Identification of antiviral roles for the exon-junction complex and nonsense-mediated decay in flaviviral infection

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West Nile virus (WNV) is an emerging mosquito-borne flavivirus, related to dengue virus and Zika virus. To gain insight into host pathways involved in WNV infection, we performed a systematic affinity-tag purification mass spectrometry (APMS) study to identify 259 WNV-interacting human proteins. RNA interference screening revealed 26 genes that both interact with WNV proteins and influence WNV infection. We found that WNV, dengue and Zika virus capsids interact with a conserved subset of proteins that impact infection. These include the exon-junction complex (EJC) recycling factor PYM1, which is antiviral against all three viruses. The EJC has roles in nonsense-mediated decay (NMD), and we found that both the EJC and NMD are antiviral and the EJC protein RBM8A directly binds WNV RNA. To counteract this, flavivirus infection inhibits NMD and the capsid–PYM1 interaction interferes with EJC protein function and localization. Depletion of PYM1 attenuates RBM8A binding to viral RNA, suggesting that WNV sequesters PYM1 to protect viral RNA from decay. Together, these data suggest a complex interplay between the virus and host in regulating NMD and the EJC.

WNV is a member of the flavivirus genus, comprising globally important emerging and re-emerging pathogens, including dengue virus (DENV), Zika virus (ZIKV), Japanese encephalitis virus (JEV) and Yellow Fever virus (YFV). Flaviviruses are small, positive-sense RNA viruses that are translated as a single polyprotein and processed into structural (capsid, prM and Env) and non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) proteins. During infection, flaviviruses utilize host machinery to carry out replication and must subvert antiviral Type I interferon and cell-intrinsic pathways. Screening strategies have provided a wealth of information regarding host restriction and susceptibility factors in WNV infection⁵–⁸. However, it is unclear if these factors interface with viral proteins to impact infection and the plethora of factors identified is probably incomplete.

Here, we combine a mass spectrometry-based approach for mapping protein–protein interactions (PPIs) with genetic screening to identify host factors that physically interact with WNV proteins and influence infection. This approach is a powerful strategy to uncover mechanisms of viral infection and subversion of cell-intrinsic restriction pathways⁹–¹¹. In total, we identified 259 WNV-interacting host proteins by co-immunoprecipitation of WNV proteins coupled with in-solution mass spectrometry¹²,¹³. As WNV is one of a larger genus of flaviviruses, we compared our WNV interactome with DENV and ZIKV and discovered a statistically significant overlap between flavivirus capsid proteins (P < 0.01).

We selected 122 host factors, including conserved capsid interactors, for short interfering RNA (siRNA) screening to determine their role in WNV, DENV and ZIKV infection. We identified 26 genes that impact WNV infection; 13 were specific to WNV, while the remaining 13 impact WNV and DENV or ZIKV. In total, we identified 40 genes with a phenotype in at least one flavivirus. Notably, eight WNV-interacting proteins impacted infection of all three viruses. We focused on PYM1, which interacts with flavivirus capsids. PYM1 is an EJC-associated protein with a role in NMD, a cellular RNA degradation pathway¹⁶–²¹. We show that flaviviruses inhibit NMD and components of both the EJC and NMD pathway are antiviral against WNV, DENV and ZIKV. EJC association with messenger RNA elicits NMD and we demonstrate that the EJC protein RBM8A binds to WNV RNA, suggesting that NMD targets viral RNA. Moreover, WNV antagonizes this process through PYM1, relocalizing the EJC and inhibiting interactions with viral RNA. Collectively, the results of our proteomics/genomics approach identifies alternative facets of the virus–host arms race.

Results

Constructing an WNV–host protein–protein map. To identify human proteins that physically interact with WNV proteins, we cloned each of the 10 WNV proteins expressing a C-terminal 2X-Strep affinity tag (Fig. 1a). Using immunoblotting, we verified expression of the affinity-tagged WNV proteins, as well as expression of DENV and ZIKV capsid, by transient transfection into HEK293 cells (Supplementary Fig. 1). Proteins not robustly detected by western blot or mass spectrometry (NS2A and NS2B) were excluded from further analyses (Supplementary Fig. 2). WNV proteins were affinity-purified and eluted to collect WNV bait and associated host prey proteins (Fig. 1b). Eluates were analysed by western blotting and silver staining (Fig. 1c), followed by in-solution mass spectrometry. In total, we identified ~7,000 co-purifying...
host proteins from HEK293 cells from quadruplicate replicates for all WNV baits (Supplementary Table 1).

**High-confidence WNV-interacting host proteins.** To prioritize reproducible, bait-specific PPIs, we analysed four replicates for each bait protein with two different APMS scoring algorithms: mass spectrometry interaction statistics (MIST)\textsuperscript{12} and COMPPASS\textsuperscript{15}. The WNV proteins with the highest number of interacting host proteins were capsid, NS4B and NS5 (Fig. 1d). To identify the pathways targeted by WNV, we performed gene ontology enrichment analysis on host proteins, revealing an enrichment of interactors associated with RNA processing, vesicle localization, and endoplasmic...
Host processes and complexes interact with WNV proteins. We used a database of known human–human protein interactions to overlay additional connections between host proteins to identify multiprotein complexes (Fig. 2). We identified several complexes that interact with WNV, including interactions between NS5 and the AP3 adaptor complex, previously shown to be required for flaviviral infection. We identified several complexes that overlay additional connections between host proteins to identify interactions with the analogous viral proteins in previous flavivirus APMS studies and 31 (~12%) affected flavivirus infection in previous genetic screens (Supplementary Table 4)24–26.

Capsid interactions are conserved between flaviviruses. To identify shared flavivirus–host protein interactions, we compared the WNV–host interactors with similar datasets from DENV (serotype 2 strain 16681) and ZIKV (French Polynesia 2013 H/FP/2013). This revealed that the most significant overlap between all three viruses was shared between capsid-interacting host proteins (P<0.01, Fig. 3a,b and Supplementary Table 5). An examination of the protein sequence similarity between the capsid proteins of these flaviviruses revealed ~40% similarity by pairwise analysis (Fig. 3c). In contrast, NS5 is the most conserved across these flaviviruses (~70%) (Supplementary Fig. 3). Although the conservation between capsid proteins is not high, the significance of overlap between interactors suggests significant structural and functional conservation between these proteins.

Capsids interact with nucleolar proteins in the nucleus. Gene ontology enrichment analysis for flavivirus capsid interactors revealed significant overlap in categories including RNA processing, ER protein processing, consistent with the intricate relationship between flaviviruses and the ER (Fig. 1e and Supplementary Table 6). We performed subcellular localization and gene ontology enrichment analysis for flavivirus capsid interactors revealed that the non-structural proteins, NS2B-NS3, NS4A, NS4B and NS5 (Fig. 2 and Supplementary Table 2). Our high-confidence interactome revealed a total of 89 (~35%) of the viral baits (grey circles), human prey (blue circles), and virus-host interactions (solid grey lines) and host–host interactions (dashed grey lines). The virus–host interaction map was derived from four biologically independent experiments.

Fig. 2 | The WNV-human PPI network. In total, 259 high-confidence WNV-human PPIs are displayed: viral baits (grey circles), human prey (blue circles), virus-host interactions (solid grey lines) and host–host interactions (dashed grey lines). The virus–host interaction map was derived from four biologically independent experiments.
NS5 proteins of DENV, JEV, and YFV are thought to localize to the nucleus, nuclear localization of WNV NS5 has not been observed.

Flavivirus (DENV, JEV and WNV) capsid proteins can localize to the nucleus, and nuclear localization has been reported for DENV, JEV and WNV capsid proteins. We confirmed that WNV, DENV and ZIKV capsids are in the nucleolus during infection, as they co-localize with known nuclear protein fibrillarin (Fig. 3f). Ectopic expression of WNV capsid also localizes to the nucleolus (Supplementary Fig. 4a). As expected, capsid also resides in the cytoplasm, consistent with the roles for capsid in viral replication. Of the 72 WNV capsid interactors shown, 21 were identified in DENV or ZIKV, and 10 of these are known to be nucleolar (Fig. 3g and Supplementary Table 6). These findings are consistent with a recently published report identifying several nucleolar proteins as interaction partners of ZIKV capsid. We focused on the nucleolar dead-box helicase protein, DDX55, and confirmed an interaction study (Fig. 3g and Supplementary Table 5). Previously, PYM1 interacted with all three flavivirus infection inhibits NMD. Therefore, we first determined if WNV, DENV or ZIKV infection alters NMD by monitoring endogenous targets. These include an NMD target generated through alternative splicing (SC35), a long 3' UTR-containing target (GABARAPL1), a 5' uORF-containing target (ASNS) and CARs, which has an unknown NMD-inducing feature. We measured the abundance of these NMD targets during infection and found an increase in SC35, GABARAPL1, CARS and ASNS over 48 hours with all three infections (Fig. 6a and Supplementary Fig. 7a). This accumulation is more pronounced and appears to be induced at earlier time points during WNV infection. We observed a less-pronounced, but statistically significant, increase in NMD targets during DENV infection. As a control, we measured the housekeeping gene, LDHA, and observed no accumulation on infection (Supplementary Fig. 7b). To establish a role for PYM1 in NMD during flavivirus infection, we show that depletion of PYM1 in WNV-infected cells results in a further accumulation of the long 3' UTR-containing NMD targets, GABARAPL1, but not SC35 (Supplementary Fig. 7c).

PYM1 (partner of Y14 and MAGOH) interacts with and promotes the recycling of the EJC proteins, MAGOH and RBM8A (Y14), which can target transcripts to NMD. Indeed, tethering PYM1, MAGOH or RBM8A to an RNA target leads to NMD-dependent degradation. We confirmed knockdown of MAGOH and confirmed that MAGOH depletion inhibits NMD (Supplementary Fig. 8a). We observed an increase in WNV, DENV and ZIKV infection in MAGOH-depleted cells, as compared to control cells (Fig. 6b). Given that PYM1 and the EJC complex are involved in NMD, we next determined if NMD itself is antiviral against flaviviruses. Therefore, we depleted a canonical and essential component of NMD, UPF1, and confirmed both knockdown (Supplementary Fig. 8b, left panel) and inhibition of NMD by accumulation of the NMD target SC35 using two distinct siRNAs (Supplementary Fig. 8b, right panel). We monitored infection in UPF1-depleted cells via RT-qPCR and observed an increase in WNV, DENV and ZIKV RNA (Fig. 6c). These data suggest that NMD is antiviral and that the interaction between WNV capsid and PYM1 may subvert this process.

We next tested whether the WNV capsid–PYM1 interaction interferes with PYM1 function. We immunoprecipitated PYM1 from uninfected cells and observed interactions with the EJC complex proteins MAGOH and RBM8A. However, these interactions are diminished on WNV infection, suggesting disruption...
**Fig. 3 | Flaviviral capsid proteins interact with overlapping host factors.**

a, An analysis of flavivirus bait-interacting proteins reveals a highly significant overlap between capsid-interacting host proteins for WNV, DENV and ZIKV. Results were derived from four biologically independent experiments. P values calculated using the hypergeometric test. b, A circos plot to visualize flavivirus capsid-interacting host protein overlap. The outermost circles are proportional to the number of capsid-interacting host proteins for each virus. Inner circles indicate the proportion of capsid-interacting proteins unique to each virus (light orange) and shared between flaviviruses (dark orange). Interconnecting purple lines indicate shared interacting host proteins, interconnecting light blue lines indicate shared gene ontology terms between interacting host proteins. c, Pairwise comparison showing the percentage of amino acid conservation between flavivirus capsid proteins. d, Heat map representing enriched KEGG pathway, gene ontology biological processes, gene ontology molecular function, reactome pathways and CORUM biological processes of the host factors interacting with indicated flavivirus capsid proteins. The colours represent statistical significance (–log10(P) value). e, Heat map visualizing the enrichment analysis of the gene ontology cellular compartment localization of WNV-interacting host proteins for each bait. The colours represent statistical significance (–log10(P) value). For d, e, P values were calculated based on the accumulative hypergeometric distribution, and the most statistically significant term within a cluster was chosen as the representative category for each cluster. For all analyses, data are derived from four biologically independent experiments. f, Localization of flavivirus capsid proteins in infected cells. WNV, DENV and ZIKV capsids are shown in green (left panels) using virus-specific capsid antibodies at 24 h post-infection. The nucleolus is shown in red (centre panel) using an antibody recognizing the nucleolar protein, fibrillarin. Merged images demonstrating the overlap between flavivirus capsid and the nucleolus are shown in the right panels, with nuclear staining (Hoescht) in blue. Images are representative of three independent biological experiments repeated with similar results. g, The WNV capsid-host interacting map indicates those host proteins that are WNV-specific (light blue circles), shared between WNV and DENV (light blue and green circles), shared between WNV and ZIKV (light blue and orange circles) and shared between all three viruses (green and orange circles). Capsid-interacting proteins that localize to the nucleolus are indicated with a thick outer line and grouped together in light pink. The capsid-interacting protein PYM1 is indicated in red. h, Immunoprecipitations (IPs) in HEK293 cells expressing the indicated constructs (WNV capsid-2X strep, DDX55-V5) were performed with Strep-Tactin resin (IBA) and blotted with the indicated antibodies. Results are representative of two biologically independent experiments. i, Infectious WNV, DENV and ZIKV viral particles from DDX55-depleted and control U2OS cells were measured by median tissue culture infectious dose (TCID50) assay in BHK (baby hamster kidney) cells 24 h post-infection. Shown is the mean ± s.e.m.; n equals three biologically independent experiments repeated with similar results. Statistical significance was calculated using an unpaired, one-tailed Student’s t-test; * P < 0.05, ** P < 0.005, *** P < 0.0005.
of these associations (Fig. 6d). We did not observe a significant change in abundance of MAGOH and RBM8A during WNV infection (Fig. 6d), but reasoned that disruption of their interactions with PYM1 may alter the localization of these proteins. Therefore, we performed fractionation experiments in uninfected and WNV-infected cells and monitored the relative abundance of MAGOH and RBM8A in the WCL, and cytoplasmic, nuclear and membrane/organelle fractions (Fig. 6e). While the total abundance of MAGOH and RBM8A is unchanged in infected WCLs, we observed decreased abundance in the cytoplasmic fraction, with a concomitant increase in the membrane/organelle fraction on infection. This suggests that WNV infection leads to sequestration of PYM1 and disruption of EJC protein function. Interestingly, we observed a change in the localization of PYM1 on infection (Fig. 6e). Given that WNV capsid binds and sequesters PYM1, attenuating its interaction with MAGOH and RBM8A, we hypothesized that depletion of PYM1 may induce this mislocalization. Indeed, we found that depletion of PYM1 results in a similar redistribution of these EJC proteins (Supplementary Fig. 8c). These data suggest that WNV capsid inhibits the function of MAGOH and RBM8A by altering the localization of these proteins.

PYM1, MAGOH and RBM8A are RNA-binding proteins involved in NMD and tethering any of these proteins to an RNA target leads to NMD-dependent degradation. Therefore, we hypothesized that one of these proteins may serve that function by directly binding WNV RNA. To test this, we used a modified cross-linking immunoprecipitation (CLIP) protocol in which we cross-linked RNA-binding proteins to RNA in WNV-infected cells, immunoprecipitated native PYM1, MAGOH or RBM8A and determined the abundance of viral RNA using RT–qPCR (Fig. 6f), and immunoprecipitated protein by immunoblot (Supplementary Fig. 8d) in the eluates. We normalized the viral RNA to a non-specific control (18S RNA) and compared the relative amount of bound viral RNA to an IgG control. These experiments revealed a specific interaction between viral RNA and RBM8A (Fig. 6f). Next, we tested whether loss of PYM1 or MAGOH might impact RBM8A binding...
PyM1 interacts with the EJC protein, MAGOH, and the canonical NMD factor, UPF1, are antiviral against WNV, DENV, and ZIKV (Fig. 6b,c). NMD targets accumulate during flaviviral infection (Fig. 6a and Supplementary Fig. 7a); altogether, these data suggest that NMD is an antiviral pathway that is antagonized by WNV. Our studies show that WNV infection interferes with the interaction between PyM1 and the EJC proteins MAGOH and RBM8A (Fig. 6d), resulting in their mislocalization (Fig. 6e). This suggests that the interaction of WNV capsid with PyM1 during infection prevents its association with MAGOH and RBM8A, thereby attenuating NMD.

Previous reports have indicated that tethering EJC proteins to RNA substrates results in degradation by NMD. We show that RBM8A binds viral RNA and that depletion of PyM1 diminishes this interaction, suggesting that PyM1 is required for this association (Fig. 6f,g). These data support a model in which NMD targets accumulate during flaviviral infection and that WNV capsid interferes with the interaction between PyM1 and the EJC proteins, resulting in the stabilization of both (Supplementary Fig. 9). Altogether, these data suggest that the EJC targets flavivirus RNA for NMD-mediated decay and that flavivirus capsids interact with PyM1 to interfere with this pathway.

**Discussion**

Here, we provide a complete APMS-generated, WNV–host PPI map. Our subsequent siRNA screen targeted a subset of factors in WNV, DENV, and ZIKV infection to identify 40 proteins that interact with viral proteins and influence flavivirus infection (Figs. 2 and 4c; Supplementary Tables 2 and 8). We identified eight proteins, five proviral (CSDA, RDH11, UBA2P2L, VCP and UBP15) and three antiviral (DDX55, G patch8, and PyM1), that both interacted with WNV proteins and had an effect on infection of all three flaviviruses. Of these eight proteins, five are RNA-binding proteins interacting with flavivirus capsids. These data underscore the importance of both flavivirus capsid–host protein interactions and RNA processing in flaviviral infection.

We focused on the interaction between PyM1 and WNV, DENV, and ZIKV capsids and showed that PyM1 is antiviral (Figs. 4c and 5a–f; Supplementary Table 8). PyM1 interacts with the EJC proteins MAGOH and RBM8A and plays a role in NMD. In addition to PyM1, the EJC protein, MAGOH, and the canonical NMD factor, UPF1, are antiviral against WNV, DENV, and ZIKV (Fig. 6b,c). NMD targets accumulate during flaviviral infection (Fig. 6a and Supplementary Fig. 7a); altogether, these data suggest that NMD is an antiviral pathway that is antagonized by WNV. Our studies show that WNV infection interferes with the interaction between PyM1 and the EJC proteins MAGOH and RBM8A (Fig. 6d), resulting in their mislocalization (Fig. 6e). This suggests that the interaction of WNV capsid with PyM1 during infection prevents its association with MAGOH and RBM8A, thereby attenuating NMD.

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Fig. 6 | NMD is inhibited by flaviviruses through targeting of the EJC and the EJC protein RBM8A binds to viral RNA. a. U2OS cells were infected with the indicated viruses at an multiplicity of infection (MOI) of 10 and analysed at the indicated time points. Two endogenous targets of the NMD mRNA surveillance pathway (SC35, left panel; GABARAPL1, right panel) were analysed by RT-qPCR. Gene expression data (gene/18S) are normalized to uninfected controls. Shown is the mean ± s.e.m.; n equals three biologically independent experiments. Significance was calculated using the ratios of the target gene to the control gene and is indicated by * P < 0.05, ** P < 0.005 using an unpaired, two-tailed Student’s t-test.

b. Relative WNV, DENV and ZIKV viral RNA were measured in control cells (siCON) and MAGOH-depleted cells using two independent siRNAs (siMAGOH-1 and siMAGOH-2) at 24 h post-infection. Infections are normalized to cells treated with siControl (siCON). Shown is the mean ± s.e.m.; n equals three biologically independent experiments. For all experiments significance was calculated using the ratios of the target gene to the control gene and is indicated by * P < 0.05, ** P < 0.005, *** P < 0.0005 using an unpaired, two-tailed Student’s t-test.

c. Relative WNV, DENV and ZIKV viral RNA was measured in control and UPF1-depleted cells using two independent siRNAs (siUPF1-1 and siUPF1-2) at 24 h post-infection. Infections are normalized to cells treated with siControl (siCON). Shown is the mean ± s.e.m.; n equals three biologically independent experiments. For all experiments significance was calculated using the ratios of the target gene to the control gene and is indicated by * P < 0.05, ** P < 0.005, *** P < 0.0005 using an unpaired, two-tailed Student’s t-test.

d. Immunoprecipitation of PYM1 from uninfected or WNV Kunjin-infected U2OS cells. Results are representative of two biologically independent experiments. Mouse IgG light chain is detected below PYM1 in the PYM1-IP fraction and is indicated by (*). e. Cellular fractionation experiments show the relative abundance of MAGOH, RBM8A, PYM1 and WNV capsid in the whole cell lysate (WCL), cytoplasmic (Cyto), nuclear (Nuc) and membrane/organellie (Mem) fractions between uninfected and WNV Kunjin-infected U2OS cells at 24 h post-infection. Tubulin (cytoplasm), Lamin B1 (nucleus) and AIF (mitochondria) are shown as fractionation markers and loading controls. The relative abundances of MAGOH and RBM8A were calculated by quantifying the bands using Quantity One software (Bio-Rad). For each compartment, the relative protein abundance in the uninfected sample was set to 1. Results are representative of two biologically independent experiments repeated with similar results. f. Antibodies specific for PYM1, MAGOH and RBM8A were used to immunoprecipitate native proteins from WNV Kunjin-infected cells. RNA was extracted from the eluates and associated viral RNA was measured by RT-qPCR (viral RNA/18S RNA). The viral RNA associated with the immunoprecipitated proteins was normalized to an IgG control and to the relative input viral RNA. Shown is the mean ± s.e.m.; n equals three biologically independent experiments. Significance was calculated using an unpaired, one-tailed Student’s t-test and is indicated by * P < 0.05, ** P < 0.005.
research suggests that other neurotropic flaviviruses, including WNV, can result in similar foetal damage43,44. Further studies are required to determine if the sequestration and altered localization of MAGOH and RBM8A during flaviviral infection contributes to the neurological defects in developing foetuses.

NMD is known to restrict some RNA viruses32,33,45. In some cases, viruses counteract the restrictive mechanism of NMD through interaction with the NMD protein UPF1 (refs. 33,46). It is unknown in this context whether the EJC plays a role in viral RNA recognition or degradation. Nevertheless, these data suggest a conserved antiviral role for this pathway32,33,46. Interestingly, other studies have shown that some viruses can hijack NMD or EJC factors to aid in viral translation, replication or infectious viral particle production47,48.

Our ongoing studies will allow us to further define the relationship between flaviviruses and NMD, as well as to explore additional mechanisms by which flaviviruses manipulate host cell processes.

Methods

Cells and viruses. HEK293 and U2OS cell lines were obtained from the American Type Culture Collection. All cultures were grown under standard conditions in Dulbecco’s Modified Eagle Medium supplemented with 10% foetal bovine serum, 100 units of penicillin, 100 μg of streptomycin and 20 mM l-αalanine-l-glutamine dipeptide (GlutaMax, Gibco). HEK293 cells were transfected using X-tremeGENE 9 (Roche Applied Science) according to the manufacturer’s instructions. U2OS cells were transfected using Fugene HD (Promega) according to the manufacturer’s instructions. BH494 cells were maintained as previously described49. The WNV, KUNV isolate (CH16332; a generous gift of R. Tesh, World Reference Center for Emerging Viruses and Arboviruses, Galveston, TX) was propagated using the same protocol as WNV, DENV-2 (NGC from BEI), and ZIKV Mexico 2016 (Mex2-B1; a generous gift from R. Tesh) were grown as previously described49. Viral titres were determined in BHK 21 cells by plaque assay.

Affinity purification and sample preparation for mass spectrometry. Viral protein encoding a C-terminal 2X Strep II affinity tag were used for affinity purification and were derived from the following strains: WNV 2000-crow 3356, DENV serotype 2 16681, ZIKV French Polynesia 2013 H/FP/2013. Affinity purification and sample preparation for mass spectrometry.

Mass spectrometry. Digested peptide mixtures were analysed by LC-MS/MS on a Thermo Scientific Velo Pro ion trap mass spectrometer system equipped with a Proxeon Easy nLC 1000 ultra-high-pressure liquid chromatography and autosampler system. Sample were injected onto a pre-column (2 cm × 100 μm internal diameter packed with ReproSil Pur C18-AQ 5 μm) and then separated with a 2-h gradient from 5% to 30% acetonitrile in 0.1% formic acid on an analytical column (10 cm × 75 μm internal diameter packed with ReproSil Pur C18-AQ 3 μm particles). The mass spectrometer collected data in a data-dependent fashion, collecting one full scan followed by 20 collision-induced dissociation MS/MS scans of the 20 most intense peaks from the full scan. Dynamic exclusion was enabled for 30 s with a repeat count of 1.

The results raw data was matched to protein sequences by the Protein Prospector algorithm. Data were searched against the SwissProt Human protein sequence database (downloaded 6 March 2012) concatenated to a decoy database where each sequence was randomized to estimate the false positive rate. The searches considered a precursor mass tolerance of 1 Da and fragment ion tolerances of 0.8 Da, and considered variable modifications for protein N-terminal acetylation, protein N-terminal acetylation and oxidation, glutamine to pyroglutamate conversion for peptide N-terminal glutamine residues, protein N-terminal methionine loss, protein N-terminal acetylation and methionine loss, and methionine oxidation, and constant modification for carbamidomethyl cysteine. Prospector data were filtered using a maximum protein expectation value of 0.01 and a maximum peptide expectation value of 0.05.

Interactome scoring and visualization. The WNV interactome was compiled through selecting bait–prey pairs with a MIST score > 0.68, computed with the weights we previously established for HCV-host interactome: reproducibility (0.36), abundance (0.09) and specificity (0.53)49,50. The network was also scored with the complementary CompPASS scoring algorithm49. Only the top 1% ComPPass weighted D-scores10 per bait were included in the final iteration of the interactome. Any WNV protein for which we did not detect the bait protein in all the replicates analysed was excluded from further study. To simplify the interactome and analyses, we manually removed all ribosomal proteins. We used a database of known human–human protein interactions to overlay additional connections between host proteins to identify multiprotein complexes (CORUM)10. The final iteration of the WNV interactome was visualized as a network representation using Cytoscape, v2.8.3 (ref. 49). The complete details of the scoring algorithm and set of identified prey proteins, including the individual scores for the abundance, reproducibility and specificity, and the final interactome can be found in Supplementary Tables 1 and 2.

Enrichment analysis. For each given gene list, pathway and process enrichment analysis was carried out with one or more of the following ontology sources: Kyoto Encyclopedia of Genes and Genomes, Gene Ontology, Reactome, Human Protein Reference Database (HPRD), Gene Ontology Molecular Function, reactome gene sets, canonical pathways and CORUM. All genes in the human genome were used as the enrichment background. Terms with P < 0.01, minimum count 3 and enrichment factor > 1.5 (enrichment factor is the ratio between observed count and the count expected by chance) were collected and grouped into clusters based on their membership similarities. More specifically, P values were calculated based on accumulative hypergeometric distribution and q values were calculated using the Benjamini–Hochberg procedure to account for multiple testing49. Kappa scores were used as the similarity metric when performing hierarchical clustering on the enriched terms; sub树 trees with similarity >0.3 were collapsed into a single tree cluster. A significant term within a cluster was chosen as the representative category for each cluster. All analyses and visualization were carried out using Metascape (http://metascape.org/gp/index.html#/main/steps1). A detailed list of all terms, results and statistics for each analysis are provided in the Supplementary data (Supplementary Tables 3, 6 and 7).

siRNA screening. U2OS cells were seeded at 2,000 cells per well in 50 μl media per well in a 384-well plate for siRNA knockdown. Two siRNAs for each selected host target (Ambion Silencer Select) were transfected with HiPerFect (Qiagen) according to manufacturer recommendations at a final siRNA concentration of 20 nM. Cells were infected 3 days after knockdown, at a density of 16,000 cells per well. WNV, DENV and ZIKV infections were done at an MOI of 0.01. Infection was measured 24 h post-infection by immunostaining and automated microscopy. Cells were fixed with 4% formaldehyde for 10 min, permeabilized with 0.1% Triton X-100 for 15 min, washed three times with PBS and blocked with 2% BSA in PBS-T. Cells were stained overnight with the mouse 4G2 (anti-envelope) antibody (1:4,000 for DENV and ZIKV, 1:12,000 for WNV Kunjin). Cells were washed three times with 2% BSA in PBS-T and incubated with 5 μg ml-1 Hoechst 33422 to identify nuclei and with an Alexa-Fluo-488 conjugated α-mouse secondary antibody (1:1,000) for 1 h at room temperature. Cells were washed three times with 2% BSA in PBS-T and imaged in 50 μl PBS. Four sites per well were imaged at ×10 magnification (ImageXpress Micro; Molecular Devices), and two wells were analysed for each condition for each replicate. Each screen was performed in duplicate. Automated image analysis (MetaXpress; Molecular Devices) was used to the images and determine the number of 4,6-diamidino-2-phenylindole-positive and -positive cells. The percentage of infected cells was calculated, averaged well by well and log transformed (TIL) to be used in the enrichment analysis. The TIL and interquartile range were calculated and used to calculate a robust z-score for each well using the following equation12: \( z = \frac{\log_{10}(\text{median}) - \log_{10}(\text{percentile})}{\text{IQR}} \). Where IQR is the interquartile range. The robust z-scores for the experimental replicates were averaged and candidates were identified as positive if the averaged robust z-score was > 1.3 or > 1.3. Cytotoxic candidates were identified based on nuclei counts and those with a > 20% decrease in cell number as compared to the average cell count were excluded from further study. The complete screening results and a complete list of siRNAs used in this study can be found in Supplementary Tables 8 and 9.

RNA isolation and RT–qPCR. Total cellular RNA was isolated using TriZol (Thermo Fisher Scientific), purified and DNase-treated using RNA Clean & Concentrator (Zymo) per manufacturer’s instructions. Complementary DNA was synthesized using 1 μg of input RNA using random hexamer primers (Life Technologies) with moloney murine leukaemia virus reverse transcriptase (M-MLV RT, Invitrogen) in a total volume of 20 μl. cDNA reactions were diluted 1:5 and 20 μl of each diluted sample was used to make a pooled reference. The pooled reference was used for subsequent 10-fold dilutions to generate a standard curve for all targets being measured. cDNA reactions were further diluted 1:1 (1:25 total dilution) and SYBR green reactions contained 5 μl of 2x Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific), 3 μl of diluted cDNA and 5 pmol of both forward and reverse primers. The RNA targets indicated in each figure were quantified by qPCR and the relative abundance of each target was calculated using the standard curve. The relative values for each transcript were normalized to a control RNA (185 ribosomal RNA or GAPDH) and compared between experimental conditions. A complete list of primers used for RT–qPCR is provided in Supplementary Table 10.
with Alexa-Fluor-488 or 594-conjugated secondary antibodies (1:1,000) for 1 h to calculate TCID50 values. Cells were washed three times with 2% BSA in PBS-T and imaged in 50 µl PBS to calculate TCID50 values.

**Articles**

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**Antibodies.** The following antibodies were used: α-Strep tag (Abcam, ab184224), α-FLAG tag (Sigma, F7425), α-V5 (Bethyl, A300-1260A), α-tubulin (Sigma, T6199), α-WNV capsid (Abcam, ab21673), α-WNV capsid (Genetex, GTX131947), α-WNV capsid (SAB3500912), α-DENV capsid (Genetex, GTX03343), α-ZIKV capsid (Genetex, GTX133317), α-fibrillarin (Thermo Fisher Scientific, A22227), α-MAGOH (Cell Signalling, 5318S), α-WNV capsid (Abcam, ab21673), α-WNV capsid (Genetex, GTX131947), α-WNV capsid (Sigma, SAB3500912), α-DENV capsid (Genetex, GTX03343), α-ZIKV capsid (Genetex, GTX133317), α-fibrillarin (Thermo Fisher Scientific, A22227), α-MAGOH (Cell Signalling, 5318S), α-Lamin B1 (Abcam, ab23128). The α-flavivirus glycoprotein (4G2) hybridoma was provided by M. Diamond (Washington University).

**Materials and Methods**

**RNAi transfection and infection studies.** For RNAi experiments, 200,000 U2OS cells were plated in a 6-well plate, and siRNAs were transfected using HiPerFect (Qiagen) according to manufacturer’s recommendations at a final concentration of 20 nM and incubated for 48–72 h. Cells were infected with WNV, DENV or ZIKV at the MOIs and time points indicated in the figure legends. Following infection, cells were collected in 1 ml Trizol for RT-qPCR experiments or washed three times in PBS and resuspended in 1 ml IP buffer for western blotting. For transfection experiments, 200,000 U2OS cells were plated in a 6-well plate and the indicate vectors were transfected using FuGene HD (Promega) and incubated for 48 h. Following transfection, cells were collected in 1 ml Trizol for RT-qPCR experiments or washed three times in PBS and resuspended in 1 ml IP buffer for western blotting.

**Plasmids.** Open reading frames from strains WNV NY 2000-crow 3356 (capsid, prMEA7TM2, M2, NS1, NS2A, NS2B, NS4B, NS5, DENV 16861 (capsid), ZIKV H/F/P/2013 (capsid) were cloned into pCDNA4_T0 with a C-terminal 2X Strep II affinity tag for expression in human cells (vector provided by S. Butcher). Western blotting was performed using a 1:1,000 dilution of α-strep (Abcam) and α-V5 (Bethyl) antibodies to detect the interaction of WNV capsid and PYM1 in the context of viral infection and to test if WNV infection disrupts the interaction between PYM1 and MAGOH or RBM2. U2OS cells were infected with WNV virus (MOI = 5) for 24 h, washed three times with PBS and lysed in IP buffer. The cellular lysates were clarified and endogenous PYM1 was immunoprecipitated by α-PYM1 antibody (Novus) with Protein A/G Agarose beads (Thermo Fisher Scientific) overnight at 4°C. The beads and bound proteins were washed three times in IP buffer and eluted with 0.2 M glycine pH 2.5 buffer, followed by neutralization with 1 M Tris pH 9 buffer. The eluates were resuspended in 2X SDS loading buffer and analysed by western blotting with the indicated antibodies.

**Confocal microscopy.** U2OS cells were plated into 12-well plates on glass coverslips at a density of 50,000 cells per well. After 24 h, cells were infected with the indicated viruses at the indicated MOI for 24 h. Cells were fixed with 4% formaldehyde, permeabilized for 10 min with 0.1% Triton X-100 and stained overnight at 4°C with the indicated antibodies. Coverslips were washed three times in PBS-T and incubated with 5 µg/ml Hoechst 33422 to identify nuclei and with an Alexa-Fluor-488-conjugated α-mouse secondary antibody (1:1,000) for 1 h at room temperature. Cells were washed three times with 2% BSA in PBS-T and imaged in 50 µl PBS to calculate TCID50 values.

**RNA immunoprecipitation.** U2OS cells were infected with WNV at an MOI of 5. Twenty-four hours post-infection, cells were trypsinized and subjected to ultraviolet crosslinking. Cells were then lysed in Buffer A (30 mM Hepes at pH 7.4, 2 mM MgOAc, 0.1% NP40) and 150 mM KOAc supplemented with phenylmethylsulfonyl fluoride (PMSF) and protease inhibitors. After centrifugation for 5 min at 500 g, the supernatant was saved as the membrane and organelle fraction. The pellet was then resuspended in Membrane Isolation Buffer supplemented with phenylmethylsulfonyl fluoride (PMSF) and protease inhibitors. After centrifugation for 5 min at 1000 g, the supernatant was saved as the cytoplasmic fraction. The pellet was then resuspended in Membrane Isolation Buffer and incubated on ice for 5 min. After centrifugation for 5 min at 8,000 g, the supernatant was removed and saved as the membrane and organelle fraction. The pellet was resuspended with Cytoplasmic/Nucleus Isolation Buffer (CYNB) and sonicated as the cytoskeletal and nuclear fraction. Cell lysates from each fraction were loaded into SDS–PAGE and protein abundance was analysed by western blotting.

**Supplementary information**

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

**Data availability**

Mass spectrometry data in this study is deposited in the PRIDE database (https://www.ebi.ac.uk/pride/archive/, Project Accession no. PXD0011728). A complete list of interaction scores are provided in Supplementary Tables 1, 2 and 5. Gene ontology enrichment analyses are provided in Supplementary Tables 3, 6 and 7. Interactors found in previous flavivirus proteomic or genetic studies are detailed in Supplementary Table 4. RNAi screening data are provided in Supplementary Table 8. Additional supporting data are available from the corresponding authors upon request.

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**GEO accession**

GSE147123
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Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

| n/a | Confirmed |
|-----|-----------|
| ☒️ | The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement |
| ☒️ | An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| ☒️ | The statistical test(s) used AND whether they are one- or two-sided |
| ☒️ | Only common tests should be described solely by name; describe more complex techniques in the Methods section. |
| ☒️ | A description of all covariates tested |
| ✗️ | A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons |
| ☒️ | A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals) |
| ☒️ | For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted |
| ☒️ | Give P values as exact values whenever suitable. |
| ☒️ | For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings |
| ☒️ | For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes |
| ☒️ | Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated |
| ☒️ | Clearly defined error bars |
| ☒️ | State explicitly what error bars represent (e.g. SD, SE, CI) |

Our web collection on [statistics for biologists](http://www.nature.com) may be useful.

Software and code

Policy information about availability of computer code

Data collection

Provide a description of all commercial, open source and custom code used to collect the data in this study, specifying the version used OR state that no software was used.

Data analysis

The results of the the raw mass spectrometry data were matched to the Protein Prospector algorithm (http://prospector.ucsf.edu/prospector/mshome.htm) and data were searched against the SwissProt Human protein sequence database (downloaded March 6, 2012). Interactor scoring was determined using the MiST (https://github.com/kr oganlab/mist) and compPASS algorithms (http://bioplex.hms.harvard.edu/download Compass.php). The WNIV-host protein-protein interaction network was generated using Cytoscape (version 2.8.3, Smoot et al., 2011) and host protein complexes were derived using CORUM (http://mips.helmholtz-muenchen.de/corum/). Heat maps depicting Gene Ontology terms and associated significance values were analyzed and visualized using Metascape (http://metascape.org/gp/index.html?main=step1). The heat map generated from siRNA screening data was visualized and analyzed using Morpheus (https://software.broadinstitute.org/morpheus/).

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Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The raw mass spectrometry data has been submitted to PRIDE (Project accession: PXD011728). A complete list of all MiST and compPASS scores for interacting host proteins are provided in Table S1 and the interactors that are above our designated threshold are in Table S2. All Gene Ontology enrichment analyses and associated significance values are provided in Tables S3, S6 and S7. The overlap with previous proteomic and genetic studies with associated PMIDs are provided in Table S4. The overlap of flavivirus capsid interactors, including MiST and compPASS scores for DENV and ZIKV-interacting host proteins that are above our designated threshold are in Table S5. Interactors selected for RNAi screening and all screening data is provided in Table S8. All siRNA and primer sequences used in this study are provided in Tables S9 and S10. Any additional data related to this manuscript will be provided upon request.

Field-specific reporting

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For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Sample size was determined using classical statistical methods for calculations of mean and significance. |
|-------------|-----------------------------------------------------------------------------------------------------|
| Data exclusions | No data was excluded from analysis. |
| Replication | All experimental findings were reliably reproduced. |
| Randomization | Not relevant to this study |
| Blinding | Not relevant to this study |

Reporting for specific materials, systems and methods

| Materials & experimental systems | Methods |
|--------------------------------|---------|
| n/a | Involved in the study |
| [ ] Unique biological materials | [ ] ChIP-seq |
| [x] Antibodies | [ ] Flow cytometry |
| [x] Eukaryotic cell lines | [x] MRI-based neuroimaging |
| [x] Palaeontology | |
| [x] Animals and other organisms | |
| [x] Human research participants | |

Unique biological materials

Policy information about availability of materials

Obtaining unique materials

All unique materials used in this study are readily available upon request.

Antibodies

Antibodies used

All antibodies used in this study are commercially available. The supplier and catalog numbers are provided in the methods section of the text.
Validation of all antibodies provided by the manufacturer as indicated in the text.

### Eukaryotic cell lines

**Policy information about cell lines**

| Cell line source(s) | ATCC |
|---------------------|------|
| Authentication      | Cell lines were obtained from ATCC without independent validation. |
| Mycoplasma contamination | All cell lines were negative for Mycoplasma contamination. |
| Commonly misidentified lines (See ICLAC register) | None used |