1 **Bat SARS-like coronavirus WIV1 encodes an extra accessory protein ORFX**

2 **involved in modulation of host immune response**

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Abstract

Bats harbor severe acute respiratory syndrome-like coronaviruses (SL-CoVs) from which the causative agent of the 2002-3 SARS pandemic is thought to have originated. However, despite a large number of genetically diverse SL-CoV sequences detected in bats, only two strains (named WIV1 and WIV16) have been successfully cultured *in vitro*. These two strains differ from SARS-CoV only in containing an extra ORF (named ORFX) between ORF6 and ORF7 with no homology to any known protein sequences. In this study, we constructed a full-length cDNA clone of SL-CoV WIV1 (rWIV1), an ORFX deletion mutant (rWIV1-ΔX), and a GFP-expressing mutant (rWIV1-GFP-ΔX). Northern blot and fluorescent microscopy indicate that ORFX was expressed during WIV1 infection. Virus infection assay showed that rWIV1-ΔX replicated as efficiently as rWIV1 in Vero E6, Calu-3, and HeLa-hACE2 cells. Further study showed that ORFX could inhibit interferon production and activates NF-κB. Our results demonstrate for the first time that the unique ORFX in the WIV1 strain is a functional gene involving modulation of host immune response, but not essential for *in vitro* viral replication.

Importance

Bats harbor genetically diverse SARS-like coronaviruses (SL-CoVs) and some of them have the potential of interspecies transmission. A unique open reading frame (ORFX) was identified in the genome of two recently isolated bat SL-CoV strains (WIV1 & 16). It will therefore be critical to clarify whether and how this protein would contribute to virulence during viral infection. Here we revealed that the unique ORFX is a functional gene involving in the modulation of host immune response, but not essential for *in vitro* viral replication. Our results provide important information for further exploration of the ORFX function in the future. Moreover, the reverse genetics system we constructed will be helpful for pathogenesis study of this group of viruses and develop therapeutics for future control of emerging SARS-like infections.

Introduction

Severe acute respiratory syndrome coronavirus (SARS-CoV) is a zoonotic pathogen that caused the SARS pandemic in 2002-3 which originated in China (1). Since then, genetically diverse SARS-like coronaviruses (SL-CoVs) have been reported in bats in China, Europe and Africa (2-11), indicating a wide geographic distribution of this group of viruses. However, most bat SL-CoVs have only been identified by sequences and are not fully characterized due to the lack of cultured viruses. Thus, their potential for transmission to, and their likely pathogenesis in domestic animals and humans remain untested. WIV1 &16 are two recently identified SL-CoV strains with high

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59 genomic similarity to human SARS-CoV. These two strains have been successfully
60 cultured *in vitro* and have been shown to use the same molecule (angiotensin
61 converting enzyme, ACE2) for cellular entry as SARS-CoV (2, 10). Recently, another
62 bat SL-CoV strain SHC014 has been demonstrated to use human ACE2 by the
63 construction of infectious cDNA clone (12). Furthermore, animal infection
64 experiments indicated that SL-CoV WIV1 and SHC014 could replicate efficiently and
65 caused low pathogenesis in ACE2 transgenic mice (12, 13). The fact that the native
66 bat SL-CoVs could use human ACE2 without any mutations indicates a high risk of
67 interspecies transmission for these and similar coronaviruses that may exist in natural
68 reservoirs.

69 Coronaviruses have the largest genomes among RNA viruses. Their genome
70 consists of a positive, single-stranded RNA around 30 kilo base nucleotides (nt) with
71 two-thirds at the 5'-end encoding genome replication proteins (ORF1ab) and
72 one-third at 3'-end encoding structural proteins including a spike glycoprotein (S), a
73 small envelope protein (E), a membrane protein (M) and a nucleocapsid protein (N).
74 Coronaviruses encode a set of ORFs expressed from full-length and
75 subgenomic-length mRNAs (sgRNAs), which have common 3' end originating at
76 distinct transcription regulatory sequences (TRS) and joined with a common leader
77 sequence encoded at the 5' end of genomic RNA (14). Currently, coronaviruses are
78 divided into the genera *alphacoronavirus*, *betacoronavirus*, *gammacoronavirus*, and a
79 proposed genus *deltacoronavirus* (15). SARS-CoV and SL-CoVs are grouped into the

same coronavirus species, SARS-related coronavirus (SARSr-CoV), within the genus *Betacoronavirus*. Besides the family-conserved genes, SARSr-CoV possesses several accessory genes including ORF3, ORF6, ORF7, ORF8 and ORF9 which are specific for this group of coronaviruses, but not essential for *in vitro* viral replication (16-18). Accessory genes in coronavirus genomes play important roles in regulating host immune response (19). The SARS-CoV ORF3a, ORF3b, and ORF6 have been reported to inhibit host interferon (IFN) response during virus infection and contribute to pathogenesis (20, 21). ORF3a and ORF7a activate NF-κB and upregulates IL8 and CCL5 production (22, 23). Bat SL-CoVs display great genetic diversity and share overall nt sequence identities of 88-95% with human SARS-CoV (2-11). Bat SL-CoV WIV1 & 16 are the closest relatives, so-far discovered, to human SARS-CoV. These two viruses are identical in genomic structures except that WIV1 &16 have an extra ORF (named ORFX) between ORF6 and ORF7 with no homology to any known protein sequences (2).

In this study, we explored the function of ORFX in modulating host immune response through eukaryotic overexpression assays and recombinant viruses generated through reverse genetics techniques.

Materials and Methods

Virus and Cells. The SL-CoV WIV1 strain (GenBank accession: KF367457) and other viruses were propagated as described previously (2). Sendai virus (SeV) strain

Cantell (kindly provided by Prof. Hanzhong Wang) was propagated in 10-day-old embryonated chicken eggs at 37 °C for 48 h (24). All experiments using live virus was conducted under biosafety level (BSL) 2 conditions. HeLa cells stably expressing human ACE2 (HeLa-hACE2) were described previously (25). 293T, Vero E6, HeLa and HeLa-hACE2 cells were grown and propagated in Dulbecco's Modified Eagle's Medium supplemented (GIBCO, Invitrogen) with 10% fetal bovine serum (Life Technologies). Calu-3 cells were grown and propagated in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 Media supplemented with 15% fetal bovine serum. Cells were grown at 37 °C in a humidified atmosphere with 5% CO_2.

Plasmids. The encoding region of the ORFX was amplified by reverse transcription (RT)-PCR from viral RNA using Superscript One-Step RT-PCR kit (Invitrogen). The amplified gene was cloned into the plasmid pCAGGS with a C-terminal HA tag (pCAGGS-ORFX) for eukaryotic expression. Reporter plasmids used included pIFNβ-Luc (expressing firefly luciferase under the control of the IFN-β promoter), pNF-κB-Luc (expressing firefly luciferase under the control of the NF-κB promoter) and pRL-TK (expressing *Renilla* luciferase under the control of the HSV-TK promoter), as well as expression plasmid for influenza virus NS1 as described previously (24). Subcellular organelle markers expressing plasmids including SecG1β-GFP (Endoplasmic Reticulum (ER) marker), B4Gal-Ti-RFP (Golgi marker),

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and Mito-YFP (mitochondria marker) were kindly provided by Prof. Yanyi Wang of the Wuhan Institute of Virology.

Viral infection assays. Vero E6, Calu-3, and HeLa-hACE2 cells were infected with viruses at a multiplicity of infection (MOI) of 1.0, 0.1, or 0.001 in 25-cm^2 flasks with 1 h adsorption period, followed by two washes with D-Hanks and culturing by adding 3 mL of medium. The viral supernatants were harvested, at 0, 2, 6, 12, 18, 24, 36, 48, and 72 h post inoculation, with 300 μL removed and 300 μL medium added back at each time point. Virus concentration was titrated by plaque assay in Vero E6 cells.

Vero E6 cells were infected by rWIV1-GFP-ΔX or mock infected. After 24 h, fluorescent micrographs was taken to check the expression of green fluorescence protein.

Cloning of WIV1 cDNAs. The virus genome was divided into 8 continuous fragments (A-G) and amplified using specific primers (primer sequences available upon request). Viral RNA was extracted from the supernatant of WIV1-infected cultures, and reverse-transcribed with M-MLV reverse transcriptase (Promega), random hexamer deoxynucleotide primers. The cDNA was denatured for 5 min at 95 °C and amplified by polymerase chain reaction (PCR) with KOD DNA polymerase (TOYOBO) for 20 cycles of 95 °C for 30 sec, 60 °C for 30 sec with 0.5 °C decrease per cycle, 68 °C for 5 min, and 15 cycles of 95 °C for 30 sec, 50 °C

for 30 sec, 68 °C for 5 min, and a final extension at 68 °C for 10 min. The amplicons were cloned into pGEM-T EASY (Promega). Besides three natural BglI sites, several BglI sites were introduced by synonymous mutations in the PCR process, to make all contiguous cDNA fragments capable of unidirectional ligation. SacII and AscI were introduced into the 5' terminus of fragment A and the 3' terminus of fragment G respectively. A poly(A) sequence (25 nt) was added to the 3' terminus of fragment G. At least three colonies of each cDNA clone were sequenced, and the one identical to or with some synonymous mutations to the reported sequence was selected for assembly.

To ablate a natural BglI site at position 1575, primers FA, F-c1575a, R-c1575a and RA were used for overlap extension PCR (OE-PCR) to introduce a synonymous mutation C1575A (primer sequences available upon request). Based on previous *in vitro* transcription tests, a synonymous mutation T27527C was also introduced to interrupt a potential T7 termination site via OE-PCR.

Strategy for modifying pBeloBAC11.

Cytomegalovirus (CMV) promoter was amplified from pcDNA3.1(+) (Thermo Fisher Scientific) with primers (forward, 5'-tgaggatcccgttgacattgattattgactag-3'; reverse, 5'-cctgactgcaggtcgactgccgcggagctctgcttatatagacc-3'). Hepatitis delta virus (HDV) ribozyme was synthesized as described (26), and amplified with primers (forward, 5'-cagtcgacctgcagtcaggcgcgccgggtcggcatggcatctcc-3'; reverse,

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5'-ctagaaggcacagctcccttagccatccgagtgg-3'). Bovine growth hormone (BGH) transcription terminal signal was amplified from pcDNA3.1(+) with primers (forward, 5'-ggatggctaagggagctgtgccttctagttgccagc-3', reverse, 5'-tgaaagcttccatagagcccaccgcatcc-3'). Then the three PCR products were ligated using OE-PCR, with BamHI and HindIII sites flanking the amplicon, and SacII and AscI sites sitting between CMV promoter and HDV ribozyme. The amplicon was then inserted into pBeloBAC11 (New England BioLabs) between BamHI and HindIII sites. The construct was designated pBAC-CMV.

Construction of infectious BAC clones of WIV1. The subclone A and subclone G were first digested with SacII and AscI (New England BioLabs) respectively, followed by treatment with CIAP (Takara), chloroform extraction and isopropanol precipitation, and then restricted with BglI (Takara). The subclones B-F were digested with BglI. pBAC-CMV was digested with SacII and AscI. All digestion products were then separated using 1% agarose gels, excised, and purified by using the Gel Extraction Kit (Omega). Digested fragments A-G and pBAC-CMV were ligated overnight at 4 °C, transformed into DH10B competent cells and plated on Chl^+ LB culture. Ten clones were screened by RFLP analysis with NcoI, StuI or HindIII. The correct clone was named as pBAC-CMV-rWIV1 **(Fig. 1)**.

Construction of WIV1 mutants. To delete ORFX, the fragment F was PCR-amplified with primer set FF (5'-acctgtgccctttggcgaggtttttaatgctactac-3') and RFox (5'-gcctctagggctcaaggataatctatctccatagg-3'). The fragment G was PCR-amplified with FGox (5'-gccctagaggcaacgaacatgaaaattattctcttcc-3') and RG (5'-actggcgcgccttttttttttttttttttttttttttgtcattctcctgagaagc-3'). This new fragment is named as Gox. These two products were then cloned into pGEM-T EASY. The two fragments were inserted into BAC along with the other fragments as described above. The rescued mutant was named as rWIV1-ΔX. To replace GFP into the open reading frame of ORFX, the F fragment was amplified with primer set FF and RFoeGFP (5'-gctcaccatagtggttcgtttatcaaggataatctatctcc-3'). The GFP gene was amplified with a primer set (5'-ccttgataaacgaaccactatggtgagcaagggcgaggagc-3' and 5'-tgcctctagggcttacttgtacagctcgtccatgcc-3'). The two PCR products were ligated by OE-PCR and the product was inserted into pGEM-T EASY. The rescued mutant was named as rWIV1-GFP-ΔX.

Transfection of infectious WIV1 BAC clones. Vero E6 cells were seeded in a 6-well plate a day in advance, and then one well was transfected with 6 μg infectious BAC plasmids constructed as above with Lipofectamine LTX and Plus Reagent (Life technologies). Virus progeny was plaque purified once. One clone was passaged once in Vero E6 cells for 72 h and used to generate a stock for future use.

204 **Restriction fragment length polymorphism.** RNA extracted from wild-type and
205 recombinant viruses were reverse-transcribed with random hexamer primers. RT-PCR
206 was used to generate five amplicons containing the five mutations designed in the
207 strategy. These amplicons included a 1124-bp amplicon (nucleotide positions
208 1312-2435) spanning a naturally occurring BglI site at nucleotide 1571 that had been
209 ablated in recombinant viruses, a 1438-bp amplicon spanning the B/C1 junction
210 (nucleotide positions 7560-8997), a 1437-bp amplicon spanning the C1/C2 junction
211 (nucleotide positions 10196-11632), a 1437-bp amplicon spanning the D/E junction
212 (nucleotide positions 16793-18229), and a 1438-bp amplicon spanning the E/F
213 junction (nucleotide positions 21908-23345) (These amplicons correspond to
214 fragment F1-F5 in **Fig. 1**). The first amplicon of wild-type WIV1 that contains
215 nucleotide 1571 can be cleaved by BglI, but the other four amplicons cannot. In
216 contrast, the five amplicons of recombinant viruses are different to those of wild-type
217 virus in the capability of being cut by BglI.
218
219 **Northern blot analysis.** The N gene was amplified with primers WIV1-NF
220 (5'-atgtctgataatggacccca-3') and WIV1-3R (5'-gtcattctcctgagaagcta-3') and used as a
221 template for probe preparation according to the description of DIG-High Prime DNA
222 Labeling and Detection Starter Kit II (Roche). Vero E6 cells were infected with
223 wild-type and recombinant viruses at an MOI of 1.0. At 24 h postinfection,
224 intracellular RNA was isolated using TRIzol reagent (Ambion). RNA (20 μg) was

precipitated, treated with 17 μL Sample Buffer (50% Formamide, 2.2 M Formaldehyde (37%), 1× MOPS) at 65 °C for 10 min, added with 3 μL 10× Dye Solution (50% glycerol, 0.25% Bromophenol Blue, 0.25% Xylene Cyanole FF), and then separated in denaturing 0.8% agarose-2.2 M formaldehyde gel at 28 V for ~17 h. The RNA was hydrolyzed by 0.05 M NaOH for 40 min, transferred to a Hybond-N+ membrane (GE Healthcare) for ~18 h, and then cross-linked to the membrane using UV light. The membrane was prehybridized, probed with a DIG-labelled probe for N gene, washed and detected according to the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche).

RT-PCR of leader-containing transcripts. Intracellular RNA was isolated from wtWIV1. A forward primer (Leader-F) located in the leader sequence, along with various reverse primers located in several ORFs, was used for amplifying leader-containing sequences (primer sequences available upon request). Leader-containing amplicons were sequenced with the corresponding reverse primers.

ORFX subcellular location. HeLa cells were transfected with ORFX expressing plasmid and cotransfected with organelle markers expressing plasmids SecG1β-GFP, B4Gal-Ti-RFP, or Mito-YFP. After 24 h, the cells were fixed and stained with a mouse anti-HA IgG (Promoter). A Cy3 conjugated goat anti-mouse IgG (Promoter) was used for secondary detection in cells expressing ER or mitochondrial markers. An

FITC conjugated goat anti-mouse IgG (Promoter) was used for secondary detection in cells expressing Golgi marker. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Staining patterns were examined with an Olympus Fluoview upright confocal microscope (Olympus).

Luciferase assays and quantitative PCR. For ORFX-mediated IFN promoter assay, 293T cells were seeded in 12-well plates and cotransfected with empty vector plasmid pCAGGS, plasmid pCAGGS-NS1 or escalating amount (100, 200, 400, 600, 800 ng) of pCAGGS-ORFX with the indicated reporter plasmids. At 24 h posttransfection, cells were infected with SeV (100 hemagglutinin units/ml) for 12 h to induce IFN production, or treated with TNFα for 1 h to activate NF-κB. Cell lysates were prepared and luciferase activity was measured using the Dual-Luciferase Assay kits (Promega) according to the manufacturer's instructions.

293T cells were transfected with empty vector, NS1 expressing plasmid, or escalating amount (100, 300, 600 ng) of ORFX expressing plasmid. After 24 h, the cells were infected with SeV (100 HAU/ml). At 12 h postinfection, the cells were lysed. The mRNA was extracted and reverse transcribed with PrimeScript RT Master Mix (Takara). The expression level of IFN-β mRNA was determined by quantification PCR using SYBR Premix Ex Taq II (Takara). The GAPDH mRNA was quantified as an inner control. 293T cells were transfected as above. After 24 h, the cells were treated with TNFα for 6 h, the cell RNA was extracted and used for

quantification of the expression of IL8 mRNA. All experiments were performed in triplicate and repeated at least three times. All primer sequences used in the quantitative PCRs will be provided upon request.

IRF3 translocation assay. 293T cells were transfected with empty vector, NS1, or ORFX expressing plasmid. After 24 h, IRF3 nuclear translocation was induced by infecting the cells with SeV for 8 h. The cells were fixed and stained with a rabbit anti-IRF3 polyclonal IgG (Proteintech) and a mouse anti-HA IgG (Promoter). An Alexa Fluor 488 conjugated donkey anti-rabbit IgG (Yeasen) and an Alexa Fluor 555 conjugated donkey anti-mouse IgG (Beyotime) were used to detect IRF3 and ORFX, respectively. The cells transfected with empty vector were stained with a rabbit anti-IRF3 polyclonal IgG and a goat anti-SeV IgG (kindly provided by Prof. Lin-Fa Wang at the Duke-NUS Graduate Medical School, Singapore), as an indication of infection efficiency. An Alexa Fluor 488 conjugated donkey anti-rabbit IgG and a Cy3 conjugated donkey anti-Goat IgG (Promoter) were used to detect IRF3 and SeV, respectively. Nuclei were stained with DAPI.

Quantification of mRNA expression of cytokines in infected Calu-3 cells. Calu-3 cells grown in 24-well plates were mock infected, or infected with rWIV1 or rWIV1-ΔX at an MOI of 5, or SeV (100 HAU/ml). The cells were lysed at 4, 12, 24, and 30 h postinfection. The mRNA expression level of IFN-β, IL6, IL8, and TNFα

was quantified by quantitative PCRs. The expression of GAPDH mRNA was measured as an inner control. All primer sequences used in the quantitative PCRs will be provided upon request. The experiment was performed twice.

IFN-β sensitivity assay. Vero E6 cells were seeded a day in advance. The cells were pretreated with 10, 100, or 1000 U/ml IFN-β (PBL, Piscataway, NJ) for 24 h, infected with wtWIV1, rWIV1, and rWIV1-ΔX at an MOI of 0.1 PFU/cell, and post treated with the same amount of IFN-β as used previously. At 24 h postinfection, the viral replication was analyzed by plaque assay. The experiment was performed in triplicate.

Statistics. The statistical significance of the obtained data was analyzed using a student's t-test in GraphPad Prism (GraphPad Software, San Diego, CA). A p-value <0.05 was considered statistically significant. Data is presented as the means ± SEM.

Results

Strategy for construction of an infectious WIV1 BAC. Originally, the genome was split into seven contiguous cDNAs (A~G) (**Fig. 1 A and C**). Due to the plasmid instability, fragment C was separated into two segments (C1 and C2). Besides three naturally-occurring BglI (GCCNNNN↓NGGC) sites, four BglI sites were successfully introduced by synonymous mutations in the genome (**Fig. 1 B**). Different asymmetric 3-nt overhangs at the junctions of each two contiguous fragments were created by

these BglI sites. The eight fragments were then linked in one direction. A SacII site was added to the 5' terminus of fragment A. A poly(A) sequence (25 nt) and an AscI site were added to the 3' terminus of fragment G. A naturally occurring BglI site at nucleotide 1571 was removed by synonymous mutations C1575A (Fig. 1B). Other unexpected synonymous mutations also occurred including T1422C, T12984C, T14213C, T17130C, C17934T and T26068G.

The plasmid pBAC-CMV was constructed by inserting with the cytomegalovirus (CMV) promoter, hepatitis delta virus (HDV) ribozyme and bovine growth hormone (BGH) transcription terminal signal sequences into pBeloBAC11, along with the introduction of the SacII and AscI sites between CMV promoter and HDV ribozyme (**Fig. 1 C**). The eight genomic fragments were inserted into pBAC-CMV in one step. Recombinant viruses could be rescued by direct transfection with the BAC constructs.

Rescue of recombinant viruses. To rescue recombinant WIV1 (rWIV1), fragments A and G were digested with SacII and AscI, respectively. Following calf intestinal alkaline phosphatase (CIAP) dephosphorylation, the two fragments, along with fragments B~F were digested using BglI and inserted into pBAC-CMV between SacII and AscI sites in one step. The constructed clone (pBAC-CMV-rWIV1) was transfected into Vero E6 cells. Cytopathic effect was observed at 72 h posttransfection. The one ablated natural BglI site and four introduced BglI sites in the rescued viral genome were confirmed by restriction fragment length polymorphism (RFLP)

analysis with BglI digestion (**Fig. 2 A**). Using this method, we also rescued an ORFX deletion mutant virus (rWIV1-ΔX) (**Fig. 2 B** lane 2), and a mutant with GFP replaced into the coding region of ORFX (rWIV1-GFP-ΔX) (**Fig. 3 A**).

ORFX is a functional gene not essential for virus replication. The one-step growth curve of the two rescued recombinant viruses (rWIV1-ΔX and rWIV1) and wild-type WIV1 (wtWIV1) determined by plaque assay showed that rWIV1-ΔX and rWIV1 both replicated to the titers close to wild-type virus (**Fig. 2 C**). The expected set of appropriately sized 10 sgRNAs including sgRNA7 (ORFX) were observed in Northern blot analysis in cells infected with wtWIV1 and rWIV1 (**Fig.2B lane 1 and lane 3**). As expected, sgRNA7 was not observed in rWIV1-ΔX infected cells (**Fig. 2 B lane 2**). Analysis of leader containing sequences indicated that all 10 sgRNA in wtWIV1 share an identical core sequence ACGAAC (**Table 1**), which further confirmed that ORFX is expressed as sgRNA7. The GFP was expressed in rWIV1-GFP-ΔX infected cells further confirmed that the open reading frame of ORFX could be expressed (**Fig. 3 A**). Subcellular location analyses showed that the ORFX protein colocalized with the ER marker, but not with the Golgi and Mitochondria markers (**Fig. 3 B**).

ORFX protein inhibits the production of IFN-β. To determine whether ORFX inhibits the induction of IFN, 293T cells were transfected with plasmids pIFNβ-Luc

and pRL-TK and the plasmid expressing ORFX, influenza virus strain PR8 NS1 (positive control) or empty vector (negative control). As expected, SeV activated IFN production in cells transfected with empty vector. The positive control, influenza virus NS1 protein, dramatically inhibited the expression from the IFN promoter. ORFX protein exhibited inhibition effect, but the effect decreased while more ORFX protein was expressed (**Fig. 4 A**). Similar results were observed for IFN-β mRNA quantification (**Fig. 4 B and C**).

IRF3 nuclear translocation assay was performed to see whether ORFX protein inhibits IFN production through inhibiting this process. 293T cells were transfected with empty vector, NS1, or ORFX expressing plasmid. After 24 h, IRF3 nuclear translocation was induced by infection of SeV for 8 h. The relative IRF3 translocation ratios were calculated for each group by counting the number of the IRF3 nuclear translocation cells (randomly selected at least 4 fields) divided by the number of total infected or transfected cells. The IRF3 nuclear translocation efficiency of each group was expressed as the percentage of their relative IRF3 translocation ratios to that of control (cells transfected with empty vector). As expected, NS1 strongly inhibited translocation of IRF3. While ORFX protein also showed inhibition of IRF3 translocation, but less efficiently (**Fig. 4 D and E**).

To further investigate the IFN inhibition activity of ORFX, the deletion mutant and wild-type recombinant virus were used to infect Calu-3 cells at an MOI of 5. Mock-infected cells were used as negative control. Calu-3 cells infected with SeV

372 were used as positive control. Samples were collected at 4, 12, 24, and 30 h
373 postinfection. The relative expression of IFN-β mRNA was determined by
374 quantification PCR and normalized to the expression of GAPDH mRNA. Compared
375 to SeV, WIV1 recombinants induced low levels of IFN-β mRNA in Calu-3 cells (**Fig.**
376 **4 F**). The ORFX deletion mutant induced a significantly higher level of IFN-β mRNA
377 than wild-type recombinant virus in infected cells at 12 h postinfection, but no
378 significant differences at 24, 30 h postinfection (**Fig. 4 F**). These results indicate that
379 ORFX protein may play a role in antagonizing IFN only in early time during WIV1
380 infection.
381
382 **ORFX deletion mutant shows increased sensitivity to IFN-β.** To further investigate
383 the effect of ORFX on the viral sensitivity of IFN, we tested the replication
384 efficiencies of wtWIV1, rWIV1 and rWIV1-ΔX in Vero E6 cells which were
385 pretreated and posttreated with IFN-β. The replication of rWIV1-ΔX was evidently
386 inhibited and reduced ~0.5 logs compared to wtWIV1 and rWIV1 at a concentration
387 of 10 and 100 U/ml IFN-β (**Fig. 4 G**). Whereas, at a higher IFN-β concentration (1000
388 U/ml), the titers of rWIV1-ΔX didn't show an obvious decrease compared to wild
389 type virus. We expected the ORFX deleted mutant would replicate less efficiently
390 than the wild-type virus in IFN competent cells. However, we did not find a
391 significant difference when we grew the two viruses in Calu-3 and HeLa-hACE2 cells,
392 even at a very low MOI of 0.001 (**Fig. 5**).

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ORFX protein activates NF-κB. NF-κB plays an important role in regulating immune response to viral infection and is also a key factor frequently targeted by viruses for taking over the host cell (27). Several proteins (Nsp1, N, and 3a) encoded by SARS-CoV have both activities in IFN antagonizing and NF-κB activation (28). In this study, we also tested whether ORFX protein could activate NF-κB. 293T cells were transfected with pNF-κB-Luc, pRL-TK, and empty vector, NS1, or escalating amount (200, 400, 600 ng) of ORFX expressing plasmid. After 24 h, the cells were mock treated, or treated with TNFα for 6 h, and luciferase activity was determined. ORFX protein obviously activates NF-κB, no matter whether the cells were treated with TNFα or not (**Fig. 6 A**). Whereas, IL8 was upregulated only when the cells were treated with TNFα (**Fig. 6 B**). However, no significant difference was observed for IL6 and IL8 transcription levels between the rWIV1-ΔX and rWIV1 infected Calu-3 cells (**Fig. 6 C and D**). A significant difference was only observed for the induction of TNFα mRNA at the late time of virus infection in which the ORFX deletion mutant induced apparently less amount of TNFα mRNA (**Fig. 6 E**).

Discussion

In this study, we have developed a fast and cost-effective method for reverse genetics of coronaviruses by combining two approaches developed by others (29, 30). Our method allows the genomes of coronaviruses to be split into multiple fragments

and inserted into a BAC plasmid with a single step. Recombinant viruses can then be efficiently rescued by direct transfection of the BAC constructs. As the genomes can be divided into multiple short fragments, mutations can be introduced into individual fragments easily (31). Using this method, we successfully rescued three recombinant viruses derived from SL-CoV WIV1 (rWIV1, rWIV1-ΔX and rWIV1-GFP-ΔX). The recombinant rWIV1 and rWIV1-ΔX replicated to titers close to wtWIV1 in Vero E6 cells (**Fig. 2 C**), suggesting that the deletion of ORFX did not affect WIV1 replication *in vitro*. Northern-blot and fluorescent microscopy further confirmed that ORFX is transcribed as sgRNA7 and translated in virus-infected cells. These results demonstrated that unique ORFX in SL-CoV WIV1 is a functional gene but not essential for virus replication. We propose that the ORFX sgRNA is the template for the translation of a novel 11-kDa accessory protein of WIV1, bringing the total number of group-specific accessory proteins to ten.

In previous studies, it has been proved that SARS-CoV group-specific accessory genes ORF3b and ORF6 inhibit host IFN production and/or signaling during virus infection and contribute to viral pathogenesis (20). It's interesting to know if the ORFX has a similar function in IFN antagonizing. In this study, ORFX protein showed inhibition effect on IFN production, but the effect decreased when more ORFX protein was expressed (**Fig. 4 A and B**). Moreover, ORFX deletion mutant had significant lower inhibition effect on IFN production than wild-type recombinant virus in infected Calu-3 cells, but only at an early time after infection (**Fig. 4 F**).

435 Furthermore, the IFN sensitivity assay indicated that the ORFX deletion mutant was
436 more sensitive to IFN-β (**Fig. 4 G**), suggesting that ORFX protein may participate in
437 subverting the antiviral state stimulated by IFN-β. All these results suggested that
438 ORFX participates in the modulation of IFN response. Previous studies showed that
439 the SARS-CoV ORF3a and ORF7a activate NF-κB and upregulate IL8 and CCL5
440 production (22, 23). In our study, we also found that overexpressed ORFX can
441 activate NF-κB through dual luciferase assay (**Fig. 6 A**). Furthermore, the level of
442 TNFα mRNA induced by wild-type recombinant virus was significantly higher than
443 those induced by ORFX deletion mutant, but only at the late stage of infection (**Fig. 6**
444 **E**). These results indicated that ORFX also participates in activation of NF-κB. We
445 noted that the IFN inhibition activity of ORFX was not dose-dependent and decreased
446 when there was more ORFX expression. One possible hypothesis is that ORFX
447 inhibits the IFN only at the early stage of infection. At the late stage, it activates
448 NF-κB which in turn stimulate IFN expression, this leads to the attenuation of its IFN
449 antagonist activity.

450 Coronavirus was previously shown to induce unfolded-protein response (UPR)
451 and ER stress in infected culture cells (32). Normally, ER is an active organelle for
452 protein folding and modification. Loss of protein folding homeostasis would cause ER
453 stress and induce UPR, leading to the activation of three ER stress transducers. These
454 transducers work in a concert to attenuate translation and improve ER folding
455 capacity to restore ER homeostasis (33). In this process, NF-κB is activated, and

456 apoptosis will be induced if ER stress prolong (32, 33). In this study, we observed that
457 the overexpression of ORFX protein lead to cell death and the decrease of *Renilla*
458 values (data not shown). This may imply that ORFX has cytotoxic effect and an
459 influence on overall protein translation. We also found that ORFX colocalizes with
460 ER marker. We hypothesize that ORFX may induce UPR and cause ER stress which
461 would activate NF-κB and induce apoptosis, promoting viral release at late stage of
462 infection.

463 It should be noted that the IFN and NF-κB detection systems used in this study
464 were derived from and performed in human cells. Since the innate immune system of
465 bats is special and probably deficient in some aspects compared to human (34). Thus,
466 it will be interesting to conduct the same studies in bat cells to determine whether
467 ORFX protein will have the same profiles as those observed in the human cell system.
468 The development of different *Rhinolophus* bat cell lines, which is the reservoir host of
469 SL-CoV, will facilitate this research in the future.

470

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598 **Figure Legends**

599 **Fig. 1 Strategy for construction of an infectious WIV1 BAC clone.** (A) Genomic
600 structure of WIV1. (B) The mutations are indicated under the stars (★). C1575A was
601 used to ablate a natural BglI site at nucleotide 1571 (▽) , and T27527C was used to
602 disrupt a potential T7 stop site. The others were for introducing BglI sites (▼). (C)
603 The WIV1 genome was split into eight contiguous cDNAs (A-G): A (nt 1-4387), B
604 (nt 4388-8032), C1 (nt 8033-10561), C2 (nt 10562-12079), D (nt 12080-17017), E (nt
605 17018-22468), F (nt 22469-27352), G (nt 27353-30309). Unique BglI sites were
606 introduced into the fragments by synonymous mutations to make these fragments
607 capable of unidirectional ligation along with native BglI sites in the genome. The
608 original nucleotides were shown upside of the flanking sequences of corresponding
609 fragments. A poly(A) sequence was added to the 3' terminus of fragment G. CMV
610 promoter, HDV ribozyme and BGH transcriptional terminal signal were inserted into
611 pBeloBAC11 between BamHI and HindIII. SacII and AscI were introduced between
612 CMV promoter and ribozyme. The fragments A-G were inserted into the pBAC-CMV
613 plasmid in a single step.
614
615 **Fig. 2 Recovery and characterization of recombinant viruses.** (A) Restriction
616 fragment length polymorphism. Amplicons flanking five mutated sites of wild-type
617 and recombinant viruses were digested by BglI. The first amplicon (F1) of wild-type
618 virus can be digested by BglI and its other four amplicons (F2~5) cannot. On the

619 contrary for amplicons of rWIV1, the first amplicon (F1) cannot be digested by BglI
620 and its other four amplicons (F2~5) can. M: DL2000 DNA ladder (Takara). (B)
621 Detection of viral genomic transcription and replication by Northern blotting. Vero E6
622 cells were infected with wild-type or recombinant viruses and intracellular RNA was
623 extracted for Northern blot analysis. Lane 1, wtWIV1; lane 2, rWIV1-ΔX; lane 3,
624 rWIV1; lane 4, uninfected control. (C) Growth kinetics of wild-type and recombinant
625 viruses. Vero E6 cells were infected with wtWIV1 (■), rWIV1 (◇), or rWIV1-ΔX
626 (▲) at an MOI of 1.0 or 0.1 PFU/cell. Cell supernatants were taken at indicated time
627 points postinfection and virus titers were determined by plaque assay in Vero E6 cells.
628
629 **Fig. 3 Expression and subcellular location of ORFX protein.** (A) The open reading
630 frame of ORFX was replaced by GFP and the recombinant virus was rescued. Vero
631 E6 cells were infected with the recombinant virus or mock infected. Green
632 fluorescence was visualized at 24 h postinfection. (B) ORFX protein with an HA tag
633 at C terminal was expressed in HeLa cells, along with SecG1β-GFP (ER marker),
634 Mito-YFP (mitochondria marker), or B4Gal-Ti-RFP (Golgi marker), respectively.
635 The cells were fixed after 24 h and stained with a mouse anti-HA IgG. A Cy3
636 conjugated goat anti-mouse IgG was used for secondary detection in cells expressing
637 ER or mitochondria marker. An FITC conjugated goat anti-mouse IgG was used for
638 secondary detection in cells expressing Golgi marker. ORFX protein showed a
639 cytoplasmic distribution and colocalized with ER maker SecG1β.

Fig. 4 ORFX protein inhibits the production of type I interferon. (A and B) 293T cells seeded in 12-well plates were transfected with 100 ng pIFN-β-Luc, 5 ng pRL-TK, and empty vector, influenza A NS1 expressing plasmid, or escalating doses (100, 200, 400, 600, 800 ng) of ORFX expressing plasmid. Empty vector was added appropriately to ensure that cells in each well were transfected with the same amount of plasmids. The cells were infected with Sendai virus (100 hemagglutinating units/ml) at 24 h posttransfection. Samples were collected at 12 h postinfection followed by dual-luciferase assay. The results were expressed as firefly luciferase value normalized to that of *Renilla* luciferase. The relative expression of IFN-β mRNA was determined by quantitative RT-PCR and normalized to the expression level of GAPDH mRNA. (C) The expression of NS1 and ORFX proteins were analysis by western blot with an antibody against HA-tag. The experiments were replicated for three times. (D and E) For IRF3 translocation assay, 293T cells were transfected with empty vector, NS1, or ORFX expressing plasmid. After 24 h, the cells were infected with Sendai virus to induce IRF3 nuclear translocation. The cells were fixed at 8 h postinfection and stained with anti-HA IgG. A Goat anti-Sendai virus polyclonal IgG was used to stain the cells transfected with empty vector. A rabbit anti-IRF3 polyclonal IgG was used to label IRF3. The white arrow indicates IRF3 nuclear translocation. The relative IRF3 translocation ratios were calculated for each group by counting the number of the IRF3 nuclear translocation cells (randomly selected at

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least 4 fields) divided by the number of total infected or transfected cells. The IRF3 nuclear translocation efficiency of each group was expressed as the percentage of their relative IRF3 translocation ratios to that of control (cells transfected with empty vector). (F) Calu-3 cells were mock infected, or infected with rWIV1, rWIV1-ΔX (MOI of 5), or SeV (100 HAU/ml). At 4, 12, 24, and 30 h postinfection, the cell RNA was extracted and used for quantification RT-PCR of the expression level of IFN-β mRNA. The experiment was replicated twice. (G) Vero E6 cells were pretreated with indicated amount of IFN-β, infected with wtWIV1, rWIV1, or rWIV1-ΔX at an MOI of 0.1 PFU/cell, and posttreated with IFN-β. Viral replication was analyzed at 24 h postinfection by plaque assay. The experiment was performed in triplicate and replicated twice. The differences between selected groups were significant with the p values less than 0.05: 0.0049 (* bars 4 and 6 in panel A), 0.0008 (** bars 6 and 7 in panel A), 0.0072 (* bars 4 and 6 in panel B), 0.018 (* bars of rWIV1 and rWIV1-ΔX in panel F), <0.0001 (*** in panel G).

Fig. 5 Comparison of viral replication efficiency in IFN competent cells between rWIV1-ΔX and rWIV1. Calu-3 (A) and HeLa-hACE2 (B) cells were infected with rWIV1 or rWIV1-ΔX at an MOI of 0.001. Samples were collected at 0, 12, 24, 36, 48, 72, 96, and 120 h postinfection. The viral titers were measured by plaque assay.

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Fig. 6 ORFX protein activates NF-κB. 293T cells were transfected with 100 ng pNF-κB-Luc, 10 ng pRL-TK, and empty vector, NS1 expressing plasmid, or escalating (200, 400, 600 ng) of ORFX expressing plasmid. After 24 h, the cells were treated with TNFα. Dual luciferase activity was determined after 6 h. The results were expressed as the firefly luciferase activity normalized to that of *Renilla* luciferase (A). The relative expression of IL8 mRNA was quantified through qRT-PCR and normalized to that of GAPDH mRNA (B). Differences between selected groups were significant with p value less than 0.05: < 0.0001 (*** bars 1 and 3 in panel A), 0.0339 (* bars 4 and 7 in panel A), 0.0002 (*** bars 4 and 6 in panel B). n.s., not significant. The experiments were performed for three times. (C to E) The RNA extracted from calu-3 cells in Fig. 4 was used for quantification of the expression of IL6 (C), IL8 (D) and TNFα (E) mRNA.

Tables

Table1 Leader-containing sequences of sgRNAs.

sgRNA	ORF(s)	Leader-containing sequence[a]	CS site
1	1a/b	GTAGATCTGTTCTCTAA**ACGAAC**TTTAAAATCTGT	67-72
2	S	GTAGATCTGTTCTCTAA**ACGAAC**ATGAAATTGTTA	21,486-21,491
3	3a/b	GTAGATCTGTTCTCTAA**ACGAAC**TTATGGATTTGT	25,263-25,268
4	E	GTAGATCTGTTCTCTAA**ACGAAC**TTATGTACTCAT	26,112-26,117
5	M	GTAGATCTGTTCTCTAA**ACGAAC**TAACTATTATTA	26,351-26,356
6	6	GTAGATCTGTTCTCTAA**ACGAAC**GCTTTCTTATTA	26,916-26,921
7	X	GTAGATCTGTTCTCTAA**ACGAAC**CACTATGTTACT	27,272-27,277
8	7a/b	GTAGATCTGTTCTCTAA**ACGAAC**ATGAAAATTATT	27,794-27,799
9	8	GTAGATCTGTTCTCTAA**ACGAAC**ATGAAACTTCTC	28,300-28,305
10	N	GTAGATCTGTTCTCTAA**ACGAAC**AAACTAAAATGT	28,672-28,677

[a] Consensus sequence is in bold.

SL-CoV infectious clone