Structural basis for *Sarbecovirus* ORF6 mediated blockage of nucleocytoplasmic transport

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The emergence of heavily mutated SARS-CoV-2 variants of concern (VOCs) place the international community on high alert. In addition to numerous mutations that map in the spike protein of VOCs, expression of the viral accessory proteins ORF6 and ORF9b also elevate; both are potent interferon antagonists. Here, we present the crystal structures of Rae1-Nup98 in complex with the C-terminal tails (CTT) of SARS-CoV-2 and SARS-CoV ORF6 to 2.85 Å and 2.39 Å resolution, respectively. An invariant methionine (M) 58 residue of ORF6 CTT extends its side chain into a hydrophobic cavity in the Rae1 mRNA binding groove, resembling a bolt-fitting-hole; acidic residues flanking M58 form salt-bridges with Rae1. Our mutagenesis studies identify key residues of ORF6 important for its interaction with Rae1-Nup98 in vitro and in cells, of which M58 is irreplaceable. Furthermore, we show that ORF6-mediated blockade of mRNA and STAT1 nucleocytoplasmic transport correlate with the binding affinity between ORF6 and Rae1-Nup98. Finally, binding of ORF6 to Rae1-Nup98 is linked to ORF6-induced interferon antagonism. Taken together, this study reveals the molecular basis for the antagonistic function of *Sarbecovirus* ORF6, and implies a strategy of using ORF6 CTT-derived peptides for immunosuppressive drug development.

Emerging genetic variants of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) present a formidable challenge for containing the COVID-19 pandemic. Variant of concern (VOC) refers to SARS-CoV-2 isolates that exhibit enhanced transmissibility¹, virulence² and immune evasion³, cause severer disease or reduce the effectiveness of current diagnostics, vaccines, and therapeutics. The Omicron variant (B.1.1.529) was recently designated as a top priority VOC because of an unprecedented large number of mutations that cluster in the spike protein⁴. Previous studies demonstrate that VOCs not only harbor mutations in key residues of the spike protein for evading neutralizing antibodies, but also elevate the suppression of innate immunity during infection by upregulating subgenomic RNA and protein levels of innate immune antagonists, such as SARS-CoV-2 ORF9b and ORF6⁶.

ORF9b and ORF6 are accessory proteins of *Sarbecovirus* including SARS-CoV-2 and SARS-CoV⁷⁸. Whereas ORF9b suppresses interferon (IFN) production by targeting the mitochondrial multifunctional adapter TOM70⁹¹⁰, ORF6 dampens antiviral immune responses by blocking bidirectional nucleocytoplasmic transport of cellular mRNAs and proteins¹¹. SARS-CoV-2 ORF6 antagonizes antiviral immunity more efficiently than SARS-CoV ORF6, providing a possible explanation for...
asymptomatic infection or delayed symptom onset in SARS-CoV-2-infected patients.

**Sarbecovirus** ORF6 targets the Rae1-Nup98 complex, a component on the cytoplasmic face of the nuclear pore complex (NPC). Previous studies suggest that the C-terminal tail (CTT) of ORF6 is crucial for its interaction with the Rae1-Nup98 complex and immune antagonistic activity. A methionine flanked by acidic residues in the ORF6 CTT is fundamental to its functions. Nonetheless, the molecular mechanism underlying ORF6-mediated blockade of nucleocytoplasmic trafficking and suppression of antiviral immunity remains elusive.

ORF6 is reminiscent of the vesicular stomatitis virus (VSV) matrix protein (M) and herpesviruses (Kaposi's sarcoma-associated herpesvirus or murine gammaherpesvirus 68 (MHV-68)) ORF10, both of which target cellular mRNA nuclear export and immune antagonistic activity. A methionine flanked by acidic residues in the ORF6 CTT is fundamental to its functions. Nonetheless, the molecular mechanism underlying ORF6-mediated blockade of nucleocytoplasmic trafficking and suppression of antiviral immunity remains elusive.

In this study, we determine the X-ray crystal structures of Rae1-Nup98 in complex with the C-terminal tails (CTT) of SARS-CoV-2 and SARS-CoV ORF6 to 2.85 Å and 2.39 Å resolution, respectively. The structure reveals that M58 residue of ORF6 CTT extends its side chain into a hydrophobic cavity in the Rae1 mRNA binding groove, resembling a bolt-ﬁtting-hole, acidic residues ﬂanking M58 form salt-bridges with Rae1. We show that key residues of ORF6 are important for its interaction with Rae1-Nup98 in vitro and in vivo. Furthermore, we demonstrate that ORF6-mediated blockade of mRNA and STAT1 nucleocytoplasmic transport correlates with the binding afﬁnity between ORF6 and Rae1-Nup98. In summary, our study reveals the molecular basis for the antagonistic function of Sarbecovirus ORF6 and implies a strategy of using ORF6 CTT-derived peptides for immuno-suppressive drug development.

**Results and discussion**

To gain structural insight into Sarbecovirus ORF6-mediated nuclear transport inhibition and innate immunity suppression, we sought to determine the crystal structure of the ORF6-Rae1-Nup98 complex. While we co-expressed a Rae1 fragment (residues 31–368) bound by the Gle2/Rae1-binding sequence (GLEBS, residues 157–213) of Nup98 in insect cells (Fig. 1a–c), ectopic expression of ORF6 proteins in insect cells or bacteria was unsuccessful, probably due to their cytotoxicity and/or the presence of a membrane anchoring helix at the NTE, which might undermine protein stability. To circumvent this problem, we synthesized a panel of peptides covering different regions in the ORF6 CTT (Fig. 1d) and measured the binding afﬁnity of these ORF6-derived peptides to Rae1-Nup98 using isothermal titration calorimetry (ITC). All peptides (peptide C1-C4, Fig. 1e–h and Supplementary Table 1) containing M58 and the surrounding acidic residues bound Rae1-Nup98 with nanomolar afﬁnity ($K_d = 240–440$ nM). Next, we

![Fig. 1](https://doi.org/10.1038/s41467-022-32489-5)
mixed Rae1-Nup98 with those peptides (molar ratio = 1:4) for crystallization, and obtained crystals of Rae1-Nup98 in the presence of peptide C3 (N'-pyQSYLDDEQPMEDDpC, SARS-CoV-2 ORF6 C3). Using a similar protocol, we investigated the interaction of a peptide derived from an equivalent region of the SARS-CoV ORF6 CTT (N'-pyQSYLDDEQPMEDDpC-SARS-CoV ORF6 C3) with Rae1-Nup98. The binding affinity of SARS-CoV-2 ORF6 C3 to Rae1-Nup98 (Kd = 240 nM, Fig. 1g, i) was slightly higher than that of SARS-CoV ORF6 C3 (Kd = 370 nM, Fig. 1g, i). Next, we co-crystallized Rae1-Nup98 with each of these two peptides. The crystals of SARS-CoV-2 ORF6 C3-Rae1-Nup98 and SARS-CoV ORF6 C3-Rae1-Nup98 diffracted the X-ray to 2.85 Å and 2.39 Å, respectively. We determined both crystal structures by molecular replacement (searching model PDB id: 4OWR). Statistics and parameters of data collection and structure refinement are summarized in Supplementary Table 2.

Our crystal structures revealed that both SARS-CoV-2 ORF6 C3 and SARS-CoV ORF6 C3 peptides target a positively charged groove (also known as a putative RNA binding groove, Fig. 2a, b) on the rim of Rae1 β-propellers, and we did not find contact between the peptides and Nup98GLEXL55SARS. We calculated the composite omit maps (with anneal refinement) for both structures. The maps clearly delineate nine residues in SARS-CoV-2 ORF6 C3 and ten residues in SARS-CoV ORF6 C3 (Fig. 2a, b). While the last residue of SARS-CoV-2 ORF6 D61 was clearly visible in the electron density map, two additional C-terminal residues Y62 and P63 following the D61 of SARS-CoV ORF6 were barely visible. Furthermore, the C-terminus of SARS-CoV ORF6 points away from the RNA binding groove of Rae1, which suggests that the two extra C-terminal residues of SARS-CoV ORF6 are not essential for binding. SARS-CoV-2 and SARS-CoV ORF6 C3 peptides bind Rae1 with the same orientation (Fig. 2c, d). In both structures, residue M58 at the ORF6 CTT extends its hydrophobic side chain into a deep hydrophobic cavity in the RNA-binding groove of Rae1, resembling a bolt-fitting hole. The acidic residues (glutamate and aspartate) flanking M58 form salt-bridges with positively charged residues (lysine and arginine) in the RNA-binding groove of Rae1. In addition, we observed several main-chain-mediated hydrogen bonds between the ORF6 CTT and Rae1, which strengthen their interactions. A proline immediately upstream of M58 introduces a bend to the peptides, stabilizing it in an ideal conformation for hydrogen bond interactions with Rae1. Specifically, the cis-configuration of P57 allows for two hydrogen-bonds between the ORF6 residue 56 (SARS-CoV-2 Q56 or SARS-CoV E56) and Rae1 K307, and between the ORF6 M58 and Rae1 R305 (Fig. 2c, d). P57 is not only invariant in Sarbecovirus ORF6 (Supplementary Fig. 1), but also present in MHV-68 ORF10 (Fig. 2e). M413 of MHV-68 ORF10 CTT is crucial for the interaction with the RNA-binding groove of Rae1-Nup98, and this methionine is preceded by P412. By contrast, VS M binds to the opposite side of Rae1-Nup98 with its NTE, and a proline adjacent to M51 is unavailable. Collectively, our analyses suggest that M58 of the ORF6 CTT is essential for binding Rae1.

Given that different viral proteins target the same cavity in Rae1 (constituted by F255, F257, W300 and R305) with a methionine residue, we denoted this cavity as the M-cavity. Among the 20 essential proteogenic amino acids, methionine is the only one with non-branched and non-aromatic hydrophobic side chain, suggesting that the M-cavity strictly selects for size and flexibility of the hydrophobic side chains, and only methionine matches the selection. Superimposing the two structures determined in this study onto the structures of VS M-Rae1-Nup98 and MHV-68 ORF10-Rae1-Nup98 demonstrates that the interaction between the crucial methionine and the M-cavity are similar (Fig. 2e, right). During our manuscript in revision, another group published the crystal structures of ORF6 CTT-Rae1-Nup98 complex from SARS-CoV-2 and SARS-CoV4. Comparing our crystal structures with theirs revealed similar features, including the specific interaction between ORF6 M58 and the M-cavity in Rae1-Nup98. Structural superimposition gave a root mean square deviation (RMSD) of 0.37 Å over 361 aligned Ca atoms for two SARS-CoV-2 ORF6 CTT-Rae1-Nup98 structures and 0.22 Å over 340 aligned Ca atoms for two SARS-CoV ORF6 CTT-Rae1-Nup98 structures (Supplementary Fig. 2). The specificity of the M-pocket for methionine is also supported by other structural investigations. The structure of the Rae1-Nup98 complex alone (PDB id: 3MMY) shows that the M-pocket of Rae1 is occupied by an irrelevant ME of an adjacent Rae1 molecule in crystal lattice. A cell-cycle arrest protein Bub3 in yeast is a structural homolog of Rae1, which also comprises a 7-bladed β-propeller (PDB id: 4BLO). Bub3 harbors a deep cavity constituted by F236, F238, W278 and R283, identical to the M-pocket in Rae1. The M-cavity of Bub3 is occupied by Mt69 at the MELT repeats5, which is similar to viral protein-Rae1 interactions.

To identify the molecular determinants governing the interaction between Sarbecovirus ORF6 and Rae1-Nup98, we synthesized a selection of peptides derived from different viral proteins and compared their binding affinity to Rae1-Nup98 using ITC (Supplementary Table 1, 3). One major difference between SARS-CoV-2 ORF6 and SARS-CoV ORF6 is the lack of a C-terminal Y62-P63 (YP) in the former. Our crystallographic studies suggested that the C-terminus YP of SARS-CoV ORF6 C3 peptides bound the Rae1-Nup98 complex (Fig. 1g, i), which is consistent with ITC results showing that SARS-CoV-2 ORF6 C3 and SARS-CoV ORF6 C3 peptides bound the Rae1-Nup98 complex (Fig. 1g, i). To investigate the contribution of YP in binding, we measured the binding affinity of a SARS-CoV-2 ORF6 C3 harboring a YP extension (SARS-CoV-2 ORF6 C3 + YP) to Rae1-Nup98. Indeed, adding a YP extension did not affect binding affinity dramatically (Kd = 0.20 μM, Fig. 3a and Supplementary Fig. 3a). Conversely, removing YP from the C-terminus of SARS-CoV ORF6 C3 (SARS-CoV ORF6 C3 -YP) increased the binding affinity by 3.4-fold (Kd = 0.11 μM, Fig. 3a and Supplementary Fig. 3b), indicating that YP might negatively modulated the binding affinity of SARS-CoV ORF6 C3 peptides to Rae1-Nup98. The different role of YP in the binding of SARS-CoV-2 ORF6 and SARS-CoV ORF6 C3 peptides to the Rae1-Nup98 complex implies that YP might affect binding in synergy with other residues specific to SARS-CoV-2 ORF6, but not to SARS-CoV ORF6.

We further carried out systematic mutagenesis to determine the role of individual residue of SARS-CoV-2 ORF6 CTT in Rae1-Nup98 binding. Mutations of the acidic residues D53A, E54A, E55A, E59A, D61A and P57A adjacent to M58 moderately reduced the binding affinity to Rae1-Nup98 by 2.4–11.5 folds (Fig. 3a, Supplementary Fig. 3c-e, Fig. 3b-d). By contrast, altering residues I60 and M58 of SARS-CoV-2 ORF6 C3 led to a greater loss of binding affinity. Whereas the I60A mutation reduced binding affinity by ~17.5 folds (Fig. 3a, e), the M58A mutation abolished the binding affinity (Fig. 3a, f), confirming the essential role of M58 in binding. To further investigate the selectivity of M58 in binding Rae1-Nup98, we replaced M58 with an arginine or a leucine. M58R mutation abolished the binding, which confirms that the hydrophobic side chain is selected by the M-cavity (Supplementary Fig. 3d). M58L still caused ~43 folds decline in binding affinity (Kd = 10.37 μM), even though leucine has similar hydrophobicity as methionine6 (Supplementary Fig. 3g). Possibly, the branched side chain of leucine could introduce steric hindrance for accessing the M-pocket. Furthermore, while SARS-CoV-2 ORF6 C3 lacking the last four residues 58-MEID-61 did not bind Rae1-Nup98, a peptide lacking the last three residues 59-EID-61 but retaining M58 showed a weak binding affinity (Kd = 87.71 μM, Supplementary Fig. 3h, i), indicating the requirement of Rae1 M-cavity for methionine. Collectively, these results provide direct evidence that the Rae1 M-cavity strictly selects methionine for binding.

We further compared the binding affinity of peptides derived from MHV-68 ORF10 CTT and VS M NTE with that of ORF6-derived peptides using ITC. MHV-68 ORF10 CTT and VS M NTE bound Rae1-Nup98 with lower affinity (Kd = 5.71 μM and 11.95 μM, Fig. 3g, h) than ORF6-derived peptides. A plausible explanation is that VS M and MHV-68 ORF10 are larger than ORF6, and they both contain other
domains that interact with Rae1-Nup98 in addition to their CTT or NTE. Consistent with our ITC results, recent papers demonstrated that the ORF6-mediated innate immunity suppression is largely dependent on the ORF6 CTTs\(^{14,17}\).

Recent studies showed that SARS-CoV-2 harboring Q56E in ORF6 exhibited elevation in anti-IFN activity\(^{14}\). To reveal the mechanism behind this phenomenon, we measured the binding affinity of SARS-CoV-2 ORF6 C3 containing Q56E to Rae1-Nup98 (Fig. 3a, Supplementary Fig. 3j). Whereas the Q56E mutation increased binding affinity to Rae1-Nup98 by ~6-folds, the Q56A mutation reduced the binding affinity with ~2-fold (Fig. 3a, i). From a structural perspective, the Q56 of SARS-CoV-2 ORF6 lies within the salt-bridging range with the K307 of Rae1; therefore, Q56E could enhance electrostatic interaction between SARS-CoV-2 ORF6 and Rae1 (Fig. 2c). Thus, our results support that the binding affinity of ORF6 CTT to Rae1-Nup98 correlates with its anti-IFN activity. Of note, SARS-CoV ORF6
were calculated: 0.24 μM. Mutations were, from left to right, SARS-CoV-2 Q56A, SARS-CoV E56Q, SARS-CoV-2 P57A, M58A, E59A, I60A and D61A. This experiment was performed in three independent experiments, with similar results. Source data are provided as a Source data file.
contains E56, presenting another key difference from SARS-CoV-2 ORF6. We found that SARS-CoV ORF6 C3 harboring E56Q lost -10.6-folds binding affinity to Rael-Nup98 (Kd = 3.92 μM, Fig. 3a, j), confirming that the negative charge of residue 56 is vital to ORF6-Rael-Nup98 interaction. Collectively, results of these experiments provide mechanistic insights into the role of ORF6 residue 56 in binding Rael-Nup98, which correlate with ORF6-mediated antagonistic activity.

It was previously reported that the positively charged groove on the rim of Rae1 β-propellers, in which VSV M, MHV68 ORF10 and Sarbecovirus ORF6 bind, is a putative RNA binding site of Rael-Nup98. Therefore, this region is also known as the RNA binding groove19,22. Because VSV M can dislocate RNA from Rael-Nup98 via competitive binding at the RNA binding groove, we speculated that Sarbecovirus ORF6 may adopt a similar mechanism. As anticipated, we found that both SARS-CoV-2 and SARS-CoV ORF6 CTTs (C3 peptides) could dislocate single-stranded (ss) RNA from the Rael-Nup98 complex in a concentration-dependent manner (0–64 μM) in our electrophoretic mobility shift assays (EMSA, Fig. 3k, l). Further, our mutagenesis studies demonstrated that several ORF6 residues important for binding the Rael-Nup98 complex were also important for ssRNA dislocation from the complex (Fig. 3m). Whereas ORF6 CTT mutants Q56A, E56Q, P57A, E59A, I60A and D61A lost their ability to dislocate ssRNA from Rael-Nup98 to different extents, mutant M58A was completely unable to compete with ssRNA for binding Rael-Nup98 (Fig. 3m). Additionally, considerably higher concentrations of VSV M NTE or MHV68 ORF10-CTT peptides (100–800 μM) were needed to achieve complete RNA dislocation comparing to that for Sarbecovirus ORF6 CTT peptides (Supplementary Fig. 4a, b). These results are consistent with our ITC assays showing that SARS-CoV-2 and SARS-CoV ORF6 ORF6 CTT bound Rael-Nup98 with higher affinity than that of VSV M NTE and MHV68 ORF10-CTT peptides.

To validate the structural and biophysical characterizations of ORF6-derived peptides and to understand the function of ORF6 as an intact protein in cells, we studied the interaction between ORF6 and Rael-Nup98 using co-immunoprecipitation (Co-IP). The Co-IP results revealed that both SARS-CoV-2 and SARS-CoV ORF6 interacted with Rael-Nup98, while introducing mutations to Sarbecovirus ORF6, reduced its binding affinity to Rael-Nup98 to different degrees (Fig. 4a), which agreed with our ITC results. M58A nearly abolished the binding of SARS-CoV-2 ORF6 to Rael-Nup98, therefore this key methionine was also essential for their interaction in cells. While I60A and P57A mutations impaired the binding of SARS-CoV-2 ORF6 to Rael-Nup98, D61A and E59A had only minor effects (Fig. 4a).

Residues 46 and 56 of SARS-CoV-2 ORF6 were identified as the determinants for its immunosuppressive activity13, although their precise functions remain unknown. While SARS-CoV-2 ORF6 contains E46, residue 46 of SARS-CoV ORF6 is a lysine. Therefore, we swapped residue 46 between these two Sarbecovirus ORF6 proteins and analyzed their binding affinities to Rael-Nup98. While E46K severely undermined the binding of SARS-CoV-2 ORF6 to Rael-Nup98, K46E enhanced the binding of SARS-CoV ORF6 to Rael-Nup98, therefore this key methionine was also essential for their interaction in cells. While I60A and P57A mutations impaired the binding of SARS-CoV-2 ORF6 to Rael-Nup98, D61A and E59A had only minor effects (Fig. 4a).

To verify that the observed GFP expression inhibition was caused by ORF6-mediated impairment of GFP mRNA in the nucleus, we quantified the nuclear to cytoplasmic (Nu/Cyto) ratio of GFP transcripts as described previously20. Overexpressing SARS-CoV-2 and SARS-CoV ORF6 proteins resulted in the accumulation of GFP mRNAs in the nucleus, as indicated by a high Nu/Cyto ratio of GFP transcripts (Fig. 4d). By contrast, the ORF6 mutants that lost the binding affinity to Rael-Nup98 failed in trapping GFP mRNA in the nucleus. SARS-CoV-2 ORF6 E46K and SARS-CoV ORF6 E56Q (i.e., mutants that lost anti-IFN activity) could not block GFP mRNA nuclear export (Fig. 4b–d). Taken together, these results provide experimental evidence that ORF6-mediated blocking of mRNA nuclear export and inhibition of GFP expression depend on the binding of ORF6 to the Rael-Nup98 complex. Disrupting this interaction impairs the function of ORF6.

In addition to blocking mRNA nuclear export, SARS-CoV-2 and SARS-CoV ORF6 proteins block STAT1 nuclear import and suppress the IFN-signaling pathway13,14,21,23,24. Therefore, we investigated the intermolecular interactions implicated in the ORF6-mediated inhibition of STAT1 import. Overexpressing SARS-CoV-2 and SARS-CoV ORF6 dramatically inhibited the IFN-sensitive response element-driven luciferase activity triggered by IFN-α and IFN-β, while ORF6 mutants that lost the binding affinity to Rael-Nup98 also lost their inhibitory activity to different extents (Fig. 4e, f). Specifically, ORF6 harboring the M58A and E46K mutation exhibited the strongest loss of inhibitory activity. The activity of the M58A mutant was similar to that of the negative control (i.e., empty vector). These results indicate that residues M58 and E46 of SARS-CoV-2 ORF6 are essential for its antagonistic activity against IFN-signaling. Finally, we investigated the ability of Sarbecovirus ORF6 in blocking STAT1 nuclear import by measuring the distribution of STAT1 in cytoplasmic and nuclear fractions. While IFN-β treatment induced
STAT1 nuclear import, overexpressing ORF6 blocked STAT1 translocation into the nucleus. Consistent with results from other experiments in this study, ORF6 mutants that lost the binding affinity to Rae1-Nup98 also lost their inhibitory activity (Fig. 4g, h). In addition, we analyzed phosphor-STAT1 translocation from the cytoplasm to the nucleus by confocal microscopy. Consistent with the results shown in Fig. 4g, IFN-β treatment triggered endogenous phosphor-STAT1 nuclear translocation in empty vector-transfected cells, while nuclear import was impaired in cells expressing either SARS-CoV-2 or SARS-CoV ORF6. Importantly, the ability of loss-of-interaction mutants of ORF6 to block phosphor-STAT1 translocation to the nuclei was severely impaired (Fig. 4i).
Although previous studies described the role of ORF6 in disrupting nucleocytoplasmic trafficking, the precise molecular mechanism adopted by ORF6 to disrupt nucleocytoplasmic trafficking remains unclear. Our study demonstrates that binding of SARS-CoV-2 ORF6 to the Rae1-Nup98 complex is a fundamental step for blocking nucleocytoplasmic transport. ORF6 may function as a “gate-keeper” that forms a steric hindrance by binding to the Rae1-Nup98 complex in the cytoplasm to the nuclear pore. Previous studies have demonstrated that SARS-CoV-2 ORF6 disrupts the interaction between Nup98 and KPNA1-KPNB1 through binding with Nup98157-213. In the current study, we did not find interaction between SARS-CoV-2/SARS-CoV ORF6 CTTs and the GLEBS motif of Nup98, implying that Sarbecovirus ORF6 may interact with other regions of Nup98 rather than the GLEBS motif. The Feng-repeats of Nup98 are possibly implicated in binding with ORF6 because the Feng-repeats are also the binding sites for KPNA1-KPNB113,33,36. Collectively, SARS-CoV-2 ORF6 might disrupt the formation of Nup98 for the KPNA1-KPNB1 complex via competitive binding with Rae1-Nup98 as previously described36. An alternative possibility is that the ORF6-Rae1-Nup98 complex forms a steric hindrance during the binding of KPNA1 to Nup98. As such, the docking of the STAT1-KPNB1 complex to Nup98 is impaired and ultimately blocks STAT1 nuclear import. Another recent study suggests that ORF6 clongs the nuclear pore via its interactions with Rae1-N up98, thereby preventing bidirectional nucleocytoplasmic transport18. In supporting this model, our structural and biochemical characterizations demonstrate that Sarbecovirus ORF6 proteins target on the RNA-binding groove in the Rae1-Nup98 complex and dislocate ssRNA from the complex, which provides evidence for the role of Sarbecovirus ORF6 in blocking RNAs nuclear export. Together, we provide here a wealth of experimental evidence demonstrating that binding of ORF6 to Rae1-Nup98 is a fundamental step for blocking nucleocytoplasmic trafficking. Blocking nucleocytoplasmic trafficking ultimately results in innate immunity suppression that facilitates CoV infection. Importantly, this work identifies key determinants in the ORF6 CTT that govern its antagonist functions. While our results support that Sarbecovirus ORF6 may functions as a “gate-keeper” that forms a steric hindrance by binding to the Rae1-Nup98 complex in the cytoplasmic side of the NPC, a recent study showed that overexpressing ORF6 in cells could displace Rae1-Nup98 from the NPC and also reduce the size of nucleus20. However, it remains unclear whether the reduction of nucleus size was caused by the dissociation of Rae1-Nup98 from the NPC. Future structural characterization of the NPC bound to intact ORF6 would better clarify mechanisms underlying ORF6-mediated blockade of nucleocytoplasmic transport.

In summary, this study provides structural basis for the hijacking of the cellular nucleocytoplasmic transport machinery by Sarbecovirus ORF6 proteins. Our results reveal atomic details for binding of SARS-CoV-2 and SARS-CoV ORF6 CTT to the Rae1-Nup98 complex, and identify key residues of the CTT that determine the binding affinity of ORF6 to Rae1-Nup98. We prove that the binding affinity of ORF6 to Rae1-Nup98 accounts for its role in nucleocytoplasmic trafficking blockade and IFNs suppression. Intriguingly, a 12-mer short peptide derived from ORF6 CTT sequence exhibited nanomolar binding affinity to Rae1-Nup98, suggesting a starting point for development of novel immunosuppressive drugs.

**Methods**

**Reagents and cells**

Reagents. All chemicals and reagents used in this study were purchased from Sigma-Aldrich unless otherwise stated. Anti-Flag antibody (Sigma-Aldrich, Cat#: F7425, 1:1000), anti-HA antibody (Sigma-Aldrich, Cat#: H6908, 1:1000), anti-Myc antibody (Sigma-Aldrich, Cat#: SB4303019, 1:1000), Anti FLAG M2 affinity Gel (Merck, Cat#: A2220), anti-Lamin A/C (Cell Signaling technology, Cat#: 4777 S, 1:2000), anti-GAPDH (Huaxing bio, Cat#: HK1828, 1:2000), anti-STAT1 (Cell Signaling technology, Cat#: 4999 S, 1:1000), anti-p-STAT1 (Cell Signaling technology, Cat#: 9177 S, 1:100), Goat anti-Mouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (Invitrogen, Cat#: A11029, 1:500), Goat anti-Rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 555 (Invitrogen, Cat#: A21248, 1:500).

Cells. HEK-293T and HEK293 cells were cultured in DMEM supplemented with 1% penicillin–streptomycin and 10% fetal bovine serum. Cell lines used in this study were not found in the BiSample database of commonly misidentified cell lines provided by the International Cell Line Authentication Committee (ICLAC). Cell lines were authenticated by ATCC and were routinely tested for mycoplasma contamination every 3 months.

**Plasmid construction**

The genes of human RNA export factor one (Rae1; residues 31–368; named by Rae1L31-368), human nucleoporin 98(Nup98) Glec2-binding sequence (GLEBS) motif (residues 157–213; denoted by Nup98157-213) SARS-CoV ORF6, SARS-CoV ORF6 and VSV M were synthesized and codon optimized for expression in insect cells and 293 T cells (Supplementary Table 4). The Rae1 and Nup98157-213 were amplified by PCR and cloned into a pFastBac Dual Vector (Invitrogen) for co-expression. While the gene of Nup98157-213 was inserted to the ORF2 (between Xhol and Kpnl restriction sites) without a tag as previously described22,23. In addition, pHA-Rae1, pMyc-Nup98, pFlag-M and pFlag-ORF6 plasmids were cloned into pCMV-HA, pCMV-Myc and

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pcDNA3.1, respectively (Supplementary Table 5). The pFlag-ORF10 have been constructed previously45.

**Protein expression and purification**

All of the recombinant proteins were expressed in insect cells using the Bac-to-Bac Baculovirus Expression System (Invitrogen). One liter Sf21 cells were infected with 30 ml recombinant baculovirus at 22 °C, 2 days after infection. The cells were harvested by centrifugation and the cell pellet was resuspended and lysed by ultrasonication in buffer containing 50 mM Tris-HCl, pH = 8.0, 150 mM NaCl, 10 mM imidazole, 10 mM β-mercaptoethanol and 1 mM PMSE. The resulting mixture was clarified by centrifugation, and the supernatant was passed through Ni-NTA resin pre-equilibrated with the lysis buffer. The target protein was stripped by elution buffer containing 300 mM imidazole. Subsequently, the N terminal His-tag was removed by TEV protease digestion and re-loaded to Ni-NTA resin to remove His tag, the flowthrough containing nontagged target protein was collected and subjected to a HiTrap Q HP column (GE Healthcare) equilibrated with the buffer containing 20 mM Tris-HCl, pH = 8.0, 75 mM NaCl, and eluted with a 10-1000 mM NaCl gradient. Under this condition, Rae1 and Nup98 does not bind to HiTrap Q HP column but further removed non-specifically bound proteins. The flowthrough fraction containing the target protein was finally purified by size-exclusion chromatography using the Superdex 200 10/300 (GE Healthcare) pre-equilibrated with a storage buffer containing 20 mM Tris pH = 8.0, 100 mM NaCl.

**Crystallization and structure determination**

To crystallize Rae131-368-Nup98157-213 with ORF6 peptide complex, the Rae131-368-Nup98157-213 was concentrated to 1 mg/ml before adding SARS-CoV-2 and SARS-CoV ORF6 peptides. The complexes of protein-peptide were reconstituted by incubating protein and peptide at a molar ratio of 1:4. After incubating at 4 °C overnight, the mixture was concentrated to ~5 mg/mL. The concentrated complex was crystallized by mixing 1 μl protein and 1 μl reservoir buffer containing 0.1 M Bis-Tris pH = 6.5, 45% Polypropylene glycol P 400 in a hanging drop vapor diffusion system at 18 °C. The crystals appeared in 3 days and grew to maximum size in about 1 week. For cryoprotection, the crystals were briefly soaked in the reservoir buffer supplemented with 20% ethylene glycol before flash-frozen in liquid nitrogen. Complete X-ray diffraction data were collected at 100 K at the Shanghai synchrotron radiation facility (SSRF) beamline BL10U2 and BL19U1, Shanghai China. X-ray intensities were processed using the XDS package41; the structure was solved by molecular replacement using the software Phaser MR in Microcal iTC200 calorimeter (MicroCal, USA) at 25 °C as previously described19,22. Briefly, a 10-mer poly (U) ssRNA was synthesized with fluorescein amide (FAM) label at the 5’ end (GenScript). The 0.2 μM ssRNA was first incubated with 2 μM Rae1-Nup98 complex in a buffer containing 10 mM Tris (pH 8.0), 150 mM NaCl, and 0.5 mM TCEP, for 15 min at room temperature. The various peptides were added to the mixture at different concentrations (0-64 μM for ORF6 CTT and mutants, 0-800 μM for ORF10 CTT and M NTE), and incubated for an additional 10 min at room temperature. The reaction samples were loaded onto a 6% native-PAGE gel and run in 45 mM Tris (pH 8.5, titrated with boric acid) buffer at 4 °C. After electrophoresis, the ssRNA was visualized using the fluorescence signal from the FAM label using a Typhoon Trio Variable Mode Imager (GE Healthcare).

**EMSA**

The EMSA assay was performed as previously described and slightly modified9,22. Briefly, a 10-mer poly (U) ssRNA was synthesized with fluorescein amide (FAM) label at the 5’ end (GenScript). The 0.2 μM ssRNA was first incubated with 2 μM Rae1-Nup98 complex in a buffer containing 10 mM Tris (pH 8.0), 150 mM NaCl, and 0.5 mM TCEP, for 15 min at room temperature. The various peptides were added to the mixture at different concentrations (0-64 μM for ORF6 CTT and mutants, 0-800 μM for ORF10 CTT and M NTE), and incubated for an additional 10 min at room temperature. The reaction samples were loaded onto a 6% native-PAGE gel and run in 45 mM Tris (pH 8.5, titrated with boric acid) buffer at 4 °C. After electrophoresis, the ssRNA was visualized using the fluorescence signal from the FAM label using a Typhoon Trio Variable Mode Imager (GE Healthcare).

**Co-IP and western blotting**

HEK-293T cells in 6 cm dish transfected with indicated plasmids were lysed in 600 μl lysis buffer [50 mM Tris-HCl, pH 8.0; 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, EDTA-free protease inhibitor cocktail (Roche 04693)] for 30 min at 4 °C. The supernatant of cell lysates was obtained by centrifugation at 15,870×g for 15 min at 4 °C. 50 μl cell lysate was taken as input, and the rest was incubated with anti-FLAG-M2 conjugated agarose for 5 h at 4 °C. The protein-bound were washed five times with lysis buffer at 4 °C and then boiled in SDS-PAGE loading buffer for western blotting. The samples were analyzed by SDS-PAGE and the indicated antibodies. All western blot images were scanned and collected using MINICHEMI (SAGECREATION).

**Nuclear and cytoplasmic RNA/protein fractionation**

Nuclear and cytoplasmic RNA/protein fractionation was extracted according to a method described previously22. Briefly, 293T cells in 12-well plates were collected by trypsinization, and further washed with cold DEPC-treated PBS three times. Then, cells were pelleted at 250 × g for 3 min at 4 °C and resuspended in RSB buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM MgCl2, 1 mM DTT). Incubated on ice for 5 min, followed by centrifugation at 250 × g for 3 min. The cell pellet was resuspended by pipetting with 100 μl lysis buffer RSBG40 (10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM MgCl2, 10% glycerol, 0.5% NP-40, 1 mM DTT). The nuclei were pelleted at 250×g for 5 min. The supernatant was saved as the cytoplasmic fraction, and centrifuged twice at 15,870×g for 10 min at 4 °C to remove any nuclei or cellular debris. The nuclei were resuspended with 1 ml RSBG40 buffer by gently pipetting, incubated on ice for 5 min, and then pelleted at 1500 rpm for 3 min. After washing with RSBG40 buffer three times, the nuclei were collected as the nuclear fraction. After nuclear and cytoplasmic fractions were separated, the extracted RNA or proteins was carried out for RT-PCR and western blotting respectively.

**Reverse transcription and real-time PCR**

The mRNA was reverse-transcribed to cDNA according to Genomic DNA Eraser Reverse Transcription Kits (Takara, catalog no. RR047A). Briefly, 1 μg of RNA was treated with DNA eraser enzyme to remove genomic DNA, and then cDNA was synthesized using PrimerScript RT enzyme mix and RT primer mix. 100 ng RNA were subjected to Real-
time PCR (Quantstudio 7, Applied Biosystems) to determine the GFP RNA transcript copy number, and the primers for RT-PCR were designed according to Gong et al.20.

**Luciferase reporter assay**

HEK-293T cells were transfected with ISRE-firefly luciferase reporter plasmid, pRL-TK (renilla luciferase) and the indicated expression plasmids. After 24 h transfection, cells were treated with IFN-β and IFN-α (500 IU/ml) for 16 h, and luciferase activity was measured. The dual luciferase assay kit (Promega E1960) was used to perform luciferase assays, and GloMax-Multi JR detection system (Promega) was used to quantify luciferase activity.

**Immunofluorescence assay (IFA)**

HEK-293 cells were seeded in 24 well plates and transfected with Flag-tagged SARS-CoV-2 and SARS-CoV ORF6 and mutants or empty vector (EV). After a 24 h transfection, cells were stimulated with IFN-β (500 IU/ml) for 1 h. Subsequently, cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 in phosphate-buffered saline (PBS). Cells were blocked and incubated with anti-FLAG and antipSTAT1 primary antibodies overnight at 4 °C. The fluorophore-conjugated secondary antibodies were diluted 1:500, and nuclei were visualized with DAPI staining. The antibodies used in this assay were as follows: FLAG antibody from Sigma-Aldrich (1:200, Cat# F7425); and pSTAT1 from Cell Signaling Technology (1:100, Cat# 9177S). Fluorescence images were obtained using a confocal microscope (A1R +, Nikon) and analyzed with NIS-Elements viewer 4.2.

**Reporting summary**

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The atomic coordinates and structure factors have been deposited in the Protein Data Bank under the accession codes: 7F60 (SARS-CoV-2 ORF6 in complexed Rae1-Nup98) and 7F90 (SARS-CoV ORF6 in complexed Rae1-Nup98). Publicly available protein atomic models with the following PDB code was used in the study: 4OWR. Other data are available from the corresponding author upon reasonable request. Source data are provided with this paper.

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Author contributions

S. C., H. D. and X. G. designed the study. S. C., H. D., H. T. and X. G. solved the structure and wrote the paper. X. G., H. T., K. Z., Q. L., L. W., B. Q. and W. H. performed experiments, analyzed the data and revised the paper. All authors reviewed the results and approved the final version of the manuscript.

Competing interests

Patents protecting the design and application of the ORF6-derived peptides (including its derivatives) described in this paper is pending by the authors of this paper, Institute of Pathogen Biology, Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, 100730, P. R. China) and CAS Key Laboratory of Infection and Immunity, CAS Center for Excellence in Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China; University of Chinese Academy of Sciences, Beijing 100049, China.

Additional information

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