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RNA interference screen for human genes associated with West Nile virus infection

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Abstract

West Nile virus (WNV), and related flaviviruses such as tick-borne encephalitis, Japanese encephalitis, yellow fever and dengue viruses, constitute a significant global human health problem¹. However, our understanding of the molecular interaction of WNV (and related flaviviruses) with mammalian host cells is limited¹. WNV encodes only 10 proteins, implying that the virus may use many cellular proteins for infection¹. WNV enters the cytoplasm through pH-dependent endocytosis, undergoes cycles of translation and replication, assembles progeny virions in association with endoplasmic reticulum, and exits along the secretory pathway^{1–3}. RNA-interference (RNAi) presents a powerful forward genetics approach to dissect virus-host cell interactions^{4–6}. Here we report the identification of 305 host proteins impacting WNV infection,

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Author Contributions

MNK, HA and EF designed the experiments; MNK, BS, EF and RA performed the screen; MNK, BS, RAK, ALB, SJE and HA analyzed the data; MNK and HS performed validations; PDU designed microscopy; FDG and PWM designed replicon experiments; SL provided PITPNM2 cDNA; AN and RJX performed bioinformatics analyses. MNK, HA, AN, RJX and EF co-wrote the paper.

using a human genome-wide RNAi screen. Functional clustering of the genes revealed a complex dependence of this virus on host cell physiology, requiring a wide variety of molecules and cellular pathways for successful infection. We further demonstrate a requirement for the ubiquitin ligase CBLL1 in WNV internalization, a post-entry role for the endoplasmic reticulum-associated degradation (ERAD) pathway in viral infection, and the monocarboxylic acid transporter MCT4 as a viral replication resistance factor. By extending this study to dengue virus, we show that flaviviruses have both overlapping and unique interaction strategies with host cells. This study provides the first comprehensive molecular portrait of WNV-human cell interactions that forms a paradigm for understanding single plus-stranded RNA virus infection, and reveals potential antiviral targets.

Host proteins previously reported to facilitate WNV infection (termed Host Susceptibility Factors, HSFs) are endosomal transport regulators and vATPase (for entry), eEF1A, TIA-1/TIAR, and HMGCR (for replication), and c-Yes (for secretion)^{2, 3, 7-10}, or to reduce WNV infection (termed Host Resistance Factors, HRFs) are components of the antiviral IRF3 pathway¹¹. In this context, a genome-scale small interfering-RNA (siRNA) based screen silencing 21,121 human genes in HeLa cells was performed to comprehensively identify the cellular proteins associated with the early stages of WNV infection from viral entry through the intracellular translation of viral RNA. Defects in the later stages of infection such as replication, assembly or secretion were not scored by the assay. The assay involved infection of gene-silenced cells with WNV for 24 h, and subsequently, a microscopy-based quantification of the cells immunostained for viral envelope protein was used to select the candidate host proteins. The screen was done in two steps: a primary screen using a pool of 4 siRNAs per gene, followed by a validation screen, testing each individual siRNA within the pool separately (for the hits selected in the primary screen) to minimize potential off-target hits (Figure 1a). The details of the assay and screen are described in the methods and Supplementary Figure 1.

The RNAi screen identified 283 HSFs and 22 HRFs (of which 273 and 21 are novel respectively) (Supplementary Tables 1 and 2). The number of HRFs constituted 7 % of the total host factors identified. The identification of (i) some of the known HSFs (vATPase, endosomal transport regulators³), and HRFs (IRF3¹¹) of WNV infection, and (ii) multiple components of macromolecular assemblies (e.g., vATPase, ERAD, focal adhesion complex), validated the reliability of our approach and the *in vitro* model. A cellular map summarizing several screen hits classified into cellular compartments and broad functional association categories is provided in Supplementary Figure 2.

Of the 283 HSFs, 195 (69%) and 193 (68%) could be classified using biological process and molecular function categories, respectively (Figure 1b and c, Supplementary Tables 3 and 4). There was a significant enrichment of genes regulating intracellular protein trafficking, cell adhesion and processes associated with the transport of ions and biomolecules. The enriched molecular function categories included hydrolases, transporters, ligases, cell adhesion molecules, membrane traffic proteins and synthases. Among the HSFs, six RNA binding proteins (e.g., RBPMS), twenty ubiquitination-related proteins (e.g., CBLL1), twenty one transcription factors (e.g., LDB1), three C-type lectins (CLEC7A, CLEC4A and CLEC4C) and five protocadherins (e.g., PCDHB5) were also present. The RNA binding protein RBPMS was reported as part of a protein network implicated in Purkinje cell degeneration¹². Strikingly, the current screen also captured seven other members (COIL, PCP4, UBE2I, LDB1, NUMBL, ATXN7L3 and USP6) interacting with RBPMS (Supplementary Figures 3a and b; 4a and b).

The screen also identified several genes previously implicated in immunity (Supplementary Tables 1 and 2). Immune related HSFs include beta-defensins (DEFB118 and DEFB129,

Supplementary Figure 5a), Rnase L inhibitor ABCE113⁻15 (Supplementary Figure 5b), LY6E, Zap70, TNFSF13B, and DUBA (OTUD5). Among the HRFs, alpha-defensin DEFA3 and IRF3 are known immune response genes. These findings highlight that defensin family members function as both viral resistance and susceptibility factors¹⁶. Knockdown of the immunophilin FKBP1B also enhanced WNV infection.

We next determined whether the genes identified from HeLa cells are expressed in tissues targeted by WNV *in vivo*, by analyzing the expression pattern of the HSFs across 79 tissues (Supplementary Figure 6). In accordance with the tissue tropism of WNV, 102 (46%) and 64 (29%) HSFs showed enriched expression in immune and CNS tissues, respectively (Wilcoxon $p < 0.05$) (Supplementary Table 5 and 6).

Among the twenty ubiquitination-related proteins identified in the screen, the ubiquitin ligase CBLL1 is known to regulate the endocytosis of cell-surface receptors, and therefore we hypothesized that CBLL1 may be involved in the cellular internalization of WNV17. CBLL1 silencing resulted in a marked reduction (82%, $p = 0.05$) of WNV-infected cells (Figure 2a, and b; Supplementary Figures 4a, b and 7a). In order to test whether CBLL1 is involved in WNV entry, we next examined the kinetics of TRITC-labeled WNV particle internalization into CBLL1 silenced cells. Strikingly, there was a ~20-fold ($p < 0.05$) reduction in the number of virus particles present within CBLL1 silenced cells when analyzed from 1–4 h post-incubation (Figure 2c and d; Supplementary Figure 7b). Moreover, virus was seen stuck on the plasma membrane of 17% of CBLL1 silenced cells (Figure 2d; Supplementary Figure 7b and 8). As expected, CBLL1 silencing did not alter WNV replicon translation (Supplementary Figure 9a). The virus internalization defect of CBLL1 silenced cells was similar to that observed in cells defective for clathrin dependent endocytosis (CDE), a pathway implicated in WNV entry (Figure 2d)^{2, 3}. CDE was ablated by targeting the clathrin adaptor AP3S2, which was also identified in our screen (Supplementary Table 1)¹⁸. Silencing of the post-entry HSF, vATPase, did not alter the internalization of virus (Figure 2d). Furthermore, consistent with the involvement of a ubiquitin ligase in WNV entry, depletion of cellular free ubiquitin pool by pretreatment with MG132 (a proteasomal inhibitor) strongly abolished WNV internalization (50 fold, $p = 0.001$) (Figure 2e; Supplementary Figure 4b). Notably, proteasome inhibition was also found to interfere with WNV infection at post-internalization steps (Supplementary Figure 9b). Proteasomal components were also identified in the screen (Supplementary Table 1). Demonstrating WNV specificity, MG132 treatment did not inhibit vesicular stomatitis virus infection (Supplementary Figure 9c), as reported previously¹⁹. Collectively, these findings demonstrate that CBLL1 and the proteasome-ubiquitin system are required for the cellular internalization of WNV.

Because the endoplasmic reticulum (ER) is implicated in the intracellular phase of flaviviral life cycle¹, we examined whether WNV co-opts ER components for infection. Network analysis anchoring on ER proteins revealed the presence of several components of ER-associated degradation (ERAD) pathway among the identified HSFs (Figure 2f). ERAD comprises more than ten proteins that retro-transport misfolded proteins from ER to the proteasome²⁰. Silencing of several key components or interactors of ERAD (HRD1, DERL2, UBE2J1/UBC6, UBE3A, SEC61G, SEC61A1, UFD1L and NSFL1C), but not other ERAD components (eg., DERL1, DERL3, HRD3, NPL4, p97), reduced WNV infected cells up to 89% (Figure 2g; Supplementary Figure 4a and b). ERAD was not required for human immunodeficiency virus - 2 infection (Supplementary Figure 10a), highlighting specificity between different viruses. To further validate these results, reduction of viral infection due to silencing of DERL2 was rescued by transfection with siRNA-resistant silent mutation-containing variant of DERL2 (Figure 2g; Supplementary Figure 10b). We also identified the recently reported ERAD component BCAP31 as an HSF21.

Functional studies revealed that ERAD is not involved in WNV internalization, endosomal transport, or RNA translation; however, there was ~10% reduction in the secretion of progeny virions in ERAD silenced cells (Supplementary Figure 11a–d, respectively). Interestingly, the Simian virus 40 has been shown recently to require the ERAD components DERL1 and SEL1L for uncoating²². Together, these results indicate that WNV infection requires a subset of ERAD components at a post-internalization step.

Among the genes whose knockdown enhanced WNV infection, the strongest phenotype was observed when MCT4 (SLC16A4), a plasma membrane transporter of monocarboxylic acids²³, was silenced. Three of the four tested siRNAs targeting MCT4 resulted in 10 fold ($p=0.01$) increase in WNV infected cells at 24 h (Figure 3a and b; Supplementary Figure 4a and b). A quantitative PCR-based time course analysis of the viral genomic RNA (plus-strand) revealed similar rate of WNV particle internalization into both MCT4-repressed and control cells (Figure 3c). However, replication started at ≤ 9 h post infection in MCT4-silenced cells, whereas in control cells it was delayed until after 12 h (Figure 3c). Consistent with this, MCT4 silenced cells (a) had 3, 10, 12 and 18 folds ($p<0.05$) more viral plus-strand RNA at 9 h, 12 h, 15 h and 24 h post infection, respectively (Figure 3c); (b) immuno-stained for WNV antigens at 9 h (not detectable in control cells until after 12 h) (Figure 3d), and (c) secreted progeny virions by 12 h, whereas control cells did not (Figure 3e). However, importantly, replication of viral genomic RNA introduced directly to the cytoplasm by-passing the entry stages, was not affected by MCT4 silencing (Supplementary Figure 12). Collectively, these observations show that the functional activity of MCT4 delays the temporal transition into the replication phase of endocytosed WNV particles.

We next examined whether the host cell interaction strategies are similar between different members of the genus *Flavivirus*, by investigating the effect of silencing all the identified WNV HSFs and HRFs in HeLa cells infected by dengue virus - 2 (DENV). We determined that 30 h post-infection of DENV is comparable to the 24 h infection of WNV (Supplementary Figure 1a and b). Silencing of thirty-six percent of the WNV HSFs reduced DENV infection, including previously implicated vATPase and UBE2I (Supplementary Table 1)^{3, 24}. In contrast, all the 22 WNV HRFs increased DENV infection (Supplementary Table 2). Further supporting pathogen specificity, only five of the host factors impacting WNV infection altered HIV-2 infection (not shown).

An assessment of enrichment for biological process categories revealed significant over-representation ($p<0.05$) of seven key processes in which HSFs are targeted by both WNV and DENV (Figure 4a), relative to their representation among all HSFs identified. We selected three pathways—ERAD, focal adhesion complex (FAC) and histone deacetylase (HDAC) – to compare the conservation between WNV and DENV. There was a near complete overlap of ERAD component usage shared by both WNV and DENV, with the single exception of HRD1 (Supplementary Figure 13a). Silencing of four genes constituting FAC core (e.g., *PXN*, *SHC1*, *PITPNM2* and *PTK2B*), and thirty-three interactors, reduced WNV infection (Figure 4b and c; Supplementary Figure 4a and b)²⁵. Reduction of WNV infection in PITPNM2 silenced cells was also rescued with siRNA resistant PITPNM2 mutant (Figure 13b). Notably, only one core FAC component (PITPNM2) and eleven interactors reduced DENV infection (Figure 4b and c). Among the nine HDAC components associated with WNV infection, four were required for DENV (Supplementary Figure 13c). These examples indicate that WNV and DENV may have evolved different sensitivities in their interaction with host proteins, and this may be reflected in the differences in their biology.

In summary, this study portrays a comprehensive genome-scale map of human proteins and cellular pathways impacting the outcome of flavivirus-host cell interactions, and presents a

potentially useful resource for further studies. Furthermore, these results may provide insights into the molecular differences in the pathogenesis of related flaviviruses, and reveal potential flaviviral therapeutic targets.

Methods Summary

RNAi screen

Any gene for which a minimum of two siRNAs reduced (HSF) or increased (HRF) the percent of infected cells by ≥ 2 -fold, and the fold change was ≥ 2 times the standard deviation (SD) of the percent control cells infected, was scored as a hit. Gene silencing that resulted in cell number decrease ≥ 2 times the SD (of controls) was considered toxic and excluded.

Immunofluorescence assay sensitivity determination

As positive controls to determine whether the immunofluorescence assay (IFA) can detect changes in viral (a) replication, the previously reported WNV replication impacting host gene HMGCR was silenced (Supplementary Figure 1c and 1d); and (b) to test whether IFA can detect changes in viral translation, host translation machinery was arrested by cycloheximide (Supplementary Figure 1e). Anti-WNV siRNA was also used as a positive control (Supplementary Table 9). Results showed that the IFA was sensitive enough to detect changes in viral RNA translation, but not later stages such as replication.

Bioinformatic analysis

Genes were categorized using PANTHER classification system²⁶. Enrichment was analyzed using hypergeometric probability distribution. For tissue expression analysis, microarray data files were obtained from the Novartis GNF human expression atlas version² resource²⁷. The protein network constructions used interaction data from the Human Protein Reference Database (HPRD)²⁸, Biomolecular Interaction Network Database (BIND)²⁹, Ingenuity pathways database (Mountainview, CA) and supplemented with functional information from the literature.

Methods

WNV RNAi screen and candidate protein selection criteria

A library of 21,121 siRNA pools targeting human genome (Dharmacon siARRAY siRNA Library, Human Genome, G-005000-05, Thermo Fsher Scientific, CO) was used. For both the primary and validation screens, HeLa cells (384-well format) were transfected (using Dharmafect 1, Dharmacon) in duplicates with siRNA (50 nM) for 72 h, infected for 24 h with West Nile (WNV strain 2741) or 30 h with dengue (DENV New Guinea C strain) viruses, fixed in 4% paraformaldehyde, immunostained with antibodies detecting viral E-proteins (TRITC labeled, anti-WNV-E antibody developed in horse, or monoclonal anti-DENV-E, Chemicon), and imaged by fluorescence microscopy (Molecular Devices, 4x magnification) at TRITC filter for virus and DAPI filter for nuclei. Infection was done at a multiplicity of infection of 0.3 for both WNV and DENV. Generally, infection was in the range of 20–30% for both WNV and DENV. As positive control of infection reduction due to gene silencing, endosomal proton pump vATPase was silenced. Cell number/well was in the range of 7000–9000. The percent infection was relatively linear in the cell number range in which the screen was performed (Supplementary Figure 1f). Each 384-well plate had additional control wells with a non-targeting control siRNA (siCONTROL non-targeting siRNA, Dharmacon) (for determining the general effect of siRNA transfection on infection), siRNA targeting PLK-1 whose silencing kills the cells (for determining general knockdown efficiency), fluorescently labeled non-targeting control siRNA (for determining transfection

efficiency), and wells with neither transfection reagent nor siRNA. Quantification of the effect of gene silencing on viral infection was done using the software Metamorph (Molecular Devices), which counted cell that were immuno-stained vs non-stained for virus antigen. Based on the infection kinetics and infection inhibition by the silencing of a host gene known to be required for the infection of both WNV and DENV (vATPase, Supplementary Figure 1b), we defined an infection reduction of 2-fold or greater at 24 h for WNV or 30 h for DENV as the threshold for hit selection. Silencing of vATPase resulted in a reduction of infection of 2.9 ± 0.3 fold compared to the controls for WNV or 2.7 ± 0.4 for DENV (Supplementary Figure 1b).

Cell lines, Virus propagation

Gene silencing and infection studies were done on low passage HeLa (ATCC# CCL-2.1) cells maintained in DMEM supplemented with 10% fetal bovine serum. West Nile (strain 2471, Dr. John Anderson, Connecticut Agricultural Experiment Station), and dengue -2 (New Guinea C strain, Dr. Aravinda de Silva, North Carolina University) viruses were grown on vero (ATCC# CRL-1586) or C6/36 (ATCC# CRL-1660) cells, respectively.

Gene knockdown verification, RNAi resistant mutant generation and phenotype rescue

For the quantitation of the target transcript reduction, pooled siRNAs corresponding to the tested genes were transfected (50 nM) to cells (or cells pre-transfected with cDNAs of genes) in 48-well plates for 3 days, total RNA was isolated using RNeasy kit (Qiagen), and cDNA was prepared by iScript kit (Biorad). Quantitative PCR (QPCR) was performed by using Sybergreen reagent (Biorad). The primers used were given in the Supplementary Table 7. To generate RNAi resistant variants of genes, four silent mutations each was introduced into those sequences of DERL2 and PITPNM2 where siRNA binds (in the expression vector **pCDNA6.2** with V5 tag), using QuickChange Mutagenesis kit (Stratagene) (Supplementary Table 7 shows the mutagenesis primer sequences). HeLa cells transfected separately with wild type or mutant copies of the genes were selected for 8 days using blasticidin, treated with siRNA for 72 h, and either WNV infection assay or Western blot (for knock down and rescue verification) was performed. Six random fields of fluorescent images (10× objective, Zeiss Axiovert 200M) of WNV infected mutant vs wild type gene expressing cells were counted to quantify and assess the rescue of viral infection by mutant genes. For Western blot, cells were lysed in 1% 50mM Tris-HCL, 150mM NaCl and Triton X-100. Western blot was performed to verify the extent of knockdown and rescue of DERL2 and PITPNM2 using anti-V5 antibodies (Invitrogen). Anti-CBLL1 antibody was obtained from Abcam.

Cytotoxicity

Cytotoxic effects of gene silencing and MG132 treatments were determined using LDH release assay kit (Roche). Supernatants of gene silenced cells were harvested at 3 days post-transfection or 1–24 h post treatment for compounds, and assayed for LDH release according to manufacturer's protocol.

Viral RNA transfection and secretion studies

Two kinds of studies were done using viral genomic RNA transfection: (a) Determination of the effect of gene silencing on viral RNA translation, and (b) Determination of the effect of gene silencing on progeny virion secretion. The RNA of a subgenomic replicon of WNV (lacking complete genes for the capsid, pre- membrane, and envelope proteins) was used for viral translation studies³⁰, while a full length viral genome was used for viral secretion studies³¹. The viral RNA was prepared as described previously³¹. The viral RNA was transiently transfected into HeLa cells by electroporation, after gene knock down with

siRNA for 3 days. Mouse hyperimmune ascetic fluid against WNV was used for the immunofluorescence of replicon transfected cells, after 14 h of transfection. For the viral secretion assay, the culture supernatants were collected at 24 h from HeLa cells electroporated with (400ng) full length WNV genomic RNA for ERAD silenced cells. As positive control for inhibition of WNV secretion, Brefeldin A (10 μ g/ml) was used, by adding 12 h post infection. To study the viral release from MCT4 silenced cells, supernatants were collected at 12 h and 24 h (post-infection), and performed a plaque formation assay. For anti-WNV siRNA (siRNA sequence is given in Supplementary Table 9) treatment of replicon silenced cells, cells are first transfected with anti-WNV siRNA for 6 h, followed by electroporation of replicon, and fixed after 30 h for IFA.

Inhibitor studies

HeLa cells were treated with 15 μ M MG132 (Biomol) or 700 μ g/ml cycloheximide (Sigma) (dissolved in DMSO) or DMSO for various time periods as described in the text or figure. For determining the role ubiquitination in viral internalization, HeLa cells were pre-treated with MG132 for 1 h, TRITC-WNV was added (MOI of 100), incubated for 1–4 h at 37°C, fixed with 4% paraformaldehyde, and confocal microscopy was performed. For determining the post-entry requirements of ubiquitination, the virus was inoculated (MOI of 0.3), incubated at 37°C, and MG132 was added at different time points. The cells were fixed and immunostained after 18 h. The final DMSO concentration was no more than 0.2% of the total culture medium.

Vesicular stomatitis virus (VSV) and HIV studies

The human immunodeficiency virus (VSV-G carrying pHXBGFP-IRES-nef, an infectious molecular clone expressing GFP; Premalata Shankar, CBRI, Harvard University) infection experiments were done by infecting gene silenced cells at an MOI of 0.3 for 24 h, followed by fluorescent imaging (10x, Zeiss) to quantify the percent infection. For the VSV experiments, cells were pre-treated for 1 h with either DMSO or 15 μ M MG132, followed by infection with VSV expressing GFP (MOI of 0.5) for 12 h. GFP-positive VSV cells were quantified by flow cytometry.

WNV entry and colocalization studies

Modification of a previous protocol was used for these studies³². Purified virus was exchanged into phosphate buffered saline (PBS, pH 7.4) through repeated cycles of concentration by centrifugation (800 $\times$ g) and dilution with PBS, using 15 ml ultrafiltration tubes (10kD, Amicon). The virus in PBS (equivalent to 0.5mg/ml protein) was incubated with tetramethyl rhodaminy isothiocyanate (TRITC, Pierce Biotechnology) (0.3mg/ml, in dimethyl formamide) for 1 h at room temperature. After removal of excess dye, labeled virus (WNV-TRITC) was immediately used for experiments. Labeling did not abolish viral infectivity. Infectious entry of WNV-TRITC was sensitive to the vATPase inhibitor Bafilomycin, and colocalized with Rab5 labeled compartments, similar to the entry mechanism of unlabeled WNV (Supplementary Figure 14a and b). For colocalization imaging, Rab5-GFP or Rab7-GFP transduced HeLa cells were used (Gift from Tatjana Dragic, Albert Einstein College of medicine). For the entry assay (or Rab5/7-GFP colocalization experiments), cells in 48 well culture plates were transfected with siRNA for 48 h, and re-plated onto glass slide bottom chambers (MatTek) in DMEM (with 5% serum and 20 mM Hepes, pH 7.4). After further 24 h, WNV-TRITC (MOI of ~100, to capture sufficient events) was added to the cells and allowed to bind for 1 h at 4 °C, and cells were shifted to 37 °C for different time periods, fixed with 4% paraformaldehyde, and confocal imaging was performed, on a LSM 510 confocal microscope equipped with a Zeiss axiovert 100 M base, using 100 $\times$ oil objective (ZeissMicroImaging, Inc.). Z-stack imaging was done

at 0.5µm sections. Virus particles within the cells were counted using the software velocity and ImageJ.

Enrichment analysis of biological process and molecular function categories

Genes were classified into biological process and molecular function terms using the PANTHER system. To assess the statistical enrichment or over-representation of these categories for the set of hits relative to the global set of genes examined in the RNAi screen, *P-values* were computed using the hypergeometric probability distribution, which was implemented in the R-language (<http://www.r-project.org/>). The hypergeometric distribution describes the probability of finding *s* or more genes associated with a particular category, in a set of *g* genes essential for WNV infection (identified from the RNAi screen), given that there are *S* genes associated with that same category in the global set of *G* genes examined in the genome-wide RNAi screen. For each category, *c* and the list of genes *l*, and *P* value was calculated as:

$$P(c, l) = 1 - \sum_{k \in \{0, 1, \dots, s\}} (C(g, k) C(G - g, S - k) / C(G, S))$$

where *G* and *g* denote the total number of unique genes screened and those identified as essential for WNV infectivity, respectively. *S* and *s* represent the number of genes from *G* and *g*, respectively, that is associated with each annotation category. The binomial coefficient is of the form $C(n, r)$. A *P*-value <0.05 was considered significant. Categories assigned with at least 10 genes are displayed in the Figure 1b and c. A similar approach was used to examine over-representation in Figure 4a, except that the assessment of enrichment for biological process categories was made relative to their representation among all HSFs identified.

Analysis of Gene Expression across 79 tissues

Microarray data files were obtained from the Novartis GNF human expression atlas version2 resource, and expression values of 33,689 probe sets from the HG-U133A (Affymetrix) platform and the GNF1H custom chip were analyzed. The dataset was normalized using global median scaling, and we filtered the data by excluding from the analysis those probe sets with 100% ‘absent’ calls (MAS5.0 algorithm) across all 79 tissues. The dataset was further filtered by setting a minimum threshold value >20 in at least one sample for each probe set and a maximum-mean expression value >100. Hierarchical clustering (centroid linkage method) was performed with Cluster 3.0 using Pearson’s correlation as the similarity metric³³. Z-score transformation was applied to each probeset across all arrays prior to generating ‘heatmaps’ for visualization using TreeView³⁴.

Constructing human protein interaction network

The protein networks construction used protein interaction data obtained from the Human Protein Reference Database (HPRD), Biomolecular Interaction Network Database (BIND), Ingenuity pathways database (Mountainview) and functional information from the literature. The network uses graph theoretical representations in which components (gene products) are depicted as nodes and interactions between components as edges. Graph layout descriptions were written in the Dot language, which implements a multi-dimensional scaling heuristic and uses an iterative solver (Newton-Raphson algorithm) that searches for low-energy configurations to optimize the graph layout when creating a virtual physical model (Spring model) for visualization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. RNAi screen and bioinformatics

(a) West Nile virus RNAi screen strategy (see text for description). (b–c) Bioinformatics classification of hits into (b) biological process and (c) molecular function categories. * Denotes categories found enriched ($p < 0.05$) relative to all the genes examined in the RNAi screen. Only categories with ten or more members are displayed.

Figure 2. CBLL1 (a–e) and ERAD (f–g) silencing reduces West Nile virus (WNV) infection (a and b) CBLL1 silencing reduces WNV immunostained cells (MOI ~0.3, 10x, Zeiss). Red (virus), blue (nucleus). (c) a time course analysis showing reduced internalization of TRITC-WNV (MOI ~100) into CBLL1 silenced cells. (d) Localization of TRITC-WNV within CBLL1 silenced cells (percentage of total cells showing the phenotypes, 20 images, ~6 cells each). Plasma membrane alone (PM only), inside the cell (In only), and both PM and inside (In+PM). AP3S2 and vATPase were controls (see text). (e) MG132 reduces WNV internalization (MOI ~100). (f) ERAD network. Yellow squares denote hits; blue circles represent other host proteins within the network neighborhood. Dotted line indicates complexes. (g) ERAD silencing reduces WNV infection (MOI ~0.3). DERL2R is RNAi resistant mutant of DERL2. For (a) and (g), the % of infected control NT-siRNA cells (~30%) was set at 100% and used to normalize the % infection of siRNA-treated cells (from six fields of ~8000 cells). Values for (c) and (e) are number of virus particles/cell (mean of 20 cells). Results are mean $\pm$ SD from a representative experiment performed in triplicates.

Figure 3. MCT4 silencing enhances WNV replication

(a and b) MCT4 silencing increases the number of WNV immunostained cells (MOI ~0.3, 10x, Zeiss). Red (virus), blue (nucleus). (c) QPCR of WNV RNA levels in control NT-siRNA and MCT4 silenced cells (ng viral RNA/beta actin). (d) immunostaining for WNV E-protein in MCT4 silenced cells. (e) WNV secretion from MCT4 silenced cells, expressed as plaque forming units/ml (PFU/ml). Values in (a) and (d) are percentage of total cells immunostained for WNV (six images having ~1000 cells each). Results are mean $\pm$ SD from a representative experiment performed in triplicates.

Figure 4. (a–c) Interaction of West Nile virus (WNV) and dengue virus (DENV) with host cells
(a) Classification of HSFs common to both WNV and DENV or specific to WNV, into biological process categories. * Denotes categories found enriched ($p < 0.05$) relative to all identified HSFs. (b) Focal adhesion complex (FAC) network. Cyan line connects the core proteins. Yellow squares (WNV specific HSFs), red squares (WNV and DENV shared HSFs), blue circles (other host proteins within the network neighborhood). (c) Effect of silencing of PXN, SHC1 and PITPNM2 on WNV and DENV infection (the % of infected control NT-siRNA cells (~30%) was set at 100% and used to normalize the % infection of siRNA-treated cells (from six fields of ~8000 cells). PITPNM2R indicates RNAi resistant

mutant of PITPNM2. Results are mean $\pm$ SD from a representative experiment performed in triplicates.