

## The nucleocapsid protein N of the 2019 novel coronavirus can function as a virus-encoded RNAi suppressor

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### Abstract

COVID-19 remains ongoing in Wuhan and other regions of China. Under the current circumstances, further elucidation of SARS-CoV-2 virology and virus-host interactions holds significant importance for infection control and the development of effective therapeutics. RNA interference (RNAi) represents an evolutionarily conserved antiviral immune mechanism in eukaryotes, and numerous viruses have been discovered to encode their own viral suppressors of RNA silencing (VSRs) as countermeasures. In this study, we demonstrate that the nucleocapsid protein (N) encoded by SARS-CoV-2 effectively suppresses RNAi triggered by shRNAs or siRNAs. Moreover, analogous to many VSRs encoded by other viruses, the SARS-CoV-2 VSR interacts with double-stranded RNA (dsRNA) *in vitro* and in human cells, exhibiting dsRNA-binding activity. Collectively, our findings indicate that the SARS-CoV-2 N protein exhibits VSR activity in human cells, which may constitute a key immune evasion factor for this novel coronavirus.

### Full Text

### Preamble

#### SARS-CoV-2-encoded Nucleocapsid Protein Acts as a Viral Suppressor of RNA interference in Cells

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**Key words:** SARS-CoV-2, nucleocapsid, antiviral RNAi, viral suppressor of RNAi

## Abstract

The SARS-CoV-2 outbreak emerged and continues to affect Wuhan and other regions in China and worldwide. Human infections with SARS-CoV-2 cause diseases ranging from mild symptoms to severe pneumonia and even death. In the current situation, a better understanding of SARS-CoV-2 virology and virus-host interactions is vital for controlling infections and developing effective therapies. RNA interference (RNAi) is an evolutionarily conserved antiviral immune mechanism in diverse eukaryotic organisms, and numerous viruses encode viral suppressors of RNAi (VSRs) as countermeasures. In this study, we uncovered that the nucleocapsid (N) protein encoded by SARS-CoV-2 effectively suppressed RNAi triggered by either small hairpin RNAs (shRNAs) or small interfering RNAs (siRNAs) in cultured human cells. Furthermore, similar to VSRs encoded by other viruses, SARS-CoV-2 N protein shows double-stranded RNA (dsRNA)-binding activity, as it interacted with *in vitro* transcribed dsRNAs in human cells. Taken together, our findings showed that SARS-CoV-2 N exhibits VSR activity in human cells, which could serve as a key immune evasion factor for SARS-CoV-2 and contribute to its pathogenicity.

## Introduction

Coronaviruses (CoVs) are large enveloped non-segmented positive-stranded RNA viruses that are broadly distributed among humans and other animal species, including bats, mice, and birds [1, 2]. Among the six pathogenic coronavirus species, CoV-229E, CoV-OC43, CoV-NL63, and CoV-HKU1 typically cause mild symptoms, whereas severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV) have caused severe respiratory disease outbreaks in the past two decades [1, 3, 4]. Since December 2019, an outbreak of mysterious pneumonia has been reported in Wuhan City, Hubei Province, China. In early January 2020, the causative agent of this mysterious viral pneumonia was identified and isolated as a novel coronavirus by several Chinese institutions in Wuhan and Beijing. This novel coronavirus was initially named 2019 novel coronavirus

(2019-nCoV) and later designated SARS-CoV-2, while the disease caused by SARS-CoV-2 was named 2019 novel coronavirus disease (COVID-19) by the World Health Organization (WHO). According to clinical observations, SARS-CoV-2 infections can cause diseases ranging from mild symptoms to severe respiratory syndromes, including pneumonia and even death, and its human-to-human transmission has also been confirmed [5, 6]. To date, the COVID-19 outbreak has caused more than 76,000 confirmed cases and has been declared a global public health emergency by WHO.

RNAi is a post-transcriptional gene silencing mechanism that is evolutionarily conserved in all eukaryotes and has been recognized as a cell-intrinsic antiviral immune defense mechanism in diverse eukaryotes including mammals [7]. In antiviral RNAi, viral infection and replication generate virus-derived dsRNA (vi-dsRNA), which can be recognized and cleaved by the host endoribonuclease Dicer into virus-derived siRNAs (vsiRNAs). These vsiRNAs are incorporated into the Argonaute protein within the RNA-induced silencing complex (RISC) to direct the destruction of cognate viral RNAs in infected cells in a sequence-specific manner [8]. As a countermeasure, viruses encode viral suppressors of RNAi (VSRs) to antagonize the RNAi pathway at different steps [9]. Nodamura virus (NoV) B2, Influenza A virus (IAV) NS1, human enterovirus A71 (EV-A71) 3A, Dengue virus 2 (DENV2) NS2A, and Semliki Forest virus (SFV) Capsid have been identified to act as VSRs that inhibit vsiRNA production and antiviral RNAi response in the context of authentic viral infections [10-15]. In addition, Wuhan Nodavirus (WhNV) B2, Ebola virus (EBOV) VP35, HIV-1 TAT, Hepacivirus core and NS2, Yellow Fever virus capsid, Chikungunya virus nsP2 and nsP3 have been found to suppress RNAi in vitro [16-22]. The widespread phenomenon of viruses encoding VSRs to suppress the RNAi pathway highlights the importance of antagonizing antiviral RNAi during the viral life cycle. Therefore, understanding how SARS-CoV-2 interacts with antiviral RNAi is important for gaining better knowledge of this novel coronavirus and may contribute to efforts to control the spread of infections and develop effective therapies.

A previous study reported that SARS-CoV nucleocapsid (N) protein displayed VSR activity in mammalian cells via a cellular reversal-of-silencing assay [23]. Given the high homology of amino acid sequences among coronavirus N proteins, it is intriguing to examine whether SARS-CoV-2 N also possesses VSR activity. Therefore, in the current study, we evaluated the role of SARS-CoV-2 N in suppressing RNAi in cultured human cells. Our findings demonstrated that SARS-CoV-2 N does possess VSR activity to inhibit RNAi response in cells, which likely acts as one of the key immune evasion factors of SARS-CoV-2 and contributes to its pathogenicity, thereby representing a potential target for antiviral therapy.

## Results

**SARS-CoV-2 N suppressed shRNA-induced RNAi in cultured human cells.** We first examined whether SARS-CoV-2 N possessed VSR activity via a classic reversal-of-silencing assay, in which enhanced green fluorescent protein (EGFP)-specific shRNA was transfected into EGFP-expressing 293T cells together with a plasmid encoding Flag-tagged SARS-CoV-2 N protein. At 48 hours post-transfection (hpt), EGFP protein levels were examined via fluorescent microscopy and western blotting, respectively. EGFP-specific shRNA expression resulted in low EGFP protein levels (Fig. 1A [Figure 1: see original paper], panel “Vec” ; Fig. 1B, lane “Vec” ), confirming the efficiency of shRNA in this RNAi system. Our data showed that expression of SARS-CoV-2 N markedly restored the protein level of EGFP (Fig. 1A, panel “N” ; Fig. 1B, lane “N” ), indicating that SARS-CoV-2 N displays VSR activity in cells. Notably, ectopic expression of EBOV VP35, a well-characterized VSR, suppressed the shRNA-induced RNAi as expected (Fig. 1B, lane “VP35” ).

Because RNAi directly results in the cleavage and degradation of target mRNAs, we further examined the VSR activity of SARS-CoV-2 N using the reversal-of-silencing system via northern blotting with a digoxin (DIG)-labeled RNA probe targeting EGFP ORF 1-400 nt. Our results showed that SARS-CoV-2 N markedly restored EGFP mRNA levels in 293T cells (Fig. 1C). Taken together, our data demonstrate that SARS-CoV-2-encoded N protein has VSR activity in cultured human cells.

**SARS-CoV-2 N sequestered dsRNAs in cells.** Having established that SARS-CoV-2 N contains VSR activity, we sought to examine the mechanism by which SARS-CoV-2 N antagonizes RNAi. During dsRNA/shRNA-induced RNAi, dsRNA/shRNA is initially recognized and cleaved by Dicer into siRNA. Previous studies also found that numerous VSRs encoded by different viruses antagonize antiviral RNAi via sequestering viral dsRNAs. Thus, we examined whether SARS-CoV-2 N can sequester dsRNA via the RNA-IP assay. In brief, 293T cells expressing Flag-tagged N or empty vector, together with EGFP-specific dsRNA (EGFP ORF 1-200nt), were lysed and immunoprecipitated with anti-Flag or mouse IgG antibodies, respectively. The RNAs extracted from the RNA-IP precipitates were then examined via northern blotting with RNA probes targeting the 1-200 nt dsRNA of EGFP. Our results showed that SARS-CoV-2 N does indeed associate with dsRNA in 293T cells (Fig. 2 [Figure 2: see original paper]), implying that the mechanism by which SARS-CoV-2 N suppresses RNAi is to sequester dsRNA in cells, which likely prevents the recognition and cleavage of viral dsRNA by Dicer.

**SARS-CoV-2 N suppressed siRNA-induced RNAi in mammalian cells.** In the process of RNAi, Dicer-cleaved siRNAs are required to assemble siRNA-incorporated RISC to direct the degradation of cognate RNAs, which is the effector step of RNAi [24]. After establishing that SARS-CoV-2 N can associate with dsRNA, we further examined whether SARS-CoV-2 N could

also suppress siRNA-induced RNAi. To this end, we co-transfected chemically synthesized EGFP-specific siRNA together with the plasmid for SARS-CoV-2 N into EGFP-expressing 293T cells. The effects of RNAi were determined via fluorescent microscopy and western blotting to detect EGFP protein expression, or via northern blotting to detect EGFP mRNA level. EGFP-specific siRNA reduced the protein and mRNA levels of EGFP, while ectopic expression of SARS-CoV-2 N efficiently restored EGFP expression at both protein and mRNA levels (Fig. 3A [Figure 3: see original paper]-C). Consistent with previous findings, EBOV VP35, which was used as a positive control, also restored EGFP expression at both protein and mRNA levels as expected (Fig. 3B and 3C). Our findings indicate that SARS-CoV-2 N can suppress siRNA-induced RNAi in cells, implying that SARS-CoV-2 N may also interfere with RISC assembly or function.

## Discussion

The emergence of the SARS-CoV-2 outbreak has caused and continues to pose a serious threat to human health and tremendous economic loss in China and across the globe, pushing us to obtain knowledge about all aspects of this novel coronavirus as quickly and comprehensively as possible. RNAi is an antiviral response conserved in all eukaryotes including mammals, and viruses encode VSRs to antagonize RNAi [24]. In this study, we found that the SARS-CoV-2-encoded structural protein N displayed VSR activity in cultured human cells. Our findings showed that SARS-CoV-2 N can antagonize RNAi at both the initiation (i.e., siRNA biogenesis) and effector (i.e., RISC assembly and target RNA cleavage) steps. Therefore, our data support the notion that N protein can act as a VSR to facilitate SARS-CoV-2 replication by shielding viral dsRNA and siRNA from Dicer cleavage and RISC assembly, respectively.

The finding that SARS-CoV-2 N suppresses RNAi in cells is consistent with the previous observation that SARS-CoV N also displayed VSR activity [23], implying that using N protein as a VSR is a common strategy for coronaviruses to antagonize antiviral RNAi. Moreover, in addition to N protein, a previous study identified that SARS-CoV 7a could suppress RNAi in mammalian cells [25], suggesting that coronaviruses may antagonize RNAi by encoding multiple VSRs. Having multiple VSRs is not uncommon, as several other RNA viruses such as EBOV, DENV, and HIV-1 have also been reported to encode more than one VSR [21, 22, 26-30]. Encoding multiple VSRs may offer these pathogenic viruses extra advantages for efficient inhibition of RNAi, highlighting the importance of antiviral RNAi for host cells in defending against viral infection.

Coronavirus N protein contains nonspecific RNA-binding activity [31, 32]. In this study, we also found that SARS-CoV-2 N could associate with dsRNA in cells. Our results that SARS-CoV-2 N suppressed RNAi by sequestering dsRNA are consistent with previous findings that coronavirus N is directly involved in viral RNA replication [33, 34]. During the viral life cycle, coronavirus N protein encapsulates viral genomic RNAs to protect the genome and co-enters the host

cell with viral genomic RNAs, indicating that N is important for viral RNA replication, especially at the initiation step [33, 34].

In summary, SARS-CoV-2 N can act as a VSR in cells at both initiation and effector steps of RNAi, thereby likely representing a key immune evasion factor of SARS-CoV-2 and contributing to the pathogenicity of this novel coronavirus. Our study extends our knowledge about the interaction between antiviral RNAi immunity and SARS-CoV-2 in a timely manner and may be helpful in efforts to control this dangerous virus.

## Materials and Methods

**Cell culture and transfection.** Human embryonic kidney 293T (HEK293T) cells were commercially obtained from the American Type Culture Collection and maintained in modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin, and 100 g/ml streptomycin at 37°C in an incubator with 5% CO<sub>2</sub>. Transfection of HEK293T cells was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

**Plasmid construction.** pRK-Flag-SARS-CoV-2 N was generated using a standard cloning protocol. The cDNA fragment of SARS-CoV-2 N was generated by reverse transcription; the RNA template was isolated from SARS-CoV-2-infected cells. pRK-Flag-EBOV VP35 was described previously [35]. The primers used in this study are shown in Table S1.

**Northern blotting.** Total cellular RNAs were extracted using Trizol reagent (Tiangen, Beijing, China) according to the manufacturer's protocol. Northern blotting was performed as previously described [12]. Briefly, 5 g of total RNAs were electrophoresed on denaturing 1.2% agarose gels containing 2.2 M formaldehyde, and then capillary transferred to Hybond-A nylon membrane (GE Healthcare). The membranes were hybridized with the indicated DIG-labeled RNA probes at 65°C for 12 h, and then incubated with anti-DIG-alkaline phosphatase antibody (Roche). The membranes were then incubated with CDP-STAR (Roche) at 37 °C for 10 min. The signals were detected by radiography on X-ray film (FujiFilm, Tokyo, Japan). The DIG-RNA probe targeting EGFP was produced via in vitro transcription. Intact 28s and 18s rRNAs were observed using ethidium bromide staining and were used as loading controls. The primers used for the preparation of RNA probes in this study are shown in Table S1.

**Western blotting.** Cells were washed twice in cold phosphate-buffered saline and lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% NP40, 0.25% deoxycholate, and a protease inhibitor cocktail (Roche). The lysates were then subjected to 12% SDS-PAGE and western blotting according to standard procedures with the relevant antibodies.

**RNA-immunoprecipitation (RNA-IP).** RNA-IP was performed as previously described [12]. Briefly, cells were lysed in lysis buffer containing 20 mM

Tris-HCl (pH 7.4), 100 mM NaCl, 2.5 mM MgCl<sub>2</sub>, 0.5% NP-40, 0.5 U/μL RNase inhibitor (Promega), and a protease inhibitor cocktail (Roche) at 4°C for 30 min. Lysates were clarified at 12,000 × g for 10 min at 4°C, and post-nuclear lysates were incubated with antibodies (anti-FLAG or anti-IgG) together with protein-A/G agarose beads (Roche) at 4°C for 4 h. The antibody-bound complexes were washed five times with the same lysis buffer. Finally, proteins or RNAs were extracted from the complexes and analyzed by western or northern blotting as described above.

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**Conflicts of interest.** The authors declare that there are no conflicts of interest.

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## Figures

**Figure 1.** SARS-CoV-2 N suppressed shRNA-induced RNAi in cultured human cells. HEK293T cells were co-transfected with plasmids encoding EGFP (0.1 µg) and EGFP-specific shRNA (0.3 µg), together with either empty vector or the plasmid encoding SARS-CoV-2 N or EBOV VP35 (1 µg each). (A) At 48 hpt, cells were observed via fluorescent microscopy. (B) Cell lysates were harvested and analyzed by western blotting with anti-EGFP, anti-FLAG, and anti-Tubulin antibodies. (C) Total RNAs were extracted and EGFP mRNA levels were examined by northern blotting with a DIG-labeled RNA probe targeting EGFP ORF 1-400nt. 18s and 28s rRNAs were used as loading controls.

**Figure 2.** SARS-CoV-2 N sequestered dsRNAs in cells. HEK293T cells were transfected with the plasmid encoding SARS-CoV-2 N or empty vector, together with EGFP-specific dsRNA. At 24 hpt, the cell lysates were subjected to RNA-IP with anti-FLAG or anti-IgG antibodies. Input and precipitated RNAs and proteins were detected by northern blotting and western blotting, respectively.

**Figure 3.** SARS-CoV-2 N suppressed siRNA-induced RNAi in cells. HEK293T cells were co-transfected with the plasmid encoding EGFP (0.1 µg) and EGFP-specific siRNA (0.3 µg), together with either empty vector or the plasmid encoding SARS-CoV-2 N or EBOV VP35 (1.0 µg each). (A) At 48 hpt, cells were observed via fluorescent microscopy. (B) Cell lysates were harvested and analyzed by western blotting with anti-EGFP, anti-FLAG, and anti-Tubulin antibodies. (C) Total RNAs were extracted and EGFP mRNA levels were examined by northern blotting with a DIG-labeled RNA probe targeting EGFP ORF 1-400nt. 18s and 28s rRNAs were used as loading controls.

## Supplementary Information

**Supplementary Table S1.** The primers and oligonucleotides used in this study.

**Primers for plasmid construction:** - SARS-CoV-2 N-FOR: GACTACAAGGACGACGATGACAAGATGTCTGATAATGGACCCCAAAATCAGC  
- SARS-CoV-2 N-REV: GTTCTGCGCCTGCAGGTTTATTAGGCCTGAGTTGAGTCAGCA

**Primers for amplification of templates for in vitro transcription dsRNAs:** - EGFP-ds200-FOR: TAATACGACTCACTATAGATGGTGAGCTAGGGCGAGGA - EGFP-ds200-REV: TAATACGACTCACTATAGGCGCTGAAGCACTGCACGC

**Primers for amplification of templates for in vitro transcription RNA probes:** - EGFP-1-400(+)Probe-F: ATGGTGAGCAAGGGCGAGGA  
- EGFP-1-400(+)Probe-R: TAATACGACTCACTATAGGCTTGTGCC-CCAGGATGTTGC - EGFP-1-400(-)Probe-F: TAATACGACTCACTATAGATGGTGAGCAAGGGCGAGGA - EGFP-1-400(-)Probe-R: GCTTGTGCC-CCAGGATGTTGC

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