Hepatitis C virus NS5B triggers an MDA5-mediated innate immune response by producing dsRNA without the replication of viral genomes.

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Running title: NS5B activates MDA5 by producing non-viral dsRNA.

Abbreviations: dsRNA, double-stranded RNA; RdRp, RNA-dependent RNA polymerase; IFN, interferon; IRF, IFN regulatory factor; RIG-I, retinoic acid-induced gene-I; TLR3, Toll-like receptor 3; MDA5, melanoma differentiation-associated gene 5; HCV, hepatitis C virus; GBV-B, GB virus B; E1, envelope 1; NS2, nonstructural protein 2; CARD, caspase recruitment domain; TRAF, TNF receptor-associated factor; ISGs, interferon-stimulated genes; PKR, protein kinase R; TERT, telomerase reverse transcriptase; siRNA, small interfering RNA; PI, propidium iodide; DAPI, 4'-6-diamino-2-phenylindole.

Keywords: Hepatitis C virus; double-stranded RNA; RNA virus; RIG-I-like receptor; innate immunity

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Abstract

During the replication of viral genomes, RNA viruses produce double-stranded RNA (dsRNA), through the activity of their RNA-dependent RNA polymerases (RdRps) as viral replication intermediates. Recognition of viral dsRNA by host pattern recognition receptors – such as retinoic acid-induced gene-I (RIG-I)-like receptors and Toll-like receptor 3 (TLR3) – triggers the production of interferon (IFN)-β via the activation of IFN regulatory factor (IRF)-3. It has been proposed that, during the replication of viral genomes, each of RIG-I and melanoma differentiation-associated gene 5 (MDA5) form homodimers for the efficient activation of a downstream signaling pathway in host cells. We previously reported that, in the non-neoplastic human hepatocyte line PH5CH8, the RNA-dependent RNA polymerase NS5B derived from hepatitis C virus (HCV) could induce IFN-β expression by its RdRp activity without the actual replication of viral genomes. However, the exact mechanism by which HCV NS5B produced IFN-β remained unknown. In the present study, we first showed that NS5B derived from another *Flaviviridae* family member, GB virus B (GBV-B), also possessed the ability to induce IFN-β in PH5CH8 cells. Similarly, HCV NS5B, but not its G317V mutant, which lacks

RdRp activity, induced the dimerization of MDA5 and subsequently the activation of IRF-3. Interestingly, immunofluorescence analysis showed that HCV NS5B produced dsRNA. Like HCV NS5B, GBV-B NS5B also triggered the production of dsRNA and subsequently the dimerization of MDA5. Taken together, our results show that HCV NS5B triggers an MDA5-mediated innate immune response by producing dsRNA without the replication of viral genomes in human hepatocytes.

Introduction

Hepatitis C virus (HCV) is an enveloped positive single-stranded RNA virus belonging to the *Flaviviridae* family. The HCV genome encodes a large polyprotein precursor of approximately 3000 amino acid residues, which is co- and post-translationally cleaved into at least ten proteins in the following order: core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B. NS5B is otherwise known as RNA-dependent RNA polymerase (RdRp), which plays an important role in the replication of viral genomes. During the replication of viral genomes, HCV frequently causes chronic hepatitis, which finally progresses to liver cirrhosis and hepatocellular

carcinoma [1, 2]. To understand how these HCV-related liver diseases occur, it will be necessary to gather more information about the host innate immune response against the replication of viral genomes.

During the replication of viral genomes, RNA viruses are known to produce double-stranded RNA (dsRNA) as viral replication intermediates via the actions of their RdRps. DsRNA is recognized by the host pattern recognition receptors, retinoic acid-induced gene I (RIG-I)-like receptors and Toll-like receptor 3 (TLR3) [3]. RIG-I-like receptors, RIG-I and melanoma differentiation-associated gene 5 (MDA5), contain the N-terminal caspase recruitment domain (CARD), the central DexD/H-box helicase domain, and the C-terminal regulatory domain. The binding of dsRNA to the helicase domain has been proposed to cause the conformational change and subsequently the dimerization of RIG-I or MDA5, respectively [4, 5, 6]. After dimerization, RIG-I or MDA5 interacts with their adaptor protein Cardif (also called IPS-1, VISA or MAVS) through the CARD–CARD interaction [7]. The interaction between RIG-I/MDA5 and Cardif recruits downstream signaling molecules such as TNF receptor-associated factor (TRAF) family members (TRAF3 and TRAF6) and IKK family members (IKKε, TBK1, and IKKα) to activate

IFN regulatory factor (IRF)-3 and NF-κB, respectively. TRAF3 and TRAF6 are E3 ubiquitin ligases, and they function as the major regulators for the activation of IRF-3 and NF-κB, respectively. Finally, phosphorylated IRF-3 forms a dimer and subsequently translocates to the nucleus to induce IFN-β.

We previously reported that HCV NS5B induced IFN-β by its RdRp activity without the replication of viral genomes in the non-neoplastic human hepatocyte lines PH5CH8 and NKNT-3 [8, 9]. However, these previous studies did not elucidate exactly how HCV NS5B induced IFN-β. In the present study, we showed that HCV NS5B triggered the MDA5-mediated innate immune response by producing dsRNA without the replication of viral genomes. Like HCV NS5B, NS5B derived from another *Flaviviridae* family member, namely GB virus B (GBV-B), also triggered both the production of dsRNA and the dimerization of MDA5, leading to the induction of IFN-β. Here, we propose the novel mechanism by which HCV NS5B triggers the MDA5-mediated host innate immune response by producing dsRNA without the replication of viral genomes.

Results

Like HCV NS5B, GBV-B NS5B possesses the ability to induce IFN-β.

We previously reported that in both PH5CH8 and NKNT-3 cells, NS5B derived from HCV could induce IFN-β by its RdRp activity without the actual replication of viral genomes [8, 9]. As one of its biological functions, IFN-β is known to show antiviral activity against HCV. By using our OR6 assay system, which is capable of measuring HCV RNA replication levels by *Renilla* luciferase activity [10], we first examined whether the culture media derived from HCV NS5B-expressing PH5CH8 cells (hereinafter PH5CH8/NS5B cells) showed antiviral activity against HCV. HCV RNA replication levels were decreased by the addition of the culture media derived from PH5CH8/NS5B cells but not the G317V mutant of HCV NS5B-expressing PH5CH8 cells (hereinafter PH5CH8/NS5B G317V cells: NS5B G317V lacks RdRp activity) in OR6 cells (Fig. 1A). Consistent with this result, interferon-stimulated genes (ISGs) such as RIG-I, MDA5, LGP2, and IRF-7 were also induced in PH5CH8/NS5B cells but not in PH5CH8/NS5B G317V cells (Fig. 1B). We next examined whether NS5B derived from GBV-B, a member of the *Flaviviridae* family, induced IFN-β mRNA in PH5CH8 cells. IFN-β mRNA (Fig. 1C) and subsequently, ISGs (Fig. 1D), were induced in GBV-B

NS5B-expressing PH5CH8 cells (hereinafter PH5CH8/HA-NS5B (GBV-B) cells), just as in HCV NS5B-expressing PH5CH8 cells (hereinafter PH5CH8/HA-NS5B (HCV) cells). These results suggested that, like HCV NS5B, GBV-B NS5B possessed the ability to induce IFN-β in PH5CH8 cells.

HCV NS5B triggered the phosphorylation and dimerization of IRF-3 to induce IFN-β.

We previously reported that HCV NS3-4A completely blocked HCV NS5B-mediated IFN-β induction in PH5CH8 cells [8]. HCV NS3-4A is known to prevent IFN-β induction to block the phosphorylation and subsequently the dimerization of IRF-3 [11]. From these results, we predicted that IRF-3 was required for HCV NS5B-mediated IFN-β induction. Quantitative RT-PCR analysis revealed that the knockdown of IRF-3 drastically decreased the induction of IFN-β mRNA in PH5CH8/NS5B cells (Fig. 2A). However, unexpectedly, we could not detect the dimerization of endogenous IRF-3 in PH5CH8/NS5B cells (Fig. 2B). One possible explanation is that phosphorylated IRF-3 is degraded after its nuclear translocation in PH5CH8/NS5B cells. By using pEGFP-C1/IRF-3 mNLS vector that abrogates the nuclear localization signal of phosphorylated

129 IRF-3 [12], we detected the phosphorylation and subsequent dimerization of exogenous
130 IRF-3 in PH5CH8/NS5B cells (Fig. 2C). Another possibility is that the phosphorylated
131 IRF-3 is regulated by TRAF3 or TRAF6 in PH5CH8/NS5B cells. The knockdown of
132 TRAF6, but not TRAF3, caused the dimerization of endogenous IRF-3 in PH5CH8/NS5B
133 cells (Figs. 2D and 2E). Consistent with the enhancement of IRF-3 dimer, the induction
134 of IFN-β mRNA was also increased by the knockdown of TRAF6 in PH5CH8/NS5B
135 cells (Fig. 2F). These results suggested that the dimerization of IRF-3 was regulated at
136 multiple steps during HCV NS5B-mediated IFN-β induction.
137
138 **HCV NS5B triggered the dimerization of MDA5 to induce IFN-β.**
139 IRF-3 is phosphorylated through a TLR3-mediated signaling pathway (M-polyIC) and/or
140 RIG-I/MDA5-mediated signaling pathway (T-polyIC) in PH5CH8 cells ([13], Fig. 2C).
141 We previously reported that, in PH5CH8 cells, HCV NS5B could induce IFN-β via TLR3
142 but not via RIG-I [9]. However, we have no information about whether HCV NS5B
143 induces IFN-β via MDA5. Quantitative RT-PCR analysis revealed that the knockdown
144 of MDA5 drastically decreased the induction of IFN-β mRNA in PH5CH8/NS5B cells

145 (Fig. 3A). The binding of dsRNA to MDA5 is proposed to cause the dimerization
146 necessary to activate its downstream signaling pathway [5, 6]. We detected a clear single
147 band of MDA5 in unstimulated PH5CH8 cells (T-polyIC (-)) while we also detected a
148 slow migrating band of MDA5 in PH5CH8 cells transfected with polyIC (T-polyIC (+))
149 (Fig. 3B), suggesting that intracellular polyIC triggers the dimerization of MDA5. The
150 dimerization of MDA5 was also detected in both PH5CH8/NS5B cells (Fig. 3B) and
151 PH5CH8/HA-NS5B (GBV-B) cells (Fig. 3C). These results suggested that HCV NS5B
152 triggered the dimerization of MDA5 to induce IFN-β.
153
154 **HCV NS5B produced dsRNA by its RdRp activity without viral genomes.**
155 By immunofluorescence analysis using a monoclonal antibody specific for dsRNA [14],
156 the intracellular viral dsRNA has been observed in cells infected by vesicular stomatitis
157 virus (VSV), encephalomyocarditis virus (EMCV), and HCV (JFH-1 strain) [15, 16]. To
158 confirm the specificity and sensitivity of immunofluorescence analysis using this
159 monoclonal antibody, we first tried to detect intracellular dsRNA in PH5CH8 cells
160 transfected with the dsRNA analog, polyIC. As a result, intracellular polyIC but not the

161 dsDNA analog, polydAdT, was detected in PH5CH8 cells (Fig. 4A). PolyIC was also
162 detected in the cytoplasm of PH5CH8 cells when it was added to the culture media (Fig.
163 4A). Interestingly, intracellular dsRNA was detected in PH5CH8/NS5B cells but not in
164 PH5CH8/NS5B G317V cells, suggesting that its RdRp activity was required for dsRNA
165 production (Fig. 4B). Intracellular dsRNA was also detected in PH5CH8/HA-NS5B
166 (GBV-B) cells as well as PH5CH8/HA-NS5B (HCV) cells (Fig. 4C). These results
167 suggested that HCV NS5B produced dsRNA by its RdRp activity without the replication
168 of viral genomes in PH5CH8 cells.
169

170 ## Discussion

171 RNA viruses produce viral dsRNA as the replication intermediates by their RdRps. HCV
172 NS5B forms the replication complex with other HCV nonstructural proteins and
173 subsequently produces viral dsRNA by its RdRp during the replication of viral genomes
174 on the lipid raft [17]. In the present study, we introduce the novel discovery that HCV
175 NS5B through its RdRp activity possesses the ability to produce dsRNA without the
176 replication of viral genomes, leading to the induction of IFN-β through an MDA5-

177 mediated innate immune response (Fig. 5). The dsRNA structure was partially composed
178 of several stem-loop structures within the NS5B-coding region [18]. In our experiments,
179 however, since the NS5B G317V mutant did not induce the dimerization of either MDA5
180 (Fig. 3B) or, subsequently, IRF-3 (Fig. 2C), we were able to exclude the possibility that
181 MDA5 recognized the dsRNA structure within the NS5B-coding region.
182 HCV NS5B has been reported to activate protein kinase R (PKR) without the replication
183 of viral genomes via its RdRp activity [19]. Since PKR is activated by dsRNA, HCV
184 NS5B-produced dsRNA was predicted to activate PKR. On the other hand, interestingly,
185 human telomerase reverse transcriptase (TERT) also possesses RdRP activity and
186 produces dsRNA that can be processed into small interfering RNA (siRNA) duplexes in
187 a Dicer-dependent manner [20, 21]. However, it is uncertain whether an siRNA duplex is
188 also generated from the HCV NS5B-produced dsRNA. RIG-I and MDA5 were previously
189 reported to recognize different lengths of dsRNA [15]: shorter dsRNA, which serves as a
190 ligand for RIG-I, and longer dsRNA, the ligand for MDA5. Since HCV NS5B-mediated
191 IFN-β induction did not require RIG-I [9], HCV NS5B may produce only long dsRNA.
192 On the other hands, both HCV NS5B and siRNA are reported to localize at the perinuclear

193 region under the transient expression in the cells, respectively [22, 23]. HCV NS5B,
194 which is not involved in the replication complex, may generate siRNA duplex via the
195 production of dsRNA at the perinuclear region. We need to further examine whether
196 siRNA duplexes are generated from HCV NS5B-produced dsRNA as has been shown for
197 human TERT.
198 At the early stage of viral infection, IRF-3 is phosphorylated at two C-terminal phospho-
199 accepter clusters (Ser385-Ser386 and Ser396-Ser398-Ser402-Thr404-Ser405), causing
200 the activation of IRF-3 (dimerization and subsequent nuclear translocation), leading to
201 the induction of IFN-β. On the other hand, to control the induction of IFN-β, IRF-3 is
202 also phosphorylated at Ser339, which causes polyubiquitination and subsequent
203 degradation in a proteasome-dependent manner [24]. In the present study, we showed that
204 the dimerization of phosphorylated IRF-3 was regulated at multiple steps. One is the
205 regulation of phosphorylated IRF-3 after its translocation to the nucleus (Fig. 2C); the
206 other is the regulation of phosphorylated IRF-3 by TRAF6 (Fig. 2E). TRAF6 possesses
207 E3 ubiquitin ligase activity and controls signal transduction by promoting K48- or K63-
208 linked ubiquitination of target host factors [25-28]. In contrast to K63-linked

209 ubiquitination, K48-linked ubiquitination is known to induce the proteasome-dependent
210 degradation of target host factors. TRAF6 forms a complex with CD40 and TRANCE-R
211 (also known as RANK) through the P-X-E-X-X-(aromatic/acidic residue) TRAF6-
212 binding motif for signal transduction [26]. Since this TRAF6-binding motif is present in
213 other IRF family members, IRF-5 (392-PFEIFF-397) and IRF-7 (211-PGEGQE-216,
214 334-PAELPD-339), IRF-5 and IRF-7, are positively regulated by TRAF6-mediated K63-
215 linked ubiquitination, respectively [27, 28]. On the other hands, since this TRAF6-
216 binding motif is also present in IRF-3 (198-PGEEWE-203), TRAF6 may negatively
217 regulate IFN-β induction through ubiquitination of phosphorylated IRF-3. We need to
218 examine further the exact mechanism by which TRAF6 controls IFN-β induction through
219 the regulation of phosphorylated IRF-3.
220 In conclusion, we suggest that HCV NS5B triggered an MDA5-mediated innate immune
221 response by producing dsRNA without the replication of viral genomes. Our findings
222 provide novel insights into the molecular mechanisms of host innate immunity against
223 HCV.
224

Materials and Methods

Cell culture

The non-neoplastic human hepatocyte line, PH5CH8 (RRID: CVCL_VL00) [29] were cultured as reported previously [30]. The human hepatoma cell line, OR6 (RRID: CVCL_VN30), which efficiently replicate genome-length HCV-O RNA with *Renilla* luciferase, were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum [10]. These cell lines were obtained from the properly maintained frozen mycoplasma-free stock in Okayama University. These cell lines were also used at low passages without further authentication or testing for mycoplasma contamination.

Construction of expression vectors

The retroviral vector pCX4bsr (accession no. AB086384) [31], which contains the resistance gene for blasticidin, was used to construct the various expression vectors. To construct pCX4bsr/NS5B G317V, the BamHI-NotI fragment of pCXbsr/NS5B G317V [8] was replaced with the BamHI-NotI region of pCX4bsr. To construct pCX4bsr/HA-

241 NS5B (HCV), the EcoRI-NotI fragment of pCXbsr/HA-NS5B (HCV) [9] was replaced
242 with the EcoRI-NotI region of pCX4bsr. To construct pCX4bsr/HA-NS5B (GBV-B),
243 DNA fragments encoding GBV-B NS5B were amplified from the infectious clone of
244 GBV-B (pGBB) [32] by PCR using KOD-plus DNA polymerase (Toyobo, Osaka, Japan)
245 [33]. pEGFP-C1/IRF-3 mNLS vector was constructed from pEGFP-C1/IRF-3 vector [13]
246 by PCR mutagenesis with primers containing base alterations. The nucleotide sequence
247 was confirmed by Big Dye termination cycle sequencing using an ABI Prism 310 genetic
248 analyzer (Applied Biosystems, Foster City, CA).
249
250 **Preparation of PH5CH8 cells stably expressing HCV or GBV-B NS5B**
251 Five retroviral vectors, pCX4bsr, pCX4bsr/NS5B, pCX4bsr/NS5B G317V,
252 pCX4bsr/HA-NS5B (HCV), and pCX4bsr/HA-NS5B (GBV-B), were used for the
253 retrovirus infection [34]. At two days post-infection, PH5CH8 cells were supplied with
254 fresh medium containing blasticidin (20 μg/mL), and the culture was continued for seven
255 days to select PH5CH8 cells expressing NS5B, NS5B G317V, HA-NS5B (HCV), and
256 HA-NS5B (GBV-B), respectively. As negative control, PH5CH8/Cont cells were also

257 prepared by the retroviral transfer of pCX4bsr. The culture media were harvested from
258 PH5CH8/NS5B cells to examine the antiviral activity as described below.
259
260 **OR6 assay system**
261 PH5CH8/NS5B cells were cultured in 10 mL of fresh medium without blasticidin for 5
262 days. The culture media of PH5CH8/NS5B cells (50 μL) was added to that (2 mL) of
263 OR6 cells, and 72 h later, *Renilla* luciferase activity was measured according to the
264 manufacturer's protocol (Promega, Madison, WI). Luciferase activity was calculated
265 relative to the activity in OR6 cells treated without IFN-β, which was set at 100. Data are
266 the means ± SD from three independent experiments.
267
268 **Western blot analysis and the antibody used in this study**
269 Preparation of cell lysates, SDS-PAGE, and immunoblotting were performed as described
270 previously [35]. Immunocomplexes were detected by a Renaissance enhanced
271 chemiluminescence assay (Perkin-Elmer Life Sciences, Waltham, MA).
272 Anti-NS5B antibody was a gift from Dr. M. Kohara. Anti-RIG-I antibody, anti-MDA5

antibody, and anti-LGP2 antibody were purchased from Immuno-Biological Laboratories (Gunma, Japan). Anti-IRF-3 antibody (FL-425) and anti-IRF-7 antibody (H-246) were purchased from Santa Cruz Biotechnology (Dallas, Tx). Anti-phospho-IRF-3 (Ser386) antibody (Upstate Biotechnology, Lake Placid, NY) was used for the detection of phosphorylated IRF-3 at Ser386. Anti-HA antibody (3F10; Roche, Mannheim, Germany) was used for the detection of HA-tagged NS5B. Monoclonal antibody (K1) specific for dsRNA was purchased from English and Scientific Consulting (Szirák, Hungary). Anti-EGFP antibody (JL-8) was purchased from Clontech (Takara Bio Inc., Shiga, Japan). Anti-TRAF3 antibody and anti-TRAF6 antibody were purchased from Cell Signaling Technology (Danvers, MA).

RNA interference and real-time LightCycler PCR

SiRNA duplexes targeting the coding regions of MDA5 (M-013041-00), IRF-3 (M-006875-01), TRAF3 (M-005252-00), TRAF6 (M-004712-00), and luciferase GL2 (D-001100-01) as a control were purchased from Horizon Discovery (Cambridge, UK). Cells were transfected with the indicated siRNA duplex using DharmaFECT transfection

289 reagent (Horizon Discovery) [36]. The extraction of total cellular RNA and real-time
290 LightCycler PCR of IFN-β were performed as described previously [37]. Data are the
291 means ± SD from three independent experiments.
292
293 **Dimerization analysis of IRF-3 or MDA5**
294 Preparation of cell lysates, native-polyacrylamide gel electrophoresis and
295 immunoblotting was performed as described previously [13]. Anti-MDA5 antibody, anti-
296 IRF-3 antibody, and anti-EGFP antibody were used to detect the activation of MDA5 and
297 IRF-3, respectively.
298
299 **Immunofluorescence analysis**
300 The intracellular localizations of NS5B and dsRNA were photographed under a confocal
301 laser-scanning microscope (LSM510; Carl Zeiss, Oberkochen, Germany) as previously
302 described [38]. Anti-HA antibody (3F10; Roche) and FITC-conjugated donkey anti-rat
303 secondary antibody were used for the visualization of HA-tagged NS5B. Monoclonal
304 antibody (K1) specific for dsRNA together with Cy2- (or FITC)-conjugated donkey anti-

305 mouse secondary antibody were used for the visualization of intracellular dsRNA.
306 Propidium iodide (PI: Sigma-Aldrich, St. Louis, MO) or 4'-6-diamino-2-phenylindole
307 (DAPI: Sigma-Aldrich) was used for the visualization of nuclei. As a control for the
308 visualization of dsRNA, PH5CH8 cells were treated with the dsRNA analog, polyIC, or
309 the dsDNA analog, polydAdT (Invivogen, Pak Shek Kok, Hong Kong).
310

311 **Statistical analysis**

312 To determine the significance of differences among groups, statistical analysis was
313 performed by using Student's t-test. Values of $P<0.05$ were considered statistically
314 significant.
315

316 **Author contributions**

317 HD designed this research, performed all experiments, and wrote the paper. MI provided
318 OR6 cells. NK provided PH5CH8 cells. HD, MI, YA, YT, and NK analyzed and
319 discussed the results. HD, MI, YA, YT, and NK reviewed the manuscript.
320

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Data availability statement

The representative data are included in this article. The data that support the findings of this study are available from the corresponding author (dansako@md.okayama-u.ac.jp) upon reasonable request.

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455

456 **Figure legends**

457 **Fig. 1. Like HCV NS5B, GBV-B NS5B induced IFN-β in PH5CH8 cells.** (A)
458 Quantitative anti-HCV analysis of the culture media by an OR6 assay system. The culture
459 media were harvested from PH5CH8/Cont (which lacks the exogenous expression of
460 NS5B), PH5CH8/NS5B, and PH5CH8/NS5B G317V (which lacks RdRp activity of
461 NS5B) cells, respectively. *Renilla* luciferase activity was measured 72 h after the addition
462 of culture media as described in the Materials and Methods section. Data are the means
463 ± SD from three independent experiments. Student's t-test: ***$P<0.001$; NS, not
464 significant versus OR6 cells added culture media derived from PH5CH8/Cont cells. (B)

Western blot analysis of ISGs in PH5CH8/NS5B cells. β-actin was used as a control for the amounts of protein loaded per lane. Representative results of at least two independent experiments are shown. (C) Quantitative RT-PCR analysis of IFN-β mRNA in PH5CH8/HA-NS5B (HCV) cells and PH5CH8/HA-NS5B (GBV-B) cells. PH5CH8/Cont cells were used as a control. The IFN-β mRNA level was calculated relative to the level in PH5CH8/HA-NS5B (HCV) cells, which was set at 1. Data are the means ± SD from three independent experiments. Student's t-test: ***$P<0.001$ versus PH5CH8/HA-NS5B (HCV) cells. (D) Western blot analysis of ISGs in PH5CH8/HA-NS5B (HCV) cells and PH5CH8/HA-NS5B (GBV-B) cells. PH5CH8/Cont cells were used as a control. β-actin was used as a control for the amounts of protein loaded per lane. Representative results of at least two independent experiments are shown.

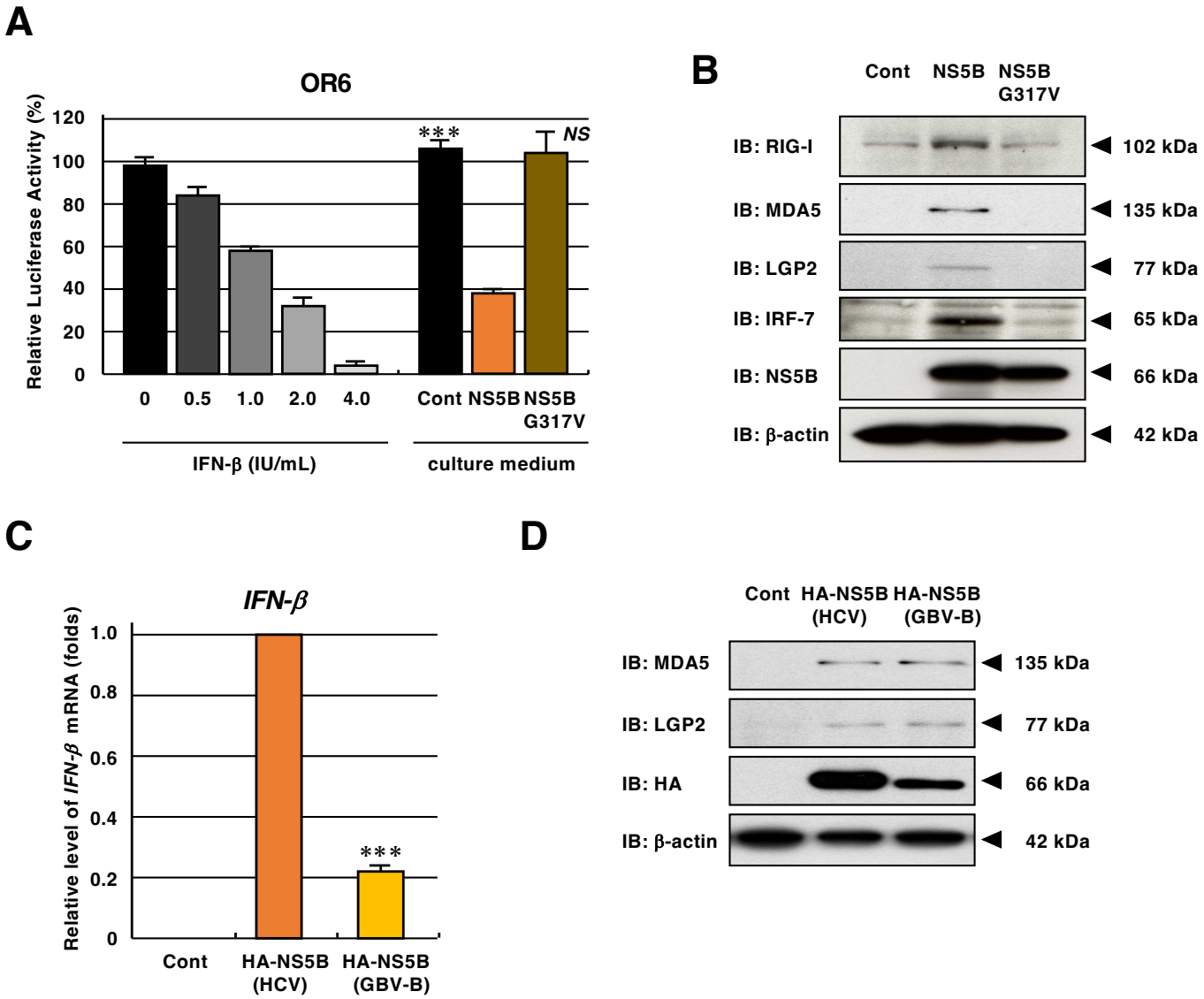
Fig. 2. HCV NS5B triggered the phosphorylation and dimerization of IRF-3 to induce IFN-β. (A) Quantitative RT-PCR analysis of IFN-β mRNA in IRF-3-knocked-down PH5CH8/NS5B cells. The levels of IRF-3 and IFN-β mRNA were calculated relative to the level in luciferase GL2-transfected PH5CH8/NS5B cells, which was set at 1,

481 respectively. Data are the means ± SD from three independent experiments. Student's t-
482 test: ***$P<0.001$ versus luciferase GL2-transfected PH5CH8/Cont, PH5CH8/NS5B, or
483 PH5CH8/NS5B G317V cells, respectively. (B) Dimerization analysis of endogenous
484 IRF-3 in PH5CH8/NS5B cells. The preparation of cell lysates and Native-PAGE were
485 performed as described in the Materials and Methods section. (C) Dimerization analysis
486 of exogenous IRF-3 in PH5CH8/NS5B cells transfected with pEGFP- C1/IRF-3 mNLS
487 vector that abrogated the nuclear localization signal of IRF-3. (D) Western blot analysis
488 of TRAF3 and TRAF6 in TRAF3 or TRAF6-knocked-down PH5CH8/NS5B cells.
489 Luciferase GL2-transfected PH5CH8/NS5B cells were used as a control. β-actin was used
490 as a control for the amounts of protein loaded per lane. (E) Dimerization analysis of
491 endogenous IRF-3 in TRAF3- or TRAF6-knocked-down PH5CH8/NS5B cells.
492 Luciferase GL2-transfected PH5CH8/NS5B cells were used as a control. Representative
493 results of two independent experiments are shown in B-E. (F) Quantitative RT-PCR
494 analysis of IFN-β mRNA in TRAF6-knocked-down PH5CH8/NS5B cells. The levels of
495 IFN-β mRNA were calculated relative to the level in luciferase GL2-transfected
496 PH5CH8/NS5B cells, which was set at 1. Data are the means ± SD from three independent

497 experiments. Student's t-test: $**P<0.01$ versus luciferase GL2-transfected
498 PH5CH8/NS5B cells.
499
500 **Fig. 3. HCV NS5B triggered the dimerization of MDA5 to induce IFN-β.** (A) Quantitative
501 RT-PCR analysis of IFN-β mRNA in MDA5-knocked-down PH5CH8/NS5B cells. The
502 levels of MDA5 and IFN-β mRNA were calculated relative to the level in luciferase GL2-
503 transfected PH5CH8/NS5B cells, which was set at 1. Data are the means ± SD from three
504 independent experiments. Student's t-test: $***P<0.001$ versus luciferase GL2-transfected
505 PH5CH8/NS5B cells. (B) Dimerization analysis of MDA5 in PH5CH8/NS5B cells. The
506 preparation of cell lysates and Native-PAGE were performed as described in the Materials
507 and Methods section. Endogenous IRF-3 was used as a control for the amounts of protein
508 loaded per lane. (C) Dimerization analysis of MDA5 in PH5CH8/HA-NS5B (HCV) cells
509 and PH5CH8/HA-NS5B (GBV-B) cells. Representative results of at least two
510 independent experiments are shown in B-C.
511
512 **Fig. 4. HCV NS5B produced dsRNA by its RdRp activity in the absence of viral genomes.**

513 (A) Immunofluorescence analysis of intercellular dsRNA in PH5CH8 cells. PH5CH8
514 cells were treated as indicated below: untreated, polyIC transfection (5μg, T-polyIC),
515 polyIC addition (50 mg/mL, M-polyIC), and polydAdT transfection (5μg, T-polydAdT),
516 respectively. The cells were stained as described in the Materials and Methods section.
517 The white bar indicates 20 μm. The white arrowhead indicates cells introducing dsRNA.
518 Green: dsRNA, Red: nucleus. (B) Immunofluorescence analysis of dsRNA produced in
519 PH5CH8/NS5B cells and PH5CH8/NS5B G317V cells. The white bar indicates 20 μm.
520 The white arrowhead indicates cells producing dsRNA. Green: dsRNA, Red: nucleus. (C)
521 Immunofluorescence analysis of dsRNA produced in PH5CH8/HA-NS5B (HBV) cells
522 and PH5CH8/HA-NS5B (GBV-B) cells. The white bar indicates 20 μm. The white
523 arrowhead indicates cells producing dsRNA. Red: dsRNA, Green: HA-NS5B, Blue:
524 nucleus. Representative results of at least two independent experiments are shown in A-
525 C.
526
527 **Fig. 5. The proposed model of IFN-β inductions through dsRNA produced by HCV NS5B.**
528 The solid lines with arrow show the canonical signaling pathway in MDA5-mediated

529 innate immune response. The dashed line with arrow shows the production of non-viral
530 dsRNA by the RdRp activity of NS5B. T-shaped dashed line shows the inhibition of IRF-
531 3 dimerization by TRAF6. Both the dashed line with arrow and T- shaped dashed line
532 show the results in the present study.



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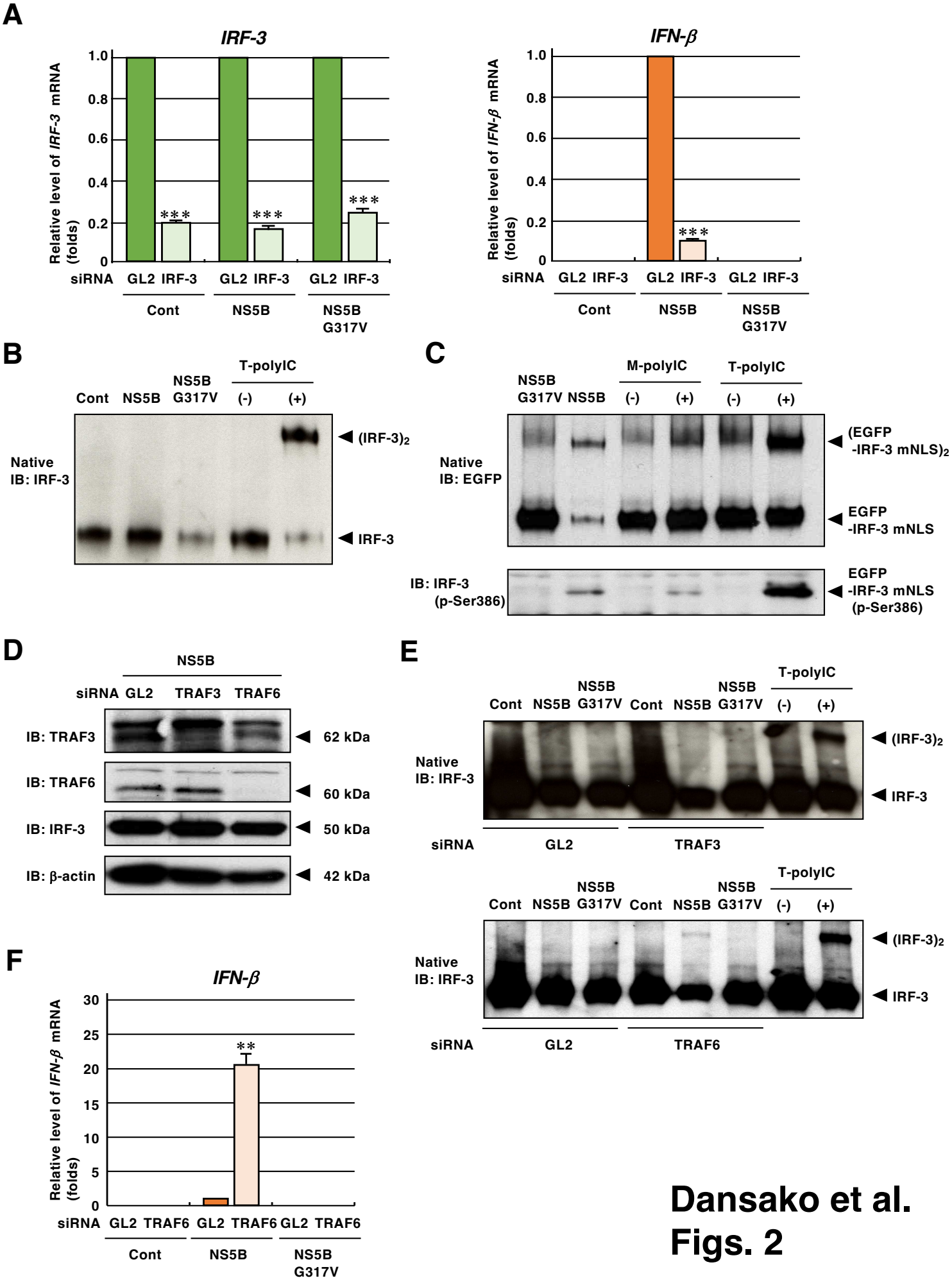
Figs. 1



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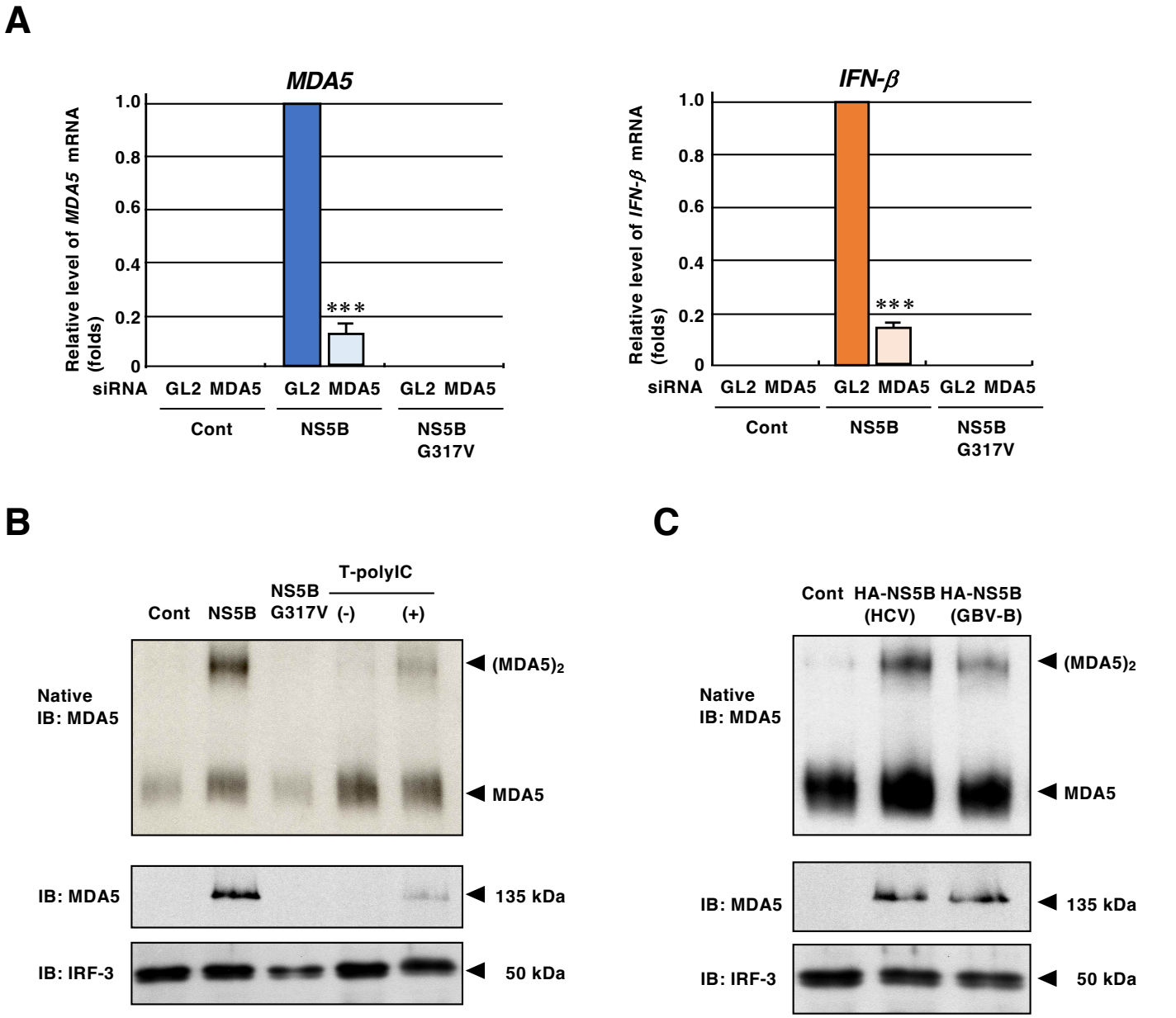

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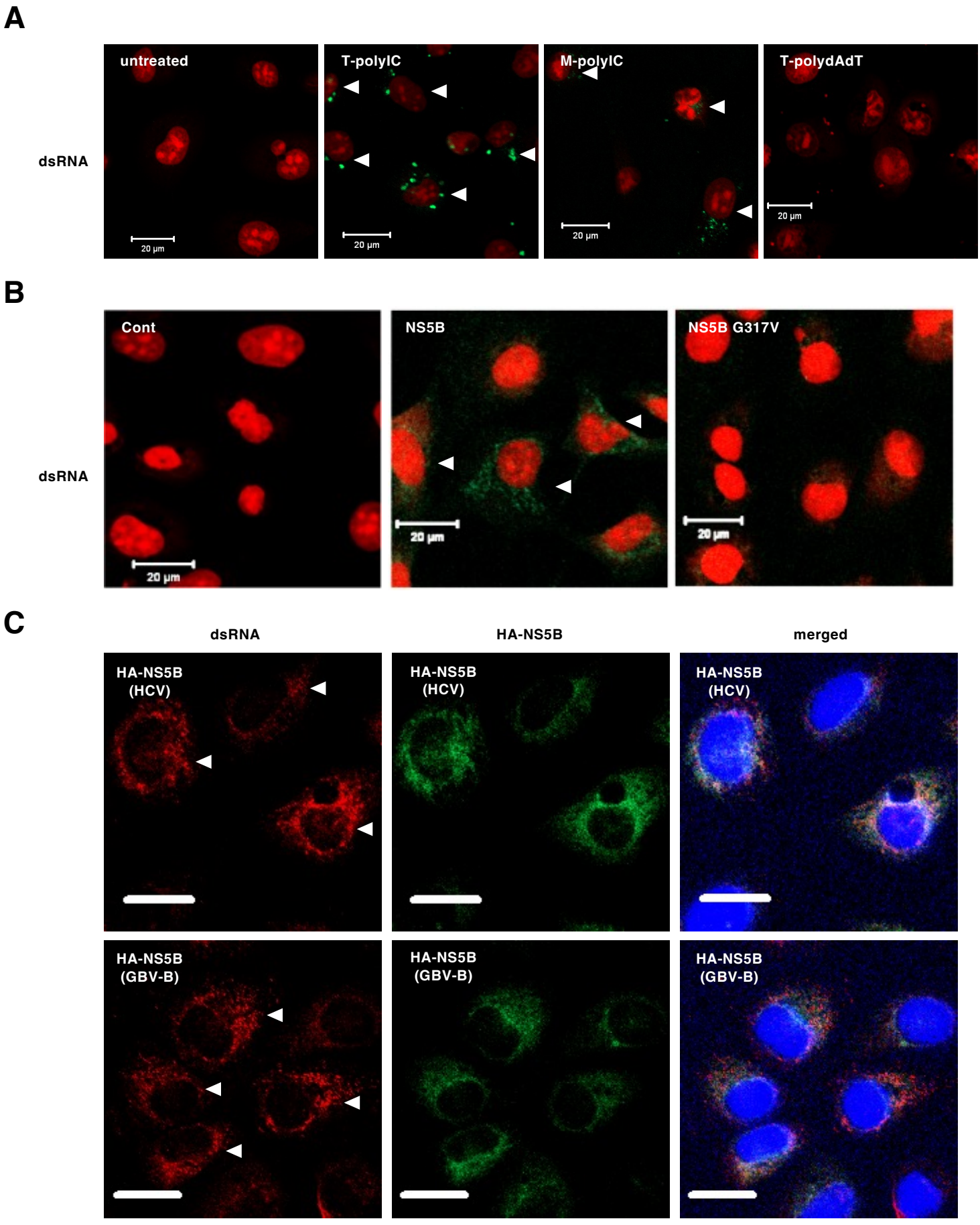
Figs. 3



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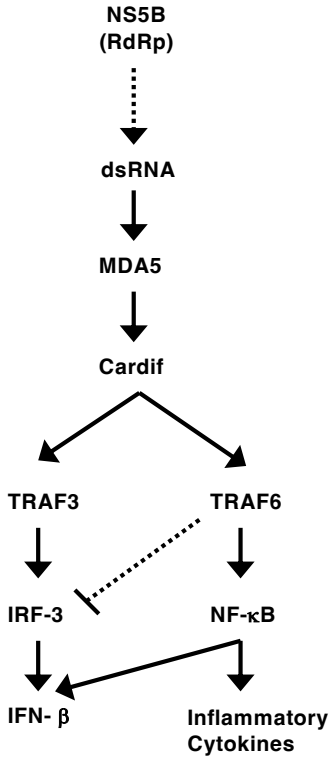

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Fig. 5