



Genome-Wide Analysis of the Indispensable Role of Non-structural Proteins in the Replication of SARS-CoV-2

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Understanding the process of replication and transcription of SARS-CoV-2 is essential for antiviral strategy development. The replicase polyprotein is indispensable for viral replication. However, whether all nsps derived from the replicase polyprotein of SARS-CoV-2 are indispensable is not fully understood. In this study, we utilized the SARS-CoV-2 replicon as the system to investigate the role of each nsp in viral replication. We found that except for nsp16, all the nsp deletions drastically impair the replication of the replicon, and nsp14 could recover the replication deficiency caused by its deletion in the viral replicon. Due to the unsuccessful expressions of nsp1, nsp3, and nsp16, we could not draw a conclusion about their *in trans*-rescue functions. Our study provided a new angle to understand the role of each nsp in viral replication and transcription, helping the evaluation of nsps as the target for antiviral drug development.

Keywords: SARS-CoV-2, nsp, viral replication and transcription, indispensable role, replicon

INTRODUCTION

The ongoing pandemic of COVID-19 caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has posed a serious threat to human health and led to heavy economic loss (V'Kovski et al., 2021). The relatively limited knowledge of this deadly virus hinders us from efficiently treating patients with COVID-19 (Feng et al., 2020). Besides the recognition and entry process (Lan et al., 2020; Yin et al., 2022), the viral replication and transcription process regulated by viral replicase proteins possesses many promising targets for antiviral strategy development (V'Kovski et al., 2021; Malone et al., 2022). Thus, the systematical analysis on the roles of proteins derived from viral replicase is an important biomedical objective.

SARS-CoV-2, belonging to the *Nidovirales* order, the *Coronaviridae* family, and the *Betacoronavirus* genus (Coronaviridae Study Group of the International Committee on Taxonomy of Viruses, 2020), is an enveloped positive-strand RNA virus. Its genome contains at least nine open reading frames (ORFs). The 5'-terminal two-thirds of the viral genome contains two open reading frames, ORF1a and ORF1ab (Chen et al., 2020). The latter is translated by a -1 ribosomal frameshifting mechanism, in which the translational complex avoids the stop codon by altering the reading frame while encountering the "slippery" sequence at the terminus of ORF1a (Pan et al., 2008). The translational products of ORF1a and ORF1ab, polyprotein 1a (pp1a) and polyprotein

1ab (pp1ab), are responsible for the viral replication and transcription. Before going to its final roles, pp1a and pp1ab are processed up to 16 non-structural proteins (nsps) by their own proteases, papain-like protease (PLpro) located in nsp3 and 3C-like protease (3CLpro) or main protease (Mpro) of nsp5 (Pan et al., 2008; Du et al., 2021; Jiang et al., 2021).

Nsp1 (approximately 180 aa in SARS-CoV-2) is a multifunctional protein capable of altering host translation (Narayanan et al., 2008a; Kamitani et al., 2009; Lokugamage et al., 2012), triggering host mRNA cleavage (Kamitani et al., 2009; Huang et al., 2011) and decay (Kamitani et al., 2006; Narayanan et al., 2008b), inhibiting the innate immune response (Zust et al., 2007; Narayanan et al., 2008b), and inducing cytokines and chemokines (Law et al., 2007). By introducing deletions in murine hepatitis viruses (MHV) and the analysis of mutant MHV, another member of the *Betacoronavirus* genus, Brockway and Denison (2005) identified a few residues in nsp1 important for viral RNA synthesis and replication, and viral protein processing. The SARS-CoV-2 nsp1 is an evolving protein, as, besides mutations, two deletions in nsp1 were identified, including the deletion of 686–694 nt (Benedetti et al., 2020) and the deletion of 500–532 nt (Lin et al., 2021b).

Nsp2's (approximately 638 aa in SARS-CoV-2) function is not well defined, while it may be involved in regulating host intracellular signaling through the interaction with prohibitin 1 (PHB1) and PHB2 (Cornillez-Ty et al., 2009). SARS-CoV-2 with the variation of nsp2 (T85I) may lead to poor replication in Vero-CCL81 cells (Pohl et al., 2021). Furthermore, the genetic deletions of nsp2 on the reverse genetics systems of MHV and SARS-CoV lead to attenuated viral growth and RNA synthesis (Graham et al., 2005).

Nsp3 (approximately 1945 aa in SARS-CoV-2) is a large multi-domain protein. It encompasses multiple functional domains, including ubiquitin-like domain, single-stranded poly(A) binding domain, C-terminal SARS-Unique domain, PLpro domain, nucleic acid-binding domain, and two transmembrane helix motifs (Jiang et al., 2021). The PLpro activity of nsp3 is responsible for releasing nsp1, nsp2, and nsp3 *per se* from the pp1a or pp1ab. Thus, it is recognized as a promising target for antiviral drug development (Shin et al., 2020). Besides protease activity, nsp3 has deubiquitinating and interferon antagonism activities (Clementz et al., 2010) and may regulate the viral replication by interacting with viral nsps, including nsp1, nsp4, nsp6, nsp10, nsp12, nsp13, nsp14, and N (Baez-Santos et al., 2015; Jiang et al., 2021). Nsp3 could also benefit viral replication by improving the inter/intra-cellular microenvironment. SARS-CoV-2 nsp3 delays the expression of IFN- β (Lei et al., 2020).

Nsp4 (approximately 500 aa in SARS-CoV-2) also has multiple transmembrane domains. Nsp4 of SARS-CoV interacts with nsp3, contributing to the endoplasmic reticulum (ER) membrane rearrangement and the assembly of double-membrane vesicles (DMVs), which play an essential role in viral replication and transcription (Sakai et al., 2017). The essential role of nsp4 was also supported by the sequence analysis on patient samples, and the nsp4 variant (E3073A) of SARS-CoV-2 is associated with a significantly reduced fever duration (Zekri et al., 2021).

Nsp5 (approximately 306 aa in SARS-CoV-2) is one of the most widely studied proteins of SARS-CoV-2. Nsp5 plays a crucial role in the maturation of viral replicase polyprotein by cleaving pp1ab at 11 sites and subsequently releasing nsp4 to nsp16 for the assembly of replication and transcription complex (RTC) (Jin et al., 2020). Nsp5 is recognized as a promising target for antiviral drug development, and more than 86 potential inhibitors of nsp5 were selected by various studies (Yan and Gao, 2021). Its structures with or without inhibitors are resolved by a myriad of studies (Mariano et al., 2020).

Nsp6 (approximately 290 aa in SARS-CoV-2) also has transmembrane domains. Together with nsp3 and nsp4, nsp6 contributes to the formation of DMV (Angelini et al., 2013). Different from the function of nsp3-nsp4 complex in pairing membranes, nsp6 majors in membrane proliferation. Nsp6 was shown to limit autophagosome expansion (Cottam et al., 2014).

Nsp7 (approximately 83 aa in SARS-CoV-2) and nsp8 (approximately 198 aa in SARS-CoV-2) function as the cofactors for RTC (Kirchdoerfer and Ward, 2019). The involvement of nsp7 and nsp8 in RTC goes through a transition from nsp7–nsp8 hexadecameric primase complex to the nsp12–nsp7–nsp8 polymerase complex, promoting RdRP efficiency of viral RNA product synthesis (Wang et al., 2020).

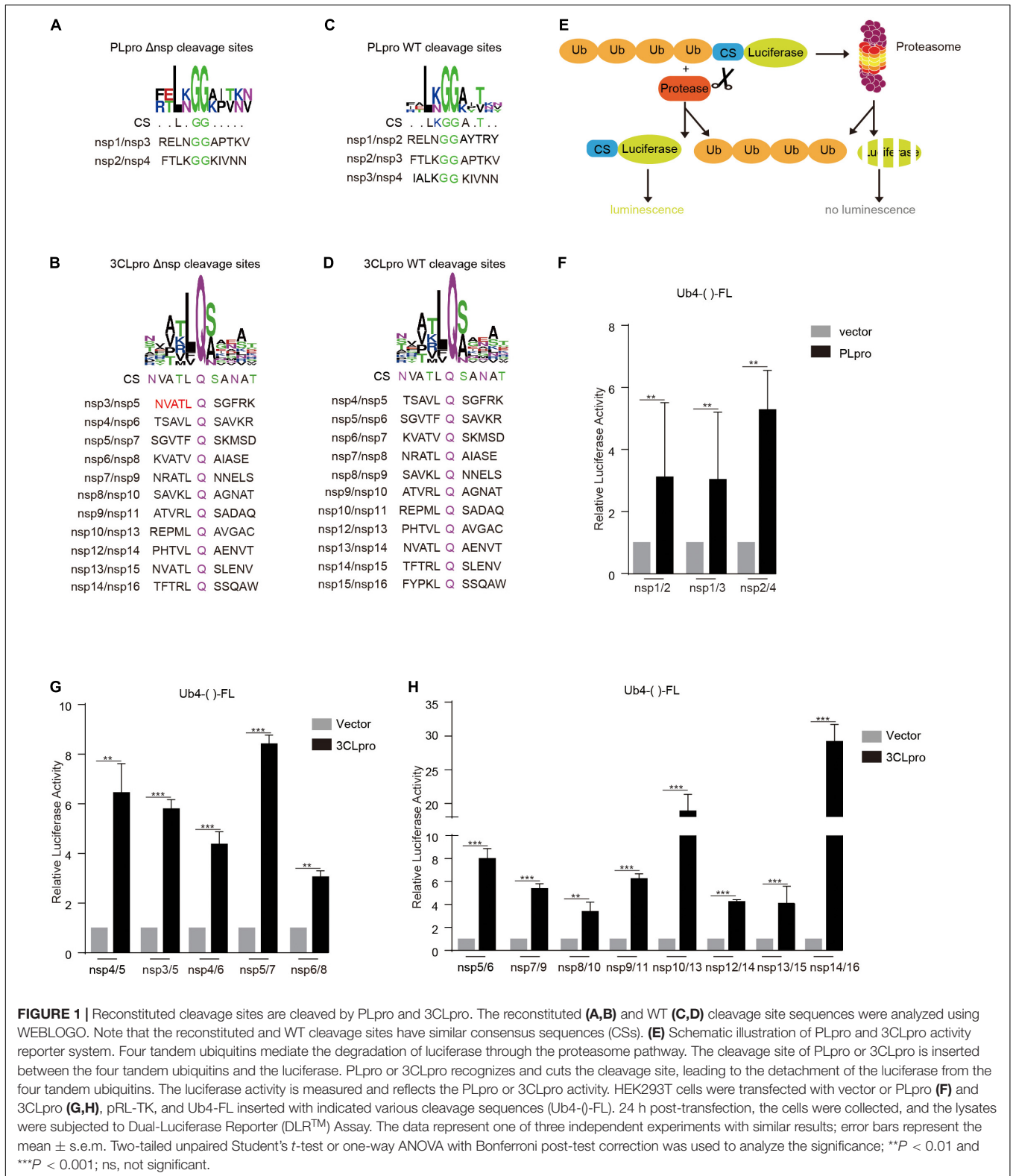
Nsp9 (approximately 113 aa in SARS-CoV-2) plays a vital role in the replication of SARS-CoV-2 through its activity in ssRNA/DNA binding ability (Littler et al., 2020), which is regulated by its dimerization (de et al., 2021) and NMPylation on its conserved site (Slanina et al., 2021). SARS-CoV-2 nsp9 stimulates type I interferon response (Lei et al., 2020).

Nsp10 (approximately 139 aa in SARS-CoV-2) was identified as an interaction partner of nsp14 and nsp16 by the genome-wide screening of intraviral protein–protein interactions (Pan et al., 2008). Nsp10 interacts with nsp14 (Lin et al., 2021a) and nsp16 (Krafcikova et al., 2020) to promote their 3'–5' exonuclease and RNA ribose-2'-O-methylation activities, respectively.

Nsp11 (approximately 13 aa in SARS-CoV-2) is a cleavage product of pp1a processed by Mpro at the nsp10/11 boundary. Nsp11 shares the same first nine amino acids with nsp12 and exhibits an intrinsically disordered protein behavior (Gadhavie et al., 2021).

Nsp12 (approximately 932 aa in SARS-CoV-2), the RNA-dependent RNA polymerase (RdRP) of SARS-CoV-2, forms the RTC with nsp7 and nsp8 (Wang et al., 2020). Mutations, S759A/D760A/D761A, at the key residues in nsp12 diminished the viral replication (Jin et al., 2021). More than 95% identical to SARS-CoV counterpart, nsp12 of SARS-CoV-2 exhibits a similar sensitivity to the inhibitory effect of remdesivir, and the decreased enzymatic activity and thermostability (Peng et al., 2020). Besides RdRP activity, SARS-CoV-2 nsp12 is responsible for viral RNA capping as a GTPase, adding a GTP nucleotide to the 5' end of viral RNA via a 5'-5' triphosphate linkage (Walker et al., 2021).

Nsp13 (approximately 601 aa in SARS-CoV-2) possesses RNA helicase and the nucleoside triphosphate hydrolase (NTPase) activities, unwinding viral RNA duplex and supplying the energy for unwinding by hydrolyzing ATP, respectively, in the replication of viral RNAs (Shu et al., 2020). Thus, RNA helicase activity of nsp13 is sensitive to the concentration of ATP. The



increased ATP concentrations promote the processivity of nsp13 in unwinding duplex RNA (Jang et al., 2020). Two mutations, Y541C and P504L, from variants of SARS-CoV-2, located in

the nucleotide-binding core of nsp13, weaken the transmission capacity of SARS-CoV-2, indicating the crucial role of nsp13 in viral replication (Wang et al., 2021).

Nsp14 (approximately 527 aa in SARS-CoV-2) has 3'-5' exoribonuclease (ExoN) and N7-guanine methyltransferase (N7-MTase) activities, which are born by N-terminal and C-terminal domains, respectively (Chen et al., 2009). The nsp14 ExoN activity is a key component of the RNA proofreading machinery, which is proposed to be essential for the stability and replication efficiency of viral genome (Becares et al., 2016). Nsp10 interacts with the N-terminal domain of nsp14, promoting ExoN activity (Ma et al., 2015). Its N7-MTase activity plays a crucial role in the synthesis of viral mRNA cap, preventing the recognition by the host cell (Becares et al., 2016). Both enzymatic activities of nsp14 are crucial for viral replication, and the mutations of D90A/E92A and D331A, impairing the ExoN and N7-MTase, respectively, drastically decreased the generation of viral genomic/subgenomic RNAs in viral replicon system (Jin et al., 2021).

Nsp15 (approximately 346 aa in SARS-CoV-2) is a uridine-specific endoribonuclease (EndoU) whose activity resides in its C-terminal domain (Frazier et al., 2021). Nsp15 plays a crucial role in viral replication, because the inactivation of EndoU by introducing mutations in nsp15 and the deletion in nsp15 drastically decreased the viral replication and recovery (Ivanov et al., 2004; Almazan et al., 2006; Kang et al., 2007; Nguyen et al., 2021).

Nsp16 (approximately 298 aa in SARS-CoV-2) mediates 2'-O-methylation of viral RNA cap structure, preventing the degradation by host nucleases (Krafcikova et al., 2020; Wilamowski et al., 2021). To perform its MTase activity, nsp16 requires nsp10 as a stimulatory factor to bind its m7GpppA-RNA substrate and S-adenosyl-L-methionine (SAM), methyl donor (Chen et al., 2011). In contrast, nsp10 also interacts with nsp14, another MTase of SARS-CoV-2, but is not required for MTase activity of nsp14 (Ma et al., 2015). SARS nsp16 plays a crucial role in the viral replication, because the elimination of the nsp16 expression by introducing the stop codon at its 5' end largely attenuated the viral replication (Almazan et al., 2006; Rohaim et al., 2021).

As summarized above, the functional characterization of nsps indicates their potential involvement in viral replication and transcription. A number of studies showed that the overexpression of some nsps could further promote the replication level of viral reverse genetics systems, indicating that these nsps play essential roles in viral replication but possibly are not sufficient in the viral replication complex, which is composed of the nsps processed from replicase polyprotein (Jin et al., 2021; Luo et al., 2021).

In this study, to clarify whether each nsp is indispensable for viral replication and transcription, we examined the replication of the viral replicon with the deletion of each nsp. To minimize the undesired impact of each nsp deletion on the viral polyprotein process, we reconstructed cleavage sites recognized by PLpro and 3CLpro and confirmed the cleavage efficiency using our PLpro/3CLpro activity reporter system. By monitoring the replication activity of viral replicons with each nsp deletion, we found that the dependencies of viral replication on each nsp varied considerably, and nsp14 can rescue the decreased replication of viral replicon caused by the loss of nsp14 *per se*.

RESULTS

The Reconstituted Cleavage Sites Can Be Efficiently Cleaved by Papain-Like Protease and 3C-Like Protease

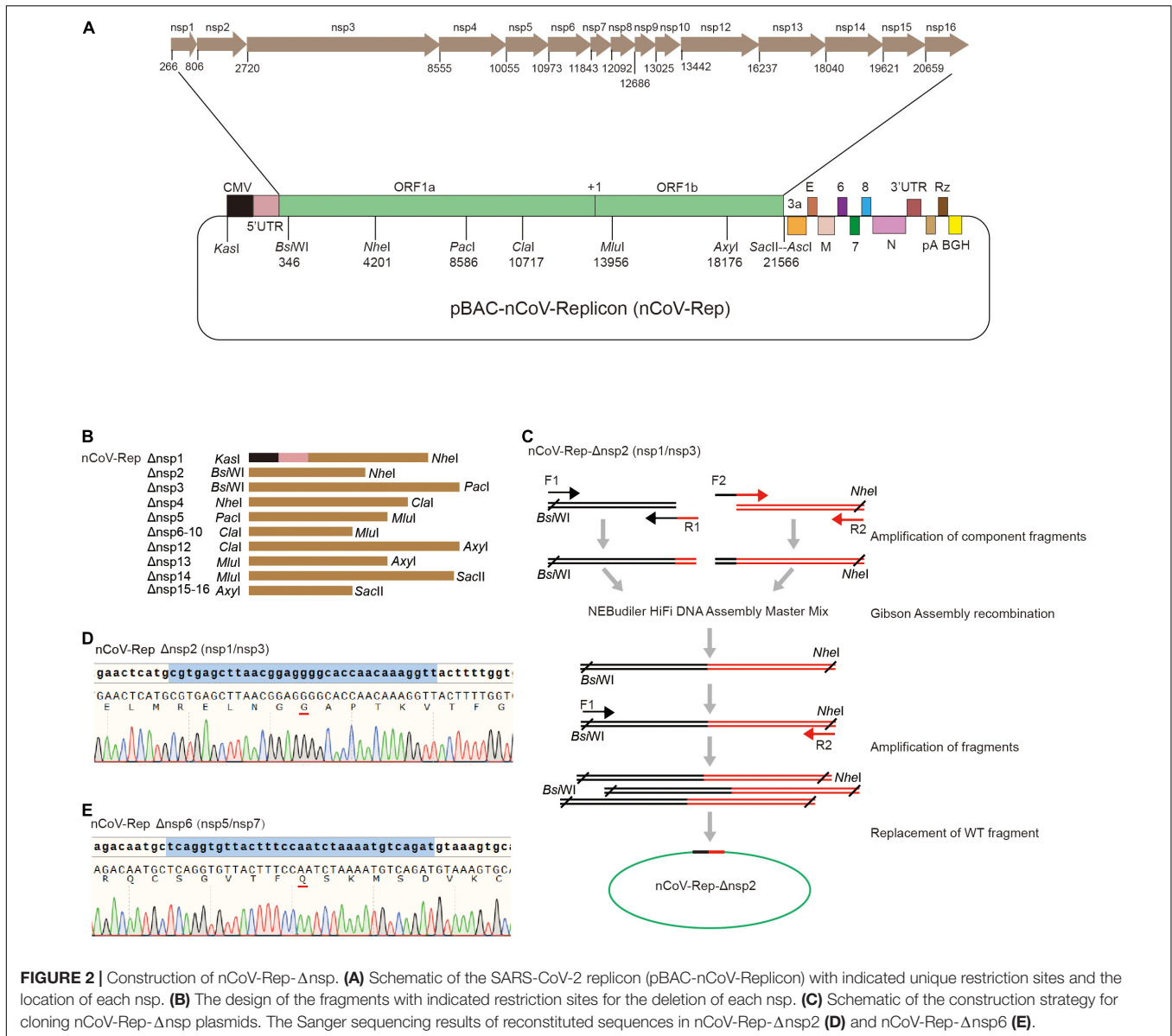
The deletion of nsp x (x is any number from 1 to 16) from the viral genome can lead to the failure to separate its adjacent upstream (nsp x -1) and downstream nsps (nsp x + 1) (Supplementary Figure 1). The functions of nsp x -1 and nsp x + 1 are likely altered in the fused form, nsp x -1-nsp x + 1. To solve this issue, we reconstituted the cleavage sites between nsp x -1 and nsp x + 1, adjacent to the removed nsp x . The reconstituted cleavage sites are composed of the C-terminal amino acid sequence of nsp x -1, double glycine (GG) or glutamine (Q), and N-terminal amino acid sequence of nsp x + 1 (Figures 1A,B). The consensus sequence of reconstituted cleavage sites is similar to that of the wild-type cleavage sites (Figures 1C,D). For nsp3/nsp5 cleavage site, we replaced the last five amino acids, IALKG, at the C-terminal end of nsp3 with NVATL to rebuild the cleavage site recognized by 3CLpro (Figure 1B).

To verify whether the reconstituted cleavage sites could be processed by PLpro and 3CLpro, we employed the 3CLpro activity reporter system developed previously by our group (Du et al., 2021; Figure 1E). In this system, the reporter gene firefly luciferase (FL) is fused with four tandem ubiquitins (Ub4), which lead to the degradation of firefly luciferase in a ubiquitin-dependent proteasome mechanism and low activity of firefly luciferase. An in-frame amino acid sequence is inserted at the *Hind*III site between four tandem ubiquitins and firefly luciferase (Ub4-()-FL). The cleavage on this inserted sequence leads to the separation of four tandem ubiquitins and firefly luciferase, which gives a robust increase in the detected activity of firefly luciferase.

We inserted all the reconstituted cleavage site sequences at the *Hind*III site and tested the cleavage efficiency by PLpro and 3CLpro. The results showed that the cleavage efficiencies on the reconstituted cleavage sites are comparable to that of WT cleavage sites, indicating that the deletion of nsps, except nsp3 with PLpro activity and nsp5 with 3CLpro activity, should not influence the process of replicase polyprotein (Figures 1F-H).

Construction of pBAC-nCoV-Replicon- Δ nsp (nCoV-Rep- Δ nsp)

Next, we constructed the replicon of SARS-CoV-2 with the deletions of nsp1 to nsp16 based on pBAC-nCoV-Replicon (nCoV-Rep), which was constructed by our group previously (Jin et al., 2021). In CMV-5' UTR-ORF1ab region of nCoV-Rep, we designed eight unique restriction sites, namely, *Kas*I, *Bsi*WI, *Nhe*I, *Pac*I, *Cl*aI, *Mlu*I, *Axy*I, and *Sac*II, which separate the viral cDNA sequence into seven replaceable segments and thus are very helpful for the reconstruction operation on the replicon (Figure 2A). For the deletion of each nsp, we first selected the segment containing the target nsp with the unique restriction sites. The segments for various nsps are described in Figure 2B.

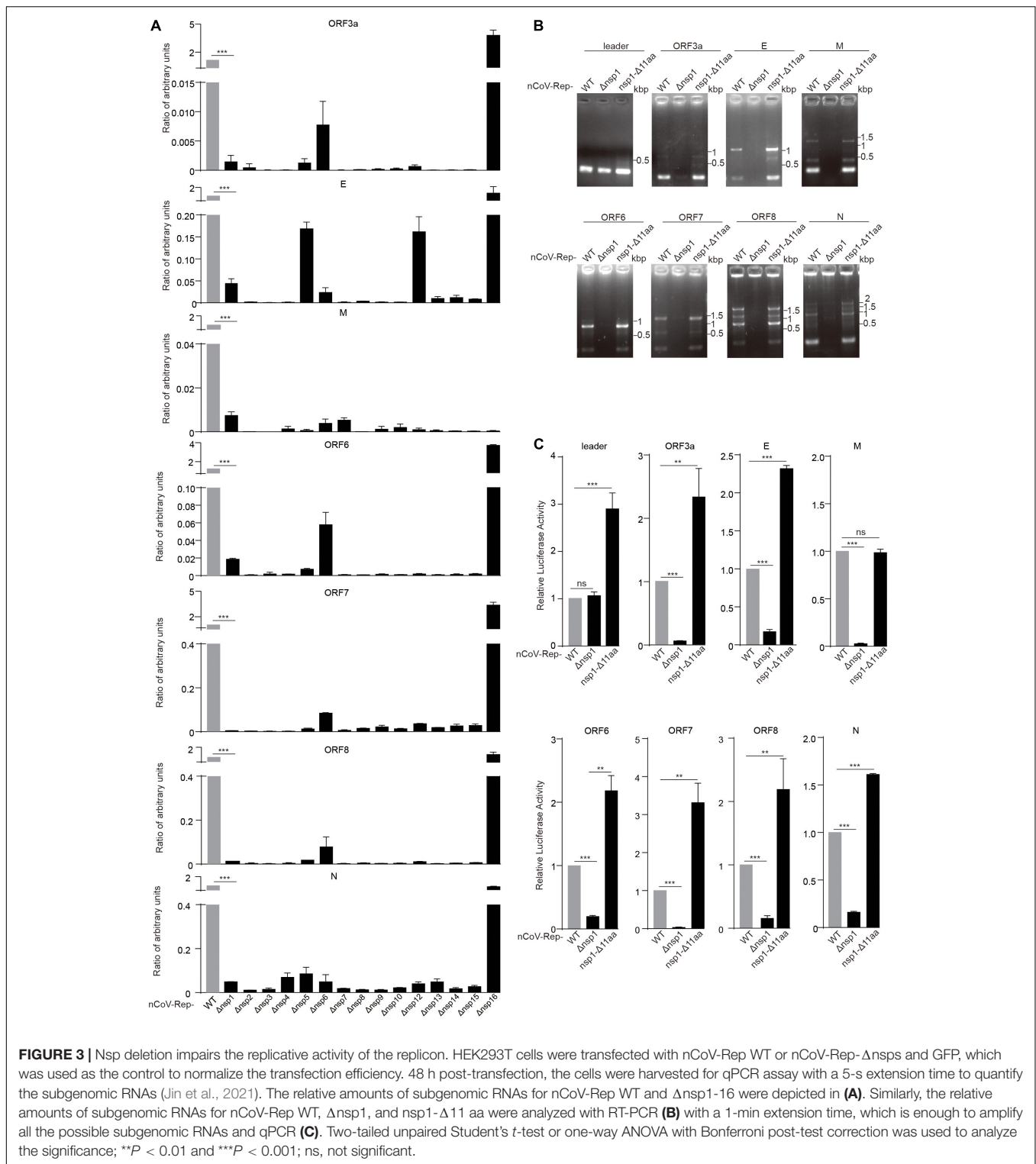


To obtain the target nsp-deleted segment, we designed middle forward (F2) and reverse (R1) primers composed of 3' terminal sequence of the upstream nsp_x-1 adjacent to the deleted nsp_x, cleavage site sequences, encoding GG or Q, and 5' terminal sequence of the downstream nsp_x + 1 adjacent to the deleted nsp_x (Figure 2C), except the primers for nsp4 deletion, which contain the mutant sequence encoding NVATL (Figure 1B). We used the primer pair of the forward primer (F1) upstream of the restriction site and the middle reverse primer (R1) and the primer pair of the reverse primer (R2) downstream of restriction site and the middle forward primer (F2) to amplify two-component fragments. We assembled two-component fragments into the segment with deletion of the target nsp using Gibson Assembly strategy and amplified the segment with the primer pair of the forward primer upstream (F1) of the restriction site and the reverse primer (R2) downstream of

the restriction site (Figure 2C). Using the unique restriction sites, we replaced WT segments with the target nsp-deleted segments and verified the segment sequence with Sanger DNA sequencing to ensure no undesired mutations (Figures 2D,E and Supplementary Figure 2).

The Deletion of Non-structural Protein 1–15 Impairs the Replicative Activity of the Replicon

To investigate the role of various nsps in viral replication and transcription, we transfected the WT replicon (nCoV-Rep) and the replicons with the deletion of various nsps (nCoV-Rep-Δnsp1 to 16) into HEK293T cells as described previously (Jin et al., 2021). We examined the subgenomic RNAs using quantitative PCR (qPCR) with the forward primer



in the leader sequence and the reverse primers in 5' UTR, ORF3a, E, M, ORF6, ORF7, ORF8, and N (Figure 3A). To only synthesize the products from the template composed of a direct fusion of leader sequence and coding regions of various ORFs, we reduced the extension time to 5 s as discussed

previously (Jin et al., 2021). The results showed that except nsp16 deletion, the synthesis of each subgenomic RNA is largely impaired, indicating that all the nsps except nsp16 could play an indispensable role in the viral replication and transcription.

Deletions in nsp1 of SARS-CoV-2 variants were reported, indicating that nsp1 may not be essential for viral replication and transcription (Lin et al., 2021b). To clarify the different impacts on viral replication between the deletion of whole nsp1 and $\Delta 500-532$ in nsp1, we constructed the replicon with the reported deletion of 11 amino acids in nsp1 (nCoV-Rep-nsp1- $\Delta 11$ aa) (Lin et al., 2021b) and examined the synthesis of subgenomic RNAs. The results showed that unlike the replicon with the deletion of the whole nsp1 (nCoV-Rep- Δ nsp1), nCoV-Rep-nsp1- $\Delta 11$ aa gave an increased synthesis of many subgenomic RNAs, in agreement with the previous report (Lin et al., 2021b; **Figures 3B,C**). This piece of data suggested that nsp1 plays an indispensable role in viral replication and transcription despite its unfavorable functions mentioned above.

Non-structural Protein14 Reconstitution Can Rescue the Impaired Replication Caused by the Non-structural Protein14 Deletion

Next, we asked whether the expression of non-replicon nsps *in trans* could rescue the impaired replication caused by the nsp deletion. We cotransfected nCoV-Rep- Δ nsp1 to 16 with nsp1 to 16 expressing plasmid into HEK293T cells. Besides quantitative PCR, we examined the N protein expression using Western blotting (WB). In agreement with the qPCR result, except nsp16, the nsp deletions largely impair N protein expression (**Figure 4** and **Supplementary Figure 3**). We found that the nsp14 could rescue the impaired viral replication caused by the nsp14 deletion, as indicated by the significantly increased expression of N protein by nsp14 expression compared with vector control. The nsp16 expression *in trans* could not increase the N expression, which is not affected by the nsp16 deletion, further supporting a dispensable role of nsp16 in viral replication and transcription (**Figure 4** and **Supplementary Figure 3**). Since we failed to detect the expression of nsp1, nsp3, and nsp16, whether these nsps could rescue the impaired viral replication by corresponding nsp deletion is still not determined.

DISCUSSION

It is well recognized that nsps derived from the replicase polyprotein of SARS-CoV-2 play crucial roles in viral replication and transcription. However, whether all these nsps are indispensable for viral replication and transcription is still not fully defined. Here, we constructed the replicon of SARS-CoV-2 with the deletion of each nsp and verified the replication activity of the mutant replicons. We found that except for nsp16, all the deletions of nsp impaired the replication of the viral replicon (**Figure 3A**). The effect caused by the deletion of nsps should not be due to the low cleavage efficiency between the two nsps adjacent to the deleted nsp, because the cleavages on the reconstructed cleavage sites are verified with our 3CLpro/PLpro activity reporter system (**Figure 1**). By restoring the nsp14 expression, the nsp14 deleted replicon regained the replication activity

and gave the expression of N gene at the 3' terminal of viral genome (**Figure 4**).

In general, our findings suggest that the viral replication relies on each nsp are varied. We found that the replicon with the deletion of nsp16 is still able to replicate itself and gives the expression of N gene, which is dependent on the discontinuous mechanism (Hussain et al., 2005). This piece of data indicating the viral 2'-O-methylation by nsp16 is likely dispensable for viral replication. Nsp16 could help the viral RNA escape from the recognition of the host innate immune system by decorating the viral RNA with 2'-O-methylation (Zust et al., 2011). We noticed an apparent increase in N gene expression, indicating that the deletion of nsp16 may promote translation efficiency. Indeed, the previous studies (Hoernes et al., 2016; Choi et al., 2018) uncovered that 2'-O-methylation decreases translation efficiency by disrupting tRNA decoding during translation elongation. Thus, we infer that besides facilitating the escape from the host immune monitoring, nsp16 could tune the expression of various subgenomic RNAs.

We found that nsp14 could rescue the replication of nsp14-deleted replicon, but the other nsps could not, indicating that nsp14 could function as a separate protein, not tightly associated with RTC as other nsps. Considering that the two reactions mediated by 3'-5' exoribonuclease (ExoN) and N7-guanine methyltransferase (N7-MTase) activities of nsp14 could perform independently, we infer that hampering the association of nsp14 with viral RTC may not be a feasible antiviral strategy.

The expression of well-known replication-associated proteins, including nsp7, nsp8, nsp9, nsp10, nsp12, nsp13, and nsp15, could not recover the impaired replication of the viral replicon with the corresponding nsp deletion, indicating that the viral RTC assembly likely prefers the *in cis* component nsps generated from the same original polyproteins. The preference is likely due to the time window and the subcellular location for the viral RTC assembly (van Hemert et al., 2008; Acheampong et al., 2022). The exogenous expression of some nsps cannot fully satisfy the requirements, so these nsps cannot restore the assembly and the proper function of viral RTC.

Three nsps containing transmembrane domains, nsp3, nsp4, and nsp6, exhibited indispensable functions in the viral replication, indicating that the membrane structure or the location of RTCs could be essential for the efficient replication of viral RNAs by providing an essential microenvironment (Cortese et al., 2020). Mutations on the cleavage sites of nsp7 to nsp10 were reported to have a different impact on the viral replication compared with the in-frame deletions of nsp7 to nsp10 coding sequences. The mutation on the nsp9-nsp10 cleavage site only resulted in an attenuated viral replication, while the deletion of the coding sequence of nsp7-nsp10 was lethal for mutant viruses, consistent with our study (Deming et al., 2007). Although no replication-associated function of nsp2 is uncovered, the deletion of nsp2 largely impaired the replication of viral replicon, consistent with the previous reports that the titers of progeny virus by SARS Δ nsp2 infection were $\sim 1\text{-log}_{10}$ reduced compared to wild-type infections (Graham et al., 2005), indicating that investigation on the nsp2's replication-associated function is a potential research direction.

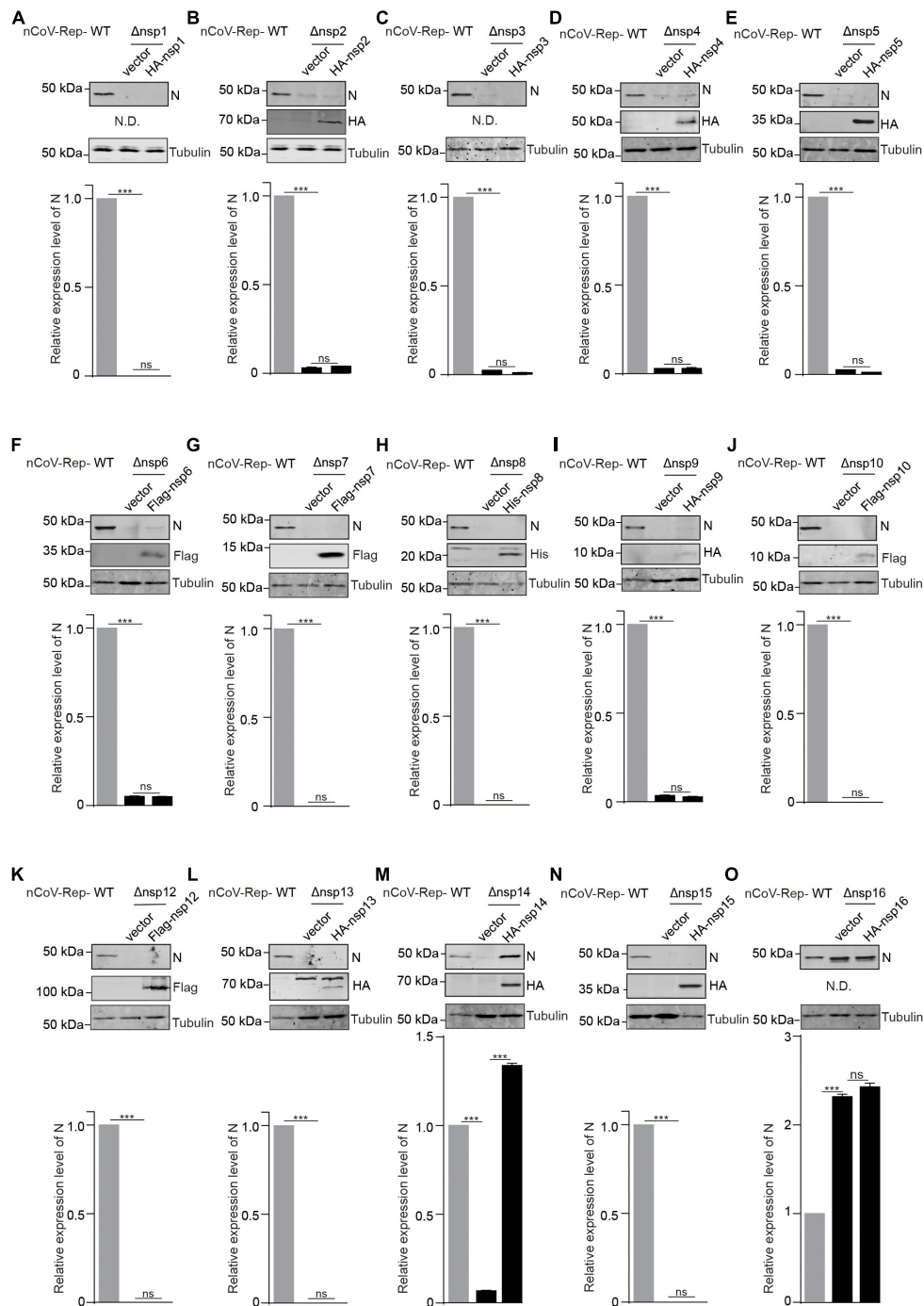


FIGURE 4 | *In trans* expression of nsp14 rescues the nsp14-deletion-induced impaired replication. (A–O) HEK293T cells were transfected with nCoV-Rep WT or nCoV-Rep-Δnsp with vector or corresponding nsp expressing plasmid. 48 h post-transfection, the cells were collected and subjected to WB, blotted with indicated antibodies. N is the nucleocapsid protein antibody. N.D. is not detected. The densitometry of immunoblot bands was determined using Image Studio™ Lite Software (LI-COR Biosciences). The amounts of N protein were normalized with tubulin. Two-tailed unpaired Student's *t*-test was used to analyze the significance; ****P* < 0.001; ns, not significant.

We acknowledge that our findings only briefly described the role of each nsp on viral replication and transcription. The conclusion drawn here based on the replicon system awaits further studies in detail using different systems, such as

recombinant live viruses. Our findings here may provide a new angle to look at the role of various nsps in viral replication, suggesting more essential nsps and associations between nsps for antiviral strategy development.

TABLE 1 | Primers for cloning cleavage sites into Ub4-FL vector.

Primer name	Forward primer (5'–3')	Reverse primer (5'–3')
<i>Hind</i> III-Δnsp2	AGCTTCGTGAGCTTAACGGAGGGGACCAACAAAGGTTA	AGCTTAACCTTTGTTGGTGCCCTCCGTTAAGCTCACGA
<i>Hind</i> III-Δnsp3	AGCTTTTCACACTCAAAGGCGGTAATAATTGTTAATAATA	AGCTTATATTAACAATTTTACCGCCTTTGAGTGTGAAA
<i>Hind</i> III-Δnsp4	AGCTTAATGTGGCAACTTTACAAAGTGGTTTTAGAAAAA	AGCTTTTTTCTAAAACCACTTTGTAAAGTTGCCACATTA
<i>Hind</i> III-Δnsp5	AGCTTACCTCAGCTGTTTTGCAGAGTGCAGTGAAAAAGAA	AGCTTTCTTTTCACTGCACCTCTGCAAAACAGCTGAGGTA
<i>Hind</i> III-Δnsp6	AGCTTTCAGGTGTTACTTTCCAATCTAAAATGTCAGATA	AGCTTATCTGACATTTTAGATTGAAAAGTAAACACCTGAA
<i>Hind</i> III-Δnsp7	AGCTTAAAGTAGCCACTGTACAGGCTATAGCCTCAGAGA	AGCTTCTCTGAGGCTATAGCCTGTACAGTGGCTACTTTA
<i>Hind</i> III-Δnsp8	AGCTTAACAGGGCAACCTTACAAAATAATGAGCTTAGTA	AGCTTACTAAGCTCATTATTTTGAAGGTTGCCCTGTTA
<i>Hind</i> III-Δnsp9	AGCTTTCTGCTGTCAAATTACAGGCTGGTAATGCAACAA	AGCTTTGTTGCATTACCAGCCTGTAATTTGACAGCAGAA
<i>Hind</i> III-Δnsp10	AGCTTGCCACAGTACGTCTACAATCAGCTGATGCACAAA	AGCTTTTGTGCATCAGCTGATTGTAGACGTAAGTGTGGCA
<i>Hind</i> III-Δnsp12	AGCTTCGCGAAGCCATGCTTCAGGCTGTTGGGGCTTGTA	AGCTTACAAGCCCCAACAGCCTGAAGCATGGGTTTCGCGA
<i>Hind</i> III-Δnsp13	AGCTTCCGCATACAGTCTTACAGGCTGAAAATGTAACAA	AGCTTTGTACATTTTCAAGCCTGTAAGACTGTATGCGGA
<i>Hind</i> III-Δnsp14	AGCTTAATGTGGCAACTTTACAAAGTTTAGAAAATGTGA	AGCTTCACATTTTCTAAAACCTTTGTAAAGTTGCCACATTA
<i>Hind</i> III-Δnsp15	AGCTTACTTTTACAAGACTTCAGCTTAGTCAAGCGTGGA	AGCTTCCACGCTTGACTAGACTGAAGTCTTGTAAAAGTA

MATERIALS AND METHODS

Cell Culture and Transfection

HEK293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific, C11960500), supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, A3160801), 100 unit/mL penicillin, and 100 μg/mL streptomycin (Thermo Fisher Scientific, 15140-122) at 37°C with 5% CO₂.

HEK293T cells were transfected at approximately 60% confluency with various nCoV-Rep vectors using Hieff TransTM Liposomal Transfection Reagent (Yeasen Biotech, Cat#40802ES03) following the manufacturer's instructions. 48 h post-transfection, the cells were subjected to various assays.

Plasmid Construction

The construction of Ub4-nsp1/3-FL to Ub4-nsp14/16-FL is described as follows: First, the oligos of the positive and negative strands (1 μL for each at the concentration of 10 μM, listed in **Table 1**) were phosphorylated with T4 polynucleotide kinase (NEB M0201S) in T4 ligase buffer (NEB M0202S) at 37°C for 30 min. Second, the oligos were denatured for 10 min at 95°C and cooled down slowly (approximately 30 min) to room temperature. Third, the Ub4-FL (Du et al., 2021) was linearized with *Hind*III and purified. Lastly, the annealed oligos were ligated with linearized Ub4-FL at the ratio of 1:10, and the ligation products were transformed into the DH5α component cells. The clones were verified with Sanger DNA sequencing (Tsingke Biotechnology Co., Ltd., Beijing).

The nCoV-Rep was constructed in our previous work (Jin et al., 2021). Considering the vectors' capacity in cloning and ability to stably maintain the foreign DNA fragments, we employed the bacterial artificial chromosome (BAC) vector to clone the full-length cDNA of SARS-CoV-2. So far, six strategies have been successfully applied to construct the reverse genetics systems of coronaviruses: the RNA recombination-based, the vaccinia virus vector-based, the yeast-based recombination system-based, the circular polymerase extension reaction-based,

BAC vector-based, and the *in vitro* ligation-based strategies. Among these strategies, BAC-based and the *in vitro* ligation-based strategies are the most widely used to construct reverse genetics systems of coronaviruses (Wang et al., 2022). Compared with the *in vitro* ligation-based strategy, the BAC-based strategy is more component in constructing the biosafe replicon and performing no live virus-involved quantitative studies on the replication and transcription of viral RNAs. To achieve the expression of viral genomic cDNA sequence in cells, we fused type II promoter CMV with the N-terminus of viral genomic cDNA and installed a transcriptional terminator BGH downstream of the C-terminus of viral genomic cDNA. To obtain the authentic 3' terminus of viral genomic RNA, we inserted the HDV ribozyme between 3' terminus of viral RNA and the BGH terminator. After the viral RNA is transcribed by the CMV promoter, the RNA sequence derived from BGH terminator can be removed through HDV ribozyme-mediated splicing mechanism, and the complete viral RNA is generated.

The constructions of nCoV-Rep-Δnsp1 to nCoV-Rep-Δnsp15 are described as follows. First, two unique restriction sites upstream and downstream of the nsp to be removed in nCoV-Rep (Jin et al., 2021) were selected. The middle forward/reverse primers (**Table 2**) include 3' terminal sequence of the nsp prior to the nsp to be removed, 5' terminal sequence of the nsp after the nsp to be removed, and the sequence generating the cleavage sites for PLpro or 3CLpro. The fragments upstream and downstream of the nsp to be moved were amplified with the primer combinations of the primer upstream of the restriction site and middle reverse primer and that of the primer downstream of the restriction site and middle forward primer, respectively. The two fragments were assembled seamlessly with NEBuilder HiFi DNA Assembly Master Mix (NEB #E2621). The assembled new fragments were amplified with the primers upstream and downstream of the restriction sites and inserted into nCoV-Rep vector to replace WT fragment between the selected two restriction sites. The DH10B component cells were employed for transformation, and the clones were verified with Sanger DNA sequencing (Tsingke Biotechnology Co., Ltd., Beijing).

TABLE 2 | Primers for constructing the fragments with the deletion of each nsp.

Primer name	Forward primer (5'–3')	Reverse primer (5'–3')
Δ nsp1	GGTAAGATGGCATACTCGCTATGTCGATAACAACCTC	CATAGCGAGTGTATGCCATCTTACCTTTTCGGTCACACCCG
Δ nsp2	GCTTAACGGAGGGGACCAACAAAGGTTACTTTTGGTG	GTTGGTGCCCTCCGTTAAGCTCAGCG
Δ nsp3	CGGTAATAATTGTTAATAATTGGTTGAGCAGTTAATTAAGTTAC	CCAATTATTAACAATTTTACCCTTTGAGTGTGAAGG
Δ nsp4	CAAAGAATGTGGCAACTTTACAAAGTGGTTTTAGAAAAATGGCATTCCC	GTAAAGTTGCCACATTCTTTGTTGTACAAACATTAAACAATTGTCTAGTAG
Δ nsp5	CAGCTGTTTTGCAGAGTGCAGTGAAGAACAATCAAGGG	CACTGCCTCTGCAAAAACAGCTGAGGTGATAGAG
Δ nsp6	GTTACTTTCCAATCTAAAATGTCAGATGTAAGTGCACATCAGTAG	CTGACATTTTAGATTGGAAAGTAACACCTGAGCATTGTCTAAC
Δ nsp7	GCCACTGTACAGGCTATAGCCTCAGAGTTTATGTTCCCTTC	CTGAGGCTATAGCCTGTACAGTGGCTACTTTGATACAAGG
Δ nsp8	GGGCAACCTTACAAAATAATGAGCTTAGTCTGTTGCACTAC	GCTCATTATTTGTAAGGTTGCCCTGTTGTCCAG
Δ nsp9	GTCAAATTACAGGCTGGTAATGCAACAGAAGTGCC	CATTACCAGCCTGTAATTTGACAGCAGAATTGGCCC
Δ nsp10	GTACGTCTACAATCAGCTGATGCACAATCGTTTTTAAACG	GTGCATCAGCTGATTGTAGACGTACTGTGGCAGCTAAAC
Δ nsp12	CCATGCTTCAGGCTGTTGGGGCTTGTGTTCTTTG	CAAGCCCCAACAGCCTGAAGCATGGGTTCCGCG
Δ nsp13	CAGTCTTACAGGCTGAAAATGTAACAGGACTCTTTAAAGATTGTAG	GTTACATTTTCAGCCTGTAAGACTGTATGCGGTGTGATACATAG
Δ nsp14	CTTTACAAAGTTTAGAAAATGTGGCTTTTAAATGTTGTAATAAAGG	CATTTTCTAAACTTTGTAAGTTGCCACATTCTACGTG
Δ nsp15	CAAGACTTCAGTCTAGTCAAGCGTGGCAACCG	CGCTTGACTAGACTGAAGTCTGTAAGTGTTCAGAGG
Δ nsp16	CCCAAATTACAAATAAACGAACAATCCGCGGGGG	GATTGTTGCTTTATTGTAATTTGGGTAAATGTTTCTACATGGCC
nsp1- Δ 11aa	GTTTCGGATGCTCGAACTGCAGAAGGCGAAGGCATTGAGTACGG	GCCTTCGAGTCTGCAGTTCGAGCATCCGAAC

The strategy to construct nCoV-Rep-nsp1- Δ 11aa was described previously for introducing mutations in nsp12 and nsp14 (Jin et al., 2021). In brief, the segment between *Bsi*WI and *Nhe*I was chosen for mutagenesis. The forward and reverse primers (Table 2) for generating the deletion of 500–532 nt (Δ 11aa) were used to amplify the fragments upstream or downstream of the mutant site. The two fragments were assembled seamlessly with NEBuilder HiFi DNA Assembly Master Mix. The assembled products were amplified by PCR and used to replace the wild-type *Bsi*WI-*Nhe*I segment.

The construction of nCoV-Rep- Δ nsp16 is relatively straightforward compared with other nsp deletion mutants. The *Axy*I-*Sac*II fragment without nsp16 was inserted into nCoV-Rep vector to replace WT fragment between *Axy*I and *Sac*II sites.

The construction of LPC-nsp1 to LPC-nsp16 is referred to as in our previous work (Jiang et al., 2021).

Dual-Luciferase Reporter Assay

One day prior to transfection, HEK293T cells (1×10^5) were plated in 24-well plates. Various Ub4-CS-FL (0.5 μ g), RL-TK (0.1 μ g), and LPC-3CLpro-HA or LPC-PLpro-HA were transfected into the cells using Hieff TransTM Liposomal Transfection Reagent. Forty eight hour post-transfection, the cells were lysed in 50 μ L 1x Passive Lysis Buffer (PLB, Promega, E1941). The activities of firefly and renilla luciferase were measured using Dual-Glo[®] Luciferase Assay System to determine the relative luciferase activities.

RNA Extraction, Real-Time Quantitative PCR, and RT-PCR

Total RNA was isolated from the HEK293T cells using TRIzol reagent (Thermo Fisher Scientific, Cat#15596026) and treated with RNase-free DNase (Takara, Dalian, China, EN0521)

according to the manufacturer's instructions. The quality of RNA was examined using the electrophoresis on 1% agarose gel, and the purity of RNA was verified on the basis of the ratio of OD260/280 on NanoDrop One (Thermo Scientific). Two μ g of total RNA was used as a template to synthesize cDNA using the cDNA synthesis kit (Thermo Fisher Scientific, Cat#K1622). The cDNAs were subjected to the real-time quantitative PCR (qPCR), which were performed on a 96TM Real Time PCR Detection System (Applied BiosystemsTM 7500), in 10 μ L reaction mixtures containing 5 μ L SYBR[®] TB Green[®] Premix Ex TaqTM (Tli RNaseH Plus) (Takara, Dalian, China, Cat#RR420A). The thermal profile consists of 30 s at 95°C, followed by 40 cycles of 5 s at 95°C and 5 s at 60°C. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative gene expression values. The details for the design of primers and qPCR conditions were described as previously (Jin et al., 2021). The RT-PCR was performed with 2 \times Hieff[®] PCR Master Mix (Yeasen, Shanghai, China, 10102ES08), extension time of PCR program is 1 min, and PCR products were examined in the 1.5% agarose gel after DNA electrophoresis.

Western Blot

Cell samples were collected and lysed in RIPA lysis buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM EDTA, and 50 mM Tris-HCl pH7.4, supplemented with cCompleteTM Protease Inhibitor Cocktail). All samples' concentration was quantified with BCA assay (Thermo Fisher Scientific, 23227) following the manufacturer's instructions. The lysates were subjected to SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were incubated in PBS containing 0.05% Tween 20 and 5% non-fat milk (blocking buffer) for 1 h at room temperature and then left in the blocking buffer containing primary antibody of HA (Proteintech, 10011878), His (Proteintech, 10004365), Flag (Proteintech, 00098867), Tubulin (Proteintech, 66031-1-Ig), or

Nucleocapsid (Sino Biological, 40143-R019) at 4°C overnight. The following day, after being washed with PBS containing 0.05% Tween 20, the membranes were incubated with appropriate secondary antibody (Goat anti-Rabbit, LI-COR, D10121-05; Goat anti-Mouse, LI-COR, D10217-05, at 1:10,000). The final blots were developed on Odyssey CLx Imaging System (LI-COR Biosciences).

Statistics

Except for specially stated, all the experiments were performed at least three times. The data analyses were finished using Student's *t*-test of SPSS (version 21.0; SPSS Inc., Chicago, IL). When *P*-value was less than 0.05, the results were considered significant.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

XP and J-AP conceived the ideas, designed the experiments, and wrote the manuscript. All authors performed experiments or data analysis, read, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2022.907422/full#supplementary-material>

Supplementary Figure 1 | Design of each nsp deletion. Each nsp was deleted by fusing its adjacent upstream and adjacent downstream nsps, except nsp1 and nsp16. The new cleavage site sequences were depicted. The deletion of nsp1 and nsp16 left a new start codon before nsp2 and a stop codon after nsp15, respectively.

Supplementary Figure 2 | Sanger sequencing results for nCoV-Rep-Δnsps. The Sanger sequencing results covering the newly formed cleavage site regions in various nCoV-Rep-Δnsps were depicted.

Supplementary Figure 3 | Investigation of *in trans*-rescue function of various nsps in the viral replication by quantifying the amount the N (related to **Figure 4**). **(A–O)** The RNA samples described in **Figures 4A–O** were subjected to real-time quantitative PCR assay. The amounts of N subgenomic RNA (sgRNA) from various samples were normalized with internal control GAPDH.

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