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3 # Translocase of outer mitochondrial membrane 70 induces interferon

4 # response and is impaired by hepatitis C virus NS3

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33 **ABSTRACT**

34 Hepatitis C virus (HCV) elevated expression of the translocase of outer mitochondrial
35 membrane 70 (Tom70). Interestingly, overexpression of Tom70 induces interferon (IFN)
36 synthesis in hepatocytes, and it was impaired by HCV. Here, we addressed the mechanism of
37 this impairment. The HCV NS3/4A protein induced Tom70 expression. The HCV NS3 protein
38 interacted in cells, and cleaved the adapter protein mitochondrial anti-viral signaling (MAVS).
39 Ectopic overexpression of Tom70 could not inhibit this cleavage. As a result, IRF-3
40 phosphorylation was impaired and IFN-β induction was suppressed. These results indicate that
41 MAVS works upstream of Tom70 and the cleavage of MAVS by HCV NS3 protease suppresses
42 signaling of IFN induction.

43

44 Type I interferon (IFN) induction is the front line of host defense against viral
45 infection. Intracellular double-stranded RNA is a viral replication intermediate and contains
46 pathogen-associated molecular patterns (PAMPS) (Saito et al., 2008) that are recognized by
47 pathogen-recognition receptors (PRRs) to induce IFN. One PRR family includes the Toll-like
48 receptors (TLRs), which are predominantly expressed in the endosome (Heil et al., 2004).
49 Another route of IFN induction takes place in the cytosol through activation of specific RNA
50 helicases, such as retinoic acid-inducible (RIG)-I and melanoma differentiation associated gene
51 5 (MDA5). The ligand for RIG-I is an uncapped 5′ triphosphate RNA, which is found in viral
52 RNAs of the *Flaviviridae* family, including hepatitis C virus (HCV), paramyxovirus, and
53 rhabdoviruses (Kato et al., 2006). MDA5 recognizes viruses with protected 5′ RNA ends, for
54 example, picornaviruses (Hornung et al., 2006). The adapter protein that links the RNA helicase
55 to the downstream MAPK, NF-κB, and IRF-3 signaling pathways is referred to as the

56 mitochondrial anti-viral signaling (MAVS) protein (Seth et al., 2005); alternative names include
57 IPS-1, interferon-promoter stimulator 1; VISA, virus-induced signaling adaptor; and CARDIF,
58 CARD adapter inducing IFN. HCV nonstructural protein 3 (NS3) possesses a serine protease
59 domain at the N terminus (amino acids (aa) 1–180) and has been found to cleave adaptor
60 proteins, MAVS at aa 508 (Meylan et al., 2005) and Toll/IL-1R domain-containing adapter
61 inducing IFN-β-deficient (TRIF at aa 372 (Ferreon et al., 2005)). These cleavages provoke
62 abrogation of the induction of the IFN pathway.

63 The translocase of the outer membrane (TOM) is responsible for initial recognition of
64 mitochondrial preproteins in the cytosol (Baker et al., 2007; Neupert and Herrmann, 2007). The
65 TOM machinery consists of 2 import receptors, Tom20 and Tom70, and, along with several
66 other subunits, comprises the general import pore (Abe et al., 2000). Recently, Tom70 was
67 found to interact with MAVS (Liu et al., 2010). Ectopic expression or silencing of Tom70,
68 respectively, enhanced or impaired IRF3-mediated gene expression and IFN-β production.
69 Sendai virus infection accelerated the Tom70-mediated IFN induction and the interaction of
70 Tom70 with MAVS. These recent findings indicated that Tom70 might be a critical mediator
71 during IFN induction (Liu et al., 2010).

72 We previously observed that HCV induces Tom70 and is related to the apoptotic
73 response (Takano et al., 2011a). However, no synergistic effect was observed for IFN induction
74 by Tom70 and HCV. Therefore, in the present study, we have investigated the mechanism of
75 modification of the Tom70-induced IFN synthesis pathway by HCV and clarified a finely
76 balanced system regulated by viral protein.

77 The expression of Tom70 protein was examined using western blotting and
78 modification by HCV was characterized (Fig.1A). The level of Tom70 protein was increased in
79 RzM6-LC cells compared with that in RzM6-0d cells (Tsukiyama-Kohara et al., 2004). The
80 full-length HCV-RNA expression was induced by 4-hydroxy-tamoxifen (100 nM) and passaged

81 for more than 44 days in RzM6-LC cells, and HCV expression was not induced in RzM6-0d
82 cells. Silencing of HCV expression by siRNA (R5; Thermo Scientific) abolished core protein
83 expression, and decreased the level of Tom70 protein expression in RzM6-LC cells (Fig.1A).
84 Silencing of Tom70 by siRNA significantly decreased the level of HCV core protein expression
85 in RzM6-LC cells (Fig.1A). The siRNA against 3-beta-hydroxysterol-delta24 reductase
86 (DHCR24) slightly decreased the level of Tom70 protein. In contrast, the control siRNA did not
87 have a significant effect on Tom70 protein expression.

88 We next examined the effects of HCV JFH-1 (Wakita et al., 2005) infection on
89 Tom70 expression (Fig. 1B). Infection with HCV significantly increased the level of Tom70
90 protein expression. We also examine the role of Tom70 in HCV replication (Fig. 1C, D).
91 Silencing of Tom70 by siRNA decreased the HCV replication in a dose dependent manner.
92 Thus, HCV induces Tom70 expression, and Tom70 is involved in viral replication.
93
94 It was recently shown that Tom70 recruits TBK1/IRF3 to mitochondria by binding to
95 Hsp90 and inducing IFN-β synthesis (Liu et al., 2010). Therefore, we examined the effects of
96 Tom70 overexpression on IFN synthesis and modification by HCV (Fig. 2). Level of IFN-β
97 mRNA synthesis was quantitated by real-time detection (RTD) PCR. Overexpression of Tom70
98 by transfection of pcDNA6-Tom70 (Takano et al., 2011a) induced IFN-β mRNA synthesis in
99 the absence of HCV after poly (I-C) treatment (RzM6-0d cells). However, the Tom70-mediated
100 induction of IFN-β mRNA transcription was impaired in the presence of HCV (RzM6-LC cells)
101 (Fig. 2A). Overexpression of Tom70 induced IFN-β mRNA synthesis in HuH-7 cells (Fig. 2B).
102 Induction of IFN-β mRNA was lower in HuH-7 cells than HepG2 based RzM6 cells, which
103 might be due to the defect in IFN induction system in HuH-7 cells (Preiss et al., 2008).
104 We have further addressed the mechanism of impairment of IFN-β mRNA

105 transcription by HCV.

106

107 To identify the viral protein that was responsible for the induction of Tom70, we
108 examined the Tom70 protein expression levels in HCV core, E1, E2, NS2, NS3/4A, NS4B,
109 NS5A, and NS5B protein-expressing cells (data not shown), and Tom70 protein expression
110 level was highest in the NS3/4A-expressing cells than was observed in cells expressing other
111 proteins (Fig. 3A, data not shown), indicating an effect of HCV NS3/4A protein on Tom70
112 expression.

113 The expression vector of Myc- and His-tagged Tom70 was transfected into the empty
114 control or NS3/4A-expressing cells and immunoprecipitated with anti-Myc antibody (Suppl Fig.
115 1A). Results showed that Myc-Tom70 was precipitated in both cells (right panel) and NS3
116 protein was specifically precipitated by anti-Myc antibody in the NS3/4A-expressing cells (left
117 panel). NS4A protein could not be detected (data not shown).

118 We next stained the NS3/4A-expressing cells with anti-NS3 and -Tom70 antibodies,
119 and observed with confocal microscopy (Suppl. Fig. 1B). The signal of NS3 protein was clearly
120 merged with that of Tom70, strongly supporting the possibility that the NS3 protein co-localizes
121 with the Tom70 protein.

122 To clarify the effect of Tom70 on NS3, we transfected NS3/4A-expressing cells with
123 the siRNA of Tom70 (Fig. 3A). Silencing of Tom70 decreased the level of NS3 protein in cells,
124 but did not influence the levels of the MAVS and NF-κB proteins. These results suggest the
125 possibility that Tom70 may increase the stability of NS3 protein in cells.

126

127 Tom70 reportedly interacts with MAVS during viral infection (Liu et al., 2010).
128 Therefore, we examined the MAVS protein in cells expressing either the control empty or
129 NS3/4A lenti-virus vector (Fig. 3B). Cleavage of MAVS (indicated as ΔMAVS) was observed

130 in NS3/4A protein-expressing cells, as was reported previously (Meylan et al., 2005).
131 Overexpression of Tom70 did not have a significant effect on the MAVS expression level and
132 did not prevent MAVS cleavage by NS3. IRF-3 phosphorylation was suppressed in
133 NS3/4A-expressing cells and was not influenced by Tom70 overexpression. The induction of
134 IFN-β was impaired in NS3/4A-expressing cells, even in the presence of Tom70 overexpression
135 (Fig. 3C). These data may indicate that MAVS exists upstream of Tom70 and that cleavage of
136 MAVS by NS3/4A impaired the downstream signaling activation of IRF-3 phosphorylation
137 (Suppl. Fig. 3).
138
139 Mitochondria provide a substantial platform for the regulation of IFN signaling. The
140 MAVS adapter protein is a member of the family of RIG-I like receptors (RLRs), which links
141 the mitochondria to the mammalian antiviral defense system (Seth et al., 2005). Proteomic
142 studies have demonstrated that MAVS interacts with Tom70 (Liu et al., 2010). This interaction
143 was accelerated by Sendai virus infection and synergized with ectopic expression of Tom70 to
144 significantly increase the production of IFN-β (Liu et al., 2010). The results of the present
145 study revealed that infection with HCV induced Tom70 expression, but the presence of HCV
146 impaired IFN induction. It has been reported that the C-terminal transmembrane domain (TM)
147 of MAVS interacts with the N-terminal transmembrane domain of Tom70 (Liu et al., 2010).
148 The HCV NS3 protein cleaves MAVS at residue 508 (Meylan et al., 2005), which should impair
149 the interaction of MAVS and Tom70. This may attenuate the downstream signaling pathway
150 (TBK-IRF3) and the induction of IFN synthesis (Suppl. Fig. 2). In our study, the level of NF-κB
151 protein was not significantly influenced by Tom70 in the presence or absence of NS3. This may
152 indicate that other pathways, such as TLR3 and downstream pathways, might compensate to
153 maintain the NF-κB protein expression level in the absence of the MAVS-Tom70 signaling
154 pathway.

Infection with HCV induced expression of Tom70, but the activation of the IFN signaling pathway was abrogated by the HCV NS3 protease. These findings indicate that recovery of the MAVS-Tom70 pathway may be a means to increase the efficacy of IFN therapy against HCV infection.

Recently, we observed that overexpression of Tom70 increased the resistance to the TNF$\alpha$-induced apoptotic response (Takano et al., 2011a), indicating that Tom70 overexpression might contribute to the apoptotic resistance of HCV-infected cells and the establishment of persistent HCV infection. Thus, Tom70 might be a novel target for the regulation of HCV infection.

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230

231 **FIGURE LEGENDS**

232 **Fig. 1**. HCV induces overexpression of Tom70 but impairs Tom70-induced IFN synthesis. **(A)**
233 RzM6 cells (HCV-) and RzM6-LC cells (HCV+) were transfected with siRNAs of control (non
234 target siRNA#3: Thermo Fisher Scientific), HCV (R5: 5'-
235 GUCUCGUAGACCGUGCAUCAuu-3'), DHCR24 (Nishimura et al., 2009), and Tom70
236 (Takano et al., 2011a). Control cells were mock-transfected. Tom70 protein was detected with
237 MAb2-243a (Takano et al., 2011a) and actin protein was detected as an internal control (lower
238 column). **(B)** HuH-7 cells were infected with HCV JFH-1 strain; Tom70 protein and actin
239 protein were detected. **(C)** The HCV replicon cels (FLR3-1; (Takano et al., 2011b)) were
240 transfected with siRNAs (control, HCV ((R7: 5'- GUCUCGUAGACCGUGCACCAuu-3')),
241 Tom70; 0.1, 0.3, 1, 3 nM) and HCV replication activity was measured with luciferase activity
242 using the Bright-Glo luciferase assay kit (Promega). Cell viability was measured using WST-8
243 (Dojindo) reagent. Ratio with those of control siRNA treatment was calculated. Vertical bars
244 were S.D. (**D**) HCV replicon cells (FLR3-1) were transfected with control, HCV (R7) and
245 Tom70 siRNAs (0.1, 0.3 nM) and Tom70, NS5A and actin proteins were detected.

246

247 **Fig. 2**. Tom70-induced IFN synthesis was impaired by HCV. **(A)** RzM6-0d cells and LC cells
248 were transfected with mock-vector, control pcDNA vector (vec.), or pcDNA-Tom70 expression
249 vector, and the amount of IFN-β mRNA was measured by RTD-PCR and normalized to the
250 amount of GAPDH mRNA using Gene expression assay kit (GE-Healthcare). Poly (I-C) (GE
251 Healthcare) (5μg) was transfected with RNAi Max reagent (Invitrogen) and IFN-β mRNA was
252 measured after 6h of poly (I-C) treatment. Vertical bars indicate S.D. $*p < 0.05$. **(B)** HuH-7 cells
253 were transfected with mock-vector, control vector, or Tom70 expression vector, and the amount
254 of IFN-β mRNA was measured by RTD-PCR and normalized to the amount of GAPDH mRNA.
255 Vertical bars indicate S.D. $*p < 0.05$.

256

257 **Fig. 3**. Silencing of Tom70 decreased the level of NS3 and cleavage of MAVS by NS3/4A
258 impaired IRF-3 phosphorylation even in the presence of Tom70. (**A**) Empty or NS3/4A-lenti
259 virus vector expressing HepG2 cells were transfected with control siRNA and Tom70 siRNA or
260 mock-transfected (non) as a control. MAVS, NS3, Tom70, and actin proteins were detected by
261 western blot. (**B)** Empty or NS3/4A-expressing HepG2 cells were transfected with control
262 pcDNA vector (vec.) and pcDNA6 (Invitrogen)-Tom70 or mock-transfected (non) as a control.
263 NS3, Tom70, phosphorylated IRF-3, MAVS, and actin proteins were examined by western blot.
264 (**C**) IFN-β mRNA was measured by RTD-PCR and normalized with GAPDH mRNA amount in
265 empty or NS3/4A expressing cells with transfection of mock (non), pcDNA-vector (vec.) or
266 pcDNA-Tom70 (Tom70). Poly (I-C) was treated, as described in the legend of Fig.2.
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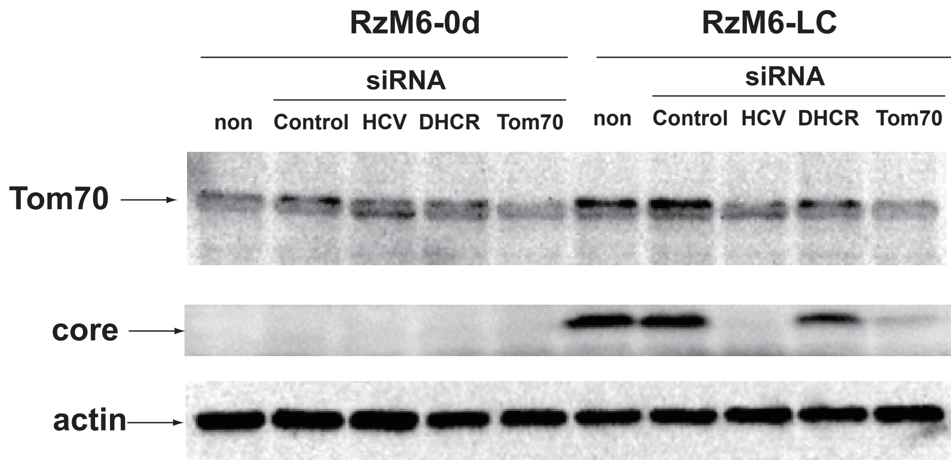
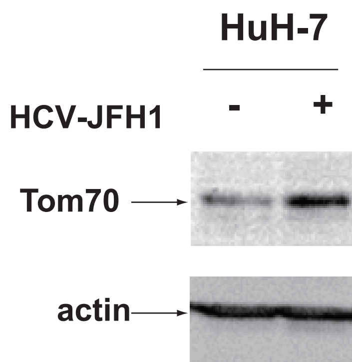
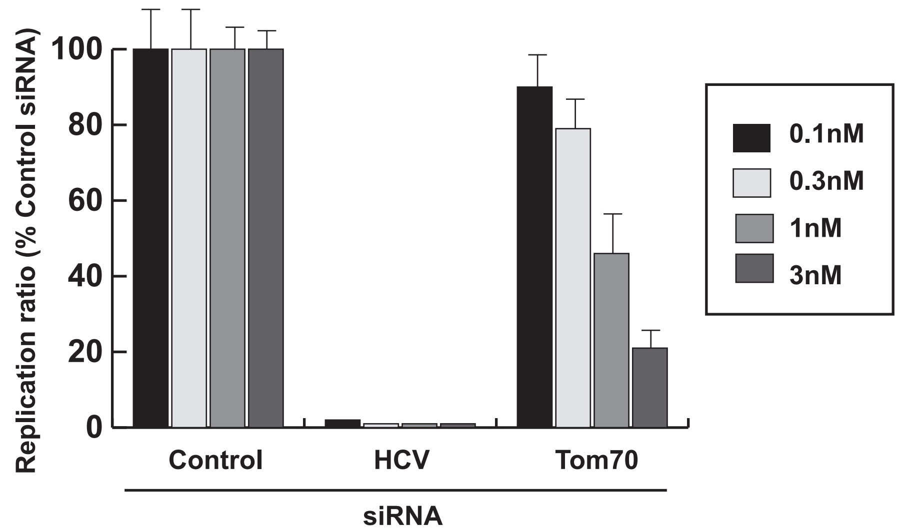
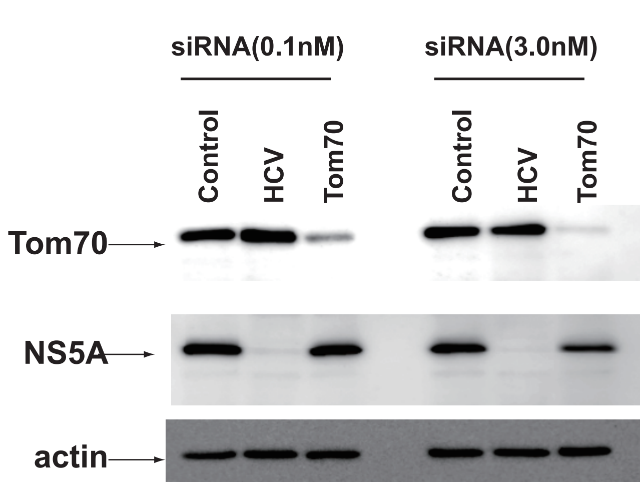


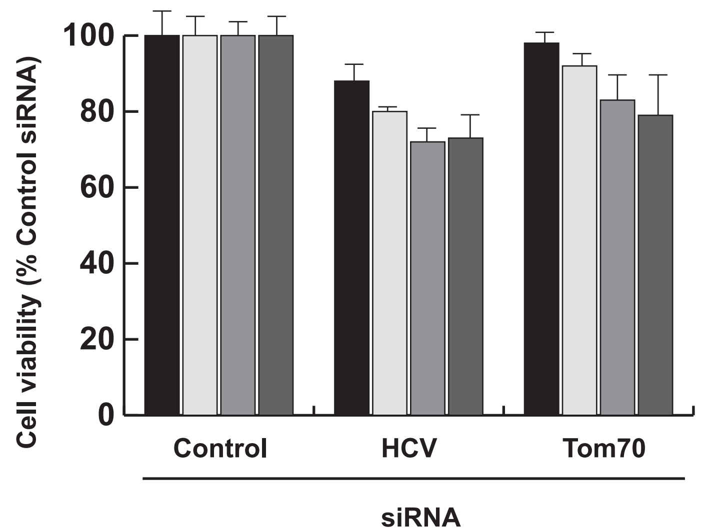
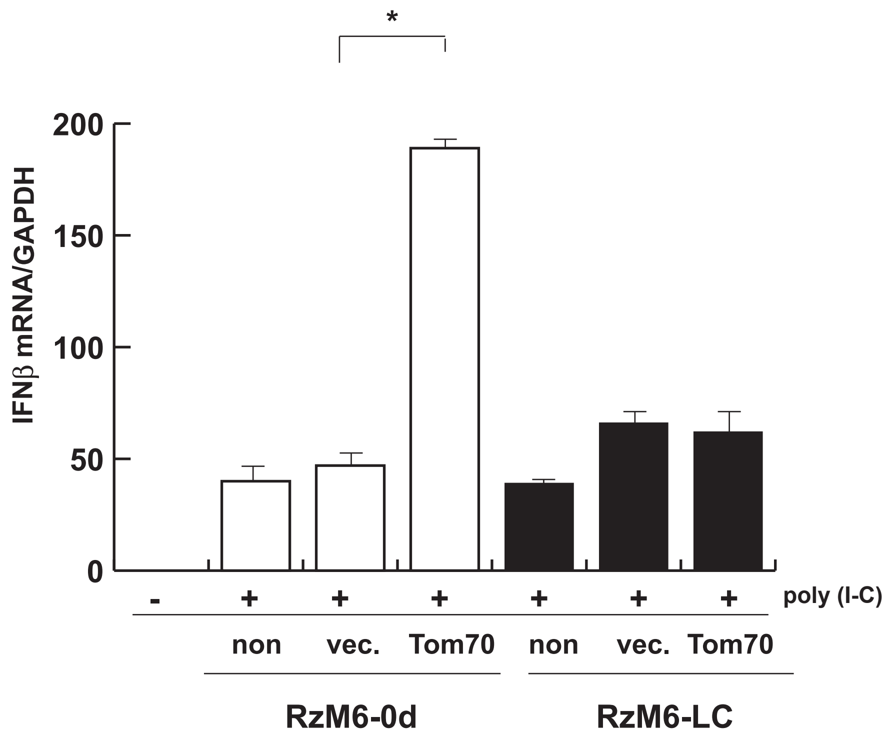




Fig.1



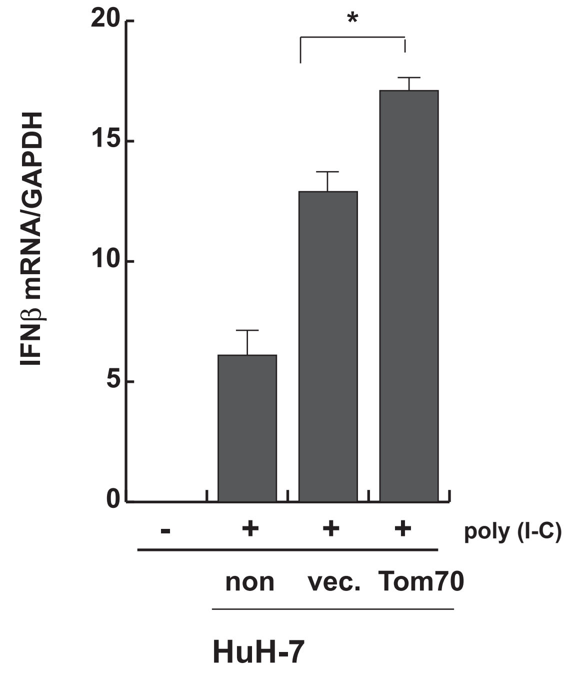




Fig.2

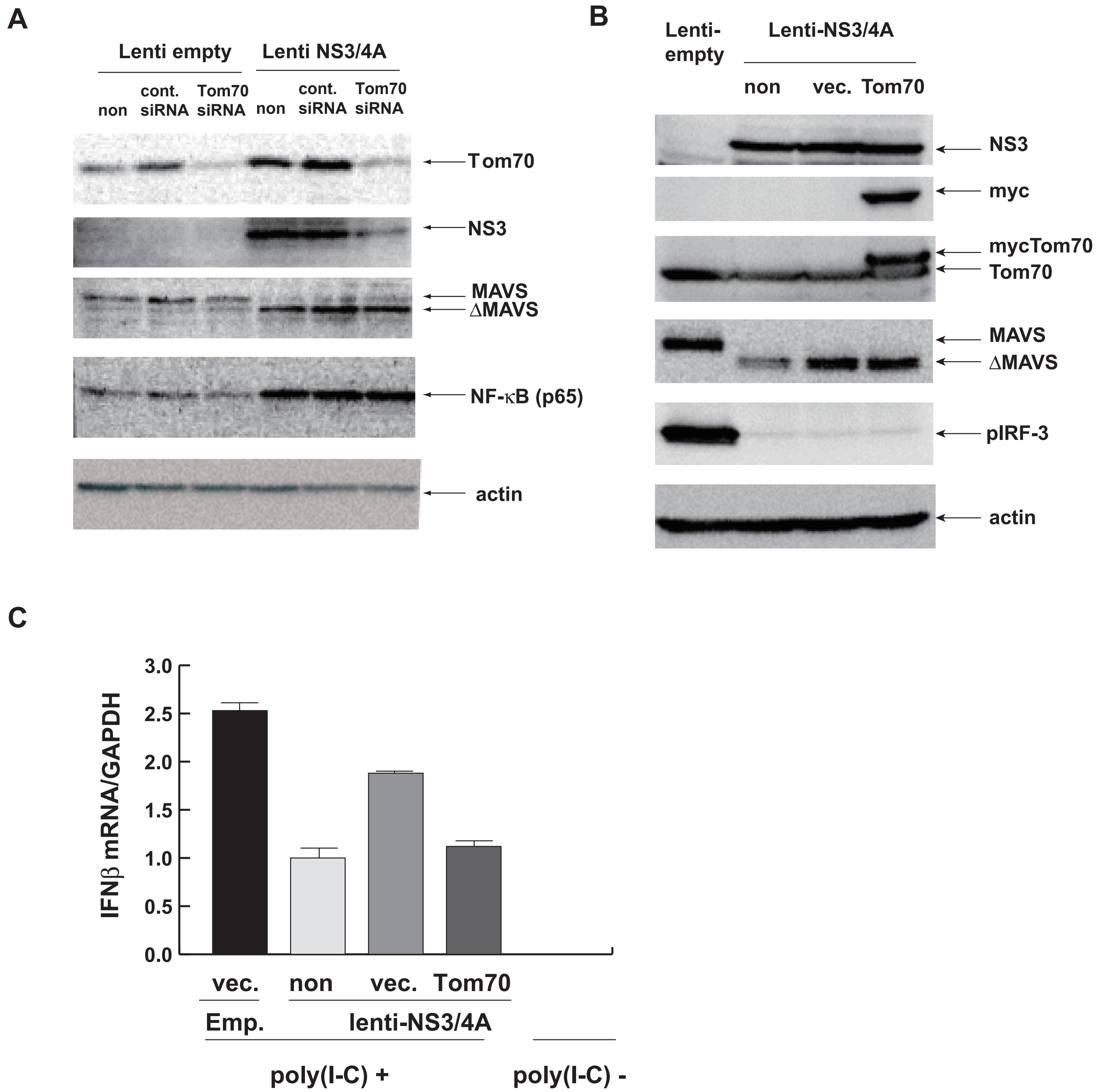



Fig.3