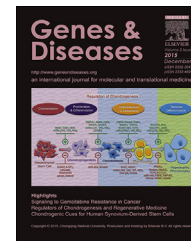




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RAPID COMMUNICATION

NSP16 promotes the expression of TMPRSS2 to enhance SARS-CoV-2 cell entry

Since the end of 2019, COVID-19 has caused worldwide pandemic. SARS-CoV-2, the culprit of this epidemic, binds to the host receptor Angiotensin converting enzyme 2 (ACE2) using spike (S) protein for cell entry. Recent studies showed that ACE2 expressed at a low level in the main target organs such as lung and bronchial tissues. How SARS-CoV-2 efficiently invades into the respiratory system or other organs with low levels of ACE2 is an urgent problem to be solved. Here, we discovered that NSP16 significantly promoted the invasion of SARS-CoV-2 pseudovirus. NSP16 could promote the formation of a STUB1-USP14 de-ubiquitination complex that regulated the ubiquitination and stability of transmembrane serine protease 2 (TMPRSS2). Inhibiting the function of this complex remarkably reduced the invasion ability of SARS-CoV-2 pseudovirus. Thus, our study demonstrates for the first time that NSPs of SARS-CoV-2 can also participate in viral invasion.

To explore the effects of non-structural proteins (NSPs) on the entry of SARS-CoV-2 into human cells, we constructed all the NSP plasmids of SARS-CoV-2, except NSP3 and NSP11. We transfected these NSP plasmids into 293T cells and then SARS-CoV-2 pseudovirus was used to infect cells. As shown in [Figure 1A](#) and [S1A](#), expression of NSP16 significantly enhanced the infection of the pseudovirus. Similar result was obtained in 293T cells stably expressing human ACE2 (293T-ACE2) ([Figure S1B](#)). We next examined the effects of NSP16 on the expression of viral receptors and cofactors: ACE2, TMPRSS2, Tyrosine-protein kinase receptor UFO (AXL) and Cathepsin L (CTSL). [Figure 1B](#) and [S1C](#) showed that NSP16 significantly increased the protein expression of TMPRSS2. Treatment with the TMPRSS2 inhibitor-Bromhexine hydrochloride (BHH) significantly attenuated the infection of SARS-CoV-2 pseudovirus induced by NSP16 ([Figure S1D](#)).

We next explore the mechanism for NSP16 in up-regulating TMPRSS2. Previous study showed that 293T cells were TMPRSS2-negative.¹ We first examined the

expression of TMPRSS2 in 293T and other cell lines. [Figure S2A](#) and [S2B](#) showed that the expression of TMPRSS2 was much lower in 293T cells than that in Caco-2 cells. However, the mRNA and protein expression of TMPRSS2 in 293T cells were still detectable and comparable to that in bronchial epithelial cells (HBE). Overexpressing NSP16 did not affect the mRNA level of TMPRSS2 significantly ([Figure S2C](#)). Then, protein stability of TMPRSS2 was examined. Cycloheximide (CHX) treatment led to significant decrease on TMPRSS2 expression at 12 h under physiological conditions, while the protein level of TMPRSS2 did not show obvious decrease under the same condition when overexpressing NSP16 ([Figure 1C](#); [Figure S2D](#)). Previous studies demonstrated that NSP16 together with NSP10 formed a complex and functioned as a 2'-O-methyltransferase.² [Figure S3A](#) showed that NSP10 did not affect the increased expression of TMPRSS2 induced by NSP16. We wondered whether NSP16 regulated the ubiquitination of TMPRSS2. Overexpressing NSP16 did not influence the total ubiquitination of TMPRSS2 ([Figure S3B](#)). However, the K48-linked ubiquitination of TMPRSS2 decreased remarkably when overexpressing NSP16, while the K63-linked ubiquitination did not significantly change ([Figure 1D](#); [Figure S3C](#)). We confirmed this result by using mutant ubiquitin plasmids (K63R and K48R). [Figure S3D](#) and [S3E](#) showed that overexpressing NSP16 decreased the ubiquitination of TMPRSS2 when transfected with ubiquitin-K63R, but not ubiquitin-K48R. On the contrary, the ubiquitination of TMPRSS2 increased when transfected with ubiquitin-K48R ([Figure S3E](#)). As the total ubiquitination of TMPRSS2 did not change when overexpressing NSP16 ([Figure S3B](#)), this result indicated that other lysine-linkage types of ubiquitination were also affected by NSP16 except K48 linkage.

Interestingly, we found that NSP16 did not interact with TMPRSS2 ([Figure S4A](#)). Then, mass spectrometry was used to explore the proteins interacted with NSP16. We discovered three E3 ligases (TRIM21, CUL2 and UBR5) and BAG2, a regulatory protein of the E3 ligase STUB1 ([Figure 1E](#) and [Table S1](#)).³ Immunoprecipitation showed that TRIM21 and STUB1 could

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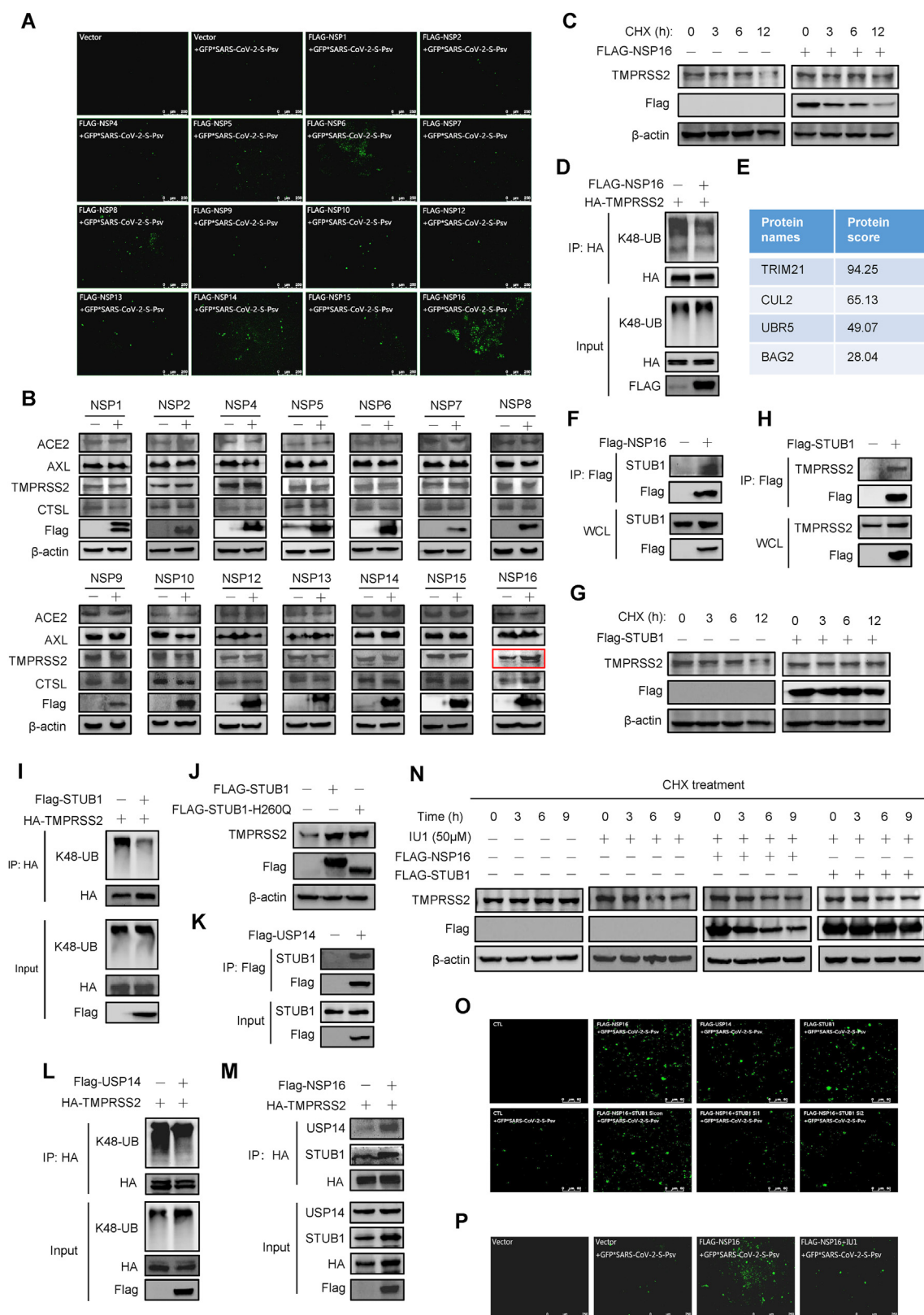


Figure 1 NSP16 promotes the expression of TMPRSS2 to enhance SARS-CoV-2 cell entry. **(A)** The NSPs of SARS-CoV-2 were transfected into 293T cells. After 24 h, the cells were seeded in a 24-well plate. After the cells adhered to the wells, the SARS-CoV-2 pseudovirus (GFP-SARS-CoV-2-S-Psv) was added and incubated for 24 h. The photographs were taken by fluorescence microscopy. **(B)** The NSPs of SARS-CoV-2 were transfected into 293T cells. After 48 h, the cells were lysed and Western blot was performed to detect the expression of the indicated proteins. **(C)** 293T cells were transfected with vector control or pCMV-FLAG-NSP16. After 48 h, the cells were treated with 25 μ g/mL cycloheximide (CHX) for different times and the expression of the indicated proteins

interact with NSP16 (Figure 1F, S4B and S4C). Overexpression or knockdown of TRIM21 did not affect the expression of TMPRSS2 (Figure S4D, E). However, overexpressing STUB1 significantly increased the expression of TMPRSS2 and knockdown of STUB1 decreased TMPRSS2 expression (Figure S4F, G). Knocking down STUB1 could eliminate the up-regulation of TMPRSS2 induced by NSP16 (Figure S4H), and STUB1 expression could stabilize TMPRSS2 protein (Figure 1G).

We next detected the interaction between STUB1 and TMPRSS2. Figure 1H and S4I showed that STUB1 and TMPRSS2 interacted with each other. Overexpressing STUB1 significantly reduced the total and K48-linked ubiquitination of TMPRSS2 (Figure 1I; Figure S4J), while the K63-linked ubiquitination was not affected (Figure S4K). These results contradicted with the traditional function of STUB1 acting as an E3-ligase. As previously mentioned, BAG2 was a regulatory protein for the E3-ligase function of STUB1.³ However, overexpressing BAG2 did not influence the expression of TMPRSS2 (Figure S4L). We also used a STUB1 mutant that lost its E3-ligase activity (STUB1-H260Q), and found that overexpressing STUB1-H260Q had similar effect on TMPRSS2 as with STUB1 wild-type (Figure 1J). We further showed that NSP16 expression could increase the interaction between STUB1 and TMPRSS2 (Figure S4M). These results indicated that STUB1 might affect ubiquitination of TMPRSS2 by regulating the function of a certain de-ubiquitinase.

We next used UbiBrowser to find the potentially interacted de-ubiquitinases with STUB1 and USP14 was found. Figure 1K showed that USP14 interacted with STUB1. Overexpressing USP14 increased the expression and stability of TMPRSS2 (Figure S5A, B). Figure 1L and S5C showed that overexpressing USP14 decreased the total and K48-linked ubiquitination of TMPRSS2, but not the K63-linked ubiquitination (Figure S5D). Knocking down STUB1 eliminated the

de-ubiquitination of TMPRSS2 mediated by USP14 (Figure S5E). We further demonstrated that NSP16 facilitated the formation of a new de-ubiquitination complex: the STUB1-USP14 complex (Figure 1M). The protein stability of TMPRSS2 was strictly regulated by this de-ubiquitination complex. Blocking the activity of USP14 using specific inhibitor-IU1 inhibited the regulatory ability of NSP16 and STUB1 on TMPRSS2 protein stability (Figure 1N). Also, knockdown of STUB1 could alleviate the enhanced protein stability of TMPRSS2 induced by NSP16 or STUB1 (Figure S5F). We next examined the effects of the STUB1-USP14 complex on the infection of SARS-CoV-2. Overexpressing NSP16 or USP14 or STUB1 all increased the infection of SARS-CoV-2 pseudovirus and knocking down STUB1 significantly attenuated NSP16-induced viral infection (Figure 1O, S6A). Inhibiting the activity of USP14 using IU1 also greatly weakened the infection of SARS-CoV-2 pseudovirus induced by NSP16 (Figure 1P, S6B).

Thus, our study demonstrated for the first time that NSPs of SARS-CoV-2 participated in viral invasion (Figure S7). One of the limitation of this study is that we only used the pseudovirus system, live viruses should be used to further confirm these results. Taken together, our study provides a reasonable explanation for SARS-CoV-2 efficiently entry into host organs with low expression of ACE2, such as lung and bronchus.

Author contributions

T. H. and W. Z. conceived and designed the study. T. H., J. L., Y. W., W. X., Q. H., Y. L., Q. P. performed the experiments. T. H. and J. L. analyzed data. T. H. and W. Z. wrote the manuscript. This manuscript was approved by all authors.

were detected by Western blot. (D) 293T cells were transfected with the indicated plasmids. After 48 h, immunoprecipitation was performed and the expressions of the indicated proteins were detected by Western blot. The K48-linked ubiquitination was detected using a K48-linkage specific polyubiquitin antibody. (E) The E3 ligases and the related proteins identified by mass spectrum were shown. (F) 293T cells were transfected with vector control or pCMV-FLAG-NSP16. After 48 h, immunoprecipitation was performed and the expressions of the indicated proteins were detected by Western blot. (G) 293T cells were transfected with vector control or pCMV6-FLAG-STUB1. After 48 h, the cells were treated with 25 μ g/mL cycloheximide (CHX) for different times and the expression of the indicated proteins were detected by Western blot. (H) 293T cells were transfected with vector control or pCMV6-FLAG-STUB1. After 48 h, immunoprecipitation was performed and the expressions of the indicated proteins were detected by Western blot. (I) 293T cells were transfected with the indicated plasmids. After 48 h, immunoprecipitation was performed and the expressions of the indicated proteins were detected by Western blot. (J) 293T cells were transfected with vector control or pCMV6-FLAG-STUB1 or pEnCMV-FLAG-STUB1-H260Q. After 48 h, Western blot was performed to detect the expression of the indicated proteins. (K) 293T cells were transfected with vector control or pCMV-FLAG-USP14. After 48 h, immunoprecipitation was performed and the expressions of the indicated proteins were detected by Western blot. (L) 293T cells were transfected with the indicated plasmids. After 48 h, immunoprecipitation was performed and the expressions of the indicated proteins were detected by Western blot. (M) 293T cells were transfected with pCMV3-HA-TMPRSS2 alone or co-transfected with pCMV3-HA-TMPRSS2 and pCMV-FLAG-NSP16. After 48 h, immunoprecipitation was performed and Western blot was performed to detect the expression of the indicated proteins. (N) 293T cells were transfected with the indicated plasmids. After 48 h, 25 μ g/mL CHX combined with or without 50 μ M IU1 was added and treated cells for different times. The expressions of the indicated proteins were detected by Western blot. (O) 293T cells were transfected with the indicated plasmids and siRNAs. After 24 h, the cells were seeded in a 24-well plate. After the cells adhered to the wells, the SARS-CoV-2 pseudovirus was added and incubated for 24 h. The photographs were taken by fluorescence microscopy. (P) 293T cells were transfected with the vector or pCMV-FLAG-NSP16. After 24 h, the cells were seeded in a 24-well plate. After the cells adhered to the wells, the SARS-CoV-2 pseudovirus was added and treated with or without IU1 (50 μ M) for 24 h. The photographs were taken by fluorescence microscopy.

Conflict of interests

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.gendis.2022.09.005>.

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