Genome-wide CRISPR screen for Zika virus resistance in human neural cells

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Zika virus (ZIKV) is a neurotropic and neurovirulent arbovirus that has severe detrimental impact on the developing human fetal brain. To date, little is known about the factors required for ZIKV infection of human neural cells. We identified ZIKV host genes in human pluripotent stem cell (hPSC)-derived neural progenitors (NPs) using a genome-wide CRISPR-Cas9 knockout screen. Mutations of host factors involved in heparan sulfation, endocytosis, endoplasmic reticulum processing, Golgi function, and interferon activity conferred resistance to infection with the Uganda strain of ZIKV and a more recent North American isolate. Host genes essential for ZIKV replication identified in human NPs also provided a low level of protection against ZIKV in isogenic human astrocytes. Our findings provide insights into host-dependent mechanisms for ZIKV infection in the highly vulnerable human NP cells and identify molecular targets for potential therapeutic intervention.

Zika virus | neural progenitors | CRISPR screen | fetal CNS infection | human pluripotent stem cells

The recent outbreaks of Zika virus (ZIKV) in French Polynesia, the Caribbean, and Central and South America have been associated with a significant incidence of central and peripheral neuropathy (1, 2). Of particular concern are the severe fetal brain abnormalities such as microcephaly, lissencephaly, and cortical calcification after maternal infection during early pregnancy (3, 4). Accumulating evidence (in vitro and in vivo) demonstrates that human neural progenitors (NPs) are particularly sensitive to ZIKV infection (5, 6). Exposure to even a low level of ZIKV can result in robust infection of NPs, intracellular buildup of viral proteins, and extracellular release of functional virions, and ultimately lead to reduced differentiation and cell death (6, 7). This finding is in contrast to other central nervous system (CNS) cell types, including neurons, astrocytes, and microglia, which are all infectible by ZIKV but do not display a level of vulnerability comparable to NPs (5, 8). We and others have recently reported that intrinsic differences between NPs and other neural cell types in interferon (IFN) activity (8), RNA binding protein level (9), and signaling pathway dependence (10) may contribute to their enhanced vulnerability.

Unbiased forward genetic screens have proved powerful in identifying the cellular factors present in the host cells involved in viral propagation (11, 12). These host factors are often beneficial for viral entry, replication, packaging, and exit, or are involved in silencing host antiviral defense mechanisms. Genetic screens have been used to identify putative host factors for flaviviruses including dengue virus (13, 14), ZIKV (13), and West Nile virus (15). These studies used cancer cells such as kidney-derived HEK293, cervical HeLa cells, hepatic Huh7, and myeloid Hap1 and showed that some common pathways mediate the infection by different subtypes of flaviviruses. However, different flaviviruses vary significantly in their cellular tropism and manifest vastly different clinical pathology in patients. To date, ZIKV is the only example of a flavivirus that causes loss of proliferation and cell death in human NPs during early cortical development, which in turn results in fetal brain abnormality. Therefore, a better understanding of the host factors required for ZIKV infection in human NPs is needed to elucidate the mechanism of neural vulnerability and to develop future treatment options.

To date, the only genome-wide screen against ZIKV used culture-adapted cancer cell lines (13). Although that study identified genes important for ZIKV replication, it is possible that key genes involved in virus replication in the relevant cell types of the CNS were not expressed in the cancer cells and that viral host genes identified in cancer cells were not active in the CNS target cell types. In the present study, we performed a genome-wide CRISPR knockout screen in human NPs to identify host factors that, when mutated, confer resistance to ZIKV-induced cell death. Genes encoding proteins involved in heparan sulfation, endocytosis, endoplasmic reticulum (ER) protein processing, Golgi function, and IFN regulation were identified as putative host factors for ZIKV strains MR766 and PRVABC59. Pharmacological manipulation further validated the involvement of these pathways in mediating

Significance

Zika virus (ZIKV) is a neurotropic and neurovirulent arbovirus that has severe detrimental impact on the developing human fetal brain. We used a genome-wide CRISPR-Cas9 knockout screen to identify ZIKV host genes in human neural progenitors. The screen identified host factors involved in heparan sulfation, endocytosis, endoplasmic reticulum processing, Golgi function, and interferon activity. Our findings provide insights into host-dependent mechanisms for ZIKV infection in the highly vulnerable human neural progenitor cells and identify molecular targets for potential therapeutic intervention.

ZIKV infection in human NPs. Host factors were identified for ZIKV infection in NPs that were not previously reported in cancer cells. Furthermore, we show that the host factors identified in NPs partially mediate ZIKV infection in isogenic human astrocytes. These results emphasize the need to study viral mechanisms in biologically relevant cells. Our findings define host-dependent mechanisms that are essential for ZIKV propagation in the highly vulnerable human neural cells and identify molecular targets for potential therapeutic intervention.

Results

CRISPR-Cas9 Screens for ZIKV Host Factors in Human NPs. Human induced pluripotent stem cells (iPSCs)-derived NPs are highly vulnerable to ZIKV infection (6). We and others have shown that the prototypic ZIKV Uganda strain MR766 (ZIKV^U) efficiently infects human NPs and leads to rapid cell death in 2D mono-cultures and 3D brain organoids (6, 8, 16). To identify host genes required for ZIKV^U-mediated toxicity, we conducted a genome-wide CRISPR knockout screen. We used a pooled lentiviral library encoding Cas9 and 187,535 single guide RNAs (sgRNAs) targeting 18,663 protein-coding human genes that included 1,503 intergenic-targeted and nontargeting control sgRNAs (Fig. 1A). NPs derived

from a wild-type human iPSC line (iPS-wt5) were transduced with the library and selected using puromycin (*SI Appendix,* Fig. S1A). These cells were then inoculated with ZIKV^U at a multiplicity of infection of 1. While ZIKV^U infection led to drastic death of the lentiviral library-carrying NPs starting from 48 h postinoculation, a small population was protected and survived. We confirmed that surviving NPs had substantially reduced ZIKV protein (Fig. 1B and *SI Appendix,* Fig. S1B) and viral RNA release (Fig. 1C) compared with ZIKV-infected wild-type NPs. Surviving NPs further showed reduced reinfection by both the initially screened MR766 strain (ZIKV^U) as well as PRVABC59, an epidemic North American strain recently isolated in Puerto Rico (ZIKV^{PR}) (17), as assessed by ZIKV protein staining 7 d postreinfection (Fig. 1D). High-throughput sequencing libraries were prepared from genomic DNA collected from surviving cells at day 8 and from an initial reference sample. The change in representation of each sgRNA in the population after ZIKV^U exposure was calculated, and sgRNAs enriched in the surviving cells were identified. We found that knockouts of genes encoding proteins involved in heparan sulfation, endosome–lysosome acidification, ER protein complexes regulating translocation and proteostasis, Golgi complexes regulating glycosylation, and negative regulators of IFN response were protective (Fig. 1E and *SI Appendix,* Table S1). *TP53*, a gene that when knocked out confers growth advantage (18, 19), was also identified.

To further validate the candidates from the genome-wide screen, we next performed a focused screen using a custom library containing sgRNAs against candidate gene knockout hits (Fig. 2A). We started by confirming that candidate genes were expressed in mock and ZIKV^U-infected NPs using RNA sequencing (Fig. 2B and *SI Appendix,* Table S2). We next constructed a secondary lentivirus CRISPR library encoding Cas9 and 3,743 sgRNAs targeting the top ~130 protective gene knockout hits, plus 499 to 5,191 control sgRNAs targeting intergenic regions or protein-coding genes whose loss should not be protective to ZIKV^U exposure (Fig. 2A and *SI Appendix,* Table S3). We transduced human NPs from two different genetic backgrounds, iPS-wt5 and WIBR3 hESCs, with the lentivirus library. As expected, a substantial fraction of lentivirus-transduced NPs survived ZIKV^U infection (*SI Appendix,* Fig. S2A), and DNA was collected on day 8 or 10 after infection and sequenced. These secondary screens confirmed that top hits from the genome-wide screen were modifiers of ZIKV^U toxicity in human NPs (Fig. 2C and *SI Appendix,* Fig. S2B).

Multiple Pathways Mediate ZIKV Infectivity in Human NPs. We targeted 11 host genes in iPS-wt5 NPs, using two independent sgRNAs targeting different regions of each gene. These genes were selected to represent the main functional categories identified through the pooled CRISPR screens. We confirmed that targeting of all 11 genes significantly reduced ZIKV^U infection as measured by viral RNA released into the medium (Fig. 3A) and viral protein levels in the NPs (*SI Appendix,* Fig. S3). We further tested the effects of targeting these host genes on infection by ZIKV^{PR}. Human NPs targeted for 10 of the 11 genes showed significantly less viral RNA release (Fig. 3B). Immunostaining for ZIKV envelope protein further confirmed reduced ZIKV^{PR} infection in NPs (Fig. 3C). NPs targeted for STAT3, a regulator of IFN response, showed reduced infection by ZIKV^U, but not by ZIKV^{PR} (Fig. 3 and *SI Appendix,* Fig. S3). These findings demonstrate that ZIKV propagation depends on multiple pathways in human NPs.

We investigated whether pharmacological modulation of these ZIKV host-factor pathways could reduce viral infection in NPs. We first examined the heparan sulfation pathway. Treatment of NPs with sodium chlorate, an inhibitor of sulfation, strongly inhibited infection by ZIKV^U and ZIKV^{PR}, as measured by viral RNA load and envelope protein staining (Fig. 4 A and B).

We noted that many genes involved in vacuolar acidification were enriched in the modifier genes, including *RNASEK, VMA21,* and 12 genes encoding subunits of vacuolar ATPase (V-ATPase).
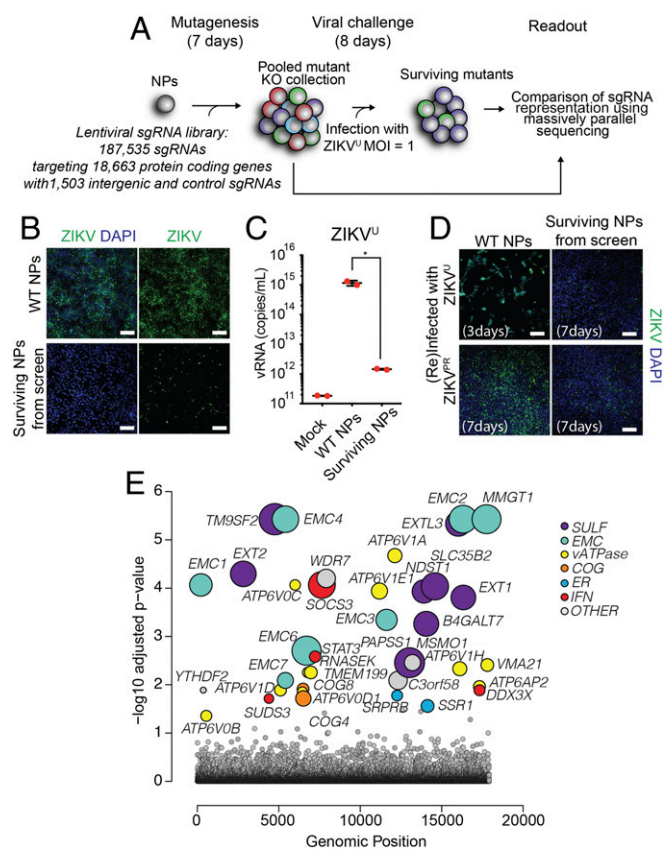

Fig. 1. Genome-wide screen for ZIKV^U resistance in human iPSC-derived NPs. (A) Schematic diagram of CRISPR screen with a genome-wide library in iPS-wt5 NPs. (B and C) Surviving NPs displayed minimal level of ZIKV protein (B) and greatly diminished viral RNA (vRNA) release into the medium (C). Control NPs were infected with multiplicity of infection (MOI) 1 ZIKV^U and collected at 48 h (B) or 72 h (C) postinfection. (Scale bars in B: 100 μm.) (D) Surviving NPs rechallenged with MOI 1 ZIKV^U and ZIKV^{PR} displayed low ZIKV infectivity at 7 d postinfection. Control NPs infected with ZIKV^U and ZIKV^{PR} were collected at 3 d and 7 d postinfection, respectively. (Scale bars in D: 100 μm.) (E) Six main pathways were identified as protective against ZIKV^U infection, upon gene knockouts. The size of the circles represents the magnitude of the increase in sgRNA representation after ZIKV^U exposure. *P < 0.05.
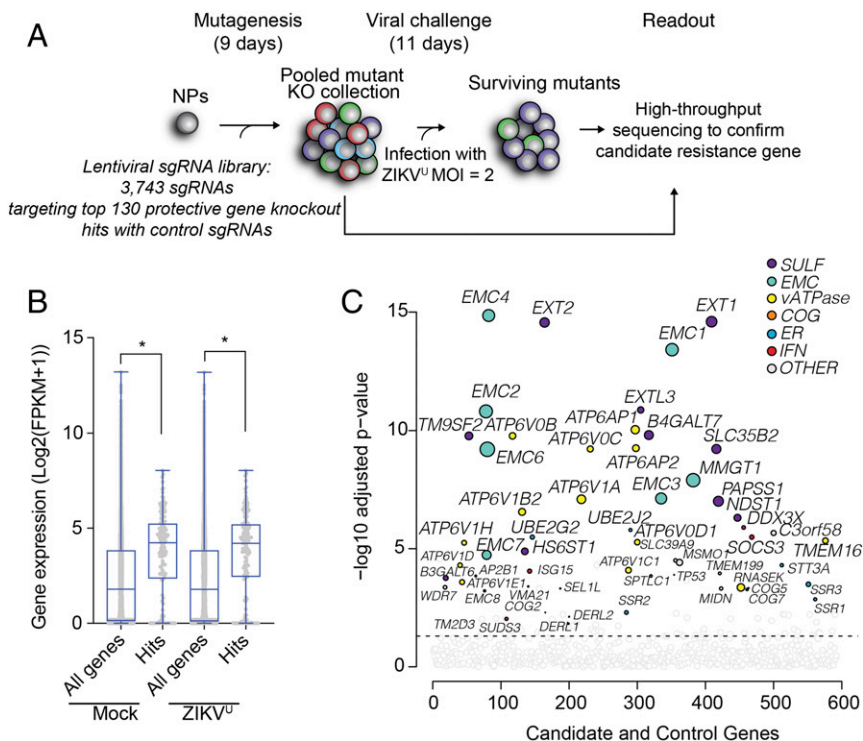
Fig. 2. Secondary CRISPR knockout screen in human NPs against ZIKV^U. (*A*) Schematic diagram of CRISPR screen with a focused library in iPS-wt5 NPs. (*B*) Focused library contained gene hits with confirmed expression in human NPs on the baseline and 24 h after multiplicity of infection (MOI) 1 ZIKV^U infection. (*C*) Focused screen in iPS-wt5 NPs identified most of the hits previously identified in the genome-wide screen. The size of the colored circles represents the increase in sgRNA representation after ZIKV^U exposure. FPKM, fragments per kilobase of exon model per million reads mapped. *$P < 0.05$.

While some of these genes were previously associated with flavivirus infection (13), most were not identified in genome-wide screens performed on immortalized cell lines (14, 15). Indeed, many of these genes belong to the group of core fitness genes for human cancer cells (20, 21). To investigate the role of the endosome–lysosome pathway in mediating ZIKV infection of human NPs, we treated infected cells with bafilomycin A1, a specific inhibitor for vacuolar-type proton pumps. Treatment at 10 and 100 nM strongly protected NPs against ZIKV^U infection (Fig. 4*C*). A significant protective effect was observed, even with a low concentration (1 nM) of bafilomycin A1 (Fig. 4*D*).

We have recently reported that low baseline IFN activity in NPs contributes to their high susceptibility to ZIKV infection (8). In the current genetic screen, we identified negative regulators of the IFN pathway such as ISG15 and SOCS3. Fig. 4 *E* and *F* shows that a 2-h treatment with IFN-γ before viral exposure was effective in reducing infection by ZIKV^U and ZIKV^{PR}. Mutations in one of these host genes, *ISG15*, has been linked to autoinflammatory disease and susceptibility to mycobacterial exposure (22, 23), consistent with these genes playing general role in controlling infection. Using CRISPR-Cas9–mediated gene editing, we generated human PSCs that lack ISG15 (Fig. 4*G* and *SI Appendix*, Fig. S4), which were then used to generate cerebral organoids. Organoids were exposed to ZIKV^{PR} on day 30 of formation and examined 12 d after infection. We found that ZIKV^{PR} efficiently infected control cerebral organoids, led to elevated apoptosis in Sox2+ NPs, and resulted in disruption of ventricle-like structures (Fig. 4 *H* and *I*). In contrast, *ISG15* mutant organoids showed reduced viral infection and preserved Sox2+ NPs and maintained their ventricle-like structures (Fig. 4 *H* and *I*). Our results are consistent with the IFN pathway being critical for regulating ZIKV infection in human NPs.

Host Genes Identified in NPs Play Some Role During ZIKV Infection of Astrocytes. In addition to NPs, other CNS cell types are also susceptible to ZIKV infection, including astrocytes, microglia, and neurons (8). Because astrocytes and NPs are both proliferative CNS cell types and closely related in lineage, we investigated whether mutation of host genes that reduced ZIKV infection in NPs could also protect astrocytes. Isogenic human astrocytes were differentiated from iPS-wt5 NPs and shown to express
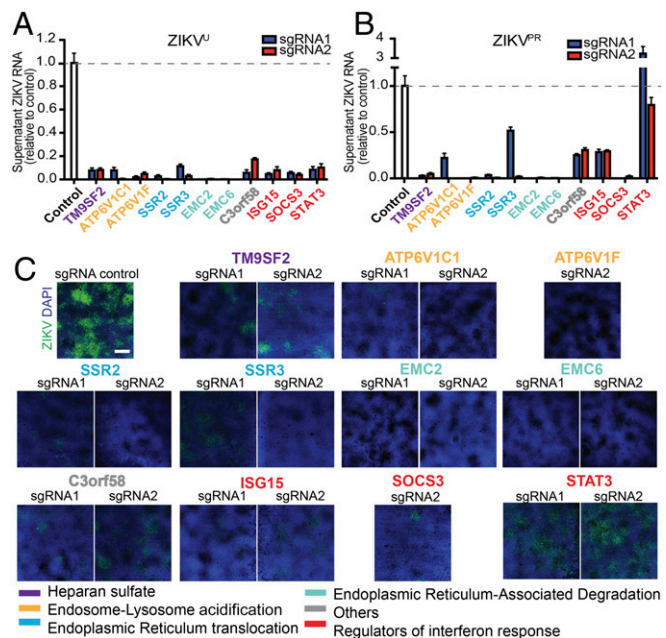

Fig. 3. Validation of ZIKV host factors with individual sgRNAs in human NPs. (*A* and *B*) Individually targeted iPS-wt5 NPs infected with multiplicity of infection (MOI) 0.5 ZIKV^U (*A*) and ZIKV^{PR} (*B*) showed reduced viral RNA released into the supernatant at 72 h postinfection, compared with ZIKV-infected control NPs. All experimental sgRNA results were significant ($P < 0.001$) compared with control sgRNA, except sgRNA-2 against STAT3 infected with ZIKV^{PR}. (*C*) Immunostaining for ZIKV envelope (green) and DAPI (blue) showed reduced infection by MOI 0.5 ZIKV^{PR} in targeted human NPs, 96 h postinfection. (Scale bar: 1,000 μm.) Three technical replicates. Results are mean ± SEM.
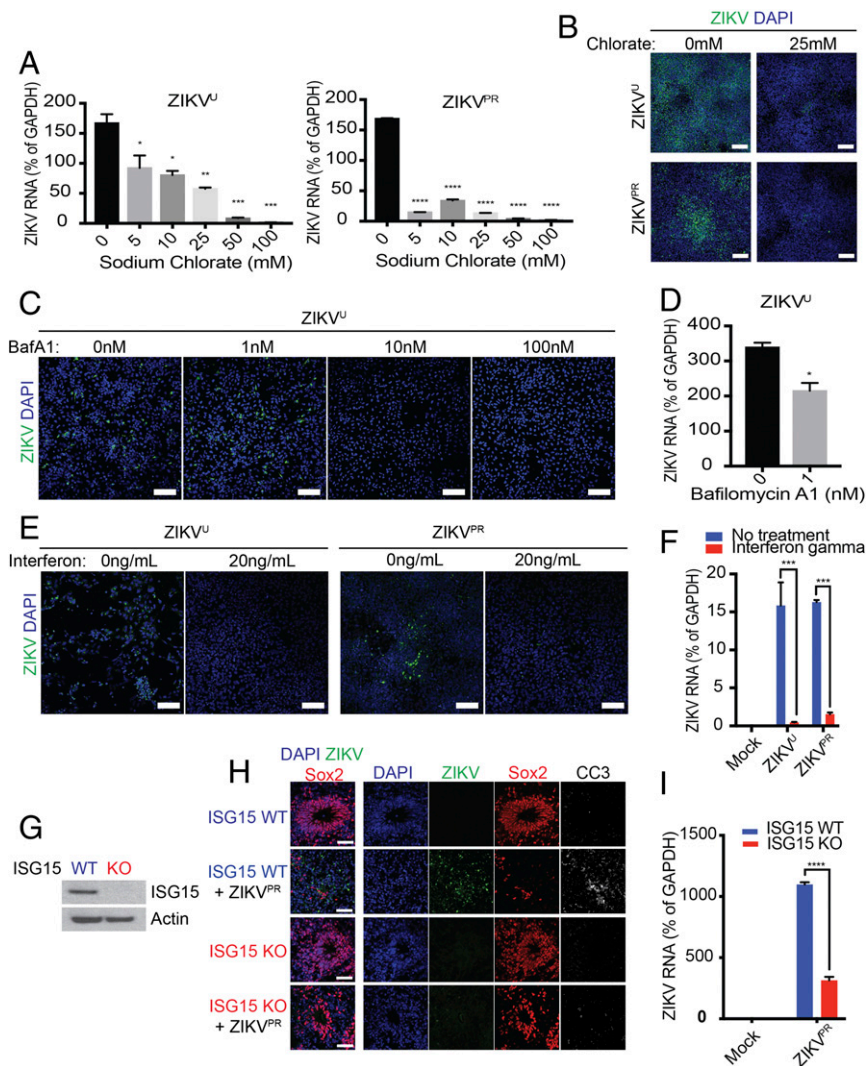
Fig. 4. Validation of host pathways using pharmacological compounds and in gene-edited brain organoids. (*A* and *B*) Depletion of cellular heparan sulfate with sodium chlorate reduced viral RNA load (*A*) and envelope protein staining (*B*) upon ZIKV^U and ZIKV^{PR} exposure. iPS-wt5 NPs were treated with multiplicity of infection (MOI) 0.1 ZIKV^U or MOI 1 ZIKV^{PR} and collected at 48 h or 72 h postinfection, respectively. (Scale bars in *B*: 100 μm.) (*C* and *D*) Blockade of acidification with bafilomycin A1 (BafA1) decreased ZIKV^U viral envelope protein staining. Low concentration (1 nM) bafilomycin A1 was sufficient to reduce ZIKV^U infection as quantified by viral RNA load (*D*). NPs were infected with MOI 0.1 ZIKV^U and collected at 24 h (*C*) or 48 h (*D*) postinfection. (Scale bars in *C*: 100 μm.) (*E* and *F*) NPs Pretreated with IFN-γ showed reduced infection by ZIKV^U and ZIKV^{PR}, as observed by viral RNA load (*E*) and ZIKV envelope protein staining (*F*). NPs were infected with MOI 1 ZIKV^U and ZIKV^{PR} and collected at 48 h (*E*) or 72 h (*F*) postinfection. (Scale bars in *E*: 100 μm.) (*G–I*) ISG15 mutant WIBR3-derived brain organoids lacked ISG15 protein (*G*) and showed reduced ZIKV^{PR} infection, as shown by ZIKV protein (*H*) and viral RNA load (*I*). (Scale bars in *H*: 100 μm.) Two technical replicates. Results are mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

canonical markers of the astrocytic lineage and be infectible by ZIKV^U and ZIKV^{PR} (Fig. 5 *A* and *B* and *SI Appendix*, Fig. S5 *A and B*). We targeted the same 11 modifier genes and measured the susceptibility of the targeted astrocytes to ZIKV infection. We found that targeting of some of the modifier genes (such as *EMC2, SSR2, SSR3, ISG15,* and *SOCS3*) in astrocytes showed reduced infection by ZIKV^U or ZIKV^{PR} (Fig. 5 *C* and *D* and *SI Appendix,* Fig. S5 *C and D*). This suggests that both common and divergent signaling pathways may mediate ZIKV infection in these related neural cell types.

Discussion

Selective vulnerability is a phenomenon often observed in neurodegenerative disorders. Subpopulations of progenitors, neurons, and glial cells can display increased susceptibility to certain genetic perturbations, environmental insults, and traumatic injuries. This leads to impairment of the development and function

of the nervous system and can have dire consequences. Bacterial and viral infections of the CNS are often limited by the blood–brain barrier (BBB), but those that breach the BBB can lead to severe neuropathy, especially when infection occurs during early fetal development. Devastating consequences are often observed after fetal infection by a group of infectious agents (toxoplasma, other agents, rubella, cytomegalovirus, and herpes simplex) collectively known as TORCH. Historically, infection by flaviviruses during pregnancy has not been linked with fetal abnormalities (1). However, the emergence of ZIKV-related fetal brain malformations highlights the need to better understand the mechanism of selective neural vulnerability. Using an isogenic human PSC-derived in vitro system, we have recently reported that ZIKV and dengue virus are capable of infecting multiple cell types of the CNS and produce vastly different responses and cytopathic outcomes (8). While human NPs, neurons, astrocytes, microglia, and endothelial cells are all infectible by ZIKV, NPs
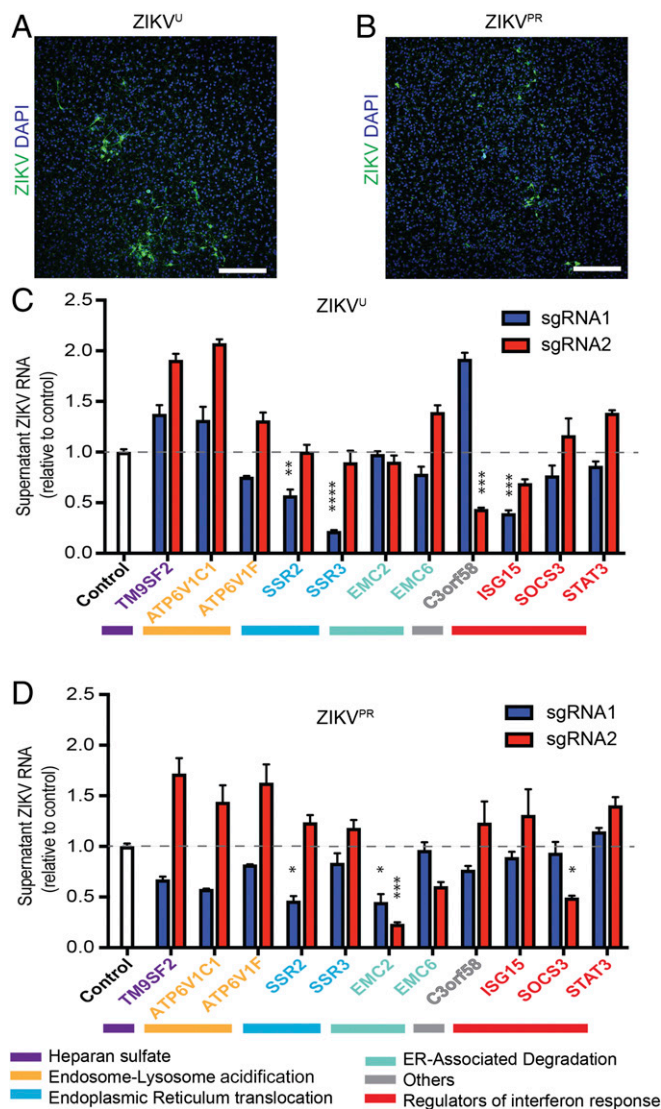
Fig. 5. Investigation of the role of ZIKV host factors in human astrocytes. (*A* and *B*) Human astrocytes derived from iPS-wt5 were susceptible to ZIKV^U (*A*) and ZIKV^{PR} (*B*) infection. Astrocytes were infected with multiplicity of infection (MOI) 0.1 ZIKV^U or MOI 0.6 ZIKV^{PR}, and collected at 48 h. (Scale bars: 1,000 μm.) (*C* and *D*) Astrocytes targeted with individual sgRNAs show varying degrees of infection by ZIKV^U (*C*) and ZIKV^{PR} (*D*), as measured by viral RNA release into the medium. Astrocytes were infected with MOI 1 ZIKV^U or ZIKV^{PR} and collected at 72 h. Experimental sgRNA results that showed significant reduction compared with control sgRNA were noted. Two technical replicates. Results are mean ± SEM. *$P < 0.05$, ***$P < 0.001$, **$P < 0.01$, ****$P < 0.0001$.

display significantly higher levels of cytopathology and cell death. Compared with astrocytes and microglia, NPs have low baseline IFN activity and display muted IFN response upon ZIKV infection, likely contributing to their vulnerability (8). It has also been reported that upon infection, RNA and protein components of ZIKV sequester key proteins that maintain neural stem and progenitor identity (9) and interfere with the strength of the growth factor signaling pathway important for proliferation (10). One of these pathogen–host interactions was recently identified to be between ZIKV NS4A and ANKLE2 (24), which has previously been linked to primary microcephaly (25). These findings suggest that vulnerability of human NPs to ZIKV is multifaceted.

In the present study, we performed a systematic evaluation of ZIKV host factors in human NPs. We found that ZIKV utilizes

multiple host cellular machineries to potentially facilitate entry (heparan sulfation, endocytosis); translation and replication of the viral genome (ER); assembly of virion; and egress from host (Golgi apparatus). Our results show that ZIKV relies on negative regulators of the IFN pathway to evade host antiviral defense. While some of these mechanisms have previously been identified in genomic screens on cancer cells against other flaviviruses, the active suppression of IFN activity was not seen in these systems. Interestingly, one of the suppressors of IFN activity is *ISG15*, a gene linked to an inherited form of autoinflammation reminiscent of Aicardi–Goutières syndrome (22, 23). Patients with *ISG15* loss-of-function mutations have mycobacterial disease but no increased susceptibility to viral infection (23). Our findings suggest that lack of ISG15 in human NPs reduces ZIKV infection and has an acute protective effect. This is consistent with the protective effect of IFN treatment in NPs (8) and the finding that *ISG15* mutant patient fibroblasts had enhanced antiviral protection (26). It has been recently reported that type I IFNs instigate ZIKV-mediated fetal demise in mice, while type III IFNs curb placental to fetal transmission (27, 28). Future investigation into the roles of different IFN pathways in the developing CNS may provide new insights into the mechanism of selective neural vulnerability.

Another category of ZIKV host factors differentially identified in our screen using human neural cells—compared with previous work done in cancer cell lines—is composed of components of the endosome–lysosome pathway. These putative ZIKV host factors include 12 genes encoding subunits of V-ATPase, as well as VMA21 and RNASEK, which facilitate V-ATPase assembly and function (29, 30). We validated that knockout of individual components of the V-ATPase (ATP6V1F, ATP6V1C1) reduced ZIKV infection in human NPs. This effect was recapitulated using bafilomycin A1, indicating that the canonical function of V-ATPase in vacuolar acidification is required for ZIKV infection. Our finding that the endosome–lysosome pathway is utilized by ZIKV is consistent with recent reports that the blockade of endocytosis using chloroquine reduced ZIKV infection (10, 31–33). Other recently identified protective small-molecule compounds against ZIKV infection have also been shown to interfere with this pathway (34, 35). We reason that many of these genes were not identified in screens using cancer cell lines because they are considered essential for cancer cells and their ablations disrupt cellular fitness (20, 21). This finding further highlights the value of investigating viral mechanism in biologically relevant cell types.

By comparing hits from our screens in NPs to previous studies in cell lines, we also highlight mechanisms that may be protective in other cell types but fail to be so in human NPs. For example, the receptor tyrosine kinase AXL was previously identified as an essential entry factor for ZIKV in human fibroblasts (36) and has been suggested to play a similar role in human neural stem cells based on expression pattern (37). However, genetic ablation of AXL was recently found to have no effect on ZIKV infection in mice (38) or in human NPs (39). AXL and other members of the receptor tyrosine kinase subfamily (TYRO3, MERTK) were not identified as protective against ZIKV in the current screens. This result is consistent with the notion that divergent entry mechanisms may be utilized by ZIKV on different cell types. The identification of genes in the heparan sulfation and endosome–lysosome pathways in the current screen supports their potential roles as attachment and entry mechanisms for ZIKV. Because different cell types may rely on different fitness genes, the current screens would be unable to identify host factors that are important for other cell types, if they are essential for the fitness of NPs. This may explain the identification of some (STT3A), but not other, members of the oligosaccharyltransferase complex as host factors in NPs. We also note that NPs used in the current study most closely resemble in vivo stem and progenitor cells from the developing dorsal forebrain. Because fetal exposure to

ZIKV has been linked to structural abnormality in many brain regions, including forebrain, midbrain, and hindbrain (3, 4), future studies using NPs bearing midbrain and hindbrain regional identity could provide further insights into cell type-specific viral–host interactions. Lastly, the current study was conducted in two hPSC lines. In future studies, this effort could be expanded to more systematically analyze the genetic and environmental variabilities that impose protection or vulnerability against ZIKV infection, shedding new light on the mechanism of viral–host interaction and potential therapeutic strategies.

While ZIKV infection during early pregnancy is correlated with overt brain malformation such as microcephaly and lissencephaly, later infection is also linked to neural structural and functional abnormalities (1, 2). The diverse tropism of ZIKV toward CNS cell types highlights its potent ability to cause harm. During human fetal brain development, as neurons and glia emerge from NPs, or as microglia migrate into the brain, these cell types are susceptible to ZIKV infection. Evidence suggests that neuronal function can be impaired by ZIKV (40), and glial cells that are less acutely impacted could serve as viral reservoirs (8, 41). In the current study, we investigated whether ZIKV host factors in NPs also mediate infection of astrocytes. We used CRISPR-Cas9 to target the putative host factors in astrocytes. Compared with NPs, astrocytes divide less frequently and may therefore display a slower rate of protein depletion. Despite this possible limitation of incomplete protein depletion, we found that a subset of the factors that protected NPs against ZIKV infection also protected astrocytes. Future therapeutic strategies exploring these shared host factors may confer broad neural protection.

Materials and Methods

For a complete description of the materials and methods used in this study, please refer to *SI Appendix, Materials and Methods*. The human iPSC line iPS-wt5 and the hESC line WIBR3 were previously described (42, 43). ZIKV strains MR766 (ZIKV^U) and PRVABC59 (ZIKV^{PR}) were obtained from American Type Culture Collection and were expanded in Vero cells or in C6/36 mosquito cells.

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