

1 **Altered m<sup>6</sup>A modification of specific cellular transcripts affects *Flaviviridae* infection**

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3 Nandan S. Gokhale<sup>1\*</sup>, Alexa B.R. McIntyre<sup>3,4\*</sup>, Melissa D. Mattocks<sup>5</sup>, Christopher L. Holley<sup>1,2</sup>,  
4 Helen M. Lazear<sup>5</sup>, Christopher E. Mason<sup>3,6,7,8†</sup>, Stacy M. Horner<sup>1,2†</sup>

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6 <sup>1</sup> Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham,  
7 NC 27705, USA

8 <sup>2</sup> Department of Medicine, Duke University Medical Center, Durham, NC 27705, USA

9 <sup>3</sup> Department of Physiology and Biophysics and the Institute for Computational Biomedicine, Weill  
10 Cornell Medicine, New York, NY 10065, USA

11 <sup>4</sup> Tri-Institutional Program in Computational Biology and Medicine, New York, NY 10065, USA

12 <sup>5</sup> Department of Microbiology and Immunology, University of North Carolina – Chapel Hill, Chapel  
13 Hill, NC 27599, USA

14 <sup>6</sup> The HRH Prince Alwaleed Bin Talal Abdulaziz Al Saud Institute for Computational Biomedicine,  
15 Weill Cornell Medicine, New York, NY 10065, USA

16 <sup>7</sup> The WorldQuant Initiative for Quantitative Prediction, Weill Cornell Medicine, New York, NY,  
17 10065, USA

18 <sup>8</sup> The Feil Family Brain and Mind Research Institute, Weill Cornell Medicine, New York, NY,  
19 10065, USA

20

21 \* Equal Contribution

22

23 † Corresponding authors:

24 Stacy M. Horner ([stacy.horner@duke.edu](mailto:stacy.horner@duke.edu))

25 Christopher E. Mason ([chm2042@med.cornell.edu](mailto:chm2042@med.cornell.edu))

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## 35 **Summary**

36 The RNA modification *N*6-methyladenosine ( $m^6A$ ) can modulate mRNA fate and thus  
37 affect many biological processes. We analyzed  $m^6A$  modification across the transcriptome  
38 following infection by dengue virus (DENV), Zika virus (ZIKV), West Nile virus (WNV), and  
39 hepatitis C virus (HCV). We found that infection by these viruses in the *Flaviviridae* family alters  
40  $m^6A$  modification of specific cellular transcripts, including *RIOK3* and *CIRBP*. During viral  
41 infection, the addition of  $m^6A$  to *RIOK3* promotes its translation, while loss of  $m^6A$  in *CIRBP*  
42 promotes alternative splicing. Importantly, we found that activation of innate immune sensing or  
43 the endoplasmic reticulum (ER) stress response by viral infection contributes to the changes in  
44  $m^6A$  modification in *RIOK3* and *CIRBP*, respectively. Further, several transcripts with infection-  
45 altered  $m^6A$  profiles, including *RIOK3* and *CIRBP*, encode proteins that influence DENV, ZIKV,  
46 and HCV infection. Overall, this work reveals that cellular signaling pathways activated during  
47 viral infection lead to alterations in  $m^6A$  modification of host mRNAs to regulate infection.

48

## 49 **Introduction**

50 Transcriptional and post-transcriptional regulation influence gene expression in cells  
51 following infection by viruses, including those in the *Flaviviridae* family. The *Flaviviridae* family of  
52 positive sense RNA viruses includes dengue virus (DENV), Zika virus (ZIKV), West Nile virus  
53 (WNV), and hepatitis C virus (HCV), all of which cause significant mortality and morbidity  
54 worldwide. The effects of *Flaviviridae* infection on human health are diverse, ranging from  
55 microcephaly and encephalitis to chronic liver disease (Holbrook, 2017; Thrift et al., 2017).  
56 Previous studies have shown broad changes in cellular transcript levels during *Flaviviridae*  
57 infection that highlight a complex relationship between viral infection and gene expression,  
58 whereby the host attempts to resist infection by up- or down-regulating relevant genes while  
59 viruses co-opt host transcription to facilitate replication and avoid host defenses (Fink et al., 2007;  
60 Kumar et al., 2016; Rosenberg et al., 2018; Sessions et al., 2013; Su et al., 2002; Zanini et al.,  
61 2018). Differential expression of proviral and antiviral host factors is therefore an important  
62 determinant of the outcome of *Flaviviridae* infection

63 Host gene expression during *Flaviviridae* infection can be tuned by post-transcriptional  
64 RNA controls (De Maio et al., 2016; Luna et al., 2015; Schwerk et al., 2015). An important post-  
65 transcriptional RNA regulatory mechanism is the chemical modification of RNA (Gilbert et al.,  
66 2016). The most prevalent internal modification of mRNA is *N*6-methyladenosine ( $m^6A$ ). The  $m^6A$   
67 epitranscriptome is controlled by specific cellular proteins. METTL3, METTL14, WTAP, and other  
68 “writer” proteins form a complex that catalyzes the methylation of adenosine residues in mRNA.

69 This protein complex targets the consensus motif DRA\*CH (where D=G/A/U, R=G/A, H=U/A/C,  
70 and \* denotes modified A) in mRNA for methylation, although how specific DRACH motifs are  
71 selected for modification is still not well understood (Meyer and Jaffrey, 2017; Shi et al., 2019;  
72 Yang et al., 2018). “Reader” RNA-binding proteins recognize m<sup>6</sup>A to modulate many aspects of  
73 mRNA metabolism, including mRNA splicing, nuclear export, stability, translation, and structure  
74 (Meyer and Jaffrey, 2017; Shi et al., 2019; Yang et al., 2018). By regulating specific transcripts,  
75 m<sup>6</sup>A plays a role in many important biological processes including circadian rhythm, cell  
76 differentiation, development, stress responses, cancer, and viral infection (Gonzales-van Horn  
77 and Sarnow, 2017; Meyer and Jaffrey, 2017; Shi et al., 2019; Yang et al., 2018).

78 Viral infection can be affected by m<sup>6</sup>A modification of either viral or host transcripts.  
79 Transcripts from both DNA and RNA viruses can be methylated, and m<sup>6</sup>A in these RNAs has  
80 been shown to have various proviral and antiviral functions (Courtney et al., 2017; Gokhale and  
81 Horner, 2017; Gokhale et al., 2016; Hao et al., 2019; Imam et al., 2018; Kennedy et al., 2016;  
82 Lichinchi et al., 2016a; Lichinchi et al., 2016b; McIntyre et al., 2018; Rubio et al., 2018; Tirumuru  
83 et al., 2016; Tsai et al., 2018; Winkler et al., 2019; Ye et al., 2017). m<sup>6</sup>A in specific cellular  
84 transcripts is also important during viral infection. For example, m<sup>6</sup>A regulates the expression of  
85 the antiviral *IFNB1* transcript induced by double-stranded DNA viruses (Rubio et al., 2018; Winkler  
86 et al., 2019). However, the role of m<sup>6</sup>A in cellular mRNA during viral infection is still not well  
87 understood, in part because of difficulties in accurately and quantitatively mapping the  
88 modification. While ZIKV, Kaposi’s sarcoma-associated herpes virus (KSHV), and human  
89 immunodeficiency 1 (HIV-1) have been reported to alter m<sup>6</sup>A modification in cellular mRNAs  
90 (Hesser et al., 2018; Lichinchi et al.; Lichinchi et al., 2016b; Tan et al., 2018), the scale of these  
91 changes has likely been overestimated (McIntyre et al., 2019). Moreover, there are almost no  
92 data on common m<sup>6</sup>A changes in host mRNA across multiple viruses, and the functional  
93 consequences of epitranscriptomic changes in cellular mRNA during viral infection have also not  
94 been examined. Therefore, better estimating the number of m<sup>6</sup>A changes and defining the  
95 consequences of altered m<sup>6</sup>A modification of cellular mRNA during viral infection are important  
96 for understanding post-transcriptional regulation of the host response to infection.

97 Here, we studied the effect of DENV, ZIKV, WNV, and HCV infection on the m<sup>6</sup>A  
98 epitranscriptome. We found that infection by all four viruses led to altered m<sup>6</sup>A modification of a  
99 set of specific cellular transcripts, and that activation of cellular pathways, including innate  
100 immunity and endoplasmic reticulum (ER) stress responses, by infection contribute to differential  
101 m<sup>6</sup>A modification and changes in translation or splicing of these transcripts. Importantly,

102 transcripts with altered m<sup>6</sup>A encode proteins that regulate infection, indicating that post-  
103 transcriptional gene regulation of mRNA by m<sup>6</sup>A has the potential to affect viral replication.

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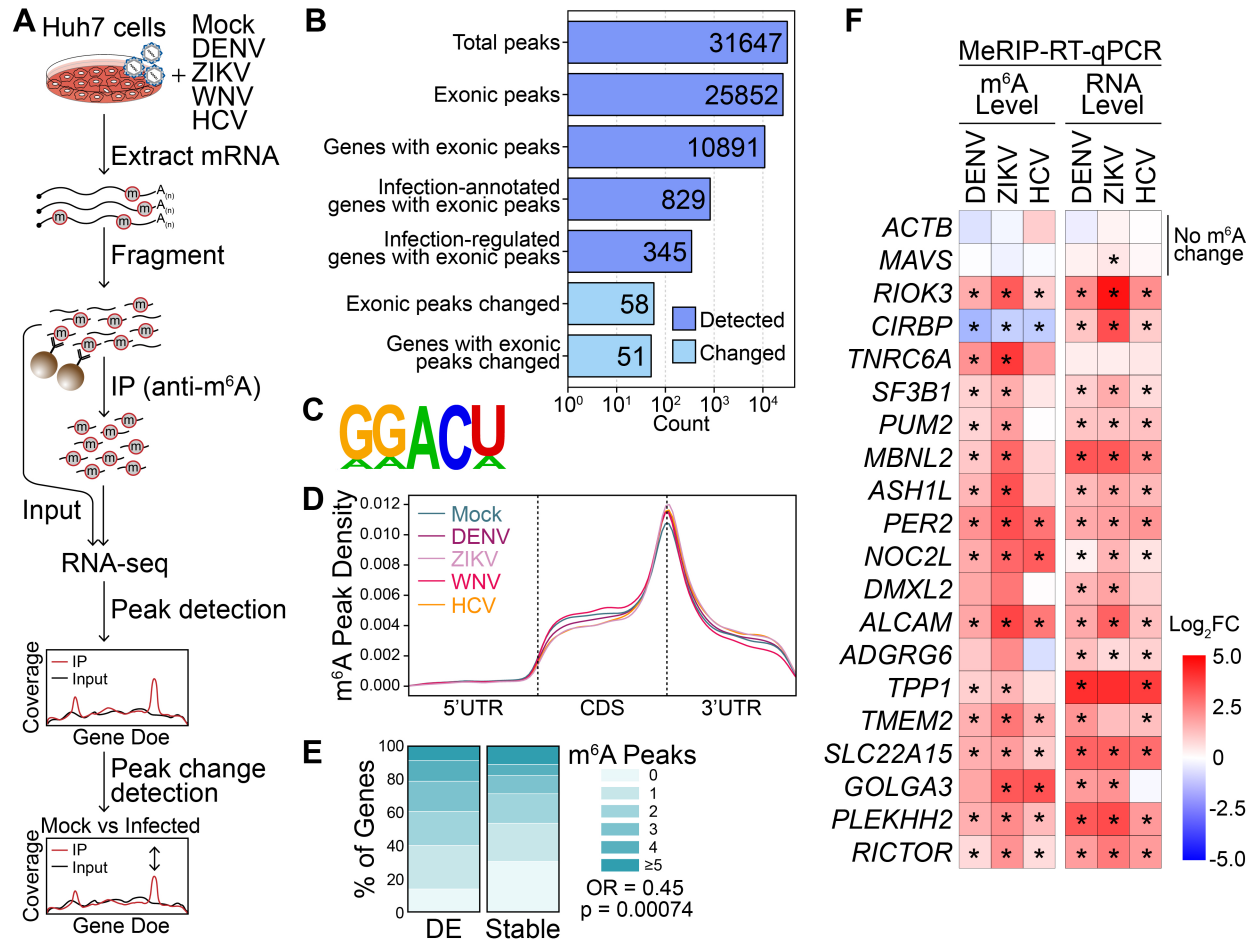
## 105 **Results**

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### 107 ***Flaviviridae* infection alters m<sup>6</sup>A modification of specific cellular transcripts.**

108 *Flaviviridae* infection leads to changes in the expression of proviral and antiviral gene  
109 products (Fink et al., 2007; Kumar et al., 2016; Rosenberg et al., 2018; Sessions et al., 2013; Su  
110 et al., 2002; Zanini et al., 2018). Since m<sup>6</sup>A can modulate RNA fate, and therefore protein  
111 expression, we hypothesized that altered m<sup>6</sup>A modification would influence expression of host  
112 genes that regulate viral infection. We therefore sought to measure changes in the m<sup>6</sup>A  
113 modification of host transcripts during *Flaviviridae* infection using methylated RNA  
114 immunoprecipitation and sequencing (MeRIP-seq) (Figure 1A). For MeRIP-seq, we used an anti-  
115 m<sup>6</sup>A antibody to enrich m<sup>6</sup>A-modified RNA fragments prior to RNA sequencing of both the input  
116 and immunoprecipitated (IP) fractions (Dominissini et al., 2012; Meyer et al., 2012). We note that  
117 this antibody can also recognize the similar modification N<sup>6</sup>,2'-O-dimethyladenosine (m<sup>6</sup>A<sub>m</sub>),  
118 which is found at lower abundance than m<sup>6</sup>A and, in mRNA, only within the 5' cap (Linder et al.,  
119 2015; Mauer and Jaffrey, 2018). We performed MeRIP-seq on RNA extracted from human Huh7  
120 liver hepatoma cells, which are permissive for infection by all four viruses. At 48 hours post-  
121 infection, 60-90% of cells were infected with DENV, ZIKV, WNV, or HCV, depending on the virus  
122 (Figure S1A). We first identified the gene expression changes in response to infection. To do this,  
123 we analyzed differential expression of genes between infected samples and uninfected controls  
124 using the input fractions from MeRIP-seq and found 50 genes that were significantly differentially  
125 expressed (DESeq2, adjusted p < 0.05, |Log<sub>2</sub>Fold Change (FC)| ≥ 2) following infection by all four  
126 viruses individually (Figure S1B-C, Table S1). Notably, although ZIKV, DENV, and WNV are  
127 known to generate acute, cytotoxic responses, while HCV leads to persistent infection (Neufeldt  
128 et al., 2018), we found that several pathways were similarly altered by all of these viruses in Huh7  
129 cells. Significantly upregulated pathways included those associated with innate immunity (such  
130 as NF-κB, TNF, and MAPK signaling) and the ER stress response, while downregulated pathways  
131 included those associated with the cell cycle (Figure S1D). These results, which we validated by  
132 RT-qPCR, are similar to what has been reported for individual *Flaviviridae* (Figure S1E) (Fink et  
133 al., 2007; Kumar et al., 2016; Rosenberg et al., 2018; Sessions et al., 2013; Su et al., 2002; Zanini  
134 et al., 2018).

**Figure 1**



135 **Figure 1: *Flaviviridae* infection alters m<sup>6</sup>A modification of specific transcripts.** (A)  
 136 Schematic of the MeRIP-seq protocol used to identify differential m<sup>6</sup>A methylation following  
 137 infection of Huh7 cells with DENV, ZIKV, WNV, and HCV. RNA was harvested at 48 hours post-  
 138 infection (hpi) and experiments were performed in triplicate. (B) The number of peaks and genes  
 139 with m<sup>6</sup>A peaks detected in ≥ 2 mock- or virus-infected samples (dark blue; MACS2 q-value <  
 140 0.05) and peaks that change during infection (light blue, |peak – gene Log<sub>2</sub>FC| ≥ 1, adjusted p <  
 141 0.05). “Infection-annotated genes” are defined as those with known annotations for the Reactome  
 142 Pathways ‘Infectious Disease’, ‘Unfolded Protein Response’, ‘Interferon Signaling’, or ‘Innate  
 143 Immune Signaling’ in the database used by fgsea. “Infection-regulated genes” are defined as  
 144 those that show a Log<sub>2</sub> fold change in gene expression ≥ 2 in RNA expression between mock-  
 145 and virus-infected samples in our data set (adjusted p < 0.05). (C) The most significantly enriched  
 146 motif identified as enriched in the MeRIP fractions across all samples (HOMER, p = 1e-831). (D)  
 147 Metagene plot of methylated DRACH motifs across transcripts in mock- and virus-infected cells.  
 148 DRACH motifs were considered methylated if they fell under a peak detected in at least two  
 149 replicates. (E) The percent of genes with m<sup>6</sup>A peaks that changed expression with infection  
 150 (|Log<sub>2</sub>FC| ≥ 2, adjusted p < 0.05, N = 137) and genes that remained stable (|Log<sub>2</sub>FC| < 0.5,  
 151 adjusted p > 0.05, N = 7627) for transcripts with mean expression ≥ 50 reads. (F) (Left) MeRIP-  
 152 RT-qPCR analysis of relative m<sup>6</sup>A level of transcripts with infection-altered m<sup>6</sup>A modification or

153 controls (*ACTB* and *MAVS*) in DENV, ZIKV, and HCV-infected (48 hpi) Huh7 cells. (Right) RNA  
154 expression of these transcripts relative to *GAPDH* (right). Values in heatmaps are the mean of 3  
155 independent experiments. \*  $p < 0.05$ , by unpaired Student's t test.

156

157 We then predicted m<sup>6</sup>A-modified regions by calling peaks in IP over input RNA-seq  
158 coverage across transcripts using MACS2 (Zhang et al., 2008). We detected a total of 31,647  
159 peaks, with 25,852 exonic peaks corresponding to 10,891 genes across all uninfected and  
160 infected samples (Figure 1B). The known m<sup>6</sup>A motif DRACH (in particular, GGACU), was enriched  
161 under the identified peaks (Figure 1C). As expected, detected peaks were most common at the  
162 end of the coding sequence and beginning of the 3' untranslated region (UTR) (Figure 1D) (Meyer  
163 and Jaffrey, 2017; Shi et al., 2019; Yang et al., 2018). We did not observe a change in the  
164 distribution of m<sup>6</sup>A across transcript regions with DENV, ZIKV, WNV, or HCV infection (Figure  
165 1D). This is in contrast to a previous report that suggested ZIKV infection was associated with  
166 increased methylation at the 5' UTRs of cellular transcripts (Lichinchi et al., 2016b); however, we  
167 also did not detect a difference in m<sup>6</sup>A distribution following ZIKV infection on reanalysis of that  
168 published data (Figure S1F). Further, following viral infection, we found only subtle changes in  
169 the overall level of m<sup>6</sup>A relative to unmodified adenosine in purified mRNA, as analyzed by liquid  
170 chromatography tandem-mass spectrometry (LC-MS/MS) of digested nucleotides, and no change  
171 in the expression of cellular m<sup>6</sup>A machinery, as analyzed by immunoblotting (Figure S1G-H).  
172 Indeed, since the expression of the methylation machinery was not changed by infection, we  
173 would not predict broad, unidirectional changes in the abundance or distribution of m<sup>6</sup>A on cellular  
174 transcripts.

175 However, functional annotation of the m<sup>6</sup>A-modified genes expressed in the infected  
176 samples did reveal an enrichment for genes with roles in infection. In total, 829 methylated genes  
177 were annotated as involved in the Reactome Pathways of "Infectious Disease", "Unfolded Protein  
178 Response", "Interferon Signaling", or "Innate Immune System" ("Infection-annotated genes"; see  
179 Methods; Figure 1B). Further, 345 genes that were differentially expressed between infected and  
180 uninfected samples were also methylated ("Infection-regulated genes"; Figure 1B). Indeed,  
181 mRNAs that changed expression with infection ( $p_{\text{adj}} < 0.05$ ,  $|\text{Log}_2\text{FC}| \geq 2$ , mean expression  $\geq$   
182 50) were more likely to have at least one m<sup>6</sup>A site than those that did not change expression ( $p_{\text{adj}} > 0.05$ ,  $|\text{Log}_2\text{FC}| < 0.5$ , mean expression  $\geq 50$ ; Fisher's exact test  $p = 0.00074$ , odds ratio =  
183 0.64) (Figure 1E). These results are consistent with previous reports that genes that undergo  
184 dynamic regulation tend to contain more m<sup>6</sup>A sites in their transcripts than stable housekeeping  
185 genes (Schwartz et al., 2014), and suggest that m<sup>6</sup>A may be an important regulator of genes  
186 implicated in infection.

187

188 We next predicted changes in m<sup>6</sup>A based on differences in IP enrichment relative to gene  
189 expression with infection by all four *Flaviviridae* members. We detected shared m<sup>6</sup>A changes in  
190 58 exonic peaks in 51 genes following infection with all viruses, most of which showed increases  
191 in m<sup>6</sup>A and occurred within the 3' UTR or coding sequence of the transcript (Figure 1B, Table S2).  
192 Whereas genes that showed changes in expression were enriched for pathways with known roles  
193 in infection (Figure S1D), genes that showed changes in methylation did not show any enrichment  
194 for functional categories relevant to infection. We and others previously showed that MeRIP-RT-  
195 qPCR can detect relative changes in m<sup>6</sup>A levels (Engel et al., 2018; McIntyre et al., 2019).  
196 Therefore, we used this method with RT-qPCR primers under the changed m<sup>6</sup>A peaks to  
197 orthogonally validate a set of 18 of the predicted m<sup>6</sup>A changes in transcripts following infection.  
198 In these and subsequent analyses, we focused on m<sup>6</sup>A changes following infection by DENV,  
199 ZIKV, and HCV. Of the 18 transcripts tested by MeRIP-RT-qPCR, 16 showed a significant change  
200 in m<sup>6</sup>A modification relative to any change in gene expression with at least two viruses, and 9 of  
201 those showed a significant change with all three viruses. The control mRNAs (*ACTB* and *MAVS*),  
202 both predicted to be stably methylated during infection, indeed showed no m<sup>6</sup>A changes (Figure  
203 1F). Most non-significant m<sup>6</sup>A changes trended towards the change predicted by MeRIP-seq  
204 (Figure 1F).

205 For our predictions of pan-viral m<sup>6</sup>A changes using MeRIP-seq (above), we compared all  
206 infected to all uninfected replicates for increased statistical power (McIntyre et al., 2019). However  
207 we also wanted to detect any peak changes unique to single viruses, and therefore, we used the  
208 same computational approach described above to identify significant peaks unique to each virus  
209 (Table S2). MeRIP-RT-qPCR validation of these putative virus-specific peaks (two per virus)  
210 showed similar changes in relative m<sup>6</sup>A modification at those peaks with infection by all three  
211 viruses tested, rather than individual virus-mediated changes (Figure S1I), suggesting that most  
212 m<sup>6</sup>A regulation occurs through processes activated in response to infection by all the *Flaviviridae*  
213 we tested. Together, our data reveal that hundreds of transcripts differentially expressed during  
214 *Flaviviridae* infection contain m<sup>6</sup>A and that infection alters m<sup>6</sup>A modification of specific host  
215 transcripts.

216  
217 ***Flaviviridae* infection alters m<sup>6</sup>A modification of *RIOK3* and *CIRBP* mRNA through distinct**  
218 **pathways.**

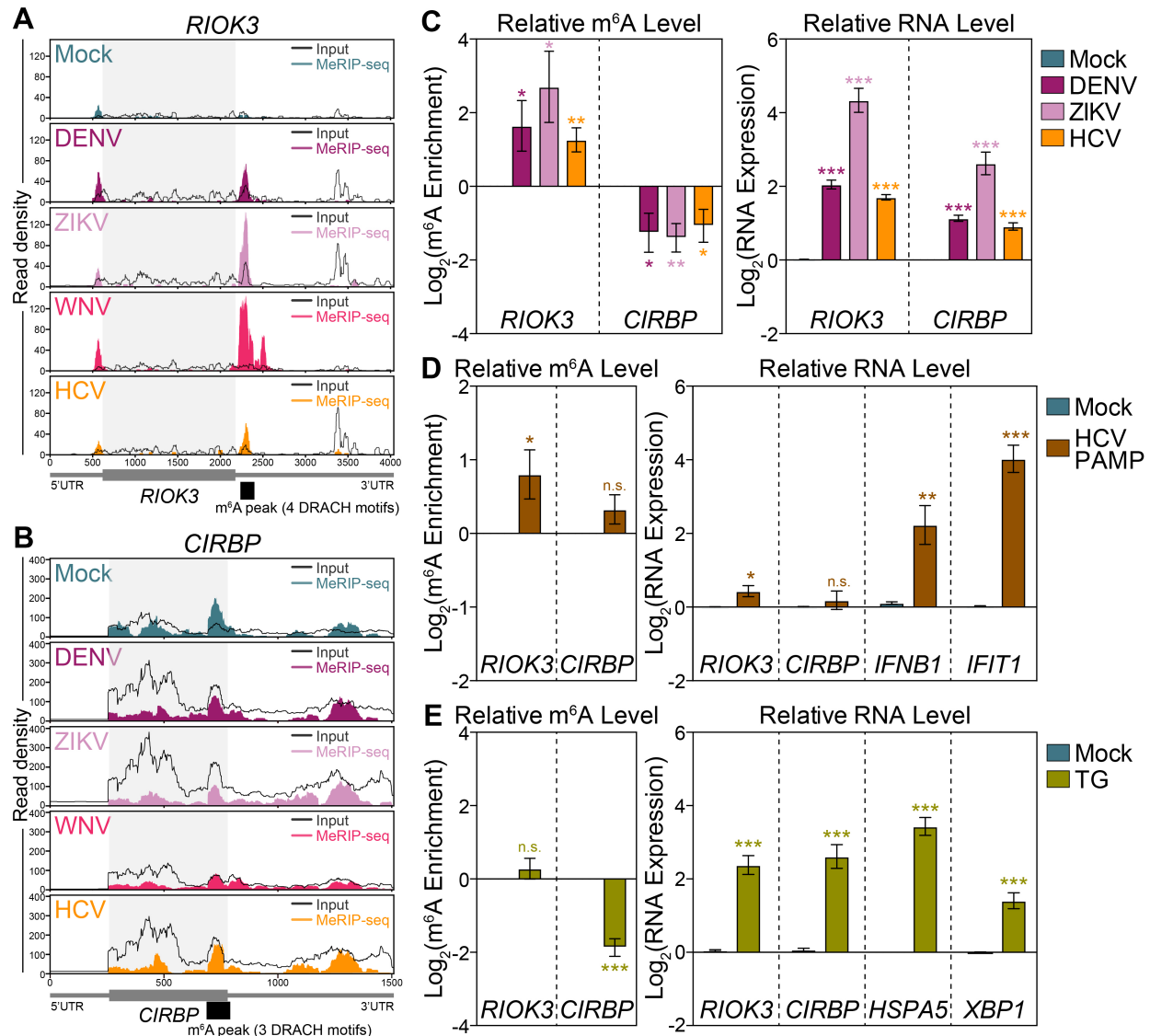
219 We focused on two specific transcripts that gain or lose m<sup>6</sup>A during infection by all viruses  
220 (DENV, ZIKV, WNV, and HCV) for further analysis: *RIOK3* (gains) and *CIRBP* (loses). *RIOK3*  
221 encodes a serine/threonine kinase and has been implicated in regulating antiviral signaling (Feng

222 et al., 2014; Takashima et al., 2015; Willemsen et al., 2017), while *CIRBP* encodes a stress-  
223 induced RNA-binding protein (Liao et al., 2017). Following viral infection, *RIOK3* mRNA gains an  
224 m<sup>6</sup>A peak in the 3' UTR close to the stop codon (Figure 2A), and *CIRBP* mRNA shows reduced  
225 m<sup>6</sup>A modification in the coding sequence of its last exon (Figure 2B). The *RIOK3* and *CIRBP*  
226 peaks span four and three DRACH motifs, respectively. Both peaks have been previously  
227 reported in published datasets, although the function of m<sup>6</sup>A on these transcripts has not been  
228 investigated; the *RIOK3* peak was identified in mouse liver tissue (Zhou et al., 2018), while the  
229 *CIRBP* peak was present in HepG2 cells (Huang et al., 2019; Zhong et al., 2018). We performed  
230 MeRIP-RT-qPCR on RNA from cells infected with DENV, ZIKV, and HCV to validate these  
231 predicted changes in m<sup>6</sup>A, as in Figure 1F. MeRIP-RT-qPCR confirmed that relative to gene  
232 expression, the m<sup>6</sup>A modification of *RIOK3* significantly increased following infection and that of  
233 *CIRBP* decreased, while *RIOK3* and *CIRBP* mRNA levels both increased following infection  
234 (Figure 1F and 2C). We found similar changes in the m<sup>6</sup>A modification of *RIOK3* and *CIRBP* in  
235 chromatin-associated RNA following ZIKV infection, suggesting that the regulation of m<sup>6</sup>A at these  
236 sites occurs co-transcriptionally (Ke et al., 2017; Slobodin et al., 2017) (Figure S2A). In uninfected  
237 cells, both *RIOK3* and *CIRBP* transcripts are bound by the m<sup>6</sup>A-binding protein YTHDF1 (Figure  
238 S2B-C). However, DENV, ZIKV, and HCV infection all increased the association of YTHDF1 with  
239 *RIOK3* mRNA and decreased its association with *CIRBP* mRNA, suggesting that YTHDF1  
240 recognizes the altered m<sup>6</sup>A modification of *RIOK3* and *CIRBP* transcripts following infection  
241 (Figure S2D).

242 We next investigated whether cellular pathways stimulated by viral infection (Figure S1D)  
243 contribute to the virally induced m<sup>6</sup>A changes in *RIOK3* and *CIRBP*. *Flaviviridae* infection drives  
244 innate immune signaling cascades which lead to transcriptional induction of interferon- $\beta$  (IFN) and  
245 antiviral interferon-stimulated genes (ISGs) (Horner and Gale, 2013; Munoz-Jordan and  
246 Fredericksen, 2010; Suthar et al., 2013). Therefore, we tested whether innate immune activation  
247 in the absence of a replicating virus alters m<sup>6</sup>A modification. We measured the relative m<sup>6</sup>A levels  
248 of *RIOK3* and *CIRBP* mRNA by MeRIP-RT-qPCR following transfection of Huh7 cells with the  
249 short, previously described, HCV pathogen-associated molecular pattern (HCV PAMP)  
250 immunostimulatory RNA (Saito et al., 2008). As expected, HCV PAMP induced expression of  
251 *IFNB1* and the ISG *IFIT1* (Figure 2D). It also increased m<sup>6</sup>A modification of *RIOK3* mRNA to a  
252 similar degree as viral infection, but it did not reproduce the decrease in *CIRBP* methylation seen  
253 with viral infection (Figure 2D). These data indicate that innate immune signaling promotes the  
254 m<sup>6</sup>A modification of *RIOK3* mRNA following infection.



**Figure 2**



255 **Figure 2: Flaviviridae infection alters m<sup>6</sup>A modification of *RIOK3* and *CIRBP* mRNA through**  
 256 **distinct cellular pathways. (A and B)** Coverage plot of MeRIP (color) and input (black) reads in  
 257 (A) *RIOK3* and (B) *CIRBP* transcripts in Huh7 cells infected with the indicated virus (48 hpi) as  
 258 determined by MeRIP-seq. Data are representative of three biological replicates. Infection-altered  
 259 m<sup>6</sup>A peaks (and the number of DRACH motifs within) are indicated in black under the transcript  
 260 map. (C) (Left) MeRIP-RT-qPCR analysis of relative m<sup>6</sup>A level of *RIOK3* and *CIRBP* in mock- and  
 261 virus-infected (48 hpi) Huh7 cells. (Right) RNA expression of *RIOK3* and *CIRBP* relative to *HPRT1*  
 262 (right). (D) (Left) MeRIP-RT-qPCR analysis of relative m<sup>6</sup>A level of *RIOK3* and *CIRBP* in mock-  
 263 and HCV PAMP-transfected (8 h) Huh7 cells. (Right) RNA expression of *RIOK3*, *CIRBP*, as well  
 264 as positive control transcripts *IFNB1* and *IFIT1* relative to *HPRT1*. (E) (Left) MeRIP-RT-qPCR  
 265 analysis of relative m<sup>6</sup>A level of *RIOK3* and *CIRBP* in mock- and thapsigargin-treated (TG; 16 h)  
 266 Huh7 cells. (Right) RNA expression of *RIOK3*, *CIRBP*, and positive control transcripts *HSPA5*

267 and *XBP1* relative to *HPRT1*. Values are the mean  $\pm$  SEM of 6 (C-D), 3 (E), or 5 (F) biological  
268 replicates. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  by unpaired Student's t test. n.s. = not significant.  
269

270 Our work and that of others have shown that the ER stress response is activated during  
271 *Flaviviridae* infection, which remodels host ER membranes to facilitate viral replication (Figure  
272 S1D) (Blazquez et al., 2014; Chan, 2014; Neufeldt et al., 2018). The ER  $\text{Ca}^{2+}$  ATPase inhibitor  
273 thapsigargin can induce a similar stress response, including increased expression of *HSPA5* and  
274 *XBP1* (Lee et al., 2012). To test whether ER stress alters the m<sup>6</sup>A modification of *RIOK3* and  
275 *CIRBP*, we measured their relative m<sup>6</sup>A levels by MeRIP-RT-qPCR following treatment of cells  
276 with thapsigargin (Figure 2E). Thapsigargin treatment increased the mRNA level of both *RIOK3*  
277 and *CIRBP*, as well as that of the positive controls *HSPA5* and *XBP1*, by about 4-fold. However,  
278 while thapsigargin treatment did not change the relative m<sup>6</sup>A level of *RIOK3*, it did reduce m<sup>6</sup>A  
279 modification of *CIRBP*, similar to what we observed with viral infection (Figure 2E). Taken  
280 together, these data reveal that innate immune and ER stress signaling, which are activated  
281 during *Flaviviridae* infection, can separately affect m<sup>6</sup>A modification of different transcripts.

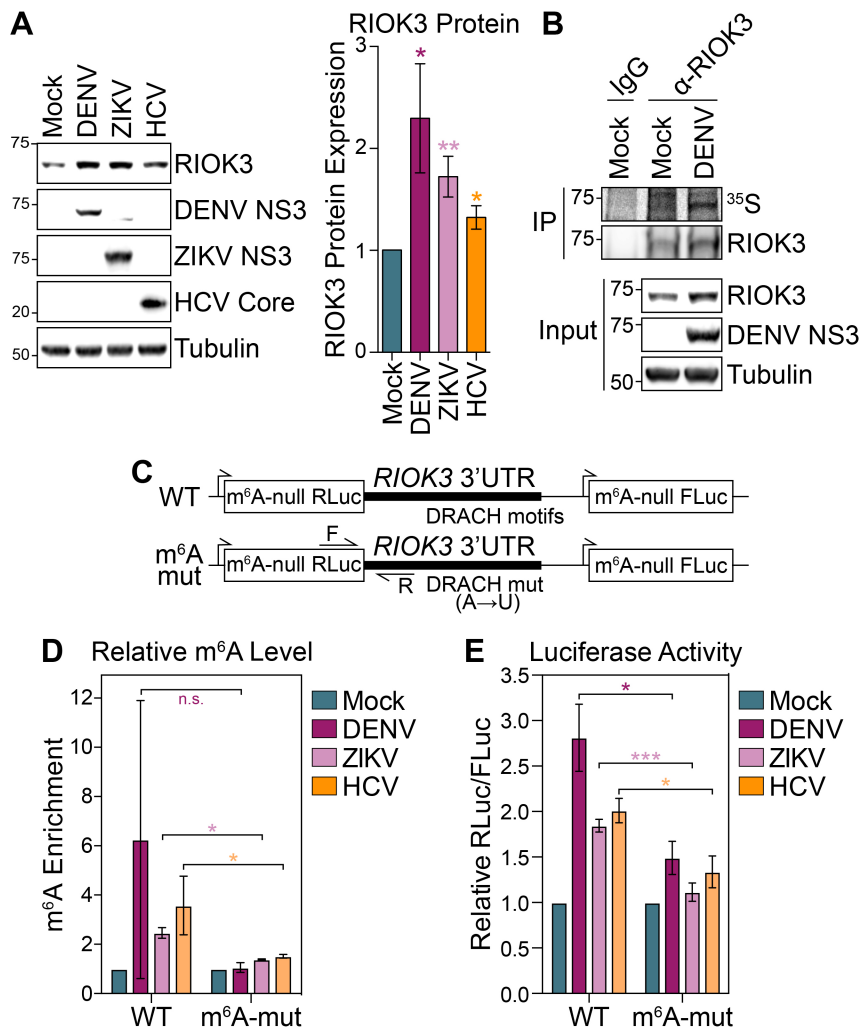
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### 283 m<sup>6</sup>A modification enhances *RIOK3* protein expression during infection

284 We next investigated the function of m<sup>6</sup>A in *RIOK3* mRNA during infection. Consistent with  
285 our finding that DENV, ZIKV, and HCV infection all increased *RIOK3* mRNA levels (Figure 2C),  
286 *RIOK3* protein expression also increased following infection (Figure 3A). m<sup>6</sup>A can alter mRNA  
287 nuclear export, stability, and translation, all of which could regulate protein expression (Meyer and  
288 Jaffrey, 2017; Yang et al., 2018). When we analyzed the mRNA levels of *RIOK3* in nuclear and  
289 cytoplasmic fractions from uninfected and infected Huh7 cells using RT-qPCR, we found no  
290 significant change in the nuclear export of *RIOK3* post-infection (Figure S3A). Similarly, we found  
291 no consistent change in the mRNA stability of *RIOK3* in uninfected and infected cells (Figure  
292 S3B). However, we did detect increased nascent translation of *RIOK3* in DENV-infected cells  
293 compared to uninfected cells as measured by <sup>35</sup>S labeling of nascent proteins followed by *RIOK3*  
294 protein immunoprecipitation, suggesting that *RIOK3* translation was increased in infected cells  
295 (Figure 3B). This is consistent with our observation that during infection *RIOK3* has increased  
296 binding to the m<sup>6</sup>A reader protein YTHDF1, which is known to promote translation of bound  
297 mRNAs under specific conditions (Figure S2D) (Han et al., 2019; Shi et al., 2018; Wang et al.,  
298 2019; Wang et al., 2015). However, global cellular translation is known to be inhibited during  
299 DENV, ZIKV, and HCV infection (Arnaud et al., 2010; Garaigorta and Chisari, 2009; Roth et al.,  
300 2017). Indeed, we observed that in Huh7 cells, infection with all three viruses induces the

301 phosphorylation of the eukaryotic translation initiation factor eIF2 $\alpha$ , which inhibits recycling of eIF2  
 302 and therefore prevents translation of RNAs that require this factor (Figure S3C) (Stern-Ginossar  
 303 et al., 2019; Wek, 2018). Together, our results suggest that m<sup>6</sup>A modification of *RIOK3* could  
 304 allow this transcript to be efficiently translated during infection, despite global inhibition of  
 305 translation.

**Figure 3**



306 **Figure 3: m<sup>6</sup>A promotes RIOK3 protein expression.** (A) (Left) Representative immunoblot of  
 307 RIOK3 protein expression in mock- and virus-infected (48 hpi) Huh7 cells. (Right) Quantification  
 308 of RIOK3 protein expression relative to tubulin from replicate experiments. (B)  
 309 Immunoprecipitation (IP) of RIOK3 from mock- and DENV-infected (48 hpi) Huh7 cells labeled  
 310 with <sup>35</sup>S for 3 hours. IP fractions were analyzed by autoradiography (<sup>35</sup>S) and immunoblotting.  
 311 Data are representative of 3 biological replicates. (C) Schematic of WT and mutant m<sup>6</sup>A-null  
 312 *Renilla* luciferase (RLuc) *RIOK3* 3' UTR reporters that also express m<sup>6</sup>A-null Firefly luciferase  
 313 (FLuc) from a separate promoter. RT-qPCR primer (F and R) locations are indicated with arrows.  
 314 (D) MeRIP-RT-qPCR analysis of relative m<sup>6</sup>A level of stably expressed WT and m<sup>6</sup>A-mut *RIOK3*

315 3' UTR reporter RNA in mock- and virus-infected (48 hpi) Huh7 cells. **(E)** Relative luciferase  
316 activity (RLuc/FLuc) in mock- and virus-infected (48 hpi) Huh7 cells stably expressing WT and  
317 m<sup>6</sup>A-mut *RIOK3* 3' UTR reporters. Relative luciferase activity in uninfected cells was set as 1 for  
318 each reporter. Values are the mean  $\pm$  SEM of 6 (A), 2 (D), or 5 (E) biological replicates. \*  $p < 0.05$ ,  
319 \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  by unpaired Student's t test. n.s. = not significant.

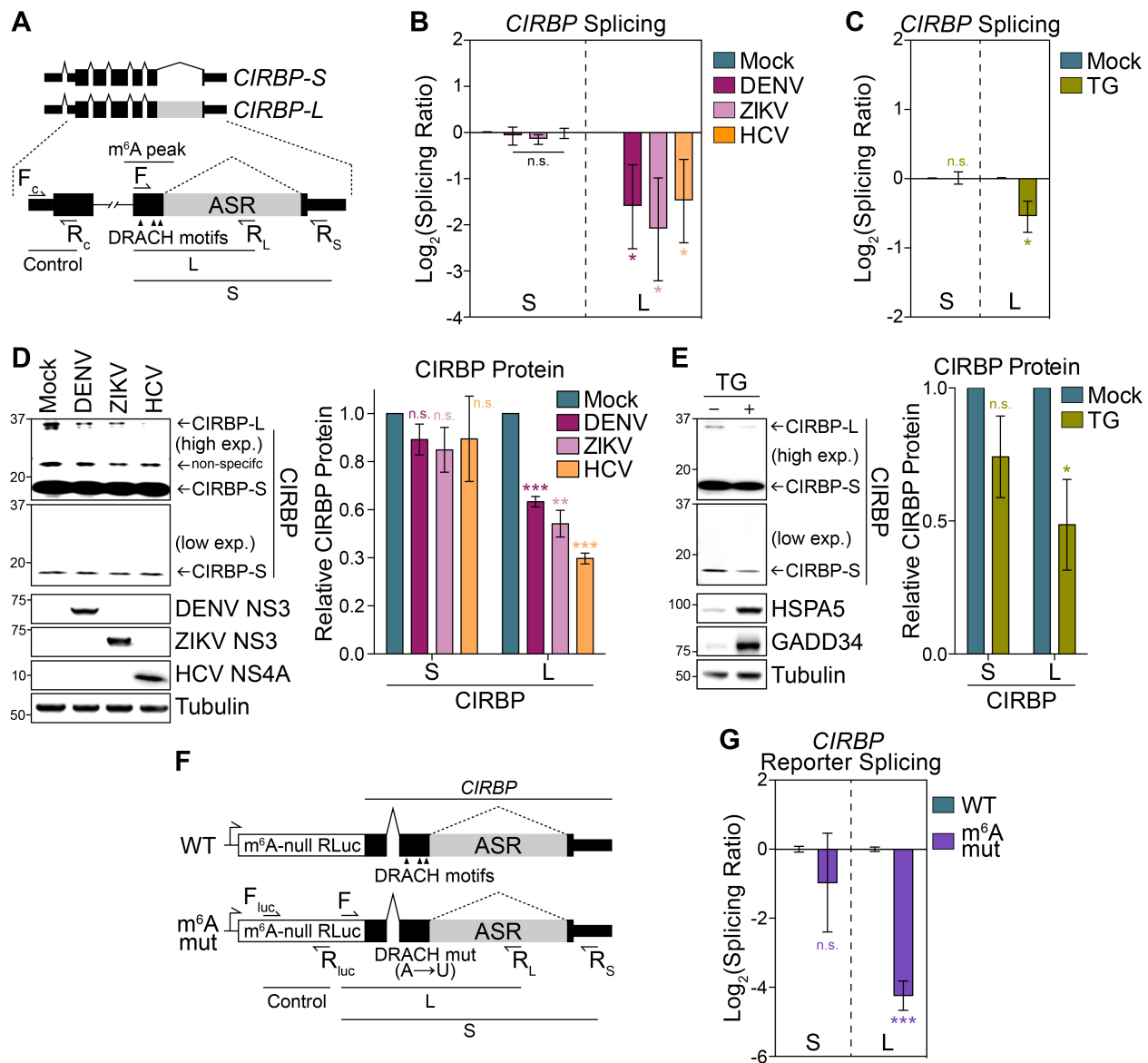
320  
321 To directly test whether m<sup>6</sup>A can promote *RIOK3* protein expression during infection, we  
322 generated Huh7 cell lines stably expressing a luciferase reporter which contains the wild type  
323 (WT) *RIOK3* 3' UTR, or an analogous 3' UTR sequence in which all putative m<sup>6</sup>A sites were  
324 abrogated by A→T mutations (m<sup>6</sup>A-mut), downstream of a *Renilla* luciferase gene in which all  
325 DRACH motifs were ablated (m<sup>6</sup>A-null) (Figure 3C). These constructs also expressed a separate  
326 m<sup>6</sup>A-null Firefly luciferase gene whose expression is not regulated by m<sup>6</sup>A. As expected, the WT  
327 *RIOK3* reporter had increased m<sup>6</sup>A modification compared to the m<sup>6</sup>A-mut *RIOK3* reporter  
328 following viral infection, as measured by MeRIP-RT-qPCR using primers that specifically amplified  
329 reporter RNA (Figure 3D). This reveals that the *RIOK3* 3' UTR sequence is sufficient for m<sup>6</sup>A  
330 addition following infection. Importantly, the relative luciferase activity of the WT *RIOK3* reporter  
331 was significantly increased compared to the m<sup>6</sup>A-mut reporter following viral infection (Figure 3E).  
332 Taken together, these data reveal that m<sup>6</sup>A modification of the 3' UTR of *RIOK3* mRNA during  
333 infection consistently promotes its translation during infection with all three *Flaviviridae* tested.

### 334 335 **m<sup>6</sup>A modification promotes alternative splicing of *CIRBP* mRNA during infection**

336 We then analyzed the function of reduced m<sup>6</sup>A modification in *CIRBP* mRNA following  
337 infection. Neither the nuclear export nor the stability of *CIRBP* mRNA were consistently affected  
338 following DENV, ZIKV, or HCV infection, suggesting that the loss m<sup>6</sup>A in *CIRBP* does not regulate  
339 these processes (Figure S4A-B). Based on our RNA-seq data, *CIRBP* encodes at least 2  
340 isoforms: (1) the dominant, short isoform (*CIRBP-S*) which encodes a 172 aa, 18 kDa protein and  
341 (2) a second, long isoform in which an intron immediately downstream of the infection-altered m<sup>6</sup>A  
342 peak and upstream of the stop codon is retained (*CIRBP-L*), resulting in a 297 aa, 32 kDa protein  
343 (Figure 4A; retained intron referred to as alternatively spliced region (ASR)). Interestingly, analysis  
344 of our RNA-seq data using MAJIQ (Vaquero-Garcia et al., 2016) to identify local splice variants  
345 suggested decreased retention of this intron during infection, which we confirmed in DENV, ZIKV,  
346 and HCV-infected cells using RT-qPCR (Figure 4B). We observed a similar reduction of intron  
347 retention following thapsigargin treatment, which we had found also reduces m<sup>6</sup>A modification of  
348 *CIRBP* (Figure 4C and 2F). Indeed, both viral infection and thapsigargin treatment significantly  
349 reduced the protein level of *CIRBP-L* containing the retained intron, while not affecting expression

350 of CIRBP-S (Figure 4D-E). To test whether reduction of m<sup>6</sup>A modification at the m<sup>6</sup>A peak in  
 351 *CIRBP* might affect alternative splicing of this transcript, we generated a splicing reporter wherein  
 352 the m<sup>6</sup>A-null *Renilla* luciferase gene was fused to the WT genomic sequence of *CIRBP* from exon  
 353 5 onwards (WT *CIRBP*) and a corresponding reporter in which the putative m<sup>6</sup>A sites in the  
 354 identified *CIRBP* m<sup>6</sup>A peak were synonymously mutated (m<sup>6</sup>A-mut *CIRBP*) (Figure 4F). Using  
 355 RT-qPCR, we found that the m<sup>6</sup>A-mut reporter had reduced intron retention compared to the WT  
 356 reporter, suggesting that the loss of m<sup>6</sup>A in *CIRBP* regulates its alternative splicing and reduces  
 357 the expression of the long isoform (Figure 4G).

**Figure 4**



358 **Figure 4: m<sup>6</sup>A promotes alternative splicing of *CIRBP*.** (A) Schematic of *CIRBP* transcript  
 359 isoforms with a focus on the alternatively spliced region (ASR). RT-qPCR primer locations are

360 indicated with arrows (F<sub>C</sub>-R<sub>C</sub>: control *CIRBP* amplicon; F-R<sub>L</sub>: long isoform specific; F-R<sub>S</sub>: short  
361 isoform specific. **(B)** RT-qPCR analysis of short (S) and long (L) *CIRBP* RNA isoform expression  
362 in mock- and virus-infected (48 hpi) Huh7 cells relative to control *CIRBP* amplicon. **(C)** RT-qPCR  
363 analysis of S and L *CIRBP* RNA isoform expression in mock- and TG-treated (16 h) Huh7 cells.  
364 **(D)** (Left) Representative immunoblot of short (CIRBP-S) and long (CIRBP-L) CIRBP protein  
365 isoforms in mock- and virus-infected (48 hpi) Huh7 cells. (Right) Quantification of CIRBP protein  
366 isoform expression relative to tubulin from replicate experiments. **(E)** (Left) Representative  
367 immunoblot analysis of CIRBP protein isoforms in mock- and TG-treated (500nM, 16 h) Huh7  
368 cells. HSPA5 and GADD34 are positive controls. (Right) Quantification of CIRBP protein isoform  
369 expression relative to tubulin from replicate experiments. **(F)** Schematic of WT and m<sup>6</sup>A-mut  
370 *CIRBP* splicing reporters. RT-qPCR primer locations (F<sub>luc</sub>-R<sub>luc</sub>: control; F-R<sub>L</sub>: long isoform specific;  
371 F-R<sub>S</sub>: short isoform specific) are indicated with arrows. **(G)** RT-qPCR analysis of *CIRBP* splicing  
372 reporter isoform expression (S and L) relative to control *RLuc* amplicon in Huh7 cells transfected  
373 with WT and m<sup>6</sup>A-mut constructs. Values are the mean ± SEM of 3 (B, D, E, G) or 5 (C) biological  
374 replicates. \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001 by unpaired Student's t test. n.s. = not significant.  
375

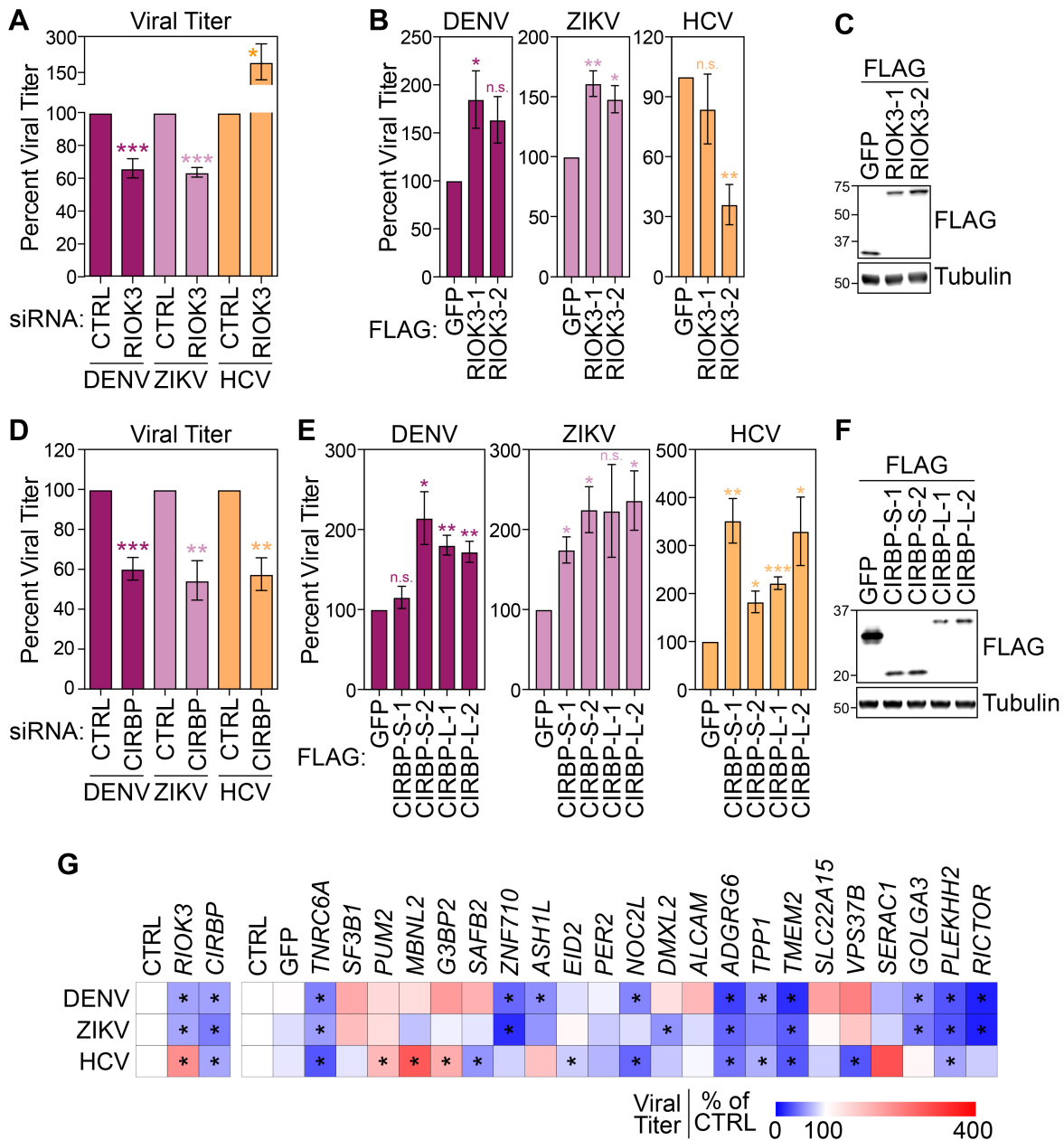
### 376 m<sup>6</sup>A-altered genes regulate *Flaviviridae* infection

377 Having found that both *RIOK3* and *CIRBP* transcripts have altered m<sup>6</sup>A modification during  
378 infection, we next tested whether their encoded protein products affect *Flaviviridae* infection. To  
379 this end, we depleted *RIOK3* and *CIRBP* in Huh7 cells using small interfering RNA (siRNA),  
380 infected these cells with DENV, ZIKV, or HCV, and then measured viral titer in the supernatant at  
381 72 hours post-infection. siRNA treatment reduced both *RIOK3* and *CIRBP* mRNA levels by ~70%  
382 and did not affect cell viability (Figure S5A). We found that *RIOK3* depletion significantly reduced  
383 the production of infectious DENV and ZIKV particles but increased the production of infectious  
384 HCV particles (Figure 5A). Consistent with these data, *RIOK3* stably overexpressed in two  
385 different clonal cell lines (*RIOK3*-1 and *RIOK3*-2) had the opposite effect on DENV, ZIKV, and  
386 HCV infectious particle production (Figure 5B-C). This suggests that *RIOK3* promotes DENV and  
387 ZIKV infection but inhibits HCV infection. In contrast, the depletion of *CIRBP* consistently reduced  
388 the production of infectious DENV, ZIKV, and HCV (Figure 5D), while overexpression of both the  
389 short and long isoforms of *CIRBP* in two different clonal cell lines (*CIRBP*-S-1 and 2, *CIRBP*-L-1  
390 and 2) increased infection by these viruses (Figure 5E-F).

391 We then performed a targeted siRNA screen to test whether other transcripts with  
392 infection-altered m<sup>6</sup>A modification affect *Flaviviridae* infection. We depleted transcripts in which  
393 we had identified m<sup>6</sup>A changes during infection (either co-regulated (Figure 1F) or virus-specific  
394 (Figure S11)), infected depleted cells with DENV, ZIKV, or HCV, and measured cell viability,  
395 relative RNA depletion levels, and the production of infectious virions in the supernatant at 48  
396 hours post-infection (Figure 5G and S5A-B). We focused only on those transcripts that were

397 depleted by at least 40% in our further analysis (21 out of 24 tested). For these, we found that  
 398 86% (18/21) regulate at least 1 virus, while 10/21 affect at least 2, and 6/21 regulate all three  
 399 viruses. For each virus, ~50% of m<sup>6</sup>A-altered transcripts that we tested significantly increased or  
 400 decreased infection. This indicates that by modifying specific transcripts that modulate infection,  
 401 m<sup>6</sup>A can tune the outcome of infection.

**Figure 5**



402 **Figure 5: Genes with infection-induced m<sup>6</sup>A alterations regulate *Flaviviridae* infection. (A)**  
 403 **Focus-forming assay (FFA) of supernatants harvested from DENV, ZIKV, or HCV-infected (72**  
 404 **hpi) Huh7 cells treated with non-targeting control (CTRL) or *RIOK3* siRNA. (B) FFA of**

405 supernatants harvested from DENV, ZIKV, or HCV-infected (72 hpi) Huh7 cells stably  
406 overexpressing FLAG-GFP or FLAG-RIOK3 (2 independent clones). **(C)** Immunoblot analysis of  
407 cell lines used in (B). **(D)** FFA of supernatants harvested from DENV, ZIKV, or HCV-infected (72  
408 hpi) Huh7 treated with CTRL or *CIRBP* siRNA. **(E)** FFA of supernatants harvested from DENV,  
409 ZIKV, or HCV-infected (72 hpi) Huh7 cells stably overexpressing FLAG-GFP or the short (FLAG-  
410 CIRBP-S) or long (FLAG-CIRBP-L) isoforms of CIRBP (2 independent clones). **(F)** Immunoblot  
411 analysis of cell lines used in (C). **(G)** Summary of data from targeted siRNA depletion experiments.  
412 Viral titers were determined by FFA of supernatants harvested from infected cells (48 hpi) treated  
413 with the indicated siRNAs. Data are presented as percentage of titer of each virus relative to cells  
414 treated with CTRL siRNA. \* indicates significance. Values are the mean  $\pm$  SEM of 4 (A and D), or  
415 3 (B, E, G) experiments. All viral infections for experiments in this figure were performed at a  
416 multiplicity of infection of 0.2. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  by unpaired Student's t test.  
417 n.s. = not significant.

418

## 419 Discussion

420 Here, we identify changes in m<sup>6</sup>A methylation of cellular mRNAs during infection by  
421 viruses in the *Flaviviridae* family, specifically DENV, ZIKV, WNV, and HCV. We observed that  
422 infection by all of these viruses leads to changes in m<sup>6</sup>A modification of a specific set of cellular  
423 transcripts, including some that encode factors that modulate *Flaviviridae* infection in Huh7 cells.  
424 We found that virus-induced pathways, including innate immune signaling and ER stress  
425 responses, contributed to altered m<sup>6</sup>A modification of at least two of these transcripts during  
426 infection. Taken together, this work suggests that m<sup>6</sup>A epitranscriptomic changes induced through  
427 cellular signaling pathways influence *Flaviviridae* infection.

428 We identified hundreds of m<sup>6</sup>A-modified transcripts that were differentially expressed  
429 during infection or that were annotated as part of cellular pathways relevant for infection. These  
430 findings suggest that m<sup>6</sup>A has the potential to post-transcriptionally regulate many genes during  
431 infection. Here, we focused on specific transcripts with virus-induced m<sup>6</sup>A changes; we identified  
432 58 peak changes in 51 transcripts following infection by DENV, ZIKV, WNV, and HCV. As our  
433 m<sup>6</sup>A change analysis pipeline controls for changes in gene expression, these data should  
434 represent true changes in m<sup>6</sup>A modification rather than changes in the expression of m<sup>6</sup>A-  
435 modified transcripts. While changes in both m<sup>6</sup>A modification and the expression of m<sup>6</sup>A-modified  
436 transcripts are biologically relevant, identifying *bona fide* m<sup>6</sup>A alterations will allow us to  
437 understand how m<sup>6</sup>A modification of cellular mRNA is regulated.

438 Our work reveals that the changes in m<sup>6</sup>A methylation of *RIOK3* and *CRIBP* can be driven  
439 by innate immune induction and the cellular response to ER stress, respectively. This suggests  
440 that these signals, and likely other infection-induced pathways, can be integrated into differential  
441 m<sup>6</sup>A methylation activity and ultimately affect m<sup>6</sup>A modification of cellular mRNAs. Indeed, it is



442 likely that other cellular signaling pathways stimulated by infection can also influence m<sup>6</sup>A  
443 modification of cellular transcripts. While changes in the expression of the m<sup>6</sup>A machinery have  
444 been shown to affect m<sup>6</sup>A modification during cancer and infection (Barbieri et al., 2017; Li et al.,  
445 2017b; Lin et al., 2016a; Rubio et al., 2018; Vu et al., 2017; Winkler et al., 2019), the expression  
446 of this machinery did not change with *Flaviviridae* infection, pointing to a different mechanism for  
447 altered m<sup>6</sup>A modification. Going forward, identifying the molecular mechanisms through which  
448 these signaling pathways lead to differential m<sup>6</sup>A modification during infection will be an important  
449 advance in understanding how the cellular m<sup>6</sup>A machinery selects specific sites for modification.

450 Thus far, our data suggest that the virus-induced m<sup>6</sup>A changes we observed occur in  
451 nascent mRNA, which is consistent with the hypothesis that m<sup>6</sup>A is added co-transcriptionally and  
452 does not dynamically change at single sites after export to the cytoplasm (Ke et al., 2017). At  
453 least three processes could modulate the selective m<sup>6</sup>A modification of specific transcripts during  
454 transcription and explain the changes we observed. First, novel interactions of the m<sup>6</sup>A writers  
455 METTL3 and METTL14 with viral-induced or stress-regulated RNA-binding proteins could target  
456 these writers to specific transcripts and lead to m<sup>6</sup>A epitranscriptomic changes during infection.  
457 For example, RBM15/15B and VIRMA can target the m<sup>6</sup>A methyltransferase complex to *Xist* long  
458 non-coding RNA or to the 3' UTRs of mRNA respectively (Patil et al., 2016; Yue et al., 2018).  
459 Second, the writers could have differential recruitment to nascent mRNAs by the histone  
460 modification H3K36me3 which marks transcriptionally active loci and is known to recruit METTL14  
461 (Huang et al., 2019). Intriguingly, the *CIRBP* locus is marked by H3K36me3 in untreated HepG2  
462 hepatocellular carcinoma cells, and its transcript contains an m<sup>6</sup>A peak at the same site as we  
463 identified in Huh7 cells (Huang et al., 2019). This suggests that infection- or ER stress-induced  
464 depletion of H3K36me3 marks at the *CIRBP* locus could result in reduced m<sup>6</sup>A modification of  
465 *CIRBP* mRNA by METTL3/METTL14. Third, changes in transcription rates, which have been  
466 inversely correlated with m<sup>6</sup>A deposition in mRNA, could also contribute to m<sup>6</sup>A modification of  
467 specific transcripts during infection (Slobodin et al., 2017). Additionally, viral infection can affect  
468 RNA structure in cellular transcripts; it is possible that altered mRNA structure could result in  
469 altered m<sup>6</sup>A modification of cellular transcripts during infection (Mizrahi et al., 2018). Perturbing  
470 cellular homeostasis by *Flaviviridae* infection therefore has the potential to reveal new insights  
471 into how m<sup>6</sup>A modification of cellular transcripts is regulated.

472 We hypothesize that during viral infection m<sup>6</sup>A regulation of RNA metabolism can lead to  
473 rapid, tunable changes in mRNA and protein abundance of host factors. Our work suggests that  
474 m<sup>6</sup>A modification promotes translation of *RIOK3* and alternative splicing of *CIRBP*. While m<sup>6</sup>A can  
475 affect mRNA nuclear export and stability, *Flaviviridae* infection did not affect these processes for

476 either *RIOK3* or *CIRBP*. m<sup>6</sup>A has also been shown to promote translation of modified mRNAs in  
477 multiple contexts by mediating interactions with m<sup>6</sup>A-binding proteins including YTHDF1  
478 (Edupuganti et al., 2017; Han et al., 2019; Huang et al., 2018; Li et al., 2017a; Lin et al., 2016b;  
479 Meyer et al., 2015; Shi et al., 2017; Shi et al., 2018; Wang et al., 2019; Wang et al., 2015). We  
480 found that increased m<sup>6</sup>A in *RIOK3* mRNA during *Flaviviridae* infection promotes its translation.  
481 Interestingly, YTHDF1 also showed increased binding to *RIOK3* during infection. Given its role in  
482 recruiting translation factors to modified transcripts and promoting translation under some  
483 conditions (Han et al., 2019; Shi et al., 2018; Wang et al., 2019; Wang et al., 2015), YTHDF1  
484 binding to m<sup>6</sup>A in *RIOK3* may allow this transcript to be preferentially translated despite eIF2 $\alpha$   
485 phosphorylation and suppression of global translation during infection (Arnaud et al., 2010;  
486 Garaigorta and Chisari, 2009; Roth et al., 2017). For *CIRBP*, we found that loss of m<sup>6</sup>A following  
487 viral infection led to reduced expression of its long isoform. m<sup>6</sup>A has been shown to regulate  
488 splicing by modulating mRNA interactions with several m<sup>6</sup>A-binding splicing factors (Alarcon et  
489 al., 2015; Liu et al., 2015; Liu et al., 2017b; Louloui et al., 2018; Xiao et al., 2016; Ye et al., 2017;  
490 Zhao et al., 2014). We hypothesize that the loss of m<sup>6</sup>A in this transcript regulates alternative  
491 splicing through changes in the interactions between splicing factors and *CIRBP*. Further  
492 investigation into any differences in the roles of the proteins encoded by these two isoforms will  
493 help reveal the downstream functional consequences of changes in m<sup>6</sup>A in this gene. How m<sup>6</sup>A  
494 regulates the fate of other mRNAs with altered modification also remains unclear, but it is possible  
495 that m<sup>6</sup>A post-transcriptionally affects the abundance of their protein products or splicing isoforms,  
496 similar to how it regulates *RIOK3* and *CIRBP*.

497 Importantly, we found that transcripts with altered m<sup>6</sup>A modification during *Flaviviridae*  
498 infection encode protein products that can influence the outcome of infection. *RIOK3* expression  
499 correlated with the abundance of DENV and ZIKV RNA in a single-cell analysis of infected Huh7  
500 cells, suggesting that it might play a role in infection by these viruses (Zanini et al., 2018). We  
501 found that *RIOK3* expression and m<sup>6</sup>A modification was increased with infection by DENV, ZIKV,  
502 WNV, and HCV. Further, we found that *RIOK3* promoted DENV and ZIKV infection, but inhibited  
503 HCV. Interestingly, *RIOK3* has been found to both positively and negatively regulate innate  
504 immune responses, by either stimulating the interaction between TBK1 and IRF3 or by  
505 phosphorylating and inactivating MDA5 (Feng et al., 2014; Shan et al., 2009; Takashima et al.,  
506 2015; Willemsen et al., 2017). The differences in the effects of *RIOK3* on DENV, ZIKV, and HCV  
507 infection could reflect the different strategies used by these viruses to inhibit host immune  
508 responses (Chen et al., 2017; Gack and Diamond, 2016; Gokhale et al., 2014). Further,  
509 Willemsen et al. found that while *RIOK3* enhanced innate immune activation, it also promoted

510 influenza A virus infection, implying that RIOK3 could have roles in infection beyond innate  
511 immunity (Willemsen et al., 2017). Unlike RIOK3, depletion of CIRBP (using siRNA that targets  
512 both the small and large isoform) decreased viral replication for DENV, ZIKV, and HCV,  
513 suggesting a consistently proviral role. Therefore, during infection, reduction in the long isoform  
514 of CIRBP through loss of m<sup>6</sup>A could inhibit infection, suggesting that this loss of m<sup>6</sup>A during  
515 infection is part of the host response to infection. CIRBP can modulate the translation of pro-  
516 inflammatory factors and have anti-apoptotic effects in response to various stresses (Liao et al.,  
517 2017), although the roles of different CIRBP isoforms remains unknown. We also tested whether  
518 other transcripts with altered m<sup>6</sup>A following *Flaviviridae* could regulate viral infection. For each  
519 virus, approximately half of the factors that we tested that were efficiently depleted showed either  
520 proviral or antiviral effects, while in total 86% had roles on any virus. These data suggest that m<sup>6</sup>A  
521 itself does not represent a proviral or antiviral mechanism during infection, but rather modulates  
522 transcripts that ultimately affect the outcome of infection by different members of the *Flaviviridae*  
523 family.

524         The scale of m<sup>6</sup>A epitranscriptomic changes with virus infection varies greatly among  
525 previous reports (Hesser et al., 2018; Lichinchi et al., 2016a; Rubio et al., 2018; Tan et al., 2018;  
526 Winkler et al., 2019). Although we identified altered m<sup>6</sup>A in 58 peaks in 51 transcripts during  
527 infection, inherent variance in transcript coverage in MeRIP-seq data means that many replicates  
528 are necessary for statistically significant detection of m<sup>6</sup>A changes (McIntyre et al., 2019). In  
529 particular, this means that our analysis, which used data from three replicates per virus, may  
530 underestimate the total number of virus-specific, altered m<sup>6</sup>A peaks. Additionally, we used a more  
531 conservative statistical approach than many previous studies to reveal only the most robust peak  
532 changes (McIntyre et al., 2019). The changes detected in MeRIP-seq peaks were validated using  
533 MeRIP-RT-qPCR; however, these data do not provide the precise ratio of modified to unmodified  
534 copies of a transcript or the exact nucleotides that are modified. Biochemical assays like  
535 SCARLET or new sequencing methods will be necessary to resolve this question in the future  
536 (Liu et al., 2019; Saletore et al., 2012).

537         In summary, we found that *Flaviviridae* infection leads to m<sup>6</sup>A changes in transcripts that  
538 can influence viral infection. We identified innate immune activation and the ER stress response  
539 as signals that can modulate m<sup>6</sup>A levels in specific cellular mRNAs. Our work indicates that post-  
540 transcriptional regulation of specific transcripts by m<sup>6</sup>A and other RNA modifications can be an  
541 important determinant of the outcome of infection. Indeed, viral infection alters the abundance of  
542 several other epitranscriptomic modifications on cellular RNA (McIntyre et al., 2018), revealing

543 that we are only at the beginning of our understanding of how m<sup>6</sup>A and other RNA modifications  
544 can influence viral infection.

545

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562

## 563 **Author contributions**

564 Conceptualization: N.S.G., A.B.R.M., C.E.M., and S.M.H. Investigation: N.S.G., A.B.R.M.,  
565 C.L.H., H.M.L., and M.D.M. Formal analysis: A.B.R.M. and N.S.G. Software: A.B.R.M.  
566 Visualization: N.S.G. and A.B.R.M. Writing – original draft: N.S.G., A.B.R.M., and S.M.H. Writing  
567 – review and editing: N.S.G., A.B.R.M., C.L.H., H.M.L., M.D.M., C.E.M., and S.M.H. Funding  
568 acquisition: N.S.G., A.B.R.M., C.L.H., H.M.L., C.E.M., and S.M.H.

569

## 570 **Competing interests**

571 C.E.M. is a cofounder and board member for Biotia and Onegevity Health, as well as an  
572 advisor or compensated speaker for Abbvie, Acuamark Diagnostics, ArcBio, Bio-Rad, DNA  
573 Genotek, Genialis, Genpro, Illumina, New England Biolabs, QIAGEN, Whole Biome, and Zymo  
574 Research.

575

## 576 **Methods**

577  
578 **Cell culture, viral stocks, and viral infection.** Huh7 and Huh-7.5 cells (gift of Dr. Michael Gale  
579 Jr., University of Washington (Sumpter et al., 2005)), 293T cells (ATCC: CRL-3216) Vero cells  
580 (ATCC: CCL-81), C6/36 (ATCC: CRL-1660) were grown in Dulbecco's modification of Eagle's  
581 medium (DMEM; Mediatech) supplemented with 10% fetal bovine serum (HyClone), 25 mM  
582 HEPES (Thermo Fisher), and 1X non-essential amino acids (Thermo Fisher), referred to as  
583 complete DMEM (cDMEM). Huh7 and Huh-7.5 cells were verified using the Promega GenePrint  
584 STR kit (DNA Analysis Facility, Duke University), and cells were verified as mycoplasma free by  
585 the LookOut Mycoplasma PCR detection kit (Sigma-Aldrich). Infectious stocks of a cell culture-  
586 adapted strain of genotype 2A JFH1 HCV were generated and titered in Huh-7.5 cells by focus-  
587 forming assay (FFA), as described (Aligeti et al., 2015). DENV2-NGC (Sessions et al., 2009),  
588 ZIKV-PR2015 (Quicke et al., 2016), and WNV-NY2000 (Diamond et al., 2003) stocks were  
589 prepared in C6/36 insect cells and titered in Vero cells, as described. For viral infections, cells  
590 were incubated in a low volume of cDMEM containing virus at a multiplicity of infection (MOI) of  
591 one for 2-3 hours (except when otherwise stated), following which cDMEM was replenished. Cells  
592 were infected for 48 hours unless otherwise described. To quantify virus, cellular supernatants  
593 were analyzed by FFA.

594  
595 **MeRIP-seq and MeRIP-RT-qPCR.** Sample preparation: Huh7 cells seeded in 15 cm plates were  
596 infected with DENV, ZIKV, WNV, or HCV (MOI 1) or left uninfected (mock-infected). At 48 hours  
597 post-infection, total RNA was extracted using TRIzol (Thermo Fisher) and treated with TURBO  
598 DNase I (Thermo Fisher). mRNA was purified from 200 µg total RNA from each sample using the  
599 Dynabeads mRNA purification kit (Thermo Fisher) and concentrated by ethanol precipitation.  
600 mRNA was fragmented using the RNA Fragmentation Reagent (Thermo Fisher) for 15 minutes  
601 and purified by ethanol precipitation. MeRIP was performed using EpiMark N6-methyladenosine  
602 Enrichment kit (NEB) according to the manufacturer's recommendations with the following  
603 modifications. Briefly, 25 µL Protein G Dynabeads (Thermo Fisher) per sample were washed  
604 three times in MeRIP buffer (150 mM NaCl, 10 mM Tris-HCl [pH 7.5], 0.1% NP-40), and incubated  
605 with 1 µL anti-m<sup>6</sup>A antibody for 2 hours at 4°C with rotation. After washing three times with MeRIP  
606 buffer, anti-m<sup>6</sup>A conjugated beads were incubated with purified mRNA with rotation at 4°C  
607 overnight in 300 µL MeRIP buffer with 1 µL RNase inhibitor (recombinant RNasin; Promega). 10%  
608 of the mRNA sample was saved as the input fraction. Beads were then washed twice with 500 µL  
609 MeRIP buffer, twice with low salt wash buffer (50 mM NaCl, 10 mM Tris-HCl [pH 7.5], 0.1% NP-  
610 40), twice with high salt wash buffer (500 mM NaCl, 10 mM Tris-HCl [pH 7.5], 0.1% NP-40), and

611 once again with MeRIP buffer. m<sup>6</sup>A-modified RNA was eluted twice in 100  $\mu$ L of MeRIP buffer  
612 containing 5 mM m<sup>6</sup>A salt (Santa Cruz Biotechnology) for 30 minutes at 4°C with rotation. Eluates  
613 were pooled and concentrated by ethanol purification. RNA-seq libraries were prepared from both  
614 eluate and 10% input mRNA using the TruSeq mRNA library prep kit (Illumina), subjected to  
615 quality control (MultiQC), and sequenced on the HiSeq 4000 instrument.

616 For MeRIP-RT-qPCR, total RNA was harvested from cells with the indicated treatments  
617 in 10 cm plates or 6-well plates. For ER-stress induction, cells seeded in 6-well plates were treated  
618 with 500 nM thapsigargin (Tocris) for 16 hours. HCV PAMP was prepared by *in vitro* transcription,  
619 as described (Beachboard et al., 2019; Saito et al., 2008). 2.5  $\mu$ g of HCV PAMP RNA was  
620 transfected into cells seeded in 6-well plates using the Mirus mRNA transfection kit. 8 hours later,  
621 RNA was extracted and MeRIP-RT-qPCR was performed like MeRIP-seq with some differences.  
622 Specifically, total RNA was prepared from cells using TRIzol, and diluted to equivalent  
623 concentrations. Then, 20-50  $\mu$ g total RNA was fragmented for 3 minutes, purified by ethanol  
624 precipitation, and resuspended in 30  $\mu$ L water. 0.1 fmol of positive control (m<sup>6</sup>A-modified *Gaussia*  
625 luciferase RNA) and negative control (unmodified *Cypridina* luciferase RNA) spike-ins supplied  
626 with the EpiMark N6-methyladenosine Enrichment kit were added to each sample. Following  
627 MeRIP as described above, eluates were concentrated by ethanol precipitation. 1  $\mu$ L input and  
628 the entire IP fractions were reverse transcribed using the iScript cDNA synthesis kit (BioRad) and  
629 subjected to RT-qPCR. Primer sequences are supplied in Table S3. Relative m<sup>6</sup>A level for each  
630 transcript was calculated as the percent of input in each condition normalized to that of the  
631 respective positive control spike-in. Fold change of enrichment was calculated with mock samples  
632 normalized to 1.

633 Data analysis: Reads were aligned using STAR (Dobin et al., 2013) to the human  
634 reference genome (hg38), combined with the appropriate virus genome for each infected sample.  
635 Differential gene expression between infected and uninfected samples was compared using  
636 DESeq2 (Love et al., 2014). UpSet plots of the intersects between genes regulated with individual  
637 viruses were generated using UpSetR (Conway et al., 2017). Gene ontology for RNA-seq  
638 changes in Figure S1D was analyzed using gProfiler, with redundant GO terms collapsed using  
639 REVIGO (Reimand et al., 2016; Supek et al., 2011). For gProfiler, upregulated genes with Log<sub>2</sub>FC  
640  $\geq 2$  and adjusted p-value  $< 0.05$  with all viruses were considered. There were very few consistently  
641 downregulated genes at Log<sub>2</sub>FC  $\leq -2$  (particularly for ZIKV), so we expanded our set to genes  
642 with smaller Log<sub>2</sub>FC  $\leq -0.5$ , downregulated by DENV, HCV, and WNV infection. For REVIGO, we  
643 allowed similarity of up to 0.5, with semantic similarity calculated using SimRel. Adjusted p-values  
644 were provided for the REVIGO calculations. Gene set enrichment analyses using fgsea in R

645 showed similar differentially regulated pathways as gProfiler (Sergushichev, 2016). “Infection-  
646 annotated” genes and peaks were summarized for Figure 1B based on gene inclusion in  
647 “Infectious disease”, “Unfolded Protein Response (UPR)”, “Interferon Signaling”, and “Innate  
648 Immune System” Reactome pathways from fgsea.

649 We called m<sup>6</sup>A peaks from MeRIP-seq using MACS2 (Zhang et al., 2008) and used all  
650 peaks detected in at least two replicates for further analysis. Motif enrichment was calculated  
651 using HOMER for Figure 1C (Heinz et al., 2010). Metagene plots for methylated DRACH motifs  
652 were plotted using a custom script. DRACH motifs were considered methylated if detected under  
653 m<sup>6</sup>A peaks in at least 2 biological replicates. Relative positions of m<sup>6</sup>A peaks within genes are  
654 based on the transcripts with the highest mean coverage per gene, as calculated with kallisto  
655 (Bray et al., 2016).

656 We identified m<sup>6</sup>A peaks changes using a generalized linear model (adapted from (Park  
657 et al., 2014)), and the QNB program (Liu et al., 2017a). In brief (see Park et al., 2014 or McIntyre  
658 et al., 2019 for more details), a generalized linear model following the equation

$$\log \mu_{ij} = \beta_0 + \beta_{IPi} X_{IPj} + \beta_{VIRi} X_{VIRj} + \beta_{IP.VIRi} X_{IP.VIRj} + \log N_j.$$

660 was fit with the following parameters for each peak i and sample j:  $X_{IP} = 1$  for immunoprecipitated  
661 samples and 0 for input samples, and  $X_{VIR} = 1$  for infected samples and 0 for mock. A library size  
662 parameter was included for normalization (N) with edgeR (Robinson et al., 2010). The full model  
663 was compared to a reduced model without the infection:IP interaction term using a likelihood ratio  
664 test of the difference between deviances, implemented through DESeq2 (Love et al., 2014) or  
665 edgeR. To control for changes in gene expression, changes in gene expression were subtracted  
666 from changes in IP peak reads for significantly modified peaks from DESeq2, edgeR, and QNB,  
667 with a threshold for absolute difference in Log<sub>2</sub> fold change of  $\geq 1$ . Significant peaks were further  
668 filtered for location within exons, DRACH motif content, and mean input read counts of  $\geq 10$  to  
669 produce the final set of 58 peak changes.

670 Peaks of interest were plotted for visual evaluation using CovFuzze ([https://github.com/al-  
671 mcintyre/CovFuzze](https://github.com/al-mcintyre/CovFuzze)) (Imam et al., 2018).

672  
673 **RT-qPCR.** The iScript cDNA synthesis kit (Bio-Rad) was used for reverse transcription of total  
674 RNA samples. RT-qPCR was performed using the Applied Biosystems QuantStudio 6 Flex real-  
675 time PCR instrument. To measure relative abundance of *CIRBP* isoforms, total RNA was reverse  
676 transcribed with the Superscript III enzyme (Invitrogen) using a gene specific primer. RT-qPCR  
677 was performed using specific primers that detect *CIRBP* isoforms. The expression of each isoform  
678 was normalized to invariant region of *CIRBP*. Primer sequences are provided in Table S3.

679  
680 **Immunoblotting.** Cell lysates were prepared in a modified RIPA buffer (10 mM Tris [pH 7.5], 150  
681 mM NaCl, 0.5% sodium deoxycholate, and 1% Triton X-100) supplemented with protease inhibitor  
682 cocktail (Sigma-Aldrich) and phosphatase inhibitor cocktail II (Millipore), and clarified by  
683 centrifugation. Protein concentration was determined by Bradford assay (Bio-Rad). 5-15  $\mu$ g of  
684 protein was resolved by SDS/PAGE and transferred to nitrocellulose membranes using the Trans-  
685 Blot Turbo System (Bio-Rad). Membranes were blocked in 5% milk in phosphate buffered saline  
686 with 0.1% Tween (PBS-T) and incubated with the relevant primary antibodies. After washing three  
687 times with PBS-T, membranes were incubated with species-specific horseradish peroxidase-  
688 conjugated antibodies (Jackson ImmunoResearch, 1:5000) or fluorescent antibodies (LI-COR,  
689 IRDye 800, 1:5000). Chemiluminescence (Clarity ECL, Bio-Rad) or fluorescence was detected  
690 on a LI-COR Odyssey Fc instrument and analyzed using the ImageStudio software. The following  
691 antibodies were used for immunoblot: anti-METTL3 (Novus Biologicals, 1:1000), anti-METTL14  
692 (Sigma-Aldrich, 1:5000), anti-FTO (Abcam, 1:1000), anti-YTHDF1 (Proteintech, 1:1000), anti-  
693 YTHDF2 (Proteintech, 1:1000), anti-YTHDF3 (Sigma-Aldrich, 1:1000), anti-ALKBH5 (Sigma-  
694 Aldrich, 1:1000), anti-WTAP (Proteintech, 1:1000) anti-FLAG M2 (Sigma-Aldrich, 1:5000), anti-  
695 tubulin (Sigma-Aldrich, 1:5000), anti-HCV NS5A (clone 9E10, gift of Charles Rice, Rockefeller  
696 University (Lindenbach et al., 2005), 1:1000), anti-RIOK3 (Proteintech, 1:1000), anti-CIRBP  
697 (Proteintech 1:1000), anti-DENV NS3 (GeneTex, 1:1000), anti-ZIKV NS3 (GeneTex, 1:1000),  
698 anti-HCV NS4A (Genscript custom (Horner et al., 2011)), 1:1000), anti-eIF2 $\alpha$  (Cell Signaling,  
699 1:1000), anti-phospho-eIF2 $\alpha$  (Cell Signaling, 1:1000), anti-GADD34 (Proteintech, 1:1000), anti-  
700 HSPA5 (Cell Signaling, 1:1000), anti-H2A.X (Cell Signaling, 1:1000), anti-U170K serum (gift of  
701 Dr. Jack Keene, Duke University, (Query and Keene, 1987), 1:1000)

702  
703 **FLAG-YTHDF RNA immunoprecipitation.** Generation of Huh7 cells stably expressing FLAG-  
704 GFP or FLAG-YTHDF1 was described previously (Gokhale et al., 2016). Cells seeded in 6-well  
705 plates were infected with DENV, ZIKV, or HCV (MOI 1). At 48 hours post-infection cells were  
706 harvested by trypsinization and lysed in polysome lysis buffer (100 mM KCl, 5 mM MgCl<sub>2</sub>, 10 mM  
707 HEPES [pH 7.0], 0.5% NP-40), supplemented with protease inhibitor cocktail (Sigma-Aldrich) and  
708 RNase inhibitor (RNasin), and cleared by centrifugation. Protein was quantified by Bradford  
709 assay, and 200  $\mu$ g ribonucleoprotein complexes were immunoprecipitated with M2 anti-FLAG  
710 conjugated magnetic beads (Sigma-Aldrich) overnight at 4°C with rotation in NT2 buffer (50 mM  
711 Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM MgCl<sub>2</sub>, 0.05% NP-40). Beads were washed five times in  
712 ice-cold NT2 buffer. Protein for immunoblotting was eluted from ten percent of beads by boiling



713 in 2X Laemmli sample buffer (Bio-Rad). RNA was extracted from ninety percent of beads using  
714 TRIzol reagent (Thermo Fisher). Equal volumes of eluted RNA were used for cDNA synthesis,  
715 quantified by RT-qPCR, and normalized to RNA levels in input samples. Fold enrichment was  
716 calculated with FLAG-GFP and mock samples set as 1.

717 **siRNA treatment and viral infectivity assays.** Cells seeded in 24-well plates were transfected  
718 with siRNA against intended targets (Qiagen, sequences provided in Table S3) using  
719 Lipofectamine RNAiMAX (Thermo Fisher) according to the manufacturer's recommendation. At  
720 24 hours post-transfection, cells were infected with DENV, ZIKV, and HCV (MOI 0.2). At 48  
721 (targeted siRNA screen) or 72 (RIOK3 and CIRBP depletion) hours post-infection, virus titer in  
722 the supernatant was measured by FFA. Serial dilutions of supernatants were used to infect naïve  
723 Vero (DENV and ZIKV) or Huh-7.5 (HCV) cells in triplicate wells of a 48-well plate. At 72 hours  
724 post-infection, cells were fixed in cold 1:1 methanol:acetone and immunostained with 4G2  
725 antibody purified in the lab from a hybridoma (for DENV and ZIKV, 1:2000), or anti-HCV NS5A  
726 (1:2000). Following binding of horseradish peroxidase conjugated secondary antibody (1:1000;  
727 Jackson ImmunoResearch), infected foci were visualized with the VIP Peroxidase Substrate Kit  
728 (Vector Laboratories) and counted at 40X magnification. Titer was calculated using the following  
729 formula: (dilution factor x number of foci x 1000) / volume of infection ( $\mu$ l), resulting in units of  
730 focus forming units / mL (FFU/mL). Depletion of siRNA targets was confirmed by RT-qPCR  
731 (primer sequences in Table S3). Cellular viability after siRNA treatment was measured by the  
732 Cell-Titer Glo assay (Promega) according to the manufacturer's recommendation.

733  
734 **Quantification of infection by immunofluorescence.** To measure percent of cells infected  
735 following viral infection, Huh7 cells seeded in 96-well plates were infected with DENV, ZIKV,  
736 WNV, or HCV (MOI 1). Cells were fixed in cold 1:1 methanol:acetone at the indicated hours post-  
737 infection, and immunostained with 4G2 antibody (DENV, ZIKV, WNV) or anti-HCV NS5A.  
738 Following binding of AlexaFluor 488-conjugated secondary antibody (Thermo Fisher) and nuclear  
739 staining with Hoechst (Thermo Fisher), cells were imaged using the Cellomics Arrayscan VTI  
740 robotic microscope. The percentage of infected cells was determined by measuring cells stained  
741 for viral antigen relative to the total number of nuclei.

742  
743 **Cell fractionation.** Fractionation of cells to isolate chromatin-associated RNA was performed as  
744 described (Ke et al., 2017). Briefly, cells were collected from 10 cm plates by trypsinization, lysed  
745 in 200  $\mu$ L cytoplasmic lysis buffer (10 mM Tris-HCL [pH 7.4], 150 mM NaCl, 0.15% NP-40) on ice  
746 for 5 minutes, and passed through 500  $\mu$ l 24% sucrose cushion by centrifugation at 12000 xG for

747 10 minutes at 4°C. The supernatant (cytoplasmic fraction) was then removed and the nuclear  
748 pellet was rinsed twice with cold phosphate buffered saline (PBS). The nuclear pellet was  
749 resuspended in 100 µL ice cold glycerol buffer (20 mM Tris-HCL [pH 7.4], 75 mM NaCl, 0.5 mM  
750 EDTA, 1 mM DTT, 125 µM PMSF, 50% glycerol). 100 µL nuclear lysis buffer (10 mM HEPES [pH  
751 7.4], 1 mM DTT, 7.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 300 mM NaCl, 1 M urea, 1% NP-40) was added  
752 to the suspension, followed by brief vortexing, and incubation on ice for 2 minutes. Samples were  
753 centrifuged for 2 minutes at 4°C at 12000 xG and the supernatant (nuclear fraction) was removed.  
754 The chromatin pellet was rinsed twice with cold PBS, resuspended in 50 µL DNase I buffer with  
755 2 U Turbo DNase I (Invitrogen), and incubated at 37°C for 30 minutes. RNA was then extracted  
756 from the chromatin fraction using TRIzol reagent and subjected to MeRIP-RT-qPCR. The  
757 cytoplasmic, nuclear, and chromatin fractions were subjected to immunoblotting to analyze  
758 fractionation.

759 For nuclear/cytoplasmic fractionation to investigate mRNA export, uninfected and infected  
760 (MOI 1) cells grown in 10 cm plates were harvested by trypsinization and lysed in 200 µL lysis  
761 buffer (10mM Tris-HCl [pH 7.4], 140 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 10 mM EDTA, 0.5% NP-40) on ice  
762 for 5 minutes. Following centrifugation at 12000 xG at 4°C for 5 minutes, the supernatant  
763 (cytoplasmic fraction) was collected, and the nuclear pellet was rinsed twice with lysis buffer. RNA  
764 was extracted from cytoplasmic and nuclear pellets using TRIzol reagent and analyzed by RT-  
765 qPCR.

766  
767 **Measurement of RNA stability.** Cells plated in 24-well plates were infected with the indicated  
768 virus (MOI 1). At 36 hours post-infection, media was changed to cDMEM containing 1 µM  
769 Actinomycin D (Sigma-Aldrich). RNA was extracted from cells at the indicated time points post-  
770 treatment using TRIzol reagent and analyzed by RT-qPCR. Data were normalized as the percent  
771 of RNA remaining at each time point after treatment, relative to that at the time of treatment.

772  
773 **Cloning of *RIOK3* and *CIRBP* and generation of stable cell lines.** All primer sequences used  
774 for cloning are provided in Table S3. *RIOK3* (NM\_003831.4) and both long (NM\_001300829) and  
775 short (NM\_001280) isoforms of *CIRBP* were cloned by PCR (HiFi PCR premix, Clontech) from  
776 cDNA from Huh7 cells prepared with the Superscript III RT kit (Thermo Fisher) using the  
777 oligo(dT)<sub>20</sub> primer. PCR products were inserted into pLEX-FLAG lentiviral vector between the *NotI*  
778 and *XhoI* sites using the InFusion HD cloning kit (Takara Bio) to generate constructs with N-  
779 terminal FLAG tags. Lentivirus was produced from 293T cells transfected with pLEX vectors and  
780 packaging plasmids psPAX2 and pMD2.G (provided by Duke Functional Genomics Facility).

781 Huh7 cells were transduced by these lentiviruses and stable cell lines expressing FLAG-RIOK3,  
782 FLAG-CIRBP-S, and FLAG-CIRBP-L were selected using puromycin (2  $\mu$ g/mL). Single cell clones  
783 were obtained by serial dilution and verified by immunoblotting. Cell lines were maintained in  
784 cDMEM containing 1  $\mu$ g/mL puromycin.

785  
786 **Reporter cloning and luciferase assays.** All primer and gBlock sequences are provided in Table  
787 S3. To generate m<sup>6</sup>A-null *RIOK3* reporters, the *Renilla* and Firefly luciferase genes in psiCheck2  
788 plasmid (Promega) were first replaced by constructs with synonymous mutations in putative m<sup>6</sup>A  
789 sites (obtained as IDT gBlocks). The wild type *RIOK3* 3' UTR was cloned from Huh7 cDNA  
790 (NM\_003831.4) and inserted after the m<sup>6</sup>A-null *Renilla* luciferase gene in the multiple cloning site  
791 of psiCheck2 between *XhoI* and *NotI* using the InFusion HD kit. m<sup>6</sup>A-mut *RIOK3* 3' UTR (in which  
792 all putative m<sup>6</sup>A sites were mutated from A to T) was obtained as a gBlock and also inserted  
793 between these restriction sites. WT and m<sup>6</sup>A-mut *RIOK3* reporter plasmids along with the pcDNA-  
794 Blast plasmid (Kennedy et al., 2015) were linearized using *BamHI* and *BglII* respectively, purified  
795 by ethanol precipitation and co-transfected into Huh7 cells in 6-well plates (90 ng reporter, 10 ng  
796 pcDNA-Blast) using FuGENE 6 transfection reagent (Promega). Cells were selected with  
797 blasticidin (0.2  $\mu$ g/mL) and single cell clones stably expressing WT and m<sup>6</sup>A-mut reporters were  
798 isolated. For MeRIP-RT-qPCR of reporter RNA, WT and m<sup>6</sup>A-mut expressing cells were plated in  
799 6-well plates, infected with the indicated virus (MOI 1), and RNA was extracted using TRIzol at  
800 48 hours post-infection. Following MeRIP as described, RT-qPCR was performed to discriminate  
801 reporter RNA using a forward primer within the *Renilla* luciferase gene and a reverse primer in  
802 the *RIOK3* 3' UTR. For luciferase assays, WT and m<sup>6</sup>A-mut expressing cells in 24-well plates  
803 were infected with the indicated virus (MOI 1) and dual luciferase assay (Promega) was performed  
804 at 48 hours post-infection according to the manufacturer's instructions. Data was normalized as  
805 the value of *Renilla* luminescence divided by Firefly luminescence, and values for mock-infected  
806 cells were set as 1.

807 To generate *CIRBP* splicing reporters, *CIRBP* exon 5 – 3' UTR (Hg38;chr19:127553-  
808 1273172) was amplified by PCR from genomic DNA. A fragment of m<sup>6</sup>A-null *Renilla* luciferase  
809 beyond the *Nrul* site and up to the stop codon was amplified by PCR with overlapping ends with  
810 *Renilla* luciferase (5'; before the *Nrul* site) and the *CIRBP* fragment (3'). These fragments were  
811 inserted into *Nrul-XhoI* digested psiCheck2 m<sup>6</sup>A-null plasmid using the InFusion HD kit. m<sup>6</sup>A-mut  
812 *CIRBP* reporter was generated by mutating the essential C in the m<sup>6</sup>A site synonymously to T  
813 using two rounds of site-directed mutagenesis with the QuikChange Lightning kit (Agilent).

814

815 **<sup>35</sup>S labeled immunoprecipitation for nascent translation.** Huh7 cells seeded in 10 cm plates  
816 were infected with DENV (MOI 1) or left uninfected. At 45 hours post-infection, media was  
817 removed and 3 mL warm methionine/cysteine-free DMEM was added to plates. After 15 minutes  
818 of incubation, 3 mL methionine/cysteine-free DMEM containing 100 mCi <sup>35</sup>S (Perkin Elmer) was  
819 added. Cells were harvested at 3 hours post-treatment and lysed in RIPA buffer. 300 µg protein  
820 was incubated with 4 µg anti-RIOK3 antibody (Proteintech) or normal rabbit IgG (Cell Signaling)  
821 in 300 µL RIPA buffer overnight at 4°C with rotation. Antibody-protein complexes were then  
822 incubated with 40 µL pre-washed protein G Dynabeads (Thermo Fisher) for 2 hours. Protein was  
823 eluted from beads in 2X Laemmli buffer. Eluates were resolved by SDS/PAGE. Gels were fixed  
824 in solution containing 50% methanol and 10% acetic acid, dried, and subjected to autoradiography  
825 on film.

826  
827 **LC-MS/MS for m<sup>6</sup>A/A determination.** mRNA was purified from 200 µg total RNA extracted from  
828 uninfected and infected Huh7 cells (MOI 1, 48 hours post-infection) using one round of polyA  
829 selection (Dynabeads mRNA purification kit; Thermo Fisher) and one round of rRNA depletion  
830 (NEBNext rRNA depletion kit, NEB). After ethanol precipitation, purified mRNA was digested into  
831 mononucleotides with nuclease P1 (Sigma-Aldrich, 2 U) in buffer containing 25 mM NaCl and 2.5  
832 mM ZnCl<sub>2</sub> for 2 hours at 37°C, followed by incubation with Antarctic Phosphatase (NEB, 5 U) for  
833 an additional 2 hours at 37°C. Nucleosides were separated and quantified using UPLC-MS/MS  
834 as previously described, except acetic acid was used in place of formic acid (Basanta-Sanchez  
835 et al., 2016).

836  
837 **Data Availability.** All raw data from MeRIP-seq analysis of uninfected and infected Huh7 cells  
838 are available through GEO (accession number: GSE130891).

839

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