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Graphical abstract

Highlights

- Experimental characterization of the microRNA-binding landscape of SARS-CoV-2 RNA
- microRNA binding has limited effect on SARS-CoV-2 infection
- SARS-CoV-2 affects the cellular microRNA interactome
- miR-15 family mRNA targets are derepressed during SARS-CoV-2 infection

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In brief
microRNAs are important post-transcriptional gene regulators hijacked by viruses. Here, Fossat et al. map the microRNA-binding landscape for SARS-CoV-2 RNA. Mutagenesis of the main binding sites and Argonaute silencing show that microRNAs are not critical for replication. However, the cellular microRNA interactome is perturbed during infection, specifically with derepression of miR-15 family targets.
Identification of the viral and cellular microRNA interactomes during SARS-CoV-2 infection

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SUMMARY

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic has had a tremendous impact worldwide. Mapping virus-host interactions is critical to understand disease progression. MicroRNAs (miRNAs) are important RNA regulators, but their interaction with SARS-CoV-2 RNA was not experimentally investigated. Here, using Argonaute (AGO) cross-linking immunoprecipitation combined with RNA proximity ligation (CLEAR-CLIP), we provide unbiased mapping of SARS-CoV-2/miRNA interactions. We identified six main regions on the viral RNA bound primarily by one specific miRNA. Targeted mutagenesis and AGO1-3 knockdown demonstrated that these interactions are not critical for virus production. Moreover, we identified perturbed regulation of cellular miRNA interactions during infection, including non-compensated viral sequestration of the miR-15 family. Transcriptome analysis further showed that miRNAs targeted by this miRNA family are derepressed. This work delineates the interphase between miRNA regulation and SARS-CoV-2 infection and further contributes to deciphering the full molecular interactome of this virus.

INTRODUCTION

Since late 2019, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) outbreak has had impactful consequences for health and economy worldwide. SARS-CoV-2 is a Betacoronavirus belonging to the Coronaviridae family. Its enveloped helical capsid contains a positive-sense single-stranded RNA genome of ~30 kb. The genome is 5’-7-methylguanosine (m7G) capped, terminates in a poly-A tail, and contains 5’ and 3’ UTRs each of ~0.3 kb. It encompasses 14 main open reading frames (ORFs), which encode at least 26 non-structural, structural, and accessory proteins through the production of eight main sub-genomic mRNAs.1–3 This genomic organization reflects a complex mechanism of gene expression regulated by cis-regulatory elements: a slippery sequence located in the vicinity of a pseudoknot triggers a ribosomal –1 frameshift responsible for the translation of ORF1b,3,4 and long-range intramolecular RNA-RNA interactions between specific transcription regulatory sequences (TRSs) support RNA template switching during replication, responsible for the production of the eight sub-genomic mRNAs.5–7 Gene expression and RNA replication are further controlled by trans-regulatory elements, in particular the 16 non-structural proteins (NSP1–NSP16) encoded by the ORF1a/b.2

To identify trans-acting cellular factors affecting SARS-CoV-2 replication, a number of genomic screens have been performed.8–15 These have resulted in the identification of more than 1,000 protein candidates,16 emphasizing the need for functional validation. Furthermore, interactions between viral proteins and cellular RNAs have been reported.11,12,17 NSP1 can inhibit translation through direct interaction with the 18S ribosomal RNA and NSP16 affects cellular mRNA splicing by interacting with the small spliceosomal nuclear RNAs U1, U2, and U4, or the small nucleolar RNA SNORD27, and that cellular RNAs have been reported.11,12,17 NSP1 can inhibit translation through direct interaction with the 18S ribosomal RNA and NSP16 affects cellular mRNA splicing by interacting with the small spliceosomal nuclear RNAs U1 and U2.17 Finally, hundreds of cellular proteins, with or without canonical RNA-binding domains, were shown to interact with SARS-CoV-2 RNA.11,12,15 While a primary role in protecting the infected host cell from apoptosis was suggested for these interactions,15 they are likely to be equally important for the antiviral response but also for how SARS-CoV-2 could affect RNA biology (splicing, translation, editing, stability, transport), as well as vesicle trafficking and granule assembly.11,12 Two studies further investigated RNA-RNA interactions in infected cells.5,7 By identifying and locating the SARS-CoV-2 RNA intramolecular interactions, they improved our understanding of the contribution of the cis-regulatory elements to the viral replication cycle. They also reported that SARS-CoV-2 RNA can directly interact with cellular RNAs such as the small spliceosomal nuclear RNAs U1, U2, and U4, or the small nucleolar RNA SNORD27, and that these interactions could contribute to modulate translation, splicing, or 2’-O-methylation.

MicroRNAs (miRNAs) are small ~22-nucleotide (nt) single-stranded RNAs. Accompanied by Argonaute (AGO) and other accessory proteins, they make up the RNA-induced silencing complex (RISC), which interacts by base-pair complementarity with specific miRNAs to exert post-transcriptional gene regulation.18 In humans, more than 60% of protein-encoding
**CLEAR-CLIP workflow**

**A** CLEAR-CLIP in cell \(\rightarrow\) in vitro

- RBP
- AGO
- miRNA
- viral RNA
- cellular RNA
- Cross-linking
- UV 254 nm
- RNA fragmentation + AGO immunoprecipitation
- RNA proximity ligation
- Gel size selection + protein digestion
- Single-stranded Chimeras
- cDNA library preparation + high throughput sequencing + in silico analysis

**B** CLEAR-CLIP Singletons

**C** RNA-Seq

**D** CLEAR-CLIP Chimeras

**E** Binding Abundance

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transcripts are regulated by miRNAs. Complementarity within the first 8 nt of the miRNA, referred to as the seed site, is generally critical for functional interaction. Functional miRNA interaction is typically exerted through binding to the 3′ UTR of miRNAs, which results in degradation or inhibition of translation. Several RNA viruses have evolved to utilize the miRNA system to their own advantage, both by conventional miRNA induced repression, e.g., to avoid immune recognition (eastern equine encephalitis virus (EEEV)/miR-142-3p22), and through non-canonical stimulation of viral RNA stability and replication by miRNA binding (hepatitis C virus [HCV]/miR-122-5p and pestiviruses/miR-17-5p23). For SARS-CoV-2, two recent studies reported that small RNAs derived from the viral genome could act as miRNAs, destabilizing target mRNAs and attenuating the interferon (IFN) response.24 However, to what degree SARS-CoV-2 RNA interacts with cellular miRNAs and any potential functional consequences have not been experimentally investigated.

Here, we used covalent ligation of endogenous AGO-bound RNAs (CLEAR)-cross-linking immunoprecipitation (CLIP) methodology, which allows unambiguous mapping of specific miRNA-binding sites, to explore SARS-CoV-2 RNA interactions with cellular miRNAs. We further investigated the functional role of these interactions through the engineering of loss- and gain-of-miRNA-binding SARS-CoV-2 mutants, as well as knockdown of AGO1, 2, and 3. Finally, we used our CLEAR-CLIP data to investigate changes to the cellular miRNA interactome during infection and performed transcriptome analyses to examine alterations in expression of the targeted mRNAs. Altogether, our results provide an experimental description of the interrelationship between SARS-CoV-2 RNA and cellular miRNAs, as well as its potential repercussion onto the regulation of cellular gene expression. They further constitute a useful resource in the effort to reveal the complete molecular interactome of SARS-CoV-2 to advance understanding of this novel coronavirus.

RESULTS

CLEAR-CLIP reveals the SARS-CoV-2 miRNA interactome

To investigate to what degree SARS-CoV-2 RNA interacts with the RISC and identify putative miRNA interactions, we infected highly permissive VeroE6 African green-monkey kidney cells with the Danish ancestral isolate DK-AHH127 and processed these for CLEAR-CLIP (Figures 1 and S1; Table S1A). Through the introduction of covalent bonds between RNA-binding proteins (RBPs) and their RNA targets using UV254, followed by cell lysis, partial RNA digestion, AGO immunoprecipitation, and RNA proximity ligation (Figure 1A), CLEAR-CLIP allows unambiguous identification of the AGO-loaded miRNAs by linkage to their cognate RNA targets (referred to as chimeric miRNAs; see also STAR Methods and Moore et al., Scheel et al., and Rozen-Gagnon et al.26,28,29). This resulted in the specific identification of AGO/miRNA-binding sites on SARS-CoV-2 RNA (Figures 1 and S1; Tables S2 and S3).

Non-chimeric singleton reads revealed specific AGO-binding sites throughout the SARS-CoV-2 RNA with enrichment in the 3′ terminal 5 kb of the genome, particularly in ORFs 3a, M, N, and the 3′ UTR (Figures 1B and S1C; Table S2). Enrichment for AGO-binding sites toward the 3′ terminus was consistent with the canonical role of the RISC binding primarily to 3′ UTRs but also with higher RNA abundance for this part of the viral RNA as assessed by RNA sequencing (RNA-seq) (Figure 1C), given that SARS-CoV-2 produces sub-genomic viral transcripts with overlapping 3′ ends.30 Chimeric reads constituted 7.3% of the total mapped reads (Figure S1B; Tables S1A and S3) and revealed the miRNA-binding landscape along SARS-CoV-2 RNA (Figures 1D, S1D and S1E; all miRNA analyses are presented by miRNA seed site family named according to representative type members). As expected, the general coverage resembled what was observed from standard singleton reads but added unambiguous identification of the specific miRNA(s) contributing to each AGO-binding event. We could further estimate the relative amount of each miRNA binding the viral RNA. Among 214 miRNA binders, the top 50 contributed 96.3% of the chimeric reads (Table S1B). Using chimeric reads, we could also estimate the relative abundance of each miRNA functionally loaded onto AGO in VeroE6 cells (Figure 1D; Table S1C). Doing so, we observed that the top-50 binders were also among the most abundant miRNAs expressed.

Six isolated regions were predominantly bound by a single type of miRNA (annotated #1–#6; Figures 1D and S1E), whereas most other regions of binding were the results of multiple overlapping...
Figure 2. SARS-CoV-2 miRNA interactome in A549hACE2 cells

(A) Mapping of CLEAR-CLIP singleton reads to the SARS-CoV-2 genome. Shown are the accumulated reads from three independent experiments (48 hpi, MOI = 0.001), normalized to the size of the accumulated libraries, and represented as RPKM. Significant AGO-binding events identified by peak-calling analysis are indicated by asterisks (*).

(B) Mapping of reads from RNA-seq to the SARS-CoV-2 genome showing the relative read depth. Shown is the average coverage (RPKM) of mapped reads from three independent experiments (48 hpi, MOI = 0.001).

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miRNA interactions. Among the six regions of interest, #1 (let-7a-5p), #2 (miR-29a-3p), #4 (miR-27a-3p), and #6 (miR-30a-5p) had perfectly conserved seed-binding sites for the miRNA identified, whereas single-nucleotide mismatches were found in regions #3 (miR-17-5p) and #5 (miR-27a-3p), which were, however, supported by supplementary auxiliary pairing (Figure 1E).

The SARS-CoV-2 miRNA interactome is conserved in human lung cells
To investigate the miRNA interactome of SARS-CoV-2 in a more relevant context, we performed CLEAR-CLIP using infected A549hACE2 human lung cells, which over-express the human SARS-CoV-2 receptor angiotensin-converting enzyme 2 (ACE2) (Figures 2 and S2; Tables S4–S6). We observed an AGO-binding landscape (Figures 2A, 2B, and S2C) comparable with the one observed in VeroE6 cells. The relative distribution and binding of miRNAs to SARS-CoV-2 RNA was also similar to what was observed in VeroE6 cells, including the same six main regions of binding, although binding to region #3 was decreased (Figures 2C, 2D, S2D, and S2E). Among 201 different miRNAs binding SARS-CoV-2 RNA in A549hACE2 cells, the top 50 contributed 98.1% of the chimeric reads (Table S4B). As for the VeroE6 cells, these were also among the most expressed miRNAs in A549hACE2 cells (Figure 2C; Table S4C).

Further analysis of the SARS-CoV-2 miRNA-binding landscape showed a clear correlation between binding and expression in both cell lines (Figures 3A and 3B). This comparison also identified miRNAs with more binding to SARS-CoV-2 RNA than expected by average, hence constituting potential candidates for functional investigation. Furthermore, the main binders were shared between VeroE6 and A549hACE2 (Figure 3C), to the extent that these were expressed in both cell types (Figure 3D). Among the top-50 binders, 36 (72%) were common (Figure 3E), supporting the existence of a conserved SARS-CoV-2 miRNA interactome between cells as distinct as VeroE6 and A549hACE2.

Two recent studies reported the production of a small SARS-CoV-2 derived RNA from the ORF7a region, CoV2-miR-O7a, in the top 50 contributed 98.1% of the chimeric reads (Table S4B). Given that CLEAR-CLIP, unlike smRNA-seq, provides information on miRNAs loaded onto AGO, an absolute requirement to infer miRNA functionality, we looked for this putative miRNA in our data. We did not detect chimeric reads containing the CoV2-miR-O7a sequence neither in VeroE6 nor in A549hACE2 cells infected with SARS-CoV-2. Furthermore, among singleton reads of 17–27 nt, fewer than 15 reads in total mapped to the proposed CoV2-miR-O7a region, and generally not with the proposed start and end coordinates (Figure S3). Altogether, these observations suggest poor loading of this putative small RNA on AGO and therefore a limited role, if any, as a functional miRNA, at least in the experimental conditions used here.

Perturbation of miRNA interactions has minimal effect on SARS-CoV-2 replication and virus production
To study the impact of miRNA/viral RNA interactions for SARS-CoV-2, we functionally investigated the importance of miRNA binding to regions #1–6. These were chosen based on interaction strength (peak size), contribution from individual miRNA binding (chimera data), and conservation between cell lines. Furthermore, high sequence conservation of the corresponding seed-binding sites was observed across SARS-CoV-2 strains (Figure S4A).

We used the DK-AHH1 SARS-CoV-2 infectious clone to introduce loss-of-binding (L) mutations (preserving encoded amino acids) in SARS-CoV-2 RNA at each of the miRNA-binding seed sites of regions #1–6 (Figure 4A). Given the inherent mismatches at the binding seed sites for region #3 (miR-17-5p) and #5 (miR-27a-3p), we further engineered gain-of-binding (G) mutants to create canonical seed-binding sites at these two positions (Figure 4A). For region #3, the mutation was synonymous, whereas, for region #5, a valine to leucine change was associated with the substitution. In vitro-transcribed wild-type (WT) and mutant genomic RNAs were then transfected into VeroE6 cells for production of titrated virus stocks. The engineered mutations did not revert after passage to naive cells. To confirm that the introduced mutations had the expected effect on miRNA binding, we performed CLEAR-CLIP on mutant-infected VeroE6 and A549hACE2 cells. As expected, miRNA binding was specifically abolished at the mutated sites for the six loss-of-binding mutants, whereas it was strongly enhanced for the two G mutants in both cell lines (Figures 4B and 4B). The large increase in binding (more than 40-fold for the 3G mutant in VeroE6 cells) was consistent with extensive auxiliary pairing observed for miR-17-5p to region #3 and for miR-27a-3p to region #5 (see Figures 1E and 2D).

We then compared the viral fitness of the different SARS-CoV-2 mutants by infecting VeroE6 and A549hACE2 cells at equal multiplicity of infection (MOI) of 0.001 with WT or mutant viral particles and followed the infection for 72 h. No major difference in intracellular viral RNA levels or production of infectious particles were observed for any of the loss-of-binding or gain-of-binding mutants, as assessed by RT-qPCR and median tissue culture infectious dose (TCID<sub>50</sub>) assay, respectively (Figures 4C and 4D).

To go beyond the specific interactions at regions #1–6 and address the role of the RISC and miRNA/viral RNA interactions for SARS-CoV-2 in general, we further investigated the course of infection in cells depleted of AGO2 by small interfering RNA
(siRNA)-mediated knockdown (KD; Figure S5A), AGO2 is the main AGO paralog functioning in the RISC, and also the one responsible for critical miRNA interactions for HCV and bovine viral diarrhea virus (BVDV). Using similar conditions of KD that were used to show its importance for these viruses and compared with cells transfected with a non-specific scrambled siRNA, no difference in SARS-CoV-2 production was observed (Figure 4E). Using the same experimental strategy (Figure S5A), we observed similar results for AGO1 and AGO3 (Figure 4E). Finally, SARS-CoV-2 infection did not substantially affect the expression of AGO1, 2, or 3 (Figures SSB and SSC), in agreement with another study. Altogether, our results confirmed specific binding of the let-7a-5p, miR-29a-3p, miR-17-5p, mir-27a-3p, and miR-30a-5p miRNAs at their corresponding binding sites in regions #1–6. They also showed that these interactions, and the activity of the RISC and other miRNAs, were not critical for SARS-CoV-2 replication or virus production.

**Figure 3. Comparison of SARS-CoV-2 miRNA interactions in VeroE6 and A549hACE2 cells**

(A) Comparison between the binding level of miRNAs interacting with SARS-CoV-2 RNA and their expression level in infected VeroE6 cells. (B) Comparison between the binding level of miRNAs interacting with SARS-CoV-2 RNA and their expression level in infected A549hACE2 cells. (C) Comparison of the binding level of miRNAs interacting with SARS-CoV-2 RNA in VeroE6 and A549hACE2 cells. (D) Comparison of miRNA expression levels in VeroE6 and A549hACE2 cells infected with SARS-CoV-2. (E) List of common and unique miRNAs binding SARS-CoV-2 RNAs among the combined top-50 binders in VeroE6 and A549hACE2 cells. Data for (A)–(D) have been obtained by post trimmed means of M values (TMM) normalization and visualized as average counts per million (CPM), from three independent experiments. Pearson’s correlation coefficients (\(R\)) and p values are indicated. For (A) and (B), miRNAs binding significantly more (in yellow) or less (in purple) to SARS-CoV-2 RNA compared with cellular RNA were identified. The miRNAs binding more to SARS-CoV-2 RNA (in yellow in A and B) are indicated in all panels and highlighted in bold in (E). miRNAs of specific focus in this study are colored (following the same color-coding used throughout the manuscript). See also Figure S3 for complementary information.
A  Seed binding site in the regions of interest for SARS-CoV-2 wild-type and mutants

<table>
<thead>
<tr>
<th>Region</th>
<th>#1</th>
<th>#2</th>
<th>#3</th>
<th>#4</th>
<th>#5</th>
<th>#6</th>
</tr>
</thead>
<tbody>
<tr>
<td>mRNA-binder</td>
<td>let-7a-3p</td>
<td>miR-23a-3p</td>
<td>miR-17-5p</td>
<td>miR-27a-3p</td>
<td>miR-27a-3p</td>
<td>miR-30a-5p</td>
</tr>
<tr>
<td>Seed binding site: Wild-type (WT)</td>
<td>ACCUCA</td>
<td>UGGUGCUA</td>
<td>GCACUGUG</td>
<td>ACUGUGA</td>
<td>ACUGUGA</td>
<td>ACGUUA/A</td>
</tr>
<tr>
<td>Loss-of-binding (L)</td>
<td>GGUACUG</td>
<td>GUGACUG</td>
<td>GUGACUG</td>
<td>GUGACUG</td>
<td>GUGACUG</td>
<td>GUGACUG</td>
</tr>
<tr>
<td>Gain-of-binding (G)</td>
<td>ACGUUA/A</td>
<td>ACGUUA/A</td>
<td>ACGUUA/A</td>
<td>ACGUUA/A</td>
<td>ACGUUA/A</td>
<td>ACGUUA/A</td>
</tr>
</tbody>
</table>

Nucleotides highlighted in pink are mismatches when binding the seed site of the miRNA of interest.

B  Mutants - Intracellular viral RNA

C  Mutants - Infectivity titer

D  AGO KD - Infectivity titer

(legend on next page)
of VeroE6 cells, five miRNAs were downregulated while 11 were upregulated (Figures 5A and S6A; Table S1D). In A549hACE2 cells, two were downregulated while three were upregulated (Figures 5A and S6A; Table S4D). miR-29a-3p was the only common miRNA family changed (upregulated) in both cell lines.

Change in abundance of a specific miRNA may not translate directly into a similar change in its interaction with cellular RNAs; e.g., due to competing binding to viral RNA. We therefore analyzed and quantified the binding level of each miRNA to its cellular targets using information from chimeric reads. We found four miRNAs for which binding to cellular RNA was decreased and another four for which it was increased upon infection of VeroE6 cells (Figures 5B and S6B; Table S1E). In A549hACE2 cells, two were decreased while none were increased (Figures 5B and S6B; Table S4E). As expected, changes in miRNA binding to cellular RNAs correlated with changes in the corresponding abundance levels for most miRNAs (compare Figures 5A and 5B). However, less binding of miR-15a-5p and, to a lesser extent, miR-17-5p in VeroE6 cells and less binding of miR-15a-5p in A549hACE2 cells were observed despite unchanged abundance levels.

Functional sequestration by RNA viruses of specific miRNAs was previously described by us and others for HCV and miR-122-5p, as well as for pestiviruses and miR-17-5p. Here, the decrease in miR-15a-5p and miR-17-5p binding to cellular RNA (Figure 5B), despite unchanged abundance levels (Figure 5A), suggested specific sequestration by SARS-CoV-2. MiR-15a-5p and miR-17-5p were among the top miRNAs binding SARS-CoV-2 RNA in VeroE6 and A549hACE2 cells (see Figures 1D, 2C, and 3). Analyzing the fraction of chimeric reads mapping to viral versus host RNA, miR-15a-5p (22.1% in VeroE6 cells; 23.8% in A549hACE2 cells) and miR-17-5p (18.3%; 19.3%) both exhibited a higher binding fraction to viral RNA than the average miRNA family (15.1% and 14.5%) (Figure 5C). Other miRNAs, such as miR-29a-3p (32.8% and 33.6%) had a particularly prominent fraction binding SARS-CoV-2 RNA (Figure 5C). However, the expression of most of these, including miR-29a-3p, was upregulated during infection (Figure 5A), which would constitute a mechanism to compensate for depletion of the miRNA pool through binding to viral RNA.

Due to its decreased binding to cellular targets in both cell lines, miR-15a-5p could constitute a molecular regulator universally affected by SARS-CoV-2 during infection and COVID-19. By analyzing AGO binding specifically at significant peaks containing canonical miR-15a-5p binding seed sites in mRNA 3’ UTRs, we indeed observed less binding to these cellular targets of miR-15a-5p during infection, again in both cell lines (Figure 5D). This further supported sequestration of this miRNA by SARS-CoV-2 RNA and the potential relevance of its deregulation during infection, although the effect was smaller than, e.g., what was observed for the HCV/miR-122-5p interaction. Altogether, these results showed that SARS-CoV-2 infection affects the cellular miRNA interactome of both VeroE6 and A549hACE2 cells by perturbing the binding of specific miRNAs. These changes are generally compensated by similar changes in abundance, with the notable exception of miR-15a-5p and miR-17-5p, which, therefore, to a certain extent, could be functionally sequestrated by the viral RNA. Noticeably, the decrease of miR-15a-5p binding to cellular targets was observed both in VeroE6 and A549hACE2 cells, suggesting that it could represent a broad mechanism of deregulation associated with SARS-CoV-2 infection.

Deregulation of miR-15a-5p targets during SARS-CoV-2 infection

Decrease in binding of miR-15a-5p to its mRNA targets during SARS-CoV-2 infection should result in their derepression. To assess this, we performed transcriptome analyses using RNA-seq on VeroE6 and A549hACE2 cells, which, as for the CLEAR-CLIP analyses, were collected 48 h after infection with MOI = 0.001. Compared with the transcriptome of non-infected cells, typical expression changes associated with SARS-CoV-2 infection were observed, including upregulation of IFN signaling and chemokine-encoding genes, in VeroE6 cells (Figure S7; Table S7). In A549hACE2 cells, with the exception of the ATF3 gene, no significant expression changes were observed after 48 h (Figure S7), consistent with the slower kinetic of SARS-CoV-2 infection in these cells compared with VeroE6 cells (see Figures 4C–4E). This observation further supported that the alteration in miR-15a-5p binding to its cellular targets is an immediate and direct consequence of SARS-CoV-2 replication through possible miRNA sequestration, and not a secondary effect induced by global gene expression changes. To allow assessment of putative expression changes of miR-15a-5p targets, we therefore also analyzed the transcriptome of A549hACE2 cells 72 h post infection. At this time point, we again observed expression changes typically associated with SARS-CoV-2 infection (Figure S7; Table S8).

We found that the set of mRNAs containing miR-15a-5p family binding seed sites in 3’ UTR significant CLIP peaks, defined as in Figure 5D above, was generally upregulated compared with all mRNAs (Figure 6A), and that this upregulation scaled with the...
A Change in miRNA abundance

B Change in miRNA binding to host RNA

C Proportion of specific miRNA binding SARS-CoV-2 RNA

D Change in AGO binding to miR-15a-5p mRNA targets

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extent of miRNA seed paring (Figure 6B). For comparison, we also looked at expression changes for miRNAs containing miR-17-5p binding sites. While not as distinct as for miR-15a-5p targets, we also observed seed-dependent upregulation in VeroE6, but no consistent regulation in A549hACE2 cells (Figure S8), in agreement with the slightly reduced binding of miR-17-5p to its targets in VeroE6 cells, but not A549hACE2 cells, during infection (Figure 5B). Logically, among the miR-15a-5p targets differentially expressed during infection, the vast majority were upregulated in both cell lines (Figure 6C). Finally, gene ontology analysis of these revealed terms related to T cell and cell death, toll-receptor biology, but also terms covering processes important for cell differentiation (Figure 7), in line with recent studies showing that the miR-15 family is important for survival and differentiation of T cells and that SARS-CoV-2 can infect CD4+ T cells. The results of our experiments would thus suggest common pathways regulated by miR-15a-5p and affected by SARS-CoV-2 between different cell types. Furthermore, while we cannot exclude that some of the gene expression changes identified during infection may be indirect, in aggregate our observations support that SARS-CoV-2 RNA reduces binding of AGO/miR-15a-5p to its mRNA targets resulting in their derepression.

**DISCUSSION**

In the current study, we have characterized the SARS-CoV-2 molecular interactome focusing on its RNA and functional interactions with miRNAs. Since the emergence of SARS-CoV-2 in late 2019, many studies have contributed to deciphering its molecular interactome, focusing efforts on the identification of protein and, to a lesser extent, RNA interactors. The study presented here experimentally assesses direct interactions between the viral RNA and the critical regulators of gene expression that are miRNAs. We identified six main binding regions principally interacting with one type of miRNA. Unfortunately, CLEAR-CLIP does not allow us to distinguish between miRNA binding to regions of identical sequence between the different SARS-CoV-2 transcripts. Thus, we could not deduce whether any discrimination between binding to ORF and UTR sequences take place for interactions found downstream of ORF1a at ~13.5 kb and upstream of the ORF10 stop codon. It would be interesting for future studies to resolve this through methodological development. Nevertheless, a more abundant miRNA-binding landscape was observed toward the 3’ end, which was consistent with higher RNA abundance in this region and miRNA regulation in general mostly occurring on 3’ UTRs. No or minimal binding was observed on the 5’ terminal leader sequence, the slippery sequence in ORF1a/b, or the nine TRSs, suggesting no particular role for miRNAs in regulating SARS-CoV-2 RNA processing.

Investigation of whether miRNA interactions at regions #1–6 were necessary for viral replication and particle production did not reveal any major difference between WT and engineered mutants. This suggests that the observed interaction of SARS-CoV-2 RNA with the RISC is neither critical for the replication cycle of the virus nor does it lead to any significant miRNA-mediated repression of viral RNA, at least in cell culture. This was further substantiated by complementary AGO depletion experiments. Nevertheless, we cannot exclude that the binding of the RISC and miRNAs to SARS-CoV-2 RNA may contribute to other aspects of the viral replication cycle during natural infection in patients. SARS-CoV-2 transcripts are believed to be 5’ m^G capped and carry a poly-A tail, which would make them prone to canonical miRNA regulation. This is unlike HCV and pestiviruses, which require direct miRNA binding (miR-122-5p and miR-17-5p family, respectively) for replication, but do not carry m^G caps and poly-A tails, and therefore can escape canonical miRNA-mediated repression. In the pursuit of full understanding of the SARS-CoV-2 genetic landscape, recent studies have reported the existence of potential SARS-CoV-2-derived miRNAs. Using CLEAR-CLIP, the gold standard for identification of functional AGO-interacting miRNAs, no reads supporting loading of such miRNAs onto AGO could be detected in our experiments, which would not suggest any functional role of significance, at least in the conditions analyzed here.

Finally, we investigated the impact of infection on cellular miRNA regulation. Compared with data obtained with other methodologies such as smRNA-seq, CLEAR-CLIP data present the unique advantage to focus only on functionally active miRNAs loaded onto AGO. We observed minimal changes to miRNA abundance levels during infection. Changes were mostly upregulation (11 out of 16 miRNAs in VeroE6 cells; three out of five in A549hACE2 cells). Furthermore, all miRNAs for which a significant change in expression was observed could interact with SARS-CoV-2 RNA, among which 13 out of 16 (for VeroE6) and four out of five (for A549hACE2) were among the top-50 binders. Interestingly, miR-29-3p, the only miRNA for which more than 30% of the molecules bound SARS-CoV-2 RNA regardless of the cell line, was also the only miRNA to be upregulated in both cell lines. We then investigated whether the change in expression of these miRNAs was associated with a change of binding to cellular targets. For VeroE6 cells, two downregulated miRNAs (miR-25-3p and miR-193a-3p) also exhibited less binding to their correspondence.
cellular targets, while only a small proportion of upregulated miRNAs (four out of 11: let-7a-5p, miR-30a-5p, miR-155-5p, and miR-181a-5p) exhibited more binding to their cellular RNA targets. In A549hACE2 cells, downregulated let-7a-5p also exhibited less binding to its cellular targets, while none of the three upregulated miRNAs exhibited more binding to cellular targets. Altogether, these observations would suggest that the interaction of certain miRNAs with SARS-CoV-2 RNA is somewhat compensated by an increase in abundance in the infected host cell and that this response mechanism is conserved between cell lines. For miR-15a-5p and miR-17-5p, however, we did observe specific sequestration by SARS-CoV-2 RNA, which was not compensated by increased expression during infection. Viral sequestration of these miRNAs could be mediated by up to about 10^4 viral RNAs per cell (this study and Lee et al.39). While based on prediction using transcriptome-profiling datasets, sponging of miRNAs by SARS-CoV-2 in COVID-19 patients has previously been suggested.39 Altogether, these observations are in line with our transcriptome expression analysis, which showed global upregulation of miR-15a-5p mRNA targets. Hence, we find that SARS-CoV-2 binds a wide range of cellular miRNAs, most of the interactions are compensated by increased cellular abundance during infection, while functional sequestration is only affecting a few miRNAs. Compared, e.g., with miR-122 sponging by HCV,34 this effect, although significant, is at smaller scale. The SARS-CoV-2 miRNA interactome therefore has parallels but also marked differences to viruses such as HCV and BVDV, which efficiently bind and sequester one specific miRNA and do so more efficiently.21-23

Finally, as the only miRNA deregulated in both cell lines analyzed in our study, miR-15a-5p constituted an miRNA of particular interest. Deregression of this miRNA has also been observed in COVID-19 patients.40 It is therefore plausible that perturbation of miR-15a-5p is a universal feature associated with SARS-CoV-2 infection. This is particularly relevant given the recently reported role of the miR-15 family in restricting T-cell cycle, survival, and memory T-cell differentiation36 and the ability of SARS-CoV-2 to infect CD4+ T cells.37 Altogether, our work and these studies suggest a possible connection between deregression of the miR-15 family and SARS-CoV-2 pathogenesis.

In conclusion, we have demonstrated that SARS-CoV-2 can interact with the RISC and deciphered its comprehensive miRNA interactome while showing that miRNA interactions are not critical for the virus. We have furthermore explored how the miRNA interactome of the cell is affected by SARS-CoV-2 infection and demonstrated that it affects the cellular activity of certain specific miRNAs, including miR-15a-5p, through possible sequestration and the deregulation of its mRNA targets. In the future, it will be important to determine whether the same variations are observed in patients and contribute to COVID-19 etiology. Finally, the work presented here contributes another important piece to the complete characterization of the SARS-CoV-2 molecular interactome as well as a useful resource for the virology and RNA biology communities. It also provides avenues to explore the molecular requirement of SARS-CoV-2 in infected patients and advance the fight against COVID-19.

**Limitations to this study**

While our results show that the miRNA-binding pattern to SARS-CoV-2 RNA is mainly driven by miRNA abundance and conserved between VeroE6 and A549hACE2 cells, suggesting that our observations could be extrapolated to other cell types, we cannot draw conclusions on the role of miRNAs not expressed in these two cell lines. Furthermore, cross-linking between RNA and proteins induced by UV254, as well as proximity ligation between miRNA and target, as part of the CLEAR-CLIP methodology are biased for certain nucleotides.26,28,41 While this would not affect relative differences between infected and non-infected cells and conclusions drawn in general, it could bias the peak size between different miRNAs. The analysis of viral perturbation of cellular miRNA regulation suggested functional sequestration of miR-15a-5p. It should be noted, though, that, compared with other cases of viral miRNA sponging, the effect on post-transcriptional gene regulation is small and accordingly may not have important biologic implications. Finally, while we have shown that the RISC and miRNAs are not critical for SARS-CoV-2 infection, we cannot exclude minor effects on the viral life cycle or roles in contexts not analyzed here.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead contact
  - Materials availability
  - Data and code availability
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Cell lines
  - Viruses
Figure 7. Gene Ontology analysis of miR-15a-5p mRNA targets upregulated during infection
Top-25 terms obtained after Gene Ontology (GO) analysis of the biological processes for the miR-15a-5p targets upregulated during infection. Terms are sorted and colored by p value, while dot size represents the gene ratio (%) for each GO term. Terms generally associated with infection are highlighted in red. For each panel and condition, n = 3 independent biological replicates.

### METHOD DETAILS
- CLEAR-CLIP
- Engineering of SARS-CoV-2 mutants
- Kinetic of infection for SARS-CoV-2 wild-type and mutants
- AGO knockdown and Western blot
- Median tissue culture infectious dose (TCID50)
- RNA extraction and RT-qPCR
- RNA-seq

### QUANTIFICATION AND STATISTICAL ANALYSIS
- Peak calling
- Differential abundance of miRNAs
- RNAseq: Differential gene expression and gene set enrichment analyses
- Measure of entropy
- qPCR analysis
SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2023.112282.

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AUTHOR CONTRIBUTIONS

DECLARATION OF INTERESTS
The authors declare no competing interests.

INCLUSION AND DIVERSITY
We support inclusive, diverse, and equitable conduct of research.

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Revised: January 24, 2023
Accepted: March 2, 2023

REFERENCES


### REAGENT or RESOURCE | SOURCE | IDENTIFIER
--- | --- | ---
**Antibodies**
Anti-mouse IgG | Jackson ImmunoResearch | Cat# 315-005-008; RRID: AB_2340035
Anti-Pan-AGO | Millipore | Cat# MABE56; RRID: AB_10807962
Anti-AGO1 | Abcam | Cat# ab300152; RRID: AB_2935781
Anti-AGO2 | Abcam | Cat# ab23831; RRID: AB_867543
Anti-AGO3 | Abcam | Cat# ab154844; RRID: AB_2935783
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Anti-rabbit IgG Alexa Fluor 488 | Thermo Fisher Scientific | Cat# A32731; RRID: AB_2633280
Anti-Spike SARS-CoV/SARS-CoV-2 | Sino Biological | Cat# 40150-D004; RRID: AB_2827983
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SARS-CoV-2 (DK-AHH1) mutant 6L | This paper | N/A
**Chemicals, peptides, and recombinant proteins**
RNasin Plus | Promega | Cat# N2615
Protease inhibitors | Roche | Cat# 693159001
RQ1 DNase | Promega | Cat# M6101
RNase I | Thermo Scientific | Cat# EN0601
Protein A Dynabeads | Invitrogen | Cat# 10002D
T4 polynucleotide kinase 3’ phosphatase minus | New England Biolabs | Cat# M0236L
T4 RNA ligase | Thermo Scientific | Cat# EL0021
Alkaline phosphatase | Roche | Cat# 10713023001
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Nitrocellulose membrane | Whatman | Cat# WHA10402525
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NaAc 3M pH 5.5 | Invitrogen | Cat# AM9740
GlycoBlue | Invitrogen | Cat# AM9516
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AccuPrime Pfx SuperMix | Invitrogen | Cat# 12344040
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Critical commercial assays

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Experimental models: Cell lines

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**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for reagents and resources should be directed to and will be fulfilled by the Lead Contact, Troels Scheel (tscheel@sund.ku.dk).

**Materials availability**
The BAC constructs generated for this study will be made available on request after completion of a Materials Transfer Agreement.

**Data and code availability**
All HTS data generated for this study have been deposited at GEO and are publicly available as of the date of publication. The GEO accession number is listed in the key resources table. This paper does not report original code. Any additional information required to reanalyse the data reported in this paper is available from the lead contact upon request.

Additional Supplemental Items are available from Mendeley Data: https://doi.org/10.17632/3n74zj2vzm.1.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Cell lines**
African green monkey VeroE6 kidney female cells were cultivated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with fetal bovine serum (FBS, 10%), penicillin (100 U/mL) and streptomycin (100 µg/mL) and sub-cultured 1:5 twice a week. Human A549hACE2 lung male cells were cultivated in Ham’s F-12K (Kaighn’s) medium supplemented with FBS (10%), penicillin (100 U/mL), streptomycin (100 µg/mL) and puromycin (0.5 µg/mL) and sub-cultured 1:3 twice a week. Both cell lines were kept at 37°C, within a humidified incubator and an atmosphere containing 5% CO₂.

**Viruses**
A SARS-CoV-2 virus strain (DK-AHH1) previously isolated and for which the sequence was cloned into a bacterial artificial chromosome (BAC) was used for this study. Mutant viruses were generated as described in the "Method details" section. Infections were initiated using media from VeroE6 cells infected with viral particles or transfected with RNA produced using the BACs. Work with infectious viruses and the laboratory in which it was performed was approved by the Danish Work Authorities.

**METHOD DETAILS**

**CLEAR-CLIP**
The procedure follows a protocol described in and adapted to the present study including minor changes. The successive steps are described below in chronological order.

**Cells seeding and infection**
1.5-2 million VeroE6 cells or 2.5 million A549hACE2 cells were seeded in one 10 cm dish. The day after, the cells were infected with wild-type or mutant SARS-CoV-2 viruses at MOI = 0.001. One single seeded 10 cm dish was used for each independent sample. Non-infected controls were prepared in parallel using similar number of cells, timeline and conditions but without the addition of viral particles.

**UV cross-linking**
48 h after infection, the cells were washed twice with 5 mL ice-cold PBS 1X and irradiated, still adherent to the dish in 1 mL ice-cold PBS 1X and while on ice, with UV254 once at 400 mJ/cm², then a second time at 200 mJ/cm². The cells were then scraped off the surface of the dish, collected and centrifuged at 500 g for 5 min at 4°C. The cell pellet was snap-frozen in liquid nitrogen. Non-cross link controls were prepared in parallel following similar steps but without UV irradiation.
Cells lysis and RNA fragmentation
The cell pellet was lysed with 1 mL ice-cold PXL 1X buffer (PBS 1X, SDS 0.1%, sodium deoxycholate 0.5%, Igepal CA-630 0.5%) complemented with RNasin Plus (Promega N2615; 400 U) and protease inhibitors (Roche 4693159001; 1 tablet per 10 mL) and incubated 10 min at 4°C. 30 μL QK1 DNase (Promega M6101) was then added, and the sample incubated for 5 min at 37°C with constant shaking at 1200 rpm. Finally, 10 μL of RNase I (Thermo Scientific EN0801) diluted 1:1000 in PXL 1X buffer was added, and the sample incubated for 5 min at 37°C. The sample was then centrifuged at 14000 g for 15 min at 4°C and the supernatant collected to proceed with the next step of the protocol using magnetic beads.

Beads preparation
The magnetic beads were prepared as follows: For each sample, 50 μL protein A Dynabeads (Invitrogen 10002D) were washed three times with ice-cold PBST buffer (PBS 1X, Tween-20 0.02%). After washing, the beads were resuspended in 50 μL PBST and incubated with 7.15 μL anti-mouse IgG (Jackson ImmunoResearch 315-005-008) for 45 min at room temperature while rotating. The beads were then washed three additional times with ice-cold PBST, resuspended in 100 μL PBST and incubated with 3 μL pan-AGO antibody (Millipore MAB56) overnight at 4°C while rotating. The following day, the beads were washed three times with ice-cold PXL 1X buffer and resuspended in 50 μL ice-cold PXL 1X. Non-AGO IgG control beads were prepared in parallel using similar quantities, conditions and steps but without the addition of the pan-AGO antibody. Beads used for pre-clearing were also prepared in parallel using similar conditions and steps but without the addition of any IgG.

Pre-clearing and immunoprecipitation
25 μL of the pre-clearing beads were added to the ~1 mL cell lysate supernatant collected before, and the mix was incubated 1 h at 4°C while rotating. The cell lysate was then separated from the pre-clearing beads on a magnet and incubated with 50 μL pan-AGO antibody or IgG control beads for 4 h at 4°C while rotating. Beads were finally washed three times with 500 μL ice-cold PXL 1X buffer, three times with 500 μL ice-cold PXL 5X buffer (PBST 5X, SDS 0.1%, sodium deoxycholate 0.5%, Igepal CA-630 0.5%), and three times with 500 μL ice-cold PNK buffer (Tris-HCl pH 7.5 50 mM, MgCl2 10 mM, Igepal CA-630 0.5%).

miRNA-RNA proximity ligation
The beads were resuspended with 80 μL of the phosphorylation-3'-minus mix (phosphorylation-3'-minus buffer 1X, ATP 1 mM, T4 polynucleotide kinase 3' phosphatase minus 40 U [New England Biolabs M0236L], RNasin Plus 80 U) and incubated for 20 min at 37°C with intermittent shaking for 15 s every 2 min at 1100 rpm, followed by one wash with 500 μL ice-cold PXL 1X buffer, one wash with 500 μL ice-cold PXL 5X buffer, and three washes with 500 μL ice-cold PNK buffer. After that, the beads were resuspended with 80 μL of the proximity ligation mix (T4 RNA ligase buffer 1X, BSA 0.1 μg/μL, T4 RNA ligase 40 U [Thermo Scientific E0021], RNasin Plus 80 U) and incubated 16 h at 16°C with intermittent shaking for 15 s every 2 min at 1100 rpm. Then, 2.5 μL of T4 RNA ligase and 9 μL of ATP 10 mM were added and incubation continued for 4–6 h, before beads were washed once with 500 μL ice-cold PXL 1X buffer, once with 500 μL ice-cold PXL 5X buffer, and three times with 500 μL ice-cold PNK buffer.

3' end linker ligation
The beads were resuspended with 80 μL of the dephosphorylation mix (dephosphorylation buffer 1X, alkaline phosphatase 3U [Roche 10,713,023,001], RNasin Plus 80 U) and incubated for 20 min at 37°C with intermittent shaking for 15 s every 2 min at 1100 rpm, followed by one wash with 500 μL ice-cold PNK buffer, one wash with 500 μL ice-cold PNK + EDTA buffer (Tris-HCl pH 7.5 50 mM, EDTA 20 mM, Igepal CA-630 0.5%), and three washes with 500 μL ice-cold PNK buffer. The beads were then resuspended with 80 μL of the 3' end linker ligation mix (T4 RNA ligase buffer 1X, BSA 0.1 μg/μL, T4 RNA ligase 40 U, RNasin Plus 80 U), 32P-end-labelled RL3 linker [Table S9] 0.15 μM (prepared extemporaneously using a standard 5'-end phosphorylation protocol and γ32P ATP) and incubated for 1 h at 16°C with intermittent shaking for 15 s every 2 min at 1100 rpm. After 1 h, 4 μL of non-radioactive P-5' RL3 linker 20 μM was added and the incubation was carried out overnight. The day after, the beads were washed once with 500 μL ice-cold PXL 1X buffer, once with 500 μL ice-cold PXL 5X buffer, and thrice with 500 μL ice-cold PNK buffer.

Isolation of AGO-miRNA/RNA complexes
The beads were resuspended with 80 μL of the phosphorylation mix (phosphorylation buffer 1X, ATP 1 mM, T4 polynucleotide kinase 40 U [New England Biolabs M0201L], RNasin Plus 80 U) and incubated for 20 min at 37°C with intermittent shaking for 15 s every 2 min at 1100 rpm, followed by one wash with 500 μL ice-cold PNK buffer, one wash with 500 μL ice-cold PNK + EDTA buffer (Tris-HCl pH 7.5 100 mM, NaCl 50 mM, EDTA 10 mM) and incubated for 20 min at 37°C with permanent shaking at 1000 rpm. The loading mix was then separated from the beads, loaded onto a Nupage 10% bis-tris polyacrylamide gel (Invitrogen NP0301BOX) and run for 3–4 h at 150–180 V according to standard protocol. Transfer was also performed according to standard protocol on nitrocellulose membrane (Whatman WHA10402525) for 1.5 h at 30 V. The radioactive signal of the membrane was assessed using the phosphoimager technology (see Figures S1A and S2A). The area containing AGO-miRNA/RNA complexes was cut out, further sectioned in smaller pieces, and incubated in 200 μL of the proteinase K mix (proteinase K [Roche 03115879001] 4 mg/mL, Tris-HCl pH 7.5 100 mM, NaCl 50 mM, EDTA 10 mM) for 20 min at 37°C with permanent shaking at 1000 rpm. Then, 200 μL of the urea mix (urea 7M, Tris-HCl pH 7.5 100 mM, NaCl 50 mM, EDTA 10 mM) was added and incubation prolonged for 20 min. Finally, 530 μL of acid-phenol:chloroform pH4.5 (Invitrogen AM9720) was added and the mix incubated for an extra 20 min at 37°C with permanent shaking at 1500 rpm, before centrifugation at 14000 g for 5 min at room temperature. The aqueous phase was then collected, mixed with 50 μL NaAc 3M pH 5.5 (Invitrogen AM9740), 0.7 μL GlycoBlue (Invitrogen AM9516), 500 μL isopropanol, 500 μL ethanol, and centrifuged overnight at −20°C.
5’ end linker ligation

The following day, the sample was centrifuged at 14,000 g for 10 min at 4°C and the supernatant discarded. The pellet was washed twice with 150 μL ice-cold 70% ethanol and centrifugation at 14,000 g for 5 min at 4°C, before air drying and resuspension in 5.9 μL water. 4.1 μL of the 5’ end linker ligation mix (1 μL T4 RNA ligase buffer 10X, 1 μL BSA 1 μg/μL, 0.1 μL T4 RNA ligase 10 U/40 U, 1 μL RNasin Plus 40 U/μL, RLSDS linker [Table S9] 0.1 μL) was then added. The mix was incubated for 4 h at 16°C. 100 μL of the RQ1 DNase mix (RQ1 DNase 1X, RQ1 DNase 5U, RNasin Plus 100 U) was then added and the mix incubated for 30 min at 4°C. After that, 300 μL of acid-phenol:chloroform pH4.5 was added and mixed with the sample before centrifugation at 14,000 g for 5 min at room temperature. Finally, the aqueous phase was collected, mixed with 50 μL NaAc 3M pH 5.5, 0.7 μL GlycoBlue, 500 μL isopropanol, 500 μL ethanol, and incubated overnight at –20°C. 

Reverse transcription (RT), PCR amplification and high throughput sequencing

The day after, the sample was precipitated and washed twice with 70% ethanol as described in the previous section, and the pellet resuspended in 8 μL water to which was precipitated and washed twice with 70% ethanol as described in the previous section, and the pellet resuspended in 8 μL water to which was added 1 μL of dNTPs (10 mM) and 2 μL of DP3 primer (5 μM). The mix was incubated for 5 min at 65°C before the addition of 9 μL of the RT mix (4 μL RT buffer 5X, 1 μL DTT 0.1M, 1 μL RNasin Plus 40 U/μL, 1 μL SuperScript III 200 UI/μL [Invitrogen 18080044], 2 μL water) and additional incubation for 45 min at 50°C, 15 min at 55°C and 5 min at 90°C. After RT, 220 μL of the first-step PCR amplification mix (1.7 μL DP3 primer [Table S9]) 100 μM, 1.7 μL DP5 primer [Table S9] 100 μM, 216.4 μL AccuPrime Pfx SuperMix [Invitrogen 12344040]) was added and 60 μL of the mix run by PCR as follow: 2 min at 95°C, 22×20 s at 95°C, 30 s at 58°C, 20 s at 68°C), 5 min at 68°C. Next, 15 μL of the PCR was visualised on 10% TBE polyacrylamide gel according to standard protocol and the region containing a smear of amplicons between 80 and 300 bp was cut out. The amplified DNA was then isolated from the gel according to standard protocol and eluted in 9 μL water to which was added 81 μL of the second-step PCR amplification mix (3 μL AS2R5 primer [Table S9] 10 μM, 3 μL MSFP3 primer [Table S9] 10 μM, 75 μL AccuPrime Pfx SuperMix). 30 μL of the mix was run by PCR as follow: 2 min at 95°C, 8 or 12 or 16×20 s at 95°C, 30 s at 58°C, 40 s at 68°C), 5 min at 68°C. After that, the PCR was visualised on 2% TAE agarose gel according to standard protocol and the region containing an amplicon present between 150 and 400 bp was cut out. The amplified DNA was then isolated from the gel according to standard protocol and eluted in 30 μL water. After quantification and verification of the quality of the library using a 2100 bioanalyzer instrument (Agilent), it was finally processed for high throughput sequencing on a MiSeq sequencer (Single end, MiSeq v3 kit reagent 150 cycles, protocol and eluted in 30 μL water. After quantification and verification of the quality of the library using a 2100 bioanalyzer instrument (Agilent), it was finally processed for high throughput sequencing on a MiSeq sequencer (Single end, MiSeq v3 kit reagent 150 cycles, Illumina MS-102-3001).

Processing and alignment of singleton and chimeric reads

Sequencing reads were primarily processed, aligned and visualized in R, using CLIPflexR tools and wrappers in combination with a number of tools of the Bioconductor suite as well as tools for biological string manipulation. See also and. In brief, to first identify singleton reads, fastq sequences were quality-filtered and read-trimmed using Trimmomatic with SLIDINGWINDOW:4:20. Prior to alignment, identical sequences were collapsed and 3’ and 5’ linkers were trimmed. Remaining reads where then aligned to the SARS-CoV-2 (isolate DK-AHH1; wild-type or with engineered mutations), the green monkey (chlSab2) or the human genome (hg38), by CLIPflexRts bowtie_align using default parameters. Finally, Unique Molecular Identifier (UMI) sequences originally contained in the 5’ RNA linker served as basis for UMI deduplication. To identify chimeric reads, we reverse mapped miRbase reference sequences of mature miRNAs to any unmapped reads, using bowtie_align with custom parameters (seedSubString = 18, maxMismatches = 0). Target sequences of potential chimera candidates were then extracted in R and mapped to SARS-CoV-2 as well as to the respective host-cell genomes. Chimera-derived miRNAs were grouped into miRNA families, based on their respective 8mer seed composition, naming the first numeric miRNA occurrence as type species, before deduplication using exact genome coordinates in combination with UMIs. Thus, the miR-15a-5p family is a compilation of miR-15a-5p, miR-15b-5p, miR-16-5p, miR-195-5p, miR-424-5p and miR-497, based off their shared seed composition. Singleton and chimera derived bedgraphs were generated using the CLIPflexR wrapper ctk_tag2profile (normalization = “rpkm”), and resulting coverage profiles were visualized in R.

Engineering of SARS-CoV-2 mutants

SARS-CoV-2 mutants were engineered using the SARS-CoV-2-DK-AHH1 reverse genetic system. This consists of the sequence of a COVID-19 associated SARS-CoV-2 virus isolate (DK-AHH1) cloned into a bacterial artificial chromosome (BAC). The mutations as described Figure 4A were introduced by targeted mutagenesis into the SARS-CoV-2 BAC using a custom-made mega-primer approach and primer sets TSO1314/TSO1331 (mutant 1L), TSO1316/TSO1332 (2L), TSO1318/TSO1333 (3L), TSO1318/TSO1427 (3G), TSO1320/TSO1334 (4L), TSO1374/TSO1335 (5L), TSO1374/TSO1428 (5G) or TSO1324/1336 (6L) (Table S9). After targeted mutagenesis and before in vitro transcription (IVT), the mutated BAC clones were fully sequenced by high throughput sequencing to confirm the presence of the desired mutations and to check the absence of any other unwanted mutations. After plasmid linearization using the NotI restriction enzyme, SARS-CoV-2 full genome length RNAs were produced by IVT from each engineered BAC and purified using the protocols described. Finally, 500,000 VeroE6 cells were transfected with 0.5-1 μg of each RNA using a standard protocol and lipofectamine 2000 transfection reagent (Invitrogen 11668027). 72 h after transfection, the medium was collected, and the presence of viral particles assessed and measured by TCID50. SARS-CoV-2 RNA was extracted and analyzed to check that the engineered mutations were still present in the viral genome. The supernatant was then used to initiate infections with controlled MOI.
Kinetic of infection for SARS-CoV-2 wild-type and mutants

50,000 VeroE6 or A549hACE2 cells were seeded in each well of a 24-well plate. The day after, the medium was replaced with 1 mL fresh medium containing the number of viral particles necessary to initiate an infection at MOI = 0.001. 24 h, 48 h or 72 h post-infection, the medium was collected, and the cells were washed twice with 0.5 mL ice-cold PBS before to be dissolved and collected in 0.5 mL of ice-cold Trizol reagent (Invitrogen 15506026). Media and Trizol samples were frozen and stored at –80°C. Wild-type and all mutants were done in parallel in three independent batches. The number of infectious particles in the media was quantified by TCID_{50}. The number of intracellular viral genomes in cells was quantified after RNA extraction and RT-qPCR from the Trizol samples.

AGO knockdown and Western blot

50,000 VeroE6 or A549hACE2 cells were seeded in one well of a 12-well plate. The day after, the cells were transfected for 6 h with scrambled siRNA (Horizon D-001210-04-05), AGO1 siRNA #1, AGO1 siRNA #2, AGO2 siRNA #1, AGO2 siRNA #2, AGO3 siRNA #1 or AGO3 siRNA #2 (Horizon) (20 nM final) (Table S9) using a standard protocol and lipofectamine RNAiMAX transfection reagent (Invitrogen 13778075). After two days, the cells were subjected to a second round of similar transfection with siRNA and infected with SARS-CoV-2 at MOI = 0.001. 24 h, 48 h or 72 h post-infection, the medium was collected, and the number of infectious particles quantified by TCID_{50}. The different conditions were done in parallel and in triplicates. Replicates were used to collect media and for TCID_{50} measurement.

To check the efficiency of the knockdown, the same exact protocol as described above was followed, excluding the infection step, and the cells were collected instead of the media. The cells were washed twice with 0.5 mL ice-cold PBS 1X before to be centrifuged at 500 g for 5 min at 4°C. The cell pellet was then snap-frozen in liquid nitrogen and used for protein extraction and fluorescent Western blot.

To check the expression of AGO1, 2 and 3 during SARS-CoV-2 infection, the same condition of infection as the CLEAR-CLIP experiment was used and the cells were collected 24 h, 48 h or 72 h post-infection. The cells were washed twice with 5 mL ice-cold PBS 1X before to be centrifuged at 500 g for 5 min at 4°C. The cell pellet was then snap-frozen in liquid nitrogen and used for protein extraction and fluorescent Western blot.

Protein extraction was done in RIPA buffer and concentration was measured using BCA assay according to standard protocols. For Western blot, 5 or 10 μg of proteins were mixed in 13 μL water. 5 μL Nupage loading buffer (Invitrogen NP0007) and 2 μL Nupage reducing agent (Invitrogen NP0004) were then added and the mix incubated at 70°C for 10 min. The sample was then loaded onto a Nupage 4–12% bis-tris polyacrylamide gel (Invitrogen NP0322BOX) or a Nupage 3–8% Tris-Acetate gel (Invitrogen EA0375BOX; for AGO3 detection in A549hACE2 cells) and run for 45 min-1 h at 150–200 V according to manufacturer instructions. Transfer was performed according to standard protocol on PVDF membrane (BioRad 162-0260) for 1.5 h at 30 V. Membrane was then incubated in blocking solution (Rockland MB-070) overnight at 4°C under constant shaking. The day after, the membrane was incubated with the primary antibody diluted in the blocking solution for 4 h, washed three times 5 min in PBST buffer (PBS 1X, Tween 20 0.1%), incubated another hour with the secondary antibody diluted in PBST, and washed three times 5 min in PBST buffer, all at room temperature and under constant shaking. The membrane was then dried using 100% methanol and its fluorescence visualised and recorded using a BioRad Chemidoc MP Imaging System. Primary antibodies used were anti-AGO1 (Abcam ab300152; 1:1000 dilution), -AGO2 (Abcam ab32381; 1:1000), -AGO3 (Abcam ab154844; 1:1000) and -GAPDH (Abcam ab111602; 1:10,000). All primary antibodies could be saved and reused for successive staining while diluted in blocking solution. This was particularly important for the anti-AGO3 antibody which only gave acceptable signal-to-noise ratio after pre-clearing by performing at least one round of incubation with proteins on membrane as described just above. Secondary antibody was anti-rabbit-Alexa Fluor 488 (Invitrogen A32731; 1:5000 dilution). Samples to be compared were loaded in equal amounts and run on the same gel.

Median tissue culture infectious dose (TCID_{50})

TCID_{50} was performed by immunostaining of four or six replicates of VeroE6 cells after serial dilution of the supernatant(s) to quantify and using anti-Spike SARS-CoV-2 (SinoBiological 40-150-D004) and -human-HRP (Invitrogen A24476) antibodies as described in.27 In details, 8,000 VeroE6 cells were seeded in each well of 96-well plate (the number of wells and 96-plates to be seeded depend on the number of samples and dilutions to quantify). The day after, the supernatant to quantify was serially diluted and 100 μL of each dilution used to infect the cells of one well, in quadruplicate or sixplicate. 72 h after infection, the media of each well were decanted and the cells fixed for 20 min in ice-cold ethanol 100% before to be washed twice with PBST buffer (PBS 1X, Tween 20 0.1%). Fixed cells were then covered and incubated with 100 μL per well of H2O2 3% for 10 min at room temperature, washed twice with PBST and incubated for at least 16 h at 4°C with 40 μL per well of anti-Spike SARS-CoV-2 antibody (SinoBiological 40-150-D004) diluted 1:5000 in PBSK buffer (BSA 1%, Skimmed milk 0.2%, PBS 1X). The antibody solution was then removed and the cells washed twice with PBST and incubated for at least 1 h at room temperature with 40 μL per well of anti-human-HRP antibody (Invitrogen A24476) diluted 1:2000 in PBSK buffer. The antibody solution was then decanted and the cells washed three times with PBST and incubated for 20–30 min at room temperature with 50 μL per well of 3,3′ diaminobenzidine tetrahydrochloride (DAB) substrate (ImmunoLogic BS04-110). Cells were then washed three times with distilled water and dried at room temperature. Each well was then imaged and recorded using an imaging system (Cellular Technology Limited). Finally, the presence (positive for DAB-staining) or absence (negative for DAB-staining) of infected cells for each well was scored and used to determine the TCID_{50} per mL based on the Reed and Muench method.
RNA extraction and RT-qPCR

For RNA extraction, 60 μL of chloroform was mixed with 300 μL of the cell containing Trizol sample and centrifuged at 15,000 g for 15 min at 4°C. The aqueous phase was then collected and processed with an RNA Clean & Concentrator-25 kit (Zymo Research R1018) according to manufacturer instruction and adding an on-column DNase I step to extract and elute total RNA in 50 μL water. Multiplex RT-qPCR simultaneously quantifying SARS-CoV-2 RNA (E gene)\(^{55,56}\) and RPS11 mRNA was performed in duplicate from 30 ng of total RNA using the TaqMan Fast Virus 1-Step Master Mix kit (Applied Biosystems 4444432). Cycling conditions were as following: Reverse transcription for 10 min at 55°C, denaturation for 3 min at 95°C, amplification for 45 cycles of (15 s at 95°C, 30 s at 58°C with detection), final cooling for 60 s at 40°C. The sequence of the primers can be found Table S9.

RNA-seq

RNA-Seq was performed from three non-infected and three infected replicates of VeroE6 and A549hACE2 cells. Infection with wild-type SARS-CoV-2 was described in the section “Kinetic of infection for SARS-CoV-2 wild-type and mutants” and RNA samples prepared as described in the section “RNA extraction and RT-qPCR”. Libraries were prepared from 200 ng total RNA (RNAi >9.5) using the TruSeq Stranded Total RNA Library Prep Human/Mouse/Rat (48 samples; Illumina 20020596) and the TruSeq RNA CD index plate (96 indexes, 96 samples; Illumina 20019792) following manufacturer instruction. After quantification and verification of the quality of the library using a 2100 bioanalyzer instrument (Agilent), it was finally processed for high throughput sequencing on a NextSeq sequencer (paired end, 2 x 75 cycles, NextSeq 500/550 high output kit v2.5 150 cycles, Illumina 20024907).

For generation of RNA-Seq coverage profiles, fastq files were quality-checked and aligned to the SARS-CoV-2 (DK-AHH1) genome, using STAR.\(^{43}\) RPKM normalized bedgraphs were computed from resulting BAM files using bamCoverage from deeptools\(^{44}\) and average coverage profiles of infected biological replicates of each of the two cell lines were visualised in R.

Quantification and Statistical Analysis

Quantification and statistical analyses were performed using the CLIPflexR package, EdgeR or GraphPad Prism 8. Analyses performed, methods of quantification and normalization, the nature and the number of controls and replicates are indicated in the “method details” section, the main text and/or the figure legends.

Peak calling

Significant AGO-binding events, as represented by peaks, were called on CLEAR CLIP singleton reads aligned to host RNA, from all replicates across conditions, using CLIPflexR homerpeaks with an approximate fragment length of 25 nts, peak size of 53 nts, minimum distance between peaks of 75 nts, separate strands, and a criteria of 4-fold enrichment over local background. Significantly enriched peaks were processed as a genomicRanges\(^{47}\) object and annotated by annotatePeak from ChIPseeker.\(^{46}\) Singleton reads per enriched peak were quantified by CLIPflexR countFromBed, and log2 fold changes were computed from TMM-normalized library sizes using edgeR.\(^{45}\)

For construction of cumulative distribution function (cdf) plots, miRNA seed binding sites were predicted from 7mer or 8mer miR-15a-5p seed matches for enriched peak sequences ±32 nucleotides. The analysis was restricted to annotated peaks containing a predicted seed site in the 3′UTR and downstream region. Further, peaks with a biological complexity of <3 across conditions, or which were annotated as miRNAs were excluded from the analysis. Cdf curves of the miR-15a-5p family was compared against all other miRNA family targets by two-sided Kolmogorov-Smirnov (K-S) test and resulting p values were reported.

Statistical peak calling was conducted on viral CLEAR-CLIP singeltons reads with similar settings as above. Furthermore, to control for background signal, each infected replicate was corrected using each of the three uninfected controls separately. Putative peaks were filtered by a requirement of at least a 4-fold enrichment over local background and mock control respectively. Furthermore, a majority criterion (>50%) was applied to select the final peaks.

Differential abundance of miRNAs

Changes in miRNA-binding to host cell miRNAs, was investigated by analysing differential abundance of either all- or only cell-bound miRNA counts inferred from chimeric reads for infected vs. non-infected cells, using edgeR. Resulting p values were adjusted for multiple testing, by the Benjamin-Hochberg procedure.

RNAseq: Differential gene expression and gene set enrichment analyses

To assess the expression of miR-15a-5p family targets, fastq files from RNAseq experiments in VeroE6 and A549hACE6 cells were quality-checked and aligned to the green monkey (chlSab2) or the human (hg38) genomes using STAR.\(^{43}\) Genomic features were assigned and summarized by featureCounts\(^{49}\) and differential expression of resulting RNAseq counts were analyzed separately for each cell-line and time point, using limma-voom in R. Results were visualized in volcano plots generated using EnhancedVolcano (v1.4) (Figure S7).

Genes predicted as miRNA gene targets, as identified from CLEAR-CLIP singleton peak analysis and seed position in the 3′UTR as described earlier, were selected for further analysis. To assess putative gene set enrichment of miR-15a-5p family gene targets (7mer or 8mer seed matches), we constructed barcodeplots and set up rotation gene set testing, comparing log2 fold changes of...
miR-15a-5p family targets to a background gene set of all additional miRNA gene targets (non-miR-15a-5p family targets) using bar-codeplot and ROAST as implemented in limma. To further emphasize the gene target enrichment and illustrate the deviation in miRNA targeting specificity, we constructed cdf curves from log$_2$ fold changes of miR-15a-5p or miR-17-5p family targets divided into either 6mer, 7mer and 8mer matches, as compared to all other miRNA gene targets by one-sided Kolmogorov-Smirnov (K-S) test and resulting p values were reported.

Finally, differentially expressed miR-15a-5p family gene targets (7mer and 8mer seed matches) were depicted in heatmaps for each condition, using ComplexHeatmap (v2.2.0). Potential functional enrichment for upregulated miR-15a-5p family gene targets was investigated by gene ontology (GO) term analysis, using topGO (v2.38.1). The gene universe was defined as all genes measured in the RNAseq experiment. Results for the top 25 terms were visualized by ggplot2, ranking and coloring terms by p value while dot size represents the gene ratio (%) for each GO term.

**Measure of entropy**
Measure of entropy (Figure S4A) was analyzed at nextstrain.org. Diversity was plotted for the miRNA binding regions using per codon Shannon entropy data acquired from nextstrain.org/ncov/gisaid/global by downloading data and selecting “diversity”. A list with the windows corresponding to the miRNA binding regions was interpolated with the discrete base/entropy values gisaid table. The plots were constructed using R. The most recent data acquired from the GISAID database correspond to the earliest version publicly available on the ninth of December 2021. Authors listed in Table S10 contributed with the submission of sequences to the database that allowed for the construction of the Shannon entropy table.

**qPCR analysis**
qPCR normalization was performed using the Roche Light Cycler 480 software (v1.5.0) using an advanced relative quantification with a one-to-one target/reference distribution. Biological triplicates were measured in technical duplicates. RPS11 was used as reference for the relative quantification of the E gene RNA in a multiplexed reaction and normalized to an in-run calibrator.