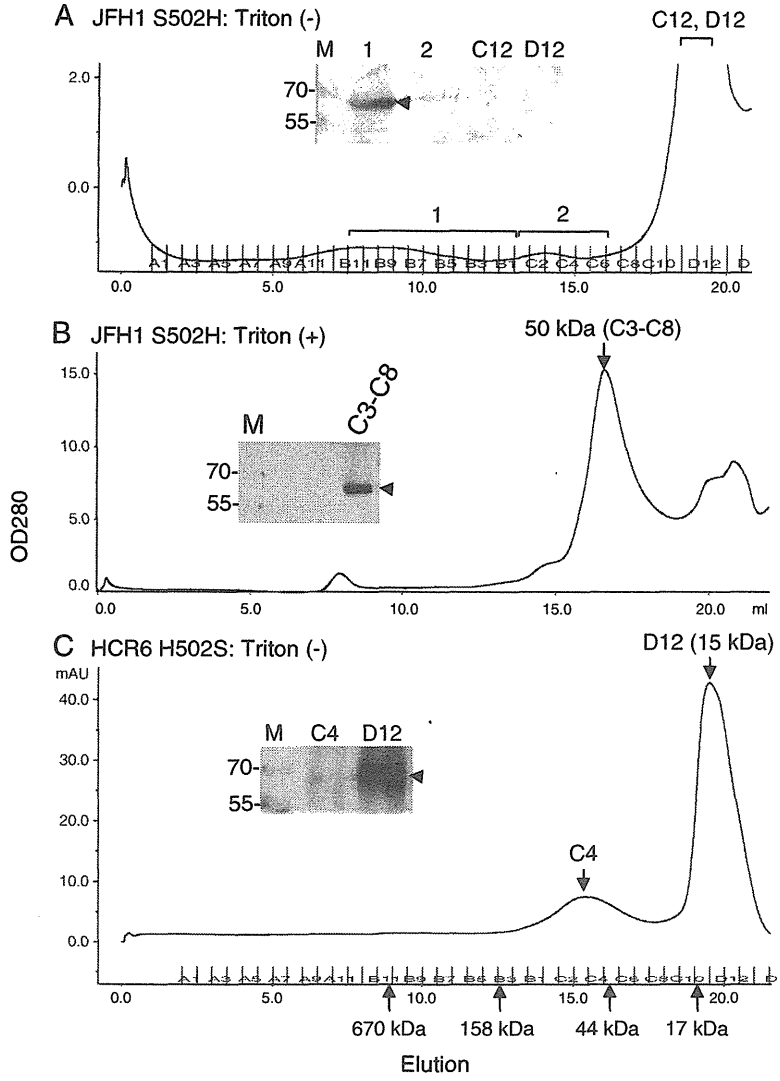


**Fig. 6.** Superdex 200 gel filtration of HCV JFH1 (2a) and HCR6 (1b) 502 mutant RNA polymerases. JFH1 (2a) S502H (A), and HCR6 (1b) H502S RdRps (C) were applied on Superdex 200 gel filtration columns in 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 3.5 mM $MnCl_2$, 1 mM DTT, and 0.2% glycerol. JFH1 (2a) RdRpS502H was also applied with 0.1% Triton X-100 (B). Inset in A: The fractions of the void volume—158 kDa (1), those of lower molecular weight fractions (2), fractions C12, and D12 of JFH1 (2a) RdRpS502H gel filtrations were precipitated with TCA and analyzed by western blot. Inset in B: Fraction C3–C8 of JFH1 (2a) RdRpS502H in Triton X-100 were precipitated with TCA and analyzed by western blot. Inset in C: Fractions C4 and D12 of HCR6 (1b) RdRpH502S were precipitated with TCA and analyzed by western blot. The position of HCV RdRp is indicated by an arrowhead. The position of the pre-stained size marker is indicated on the left side of the blots.

is important for the intermolecular interaction of HCV 1b RdRp (Qin et al., 2002). HCV RdRps without the C-terminal hydrophobic domain were soluble in high-salt buffer (>300 mM NaCl; Fig. S1) (Ferrari et al., 1999). The shift to the delayed elution of gel-filtration of HCR6 (1b) RdRpwt and JFH1 (2a) RdRp S502H with Triton X-100, and HCR6 (1b) RdRpH502S may come from the interaction of the RdRps with Superdex200 gel matrix induced by the mutations and Triton X-100.

Our data of HCV RdRp oligomerization at 502H (Fig. 6) are in agreement with those by Qin et al. (Qin et al., 2002), but are contradictory to those obtained by more sensitive methods (fluorescence resonance energy transfer [FRET] and yeast two-hybrid system) (Wang et al., 2002; Clemente-Casares et al., 2011). Interactions between a charged amino acid (His) and an aromatic residue (Trp) (Fernandez-Recio et al., 1997; Matthews et al., 1997; Takeuchi et al., 2003), or His–Glu interactions (Marti and Bosshard, 2003), are often found in proteins. JFH1 (2a) RdRpwt did not form dimers (Chinnaswamy et al., 2010). 502H may interact with 125 W in αF (Clemente-Casares et al., 2011), but not with 18E (Qin et al., 2002). This interaction is dissociated both with high-salt (Fig. S1) and with Triton X-100 (Figs. 5 and 6). Taken together, 502H of HCV 1b RdRp is important for oligomer formation in transcription (physiological salt) conditions. Besides the oligomerization using 502H, the αF and αT helixes of HCV RdRp, which were proposed to be involved in oligomerization (Clemente-Casares et al., 2011), may also be involved in oligomerization of the molecules in transcription condition. The 502 mutations in HCR6 (1b) and JFH1 (2a) RdRps are likely to affect the structure of the template channel by affecting the helix structures of the thumb domain (Bressanelli et al., 2002; Chinnaswamy et al., 2008) because the polymerase and RNA template binding activity of these mutant RdRps was not activated by Triton X-100 (Figs. 3 and 4). These findings indicate the importance around amino acid 502H for HCV 1b RdRp structure. However, these data contradict to the previous reports (Qin et al., 2002; Clemente-Casares et al., 2011).

Comparing the polymerase and template RNA-binding activity of JFH1 (2a) and HCR6 (1b) RdRps with and without Triton X-100,

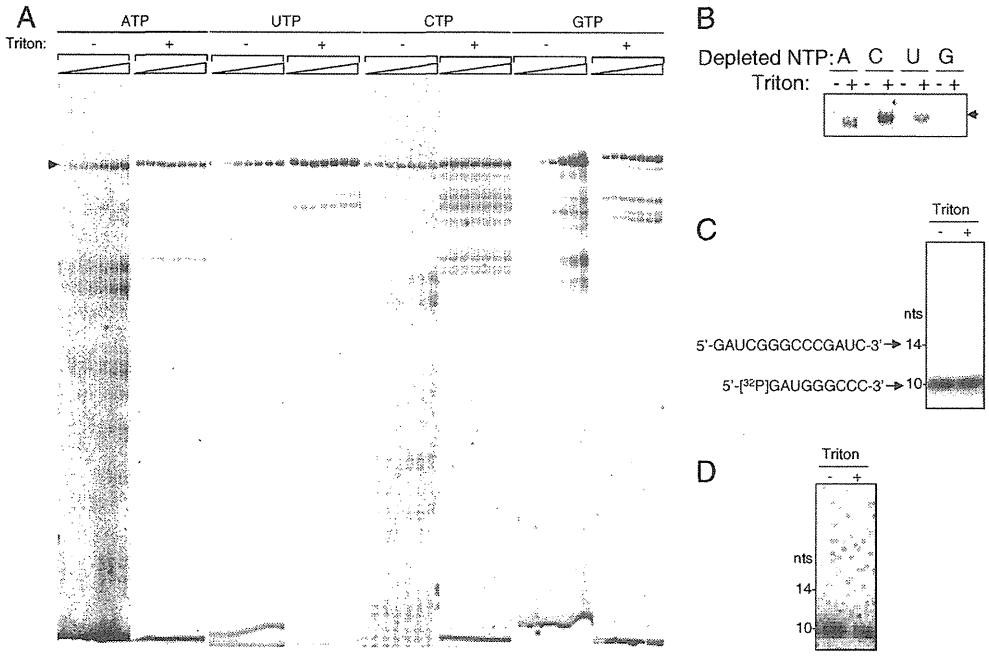


**Fig. 7.** Effect of substrate concentrations on in vitro transcription of HCR6 (1b) wild-type RNA polymerase, and TNTase activity in the presence of Triton X-100. A: Effect of nucleotide concentration on HCV HCR6 (1b) RdRpwt in vitro transcription with (+) and without (−) 0.02% Triton X-100. The concentration of ATP, UTP, and CTP varied from 1 to 50 μM, and that of GTP varied from 5 to 500 μM. B: Effect of nucleotide depletion on HCV HCR6 (1b) RdRpwt in vitro transcription with (+) and without (−) 0.02% Triton X-100. The position of 184-nt products is indicated by an arrowhead (A and B). C: TNTase activity of HCV HCR6 (1b) RdRpwt. 5′-[$^{32}$P]sym/sub was transcribed by HCV HCR6 (1b) RdRpwt with (+) and without (−) 0.02% Triton X-100. D: TNTase activity of HCV HCR6 (1b) RdRpwt. sym/sub was transcribed with [$^{32}$P]UTP by HCV HCR6 (1b) RdRpwt with (+) and without (−) 0.02% Triton X-100. The position of 10-nt sym/sub and 14-nt is indicated on the left.

RdRp which formed oligomer using 502H did not show high polymerase activity (Figs. 3–6). The inactive oligomer may be a part of the reason why a small fraction, less than 1%, of the purified HCV BK RdRp which belonged to 1b participated productively in transcription in vitro (Carroll et al., 2000). Taking together the data obtained by FRET (Clemente-Casares et al., 2011) and yeast two-hybrid systems (Wang et al., 2002), dynamic intermolecular interactions may occur under transcription conditions through the αT helix where amino acid 502 is located. The reason why only 1b RdRp was activated with Triton X-100 although RNA binding of all the RdRps tested was enhanced with Triton X-100, is not clear.

In case of JFH1 (2a) RdRp, the interaction with Triton X-100 may be different from that of HCR6 (1b) RdRp because it was not activated with Triton X-100 (Figs. 1 and 3), and because its gel-filtration profile was not affected with Triton X-100 (Fig. 5). This may be the reason of the inhibition of polymerase activity of JFH1 (2a) RdRpS502H by Triton X-100 although it was also disrupted to monomer (Figs. 3 and 6).

Triton X-100 activated only HCV 1b RdRp (Figs. 3 and 4). The closed conformation of HCV RdRp is required for de novo initiation (Chinnaswamy et al., 2008). With and without Triton X-100, JFH1 (2a) RdRpwt showed as high polymerase activity as HCR6 (1b) RdRpwt did with Triton X-100 (Fig. 3B). The very closed conformation of JFH1 (2a) RdRp is proposed to facilitate de novo initiation and high polymerase activity (Simister et al., 2009). Triton X-100 may also help the conformational change of HCR6 (1b) RdRp to the very closed conformation like that of JFH1 (2a) RdRp during transcription initiation.

HCV RdRp was co-purified with nucleic acids (Figs. S1 and S2). The contaminating nucleic acids were removed from HCV RdRp by high salt treatment. The contaminating nucleic acids carry proteins that have affinity to them, which misleads HCV in vitro transcription data. They also oligomerize HCV RdRp by crosslinking them. In a similar way, the contaminating nucleic acids in HCV RdRp preparations may mislead the binding data of HCV RdRp with other proteins.

From the activation kinetics of the detergents (Fig. 1, Table 1), the polymerase activation of 1b RdRp is likely to depend on the micelle formation of the detergent and on the direct interaction between RdRp and the detergents. The reason why the non-ionic detergent nOG did not activate the HCV RdRp is not known (Figs. 1 and 2).

The interaction mechanism of Triton X-100 and HCV 1b RdRp may be similar as that of sphingomyelin and HCV 1b RdRp because their activation kinetics were similar and the activated genotype was the same (Weng et al., 2010). Sphingomyelin activated only HCV 1b, but did not activate 1a or 2a RdRps. Both the activation curve of sphingomyelin and that of Triton X-100 showed the linear increase of polymerase activity. Then, sphingomyelin reached plateau at 20 molecules, and Triton X-100 reached plateau around its CMC.

Data about TNTase activity of HCV RdRp are controversial (Behrens et al., 1996; Ranjith-Kumar et al., 2001, 2004; Vo et al., 2004). In our system, TNTase activity was not detected with or without Triton X-100 (Figs. 7C and D).

GTP binds to HCV RdRp both as substrate and as a component of RdRp (Bressanelli et al., 2002). The apparent *Km* for GTP with Triton X-100 indicated that the substrate affinity dropped as low as to lose fidelity (Table 1, Figs. 7A and B). Triton X-100 may have affected the substrate-binding although its mechanism is not clear. HCV 1b full-length RdRp transcription activity obtained with CHAPS (Wang et al., 2002) might be that without fidelity as shown with Triton X-100. Detergents should not be used while screening substrate inhibitors of HCV RdRp. These data indicate that caution should be exercised while using detergents in anti-HCV RdRp drug screening tests.

Supplementary materials related to this article can be found online at doi:10.1016/j.gene.2012.01.044.

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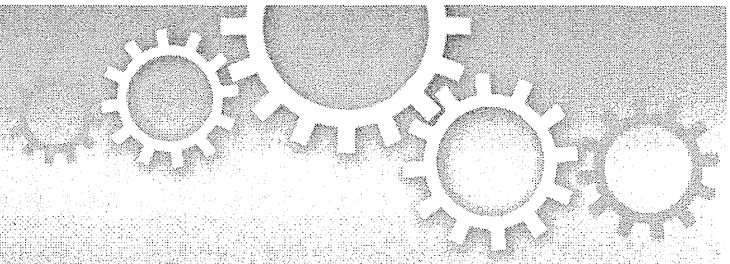

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# An orally available, small-molecule interferon inhibits viral replication

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**Most acute hepatitis C virus (HCV) infections become chronic and some progress to liver cirrhosis or hepatocellular carcinoma. Standard therapy involves an interferon (IFN)-α-based regimen, and efficacy of therapy has been significantly improved by the development of protease inhibitors. However, several issues remain concerning the injectable form and the side effects of IFN. Here, we report an orally available, small-molecule type I IFN receptor agonist that directly transduces the IFN signal cascade and stimulates antiviral gene expression. Like type I IFN, the small-molecule compound induces IFN-stimulated gene (ISG) expression for antiviral activity *in vitro* and *in vivo* in mice, and the ISG induction mechanism is attributed to a direct interaction between the compound and IFN-α receptor 2, a key molecule of IFN-signaling on the cell surface. Our study highlights the importance of an orally active IFN-like agent, both as a therapy for antiviral infections and as a potential IFN substitute.**

Hepatitis C virus (HCV) infection affects 170 million people worldwide[1], and most acute HCV infections become chronic, with some progression to liver cirrhosis or hepatocellular carcinoma. HCV has a plus-strand RNA genome that encodes both structural proteins and the nonstructural (NS) proteins 2, 3, 4A, 4B, 5A and 5B. Current standard therapy against chronic HCV infection includes the use of host factor-targeting pegylated interferon (PEG-IFN)-α and ribavirin[2], which is effective in only 50% of patients chronically infected with HCV genotype 1[3]. The main causes of this low rate of efficacy may be (i) single-nucleotide polymorphisms (SNPs) in the upstream region of the *IL28B* gene and (ii) low compliance with the therapy, which must be administered subcutaneously. Regarding the first cause—SNPs—the host factors that are important in the early response to therapy remain unknown. However, recent studies report that genetic variants near *IL28B*, which encodes IFN-λ3 (interleukin 28B), correlate with the response to treatment of chronic hepatitis C infection using IFN-α/ribavirin combination therapy[4–7]. Patients with an rs12979860 SNP genotype of CC are reported to have a stronger response to IFN therapy (up to an 80% sustained virological response (SVR) rate with the combined therapy) than those with TC or TT genotypes[4]. Regarding the lack of compliance, the current therapy using recombinant IFN is a weekly injectable formulation that is unstable, requires refrigeration, and is expensive and complex to administer. Furthermore, therapy is often poorly tolerated as a result of the presence of many adverse effects, including flu-like symptoms, hematological abnormalities and adverse neuropsychiatric events, any of which may require early discontinuation[8]. These side effects may result in dose modifications that lead to less-than-optimal responses.

Recent trends in drug development focus on drugs targeted against viral proteins such as NS 3/4A serine protease, RNA helicase, NS4B, NS5A, and NS5B RNA-dependent RNA polymerase[9]. Very recently, two NS3/4A protease inhibitors, telaprevir and boceprevir, have been approved as new anti-HCV agents. Adding such an inhibitor to the standard therapy in the ADVANCE[10] and the SPRINT-2[11] trials achieved significantly higher SVR rates, but the issue still remains that using these inhibitors without injectable IFN possibly yields clinical resistance[12]. To overcome this problem and alleviate the low compliance outlined above, an orally available IFN would be valuable because the dosing regimen is less complex.

IFNs induce the expression of a subset of IFN-stimulated genes (ISG)[13], some of which show antiviral activity or are involved in lipid metabolism, apoptosis, protein degradation and inflammation[14]. IFNs are not only effective

against HCV infection, but are also essential for innate immunity. Broadly speaking, type I IFNs (IFN-α and -β) bind to their receptor, causing the phosphorylation and activation of JAK1 and Tyk2, which is followed by the phosphorylation of signal transducers and activators of transcription (STATs) and subsequent ISG expression. To activate the JAK/STAT pathway, IFN-α requires the IFN-α/β receptor, which consists of 2 subunits, IFN-α receptor (IFNAR) 1 and IFNAR2. These IFNAR subunits together form a heterodimer upon IFN stimulation. This association of IFNAR2 and IFNAR1 is required to mediate the antiviral, antiproliferative, and apoptotic effects of type I IFNs[15–17] because the dimerization of IFNARs induces the phosphorylation of the receptor-associated tyrosine kinases, JAK1 and Tyk2, and the phosphorylated JAK kinases then phosphorylate STAT1 and STAT2. In turn, phosphorylated STAT1 and STAT2 bind to IRF9 to form the transcriptional activator IFN-stimulating gene factor 3 (ISGF3) that induces the expression of a subset of ISGs[13].

Using quantitative high-throughput screening (HTS), we identified in this study a novel small molecule that acts like IFN by directly interacting with the type I IFN receptor to drive ISG expression. Our results indicate that oral administration of the small-molecule IFN agonist stimulates ISG expression in mice, and that the ISG expression from this small-molecule IFN provides antiviral activity, indicating that the compound may be a potential therapeutic IFN substitute.

## Results

**Identification of antiviral small-molecule IFN agonists by high-throughput chemical library screening.** HCV replicon cells, which were established ten years ago[18] using a cell line that expresses the HCV genotype 1b subgenomic replicon[19], possess a luciferase gene as a reporter optimized for use in a robust HTS system. The HTS system provides in-depth analysis of primary screening results, including detailed information regarding potency, efficacy and structure-activity relationships. IFNs show strong inhibition using this system and have been used as a positive control in the assay. Many compounds, including HCV protease inhibitors and HCV polymerase nucleoside/non-nucleoside analogs, have been assessed and are being developed for clinical testing. Analysis of data from the combination of target/counterscreen HTS, data from other assays measuring cellular toxicity, *in vitro* sphingolipid biosynthesis and HCV enzymatic activity (including protease and polymerase) allowed us to select compounds with potentially novel modes of action from the primary screen.

A secondary IFN signal assay, using a luciferase reporter gene which was located downstream of the IFN-stimulated response element (ISRE), eliminated assay-related false-positive compounds. Of the remaining anti-HCV replicon compounds, one of the most active was an imidazonaphthyridine with the structural formula 8-(1, 3, 4-oxadiazol-2-yl)-2, 4-bis (trifluoromethyl) imidazo [1, 2-a] [1, 8] naphthyridine (RO4948191, hereinafter RO8191) (Fig. 1a). This compound strongly suppressed HCV replicon activity at 72 h in a dose-dependent manner (Fig. 1b, left graph) without inducing host cell toxicity, as measured by the WST-8 (Fig. 1b, right graph) and CellTiter-Glo assays (data not shown). The $IC_{50}$ (50% inhibitory concentration) of the compound in an anti-HCV replicon assay was 200 nM. The compound suppressed viral replication within 24 h and showed even more effective inhibition, without cytotoxicity, after 7 days (Supplementary Fig. 1). In addition, the HCV RNA replicon levels significantly decreased after incubation with the compound for 72 h, as determined by real-time reverse transcription (RT)-polymerase chain reaction (PCR) analysis (Fig. 1c). Immunostaining showed that levels of the proteins HCV NS3 and NS4A, which are localized mainly in the perinuclear region of the replicon cells, were also reduced after RO8191 treatment for 24 h (Fig. 1d). This treatment also resulted in the disappearance of viral proteins such as NS3, NS4A/B, and NS5A/B, as shown by western blot analysis (Fig. 1e). The luciferase activity of HCV subgenomic genotype 2 replicon cells (JFH1, data not shown) and, surprisingly, the HCV viral titer of JFH1[20] in a Huh-7/K4 cell line were also reduced by RO8191 treatment (Fig. 1f).

**RO8191 induces IFN signals, ISGs expression and JAK/STAT phosphorylation.** To clarify whether RO8191 shows inhibitory activity against another RNA virus, we tested its action in encephalomyocarditis virus (EMCV)-infected A549 cells. RO8191 showed a cell-protective activity against EMCV infection similar to that of IFN-α (Fig. 2a). Because IFN-α is the most common host cell factor to exert its antiviral activity against HCV[21,22] by inducing ISG expression[13], we compared the gene expression profiles of IFN-α and RO8191 by conducting a global-scale DNA microarray analysis to identify genes, especially ISGs[23], that were regulated by RO8191. As expected, RO8191 increased the expression of some ISGs (Supplementary Fig. 2 and Supplementary Table 1). DNA microarray analysis showed that RO8191 induced the expression of IP-10 (CXCL10), known as an ISG, RO8191 did not induce the genes encoding inflammatory cytokines and chemokines (Supplementary Fig. 3a). And, a reporter gene assay was performed using an NF-κB reporter gene. We transiently transfected the reporter gene to HCV-naïve HuH-7 cells, and then treated them with RO8191 or TNF-α. In Huh-7 cells, NF-κB reporter gene was activated by TNF-α treatment, but not by RO8191 (Supplementary Fig. 3b). These results indicate that RO8191 specifically induces the IFN-relevant gene expressions.

Real-time RT-PCR analysis also revealed that RO8191 induced many ISGs similar to those expressed in IFN-α-treated cells (Fig. 2b and Supplementary Table 2), suggesting that the antiviral mechanism of RO8191 depends on ISG expression. In addition to HCV replicon cells, we tested the compound in several cancer cell lines and normal human primary hepatocytes (Hc cells). Real-time RT-PCR analysis showed that RO8191 induced ISG expression in cultured cell lines and human primary hepatocytes (Supplementary Table 3 and Supplementary Fig. 4). These results suggest that RO8191 induces an IFN signal, and that the application in clinical of RO8191 is not limited to suppressing HCV infection.

As mentioned earlier, type I IFNs phosphorylate JAK kinases and STAT proteins by inducing a heterodimerization of both IFNAR1 and IFNAR2, and the complexes thus formed transduce signals from IFN. Since RO8191 induces ISGs in a similar profile to IFN-α, we examined the phosphorylation of IFN signaling molecules. Immunoblotting analysis was performed to detect phosphorylated tyrosine (Tyr) and serine (Ser) residues of the STATs and JAK kinases using cell lysate that was treated with 50~5000 times the HCV replicon $IC_{50}$s of RO8191, IFN-α, IFN-β, and IFN-γ (type II IFN). The degree to which both RO8191 and IFN-β phosphorylated STAT1, STAT5, and STAT6 was similar, as shown in Fig. 2c, and the phosphorylation level of STAT2 by RO8191 was quite similar to that of IFN-α. Interestingly, STAT3 and JAK1 were more strongly phosphorylated by RO8191 than by IFN-α, -β, or -γ. On the other hand, Tyk2 was phosphorylated by type I IFNs, but not by IFN-γ or RO8191, indicating that Tyk2 is dispensable for RO8191 activity (Fig. 2c). Taken together, the phosphorylation profile of STAT proteins by RO8191 is generally similar to that of type I IFNs, and the phosphorylation profiles of STAT1–3, 5, 6, and JAK1 in HCV replicon cells treated with RO8191 or type I IFNs suggest a common mechanism that differs from the mechanism in cells treated with type II IFN.

In addition to imiquimod, an IFN inducer and a Toll-like receptor (TLR) 7 agonist[24], small-molecule ligands recognized by TLRs and RIG-I–like receptors are known to induce ISG expression by inducing IFN[25]. The chemical structure of RO8191 is similar to imiquimod so we examined whether RO8191 has a mechanism of activity like imiquimod. However, imiquimod did not affect HCV replicon cells (Supplementary Fig. 5a) and, moreover, did not stimulate

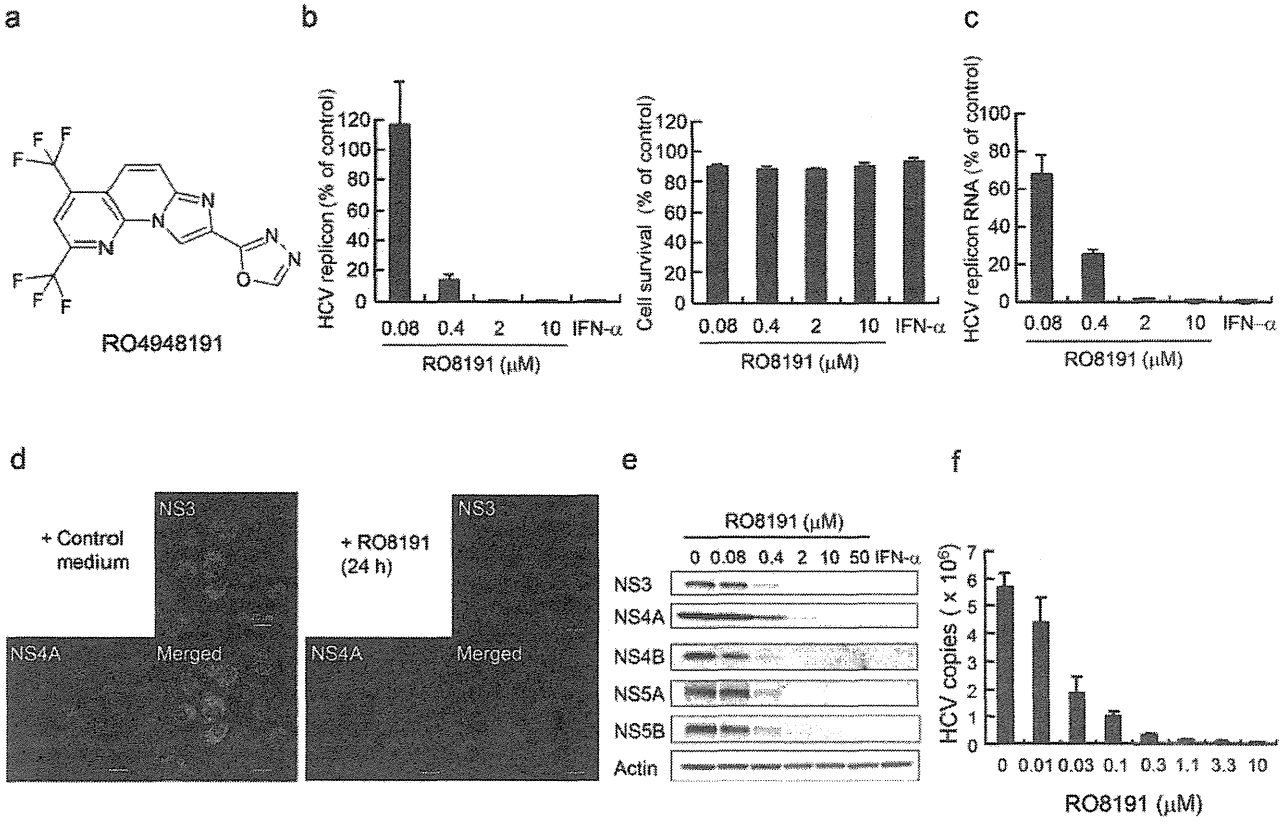


**Figure 1 | Identification of a small molecule that inhibits HCV replication.** (a) The chemical structure of RO8191. (b) After treatment with various concentrations of RO8191 or 100 IU/mL IFN-α for 72 h, HCV replication levels were examined using a luciferase assay (left graph), and cell viabilities were determined using a WST-8 assay (right graph). The mean values and their SDs were recorded for treated cells as a percentage of the values for untreated cells, and the values represent the means of 3 independent experiments. (c) Total RNA was extracted from HCV replicon cells cultured with the indicated concentration of RO8191 or 100 IU/mL IFN-α for 72 h; HCV RNA levels were analyzed using real-time RT-PCR. The mean values and their SDs were recorded for treated cells relative to the mRNA levels of β-actin, and are shown as a percentage of untreated cells. The values represent the means of 3 independent experiments. (d) HCV replicon cells were treated with control medium (left panels) or 10 μM RO8191 (right panels) for 24 h and immunostained with Hoechst 33452 (blue), anti-NS3 antibody (green), and anti-NS4A antibody (red). The results were then merged (yellow). (e) HCV replicon cells were treated with the indicated concentrations of RO8191 or 100 IU/mL IFN-α for 72 h. Whole cell lysates were immunoblotted with antibodies specific to the indicated HCV NS proteins. (f) After infection with the HCV JFH1 strain, Huh-7/K4 cells were treated with the indicated concentrations of RO8191 for 72 h. Total RNA was extracted, and the HCV RNA levels were analyzed using quantitative real-time RT-PCR.

STAT1 phosphorylation, whereas RO8191 caused prolonged STAT1 activation (for up to 8 h post-treatment; Supplementary Fig. 5b). Next, RO8191 is actually a small-molecule and we could not exclude the possibility that the antiviral activity might be induced through TLRs. To confirm that, we tested ISG induction using Tlr3, 4, 7 and 9 knockout (KO) mouse embryo fibroblasts (MEF)[24,26–28]. We treated the MEFs with RO8191 or murine IFN-αA for 8 h and both induced Oas1b mRNA in wild type and Tlr-KO MEFs (Supplementary Fig. 5c), demonstrating that RO8191 induces ISG expressions independently of TLRs. To exclude the possibility that RO8191 acts by inducing type I IFN, we examined its effects in Vero cells that lack the IFN gene locus[29,30]. Whereas imiquimod did not show IFN-like effects in Vero cells, RO8191 and IFN-α induced the ISRE activation (Supplementary Fig. 5d), indicating that RO8191 acts independently of the inducing IFN and is quite distinct from imiquimod.

**RO8191 exerts antiviral activity dependent on IFNAR2/JAK1, but is independent of IFNAR1/Tyk2.** IFNs require IFN receptor subunits for their activity, and we hypothesized that RO8191 uses the IFN receptor to exert anti-HCV activity. To determine the contributions of IFNAR1 and IFNAR2 toward the anti-HCV replicon activity of RO8191, we suppressed the expression of these receptors using specific siRNA and treated the cells with RO8191 or IFN-α. The knockdown efficiency was determined using RT-PCR in the HCV replicon cells transfected with each siRNA, as shown in Supplementary Fig. 6. As expected, a knockdown of each receptor subunit decreased the antiviral activity of IFN-α (Fig. 3a and b, Supplementary Fig. 7a and b). IFNAR2 knockdown attenuated the antiviral activity of RO8191 (Fig. 3b and Supplementary Fig. 7b) but, interestingly, IFNAR1 knockdown did not change the antiviral activity (Fig. 3a and Supplementary Fig. 7a), suggesting that RO8191 is independent of IFNAR1. To address whether IFN-α receptor contributes to RO8191 signaling, we evaluated RO8191 using Ifnar1-KO MEF cells[31]. We treated Ifnar1-KO MEFs with 50 μM RO8191 or 1,000 IU/mL of murine IFN-αA for 8 h and analyzed the expression of murine *Oas1b* mRNA using real-time RT-PCR. Murine IFN induced murine *Oas1b* mRNA only in wild type MEFs, not in Ifnar1-KO MEFs, although RO8191 induced *Oas1b* in both wild type and Ifnar1-KO MEFs (Supplementary Fig. 8). Therefore, RO8191 induces IFN-like activity even in Ifnar1-KO MEFs.

Since IFNAR2 knockdown reduced RO8191 activity, we focused on and analyzed the IFNAR2 function using RO8191. First, U5A cells, which do not respond to IFN-α because of the lack of

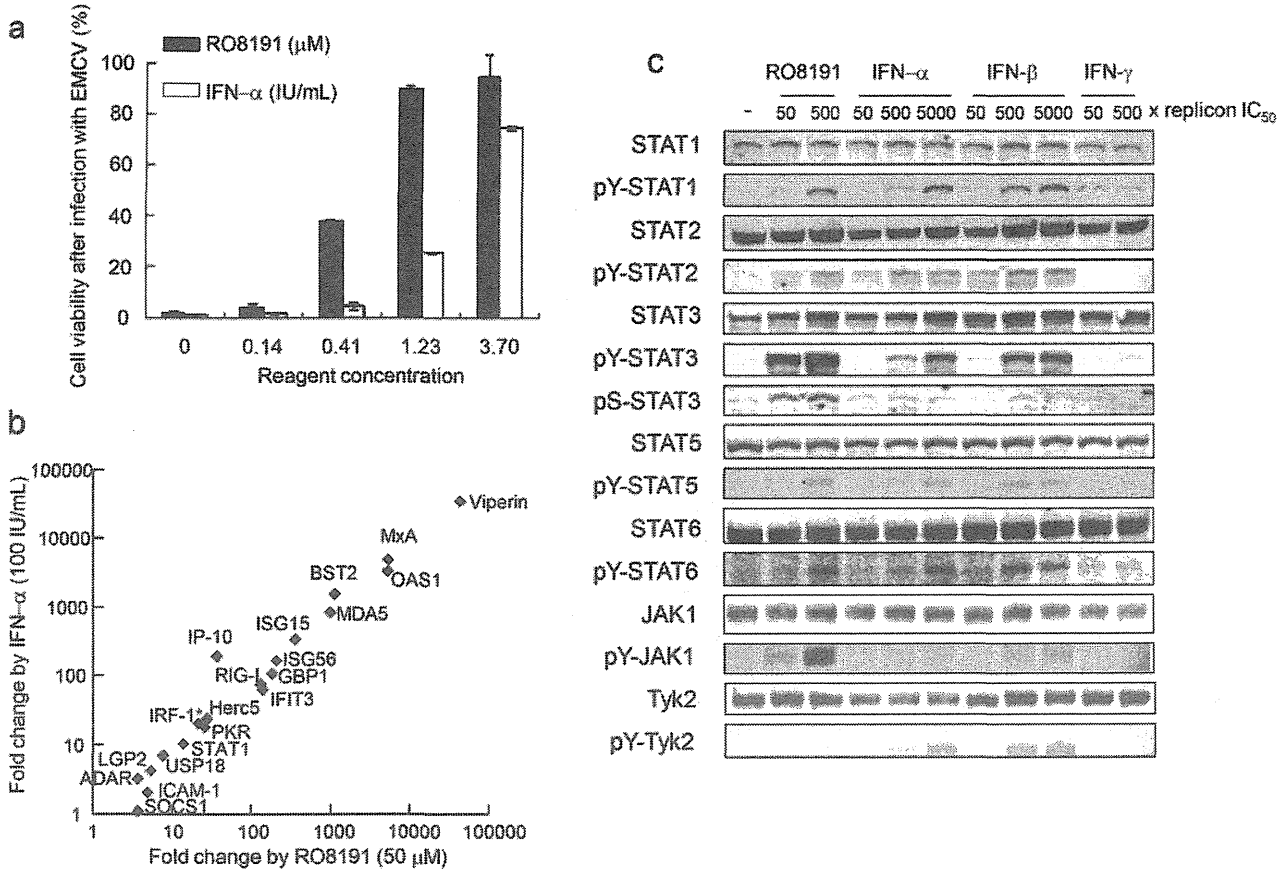


**Figure 2 | RO8191 activates JAK/STAT and induces IFN-like signals.** (a) The cytopathic effect of EMCV infection was inhibited by the indicated concentrations of RO8191 or IFN-α. Viable cells were stained with crystal violet. The data shown are the mean values and SDs based on experiments performed in quadruplicate. (b) ISG expression levels were measured using real-time RT-PCR. Total RNA was extracted from HCV replicon cells cultured in the presence of 50 μM RO8191 or 100 IU/mL IFN-α for 2 or 8 h and known ISGs were analyzed. Asterisk indicates 2-h treatment with agents. The values shown are the mean fold change induction compared to the mRNA level of human *β-actin* and the fold change induction compared to untreated cells. (c) HCV replicon cells were treated with various concentrations of the indicated agents for 15 min. Total lysates were immunoblotted with antibodies to STATs or JAK kinases. The HCV replicon $IC_{50}$ of IFN-α was 0.4 IU/mL, that of IFN-β was 3 IU/mL, and that of IFN-γ was 0.3 ng/mL.

IFNAR2 expression[32], were treated with RO8191. Although RO8191 and IFN-α did not induce *OAS1* expression in the U5A cells, IFNAR2-overexpressing U5A cells were successfully complemented, and this conferred susceptibility to both RO8191 and IFN-α (Fig. 3c and d). RO8191 also induced the *OAS1* gene in HT1080 and 2fTGH cells, the parental cell lines of U5A cells (Supplementary Table 3, lowest and second lowest rows). Second, to elucidate whether RO8191 directly interacts with IFNAR2, we obtained a recombinant IFNAR2 extracellular domain (ECD) protein (N-terminal half of the protein, amino acids from Ile27- Lys243). The protein was subjected to surface plasmon resonance (SPR) spectroscopy using a Biacore system to directly evaluate the binding activity of the recombinant protein with its possible ligands, RO8191 and IFN-α. The IFNAR2 ECD protein was fixed on the surface of the CM7 sensor chip by amine coupling, and PEG-IFN-α2a and RO8191 were injected as analytes. We comparatively analyzed 0.31 and 0.63 μM of RO8191, and both concentrations showed similar binding affinities of 480.5 and 484.5 nM, respectively (Fig. 3e), whereas a chemically derivatized compound of RO8191 that cannot inhibit HCV replication did not bind to IFNAR2 ECD (data not shown). The SPR results were consistent with 1:1 binding and showed an interaction between RO8191 and the IFNAR2 ECD protein in a dose-dependent manner, indicating that the compound and IFNAR2 may directly interact on the cell surface. In addition to the anti-replicon activity, the phosphorylation of STAT1 by RO8191 was also repressed by IFNAR2 knockdown (Fig. 3g), but the knockdown of IFNAR1 did not inhibit the STAT1 phosphorylation (Fig. 3f), indicating that RO8191 requires IFNAR2 but not IFNAR1 to achieve activity. Similarly, the knockdown of JAK1 attenuated the activity of RO8191 and IFN-α (Fig. 4a and Supplementary Fig. 9a), while, in contrast, Tyk2 and JAK2 were not required for RO8191 activity (Fig. 4b, Supplementary Fig. 9b and c). The phosphorylation of STAT1 by RO8191 was also inhibited by a knockdown of JAK1 (Supplementary Fig. 10a), but not by a knockdown of Tyk2 (Supplementary Fig. 10b), indicating JAK1 essentiality for the antiviral activity of RO8191. To confirm Tyk2-independency, we used U1A (Tyk2-deficient) cells and the parental cell, 2fTGH[33], and treated them with 50 μM RO8191 or 100 IU/mL IFN-α for 8 h to analyze the expression of *OAS1*. As expected from previous reports[34], IFN-α induced the *OAS1* expression only in 2fTGH cells but not in U1A cells; however, RO8191 induced the expression in both 2fTGH and U1A cells significantly (Supplementary Fig. 11). Thus, RO8191 activates ISGs in a JAK1-dependent and Tyk2-independent manner; on the other hand, IFN-α depends on both factors. Next, we conducted a knockdown of the transcription factors related to type I IFNs activity, STAT1, STAT2 and IRF-9, to clarify whether RO8191 required these factors for antiviral activity. As expected, STAT1-siRNAs partially blocked IFN-γ activity (Supplementary Fig. 9d),

Table 1 | ISG expression in the livers of RO8191 treated mice. ISG expression levels were measured using real-time RT-PCR. Values are listed relative to the mRNA levels of rodent *Gapdh* and represent the mean fold change induction compared to vehicle-administered mice. Twenty-four hours after oral administration of 30 mg/kg RO8191 or vehicle (including 10% dimethyl sulfoxide and 10% Cremophor) to mice, total RNA was extracted from the mouse livers, and the mRNA levels of murine ISGs were measured using real-time RT-PCR. The data shown are the means and SDs of 4 mice per group. The data were statistically analyzed using Student's *t*-test, and differences were considered significant at p values < 0.05

| Gene | Entrez ID | Fold change ± SD | | | p-value |
|---|---|---|---|---|---|
| murine Oas1 | NM_001083925 | 3.0 | ± | 0.72 | 0.003 |
| murine Mx1 | NM_010846 | 2.1 | ± | 0.15 | 0.0003 |
| murine Pkr | NM_011163 | 1.4 | ± | 0.21 | 0.009 |
| murine Cxcl10 | NM_021274 | 1.7 | ± | 0.63 | 0.097 |
| murine Ifit3 | NM_010501 | 2.5 | ± | 0.48 | 0.001 |
| murine Isg15 | NM_015783 | 2.3 | ± | 0.41 | 0.002 |
| murine Mda5 | NM_027835 | 1.6 | ± | 0.22 | 0.003 |
| murine Rig-i | NM_172689 | 2.1 | ± | 0.16 | 0.00003 |
| murine Socs1 | NM_009896 | 2.6 | ± | 1.04 | 0.057 |
| murine Stat1 | NM_009283 | 1.8 | ± | 0.21 | 0.001 |
| murine Usp18 | NM_011909 | 2.6 | ± | 0.69 | 0.017 |

which is mediated by STAT1 homodimers[35]. STAT1 was significantly phosphorylated by both RO8191 and IFN-α (Fig. 2c); however, the STAT1 knockdown affected neither RO8191 nor IFN-α activity (Fig. 4c and Supplementary Fig. 9d). We also analyzed the impact of STAT1 knockdown on *OAS1* induction by RO8191, and found *OAS1* induction by RO8191 was inhibited (Supplementary Fig. 12). STAT2 and IRF9 knockdown attenuated the inhibitory activity of both RO8191 and IFN-α (Fig. 4d and e and Supplementary Fig. 9e and f). In summary, RO8191 only binds to IFNAR2 and activates JAK1, STATs, and IRF9, thereby exhibiting type I IFN-like activity (Fig. 4f).

**RO8191, an IFNAR2 agonist, stimulates IFN signals in mice.** To evaluate whether RO8191 could be a clinical lead for drug development, we studied the effects of RO8191 on IFN signaling in mice. The compound or vehicle was orally administered to mice and, 24 h after treatment, the liver was removed and examined. The antiviral genes *Oas1b*, *Mx1*, and *Pkr* were significantly induced in the livers of mice treated with RO8191 (Table 1). As expected, gene homologs that were induced in the livers of HCV patients treated with PEG-IFN-α2b[36] were also induced in mouse liver (*Ifit3*, *Isg15*, *Mda5*, *Rig-I*, *Socs1* and *Stat1*; Table 1). In addition, genes that had previously been reported to be induced by IFN-β in mouse liver[37] were also induced in the livers of RO8191-treated mice (*Cxcl10*, *Ifit3*, *Isg15*, *Socs1* and *Usp18*; Table 1,). We also measured inflammatory cytokine and chemokine expressions, and RO8191 did not significantly induce the expression of these genes (Supplementary Table 4). To evaluate anti-HCV activity of RO8191 *in vivo*, RO8191 was orally administered to HCV-infected humanized liver mice[38]. The results of this humanized liver mice study showed that RO8191 reduced HCV titer *in vivo* (Supplementary Fig. 13). These data show that RO8191 stimulates IFN signaling and is an orally available agent in mice.

## Discussion

In this study, we identified a small-molecule IFN receptor agonist, RO8191, by quantitative HTS of a chemical library. This compound showed antiviral activity against both HCV and EMCV, suggesting a broad spectrum of target viruses. To learn more about the possible mechanism of action of IFN signal induction by RO8191, we investigated IFN-induced signaling and ISG induction by the small-molecule compound *in vitro* and *in vivo*. A comparison of microarray expression profiles in HCV replicon cells stimulated by IFN-α or RO8191 indicates that the IFN signal was induced not only by IFNs, but also by the small-molecule compound (Fig. 2b and Supplementary Fig. 2). Thus, this compound is an IFN-α-like small molecule, but the mechanism of the RO8191 antiviral activity remained unknown. Therefore, we examined the JAK/STAT activation pathway, which includes key players in the IFN signaling cascade.

Like type I IFNs, RO8191 significantly phosphorylates and activates STATs, in particular, STAT1 and STAT2 (Fig. 2c). Intriguingly, in HCV replicon cells, STAT1 expression knockdown did not affect the antiviral activity of RO8191 or IFN-α, although IFN-γ activity was inhibited (Fig. 4c). These data suggest that, in addition to inducing similar gene expression, RO8191 and IFN-α exhibit similar STAT phosphorylation profiles. Although RO8191- and IFN-α-mediated antiviral activity remained constant when STAT1 expression was reduced, this could be because IFN-α signaling in HuH-7 cells requires minimal amounts of STAT1 protein and STAT1 expression was not reduced below such a critical threshold by siRNA in our system. In contrast, the inhibitory activity of RO8191 was attenuated to the same extent as that of IFN-α when the expression of other components of ISGF3 (STAT2 and IRF-9) were reduced by siRNA (Fig. 4d and e). Incidentally, STAT1 siRNA did not attenuate RO8191 or IFN activity in EMCV-infected A549 cells (supplementary Fig.14), which supports the notion that STAT2 is an essential component of type I IFN signaling[39]. Type I IFN stimulates the formation of other STAT-containing complexes, including STAT1:STAT1, STAT3:STAT3 and STAT5:STAT5 homodimers, as well as STAT1:STAT3 and STAT2:STAT6 heterodimers[40–42]. Like IFN, RO8191 induced the phosphorylation of STAT1 and STAT2, which function as a gateway to the type I IFN signal cascade, and stimulated the phosphorylation of STAT3, 5 and 6. Another possible cause for the fact that STAT1 knockdown did not show any effect on RO8191 inhibition could be compensation by these IFN signaling-stimulated STAT complexes. This finding matches the recent report by Perry *et al.* on the STAT dependency of IFN activity against Dengue virus, that belongs to flavivirus[43]. They showed that STAT2 mediates IFN antiviral signals even in STAT1 KO cells and they discussed the possibility that other STAT family proteins would compensate for STAT1 deficiency. In summary, with regards to the activation of transcription factors and ISG expression, RO8191 and IFN-α mediate the same pathway.

IFNs activate JAK kinases via IFN receptors to induce STAT phosphorylation. RO8191 robustly phosphorylated JAK1 (Fig. 2c) in comparison with IFN-α or -β and therefore we focused on IFNAR2 (a JAK1-binding subunit of the type I IFN receptor). As with IFN-α, the activity of RO8191 was inhibited by IFNAR2 knockdown (Fig. 3b). The suggestion that IFNAR2 is an essential molecule for RO8191-induced signal transduction is supported by the fact that an IFNAR2-deficient cell line, U5A, did not respond to RO8191. Furthermore, after IFNAR2 expression had been complemented in the U5A cells, ISG induction by both RO8191 and IFN-α recovered (Fig. 3c and d). The mechanism was directly explained by SPR spectroscopy, which showed an interaction between RO8191 and the IFNAR2 ECD (Fig. 3e). RO8191 strikingly phosphorylates the IFNAR2-associated kinase JAK1, when compared to other IFN-treated cell lysates (Fig. 2c). JAK1 siRNA expression inhibited RO8191 activity (Fig. 4a), indicating that JAK1 is also an essential molecule for RO8191 activity. Interestingly, RO8191 activity remains static when IFNAR1 expression is knocked down, unlike IFN-α activity (Fig. 3a). The IFNAR1-binding-kinase Tyk2 is not required for RO8191 activity (Fig. 4b) and Tyk2 was not phosphorylated (Fig. 2c) by RO8191. Also, RO8191 induced its signal even in the Ifnar1 KO MEF and the Tyk2-deficient cell line (Supplementary Fig.

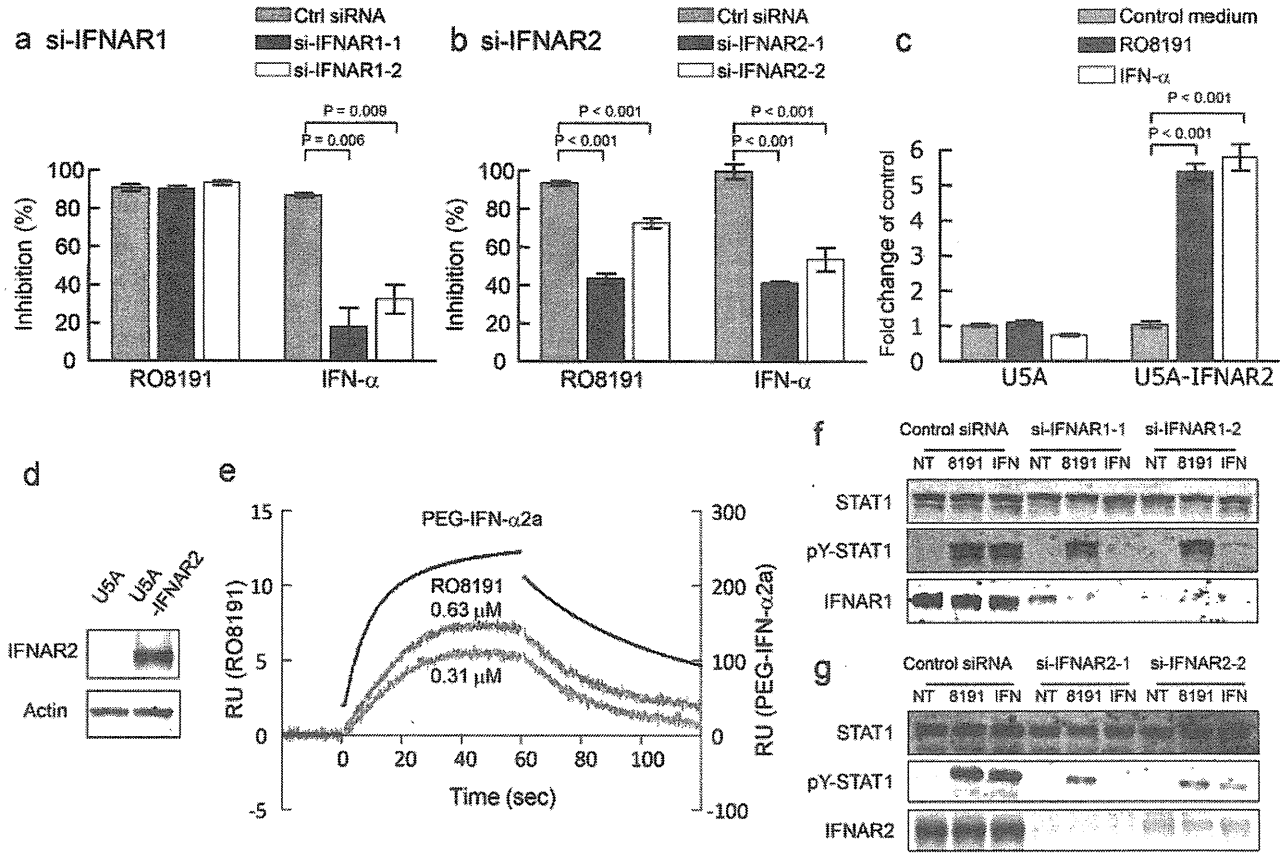


**Figure 3 | RO8191 requires and binds IFNAR2.** (a, b) The anti-HCV replicon activity of RO8191 was attenuated by knockdown of IFNAR2 (b), but not IFNAR1 (a). Inhibition of HCV replicon replication by each agent is shown (the mean and SD from 3 experiments). The HCV replicon cells were transfected with 50 nM of the indicated siRNAs (blue, red, and yellow bars). Forty-eight hours after transfection, the HCV replicon cells were treated with 1.5 μM RO8191 or 3 IU/mL IFN-α for 24 h. Twenty-four hours after treatment with each agent, the replication levels of HCV RNA were analyzed using a luciferase assay. (c, d) U5A cells that lack IFNAR2 were transfected with either an empty vector or a vector expressing the *IFNAR2* gene. (c) Forty-eight hours after transfection, the cells were treated with 50 μM RO8191 (red bars) or 100 IU/mL IFN-α (yellow bars). After an additional 8 h of incubation, total RNA was extracted from the U5A cells, and the *OAS1* mRNA level was measured using real-time RT-PCR. The values shown are relative to the mRNA level of human *β-actin*. (d) Forty-eight hours after transfection, the cells were lysed, and the whole cell lysates were immunoblotted with the indicated antibodies. (e) Real-time kinetic SPR analysis of the binding of RO8191 to the IFNAR2 ECD (red and blue lines). The results are consistent with 1:1 binding. PEG-IFN-α2a was also injected as a positive interacting control for IFNAR2 (black line, $K_D$: 30 nM). (f, g) The phosphorylation of STAT1 was attenuated by a knockdown of IFNAR2 (g) but not IFNAR1 (f). The HCV replicon cells were transfected with the indicated siRNAs (10 nM). Forty-eight hours after transfection, the cells were treated for 15 min with 10 μM RO8191 or 200 IU/mL IFN-α. The total lysates were subjected to western blot analysis to analyze the phosphorylated and total protein levels of STAT1. The data were statistically analyzed using Student's *t*-test.

8 and 11). IFN-α induces a signal via IFNAR1/Tyk2 and IFNAR2/JAK1 although RO8191 and IFN-α induce common ISGs (Fig. 2b and Supplementary Fig. 2), RO8191 activity was dependent only on IFNAR2.

We therefore propose a novel model of the induction of IFN-like signal transduction by this small molecule (Fig. 4f). So far, the IFNAR2 homodimer has been suggested to play various roles in IFN signal transduction[34,44,45], and RO8191 would induce the ISG expression via such IFNAR2 homodimer. For type I IFN, both IFNAR1 and IFNAR2 cooperate and induce phosphorylation of STATs via JAK1 and Tyk2. Conversely, for RO8191, IFNAR2 alone, as a homodimer, activates JAK1 phosphorylation and subsequent STATs activation. Experiments using siRNA and deficient cells have also shown that IFNAR1 and Tyk2 were not required to induce antiviral activity in the RO8191 compound pathway. These findings suggest a novel aspect of the IFN signaling pathway that may contribute to the understanding of other molecular signaling in IFN pathways.

RO8191 is a small molecule whose oral administration is feasible and effective in a murine model (Table 1 and Supplementary Fig. 13). In the chimeric mice study, the anti-HCV effect of RO8191 and PEG-IFN was similar in that they both showed strong activity at day 1 after treatment with subsequent weak suppression of HCV replication possibly due to the immunodeficiency of chimeric mice. Further development of RO8191 by using rational chemical modifications is therefore required to produce more potent molecules for testing as an antiviral molecule which will substitute current recombinant IFN. Although RO8191 has the potential to cause IFN-like adverse effects, further development of the small-molecule agonist offers the advantages of inexpensive production cost, convenient oral administration, dose-control to reduce some adverse effects, and potentially increased activity versus current recombinant IFNs.

Whereas oral NS3 protease inhibitors in monotherapy development yield resistant viruses[46,47], these protease inhibitors show a significantly high rate of SVR when combined with PEG-IFN[10,11] and a NS5B polymerase inhibitor also shows additive efficacy in combination with PEG-IFN[48]. In addition to the results of the *in vivo* study, we found that RO8191 induced ISGs at a level similar to IFN-α in human primary hepatocytes (Supplementary Fig. 4); we therefore expect that RO8191 will show IFN-like activity in clinical use. As an

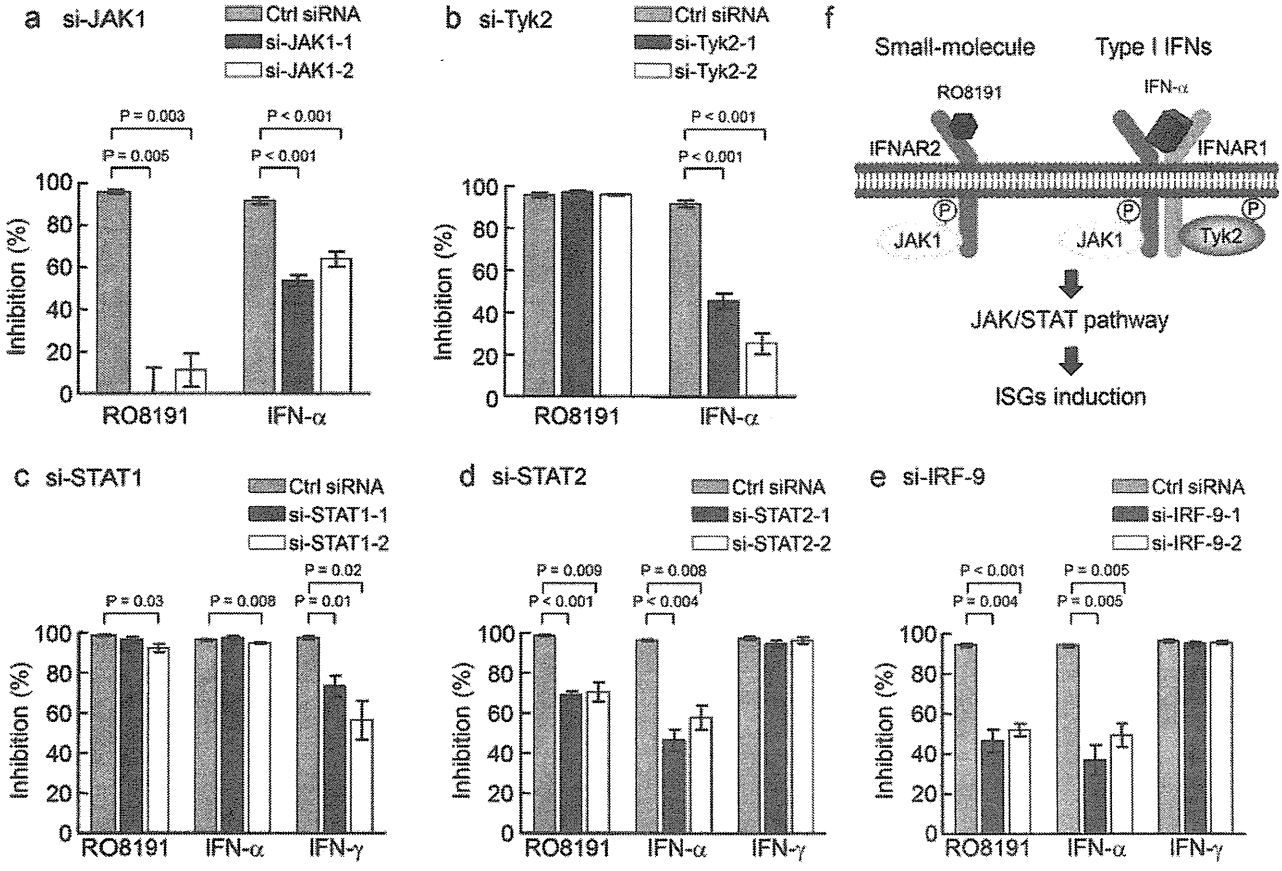


**Figure 4 | RO8191 activates a novel IFN-like pathway.** (a–e) The inhibition of HCV replicon replications by each agent is shown (mean and SD). The HCV replicon cells were transfected with 50 nM of the indicated siRNAs (blue, red, and yellow bars). (a, b) The anti-HCV replicon activity of RO8191 was reduced by knockdown of JAK1 (a) but not Tyk2 (b). Forty-eight hours after transfection, the HCV replicon cells were treated with 1.5 μM RO8191 or 3 IU/mL IFN-α. Twenty-four hours after treatment with each agent, the replication levels of HCV RNA were analyzed using a luciferase assay. (c–e) The anti-HCV replicon activity of RO8191 was reduced by knockdowns of STAT2 (d) and IRF9 (e), but not STAT1 (c). Forty-eight hours after transfection, the HCV replicon cells were treated with 1.5 μM RO8191, 3 IU/mL IFN-α, or 50 ng/mL IFN-γ. Twenty-four hours after treatment, the replication levels of HCV RNA were analyzed using a luciferase assay. (f) A schematic showing the pathways of RO8191 and IFN-α. The data were statistically analyzed using Student's *t*-test, and differences were considered significant at p values $< 0.05$.

alternative strategy to protease/polymerase inhibitors with PEG-IFN, the combined use of these direct-acting antiviral agents with RO8191 in a new oral regimen may help overcome some of the delivery problems associated with current IFNs. SVR rates of individuals infected with HCV genotype 1 have increased from 5-20% with IFN monotherapy and up to 50% with a combination of IFN and ribavirin. However, the refractory patients in this therapy constitute an unmet medical need. Thus, the development of a novel IFN receptor agonist, used alone or in combination with direct-acting antiviral drugs, will add a new milestone to the treatment of chronic hepatitis C. In addition to HCV infection, type I IFNs have been approved for the treatment of multiple clinical conditions, including hairy cell leukemia, malignant melanoma, AIDS-related Kaposi's sarcoma, multiple sclerosis, and chronic hepatitis B[49]. Thus, R08191 shows strong potential as a lead compound for IFN substitutes.

## Methods

**Cell culture, mice, and reagents.** The #Huh7/3-1 cell line, which expresses HCV replicons, was a kind gift from F. Hoffmann-La Roche. The cells were cultured in 0.5 mg/mL G418-containing Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum (FBS, HyClone). The replicon construct was derived from pFK-I377neo/NS3-3′/WT, as previously reported[19]. Hc cells (DS Pharma Biomedical) were cultured in CSC Complete Defined Serum-Free Medium (Cell Systems Corporation) supplemented with SF4ZR-500-D Rocket Fuel. Tlr KO MEFs were purchased from OrientalBioService, Inc. Ifnar1 KO MEF was kindly gifted by Prof. Takaoka. 2fTGH, and U1A and U5A cells were kindly gifted by Prof. Stark. Culture conditions for the other cell lines are shown in Supplementary Table 5. Six-week-old C57BL/6J mice were obtained from Charles River Laboratories. Chimeric mice harboring a functional human liver cell xenograft were purchased from PhoenixBio. The protocol was reviewed by the Institutional Animal Care and Use Committee of Chugai Pharmaceutical Co., Ltd. and all mouse experiments were performed in accordance with the Guidelines for the Accommodation and Care of Laboratory Animals promulgated in Chugai Pharmaceutical Co., Ltd. Recombinant human IFN-α2a was a kind gift from F. Hoffmann-La Roche. Recombinant murine IFN-αA and human IFN-β1a were purchased from PBL Interferon Source. Recombinant TNF-α and IFN-γ were purchased from R&D Systems. Imiquimod was purchased from LKT Laboratories. JAK inhibitor I was purchased from Merck.

**Luciferase assay.** Luciferase activity was quantified using the Steady-Glo Luciferase assay system (Promega) and the EnVision 2013 Multilabel Reader (PerkinElmer).

**WST-8 assay.** The viability of drug-treated Huh-7 cells was determined using a WST-8 cell counting kit (Dojin Laboratories).

**Real-time RT-PCR.** Total RNA was extracted using Rneasy (Qiagen), and cDNA was synthesized using a Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science). Gene expression was measured using the LightCycler 480 System and LightCycler 480 Probes Master (Roche Applied Science). The amplification used 50 cycles of: 95°C for 5 min, 95°C for 10 s, and 60°C for 30 s. Human β-actin or rodent glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Applied Biosystems) expression was used as the endogenous reference for each sample. Primers and TaqMan probes for genes were designed using the Universal Probe Library Assay Design Center (Roche Applied Science; Supplementary Table 6). The probes used were from the Roche Universal Probe Library (Roche Applied Science). The samples

were run in triplicate for each target gene, and each reference gene was used as an internal control.

**Western blotting and immunostaining.** Cells were lysed in CelLytic M Cell Lysis Reagent (Sigma-Aldrich) containing Protease Inhibitor Cocktail (Sigma-Aldrich) and PhosSTOP (Roche Applied Science). Rabbit polyclonal antibodies against STAT1, STAT3, STAT6, pY701-STAT1, pY690-STAT2, pY705-STAT3, pS727-STAT3, pY694-STAT5, pY641-STAT6, pY1022/1023-JAK1, and pY1054/1055-Tyk2 were purchased from Cell Signaling Technology. Rabbit polyclonal antibodies against actin, STAT2 and STAT5 were purchased from Santa Cruz Biotechnology. Anti-Tyk2 rabbit polyclonal antibody was purchased from Upstate. Anti-IFNAR1 mouse monoclonal (MAB245) and anti-IFNAR2 sheep polyclonal antibodies were purchased from R&D Systems. Anti-NS3, anti-NS5A, and anti-NS5B rabbit polyclonal antibodies were a kind gift from F. Hoffmann-La Roche. Anti-NS4A and anti-NS4B mouse monoclonal antibodies were a kind gift from the Tokyo Metropolitan Institute of Medical Science. Proteins were detected using the Odyssey Infrared Imaging System (LI-COR). For immunostaining analysis, the cells were fixed on a 35-mm glass-based dish (Iwaki) with 4% paraformaldehyde, blocked using 5% fetal bovine serum in phosphate-buffered saline, and then incubated with anti-NS3 and anti-NS4A antibodies. The cells were then washed and incubated with Alexa488-labeled anti-rabbit IgG and Alexa568-labeled anti-mouse IgG (Molecular Probes) and analyzed using confocal laser microscopy.

**JFH-1 antiviral assay.** A cured K4 cell line derived from HuH-7 HCV replicon cells was maintained in DMEM supplemented with 10% fetal calf serum (FCS), high-glucose nonessential amino acids, and HEPES (Invitrogen). The JFH-1/K4 cell line, which was persistently infected with the HCV JFH-1 strain, was maintained under the same conditions as the cured K4 cell line. For the anti-HCV assay of JFH-1/K4 cells persistently infected with the JFH-1 strain, JFH-1/K4 cells were seeded in a 24-well tissue culture plate containing DMEM supplemented with 10% FCS, high-glucose nonessential amino acids, and HEPES (Invitrogen). After overnight incubation, serial dilutions of reagent in growth medium were added. After 72 h, total RNA was purified from the JFH-1/K4 cells using Isogene (Nippon Gene). HCV-RNA was quantified by real-time PCR as previously reported[50].

**EMCV cytopathic effect assay.** This assay was performed on A549 cells seeded in a 96-well tissue culture plate containing DMEM supplemented with 10% FBS. After overnight incubation, the indicated concentrations of each reagent were added to the growth medium. After 12 h, 100 $TCID_{50}$/mL EMCV was added, and after another 48 h, viable cells were stained with 0.5% crystal violet. RNAi experiment using EMCV was also performed on A549 cells seeded in a 96-well tissue culture plate containing DMEM supplemented with 10% FBS. We transfected STAT1- or STAT2-siRNA to A549 cells, and after 72 h we infected EMCV to the cells and treated them with 1 μM RO8191 or 2 IU/mL IFN. After additional 48 h incubation, we evaluated the cell viability by staining with crystal violet.

**GeneChip and data analysis.** Total RNA was extracted from $10^7$ HCV replicon cells cultured for 8 h in the presence of 2 μM RO8191 or 4 IU/mL IFN-α with TRIzol Reagent (Invitrogen). Reverse transcription, RNA labeling (5 μg of total RNA), hybridization to Human Genome U133 Plus 2.0 Arrays (Affymetrix), and scanning were performed according to the manufacturer's instructions (Affymetrix, http://www.affymetrix.com). GC-RMA (GeneChip Robust Microarray Analysis) algorithms were used to generate scaled gene expression values. The fold change compared to untreated cells was calculated, and probe sets were selected for genes that were at least 2.0-fold upregulated in RO8191- and IFN-α-treated cells relative to the control cells.

**RNA interference.** For all double-stranded RNAs, ON-TARGET Plus siRNA reagents (Dharmacon) were used (Supplementary Table 7). The siRNAs were transiently transfected using Lipofectamine RNAiMAX Transfection reagent (Invitrogen) according to the manufacturer's protocols for reverse transfection.

**Plasmids and transfection.** ISRE and NF-κB reporter gene were purchased from Clontech. *IFNAR2* was cloned into a pCOS2 vector[51] harboring the EF1α promoter. Plasmids were transfected using FuGENE HD (Roche Applied Science) according to the manufacturer's instructions.

**SPR measurements.** SPR binding studies were performed using a Biacore T100. Recombinant IFNAR2 ECD protein was purchased from R&D Systems. The protein (1 mg/mL) was diluted 1:20 with 10 mM sodium acetate buffer (pH 5.0) and mixed with 2 μM RO8191 for stabilization of the binding site. The mixture was immobilized on a Series S sensor chip CM7 using amine coupling. RO8191 and PEG-IFN-α2a (Chugai Pharmaceutical) were injected onto the sensor chip at a flow rate of 0.03 mL/min. Response curves were generated by subtraction of the background signal generated simultaneously on a control flow cell. Kinetic parameters were obtained by global fitting of the sensorgrams to a 1:1 model using Biacore T100 Evaluation Software, version 2.0.1.

**Humanized liver mice study.** The chimeric mice were generated by transplanting human primary hepatocytes into severe combined immunodeficient (SCID) mice carrying the urokinase plasminogen activator transgene controlled by an albumin promoter[38]. The chimeric mice used in this study were applied from Inoue *et al.*[52], and had a high substitution rate of human hepatocytes. Six weeks after hepatocyte transplantation, patient serum containing $10^6$ copies of HCV genotype 1b was intravenously injected into each mouse. HCV titer reached approximately $10^8$ copies/mL and was stable after 4 weeks of HCV injection and persistently infected for 12 weeks. Here, we used mice after 5 weeks post infection and tested for 2 weeks. The mice were treated for 14 days with RO8191 30 mg/kg/day orally or PEG-IFN-α2a 30 μg/kg subcutaneously twice weekly. HCV RNA in serum was extracted using the acid guanidinium-phenol-chloroform method. Quantification of HCV RNA was performed using real-time RT-PCR based on TaqMan chemistry, as described[50]. HCV inoculations, drug administration, blood collection, and killing were performed under ether anesthesia. Blood samples were taken from the orbital vein and sera were immediately isolated. The protocols for animal experiments were approved by the local ethics committee. The animals received humane care according to NIH guidelines. Patients gave written informed consent before sampling.


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## Author contribution statement

H.K., K.O., Y.O., H.Y., Y.H., A.O. and N.H. performed the experiments; H.K., K.O., H.O. and M.A. analyzed the data; G.F. and W.A. provided experimental materials and input into the data analysis; H.S., A.K., M.K., T.T., N.S., G.F., Y.A., M.A. and M.S. provided expert information; and H.K. and M.S. wrote the manuscript.

## Additional information

**Supplementary information** accompanies this paper at http://www.nature.com/scientificreports

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# HSC90 is required for nascent hepatitis C virus core protein stability in yeast cells

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ABSTRACT

**Hepatitis C virus core protein (Core) contributes to HCV pathogenicity. Here, we demonstrate that Core impairs growth in budding yeast. We identify HSP90 inhibitors as compounds that reduce intracellular Core protein level and restore yeast growth. Our results suggest that HSC90 (Hsc82) may function in the protection of the nascent Core polypeptide against degradation in yeast and the C-terminal region of Core corresponding to the organelle-interaction domain was responsible for Hsc82-dependent stability. The yeast system may be utilized to select compounds that can direct the C-terminal region to reduce the stability of Core protein.**


## 1. Introduction

Chronic hepatitis C virus (HCV) infection causes liver cirrhosis and hepatocellular carcinoma [1]. Approximately 170 million individuals have been infected by HCV and are at risk for viral hepatitis, cirrhosis, and hepatocellular carcinoma [2]. HCV has a positive-strand RNA genome that encodes a polyprotein of ~3000 amino acids. The polyprotein is cleaved to yield the structural core, E1 and E2 polypeptides and seven non-structural polypeptides [3]. HCV core protein exhibits RNA-binding activity and is the major component of the viral nucleocapsid [4]. During HCV polypeptide synthesis, the core protein (Core, found at the N-terminus) is cleaved by a signal peptidase, releasing a protein 191 amino acids in length (Fig. 1A). The C-terminus of Core is further cleaved by a signal peptide peptidase at the endoplasmic reticulum (ER) membrane [5], which is required for virus production [6]. Generation of the mature Core (aa 1–177, $Core^{177}$) enables it to translocate to lipid droplets [7], where virion assembly is thought to occur. In addition to the direct role of the Core in viral production, expression of the Core induces lipid accumulation in hepatocytes and may be responsible for HCV-associated steatosis [8]. The Core has been implicated in the modulation of cellular processes that include lipid droplet reorganization, apoptosis, transformation, and host cell gene expression [9]. Thus, compounds that inhibit Core function or that reduce the stability of Core may also prevent the progression of HCV pathogenesis and viral production.

In this study, we established a system to screen compounds that reduce the stability of Core in the budding yeast *Saccharomyces cerevisiae*. We identified HSP90 (heat shock protein 90) inhibitors that reduce Core stability. Our results reveal that HSC90 (Hsc82) is required for the stability of the nascent Core in yeast cells. We found that the C-terminal domain of Core contributes to the stability of Core in yeast cells. The yeast system presented herein, which can be applied to high-throughput screening, may be useful for identifying for compounds that can direct a C-terminal domain to reduce the stability of the Core.

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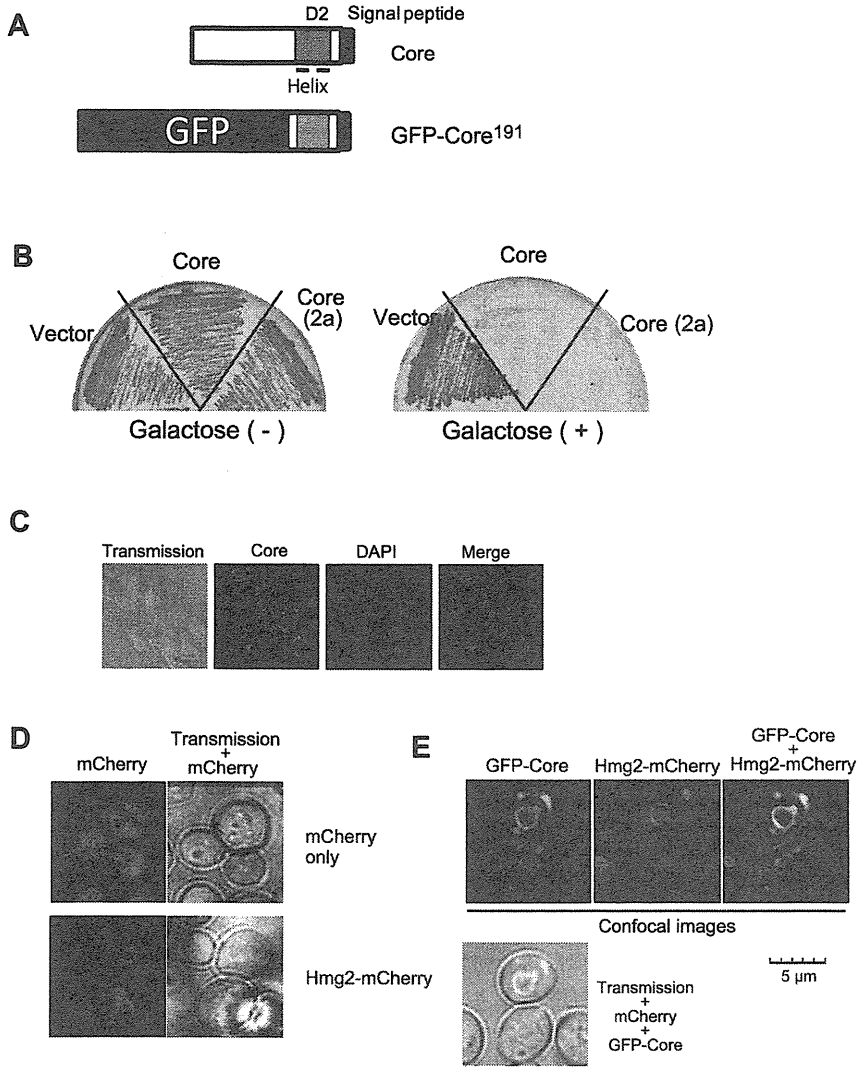


**Fig. 1.** HCV core protein-induced growth inhibition of yeast cells. (A) Schematic diagram of Core and GFP-Core[191]. Core protein contains a D2 domain (aa 118–171, shadowed boxes) including two helix structure and a Core-E1 signal peptide (aa 178–191, closed box). (B) Yeast cells carrying pRS425 (Vector), pRS425-GAL1-core (1b, aa 1–191) (Core) and pRS425-GAL1-core (2a, aa 1–191) (Core (2a)) were cultured on SR agar plates with (galactose +) and without (galactose −) 3% galactose for 60 h at 30 °C. (C) Core was induced in yeast cells carrying pRS425-GAL1-core for 2 h, fixed and examined using immunofluorescence assays. Nuclear staining was performed using DAPI (4′,6-diamidino-2-phenylindole, 2HCl). (D) The ER marker Hmg2-mCherry was localized to the perinuclear region. Confocal images of mCherry and Hmg2-mCherry fluorescence in wild-type yeast cells were monitored (ex. 543 nm; em. BA560IF). The confocal images were overlaid on a corresponding transmission image. (E) The GFP-fused Core D2 domain (GFP-Core, green) co-localized with Hmg2-mCherry (red). Confocal images of GFP were monitored (ex. 488 nm; em. BA505–525). Co-localization of GFP and mCherry and the transmission overlay are shown.

## 2. Materials and methods

### 2.1. Yeast strains, media, reagents and yeast transformation

Yeast cells were grown in a synthetic raffinose (SR dropout) medium [1.67% Bacto™ yeast nitrogen base lacking aminoacids (Difco) with 2% raffinose] supplemented with 0.04 mg/ml adenine and amino acids [SR dropout; [10]] at 30 °C. *S. cerevisiae* strains used in this study are indicated in the Supplemental Information.

### 2.2. Construction of plasmids for expression of HCV Core in yeast

To induce expression of the Core in yeast, we utilized a multicopy plasmid containing the *GAL1* promoter and the GAPDH terminator region (pKT10-GAL1) and a pRS425 [11] derivative containing the same *GAL1* promoter-GAPDH terminator region (pRS425-GAL1). The Core of HCV (1b) (hepatitis C virus isolate HCR6; GenBank accession no. AY045702) and pJFH1 (hepatitis C virus isolate JFH1; GenBank accession no. AB047639) were used. There are 11 amino acid differences within the D2 and the signal peptide between JFH1 and HCR6.

### 2.3. Screening for anti-Core chemicals

We examined the effects of various compounds isolated from microorganisms and known antibiotics (a library constructed in-house at the Kitasato Institute) as described in the Supplemental Information.

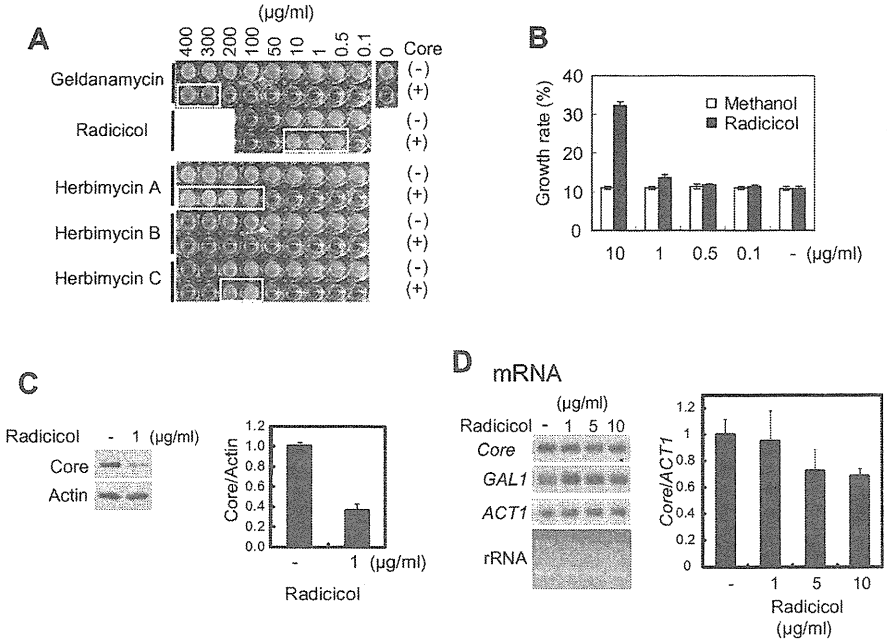


**Fig. 2.** HSP90 inhibitors reverse Core-induced growth inhibition in yeast cells. (A) Yeast cells carrying the pRS425-GAL1-core plasmid were cultured with (+) and without (−) 3% galactose plus geldanamycin, herbimycin A, herbimycin B, herbimycin C (0.1–400 μg/ml), or radicicol (0.1–100 μg/ml) as indicated for 72 h at 30 °C. As a control, cells were cultured without the antibiotics (0). (B) Growth recovery% in the presence of 10 μg/ml radicicol for 36 h at 30 °C. The percentage of the growth recovery was 35%. The data shown are the mean ± S.D. of three independent experiments. (C) Effect of radicicol on the level of Core protein in yeast cells. Yeast cells carrying the pRS425-GAL1-core plasmid were cultured with (+) or without (−) 1 μg/ml radicicol in the presence of 3% galactose for 72 h at 30 °C. Photographs of immunoblots using Core-specific and actin-specific antibodies. (D) Effects of radicicol (1, 5 and 10 μg/ml) on the level of Core RNA as determined by northern blotting. Total RNA was prepared from yeast cells 2 h after induction of Core protein expression. Effects of radicicol on galactose-induced Gal4-dependent transcription of Core (*Core*) and *GAL1* (*GAL1*) were examined by hybridization with probes to detect Core, *GAL1* and *ACT1* RNAs. Ribosomal RNA was visualized by staining with ethidium bromide.

### *2.4. Preparation of cell lysates and western blotting*

We examined the Core by western blotting as described previously [12] and in the Supplemental Information. We used primary antibodies specific for actin (sc-1616; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Hsp90 (K41110, from Dr. Nemoto) [13,14], and Core (515S) [15].

### *2.5. Analysis of the distribution of the Core in yeast cells using GFP-core proteins and immunofluorescence*

Core in yeast were examined by indirect immunofluorescence as described previously [16] and in the Supplemental Information. Yeast cells expressing core proteins carrying pKT10-GAL1-GFP-core$^{191}$ and pKT10-GAL1-GFP-core$^{177}$ were analyzed using confocal microscopy (Olympus FV1000).

### *2.6. Statistical analysis*

Multiple independent replicates ($n = 3$, except as indicated) were performed for each experiment, and data are presented as the mean of three independent experiments with the standard deviation (SD).

## 3. Results

### *3.1. Inhibition of yeast cell growth by expressing Core*

Yeast cell growth was significantly inhibited when the full-length core of HCV 1b (aa 1–191; Core) was expressed under the control of the inducible *GAL1* promoter (Fig. 1B). Core of the HCV 2a (JFH1) genotype had a similar effect, suggesting that the conserved HCV core structure from different strains may be responsible for the core protein's effect on yeast cells. The C-terminal region of Core includes a domain (D2: aa 118–171) (Fig. 1A) that is responsible for association with the ER and lipid droplets [5] and a Core-E1 signal peptide (aa 178–191) [17]. We examined the distribution of Core on ER in yeast. Immunofluorescent microscopy indicated that Core localized on the periphery of the nucleus (Fig. 1C). The D2 domain fused to GFP (GFP-Core) co-localized with Hmg2 as an ER marker [18] (Hmg2-mCherry; Fig. 1D) in live yeast cells (Fig. 1E) and showed a reticular fluorescence pattern and cell periphery.

### *3.2. Inhibitors of HSP90 reduce Core stability in yeast cells*

We postulated that if the proliferation of yeast cells expressing Core could be restored by treatment with certain compounds, it might provide clues regarding the cytotoxic effect of Core on yeast cells. We screened various antibiotics isolated from fungi and actinomycetes and found that inhibitors of HSP90 were able to suppress Core toxicity. The compounds determined to exhibit this suppressive activity included geldanamycin (300 and 400 μg/ml), radicicol (0.5, 1 and 10 μg/ml), herbimycin A (100–400 μg/ml) and herbimycin C (100 and 200 μg/ml), but not herbimycin B (Fig. 2A). We observed that treatment with radicicol (>50 μg/ml) and herbimycin C (>300 μg/ml) had cytotoxic effects, even under galactose-free conditions, when no Core protein was produced [Fig. 2A; Core (−)]. Radicicol was more effective at lower concentrations than the other inhibitors, so we focused on its effects (Fig. 2A). Treatment with 10 μg/ml radicicol for 36 h restored yeast cell growth (Fig. 2B). HSP90 inhibitors inhibit HSP90/HSC90 ATPase activity by competing with ATP for binding and thereby eliminate HSP90/HSC90 chaperone activity [19]. This chaperone activity may affect expression of the Core. The level of the Core in

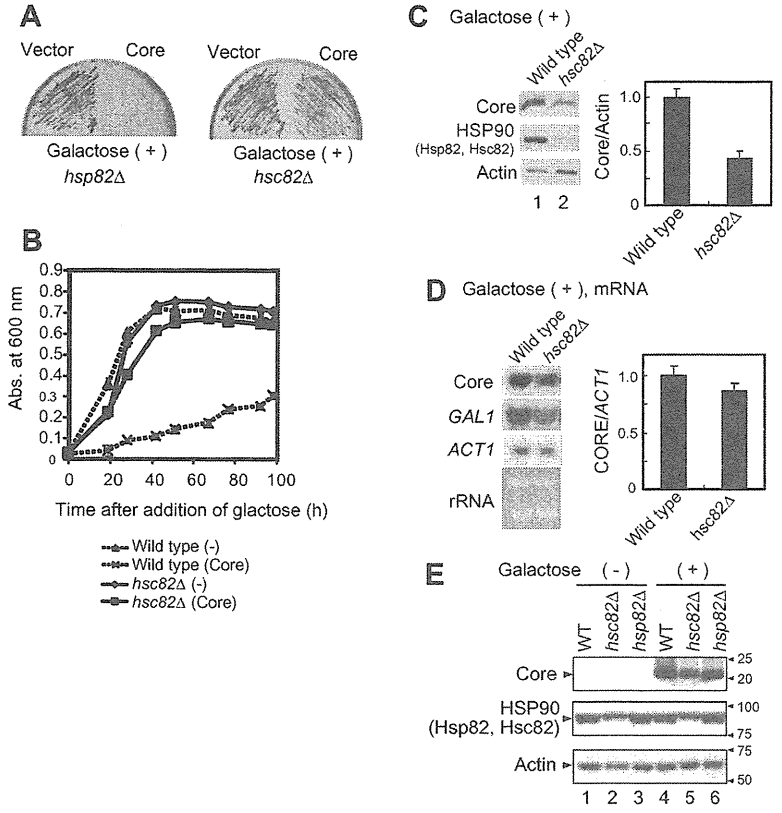


**Fig. 3.** Effects of HSP90 on the stability of Core in yeast cells. (A) Disruption of *HSC82*, but not *HSP82* reversed the growth inhibition induced by Core. (B) Growth curve of core-expressing *hsc82*Δ yeast cells. We examined the time-dependent absorbance at 600 nm of BY4742 (Wild type) and *hsp82*Δ yeast cells carrying pRS425-GAL1-core for 96 h after addition of 3% galactose (Core) or without addition of galactose (−). (C and D) Effects of *HSC82* disruption on Core (C) and Core RNA (D) levels in yeast cells that were monitored as in Fig. 2. (E) Effects of the levels of all Hsp82/Hsc82 proteins on Core levels. Mutant yeast cells with a disrupted *HSC82* or *HSP82* gene harboring pRS425-GAL1-core were cultured with (+) or without (−) 3% galactose for 72 h (A) or 2 h (C–E) at 30 °C. We used a monoclonal antibody that reacts with the Ic epitope common to Hsc82 and Hsp82 [14].

radicicol-treated yeast cells (1 μg/ml) was 36% of control levels (Fig. 2C) whereas the level of Core mRNA did not change (Fig. 2D).

### 3.3. The HSC90 ortholog Hsc82 is responsible for the Core toxicity

An HSP90 family member (HSP90/HSC90) is a highly conserved molecular chaperone that can induce the maturation of various proteins, including kinases, transcription factors, and proteins, that function in signal transduction (for review, see [20]). To identify the *HSP90* gene that affects Core levels, we disrupted HSP90 family member (*HSP82* and *HSC82*) genes in yeast [21] and examined growth in the presence of Core. Disruption of *HSC82* (*hsc82*Δ) but not of *HSP82* (*hsp82*Δ) alleviated some of the negative effects of the Core on cell growth (Fig. 3A). The effect of *HSC82* disruption was also clear in liquid culture: the growth rate of Core-expressing *hsc82*Δ cells was similar to that of wild-type cells and of *hsc82*Δ cells not expressing Core (Fig. 3B). In addition, the Core levels in *hsc82*Δ cells decreased to 44% of the level in control cells (Fig. 3C), but the level of Core mRNA was only slightly lower (Fig. 3D). The total levels of HSP90 family proteins (Hsc82 and Hsp82) decreased to 18% of the levels in control cells (Fig. 3C, compare lanes 1 and 2 of the left panel) after disruption of the *HSC82* gene, indicating that the principal HSP90 family protein (at steady state) in yeast is Hsc82, as previously reported [21]. Furthermore, neither heat-inducible Hsp82 protein levels (compare lanes 2 and 5 in Fig. 3E) nor total HSP90 family protein levels (compare lanes 1 and 4) were increased in response to induced Core expression. There was a correlation between the effects of radicicol and disruption of *HSC82*, suggesting that total HSP90 family protein levels (activity) may be involved in modulating Core levels and thereby contribute to the Core protein's growth-inhibitory effects.

### 3.4. An HSP90 family protein is required for stability of the nascent Core

To examine whether the Core degradation rate is affected by disruption of *HSC82*, we treated cells expressing the Core with cycloheximide (CHX) and examined the time-dependent decay of the core protein. As shown in Fig. 4A, the Core degradation rate was significantly greater when *HSC82* was disrupted. This greater degradation rate was also observed when cells were pretreated with radicicol prior to CHX treatment (Fig. 4B). In contrast, there was no significant difference in the Core degradation rate when cells were treated with radicicol and CHX simultaneously (Fig. 4C). These results suggest that the degradation rate of nascent Core may be enhanced when the levels of HSP90 family members are reduced or the activity of HSP90 family members is inhibited. However, the stability of Core produced in the presence of a wild-type level of activity of HSP90 family members was not affected by HSP90 inhibitors. Our results suggest that HSP90/HSC90 may be essential for protecting nascent Core from degradation during protein synthesis.

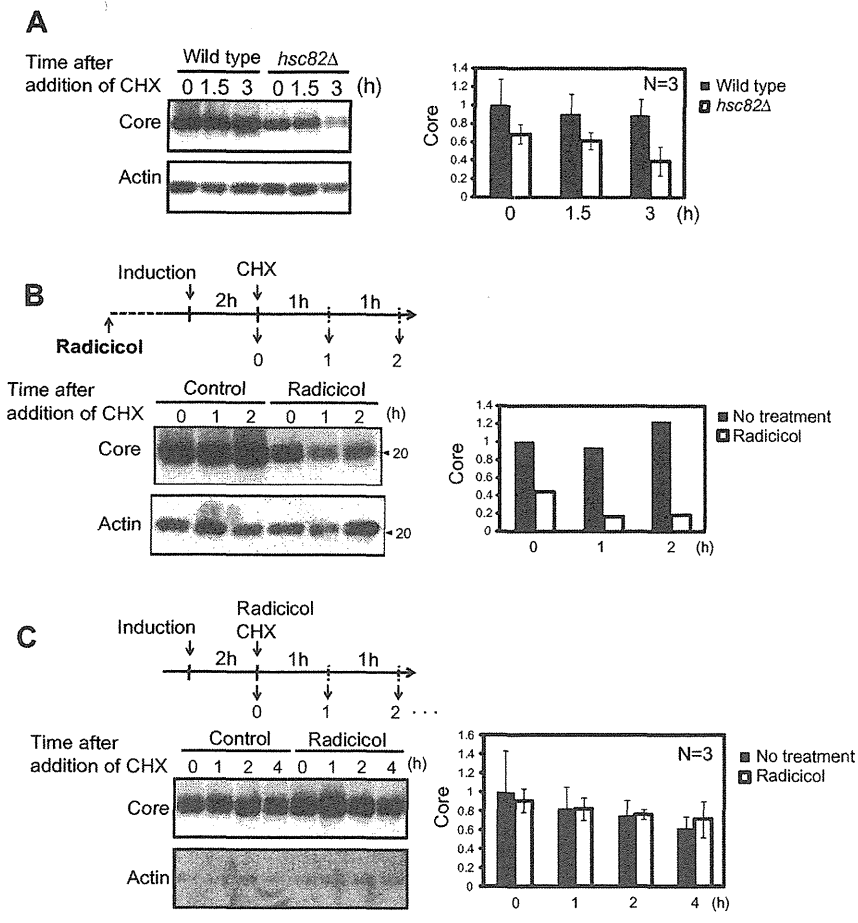


**Fig. 4.** Instability of nascent Core in yeast cells with reduced HSP90 activity. (A) Effect of *HSC82* disruption on Core stability. We cultured wild-type and *hsc82*Δ yeast cells carrying pRS425-GAL1-core with 3% galactose for 2 h (0 h) and then further incubated (chased) the cells with 300 mM cycloheximide (CHX) for 1.5 h and 3 h. Core and actin were separated by SDS-PAGE and quantified by western blotting. (B) Pretreatment with radicicol had an effect similar to that of *hsc82* disruption. Wild-type yeast cells carrying pRS425-GAL1-core were treated with radicicol (1 μg/ml) for 12 h and then cultured with 3% galactose for 2 h. Core was chased as in (A). (C) Simultaneous treatment with radicicol and CHX did not alter Core stability. We treated wild-type yeast cells expressing Core with radicicol (1 μg/ml) and CHX (0.1 mg/ml) to examine their effects on Core stability. Means from experiments performed in triplicate are indicated (A and C), and representative data (B) are presented in bar graphs.

### *3.5. The Core D2 domain contributes to HSC90-dependent stability and association with the ER*

The Core-E1 signal peptide in the full-length Core is efficiently cleaved by a signal peptide peptidase to form Core$^{177}$ in human hepatoma cells [17]. This Core-processing activity is significantly lower in yeast (data not shown). Thus, we expressed Core$^{177}$ and examined its stability. We found that yeast cell growth was also impaired by Core$^{177}$ (Fig. 5A). As shown in Fig. 5B, we discovered that the rate of Core$^{177}$ degradation was accelerated in *HSC82*-disrupted cells. The D2 domain includes two amphipathic α-helices with the potential ability to interact with lipid droplets as well as the ER [9]. We examined whether the Core D2 domain is responsible for Hsc82-dependent stability of the Core. The degradation rate of GFP fused to a region containing the D2 domain and the signal peptide (aa 125–191; GFP-Core) and GFP fused to the D2 domain with the processed signal peptide (aa 125–177; GFP-Core$^{177}$) increased twofold when *HSC82* was disrupted (Figs. 5B–D). Interestingly, the Hsc82-dependent stability of Core$^{177}$ was comparable to that of GFP-Core$^{177}$. However, GFP-Core was the most stable form in the absence of Hsc82 (Fig. 5E).

We examined the distribution of GFP-Core$^{177}$ in yeast. GFP-Core$^{177}$ was detected in small particles in the perinuclear region, although it also co-localized with Hmg2-mCherry (Fig. 6A). These results suggest that the ER-D2 domain interaction might affect the distribution of ER-localized Hmg2 proteins or the structure of the ER membrane itself. In addition to the observed puncta, GFP-Core$^{177}$ was distributed throughout the cytoplasm (compare Fig. 6A and Fig. 1E). Next, to demonstrate the effect of *HSC82* disruption on the distribution and degradation of GFP-Core proteins, we obtained time-lapse fluorescent images of GFP-Core after suppressing its expression; the *GAL4* promoter was suppressed by addition of glucose (Figs. 6B and C). Disruption of *HSC82* did not affect the localization of either GFP-Core or GFP-Core$^{177}$, but the degradation rates were again enhanced. Our results suggest a contribution of the D2 domain to the ER localization and the HSP90/HSC90-dependent stability of the Core. In fact, the intracellular levels (Fig. 7A) and the stability of a truncated Core (aa 1–151; Core$^{151}$; Fig. 7B), in which a half of D2 was removed, were significantly decreased (the half-life was less than 0.5 h). The yeast cell growth was not affected by expression of Core$^{151}$ (Fig. 7B). Thus, the stabilities (expression levels) of core might determine the growth inhibition.

## 4. Discussion

Here, we demonstrate that the Core impairs growth in yeast and that HSC90 is required for Core stabilization in yeast cells. Our

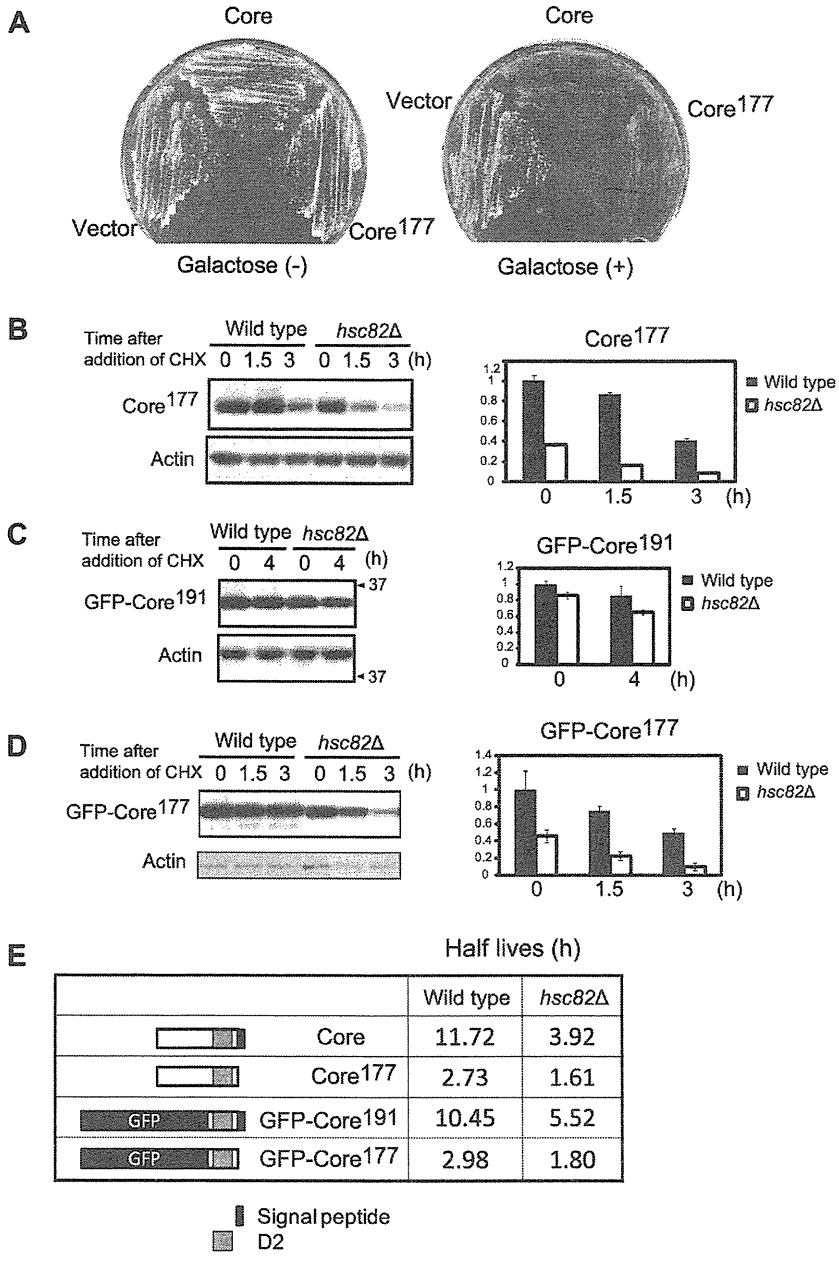


**Fig. 5.** The stability of the organelle association domain (D2 domain) of the Core depends on HSP90 activity. (A) Effect of yeast cell growth by Core$^{177}$ was examined as described in legend to Fig. 1. (B–D) The stability of Core$^{177}$ (B), GFP fused to the D2 domain with the signal peptide (C) and the GFP fused to the D2 domain (D) were all lower in *hsc82Δ* cells. Log-phase cultures of wild-type and *hsc82Δ* cells each harboring the corresponding plasmids were cultured with galactose for 2 h and then chased after treatment with CHX, as in Fig. 4. (E) Half-lives of Core, Core$^{177}$, GFP-Core$^{191}$ and GFP-Core$^{177}$ in wild-type and *hsc82Δ* cells. The half-lives were determined in chase experiments (Figs. 4 and 5B–D).

previous results involving expression profiling of yeast suggest that Core induces the expression of genes involved in the ER stress response, but not the heat stress response that induces molecular chaperons [22]. Furthermore, Core did not increase the level of a heat-inducible HSP90 (Hsp82; Fig. 3E). Thus, Core and Core$^{177}$ expressed in the yeast cells may fold correctly, accumulate on the ER, and induce cellular responses involved in the observed growth inhibition. Growth of yeast cells were not significantly affected by GFP-fused D2 peptides (GFP-Core$^{191}$ and GFP-Core$^{177}$; data not shown). Consequently, it is possible that some characteristics of N-terminal region such as oligomerization [4] also may impact on the yeast cell growth. Several reports indicate that expression of Core disturbs cellular signaling and enhances the cell growth [23,24]. It is possible that higher level of Core expression in yeast cells inhibits intrinsic functions of ER (manuscript in preparation).

We showed that treatment with HSP90 inhibitors and disruption of *HSC82* reduced the stability of the nascent Core protein, but pre-existing Core protein was not affected by HSP90 inhibitors. Steady-state HSP90 family protein levels, which derive from the *HSC82* gene, could determine the stability (amount) of the Core, causing growth inhibition in yeast cells. We also demonstrated that the stability of nascent Core and Core$^{177}$ were increased in

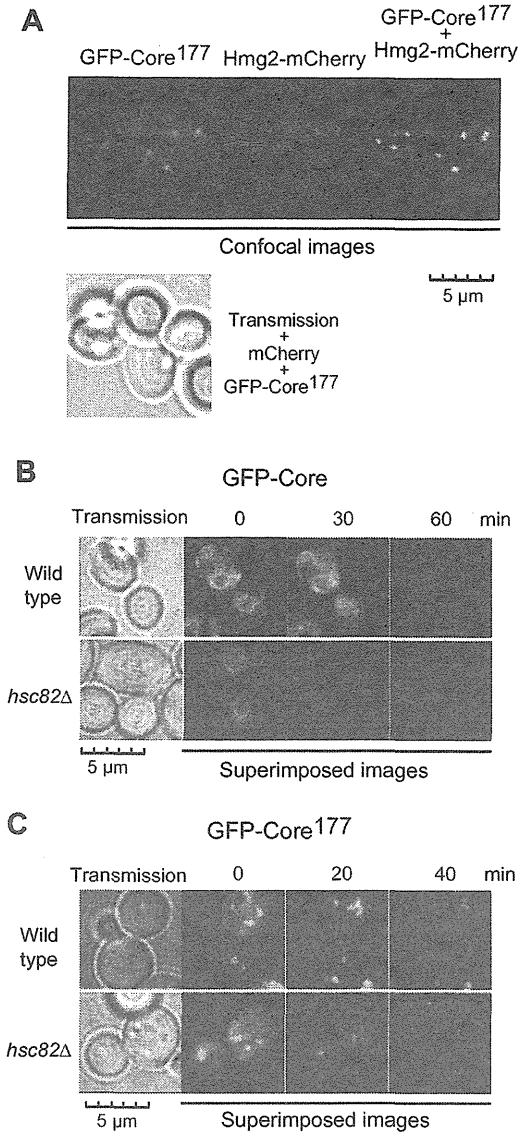


**Fig. 6.** Requirement of HSC82 for ER localization of Core D2 region in yeast. (A) The GFP-Core$^{177}$ co-localized with the ER marker Hmg2-mCherry. Confocal images were monitored as described in legend to Fig. 1. Co-localization of GFP and mCherry and the transmission overlay are shown. (B and C) Hsc82 is required for extended Core localization to the ER. We monitored the time-dependent extinction of GFP-Core$^{191}$ (0, 30, 60 min; D) and GFP-Core$^{177}$ (0, 20, 40 min; E) expressed in wild-type (upper panels) and *hsc82Δ* (lower panels) cells after production of GFP-Core proteins was stopped by switching from galactose to glucose in the medium (time 0). We acquired a series of 20 GFP images (0.26 μm *Z* dimension) and overlaid them (superimposed images).

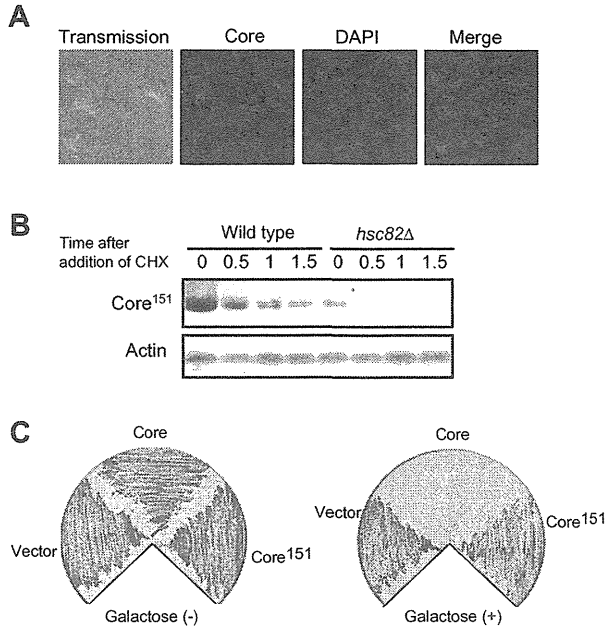


**Fig. 7.** ER localization and stability of Core$^{151}$. (A) Localization of Core$^{151}$ was examined as described in legend to Fig. 1. (B) The stability of Core$^{151}$ was examined as described in legend to Fig. 5. (C) Growth of yeast cells carrying pRS425-GAL1-core (1b, aa 1–151) (Core$^{151}$) were examined as described in legend to Fig. 1.

an Hsc82-dependent manner and that the half-lives of GFP-fused D2 peptides (GFP-Core, GFP-Core$^{177}$) were comparable to those of Core and Core$^{177}$ (Fig. 5E). Our results suggest that Hsc82 activity might play a role in ensuring that the D2 peptide folds correctly, allowing the Core and Core$^{177}$ to be stabilized. Our results are consistent with the finding that the D2 domain is essential for the stability of the mature Core (Core$^{177}$), conferring protection from degradation in mammalian cells [5]. We failed to detect a direct interaction between Core and Hsp82/Hsc82 in yeast cells using immunoprecipitation (data not shown). Further studies are required to elucidate the Hsc82-dependent mechanism involved in stabilization of the Core peptide chain during translation, including the potential requirement for a co-chaperone. In this aspect, one of the Hsp70 co-chaperon Ydj1 may be such a candidate: Ydj1 can interact with HSP90-client proteins [25] and protect nascent chains against degradation [26].

Thus far, we have no direct evidence of the effect of HSC90 on the stability of the D2 domain of Core in human hepatoma Huh7 cells. It is possible that the result demonstrated here may be specific to yeast cells. Nevertheless, the D2 domain is shown to be responsible for stability of Core in mammalian cells [27], thereby the yeast system presented herein, which can be applied to high-throughput screening, may be useful for identifying (screening or validation) for compounds that can direct the nascent D2 peptide to reduce the stability of the Core. Such compounds may represent possible drug candidates for prevention of the progression of HCV pathogenesis and HCV production.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.05.023.

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