Large scale discovery of coronavirus-host factor protein interaction motifs reveals SARS-CoV-2 specific mechanisms and vulnerabilities

Viral proteins make extensive use of short peptide interaction motifs to hijack cellular host factors. However, most current large-scale methods do not identify this important class of protein-protein interactions. Uncovering peptide mediated interactions provides both a molecular understanding of viral interactions with their host and the foundation for developing novel antiviral reagents. Here we describe a viral peptide discovery approach covering 23 coronavirus strains that provides high resolution information on direct virus-host interactions. We identify 269 peptide-based interactions for 18 coronaviruses including a specific interaction between the human G3BP1/2 proteins and an ΦxFG peptide motif in the SARS-CoV-2 nucleocapsid (N) protein. This interaction supports viral replication and through its ΦxFG motif N rewires the G3BP1/2 interactome to disrupt stress granules. A peptide-based inhibitor disrupting the G3BP1/2-N interaction dampened SARS-CoV-2 infection showing that our results can be directly translated into novel specific antiviral reagents.
NA viruses such as the Ebola, dengue, and coronaviruses cause a variety of diseases and constitute a continuous threat to public health. The coronaviruses are the largest single-stranded RNA viruses known and their genomic RNA encodes around 30 viral proteins\(^1\). During infection, each viral protein performs unique functions and interacts with a range of cellular host factors to allow viral proliferation and immune escape\(^2,3\). Precise disruption of viral-host factor interactions is an attractive strategy for developing novel antiviral reagents. The advantage of targeting these interactions is that resistance is less likely to develop and furthermore as the same host factor can be used by multiple viruses such reagents may provide broader spectrum activity. Numerous large-scale mass spectrometry (MS) based interaction screens\(^3,5\), as well as CRISPR based screens\(^6,10\) have been conducted to uncover host factor interactions and dependencies for SARS-CoV-2 allowing repurposing of drugs against human targets\(^11\). Although these methods have been transformative in our understanding of SARS-CoV-2 biology the molecular detail provided by these methods is not always sufficient to readily transform the results into novel antiviral reagents. Experimental approaches that would complement the existing powerful methods and provide a more detailed view of viral interactions with host factors could accelerate the development of new antivirals.

An attractive class of protein interactions that can be inhibited for therapeutic purposes are viral short linear interaction motifs (SLiMs) that bind to defined pockets on globular domains of the host factor\(^13,14\). SLiMs are short peptide motifs in unstructured regions of proteins and typically contain 2–4 amino acid binding determinants within a 10 amino acid stretch\(^15,16\). Viruses extensively use SLiMs to hijack cellular host factors and SLiMs can readily evolve through mutations in unstructured regions allowing viruses to interact with novel host factors\(^17–19\). Despite the importance of SLiMs for understanding viral biology, they are not uncovered by most current large-scale methods\(^15,16\). Peptidomimetic peptide-phage display (ProP-PD) provides the opportunity to identify novel SLiM-based interactions and binding sites at high resolution\(^20\). As shown in a small scale pilot study on C-terminal peptides of viral proteomes, it can be used to faithfully capture SLiM-based host-pathogen interactions\(^21\). Here we describe a novel phage-based viral peptide library to map SLiMs from 23 coronaviruses mediating host factor interactions (Fig. 1a). This approach allows the simultaneous pan-viral identification of SLiM-based interactions with high resolution of the binding sites. We document the power of this approach by identifying novel SARS-CoV-2 specific SLiM mediated host factor interactions and directly translate our screening results into novel mechanistic insights and pinpoint a potential target for antiviral intervention.

**Results**

**A pipeline for viral SLiM discovery.** We exploited recent developments of the ProP-PD technology\(^20,22\) and established a pipeline to identify RNA virus SLiMs binding to host factors. Briefly, the pipeline consists of purifying protein domains and screening these against a novel phage display library displaying the unstructured parts of viral proteins. Following a number of selection cycles enriched phages are sequenced to identify the viral SLiMs binding a specific bait (Fig. 1a). We designed a unique phage display library (RiboVD library) that tile the unstructured regions of 1074 viral proteins from 229 RNA viruses, including SARS-CoV-2, SARS-CoV, MERS-CoV and 20 additional coronavirus strains. This library represents 19,549 unique 16 amino acid long peptides that are multivalently displayed on the major coat protein of the filamentous M13 phage. We scanned a published host factor interactome for the SARS-CoV-2 viral proteins and recombinantly produced 57 domains from 53 cellular proteins reported to interact with SARS-CoV-2\(^2\). As transient SLiM-based protein interactions might be lost during purifications of viral proteins for subsequent mass spectrometry analysis, we screened an additional set of 82 peptide-binding domains. These domains were chosen because they were efficiently expressed and purified in E. coli\(^22\) and at least 27 of them have previously been reported to act as viral-host factors and to be hijacked by SLiMs from viral proteins. In total, 139 recombinantly expressed and purified human bait proteins (Supplementary Data 1) were used in selections against the RiboVD library. Enriched phage pools were analyzed by next-generation sequencing (NGS) to identify viral peptides that bound to the bait. In light of the ongoing COVID-19 epidemic, we chose to focus on the interactions mediated by coronavirus proteins in the following, which represent 13% of the total number of interactions identified in the screen. Interaction data for the remaining RNA viruses will be published elsewhere.

We uncovered 269 putative SLiM-based interactions with 104 interactions identified using the 57 MS identified SARS-CoV-2 host factor baits and 165 interactions identified using the 82 peptide-binding domains. The interactions covers 44 domains from 42 human proteins and 64 viral proteins from 18 coronavirus strains (Supplementary data 2). Of these, 117 (43%) interaction pairs involved human coronavirus proteins. We validated 27 interactions using fluorescence polarization (FP) affinity measurements (Fig. 1b, Supplementary Fig. 1 and Supplementary Data 3). This N terminal region of the E protein from all three strains binds to the FERM domains of Ezrin and Radixin for these three coronaviruses. For instance, NSP14 of all three strains has a YxxL motif that binds to the clathrin coat adaptor protein AP2M1 with high affinity (Fig. 1b, Supplementary Fig. 1 and Supplementary Data 3), which may be linked to the trafficking of the viral protein or blocking of endocytosis of host proteins\(^23,25\). The N-terminal region of the E protein from all three strains binds to the FERM domains of Ezrin and Radixin via a recently established [FY][xFILV] SLiM\(^26\). Interestingly, our data show that the FERM domains also bind to NSP3 of SARS-CoV and SARS-CoV-2, thus, they can be targeted by distinct viral proteins. The SARS-CoV NSP3 FERM binding site overlaps with a [FWY][x][ILV] binding site for the ATG8 domains of the autophagy-related MAP1LC3A-C proteins. As an example of strain-specific interactions, we found that an N-terminal peptide from the Nucleocapsid (N) proteins from SARS-CoV-2 and SARS-CoV bound to the NTF2 domain of the homologous G3BP1 and G3BP2 proteins (G3BPs) with high affinity (Fig. 1b, c, Supplementary Fig. 1 and Supplementary Data 3). This N peptide contains an ΦxFG SLiM (where Φ is a hydrophobic residue) that resembles motifs in USP10 and UBA2PL and in the alphavirus nsP3 protein known to bind a hydrophobic pocket in the NTF2 domain of G3BP\(^27,30\). The ΦxFG SLiM is also present in the N proteins from bat beta coronaviruses and consistently the corresponding bat HKU5 peptide was identified in our screen (Supplementary Fig. 2 and Supplementary Data 2).

To pinpoint therapeutically relevant host protein-viral SLiM interactions we screened three of the identified peptide motifs for antiviral activity. To this end, we generated lentiviral vectors expressing GFP fused to four copies of one viral SLiM reasoning that this would inhibit binding of the corresponding full-length SARS-CoV-2 protein to the specific host factors through competition. As a control we used GFP fused to SLiMs containing...
mutations in the binding motif. The host proteins targeted by viral peptides were G3BPs (SARS-CoV-2 N), Ezrin and Radixin (SARS-CoV-2 E and NSP3), and the MAP1LC3s (NSP3). VeroE6 cells were first transduced with the lentiviruses and 3 days later infected with SARS-CoV-2 and viral titer determined after 16 h. This revealed that the G3BP-binding peptide from the N protein decreased viral titer 3.4-fold (Fig. 2a). To obtain a more potent inhibition of the SARS-CoV-2 N-G3BP interaction, we used a 25
amino acid residue peptide from Semliki Forest virus (SFV) nsP3 containing two continuous FGDF like SLiMs that has previously been shown to bind G3PBs with high affinity. Remarkably, this peptide binds approximately 10-fold stronger than the SARS-CoV-2 N peptide to both G3BP1 and G3BP2 (KD = 4 μM vs KD = 0.3 μM, Fig. 2b and Supplementary Fig. 3a). We constructed “G3BP inhibitors” (G3BPi) by fusing sequences encoding one or three copies of wild type (wt) or mutated (ctrl) SFV nsP3 SLiMs to GFP. As expected, mass spectrometry analysis confirmed that the major cellular targets of the G3BPi are the G3BPs (Fig. 2c, Supplementary Fig. 3b and Supplementary Data 4). Furthermore, expression of the G3BPi wt but not G3BPi ctrl prevented the binding of SARS-CoV-2 N to G3BP1 in cells (Fig. 2d). Consistent with these binding and competition data, lentiviral mediated
expression of the G3BP1 in VeroE6 cells potently inhibited SARS-CoV-2 proliferation after 16 h of infection (Fig. 2a). An effect of the G3BP1 was also evident in assays monitoring viral infection rates or replication (Fig. 2e, f). In a cell-based transfection assay monitoring assembly and release of virus-like particles mutating the G3BP-binding motif in N had no effect (Supplementary Fig. 3d). Thus, the approach presented here is useful for identifying important virus-host factor interactions that inhibit viral proliferation when disrupted.

The N-G3BP1/2 interaction supports SARS-CoV-2 infection.

The above results prompted us to further investigate the N-G3BP interaction and its function during infection. The coronavirus N protein is important for viral replication, as well as packaging of the viral RNA. The G3BPs are multi-functional RNA-binding proteins best known for their essential roles in innate immune signaling and the assembly and dynamics of cytosolic stress granules. Stress granules are large protein-RNA assemblies formed in response to various stresses and viral infections. The G3BPs have turned out to be major targets for viral interference and several viral proteins have been shown to recruit G3BP1 to support viral replication and/or to inhibit stress granules formation. Of note, the herpesviruses and alphaviruses have been shown to recruit G3BPs by SLiMs having resemblance to the sequence in N. Stress granules (Fig. 3d). However, in cells with high levels of N, this protein and viral dsRNA co-localize with G3BP1 to stress granules. Once N concentrations reach a certain threshold, this disrupts stress granule formation and instead N and viral dsRNA co-localize with G3BP1 in these structures. N-terminal phosphorylation of the G3BP motif in N protein (below 10,000 fluorescent units) a large proportion of cells had multiple G3BP1 foci (Fig. 3e). In cells with low levels of N, this protein and viral dsRNA co-localized with G3BP1 to stress granules (Fig. 3d). In infected cells with low levels of N protein from HKU1-CoV did not bind G3BPs, consistent with the ProP-PD results (Supplementary Fig. 2 and Supplementary Fig. 3e). Consistently, the ΦxFG motif resulted in the specific co-localization of mCherry tagged N protein from SARS-CoV and SARS-CoV-2 to arsenite-induced stress granules in cells expressing YFP-tagged G3BP1 (Fig. 3a).

The N ΦxFG motif affects stress granule formation.

Recent publications have reported that SARS-CoV-2 N induces stress granule disassembly but the mechanistic basis of this is unclear. To investigate the effect of the SARS-CoV-2 N ΦxFG motif on endogenous stress granule formation we overexpressed YFP-tagged N wt or N A in HeLa cells and stained for endogenous G3BP1 following arsenite treatment. Quantifying the intensity of cytoplasmic G3BP1 foci in cells positive for YFP revealed that WT expression disrupted stress granule formation more efficiently when the G3BP-binding motif was intact (Fig. 3b, c). Thus, the N-terminal ΦxFG motif of the N protein constitutes the main determinant of G3BP-binding and stress granule disassembly. We next analyzed G3BP1 foci formation and cellular localization of viral dsRNA in relation to N protein expression levels in VeroE6 cells after six hours of SARS-CoV-2 infection (Fig. 3d, e). At this timepoint, a mixture of early and later stage infected cells is observed. In mock-treated cells, we detected no cells with more than two G3BP1 foci and based on this we set the background threshold at three G3BP1 foci per cell (Supplementary Fig. 3f). In infected cells with low levels of N protein (below 10,000 fluorescent units) a large proportion of cells had multiple G3BP1 foci (Fig. 3e). In cells with low levels of N, this protein and viral dsRNA co-localized with G3BP1 to stress granules. In infected cells with low levels of N protein (below 10,000 fluorescent units) a large proportion of cells had multiple G3BP1 foci (Fig. 3e). In cells with low levels of N, this protein and viral dsRNA co-localized with G3BP1 to stress granules (Fig. 3d). However, in cells with high levels of N, protein only two out of 11 cells had G3BP1 foci above threshold levels. Collectively our results suggest that low levels of N protein are insufficient to disrupt stress granule formation and instead N and viral dsRNA co-localize with G3BP1 in these structures. Once N concentrations reach a certain threshold, this disrupts stress granules, and this depends on the ΦxFG motif. A possible interpretation of these observations is that SARS-CoV-2 takes advantage of the stress granule RNA machinery during the earlier stages of infection. Consistently, dsRNA and N co-localize with G3BP1 foci and when the N-G3BP interaction is inhibited a reduction of viral replication is observed (Figs. 2f and 3d).

N rewires the G3BP1/2 interaction through the ΦxFG motif.

To understand how N could affect stress granule formation and G3BP function through the ΦxFG motif we set out to identify cellular G3BP interactors with similar binding motifs. To this end, we screened a novel ProP-PD library that displays the
**Fig. 3 Interaction between SARS-CoV-2 N and G3BP1/2 affects stress granule formation.**

**a** Live cell microscopy analysis of HeLa cells co-transfected with YFP-G3BP1 and mCherry tagged N proteins from the viral strains indicated. **b** Effect of SARS-CoV-2 N wt and N 2A on arsenite-induced stress granule formation as measured by immunofluorescence of endogenous G3BP1. **c** Quantification of G3BP1 foci intensity from **b**. Red bar indicates median intensity, and each circle represents the intensity of one G3BP1 foci. At least five foci from 10 cells were measured. **d** Immunofluorescence analysis of G3BP1, N and viral dsRNA in SARS-CoV-2 infected VeroE6 cells 6 h postinfection. **e** Effect of N levels on G3BP1 foci formation in SARS-CoV-2 infected cells. Each circle represents one cell analysed automatically from one experiment done in duplicate (n = 51). Microscopy images shown are representatives of three independent experiments for **a** and **b**. Scale bars are 10 μM. Source data are provided as a Source Data file.
intrinsically disordered regions of the human proteome against the NTF2 domains from G3BP1 and G3BP2. The combined data set includes 72 peptides from 57 proteins with the majority of sequences containing a ΦxFG motif (Φ[FILV]), thus resembling the sequence in the N protein (Fig. 4a and Supplementary Data 5). Nineteen of the proteins uncovered by the screen are core stress granule proteins, including known peptide motifs in USP10 and UBA2L, but also peptides from stress granule proteins that have not previously been reported to contain ΦxFG motifs (Fig. 4a, b). The screen also uncovered a peptide from Caprin-1, which has been shown to bind G3BPs but does not match the consensus sequence (Fig. 4a). This suggests that G3BPs serve as major hubs for stress granule biology in part by interacting with ΦxFG like motifs residing in several stress...
granule components. However, the screen also returned many peptides in proteins with roles outside of stress granule biology, such as TRIM25 and IRF7 (antiviral interferon signaling)\(^2\), and DDIT3 (endoplasmic reticulum stress)\(^4\). FP measurements were used to confirm binding between the purified NTF2 domain of G3BP2 and several identified peptides originating from TRIM25, DDIT3, UBAP2L, Caprin-1, USP10, and PRRC2B (Fig. 4c and Supplementary Fig. 4a). Furthermore, we biochemically validated a number of the G3BP-binding motifs in the context of the full-length proteins (Supplementary Fig. 4b). Caprin-1 and UBAP2L co-localized with G3BP1 in stress granules after arsenite treatment (Supplementary Fig. 4c). Conversely, no stress granule localization was observed for TRIM25 and DDIT3 (Supplementary Fig. 4c) further supporting the notion that the G3BPs also have cellular roles beyond stress granule biology\(^5\).

The N protein is a highly expressed viral protein\(^5\) during infection so we hypothesized that it would compete with host cell proteins containing \(\Phi\)xFG SLiMs for binding to G3BPs. Consistently, FP measurements revealed competition between the N \(\Phi\)xFG peptide and all of the 7 peptides we tested for interaction with G3BP2 (Fig. 4c). Next, immunopurifications of full-length YFP-tagged TRIM25, DDIT3, Caprin-1, and UBAP2L in the presence of either a N wt peptide or a N 3 A peptide where the \(\Phi\)xFG motif is mutated to AxA (Supplementary Fig. 4d). As expected, the N wt peptide disrupted interactions to G3BP1 thus validating a direct competition between the N \(\Phi\)xFG peptide motif and four G3BP-binding proteins (Fig. 4d). The observed competition between the viral N \(\Phi\)xFG peptide and UBAP2L for binding to G3BP1 is particularly interesting since UBAP2L is required for stress granule assembly through a direct interaction to the G3BPs via its \(\Phi\)xFG like motif\(^2\). This suggests a mechanistic basis for the ability of the N protein to inhibit stress granule formation.

Given the high levels of N during infection, we speculated that it could mediate a general rewiring of the G3BP interactome through its \(\Phi\)xFG motif. To test this on a global scale, we purified G3BP1-YFP from HeLa cells and added either N wt or the N 3 A mutated peptide as competitors for cellular proteins. Quantitative label-free mass spectrometry allowed us to determine the proteins being specifically displaced by the N wt peptide (Fig. 4e and Supplementary Data 4). This revealed specific displacement of 59 proteins, including several core stress granule components. In addition, the N peptide also displaced a large number of nuclear pore complex components, heat shock chaperones of the Hsp70 family and proteins of the ASC-1 and CTLH complexes. Except the CTLH components, all of these proteins have been reported to localize to stress granules\(^2\,7\,28\,30\,33\). The displacement of nucleoporins from G3BP1 by the N peptide suggests that FG motifs, which are abundant in nucleoporins\(^34\), might recruit them to stress granules through direct interaction to the NTF2 domain of the G3BPs. Consistently, two \(\Phi\)xFG like motifs from nucleoporins were selected in the G3BP Pro-PD screen (Fig. 4a, b and Supplementary Data 5). Importantly, Nup62, which we identify in our MS competition screen (Fig. 4e) has been shown to be required for efficient SARS-CoV-2 infection\(^3\). It is possible that N displaces Nup62 from G3BPs to make it accessible for other viral processes. Together, we show that the N protein modulates the G3BP1/2 host interactome through its \(\Phi\)xFG motif by competing with numerous cellular \(\Phi\)xFG containing proteins. Our G3BP motif and mass spectrometric screens provide a rich resource for the future dissection of basic stress granule biology and G3BP signaling in general.

Discussion

Collectively, we describe a potentially therapeutic relevant interaction between the \(\Phi\)xFG SLiM in SARS-CoV-2 N and the G3BP proteins. Our results reveal that the N protein during infection hijacks G3BPs to viral replication centers likely to facilitate replication and possibly other aspects of viral RNA metabolism. The disruption of stress granules at later stages of infection could also dampen the cellular antiviral response. Consistent with this idea we identify \(\Phi\)xFG motifs in TRIM25, MEX3C and IRF7 that are key components of the G3BP-RIG-1 antiviral interferon pathway\(^2\,42\,55\,57\).

By screening the intrinsically disordered regions of 229 RNA viruses against a host factor in one we uncovered both common principles shared by several viruses as well as interactions specific for a given strain. We show that the SLiMs can be screened for antiviral activity to pinpoint therapeutically relevant interactions. Given the high-resolution binding site information provided by the ProP-PD, this can guide the development of agents targeting these interactions. Peptide-based inhibitors are highly specific but a challenge for peptides as compared to small molecules is their poor pharmacokinetic properties, which may be improved through peptide modifications\(^58\,60\). The clinical use of the HIV peptide inhibitor enfuvirtide (T20) demonstrate that the use of peptides represents a viable strategy for the development of therapeutic antiviral agents\(^61\), although the T20 peptide does not need to cross the cell membrane to elicit an effect. As far as we know, no cell-permeable peptide is used in the clinic, but there are several ongoing clinical trials\(^62\). The ligands described here may thus represent potential starting points for the development of antiviral agents.

Our approach is easily applied to other relevant host factors and the library can be readily updated to incorporate novel RNA viruses emerging in the future. In this work, 139 recombinant host protein baits were produced for the RibovD library screen. Still, this set of baits only represents a small fraction of host protein domains expected to recognize virally encoded SLiMs. Thus, the production of recombinant protein baits in considerable numbers and of sufficient purity constitute a bottleneck of the ProP-PD approach. Nevertheless, we foresee that this approach can be a powerful tool for future investigations of virus interactions with cellular host factors and for developing novel antivirals.
The phage library was designed using a previously described design pipeline. Following this pipeline, the first step was to define the RNA virus search space. The search space was defined as the UniProt reference proteomes of the mammalian and avian RNA viruses (RiboVizia; taxonomic identifier: 2559987) and a representative proteome from RNA virus clades without a reference proteome (A complete list of the viral strains in the library is available at http://slim.icr.ac.uk/phage_libraries/rna_viruses/species.html). This set of 229 viral strains was analysed to define intracellular and intrinsically disordered protein regions. First, the UniProt defined transmembrane and the extracellular regions of transmembrane proteins were removed. Next, the intrinsically disordered region of the remaining search space was defined as follows: (i) Surface Accessibility Scores of homology mapped structures; and (ii) Disorder Predictions using IUPred.

The phage pellets were dissolved in phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 95 mM Na2HPO4, 15 mM KH2PO4, pH 7.5). The resulting phage library was re-amplified in 23 strains including SARS-CoV-2, SARS-CoV, and MERS-CoV. The details of the library designs including the viral strains, proteins, peptides and statistics are available at http://slim.icr.ac.uk/phage_libraries/rna_viruses/species.html.

The resulting library contained 19,549 unique peptides from 1074 proteins (i.e., the number of peptide sequences designed to be in the library was 22,171). The library was screened using a nucleotide removal kit (Qiagen), phosphorylated using T4 polynucleotide kinase (Thermo Scientific) for 1 h at 37°C and annealed to phagemid DNA oligonucleotide deprotected (5°C CAGCCTCTTCATCTGGC and 3°C GGCTGAGGAGTCGCGGAG) were added to each end of the oligonucleotide design. Finally, oligonucleotides were checked for SmaI restriction sites (GGGCCC or CCCGGG) or self-annealing. The peptide sequences designed to be in the library were further purified by reverse IMAC or by using a HiTrap Glutathione Sepharose 4 Fast Flow media (Cytiva) following the manufacturer’s protocol. The resulting phage pool was used as an in-phage for the annotation of protein regions built on the framework of the PSSMSearch tool.

The selected peptide sequences were mapped to the viral or human proteomes with PepTools and annotated with information of the bait and prey (known interaction or shared localisation and functional terms). To assess the quality of the phage library, we analyzed the coverage percentage of the phage library using the design library using the NGS results of non-challenged library phage pools and the estimation of the number of unique peptides sequenced. 95.5% of the peptide sequences designed to be in the library were confirmed by the NGS analysis of the naïve phage library.

Analysis of the ProP-PD selection data. Following our recently outlined ProP-PD data analysis approach, four metrics were used to rank the peptides i) NGS read counts, ii) peptide occurrence in replicated selections, iii) number of overlapped peptides, iv) motif match. The four metrics were then combined into a single score called ‘Confidence level’ forming 3 categories: high (4 metric criteria matched); medium (2 to 3 criteria matched); and low (only 1 metric is matched). Due to the relatively small size of the phage library, the coronavirus dataset was further filtered for target-specific ligands (occurring in less than 10 unrelated selections) (Supplementary data 2). The GPBPs HD2 P8 data were combined and a joint confidence score was calculated (Supplementary data 5). The peptide data were combined with available information on stress granule localized proteins from mass spectrometry of purified mammalian stress granules, from other studies based on the information listed in the HIPPIE database. Each ProP-PD selected peptide was annotated with the above data and a count of the number of sources of evidence of stress granule localisation.

Protein purification. *E. coli* BL21-Gold(DE3) (Agilent Technology) transformed with the plasmids encoding the His-GST-tagged proteins (Supplementary Data 1) were grown in 500 mL 2YT at 37°C until an OD600 of 0.6 and 0.8. Protein expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and allowed to proceed for 20 h at 18°C. The pellet was dissolved in phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 95 mM NaH2PO4, 15 mM KH2PO4, pH 7.5). The resulting phage library was re-amplified and stored at ~80°C in the presence of 10% glycerol.

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**FP affinity determinations.** FP experiments were carried out using a SpectraMax iDS multimode microplate reader (Molecular Devices) using a black half area 96-well plate (Corning, USA #9093) with a total volume of 50 μL. The settings used were 485 nm for excitation and 535 nm for emission. Peptides were from GeneCust (France) with a purity of over 95%. FITC-labeled peptides were dissolved in dimethyl sulfoxide (DMSO). Unlabelled peptides were dissolved in phosphate buffer (50 mM phosphate buffer pH 7.5 and 1 mM EDTA). All measurements were performed at least in triplicates. For saturation binding experiments, proteins were serially diluted in the reducing agent DTT or TCEP. All measurements were performed at least in triplicate. For saturation binding experiment, proteins were serially diluted in the reducing agent DTT or TCEP. All measurements were performed at least in triplicate. For saturation binding experiment, proteins were serially diluted in the reducing agent DTT or TCEP.

The data of the displacement experiment were fitted to a sigmoidal dose-response (2):

\[ Y = \frac{Top - Bottom}{1 + \left(\frac{X}{IC_{50}}\right)^{n}} \]

in which \( Y \) is the observed signal, \( X \) is the log of the variable concentration of the FITC-labeled probe peptide, \( X \) is the varying protein concentration, \( A \) is the signal amplitude divided by the peptide concentration and \( B \) is the plateau value obtained for the unbound probe peptide.

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**Cell culture, virus, and reagents.** HeLa and HEK293 cells were maintained in DMEM GlutaMax containing 10% FBS, 100 μL penicillin, 100 mg/ml streptomycin, and 10% FCS (all from Thermo Fisher Scientific). Stable HEK cell lines were generated using the T-Rex doxycycline inducible FpIn-System (Invitrogen) and supplemented with like HeLa cells with the addition of 5 μg/mL Lasticidin and 100 μg/mL Hygroycin B. Caco2 cells were kept in MEM ( Gibco®, 11095-080) supplemented with 20% FBS, penicillin-streptomycin solution (Sigma, P4333), 1 mM sodium pyruvate (ThermoScientific®, 11360039), and nonEssential amino acids (Gibco®, 1141035). VeroE6 cells were cultured in DMEM (Sigma) supplemented with 5% fetal bovine serum (FBS), 100 μL of penicillin and 100 μg/ml streptomycin (Gibco®). The patient isolate SARS-CoV-2/01/human/2020/SWE accession no/GeneBank no. MT000000.1 was provided by the Public Health Agency of Sweden. The SARS-CoV-2 passage number 4 was cultured and titrated in VeroE6 cells. e. coli DH5 making and propagated using standard microbiological procedures. The following drug concentrations were used: sodium asartine 0.5 mM, doxycycline 10 mg/mL unless otherwise stated.

**Expression constructs and cell line generation.** Standard cloning techniques were used throughout. All N proteins and variants were generated by gene synthesis (Geneart). To generate the YFP-GrpIB inhibitor, the double FGD motif from Semliki Forest virus mp9 (RTFRNKLFPTGDFEDEHVLALASGTGDFEEDVL) or a control inhibitor (RTFRNKLPTADGDFEDEHVLALASGTGADADDVL) was fused to the C-terminus of YFP by cloning the DNA encoding this into the pcDNA3/EPT/TO vector (Invitrogen). DNA encoding the GrpIB sequences was purchased from GeneArt, Life Technologies. All constructions were fully sequenced. The following point mutations were introduced using quick-change mutagenesis to uncouple binding to G3BP: TRIM25 (R406A G407A), DDIT3 (F10A G11A), CAPRIN-1 (Y370S N371K F372S I373T), UBAP2L (F518L F523G). See supplementary material for names and sequences of primers used in this study. Detailed mutagenesis and cloning strategies are available upon request.

**Lentivirus production, transductions, and virus infection.** Transfer plasmids for lentiviral transduction were ordered from GenScript. To generate transfer plasmids, four copies of inhibitory peptide (three copies for the Semliki Forest virus) or peptide controls with the binding motifs mutated were fused to C-terminus of eCFP. The plasmids were cloned to pUM4-EGFP vector (David Sabatini lab, Addgene plasmid #19319).

**Antibodies.** The following antibodies were used at the indicated dilutions: c-Myc (1:1000, Santa Cruz Biotechnology, sc-40), rabbit anti-GrpIB (WB 1:1000, Cell Signaling Technology, #17799S), mouse anti-GrpIB (IF 1:1000, Abcam, ab36574), GFP-Booster_-auto488 (IF 1:300, ChromoTek), mouse anti-GFP (WB 1:1000, Roche, 11814460001), rabbit anti-GFP (WB 1:1000, in-house), rabbit anti-SARS-CoV-2 nucleoprotein (WB 1:2500, Sino Biological Inc., 926-3211), goat antiserum anti-Mouse IgG (WB 1:1000, Thermo Fisher Scientific, A21200), mouse APC-conjugated antibody directed against dsRNA J2 (IF 1:200, Scioncs, 10010500) mouse anti-3xFlag M2 (WB 1:2500, Sigma, F1804), rabbit anti-Tubulin (WB 1:4000, Abcam, ab6066), antirabbit SARS-CoV-2 nucleoprotein (1:500; Invitrogen, MA5-29981), antirabbit GrpIB (1:1000; Santa Cruz Biotechnology, sc-363338), antirabbit GAPDH (1:1000; SantaCruzBiotechnology, sc-47724), donkey antirabbit IgG (Invitrogen, A32794), goat antirabbit HRP conjugated antibodies (1:2000, Thermo Fisher Scientific). Virec foci were then stained by incubation with TrueBlue peroxidase substrate for 30 min (KPL, Gaithersburg, MD).

**DNA isolation, cDNA synthesis, and qPCR.** Total RNA was isolated from cells and 400 ng was used to synthesize cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s instructions. qPCR primers were designed using primers from qPCR Primer Design (Qiagen Cat #300001 PPO40269 A) and the qPCRbio SyGreen Mix Hi-ROX kit (PCRBIOSYSTEMS). SARS-CoV-2 transcripts were detected using forward (GTCATGTGTGGCGGTTTCG G) and reverse (CAACAGCTTTTCAAGGGTGTG) primers and probe (FAM-CACGGTGGAACCTCACTGGAGATC-BHQ1 and the qPCRbio Probe Mix Hi-ROX kit (PCRBIOSYSTEMS). qPCR was run using a StepOnePlus fast real-time PCR system (Applied Biosystems).

**Live cell imaging.** Live-cell analysis was performed on a Deltavision Elite system using a x 40 oil objective with a numerical aperture of 1.35 (GE Healthcare). The DeltaVision Elite microscope was equipped with a CoolSNAP HQ2 camera (Photometrics). Cells were seeded in eight-well dishes (Ibidi) and before filming, the media was changed to Leibovitz’s L-15 (Life Technologies). Approximately 200 cells were recorded for the longest time indicated. For transfection experiments, DNA constructs were transfected into HeLa cells using lipofectamine 2000 (Life Technologies) 24 h prior to analysis.

**Immunoprecipitation.** Cells were lyzed in lysis buffer (150 mM NaCl, 50 mM Tris pH 7.4, 1 mM EDTA, 1 mM DTT, 0.1% NP40) supplemented with protease and phosphatase inhibitors (Roche) for 25 min on ice. Lysates were cleared for 15 min at 20000 g x g and incubated with 20 μL prequilliated GFP-trap or Myc-trap beads (Clontech, Cat #211173) for 45 min at 4°C. Followed by 3 washes with lysis buffer, the beads were either eluted in 25 μL 2x LDS sample buffer (Novex, Life Technologies), boiled for 5 min, separated by SDS-PAGE and analyzed by Western blotting (LI-COR ImageStuido v. 3.1.4 used for analysis) with the indicated antibodies or subjected to quantitative mass spectrometry as described in the AP-MS section. To generate the indicated peptides were added to cells lysates for 30 min at 4°C before incubated with GFP-trap beads. For uncropped Western blots, see Supplementary Fig. 5.
Immunoprecipitation of SARS-CoV/SARS-CoV2/MERS-CoV N proteins. Two 15 cm² dishes were seeded with HeLa cells at 20% confluency. On the following day, cells were transfected with 2.5 μg pClN1-200 TRID; Alexa Fluor 647-2XME, pCDNA 5/FRT/TO Myc SARS CoV-2 N and N 2 A was transfected into HEK293T cells using GeneJuice (Novagen, Darmstadt, Germany) following the manufacturer’s protocol (12 μg of DNA/ sample in total). Cells and Virus-Like Particles (VLPs) were collected 24 h after transfection. Cells were lysed (0.5 M Tris-HCl pH 8, 1 M NaCl, 1% Triton X-100) and supernatant of transfected cells was collected, and concentrated by ultra-centrifugation (100,000 x g, 90 min 4 °C, SW41, Beckman Coulter, Brea, CA). The pellet was resuspended in reducing Laemmli SDS-PAGE sample buffer. Proteins were separated with SDS-PAGE and Western blot analysis was performed using antibodies against SARS-CoV-2 nucleocapsid, 3xFlag M2 (Sigma, F1804) and tubulin (Abcam, ab6046).

Affinity purification and mass spectrometry (AP-MS). Partial on-bead digestion was used for peptide elution from GFP-Trap Agarose (Chromotekt). Briefly, 100 μL of elution buffer (2 M urea; 2 mM DTT; 20 μg/ml trypsin; and 30 μM Tris, pH 7.5) was added and incubated at 37 °C for 30 min. Samples were alkylated with 25 mM CAA and digested overnight at room temperature before the addition of 1% trifluoroacetic acid (TFA) to stop digestion. Peptides were desalted and purified with styrene-divinylbenzene reversed-phase sulfonate (SDB-RPS) StageTips. Briefly, two layers of SDB-RPS were prepared with 100 μL wash buffer (0.2% TFA in water) and supernatant of transfected cells was collected, and concentrated by ultra-centrifugation (100,000 x g, 90 min 4 °C, SW41, Beckman Coulter, Brea, CA). The pellet was resuspended in reducing Laemmli SDS-PAGE sample buffer. Proteins were separated with SDS-PAGE and Western blot analysis was performed using antibodies against SARS-CoV-2 nucleocapsid, 3xFlag M2 (Sigma, F1804) and tubulin (Abcam, ab6046).

LC-MS analysis. Liquid chromatography-mass spectrometry (LC-MS) analysis was performed with an EASY-nLC 1200 system (Thermo Fisher Scientific) connected to a trapped ion mobility spectrometry quadrupole time-of-flight mass spectrometer (timsTOF Pro, Bruker Daltonik GmbH, Germany) with a nano-electrospray ion source (Captive spray, Bruker Daltonik GmbH). Peptides were loaded on a 50 cm in-house packed HPLC-column (75 μm inner diameter packed with 1.9 μm ReproSilPur C18-AQ silica beads, Dr. Maisch GmbH, Germany). Peptides were separated using a linear gradient from 5-30% buffer B (0.1% formic acid, 80% ACN in LC-MS grade H2O) in 45 min followed by an increase to 60% buffer B for 7 min, then to 95% buffer B for 5 min and back to 5% buffer B in the final 5 min. A consisted of 0.1% triethylamine adjusted to pH 8 with LC-MS grade H2O. The total gradient length was 60 min. We used an in-house made column oven to keep the column temperature constant at 60 °C.

Mass spectrometric analysis was performed essentially as described in Brunner et al.23,24 in data-dependent (ddaPASEF) mode for ddaPASEF, 1 MS1 survey TIMS-MS and 10 PASEF MS/MS scans were acquired per acquisition cycle. Ion accumulation and ramp time in the dual TIMS analyzer was set to 100 ms each and we analyzed the ion mobility range from 1K/0 = 1.6 V cm⁻² to 0.6 V cm⁻². Precursor ions for MS/MS analysis were isolated with a 2 Th window for m/z < 700 and 3 Th for m/z > 700 in a total m/z range of 100-1,700 by synchronizing quadrupole switching every 120 ms. Precursor ions of the TIMS device. The collision energy was lowered linearly as a function of increasing mobility starting from 59 eV at 1K/0 = 1.6 V cm⁻² to 20 eV at 1K/0 = 0.6 V cm⁻². Single charged precursor ions were excluded with a polygon filter (out of control, Bruker Daltonik GmbH). Precursors for MS/MS were picked at an intensity threshold of 1,000 arbitrary units (a.u.) and resequenced until reaching a 'target value' of 20,000 a.u. taking into account a dynamic exclusion of 40 s elution.

Data analysis of proteomic raw files. Mass spectrometric raw files acquired in ddaPASEF mode were analyzed with MaxQuant (version 1.6.7.0)25,26. The Uniprot database (2019 release, UP000005600_9606) was searched with a peptide spectral match (PSM) and protein level FDR of 1%. A minimum of seven amino acids was required including N-terminal acetylation and methionine oxidation as variable modifications and cysteine carbamidomethylation as fixed modification. Enzyme specificity was set to trypsin with a maximum of two allowed missed cleavages. The first and main search mass tolerance was set to 70 ppm and 20 ppm, respectively. Peptide identifications by MS/MS were transferred by matching four-dimensional isotope patterns between the runs (MBR) with a 0.7-min retention-time match window and a 0.05 1/K0 ion mobility window. Label-free quantification was performed with the MaxLFQ algorithm72 and a minimum ratio count of two. A minimum ratio count of two.

Bioinformatic analysis of LC-MS data. Proteomics data analysis was performed with Perseus73 and within the R environment (https://www.r-project.org/). MaxQuant output tables were filtered for ‘Reverse’, ‘Only identified by site modification’, ‘Potential contaminants’ before data analysis. Missing values were imputed after stringent data filtering and based on a normal distribution (width = 0.65). For statistical testing. For comparisons (two-sided paired t test), we applied a permutation-based FDR of 5% to correct for multiple hypothesis testing including an ym value of 0.1.
Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository 6 with the dataset identifier PXD025410 available at: http://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD025410. A complete list of the viral strains in the library is available at: http://slim.icr.ac.uk/phageibraries/rna_viruses/species.html. Transmembrane and extracellular regions of transmembrane proteins were identified using UniProt (https://www.uniprot.org). The details of the library designs including the viral strains, proteins, peptides and statistics are available at: http://slim.icr.ac.uk/phageibraries/rna_viruses/species.html. For peptide mapping and annotation, an RNA virus search database was added to the PepTools (http://slim.icr.ac.uk/tools/peptools/) webserver. The identity of proteins localizing to stress granules was retrieved from the HIPPIE database (http://dbdm01.0adv.unizh-marz.de/michaelf/hippie/). Intrinsic disorder predictions were made using IUPred (https://iupred.elte.hu/). Raw data is provided as source data for Figs. 2a, e, f, 3c, e and Supplementary Figs. 3f and 4c. Source data are provided with this paper.

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Reporting summary

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

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Competing interests

J.N. is on the scientific advisory board for Orion Pharma. The other authors declare no competing interests.

Additional information

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