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The Nucleocapsid Protein of Coronaviruses Acts as a Viral Suppressor of RNA Silencing in Mammalian Cells

Running title: Coronavirus N as VSR in Mammalian cells

Lei Cui, Haiying Wang, Yanxi Ji, Jie Yang, Shan Xu, Xingyu Huang, Zidao Wang, Lei Qin, Po Tien, Xi Zhou, Deyin Guo[#], Yu Chen[#]

State Key Laboratory of Virology, College of Life Sciences, Wuhan University, Wuhan, Hubei 430072, China

[#]Address correspondence to Deyin Guo, dguo@whu.edu.cn

[#]Address correspondence to Yu Chen, chenyu@whu.edu.cn

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Abstract

RNA interference (RNAi) is a process of eukaryotic posttranscriptional gene silencing that functions in antiviral immunity in plants, nematodes, and insects. However, recent studies provided strong supports that RNAi also plays a role in antiviral mechanism in mammalian cells. To combat RNAi-mediated antiviral responses, many viruses encode viral suppressors of RNA silencing (VSR) to facilitate their replication. VSRs have been widely studied for plant and insect viruses but only few have been defined for mammalian viruses currently. Here, we identified a novel VSR from coronaviruses, a group of medically important mammalian viruses including Severe acute respiratory syndrome coronavirus (SARS-CoV), and showed that the nucleocapsid protein (N protein) of coronaviruses suppresses RNAi triggered by either short hairpin RNAs (shRNAs) or small interfering RNAs (siRNAs) in mammalian cells. Mouse hepatitis virus (MHV) is closely related to SARS-CoV in the family *Coronaviridae* and was used as a coronavirus replication model. The replication of MHV increased when the N proteins were expressed *in trans*, while knockdown of Dicer1 or Ago2 transcripts facilitated the MHV replication in mammalian cells. These results support the hypothesis that RNAi is a part of the antiviral immunity responses in mammalian cells.

Importance

RNAi has been well known to play important antiviral roles from plants to invertebrates. However, recent studies provided strong supports that RNAi is also

involved in antiviral response in mammalian cells. An important indication for RNAi-mediated antiviral activity in mammals is the fact that a number of mammalian viruses encode potent suppressor of RNA silencing (VSR). Our current results demonstrate that coronaviruses N protein could function as a VSR through its dsRNA binding activity. Mutational analysis of N protein allowed us to find out the critical residues for the VSR activity. Using the MHV-A59 as the coronavirus replication model, we showed that ectopic expression of SARS-CoV N protein could promote MHV replication in RNAi-active cells but did not in RNAi depleted cells. These results indicate that coronaviruses encode a VSR that functions in the replication cycle, and provide further evidences to support that RNAi-mediated antiviral response exists in mammalian cells.

Introduction

RNA interference (RNAi) is originally regarded as a mechanism of eukaryotic posttranscriptional gene regulation mediated by small interfering RNA (siRNA) induced sequence-specific RNA degradation (1). It is also well known to exert as an important antiviral defense mechanism in a wide range of organisms, from plants to invertebrates (2). During the virus infection, the virus-derived long double-stranded RNA (dsRNA) is cleaved by RNAIII-like endonuclease (named Dicer) into approximately 21- to 23-nucleotide (nt) siRNA, which is incorporated into the RNA-induced silencing complex (RISC) and activates the antiviral RNAi for viral RNA degradation. In mammalian cells, although the activation of RNAi by synthetic siRNA or short hairpin RNA (shRNA) is widely used as a tool for gene knockdown

and antiviral treatment, the RNAi-mediated antiviral mechanism has been debated for a long time (3), because the interferon (IFN) response of the innate immune system is well known as the dominant antiviral mechanism (4). However, more and more evidence has provided strong support for the existence of a natural RNAi-mediated antiviral response in mammals (5). Moreover, recent studies showed that in undifferentiated cells and immature mice, the RNAi-mediated antiviral response is essential (6-8).

To overcome the RNAi-mediated antiviral defense, viruses have evolved to encode viral suppressor of RNA silencing (VSR) (9, 10). For example, in plant viruses, rice hoja blancavirus NS3, tombusvirus P19 and tomato aspermy virus 2b bind to long dsRNA or siRNA to block RNAi (11-13). Turnip crinkle virus (TCV) P38 and cauliflower mosaic virus (CaMV) P6 disrupt the components of RNAi machinery (14, 15). In insect viruses, flock house virus (FHV) B2 blocks RNAi by dsRNA binding (16, 17), and Wuhan nodavirus (WhNV) B2 was identified as VSR by targeting both dsRNAs and Dicer-2 (18, 19). Though the majority of VSRs have been identified in plant and invertebrate viruses, several mammalian viruses were shown to encode VSRs. For instance, Ebola virus (EBOV) VP35, influenza A virus NS1, vaccinia virus E3L and Nodamura virus (NoV) B2 act as VSRs by binding dsRNA (20-23). Hepatitis C virus (HCV) core and HIV-1 Tat block RNAi by inhibiting the activity of Dicer (24, 25). Interestingly, all VSRs identified from mammalian viruses possess IFN or protein kinase R (PKR) antagonistic properties and are essential for replication and pathogenesis, suggesting that RNAi and other innate antiviral responses are

interrelated (26-28).

Coronaviruses (CoVs) are the largest positive single-strand RNA (+ ssRNA) viruses carrying an RNA genome of 26.2-31.7 kb that infect a wide range of mammalian and avian species (29). It is reported that coronaviruses generate significant amount of dsRNAs as replicative and transcriptive intermediates (30, 31). Therefore, it might be the target of Dicer and thus induce RNAi-mediated antiviral responses. An indirect evidence was shown that severe acute respiratory syndrome coronavirus (SARS-CoV) accessory protein 7a was identified as a VSR (32). However, 7a protein is not essential for viral replication and transcription at least in cell culture and tested animal models and is unique to SARS-CoV (33, 34). Consequently, it is interesting to know whether there is another VSR commonly encoded among coronaviruses family. In this study, we screened the viral proteins of SARS-CoV as a representative by the reversal-of-silencing assay and identified the nucleocapsid (N) protein as a novel VSR, which is conserved and encoded by all the coronaviruses.

N protein is a basic protein (with typical pIs of ~10) and has nonspecific binding activity toward nucleic acids, including ssRNA, single-stranded and double-stranded DNA (35, 36). It encapsulates viral genomic RNA (gRNA) to protect the genome and enters the host cell together with the viral RNA to facilitate its replication (37-40). Furthermore, we have reported that the N protein antagonizes IFN-β by targeting the initial pattern recognition receptor (PRR)-RNA-recognition step and the C-terminal domain (CTD) is critical for this antagonism (41). Other studies also revealed that the SARS-CoV N protein contains two distinct RNA-binding domains (N-terminal

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domain, NTD and CTD) linked by a poorly structured linkage region (Linker) containing a serine/arginine-rich (SR-rich) domain (SRD) (42-45). The CTD spanning residues 248-365 shows stronger nucleic acid-binding activity than the NTD (36, 45, 46) and the basic region between residues 248-280 of CTD forms a positively charged groove that represents a likely binding region for RNA (46). Here, we demonstrated that N protein of coronaviruses could efficiently inhibit Dicer-mediated dsRNA cleavage and post-Dicer activities by sequestering dsRNAs and siRNAs. Furthermore, we showed that N protein deficient in the RNAi inhibition activity was unable to promote the replication of mouse hepatitis virus (MHV) comparing with the wild type N protein and knockdown of Dicer1 or Ago2 enhanced the MHV replication. Our studies identified a novel coronaviral VSR and provide new evidence on the existence of RNAi-mediated antiviral response in mammalian cells.

Materials and Methods

Plasmids and RNAs

For RNAi suppression assays in mammalian cells, the plasmid eGFP-C1 (Clonetech) was used to express the enhanced green fluorescent (eGFP) protein. The eGFP specific shRNA (shGFP) and control shRNA targeting luciferase (shLuc) with the indicated sequences in Table S1 were cloned to vector pSuperRetro driven by H1 polymerase III promoter. The plasmid pCMV-tag2b-N expressing SARS-CoV N protein and the deletion mutants with flag tag were constructed in our previous work (41). The open reading frame 6 (ORF 6) expression plasmid with HA tag was kindly

provided by Dr. Stanley Perlman. Point mutations were introduced into the N coding region by PCR mediated mutagenesis, with appropriate primers containing the desired nucleotide changes (Table S2) and subsequently selected by Dpn1 digestion. The coding sequences of MERS-CoV N protein was chemically synthesized and cloned into the pCMV-tag2b vector. Plasmids expressing N protein of coronaviruses MHV, PEDV and TGEV were gifts from Dr. Shaobo Xiao. The NoV B2 expressing plasmid with myc tag was provided by Dr Christopher S. Sullivan. For RNAi suppression assays in Drosophila S2 cells, the eGFP reporter gene and the FHV B2 were constructed into the insect expression vector pAc5.1/V5-HisB. SARS-CoV N protein and ORF6 were inserted into the EcoRI/NotI sites of pAc5.1/V5-HisB. Nonstructural protein 14 (nsp14) cloned in pAc5.1/V5-HisB were inserted in to the NotI/XhoI sites. The primers are shown in Table S2. Full-length cDNA of FHV RNA1 and RNA1-ΔB2 (T2739C and C2910A) were described previously (17). In addition, the siRNAs targeting eGFP (siGFP) were prepared by chemical synthesis (Invitrogen) while siRNAs targeting mouse Dicer1 and Ago2 were ordered in QIAGEN. The oligonucleotides are shown in Table S3. The 244-bp dsRNA for eGFP silencing in Drosophila S2 cells was generated by in vitro transcription using MEGAscript Kits (Ambion).

Cell culture and transfection

Human embryonic kidney 293T cells (HEK293T), mouse Neuro-2a cells (gifts from Dr. Yan Zhou) and L2 cells (gifts from Dr. Rong Ye) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml

144 penicillin and 100 μg/ml streptomycin. Drosophila S2 cells were cultured in semi
145 suspension at 27°C in Schneider's insect medium (Gibco, Carlsbad, CA)
146 supplemented with 10% fetal bovine serum (Gibco) (18). HEK293T cells were seeded
147 on 12-well dishes and grown overnight to reach 50% confluence followed by
148 transfection with standard calcium phosphate precipitation method. Transfection of
149 Drosophila S2 cells was conducted by using FuGene HD reagent (Roche, Basel,
150 Switzerland) when the cells were grown to reach 80% confluence, according to the
151 manufacturer's protocol. Neuro-2a and L2 cells were seeded on 12-well dishes and
152 grown overnight to reach 1×10^6 followed by Lipofectamine 2000 (Invitrogen)
153 transfection. In dose-dependent experiments, empty control plasmid was added to
154 ensure that each transfection received the same amount of total DNA.

155 **Western blotting analysis**

156 Cells were harvested in cell lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl,
157 1% NP40, 0.25% deoxycholate and a protease inhibitor cocktail (Roche)], and the
158 extracts were then subjected to SDS-PAGE and Western blotting, according to our
159 standard procedures (47).The blots were exposed to luminescent image analyzer
160 LAS4000 (FUJIFILM). The antibodies used in this study were as follows:
161 anti-β-actin (Proteintech Group), HRP conjuncted anti-eGFP (Santa Cruz
162 Biotechnology; 1:2000), anti-Flag and anti-HA (Sigma; 1:5000), and anti-Myc
163 (Roche; 1:2000).

Northern blotting analysis

Total RNA was extracted from cells using TRIzol reagent (Invitrogen), according to the manufacturer's protocol. For eGFP mRNA detection, 20 µg RNA was subjected to electrophoresis in 1.2% denaturing agarose gels containing 2.2M formaldehyde. The separated RNAs were transferred onto a Hybond N+ nylon membrane (GE Healthcare, Waukesha, WI) and then cross-linked by exposure to UV light. For siRNA detection, 10 µg low-molecular-weight RNAs extracted from cells using RNAiso (Takara) were separated on a 12% polyacrylamide gel with 7 M urea, and transferred to Hybond N+ nylon membranes by electro blotting using a semidry blotting apparatus. The hybridization with DIG-labeled probes and DIG chemiluminescent detection were conducted with DIG Northern Starter Kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instruction. The blots were exposed to luminescent image analyzer LAS4000 (FUJIFILM). The probe for detection of eGFP mRNA was complementary to the eGFP ORF region of nucleotides 1 to 500 (for experiments in mammalian cells) or 501 to 720 (for experiments in insect cells). The probe for detection of FHV RNA1 and subgenomic RNA3 specifically targets to the B2 coding region 2738 nt -3058 nt. For eGFP shRNA and siRNA detection, the sense sequence of eGFP was used to probe the antisense moiety of shRNA and siRNA of eGFP. All probes were labeled with digoxigenin (DIG)-UTP by in vitro transcription using DIG Northern Starter Kit. The templates were made from PCR amplification or annealing with oligonucleotides listed in Table S1. Ribosome RNAs (rRNAs) or

low-molecular-weight RNAs were visualized by staining with ethidium bromide.

Expression and purification of recombinant proteins

The coding sequences of SARS-CoV N protein and WhNV B2 were PCR amplified and inserted into the BamHI/NotI sites of pGEX-6P-1. BL21 *Escherichia coli* (Invitrogen) transformed with the expression plasmids were grown to the log phase and induced with 0.6 mM isopropyl-β-D-thiogalactopyanoside (IPTG) followed by incubation at 16°C for 12 hr. After harvest by centrifugation, the bacterial pellet was lysed with the lysis buffer [50 mM Tris-HCL (PH 8.0), 150 mM NaCl, 1mM EDTA, 1mM DTT 0.1 mg/ml lysozyme, 0.05% NP40] and the recombinants proteins were purified with glutathione resin (GenScript) according to manufacturer's instruction and stored at -80 °C.

Gel shift assay and RNase III-mediated cleavage assays

244-bp DIG-labeled dsRNA, 500 bp dsRNA and 500 nt ssRNA were generated by in vitro transcription using DIG RNA Labeling Mix (Roche). Gel shift assays for RNA binding were performed using 15μM of GST, 15μM of GST-WhNV B2 or increasing concentrations of GST-N up to 15 μM and 0.2 pmol of DIG-labeled RNAs in a 20 μl reaction system containing 50 mM Tris-HCL (PH 7.4), 75 mM NaCl, 1 mM EDTA, 1 mM DTT and 20 U RNA inhibitor (Fermentas). After incubation for 30 min at 25°C, reaction mixtures were separated on 1.2% Tris-borate-EDTA (TBE)-agarose gel and subjected to Northern blotting for DIG signal detection. In gel shift assay for siRNA binding, 0.2 pmol 5'Hex labeled siRNA was incorporated to the reaction. The reaction

mixtures were separated on 4% native polyacrylamide gel electrophoresis following by fluorescent detection with Typhoon 9200 (Amersham Biosciences).

RNase III cleavage inhibition assay were conducted using DIG-labeled 500 bp dsRNA and RNase III (Invitrogen) as described (19). The assay was performed in 20 µl reaction system containing 0.2 pmol DIG-labeled 500 bp dsRNA, 2 µl 10× RNase III reaction buffer (Invitrogen) and 15 µM GST, GST-WhNV B2 or GST-N. After 30 min of preincubation at 25°C, 1 U of RNase III was added and the reaction mixtures were incubated at 37°C for 30min. Reaction products were resolved by 1.2% TBE-agarose gel electrophoresis and then subjected to Northern blotting for DIG signal detection.

Sequence alignment and analysis of coronavirus N protein

The Clustal X program V2.0 was used to align the sequences of coronavirus N protein N. The resulting file was transferred to GENDOC to prepare for the graphic figures. Sequences for SARS-CoV N proteins were collected from the following genome sequences: SARS-CoV, strain Tor2, NC_004718; MERS-CoV-Jeddah-human-1, KF958702.1); Bat-CoV strain HKU9-1 BF_005I, NC_009021; MHV-A59, NC_001846; IBV strain Beaudette, NC_001451; TGEV strain PUR46-MAD, NC_002306; PEDV Strain CV777, NC_003436; H-CoV strain 229E, NC_002645.

Virus infection, and Real-time PCR

24 hrs or 48 hrs post transfection, Neuro-2a cells or L2 cells were infected with MHV strain A59 at an MOI of 0.1. At 16 hrs post infection, supernatants were collected and

the virus titer was determined by plaque assay on L2 cells (48). For mRNA detection, total RNA was isolated from cells with TRIzol reagent. The RNA was reverse transcribed to first strand cDNA using M-MLV reverse transcriptase (Promega). The SYBR Green master mix (Roche) was used for real-time PCR. Mouse Gapdh mRNA was used as internal control. The primers used in this study were shown in Table S3.

Results

Identification of coronaviral N protein as a VSR in mammalian cells

To evaluate whether coronaviruses encode a VSR, we screened the proteins encoded by SARS-CoV using the reversal-of-silencing assay in HEK293T cells (Fig. 1A, 1B and 1C). The NoV B2, a well known VSR in mammalian cells (23), was used as a positive control. The expression level of enhanced green fluorescent protein (eGFP) reporter gene was tested by Western blotting and fluorescence microscopy, and the relative eGFP reversion activities of each protein towards NoV B2 were calculated. As shown in Fig. 1E, SARS-CoV N protein could efficiently revert the expression of RNAi-silenced eGFP as well as NoV B2. In contrast, the accessory protein 7a, which was reported as a VSR of SARS-CoV (32), showed extremely limited VSR activity in the screening assays. We detected the protein expression level of some constructs including nsp7-16, N, 7a, ORF6, M and B2, and the results showed that most of them were readily detectable but at variable levels (Fig. 1D). Therefore, the VSR activity and its strength observed in the initial screening were not conclusive and had to be verified by further systematic experiments. Because SARS-CoV N showed relatively

248 higher VSR activity in the reversal-of-silencing assay, we continued to characterize
249 SARS-CoV N as a potential VSR.

250 To confirm the RNAi suppressor activity of SARS-CoV N protein, we assayed the
251 capability of N protein in suppressing shRNA-induced eGFP silencing at protein and
252 mRNA level. The eGFP specific shRNA (shGFP) caused a strong decrease of eGFP
253 expression (Fig. 2A and 2B) and transcription (Fig. 2C) compared to the irrelevant
254 shRNA that targets luciferase (shLuc). The nsp14 with non-specific RNA binding
255 activity (49) and ORF6 protein with IFN antagonistic activity (50) were used as
256 negative controls. Notably, transfection of the N expression plasmid remarkably
257 resurrected the expression of RNAi-silenced eGFP at both protein and mRNA level as
258 well as B2, but ORF6 and nsp14 could not (Fig. 2A, 2B and 2C), indicating that N
259 suppressed the shRNA-induced RNA silencing of eGFP. Importantly, N protein did
260 not affect the expression efficiency of eGFP in the absence of shRNA (Fig. 2D and
261 2E), confirming that N protein inhibited the effects of shGFP-mediated RNAi rather
262 than promoted eGFP transcription or translation. To exclude that the observed effects
263 specifically exists in the eGFP reporter system, the RNAi suppression activity of N
264 protein was determined in an endogenous RNAi system (Fig. 2F). We demonstrated
265 that the presence of N protein restored the endogenous VHL expression silenced by
266 VHL-specific shRNA (shVHL), whereas the VHL expression level was not affected
267 in mock vector (pLko.1) transfected cells (Fig. 2F). Since the RNAi pathway is
268 conserved from plants to animals, some VSRs of mammalian viruses are also
269 functional in insect cells such as influenza A virus NS1 and vaccinia virus E3L (22).

We further confirmed the VSR activity of SARS-CoV N protein using FHV RNA1 replication system (Fig. 2G). Transfection of pRNA1 led to the self-replication of FHV RNA1 and the transcription of RNA3 in S2 cells. FHV B2 was well known to inhibit RNA silencing induced by virus RNA replication. B2-deficient mutant (pRNA1-△B2) failed to accumulate FHV RNA1 and RNA3. This defect could be partially rescued by co-transfection with plasmids either expressing SARS-CoV N protein or FHV B2 (Fig. 2G). To explore whether the RNAi inhibition activity of N protein is universal among coronaviruses, the N proteins from alpha coronavirus (Porcine epidemic diarrhea virus, PEDV; Transmissible gastroenteritis virus, TGEV) and beta coronavirus (SARS-CoV; Middle east respiratory syndrome coronavirus, MERS-CoV; MHV) were tested. As shown in Fig. 2H, the indicated coronavirus N proteins inhibited shRNA-induced RNAi in varying degrees in the reversal-of-silencing system, where, MERS-CoV N showed notably low VSR activity.

To investigate whether the VSR activity is dependent on the protein expression levels, the increasing amount of SARS-CoV N was tested using the reversal-of-silencing assay (Fig. 3A and 3B). The reversal effect of eGFP silencing increased progressively both at protein and mRNA level along with the gradual increase of the transfected N-expressing plasmid (Fig. 3A). We also detected the eGFP expression at different time points, and the results showed that reversal of eGFP silencing could be observed as early as 24 hrs post transfection and was more effective at 48 and 72 hrs (Fig. 3C), indicating that the VSR activity was dependent on the expression level of SARS-CoV

N protein.

N protein inhibits Dicer-mediated siRNA generation and siRNA-induced RNAi

The shRNA-induced RNAi pathway requires Dicer-mediated dsRNA cleavage into siRNA. To find out whether N protein blocks the aforementioned step, small RNAs harvested from HEK293T cells were subjected to Northern blotting analysis with DIG-labeled probes which recognized both shRNA and siRNA (Fig. 4A and 4B). In the presence of SARS-CoV N protein, an increase in the ratio of shRNA to 21-nt Dicer-processed siRNA was detected. However, the increase was more obviously in the presence of NoV B2 (Fig. 4A and 4B). This phenomenon is consistent with the eGFP expression and its mRNA level observed in the reversal-of-silencing assays (Fig. 2). To further demonstrate whether N protein could block RNAi induced by siRNA (the post-Dicer product), 40 nM eGFP-specific siRNA (siGFP) was used to induce a remarkable reduction of eGFP expression (Fig. 4C). As shown in Fig. 4D and 4E, the decrease of eGFP expression and its mRNA level in HEK293T cells was partially inhibited in the presence of SARS-CoV N protein comparing with NoV B2. We investigated the capability of RNA silencing repression of N protein in insect cells. Consistent with the phenomenon observed in cells, the presence of SARS-CoV N protein efficiently abolished the eGFP-specific RNAi induced by dsRNA (dsRNA-GFP) while remarkably inhibited the siGFP-induced RNAi in Drosophila S2 cells (Fig. 4F). In conclusion, the N protein could inhibit the RNA silencing in mammalian and insect cells at both Dicer-processing and post-Dicer stages with different efficiency.

N protein directly binds to RNAs and prevents dsRNA from RNase III-mediated cleavage in vitro.

A number of VSRs could sequester RNA duplexes from Dicer cleavage and the incorporation of siRNA into RISC by dsRNA binding activity, such as NoV B2, EBoV VP35 and influenza virus NS1 (20, 22, 23). To investigate the mechanism of N protein as a VSR, we adopted a gel shift assay after the incubation of recombinant GST-tagged SARS-CoV N protein (GST-N) and WhNV B2 (GST-B2) with DIG-labeled 500 nt ssRNA (Fig. 5A), DIG-labeled 244 bp dsRNA (Fig. 5B) and synthetic 5'-HEX labeled 21 nt siRNA (Fig. 5C), which mimic viral ssRNA, cellular pre-Dicer dsRNA and post-Dicer siRNA, respectively. As shown in Fig. 5A to 5C, the shifting amount of all labeled RNAs increased as more SARS N protein was used in the reaction up to 15 μM, while the WhNV B2 could not bind ssRNA. Interestingly, the mobility of labeled ssRNA (Fig. 5A) and dsRNA (Fig. 5B) decreased as more SARS-COV N protein used, except that of labeled siRNA (Fig. 5C), suggesting that one molecule of long RNAs (ssRNA and dsRNA) was bound by the multiple N proteins. Subsequently, we examined the possibility of SARS-COV N protein to protect dsRNA from RNase III-mediated cleavage. RNase III is a Dicer homolog and was widely used as the Dicer substitute *in vitro* as previously reported (51). As shown in Fig. 5D, in gel shift assay, 500 bp dsRNA formed a high-molecular-weight complex with GST-N as well as GST-B2, and therefore was protected from RNase III digestion. However, without the protection of GST-N or GST-B2, dsRNA was digested into 21-to 23-nt siRNA. These data illustrated that N protein could directly

bind dsRNA to prevent the digestion of Dicer and partially sequester siRNA at post-Dicer stages.

Positively charged residues Lys 258 and Lys 262 of SARS-CoV N protein are critical for the RNAi repression activity.

Mutagenesis and alanine scanning analyses were performed to identify the critical domain or amino acid (aa) required for the VSR activity of SARS-CoV N protein. The domain architecture of SARS-CoV N protein is shown in Fig. 6A. Based on domain truncation analysis, we found that the N-terminal (1-181 aa) and the C-terminal (365-422 aa) of SARS-CoV N protein are not required for RNAi inhibition activity (Fig. 6B), suggesting that the functional VSR region might be located at the linkage region (Linker) and CTD of SARS-CoV N protein (182-365 aa). The expression of truncated proteins was evaluated (Fig. 6C). Previous studies revealed that this region possesses an SR-rich domain (182-227 aa) and a positively charged groove (248-280 aa) (Fig. 6A), which might employ positively charged residues arginine and lysine to mediate electrostatic interaction with the phosphate backbone of RNAs (36, 46, 52, 53). Therefore, multiple sequence alignments of the SR-rich domain (data not shown) and the positive charged groove of CTD of coronaviruses N protein (Fig. 6A) were performed, and the single or double mutations of conserved arginine and lysine were analyzed by the reversal-of-silencing assay in HEK293T cells as shown in Fig. 6D and Fig. 6E. Further mutational analysis revealed that mutations of conserved Lys 258 and Lys262 in CTD of N protein (K258A and K262A) completely abolished its ability to repress RNAi induced by shRNA in HEK293T cells (Fig. 6F). Taken together,

these data show that the positively charged residues Lys 258 and Lys 262 are critical for the RNAi repression activity of SARS-CoV N protein, and these results support the importance of RNA-binding activity of protein N in RNAi suppression.

SARS-CoV N protein and NoV B2 promote the replication of coronavirus MHV

To demonstrate the physiologic function of coronaviruses N protein as a VSR *in vivo*, we used the coronavirus MHV strain A59, which belongs to the same virus group as that of SARS-CoV, as a model to investigate the influence of N protein and its mutants on virus replication. We and others have reported that the multiple functional N protein is essential for coronavirus, and the expression of N protein either *in cis* or *in trans* could facilitate the viral replication (40, 54, 55). Therefore, SARS-CoV N protein, N mutants and NoV B2 provided *in trans* were tested in the *in vivo* system. As shown in Fig. 7A to 7D, the MHV titer was up-regulated 3-5 fold by SARS-CoV N protein in a dosage-dependent manner in Neuro-2a cells (Fig. 7A), while the VSR inactive mutant K258/262A failed to promote the replication of MHV (Fig. 7B). In contrast, the VSR activity irrelevant mutant K267A (Fig. 6E) exhibited similarly promotion as wild type N protein (Fig. 7B). Impressively, the typical VSR NoV B2 could also facilitate the replication of MHV (Fig. 7B), further suggesting that the increase of MHV replication may be due to the general suppression of RNAi in host cells. The MHV titers were checked by the plaque assays in L2 cells (Fig. 7D). These results were also confirmed by analyzing the synthesis of viral gene 7 mRNA (mRNA 7) using RT-PCR in Neuro-2a cells (Fig. 7C), and the expression of corresponding proteins were evaluated (Fig. 7E). As shown in Fig. 7D and 7E, the effect of

SARS-CoV N protein on the enhancement of MHV growth was also dose-dependent, consistent with the previous observation (Fig. 3A and 3B). In the above assays, the steady-state protein level of K258/262A mutant was similar to that of wild type N protein with the transfection dosage of 300 ng DNA (Fig. 7E); however, the mutant K258/262A could not promote MHV growth, but 300 ng wild type N protein could still significantly enhance virus replication (Fig. 7A, 7D and 7E). Therefore, the deficiency of K258/262A mutant in VSR activity was not due to reduced expression level.

Coronaviruses N proteins have been reported to have IFN-β response inhibition activity in IFN induction systems (41, 50, 56), suggesting that N might also promote MHV replication through antagonizing IFN responses in host cells. However, previous studies on coronavirus infections showed that MHV induces a minimal type I IFN response in several cell types, and the expression of IFN-β is undetectable (57-60). When Neuro-2a cells were treated with dsRNA analog poly (I:C), low level of IFN-β was produced, but MHV infection did not induce expression of IFN-β (Fig. 7F). We also analyzed the transcription of the *Ifn-β* mRNA and its downstream gene *Isg56* mRNA that represent the expression level of type I IFN. As shown in Fig. 7G and 7H, the mRNA level of *Ifn-β* and *Isg56* are neither reduced compared with Nov B2 and vector control nor significantly changed with N protein and its mutants, which ruled out the possibility that the N protein increased MHV production by antagonizing IFN-β signaling pathway. These results demonstrated that SARS-CoV N protein could promote the virus replication independently of IFN signaling pathway and the

critical residues Lys 258 and Lys 262 of SARS-CoV N protein for VSR activity are involved in facilitating MHV replication by inhibiting the RNAi-mediated antiviral mechanism. Moreover, the irrelevant viral protein NoV B2 could also promote the replication of MHV, indicating that the MHV replication might be sensitive to the RNAi-mediated antiviral response.

Knockdown of either Dicer1 or Ago2 facilitated the replication of MHV

To further investigate the function of RNAi-mediated antiviral response when the IFN-mediated antiviral response is highly repressed by coronaviruses, the Dicer1 and Ago2 transcripts in mouse Neuro-2a and L2 cells were knocked down by synthetic siRNAs (siDicer1 and siAgo2). As shown in Fig. 8, the mRNA levels of *Dicer1* and *Ago2* were markedly reduced in cells transfected with siDicer1-6 and siAgo2-4, respectively (Fig. 8A and Fig. 8C), while the *Ifn-β* mRNA was not significantly affected (Fig. 8B and Fig. 8D). The RNAi deficient Neuro-2a cells were still competent in IFN production (Fig. 9A), indicating that RNAi depletion did not non-specifically abolish IFN pathway. In RNAi-deficient Neuro-2a cells, either in the presence or absence of SARS-CoV N protein, the replications of MHV were increased to the same level as in RNAi pathway healthy cells provided with SARS N protein (Fig. 9B and 9C). Accordingly, there was no reduction in the mRNA level of *Isg56* observed in the presence of SARS-CoV N (Fig. 9D), indicating that the increase of MHV replication was affected by the attenuated RNAi pathway but not by suppression of type I IFN response. In fact, the mRNA level of *Isg56* increased to some extent whereas the replication of MHV was still promoted in Ago2-knockdown

cells (Fig. 9B to 9D), strongly suggesting that RNAi knockdown was involved in these cells. Moreover, the differences of MHV replication promotion between wild-type SARS-CoV N protein, NoV B2 and mutant K285/262A of SARS-COV N protein observed in Neuro-2a cells (Fig. 7) were eliminated in RNAi knockdown cells by siDicer1 or siAgo2, respectively (Fig. 9E, 9F, 9H and 9I), while expression of these proteins did not result in different IFN responses (Fig. 9G and 9J). The VSR activity was also analyzed in L2 cells that are highly IFN-sensitive. Both healthy and RNAi pathway-deficient L2 cells could produce high level of IFN-β (up to 10000 pg/ml) when infected by Sendai virus (SeV), but the production of IFN-β was still undetectable when infected by MHV (Fig. 9K). In RNAi-competent L2 cells (treated with siGFP), N protein could significantly increase MHV growth (Fig. 9L). Interestingly, when the RNA pathway was depleted by siDicer1-6 or siAgo2-4, the increment of MHV growth was kept in the absence of N or in the presence of VSR-deficient mutant K258/262A (Fig. 9L). Together, the observations in L2 cells indicate that either suppression of RNAi by SARS-CoV N or depletion of RNAi pathway by siRNAs could lead to increment of MHV growth. These results were consistent with that of Neuro-2a cells.

Taken together, these results demonstrate that SARS-CoV N protein is a novel VSR and the RNAi pathway was involved in anti-coronavirus response in mammalian cells.

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Discussion

Mammalian RNA viruses have evolved many mechanisms to protect their genomic RNAs and dsRNAs generated as replicative and transcriptive intermediates (30, 31, 59, 61) from host cell recognition. For examples, they associate the replication-transcription complex (RTC) with the double membrane vesicles (DMV) to form a protective microenvironment which prevents viral RNA from being detected by host cell sensors (62, 63); encode several RNA processing enzymes to undergo RNA modifications such as RNA capping to mimic the host cell RNAs (49, 64); and adopt RNA binding proteins to protect viral RNAs (65, 66). However, virus-derived small RNAs were still detectable in 41 human cell lines infected with 6 different RNA viruses respectively (5). Moreover, several reports showed that virus-specific siRNAs accumulated in infected mammalian cells, and recently the specific siRNAs of Encephalomyocarditis virus (EMCV) and Nodamura virus (NoV) were deep sequenced (6, 7). Therefore, in mammalian cells which possess functional RNAi pathway, the sequence-specific RNA degradation would still threaten the abundant viral mRNA in cytoplasm if without VSR during virus infection, especially for coronavirus, the largest RNA virus as known. In this study, we showed the evidence that coronaviruses also possess VSR, which was identified as N protein that shares similar structural architecture and gRNA/subgenomic RNA (sgRNA) binding activity to enhance the viral transcription, replication and assembly among coronaviruses (37, 38, 40, 65, 66). It is abundantly produced in infected cells to support its multiple functions as well as VSR. Besides the ssRNA binding activity, we showed that N

protein also possesses the dsRNA and siRNA binding activities. Moreover, SARS-CoV N protein could bind more efficiently with long ssRNA and dsRNA than siRNAs (Fig. 5), which may be resulted from cooperative binding of multiple N protein monomers. Coronavirus possess the largest single stranded genomic RNA and abundant subgenomic RNAs and dsRNA intermediates (29-31, 61). Therefore, we propose a hypothesis that the coronaviruses N protein might protect viral RNA from RNAi-mediated gene silencing at three stages (Fig. 10): (1) binding viral ssRNAs to prevent the positive- and negative-sense genomic or subgenomic RNAs from formation of unnecessary intramolecular and intermolecular dsRNA; (2) shielding virus-derived dsRNA from Dicer cleavage through dsRNA binding activity; (3) binding to virus-derived siRNA to interfere with RISC assembly.

In this study, we showed that the VSR activity was highly dependent on the protein expression levels (Fig. 3A and 3B; Fig. 7D and 7E). In the initial screening assays for putative VSRs (Fig. 1), we did not evaluate the protein expression levels of different constructs, and therefore effects observed in the reversal-of-RNA silencing could not be directly compared and may result in either false-positive or false-negative outcomes. For example, SARS-CoV 7a, previously reported as a VSR (32), did not show apparent VSR activity in our assay system. Notably, the MERS-CoV N protein expression level is higher than that of SARS-CoV N protein, while the VSR activity of MERS-CoV N protein is less than that of SARS-CoV N protein (Fig. 2H). These results suggest that the N proteins of coronaviruses may have different intrinsic activities in suppressing RNAi. We speculate that the low VSR activity of

MERS-CoV N might be compensated by a second VSR and one potential candidate could be the dsRNA-binding IFN antagonist protein 4a (67).

Previous studies revealed that the three distinct and highly conserved domains of N proteins (NTD, SRD and CTD) could bind with viral RNAs in different coronaviruses (42, 66, 68, 69). The NTD has been found to associate with a stem-loop structure located at the 3' end of RNA genome, and the CTD was involved in the specific binding of coronaviral packaging signal within nonstructural protein nsp15 region (43, 70, 71). The critical residues Lys 258 and Lys 262 of SARS-CoV N protein for VSR identified in current study are located in the positive charged groove of CTD (Fig. 6). Moreover, Lys 258 was previously reported as a significant determinant of SARS-CoV N protein CTD binding affinity towards oligo nucleotides (36). Here, we chose SARS-CoV N protein to promote MHV because SARS-CoV is closely related to MHV on one side while the CTD of SARS-CoV N protein cannot enhance the specific selective packaging of MHV gRNA to facilitate the MHV replication (71). Therefore, that the VSR inactive mutant K258/262A of SARS-CoV N protein led to the attenuation of MHV replication might imply that the sufficient VSR activity is important for the coronaviruses replication.

Although the IFN-mediated antiviral response is important for mammalian cells, a lot of studies have indicated that MHV is a poor inducer of type I IFN response (59). Moreover, MHV is resistant to the pretreatment of IFN-α/β and does not induce the generation of IFN-β in various cell types, suggesting that it can suppress IFN signaling pathway or its downstream gene effects at multiple level (56, 58). In this

study, we showed that MHV infection did not induce the production of IFN-β in both mouse Neuro-2a and L2 cells (Fig. 7F and 9A). In primary cells, IFN is induced by MHV infection in plasmacytoid dendritic cells (pDC) and macrophages (57, 72, 73), but not in neurons, astrocytes and hepatocytes (57). Therefore, we used coronavirus MHV strain A59 as a replication model, which might to some extent avoid the impact of IFN response, to investigate the influence of N protein and its mutants on virus replication. Here we demonstrate that SARS-CoV N protein could promote MHV replication both in Neuro-2a cells and L2 cells, the former being low responsive to IFN induction while the latter highly sensitive (Fig. 7 and Fig. 9). We also test the VSR activity of N protein in IFN-deficient insect cells (Fig. 2G). These results indicate that the VSR activity of SARS-CoV N protein is independent of either cell type or interferon pathway. Moreover, the depletion of critical components Dicer1 or Ago2 of the RNAi pathway in Neuro-2a and L2 cells results in significant upregulation of MHV replication, which was not dependent on the changes of interferon response (Fig. 9), further strengthen the role of SARS-CoV N protein in IFN-independent RNAi suppression.

In the study of Schelle and colleagues (74), the observed role of CoV N protein in the trans-complementation is most likely related to the virus recovery step before viral replication, whereas in our experimental system, we are dealing with a wild type virus that does not require trans-complementation for virus recovery, and thus the role of SARS-CoV N protein we observed is its effect on virus replication. The modest increment of MHV replication by SARS-CoV N protein suggests that the natural N

protein of MHV may already exert rather effective VSR function and exogenous SARS-CoV N protein may further increase the anti-RNAi effect. Interestingly, NoV B2, which has no correlation and structural or sequence similarities with SARS-CoV N, exhibited the same ability in stimulating MHV replication. These results indicated that the MHV replication is sensitive to the RNAi-mediated antiviral response. However, there has been no direct evidence for existence of CoV-derived siRNAs in infected cells. Generation of a VSR-defective MHV mutant may help to detect MHV-derived siRNAs in mammalian cells. Currently, viral siRNAs have been discovered in cell culture for 6 RNA viruses and in vivo for Encephalomyocarditis virus (EMCV) and NoV (5-7). Our studies provide new evidence for the functional RNAi-mediated antiviral response in addition to the IFN-mediated innate immunity in mammalian cells. These two important antiviral mechanisms might work in turn in different situations and locations to defense the virus infection, which constantly provide a positive selection toward VSR and viral IFN-antagonist during the tachytelic evolution of viruses.

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766 **Figure Legend**

767 **Fig. 1 Screening of potential VSR of SARS-CoV by reversal-of-silencing assays.**
768 293T cells were co-transfected with plasmids encoding eGFP reporter (125 ng),
769 eGFP specific shRNA (shGFP) (1 μg) or luciferase specific shRNA (shLuc) (1 μg)
770 and viral protein of SARS-CoV (500 ng), respectively. (A to C) The expression level
771 of eGFP was determined by Western blotting 72 hrs post transfection. Empty vector
772 (Vec) and NoV B2 (B2) were used as mock control and positive control. The shLuc
773 was irrelevant silencing control. β-actin was used as loading control. (D) The
774 expression of SARS-CoV-encoded proteins as indicated was detected by Western
775 blotting. (E) The relative eGFP reversion activity of different viral proteins in (A to
776 C) was normalized by that of typical VSR NoV B2 control and was shown in the bar
777 diagram.

778 **Fig. 2 N protein of coronaviruses represses shRNA-induced RNAi in**
779 **mammalian cells.** (A) to (C) HEK293T cells were co-transfected with plasmids of
780 125 ng encoding eGFP reporter, eGFP-specific shRNA (shGFP) or
781 luciferase-specific shRNA (shLuc) of 1 μg and Flag-tagged SARS-CoV N, nsp14,
782 ORF6 or Myc-tagged NoV B2 of 300 ng respectively. The expression of eGFP
783 reporter was analyzed 72 hrs after co-transfection. (A) The intensity of eGFP was
784 observed under fluorescence microscopy. (B) Cell lysates were harvested and
785 analyzed by Western blotting. (C) Cellular total mRNAs were harvested and
786 analyzed by Northern blotting. (D) to (E) HEK293T cells were co-transfected with

plasmids encoding eGFP and SARS-CoV N protein or NoV B2 respectively. EGFP fluorescence, protein and mRNA level were determined by fluorescence microscopy (D), Western blotting and Northern blotting (E), respectively. (F) HEK293T cells were co-transfected with plasmids of mock vector pLko.1 (1 μg) or shRNA targeting endogenous gene VHL (shVHL) in the presence or absence of SARS-CoV N protein (300 ng). 72 hrs post transfection, cells were harvested and subjected to Western blotting to determine the endogenous VHL expression. (G) S2 Cells were transfected with 0.03 μg pFR1 or 0.6 μg pFRNA1-ΔB2 and with SARS-CoV N or FHV B2 as indicated above. 48 hrs post transfection, FHV RNA transcription was induced by incubation with $CuSO_4$ at 0.5 mM. 24 hrs after induction, cellular total mRNA was harvested for Northern blot analysis by a probe recognizing FHV RNA1 and 3. The band between RNA1 and RNA3 represents the mRNA transcribed from B2 expression plasmid. (H) HEK293T cells were co-transfected with plasmids encoding eGFP, shLuc or shGFP and N proteins of different coronaviruses (Flag-tagged N proteins of SARS-CoV and MERS-CoV, and HA-tagged N proteins of MHV, PEDV, and TGEV), respectively. The eGFP expression level and mRNA level were determined by Western blotting and Northern blotting. Empty vector (Vec, Vec1 or Vec2), nsp14 and ORF6 were used as negative controls while NoV B2 as positive control. The shLuc was irrelevant control shRNA. β-actin and ribosome RNAs (rRNAs) were used as loading controls for Western blotting and Northern blotting, respectively.

Fig. 3 N protein represses shRNA-induced RNAi in a dose-dependent and

time-dependent manner in mammalian cells. Increasing amount of the plasmid expressing SARS-CoV N protein was transfected into HEK293T cells as indicated in the upper panel in the reversal-of-silencing assay. 72 hrs after transfection, (A) Western blotting and (B) Northern blotting were performed to determine the eGFP protein and mRNA level, respectively. (C) Northern blotting (upper panel) and Western blotting (bottom panel) were performed 24 hrs, 48 hrs and 72 hrs post transfection, respectively.

Fig. 4 N protein inhibits the production of siRNA and RNAi in both mammalian and insect cells. (A) HEK293T cells were co-transfected with plasmid as indicated above, 72hrs after transfection, small RNAs were harvested from the cells and probed with DIG-labeled oligonucleotides that correspond to the target sites of siRNA produced from shGFP. Locations of bands corresponding to shRNA and siRNA are indicated with cartoons on the right side. Short exposure and long exposure are shown on the left and right, respectively. An ethidium bromide-stained gel of low-molecular-weight RNA is shown as a loading control. Neg. represents the mock control transfected with shLuc. (B) The ratio of shRNA to siRNA in (A) were quantified based on the corresponding exposure signals and shown in the bar diagram. (C) EGFP expression plasmid (200 ng) was co-transfected with multiple concentrations (5nM to 40 nM) of synthetic eGFP specific siRNA (siGFP) to confirm the effective siGFP concentration in HEK293T cells. 72 hrs after transfection, cell lysates were harvested to determine the reduction in eGFP expression by Western blotting. (D) to (E) SARS-CoV N protein inhibits siRNA

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induced RNAi. HEK293T cells were transfected with plasmids as indicated above. 72 hrs after transfection, eGFP fluorescence (D), protein (E) and mRNA (F) were detected as described in Fig. 2. (F) SARS-CoV N protein inhibits RNAi in Drosophila S2 cells. EGFP specific dsRNA (dsRNA-GFP) and siGFP were used to induce RNAi in S2 cells. The mRNA of eGFP was detected by Northern blotting. Empty vector (Vec), nsp14 and ORF6 were used as negative controls. NoV B2 and FHV B2 were used as positive control. Rp49 was used as a loading control.

Fig. 5 N protein binds to RNAs and inhibits the Dicer-like RNase III cleavage reaction in vitro. Increasing amount of purified GST-tagged SARS-CoV N protein (GST-N) from 0 to 15μM was incubated with 0.2 pmol 500 nt DIG-labeled ssRNA (A) or 244 bp DIG-labeled dsRNA (B) at 25ºC for 30 min respectively. Complexes were separated on 1.2% TBE-agarose gel and subjected to Northern blotting. The free ssRNA and dsRNA are indicated at the left side. (C) GST-N up to 15μM was incubated with 0.2 pmol 5'-HEX labeled 21 nt siRNA as described in (A and B). Complexes were applied to 4% native polyacrylamide gel and the fluorescent signal was visualized by Typhoon 9200. The free siRNA is indicated at the left side. (D) DIG-labeled 500 bp dsRNA was incubated with purified proteins as indicated above at 25°C for 30 min before the processing of Dicer-like RNase III at 37°C for 30 min. The reaction products were separated on 1.2% TBE–agarose gel and subjected to Northern blotting. The free dsRNA is indicated at the left side and the cleaved dsRNA is indicated at the right side. The shifted protein-RNA complexes are indicated by black arrows at the right side. The protein GST and GST-tagged WhNV B2 (GST-B2)

853 were used as mock control and positive control.

854 **Fig. 6 Conserved residues LysK258 and LysK262 of SARS-CoV N protein are**
855 **critical for RNAi activity.** (A) The schematic diagram of the domain architecture of
856 the SARS-CoV N protein and multiple sequence alignment of CTD spanning
857 residues 248-281 of coronaviruses N proteins. The conserved residues are indicated
858 by solid black box. NTD, N-terminal domain; CTD, C-terminal domain; SR-rich,
859 rich in serine and arginine; Linker, linkage region. (B) and (C) Mapping of critical
860 residues of SARS-CoV N protein for VSR activity. The RNAi repression activity of
861 Flag-tagged truncations of SARS-CoV N protein were analyzed by
862 reversal-of-silencing assay as described in Fig. 2B. The expression of truncated
863 SARS-CoV N proteins was detected by Western blotting (C). (D) to (F) The RNAi
864 repression activity of Flag-tagged mutants of SARS-CoV N protein were analyzed
865 by the reversal-of-silencing assay as described in Fig. 2. (F) The mutants K257A,
866 K258A, K262A and R263A were further analyzed based on the results from (E).

867 **Fig. 7 SARS-CoV N protein promotes MHV replication when provided in trans.**
868 (A) to (B) Mouse Neuro-2a cells were transfected with plasmids as indicated and
869 infected with MHV strain A59 at multiple of infection (MOI) 0.1 at 24 hr post
870 transfection. 16 hrs after infection, culture supernatants were collected and subjected
871 to plaque assay on L2 cells to determine the MHV titers. The relative titer fold of
872 MHV were quantified and showed in the bar diagram. The actual virus titers are in
873 indicated (A) to show MHV replication efficiency. (C) Total RNAs were extracted
874 from the transfected cells and subjected to RT-PCR using the primers targeting the

875 subgenomic RNA7 of MHV. Data were normalized to the abundance of endogenous
876 mouse Gapdh mRNA. (D) The virus plaque formation was analyzed on L2 cells at a
877 dilution of 10^{-6}. (E) Protein expression levels in transfected cell lysates were detected
878 by Western blotting with indicated antibodies. β-actin was used as a loading control.
879 (F) Neuro-2a cells were infected with MHV strain A59 at an MOI of 0.1 or
880 transfected with 2μg poly (I:C). 16 hrs post infection or transfection, IFN-β
881 production was determined by ELISA. (G) to (H) Total RNAs were extracted from the
882 transfected cells as described in (B) and subjected to RT-PCR to detect mRNA of *Ifn-β*
883 and *Isg56*. Error bars indicate means and standard deviations of triplicate experiments.
884 * $p < 0.05$, ** $p < 0.01$ and no significant (ns) (by unpaired Students's *t*-test).

885 **Fig. 8 Screen for siRNAs targeting mouse Dicer1 and Ago2.** Mouse Neuro-2a
886 cells were transfected with 40 nM siDicer1 (A and B) or siAgo2 (C and D). 48 hrs
887 post transfection, total cellular mRNAs were extracted and subjected to RT-PCR to
888 determine the mRNA levels of Dicer1 (A), Ago2 (C) and Ifn-β (B and D). siGFP was
889 used as the negative control. Data were normalized to the abundance of internal
890 mouse GAPDH mRNA. Error bars indicate means and standard deviations of
891 triplicate experiments.

892 **Fig. 9 Knockdown of Dicer1 or Ago2 facilitated MHV replication.** (A) Mouse
893 Neuro-2a cells were transfected with siRNAs as indicated above, and 48 hrs post
894 transfection, cells were infected with MHV at an MOI of 0.1 or transfected with 2 μg
895 poly (I:C). 16 hrs post infection or transfection，IFN-β production was determined by
896 ELISA. (B) to (J) Mouse Neuro-2a cells were co-transfected with 40 nM siRNA and

500 ng protein expression plasmids as indicated and infected with MHV strain A59 at MOI of 0.1 at 48 hrs post transfection. 16 hrs post infection, culture supernatants were collected and subjected to plaque assays on L2 cells to determine the MHV titers. (B), (E) and (H) The relative titer fold of MHV were quantified and shown in the bar diagrams. Total RNAs were extracted from the transfected cells and subjected to RT-PCR to determine the indicated mRNA levels. MHV RNA7 level was shown in (C), (F) and (I). *Isg56* mRNA level was shown in (D), (G) and (J). (K) L2 cells were transfected with siRNAs as indicated above, and 48 hrs post transfection, cells were infected with MHV or SeV. 16 hrs post infection, IFN-β production was determined by ELISA. (L) L2 was transfected and infected similarly as described for Neuro-2a cells. The MHV titers were determined by plaque assay. Empty vector (Vec) and siGFP were used as the negative control. Data were normalized to the abundance of endogenous mouse Gapdh mRNA. Error bars indicate means and standard deviations of triplicate experiments.

Fig. 10 Model for the suppression of RNAi in mammalian cells by coronaviruses N protein. Following the entry and un coating of coronaviral virions, the single-stranded genomic RNA (gRNA) is protected by N proteins and serves as a template for the synthesis of negative-strand gRNA and a set of subgenomic RNA (sgRNA). The (-) gRNA and (-) sgRNA are replicated to generate full length gRNA and a set of (+) sgRNA. The virus-derived double-stranded RNA (dsRNA) could be generated during the viral transcription and replication. The sgRNA and gRNA sequence may also form intramolecular hairpin structure. These viral dsRNA may be

recognized by Dicer, consequently triggering antiviral RNAi. Coronaviruses N proteins may repress the RNAi at 3 different stages as indicated.