

1 **Hypoxia reduces cell attachment of SARS-CoV-2 spike protein**
2 **by modulating the expression of ACE2 and heparan sulfate**

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16 **ABSTRACT**

17 A main clinical parameter of Covid-19 pathophysiology is hypoxia. Here we show that hypoxia
18 decreases the attachment of the receptor binding domain (RBD) and the S1 subunit (S1) of the
19 spike protein to epithelial cells. In Vero E6 cells, hypoxia reduces the protein levels of ACE2,
20 which might in part explain the observed reduction of the infection rate. However, hypoxia also
21 inhibits the binding of the spike to human lung epithelial cells lacking ACE2 expression,
22 indicating that hypoxia modulates the expression of additional binding partners of SARS-CoV-2.
23 We show that hypoxia also decreases the total cell surface levels of heparan sulfate, a known
24 attachment receptor of SARS-CoV-2, by reducing the expression of syndecan-1 and
25 syndecan3, the main proteoglycans containing heparan sulfate. Our study indicates that
26 hypoxia acts to prevent SARS-CoV-2 infection, suggesting that the hypoxia signaling pathway
27 might offer therapeutic opportunities for the treatment of Covid-19.

28 INTRODUCTION

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30 By the end of 2020, SARS-CoV-2 has infected over 54 million people and caused >1.3 million
31 deaths. There is an urgent medical need of developing efficacious and safe vaccines and
32 therapeutic approaches. A common feature of Covid-19 patients is moderate to severe hypoxia
33 (Caputo et al., 2020). Currently, the impact of hypoxia on SARS-CoV-2 infectivity and Covid-19
34 pathogenesis is unclear, but there are emerging indications that low oxygen concentrations
35 might prevent infection or disease severity (Couzin-Frankel, 2020). This notion is supported by
36 the fact that epidemiological data indicates that Covid-19 severity decreases in high altitude
37 areas (Arias-Reyes et al., 2020). The spike protein of SARS-CoV-2 interacts with both ACE2
38 and cellular heparan sulfate (HS) through the receptor binding domain (RBD) (Clausen et al.,
39 2020; Walls et al., 2020; Yan et al., 2020). ACE2 expression is regulated by hypoxia on a cell
40 type- and time-dependent manner (Paizis et al., 2005; Zhang et al., 2009). Syndecans, the main
41 cellular HS-containing proteoglycans (HSPGs), are also differentially regulated by hypoxia. For
42 example, the expression of syndecan-1 (sdc-1) in the lungs is reduced under hypoxia (Asplund
43 et al., 2009; Wu et al., 2020). Our hypothesis is that hypoxia influences the cellular attachment
44 of SARS-CoV-2 into host cells by modulating the expression of viral entry and attachment
45 receptors on epithelial cells.

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47 RESULTS

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49 ***Hypoxia reduces the binding of RBD and S1 to epithelial cells and decreases ACE2*** 50 ***protein levels***

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52 In order to understand if hypoxia affects the cellular binding of the SARS-CoV-2 spike protein,
53 we subjected human lung epithelial cells (NCI-H460) or Vero E6 cells to different oxygen
54 concentrations (21% vs. 1% oxygen) and interrogated the binding of RBD and S1. Figure 1A
55 shows a significant decrease of the binding of RBD and S1 after culturing the target cells under
56 hypoxia (1% oxygen) for 24 or 48 hours. To further explore the mechanism responsible for the
57 observed reduced binding of the spike, we assessed *ACE2* gene expression on both cell lines
58 by quantitative PCR (Q-PCR), showing that only Vero E6 cells presented detectable levels of
59 *ACE2* transcripts (Figure 1B). Hypoxia significantly increases *ACE2* transcription on Vero E6
60 cells (Figure 1C), as expected (Paizis et al., 2005; Shenoy et al., 2020). However, total levels of
61 *ACE2* protein decrease after hypoxia exposure (Figure 1D), which might partially explain the
62 reduced cellular binding of RBD. Indeed, Vero E6 cells cultured under hypoxia show a
63 decreased rate of infection with pseudotyped lentiviral particles that were engineered to express
64 the full length spike of SARS-CoV-2 (Figure 1E). However, the spike is also able to bind to NCI-
65 H460, a cell line that contains significantly less abundance of *ACE2* transcripts compared to
66 Vero E6 cells (Figure 1B) and does not express detectable levels of *ACE2* protein (Figure 1D).
67 The interaction between the spike and NCI-H460 cells is strongly inhibited by hypoxia (Figure
68 1A). Altogether, these findings demonstrate the presence of additional SARS-CoV-2 binding
69 factors that are regulated by hypoxia.

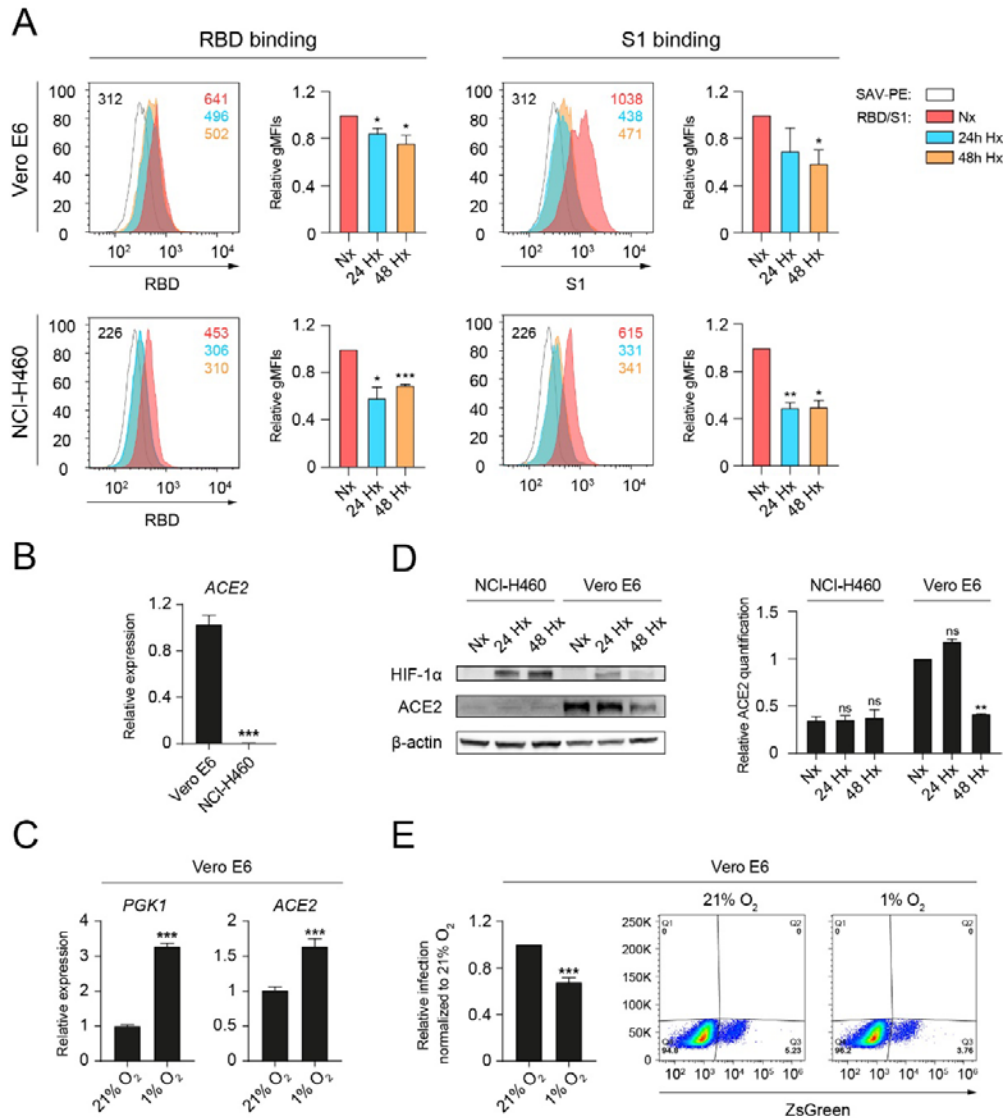
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71 ***Hypoxia decreases the total level of heparan sulfate on the cell surface***

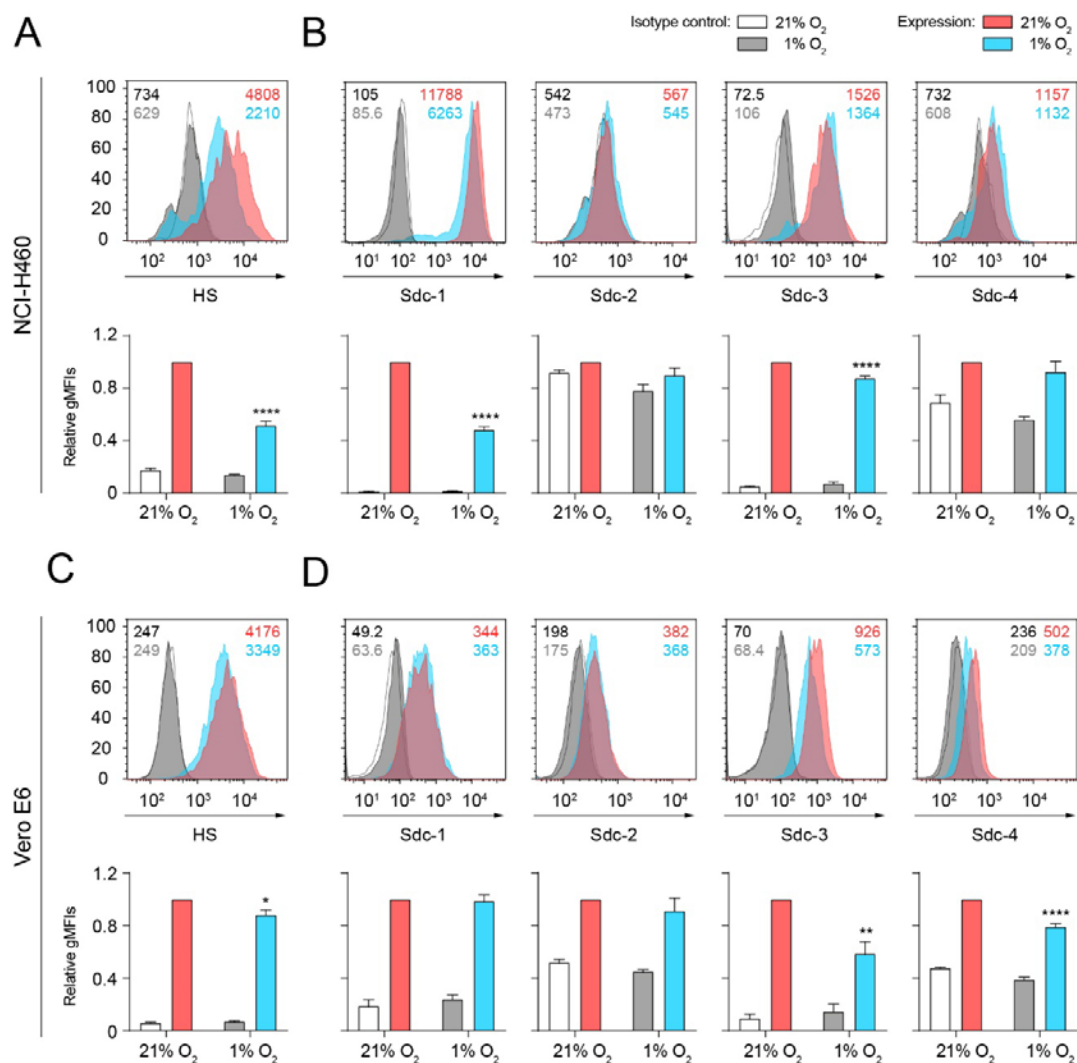
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73 We then explored if the levels of cellular HS, a known necessary host attachment factor for
74 SARS-CoV-2, were altered under hypoxia (Figure 2). Hypoxia significantly decreases the total
75 level of cell surface HS on NCI-H460 (Figure 2A) and Vero E6 (Figure 2C) cells. We then
76 assessed the expression of the members of the syndecan family, the main HSPGs on the cell
77 surface, by flow cytometry. Among them, sdc-1 and syndecan-3 (sdc-3) are the most abundant
78 on both cell lines. Hypoxia downregulates the levels of sdc-1 on human lung epithelial cells both
79 at transcriptional (data not shown) and protein levels (Figure 2B), suggesting that sdc-1 is the
80 main contributor to the observed reduction of the total HS pool under hypoxia on NCI-H460

81 cells. Vero E6 cells also express prominent levels of cell surface sdc-3 that decrease after
 82 hypoxia exposure (Figure 2D).
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 86 **Figure 1. Hypoxia reduces the binding of RBD and S1 to epithelial cells and decreases**
 87 **ACE2 protein levels.** (A) Binding of the receptor binding domain (RBD) (left) or S1 subunit (S1)
 88 (right) to Vero E6 (top, n=3, unpaired t test) or NCI-H460 (bottom, n=2, unpaired t test)
 89 cells cultured under normoxia (21% oxygen) or hypoxia (1% oxygen) for 24 and 48 hours,
 90 measured by flow cytometry. A representative histogram indicating the geometric mean
 91 fluorescent intensity (gMFI) value for each condition is shown. (B) Relative ACE2 gene
 92 expression on Vero E6 and NCI-H460 measured by Q-PCR (n=3, unpaired t test). (C)
 93 Relative gene expression of PGK1 (left) and ACE2 (right) on Vero E6 cells cultured
 94 under normoxia or hypoxia for 24 hours (n=3, unpaired t test). (D) (Left) Western
 95 blot of HIF-1α, ACE2 and β-actin on Vero E6 and NCI-H460 cells cultured under
 96 normoxia or hypoxia for the indicated time points. (Right) Relative quantification
 97 of ACE2 protein expression by densitometry (n=2, 2-way ANOVA). (E) Relative
 98 infection of Vero E6 cells with pseudotyped viral particles expressing the spike protein
 99 of SARS-CoV-2, measured by flow cytometry based on ZsGreen expression (n=4
 100 independent experiments). Error bars represent SEM. Asterisks represent p values (*, ≤ 0.05; **, < 0.01; ***, < 0.001).



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Figure 2. Hypoxia decreases the total level of heparan sulfate on the cell surface. (A, B) Total heparan sulfate levels **(A)** and expression of Sdc-1 to Sdc-4 **(B)** on NCI-H460 cells cultured under normoxia (21% oxygen) or hypoxia (1% oxygen) for 24 hours, measured by flow cytometry. *(Top)* Histograms show representative results, numerical gMFI values are depicted. *(Bottom)* Bar graphs represent relative gMFI values of a pool of two independent experiments (2-way ANOVA). **(C, D)** The same experiments shown in **(A)** and **(B)** performed in Vero E6 cells. Error bars represent SEM, and asterisks represent p values (*, ≤ 0.05 ; **, < 0.01 ; ****, ≤ 0.0001).

111 DISCUSSION

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113 Covid-19 disease is associated with hypoxia (Caputo et al., 2020), which results on decreased
114 oxygen availability for tissues. This phenomenon affects the susceptibility of host cells to viral
115 infections (Gan and Ooi, 2020) and shapes immune responses (Palazon et al., 2014). Here we
116 describe the binding ability of the spike of SARS-CoV-2 to epithelial cells under different oxygen
117 concentrations.

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119 We show that ACE2, the main SARS-CoV-2 entry receptor, is downregulated under hypoxia.
120 Moreover, hypoxia reduced the infection rate of Vero E6 cells by pseudotyped SARS-CoV-2
121 lentiviral particles. The observed reduction of ACE2 protein level contrasts with the early
122 transcriptional increase of this gene, a well-documented observation that likely results from the
123 binding of the hypoxia inducible factor (HIF) transcription factors to hypoxia response elements
124 on the ACE2 promoter (Liu et al., 2020). The observed discrepancy at the RNA vs. protein
125 ACE2 levels might be explained by protein degradation, cleavage (Heurich et al., 2014) or time-
126 dependent adaptation to hypoxia. Apart from ACE2, other cell surface proteins contribute to the
127 initial attachment of the virus to the host cells through the interaction of the RBD with HS
128 polysaccharides (Clausen et al., 2020) that decorate HSPGs on the cell surface. Hypoxia
129 decreases the total cell level of HS that is available for the binding of the spike. This reduction
130 might be explained by the hypoxic downregulation of sdc-1 or sdc-3, the most abundant HSPGs
131 present on the cell models tested herein.

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133 In summary, we show that hypoxia decreases the binding of the spike to epithelial cells by at
134 least two different mechanisms: 1) decreasing the level of ACE2 (entry receptor); and 2)
135 reducing the level of HS within the HSPGs (attachment factors) on the cell surface. The
136 significance of these findings at physiopathological oxygen levels remains unexplored. In this
137 context, elucidating the role of the HIF signalling pathway might unlock novel therapeutic targets
138 that, when modulated, reduce the initial virus-host interaction and viral load.

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140 MATERIALS AND METHODS

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142 *Key Resources Table*

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Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Antibody	ACE2	Bioss antibodies	Cat#: bsm-52614R	1:200 dilution
Antibody	Anti-HS	Amsbio	Cat#: 370255-S	1:200 dilution. Clone F58-10E4.
Antibody	Anti-IgM-FITC	Miltenyi Biotec	RRID:AB_10829949	1:500 dilution
Antibody	Anti-Mo-HRP	Cell Signaling	Cat#: S301677076S	1:5000 dilution
Antibody	Anti-Rb-HRP	Cell Signaling	Cat#: S301677074S	1:5000 dilution
Antibody	HIF-1 α	Novus	Cat#: NB100-449	1:100 dilution
Antibody	Syndecan-1	BD	Cat#: 565943	1:100 dilution
Antibody	Syndecan-2	R&D	Cat#: FAB2965P	1:100 dilution
Antibody	Syndecan-3	R&D	Cat#: FAB3539A	1:100 dilution
Antibody	Syndecan-4	R&D	Cat#: FAB291819	1:100 dilution
Cell culture medium	DMEM	Gibco	Cat#: 41966-029	10% FBS and 1% PS antibiotic

Cell culture medium	MEM	Gibco	Cat#: 31095-029	10% FBS and 1% PS antibiotic
Cell culture medium	RPMI	Gibco	Cat#: 61870044	10% FBS and 1% PS antibiotic
Cell line	HEK 293T	Takara	Cat#: 632180	
Cell line	NCI-H460	DSMZ	RRID:CVCL_0459	
Cell line	Vero E6	ATCC	RRID:CVCL_0574	
Chemical compound, drug	7-AAD	BD	RRID:AB_2869266	
Chemical compound, drug	BSA	Sigma	Cat#: A9647	
Chemical compound, drug	Cell dissociation buffer	Thermo	Cat#: 13151-014	
Chemical compound, drug	LDS sample buffer	Invitrogen	Cat#: NP0007	
Chemical compound, drug	M-MLV reverse transcriptase	Thermo	Cat#: 28025-013	
Chemical compound, drug	PageRuler	Thermo	Cat#: 26619	
Chemical compound, drug	PBS	Fisher	Cat#: BP3994	
Chemical compound, drug	Random primers	Thermo	Cat#: 58875	
Chemical compound, drug	Skin milk	Millipore	Cat#: 70166	5%
Chemical compound, drug	Streptavidin PE	Thermo	Cat#: 12-4317-87	1:200 dilution
Chemical compound, drug	SYBR Green	Quantabio	Cat#: 95056-500	
Chemical compound, drug	Tween-20	Sigma	Cat#: 9005-64-5	0,5%
Commercial assay or kit	0.45 µm filter	VWR	Cat#: 514-0063	
Commercial assay or kit	7.5% Mini-PROTEAN TGX gels	BioRad	Cat#: 4561023	
Commercial assay or kit	96-well plates	Thermo	Cat#: 249570	
Commercial assay or kit	BCA Protein Assay kit	Thermo	Cat#: 23227	
Commercial assay or kit	Clarity Max ECL substrate	BioRad	Cat#: 170506	
Commercial assay or kit	jetPEI kit	Polyplus-transfection	Cat#: 101-10N	
Commercial assay or kit	Lenti-X Concentrator	Takara	Cat#: 631231	
Commercial assay or kit	NucleoSpin RNA kit	Macherey-Nagel	Cat#: 740955.250	
Commercial assay or kit	PVDF membrane	BioRad	Cat#: 1704156	
Peptide, recombinant protein	Biotinylated RBD	Acrobiosystems	Cat#: SPD-C82E9	20 µg/mL
Peptide, recombinant protein	Biotinylated S1	Acrobiosystems	Cat#: S1N-C82E8	20 µg/mL

Plasmid	HDM-Hgpm2	Bei resources	Cat#: NR-52517	1.27 µg
Plasmid	HDM-tat1b	Bei resources	Cat#: NR-52518	1.27 µg
Plasmid	Lentiviral backbone (ZsGreen)	Bei resources	Cat#: NR-52520	5.79 µg
Plasmid	pRC-CMV-Rev1b	Bei resources	Cat#: NR-52519	1.27 µg
Plasmid	SARS-CoV-2 spike protein	Bei resources	Cat#: NR-52514	1.97 µg
Software	FlowJo	BD	RRID:SCR_008520	Version 10
Software	ImageJ	ImageJ	RRID:SCR_003070	
Software	QuantStudio	Thermo	RRID:SCR_018712	Version 1.3

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Cell Culture. The NCI-H460 cell line was obtained from the DSMZ (German Collection of Microorganisms and Cell Lines Cat#: ACC737) and cultured in RPMI medium 1640 GlutaMAX (Gibco Cat#: 61870044) according to standard culture protocols. Vero E6 cells (ATCC Cat#: CRL-1586) were kindly provided by Nicola G.A. Abrescia (CIC bioGUNE) and cultured in MEM (Gibco Cat#: 31095-029). HEK 293T cells (Takara Bio Inc. Cat#: 632180) were cultured in DMEM (Gibco Cat#: 41966-029). Medium was supplemented with 10% FBS and 1% Penicillin-Streptomycin. Hypoxia cultures were performed at 1% oxygen and 5% CO₂ in an In Vivo2 400 hypoxic station (Ruskin Technologies).

Binding of RBD and S1 to the cell surface. After culture, cells were collected with cell dissociation buffer (Thermo Fisher Cat#: 13151-014), distributed in 96-well polystyrene conical bottom plates (Thermo Fisher Cat#: 249570) and washed with PBS (Fisher BioReagents Cat#: BP3994) containing 0.5% BSA (Sigma Aldrich Cat#: A9647) (blocking buffer). Cells were incubated with biotinylated S1 (Acrobiosystems Cat#: S1N-C82E8) or RBD (Acrobiosystems Cat#: SPD-C82E9) proteins (20 µg/mL) in blocking buffer for 30 minutes at 4°C. After incubation, cells were washed with 200 µL of blocking buffer and incubated with streptavidin-PE (Thermo Fisher Cat#: 12-4317-87) diluted in 100 µL of blocking buffer (1:200 dilution). All centrifugation steps were performed at 300 x g for 5 min (4°C).

Generation of pseudotyped lentiviral particles. Pseudotyped lentiviral particles were generated by transfecting HEK 293T cells as described in (Crawford et al., 2020), using a five plasmid system kindly provided by June Ereño (CIC bioGUNE). Confluent HEK 293T cells (50-70%) were transfected with plasmids encoding for the lentiviral backbone (containing a CMV promoter to express ZsGreen) (NR-52520, 5.79 µg), the SARS-CoV-2 spike protein (NR-52514, 1.97 µg), HDM-Hgpm2 (NR-52517, 1.27 µg), pRC-CMV-Rev1b (NR-52519, 1.27 µg) and HDM-tat1b (NR-52518, 1.27 µg), using the jetPEI kit (Polyplus-transfection Cat#: 101-10N). 48 hours after transfection, the viruses were harvested from the supernatant, filtered through a 0.45 µm filter (VWR Cat#: 514-0063), concentrated using Lenti-X Concentrator (Takara Bio Inc. Cat#: 631231) and stored in PBS at -80°C until use.

Titration of the pseudotyped lentiviral particles and infection of Vero E6 cells. Viral titration was performed in Vero E6 cells as described in (Crawford et al., 2020). For single-infection experiments, 150.000 cells were seeded in 6-well plates, left attaching to the bottom overnight in normoxia (21% O₂) and incubated for additional 24 hours under normoxia or hypoxia (1% O₂). After that, cells were incubated with the pseudotyped lentiviral particles (MOI=0.1) for 48 hours under normoxic conditions. The infected cells expressed ZsGreen, allowing the detection by flow cytometry.

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184 **Flow cytometry.** Cells were collected, washed with blocking buffer and incubated with
185 fluorochrome-conjugated antibodies (1:100 dilution in blocking buffer) against syndecan-1 (BD
186 Biosciences Cat#: 565943), syndecan-2 (R&D Systems Cat#: FAB2965P), syndecan-3 (R&D
187 Systems Cat#: FAB3539A) and syndecan-4 (R&D Systems Cat#: FAB291819) for 15 minutes at
188 4°C. Total heparan sulfate (HS) was measured by flow cytometry after incubation for 30 minutes
189 at 4°C with an anti-HS primary antibody (Amsbio Cat#: 370255-S; clone F58-10E4) (1:200
190 dilution in blocking buffer). After that, cells were washed and incubated with an anti-IgM FITC-
191 conjugated secondary antibody (Miltenyi Biotec Cat#: 130-095-906) (1:500 dilution in blocking
192 buffer). 7-AAD (BD Biosciences Cat#: 51-68981E) was used to discriminate alive cells. Finally,
193 cells were washed twice, resuspended in 200 μ L of blocking buffer and acquired on a
194 FACSymphony cytometer (BD Biosciences). The results were analyzed using FlowJo version
195 10 (BD Biosciences).

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197 **Q-PCR.** Total RNA was isolated using the NucleoSpin RNA kit (Macherey-Nagel Cat#: 740955.250). The cDNA was synthesized by RT-PCR from 1 μ g of purified RNA with the M-
198 MLV reverse transcriptase (Thermo Fisher Cat#: 28025-013) and random primers (Thermo
199 Fisher Cat#: 58875). The Q-PCR reactions were conducted in triplicate on a ViiA 7 Real-Time
200 PCR system (Thermo Fisher) from 1 μ L of cDNA using the PerfeCTa SYBR Green SuperMix
201 reagent (Quantabio Cat#: 95056-500) and gene-specific primers. The amplification program
202 consisted of initial denaturation at 95°C for 3 min followed by 40 cycles of denaturation at 95°C
203 for 15 s, annealing at 60°C for 60 s and extension at 72°C for 60 s. Data were analyzed using
204 the QuantStudio software version 1.3 (Thermo Fisher). The relative quantification in gene
205 expression was determined using the $2^{-\Delta\Delta Ct}$ method by using the *RPLP0* gene as a house-
206 keeping gene (Fw: 5'-CGACCTGGAAGTCCAACACTAC-3' and Rv: 5'-ATCTGCTGCATCTGCTTG-
207 3'). *PGK1*, a well-established target gene of HIF-1 α , was used as a control for the hypoxia
208 condition (Fw: 5'- CCGCTTTCATGTGGAGGAAGAAG-3' and Rv: 5'-
209 CTCTGTGAGCAGTGCCAAAAGC-3'). The primer sequences for *ACE2* and *SDC1* expression
210 on human NCI-H460 cells were previously described by (Ma et al., 2020) and (Reynolds et al.,
211 2016), respectively. As these oligos presented several mismatches with the reference sequence
212 of the African green monkey (Vero E6 cells), we designed the following primers to amplify the
213 *ACE2* (Fw: 5'-AGCACTCACGATTGTTGGGA-3' and Rv: 5'- CCACCCCAACTATCTCTCGC-3')
214 and *SDC1* (Fw: 5'-GGGGCTCATCTTTGCTGTGT-3' and Rv: 5'-
215 AAGGAGTAGCTGCCTTCGTC-3') genes.

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217 **Western Blot.** Total cell lysates were collected using RIPA buffer and quantified with the BCA
218 Protein Assay kit (Thermo Fisher Cat#: 23227). Samples were mixed with LDS sample buffer
219 (Invitrogen Cat#: NP0007) containing DTT, boiled for 15 minutes, separated in a 7.5% Mini-
220 PROTEAN TGX precast protein gel (BioRad Cat#: 4561023) and transferred to a 0.2 μ m PVDF
221 membrane (BioRad Cat#: 1704156) using a Trans-Blot Turbo transfer system (BioRad).
222 PageRuler protein ladder (Thermo Fisher Cat#: 26619) was used to estimate the molecular
223 weight of the proteins. The membranes were blocked for 1 hour in 5% skim milk (Millipore Cat#: 70166)
224 and 0.5% Tween-20 (Sigma Aldrich Cat#: 9005-64-5) diluted in PBS. The membranes
225 were probed overnight at 4°C with primary antibodies diluted in PBS containing 5% BSA and
226 0.5% Tween-20, washed five times with PBS (containing 0.5% Tween-20), incubated for 1 hour
227 at RT with the corresponding secondary HRP-conjugated antibodies (1:5000 diluted in 5% skim
228 milk and 0.5% Tween-20 diluted in PBS) and washed for five additional times. The membranes
229 were probed with antibodies against HIF-1 α (Novus Biologicals Cat#: NB100-449), ACE2 (Bioss
230 antibodies Cat#: bsm-52614R) and β -actin (Cell Signaling Cat#: D6A8). The HRP-conjugated
231 secondary antibodies against mouse (Cat#: S301677076S) and rabbit (Cat#: S301677074S)
232 were obtained from Cell Signaling. Chemiluminescence detection was performed using Clarity
233 Max Western ECL Substrate (BioRad Cat#: 170506) on an iBright system (Invitrogen). Band
234 densitometry was performed using ImageJ (<https://imagej.nih.gov/ij/>).

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238

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241

242 **COMPETING INTERESTS**

243

244 The authors declare no competing interest.

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