

# Gene regulatory function and cellular partners of SARS-associated coronavirus nucleocapsid protein

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## KEY MESSAGES

1. SARS coronavirus (SARS-CoV) nucleocapsid (N) protein expressed in cultured human cells was predominantly found in the cytoplasm and was competent in repressing the transcriptional activity driven by interferon-stimulated response elements. Expression of N protein did not influence the transcription from FGL2 promoter. N protein did not modulate the expression of FGL2 mRNA or protein in transfected or SARS-CoV-infected cells.
2. SARS-CoV N and M proteins inhibit gene transcription of type I interferons through different mechanisms. M protein potently antagonises the activation of interferon-stimulated response element-dependent

transcription by RIG-I, MDA5, TBK1, IKK $\epsilon$ , and VISA, whereas N protein has no influence on these stimuli. The expression of M protein prevents the formation of TRAF3-TANK-TBK1/IKK $\epsilon$  complex.

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## Introduction

Severe acute respiratory syndrome (SARS) is a fatal infectious disease that spread in China and around the world in 2003. The primary aetiological agent was the SARS coronavirus (SARS-CoV), close relatives of which have been found in various bats.<sup>1</sup>

Coronavirus nucleocapsid (N) protein is a relatively conserved structural protein that binds genomic RNA and plays an important role in viral RNA synthesis, viral assembly, and formation of RNA replication complex.<sup>2</sup> Intracellularly, coronavirus N protein localises to the cytoplasm where it interacts with M protein to form an icosahedral core. In addition, N proteins from mouse hepatitis virus (MHV) and several other coronaviruses have also been shown to localise to the nucleolus to exert an impact on cell cycle progression.<sup>3</sup> In line with this, MHV N protein regulates the expression of cellular genes such as FGL2.<sup>4</sup> FGL2 encodes a prothrombinase termed fibrinogen-like protein 2 that causes vascular thrombosis and fibrin deposition.

Although nucleolar localisation has also been suggested, SARS-CoV N protein is more frequently found in the cytoplasm.<sup>5</sup> Interestingly, its gene regulatory function has also been documented in the context of AP-1-, NF- $\kappa$ B-, Smad-, and CCAAT/enhancer binding protein (C/EBP)-dependent transcription,<sup>6,7</sup> and interferon production.<sup>8</sup> Fibrosis

and vascular thrombosis in the lung are also observed commonly in patients with SARS. In line with this, SARS-CoV N protein has been shown to regulate the expression of human FGL2 gene.<sup>9</sup> In addition to N protein, the inhibition of interferon production and signalling by SARS-CoV is thought to be mediated through other viral structural and non-structural proteins ORF3b, ORF6, nsp1, and PLpro.<sup>10</sup> In this way, SARS-CoV counteracts a major component of the host antiviral innate immunity at multiple levels.

This project aims to shed light on the gene regulatory function and cellular partners of SARS-CoV N protein. We found that N protein does not modulate FGL2 but inhibits transcription from interferon gene promoters through a mechanism distinct from M protein, and impeded the formation of TRAF3-TANK-TBK1/IKK $\epsilon$  complex. Our findings point to a new model in which SARS-CoV circumvents the production of type I interferons.

## Methods

N gene and other viral genes of SARS-CoV were subcloned and expressed in cultured mammalian cells. Properties of N and M protein were characterised in N or M gene-transfected and SARS-CoV-infected cells using Western blotting, luciferase reporter assay, confocal immunofluorescence microscopy, and immunoprecipitation.

## Results

### Expression and gene regulatory activity of SARS-CoV N protein

The distinct subcellular localisation patterns of MHV and SARS-CoV N proteins suggest that they might serve different functions inside the cell. Gene regulatory activity of SARS-CoV N protein has recently been demonstrated in different models. As a first step to characterise its possible roles in cellular pathogenesis, we expressed SARS-CoV N protein in HEK293 cells. Western blot analysis indicated that N protein was abundantly expressed in transfected cells.

### SARS-CoV N protein did not influence transcription of FGL2 gene in transfected cells

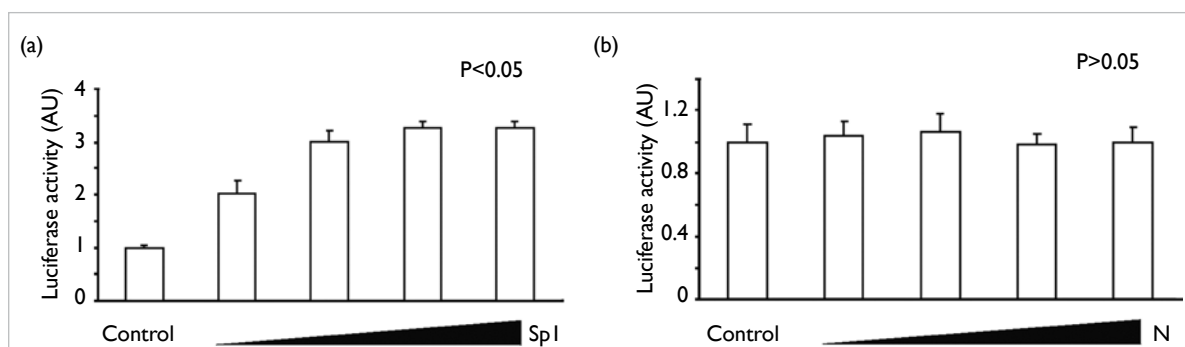
The stimulation of FGL2 promoter by SARS-CoV N protein has implications not only in the pathogenesis of SARS, but also in the development of therapeutics. To shed light on whether and how SARS-CoV N protein might activate transcription of the FGL2 gene, we constructed reporter plasmid pFGL2-Luc, in which the expression of firefly luciferase is under the control of FGL2 promoter. To confirm the activity of this construct, we cotransfected it into HEK293 cells with an expression vector for Sp1, a known activator of FGL2 promoter. A more than two-fold stimulation of reporter expression by Sp1 demonstrated that pFGL2-Luc sensitively reflected intracellular activity of FGL2 promoter (Fig 1a). As a control for proper expression and activity of SARS-CoV N protein in HEK293 cells, we found that N protein was fully competent to repress ISRE transcriptional activity that controls interferon production.

Nonetheless, when we cotransfected pFGL2-Luc and an expression plasmid for SARS-CoV N

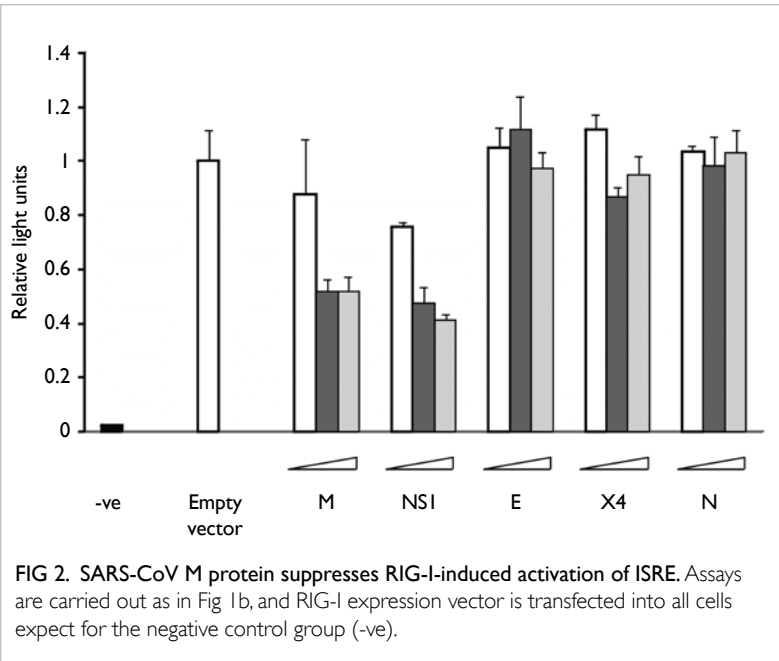
protein into HEK293 cells, an induction of reporter activity was not observed (Fig 1b). To further characterise the influence of N protein on expression of the endogenous FGL2 gene, we analysed the steady-state amounts of FGL2 transcript in HEK293 cells overexpressing Sp1 or N protein. While the expression of Sp1 correlated with elevation of FGL2 transcript above the basal level, expression of N protein did not alter the relative amount of FGL2 mRNA in transfected cells. Consistently, the accumulation of Sp1 protein in the cell was associated with an abrupt increase in FGL2 protein level, whereas increased expression of N protein had no influence on the steady-state amount of FGL2. Thus, SARS-CoV N protein did not induce expression of FGL2 transcript or protein in transfected cells. We also showed that SARS-CoV N protein did not affect the expression of FGL2 in SARS-CoV-infected cells.

### SARS-CoV N and M proteins counteract type I interferon production through different mechanisms

SARS-CoV N protein antagonises the production of type I interferons.<sup>8</sup> In our study to characterise the gene regulatory function of N protein, we included M protein as a control and found serendipitously that M protein was a more potent inhibitor of interferon  $\beta$  promoter when compared with N protein. To compare the mechanisms by which N and M proteins counteract interferon induction and signalling in cultured cells, we used RIG-I, a well-studied cytoplasmic sensor of dsRNA and activator of interferon production and signalling, to stimulate ISRE activity and then assessed the influence of N and M. Interestingly, coexpression of N did not modulate RIG-I-activated interferon production, whereas M was able to suppress RIG-I activity in a dose-dependent manner (Fig 2). Notably, this activity of M is similar to that of influenza A virus NS1, but



**FIG 1.** Expression of SARS-CoV N protein does not stimulate FGL2 promoter. HEK293 cells are transfected with 100 ng pFGL2-Luc plus progressively increasing amounts (100, 200, 400, and 600 ng) of (a) Sp1 expression plasmid and (b) SARS-CoV N protein expression plasmid, respectively. Control cells are transfected with pFGL2-Luc and empty vector.



not SARS-CoV E or ORF7A (X4) proteins. We also found that while M protein inhibited the ability of MDA5, TBK1, IKK $\epsilon$  and VISA to activate ISRE, N had no influence on any of these stimuli (data not shown). Hence, SARS-CoV N and M proteins suppress type I interferon production through different mechanisms.

### SARS-CoV M protein counteracts type I interferon production by disrupting the formation of TRAF3-TANK-TBK1/IKK $\epsilon$ complex

Since M protein displayed a unique pattern to inhibit RIG-I, we next investigated the mechanism by which it modulates interferon induction and signalling. Co-immunoprecipitation and confocal immunofluorescence microscopy revealed that M associated with RIG-I, TRAF3, TBK1, and IKK $\epsilon$ . Because the formation of TRAF3-TANK-TBK1/IKK $\epsilon$  complex is an essential step in stimulus-induced activation of IRF3 and IRF7, we explored whether expression of M might influence this step. Notably, the interactions of TRAF3 with TBK1, IKK $\epsilon$ , and TANK were evident in the absence of M. In contrast, these three pairs of interaction were abolished in M-expressing cells. In other words, the expression of M prevented the formation of TRAF3-TANK-TBK1/IKK $\epsilon$  complex, thereby inhibiting IRF3/7 phosphorylation and activation.

## Discussion

We showed that SARS-CoV N protein did not

modulate transcription of the human FGL2 gene. Our findings are opposed to those of a recent report on the induction of FGL2 promoter through activation of C/EBP $\alpha$  by SARS-CoV N protein.<sup>9</sup> It is noteworthy that the human FGL2 promoter used in our study contained all *cis* regulatory elements described by others including the C/EBP site.<sup>9</sup> In addition, the utility of our pFGL2-Luc construct was further supported experimentally by the significant activation induced by cellular Sp1 transcription factor (Fig 1a). Although we do not understand whether different experimental systems might explain different observations, our demonstration of the unaltered expression of FGL2 protein in infected cells might be more biologically relevant to SARS-CoV.

SARS-CoV N protein is structurally and functionally related to MHV N protein. As such, both proteins are capable of multimerisation and RNA binding.<sup>11</sup> In addition, both proteins have gene regulatory activity and can repress interferon production.<sup>10</sup> Nonetheless, SARS-CoV N protein also possesses properties that are not shared with its MHV counterpart. For example, SARS-CoV N protein is localised predominantly to the cytoplasm, whereas MHV N protein was found in the nucleolus.<sup>5</sup> In addition, SARS-CoV N protein did not activate FGL2 transcription. FGL2 is unlikely to be involved in the pathogenesis of SARS.

We compared the molecular mechanisms by which SARS-CoV N and M proteins antagonise type I interferon production and signalling. We found that M protein physically interacted with RIG-I, TBK1, and IKK $\epsilon$ . In addition, M protein disrupted the formation of TRAF3-TANK-TBK1/IKK $\epsilon$  complex to inhibit IRF3/7 phosphorylation and activation. Our work not only reveals a new interferon antagonist encoded by SARS-CoV, but also provides a new mechanism for SARS-CoV modulation of interferon production and signalling. Our findings are generally consistent with the concept that SARS-CoV encodes multiple structural and non-structural proteins to counteract the host antiviral response.<sup>10</sup> In addition, our results have implications in the rational design and use of anti-SARS CoV agents.

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Results of this study have been published in:

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