Mosquito-borne flaviviruses such as dengue virus (DENV) and Zika virus (ZIKV) severely impact global health with an estimated 100 million individuals suffering from DENV-induced illness alone\(^3\). Gaining insights into the mechanisms by which flaviviruses exploit their host environment to promote viral propagation could yield targets for host-directed therapies to combat infections\(^4\). Flaviviruses enter cells through receptor-mediated endocytosis and following membrane fusion the viral genomes are released into the cytoplasm. The approximately 11 kilobase (kb) flavivirus genomic RNA encodes a single viral polyprotein, which is subsequently cleaved into mature structural and non-structural proteins. Biogenesis of the flaviviral proteins, which occurs at endoplasmic reticulum (ER) membranes, is not trivial due to the size of the polyprotein (approximately 3,300 amino acids), the occurrence of multiple transmembrane regions, and the cotranslational cleavage by viral and cellular proteases. After an initial round of translation, the RNA serves as a template for RNA replication, which occurs in close association to the ER membrane and primarily requires the NS5 RNA-dependent polymerase and NS3 helicase with involvement of other non-structural proteins and poorly defined host factors. The viral RNA–protein interactions are essential for recruiting and retaining the RNA at these ER sites, and for mobilizing the cellular factors required for translation, replication and packaging\(^5\). Although the viral RNA (vRNA) constitutes a central molecular hub during flavivirus infection, the precise molecular details have yet to be unravelled\(^6\). A global survey of cellular RNA-binding proteins (RBPs) interacting with vRNAs during infection would provide molecular insights into the composition and function of the ribonucleoprotein machines that drive vRNA translation and replication.

Results

Unbiased discovery of the flaviviral genomic RNA–protein interactome. To define the compendium of host proteins that associate with the positive-strand vRNA, we implemented comprehensive identification of RNA-binding proteins by mass spectrometry (ChIRP-MS)\(^7\). The human hepatoma cell line Huh7.5.1, which supports high levels of flaviviral replication, was infected with either DENV-2 or ZIKV at a multiplicity of infection (m.o.i.) of 0.1 for 48 h. The infected cells were subsequently crosslinked with formaldehyde to preserve the in-cell interactions between proteins and RNAs and stabilize the ribonucleoprotein complexes. Biotinylated oligonucleotides (Supplementary Table 1) were used to specifically enrich for DENV or ZIKV RNA and the recovered proteins were subjected to label-free quantitative liquid chromatography with tandem mass spectrometry (LC–MS/MS; Fig. 1a). We recovered roughly 50% of the vRNA across the full length of each vRNA, suggesting robust sampling of the total cellular vRNAs, while strongly depleting highly abundant host RNAs such as 7SK and ribosomal RNA, as shown by analysis of the quantitative PCR with reverse transcription (qRT–PCR; Supplementary Fig. 1a). The tiled probes were also able to capture full-length DENV-2 RNA from the total RNA of infected Huh7.5.1 cells while depleting all other cellular RNAs (Supplementary Fig. 1b,c). Together, these experiments provide strong evidence that ChIRP enrichment for DENV and ZIKV RNA is specific and efficient.

Investigation of the ChIRP-MS revealed extensive coverage of both DENV and ZIKV polyproteins including the structural (C, PrM and E) and non-structural (NS1–NS5) viral proteins (Fig. 1b–d). Analysis of the peptide coverage per protein length revealed that the viral NS3 and NS5 were the most abundant recovered proteins.
In addition to the virally encoded polyproteins, we identified 464 high-confidence hits from the human proteome that were specifically and reproducibly associated with DENV or ZIKV RNA (false-discovery rate (FDR) < 0.01, SAINT score > 0.99 and enrichment > 2 fold over the uninfected control ChIRP-MS; Fig. 1e, Supplementary Fig. 1d), which is consistent with these being RBPs that directly bind the flaviviral RNA. Our results are thus in line with ChIRP-MS enriching most strongly for RBPs but also for proteins present in functionally relevant RNA–protein complexes that do not directly interact with the RNA. In addition to the virally encoded polyproteins, we identified 464 high-confidence hits from the human proteome that were specifically and reproducibly associated with DENV or ZIKV RNA (false-discovery rate (FDR) < 0.01, SAINT score > 0.99 and enrichment > 2 fold over the uninfected control ChIRP-MS; Fig. 1e, Supplementary Fig. 1d), which is consistent with these being RBPs that directly bind the flaviviral RNA. Our results are thus in line with ChIRP-MS enriching most strongly for RBPs but also for proteins present in functionally relevant RNA–protein complexes that do not directly interact with the RNA. In addition to the virally encoded polyproteins, we identified 464 high-confidence hits from the human proteome that were specifically and reproducibly associated with DENV or ZIKV RNA (false-discovery rate (FDR) < 0.01, SAINT score > 0.99 and enrichment > 2 fold over the uninfected control ChIRP-MS; Fig. 1e, Supplementary Fig. 1d), which is consistent with these being RBPs that directly bind the flaviviral RNA. Our results are thus in line with ChIRP-MS enriching most strongly for RBPs but also for proteins present in functionally relevant RNA–protein complexes that do not directly interact with the RNA.
The ChIRP-MS was highly consistent across the biological triplicates, confirming a strong intra-probe set reproducibility (Supplementary Fig. 1e,f). The enrichment of specific host factors recovered with DENV or ZIKV RNA were positively correlated (Pearson's $r = 0.67$; Fig. 1e). RNA-binding proteins previously implicated in the antiviral response against DENV infection—including MOV10 (ref. 13), YBX1 (ref. 13) and ADAR$^3$—as well as proteins with pro-viral functions, such as SNBD1 (ref. 14), were among the highest-scoring candidates. Gene Ontology annotation revealed the strongest enrichment for the membrane component of cells (Fig. 1f). Protein-domain analysis of the hits was enriched for RNA-binding domains and the majority of the hits (approximately 75%) overlapped with a comprehensive list of mammalian RBPs$^1$ (Fig. 1g and Supplementary Table 2). Given the role of the ER membrane in flaviviral translation and the Gene Ontology enrichment, we examined ER-localized proteins in the ChIRP-MS data. The ER-localized proteins were enriched with high statistical significance ($P < 0.0001$, Fischer's exact test) in the ChIRP-MS dataset (31/464) when compared with the fraction of ER-localized proteins expressed in Huh7.5.1 cells (421/18,199; Methods and Supplementary Tables 3,4). These data reinforce the idea that ChIRP-MS retrieves RBPs that associate with flaviviral RNA and that several of these are ER-proteins.

To determine the specificity of the RBPs for flaviviruses, we performed ChIRP-MS on an unrelated single-stranded RNA virus from the picornavirus family (rhinovirus (RV), strain RV-B14). We recovered 350 host proteins associated with rhinovirus RNA and that several of these are ER-proteins.

Global characterization of RNAs associated with RRBP1 and vigilin. To examine the interaction of RRBP1 and vigilin with viral and cellular RNA during infection, we performed infrared crosslinking (irCLIP) RNAse-sensitive signal that was specific to their respective RRBP1 and ZIKV KO screens with the ChIRP-MS resulted in ten co-occurring proteins (Fig. 2b and Supplementary Table 6). Six subunits of the OST complex (STT3A, STT3B, RN2 and MAGT1) were top hits in this dataset, both associating with the vRNA and of central importance for viral replication$^8$ (Fig. 2b). ASCC3 and SND1 were previously described to be important as a regulator of the cellular transcriptional response to flavivirus infection and as directly binding to vRNA, respectively$^{14,20}$. Finally, two factors, vigilin and ribosome-binding protein 1 (RRBP1), that have not been previously linked to flavivirus infection stood out as among the most enriched in the ChIRP-MS (Supplementary Table 2 and Fig. 2b). We therefore focused on characterizing the molecular properties of these RBPs as well as defining the stages of the flaviviral life cycle at which these RBPs act.

RRBP1 and vigilin interact at the ER. RRBP1/p180 is a highly expressed RBP anchored to the ER via a N-terminal transmembrane domain that can act as a messenger RNA receptor at the ER$^5$. Vigilin promotes the translation of a subset of secretory mRNAs at the ER but, in contrast to RRBP1, lacks a transmembrane domain to anchor it to the ER$^2$. Confocal microscopy revealed that both RRBP1 and vigilin associate with each other, we performed co-immunoprecipitation (co-IP) experiments. We found that vigilin co-immunoprecipitated with RRBP1, suggesting that this interaction was RNA dependent (Fig. 3c and Supplementary Fig. 4b). Treatment with RNase A markedly reduced the co-recovery of vigilin with RRBP1, suggesting that both RRBP1 and vigilin are engaged in an RNA-dependent manner at the ER close to the positive-stranded vRNA.
**Fig. 2 | Intersection of ChIRP-MS with genome-wide CRISPR screens nominates functionally relevant pro-viral host proteins.**

a, Genome-scale CRISPR KO screens of all four DENV serotypes (DENV-1<sub>Vtalke</sub>, DENV-2<sub>429557</sub>, DENV-3<sub>Philippines/H871856</sub> and DENV-4<sub>BC287/97</sub>) in Huh7.5.1 cells. The genetic screens were independently performed for each serotype, analysed with MAGeCK and combined to obtain the robust rank aggregation (RRA) significance scores (y axis). The 50 most-enriched genes were coloured and grouped by function.

b, Scatter plot depicting the enrichment scores of high-confidence ChIRP-MS DENV hits (x axis) and the 200 top-scoring hits from DENV CRISPR genetic screens (y axis). Common hits shared by both the DENV genetic screens and DENV ChIRP-MS are indicated in red (vigilin), blue (RRBP1) and purple (others).

c, Western blot analysis of WT and clonal RRBP1-KO (top) or vigilin-KO (bottom) Huh7.5.1 cells. Representative western blot of n = 2 biologically independent replicates showing similar results.

d, Analysis by qRT-PCR of WT and RRBP1-KO cells infected with DENV (48 h.p.i.; m.o.i. of 0.1), ZIKV<sub>48h.p.i.</sub> (48 h.p.i.; m.o.i. of 0.1), POWV<sub>48h.p.i.</sub> (48 h.p.i.; m.o.i. of 0.1) or CHIKV<sub>24h.p.i.</sub> (24 h.p.i.; m.o.i. of 0.01). e, Analysis by qRT-PCR of WT and vigilin-KO cells infected as in d. The WT datasets for POWV in d, e were derived from the same experiments.

f, Western blot analysis of the cell lysates of DENV-2<sub>429557</sub>-infected (72 h.p.i.; m.o.i. of 0.1) WT, RRBP1-KO and vigilin-KO Huh7.5.1 cells probed with antibodies to DENV prM and NS3. Representative western blot of n = 4 biologically independent replicates showing similar results.

g, Titres of the infectious particles produced from WT, RRBP1-KO and vigilin-KO Huh7.5.1 cells infected with DENV-2<sub>429557</sub> for 72 h at an m.o.i. of 0.1. d, e, g, The data represent the mean ± s.e.m. of n = 3 independent biological replicates, except POWV, where n = 4 independent biological replicates. All P values were determined by two-tailed, unpaired t-tests using GraphPad Prism; n.s., not significant.
analysis of proteins co-enriched with RRBP1 or vigilin irCLIP enrichments near their molecular weight confirmed their specificity (Supplementary Fig. 5c,d). Sequencing of the enriched RNAs revealed that RRBP1 had a preference for binding ribosomal RNA (66% rRNA) over messenger/non-coding RNA (33% mRNA/ncRNA; Fig. 4a), which is in line with its known direct association with ribosomes26. RRBP1 crosslinks to many sites across rRNAs with a strong peak in the 18S rRNA, adjacent to the RRBP1-bound position at the mRNA localization of these mRNAs following infection (Supplementary Fig. 6a). Interestingly, although vigilin has a more restricted rRNA-binding pattern, its major binding site is on H16 of the 18S rRNA, adjacent to the RRBP1-bound position at the mRNA entry channel (Fig. 4b).

Infection with DENV or ZIKV resulted in the appearance of reads derived from the viral positive-stranded genome for both RRBP1 and vigilin. The change in binding profile was especially apparent for vigilin, where 75% and 49% of all crosslinks mapped to the vRNA of DENV and ZIKV, respectively (Fig. 4a). Globally, for both RRBP1 and vigilin, RT stops mapping the mRNAs were depleted in intronic regions, suggesting a preference for mature regions (Fig. 4a). Vigilin preferentially bound exonic regions, whereas RRBP1 binding was enriched for binding to exons as well as to the 5′ and 3′ untranslated regions (UTRs; Fig. 4c). For both RRBP1 and vigilin, Gene Ontology analysis of the bound mRNAs revealed terms related to membrane-bound and secreted proteins known to be highly expressed in hepatic cells (Supplementary Tables 7 and 8)29,29. For RRBP1, in the context of infection, there was weaker enrichment for membrane terms, whereas there was a gain of novel terms such as cytosol and ribosome, suggesting differential localization of these mRNAs following infection (Supplementary Fig. 5e). For vigilin, the enrichments were quite similar between infected and uninfected cells (Supplementary Fig. 5f).

We next visualized the RT stops mapping to DENV or ZIKV RNA. RRBP1 crosslinked across the full-length positive-strand vRNA with RT stops extending into the 5′ and 3′ UTRs (Fig. 4d and Supplementary Fig. 6a). In contrast, vigilin bound to the coding region but markedly fewer RT stops were observed in the 5′ and 3′ UTRs (Fig. 4d). This pattern was similar to what was observed with cellular mRNAs (Fig. 4c and Supplementary Fig. 6b,c) and is in line with the previously reported preference for binding to coding regions27. We observed rather uniform binding throughout the vRNA without apparent hotspots for both RBPs. On a per nucleotide basis, RRBP1 and vigilin binding was positively correlated on the DENV and ZIKV genomes (r = 0.79 and 0.84, respectively; Supplementary Tables 7 and 8) but did not correlate to complementary DNA truncations from RNA sequencing (RNA-seq) of the vRNA, which suggests that the irCLIP profiles are specific (Supplementary Tables 7 and 8). This broad ‘coating’ of the vRNA is reminiscent of how other RBPs, such as FMRP, bind actively translating mRNAs83. Together, the comparative RNA-binding profiles of RRBP1 and vigilin show that both proteins engage cellular rRNA and secretory mRNAs, with RRBP1 demonstrating a higher proportion of rRNA binding. During infection, both RBPs bind flavivirus
RNA and the majority of RT stops retrieved for vigilin are of flaviviral origin.

**RRBP1 and vigilin are required for the optimal translation and replication of DENV.** We further defined the step(s) at which the RBPs act in the viral life cycle by utilizing a luciferase-expressing DENV (DENV-Luc). First, we generated additional RRBP1 and vigilin clonal KO HEK293 cell lines to mitigate potential cell-type-specific effects (Supplementary Fig. 7a,b). RRBP1 and vigilin deficiency resulted in decreased luciferase expression throughout the infection cycle for DENV-Luc but not for the unrelated Coxsackievirus B3 virus expressing luciferase (Fig. 5a–d). Re-expression of RRBP1 and vigilin rescued, at least partially, the defect in flavivirus translation and replication, indicating that it is specific to the KO. To separate the translation and replication phase of the viral life cycle from the viral entry and uncoating steps, we transfected in vitro transcribed DENV replicon, in which the structural proteins are replaced with the Renilla luciferase gene. Compared with wild-type (WT) cells, the KO of both RBPs resulted in decreased luciferase expression throughout the time course (Fig. 5c,f). Because viral entry was bypassed in this experiment, these results suggest that RRBP1 and vigilin promote optimal viral translation and replication rather than viral entry.

Viral translation and replication are intricately linked: after the initial translation, viral non-structural proteins are produced that replicate genomic RNA, which in turn produces more mRNA templates resulting in increased translation. A larger contribution of initial translation is expected early in infection, whereas it is a combination of viral translation and replication at later time points. We performed DENV-Luc infections in the presence or absence of the DENV RNA replication inhibitor MK0608 to examine this in more detail and to assess the respective contributions of RRBP1 and vigilin to these phases. Control experiments confirmed that the luciferase signal at 8 h post-infection (h.p.i.) in the presence of MK0608 represents initial viral translation, whereas the bulk of the signal at 36 h.p.i. (in the absence of MK0608) is due to the subsequent RNA replication and translation (Supplementary Fig. 7c,d). Compared with the WT, RRBP1 deficiency resulted in an approximately
Fig. 5 | RRBP1 and vigilin modulate DENV translation and replication.  

**a, b.** Time-course DENV-Luc infection assays. WT, RRBP1-KO and RRBP1-KO + RRBP1 cDNA rescue (a) or WT, vigilin-KO and vigilin-KO + vigilin cDNA rescue (b) HEK293FT cells were infected with DENV-Luc (m.o.i. of 0.01) and harvested at the indicated time points. Virus infectivity was then determined by measuring the Renilla luciferase expression of the infected cells.  

**c, d.** Time-course CVB3-Luc infection assays. WT, RRBP1-KO and RRBP1-KO + RRBP1 cDNA rescue (c) or WT, vigilin-KO and vigilin-KO + vigilin cDNA rescue (d) HEK293FT cells were infected with CVB3-Luc (m.o.i. of 1) and harvested at the indicated time points.  

**e, f.** Luciferase expression of luciferase-encoding DENV replicon RNA in WT and RRBP1-KO (e) or WT and vigilin-KO (f) HEK293FT cells over the indicated time points post-electroporation of the replicon RNA. 

| Time post-infection (h) | Luciferase expression 8 h | Luciferase expression 36 h |
|------------------------|--------------------------|---------------------------|
| 0                      | n.s.                     | n.s.                      |
| 2                      | n.s.                     | n.s.                      |
| 4                      | n.s.                     | n.s.                      |
| 6                      | n.s.                     | n.s.                      |
| 8                      | n.s.                     | n.s.                      |
| 10                     | n.s.                     | n.s.                      |

We therefore conclude that the role of RRBP1 is more pronounced during the early stages of infection, whereas vigilin plays a more significant role at the later stages of infection.

twofold reduction of luciferase expression at 8 h.p.i., whereas vigilin deficiency did not decrease luciferase expression (Fig. 5g). However, vigilin-KO showed a more severe phenotype than RRBP1-KO at 36 h.p.i. (7× versus 3× reduction, respectively). We therefore conclude that the role of RRBP1 is more pronounced during the early stages of infection, whereas vigilin plays a more significant role at the later stages of infection.

**Cellular RBPs contribute to DENV genomic RNA stability.** We generated an isogenic cell line that is deficient in both RRBP1 and vigilin in Huh7.5.1 cells to further characterize the role of RRBP1 and vigilin (Fig. 6a). We observed a greater decrease in luciferase expression in the single RRBP1-KO cells compared with vigilin-KO cells early in infection and the reverse pattern later in infection with DENV-Luc, corroborating the RBP-KO viral phenotypes in HEK293...
stimulate vRNA replication, at least partially, by promoting translation of the viral polyprotein. In addition to translation, both vigilin and RRBP1 can also act on the stability of their target mRNAs\textsuperscript{31,35}. To assay for vRNA accumulation, we used MK0608 to inhibit viral replication and northern blotted against the DENV 3' UTR, which allows the detection of vRNA decay (Fig. 6c). We observed that the accumulated DENV genomic RNA was relatively stable in the WT cells for up to 24 h following MK0608 treatment (Fig. 6c and Supplementary Fig. 8a). In contrast, the decay rate of the genomic RNA was accelerated in the absence of both RRBP1 and vigilin (Fig. 6c and Supplementary. 8b). Together, our data indicate that RRBP1 and vigilin promote optimal flavivirus infection and have roles in vRNA translation, replication and stability.

**Discussion**

Our results provide detailed insights into the molecular identity of the host machineries engaged by flaviviral RNA during infection. We have comprehensively mapped the interactions between the flaviviral RNA genome and the human cellular proteome during viral infection using ChIRP-MS. Intersecting this dataset with a core set of genes identified in our genetic screens using all serotypes of DENV and multiple strains of ZIKV (Supplementary Table 9) highlighted the importance of ER-localized RBPs for flavivirus infection. One example of an ER-localized RBP was RRBP1—which has a short luminal domain, a transmembrane domain, and a large domain facing the cytosol that is highly basic and contains a decapeptide tandem-repeat motif\textsuperscript{37}. RRBP1 can act as a minor polysome receptor at the ER membrane\textsuperscript{38} and it can also bind certain mRNAs in a ribosome-independent fashion\textsuperscript{39}. Our data support these non-mutually exclusive views of RRBP1 function: the majority of RT stops identified from the RRBP1 irCLIP are indeed from the rRNA but nearly one-third of binding maps to mRNAs enriched for secretory protein transcripts (for example, APOB and AFP).

Vigilin is an evolutionarily conserved RBP that interacts with RNA through its KH domains. Although vigilin has been reported to be predominantly cytosolic, associating with free ribosomes\textsuperscript{34}, we detected a portion of vigilin in the ER fraction, thus corroborating previous observations of its association with ribosomes at the rough ER\textsuperscript{39,40}. We found that vigilin directly binds to rRNA and is preferentially enriched for binding to a subset of cellular mRNAs that encode secretory proteins, indicating a potential role in translation. This is consistent with the emerging view of vigilin as a translational enhancer for a subset of mRNAs of the secretory pathway\textsuperscript{22,41}. Our results indicate that DENV and ZIKV co-opt vigilin to promote infection.

Determining the protein interactome of RNA viruses has been a long-standing question of the field and is of interest to many laboratories. Two recent reports that utilized ultraviolet (UV) crosslinking and DENV RNA pulldown\textsuperscript{34,43} found 12 and 93 host RBPs, respectively, that partially overlap with our ChIRP-MS data (Supplementary Table 10). We used ChIRP-MS as a robust platform to discover the protein interactomes of vRNA. We were careful to verify that the enrichment procedure would recover proteins binding across the entire length of the 11-kb viral genome as well as sample a majority of the vRNA from infected cells. These quality controls ensured that the resulting proteomic data would be as robust and complete as possible. Further, as a discovery tool, we opted for chemical crosslinking, which provides the context of RBP complexes associated with the target RNA.

Our study of the flavivirus RNA interactome is a valuable resource that provides an RNA-centric perspective on viral infection, complementing other large-scale approaches that map virus–host interactions. The strategy employed here, integrating RNA–protein interactome data with genome-scale KO screening, is a generalizable strategy for the study of the complex interactions of cellular proteins with other RNA viruses. Critically, rigorous
validation through isogenic KO of host factors and direct but unbi-
ased assessment of their RNA interactomes with irCLIP provides an
accurate assessment of the design of the genetic screens but can readily be modified
to include other large-scale approaches that identify proteins with
antiviral activities44.

Methods

Cell lines, reagents and generation of KO cells. HAP1 cells were derived from the
near-haploid chronic myeloid leukaemia cell line KBM7 (ref. 17). The HAP1 cells
were cultured in IMDM supplemented with 10% heat-inactivated fetal
bovine serum (HI-FBS), penicillin-streptomycin and l-glutamine. BHK-21 (ATCC,
HEK293FT (Thermo Fischer Scientific), H1-HeLa cells (ATCC), Huh7.5.1 (ATCC),
Huh7.5.1 (gift from F. Chisari), RD (ATCC), BHK-21 (ATCC), Vero (ATCC) cells
and their KO derivatives were cultured in DMEM media supplemented with 10%
HI-FBS, 1X penicillin-streptomycin and 1X l-glutamine. C6/36 cells (ATCC) were
purchased from ATCC and cultured in Leibovitz’s L-15 medium supplemented with
penicillin-streptomycin, t-glutamine and 10% HI-FBS. Cycloheximide was
purchased from Sigma-Aldrich. MK0608 (7-deaza-2’-C-methylenadenosine) was
purchased from Carbosynth.

A CRISPR-Cas9 strategy was employed to generate RRBP1- and vigilin KO
cell lines. The CRISPR guide RNA sequences were designed using the Zhang lab
CRISPR design tool (http://crispr.mit.edu) and the corresponding oligos were
purchased from Integrated DNA Technologies. The oligos were cloned into the
eCas9 expressing PX458 guide RNA plasmid (Addgene, cat. no. 48138) generated
by the Zhang lab as previously described18. The cloning products were transfected
into H1-HeLa cells using Lipofectamine 3000 (ThermoFisher Scientific) and subsequently single-cell sorted based on GFP expression into
96-well plates using a BD Influx cell sorter at the Stanford Shared FACS facility.
Clonal cell lines were allowed to expand from a single cell and genomic DNA was
isolated for sequencing-based genotyping of targeted alleles. For this, a 300–300
base pair region that encompassed the guide RNA-targeted site was amplified and
the sequence. The PCR product was electrophoresed on a sequencing gel. Subclones were chosen where all alleles were mutated with insertions or deletions
that were not a factor of three. Knock-out subclones verified by genotyping were
further confirmed by western blotting using antibodies against RRBP1 (Bethyl
Laboratories, A303-996A) or vigilin (Bethyl Laboratories, A303-971A). The guide
RNA primers are listed in Supplementary Table 1 and were cloned into the
PX458 plasmid. RRBP1-vigilin double-KO Huh7.5.1 cell lines were generated by
transfecting verified RRBP1-KO cells with the same PX458 plasmid containing
guide RNA used earlier to knock out vigilin. Double-KO selection and
characterization was performed by western-blot analysis on single-cell FACS-
sorted clonal cells.

Viru
s strains, serotypes, reporter viruses and replicon. DENV-2 infectious clone
16681 was a gift from K. Kirkegaard (Stanford University). DENV-2 derived
from infectious clone 16681 was a cell culture (HAP1 cells) adapted strain7.18.
DENV-2 (strain 16681) (isolated in 1997 from a human in India; cat.
no. NR-3872), DENV-2 (MO1005) (isolated in 2005 in Mexico; cat.
no. NR-12316), DENV-2 (strain ZIKV-16681) (isolated in 1956 from human serum in
the Philippines; cat. no. NR-80), DENV-2 (strain ZIKV-16681) (isolated in
2006 from a human in Vietnam; cat. no. NR-44088) and DENV-4 (strain ZIKV-16681)
(isolated in 1997 from a human in Mexico; cat. no. NR-3806), ZIKV19-20 (isolated in
2015 from a human in Puerto Rico; cat. no. NR-50240) were obtained from BEI
resources (NIAID, NIAID). ZIKV21-24 (isolated in 2015 from a human in
Porto Alegre, Brazil; cat. no. NR-50240) was obtained from BIPI laboratories (NIAID, NIAID). ZIKV21-24 (isolated in 2015 from a human in Porto Alegre, Brazil; cat. no. NR-50240) was obtained from BIPI laboratories (NIAID, NIAID). ZIKV21-24 (isolated in 2015 from a human in Porto Alegre, Brazil; cat. no. NR-50240) was obtained from BIPI laboratories (NIAID, NIAID). ZIKV21-24 (isolated in 2015 from a human in Porto Alegre, Brazil; cat. no. NR-50240) was obtained from BIPI laboratories (NIAID, NIAID). The construct was based on pD2/IC-30P, which contains a full-length infectious clone encoding DENV-247 which encodes an H4399 mutation of the envelope protein (E) was introduced that enhanced viral infection in mammalian cells using the
QuickChange site-directed mutagenesis kit (Agilent Technologies) and the primers
in Supplementary Table 1. We gene-synthesized a fragment containing the
T7-polymersase promoter sequence followed by the first 104 nucleotides of the C
coding region in frame with Renilla luciferase and FMDV 2A. This fragment
was PCR amplified, and digested into pD2/IC-30P with Sall and Spel to create pDENV-
PRV ABC59 (isolated in 2015 from a human in Puerto Rico; cat. no. NR-50240)
with the lysis buffer from the Ambion Power SYBR Green Cells-to-Ct kit (cat. no.
AM1334) and transfecting BHK-21 cells using Lipofectamine 2000. Filtered
supernatant of transfected BHK-21 cells was used to infect Huh7.5.1 cells.

Construction of the DENV WT replicon was described previously24 and
was purchased as pDENV-Luc-ex, except that the Renilla luciferase coding region
was directly followed by the DENV ORF starting at the signal peptide preceding NS1,
deleting the structural proteins. The construct was based on pD2/IC-30P which
contains a full-length infectious clone encoding DENV-247. We gene-synthesized
a fragment containing the T7-polymersase promoter sequence followed by the
first 104 nucleotides of the C coding region in frame with Renilla luciferase and
FMDV 2A followed by the DENV ORF starting at the signal peptide preceding NS1 up to an internal Hpal site. This fragment was released by Sacl (preceding the
T7 promoter) and Hpal, and cloned in pD2/IC-30P in a three-point ligation with
KpnI/Sacl and KpnI/Hpal fragments. The primer sequences are listed in
Supplementary Table 1.

Constructs and packaging of lentivirus. To generate a lentiviral construct
expressing GFP–RRBP1, the CDNA construct (provided by A. Palazzo, University
of Toronto)25 was used as the template to generate a PCR product using primers
in Supplementary Table 1. The amplified PCR product was cloned into the
Gibson assembly reaction kit (New England Biolabs) into the plLenti-CMV-
Puro-Durox (w118-1) that was EcoRV digested. Vigilin cDNA (also known as
HDLPB; MGC cDNA BC001179) was purchased from GE DHarmaco. The CDNA was
amplified in two separate fragments using primers listed in Supplementary Table 1.
Both PCR fragments were cloned by Gibson Assembly (New England Biolabs)
into the EcoRV-digested third generation lentiviral Gateway destination vector plenti-
CMV-Puro-Durox (w118-1), which drives transgene expression by a CMV promoter
and harbours a puromycin-resistant gene as a selectable marker.

ER–GFP was engineered according to a previously reported construct26.
Enhanced GFP (EGFP) fused with an N-terminal signal peptide of calreticulin
and a C-terminal KDEL ER retention sequence was synthesized in two fragments
(Enhanced GFP and DNA Technologies) and cloned by Gibson Assembly (New
England Biolabs) into the plLenti-CMV-Puro-Durox (w118-1) expression vector.

Lentiviral or retroviral transduction was used to create stable cell lines
expressing a selected gene of interest. The respective genes of interest were cloned
into the plLenti-CMV-Puro-Durox vector (w118-1; a gift from E. Campeau).
Lentiviral transduction of the KO cell lines and packaging of the viral
plasmid with a mixture of AvPR, VSV-G and pAdVantage packaging plasmids into
HEK293FT cells using FuGENE HD (Promega). The lentivirus was harvested from
the supernatant and filtered through a 0.45-μm filter 48 h post-transfection.
We then added 1Xprotamine sulphate to the lentivirus before transducing the
respective cell lines overnight. Cells stably expressing the gene of interest were
selected by treatment with 1–4μg ml-1 puromycin over 2 d (InvivoGen) along
with untransduced cells as negative controls. A lentivirus carrying either the
mCherry (RFP) gene or the empty plLenti-CMV-Puro-Durox vector was used as a control
for RRBP1 and vigilin complementation in the KO cell lines, respectively.

Quantitation of virus infectivity by qRT–PCR. Experiments where the RNA
loads were determined by quantitative PCR (qPCR) were performed as follows:20,000
HEK293FT, Huh7.5.1 cells or their derivatives were seeded in triplicate in
96-well plates 1 d before infection. The cells were infected the next day with the
indicated virus and m.o.i. The cells were lysed at the indicated times post-infection
with the lysis buffer from the Ambion Power SYBR Green Cells-to-C kit (cat. no.
440295). Reverse transcription and qPCR were performed according to the Celi-
to-C, kit instructions on a Bio-Rad CFX Connect qPCR machine. All C, values
were normalized to the expression values of 18S rRNA. The qRT–PCR primers are
listed in Supplementary Table 1.

POWV infection, RNA extraction and qRT–PCR analysis. Cells were seeded in
triplicate in 1X104 cells well-1 in a 24-well plate and incubated overnight.
Following incubation, the DMEM medium was aspirated and the cells were washed
twice with PBS. The cells were then infected with POWV (LB strain) at an m.o.
1. At 48 h.p.i., the POWV-infected cells were washed twice with PBS and
the cells were lysed in 350 μl RLT buffer (Qiagen). Total RNA was extracted using an
RNeasy mini kit (Qiagen). Complementary DNA was synthesized from 400 ng of total RNA target using the iScript cDNA synthesis kit (Bio-Rad). Primers targeting the positive-strand POWV genome (Supplementary Table 1) were used to quantify the POWV transcripts as a measure of viral load. The 18S rRNA gene was used as a control to normalize results. After reverse transcription, the samples were re-extracted with 1 ml chloroform (inverting ten times to mix; no vortexing) in the same 2 ml Phase Lock Gel tube and centrifuged for 2 min at 13,000 r.p.m. The aqueous layer was then transferred to a new 2 ml Heavy Phase Lock Gel tube and extracted again with an additional 1 ml chloroform. After a 2 min centrifugation at >13,000 r.p.m., the aqueous layer was transferred to a siliconized 1.5 ml tube and precipitated overnight at −20°C with 10 μl 3 M sodium acetate, 0.1 μl RNase-free diethylpyrocarbonate and 7 μl isopropanol. The resulting RNA was washed twice with 75% ethanol, air dried for 5 min and eluted in 14 μl water.

 Detection of DENV proteins using immunoblotting. Wild-type Huh7.5.1 cells and their derivatives were seeded in quadruplicate at 1 × 105 cells well−1 in a 24-well plate and incubated overnight. The cells were then infected with DENV, at an m.o.i. of 0.1. The cells were harvested 72 h post-infection using RIPA buffer (TEKNOVA) supplemented with Laemmli sample buffer (Bio-Rad) and 5% β-Mercaptoethanol (Bio-Rad). The cell lysates were then boiled for 10 min and separated by SDS–PAGE on a pre-cast 4–15% polyacrylamide gel (Bio-Rad) in a Bio-Rad Mini-PROTEAN TetraSDS Cell (Bio-Rad). The gels were then incubated with ECL solution (Chemiluminescent substrate or Dura Extended duration substrate (Thermo Fisher Scientific)) overnight with primary antibody diluted in blocking buffer on a rocker at 4°C. The primary antibodies were subsequently detected by incubating the membranes for 1 h at room temperature with secondary anti-mouse or anti-rabbit antibodies conjugated to horseradish peroxidase (Thermo Fisher Scientific). The bound proteins were detected by incubating with SuperSignal West Pico PLUS chemiluminescent substrate or Dura Extended duration substrate (Thermo Fisher Scientific) peroxide solutions and visualized on a Bio-Rad ChemiDoc Touch imaging system. The following primary antibodies were used: anti-p-mR (Genetex, GTX128092) at a dilution of 1:2,500, anti-N (Genetex, GTX124252) at a dilution of 1:2,500 and anti-GAPDH (Genetex, GTX627408) at dilution of 1:5,000.

Luciferase reporter virus and DENV replicon luciferase assays. For the luciferase reporter virus assays, HEK293FT or Huh7.5.1 cells were seeded in 96-well plates (20,000 cells per well−1), transfected with the luciferase-expressing DENV replicon assay master mix and 0.4 μl P3solexa/P6solexa oligo mix and amplified as follows: 98°C for 10 s, 35 cycles of 98°C for 10 s, 55°C for 5 s and 72°C for 30 s, then quenched on ice. The RRBP1 (Bethyl, A303-996A), vigilin (Bethyl, A303-971A) and IgG (Thermo Fisher Scientific, 2-6102) immunoprecipitations were performed using 15 μg of each antibody with 50 μl Protein A Dynabeads (Thermo Fisher Scientific) for 8 h at 4°C with rotation. The samples were sequentially washed (1 min each wash) at 25°C in 1 ml volumes as follows: 1× high stringency buffer (15 mM Tris–HCl, pH 7.5, 5 mM EDTA, 2.5 mM EGTA, 1% Triton X-100, 1% sodium deoxycholate, 120 mM NaCl and 25 mM KCl), 1× high salt buffer (15 mM Tris–HCl, pH 7.5, 5 mM EDTA, 2.5 mM EGTA, 1% Triton X-100, 1% sodium deoxycholate and 1 M NaCl) and 2×NT2 buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40). The remaining IP complexes were desalted following the NT2 washes with 74 T4 PNK (NEB) for 45 min in an Appendorf Thermomixer at 37°C, 15 s at 1,400 r.p.m., 90 s of rest in a 30 μl reaction, pH 6.5, containing 10 U T4 PNK, 0.1 μl SuperRXase-IN (Thermo Fisher Scientific) and 6 μl PEG 400 (16.7% final). The desalted immunoprecipitates were then resuspended once with NT2 buffer and incubated at 37°C for 30 min. The resuspended complexes were then incubated overnight with T4 RNA ligase 1 (NEB) in an Appendorf Thermomixer at 16°C, 15 s at 1,400 r.p.m., 90 s of rest in a 60 μl reaction containing 10 U T4 RNA ligase, 1.5 pmol pre-adenylated-IR800-3′-biotin DNA-adaptor, 0.1 μl SuperRXase-IN and 6 μl of PEG 400 (16.7% final). The following day, the samples were resuspended once with 500 μl NT2 buffer and resuspended in 30 μl of 20 mM dithiothreitol (DTT) and 1 μg (10 μl) of Thermo Fisher Scientific NT2 buffer. The samples were then incubated overnight with T4 RNA ligase 1 (NEB). The quality of the immunoprecipitated protein complexes was determined by gel electrophoresis on E-gel 1.2% agarose with SYBR Safe (Life Technologies), an annealing temperature with ampicillin of the expected size with no non-specific amplification was chosen. The qPCR reactions were performed on the Applied Biosystems QuantStudio 6 Flex real-time system (Life Technologies) in Micro-Amp optical 384-well reaction plates (Life Technologies). The QuantStudio real-time PCR software (v.3.1) and the ΔΔCt method were used to calculate the relative numbers of POWV transcripts.

Analysis of iCLIP data. The iCLIP data were processed using the FAST-iCLIP pipeline (https://github.com/ChangLab/FAST-iCLIP/tree/site). PCR duplicates were removed using unique molecular identifiers in the RT primer region. The adaptor and barcode sequences were trimmed and reads were mapped step-wise to viral (DENV or ZIKV), repetitive and finally non-repetitive (GRCh38) genomes. Bowtie2 indexes were generated using the ‘bowtie2-build’ command in Bowtie2.
for the DENV (KU725663.1) and ZIKV (KU010121.3) RNA genome sequences. The specific parameters used for the FASTQ-CLIP pipeline were as follows: -1 18 (trims 17 nt from the 5’ end of the read), -1 16 (includes all reads longer than 16 nt), --minMAPQ 20 (minimum MAPQ score from bowtie2 mapping: unique mapping only), --tr 2,3 (repetitive genome) and --tn 2,3 (non-repetitive genome) RT stop intersection (n,m; where n = unique mapping only, –tr 2,3 (repetitive genome) and –tn 2,3 (non-repetitive genome) RT stop intersection (n,m; where n = unique mapping only, –tr 2,3 (repetitive genome) and –tn 2,3 (non-repetitive genome)). The peaks of the RT stops were called on the biologically replicated intersection of the RT stop positions using Count peaks (http://count.readthedocs.io/en/latest). The command line was as follows: Count peaks genome.v21.annotation.ogafit.rnaInt_dramaOut_3.rnaInt_dramaOut_5.bed --CountForCPEaks.bed --iCountScoreXscore 'from Out_iCpeaks.bed' were then annotated with HOMER (http://homer.ucsd.edu/homer) using the following command: annotatePeaks.pl Out_iCpeaks.bed hg38> Out_iCpeaks_hg38_HOMERAnno.txt --annStatsOut_iCpeaks_hg38_HOMERAnno.stats.tat.

CLIP mass spectrometry. Cells were grown and UV crosslinked, and lysates were generated, RNAse A treated, immunoprecipitated and washed as described for CLIP. No dephosphorylation or RNA ligation took place, but the RBP–RNA complexes were denatured and run in SDS–PAGE gels as per the CLIP procedure. After SDS–PAGE, the proteins were transferred onto a Colloidal blue staining kit (Thermo Fisher Scientific) as per the manufacturer's instructions. The stained gels were visualized with the Odyssey CX Laser scanner and regions of each lane were excised based on where the predicted BMP–RNA complex would migrate.

The gel slices were prepared for mass spectrometry by sequentially rinsing in 200 μL of 25 mM ammonium bicarbonate (AmBic), 100% acetonitrile (ACN; Thermo Fisher Scientific) and flash drying. Lysates were resuspended in 50 mM ammonium bicarbonate (AmBic). The samples were reduced by adding 200 μL of 5 mM DTT in 50 mM AmBic and incubating at 65 °C for 35 min. The reduction buffer was discarded and the samples were cooled to room temperature. Alkylation was achieved by adding 200 μL of 25 mM iodoacetamide in 50 mM AmBic for 20 min at 25 °C in the dark. The alkylation buffer was discarded; the samples were rinsed once in 200 μL of 50 mM AmBic and then washed twice for 10 min each in 200 μL freshly prepared 50% ACN in 50 mM AmBic. After each wash, the supernatant was discarded and the samples were dried for 3 h using a SpeedVac. Once dry, the proteins were digested by adding 100 ng trypsin in 200 μL of 50 mM AmBic for 16 h at 37 °C. The samples were subsequently acidified by adding formic acid to a final concentration of 2.5% and incubating at 37 °C for 45 min. Finally, the samples were desalted using HyperSep filter plates with a 5–7 μm bed volume (Thermo Fisher Scientific) following the manufacturer's instructions. The samples were eluted three times in 100 μL 80% ACN in 2.5% formic acid, dried on a SpeedVac and resuspended in 10 μL 0.1% formic acid for mass-spectrometry analysis. Desalted peptides were analysed by tandem mass spectrometry (LC–MS/MS).

The samples were separated using a 20-cm reversed phase column fabricated in-house (inner diameter of 100 μm, packed with ReproSil-Pur C18-AQ 3.0 μm resin (Dr. Maisch GmbH)), which was equipped with a laser-pulled nano-electrospray emitter tip. Peptides were eluted at a flow rate of 400 nL/min using a two-step linear gradient of 2–25% buffer B in 70 min and 25–40% B in 20 min (buffer A: 0.2% formic acid and 5% DMSO in water; buffer B: 0.2% formic acid and 5% DMSO in ACN) in an Eksigent expert nanoLC-425 system (AB Sciex). The peptides were ionized with electrospray ionization into an Orbitrap Elite Hybrid Ion Trap–Orbitrap mass spectrometer (Thermo Fisher Scientific). The instrument method parameters were as follows: MS1 resolution, 60,000 at 400 m/z; scan range, 340–1,600 m/z. The top 20 most-abundant ions were subjected to collision-induced dissociation with a normalized collision energy of 35%, activation q of 0.25 and precursor isolation width of 2 m/z. Dynamic exclusion was enabled with a repeat count of one, a repeat duration of 30 s and an exclusion duration of 90 s. The samples were then scaled with a factor of 1,000 for readability. Next, we consolidated data across biological replicates. We filtered identified proteins so that a given protein was required to be present with at least one spectrum in each biological replicate. Only the surviving proteins were averaged across the biological replicates. Finally, a combined table was generated across all experimental data as per the previous steps. For each protein identified, we output the following information: common gene name, UniProt ID, comma-separated values of the raw spectra from each technical and biological replicate, the average normalized spectra from each experiment (with an added correction factor of one to avoid any zeroes for subsequent analyses), the average normalized spectra divided by the number of amino acids in the protein and the total amino acids in that protein.

Comprehensive identification of RNA-binding proteins by mass spectrometry. DENV-, ZIKV- and RV-targeting probes were designed using online tools available at https://bioinforme.stellaris/c/ (with a repeat masking setting of three and even coverage of the whole transcript). The full probe sequences are available in Supplementary Table 1. Oligos were synthesized with 3’ biotin-TEG modification at the Stanford Protein and Nucleic Acid Facility.

ChIRP-MS was performed largely as described in Chu and colleagues. Huh7.5.1 cells (9 × 10^5 × 10^5) were seeded and infected the following day with DENV-2 (16681 strain- Hap1 adapted), ZIKV PRVAC59 at an m.o.i. of 0.1 or mock-treated in triplicate (3 × 3 = 9 flasks per condition). For RV, 2 × 10^5 × 10^5 H1-Lac cells were seeded and infected the next day at an m.o.i. of 1 or mock infected with UV-inactivated and infected with RV after infection and infected once with 10 mL PBS per flask, trypsinized, pelleted at 1,400 r.p.m. for 5 min and washed twice with PBS. The cells were then resuspended in 3% formaldehyde containing PBS and rocked for 30 min at 25 °C. Chemical crosslinking was stopped by the addition of glycine to a final concentration of 125 mM for 5 min at 25 °C. The crosslinked cells were pelleted at 2,000 r.p.m. for 5 min (the supernatant was reserved for future experiments). The RV protein pellets were washed once with 1 mM EDTA and 15% formamide was added for every millilitre of sonicated lysate and the lysates were pre-cleared by adding 30 μL washed MyOne C1 beads per millilitre at 37 °C for 30 min on rotation. Beads were removed twice from the lysate using a magnetic stand; for this and all subsequent magnetic stand steps we allowed >1 min of separation before removing any supernatant. Next, 1.5 μL of 100 μM CHIRP Probe Poos was added per millilitre of lysate. CHIRP Probe Poos (Supplementary Table 1) for control, DENV or ZIKV enrichments were made by mixing equal volumes of 99 (DENV + ZIKV), 50 (DENV) or 49 (ZIKV) individual antisense oligos to 100 μM (final concentration of 1.01, 2.04 μM for each probe, respectively). The RV pool was made by mixing equal volumes of 50 individual antisense oligos targeting the RV genome at 100 μM (2 μM final concentration for each probe) and was used for the RV-uninfected control. After mixing, hybridization took place on rotation for 16 h at 37 °C. Subsequently, 150 μL washed MyOne C1 beads per millilitre of lysate were added to each sample and rotated for 24 h. The enriched RNA was resuspended on the beads with a magnetic stand and the beads were washed 5 × 2 min in 1 mL CHIRP wash buffer (2× SSC solution (Thermo Fisher Scientific) and 0.5% SDS) at 37 °C. To elute the enriched proteins, the beads were collected on a magnetic stand, resuspended in CHIRP biotin elution buffer (12.5 mM biotin, 7.5 mM HEPES, pH 7.9, 75 mM NaCl, 1.5 mM EDTA, 0.13% SDS, 0.075% sarkosyl and 0.02% sodium deoxycholate), mixed at 25 °C for 20 min on rotation and at 65 °C for 15 min with shaking. The eluent was transferred to a fresh tube and the beads were eluted again. The two eluents were pooled (about 1,200 μL) and residual beads were removed again using the magnetic stand. Trichloroacetic acid (25% of the total volume; 300 μL) was added to the clean eluent, vortexed and the samples were then placed on ice for ≥2 h. The next day, the samples were pelleted at 21,000 g at 4 °C for 15 min. The supernatant was carefully removed and the protein pellets were washed once with ice-cold acetone. The samples were spun at 21,000 r.c.f. for 5 min at 4 °C. The aceton supernatant was removed, the tubes were briefly centrifuged again and—after the removal of the residual aceton—the pellet was left to air-dry on the bench top. The proteins were then solubilized in 1xLDS buffer in NT2 with 20 mM DTT and boiled at 95 °C for 30 min with occasional mixing for reverse-crosslinking.

The protein samples were size-separated on bis–Tris SDS–PAGE gels (Bio-Rad), fixed and stained with colloidal blue, and prepared for mass-spectrometry analysis as described for 'CLIP mass spectrometry'. Biological triplicates were performed for each infected and uninfected condition. DENV or ZIKV CHIRP MS, each replicate was cut into four slices in the SDS–PAGE and prepared independently (total of four mass spectrometry runs per biological replicate). For RV CHIRP-MS, one replicate of RV-infected and uninfected H1-Lac cells was used and split across four independent gel slices. CHIRP-MS data were searched with Byonic and processed as per the custom python script described in 'CLIP mass spectrometry'. Principal
ChIRP qRT–PCR. Cells were grown, infected, crosslinked and sonicated as described earlier. After sonication, 1% of the lysate was removed and saved as an ‘input’ sample. The lysates were again processed as earlier for pre-clearing, hybridization, MyOne C1 capture and bead washing. After washing, 1% of each sample was removed as an ‘enriched’ fraction. The enriched fractions were collected while the MyOne C1 beads were fully resuspended in ChIRP wash buffer. ChIRP PK buffer (10 mM Tris–HCl pH 7.0, 100 mM NaCl, 1 mM EDTA, 0.2% SDS) was added to the input and enriched samples to a final volume of 95 μL. Proteinase K (5 μL of 20 mg mL⁻¹) was then added with shaking at 35°C for 45 min to digest the protein. RNA was extracted by adding 500 μL TRIzol (Thermo Fisher Scientific), incubating at 55°C for 5 min and then adding 100 μl chloroform directly mixing each sample by vortexing for 7 s. The samples were incubated at 25°C for 5 min and then centrifuged at 12 000 r.p.m. for 15 min at 4°C. The aqueous layer was carefully removed from each sample, mixed with twice the volume of ethanol and purified using a RNA Clean & Concentrator-25 kit (Zymo Research) as per the manufacturer’s instructions. All RNA samples were DNase treated using Turbo RNA-free DNA-free kit (Thermo Fisher Scientific). SuperScript VILLO (Thermo Fisher Scientific) was used to generate cDNA according to manufacturer’s instructions. The qPCR analyses were performed on the CFX96 Touch real-time PCR detection system (Bio-Rad). All of the primers used are provided in Supplementary Table 1.

ChIRP and RNA Bioanalyzer. Cells were grown, infected and the RNA was harvested as described earlier. This RNA was either analysed directly using a RNA Pico Bioanalyzer chip (Agilent Technologies) or by denaturing gel electrophoresis. A formaldehyde–agarose gel was made using the NorthernMax kit (Thermo Fisher Scientific) as per the manufacturer’s protocol. The RNA samples were denatured in 0.5× Loading Gel Loading Buffer II (Thermo Fisher Scientific) and 1× SYBR Gold (Thermo Fisher Scientific) at 55°C for 10 min, cooled on ice for 3 min and then loaded into the gel. After running at 110 V for 35 min, the RNA was imaged using a UV transilluminator.

RNA-seq from iCLIP samples. Input material for RNA-seq was obtained from the same lysates generated for the iCLIP experiment. After lysis and sonication, as described earlier, 100 μL lysate from each biological duplicate of the uninfected and DENV- or ZIKV-infected cells was removed. Proteins were digested and RNA extracted with Proteinase K and TRIzol as described in the ‘ChIRP qRT–PCR’ section. Ribosomal RNA was depleted using a RiboMinus transcriptome isolation kit (Hiromi/mouse; Thermo Fisher Scientific) as per the manufacturer’s instructions starting with 5 μg total RNA per sample. Samples depleted of rRNA were fragmented using the RNA fragmentation reagent (Thermo Fisher Scientific) at 90°C for 30 s. After fragmentation, the RNA samples were purified using a RNA Clean & Concentrator-5 kit (Zymo Research) as described in the ‘ChIRP qRT–PCR’ section. The RNA samples were re-dissolved in 5 μl water and used for the next steps. 3′ ends were repaired by the addition of 0.5 μl 10× T4 PNK buffer (New England Biolabs), 1 μl T4 PNK, 1 μl FastAP (Thermo Fisher Scientific), 1 μl RiboLock (Thermo Fisher Scientific) and 1.5 μl water for 45 min at 37°C. Next, a 3′-adapter was ligated to the RNA samples by directly adding 1 μl 10× T4-RNL1 buffer (New England Biolabs), 1 μl T4-RNL1 (New England Biolabs), 1 μl of 100 mM DTT, 0.75 μl of 3 μM iCLIP 3′-adapter and 6 μl 50% PEG8000 (New England Biolabs) for 4 h at 25°C. After the completion of the ligation reaction, unligated 3′-adaptors were digested by directly adding 2.5 μl Rec-Jf (New England Biolabs), 1.25 μl 5′-deadenylase (Epitect), 3 μl 10×5'-deadenylase buffer (Epitect) and incubating for 1 h at 37°C. The samples were purified using a RNA Clean & Concentrator-5 kit (Zymo Research) as above. The ligated and purified RNA samples were processed further through the next steps as per the final steps of the iCLIP procedure described earlier. The sequences were then sequenced on the NextSeq 500 platform (Illumina) after library quantification and pooling. Data were processed for mapping as per the iCLIP pipeline; however, RT stops were not isolated after mapping. Instead the featureCount tool of the Subread (http://subread.sourceforge.net/Main.html), to apply a statistical confidence filter to refine the ChIRP-MS hits. Individual biological triplicates for mock-, DENV- or ZIKV-infected ChIRP-MS hits were used as input data for the Subread software with default settings for the FDR calculations.

Immunofluorescence and RNA fluorescence in situ hybridization. Huh7.5.1 cells (80,000) were seeded on poly-l-lysine-coated glass coverslips in a 24-well format. The cells were infected the following day with either DENV-2 or ZIKV (Puerto Rico) at an m.o.i. of 1 for 24 h. The cells were fixed with 4% formaldehyde (Sigma), washed with PBS and permeabilized using the Immunofluorescence application solution (kit according to the manufacturer’s recommendations) before the SNAI (http://smart-apms.sourceforge.net/Main.html), to apply a statistical confidence filter to refine the ChIRP-MS hits. Individual biological triplicates for mock-, DENV- or ZIKV-infected ChIRP-MS hits were used as input data for the SNAI software with default settings for the FDR calculations.

Native co-IP. Huh7.5.1 cells were seeded in six-well plates and infected with DENV-2 at an m.o. of 0.1 or with no virus as described earlier. Protein-G beads were pre-conjugated to rabbit-IgG (Thermo Fisher Scientific, 02-602) or anti-RRBP1 (Bethyl, A303-996A) antibodies; 5 μg antibody and 10 μl Protein G beads were used per immunoprecipitation reaction. After 4 h of infection, protein lysate was generated by adding 650 μl co-IP lysis buffer (10 mM HEPES, 2 mM MgCl₂, 10 mM KCl, 0.5% NP-40, 0.5 mM EDTA and 150 mM NaCl) to each well, disrupting the cells in a cell lifter (Thermo Fisher Scientific) and centrifuging at 300 x g at 4°C for 10 min. The supernatant was collected and is the same lysate that was used for the iCLIP experiment. The RNA was extracted using TRIzol (Life Technologies) and purified using a RNA Clean & Concentrator-5 kit (Zymo Research) as described for the iCLIP protocol. The RNase-treated samples were generated by adding 1 μl of 1 μg/mL RNase A to the lysate during the immunoprecipitation at 4°C. After 3 h, each sample was washed three times with 750 μl co-IP lysis buffer and once with 750 μl NT2 buffer. The enriched proteins were subsequently analysed by western blotting.

Genome-scale CRISPR–Cas9 KO screens. Genomic CRISPR–Cas9 models were generated in Huh7.5.1 cells as previously described (37). Briefly, stable Cas9-expressing Huh7.5.1 WT cells were engineered by transducing lentiCas9-Blunt and selected using blasticidin. Subsequently, 300 × 10⁵ Cas9-expressing Huh7.5.1 cells were transduced with the lentivector–Guide–Puro from the GeCKOv2 library (51) at an m.o.I. of 0.3. Puromycin-resistant cells were selected, pooled and expanded. These mutated cells were ready to be used for CRISPR genetic screens at 10 d post-transduction. Mutagenized cells — 65 × 10⁶ per library (A and B) — were seeded for 16 h and then subjected to the following DENV infections: DENV-1 1 × 10⁶ (m.o.I. = 0.4 plaque-forming units (p.f.u.) per cell), DENV-2 1 × 10⁶ (m.o.I. = 0.5 p.f.u. per cell), DENV-3 1 × 10⁶ (m.o.I. = 0.005 p.f.u. per cell) and DENV-4 1 × 10⁶ (m.o.I. = 0.1 p.f.u. per cell). Cytopathic effects were observed as early as 4 d post-infection. Populations of virus-resistant cells were harvested at 10 d post-infection. Uninfected starting populations of mutated cells were used as the unselected reference. Total genomic DNA from both virus-resistant and uninfected cells was extracted using a QIAamp DNA mini kit (Qiagen). The inserted guide RNA sequences were retrieved from the genomic DNA by PCR amplification using the primers F1 and R1 (Supplementary Table 1). The PCR products were further
barcoded by an additional round of PCR amplification using the specific primers listed in Supplementary Table 1. The barcoded PCR products were then purified and subjected to next-generation sequencing on a NextSeq platform (Illumina) using a 2 × 100 bp-2 × 100 bp run on a NextSeq platform (Supplementary Table 1). The sequencing data were processed and analysed using the MAGeCK algorithm to determine the ranking of each hit by taking the following criteria into account: the number of sequencing reads per unique guide, the number of unique guide RNA per gene (that is, 0–6) and the enrichment of a particular guide RNA in comparison to uninfected cell populations. The sequencing reads were subjected to preprocessing to remove contaminating reads. The processing steps included quality trimming, removal of barcode sequences, removal of sequencing adapters and removal of read pairs that were reverse complementary. Next, the reads were aligned to the ZIKV haplotypic genome of the ZIKV FLR (Colombia strain). The reads were then subjected to additional processing steps. The reads were filtered for reads with high mapping quality (MAPQ) scores, which were then used to call single-nucleotide variants (SNVs). The reads were then mapped to the ZIKV FLR (Colombia strain) genome using Bowtie and enrichment of independent insertions was calculated as previously described.  

Haploid genetic screens. The haploid genetic screens were performed as previously described. Briefly, 5 × 100 × 106 gene trap mutagenized HAP1 cells were seeded and infected with the following ZIKV strains (m.o.i. of 1): ZIKV+1 (Colombia strain), ZIKV+287,368 and ZIKV+1,128. The medium was aspirated 48 h post-infection and replaced with fresh IMDM medium containing 10% FBS. Clear cytopathic effects were observed at 2 and 3 d after infection, leading to death of the majority of cells. Resistant HAP1 colonies were harvested 10 d after infection (yield of approximately 30 × 106 cells per virus strain) and the genomic DNA was isolated. Gene trap insertion sites were determined by linear amplification of the genomic DNA-flanking regions of the gene trap DNA insertion sites using linear-amplification mediated PCR. Briefly, genomic DNA from selected unselected populations (approximately 40 × 106 cells per condition) were isolated using a QIAamp mini kit (Qiagen). The isolated genomic DNA was then separately digested using the restriction enzymes Msel and SpeI, and the resulting digested DNA products from both digestion reactions were pooled for each condition. A linear PCR using biotinylated primers recognizing the long terminal repeat (Supplementary Table 1) and the gene trap vector were performed using the 3′ PCR kit from Invitrogen. A DNA linker primer (Supplementary Table 1) was then ligated to the linear-amplification mediated PCR products on beads using the Circilgase II kit (Epicentre). Biotinylated PCR products were then used in magnetic streptavidin beads provided with the Dylna kiloBaseBinder kit (Invitrogen). A final PCR using primer sets with Solexa adapter sequences (Supplementary Table 1) and bar codes was then used to amplify the isolated fragments with different sizes. The final PCR products were checked on a 2% agarose gel and sent for sequencing on an Illumina NextSeq platform. The reads were aligned to the human genome using Bowtie and enrichment of independent insertions was calculated as previously described. The P value (corrected for FDR) for each gene identified in the screen was determined using a one-sided Fisher’s exact test run in the R software environment. If the P value was lower than the R software could report, the corrected P value was set to the smallest non-zero normalized floating-point number R could report, that is, ~1 × 10−40. The screens were individually compared with the unselected dataset or the fastq files were first merged to detect genes common to the unselected dataset or the unspliced dataset. The reads were then mapped to the ZIKV haplotypic genome screens were analysed using DAVID (https://david.ncifcrf.gov/tools.jsp). 

RNA stability and northern blot analysis. The 3′ UTR of the DENV-2 106 gene was inserted into the pCR Blunt plasmid (Thermo Fisher Scientific). This plasmid served as a template to generate northern blot probes that were used to detect mRNA translation. The probes were then hybridized to the northern blot and subjected to next-generation sequencing on a NextSeq platform (Illumina) using a 2 × 100 bp sequencing run. The sequencing data were processed and analysed using the MAGeCK algorithm to determine the ranking of each hit by taking the following criteria into account: the number of sequencing reads per unique guide, the number of unique guide RNA per gene (that is, 0–6) and the enrichment of a particular guide RNA in comparison to uninfected cell populations. The separate next-generation sequencing reads reports were used to determine the stability of the mRNA translation.  

β2-microglobulin gene 3′ UTR. The β2-microglobulin gene 3′ UTR was inserted into the pCR-Blunt plasmid (Thermo Fisher Scientific). This plasmid served as a template to generate northern blot probes that were used to detect mRNA translation. The probes were then hybridized to the northern blot and subjected to next-generation sequencing on a NextSeq platform (Illumina) using a 2 × 100 bp sequencing run. The sequencing data were processed and analysed using the MAGeCK algorithm to determine the ranking of each hit by taking the following criteria into account: the number of sequencing reads per unique guide, the number of unique guide RNA per gene (that is, 0–6) and the enrichment of a particular guide RNA in comparison to uninfected cell populations. The separate next-generation sequencing reads reports were used to determine the stability of the mRNA translation.
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Author contributions

Y.S.O., K.M. and R.A.F. were responsible for the design and execution of experiments, data analysis and manuscript preparation. M.A.M. and P.S. performed and analysed the northern blot assays. J.D. carried out the MAGeCK analysis for all CRISPR screening results. J.K.L. and N.R. assisted with the proteomic experiments. N.V.B. and K.K. were responsible for the immunofluorescence and fluorescent in situ hybridization targeting results. J.K.L. and N.R. assisted with the proteomic experiments. N.V.B. and K.K. were responsible for the immunofluorescence and fluorescent in situ hybridization targeting assays. A.G.J assisted with the preparation of manuscript. A.S.P and C.D.M. helped with the preparation of reagents, cell lines and viruses. L.M., J.M.G. and M.E.B. were responsible for the infection assays and analyses involving POWV. J.E.C. and C.R.B. supervised the research, acquired funding, interpreted data and prepared the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Data collection

- irCLIP data were collected in biological duplicate and generated on Illumina NextSeq 500 instrument. ChIRP-MS data were collected in biological triplicate and generated on Thermo Orbitrap Fusion instrument. CRISPR-Cas9 KO screens and Haploid Genetic screens data were generated on Illumina NextSeq 500 instrument.

Data analysis

- MAGECK (version 0.5.4) (PMID 25476605) was used for CRISPR-Cas9 screen analyses.
- Deep sequencing data from Haploid Genetic Screen was analyzed as previously described (PMID: 27383987).
- Imagej colocalization algorithm COLOC2 (https://github.com/fiji/Colocalisation_Analysis/releases/tag/Colocalisation_Analysis-3.0.0) was used to quantify colocalization between proteins and/or viral RNAs.
- SAINTq software (http://saaint-apms.sourceforge.net/Main.html) was used to apply a statistical confidence filter to refine the ChIRP-MS hits.
- The Database for Annotation, Visualization and Integrated Discovery, DAVID (https://david.ncifcrf.gov/tools.jsp) was used for gene ontology (GO) analysis.
- irCLIP data were analyzed using a custom script available at: https://github.com/ChangLab/FAST-iCLIP/tree/lite.

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Raw and processed sequencing data will be deposited on GEO: GSE109194

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| Sample size | For each experimental type, an appropriate number of data points or experiments were collected. For example, deep sequencing experiments were performed in duplicate, while qRT-PCR experiments were performed in at least triplicate. |
| Data exclusions | No relevant data were excluded. |
| Replication | As no data were excluded, all values from each experiment are presented and the variation can be seen in the figures. Statistical analysis as described throughout the manuscript provided the ability to confidently assess differences between different experimental conditions. |
| Randomization | No randomization was used. |
| Blinding | No blinding was used. |

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Materials & experimental systems

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- Animals and other organisms
- Human research participants
- Clinical data

Methods

- n/a
- ChIP-seq
- Flow cytometry
- MRI-based neuroimaging

Antibodies

- Mouse anti-GAPDH, GeneTex, catalog # GTX627408
- Mouse anti-P84, GeneTex, catalog # GTX70220
- Rabbit anti-DENV prM, GeneTex, catalog # GTX128092
- Rabbit anti-DENV NS3, GeneTex, catalog # GTX124252
- Rabbit anti-RRBP1, Bethyl Laboratories, catalog # A303-996A
- Rabbit anti-Vigilin, Bethyl Laboratories, catalog # A303-971A
- Rabbit anti-RPN1, Bethyl Laboratories, catalog # A305-026A
- Rabbit anti-beta tubulin, Abcam, catalog # ab97872
- IgG, Thermo Fisher Scientific, catalog # 02-6102
- Mouse GFP tag antibody, Thermo Fisher Scientific, catalog # MA5-15256
- Goat anti-mouse IgG (HRP), GeneTex, catalog # GTX213111-01
- Goat anti-rabbit IgG (HRP), GeneTex, catalog # GTX213110-01
- Alexa Fluor 488 nm, Thermo Fisher Scientific, catalog # A11034
Validation

Antibody validation was accomplished by the provider.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

American Type Culture Collection (ATCC): H1-HeLa (CRL-1968), rhabdomyosarcoma (RD) (CCL-136), Vero cells (CCL-81), C6/36 cells (CRL-1660). Thermo Fisher Scientific: 293FT (R70007). Huh7.5.1 was provided by Frank Chisari Lab. HAP1 cells was originally generated by Jan Carette and Thijn Brummelkamp (PMID: 21866103). BHK-21 cells was provided by Karla Kirkegaard lab.

Authentication

Authentication was accomplished by the provider.

Mycoplasma contamination

All cell lines tested negative for mycoplasma contamination.

Commonly misidentified lines

N/A

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