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1 # Severe Acute Respiratory Syndrome Coronavirus 2
2 (SARS-CoV-2) Membrane (M) Protein Inhibits Type I and III
3 Interferon Production by Targeting RIG-I/MDA-5 Signaling
4
5
6 Yi Zheng [1], Meng-Wei Zhuang [2], Lulu Han [1], Jing Zhang [2], Mei-Ling Nan [2], Chengjiang
7 Gao [1], Pei-Hui Wang [2, 3]
8
9 [1] Key Laboratory of Infection and Immunity of Shandong Province, Department of
10 Immunology, School of Basic Medical Sciences, Cheeloo College of Medicine, Shandong
11 University, Jinan 250012, China
12
13 [2] Advanced Medical Research Institute, Cheeloo College of Medicine, Shandong
14 University, Jinan 250012, China
15
16 [3] Suzhou Research Institute, Shandong University, Shandong University, Suzhou, Jiangsu
17 215123, China
18
19
20 *Correspondence: Chengjiang Gao (cgao@sdu.edu.cn) or Pei-Hui Wang
21 (pei-hui.wang@sdu.edu.cn or pei-hui.wang@connect.hku.hk)
22
23 These authors contributed equally: Yi Zheng, Meng-Wei Zhuang
24

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1 # Abstract

2 The coronavirus disease 2019 (COVID-19) caused by Severe acute
3 respiratory syndrome coronavirus 2 (SARS-CoV-2) has quickly spread
4 worldwide and has infected more than ten million individuals. One of the typical
5 features of COVID-19 is that both type I and III interferon (IFN)-mediated
6 antiviral immunity are suppressed. However, the molecular mechanism by
7 which SARS-CoV-2 evades this antiviral immunity remains elusive. Here, we
8 report that the SARS-CoV-2 membrane (M) protein inhibits the production of
9 type I and III IFNs induced by the cytosolic dsRNA-sensing pathway of
10 RIG-I/MDA-5-MAVS signaling. The SARS-CoV2 M protein also dampens type
11 I and III IFN induction stimulated by Sendai virus infection or poly (I:C)
12 transfection. Mechanistically, the SARS-CoV-2 M protein interacts with RIG-I,
13 MAVS, and TBK1 and prevents the formation of a multi-protein complex
14 containing RIG-I, MAVS, TRAF3, and TBK1, thus impeding IRF3
15 phosphorylation, nuclear translocation, and activation. Consequently, the
16 ectopic expression of the SARS-CoV2 M protein facilitates the replication of
17 vesicular stomatitis virus (VSV). Taken together, the SARS-CoV-2 M protein
18 antagonizes type I and III IFN production by targeting RIG-I/MDA-5 signaling,
19 which subsequently attenuates antiviral immunity and enhances viral
20 replication. This study provides insight into the interpretation of the
21 SARS-CoV-2-induced antiviral immune suppression and sheds light on the
22 pathogenic mechanism of COVID-19.

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1 **Keywords**: SARS-CoV-2, COVID-19, M protein, antiviral immunity, IFNs.

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1 # Introduction

2 The coronavirus disease 2019 (COVID-19), caused by severe acute
3 respiratory syndrome coronavirus 2 (SARS-CoV-2), has caused a large
4 number of infections and fatalities worldwide, representing an acute and
5 rapidly developing global health crisis. SARS-CoV-2 show 79.5% identity with
6 SARS-CoV-1 and approximately 50% identity with MERS-CoV at the
7 whole-genome level.[1-3] SARS-CoV-2, together with SARS-CoV-1 and Middle
8 East respiratory syndrome coronavirus (MERS-CoV), belongs to the genus
9 betacoronavirus in the Coronaviridae family. Coronaviruses are
10 single-stranded positive-sense RNA viruses carrying the largest genomes
11 (26-32 kb) among all RNA virus families, with a wide range of vertebrate
12 hosts.[4] The coronavirus transcripts have a 5′-cap structure and a 3′ poly(A) tail.
13 SARS-CoV-2 is an enveloped virus with a genome of ~30 kb. Upon entry into
14 host cells, the viral genome is used as the template for replication,
15 transcription, and the synthesis of positive-sense genomic RNA (gRNA) and
16 subgenomic RNAs. The gRNA is packaged by the structural proteins, namely,
17 the spike, membrane (M), and envelope proteins, to assemble progeny virions.
18 [5] Similar to SARS and MERS, COVID-19 may be a life-threatening disease,
19 which typically begins with pneumonia.[3] SARS-CoV-1 has infected
20 approximately 8,000 people with an ~11% fatality rate worldwide during
21 2002-2003, and MERS-CoV has infected ~2,500 people with a ~36% fatality
22 rate since 2012;[6] after its outbreak in December 2019, SARS-CoV-2 has

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1 infected 14,974,446 individuals and caused 617,254 deaths as of July 22,
2 2020, according to the COVID-19 Dashboard by the Center for Systems
3 Science and Engineering (CSSE) at Johns Hopkins University
4 (https://coronavirus.jhu.edu/map.html). One of the hallmarked clinical feathers
5 of COVID-19 is the poor protective immunity with high levels of
6 pro-inflammatory cytokines, suggesting that the host immune system may be
7 involved in COVID-19 pathogenesis.[7]
8
9 Innate immunity is the first line of host defense against viruses, initiated by the
10 recognition of pathogen-associated molecular patterns (PAMPs), such as
11 single-stranded RNA (ssRNA), double-stranded RNA (dsRNA), and DNA,
12 which will trigger the production of type I interferon (IFN-α/β) and type III IFN
13 (IFN-λ1/2/3) in infected cells. [8,9] The Toll-like receptor 3 (TLR3) senses dsRNA
14 in the endosome, while the retinoic acid-inducible gene I (RIG-I) and
15 melanoma differentiation-associated gene 5 (MDA-5) are cytosolic receptors
16 for dsRNA.[9] Upon recognition of dsRNA, TIR-domain-containing
17 adapter-inducing IFN-β (TRIF) will be recruited to the cytoplasmic domain of
18 TLR3. TRIF further associates with receptor-interacting protein 1 (RIP1), TNF
19 receptor-associated factor 6 (TRAF6), and TANK-binding kinase 1 (TBK1).
20 RIP1 and TRAF6 involved in the activation of the NF-κB pathway, whereas
21 TBK1 directly phosphorylates transcription factor IRF3, which subsequently
22 translocates to the nucleus, leading to the induction of IFNs and other

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1 pro-inflammatory cytokines.[9,10] When viruses enter into host cells, the viral
2 dsRNA is recognized by RIG-I/MDA-5, which will initiate an antiviral signaling
3 cascade by interacting with mitochondrial antiviral signaling (MAVS, also called
4 IPS-1/VISA/Cardif). MAVS then activates IκB kinase α/β (IKK) and TBK1/IKKε,
5 which in turn activates the transcription factors NF-κB and IRF3, respectively,
6 to induce IFNs and other pro-inflammatory cytokines. [11] The cytosolic DNA
7 sensor cyclic GMP-AMP (cGAMP) synthase (cGAS) can recognize dsDNA and
8 produce 2'-3' cGAMP which can bind to the stimulator of interferon genes
9 (STING) and then activate TBK1 and IRF3, leading to IFN production.[12] The
10 binding of the type I or III IFNs to their specific receptors, the type I IFN
11 receptor (IFNAR) and the type III IFN receptor (IFNLR), respectively, triggers
12 the activation of the receptor-associated Janus kinase 1 (JAK1)/tyrosine
13 kinase 2 (TYK2), which stimulates the phosphorylation of STAT1 and
14 STAT2.[9,13] JAK2 also participates in type III IFN-induced STAT
15 phosphorylation.[14] The activated STAT1/STAT2 heterodimers associate with
16 IRF9 to form IFN-stimulated gene factor 3 (ISGF3), which in turn translocates
17 into the nucleus and binds the IFN-stimulated response element (ISRE) in
18 gene promoters, thus driving the expression of IFN-stimulated genes (ISGs)
19 that confer antiviral abilities on host cells. Type I and III IFNs induce a similar
20 ISG signature, with type I IFN signaling leading to a more rapid induction and
21 decline in ISG expression.[9,13]
22

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1 SARS-CoV-2 is a novel emerging coronavirus that is a global threat. How this
2 virus is recognized by the innate immune system is currently unknown.
3 However, studies from other coronaviruses indicated that RIG-I/MDA5
4 participates in the sensing of coronaviruses.[15] TLR3 has been shown to be
5 involved in the sensing of SARS-CoV-1 in a mouse model.[8] SARS-CoV-2 has
6 similar replication intermediates containing dsRNAs that can serve as
7 RIG-I/MDA5 or TLR3 ligands. Therefore, SARS-CoV-2 is likely detected by
8 these dsRNA sensors, which would induce the production of IFNs and other
9 pro-inflammatory cytokines.[8]
10
11 Host antiviral immunity may provide the selective pressure on viruses, which
12 has thus resulted in distinct strategies in viruses, including coronaviruses, for
13 counteracting IFN responses. The viral-encoded IFN antagonists are common
14 strategies to evade host antiviral immunity.[8] SARS-CoV-1-encoded proteins,
15 such as nonstructural protein 1, the papain-like protease domain in NSP3,
16 ORF3b, ORF6, M protein, and the nucleocapsid protein, have been attributed
17 with being the cause of the antagonism of the IFNs and ISGs.[8] The common
18 respiratory virus, influenza A virus, also encodes the IFN antagonist
19 nonstructural protein 1, which blocks the initial detection by the PRR through
20 binding and masking aberrant RNA produced during infection.[6] The generation
21 of IFN antagonists by viruses is a common strategy to evade host antiviral
22 immunity.[8] One of the striking clinical features of COVID-19 is that antiviral

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1 immunity is drastically impaired.[6,16] SARS-CoV-2 infection induces low levels
2 of type I and III IFNs with a moderate ISG response.[6] Type I and III IFNs have
3 been supplied or combined together with other drugs to treat patients with
4 COVID-19, which is shown to be effective in suppressing SARS-CoV-2
5 infection and may effectively prevent COVID-19.[17-20] In Vero cells, the
6 replication of SARS-CoV-2 is dramatically reduced when pretreated with
7 recombinant IFN-α or IFN-β. SARS-CoV-2 has been shown to be more
8 sensitive than many other human pathogenic viruses, including
9 SARS-CoV-1.[19,21] Treatment with type I and III IFNs can dramatically inhibit
10 SARS-CoV-2 replication in primary human airway epithelial cells with the
11 corresponding induction of ISGs.[22] The role of type III IFNs in the suppression
12 of virus replication has been highlighted.[23,24] Type III IFN shows a better
13 treatment option than type I IFN for influenza A virus-induced disease for its
14 induction of antiviral immunity without the pro-inflammatory responses.[25,26]
15 However, in some cases, the type I and III IFN treatment promote the
16 replication of SARS-CoV-2, and this phenotype was more pronounced in
17 certain SARS-CoV-2 isolates.[27] Two recent studies showed that ACE2, the
18 cellular receptor of SARS-CoV-2, may act as a novel ISG upregulated by type I
19 IFNs to facilitate SARS-CoV-2 infection. ACE2 is indeed upregulated by IFN
20 treatment and SARS-CoV-2 infection, which has been observed in COVID19
21 patients.[28,29] Therefore, the interactions between SARS-CoV-2 and the host
22 type I and III IFN responses merit extensive investigations.

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1

2 Overall, in most conditions, the host type I and III IFNs play an important role in
3 restricting the infection and replication of SARS-CoV-2. To combat this,
4 SARS-CoV-2 likely encodes multiple viral proteins to antagonize IFN
5 responses akin to other coronaviruses. Because of its recent emergence,
6 there is a paucity of information regarding the interaction between host antiviral
7 immunity and SARS-CoV-2 infection. Thus, dissection of the evasion
8 mechanism of type I and III IFN responses by SARS-CoV2 will contribute to
9 the understanding of the pathogenesis of COVID-19 and a corresponding
10 treatment. Here, we report that the SARS-CoV-2 M protein acts as an
11 antagonist of both types I and III IFNs by affecting the multi-protein complex
12 formation of RIG-I-MAVS-TRAF3-TBK1 signalosome. The SARS-CoV-2 M
13 protein inhibits type I and III IFN production by Sendai virus (SeV) infection,
14 poly (I:C) transfection, and the overexpression of the RIG-I/MDA-5 pathway
15 signaling molecules. The ectopic expression of the SARS-CoV-2 M protein
16 facilitates the replication of vesicular stomatitis virus (VSV). This study reveals
17 a previously undiscovered mechanism of SARS-CoV-2 in evading host
18 antiviral immunity, which may partially explain the clinical features of impaired
19 antiviral immunity in COVID-19 patients and provide insights into the viral
20 pathogenicity and treatment.

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1 # Materials and methods

2 **Cell culture and transfection**

3 HEK293, HEK293T, HeLa, and Vero-E6 cells were cultured in Dulbecco's
4 modified Eagle's medium (DMEM, Gibco, USA) with 10% heat-inactivated fetal
5 bovine serum (FBS, Gibco, USA). All cells were cultured at 37°C in a
6 humidified incubator with 5% CO_2. The plasmids were transfected into
7 HEK293, HEK293T, and HeLa cells by Polyethylenimine 'Max' (Polysciences,
8 Inc., Germany). Poly (I:C) (Sigma P1530, USA) was transfected into cells
9 using Lipofectamine 2000 (Thermo Fisher, USA) as described previously. [29]
10

11 **Antibodies and reagents**

12 Rabbit anti-DYKDDDDK Tag (D6W5B), rabbit anti-IRF3 (D83B9), rabbit
13 anti-pIRF3 (4D46), rabbit anti-TBK1 (3031S), rabbit anti-pTBK1 (D52C2), and
14 rabbit anti-TRAF3 were from Cell Signaling Technology (USA); Mouse
15 anti-MAVS was from Santa Cruz Biotechnology (USA); Mouse anti-actin and
16 rabbit anti-calnexin were from proteintech (Wuhan, China); Mouse anti-Flag
17 M2 was from Sigma Aldrich (USA); Mouse anti-Myc (9E10) Ab was from
18 Origene (USA); Rabbit anti-GM130 was from Abcam (United Kingdom); Mouse
19 anti-HA was from MDL biotech (China). Protein A/G beads were from Santa
20 Cruz Biotechnology, and the anti-Flag magnetic beads were from Bimake
21 (USA). Alexa Fluor 488 goat anti-rabbit IgG secondary antibody, Alexa Fluor
22 568 goat anti-mouse IgG secondary antibody, Alexa Fluor 488 goat anti-mouse

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1 IgG secondary antibody, and Alexa Fluor 568 goat anti-rabbit IgG secondary
2 antibody were from Thermo Fisher Scientific (USA).
3
4 **Constructs and plasmids**
5 The RIG-I, RIG-IN, MDA-5, MAVS, TBK1, IKKε, IRF3-5D, TRIF, and STING
6 genes were cloned into pcDNA6B-Flag, pcDNA6B-Myc, pcDNA6B-V5,
7 pCAG-Flag or pCMV-HA-N expression vectors using standard molecular
8 cloning methods as described in our previous publications.[30-32] The IFN-β
9 luciferase reporter plasmid pGL3-IFN-β-Luc vector was constructed in our
10 previous study.[33,34] The IFN-λ1 luciferase reporter plasmid pGL3-IFN-λ1-Luc
11 was constructed by inserting the 1000-bp promoter region of human IFN-λ1
12 (nucleotides −1000 to +1, with the translation start site set as 1) into
13 pGL3-Basic (Promega, USA) according to methods outlined in previous
14 studies.[13,34] The ISG luciferase reporter plasmid pISRE-Luc vector was
15 purchased from Clontech (USA). The SARS-CoV-2 M protein gene (NCBI
16 access No. MN908947) was synthesized (GENERAL BIOL, China) and
17 subcloned into the pCAG-Flag expression vector. The primers used in plasmid
18 construction are listed in Supplemental Table 1.
19
20 **Real-time quantitative PCR**
21 Total RNA isolated with TRIzol reagent (Invitrogen) was reverse-transcribed
22 into first-strand cDNA with the HiScript III 1st Strand cDNA Synthesis Kit with

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1 gDNA wiper (Vazyme, China) as per the manufacturer's instructions. Real-time
2 quantitative PCR (RT-qPCR) assays were performed using the SYBR
3 Green-based RT-qPCR kit UltraSYBR Mixture (CWBIO, China) with a Roche
4 LightCycler96 system as per the manufacturer's instructions. The relative
5 abundance of the indicated mRNA was normalized to that of GAPDH. A
6 comparative C_T method ($\Delta\Delta C_T$ method) was used for the calculation of fold
7 change in gene expression as described previously.[32,35] The primers used in
8 RT-qPCR analysis are listed in Supplemental Table 1.
9
10 **Luciferase reporter assays**
11 To determine the activation of the luciferase reporters, including IFN-β-Luc,
12 IFN-λ1-Luc, and ISRE-Luc, by the proteins indicated in each experiment, a
13 dual-luciferase reporter assay was performed as described in our previous
14 studies.[32,35] Briefly, approximately 0.5 x 10^5 HEK293T cells were seeded in
15 48-well plates and transfected 12 hours later with the luciferase reporter
16 plasmid and the expression vector plasmids of RIG-I, RIG-IN, MDA-5, MAVS,
17 TBK1, IKKε, IRF3-5D, TRIF, and STING, alone or together with the plasmid
18 expressing the SARS-CoV-2 M protein, as indicated in the experiments. The
19 pRL-TK Renilla luciferase reporter (Promega, USA) was cotransfected to
20 normalize the transfection efficiency and serve as an internal control. Thirty-six
21 hours after transfection, the cells were harvested and lysed to assess the
22 luciferase activities using the Dual-Luciferase Reporter Assay Kit (Vazyme,

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China) according to the manufacturer's protocol. The luciferase activity was measured in a Centro XS3 LB 960 microplate luminometer (BERTHOLD TECHNOLOGIES, Germany). The relative luciferase activity was calculated by normalizing firefly luciferase activity to that of Renilla luciferase.

Viruses and infection

VSV-enhanced green fluorescent protein (eGFP) and SeV were used to infect HeLa, HEK293, or HEK293T cells as described in our previous publications.[30-32] Briefly, before infection, prewarmed serum-free DMEM medium at 37°C was used to wash the target cells, after which the virus was diluted to the desired multiplicity of infection (MOI) in serum-free DMEM and incubated with the target cells for 1-2 hours. At the end of the infection, the virus-medium complexes were discarded, and DMEM containing 10% FBS was added.

Coimmunoprecipitation and immunoblotting

For coimmunoprecipitation assays, HEK293T cells were collected 24 hours after transfection and lysed in lysis buffer [1.0% (v/v) NP-40, 50 mM Tris-HCl, pH 7.4, 50 mM EDTA, 0.15 M NaCl] supplemented with a protease inhibitor cocktail (Sigma, USA) and a phosphatase inhibitor cocktail (Sigma, USA) as described in our previous publications.[30,31] After centrifugation for 10 min at 14,000 g, the supernatants were collected and incubated with the indicated

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1 antibodies, followed by the addition of protein A/G beads (Santa Cruz, USA),
2 anti-Flag magnetic beads (Bimake, USA), or anti-Myc magnetic beads
3 (Bimake, USA). After incubation overnight at 4℃, the beads were washed four
4 times with lysis buffer. The immunoprecipitates were eluted by boiling with
5 2×SDS loading buffer containing 100 mM Tris-HCl pH 6.8, 4% (w/v) SDS, 20%
6 (v/v) glycerol, 0.2% (w/v) bromophenol blue, and 1% (v/v) 2-mercaptoethanol.
7
8 For immunoblot analysis, the M-PER Protein Extraction Reagent (Pierce, USA)
9 supplemented with a protease inhibitor cocktail (Sigma, USA) was used to lyse
10 the cells. The protein concentrations in the extracts were measured with a
11 bicinchoninic acid assay (Pierce, USA) and were made equal in the different
12 samples with extraction reagent. Total cell lysates or immunoprecipitates
13 prepared as described above were electrophoretically separated by
14 SDS-PAGE, transferred onto a polyvinylidene difluoride membrane (Millipore,
15 Germany), blocked with 3% (w/v) bovine serum albumin (BSA), probed with
16 the indicated primary antibodies and corresponding secondary antibodies, and
17 visualized with the ECL Western Blotting Detection Reagents (Pierce, USA).
18
19 **Confocal immunofluorescence microscopy**
20 Confocal immunofluorescence microscopy studies were performed as
21 described in our previous publications.[30,31] Briefly, HeLa cells were grown on
22 12-well slides one day before transfection with the indicated plasmids.

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1 Transfected or infected HeLa cells were then fixed in 4% paraformaldehyde,
2 permeabilized with 0.2% Triton X-100, and blocked with phosphate-buffered
3 saline (PBS) containing 5% horse serum and 1% BSA. The fixation,
4 permeabilization, and blocking buffers were all purchased from Beyotime
5 Biotechnology (China). The cells were then reacted with the indicated primary
6 antibodies at 4°C overnight, rinsed, and reacted with corresponding secondary
7 antibodies (Invitrogen, USA). The nuclei were counterstained with DAPI
8 (Abcam, USA). Images were taken with a Zeiss LSM780 confocal microscope
9 (Germany).
10
11 **Viral plaque assays**
12 Viral plaque assays were performed on Vero-E6 cells to measure the titer of
13 VSV-eGFP as described in our previous study.[32] Briefly, Vero cells were
14 seeded on 24-well plates. The next day, the cells at approximately 100%
15 confluency were infected with serial dilutions of VSV-eGFP for 30 min. After
16 infection, the medium was replaced with DMEM containing 0.5% agar and 2%
17 FBS. After the agar overlay turned solid, the cells were cultured for 20-24
18 hours, after which the cells were fixed with a 1:1 methanol-ethanol mixture for
19 30 min. After removing the solid agarose-medium mix, the cells were stained
20 with 0.05% crystal violet, and the plaques on the monolayer were then counted
21 to calculate the virus titer.
22

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1 **Bioinformatics analysis**

2 The transmembrane motifs were predicted with the TMHMM server version 2.0
3 (http://www.cbs.dtu.dk/services/TMHMM/).

4

5 **Statistics**

6 The results are representative of three independent experiments and are
7 presented as the mean ± SEM. For statistical analysis, two-tailed unpaired
8 Student's t-tests were performed by GraphPad Prism 8.0 and Microsoft Excel.
9 The P values are presented within each figure or figure legend. In all cases, a
10 value of $P < 0.05$ was considered to be statistically significant.

11

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1 # Results

2 **The SARS-CoV-2 M protein inhibits type I and III IFN induction by SeV**
3 **and poly (I:C)**

4 To explore whether the SARS-CoV-2 M protein affects type I and III IFN
5 production, HEK293T cells expressing the SARS-CoV-2 M protein were
6 infected with SeV or transfected with a dsRNA mimic, poly (I:C). The
7 expression of IFN-β, IFN-λ1, and two ISG, ISG56 and CXCL10, were
8 determined by RT-qPCR. The results indicated that both SeV infection and
9 poly (I:C) transfection strongly stimulated the expression of IFN-β, IFN-λ1,
10 ISG56, and CXCL10 in the control HEK293T cells (Fig. 1). In HEK293T cells
11 expressing the SARS-CoV-2 M protein, the induction of IFN-β, IFN-λ1, ISG56,
12 and CXCL10 by SeV and poly (I:C) was significantly suppressed compared
13 with that in the HEK293T cells transfected with empty vector (Fig. 1). Therefore,
14 the SARS-CoV-2 M protein inhibits the SeV- and poly (I:C)-induced
15 upregulation of IFN-β, IFN-λ1, ISG56, and CXCL10, suggesting that it is
16 involved in antagonizing the IFN response.

17

18 **The SARS-CoV-2 M protein dampens the cytosolic dsRNA-sensing**
19 **pathway mediated by RIG-I/MDA-5 signaling**

20 RIG-I and MDA-5 are cytosolic dsRNA sensors, which participate in the
21 recognition of SeV- and poly (I:C) and the subsequent induction of type I and III
22 IFNs. To further confirm the inhibitory effect of the SARS-CoV-2 M protein on

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1 type I and III IFN expression, we employed luciferase reporters of type I and III
2 IFNs and ISGs to determine whether the SARS-CoV-2 M protein also
3 interferes with the activation of the cytosolic RNA-sensing pathway induced by
4 the overexpression of the RIG-I/MDA-5 pathway components. The results from
5 IFN-β luciferase reporter (IFN-β-Luc) assays showed that overexpression of
6 the SARS-CoV-2 M protein significantly suppressed the activities of IFN-β-Luc
7 induced by RIG-IN (an active form of RIG-I), MDA-5, MAVS, TBK1, and IKKε
8 but had no effect on IRF3-5D (an active form of IRF3)-, TRIF-, or
9 STING-induced IFN-β-Luc activation (Fig. 2). Similarly, we observed that the
10 SARS-CoV-2 M protein decreased the activities of IFN-λ1-Luc (IFN-λ1
11 luciferase reporter) and ISRE-Luc (ISG luciferase reporter) induced by RIG-IN,
12 MDA-5, MAVS, TBK1, and IKKε instead of IRF3-5D, TRIF, or STING (Fig. 2).
13 Consistently, in HEK293T cells expressing the SARS-CoV-2 M protein, the
14 induction of ISG56 by RIG-I, MDA-5, MAVS, TBK1, and IKKε rather than
15 IRF3-5D, TRIF, or STING was significantly restrained (Supplemental Fig. 1).
16 Taken together, the SARS-CoV-2 M protein can impair the activation of the
17 RIG-I/MDA-5-dependent cytosolic dsRNA-sensing pathway but does not affect
18 the TLR3-TRIF signaling-mediated endosome dsRNA-sensing pathway or the
19 cGAS-STING-mediated cytosolic DNA-sensing pathway. Because TBK1 is
20 also downstream of TLR3-TRIF and cGAS-STING signaling, the SARS-CoV-2
21 M protein might thus inhibit the dsRNA-induced IFN production at the step
22 upstream of TBK1.

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1

2 **Subcellular localization of the SARS-CoV-2 M protein**

3 The SARS-CoV-2 M protein is predicted to possess three transmembrane
4 motifs at the N terminus (Supplementary Fig. 2). Therefore, it is of interest to
5 determine the subcellular localization of the SARS-CoV-2 M protein. To
6 address this issue, a Flag-tagged SARS-CoV-2 M protein was overexpressed
7 in HeLa cells, reacted with the Flag antibody and then stained with a
8 fluorescence-labeled secondary antibody. The subcellular localization of the
9 SARS-CoV-2 M protein was observed by confocal microscopy. The results
10 indicated that the SARS-CoV-2 M protein showed almost no localization with
11 mitochondria markers but was primarily localized to the endoplasmic reticulum
12 (ER) and Golgi (Fig. 3a-c). The mitochondria, ER, and Golgi are important
13 platforms for the multi-protein complex formation and signaling transduction of
14 the RIG-I/MDA-5 pathway, and therefore we assessed the colocalization of the
15 SARS-CoV-2 M protein with RIG-I, MDA-5, MAVS, TRAF3, and TBK1. The
16 results showed that the SARS-CoV-2 M protein showed a strong colocalization
17 signal with TBK1 but had a partial colocalization signal with RIG-I, MDA-5, and
18 MAVS (Fig. 3d-h).

19

20 **The SARS-CoV-2 M protein interacts with RIG-I, MDA-5, MAVS, and TBK1**

21 To determine how the SARS-CoV-2 M protein affects IFN signaling activation,
22 coimmunoprecipitation experiments between the SARS-CoV-2 M protein and

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1 the RIG-I-like receptor signaling molecules were performed. We observed the
2 colocalization of the SARS-CoV-2 M protein with RIG-I, MDA-5, MAVS, and
3 TBK1, and thus we examined the interactions between the SARS-CoV-2 M
4 protein with RIG-I, MDA-5, MAVS, and TBK1. In HEK293T cells, the
5 SARS-CoV-2 M protein was coexpressed with RIG-I, MDA-5, MAVS, TBK1,
6 and IRF3 (Fig. 4), after which coimmunoprecipitation experiments were
7 performed. We found that RIG-I, MDA-5, MAVS, and TBK1 but not IRF3 could
8 be detected in the SARS-CoV-2 M protein immunoprecipitates (Fig. 4), and
9 thus the SARS-CoV-2 M protein can associate with RIG-I, MDA-5, MAVS, and
10 TBK1 rather than IRF3. Consistent with the coimmunoprecipitation results, we
11 observed a strong colocalization between the SARS-CoV-2 M protein and
12 TBK1 and a partial colocalization between the SARS-CoV-2 M protein and
13 RIG-I, MDA-5, or MAVS (Fig. 3).
14

15 **The SARS-CoV-2 M protein prevents RIG-I-MAVS, MAVS-TBK1, and**
16 **TRAF3-TBK1 interactions and inhibits IRF3 phosphorylation**

17 We observed the interaction between the SARS-CoV-2 M protein and RIG-I,
18 MDA-5, MAVS, and TBK1 (Fig. 3a-d). However, an association between the
19 SARS-CoV-2 M protein and IRF3 was unable to be detected (Fig. 3e). IRF3
20 activation and subsequent IFN activation relies on the assembly of a
21 multi-protein complex containing the dsRNA sensor RIG-I/MDA-5, MAVS,
22 TRAF3, and TBK1. Since the SARS-CoV-2 M protein can interact with RIG-I,

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1 MDA-5, MAVS, and TBK1, it of interest to investigate whether the
2 SARS-CoV-2 M protein can affect RIG-I/MDA-5-MAVS-TRAF3-TBK1 complex
3 formation, which is essential for IRF3 activation and IFN induction. The
4 expression plasmids of the SARS-CoV-2 M protein and plasmids expressing
5 RIG-I or MDA-5 were cotransfected into HEK293T cell, 24 hours later, MAVS
6 antibodies were used to perform coimmunoprecipitation. When the
7 SARS-CoV-2 M protein was overexpressed, the binding between RIG-I and
8 MAVS was reduced (Fig. 5a, lanes 2 compared to lane 3); however, in the
9 same condition, the interaction between MDA-5 and MAVS was not affected
10 (Fig 5b, lanes 2 compared to lane 3), indicating that the SARS-CoV-2 M
11 protein impedes the complex formation of RIG-I and MAVS but has no effect
12 on the interaction between MDA-5 and MAVS. When using the TBK1 antibody
13 to perform endogenous coimmunoprecipitation, MAVS was detected in the
14 TBK1 immunoprecipitates (Fig. 5c). Moreover, when the SARS-CoV-2 M
15 protein was overexpressed in HEK293T cells, TBK1-associated MAVS was
16 apparently reduced compared with the cells without SARS-CoV-2 M protein
17 expression (Fig. 5c, lanes 1 and 3 compared to lane 2; Fig. 5d, lane 4
18 compared to lane 2), suggesting that the SARS-CoV-2 M protein might
19 preferentially reduce the complex formation of MAVS with TBK1. Notably,
20 when the protein complex of TBK1 and MAVS was reduced, IRF3
21 phosphorylation induced by RIG-IN was correspondingly impaired (Fig 5c, lane
22 3 compared to lane 2). Similarly, the endogenous coimmunoprecipitation of

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1 TBK1 indicated that the overexpression of the SARS-CoV-2 M protein
2 impeded the endogenous association between TRAF3 and TBK1 (Fig. 5d,
3 lane 4 compared to lane 2) and thus correspondingly suppressed IRF3
4 phosphorylation (Fig. 5d, lane 4 compared to lane 2). Previous studies have
5 shown that the SARS-CoV-1 M protein and MERS-CoV M protein can diminish
6 the association between TBK1 and TRAF3 to interfere with IFN production.[36,37]
7 Here, we found that the overexpression of the SARS-CoV-2 M protein not only
8 decreased the interaction between TBK1 and TRAF3 but also reduced the
9 binding between RIG-I and MAVS and the binding between MAVS and TBK1,
10 suggesting that the SARS-CoV-2 M protein affects the multi-protein complex
11 formation of RIG-I-MAVS-TRAF3-TBK1 and the subsequent IRF3
12 phosphorylation.
13
14 **The SARS-CoV-2 M protein suppresses SeV-induced IRF3**
15 **phosphorylation and nuclear translocation.**
16 The phosphorylation and nuclear translocation of IRF3 is the hallmark of its
17 activation, which is essential for type I and III IFN induction during virus
18 infection. IRF3 phosphorylation is the prerequisite for its nuclear translocation
19 and IFN transcription. Therefore, we next investigated the effect of the
20 SARS-CoV-2 M protein on IRF3 phosphorylation. The results from RT-qPCR
21 analysis (Fig. 1) and a luciferase reporter assay (Fig. 2) have indicated that the
22 SARS-CoV-2 M protein could inhibit the induction of type I and III IFN and that

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1 the overexpression of the SARS-CoV-2 M protein reduced the IRF3
2 phosphorylation induced by RIG-IN (Fig. 5c, d). However, whether the
3 SARS-CoV-2 M protein affects IRF3 phosphorylation in a real virus infection is
4 unknown, which may contribute to our understanding of the role of the
5 SARS-CoV-2 M protein in SARS-CoV-2 infection. Because of the lack of a
6 biosafety level 3 laboratory, we used another RNA virus, SeV, as a surrogate
7 for SARS-CoV-2 to perform the virus infection studies. To address the effect of
8 the SARS-CoV-2 M protein on virus-induced IRF3 phosphorylation, control
9 HeLa cells and HeLa cells expressing the SARS-CoV-2 M protein were
10 infected with SeV. The immunoblotting results indicated that SeV infection
11 could induce the phosphorylation of IRF3 in HeLa cells, while the
12 phosphorylation of IRF3 was obviously decreased in HeLa cells expressing the
13 SARS-CoV-2 M protein (Fig. 6a). Therefore, the SARS-CoV-2 M protein can
14 inhibit IRF3 phosphorylation induced by SeV infection. In contrast, TBK1
15 phosphorylation was not affected in HeLa cells expressing the SARS-CoV-2 M
16 protein (Fig. 6a).
17
18 The phosphorylation of IRF3 is a pivotal step for its nuclear translocation to
19 initiate the transcription of type I and III IFNs. Because we observed that the
20 SARS-CoV-2 M protein suppressed SeV-induced IRF3 phosphorylation (Fig.
21 6a), we next examined the effect of the SARS-CoV-2 M protein on
22 SeV-induced IRF3 nuclear translocation. In HeLa cells, IRF3 was evenly

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1 distributed in both the nucleus and the cytosol in the absence or presence of
2 the SARS-CoV-2 M protein without viral infection (Fig. 6b), and SeV infection
3 strongly stimulated the nuclear translocation of IRF3 in HeLa cells transfected
4 with the control vector (Fig. 6b). However, SeV-induced IRF3 nuclear
5 translocation was significantly decreased when the SARS-CoV-2 M protein
6 was expressed in HeLa cells, compared with the control cells (Fig. 6b, c). Thus,
7 the SARS-CoV-2 M protein exerts its inhibitory function of type I and III IFNs by
8 preventing IRF3 phosphorylation and nuclear translocation.
9
10 **The SARS-CoV-2 M protein promotes viral replication**
11 We have shown that the SARS-CoV-2 M protein can suppress type I and III
12 IFN-induced antiviral immunity. Next, it was of interest to determine the role of
13 the SARS-CoV-2 M protein in viral replication. VSV is commonly used as a
14 model virus to study the effect of IFN on viral replication. VSV-eGFP was used
15 to infect HEK293 cells transfected with empty vector or the SARS-CoV-2 M
16 protein plasmid. With fluorescence microscopy, we observed that there were
17 more VSV-eGFP-positive cells in the SARS-CoV-2 M protein-expressing
18 samples than in the control cells transfected with an empty vector (Fig. 7). In
19 the culture supernatant of HEK293 cells expressing the SARS-CoV-2 M
20 protein, the titer of VSV-eGFP was much higher than that of the HEK293 cells
21 transfected with an empty vector (Fig. 7). Moreover, there was more eGFP
22 protein expression in the HEK293 cells expressing the SARS-CoV-2 M protein

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1 than in the HEK293 cells transfected with an empty vector (Fig. 7). Thus, the
2 overexpression of the SARS-CoV-2 M protein facilitates the replication of
3 VSV-eGFP.
4

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1 # Discussion

2 Our previous studies have shown that innate antiviral immunity may play an
3 important role in the SARS-CoV-2 clearance observed in COVID-19 patients.[38]
4 The dysregulation of innate antiviral immunity and inflammatory responses by
5 SARS-CoV-2 is strongly responsible for COVID-19-induced human death.[6,39]
6 Type I and III IFNs are typically suppressed in COVID-19 patients, but the
7 molecular mechanism of this phenomenon caused by SARS-CoV-2 still needs
8 to be elucidated.[6,23] Here, we reported that the SARS-CoV-2 M protein can
9 target the cytosolic RNA-sensing pathway of RIG-I/MDA-5 signaling to block
10 the activation of type I and III IFN responses and thus inhibit host antiviral
11 immunity.
12
13 The M proteins of SARS-CoV-1 and MERS-CoV were reported to suppress
14 type I IFN production by interacting with TRAF3 to disrupt TRAF3-TBK1
15 association. [36,37,40] However, the IFN-antagonizing activities of the MERS-CoV
16 M protein seem to be extremely low. The SARS-CoV-1 M protein interacts with
17 TBK1, but the MERS-CoV M protein cannot be detected to associate with
18 TBK1, suggesting that these two highly pathogenic coronaviruses engage
19 distinctive mechanisms in IFN suppression. Moreover, the M protein of the
20 human coronavirus HKU1 does not impact type I IFN production, indicating
21 that the IFN antagonistic property of the M proteins is not evolutionarily
22 conserved in human coronaviruses.[36,37,40] Importantly, one recent study found

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1 that the SARS-CoV-2 M protein cannot inhibit type I IFN and ISG production.[41]
2 The differences between the M proteins of these coronaviruses in
3 antagonizing IFN signaling drive us to study the function and mechanism of the
4 SARS-CoV-2 M protein in evading innate antiviral immunity. We first examined
5 the effect of the SARS-CoV-2 M protein on the activation of the cytosolic
6 dsRNA-sensing pathway of RIG-I/MDA-5-MAVS signaling and the endosome
7 dsRNA-sensing pathway of TLR3-TRIF signaling. The results demonstrated
8 that the SARS-CoV-2 M protein can significantly inhibit the activation of type I
9 and III IFN production induced by the RIG-I/MDA-5 signaling components,
10 including RIG-I, MDA-5, MAVS, TBK1, and IKKε, but not by TRIF, the adaptor
11 protein of the TLR3-TRIF signaling, or by STING, the adaptor protein of the
12 cGAS-STING signaling (Fig. 2). These results indicated that the SARS-CoV-2
13 M protein specifically targets the RIG-I/MDA-5-dependent RNA-sensing
14 pathway but not the TLR3-TRIF-dependent endosome dsRNA-sensing
15 pathway or the cGAS-STING-dependent cytosolic dsDNA-sensing pathway.
16 Furthermore, coimmunoprecipitation studies showed that the SARS-CoV-2 M
17 protein interacts with RIG-I, MDA-5, MAVS, and TBK1 but not with IRF3 (Fig.
18 4). The overexpression of the SARS-CoV-2 M protein could prevent the
19 complex formation of RIG-I-MAVS, MAVS-TBK1, and TRAF3-TBK1 (Fig. 5).
20 Thus, the SARS-CoV-2 M protein exerts its inhibitory effect on IFN production
21 by impeding the formation of protein complex involving RIG-I, MAVS, TRAF3,
22 and TBK1, which are essential for IRF3 activation and IFN production. We also

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1 found that the SARS-CoV-2 M protein can inhibit the phosphorylation of IRF3
2 but not that of TBK1 (Fig. 6a), which may explain why the SARS-CoV-2 M
3 protein interacts with TBK1 but cannot suppress TRIF- or STING-induced IFN
4 production. This finding also suggests that the SARS-CoV-2 M protein does
5 not directly target TBK1 but acts upstream of TBK1.
6
7 Although SARS-CoV-1 and MERS-CoV M proteins were shown to inhibit IRF3
8 phosphorylation, whether they also affected the translocation of IRF3 into the
9 nucleus was unknown. [36,37,40] To extend the understanding of the biological
10 function of the SARS-CoV-2 M protein, we observed that after SeV infection,
11 IRF3 was mainly retained in the cytosol in HeLa cells expressing the
12 SARS-CoV-2 M protein; in addition, in control HeLa cells that did not express
13 the SARS-CoV-2 M protein, IRF3 was dominantly translocated into the nucleus
14 upon SeV infection (Fig. 6b, c). Thus, the SARS-CoV-2 M protein can
15 powerfully inhibit the nuclear localization of IRF3 induced by SeV. Consistently,
16 IRF3 phosphorylation in HeLa cells expressing the SARS-CoV-2 M protein
17 was also attenuated compared with those cells expressing an empty vector
18 (Fig. 6a).
19
20 The SARS-CoV-2 M protein possesses three transmembrane motifs
21 (Supplementary Fig. 2). We showed that it could colocalize with markers of the
22 ER and Golgi apparatus (Fig. 3a-c), which are important signaling platforms for

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1 innate antiviral immunity.[42,43] In addition, we observed that the SARS-CoV-2 M
2 protein showed colocalization with TBK1, and partial colocalization with RIG-I,
3 MDA-5, and MAVS but not with IRF3 (Fig. 3d-h; Fig. 6b), and these
4 observations were also consistent with our coimmunoprecipitation results that
5 the SARS-CoV-2 M protein interacted with RIG-I, MAVS, and TBK1 but not
6 with IRF3 (Fig. 4). Moreover, we found that the SARS-CoV-2 M protein
7 impeded the formation of the multi-protein complex of RIG-I, MAVS, and TBK1
8 (Fig. 5a-d). An interesting question is whether this inhibition results from
9 retention in a cellular compartment that blocks translocalization. A previous
10 study has suggested that the translocalization of TBK1 from the ER or
11 mitochondria to the Golgi fragments is pivotal for SeV-induced IFN activation.
12 [43] Since the SARS-CoV-2 M protein is localized in both the ER and the Golgi,
13 its inhibitory effect is likely achieved through inhibiting TBK1 translocation from
14 the ER to the Golgi compartments and subsequently affecting the formation of
15 TBK1-containing Golgi fragments. [42,43] Therefore, whether TBK1-containing
16 Golgi fragment formation is affected by the SARS-CoV-2 M protein warrants
17 further analysis, which may represent as a novel mechanism by which
18 virus-encoded proteins render IFN production inefficient.
19
20 The M proteins of SARS-CoV-1 and MERS-CoV were reported to inhibit type I
21 IFN production.[36,37,40] However, the role of the coronavirus M protein in viral
22 replication still needs further investigation. The results from the fluorescence

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1 microscopy, plaque assays, and immunoblotting indicated that the replication
2 of VSV-eGFP was significantly enhanced in HEK293T cells expressing the
3 SARS-CoV-2 M protein (Fig. 7). Thus, the SARS-CoV-2 M protein likely
4 promotes viral replication by suppressing the host IFN responses.
5
6 Compared with the previous studies on the function of coronavirus M proteins
7 in antagonizing IFNs, we provided several novel findings. First, we showed for
8 the first time that the SARS-CoV-2 M protein suppresses both type I IFN and III
9 IFN production, but the previous studies were restricted to the effect of
10 coronavirus M protein on type I IFN. This finding may explain the advantage of
11 type III IFNs in curing COVID-19.[24] Second, the SARS-CoV-1 and MERS-CoV
12 M proteins have been reported to impede TRAF3-TBK1 complex formation. In
13 our study, we found that the SARS-CoV-2 M protein not only prevented
14 TRAF3-TBK1 complex formation but also impaired the formation of the
15 multi-protein complex of RIG-I-MAVS-TBK1, which is essential for IRF3
16 phosphorylation, translocation, and IFN transcription. Third, we also observed
17 that the SARS-CoV-2 M protein specifically inhibited RIG-I/MDA-5-MASV
18 signaling rather than TLR3-TRIF or cGAS-STING signaling, which may provide
19 more precise targets for potential COVID-19 treatment. In addition, we
20 reported that the SARS-CoV-2 M protein inhibited IRF3 phosphorylation and
21 nuclear translocation activated by SeV infection. Last, although the
22 SARS-CoV-1 and MERS-CoV M proteins were reported to suppress type I

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1 IFNs, their roles in viral replication are still need to be addressed. In our study,
2 we demonstrated that the overexpression of the SARS-CoV-2 M protein could
3 prevent the replication of VSV-eGFP.
4
5 Although we provide ample results to demonstrate that the SARS-CoV-2 M
6 protein inhibits type I and III IFN production, we are aware that the ectopic
7 expression of the viral protein is different from a real infection for the study of
8 its biological function. Since the M protein is a structural protein and
9 indispensable for virion assembly, constructing an M-null SARS-CoV-2 virus is
10 currently not possible. Identification of a mutant of the SARS-CoV-2 M protein
11 that loses IFN suppression activities but does not affect virion assembly merits
12 further investigations. In addition, such a mutant may provide guidelines for the
13 future production of an M-defective mutant virus of SARS-CoV-2, which will
14 help to contribute to our understanding of SARS-CoV-2 M in antagonizing IFN
15 in a real viral infection context.
16
17 The administration of type I or III IFNs alone or together with other drugs has
18 resulted in a reduced virus titer, a limited inflammatory response, and mild
19 clinical disease in both animal models and patients infected by SARS-CoV-1 or
20 SARS-CoV-2.[9,24] Multiple proteins encoded by coronaviruses were shown to
21 counteract the IFN response, although the mechanistic details of their actions
22 for most of these proteins have not been well documented.[8,41] A better

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1 understanding of the viral IFN antagonists in the pathogenesis of SARS-CoV-2
2 has important implications in the development of new antiviral drugs and
3 vaccines. In this study, for the first time, we studied the function of the
4 SARS-CoV-2 M protein in counteracting the IFN-mediated innate antiviral
5 immunity and its role in viral replication. Our investigation extends the
6 understanding of antiviral immunity evasion strategies employed by
7 SARS-CoV-2 and reveals a novel mechanistic insight on the mechanisms of
8 IFN inhibition by coronavirus M protein, thus shedding light on the interactions
9 between human antiviral immunity and coronavirus infection in the
10 pathogenesis of COVID-19.
11

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13

14 ## Contributions

15 C.G. and P.-H.W. conceptualized the study. Y.Z., M.-W.Z., L.H., and J.Z.
16 performed the experiments. P.-H.W. wrote the first draft of manuscript. All of
17 the authors contributed to revising the manuscript and approved the final
18 version for publication.

19

20 ## ORCID

21 Chengjiang Gao https://orcid.org/0000-0002-9365-4497
22 Pei-Hui Wang https://orcid.org/0000-0001-6853-2423

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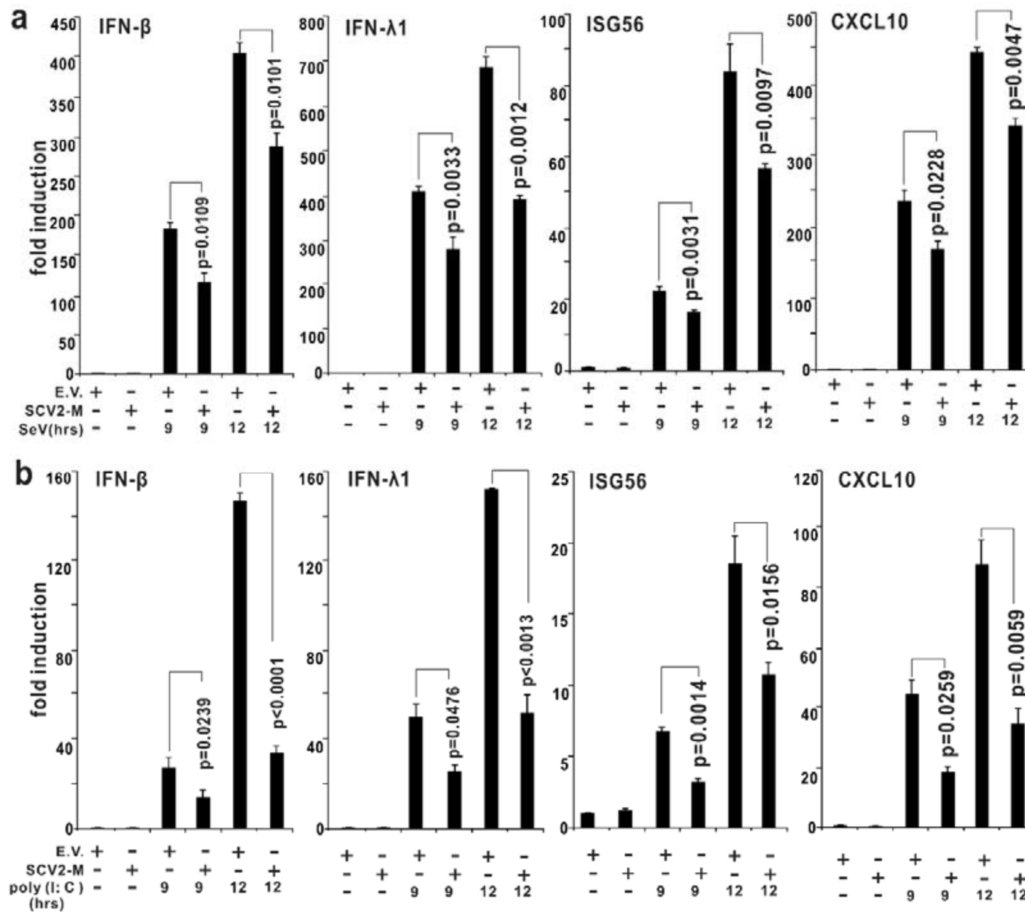
1 # Figure legends



2

3 **Figure 1. The SARS-CoV-2 M protein inhibits the induction of IFN-β,**

4 **IFN-λ1, ISG56, and CLXL10 by SeV infection and poly (I:C) transfection.**

5 HEK293T cells cultured in 24-well plates (0.8-1 x 10^5 per well) were

6 transfected with a pcDNA6B empty vector (E.V., 500 ng) or an SARS-CoV-2 M

7 protein-expressing plasmid (500 ng). Twenty-four hours after transfection, the

8 cells were stimulated with SeV infection (50 HA/mL) or poly (I:C) (1000 ng/mL)

9 transfection as indicated, and at 9 and 12 hours after stimulation, the cells

10 were harvested for RNA extraction and subsequent RT-qPCR analysis. Three

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1 independent biological replicates were analyzed; the results of one
2 representative experiment are shown, and the error bars indicate SEM. The
3 statistical significance is shown as indicated. SARS-CoV-2 M protein, SCV2-M;
4 hours, hrs.
5

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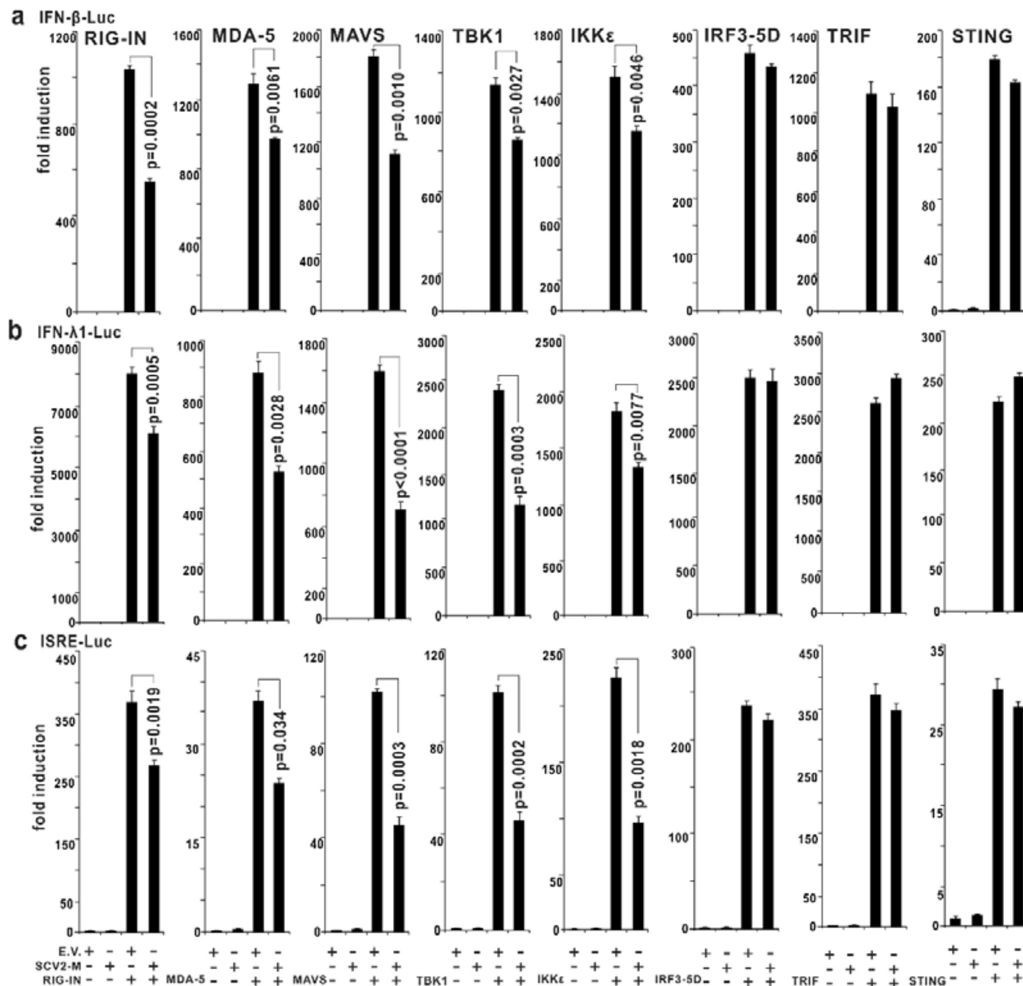

1

2 **Figure 2. The SARS-CoV-2 M protein suppresses the luciferase reporters**

3 **of type I and III IFNs and ISGs.** The pcDNA6B empty vector (E.V.) and the

4 SARS-CoV-2 M protein-expressing plasmid (100 ng) were transfected with the

5 indicated combinations of plasmids expressing RIG-IN (100 ng), MDA-5 (100

6 ng), TBK1 (100 ng), IKKε (100 ng), IRF3-5D (100 ng, an active form of IRF3),

7 TRIF (100 ng, component of TLR3-TRIF pathway), or STING (100 ng,

8 component of cGAS-STING pathway) into HEK293T cells cultured in 48-well

9 plates (0.5 x 10^5 per well). Plasmids containing IFN-β-Luc (45 ng, the IFN-β

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1 luciferase reporter), IFN-λ1-Luc (45 ng, the IFN-λ1 luciferase reporter), or
2 ISRE-Luc (45 ng, the IFN-stimulated response element luciferase reporter)
3 were also transfected for indicating the activation of type I IFNs, type III IFNs,
4 or ISGs, respectively. The pRL-TK (5 ng) was transfected into each well as an
5 internal control. The pcDNA6B empty vector was used to balance the total
6 amount of plasmid DNA in the transfection. Dual-luciferase assays were
7 performed 36 hours after transfection. Three independent biological replicates
8 were analyzed; the results of one representative experiment are shown, and
9 the error bars indicate SEM. The statistical significance is shown as indicated.
10 SARS-CoV-2 M protein, SCV2-M.
11

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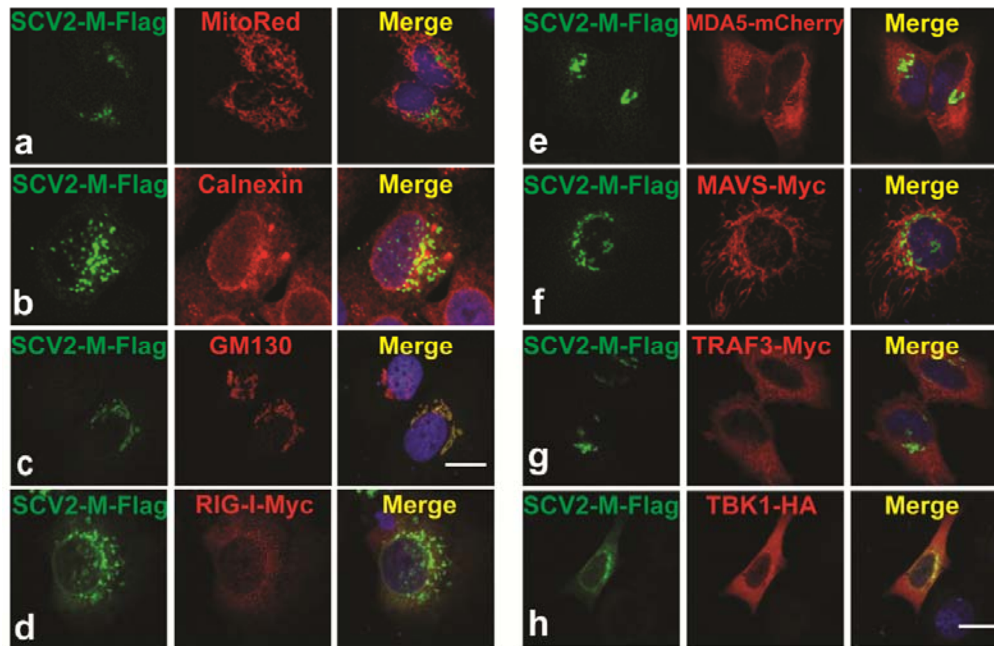

1 Figure 3

2 **Figure 3. Subcellular localization of the SARS-CoV-2 M protein**. HeLa cells
3 seeded on 12-well coverslips were transfected with the indicated plasmids.
4 Twenty hours later, the cells are fixed, blocked, and then incubated with a
5 rabbit anti-Flag antibody and a mouse antibody against the corresponding
6 organelle marker (**a-c**) or the indicated protein (**d, f-h**). Subsequently, the
7 proteins were stained with a fluorescence-labeled secondary antibody.
8 Nucleus were visualized with DAPI (blue). Confocal imaging results are
9 representative of two independent experiments. Scale bar, 10 μm. MitoRed,
10 mitochondria marker; Calnexin, ER marker; CM130, Golgi marker;
11 SARS-CoV-2 M protein, SCV2-M.
12

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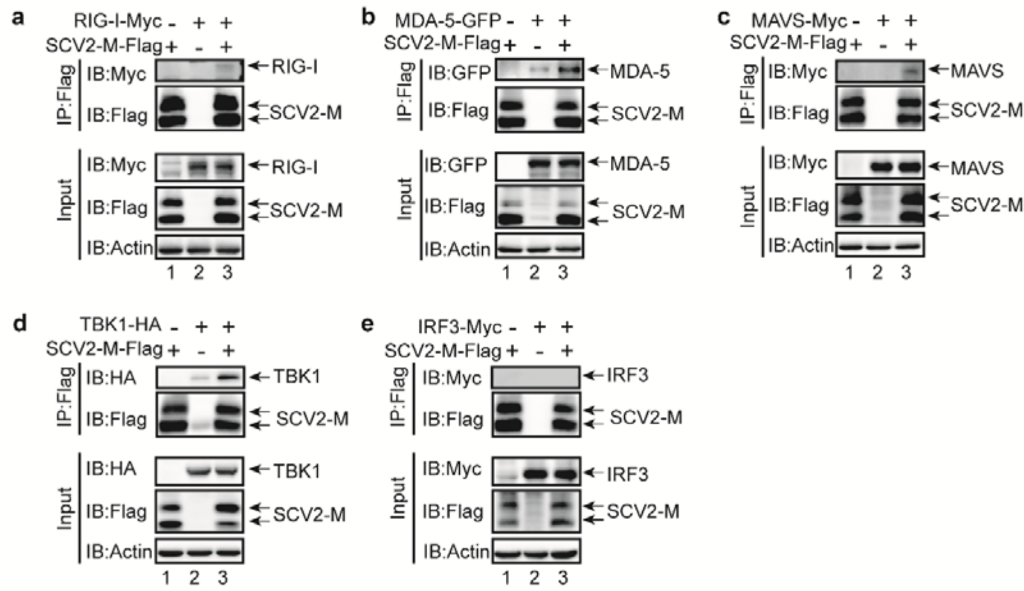

1

2 **Figure 4. The SARS-CoV-2 M protein interacts with RIG-I (a), MDA-5 (b),**
3 **MAVS (c), and TBK1 (d) but not with IRF3 (e).** The HEK293T cells were
4 transfected with the indicated plasmids for twenty-four hours before
5 coimmunoprecipitation by the anti-Flag magnetic beads. The pcDNA6B empty
6 vector was used to balance the total amount of plasmid DNA in the transfection.
7 The input and immunoprecipitates were immunoblotted with the indicated
8 antibodies. Immunoblotting results are representative of two independent
9 experiments. SARS-CoV-2 M protein, SCV2-M.
10

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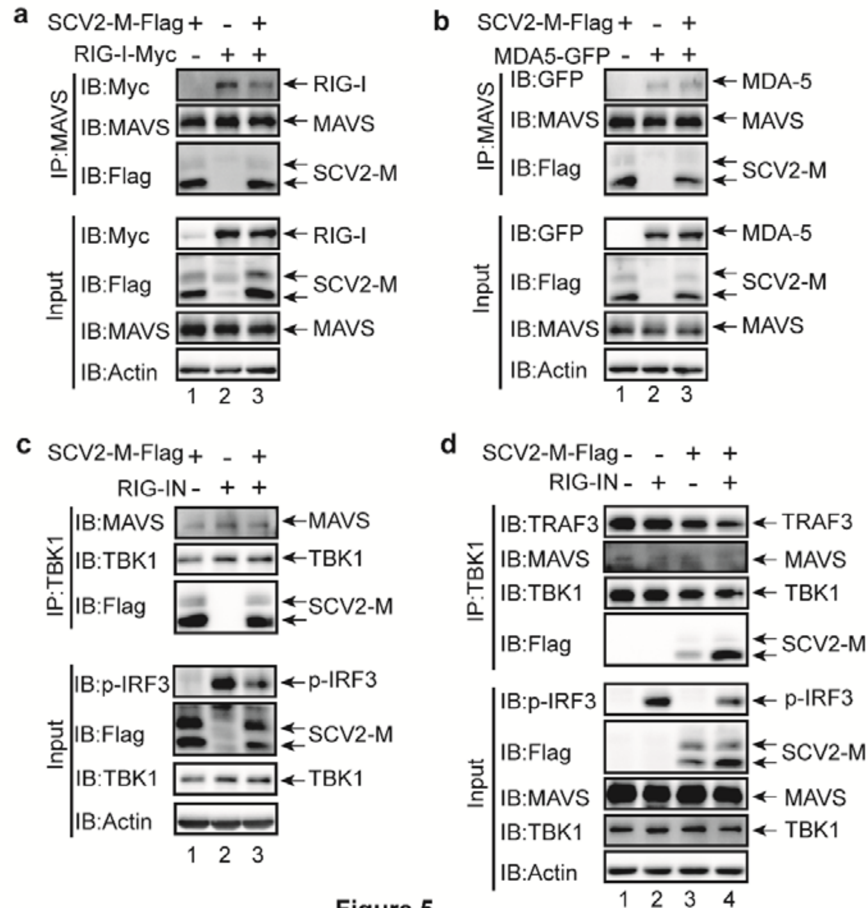

1

2 **Figure 5. The SARS-CoV-2 M protein prevents the multi-protein complex**

3 **formation involving RIG-I, MAVS, TRAF3, and TBK1.** The SARS-CoV-2 M

4 protein inhibits the RIG-I-MAVS (**a**), MAVS-TBK1 (**c**), and TRAF3-TBK1 (**d**)

5 interactions. The HEK293T cells were transfected with the indicated plasmids

6 for 24 h before coimmunoprecipitation by the MAVS (**a-b**) or TBK1 (**c-d**)

7 antibody. The pcDNA6B empty vector was used to balance the total amount of

8 plasmid DNA in the transfection. The input and immunoprecipitates were

9 immunoblotted with the indicated antibodies. Immunoblotting results are

10 representative of two independent experiments. SARS-CoV-2 M protein,

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1 SCV2-M.

2

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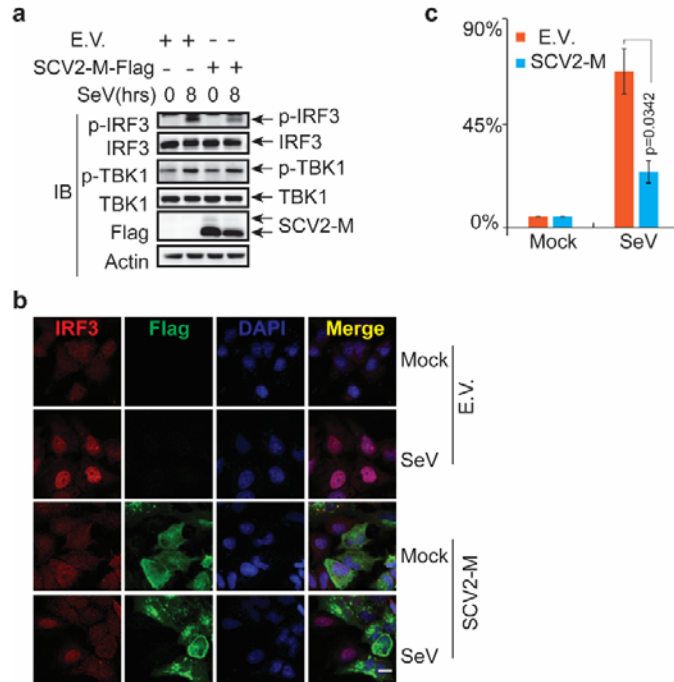

1 Figure 6

2 **Figure 6 The SARS-CoV-2 M protein suppresses IRF3 phosphorylation**
3 **and nuclear translocation. a** The SARS-CoV-2 M protein affects the
4 phosphorylation of IRF3 upon SeV infection. HeLa cells seeded on 6-well
5 plates were transfected with the plasmid of pCAG-Flag empty vector or the
6 plasmid of Flag-tagged SARS-CoV-2 M protein for 20 hours before infection
7 with SeV (50 HA/mL). At the indicated time points, the cells were harvested
8 and processed for immunoblotting with the indicated antibodies. **b** The
9 SARS-CoV-2 M protein prevents the nuclear translocation of IRF3. HeLa cells
10 seeded on 12-well coverslips were transfected with the plasmid of pCAG-Flag
11 empty vector or the plasmid of Flag-tagged SARS-CoV-2 M protein for 20
12 hours before infection with SeV. After infection for 8 hours, the slides were
13 harvested and processed for immunofluorescence with a mouse anti-Flag

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1 antibody and a rabbit anti-IRF3 antibody. Scale bar, 10 μm. **c** Quantification of
2 the percentage of IRF3 in the nucleus upon SeV infection. IRF3 present in the
3 nucleus from 50 cells within each group from (**b**) was counted and calculated.
4 Immunoblotting and confocal imaging results are representative of two
5 independent experiments. Empty vector, E.V.; SARS-CoV-2 M protein,
6 SCV2-M; hours, hrs.
7

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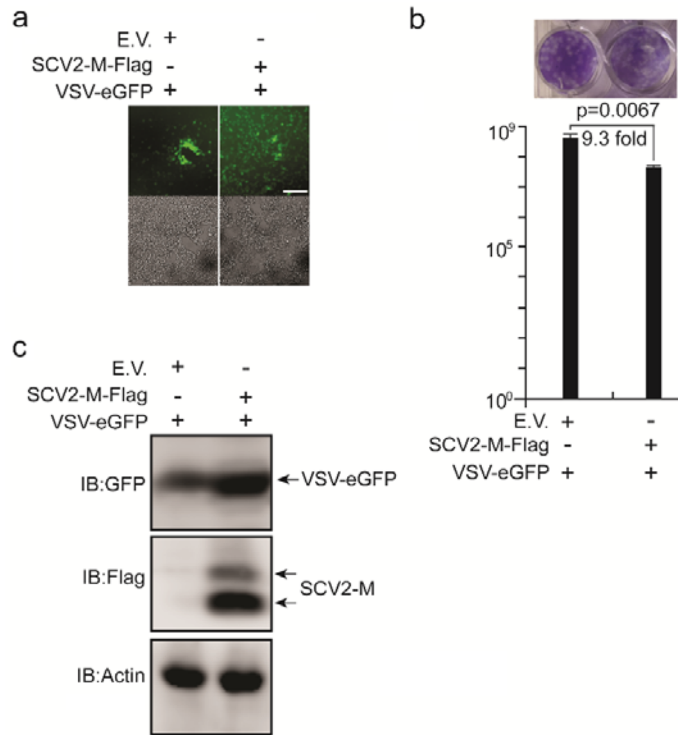

Figure 7

1

2 **Figure 7 The SARS-CoV-2 M protein facilitates viral replication.** The
3 HEK293T cells were transfected with plasmids as indicated, and 24 hours later,
4 the cells were infected with VSV-eGFP (MOI=0.001). Twelve hours after
5 infection, the GFP-positive cells were observed (**a**), and the culture
6 supernatant (20 hours post-infection) was collected for plaque assays to
7 measure the titer of extracellular VSV-eGFP (**b**). Confocal imaging results are
8 representative of two independent experiments. Scale bar, 50 μm. Three
9 independent biological replicates were analyzed; the results of one
10 representative experiment are shown, and the error bars indicate SEM. The
11 statistical significance is shown as indicated. **c** The replication of intracellular
12 VSV-eGFP in the cell lysate (20 hours post-infection) was determined by
13 immunoblotting using an anti-GFP antibody. Empty vector, E.V.; SARS-CoV-2

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1 M protein, SCV2-M.

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1 **Supplemental Table S1. Primers used in this study**

Primer name	Sequence (5′-3′)	Usage
GAPDH-F	GGAGCGAGATCCCTCCAAAAT	RT-qPCR
GAPDH-R	GGCTGTTGTCATACTTCTCATGG	
IFN-β-F	TTGCTCTCCTGTTGTGCTTC	RT-qPCR
IFN-β-R	AAGCCTCCCATTCAATTGCC	
IFN-λ1-F	GAGGCCCCCAAAAAGGAGTC	RT-qPCR
IFN-λ1-R	AGGTTCCCATCGGCCACATA	
ISG56-F	CTAAGCAAAACCCTGCAGAAC	RT-qPCR
ISG56-R	TCAGGCATTTCATCGTCATC	
CXCL10-F	GTGGCATTCAAGGAGTACCTC	RT-qPCR
CXCL10-R	GACCTTTCCTTGCTAACTGCT	
SCV2-M-F	GCACAGTGGCGGCCGCTCGAGGCCACCATGGCAGATTCCAACGGTAC	expression
SCV2-M-R	GTCATCCTTGTAATCTCTAGACTGTACAAGCAAAGCAATATTG	plasmid
IFN-λ1-Luc-F	GGGGTACCTAAACCAATGGCAGAAGCTCC	luciferase
IFN-λ1-Luc-R	GAAGATCTGGCTAAATCGCAACTGCTTCCCCAG	reporter

2 F: forward primer. R: reverse primer.

3

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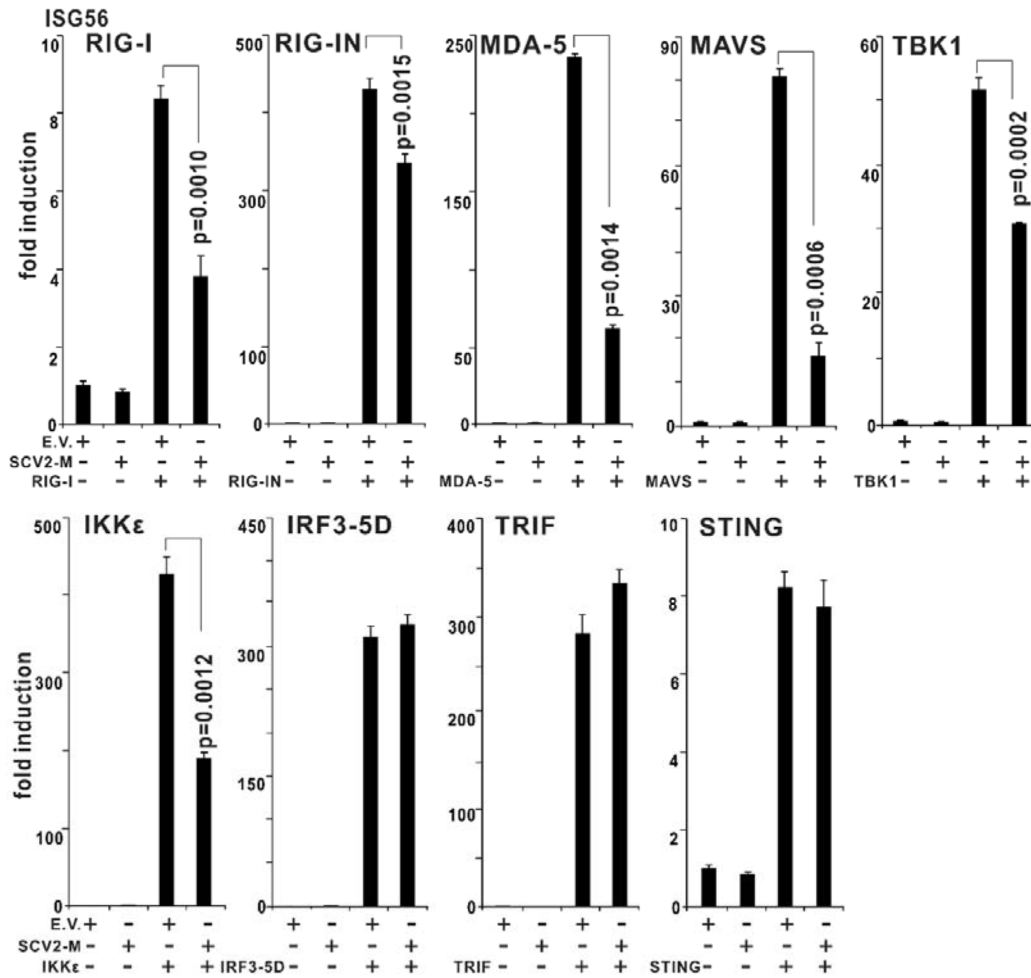

Supplemental Figure 1. The SARS-CoV-2 M protein impairs IFN-stimulated 56 (ISG56) production induced by the RIG-I/MDA-5 pathway but not by the TLR3-TRIF pathway or the cGAS-STING pathway. The pcDNA6B empty vector (E.V.) and plasmid expressing SARS-CoV-2 M protein (SCV2-M, 300 ng) were transfected with the indicated combinations of plasmids expressing RIG-IN (200 ng), MDA-5 (200 ng), TBK1 (200 ng), IKKε (200 ng), IRF3-5D (200 ng), TRIF (200 ng), or STING (200 ng) into HEK293T cells cultured in 24-well plates (0.8-1 x 10^5 per well). Thirty-six hours later, the cells were harvested for RNA extraction and subsequent RT-qPCR analysis of the ISG56 induction. Three independent biological replicates were analyzed; the results of one representative experiment are shown, and the error bars indicate SEM. The statistical significance is shown as indicated. Empty vector, E.V.; SARS-CoV-2 M protein, SCV2-M.

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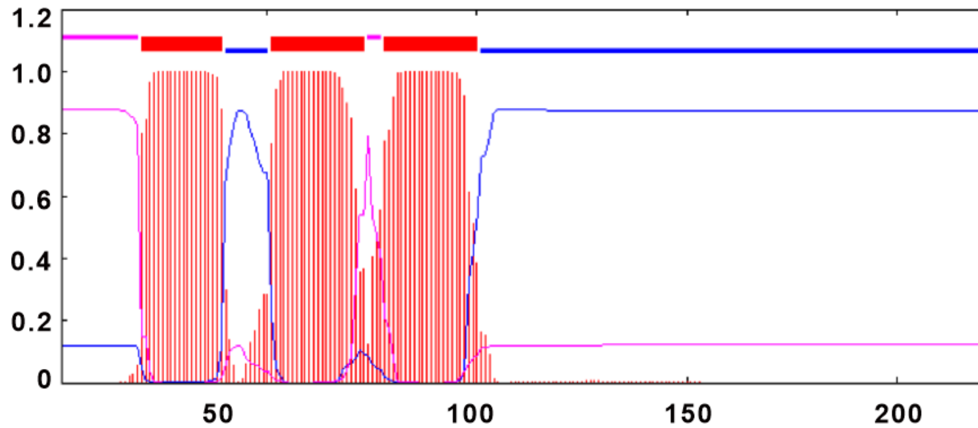

b

>SARS-CoV-2 M protein

MADSNGTITVEELKKLLEQWNLVIGFLFLTWICLLQFAYANRNRFLYIIK
LIFLWLLWPVTLACFVLAAVYRINWITGGIAIAMACLVGLMWLSYFIASF
RLFARTRSMWSFNPETNILLNVPLHGTILTRPLLESELVIGAVILRGHLR
IAGHHLGRCDIKDLPKEITVATSRTLSYYKLGASQRVAGDSGFAAYSRY
RIGNYKLNTDHSSSSDNIALLVQ

Supplemental Figure 2. The SARS-CoV-2 M protein is predicated to possess three transmembrane motifs. (A) The transmembrane motifs of the SARS-CoV-2 M protein were predicted with the TMHMM server, version 2.0. (B) The transmembrane motifs of SARS-CoV-2 M protein were labeled with a red box.