

1 **Coronavirus endoribonuclease nsp15 induces host cellular protein**
2 **synthesis shutoff**

3 **Short title: CoV nsp15 induces cellular protein synthesis shutoff**

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27 **Abstract:**

28 The endoribonuclease (EndoU) nsp15 of coronaviruses plays an important role in evasion of
29 host innate immune responses by reducing the abundance of viral double-stranded RNA,
30 whereas less is known about potential host cellular targets of nsp15. In this study, we show that
31 cellular protein synthesis is inhibited upon over-expression of nsp15 from four genera of
32 coronaviruses and this is accompanied by nuclear retention of the poly(A) binding protein
33 cytoplasmic 1 (PABPC1). We also show that the EndoU activity of nsp15 is indispensable for
34 both, inhibition of protein synthesis and PABPC1 nuclear relocation. FISH analysis using oligo-
35 dT probes, revealed an overlap between the localization of cellular mRNA and that of
36 overexpressed nsp15 in some cells, suggesting that, when expressed alone, nsp15 may target
37 host mRNA. When investigating the association of nsp15 on protein shut off in the context of
38 a viral infection, we observed that the γ -coronavirus infectious bronchitis virus (IBV), induced
39 host translation shutoff in an p-eIF2 α -independent manner and mainly retained PABPC1 in the
40 cytoplasm, whereas the nsp15 EndoU-deficient IBV accumulated viral dsRNA and caused p-
41 PKR-p-eIF2 α -dependent host protein translation shutoff, accompanied with PABPC1 nuclear
42 relocation or stress granule (SG) localization. This phenomenon suggests that during infection
43 with wild type IBV, although the cellular translation is inhibited, initiation of viral mRNA
44 translation leads to PABPC1 binding to viral mRNA, thereby preventing its nuclear entry;
45 during infection with nsp15 EndoU-deficient IBV however, the eIF2 α -dependent host protein
46 translation shutoff prevents both host and viral mRNA translation initiation, releasing PABPC1
47 from binding to cytosolic and viral mRNA, thereby relocating it to the nucleus or to SG.
48 Altogether, this study reveals unique yet conserved mechanisms of host protein shutoff that add
49 to our understanding of how coronaviruses regulate host protein expression through a
50 mechanism that involves catalytically active nsp15 EndoU, and describes how nsp15 may target
51 both, viral and host mRNA.

52 **Author summary**

53 It has been reported that coronavirus infection suppresses host protein translation, α - and
54 β - coronavirus nsp1 is responsible for inhibition of host gene expression. However, for γ - and
55 δ -coronavirus, there is no nsp1 and the underlying mechanisms by which virus regulates host
56 translation are not well characterized. Here, we show that coronavirus endoribonuclease nsp15
57 is responsible for the inhibition of host translation by targeting to host factors, meanwhile it
58 helps virus bypass the PKR-eIF2 α mediated host translation shutoff, which is harmful for virus
59 gene expression, by reducing the accumulation of viral dsRNA. This novel finding gives insight
60 how does nsp15 targets to both host factors and viral RNA, to facilitate virus replication.
61 Moreover, the novel function of nsp15 is found to be conserved among coronaviruses, revealing
62 the essential role of this endoribonuclease in hijacking host translation machinery for virus
63 replication.

64 **Introduction**

65 Coronaviruses are classified into four genera, α , β , γ and δ . They are enveloped, positive-
66 sense, single-stranded RNA viruses possessing the largest known RNA genome (approximately
67 25 to 32 kilobases) [1]. Over two-thirds of the genome at the 5'-end comprises the open reading
68 frames ORF1a and ORF1b; the translation of ORF1b requires a programmed -1 ribosomal
69 frameshifting mechanism [2]. Upon entry into host cells, coronavirus genome serves as the
70 template for the translation of polyproteins 1a and 1ab, which are cleaved by internal papain-
71 like protease (nsp3) and 3C-like protease (nsp5), to produce non-structural proteins (nsp) [3].
72 The α and β coronaviruses encode 16 nsps (nsp1 to nsp16) [4]; while the γ and δ coronaviruses
73 only encode 15 nsps (nsp2-nsp16) and lack the most N-terminal cleavage product nsp1 [5, 6].
74 A number of nsps contain domains involved in transcription and replication of viral RNA,
75 including the RNA-dependent RNA polymerase (RdRp) nsp12, the template-primer providers
76 nsp7 and nsp8, the RNA helicase/5'-triphosphatase nsp13, the exoribonuclease nsp14, the
77 endoribonuclease (EndoU) nsp15, and the RNA-cap methyltransferase nsp14 and nsp16 [7-13].
78 Amongst them, RdRp nsp12 plays a central role to drive the replication and transcription of
79 viral RNA, while the other nsps play a supportive role.

80 It has been reported that the highly conserved EndoU nsp15 is an integral component of
81 the replication and transcription complex (RTC) [14-16], where it possesses uridylate-specific
82 endonucleolytic activity on viral RNA. The RTC is accommodated in virus-induced double
83 membrane vesicles (DMV) during SARS-CoV-2, SARS-CoV, MERS-CoV or MHV infection
84 [17-21], or in zippered ER and spherules single membranes during IBV infection [22], which
85 is formed by the transmembrane protein nsp3, nsp4, and nsp6 [23, 24]. The viral ligand double
86 stranded (ds)RNA, an intermediate product of viral replication, is as well present in the virus-
87 induced remodelled intracellular membrane structures [19, 25]. Nsp15 activity contributes to
88 keeping the amount of dsRNA low to evade the detection by host cell sensors. Such role for
89 nsp15 has been reported for MHV, HCoV-229E, PEDV, IBV [26-29], and likely also for SARS-
90 CoV-2 [30, 31]. While the role of nsp15 in innate immune escape is well established, its effect
91 on host cells remains poorly understood. In our previous study, we observed that nsp15
92 suppresses the chemically- or physically-induced formation of stress granules (SG), and
93 promotes nuclear accumulation of the cytoplasmic poly(A) binding protein (PABPC1) [29].
94 This observation suggests that nsp15 not only targets viral RNA, but also regulates the host
95 functions by targeting some unknown host substrates.

96 In order to successfully replicate, coronaviruses employ a range of strategies to escape or
97 antagonize the host immune responses [32]. Inhibition of host gene expression is not only an
98 alternative strategy to antagonize the host innate immune response by reducing the synthesis

99 anti-viral proteins, but also a smart way to hijack the host translation machinery to facilitate the
100 translation of viral mRNA instead. The process of eukaryotic gene expression includes
101 transcription, RNA processing, nuclear export of RNA, protein translation, and post-
102 translational modification [33]. Viruses may suppress the host gene expression by reducing the
103 levels of host mRNA or preventing their association with ribosomes or translation initiation
104 factors. For example, poliovirus 3C protease inhibits RNA polymerase II mediated transcription
105 initiation by cleavage of transcription activator Oct-1 [34, 35]; influenza A virus (IAV)
106 polymerase acidic (PA) protein snatches the capped primers from nascent host transcripts for
107 the synthesis of viral mRNA [36]; IAV NS1 inhibits polyadenylation of cellular precursor
108 mRNA (pre-mRNA) and prevents the nuclear export of cellular mRNA [37-39]; human
109 immunodeficiency virus (HIV) viral protein R (VPR) inhibits the splicing of host pre-mRNA
110 [40]; herpes simplex virus (HSV) infected cell protein 27 (ICP27) blocks host transcription
111 termination [41]; poliovirus 2A protease affects cellular mRNA nuclear export by mediating
112 nucleoporin cleavage [42]; poliovirus 3C proteinase cleaves PABP to inhibit translation
113 initiation [43]. It has been reported that several α - and β -coronaviruses, through their nsp1,
114 hijack the host translation machinery by repressing host mRNA transcription in the nucleus [44]
115 , preventing the nuclear export of host mRNA [45, 46], degrading host mRNA in the nucleus
116 and cytoplasm [44, 47-50], and inhibiting host mRNA translation by interfering ribosome [49,
117 51, 52]. However, for the γ -coronavirus IBV and δ -coronavirus PDCoV, which lack nsp1,
118 although host protein translation shutoff is also observed, the protein(s) potentially involved in
119 are not well characterized [53-55], although the IBV 5b and S were identified to be associated
120 with translation inhibition [56, 57].

121 Several viral endonucleases play a role in regulating not only viral RNA but also host gene
122 expression by targeting host mRNA for degradation. For example, the α -*herpesvirinae* herpes
123 simplex virus (HSV) virion host shutoff (Vhs) protein [58-60], the γ -*herpesvirinae* Kaposi's
124 sarcoma-associated herpesvirus (KSHV) shutoff and exonuclease (SOX protein) [61-63],
125 Epstein Barr virus (EBV) BGLF5 protein [64-66], and murine herpesvirus 68 (MHV-68)
126 muSOX protein [61, 67], possess endonuclease activity and inhibit synthesis of cellular proteins
127 by promoting the global mRNAs degradation [61, 63, 68, 69]. As mentioned, the IAV cap-
128 dependent endonuclease PA is associated with the RNA polymerase complex and is responsible
129 for snatching capped oligonucleotides from cellular pre-mRNAs and for using them as primers
130 for the synthesis of viral mRNAs, thereby helping viral gene expression while suppressing host
131 mRNA maturation [36, 70, 71]; moreover, IAV encodes another endonuclease, PA-X, that

132 selectively degrades host RNAs and usurps the host mRNA processing machinery to destroy
133 nascent mRNAs and limit host gene expression [72-74].

134 Here we investigated the ability of EndoU nsp15 of IBV and of other coronaviruses to
135 inhibit cellular protein synthesis. We provide evidences that nsp15 inhibits host protein
136 translation through a mechanism that involves targeting host factors involved in the translation
137 complex, including host mRNA and PABPC1; a mechanism for which the EndoU activity is
138 indispensable. In the context of IBV infection, nsp15, probably in concert with other IBV
139 proteins, trigger an eIF2 α -independent host translation shutoff. When nsp15 EndoU activity is
140 deficient, a higher amount of viral dsRNA accumulates in the cells [29], the nsp15 deficient
141 IBV inhibits host protein translation through a PKR-eIF2 α -dependent mechanism. Altogether,
142 our findings unveil new, yet conserved strategies shared among coronaviruses, to regulate host
143 gene and protein expression and the role of the EndoU nsp15 therein.

144 **Results**

145 **IBV nsp15 inhibits exogenous protein synthesis but does not affect mRNA levels**

146 We previously reported that IBV nsp15 interferes with the chemically- and physically-induced
147 SGs formation, possibly by targeting the host translation machinery [29]. Furthermore, during
148 a screening of IBV-encoded type I IFN antagonists using a luciferase reporter system, we
149 observed that IBV nsp15 reduced the expression not only of the IFN β promotor-driven
150 luciferase, but also the expression of the co-transfected plasmids encoding HA-tagged MAVS
151 (HA-huMAVS) in 293T cells (**supplementary Fig 1**). These data prompted us to hypothesize
152 that IBV nsp15 interferes with the host translation system. It was noted that 5a and E also
153 suppressed the co-transfected huMAVS expression, meanwhile reduced the expression of
154 IFN β -driven luciferase. This result indicates IBV encodes several proteins involved in
155 inhibition of protein expression.

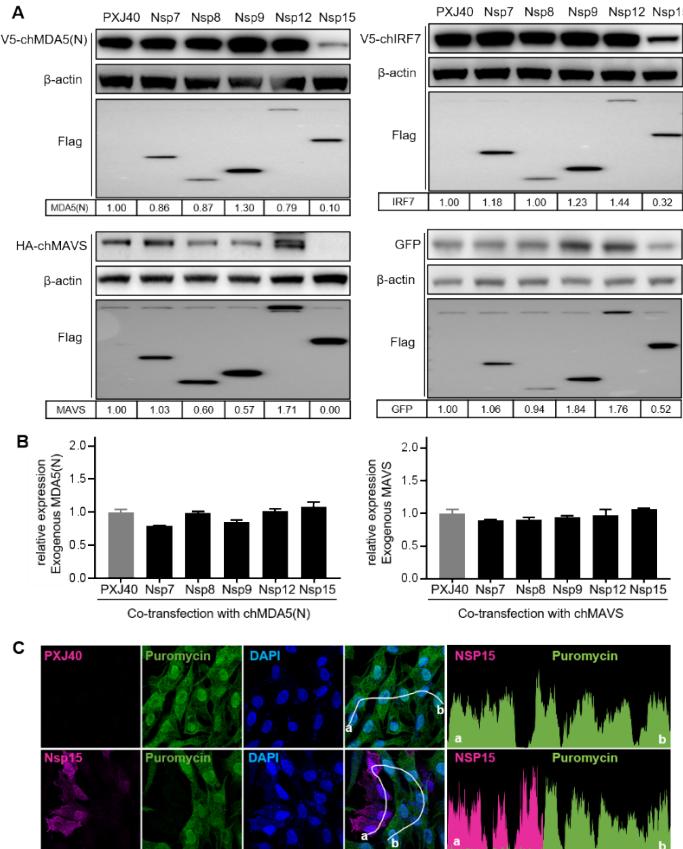
156 To examine whether IBV nsp15 indeed interferes with the host protein synthesis, DF-1
157 cells were co-transfected with plasmids encoding Flag-tagged IBV nsp15 and V5-tagged
158 constitutively active form (N-terminal domain) of chicken MDA5 [V5-chMDA5(N)] [75],
159 nsp15 and HA-chMAVS [76], nsp15 and V5-tagged chicken interferon regulatory factor 7 (V5-
160 chIRF7) [77], or nsp15 and enhanced green fluorescent protein (EGFP). Vector PXJ40, Flag-
161 tagged nsp7, nsp8, nsp9, and nsp13 were co-transfected with corresponding plasmids as control.
162 Western blot analysis showed that overexpression of nsp15 strongly suppressed the protein
163 levels of V5-chMDA5(N), HA-chMAVS, V5-chIRF7, and also EGFP to various degrees (**Fig**
164 **1A**), whereas overexpression of nsp7, nsp8, nsp9, nsp12, or transfection of PXJ40 did not.

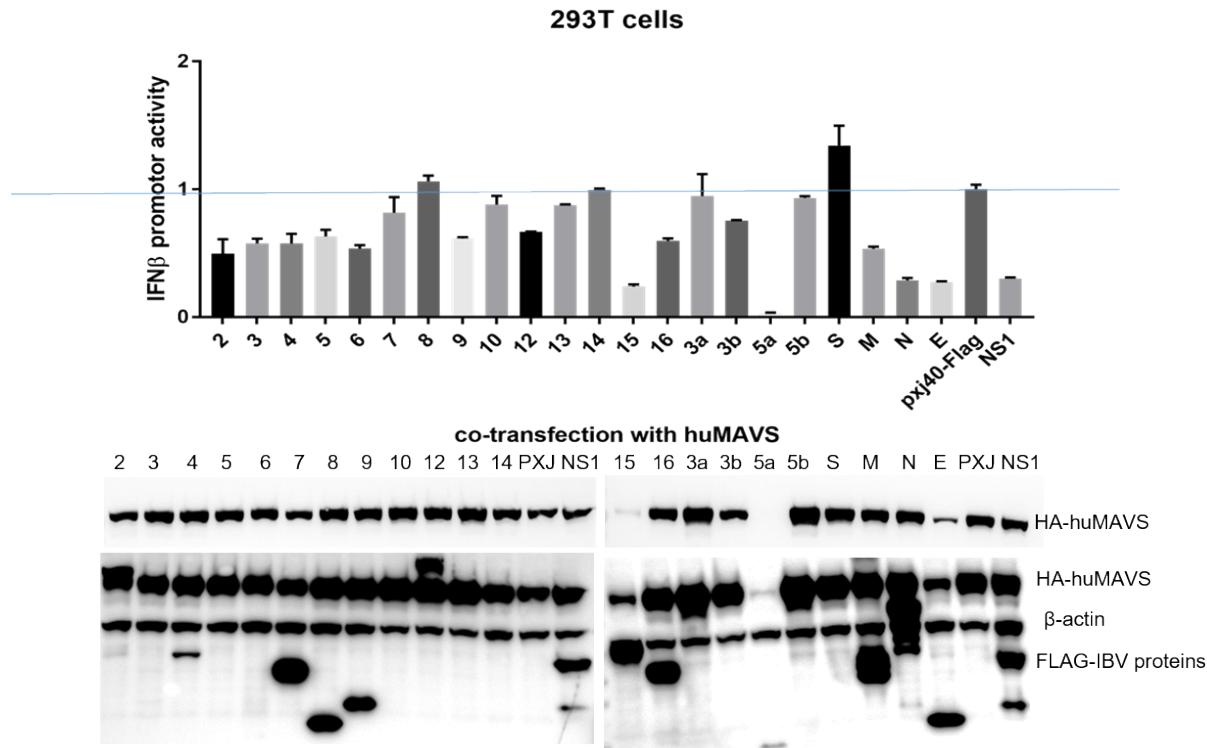
165 These results confirm that nsp15 inhibits the expression of exogenous proteins encoded by co-
166 transfected plasmids.

167 Considering the EndoU activity, we next asked whether the effects of IBV nsp15 on
168 exogenous protein expression were mediated by reducing mRNA levels. To this end, we
169 quantified the transcripts derived from the co-transfected plasmids encoding V5-chMDA5(N)
170 or HA-chMAVS. To prevent amplification of endogenous transcripts, primers were designed
171 to span the V5 or HA tag sequence. Using random primers for an unbiased cDNA synthesis,
172 the quantitative RT-PCR analysis showed that nsp15 did not affect mRNA levels of transcripts
173 derived from co-transfected chMDA5(N) or chMAVS (**Fig 1B**). These data are also consistent
174 with our previous report showing that during IBV infection no effect on host mRNA stability
175 was observed [53].

176 Since the nsp15 induced inhibition of exogenous protein expression was not restricted
177 to proteins involved in anti-viral responses but was also observed for EGFP, we next asked
178 whether the effect on protein synthesis is universal to host endogenous proteins. To test this,
179 we determined the signal of puromycin-labelled, *de novo* synthetized, endogenous peptides.
180 Puromycin is an analogue of tRNA that binds to growing peptide chains and causes the release
181 of premature peptide chains [78]. Therefore, the signal of puromycin-labelled peptides, detected
182 with an anti-puromycin antibody, represents the number of peptides *de novo* synthesized during
183 the period of puromycin treatment. In all nsp15-expressing cells, puromycin labelling signal
184 was strongly reduced, while this was not observed in nsp15 non-expressing cells, or cells
185 transfected with the control PXJ40, indicating that nsp15 indeed suppresses *de novo* protein
186 synthesis (**Fig 1C**). Together, these results show that overexpression of nsp15 inhibits the global
187 synthesis of endogenous proteins.

188





214
215
216 **Supplementary Fig 1. IBV nsp15 downregulates MAVS-mediated IFN β induction and the**
217 **expression of co-transfected plasmid.** 293T cells were seeded in 48 well plates at 3×10^4
218 cells/well (for luciferase assay) or 12 well plates at a density of 10^5 cells/well (for Western blot).
219 Cells were co-transfected with Flag-tagged IBV proteins (nsp2, nsp3, nsp4, nsp5, nsp6, nsp7,
220 nsp8, nsp9, nsp10, nsp12, nsp13, nsp14, nsp15, nsp16, 3a, 3b, 5a, 5b, S, M, N, E) together with
221 HA-huMAVS, the reporter plasmid encoding firefly luciferase driven by the inducible IFN β
222 promoter, and the control plasmid pRL-TK encoding Renilla luciferase driven by the
223 constitutive HSV TK promoter. The PXJ40-Flag and IAV Flag-tagged NS1 were included in
224 parallel experiment control. After 24 h, cells in 48 well plates were lysed, the firefly and Renilla
225 luciferase activities were measured. The IFN β promoter activity was normalized to Renilla and
226 presented relative to the PXJ40-Flag control. Bars indicated the average of two co-transfection
227 experiments performed independently. Cells in 12 well plates were lysed and subjected to
228 Western blot to verify the protein expression. The membranes were first probed with an anti-
229 HA antibody to detect huMAVS, followed by re-probing with an anti-Flag antibody to detect
230 IBV proteins and IAV NS1, and then probed with an anti-actin antibody to detect actin as
231 loading control.

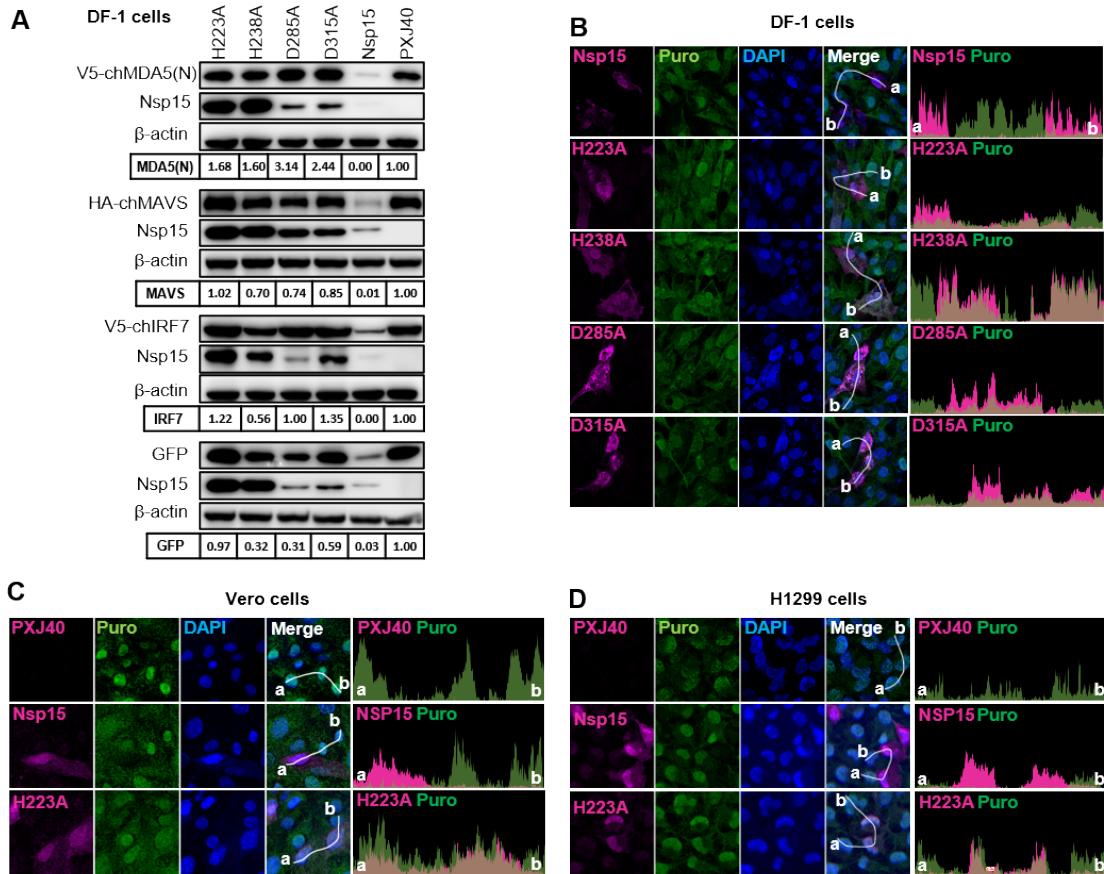
232
233 **The catalytic activity and oligomeric structure of IBV nsp15 are indispensable for**
234 **inhibition of *de novo* protein synthesis**

235 The two conserved catalytic residues for IBV nsp15's EndoU activity reside at histidine
236 residues H223 and H238, and the two residues critical for oligomerization resides at aspartic

237 acid D285 and D315. We previously reported that also for IBV, the EndoU activity of nsp15 is
238 required to limit the accumulation of dsRNA intermediates in the cells, thereby escaping host
239 recognition and delaying IFN β production [29]. To investigate whether the EndoU activity is
240 required also for the observed effects on *de novo* protein synthesis inhibition, we made use of
241 previously generated mutated nsp15 in which either one of the catalytic histidine (H) or one of
242 the aspartic acid (D) residues, were substituted by an alanine (A) residue (H223A, H238A,
243 D285A, D315A) [29]. Western blot analysis revealed that mutating the catalytic or the
244 oligomerization core residues largely abolished the inhibiting effect of nsp15 on exogenous
245 protein expression [chMDA5(N), chMAVS, chIRF7, EGFP] (**Fig 2A**), suggesting that the
246 EndoU activity and oligomerization of nsp15 are indeed indispensable for its inhibition on
247 protein synthesis. In addition, the expression level of wild type nsp15 was lower than the levels
248 of mutated nsp15 (**Fig 2A**), suggesting that the effect on cellular protein synthesis also affected
249 the expression of nsp15 itself. These data confirm that the inhibitory effect of nsp15 on the
250 cellular protein synthesis requires its EndoU activity and oligomeric structure. The expression
251 levels of the oligomerization-deficient nsp15 (D285A, D315A) were lower than those of the
252 catalytic-deficient nsp15, suggesting that the inability to oligomerize may affect protein
253 stability. Although far less profound than observed for wild type nsp15, the H238A mutation
254 also resulted in a somewhat lower expression of co-transfected chIRF7, and the H238A, D285A
255 and D315A mutations resulted in a lower expression of co-transfected EGFP (**Fig 2A**),
256 suggesting that inactivation of single catalytic/oligomerization domain might not be sufficient
257 to completely abolish the effect of nsp15 on protein expression.

258 We then examined the effect of wild type and mutated nsp15 on endogenous *de novo*
259 protein synthesis by analysing the fluorescence intensity of the puromycin-labelled peptides
260 signals. Indirect immunofluorescence analysis in DF-1 cells showed that, compared to the cells
261 not expressing nsp15, wild type nsp15 expressing cells displayed a strongly reduced puromycin
262 signal ; however, this was not observed in cells expressing mutated nsp15 (**Fig 2B**),
263 demonstrating that EndoU activity and oligomerization structure are indeed indispensable for
264 the inhibiting effect on cellular protein synthesis. We further assessed the inhibiting effect of
265 nsp15 on cellular protein synthesis in Vero cells and H1299 cells, which are permissive cell
266 lines for the IBV-Beaudette strain. Consistent with the results in DF-1 cells, indirect
267 immunofluorescence analysis showed that the presence of wild type nsp15, but not of catalytic-
268 deficient H223A nsp15, led to lower puromycin labelling signal in Vero and H1299 cells (**Fig**
269 **2C**). Altogether, these results demonstrate that the inhibiting effect on exogenous and

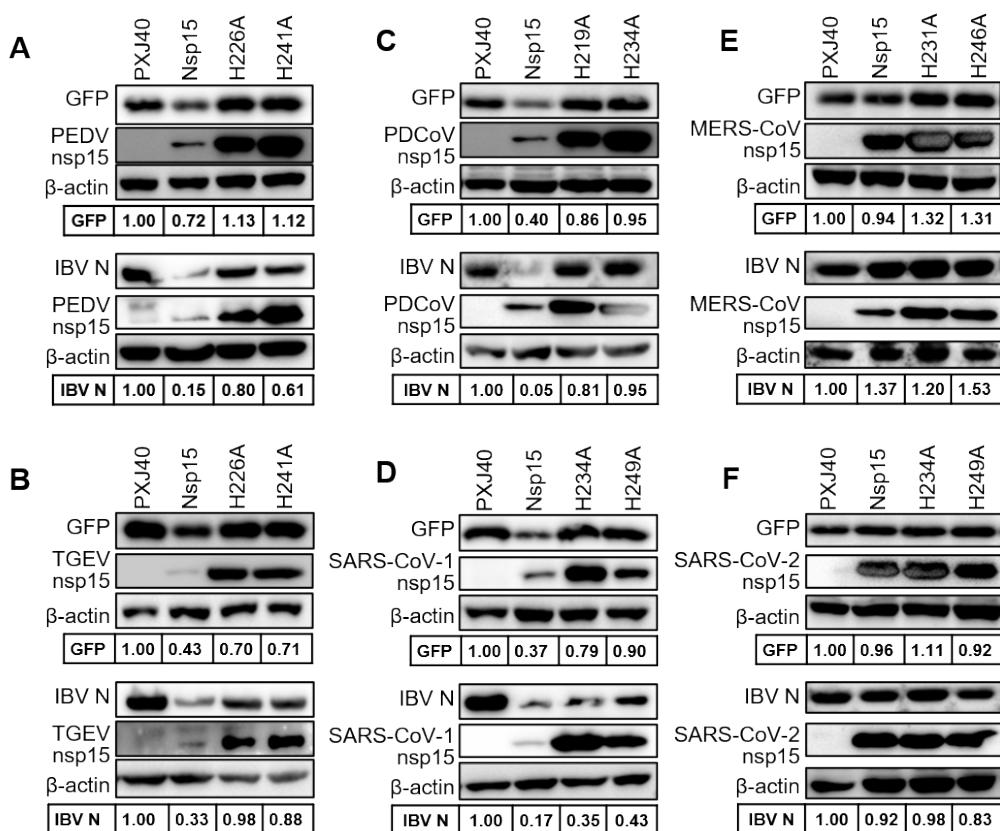
270 endogenous protein synthesis by IBV nsp15 is a generalized inhibitory effect on cellular protein
271 synthesis, and not restricted to cell types.



272
273 **Fig 2. The catalytic activity and oligomeric structure of IBV nsp15 are indispensable for**
274 **the inhibition of *de novo* protein synthesis in chicken cells as well as mammalian cells.** (A)
275 Plasmid encoding Flag-tagged catalytic-deficient nsp15 H223A, H238A, oligomerization-
276 deficient nsp15 D285A, D315A, wild type nsp15, and the vector PXJ40, were each co-
277 transfected with plasmids encoding V5-chMDA5(N), HA-chMAVS, V5-IRF7, or EGFP into
278 DF-1 cells. After 24 h, Western blot analysis was performed using corresponding antibodies.
279 β-actin was detected as loading control. Density of the bands was analysed by Image J,
280 normalized to the signal of β-actin, and the ratio was presented relative to the density detected
281 in the corresponding PXJ40 transfected cells. (B) DF-1 (C) Vero and H1299 cells, were
282 transfected with the plasmid encoding wild type or mutated nsp15 and treated with puromycin
283 (5 µg/ml) for 1 h at 23 h post-transfection (h.p.t), to label the *de novo* synthesized peptides.
284 Indirect immunofluorescence was performed to detect nsp15 (magenta), puromycin (green),
285 and nuclei (DAPI, blue). Fluorescence intensity of nsp15 and puromycin signal along the white
286 line (from a to b) is indicated in the right panel (histogram plot).

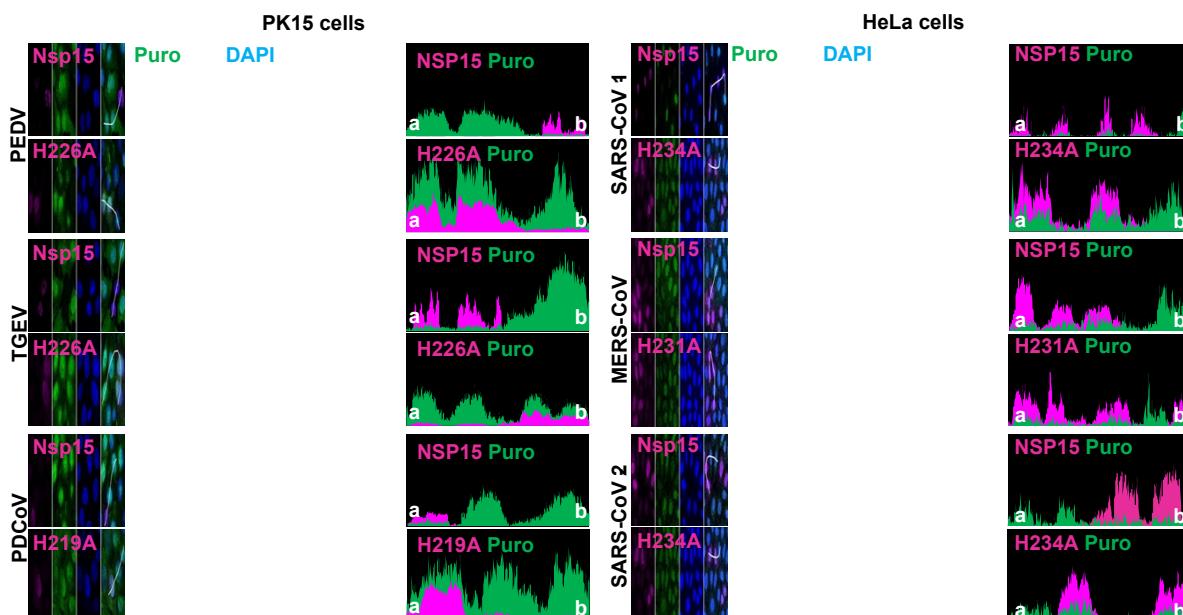
287
288 **Inhibition of *de novo* protein synthesis is a conserved feature of nsp15 from different**
289 **genera of coronaviruses**

290 We previously reported on the conserved activity of catalytic histidine residues of nsp15 on
291 inhibition of SG formation, including nsp15 from IBV, PEDV, TGEV, PDCoV, SARS-CoV-1,
292 MERS-CoVs, and SARS-CoV-2 [29]. This prompted us to investigate whether the observed
293 inhibitory effect of IBV nsp15 on protein synthesis is conserved among nsp15 from different
294 genera of coronaviruses and whether such function is dependent on the EndoU catalytic activity.
295 For this purpose, the expression plasmids for wild type and catalytic-deficient nsp15 from the
296 above-mentioned coronaviruses were co-transfected with a plasmid encoding EGFP or IBV N
297 in Vero cells. Western blot analysis revealed that wild type nsp15 of PEDV, TGEV, PDCoV
298 and SARS-CoV-1 reduced the expression EGFP and IBV N, while catalytic-deficient nsp15
299 did not (Fig 3A-D). Conversely, nsp15 of MERS-CoV and SARS-CoV-2 did not show a
300 pronounced effect on EGFP or IBV N expression (Fig 3E-F). These data suggest that,
301 consistently with the data for IBV nsp15 (Fig 1), also nsp15 from most of the tested
302 coronaviruses can suppress exogenous protein expression.



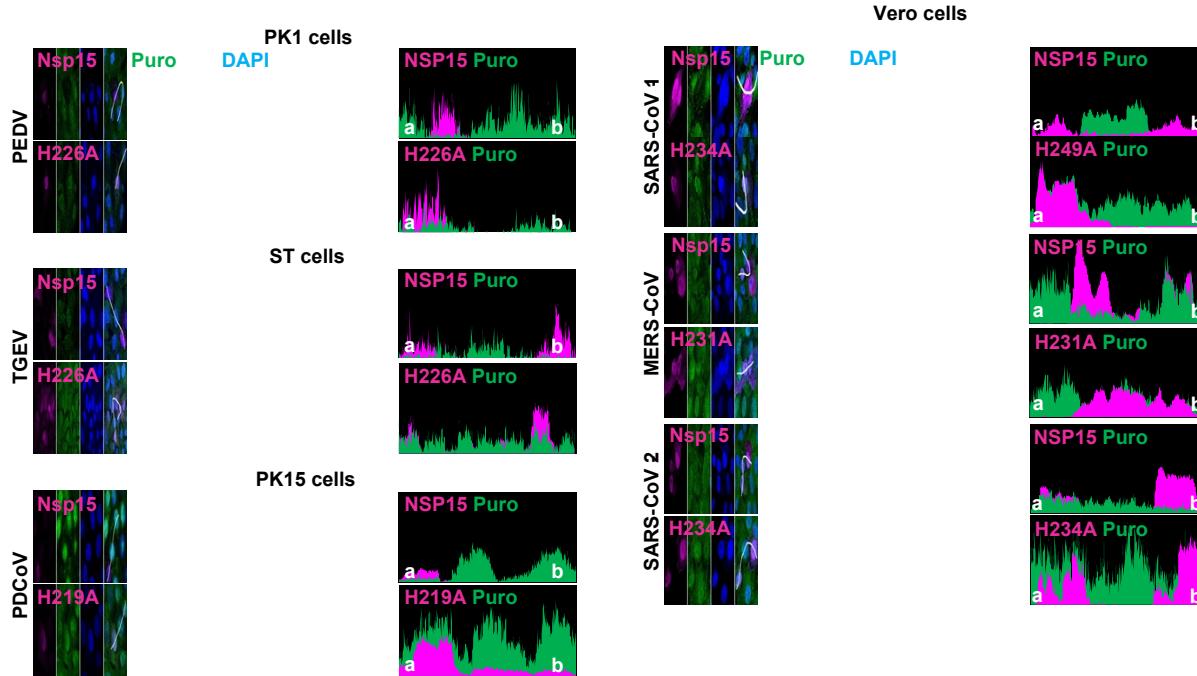
303 **Fig 3. Nsp15 from most of coronaviruses inhibits expression of exogenous transfected**
304 **plasmids.** The plasmid encoding wild type or catalytic-deficient nsp15 from the indicated
305 coronaviruses was co-transfected with the plasmid encoding EGFP or IBV N into Vero cells.
306 After 24 h, Western blot analysis was performed using corresponding antibodies. β-actin was
307 detected as loading control. Density of the bands of EGFP or IBV N were analysed by Image
308 J, normalized to the signal of β-actin and presented relative to the PXJ40 group.

310 Next, we investigated whether nsp15 from different genera of coronaviruses also
311 suppress endogenous protein expression. Consistent with the result on IBV nsp15 (**Fig 2B-D**),
312 indirect immunofluorescence analysis revealed that PK15 cells expressing wild type nsp15 of
313 PEDV, TGEV, PDCoV and HeLa cells expressing wild type nsp15 of SARS-CoV-1 displayed
314 weaker puromycin labelling signals than cells not expressing nsp15 (**Fig 4**). This effect was
315 largely abolished in catalytic-deficient nsp15 expressing cells (**Fig 4**). Consistent with the data
316 in **Fig 3**, cells expressing nsp15 of MERS-CoV and nsp15 of SARS-CoV-2 also showed a
317 reduction in puromycin signal compared to cells not expressing nsp15, but the effect was less
318 pronounced than the reduction afforded by nsp15 from other coronaviruses, suggesting that
319 nsp15 of MERS-CoV and SARS-CoV-2 might work somewhat differently from other
320 coronaviruses nsp15. Similar effects of nsp15 on puromycin staining were observed when
321 nsp15 of PEDV, TGEV, PDCoV were expressed in PK1, ST, and PK15 cells, respectively, and
322 nsp15 of SARS-CoV-1, MERS-CoV and SARS-CoV-2 were expressed in Vero cells
323 (**Supplementary Fig 2**), suggesting the inhibition of host protein translation by nsp15 is general
324 and not restricted to specific cell types.



325
326 **Fig 4. Nsp15 from different genera of coronavirus inhibits *de novo* protein synthesis.**
327 Porcine kidney 15 (PK15) cells were transfected with wild type nsp15 or the corresponding
328 catalytic-deficient nsp15 from porcine coronaviruses PEDV, TGEV or PDCoV, human HeLa
329 cells were transfected with wild type nsp15 or the corresponding catalytic-deficient nsp15 from
330 human coronaviruses SARS-CoV-1, MERS-CoV or SARS-CoV-2. At 23 h.p.t, cells were
331 treated with puromycin (5 μ g/ml) for 1 h. Indirect immunofluorescence was performed using
332 anti-Flag to detect nsp15 (magenta), anti-puromycin to detect puromycin-labelled *de novo*
333 synthesized peptides (green), and DAPI to visualize nuclei (blue). Fluorescence intensity of

334 nsp15 and puromycin signal along the white line (from a to b) is indicated in the right panel
335 (histogram plot).

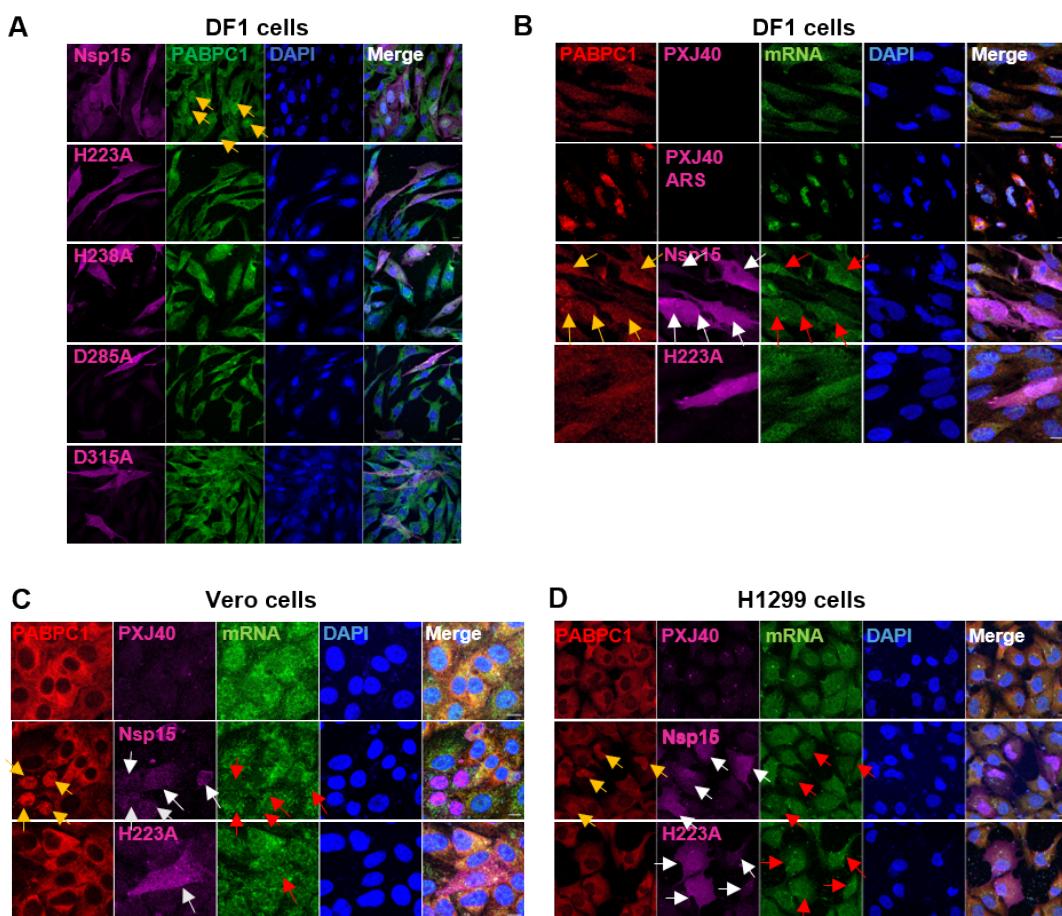


336
337 **Supplementary Fig 2. Nsp15 from different genera of coronavirus inhibits *de novo* protein
338 synthesis.** The porcine (PK1, ST and PK15) cells and Vero cells were transfected with wild
339 type nsp15 or the corresponding catalytic-deficient nsp15 from the indicated coronaviruses. At
340 23 h.p.t, cells were treated with puromycin (5 μ g/ml) for 1 h. Indirect immunofluorescence was
341 performed using anti-Flag to detect nsp15 (magenta), anti-puromycin to detect puromycin-
342 labelled *de novo* synthesized peptides (green), and DAPI to visualize nuclei (blue).
343 Fluorescence intensity of nsp15 and puromycin signal along the white line (from a to b) is
344 indicated in the right panel (histogram plot).

345
346 **Nsp15 from different genera of coronavirus alters the subcellular distribution of PABPC1
347 but does not significantly affect cellular mRNA localization**

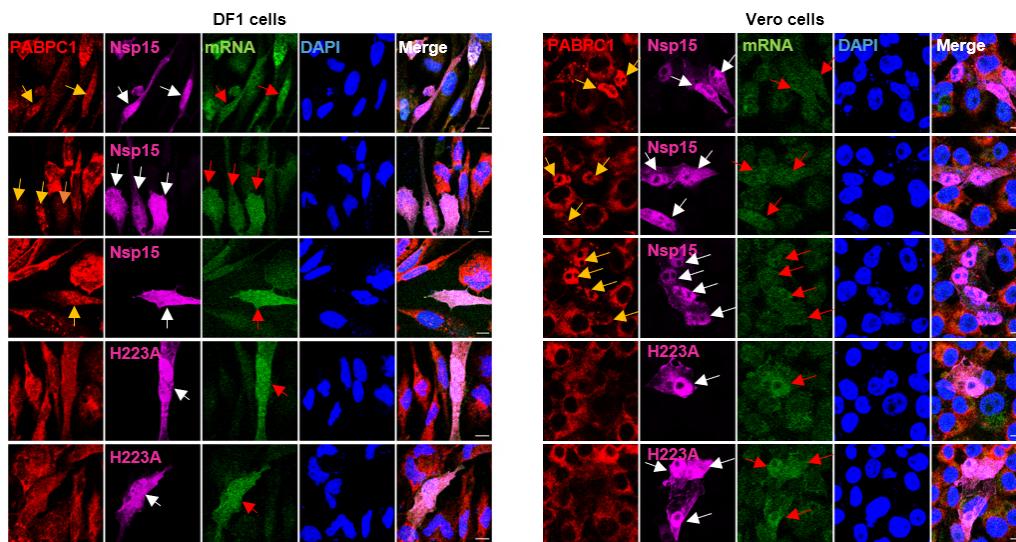
348 Considering that IBV nsp15 alone has a profound effect on *de novo* protein synthesis (**Fig 1**
349 and **Fig 2**) but that such effect is not mediated by direct degradation of mRNA (**Fig 1B**), we
350 next investigated whether nsp15 may rather act on mRNA localization by for example
351 preventing mRNA export from the nucleus to the cytoplasm leading to reduced synthesis of
352 host proteins, as previously reported for Influenza virus NS1 protein [39]. mRNA shuttling is
353 tightly regulated by RNA binding proteins such as PABPC1 [79-81]. We previously showed
354 that under stress conditions [heat shock or sodium arsenite (ARS) treatment] the ability of nsp15
355 to relocate PABPC1 into the nucleus is a conserved feature among different genera of
356 coronaviruses [29], we now ask whether this is accompanied by changes in mRNA distribution.
357 We used a cross-reacting PABPC1 antibody that allowed us to visualize the localization of
358 PABPC1 in DF-1 cells as well as mammalian cells, concomitantly to the distribution of mRNA

359 by fluorescence *in situ* hybridization (FISH) using fluorescently labelled oligo (dT) probes.
360 Consistent with our previous study, overexpression of IBV wild type nsp15, but not catalytic-
361 deficient or oligomerization-deficient IBV nsp15, caused PABPC1 nuclear localization (yellow
362 arrow) in some but not all nsp15-expressing DF-1 cells (**Fig 5A**), but this was not accompanied
363 by accumulation of mRNA in the nucleus (red arrows), as otherwise observed in the positive
364 control group treated with ARS (**Fig 5B**). The nuclear relocation of PABPC1 (yellow arrows)
365 in nsp15-expressing cells was more obvious in Vero and H1299 cells than in DF-1 cells (**Fig**
366 **5B-D**), and again no obvious nuclear retention of mRNA (red arrows) was observed (**Fig 5B-**
367 **D**). Closer observation revealed that in some cells, despite the lack of correlation between
368 PABPC1 (yellow arrows) and mRNA distribution (red arrows), an apparent overlap between
369 nsp15 or nsp15-H223A (white arrows) and mRNA (red arrows) could be observed in DF-1,
370 H1299, and Vero cells (**Fig 5B, 5D, supplementary Fig 3**), hinting at the possibility that nsp15
371 may bind to host mRNA.



372
373 **Fig 5. IBV nsp15 alters the localization of PABPC1 but not that of cellular mRNA.** (A)
374 Plasmids encoding wild type IBV nsp15 or the catalytic/oligomerization-deficient nsp15
375 (H223A, H238A, D285A, D315A) were transfected into DF-1 cells. After 24 h, indirect
376 immunofluorescence was performed to visualize nsp15 (magenta), PABPC1 (green), and

377 nucleus (blue). White arrows indicate cells that express wild type nsp15 and have nuclear
378 accumulation of PABPC1. (B-C) DF-1, Vero, or H1299 cells were transfected with plasmids
379 encoding IBV nsp15 or catalytic-deficient nsp15 H223A, or the empty PXJ40. After 24 h, *in*
380 *situ* hybridization of mRNA was performed using oligo dT probes (green) followed by indirect
381 immunofluorescence to detect IBV nsp15 (magenta) and PABPC1 (red). Nuclei were labelled
382 by DAPI (blue). White arrows indicate the cells that express IBV nsp15, red arrows indicate
383 the distribution of mRNA, yellow arrows indicate the cells with PABPC1 nuclear relocation.
384 Treatment with 1 mM ARS for 30 min was used as positive control for stimulation of PABPC1
385 and mRNA nuclear localization.



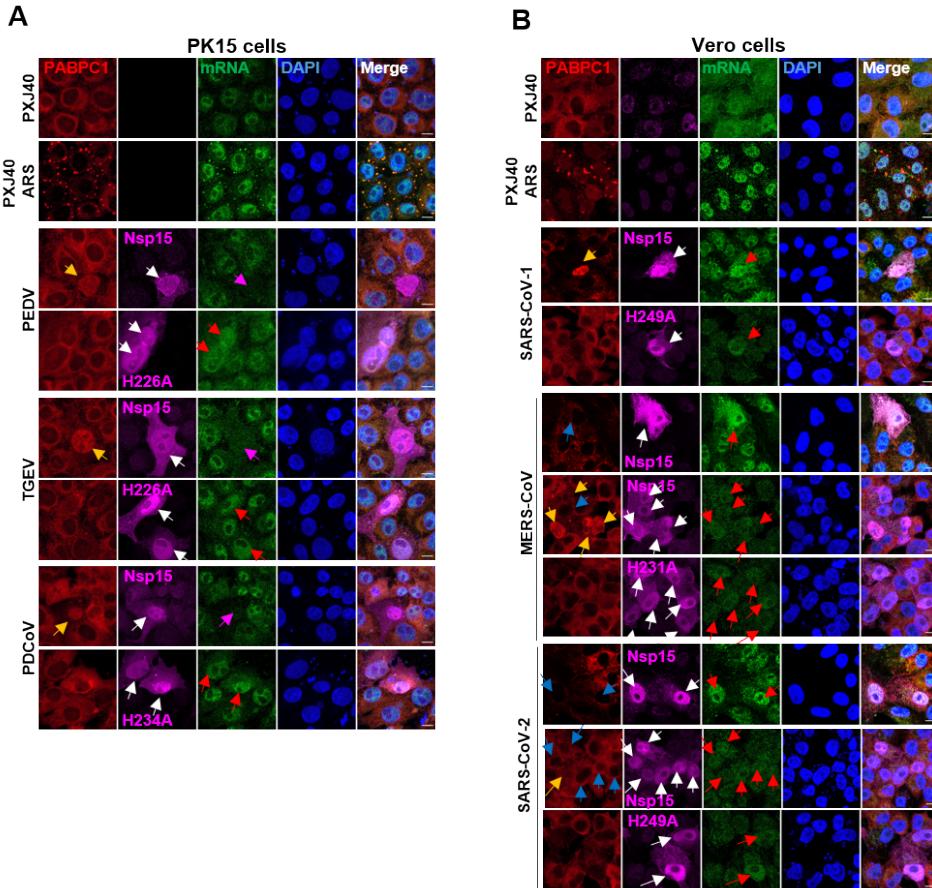
386
387 **Supplementary Fig 3. Distribution of IBV nsp15 overlaps with that of mRNA.** Wild type
388 IBV nsp15 or the catalytic/oligomerization-deficient nsp15 (H223A, H238A, D285A, D315A)
389 was transfected into DF-1 or Vero cells. After 24 h, *in situ* hybridization followed by indirect
390 immunofluorescence was performed to visualize PABPC1 (red), nsp15 (magenta), cellular
391 mRNA (green) and nuclei (blue). Yellow arrows indicate cells that express wild type nsp15
392 with nuclear accumulation of PABPC1. White arrows and red arrows indicate cells expressing
393 either wild type or mutated nsp15 in which location of nsp15 strongly overlaps with that of
394 mRNA.

395 After observing that the inhibitory effect on cellular protein synthesis is conserved
396 among nsp15 from most coronaviruses (**Fig 3-4**), and that IBV nsp15 alters PABPC1
397 localization to nucleus without apparent effects on cellular mRNA distribution (**Fig 5**), we next
398 investigated whether nuclear localization of PABPC1 in cells expressing nsp15 of PEDV,
399 TGEV, PDCoV, SARS-CoV-1, MERS-CoV, or SARS-CoV-2 (as previously reported [29]) is
400 accompanied by changes in mRNA distribution. In agreement with previous observation [29],
401 also in PK15 cells and Vero cells, ARS treatment led to redistribution and colocalization of
402 PABPC1 and mRNA to ARS induced cytoplasmic SGs (**Fig 6**). Whereas, similar to what was
403 observed for IBV nsp15, overexpression of wild type, but not of the catalytic-deficient nsp15

404 of PEDV, TGEV and PDCoV, caused nuclear accumulation of PABPC1 (indicated by yellow
405 arrows) but not of mRNA (indicated by red arrows) (**Fig 6A**). Interestingly, the mRNA signal
406 was clearly lower (magenta arrows) in PEDV, TGEV and PDCoV nsp15 expressing cells that
407 displayed nuclear relocation of PABPC1 (yellow arrows) than in cells that did not show this
408 nuclear relocation, suggesting degradation of cellular mRNA. In Vero cells, wild type but not
409 catalytic-deficient nsp15 of SARS-CoV-1 led to nuclear localization of PABPC1 (yellow arrows)
410 and this was not accompanied by a decrease or re-distribution of mRNA signal (red arrows)
411 (**Fig 6B**). In line with the observed different effects of MERS-CoV and SARS-CoV-2 nsp15 on
412 protein synthesis in **Fig 3**, **Fig 4**, **supplementary Fig 2**, only part of the cells expressing nsp15
413 of MERS-CoV and SARS-CoV-2 showed an altered localization of PABPC1 (yellow arrows)
414 (**Fig 6B**); PABPC1 remained in cytoplasm in some nsp15 expressing cells (blue arrows). Again,
415 in most nsp15-expressing cells, co-localization between nsp15 and mRNA signal or between
416 nsp15-H238A and mRNA was observed (red arrows) (**Fig 6B**), suggesting binding of nsp15 to
417 mRNA.

418 Altogether these data suggest that subcellular redistribution to the nucleus of PABPC1
419 can be triggered by nsp15 of different genera of coronaviruses, and that this is dependent on the
420 catalytic activity of nsp15. Furthermore, the PEDV, TGEV, or PDCoV nsp15-associated
421 nuclear localisation of PABPC1 is accompanied by a weaker mRNA signal, possibly suggesting
422 depletion or degradation of host mRNA by nsp15. Conversely, SARS-CoV-1, MERS-CoV and
423 SARS-CoV-2 nsp15-mediated nuclear relocation of PABPC1 is not accompanied by changes
424 in host mRNA distribution. Finally, overlap between nsp15 and mRNA signal was observed in
425 some cells, suggesting nsp15 might competitively binds to host mRNA which in turns triggers
426 relocation of PABPC1 into the nucleus.

427



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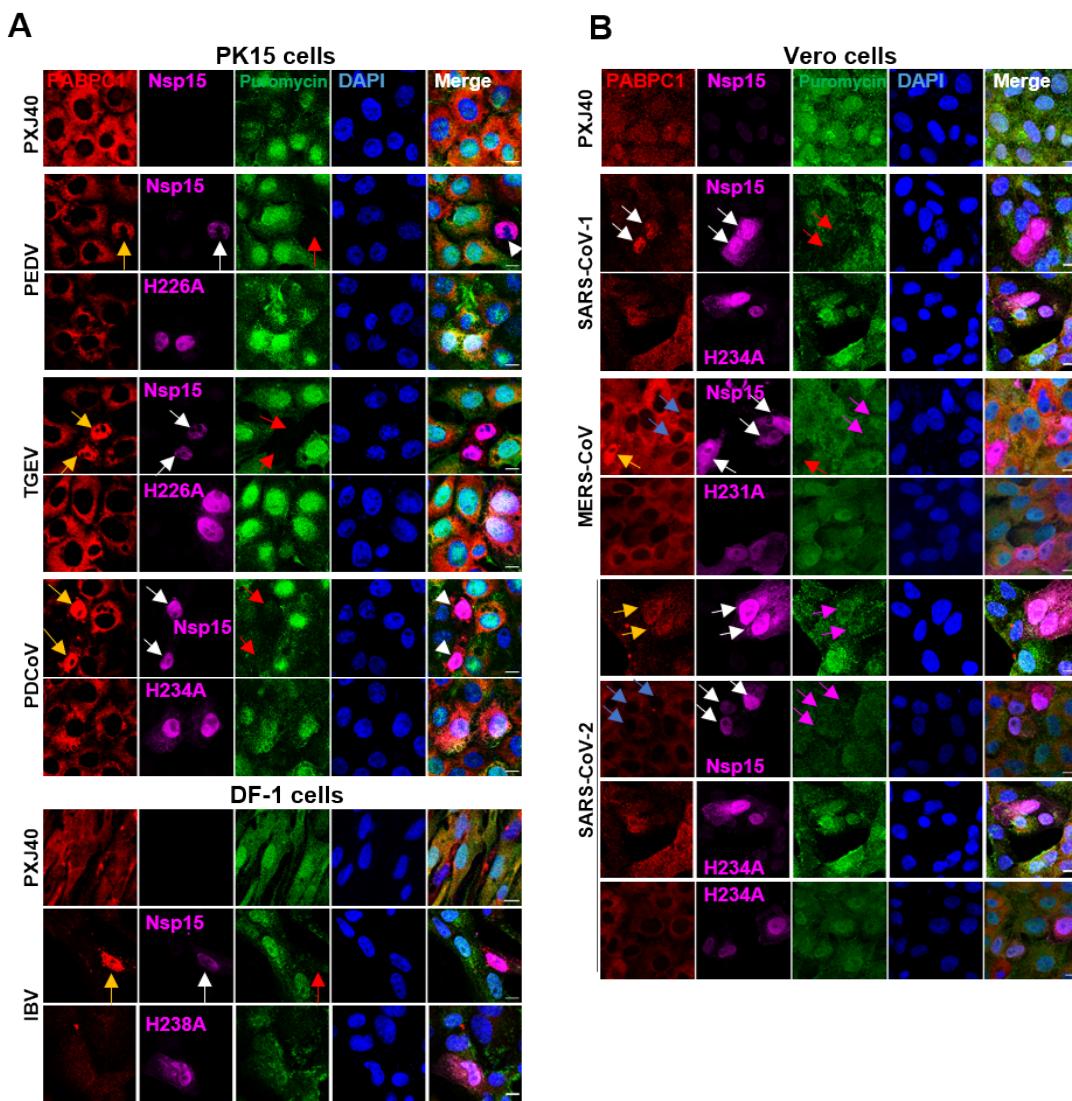
429 **Fig 6. Nsp15 of PEDV, TGEV, PDCoV and SARS-CoV-1 alters the localization of**
430 **PABPC1 but not that of cellular mRNA, nsp15 of MERS-CoV, SARS-CoV-2 does so in**
431 **most but not all cells.** Wild type nsp15 or catalytic-deficient nsp15 of the indicated
432 coronaviruses was transfected into the indicated cell line. After 24 h, indirect
433 immunofluorescence was performed to reveal the location of nsp15 (magenta), PABPC1 (green)
434 and nucleus (blue). White arrows indicate the cells expressing nsp15, yellow arrows indicate
435 the cells expressing nsp15 with a nuclear localization of PABPC1, blue arrows indicate the cells
436 expressing nsp15 without nuclear localization of PABPC1, magenta arrows indicate the nsp15
437 expressing cells with weaker mRNA signal, red arrows indicate cells with overlapping of nsp15
438 and mRNA distribution. Treatment with 1 mM ARS for 30 min was used as positive control
439 for stimulation of PABPC1 and mRNA nuclear localization.

440

441 **Nsp15 causes nuclear localization of PABPC1 accompanied by inhibition of cellular** 442 **protein synthesis by targeting cytosolic factors**

443 Next, we examined whether the nsp15-mediated PABPC1 nuclear relocation is associated with
444 cellular protein synthesis shutoff. Overexpression of wild type, but not catalytic-deficient nsp15
445 from PEDV, TGEV, PDCoV, SARS-CoV-1 and IBV, altered the localization of PABPC1
446 (yellow arrows) and this was associated with a strong reduction in puromycin signal (Fig 7, red
447 arrows). In agreement with the data in Fig 6, in cells expressing nsp15 of MERS-CoV and
448 SARS-CoV-2, nuclear localization of PABPC1 was observed in some but not all cells (Fig 7B).

449 In the MERS-CoV nsp15-expressing cells with nuclear PABPC1 localization, a weaker
450 puromycin signal was observed (**Fig 7B**, red arrows), whereas in nsp15-expressing cells in
451 which a change in localization of PABPC1 did not occur, no effect on puromycin signal was
452 observed (**Fig 7B**, magenta arrows). In the SARS-CoV-2 nsp15 expressing cells, no obvious
453 effect on puromycin signal was observed, no matter PABPC1 enters nucleus or retains in
454 cytoplasm (**Fig 7**, magenta arrows). As expected, catalytic-deficient mutants of all nsp15
455 neither changed PABPC1 distribution, nor reduced the puromycin signal, demonstrating that
456 the catalytic activity is required for both, PABPC1 redistribution and cellular translation shutoff.
457 These results, combined with those in **Fig 2**, **Fig 4** and **Fig 6**, demonstrate that overexpression
458 of nsp15 triggers nuclear retention of PABPC1 and that this is associated with a strong
459 inhibition of *de novo* protein synthesis. The SARS-CoV nsp15 is an exception for which does
460 not obviously inhibits the *de novo* protein synthesis in Vero cells.



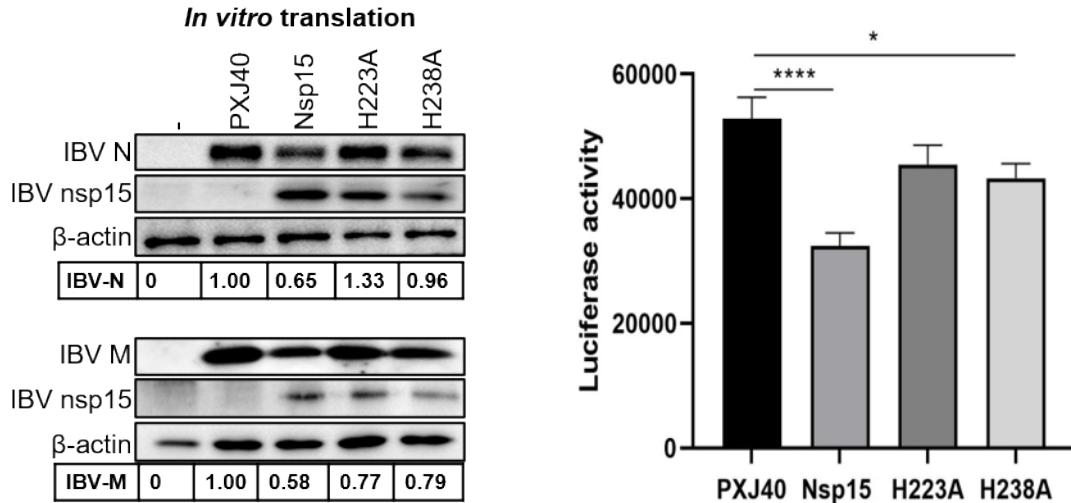
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463 **Fig 7. The nsp15-mediated relocation of PABPC1, is accompanied by inhibition of *de novo***
464 **protein synthesis.** The indicated cell lines were transfected with PXJ40 or with a plasmid
465 encoding wild type or the corresponding catalytic-deficient Flag-tagged nsp15 of the indicated
466 coronaviruses. After 23 h, puromycin labelling (5 μ g/ml) was performed for 1 h. Indirect
467 immunofluorescence was performed using anti-Flag (magenta), anti-PABPC1 (red), and anti-
468 puromycin (green) antibodies. The nuclei were stained with DAPI (blue). White arrows indicate
469 nsp15 expressing cells, yellow arrows indicate nsp15-expressing cells with PABPC1 nuclear
470 retention, blue arrows indicate nsp15-expressing cells without PABPC1 nuclear retention, red
471 arrows indicating cells with PABPC1 nuclear retention and reduced puromycin labelling,
472 magenta arrows indicate cells expressing nsp15 of MERS-CoV and SARS-CoV-2 in which
473 puromycin labelling.

474

475 Next, we asked whether nsp15-induced protein translation shutoff may be caused by the
476 ability of nsp15 to target cytosolic or nuclear factors or both. To this end, first a nucleus-free *in*
477 *vitro* translation system (Rabbit Reticulocyte Lysate) was employed. Upon *in vitro* translation,
478 IBV nsp15 reduced the expression of IBV N, IBV M, or luciferase, whereas catalytic-deficient
479 nsp15 H223A and H238A did not (Fig 8). These data indicate that inhibition of translation by
480 nsp15 can occur in the absence of nuclear factors, and that cytosolic factors involved in protein
481 translation might be targeted by nsp15.

482 The observation that in nsp15-expressing cells PABPC1 nuclear retention is accompanied
483 by protein shutoff but not by mRNA relocation, and that in some cells nsp15 localization largely
484 overlaps with that of mRNA, combined with the observation that nsp15 may target cytosolic
485 factors, suggests that nsp15 may interact with cellular mRNA itself or with (translation)
486 complexes associated with the mRNA. The association of nsp15 to these complexes may
487 compete or interfere with PABPC1 binding to the mRNA causing PABPC1 nuclear import,
488 thereby leading to host protein translation shutoff. The targeting to host mRNA by nsp15 is
489 further supported by the inability of catalytic-deficient nsp15 to trigger PABPC1 nuclear
490 relocation and host-protein shutoff.



491

492 **Fig 8. IBV nsp15 targets cytoplasmic as well as nuclear factors to inhibit protein**
493 **translation.** Plasmid encoding wild type or catalytic-deficient IBV nsp15 and reporter plasmid
494 encoding IBV N or IBV M, or luciferase DNA, were co-incubated with Rabbit Reticulocyte
495 Lysate for 1 h followed by Western blot analysis or luciferase assay. Density of the bands
496 corresponding to the reporter proteins was normalized to the signal of β-actin and presented
497 relative to the sample transfected with the empty vector PXJ40.

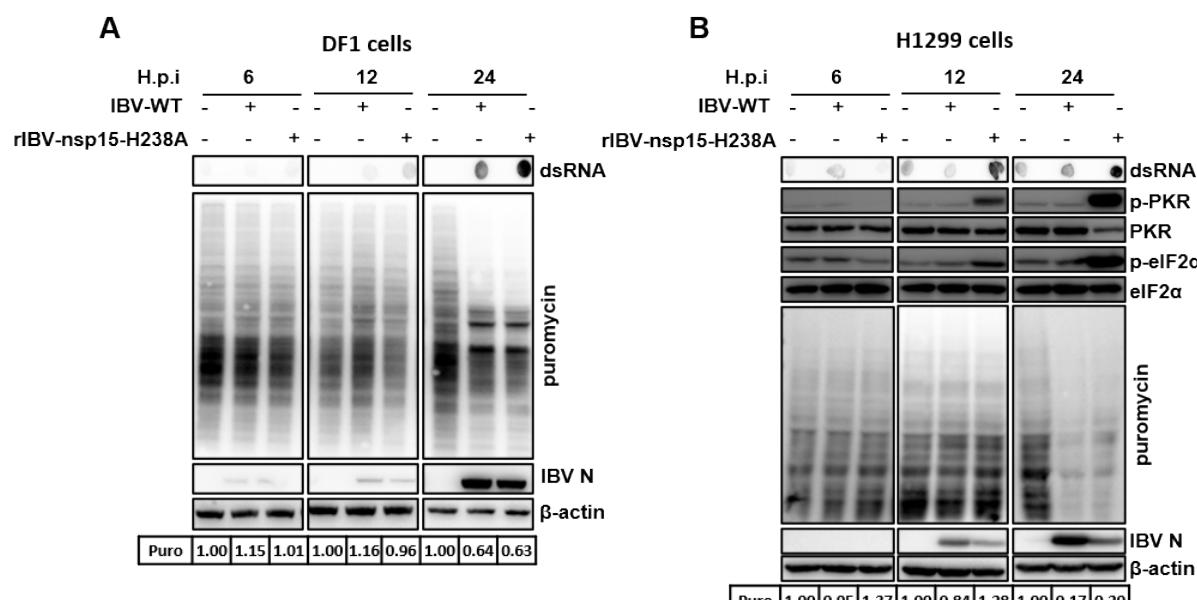
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499 **Wild type IBV and the catalytic-deficient nsp15 mutant both induce host protein**
500 **expression shutoff, but via different mechanisms**

501 After having assessed that nsp15 may be involved in regulation of host protein translation,
502 possibly through a mechanism involving targeting cytosolic mRNA and relocation of PABPC1,
503 we next evaluated the effect of nsp15 on host protein translation in the context of a virus
504 infection. To this end, we used the previously reported catalytic-deficient nsp15 recombinant
505 IBV (rIBV-nsp15-H238A), with an Alanine substitution in the nsp15 catalytic domain H238
506 [29]. Western blot analysis after infection with wild type IBV (IBV-WT) or with the rIBV-
507 nsp15-H238A mutant showed that, compared to uninfected cells, both viruses reduced
508 puromycin labelling in DF-1 (Fig 9A) as well as H1299 cells (Fig 9B). IBV-WT did trigger a
509 translational shutoff, however, it did not activate the PKR-eIF2 α pathway, as dsRNA levels
510 remained low until 24 h.p.i. and the phosphorylation levels of dsRNA sensor PKR and
511 translation initiation factor eIF2 α did not increase (Fig 9B), in agreement with our previous
512 reports [29, 57, 82]. Thus, nsp15 helps virus degrade the viral dsRNA to escape the PKR-eIF2 α
513 dependent translation shutoff and subsequent stress response [29], which is detrimental for
514 virus replication; in contrast, it might induce translation shut off through PKR-eIF2 α
515 independent strategies.

516 Although rIBV-nsp15-H238A replicating less (assessed by the lower viral N protein
517 expression), it reduced *de novo* protein synthesis to a similar extent as IBV-WT did (**Fig 9**). In
518 agreement with previous report [29], infection with rIBV-nsp15-H238A leads to accumulation
519 of higher levels of intracellular dsRNA intermediates than infection with IBV-WT, and this is
520 accompanied by high levels of p-PKR as well as p-eIF2 α (**Fig 9B**), ultimately leading to SGs
521 formation and activation of the type I IFN response [29]. This, combined with our current data
522 on puromycin labelling (**Fig 9**), confirms that rIBV-nsp15-H238A, but not IBV-WT, triggers a
523 PKR-eIF2 α -dependent translational shutoff that might impair both, host and viral protein
524 translation initiation. This host-mediated translation initiation checkpoint shutoff triggered by
525 rIBV-nsp15-H238A apparently did not benefit the virus, as synthesis of the viral N protein was
526 lower compared to rIBV-WT. Therefore, the PKR-eIF2 α -dependent translational shutoff,
527 together with the induction of type I IFN [29], are responsible for the lower replication of rIBV-
528 nsp15-H238A.

529 Taken all together, we hypothesize that both IBV-WT and rIBV-nsp15H238A cause host
530 translation shutoff, but via different mechanisms: (1) IBV-WT controls the accumulation of
531 viral dsRNA and therefore does not trigger the PKR-eIF2 α pathway for >24h, but triggers a
532 virus-mediate host translational shutoff that likely involves nsp15 and other viral proteins
533 including E protein, the accessory protein 5a and 5b through a yet unidentified mechanism.
534 (**Supplementary Fig 1, Fig 1, Fig 2, Fig 7, Fig 8**) [57]; (2) in the absence of nsp15 EndoU
535 activity, rIBV-nsp15-H238A is unable to control the amount of viral dsRNA and induces
536 translation shutoff through a host-mediated route involving the activation of the host PKR-
537 eIF2 α -mediated pathway [29].



539 **Fig 9. Both IBV-WT and rIBV-nsp15-H238A downregulate cellular protein synthesis but**
540 **through different mechanisms.** (A) DF-1 cells or (B) H1299 cells were infected with IBV-
541 WT or rIBV-nsp15H1238A at an MOI of 1. At 6, 12, 24 h.p.i., cells were treated with
542 puromycin (5 µg/ml) for 1 h, followed by western blot analysis to detect puromycin-labelled *de*
543 *novo* peptides, IBV-N protein, and β-actin. Density of the puromycin labelled proteins was
544 normalized to the signal of β-actin. Ratio of the puromycin-labelled *de novo* peptides of the
545 infected cells (+) to that of the uninfected cells (-) at the same time h.p.i. is shown. (B) H1299
546 cells were infected as described above followed by dot blot analysis to detects dsRNA and
547 western blot analysis to detect p-PKR, PKR, p-eIF2α, eIF2α.

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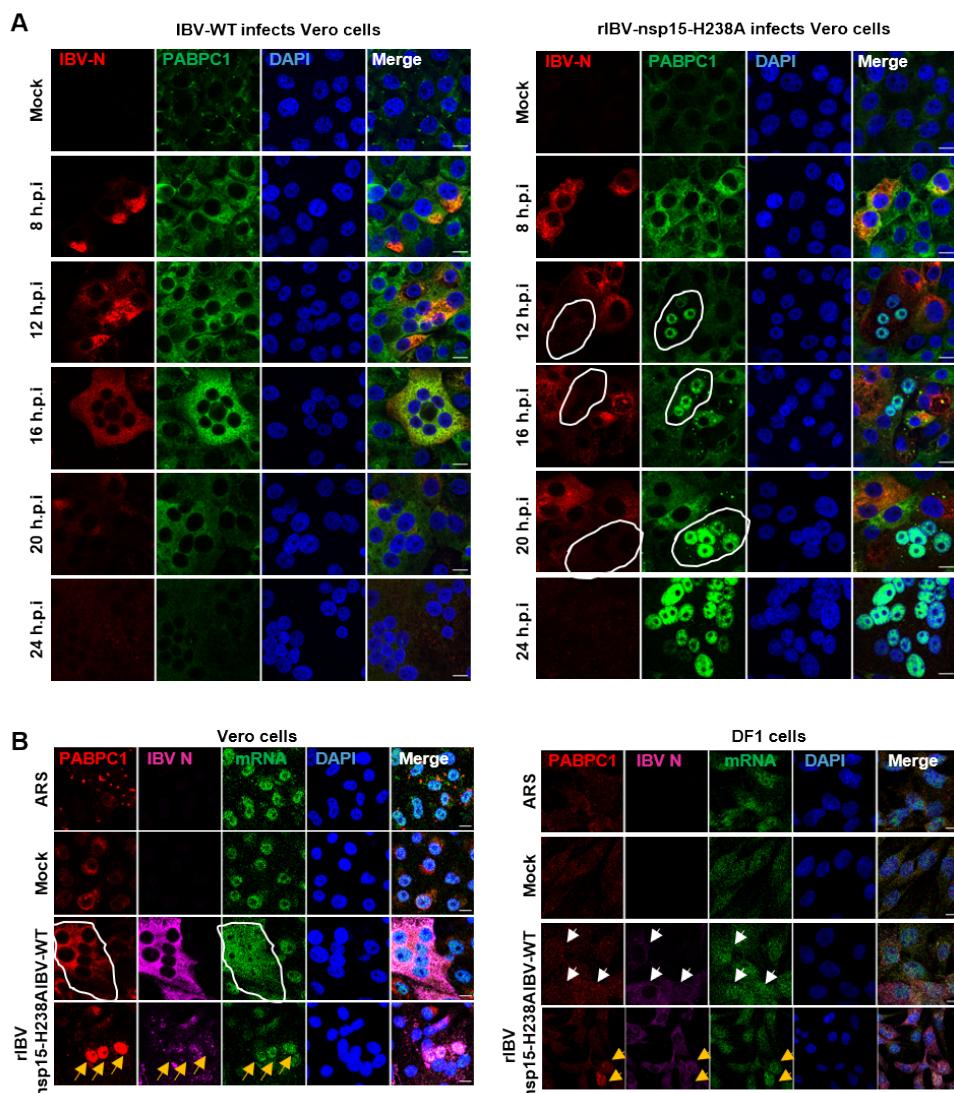
549 **PABPC1 and mRNA nuclear relocation occurs upon rIBV-nsp15-H238A but not wild**
550 **type IBV infection**

551 As we observed after transfection a correlation between PABPC1 nuclear retention and
552 inhibition of *de novo* protein synthesis only in cells overexpressing wild type but not catalytic-
553 deficient nsp15 (**Fig 7**), while we observed a translation shutoff in both, IBV-WT- and rIBV-
554 nsp15-H238A-infected cells (**Fig 9**), we next examined the localization of PABPC1 in cells
555 infected with these two viruses. No changes in PABPC1 distribution were observed in most
556 IBV-WT-infected cells at all time points after infection (**Fig 10A**, left panel), from 12 h.p.i.
557 onwards, only few infected cells display the PABPC1 puncta aggregates colocalized with the
558 SG core protein G3BP1 (**Supplementary Fig 4**), consistent with previous report [29]; in some
559 but not all rIBV-nsp15-H238A-infected cells, from 12 h.p.i. onwards, nuclear localization of
560 PABPC1 (**Fig 10A** right panel, **Supplementary Fig 5** right panel) or PABPC1 puncta
561 aggregates colocalized with SG marker protein G3BP1 were observed (**Supplementary Fig 4**,
562 **Supplementary Fig 5** right panel). As PABPC1 is involved in protein translation initiation in
563 the cytoplasm, nuclear relocation or SG localization of PABPC1 after rIBV-nsp15-H238A
564 infection, further suggests that rIBV-nsp15-H238A triggers a translation shutoff via a host-
565 mediated mechanism. This host-mediated translation shutoff is detrimental to the virus as
566 shown by the weaker viral N protein signal observed in rIBV-nsp15-H238A-infected cells with
567 PABPC1 nuclear retention compared to infected cells without PABPC1 nuclear retention (**Fig**
568 **10A**, right panel, yellow cycles).

569 FISH analysis further showed that upon infection with IBV-WT in both, Vero and DF-1
570 cells, most mRNA is homogeneously distributed throughout the cells (**Fig 10B**, white circle or
571 white arrows, **Supplementary Fig 4** left panel), whereas infection with rIBV-nsp15-H238A
572 triggers PABPC1 nuclear retention or SG localization, and this is accompanied by mRNA

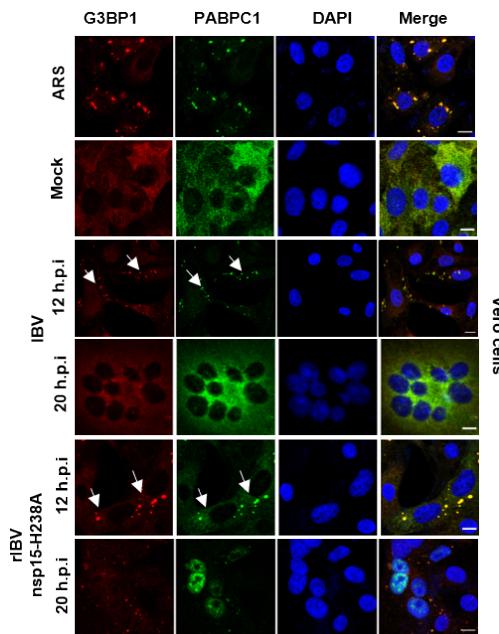
573 nuclear accumulation and aggregates to SGs (**Fig 10B**, yellow arrows, **Supplementary Fig 4**
574 right panel). These results suggest that in IBV-WT-infected cells, viral mRNA translation
575 occurs; the presence of viral mRNA causes PABPC1 to bind and be retained in the cytoplasm;
576 in rIBV-nsp15-H238A-infected cells however, the host-mediated translation shutoff halts both,
577 host and viral mRNA translation, thereby releasing PABPC1 from cytosolic host and viral
578 mRNA, redirecting it to the nucleus; the nuclear relocation of PABPC1 further retains the host
579 mRNA in the nucleus.

580



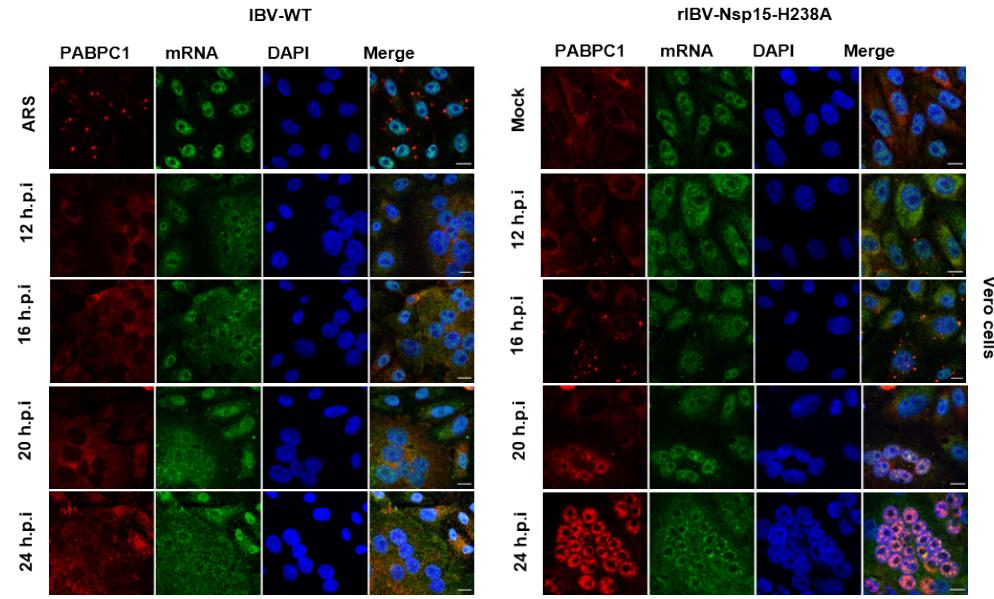
581
582 **Fig 10. Infection with rIBV-nsp15-H238A, but not with IBV-WT, triggers nuclear**
583 **retention of both, PABPC1 and mRNA and is associated with lower viral protein synthesis.**
584 (A) Vero cells were infected with IBV-WT (left panel) or rIBV-nsp15-H1238A (right panel) at
585 an MOI of 1. At 8, 12, 16, 20 and 24 h.p.i, indirect immunofluorescence was performed to
586 detect IBV-N protein (red), PABPC1 (green) and nuclei were stained with DAPI (blue). White
587 circles indicate the rIBV-nsp15-H238A-infected cells that show nuclear localization of

588 PABPC1 and weaker IBV-N signal compared to rIBV-nsp15-H238A-infected cells that do not
589 display nuclear localization of PABPC1. (B) Vero and DF-1 cells were infected with IBV-WT
590 or rIBV-nsp15-H1238A at an MOI of 1. At 16 h.p.i., FISH and indirect immunofluorescence
591 were performed to detected PABPC1(red), IBV N (Purple), and mRNA (green). Nuclei were
592 stained with DAPI (blue). White circle and white arrows indicate IBV-WT-infected cells in
593 which no relocation of PABPC1 and a homogeneous distribution of mRNA was observed;
594 yellow arrows indicate rIBV-nsp15-H238A-infected cells that show nuclear localization of both,
595 PABPC1 and mRNA.



596
597 **Supplementary figure 4. Infection with rIBV-nsp15-H238A, but not with IBV-WT, triggers PABPC1 nuclear retention. In the infected cells with SG formation, PABPC1**
598 **aggregates to SG.** (A) Vero cells were infected with IBV-WT or rIBV-nsp15-H1238A at an
599 MOI of 1. At 12 and 20 h.p.i., indirect immunofluorescence was performed to detect G3BP1
600 (red), PABPC1 (green) and nuclei were stained with DAPI (blue). White arrows indicate the
601 infected cells with SG formation and SG localization of PABPC1.
602

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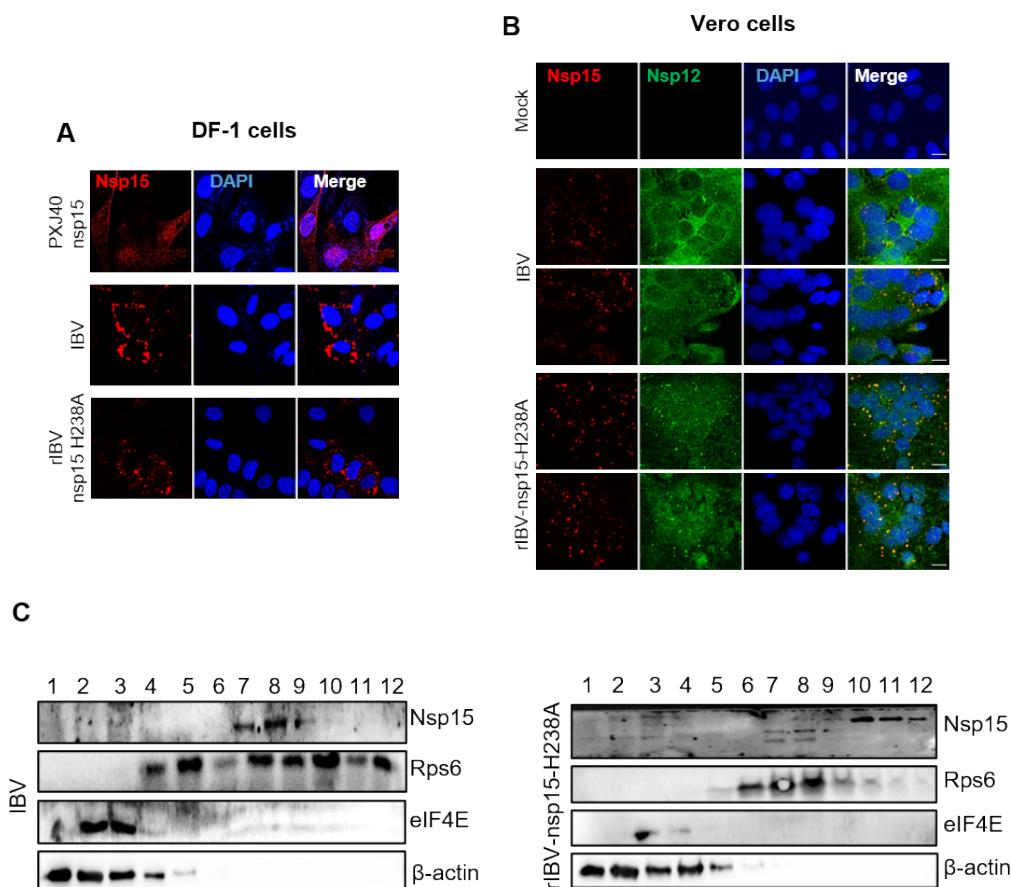


Supplementary figure 5. Infection with rIBV-nsp15-H238A, but not with IBV-WT, triggers nuclear retention or SG localization of both PABPC1 and mRNA. (A) Vero cells were infected with IBV-WT (left panel) or rIBV-nsp15-H1238A (right panel) at an MOI of 1. At 12, 16, 20 and 24 h.p.i, FISH and indirect immunofluorescence were performed to detected PABPC1(red) and mRNA (green). Nuclei were stained with DAPI (blue).

IBV nsp15 exhibits different subcellular localization after plasmid transfection and IBV infection

Subcellular localization is one of the key determinants for proteins access to their interacting partners, which provides important clues about proteins' function. Thus, we examined the subcellular localization of IBV nsp15 expressed upon plasmid transfection and during IBV infection in DF-1 cells. Indirect immunofluorescence showed that exogenous Flag-tagged nsp15 localized to the cytoplasm, and likely also the nucleus (Fig 11A, upper panel; also visible in Fig 2, Fig 5, and Fig 7); however, upon IBV-WT or rIBV-nsp15-H238A infection, perinuclear aggregates of nsp15 or nsp15-H238A can be observed (Fig 11A, middle and low panels). These results show that nsp15 has different subcellular localizations in the context of plasmid transfection and virus infection. We hypothesize that during virus infection, nsp15 usually interacts with other viral nsps or viral RNA and locates to RTC to help virus genome replication and reduce viral dsRNA load [29], whereas when overexpressed, it cannot associate to RTC (owing to the absence of viral RNA and of other viral proteins) and is able to distribute throughout the cytoplasm and nucleus. The co-localization of nsp15 and RTC core protein RdRp nsp12 in Fig 11B further suggests that nsp15 indeed localizes to RTC during virus infection.

629 We next asked whether nsp15 is associated to the translation machinery ribosomes and/or
630 polysomes. For this purpose, Vero cells were infected with IBV-WT or rIBV-nsp15-H238A.
631 After 18 h.p.i., cells were treated with translation elongation inhibitor cycloheximide (CHX) to
632 immobilize ribosomes, lysed, and then subjected to a 7–47% sucrose density gradient
633 ultracentrifugation. The fractions were analysed by Western blot to assess the distribution of
634 nsp15 in the gradient fractions. The eIF4E protein was detected at fractions 1-4, and the
635 ribosomal S6 protein (Rps6) was detected at fractions 4-11, representing mono-ribosomes and
636 polysomes, respectively. In both, IBV-WT- and rIBV-nsp15-H238A-infected cells, nsp15 or
637 nsp15-H238A was co-fractionated with Rsp6 at fraction 5-11, confirming its association with
638 the host translation machinery, polysomes. It was noted that rIBV-nsp15-H238A infection led
639 to a shift of Rsp6 to the lighter fractions 5-9, likely due to the host mediated translation shutoff
640 (p-eIF2 α -mediated translation initiation shutoff) which results in fewer polysomes and lower
641 translation efficiency. It was worth noting that in rIBV-nsp15-H238A-infected cells, nsp15-
642 H238A was detected in fractions 7-9, at the expected apparent molecular weight, whereas when
643 detected in fractions 10-12, it displayed a higher apparent molecular weight, due to a yet
644 unknown mechanism.



646 **Fig 11. IBV nsp15 exhibits different subcellular localization after plasmid transfection**
647 **and virus infection.** (A) DF-1 cells were transfected with a plasmid coding Flag-tagged nsp15
648 (PXJ40F-nsp15). At 24 h.p.t, indirect immunofluorescence was performed with a chicken anti-
649 Flag-tag antibody (red). Nuclei were stained with DAPI (blue). (B) Vero cells were infected
650 with IBV-WT or rIBV-nsp15-H238A at an MOI=1. At 18 h.p.i, indirect immunofluorescence
651 was performed with a mouse anti-IBV-nsp15 monoclonal antibody (red), a rabbit anti-IBV-
652 nsp12 polyclonal antibody (green) and the nuclei were stained with DAPI (blue). (C) Vero cells
653 were infected with IBV or rIBV-nsp15-H238A with an MOI of 1. At 18 h.p.i., cells were treated
654 with 100 μ g/mL cycloheximide (CHX) for 15 min at 37°C and subjected to 7–47% sucrose
655 density gradient ultracentrifugation (38,000 rpm for 3 h), and the fractions were analysed by
656 Western blot to detect nsp15, Rsp6, eIF4E, and β -actin (left panel).

657

658 **Discussion**

659 Several viruses encode ribonuclease and employ fine-tuned tactics to control viral as well as
660 host mRNA expression balancing viral and host protein expression [73, 83-85]. The conserved
661 EndoU nsp15 is the unique genetic marker of *Nidovirale*, as it is not present in other RNA virus
662 families. In our previous study, nsp15 was found to suppress SG formation, either by preventing
663 the accumulation of viral dsRNA or by targeting unknown host factors [29]. Thus, the role of
664 nsp15 was shown to be more complex than originally believed, primarily because it has the
665 potential to act on virus as well as cellular substrates. Although the role of nsp15 on the
666 regulation of viral RNA is well studied [26, 27, 29, 86], the cellular substrates targeted by nsp15
667 are not yet known. In this study, by transfecting nsp15-encoding plasmids into eukaryotic cells,
668 we show that nsp15 of different genera of coronaviruses inhibits the global protein synthesis,
669 and that this inhibition is accompanied by the re-localization of the poly(A) tail binding protein
670 PABPC1 to the nucleus. These activities were largely abolished when expressing catalytic-
671 deficient nsp15, demonstrating the involvement of EndoU activity in the protein translation
672 shutoff. This is the first report that coronavirus encoded EndoU is involved in regulation of host
673 gene expression.

674 Protein translation initiation is a major step that determines the efficiency of host protein
675 synthesis and viral protein synthesis [87]. PABPC1 is a nucleocytoplasmic shuttling protein
676 that is predominantly located in the cytoplasm to help translation initiation by simultaneously
677 interacting with the mRNA poly(A) tail and the eukaryotic initiation factor 4F complex (eIF4F),
678 which binds to 5' cap of mRNA and is part of the translation complex (TC); after bringing the
679 mRNA-TC to ribosomes, PABPC1 is released and can bind the importin α/β complex, which
680 mediates nuclear import of PABPC1 [80, 88-90]. In response to various pathogenic and non-
681 pathogenic stressors, PABPC1 relocates to the nucleus or aggregates to the SGs [80, 91-93].

682 High levels of PABPC1 after nuclear relocation promote hyperadenylation and nuclear
683 retention of mRNA [94], thereby restrict general gene expression.

684 In this study, we observed nuclear relocation of PABPC1 associated with protein
685 translation shutoff in all nsp15 over-expressing cells. Considering that PABPC1 is able to
686 shuttle between the nucleus and the cytoplasm [79] and that previous reports showed that
687 blocking mRNA export from the nucleus to the cytoplasm usually causes nuclear retention of
688 PABPC1 [80], we asked whether the nsp15 associated nuclear relocation of PABPC1 is the
689 result of inhibition of PABPC1 nuclear export or enhanced nuclear import. *In situ* hybridization
690 detecting poly(A) mRNA transcripts, did not reveal nuclear retention of mRNA in nsp15-
691 expressing cells. This indicates that EndoU nsp15 does not block mRNA nuclear export to
692 retain PABPC1 in the nucleus, and that nuclear relocation of PABPC1 is therefore the result of
693 enhanced nuclear import. Nuclear import of PABPC1 is dependent on interaction with
694 importin- α/β complex [80]. The motifs of PABPC1 that bind to importin- α/β complex are the
695 same that recognize and bind mRNA [80, 95]; therefore, the dissociation from cytoplasmic
696 mRNA leads to the exposure of PABPC1's nuclear import signal and shuttling to the nucleus
697 [80, 95], resulting in the observed inhibition of cellular protein expression. The dissociation of
698 PABPC1 from cytoplasmic mRNA may be caused by the binding of nsp15 to (host) mRNA,
699 thereby competing with PABPC1, as suggested by the general co-localization of nsp15 from all
700 investigated coronaviruses with cellular mRNA. Furthermore, specifically in PK15 cells
701 expressing nsp15 of porcine coronaviruses (TGEV, PEDV, PDCoV), a reduction in cellular
702 mRNA signal was observed, supporting the possibility that nsp15 from some coronaviruses
703 may not only bind, but also degrade host mRNA. Taken together, the relocation of PABPC1 to
704 the nucleus, likely caused by the competition for cellular mRNA binding and/or mRNA
705 degradation by nsp15, may therefore be the consequence, not the cause, of translation shutoff.
706 This is further supported by the nucleus-free *in vitro* translation study, showing that inhibition
707 of protein translation by nsp15 is not dependent on PABPC1 nucleus entry.

708 To assess whether nsp15 mediates host protein translation shutoff also during a virus
709 infection, we infected cells with wild type IBV (IBV-WT) and the catalytic-deficient nsp15-
710 H238A recombinant IBV (rIBV-nsp15-H238A). Interestingly, we found that both viruses
711 inhibited host protein synthesis but via different mechanisms. IBV-WT inhibited host protein
712 expression in an eIF2 α check point-independent manner likely through a yet unknown virus-
713 mediated mechanism, while rIBV-nsp15-H238A triggered host protein shutoff in a manner
714 dependent on the activation of the dsRNA-PKR-eIF2 α pathway, due to the accumulation of
715 higher levels of dsRNA during this mutant virus infection. Surprisingly, nuclear retention of

716 PABPC1 typical of nsp15-expressing cells, was not observed in IBV-WT-infected cells,
717 whereas PABPC1 nucleus accumulation or SG aggregation was clearly observed in rIBV-
718 nsp15-H238A-infected cells. The apparent contradiction with respect to PABPC1 re-
719 localization between transfection and infection conditions might be explained by the presence
720 of viral RNA during infection. Infection with IBV-WT causes host protein translation shutoff,
721 but viral mRNAs (with poly(A) tail) may still bind to cytoplasmic PABPC1 for their own
722 translation [96], thereby retaining PABPC1 in the cytoplasm. Infection with rIBV-nsp15-
723 H238A, however, results in the accumulation of dsRNA due to the loss of nsp15 EndoU activity;
724 this causes the activation of the PKR-eIF2 α pathway, which in turn causes both, global mRNAs
725 translation shuts off and aggregation of host and/or viral mRNAs to SGs, after which PABPC1
726 is released from host/viral mRNA and shuttles to the nucleus. This hypothesis is supported by
727 previous studies showing the low abundance of cytoplasmic mRNA releases PABPC1 into
728 nucleus [62, 95, 97]. Intriguingly, in rIBV-nsp15-H238A-infected cells, PABPC1 nuclear
729 localization was accompanied by lower IBV-N protein signal compared to cells without
730 PABPC1 nuclear relocation. This might be due to the stalled viral mRNA translation that
731 releases more PABPC1 which in turn can enter into the nucleus. This phenomenon further
732 suggests that re-localization of PABPC1 to the nucleus is a consequence of stalled translation
733 initiation and dissociation of PABPC1 from mRNA.

734 The eIF2 α phosphorylation-induced host shutoff inhibits both host and viral protein
735 synthesis and is an anti-viral defence mechanism of the host [98, 99], while the eIF2 α -
736 independent translation shutoff might specifically regulate the host protein expression [70].
737 Thus, wild type IBV might specifically shutoff host protein expression by hijacking the host
738 translation machinery through a yet unknown mechanism, in which nsp15, 5a, 5b, E, and S
739 might be involved in (supplementary Fig 1) [56, 57]. Kint et al showed that the 5b is
740 indispensable for host translation shut off by using the 5b null IBV [57], and Xiao demonstrated
741 that IBV and SARS-CoV S protein bind to eIF3F and this interaction led to the inhibition of
742 translation of a reporter gene [56]. Thus, we speculate that nsp15 inhibits host translation
743 together with other viral proteins. However, rIBV-nsp15-H238A may cause translation shutoff
744 through a host-mediated mechanisms based on phosphorylation of eIF2 α caused by
745 accumulation of dsRNA. The high level of phospho-eIF2 α , induced by dsRNA activation of
746 the PKR-eIF2 α pathway, hampers the translation initiation step (Met-tRNA recruiting). This
747 effectively stops both, host and viral protein translation, resulting in lower viral replication
748 reflected by the reduced expression of the viral N protein. Nevertheless, the various
749 mechanisms of translation shutoff triggered with or without nsp15 EndoU activity during virus

750 infection, indicate that nsp15 plays a role in regulating protein translation in a way that benefits
751 viral replication: by promoting the host translation shutoff via targeting to cytoplasmic factors
752 and by avoiding the activation eIF2 α -dependent translation shutoff via reducing the dsRNA
753 formation.

754 Subcellular localization is one of the determinants for proteins to properly exert their
755 functions. When over-expressed, nsp15 has a dispersed cytoplasmic and nuclear localization as
756 no viral RNA or viral proteins are present to interact with or to recruit it to RTC. The
757 cytoplasmic and nuclear distribution, enable nsp15 to target cellular substrates in the cytoplasm
758 and the nucleus. During virus infection however, IBV nsp15 (**Fig 11**) and MHV nsp15 [100]
759 display a perinuclear aggregated localization, colocalized with RdRp nsp12, the catalytic centre
760 of the RTC [101]. Endoplasmic reticulum (ER) membrane transformation termed DMV during
761 SARS, MERS or MHV infection, or zippered ER and spherules single membranes during IBV
762 infections [22-24, 102-104], and collectively referred to as membrane rearrangements, provide
763 a viral RNA replication microenvironment by accommodating the RTC. The association of
764 coronavirus nsp15 with RTC implies that this EndoU may locate inside ER membrane
765 rearrangements, as also suggested by IBV nsp15 perinuclear localization during infection and
766 its co-localization with RTC-associated proteins like nsp12. Inside membrane rearrangements,
767 nsp15 EndoU may cleave viral RNAs to reduce dsRNA accumulation. The membrane
768 rearrangements are connected to each other, there is contiguity with the membrane donor of the
769 ER [24], and the ribosomes are associated with the outer membrane [105-107]. Recently,
770 crytomography revealed that DMVs of MHV contain membrane spanning structures, a
771 hexameric, crown-shaped pore complex surrounding a central channel that would allow RNA
772 and protein transport [108]. The nucleocapsid structure was visualized on the cytosolic side of
773 the pore, suggesting that the RNA is encapsidated following the export from DMVs [108]. Thus,
774 although nsp15 is associated with the RTC or membrane vesicles during virus infection, it is
775 still possible for it to locate outside membrane vesicles and to target host mRNA or the
776 ribosomes in the outer membrane of vesicles, thereby interfering with host translation and this
777 was also confirmed by our fractionation studies showing that nsp15 is associated to the
778 translation machinery, and specifically to polysomes. Investigation into the kinetics of nsp15
779 production and formation of membrane rearrangements is necessary to track the location of
780 nsp15 and analyse its roles during virus infection. In addition, regulation by other viral proteins
781 interacting with nsp15 can also be a way to fine-tune nsp15's roles during virus infection. We
782 attempted to investigated whether nsp15 specifically inhibits host mRNA translation or also
783 suppresses viral mRNA translation, by constructing the plasmid containing viral genomic 5'

784 leader sequence, 5' UTR (untranslated region), transcription regulation sequence, IBV N gene,
785 to mimic viral subgenomic mRNA structure; however, the translation of this construct was still
786 suppressed by co-transfected with IBV nsp15, probably due to lacking the 3' sequence
787 downstream of N gene. We will further construct the plasmid containing 5' end and 3' end of
788 viral subgemomic mRNA, to fully mimic viral mRNA structure, for checking whether nsp15
789 viral mRNA translation.

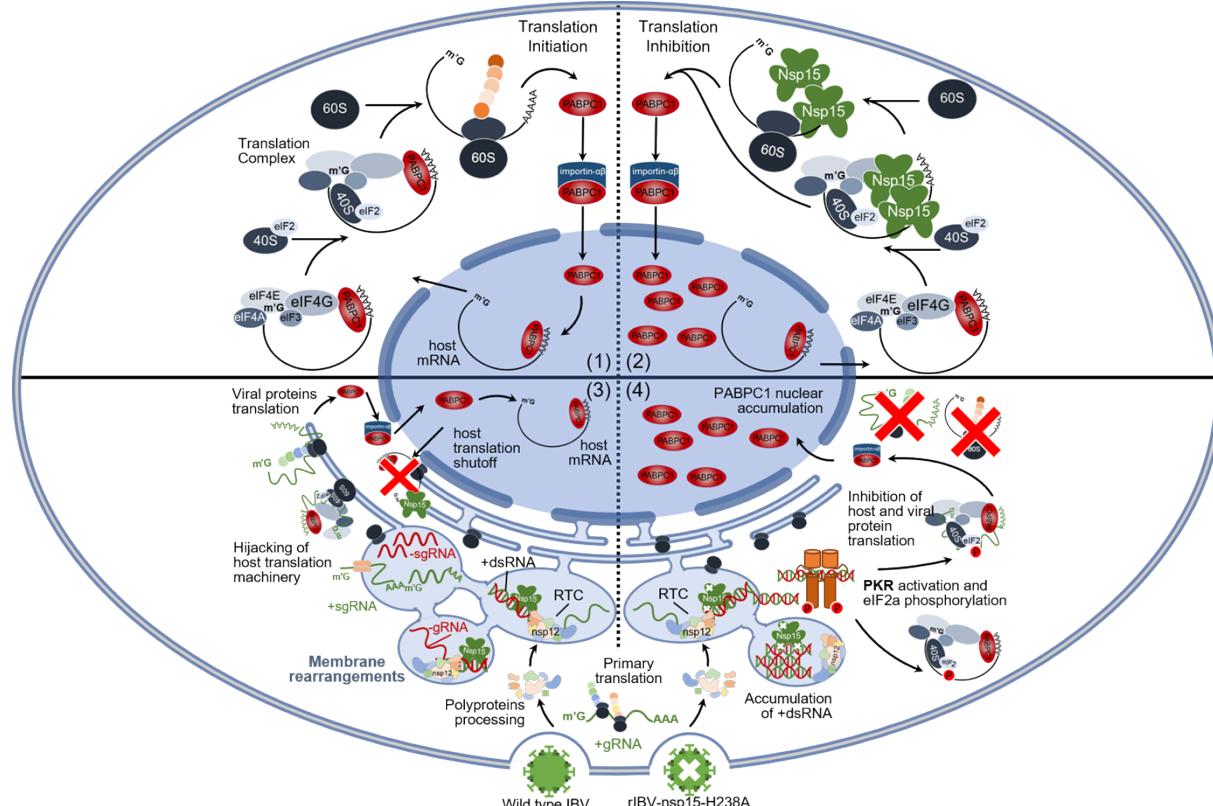
790 Inhibiting host antiviral gene expression is an important strategy for viruses to
791 antagonize the host innate immune response. Previous studies have reported that coronaviruses
792 shut down host translation by various mechanisms. α - and β -coronaviruses employ nsp1 to
793 trigger host translation shutoff via multiple strategies [109]: interference with ribosomal
794 function [44, 110, 111], endo-nucleolytic cleavage of 5' capped non-viral mRNA that triggers
795 its degradation [44, 48, 50, 68], interference with nucleocytoplasmic transport of host mRNA
796 leading to its nuclear retention [45, 46], or halting translation of host mRNA by targeting
797 mRNA derived from the nucleus [48]. SARS-CoV-2 nsp14 and nsp16 also inhibit host
798 translation, through a mechanism in which nsp16 suppresses global mRNA splicing and
799 prevents the production of mature host mRNA [112, 113]. We previously reported that IBV, a
800 γ -coronavirus, which lacks nsp1, also inhibits host translation through a yet unknown
801 mechanisms that likely involves accessory protein 5b [53]. In the current study, by comparing
802 the activity of wild type and catalytic-deficient nsp15 in the context of a viral infection or when
803 over-expressed alone in eukaryotic cells after transfection, we were able to show that, besides
804 the well characterized activity of nsp15 on viral dsRNA, nsp15 of IBV and of other
805 coronaviruses exerts additional functions by also targeting host substrates, ultimately leading
806 to suppression of host protein synthesis. The role of nsp15 in the regulation of host and viral
807 protein expression is summarized in the model shown in **Fig 12**: (0) under steady-state
808 conditions, newly transcribed mRNA is bound by PABPC1 in the nucleus, the complex shuttles
809 to the cytoplasm where it immediately interacts with elongation initiation factors (eIF) part of
810 the translation complex (TC), which in turn recruit first the 40S and then the 60S ribosomal
811 subunits to start translation; translation initiation releases the mRNA from PABPC1 which
812 binds to importin- α/β complex to shuttle back into the nucleus, where the cycle begins again.
813 (1) When expressed alone (after transfection of nsp15-coding plasmids), nsp15 targets host
814 substrates involved in mRNA translation by possibly targeting mRNA itself and/or factors
815 associated to the TC, leading to dissociation of PABPC1 from cellular mRNA, its re-
816 localization to the nucleus; for all this, the EndoU activity of nsp15 is indispensable. (2) Wild
817 type IBV induces a host translation shutoff that specifically restricts host and benefits viral

818 protein synthesis by hijacking the translation machinery; for this, nsp15 and the previously
819 reported accessory protein 5b, 5a and E in our screen data (supplementary Fig 1), might both
820 be involved. (3) Catalytic-deficient-nsp15-IBV can no longer regulate the levels of viral dsRNA
821 [29] and therefore triggers a dsRNA-PKR-eIF2 α -mediated host shutoff that hampers both, host
822 and viral mRNA translation, causes nuclear re-localization of PABPC1, and restricts viral
823 replication. The perinuclear localization of nsp15 during IBV infection, suggest that it can be
824 associated to membrane rearrangements (zipped ER or spherules) that would give it access not
825 only to viral RNAs to regulate the abundance of dsRNA [22], but also ribosomes and/or host
826 mRNA normally associated to the same membrane rearrangements.

827

828 This is a novel finding on the role of nsp15 to regulate host and viral gene expression,
829 and increases our understanding on the regulation mechanisms of host translation by
830 coronaviruses. Since the EndoU are conserved genetic marker of *Nidovirales*, the mechanisms
831 of this EndoU as host translation suppressor may be a breakthrough in finding common
832 strategies employed by coronaviruses, even by nidoviruses.

833



835 **Fig 12. Working model of the mode of action on host protein translation of nsp15 when**
836 **expressed alone or in the context of IBV infection.** (1) host mRNA translation and PABPC1
837 turnover under normal conditions; (2) host mRNA translation upon overexpression of wild type

838 IBV nsp15 leading to host-translation shutoff and nuclear accumulation of PABPC1; (3)
839 simplified overview of the events occurring upon wild type IBV infection, ultimately leading
840 to host-translation shutoff through a hijacking of the host translation machinery in favour of
841 viral protein translation. (4) simplified overview of the events occurring upon catalytic mutant
842 rIBV-nsp15-H238A infection. Catalytic-deficient nsp15 is no longer able to control the levels
843 of viral dsRNA intermediates, leading to a dsRNA-PKR-eIF2 α -mediated host-protein shutoff
844 that limits both, host and viral protein synthesis, thereby leading to nuclear accumulation of
845 PABPC1. For details refer to the main text.

846 **Materials and Methods**

847 **Cells and Viruses**

848 Human non-small cell lung carcinoma H1299 cells were purchased from Cell Bank of Chinese
849 Academy of Sciences (Shanghai, China). Chicken embryo fibroblasts DF-1 cells (ATCC®
850 CRL-12203™), African green monkey kidney epithelial Vero cells (ATCC®CCL-81™) and
851 human embryonic kidney HEK293T cells (ATCC® CRL-3216™) were purchased from ATCC.
852 Porcine kidney epithelial cells (PK15) were provided from Prof. Hongjun Chen (Shanghai
853 Veterinary Research Institute, CAAS, China). LLC-PK1 and ST cells were provided by Prof.
854 Tongling Shan (Shanghai Academy of Agricultural Sciences, CAAS). H1299 cells were
855 maintained in Roswell Park Memorial Institute 1640 medium (RPMI, 21875034, Gibco™)
856 supplemented with 10% (v/v) foetal bovine serum (FBS, Gibco). The rest of the cell lines were
857 grown in Dulbecco's modified eagle medium (DMEM, Gibco™) containing 10% fetal bovine
858 serum (FBS, Gibco).

859 A mammalian cell adapted Beaudette IBV strain obtained from Prof Dingxiang Liu
860 (Huanan Agricultural University, China) [114] was used in this study, as this IBV strain can be
861 propagated in the DF-1 cells as well as in some mammalian cells, including Vero and H1299
862 cell lines [115]. The recombinant virus rIBV-nsp15-H238 was retrieved and its generation
863 described in details our previous study [116].

864

865 **Plasmids**

866 V5-tagged constitutively active form (N-terminus 1-1920 bp) of chicken MDA5 [V5-
867 chMDA5(N)] and full-length chicken IRF7 (V5-chIRF7) were respectively cloned into pcDNA
868 3.1 vector and HA-tagged full-length chicken MAVS was cloned into the pCAGGS vector
869 (provided by Yuqiang Cheng) [117]. The epitope tag is located at the C-terminus of the inserted
870 gene. The plasmid pEGFP-N1 encoding enhanced green fluorescent protein (EGFP) was
871 provided by Prof Yingjie Sun (Shanghai Veterinary Research Institute, CAAS, China).

872 Construction of plasmids encoding IBV nsp2, nsp3, nsp4, nsp5, nsp6, nsp7, nsp8, nsp9, nsp10,
873 nsp12, nsp13, nsp14, nsp15, nsp16, 3a, 3b, 5a, 5b, S, E, M, N, IAV NS1, PEDV nsp15, TGEV
874 nsp15, SARS-CoV-1 nsp15, SARS-CoV-2 nsp15, and the catalytic-deficient mutants of the
875 above nsp15 were cloned into vector PXJ40F, as described previously [29]. MERS-CoV nsp15
876 cDNA was purchased from Sangon Biotech, PDCoV-nsp15 cDNA was provided by Prof.
877 Tongling Shan (Shanghai Veterinary Research Institute), and both were inserted into a PXJ40F
878 vector. The oligomerization-deficient mutants of IBV nsp15 (IBV nsp15-D285A and IBV
879 nsp15-D315A), and the catalytic-deficient mutants of MERS-CoV nsp15 and PDCoV nsp15
880 (MERS-CoV nsp15-H231A and PDCoV nsp15-H219A), were cloned using Mut Express II
881 Fast Mutagenesis Kit V2 (C214, Vazyme). The mutagenesis primers were: for IBV nsp15-
882 D285A, 5'-TGTTGTggcTTTACTGCTTGATGATTCTTAGAACTTC-3' (F) and 5'-
883 GCAGTAAAgccACAAACAGTACACACTTGCTTGTAA-3' (R); for IBV nsp15-D315A, 5'-
884 GTGTCAATTgctTACCATAGCATAAATTATGACTTGG-3' (F) and 5'-
885 TGGTAaggcAATTGACACTGTTACAACCTTGACTT-3' (R); for MERS-CoV nsp15-
886 H231A, 5'-TTTGAGggcGTAgGTCTATGGAGACTTCTCTCATACTACG-3'(F) and 5'-
887 AGACTACggcCTCAAAAGCATAGTTTCCAAGCC-3'(R); for PDCoV nsp15-H231A, 5'-
888 CGGAACTgccACACTTATCTCACTAGTTAAAAACAAGTTG-3' (F) and 5'-
889 TAAGTGTggcAGTTCCGCCAATGACTGGACTG -3' (R). The underlined sequences were
890 the targeted sites for the mutations.

891

892 Primary antibodies

893 Mouse anti-V5 (Thermo fisher scientific, #R961-25, horseradish peroxidase HRP-conjugated),
894 mouse anti-HA (MBL, #M180-7, HRP-conjugated), mouse anti-Flag (MBL, #M185-7, HRP-
895 conjugated), were diluted by 1:2500 for Western Blot; mouse anti- β -actin (CST, #3700S),
896 rabbit anti-GFP (CST, #2956), chicken anti-Flag (Gentaur, #AFLAG), rabbit anti-
897 phosphorylated PKR (Abcam, #ab32036), rabbit anti-PKR (CST, #12297), rabbit anti-
898 phosphorylated eIF2 α (CST, #3398), rabbit anti-eIF2 α (CST, #5324), anti-RPS6 rabbit mAb
899 (CST, #2217) and eIF4E rabbit mAb (CST, #2067), were diluted by 1:1000 dilution for Western
900 Blot; rabbit anti-IBV N (provided by Prof Dingxiang Liu, South China Agricultural University,
901 China) was diluted by 1:2000 for Western blot; rabbit anti-human PABPC1 (Abcam, #Ab21060,
902 cross-reacts against chicken PABPC1 in DF-1 cells), rabbit anti-IBV-N, anti-IBV nsp12 rabbit
903 pAb (provided by Prof Dingxiang Liu, South China Agricultural University, China), anti-IBV
904 nsp15 mouse mAb (provided by Dr. Min Liao's lab, Zhejiang University, China), were diluted
905 by 1:500 for immunofluorescence; mouse anti-puromycin (Sigma-Aldrich, #MABE343) was

906 diluted by 1:25000 for Western blot and 1:10000 for immunofluorescence; anti-dsRNA mouse
907 mAb J2 (Scicons, #10010200) was diluted by 1:1000 for dot blot analysis.

908

909 **Secondary antibodies**

910 Goat anti-rabbit IgG (H+L) (ABclonal, #AS014, HRP-conjugated) and goat anti-mouse IgG
911 (H+L) (ABclonal, #AS003, HRP-conjugated) were diluted by 1:5000 for Western blot or dot
912 blot; goat anti-chicken IgY (H+L) (Invitrogen, # A-11041, Alexa Fluor 568-conjugated), goat
913 anti-mouse IgG (H+L) (Invitrogen, #A-11029, Alexa Fluor 488-conjugated), goat anti-rabbit
914 IgG (H+L) (Invitrogen, #A-11034, Alexa Fluor 488-conjugated) were diluted by 1:500 for
915 immunofluorescence.

916

917 **Chemicals**

918 Puromycin (Merck, #58-58-2, reconstituted with sterile H₂O to 50 mg/mL as stock solutions);
919 Fugene (HD) (Promega, #E2311); Opti-MEM (GibcoTM, #31985062); TRIzol reagent (Life
920 Technologies, #15596018); M-MLV (Promega, #M1701); Random primers (InvitrogenTM,
921 #48190011); SYBR green master mix (Dongsheng Biotech, #P2092); 4%-20% gradient
922 SurePAGE gel (GenScript, #M00657); Tris-MOPS-SDS running buffer (GenScript, #M00138);
923 Biotin-oligo d(T) (Promega, #Z5261, 0.2 μmol/L for mRNA FISH); Diethyl Pyrocarbonate
924 (DEPC)-treated water (InvitrogenTM, #4387937); Paraformaldehyde (Sigma-Aldrich, #158127);
925 Triton X-100 (Sigma-Aldrich, #X100); BSA (Sigma-Aldrich, #A2153); SSC (InvitrogenTM,
926 #15557044); PBS (Sigma-Aldrich, #P3813); Dithiothreitol (DTT) (Thermo ScientificTM,
927 #R0861); RNase inhibitor (Promega, #N2611); Streptavidin (InvitrogenTM, # SA1001, FITC -
928 conjugated, 1:500 dilution for mRNA FISH); Diamidino-2-phenylindole (DAPI) (Thermo
929 Scientific, #62247, 1:1000 dilution for IFA); Mounting medium (Sigma-Aldrich, #C9368);
930 ARS (Sigma-Aldrich, #S7400).

931

932 **Plasmid transfection**

933 Transient transfection using Fugene (HD) was described previously [29]. Fugene was used to
934 transfect all cell lines employed in this study. Briefly, plasmid(s) and Fugene HD (M/V=1:3)
935 were mixed in Opti-MEM. After incubation for 15 min at room temperature, the mixture was
936 added to the cultured cells.

937

938 **Dual luciferase assay**

939 Plasmids encoding IBV proteins (300 ng) (CMV promoter), human/chicken MDA5 (200 ng)
940 (CMV promoter) or human/chicken MAVS (200 ng) (SV40 promoter), IFN β promoter driven
941 Firefly luciferase reporter (100 ng) and Renilla luciferase reporter pRL-TK (50 ng) (HSV TK
942 promoter) were co-transfected into cells in a 24-well plate. To make the different IBV protein
943 groups more comparable, the plasmids, except those encoding IBV proteins were mixed, and
944 divided in equal volumes to which the plasmid coding each of the IBV protein was added in.
945 Each co-transfection group was repeated twice. At 24 h.p.t., cells were lysed using the passive
946 lysis buffer supplied by the Dual-Luciferase[®] Reporter Assay System (Promega, #E1910).
947 Measurement of firefly luciferase activity by adding LAR II, and measurement of Renilla
948 luciferase activity by adding Stop & Glo[®] were performed according to the manufacturer
949 instructions using a luminometer reading (Cytation 5 imaging multimode reader, Biotek).

950

951 **Western blotting analysis**

952 Briefly, cells transfected with plasmids for 24 h were lysed in lysis buffer and the cell lysates
953 were resolved on 10% SDS-PAGE. To separate IBV-nsp7 (23 kDa), nsp8 (12 kDa), nsp9 (15
954 kDa), nsp12 (106 kDa), nsp15 (37 kDa) on the same gel, a commercial 4-20% acrylamide
955 gradient SurePAGE gel was used. Different from SDS-PAGE gel electrophoresis, SurePAGE
956 gel electrophoresis was performed using 1X Tris-MOPS-SDS running buffer. The proteins
957 separated by the SDS-PAGE were transferred to a nitrocellulose membrane (GE life Sciences).
958 The membrane was then incubated for 1 h at room temperature or overnight at 4 °C in blocking
959 buffer (5% non-fat milk powder diluted in TBST (20 mM Tris, 150 mM NaCl, 0.1% Tween[®]
960 20 detergent), incubated with primary antibody diluted in blocking buffer, washed three times
961 in TBST, incubated with HRP-conjugated secondary antibody diluted in blocking buffer, and
962 again washed three times in TBST. Finally, signal was detected using a Tanon 4600
963 Chemiluminescent Imaging System (Bio Tanon, China) after development with luminol
964 chemiluminescence reagent kit (Share-bio, China).

965

966 **Puromycin labelling**

967 Puromycin resembles the 3' end of tRNA and binds to growing peptide chains during translation,
968 causing the stop of protein synthesis and release of premature polypeptides containing
969 puromycin [78]. Cells were transfected with plasmid for 24 h or infected with IBV for indicated
970 time, the cells were incubated with 5 μ g/mL puromycin for 1 h at 37°C. The same amounts of
971 6 well plate cultured cells were lysed for western blotting analysis, or the cells cultured in 4
972 well chamber slide were fixed for indirect immunofluorescence assay.

973

974 **Sodium arsenite (ARS) treatment**

975 Cells were seeded in six well culture plates and treated with 1 mM ARS for 30 min before being
976 collected for mRNA *in situ* hybridization and indirect immunofluorescence analysis.

977

978 **Indirect immunofluorescence assay**

979 Briefly, cells were fixed with 4% paraformaldehyde (diluted in PBS) for 15 min at room
980 temperature (20-22°C), permeabilized with 0.5% Triton X-100 (diluted in PBS) for 15 min at
981 room temperature, and incubated in blocking buffer (3% BSA diluted in PBS) for 1 h at 37°C.
982 Cells were washed with PBS three times (5 min each) at the intervals of above steps on a shaker.
983 Cells were then incubated with the primary antibody diluted in blocking buffer for 1 h at 37°C,
984 followed by incubation with FITC- or TRITC-conjugated secondary antibody diluted in
985 blocking buffer for 1 h at 37°C. In case of double staining, cells were then incubated with the
986 other unconjugated primary antibody, followed by incubation with the corresponding FITC- or
987 TRITC-conjugated secondary antibody. At the intervals of each incubation step, cells were
988 washed three times (5 min each) with PBS buffer containing 0.2% Triton X-100 on a shaker.
989 DAPI was then applied to stain the nuclei for 7 min at room temperature. Finally, cells were
990 washed three times with PBS, mounted onto glass slides using prolong gold antifade mountant
991 (Invitrogen), and examined by Zeiss LSM880 confocal microscope.

992

993 **Indirect immunofluorescence and mRNA fluorescence *in situ* hybridization (FISH)**

994 To combine indirect immunofluorescence and mRNA FISH, cells were fixed for 15 min (4%
995 paraformaldehyde in DEPC-treated PBS), permeabilized for 15 min (0.5% Triton X-100 in
996 DEPC-treated PBS), and blocked for 1 h (3% BSA in DEPC-treated PBS), followed with endo-
997 biotin blocking using a blocking kit (Invitrogen, #E21390) according to the manufacture
998 instructions [118]. Cells were then incubated for 1 h at 37°C with the primary antibody. In case
999 of double staining, the other primary antibody was then incubated in the same way. At the
1000 intervals of each incubation step, cells were washed three times with DEPC-treated PBS
1001 containing 0.2% Triton X-100. Cells were again fixed with 4% paraformaldehyde and washed
1002 three times with DEPC-treated PBS. Cells were then equilibrated in 2×SSC (1mg/mL t-RNA ,
1003 10% detran sulfate and 25% formamide) for 15 min at 42°C, followed by hybridization of
1004 biotin-oligo d(T) with the poly(A) tail of mRNA for approximately 12 h at 42°C in a humid
1005 environment. Biotin-oligo d(T) (0.2 μmol/L) were diluted in DEPC-treated PBS containing 0.2%

1006 TritonX-100, 1 mM DTT, and 200 units/ml RNase inhibitor. After the hybridization step,
1007 samples were washed with 2× SSC for 15 min and then with 0.5× SSC for 15 min, at 42°C on
1008 a shaker. Cells were again fixed with 4% paraformaldehyde and washed with DEPC-treated
1009 PBS. Cells were then incubated with Alexa Fluor-conjugated secondary antibodies for 30 min,
1010 and then with FITC-conjugated streptavidin for 30 min at 37°C. At the intervals of each step,
1011 cells were washed with DEPC-treated PBS containing 0.2% Triton X-100 three times. DAPI
1012 was then applied to stain the nuclei for 7 min at room temperature. Cells were washed again
1013 three times and mounted onto glass slides using mounting reagent. Cells were examined by
1014 Zeiss LSM880 confocal microscope.

1015

1016 ***In vitro* translation**

1017 0.5 µg of PXJ40-IBV-nsp15, 0.5 µg of reporter gene (PXJ40-IBV-N, PXJ40-IBV-M, or T7
1018 luciferase control DNA), 40 µl TnT® Quick Master Mix (L1170, Promega), 1 µl Methionine
1019 (1 mM), nuclease-free water (to a final volume of 50 µl) were gently mixed by pipetting. The
1020 above mixture was then incubated for 90 min at 30°C. Samples of the translation reaction
1021 products were analysed by Western blot to detect protein expression level, or by luciferase assay
1022 to detect luciferase activity. For luciferase assay, 2.5 µl of translation reaction products and 50
1023 µl of Luciferase Assay Reagent (Promega) were mixed by gently pipetting and subjected to
1024 luminometer reading (Cytation 5 imaging multimode reader, Biotek).

1025

1026 **Quantitative RT-PCR analysis**

1027 Total cellular RNAs were extracted using Trizol reagent. cDNAs were synthesized
1028 using M-MLV reverse transcriptase system. To prime the amplification of mRNAs in case their
1029 poly(A) tail is cleaved, random primers were used to synthesize cDNAs. The primers for
1030 quantitative PCR analysis were: for exogenous chicken *MDA5(N)*, 5'-
1031 AAAACGCAAGGAACGTGTCTG-3' (F) and 5'-GACCGAGGAGAGGGTTAGGG-3' (R);
1032 for exogenous chicken *MAVS*, 5'-ACATCCTTCCAGCTGTTGGC-3' (F) and 5'-
1033 CGTAATCTGGAACATCGTATGGG-3'(R); for chicken β -*actin*, 5'-
1034 CCAGACATCAGGGTGTGATGG-3' (F) and 5'-CTCCATATCATCCCAGTTGGTGA-
1035 3'(R). The underlined reverse primers target the V5 or HA epitope tag's DNA sequence. In this
1036 way, mRNA transcribed from the plasmids were specifically amplified.

1037

1038 **dsRNA dot blot analysis**

1039 The accumulation of dsRNA was detected via dot blot analysis using an anti-dsRNA J2
1040 antibody. Briefly, total cellular RNAs were extracted using Trizol reagent. RNA (2 µg) of each
1041 group was spotted on a Hybond-N+ membrane (GE Healthcare), followed by UV cross-linking
1042 (120 mJ/cm²) using SCIENTZ 03-II (Scientz Biotech). The membrane was then blocked in 5%
1043 non-fat milk dissolved in DEPC-treated TBS, incubated with mouse anti-dsRNA J2 antibody
1044 overnight at 4°C or for 1 h at room temperature, followed by incubation with the HRP
1045 conjugated goat anti-mouse secondary antibody for 1 h at room temperature. At the intervals of
1046 each incubation step, the membrane was washed three times with washing buffer (0.1%
1047 Tween® 20 detergent diluted in TBS). The dsRNA signals were detected using Tanon 4600
1048 Chemiluminescent Imaging System after development with luminol chemiluminescence
1049 reagent kit.

1050

1051 **Polysome profile analysis**

1052 Vero cells were infected with 1 MOI of IBV or rIBV-nsp15-H238A for 16 h, then incubated
1053 with 100 µg/mL cycloheximide (CHX) (Selleck, Catalog No. S7418) for 15 min at 37°C to
1054 block translation elongation. Cells were washed and lysed by polysome extraction buffer (20
1055 mM Tris-HCl pH 7.5, 100 mM KCl, 5 mM MgCl₂, 0.5% Nonidet P-40) containing 100 µg/mL
1056 of CHX, protease inhibitors and RNase inhibitors. Lysates were clarified by centrifugation at
1057 12,000 × g for 15 min at 4°C, and supernatants were resolved on a linear sucrose gradient (7–
1058 47% in buffer containing 20 mM Tris-Cl, pH 8.0, 140 mM KCl, 1.5 mM MgCl₂, 1 mM DTT,
1059 1 mg/mL heparin) by centrifugation at 38,000 × g at 4°C for 3 h. After centrifugation, each 1 ml
1060 fraction was collected and analysed by Western blot analysis.

1061

1062 **Densitometry**

1063 Image J program (NIH, USA) was used to quantify the intensities of corresponding bands of
1064 western blot, dsRNA dot blot, the intensity of puromycin signal in immunofluorescence image,
1065 and Pearson's correlation coefficient of signals in immunofluorescence image.

1066

1067 **Statistical analysis**

1068 Data and statistical analysis were performed with Graphpad Prism 8 software. Significance was
1069 determined by ONE-Way ANOVA followed by Tukey's post-hoc test. *p* < 0.05 was considered
1070 significant.

1071

1072 **Data availability statement**

1073 All relevant data are within the paper and its Supporting Information files.

1074

1075 **Acknowledgments**

1076 We would like to thank Prof. Dingxiang Liu (South China Agricultural University, China) for
1077 providing the IBV Beaudette strain and the rabbit anti-IBV-nsp12 polyclonal antibody, and for
1078 his excellent scientific advice. We also grateful to Prof. Bin Li (Jiangsu Academy of
1079 Agricultural Sciences, China) for providing TGEV nsp15 cDNA, Prof. Tongling Shan
1080 (Shanghai Academy of Agricultural Sciences, CAAS) for providing LLC-PK1 and ST cells,
1081 pDCoV cDNA, Dr. Min Liao (Zhejiang University, China) for providing IBV nsp15
1082 monoclonal antibody.

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1084 **Disclosure**

1085 The authors have no financial conflict of interest.

1086

1087 **Grant support**

1088 This study was supported by the National Key Technologies Research and Development
1089 Program (No. 2021YFD1801104), the National Natural Science Foundation of China
1090 (32172834, 31772724).

1091

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