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2 **Suppressor of Cytokine Signaling 3 (SOCS3) Suppresses**

3 **Hepatitis C Virus Replication in an mTOR-dependent Manner**

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25

26 **Abstract**

27 We and others have observed that hepatic levels the suppressor of cytokine signaling 3
28 (SOCS3) are significantly higher in chronic hepatitis C, particularly among persons who
29 are non-responders to subsequent IFN treatment. However, the relationship between
30 SOCS3 and Hepatitis C virus (HCV) replication remains unclear. Given its putative role,
31 we hypothesized that SOCS3 is permissive for viral replication. We therefore used the
32 genotype 1b full-length HCV replicon OR6 cell line, and the genotype 2a HCV full-
33 length JFH1 infection system to analyze the effect on HCV replication of SOCS3
34 overexpression and shRNA mediated knockdown. We further analyzed the role of mTOR
35 in SOCS3's effects by treating selected cells with rapamycin. OR6 cells and JFH1-
36 infected Huh7.5.1 cells expressed significantly less SOCS3 than control cells.
37 Furthermore, inhibition of HCV replication with the HCV protease inhibitor BILN 2061
38 restored SOCS3 protein levels. SOCS3 overexpression in OR6 cells and JFH1 infected
39 Huh7.5.1 cells resulted in significantly lower HCV replication than in the control cells,
40 despite SOCS3-related inhibition of STAT1 phosphorylation and type I IFN signaling. In
41 contrast, JFH1-infected, stable SOCS3 knockdown cells expressed more HCV than did
42 control cells. shSOCS3 also knocked down mTOR and phospho–mTOR. The mTOR
43 inhibitor rapamycin reversed SOCS3's inhibitory effects. In independent lines of
44 investigation, SOCS3 unexpectedly suppresses HCV replication in an mTOR-dependent
45 manner. These findings suggest that increased SOCS3 levels consistently observed in
46 chronic IFN nonresponders may reflect a compensatory host antiviral response to
47 persistent infection and that manipulation of SOCS3/mTOR may offer benefit against

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48 HCV infection.

49 Introduction

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51 Hepatitis C virus (HCV) is a small, enveloped plus-strand RNA virus in the genus
52 Hepacivirus and the family Flaviviridae. HCV has a 9.6 kb genome that encodes
53 structural (core, E1, E2) and non-structural proteins (NS2-NS5B) (1). The HCV life cycle
54 and host–virus interactions that determine the outcome of infection have been difficult to
55 study because small animal models of HCV infection are not available, and cell culture
56 models were not developed until recently. The development of subgenomic and genomic
57 replicons provided a major breakthrough to the understanding of HCV replication and
58 viral-cell interactions. Recently, several groups have created a robust genotype 2a HCV
59 full-length replication system (23, 27). Huh-7.5.1 cells transfected with *in vitro*
60 transcribed JFH-1 genomic RNA will successfully secrete virions. Therefore, transfection
61 of JFH-1 RNA into the Huh-7.5.1-derived cells allows for the recovery of a viable JFH-1
62 virus that can then be serially passaged and used for infection-based experimentation.

63 The suppressor of cytokine signaling (SOCS; also known as JAB and SSI) family
64 consists of 8 members: SOCS1 through SOCS7, and the cytokine-inducible src homology
65 2 domain-containing protein (CIS) (14, 25). SOCS1 SOCS2, SOCS3, and CIS mRNAs
66 are induced by a variety of cytokines and growth factors such as interferon-gamma, IL-2,
67 IL-3, IL-6, erythropoietin, prolactin, but the correlation varies amongst tissue types (14,
68 22). SOCS family members modulate signaling by several mechanisms, which include
69 inactivation of the Janus Kinases (JAKs) by blocking access to both the signal transducers
70 and activators of transcription (STATs) and nuclear factor κB (NFκB) -mediated
71 pathways.

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72 SOCS3 is one of the negative regulators of cytokine signaling that function via the
73 JAK/STAT pathway (5, 17, 20). SOCS1 induces STAT3 phosphorylation, while SOCS3
74 strongly interacts with activated cytokine receptors, such as gp130, to negatively regulate
75 STAT3 phosphorylation (19). SOCS3 uses its Src homology 2 proteins domain to bind
76 the cytokine receptor with high affinity to attenuate the activity of JAKs via its kinase
77 inhibitory region in order to abolish STAT3 phosphorylation (4, 22).

78 Bode et *al*. have reported that SOCS3 can be induced by HCV core protein and
79 suppress JAK-STAT signaling to block the IFN-induced formation of ISGF3 in cell
80 culture (2). We and others have observed that levels of hepatic SOCS3 are significantly
81 higher in chronic hepatitis C (CHC), particularly amongst persons who are nonresponders
82 to subsequent IFN treatment (7, 13, 24). Patients with high TNF alpha levels have a poor
83 response to IFN alpha therapy; it has been proposed that this may occur via induction of
84 SOCS3 proteins that interfere with the interaction between the IFN alpha receptor and its
85 signaling proteins (24).

86 Previous studies have found that HCV core protein can induce SOCS3 expression,
87 and inhibit Phospho-STAT1 expression (2). The authors hypothesized that elevation of
88 SOCS3 levels and inhibition of Phospho-STAT1 impaired antiviral activity of IFN. Thus,
89 high levels of SOCS3 in the CHC patients may be a reason for a decrease or lack in
90 responsiveness to subsequent IFN therapy (7, 24). However, these hypotheses have not
91 been confirmed experimentally.

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95 ## MATERIALS AND METHODS

96 **Cell culture and virus.** Huh7.5.1 cells (27) were grown in Dulbecco's Modified Eagle's
97 Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Infectious JFH1
98 and JFH1-GND mutation plasmid was obtained from Dr. Takaji Wakita and infected as
99 previously described (23). A multiplicity of infection (m.o.i) of 0.01 ffu/cell was used for
100 infection in this study. OR6 cells (HCV replicon that stably harbors full-length genotype
101 1b HCV RNA and co-expresses *Renilla* luciferase) (8) were grown in 10% FBS
102 supplemented with 500 μg/ml of Geneticin G418 (Promega, Madison, WI). To prepare
103 cured cells, OR6 cells were treated with 50 ng/ml PEG-IFN for two weeks. After this
104 treatment period, the cured OR6 cells were grown in 10% FBS with DMEM for another
105 two weeks. *Renilla* luciferase assay and Western blot for HCV core confirmed the
106 absence of HCV replication in cured OR6 cells.

107 **Plasmids and transfection**. pCDNA3-Myc and pCDNA3-Myc-hSOCS3 were
108 generous gifts from the laboratory of Jie Chen (12), and the full-length HCV core
109 construct pC191 was a kind gift from Tetsuro Suzuki (National Institute of Infectious
110 Diseases, Japan) (21). Huh7.5.1, OR6 or cured OR6 cells grown on 12-well plates at
111 60%-70% confluence were transfected with 2 μg of pCDNA3-Myc, pCDNA3-Myc-
112 hSOCS3 using Fugene HD according to the manufacturer's protocol. For OR6 or cured
113 OR6 cells, Western blot or RT-PCR were performed 72 hours after transfection. For
114 Huh7.5.1 cells infected with JFH1 inoculum Western blot or RT-PCR were performed 48
115 hours after transfection. Two mcg of the empty vectors pCAG or pCMV, or vectors
116 expressing HCV core pC191, ST proteins (containing core, E1, E2), NS3-4A, NS4B,

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117 NS5A, and NS5B were transfected into Huh7.5.1 cells using the same methods. Western
118 blot was performed 72 hours post-transfection.

119 **Western blotting.** Cells were lysed using a radioimmune precipitation assay (RIPA)
120 buffer containing 0.1% SDS, 0.5% NP-40, 10 mM Tris-HCl (pH 7.4), 1 mM
121 ethylenediaminetetraacetic acid (EDTA), and 150 mM NaCl. Whole cell lysates were
122 sonicated, boiled at 95º C for 5 min, and chilled on ice for 10 minutes. Proteins were
123 separated by SDS-PAGE with NuPAGE Novex pre-cast 4-12% Bis-Tris gradient gels
124 (Invitrogen, Carlsbad, CA) and transferred to PVDF membranes. The primary antibodies
125 include mouse anti-STAT1, rabbit anti-Phospho-STAT1 (Tyr701), rabbit anti-mTOR,
126 rabbit anti-Phospho-mTOR (Ser2448) (Cell Signaling Technology, Inc., Beverly, MA),
127 mouse anti-HCV core, NS5B (Affinity BioReagents Inc., Golden, CO), mouse anti-HCV
128 NS3, NS5A, NS4A, NS4B (Virogen, Inc., Watertown, MA), mouse anti-HCV E1, E2
129 (Austral Biologicals, San Ramon, CA), mouse anti-SOCS3 (for endogenous
130 SOCS3)(Abcam, Inc., Cambridge, MA), rabbit anti-SOCS3 (for exogenous SOCS3)
131 (AnaSpec, Inc., San Jose, CA), and mouse anti-β-actin(Sigma, Inc., St Louis, MO). The
132 secondary antibodies were HRP-conjugated ECL donkey anti-rabbit IgG, or HRP-
133 conjugated ECL sheep anti-mouse IgG (Amersham Biosciences, Piscataway, NJ). The
134 ECL Western Blotting Detection Kit (Amersham Biosciences, Piscataway, NJ) was used
135 to detect chemiluminescent signals.

136 **JFH1 and JFH1-GND mutation RNA synthesis and transfection.** *In vitro* synthesis
137 of JFH1 and JFH1-GND mutant HCV RNA, and RNA transfections were performed as
138 described previously (23, 27).

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139 **Immunoprecipitation assay.** Huh7.5.1 cells were grown in 10-cm plates. Cells at 5
140 days post-JFH1 infection and mock infected cells were lysed using a radioimmune
141 precipitation assay (RIPA) buffer containing 0.1% SDS, 0.5% NP-40, 10 mM Tris-HCl
142 (pH 7.4), 1 mM ethylenediaminetetraacetic acid (EDTA), 150 mM NaCl, and
143 supplemented with protease inhibitor cocktail (10 μl/ml lysis buffer) (Sigma, St Louis,
144 MO). Protein A/G beads were incubated with mouse anti-SOCS3 (Abcam, Inc.,
145 Cambridge, MA) at 4°C rolling for 2 hours. After incubation, the beads were washed
146 three times with PBS buffer. Cell lysates were incubated with anti-SOCS3 banding beads
147 at 4°C rolling for 2 hours. After incubation, the beads were washed three times with PBS
148 buffer. The precipitates were then boiled for 5 min in Laemmli sample buffer and run on
149 a 4-12 % SDS-PAGE gel. Western blotting was performed as described. Mouse anti-
150 SOCS3 (Abcam, Inc., Cambridge, MA) and mouse anti-ubiquitin (Cell Signaling
151 Technology, Inc., Beverly, MA) were used as primary antibodies.
152 **Renilla luciferase assay and luciferase reporter gene assay.** OR6 cells were seeded
153 at 5,000 cells/well in 96 well plates. After 24 hours, cells were transfected with
154 pCDNA3-Myc or pCDNA3-Myc-hSOCS3 using Fugene HD following the
155 manufacturer's protocol. HCV replication in OR6 cells were determined by monitoring
156 *Renilla* luciferase activity (Promega, Madison, WI) (8). Gene expression was monitored
157 by the Promega dual-luciferase reporter assay system (Promega, Madison, WI). To
158 monitor IFN signaling directed by the IFN-stimulated response element (ISRE), the
159 plasmids pISRE-luc (500 ng/well) expressing firefly luciferase and pRL-TK (50 ng/well)
160 expressing *Renilla* luciferase were cotransfected with the appropriate plasmid (2 μg/well)

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161 and relative luciferase activity was assessed. Relative luciferase activity was calculated
162 by dividing the firefly luciferase value by the *Renilla* luciferase value.
163 **Cell Viability Assay.** Huh7.5.1 cells or OR6 cells were seeded in 96-well plates. Cells
164 were treated according to different experiment design. Cell viability was monitored using
165 the Cell Titer-Glo Luminescent Cell Viability Assay (Promega, Madison, WI) Kit
166 according to the manufacturer's protocol.
167 **Real-time polymerase chain reaction.** HCV RNA, SOCS3 and beta-actin messenger
168 RNA were measured according to the method of Castet et al (3). Briefly, total cellular
169 and viral RNA was isolated postinfection using RNeasy Mini columns (QIAGEN) with
170 on-column DNase digestion, reverse transcribed by random priming with the High
171 Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Foster City, CA), and
172 then quantitated by real-time PCR using the DyNAmo HS SYBR Green qPCR kit
173 (Finnzyme; Espoo, Finland).
174 **Establishment of a Huh7.5.1 cell line with stably knocked-down expression of**
175 **SOCS3**. Sequences targeting the SOCS3 gene were selected to generate a short hairpin
176 RNA using and the following target sense 5'- TCGGGAGTTCCTGGACCAGTA-3' were
177 used (28). The short interference RNA (siRNA) expression vector for SOCS3,
178 pcPUR+U6-SOCS3i, was obtained from Takara (Takara Holdings Inc, Japan) and
179 designed according to the manufacturer's instructions (iGENE Therapeutics, Tsukuba,
180 Japan) using pcPUR+U6i cassette vector (iGENE Therapeutics), which was previously
181 described (10, 16). The pcPUR+U6-GFPi cassette vector was served as the control
182 (control vector). These vectors were introduced into Huh7.5.1 cells to establish stable
183 SOCS3 knocked-down cells. Briefly, the targeting or the control vectors were transfected

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184 into Huh7.5.1 cells using Fugene HD according the manufacturer's protocol, and
185 puromycin (Sigma, Steinheim, Germany) resistant clones or cell pools were selected as
186 stable transfectants. Knockdown of SOCS3 was confirmed by Western blotting.

187 **Statistics.** Data analysis was carried out using the Student's t test with pooled
188 variance. Data were expressed as an average of at least quadruplicate, unless stated
189 otherwise. The significance of differences was calculated by two-way ANOVA. In all
190 analyses, values of $P < 0.05$ were considered statistically significant.

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207 **RESULTS**

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209 **HCV replication decreases SOCS3 protein expression.** Our previous studies have
210 found that nonresponders to peginterferon and ribavirin exhibit higher pretreatment
211 hepatic SOCS3 expression than responders in patients with chronic HCV infection (13),
212 In view of these findings, we hypothesized that SOCS3 has permissive effects on HCV
213 replication. To establish the relationship between HCV replication and SOCS3, we used
214 HCV genotype 1b full-length replicon (OR6) cells and JFH1 infection cells to analyze
215 SOCS3 levels. As Figure 1A shows, Huh7 cells and cured OR6 cells exhibited high
216 expression of SOCS3, while the HCV replicon expressing OR6 cells had nearly
217 undetectable levels of SOCS3. Rescue of low SOCS3 levels occurred after treating OR6
218 cells with the HCV protease inhibitor, BILN 2061 (Fig. 1B). Furthermore, a time course
219 of JFH1-infected Huh7.5.1 cells showed an increase in HCV core protein expression with
220 a corresponding decrease in SOCS3 levels (Fig. 1C). Treatment of the JFH1-infected
221 Huh7.5.1 cells with BILN 2061 restored SOCS3 protein levels and decreased expression
222 of HCV core protein (Fig. 1D).

223 **SOCS3 is degraded in a ubiquitination- and proteasome-dependent manner.** It
224 has been previously reported that HCV core protein increases SOCS3 mRNA levels (2),
225 and that SOCS3 mRNA levels are increased in chimpanzees infected with HCV for 3
226 days (7). In contrast, in our experiments, we observed decreased levels of SOCS3 protein
227 in HCV replicon cells, whereas in JFH1 infected cells SOCS3 levels did not decline in
228 the first 3 days despite high levels of HCV RNA and core protein expression. To explore
229 these differences, we also examined the kinetics of SOCS3 mRNA expression from 1 to
230 28 days following HCV JFH1 infection. Interestingly, we found SOCS3 mRNA levels

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231 were significantly higher than in mock infected cells (Fig. 2A), and that SOCS3 mRNA
232 kinetics mirrored HCV JFH1 infection kinetics (Fig. 2B). We also compared SOCS3
233 mRNA levels in both OR6 cells and cured OR6 cells lacking the HCV replicon and found
234 no significant difference between the two groups (Fig. 2C). These data suggest
235 discordance between SOCS3 mRNA and protein levels. To elucidate the basis for this
236 observed discordance, we examined SOCS3 levels after treatment with the proteasome
237 inhibitor MG132 in both OR6 cells and JFH1-infected cells. Treatment with MG132
238 blocked the inhibitory effect of HCV on SOCS3 protein levels both in OR6 replicon (Fig.
239 2D) and JFH1 infected cells (Fig. 2E). These data suggest that the decrease in SOCS3
240 protein levels in JFH1 infected cells and OR6 cells are attributable to proteasome-
241 dependent degradation of SOCS3 in the presence of HCV. To test the hypothesis that the
242 degradation of SOCS3 is dependent on ubiquitination, we immunoprecipitated SOCS3
243 and used Western blot to detect ubiquitinated SOCS3. Figure 2F shows that JFH1
244 infection significantly increased the ubiquitination of SOCS3 compared to mock
245 infection. These results suggest that JFH1 degrades SOCS3 through a ubiquitination- and
246 proteasome-dependent pathway.

247 **HCV individual protein constructs and transfection of a replication-defective**
248 **viral RNA do not alter SOCS3 expression.** To determine how HCV influences SOCS3
249 protein expression, we overexpressed HCV core, E1, E2, NS3, NS4A, NS4B, NS5A, and
250 NS5B proteins in Huh7.5.1 cells as individual proteins using expression constructs. Fig
251 3A shows that SOCS3 levels were not altered by HCV core, E1, E2, NS3, NS4A, NS4B,
252 NS5A, and NS5B overexpression compared to cells transfected with empty vector
253 (pCAG and pCMV). We also transfected JFH1-GND mutant RNA and JFH1 RNA. As

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254 Figure 3B shows, replication defective JFH1-GND mutant RNA did not alter SOCS3
255 levels. These findings indicate that individually expressed HCV core, E1, E2, NS3,
256 NS4A, NS4B, NS5A, and NS5B proteins do not induce SOCS3, and further that HCV
257 replication appears to be required to induce SOCS3.

258 **SOCS3 overexpression downregulates HCV replication.** To determine whether
259 SOCS3 influences HCV replication, we overexpressed SOCS3 in OR6 replicon cells and
260 JFH1 infected Huh7.5.1 cells. Using Western blotting, Renilla luciferase assay, and RT-
261 qPCR to assess HCV replication in the OR6 replicon cells, we found that SOCS3
262 overexpression downregulated core protein levels (Fig. 4A), decreased HCV replication
263 by *Renilla* Luciferase Assay (rluc/cell viability, SOCS3: 1.6, control cells: 3.3, p=0.0012)
264 (Fig. 4B), and HCV RNA by RT-qPCR (HCV/actin, SOCS3: 2.1, control cells: 5.1,
265 P=0.028) (Fig. 4C), respectively. In addition, JFH1-infected Huh7.5.1 cells
266 overexpressing SOCS3 exhibited decreased HCV core protein (Fig. 5A) and HCV-RNA
267 levels (HCV/actin, SOCS3: 1.0, control cells: 2.4, P=0.043) (Fig. 5B). Interestingly,
268 SOCS3 overexpression also decreased phospho-STAT1 expression in both OR6 cells
269 (Fig. 4A) and JFH1 infected cells (Fig. 5A), and also decreased IFN stimulated ISRE
270 activity (Fig. 4D, 5C). These results suggest that SOCS3 mediated downregulation of
271 HCV replication occurs despite apparent inhibition of conventional type I IFN signaling.
272 They collectively implicate an alternative antiviral pathway induced by SOCS3.

273 **Knockdown of SOCS3 increases HCV replication.** Our findings strongly support
274 the unexpected finding that overexpression of SOCS3 results in a corresponding decrease
275 in HCV replication. However, to further confirm our findings, we used shSOCS3 to
276 knockdown protein expression in Huh7.5.1 cells. Based on our findings, we anticipated

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277 that knockdown of SOCS3 would enhance HCV replication. Indeed, we observed a
278 significantly higher level of HCV core and NS3 proteins in the knockdown cells when
279 compared to control cells (Fig. 6A). Similarly, we also observed significantly higher
280 HCV RNA levels over a 5-day time course in SOCS3 knockdown cells versus control
281 cells using three stable shSOCS3 (Fig. 6B).

282 Previous studies have investigated the relationship between HCV RNA replication
283 and PI3 kinase and mTOR signaling. PI3 kinase has been shown to be activated by a
284 direct interaction between NS5A and the p85 regulatory subunit of PI3K as well as by the
285 enhanced expression of N-Ras in HCV replicon cells. N-Ras-mediated PI3K activation
286 resulted in mTOR activation, and when mTOR was knocked down, HCV abundance was
287 enhanced (15). Ishida et al. also found HCV RNA replication to be suppressed by the
288 mTOR substrate P70 S6K (9). Thus, the evidence supports that activation of the mTOR
289 signaling pathway suppresses HCV replication. To determine whether SOCS3 levels
290 correlate with mTOR levels in the context of HCV infection, we assayed mTOR and
291 Phospho-mTOR levels in shSOCS3 treated, JFH1-infected Huh7.5.1 cells. Indeed, we
292 found that knockdown of SOCS3 also knocked down both mTOR and Phospho-mTOR
293 levels (Fig. 6A).

294 **SOCS3 downregulates HCV replication through the mTOR pathway.** Given our
295 finding that knockdown of SOCS3 also decreased mTOR expression, we suspected that
296 SOCS3 may downregulate HCV replication through the mTOR pathway. To test this
297 hypothesis, we overexpressed SOCS3 in the presence of the mTOR inhibitor rapamycin.
298 We found that inhibition of mTOR could reverse SOCS3's inhibitory effects on HCV

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299 replication in the OR6 replicon cells and JFH1-infected Huh7.5.1 cells (Fig. 7A, B and
300 Fig. 8A, B).
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DISCUSSION

320 We and others have found SOCS3 to be highly expressed in IFN nonresponsive
321 patients (7, 13, 18, 24). Persico *et al*. also found that HCV genotype 1b-infected patients
322 exhibit higher expression of SOCS3 than genotype 2a patients (18). They hypothesized
323 that SOCS3 overexpression may explain why IFN genotype 1b patients do not respond to
324 IFN, and suggest that reducing SOCS3 gene expression may be an approach aimed treat
325 HCV infection (18). However, the hypothesis was based on clinical observation, and
326 lacked substantiation by biological experimentation. In this study, we used the HCV
327 genotype 1b replicon OR6 (8) and HCV infectious model genotype 2a JFH1 (23) to
328 investigate the relationship between SOCS3 and HCV replication. We found that HCV
329 replication does not decrease SOCS3 mRNA levels, but rather decreases SOCS3 protein
330 levels in a proteasome-dependent manner. Interestingly, we found that SOCS3
331 overexpression actually suppresses HCV replication, and does so despite SOCS3's
332 inhibition of classical type I IFN signaling. We confirmed the reciprocal relationship
333 between SOCS3 and HCV replication by knocking down SOCS3, which leads to a
334 significant increase in HCV replication as assayed through Western blotting and real time
335 PCR. Furthermore, SOCS3 levels were restored using the HCV protease inhibitor BILN
336 2061.

337 In addition, we found that knock down of SOCS3 also down regulated mTOR and
338 phospho-mTOR protein levels and that the mTOR inhibitor rapamycin reversed SOCS3's
339 antiviral effects. Taken together, these data indicate that SOCS3 downregulates HCV
340 replication in an mTOR-dependent manner.

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341 HCV is remarkably successful in its ability to establish persistent infection that,
342 unless interrupted by interferon (IFN)-based therapy, will continue for the lifetime of the
343 individual and present opportunities for further transmission within the human population.
344 This success is linked to an ability of HCV to evade and antagonize the immune response
345 of the host and to resist the antiviral actions of IFN therapy. HCV evades the host
346 response through a complex combination of processes that include signaling interference
347 and continual viral genetic variation. These evasion strategies support persistent infection
348 and the spread of HCV (6). It is the hepatic host response that imposes initial immune
349 defenses against HCV infection. The host response is triggered when a pathogen-
350 associated molecular pattern (PAMP) presented by the infecting virus is recognized and
351 engaged by specific PAMP receptor factors expressed in the host cell, initiating signals
352 that ultimately induce the expression of antiviral effectors genes.

353 Several groups have speculated that elevated SOCS3 expression inhibits Phospho-
354 STAT1 expression, which impairs the IFN defense pathway (7, 13, 18, 24). In addition, it
355 has been reported that HCV core protein induces SOCS3 expression in cell lines,
356 resulting in impaired IFN and specifically STAT1 signaling (2, 11, 26). However, we
357 found that Huh7.5.1 cells expressing HCV core protein did not alter SOCS3 levels.
358 Interestingly, SOCS3 overexpression in HCV genotype 1 replicon cells and JFH1
359 infection did result in inhibition of IFN-induced STAT1 phosphorylation, and also
360 blocked IFN stimulated ISRE activity as shown by ISRE reporter assays (Fig. 4D, 5C).
361 Despite this block of IFN-induced P-STAT1 and ISRE activity, overexpression of
362 SOCS3 still clearly produced an inhibitory effect on HCV replication, suggesting that
363 SOCS3's antiviral actions are mediated by independent pathway. We instead found that

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364 SOCS3 stimulation of mTOR, which is independent of IFN signaling (9), may overcome
365 any classical IFN blockade and produce a net antiviral effect against HCV.

366 While it is formally possible that higher levels of SOCS3 are required for more robust
367 inhibition of the Jak-STAT pathway, we still observed an *antiviral* effect at these levels
368 of SOCS3 expression. Our data thus point to an unexpected antiviral action of SOCS3 in
369 HCV infection, one mediated by the mTOR pathway. Furthermore, in a bona fide HCV
370 infection model, there does not appear to be an impairment of IFN's antiviral actions by
371 SOCS3.

372 What about the reciprocal effect of HCV on SOCS3 levels? We consistently observed
373 a decrease of SOCS3 protein levels with prolonged HCV infection in multiple model
374 systems. The apparent dissociation of protein levels with increased mRNA levels
375 suggests an alteration in the posttranscriptional stability of SOCS3 mRNA, or possibly
376 increased degradation of SOCS3 protein. The finding that the proteasome inhibitor
377 MG132 restored SOCS3 protein levels in the face of HCV supports the explanation that
378 HCV infection promotes degradation of SOCS3 protein through a ubiquitination-
379 dependent, proteasomally–mediated pathway. That this is an HCV-dependent
380 phenomenon is reinforced by our finding that administration of the direct antiviral agent
381 BILN 2061 restored levels of SOCS3. This decrease in SOCS3 also appears to depend on
382 HCV replication, since expression of individual HCV core, E1, E2, NS3, NS4A, NS4B,
383 NS5A, and NS5B proteins and the replication-defective JFH1-GND RNA did not
384 reproduce these effects. Thus, HCV replication appears to be associated with decreased
385 SOCS3 levels, which, given the net antiviral effect of SOCS3, could support the
386 persistent infection state.

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387 How can these experimental findings be reconciled with the consistent observation of
388 higher SOCS3 levels in patients who subsequently fail to respond to IFN (7, 12, 17, 23)?
389 As with IFN-stimulated genes, many of whose protein levels are increased in
390 nonresponder patients, this apparently paradoxical finding suggests that there is a net
391 block to antiviral effector genes, and that some antiviral genes may be upregulated in a
392 compensatory manner in response to impaired antiviral defenses elsewhere in the
393 pathway. Alternatively, other, yet to be elucidated antagonistic ISGs may alter the net
394 antiviral state. Exploration of these hypotheses awaits identification of the key IFN-
395 stimulated genes responsible for clearance of HCV.

396 In conclusion, we have demonstrated that SOCS3 suppresses HCV replication in an
397 mTOR-dependent manner, and that this IFN-independent mechanism represents the
398 dominant pathway for SOCS3 downregulation of HCV replication. Efforts to enhance
399 SOCS3 and mTOR function may be a useful adjunctive strategy to control HCV infection.

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406 construct); and Dr Jie Chen, University of Illinois, Urbana, Illinois (pCDNA3-Myc and
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537 ## Figure Legends

538

539 **Figure 1. HCV replication decreases SOCS3 protein expression**

540 Huh7 cells, HCV cured OR6 cells, OR6 cells, Huh7.5.1 cells, and JFH1-infected
541 Huh7.5.1 cells (6, 12 hours, 1, 2, 3, 5, 7 days) were cultured and lysed for Western
542 blotting analysis as described in the *Methods* section. We treated selected cells with peg-
543 IFN 100 U/ml for 10 min (B, D) before lysis. To inhibit HCV replication, we treated
544 cells with 1.6 μM BILN 2061 (final concentration) for 5 days, and then harvested the
545 cells (B, D). Huh7.5.1 cells were infected with JFH1 for 3 days before treatment with
546 BILN 2061 (D). A. HCV replication decreases SOCS3 levels in OR6 HCV replicon cells.
547 B. Inhibition of HCV replication restores SOCS3 levels in the OR6 replicon. C. HCV
548 infection decreases SOCS3 levels. Three independent experiments were performed and
549 analyzed. Top: Representative Western blot. Middle and Bottom: SOCS3 (middle) or
550 core (bottom) expression levels in the Western blot were quantified by densitometry
551 analysis and the ratio to beta-actin was calculated by setting the value of mock infection
552 (middle, SOCS3) or 24 hours post infection (bottom, core) as one. Results are expressed
553 as mean ± SD (n=3). SOCS3 expression levels at 5 and 7 days post-infection compared to
554 mock infection, * $p<0.001$. Core expression levels at 2, 3, 5, and 7 days pi did not change
555 significantly. D. Inhibition of HCV replication restores SOCS3 levels in JFH1-infected
556 cells.

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558 **Figure 2. SOCS3 is degraded in a ubiquitination- and proteasome-dependent**
559 **manner.**
560 Huh7.5.1 cells were infected with JFH1 virus or no-JFH1 virus mock-infected medium.
561 At day 1, 2, 3, 5, 7, 14, 21, 28 post-infection cells were collected, total RNA were
562 isolated and HCV RNA and SOCS3 mRNA were quantified using RT-qPCR as described
563 in the *Methods* section. Cured OR6 cells and OR6 cells were also treated using the same
564 method. Beta-actin mRNA quantification was used for normalization. Data are
565 represented as mean ± SD. Each experiment was performed in triplicate. To determine
566 whether SOCS3 proteins were degraded during HCV replication, the proteasome
567 inhibitor MG132 (20 μM for 24 hours) was added to cured OR6 cells and OR6 cells, and
568 JFH1- and mock-infected cells (5 days post-infection). To determine whether SOCS3
569 protein is degraded through ubiquitination-mediated disposal by the proteasome, cells at
570 5 days post-JFH1 and mock infection were immunoprecipitated using mouse anti-SOCS3
571 and detected on Western blot using SOCS3 and ubiquitin antibodies. A. Time course of
572 SOCS3 mRNA levels in JFH1 infected and mock-infected Huh7.5.1 cells, * $p<0.003$, **
573 $p<0.05$. B. Time course of intracellular HCV RNA in JFH1 infected or mock infected
574 Huh7.5.1 cells. C. HCV cured OR6 cells and OR6 cells SOCS3 mRNA levels. D.
575 Addition of the proteasome inhibitor MG132 blocks the degradation of SOCS3 in OR6
576 cells. E. Addition of the proteasome inhibitor MG132 blocks the degradation of SOCS3
577 in JFH1 infected cells. F. JFH1 infection increased ubiquitinated SOCS3.
578
579 **Figure 3. HCV individual protein constructs and transfection of a replication-**
580 **defective viral RNA do not alter SOCS3 expression**

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581 A. Transfection of Huh7.5.1 cells with the empty vector pCAG and pCMV, or pCMV-
582 driven vectors containing full-length HCV Core (pCAG-C191), structural proteins
583 (containing core-E1-E2), NS3-4A, NS4B, NS5A, and NS5B was performed for 72 hours
584 before lysing cells for Western blot. Lane 1: pCAG empty vector, lane 2: pCMV empty
585 vector, lane 3: full-length HCV core pCAG-C191, lane 4: pCMV-HCVst (containing
586 core-E1-E2), lane 5: pCMV NS3-4A, lane 6: pCMV NS4B, lane 7: pCMV NS5A, lane 8:
587 pCMV NS5B. B. At the different times indicated post-transfection with JFH1 and mutant
588 JFH1-GND RNA, Huh7.5.1 cells were lysed using RIPA buffer and Western blot
589 performed as described in *Methods*.
590
591 **Figure 4. SOCS 3 downregulates HCV replication in the OR6 replicon**
592 OR6 cells were transfected with pCDNA3-Myc or pCDNA3-Myc-hSOCS3 plasmids, and
593 72 hours later the cells were lysed for Western blotting, *Renilla* luciferase Assay, RT-
594 qPCR according to *Methods*. To determine whether SOCS3 influences the IFN signaling
595 pathway, the ISRE-luc reporter plasmid was co-transfected with pCDNA3-Myc or
596 pCDNA3-Myc-hSOCS3 plasmids. Luciferase assays were performed according to
597 *Methods*. Peg-IFN at a dose of 100 U/ml was added 24 hours before cells were harvested
598 (A, C, D, E). The cells were seeded in 96-well plates and cell viability assays performed
599 according to *Methods*. A. Western blotting revealed that overexpression of SOCS3
600 decreased HCV core protein levels. To distinguish between endogenous and exogenous
601 expressed SOCS3 we used mouse anti-SOCS3 for endogenous SOCS3, and rabbit anti-
602 SOCS3 for exogenous SOCS3. B. *Renilla* luciferase Assay results show that
603 overexpression of SOCS3 decreases HCV RNA replication. OR6 cells (5000 cells/well)

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604 were plated in a 96-well plate for one day prior to being transfected with pCDNA3-Myc
605 or pCDNA3-Myc-hSOCS3. Results were normalized to cell viability. Data represent the
606 mean ± SD of three independent experiments,*p=0.0012. C. HCV RNA measured by RT-
607 qPCR. Results were normalized to beta-actin. Data represent the mean ± SD of three
608 independent experiments, *p=0.028. D. Luciferase assays results show that
609 overexpression of SOCS3 decreases IFN stimulated ISRE-luciferase activity. Results
610 were normalized to cell viability. Data represent the mean ± SD of three independent
611 experiments, *p=0.0008. E. Cell viability assay.
612
613 **Figure 5. SOCS 3 downregulates HCV replication in JFH1-infected Cells**
614 After 24 hours transfection with pCDNA3-Myc or pCDNA3-Myc-hSOCS3 plasmids
615 Huh7.5.1 cells were infected with JFH1. At 72 hours post infection, the Western blotting
616 and Q-PCR were performed according to the *Methods* section, as described. To determine
617 whether SOCS3 influences the IFN signaling pathway, the ISRE-luc reporter plasmid
618 was co-transfected with pCDNA3-Myc or pCDNA3-Myc-hSOCS3 plasmids. Luciferase
619 assays were performed according to *Methods*. Peg-IFN at a dose of 100 U/ml was added
620 to the cells for 24 hours before cells were collected. The cells were seeded in 96-well
621 plates and cell viability assays performed according to *Methods.* A. Overexpression of
622 SOCS3 decreases HCV core proteins levels, as assayed by Western blotting. To
623 distinguish between endogenous and exogenous expressed SOCS3 we used mouse anti-
624 SOCS3 for endogenous SOCS3, and rabbit anti-SOCS3 for exogenous SOCS3. B.
625 Overexpression of SOCS3 decreases HCV RNA replication, as exhibited by HCV RNA
626 measured by RT-qPCR. Results were normalized to beta-actin (data represent the mean ±

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627 SD of three independent experiments. *p=0.034). C. Overexpression of SOCS3 decreases
628 IFN stimulated ISRE-luciferase activity. Results were normalized to pRL-TK *Renilla*
629 luciferase. Data represent the mean ± SD of three independent experiments. *p=0.0002.
630 D. Cell viability assay.
631
632 **Figure 6. SOCS3 knockdown increases HCV replication in JFH1-infected cells**
633 Stable shSOCS3 knockdown in Huh7.5.1 cells was established according to the *Methods*.
634 We infected with inoculum from Huh7.5.1 JFH1 harboring cells and transfected cells
635 with U6shGFP knockdown control cells, pU6 empty vector and U6shSOCS3 knockdown
636 cells. A. Seventy-two hours post infection, cells were collected for Western blot. Lane 1,5:
637 pU6 shGFP, lane 6: pU6 empty vector, Lane 2, 7: stable pU6shSOCS3 cells line 1, Lane
638 3, 8: stable pU6shSOCS3 cells line 2, Lane 4, 9: stable pU6shSOCS3 cells line 3. The
639 results show that knockdown of SOCS3 increases HCV core and NS3 protein levels.
640 Knock down of SOCS3 expression also downregulates mTOR protein expression. B.
641 After infection for 1, 2, 3, and 5 days, cells were collected and RNA purified, HCV RNA
642 measured by Q-PCR, results were normalized to beta-actin. Data represent the mean ±
643 SD of three independent experiments. *p< 0.004, ** p< 0.002. HCV RNA replication
644 was significantly increased in shSOCS3 knock down cells compared to control shGFP
645 knockdown cells.
646
647 **Figure 7. mTOR inhibition reverses SOCS3's inhibitory effects on HCV replication**
648 **in the OR6 replicon**

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649 The OR6 cells and OR6 cured cells were transfected with pCDNA3-Myc or pCDNA3-
650 Myc-hSOCS3 plasmids for 24 hours, and then treated with 100 nM rapamycin (final
651 concentration). After 72 hours the addition of the pCDNA3-Myc or pCDNA3-Myc-
652 hSOCS3 constructs, the cells were collected and lysed for Western blotting and *Renilla*
653 luciferase Assay. The cells were seeded in 96-well plates and cell viability assays
654 performed according to *Methods*. A. The mTOR inhibitor rapamycin reverses SOCS3's
655 inhibit HCV core proteins levels, as assayed by Western blotting. To distinguish between
656 endogenous and exogenous expressed SOCS3, we used mouse anti-SOCS3 for
657 endogenous SOCS3, and rabbit anti-SOCS3 for exogenous SOCS3. B. Rapamycin
658 reverses SOCS3's inhibition of HCV RNA replication. The *Renilla* luciferase Assay
659 results were normalized for cell viability. Data represent the mean ± SD of three
660 independent experiments. *p=0.00004, **p=0.00003. C. Cell viability assay.
661
662 **Figure 8. mTOR inhibition reverses SOCS3's inhibitory effects on HCV replication**
663 **in JFH1-infected cells**
664 Huh7.5.1 cells were transfected with pCDNA3-Myc or pCDNA3-Myc-hSOCS3 plasmids.
665 24 hours after transfection the cells were mock infected or infected with JFH1 inoculum,
666 and treated with 100 nM rapamycin. 72 hours after the addition of the pCDNA constructs,
667 the cells were lysed for Western blotting and RT-qPCR. The cells were seeded in 96-well
668 plates and cell viability assays performed according to *Methods*. A. Western blotting
669 results show that mTOR inhibitor rapamycin reverses SOCS3's inhibition of HCV core
670 protein levels. To distinguish between endogenous and exogenous expressed SOCS3, we
671 used mouse anti-SOCS3 for endogenous SOCS3, and rabbit anti-SOCS3 for exogenous

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672 SOCS3. B. The mTOR inhibitor rapamycin reverses SOCS3's inhibition of HCV RNA
673 replication. HCV RNA was measured by Q-PCR, results were normalized to beta-actin.
674 Data represent the mean ± SD of three independent experiments. *p=0.04, **p=0.004. C.
675 Cell viability assay.
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698 **Figure 1. HCV replication decreases SOCS3 protein levels**

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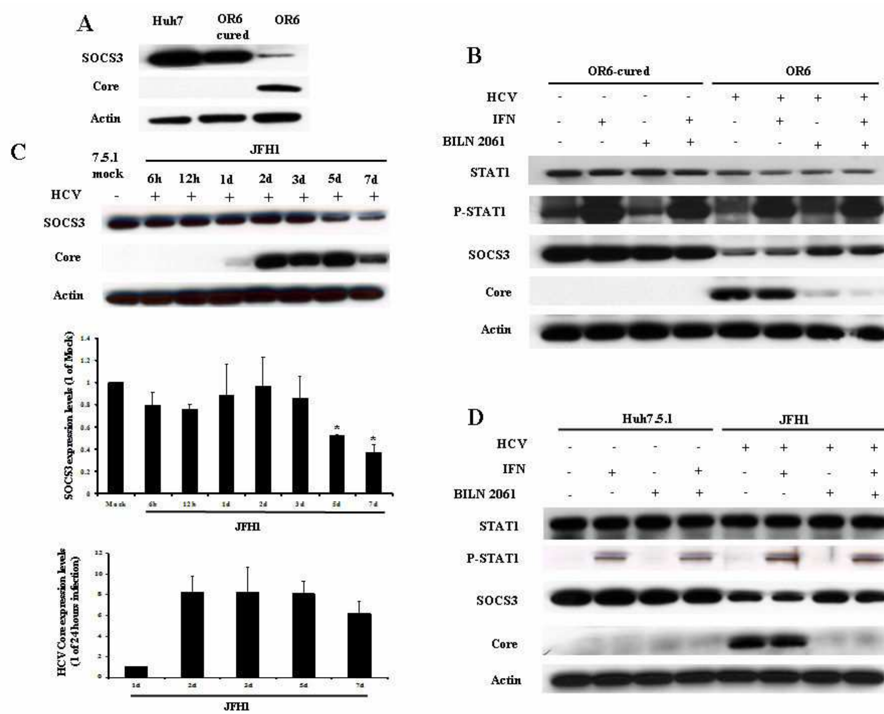

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710 **Figure 2. SOCS3 is degraded in a ubiquitination- and proteasome-dependent**

711 **manner**

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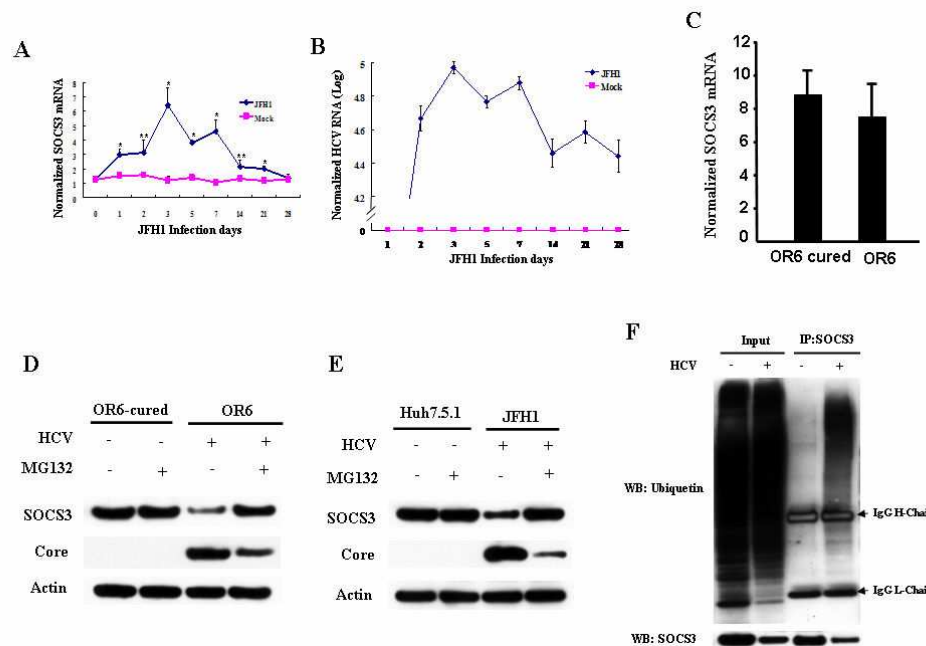

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722 **Figure 3. HCV individual protein constructs and transfection of a replication-**

723 **defective viral RNA do not alter SOCS3 expression**

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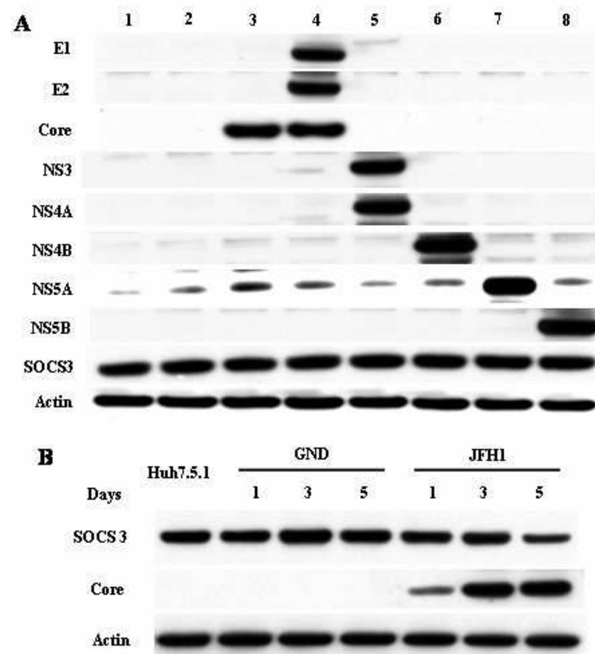

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735 **Figure 4. SOCS 3 downregulates HCV replication in the OR6 replicon**

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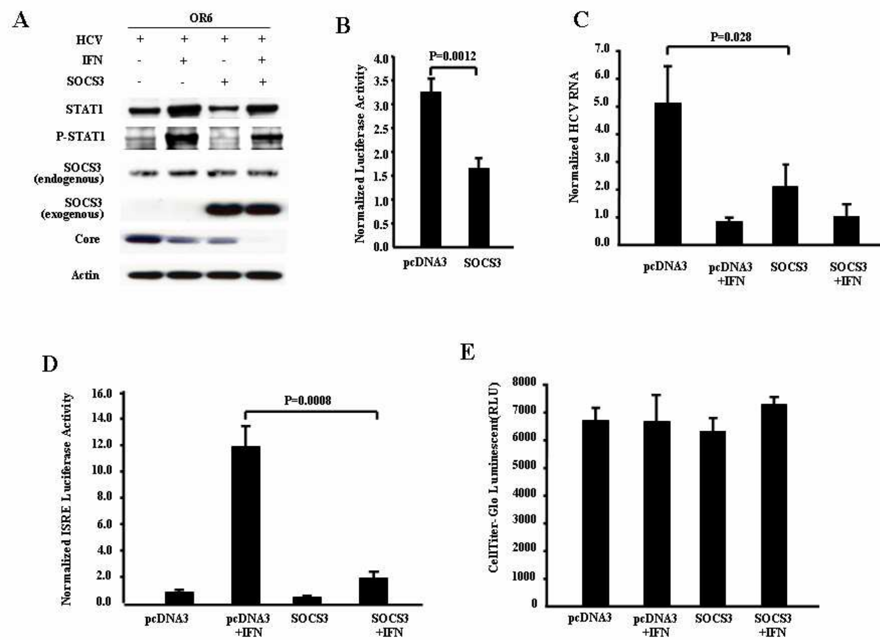

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747 **Figure 5. SOCS 3 downregulates HCV replication in JFH1-infected Cells**

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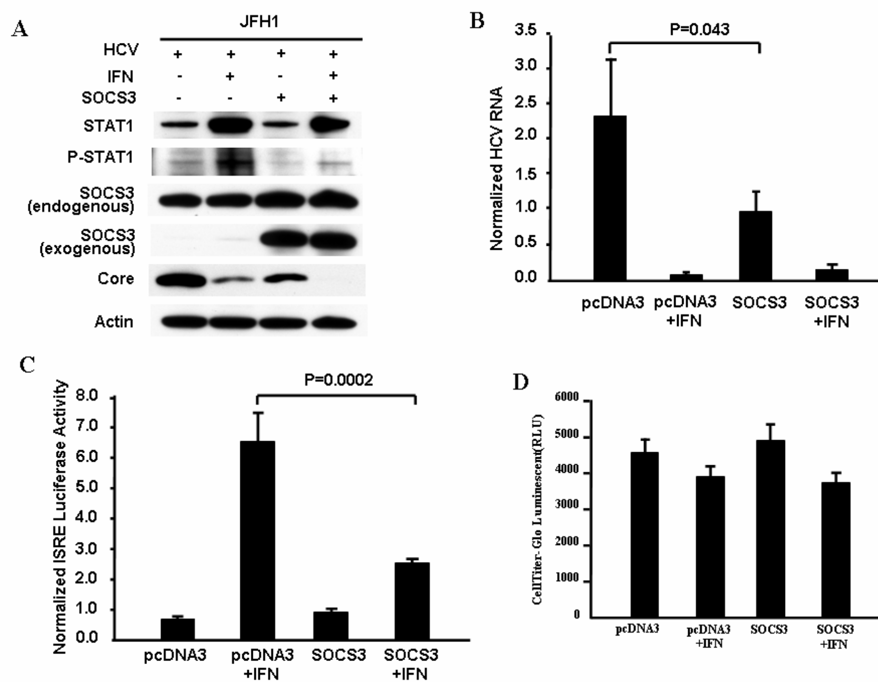

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759 **Figure 6. SOCS3 knockdown increases HCV replication in JFH1-infected cells**

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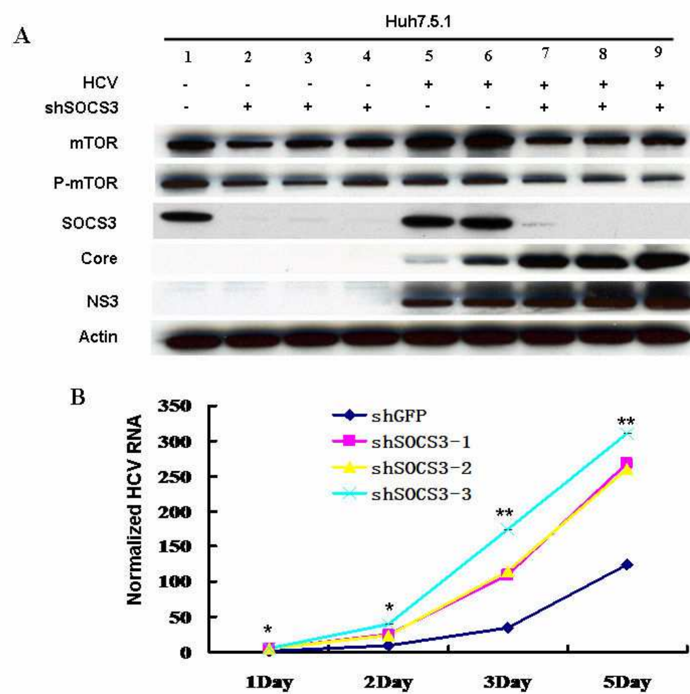

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771 **Figure 7. mTOR inhibition reverses SOCS3's inhibitory effects on HCV replication**

772 **in the OR6 replicon**

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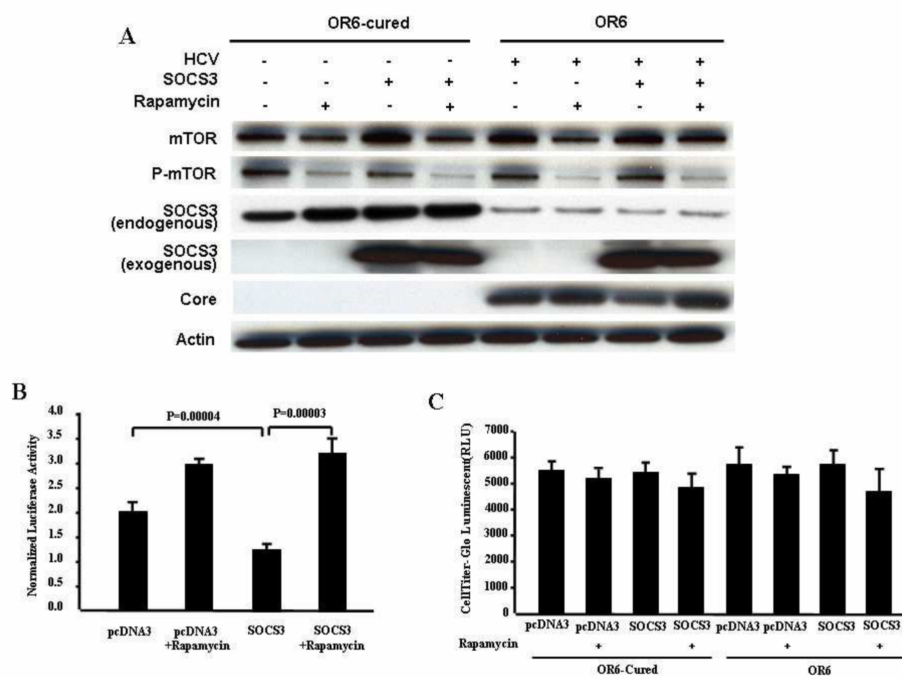

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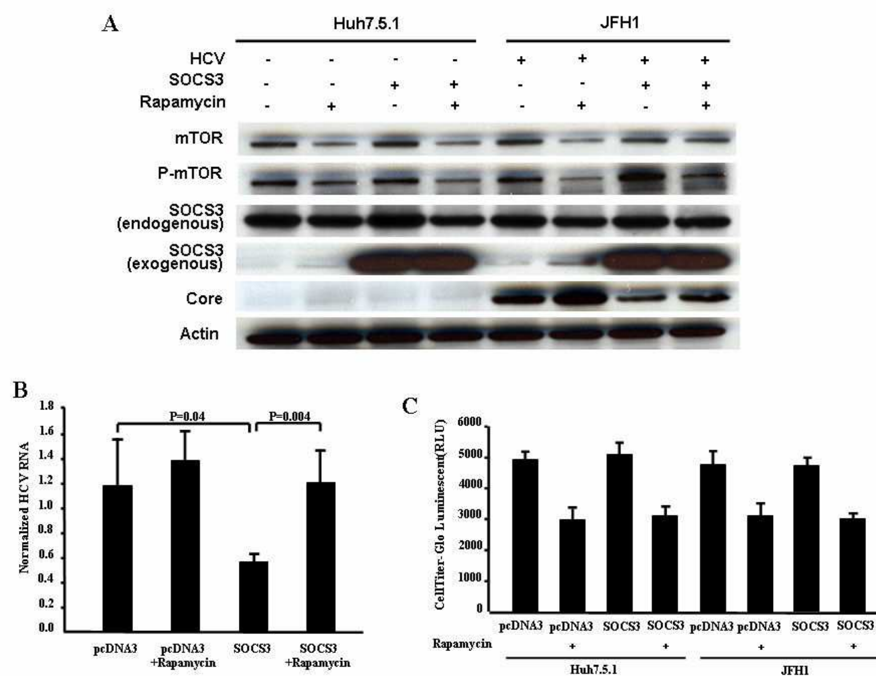

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