phosphatase (Takara Biochemicals) first for 90 min at 37 °C and then for another 10 min at 80 °C. Sequencing reactions were performed with the use of a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems). After purification with MultiScreen-HV (Millipore) and Sephadex G-50 Fine (GE Healthcare UK Ltd.), the reaction products were applied to the Applied Biosystems 3730 DNA Analyzer.

In the variation screening, three SNPs (rs8103142, rs28416813 and rs4803219) and a few infrequent variations were detected. We then typed these SNPs in all of the 314 patients.

Statistical analysis. The observed association between a SNP and response to PEG-IFN-α/RBV treatment was assessed by χ^2 test with a two-by-two contingency table in three genetic models: allele frequency model, dominant-effect model and recessive-effect model. SNPs on the X chromosome were removed because gender was not matched between the NVR group and the VR group. A total of 621,220 SNPs passed the QC filters in the GWAS stage; therefore, significance levels after the Bonferroni correction for multiple testing were $P = 8.05 \times 10^{-8}$ (0.05/621,220) in the GWAS stage and $P = 0.0031$(0.05/16) in the replication stage. None of the 16 markers genotyped in the replication stage showed deviations from Hardy-Weinberg equilibrium in the VR group ($P > 0.05$).

The inflation factor λ was estimated based on the median χ^2 and revealed to be 1.029 (median) and 1.011 (mean), suggesting that the population substructure should not have any substantial effect on the statistical analysis (**Supplementary Fig. 1**). In addition, the principal component analysis on the 142 patients (78 NVR samples and 64 VR samples) analyzed in the GWAS stage together with the HapMap samples also revealed that the effect of population stratification was negligible (**Supplementary Fig. 2**).

For the replication study and the high-density association mapping, 16 SNPs were selected from the region of ~40 kb (chr. 9, nucleotide positions 44421319–44461718; build 35) containing the significantly associated SNPs (rs12980275 and rs8099917) in the GWAS stage by analyzing, using Haploview software, LD and haplotype structure based on the HapMap data for individuals of Japanese descent. These SNPs included tagging SNPs estimated on the basis of haplotype blocks, SNPs located within the *IL28B* and *IL28A* genes (rs11881222 and rs576832, respectively) and the significantly associated SNPs identified in the GWAS stage (**Supplementary Table 1**). On the basis of the genotype data from the total of 314 patients in the GWAS stage and replication stages, haplotype blocks were estimated using the four-gamete rule, and three blocks were observed (**Fig. 2**). Association of haplotype with response to PEG-IFN-α/RBV treatment was analyzed using Haploview software.

The logistic regression model was used to assess the factors associated with NVR. STATA 10 (Statacorp LP) was used for all analysis. Age, platelet count, and aminotransferase (ALT) and HCV-RNA levels were applied as continuous variables.

Real-time quantitative RT-PCR for *IL28* gene. A layer of mononuclear cells was collected via Ficoll from peripheral blood. Total RNA was isolated using the RNeasy Mini Kit and the RNase-Free DNase Set (Qiagen) according to the manufacturer's protocol. First-strand cDNA was synthesized using SuperScript II reverse transcriptase with Oligo $(dT)_{12-18}$ primer (Invitrogen). The relative quantification of the target gene was determined using Custom TaqMan Gene Expression Assays, and the expression of glyceraldehyde-3-phosphate dehydrogenase was used to normalize the gene expression level (Applied Biosystems) according to the manufacturer's protocol. The data were analyzed by the $2[-\Delta\Delta C_t]$ method using Sequence Detector version 1.7 software (Applied Biosystems). A standard curve was prepared by serial tenfold dilutions of

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human cDNA. The curve was linear over 7 logs with a correlation coefficient of 0.998. The specific detection of *IL28B* in real-time PCR is hard to establish, because the nucleotide differences between *IL28A* and *IL28B* consist of only 9 nucleotides scattered throughout the gene. Primers and probes are designed for the *IL28* gene (**Supplementary Table 6**).

URLs. The results of the present GWAS have been registered at a public database: https://gwas.lifesciencedb.jp/cgi-bin/gwasdb/gwas_top.cgi.

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Inhibition of intracellular hepatitis C virus replication by nelfinavir and synergistic effect with interferon-α

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SUMMARY. Liver diseases associated with hepatitis C virus (HCV) infection have become the major cause of mortality in patients with human immunodeficiency virus (HIV) infection since the introduction of highly active anti-retroviral therapy. HCV-related liver disease is more severe in HIV-infected patients than in non-HIV-infected patients, but the standard therapies used to treat chronic hepatitis C in HCV/HIV coinfected patients are the same as those for patients infected with HCV alone. HIV protease inhibitors might have potential to down-regulate HCV load of HCV/HIV coinfected patients. In this study, we evaluated the effects of nelfinavir on intracellular HCV replication using the HCV replicon system. We constructed an HCV replicon expressing a neomycin-selectable chimeric firefly luciferase reporter protein. Cytotoxicity and apoptosis induced by nelfinavir were assessed and synergism between nelfinavir and interferon (IFN) was calculated using CalcuSyn analysis. Nelfinavir dose-dependently repressed HCV replication at low concentrations (IC_{50}, 9.88 μmol/L). Nelfinavir failed to induce cytotoxicity or apoptosis at concentrations that inhibited HCV replication. Clinical concentrations of nelfinavir (5 μmol/L) combined with IFN showed synergistic inhibition of HCV replication in our replicon model. Our results suggest that the direct effects of nelfinavir on the HCV subgenome and its synergism with IFN could improve clinical responses to IFN therapy in HCV/HIV coinfected patients.

Keywords: hepatitis C virus, human immunodeficiency virus, nelfinavir.

INTRODUCTION

Patients with human immunodeficiency virus (HIV) infection are frequently coinfected with hepatitis C virus (HCV), because these viruses have similar routes of transmission, including blood transfusion, intravenous drug use and sexual contact [1,2]. The optimal therapy for HIV infection is highly active anti-retroviral therapy (HAART), which combines HIV reverse transcriptase inhibitors, often with HIV protease inhibitors. Since the introduction of HAART, the morbidity and mortality associated with HIV infection have declined. This reduction in mortality due to opportunistic infections has made HCV-associated liver disease the leading causes of mortality [3].

Several studies have reported that HCV-related liver disease is more severe in HIV-infected patients than in non-HIV-infected patients [4,5]. The severity of liver disease increases as the immunodeficiency progresses and HIV seropositivity accelerates the progression of liver fibrosis related to chronic hepatitis C [6,7]. In addition, many studies have documented that HIV/HCV coinfected patients have higher HCV loads than do HCV monoinfected controls [8–10]. However, the standard therapies used to treat chronic hepatitis C in HCV/HIV coinfected patients are the same as those for patients infected with HCV alone [11].

HAART has been reported to reduce serum HCV RNA levels accompanied by immune improvement [12], but the decrease in HIV viral load was associated with a persistent and significant increase in HCV viral load [13]. There is no consistent evidence that HAART results in suppression of HCV viraemia, suggesting that multiple factors may be affecting viral load in coinfected patients [14,15]. However,

Abbreviations: CI, combination index; HAART, highly active anti-retroviral therapy; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IFN, interferon; LDH, lactate dehydrogenase; MTS, 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl) tetrazolium inner salt; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end-labelling.

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Trimoulet *et al.* showed that patients treated with HAART that included protease inhibitors had significantly lower intrahepatic HCV loads than those treated with HAART without protease inhibitors [16].

Liver injury has been reported to be a potential side-effect of HAART [17] and potential hepatotoxicity of HIV protease inhibitors had been well realized before these drugs were licensed for the first time [18]. Concomitant hepatic damage prior to the start of HAART is an important risk factor, which can intensify hepatotoxic side-effects of HAART. The presence of chronic hepatitis C has been reported to increase the risk of HAART-associated hepatotoxicity (relative risk, 2.46; 95% confidence interval, 1.43–4.24) [19]. The mechanisms underlying the association of HCV and hepatotoxicity remain unclear, but in some patients liver enzyme elevations may be a manifestation of immune reconstitution that follows antiretroviral therapy. After immune recovery, CD4+ cell counts rise and the ability of T cells to identify and lyse HCV-infected hepatocytes may be increased [20]. The differences in the potential for hepatotoxicity have been reported to exist among the commercially available protease inhibitors and nelfinavir was associated with the low rate of severe hepatotoxicity among patients coinfected with hepatitis viruses [21].

HIV protease is a small, dimeric aspartyl protease that specifically cleaves the polyprotein precursors encoding the structural proteins and enzymes of the virus. This proteolytic activity is absolutely required for the production of mature, infectious virions. HIV protease inhibitors block HIV maturation and show remarkable antiviral potency [22]. It has recently been reported that HIV protease inhibitors also have nonviral effects on the host cells, beyond their effect of blocking HIV protease enzymatic activity [23]. NF-κB is central to the overall immune response through its ability to activate genes coding for regulators of apoptosis and cell proliferation [24]. The HIV protease inhibitor nelfinavir has been shown to regulate NF-κB activation [25].

In the present study, we investigated the action of nelfinavir alone, or in combination with interferon (IFN), on HCV replication using the replicon system.

MATERIALS AND METHODS

Cell culture

The human hepatoma cell line, Huh7, was maintained in Dulbecco's modified minimal essential medium (Sigma, St Louis, MO, USA) supplemented with 2 mM L-glutamine and 10% fetal calf serum at 37 °C under 5% CO_2. Huh7 cells expressing the HCV replicon were cultured in medium containing 500 μg/mL G418 (Nakalai Tesque, Kyoto, Japan).

HCV replicon constructs and transfected cell lines

An HCV subgenomic replicon plasmid, pHCVIbneo-delS (designated pRep-N), was derived from an infectious HCV clone, HCV-N, genotype Ib [26]. The replicon, pRep-N was reconstructed by substituting the neomycin phosphotransferase gene with a fusion gene comprising firefly luciferase and neomycin phosphotransferase (pRep-Feo) [27–29]. RNA was synthesized from pRep-Feo using T7-polymerase (Promega, Madison, WI, USA) and transfected into Huh7 cells. After culture in the presence of G418, cell lines stably expressing the replicon were established. We have previously reported that firefly luciferase activities of Feo replicon-expressing cells correlated well with HCV NS3, NS4A and NS5A protein expression levels and with replicon RNA expression levels [27].

Treatment with IFN and nelfinavir

Recombinant human IFN-α-2b (Schering-Plough, Kenilworth, NJ, USA) and purified nelfinavir (Japan Tobacco Inc., Tokyo, Japan) were used. IFN and nelfinavir treatment schedules were as described in the results.

Luciferase assays

Luciferase activity was quantified using a luminometer (Lumat LB9501; Promega) and the Bright-Glo Luciferase Assay System (Promega). Typically, 5×10^3 cells/well, plated onto 24-well plates and cultured for 48 h, were lysed with 100 μL 1× Glo luciferase Buffer (Promega), and the luciferase activity in 100 μL of the lysate was measured by adding an equal volume of Bright-Glo Luciferase Assay Reagent (Promega). Assays were performed in triplicate, and the results were expressed as mean ± SD relative light units.

Western blot analysis

Cells were lysed in buffer containing 62.5 μM Tris–HCl (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, 6% 2-mercaptoethanol and 0.01% bromophenol blue. Equal amounts of protein (10 μg) were subjected to electrophoresis on sodium dodecyl sulfate-polyacrylamide gels (Invitrogen, Carlsbad, CA, USA), followed by transfer to a polyvinylidene difluoride membrane (Roche, Basle, Switzerland) and sequential probing with a monoclonal anti-NS5A antibody (Virogen, Watertown, MA, USA) and β-actin antibody (Thermo Fisher Scientific, Fremont, CA, USA), respectively. The bands were visualized using an enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ, USA).

Cytotoxicity assay

Lactate dehydrogenase (LDH) tests and 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl)tetrazolium inner salt (MTS) reduction assays were performed to investigate cytotoxicity and cell viability. LDH levels were measured in the supernatants using the LDH-Cytotoxic Test (Wako Pure Chemical Industries, Osaka, Japan), according