SARS-CoV-2 ORF6 disrupts innate immune signalling by inhibiting cellular mRNA export

(Short title: ORF6 blocks mRNA export)

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Abstract

21   SARS-CoV-2 is a betacoronavirus and the etiological agent of COVID-19, a

22   devastating infectious disease. Due to its far-reaching effect on human health, there is an urgent

23   and growing need to understand the viral molecular biology of SARS-CoV-2 and its interaction

24   with the host cell. SARS-CoV-2 encodes 9 predicted accessory proteins, which are presumed

25   to be dispensable for *in vitro* replication, most likely having a role in modulating the host cell

26   environment to aid viral replication. Here we show that the ORF6 accessory protein interacts

27   with cellular Rae1 to inhibit cellular protein production by blocking mRNA export. We utilised

28   cell fractionation coupled with mRNAseq to explore which cellular mRNA species are affected

29   by ORF6 expression and show that ORF6 can inhibit the export of many mRNA including

30   those encoding antiviral factors such as IRF1 and RIG-I. We also show that export of these

31   mRNA is blocked in the context of SARS-CoV-2 infection. Together, our studies identify a

32   novel mechanism by which SARS-CoV-2 can manipulate the host cell environment to supress

33   antiviral responses, providing further understanding to the replication strategies of a virus that

34   has caused an unprecedented global health crisis.

35

Author Summary

37   SARS-CoV-2 is the virus responsible for the current COVID-19 pandemic. Coronaviruses, like

38   SARS-CoV-2, replicate their genome in the cytoplasm of the host cell by hijacking the cellular

39   machinery. In addition to structural proteins and viral enzymes, SARS-CoV-2 encodes 9

40   accessory proteins. Although these are not required for in vitro replication, they are thought to

41   modulate the host cell environment to favour viral replication. In this work, we show that the

42   ORF6 accessory protein can supress cellular protein production by blocking mRNA nuclear

43   export through interacting with the cellular protein Rae1, a known mRNA export factor. We

44 also investigated which cellular mRNAs were retained in the nucleus when ORF6 was

45 overexpressed. Interestingly, we found that ORF6 inhibited the export of many different

46 mRNAs, including those encoding antiviral factors, like IRF1 and RIG-I, even in the absence

47 of stimulation by interferon. Importantly, we found that the export of these mRNAs was

48 similarly affected in the context of SARS-CoV-2 infection. Therefore, we believe we have

49 identified a novel mechanism that SARS-CoV-2 uses to suppress antiviral responses in order

50 to make the cell more permissive to infection.

51

Introduction

53 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is an enveloped

54 single-stranded, positive-sense RNA virus of the *Betacoronovirus* genus and is the etiological

55 agent of the highly infectious disease, COVID-19. SARS-CoV-2 replicates in the epithelial

56 cells of the respiratory tract causing extensive respiratory symptoms, with severe cases leading

57 to mortality [1-4]. SARS-CoV-2 expresses four structural proteins, spike (S), membrane (M),

58 envelope (E) and nucleoprotein (N) and 16 non-structural proteins (nsps), as well as nine

59 predicted accessory proteins; ORF3a, ORF3b, ORF6, ORF7a, ORF7b, ORF8, ORF9b, ORF9c

60 and ORF10 [2, 5]. These accessory proteins are presumed dispensable for *in vitro* replication

61 and have been suggested to play a role in pathogenicity and host cell modulation [6, 7]. Little

62 is known about the function of these accessory proteins and how they affect the cellular

63 environment to facilitate efficient SARS-CoV-2 replication.

64 SARS-CoV-2 shares a high degree of homology with the etiological agent of the 2003

65 SARS outbreak, SARS-CoV (hereafter known as SARS-CoV-1). Indeed, the accessory

66 proteins of these two betacoronaviruses have high homology, with ORF3b and ORF6 being

67 the most divergent. ORF6 from SARS-CoV-1 is a 7.3 kDa protein, which has been reported to

68 inhibit IFN signalling by disrupting the nuclear import of STAT1/STAT2 and IRF3 [8, 9]. This

3

69   is dependent on ORF6 interacting with the cellular Rae1-Nup98 complex [10], which spans

70   the nuclear envelope, providing a gateway between the nucleus and cytoplasm, and helps

71   shuttle proteins and mRNA between the two compartments [11, 12]. Other viruses have also

72   been shown to disrupt this nuclear export pathway to aid viral propagation. VSV matrix (M)

73   and herpesvirus ORF10 both inhibit mRNA nuclear export by disrupting Nup98-Rae1

74   function, while Influenza virus NS1 renders cells more permissive to replication by

75   downregulating Nup98 expression thereby inhibiting Rae1-dependent mRNA nuclear export

76   [13-16]. As coronaviruses transcribe their genome in the cytoplasm, they are not dependent on

77   nuclear export function for viral replication. Indeed, NSP1 proteins from coronaviruses have

78   been shown to inhibit the NXF1 mRNA export pathway, potentially disrupting the expression

79   of a range of cellular proteins [17].

80   Given the potential importance of the SARS-CoV-2 accessory proteins we set out to

81   investigate their functions. Strikingly, we found that ORF6 inhibited protein production and

82   we show that in addition to disrupting STAT1/2 import, ORF6 can also block the export of

83   cellular mRNA from the nucleus into the cytoplasm, effectively blocking protein translation.

84   In agreement with other very recent studies of SARS-CoV-2 ORF6, this is also dependent on

85   interaction with the Nup98-Rae1 complex [10, 18, 19]. Here, we utilise cellular fractionation

86   and mRNAseq to identify the mRNA species blocked by ORF6. We show that ORF6 inhibits

87   export of a broad range of mRNAs, in particular several interferon-upregulated genes that

88   encode antiviral factors, including RIG-I and IRF1. This shows that SARS-CoV-2 ORF6 can

89   disrupt host cell innate immune signalling by blocking mRNA nuclear export and suggests that

90   ORF6 may function to inhibit the earliest stages of innate signalling by downregulating

91   expression of pathogen recognition receptors (PRRs) and antiviral transcription factors, such

92   as IRF1.

93

Results

95

SARS-CoV-2 ORF6 Inhibits protein expression

To investigate the role of SARS-CoV-2 accessory proteins, we first generated a panel of HIV-1 virus-like-particles (VLPs) encoding each open-reading frame (ORF) to transduce a range of different cell lines to express each accessory protein individually. VLPs were synthesised by co-transfecting HEK293T cells with plasmids encoding HIV-1 Gag-Pol (pCMVΔR8.91), VSV-G (pVSV-G) and a pLVX-StrepII-ORF vector, encoding either ORF3a, ORF3b, ORF6, ORF7a, ORF7b, ORF8, ORF9b, ORF9c or ORF10. VLPs titres were determined after 72 hours and at least 10^4 infectious units/ml were detected for all VLPs except for VLP encoding ORF6 which had undetectable infectious units (Fig 1A, upper panel). To determine if this was due to decreased infectivity or decreased particle production, transfected HEK293T cell lysates were analysed by immunoblotting for the HIV-1 structural polyprotein, Gag. Gag was detectable in all cells, except for cells transfected with pLVX-StrepII-ORF6 (Fig 1A, lower panel). This suggested that ORF6 expression resulted in inhibition of Gag production preventing formation of VLPs.

To determine if there was a dose-dependent effect on Gag expression and VLP production, we next generated GFP-reporter HIV-1 VLPs in the presence of increasing amounts of ORF6 or ORF9b as a control. Transfected HEK293T cell lysates were analysed by immunoblotting for HIV-1 Gag and ORF6/ORF9b. Gag expression was reduced in a dose-dependent manner only with increasing amounts of ORF6 (Fig 1B). VLPs generated from these transfections were used to transduce HeLa cells and the percentage of cells expressing GFP was determined after 72 hours by flow cytometry to measure VLP titre. As with Gag expression, HIV-1 VLP titres were severely reduced in a dose dependent manner compared to

118   the control when produced in the presence of ORF6 (Fig 1C, yellow lines) but not ORF9b (Fig

119   1C, blue lines).

120   To determine if ORF6 inhibition was specific to HIV-1 Gag expression, we next

121   examined the effect of ORF6 on the expression of MLV Gag and mApple fluorescent protein,

122   which have completely different protein sequences to HIV-1 Gag. As with HIV-1 Gag, ORF6

123   inhibited MLV Gag expression and VLP production in a dose-dependent manner (S1A and B

124   Figs). Furthermore, when HEK293T cells were co-transfected with plasmids expressing

125   mApple and each SARS-CoV-2 ORF and the median fluorescent intensity (MFI) of mApple

126   was measured by flow cytometry, ORF6 reduced the MFI approximately 3-fold compared to

127   the control (Fig 1D, upper panel). None of the other ORFs had an effect on the MFI of mApple

128   (Fig 1D, upper panel), although immunoblotting revealed very low expression of ORF3b and

129   ORF10 (Fig 1D, lower panel), which has also been observed by others [5]. Taken together our

130   results suggest that ORF6 can inhibit expression of multiple proteins expressed from a vector.

131   Previous work has shown that the C-terminal region of ORF6 is important for inhibiting

132   IRF3 and STAT nuclear import [5, 8, 10]. To determine if this region is also important for

133   ORF6-dependent inhibition of protein expression, we introduced alanine substitutions at

134   residues in the C-terminus that were conserved between SARS-CoV-2 and SARS-CoV-1

135   ORF6 proteins, E55A, M58A, E59A or D61A (Fig 1E, highlighted in red). HEK293T cells

136   were co-transfected with plasmids encoding each mutant ORF6 or wild type ORF6 from either

137   SARS-CoV-2 or SARS-CoV-1 together with HIV-1 Gag and cell lysates were analysed by

138   immunoblotting (Fig 1F). As before, ORF6(CoV-2) (Fig 1F, lane 2) reduced Gag expression

139   compared to the ORF9b control (lane 1). In addition, ORF6(CoV-1) (lane 7) and mutant E55A

140   (lane 3) were also able to inhibit Gag expression. However, ORF6 mutants M58A (lane 4),

141   E59A (lane 5) and D61A (lane 6) had a much weaker effect on Gag expression suggesting that

142   residues M58, E59 and D61 are essential for reducing protein expression. ORF6 mutants

143   M58A, E59A and D61A also had a much weaker effect on mApple expression compared to

144   wild type ORF6 (Fig S1C).

145

ORF6 inhibits protein expression by interfering with Rae1

147   There are several ways that ORF6 could block protein expression, for example inhibiting

148   transcription, mRNA nuclear export or translation. Biochemical studies have indicated that

149   SARS-CoV-2 ORF6 interacts with the Nup98-Rae1 complex [5] which is important for mRNA

150   nuclear export. Specifically, Rae1 helps shuttle nascent mRNA into the cytoplasm for

151   translation [11, 12, 20]. Therefore, to investigate whether ORF6 could block protein expression

152   by interfering with the Nup98-Rae1 complex, we overexpressed Rae1 in HEK293T cells

153   together with HIV-1 Gag and ORF6. Fig 2A shows that expressing exogenous Rae1 partially

154   rescued Gag expression in the presence of ORF6 (Fig 2A, compare lanes 2 and 3). Relief from

155   ORF6 inhibition could be further enhanced by introduction of a mutation known to affect the

156   inhibitory interaction between the VSV M protein and Rae1 [13]. Whilst expression of

157   Rae1(T306I) had a similar effect to overexpressing wild type Rae1 (lane 5), expression of

158   Rae1(R305G) fully rescued Gag expression to the levels seen in the ORF9b control (compare

159   lane 4 with lane 1). This suggests that ORF6 interacts with Rae1 to inhibit protein expression

160   and that R305 is important for the interaction.

161   To confirm the interaction of ORF6 with Rae1 and examine the interaction with Nup98,

162   we performed co-immunoprecipitations (co-IP). HEK293T cells were co-transfected with

163   plasmids expressing Strep-tagged GFP or ORF6 with Nup98 and/or HA-Rae1. After 48 hrs,

164   cells were lysed and GFP or ORF6 immunoprecipitated with anti-Strep antibody. Input lysates

165   and precipitated proteins were analysed by immunoblotting for Strep, HA and Nup98. Both

166   GFP and ORF6 were detected in the eluate, confirming successful immunoprecipitation (Fig

167   2B). The GFP control did not co-immunoprecipitate with either HA-Rae1 or Nup98. However,

168   HA-Rae1 was detectable in the eluate when co-expressed with ORF6, confirming an

169   interaction between these two proteins. Rae1 was detectable whether Nup98 was co-expressed

170   or not, suggesting that the ORF6 interaction with Rae1 is not dependent on Nup98. Conversely,

171   Nup98 was only detectable in the eluate when co-expressed with both ORF6 and Rae1. ORF6

172   and Rae1 also co-immunoprecipitated with a small amount of endogenous Nup98 (Fig 2B, lane

173   5). This suggests that the interaction between Nup98 and ORF6 is not direct but is instead

174   dependent on Rae1. Importantly, the Rae1(R305G) mutation that conferred resistance to

175   ORF6-dependent inhibition of protein expression (Fig 2A) did not co-immunoprecipitate with

176   ORF6 (Fig 2C) and Nup98 was also not present under these conditions, confirming that Nup98

177   interacts with ORF6 via Rae1. We also tested whether the ORF6 mutants could co-precipitate

178   Rae1 (Fig 2D). As expected, ORF6 and ORF6(E55A), which both inhibited Gag expression

179   (Fig 1F), co-immunoprecipitated with Rae1 whilst ORF6(M58A) did not (Fig 2D).

180   Additionally, we also observed that ORF6(CoV-1) co-immunoprecipitated with Rae1 (S2A

181   Fig), and that both ORF6 homologues co-immunoprecipitated with endogenous Rae1 (S2B

182   Fig). Overall, this implies that the effect of ORF6 on protein expression is mediated through

183   an interaction with Rae1, suggesting that the block is to mRNA export.

184

ORF6 inhibits the nuclear export of cellular mRNA

186   To investigate the effect of ORF6 on mRNA export directly, we first set up an assay to separate

187   nuclear (Nucl) and cytoplasmic (Cyto) fractions and measure mRNA levels in each by qPCR.

188   A higher ratio of Nucl:Cyto mRNA compared to the control would suggest that the mRNA is

189   being retained in the nucleus. As a proof-of-principle, we treated cells with Leptomycin B

190   (LMB), which inhibits Chromosomal Maintenance 1 (CRM1)-dependent mRNA export [21]

191   (S3 Fig). HEK293T cells were transfected with a plasmid expressing GFP, treated with LMB

192   for 16 hrs and then fractionated. LMB clearly reduced GFP expression in cells (S3A and B

193   Fig). Absolute levels of GFP mRNA were determined by qPCR in the Nucl and Cyto fractions

194   (S3C Fig). Although total GFP RNA levels were reduced, LMB-treated cells also had a higher

195   Nucl:Cyto GFP mRNA ratio compared to untreated cells, at 0.93 compared to 0.35 (S3D Fig).

196   Like LMB-treated cells, cells expressing ORF6 also reduced GFP expression (S3E and F Fig)

197   and increased the ratio of Nucl:Cyto GFP mRNA (S3G and H Fig), while cells expressing

198   ORF6(M58A) had a similar Nucl:Cyto GFP mRNA as the control cells expressing ORF9b.

199   Overall, this indicates that ORF6 inhibits nuclear export of GFP mRNA, in a Rae1-dependent

200   manner.

201   So far, we have shown that ORF6 can inhibit exogenous protein mRNA nuclear export

202   and that this likely accounts for the block to protein production. To investigate whether ORF6

203   also inhibits endogenous cellular mRNA export, and to determine whether specific proteins are

204   affected, we performed mRNAseq on nuclear and cytoplasmic fractions of HEK293T cells

205   either untransfected (mock) or transfected with ORF6 or GFP. Transfections were performed

206   in duplicate and after 24 hrs, one sample for each transfection was treated with IFN-β (1,000

207   units/ml) for 16 hrs. Cells were then fractionated and a sample was taken for protein analysis.

208   RNA was extracted from the remaining fraction and mRNA libraries were prepared and

209   sequenced (Fig 3A). Lysates were analysed by immunoblotting with anti-Strep antibodies to

210   confirm ORF6/GFP expression, HSP90 (cytoplasmic marker) and lamin B1 (nuclear marker),

211   to confirm that cellular fractionation was successful and phosphorylated STAT1 (pSTAT-

212   Y701) to confirm activation of the type-I IFN signalling cascade (S4A Fig).

213   Next, we determined the distribution of mRNA between the cytoplasm and nucleus

214   during ORF6 and GFP expression without and with IFN-β treatment compared to the

215   untransfected cells (S4B Fig). ORF6 was then further compared to GFP to determine which

216   mRNA species were specifically enriched in the nucleus or cytoplasm in the presence of ORF6

217   (Fig 3B). A positive fold change (FC) indicates that an mRNA species is enriched in the

218   nucleus, while a negative FC suggests that an mRNA species is enriched in the cytoplasm. In

219   total, in the absence of IFN, 2,431 mRNA species were enriched in the nucleus with ORF6,

220   while only 180 mRNA species were enriched in the cytoplasm, compared to the control (Fig

221   3B, left panel, highlighted in yellow). With IFN-β treatment, 2,128 mRNA species were

222   enriched in the nucleus with ORF6, while 81 were enriched in the cytoplasm, compared to the

223   control (Fig 3B, right panel). There was considerable overlap (1,765 mRNA) between the

224   mRNA species enriched in the nucleus in both conditions (Fig 3C). These genes were found to

225   include a wide range of Gene Ontology (GO) terms (Fig 3D), highlighting their breadth of

226   function. These data suggest that ORF6 not only inhibits mRNA export of exogenous mRNA

227   such as GFP but, more importantly, also inhibits the nuclear export of a broad range of cellular

228   mRNA.

229

ORF6 inhibits the nuclear export of IFN-upregulated mRNA

231   As many mRNA were retained in the nucleus, we decided to investigate whether genes with

232   potential anti-viral functions were among them. Firstly, we identified potential ISGs in our

233   dataset by determining which mRNA species were upregulated by IFN-β treatment in cells

234   transfected with our control plasmid (S5A Fig). We found 134 mRNAs species that were

235   significantly upregulated in either the cytoplasm and/or nucleus after IFN-β treatment and

236   labelled them interferon upregulated genes (IUGs) (Fig 4A and S5B Fig). Importantly, these

237   IUGs encoded many proteins with roles in inhibiting viral replication and immune regulation

238   (S5C Fig) and included well characterised ISGs like BST2 and IFITM1. Next, as ORF6 has

239   been reported to inhibit IFN signalling, we measured mRNA expression in the presence of

240   ORF6 compared to control cells in the context of IFN-β treatment to determine if the expression

241   of any of these identified IUGs was modulated by ORF6. Interestingly, of the 134 IUGs we

242   identified, 69 IUGs were significantly downregulated in the cytoplasm in the presence of ORF6

243   (yellow tones) and 2 were significantly upregulated (dark blue tones) (Fig 4A, top panel). In

244   the nuclear fraction, 62 IUGs were significantly downregulated in the presence of ORF6 while

245   10 were significantly upregulated (Fig 4A, bottom panel). In total, 79 unique IUGs were

246   significantly downregulated by ORF6, while 11 unique IUGs were upregulated (S5D Fig). This

247   supports the notion that ORF6 can inhibit IUG mRNA expression. However, in addition to the

248   IUGs, ORF6 modulated the expression of many other mRNA compared to the control. S5E Fig

249   summarises the total number of mRNA species upregulated or downregulated in the nucleus

250   and/or cytoplasm by ORF6 in IFN treated and untreated cells.

251   Next, we further investigated the effect of ORF6 on the IUGs we had identified.

252   Intriguingly, of the 2,431 mRNA species that were enriched in the nucleus by ORF6 in the

253   absence of IFN treatment (Fig 3B), 24 were identified as IUGs (Fig 4B), including the antiviral

254   factors TRIM69, BST2, ZNFX1, IRF1, and DDX58 (RIG-I) (Fig 4C, left panel). This suggests

255   that ORF6 blocks the mRNA export of IUGs even when they are expressed at a basal level,

256   which has significant implications for the antiviral state of the host cell. Furthermore, after IFN

257   stimulation, ORF6 enriched 11 IUGs in the nucleus including TRIM38, SOCS1, IFITM2,

258   SLFN5 and ZNFX1 (Fig 4C, right panel). Six of the IUGs were enriched in the nucleus in both

259   IFN-treated and untreated cells (Fig 4B). Altogether, we identified 29 IUGs that were retained

260   in the nucleus in the presence of ORF6 (Fig 4B) suggesting that in addition to inhibiting ISG

261   expression, ORF6 can disrupt innate signalling by preventing the nuclear export of mRNA

262   species that encode key antiviral factors that are both basally expressed and induced by IFN-β,

263   like RIG-I, IRF1 and ZNFX1.

264

SARS-CoV-2 inhibits the export of cellular mRNA

266   Our results demonstrate that expression of ORF6 in cells inhibits mRNA nuclear export with

267   the effect of inhibiting protein expression. However, in the context of a viral infection, another

268   viral protein may counteract the actions of ORF6. Therefore, to investigate whether mRNA

269   export is also blocked during a SARS-CoV-2 infection, we analysed the nuclear accumulation

270   of specific mRNAs in infected cells. Vero cells were infected with one of four SARS-CoV-2

271   variants: Eng/2, Alpha, Beta or Delta at a high MOI for 48 hrs, after which cells were either

272   fixed, permeabilised and probed with an anti-ORF6 antibody or separated into Nucl and Cyto

273   fractions and processed for protein and RNA extraction. For all infections, ORF6

274   predominantly localised to the cytoplasm with some perinuclear staining (Fig 5A). The pattern

275   of ORF6 expression was less diffuse in SARS-CoV-2(delta) infected cells than the other

276   infections. We also saw some co-localisation of ORF6 with Nup98 (S6A Fig). Immunoblot

277   analysis of cell lysates for HSP90 (cytoplasmic) and Histone H3 (nuclear) confirmed that

278   fractionation of samples was successful (S6B Fig). ORF6 was also detected predominately in

279   the cytoplasm for most infections, although in two independent infections with SARS-CoV-

280   2(delta) there was more ORF6 in the nuclear fraction (S6B and D Fig). This was probably

281   linked to the poor viability of SARS-CoV-2(delta)-infected cells. In order to investigate mRNA

282   export, we chose eight genes to analyse from our mRNAseq data: We identified three mRNAs

283   that were most enriched in the nucleus in the presence of ORF6 (FAM222A, SPOCK1,

284   SERTAD1, together classed "non-antiviral") (S6E Fig, red dots) as well as four mRNA

285   encoding antiviral IUGs that were significantly enriched in the nucleus (RIG-I, IRF1, ZNFX1

286   and TRIM38) (Fig 4B, red dots). GAPDH was not significantly enriched in the nucleus with

287   ORF6 and so was used as a control. Overall, all four SARS-CoV-2 variants increased the

288   average Nucl:Cyto ratio of FAM222A, SPOCK1 and SERTAD1 mRNAs compared to

289   uninfected cells (Fig 5B) while the Nucl:Cyto ratio of GAPDH mRNA remained constant. This

290   suggests that ORF6 can block the export of these mRNA species in the context of SARS-CoV-2

291   infection. Strikingly, there was a large increase in the average Nucl:Cyto ratio of both RIG-I

292   and IRF1 mRNA after infection with all four variants (Fig 5B). ZNFX1 was also enriched in

293   the nucleus for all infections except SARS-CoV-2 (delta). However, TRIM38, which was only

294   enriched in the nucleus by ORF6 after IFN treatment (Fig 4B, right panel), was only marginally

295   enriched in the nucleus after infection. Overall, we observed that SARS-CoV-2 infection

296   induced retention of mRNA in the nucleus similar to ORF6 expression alone and that export

297   of mRNAs involved in transcription (SERTAD1) and immune regulation (RIG-I and IRF1)

298   was inhibited, suggesting that ORF6 has a broad effect on cellular pathways during infection.

299

Discussion

301   Coronaviruses encode several accessory proteins which are dispensable for *in vitro*

302   replication but are presumably important for replication *in vivo* and disease pathogenicity [7].

303   This could make them good therapeutic targets. These proteins are not well characterised for

304   SARS-CoV-2, so we decided to investigate their function. To do this, we generated a panel of

305   lentiviral VLPs encoding each individual accessory protein with which to introduce each

306   protein into various cells for defined functional assays. Interestingly however, we were

307   repeatedly unable to generate VLPs encoding ORF6 (Fig 1A). We therefore determined how

308   ORF6 inhibits VLP production. We showed that ORF6 not only supresses expression of the

309   HIV-1 structural protein Gag, which is required for VLP synthesis, but it also reduced MLV

310   Gag (S1 Fig) and mApple expression (Fig 1D), implying that ORF6 blocks protein expression

311   more broadly, in a sequence-independent manner.

312   Protein expression could have been inhibited via multiple mechanisms, however, ORF6

313   has been reported to interact with Rae1 and Nup98 which are involved in nuclear import and

314   export suggesting that ORF6 may modulate mRNA nuclear export resulting in decreased

315   protein expression. We confirmed that ORF6 from both SARS-CoV-2 and SARS-CoV-1

316   interacted with Rae1, and that this interaction was important for the block to protein expression

317   as mutants that were unable to interact also failed to inhibit protein expression (Figs 2 and 1F).

13

318   Furthermore, we showed directly that ORF6 did indeed inhibit mRNA export (Figs 3 and 4).

319   Interestingly, the herpesvirus ORF10 protein and the VSV M protein also interact with the

320   same region of Rae1 [13, 15, 22] and block mRNA nuclear export. VSV M broadly inhibits

321   the loading of cellular mRNA to the Rae1-Nup98 complex, while herpesvirus ORF10 can still

322   form a complex with cellular mRNAs. Here, ORF6 only interacted with Nup98 indirectly

323   through Rae1, similar to herpesvirus ORF10. ORF6 has been reported to dislocate Rae1 and

324   Nup98 from the NPC, but whether this mislocalisation causes the mRNA export block or is a

325   consequence of mRNA export disruption is not known [19].

326   Blocking cellular mRNA nuclear export, thereby reducing translation of cellular

327   proteins, would be advantageous for viral replication as SARS-CoV-2 replicates in the

328   cytoplasm and would therefore be able to exploit the limited cellular translational machinery

329   in favour of viral translation (Fig 6A). In addition, by blocking mRNA export, ORF6 can also

330   affect other cellular processes beyond export, as inhibiting the translation of key cellular

331   proteins will modulate their downstream processes. Excitingly, using RNAseq, we have

332   revealed that ORF6 can prevent the nuclear export of mRNAs encoding antiviral factors (Fig

333   4), both after IFN treatment, for example IFITM2 and ZNFX1, and more provocatively, in

334   untreated cells. This implies that ORF6 can modulate the basal, steady state level of immunity

335   in cells. Indeed, DDX58(RIG-I) and ZNFX1, which were enriched in the nucleus following

336   both ORF6 expression in untreated cells (Fig 4) and during SARS-CoV-2 infection (Fig 5), are

337   key pattern recognition receptors (PRRs) that detect viral dsRNA [23, 24]. ZNFX1 has been

338   postulated to be involved in the very early stages of IFN signalling, as one of the earliest viral

339   sensors, due to its higher constitutive expression compared to both MDA5 and RIG-I [23].

340   Downregulation of these sensors would prevent the induction of an antiviral state and allow

341   SARS-CoV-2 to replicate undetected (Fig 6B). The constitutively expressed transcription

342   factor, IRF1, which helps regulate basal expression of antiviral factors like BST2 and RIG-I

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343   [25, 26] was also targeted by ORF6. IRF1 mRNA is dependent on Nup98 and Rae1 for export

344   and its corresponding protein has a relatively short half-life of around 20-40 mins [16, 27, 28].

345   Inhibiting IRF1 mRNA export would therefore have major consequences for the induction of

346   innate immunity [16, 29, 30]. Interestingly, ORF6 from SARS-CoV-1 is thought to be

347   packaged into virions and, thus, the protein is present in the cell as soon as virions infect.

348   Incoming ORF6 could therefore downregulate PRRs before viral replication generates dsRNA

349   intermediates, impeding the earliest antiviral warning system whilst also restricting the

350   expression of further antiviral factors that should induce an antiviral state in the cell [31, 32].

351   It would be interesting to know if SARS-CoV-2 ORF6 is also packaged into virions.

352   ORF6 from SARS-CoV-2 and SARS-CoV-1 have been shown to inhibit ISG and type-I

353   IFN expression by preventing STAT1/2 and IRF3 nuclear translocation [9, 10, 14, 33, 34].

354   Another recent study used immunofluorescence to show that mRNA accumulated in the

355   nucleus of ORF6 expressing cells [18]. Here, we show that ORF6 alone, or in the context of a

356   viral infection, can inhibit nuclear export of a broad range of mRNA, including mRNA of

357   antiviral proteins and transcription factors, which could have a downstream effect on ISG

358   expression. Indeed, IRF1 regulates the expression of a range of ISGs by acting as a positive

359   regulator of the JAK/STAT signalling pathway (Fig 6) [35]. Although ORF6 clearly inhibits

360   mRNA nuclear export, the multi-functional SARS-CoV-2 protein, NSP1, has also recently

361   been reported to inhibit mRNA export by blocking NXF1 docking to the NPC [17]. This

362   redundancy in targeting mRNA export suggests that blocking this pathway gives the virus a

363   clear survival advantage [36]. Further work will investigate how ORF6 and NSP1 complement

364   each other and whether this can be exploited in new therapeutic strategies directed against

365   Sarbecoviruses [17, 37].

366

Materials and Methods

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368

Cell lines

370   HEK293T, HeLa, Mus dunni tail fibroblast (MDTF) and Vero cell lines were maintained in

371   Dulbecco's modified Eagle medium (DMEM, Thermo Fisher), supplemented with 10% heat-

372   inactivated foetal bovine serum (FBS, Biosera) and 1% Penicillin/Streptomycin (Sigma). Cells

373   were grown in a humified incubator at 37°C and 5% CO_2. Stock cells were tested for

374   mycoplasma contamination and authenticated by short-tandem repeat (STR) profiling.

375

Plasmids and site-directed mutagenesis

377   SARS-CoV-2 ORF accessory proteins were expressed in cells via transfection or transduction

378   with retroviral VLPs. The plasmids used to generate HIV-1 and MLV VLPs for transduction

379   of ORFs, pCMVΔ8.91 and pHIT60 respectively, and pVSV-G have been described before [38,

380   39]. For expression of each SARS-CoV-2 ORF accessory protein, two different plasmids were

381   used. For the initial experiments on Gag expression (Figs 1A-C and S1A and B Figs) and ORF6

382   co-expression with Rae1 (Fig 2A), a pLVX-IRES-Puro vector encoding an individual ORF

383   was used. The ORF genes were initially synthesised by GeneArt (Thermo Fisher) and first

384   cloned into a pTriEX-6 plasmids using XmaI and SacI restriction sites. The pLVX-StrepII-

385   ORF-IRES-Puro plasmids were then cloned using the NEBuilder HiFi DNA Assembly Cloning

386   Kit (NEB) from the pTriEX-6 plasmids, using the primers listed in Table 1. For the remaining

387   assays that required ORF detection or immunoprecipitation, a pLVX-EF1α-SARS-CoV-2-

388   ORF-2xStrep/2xStrep-ORF -IRES-Puro encoding one of the nine ORFs or pLVX-EF1α-eGFP-

389   2xStrep-IRES-Puro plasmid was used, referred to as twin-strep-tag. These plasmids were

390   kindly gifted from Nevan Krogan (Addgene plasmid #141383-4, #141387-90, #141392-95);

391   http://n2t.net/addgene:141383-4, 141387-90, 141392-95; RRID:Addgene_141383-4, 141387-

392   90, 141392-95) [5]. To generate the pLVX-EF1α-CoV-1-ORF6-2xStrep-IRES-Puro plasmid,

16

393   the ORF6(CoV-1)-2XStrep gene was synthesised by GeneArt and cloned in using the EcoRI

394   and BamHI restriction sites. ORF6 mutations were introduced into the pLVX-StrepII-SARS-

395   CoV-2-ORF6-IRES-Puro   and   pLVX-EF1α-SARS-CoV-2-ORF6-2xStrep-IRES-Puro

396   plasmids using the QuickChange II-XL site-directed mutagenesis kit (Agilent) according to the

397   manufacturer's instructions using SDM primers listed below. The following plasmids were

398   sourced from Addgene, psfGFP-N1 (Addgene, #54737) and pmApple-N1 (Addgene, #54567).

399   pCMVsport6Rae1 (MGC:117333) was purchased from Horizon Discovery Biosciences

400   Limited. Nup98 was amplified from a HeLa cDNA library from Clontech/Takara. Affinity tags

401   were introduced by NEBuilder HiFi DNA Assembly Cloning Kit (NEB) into the expression

402   vectors encoding Rae1 and Nup98 using primers in Table 1. Sequences were verified by Sanger

403   sequencing (Source Bioscience).

404

405   **Table 1. Sequences of Primers used for cloning**

Mutation/ Modification	Primer	Sequence (5′-3′)
E55A	FWD	*cagttggacgaggCgcagcctatgg*
	REV	*ccataggctgcGcctcgtccaactg*
M58A	FWD	*gaggagcagcctGCggaaatagacctc*
	REV	*gaggtctatttccGCaggctgctcctc*
E59A	FWD	*gagcagcctatggCaatagacctcgaag*
	REV	*cttcgaggtctattGccataggctgctc*
D61A	FWD	*ctatggaaatagCcctcgaaggcgg*
	REV	*ccgccttcgaggGctatttccatag*
R305G	FWD	*gacaaagatgccGgaacaaaactaaaaac*
	REV	*gtttttagttttgttcCggcatctttgtc*
T306I	FWD	*caaagatgccagaaTaaaactaaaaac*
	REV	*gttttttagttttAttctggcatctttg*
RT305/306GI	FWD	*gacaaagatgccGgaaTaaaactaaaaac*
	REV	*gttttttagttttAttcCggcatctttgtc*
HA-RAE1	FWD	*caggttcaaaatgTACCCATACGATGTTCCAGATTACGCTagcctgtttggaac*
	REV	*gttccaaacaggctAGCGTAATCTGGAACATCGTATGGGTAcattttgaacctg*
Nup98-MYC	FWD	*gagcccagaggtgGAACAGAAACTGATCTCTGAAGAAGACCTGtaggaattctgcag*
	REV	*ctgcagaattcctaCAGGTCTTCTTCAGAGATCAGTTTCTGTTCcacctctgggctc*
pTriEX-6	FWD	*tagaggatctatttccggtgaattcACCATGGCAAGCTGGAGC*
	REV	*ggggggagggagaggggcgggatccATGAGCGGAACTACCGCG*

Virus-like particles (VLP) production

HIV-1 and MLV virus-like particles were generated by co-transfecting HEK293T cells with plasmids, pCMVΔ8.91 (HIV-1) or pHIT60 (MLV) and pVSV-G using lipofectamine 2000 (11668019, Invitrogen). For generating GFP reporter VLPs, pCSGW (HIV-1) or pczCFG2fEGFPf (MLV) were also added [39, 40]. The plasmids were used at a ratio of 1:1:1. After 16 hrs, cells were treated with 10 mM sodium butyrate for 8 hrs, and supernatant containing VLPs harvested 24 hrs later. To measure viral titres, HeLa cells were transduced with serially diluted supernatant containing the VLPs. After 72 hrs, cells were selected with media containing puromycin (1 μg/ml) for 10 days, after which cells were washed, fixed, and stained with methanol containing crystal violet. Colonies were counted and viral titre calculated.

Immunoblotting

Cells were lysed in ice cold radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher) or IP buffer (50 mM Tris-HCl, pH 7.4 at 4 °C, 150 mM NaCl, 1 mM EDTA supplemented with 0.5% Nonidet P40) containing protease inhibitor cocktail (Roche) and DNAse (#88701, Thermo Fisher). Protein lysates were boiled at 95 °C for 5 mins in 6x SDS sample buffer (Thermo Scientific). Proteins were separated on 4-12% Bis-Tris SDS-PAGE gels (Thermo Fisher) or 4-20% mini-PROTEAN TGX (Bio-Rad) and transferred to a PVDF membrane. Primary antibodies used were: anti-HIV-1 p24 (ARP432, CFAR), anti-GFP (sc-9996, SCBT), anti-HA (C29F4, CST), anti-Strep-tag (34850, Qiagen), anti-HSP90 (4874, CST), anti-Histone H3 (14269, CST), anti-alpha-Tubulin (VMA00051, BIO-RAD), anti-Lamin B1 (66095-1-Ig, Proteintech), anti-Nup98 (C39A3, CST), anti-Rae1 (ab124783, Abcam), anti-pSTAT(Y701) (D4A7, CST), anti-ORF6/9b (MRC PPU, Dundee University). Secondary antibodies include,

18

431   anti-mouse (61-6520, Thermo Fisher), anti-sheep (ab6747, Abcam) and anti-rabbit (31460,

432   Thermo Fisher) HRP-conjugated; anti-mouse IRDye 680RD/800CW (926-68072/926-32212,

433   Li-Cor) and anti-rabbit IRDye 700CW/800CW (926-68073/926-32213, Li-Cor). Blots were

434   imaged on a Chemidoc MP imaging system (Bio-Rad) or Odyssey Clx imaging system

435   (LICOR).

436

Immunoprecipitation

438   HEK293T cells were grown in 10 cm^2 culture plates transfected and incubated for 16 hrs after

439   which media was replaced. After a further 8 hrs, cells were harvested in Pierce IP lysis buffer

440   (Thermo Fisher), supplemented with protease inhibitor cocktail and Pierce DNAse (Thermo

441   Fisher). Twin-strep fusion proteins were immunoprecipitated with MagStrep "type3" XT beads

442   (IBA) as per the manufacturer's instructions. Proteins were eluted with biotin (50 mM) and

443   boiled in 6x SDS sample buffer.

444

Subcellular fractionation

446   HEK293T cells were transfected with psfGFP-N1 and either treated with LMB (Merck) at

447   5nM; or co-transfected with plasmids expressing pLVX-EF1α-ORF6, ORF6(M58A) or ORF9b

448   [5]. At the indicated times post-transfection, 1x10^6 cells were harvested and processed with the

449   PARIS kit (Life Technologies) following the manufacturer's instructions. Parallel, samples

450   were lysed as total cell lysates or were fractionated into cytoplasm and nuclear fractions. Each

451   sample was then split in two: half was used for protein analysis and half was used for RNA

452   extraction. Protein content in the total cell lysate and in the fractions was measured by BCA

453   assay (Thermo Fisher) using a FLUOstar Omega plate reader (BMG Labtech). An equivalent

454   amount of each fraction compared to the total cell lysate was analysed by immunoblotting.

455

Quantitative PCR

456

457 To remove possible DNA contamination, extracted RNA from the nuclear and cytoplasmic

458 fractions was treated with DNase using the DNA-free kit (Invitrogen) and following the

459 manufacturer's instructions. Then, 100ng of RNA was converted to cDNA for 1h at 37 °C

460 using the Omniscript RT kit (Qiagen) and random primers (Promega). Quantitative PCR

461 (qPCR) analysis was performed in TaqMan Fast Advanced master mix (Thermo Fisher) with

462 900nM primers and 250nM probes, or 1X Taqman gene expression assays. The reactions were

463 performed on a 7500 fast real-time PCR system (Applied Biosystems) using standard cycling

464 conditions: 50 °C for 2min, 95 °C for 10min followed by 40 cycles of 95 °C for 15s and 60 °C

465 for 1min. To calculate DNA copy numbers, standard curves were generated from serial

466 dilutions of the indicated cDNA in water. The following primers and probes were used; sfGFP:

467 *for* 5'-GCGCACCATCAGCTTCAAGG, *rev* 5'-GTGTCGCCCTCGAACTTCAC and *probe*

468 5'-*FAM*-CGGCACCTACAAGACCCGCGC-*TAMRA*.

469

mRNAseq

470

471 The quality of extracted RNA was checked by Agilent TapeStation 42000 on Agilent RNA

472 ScreenTape (Agilent technologies) before proceeding to library preparation. Only samples with

473 a RINe of 7 or higher were used for Library preparation. RNA-seq libraries were generated

474 using the NEBNext Ultra Directional RNA Library Prep kit for Illumina with NEBNext®

475 Poly(A) mRNA Magnetic Isolation Module (both New England BioLabs). Samples were

476 adjusted to 400 ng total RNA and spiked with diluted 1/100 ERCC Spike-In Mix (4456740,

477 Invitrogen) and Drosophila melanogaster, embryo Poly A+ RNA, 5 μG (636224-Takara) using

478 1 μl from each. PCR Enrichment of Adaptor Ligated DNA was run with 12 cycles and SPRI

479 beads (Beckman) were used for library clean up. Agilent DNA Screen tapes (Agilent

480 Technologies) were used to check library size and quality. Samples were sequenced on an

481   Illumina Novaseq 6000 platform with 180 pM loading concentration in single-end run using

482   100-bp reads. Enriched GO terms (Biological process) were filtered and mapped using the

483   REViGO tool available at http://revigo.irb.hr/ [41]. The raw RNAseq data from this study can

484   be accessed from ENA via accession PRJEB49943.

485

mRNAseq Data Analysis

487   A custom genome joining the Human (Ensembl GRCh38.100) and D. melanogaster (Ensembl

488   BDGP6.104) genomes was prepared, and reads were trimmed, aligned, and counts produced

489   using the nf-core rnaseq pipeline [42]. Normalisation and differential expression analyses were

490   conducted within DESeq2 [43]: normalisation factors for gene-level read counts were produced

491   using counts for the D. melanogaster spike-in controls and differential gene expression

492   calculations were subsequently performed for Human genes alone using the pre-calculated size

493   factors. Independent hypothesis weighting was conducted to optimise the power of p value

494   filtering using the IHW package [44] and Log2 fold-change shrinkage was performed using

495   ashr package [45]. Result tables were subsequently filtered on (IHW-adjusted) $q<0.05$.

496

SARS-CoV-2 Infections

498   The following viruses were used in the infections: The Wuhan-like reference isolate (referred

499   to as the Eng/2) was the hCoV-19/England/02/2020, obtained from the Respiratory Virus Unit,

500   Public Health England, UK (GISAID EpiCov accession EPI_ISL_407073). The B.1.1.7

501   (Alpha) isolate was the hCoV-19/England/204690005/2020, which carries the D614G, Δ69–

502   70, Δ144, N501Y, A570D, P681H, T716I, S982A, and D1118H mutations [46], obtained from

503   Public Health England (PHE), UK, through Prof. Wendy Barclay, Imperial College London,

504   London, UK. The B.1.351 (Beta) virus isolate was the 501Y.V2.HV001, which carries the

505   D614G,L18F,D80A, D215G, Δ242-244, K417N, E484K, N501Y, A701V mutations, and was

506 kindly provided by Prof. Alex Sigal and Prof. Tulio de Oliveira [47], sequencing of viral

507 isolates received identified the Q677H and R682W mutations at the furin cleavage site in

508 approximately 50% of the genomes, which was maintained upon passage in cell culture. The

509 B.1.617.2 (Delta) isolate was MS066352H (GISAID accession number EPI_ISL_1731019),

510 which carries the T19R, K77R, G142D, Δ156-157/R158G, A222V, L452R, T478K, D614G,

511 P681R, D950N, and was kindly provided by Prof. Wendy Barclay, Imperial College London,

512 London, UK through the Genotype-to-Phenotype National Virology Consortium (G2P-UK).

513 Viruses were titrated by plaque assay on Vero E6 cells. Serial ten-fold dilutions of virus are

514 incubated for 40 mins at RT on a well of confluent Vero E6 cells. The virus inoculum is then

515 removed, and the cells overlayed with a semisolid overlay containing 1.2% Avicel in MEM.

516 Cells are incubated for two days at 37 °C, 5 % CO_2 after which the overlay is removed. Cells

517 were fixed and stained with 4 % paraformaldehyde containing 0.2 % toluidine blue for at least

518 30 mins. For the infections, Vero E6 cells were seeded 24 hrs before use to achieve 80 - 90%

519 confluency. Cells were washed, medium was removed from each well and 1 ml of serum-free

520 DMEM added. The 1 ml of serum-free DMEM was removed and 500 µl virus diluted in virus

521 growth medium was added to achieve an MOI of ≥1. Plates were incubated for approx. 30 mins

522 at RT and then 1 ml virus growth medium was added to each well. Plates were incubated for

523 24 hrs before RNA isolation and qPCR as described above using Taqman gene expression

524 assays (Thermo Fisher) as follows: FAM222A (Hs00757936_m1), SPOCK1

525 (Hs00270274_m1), SERTAD1 (Hs00203547_m1), ZNFX1 (Hs01105231_m1), TRIM38

526 (Hs00197164_m1), RIG-I (Hs01061433_m1), GAPDH (Hs02786624_g1) and IRF1

527 (Hs00971964_g1).

528

529 **Immunofluorescence**

530 HEK293T cells were grown on glass coverslips and transfected or infected with SARS-CoV-

531 2. Cells were fixed in 4% PFA in PBS for 10 mins at RT. Cells were then permeabilised with

532 0.1% triton in PBS and blocked in 3% BSA in PBS. Cells were labelled with the following

533 primary antibodies, anti-Nup98 or anti-ORF6 diluted in 4% BSA in PBS for 1 hour at RT.

534 Cells were then incubated with the following secondary antibodies, donkey anti-rabbit-AF568

535 (ab175692, Abcam) and donkey anti-sheep AF568 (A-21099, Invitrogen) diluted in 4% BSA

536 in PBS for 1 hour at RT. Coverslips were washed and mounted on glass coverslips with

537 ProLong Gold Antifade Mountant with DAPI (Thermo Fisher) and imaged with the Leica SP5

538 microscope and 100X 1.3NA oil immersion objective (Leica).

539

Statistics

541 Statistical analysis was performed using GraphPad Prism 9 software. (*, $P < 0.05$; **, $P < 0.01$;

542 ***, $P < 0.001$; ****, $P < 0.0001$, ns – not significant).

543

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552

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561

562

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Figure Legends

Fig 1. SARS-CoV-2 ORF6 inhibits protein expression.

(A) To generate VLPs, HEK293T cells were co-transfected with plasmids encoding HIV-1 Gag-Pol, VSV-G and a pLVX-StrepII-ORF vector encoding either SARS-CoV-2 ORF3a, ORF3b, ORF6, ORF7a, ORF7b, ORF8, ORF9b, ORF9c or ORF10 or an empty vector control. VLPs were harvested and titrated in HeLa cells. The infectious units/ml are shown in the bar graph. Points indicated independent biological repeats. Transfected cell lysates were separated by SDS-PAGE and analysed for Gag(HIV-1) and HSP90 by immunoblotting. (B,C) HEK293T cells were transfected with plasmids encoding HIV-1 Gag-Pol, VSV-G and either increasing amounts of pLVX-StrepII-ORF6 or pLVX-StrepII-ORF9b as well as a GFP reporter plasmid, pCSGW. (B) Transfected cell lysates were separated by SDS-PAGE and analysed for Gag(HIV-1), ORF6 or ORF9b and HSP90 by immunoblotting. (C) HeLa cells were infected with increasing amounts of HIV-1 VLPs. Three days post infection, the percentage of GFP positive cells was determined by flow cytometry. Graph shows the mean and range of two biological repeats. (D) HEK293T cells were transfected with the pLVX-EF1α plasmid encoding strep-tagged GFP(Control), ORF3a, ORF3b, ORF6, ORF7a, ORF7b, ORF8, ORF9b, ORF9c or ORF10 and a plasmid encoding mApple. After 48h, cells were fixed and the mApple median fluorescent intensity (MFI) measured by flow cytometry. The fold change (FC) in MFI is plotted relative to the control for independent biological repeats. Error bars show SEM.

751 Transfected cell lysates were analysed by SDS-PAGE and immunoblotting with anti-strep. (E)

752 Amino acid alignment of the C-terminus region of ORF6 from SARS-CoV-2 and SARS-CoV-1

753 with conserved residues highlighted in red. (F) HEK293T cells were transfected with pLVX-

754 EF1α encoding strep-tagged ORF9b, ORF6(CoV-2), ORF6(CoV-1) or indicated ORF6(CoV-

755 2) mutant and a plasmid encoding HIV-1 Gag-Pol. After 24h, cell lysates were separated by

756 SDS-PAGE and analysed for Gag(HIV-1), Strep and HSP90 by immunoblotting.

757

758 **Fig 2. ORF6 inhibits protein expression by interfering with Rae1.**

759 (A) HEK293T cells were co-transfected with plasmids encoding HIV-1 Gag-Pol, ORF6 or

760 ORF9b and Rae1 or indicated Rae1 mutant. Mock cells were untransfected. After 48h, cell

761 lysates were separated by SDS-PAGE and analysed for Gag(HIV-1), Rae1, ORF6 or ORF9b

762 and HSP90 by immunoblotting. (B) HEK293T cells were transfected with plasmids encoding

763 Twin-Strep-tagged GFP or ORF6 and Nup98 and/or HA-Rae1. After 24h, Twin-Strep-tagged

764 proteins were immunoprecipitated with MagStrep beads and proteins eluted with biotin. Input

765 lysates and eluate were separated by SDS-PAGE and analysed by immunoblotting for Strep,

766 Nup98 and HA. (C) HEK293T cells were transfected with plasmids encoding Twin-Strep-

767 tagged GFP or ORF6, Nup98 and either HA-Rae1(WT) or HA-Rae1(R305G). After 24h, Twin-

768 Strep-tagged proteins were immunoprecipitated and analysed as in (C). (D) HEK293T cells

769 were transfected with plasmids encoding Twin-Strep-tagged proteins; ORF6(CoV-2),

770 ORF6(E55A), ORF6(M58A) or GFP and HA-Rae1(WT). After 24h, Twin-Strep-tagged

771 proteins were immunoprecipitated with MagStep beads and input lysates and eluates separated

772 by SDS-PAGE and analysed by immunoblotting for Strep and HA.

773

774 **Fig 3. ORF6 inhibits the nuclear export of cellular mRNA.**

775   HEK293T cells were transfected with pLVX-EF1α-GFP or pLVX-EF1α-ORF6 or left

776   untransfected (mock). After 24h, cells were either treated with IFN-β (1,000 units/ml) for 16

777   hrs or left untreated. Cells were then harvested and fractionated into nuclear (Nucl) and

778   cytoplasmic (Cyto) fractions. Cells were either lysed for immunoblot analysis (see S4A Fig)

779   or RNA was extracted for mRNAseq. (A) Schematic of experiment design. (B) The log2-fold

780   change (Log2-FC) in mRNA abundance was calculated between the Nucl and Cyto fractions

781   for both GFP and ORF6 expressing cells, without and with IFN treatment (see S4B Fig). The

782   log2-FC in mRNA abundance was then directly compared between ORF6 and GFP, without

783   (left panel) and with (right panel) IFN, to determine which mRNAs were specifically enriched

784   in the nucleus or cytoplasm by ORF6. The log-2FC was weighted against the adjusted *p*-value

785   (shrunken Log2-FC) to show significantly enriched mRNAs (yellow points). The number of

786   mRNAs significantly enriched is shown. The mRNA count was normalised and averaged

787   between the three biological repeats. (C) Venn diagram of the number of mRNAs that were

788   significantly enriched in the nucleus by ORF6 with and without IFN. (D) mRNAs significantly

789   enriched by ORF6 without IFN treatment were analysed for enriched GO terms, which were

790   filtered and mapped using the REVIGO tool. Highly similar GO terms are grouped and linked.

791   Colour intensity reflects significance levels.

792

793   **Fig 4. ORF6 inhibits the nuclear export of IFN-upregulated mRNA.**

794   (A) Interferon upregulated genes (IUGs) were identified as mRNAs that were significantly

795   upregulated after IFN treatment compared to untreated cells (see S5A Fig). These IUGs were

796   then compared to the list of mRNAs that were significantly upregulated or downregulated by

797   ORF6 compared to the GFP control (see S5E Fig), in either the Cyto (upper panel) or Nucl

798   (lower panel) fractions. The heat maps show the relative Log2-Fold Change in IUG expression

799   in cells expressing GFP (Control) or ORF6 after IFN treatment, for three biological repeats.

800   (B) Venn diagram showing the number of IUG mRNAs that were significantly enriched in the

801   nucleus by ORF6 with and without IFN treatment. (C) The log2-fold change (Log2-FC) in

802   mRNA abundance was calculated between the Nucl and Cyto fractions for both GFP and ORF6

803   expressing cells, without and with IFN treatment, as in Fig 3B, to determine which mRNA

804   were specifically enriched by ORF6. The log-2FC was weighted against the adjusted *p*-value

805   (shrunken Log2-FC) to show significantly enriched mRNA (yellow points). IUGs are

806   highlighted in red and labelled.

807

808   **Fig 5. SARS-CoV-2 inhibits the export of cellular mRNA.**

809   Vero cells were inoculated with four SARS-CoV-2 variants (Eng/2, Alpha, Beta, Delta) at a

810   MOI ≥ 1 for 48h. (A) Cells were fixed and analysed by immunofluorescence with anti-ORF6

811   and DAPI. (B) Cells were fractionated, RNA was extracted, and cDNA was generated. mRNA

812   levels of four non-antiviral (orange) and four antiviral (blue) proteins in both Nucl and Cyto

813   fractions were measured by qPCR. The ratio of Nucl to Cyto mRNA was calculated and plotted.

814   Points indicate individual biological repeats and error bars show the mean ± SEM. Significance

815   was determined by multiple paired t test.

816

817   **Fig 6. ORF6 inhibits mRNA export to favour viral translation and supress innate**

818   **signalling.**

819   (A) Incoming or expressed ORF6 (yellow rectangles) binds to the Nup98-Rae1 complex

820   (grey/brown) blocking the export of cellular mRNA (black lines). This subsequently reduces

821   the cellular mRNA pool, increasing the availability of cellular translational machinery for viral

822   translation as well as decreasing the expression of cellular proteins. (B) Incoming or expressed

823   ORF6 (yellow) binds to the Nup98-Rae1 complex (grey/brown), inhibiting export of cellular

824   mRNA encoding IRF1 (orange). This prevents the translation of IRF1, blocking IRF1

825 regulation of the transcription of additional steady state antiviral factors, like RIG-I and BST-

826 2. ORF6 also inhibits nuclear export of mRNA encoding RIG-I (green), preventing detection

827 of viral dsRNA produced during coronavirus replication. This helps reduce IRF1/3/7 activity

828 and subsequent transcription of IFNα/β (purple), which prevents IFNα/β inducing an antiviral

829 state in an autocrine and paracrine manner.

830

Supporting information

832 **S1 Fig. SARS-CoV-2 ORF6 Inhibits MLV Gag expression.**

833 (A, B) To generate MLV VLPs, HEK293T cells were transfected with plasmids encoding

834 Gag-Pol(MLV), VSV-G and either increasing amounts of pLVX-StrepII-ORF6 or pLVX-

835 StrepII-ORF9b as well as a GFP reporter plasmid, pczCFG2fEGFPf. As a control, VLPs

836 were generated with an empty pLVX vector. (A) Transfected cells were lysed and analysed

837 by SDS-PAGE and immunoblotting for Gag(MLV), ORF6 or ORF9b and HSP90. (B) MDTF

838 cells were infected with increasing amounts of MLV VLPs. Three days post infection, the

839 percentage of GFP positive cells was determined by flow cytometry. Graph shows the mean

840 and range of two biological repeats. (C) HEK293T cells were transfected with the pLVX-

841 EF1α plasmid encoding either ORF6(CoV-1), ORF6(CoV-2) or the indicated ORF6 mutant

842 as well as a plasmid encoding mApple. After 48h, cells were fixed and the mApple MFI

843 measured by flow cytometry. The fold change (FC) in MFI is plotted relative to the control

844 for independent biological repeats. Error bars show SEM. Transfected cell lysates were

845 analysed by SDS-PAGE and immunoblotting with anti-ORF6.

846

847 **S2 Fig. ORF6 interacts with endogenous Rae1.**

848 (A) HEK293T cells were transfected with plasmids encoding Twin-Strep-tagged GFP,

849 ORF6(CoV-2) or ORF6(CoV-1) and HA-Rae1. After 24h, tagged proteins were

850   immunoprecipitated with MagStrep beads and proteins eluted with biotin. Input lysates and

851   eluate were separated by SDS-PAGE and analysed by immunoblotting for Strep, Nup98 and

852   HA. (B) HEK293T cells were transfected with the pLVX-EF1α plasmid encoding Twin-

853   Strep-tagged GFP or ORF6. After 24h, tagged proteins were immunoprecipitated with

854   MagStrep beads and proteins eluted with biotin. Input lysates and eluate were separated by

855   SDS-PAGE and analysed by immunoblotting for endogenous Rae1 and anti-Strep.

856

857   **S3 Fig. ORF6 inhibits GFP mRNA export in a Rae1-dependent manner.**

858   (A-D) HEK293T cells were transfected with a plasmid expressing GFP or left untransfected

859   (mock) and either treated with 5 nM Leptomycin B (LMB) or left untreated. After 16h, cells

860   were either harvested as a total cell lysate (T) or fractionated into Nucl (N) and Cyto (C)

861   fractions. Total cell lysates and cellular fractions were divided into two for either protein

862   analysis (B) or RNA extraction (C,D). (A) Low magnification fluorescent images showing

863   GFP expression in transfected cells before harvest. (B) Protein levels were quantified by

864   BCA assay, proportional amounts of the fractions related to the total cell lysate were analysed

865   by immunoblotting for GFP, HSP90 (cytoplasmic marker) and Histone H3 (nuclear marker).

866   (C) RNA from Nucl and Cyto fractions was converted into cDNA and GFP mRNA copy

867   numbers measured by qPCR. (D) The ratio of Nucl to Cyto GFP mRNA was calculated and

868   plotted for individual biological repeats. Error bars show the mean ± SEM. (E-H) HEK293T

869   cells were co-transfected with plasmids encoding either ORF6, ORF6(M58A) or ORF9b and

870   GFP, or left untransfected (mock). After 32h, cells were either harvested as a total cell lysate

871   (T) or fractionated into Nucl (N) and Cyto (C) fractions. Total cell lysates and cellular

872   fractions were divided into two for either protein analysis (F) or RNA extraction (G,H). (E)

873   Low magnification fluorescent images showing GFP expression in transfected cells before

874   harvest. (F) Protein levels were quantified by BCA assay, proportional amounts of the

875   fractions related to the total cell lysate were analysed by immunoblotting for Strep, GFP,

876   HSP90 (cytoplasmic marker) and Histone H3 (nuclear marker). (G) RNA from Nucl and

877   Cyto fractions was converted into cDNA and GFP mRNA copy numbers measured by qPCR.

878   (H) The ratio of Nucl to Cyto GFP mRNA was calculated and plotted for individual

879   biological repeats. Error bars show the mean ± SEM. Significance was calculated by one-way

880   ANOVA with Turkey's post hoc test, $*p<0.05$, $**p<0.01$, ns – non-significant.

881

882   **S4 Fig. ORF6 inhibits the nuclear export of cellular mRNA.**

883   (A) The cell fractionations used in Fig 3A, and B were analysed by immunoblotting with

884   anti-Strep to label ORF6 and GFP, anti-pSTAT(Y701) to confirm activation of the type-I IFN

885   signalling cascade and anti-HSP90 (cytoplasmic marker) or anti-Lamin B1 (nuclear marker)

886   to confirm successful fractionation. The analysis of three biological repeats is shown. (B)

887   From the mRNAseq data in Fig 3. The log2-fold change (Log2-FC) in mRNA abundance was

888   compared between the Nucl and Cyto fractions for both GFP and ORF6 expressing cells,

889   without (upper panels) and with IFN treatment (lower panels). The log-2FC was weighted

890   against the adjusted p-value (shrunken Log2-FC) to show mRNAs that are significantly

891   enriched (GFP; black, ORF6; yellow) in either the cytoplasm or nucleus. The number of

892   mRNAs significantly enriched are shown. The mRNA count was normalised and averaged

893   between three biological repeats.

894

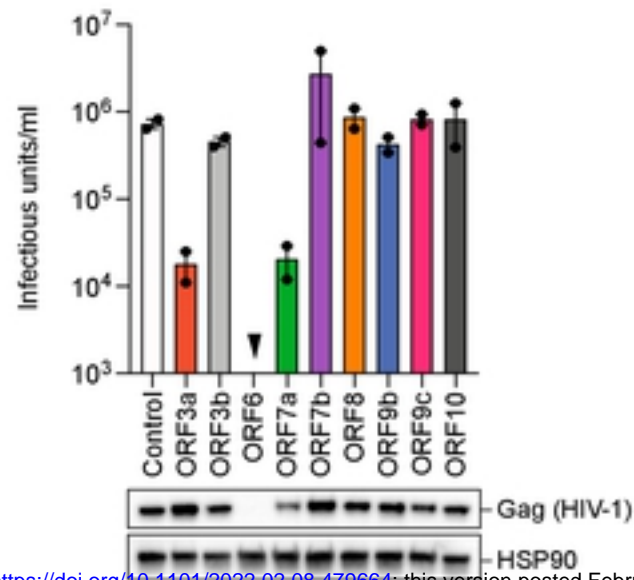895   **S5 Fig. ORF6 inhibits the nuclear export of IFN-upregulated mRNA.**

896   (A) From the mRNAseq data in Fig 3. The Log2-FC in mRNA abundance was compared

897   between cells treated with IFN or left untreated in both the Cyto (left panel) and Nucl (right

898   panel) fractions. The log-2FC was weighted against the adjusted p-value (shrunken Log2-FC)

899   to show mRNA species that are significantly upregulated or downregulated (blue). The

900   number of mRNAs upregulated is shown. These were designated as Interferon upregulated

901   genes (IUGs). (B) Venn diagram showing the number of IUGs significantly upregulated in

902   Cyto. and Nucl. fractions. (C) GO ontology enrichment analysis of significantly upregulated

903   IUGs from A. in both the Cyto and Nucl fractions. (D) Venn diagram showing the number of

904   IUGs significantly downregulated (red) or upregulated (green) by ORF6 in Cyto. and Nucl.

905   fractions, as described in Fig 4A. (E) From the mRNAseq data in Fig 3. The log2-fold change

906   (Log2-FC) in mRNA abundance was compared between ORF6 and GFP expressing cells in

907   the Cyto. and Nucl fractions, without (upper panel) and with IFN treatment (lower panel).

908   This log2-FC was weighted against the adjusted *p*-value (shrunken Log2-FC) to identify

909   significantly upregulated/downregulated mRNAs (in yellow), with the number of mRNAs

910   shown. The mRNA count was normalised and averaged between the three biological repeats.

911

912   **S6 Fig. SARS-CoV-2 inhibits the export of cellular mRNA**

913   (A) Vero cells that were inoculated with SARS-CoV-2(beta) in Fig 5A were fixed,

914   permeabilised and analysed by immunofluorescence with anti-Nup98, anti-ORF6 and DAPI.

915   (B-D) Cell lysates from Fig 5B were analysed by immunoblotting for Strep to label ORF6,

916   Nsp2, HSP90 (Cytoplasmic marker) and Histone H3 (Nuclear marker) to confirm successful

917   fractionation. The analysis of three biological repeats are shown. (E) The plot shown in Fig

918   3B (right panel) highlighting the enrichment of the mRNA encoding the four non-antiviral
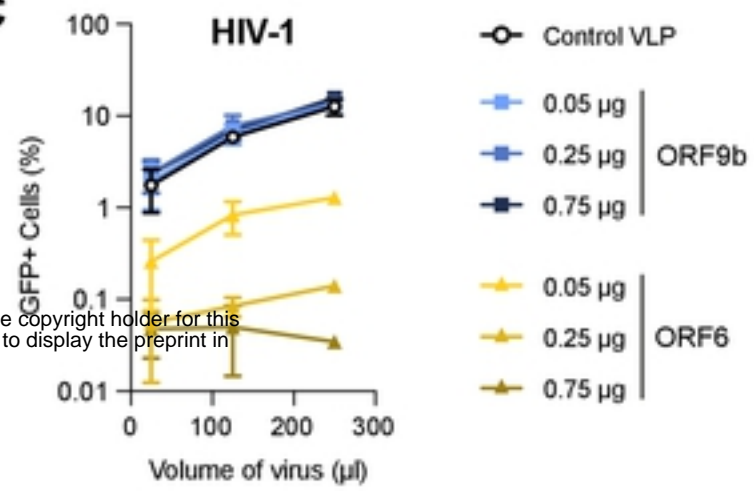
919   proteins that were quantified by qPCR in Fig 5B.

A

B

C

D

E

F

A

B

C

D

A

Mock / Control / ORF6

293T

24 hrs
-/+ IFNβ
16 hrs

Nucl. | Cyto.

Protein | RNA | Protein | RNA

WB | mRNAseq | WB | mRNAseq

B

ORF6 v Control (GFP)

- IFN | + IFN

Shrunken Log2-FC

2431 Enriched in the Nucl.

2128 Enriched in the Nucl.

180 Enriched in the Cyto.

81 Enriched in the Cyto.

Mean of Normalised Counts | Mean of Normalised Counts

C

mRNA enriched
in the Nucl. by ORF6 (2,765)

- IFN   + IFN

666 | 1,765 | 363

D

p-Value
(-Log10)

31.4

1.33

metabolic process

biosynthetic process

cellular nitrogen compound metabolic process

metabolic process

organic cyclic compound metabolic process

primary metabolic process

cellular metabolic process

heterocycle metabolic process

organic substance metabolic process

metabolic process

transcription, DNA-templated

mRNA metabolic process

organic cyclic compound biosynthetic process

RNA metabolic process

RNA biosynthetic process

nucleobase-containing compound biosynthetic process

cellular macromolecule biosynthetic process

macromolecule biosynthetic process

aromatic compound biosynthetic process

nucleobase-containing compound metabolic process

nucleic acid metabolic process

cellular macromolecule metabolic process

cellular nitrogen compound biosynthetic process

heterocycle biosynthetic process

gene expression

organic substance biosynthetic process

protein ubiquitination

protein modification by small protein conjugation or removal

negative regulation of transcription, DNA-templated

regulation of transcription, DNA-templated

regulation of transcription by RNA polymerase II

regulation of metabolic process

positive regulation of RNA metabolic process

regulation of cellular process

endosome organization

protein-DNA complex assembly

chromatin assembly or disassembly

protein-DNA complex subunit organization

A

Interferon regulated genes (IUGs)

B

C ORF6 v Control (GFP)

Interferon Upregulated
Genes (IUGs) (134)
IFN-β vs untreated

A

B