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2	The SARS-CoV-2 Spike Protein Activates the Epidermal Growth Factor Receptor-
3	Mediated Signaling
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#### 24 Abstract

25

#### 26 **Objectives:**

The coronavirus disease-19 (COVID-19) pandemic is caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). At the molecular and cellular levels, the SARS-Cov-2 uses its envelope glycoprotein, the spike S protein, to infect the target cells in the lungs via binding with their transmembrane receptor, the angiotensinconverting enzyme 2 (ACE2). Here, we wanted to invesitgate if other molecular targets

32 and pathways may be used by SARS-Cov-2.

#### 33 Methods:

We investigated the possibility for the spike 1 S protein and its receptor-binding domain (RBD) to target the epidermal growth factor receptor (EGFR) and its downstream signaling pathway *in vitro* using the lung cancer cell line (A549 cells). Protein expression and phosphorylation was examined upon cell treatment with the recombinant full spike 1 S protein or RBD.

## 39 **Results:**

We demonstrate for the first time the activation of EGFR by the Spike 1 protein associated with the phosphorylation of the canonical ERK1/2 and AKT kinases and an increase of survivin expression controlling the survival pathway.

#### 43 **Conclusions**:

44 Our study suggests the putative implication of EGFR and its related signaling pathways

in SARS-CoV-2 infectivity and Covid-19 pathology. This may open new perspectives in

the treatment of Covid-19 patients by targeting EGFR.

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## 48 Introduction

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The new COVID-19 disease was identified for the first time in December 2019 in the province of Wuhan in China and it is caused by a new member of coronavirus family called SARS-CoV-2 (Wang et al., 2020; Zhu et al., 2020; Hu et al., 2021). According to the latest statistics, over 111 million COVID-19 cases were reported causing around 2.5 million deaths worldwide (https://www.who.int/emergencies/diseases/novel-coronavirus-2019).

56 The molecular and cellular basis of the SARS-CoV-2 infection implies the renin-57 angiotensin system (RAS) and more importantly its angiotensin-converting enzyme 2 58 (ACE2) (Li et al., 2003; Tolouian et al., 2020; Hoffmann et al., 2020; Yan et al., 2020; 59 Tai et al., 2020; Sriram et al., 2020; Hu et al., 2021). ACE2 is a host membrane-bound 60 metallopeptidase with the catalytic site oriented extracellularly is mostly expressed in 61 lung, heart, kidney, brain, and gut. In contrast to ACE which converts angiotensin I to the active vasoconstrictor, angiotensin II (AngII), ACE2 breaks down AngII to 62 63 angiotensin-(1–9 and 1–7), which are potent vasodilators, and considered as a negative regulator of RAS (Lavoie & Sigmund, 2003). The implication of ACE2 in COVID-19 is 64 65 thought to occur mostly in the very early stages of the viral infection and COVID-19 pathology. Indeed, SARS-CoV-2-S protein, the spike glycoprotein (protein S), on the 66 virion surface has been reported to bind the extracellular domain of ACE2 which is used 67 68 as a co-receptor for target cell recognition and membrane fusion during the infection 69 process (Li et al., 2003; Tolouian et al., 2020; Hoffmann et al., 2020; Yan et al., 2020; 70 Tai et al., 2020; Sriram et al., 2020a; Hu et al., 2021). ACE2 constitutes the main entry 71 gate for other coronaviruses including the SARS-CoV (Yan et al., 2020). In addition, in

72 vivo studies showed a nice correlation between COVID-19 infection and the relative 73 expression of ACE2 (positive) and its activity (negative). Furthermore, the receptor-74 binding domain (RBD) in SARS-CoV-2 S protein has been identified and shown to bind 75 strongly to human and bat ACE2 receptors (Tai et al., 2020). The purified human 76 recombinant RBD showed a potent competitive action on the binding and, hence, the 77 attachment of SARS-CoV-2 RBD to ACE2-expressing cells and their infection by the 78 pseudo virus (Tai et al., 2020; Hu et al., 2021). Thus, the RBD constitutes the most 79 antigenic entity of the S protein used for the development of vaccines to prevent from 80 SARS-CoV-2 infection. Several spike-protein and RBD-based vaccines are in clinical 81 trial (Krammer, 2020).

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83 The disease severity and mortality of COVID-19 have been reported to be increased in 84 patients suffering from other chronic diseases such as cancer, diabetes, hypertension 85 and cardiovascular problems. This was further evidenced in the patients who have been 86 treated with anti-hypertensive drugs such as ACE inhibitors (ACEIs) and angiotensin receptor blockers (ARBs)(Sriram et al., 2020a; Mourad & Levy, 2020; Guo et al., 2020; 87 Gurwitz, 2020; Oliveros et al., 2020; South et al., 2020; Patel & Verma, 2020; 88 89 Sommerstein et al., 2020). In parallel, studies have showed that ACEIs and ARBs 90 resulted in an upregulation of ACE2 and favoring the entry and replication of the virus 91 (Sommerstein et al., 2020). By contrast, SARS-CoV-2 by targeting ACE2 on the target 92 cells causes down-regulation and inactivation of the latter. This downregulation generates an imbalance in favor of an over accumulation of Angll, a potent 93 94 vasoconstrictor. Such effect was shown to increase the oxidative damage which leads

95 to inflammation and pulmonary fibrosis (Mascolo et al., 2020). Moreover, inflammation, 96 cytokine storm, and thrombosis associated with pulmonary injury constitute other 97 important clinical features of COVID-19 pathology. This suggests the implication of 98 other molecular actors such as the protease thrombin via its proteinase-activated 99 receptors (PARs), the purinergic receptors, cytokine receptors, and lipid mediators. 100 Thus, the inhibition of these pathways has been proposed as a promising therapeutic 101 approach to prevent thrombotic and inflammatory processes during COVID-19 102 pathology (Sriram et al., 2020b; Lee et al., 2021).

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105 Although large body of evidence using *in vitro* studies and *in silico* data strongly support 106 the thesis that ACE2 is necessary for SARS-CoV-2 entry, still, we cannot rule out that 107 additional factors and/or alternative cell surface receptors may be also implicated in SARS-CoV-2 entry (Zamorano & Grandvaux, 2020). Among these possible receptors, 108 109 the G protein-coupled receptors (GPCRs) and receptor tyrosine kinase (RTKs) 110 constitutes the valid candidates based on their tissue abundance and pivotal roles in 111 human and animal physiology. Indeed, several previous studies reported the hijacking 112 of GPCRs and RTKs and their function by various pathogens during pathogenesis. This 113 includes microbial pathogens such as bacteria and viruses such as the SARS-CoV and 114 the adrenergic receptor and the epidermal growth factor receptor (EGFR) as the targets 115 (Wiedemann et al., 2016; Venkataraman & Frieman, 2017; Mitchell et al., 2019). Thus, 116 we hypothesize that during SARS-CoV-2 infectivity, the virus may also use EGFR 117 expressed on the epithelial lung cells as the receptor/co-receptor target for its entry.

Here, we have examined the effect of the SARS-COV-2 full-length and DRB Spike 1

- 119 protein on the activation of EGFR and its related downstream signaling pathways
- 120 consisting of AKT and ERK1/2 phosphorylation in the lung cancer cells (A549).

#### 122 Materials and Methods

#### 123 Cell culture, chemicals and antibodies

The lung cancer cells (A549) used in this study were obtained from Cell Line Service 124 125 (CLS)-GmbH and were maintained in RPMI (Cat. # 00506 Gibco, Life Technologies, 126 Rockville, UK) complemented with 10% fetal bovine serum (FBS) (Cat. # 02187 Gibco, 127 Life Technologies, Rockville, UK) and 100 U/ml penicillin streptomycin glutamine (Cat. # 128 01574 Gibco, Life Technologies, Rockville, UK). AG1478 (Cat. # 141438) was 129 purchased from Abcam, Cambridge, UK. Antibodies against phospho-EGFR (Cat. # 4407), EGFR (Cat. # 4267), phospho-ERK1/2 (Cat. # 9106), ERK1/2 (Cat. # 4695), 130 phospho-AKT (Cat. # 9271), AKT (Cat. # 9272), Survivin (Cat. # 2803), anti-mouse 131 132 (Cat. # 7076), and anti-rabbit (Cat. # 7074) were purchased from Cell Signaling, Technology, Danvers, Massachusetts USA. SARS-COV-2 spike protein 1 (Cat. # 133 134 DAGC091) was purchased from Creative Diagnostics, Shirley, NY, USA. SARS-COV-2 135 spike RBD protein (Cat. # 40592-V08B) was purchased from Sino Biological, Beijing China. Human EGF Recombinant Protein (Cat. # PHG0313) was purchased from 136 137 Gibco, Life Technologies, Rockville, UK.

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### 139 Whole Cell extract and Western Blotting analysis

140 Cells (0.5 x 10<sup>6</sup>) were seeded per well in 60 mm culture dish and cultured for 24h before 141 treatment. After treatment with or without EGF, spike 1 protein or Spike RBD protein, 142 cells were washed twice with ice-cold PBS, scraped, pelleted, and lysed in RIPA buffer 143 (Pierce) supplemented with protease inhibitor cocktail (Roche) and phosphatase 144 inhibitor (Roche). After incubation for 30 min on ice, cell lysates were centrifuged at

145 14,000 rpm for 20 min at 4°C. Protein concentration of lysates was determined by BCA 146 protein assay kit (Thermo Scientific) and the lysates were adjusted with lysis buffer. 147 Aliguots of 25 µg of total cell lysate were resolved onto 6-15% SDS-PAGE. Proteins 148 were transferred to nitrocellulose membranes (Thermo Scientific) and blocked for 1 h at 149 room temperature with 5% non-fat dry milk in TBST (TBS and 0.05% Tween 20). 150 Incubation with specific primary antibodies was performed in blocking buffer overnight at 151 4°C and this was true for all the primary protein antibodies used in this study. Notice that 152 all the lysates were freshly loaded when different phospho-proteins were analyzed. 153 However, the same membrane of the phospho-protein was stripped to examine the 154 corresponding total protein loaded except for pERK1/2 where the proteins were always 155 freshly loaded due to stronger anti-pERK1/2 binding. Moreover, a β-actin blot was also 156 used in parallel to double check that similar amount of proteins were loaded in every 157 gel's lane. Horseradish peroxidase-conjugated anti-IgG was used as secondary 158 antibody. Immunoreactive bands were detected by ECL chemiluminescent substrate 159 (Thermo-Scientific) and chemiluminescence was detected using the LiCOR C-DiGit blot 160 scanner and Image Studio Light Software.

## 162 **Results**

#### 163 Spike and RBD activate EGFR, AKT, and ERK1/2 in A549 cells

First, we examined the effect of Spike 1 and RBD on the phosphorylation status of 164 165 EGFR and its related kinases, AKT and ERK1/2, in lung cancer cells (A549) (Figure 1). 166 The treatment of cells for 5 minutes with Spike (2.5 µg/ml) promoted a strong phosphorylation of EGFR, AKT, and ERK1/2, which was either similar (EGFR and 167 168 ERK1/2) or even higher (AKT) to that promoted by stimulation with the maximal dose of 169 EGF (5 µg/ml) (Figure 1). On the other hand, RBD (5 µg/ml) under a similar condition 170 showed almost no effect on EGFR phosphorylation while it significantly induced AKT 171 and ERK1/2 phosphorylation (Figure 1). This result suggests the specific targeting of 172 EGFR by the full spike 1 protein of SARS-COV-2 but not by its RBD. Also, our data 173 suggest that RBD-mediated AKT and ERK1/2 activation is independent on EGFR 174 activation and that other molecular targets and/or intracellular mechanisms may be 175 involved.

We next performed time-course analysis with both Spike 1 and RBD on the 176 177 phosphorylation status of EGFR and ERK1/2. For this, A549 cells were treated with Spike 1 (2.5 µg/ml) (Figure 2A) or RBD (5 µg/ml) (Figure 2B) for 5, 15, 30, and 60 178 179 minutes. Stimulation with EGF (5 µg/ml) as a positive control was carried out for 15 180 minutes. As shown in Figure 2A, Spike 1 induced a rapid and a transient 181 phosphorylation of EGFR and ERK1/2 with a pic at 5 minutes of stimulation and sharp 182 decrease after 15 minutes. Spike 1-mediated AKT activation, on the other hand, was 183 also observed upon 5 minutes of stimulation but this was more sustained in time 184 (Figure 2A). For RBD, there was no clear EGFR phosphorylation induced regardless of

the stimulation time (Figure 2B). This is consistent with our observation in Figure 1B.

186 However, RBD promoted rapid and strong activation of both ERK1/2 and AKT that 187 remained persistent even after 60 minutes of stimulation (**Figure 2B**).

Together, these data further confirm the specific activation of EGFR by Spike 1, but not RBD, but they also demonstrate the implication of additional and/or different mechanisms in AKT and ERK1/2 activation whether cells were activated by Spike 1 or RBD.

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### 193 Activation of AKT by Spike 1 and RBD is EGFR-dependent

194 Next, we decided to further explore the targeting of EGFR and its related downstream 195 AKT survival pathway by Spike 1 and RBD by using the selective EGFR antagonist, AG1478. As shown in **Figure 3A**, the pre-treatment of A549 cells with AG1478 (10  $\mu$ M) 196 197 fully blocked EGFR phosphorylation induced by stimulation with EGF (5 µg/ml) 198 indicating the specificity of the response. We found that, AG1478 completed abolished 199 AKT phosphorylation induced by EGF, Spike 1, or RBD (Figure 3B). This finding 200 strongly suggests that Spike 1- and RBD-mediated AKT phosphorylation is dependent 201 on EGFR activation.

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#### 203 Effects of Spike 1 and RBD on the cell survival marker, survivin

Since EGFR, AKT, and ERK1/2 pathways are known for their role in cell proliferation and cell survival, we wanted to link our data on the phosphorylation status of these proteins especially EGFR and AKT with the induction of the anti-apoptotic protein, survivin, which belongs to the inhibitor of apoptosis (IAP) family and considered as the

208 key marker for the activation of the survival pathway in cells. Moreover, the activation of 209 survivin was shown to be induced by both AKT and ERK1/2 signaling in cells. For this, 210 we wanted to test whether Spike- and RBD-mediated activation of ERK1/2 and AKT 211 was associated with the induction of surviving expression. Toward this, A549 cells were 212 treated with Spike 1 (2.5  $\mu$ g/ml) (Figure 4A) or RBD (5  $\mu$ g/ml) (Figure 4B) for 5, 15, 30, 213 and 60 minutes using the stimulation 15 minutes with EGF (5 µg/ml) as a positive 214 control and the level of surviving was determined by Western Blot. We found that both 215 Spike 1 (Figure 4A) and RBD (Figure 4B) induced a marked increase of survivin 216 expression in a time-dependent manner with a maximal response at 30 and 60 minutes. 217 These observations demonstrate that Spike 1- and RBD-promoted EGFR/AKT pathway 218 in A549 cells is associated with the activation of survivin that may promote the survival 219 of the SARS-Cov-2-infected lung cancer cells.

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#### 221 Discussion

222 In this study, we report for the first time the possible targeting of EGFR and its related 223 downstream signaling pathways by SARS-Cov-2 Spike 1 protein and its RBD in lung 224 cancer cells (A549). We demonstrated that Spike 1-induced AKT activation occurred in 225 EGFR-dependent manner since it was drastically blocked by AG1478. On the other 226 hand, we found that Spike 1 and RBD also elicited the activation of the survival pathway 227 in A549 cells. Indeed, both Spike 1 and RBD induced the expression and the activation 228 of the anti-apoptotic protein, survivin, which belongs to the inhibitor of apoptosis (IAP) 229 family and considered as the key marker for the activation of the survival pathway in 230 cancer cells. Such a response was very consistent with the phosphorylation of AKT in these cancer cells. This may constitute a solid molecular and cellular rationale to 231

explain the increased risk of infectivity by SARS-Cov-2 and its severity in cancer
patients as recently reported by several groups (Albiges et al., 2020; Dai et al., 2020).

234 Regarding the implication of our findings in the pathophysiology of COVID-19, recent 235 studies showed that cancer patients were more vulnerable to the SAR-COV-2 infection. 236 Although COVID-19 was reported to have low death rate ~2% in the general population, 237 patients with cancer and COVID-19, have at least 3-fold increase in the death rate. Dai 238 et al., 2020 showed that patients with lung cancer, gastrointestinal cancer or breast 239 cancer had the highest frequency of critical symptoms including highest death rates. 240 Patients with lung cancer and gastrointestinal cancer had a death rate of 18.18% and 241 7.69%, respectively (Dai et al., 2020). Interestingly, they showed that cancer patients 242 that received targeted therapy that includes the EGFR-tyrosine kinase inhibitors showed 243 the lowest death rate compared to cancer patients who received immunotherapy, 244 chemotherapy or surgery (Albiges et al., 2020). Another study carried out in Gustave 245 Roussy Cancer Centre (France) by Albiges et al., 2020 showed that 27% of the cancer 246 patients with COVID-19 developed clinical worsening and 17.4% died (Albiges et al., 247 2020). Here we showed that SAR-COV-2 activates the EGFR and its downstream 248 signaling pathways controlling cell survival and proliferation. We also found that the 249 inhibition of EGFR abolished the SARS-COV-2 activation of AKT.

At the molecular level, our *in vitro* data provide, for the first time, the evidence that SARS-Cov-2 Spike 1 protein activating EGFR and its downstream signaling pathways, AKT and ERK1/2. This is very consistent with the well-established concept of the hijacking of cell surface receptors and their activity/signaling by pathogens including viruses (Sodhi et al., 2004; Zhang et al., 2016; ; Ranadheera et al., 2018; Mitchell et al.,

255 2019) and bacteria (Coureuil et al., 2010; Wiedemann et al., 2016). This implies that 256 pathogens use GPCRs and RTKs at the cell surface of the target cells during the 257 infection process leading to their entry in the target cells. Interestingly, previous studies 258 also showed the role of EGFR and its downstream signalling pathways in 259 viruses/bacteria pathogenicity being consistent with our findings on SARS-Cov-2 spike 260 protein (Mitchell et al., 2019; Wiedemann et al., 2016). Indeed, EGFR was shown to be 261 important during influenza infection (Mitchell et al., 2019). In addition, similarly to our 262 data, the Salmonella Rck membrane protein has been reported to bind and to activate 263 EGFR and its mediated signalling resulting in receptor/bacteria co-internalization and 264 cell infection (Wiedemann et al., 2016). This also occurs with GPCRs since both viruses 265 and bacteria were demonstrated to bind and activate GPCRs resulting in the co-266 internalization of viruses (Sodhi et al., 2004) or bacteria (Coureuil et al., 2010; 267 Wiedemann et al., 2016) with the target receptors. As stated above, we showed that the 268 Spike 1-induced AKT activation occurred in EGFR-dependent manner in A549 cells 269 since it was blocked by EGFR blockade (AG1478). This supports the conclusion that 270 the activation by Spike 1 of the AKT/survival axis depends on EGFR activation (Figure 5). Moreover, the differential effects of Spike 1 and RBD suggests different mode of 271 272 activation and/or molecular pathways involved. One possible explanation is that Spike 1 273 may directly target EGFR while RBD uses other targets at the cancer cell surface 274 including ACE2 resulting in AKT and ERK1/2 phosphorylation independently on EGFR 275 (**Figure 5**). Overall, our data are consistent with direct targeting of EGFR/AKT pathway, 276 but this does not rule out the alternative pathway consisting of the implication of the 277 canonical ACE2 pathway via transactivation of EGFR at the cell surface or intracellular

crosstalk between their intracellular pathways (**Figure 5**). Of course, further studies are required to demonstrate whether Spike 1 protein directly binds to EGFR or not and what would be the implication of the canonical ACE2 pathway. Even though, we did not investigate all the aspects of EGFR-dependent pathways and SARS-Cov-2 infectivity, we believe that our data will pave the way towards further investigation the exact role of EGFR in SARS-Cov-2 infection and pathogenicity.

# 285 **Author Contributions:**

AP, HA, MK performed the Western blot experiments; KA and AEH helped analyzing the data; RI and MAA designed the project, analyzed the data and wrote the manuscript. All authors reviewed the manuscript.

289

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- 292 and R.I.
- 293

# 294 **Conflicts of Interest:**

295 The authors declare no conflict of interest.

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# 297 Ethical approval statement:

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299 The authors declare no ethical approval was needed for this study.

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438 Figures Legend

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Figure 1. Effect of SARS-COV-2 Spike 1 protein on EGFR, AKT and ERK1/2 activation. A549 cells were treated or not with EGF (5 μg/mL) for 15 min or with fulllength Spike 1 (2.5 μg/mL) or with RBD (5 μg/mL) protein for 5 min then whole-cell extracts were subjected to western blot analysis for pEGFR, pAKT, pERK1/2 and their respective total levels.

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Figure 2. Time-course accumulation of pEGFR and pERK1/2 in A549 cells treated with full length Spike 1 protein and its RBD. Cells were treated or not with 2.5 $\mu$ g/mL of full length Spike 1 protein (**A**) or 5 $\mu$ g/mL of RBD (**B**) at different times as indicated (5, 15, 30 and 60 min) and the protein levels of pEGFR and pERK1/2 and their respective total levels were determined by western blot. The treatment with EGF (5  $\mu$ g/mL) was carried out for 15 min.

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Figure 3. Blockade of EGFR inhibits SARS-COV-2 spike 1-mediated activation of
AKT in A549 cells. A549 were first pre-treated with AG1478 (10 μM) for 15 min prior to
treatment with EGF (A and B), full-length spike 1 protein (B), or RBD (B) as described
in Figure 1. Whole cell extracts were resolved on SDS-PAGE and protein levels were
determined by western blot.

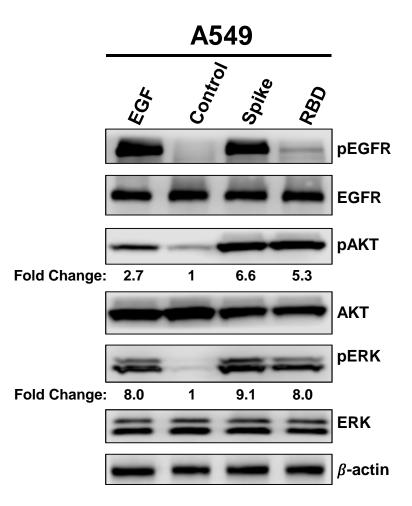
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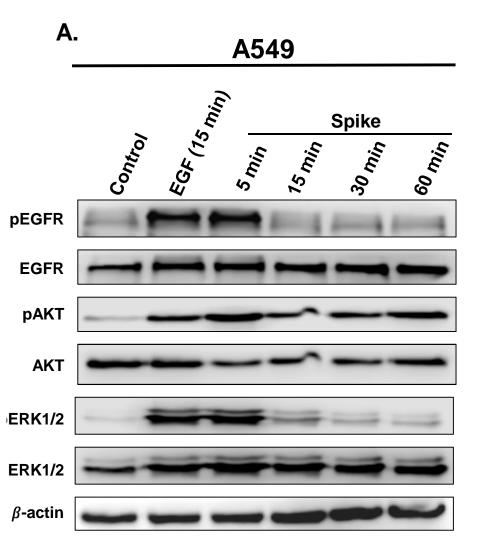
# 459 Figure 4. SARS-COV-2 full length spike 1 protein and its RBD promote survivin

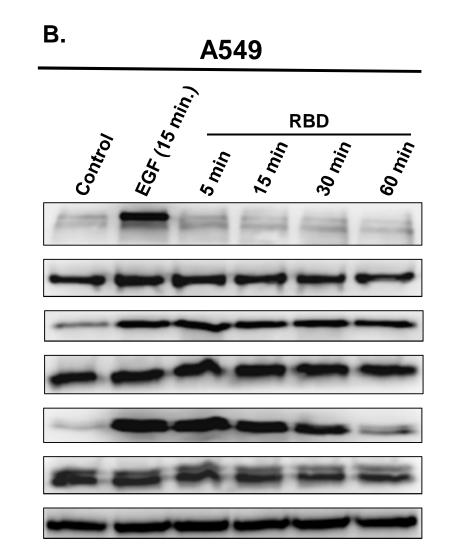
460 **expression in A549 cells.** A549 cells were treated or not with EGF ( $5 \Box \mu g/mL$ ) (**A** and 461 **B**), full-length spike 1 protein ( $2.5 \Box \mu g/mL$ )(**A**), or RBD ( $5 \Box \mu g/mL$ )(**B**) at different times 462 (5, 15, 30 and 60 min) as indicated and the protein level of survivin was determined by 463 western blot.

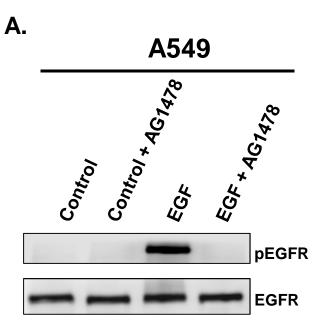
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465 Figure 5. Speculative model for the targeting of EGFR and its downstream 466 signaling by SARS-COV-2 spike 1 protein in A549 cells.

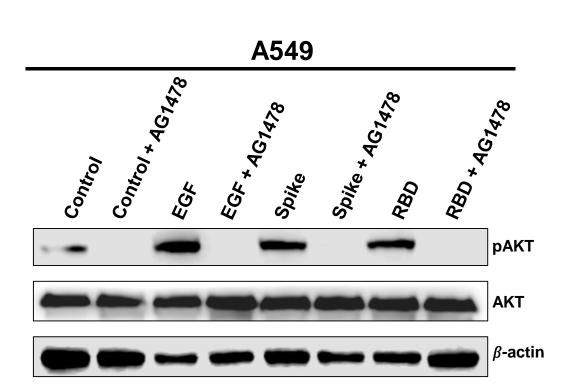








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