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## **HCoV-229E spike protein fusion activation by trypsin-like serine proteases is mediated by proteolytic processing in the S2' region**

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1 **HCoV-229E spike protein fusion activation by trypsin-like serine proteases is mediated**  
2 **by proteolytic processing in the S2' region.**

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14

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17

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21

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25 The GenBank accession number for the France/Lille/2014 spike protein sequence is :  
26 MH048989

27

28 **Abstract**

29  
30 Human coronavirus 229E (HCoV-229E) is responsible for common colds. As other  
31 coronaviruses, HCoV-229E exploits cellular proteases to activate its fusion mediated by the  
32 spike protein. We analysed the proteolytic processing of the HCoV-229E spike protein by  
33 trypsin-like serine proteases leading to activation of the fusion process. Unlike other  
34 coronaviruses, HCoV-229E fusion activation appears to be a one step process. Indeed,  
35 cleavage of the S1/S2 interface does not seem to be a pre-requisite, and the fusion activation  
36 strongly relies on the S2' region, with arginine residue 683 acting as the recognition site.

37  
38 **Main text**

39  
40 *Coronavirinae* are enveloped virus with a long positive sense RNA genome. As for other  
41 enveloped viruses, they need to fuse their viral envelope with a host cell membrane to deliver  
42 their genome to the target cell. Coronavirus spike proteins are major mediators of virus entry  
43 and fulfil a dual function by mediating receptor binding and fusion. Spike proteins consist of  
44 two distinct domains carrying these functions. The amino-terminal subunit, called S1 domain,  
45 is responsible for receptor binding, whereas the C-terminal part, called S2 domain, contains  
46 the fusion machinery. Proteolytic priming of coronavirus spike protein is a key feature of the  
47 fusion process, and coronaviruses exploit a wide variety of host proteases including  
48 cathepsins, furin and members of the transmembrane serine protease (TTSP) family [1] to  
49 achieve this requirement. It is likely that the proteolytic processing differs according to the  
50 protease used. Depending on the cellular localization of the protease used to activate the  
51 fusion, entry can occur either at the cell surface or after internalization of the virus by  
52 endocytosis. For SARS-CoV spike protein, two cleavage events mediated by trypsin have  
53 been identified. The SARS-CoV virion harbors an uncleaved spike protein at its surface. First,  
54 the spike protein is primed by cleavage of the S1 and S2 domain, then to fully activate fusion,  
55 a second cleavage occurs at a position called S2' (R797) exposing the fusion peptide [2, 3].  
56 Activation of the SARS-CoV spike protein fusion by elastase also involves recognition of a  
57 cleavage site in the S2' region [4]. The importance of the S2' cleavage has also been  
58 demonstrated for MERS-CoV and MHV [5, 6].  
59 HCoV-229E was identified in 1966 in students suffering from common cold [7], but children,  
60 elderly and immune-compromised persons can develop a more severe disease [8]. It has been  
61 shown that HCoV-229E requires endosomal cathepsin L activity to infect Hela cells [9].  
62 However, Shirato et al. have recently shown that clinical isolates of HCoV-229E

63 preferentially uses the transmembrane protease serine 2 (TMPRSS2), a trypsin-like protease,  
64 rather than endosomal cathepsin for entry [10]. TMPRSS2 is known to be present at the  
65 surface of the human airway epithelia and is likely the main activator of HCoV-229E fusion  
66 *in vivo*. In addition, TMPRSS2-mediated entry at the cell surface protects the virus from the  
67 inhibitory effects of interferon-induced transmembrane proteins (IFITMs) known to inhibit  
68 entry of several enveloped virus [11]. However, the detailed proteolytic processing of HCoV-  
69 229E spike protein to activate its fusion remains to elucidate.

70 To investigate this proteolytic maturation, we first cloned the spike protein sequence from a  
71 patient infected by HCoV-229E hospitalized in Lille, France in 2014 (Spike  
72 France/Lille/2014, accession number MH048989). The codon-optimized sequence of the  
73 France/Lille/2014 spike protein was inserted into the pCDNA3.1(+) vector and fused with a  
74 sequence encoding a C-terminal VSVG tag to facilitate the detection of the protein in  
75 immunoblot. Then, we analysed the proteolytic processing of the spike protein required for  
76 entry mediated by trypsin/trypsin like protease, focusing on the role of the S1/S2 junction and  
77 the S2' region. The sequence of the French isolate spike protein shows 99% identity with the  
78 spike of the Niigata, 0349 and USA/932-72/1993 isolates and only 94% of identity with the  
79 VR-740 laboratory strain. HCoV-229E spike protein contains 3 arginine residues in the S2'  
80 region (R679, R683 and R687) which are conserved in different strains of HCoV-229E,  
81 except for R679 that is absent in the VR-740 laboratory strain (Fig. 1(a)). First, to confirm the  
82 role of the HCoV-229E spike protein S2' region in entry, we inserted a furin cleavage site  
83 (Fur687) in the spike protein (Fig.1(a)), at the N-terminal position of the potential fusion  
84 peptide. To analyse the effect of the mutation in the context of viral particles, we used a  
85 murine leukemia virus (MLV)-based pseudotyping system that produces particles inducing  
86 luciferase expression after transduction of permissive cells. Pseudoparticles fully mimic the  
87 functionality of the wild-type virus in terms of cell entry and were used because of their  
88 technical advantage. Huh-7 cells were inoculated with pseudoparticles harboring the wild type  
89 protein or the Fur687 mutant (Fig 1(b)). Neutralization of endosomal pH by ammonium  
90 chloride treatment inhibited transduction by HCoV-229E pseudoparticles showing that  
91 HCoV-229E infects Huh-7 cells via the endosomal route and uses low-pH active endosomal  
92 protease to mediate its fusion. As shown in Figure 1(b), pseudotyped virions with HCoV-  
93 229E spike protein containing a furin cleavage site at the S2' position were insensitive to  
94 endosomal pH neutralization, but were affected by furin convertase inhibitor. This result  
95 shows that the spike protein with the furin cleavage site does not rely on endosomal cathepsin  
96 for entry but relies on furin and this confirms the involvement of the S2' region for fusion

97 activation of HCoV-229E, as observed for other coronaviruses [2, 5, 6, 12]. It is interesting to  
98 note that the spike proteins incorporated on the pseudotyped virions were not cleaved by furin  
99 in producing cells, indicating that the protein is not processed during exit of the producer cell  
100 but during entry into the target cell (Fig 1(d)). This suggests that during exit and secretion, the  
101 cleavage site is inaccessible to furin and that during entry, conformational change induced by  
102 receptor binding may expose the cleavage site. To investigate the role of the S1/S2 junction  
103 and the specific role of the 3 arginine residues located in the S2' region (R679, R683 and  
104 R687) in the proteolytic activation of HCoV-229E spike protein by trypsin-like serine-  
105 proteases, the arginine residues present at S1/S2 and at S2' were mutated individually  
106 (R565N, R679N, R683N or R687N). We chose to mutate the arginine residues into  
107 asparagine to minimize the effect of the mutations on the conformation/folding of the protein.  
108 As seen in Fig 1(c), these arginine residues were not implicated in entry when the virions use  
109 the endocytic pathway to invade the cells. The endosomal route of entry of the different  
110 mutants was confirmed by inhibition of their entry by ammonium chloride treatment (Fig  
111 1(c)). Indeed, when HCoV-229E is internalized, the virus is dependent on the endosomal  
112 cathepsin L to mediate its fusion. However, unlike other proteases, cathepsin L has a limited  
113 prime-site specificity in terms of recognition sites and the precise sites of proteolysis by  
114 cathepsin L remains unclear.

115 In 2005, Simmons et al. showed that SARS-CoV fusion can be induced at the cell surface  
116 when virions are bound to the plasma membrane at 4°C and briefly treated by exogenous  
117 protease such as trypsin [13]. Particles pseudotyped with the different S1/S2 and S2' arginine  
118 spike mutants were bound at the surface of Huh-7 cells treated with ammonium chloride to  
119 neutralize the endosomal pH and block the endosomal entry pathway. Then, fusion was  
120 induced by a brief trypsin treatment (Fig 2(a)). For the mutant R683N, trypsin treatment was  
121 not able to bypass the requirement for cathepsin L activity during the entry process,  
122 suggesting that proteolysis by trypsin occurs at R683 residue. Mutations of R679 or R687 had  
123 no effect on entry mediated by trypsin. Spike proteins of field strains of HCoV-229E contain  
124 an arginine at the position 679 whereas the laboratory strain VR-740 contains a threonine.  
125 Field strains were reported to use preferentially the TPPS pathway [10], this result suggest  
126 that acquisition of the arginine residue at the position 679 is not involved in this adaptation. It  
127 is surprising that mutation of R687 had no effect on entry mediated by trypsin. Indeed, R687  
128 is believed to be located at the N-terminus of the fusion peptide and the arginine residue  
129 located at the same position in the SARS-CoV spike protein is the major recognition site of  
130 trypsin. Unlike SARS-CoV, mutation of the arginine residue located at the S1/S2 junction

131 (R565N) has little effect on entry mediated at the cell surface by trypsin. *In vivo*, the major  
132 route of entry of respiratory coronavirus is believed to be mediated by the TTPS pathway. To  
133 confirm our results observed with trypsin, we expressed the human TMPRSS2 in Huh-7 cells  
134 with a lentiviral vector. Transduction of cells with a lentivirus expressing the human  
135 TMPRSS2 resulted in an increase in transduction by particles pseudotyped with the wild type  
136 S protein which was insensitive to ammonium chloride treatment (Fig 2(b)). As previously  
137 observed for the SARS-CoV [13, 14], HCoV-229E pseudotype entry mediated at the cell  
138 surface was more efficient than entry by the endocytic pathway. As seen with infection  
139 mediated by trypsin, mutation of the R683 abolished the capacity of the virus to use  
140 TMPRSS2 to infect the cells since its infectivity was sensitive to ammonium chloride  
141 treatment of the cells (Fig 2(c)). To further confirm that cleavage mediated by trypsin-like  
142 serine protease occurs at R683N, HEK293T cells transiently expressing the wild type or the  
143 different mutant proteins were treated by trypsin for 1h and then proteins at the cell surface  
144 were biotinylated at 4°C. After precipitation of biotinylated protein with streptavidin-  
145 conjugated beads, cleavage products were analysed in western blotting with an antibody  
146 directed against a C-terminal tag of the protein. Spike proteins with a furin cleavage site  
147 introduced at the S1/S2 boundary (Fur565) or at the S2' position (Fur687) were used as  
148 controls (Fig. 3). Cleavage by furin between the S1 and S2 domain gave rise to a band with a  
149 size below 135 kDa corresponding to the S2 domain. Cleavage at the S2' position (Fur687)  
150 induces a cleavage product with a smaller size below 100 KDa. Contrary to other  
151 coronaviruses for which trypsin induces mainly a processing at the S1/S2 junction, cleavage  
152 of the wild-type HCoV-229E spike protein by trypsin resulted in the appearance of a cleavage  
153 product in western blot with a size similar to the one observed for the mutant Fur687,  
154 corresponding to the cleavage of the protein at the S2' position. Mutation of R683 abolished  
155 the cleavage induced by trypsin confirming the proteolytic processing of the spike protein at  
156 this position by trypsin. The absence of any S2 fragment detection suggests that either the S2  
157 fragment is very short-lived, being rapidly processed at the S2' position or that the S1/S2  
158 cleavage is not required for the S2' cleavage. If the S2 fragment was short-lived, then  
159 mutation of R683 should lead to the accumulation of the intermediate cleavage product.  
160 However, we did not observe any accumulation of cleavage product of bigger size when R683  
161 was mutated, arguing again against the requirement of the S1/S2 cleavage to prime the  
162 cleavage at the S2' position as shown for the SARS-CoV and MERS-CoV spike proteins [2,  
163 6].

164 Taken together, our results show that activation of the fusion process by trypsin and trypsin-  
165 like serine protease, particularly TMPRSS2 expressed in human airway epithelia, strongly  
166 rely on the R683 residue as a cleavage site. It has been shown that the position of the cleavage  
167 site in the S2' region of the SARS-CoV spike protein can be shifted from the position relative  
168 to the fusion peptide but with some effects on fusion activation and infection. Indeed, for the  
169 SARS-CoV spike protein, cleavage at the N-terminus of the fusion peptide results in  
170 improved fusion and infection capacities [4]. For HCoV-229E spike protein, the cleavage site  
171 by trypsin is not located at the N-terminus of the fusion peptide but is present 3 residues  
172 upstream. For the SARS-CoV spike protein, even if the location of the cleavage site presents  
173 some flexibility, certain position in the vicinity of the fusion peptide were also not accessible  
174 to protease. Indeed, no cleavage product was observed when R683 was mutated (Fig 3),  
175 suggesting that both arginine residues 679 and 687 were not accessible to the protease,  
176 otherwise, if these residues could be cleaved but without being functional to activate the  
177 fusion, the same band would have been observed with the R683N mutant.

178

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180 The authors declare no conflict of interest.

181

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185

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189

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230

## 231 **FIGURE LEGEND**

### 232 **Figure 1**

233 (A) Alignment of the S1/S2 and S2' region of different coronavirus spike protein (HCoV-  
234 229E isolated in France, 2014, HCoV-229E Niigata/01/08, HCoV-229E 0349, HCoV-229E  
235 USA/932-72/1993, HCoV-229E laboratory strain VR-740, MERS-CoV and SARS-CoV).  
236 R565 at the S1/S2 boundary is in red and R679, R683 and R687 are presented in green. For



237 SARS-CoV and MERS-CoV the arginine at the S2' position is also presented in green. The  
238 amino sequence between the S1/S2 boundary and the S2' region is not totally presented and  
239 the omitted sequence is depicted by a dotted line. (B) MLV-based particles pseudotyped with  
240 the wild type spike protein (WT) or with a spike protein in which a furin cleavage site was  
241 introduced in the S2' region (Fur687) were produced and used to inoculate Huh-7 cells in  
242 presence or absence of 25 mM NH<sub>4</sub>Cl or 5 μM of furin inhibitor. Results are presented as the  
243 percentage of transduction in absence of drug (DMSO) and are expressed as the mean of three  
244 independent experiments. Error bars represent the standard error of the means (SEM). Data  
245 were analysed by using an ANOVA test (\*\*\* P<0.001). (C) Endosomal entry of particles  
246 pseudotyped with the mutated spike proteins. Pseudoparticles were produced and used to  
247 inoculate Huh-7 cells at 37°C in presence or absence of 25 mM NH<sub>4</sub>Cl. 48h later, the cells  
248 were lysed and luciferase activity was measured. Results are presented as relative light units  
249 (RLU) and are expressed as the mean of five independent experiments. Error bars represent  
250 the standard error of the means (SEM). Data were analysed by using an ANOVA test (\*\*\*  
251 P<0.001). (D) Analysis of pseudoparticles formation. Pseudoparticles were produced with the  
252 different mutated spike proteins and concentrated on sucrose cushion, then pseudoparticles  
253 were lysed and the production was analysed by western blotting with anti-VSVG antibody to  
254 detect the spike protein and anti-gag antibody.

255

## 256 **Figure 2**

257 (A). Trypsin-mediated entry at the cell surface. Particles pseudotyped with the different  
258 mutated spike proteins were bound at the cell surface at 4°C in presence of 25 mM NH<sub>4</sub>Cl  
259 and were treated with a 5 min incubation with 2 μg/ml of trypsin to induce fusion. 48h later,  
260 the cells were lysed and luciferase activity was measured. Results are presented as the  
261 percentage of transduction observed with particles pseudotyped with the wild type protein and  
262 are expressed as the mean of three independent experiments. Error bars represent the standard  
263 error of the means (SEM). Data were analysed by using an ANOVA test (\*\* P<0.01) (B)  
264 Huh-7 cells were transduced or not with a lentivirus expressing the human TMPRSS2. Then  
265 cells were infected with particles pseudotyped with the wild type spike protein in presence or  
266 absence of 25 mM NH<sub>4</sub>Cl. Results are presented as relative light units (RLU) and are  
267 expressed as the mean of three independent experiments. Data were analysed by using an  
268 ANOVA test (\*\*\* P<0.001). (C) Huh-7 cells transduced or not with a lentivirus expressing  
269 TMPRSS2 were inoculated with particles pseudotyped with the different mutated spike  
270 proteins. Results are presented as the percentage of infection observed with particles

271 pseudotyped with the wild type protein and are expressed as the mean of three independent  
272 experiments. Error bars represent the standard error of the means (SEM). Data were analysed  
273 by using an ANOVA test (\*\*\*) P<0.001).

274

275 **Figure 3**

276 Spike protein cleavage with trypsin. HEK293T cells were transfected with plasmid encoding  
277 the different mutated spike protein. 48h later, cells were treated for 1h with 2µg/ml of trypsin  
278 at 37°C. Then cell surface protein were biotinylated at 4°C and the cells were lysed.  
279 Biotinylated protein were precipitated with streptavidin-conjugated beads and analysed by  
280 western blotting with the C-terminal part of the protein detected with an anti-VSVG antibody.

281

282

Figure 1 :

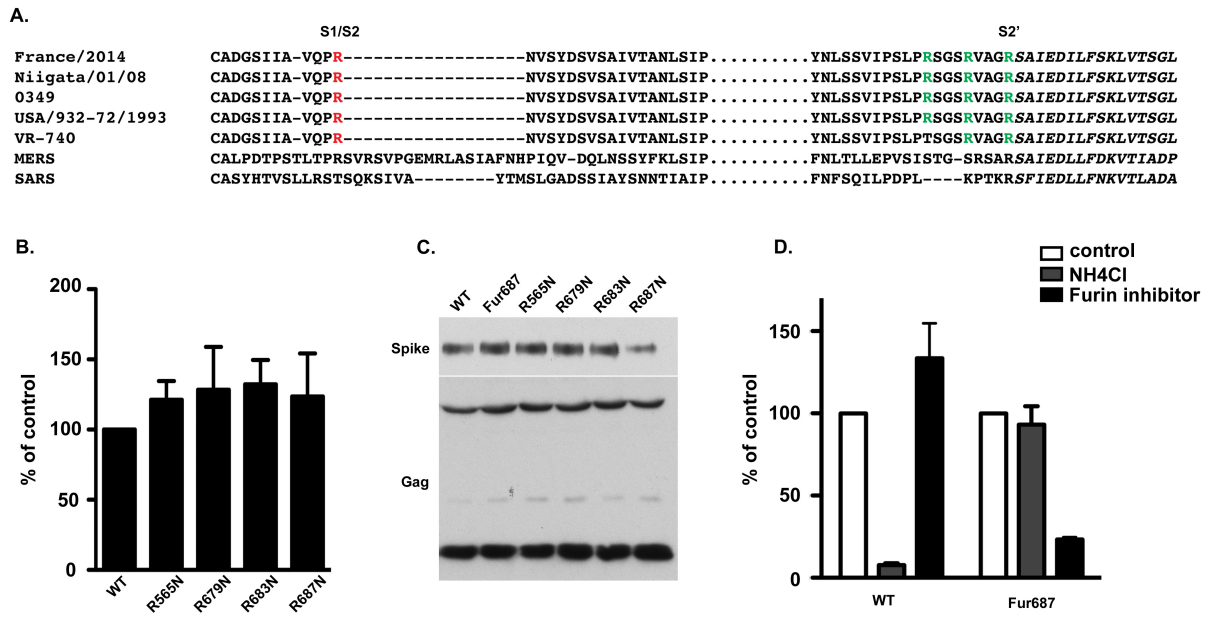


Figure 2 :

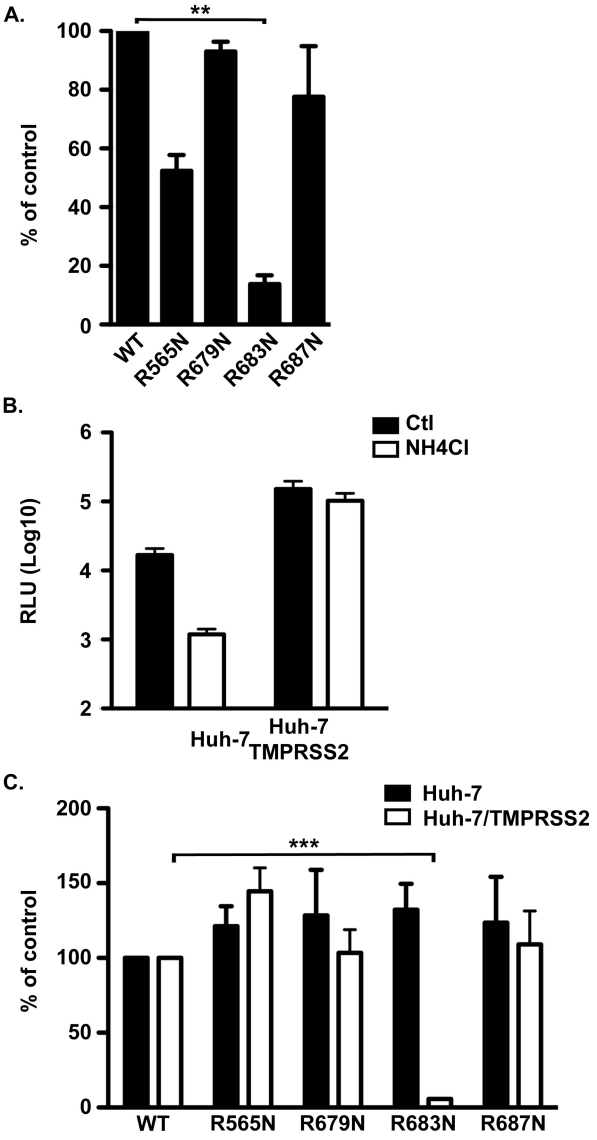


Figure 3 :

