A global map of the Zika virus phosphoproteome reveals host-driven regulation of viral budding

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Abstract

Flaviviruses are enveloped, positive-strand RNA viruses that cause millions of infections in the human population annually. Although Zika virus (ZIKV) had been detected in humans as early as the 1950s, its reemergence in South America in 2015 resulted in a global health crisis. While flaviviruses encode 10 proteins that can be post-translationally modified by host enzymes, little is known regarding post-translational modifications (PTMs) of the flavivirus proteome. We used mass spectrometry to comprehensively identify host-driven PTMs on the ZIKV proteome. This approach allowed us to identify 43 PTMs across 8 ZIKV proteins, including several that are highly conserved within the *Flavivirus* genus. Notably, we found two phosphosites on the ZIKV envelope protein that are functionally important for viral propagation and appear to regulate viral budding. Additionally, we discovered 115 host kinases that interacted with ZIKV proteins and determined that Bosutinib, an FDA-approved tyrosine kinase inhibitor that targets ZIKV interacting host kinases, impairs ZIKV growth. Thus, we have defined a high-resolution map of host-driven PTMs on ZIKV proteins as well as cellular interacting kinases, uncovered a novel mechanism of host driven-regulation of ZIKV budding, and identified an FDA-approved inhibitor of ZIKV growth.

Introduction

ZIKV is a mosquito-borne, enveloped, positive-strand RNA virus that encodes three structural proteins (capsid, C; membrane, prM; and envelope glycoprotein, E) and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5; Fig 1A). ZIKV was first discovered in 1947 and confirmed as a human pathogen in 1952 (1). ZIKV belongs to the *Flavivirus* genus, which has a large disease burden on the human population because of notable members like dengue (DENV), West Nile (WNV), and yellow fever virus (YFV). Though considered a benign disease for most of its known history, ZIKV emerged in South America in 2015 and resulted in a global health crisis that lasted for more than a year and caused over 1.6 million infections (2). Most ZIKV infections are asymptomatic, while approximately 20% of those infected experience mild disease. In rarer cases, detrimental neurological complications arise in the form of Guillain-Barré syndrome as well as fetal microcephaly (3, 4). Infants afflicted with microcephaly because of ZIKV infection suffer developmental and health consequences into childhood often with no countermeasures available; those born with severe microcephaly require constant care.

Surveys of post-translational modifications (PTMs) on viral proteins have previously been used to understand how viruses affect cells and vice versa. Viral proteins are multifunctional and PTMs are an important host-driven process that can regulate viral protein functionality. Discovery of PTMs can potentially elucidate novel viral protein functions. For example, in human immunodeficiency virus and lymphocytic choriomeningitis virus (LCMV), phosphorylation of viral proteins has been shown to be important in key processes like viral particle budding and regulation of budding of different classes of viral particles (5-8). There have been a small number of flavivirus phosphorylation sites discovered which demonstrate that

phosphorylation of viral proteins can influence the host's innate immune system or evade it, affect functionality of a viral protein, impact viral protein-protein interactions, or regulate the replication of viral RNA (9-13). These important discoveries emerged from studies of NS5 proteins of several species of flaviviruses. However, there remains an expansive terrain of other flavivirus proteins. Importantly, identification of novel PTMs and their functional relevance is anticipated to reveal unexplored avenues for pharmacological therapeutics in these diseases. To address this gap in knowledge, herein we performed a large-scale mass spectrometry analysis of the ZIKV proteome to broadly map the PTM landscape of ZIKV. Additionally, we mapped host kinases that interact with the ZIKV proteome and tested whether FDA-approved inhibitors of interacting kinases can be used to inhibit ZIKV growth. Finally, we functionally interrogated a subset of the identified PTMs to determine their importance for ZIKV propagation and, where relevant, mapped the stage of the viral life cycle impacted.

Results

Discovery of post-translational modifications (PTMs) on the Zika virus proteome

A primary goal of this study was to identify host-driven PTMs that occur on ZIKV proteins produced by mammalian cells, either in the setting of cell-free virions or within cells. Where possible, we wished to examine PTMs in the setting of authentic ZIKV infection. At the time of this study, the only suitable antibody available for purification of a ZIKV protein, expressed in the context of an infected cell, was 4G2, which recognizes the ZIKV envelope (E) protein (14). This antibody was used to purify intracellular ZIKV E and its interacting host partners from infected cells (Figs 1B and 1C) or E-decorated, cell-free virions released from these same infected cells (Figs 1B and 1D). Note that immunopurified ZIKV particles contained the

structural proteins E, prM, and C (Fig 1D and Supplementary Information 1). We also isolated cell-free virions from infected mosquito cells via banding on a potassium tartrate density gradient (Fig S1A). To circumvent the lack of antibodies suitable for immunoprecipitation of the remaining ZIKV proteins from cells, we used plasmids to express streptavidin-binding protein (SBP)-tagged ZIKV proteins. This allowed for efficient capture of SBP-tagged viral proteins via magnetic streptavidin beads (Figs 1B, 1E, S2, S3, and S4). To increase the likelihood of detecting phosphorylation events, transfected cells were treated with either H₂O₂, Calyculin A, or DMSO to inhibit tyrosine phosphatases, serine/threonine phosphatases, or to act as a control, respectively (Figs 1B, 1E, S2, S3, and S4). Samples derived from immunoprecipitation and affinity purification were subjected to SDS-PAGE to orthogonally separate protein components. Gel lanes were cut into multiple sections, and each piece was subjected to in-gel trypsin digestion. Meanwhile, ultracentrifuged purified virus from mosquito cells (Fig S1B) was digested with trypsin using filter-assisted sample preparation. All resulting tryptic peptides were subjected to liquid chromatography tandem mass spectrometry for identification of PTMs or interacting host proteins as described in the Methods and previously (8, 15). Tandem mass spectra for discovered phosphorylation and ubiquitination sites are available in Supplementary Information 2 (spectra for ZIKV E T351 from virions is available in Figs S1C, S1D).

We identified 18 phosphorylation sites and 5 ubiquitination sites on ZIKV E (Table 1, Table S1, Figs 2A, and 2B). Two of these phosphorylation sites, Y61 and T351, were discovered in the context of infection (Y61 from infected Vero E6 cells and T351 from mosquito cell-derived ZIKV particles (Fig S1D). T351 was also found on ZIKV E expressed from plasmid in HEK293T cells. The remaining PTMs were identified from plasmid-expressed viral proteins. The locations of Y61, T351, and other PTMs found on ZIKV E are shown in Figs 2A and 2B.

Y61 is located in Domain II of E, which is the region responsible for dimerization (16). T351 is located in Domain III, which is often a target of neutralizing antibodies (16). PTMs on E were also mapped using electrostatically labeled models (Fig S5). Figure S5A shows one “face” of the ZIKV E dimer as largely positively charged while Figure S5B shows the other largely negatively charged face. In contrast, the border of the E dimer is mostly neutral (Fig S5C). The majority of ZIKV E phosphosites were found on the neutral border of the envelope protein (Fig S5C). The two phosphorylation sites on M, S16 and T18, are positioned in close proximity to one another at a junction of where E and M associate (Fig 2C). Interactions between the M S16 and T18 phosphorylated residues with E are hard to resolve, however, because both sites are in flexible regions of M and are interacting with flexible regions of E (Fig S6A).

We identified 11 phosphorylation sites and one ubiquitination site on NS1 (Table 1, Table S1). Location mapping of PTMs on NS1 revealed that approximately half of the modified amino acid residues were located in the beta-roll or beta ladder domains of NS1 (Fig 2D) important for NS1 intracellular dimerization (17) and host protein interaction (18), respectively. We were unable to successfully express and isolate adequate quantities of NS2A or NS3 to analyze for PTMs (Fig S7). Mass spectrometry revealed a phosphorylated serine at position 71 on the NS2B protein (Table 1, Table S1), a small protein known to associate with NS3 to form the ZIKV protease complex (Fig 2E). There were no PTMs found on NS4A despite an abundance of NS4A peptides observed by mass spectrometry. One phosphorylation site was detected on NS4B, though the precise location could not be determined with certainty. Our mass spectrometry data suggest its location to be at either S217, S218, or T219 (Table 1, Table S1). There are currently no models of ZIKV NS4B so we were unable to depict these sites within the protein structure. Last, we discovered four phosphorylation sites on NS5 (Table 1, Table S1). Of

these, two were located in the RNA-dependent RNA polymerase region of NS5 and two in the methyl-transferase region (Fig 2F).

To determine how conserved the discovered ZIKV protein PTMs might be with other flaviviruses, we constructed a multiple sequence alignment (MSA) that included viruses spanning the *Flavivirus* genus and chose flaviviruses known to cause human disease (Supplementary Information 3; virus list also noted in the Methods section). In total, we compared 15 different flaviviruses representing tick- or mosquito-borne viruses, viruses with no known vectors, and those considered emerging pathogens. Amino acid residues were defined as conserved if they were found in at least seven of the 15 flaviviruses in our panel. Of the 43 discovered PTMs, 14 met this degree of conservation across the *Flavivirus* genus and five were conserved across nearly all flavivirus subfamilies, a result that suggests they may be fundamentally important for many species of flaviviruses (Table 1). Notably, four sites were found only in ZIKV isolates (Table 1).

Tyrosine 61 and threonine 351 on the ZIKV E protein are critical for viral growth

We sought to determine whether the PTMs identified were important for viral propagation. We focused on two sites, Y61 and T351 on ZIKV E, because they were found in both infection and plasmid expression settings. Y61 was discovered from infected cells while T351 was found in virions and on plasmid-expressed ZIKV E. Further, these sites represented residues that are either highly conserved in flaviviruses (Y61) or unique to ZIKV (T351) (Table 1; Supplementary Information 3). To evaluate the importance of these sites for viral propagation, we used reverse genetics (19) to recover infectious ZIKV clones encoding mutations at these sites that would

either block or mimic phosphorylation (Fig 3A). We then used these infectious clones in multi-step growth curves to determine how each change impacted viral fitness.

ZIKV E Y61 was mutated to a negatively-charged glutamic acid (Y61E) to reflect a permanent state of phosphorylation (i.e. a phosphomimetic) and to a phenylalanine (Y61F) to represent a non-phosphorylatable state. We recovered the non-phosphorylatable infectious clone Y61F but, interestingly, the phosphomimetic clone Y61E appeared to be nonviable as assessed by plaque assay (Fig 3B). Recovery of a Y61E clone was attempted three times without success, despite successful recovery of a WT control clone each time. To account for the possibility that this virus could be propagating but unable to form plaques (and therefore undetectable by plaque assay), we screened for the presence of infectious virus by focus assay (Fig 3C). In parallel, to account for the possibility that the virus was replicating but not with sufficient kinetics to be detectable by focus or plaque assay after the initial recovery passage, we blind-passaged Y61E three times. This approach led to the successful isolation of an infectious clone capable of forming foci beginning at passage 3 (Fig 3C). However, sequencing of this infectious clone at passage 3 revealed that the glutamic acid (Y61E) had mutated to a valine (Y61V), suggesting that there is selection pressure to avoid a permanent negative charge at position 61.

To address whether phosphorylation of Y61 influences viral fitness, we performed a multi-step growth curve comparing the phosphomutant clones Y61F and Y61V to a wild-type clone. Interestingly, both the Y61F and Y61V infectious clones grew with accelerated kinetics and produced higher quantities of infectious virus compared to the wild-type clone (Fig 3D; Y61F $p = 0.006$).

ZIKV E T351 was mutated to an aspartic acid (T351D) to mimic a state of phosphorylation or an alanine (T351A) to act as a non-phosphorylatable mutant. Infectious

clones of each mutant were successfully recovered (Fig 3B) and multi-step growth curves were performed. There was no difference between the growth of the phosphomimetic T351D mutant and the wild-type clone (Fig 3E; $p = 0.964$). Notably, the growth of the T351A non-phosphorylatable mutant was impaired compared to WT virus or the T351D mutant (Fig 3E; $p = 0.0009$). After 72 hours, the titers for the T351D and the wild-type infectious clones decreased more rapidly compared to T351A. This could be due to the fact that the wild-type and T351D clones caused more cytopathic effect at this time point and therefore the cells could not support further rounds of infection.

In summary, both the Y61 and T351 sites appear important for viral propagation. Our results suggest that blocking phosphorylation at Y61 enhances viral growth while blocking phosphorylation at T351A impairs it.

Y61 and T351 on ZIKV E are required for efficient Zika virus budding

To determine the life cycle stage impacted by ZIKV E Y61 or T351, we next tested how a phosphomimetic or non-phosphorylatable mutation at each site would impact viral budding. To do so, we used a virus-like particle (VLP) assay that features a single plasmid that expresses ZIKV prM and E. Once expressed, these two proteins form ZIKV VLPs that bud from cells and can be detected in supernatant (20). The plasmid was mutated at ZIKV E Y61 to Y61E or Y61F and at T351 to T351A or T351D. Compared to the wild-type control, VLP budding efficiency was decreased when T351 was mutated to alanine (T351A) but not aspartic acid (T351D) (Fig 3F; $p = 0.04$). Mutation of Y61 to F also reduced budding efficiency (Fig 3F, $p = 0.006$). Interestingly, we were unable to express ZIKV E protein when Y61 was mutated to Y61E despite performing two bacterial transformations and evaluating several clones from each

transformation. This result, combined with our inability to isolate genetically stable Y61E infectious clones (Fig 3C), suggests that constitutive phosphorylation at Y61 is not favorable either for protein expression or viral growth. Taken together, our findings suggest that both the Y61 and T351 phosphosites are required for efficient ZIKV budding.

Host protein kinases interact with the Zika virus proteome

We next wished to determine the host kinases that associate with ZIKV proteins. The immunoprecipitation and affinity purification performed in Figs 1C, 1D, 1E, S2, S3, and S4 not only captured ZIKV proteins, but also their host cellular protein partners. Thus, we were able to identify host kinases that interacted with ZIKV C, M, E, NS1, NS2B, NS4A, and NS4B. We used Scansite 4.0 software to predict kinases that might phosphorylate ZIKV proteins at the sites of phosphorylation identified in this study (Table 1). Intriguingly, some of the predicted kinases were interacting partners of ZIKV proteins (Table 1, highlighted in purple). Other predicted kinases, although not found by mass spectrometry to be interacting partners, either have shared homology with or are found in the same cascades as interacting kinases (Table 1, highlighted in blue). In total, we found 115 cellular kinases that interacted with ZIKV proteins (Table S2). Figure 4A shows the top 10 most abundant kinases, determined by number of tryptic peptides detected from a given viral protein during mass spectrometry. Most interacting kinases were found to interact with two or more viral proteins. Five kinases interacted with 4 ZIKV proteins found to have PTMs. These kinases were AURKB, CAMK2B, CDK2, PLK1, UCK2 (Aurora Kinase B, Calcium/Calmodulin Dependent Protein Kinase II Beta, Cyclin-dependent kinase 2, polo-like kinase 1, Uridine-Cytidine Kinase 2, respectively; Fig 4A, black circles). AURKB was

predicted by Scansite 4.0 to interact with ZIKV M and E, CAMK2B was predicted to interact with M, and PLK1 was predicted to interact with E, NS1, NS4B, and NS5 (Table 1, Table S2).

Inhibition of host cell kinases by Bosutinib impairs Zika virus growth

Considering that there are no FDA approved treatments for ZIKV, we next tested whether selected FDA-approved kinase inhibitors might be effective in restricting ZIKV growth. We focused on a panel of tyrosine and serine/threonine inhibitors to see if they could impact ZIKV growth. At 10 μ M, the tyrosine kinase inhibitors Bosutinib and Defactinib both reduced ZIKV titers (Fig 4B; $p = 0.03$, $p = 0.02$, respectively) while Sunitinib and Imatinib did not (Fig 4B; $p = 0.17$, $p = 0.35$, respectively). To determine the IC_{50} of each drug, we constructed concentration-response curves. To account for drug cytotoxicity, we performed simultaneous MTT assays on cells that had been treated with each kinase inhibitor at the equivalent concentrations. Although Defactinib inhibition of ZIKV infectious virus production reached levels as high as 86%, at the same concentration of drug, cell toxicity measured 73% (Fig 4C). At Defactinib's IC_{50} of 4.5 μ M, cell toxicity was approximately 46%. The fitted curve of inhibition and cell toxicity closely followed each other, suggesting that most of the viral inhibition may have been due to nonviable cells. After determining the CC_{50} of Defactinib to be 7.53 μ M, we calculated the selectivity index for Defactinib to be 1.7. The IC_{50} of Bosutinib was 5.5 μ M. In this case, at the same doses of drug, cell toxicity was approximately 17%. At higher concentrations, Bosutinib completely inhibited viral growth while cell toxicity remained at approximately 30% (Fig 4D). Given Bosutinib's IC_{50} and CC_{50} (17.43 μ M), we calculated the selectivity index to be 3.14. These results suggest that Bosutinib impairs ZIKV growth and that host cell kinases may be tractable antivirals for ZIKV.

Discussion

Little is known regarding the diversity of host-driven PTMs that occur on flavivirus proteins or how such modifications regulate the functionality of these proteins. We addressed this deficiency by providing the first comprehensive map of phosphorylation and ubiquitination sites found on a flavivirus proteome. A major finding was that selected phosphorylation sites on the ZIKV E protein appear critical for ZIKV propagation, likely at the stage of viral assembly and release. We also identified a network of host kinases that interact with ZIKV proteins, with many of these kinases predicted to target the newly identified phosphosites. Notably, our data suggest that inhibition of host kinases may be an effective approach to restrict ZIKV growth.

Flavivirus budding occurs at the endoplasmic reticulum and is largely driven by prM and E. This pair of structural proteins together induce curvature in the ER membrane leading to the formation of spherical budded virions that contain the encapsidated viral genome (21). We show that the ZIKV envelope glycoprotein (ZIKV E) has at least two phosphosites, Y61 and T351, which are involved in the budding stage (Fig 3F). Intriguingly, while mutation of these sites to a non-phosphorylatable residue blocks efficient budding in a VLP assay (Fig 3F), infectious clones of ZIKV encoding these same mutations have opposing effects – Y61F leads to enhanced infectious virus production while T351A does the opposite (Figs 3D-E). To our knowledge, phosphorylation of a flavivirus protein has not been implicated in the budding process. Thus, our data suggests that phosphorylation of E may play a regulatory role in ZIKV budding. Moreover, considering the conservation of Y61 in other pathogenic flaviviruses (Table 1, Supplementary Information 3), this PTM could be fundamentally important for flavivirus budding. Additionally, two ubiquitination sites on ZIKV E were recently shown to be required for viral entry and

replication (22). Notably, one of these ubiquitination sites, K281, was detected in the present study (Table 1, Table S1).

Phosphosite Y61 is found in domain II of ZIKV E, the domain responsible for ZIKV E dimerization (16). The inability of the phosphomimetic Y61E infectious clone to efficiently grow over the first two passages following reverse genetics rescue coupled with its conversion at this residue from a glutamic acid to valine by passage 3 (Fig 3C, 3D) suggest that the negatively charged amino acid is not favorable. Indeed, there may be pressure at position 61 to be either a non-charged or hydrophobic amino acid. A zoomed-in view of the ZIKV model reveals that Y61 is near an asparagine (N207) and glutamic acid (E262) (Fig S6B). In the absence of phosphorylation, it is possible that the N207 and E262 residues are interacting. A large phosphate group on Y61 would possibly disrupt this interaction and perhaps repel the negative charge on E262, providing a possible explanation for why a non-charged state is optimal at this position. This preference is further supported by the fact that we readily rescued the nonphosphorylatable mutant, Y61F, which has a hydrophobic, non-charged phenylalanine at position 61.

The hydrophobic amino acid changes (Y to F or V) at position 61 resulted in ZIKV infectious clones that outperformed wild type ZIKV in reaching peak titers (Fig 3D). We interpret this finding in several ways. First, phosphorylation of ZIKV E at Y61 is either not favorable to the virus or is beneficial at very specific time point(s) in the ZIKV life cycle. If the latter scenario is at work, our growth curve data may not be sufficient to resolve this idea because the infectious clones employed contained static mutations at this position. The negatively-charged phosphosite may be important in aiding a specific purpose, such as encouraging a conformational change for a period and then returning to an unphosphorylated

state. When mutated to Y61E, ZIKV E no longer expressed from a plasmid, suggesting that the ZIKV envelope protein containing a negatively-charged glutamic acid at this position is too unstable for expression or may be quickly marked for degradation. Interestingly, the Y61 site is highly conserved among flaviviruses. A multiple sequence alignment (Supplementary Information 3) shows that flaviviruses such as WNV, YFV, Japanese encephalitis virus (JEV), and Saint Louis encephalitis virus (SLEV) also contain a tyrosine at this position, suggesting that maintaining control over the charge of this tyrosine likely benefits these flaviviruses. DENV contains an isoleucine at this position and more distantly related flaviviruses contain a leucine, both of which share properties with tyrosine and phenylalanine in that they are also non-charged, hydrophobic amino acids.

Another potential interpretation of Y61F and Y61E/Y61V mutations resulting in higher peak infectious titers is the possibility that the phosphorylation of Y61 drives the production of defective interfering particles (DIPs), but not infectious particles. Under this scenario, blocking phosphorylation at Y61 would dampen the output of DIPs. The decrease in DIPs would result in a greater number of permissive cells available to the infectious particles produced (i.e. more replication space in the cell culture dish for infectious virions would equate to a greater infectious virus output). There is a precedent for this hypothesis in our previous studies of the mammarenavirus lymphocytic choriomeningitis virus (LCMV) (6, 23). In the case of LCMV, phosphorylation of Y88 on the viral matrix protein - the protein that drives viral budding - uniquely promotes DIP production. Y88 sits within the PPXY late domain on the LCMV matrix protein. Mutation of Y88 to a nonphosphorylatable amino acid (i.e. Y88F) blocked the production of DIPs and led to increased kinetics of infectious virus production. Notably, similar to Y61, mutation of Y88 to a nonphosphorylatable amino acid resulted in impaired VLP

production (Fig 3F). This could suggest that the impairment in budding observed in both LCMV Y88F and ZIKV Y61F is reflective of DI particle budding, but not infectious particles.

Finally, the enhanced infectious virus production observed following inhibition of Y61 phosphorylation could also be due to the blockage of conformational changes of ZIKV E necessary for virus particle maturation. Flavivirus virion populations are composed of a mixture of mature and immature virus particles. For ZIKV particles to mature, the host protease furin has to cleave prM in the trans-Golgi network. This cleavage is contingent on a ZIKV E conformational change that is triggered by the acidic environment of the trans-Golgi network, exposing the cleavage site on prM (24). It is possible that phosphorylation of Y61 or the presence of a negatively-charged amino acid at position 61 would allosterically block this conformational change and shift the population of mature particles to immature particles. Conversely, a lack of a negative charge at this site (i.e. Y61F or Y61V in Fig 3) may promote the necessary conformational changes to occur at a higher rate, shifting the population of viral particles to mature, infectious particles.

Phosphorylation of ZIKV E at position T351 was observed both in purified virions and in cells. T351 sits within Domain III of ZIKV E, which contains motifs for cellular receptor binding and is often the target of neutralizing antibodies (16). A negative charge at this position, either from a phosphorylated threonine or an aspartic acid substitution, was optimal for growth (Fig 3E). Similarly, a negatively charged aspartic acid in the budding assay resulted in the maintenance of virus-like particle budding. Conversely, mutation to an alanine, the nonphosphorylatable mutant, resulted in a strong reduction in virus-like particle budding (Fig 3F), suggesting that host-driven phosphorylation of T351 promotes ZIKV budding. Intriguingly, phosphorylation of T351 was discovered both on ZIKV E isolated from human cells as well on

virions derived from mosquito cells, suggesting that not only is phosphorylation of T351 important in the host, but that it may also play a role in the virus vector. Future studies could focus on confirming the functional importance of T351 and other discovered phosphosites, such as Y61, in mosquito cells, as well as evaluating whether phosphomutant viruses from mosquito cells impact entry or infectivity in human cells. Because there may be differences in human and arthropod kinomes, it is possible that the ZIKV phosphoproteome in vector cells would diverge from our dataset in mammalian cells. Additionally, it may be that optimal viral fitness requires different patterns of regulatory phosphorylation in the cells of mammalian and arthropod hosts. Therefore, it would be informational to map the phosphoproteome of ZIKV in mosquito cells for comparison with this dataset as well as for comparison of the roles of phosphorylation in both cell types.

Previous studies have shown that host-driven phosphorylation of the flavivirus NS5 protein is critical for the flavivirus viral life cycle. For example, phosphorylation of YFV NS5 at a highly conserved site (S56) is required for YFV to evade the cap-dependent innate immune response in the host cell (9). Blocking the phosphorylation of another conserved flavivirus residue, T449 on DENV NS5, results in a nonfunctional protein (25). Other key studies linked phosphorylation of NS5 or Hepatitis C virus NS5A to important functions such as its interaction with NS3 (10, 26), localization during different stages of infection (26), and viral particle assembly and viral replication (11, 27). In some instances, phosphorylation of NS5 was detected but not the specific residues driving the observed phenotype (10, 26). The highly conserved NS5 phosphosites identified in our study may be responsible for driving these important functions (Table 1). Indeed, we have uncovered several highly conserved phosphosites in the ZIKV

proteome that may help guide the discovery of new functions for these viral proteins or the mechanisms by which these functions are regulated.

Our mass spectrometry analysis revealed 115 host kinases (Table S2) that interacted with one or more of the 8 ZIKV proteins probed, which raises the possibility that some subset of these interacting kinases may be responsible for phosphorylation of these viral proteins. Treating cells after infection with Bosutinib, an FDA-approved tyrosine kinase inhibitor, inhibited ZIKV growth (Fig 4B-C). Indeed, a recent study by Valencia *et al.* confirmed our findings in the setting of ZIKV infection in BHK-21 cells (28). One possibility is that Bosutinib is acting on CDK2, a kinase found to interact with 5 ZIKV proteins (4 of which were phosphoproteins) (Fig 4A). Scansite 4.0 did not predict that CDK2 would phosphorylate any of the ZIKV phosphoproteins, however, host kinases can phosphorylate viral proteins at non-canonical motifs (29). Thus, although not predicted, it is possible that CDK2 phosphorylates ZIKV proteins. It is also important to consider that the concentration at which kinase inhibitors are administered will influence their specificity; further studies are needed to identify the exact mechanism by which Bosutinib decreases viral titers. Additional studies using molecules related to Bosutinib may improve efficacy and specificity for blocking specific kinases. Collectively, these data imply that blocking phosphorylation by host kinases is a potential antiviral strategy.

Several interacting kinases (ATM, ATR, and mTOR) discovered in our study were the targets of early drug screens against ZIKV. Specifically, Cherry *et al.* used inhibitors VE-822 and WAY-600 to robustly block ZIKV infection (30). Intriguingly, VE-822 targets ATM and ATR, which we found to be interacting partners of M (ATM, ATR) or E (ATM) (note that ATM is predicted to phosphorylate S16 on ZIKV M; Table 1). WAY-600 targets mTOR, which we found to be an interacting partner of phosphoproteins M and E as well as NS4A (Fig 4A).

Interestingly, mTOR was the most abundant kinase found to interact with any of the ZIKV proteins (Table S2). Its importance in ZIKV was found early on when it was discovered that ZIKV proteins can suppress the Akt-mTOR pathway, leading to impairment of neurogenesis and upregulation of autophagy, which is beneficial for ZIKV during replication (31, 32). Another study found an overall downregulation of the AKT/mTOR pathway during ZIKV infection (33). Due to ZIKV M associating with high levels of mTOR (see peptide counts in Table S2), we theorize that ZIKV M could be acting as a sink for mTOR, providing an explanation for why the pathway is downregulated and there is less phosphorylation of mTOR targets observed during ZIKV infection (33).

In summary, we have detailed a high-resolution map of host-driven PTMs on ZIKV proteins, uncovered a novel mechanism by which ZIKV budding may be regulated, and identified an FDA-approved drug that could be repurposed for the treatment of ZIKV infection. The dataset described here will be useful to the field for the discovery of novel ZIKV protein functions and the mechanisms by which these functions are regulated. Our work further suggests that targeting host kinases may be an effective antiviral strategy and provides potential targets.

Materials and Methods

Cells and viruses

HEK 293T/17 cells (CRL-11268), referred to as HEK293T throughout, and A549 cells (CCL-185) were procured from the American Type Culture Collection (ATCC, Manassas, VA). Vero E6 cells were kindly given by J. L. Whitton (The Scripps Research Institute, La Jolla, CA). HEK293T cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 1% HEPES buffer solution, 1%

MEM nonessential amino acid solution, and 1% GlutaMAX, from Thermo Fisher Scientific (Waltham, MA). A549 cells were maintained in DMEM-F12 (Thermo Fisher) containing 10% FBS and 1% penicillin-streptomycin. Vero E6 cells were maintained in DMEM containing 10% FBS, 1% penicillin-streptomycin, and 1% HEPES buffer solution. Cell lines described above were incubated at 37°C with 5% CO₂. *Aedes albopictus* mosquito cells (C6/36; a kind gift of Prof Alain Kohl, MRC-University of Glasgow Centre for Virus Research) were maintained in Leibovitz's L15 media (Gibco) containing 10% FBS (Gibco), 10% triptose phosphate broth (Gibco), and 1% pen/strep (Gibco) and incubated at 28°C. ZIKV strain BeH819015, a Brazilian isolate (GenBank: KU365778.1), was generously provided by the Baric Lab (University of North Carolina, Chapel Hill, North Carolina, USA) (19). The Zika virus strain used to infect mosquito cells was ZIKV/H.sapiens/Brazil/PE243/2015 (Recife, Brazil) (34). Working stocks of infectious ZIKV, including recombinant ZIKV clones generated via reverse genetics (see below for details on recovery), were grown on Vero E6 cells and titered by standard plaque assay.

Plasmids

Several plasmids were used for expression of ZIKV proteins. The nucleotide sequences of ZIKV ORFs used in this study were derived from ZIKV strain H/PF/2013 (NCBI gene identifier number AHZ13508). The Heise lab (University of North Carolina, Chapel Hill, North Carolina, USA) provided their pOME plasmid-based library that permits expression of each ZIKV ORF containing a C-terminal 3XFLAG tag (35). To enable streptavidin-based affinity purification of ZIKV proteins, the ZIKV C, E, M, NS1, NS2B, and NS4A ORFs encoded in the pOME plasmids were subcloned into our modified pCAGGS expression vector (36, 37) such that they would encode a C-terminal streptavidin binding peptide (SBP) (MDEKTTGWRGGHVVEGLAGELEQLRARLEHHPQGQREP) in place of the 3XFLAG tag.

Each ZIKV ORF was fused to the SBP tag through an 18 base pair linker. The nucleotide sequence of the ZIKV genes was amplified by PCR from the pOME vectors with forward primers that contained a 5' overhang containing a Gateway AttB1 site and a Kozak sequence and reverse primers containing an overhang with the linker sequence (all primers used are listed in Supplementary Information 4). The SBP tag was amplified from a previously generated in-house plasmid via PCR using the forward primer SBP (5'-GCAGCTGGAGGTGGAGGTATGGACGAAAAAACCACCGGT-3'), which has a 5' overhang containing the linker sequence, and the reverse primer SBP (5'-ACCACTTTGTACAAGAAAGCTGGGTCTTACGGTTCACGCTGACCCTGCGG-3'), which contains a 3' overhang with a stop codon preceding an AttB2 sequence. The two PCR products (each respective amplified ZIKV gene and the amplified SBP tag) were fused by PCR using the appropriate ZIKV gene forward primer and the SBP reverse primer. The resulting cassette was subcloned into the modified pCAGGS vector using the Gateway cloning system (Invitrogen) following the manufacturer's instructions. pOME plasmids encoding ZIKV NS2A, NS3, NS4B, or NS5, each with the same C-terminal linker and SBP tag as described directly above, were generated by GenScript Biotech Corporation (Piscataway, NJ, USA) but were maintained in the original pOME vector. Plasmids for the ZIKV reverse genetics system were kindly provide by the Baric Lab as described in Widman *et al.* (19). The plasmid from the Baric system which contained the ZIKV E gene was modified by GenScript to include the Y61F, Y61E, T351A, and T351D mutations. The plasmid used for the virus-like-particle budding assay, which encodes the ZIKV prM and E genes, is described here (20) and was generously provided by Emergent BioSolutions, Inc. This plasmid was also modified by GenScript to include the include the Y61F, Y61E, T351A, and T351D mutations. All plasmid sequences were verified by DNA sequencing.

Identification of post-translational modifications of ZIKV proteins by mass spectrometry

To identify phosphorylation sites on ZIKV proteins via mass spectrometry, three general approaches were taken. In the first approach, to purify ZIKV E, Vero E6 cells were infected with ZIKV at an MOI of 0.1 and 72 hours later cells were collected, and lysed in Triton buffer consisting of 1% Triton X-100, 0.5% NP40, 140mM NaCl, and 25mM Tris-HCl containing a protease inhibitor cocktail (04693159001, Roche Applied Science, Indianapolis, IN) as well as PhosStop phosphatase inhibitor cocktail (04906837001, Roche Applied Science). The resulting protein lysate was incubated with anti-ZIKV E 4G2 antibody at 4°C, overnight. The next day, magnetic Protein G Dynabeads (ThermoFisher Scientific, Waltham, MA) were added to the lysate-antibody mixture and incubated at 4°C, rotating for at least 4 hours (but not more than 8 hr). Beads were then magnetically isolated, gently washed 3 times in Triton buffer, magnetically isolated for a final time and then boiled in Laemmli buffer (62.5 mM Tris-HCl, 10% glycerol, 2% sodium dodecyl sulfate and 0.01% bromophenol blue (B392, Fisher Scientific, Pittsburgh, PA) for 5 minutes to release antibody, ZIKV E protein, and associated host or viral protein partners. Similarly, ZIKV virions were immunopurified from infected Vero E6 cell media 96 hours post-infection using Dynabeads coated with anti-ZIKV E 4G2 antibody then lysed using 10X Triton buffer containing the same protease and phosphatase inhibitor cocktails described directly above.

To purify ZIKV proteins expressed from plasmid, our second approach was to transfect HEK293T cells with modified pCAGGS plasmid expressing ZIKV C, M, E, NS1, NS2B, NS4A, NS4B, or NS5 proteins with a C-terminal SBP tag in each case. 5×10^5 cells per well in 6-well plates were transfected with 2 μ g of plasmid using 8 μ g of polyethylenimine (23966, Polysciences, Inc., Warrington, PA), which was reconstituted at 1 mg/mL. Two days later,

transfected cells were treated either with DMSO (D2650, Sigma-Aldrich, Saint Louis, MO), H₂O₂, (Thermo Fisher) or Calyculin A (Cell Signaling Technology, Danver, MA) for 20 minutes. Cells were collected and lysed with Triton buffer (same formulation described above) and then subjected to affinity purification using magnetic MyOne Streptavidin T1 beads (65601, Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, magnetic beads were washed in buffer several times, added to protein lysates from transfected cells, and then rotated overnight at 4°C. The next day, the beads were magnetically isolated and washed several times with lysis buffer containing protease and phosphatase inhibitors and then boiled in Laemmli buffer for 5 minutes to release SBP-tagged ZIKV proteins from the streptavidin beads.

Infected cell lysates, transfected cell lysates, and virion protein lysates were separated on 4-20% Tris-Glycine polyacrylamide gels (EC60255, Invitrogen). To visualize protein bands for excising and mass spectrometry processing, gels were stained with Coomassie (40% methanol, 20% acetic acid, and 0.1% Brilliant Blue R (B7920, Sigma-Aldrich)). Gels were destained with a solution of 30% methanol and 10% acetic acid and imaged using a Canon Canoscan 8800F scanner. Sample lanes were cut into a series of gel pieces containing prominent protein bands or band groups. After excision, gel pieces were further cut into 1 mm cubes and processed. Chemicals used for processing were purchased from Thermo Fisher Scientific. Gel pieces were rinsed with HPLC-grade water and then incubated with destain solution (50 mM ammonium bicarbonate and 50% acetonitrile) for 30 minutes at 37°C. Destain was removed and gel pieces were dehydrated by incubating twice with 100% acetonitrile for 5 minutes. The gel pieces were reduced with 25 mM dithiothreitol in 50 mM ammonium bicarbonate for 30 minutes at 55°C. After cooling, gel pieces were dehydrated with 100% acetonitrile for 5 minutes and then alkylated with 10 mM iodoacetamide in 50 mM ammonium biocarbonate for 45 minutes at room

temperature, while protected from light. Gel pieces were washed twice in destain solution for 5 minutes, dehydrated with 100% acetonitrile, then rehydrated with water for 10 minutes. Gel pieces were further dehydrated with two 5-minute incubations in 100% acetonitrile. All liquid was removed and gel pieces were left to incubate at room temperature to evaporate trace acetonitrile. Gel pieces were rehydrated with a solution of 12.5 ng/ μ L sequencing-grade, modified trypsin (V5111, Promega) in 50 mM ammonium bicarbonate on ice for 30 minutes, before digesting overnight at 37°C. Peptides were extracted with a solution of 2.5% formic acid in 50% acetonitrile while spinning in a microcentrifuge at 13,000 rpm for 10 minutes. The supernatant was removed and saved while the gel pieces were subjected to further extraction and rinsing with 100% acetonitrile. The second extraction was combined with the initial extraction. All solvent was removed from the extracts using a vacuum centrifuge at 37°C. The peptides were resuspended in 2.5% formic acid, 2.5% acetonitrile prior to mass spectrometry analysis. Dried peptides were resuspended in Solvent A (2.5% MeCN, 0.15% formic acid (FA)) and separated using the Easy n-LC 1200 across 15-cm columns packed in-house with 2.7 μ m C18 packing material prior to analysis on the Q Exactive Plus mass spectrometer fitted with a Nanospray Flex ion source (2.2 kV) and supplied with Thermo Xcalibur 4.0 software. Peptides were eluted using a 0 - 50% gradient of Solvent B (80% MeCN, 0.15% FA) over 60 min, followed by 10 minutes at 95% Solvent B. The precursor scan (scan range = 360–1700 m/z , resolution = 7.0×10^4 , maximum IT = 100 ms) was followed by HCD fragmentation spectra in a Top-10 approach (resolution = 3.5×10^4 , AGC = 5.0×10^4 , maximum IT = 50 ms, isolation window = 61.6 m/z , normalized collision energy = 26%, dynamic exclusion = 30 s). Raw spectra were searched against a forward and reverse database of tagged ZIKV proteins with common contaminants added using SEQUEST with a precursor mass tolerance of ± 5 PPM and a

fragment ion tolerance of ± 0.006 Da. The following differential modifications were permitted: phosphorylation of serine, threonine and tyrosine (± 79.9663 Da), ubiquitylation of lysine (± 114.0429 Da), oxidation of methionine (± 15.9949 Da), carboxyamidomethylation of cysteine (± 57.0215 Da), and acrylamidation of cysteine (± 71.0371 Da). No enzyme was indicated in the original search to expand the database, and the resulting hits were filtered for tryptic peptides. Spectra of phosphopeptide hits were manually assessed and compared to their unphosphorylated counterparts to confirm hits and determine phosphorylation site localization. To identify bound human interactors, raw data were searched against a forward and reverse human database containing common contaminants via SEQUEST. Trypsin was specified as the cleavage agent with two missed cleavages permitted. Mass tolerances and allowed modifications were as stated above. Host proteins were considered ZIKV interactors if they were identified by two or more unique peptides in experimental conditions and were not present in the controls. Proteins were also considered ZIKV interactors in cases where proteins were >5 -fold enriched in experimental over control conditions.

In our third approach, which led to the identification of ZIKV E T351 from virions, C6/36 cells were infected with ZIKV at an MOI of 0.1 and then incubated at 28°C. Four days later, the supernatant was harvested and clarified by centrifugation at 5000 rpm. The clarified supernatant was concentrated using 100,000 MW cut-off Centricon concentrators (Millipore). The concentrate was underlaid with a 5 mL sucrose cushion (24% w/v in 1x NTE buffer; NaCl-Tris-EDTA) and ultracentrifuged at 105,000 g in a Surespin 630 Rotor (Thermo Scientific) for 1.5 hours. The pellet-associated virus was resuspended in NTE, then overlaid onto a 10-35% (w/v) / density gradient of potassium tartrate in 30% glycerol in NTE, prepared using a Gradient Master (Biocomp Instruments). Virions were banded by ultracentrifugation at 175,000 g

overnight and collected by pipetting from the top of the gradient. Next, the virus-containing fraction was removed from the gradient, placed in 100,000 MW cut-off dialysis units, and dialyzed several times using 1× NTE to remove the potassium tartrate and glycerol. After dialyzing overnight, virus was concentrated using 100,000 MW cut-off Centricon concentrators (Millipore).

Concentrated ZIKV virions were inactivated at 70°C in 4M final urea in TEAB and then prepared for liquid chromatography and tandem mass spectrometry (LC-MS/MS) as described (38). Briefly, the sample was reduced, alkylated, and then digested with trypsin and LysC using filter-assisted sample preparation (FASP) (39). Peptides were separated by reversed-phase chromatography using a 2h gradient on an Ultimate 3000 RSLCnano HPLC system (Dionex). This was run in direct injection mode and coupled to a Q Exactive mass spectrometer (Thermo), running in ‘Top 10’ data-dependent acquisition mode with fragmentation by higher-energy collisional dissociation (HCD). Charge state + 1 ions were rejected and fragmentation and dynamic exclusion with 40 s was enabled. Mass spectra were annotated in MaxQuant version 1.6.3.4 (40) with reference proteomes from Zika virus isolate ZIKV/H.sapiens/Brazil/PE243/2015 (GenBank KX197192.1) and *Aedes aegypti* (UniProt UP000008820, accessed on 2018-10-26) and the MaxQuant common contaminants list. Standard settings were used, with the enzyme set as trypsin/P and fixed modifications set as carbamidomethyl (C). Variable modifications were oxidation (M), acetyl (Protein N-ter), phospho (STY) and, to account for artefactual modifications when inactivating in urea, carbamyl (KRC). Modified spectra were considered where they could be identified in the absence of artefactual carbamylation. Data from this third approach can be found at <https://researchdata.gla.ac.uk/1222/>.

Multiple sequence alignment

A multiple sequence alignment (MSA) of flavivirus FASTA-formatted sequences was generated using the MUSCLE (Multiple Sequence Comparison by Log-Expectation) algorithm (41) as implemented in the NIAID Virus Pathogen Database and Analysis Resource (ViPR) through the web site at <http://www.viprbrc.org/> (42). The Uclust parameter was set as a MUSCLE pre-processor to improve both speed and quality of alignment. The alignment visualization with PTM annotations was created in Jalview 2.11.1.4 (43). The following flaviviruses were aligned: ZIKV H/PF/2013 (AHZ13508), ZIKV Uganda (ABI54475), ZIKV SPH2015 (ALU33341), ZIKV Paraiba (ANH10698), ZIKV USA UT (AOO19564), ZIKV BeH819015 (AMA12085), DENV 1 NP059433), DENV 2 (NP056776), DENV 3 (YP001621843), DENV 4 (NP073286), JEV (Japanese encephalitis virus; NP059434), SLEV (Saint Louis Encephalitis Virus; YP001008348), USUV (Usutu virus; AAS59401), WNV 1(YP001527877), WNV 2 (NP041724), AHFV (Alkhurma hemorrhagic fever virus; AAL08421), KFDV (Kyasanur forest disease virus, AAQ91607), OHFV (Omsk hemorrhagic fever virus, AAQ91606), TBEV (tick-borne encephalitis virus; NP043135), ENTV (Entebbe bat virus; AAV34153), MMLV (Montana myotis leukoencephalitis virus, CAC82713), LAMV (Lammi virus, ACR56717), YFV (NP041726), and PCV (Palm Creek virus, AGG76014).

SDS-PAGE and western blotting

Protein lysates were diluted in Laemmli buffer, boiled for 5 minutes, then separated on NuPAGE 4–12% Bis-Tris gels with MES buffer. Protein was transferred to nitrocellulose membranes using iBlot gel transfer stacks (IB301001 or IB301002, Invitrogen) and the Invitrogen iBlot Device according to manufacturer instructions. Membranes were blocked with 5% milk in PBS for 1 hour and incubated overnight with primary antibody diluted in PBS which contained 5%

milk and 0.2% Tween 20 (BP337, Fisher Scientific). The following day the membrane was rinsed several times with TBST (tris-buffered saline with 0.1% Tween 20) then incubated for 1 hour with secondary antibody diluted in PBS containing 5% milk, 0.2% Tween 20, and 0.02% SDS. After secondary antibody incubation, the membrane was washed several times in TBST then washed in PBS before imaging on a LI-COR Odyssey CLx system.

Primary antibodies were used for western blotting at the following concentrations: mouse anti-ZIKV E (4G2) (1:10,000) (generously provided by Baric Lab), mouse anti-streptavidin binding peptide (MAB10764, Millipore, Billerica, MA) (1:10,000), rabbit anti-actin (A2066, Sigma-Aldrich) (1:5,000), and mouse anti-actin (A5441, Sigma-Aldrich) (1:5,000). For quantitative western blotting, the following secondary antibodies from LI-COR were used at a 1:20,000 dilution: IRDye 800CW goat anti-mouse (926–32210) and IRDye 680LT goat anti-rabbit (926–68021).

Generation of mutant ZIKV clones

We used the Baric lab reverse genetics system to generate phosphomimetic and non-phosphorylatable mutants as described in Widman *et al.* (19). ZIKV E was mutated at Y61 to Y61E and Y61F and separately ZIKV E T351 was mutated to T351D and T351A. The Baric reverse genetics system is composed of 4 plasmids, Fragments A through D, that encode the ZIKV proteome. Fragment A was modified by GenScript from thymine to guanine at nucleotide position 1599 and thymine to adenine at position 1601 to generate the Y61E phosphomimetic while nucleotide 1600 was modified from adenine to thymine for the Y61F non-phosphorylatable mutant. Nucleotides 2470 and 2471 in Fragment A were modified from adenine and cytosine to guanine and adenine, respectively, for the T351D phosphomimetic while position 2470 was modified from adenine to guanine for the T351A non-phosphorylatable

mutant. Infectious recombinant ZIKV clones were generated as described in Widman *et al.* (19). Four plasmids containing Fragments A-D, respectively, which encode for the ZIKV genome, were digested with BamHI and Bsu36I for Fragment A, Bsu36I and BstXI for Fragment B, BstXI and SfiI for Fragment C, and SfiI, SmaI, AlwNI for Fragment D. Restriction enzymes were all acquired from New England Biolabs (Ipswich, MA). The four digested fragments were ligated with T4 DNA ligase purchased from New England Biolabs. Ligated DNA was precipitated with chloroform then *in vitro* transcribed using the mMESSAGE mMACHINE™ T7 ULTRA Transcription Kit from ThermoFisher Scientific. The transcribed product was then electroporated into Vero E6 cells using a Gene Pulser Xcell (Bio-Rad Laboratories, Hercules, CA) and incubated at 37°C for 4 days. Cell media was collected after 4 days and passaged onto a new monolayer of Vero E6 cells. Cell media collected from this passage was described as “passage 1/ p.1”. Clarified cell media was titered by plaque assay using Vero E6 cells.

Virus growth curves

For the Y61F and Y61E mutants, six-well plates were seeded with 2×10^5 Vero E6 cells per well and infected the following day at an MOI of 0.001. For the T351A and T351D mutants, 12-well plates were seeded with 2×10^4 A549 cells and infected the following day at an MOI of 0.01. Cell media was collected at indicated time points from separate wells. Cell media was clarified by centrifugation at 1200 RPM for 5 minutes, stored at -80°C, and then titered by plaque assay (see plaque and focus assays below for details).

Plaque and focus assays

Plaque assays were used to measure infectious virus titers in several experiments as follows. Six-well plates were seeded with 2.5×10^5 Vero E6 cells per well and the following day inoculated with 10-fold serial dilutions of virus. Plates were rocked every 15 minutes for 1 hour to allow for

viral absorption at 37°C, the cells were overlaid with a solution of 0.7% agarose (20–102, Apex Industrial Chemicals, Aberdeen, United Kingdom) in Vero E6 growth media. Wells with agarose plugs were fixed 3 days later with a solution of 2.5% formaldehyde (1635-4L, Sigma-Aldrich) in 3x PBS. Following removal of the agarose, the fixed cell monolayers were stained with 0.1% crystal violet (C581-100, Fisher Scientific) and 2.1% ethanol in water.

Focus assays were used to assess the presence of the Y61E infectious clones after several viral passages. 24-well plates were seeded with 2×10^4 Vero E6 cells per well and the following day were inoculated with 10-fold serial dilutions of infectious viral clones. Plates were incubated for 1 hour at 37°C with rocking every 15 minutes. After absorption, cells were overlaid with 0.01% methylcellulose in Vero E6 media (see Cells and Viruses for media detail) and were left in a 37°C incubator for 3 days. After 3 days the overlay was removed and cell monolayers were fixed with 4% formaldehyde (28906, Thermo Fisher Scientific) at room temperature, washed, then permeabilized with 0.5% Triton X-100 in PBS. Cell monolayers were then blocked with 5% milk in PBS and thereafter incubated with 4G2 antibody (1:10,000) diluted in 5% milk in PBS for 1 hour at 37°C. Cells were washed with PBS and then incubated with goat anti-mouse secondary antibody at a 1:2000 (5450-0011, LGC Clinical Diagnostics, Inc., Milford, MA) dilution in 5% milk in PBS for 1 hour at 37°C. Cells were washed again and then incubated with TrueBlue Peroxidase Substrate (5510-0030, LGC Clinical Diagnostics, Inc.) until the formation of blue foci and reaction was stopped by removing the substrate.

Virus-like particle (VLP) release assay

To determine the efficiency of VLP formation and release when ZIKV E contains mutations at Y61 or T351, 5×10^5 HEK293T cells in 6-well plates were transfected with 2 µg of the modified PaxVax plasmids (see Plasmids for details) using 8 µg of PEI per well. Twenty-four hours after

transfection, fresh cell media was added to wells. 48 hours after transfection, cells and cell media were collected. Cell media was clarified by centrifugation for 3 minutes at 1500 RPM and then VLPs were lysed with 10X Triton buffer for a final dilution of 1X lysis buffer. Cells were collected with cold PBS, centrifuged for 3 minutes at 1500 RPM, and then lysed with Triton buffer. Cells and cell media were subjected to quantitative western blotting for the detection of ZIKV E by way of 4G2 antibody (concentration of 1:10,000). To calculate efficiency of VLP release, each ZIKV E protein value found in cells was first normalized to actin values in cells (rabbit anti-actin antibody at 1:5000). VLP release efficiency was then calculated as the quotient of the E protein quantity in VLPs divided by the quantity of E in cells $[(E_{\text{mutant VLP}} / E_{\text{mutant cells}}) / (E_{\text{WT VLP}} / E_{\text{WT cells}}) * 100]$.

Kinase inhibition assays

To determine if treating cells at the time of infection with various kinase inhibitors would impact viral growth, viral challenge assays were performed. First, to screen a panel of inhibitors at a fixed concentration, 2.5×10^5 A549 cells were seeded in 6-well plates. The next day cells were infected at an MOI of 0.1 with ZIKV. After an hour of absorption at 37°C, the inoculum was removed and cells were treated with 10 µM of Sunitinib (B1045, APEX BIO Technology LLC, Houston, TX) Bosutinib (PZ0192, Sigma-Aldrich), Defactinib (B4800, APEX BIO), Imatinib (9084S, Cell Signaling Technology), or DMSO vehicle control in HEK293T media. The infection proceeded for 48 hours then cell media was collected, clarified by centrifugation, stored at -80°C, and then titered by plaque assay. The percent inhibition was determined by normalizing titers of drug-treated wells to the DMSO control well titers and multiplying by 100. Second, to determine the IC₅₀ of Bosutinib or Defactinib, a dose response curve was set up with drugs at concentrations of 30, 20, 15, 10, 7.5, 5, and 1.25 µM as well as DMSO. 2.8×10^4 A549 cells

were seeded into 48 well plates. One day later, cells were infected at an MOI of 0.1. One hour after absorption, the inoculum was removed and cells were treated with A549 media containing drug at the listed concentrations. After 48 hours, cell media was collected, clarified by centrifugation, stored at -80°C, and then titered by plaque assay. To determine the cell toxicity of each drug, an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (M6494, Thermo Fisher Scientific) was conducted in parallel to the infection and kinase inhibitor treatment according to manufacturer instructions. A549 cells were set up in 48 well plates and treated with the listed concentrations of Bosutinib. After 48 hours, cell media was removed and replaced with 1.2 mM MTT in PBS for 4 hours at 37°C. After incubation, MTT was removed and DMSO was added to each well, incubated for 10 minutes at 37°C, and absorbance was read at 540 nm. Cell viability was determined by first normalizing the absorbance readings of drug-treated cells to the DMSO control wells. Cell toxicity was determined by subtracting the value of cell viability from 100. To determine the IC₅₀ (half-maximal inhibitory concentration) of Bosutinib and Defectinib, we used [inhibitor] versus normalized response curve fitting in Prism 8.0. To determine CC₅₀ (cytotoxic concentration causing death to 50% of cells), the same Prism 8.0 curve fitting was applied to our MTT assay data (inhibitor versus cell toxicity). The selectivity index (SI) of each drug was calculated by dividing the CC₅₀ by the IC₅₀ of each drug.

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Conflict of Interests

The authors declare that they have no conflict of interest.

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Figure Legends

Figure 1. Discovery of post-translational modifications on the ZIKV proteome.

(A) Depictions of i) a ZIKV particle with its structural proteins indicated and ii) the organization of the ZIKV positive-strand RNA genome and its encoded ORFeome.

(B) Overview of the workflow for discovery of post-translational modifications on ZIKV proteins in the settings of viral infection or plasmid-based expression of ZIKV proteins. IP, immunoprecipitation; AP, affinity purification.

(C, D, and E) Western blots and Coomassie stained SDS-page gels of affinity purified ZIKV E from ZIKV infected cells (C), authentic ZIKV particles (D), or the indicated SBP-tagged ZIKV proteins (E). For (C and D), Vero E6 cells were infected with ZIKV strain BeH819015 at an MOI of 0.1. Cells (C) and media (D) were collected 72 hours and 96 hours later, respectively. In (C), infected cells were lysed in Triton-X buffer and intracellular ZIKV E was purified using the anti-E antibody 4G2 and magnetic protein G beads. As a control, we performed immunoprecipitation of uninfected (mock) cellular lysates with the 4G2 antibody. In (D), viral particles were immunoprecipitated from clarified supernatants using the 4G2 antibody and magnetic protein G beads. Control conditions included (i) immunoprecipitation of virus-containing supernatants with a non-specific mouse IgG antibody and (ii) immunoprecipitation of an uninfected (mock) supernatant with the 4G2 antibody. For (E), to purify ZIKV proteins expressed from plasmid, we engineered plasmids to encode the indicated ZIKV ORFs with a C-terminal streptavidin-binding protein (SBP) tag. HEK293T cells were transfected with a plasmid encoding the indicated SBP-tagged ZIKV protein or, as a control, an empty vector (V). Two days after transfection, the cells were lysed in Triton-X buffer and ZIKV proteins were affinity purified using streptavidin-coated magnetic beads (labeled as Anti-SBP in panels). In panels (C-

E), for both antibody- and streptavidin-based purifications, captured protein mixtures were orthogonally separated using SDS-PAGE, followed by in-gel processing for subsequent mass spectrometry analysis to discover post-translational modifications or interacting host proteins. The molecular weight in kDa, viral proteins (yellow text and arrows), and immunoglobulin bands are labeled. Note that the asterisks in (E) indicate monomeric streptavidin that was eluted off the streptavidin beads when boiled.

Figure 2. Location of post-translational modification sites on ZIKV proteins. Phosphosites and ubiquitination sites are marked in green and light purple, respectively. For each ZIKV protein, individual domains or specific features are color coded with the text label displayed in the matching color in each instance.

(A and B) Location of the Y61 and T351 phosphosites on ZIKV E (A) or location of all post-translational modifications on ZIKV E (B). In both (A and B), PDB ID: 5JHM was used and one copy of E is shown.

(C) ZIKV E - M heterodimer (PDB ID: 5IZ7). Y61 and T351 are labeled on the E ectodomain. ZIKV M phosphosites S16 and T18 are labeled on the M protein.

(D) PTM locations on ZIKV NS1 (PDB ID: 5GS6).

(E) ZIKV NS2B – NS3 protease complex shown in close conformation (PDB ID: 5GPI) with labeled phosphosites on ZIKV NS2B.

(F) ZIKV NS5 (PDB ID: 5TMH) phosphosites. RdRp, RNA-dependent RNA polymerase; MTase, methyltransferase domain.

Figure 3. Tyrosine 61 and threonine 351 on the ZIKV E protein are critical for viral propagation at the stage of viral budding.

(A-C) Overview of the reverse genetics approach used to recover infectious ZIKV clones bearing mutations at Y61 or T351 in the E protein (A). For each recovery attempt, full-length infectious RNAs were transcribed from a cDNA template of the viral genome. The RNAs were electroporated into Vero E6 cells and 3 days later supernatants from these cultures were screened for the presence of infectious ZIKV particles by either viral plaque assay (B) or focus assay (C). In (C), the rZIKV E Y61E clone was passaged 2 additional times due to the absence of detectable plaques or foci after passage 1. The Y61E passage 3 material showed infectious virus and this rLCMV had mutated from a glutamic acid to a valine at position 61.

(D and E) The growth kinetics of the indicated rZIKV E Y61 mutants (D) or T351 mutants (E) was determined by infecting Vero E6 cells at an MOI of 0.001 (D) or A549 cells at an MOI of 0.01 (E) with these viruses and measuring the infectious particles released by plaque assay at 24, 48, 72, 96, 120, or 144 hours after infection. Data represent the mean $\pm$ SEM from three (D) or two (E) independent biological replicates. For statistical analysis, data were analyzed via mixed-effect analysis (two-way ANOVA) with Holm-Sidak's test for multiple comparisons in Prism 8.0.

(F) The budding activity of WT, Y61- or T351-mutant E proteins was measured by a virus-like particle (VLP) assay. VLPs were generated by transfection of HEK293T cells with plasmid that encodes ZIKV prM and E (either WT E or the indicated E mutants). The quantity of ZIKV E released into the supernatant vs retained in cells was measured by quantitative western blot and budding efficiency was determined as described in the Methods. Data represent the mean $\pm$ SEM

from three (Y61 mutants) or four (T351 mutants) independent biological replicates. Statistical significance was determined in Prism 8.0 software using a one-way ANOVA.

For the indicated statistical tests in (D-F): *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 4. Host kinase interactions the ZIKV proteome and effects of kinase inhibitors on ZIKV growth.

(A) Host kinases that interact with ZIKV proteins. Shown are kinases that had the greatest number of tryptic peptides (top 10 for each ZIKV protein) detected by mass spectrometry and/or were predicted by Scansite 4.0 to phosphorylate one or more of the phosphosites identified in this study. Black circles denote kinases that interacted with 5 viral proteins. ZIKV proteins with detectable phosphosites in this study are indicated with a “P”. Drugs known to inhibit specific kinases are listed; drugs with FDA approval are bolded.

(B) Efficacy of kinase inhibitors in restricting ZIKV growth. A549 cells were infected with ZIKV at a MOI of 0.1 and 1 hour later, the inoculum was removed and cells were treated with 10 μ M of the indicated drug or, as a control, DMSO (vehicle). Two days later infectious virus measured in the supernatants via plaque assay. Data represent mean $\pm$ SEM from three independent biological replicates. Statistical significance was determined in Prism 8.0 software via one-way ANOVA with Holm-Sidak’s test for multiple comparisons. *, $P < 0.05$

(C and D) IC₅₀ determination for Defactinib and Bosutinib inhibition of ZIKV growth. A549 cells were infected with ZIKV at a MOI of 0.1 and 1 hour later, the inoculum was removed and cells were treated with the indicated doses of Bosutinib or Defactinib. Two days later infectious virus was measured in the supernatants via plaque assay. In parallel, the cytotoxicity of each drug at these same doses was determined via MTT assay as described in the Methods. Data

represent mean $\pm$ SEM from three independent biological replicates. The resulting IC₅₀ and selective index (SI) values are listed for each drug. Calculations of both values are described in Methods.

Figure S1. Identification of ZIKV E phosphosite T351 in virions from mosquito cells. C6/36

mosquito cells were inoculated with ZIKV at an MOI of 0.1. Four days later, cell-free ZIKV particles were collected, concentrated, and purified by banding on a potassium tartrate density gradient. (A) Aliquots of gradient fractions were separated by SDS-PAGE and stained with Coomassie Brilliant Blue; the position of ZIKV E is shown. Virus-containing fractions then were dialyzed and concentrated. (B) Negative stain transmission electron micrograph showing concentrated ZIKV virions prepared using this process (from an infection at MOI of 1.0, harvested at 72 h post-infection). Concentrated ZIKV particles were digested with trypsin and LysC and analyzed using mass spectrometry. Annotated mass spectra are shown for ZIKV E T351 in (C) unmodified site and (D) phosphorylated forms; ph/@ = phosphorylation.

Figure S2. Purification of plasmid-expressed ZIKV M, C, or E in the setting of phosphatase

inhibition for mass spectrometry analysis. Western blots and Coomassie stained SDS-page gels of purified SBP-tagged ZIKV proteins. HEK293T cells were transfected with a plasmid encoding the indicated SBP-tagged ZIKV protein or, as a control, an empty vector (V). Two days after transfection, cells were treated with DMSO, H₂O₂, or Calyculin A for 30 minutes and then lysed in Triton-X buffer. SBP-tagged ZIKV proteins were affinity purified using streptavidin-coated magnetic beads and the captured protein mixtures were orthogonally separated using SDS-PAGE, followed by in-gel processing for subsequent mass spectrometry analysis to

discover post-translational modifications or interacting host proteins. The molecular weight in kDa, viral proteins (yellow text and arrows), and immunoglobulin bands are labeled. Note that the asterisks in indicate monomeric streptavidin that was eluted off of the streptavidin beads when boiled.

Figure S3 Purification of plasmid-expressed ZIKV NS1, NS4A, and NS2B in the setting of phosphatase inhibition for mass spectrometry analysis. Western blots and Coomassie stained SDS-page gels of purified SBP-tagged ZIKV proteins. HEK293T cells were transfected with a plasmid encoding the indicated SBP-tagged ZIKV protein or, as a control, an empty vector (V). Two days after transfection, cells were treated with DMSO, H₂O₂, or Calyculin A for 30 minutes and then lysed in Triton-X buffer. SBP-tagged ZIKV proteins were affinity purified using streptavidin-coated magnetic beads and the captured protein mixtures were orthogonally separated using SDS-PAGE, followed by in-gel processing for subsequent mass spectrometry analysis to discover post-translational modifications or interacting host proteins. The molecular weight in kDa, viral proteins (yellow text and arrows), and immunoglobulin bands are labeled. Note that the asterisks in indicate monomeric streptavidin that was eluted off streptavidin beads when boiled.

Figure S4. Purification of plasmid expressed ZIKV NS4B and NS5 in the setting of phosphatase inhibition for mass spectrometry analysis. Western blots and Coomassie stained SDS-page gels of purified SBP-tagged ZIKV proteins. HEK293T cells were transfected with a plasmid encoding the indicated SBP-tagged ZIKV protein or, as a control, an empty vector (V). Two days after transfection, cells were treated with DMSO, H₂O₂, or Calyculin A for 30 minutes

and then lysed in Triton-X buffer. SBP-tagged ZIKV proteins were affinity purified using streptavidin-coated magnetic beads and the captured protein mixtures were orthogonally separated using SDS-PAGE, followed by in-gel processing for subsequent mass spectrometry analysis to discover post-translational modifications or interacting host proteins. The molecular weight in kDa, viral proteins (yellow text and arrows), and immunoglobulin bands are labeled. Note that the asterisks in indicate monomeric streptavidin that was eluted off of the streptavidin beads when boiled.

Figure S5. PTMs labeled on an electrostatic model of ZIKV E dimer (PDB ID: 5JHM). Blue indicates positive charges while red indicates negative charges. (A) Electrostatic view of ZIKV E dimer. (B) rotated to the negatively charged “face”. (C) “Side-view” positioning showing the neutral border of electrostatically labeled ZIKV E dimer along with location of post-translational modification.

Figure S6. ZIKV E Y61 interactions with neighboring residues and a view of the junction of ZIKV E and M. (A) Zoomed in view of the junction of ZIKV E (yellow) and M heterodimer (dark purple) with M phosphosites labeled in green. (B) Zoomed in view of Y61 on ZIKV E. Y61 is labeled in green while residues in close proximity, N207 and E262, are labeled in yellow.

Figure S7. NS2A and NS3 did not express at sufficiently high levels for mass spectrometry analysis. Attempts were made to express and purify NS2A and NS3 via plasmid transfection of mammalian cells. HEK293T cells were transfected with plasmids that encoded ZIKV proteins with either a C-terminal 3x FLAG-tag (A) or a SBP tag (B). Two days later, cells were lysed and

1061 tag-based protein purifications were attempted with an anti-FLAG antibody and magnetic protein
1062 G beads (A) or magnetic streptavidin beads (B).

1063

1064

1065 **Table 1. Phosphorylation and ubiquitination sites on the ZIKV proteome.**

ZIKV Protein	Post-translational modifications	Treatment ^a			Predicted Kinases/Interacting kinases/ Closely-related kinases discovered ^b
		DMSO	H ₂ O ₂	Calyculin A	
C	None found				
M	S16# ^c			✓	PRKAA1, NEK1, NEK3, NEK4, NEK5, NEK8, NEK10 (low), CLK2 , ATM , PRKDC (min)
	T18			✓	GSK3B , AKT1, PRKACG (low), CAMK2B , PRKCE , PRKAA1, AURKA , AURKB (min)
E	Y61 ^{Ad}				FGR
	S142	✓	✓	✓	GSK3B , AURKB , NEK3 (min)
	S149	✓	✓	✓	PRKAA1, NEK5 (min)
	T156	✓			GSK3B , PLK1 , PRKAA1 (min)
	S173 [^]	✓	✓	✓	CDK1 , CDK5 (low), MAPK3 (min)
	T179		✓	✓	PLK1 , PRKCD , PRKCE , PRKAA1, AURKA , NEK6 , NEK7 , NEK10 (min)
	S185#	✓		✓	
	T205			✓	NEK7 (med), NEK6 , NEK8, NEK9 (low), NEK 10 (min)
	T231	✓	✓	✓	NEK6 , MAPK3 (min)
	T233		✓	✓	CDK1 , CDK5 (low) MAPK3 (low)
	T254			✓	PRKACG , AURKB (med), PRKCD , PRKAA1, AURKA (low), CAMK2G , PRKCE , PRKD1, NEK2, NEK4, NEK5 (min)
	S306 [^]			✓	PLK1 , AKT1, AURKA (min)
	T335	✓	✓	✓	CSNK1G2 (min)
	T351#	✓	✓	✓	CAMK2G (min)
	T353	✓	✓	✓	CDK1 , CDK5 , MAPK3 (low)
	T360 ^{AAe}			✓	AKT1 (low), PRKD1, MAPK3 (min)
	T366			✓	
	S368			✓	
	S372			✓	NEK5 (low), CSNK1G2 , PLK1 (min)
	K93 [^]	✓	✓	✓	
	K118 [^]		✓		
	K246	✓			
	K281 [^]	✓	✓		
	K290	✓	✓		
NS1	T17# ^{*f}	✓			PRKD1, PRKCZ (low) AKT1, PRKACG , PRKCA , PRKCD (min)
	Y22 [*]	✓			FGR, LCK, SCR (min)
	S132	✓	✓	✓	PLK1 (med), NEK5 (low), CAMK2G , PRKCD , PDPK1, NEK1, NEK5, NEK9 , NEK10 (min)
	Y175		✓		INSR (min)
	S176	✓		✓	PLK1 (low)

	T186			✓	CDK1 (min)
	Y200		✓		NEK10 (min)
	T233**g		✓		CSNK1G2 (min)
	S239**^^		✓		
	S246^			✓	PRKD1 (min)
	S348^^	✓	✓	✓	NEK2 (med), NEK1, NEK3, NEK4, NEK8, NEK10 (low), NEK5, NEK6, NEK9 (min)
	K141		✓		
NS2B	S71^		✓	✓	CDK1, CDK5 (low)
NS4A	None found				
NS2A	No expression				
NS3	No expression				
NS4B	S217# ^h /S218# ^h /T219#		✓		PRKAA1 (low), NEK6, NEK8, NEK9, GSK3B, PLK1, PDPK1, CDK1 (min)
NS5	S234			✓	CDK1 (min)
	S237^^			✓	PRKAA1, PRKDC, NEK3, NEK10 (min)
	T292^	✓			PRKCD, PRKCE, NEK6, NEK7, NEK8 (min)
	S663^^	✓	✓	✓	CLK2 (min)

1066 ^aCell treatment conditions (DMSO, H₂O₂, Calyculin A, or, in the case of Y61, no treatment) under
1067 peptides were detected are indicated with check marks.

1068 ^bNames in black text represent kinases predicted by Scansite 4.0 to phosphorylate the
1069 corresponding residue listed in a given row. Purple text represents predicted kinases that were also
1070 interacting partners of the indicated viral protein for a particular row. Blue text represents predicted
1071 kinases that were not found to interact with viral proteins but are in the same kinase cascade or has
1072 shared homology with an interacting kinase. Predicted kinases are followed by Scansite 4.0
1073 stringency levels (high, med/medium, low, or min/minimum). High, medium, low, minimum
1074 stringency indicates that the motif identified in the ZIKV polypeptide sequence is within the top
1075 0.2%, 1%, 5% and 15% of sequence matches, respectively.

1076 ^c†Phosphosite conserved only among ZIKV strains.

1077 ^d^Phosphosite conserved in 7 or more of the 15 *Flavivirus* genus members analyzed.

1078 ^e^^ Phosphosite highly conserved among 13 or more *Flavivirus* genus members analyzed.

1079 -For full details of the amino acid conservation analysis related to ^{c,d,e}, see the “multiple sequence
 1080 alignment” section of the Methods.

1081 ^{f*}Mass spectrometry data not sufficient to distinguish between NS1 sites T17 and Y22; there was
 1082 evidence for both sites.

1083 ^{g**}Evidence for both NS1 T233 and S239 sites.

1084 ^{h##}Phosphosite was located to one of these three locations.

Figure 2

Figure 3

Figure 4

Figure S1

Figure S2

Figure S3

Figure S4

Figure S5

Figure S6

A

ZIKV M S16 and T18 magnified

B

ZIKV E Y61 magnified

Figure S7