1 **Contribution of SARS-CoV-2 accessory proteins to viral pathogenicity in**
2 **K18 hACE2 transgenic mice**

3 Jesus Silvas, Desarey Morales-Vasquez, Jun-Gyu Park, Kevin Chiem, Jordi
4 B. Torrelles, Roy Neal Platt, Tim Anderson, Chengjin Ye[#], and Luis Martinez-
5 Sobrido[#]

6

7 Texas Biomedical Research Institute, San Antonio, TX, USA

8

9 [#] To whom correspondence should be addressed:

10 Dr. Chengjin Ye (cye@txbiomed.org)

11 Dr. Luis Martinez-Sobrido (lmartinez@txbiomed.org)

12 Texas Biomedical Research Institute

13 8715 West Military Drive

14 San Antonio, Texas, 78277

15

16

17 **Keywords:** SARS-CoV-2; COVID-19; ORF3a; ORF6; ORF7a; ORF7b; ORF8;
18 hACE2; pathogenesis, K18 hACE2 transgenic mice.

19

20 **Running title:** Characterization of SARS-CoV-2 ORF deficient viruses

21

22

23

24

25

ABSTRACT

Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2) is the viral pathogen responsible for the current coronavirus disease 2019 (COVID-19) pandemic. To date, it is estimated that over 113 million individuals have been infected with SARS-CoV-2 and over 2.5 million human deaths have been recorded worldwide. Currently, three vaccines have been approved by the Food and Drug Administration for emergency use only. However much of the pathogenesis observed during SARS-CoV-2 infection remains elusive. To gain insight into the contribution of individual accessory open reading frame (ORF) proteins in SARS-CoV-2 pathogenesis, we used our recently described reverse genetics system approach to successfully engineer recombinant (r)SARS-CoV-2, where we individually removed viral 3a, 6, 7a, 7b, and 8 ORF proteins, and characterized these recombinant viruses *in vitro* and *in vivo*. Our results indicate differences in plaque morphology, with ORF deficient (Δ ORF) viruses producing smaller plaques than those of the wild-type (rSARS-CoV-2/WT). However, growth kinetics of Δ ORF viruses were like those of rSARS-CoV-2/WT. Interestingly, infection of K18 human angiotensin converting enzyme 2 (hACE2) transgenic mice with the Δ ORF rSARS-CoV-2 identified ORF3a and ORF6 as the major contributors of viral pathogenesis, while Δ ORF7a, Δ ORF7b and Δ ORF8 rSARS-CoV-2 induced comparable pathology to rSARS-CoV-2/WT. This study demonstrates the robustness of our reverse genetics system to generate rSARS-CoV-2 and the major role for ORF3a and ORF6 in viral pathogenesis, providing important information for the generation of attenuated forms of SARS-CoV-2 for their implementation as live-

attenuated vaccines for the treatment of SARS-CoV-2 infection and associated COVID-19.

IMPORTANCE

Despite great efforts put forward worldwide to combat the current coronavirus disease 2019 (COVID-19) pandemic, Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2) continues to be a human health and socioeconomic threat. Insights into the pathogenesis of SARS-CoV-2 and contribution of viral proteins to disease outcome remains elusive. Our study aims to determine the contribution of SARS-CoV-2 accessory open reading frame (ORF) proteins in viral pathogenesis and disease outcome, and develop a synergistic platform combining our robust reverse genetics system to generate recombinant (r)SARS-CoV-2 with a validated rodent model of infection and disease. We demonstrated that SARS-CoV-2 ORF3a and ORF6 contribute to lung pathology and ultimately disease outcome in K18 hACE2 transgenic mice, while ORF7a, ORF7b, and ORF8 have little impact on disease outcome. Moreover, our combinatory platform serves as the foundation to generate attenuated forms of the virus to develop live-attenuated vaccines for the treatment of SARS-CoV-2.

75

76

77 INTRODUCTION

78 Coronaviruses are enveloped, positive sense, single-stranded RNA
79 viruses that cause both mild and lethal upper respiratory illness in humans (1-
80 3). Highly pathogenic and lethal Severe Acute Respiratory Syndrome (SARS-
81 CoV) and Middle Eastern Respiratory Syndrome (MERS-CoV) coronaviruses
82 were first identified in early 2000 and 2010 as spill-over events to humans
83 from horse-shoe bats and dromedary camels, respectively (2, 4, 5). In
84 December 2019, a respiratory infectious disease disseminated throughout the
85 Chinese city of Wuhan and by Spring of 2020, it had spread worldwide (6). A
86 novel coronavirus, Severe Acute Respiratory Syndrome coronavirus-2
87 (SARS-CoV-2) was identified as the etiological agent of this new coronavirus
88 disease 2019 (COVID-19) pandemic (6-8).

89 The SARS-CoV-2 genome encodes for 16 non-structural proteins (NSPs)
90 and 6 accessory proteins each encoded by independent open reading frames
91 (ORFs) (9). It has been described that both coronavirus NSP and ORF
92 proteins play important roles in viral replication and transcription, evasion of
93 host immune responses, and viral dissemination (10, 11). In particular, SARS-
94 CoV ORF3a has been implicated as an inducer of membrane rearrangement
95 and cell death (12-14). SARS-CoV ORF3b, ORF6 and nucleocapsid (N)
96 proteins have been described to counteract the host immune interferon (IFN)
97 responses (15). Moreover, SARS-CoV 7a protein has also been shown to
98 inhibit cellular protein synthesis and activation of p38 mitogen-activated
99 protein kinase (16). Conversely, coronavirus ORF7b has been reported to

have a Golgi localization signal, where it becomes incorporated into the virion, however no additional studies have delved into any further functions (17, 18). Furthermore, in regard to SARS-CoV-2, early clinical isolates identified a 382-nucleotide deletion leading to a truncated ORF7b and removal of the ORF8 transcription signal (19). Lastly, SARS-CoV-2 ORF8 has been postulated to play a minor role in disease outcome, as natural SARS-CoV-2 isolates containing deletions in the ORF8 has been isolated from individuals presenting COVID-19 (20). Nevertheless, only a limited number of studies have examined the contribution of coronavirus NSP or ORF proteins to pathogenesis and disease outcome in animal models of infection.

To examine the contribution of accessory proteins in the pathogenicity of SARS-CoV-2, we used our previously described reverse genetics approach based on the use of a bacterial artificial chromosome (BAC) to rescue rSARS-CoV-2 with deletions of individual accessory ORF proteins (21). We were able to successfully rescue recombinant (r)SARS-CoV-2 deficient in ORF3a, ORF6, ORF7a, ORF7b, and ORF8 individually. The *in vitro* characterization of each deficient ORF (Δ ORF) virus revealed a distinct difference in plaque phenotype in comparison to the recombinant wild-type SARS-CoV-2 (rSARS-CoV-2/WT). This phenotypic change, however, did not affect the viral growth kinetics since all Δ ORF rSARS-CoV-2 replicated similarly to rSARS-CoV-2/WT. Interestingly, we observed changes in pathogenesis between the WT and Δ ORF rSARS-CoV-2 in the established K18 human angiotensin converting enzyme 2 (hACE2) transgenic mouse model of SARS-CoV-2 infection (22). In contrast to the WT, ORF Δ 7a, ORF Δ 7b, and ORF Δ 8 rSARS-CoV-2, the ORF Δ 3a and the ORF Δ 6 rSARS-CoV-2 induced less pathology

and had a 75% and 50% survival rate, respectively. Furthermore, both ORFΔ3a and ORFΔ6 rSARS-CoV-2 had lower viral titers (10^2 PFU/ml) at 2 days post-infection (d p.i.), and by 4 d p.i. were no longer detected in nasal turbinates. In contrast, ORFΔ6 viral replication in the lungs reached 10^5 PFU/ml at 2 d p.i. and only decreased by $\sim 2\text{-log}_{10}$ at 4 d p.i. ORFΔ3a replication only reached 10^2 PFU/ml at 2 d p.i. and was not detected by 4 d p.i. in the lungs. Both the ORFΔ7a and ORFΔ7b rSARS-CoV-2 induced similar pathology to rSARS-CoV-2/WT and had a 25% survival rate. By merging our *in vitro* and *in vivo* data, we have been able to generate insights into the contribution of SARS-CoV-2 accessory ORF proteins in the pathogenesis and disease outcome of SARS-CoV-2 infection. These essential data also paved the way for further designing and developing live-attenuated vaccines against SARS-CoV-2.

150

151

152

153 **RESULTS**

154 **Generation of BACs with deletions in individual accessory ORF** 155 **proteins**

156 The SARS-CoV-2 genome, which was divided into 5 fragments and
157 chemically synthesized, was assembled into a single BAC that led to efficient
158 virus rescue after transfection into Vero E6 cells using LPF2000 (21).
159 Fragment 1 included the SARS-CoV-2 ORF accessory proteins. Using
160 standard gene engineering approaches, we systematically deleted,
161 individually, ORF3a, ORF6, ORF7a, ORF7b, or ORF8 from fragment 1 using
162 PCR and primer pairs containing BsaI type IIS restriction endonuclease sites.
163 After being confirmed by Sanger sequencing (data not shown), fragment 1
164 containing the individual deletions in the ORF3a, ORF6, ORF7a, ORF7b, or
165 ORF8 accessory proteins were reassembled into the BAC (**Figure 1**).

166 **Rescue of Δ ORF rSARS-CoV-2**

167 Each BAC with individual deletions in the accessory ORFs were
168 transfected into Vero E6 cells for the recovery of Δ ORF rSARS-CoV-2,
169 according to our previously described protocol (21). At 72 h post-transfection,
170 tissue culture supernatants (P0) were collected to inoculate fresh Vero E6
171 cells (P1). Supernatants were then collected from P1 at 72 hours post-
172 infection (h p.i.) and viral titers, defined as plaque forming units/milliliter
173 (PFU/ml), were determined as previously described (21). To verify rescue of
174 each Δ ORF rSARS-CoV-2, indirect immunofluorescence was performed using

antibodies directed at the nucleocapsid (N) and spike (S) proteins (**Figure 2A**). We next verified the individual deletion of each ORF in the rSARS-CoV-2 using RT-PCR procedures to amplify the viral N gene (control), and the regions which cover the corresponding individual ORF deletions (**Figure 2B**). All the Δ ORF rSARS-CoV-2, and rSARS-CoV-2/WT, produced a RT-PCR product of approximately 1.2 kb corresponding to the N gene, whereas amplified regions that cover the corresponding ORF deletions were smaller in the Δ ORF rSARS-CoV-2 as compared to rSARS-CoV-2/WT (**Figure 2B**), demonstrating the deletion of the individual ORFs in the viral genomes. Individual deletions of the viral proteins in the Δ ORF rSARS-CoV-2 were further confirmed by Sanger sequencing of PCR products (**Figure 2C**) and by full sequencing of the viral genome (accession number PRJNA707072). These data demonstrate that each Δ ORF rSARS-CoV-2 contained the individual deletion of their respective ORF accessory proteins.

Characterization of Δ ORF rSARS-CoV-2 *in vitro*

We next proceeded to characterize each Δ ORF rSARS-CoV-2 *in vitro* (**Figure 3**). Previous studies have shown that manipulation of the viral genome can affect the phenotype of viral plaques, therefore as an initial step we proceeded to investigate if deletion of individual accessory ORFs had an impact on rSARS-CoV-2 plaque phenotype (23), as some studies have linked changes in plaque phenotype to attenuation or disease outcome (24, 25). Interestingly, we noticed an effect in the plaque phenotype of all Δ ORF rSARS-CoV-2 as compared to rSARS-CoV-2/WT in all times p.i. studied (24, 48, 72, and 96 h) (**Figure 3A**). Next, we compared the growth kinetics of the Δ ORF rSARS-CoV-2 to those of the rSARS-CoV-2/WT in Vero E6 cells. To

this end, viral titers in the tissue culture supernatants from Vero E6 cells infected (MOI, 0.01) with WT or Δ ORF rSARS-CoV-2 collected at 12, 24, 48, 72, and 96 h p.i. were determined by plaque assay (**Figure 3B**). No statistically significant differences between WT and ORF Δ 3a, ORF Δ 6, and ORF Δ 8 rSARS-CoV-2 were observed at any times p.i., except for the replication of ORF Δ 7a and ORF Δ 7b rSARS-CoV-2 that was significantly different ($\sim 1 \log_{10}$) than those of rSARS-CoV-2/WT at 12 and 24 h p.i. (**Figure 3B**). Peak viral titers for all Δ ORF rSARS-CoV-2 and rSARS-CoV-2/WT were observed between 24 and 48 h p.i., with viral titers decreasing ($\sim 1 \log_{10}$) at later times p.i., consistent with previous studies with SARS-CoV-2 natural isolates (26, 27). Altogether, these results suggest that despite slight differences in plaque phenotype, Δ ORF rSARS-CoV-2 have similar replication kinetics than rSARS-CoV-2/WT in Vero E6 cells.

Characterization of Δ ORF rSARS-CoV-2 *in vivo*

Coronavirus ORF accessory proteins have been implicated as virulence factors and contribute to both pathogenicity and disease outcome (12, 13, 20, 28-30). Therefore, we proceeded to further investigate the contribution of SARS-CoV-2 ORF3a, ORF6, ORF7a, ORF7b, and ORF8 to viral pathogenicity and disease outcome in our previously established K18 hACE2 transgenic mouse model of SARS-CoV-2 infection and COVID-19 disease (22). Four-to-six-week-old female mice (n=4) were mock (PBS)-infected, infected (10^5 PFU) with rSARS-CoV-2/WT, or with each of the Δ ORF rSARS-CoV-2, and observed for 14 days for morbidity (body weight loss) and mortality (survival) (**Figure 4**). Our results indicate a similar decrease in body weight percentage up to 5 d p.i., from which ORF Δ 3a and ORF Δ 7b rSARS-

CoV-2 infected mice began to recover (**Figure 4A**). Interestingly, mice infected with ORF Δ 6 and ORF Δ 7a rSARS-CoV-2 continued to lose body weight until 7 and 8 d p.i., respectively, and start to recover (**Figure 4A**). All mice infected with WT or ORF Δ 8 rSARS-CoV-2 succumbed to viral infection by 6 and 7 d p.i., respectively (**Figure 4B**). Our continued observations of mice infected with ORF Δ 3a, ORF Δ 6a, ORF Δ 7a, and ORF Δ 7b rSARS-CoV-2 identified survival rates of 75%, 50%, 25%, and 25%, respectively (**Figure 4B**). Upon full comparison, it is worth noting that despite the increased morbidity observed in mice infected with the ORF Δ 3a rSARS-CoV-2, 3 out of 4 mice survived viral infection, suggesting ORF3a could play an important role in viral pathogenesis.

We next evaluated viral replication in nasal turbinates and lungs of K18 hACE2 transgenic mice infected with the WT or Δ ORF rSARS-CoV-2 at 2 and 4 d p.i. (**Figure 5**). At 2 d p.i. WT and ORF Δ 6 rSARS-CoV-2 were detected in nasal turbinates in all mice, with rSARS-CoV-2/WT reaching titers of up to 5×10^3 PFU/ml, while ORF Δ 6 rSARS-CoV-2 peaked at 5×10^2 PFU/ml. We only detected ORF Δ 3a rSARS-CoV-2 (10^2 PFU/ml) in 50% of infected mice while ORF Δ 7b rSARS-CoV-2 replicated up to 3×10^3 PFU/ml in 75% of infected mice. Only 50% of mice had detectable levels (range between 0.5 - 1×10^3 PFU/ml) of ORF Δ 8 rSARS-CoV-2 (**Figure 5A**). Interestingly, out of all the Δ ORF rSARS-CoV-2 tested, ORF Δ 7a replicated in the nasal turbinate to levels (5×10^4 PFU/ml) higher than those observed with rSARS-CoV-2/WT. By 4 d p.i. ORF Δ 3a and ORF Δ 6 rSARS-CoV-2 were no longer detected in nasal turbinates, while 75% of mice infected with rSARS-CoV-2/WT had viral titers

ranging between 5×10^1 to 5×10^2 PFU/ml. ORF Δ 7a, ORF Δ 7b and ORF Δ 8 rSARS-CoV-2 replicated to lower levels ($\sim 5 \times 10^1$ PFU/ml) than rSARS-CoV-2/WT (**Figure 5A**). In the lungs WT, ORF Δ 6 and ORF Δ 7a rSARS-CoV-2 were detected at levels of $\sim 10^5$ PFU/ml at 2 d p.i. (**Figure 5B**). Viral titers of ORF Δ 7b and ORF Δ 8 ($\sim 10^4$ PFU/ml) and ORF Δ 3a ($\sim 0.5 \times 10^2$ PFU/ml) rSARS-CoV-2 were lower than those of rSARS-CoV-2/WT (**Figure 5B**). Interestingly, by 4 d p.i., ORF Δ 3a rSARS-CoV-2 was no longer detected in the lungs while WT, ORF Δ 6, ORF Δ 7a, ORF Δ 7b, and ORF Δ 8 rSARS-CoV-2 only decreased $\sim 1 \log_{10}$ to those observed by 2 d p.i. (**Figure 5B**).

Lastly, to evaluate the impact of viral infection in the lungs of infected animals, we performed gross pathology analysis on lungs collected at 2 and 4 d p.i. (**Figure 6A**). Both WT and Δ ORF rSARS-CoV-2 induced similar pathology at 2 d p.i., with WT, ORF Δ 7a, and ORF Δ 7b rSARS-CoV-2 inducing pathological lesions in more than 50% of lung area (**Figure 6B**). Intriguingly, by 4 d p.i. only WT and ORF Δ 8 rSARS-CoV-2-infected lungs maintained pathological lesions, while ORF Δ 3a, ORF Δ 6, ORF Δ 7a, ORF Δ 7b rSARS-CoV-2 infected lungs appear to recover from lesions observed at 2 d p.i. Altogether, these *in vivo* results provide a new insight into the contribution of SARS-CoV-2 accessory ORF proteins in viral pathogenesis and disease, suggesting a major role of ORF3a and ORF6 and a less impact of ORF7a, ORF7b, and ORF8 in virulence and disease outcome.

274

275

276

277

278

279 **DISCUSSION**

280 Viral proteins contribute to different aspects of disease outcome and
 281 pathogenesis during the course of viral infections (20, 28, 29). Reverse
 282 genetics systems allow for the deletion of viral proteins to gain insight into
 283 their contribution to viral replication, pathogenesis, transmission, and/or in
 284 disease outcome, among others (31-34). We have recently established a
 285 *state-of-the-art* reverse genetics system for the efficient rescue of rSARS-
 286 CoV-2/WT (21, 22) that we have used to generate Δ ORF rSARS-CoV-2 and
 287 interrogate the contribution of the ORF3a, ORF6, ORF7a, ORF7b, and ORF8
 288 accessory proteins in viral replication *in vitro* and *in vivo*. Similar studies have
 289 provided insight into the contribution of accessory ORF proteins in SARS-CoV
 290 infection *in vitro*, and have identified a variety of functions, ranging from
 291 inhibition of host immune responses to activation of host cell death pathways
 292 (12-14, 17, 30, 31, 35-37). However, to date, no study has provided insight
 293 into the role of SARS-CoV-2 ORF accessory proteins in viral replication *in*
 294 *vitro* and/or in pathogenesis *in vivo*. Our study using robust genetics systems
 295 and the validated K18 hACE2 transgenic mouse model of SARS-CoV-2
 296 infection provides us with useful information on the contribution of SARS-CoV-
 297 2 ORFs 3a, 6, 7a, 7b, and 8 accessory proteins to viral pathogenesis.

In this study, we generated rSARS-CoV-2 deficient in ORFs 3a, 6, 7a, 7b, or 8 accessory proteins (**Figure 1**) and characterized them both *in vitro* (**Figures 2 and 3**) and *in vivo* (**Figures 4-6**). Importantly, we have been able to demonstrate the ORF deficient nature of these rSARS-CoV-2 (**Figure 2**). During our initial *in vitro* characterization, we first identified that ORF proteins contributed to early dissemination and formation of detectable viral plaques in Vero E6 cell monolayers: viruses lacking 3a, 7a, 7b, and 8 ORF proteins developed smaller plaques than rSARS-CoV-2/WT (**Figure 3**). This was a surprising finding, as we only observed ~1 log₁₀ difference in growth kinetics between the WT and any of the Δ ORF rSARS-CoV-2 (**Figure 3**).

To further determine the contribution of each ORF accessory protein in SARS-CoV-2 pathogenesis, we used our recently established K18 hACE2 transgenic mouse model (**Figure 4**) (22). This particular *in vivo* study is the first, to our knowledge, that analyzes the contribution of SARS-CoV-2 ORF accessory proteins in viral infection in a validated animal model of infection. We observed a broad range of morbidity and survival outcomes between each of the Δ ORF rSARS-CoV-2 as compared to rSARS-CoV-2/WT (**Figure 4**). SARS-CoV-2 ORF accessory proteins are encoded in the following order: 3a, 6, 7a, 7b, and 8. Our data collectively showed greatest survival in mice infected with ORF Δ 3a (75%) and 100% mortality with the ORF Δ 8 rSARS-CoV-2. In this regard, a natural SARS-CoV-2 variant with a deletion in the ORF8 has been recently isolated from patients presenting COVID-19 symptoms (20). Thus, our results with the ORF Δ 8 rSARS-CoV-2 in the K18 hACE2 transgenic mouse model correspond to those observed in people infected with a natural ORF Δ 8 SARS-CoV-2 isolate (20). Our findings with

ORF Δ 3a and ORF Δ 6 rSARS-CoV-2 warrant further characterization as there is limited insight into mechanistic exploitation of host pathways by ORF3a (12, 13, 30, 35, 36). Since it is well known that SARS-CoV-2 ORF6 is a potent inhibitor of the host innate immune response (11, 29), we were expecting low viral load and an increased immune response; however, since SARS-CoV N protein also inhibits host immune responses (15, 38), it is plausible that SARS-CoV-2 N protein may have a similar function and is responsible of counteracting host innate immune and inflammatory responses. Even though our studies did not analyze cytokine and chemokine production, the gradual recovery of mice infected with ORF Δ 6 rSARS-CoV-2 are suggestive of viral clearance over time. Analysis of innate and adaptive immune responses would be important, as well as modulation of IFN secretion, ion signaling channels, and cellular apoptotic and/or necrosis pathways, to determine the contribution of these ORF accessory proteins in viral pathogenesis *in vivo*. This is currently the focus of our ongoing *in vivo* studies with these Δ ORF rSARS-CoV-2. Overall, similar to what was described with rSARS-CoV, our study highlights that SARS-CoV ORFs 3a, 3b, 6, 7a, and 7b had no significant impact on viral replication *in vivo* (31), but ORF 3a seems to be involved in virulence, as its absence decreases SARS-CoV-2 virulence.

Overall, this study demonstrates the robustness of our BAC-based reverse genetics approach to generate rSARS-CoV-2, including those with deletions of ORF accessory proteins, and provides information on the contribution of ORFs 3a, 6, 7a, 7b, and 8 accessory proteins in viral fitness *in vitro* (Vero E6 cells) and *in vivo*, in our recently established K18 hACE2 transgenic mouse model of SARS-CoV-2 infection and COVID-19 disease

(22). Importantly, information from this study also provides novel insights for the generation of attenuated forms of SARS-CoV-2 for the development of live-attenuated vaccines for the treatment of this important respiratory pathogen, and its associated COVID-19 disease.

MATERIALS AND METHODS

Biosafety

All the *in vitro* and *in vivo* experiments with infectious natural isolate or rSARS-CoV-2 were conducted under appropriated biosafety level (BSL) 3 and animal BSL3 (ABSL3) laboratories, respectively, at Texas Biomedical Research Institute. Experiments were approved by the Texas Biomed Institutional Biosafety (IBC) and Animal Care and Use (IACUC) committees.

Cells

African green monkey kidney epithelial cells (Vero E6, CRL-1586) were obtained from the American Type Culture Collection (ATCC, Bethesda, MD) and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% (v/v) fetal bovine serum, FBS (VWR) and 100 units/ml penicillin-streptomycin (Corning).

Reverse genetics system

The BAC harboring the entire viral genome of SARS-CoV-2 USA-WA1/2020 (Accession No. MN985325) was described previously (21). Deletion of individual accessory ORF proteins was achieved in viral fragment 1 by using inverse PCR and primer pairs containing a BsaI type IIS restriction

endonuclease site. All the primer sequences are available upon request. Fragments including the individual deletion of accessory ORF proteins were reassembled into the BAC using BamHI and RsrII restriction endonucleases.

Rescue of Δ ORF rSARS-CoV-2

Virus rescues were performed as previously described (21). Briefly, confluent monolayers of Vero E6 cells (10^6 cells/well, 6-well plates, triplicates) were transfected with 4.0 μ g/well of SARS-CoV-2 BAC using Lipofectamine 2000. After 24 h, transfection media was exchanged for p.i. media [DMEM supplemented with 2% (v/v) FBS], and cells were split and seeded into T75 flasks 48 h post-transfection. After incubation for another 72 h, tissue culture supernatants were collected, labeled as P0 and stored at -80°C . After viral titration of the supernatant, the P0 virus was used to infect fresh Vero E6 cells at multiplicity of infection (MOI) of 0.0001 to make new viral stocks. Tissue culture supernatants were collected 72 h p.i., aliquoted, labeled as P1, and stored at -80°C for future use.

Immunofluorescence assay (IFA)

Vero E6 cells (10^5 cells/well, 24-well plate format, triplicates) were mock-infected or infected (MOI of 3) with WT or Δ ORF rSARS-CoV-2. At 24 h p.i, cells were fixed with 10% formaldehyde solution at 4°C overnight and permeabilized using 0.5% (v/v) Triton X-100 in PBS for 15 min at room temperature. Cells were incubated overnight with 1 μ g/ml of a SARS-CoV N protein cross-reactive polyclonal antibody at 4°C and a SARS-CoV-2 spike (S) cross-reactive monoclonal antibody (3B4), washed with PBS, and stained with a FITC-labeled goat anti-mouse IgG (1:200) and Rhodamine-labeled goat anti-rabbit IgG (1:200). Nuclei were visualized by DAPI staining. After

washing with PBS, cells were visualized and imaged with an EVOS microscope (ThermoFisher Scientific).

Plaque assay and immunostaining

Confluent monolayers of Vero E6 cells (10^6 cells/well, 6-well plate format, triplicates) were infected with serial diluted viruses for 1 h at 37°C. After viral adsorption, cells were overlaid with p.i. media containing 1% low melting agar and incubated at 37°C. At 72 h p.i., cells were fixed overnight with 10% formaldehyde solution. For immunostaining, cells were permeabilized with 0.5% (v/v) Triton X-100 in PBS for 15 min at room temperature and immunostained using the SARS-CoV N protein cross-reactive monoclonal antibody 1C7C7 (1 µg/ml) and the Vectastain ABC kit (Vector Laboratories), following the manufacturers' instruction. After immunostaining, plates were visualized on a Chemi-Doc (Bio-Rad).

Virus growth kinetics

Confluent monolayers of Vero E6 cells (10^6 cells/well, 6-well plate format, triplicates) were infected (MOI of 0.01) with WT or Δ ORF rSARS-CoV-2. After 1 h virus adsorption at 37°C, cells were washed with PBS and incubated in p.i. media at 37°C. At the indicated times after infection, viral titers in tissue culture supernatants were determined by plaque assay and immunostaining using the SARS-CoV N protein cross-reactive monoclonal antibody 1C7C7, as previously described (39) .

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from Vero E6 cells (10^6 cells/well, 6-well plate format) infected (MOI of 0.01) with WT or Δ ORF rSARS-CoV-2 was extracted with TRIzol Reagent (Thermo Fisher Scientific) according to the manufacturer's

instructions. RT-PCR amplification was performed using Super Script II Reverse transcriptase (Thermo Fisher Scientific) and Expanded High Fidelity PCR System (Sigma Aldrich). The amplified DNA products were subjected to 0.7% agarose gel analysis and the gel-purified PCR fragments were subjected to sanger sequencing (ACGT). All primer sequences used for RT-PCR are available upon request. Methods for Illumina library preparation, sequencing, and analysis of recombinant viruses follow those previously described (21).

Mice

Specific-pathogen-free, 4–8 week-old, female B6.Cg-Tg(K18-ACE2)2PrImn/J (Stock No: 034860) K18 hACE2 transgenic mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Our previous studies did not show significant sex dependent differences in morbidity, mortality and viral titers (22). K18 hACE2 transgenic mice were maintained in micro-isolator cages at ABSL-3 and provided sterile water and chow *ad libitum* and acclimatized for one week prior to experimental manipulation. For morbidity and mortality studies, a total of 20 females were used (n=4/group), whereas a total of 40 female mice (n=4 per group) were used for viral titers at 2 and 4 d p.i.. For the mock infected control groups in the morbidity and mortality studies, 2 female K18 hACE2 transgenic mice were used and a total of 4 (n=2 per time-point), were used as mock-infected controls for gross pathology analysis.

Mouse infection and sample processing

K18 hACE2 transgenic mice were either mock (PBS)-infected or infected intranasally (i.n.) with 10^5 PFU of WT or Δ ORF rSARS-CoV-2 in a final volume

of 50 μ L following isoflurane sedation. After viral infection, mice were monitored daily for 14 days for morbidity (body weight loss) and mortality (survival). Mice showing >25% loss of their initial body weight were defined as reaching experimental endpoint and humanely euthanized. For viral titers and gross pathology analysis, K18 hACE2 transgenic mice were infected as above but euthanized at 2 or 4 d p.i. Nasal turbinates and lung tissues were harvested and homogenized in 1 ml of PBS using a Precellys tissue homogenizer (Bertin Instruments) for viral titrations. Tissue homogenates were centrifuged at $21,500 \times g$ for 5 min and supernatants were collected for determination of viral titer.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.3. For multiple comparisons, a 2-way ANOVA with multiple comparisons was performed.

473

474

475

476

477

478

479

480

481

482 **ACKNOWLEDGEMENTS**

483 We want to thank Dr. Thomas Moran at the Icahn School of Medicine at
484 Mount Sinai for providing us with the SARS-CoV cross-reactive N monoclonal
485 antibody 1C7C7. We also want to thank BEI Resources for providing the
486 SARS-CoV-2 USA-WA1/2020 isolate (NR-52281). Finally, we would also like
487 to thank members at our institutes for their efforts in keeping them fully
488 operational during the COVID-19 pandemic and the BSC and IACUC
489 committees for reviewing our protocols in a time efficient manner. We would
490 like to dedicate this manuscript to all COVID-19 victims and to all heroes
491 battling this disease.

492

493

494

495

496

497

498

499

500

501

502

503

504

505

506

507

508 REFERENCES

- 509 1. Cui J, Li F, Shi ZL. 2019. Origin and evolution of pathogenic
510 coronaviruses. *Nat Rev Microbiol* 17:181-192.
- 511 2. de Wit E, van Doremalen N, Falzarano D, Munster VJ. 2016. SARS
512 and MERS: recent insights into emerging coronaviruses. *Nat Rev*
513 *Microbiol* 14:523-34.
- 514 3. Woo PC, Lau SK, Lam CS, Lau CC, Tsang AK, Lau JH, Bai R, Teng JL,
515 Tsang CC, Wang M, Zheng BJ, Chan KH, Yuen KY. 2012. Discovery of
516 seven novel Mammalian and avian coronaviruses in the genus
517 deltacoronavirus supports bat coronaviruses as the gene source of
518 alphacoronavirus and betacoronavirus and avian coronaviruses as the
519 gene source of gammacoronavirus and deltacoronavirus. *J Virol*
520 86:3995-4008.

- 521 4. Haagmans BL, Al Dhahiry SH, Reusken CB, Raj VS, Galiano M, Myers
522 R, Godeke GJ, Jonges M, Farag E, Diab A, Ghobashy H, Alhajri F, Al-
523 Thani M, Al-Marri SA, Al Romaihi HE, Al Khal A, Bermingham A,
524 Osterhaus AD, AlHajri MM, Koopmans MP. 2014. Middle East
525 respiratory syndrome coronavirus in dromedary camels: an outbreak
526 investigation. *Lancet Infect Dis* 14:140-5.
- 527 5. Luk HKH, Li X, Fung J, Lau SKP, Woo PCY. 2019. Molecular
528 epidemiology, evolution and phylogeny of SARS coronavirus. *Infect*
529 *Genet Evol* 71:21-30.
- 530 6. López-Ortiz E, López-Ortiz G, Mendiola-Pastrana IR, Mazón-Ramírez
531 JJ, Díaz-Quirón JA. 2020. From the handling of an outbreak by an
532 unknown pathogen in Wuhan to the preparedness and response in the
533 face of the emergence of Covid-19 in Mexico. *Gac Med Mex* 156:132-
534 137.
- 535 7. Ralph R, Lew J, Zeng T, Francis M, Xue B, Roux M, Toloue
536 Ostadgavahi A, Rubino S, Dawe NJ, Al-Ahdal MN, Kelvin DJ,
537 Richardson CD, Kindrachuk J, Falzarano D, Kelvin AA. 2020. 2019-
538 nCoV (Wuhan virus), a novel Coronavirus: human-to-human
539 transmission, travel-related cases, and vaccine readiness. *J Infect Dev*
540 *Ctries* 14:3-17.
- 541 8. Malik YA. 2020. Properties of Coronavirus and SARS-CoV-2. *Malays J*

542 Pathol 42:3-11.

543 9. Fernandes JD, Hinrichs AS, Clawson H, Gonzalez JN, Lee BT, Nassar
544 LR, Raney BJ, Rosenbloom KR, Nerli S, Rao AA, Schmelter D, Fyfe A,
545 Maulding N, Zweig AS, Lowe TM, Ares M, Corbet-Detig R, Kent WJ,
546 Haussler D, Haeussler M. 2020. The UCSC SARS-CoV-2 Genome
547 Browser. Nat Genet 52:991-998.

548 10. Yuan S, Peng L, Park JJ, Hu Y, Devarkar SC, Dong MB, Shen Q, Wu
549 S, Chen S, Lomakin IB, Xiong Y. 2020. Nonstructural Protein 1 of
550 SARS-CoV-2 Is a Potent Pathogenicity Factor Redirecting Host Protein
551 Synthesis Machinery toward Viral RNA. Mol Cell 80:1055-1066.e6.

552 11. Lei X, Dong X, Ma R, Wang W, Xiao X, Tian Z, Wang C, Wang Y, Li L,
553 Ren L, Guo F, Zhao Z, Zhou Z, Xiang Z, Wang J. 2020. Activation and
554 evasion of type I interferon responses by SARS-CoV-2. Nat Commun
555 11:3810.

556 12. Issa E, Merhi G, Panossian B, Salloum T, Tokajian S. 2020. SARS-
557 CoV-2 and ORF3a: Nonsynonymous Mutations, Functional Domains,
558 and Viral Pathogenesis. mSystems 5.

559 13. Ren Y, Shu T, Wu D, Mu J, Wang C, Huang M, Han Y, Zhang XY, Zhou
560 W, Qiu Y, Zhou X. 2020. The ORF3a protein of SARS-CoV-2 induces
561 apoptosis in cells. Cell Mol Immunol 17:881-883.

562 14. Freundt EC, Yu L, Goldsmith CS, Welsh S, Cheng A, Yount B, Liu W,

563 Frieman MB, Buchholz UJ, Screaton GR, Lippincott-Schwartz J, Zaki
564 SR, Xu XN, Baric RS, Subbarao K, Lenardo MJ. 2010. The open
565 reading frame 3a protein of severe acute respiratory syndrome-
566 associated coronavirus promotes membrane rearrangement and cell
567 death. *J Virol* 84:1097-109.

568 15. Kopecky-Bromberg SA, Martínez-Sobrido L, Frieman M, Baric RA,
569 Palese P. 2007. Severe acute respiratory syndrome coronavirus open
570 reading frame (ORF) 3b, ORF 6, and nucleocapsid proteins function as
571 interferon antagonists. *J Virol* 81:548-57.

572 16. Kopecky-Bromberg SA, Martinez-Sobrido L, Palese P. 2006. 7a protein
573 of severe acute respiratory syndrome coronavirus inhibits cellular
574 protein synthesis and activates p38 mitogen-activated protein kinase. *J*
575 *Virol* 80:785-93.

576 17. Schaecher SR, Mackenzie JM, Pekosz A. 2007. The ORF7b protein of
577 severe acute respiratory syndrome coronavirus (SARS-CoV) is
578 expressed in virus-infected cells and incorporated into SARS-CoV
579 particles. *J Virol* 81:718-31.

580 18. Schaecher SR, Diamond MS, Pekosz A. 2008. The transmembrane
581 domain of the severe acute respiratory syndrome coronavirus ORF7b
582 protein is necessary and sufficient for its retention in the Golgi complex.
583 *J Virol* 82:9477-91.

- 584 19. Su YCF, Anderson DE, Young BE, Linster M, Zhu F, Jayakumar J,
585 Zhuang Y, Kalimuddin S, Low JGH, Tan CW, Chia WN, Mak TM,
586 Octavia S, Chavatte JM, Lee RTC, Pada S, Tan SY, Sun L, Yan GZ,
587 Maurer-Stroh S, Mendenhall IH, Leo YS, Lye DC, Wang LF, Smith
588 GJD. 2020. Discovery and Genomic Characterization of a 382-
589 Nucleotide Deletion in ORF7b and ORF8 during the Early Evolution of
590 SARS-CoV-2. mBio 11.
- 591 20. Young BE, Fong SW, Chan YH, Mak TM, Ang LW, Anderson DE, Lee
592 CY, Amrun SN, Lee B, Goh YS, Su YCF, Wei WE, Kalimuddin S, Chai
593 LYA, Pada S, Tan SY, Sun L, Parthasarathy P, Chen YYC, Barkham T,
594 Lin RTP, Maurer-Stroh S, Leo YS, Wang LF, Renia L, Lee VJ, Smith
595 GJD, Lye DC, Ng LFP. 2020. Effects of a major deletion in the SARS-
596 CoV-2 genome on the severity of infection and the inflammatory
597 response: an observational cohort study. Lancet 396:603-611.
- 598 21. Ye C, Chiem K, Park JG, Oladunni F, Platt RN, 2nd, Anderson T,
599 Almazan F, de la Torre JC, Martinez-Sobrido L. 2020. Rescue of SARS-
600 CoV-2 from a Single Bacterial Artificial Chromosome. mBio 11:e02168-
601 20.
- 602 22. Oladunni FS, Park JG, Pino PA, Gonzalez O, Akhter A, Allué-Guardia
603 A, Olmo-Fontánéz A, Gautam S, Garcia-Vilanova A, Ye C, Chiem K,
604 Headley C, Dwivedi V, Parodi LM, Alfson KJ, Staples HM, Schami A,

605 Garcia JI, Whigham A, Platt RN, Gazi M, Martinez J, Chuba C, Earley
606 S, Rodriguez OH, Mdaki SD, Kavelish KN, Escalona R, Hallam CRA,
607 Christie C, Patterson JL, Anderson TJC, Carrion R, Dick EJ, Hall-
608 Ursone S, Schlesinger LS, Alvarez X, Kaushal D, Giavedoni LD, Turner
609 J, Martinez-Sobrido L, Torrelles JB. 2020. Lethality of SARS-CoV-2
610 infection in K18 human angiotensin-converting enzyme 2 transgenic
611 mice. Nat Commun 11:6122.

612 23. Kato F, Tajima S, Nakayama E, Kawai Y, Taniguchi S, Shibasaki K,
613 Taira M, Maeki T, Lim CK, Takasaki T, Saijo M. 2017. Characterization
614 of large and small-plaque variants in the Zika virus clinical isolate
615 ZIKV/Hu/S36/Chiba/2016. Sci Rep 7:16160.

616 24. Kim YI, Murphy R, Majumdar S, Harrison LG, Aitken J, DeVincenzo JP.
617 2015. Relating plaque morphology to respiratory syncytial virus
618 subgroup, viral load, and disease severity in children. Pediatr Res
619 78:380-8.

620 25. Goh KC, Tang CK, Norton DC, Gan ES, Tan HC, Sun B, Syenina A,
621 Yousuf A, Ong XM, Kamaraj US, Cheung YB, Gubler DJ, Davidson A,
622 St John AL, Sessions OM, Ooi EE. 2016. Molecular determinants of
623 plaque size as an indicator of dengue virus attenuation. Sci Rep
624 6:26100.

625 26. Jureka AS, Silvas JA, Basler CF. 2020. Propagation, Inactivation, and

626 Safety Testing of SARS-CoV-2. *Viruses* 12.

627 27. Case JB, Bailey AL, Kim AS, Chen RE, Diamond MS. 2020. Growth,
628 detection, quantification, and inactivation of SARS-CoV-2. *Virology*
629 548:39-48.

630 28. Shi CS, Nabar NR, Huang NN, Kehrl JH. 2019. SARS-Coronavirus
631 Open Reading Frame-8b triggers intracellular stress pathways and
632 activates NLRP3 inflammasomes. *Cell Death Discov* 5:101.

633 29. Yuen CK, Lam JY, Wong WM, Mak LF, Wang X, Chu H, Cai JP, Jin DY,
634 To KK, Chan JF, Yuen KY, Kok KH. 2020. SARS-CoV-2 nsp13, nsp14,
635 nsp15 and orf6 function as potent interferon antagonists. *Emerg*
636 *Microbes Infect* 9:1418-1428.

637 30. Majumdar P, Niyogi S. 2020. ORF3a mutation associated with higher
638 mortality rate in SARS-CoV-2 infection. *Epidemiol Infect* 148:e262.

639 31. Yount B, Roberts RS, Sims AC, Deming D, Frieman MB, Sparks J,
640 Denison MR, Davis N, Baric RS. 2005. Severe acute respiratory
641 syndrome coronavirus group-specific open reading frames encode
642 nonessential functions for replication in cell cultures and mice. *J Virol*
643 79:14909-22.

644 32. Kalveram B, Lihoradova O, Indran SV, Ikegami T. 2011. Using reverse
645 genetics to manipulate the NSs gene of the Rift Valley fever virus MP-
646 12 strain to improve vaccine safety and efficacy. *J Vis Exp*

- doi:10.3791/3400:e3400.
33. Nishiyama S, Slack OA, Lokugamage N, Hill TE, Juelich TL, Zhang L, Smith JK, Perez D, Gong B, Freiberg AN, Ikegami T. 2016. Attenuation of pathogenic Rift Valley fever virus strain through the chimeric S-segment encoding sandfly fever phlebovirus NSs or a dominant-negative PKR. *Virulence* 7:871-881.
34. Theriault S, Groseth A, Artsob H, Feldmann H. 2005. The role of reverse genetics systems in determining filovirus pathogenicity. *Arch Virol Suppl* doi:10.1007/3-211-29981-5_13:157-77.
35. Bianchi M, Borsetti A, Ciccozzi M, Pascarella S. 2021. SARS-Cov-2 ORF3a: Mutability and function. *Int J Biol Macromol* 170:820-826.
36. Miao G, Zhao H, Li Y, Ji M, Chen Y, Shi Y, Bi Y, Wang P, Zhang H. 2021. ORF3a of the COVID-19 virus SARS-CoV-2 blocks HOPS complex-mediated assembly of the SNARE complex required for autolysosome formation. *Dev Cell* 56:427-442.e5.
37. Siu KL, Yuen KS, Castaño-Rodriguez C, Ye ZW, Yeung ML, Fung SY, Yuan S, Chan CP, Yuen KY, Enjuanes L, Jin DY. 2019. Severe acute respiratory syndrome coronavirus ORF3a protein activates the NLRP3 inflammasome by promoting TRAF3-dependent ubiquitination of ASC. *FASEB J* 33:8865-8877.
38. Hu Y, Li W, Gao T, Cui Y, Jin Y, Li P, Ma Q, Liu X, Cao C. 2017. The

668 Severe Acute Respiratory Syndrome Coronavirus Nucleocapsid Inhibits
669 Type I Interferon Production by Interfering with TRIM25-Mediated RIG-I
670 Ubiquitination. J Virol 91.
671 39. Chiem K, Morales Vasquez D, Park JG, Platt RN, Anderson T, Walter
672 MR, Kobie JJ, Ye C, Martinez-Sobrido L. 2021. Generation and
673 Characterization of recombinant SARS-CoV-2 expressing reporter
674 genes. J Virol doi:10.1128/JVI.02209-20.

675

676

677

678

679

680

681

682

683

684

685

686

687

688

689 **FIGURE LEGENDS**

690 **Figure 1. Genome organization of WT and Δ ORF rSARS-CoV-2:** SARS-
691 CoV-2 genome includes ~29.8 kb nucleotides among which ~21.5 kb encodes

the ORF1a and ORF1b replicase. The rest of the ~8.3 kb viral genome encodes the structural spike (S), envelope (E), matrix (M), and nucleocapsid (N) proteins; and the accessory 3a, 6, 7a, 7b, 8, and 10 ORF proteins. Individual deletions of the ORF accessory proteins were introduced into the BAC for rescue of rSARS-CoV-2. Schematic representation not drawn to scale.

Figure 2. Rescue of Δ ORF rSARS-CoV-2. A) IFA: Vero E6 cells (24-well plate format, 10^5 cells/well, triplicates) were mock-infected or infected (MOI of 3) with WT, ORF Δ 3a, ORF Δ 6, ORF Δ 7a, ORF Δ 7b, or ORF Δ 8 rSARS-CoV-2. At 24 h p.i., cells were fixed and immunostained with a cross-reactive polyclonal antibody against SARS-CoV N protein and a cross-reactive monoclonal antibody against SARS-CoV S protein (3B4). Rhodamine red goat anti-rabbit and FITC rabbit anti-mouse secondary antibodies were used and nuclei were visualized by DAPI. Scale bars, 100 μ m. **B) RT-PCR:** Vero E6 cells (6-well plate format, 10^6 cells/well) were mock-infected or infected (MOI of 0.1) with WT and Δ ORF rSARS-CoV-2 and total RNA was extracted at 24 h p.i. Regions in the viral genome corresponding with the deletion were amplified and the N gene was amplified as an internal control. All the products were separated on a 0.8% agarose gel. **C) Sequencing:** RT-PCR products from panel B were gel-purified and subjected to Sanger sequencing. The consensus sequences in the genome of both WT and Δ ORF rSARS-CoV-2 downstream the deleted gene are indicated in blue, the intergenic region between viral genes are shown in yellow, the genes deleted in the Δ ORF rSARS-CoV-2 are indicated in green, and the viral genes upstream the deleted ORFs in the rSARS-CoV-2 are shown in red.

Figure 3. *In vitro* characterization of WT and Δ ORF rSARS-CoV-2. A)

Plaque phenotype: Vero E6 cells (6-well plate format, 10^6 cells/well) were infected with WT, ORF Δ 3a, ORF Δ 6, ORF Δ 7a, ORF Δ 7b, or ORF Δ 8 rSARS-CoV-2 and overlaid with media containing agar. Plates were incubated at 37°C and monolayers were immunostained with an anti-N protein SARS-CoV cross-reactive monoclonal antibody 1C7C7 at indicated h p.i. **B) Multicycle growth kinetics:** Vero E6 cells (6-well plate format, 10^6 cells/well, triplicates) were infected (MOI 0.01) with WT and Δ ORF rSARS-CoV-2 and incubated 37°C. At the indicated h p.i. Tissue culture supernatants from infected cells were collected and viral titers were determined by plaque assay (PFU/ml) and immunostaining using the anti-N SARS-CoV cross-reactive monoclonal antibody 1C7C7. Data represent the means $\pm$ standard deviations (SDs) of the results determined in triplicate wells. Dotted black lines indicate the limit of detection (LOD, 100 PFU/ml). *P < 0.05: using the Student T test.

Figure 4. Infection of K18 hACE2 transgenic mice with WT and Δ ORF

rSARS-CoV-2: K18 hACE2 transgenic female 6-8-week-old mice were mock (PBS)-infected or infected (i.n.) with 10^5 PFU of WT and Δ ORF rSARS-CoV-2 (n=4/group). **A)** Body weight and **B)** survival were evaluated at the indicated d p.i. Mice that lost >25% of their initial body weight were humanely euthanized. Error bars represent SDs of the mean for each group.

Figure 5. Replication of WT and Δ ORF rSARS-CoV-2 in nasal turbinate

and lungs: K18 hACE2 transgenic female 6-8-week-old mice were mock (PBS)-infected or infected (i.n.) with 10^5 PFU of WT and Δ ORF rSARS-CoV-2 (n=8/group). Mice were sacrificed at 2 (n=4/group) and 4 (n=4/group) d p.i. and viral titers nasal turbinate and lung were determined by plaque assay

(PFU/ml) and immunostaining using the cross-reactive SARS-CoV 1C7C7 N protein monoclonal antibody. Viral titers in the nasal turbinate **(A)** and lungs **(B)** are shown. Symbols represent data from individual mouse, and bars the geometric means of viral titers. Dotted lines indicate limit of detection (LOD, 10 PFU/ml). ND, not detected; @, not detected in 1 mouse; #, not detected in 2 mice; &, not detected in 3 mice. Negative results of the PBS-infected mice are not plotted.

Figure 6. Gross pathology analysis of lungs from K18 hACE2 transgenic mice infected with WT and Δ ORF rSARS-CoV-2: Lungs from 6-to-8-week-old female K18 hACE2 transgenic mice (n=8/group) mock (PBS)-infected or infected (i.n.) with 10^5 PFU of WT or Δ ORF rSARS-CoV-2 were harvested at 2 (n=4/group) or 4 (n=4/group) d p.i. and imaged **(A)**, and scored for viral induced lesions **(B)**. Calculation of total lung surface area affected by viral induced lesions. Scale bars, 3 cm.

A)

B)

A)

B)