

1 **The P681H mutation in the Spike glycoprotein confers Type I interferon**
2 **resistance in the SARS-CoV-2 alpha (B.1.1.7) variant**

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20 **SUMMARY**

21 **Variants of concern (VOCs) of severe acute respiratory syndrome coronavirus**
22 **type-2 (SARS-CoV-2) threaten the global response to the COVID-19 pandemic.**
23 **The alpha (B.1.1.7) variant appeared in the UK became dominant in Europe and**
24 **North America in early 2021. The Spike glycoprotein of alpha has acquired a**
25 **number mutations including the P681H mutation in the polybasic cleavage site**
26 **that has been suggested to enhance Spike cleavage. Here, we show that the**
27 **alpha Spike protein confers a level of resistance to the effects of interferon- β**
28 **(IFN β) in lung epithelial cells. This correlates with resistance to restriction**
29 **mediated by interferon-induced transmembrane protein-2 (IFITM2) and a**
30 **pronounced infection enhancement by IFITM3. Furthermore, the P681H**
31 **mutation is necessary for comparative resistance to IFN β in a molecularly**
32 **cloned SARS-CoV-2 encoding alpha Spike. Overall, we suggest that in**
33 **addition to adaptive immune escape, mutations associated with VOCs also**
34 **confer replication advantage through adaptation to resist innate immunity.**

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38 INTRODUCTION

39 Both SARS-CoV-1 and SARS-CoV-2 enter target cells through the interaction of their
40 Spike proteins with the angiotensin converting enzyme 2 (ACE2) cell surface
41 receptor. Upon attachment and uptake, the Spike glycoprotein trimer is cleaved by
42 cellular proteases such as cathepsins and TMPRSS family members at two positions
43 – the S1/S2 junction and the S2' site – to facilitate the activation of the fusion
44 mechanism. Similar to more distantly related beta-CoVs, but so far unique in known
45 Sarbecoviruses, the SARS-CoV-2 glycoprotein contains a polybasic furin cleavage
46 site (FCS) with a (681-PRRAR-685) sequence at the S1/S2 junction. This allows the
47 Spike precursor to be processed to the S1 and S2 subunits by furin-like proteases
48 before viral release from the previously infected cell(Hoffmann et al., 2020). This
49 leads to a proportion of processed Spikes to be present on the virion before
50 engagement with the target cell, allowing for rapid activation and fusion at or near
51 the cell surface by TMPRSS2. The importance of the FCS is highlighted by the
52 observations that it enhances SARS-CoV-2 replication specifically in airway epithelial
53 cells and is essential for efficient transmission in animal models (Peacock et al.,
54 2021a).

55

56 The alpha variant of SARS-CoV-2 arose in the South-East of England in autumn
57 2020, and rapidly spread across the world in the first months of 2021. Various
58 studies suggested that alpha had an increased transmissibility between
59 individuals(Lindstrom et al., 2021; Mok et al., 2021; Tanaka et al., 2021). Alpha
60 contains nine amino acid residue changes in Spike including a deletion of amino acid
61 residues H and V in the N-terminal domain at position 69/70, thought to increase
62 Spike incorporation into virions, a single amino acid deletion of Y144 (thought to

63 assist NTD antibody neutralization escape), and a N501Y mutation in the RBD which
64 enhances ACE2 binding affinity (Meng et al., 2021),(Chi et al., 2020). Together these
65 changes have been shown to reduce efficiency of neutralization by some
66 antibodies(Graham et al., 2021). Alpha also acquired a P681H change in the FCS
67 which has been shown to increase the accessibility of the site by furin leading to
68 enhanced cleavage(Mohammad et al., 2021),(Zhang et al., 2021). The alpha variant
69 Spike has also recently been reported to mediate more efficient cell-to-cell fusion
70 and syncytia formation (Michael Rajah et al., 2021),(Sanches et al., 2021).

71

72 We and others have previously found that SARS-CoV-2 is variably sensitive to entry
73 inhibition by the interferon-regulated IFITM family (Winstone et al., 2021),(Shi et al.,
74 2021). the three members of the family form multimeric complexes and have antiviral
75 activity against diverse enveloped viruses by blocking fusion of the viral and cellular
76 membranes (Bailey et al., 2014; Shi et al., 2017). While IFITM1 localizes primarily to
77 the plasma membrane, IFITM2 and IFITM3 are internalized via a conserved YxxΦ
78 endocytic motif to occupy distinct and overlapping endosomal compartments(Jia et
79 al., 2012; Jia et al., 2014). The sensitivity of a given virus to individual IFITM proteins
80 is largely determined by their route of cellular entry. We have previously shown that
81 for a prototypic Wuhan-like SARS-CoV-2 isolate from early 2020, IFITM2 reduced
82 viral entry and contributed to type I interferon (IFN-I)-induced inhibition in human
83 cells(Winstone et al., 2021). Sensitivity to IFITM2 could be markedly enhanced by
84 deletion of the FCS, suggesting that furin processing ameliorated SARS-CoV-2
85 sensitivity to IFITM2-restriction at least to some extent. We therefore postulated that
86 the altered cleavage site of alpha may have consequences for its sensitivity to IFN-I
87 and IFITMs. Here, we demonstrate that the Spike of the alpha variant is less

88 sensitive to restriction by IFN β and IFITMs in A549-ACE2 and Calu-3 cells.
89 Furthermore, this resistance correlates with the enhanced polybasic site as reversion
90 of this cleavage site increases the alpha variant's sensitivity to IFITM restriction.
91 Finally, we demonstrate that the H681P reversion in the full-length virus confers
92 IFN β sensitivity to alpha and suggest that part of this phenotype is driven by IFITMs.

93

94 **RESULTS**

95 **The Spike proteins of currently circulating variants display differing** 96 **sensitivities to IFITMs in A549-ACE2 cells**

97 Previously we have shown that viral entry mediated by the original Wuhan-1 Spike
98 pseudotyped lentiviral particle (PLV) or the England 02 isolate (hCoV-
99 19/England/02/2020) was inhibited by IFITM2 in A549-ACE2 cells, and that this
100 effect correlated in part with the IFN β sensitivity of the virus(Winstone et al., 2021).
101 Over 2020 and 2021 several major variants of concern (VOCs) have arisen – alpha
102 (B.1.1.7) in the UK, beta (B.1.351) in South Africa, gamma (P1) in Brazil, and delta
103 (B.1.617.2) in India. We wanted to compare the sensitivity of viral entry of the alpha,
104 beta, gamma and delta to the presence of IFITM proteins, given that these variants
105 have several changes in the Spike sequence (Figure 1A). Initially, PLVs bearing the
106 spike protein of each variant to test whether they were restricted by IFITMs
107 overexpressed on A549-ACE2 cells (Figure 1B-G). First, we confirmed that the
108 D614G mutation in the parental Wuhan-1 Spike that became prevalent in the first
109 wave of the pandemic displays a similar IFITM phenotype to the previously
110 characterised SARS-CoV-2 Spike (Wuhan-1) (Figure 1B, 1C)(Winstone et al., 2021).
111 The addition of D614G to the Wuhan-1 Spike had no effect on IFITM1 and IFITM2
112 sensitivity of PLV entry, while we observe a slight enhancement in the presence of

113 IFITM3. We next compared the IFITM sensitivities of the major global VOCs. The
114 alpha Spike appeared completely insensitive to IFITMs 1, 2 or 3 whilst beta, gamma
115 and delta still retain some sensitivity to IFITMs 1 and/or 2 (Figure 1B-G). None of the
116 VOCs were restricted by IFITM3 and, interestingly, we noted that IFITM3 appeared
117 to markedly enhance entry mediated by the alpha variant Spike. Next, we pre-treated
118 A549-ACE2-IFITM3 cells with cyclosporin H as this compound is known to drive
119 IFITM3 to degradation (Petrillo et al., 2018), and showed that this led to a specific
120 abolishment of the enhanced infection by alpha PLVs (Supplemental Figure 1).
121 Henceforth, we selected alpha to further investigate and determine the mechanism
122 of IFITM resistance.

123 Next, to confirm whether IFITM sensitivity results seen with the alpha spike
124 PLVs could be recapitulated with the full-length virus (Figure 2A), we infected A549-
125 ACE2 cells (stably expressing IFITMs or not) with either the Wuhan-like England/02
126 isolate or alpha at an MOI of 0.01 and measured infection by qPCR of E copies in
127 the infected cells 48 hours later (Figure 2B). As expected, replication of England/02
128 was significantly reduced in IFITM2 expressing cells over 48h. By contrast alpha
129 replicated as well in IFITM2-expressing cells and to a significantly higher level in
130 IFITM3-expressing cells in comparison to the control A549-ACE2 cells. This
131 confirmed that alpha SARS-CoV-2 is resistant to the effects of IFITMs 1 and 2 and
132 enhanced by IFITM3 on viral entry. Previous reports have suggested that the alpha
133 spike is more efficiently cleaved than Wuhan-like isolates due to the presence of the
134 P681H mutation optimising the accessibility of the FCS (Mohammad et al., 2021;
135 Zhang et al., 2021) We immunoblotted our viral stocks of England-02 and alpha and
136 confirmed that the alpha has more processed spike on the virions (Figure 2C, right
137 panel), although it was not as clearly discernible a difference in processing on PLVs

138 (Figure 2C, left panel). However, PLV infection in A549-ACE2 cells pre-treated with
139 E64D, a cathepsin inhibitor, that inhibits the entry of SARS-CoV-2 in endosomal
140 compartments because it cleaves S1/S2 junctions that have not been processed by
141 furin. This showed that the alpha variant Spike mediated entry of PLVs is markedly
142 less sensitive to this endosomal protease inhibition, when compared with the
143 Wuhan-1/D614G Spike, indicating differences in the site of virus entry mediated
144 consistent with enhanced Spike processing by furin in the producer cell
145 (Supplementary Figure 2).

146 **The alpha variant is less sensitive to IFN β than a wave 1 isolate**

147 While previous data has indicated that the original Wuhan-like SARS-CoV-2 virus
148 can delay pattern recognition of viral RNA in target cells, its replication highly
149 sensitive to exogenous interferon treatment in culture, in part determined by
150 IFITM2(Jouvenet, 2021). Having confirmed that the alpha variant is resistant to
151 IFITMs expression when ectopically expressed in cells, we then tested if alpha is
152 also resistant to the effects of IFN β , as suggested(Guo et al., 2021; Thorne et al.,
153 2021). Indeed, we found from measuring supernatant viral RNA 48 hours after
154 infection of both A549-ACE2 cells and the human lung epithelial cell line Calu3,
155 which more faithfully represent target cells in the respiratory tract, that alpha is more
156 resistant than England/02 to pre-treatment with increasing doses of IFN β (Figure 3A,
157 3B). We have also confirmed this phenotype by measuring viral RNA in cell lysates,
158 and further extending these observations to two clinical isolates of alpha isolated
159 (clinical isolates 10 and 28; Figure 3C). Thus in comparison to a representative
160 example of Wuhan-1-like SARS-CoV-2, the alpha variant has a marked resistance
161 to type I interferon.

162 **The P681H mutation is necessary for conferring IFITM and IFN β resistance in**
163 **alpha**

164 Our previous data indicated IFITM sensitivity of SARS-CoV-2 Spike can be
165 increased by deleting the FCS. Given that alpha and delta Spikes have acquired
166 mutations at P681 to H or R respectively, and these enhance Spike cleavage to
167 S1/S2 (Supplementary figure 3), we hypothesized that P681H might be a
168 determinant of resistance to IFN and IFITM. First we confirmed that none of the other
169 individual alpha-Spike defining mutations were sufficient to confer IFITM resistance
170 to a Wuhan/D614G Spike in PLVs (Supplementary figure 4 A–F). By contrast a
171 P681H mutation in the Wuhan-1/D614G Spike was sufficient to abolish IFITM2-
172 mediated inhibition PLVs entry, but not to fully confer the IFITM3-mediated
173 enhancement phenotype (Figure 4A). As expected, deletion of the HRRRA cleavage
174 site in the alpha Spike conferred potent sensitivity to IFITM2 and reduced the level of
175 enhancement we showed with IFITM3. Finally, we reverted the mutation in alpha to
176 H681P and showed it gained IFITM2 sensitivity. Thus, the H681P mutation in the
177 alpha spike confers IFITM resistance consistent with its enhanced furin cleavage.
178 However delta bears a P681R mutation yet is not fully IFITM resistant (Figure 1G),
179 implying that the P681H phenotype may be context dependent. Indeed, we found
180 that combining the P681H mutation in the Wuhan spike with the deletion at position
181 69-70 in the NTD found in the alpha variant but not delta was sufficient to fully confer
182 an alpha spike phenotype to D614G (Supplementary figure 4G). This suggests that
183 the H681 confers IFITM resistance in the context of other adaptations in the alpha
184 Spike that are thought to affect the conformation of S1 and the interaction with
185 ACE2.

186 Having established that P681H change is necessary for the resistance of the
187 alpha variant Spike to IFITMs, we next wanted to address if this was also a
188 determinant for the resistance to type-I IFN resistance of the virus itself. We
189 constructed a recombinant molecular clone of SARS-CoV-2 Wuhan-1 encoding
190 Spike from the alpha variant. This virus essentially mimicked the resistance of the
191 alpha variant itself to IFN β in comparison to a representative Wuhan-1 like virus,
192 England-02, demonstrating that the alpha Spike alone is sufficient to confer a level
193 type I IFN resistance in A549-ACE2 cells (Figure 4B). We then took this recombinant
194 virus and reverted the amino acid residue H681 to a proline. Importantly, this single
195 point mutation was sufficient to confer a significant sensitivity to IFN β in Calu3 cells
196 (Figure 4C). Lastly, we wanted to confirm whether siRNA knockdown of IFITM2 was
197 sufficient to rescue the IFN sensitivity of the Wuhan(B.1.1.7 Spike H681P) virus. We
198 therefore knocked down IFITM2 expression using siRNA in A549-ACE2 cells and
199 treated the cells with IFN β as before. We found that the H681P reverted virus was
200 rescued from IFN β restriction during IFITM2 knockdown, meanwhile the
201 Wuhan(B.1.1.7 Spike) virus was unaffected, consistent with this virus being resistant
202 to IFITM2 restriction. Thus, this confirmed that the Spike protein of the alpha variant
203 of SARS-CoV-2 is a determinant of type-I IFN resistance and that the P681H
204 mutation is necessary for this.

205

206 **DISCUSSION**

207 Here we have shown that the Spike protein of the alpha variant of SARS-CoV-2 is
208 resistant to IFN-I. Furthermore, we show that this maps to the histidine residue
209 adjacent to the FCS that has been mutated from the parental proline, which has
210 been shown to enhance Spike cleavage at the S1/S2 boundary (Peacock et al.,

211 2021b). This residue is necessary to confer resistance to IFITM2 and enhancement
212 by IFITM3, and as we demonstrated in our previous study(Winstone et al., 2021),
213 confirms that the FCS in Spike modulates IFITM entry restriction. Changes in the
214 FCS would be predicted to increase the efficiency of viral fusion and entry at or near
215 the plasma membrane, avoiding endosomal compartments where IFITMs 2 and 3
216 predominantly reside. Consistent with this, we show that the alpha Spike, as a PLV,
217 is less sensitive to the cathepsin inhibitor E64D. Thus we propose that these
218 changes in the alpha Spike have, in part, arisen to resist innate immunity.

219 At least two preprints suggest that variants of SARS-CoV-2 have begun to
220 evolve further resistance to interferon-induced innate immunity (Guo et al., 2021;
221 Thorne et al., 2021). In one, viral isolates over the pandemic show a reduced
222 sensitivity to type I interferons in culture(Guo et al., 2021); in a second the alpha
223 variant has a significantly reduced propensity to trigger pattern recognition in
224 epithelial cells(Thorne et al., 2021). In contrast, another study shows no difference in
225 IFN sensitivity of the new variants in African green monkey Vero-E6 cells(Michael
226 Rajah et al., 2021), although species-specificity in viral sensitivity to ISGs is a well
227 characterized trait. The SARS-CoV-2 genome contains multiple mechanisms to
228 counteract host innate immune responses, and much remains to be learned about
229 the mechanisms deployed by this virus and its relatives. While many reports on
230 SARS-CoV-2 evolution have naturally focussed on the pressing concern of potential
231 for vaccine escape, it is very unlikely that all selective adaptations that we see
232 arising in VOCs can be solely due to escape from adaptive immunity. The alpha
233 variant Spike, for example, only displays a minor reduction in sensitivity to
234 neutralizing antibodies (NAbs) (Graham et al., 2021; Mahase, 2021; Planas et al.,
235 2021; Shen et al., 2021). However, this VOC had a considerable transmission

236 advantage, with suspicions that it may have arisen in an immunocompromised
237 individual with a persistent infection giving ample time for changes to be selected
238 that further evade innate immunity, including those that target viral entry (Corey et al.,
239 2021; Kemp et al., 2021).

240 In terms of IFITM resistance of VOC Spike proteins, so far we have only seen
241 marked change in phenotype for the alpha variant. This is despite the fact that delta,
242 which has superseded alpha in many places around the world in 2021, also shows
243 an adaptation for enhanced S1/S2 cleavage with an P681R change (Liu et al., 2021;
244 Peacock et al., 2021b). This would suggest that efficient cleavage of S1/S2 is
245 necessary but not sufficient for IFITM resistance, and indeed our data implicate a
246 context dependency of the NTD deletion at 69/70. While unique to the alpha VOC,
247 the 69/70 deletion has been observed in persistent infection of immunosuppressed
248 individuals and is thought to enhance viral fitness and Spike stability (Meng et al.,
249 2021). While deletions in the NTD do affect NAb binding, it is primarily the 144
250 deletion in the alpha Spike that escapes neutralization by NTD-directed Nabs (Chi et
251 al., 2020) and we show that this has no impact on IFITM sensitivity. By contrast, the
252 more pronounced antibody evasion by the beta, gamma and delta variants is related
253 to mutations in the major neutralizing epitopes of the RBD, suggesting that they may
254 well have been driven by antibody escape (Planas et al., 2021; Zhou et al., 2021).
255 Viral glycoproteins are dynamic structures that shift through large-scale
256 conformational changes while interacting with their cognate receptors mediating viral
257 membrane fusion. Such context dependency is therefore likely to be complex and
258 will arise under competing selective pressures. Indeed, we have previously shown
259 that the HIV-1 envelope glycoprotein of transmitted viruses is IFITM insensitive and
260 this contributes to their overall type I IFN resistance (Foster et al., 2016). As HIV-1

261 infection progresses over the first 6 months in an infected person, the circulating
262 variants increase in IFN/IFITM sensitivity and this is determined by adaptive changes
263 in Env that resist the early neutralizing antibody response (Fenton-May et al., 2013).
264 Such escape has structural and functional implications for such dynamic proteins
265 that may impact upon receptor interactions and route of entry into the target cell.

266 While furin cleavage of the SARS-CoV-2 Spike reduces its IFITM sensitivity,
267 other interferon-induced proteins may contribute to this phenotype. The guanylate
268 binding protein family, and particularly GBP2 and GBP5, have been shown to have a
269 general antiviral activity against enveloped viruses by dysregulating furin processing
270 of diverse viral and cellular proteins (Braun et al., 2019). Similarly, IFITM
271 overexpression in HIV-infected cells can lead to their incorporation into virions and in
272 some cases promote defects in glycoprotein incorporation (Tartour et al., 2014).
273 Future studies will confirm whether either of these mechanisms are involved in the
274 IFN-resistance associated with the P681H mutation in alpha.

275 In summary, the spike protein of SARS-CoV-2 alpha increases resistance to
276 IFN-I and this correlates with the P681H mutation. Furthermore, this correlates with
277 resistance to IFITM-mediated entry restriction. This suggests that in addition to
278 adaptive immune escape, fixed mutations associated with VOCs may well also
279 confer replication and/or transmission advantage through adaptation to resist innate
280 immune mechanisms.

281

282

283 **MATERIALS AND METHODS**

284 **Cells and plasmids**

285 HEK293T-17 (ATCC, CRL-11268TM), Calu-3 (ATCC, HTB-55TM), A549-ACE2, Vero-
286 E6, Vero-E6-TMPRSS2 and A549-ACE2 expressing the individual IFITM proteins
287 were cultured in DMEM (Gibco) with 10% FBS (Invitrogen) and 200µg/ml Gentamicin
288 (Sigma), and incubated at 37°C, 5% CO₂. ACE2, TMPRSS2, and IFITM stable
289 overexpression cells were generated as previously described (Winstone et al.,
290 2021).

291 Codon optimised SARS-CoV-2 Wuhan Spike and ACE2 were kindly given by Dr.
292 Nigel Temperton. Codon optimised variant Spikes (B.1.1.7, B.1.351) were kindly
293 given by Dr. Katie Doores. Codon optimised variant Spikes (P1, B.617.2) were kindly
294 given by Professor Wendy Barclay. Plasmid containing TMPRSS2 gene was kindly
295 given by Dr. Caroline Goujon. Spike mutants were generated with Q5® Site-Directed
296 Mutagenesis Kit (E0554) following the manufacturer's instructions, and using the
297 following forward and reverse primers:

298 D614G (GCTGTACCAGGGCGTGAATTGCA, ACGGCCACCTGATTGCTG)
299 B.1.351. Δ242-244 (ATTTTCATATCTTACACCAGGC, ATGCAGGGTCTGGAATCTG)
300 D614G P681H (GACCAATAGCcacAGAAGAGCCAGAAGC,
301 TGGGTCTGGTAGCTGGCG)
302 B117 ΔHRRA (AGAAGCGTGGCCAGCCAG, GCTATTGGTCTGGGTCTGGTAG)
303 B117 H681P (GACCAATAGCcccAGAAGAGCCAG, TGGGTCTGGTAGCTGGCG)
304 Δ144 (CATAAGAACAACAAGAGC, ATAAACACCCAGGAAAGG), N501Y
305 (CCAGCCTACctacGGCGTGGGCT, AAGCCGTAGCTCTGCAGAG), E484K
306 (TAATGGCGTGAACGGCTTCAATTGCTACTT, CACGGTGTGCTGCCGGCC).

307 A549 stable cell lines expressing ACE2 (pMIGR1-puro), and IFITMs (pLHCX) were
308 generated and selected as described previously (Winstone et al., 2021).

309 **Production of Pseudotyped Lentiviral Vectors (PLVs) and infection**

310 HEK293T-17 were transfected with firefly luciferase expressing vector (CSXW), HIV
311 gag-pol (8.91) and Spike plasmid with PEI-max as previously described (Winstone et
312 al., 2021). Viral supernatant was then used to transduce each cell line of interest and
313 readout measured by Luciferase activity 48 hours later (Promega Steady-Glo®
314 (E2550)).

315 **Cyclosporin H assay**

316 Cells were pre-treated with 30µM of Cyclosporin H (Sigma, SML1575) for 18 hours.
317 Cells were then infected with PLVs and viral entry quantified by Luciferase activity 48
318 hours later.

319 **Passage and titration of SARS-CoV-2**

320 PHE England strain 02/2020 was propagated in Vero-E6-TMPRSS2 cells and titre
321 was determined by plaque assay (Winstone et al., 2021). Vero-E6-TMPRSS2 were
322 infected with serial dilutions of SARS-CoV-2 for 1h. Subsequently, 2X overlay media
323 (DMEM + 2% FBS + 0.1% agarose) was added, and infected cells were fixed 72
324 hours after infection and stained with Crystal Violet. Plaques were counted and
325 multiplicity of infection calculated for subsequent experiments. A replication-
326 competent alpha variant was kindly provided by Professor Wendy Barclay (Imperial
327 College London)(Brown et al., 2021). All virus stocks were sequence confirmed in
328 the Spike gene at each passage to ensure no loss of the FCS.

329 **Generation of recombinant full-length viruses**

330 We used the previously described Transformation-Associated Recombination
331 (TAR) in yeast method(Thi Nhu Thao et al., 2020), with some modifications, to
332 generate the mutant viruses described in this study. Briefly, a set of overlapping
333 cDNA fragments representing the entire genomes of SARS-CoV-2 Wuhan isolate
334 (GenBank: MN908947.3) and the B.1.1.7 alpha variant were chemically synthesized
335 and cloned into pUC57-Kan (Bio Basic Canada Inc and Genewiz, respectively). The
336 cDNA fragment representing the 5' terminus of the viral genome contained the
337 bacteriophage T7 RNA polymerase promoter preceded by a short sequence stretch
338 homologous to the *XhoI*-cut end of the TAR in yeast vector pEB2(Gaida et al., 2011).
339 The fragment representing the 3' terminus contained the T7 RNA polymerase
340 termination sequences followed by a short segment homologous to the *Bam*HI-cut
341 end of pEB2.

342 To generate Wuhan virus carrying the alpha variant spike, a mixture of the
343 relevant synthetic cDNA fragments of the Wuhan and alpha variants was co-
344 transformed with *XhoI*-*Bam*HI-cut pEB2 into the *Saccharomyces cerevisiae* strain
345 TYC1 (MATa, ura3-52, leu2 Δ 1, cyh2^r, containing a knockout of DNA Ligase 4)
346 (Gaida et al., 2011) that had been made competent for DNA uptake using the LiCl₂-
347 based Yeast transformation kit (YEAST1-1KT, Merck). The transformed cells were
348 plated on minimal synthetic defined (SD) agar medium lacking uracil (Ura) but
349 containing 0.002% (w/v) cycloheximide to prevent selection of cells carrying the
350 empty vector. Following incubation at 30°C for 4 to 5 days, colonies of the yeast
351 transformants were screened by PCR using specific primers to identify those
352 carrying plasmid with fully assembled genomes. Selected positive colonies were
353 then expanded to grow in 200 ml SD-Ura dropout medium and the plasmid
354 extracted. Approximately 4 μ g of the extracted material was then used as template to

355 *in vitro* synthesized viral genomic RNA transcripts using the Ribomax T7 RNA
356 transcription Kit (Promega) and Ribo m7G Cap Analogue (Promega) as per the
357 manufacturer's protocol. Approximately 2.5 µg of the *in vitro* synthesized RNA was
358 used to transfect ~6 x10⁵ BHK-hACE2-N cells stably expressing the SARS-CoV-2 N
359 and the human ACE2 genes(Rihn et al., 2021) using the MessengerMax lipofection
360 kit (Thermo Scientific) as per the manufacturer's instructions. Cells were then
361 incubated until signs of viral replication (syncytia formation) became visible (usually
362 after 2-3 days), at which time the medium was collected (P0 stock) and used further
363 as a source of rescued virus to infect VERO E6 cells to generate P1 and P2 stocks.
364 Full genome sequences of viruses collected from from P0 and P1 stocks were
365 obtained in order to confirm the presence of the desired mutations and exclude the
366 presence of other spurious mutations. Viruses were sequenced using Oxford
367 Nanopore as previously described(da Silva Filipe et al., 2021).

368 To generate Wuhan virus carrying alpha spike gene with the H681P mutation,
369 we first introduced this mutation into the relevant alpha variant cDNA fragment by
370 site-directed mutagenesis. This fragment was combined with those described above
371 and the mixture was then used to generate plasmid pEB2 carrying the cDNA
372 genome of Wuhan encoding the alpha spike H681P by the TAR in yeast procedure.
373 The virus rescue and subsequent characterisation were performed as described
374 above.

375 **Generation of Clinical Viral Isolates**

376 Viruses were isolated on Vero.E6 cells (ATCC CRL 1586™) from combined naso-
377 oropharyngeal swabs submitted for routine diagnostic testing by real-time RT-PCR
378 and shown to be from the B.1.1.7 (alpha) variant by on-site whole-genome
379 sequencing (Oxford Nanopore Technologies, Oxford, UK) (Pickering et al., 2021).

380 Infected cells were cultured at 37°C and 5% CO₂, in Dulbecco's modified Eagle's
381 medium (DMEM, Gibco™, Thermo Fisher, UK) supplemented with 2% foetal bovine
382 serum (FBS, Merck, Germany), pen/strep and amphotericin B.

383 All work performed with full-length SARS-CoV-2 preparations, as well as isolation
384 and propagation of viral isolates from swabs, was conducted inside a class II
385 microbiological safety cabinet in a biosafety level 3 (BSL3) facility at King's College
386 London.

387 **Infection with replication competent SARS-CoV-2**

388 1.5×10^5 A549-ACE2 cells were infected for 1 hour at 37°C with SARS-CoV-2
389 replication competent viruses at MOI 0.01 or 500 E gene mRNA copies/cell. 2×10^5
390 Calu-3 cells were infected for 1h at 37°C with SARS-CoV-2 replication competent
391 viruses at 5000 copies/cell. Media was replaced and cells were incubated for 48
392 hours at 37°C, after which cells or supernatant were harvested for RNA extraction or
393 protein analysis.

394 **Interferon assays**

395 Cells were treated with different doses of IFN β (PBL Assay Science, 11415-1) for 18
396 hours prior infection. The following day media was replaced, and the infection
397 performed as described above. Viral RNA levels in cells or supernatants were
398 measured 48 hours after infection by RT-qPCR .

399 **siRNA knockdown of IFITM2**

400 A549-ACE2 cells were reverse transfected using 20pmol of Non-targeting siRNA (D-
401 001206-13-20) or IFITM2 siRNA (M-020103-02-0010) and 1 μ L of RNAi max
402 (Invitrogen). Cells were incubated for 24h prior to a second round of reverse

403 transfection. 8h later, cells were treated with different doses of IFN β . Following 18h
404 of IFN treatment cells were infected with full-length viruses as previously described.

405 **RT-qPCR**

406 RNA from infected cells was extracted using QIAGEN RNeasy (QIAGEN RNeasy
407 Mini Kit, 74106) following the manufacturer's instructions. 1 μ L of each extracted
408 RNA was used to performed one step RT-qPCR using TaqMan Fast Virus 1-Step
409 Master Mix (Invitrogen). The relative quantities of envelope (E) gene were measured
410 using SARS-CoV-2 (2019-nCoV) CDC qPCR Probe Assay (IDT DNA technologies).
411 Relative quantities of E gene were normalised to GAPDH mRNA levels (Applied
412 Bioscience, Hs99999905_m1).

413 Supernatant RNA was extracted using RNAAdvance Viral XP (Beckman) following the
414 manufacturer's instructions. 5 μ L of each RNA was used for one-step RT-qPCR
415 (TaqManTM Fast Virus 1-Step Master Mix) to measured relative quantities of E and
416 calibrated to a standard curve of E kindly provided by Professor Wendy Barclay.

417 **SDS-PAGE and Western blotting**

418 Cellular samples were lysed in reducing Laemmli buffer at 95 $^{\circ}$ C for 10 minutes.
419 Supernatant or viral stock samples were centrifuged at 18,000 RCF through a 20%
420 sucrose cushion for 1 hour at 4 $^{\circ}$ C prior to lysis in reducing Laemmli buffer. Samples
421 were separated on 8–16 % Mini-PROTEAN[®] TGXTM Precast gels (Bio-Rad) and
422 transferred onto nitrocellulose membrane. Membranes were blocked in milk prior to
423 detection with specific antibodies: 1:1000 ACE2 rabbit (Abcam, Ab108209), 1:5000
424 GAPDH rabbit (Abcam, Ab9485), 1:5000 HSP90 mouse (Genetex, Gtx109753), 1:50
425 HIV-1 p24Gag mouse (48 ref before) 1:1000 Spike mouse (Genetex, Gtx632604),

426 1:1000 anti-SARS-CoV-2 N rabbit (GeneTex, GTX135357). Proteins were detected
427 using LI-COR and ImageQuant LAS 4000 cameras.

428 **Ethics**

429 Clinical samples were retrieved by the direct care team in the Directorate of
430 Infection, at St Thomas Hospital, London, UK, and anonymised before sending to the
431 King's College London laboratories for virus isolation and propagation. Sample
432 collection and studies were performed in accordance with the UK Policy Framework
433 for Health and Social Care Research and with specific Research Ethics Committee
434 approval (REC 20/SC/0310).

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453 **AUTHORS CONTRIBUTION**

454 Experiments were performed by MJL, HW, HDW and AD. SP, RPG, LS and GN
455 collected, sequenced and isolated clinical viral isolates. MP, AHP, GDL, VMC, WF,
456 NS, and RO generated reverse genetics-derived viruses. MJL, HW, HDW and AD
457 analysed data. CMS provided reagents, funding support and advice. HW, MJL and
458 SJDN analysed the data and wrote the manuscript. All authors edited the manuscript
459 and provided comments.

460

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596 **FIGURE LEGENDS**

597 **Figure 1. IFITM sensitivity of SARS-CoV-2 variants of concern.** A) Schematic of
598 Spike protein domains of the different variants of concern relative to the original
599 Wuhan Spike sequence: alpha, beta, gamma and delta. The different mutations
600 between the variants are represented in red. B-G) IFITM sensitivity of Wuhan,
601 D614G, alpha, beta, gamma and delta PLVs in A549-ACE2 cells stably expressing
602 the individual IFITMs. PLV entry was quantified by Luciferase activity 48 hours after
603 infection and normalized to control cells. Data shown are mean \pm SEM, n=3.
604 Statistics were calculated in Prism using *t*-test, stars indicate significance between
605 control cell and individual IFITM (*P=0.05).

606

607 **Figure 2. The alpha variant of SARS-CoV-2 is resistant to IFITMs.** A) D614G and
608 Alpha PLVs infection of A549-ACE2 cells stably expressing the individual IFITMs.
609 Infection was quantified by Luciferase activity 48 hours later and normalized to
610 control cells. Data shown are mean \pm SEM, n=3. Statistics were calculated in Prism
611 using *t*-test, stars indicate significance between control cell and individual IFITM
612 (*P=<0.05). B) Infection of A549-ACE2 stably expressing the individual IFITMs with
613 England 02 and alpha full-length viruses at MOI 0.01. Infection was quantified by RT-
614 qPCR of E gene relative to GAPDH 48 hours later; graph represents E mRNA levels
615 relative to GAPDH. Data shown are mean \pm SEM, n=3. Statistics were calculated in
616 Prism using *t*-test, stars indicate significance between control cell and individual
617 IFITM (*P=<0.05). C) Western blot from representative D614G and alpha PLVs
618 produced in HEK293T/17 cells, and virions from full-length England-02 and alpha
619 viruses. Virions were purified through a 20% sucrose gradient.

620

621 **Figure 3. The alpha variant is resistant to IFN β .** A) England 02 and alpha full-
622 length virus infection in A549-ACE2 cells pre-treated with IFN β . Cells were pre-
623 treated with increasing concentrations of IFN β for 18 hours prior to infection with
624 either virus at 500 E mRNA copies/cell. Infection was quantified by RT-qPCR of E
625 mRNA from the supernatant 48 hours later and normalised to the un-treated control.
626 Data shown are mean \pm SEM, n=3. Statistics were calculated in Prism using *t*-test,
627 stars indicate significance between the different viruses at individual IFN
628 concentrations (*P=<0.05). B) England 02 and alpha full-length virus infection in
629 Calu-3 cells pre-treated with IFN β . Cells were pre-treated with increasing
630 concentrations of IFN β for 18 hours prior infection with either virus at 5000 E
631 copies/cell. Infection was quantified by RT-qPCR of E mRNA from the supernatant
632 48 hours later and normalised to the un-treated control. Data shown are mean \pm
633 SEM, n=3. Statistics were calculated in Prism using *t*-test, stars indicate
634 significance between the different viruses at individual IFN concentrations
635 (*P=<0.05). C) England 02 and clinical isolates of alpha full-length virus infection in
636 Calu-3 cells pre-treated with IFN β and harvested as in A and B. Cells were pre-
637 treated with increasing concentrations of IFN β for 18 hours prior to infection with
638 either virus at 5000 E copies/cell. Infection was quantified by RT-qPCR of cellular E
639 mRNA relative to GAPDH 48 hours later and normalised to the un-treated control.
640 Data shown are mean \pm SEM, n=3. Statistics were calculated in Prism using *t*-test,
641 stars indicate significance between the different viruses at individual IFN
642 concentrations (*P=<0.05).

643

644 **Figure 4. The P681H mutation is necessary but not sufficient for IFITM**
645 **resistance, and necessary and sufficient for IFN β resistance.** A) D614G,

646 D614G- P681H, alpha, alpha-ΔHRRA, and alpha-H681P PLVs infection in A549-
647 ACE2 cells stably expressing the individual IFITMs. PLVs entry was quantified by
648 Luciferase activity 48 hours later and normalized to control cells. Data shown are
649 mean \pm SEM, n=3. Statistics were calculated in Prism using t-test, black stars
650 indicate significance relative to the control cells, red stars indicate significance
651 between alpha and alpha-H681P in IFITM2 cells (*P=<0.05). B) England 02 , alpha,
652 and Wuhan-alpha Spike full-length virus infection in A549-ACE2 cells pre-treated
653 with IFN β . Cells were pre-treated with increasing concentrations of IFN β for 18 hours
654 prior to infection with either virus at 500 E copies/cell. Infection was quantified by RT-
655 qPCR of E mRNA in the supernatant 48 hours later and normalised to the un-treated
656 control. Data shown are mean \pm SEM, n=3. Statistics were calculated in Prism using
657 t-test, stars indicate significance between the different viruses at individual IFN
658 concentrations (*P=<0.05). C) Wuhan(B.1.1.7 spike) and Wuhan(B1.1.7 spike
659 H681P) Spike full-length virus infection in Calu-3 cells pre-treated with IFN β . Cells
660 were pre-treated with increasing concentrations of IFN β for 18 hours prior to
661 infection with either virus at 5000 E copies/cell. Infection was quantified by RT-qPCR
662 of E mRNA in the supernatant 48 hours later and normalised to the un-treated
663 control. Data shown are mean \pm SEM, n=3. Statistics were calculated in Prism using
664 t-test, stars indicate significance between the different viruses at individual IFN
665 concentrations (*P=<0.05). D) A549-ACE2 cells were transfected with siRNAs
666 against non-targeting control or IFITM2 for 24 hours and then treated with IFN β for
667 18 hours prior to infection with Wuhan(B.1.1.7 spike) or Wuhan(B.1.1.7 spike
668 H681P) at 500 copies/cell. Infection was quantified by RT-qPCR of E gene relative to
669 GAPDH 48 hours later; graph represents E mRNA levels relative to GAPDH. Data
670 shown are mean \pm SEM, n=3. Statistics were calculated in Prism using t-test, stars

671 indicate significance between the different viruses at individual IFN concentrations
672 (* $P < 0.05$).

673

674 **Supplementary figure 1. Cyclosporin H treatment abolishes IFITM3**

675 **enhancement of alpha PLVs.** A) D614G PLVs pre-treated with Cyclosporin H.

676 A549-ACE2s stably expressing the individual IFITMs were pre-treated with 30 μ M of

677 Cyclosporin H for 18 hours prior to infection with D614G PLVs. Infection was

678 quantified by Luciferase activity 48 hours after infection and normalized to control

679 cells. Data shown are mean \pm SEM, $n=3$. Statistics were calculated in Prism using t -

680 test (* $P < 0.05$). B) A549-ACE2s stably expressing the individual IFITMs were pre-

681 treated with 30 μ M of Cyclosporin H for 18 hours prior to infection with alpha PLVs.

682 Infection was quantified by Luciferase activity 48 hours after infection and normalized

683 to control cells. Data shown are mean \pm SEM, $n=3$. Statistics were calculated in

684 Prism using t -test, stars indicate significance between IFITM3 mock and IFITM3

685 CsH (* $P < 0.05$).

686

687 **Supplementary figure 2. alpha PLVs are less sensitive to inhibition by E64D**

688 **than D614G PLVs.** A549-ACE2 cells were pre-treated with increasing

689 concentrations of E64D for 1 hour prior infection with D614G or alpha PLVs. PLV

690 entry was quantified by Luciferase activity 48 hours after infection and normalized to

691 control cells. Data shown are mean \pm SEM, $n=3$.

692

693 **Supplementary figure 3. Spike processing of full-length virus and cleavage site**

694 **PLVs mutants.** A) England-02, alpha, Wuhan(B.1.1.7 Spike), Wuhan(B.1.1.7 Spike

695 H681P) were purified through 20% sucrose and immunoblotted for Spike and N

696 proteins. B) PLVs expressing different Spike mutants were produced in HEK293T-17
697 cells and cell lysates and supernatant immunoblotted for gag and Spike. Supernatant
698 was purified through 20% sucrose.

699

700 **Supplementary figure 4. IFITM sensitivity of individual alpha Spike mutations.**

701 A–F) PLVs with individual alpha mutations were used to infect A549-ACE2 cells
702 stably expressing the individual IFITMs. Infection was quantified by Luciferase
703 activity 48 hours after infection and normalized to control cells. Data shown are
704 mean \pm SEM, n=3. Statistics were calculated in Prism using *t*-test, stars indicate
705 significance between each PLVs control cell and individual IFITM (*P=<0.05).

706

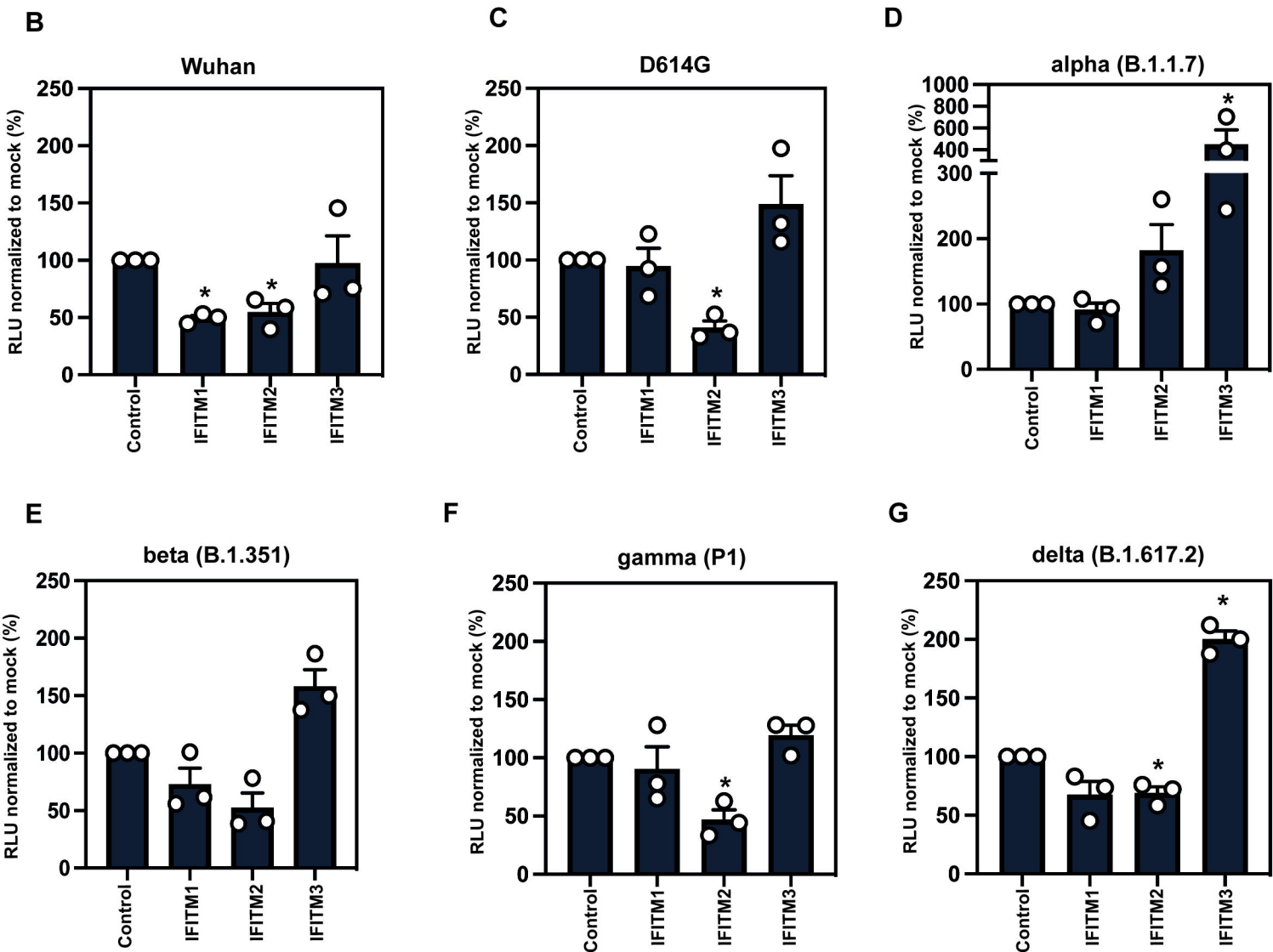
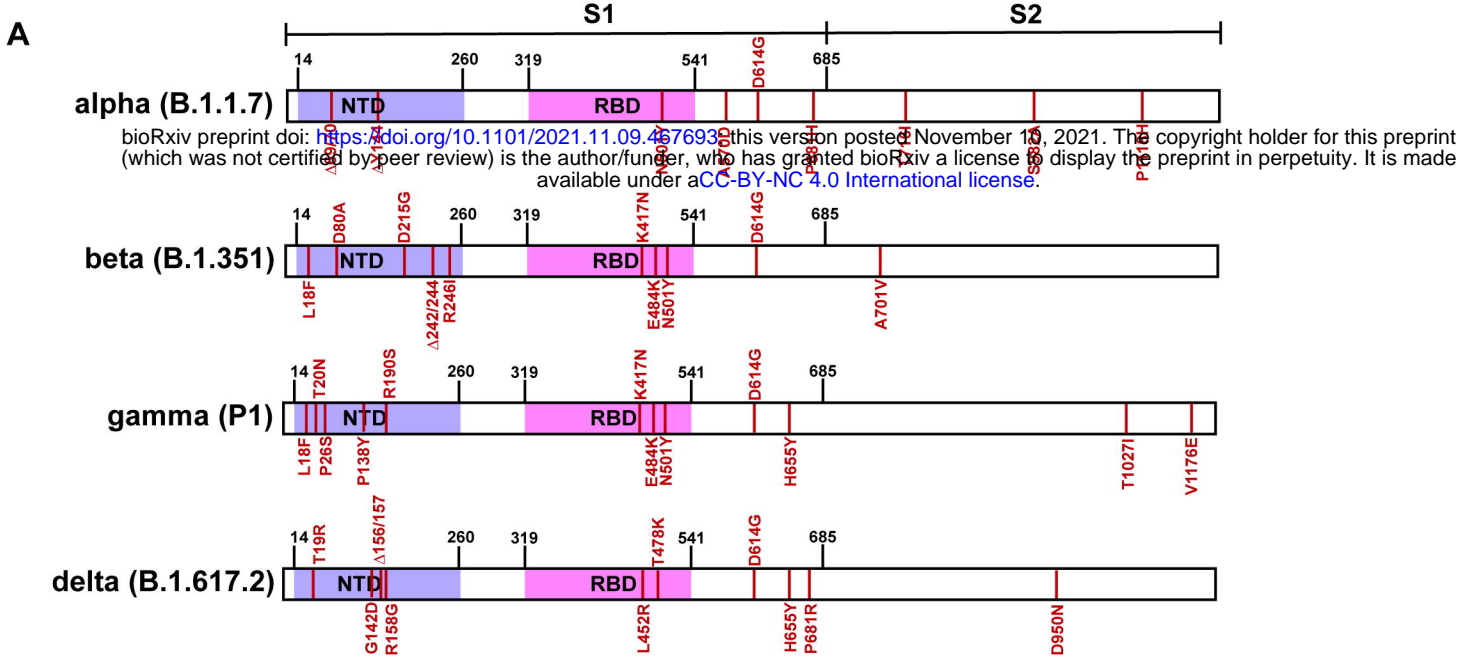


Figure 1

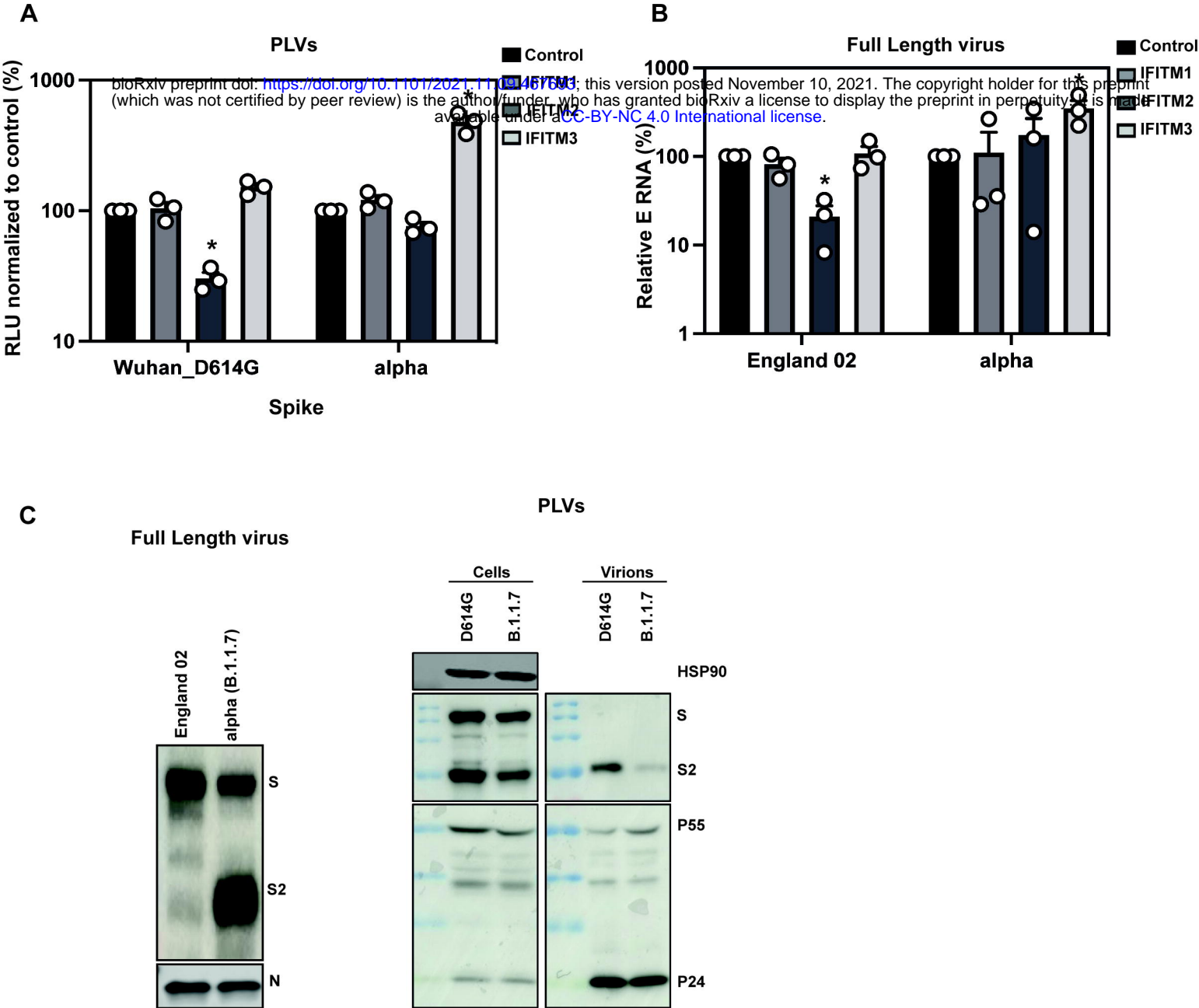


Figure 2

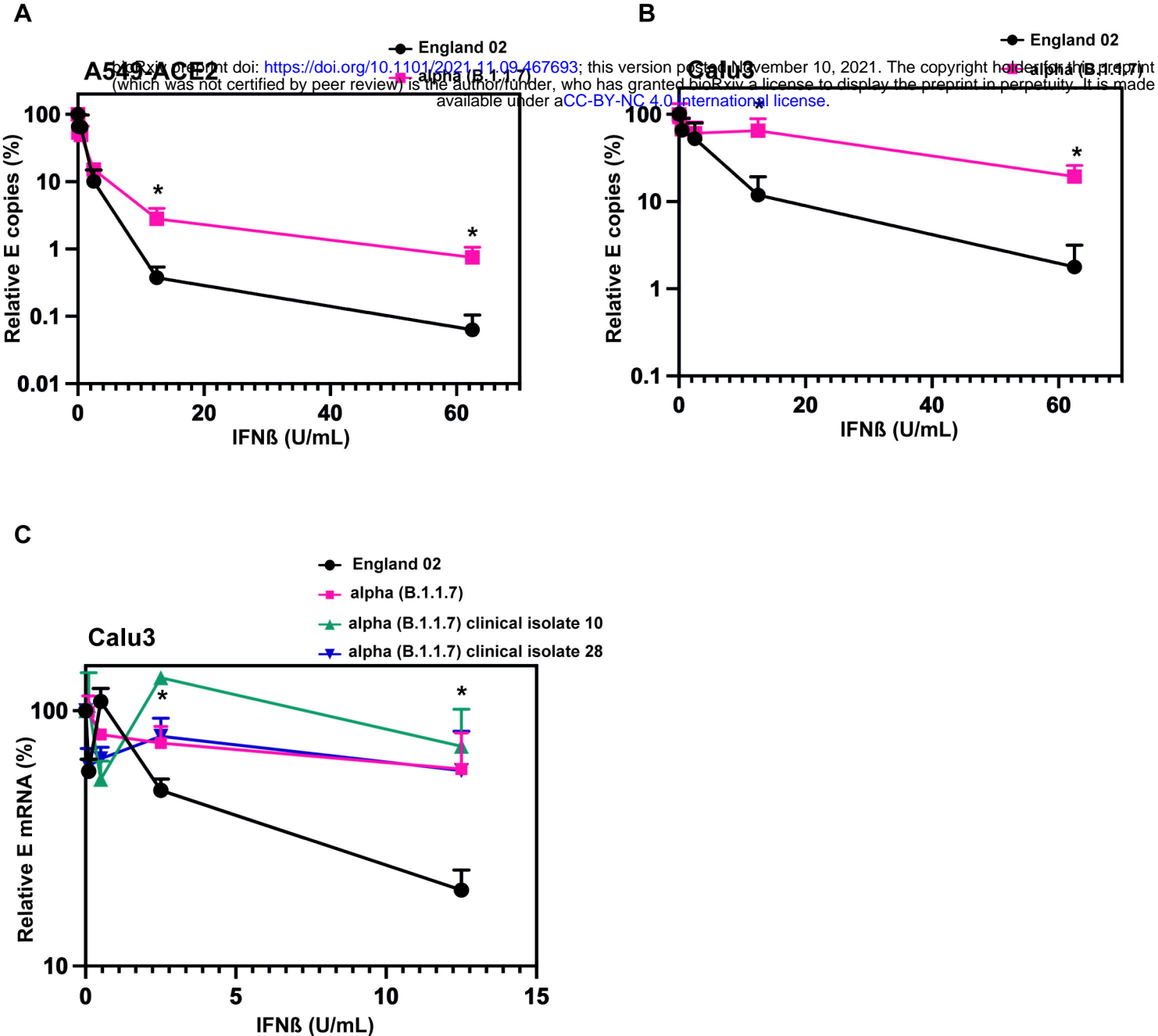


Figure 3

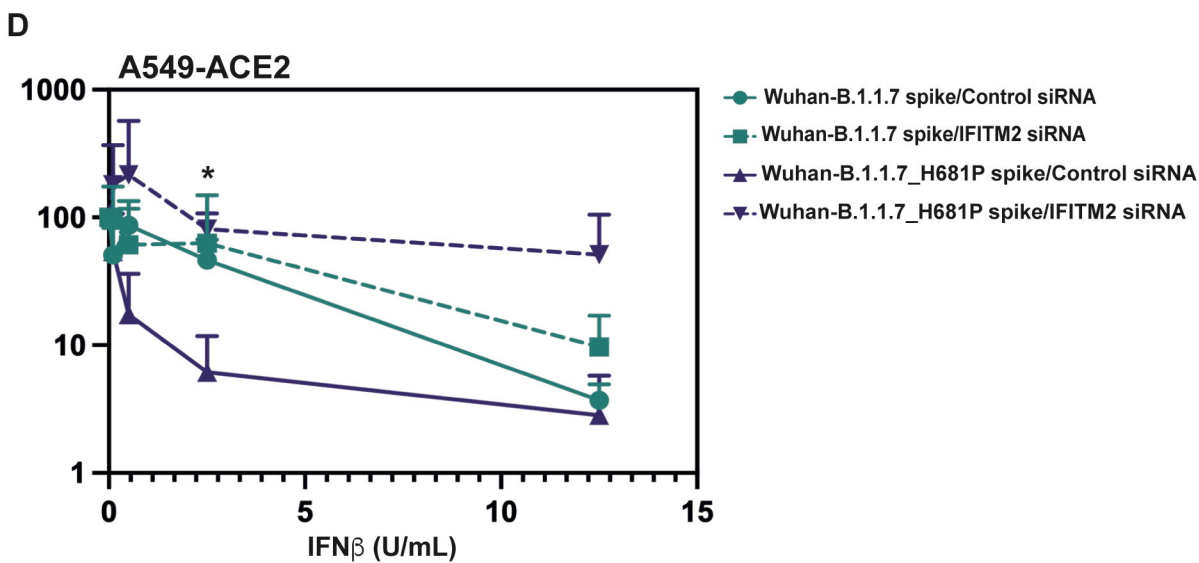
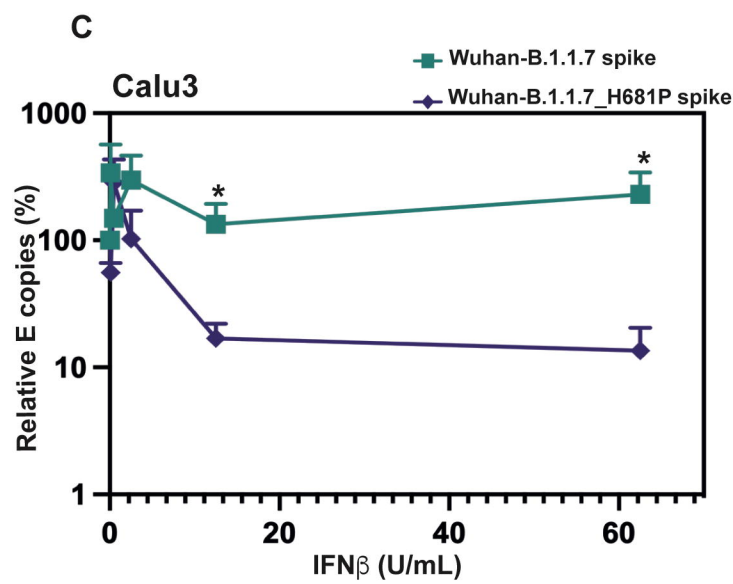
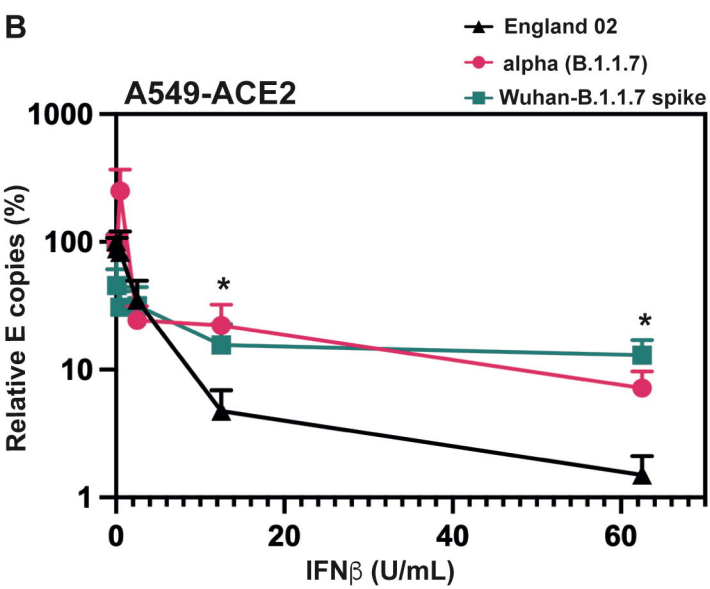
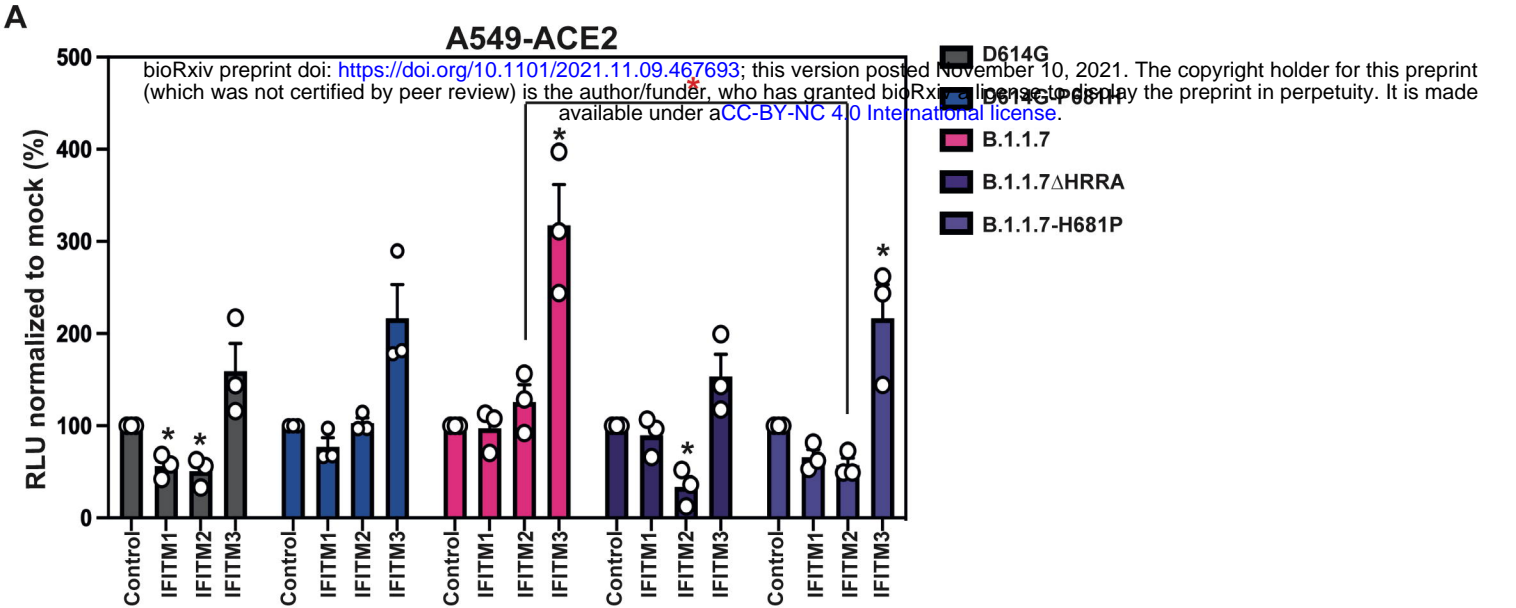
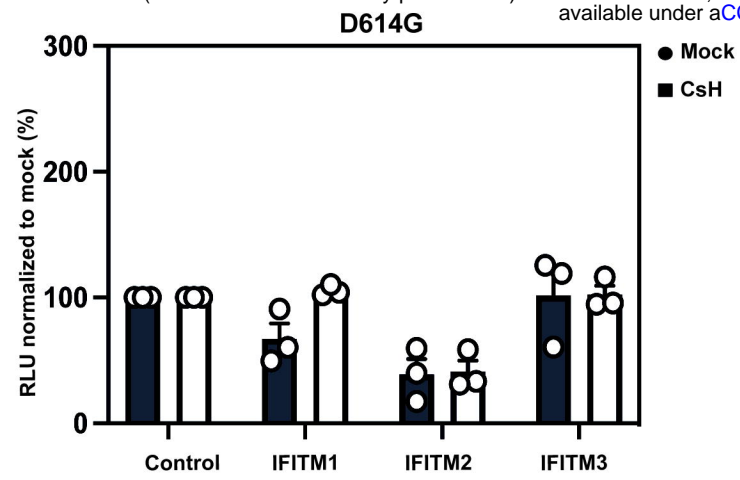
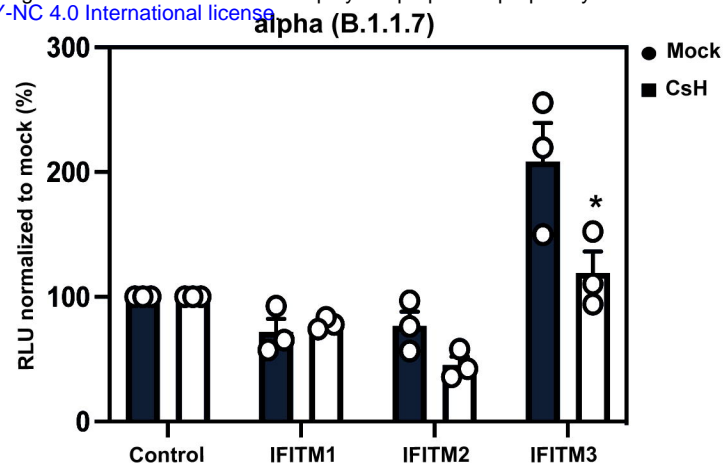


Figure 4

A

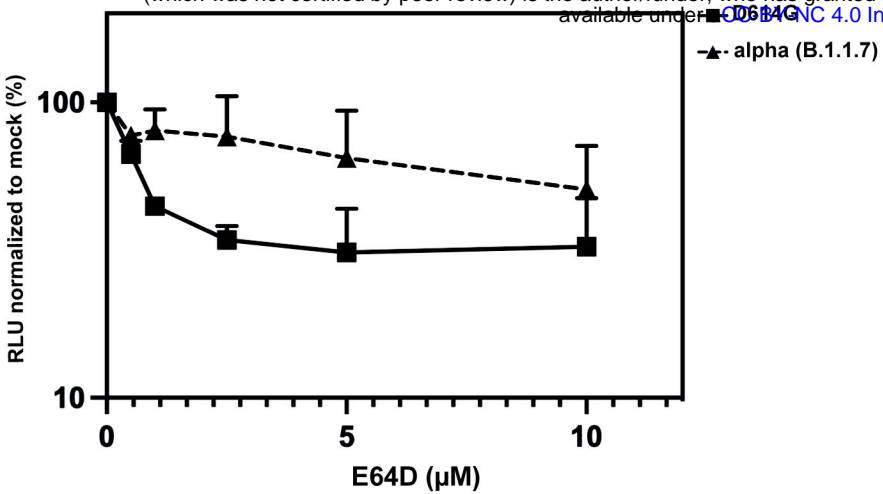


B



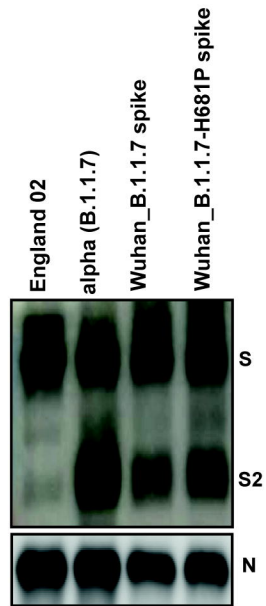
A

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**B**