Received: 27 November 2021 | Revised: 3 March 2022 | Accepted: 7 March 2022

DOI: 10.1002/wrna.1727

FOCUS ARTICLE

WIREs RNA WILEY

RNA–protein interactomes as invaluable resources to study RNA viruses: Insights from SARS CoV-2 studies

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Funding information
The National Science Centre, Grant/Award Numbers: 2018/30/E/NZ3/00624, 2020/37/B/NZ3/03633; The Polish National Agency for Academic Exchange, Grant/Award Number: PPN/PPO/2019/1/00035/U/0001

Edited by: Jeff Wilusz, Editor-in-Chief

Abstract

Understanding the molecular mechanisms of severe respiratory syndrome coronavirus 2 (SARS-CoV-2) infection is essential for the successful development of therapeutic strategies against the COVID-19 pandemic. Numerous studies have focused on the identification of host factors and cellular pathways involved in the viral replication cycle. The speed and magnitude of hijacking the translation machinery of host mRNA, and shutting down host transcription are still not well understood. Since SARS-CoV-2 relies on host RNA-binding proteins for the infection progression, several efforts have been made to define the SARS-CoV-2 RNA-bound proteomes (RNA–protein interactomes). Methodologies that enable the systemic capture of protein interactors of given RNA in vivo have been adapted for the identification of the SARS-CoV-2 RNA interactome. The obtained proteomic data aided by genome-wide and targeted CRISPR perturbation screens, revealed host factors with either pro- or anti-viral activity and highlighted cellular processes and factors involved in host response. We focus here on the recent studies on SARS-CoV-2 RNA–protein interactomes, with regard to both the technological aspects of RNA interactome capture methods and the obtained results. We also summarize several related studies, which were used in the interpretation of the SARS-CoV-2 RNA–protein interactomes. These studies provided the selection of host factors that are potentially suitable candidates for antiviral therapy. Finally, we underscore the importance of RNA–protein interactome studies in regard to the effective development of antiviral strategies against current and future threats.

This article is categorized under:

RNA Interactions with Proteins and Other Molecules > Protein-RNA Interactions: Functional Implications

RNA in Disease and Development > RNA in Disease

RNA Methods > RNA Analyses in Cells

KEYWORDS

COVID-19, RNA interactomes, RNA-binding proteins, RNA–protein interactions, SARS-CoV-2

1 | INTRODUCTION

COVID-19 pandemic intensified efforts into the research on pathogenicity of SARS-CoV-2, to understand the mechanisms underlying its infection and design therapeutic strategies to limit its spread and virulence. SARS-CoV-2 belongs to the beta genera of the *Coronaviridae* family, that comprises enveloped, positive-strand RNA viruses (Cui et al., 2019). It has large capped and polyadenylated genome of around 30 kb, that contains at least 11 open reading frames (ORFs) and generates tiered set of subgenomic RNAs (sgRNAs) (D. Kim, Lee, et al., 2020). Similar to other RNA viruses, SARS-CoV-2 relies on the host cellular infrastructure and uses different strategies to overtake it for its own advantage. In the early phase of infection, upon entering the cell the virus recruits host translation machinery to produce non-structural proteins (NSP) that arrange favorable cellular conditions for efficient replication of viral genome. It appears that NSP1, the first translated viral protein, is a virulence factor with a key role in the shutdown of host translation and stimulation of host mRNA degradation (Finkel et al., 2021; Schubert et al., 2020). NSP1 was also shown to mediate retention of host mRNA in the nucleus by direct interaction with the nuclear export receptor heterodimer NXF1–NXT1 (Zhang et al., 2021). It results in decreased immune response due to insufficient production of innate immunity factors that could alert the immune system upon infection. NSP1 is also present in other coronaviruses, where it was shown to act in an analogous way, that is, by targeting cellular processes to inhibit gene expression and downregulate type I interferon response (Narayanan et al., 2008). Nonetheless, RNA viruses encode a limited number of proteins, and they need to co-opt various host factors to assure the continuity of their spread. This interplay is by large defined by host-encoded RNA-binding proteins (RBPs). During infection many host RBPs are repurposed by viral RNA (vRNA) to guide it through their lifecycle, including RNA translation, synthesis, genome modification, localization, packaging, and release

TABLE 1 Examples of host RBPs with known function in the regulation of RNA viruses' lifecycle and/or antiviral immune response of host cells

Regulated process	RNA-binding proteins	RNA viruses
Translation of viral RNA	CSDE1, IFIT1, eIF3, UPF1, GEMIN5, PCBP2	HCV (Flynn et al., 2015) WNV (Daffis et al., 2010) HIV-1 (Rao et al., 2018) SINV (Garcia-Moreno et al., 2019)
Replication of viral RNA	EWSR1, SIGMAR1, TMEM41B, IFI16, DDX56, YTHDF1	CHIKV (B. Kim, Arcos, et al., 2020) SINV (Garcia-Moreno et al., 2019) HCV (Friesland et al., 2013; Oakland et al., 2013) WNV (Daffis et al., 2010; Reid & Hobman, 2017)
Sequestration in stress granules	DDX3, MOV10, G3BP1, TIAL1	IAV (Thulasi Raman et al., 2016) HIV-1 (Furtak et al., 2010) CHIKV (Matkovic et al., 2019) SeV, VSV (Yang et al., 2019)
Transport of viral RNA	MYH9, MAP4	HCV (Gerold et al., 2015) HIV-1 (Gallo & Hope, 2012) SARS-CoV-2 (Miao et al., 2021)
Virions assembly and release	RAB7A, RAB10, RAB14, DDX56, YBX1, Caprin1, hnRNPK	HIV-1 (Caillet et al., 2011) *Flaviviridae*: DENV, WNV, ZIKV, JEV (Diosa-Toro et al., 2020)
Degradation of viral RNA	OAS/RNaseL, XRN1, XRN2	HCV (Li et al., 2015, p. 1; Malathi et al., 2010) WNV, SINV, IAV (Li et al., 2016, p. 3)
Modulation of immune response	PKR, ADAR, MDA5, RIG-I, TLR3, METTL3, METTL14	*Flaviviridae*: HCV, ZIKV, DENV, WNV (Gokhale et al., 2016; Tsai et al., 2009) HMPV (Lu et al., 2020) SARS-CoV-2 (Sa Ribero et al., 2020) different RNA viruses generating intermediate dsRNA (Dauber & Wolff, 2009)

Abbreviations: CHIKV, Chikungunya virus; DENV, Dengue virus; HCV, Hepatitis C virus; HIV, human immunodeficiency virus; HMPV, human metapneumovirus; IAV, influenza A virus; JEV, Japanese encephalitis virus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SeV, Sendai virus; SINV, Sindbis virus; VSV, vesicular stomatitis virus; WNV, West Nile virus; ZIKV, Zika virus.

(Table 1). On the other hand, a subset of specialized RBPs act as antiviral factors (Table 1) at the frontline of cellular antiviral defense mechanisms, for example, by detecting pathogen-associated molecular patterns, such as double-stranded RNA intermediates (dsRNA) or RNAs with 5′ triphosphate ends (Barbalat et al., 2011; Vladimer et al., 2014). Over the course of their lifecycle, viruses need to balance the usage of the host resources, in order to provide efficient replication and to evade the host immune response.

To what extend are the host cell resources, beyond ribosomes and translation machinery, engaged to facilitate the SARS-CoV-2 spread? What differentiates SARS-CoV-2 strategies from those of other Coronaviruses, to assure its exceedingly high transmission and infection rates? How does the virus escape from the host innate immunity response? The systematic, high-throughput studies on SARS-CoV-2 RNA–host proteome interactions have provided new insights in the attempts to answer these questions. RNA–protein interactome studies and devoted methodology have been developing rapidly over the last decade. The reason is twofold: one lies in the urge of understanding of certain RBPs, or RBPs as a group, in the regulation of gene expression programs in the subcellular compartments, for example, their involvement in liquid-phase separating membranelles organelles, the second reason is to shed more light on the functions of specific classes of RNAs, for example, long non-coding RNAs, by revealing their interacting partners. Several types of methods have been developed for the capture and identification of RNA–protein interactions in vivo. Among the methods devoted to characterizing protein interactors of a given (target) RNA one can distinguish two general types of in vivo approaches, that is, (i) those, which employ crosslinking of RNA–protein contacts in live cells and the subsequent antisense oligonucleotide capture of target RNA coupled with mass spectrometry, and (ii) methods which do not employ crosslinking, but instead rely on genetically engineered proximity-dependent protein labeling systems, and/or introduce tags and baits into both target RNA and proteins. Both lines of approaches have been used to uncover the biology behind several abundant regulatory RNAs, such as the long non-coding RNAs *XIST* (Chu et al., 2015; McHugh et al., 2015), *MALAT1* (Spiniello et al., 2018), or *NORAD* (Munschauer et al., 2018), and recently they have found their application in the studies of RNA viruses. For instance, RNA–protein interaction detection (RaPID) protocol, which relies on the proximity-dependent labeling of proteins interacting with BoxB-labeled target RNA, has been used to identify host proteins that bind Zika virus (ZIKV) RNA within its untranslated region (UTR) sequences (Ramanathan et al., 2018). BoxB stem loops recruit the RaPID protein (biotin ligase), thereby enabling biotinylating of proteins bound to the flanked adjacent ZIKV RNA motif and purification biotin-tag proteins and their analysis with mass spectrometry (MS) (Ramanathan et al., 2018). As for the specific capture of proteins directly interacting with SARS-CoV-2 RNA, to date, several methods involving crosslinking of the viral genomic and subgenomic RNAs to interacting proteins have been applied. Here, we summarize these studies, focusing on their technical aspects and highlighting novel insights they offer on SARS-CoV-2 biology.

2 | INSIGHTS INTO SARS-COV-2 BIOLOGY FROM VIRUS RNA–HOST PROTEIN STUDIES

2.1 | Approaches

Understanding the molecular mechanisms of the SARS-CoV-2 infection is essential to developing devoted antiviral pharmacological strategies. Several methods have been applied to capture and identify SARS-CoV-2 RNA–protein interactions in human or monkey cell lines (Figure 1). All of them employ crosslinking of RNA–protein contacts in live cells and antisense oligonucleotide capture of target RNA coupled with MS. Among these methods, one can find such protocols like RNA Antisense Purification and quantitative Mass Spectrometry (RAP-MS) (McHugh et al., 2015), Comprehensive Identification of RNA-binding Proteins by Mass Spectrometry (ChIRP-MS) (Chu et al., 2015; Chu & Chang, 2018), or Hybridization Purification of RNA–protein complexes and Mass Spectrometry (HyPR-MS) (Spiniello et al., 2018). Conceptually similar, these methods share suchlike general workflows, yet feature distinctions in certain designs and related steps in their protocols, which may influence the identification of target RNA-binding proteins.

In all the SARS-CoV-2 RNA–protein interactome capture approaches the experimental procedure begins with the infection of a model cell line with the virus (Figure 1a), followed by crosslinking of proteins-RNA contacts, typically achieved by irradiation of cells with UV light at a defined wavelength, or by treatment with a chemical crosslinking agent, such as formaldehyde (FA) (Figure 1b). The goal of crosslinking is to stabilize RNA–protein contacts occurring in vivo by introducing a covalent bond between the interactors. That allows to isolate crosslinked RNA–protein complexes in stringent denaturing conditions, and to reduce the background formed by (non-specific and non-covalent)

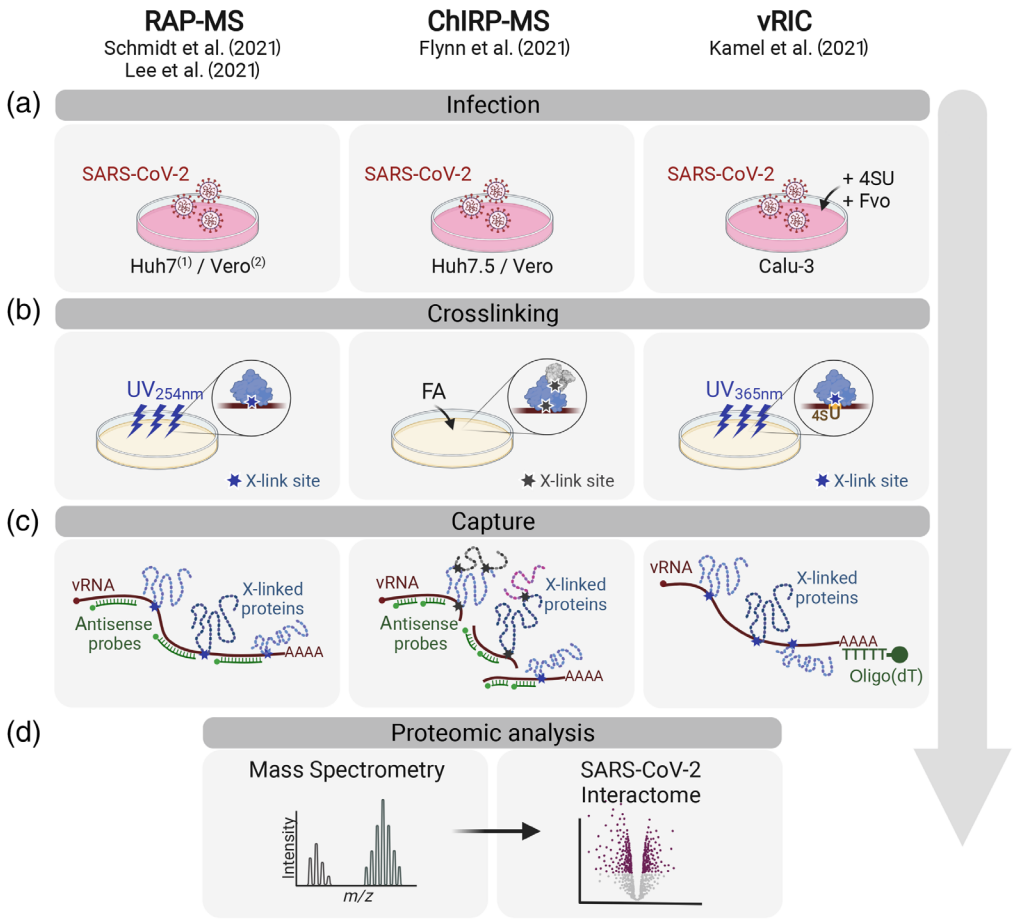

FIGURE 1 Outline of methods used for the identification of SARS-CoV-2 RNA–protein interactomes. Key steps include: (a) infection of cultured cells with SARS-CoV-2; (b) crosslinking of protein–RNA interactions either with UV light or chemical crosslinking reagent, that is, FA; (c) capture of SARS-CoV-2 RNA–protein complexes with biotinylated DNA oligonucleotides (antisense or oligo[dT] probes marked with green), isolation and purification of RNA–protein complexes is performed in denaturing conditions; (d) proteomic data acquisition and analysis. Huh7, Vero, Calu-3—cell lines used in the respective studies; 4SU—photoactivatable nucleotide analog 4-thiouridine; Fvo—flavopiridol, the RNA Polymerase II-specific inhibitor of transcription; UV_{254nm}—ultraviolet light of 254 nm wavelength; FA—formaldehyde; UV_{365nm}—ultraviolet light of 365 nm wavelength; vRNA- viral RNA; X-linked—crosslinked

protein contaminants. In contrast to the UV-based crosslinking, which selectively stabilizes RNA–protein interactions, FA crosslinking can additionally fix protein–protein and DNA–protein contacts (Figure 1b). Selectivity of UV-based crosslinking, makes it a preferable approach to analyze direct RNA–protein interactions. On the other hand, by stabilizing the protein–protein contacts, FA-based crosslinking enables the capture of whole protein complexes that could interact with a given RNA (Figure 1b). There are additional considerations: the efficiency of UV crosslinking is genially low (~5%) and biased toward certain nucleotides and amino acid residues. In particular, pyrimidines are more photoactivated than purines, and, cysteine, lysine, phenylalanine, tryptophan and tyrosine residues are crosslinked with the highest efficiency, whereas histidine, glutamic acid, and aspartic acid crosslink with moderate efficiency (Urdaneta & Beckmann, 2020). UV crosslink at 254 nm (UVC) is irreversible. FA crosslink was shown to be more effective for crosslinking dsRNA to dsRNA-binding proteins than UV, and is reversible (Chu & Chang, 2018; B. Kim et al., 2017).

The four major steps shared by all the methods used in SARS-CoV-2 RNA–protein interactome studies constitute: isolation of the RNA–protein complexes from lysed, infected cells (Figure 1a), purification of the complexes in denaturing conditions (Figure 1b), elution of RNA-bound protein accompanied by RNA digestion (Figure 1c), and finally proteomic analysis with MS (Figure 1d). For the isolation procedure, the RNA together with bound proteins are purified from lysed cells in an affinity capture approach, which typically involves set of biotinylated DNA antisense probes complementary to the given RNA, allowing for targeted RNA capture on the streptavidin-coated beads.

Both RAP-MS and ChIRP-MS methods, which employ the antisense probe capture strategy, were used to identify the SARS-CoV-2 RNA-bound proteome (Flynn et al., 2021; Lee et al., 2021; Schmidt et al., 2021). Schmidt and colleagues applied RAP-MS to identify proteins that directly bind to the SARS-CoV-2 RNAs during infection of Huh7 cells. Following UV irradiation of SARS-CoV-2-infected cells, a set of DNA oligonucleotides complementary to the viral genomic and subgenomic mRNAs was used to isolate the RNAs and bound proteins. In a similar manner, RAP-MS was utilized by Lee et al. to capture the RNA–protein interactomes of SARS-CoV-2 and HCoV-OC43 viruses from infected Vero, Calu-3, and Lenti-X 293 T cells. ChIRP-MS capture the SARS-CoV-2 RNA–protein interactome was performed in VeroE6 and Huh7.5 cells. One major distinction of this method from RAP-MS is the use of chemical (FA) crosslinking to stabilize the RNA–protein interactions. Another distinction is antisense probe design (Table 2).

The efficiency and specificity of antisense oligonucleotide capture methods heavily relies on probe design and optimization of probe hybridization conditions. Typically, the use of multiple probes leads to increased capture efficiency, but can result in a higher noise due to off-targets. Conversely, the use of fewer probes may result in increased specificity, at the cost of capture efficiency due to the possible limited accessibility of the probe-targeted region occupied by RBPs. Both RAP-MS and ChIRP-MS methods applied for the capture of the SARS-CoV-2 RNA–protein interactomes involved sets of multiple probes antisense to regions within the viral genomic and subgenomic RNAs. While both RAP-MS applications (Lee et al., 2021; Schmidt et al., 2021) used 90 nt probes, the ChIRP-MS protocol employed a set of 108 short (20 nt-long) probes (Flynn et al., 2021). A significant difference in the probe design also existed between the two RAP-MS applications. While the method used by Schmidt et al. involved 67 probes targeting viral genomic sites at a distance interval of around 400 nt, each, Lee et al. used a much bigger number of >700 probes, to achieve the highly dense coverage of the viral genome. Of note, HyPR-MS protocol utilizes FA crosslinking and antisense oligonucleotide capture for identification of protein interactors of given RNAs. In comparison with ChIRP-MS and RAP-MS, HyPR-MS employs only two to three probes of 20–30 nt at length which are designed to bind single-stranded regions on the target RNA (as predicted by Mfold tool) (Spiniello et al., 2018). HyPR-MS has not been applied for the SARS-CoV-2 RNA–protein interactome capture, but the method has been proven useful for identification of the HIV-1 RNA-bound proteome in infected Jurkat CD4+ T-cells (Knoener et al., 2021).

A different strategy for the SARS-CoV-2 RNA–protein interactome capture was presented in the work of Kamel et al. Instead of using biotinylated antisense probes to purify the viral RNA and crosslinked proteins, this method involved oligo(dT) pulldown of polyadenylated RNAs from SARS-CoV-2-infected Calu-3 cells (Kamel et al., 2021). To preferentially capture proteins bound to the polyadenylated viral genomic RNA, but not host mRNA, Kamel and colleagues introduced the RNA Polymerase II-specific inhibition of host transcription with flavopiridol, followed by a pulse labeling of newly synthesized viral RNAs with the photoactivatable nucleotide analog 4-thiouridine (4SU). With the host RNA transcription temporarily inhibited, and viral RNA polymerases unaffected by the inhibitor, 4SU was predominantly incorporated into the nascent viral RNAs. The increased capacity of 4SU to absorb UV light enables the UV-crosslinking to be done at 365 nm, the wavelength unabsorbed by natural nucleotides, which results in a specific crosslinking of proteins to RNAs labeled with 4SU. In such conditions, the SARS-CoV-2 RNA-bound proteins from infected, pre-treated Calu-3 cells were captured with oligo(dT)-conjugated beads, and analyzed with mass spectrometry. The method was named viral RNA interactome capture (vRIC) (Kamel et al., 2021). The results were overlaid with the dataset of RBPome responsive to Sindbis virus (SINV) infection of HEK293 cells, a study performed with comparative RNA interactome capture (cRIC) by the same group (Garcia-Moreno et al., 2019). cRIC relies on UVC crosslink, and RBPome identification via capture on oligo (dT)-pulled down polyadenylated RNAs from infected vs. non-infected cells grown on the SILAC media.

One notable utility of the selective 4SU-labeling of nascent viral RNAs lies in the possibility to time 4SU-labeling to a specific stage during the infection, allowing to capture host proteins engaged by the nascent, 4SU-labeled viral RNA. This is particularly useful for studying early stages of the infection, characterized by intensive vRNA replication, allowing for more effective 4SU incorporation into nascent vRNA. Alternatively, an infection of 4SU-free cells, with a virus whose RNA is pre-labeled with 4SU (as a result of a previous infection of 4SU-labeled cells), could allow detection of host factors that bind to vRNA upon virus entry and uncoating, in the stage of early infection preceding vRNA replication. This kind of strategy was used to identify the host factors binding the pre-replicated genome of Chikungunya virus (CHIKV) within the first minutes of the infection (B. Kim, Arcos, et al., 2020). The procedure used was termed Viral Cross-linking and Solid-phase Purification (VIR-CLASP). Notably, VIR-CLASP employed a sequence-independent technique for the capture of 4SU-labeled RNA and crosslinked proteins, which involved a solid-phase purification under protein-denaturing conditions using Solid-Phase Reversible Immobilization (SPRI) beads (B. Kim, Arcos, et al., 2020). A possible confounding factor for the methods based on selective 4SU-labeling of viral RNA could be the

TABLE 2 Summary of studies on SARS-CoV-2 RNA–protein interactome

Study	Schmidt et al., 2021	Lee et al., 2021	Flynn et al., 2021	Kamel et al., 2021
Method	RNA antisense purification coupled with mass spectrometry (RAP–MS)	RNA antisense purification coupled with mass spectrometry (RAP–MS)	Comprehensive identification of RNA-binding proteins by mass spectrometry (ChIRP-MS)	Viral RNA interactome capture (vRIC)
Crosslink	UVC (254 nm)	UVC (254 nm)	Formaldehyde (3%)	UVA (365 nm)
Probe design	SARS-CoV-2 RNA-specific $N = 67$ 90-mers (non-overlapping)	SARS-CoV-2 RNA-specific $N = 707$ (set1) $N = 275$ (set2) 90-mers (overlapping)	SARS-CoV-2 RNA-specific $N = 108$ 20-mers	Oligo(dT)
Cell line(s)	Huh7 (human hepatoma)	Vero E6 (Monkey African Green kidney, epithelial)	Huh7.5 Vero-E6	Calu-3 (human lung adenocarcinoma, epithelial)
Timepoint for probing vRNA-host protein interactions	24 hpi	24 hpi	24, 48 hpi	24 hpi
# Identified host proteins	104	109	309 (Huh7.5: 229, Vero E6: 163)	133
# Identified viral proteins	13	9	13	6

Abbreviation: hpi, hours postinfection.

cytotoxicity resulting from the host RNA transcription inhibition and/or 4SU incorporation (Burger et al., 2013). Both possible effects can impact the infection progression and, subsequently, the obtained proteomic data, therefore, the experiments in such a setup require systematic optimization and multistep controls.

To summarize, there are core similarities in the underlying concepts and workflows of the methods used for studying SARS-CoV-2 RNA–protein interactomes in human (or monkey) cells as well as important distinctions in their detailed protocols. The latter involves: crosslinking method used, probe number and design, and notably, the use of different cell lines as a model of the infection (Table 2).

2.2 | Biological relevance

The main output from SARS-CoV-2 RNA–protein interactome studies are lists of host and/or viral proteins captured as interactors of vRNA, namely genomic RNA and sgRNA. For each of these studies, the obtained lists of proteins were composed mainly of known RBPs, and factors involved in RNA processing. The set of 104 human- and 13 viral SARS-CoV-2 RNA interactome proteins captured in RAP-MS approach by Schmidt et al., was enriched in protein groups involved in translation, nonsense-mediated decay (NMD), co-translational protein targeting to the membrane, and viral transcription (Schmidt et al., 2021). These results are consistent with known aspects of the coronavirus RNA biology, such as the endoplasmic reticulum (ER) membrane-localized viral mRNA translation (Miller & Krijnse-Locker, 2008) and the antiviral role of the NMD (Wada et al., 2018). Among the 109 SARS-CoV-2 RNA interactome proteins identified by Lee et al., another study that utilized RAP-MS, were several factors involved in mRNA processing, export, and stability control, as well as proteins that localize to paraspeckles and cytoplasmic stress granules, hinting at a possibility of vRNA sequestration in response to infection (Lee et al., 2021). The proteomic data obtained by Flynn and colleagues with ChIRP-MS resulted in two datasets of 163 and 229 host proteins from virus-infected Vero E6 and Huh7.5 cell lines, respectively. In line with the RAP-MS reports, the obtained sets were enriched in RNA helicases, RNA processing and modification enzymes, as well as intracellular trafficking and vesicle proteins, and cytoskeleton components (Flynn et al., 2021). vRIC provided a list of 139 RBPs associated RNA metabolism, antiviral response (e.g., RIG-I pathway), cytoplasmic granule assembly (stress granules and P bodies), and virus biology (Kamel et al., 2021).

To gain understanding if and how SARS-CoV-2 RNA–protein interactomes agreed with each other across different studies and cell lines, we overlapped the lists of reported RBPs (Figure 2). Out of the total number of 397 proteins identified across all four SARS-CoV-2 RNA–protein interactome datasets, 126 (32%) were present in at least two, 46 (12%) were listed in at least three, while a subset of 21 (5%) factors was shared by all four datasets (Figure 2a). In another comparison focusing on the three approaches that utilized antisense oligonucleotide capture (RAP-MS and ChIRP-MS), out of $n = 344$ proteins identified, 86 were present in at least two of them, while 25 were shared by all three datasets (Figure 2b). While in a one-to-one comparison the list of SARS-CoV-2 RNA interactome proteins from the ChIRP-MS-based approach shared the most common factors with every other method, it also contained the highest number of uniquely-identified factors, being the largest dataset of all four. This likely corresponds to the formaldehyde crosslinking strategy uniquely employed by ChIRP-MS, which in addition to stabilizing direct RNA–protein contacts, also leads to preservation of protein–protein interactions. The list of common SARS-CoV-2 RNA protein interactors, present in at least three out of four datasets ($n = 46$ proteins), consisted of factors functionally related to cellular processes implicated in lifecycle of RNA viruses and its sub-compartmentalization as well as in the host response to viral

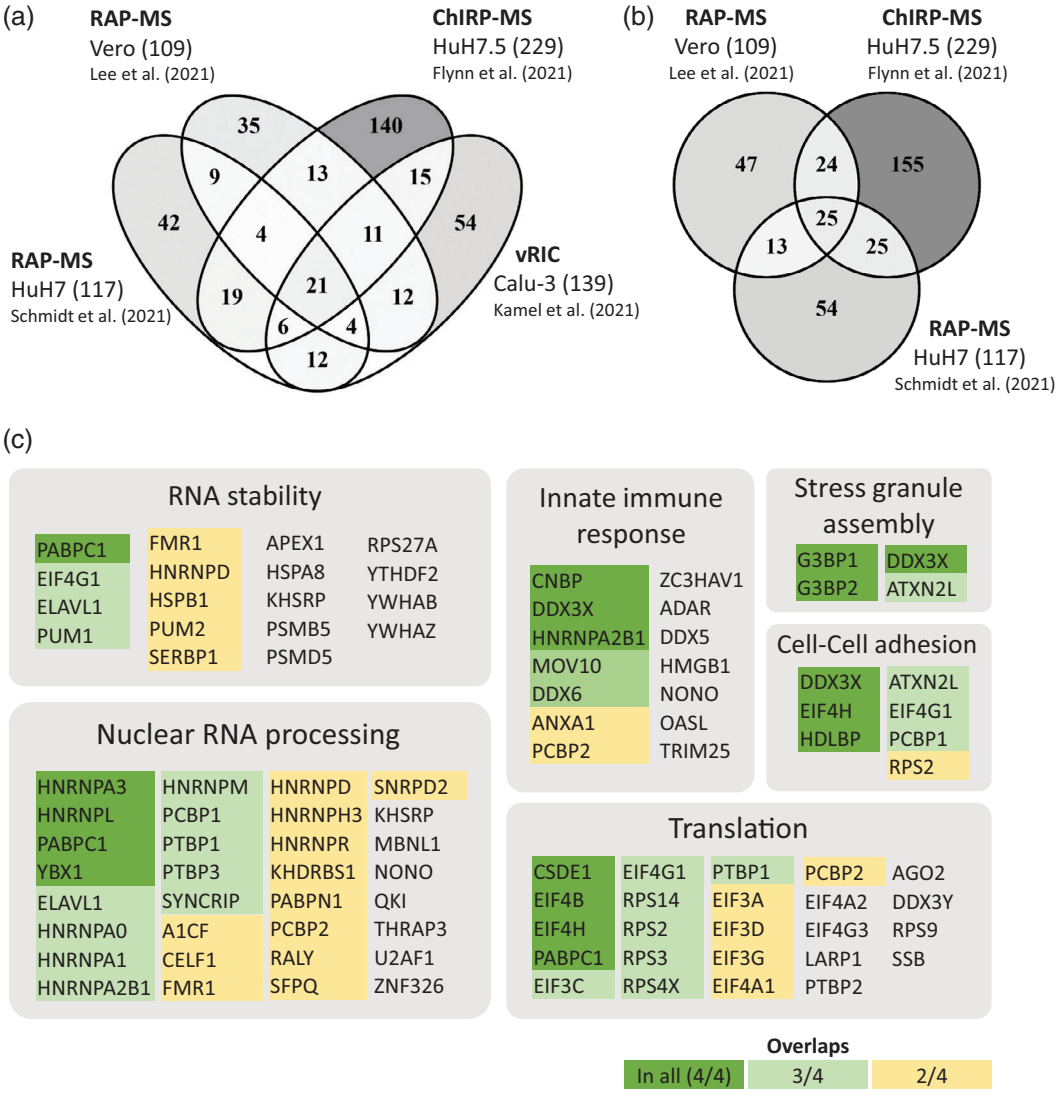

FIGURE 2 Comparison of SARS-CoV-2 RNA–protein interactome studies in terms of lists of host RBPs identified as associated with vRNA. The Venn diagrams represent the number of identified proteins common and distinct among the analyzed SARS-CoV-2 RNA interactome studies. (a) Comparison of all four datasets. (b) Comparison of three datasets obtained in studies relying on antisense oligonucleotide capture. (c) Selected functionally-related protein groups listed in the datasets defined as "SARS-CoV-2 RNA–protein interactome", organized based on their role in molecular processes associated with the viral infection. Colors indicate the proteins identified in common between the datasets based on the degree of overlap

infection (Figure 2c, green highlight). Some examples include members of the heterogeneous ribonucleoprotein (HNRNP) family, engaged in multiple RNA-metabolic processes, RNA helicases DDX3X and MOV10, and factors implicated in the assembly of stress granules (e.g., G3BP1, G3BP2), the sites of vRNA sequestration formed to suppress viral replication and translation (Ciccosanti et al., 2021; Onomoto et al., 2014). The overall relatively modest overlap between all four datasets most likely is associated with the use of different cell lines, capture methods and experimental conditions across the studies (Table 2). Notably, many proteins were uniquely listed in one out of four SARS-CoV-2 RNA-bound proteomes, several of which have a documented role in viral infection. Some notable examples include: the E3 ubiquitin ligase TRIM25, required for activity of Zinc-finger antiviral protein ZAP, non-muscle myosin heavy chain 9 (MYH9) protein, involved in vRNA transport, RNA-editing enzyme ADAR, implicated in deamination of the SARS-CoV-2 RNA (Di Giorgio et al., 2020), or YTHDF2, the reader of N^6-methyladenosine (m^6A), a known modification within vRNA (Tan & Gao, 2018). Apart from the mentioned methodical and experimental variables, the high number of uniquely listed proteins might be associated with capturing transient interactions occurring at the particular stages of the infection. Thereby, the combined results from all these individual studies are valuable for the comprehensive understanding of the SARS-CoV-2 RNA–host proteins interactions.

Comprehensive interpretation of SARS-CoV-2 RNA–protein interactome data requires downstream analyses that would allow for evaluation of the RNA interactome capture results, and functional characterization of host factors with putative roles in viral infection. Thereby, a critical part of the SARS-CoV-2 RNA–protein interactome studies was devoted to characterizing the captured proteins for the sake of searching of host RBPs with putative roles in the infection, and those which could be considered as potential targets in the design of antiviral therapies. Such characterization included not only functional enrichment analyses (already mentioned), but also comparisons with other related datasets, for example, generated with CRISPR perturbation screens, viral-host protein interaction networks, or interrogation with the drug-gene interaction databases. Worth mentioning, some datasets were compared also to other viral infections (see Section 3).

For two proteins captured with RAP-MS upon SARS-CoV-2 infection by Schmidt et al., their RNA targets were mapped with enhanced crosslinking immunoprecipitation method (eCLIP) (Schmidt et al., 2021; Van Nostrand et al., 2016). Cellular nucleic acid-binding protein (CNBP), an antiviral regulator required for innate immune response activation, was shown to bind several sites within SARS-CoV-2 RNA (Schmidt et al., 2021), and was independently reported to affect cell survival after SARS-CoV-2 infection (Wei et al., 2021). CNBP was also one of the 21 proteins identified in all four SARS-CoV-2 RNA interactome datasets (Figure 2c). The other candidate tested with eCLIP, La-related protein 1 (LARP1), also independently reported to interact with the nucleocapsid protein of SARS-CoV-2 (Gordon, Jang, et al., 2020), was shown to bind RNA of both the host cell and the virus, including a region at the 5′ end of the SARS-CoV-2 genome, similar to a 5′-terminal motif of human mRNAs, to regulate their translation (Schmidt et al., 2021).

With another comparative analysis, the SARS-CoV-2 RNA-binding proteins were also intersected with proteomic data from the SARS-CoV-2-infected cells and IFN-β-treated cells (Kerr et al., 2020). The results pointed toward (and confirmed) the role of MAPK and JAK–STAT signaling pathways activation in response to SARS-CoV-2 infection, and highlighted the role of interferon-stimulated genes upregulation through JAK–STAT pathway (Kerr et al., 2020; Sa Ribero et al., 2020).

In a series of genome-wide CRISPR perturbation screens, the proteins identified in the SARS-CoV-2 RNA interactomes were tested for their effect on infection progression and cell survival upon SARS-CoV-2 infection (Lee et al., 2021; Schmidt et al., 2021; Wei et al., 2021). Based on the perturbation analyses, the proteins can be classified as either pro-viral (i.e., inhibitory effect on infection progression and overall better cell survival) or anti-viral (i.e., when perturbed protein expression correlates with increased infection progression and severity). Out of 104 host proteins identified in SARS-CoV-2 RNA interactome by Schmidt et al., 11 were reported to have a significant effect on cell death rate upon SARS-CoV-2 infection, including cellular nucleic acid-binding protein (CNBP), polyadenylate-binding protein 1 (PABPC1), and cold shock domain-containing protein E1 (CSDE1), all of which were independently captured in all four interactome approaches (Figure 2).

Among the SARS-CoV-2 RNA interactome proteins identified by Lee et al., a series of follow-up siRNA-knockdown (KD) experiments were done for selected RBPs, which were upregulated upon the SARS-CoV-2 infection or IFN-β treatment (Lee et al., 2021). KDs revealed several possible direct suppressors of the infection, including PARP12, TRIM25, ZC3HAV1, CELF1, and SHFL, as well as LARP1 and CNBP, the last one validated in the eCLIP experiments by Schmidt et al. Lee et al. also identified several RBPs with potential pro-viral activity, including the translation factors

EIF3A, EIF3D, and CSDE1, which could be recruited to facilitate the translation initiation of the viral gRNA and sgRNAs.

Flynn et al. performed a series of genome-wide and targeted CRISPR screens of the SARS-CoV-2 RNA interactome proteins, to validate whether the proteins identified with ChIRP-MS-based approach were actively involved in the viral infection progression. The analysis revealed host factors related to mitochondria and proteasome function, consistent with the proposed role of mitochondria as general platforms for anti-SARS-CoV-2 immunity (Flynn et al., 2021) and evidence for mitochondrial proteins impinging coronavirus proliferation (Gordon, Hiatt, et al., 2020), as well as with the reported leverage of the proteasome function occurring during coronavirus infection (Raaben et al., 2010). Overall, concluding on CRISPR and KD screens performed for SARS-CoV-2 RNA interactome proteins, it appears that the majority of tested RBPs interact with the viral RNA to act as repressors of the infection, with only a subset recruited by the virus to facilitate the infection progression.

An important objective of SARS-CoV-2 RNA–protein interactome studies has been to provide a resource that could aid the selection of host factors for the development of antiviral strategies. SARS-CoV-2 RNA interactomes were intersected with protein-interaction databases, to select proteins that are known targets of pharmacological compounds, and to test the effects of those compounds on viral infection. Such measurements have been done for selected SARS-CoV-2 RNA interactome proteins, and revealed several cellular processes and signaling pathways, whose pharmacological inhibition interferes with viral replication, including translation initiation and elongation, stress granule assembly, mTOR signaling, the interferon response (Blanco-Melo et al., 2020; Bouhaddou et al., 2020). For example, strong antiviral effects were observed for compounds targeting epidermal growth factor receptor EGFR, through inhibition of MAPK signaling cascade and mTORC signaling (Klann et al. 2020), as well as when targeting the elongation factor-1A (eEF1A), which interfered with cap-dependent translation of viral proteins (White et al. 2021). Schmidt et al. found that drug-induced inhibition of selected SARS-CoV-2 RNA interactome proteins, including PPIA, ARP2/3, and ATP1A1, leads to a dose-dependent reduction in viral replication (Schmidt et al., 2021). Kamel et al. observed a strong inhibition of SARS-CoV-2 protein synthesis upon pharmacological targeting of heat shock protein 90 (HSP90) and Insulin-like growth factor 2 (IGF2BP1), consistently with reports on the anti-SARS-CoV-2 effects of HSP90 (Wyler et al. 2021). Overall, the inhibition assays of druggable targets identified in SARS-CoV-2 RNA–protein interactomes, confirmed that these datasets provide a valuable resource of viable targets for the development of antiviral therapies.

3 | USE OF HIGH-THROUGHPUT MOLECULAR TECHNIQUES FOR STUDYING INTERACTOMES OF OTHER RNA VIRUSES

SARS-CoV-2 RNA–protein interactomes were compared with RPBome datasets revealed as associated with the ongoing infection caused by other RNA viruses. Based on a comparison of the SARS-CoV-2- and SINV-induced changes to the RNA-bound proteome reported by Kamel et al. and Garcia-Moreno et al., both types of viruses caused upregulation of over 90 common RBPs (Kamel et al., 2021). These group included factors involved in the antiviral response, such as TRIM25, TRIM56, ZC3HAV1, DHX36, and GEMIN5. Interestingly, a few of these antiviral factors were impaired in the SARS-CoV-2 infection, including the RNA-editing enzyme ADAR, and the nonsense-mediated decay helicase UPF1 (Kamel et al., 2021).

Application of high-throughput, genome- and proteome-wide technologies in molecular virology is not limited to SARS-CoV-2. VIR-CLASP method was proved useful for the identification of hundreds of proteins bound to CHIKV and influenza A virus (IAV) genomes. For instance, CHIKV RNA was found to bind the lipid-modifying enzyme fatty acid synthase (FASN), which enhances its replication (B. Kim, Arcos, et al., 2020). In another study, the authors performed affinity purification coupled with mass spectrometry for mapping RNA–protein interactions between several different strains of IAVs in human cell line (HEK293T) (Wang et al., 2017). Comparative analyses led to identification of unique as well as shared host factors that regulate innate immunity and control infection progression. Among shared proteins, the authors reported plakophilin 2 (PKP2) as a natural inhibitor of IAV polymerase complex. The analysis of unique, strain-specific RNA–host protein interactions provided insights into mechanisms underlying de novo virulence (Wang et al., 2017). Another orthogonal method for studying RNA–protein interactions, RaPID, was used for identifying RNA interactome of ZIKV. The study of Ramanathan et al. showed that QKI protein, which is highly expressed in neuronal progenitor cells, interacts with the non-coding region of the ZIKV RNA and is important for virus replication (Ramanathan et al., 2018). Ooi et al. used CHIRP-MS method, to study RNA–protein interactomes of two viruses from *Flaviviridae* family, including ZIKV and Dengue virus (DENV). The study highlighted the importance of ER-localized

RBPs for flavivirus infection. Both viruses need vigilin, an evolutionary conserved RBP, to promote infection (Ooi et al., 2019).

Next to RNA-centric approaches, methods based on identification of protein–protein interaction (PPI), provided important insights into infection mechanisms of RNA viruses. Gestuveo et al. studied protein networks in ZIKV infection by creating an *Aedes aegypti* cell line with stable expression of ZIKV capsid proteins to study virus-vector interactions. The study identified 157 interactors, including eight of potentially pro-viral activity during ZIKV infection in mosquito cells. Authors demonstrated that ZIKV capsid interacts with TER94/VCP in order to establish viral infection in both mosquito and human cells (Gestuveo et al., 2021). Last but not least, host-virus PPI networks have been investigated for SARS-CoV-2 and have proven valuable for illustrating the complex nexus between viral and cellular proteins (Banerjee et al., 2020; Gordon, Jang, et al., 2020; Sadegh et al., 2020; Stukalov et al., 2021). In regard to *Coronaviridae* family, Hoffman et al. used published SARS-CoV-2 PPI interactome data (Gordon, Jang, et al., 2020) to design high-coverage CRISPR-Cas9 library targeting >330 proteins (Hoffmann et al., 2021). This functional screen was applied in SARS-CoV-2 infected cells at two temperatures (33°C or 37°C) along with three related, seasonal human coronaviruses: HCoV-229E, HCoV-NL63, and HCoV-OC43. They were able to identify RAB10, RAB14 as critical SARS-CoV-2 host factors, together with multiple pan-coronavirus factors being involved in cholesterol homeostasis. It was shown that targeting of SCAP, key regulator of sterol regulatory element binding protein (SREBP) maturation, impairs the infection capability of all four coronaviruses. This observation may be useful for developing broad-spectrum anti-coronavirus treatments. An interesting observation on RAB10 and RAB14 to be indispensable for SARS-CoV-2 (in turn RAB2A and RAB7A appeared to be critical for HCoV-229E, HCoV-OC43, and HCoV-NL63), implies potential coronavirus-specific differences in Rab GTPase requirements. The authors speculate that different coronaviruses might differentially rely on specific Rab GTPases and utilize different entry pathways in a context-dependent manner (Hoffmann et al., 2021).

Taking together, the application of system-wide approaches for studying viral RNA–host proteins interplay has delivered tremendous advances in our understanding of virus biology as well as the regulatory roles of RBPs. Identification of RBPs and pathways commonly and uniquely used by specific RNA viruses to provide efficient replication and spread can lead to the development of broad-spectrum antivirals against current and future threats.

4 | CONCLUSIONS

COVID-19 pandemic with its acute hit and quick spread has had a profound impact on society in multiple aspects of life. One of the immediate actions taken was the repurposing of the research in many laboratories all over the globe to study SARS-CoV-2, its genome, entry, mechanisms of hijacking host translation machinery, the immune response of the host, among others. SARS-CoV-2 RNA–protein interactome studies enrich that list with a wealth of data that shed more light on the processes from the interface of viral RNA–host proteins interactions. Importantly, the lists of human proteins interacting with SARS-CoV-2 RNA offer a guide for the development of novel, perhaps virus-specific, antiviral strategies or target-instructed drug repurposing. On the other hand, by comparing with RNA–protein interactomes of other RNA viruses, these data may help to identify RBPs of pan-viral regulatory functions, and putative targets for broad-spectrum antiviral therapies. SARS-CoV-2 RNA–protein interactome datasets were extended by integrating them with CRISPR and knockdown perturbation screens, drug-gene interaction databases, viral-host protein–protein interaction networks. From another angle, comparisons performed on RNA interactomes of SARS-CoV-2 and the other positive-sense RNA viruses provided several new insights into the "molecular arms race" between the virus and host. All these lines of evidence are convincing for the notion that systems biology approaches are providing new exciting insights into the molecular virology, with a great hope to instruct new, effective antiviral therapies. SARS-CoV-2 RNA–protein interactome studies showcased the utility of RNA interactome studies to uncover the pathomechanism of RNA viruses and hold promise to accelerate the treatment design process of emerging infections caused by RNA viruses in the future.

CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

AUTHOR CONTRIBUTIONS

Marcin Koliński: Formal analysis (lead); visualization (lead); writing – original draft (equal). **Ewelina Kałużna:** Visualization (supporting); writing – original draft (equal). **Monika Piwecka:** Conceptualization (lead); formal

analysis (supporting); funding acquisition (lead); supervision (lead); writing – original draft (supporting); writing – review and editing (lead).

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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How to cite this article: Koliński, M., Kałużna, E., & Piwecka, M. (2022). RNA–protein interactomes as invaluable resources to study RNA viruses: Insights from SARS CoV-2 studies. *Wiley Interdisciplinary Reviews: RNA*, e1727. https://doi.org/10.1002/wrna.1727