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# Coronavirus nonstructural protein 1: Common and distinct functions in the regulation of host and viral gene expression



Krishna Narayanan<sup>a,\*</sup>, Sydney I. Ramirez<sup>b,1,2</sup>, Kumari G. Lokugamage<sup>a,2</sup>, Shinji Makino<sup>a,\*\*</sup>

<sup>a</sup> Department of Microbiology and Immunology, The University of Texas Medical Branch at Galveston, Galveston, TX 77555-1019, United States

<sup>b</sup> Department of Pathology, The University of Texas Medical Branch at Galveston, Galveston, TX 77555-1019, United States

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## ABSTRACT

The recent emergence of two highly pathogenic human coronaviruses (CoVs), severe acute respiratory syndrome CoV and Middle East respiratory syndrome CoV, has ignited a strong interest in the identification of viral factors that determine the virulence and pathogenesis of CoVs. The nonstructural protein 1 (nsp1) of CoVs has attracted considerable attention in this regard as a potential virulence factor and a target for CoV vaccine development because of accumulating evidence that point to its role in the down-regulation of host innate immune responses to CoV infection. Studies have revealed both functional conservation and mechanistic divergence among the nsp1 of different mammalian CoVs in perturbing host gene expression and antiviral responses. This review summarizes the current knowledge about the biological functions of CoV nsp1 that provides an insight into the novel strategies utilized by this viral protein to modulate host and viral gene expression during CoV infection.

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## 1. Introduction

Coronaviruses (CoVs) are found in a large variety of animal species, including humans, and primarily cause respiratory and enteric diseases (Weiss and Navas-Martin, 2005). In humans, coronaviruses usually cause a mild respiratory disease, like the common cold (Falsey et al., 1997; van der Hoek et al., 2006). However, the identification of severe acute respiratory syndrome CoV (SARS-CoV) as the etiological agent of the SARS epidemic in 2003 and the recent discovery of Middle East respiratory syndrome CoV (MERS-CoV) as the causative agent of MERS, a viral respiratory disease first reported in Saudi Arabia in 2012, have highlighted the potential for zoonotic transmission of highly pathogenic CoVs to humans causing severe diseases in the human population (Drosten et al., 2003; Ksiazek et al., 2003; Perlman and Dandekar, 2005; Perlman and

Netland, 2009; Rota et al., 2003; van Boheemen et al., 2012; Zaki et al., 2012).

CoVs belong to the order *Nidovirales* in the family *Coronaviridae*, and are currently classified into four genera, Alphacoronavirus, Betacoronavirus, Gammacoronavirus and Deltacoronavirus ( $\alpha$ -CoV,  $\beta$ -CoV,  $\gamma$ -CoV and  $\delta$ -CoV) in the subfamily *Coronavirinae* (de Groot et al., 2011; Gorbalenya et al., 2004; Snijder et al., 2003; Woo et al., 2010, 2012). The  $\alpha$ -CoVs and  $\beta$ -CoVs are predominantly found in mammals and include several pathogenic human CoVs such as HCoV-229E, HCoV-HKU1, HCoV-OC43, HCoV-NL63, SARS-CoV and MERS-CoV (Drexler et al., 2010; Drosten et al., 2003; Isaacs et al., 1983; Ksiazek et al., 2003; Larson et al., 1980; Vabret et al., 2003, 2008; Wertheim et al., 2013; Zaki et al., 2012). The  $\gamma$ -CoVs and  $\delta$ -CoVs are primarily detected in birds. Bats appear to be the natural reservoir involved in the evolution and dissemination of many mammalian CoVs (Carrington et al., 2008; Chan et al., 2013; Chu et al., 2008; Gloza-Rausch et al., 2008; Poon et al., 2005; Reusken et al., 2010; Tang et al., 2006).

CoVs possess a large, single-stranded, positive-sense RNA genome that range in length from 27 to 32 kb, the largest among any of the RNA viruses (Lee et al., 1991; Lomniczi, 1977; Lomniczi and Kennedy, 1977). The 5'-most gene of the CoV genome, gene 1, occupies about two-thirds of the genome and consists of two large overlapping open reading frames (ORFs), ORF 1a and ORF 1b, with a ribosomal frameshifting signal at the junction of the two ORFs

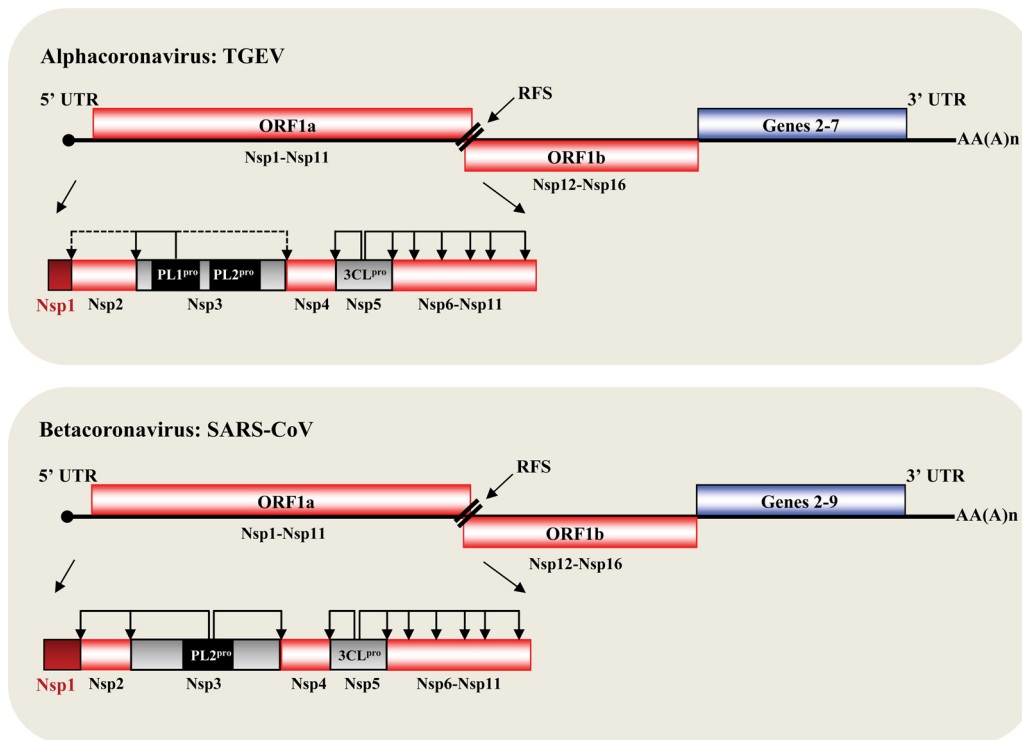
\* Corresponding author. Tel.: +1 409 772 8172; fax: +1 409 772 5065.

\*\* Corresponding author. Tel.: +1 409 772 2323; fax: +1 409 772 5065.

E-mail addresses: [krnaraya@utmb.edu](mailto:krnaraya@utmb.edu) (K. Narayanan), [syiramir@utmb.edu](mailto:syiramir@utmb.edu) (S.I. Ramirez), [kglokuga@utmb.edu](mailto:kglokuga@utmb.edu) (K.G. Lokugamage), [shmakino@utmb.edu](mailto:shmakino@utmb.edu) (S. Makino).

<sup>1</sup> These authors contributed equally to this study.

<sup>2</sup> Tel.: +1 409 772 8172.



**Fig. 1.** Genome organization and proteolytic processing of ORF1a polyprotein of selected members in the  $\alpha$ -CoV and  $\beta$ -CoV genera of Coronaviridae family. The open reading frames (ORFs) 1a and 1b constitute gene 1. One member of each genus is shown as a representative example. The sites in ORF1a polyprotein processed by the viral proteinases, PL<sup>pro</sup> and 3CL<sup>pro</sup>, are indicated. In the upper panel, the dotted lines indicate the putative processing at the nsp1–nsp2 and nsp3–nsp4 cleavage sites by TGEV PL1<sup>pro</sup>. The predicted activity of TGEV PL1<sup>pro</sup> at these cleavage sites remains to be determined. RFS: ribosomal frameshift; UTR: untranslated region. The figure is not drawn to scale.

(Fig. 1) (Bredenbeek et al., 1990; Brian and Baric, 2005; Gorbalenya, 2001; Lee et al., 1991; Ziebuhr, 2005). Upon entry into host cells, the incoming viral genome is translated to produce two large precursor polyproteins 1a (pp1a) and 1b (pp1ab) that are processed by ORF 1a-encoded viral proteinases, papain-like proteinase (PL<sup>pro</sup>) and 3C-like proteinase (3CL<sup>pro</sup>), into 16 mature nonstructural proteins (nsp1–nsp16, numbered according to their order from the N-terminus to the C-terminus of the ORF 1 polyproteins) (Ziebuhr, 2005). Many of the nsps perform essential functions in viral RNA replication and transcription (Bhardwaj et al., 2004; Cheng et al., 2005; Fan et al., 2004; Imbert et al., 2006; Ivanov et al., 2004a,b; Minskaia et al., 2006; Saikatendu et al., 2005; Snijder et al., 2003). Besides the RNA-dependent RNA polymerase, helicase and proteases, some of the nsps are RNA-processing enzymes such as poly (U)-specific endoribonuclease, 3'-5' exoribonuclease, ribose 2'-O methyltransferase, adenosine diphosphate-ribose-1''-phosphatase and cyclic nucleotide phosphodiesterase (Lee et al., 1991; Snijder et al., 2003; Thiel et al., 2003; Ziebuhr, 2005). The enzymatic activities and the functional domains of many of these essential nsps are predicted to be conserved between the different genera of CoVs, indicating their importance in viral replication (Snijder et al., 2003; Thiel et al., 2003). In addition to these nsps with defined functions, there are several nsps whose biological functions and roles in CoV life cycle still remain to be characterized.

While nsp3–nsp16 from different CoV genera share several conserved functional domains, the N-terminal region of the ORF 1 polyprotein, especially the nsp1 sequence, is highly divergent among CoVs (Connor and Roper, 2007; Snijder et al., 2003; Thiel et al., 2003). Nsp1 is the most N-terminal cleavage product released from the ORF 1a polyprotein by the action of PL<sup>pro</sup> (Fig. 1) (Ziebuhr, 2005). Among the four CoV genera, only  $\alpha$ -CoVs and  $\beta$ -CoVs encode nsp1 (Fig. 1), whereas  $\gamma$ -CoVs and  $\delta$ -CoVs lack nsp1 and thus, their gene 1 encodes only 15 nsps (nsp2–nsp16) (Snijder et al., 2003; Woo et al., 2010; Ziebuhr, 2005; Ziebuhr et al., 2007). The nsp1 of

$\alpha$ -CoVs share no significant sequence similarity with  $\beta$ -CoV nsp1 and their sizes are also different (Connor and Roper, 2007; Jansson, 2013). Based on the comparative sequence analysis of the genomes of different CoVs, nsp1 could be considered as one of the genus-specific markers (Snijder et al., 2003). Furthermore, bioinformatics analysis of the primary amino acid sequence of nsp1 does not reveal any known cellular or viral homologs, other than in CoVs, and also rules out the presence of any obvious functional protein motifs in nsp1 (Connor and Roper, 2007). These intriguing features of the primary amino acid sequence of nsp1 combined with the fact that all the known mammalian CoVs, including the pathogenic human CoVs, encode nsp1 has put a spotlight on this protein, especially since the SARS epidemic in 2003. Indeed, nsp1 has garnered considerable attention, as evidenced by the growing body of literature aimed at delineating its structure, biological functions and importance in CoV replication and pathogenesis. Numerous studies have revealed interesting biological properties of nsp1 and also highlighted novel mechanisms of regulation of host and viral gene expression by nsp1 (Table 1). An emerging theme from these studies is that the nsp1 of  $\alpha$ -CoVs and  $\beta$ -CoVs exhibit remarkably similar biological functions, despite the lack of overall sequence similarity and known protein motifs, suggesting its importance in the life cycle of these different lineages of CoVs.

In this review, we will summarize the current knowledge about the properties of CoV nsp1 and describe studies that highlight the common as well as distinct biological functions of  $\alpha$ -CoV and  $\beta$ -CoV nsp1 in the regulation of host and viral gene expression.

## 2. CoV nsp1: general features and biological functions

### 2.1. $\alpha$ -CoV nsp1

The nsp1 of  $\alpha$ -CoVs is about 110 amino acids in length (Almeida et al., 2007; Jansson, 2013). In HCoV-229E, the processing of

**Table 1**  
Summary of the biological functions of coronavirus nsp1.

Nsp1	Length (amino acids)	Function(s)	Reference(s)
<b><u>α-CoV</u></b>			
TGEV	110	• Translation inhibition	• Huang et al., 2011a
HCoV-229E	110	• Inhibition of reporter gene expression from constitutive and inducible promoters	• Züst et al., 2007; Wang et al., 2010
HCoV-NL63	110		
<b><u>β-CoV</u></b>			
	<b>Lineage</b>		
MHV	A 245	• Induction of cell cycle arrest • Inhibition of reporter gene expression from constitutive and inducible promoters • Inhibition of type I IFN signaling*	• Chen et al., 2004 • Züst et al., 2007 • Lei et al., 2013
SARS-CoV	B 180	• Inhibition of type I IFN induction and signaling* • Translation inhibition*  • Induction of host mRNA cleavage • Induction of host mRNA decay* • Induction of cytokines and chemokines	• Kamitani et al., 2006; Wathelet et al., 2007; Narayanan et al., 2008 • Narayanan et al., 2008; Kamitani et al., 2009; Lokugamage et al., 2012 • Kamitani et al., 2009; Huang et al., 2011b • Kamitani et al., 2006; Narayanan et al., 2008 • Law et al., 2007; Pfefferle et al., 2011
Bat CoV:Rm1	B 180	• Inhibition of host protein synthesis • Induction of host mRNA decay • Inhibition of type I IFN and IFN-stimulated gene induction	• Tohya et al., 2009
Bat CoV:133	C 195		
Bat CoV:HKU9-1	D 175		

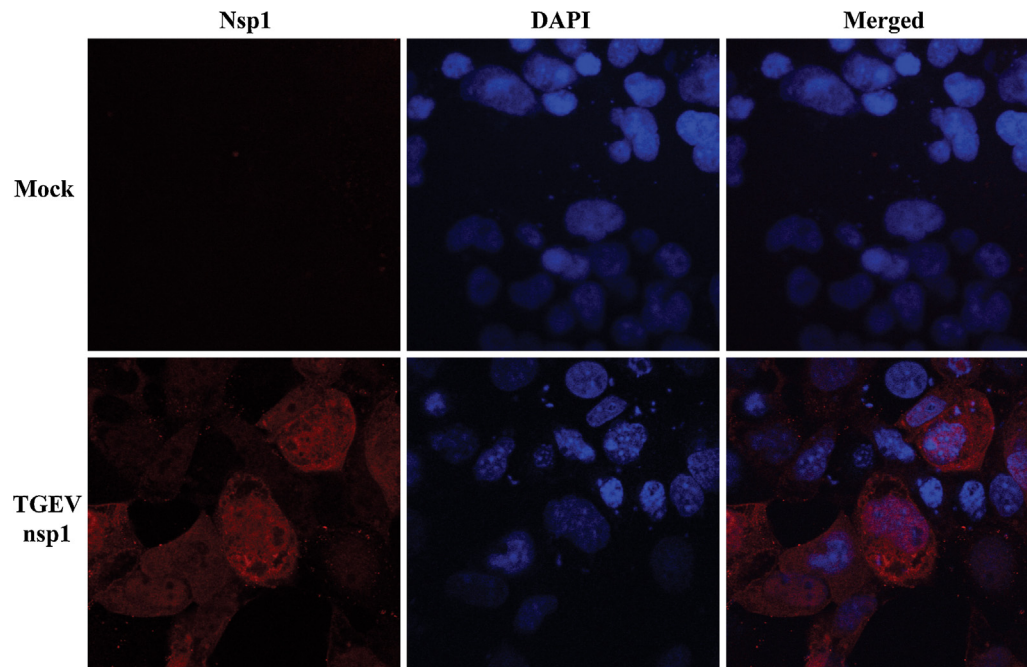
\* Denotes function(s) validated in virus infection

the ORF1a polyprotein by PL1<sup>Pro</sup> releases nsp1 as an amino-terminal 9-kDa protein along with nsp2, an 87-kDa protein (Herold et al., 1998; Ziebuhr et al., 2001). Based on sequence comparison, this pattern of processing, resulting in the release of nsp1 (also known as p9), was also predicted for another closely related α-CoV, transmissible gastroenteritis virus (TGEV) (Galan et al., 2005). Comparative amino acid sequence analysis of the nsp1 of different α-CoVs revealed that HCoV-229E nsp1 shares moderate sequence identities of 60% and 52% with HCoV-NL63 and porcine epidemic diarrhea virus nsp1, respectively (Huang et al., 2011a). However, TGEV nsp1 has only 32% amino acid sequence similarity with HCoV-229E nsp1 but shares high sequence identities of 97% and 93% with the nsp1 of porcine respiratory coronavirus and feline infectious peritonitis virus, respectively (Huang et al., 2011a). A high-resolution crystal structure of TGEV nsp1 combined with alignment of nsp1 sequences from different α-CoVs provided additional information about the spatial location of the evolutionarily conserved regions of α-CoV nsp1 (Jansson, 2013). The TGEV nsp1 structure is defined by an irregular six-stranded β-barrel flanked by an α-helix and many of the conserved residues form the hydrophobic core of the β-barrel fold, which was suggested to be more important for the structural stability of nsp1 rather than for its function (Jansson, 2013). In addition, two highly conserved areas map to the surface of TGEV nsp1 structure, which are thought to be potentially important for the functional interaction of TGEV nsp1 with other proteins (Jansson, 2013). However, neither the three-dimensional structure of TGEV nsp1 nor

the sequence analysis of nsp1 from different α-CoVs revealed any conserved protein motifs or domains that could provide significant clues about the functions of α-CoV nsp1. Analysis of the subcellular localization of transiently expressed TGEV nsp1 in human embryonic kidney (HEK) 293 cells using confocal microscopy showed the distribution of TGEV nsp1 in both the nucleus and the cytoplasm (Fig. 2). The primary sequence analysis of TGEV nsp1 did not reveal any canonical nuclear localization signal. TGEV nsp1 could diffuse freely into the nucleus because of its small molecular weight (~9 kDa), which is below the size exclusion limit of the nuclear pore complex (Gorlich, 1998; Silver, 1991).

A limited number of studies have explored the biological functions of α-CoV nsp1, primarily using transient gene expression and cell-free in vitro translation systems. In mammalian cells, both HCoV-229E and HCoV-NL63 nsp1 inhibit the expression of reporter genes, under the control of constitutive promoters, like SV40, HSV-TK and CMV, as well as inducible promoters of innate immune response genes, like interferon (IFN)-β and IFN-stimulated gene (ISG)15, carrying the IFN-stimulated response element (ISRE) (Wang et al., 2010; Züst et al., 2007). Similarly, TGEV nsp1 also inhibits the expression of SV40-promoter-driven reporter gene in HEK 293 cells as well as in swine testis (ST) cells, which support TGEV replication (Huang et al., 2011a). Furthermore, TGEV nsp1 strongly inhibits host protein synthesis without affecting the stability of host mRNAs and also suppresses the translation of several different reporter mRNAs in a cell-free in vitro translation system like HeLa S10 extract (Huang et al., 2011a). Intriguingly, TGEV





**Fig. 2.** Analysis of the subcellular localization of TGEV nsp1 using confocal microscopy. HEK 293 cells, grown on 4-well Lab-Tek II chamber slides (Nalgene Nunc International), were transfected with a TGEV nsp1 expressing plasmid, pCAGGS-nsp1 (lower row) or an empty pCAGGS plasmid (mock; upper row). At 24 h post-transfection, the cells were fixed in 4% paraformaldehyde for 15 min and permeabilized with 0.1% Triton X-100. Subsequently, the cells were subjected to immunofluorescence analysis using a TGEV-nsp1 specific primary antibody, which was raised by immunizing rabbits with purified full-length C-terminally myc-tagged TGEV nsp1 protein, followed by Alexa Fluor 594-conjugated secondary antibody (Molecular Probes) and DAPI counterstaining (Cell Signaling Technology). Images were collected using a Zeiss LSM-510 META confocal laser-scanning microscope with a 100X oil immersion lens and processed with the LSM image browser (Zeiss) and ImageJ (NIH) software program. No signal was detected in the negative control (mock; upper row), confirming the specificity of the anti-TGEV nsp1 antibody.

nsp1 lacks the ability to inhibit the translation of reporter mRNAs in the rabbit reticulocyte lysate (RRL) *in vitro* translation system but regains its translation inhibition activity in RRL supplemented with HeLa S10 extract or HeLa S100 postribosomal supernatant, derived from HeLa S10 extract after centrifugation (Huang et al., 2011a). Collectively, these data indicate the presence of a host factor(s) in HeLa cell extracts and cultured cells that is utilized by TGEV nsp1 to exert its inhibitory effect on translation. The mechanisms of inhibition of reporter gene expression, host protein synthesis and mRNA translation by  $\alpha$ -CoV nsp1 have not been elucidated as yet. Some clues are provided by the observation that both HCoV-229E and HCoV-NL63 nsp1 associate with the ribosomal protein S6, which is located in the mRNA binding site of the 40S ribosomal subunit, a central component of the cellular translation apparatus (Wang et al., 2010; Williams et al., 2003). Interestingly, TGEV nsp1 does not bind to the 40S ribosomal subunit, highlighting a possible mechanistic difference between the nsp1 of TGEV and other  $\alpha$ -CoVs (Huang et al., 2011a). The contribution of these biological activities of  $\alpha$ -CoV nsp1 toward the regulation of host gene expression during CoV infection remains to be determined.

## 2.2. $\beta$ -CoV nsp1

The  $\beta$ -CoV genus contains four different lineages, A–D. The size of  $\beta$ -CoV nsp1 varies among the viruses of different lineages within this genus. The nsp1 of viruses in lineage A of  $\beta$ -CoV ( $\beta$ -CoV<sub>A</sub>), which include mouse hepatitis virus (MHV), bovine coronavirus (BCoV) and the human coronaviruses, HCoV-OC43 and HCoV-HKU1, has about 245 amino acid residues and is also known as p28 (Almeida et al., 2007; Jansson, 2013). The nsp1 of SARS-CoV and Rm1, a SARS-like bat CoV, which belong to the lineage B of  $\beta$ -CoV ( $\beta$ -CoV<sub>B</sub>), contains 180 amino acid residues (Almeida et al., 2007; Jansson, 2013; Tohya et al., 2009). In lineage C  $\beta$ -CoVs ( $\beta$ -CoV<sub>C</sub>), which include MERS-CoV and several bat CoVs, such as HKU4-1, 133

and HKU5-5, the nsp1 is about 195 amino acids in length whereas in lineage D  $\beta$ -CoVs ( $\beta$ -CoV<sub>D</sub>) that includes the bat CoV strains, HKU9-1, HKU9-2, HKU9-3 and HKU9-4, it is about 175 amino acids long (Almeida et al., 2007; Tohya et al., 2009). There is only a limited degree of amino acid sequence homology among the nsp1 of different  $\beta$ -CoVs belonging to these four lineages. For example, SARS-CoV nsp1 has an amino acid sequence similarity of only 20.6% and 17.3% with MHV nsp1 and BCoV nsp1, respectively, whereas it shares a high sequence identity of 92.2% with Rm1, which belongs to the same lineage as SARS-CoV (Tohya et al., 2009). Similarly, the nsp1 of the bat CoV strains, 133 and HKU9-1, share low sequence identities of 19.7% and 30.9% with SARS-CoV nsp1 (Tohya et al., 2009). In MHV and BCoV, nsp1 is released as an N-terminal 28-kDa protein (p28) along with nsp2, a 65-kDa protein (p65) after the proteolytic processing of the ORF1a polyprotein by PL1<sup>PRO</sup> (Denison et al., 1995, 2004). Similarly, in SARS-CoV, PL1<sup>PRO</sup>-mediated cleavage at the consensus cleavage site LXGG in the ORF1a polyprotein liberates the analogous nsp1 and nsp2 as 20-kDa and 70-kDa proteins, respectively (Prentice et al., 2004). Both MHV and SARS-CoV nsp1 are localized exclusively in the cytoplasm of virus-infected cells (Brockway et al., 2004; Kamitani et al., 2006).

Several studies have investigated the biological functions of  $\beta$ -CoV nsp1. MHV nsp1 expression in mammalian cells, including the murine 17Cl-1 and NIH 3T3 cells as well as the human embryonic lung fibroblast LU cells, inhibits cell proliferation and induces cell cycle arrest in G<sub>0</sub>/G<sub>1</sub> phase (Chen et al., 2004). In transient expression studies, like HCoV-229E nsp1, MHV nsp1 also inhibit the expression of reporter genes under the control of constitutive and inducible promoters such as SV40, IFN- $\beta$  and ISRE, suggesting a functional similarity between the nsp1 of CoVs belonging to different phylogenetic lineages (Zust et al., 2007). Additionally, deletion of the C-terminal region of MHV nsp1 abolishes its activity to inhibit reporter gene expression, revealing the importance of this region for its function (Zust et al., 2007). Most importantly, this seminal

study used a recombinant MHV encoding a truncated nsp1, carrying a deletion of 99 nucleotides (nts) in the nsp1-coding sequence, to examine the role of nsp1 in the virulence and pathogenesis of MHV in a murine model of CoV infection (Zust et al., 2007). The replication of this mutant virus, MHV-nsp1 $\Delta$ 99, was similar to wild-type MHV in cultured cells, including primary professional antigen-presenting cells, such as dendritic cells and macrophages, but its growth was severely attenuated *in vivo*, implying that MHV nsp1 is a major virulence factor. Strikingly, compared to the severely attenuated growth of MHV-nsp1 $\Delta$ 99 in wild-type mice, the replication of MHV-nsp1 $\Delta$ 99 was restored almost to the levels of wild-type MHV in type I IFN receptor-deficient mice. These data strongly indicated the role of nsp1 in facilitating the efficient replication of MHV in wild-type mice by counteracting the type I IFN system. Interestingly, inoculation with MHV-nsp1 $\Delta$ 99 also protected mice against challenge with wild-type MHV, highlighting its potential to be developed as a novel type of live-attenuated CoV vaccine (Zust et al., 2007). More evidence for MHV nsp1 as a major virulence factor was provided in a study that examined the function of a relatively conserved amino acid sequence, LLRKxGxKG, in MHV nsp1 that is also found in SARS-CoV nsp1 (Lei et al., 2013). A mutant MHV, MHV-nsp1-27D, carrying a deletion of this sequence in nsp1, has similar growth kinetics in 17Cl-1 cells, but was highly attenuated in mice (Lei et al., 2013). The mechanism by which MHV nsp1 inhibits host gene expression, including the type I IFN system, remains to be elucidated.

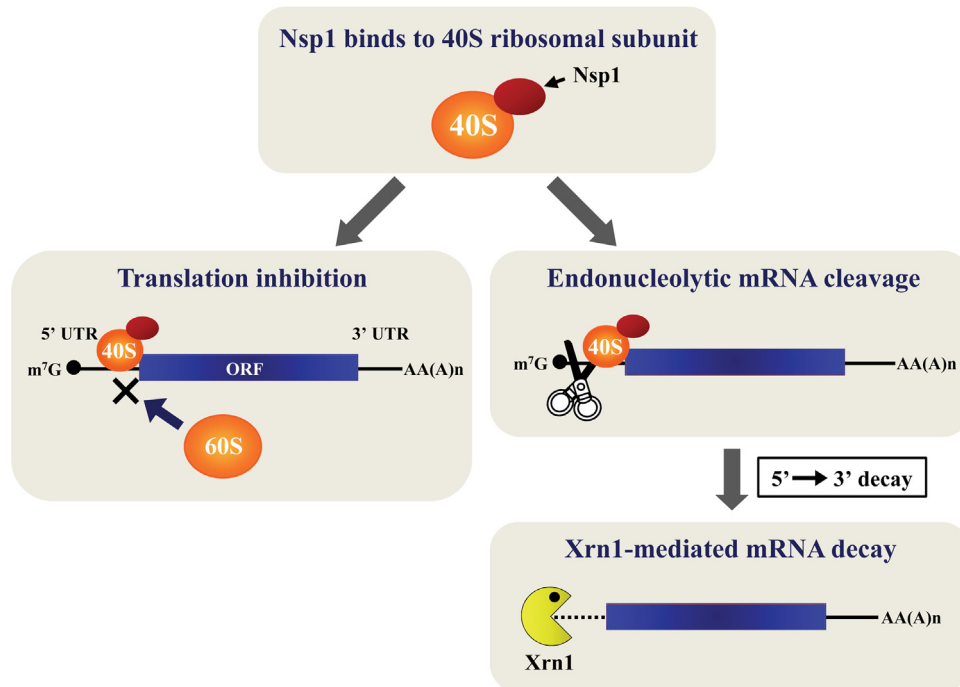
SARS-CoV nsp1 has been the focus of many research efforts, including our group, that have revealed some novel and interesting biological properties. SARS-CoV nsp1 is one of the most well characterized nsp1 of CoVs, both in terms of its biological functions and mode of action. Using transient gene expression in mammalian cells, SARS-CoV nsp1 was the first CoV nsp1 that was shown to block the expression of reporter gene under the control of constitutive promoters as well as the inducible IFN- $\beta$  promoter (Kamitani et al.,

2006). Subsequently, functionally similar activities were demonstrated for other  $\alpha$ -CoV and  $\beta$ -CoV nsp1. A detailed characterization of the mechanism of SARS-CoV nsp1-mediated inhibition of gene expression revealed a novel mode of action and also identified specific amino acid residues in nsp1 that are important for its functions (Huang et al., 2011b; Kamitani et al., 2009; Lokugamage et al., 2012).

SARS-CoV nsp1 employs a two-pronged strategy to inhibit host gene expression by targeting the translation and stability of cellular mRNAs; through its tight association with the 40S ribosomal subunit, a key component of the cellular translation machinery, nsp1 inhibits mRNA translation and also induces an endonucleolytic RNA cleavage in the 5'-UTR of cellular mRNAs (Fig. 3) (Huang et al., 2011b; Kamitani et al., 2009). The outcome of this cleavage is the accelerated turnover of cellular mRNAs as the internally cleaved mRNAs are subsequently degraded by the cellular Xrn1-mediated 5'-3' exonucleolytic mRNA decay pathway (Fig. 3) (Gaglia et al., 2012). The binding of SARS-CoV nsp1 to the 40S ribosomal subunit serves two important roles: it allows nsp1 to inactivate the translation function of the ribosome and also gain access to actively translating host mRNAs. A biologically inactive nsp1, nsp1-mt, carrying the mutations K164A and H165A in the C-terminal region of SARS-CoV nsp1, is unable to bind the 40S subunit, demonstrating the requirement of these amino acid residues and the association of SARS-CoV nsp1 with the 40S subunit for its functions (Narayanan et al., 2008).

Interestingly, SARS-CoV nsp1 does not possess any intrinsic nuclease activity and possibly, recruits a cellular endonuclease for inducing mRNA cleavage (Huang et al., 2011b; Kamitani et al., 2009). The identity of this putative cellular endonuclease is still unknown. Furthermore, SARS-CoV nsp1 induces a template-dependent endonucleolytic cleavage of mRNAs but does not target any specific nucleotide sequence in the mRNA substrate (Huang et al., 2011b). In cell-free *in vitro* translation systems, SARS-CoV nsp1 induces an endonucleolytic RNA cleavage in the 5'-UTR of

### Two-pronged strategy of SARS-CoV nsp1 to inhibit host protein synthesis



**Fig. 3.** Two-pronged strategy of SARS-CoV nsp1 to inhibit host gene expression. SARS-CoV nsp1 gains access to host mRNAs through its association with the 40S ribosomal subunit. Consequently, nsp1 inhibits the translation of capped cellular mRNAs by primarily blocking the steps involved in the formation of elongation-competent 80S initiation complex. Nsp1 also recruits a cellular endonuclease to induce an endonucleolytic RNA cleavage in the 5'-UTR of host mRNAs that subsequently results in the accelerated degradation of the 5'-truncated intermediate by the cellular Xrn1-mediated 5'-3' exonucleolytic mRNA decay pathway.

capped mRNAs and within the ribosome loading region of mRNAs carrying picornavirus type I and type II internal ribosome entry sites (IRESes), whereas mRNAs carrying the IRESes of cricket paralysis virus, hepatitis C virus or classical swine fever virus are resistant to nsp1-induced RNA cleavage (Huang et al., 2011b). It has been proposed that the template-dependent nature of SARS-CoV nsp1-induced mRNA cleavage could be due to differences in the requirement of translation initiation factors and mechanism of translation initiation among capped cellular mRNAs and mRNAs with different IRESes (Huang et al., 2011b). However, the exact mechanism underlying the template-dependent mRNA cleavage by SARS-CoV nsp1 still remains to be clarified.

A nuclear magnetic resonance (NMR) structure of the N-terminal region of SARS-CoV nsp1 revealed some unique structural features, including a complex irregular  $\beta$ -barrel fold (Almeida et al., 2007). This study also alluded to the potential role of positively charged residues exposed on the surface of SARS-CoV nsp1 in the mRNA degradation activity of SARS-CoV nsp1 (Almeida et al., 2007). In line with this possibility, a mutated SARS-CoV nsp1, carrying alanine substitution of the charged residues R124 and K125 that are exposed on the surface of nsp1, lacks the mRNA cleavage function but retains the translation inhibition activity, implying the importance of these residues for the mRNA cleavage property of SARS-CoV nsp1 (Lokugamage et al., 2012). The isolation of this cleavage-defective (CD) mutant of SARS-CoV nsp1, nsp1-CD, demonstrates that the translation inhibition function of SARS-CoV nsp1 is independent and separable from its mRNA cleavage activity (Lokugamage et al., 2012). Furthermore, it was shown that SARS-CoV nsp1 inhibits the translation of mRNAs at the initiation step by targeting multiple stages, depending on the different mechanisms of initiation operating on capped and IRES-driven mRNA templates (Lokugamage et al., 2012).

SARS-CoV nsp1 also blocks the activation of IFN-inducible genes by inhibiting the virus- and IFN-dependent antiviral signaling pathways (Kamitani et al., 2006; Wathelet et al., 2007). The introduction of mutations R124S and K125E in SARS-CoV nsp1 significantly reduced the ability of nsp1 to inhibit the antiviral signaling pathways, further highlighting the importance of these residues for the inhibitory activity of SARS-CoV (Wathelet et al., 2007). An exhaustive mutational analysis of SARS-CoV nsp1, specifically targeting the solvent exposed residues of nsp1, suggested that the inhibition of host gene expression and antiviral signaling pathways could be mediated by distinct but overlapping regions of nsp1 interacting with different host factors (Jauregui et al., 2013). In a genome-wide yeast two-hybrid screen, several members of the immunophilin and calcipressin families were identified as interacting partners of SARS-CoV nsp1 (Pfefferle et al., 2011). This study also showed that nsp1 expression as well as SARS-CoV infection strongly enhanced signaling through the Calcineurin/NFAT pathway, which is modulated by immunophilins and plays an important role in immune cell activation (Pfefferle et al., 2011). Along similar lines, SARS-CoV nsp1 expression induced the secretion of chemokines, such as CCL5, CXCL10 and CCL3, in human lung epithelial cells (Law et al., 2007). However, the involvement of nsp1 in the induction of chemokines in the context of SARS-CoV infection remains to be validated. Nevertheless, these studies suggest a possible role for nsp1 in immune dysregulation, as observed in later stages of SARS.

Most importantly, studies have also investigated the role of SARS-CoV nsp1 in the context of virus replication in cell culture (Narayanan et al., 2008; Wathelet et al., 2007). Notably, experiments using a mutant SARS-CoV (SCoV-mt), encoding the biologically inactive nsp1 (nsp1-mt), showed that nsp1 suppresses the expression of host genes, including the innate immune response genes like type I IFN, ISG15 and ISG56, by inhibiting host protein synthesis and promoting the degradation of host mRNAs in SARS-CoV-infected cells (Narayanan et al., 2008). Furthermore, the

replication of a mutant SARS-CoV, encoding nsp1 with the mutations R124S and K125E, was strongly attenuated in cells with an intact IFN response (Wathelet et al., 2007). Collectively, these studies highlight the role of SARS-CoV nsp1 in regulating the innate immune response during virus infection and also lend further support to the notion that SARS-CoV nsp1 is a potential virulence factor that contributes to viral pathogenesis.

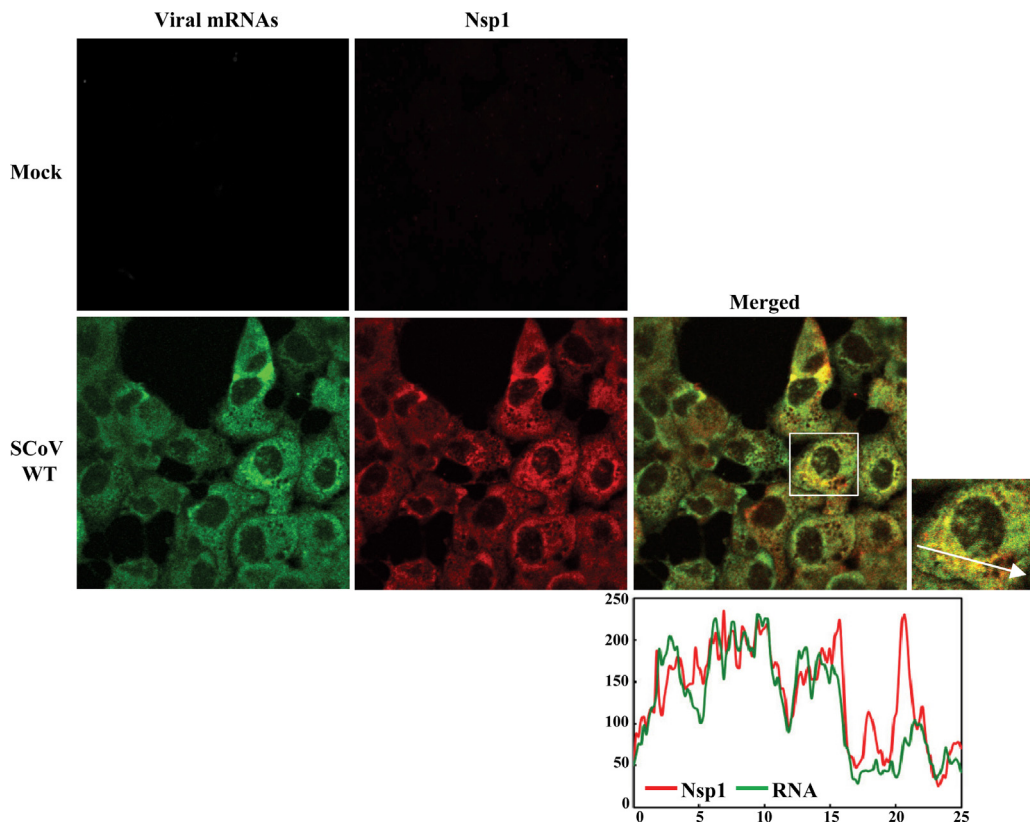
It is worth noting that the nsp1 of bat CoVs belonging to different  $\beta$ -CoV lineages also exhibit functional similarities with SARS-CoV nsp1 (Tohya et al., 2009). Nsp1 of the bat CoV strains, Rm1, 133 and HKU9-1, belonging to  $\beta$ -CoV<sub>B</sub>,  $\beta$ -CoV<sub>C</sub> and  $\beta$ -CoV<sub>D</sub> lineages, respectively, also displayed an ability to inhibit host protein synthesis and promote host mRNA degradation in mammalian cells (Tohya et al., 2009). In addition, expression of these bat CoV nsp1 in *trans* inhibits the induction of type I IFN and IFN-stimulated genes in cells infected with the IFN-inducing SCoV-mt (Tohya et al., 2009). However, these bat CoV nsp1 had differential inhibitory activities, indicating possible differences in their mechanism of action (Tohya et al., 2009). Nevertheless, the evidence of a conserved biological function among the nsp1 of SARS-CoV and bat CoVs in the  $\beta$ -CoV genus could have potentially significant implications, considering the identification of bats as the natural reservoir of several  $\alpha$ -CoVs and  $\beta$ -CoVs, including those closely related to SARS-CoV, and the potential of their virome as the source of emerging human CoVs (Smith and Wang, 2013).

### 3. CoV nsp1: roles in the regulation of viral gene expression

Multiple lines of evidence have suggested the role of CoV nsp1 in regulating viral replication and gene expression. A point mutation, introduced into the TGEV genome by reverse genetics, at the predicted PL1<sup>PRO</sup> cleavage site that severely affected the processing of the ORF1a polyprotein and the release of the PL1<sup>PRO</sup> cleavage products, including nsp1, also caused a drastic reduction in the efficiency of infectious TGEV rescue from cDNA (Galan et al., 2005). In addition, the recovered viruses had a small-plaque-size phenotype and also showed a rapid reversion of the introduced mutation to the original wild type TGEV sequence (Galan et al., 2005). These data suggested the importance of the proteins liberated by PL1<sup>PRO</sup>-mediated cleavage, which includes nsp1, in TGEV replication.

Mutagenesis of the coding region of MHV nsp1 in the viral genome, using reverse genetics, identified two domains in nsp1 that are important for virus replication (Brockway and Denison, 2005). Deletions in the N-terminal half of nsp1 were lethal and infectious viruses could not be recovered (Brockway and Denison, 2005). Furthermore, introduction of point mutations in the N-terminal region of nsp1 resulted in the recovery of viable MHV mutants that exhibited replication defects in cell culture, suggesting the presence of critical replication determinants within the N-terminal half of MHV nsp1 that are required for optimal virus replication (Brockway and Denison, 2005). It should be noted that these replication elements could be an RNA structure formed by sequences within this region of MHV nsp1 and/or a functional moiety in the nsp1 protein. Mutations in the C-terminal half of MHV nsp1, harboring the PL1<sup>PRO</sup> cleavage site, that abolished the release of nsp1 from the ORF1a polyprotein showed that the cleavage between nsp1 and nsp2 is not required for virus replication (Brockway and Denison, 2005; Denison et al., 2004). However, the rescued MHV mutants formed small plaques and exhibited delayed growth kinetics with reduced viral RNA synthesis and peak viral titers, indicating the importance of the PL1<sup>PRO</sup>-released proteins, including nsp1, in MHV replication (Brockway and Denison, 2005; Denison et al., 2004). The potential role of nsp1 in MHV replication is also suggested by the localization of MHV nsp1 to viral replication complexes during virus infection and its interaction with nsp7





**Fig. 4.** Viral mRNAs are not spatially separated from nsp1 in SARS-CoV-infected cells. RNA fluorescent in situ hybridization (RNA-FISH) and confocal microscopy analyses of viral mRNAs and nsp1 in SARS-CoV-infected cells. Vero E6 cells, grown on 4-well Lab-Tek II chamber slides (Nalge Nunc International), were either mock-infected (Mock) or infected with wt SARS-CoV (SCoV-WT) at a multiplicity of infection (MOI) of 5. At 12 h p.i., the cells were fixed in 4% paraformaldehyde for 16 h at 4 °C and permeabilized with 0.5% Triton X-100 for 5 min at room temperature. Subsequently, the cells were precipitated with 70% ethanol at 4 °C overnight and subjected to RNA-FISH and immunofluorescence analyses. A digoxigenin (DIG)-labeled antisense riboprobe, corresponding to the nucleotides (nt) 29,084–29,608 at the 3'-end of the SARS-CoV genome, was used to detect viral mRNAs, including mRNAs 1–9. Viral mRNAs (green) were visualized with a primary sheep anti-DIG antibody and Alexa488-conjugated anti-sheep secondary antibody (Invitrogen). Nsp1 (red) was visualized using an affinity-purified rabbit anti-nsp1 polyclonal antibody, which was raised by immunizing rabbits with purified full-length nsp1 protein, followed by Alexa594-conjugated secondary antibody (Invitrogen). Images were collected using a Zeiss LSM-510 META confocal microscope with a 63 $\times$ , 1.40 numerical aperture oil immersion lens and processed with the LSM image browser and Metamorph software (Molecular Devices, Downingtown, PA). In the lower panel (SCoV-WT), the inset to the right of the merged image represents the indicated region (small square panel) in the merged image. The histogram displays the fluorescence signal intensities along the white arrow in the inset, which is a representative line for a single line scan showing the fluorescence signal intensity (y axis) in both channels (red and green) in an arbitrary scale versus distance (x axis) (in pixel) over that line.

and nsp10, two ORF1a-derived proteins that have been shown to play critical roles in regulating viral RNA synthesis and polyprotein processing (Brockway et al., 2004; Deming et al., 2007). MHV nsp1 is associated with intracellular membranes and displays a temporal pattern of subcellular localization during virus infection (Brockway et al., 2004). At early times postinfection, MHV nsp1 colocalizes with viral proteins that are components of the replication complex that include nsp7, nsp10, nsp12 (RNA-dependent RNA polymerase), nsp13 (helicase), and the viral nucleocapsid protein, N (Brockway et al., 2004). At late times postinfection, MHV nsp1 localizes to sites of M protein accumulation, hinting at its possible role in virion assembly (Brockway et al., 2004).

BCoV nsp1 has been shown to be an RNA-binding protein that interacts with cis-acting replication elements in the 5' untranslated region (UTR) of the BCoV genome, implying its potential role in the regulation of viral translation or replication (Gustin et al., 2009). SARS-CoV nsp1 also binds to a stem-loop structure, SL1, in the 5'-UTR of SARS-CoV genome and it has been suggested that this interaction enhances virus replication (Tanaka et al., 2012). In both BCoV and MHV, there is evidence of a long-range RNA-RNA interaction between the 5'-UTR and the coding region of nsp1 (Guan et al., 2012). Furthermore, synthesis of a nascent BCoV nsp1 protein carrying the N-terminal amino acid sequence WAPEFPWM, which is conserved among the nsp1 of  $\beta$ -CoV<sub>A</sub>, is required in *cis* for BCoV

defective interfering (DI) RNA replication (Su et al., 2014). Taken together, these studies point toward a functional link between nsp1 and CoV replication.

The effect of SARS-CoV nsp1 on viral gene expression has been studied primarily using cell-free *in vitro* translation systems (Huang et al., 2011b; Lokugamage et al., 2012). Strikingly, nsp1 efficiently inhibits the translation of SARS-CoV mRNAs in RRL and HeLa extracts but unlike host mRNAs, nsp1 does not induce the endonucleolytic cleavage of viral mRNAs in the RRL system (Huang et al., 2011b); the presence of the 5'-end leader sequence, a common feature of all the viral mRNAs, protects the viral mRNAs from nsp1-induced RNA cleavage (Huang et al., 2011b). Nsp1 inhibits the translation of SARS-CoV mRNAs at the initiation stage by primarily blocking the steps involved in the conversion of the 48S initiation complex into the elongation-competent 80S initiation complex (Lokugamage et al., 2012).

To clarify the effect of nsp1 on viral mRNA translation in SARS-CoV-infected cells and gain a better understanding of the interplay between viral and host gene expression in SARS-CoV infection, we examined the effect of nsp1 on viral gene expression in SARS-CoV-infected cells.

To investigate the possibility that viral mRNAs are spatially separated from nsp1 in infected cells that would facilitate the escape of viral mRNAs from the nsp1-induced translation

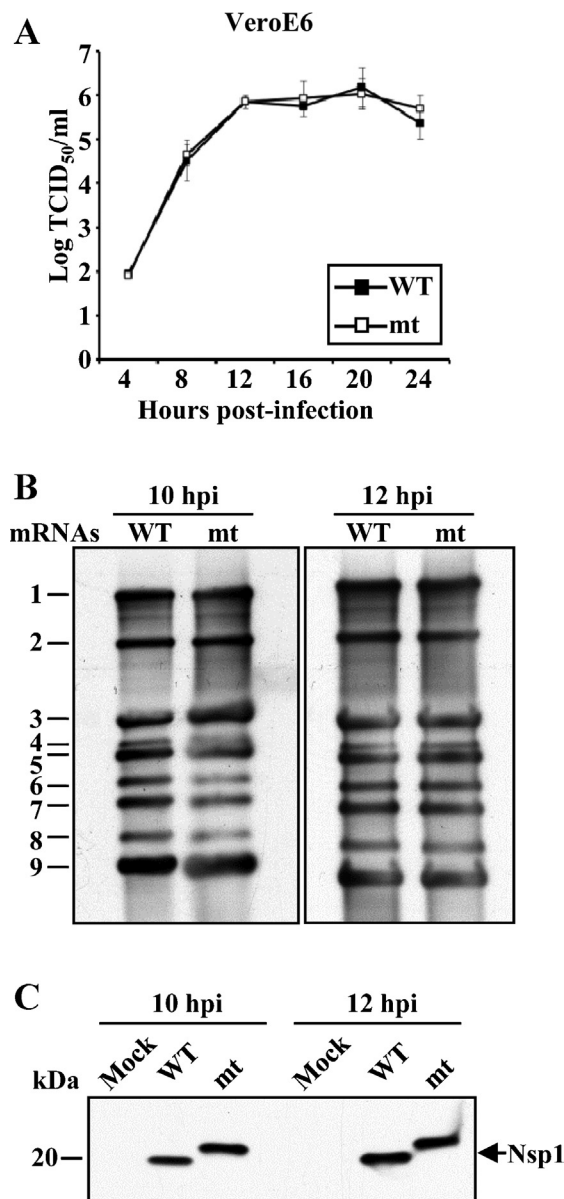


inhibition, we examined the localization of viral mRNAs and nsp1 in SARS-CoV-infected cells by combining RNA fluorescent in situ hybridization (RNA-FISH) and immunofluorescence analyses to detect viral mRNAs and nsp1, respectively (Fig. 4). No signals were detected in mock-infected cells (Fig. 4, upper panel), confirming the specificity of the riboprobe and anti-nsp1 antibody. In SARS-CoV-infected cells, we observed areas of colocalization of nsp1 with viral mRNAs, as demonstrated by the similar fluorescence histogram patterns of viral mRNAs and nsp1 (Fig. 4, lower panel). This data implied that nsp1 was not excluded from the sites of viral mRNA translation in SARS-CoV-infected cells.

The above data led us to hypothesize that viral mRNA translation is also susceptible to inhibition by nsp1 in SARS-CoV-infected cells. This hypothesis predicts that the translation of viral mRNAs would be less efficient in SARS-CoV-infected cells than in SARS-CoV-mt-infected cells, because of the abortive translation of viral mRNAs, caused by the translationally-inactive 48S-nsp1 complex, and the reduced pool of biologically active 40S subunits in SARS-CoV-infected cells, due to nsp1-induced inactivation of the 40S subunit. Both SARS-CoV and SARS-CoV-mt displayed similar replication kinetics with similar virus yields and levels of accumulation of viral mRNAs (Fig. 5A and B). While the level of nsp1-mt was marginally higher than nsp1 at 10 h postinfection (p.i.), the levels of nsp1 and nsp1-mt were similar at 12 h p.i. (Fig. 5C). As described previously (Narayanan et al., 2008), nsp1-mt migrated slightly slower than nsp1 (Fig. 5C).

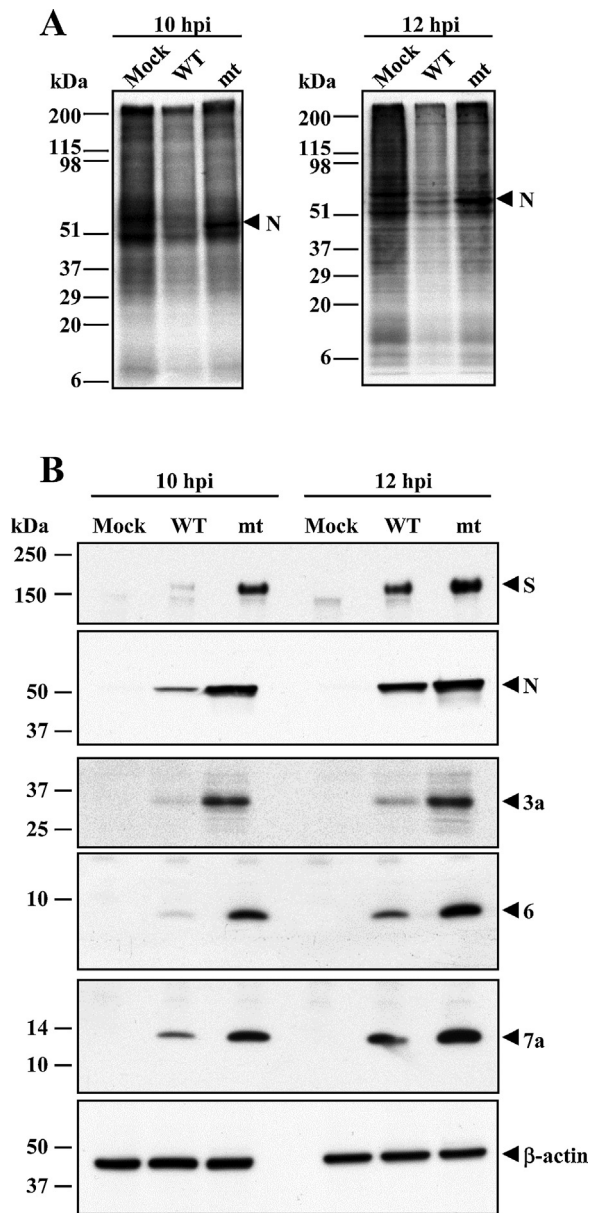
Next, we performed metabolic pulse-radiolabeling experiments to tease out the effect of nsp1 on viral mRNA translation in infected cells. SARS-CoV replication induced a more prominent inhibition of host protein synthesis than SARS-CoV-mt replication in infected cells, as reported previously (Fig. 6A) (Narayanan et al., 2008). The synthesis of the major viral structural protein, N, was markedly lower in SARS-CoV-infected cells than that in SARS-CoV-mt-infected cells at both 10 h and 12 h p.i. (Fig. 6A), despite the similar levels of accumulation of mRNA 9, encoding N protein, in SARS-CoV and SARS-CoV-mt-infected cells (Fig. 5B). These data strongly indicated that nsp1, but not nsp1-mt, inhibited the translation of mRNA 9. Radiolabeled M protein, another major viral structural protein, was not detected due to M protein aggregation, caused by the incubation of samples at 100 °C for 15 min in SDS-sample buffer for the complete inactivation of SARS-CoV infectivity (Lee et al., 2005; Sturman et al., 1980). Western blot analysis of the cell extracts clearly showed that the levels of the viral structural proteins, N and S, and the accessory proteins, 3a, 6 and 7a, were lower in SARS-CoV-infected cells than in SARS-CoV-mt-infected cells (Fig. 6B). In summary, despite the similar levels of accumulation of viral mRNAs encoding these proteins in SARS-CoV and SARS-CoV-mt-infected cells, the synthesis of N protein and the accumulation of viral structural and accessory proteins were markedly lower in SARS-CoV-infected cells than in SARS-CoV-mt-infected cells. These data support our hypothesis that nsp1 inhibits the translation of viral mRNAs in SARS-CoV-infected cells.

Our seemingly counter-intuitive finding that SARS-CoV nsp1 inhibited the translation of viral mRNAs in SARS-CoV-infected cells is nevertheless consistent with the following observations. Nsp1 was not spatially separated from viral mRNAs in SARS-CoV-infected cells (Fig. 4). Nsp1 inhibits the translation of SARS-CoV mRNA 9 in RRL primarily by blocking the steps involved in the conversion of the 48S complex into the 80S complex, which suggests that the nsp1-40S complex can load onto viral mRNAs to form the 48S complex in infected cells (Lokugamage et al., 2012). Nsp1 associates tightly with the 40S subunit, indicated by the resistance of this interaction to stringent high-salt treatment conditions that is known to cause the dissociation of translation initiation factors and the 60S subunit from the 40S subunit (Kamitani et al., 2009; Merrick, 1979); this data suggests that the inactivation of 40S



**Fig. 5.** Viral growth kinetics and mRNA accumulation are similar in SARS-CoV and SARS-CoV-mt-infected cells. Vero E6 cells were either mock-infected (Mock) or infected with wt SARS-CoV (WT) or SARS-CoV-mt (mt), carrying the mutations K164A and H165A in nsp1, at an MOI of 5. (A) Culture supernatants were collected at the indicated times p.i., and virus titers were determined by TCID<sub>50</sub> analysis in Vero E6 cells. The results represent the average of three independent experiments. (B) Total RNAs were extracted at 10 h and 12 h p.i. The viral mRNAs were detected by Northern blot analysis using a riboprobe that binds to the 3'-end of SARS-CoV genome, as described in the legend for Fig. 1. Representative data from three independent experiments are shown. (C) At 10 h and 12 h p.i., total proteins were extracted, and Western blot analysis was performed to detect Nsp1 protein using a rabbit anti-nsp1 polyclonal antibody. Representative data from three independent experiments are shown.

subunit by nsp1 could lead to a reduction in the pool of biologically active 40S subunits in infected cells. Based on these data, we propose that the non-productive interaction of nsp1-40S complex with viral mRNAs in infected cells that leads to abortive translation and the nsp1-induced reduction in the pool of biologically active 40S subunits both contribute to the inhibition of viral mRNA translation by nsp1 in SARS-CoV-infected cells. Importantly, because viral mRNAs are resistant to nsp1-induced mRNA cleavage (Huang et al., 2011b), the translationally-competent intact viral mRNAs



**Fig. 6.** SARS-CoV nsp1 inhibited the translation of viral mRNAs in infected cells. Vero E6 cells were either mock-infected (Mock) or infected with wt SARS-CoV (WT) or SARS-CoV-mt (mt), carrying the mutations K164A and H165A in nsp1, at an MOI of 5. (A) Cells were radiolabeled for 15 min with 500  $\mu$ Ci of Tran[ $^{35}$ S] label (MP Biomedicals) at 10 h and 12 h p.i. Equivalent amounts of the intracellular proteins were analyzed on a 12% SDS-PAGE and visualized by autoradiography. Arrowhead indicates the position of N protein. Representative data from three independent experiments are shown. (B) Total proteins, extracted at 10 h and 12 h p.i. in (A), were subjected to Western blot analysis to detect the viral structural and accessory proteins, S, N, 3a, 6 and 7a. Anti-SARS-CoV S antibodies (IMG-541; Imgenex and AP6000a; Abgent) and anti-SARS-CoV N antibody (IMG-548; Imgenex) were used to detect S and N proteins, respectively. Rabbit anti-S-CoV 3a, 6 and 7a antibodies were used to detect 3a, 6 and 7a proteins, respectively, as described previously (Huang et al., 2006a,b, 2007). Anti-actin antibody (Santa Cruz Biotechnology) was used to detect  $\beta$ -actin and the detection of similar amounts of  $\beta$ -actin in both SARS-CoV and SARS-CoV-mt-infected cells confirmed the loading of similar amounts of cell extracts in each lane. Representative data from three independent experiments are shown.

are most probably translated by the biologically active nsp1-free 40S subunits to produce viral proteins in SARS-CoV-infected cells.

Both SARS-CoV and SARS-CoV-mt exhibited similar replication kinetics with similar virus yields, despite the reduced level of accumulation of viral structural and accessory proteins in SARS-CoV-infected cells compared to SARS-CoV-mt-infected cells. These

data imply that even though nsp1 inhibited viral mRNA translation, the reduced levels of viral structural proteins in SARS-CoV-infected cells are still sufficient and above the threshold of viral proteins required for optimal virus replication and assembly. In contrast to the structural and accessory proteins, the levels of accumulation of nsp1 and viral mRNAs were similar in SARS-CoV- and SARS-CoV-mt-infected cells. We speculate that the efficient synthesis of nsp1, which is translated from mRNA 1, the viral genomic RNA, as part of the replicase polyproteins, occurs prior to the translation of viral subgenomic mRNAs encoding the viral structural and accessory proteins, and during the early stages of infection when mRNA 1 is undergoing translation, the levels of nsp1 are not sufficient to inhibit the synthesis of replicase proteins, including nsp1, resulting in the similar accumulation of nsp1 and viral mRNAs in SARS-CoV and SARS-CoV-mt-infected cells.

A study by Tanaka et al. suggested that a specific interaction of nsp1 with the 5' untranslated region (UTR) of SARS-CoV mRNA protects viral mRNAs from nsp1-mediated translational shutoff in SARS-CoV-infected cells (Tanaka et al., 2012). In addition, the authors also speculated that nsp1 promotes viral protein synthesis and viral RNA replication through this interaction because this effect was not observed with a mutated nsp1 protein, carrying R124A mutation that abolished its interaction with the 5' UTR of viral mRNA (Tanaka et al., 2012). Our data that viral protein synthesis and accumulation are lower in SARS-CoV-infected cells than in SARS-CoV-mt-infected cells do not support the possibility that the interaction of nsp1 with viral mRNA augments viral mRNA translation, because the putative nsp1-mediated enhancement of viral protein synthesis would have resulted in a more efficient production of viral proteins in SARS-CoV-infected cells than in SARS-CoV-mt-infected cells. Based on our studies with the cleavage-defective nsp1 mutant, nsp1-CD, carrying R124A and K125A mutations, the nsp1R124A mutant most probably lacked the host mRNA cleavage function but retained the ability to bind and inactivate the 40S subunit (Lokugamage et al., 2012). Accordingly, in the study by Tanaka et al., the nsp1-mediated enhancement of viral protein synthesis could be an indirect consequence of the degradation of host mRNAs, induced by nsp1 but not by the nsp1R124A mutant, that would eliminate the competition between viral and host mRNAs for the limiting amounts of translationally-competent 40S subunits, thereby tilting the balance in favor of viral mRNA translation. Furthermore, the nsp1-induced degradation of host mRNAs could also liberate the translation initiation factors from host mRNAs that can be utilized by the intact viral mRNAs in SARS-CoV-infected cells. Therefore, it is conceivable that the cleavage of host mRNAs by nsp1 and the resistance of viral mRNAs to nsp1-induced RNA cleavage are strategies that SARS-CoV could have evolved to compensate for the inhibition of viral mRNA translation by nsp1 thereby facilitating the production of viral proteins in SARS-CoV-infected cells.

#### 4. Concluding remarks

The accumulated knowledge of CoV nsp1 has revealed conserved functions and divergent mechanisms among different CoVs to block host gene expression and antagonize host innate immune responses that provide an insight into the expanding repertoire of novel viral strategies of immune evasion. In addition, it also highlights functionally significant correlations between the nsp1 of CoVs belonging to different genera, despite the lack of obvious primary sequence homology with each other, suggesting their evolutionary relatedness and role in the adaptation of CoVs to different host species. The fact that nsp1 of different CoVs share a common biological function to inhibit host gene expression, but use different modes of action to exert this function, has also raised some

important questions about the impact of these functions and divergent mechanisms on the virulence and pathogenesis of emerging human CoVs. For example, what is the contribution of the mRNA degradation activity of SARS-CoV nsp1 to the virulence of SARS-CoV? Studies to examine the role of nsp1 as a potential virulence factor *in vivo* are now feasible with the availability of SARS-CoV nsp1 mutants and suitable mouse model systems, which will yield valuable information for the rational design of live-attenuated vaccines against CoVs (McCray et al., 2007; Roberts et al., 2007; Tseng et al., 2007). Also, does the nsp1 of MERS-CoV, another highly pathogenic human CoV belonging to a different  $\beta$ -CoV lineage,  $\beta$ -CoV<sub>C</sub>, possess similar functions, including the mRNA cleavage property, as SARS-CoV nsp1? A recent study comparing the regulation of global ISG responses by highly pathogenic respiratory viruses, including SARS-CoV and MERS-CoV, has suggested a possible mechanistic divergence among the two CoVs in antagonizing the host IFN response (Menachery et al., 2014). It will be of great interest to explore the role of MERS-CoV nsp1 in modulating the host immune responses during MERS-CoV infection. Overall, CoV nsp1, with its intriguing properties and characteristics, is an exciting avenue for future research that could potentially lead to the discovery of novel players and pathways of host gene regulation.

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