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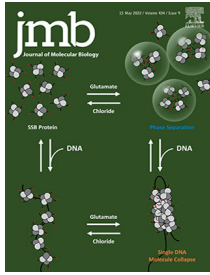
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# SARS-CoV-2 Nucleocapsid Protein Targets a Conserved Surface Groove of the NTF2-like Domain of G3BP1

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## Abstract

Stress granule (SG) formation mediated by Ras GTPase-activating protein-binding protein 1 (G3BP1) constitutes a key obstacle for viral replication, which makes G3BP1 a frequent target for viruses. For instance, the SARS-CoV-2 nucleocapsid (N) protein interacts with G3BP1 directly to suppress SG assembly and promote viral production. However, the molecular basis for the SARS-CoV-2 N – G3BP1 interaction remains elusive. Here we report biochemical and structural analyses of the SARS-CoV-2 N – G3BP1 interaction, revealing differential contributions of various regions of SARS-CoV-2 N to G3BP1 binding. The crystal structure of the NTF2-like domain of G3BP1 (G3BP1<sub>NTF2</sub>) in complex with a peptide derived from SARS-CoV-2 N (residues 1–25, N<sub>1–25</sub>) reveals that SARS-CoV-2 N<sub>1–25</sub> occupies a conserved surface groove of G3BP1<sub>NTF2</sub> via surface complementarity. We show that a  $\phi$ -X-F ( $\phi$ , hydrophobic residue) motif constitutes the primary determinant for G3BP1<sub>NTF2</sub>-targeting proteins, while the flanking sequence underpins diverse secondary interactions. We demonstrate that mutation of key interaction residues of the SARS-CoV-2 N<sub>1–25</sub> – G3BP1<sub>NTF2</sub> complex leads to disruption of the SARS-CoV-2 N – G3BP1 interaction *in vitro*. Together, these results provide a molecular basis of the strain-specific interaction between SARS-CoV-2 N and G3BP1, which has important implications for the development of novel therapeutic strategies against SARS-CoV-2 infection.

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## Introduction

The Covid-19 pandemic caused by the novel Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) is posing a grave threat to global public health, resulting in ~28 million confirmed cases and ~5 million deaths reported to date. SARS-CoV-2 is a beta-coronavirus containing a ~30 kb, positive-sense single-stranded RNA genome, which makes it one of the largest genomes of all known RNA viruses. The SARS-CoV-2 genome is organized into 14 open reading frames (ORFs) encoding 27 proteins that are functionally divided into non-structural proteins (NSPs), structural proteins and accessory proteins. The structural proteins include Spike

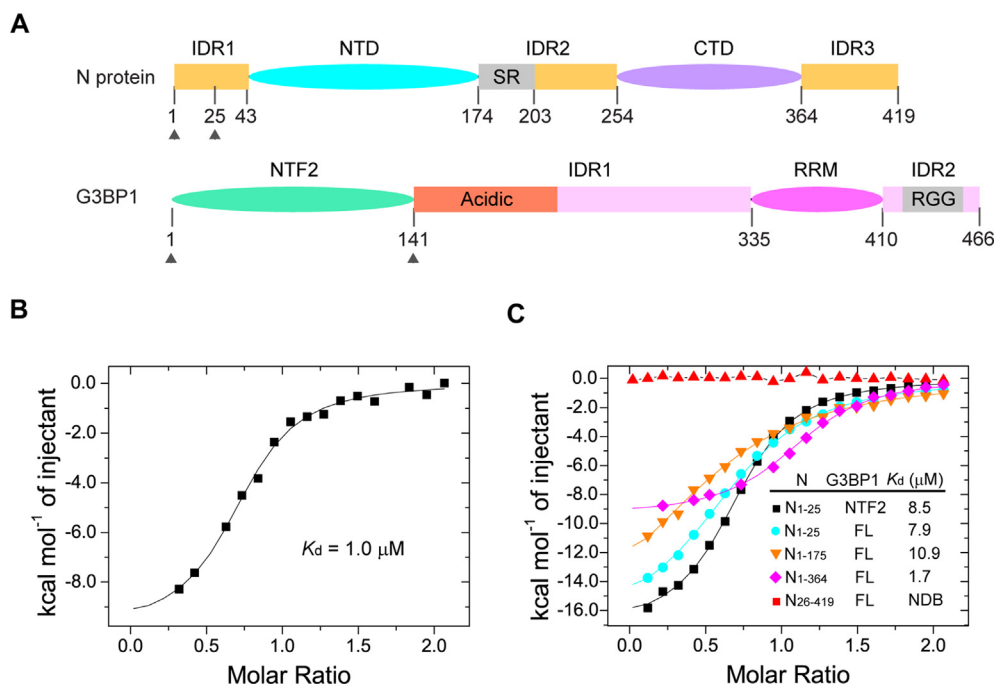
protein (S), Envelope protein (E), Membrane protein (M) and Nucleocapsid protein (N).<sup>1</sup> Among these, the N protein is essential for virion assembly and viral RNA synthesis.<sup>2,3</sup> In addition, the N protein modulates a variety of host cellular activities, such as actin reorganization, cell cycle progression, apoptosis, and immune response<sup>4</sup>. The multifunctionality of the N protein makes it an attractive target for drug intervention against Covid-19.<sup>5</sup>

Recent studies have further demonstrated that SARS-CoV-2 N plays a role in disassembling host stress granule (SG) through an interaction with Ras GTPase-activating protein-binding protein 1 (G3BP1) and 2 (G3BP2).<sup>6,7</sup> SGs are dynamic ribonucleoprotein (RNP) assemblies formed in eukaryotic cells in response to various

stresses, such as oxidative stress and viral infection,<sup>8,9</sup> with G3BP1/2 serving as key SG-nucleating agents.<sup>10–15</sup> Through restriction of protein synthesis and insulating viral mRNAs, SG serves as a key defense mechanism for the host to counter viral attack.<sup>16</sup> G3BP1/2 contains an N-terminal nuclear transport factor 2-like (referred to as NTF2 herein) domain responsible for protein interaction and a C-terminal RRM domain responsible for RNA binding (Figure 1(A)). The NTF2 domain is followed by the first intrinsically disordered region (IDR1) harboring an acidic segment, while the RRM domain is followed by an Arg-Gly-rich (RGG) region-containing IDR (IDR2) (Figure 1(A)).<sup>17</sup> The G3BP1- and G3BP2-mediated SG assembly and disassembly is dynamically regulated via a network of competing protein and RNA interactions involving the NTF2 domain, the RRM domain and the IDR2, as described by the network theory proposed recently.<sup>10,18,19</sup> It has been demonstrated that the SG-associated protein Caprin1, containing G3BP1/2 NTF2-binding, oligomerization and RNA-interaction modules, provides multiple intermolecular contacts (valences) to expand the protein-RNA interaction network within the SG, thereby promoting SG condensation.<sup>19,20</sup> In contrast, another SG-associated protein USP10, which possesses a G3BP1/2 NTF2-binding module but lacks an oligomerization or

RNA-binding module, serves as a valence cap to inhibit the SG formation.<sup>19,20</sup> Given its important role in the formation of SG, the G3BP1/2 protein has become a recurrent target for viral proteins to suppress the SG assembly.<sup>21–24</sup> For instance, the non-structural protein 3 (nsP3) from the Old World alphavirus recruits G3BP1 to viral replication complex via an interaction with the NTF2 domain, leading to disruption of SG in virus-infected cells.<sup>21,25,26</sup> Likewise, SARS-CoV-2 N was shown to partition into stress granules and interacts with the NTF2 domains of G3BP1 and G3BP2, resulting in inhibition of SG assembly to promote viral infection in complementation-based assay.<sup>7,27</sup> However, the underlying mechanism of SARS-CoV-2 N-mediated SG disassembly is unclear.

The crystal structure of the G3BP1 NTF2 domain (G3BP1<sub>NTF2</sub>) in complex with a peptide derived from nucleoporin, Semliki Forest virus (SFV) nsP3 or Caprin1 has been reported.<sup>26,28,29</sup> Common to these structures is an insertion of a phenylalanine residue from the G3BP1<sub>NTF2</sub>-interacting peptide into a hydrophobic pocket formed by the G3BP1<sub>NTF2</sub> domain. On the other hand, these structures show large divergence for the Phe-flanking regions of the peptide sequence, suggesting high diversity for the G3BP1<sub>NTF2</sub>-mediated protein interactions.



**Figure 1. Biochemical characterization of the interaction between SARS-CoV-2 N and G3BP1.** (A) Domain architecture of SARS-CoV-2 N and G3BP1, with individual domains color coded. The SR-rich region within the IDR2 of SRS-CoV-2 N, the acidic region within the IDR1 of G3BP1 and the RG-rich region (RGG) within the IDR2 of G3BP1 are labeled. The protein fragments (residues 1–25 of SARS-CoV-2 N protein and 1–139 of G3BP1) used for crystallographic study are delimited by arrows. (B) ITC binding assays for full-length SARS-CoV-2 N and G3BP1. (C) ITC binding assays for the truncated fragments of SARS-CoV-2 N and G3BP1. FL, full length; NDB, no detectable binding.

SARS-CoV-2 N consists of an N-terminal domain (NTD) responsible for RNA binding and a C-terminal domain (CTD) that mediates both RNA binding and dimerization (Figure 1(A)).<sup>30–32</sup> Both the NTD and CTD domains are flanked by IDRs to reinforce their RNA-binding activities,<sup>2,33</sup> with a serine-arginine (SR) rich segment located within the second IDR (IDR2) subjected to posttranslational modification for modulation of RNA binding.<sup>34,35</sup> Recent biochemical and cellular evidence indicated that the interaction between SARS-CoV-2 N protein and G3BP1<sub>NTF2</sub> critically depends on the N-terminal IDR (IDR1) of SARS-CoV-2 N protein<sup>7,27,36,37</sup>. Deletion of the first 50 residues of SARS-CoV-2 N led to disruption of SG inhibition and reduced viral production,<sup>7</sup> suggesting an important role of the IDR1 of SARS-CoV-2 N in viral infection. Consistently, introduction of an I15A/T16A/F17A/G18A quadruple mutation to the IDR1 of SARS-CoV-2 N led to abolished G3BP1 binding in an *in vitro* binding assay, indicating an ITFG motif-dependent interaction.<sup>37</sup> However, due to the lack of structural information, the molecular basis for the interaction between SARS-CoV-2 N and G3BP1 remains elusive.

To determine the molecular basis of the SARS-CoV-2 N – G3BP1 interaction, we solved the crystal structure of the complex between the G3BP1<sub>NTF2</sub> domain and a peptide derived from the IDR1 of SARS-CoV-2 N (comprising residues 1–25, N<sub>1–25</sub>) at a resolution of 2.35 Å. The structure revealed surface complementarity and hydrophobic groove-insertion mechanisms dominating the SARS-CoV-2 N<sub>1–25</sub> – G3BP1<sub>NTF2</sub> interaction. Comparative structural analyses of SARS-CoV-2 N – G3BP1<sub>NTF2</sub> and other G3BP1<sub>NTF2</sub> complexes further revealed a consensus  $\phi$ xF motif as a primary G3BP1<sub>NTF2</sub>-binding determinant, which insert into a surface groove of G3BP1<sub>NTF2</sub> that is conserved among the NTF2 domains. On the other hand, the  $\phi$ xF-flanking sequences underpin diverse secondary interactions among various G3BP1<sub>NTF2</sub> complexes, raising a possibility for the development of specific inhibitors toward the SARS-CoV-2 N – G3BP1 interaction. The  $\phi$ xF motif is conserved in SARS-CoV and SARS-CoV-2 but not in many other coronavirus strains, suggesting a strain-specific G3BP1<sub>NTF2</sub> interaction. This study provides a framework for molecular understanding of the targeting of G3BP1 by SARS-CoV-2 N, with important implications in development of therapeutic interventions against SARS-CoV-2 infection.

## Results

### Biochemical characterization of the SARS-CoV-2 N – G3BP1/2 interaction

To identify the interaction elements between SARS-CoV-2 N and G3BP1, we performed

isothermal titration calorimetry (ITC) on full-length or truncated fragments of SARS-CoV-2 N and G3BP1. Full-length SARS-CoV-2 N and G3BP1 interact strongly, with a dissociation constant ( $K_d$ ) of 1.0  $\mu$ M (Figure 1(B) and Figure S1(A)), and enthalpy and entropy changes of  $\Delta H = -9.9$  kcal/mol and  $\Delta S = -5.9$  cal/mol/deg, respectively (Table S1). Similar binding thermograms were observed when full-length G3BP1 was titrated with the C-terminal IDR3-truncated SARS-CoV-2 N (residues 1–364, N<sub>1–364</sub>), which gave a  $K_d$  of 1.7  $\mu$ M (Figure 1(C) and Figure S1(B)), and enthalpy and entropy changes of  $\Delta H = -9.4$  kcal/mol and  $\Delta S = -5.4$  cal/mol/deg, respectively (Table S1). Next, we titrated full-length G3BP1 with the SARS-CoV-2 N fragment encompassing the IDR1 and NTD but not the CTD and IDR2 (residues 1–175, N<sub>1–175</sub>). We observed a  $K_d$  of 10.9  $\mu$ M (Figure 1(C) and Figure S1(C)), which is  $\sim 11$ - and  $\sim 6$ -fold weaker than those of full-length N or N<sub>1–364</sub>, respectively. Interestingly, comparison of the titration parameters for full-length SARS-CoV-2 N, N<sub>1–364</sub> and N<sub>1–175</sub> reveals that removal of the IDR2 and CTD led to increased reductions for both enthalpy ( $\Delta H = -18.2$  kcal/mol) and entropy ( $\Delta S = -38.2$  cal/mol/deg) (Table S1), suggesting that, in the context of full-length N or N<sub>1–364</sub>, the CTD and/or IDR2 contribute to the G3BP1 binding by reducing the entropic cost of the complex formation. Furthermore, we titrated full-length G3BP1, the G3BP1<sub>NTF2</sub> domain and the G3BP2<sub>NTF2</sub> domain with the peptide derived from the first 25 residues of the IDR1 of SARS-CoV-2 N protein (N<sub>1–25</sub>), which gave  $K_d$ s of 7.9  $\mu$ M, 8.5  $\mu$ M and 10.9  $\mu$ M, respectively (Figure 1(C) and Figure S1(D–F)), consistent with the previous observations that the IDR1 of SARS-CoV-2 N protein is primarily responsible for its interaction with G3BP1/2.<sup>7,27,36,37</sup> Notably, the enthalpy and entropy changes associated with the titrations by the N<sub>1–25</sub> peptide are equivalent to those of SARS-CoV-2 N<sub>1–175</sub> within experimental error (Table S1), suggesting a minimal effect of the NTD of SARS-CoV-2 N in G3BP1 binding. Finally, titration of full-length G3BP1 with SARS-CoV-2 N<sub>26–419</sub> showed no appreciable binding (Figure 1(C) and Figure S1(G)), indicating that the N<sub>1–25</sub> is indispensable for the SARS-CoV-2 N – G3BP1 interaction.

Together, these observations identified that the SARS-CoV-2 N<sub>1–25</sub> and the G3BP1<sub>NTF2</sub> domain as the major elements for the SARS-CoV-2 N protein – G3BP1 interaction, whereas the CTD and/or IDR2 of SARS-CoV-2 N protein contribute to the interaction entropically.

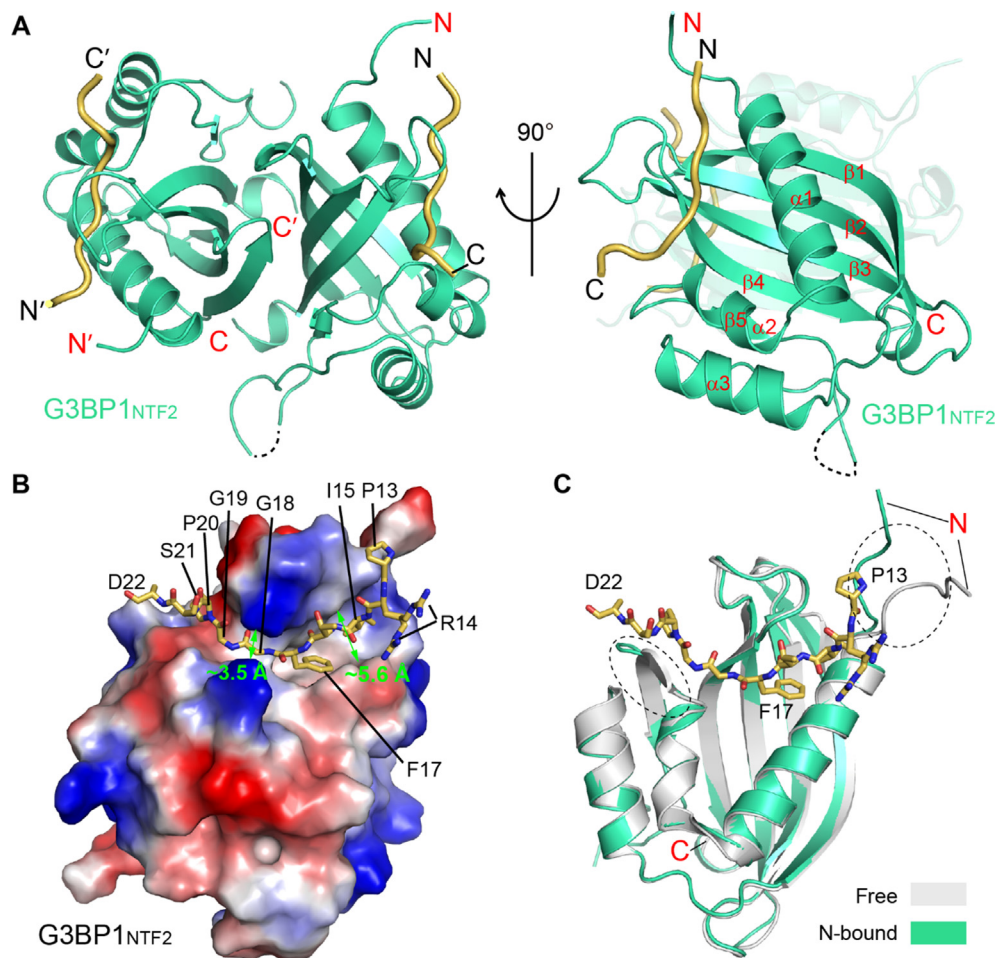
### Crystal structure of the SARS-CoV-2 N<sub>1–25</sub> – G3BP1<sub>NTF2</sub> complex

We then solved the crystal structure of the G3BP1<sub>NTF2</sub> domain bound to the SARS-CoV-

2 N<sub>1-25</sub> peptide at 2.35 Å resolution (Figure 2(A) and Table S2). The SARS-CoV-2 N<sub>1-25</sub> – G3BP1<sub>NTF2</sub> complex belongs to the P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub> space group, with each asymmetric unit containing a G3BP1<sub>NTF2</sub> homodimer bound to the N<sub>1-25</sub> peptide in a G3BP1<sub>NTF2</sub>:N<sub>1-25</sub> ratio of 2:2. We were able to trace nearly the entire G3BP1<sub>NTF2</sub> domain, spanning residues V2-F138 (except for S47) (Figure 2(A)), as well as residues P13-D22 of the SARS-CoV-2 N<sub>1-25</sub> peptide (Figure S2(A)). Note that this segment of SARS-CoV-2 N is strictly conserved in SARS-CoV N, but not in the counterparts of related Middle East Respiratory Syndrome Coronavirus (MERS-CoV) or other coronaviruses (Figure S2(B)).

As previously characterized,<sup>26,29,38</sup> the G3BP1<sub>NTF2</sub> domain is dominated by a five-stranded antiparallel β-sheet, preceded by three

α-helices packed on one side of the β-sheet (Figure 2(A)). Two of the G3BP1<sub>NTF2</sub> domains further undergo a face-to-face β-sheet stacking with each other to form a homodimer. On the outer face of the β-sheet of each G3BP1<sub>NTF2</sub> monomer, the α1- and α2-helices join with the loop connecting β3 and β4 (I<sub>β3β4</sub>) to form a long surface groove, cradling the extended SARS-CoV-2 N<sub>1-25</sub> peptide (Figure 2(B)). The association of SARS-CoV-2 N<sub>1-25</sub> peptide with the G3BP1<sub>NTF2</sub> is underpinned by their strong surface complementarity (Figure 2(B)). Notably, the bulky side chains of N<sub>1-25</sub> I15 and F17 are accommodated by a ~5.6 Å-wide groove, whereas the downstream residues G18 and G19 snug into a ~3.5 Å-wide groove (Figure 2(B)). In fact, our structural modeling analysis indicated that replacement of N<sub>1-25</sub> G18 with bigger-sized threonine would lead to a steric clash with G3BP1<sub>NTF2</sub> K123 (Figure S2



**Figure 2. Structural overview of the SARS-CoV-2 N<sub>1-25</sub> – G3BP1<sub>NTF2</sub> complex.** (A) Orthogonal views of the SARS-CoV-2 N<sub>1-25</sub> – G3BP1<sub>NTF2</sub> complex, with SARS-CoV-2 N<sub>1-25</sub> colored in yellow orange and G3BP1<sub>NTF2</sub> colored in green. The traceable N- and C-termini of SARS-CoV-2 N<sub>1-25</sub> or G3BP1<sub>NTF2</sub> are labeled with “N” and “C”, respectively. The region (residue S47) with untraceable electron density is shown in dashed line. (B) Electrostatic surface view of the G3BP1<sub>NTF2</sub> domain bound to the SARS-CoV-2 N<sub>1-25</sub> peptide (stick representation). The widths of two distinct groove regions of G3BP1<sub>NTF2</sub> are marked. For clarity, only one monomer of the G3BP1<sub>NTF2</sub> homodimer is shown. (C) Structural overlay of the G3BP1<sub>NTF2</sub> domain, free (grey) and in complex with SARS-CoV-2 N<sub>1-25</sub> (green). The two structurally divergent regions are circled with dotted lines.

(C)). Together, the association of SARS-CoV-2 N<sub>1–25</sub> with G3BP1<sub>NTF2</sub> results in a buried surface area of ~571 Å<sup>2</sup>. Structural superposition of the SARS-CoV-2 N<sub>1–25</sub>-bound G3BP1<sub>NTF2</sub> with the previously reported apo form<sup>38</sup> gives a root-mean-square deviation (RMSD) of 0.43 Å over 225 aligned C $\alpha$  atoms (Figure 2(C)), indicative of high similarity. The most divergent regions include the N-terminal tail and the loop connecting  $\alpha$ 2 and  $\beta$ 2, both of which are involved in the interaction with the N<sub>1–25</sub> peptide (Figure 2(C)). These observations highlight that the G3BP1<sub>NTF2</sub> domain adopts a preconfigured conformation for the interaction with N<sub>1–25</sub> peptide.

### Structural details of the SARS-CoV-2 N<sub>1–25</sub> – G3BP1<sub>NTF2</sub> interaction

The assembly of G3BP1<sub>NTF2</sub> with SARS-CoV-2 N<sub>1–25</sub> is mediated by a network of hydrogen-bonding and van der Waals contacts (Figure 3(A, B)). Of note, the aromatic ring of SARS-CoV-2 N<sub>1–25</sub> F17 inserts into the hydrophobic cavity formed by the side chains of G3BP1<sub>NTF2</sub> V11, F15, Q18, F33 and F124 (Figure 3(A–D)). The side chain of N<sub>1–25</sub> I15 is positioned in parallel with that of N<sub>1–25</sub> F17, engaging in nonpolar contacts with the side chains of G3BP1<sub>NTF2</sub> P6, L10 and V11 (Figure 3(B, D)). The side chains of N<sub>1–25</sub> P13, R14 and T16 point away from G3BP1<sub>NTF2</sub>, with N<sub>1–25</sub> P13 surrounded by the side chains of G3BP1<sub>NTF2</sub> A121 and N122, the guanidinium of N<sub>1–25</sub> R14 positioned within a distance for an electrostatic contact with the sidechain carboxylate of G3BP1<sub>NTF2</sub> E14, and N<sub>1–25</sub> T16 in proximity with the side chain of G3BP1<sub>NTF2</sub> V120 (Figure 3(A, B)). Along one side of the groove, the N<sub>1–25</sub> I15–G18 segment pairs in parallel with the C-terminal end of G3BP1<sub>NTF2</sub>  $\beta$ 5 (residues A121–Y125), involving both direct and water-mediated main chain hydrogen bonds (Figure 3(A, B)). On the other side of the groove, G3BP1<sub>NTF2</sub> Q18, R32 and K123 form water-mediated or direct hydrogen bonds with the backbone of N<sub>1–25</sub> F17, G18 and G19, respectively (Figure 3(A, B)). Additional intermolecular interactions involve the van der Waals contacts between the backbone of N<sub>1–25</sub> G18–S21 and the side chains of G3BP1<sub>NTF2</sub> F33, Q58, E117 and Y125 (Figure 3(A, B)).

To test the structural observation, we selected several key SARS-CoV-2 N<sub>1–25</sub> – G3BP1<sub>NTF2</sub> interacting residues for mutagenesis and performed ITC binding assays. Mutation of N<sub>1–25</sub> I15 or T16 each to alanine reduced the binding by > 10-fold and ~3-fold, respectively (Figure 3(E) and Figure S3(A, B)). Mutation of N<sub>1–25</sub> F17 to alanine or asparagine abolished the interaction between SARS-CoV-2 N<sub>1–25</sub> and G3BP1<sub>NTF2</sub> (Figure 3(E) and Figure S3(C, D)), supporting the role of these residues in the G3BP1<sub>NTF2</sub> interaction. Furthermore, we observed that introducing the N<sub>1–25</sub> G18T mutation led to ~6-fold reduction of the binding affinity (Figure S3(E)),

supporting the notion that surface complementarity underpins the specific interaction between SARS-CoV-2 N<sub>1–25</sub> and G3BP1<sub>NTF2</sub>. Conversely, we also observed impairment of the SARS-CoV-2 N<sub>1–25</sub> – G3BP1<sub>NTF2</sub> interaction by several G3BP1<sub>NTF2</sub> mutations: Introducing G3BP1<sub>NTF2</sub> V11A and F124A mutations reduced the binding by ~10- and ~20-fold, respectively, while introducing the G3BP1<sub>NTF2</sub> F15A mutation abolished the binding.

It is worth noting that introduction of the G3BP1<sub>NTF2</sub> Q18A or F33A mutations led to no appreciable change in binding (for Q18A) or even slightly enhanced the binding affinity (for F33A). The G3BP1<sub>NTF2</sub> Q18A mutation modestly reduced both the enthalpy ( $\Delta H = -15.1$  kcal/mol for Q18A vs  $-17.6$  kcal/mol for wild-type) and entropy ( $\Delta S = -27.8$  cal/mol/degree for Q18A vs  $-35.0$  cal/mol/degree for wild-type) effects, whereas the G3BP1<sub>NTF2</sub> F33A mutation led to an increase of both enthalpy ( $\Delta H = -21.3$  kcal/mol for Q18A vs  $-17.6$  kcal/mol for wild-type) and entropy ( $\Delta S = -51.6$  cal/mol/degree for F33A vs  $-35.0$  cal/mol/degree for wild-type) effects. These enthalpy–entropy compensation effects, the origin of which is currently unclear, suggest a certain extent of structural plasticity of the N<sub>1–25</sub> F17-binding pocket of the G3BP1<sub>NTF2</sub> domain.

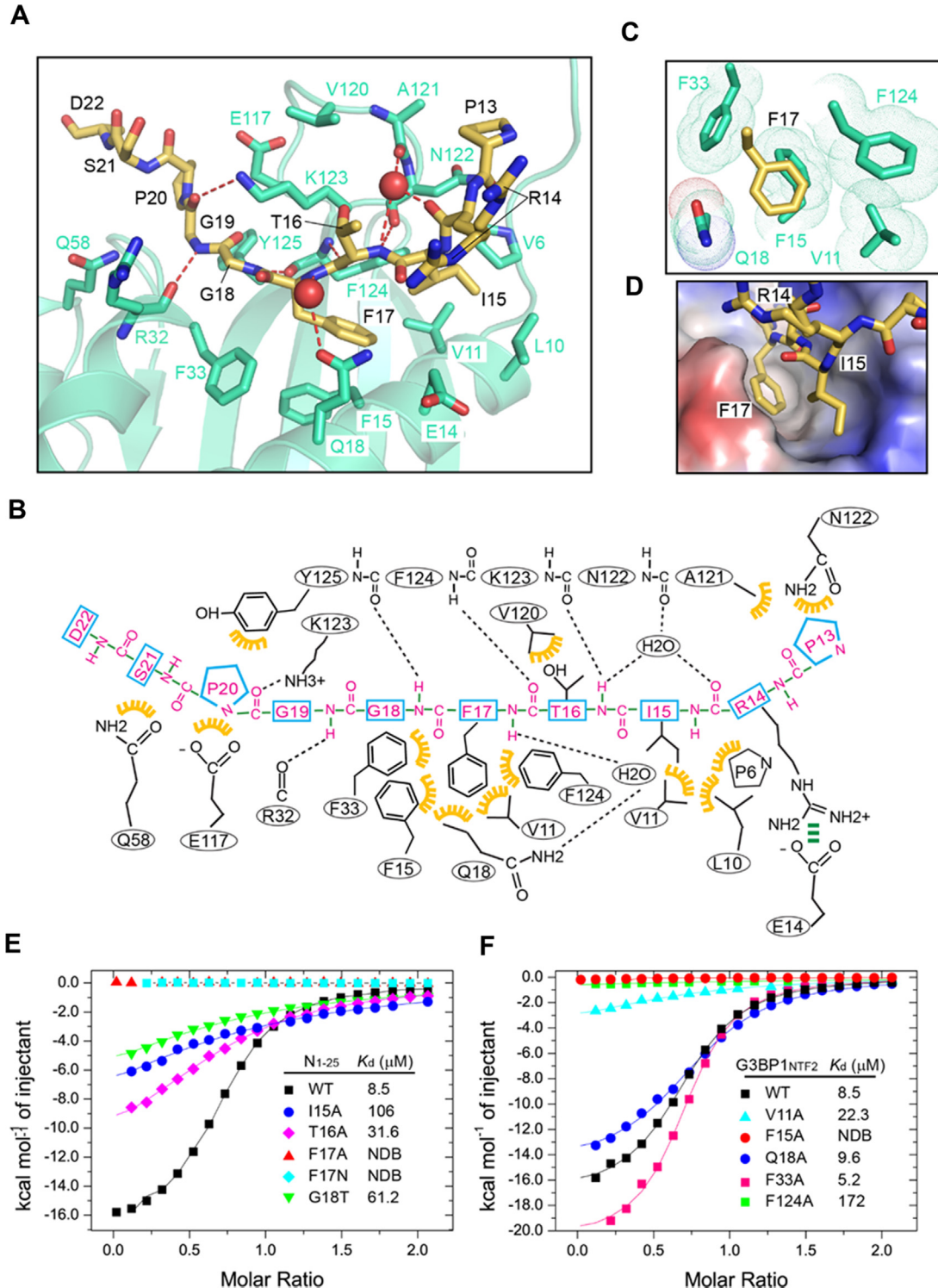
### Distinct intermolecular interaction mechanisms among G3BP1 complexes

Structural comparison of the G3BP1<sub>NTF2</sub> domain bound to SARS-CoV-2 N<sub>1–25</sub> with that bound to a peptide derived from the SFV nsP3 protein (residues 449–471, nsP3<sub>449–471</sub>) or a peptide derived from Caprin1 (residues 363–382, Caprin1<sub>363–382</sub>) reveals different binding stoichiometry: In the SFV nsP3<sub>449–471</sub> – G3BP1<sub>NTF2</sub> complex, two FGDF motifs of SFV nsP3 each bind to one G3BP1<sub>NTF2</sub> homodimer, thereby tethering the G3BP1 molecules into a poly-complex<sup>26</sup>; in contrast, in the SARS-CoV-2 N<sub>1–25</sub> – G3BP1<sub>NTF2</sub> and Caprin1<sub>363–382</sub> – G3BP1<sub>NTF2</sub> complexes, the G3BP1<sub>NTF2</sub> domain binds to the N<sub>1–25</sub> or Caprin1<sub>363–382</sub> peptide in a 2:2 binding mode (Figure 4(A, B)). Nevertheless, residues I15–G18 of N<sub>1–25</sub>, L449–G452 of the nsP3<sub>449–471</sub> peptide and Y370–I373 of the Caprin1<sub>363–382</sub> peptide are anchored to the surface groove of the G3BP1<sub>NTF2</sub> domain in a similar fashion (Figure 4(A–C) and Figure S4(A)), with the aromatic rings of N<sub>1–25</sub> F17, nsP3<sub>449–471</sub> F451 and Caprin1<sub>363–382</sub> F372 embraced by the same aromatic cage of the G3BP1<sub>NTF2</sub> domain (Figure 4(A, B) and Figure S4(A, B)). In addition, nsP3<sub>449–471</sub> L449 and Caprin1<sub>363–382</sub> Y370 insert their side chains into the groove similarly as the corresponding N<sub>1–25</sub> I15 (Figure 4(C, D)). Beyond this region, nsP3<sub>449–471</sub> and Caprin1<sub>363–382</sub> interact with the G3BP1<sub>NTF2</sub> domain in a distinct mode than that of N<sub>1–25</sub> (Figure 4(A, B)). Unlike the N<sub>1–25</sub> peptide that occupies

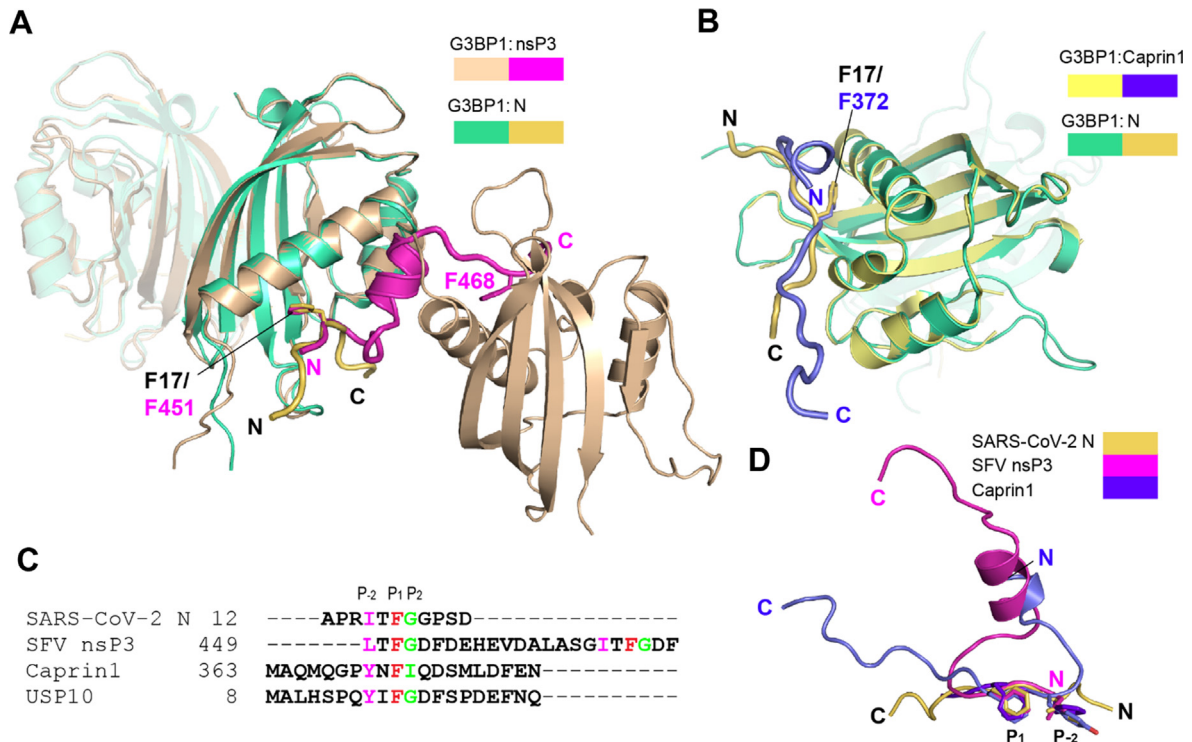
the entire groove, the nsP3<sub>449-471</sub> peptide exits from the surface groove at the D453 site to engage in a secondary contact with the  $\alpha$ 1-helix of G3BP1<sub>N<sub>TF</sub>2</sub> via helical packing (Figure 4(A) vs Figure 2(B)). Subsequently, the C-terminal segment of nsP3<sub>449-471</sub> extends into another G3BP1<sub>N<sub>TF</sub>2</sub> homodimer and presents residue F468 for a groove-insertion interaction like that of F452

(Figure 4(A)). Similar to nsP3<sub>449-471</sub>, Caprin<sub>363-382</sub> forms a helical turn at its N-terminal end to interact with the  $\alpha$ 1-helix of the G3BP1<sub>N<sub>TF</sub>2</sub> domain (Figure 4 (B)), albeit in an opposite direction (Figure 4(D)).

Together, our structural and sequence analyses of the G3BP1<sub>N<sub>TF</sub>2</sub>-binding peptides reveal a  $\varphi$ x $\varphi$  (  $\varphi$  denotes a hydrophobic amino acid) consensus motif, in which an invariable phenylalanine







**Figure 4. Structural comparison of SARS-CoV-2 N<sub>1-25</sub> – G3BP1<sub>NTF2</sub> with other G3BP1<sub>NTF2</sub> complexes.** (A) Structural overlay between the SARS-CoV-2 N<sub>1-25</sub> (yellow orange) – G3BP1<sub>NTF2</sub> (green) and the SFV nsP3<sub>449-471</sub> (magenta) – G3BP1<sub>NTF2</sub> (wheat) complex. The phenylalanine residues inserting into the hydrophobic pocket of G3BP1<sub>NTF2</sub> are labeled and shown in stick representation. (B) Structural overlay between the SARS-CoV-2 N<sub>1-25</sub> (yellow orange) – G3BP1<sub>NTF2</sub> (green) and the Caprin1<sub>363-382</sub> (slate) – G3BP1<sub>NTF2</sub> (yellow) complex. The phenylalanine residues inserting into the hydrophobic pocket of G3BP1<sub>NTF2</sub> are labeled and shown in stick representation. (C) Sequence alignment of the G3BP1<sub>NTF2</sub>-interacting peptides, with the P<sub>-2</sub>, P<sub>1</sub> and P<sub>2</sub> sites colored in magenta, red and green, respectively. (D) Structural alignment of the G3BP1<sub>NTF2</sub>-interacting peptides, with the P<sub>-2</sub> and P<sub>1</sub> sites, as well as the N- and C-termini, labeled.

(designated as P<sub>1</sub> site) and a bulky hydrophobic amino acid at the P<sub>-2</sub> site dominate the G3BP1<sub>NTF2</sub> binding via a groove-insertion mechanism (Figure 4(C, D)). In addition, the P<sub>2</sub> site is populated with a small amino acid (e.g. glycine), except for that in the Caprin1<sub>363-382</sub> peptide, which contains an isoleucine (I373), coinciding with its structural divergence from the SARS-CoV-2 N<sub>1-25</sub> and nsP3<sub>449-471</sub> peptides (Figure 4(B, D)). The diverse sequence

composition at the φxF-flanking regions introduces secondary protein interactions underpinning various binding outcomes of the G3BP1<sub>NTF2</sub>-interaction partners. Along the line, a recent study reported that residues K36, K50, K59 and K64 of G3BP1 are subjected to ubiquitination in response to heat shock in cells, leading to disassembly and autophagy-independent degradation of SG.<sup>39</sup> Structural inspection of the SARS-CoV-2 N<sub>1-25</sub> – G3BP1<sub>NTF2</sub>, SFV nsP3<sub>449-471</sub> – G3BP1<sub>NTF2</sub> and

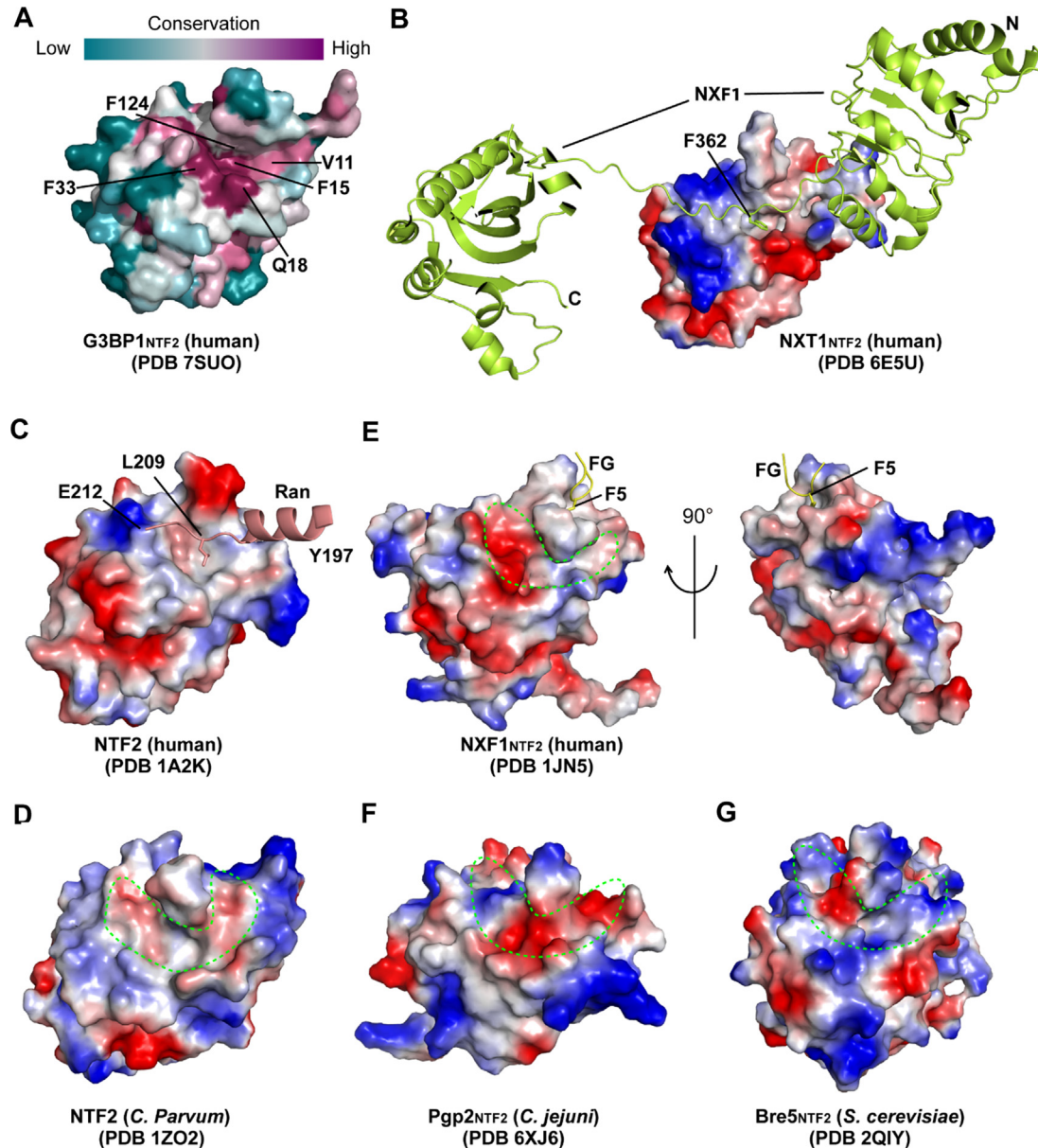


**Figure 3. Structural and biochemical characterizations of the SARS-CoV-2 N<sub>1-25</sub> – G3BP1<sub>NTF2</sub> interaction.** (A) Close-up view of the SARS-CoV-2 N<sub>1-25</sub> – G3BP1<sub>NTF2</sub> interaction. The interacting residues are shown in stick representation. The hydrogen bonds are shown as dashed lines. The water molecules are shown as red spheres. (B) Schematic view of the SARS-CoV-2 N<sub>1-25</sub> – G3BP1<sub>NTF2</sub> interaction. Hydrogen bonds and electrostatic interactions are indicated by black and green dashed lines, respectively, and van der Waals contacts are indicated by yellow gears. (C) Close-up view of the hydrophobic pocket harboring residue F17 of SARS-CoV-2 N<sub>1-25</sub>. The van der Waals radii of the G3BP1<sub>NTF2</sub> residues are shown in dots. (D) Close-up view of the electrostatic surface of G3BP1<sub>NTF2</sub> harboring the side chains of I15 and F17 of SARS-CoV-2 N<sub>1-25</sub>. (E) ITC binding assays for wild-type G3BP1<sub>NTF2</sub> titrated with wild-type or mutant SARS-CoV-2 N<sub>1-25</sub> peptide. (F) ITC binding assays for wild-type or mutant G3BP1<sub>NTF2</sub> titrated with the wild-type SARS-CoV-2 N<sub>1-25</sub> peptide. NDB, no detectable binding.

Caprin1<sub>363–382</sub> – G3BP1<sub>NTF2</sub> complexes indicate that the potential ubiquitination sites of G3BP1 are largely exposed in these complexes (Figure S4 (C–E)). One exception lies in G3BP1 K59 in the Caprin1<sub>363–382</sub> complex, which becomes partially shielded from solvent by the Caprin1<sub>363–382</sub> binding (Figure S4(E)). These observations imply that the Caprin1 binding may affect the ubiquitylation of G3BP1 differently than the SARS-CoV-2 N<sub>1–25</sub> and SFV nsP<sub>3449–471</sub> bindings.

### The SARS-CoV-2 N<sub>1–25</sub>-binding groove is recurrently present in NTF2 domains

The NTF2 domain is present in a wide array of proteins from diverse species.<sup>40</sup> Sequence analysis of the G3BP1<sub>NTF2</sub> domain from selected model species (Figure S5(A)) or using the ConSurf server<sup>41</sup> reveals that the SARS-CoV-2 N<sub>1–25</sub>-binding groove evolves more slowly than the surrounding area, with the N<sub>1–25</sub> F17-binding site falling into the most



**Figure 5. Structural evolution of the NTF2 domains.** (A) Color-coded sequence conservation of the G3BP1<sub>NTF2</sub> domain, analyzed using the ConSurf server (<https://consurf.tau.ac.il>). (B) Electrostatic surface of the NXT1<sub>NTF2</sub> domain bound to the LRR (NXF1<sub>LRR</sub>)-NTF2 (NXF1<sub>NTF2</sub>) domain linker of the NXF1 protein (limon), with the side chain of NXF1 F362 shown in stick representation. (C) Electrostatic surface of human NTF2 domain bound to residues 197–212 of one Ran molecule (light pink), with the side chain of Ran L209 shown in stick representation. For clarity, the rest of Ran structure is not shown. (D–G) Electrostatic surface of *C. parvum* NTF2 (D), human NXF1<sub>NTF2</sub> (E), *C. jejuni* Pgp2<sub>NTF2</sub> (F) and *S. cerevisiae* Bre5<sub>NTF2</sub> (G), with the individual surface grooves circled by dotted lines. Note that human NXF1<sub>NTF2</sub> binds to the FG peptide through a hydrophobic pocket positioned separately from the groove.

conserved region (Figure 5(A)). Furthermore, structural survey of the 19 NTF2-containing proteins in the protein data bank reveals that the surface groove is evolutionarily preserved in the NTF2 domain of many other proteins. For instance, the NTF2 domain of nuclear transport factor 2-related export protein 1 (NXT1<sub>NTF2</sub>) forms a surface groove in the corresponding region to harbor the linker sequence connecting the leucine-rich repeat (LRR) and NTF2 domains of nuclear RNA export factor 1 (NXF1), resulting in formation of a heterodimer for mRNA export (Figure 5(B)).<sup>42</sup> Likewise, human NTF2 protein and its counterpart from *C. parvum*, the founding members of the NTF2 family, also form a surface groove in the corresponding region (Figure 5(C, D)).<sup>43,44</sup> As with the G3BP1<sub>NTF2</sub> domain, the NXT1 and NTF2 proteins form a hydrophobic cavity at the center of the groove, harboring NXF1 F362 and Ran L209, respectively (Figure 5(B, C)). These observations suggest groove insertion as a conserved interaction mechanism for the NTF2 domains.

A similar surface groove is formed in the NTF2 domain of some other proteins, such as human NXF1, *C. jejuni* PgP2 and *S. cerevisiae* Bre5 (Figure 5(E–G)).<sup>28,45,46</sup> However, the electrostatic and steric properties of these grooves diverge substantially from that of the G3BP1<sub>NTF2</sub> domain (Figure 5(E–G)), implying different interaction mechanisms associated with the groove. Indeed, it was shown that the NXF1<sub>NTF2</sub> domain binds to the phenylalanine (F5) of an FG peptide derived from the nucleoporins (NUPs) repeat via a hydrophobic pocket distant to the surface groove corresponding to the N<sub>1–25</sub>-binding site of the G3BP1<sub>NTF2</sub> domain (Figure 5(E)).<sup>28</sup>

Taken together, these observations suggest that the SARS-CoV-2 N<sub>1–25</sub>-binding groove serves as a recurrent structural feature of the NTF2 domain, which is subjected to electrostatic and steric fine tuning for diverse protein interaction behaviors.

## Discussion

SARS-CoV-2 N becomes an increasingly attractive drug target not only because it plays a multifaceted role in packing of viral genome, virion assembly and viral transcription<sup>4,47</sup>, but also due to the fact that it helps break host defense through inhibition of the G3BP1/2-mediated SG formation in cells.<sup>7</sup> Development of an effective therapeutic strategy against this viral factor necessitates a detailed understanding of its molecular mechanism. Through combined structural and biochemical analyses, this study establishes the structural basis for the interaction between SARS-CoV-2 N and the G3BP1<sub>NTF2</sub> domain, providing a framework for the development of novel therapeutic strategies against COVID-19 and related viral infection.

## Molecular basis for the inhibition of G3BP1-mediated SG formation by SARS-CoV-2 N protein

The G3BP1-mediated SG formation is underpinned by a heterologous network of interactions involving the protein contacts mediated by the G3BP1<sub>NTF2</sub> domain and the RNA bindings mediated by the G3BP1<sub>RRM</sub> domain.<sup>10–15</sup> As proposed in a network-based model,<sup>19</sup> the interaction of G3BP1<sub>NTF2</sub> with the “bridge” protein Caprin1, which possesses both protein and RNA binding-modules, creates multiple valences for the interaction network, thereby promoting the SG assembly.<sup>19</sup> In contrast, the interaction with USP10, which is classified as “valence cap” due to lack of multivalent interaction module, serves to halt the propagation of the interaction network inside the SG.<sup>20</sup> Surprisingly, the interaction of SARS-CoV-2 N with G3BP1 leads to inhibition of G3BP1-mediated liquid–liquid phase separation *in vitro* and in cells,<sup>7,37</sup> even though the former contains both protein and RNA interaction modules.

Through fragment-based ITC binding assays, this study identified that the interaction between SARS-CoV-2 N and G3BP1 is primarily mediated by N<sub>1–25</sub> and the G3BP1<sub>NTF2</sub> domain. In addition, the C-terminal regions of SARS-CoV-2 N, including IDR2 and/or CTD, contribute to the interaction by reducing the entropic cost of the complex formation. Note that both G3BP1 and SARS-CoV-2 N assume a dimeric form in solution, which presumably contributes to the fact that full-length SARS-CoV-2 N protein shows a higher G3BP1-binding affinity than N<sub>1–25</sub>. The observation that both the N- and C-terminal regions of SARS-CoV-2 N contribute to the G3BP1 binding further suggests a multivalent engagement between SARS-CoV-2 N and G3BP1, by which the C-terminal region of SARS-CoV-2 N-mediated secondary interaction may interfere with the SG-nucleating activity of G3BP1. In support of this notion, a recent study showed that the SARS-CoV-2 N<sub>1–175</sub> fragment exhibits a reduced SG inhibition activity than full-length SARS-CoV-2 N.<sup>7</sup> In addition, given the fact that the SARS-CoV-2 N and Caprin1 interact with G3BP1 in a mutually exclusive manner, it is conceivable that the interaction between SARS-CoV-2 N<sub>1–25</sub> and G3BP1<sub>NTF2</sub> also serves to inhibit the association of G3BP1 with Caprin1, providing another mechanism in attenuating the SG formation. A detailed mechanism by which SARS-CoV-2 N protein inhibits G3BP1-mediated SG formation remains to be investigated.

## A groove-insertion mechanism dictating the SARS-CoV-2 N<sub>1–25</sub> – G3BP1<sub>NTF2</sub> interaction

The structure of the SARS-CoV-2 N<sub>1–25</sub> – G3BP1<sub>NTF2</sub> complex reveals strong surface complementarity between residues 13–22 of SARS-CoV-2 N<sub>1–25</sub> and the surface groove of

G3BP1<sub>NTF2</sub>. Most notably, the P<sub>1</sub>-F17 of SARS-CoV-2 N<sub>1–25</sub> inserts its aromatic ring into a hydrophobic pocket at the center of the surface groove, which is flanked by a parallel sidechain insertion of P<sub>2</sub>-I15. This dual groove-insertion mode is reminiscent of what was previously observed for the complexes of G3BP1<sub>NTF2</sub> with SFV nsP3 and Caprin1,<sup>26,29</sup> attesting the  $\phi$ xF motif as a primary determinant for the G3BP1<sub>NTF2</sub>-mediated protein interaction. In addition, the embedding of N<sub>1–25</sub> P<sub>2</sub>-G18 in a narrow region of the surface groove permits a parallel pairing between N<sub>1–25</sub> I15-G18 and G3BP1<sub>NTF2</sub>  $\beta$ 5-strand, in line with the previous observation that a P<sub>2</sub>-glycine is favored by the G3BP1<sub>NTF2</sub> domain.<sup>21,26</sup> Note that these G3BP1-binding sites of N<sub>1–25</sub> is highly conserved among different variants of SARS-CoV-2 reported to date,<sup>48</sup> suggesting that the SARS-CoV-2 N<sub>1–25</sub> – G3BP1<sub>NTF2</sub> interaction might constitute a critical factor for SARS-CoV-2 infection.

Unlike the Caprin1 and SFV nsP3 peptides that diverge their  $\phi$ xF-flanking regions out of the surface groove to engage helical packing with the  $\alpha$ 1-helix of G3BP1<sub>NTF2</sub>, the  $\phi$ xF-flanking regions of SARS-CoV-2 N<sub>1–25</sub> peptide remain bound to the groove. Through surface complementarity, residues P13-R14 and G19-S21 of N<sub>1–25</sub> are anchored to the two ends of the groove, engaging van der Waals and/or hydrogen-bonding interactions with G3BP1<sub>NTF2</sub>, respectively. These secondary binding sites in the SARS-CoV-2 N<sub>1–25</sub> – G3BP1<sub>NTF2</sub> complex may serve as attractive targets for the development of therapeutic agents that selectively inhibit the SARS-CoV-2 N – G3BP1 association.

### NTF2 domain, a platform for diverse protein–protein interactions

The NTF2 domain represents an evolutionarily divergent protein interaction module involved in various cellular activities, such as SG formation<sup>7,37,10–15</sup> and mRNA transport.<sup>42</sup> This study, through structural survey of the NTF2 domains from diverse proteins, uncovers that the surface groove of G3BP1<sub>NTF2</sub> represents a recurrent feature of the NTF2 domains. Common to many of these NTF2 domains is the formation of a hydrophobic cavity at the center of the groove, which provides a primary docking site for a bulky, hydrophobic side chain from host or viral factors. On the other hand, the NTF2-interacting proteins, such as SARS-CoV-2 N, Caprin1 and NXF1, often extend beyond the hydrophobic pocket for secondary contacts, which presumably contributes to their distinct binding affinity and specificity. In this context, the structure of the SARS-CoV-2 N<sub>1–25</sub> – G3BP1<sub>NTF2</sub> complex not only provides a framework for understanding how SARS-CoV N and SARS-CoV-2 N have evolved to target the G3BP1/2, but also

provides a basis for identification of other host factors that can potentially be targeted by the SARS-CoV-2 N.

## Experimental procedures

### Cloning, expression and purification of proteins

The DNA fragment encoding SARS-CoV-2 N, codon optimized for bacterial expression, was synthesized from Thermo Fisher Scientific. The cDNAs for full-length human G3BP1 and G3BP2 were purchased from DNASU plasmid repository (<https://dnasu.org>). The SARS-CoV-2 N<sub>1–25</sub> peptide was cloned in a modified pRSF vector, in which N<sub>1–25</sub> was preceded by an N-terminal His<sub>6</sub>-SUMO tag and ULP1 (ubiquitin-like protease) cleavage site. Full-length SARS-CoV-2 N, full-length G3BP1, G3BP1<sub>NTF2</sub> (residues 1–139) and G3BP2<sub>NTF2</sub> (residues 1–139) were cloned into an in-house His<sub>6</sub>-MBP vector, preceded by a TEV cleavage site. The plasmids were transformed into BL21 RIL (DE3) cell strain (Agilent technologies) for protein expression. The transformed cells were first grown at 37 °C until cells attained an A<sub>600</sub> of 0.8. The temperature was then lowered to 16 °C, after which the cells were induced by addition of 0.1 mM isopropyl  $\beta$ -D-galactoside and continued to grow overnight. For His<sub>6</sub>-SUMO-tagged N<sub>1–25</sub>, the fusion protein was purified using a Ni-NTA affinity column, followed by removal of the His<sub>6</sub>-SUMO tag with ULP1 protease and further purification by size-exclusion chromatography on a HiLoad 16/600 Superdex 75 pg column (GE Healthcare) pre equilibrated with 25 mM Hepes (pH 7.5), 0.3 M NaCl and 2 mM DTT. His<sub>6</sub>-MBP-tagged full-length G3BP1, G3BP1<sub>NTF2</sub> and G3BP2<sub>NTF2</sub> proteins were purified sequentially through Ni-NTA chromatography, Q HP column (GE Healthcare) followed by TEV protease treatment. Samples were then subjected to a second round of Ni-NTA chromatography for tag removal. Finally, the proteins were purified by size-exclusion chromatography on a HiLoad 16/600 Superdex 200 pg column (GE Healthcare) pre equilibrated with 25 mM Hepes (pH 7.5), 300 mM NaCl, and 2 mM DTT. The purified proteins were confirmed by SDS-PAGE, concentrated to ~20 mg/mL and stored at –80 °C for further use.

### Crystallization, X-ray data collection and structure determination

For crystallization, ~6 mg/mL G3BP1<sub>NTF2</sub> protein dissolved in 25 mM Hepes (pH7.5), 300 mM NaCl and 2 mM DTT was mixed with the N<sub>1–25</sub> peptide in a 1:2 molar ratio. The crystallization condition for the SARS-CoV-2 N<sub>1–25</sub> – G3BP1<sub>NTF2</sub> complex

was initially identified through sparse-matrix screening (Hampton Research Inc.) at 4 °C. The crystals were then reproduced by hanging-drop vapor diffusion method under the same temperature, from drops mixed from 1  $\mu$ L of SARS-CoV-2 N<sub>1–25</sub> – G3BP1<sub>NTF2</sub> complex and 1  $\mu$ L of precipitant solution containing 20% propan-2-ol, 0.1 M MES monohydrate (pH 6.0), 20% PEG MME 20,000. Crystals were soaked for one minute in a cryoprotectant solution, comprised of crystallization buffer and 30% glycerol, before flash frozen in liquid nitrogen. The X-ray diffraction data for the SARS-CoV-2 N<sub>1–25</sub> – G3BP1<sub>NTF2</sub> complex were collected on the BL 5.0.3 beamline at the Advanced Light Source, Lawrence Berkeley National Laboratory. The diffraction data were indexed, integrated and scaled using the HKL3000 program<sup>49</sup>. The structure was solved using the molecular replacement method in PHASER<sup>50</sup> with the structure of human G3BP1<sub>NTF2</sub> in complex with SFV nsP3<sub>449–471</sub> (PDB ID: 5FW5) as search model. The structure was improved by iterative model building and refinement with Coot<sup>51</sup> and PHENIX<sup>52</sup> software packages. The same R-free test set was used throughout the refinement. The statistics for data collection and structural refinement of the SARS-CoV-2 N<sub>1–25</sub> – G3BP1<sub>NTF2</sub> complex is summarized in Table S2.

### Isothermal titration calorimetry

A MicroCal iTC200 system (GE Healthcare) was used to conduct the ITC measurements. All proteins and peptides were dialyzed against buffer containing 25 mM Hepes (pH 7.5) and 300 mM NaCl before titration. For titration of G3BP1<sub>NTF2</sub> or G3BP2<sub>NTF2</sub> with SARS-CoV-2 N<sub>1–25</sub>, 0.1 mM human G3BP1<sub>NTF2</sub> or G3BP2<sub>NTF2</sub> and 1 mM SARS-CoV-2 N<sub>1–25</sub> were used. For the rest of titrations, 0.03 mM full-length G3BP1 and 0.3 mM SARS-CoV-2 N, full-length or fragments, were used. A total of 20 injections with a spacing of 180 s and a reference power of 5  $\mu$ cal/s were performed at 25 °C. The ITC curves were processed with ORIGIN (MicroCal) software by using a one-site fitting model.

**CRedit authorship contribution statement.** Mahamaya Biswal: Conceptualization, Investigation, Visualization, Data curation. Jiuwei Lu: Investigation. Jikui Song: Conceptualization, Supervision, Investigation, Visualization, Writing – review & editing.

### DATA AVAILABILITY

Coordinates and structure factors for the SARS-CoV-2 N<sub>1–25</sub>–G3BP1<sub>NTF2</sub> complex have been deposited in the Protein Data Bank under accession code 7SUO.

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### Conflict of Interest

The authors declare no competing interest.

### Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jmb.2022.167516>.

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