Infectious bronchitis coronavirus limits interferon production by inducing a host shutoff that requires accessory protein 5b.

Running title: **IBV-5b limits IFN production through host shutoff**

Joeri Kint[1,2,†], Martijn A. Langereis[3,*], Helena J. Maier[4], Paul Britton[4], Frank J. van Kuppeveld[3], Joseph Koumans[2], Geert F Wiegertjes[1], Maria Forlenza[1,#]

[1]Cell Biology and Immunology Group, Wageningen Institute of Animal Sciences, Wageningen University, Wageningen, The Netherlands.
[2]MSD Animal Health, Bioprocess Technology & Support, Boxmeer, The Netherlands
[3]Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands
[4]Avian Viral Diseases, The Pirbright Institute, Compton Laboratory, United Kingdom
† Current address: Biotype Diagnostic GmbH, Dresden, Germany
* Current address: MSD Animal Health, Discovery & Technology Research, Boxmeer, The Netherlands

[#] Correspondence should be addressed to M.F. (maria.forlenza@wur.nl)

Word counts

Abstract	206
Importance	126
Text	4479 (excl references, table footnotes, and fig. legends).

Abstract

During infection of their host cell, viruses often inhibit production of host proteins, a process which is referred to as host shutoff. By doing this, viruses limit production of antiviral proteins and increase production capacity for viral proteins. Coronaviruses from the *Alpha-* and *Betacoronavirus* genera, such as severe acute respiratory syndrome coronavirus (SARS-CoV) establish host shutoff via their non-structural protein 1 (nsp1). The genomes of *Gamma-* and *Deltacoronaviruses* however do not encode nsp1, and it has been suggested that these viruses do not induce host shutoff. Here we show that infectious bronchitis *Gammacoronavirus* (IBV) does induce host shutoff and we find that its accessory protein 5b is indispensable for this function. Importantly, we found that 5b-null viruses, unlike wild type viruses, induce production of high concentrations of type I interferon protein *in vitro*, indicating that host shutoff by IBV plays an important role in antagonizing the host's innate immune response. Altogether we demonstrate that 5b is a functional equivalent of nsp1 thereby answering the long-standing question whether lack of nsp1 in *Gammacoronaviruses* is compensated for by another viral protein. As such, our study is a significant step forward in the understanding of coronavirus biology and closes a gap in the understanding of some IBV virulence strategies.

Importance

Many viruses inhibit protein synthesis of their host cell to enhance virus replication and antagonize anti-viral defense mechanisms. This process is referred to as 'host-shutoff'. We have studied gene expression and protein synthesis in chicken cells infected with the important poultry pathogen, infectious bronchitis virus (IBV). We show that IBV inhibits synthesis of host proteins, including that of type I interferon, a key component of the antiviral response. The IBV-induced host shutoff however, does not require degradation of host RNA. Furthermore, we demonstrate that accessory protein 5b of IBV plays a crucial role in the onset of the host shutoff. Our findings suggest that inhibition of host protein synthesis is a common feature of coronaviruses and primarily serves to inhibit the antiviral response of the host.

52 **Introduction**

53 Viruses are dependent on the host-cell machinery for translation of their proteins. To maximize
54 production of viral proteins and limit production of antiviral proteins, viruses have evolved
55 strategies to interfere with the host-cell machinery at various levels (1). *Betacoronaviruses*, such
56 as mouse hepatitis coronavirus (MHV), severe acute respiratory syndrome coronavirus (SARS-
57 CoV) and several bat coronaviruses limit host-translation using virus-encoded nsp1 (2-4), which
58 induces degradation of host, but not viral RNA (3, 5, 6). Alternatively, nsp1 of transmissible
59 gastroenteritis coronavirus (TGEV, genus *Alphacoronavirus)* inhibits host-translation through an
60 unknown mechanism that does not seem to involve degradation of host mRNA (4, 7).

61

62 In addition to inhibiting host-translation, nsp1 also counteracts the innate immune response
63 (Reviewed in (8)). Observations on recombinant SARS-CoV expressing a truncated nsp1 indicate
64 that inhibition of translation by nsp1 limits production of cytokines, as the mutant virus induced
65 considerably higher production of IFN in HEK 293 cells than the parental virus (9). In addition,
66 this virus was attenuated in IFN competent cells (10). In line with this, MHV-nsp1-mutant
67 viruses were severely attenuated in IFN competent, but not in type I IFN receptor-deficient
68 ($IFNAR^{-/-}$) mice (11, 12). Contrary to the SARS-CoV nsp1 mutant virus, the MHV-nsp1 mutant
69 did not elicit more production of IFNα, but was significantly more sensitive to treatment with
70 IFNα in macrophages than the parental virus (11). Experimental data suggest that nsp1 from
71 *Alpha*- and *Betacoronaviruses* inhibit reporter-gene expression driven by an Interferonβ (Ifnβ)-
72 promoter as well as from an IFN-inducible promoter (4, 10, 12-14).

73

74 Although nsp1 of *Alpha*- and *Betacoronaviruses* exhibit remarkably similar biological functions,
75 they are different in size and lack significant similarity in protein sequence (15, 16). Interestingly,
76 of the four coronavirus genera, only *Alpha*- and *Betacoronaviruses* encode nsp1 (17-20). This
77 observation has prompted the question whether or not *Gamma*- and *Deltacoronaviruses* inhibit
78 translation of host mRNA and if so, which viral protein is involved. Wang *et al.* found that

Gammacoronavirus infectious bronchitis virus (IBV*)* does not reduce translation of host proteins (21), although the same group reported earlier that the spike protein of IBV inhibits host-translation through interaction with eIF3f (22). Because of these conflicting reports, it has remained unclear whether or not IBV uses a host shutoff mechanism to enhance virus replication. In this study we show that IBV inhibits synthesis of host proteins, including that of type I interferons, and we present evidence that accessory protein 5b is, at least partly, responsible for the IBV-induced host shutoff. Similar to *Alphacoronavirus* TGEV, inhibition of protein synthesis by IBV does not involve degradation of host mRNA. Taken together, our results suggest that *Gammacoronavirus* accessory protein 5b acts as the functional equivalent of *Alpha-* and *Betacoronaviruses* nsp1. As such, this study closes a gap in the understanding of *Gammacoronaviruses* virulence strategies and shows that evolutionarily distant coronaviruses use similar strategies to manipulate host cells.

92 **Materials and methods**

93

94 **Cells**

95 Chicken embryonic kidneys were aseptically removed from 17- to 19-day-old chicken embryo's
96 (Charles River, SPAFAS). A cell suspension was obtained by trypsinization for 30 min at 37°C
97 and filtered through a 100 µm mesh. The resulting chicken embryo kidney (CEK) cells were
98 seeded at 4 x 10^5 cells/cm^2 in 199 medium (Invitrogen) supplemented with 0.5% fetal bovine
99 serum (FBS, SAFC) and 1% PenStrep (Gibco, Invitrogen). DF-1, Vero and CEC-32 cells were
100 cultured in DMEM (Gibco, Invitrogen) supplemented with 10% FBS and 1% PenStrep. All cells
101 were incubated in a humidified incubator at 37°C and 5% CO_2.

102

103 **Viruses**

104 IBV-M41, IBV-QX and IBV-Italy-O2, Rift Valley Fever Virus clone 13 (RVFV Cl13) were obtained
105 from Merck Animal Health, Boxmeer, The Netherlands. Sindbis virus (SinV) was a kind gift from
106 G. P. Pijlman, (Laboratory of Virology, Wageningen University). IBV Beaudette, strain Beau-R, as
107 well as the generation of the ScAUG3a, ScAUG3b, ScAUG5a, ScAUG5b, ScAUG3ab and
108 ScAUG5ab Beau-R null viruses has been published previously (23-25). In these mutant IBV
109 viruses, the start codons of the indicated accessory genes were mutated to stop codons. All IBV
110 viruses were amplified and titrated on the cells in which the experiment was carried out. SinV
111 was amplified on BHK cells and titrated on CEK cells. RVFV Cl13 was amplified and titrated on
112 Vero cells.

113

114 **cDNA synthesis, RNA isolation and gene expression analysis**

115 Total RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's
116 instructions, including an on-column DNase treatment (Qiagen). Approximately 8 x 10^5 CEK cells
117 were lysed in RLT buffer (Qiagen) at various time points after infection. RLT cell lysis buffer was
118 spiked with 1 ng/sample of luciferase mRNA (Promega) immediately prior to RNA isolation as

external reference gene for normalization during the gene expression analysis. An external reference gene was used for normalization because none of the endogenous genes tested was suitable as housekeeping genes during viral infections. Prior to cDNA synthesis, a second DNase treatment was performed using amplification grade DNase I (Invitrogen), and subsequently 0.5 - 1.0 µg RNA was used for cDNA synthesis using SuperScript III (Invitrogen) and random hexamer primers. cDNA samples were diluted 1:50 in nuclease-free water before real-time quantitative PCR analysis on a Rotor-Gene 6000 (Corbett Research), using Brilliant SYBR Green quantitative PCR (Stratagene) and primers as listed in Table 1 (26-31). Cycle thresholds and amplification efficiencies were calculated by the Rotor-Gene software (version 1.7) using the comparative quantitation method. The relative expression ratio of the target gene was calculated using the average reaction efficiency for each primer set and the cycle threshold (C_t) deviation of sample vs. control at time point 0h, as previously described (32). Because expression of various housekeeping genes was unstable during virus infections at time points later than 24 h (data not shown), gene-expression ratios were normalized using an external reference gene (luciferase).

Chicken type I IFN bioassay.

Bioactive chicken type I interferon (chIFN) was measured using a bioassay based on the CEC-32 quail reporter cell line expressing luciferase under the control of the chicken *mx* promoter (33) (kindly provided by Prof. Peter Staeheli). Briefly, CEC-32 cells were incubated with serial dilutions of chIFN-containing samples for 6 hours, after which luciferase activity was quantified and IFN concentrations calculated using a chIFN standard. To avoid influence of virus on the assay, samples were heat inactivated at 56°C for 30 min, which did not influence bioactivity of type I chIFN.

Luciferase expression assay

Before seeding at 100.000 cells/well in 96 well plates, CEK cells were electroporated using the Amaxa nucleofectorII (solution V, program W001), applying 2 µg pGL3-Firefly luciferase reporter

plasmid (pGL3-FFluc) per 4 million cells. Vero and DF-1 cells at 80 - 90% confluence in 96 well plates were transfected with 100 ng pGL3-FFluc per well using FuGENE HD (Promega) at a 1:3 ratio of DNA:FuGENE according to manufacturers' specifications. At 24 hours post transfection, cells were infected with IBV M41 (CEK) or Beau-R (DF-1 and Vero) and 22 hours later, luciferase activity was quantified using the Bright-Glo Luciferase Assay (Promega) and a Filtermax F5 luminometer (Molecular Devices).

Transfection of accessory proteins and cytotoxicity assay

Plasmids encoding FLAG-tagged accessory proteins were constructed as follows. Accessory genes 3a, 3b, 5a and 5b were PCR amplified using Taq polymerase (Invitrogen) using template cDNA from IBV-infected samples. The primers used for the cloning are listed in Table 1. PCR products were ligated into pFLAG-CMV-2 (Sigma Aldrich) at the EcoRI site after which sequences were verified. Vero and DF-1 cells at 80 - 90% confluence, were transfected using FuGENE HD (Promega) at a 1:3 ratio of DNA:FuGENE in 96 well plates according to manufacturers' specifications using 10 ng pRL-SV40 Renilla luciferase plasmid and 90 ng pFLAG-Beau-R 3a/3b/5a/5b-GFP or pEGFP-MHV-nsp1 per well. At 18 hours post transfection, luciferase activity was quantified using the Renilla Luciferase Assay (Promega) and a Filtermax F5 luminometer (Molecular Devices). In parallel wells, cytotoxicity of accessory proteins was quantified using the CellTiter 96 cell proliferation assay (Promega). At 18 hours post transfection, 20 µl AQueous one solution was added per well and incubated at 37°C for 4 hours after which absorbance at 485 nm was measured using a FilterMAx F5 luminometer. The absorbance value for 0% cell viability was established by incubating non-transfected cells for 15 minutes in 2% Triton X-100 (BioRad) in medium, prior to addition of the Aqueous one solution.

To visualize expression of Beau-R accessory proteins, the aforementioned transfection method was used to transfect Vero cells at 60% confluency, cultured on 8 well Lab-Tek #1.0 borosilicate coverglasses (Sigma-Aldrich). At 18 hours post-transfection, cells were fixed with 3.7% paraformaldehyde and permeabilized using 0.1% Triton X-100 in PBS. FLAG-tagged accessory

proteins were detected using anti-FLAG M2 antibody (Sigma-Aldrich) and visualized using Alexa-488 labelled goat-anti mouse antibody (Invitrogen). Antibodies were diluted 1:1000 in phosphate-buffered saline (PBS) supplemented with 5% FBS. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Cells were imaged using a Zeiss Primo Vert microscope and Axiovision software. Image overlays were obtained in ImageJ.

Host mRNA stability assay

Stability of host mRNAs was quantified by comparing the fold change in gene expression between infected and non-infected cells after treatment with 10 µg/ml Actinomycin D (ActD, Sigma-Aldrich). To this end, CEK cells were infected with Beau-R at MOI 10 or mock treated, and 5 hours later ActD was added to all cells and incubation was continued for an additional 6 hours. Before (t = 0h) and after ActD treatment (t = 6h), samples were taken for RNA isolation, cDNA synthesis and RT-qPCR. mRNA stability was defined as the fold change of gene expression after ActD treatment. All fold changes were calculated relative to t = 0h and normalized to an external reference gene (luciferase) which was added as mRNA to the RLT lysis buffer.

Radioactive labelling

Approximately 2 x 10^5 Vero cells were seeded in 6-well clusters and 24 hours later infected with either Beau-R or ScAUG3ab, ScAUG5ab, ScAUG5a, ScAUG5b Beau-R null viruses at a MOI of 20. Cell lysates were collected at 6, 12, and 24 hours post infection. At indicated time points, cells were starved in Methionine- and Cysteine-deficient medium for 30 minutes and incubated with ^{35}S-trans-label (Amersham) for 15 minutes. Cells were washed three times in phosphate-buffered saline, trypsinised, spun down and lysed in TEN-L buffer (40 mM Tris-HCl pH7.4, 150 mM NaCl, 10 mM EDTA, 1% NP40 and protease inhibitor cocktail [Roche]). Lysates were cleared for 15 minutes at 20.000 x g and supernatants were used for sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent fluorography as previously described (2). Sample quantities loaded on the SDS-PAGE gel were adjusted so each slot

contained equal scintillation counts. For quantification of protein translation levels, radioactive signal from the gel was imaged using a Storm 860 PhosphorImager (Molecular Dynamics). Signal intensity in the images was quantified using ImageJ software.

Statistics

Statistical analyses were performed in GraphPad Prism 6.0. Significant differences were determined using an unpaired t test or a one-way ANOVA, followed by a Bonferroni post-hoc test or a two-way ANOVA, followed by a Dunnet multiple comparison test.

Results

IBV inhibits production of type I IFN

To characterize production of type I interferon (IFN) by chicken cells in response to virus infection, primary chicken embryo kidney (CEK) cells were infected with IBV, RVFV Cl13 or Sindbis virus (SinV). Subsequently, *Ifnβ* mRNA was quantified, as this is the primary IFN gene transcribed in response to viral infection of CEK cells and a proxy for innate immune activation (28). Infection of CEK cells with IBV, RVFV Cl13 and SinV induced production of *Ifnβ* mRNA as early as 6hpi (Fig 1A). However, IFN protein was only detected in the supernatant of cells infected with RVFV Cl13 and Sindbis (Fig 1B). The supernatant of IBV-infected cells contained almost no IFN, although the virus replicates well in these cells (Fig 1C) indicating that IBV inhibits production of type I IFN protein. To assess whether inhibition of IFN production is a common feature of IBV, we investigated IFN production by CEK cells upon infection with five serotypes of IBV. We found that at 24 hours post infection (hpi) all serotypes induced production of *Ifnβ* mRNA (Fig 1D), but no IFN protein was detected in the cell culture supernatant (Fig 1E). At 48 hpi, cytopathic effect (CPE) was extensive (data not shown) and low concentrations of IFN (30 to 180 U/ml) were detected in the supernatant of cells infected with IBV strains M41, It02, QX and 4/91. A concentration of 1000 U/ml was detected in the supernatant of cells infected with strain Beau-R, which showed even more extensive CPE. Titration of cell-supernatants showed that the titer of the cell culture adapted Beau-R strain was higher than that of the other IBV strains (Fig 1F), which could explain why Beau-R induced higher production of IFN. Notably, *Ifnβ* mRNA levels were not higher in Beau-R infected cells, suggesting that Beau-R allows more production of IFN protein than the other serotypes studied. Taken together, we conclude that IBV efficiently inhibits production of IFN protein by primary chicken kidney cells during the first 24 hours of infection.

IBV inhibits translation of host proteins

The observation that IBV-infected cells synthesize *Ifnβ* mRNA but do not produce IFN protein, suggests that IBV infection might inhibit synthesis of host proteins. To test this hypothesis, a ^{35}S labelling experiment was performed, which showed that in IBV-infected Vero cells synthesis of host-proteins is severely reduced at both 12 and 24 hpi (Fig 2A). In addition, we found that IBV infection reduced luciferase expression from a constitutively active promoter (Fig 2B) in three different cell types, i.e. Vero, CEK and DF-1 cells. Taken together, these results indicate that IBV inhibits synthesis of host proteins. The *Betacoronaviruses* SARS-CoV and MHV also inhibit synthesis of host-proteins, and in cells infected with these viruses, degradation of host mRNA was observed (5, 6). To investigate whether the mechanism by which IBV inhibits host-protein synthesis is comparable to SARS and MHV, we investigated the stability of host mRNA in IBV-infected cells. To this end, we compared mRNA levels before and after inhibition of *de novo* mRNA transcription with Actinomycin D (ActD) in IBV-infected and non-infected (mock) cells (Fig 2C). Using RT-qPCR we quantified mRNA and calculated the percentage of mRNA that remained after 6hrs of treatment with ActD. The percentage of mRNA that remains after ActD treatment is a function of the stability of that specific mRNA and the duration of the treatment. We quantified mRNA levels of housekeeping genes (*Gapdh, Eef1α1* and *Rpl17*) as well as genes involved in the innate anti-viral response. Upon ActD treatment, mRNA levels for most genes decreased between 30 and 90 % in both IBV-infected and non-infected cells. For several immune-related genes involved in anti-viral responses (*Irf3, Mda5, Tlr3, Isg20, Ifnβ*) mRNA levels were less reduced in IBV-infected cells. In conclusion, no evidence was found to support the hypothesis that IBV-infection decreases stability of host mRNAs. As such, degradation of host mRNA is most probably not the mechanism by which inhibition of host-protein synthesis in IBV-infected cells occurs.

Accessory protein 5b inhibits synthesis of host-proteins

Unlike *Alpha-* and *Betacoronaviruses,* the genome of *Gammacoronaviruses* does not encode for an nsp1 homologue, consequently another viral protein must be responsible for inhibition of host-protein synthesis. Plausible candidate proteins that could fulfill this function are the genus-specific accessory proteins. Therefore, we investigated the inhibitory potential of the four IBV accessory proteins on protein synthesis. Plasmids were constructed that encode individual IBV accessory proteins with an N-terminal Flag tag (Fig 3A). These plasmids were transfected into Vero and DF-1 cells together with a plasmid that encodes Renilla luciferase (Fig 3B and C). It was observed that co-transfection of the plasmid encoding accessory protein 3a moderately reduced luciferase activity in DF-1 cells (Fig 3B), whereas co-transfection of accessory protein 3b slightly increased luciferase activity in both DF-1 and Vero cells (Fig 3B and C). Importantly, the only plasmids that significantly reduced luciferase expression in both DF-1 and Vero cells were those encoding the accessory protein 5b of IBV and nsp1 of MHV. The reduction in expression of Renilla luciferase was not due to cytotoxic effects of either the transfection procedure or the plasmids (Fig 3B and C, right Y-axis). Taken together, these over-expression studies suggest that accessory protein 5b plays the most prominent role in the reduction of host-protein production.

To investigate whether accessory protein 5b is involved in inhibition of host-protein production during IBV-infection, cells were infected with Beau-R or Beau-R accessory gene-null mutant viruses (ScAUG) and *de novo* protein synthesis was measured using ^{35}S pulse-labelling at 6, 12 and 24 hpi (Fig 4A). Fig 4A shows three regions that contain mostly host-proteins, indicated with 1, 2 and 3. Quantification of the ^{35}S signal in these regions was used to measure *de novo* synthesis of host proteins in virus and mock infected cells at 12 and 24 hpi (Fig 4B and C). The results of the quantification indicated that all viruses except ScAUG5b and ScAUG5ab decreased translation of host proteins at both 12 and 24 hpi. Next we investigated whether increased synthesis of host proteins in ScAUG5b- and ScAUG5ab-infected cells corresponds to a decrease

in synthesis of viral proteins. To do this, we quantified the ^{35}S signal of the Spike (S), nucleocapsid (N) and membrane (M) proteins of IBV and found synthesis of these proteins to be comparable between all viruses (Fig 4D). Overall, these results indicate that accessory protein 5b is required for inhibition of host protein synthesis by IBV.

Accessory protein 5b inhibits production of IFN

Subsequently, we explored whether 5b could be responsible for the lack of IFN production by IBV-infected cells observed in figure 1. To investigate the role of 5b, we quantified production of type I IFN (IFN) by DF-1 cells infected with Beau-R or accessory gene-null mutant viruses (Fig 5A). We found that infection with ScAUG5b as well as ScAUG5ab resulted in significantly higher production of IFN by DF-1 cells compared to infection with Beau-R. In fact, ScAUG5b-infected cells produced 55 and 30 times more IFN at 36 and 48 hpi, respectively. To verify the relevance of this finding, the experiment was repeated in primary (immunocompetent) CEK cells, where we found that both ScAUG5b- and ScAUG5ab-infected cells produced up to 15 times more IFN than Beau-R-infected cells (Fig 5B). It was also found that levels of *Ifnβ* mRNA were significantly higher in cells infected with any of the accessory gene null-viruses (Fig 5C), which is consistent with findings from a previous study (28). Despite the overall increase in *Ifnβ* mRNA transcription observed in cells infected with any of the accessory gene null-viruses, only in 5b-mutant virus infected cells increased IFN protein levels were detected. This observation is consistent with the hypothesis that 5b inhibits translation of *Ifnβ* mRNA. At 36 hpi, when IFN levels were significantly higher for the accessory gene-null viruses than for Beau-R, the virus titer of all accessory gene-null viruses was moderately lower compared to that of Beau-R (Fig 5D). These growth-characteristics are generally in line with previous observations (24). Taken together, our results indicate that accessory protein 5b plays a major role in the inhibition of general host protein synthesis, thereby inhibiting production of IFN by IBV-infected cells.

314 **Discussion**

315 The type I IFN response has been shown to be important for clearance of coronavirus infection *in*
316 *vivo,* and coronaviruses have evolved multiple mechanisms to delay and antagonize it (reviewed
317 in (34)). One of the strategies of *Alpha*- and *Betacoronaviruses* is to inhibit production of host
318 proteins, including type I IFN, via the viral nsp1 protein. (2-4, 7). *Gamma*- and
319 *Deltacoronaviruses* lack nsp1 and it is therefore unclear whether and how these viruses
320 antagonize host-translation. Previously, we demonstrated that the IBV *Gammacoronavirus* elicits
321 a remarkable induction of transcription of *Ifnβ* mRNA in avian cells but that this is delayed with
322 respect to the peak of viral replication. Here we further show that IBV-induced *Ifnβ* transcription
323 does not lead to production of significant levels of IFN protein until well after the onset of
324 transcription. In fact we found that IBV inhibits IFN production by blocking host-translation,
325 better known as host shutoff, and we show that accessory protein 5b is required for this function.
326 Various *Alpha*- and *Betacoronaviruses* have been shown to induce host shutoff (2, 3, 7), and the
327 only study on a *Gammacoronavirus* (IBV) reported absence of host shutoff in IBV-infected cells
328 (21). Our observation that IBV infection induces transcription of *Ifnβ*, but not production of IFN
329 protein, prompted us to re-evaluate whether IBV induces host shutoff. Using the same cell line,
330 and the same IBV strain that Wang *et al* (21) used, we find that IBV-infection severely reduces
331 synthesis of host proteins. The apparent discrepancy between our results and those of Wang *et*
332 *al.*, is probably caused by the application of a higher MOI in our case (20 instead of 2).
333 During *Beta*- but not *Alphacoronavirus*-infection, host shutoff is accompanied by degradation of
334 host mRNAs (5, 6). To investigate whether IBV infection induces degradation of mRNA, we
335 inhibited transcription in IBV-infected and non-infected cells with Actinomycin D and quantified
336 the decrease in host-mRNA levels. In contrast to infection with MHV and SARS-CoV (5, 6), we
337 did not observe a decrease in stability of host mRNAs. In fact, a subset of mRNAs, including *Irf3,*
338 *Mda5, Tlr3, Isg20, Ifnβ* appeared to show increased stability in IBV infected cells. The reason for
339 the increased stability of these innate-immune mRNAs is unclear, but it could be the result of
340 transcription induced in response to IBV-infection. An alternative explanation is that during IBV-

infection a subset of mRNAs are recruited to structures such as stress granules (SG). SG are temporary repositories of mRNAs and they are formed in response to stress-induced translational arrest (35, 36). SG have been shown to prevent degradation of mRNAs by cellular ribonucleases, and increased phosphorylation of eIF2α, which frequently accompanies virus-induced host shutoff, is one of the triggers for SG formation (37). Many viruses have been shown to modulate formation of SG, but in most cases it is unclear whether SG formation is beneficial to the host or to the virus (reviewed in (38)). Stress-granules have been observed in TGEV and MHV infected cells (2, 39) and in the case of TGEV, formation of SG coincided with decreased viral RNA synthesis, suggesting that SGs are detrimental to virus replication. The observation that IBV does not decrease mRNA stability may suggest that IBV induces host-shutoff via a mechanism similar to *Alphacoronaviruses*, which also do not induce degradation of host-mRNA (4, 7).

For both *Alpha*- and *Betacoronaviruses*, the viral nsp1 protein was shown to be essential for establishment of host shutoff. The genomes of *Gamma*- and *Deltacoronaviruses* do not encode nsp1, so it is unclear which IBV protein could play a role in IBV-induced host shutoff. We speculated that one of the accessory proteins might be involved in the IBV induced host shutoff. In a previous study we showed that absence of IBV accessory proteins 3b increases production of type I interferon protein late (≥36 hpi) during infection (28). In addition to this, it was shown that accessory protein 3a confers resistance to IFN, through an unknown mechanism (40). To investigate the influence of individual IBV accessory proteins on gene expression, we overexpressed each of the four accessory proteins, and found that not 3a or 3b, but only 5b decreased expression from a constitutive promoter in both a chicken and a mammalian cell line. Inhibition by IBV 5b was less pronounced than inhibition by MHV-nsp1; similar differences in inhibition-efficiency were reported for nsp1 proteins from various *Betacoronaviruses* (4). Next, we investigated the effect of 5b in the context of a virus infection using mutant viruses that do not express one or more accessory proteins. Using radioactive labelling of *de novo* protein synthesis, we found that both 5a/5b-null (ScAUG5ab) and 5b-null (ScAUG5b) viruses were less

efficient at inhibiting host translation than the parental virus. The difference in inhibition-efficiency between the 5b-null virus and the parental virus, was comparable to the difference previously found between wildtype SARS-CoV and SARS-CoV nsp1-mutant virus (9).

We then examined whether inhibition of host-protein synthesis by 5b is responsible for the block in IFN production observed in IBV-infected chicken cells. We found that both 5a/5b- and 5b-null viruses induced up to 90 times higher production of type I IFN in chicken cells than the parental virus. The extent of the difference of IFN production is comparable to previous observations on SARS-CoV nsp1 mutant virus (9). The functional resemblance between IBV-5b and nsp1, prompted us to compare the two proteins. Amino acid sequence alignment of 5b and nsp1 proteins from various viruses, yielded no significant similarity (< 20%, data not shown), Additionally, phylogenetic analysis showed that nsp1 proteins from both *Alpha-* and *Betacoronaviruses* do not cluster with any of the four *Gammacoronavirus* accessory proteins (data not shown). Although 5b and nsp1 do not show sequence similarities, they both evolved to fulfill the same biological function; i.e. inhibition of host translation. The *Coronavirinae* split two million years ago (41) to yield the ancestor of all *Alpha-* and *Betacoronaviruses* and the ancestor of both *Gamma-* and *Deltacoronaviruses*. The question that remains to be answered is whether the common ancestor of all coronaviruses encoded nsp1 or 5b. The lack of sequence homology between 5b and nsp1, their differential location in the genome and the evolutionary history of the viral genomes carrying them, suggests that their functional homology is the result of convergent evolution. Interestingly, nsp1 is the first protein to be synthesized in *Alpha-* and *Betacoronavirus* infected cells, because it is translated directly from genomic viral RNA. In contrast, 5b, can only be translated from subgenomic RNAs that are produced only later during infection. This may indicate that inhibition of host protein synthesis during the initial stages of infection may not be required for IBV *Gammacoronavirus*.

The finding that 5b and nsp1 are functionally equivalent answers the long-standing question whether lack of nsp1 in *Gammacoronaviruses* is compensated for by another viral protein. As

such, our study is a significant step forward in the understanding of coronavirus biology. Although the mechanism by which 5b inhibits host-translation remains to be investigated, our *in vitro* results indicate that it may be an important virulence factor of *Gammacoronaviruses* and a potential target for the rational design of live-attenuated virus vaccines against this important pathogen.

Acknowledgements

The authors would like to thank Gorben Pijlman and Jelke Fros from the Laboratory of Virology, Wageningen University for sharing of reagents and facilities. Petra Ruemmele from the Animal Service Department of MSD Animal Health for isolation of CEK cells, and Erwin van de Born for critical revision of this manuscript. This work was financially supported by MSD Animal Health, Bioprocess Technology & Support, Boxmeer, The Netherlands. Helena Maier and Paul Britton were supported by The Pirbright Institute and the Biotechnology and Biological Sciences Research Council (BBSRC). Martijn Langereis was supported by a Veni grant (NWO-863.13.008) from the Netherlands Organization for Scientific Research. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

411 **References**

412 1. **Walsh D, Mohr I.** 2011. Viral subversion of the host protein synthesis machinery. Nature
413 reviews microbiology **9:**860-875.
414 2. **Raaben M, Groot Koerkamp MJ, Rottier PJ, de Haan CA.** 2007. Mouse hepatitis
415 coronavirus replication induces host translational shutoff and mRNA decay, with
416 concomitant formation of stress granules and processing bodies. Cell Microbiol **9:**2218-
417 2229.
418 3. **Kamitani W, Narayanan K, Huang C, Lokugamage K, Ikegami T, Ito N, Kubo H,**
419 **Makino S.** 2006. Severe acute respiratory syndrome coronavirus nsp1 protein
420 suppresses host gene expression by promoting host mRNA degradation. Proc Natl Acad
421 Sci U S A **103:**12885-12890.
422 4. **Tohya Y, Narayanan K, Kamitani W, Huang C, Lokugamage K, Makino S.** 2009.
423 Suppression of host gene expression by nsp1 proteins of group 2 bat coronaviruses. J
424 Virol **83:**5282-5288.
425 5. **Huang C, Lokugamage KG, Rozovics JM, Narayanan K, Semler BL, Makino S.** 2011.
426 SARS coronavirus nsp1 protein induces template-dependent endonucleolytic cleavage of
427 mRNAs: viral mRNAs are resistant to nsp1-induced RNA cleavage. PLoS Pathog
428 **7:**e1002433.
429 6. **Kamitani W, Huang C, Narayanan K, Lokugamage KG, Makino S.** 2009. A two-
430 pronged strategy to suppress host protein synthesis by SARS coronavirus Nsp1 protein.
431 Nat Struct Mol Biol **16:**1134-1140.
432 7. **Huang C, Lokugamage KG, Rozovics JM, Narayanan K, Semler BL, Makino S.** 2011.
433 Alphacoronavirus transmissible gastroenteritis virus nsp1 protein suppresses protein
434 translation in mammalian cells and in cell-free HeLa cell extracts but not in rabbit
435 reticulocyte lysate. J Virol **85:**638-643.
436 8. **Narayanan K, Ramirez SI, Lokugamage KG, Makino S.** 2014. Coronavirus
437 nonstructural protein 1: Common and distinct functions in the regulation of host and
438 viral gene expression. Virus Res doi:10.1016/j.virusres.2014.11.019.
439 9. **Narayanan K, Huang C, Lokugamage K, Kamitani W, Ikegami T, Tseng CTK, Makino**
440 **S.** 2008. Severe acute respiratory syndrome coronavirus nsp1 suppresses host gene
441 expression, including that of type I interferon, in infected cells. J Virol **82:**4471-4479.
442 10. **Wathelet MG, Orr M, Frieman MB, Baric RS.** 2007. Severe acute respiratory syndrome
443 coronavirus evades antiviral signaling: role of nsp1 and rational design of an attenuated
444 strain. J Virol **81:**11620-11633.
445 11. **Zust R, Cervantes-Barragan L, Kuri T, Blakqori G, Weber F, Ludewig B, Thiel V.** 2007.
446 Coronavirus non-structural protein 1 is a major pathogenicity factor: implications for the
447 rational design of coronavirus vaccines. PLoS pathogens **3:**e109.
448 12. **Lei L, Ying S, Baojun L, Yi Y, Xiang H, Wenli S, Zounan S, Deyin G, Qingyu Z, Jingmei L,**
449 **Guohui C.** 2013. Attenuation of mouse hepatitis virus by deletion of the LLRKxGxKG
450 region of Nsp1. PLoS One **8:**e61166.
451 13. **Zust R, Cervantes-Barragan L, Habjan M, Maier R, Neuman BW, Ziebuhr J, Szretter**
452 **KJ, Baker SC, Barchet W, Diamond MS, Siddell SG, Ludewig B, Thiel V.** 2011. Ribose
453 2'-O-methylation provides a molecular signature for the distinction of self and non-self
454 mRNA dependent on the RNA sensor Mda5. Nat Immunol **12:**137-143.

14. **Wang Y, Shi H, Rigolet P, Wu N, Zhu L, Xi XG, Vabret A, Wang X, Wang T.** 2010. Nsp1 proteins of group I and SARS coronaviruses share structural and functional similarities. Infect Genet Evol **10:**919-924.
15. **Connor RF, Roper RL.** 2007. Unique SARS-CoV protein nsp1: bioinformatics, biochemistry and potential effects on virulence. Trends Microbiol **15:**51-53.
16. **Jansson AM.** 2013. Structure of alphacoronavirus transmissible gastroenteritis virus nsp1 has implications for coronavirus nsp1 function and evolution. J Virol **87:**2949-2955.
17. **Armesto M, Cavanagh D, Britton P.** 2009. The replicase gene of avian coronavirus infectious bronchitis virus is a determinant of pathogenicity. PLoS One **4:**e7384.
18. **Woo PC, Lau SK, Lam CS, Lai KK, Huang Y, Lee P, Luk GS, Dyrting KC, Chan KH, Yuen KY.** 2009. Comparative analysis of complete genome sequences of three avian coronaviruses reveals a novel group 3c coronavirus. J Virol **83:**908-917.
19. **Cao J, Wu CC, Lin TL.** 2008. Complete nucleotide sequence of polyprotein gene 1 and genome organization of turkey coronavirus. Virus Res **136:**43-49.
20. **Ziebuhr J, Schelle B, Karl N, Minskaia E, Bayer S, Siddell SG, Gorbalenya AE, Thiel V.** 2007. Human coronavirus 229E papain-like proteases have overlapping specificities but distinct functions in viral replication. J Virol **81:**3922-3932.
21. **Wang X, Liao Y, Yap PL, Png KJ, Tam JP, Liu DX.** 2009. Inhibition of protein kinase R activation and upregulation of GADD34 expression play a synergistic role in facilitating coronavirus replication by maintaining de novo protein synthesis in virus-infected cells. J Virol **83:**12462-12472.
22. **Xiao H, Xu LH, Yamada Y, Liu DX.** 2008. Coronavirus spike protein inhibits host cell translation by interaction with eIF3f. PLoS One **3:**e1494.
23. **Hodgson T, Britton P, Cavanagh D.** 2006. Neither the RNA nor the proteins of open reading frames 3a and 3b of the coronavirus infectious bronchitis virus are essential for replication. Journal of virology **80:**296-305.
24. **Casais R, Davies M, Cavanagh D, Britton P.** 2005. Gene 5 of the avian coronavirus infectious bronchitis virus is not essential for replication. Journal of virology **79:**8065-8078.
25. **Casais R, Thiel V, Siddell SG, Cavanagh D, Britton P.** 2001. Reverse genetics system for the avian coronavirus infectious bronchitis virus. J Virol **75:**12359-12369.
26. **Drosten C, Gottig S, Schilling S, Asper M, Panning M, Schmitz H, Gunther S.** 2002. Rapid detection and quantification of RNA of Ebola and Marburg viruses, Lassa virus, Crimean-Congo hemorrhagic fever virus, Rift Valley fever virus, dengue virus, and yellow fever virus by real-time reverse transcription-PCR. J Clin Microbiol **40:**2323-2330.
27. **Cristea IM, Rozjabek H, Molloy KR, Karki S, White LL, Rice CM, Rout MP, Chait BT, MacDonald MR.** 2010. Host factors associated with the Sindbis virus RNA-dependent RNA polymerase: role for G3BP1 and G3BP2 in virus replication. J Virol **84:**6720-6732.
28. **Kint J, Fernandez-Gutierrez M, Maier HJ, Britton P, Langereis MA, Koumans J, Wiegertjes GF, Forlenza M.** 2015. Activation of the chicken type I interferon response by infectious bronchitis coronavirus. J Virol **89:**1156-1167.
29. **Li YP, Handberg KJ, Juul-Madsen HR, Zhang MF, Jorgensen PH.** 2007. Transcriptional profiles of chicken embryo cell cultures following infection with infectious bursal disease virus. Archives of virology **152:**463-478.

30. **Villanueva AI, Kulkarni RR, Sharif S.** 2011. Synthetic double-stranded RNA oligonucleotides are immunostimulatory for chicken spleen cells. Dev Comp Immunol **35:**28-34.
31. **Daviet S, Van Borm S, Habyarimana A, Ahanda M-LE, Morin V, Oudin A, Van Den Berg T, Zoorob R.** 2009. Induction of Mx and PKR Failed to Protect Chickens from H5N1 Infection. Viral Immunology **22:**467-472.
32. **Forlenza M, Kaiser T, Savelkoul HF, Wiegertjes GF.** 2012. The use of real-time quantitative PCR for the analysis of cytokine mRNA levels. Methods Mol Biol **820:**7-23.
33. **Schwarz H, Harlin O, Ohnemus A, Kaspers B, Staeheli P.** 2004. Synthesis of IFN-beta by virus-infected chicken embryo cells demonstrated with specific antisera and a new bioassay. Journal of interferon & cytokine research **24:**179-184.
34. **Kindler E, Thiel V.** 2014. To sense or not to sense viral RNA: essentials of coronavirus innate immune evasion. Current Opinion in Microbiology **20:**69-75.
35. **Kedersha N, Ivanov P, Anderson P.** 2013. Stress granules and cell signaling: more than just a passing phase? Trends Biochem Sci **38:**494-506.
36. **Kedersha N, Anderson P.** 2009. Regulation of translation by stress granules and processing bodies. Prog Mol Biol Transl Sci **90:**155-185.
37. **Kedersha NL, Gupta M, Li W, Miller I, Anderson P.** 1999. RNA-binding proteins TIA-1 and TIAR link the phosphorylation of eIF-2 alpha to the assembly of mammalian stress granules. J Cell Biol **147:**1431-1442.
38. **White JP, Lloyd RE.** 2012. Regulation of stress granules in virus systems. Trends Microbiol **20:**175-183.
39. **Sola I, Galan C, Mateos-Gomez PA, Palacio L, Zuniga S, Cruz JL, Almazan F, Enjuanes L.** 2011. The polypyrimidine tract-binding protein affects coronavirus RNA accumulation levels and relocalizes viral RNAs to novel cytoplasmic domains different from replication-transcription sites. J Virol **85:**5136-5149.
40. **Kint J, Dickhout A, Kutter J, Maier HJ, Britton P, Koumans J, Pijlman GP, Fros JJ, Wiegertjes GF, Forlenza M.** 2015. Infectious Bronchitis Coronavirus Inhibits STAT1 Signaling and Requires Accessory Proteins for Resistance to Type I Interferon Activity. J Virol **89:**12047-12057.
41. **Wertheim JO, Chu DK, Peiris JS, Kosakovsky Pond SL, Poon LL.** 2013. A case for the ancient origin of coronaviruses. J Virol **87:**7039-7045.

533 **Figure legends**

534

535 **FIG 1. IBV induces transcription of Ifnβ, but limits production of interferon protein.**

536 Chicken embryo kidney (CEK) cells were infected with IBV-M41 (MOI 5), Sindbis (MOI 1) or

537 RVFV Cl13 (MOI 5). At the indicated time points (A) *Ifnβ* mRNA, (B) extracellular IFN protein and

538 (C) total viral RNA in the supernatant were quantified. (D - F) CEK cells were infected with the

539 indicated strains of IBV (MOI 1) and (D) *Ifnβ* mRNA (E) IFN protein and (F) virus titers were

540 determined at 24 and 48 hpi. Values represent the results of one experiment, which was

541 performed twice with comparable results.

542

543 **FIG 2. IBV induces host shutoff, without degradation of host mRNA.**

544 (A) Vero cells were infected with Beau-R (MOI 20), and at 6, 12 and 18 hours post infection (hpi)

545 newly synthesized proteins were radioactively labelled for one hour with ^{35}S methionine. Cells

546 were subsequently lysed and proteins were separated using SDS-PAGE after which ^{35}S was

547 visualized using a phosphorimager. Virus proteins are indicated with S, N and M. (B) cells were

548 electroporated (CEK) or transfected (DF-1 and Vero) with pGL3 SV40-Firefly luciferase plasmid

549 and 24 h later infected with IBV-M41 (MOI 10). At 22 hpi, luciferase activity was quantified. Bars

550 represent mean luciferase activity of triplicate measurements from two experiments. Error bars

551 indicate standard deviation and asterisks indicate statistically significant differences ($P < 0.0001$)

552 compared to mock-infected cells, as determined using an unpaired Student's t-test. (C) CEK cells

553 were infected with Beau-R (MOI 10) and at 5 hpi, transcription was inhibited using Actinomycin

554 D (ActD, 10 µg/ml) for an additional 6 h, after which mRNA was quantified using RT-qPCR. Bars

555 indicate the mean percentage of mRNA remaining after Act D treatment from triplicate wells of a

556 representative example of two biological replicates. Error bars indicate standard deviation.

557

558

FIG 3. Accessory protein 5b inhibits luciferase activity in DF-1 and Vero cells

Vero cells were transfected with plasmids expressing Flag-tagged accessory proteins and 22 h later proteins were detected using a Flag-specific antibody. (A) Detection of IBV accessory proteins. (B) DF-1 and (C) Vero cells were seeded in 96 well plates and transfected with 10 ng Renilla luciferase-expressing plasmid plus 90 ng of plasmid expressing the indicated accessory protein of Beau-R, MHV nsp1 or empty plasmid (ctrl). At 18 hours post transfection, luciferase activity was quantified and plotted on the left Y-axis. In parallel wells, cytotoxicity of each construct was investigated using the cell titer 96 cytotoxicity assay. Results are plotted on the right Y-axis. All values represent the mean of quadruplicate measurements from two independent experiments. Error bars indicate standard deviation and asterisks indicate significant differences ($P < 0.001$) compared to the control, as assessed by a one-way ANOVA followed by a Bonferroni post-hoc test.

FIG 4. Accessory protein 5b is required for induction of host shutoff

(A) Vero cells were mock treated or infected with the indicated mutant viruses not expressing one or two accessory proteins (MOI 20). At the indicated time points after infection, de novo synthesized proteins were labelled with ^{35}S methionine for one hour. Subsequently, cells were lysed and proteins were separated using SDS-PAGE and ^{35}S-labelled proteins were visualized using a phosphorimager. Areas containing host proteins are indicated with H1 - 3. Viral Spike (S), nucleocapsid (N) and Membrane (M) proteins are also indicated. Host protein synthesis at 12 hpi (B) and at 24 hpi (C) was approximated by quantification of ^{35}S signal intensity in the areas H1 - 3 indicated in (A) and expressed as ratio relative to mock-infected cells at 6 hpi. (D) Synthesis of IBV-proteins was approximated by quantification of ^{35}S signal intensity of IBV-S, -N and -M proteins at 12 hpi relative to Beau-R infected cells at 6 hpi. Bars indicate the mean of the three values determined for either virus or host proteins at the indicated time point and error bars indicate standard deviation. Asterisks indicate significant differences ($P < 0.001$) compared to mock, as assessed by one-way ANOVA followed by a Bonferroni post-hoc test.

586 **FIG 5. Host shutoff induced by accessory protein 5b limits production of IFN**

587 A) DF-1 and (B - D) CEK cells were infected (MOI 0.1) with Beau-R or Beau-R-mutant viruses
588 that do not express the indicated accessory proteins. (A and B) IFN protein in the supernatant
589 (C) *Ifnβ* mRNA and (D) virus in the supernatant was quantified. Values represent the mean of a
590 representative experiment performed in triplicate and error bars indicate standard deviation.
591 Asterisks indicate significant differences ($P < 0.01$) compared to the parental Beau-R virus, as
592 assessed by a two-way ANOVA followed by a Dunnet multiple comparison test.

Fig.1

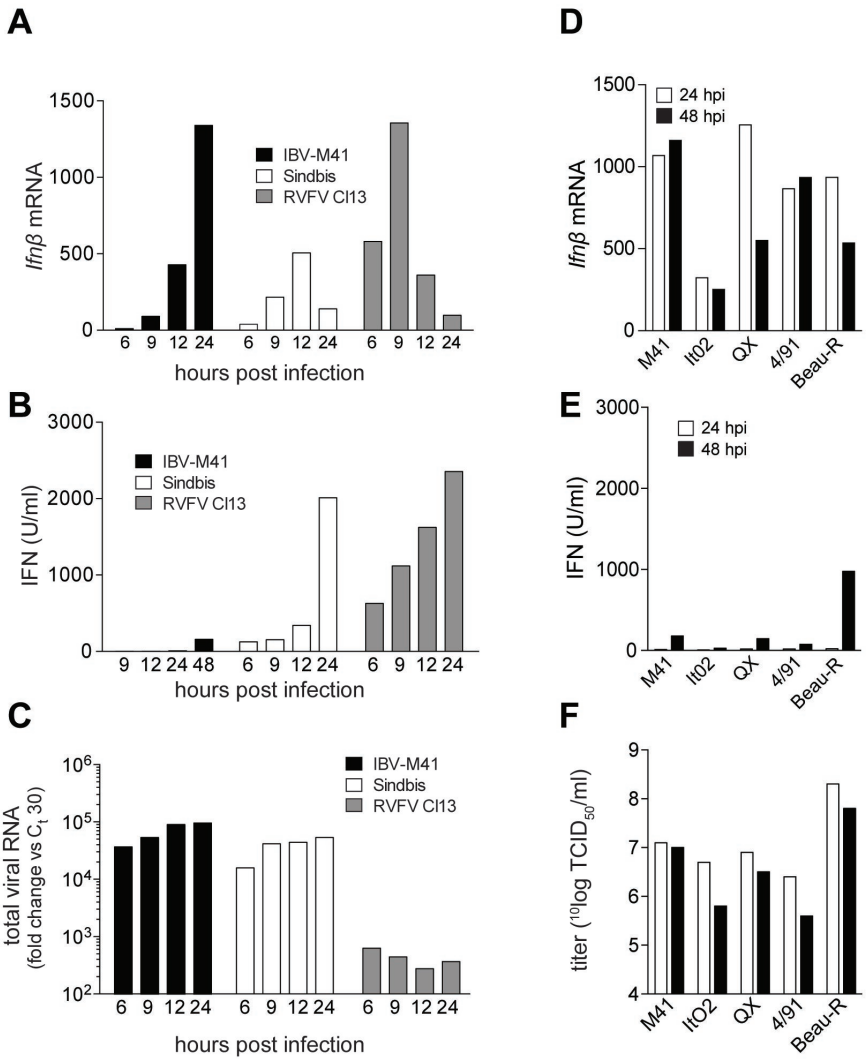


Fig.2

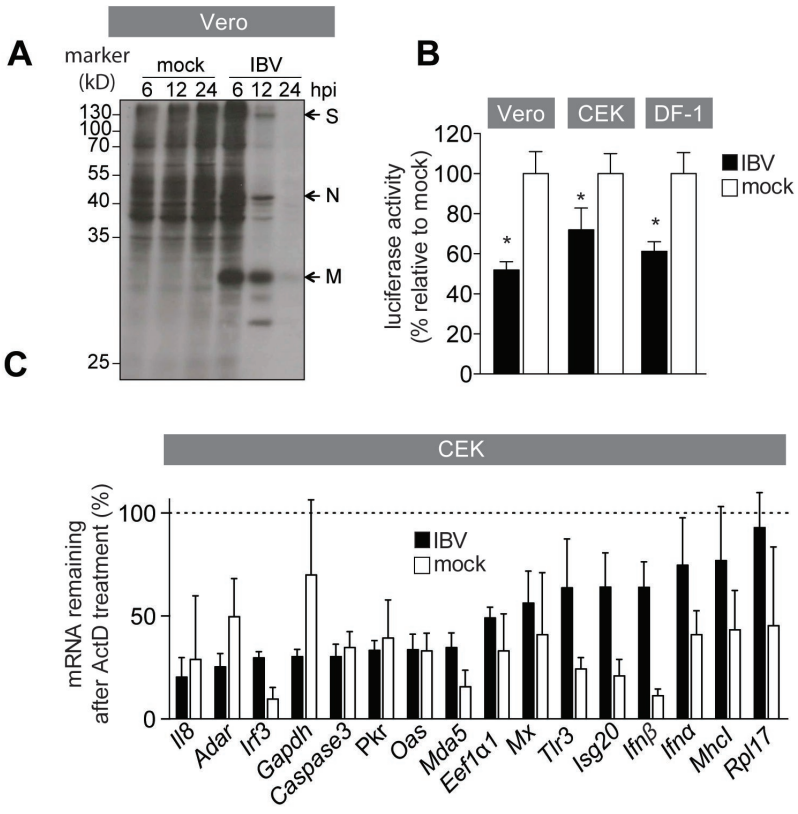


Fig.3

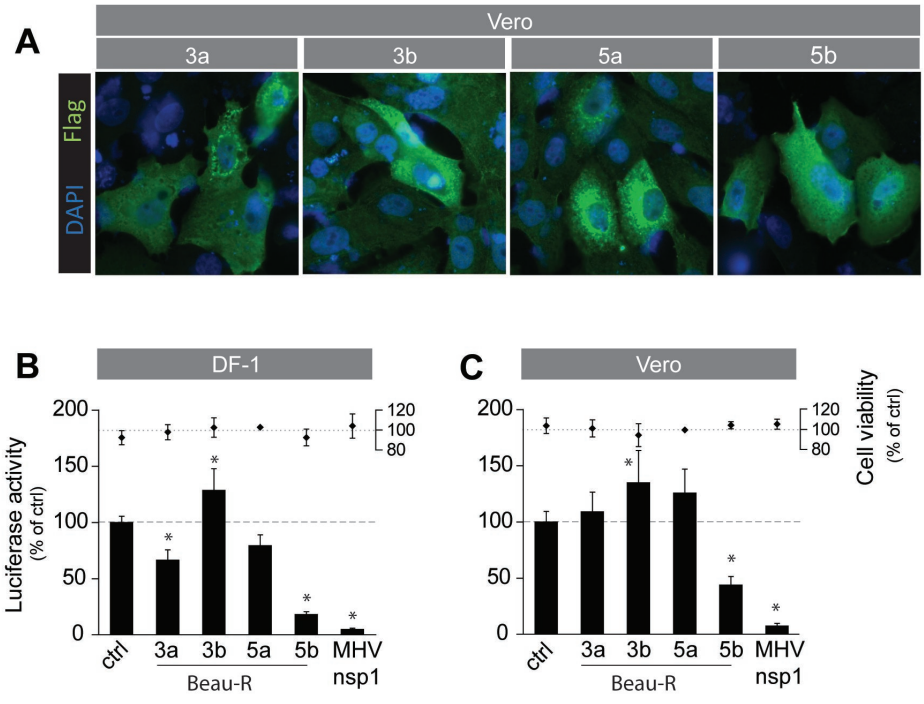
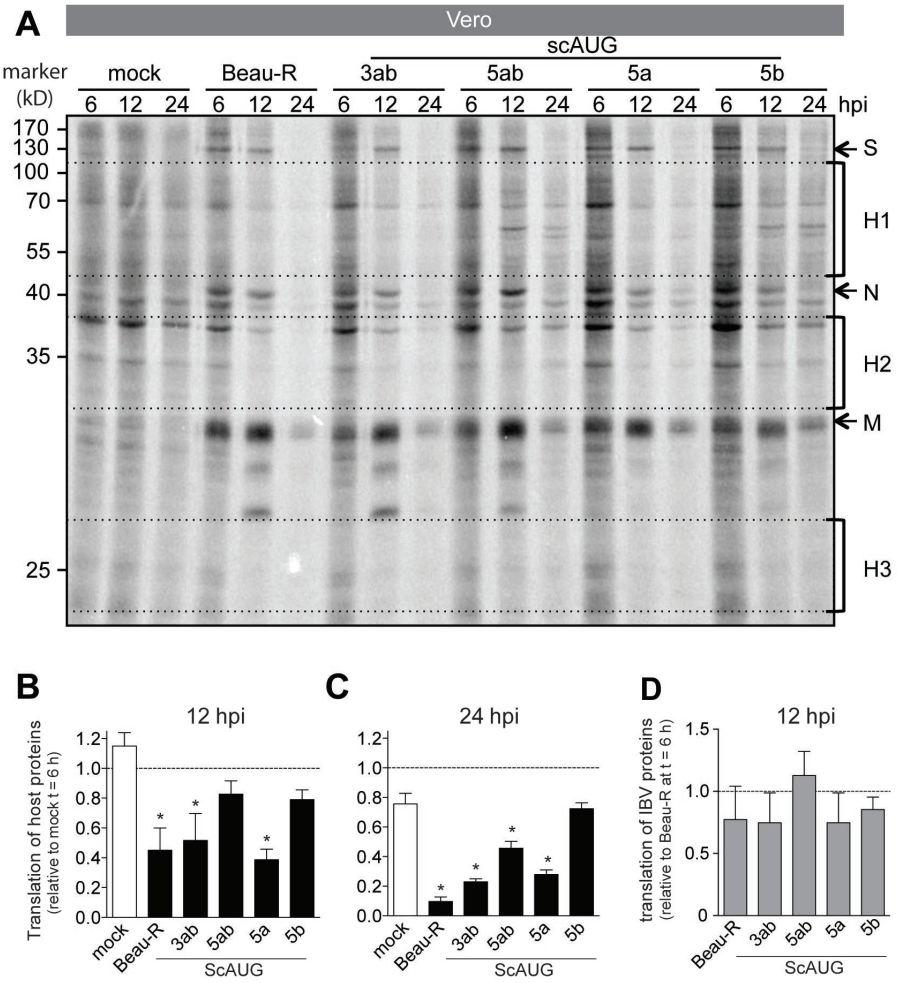


Fig.4



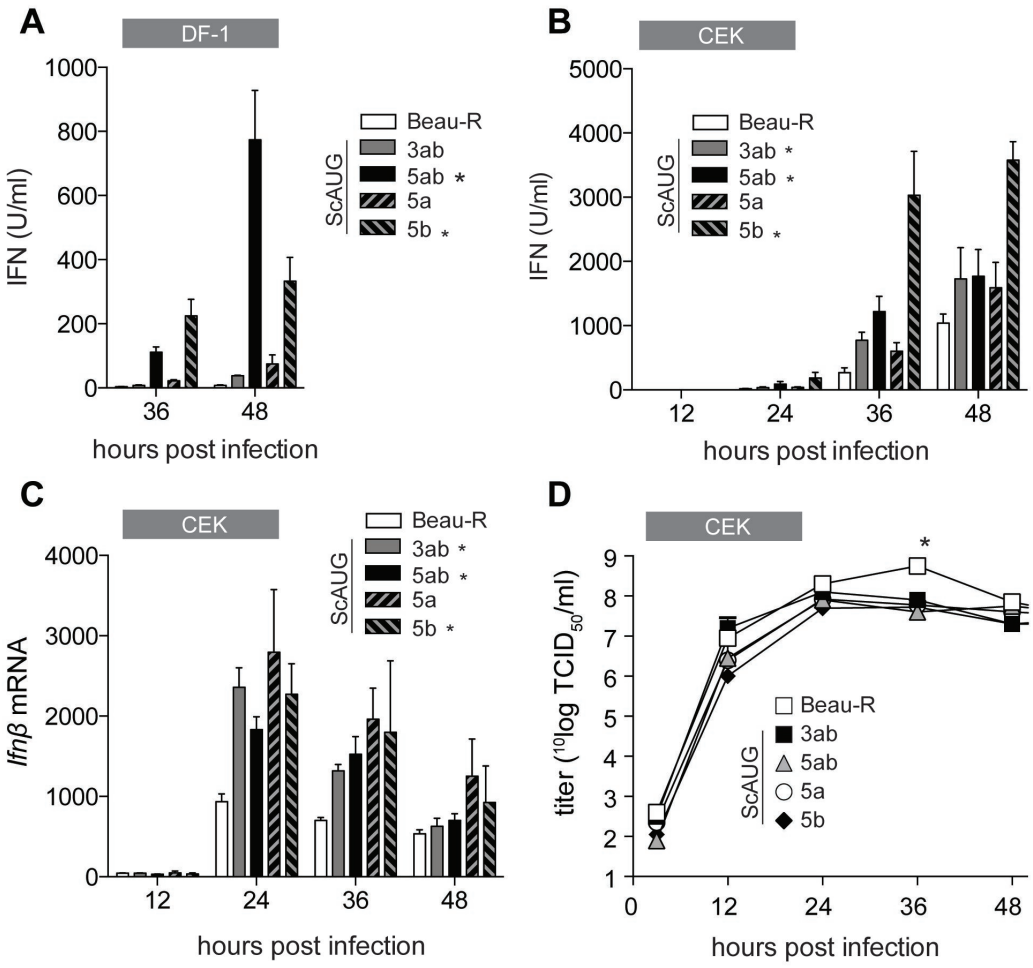


TABLE 1 primers used in this study

Gene product	Orientation	sequence (5'-3')	Accession no.	Reference
Ifnβ	FW	GCTCTCACCACCACCTTCTC	NM_001024836	6
	RV	GCTTGCTTCTTGTCCTTGCT		
Ifnα	FW	ATCCTGCTGCTCACGCTCCTTCT	XM_004937096	1
	RV	GGTGTTGCTGGTGTCCAGGATG		
Irf3	FW	CAGTGCTTCTCCAGCACAAA	NM_205372	
	RV	TGCATGTGGTATTGCTCGAT		
Tlr3	FW	TCAGTACATTTGTAACACCCCGCC	NM_001011691	1
	RV	GGCGTCATAATCAAACACTCC		
Mda5	FW	TGGAGCTGGGCATCTTTCAG	GU570144	6
	RV	GTTCCCACGACTCTCAATAACAGT		
Mx	FW	TTGTCTGGTGTTGCTCTTCCT	GQ390353	6
	RV	GCTGTATTTCTGTGTTGCGGTA		
Oas	FW	CACGGCCTCTTCTACGACA	NM_205041	2
	RV	TGGGCCATACGGTGTAGACT		
Il8	FW	TTGGAAGCCACTTCAGTCAGAC	NM_205498	2
	RV	GGAGCAGGAGGAATTACCAGTT		
Pkr	FW	CCTCTGCTGGCCTTACTGTCA	NM_204487	3
	RV	AAGAGAGGCAGAAGGAATAATTTGCC		
Adar	FW	TGTTTGTGATGGCTGTTGAG	AF403114	6
	RV	AGATGTGAAGTCCGTGTTG		
Mhc-I	FW	CTTCATTGCCTTCGACAAAG	NM_001031338	2
	RV	GCCACTCCACGCAGGT		
Isg20	FW	TCTGGAAAGGTGGTGGTT	EU602349	
	RV	AAGGGGATTTTGGATGTGT		
Caspase 3	FW	GTTAGAAACGCAAACCTGA	NM_204725	
	RV	TGAAGATACGAAACCAAACCA		
Rpl17	FW	TGGATTCTCTGGTGATTGAG	XM_004949013	
	RV	CTTCTTCCTCTGGCTTGG		
Gapdh	FW	CATCACAGCCACACAGAAG	NM_204305	
	RV	GGTCAGGTCAACAACAGAGA		
Eef1α1	FW	CTGATTGTGCTGTCCTGATT	NM_204157	
	RV	TTCGTATCTCTTCTGGCTGT		
RVFV	FW	AAAGGAACAATGGACTCTGGTCA	AF134508	4
	RV	CACTTCTTACTACCATGTCCTCCAAT		
SinV	FW	CCCAGGAACCCGCAAGTATG	GM893992	5
	RV	CGTGAGGAAGATTGCGGTTC		
IBV-N	FW	GAAGAAAACCAGTCCCAGA	AY851295	6
	RV	TTACCAGCAACCCACAC		
Luciferase	FW	TGTTGGGCGCGTTATTTATC	X65316	6
	RV	AGGCTGCGAAATGTTCATACT		

3a	FW	GCCGC*GAATTC*GATGATCCAAAGTCCCACG
	RV	TATCGAT*GAATTC*GC**TTA**GTCTAGACTGTGCCAAAGG
3b	FW	GCCGC*GAATTC*AATGTTAAACTTAGAAGTAATTATTGAAACTG
	RV	TATCGAT*GAATTC*GC**TTA**TTCAATAAATTCATCATCACC
5a	FW	GCCGC*GAATTC*AATGAAATGGCTGACTAGTTTTG
	RV	TATCGAT*GAATTC*GC**TCA**TGCCAGCGATTGGGTGG
5b	FW	GCCGC*GAATTC*AATGAATAATAGTAAAGATAATCCTTTTCG
	RV	TATCGAT*GAATTC*GC**CTA**GTTTAATGACTGGCGCTG

FW, forward; RV, reverse

EcoRI sites are italicized, start codons are underlined, stop codon are in bold.

1