Identification of Zika Virus and Dengue Virus Dependency Factors using Functional Genomics

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Keywords
Zika virus, dengue virus, yellow fever virus, flavivirus, arbovirus, EMC, AXL, CRISPR/Cas9 screen, MORR, RNAi screen

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Highlights

- RNAi and CRISPR/Cas9 screens were used to find flavivirus dependencies
- The screens recovered host factors involved in endocytosis and heparin sulfation
- The EMC is required by DENV and ZIKV in the early stages of replication
- These studies give a systems-wide view of human-flavivirus interactions

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In Brief

Savidis et al. identify DENV and ZIKV dependencies using orthologous RNAi and CRISPR/Cas9 approaches. Multiple host factors involved in endocytosis and transmembrane protein processing, including the endoplasmic reticulum membrane complex (EMC), are important for flaviviral replication. Together, their studies generate a systems-wide view of human-flavivirus interactions.
Identification of Zika Virus and Dengue Virus Dependency Factors using Functional Genomics

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SUMMARY

The flaviviruses dengue virus (DENV) and Zika virus (ZIKV) are severe health threats with rapidly expanding ranges. To identify the host cell dependencies of DENV and ZIKV, we completed orthogonal functional genomic screens using RNAi and CRISPR/Cas9 approaches. The screens recovered the ZIKV entry factor AXL as well as multiple host factors involved in endocytosis (RAB5C and RABGEF), heparin sulfation (NDST1 and EXT1), and transmembrane protein processing and maturation, including the endoplasmic reticulum membrane complex (EMC). We find that both flaviviruses require the EMC for their early stages of infection. Together, these studies generate a high-confidence, systems-wide view of human-flavivirus interactions and provide insights into the role of the EMC in flavivirus replication.

INTRODUCTION

The New Millennium has brought a rapid expansion of human flavivirus infections, including dengue virus (DENV), yellow fever virus (YFV), West Nile virus (WNV), and Zika virus (ZIKV) (Bhatt et al., 2013). Given that global warming is predicted to enlarge the range of the insect vectors that carry these viruses, it is critical that we understand the biology of these viruses in order to design effective therapies against them. DENV and ZIKV are single-stranded, positive-sense RNA viruses that are transmitted to humans by Aedes mosquitos. Both are fast-growing health threats that are producing an escalating number of infections in the Americas and worldwide.

Each year, 390 million people are infected with DENV, with 500,000 individuals hospitalized with severe dengue, the majority of those being young children (Bhatt et al., 2013). ZIKV, first isolated from an infected macaque in Uganda in 1947, suddenly emerged in Micronesia in 2007 and expanded its range to Southeast Asia. In May 2015, ZIKV was identified in Brazil coincident with an upsurge in neurologic and fetal abnormalities. With its rapid spread to Central and South America, ZIKV has emerged as a severe health threat by virtue of its fast-paced global spread and associated morbidities, including microcephaly and Guillain-Barre syndrome. (D’Ortenzio et al., 2016; Driggers et al., 2016; Haug et al., 2016; Lazear and Diamond, 2016; Musso and Gubler, 2016; Rasmussen et al., 2016). These events have led to ZIKV being declared a public health emergency by the World Health Organization. Recent animal models have demonstrated that ZIKV infects the placentas of pregnant mice, with transmission to fetal mice resulting in death or severe growth impairment (Cugola et al., 2016; Miner and Diamond, 2016; Miner et al., 2016; Li et al., 2016). There are no specific therapies for flavivirus infection, although a DENV vaccine has recently been approved in some countries. There is no approved vaccine or therapy for ZIKV infection.

Flavivirus replication begins with the virus binding to host cell receptors and undergoing endocytosis (Fernandez-Garcia et al., 2009). A number of proteins have been implicated to facilitate DENV attachment and entry, including TIM1 and AXL (Jemiility et al., 2013; Meertens et al., 2012; Morizono and Chen, 2014; Perera-Lecoin et al., 2014; Richard et al., 2015), the latter having also been identified as an important ZIKV entry factor (Hamel et al., 2015). Subsequent to initial viral entry, late endosomal acidification triggers the fusion of host and viral membranes and permits the virus’ positive sense RNA genome (viral RNA [vRNA]) to enter the host cell cytosol. Upon cytosolic entry, the vRNA is translated into a large polyprotein on the rough endoplasmic reticulum (RER). This polyprotein is processed by both host and viral proteases into three structural proteins (premembrane [prM], capsid [C], and the glycoprotein envelope [E protein]), and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). DENV has been demonstrated to extensively remodel the ER into replication centers (RCs), where progeny viruses are created. The newly synthesized flaviviruses then traffic from the RER to the cell surface via the Golgi.
Figure 1. MORR Screens for Identifying Human Proteins that Modulate DENV Replication

(A) Schematic workflow diagram of the DENV-HF screen.

(B) MAGI HeLa cells were transfected with the indicated negative (non-targeting [NT]) and positive (ATP6V0B, IFITM3) controls for 72 hr and then infected with DENV2-NGC (MOI 0.5) for 30 hr. Cells were then fixed, permeabilized, and immunostained with anti-E protein antibody (4G2, green) or stained for DNA (blue). Percent infected cells ± SD are shown at 4 x magnification.

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undergo exocytosis, thus spreading the infection to neighboring cells.

The flaviviruses have a complex lifecycle that relies on the host cell’s resources. Earlier efforts have addressed the role of arthropod DENV-host factors (DENV-HFs) (Sessions et al., 2009) and human factors required by YFV (Le Sommer et al., 2012) and WNV (Krishnan et al., 2008; Ma et al., 2015). Nonetheless, fundamental questions regarding how human proteins modulate flavivirus replication, including ZIKV infection, remain. Therefore, we investigated human-flavivirus interactions with a combined functional genomic approach employing multiple orthologous RNAi reagent (MORR) screens for DENV replication together with a pooled CRISPR/Cas9 cell survival enrichment screen to find host factors that are required for ZIKV replication. To limit false positives, we used gene-expression filtering to remove host cell candidates that were not expressed in the cells used for the screens. To address some of the shortcomings of functional genomics (false positives and false negatives), we integrated the MORR screen datasets using an existing RNAi analysis program, RIGER (Luo et al., 2008), which quantitatively integrates the MORR screen datasets using an existing RNAi analysis program. RIGER supports the enrichment of multiple pathways and molecular complexes in the high-confidence set (e.g., the vacuolar ATPase [vATPase], the OST complex, and endocytic trafficking; Figures S1A–S1D; Tables S1 and S2), arguing that the majority of these candidates are true positives. Overlap of specific genes between our high-confidence list with either a Drosophila-based DENV-HF screen (Sessions et al., 2009) or a human-cell-based whole-genome screen using WNV (Krishnan et al., 2008) was low (discussed below). Consistent with known DENV dependencies, all of the screens independently identified various vATPase components, and two or more screens also detected a role in replication for SEC61B, an endoplasmic reticulum translocon component, and SCFD1, which is necessary for transmembrane protein processing. The low specific gene overlap between the screens may reflect different screening strategies and reagents and is consistent with previous screen comparisons revealing that, although pathways and complexes are more likely to be detected across screens, the overlap of specific genes is less pronounced. Although we did find significant overlap in pathways and gene clusters when comparing our own Silencer Select and SMARTpool library screens, the exact gene overlap was low (13%–14%; Table S2). This is consistent with the differences between the various siRNA libraries influencing the genes recovered. Therefore, our use of multiple orthologous RNAi reagents is useful in attempting to saturate a given screen.

RIGER Analysis of the MORR DENV-HF Screens

In an attempt to address the caveats of RNAi, false negatives, and false positives, we employed the MORR screening approach and used an existing informatics program, RIGER (Luo et al., 2008), to integrate the primary screen data into rankings of dependency factors (DFs; RIGER3-DF) or competitive factors (CFs; RIGER3-CF; Table S3). We compared the RIGER and primary screen rankings for their ability to detect genes in the EMC, OST complex or cumulative comprehensive coverage. A comparison of >1,000 siRNA pools from the Silencer Select and SMARTpool libraries demonstrated <5% similar siRNAs showing that the reagents are largely orthologous. The SMART-Rev library is based on a more recent RefSeq database annotation and thus in part supplements the earlier designed SMARTpool library with which it shares <10% siRNA sequence overlap. siRNA pools were chosen as hits if the percentage of infected cells was >50% or >150% of the plate mean and cell number was ≥50% of the plate mean. Selected DENV-HF candidate pools were validated by independently testing the individual oligos from each pool (Table S1). In the validation round, pools with two or more siRNAs that satisfied these criteria were denoted as higher confidence because such results are most consistent with the depletion of the intended target (Echeverri et al., 2006). Collectively, these screens identified over 150 high-confidence candidate DENV-HFs that were validated with two or more individual siRNAs (Tables S1 and S2). The validity of this approach was supported by the enrichment of multiple pathways and molecular complexes in the high-confidence set (e.g., the vacuolar ATPase [vATPase], the OST complex, and endocytic trafficking; Figures S1A–S1D; Tables S1 and S2), arguing that the majority of these candidates are true positives. Overlap of specific genes between our high-confidence list with either a Drosophila-based DENV-HF screen (Sessions et al., 2009) or a human-cell-based whole-genome screen using WNV (Krishnan et al., 2008) was low (discussed below). Consistent with known DENV dependencies, all of the screens independently identified various vATPase components, and two or more screens also detected a role in replication for SEC61B, an endoplasmic reticulum translocon component, and SCFD1, which is necessary for transmembrane protein processing. The low specific gene overlap between the screens may reflect different screening strategies and reagents and is consistent with previous screen comparisons revealing that, although pathways and complexes are more likely to be detected across screens, the overlap of specific genes is less pronounced. Although we did find significant overlap in pathways and gene clusters when comparing our own Silencer Select and SMARTpool library screens, the exact gene overlap was low (13%–14%; Table S2). This is consistent with the differences between the various siRNA libraries influencing the genes recovered. Therefore, our use of multiple orthologous RNAi reagents is useful in attempting to saturate a given screen.

(c) The results of the DENV-HF screens with the siRNA pools ranked in order of their normalized percent infection (log2 scale). The highlighted genes are selected hits from each respective library.

(d) Based on the RIGER3 screen dataset, a hypothetical model cell was created highlighting the DENV lifecycle as well as where 259 of the DENV-HFs might function on the basis of the available literature (Table S3).
the EC (Figure S2A). For this, we calculated the area under the curve (AUC) generated by plotting the percentage of complex-related genes from the total set that is detected moving from the top down along the respective gene rankings (Figure S2A; Table S3). The AUC analysis shows graphically how well an siRNA library or RIGER analysis performed in identifying the expected components. The AUC analyses show that the Silencer Select screen was superior, outperforming both the RIGER analysis and the SMARTpool screen for both the EMC and OST complex. For the EC, the RIGER analyses were more efficient than the independent screens, revealing its usefulness. Using the flavivirus replication cycle as a starting point and with the referenced literature as a guide, 259 (137 DFs and 122 CFs) of the top 150 of both the DF and CF RIGER3 gene lists expressed in the HeLa cell were assembled into a speculative model of how they might modulate viral infection (Figure 1D; Table S3).

**DENV-HF Pathways and Complexes**

The screens enriched for several pathways and complexes needed by the virus (e.g., OST, conserved oligomeric complex of Golgi, and nonsense-mediated decay pathway), whose components scored across the primary screen datasets (Figures S1 and S2; Tables S2 and S3). For the OST complex, DENV required seven of eight components (Figure S1D; Table S2). A similarly strong enrichment was seen with the vATPase, with 13 subunits needed by the virus. In addition to DENV-HFs, the screens also identified the EC as well as others, whose loss enhanced viral replication seven of eight components (Figure S1D; Table S2). A similarly strong enrichment was scored across the primary screen datasets (Figures S1 and S2; Tables S2 and S3). For the OST complex, DENV required seven of eight components (Figure S1D; Table S2). A similarly strong enrichment was seen with the vATPase, with 13 subunits needed by the virus. In addition to DENV-HFs, the screens also identified the EC as well as others, whose loss enhanced viral replication seven of eight components (Figure S1D; Table S2). For this, we calculated the area under the curve (AUC) generated by plotting the percentage of complex-related genes from the total set that is detected moving from the top down along the respective gene rankings (Figure S2A; Table S3). The AUC analysis shows graphically how well an siRNA library or RIGER analysis performed in identifying the expected components. The AUC analyses show that the Silencer Select screen was superior, outperforming both the RIGER analysis and the SMARTpool screen for both the EMC and OST complex. For the EC, the RIGER analyses were more efficient than the independent screens, revealing its usefulness. Using the flavivirus replication cycle as a starting point and with the referenced literature as a guide, 259 (137 DFs and 122 CFs) of the top 150 of both the DF and CF RIGER3 gene lists expressed in the HeLa cell were assembled into a speculative model of how they might modulate viral infection (Figure 1D; Table S3).

**DENV-HF MORR Screens Have False Positives and False Negatives**

Comparison of candidate lists from similar siRNA screens reveals shared pathways and complexes, but in the majority of instances, there is low exact gene overlap (Perreira et al., 2015, 2016; Zhu et al., 2014). The MORR DENV-HF screens behaved similarly, with a low percentage of same-gene overlap detected in the primary screens (for DFs, with ≤ 50% plate mean infected cells and ≥ 50% plate mean number of cells: Silencer Select 13.5%, SMARTpool 14.4%, and SMART-Rev 10.6%; Table S2). A comparison across the screens for siRNAs targeting components of the OST complex, the EC, or the EMC showed estimated false negative rates ranging from 25% to 50% (Table S2). We generated a common candidate list for genes that scored in two or more of the screens (Table S2). To minimize the effects of false positives and off-target effects, we performed microarray analysis (Affymetrix GeneChip human 2.0 ST array) to determine the genes expressed in the HeLa cells (Tables S1, S2, and S3). The microarray probe-set values were matched to the genes present in the siRNA libraries: 17,070 (80.8%) and 17,168 (79.5%) genes with expression data in the SMARTpool and Silencer Select screens. The median of the negative control intron probe set served as a cutoff for gene expression, producing a list of 12,461 common genes in the siRNA libraries that are expressed in the HeLa cells.

**A CRISPR/Cas9 Screen to Identify ZIKV Host Factors**

The advent of bacterially derived CRISPR/Cas9 gene modulation has revolutionized the field of in vitro mammalian pooled genetic screening (Hsu et al., 2014; Sanjana et al., 2014; Shalem et al., 2014, 2015). In optimization experiments, we found that the ZIKV MR766 strain kills >95% of H1-HeLa cells in ~8 days at an MOI of 5, with other ZIKV strains not being as cytopathic, making this combination of virus and the host cell ideal for a pooled CRISPR/Cas9 screen (data not shown). Therefore, to find additional flavivirus host factors, we performed a CRISPR/Cas9 screen using the H1-HeLa cell line and ZIKV MR766 (Figure 3A). We first stably expressed a human-codon-optimized cDNA of S, pyogenes Cas9 in a population of HeLa cells (Shalem et al., 2014). Next, we stably transduced the H1-HeLa-Cas9 cells at an MOI of 0.2 with a complex lentiviral pool expressing the human GeCKO v.2 single-guide RNA (sgRNA) library (Shalem et al., 2014), which targets 19,052 genes in the human genome with six sgRNAs per gene across two half-libraries (libraries A and B). Libraries A and B each possess three unique sgRNA per gene and these half-libraries were used in parallel to screen for ZIKV host factors in independent triplicate screens. The Cas9-expressing H1-HeLa cells were infected with ZIKV MR766 at an MOI of 5 and cultured for 8 days until >95% of the cells were dead, as determined by their detachment from the plate surface as seen with an inverted microscope. The surviving cells, appearing as tightly clustered clonal colonies, were expanded, and genomic DNA was prepared. Proviruses containing the sgRNA stably integrated into each of the surviving cells were amplified and identified from genomic DNA using PCR and next-gen sequencing. Similar to RNAi screens, we used the reagent redundancy principle (Echeverri et al., 2006) to select for candidate genes which had >6 sequencing reads for three or more independent sgRNAs (Table S4). We then repeated the screen and compared the data to identify candidates that are high confidence in both screens (Table S4). There was a high degree of sgRNA and exact-gene overlap among the top candidates found by the independent screens (Figure S2B; Table S4), suggesting that the screen was approaching saturation under these conditions.

**EMC Is Required for the Replication of ZIKV, DENV, and YFV**

Among the top ZIKV-DF candidates (>4 independent sgRNAs, with >6 sequencing reads per sgRNA; Table S4) from the CRISPR/Cas9 screens was the recently published ZIKV entry factor, AXL (six of six sgRNAs) (Hamel et al., 2015; Jemielity...
Figure 2. The EMC Is Needed for DENV Replication

(A) MAGI HeLa cells were transfected with the indicated Silencer Select siRNAs (Table S1) for 72 hr and then infected with DENV2-NGC (MOI 0.5). At 48 hr post infection, the cells were fixed, permeabilized, and immunostained for the viral E protein. Imaging analysis software was used to determine the percentage infection. Values indicate the mean infected cells normalized to the NT negative control siRNA-transfected samples of n = 3 independent experiments ± SD.

(B) Schematic of the EMC with components 1–10 depicted as circles whose relative sizes are proportional to their respective molecular weights (Table S1). Components 8 and 9 are shown as one subunit because of their similarity and variable expression. Subunits are indicated in color if they scored in one or more of the DENV-HF MORR screens.

(C) MAGI HeLa cells were transfected with the indicated pooled Silencer Select siRNAs (three siRNAs per gene; Table S1) for 72 hr and then infected with DENV2-NGC (MOI 0.5). At 48 hr post infection, the cells were fixed, permeabilized, and immunostained for the viral E protein. The nuclei of the cells were also stained for DNA using Hoechst 33342 dye (blue). Imaging analysis software was used to determine the percentage infection (numbers shown ± SD). Values indicate the mean percent infected cells of n = 3 independent experiments ± SD at 4× magnification.

Results throughout are the mean of three independent experiments ± SD.
Figure 3. CRISPR/Cas9 Screens to Identify Human Proteins that Modulate ZIKV Replication

(A) Schematic workflow diagram of the ZIKV-HF CRISPR/Cas9 screen.

(B) Ranking of genes from the ZIKV-HF CRISPR/Cas9 screen. Relative sgRNA frequencies were detected using next-gen sequencing (pooled data from two independent screens) were plotted against relative frequencies of sgRNAs in the unselected starting population of H1-HeLa cells containing the human GeCKO v2.0 library. For top-ranking genes, sgRNA frequencies are highlighted.

(C) Ranking of genes from the ZIKV-HF CRISPR/Cas9 screen. Next-gen sequencing reads from two independent screens were mapped to the human GeCKO v2.0 library using bowtie2. Only sgRNAs with six or more sequencing reads were used for analysis. Identified genes were ranked according to the number of total reads from the two screens combined and according to the number of retrieved independent sgRNAs per gene (the human GeCKO v2.0 library contains six independent sgRNAs per gene). Gene symbols are shown for a selection of the highest-ranking genes.

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et al., 2013; Meertens et al., 2012; Perera-Lecoin et al., 2014), providing both an unbiased confirmation of AXL’s role in ZIKV replication as well as an attestation to the validity of the screening approach (Figures 3B and 3C; Table S4). The screen also recovered candidate ZIKV-HFs involved in endocytosis (RABSC, RABGQEF1, WDR7, and ZFYVE20), transmembrane protein production (the translocon-associated components SSR2 and SSR3), and post-translational modification (STT3A, EXT1, and EXT13) as well as two microRNAs, HSA-MIR-451A and HSA-MIR-4518 (Figures 3B and 3C; Table S4).

Consistent with the MORR DENV-HF screens, the CRISPR/Cas9 ZIKV-HF screen effectively saturated the EMC, finding all nine components with three or more sgRNAs, including ≥5 sgRNAs out of six total for EMC1–EMC6 (Figure 3D; Table S4). Importantly, the EMC was needed for the replication of a more recently isolated ZIKV strain provided by a pediatric patient in Cambodia in 2010 based on the complex’s depletion leading to a marked decrease in the levels of either viral double stranded RNA (dsRNA) or E protein in H1–HeLa cells (Figures 3E and S3A), with similar results seen using A549 adenocarcinoma epithelial cells (data not shown).

The role of 12 of the top candidates found in the ZIKV-HF screen was then assessed in a replication assay (E protein expression) using three independent siRNAs targeting each gene (Figure 4A). In matched studies, we determined the level of mRNA target depletion produced by each of the siRNAs (Figure 4B). These paired assays showed a strong correlation between the level of mRNA knockdown and the observed phenotype. All of the ZIKV-HF candidates, including EMC1–EMC6, decreased E protein levels to ≤50% of control with two or more siRNAs, and this effect was seen with multiple strains of ZIKV (MR766 Africa 1947, ZIKV FSS3025 Cambodia 2010, and ZIKV Puerto Rico 2015) as well as DENV2-NGC. Low-passage clinical isolates of DENV1 and DENV4 from Thailand, as well as the YFV vaccine strain YF17D, also required the EMC for replication (Figure 4C).

A Role for the EMC in Early DENV and ZIKV Replication

These data revealed that the EMC was needed for DENV and ZIKV replication. In contrast, WNV has been shown to not require the EMC for its replication, with loss of the complex resulting in an increase in viral replication intermediates in some instances (Ma et al., 2015). Although the role of the EMC in WNV replication remains to be tested in our system, these data suggest that the DENV- and ZIKV-HF screens reported here have identified a previously unappreciated role for the EMC in flavivirus replication.

To determine where in the DENV and ZIKV lifecycles the EMC is needed, we tested how depletion of either EMC1, EMC2, EMC4, or EMC5 impacted the early stages of DENV infection using synchronized time course infectivity experiments. To monitor viral infection in these assays, we employed immunostaining for E protein combined with a fluorescence-in-situ-hybridization-based imaging assay (viewRNA) for vRNA (Feeley et al., 2011). DENV2-NGC (MOI 50) was first incubated on ice with siRNA-transfected H1–HeLa cells to allow viral binding but not endocytosis. Warm media was then added, and, at the given times, the cells were washed with cold PBS, incubated with or without trypsin (T), fixed, processed, and confocally imaged using either an antibody that binds E protein (4G2) or a probe set that recognizes DENV or ZIKV RNA (ViewRNA). The trypsin step is designed to remove the virus remaining at the cell surface after warming has triggered the viruses’ endocytosis. Within the time course of this imaging assay (0 to 90 min post infection), the viral signals arise predominantly from the incoming viruses that are either surface bound, trafficking within the endosomal pathway, or have recently fused and transferred their contents, including the vRNA, into the cytosol. However, we cannot rule out the possibility that in addition to the vRNA that has recently entered the cytosol from incoming viruses, this assay is also detecting the products of de novo vRNA transcription that has occurred at 60 and 90 min post infection. Given our experiences using the viewRNA assay to detect the RNA of other viruses, e.g., influenza (Chin et al., 2015; Feeley et al., 2011), it is unlikely that this assay would detect vRNA within intact virions due to their limited accessibility to the probes. In support of this, our attempts to detect vRNA fixed to the cell surface immediately prior to warming and infection (time zero) in samples that were not treated with trypsin (−T) showed little to no vRNA signal (data not shown).

At 0 min with no trypsin treatment, the levels of surface-bound virus were not appreciably altered by loss of the EMCs (Figures 5A–5C and S3B). In contrast, at 40 min post infection, loss of the EMC decreased the levels of both intracellular E protein and vRNA as compared to the control cells. A small amount of virus was seen arrested at or very near the surface of the EMC-depleted cells at 40 min post infection subsequent to trypsin treatment; therefore, we attempted to rescue DENV2-NGC entry using acidified buffer. However, this did not produce an observable effect (data not shown). Similar results were obtained using ZIKV MR766, with EMC depletion decreasing intracellular vRNA levels but not the levels of bound virus at 0 min (Figures S4A–S4D). The depletion of AXL also resulted in less intracellular E and vRNA apparent at both 60 and 90 min post infection but little to no decrease in the levels of surface bound virus at time zero (Figures S5A–5E). The lack of an effect on viral binding with AXL depletion seen with this confocal imaging-based assay was surprising given its known role as a viral entry factor; however, it is partly consistent with a published study that detected only a modest loss, ~20%, of DENV binding to host cells in the

(D) Schematic of the EMC with components 1–10 depicted as circles whose size is representative of their relative molecular weights. Components 8 and 9 are shown as one subunit because of their similarity and variable expression. Subunits are indicated in color if they scored in two or more of ZIKV-HF CRISPR/Cas9 screens repeats with the indicated number of independent sgRNAs (right).

(E) H1–HeLa cells were transfected with the indicated pooled Silencer Select siRNAs (three siRNAs per gene; Table S1) for 72 hr, then infected with ZIKV Cambodia (moi 0.5). At 48 hr post infection the cells were fixed, permeabilized, and immunostained for viral double-stranded RNA (dsRNA; recombinant J [J] antibody, green). The nuclei of the cells were also stained for DNA using Hoechst 33342 dye (blue). Imaging analysis software was used to determine the percentage infection (numbers shown ± SD). Values indicate the mean percent infected cells of n = 3 independent experiments ± SD shown at 4× magnification. Results throughout are the mean of three independent experiments ± SD.
Figure 4. ZIKV-HF Are Required for the Replication of Multiple Viral Strains

(A) H1-HeLa cells were transfected with the indicated Silencer Select siRNAs (three independent siRNAs [#1, #2, and #3] per gene; Table S1) for 72 hr and then infected with the indicated flaviviruses (MOI 0.3–1). At 48 hr post infection, the cells were fixed, permeabilized, and immunostained for the viral E protein. Imaging analysis software was used to determine the percentage infection. Values indicate the mean percent infected cells of n = 3 independent experiments ± SD.

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presence of an anti-AXL antibody using a flow-cytometry-based assay (Meertens et al., 2012).

These studies showed that DENV and ZIKV infection was halted early in the setting of lowered EMC components or with the loss of AXL but did not impact viral binding under these settings. Therefore, we modified the above assay by omitting the trypsin step at 80 min post infection in order to assess what happens to the bound virus we observed at time zero in the EMC- and AXL-depleted cell samples. We also omitted a permeabilization step in the no-trypsin (-T) 80 min post infection samples so as to preferentially detect cell-surface-bound viruses. (B) mRNA was made from cells in (A), and qPCR was performed in order to determine the relative abundance of the indicated mRNAs. Values indicate the mean expression normalized to the NT control.

Results throughout are the mean of three independent experiments ± SD.

Figure 5. Loss of the EMC Halts DENV Early in Replication

(A) H1-HeLa cells were transfected with the indicated pooled Silencer Select siRNAs (three siRNAs per gene, vATP = vATPase subunit ATP6V0B) for 72 hr and then incubated with DENV2-NGC on ice (MOI 100). The viral supernatant was removed, and warm media was added at time zero. At the indicated time points after warming, cells were washed and treated with (+) or without (−) a 1 min incubation with trypsin (T) to remove or leave surface-bound virus. The cells were then fixed, permeabilized, and immunostained with anti-E antibody (4G2, green) and confocally imaged (63×). At 40 min post warming, vRNA was also detected in matched samples using a viewRNA probe set (red). Cellular DNA was stained with DAPI (blue). The scale bar represents 20 μM. Corresponding differential interference contrast (DIC) images are provided in Figure S3B.

(B and C) Quantitation of DENV E and DENV RNA signal intensity normalized to the NT control is provided (normalized E or vRNA mean intensity ± SD) for R12 cells from each of n = 3 experiments.

These studies showed that, at 80 min post infection, the majority of the ZIKV MR766 remained bound to the cell surface of the EMC and AXL siRNA-transfected cells, whereas, in the negative control, the vast majority of the surface-bound virus had undergone endocytosis and entered the cell’s interior (Figures 6A–6C).

Given these data, we determined whether the EMC was involved with the cell-surface levels of either AXL or heparin sulfate, both of which are important for the initial stages of DENV and ZIKV replication (Jemielity et al., 2013; Meertens et al., 2012; Richard et al., 2015). Depletion of EMC1, EMC2, EMC4, or EMC5 did not decrease surface expression of either AXL or heparin sulfate (Figures S6A and S6B). We also evaluated the levels of a broad range of surface protein glycosylation moieties using flow cytometry and a panel of conjugated lectins and found no differences between the negative control and the EMC-depleted cells (data not shown). Together, these data suggest that the loss of the EMC blocks DENV and ZIKV replication after the initial virus binding to the host cell but prior to the virus’ endocytosis; however, given the limitations of this imaging assays noted above, we cannot rule out the possibility that a portion of the block to infection involves the inhibition of the early amplification of vRNA in the cytosol. This last possibility will require further evaluation in future studies.

(B) mRNA was made from cells in (A), and qPCR was performed in order to determine the relative abundance of the indicated mRNAs. Values indicate the mean expression normalized to the NT control.

(C) Experiments performed as in (A) using the YF17D and low-passage DENV1 and DENV4 viruses. Results throughout are the mean of three independent experiments ± SD.
using additional assays such as flavivirus replicon-expressing cells.

**The EMC Associates with the OST Complex**

The EMC is conserved from yeast to humans, participates in ER protein folding response (with its loss eliciting the unfolded protein response (UPR) [Jonikas et al., 2009]), and is needed for ER-to-mitochondrial lipid trafficking (Lahiri et al., 2014) and the expression of several multi-pass transmembrane proteins, including rhodopsin, in the *Drosophila* eye (Satoh et al., 2015) and the acetylcholine receptor in *C. elegans* (Richard et al., 2013). EMC1–EMC6 were shown to form a complex after their identification in a screen in yeast for ER protein folding factors (Jonikas et al., 2009). EMC7–EMC10 were added to these initial functionally defined EMC components based on proteomic studies (Christianson et al., 2012). Our data now demonstrate that, in addition to WNV-induced cell death (Ma et al., 2015), the loss of the EMC arrests DENV and ZIKV early in their replication cycles. To further elucidate the role of the EMC in viral infection and host-cell biology, we identified proteins that associate with EMC1, EMC2, EMC4, or EMC5, using affinity purification coupled to mass spectrometry (AP-MS). EMC1, EMC2, EMC5–HA, and EMC4–FLAG fusion protein expression in H1-HeLa cells were induced for 72 hr with doxycycline, and affinity purification (AP) was performed (Figures 7A–7D). Co-purified proteins were then identified with shotgun-proteomics. Proteins associating with EMC components (hits) have increased total spectral counts from affinity purifying EMC components compared to vector control cells (Figure 7E; Table S5). These experiments identified multiple components of the translocon and OST complexes, including both STT3A and STT3B subunits, RPN1/RPN2, and DDOST, as associating with the EMC (Figures 7E–7G; Table S5). Glycolysis and glucose-metabolism pathway components are the most enriched (23 of 72 proteins). The next most represented pathway is the citric acid cycle and electron transport, where 26 of 155 proteins are present in the EMC affinity purifications. Protein metabolism (57 of 687), protein processing in the ER (23 of 169), and protein folding (CCT/TriC) pathways (8 of 21) are also highly represented.

The higher-order assembly of the EMC proteins with one another, and how such differing assemblages might function, is poorly understood. Consistent with the notion that different EMC components have distinct functions, proteins that co-purify with EMC4 showed the least amount of overlap with those associating with EMC1, EMC2, and EMC5. EMC4’s affinity purification alone co-purified EMC2, whereas EMC1, EMC2, and EMC5 all associated with multiple other EMC components, including...
EMC7, EMC8, and EMC10. These data suggest that EMC1, EMC2, and EMC5 are core components of the EMC complex, whereas EMC4’s association may be more peripheral or restricted to a particular variant. Consistent with this idea, EMC4 alone co-purified with multiple COP1 (COPB1, COPB2, and COPG1) and COPII (SEC13, SEC23A, and SEC22) components, which are coat-protein complexes responsible for anterograde and retrograde transport to and from the ER to the Golgi (Brandizzi and Barlowe, 2013), suggesting that this subunit is involved in trafficking between these closely interacting organelles.

**DISCUSSION**

The rapid spread of flavivirus infections represents a severe and escalating threat to global health (Lazar and Diamond, 2016). To obtain a systems-level understanding of DENV and ZIKV host factor dependencies and identify potential therapeutic targets, we completed orthogonal genomic(bs) screening using both MORR/RIGER and CRISPR/Cas9 strategies coupled with validation experiments and proteomics. We chose this combinatorial approach in order to take advantage of the best that each of the theses technologies has to offer, thereby offsetting the caveats of genetic screening, false positives, and false negatives. This combined functional genomic approach generated a comprehensive overview of flavivirus dependencies in human cells. The strong overlap of enriched complexes and pathways between the MORR RNAi screens and the CRISPR/Cas9 screens, as well as the CRISPR/Cas9 screens themselves, suggest that the flavivirus host factor screens are approaching saturation (Tables S6 and S7). These datasets will now hopefully provide a useful resource in helping the research community find and exploit flavivirus weaknesses.

Both screening approaches (MORR and CRISPR/Cas9) enriched for common complexes and pathways, including the EMC; however, it was only in the CRISPR/Cas9 screen that the reported ZIKV and DENV entry factor AXL was detected as important for viral replication (Hamel et al., 2015). This is consistent with pooled screens using cell survival as a readout displaying limited sensitivity (few candidates recovered) but excellent specificity in finding host genes that act very early in viral replication, for instance host factors needed for viral entry (Perreira et al., 2016). In contrast, RNAi screens generate a larger list of host factor candidates contributing to a broader understanding of viral requirements; however, this comes at the high cost of increased false leads. Therefore given these complementary strengths and weaknesses, a combined RNAi and CRISPR/ Cas9 screening effort is likely to yield the most comprehensive insight into human-viral interactions.

**Overlap with Flaviviral Screens**

siRNA screens for both WNV and YFV host factors in human cells, as well as a Drosophila cell-based siRNA screen for DENV host factors, have been previously published (Krishnan et al., 2008; Kwon et al., 2014; Le Sommer et al., 2012; Ma et al., 2015; Sessions et al., 2009). We compared our MORR screen datasets with the published candidate lists from these efforts (Table S6). We found that the overlap between these efforts was low, most likely due to false positives and false negatives that vary significantly between siRNA libraries and platforms (Table S6). The greatest overlap was with the fly-based screen (Sessions et al., 2009), which, although surprising, may speak to lower levels of artifacts being produced using that approach. Notably, neither AXL nor the other published DENV receptor TIM1 (Jemielity et al., 2013; Meertens et al., 2012) were recovered as a viral dependency factor in any of these RNAi-based efforts.

A comparison between the published flavivirus screens and the ZIKV CRISPR/Cas9 screen for overlap showed that the WNV CRISPR/Cas9 screen detected EMC2, 3 and 7 and the translocon-associated protein, SSR2, as being needed for WNV cytopathicity (Ma et al., 2015). The YFV siRNA screen found EMC3, WDR7 (endocytosis), EXT1 (heparin sulfation) to be needed for viral replication (Le Sommer et al., 2012); with the requirement for EMC3 in YFV replication being consistent with the results presented here. WDR7 was also needed for WNV replication in the siRNA-based screen, along with SH3GLB2 and the vATPase subunit, ATP6V0A1, which are both involved in endocytosis (Krishnan et al., 2008).

As discussed, the WNV CRISPR/Cas9 screen found that three EMC subunits (EMC2, EMC3, and EMC7) were necessary for virus-induced cell death but not for viral replication (Ma et al., 2015), which is in contrast to what we had found with DENV, ZIKV, and YFV. The lack of detection of any EMC subunit in the WNV siRNA replication screen (Krishnan et al., 2008), together with the EMC-modulating viral cytopathicity, but not viral replication, as shown with the WNV CRISPR/Cas9 screen, are consistent with the notion that the EMC plays an important but differing role in the WNV lifecycle as compared to those of DENV, ZIKV, and YFV.

**Overlap with Related Screens**

A role for the EMC in yeast was discovered using a genetic screen to find genes needed for phospholipid (PL) transfer from the ER to the mitochondria (Lahiri et al., 2014). A comparison of the yeast PL and ZIKV CRISPR/Cas9 screens’ respective candidate lists showed that only the four EMC components were shared in common, arguing against a shared pathway in PL transfer underlying the observed phenotype (Table S6). The flaviviruses enter host cells via clathrin-medi- ated endocytosis (CME), and the loss of the EMC blocks flavivirus infection early, after viral binding but likely before viral entry. Therefore, we checked for any overlap between the ZIKV CRISPR/Cas9 high-confidence dataset and one from a whole-genome siRNA screen for CME using the SMARTpool library and human cells (Kozik et al., 2013). This comparison revealed one common gene, LRRC29. The lack of overlap between our screens and the CME RNAi screen, together with the EMC only registering as needed for select flaviviruses and not any other virus we or others have investigated (HIV-1, HRV, IAV, and HCVl; Table S6) strongly suggests that the block in flaviviral replication found in the EMC-depleted cells is rather unique to this subset of viruses and does not involve a general disruption of CME. In support of this, YF17D has recently been shown to enter via a clathrin-independent pathway (Fernandez-Garcia et al., 2016).
Figure 7. Identification of EMC-Interacting Proteins and Pathways using AP-MS

(A) EMC1, EMC2, and EMC5-HA expression in H1-HeLa cells after induction with 5 μg/ml doxycycline and immunostaining with anti-HA antibody.

(B) EMC4-FLAG expression in H1-HeLa cells after induction with 5 μg/ml doxycycline and immunostaining with anti-FLAG antibody.

(C) Whole-cell lysates from cells in (A) showing EMC1, EMC2, and EMC5-HA expression. Actin (ACTB) serves as a loading control.

(D) Whole-cell lysates from cells in (B) showing EMC4-FLAG expression. ACTB serves as a loading control.

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<th>Enriched Pathway</th>
<th>Number of Proteins (% of Pathway)</th>
<th>p-Value</th>
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<td>Purine nucleotides de novo biosynthesis</td>
<td>12 (21.1%)</td>
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The Role of AXL in Flavivirus Replication

The phosphatidyl serine (PS) binding transmembrane proteins AXL and TIM1 have been shown to be required for the entry of a broad range of viruses, including DENV and ZIKV, via a general interaction with the PS present on the viral membrane (Hamel et al., 2015; Jemielity et al., 2013; Meertens et al., 2012). Although multiple screens for flavivirus host factors have not detected these proteins, including our own MORR screens, the ZIKV CRISPR/Cas9 found AXL to be one of the strongest candidates in terms of both orthogonal sgRNAs as well as total number of reads. To date, no flavivirus host-factor screen has detected TIM1 as important for viral replication, indeed we did not find it to be expressed in the HeLa cells used in these studies (Table S1; data not shown). We found the ZIKV requirement for AXL to occur early in infection, after viral binding but prior to viral entry into the endosomal pathway and/or the early amplification of vRNA in the cytosol. Interestingly, the requirement for AXL was considerably less with the replication of DENV and YF17D, demonstrating that, for this subset of flaviviruses, an AXL- and TIM-1-independent means of viral entry exists.

EMC’s Cellular Function

The EMC was first identified through a genetic interaction screen in yeast as playing a critical role in protein folding, the loss of which induced the UPR (Jonikas et al., 2009). More recently, the EMC has been postulated to serve as a tethering mechanism that facilitates PL transfer from the ER to the mitochondria in yeast (Lahiri et al., 2014) and to mediate the folding of multi-pass, but not single-pass, proteins in the ER (Richard et al., 2013; Satoh et al., 2015). We became interested in the EMC because it was the top complex enriched for in all of our flavivirus MORR and CRISPR/Cas9 screens. This viral dependency on the EMC was unique among the multiple viruses we and others have investigated in human-cell-based screens, suggesting a specific role for the EMC in the flavivirus lifecycle (Tables S6 and S7). To improve our understanding of the EMC, we used EMC proteins as baits in AP-MS studies to find proteins with which they associate. These studies showed that the EMC interacts with both the translocon and the OST complex. Given the known close interaction between the translocon and OST (Rapoport, 2007) and the role of the EMC in ER protein folding (Jonikas et al., 2009), our data suggest that, similar to the OST complex, the EMC is poised near the intraluminal opening of the translocon, where it would have immediate access to the nascent transmembrane protein being extruded into the ER lumen. Additional EMC interactions included multiple nuclear-membrane-associated proteins as well as mitochondrial proteins (Table SS). Although the EMC has been thought to reside predominantly in the ER, these studies suggest that it may also contribute to the functions of additional organelles, warranting further investigation.

The Role of the EMC in Flavivirus Infection

The UPR has been implicated in modulating flavivirus replication (Blázquez et al., 2014; Peña and Harris, 2011, 2012), in particular during the generation of viral RCs beginning several hours post infection. We therefore compared our gene lists with those of the yeast screen that first identified the EMC as being important for protein folding because its loss induces the UPR (Jonikas et al., 2009). Interestingly, we did not identify a strong overlap in genes that were found to play a role in the induction of the UPR in yeast and those that are required for flavivirus replication (Table S6). These findings point away from a triggering of the UPR as the mechanism underlying the EMC-dependency of flaviviruses. Indeed, this comparison leaves open the possibility that the EMC’s role in transmembrane protein folding is needed for the expression of one or more additional glycosylated multi-pass transmembrane proteins required for DENV, ZIKV, and YFV replication.

A CRISPR/Cas9 screen found that the EMC was needed for WNV-induced cytopathicity but not for viral replication (Ma et al., 2015). Although we have not tested these observations directly in our system, they suggest that there are at least two distinct roles for the EMC in flaviviral infection: an early one, potentially acting at viral entry and impacting DENV, ZIKV, and YFV, and a second later one which may result in the death of cytopathic flavivirus-infected cells. EMC’s role in cell death appears to predominate in WNV replication, but we note that both roles (replication and cell death) could contribute to what we observe with ZIKV, which is also cytopathic. An early requirement for the EMC could potentially occur via the loss of an additional human protein or lipid that is needed for the early stages of viral replication and/or the triggering of an anti-viral state; if the latter is the case, then the induced phenotype would act in a restricted manner because we and others have not found that the loss of the EMC inhibits the replication of the diverse viruses interrogated via genetic screening. In addition, multiple EMCS are themselves predicted to be transmembrane proteins, including EMC1, EMC3, EMC4, and EMC5, and so there also exists the possibility that one or more of the EMCS are interacting directly with the flaviviruses, potentially as entry factors. WNV is more closely related to DENV and ZIKV than YFV (Blitvich and Firth, 2015), arguing against the differing roles of the EMC being explained by phylogeny. DENV, ZIKV, and YFV are all transmitted by Aedes mosquitoes, whereas WNV is transmitted by mosquitoes of the genus Culex, suggesting that a vector-specific viral adaptation may have resulted in these two distinct roles for the EMC in human cells. A prediction arising from this notion is that Japanese encephalitis virus, which is also transmitted by Culex mosquitoes, should not require the EMC for its early replication. Additional studies are now needed in order to more fully elucidate the differing actions of the EMC in flavivirus infection. Collectively, these results provide an early evaluation of the role of the EMC in the viral lifecycle (Blitvich and Firth, 2015; Reports (2016), http://dx.doi.org/10.1016/j.celrep.2016.06.028

(E) EMC interaction networks revealed through AP-MS of EMC1, EMC2, EMC4, and EMC5 to identify interacting proteins. The top 20 enriched proteins for each EMC subunit (blue) were used to generate networks in Cytoscape 3.1 (Table S5). Proteins that interacted with two or more EMCS are shown in yellow.

(F) Pathway enrichment of proteins identified in (E) using ConsensusPath DB.

(G) Schematic of the OST complex, with components depicted in color if they were found to be enriched (p ≤ 0.5) in AP-MS experiments involving EMC1, EMC2, EMC4, or EMC5 versus the negative control samples.
Huang et al., 2014) together with a comprehensive and systematic view of human-flavivirus interactions intended to serve as a useful resource in combating these emerging viral infections.

EXPERIMENTAL PROCEDURES

MORR RNAi Screens

The RNAi screens were done in triplicate using four siRNA libraries:

SMARTpool, Dharmacon (21,121 pools, three oligos per pool), Silencer Select, Ambion (21,584 siRNA pools, three oligos per pool), and Dharmacon ReSeq27 Reversion Pools (4,506 siRNA pools, four oligos per pool). We created a high-throughput image-based screening platform to find host factors needed for DENV replication as follows: human cervical cancer cells (MAGi cells, NIH AIDS Reagent Repository) were transiently transfected with siRNA (50 nM concentration) using a reverse transfection protocol employing 0.44% Oligofectamine (siRNA delivery reagent, Invitrogen) in a 384-well plate format. After 72 hr of siRNA-mediated gene knockdown, the cells were infected with DENV2-NGC (VR-1584) at an MOI of 0.5. At 30 hr post infection, the cells were fixed with formalin, permeabilized with 0.1% Triton X-100, and immunostained using the 4G2 monoclonal antibody against the E protein. The cells were then incubated with an Alexa Fluor 488 goat anti-mouse secondary antibody and stained for DNA with Hoechst 33342. The cells were then incubated with an Alexa Fluor 488 goat anti-mouse secondary antibody and stained for DNA with Hoechst 33342. The cells were imaged on an automated Image Express Micro microscope at 4× magnification. Images were analyzed with MetaXpress in order to determine the total cells per well and the percentage of infected cells in each well. Positive control siRNA SMARTpools targeting the V-ATPase subunits ATP6V0B or IFITM3 were used for the high-throughput screen. siRNA pools were classified as hits (decreased infection) if the average of the triplicate plates showed that the percentage of infected cells was >50% of the plate mean and the cell number was not >50% of the plate mean. Pools which increase infection by >150% of the plate mean were also selected as hits (increased infection). Next, in the validation round each siRNA from selected hit pools (Ambion, three siRNAs, and Dharmacon, four siRNAs) was screened individually as above. In the validation datasets, pools with two or more individual siRNAs that met the above criteria were deemed higher confidence, as per the reagent redundancy principle.

RIGER Analysis

Z score robust normalization was applied prior to using the RIGER algorithm as previously described (RNAi Gene Enrichment Ranking, GENE-E [Luo et al., 2008]). Three RIGER methods were employed: second best (SB) method, weighted sum (WS) method, and the Kolmogorov-Smirnov (KS) method, all of which provide a p value for the phenotypic significance of each gene. To compare the individual RIGER analyses to one another and the individual DENV-HF screen datasets (Silencer Select, endoribonuclease prepared siRNA [esRNA], SMARTpool), we used several test sets of complexes or pathways. For these comparisons, AUCs were derived by integration with Microsoft Office employing the following equation: \( \text{AUC} = \frac{\text{SUMPRODUCT}(Y1:YL-1,1:Y2:YL) + \text{SUMPRODUCT}(X1:XL-1,1:X2:XL)}{2} \), where \( X \) and \( Y \) are a plotted X and Y value, respectively, with the subscript representing each coordinate \( X \) and \( Y \) representing the last value. Thus, \( L-1 \) represents the second to last value. If a component was not in a dataset, then it was taken out of that dataset; in this way, alterations were made in the total number of dataset parts so that, at the end of the dataset, the percentage of the complex was 100%.

SUPPLEMENTAL INFORMATION

Supplemental Information contains Supplemental Experimental Procedures, six figures, and seven tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.06.028.

AUTHOR CONTRIBUTIONS


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