SARS-CoV-2 ORF3a inhibits cGAS-STING-mediated autophagy flux and antiviral function

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Abstract
Recognizing aberrant cytoplasmic dsDNA and stimulating cGAS-STING-mediated innate immunity is essential for the host defense against viruses. Recent studies have reported that SARS-CoV-2 infection, responsible for the COVID-19 pandemic, triggers cGAS-STING activation. cGAS-STING activation can trigger IRF3-Type I interferon (IFN) and autophagy-mediated antiviral activity. Although viral evasion of STING-triggered IFN-mediated antiviral function has been well studied, studies concerning viral evasion of STING-triggered autophagy-mediated antiviral function are scarce. In the present study, we have discovered that SARS-CoV-2 ORF3a is a unique viral protein that can interact with STING and disrupt the STING-LC3 interaction, thus blocking cGAS-STING-induced autophagy but not IRF3-Type I IFN induction. This novel function of ORF3a, distinct from targeting autophagosome-lysosome fusion, is a selective inhibition of STING-triggered autophagy to facilitate viral replication. We have also found that activation of bat STING can induce autophagy and antiviral activity despite its defect in IFN induction. Furthermore, ORF3a from bat coronaviruses can block bat STING-triggered autophagy and antiviral function. Interestingly, the ability to inhibit STING-induced autophagy appears to be an acquired function of SARS-CoV-2 ORF3a, since SARS-CoV ORF3a lacks this function. Taken together, these discoveries identify ORF3a as a potential target for intervention against COVID-19.

KEYWORDS
coronavirus, innate immunity, immune responses, SARS coronavirus, virus classification

1 | INTRODUCTION

The COVID-19 pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has swept the world since 2019, causing an epic public health crisis and posing a tremendous economic burden.1,2 To date, the rampant spread of SARS-CoV-2 has caused over 555 million infections and 6.3 million deaths worldwide.3 Thus, it is urgent that we clearly delineate the pathogenesis of SARS-CoV-2 and identify efficient therapies to antagonize this potentially deadly virus.

The DNA pattern recognition receptor cyclic GMP-AMP synthase (cGAS) is a key sensor for recognizing viral infection in the cytoplasm of
cells. Recent studies have demonstrated that SARS-CoV-2 infection disrupts mitochondrial homeostasis, inducing mitochondrial DNA (mtDNA) release. It also causes syncytia formation, which leads to disrupting mitochondrial homeostasis, inducing mitochondrial DNA defenses and the counteracting responses by diverse viruses, including SARS-CoV-2, have been widely reported. Apparently, STING-mediated Type I IFN is dampened in bats, which are often commensal with multiple viruses, including coronaviruses. Bat coronaviruses (CoVs) are highly conserved when compared to SARS-CoV-2 and are believed to be the most likely origin of SARS-CoV-2. These observations have led us to speculate that the Type I IFN-independent function of STING may play an important role in antiviral responses.

Autophagy is a fundamental and highly conserved intracellular degradation process in eukaryotes for eliminating damaged organelles, protein aggregates, and invading pathogens. cGAS-STING-induced autophagy is a primordial Type I IFN-independent autophagy that can significantly inhibit the replication of viruses, but little is known about how viruses thwart this cGAS-STING-triggered autophagy. We now propose that to facilitate its survival, SARS-CoV-2 may have evolved evasion strategies to suppress autophagy. In the present study, by screening reported STING suppressors and SARS-CoV-2-encoded proteins, we have identified ORF3a as a potent inhibitor of STING-mediated autophagy that facilitates viral replication. In addition, we have found that bat-CoV ORF3a can antagonize bat STING-triggered autophagy, and this suppression can be neutralized by a small molecule inhibitor, Tpen. Notably, among human coronaviruses, only SARS-CoV and SARS-CoV-2 encode ORF3a. We found that, as compared to SARS-CoV-2, SARS-CoV ORF3a is less effective in defending against STING-mediated autophagy, which may explain the epidemic potential of SARS-CoV-2. Taken together, our results indicate the unique role of the SARS-CoV-2 ORF3a in suppressing STING-mediated autophagy, which benefits viral replication and also provides potential targets for the development of therapeutic strategies against SARS-CoV-2.

2 | MATERIALS AND METHODS

2.1 | Viruses and cell lines

HEK293T cells (ATCC, CRL-3216) and HeLa cells (ATCC, CCL-2) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin solution. EV-A71 and HSV-1 GFP were prepared as previously described.13

2.2 | Plasmids, antibodies and reagents

mCherry-GFP-LC3 (#22418) and mCherry-LC3 (#40827) were purchased from Addgene. Nematostella vectensis, Myotis davidii, Rhinolophus sinicus, Mus musculus, Gallus gallus, Xenopus tropicalis, and Danio rerio STING-Flag; HPV E7-HA (NC_001357.1), Adenovirus E1A-HA (NC_001405.1), KHSV vIRF1-HA (NC_009333.1), HCV NS4B-HA (YP_009709868.1), HCMV UL82-HA (P06726.2), YFV NS4B-HA (NP_776008.1), DENV NS2B3-HA (P14340.2), ZC45 ORF3a-HA (MG772933.1), RaTG ORF3a-HA (QHR63301.1), and ORF3a chimeras were obtained from Genery Biotech Co., Ltd., CN, and cloned into VR1012. All the coronavirus protein constructs, Homo sapiens STING wild type and truncation plasmids, Myc-cGAS, IFNa, and NF-kB luciferase reporter plasmids and Vpx-HA were described previously.14 Plasmids were verified by sequencing before transfection using the Hieff TransTM Liposomal Transfection Reagent (YeasenBiotech, CN) according to the manufacturer’s instructions.

The following antibodies were used for western blot analysis or immunofluorescence: anti-Flag (Sigma, F3165), anti-Myc (Millipore, 05-724), anti-p62 (MBL, PM045), anti-LC3B (Sigma, L7543), anti-GAPDH (Proteintech, 60004-1-g), anti-HA (Invitrogen, 71-5500), anti-Histone (GenScript, A01502), anti-IFIT3 (Proteintech, 15201-2-AP), anti-iRIF3-p (Cell Signal, 4974), GM130 (abcam, ab52649), DAPI (Sigma, 28718-90-3), bafilomycin A1 (MCE, HY-10055), anti-Flag M2 Affinity Gel (Sigma, A2220), Alexa Fluor 488 AffiniPure Goat Anti-Mouse IgG (H + L) (Yeasen, 33106ES60), and Alexa Fluor 647 AffiniPure Goat Anti-Rabbit IgG (H + L) (Yeasen, 33113ES60). HRP-conjugated secondary antibodies (anti-mouse or anti-rabbit) were purchased from Huabio.

2.3 | Virus infection and quantitation

HEK293T cells were transfected with indicated plasmids and then infected with HSV-1 GFP and EV-A71. At 24 h postinfection, the cells were washed with cold PBS, and HSV-1 GFP was measured by flow cytometry according to the manufacturer’s instructions. EV-A71-infected cells were observed for morphological changes and photographed by light microscopy at 100× magnification. The viral RNA levels of EV-A71 were evaluated by real-time PCR using SYBR Green. EV-A71-VP1 primers: Forward, 5′-CAAGGGATGGTACTGGAAGT-3′; Reverse, 5′-GATCGGTAGAGGTAGTGGAAA-3′.

2.4 | Dual-luciferase assays

HEK293T cells were transfected with 100 ng of the Firefly luciferase reporter plasmids for the IFNa promoter and NF-kB response element; 5 ng of Renilla luciferase control plasmid (pRL-TK); and the indicated amounts of the expression plasmids per well. Dual-luciferase activity was measured as previously described.

2.5 | Confocal microscopy

HeLa cells were transfected with the indicated plasmids, with or without bafilomycin-A1. Images were captured as previously described.
2.6 Flow cytometry

After HSV-1 GFP infection, cells were washed with cold PBS and analyzed on a DxFLEX flow cytometer (Beckman Coulter). Apoptosis was evaluated using an Annexin V-APC/7AAD apoptosis kit (Multi Sciences Biotech, CN). A DxFLEX flow cytometer (Beckman Coulter) was used to quantify apoptosis detection, and all the data analysis was performed using FlowJo software.

2.7 Co-immunoprecipitation (Co-IP)

HEK293T cells were seeded into 6-cm dishes and transfected with the indicated plasmids using Hieff TransTM Liposomal Transfection Reagent (YeasenBiotech), with or without bafilomycin-A1. Immunoblotting and co-immunoprecipitation were performed as previously described.\(^\text{14}\)

2.8 Immunoblot band quantitation

Quantification of band intensities was performed using ImageJ (version 1.50i).

2.9 Statistical analysis

Data analyses were performed using GraphPad Prism 6.0 software. All data are shown as means ± SD. The statistical significance analyses were performed using a two-sided unpaired t-test (p values) with exact values.

3 RESULTS

To investigate the involvement of the cGAS-STING signaling pathway across phylogenetic kingdoms in innate immune activation and antiviral defense, we employed diverse STING molecules from \textit{Homo sapiens} to \textit{Nematostella vectensis} (Figure 1A). Using established experimental systems for the detection of cGAS-STING-triggered innate immune activation, we discovered that different STING molecules exhibited different activity in terms of stimulating down-regulated innate immune activation, we discovered that different STING molecules exhibited different activity in terms of stimulating down-regulated innate immune activation, we discovered that different STING molecules exhibited different activity in terms of stimulating down-regulated innate immune activation, we discovered that different STING molecules exhibited different activity in terms of stimulating down-regulated innate immune activation, we discovered that different STING molecules exhibited different activity in terms of stimulating down-regulated innate immune activation, we discovered that different STING molecules exhibited different activity in terms of stimulating down-regulated innate immune activation, we discovered that different STING molecules exhibited different activity in terms of stimulating down-regulated innate immune activation, we discovered that different STING molecules exhibited different activity in terms of stimulating down-regulated innate immune activation, we discovered that different STING molecules exhibited different activity in terms of stimulating down-regulated innate immune activation, we discovered that different STING molecules exhibited different activity in terms of stimulating down-regulated innate immune activation, we discovered that different STING molecules exhibited different activity in terms of stimulating down-regulated innate immune activation, we discovered that different STING molecules exhibited different activity in terms of stimulating down-regulated innate immune activation, we discovered that different STING molecules exhibited different activity in terms of stimulating down-regulated innate immune activation, we discovered that different STING molecules exhibited different activity in terms of stimulating down-regulated innate immune activation, we discovered that different STING molecules exhibited different activity in terms of stimulating down-regulated innate immune activation, we discovered that different STING molecules exhibited different activity in terms of stimulating down-regulated innate immune activation, we discovered that different STING molecules exhibited different activity in terms of stimulating down-regulated innate immune activation, we discovered that different STING molecules exhibited different activity in terms of stimulating down-regulated innate immune activation, we discovered that different STING molecules exhibited different activity in terms of stimulating down-regulated innate immune activation, we discovered that different STING molecules exhibited different activity in terms of stimulating down-regulated innate immune activation, we discovered that different STING molecules exhibited different activity in terms of stimulating down-regulated innate immune activation, we discovered that different STING molecules exhibited different activity in terms of stimulating down-regulated innate immune activation.

showed a significant inhibition of HSV-1 replication, suggesting that the cGAS-STING antiviral defense is independent of NF-κB induction. A unique and important feature of the cGAS-STING pathway is the robust activation of autophagy in addition to the induction of interferons and NF-κB responses.\(^\text{12}\) We observed that overexpression of cGAS-STING induced the activation of autophagic flux, as monitored by p62 degradation and LC3 lipidation (Figure 1E,F). Notably, STING from all the various species examined was capable of triggering autophagy induction (Figure 1E,F) and efficiently restricted HSV-1 replication (Figure 1C,D). Thus, the cGAS-STING-triggered antiviral defense appeared to be independent of IFN and NF-κB signaling. Moreover, the induction-related autophagy of cGAS-STING appeared to be an evolutionarily conserved activity that played a dominant role in antagonizing viral replication.

All viruses have developed efficient evasion mechanisms to survive. In previous studies we have demonstrated that SARS-CoV-2 proteins are capable of a unique and complementary suppression of cGAS-STING and RNA sensing-triggered innate immune responses.\(^\text{14}\) Since cGAS-STING-triggered autophagy is prominent in antiviral replication,\(^\text{12}\) we conducted screening to determine whether any viral proteins could potentially target the STING-autophagy axis. For this purpose, we synthesized viral proteins from RNA viruses (SARS-CoV-2 ORF3a, HCV NS4B, DENV NS2B3, and YFV NS4B), DNA viruses (HPV E7, HCMV UL82, AdV E1A, and KHSV vIRF1), and retrovirus (HIV-2/SIV Vpx) that have been reported to associate with STING and subsequently disrupt STING’s downstream signalosome assembly. To further exclude the effect of STING-mediated interferon activity against DNA viruses, we generated a construct with a STING N-terminal tail truncation (STING dCTT) that is defective in the stimulation of Type I IFN and Type I IFN-stimulated gene expression\(^\text{15}\) but functional in terms of autophagy induction\(^\text{12}\) (Figure 2A). Surprisingly, the SARS-CoV-2 ORF3a could be distinguished from various other viral proteins in terms of impeding cGAS-STING-mediated p62 degradation, as shown in Figure 2B,C. It has been reported that SARS-CoV-2 3CL can also inhibit cGAS-STING signaling.\(^\text{14}\) Therefore, we asked whether other SARS-CoV-2 structural and accessory proteins (Figure 2D) might be involved in STING-based suppression of autophagy induction. For this purpose, we co-expressed cGAS-STING dCTT with each individual SARS-CoV-2 viral protein in HEK293T cells and discovered that only ORF3a significantly restored p62 expression (Figure 2E,F). We observed an interaction of ORF3a with STING as well as STING dCTT, both of which are competent to induce autophagy (Supporting Information: Figure S1a and Figure 2A). Moreover, we detected intracellular co-localization of STING and ORF3a (Supporting Information: Figure S1b). STING activation induces co-localization of STING and LC3 which is important for STING-triggered autophagy.\(^\text{16}\) We discovered that ORF3a inhibited the co-localization between STING and LC3 (Figure 2G). Consistent with this observation, we also discovered that ORF3a inhibited the interaction between STING and LC3-II (Supporting Information: Figure S2a).

Furthermore, ORF3a efficiently restored the replication of HSV-1 (Figure 2H–J) and EV-A71 (Supporting Information: Figure S3a,b),
which had been repressed in the presence of cGAS-STING dCTT. We have previously demonstrated that SARS-CoV-2 ORF3a selectively inhibits cGAS-STING-NF-κB signaling, but not the RIG-I-like Receptor (RLR) pathway, implying that the cGAS-STING pathway is a crucial barrier during SARS-CoV-2 infection and that SARS-CoV-2 ORF3a is a potent inhibitor of cGAS-STING. In addition, the SARS-CoV-2 ORF3a has a Cys-rich region with the potential to bind zinc. Interestingly, we found that N,N,N',N'-tetrakis
FIGURE 2  SARS-CoV-2 ORF3a is a potent and unique inhibitor of STING-mediated autophagy among viral proteins from RNA viruses, DNA viruses, and retrovirus. (A) STING-dCTT can induce autophagy despite its inability to activate the IRF3 pathway. HEK293T cells were transfected with Myc-cGAS and STING-Flag or STING dCTT-Flag for 24 h. The cells were harvested, and the indicated proteins were analyzed by immunoblotting with anti-IFIT3, anti-p-IRF3, anti-p62, anti-LC3, anti-Myc, anti-Flag, and anti-GAPDH. Results are shown for n = 3 independent experiments (representative immunoblotting results are shown). (B, C) Among other viral proteins reported to cause cGAS-STING signaling inhibition, only SARS-CoV-2 ORF3a antagonizes cGAS-STING dCTT-induced autophagy. HEK293T cells were transfected with Myc-cGAS and STING dCTT-Flag expression vectors in the presence or absence of vectors expressing viral proteins as indicated. Cells were harvested 24 h after transfection. The indicated proteins were analyzed by immunoblotting with anti-p62, anti-LC3, anti-Myc, anti-Flag, anti-HA, and anti-GAPDH (B). Relative p62 and LC3-I/LC3-II levels in (B) were quantified with ImageJ software (C). cGAS-STING dCTT alone served as a control and was set to 100%. Statistical significance was determined by two-sided unpaired t-test. Means and SDs are presented. Results are shown for n = 3 independent experiments. (D) Schematic diagram of the genomic organization and encoded proteins of SARS-CoV-2 from the 2019 Wuhan-Hu-1 strain (MN908947.3). (E, F) ORF3a is the only protein among SARS-CoV-2 encoded proteins to antagonize cGAS-STING dCTT-induced autophagy. HEK293T cells were cotransfected with the Myc-cGAS and STING dCTT-Flag expression vectors in the presence or absence of SARS-CoV-2 encoded proteins. Cells were harvested 24 h after transfection. The indicated proteins were analyzed by immunoblotting with anti-p62, anti-LC3, anti-Myc, anti-Flag, anti-HA, and anti-GAPDH (B). Relative p62 and LC3-I/LC3-II levels in (B) were quantified with ImageJ software (C). cGAS-STING dCTT alone served as a control and was set to 100%. Statistical significance was determined by two-sided unpaired t-test. Means and SDs are presented. Results are shown for n = 3 independent experiments. (G) SARS-CoV-2 ORF3a disrupts cGAS-STING-LC3 co-localization specifically. HeLa cells were transfected as shown and treated with bafilomycin-A1 (0.2 μM). After 24 h, the cells were visualized by confocal microscopy. Scale bars, 20 μm. Results are shown for n = 3 independent experiments (representative images are shown). (H–J) HSV-1 GFP replication suppressed by cGAS-STING dCTT activation is partially restored by SARS-CoV-2 ORF3a. HEK293T cells were cotransfected with Myc-cGAS and STING dCTT-Flag expression vectors in the presence or absence of SARS-CoV-2 ORF3a for 20 h and subsequently infected with HSV-1 GFP for 24 h. GFP-positive cells were analyzed by FACS (H). The percentage of GFP-positive cells (I) and relative HSV-1 GFP inhibition (J) are shown. Empty vector served as a control (I), and HSV-1 GFP inhibited by cGAS-STING dCTT served as a positive control and was set to 100% (J). Statistical significance was determined by two-sided unpaired t-test. Means and SDs are presented. Results are shown for n = 3 independent experiments.
Bat-CoV ORF3a can antagonize bat STING-mediated induction of autophagy. (A) Phylogenetic tree of ORF3a from SARS-CoV-2, SARS-CoV, and bat-CoVs. Reference sequences of representative molecules: SARS-CoV-2 (MN908947.3), SARS-CoV (AY278741.1), bat RaTG13 (QHR63301.1), bat ZC45 (MG772933.1). Phylogenetic analysis was performed with the MEGA X program by the neighbor-joining method on the basis of the Kimura two-parameter model. (B) ORF3as from SARS-CoV-2 and bat-CoV antagonize autophagy induction through homologous STING activation. HEK293T cells were cotransfected with Myc-cGAS and human STING (HsaSTING), Myotis davidii STING (MydSTING), or Rhinolophus sinicus STING (RssSTING) expression vector in the presence or absence of vectors expressing SARS-CoV-2 ORF3a and bat ZC45 ORF3a. Cells were harvested 24 h after transfection. The indicated proteins were analyzed by immunoblotting with anti-p62, anti-LC3, anti-Myc, anti-Flag, anti-HA, and anti-GAPDH. Results are shown for n = 3 independent experiments (representative immunoblotting results are shown). (C, D) ORF3a from bat-CoV RaTG inhibits bat STING dCTT-triggered autophagy induction. HEK293T cells were cotransfected with Myc-cGAS and human STING (HsaSTING), Myotis davidii STING (MydSTING), or Rhinolophus sinicus STING (RssSTING) expression vector in the presence or absence of vectors expressing bat RaTG ORF3a. Cells were harvested 24 h after transfection. The indicated proteins were analyzed by immunoblotting with anti-p62, anti-LC3, anti-Myc, anti-Flag, anti-HA, and anti-GAPDH. Results are shown for n = 3 independent experiments (representative immunoblotting results are shown). (E, F) RssSTING dCTT activation suppresses HSV-1 GFP replication, which can be partially restored by ORF3a from bat CoVs. HEK293T cells were transfected with bat RaTG ORF3a or bat ZC45 ORF3a alone, or cotransfected with Myc-cGAS and RssSTING dCTT in the presence or absence of vectors expressing bat RaTG ORF3a and bat ZC45 ORF3a for 20 h, and subsequently infected with HSV-1 GFP for 24 h. GFP-positive cells were analyzed by FACS. The relative HSV-1 GFP positivity is shown in (F). Empty vector with HSV-1 GFP infection served as a control and was set to 100%. Statistical significance was determined by two-sided unpaired t-test. Means and SDs are presented. Results are shown for n = 4 independent experiments.
HEK293T cells were cotransfected with Myc-harbor compromised in their ability to restore HSV-1 GFP replication suppressed by cGAS-STING dCTT when compared to SARS-CoV-2 ORF3a. HEK293T cells were cotransfected with Myc-cGAS and STING dCTT-Flag in the presence or absence of vectors expressing SARS-CoV-2 ORF3a, SARS-CoV ORF3a, and chimeric ORF3a S2-S1 for 20 h, then infected with HSV-1 GFP for 24 h. GFP-positive cells were analyzed by FACS (K). The relative HSV-1 GFP positivity is shown in (L). The empty vector with HSV-1 GFP infection served as a control and was set to 100%. Statistical significance was determined by two-sided unpaired t-test. Means and SDs are presented. Results are shown for n ≥ 3 independent experiments. (M) A model representing the mechanism of SARS-CoV-2 ORF3a-mediated suppression of cGAS-STING-autophagy.
(2-pyridylmethyl)-ethylenediamine (TPEN), a lipid-soluble zinc metal chelator, was able to block ORF3a function (Supporting Information: Figure S2b). Taken together, these data indicate that, among diverse viral proteins, SARS-CoV-2 ORF3a is a unique and potent inhibitor of the STING-autophagy axis that facilitates efficient viral replication and helps viral pathogenesis.

Bats serve as reservoirs for many viruses, including coronaviruses. STING molecules in all the bats examined to date have proved defective for the induction of Type I IFN and have compromised antiviral activity. We have now demonstrated that STING from the bats Rhinolophus sinicus (RssSTING) and Myotis davidii (MydSTING) can induce autophagy (Figure 1E,F). Interestingly, though, diverse ORF3a molecules encoded by bat coronaviruses (Figure 3A) have evolved an evolutionarily conserved suppression of bat STING dCTT-triggered autophagy (Figure 3B–D). Functionally, bat ORF3as, including RaTG16 and ZC45, were effective in rescuing HSV-1 suppression induced by RssSTING dCTT-mediated autophagy induction (Figure 3E,F).

Since, ORF3a is only encoded by SARS-CoV and SARS-CoV-2 among human coronaviruses, and this ORF is conserved in terms of the induction of apoptosis (Figure 4A–C) and antagonism of cGAS-STING-mediated NF-κB activity (Figure 4D), we wondered whether SARS-CoV and SARS-CoV-2 ORF3a would both be able to suppress STING-mediated autophagy. Surprisingly, we discovered that the ORF3a derived from SARS-CoV was less capable of inhibiting STING-triggered p62 degradation (Figure 4E,F), and autolysosome formation (Supporting Information: Figure S4a,b) than was SARS-CoV-2 ORF3a. To pursue this question further, we constructed an ORF3a S2 (Supporting Information: Figure S4a,b) but maintained the ability to inhibit STING-triggered autophagy (Supporting Information: Figure S4e,f). Consistently, SARS-CoV-2 ORF3a and the S1-S2 chimera showed stronger neutralization of cGAS-STING dCTT autophagy-mediated HSV-1 inhibition than did SARS-CoV ORF3a or the chimeric ORF3a S2-S1 (Figure 4I–L and Supporting Information: Figure S4g,h). This unusual effect of the SARS-CoV-2 ORF3a may have contributed to some of the unique biological properties of the SARS-CoV-2.

4 | DISCUSSION

Evasion of host innate immunity is essential for the survival of viruses. We have shown that SARS-CoV-2 infection triggers cGAS-STING activation, which leads to the generation of Type I IFN and autophagy-mediated antiviral activity. The means by which viruses evade cGAS-STING-triggered, autophagy-mediated antiviral activity is poorly understood. ORF3a is a virion-associated protein, and thus it plays an important role in the early stage of SARS-CoV-2 infection. We observed here that SARS-CoV-2 ORF3a was able to block cGAS-STING-induced autophagy and its antiviral activity (Figure 2B–J). Surprisingly, SARS-CoV ORF3a had an impaired ability to block cGAS-STING-induced autophagy and the resulting antiviral activity (Figure 4E–L), although ORF3a from both SARS-CoV and SARS-CoV-2 maintained the ability to induce apoptosis (Figure 4A–C) and NF-κB inhibition (Figure 4D).

SARS-CoV-2 ORF10 has also been reported to inhibit cGAS-STING-induced autophagy. Unlike ORF10, which also affects STING foci formation and IRF3 activation, ORF3a specifically affects cGAS-STING-induced autophagy, without affecting STING foci formation or IRF3 activation (Figure 4M).

Bat STING is defective in stimulating IRF3-Type I IFN activation (Figure 1B); however, we have now discovered that bat STING activation can still induce autophagy and antiviral activity (Figure 1C–F). On the other hand, ORF3a from bat coronavirus has the ability to suppress bat STING-triggered autophagy and its antiviral function (Figure 3B–F), even though STING-mediated autophagy is a primordial, conserved antiviral activity. The ability to inhibit STING-induced autophagy appears to be an acquired attribute of SARS-CoV-2 and bat coronavirus ORF3a, and this inhibition is sensitive to a small-molecule inhibitor, namely TPEN (Supporting Information: Figure S2b). Thus, our study identifies ORF3a as a potential drug target for intervention against COVID-19.

AUTHOR CONTRIBUTIONS
Jiaming Su, Si Shen, and Ying Hu carried out most of the biochemical experiments, with help from Yanpu Wang, Shiqi Chen, and Leyi Cheng. Yanpu Wang conducted the immunostaining and confocal microscopy experiments. Yong Cai and Wei Wei contributed the key reagents. Jiaming Su, Si Shen, and Yajuan Rui performed the viral infection experiments and plasmid construction. Xiao-Fang Yu, Yajuan Rui, and Yanpu Wang contributed to the supervision and data analysis. Xiao-Fang Yu directed the project, analyzed the data, and wrote the paper, with help from all of the authors.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT
All data, materials, and methods are included in the article.

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SUPPORTING INFORMATION
Additional supporting information can be found online in the Supporting Information section at the end of this article.

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