

1 **Abstract**

2 The SARS-CoV-2 virus causes severe acute respiratory syndrome (COVID-19) and has
3 rapidly created a global pandemic. Patients that survive may face a slow recovery with
4 long lasting side effects that can afflict different organs. SARS-CoV-2 primarily infects
5 epithelial airway cells that express the host entry receptor Angiotensin Converting
6 Enzyme 2 (ACE2) which binds to spike protein trimers on the surface of SARS-CoV-2
7 virions. However, SARS-CoV-2 can spread to other tissues even though they are
8 negative for ACE2. To gain insight into the molecular constituents that might influence
9 SARS-CoV-2 tropism, we determined which additional host factors engage with the viral
10 spike protein in disease-relevant human bronchial epithelial cells (16HBE^{o-}). We found
11 that spike recruited the extracellular proteins laminin and thrombospondin and was
12 retained in the endoplasmatic reticulum (ER) by the proteins DJB11 and FBX2 which
13 support re-folding or degradation of nascent proteins in the ER. Because emerging
14 mutations of the spike protein potentially impact the virus tropism, we compared the
15 interactome of D614 spike with that of the rapidly spreading G614 mutated spike. More
16 D614 than G614 spike associated with the proteins UGGT1, calnexin, HSP7A and
17 GRP78/BiP which ensure glycosylation and folding of proteins in the ER. In contrast to
18 G614 spike, D614 spike was endoproteolytically cleaved, and the N-terminal S1 domain
19 was degraded in the ER even though C-terminal 'S2 only' proteoforms remained
20 present. D614 spike also bound more laminin than G614 spike, which suggested that
21 extracellular laminins may function as co-factor for an alternative, 'S2 only' dependent
22 virus entry. Because the host interactome determines whether an infection is
23 productive, we developed a novel proteome-based cell type set enrichment analysis
24 (pCtSEA). With pCtSEA we determined that the host interactome of the spike protein
25 may extend the tropism of SARS-CoV-2 beyond mucous epithelia to several different
26 cell types, including macrophages and epithelial cells in the nephron. An 'S2 only'
27 dependent, alternative infection of additional cell types with SARS-CoV-2 may impact
28 vaccination strategies and may provide a molecular explanation for a severe or
29 prolonged progression of disease in select COVID-19 patients.

30

31

32 **Keywords:** SARS-CoV-2 strains, proteome analysis, protein folding, protein quality
33 control, extracellular matrix, bottom up mass spectrometry, virus variants, CoPIT,
34 bioinformatic analysis of proteomes, virus host interactions, coronavirus, biochemical
35 reaction networks, virus replication, structural biology modeling, cellular microbiology,
36 protein folding, unfolded protein response, cell ontology, brain tissue, monocytes, mast
37 cells, dendritic cells, astrocytes, oligodendrocytes, megakaryocytes, muscle, fibroblasts,
38 emerging spike variants

1 Introduction

2 The novel β -coronavirus SARS-CoV-2 emerged in 2019, spread rapidly and has created
3 a global pandemic. While SARS-CoV-2 shows DNA sequence similarity to SARS-CoV¹
4 and to the bat β -coronavirus RaTG13, the infectivity of SARS-CoV-2 and zoonosis in
5 humans was likely caused by an alteration in virus to host protein-protein
6 interactions. A small number of very specific β -coronavirus to host protein interactions
7 are required to infect human cells and complete replication. A comparison of the SARS-
8 CoV-2 genome with the RaTG13 genome shows that only a few changes in the amino
9 acid sequence of the virus entry protein (the spike protein) may have enabled SARS-
10 CoV-2 infection and transmission in humans¹.

11 The virus to host interactome determines the virus' infectivity and thus, tropism. In
12 SARS-CoV-2, the receptor binding domain (RBD) of the spike protein binds to the host
13 receptor human angiotensin converting enzyme 2 (ACE2) to initiate virus
14 entry^{2,3}. Upon binding, high levels of the endoprotease TMPRSS2 cleave the spike
15 protein (S) at the second polybasic cleavage site into S1 and S2' to allow for membrane
16 fusion. Once endocytosed, S2' mediated fusion of the virion with the endosomal
17 membrane releases the viral RNA genome into the host cytoplasm⁴. Both ACE2 and
18 TMPRSS2 are expressed in cells of the upper airways (bronchia and trachea), digestive
19 tract (esophagus, ileum, colon, gallbladder) and common bile duct⁵ where they confer
20 high transmissibility, which may enable SARS-CoV-2 to linger in patients with long-
21 lasting side effects that are not fully explained⁶. It is also possible that a substantial
22 number of SARS-CoV-2 cell infections occur without ACE2/TMPRSS2-mediated cell
23 entry, albeit with much lower infectivity^{4,7}. An alternate cell entry may occur when
24 cathepsins B/L in the lysosome cleave spike priming it for membrane fusion⁸. Since
25 the spike protein decorates SARS-CoV-2 virions as trimers, the proteoform composition
26 of mature spike trimers likely determines the mechanism and specificity of any
27 alternative, ACE2/TMPRSS2 independent cell entry. However, the precise role of spike
28 proteoforms and their host interactomes in alternative paths for cell entry is unknown.

29 Open reading frames (ORF) that are encoded in the viral RNA genome are translated
30 into virus proteins which replicate and efficiently pack the viral genome into nascent viral
31 particles and support the egress of mature virus particles⁹. Indeed, the virus
32 interactome is primarily derived from virus-virus rather than virus-host interactions¹⁰,
33 and interactions with host proteins might be actively avoided to prevent any impairment
34 of virus particle formation. However, individual viral proteins target cellular proteins to
35 ward off detection by the immune system¹¹ or to nudge the cellular translation
36 machinery towards a preferential translation of viral RNA transcripts¹². Additionally, the
37 host interactome coordinates glycosylation^{13,14}, endoproteolytic cleavage¹⁵, and
38 trimerization¹⁶ of the maturing spike protein which in turn influences selectivity and
39 efficiency of viral cell entry in the next infection cycle¹⁷.

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1 Here, we identified the virus to host interactome of select SARS-CoV-2 ORFs in human
2 bronchial epithelial 16HBEo⁻ cells¹⁸. We devised a novel proteome-based cell type set
3 enrichment analysis (pCtSEA) from the frequently used gene set enrichment analysis
4 (GSEA) in transcriptomics¹⁹⁻²¹, and revealed an expanded tropism of SARS-CoV-2
5 based on the host interactome of the spike protein. Several cell types that we identified
6 are ACE2 negative but might harbor a proteome that allows infection with SARS-CoV-
7 2. Further, we compared the host interactome of the D614 spike protein with the host
8 interactome of the G614 spike. G614 yields approximately 5 fold more spike protein per
9 mature virus particle than D614²² which increases transmissibility and infectivity²³;
10 however, the mutation does not influence the severity of COVID-19 symptoms. Here
11 we show that host proteins in 16HBEo⁻ cells processed D614 less efficient than G614
12 spike proteoforms. The differential processing of spike revealed that host interactors
13 bound to the spike proteoform S2 might be important for an alternative virus cell entry.

14 Results

15 15 of the 28 open reading frames (ORFs) in SARS-CoV-2 were expressed in human
16 bronchial epithelial cells (16HBEo⁻). Bronchial epithelial cells serve as the main entry
17 point of the virus into the lower respiratory tract and also provide the first line of defense
18 against viral respiratory infections²⁴. We performed immunoprecipitations of the
19 2xStrep tagged viral proteins (Gordon *et al.*²⁵) and controls (2xStrep-GFP and MOCK)
20 in biological triplicates and measured each in technical duplicates, yielding a total of 166
21 mass spectrometry experiments (Data availability). Bait proteins with interactors were
22 immunoprecipitated using the CoPIT²⁶ method and digested with the endoprotease
23 trypsin. The resulting peptides were bound to C18 reversed phase resin (PepSep) and
24 separated by high pressure liquid chromatography (Evosep). Peptide identification with
25 a timsTOFpro mass spectrometer (Bruker) and data analysis with SAINTexpress²⁷
26 (Sum SpC ≥ 5, detection in ≥ 3 replicate experiments, and ≥ 5 fold above background)
27 resulted in an interactome comprising 15 viral and 189 host proteins (Figure 1A,
28 Extended data network 1, Supplementary data 1). 41 of 189 (22%) host proteins
29 interacted with more than one virus protein. Unique host-virus interactions were
30 detected for 12 of the 15 viral proteins: orf3a (2 host proteins), orf9b (5), E (1), orf7a (2),
31 nsp2 (2), nsp7 (3), nsp10 (5), or nsp13 (3). Nsp5-C145A, orf7b, orf8, and M protein
32 each recruited more than 10 unique host proteins. The spike protein associated with
33 4 unique human proteins (TR150, LAMC2, FBX2, ERH) in addition to LAMB3, CALX,
34 and TSP1 which were also enriched in immunoprecipitations with M and orf7b. None of
35 the human proteins that bound to nsp9 and N were unique to either viral protein.

36 The overlap of the host proteins with two recently published host interactomes in human
37 embryonic kidney HEK293 cells²⁵ and lung carcinoma A549 cells²⁸ (Figure 1B) was
38 minimal. Common to all three interactomes were the endolysosomal trafficking proteins
39 VSP39 (orf3a) and RAB14 (orf9b) as well as the chloride channel CLCC1, which might

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1 be involved in regulation of the unfolded protein response (UPR) ²⁹, and the
2 mitochondrial import receptor TOM70, which is targeted by orf9b to suppress type 1
3 interferon responses ¹¹. The cell lines 16HBEo⁻ and A549 are lung airway derived, and
4 the overlap (26 proteins, 2.5 %) was larger than between 16HBEo⁻ and HEK293
5 (9 proteins, 1.8 %). Therefore, we conclude that very specific virus to host protein
6 interactions exist and that the extended virus-host interactome depends on the cellular
7 proteome and cell type in which SARS-CoV-2 proteins were expressed. The few
8 common SARS-CoV-2 host-virus protein-protein interactions are most likely necessary
9 for successful virus replication.

10 ***The SARS-CoV-2 spike interactome in 16HBEo⁻ cells***

11 Because the spike protein is required for successful infection, we analyzed the host
12 interactome of spike in more detail. Two interactors, laminin- β 3 (LAMB3) and laminin- γ 2
13 (LAMC2), are extracellular glycoproteins that may impact virus cell entry or, conversely,
14 may be functionally impaired upon SARS-CoV-2 infection. Locally confined skin
15 blistering has been detected in conjunction with SARS-CoV-2 infection and in the
16 presence of spike protein ³⁰, and functional impairment of laminin- β 3 or laminin- γ 2 can
17 cause skin blistering (epidermolysis bullosa) ^{31,32} or failure of nephrons in the kidney
18 (Pierson syndrome) ^{33,34}. Laminin- β 3 can serve as co-receptor for the entry of Vaccinia
19 virus or HPV into human cells ^{35,36}.

20 We further found that spike binds to thrombospondin-1 (TSP1). TSP1 is present in the
21 extracellular space and ER, and is involved in the mucosal innate immune
22 response ³⁷. TSP1 includes a C-terminal L-lectin binding domain ³⁸ which may
23 competitively inhibit a subsequent viral infection of cells as observed for
24 HIV ³⁹. Increased levels of TSP1 induce the unfolded protein response (UPR) ⁴⁰. Key
25 to the UPR is the ER chaperone GRP78/BiP (HSPA5), which also binds to the spike
26 protein. GRP78/BiP might facilitate infection of epithelial cells with SARS-CoV-2 as
27 observed for the related β -coronavirus MERS-CoV ⁴¹.

28 The extended spike interactome included DJB11. DJB11 is a co-chaperone of
29 GRP78/BiP which binds to nascent polypeptides and stimulates GRP78/BiP dependent
30 protein folding in the ER, or targets misfolded proteins to ERAD ⁴². Spike proteins also
31 interacted with calnexin (CALX), which retains glycoproteins in the ER that require
32 refolding. Calnexin was previously found to bind to the C-terminus of SARS-CoV spike
33 protein ⁴³. The F-box only protein 2, FBX2, binds N-glycosylated proteins with high-
34 mannose oligosaccharides as part of the SKP1-CUL1-F-box E3 ligase protein
35 complex ⁴⁴ and targets glycoproteins to the ERAD. For example, FBX2 degrades the
36 viral glycoprotein of the Epstein Barr virus (EBV), limiting infection of epithelial cells ⁴⁵.

37 ***Differences between the interactome of spike G614 to D614***

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1 After a zoonosis, viruses continue to adapt to a new host by acquiring additional
2 mutations in the virus entry protein sequence mainly to either evade the host's immune
3 response or increase transmissibility. To gain insight into whether host interactors of
4 the spike protein are altered in different SARS-CoV-2 strains, we determined the host
5 interactome of D614G mutated spike. Mammalian expression vectors with C-terminal
6 single or N- and C-terminal double flag tagged D614 or G614 spike cDNA were
7 transiently transfected into 16HBE⁻ cells and spike protein was immunoprecipitated
8 48 h later. Host interactors of D614 spike protein were directly compared to host
9 interactors of G614 spike protein with CoPIT^{26,46} (Table 1). G614 spike associated with
10 SRP72 and high mobility group proteins (HMG). G614 but not D614 spike bound the
11 signal recognition particle 72 (SRP72) which regulates cellular translation of membrane
12 proteins. SRP72 relocates the translation complex with the nascent polypeptide from
13 the cytoplasm to the rER. High mobility group proteins HMGB1 bound to G614 spike
14 preferentially and HMGN2 or HMGB2 bound exclusively. High mobility group proteins
15 are involved in a range of cellular pathways from proliferation to inflammation, and
16 extracellular accumulation of HMGB1 stimulates the innate immune response to HCV⁴⁷.

17 D614G mutation altered the stoichiometry of the main proteoforms of spike. Spike
18 proteoforms include S, the N-terminal S1 and the C-terminal S2 or S2' fragment which
19 result from endoproteolytic cleavage at the first polybasic (furin) or second (TMPRSS2)
20 cleavage site, respectively (Figure 2A). S1 was below the level of detection in D614
21 spike expressing cells, whereas the mutation D614G prevented degradation of S1 and
22 stabilized the ratio of S1 : S2 at close to 1:1, as described previously²² (Figure 2B). S2
23 was present in similar amounts to S (S2:S ~ 1:1) in both D614 and G614 spike
24 expressing cells.

25 We found that heat shock cognate 71 kDa protein (HSP7C), calnexin (CALX), and UDP-
26 glucose:glycoprotein glucosyltransferase 1 (UGGT1) were enriched by a factor of two or
27 more in D614 over G614 spike immunoprecipitations despite the absence of S1 in D614
28 spike expressing 16HBE⁻ cells (Table 1). In addition, tubulin- β (TUBB, TUBB4B),
29 filamin-A (FLNA), plectin (PLEC), and laminin- β 3 (LAMB3) were increased in D614 over
30 G614 spike immunoprecipitations. The difference in binding can only be explained if
31 host interactors bind to one or several cryptic motifs in S2 that are accessible only in the
32 absence of S1 or alternatively in S with RBD in 'open' conformation (Figure 2C). RBD
33 has previously been estimated to be in an 'open' conformation in an estimated 28% of
34 spike proteins⁴⁸. Here, we used Covalent Protein Painting (CPP)⁴⁹ on lysine residue
35 P0DTC2#K97 in spike, and found that RBD is in an open confirmation in ~ 20 % of
36 G614 spike. Because D614 spike sheds S1, the number of 'S2 only' proteoforms is
37 increased by approximately 33% which in turn increases the accessibility of the cryptic
38 motifs by ~ 1.8-fold. Thus, cellular proteins that are enriched in D614 spike
39 immunoprecipitations may bind to S2 at cryptic binding motifs that are accessible in 'S2

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1 only' proteoforms that have become available by the increased shedding of S1 and in
2 'open', not 'closed', RBD conformations of S.

3 HSP7C, CALX, and UGGT1 all retain nascent proteins in the ER to ensure proper
4 folding and refolding, or channel misfolded proteins to the ERAD. Spike D614 also
5 bound more of the stress induced molecular chaperone GRP78/BiP. Western blots
6 using a second set of biological replicate immunoprecipitations showed higher variability
7 of GRP78/BiP in G614 than in D614 immunoprecipitations (Figure 3A). GRP78/BiP is
8 exported with SARS-CoV-2 virions during virus egression⁹ – potentially through the
9 lysosomal pathway – and may facilitate SARS-CoV-2 cell infection as an alternative
10 host factor that aids in virus cell entry⁵⁰. Recent amino acid sequence homology
11 comparisons suggest GRP78/BiP may bind to a region in RBD in S1^{51,52} that
12 encompasses the amino acid E484 in spike. Mutation (E484K) has been associated
13 with SARS-CoV-2 mutant strains that can escape an initial humoral immune response
14 to SARS-CoV-2⁵³.

15 Further, we focused on the interaction of D614G spike with calnexin. Western blot
16 analysis confirmed that less calnexin associated with G614 than D614 spike
17 (Figure 3B), as observed with CoPIT (Figure 3C). We used immunostaining to
18 determine the localization of spike with calnexin in 16HBEo⁻ cells (Figure 3D). A
19 quantitative analysis of the immunofluorescence images revealed that less calnexin
20 localized with G614 spike than with D614 spike. Moreover, G614 but not D614 spike
21 was visible in (calnexin-negative) cellular substructures which tended to be closer to the
22 cell membrane. Thus, maturing G614 spike might escape the ER quality control better
23 than D614 spike that includes high levels of spike 'S2 only' proteoforms.

24 ***Virus tropism based on the virus-host interactome***

25 Tropism, defined as the cell types and tissues that can be infected by a virus, is
26 established by the host interactome of a virus, especially in the event of zoonosis. We
27 wanted to know whether the protein interactions of spike – and more specifically of 'S2
28 only' proteoforms in D614 spike – may lead to an ACE2 independent entry into cell
29 types previously not associated with SARS-CoV-2 infection. To answer this question
30 we devised a novel, proteome-based cell type set enrichment analysis (pCtSEA) to
31 uncover SARS-CoV-2 virus tropism.

32 First, we sought to find out which lung cell types are most likely the target of SARS-
33 CoV-2 based on the host proteins we identified with the 2xStrep tagged spike protein
34 (Figure 4A). We used standard cell culture conditions to maintain proliferating cells;
35 however, a subset constantly undergoes terminal differentiation, and ACE2 expression
36 can be detected by RT-PCR⁵⁴. We determined the Pearson's correlation of spike
37 protein interactors with the digital gene expression (dge) in single cells isolated from
38 human lung tissue⁵⁵. The original analysis included a large number of non-annotated
39 single cells (unknown) and seven distinct lung cell types (basal, ionocytes, brush,

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1 secretory, FOXN4⁺, ciliated, and SLC16A7⁺ cells) based on differences in dge. Single
2 cells with a correlation of $p > 0.4$ were sparsely scattered in non-annotated cells, basal
3 and SLC16A7⁺ cells in the original single cell plot of the dataset ⁵⁵. Overall, the dge in
4 ciliated cells matched ($p > 0.5$) more frequently with the host interactome than with any
5 other cell type. This initial analysis indicated that ciliated epithelial cells in the lung are
6 likely the cell type that is most susceptible to a productive SARS-CoV-2 infection in the
7 lung.

8 Next, we improved pCtSEA so we could examine a larger panel of human single cells ⁵⁶
9 using a sum of host proteins identified either with flag or 2xStrep tagged spike
10 protein. The interactome matched 918 dge patterns out of 643,636 single cell
11 measurements with a Pearson's correlation of $p > 0.1$. Sixty-seven of the 918 single
12 cells were macrophages (hypergeometric test p-value $< 9 \times 10^{-5}$). Macrophages were
13 significantly enriched in a two-sample Kolmogorov-Smirnov goodness of fit test of all
14 rank ordered single cells that passed an initial Pearson's correlation of $p > 0.1$ (K-S p-
15 value < 0.001 , Figure 4B). Seventeen additional cell ontology terms were significantly
16 enriched (K-S p-value < 0.001) within all 117 cell ontology terms that were represented
17 by the 918 single cells (Supplementary data 2). The remaining 99 cell types were
18 depleted or represented by only few single cells.

19 The 18 cell types and cell ontology terms clustered in three different groups in a two-
20 dimensional, uniform manifold approximation and projection map (UMAP) plot based on
21 the single cell transcriptomes that overlapped with the host interactome of the spike
22 protein (Figure 4C). Each group in the UMAP included one cell type that was
23 significantly enriched when compared to a random distribution of p-values obtained by
24 permutating the cell types in the dataset (normalized enrichment score). The significant
25 cell ontology terms were (1) 'distal tubule' of the nephron or (2) 'macrophage' or (3)
26 'mucous' tissue-associated cells. Two of these three groups comprised cell types of
27 similar cell ontology. For example, the group with the cell type 'macrophage' also
28 included 'dendritic cells' which are both mononuclear phagocytes of the immune
29 system ⁵⁷. Cell types like 'distal tubule' and 'loop of Henle' of the nephron clustered
30 together in the second group. Finally, monocytes and hepatocytes and cells of
31 undefined cell ontology ('unknown') clustered with the cell ontology term
32 'mucous'. Secretory cell types, which are characteristic of mucous tissues such as the
33 sinus, bronchial, lung, and digestive tract epithelia, are ACE2/TMPRSS2 positive ⁵. A
34 subset of the cell types is ACE2/TMPRSS2 negative and therefore potentially prone to
35 SARS-CoV-2 infection in the presence of 'S2 only' proteoforms. Thus, an in-depth
36 analysis of the SARS-CoV-2 spike protein interactome with pCtSEA identified three
37 different groups of cell types that are potentially prone to a productive SARS-CoV-2
38 infection.

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1 pCtSEA incorporates relative abundance of host interactors as a measure for cell type
2 enrichment. We compared the relative expression levels of host interactors between
3 the three groups (Figure 5). Laminin LAMB3 and LAMC2 were increased in the group
4 labeled ‘mucous’ over ‘distal tubule’ and ‘macrophage’. A lack of filamin A (FLNA) and
5 reduced relative levels of calnexin (CANX) but increased plectin (PLEC) characterized
6 the group ‘distal tubule’. Thus, spike may preferentially disrupt hemi- and desmosomes
7 formed by plectin in infected cells in the nephron. Likewise, spike might disrupt
8 membrane anchoring of the actin cytoskeleton (FLNA) in infected cells within both
9 groups cell types ‘macrophage’ and ‘mucous’.

10 Discussion

11 This study revealed a small number of host proteins that specifically interact with the
12 SARS-CoV-2 spike protein. A subset of spike protein interactors points to an
13 ACE2/TMPRSS2 independent cell entry which might explain an infection of
14 ACE2/TMPRSS2 negative tissues with SARS-CoV-2. We found three major
15 proteoforms encompassing full length, non-cleaved spike protein (S) or
16 endoproteolytically cleaved spike protein (S1-S2/S2’) or ‘S2 only’ without S1. All
17 proteoforms are expressed in 16HBEo⁻ cells and presumably present on SARS-CoV-2
18 virus particles following infection of ciliated epithelial cells. Because S1 harbors the
19 RBD, ACE2 is the preferred entry receptor during initial virus infection. However, the N-
20 terminal domain NTD in S1 of several different coronaviruses has been shown to bind to
21 glycosaminoglycans⁵⁸ or sialoglycans on glycoproteins and gangliosides turning them
22 into co-receptors or potential restriction factors⁵⁹. We confirmed that D614 spike yields
23 more ‘S2 only’ proteoforms, as observed previously. We speculate that the FBX2
24 associated ubiquitination complex may preferentially recognize N-glycosylated, high-
25 mannose S1 at N234¹⁴ in D614 over G614 spike, which may target it more efficiently to
26 ERAD. Further, we suspect that S2 in the absence of S1 may expose an otherwise
27 cryptic receptor binding site which enables an alternative virus entry. Antibodies that
28 bind to S2 with very high affinity and neutralize SARS-CoV-2 virus entry have been
29 identified³³ and may confer protection against severe COVID-19⁶⁰. Thus, we propose
30 that loss of S1 alters the proteoform composition of spike in trimers, which in turn allows
31 for infection of cells through an alternative, ACE2-independent virus entry. We
32 speculate that extracellular laminins may serve as additional co-receptors to increase
33 virus uptake.

34 This interpretation is supported by a pCtSEA analysis of the host interactome of
35 spike. pCtSEA indicated that epithelial cells in the nephron and macrophages might get
36 infected with SARS-CoV-2 in addition to lung epithelial cells. Notably, the immune cells
37 identified with pCtSEA may be prone to antibody-dependent enhancement (ADE) of
38 infection with β -coronaviruses⁶¹ in the absence of ACE2. The cell types identified with
39 pCtSEA reside in several different human tissues including muscle and brain

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1 (oligodendrocytes and astrocytes). We speculate that an expanded tropism of SARS-
2 CoV-2 might shed light on a prolonged progression of COVID-19 in select patients. A
3 pCtSEA analysis can indicate whether a virus-host interactome in its native reservoir
4 may permit a zoonosis, as observed in the COVID-19 pandemic. Indeed, when the
5 virus did not co-evolve with the host, a high variability of host-virus protein-protein
6 interactions may arise from introducing viral proteins into a novel, differently equilibrated
7 host environment. Hence, it might explain the high variability in host interactors of
8 SARS-CoV-2 that were identified in different cell lines and tissues^{25,28,62}.

9 Our data analysis suggests that SARS-CoV-2 may utilize two alternate paths for cell
10 entry: one path exploits ACE2 receptor binding, and the other path may be based on
11 'S2 only' proteoforms that may bind to laminins as coreceptors. As a consequence, an
12 adaptive immune response that targets almost exclusively antigenic S1 may fail to
13 completely curb severe or persistent infections with SARS-CoV-2. Therefore, the host
14 interactome of spike suggests that immunization strategies might provide increased
15 protection against COVID-19 if the 'S2 only' proteoform is also used as immunogen.

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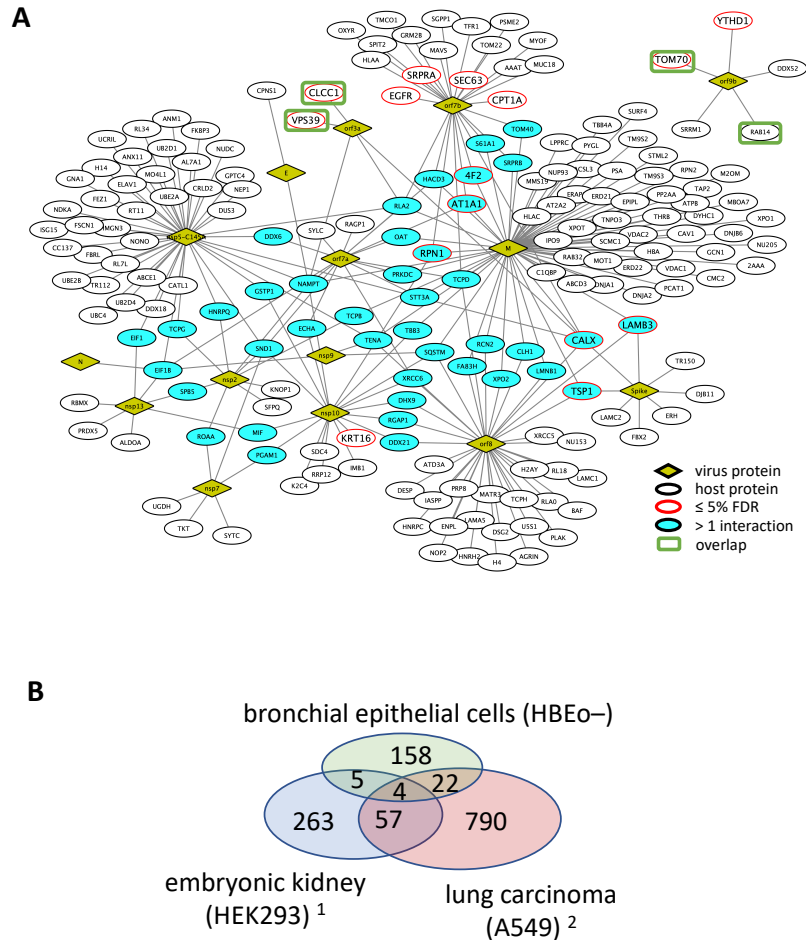
| Protein symbol | Ratio (G614/D614) | p-value | UniProt KB | Protein |
|----------------|-------------------|---------|------------|--|
| SRP72 | G614 only | 0.00 | O76094 | Signal recognition particle subunit SRP72 |
| HMGB2 | G614 only | 0.00 | P26583 | High mobility group protein B2 |
| HMGB1 | 1.97 | 0.09 | P09429 | High mobility group protein B1 |
| HMGN2 | 3.00 | 0.00 | P05204 | Non-histone chromosomal protein HMG-17 |
| TBB5 | 0.69 | 0.09 | P07437 | Tubulin beta chain |
| PLEC | 0.68 | 0.08 | Q15149 | Plectin |
| TBB4B | 0.65 | 0.06 | P68371 | Tubulin beta-4B chain |
| ADT2 | 0.63 | 0.05 | P05141 | ADP/ATP translocase 2 |
| FLNA | 0.63 | 0.05 | P21333 | Filamin-A |
| LAMB3 | 0.59 | 0.03 | Q13751 | Laminin subunit beta-3 |
| BIP | 0.56 | 0.02 | P11021 | Endoplasmic reticulum chaperone BiP |
| HSP7C | 0.55 | 0.02 | P11142 | Heat shock cognate 71 kDa protein |
| CALX | 0.50 | 0.01 | P27824 | Calnexin |
| UGGG1 | 0.48 | 0.00 | Q9NYU2 | UDP-glucose:glycoprotein glucosyltransferase 1 |

1
2 **Table 1: Differential binding of host interactors with spike protein variants G614**
3 **versus D614.** Rows highlighted in red are host interactors that are enriched in
4 immunoprecipitations of G614 spike whereas host interactors that preferentially bind to
5 D614 are highlighted in green. The columns indicate the protein symbol (UniProt), the
6 ratio of differential presence and the p-value of detection as determined with
7 CoPIT. The UniProt identifier and name of each protein are listed separately.

8

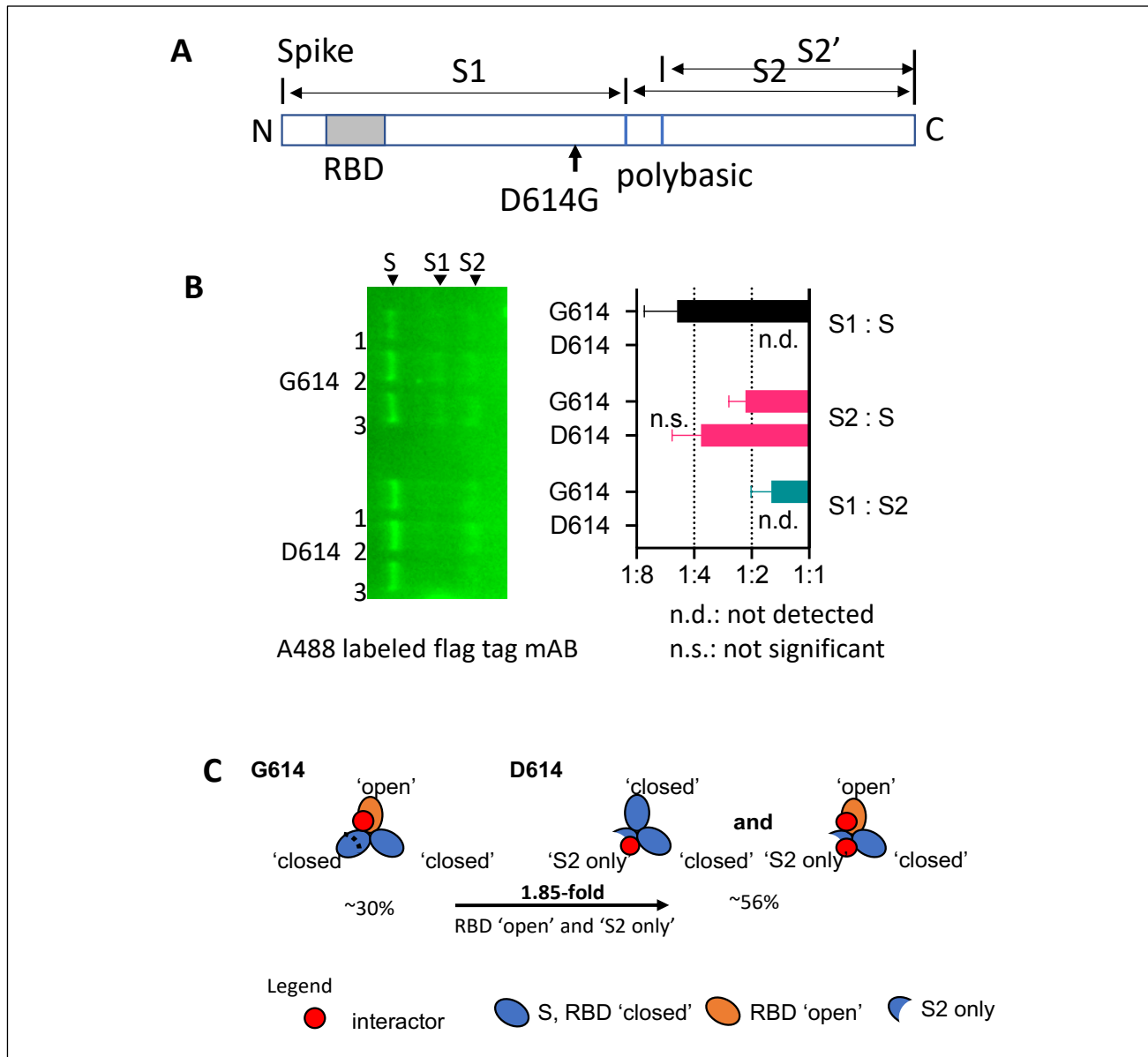
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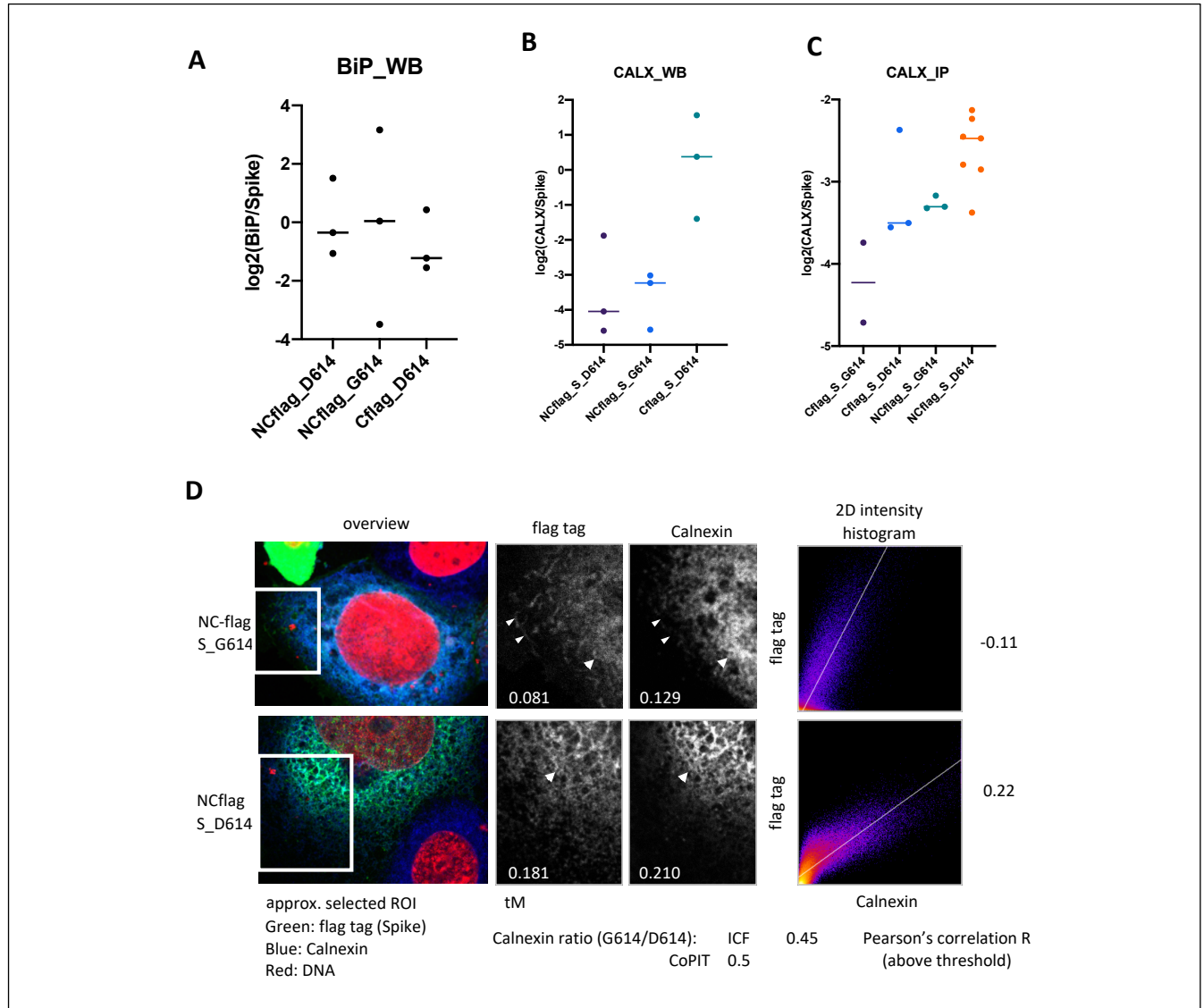


1

2 **Figure 1 Interactome of select SARS-CoV-2 ORFs with host proteins in 16HBEo⁻**
 3 **cells. (A)** The edges in the protein-protein network indicate host proteins (ovals) that
 4 were identified in immunoprecipitations of viral proteins (diamonds). Host interactors
 5 that associated with more than one host protein or that are of high confidence
 6 (BFDR \leq 5%) are highlighted in turquoise and red, respectively. **(B)** The Venn diagram
 7 shows the overlap of the host interactomes in HEK293²⁵, A549²⁸ and 16HBEo⁻ cells
 8 (this study). The green rectangles in **(A)** indicate three of the four host interactors that
 9 were identified in all three cell lines.

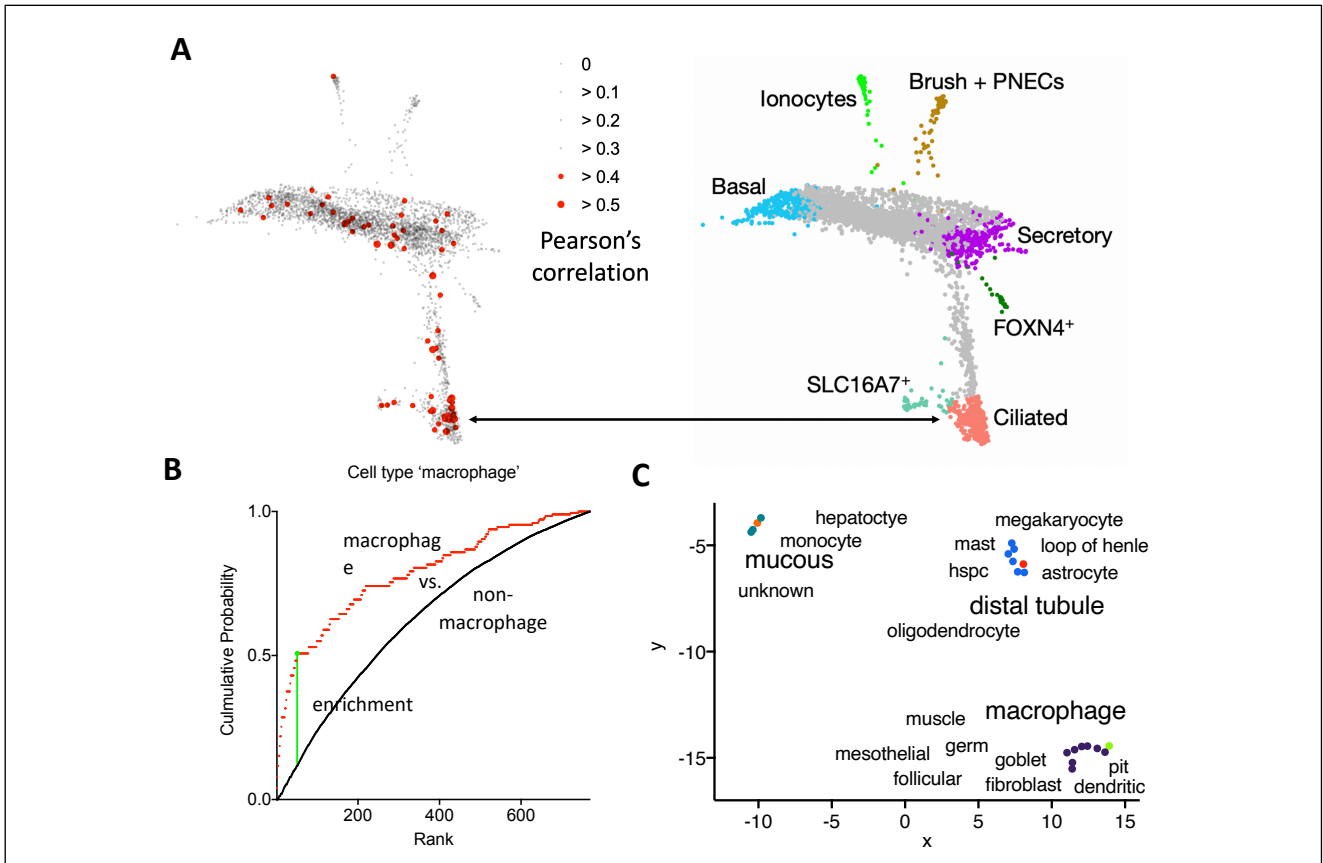


1
2 **Figure 2: (A)** Schematic of the spike protein and polybasic cleavage sites. Full-length
3 spike protein (S) can be cleaved into an N-terminal proteoform S1 and two different C-
4 terminal proteoforms S2 and S2' which are subsumed in 'S2 only'. **(B)**
5 Immunodetection of flag tagged spike proteoforms with Alexa 488 labeled flag tag
6 mAb. The bar graph indicates the relative abundance of full-length spike S and of the
7 endoproteolytic cleavage products, the spike proteoforms S1 and S2/S2'. Full-length
8 spike (S) harbors one anti-flag tag antibody binding site at the N- and C-terminus. **(C)**
9 The schematic indicates the presence of host interactor binding sites on S that overlap
10 between 'S2 only' and the RBD 'open' conformation in S. Percentages indicate the
11 increase (~2 fold) in 'S2 only' binding site availability in D614 over G614 spike.



1 **Figure 3: Interaction of spike with BiP and Calnexin.** (A) The plot shows the
 2 relative abundance of GRP78/BiP to spike in three independent immunoprecipitations of
 3 spike. (B) Calnexin was detected and quantified in spike G614D immunoprecipitations
 4 in different biological replicate experiments by Western blot (B) or mass spectrometry
 5 (C). Calnexin was detected by chemiluminescence (horseradish peroxidase coupled
 6 secondary antibody). Horizontal bar: Median. (D) Confocal images were analyzed for
 7 colocalization of spike with calnexin in immunofluorescence stained 16HBEo- cells in
 8 the regions of interest depicted in the overview. Arrows indicate colocalization of spike
 9 with calnexin whereas arrowheads point out spike protein that did not colocalize with
 10 calnexin. The colocalization of spike and calnexin above threshold was quantified with
 11 Mander's overlap coefficient (tM) and Pearson's correlation.

12

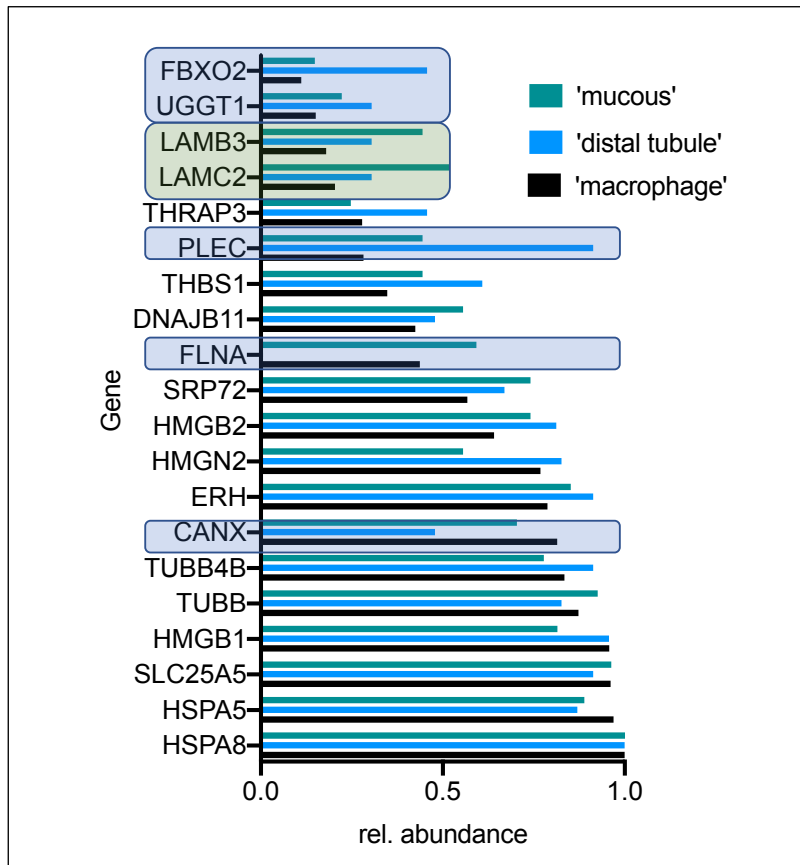


1

2 **Figure 4: pCtSEA analysis of the SARS-CoV-2 tropism based on the spike protein**
 3 **interactome. (A)** The scatter plot highlights the limited overlap of the host interactome
 4 with single cell transcriptomes of different lung cell types (adopted from ⁵⁵). The host
 5 interactome of spike was correlated with the digital gene expression levels of single lung
 6 cell transcriptomes taking the presence and relative abundance of interactors into
 7 account. **(B)** The plot of the cumulative probability shows the weighted empirical
 8 distribution of macrophage cells in which the transcriptome correlated with the spike
 9 host cell interactome (red line), whereas the black line is the empirical distribution of all
 10 'non-macrophage' cell types with $p > 0.1$. The green vertical line indicates the
 11 suprema. **(C)** Additional cell types that may be prone to SARS-CoV-2 infection are
 12 plotted in a uniform manifold approximation and projection (UMAP) which shows three
 13 clusters based on a differential presence of host interactors in the single cell
 14 transcriptomes.

15

1



2 **Figure 5:** The bar graph indicates the relative abundance of gene transcripts in the
3 three groups labeled 'mucous', 'distal tubule' and 'macrophage' that were identified
4 initially with pCtSEA. Genes that deviate in expression levels relative to the group
5 'macrophages' are boxed in light blue or green for the groups 'distal tubule' or 'mucous',
6 respectively.

7

1 **Methods**

2 **Expression plasmids**

3 All SARS-CoV-2 ORF expression plasmids were obtained from the laboratory of Nevan
4 Krogan⁶³. Viral ORFs were provided as inserts into the vector backbone pLVX-
5 EF1alpha-2xStrep-IRES-Puro except for the spike protein cDNA which was cloned into
6 pTwist vector and extended at the C-terminus with a 2xStrep tag. Expression vectors
7 for the N-terminal or N- and C-terminal flag tagged spike protein variants D614 and
8 G614 were kindly provided by Hyeryun Choe's laboratory^{25,64}.

9 **Cell culture, transfection and transduction**

10 HEK293T cells were grown at 37 °C and 5 % CO₂ in DMEM supplemented with 10%
11 tetracycline-free fetal bovine serum and 1% Penicillin-Streptomycin (Invitrogen). Cells
12 were transfected with the LentiX VSV-G transfection mix (Clontech) and different SARS-
13 CoV-2 ORFs (cloned into pLX vector). VSV-G pseudo-typed lentiviral particles were
14 collected 48 h later and filtered through a 0.22 µm filter. Human bronchial epithelial
15 cells (16HBEo-) were transduced with VSV-G pseudo-typed lentiviral particles in the
16 presence of 3 µg/ml polybrene (Millipore). Expression vectors encoding the spike
17 protein cDNA were directly transfected into 16HBEo- with Lipofectamine P3000
18 according to manufacturer's recommendations (Invitrogen). Transduced and
19 transfected 16HBEo- cells were grown at 37 °C and 5 % CO₂ for additional 48 h prior to
20 harvest.

21 **Immunoprecipitation**

22 Cells were lysed in TNI lysis buffer (50 mM Tris pH 7.5, 250 mM NaCl, 1 mM EDTA,
23 0.5 % Igepal CA-630, 1 x Complete ULTRA EDTA-free protease inhibitor mix (Roche),
24 1 x HALT Phosphatase inhibitor mix (Thermo) for 20 min at 4 °C and further processed
25 for immunoprecipitation according to⁴⁶. 2xStrep tagged SARS-CoV-2 ORFs were
26 immunoprecipitated using 30 µl of Megastrep type 3 XT beads (IBA LifeSciences,
27 Germany), and flag-tagged SARS-CoV-2 ORFs were immunoprecipitated using Anti-
28 DYKDDDDK Magnetic Agarose (Pierce) at 4 °C for 16 h. Beads were washed 3 x with
29 10 volumes of TNI buffer (50 mM Tris pH 7.5, 250 mM NaCl, 0.5 % Igepal CA-630) and

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1 2 x with 10 volumes of TN buffer (50 mM Tris pH 7.5, 250 mM NaCl, 1 mM EDTA)
2 before elution with 8 M urea, 5 mM TCEP, 0.1 % (w:v) Rapigest (Waters) for 15 min at
3 60 °C.

4 **Digestion, sample purification and mass spectrometry**

5 Eluted proteins were alkylated with chloroacetamide (10 mM, 30 min, 37 °C) and
6 digested at 37 °C for 16 h in 100 mM Tris HCl, pH 7.2, in the presence of 0.5 to 0.8 M
7 urea with the endoprotease Trypsin (Promega). Peptides were subsequently bound to
8 the reversed phase in Evotips (Evosep) and washed according to manufacturer's
9 recommendations. Each immunoprecipitation was measured in technical
10 duplicate. Following elution and chromatographic separation of peptides on a 15 cm
11 ReproSil C18 column (3 µm, 120 Å, id 100 µm, PepSep) with a 45 min gradient
12 (Evosep), peptides were detected and fragmented with a timsTOFpro mass
13 spectrometer (Bruker) with PASEF enabled ⁶⁵.

14 **Data analysis**

15 Fragment ion mass spectra were extracted from timsTOFpro raw data files and stored
16 in the ms2.txt flat-file format. Fragment ion spectra were searched with Prolucid ⁶⁶ with
17 the forward and reversed human UniProt database v.07-01-2020, and filtered with
18 DTASelect ⁶⁷ to a false discovery rate of < 1 %. Immunoprecipitation experiments were
19 filtered with SAINTexpress to remove non-specific background proteins ⁶⁸
20 (Supplementary Table 1). The final virus-host protein-protein interactome includes all
21 2xStrep tagged virus-host protein interactions. Due to a different background of
22 contaminants of flag versus 2xStrep tag, flag-tag immunoprecipitations were not further
23 considered for the spike protein in the final network of virus-host interactions
24 immunoprecipitations (Figure 1A). Spike protein immunoprecipitations of the variants
25 D164 and G614 were directly compared with CoPIT ²⁶. Interactors present in ≥ 2
26 biological replicates and detected as differentially expressed with a cut off value of 0.1
27 (CoPITgenerator) were further analyzed. Keratins are often observed as contaminants
28 in immunoprecipitation experiments from epithelial cell lines and therefore were
29 excluded from further analysis.

30 **Immunofluorescence detection and protein co-localization**

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1 48 hours post transfection cells were washed with 1x PBS, fixed in 4%
2 paraformaldehyde/1x PBS for 15 min at RT, permeabilized with 0.1 % Triton X-
3 100/1xPBS for 10 min at RT, washed three times with 1x PBS and incubated with 5 %
4 horse serum/1xPBS for 1 h at 20 °C before incubating with the following antibodies (Cell
5 Signaling Laboratories): anti-calnexin (clone C5C9), anti-BiP (clone C50B12), anti-
6 Hsp90a (clone D1A7), anti-ZO1 (clone D7D12), anti-ZO2 (2847), anti-ZO3 (D57G7),
7 anti-claudin 1 (D5H1D), anti-CD2AP (2135), anti-afadin (clone D1Y3Z) or Alexa
8 Fluor 488 anti-DYKDDDDK (flag) tag monoclonal antibody (L5, Life Technologies). The
9 plasma membrane was stained with CF594 dye conjugated wheat germ agglutinin
10 (Biotium), while ER was stained with the ER Cytopainter staining kit (AbCam) according
11 to manufacturer recommendations. Images were taken with a Zeiss LSM 780 confocal
12 laser scanning microscope and analyzed using Fiji/ImageJ (v.2.1.0/1.53c).

13 **Western blotting**

14 One volume of 2x SDS sample loading buffer was added to samples in TN buffer
15 (1:1; v:v) and samples were heated to 50°C for 15 min. Proteins were separated on
16 NuPAGE 4-12% Bis-Tris gels (Life technologies) before transferring proteins onto
17 Nitrocellulose membranes and incubating with anti-BiP (C50B12, Cell Signaling
18 Laboratories (CSL)), anti-ZO1 (D7D12, CSL), anti-ZO2 (2847, CSL) or Alexa Fluor 488
19 conjugated anti-DYKDDDDK Tag monoclonal antibody (L5, Sigma) in 5 % BSA,
20 1x PBS, 0.05 % Tween-20. Signals were detected either using Radiance Q ECL
21 reagent (Azure Biosystems) after washing and incubation with HRP coupled anti-rabbit
22 antibodies (CSL) or imaged directly using an Azure 600 Western blot imaging
23 system. Western blot images were quantified in Fiji/ImageJ.

24 **Proteome based Cell Type Set Enrichment Analysis (pCtSEA)**

25 The input dataset for a pCtSEA analysis can be the host interactome of SARS-CoV-2 or
26 any proteome that is listed with a UniProt identifier and an abundance value (Spectral
27 counts, SpC). Importantly, pCtSEA assumes that at least a partial stoichiometric
28 relationship exists between the relative abundance of mRNA transcripts in cells the
29 proteins in the interactome. First, single cell transcriptomes are filtered for the
30 expression of ≥ 10 genes that are also present in the proteome. All protein abundances

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1 are correlated with the single cell digital gene expression (dge) values (Pearson's
2 correlation), and each cell type or cell ontology term with ≥ 10 cells that pass the
3 Pearson's correlation threshold of $p > 0.1$ is further evaluated. pCtSEA calculates the p-
4 value of the hypergeometric test and an enrichment score based on a two-sample
5 Kolmogorov-Smirnov goodness of fit test (K-S test) in which the empirical cumulative
6 distribution function of cells specific for a cell type is compared to the remaining cells
7 when rank ordered by their correlation values. In addition, the value and rank position
8 of the supremum is reported. Furthermore, each cell type enrichment score is
9 differentiated from a population of enrichment scores (suprema) that is obtained in an
10 iterative process in which cell type labels are randomly permuted, yielding an
11 empirical p-value that indicates the significance of enrichment. Finally, the total
12 distribution of all enrichment scores of all cell types is corrected for multiple hypothesis
13 testing and a false discovery rate (FDR) associated with each cell type.

14 Cell types that are significantly enriched (K-S test) are clustered based on the relative
15 overlap of the proteins that correlated with the single cell gene expression pattern using
16 a uniform manifold approximation and projection (UMAP). pCtSEA is accessible as web
17 tool: <http://pctsea.scripps.edu>.

18

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21 D614G. The thank Claire Delahunty for critically reading the manuscript.

22

23 **Author Contributions**

24 C.B. and S.P. designed and executed the experiments. J.D. analyzed the samples on
25 the mass spectrometer, R.P. performed data analysis of mass spectrometric data. C.B.
26 conceived pCtSEA and S.M.-B. realized pCtSEA. J.R.Y. provided funding and
27 instrumentation. C.B. performed data analysis, prepared figures and wrote the
28 manuscript.

29 **Competing financial interest**

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1 The authors declare no competing financial interests.

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4 awarded to John R. Yates III.

5

6 **Extended data network 1: The virus-host protein network of select SARS-CoV-2**
7 **open reading frames in 16HBEo-cells is depicted.** The bipartite network is available

8 at the url: <http://ndexbio.org/#/network/cb71d1f8-12fa-11eb-9eee->

9 [0ac135e8bacf?accesskey=1713beeb8f740c163894126d8346ea4c20edbf1e4910f4da41](http://ndexbio.org/#/network/cb71d1f8-12fa-11eb-9eee-0ac135e8bacf?accesskey=1713beeb8f740c163894126d8346ea4c20edbf1e4910f4da41c6bdd153f673b5)
10 [c6bdd153f673b5](http://ndexbio.org/#/network/cb71d1f8-12fa-11eb-9eee-0ac135e8bacf?accesskey=1713beeb8f740c163894126d8346ea4c20edbf1e4910f4da41c6bdd153f673b5)

11 **Supplementary data 1:** The table lists all proteins detected in the IP-MS experiments
12 and their respective scores upon analysis with SAINTexpress for the probability of a
13 virus-protein interaction.

14 **Supplementary data 2:** pCtSEA analysis of the host interactome of spike in 16HBEo-
15 cells.

16 **Data availability:** Mass spectrometric fragmentation ion spectra and quantification files
17 can be accessed in Massive (MSV000086433) or ProteomeExchange
18 (PXD022457). Access will be made available upon request by reviewers.

19

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21

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