Whole Genome CRISPR Screening Strategy to Identify Genes Contributing to SARS-CoV-2 Spike and VSV-G Mediated Entry.

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Abstract

Background Understanding the cellular host factors that promote and inhibit viral entry is important for identifying viral countermeasures. CRISPR whole genome screens can be used to rapidly discover host factors that contribute to or impair viral entry. However, when using the live viruses and cellular lethality for selection, these screens can identify an overwhelming number of genes without specificity for the stage of the viral infection cycle. New screening methods are needed to identify host machinery contributing to specific steps of viral infection. Here, we developed a CRISPR whole genome screen and counter screen strategy based on a pseudoviral platform that allowed identification of genes specific to SARS-CoV-2 spike and vesicular stomatitis virus glycoprotein VSV-G mediated entry. **Methods** To focus the screen onto the entry step, we used non-lytic fluorescent reporters in combination with a comparative counter screen strategy to distinguish host genes affecting the pseudoviral reporter from those unique to envelope-mediated entry. Screening of SARS-CoV-2 spike and VSV-G on the same lentiviral pseudovirus allowed identification of entry-specific genes relative to genes associated with retro-transcription, integration, and reporter expression from the lentiviral pseudovirus. Second, a Cre-Gag fusion protein in the pseudovirus was used to bypass retro-transcription and integration by directly activating a floxed GFP reporter upon entry to reduce the number of gene hits and increase specificity for viral entry. **Results** Our approach correctly identified SARS-CoV-2 and VSV-G receptors ACE2 and LDLR, respectively and distinguished genes associated with retroviral reporter expression from envelope-mediated entry. Moreover, the CRE-Gag fusion/flox reporter increased the screen specificity for viral entry associated genes. Validation of a few hits demonstrates that this approach distinguishes envelope-specific host factors from genes affecting reporter expression. **Conclusion** Overall, this approach provides a new strategy for identifying host genes influencing viral entry without the confounding complexity of live-viral screens which produce long gene lists associated with all aspects of viral pathogenesis and replication. This approach provides a pathway for increasing the specificity of CRISPR whole genome screens for identifying host genes contributing to specific steps in viral infection.

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Short title: CRISPR screen for SARS-CoV-2 entry.

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Keywords: Sars-CoV-2, COVID-19, pseudovirus, CRISPR, VSV-G, screen.

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of the COVID-19 pandemic, has claimed over 6 million deaths worldwide and has disrupted the global economy (COVID-19 Dashboard by the Center for Systems Science and Engineering (CSSE) at Johns Hopkins University, accessed July 21, 2023). Mutations in the spike envelope protein of SARS-CoV-2 continue to accumulate contributing to new variants that may have altered infectivity. Additionally, new viruses will emerge with envelopes that influence their transmissibility and pathogenesis as evidenced by the emergence of new SARS-CoV-2 variants and the need for revised vaccines (Harvey et al., 2021; Mannar et al., 2022; Volz et al., 2021; L. Zhang et al., 2020). Thus, developing countermeasures requires new methods that can rapidly identify host factors affecting the viral infection.

CRIPSR/Cas9 has emerged as a powerful tool for identifying host factors affecting the viral life cycle. CRISPR screens against SARS-CoV-2, provide insight into these factors, however, nearly all screens run to date rely on cellular lethality leading to the depletion of host cells bearing all kinds of mutations that decrease viability during infection. These screens are therefore complicated by large numbers of host factors affecting all steps of the viral life cycle including entry, replication, pathogenesis and egress as well as host viability factors influencing energy metabolism, macromolecular synthesis and cell cycle regulation (Baggen et al., 2021; Daniloski et al., 2021; Schneider et al., 2021; Wang et al., 2021; Wei et al., 2021). The lack of specificity

in current screening strategies leads to a biased selection of target genes for validation, based on investigator preference, or costly and time-consuming rational validation screens (Baggen et al., 2021; Daniloski et al., 2021; Han et al., 2017; Schneider et al., 2021; Wang et al., 2021; Wei et al., 2021). Replication incompetent pseudoviral vectors, can deliver reporters without triggering cell death and therefore provide a strategy for isolating genes associated with viral entry. Indeed, lentivirus pseudotyped with SARS-CoV-2 spike protein have demonstrated great utility in the pandemic, serving as the basis for viral neutralization assays (Neises et al., 2021). Also, replication competent vesicular stomatitis virus (VSV) expressing SARS-CoV-2 Spike has been used in previously published CRISPR screens (Wei et al., 2021). Yet, improved strategies for identifying host factors affecting viral entry are needed.

Here we describe proof-of-concept findings for a method for identifying genes affecting viral entry of SARS-CoV-2 and VSV-G into target cells. Using the Brunello library, we mutagenized 293T-Ace2 cells and then screened them for entry of SARS-CoV-2 spike and VSV-G pseudoviruses with a GFP reporter (Doench et al., 2016). Comparative analysis of these screens demonstrated the feasibility of focusing CRISPR whole-genome screens down to genes influencing the specific step of envelope mediated entry and allowed identification of genes that were responsible for retro-transcription of reporter genes. Moreover, inclusion of a recently developed Gag-Cre auto-cleaving fusion into the VSV-G pseudoviruses and screening in 293T cells bearing a floxed fluorescent protein reporter increased the specificity for the genes identified to viral entry (Esposito et al., 2016; P. E. Mangeot et al., 2019). This method identified the known ACE2 receptor as a top hit gene in our SARS-CoV-2 spike screen and LDL receptor as one of the top hits in our VSV-G-GagCre screens as well as other putative genes. Overall, this strategy provides a new approach for identifying host genes specific to envelope mediated entry and will be of use for new studies targeting viral entry.

Results

Genome-wide CRISPR screening strategy to selectively identify genes promoting and inhibiting envelope mediated entry.

Our strategy for genome-wide loss of function screens to identify genes facilitating and inhibiting viral entry is illustrated in Figure 1A. 293T cells or variants (described below) were mutagenized by transduction with the Brunello pooled sgRNA library containing 76,441 single gRNA targeting 19,114 human genes (Doench et al. 2016). After 72h of infection with the Brunello library, we then selected these cells with puromycin for 5 days and cultured 5 more days to allow for protein depletion. Reporter viruses consisted of lentiviral vectors that deliver mNeonGreen pseudotyped with SARS-CoV-2 spike (Spike-mNG) or VSV-G (VSVG-mNG), with the rationale that comparative analysis of genes associated with entry would be distinct between screens and that genes influencing expression of the reporter, such as viral integration would be common (Figure 1A). Additionally, a Gag-Cre fusion protein, that undergoes cleavage upon viral maturation, thus upon fusion with the target cell membrane, Cre is able to activate a floxed dsRED/eGFP reporter allowing further separation of host genes associated with viral entry from those that may influencing reporter expression (Figure 1B) (Esposito et al., 2016; P. E. Mangeot et al., 2019). After 48h of infection with the virus we collected the fractions of the total transduced population and cells sorted on high and low/no GFP fluorescence by fluorescent activated cell sorting (FACS). We then extracted genomic DNA from the sorted cells and PCR amplified the sgRNA for next generation high throughput sequencing (Figure 1A). Analysis using MAGeCK provided sgRNA frequencies in the high and low fractions for statistical comparison of gene-function associations (Chen et al., 2018; Li et al., 2014). The raw read counts from these screens can be found in NCBI GEO (accession number: **GSE206996**).

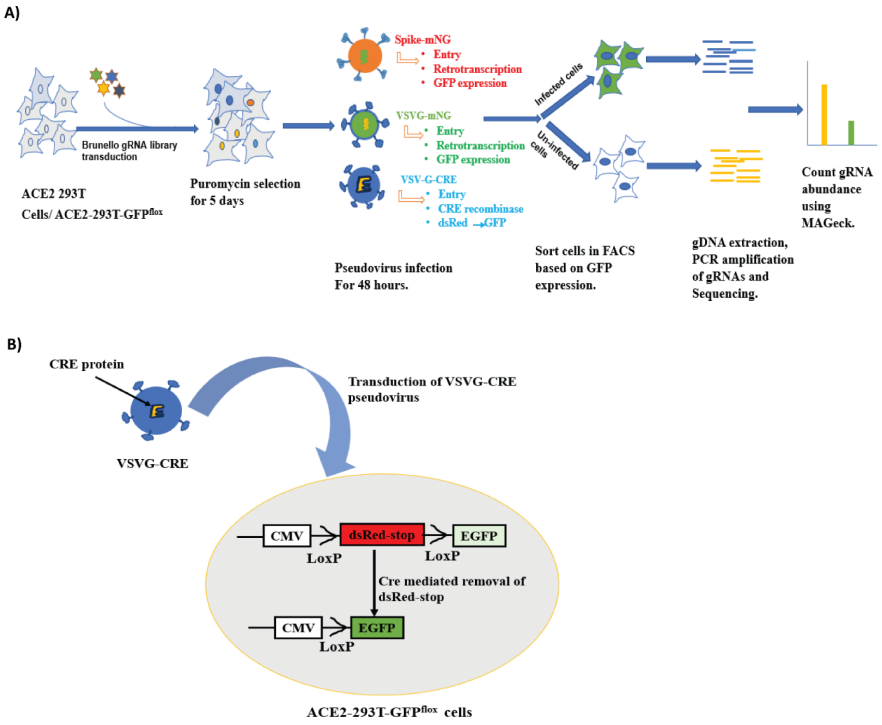


Figure 1: Whole genome screening strategy for entry of SARS-CoV-2-S, VSVG-mNG and VSVG-CRE pseudoviruses. A) ACE2 293T or ACE2-293T-GFP^{flox} cells were transduced with the Brunello sgRNA genome wide CRISPR library. These cells were selected with puromycin to kill off cells that did not integrate the sgRNA cassette. 5 days following selection, these cells were infected with SARS-CoV-2 Spike, VSV-G or VSV-G-Cre pseudoviruses and allowed 48h for reporter expression. The cells were sorted using FACS, genomic DNA was extracted, sgRNA sequences were amplified by PCR, sequenced by next generation sequencing and sgRNA abundance in the sample was quantified by MAGeCK. **B)** Representation of Cre-LoxP system in ACE2-293T-GFP^{flox} cells. The cells express dsRed and after excision of dsRed-stop by Cre recombinase, the cells start to express EGFP.

Development and validation of reporter pseudovirus in ACE2-293T with a floxed reporter.

Given that HEK 293T cells do not express ACE2, we transduced HEK 293T cells with lentivirus containing human ACE2 cDNA for stable expression of ACE2 gene and sorted these cells after 7 days of transduction to get uniform population of ACE2 expressing cells (Figure 2A). For the Gag-CRE fusion, a cell line was generated by lentiviral integration of a floxed dsRED/eGFP reporter which were sorted to produce a pure population of dsRED expressing ACE2-293T (Figure 2B). In all three cases, green fluorescence is a readout of viral entry. To create a screen that was sensitive to cells that contained genetic knock outs that blocked viral entry, we used viral titers that resulted in transduction of more than 90% of the ACE2-293T cells. To compensate for Spike-mNG pseudovirus having consistently lower titers than the VSVG-mNG we concentrated Spike-mNG by 50-fold. With this concertation step, all three pseudoviruses produced similar transduction frequencies with over 90% of the cells transduced (Figure 2C).

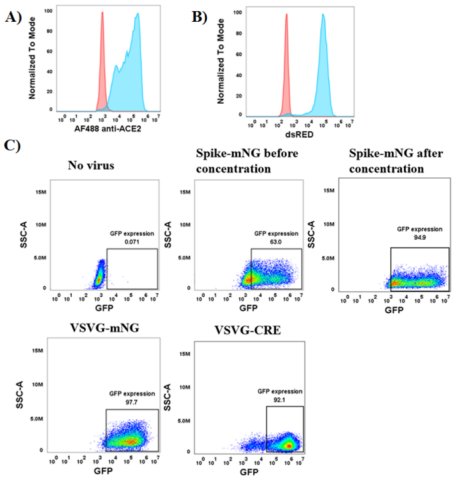


Figure 2: Generation of ACE2 293T and CRE reporter ACE2 293T cell lines and their characterization. A) 293T cells were transduced with lentivirus containing ACE2 cDNA for stable ACE2 293T cell line production. Cells were sorted after 72h of transduction after staining for ACE2 using AF488 conjugated anti-ACE2 antibody. The histogram shows ACE2 expression in these cells after sorting, red is the parental 293T cells and cyan is the ACE2 expressing 293T cells. **B)** ACE2 293T cells were transduced with lentivirus containing CRE reporter gene and cells were selected with blasticidin to get the uniform population of dsRED expression. **C)** GFP expression quantified after infection of ACE2 293T with Spike-mNG and VSVG-mNG or ACE2 293T-GFP^{floxed} with VSVG-CRE pseudoviruses. Spike-mNG pseudovirus was concentrated to allow for titers similar to VSVG-mNG.

Genes affecting viral entry were selectively identified with enhanced resolution with the CRE/floxed reporter over the lentiviral reporter.

We predicted that the known envelope receptors would be discovered by the screen with signal strength specificity of the selection. In other words, as the specificity of the molecular events is increased toward viral entry and fewer genes associated with other viral activities affect the selection, the frequency of sgRNAs targeting genes that influence entry will be enriched as there will be fewer competing gene sgRNA sequencing amplicons in the low-infection population. Thus, we expected that that the normalized Log fold change, a normalized frequency count and the primary statistical output of MaGeCK robust rank aggregation (RRA) score will increase as the pathway specificity increases, resulting in fewer genes affected (Li et al., 2014). Indeed, ACE2 was identified for Spike-mNG entry but not for VSVG-mNG or VSVG-CRE (Figure 3). Even though ACE2 expression in the ACE2-293T was driven from a virally integrated cDNA, the sgRNAs in the Brunello library were able to cut and disable the gene thereby preventing entry of Spike-mNG as expected. 293T cells without the ACE2 transgene were highly resistant to the reporter virus prior to expression of ACE2 (unpublished data, (Ou et al., 2020). We attempted to conduct the comparative screen using Spike-CRE, however we were unable to generate sufficient Spike-CRE reporter virus. In the case of VSVG-CRE, our screen identified the low-density lipoprotein receptor (LDLR, Figure 3C) which is a known receptor for VSV-G (Finkelshtein et al., 2013; Nikolic et al., 2018). The VSVG-mNG screen did not identify the LDLR (Figure 3B) with the LDLR producing a modes LFC (0.83) and weakly significant RRA score (0.0023) and rank of 205, which is trending toward importance for viral entry but much weaker than in the VSVG-CRE which had a LFC (1.32) and a highly significant RRA score ($9.23*10^{-6}$) and is ranked 13th. We interpret this improvement in the detection of the LDLR within the VSVG-CRE reporter screen over the VSV-G-mNG screen due to the reduction in overall noise arising from the additional genes influencing retro transcription and viral integration present in the VSVG-mNG screen, but not in the VSVG-CRE screen.

To compare the effect of the reporter with the same envelope, we plotted the negative Log (RRA) of VSVG-

mNG against negative Log (RRA) of VSVG-CRE for the top 15 ranked genes for loss of viral reporter expression in each screen (Figure 3D). Similarly, we plotted the Log (RRA) for VSVG-mNG against Spike-mNG to allow for comparison of the same reporter with different envelopes (Figure 3E). In these plots, genes strongly affecting both screens will appear in the upper righthand corner. In both plots, SLC35B2 appeared in the upper left indicating a role for facilitating viral entry (Figure 3D and 3E). This Golgi associated protein is a transporter for 3'-phosphoadenosine 5'-phosphosulfate and is required for sulfation of heparan sulfate proteoglycans and has been shown to contribute to entry for SARS-CoV-2, seasonal coronavirus, and a wide range of other viruses, likely by heparan sulfate mediated recruitment of viral particles to the cell surface (Clausen et al., 2020; Park et al., 2017; Schneider et al., 2021; Tandon et al., 2021; Wang et al., 2021; Q. Zhang et al., 2020).

The ER chaperone Gp96 (HSP90B1) was identified in VSVG-mNG and VSVG-CRE screens, but not in the Spike-mNG screen indicating that it is specific to VSV-G mediated entry (Figure 3). Prior work has shown that Gp96 facilitates the correct folding of proteins and is essential for the presence of functional LDLR at the cell surface and entry of VSV (Hastie et al., 2013). Its absence in our screen against Spike-mNG is consistent with it not being identified in other SARS-CoV-2 screens (Baggen et al., 2021; Daniloski et al., 2021; Schneider et al., 2021; Wang et al., 2021; Wei et al., 2021) and is consistent with HSP90B1 being a host factor associated with the cell surface expression of the LDLR and VSV-G mediated entry.

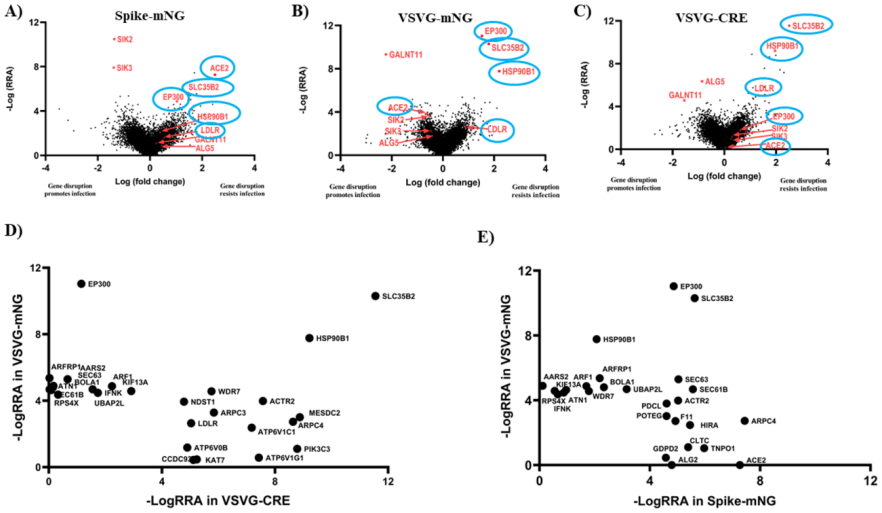


Figure 3: Genome wide CRISPR screen against pseudoviruses reveals host factors necessary for entry into target cells. Volcano plot of negative Log (RRA) versus Log (fold change) of the **A)**SARS-CoV-2-Spike screen, **B)** VSVG-mNG screen, and **C)**the VSVG-Cre screen. The circled genes are highlighted based on biological function and subsequent analysis. **D)** The top 15 genes in VSVG-mNG plotted versus the top 15 genes in VSVG-CRE by negative Log (RRA). **E)** The top 15 genes in VSVG-mNG plotted versus the top 15 genes in Spike-mNG by negative Log (RRA).

Endocytic and actin genes were most frequently detected by the VSVG-CRE screen.

With the goal of uncovering the host that contributes to viral entry, we compared the three screens for their ability to identify genes that contribute to viral entry from the cell surface. We observed that the ARP2/3 complex was prominent in the VSVG-Cre and Spike-mNG screens, with ACTR2 (both), ARPC4 (both) and ARPC3 (VSVG-Cre only), appearing in the top 15 genes (Figure 3D and 3E). The ARP2/3 complex consists of 7 proteins and is well-known for nucleating and branching actin (Goley & Welch, 2006; Mullins et al., 1998; Pollard, 2007; Robinson et al., 2001; Rouiller et al., 2008), which can aid in endosome formation contribute to SARS-CoV-2 infection (Daniloski et al., 2021; Schmidt et al., 2021; Zhu et al., 2021). The ARP2/3 complex also is important for entry of VSV and a wide range of other viruses (Paluck et al.,

2021; Zhu et al., 2021). Given this finding, we expected that the VSVG-CRE screen would be superior at identifying genes contributing to viral entry. Indeed, plotting RRA for VSVG-CRE against VSVG-mNG showed that the VSVG-CRE screen placed multiple subunits of the Arp2/3 at the top, VSVG-mNG was more likely to identify genes associated with protein synthesis and gene expression (such as SEC61B, SEC63 and ARF1) (Figure 3D).

Additionally, the VSVG-CRE screen identified multiple subunits (ATP6V1C1, ATP61G1 and ATP6V0B) of the proton-transporting v-type ATPase complex: ATP6V0D1, ATP6AP2, ATP6V1C1, ATP6V1G1, CCDC115, ATP6V0B, APT6AP1 which are required for endosome acidification and support VSV-G membrane fusion (Breton & Brown, 2013; Icho et al., 2022; Lafourcade et al., 2008). However, we have not identified this pathway in other two screens even though as it has been shown that this complex is important SARS-CoV-2 entry (Icho et al., 2022). In addition, the VSVG-CRE screen identified MESDC2 (Figure 3D) an ER chaperone protein for LDLR folding for proper localization to the plasma membrane (Culi et al., 2004; Hsieh et al., 2003). Taken together, these findings indicate that bypassing the expression of a lentiviral reporter increased the frequency of identifying genes directly contributing to viral endocytosis and entry.

Our VSVG-mNG and Spike-mNG screens identified SEC63 and SEC61B, representing two of the three components of the translocon pore (Linxweiler et al., 2017). The SEC complexes are located in the plasma membrane of the ER and play an important role in the translocation of newly synthesized polypeptide into the ER. The VSVG-mNG screen, also identifies secretory related proteins ARF1, ARFRP1 and KIF13A (Figure 3D) which contribute to trafficking of proteins from the trans-Golgi network to the plasma membrane (Donaldson & Jackson, 2011). Together, these hits may reflect a requirement of an intact secretory pathway transporting ACE2 or LDLR to the plasma membrane.

The use of pseudoviral delivered CRE and floxed reporter distinguished genes associated with lentiviral reporter expression.

In addition to increased sensitivity for detecting genes associated with entry when using the CRE reporter, we also found that the use of the CRE reporter bypassed genes influencing retro-transcription. Specifically, EP300 was a top hit in both the Spike-mNG and VSVG-mNG screen but not in the VSVG-CRE screen (Figure 3D and 3E). EP300 is an acetyltransferase that acetylates HIV-1 integrase to facilitate integration of viral genome into host genome (Cereseto et al., 2005). In the VSVG-CRE screen, however, this gene is not detected indicating that direct delivery of the CRE protein bypassed the steps required for lentiviral reporter expression. This finding, in conjunction with the strong detection of LDLR as the known VSV-G receptor, highlights the benefits of using the CRE to closely link to viral entry with reporter activation without introducing complexities arising from other viral functions, including retroviral reporters.

Validation of candidate hits using one vector system shows the strength of the CRISPR-Cas9 whole genome screening.

To validate the candidate hits from our screen we generated single gene knockouts in ACE2-293T and ACE2-293T-Cre cells by introducing a one vector Cas9 endonuclease and single gRNA (Stringer et al., 2019) Figure 4A). We evaluated this approach by targeting and staining cells for ACE2 expression. Knockout of ACE2 in both ACE2-293T and ACE2-293T-Cre reporter cells was highly efficient, >95% (Figure 4A and 4B). As expected, knockout of ACE2 from ACE2-293T and ACE2-293T-Cre abolished the infection by both Spike-mNG and Spike-Cre (Figure 4). ACE2 KO did not affect VSVG-mNG which was anticipated; however, it did lead to a slight drop in infectivity of VSVG-Cre (Figure 4E and 4F).

We evaluated gene disruptions of SLC35B2, HSP90B1 and EP300, which reflect three distinct molecular steps in viral infection and reporter expression, with SLC35B2 affecting broad recruitment of viral particles to the cell surface and HSP90B1 affecting LDLR expression and hence VSV-G attachment and EP300 affecting lentiviral integration and reporter gene expression. As expected, the SLC35B2 KO dropped the infection of both Spike and VSVG mediated entry (Figure 4), consistent with heparan sulfate proteoglycan being important for general viral attachment to the cell surface (Guimond et al., 2022). The ER chaperone Gp96 (HSP90B1) was identified in both VSVG-mNG (Rank#3) and VSVG-CRE (Rank#2) as top hit but not

in Spike-mNG (Rank#607). As anticipated, HSP90B1 KO reduced VSVG-mNG and VSVG-Cre mediated entry without effect on Spike-mediated infection (Figure 4). The acetyltransferase EP300 was identified as a top hit in both Spike-mNG and VSVG-mNG screens, but not a hit in VSVG-Cre consistent with its previously identified role in lentiviral integration (Cereseto et al., 2005). As expected, EP300 KO reduced VSVG-mNG reporter expression. However, it unexpectedly decreased VSVG-Cre reporter expression slightly and had no effect on Spike-mNG reporter expression (Figure 4). We are unclear as to the reason for this reproducible discrepancy; however, one possibility is that the titers used in the screen may have been slightly lower than in the validation experiment, which somehow allowed Spike-mNG to bypass the effect of the EP300 knockout.

Identification of putative genes that natively inhibit SARS-CoV2 and VSVG entry.

We identified salt inducible kinase 2 and 3 (SIK2 and SIK3) as top negative regulators of Spike-mNG entry (Figure 3A). These serine/threonine protein kinases belonging to the AMPK protein family, and they have been shown to regulate glucose metabolism, mTORC signaling and hormonal signaling (Bricambert et al., 2010; Csukasi et al., 2018; Yang et al., 2013; Zhang et al., 2016). However, the mechanism by which these kinases might inhibit spike-mediated entry is unclear and we have not validated this finding. Additionally, GALNT11 was identified as a suppressing VSVG-mNG and VSVG-CRE entry and ALG5 in suppressing VSVG-CRE (Figure 3B and 3C). GALNT11 is an N-Acetylgalactosaminyltransferase and is involved in O-linked glycosylation of proteins in the Golgi, whereases ALG5 is involved in N-linked glycosylation of proteins (Imbach et al., 1999; Schwientek et al., 2002).

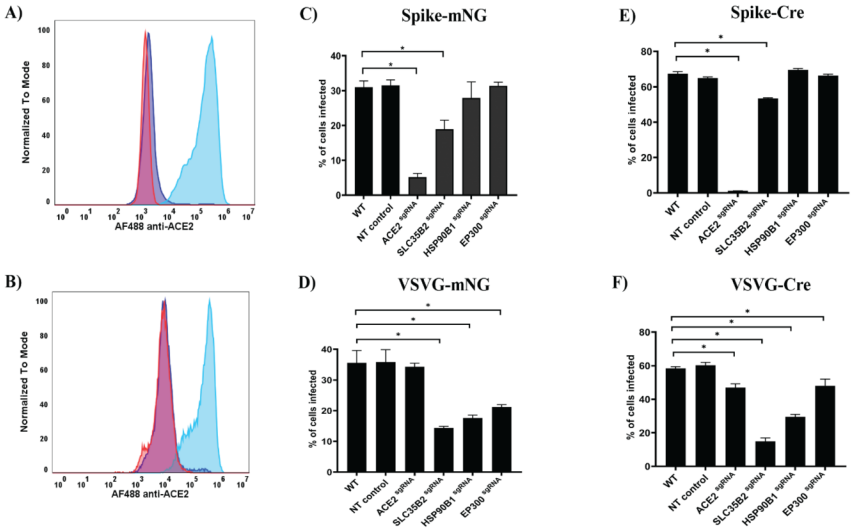


Figure 4: Validation of key genes identified in CRISPR-Cas9 whole genome screening using the one vector system. A) ACE2 staining on the ACE2 KO cells using AF488 conjugated anti-ACE2 antibody (dark blue) which is similar to unstained (magenta) versus WT ACE2 positive cells (light blue). B) ACE2 staining on the ACE2-293T-CRE reporter cells following transduction with the ACE2 targeting sgRNA (dark blue) relative to WT (light blue) and unstained (magenta). C-F) Effect of mutants on the entry of mNG and Cre reporter viruses. The error bar represents three biological replicates. One-way ANOVA and Šídák's multiple comparisons test were performed to do statistical analysis between the WT and the mutants. Bar shows mean ± SD (n= 3). * Denotes $p<0.05$ for comparison between WT and the mutants.

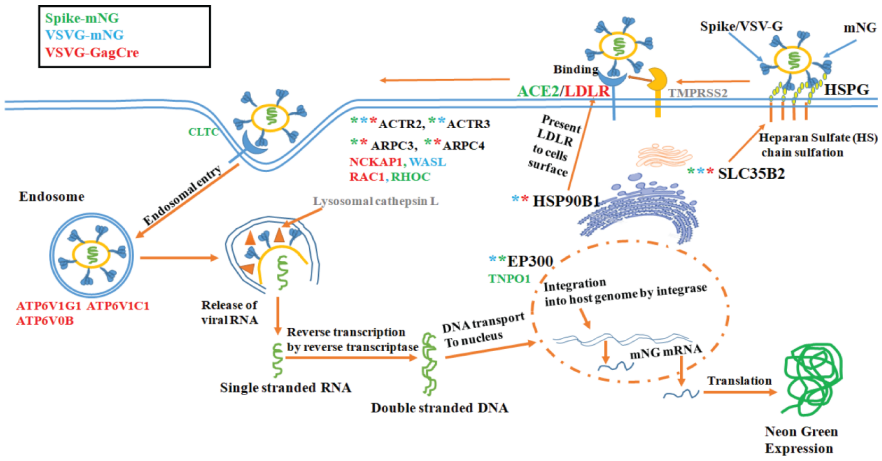
Discussion

The identification of host genes contributing to viral entry is an important step in understanding the pathogenesis of viruses and identify in new therapeutic strategies. Several whole genome CRISPR screens against live SARS-CoV-2 identified host factors contributing to infection (Baggen et al., 2021; Daniloski et al., 2021;

Schneider et al., 2021; Wang et al., 2021; Wei et al., 2021). Here we have devised a strategy using lentiviral pseudoviruses focusing on the selection strategy on the viral entry step at a level compatible with biosafety level-2 laboratories.

For the analysis of SARS-CoV-2 entry, the lentiviral pseudovirus is advantageous as it has a similar size and shape ˜100 nm in diameter (Illanes-Álvarez et al., 2021; Rein, 2019), making it compatible with similar endocytic mechanisms. While, VSV based pseudovirus has also been used for SARS-CoV-2 screens (Wei et al., 2021), this strategy has the limitation that VSV is larger than SARS-CoV-2 and has a bullet shape that is ˜200 nm in length (Cureton et al., 2010). Our study, however, did not determine if this difference results in any changes in the viral entry.

In addition to improving the stringency for CRISPR screens on viral entry, the use of the CRE-Gag fusion protein focuses the gene hits onto entry related processes rather than genes affecting reporter expression. This is because CRE reporter can be activated directly by the CRE recombinase protein provided by the viral particles without the need of the integration process (Esposito et al., 2016; Philippe E. Mangeot et al., 2019).



To compare genes detected by the different screens, diagramed their subcellular localization and known functions (Figure 5), using publication and web databases including STRINGDB (string-db.org) and GeneCards (genecard.org). Broadly, our screens identified genes affecting viral attachment, the formation of endosomes including Arp2/3, endosome acidification ATP6V genes and expression of downstream reporters.

Figure 5: Top ranked genes (FDR < 0.3) in three different screens are involved in key elements of the viral life cycle. Schematic diagram of the viral envelope mediated entry in target cells, endosome formation, endosome acidification and release of viral genome in the cytoplasm, reverse transcription and integration and translation of reporter gene expression. The color of asterisks represents the corresponding screen condition. Three colors represent that the genes are present in all three screen conditions. These genes were known to have an effect on the entry of viral particles and were put in the diagram based on their function and location in the cell.

Conclusion

While previous screens used live coronaviruses or pseudoviruses, largely focused on viability for selection, here we have used SARS-CoV-2 spike and VSV-G enveloped pseudoviruses to focus a screen/counter screen strategy for viral entry (Baggen et al., 2021; Daniloski et al., 2021; Schneider et al., 2021; Wang et al., 2021; Wei et al., 2021). Our results indicate that delivery of CRE protein in the virion helped distinguish genes that play role in entry rather than retro-transcription from lentiviral reporters. Further development of this approach will be needed along to include CRE reporter in SARS-CoV-2 Spike pseudotyped cells. Thus, we

view our screen is a proof of concept and that higher resolution data can be obtained by increasing the numbers of cells analyzed. Our screen identified novel genes that were not found in previously published screens suggesting that refining the selection strategy for screening should make it possible to identify mechanisms by which host genes facilitate and inhibit viral entry.

Methods

Preparation of ACE2 293T cell line

ACE2 expressing human embryonic kidney cells (HEK293T) were generated by lentiviral transduction using pLENTI-hACE2-HygR (a gift from Raffaele De Francesco; Addgene plasmid#155296) (Notarbartolo et al., 2021). 72h after transduction the cells were FACS sorted based on the ACE2 expression detected with anti-hACE2 Alexa Fluor 488 conjugated antibodies (catalog #Fab9332G, clone#535919; R&D Systems). A CRE reporter was inserted into ACE2 293T cells using pLenti- V6.3 lox-dsRED-stop-lox-eGFP-blas (a gift from Ewa Snaar-Jagalska, Addgene plasmid #106171) (Heitzer et al., 2019). After selection with the Blasticidin, these cells were then FACS sorted based on their dsRED intensity.

Preparation of viral particles for screening

Spike-mNG pseudovirus was generated using pCMV14-3X-Flag-SARS-CoV-2 S (a gift from Zhaohui Qian Lab (Addgene plasmid #145780) (Ou et al., 2020). This vector carries a codon-optimized complementary DNA that encodes SARS-CoV-2-Spike glycoprotein (Wuhan 2019) along with a C-terminal 19 amino acid deletion. We performed site directed mutagenesis to make create the D614G mutation in spike and named it pCMV-SD614G. Spike-mNG pseudovirus was made by co-transfecting Lenti-X 293T (TakaraBio) cells with packaging vector psPAX2 (psPAX2 was a gift from Didier Trono, Addgene plasmid #12260), pLJM1-Lck-mNeonGreenvector and pCMV-SD614G. VSVG-mNG pseudovirus was made the same way except the envelop vector pCMV-VSV-G was used for the envelope instead of pCMV-SD614G (pCMV-VSV-G was a gift from Bob Weinberg, Addgene plasmid #8454) (Stewart et al., 2003). To make GagCre-VSVG viral particles, we transfected Lenti-X 293T cells with the vector pCMV-VSV-G and GagCre vector (GAG-CRErec was a gift from Philippe Mangeot & Théophile Ohlmann & Emiliano Ricci, Addgene plasmid #119971) (P. E. Mangeot et al., 2019). All the viruses were harvested 48h after transfection.

CRISPR- Cas9 genetic screening in ACE2 293T cells

Lentiviral Brunello CRISPR KO library was purchased from Addgene at a titer of 1.4*10ˆ7 TU/mL (a gift from David Root and John Doench Addgene #73178) (Doench et al., 2016). 40*10ˆ6 ACE2 293T cells were transduced with the Brunello library at an MOI of 0.3 to enable [?]100-fold gRNA representation. 72h after transduction with the viral library the cells were selected with puromycin at 1ug/mL for 5 days and cultured for 5 more days to allow for the depletion of proteins from the cells. After that, cells were plated in 10cm dishes for transduction with the viral particles for screening. 2*10ˆ6 cells in each 10cm dish were plated for 10 dishes for each of the screening condition the day before transduction. The spike pseudovirus was concentrated using the LV concentrator (Catalog #631213, Takara Bio) on the day of transduction. After 48h of transduction with the pseudoviruses the cells were FACS sorted based on mNG or GFP fluorescence into infected and uninfected groups.

Genomic DNA isolation and amplification

Genomic DNA was isolated from the sorted cells using GenJet genomic DNA purification kit (Catalog #K0721). The sgRNA was amplified by PCR using Thermo Scientific Phire Green Hot Start II PCR master mix (Thermo Scientific, Catalog# F126S). For PCR amplification, genomic DNA samples were divided into 50uL PCR reaction. Each reaction consists of 25uL PCR master mix, 0.71uL of 14uM P5 stagger primer mix and 5uL of 14uM uniquely barcoded P7 primer mix, and rest of the volume was gDNA solution. All the P5 and P7 primers are shown in supplemental file. PCR cycling setting: initial denaturation 30 seconds at 98C; then 20 seconds at 98C, 15 seconds at 54C, 20 seconds at 72C for 25 cycles followed by 2 minutes extension at 72C. The pooled PCR products were run on a 2% agarose gel and correct band was extracted and purified

with the Wizard SV gel and PCR clean-up system (Promega, catalog #A9281). All the sequencing primers are in Supplemental Table 1.

Sequencing and read count analysis

Equal molar amounts PCR gel-purified samples were pooled, denatured and diluted to 1.8pM, of which 1.3 ml was sequenced on a NextSeq 550 using a high output 75 cycle sequencing kit (Illumina). This yielded between 35 and 62 million reads per sample. Sequencing reads were aligned to the Brunello library file and counted using MAGeCK (Chen et al., 2018; Li et al., 2014). The raw read counts table are in NCBI GEO (accession number **GSE206996**).

Validation experiment with the reporter virus

One vector system containing both Cas9 and sgRNA was used to make the KO lentivirus to disrupt the genes in both ACE2 293T cells and ACE2-293T-Cre reporter cells. The one vector system LentiCRISPR v2 was a gift from Brett Stringer (Addgene plasmid #98290). The following sgRNA sequences were used for the validation experiment- ACE2 (AACATCTTCATGCCTATGTG), SLC35B2 (GCACTCGGTTCATTAGCACC), HSP90B1 (AGACCACGTGGAGCAGATGT), EP300 (ATGGTGAACCATAAGGATTG), NT control (ACAGCCCTCACGAGCCCGAA). The reporter viruses were made following the same procedure that was followed to make reporter virus for the whole genome screening. The mutants were transduced with reporter viruses and incubated for 48 hours. After 48 hours of incubation the cells were run through flow cytometer to measure the infection.

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Author Contribution

MSH and JGK conducted experiments and analyzed the data. ADH conceived the idea and guided experimental design and data analysis. MSH, JGK and ADH wrote the manuscript.

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Availability of data and materials

All raw read count tables are available to the public at NCBI GEO with ascension number **GSE206996. https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE206996**

Declarations

Ethics approval and consent to application

Not applicable

Consent for publication

Not applicable

Competing interest

The authors declare that they have no competing interest.

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