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1	Development of a Single-cycle Infectious SARS-CoV-2 Virus Replicon Particle System			
2	for use in BSL2 Laboratories			
3				
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18 19 20 21 22 23 24 25 26 27 28 29 30 31 32	Running title: Single-cycle Infectious SARS-CoV-2 Replicon			

#### 33 Abstract

34 Research activities with infectious severe acute respiratory syndrome associated coronavirus 2 (SARS-CoV-2) are currently permitted only under biosafety level 3 (BSL3) containment. Here, 35 we report the development of a single-cycle infectious SARS-CoV-2 virus replicon particle 36 37 (VRP) system with a luciferase and green fluorescent protein (GFP) dual reporter that can be 38 safely handled in BSL2 laboratories to study SARS-CoV-2 biology. The Spike (S) gene of SARS-CoV-2 encodes for the envelope glycoprotein, which is essential for mediating infection 39 40 of new host cells. Through deletion and replacement of this essential S gene with a luciferase 41 and GFP dual reporter, we have generated a conditional SARS-CoV-2 mutant ( $\Delta$ S-VRP) that produces infectious particles only in cells expressing a viral envelope glycoprotein of choice. 42 43 Interestingly, we observed more efficient production of infectious particles in cells expressing 44 vesicular stomatitis virus (VSV) glycoprotein G ( $\Delta$ S-VRP(G)) as compared to cells expressing other viral glycoproteins including S. We confirmed that infection from  $\Delta$ S-VRP(G) is limited to 45 46 a single round and can be neutralized by anti-VSV serum. In our studies with  $\Delta$ S-VRP(G), we 47 observed robust expression of both luciferase and GFP reporters in various human and murine 48 cell types, demonstrating that a broad variety of cells can support intracellular replication of 49 SARS-CoV-2. In addition, treatment of  $\Delta$ S-VRP(G) infected cells with anti-CoV drugs 50 remdesivir (nucleoside analog) or GC376 (CoV 3CL protease inhibitor) resulted in a robust 51 decrease in both luciferase and GFP expression in a drug-dose and cell-type dependent 52 manner. Taken together, we have developed a single-cycle infectious SARS-CoV-2 VRP 53 system that serves as a versatile platform to study SARS-CoV-2 intracellular biology and to 54 perform high throughput screening of antiviral drugs under BSL2 containment. 55

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#### 64 Importance

- <sup>65</sup> Due to the highly contagious nature of SARS-CoV-2 and the lack of immunity in the human
- 66 population, research on SARS-CoV-2 has been restricted to biosafety level 3 laboratories. This
- 67 has greatly limited participation of the broader scientific community in SARS-CoV-2 research
- and thus has hindered the development of vaccines and antiviral drugs. By deleting the
- 69 essential Spike gene in the viral genome, we have developed a conditional mutant of SARS-
- 70 CoV-2 with luciferase and fluorescent reporters, which can be safely used under biosafety
- 71 level 2 conditions. Our single-cycle infectious SARS-CoV-2 virus replicon system can serve as
- 72 a versatile platform to study SARS-CoV-2 intracellular biology and to perform high throughput
- 73 screening of antiviral drugs under BSL2 containment.
- 74

#### 75 Introduction

76 Due to the highly contagious nature of severe acute respiratory syndrome associated coronavirus 2 (SARS-CoV-2) and the lack of sufficient immunity in the population, research on 77 78 SARS-CoV-2 is permitted only under BSL3 containment, which significantly limits SARS-CoV-79 2 research only to institutions with BSL3 infrastructure. In addition, due to physical limitations, 80 high throughput screening of antiviral drugs can be impractical under BSL3 containment (1, 2). Fortunately, research with attenuated or conditional mutants of BSL3/BSL4 pathogens is 81 82 allowed under lower containment upon demonstration of attenuation and safety (3). These 83 include low pathogenic H5N1 (lacking the multibasic site in HA), an Ebola virus conditional mutant (VP30 deletion mutant), and Yersinia pestis conditional mutants, etc (4-6). Thus, to 84 enable studies with SARS-CoV-2 under BSL2 containment, SARS-CoV-2 spike (S) 85 86 pseudotyped HIV lentivirus particles or recombinant vesicular stomatitis virus (VSV) with the native G gene replaced with the S gene have been developed and can be used to study the 87 88 viral entry process and to identify viral entry inhibitors (7-9). In addition, several SARS-CoV-2 89 replicon reporter systems that retain the minimal viral genes necessary for intracellular 90 replication have been developed (10-12). Moreover, conditional deletion mutants of SARS-91 CoV-2 capable of replicating only in complemented cells expressing viral proteins have been 92 reported (N gene or ORF3a/E genes deleted) (13, 14). A recent study reported a single cycle 93 infectious particle system through co-expression of viral S, M, E and N proteins along with a 94 luciferase reporter carrying cis-acting elements (packaging sequences) of SARS-CoV-2 in the 95 3'UTR (15). The generation of these SARS-CoV-2 tools has allowed us to safely study various 96 aspects of SARS-CoV-2 biology under BSL2 containment.

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98 Here, we report the development of a single-cycle infectious SARS-CoV-2 virus replicon 99 particle system ( $\Delta$ S-VRP) with a luciferase and green fluorescent protein (GFP) reporter. Using 100 a bacterial artificial chromosome based reverse genetics system, we replaced the essential 101 spike gene of SARS-CoV-2 with a luciferase (Luc) and GFP dual reporter ( $\Delta$ S-Luc-GFP) (16). 102 Co-transfection of the  $\Delta$ S-Luc-GFP bacmid with a VSV-G expressing plasmid resulted in 103 efficient production of infectious VRPs ( $\Delta$ S-VRP(G)), which can be further amplified in VSV-G 104 transfected cells. As expected, control vector transfected cells failed to produce any infectious 105  $\Delta$ S-VRPs, demonstrating that VRP infection is restricted to a single cycle.  $\Delta$ S-VRP(G) stocks 106 produced from VSV-G expressing cells showed robust infection and expression of both

107 reporters in various murine and human cell lines, indicating that various cell types are 108 permissive to SARS-CoV-2 replication. Importantly, treatment of  $\Delta$ S-VRP(G) infected cells with 109 antivirals Remdesivir (nucleoside analog) or GC376 (CoV 3CL protease inhibitor) resulted in a 110 robust and drug-dose dependent decrease in both luciferase and GFP reporter activity, 111 demonstrating that this platform can be useful for antiviral drug screening. Taken together, our 112 studies demonstrate that this  $\Delta$ S-VRP system with a dual reporter can serve as a versatile tool 113 to investigate SARS-CoV-2 biology and perform antiviral drug screening under BSL2 114 containment.

115

#### 116 Results

### 117 Design of a single-cycle infectious SARS-CoV-2 virus replicon particle system

118 To safely study SARS-CoV-2 biology under BSL2 containment, we deleted and replaced the 119 essential viral spike (S) ORF with a tandem Gaussia luciferase and Neon Green GFP dual 120 reporter separated by the porcine teschovirus 2A ribosome skipping signal under the control of 121 the S gene transcription regulatory sequence ( $\Delta$ S-Luc-GFP; Fig 1A). Similar to other viral replicons, the  $\Delta$ S-Luc-GFP genome can undergo normal transcription and replication upon 122 123 transfection into cells, yet is unable to produce infectious virus particles due to the lack of the S gene. As such, formation of infectious particles carrying the  $\Delta$ S-Luc-GFP genome requires 124 125 expression of the S protein or other viral glycoprotein(s) in producer cells. We rescued infectious VRPs by co-transfecting the ΔS-Luc-GFP bacmid with a VSV-G expression plasmid 126 127 into a mixture of 293T/Huh7.5 cells ( $\Delta$ S-VRP(G); Fig 1B). We observed a significant increase in luciferase activity in the supernatants and GFP expression in cells transfected with  $\Delta$ S-Luc-128 129 GFP bacmid over time as compared to control vector transfected cells (Fig 1C-D). On day 6 130 post-transfection, the 293T/Huh7.5 cell mixture was transfected again with a VSV-G 131 expression plasmid, which resulted in a significant increase in both luciferase activity and the numbers of GFP expressing cells. These data show that  $\Delta$ S-Luc-GFP replicated efficiently in 132 133 cells, and additional expression of VSV-G presumably increased the spread of  $\Delta$ S-Luc-GFP 134 genome to new cells. To produce  $\Delta$ S-VRP(G) working stocks, supernatants from  $\Delta$ S-VRP(G) 135 rescue transfections were used as seed stocks to infect VSV-G expressing Huh7.5 cells. Next, 136 we evaluated the infectivity of amplified  $\Delta$ S-VRP(G) stocks by measuring the expression of viral nucleoprotein (N) and GFP in a human lung epithelial cell line (A549) expressing human 137 138 angiotensin-converting enzyme 2 (hACE2) (A549-hACE2). At 18hpi, immunofluorescence

analysis showed GFP expression in N positive cells (Fig 1E). Furthermore, western blot analysis showed expression of N protein but not S protein in  $\Delta$ S-VRP(G) infected cells (Fig 1F). Wild-type SARS-CoV-2 infected A549-hACE2 cell lysates were included as controls. Taken together, we have successfully established a SARS-CoV-2 VRP system with a dual reporter that can be easily propagated in VSV-G expressing cells.

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#### 145 ΔS-VRP(G) infection is restricted to a single cycle and neutralized by anti-VSV sera

Next, we compared the infectivity of  $\Delta$ S-VRPs produced in Huh7.5 cells expressing 146 147 glycoproteins from diverse viral families including SARS-CoV-2 S and observed more efficient 148 production of infectious  $\Delta$ S-VRPs only in cells expressing VSV-G (data not shown); hence, our 149 subsequent experiments were performed with  $\Delta$ S-VRP(G) particles. Next, to demonstrate that 150 infection from  $\Delta$ S-VRP(G) is restricted to a single round, we transferred the supernatants from 151 △S-VRP(G) infected Huh7.5 cells (No VSV-G transfection; R2 sup) onto fresh Huh7.5 cells and observed no GFP or Luc expression. These results demonstrate that infection with  $\Delta$ S-VRP(G) 152 153 is limited to a single cycle (Fig 2A).

154

155 Next, to assess the reproducibility of the  $\Delta$ S-VRP(G) system, we tested the infectivity of 3 independent preparations of  $\Delta$ S-VRP(G) stocks in Huh7.5 cells and observed similar levels of 156 157 luciferase activity across different preparations (Fig 2B). In addition,  $\Delta$ S-VRP(G) preparations 158 were stable during storage at -80C and retained infectivity at levels similar to fresh  $\Delta$ S-VRP(G) 159 preparations (Fig 2C). Moreover, we observed a dose dependent increase in the infectivity of 160  $\Delta$ S-VRP(G) in Huh7.5 cells. Next, to demonstrate that  $\Delta$ S-VRP(G) infection is solely mediated through the VSV-G glycoprotein, we tested if  $\Delta$ S-VRP(G) infection can be inhibited in the 161 presence of anti-VSV serum (Fig 2D-E). Infectivity of  $\Delta$ S-VRP(G) in Huh7.5 cells was 162 significantly reduced in the presence of anti-VSV serum as compared to control serum, 163 164 indicating that  $\Delta$ S-VRP(G) infection is mediated through the VSV-G protein. Taken together, 165 we have developed a robust single-cycle infectious SARS-CoV-2  $\Delta$ S-VRP(G) dual reporter system that can be safely used under BSL2 containment. 166 167

# ΔS-VRP(G) dual reporter system is a versatile tool to study SARS-CoV-2 biology in different cell types

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172 cell types of human and murine origin (Fig 3A-D). We observed robust replication of  $\Delta$ S-Luc-GFP in various cell types from different species including lung epithelial cells, kidney cells, 173 174 monocytic cell lines, lymphocytes, macrophages and dendritic cells. These results indicate that 175 several human and murine cell types are permissive to replication of SARS-CoV-2. 176 Interestingly, we observed differences in the levels of replication in different cells types, 177 indicating that cell specific host factors may regulate the intracellular replication steps of 178 SARS-CoV-2. 179 180 Next, to investigate if the  $\Delta$ S-VRP(G) dual reporter system can be useful for the assessment of 181 host antiviral responses, we infected murine bone marrow derived macrophages (BMDM) and 182 dendritic cells (BMDC), and measured expression of the viral N gene and various host antiviral

As  $\Delta$ S-VRP(G) infection of host cells is independent of SARS-CoV-2 host receptor ACE2

expression, we evaluated the robustness of the  $\Delta$ S-VRP(G) dual reporter system in various

183 genes. We observed robust induction of various antiviral genes including  $Ifn\alpha$ ,  $Ifn\beta$ , Mx1, Isg15 184 and Tnf $\alpha$  in BMDMs and to a lesser extent in BMDCs (Fig 3E). Taken together, these results demonstrate that the  $\Delta$ S-VRP(G) dual reporter system is a versatile tool to study SARS-CoV-2 185

186 biology as well as host immune responses in various cell types.

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#### $\Delta$ S-VRP(G) dual reporter system can be valuable for antiviral drug screening 188

189 Next, to test if the  $\Delta$ S-VRP(G) dual reporter system can be a useful platform for testing anti-190 SARS-CoV-2 drugs, we assessed the effects of well-known anti-CoV drugs Remdesivir and GC376 on  $\Delta$ S-Luc-GFP replication (Fig 4). Huh7.5 or A549 cells infected with  $\Delta$ S-VRP(G) were 191 192 treated with different concentrations of Remdesivir or GC376 starting at 2hpi. At 18hpi, both 193 luciferase activity in the supernatants and GFP expression in infected cells were compared 194 between different treatment groups. In Huh7.5 cells, we observed a dose dependent decrease 195 in luciferase activity along with a concomitant decrease in GFP expression in both Remdesivir 196 and GC376 treatment groups as compared to DMSO treated cells (Fig 4A and 4C; Remdesivir 197 IC50 26.7nM and GC376 IC50 17.3nM). We also observed a similar dose dependent inhibition 198 of ΔS-Luc-GFP replication in A549 cells treated with either Remdesivir or GC376 (Fig 4B and 199 4D; Remdesivir 55.2nM and GC376 2300 nM); Interestingly, inhibition of  $\Delta$ S Luc-GFP 200 replication by Remdesivir and GC376 was less pronounced in A549 cells as compared to 201 Huh7.5 cells, indicating cell type specific differences in the activity of antiviral drugs. These

results demonstrate that the ΔS-VRP(G) dual reporter system can be useful for rapid screening
 of antiviral drugs against SARS-CoV-2 under BSL2 containment. Taken together, we have
 successfully established a SARS-CoV-2 VRP dual reporter platform that allows for safe
 investigation of SARS-CoV-2 biology, host interactions, and antiviral responses, as well as for
 high throughput screening of anti-CoV drugs, under BSL2 containment.

#### 208 Discussion

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209 Studies with highly pathogenic viruses can be safely performed in biological laboratories with 210 specialized biocontainment procedures. Through the generation of conditional replicating 211 mutants or attenuated mutants, we can safely study some aspects of virus biology in standard 212 BSL2 or BSL2+ facilities. Currently, research activities with SARS-CoV-2 are restricted to 213 BSL3 facilities that mostly have limited capabilities. To overcome this limitation, we have 214 developed a conditional mutant of SARS-CoV-2 through deletion and replacement of the 215 essential viral S glycoprotein gene with a luciferase-GFP dual reporter, leaving the remaining 216 SARS-CoV-2 genome intact. Through co-expression of VSV-G protein in trans, we have 217 successfully generated single-cycle infectious SARS-CoV-2 replicon particles carrying the  $\Delta S$ Luc-GFP genome. Various human and murine cells infected with  $\Delta$ S-VRP(G) showed robust 218 219 expression of both luciferase and GFP reporters, indicating that these cell lines are permissive 220 to intracellular replication of SARS-CoV-2. In addition, as viral entry of  $\Delta$ S-VRP(G) particles is 221 mediated through the G protein,  $\Delta$ S-VRP(G) can efficiently infect a variety of human and 222 murine cell types lacking the SARS-CoV-2 ACE2 receptor. Importantly, the  $\Delta$ S-VRP(G) dual 223 reporter system showed robust responsiveness to treatment with Remdesivir or GC376, 224 demonstrating that the  $\Delta$ S-VRP(G) dual reporter system can be useful for high-through put 225 screening of antiviral drugs against SARS-CoV-2.

226

Several groups have reported the development of SARS-CoV-2 replicon systems with either luciferase or GFP reporters, and the majority of these replicon systems require transfection of replicon DNA or RNA into cells, which may limit studies to cell types with higher transfection efficiency. In addition, there might be a time lag between nucleic acid transfection and optimal reporter expression. One advantage of our  $\Delta$ S-VRP(G) dual reporter system is that large quantities of  $\Delta$ S-VRP(G) can be easily amplified in VSV-G expressing Huh7.5 cells, and can be used to investigate SARS-CoV-2 biology in a variety of cell types. In addition to replicons,

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234 conditional SARS-CoV-2 mutants with GFP or luciferase reporters have been developed. Ju, 235 et al. generated a mutant lacking a portion of the essential N gene, thereby limiting replication 236 to cells expressing N (14). Zhang, et al. generated a conditional mutant lacking the ORF3a/E 237 genes, which restricted replication to cells stably expressing ORF3a and E proteins (13). As an 238 additional safety measure, these authors elegantly modified all TRS sites to restrict the 239 potential emergence of replication competent virus through recombination with wild-type 240 SARS-CoV-2. Another advantage of our ∆S-VRP(G) system is that VSV-G mediated delivery 241 of the  $\Delta$ S-Luc-GFP genome occurs independently of the ACE2 receptor. Indeed, a vast 242 majority of human and murine cell types supported  $\Delta$ S-Luc-GFP replication, albeit with some 243 differences in the levels of replication. It is possible that the observed variations in  $\Delta$ S-Luc-GFP 244 replication can be in part due to inherent differences in VSV-G mediated entry into various cell 245 types. Finally, as the  $\Delta$ S-VRP(G) system contains both luciferase and GFP reporters, we observed a strong co-relation between luciferase activity and GFP expression across different 246 247 treatment groups in our studies with remdesivir and GC376. The presence of a dual reporter 248 allows for rapid elimination of false positive hits that directly inhibit luciferase or interfere with 249 GFP fluorescence during high-throughput antiviral drug screening. 250

In our studies, we observed robust luciferase and GFP expression in cells infected with  $\Delta$ S-251 252 VRP(G) as compared to  $\Delta$ S-VRP produced in cells expressing other viral glycoproteins 253 including SARS-CoV-2 S (data not shown). We speculate that this is in part due to the higher 254 efficiency of VSV-G protein in mediating viral entry as compared to other viral glycoproteins. It 255 is not completely clear how VSV-G is incorporated onto  $\Delta$ S-VRPs to mediate infection. VSV-G 256 has been reported to be localized to intracellular compartments, such as the endoplasmic 257 reticulum-golgi, as well as on the plasma membrane. As SARS-CoV-2 assembly and budding 258 is thought to occur in the endoplasmic reticulum-golgi intermediate compartment (ERGIC), it is 259 possible that intracellularly expressed VSV-G is incorporated onto VRP membranes during 260 canonical SARS-CoV-2 budding in the ERGIC. In agreement with VSV-G dependent delivery of the  $\Delta$ S Luc-GFP genome,  $\Delta$ S-VRP(G) infection was neutralized in the presence of anti-VSV 261 262 sera. Our future studies will determine if VSV-G incorporation onto  $\Delta$ S-VRPs occurs in 263 intracellular compartments.

264

In conclusion, we have developed a SARS-CoV-2 VRP platform with a dual reporter that can serve as a versatile tool to study SARS-CoV-2 host biology under BSL2 containment. Importantly, we observed robust and dose dependent changes in both luciferase and GFP expression upon treatment with anti-CoV drugs, demonstrating that this  $\Delta$ S-VRP(G) platform can be suitable for safe high throughput screening of antivirals against SARS-CoV-2.

#### 271 Materials and Methods

#### 272 Biosafety statement

Studies with infectious SARS-CoV-2 and  $\Delta$ S-VRP(G) viruses were approved by Ulowa IBC and CCOM BSL3 Oversight Committee (Protocol # 210053). Initial validation and safety studies with  $\Delta$ S-VRP(G) were performed in a BSL3 laboratory. After review of the safety data, both Ulowa IBC and the NIH Office of Science Policy approved the use of  $\Delta$ S-VRP(G) under BSL2+ containment at Ulowa.

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### 279 Ethics statement

All studies were performed in accordance with the principles described by the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of laboratory animals in biomedical research. The protocol for isolating anti-VSV serum from mice was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Iowa (Animal Protocol #1062127).

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#### 286 Anti-VSV serum

Antisera against VSV was produced in-house by immunizing C57BL/6J mice (6 weeks old) with 2x10<sup>8</sup> PFU of live VSV-GFP virus via intramuscular route. On day 14, mice were boosted with the same dose of VSV-GFP virus. At 4 weeks post-immunization, serum was collected by cardiac puncture and used in neutralization experiments after heat inactivation.

291

# 292 Cell lines and primary cells

Human lung epithelial cells (A549), human embryonic kidney cells (HEK293T), hepatocellular
carcinoma cell line (Huh7.5), mouse endothelial cell line (MS1), mouse lung epithelial cells
(LA4), mouse macrophage cell line (Raw 264.7), and African green monkey kidney epithelial
cells (Vero) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented wth
10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (10,000U/ml). Primary bone

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marrow derived macrophages (BMDM) and dendritic cells (BMDC) were generated from bone
marrows isolated from C57BL/6J mice (Jackson labs) by culturing in the presence of IL-4 and
GMCSF or IL-4 and L929 conditioned media, respectively (17, 18).

# 301

#### 302 Generation of ΔS-Luc-GFP bacmid

303 SARS-CoV-2 reverse genetics system based on the sequence of Wuhan-Hu-1/2019 isolate 304 (Accession number: NC 045512) was designed and assembled into a pBeloBac11 vector as 305 previously described for other coronaviruses (16). Our SARS-CoV-2 bacmid carries a unique 306 engineered SanDI restriction site in the NSP15 gene and a naturally occurring unique BamHI 307 restriction site near the 3' end of the S gene (70 nucleotides before ORF3a TRS). A gene 308 fragment representing the dual reporter under S TRS was chemically synthesized as a 1.7kb 309 gBlock fragment (Integrated DNA Technologies) in the following in-frame arrangement of 310 reporters: S gene TRS, S signal peptide (MFVFLVLLPLVSSQC), Gaussia luciferase, porcine 311 teschovirus 2A site (ATNFSLLKQAGDVEENPGUP), and Neon Green GFP reporter. The 312 genomic sequence between the SanDI site and the S TRS was PCR amplified using PrimeStar 313 Max (Clontech). Both fragments were combined together by fusion PCR and subsequently 314 cloned into the SARS-CoV-2 bacmid cut with SanDI and BamHI enzymes using the HiFi 315 assembly system (NEB). Assembly mixtures were transformed into DH10Bac and individual △S-Luc-GFP bacmid clones were identified by restriction digestion of bacmid DNA. 316 317

#### 318 **Rescue and amplification of \DeltaS-VRP(G)**

319 To rescue recombinant  $\Delta$ S-VRP(G) virus, a mixture of 293T cells/Huh7.5 cells in suspension 320  $(1 \times 10^6 \text{ cells each cell type})$  were transfected with 4µg of  $\Delta$ S-Luc-GFP bacmid and 1µg of VSV-G plasmid (Addgene #138479) using PEI transfection reagent (Polysciences; DNA:PEI ratio 321 322 1:4). After 5hrs post-transfection, the transfection mixture containing media was replaced with 323 DMEM/2%FBS media. At 72-96hrs post-transfection, supernatants were collected and kept as  $\Delta$ S-VRP(G) seed stocks. To amplify  $\Delta$ S-VRP(G), Huh7.5 cells seeded in 150mm plates 324  $(1.5 \times 10^7)$  were transfected with 40  $\mu$ g of VSV-G plasmid using PEI reagent. At 5hrs post-325 326 transfection, the transfection mixture containing media was replaced with DMEM/10% FBS 327 media. On the following day, 1ml of ∆S-VRP(G) seed stock was added to VSV-G transfected 328 Huh7.5 cells and incubated for 2hrs. After washing with PBS, infected Huh7.5 cells were placed in 25ml of DMEM/2% FBS, and monitored for GFP expression and cytopathic effects 329

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(CPE). At 48-72h post infection, supernatants were collected, clarified of debris, aliquoted and
 stored at -80C. These stocks were used to perform subsequent infection experiments.

# 333 **△S-VRP(G) infection**

Indicated cell types were seeded in 12-well (1-2x10<sup>5</sup> per well) or 6-well plates (5-8x10<sup>5</sup> per 334 well) a day prior to infection and infected with 0.5ml or 1ml of  $\Delta$ S-VRP(G) stock, respectively. 335 336 After 2hr incubation, viral inoculum was removed and replaced with DMEM/2%FBS media, 337 after two PBS washes. At 18hpi, luciferase activity in the supernatant and GFP expression in the cells were measured. For measurement of luciferase activity, 50ul of supernatant was 338 339 mixed with 50ul of Renilla luciferase substrate (Promega) and immediately measured in a 340 GloMax 20/20 single tube luminometer. GFP images were captured with a Nikon Eclipse 341 microscope using a 20X objective at an exposure time of 600 mSec.

# 342

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# 343 Remdesivir and GC376 inhibition

A549 or Huh7.5 cells seeded at a density of  $1.5 \times 10^5$  per well (12-well plate) a day prior were infected with 0.5ml of  $\Delta$ S-VRP(G) stock. After 2hpi, cells were washed with PBS and placed in DMEM/2%FBS media with the indicated amounts of drug. Measurement of luciferase activity and GFP expression were performed as described above at 18hpi.

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# 349 Immunofluorescence

A day prior to infection, A549 cells were seeded onto glass coverslips at 1x10<sup>5</sup> cells/well in a 350 351 24-well plate in Opti-Modified Eagle Medium (Opti-MEM) and infected with 0.5ml of  $\Delta$ S-352 VRP(G). After 2hrs, viral inoculum was replaced with Opti-MEM. At 18hpi, infected cells were washed twice with PBS and fixed with 4% Paraformaldehyde (Electron Microscopy Sciences) 353 354 in PBS for 5 min at room temperature. After washing with PBS, fixed cell were permeabilized 355 with 0.3% Triton X-100 in PBS for 5 min, washed with PBS and incubated in a blocking buffer 356 consisting of 1% BSA, 0.5% fish gelatin (Sigma), and 0.01% Tween20 (Sigma) in PBS for 30 357 min. Permeabilized cells were incubated in blocking buffer with mouse anti-nucleoprotein 358 antibody (1:1000 from Sino Biological) for 1hr followed by staining with goat anti-mouse 359 Alexa647 secondary antibody for 30min. Coverslips were mounted onto microscopy slides with 360 ProLong Gold Antifade Mountant with DAPI (Invitrogen). All incubations involving antibody staining were performed with gentle shaking at room temperature. Images were acquired on a 361

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Leica DFC7000T microscope under 63x oil-immersion objective using Leica software and
 processed using ImageJ software.

#### 364 Western blot analysis

A549 cells seeded in 6-well plates at  $1 \times 10^6$  cells/well were infected with 1ml of ΔS-VRP(G) and at 18hpi, cells were lysed in RIPA buffer and protein samples were separated on a 4-15% gradiatent SDS-PAGE gel (Bio-Rad). As controls, A549-hACE2 cells were infected with SARS-COV-2 (Wuhan-Hu-1/2019) at an MOI of 3 and lysed in RIPA buffer. Western blot analysis was performed following the transfer of proteins onto a nitrocellulose membrane using mouse antinucleoprotein or S antibody (1:1000 from Sino Biological) and goat α-mouse secondary antibody conjugated to horseradish peroxidase (#GENA931, Sigma).

#### 372 Quantitative RT-PCR analysis

BMDM and BMDC seeded at a density of  $2x10^6$  cells/well were infected with 2ml of  $\Delta$ S-373 VRP(G) and at 18hpi, total RNA from infected cells were extracted using PureLink RNA 374 375 extraction kit according to manufacturer's instructions. Residual genomic DNA contamination 376 was removed by DNase I (Invitrogen Cat #12185010) treatment. cDNA was synthesized using 377 Superscript IV Reverse Transcriptase (Invitrogen Cat #18090010) and Oligo d(T) (Invitrogen) 378 and qPCR analysis was performed using SYBR Green PCR Master Mix (Applied Biosystem 379 Cat #4368702) with technical duplicates and gene specific primers (19). 18S RNA was used as 380 an endogenous house housekeeping gene to calculate delta delta cycle thresholds. gPCR 381 primers are as follows - SARS-CoV-2 N forward: CAATGCTGCAATCGTGCTAC and reverse 382 GTTGCGACTACGTGATGAGG; mouse lfna1 forward: TCAAAGGACTCATCTGCTGCTTG 383 and reverse CCACCTGCTGCATCAGACAAC; mouse Ifn $\beta$  forward: CAGCTCCAAGAAAGGACGAAC and reverse: GGCAGTGTAACTCTTCTGCAT; Mx1 forward: 384 GACCATAGGGGTCTTGACCAA and reverse: AGACTTGCTCTTTCTGAAAAGCC; lsg15 385 forward: GGTGTCCGTGACTAACTCCAT and reverse: TGGAAAGGGTAAGACCGTCCT: Tnf $\alpha$ 386 387 forward: GACGTGGAACTGGCAGAAGAG and reverse: TTGGTGGTTTGTGAGTGTGAG;

- 388 18S forward: AAACGGCTACCACATCCAAG and reverse: CCTCCAATGGATCCTCGTTA.
- 389 Results are represented as fold expression relative to mock samples.

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# 391 Statistical analysis

392	Significance of data point	s was assessed using	g the unpaired Stude	ent's t-test. * denotes p-
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393 value <0.05 and ns denotes non-significant.

# 394

# 395 Acknowledgements

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#### 423 Figure Legends

424 Fig 1. Development of single-cycle infectious SARS-CoV-2 replicon system with a dual reporter. (A) Schematic representation of SARS-CoV-2 and  $\Delta$ S Luc-GFP SARS-CoV-2 425 genomes. The S gene in the SARS-CoV-2 genome was replaced with a luciferase and GFP 426 427 dual reporter. (B) Generation and amplification of  $\Delta S$  virus replicon particles. Left: 428 293T/Huh7.5 cell mixture was transfected with ΔS Luc-GFP bacmid and VSV-G plasmid. 429 Right: Supernatants from  $\Delta$ S-VRP(G) rescue transfections were amplified in Huh7.5 cells 430 transfected with VSV-G plasmid. (C-D) Kinetics of luciferase and GFP expression during 431 rescue transfection process. 293T/Huh7.5 cells were co-transfected with ΔS Luc-GFP bacmid 432 and VSV-G plasmid, and at various days post-transfection, luciferase activity in the supernatants and GFP expression in the cell mixture were assessed. On day 6 post-433 434 transfection, 293T/Huh7.5 cells were again transfected with additional VSV-G plasmid. (C) Luciferase values and (D) GFP expression are shown at the indicated time points. Luciferase 435 436 activity is shown for 3 independent Bac clones. GFP expression is shown for Bac clone #7. (E) 437 Immunofluorescence imagining of GFP and nucleoprotein expression in ΔS-VRP(G) infected cells. A549-hACE2 cells were infected with  $\Delta$ S-VRP(G) and at 18hpi, cells were stained with 438 439 anti-N antibody and imaged. (F) Western blot analysis of nucleoprotein expression in  $\Delta S$ -440 VRP(G) infected cells. A549-hACE2 cells were infected with  $\Delta$ S-VRP(G) and at 18hpi, cell 441 lysates were collected and analyzed for nucleoprotein and spike protein expression by western blot. Cell lysates from wild type SARS-CoV-2 infected cells were included as controls. β-actin 442 443 levels are shown as loading controls.

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Fig 2. Characterization of  $\Delta$ S-VRP dual reporter system. (A)  $\Delta$ S-VRP(G) infection is 446 447 restricted to a single round. Huh7.5 cells were infected with  $\Delta$ S-VRP(G) and at 2hpi, cells were 448 washed and incubated in fresh media. At 48h, supernatants (Round 2 - R2 sup) were collected 449 and added to new Huh7.5 cells. At 18hpi, luciferase activity in the supernatant was measured. 450 (B) Comparison of infectivity of different  $\Delta$ S-VRP(G) preparations. Huh7.5 cells were infected with 3 independent preparations of  $\Delta$ S-VRP(G) and luciferase activity was measured at 18hpi. 451 (C) Infectivity of  $\Delta$ S-VRP(G) stored at -80C. Huh7.5 cells were infected with  $\Delta$ S-VRP(G) stored 452 453 at -80C or fresh preparations and luciferase activity was measured at 18hpi. (D-E) 454 Neutralization of ΔS-VRP(G) infection by anti-VSV sera. Sera from Control (Cntrl) and VSV-455 infected mice were pre-incubated with  $\Delta$ S-VRP(G) for 1 hour and subsequently incubated with

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Huh7.5 cells for 2 hours. Luciferase and GFP expression were assessed at 18hpi. For panels
B-E, luciferase activity in the supernatants was measured and is shown as relative light units
(RLU). For panel G, luciferase activity was normalized to No Treatment control and is shown
as percentage (%) of No Treatment control.

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# **Fig 3. Permissiveness of human and murine cells to ΔS Luc-GFP replication.** The

indicated human and murine cells were infected with  $\Delta$ S-VRP(G) and at 18hpi, luciferase activity and GFP expression were measured. (A-B) Luciferase expression in human and murine cell lines. (C-D) GFP expression in human and murine cell lines. (E-F) Primary BMDM and BMDC were infected with  $\Delta$ S-VRP(G) and at 18hpi, expression of viral N mRNA and host antiviral genes were measured by qRT-PCR. (E) Viral N mRNA expression and (F) host antiviral gene expression.

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470 Fig 4. ΔS-VRP(G) reporter system is suitable for antiviral drug screening. Huh7.5 or
 471 A549 cells were infected with ΔS-VRP(G) virus and at 2hpi, infected cells were treated with the

indicated concentrations of Remdesivir or GC376 dissolved in DMSO. At 18hpi, GFP

473 expression and luciferase activity were measured. (A-B) Assessment of effects of Remdesivir

treatment on  $\Delta$ S Luc-GFP replication in Huh7.5 and A549 cells. (C & D) Assessment of effects

of GC376 treatment on  $\Delta$ S Luc-GFP replication in Huh7.5 and A549 cells. Luciferase values

are normalized to DMSO control and shown as % DMSO control.

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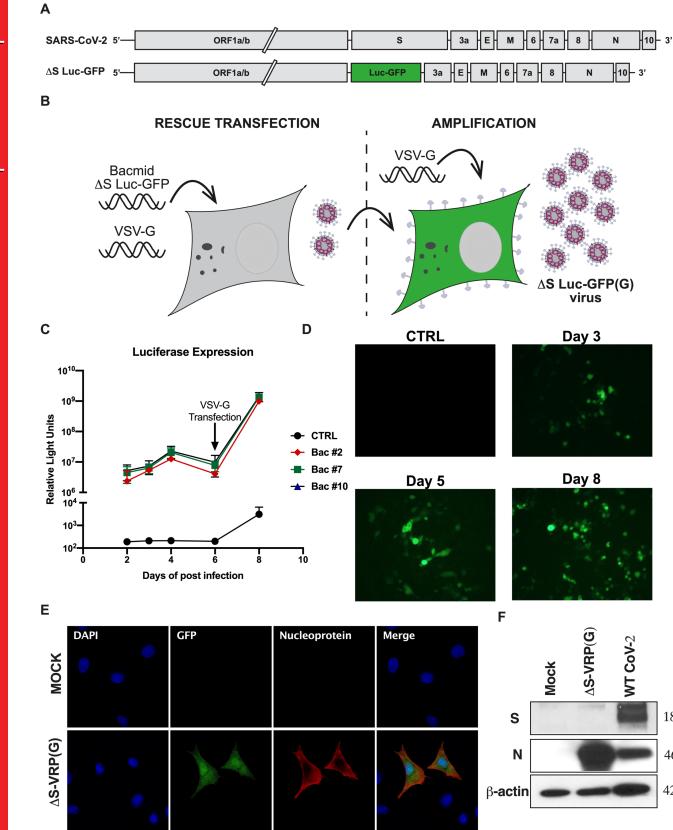
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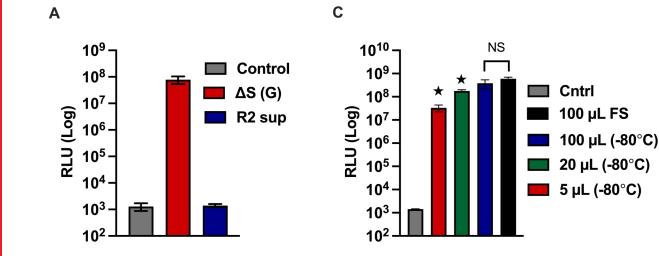


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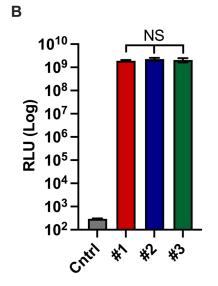
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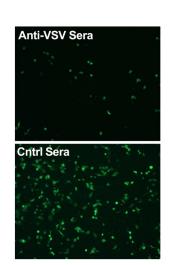
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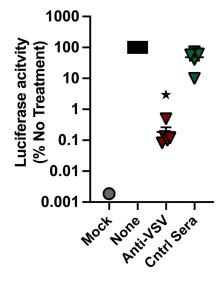


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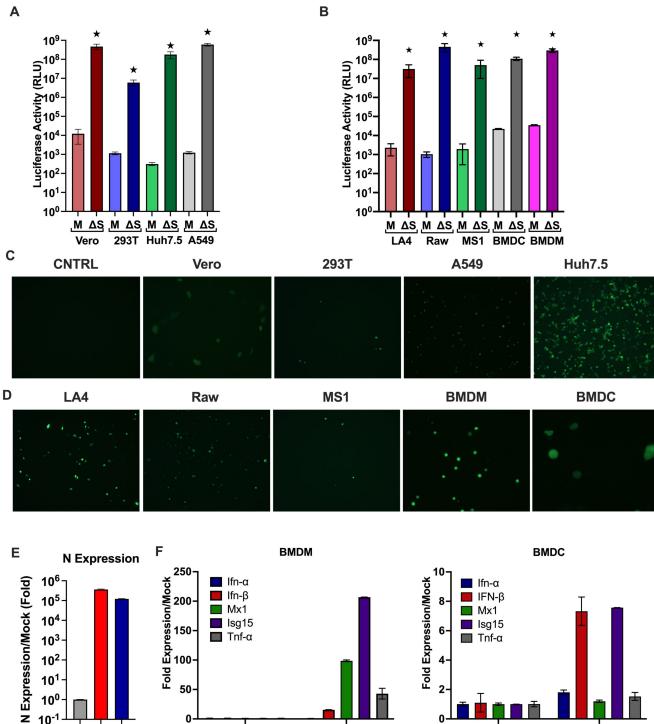




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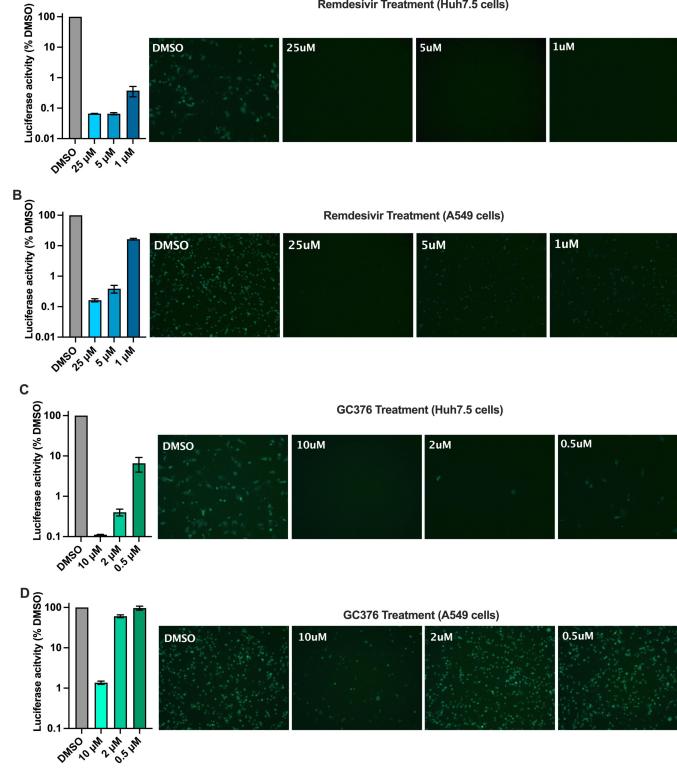
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Remdesivir Treatment (Huh7.5 cells)

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