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Letters

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The Unique Cofactor Region of Zika Virus NS2B−NS3 Protease Facilitates Cleavage of Key Host Proteins

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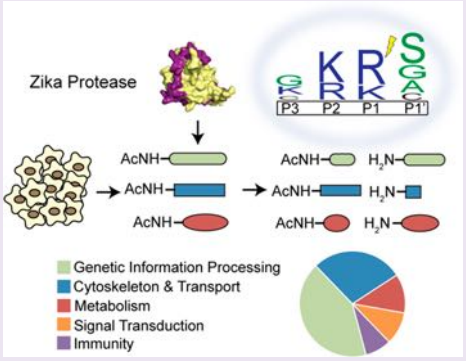
Supporting Information

ABSTRACT: Zika virus is an emerging mosquito-borne pathogen capable of severely damaging developing fetuses as well as causing neurological abnormalities in adults. The molecular details of how Zika virus causes pathologies that are unique among the flavivirus family remain poorly understood and have contributed to the lack of Zika antiviral therapies. To elucidate how Zika virus protease (ZVP) affects host cellular pathways and consequent pathologies, we used unbiased N-terminomics to identify 31 human proteins cleaved by the NS2B−NS3 protease. In particular, autophagy-related protein 16-1 (ATG16L1) and eukaryotic translation initiation factor 4 gamma 1 (eIF4G1) are dramatically depleted during Zika virus infection. ATG16L1 and eIF4G1 mediate type-II interferon production and host-cell translation, respectively, likely aiding immune system evasion and driving the Zika life cycle. Intriguingly, the NS2B cofactor region from Zika virus protease is essential for recognition of host cell substrates. Replacing the NS2B region in another flavivirus protease enabled recognition of novel Zika-specific substrates by hybrid proteases, suggesting that the cofactor is the principal determinant in ZVP substrate selection.



The recent emergence of Zika virus (ZIKV) gained worldwide attention due to its association with neurological defects, including microcephaly in newborns and Guillain-Barré syndrome in adults.[1,2] These associated pathologies are unique among the flavivirus family, which includes dengue virus (DENV) and West Nile virus (WNV), and are likely related to the ability of ZIKV to persist in semen[3] and cross placental barriers.[4] Over 2 billion people live in regions with climates suitable to support ZIKV-transmitting *Aedes* mosquitos,[5] yet there is currently no suitable vaccine or drug available against this virus. Moreover, vaccine approaches for ZIKV could be challenging due to the possible phenomenon of antibody-dependent enhancement, wherein non-neutralizing antibodies for one flavivirus inadvertently increase infectivity of another flavivirus, causing more serious symptoms.[6] However, even if a suitable vaccine is implemented, antivirals will still be necessary to decrease the incidence of pathologies associated with ZIKV infection.

The ZIKV genome is a positive-sense RNA that is translated by the host cell machinery into a single-chain polyprotein precursor comprising both structural and nonstructural (NS) components. Flavivirus replication requires the activity of the encoded viral protease, which along with host proteases cleaves the polyprotein, releasing mature viral proteins and allowing formation of the replication complex.[7] Four enzymatic activities are associated the NS proteins: the protease (NS2B−NS3pro, ZVP), helicase (NS3),[8] and methyltransferase and RNA polymerase (NS5).[9] Each of these enzymes is a potential drug target for ZIKV antivirals, but due to its indispensable role in the production of both nonstructural and enzymatic components of the virus, the protease is a particularly compelling target. Indeed, FDA-approved protease-directed therapies toward HIV[10] and hepatitis C virus[11] have proven highly efficacious and thereby establish viral proteases as effective targets to combat viral infection.

Targeting viral proteases impacts their action on both the viral polyprotein and host proteins. The crystal structure of ZVP[12] (pdb 5LC0) in complex with a boronate inhibitor revealed the expected chymotrypsin-like fold with the NS2B cofactor wrapping around the protease core to participate directly in substrate binding (Figure 1a) at the S2 and S3 specificity subsites[13] (Figure 1b). To define the role of ZIKV

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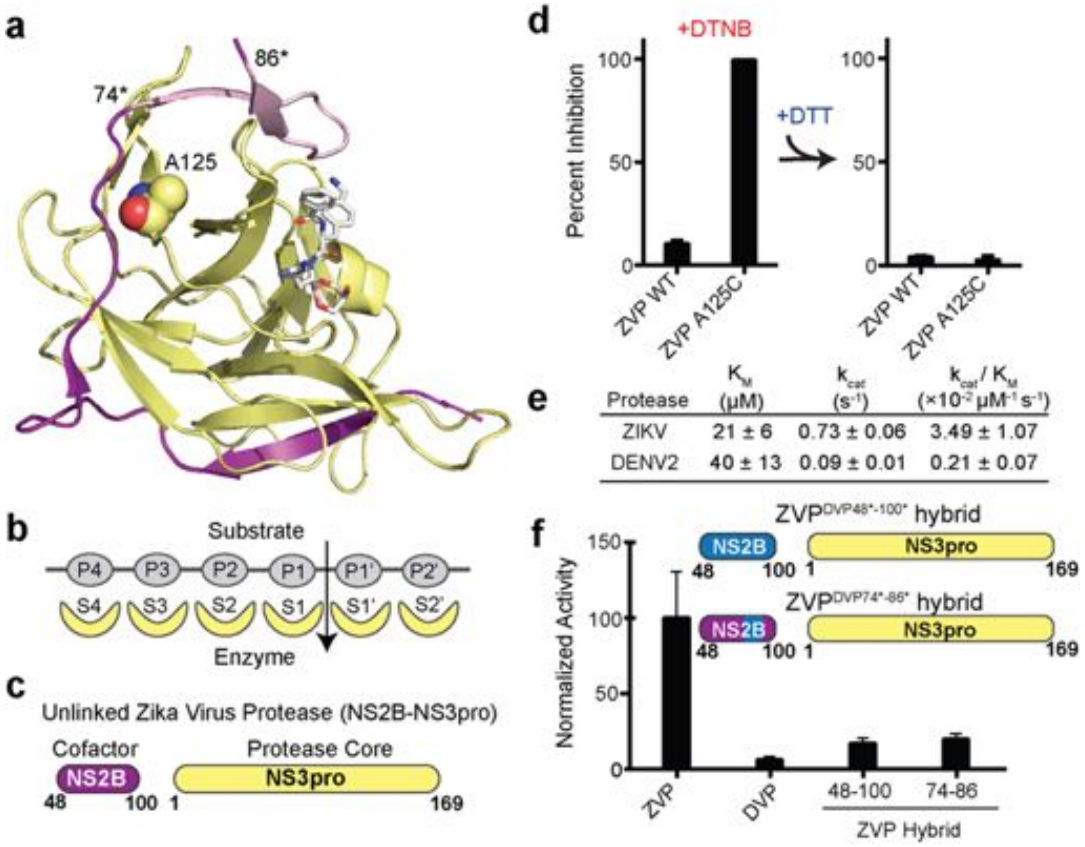

Figure 1. ZVP maintains the fold and allosteric site of flaviviral proteases, but shows enhanced catalytic activity. (a) Crystal structure of ZVP (NS2B in purple, NS3pro in yellow, pdb: 5LC0) in complex with a boronate inhibitor (white sticks) at the active site, highlights the previously identified Ala125 allosteric site (spheres) from DVP and the C-terminal region of NS2B important for catalysis (pink). (b) Schechter and Berger nomenclature defines protease specificity.[13] The enzymatic specificity subsites (S) act on the substrate peptide sites (P). Cleavage occurs between the P1 and the P1' position on substrates. (c) An unlinked protein complex was generated by co-expressing residues 48*−100* of ZIKV NS2B and residues 1−169 of ZIKV NS3pro. (d) Mutant ZVP A125C is completely inhibited after incubation with cysteine-reactive small molecule DTNB. The inhibition is reversible upon addition of the reductant DTT. These data validate the Ala125 allosteric site conservation between DVP and ZVP. (e) ZVP activity is much higher than DVP using Boc-GRR-AMC substrate. (f) Hybrid ZVP NS3pro enzymes co-expressed residues 1−169 from ZVP NS3 core with either the entire DVP NS2B cofactor residues (blue) 48*−100* or 74*−86*. Replacing the ZVP cofactor with the DVP cofactor resulted in a significant decrease in activity, demonstrating the importance of the NS2B cofactor to inherent ZVP catalytic function.

NS2B−NS3 protease (ZVP) in virus−host interactions at the cellular level, we designed a novel construct that coexpresses NS3 protease core with its cofactor (Figure 1c).

ZVP is highly similar to the DENV NS2B−NS3 protease (DVP) with respect to the overall fold and catalytic machinery. We also observed that a previously identified allosteric site centered at Ala125 in DVP[14] is conserved in ZVP (Figure 1d). Despite these similarities, we and others[12] have found that ZVP is significantly more active (16-fold) than DVP at turning over GRR-AMC substrate (Figure 1e, Table S1a). Enhanced activity may reflect the need for rapid proteolysis to overcome host responses or for rapid virus maturation for replication and escape. NS2B residue Asp83* (*NS2B nomenclature) was previously predicted to be responsible for this heightened activity, but catalytic efficiency decreased only 2-fold in the D83N* substitution.[12] Furthermore, ZVP D83N* remained more active than WNV protease and DVP, suggesting that D83* alone is not responsible for the increased activity of ZVP, though it may have the ability to influence substrate specificity.[15] To investigate the contribution of the intact cofactor (NS2B), which is 41% identical in DVP and ZVP (Figure S1), we coexpressed the ZVP NS3 core with the DVP cofactor (Figure 1f). The catalytic efficiency of this hybrid $ZVP^{DVP48*-100*}$ decreased ∼6-fold relative to WT ZVP (Figure 1f, Table S1a). Including only the C-terminal portion of DVP cofactor in the hybrid, $ZVP^{DVP74*-86*}$ equally diminished the ZVP activity, suggesting that the C-terminal region of the cofactor is important for the enhanced catalytic activity of ZVP despite it contributing only to the S2 and S3 specificity subsites and not to the region housing the catalytic triad.

Given the unique features we observed in the catalytic rates for ZVP, we hypothesized that identifying host proteins for this viral protease would provide insights into ZIKV-specific pathologies, as proteolytic cleavage of various substrates can lead to either a gain or loss of function. A number of proteomic technologies enable the assessment of cleaved protein substrates across the entire human proteome.[16−19] We applied the subtiligase-mediated N-terminomics approach,[20] which allowed identification of the exact sites of cleavage within ZVP target proteins as well as the overall ZVP specificity (Figure 2a,b). Because viral infection often induces the rapid degradation of many host cell substrates, our approach of assessing uninfected cells has the significant advantage of allowing a more comprehensive compilation of ZVP substrates, which would be otherwise degraded and missed by N-terminal labeling in infected cell lysates. To investigate cleavage of host

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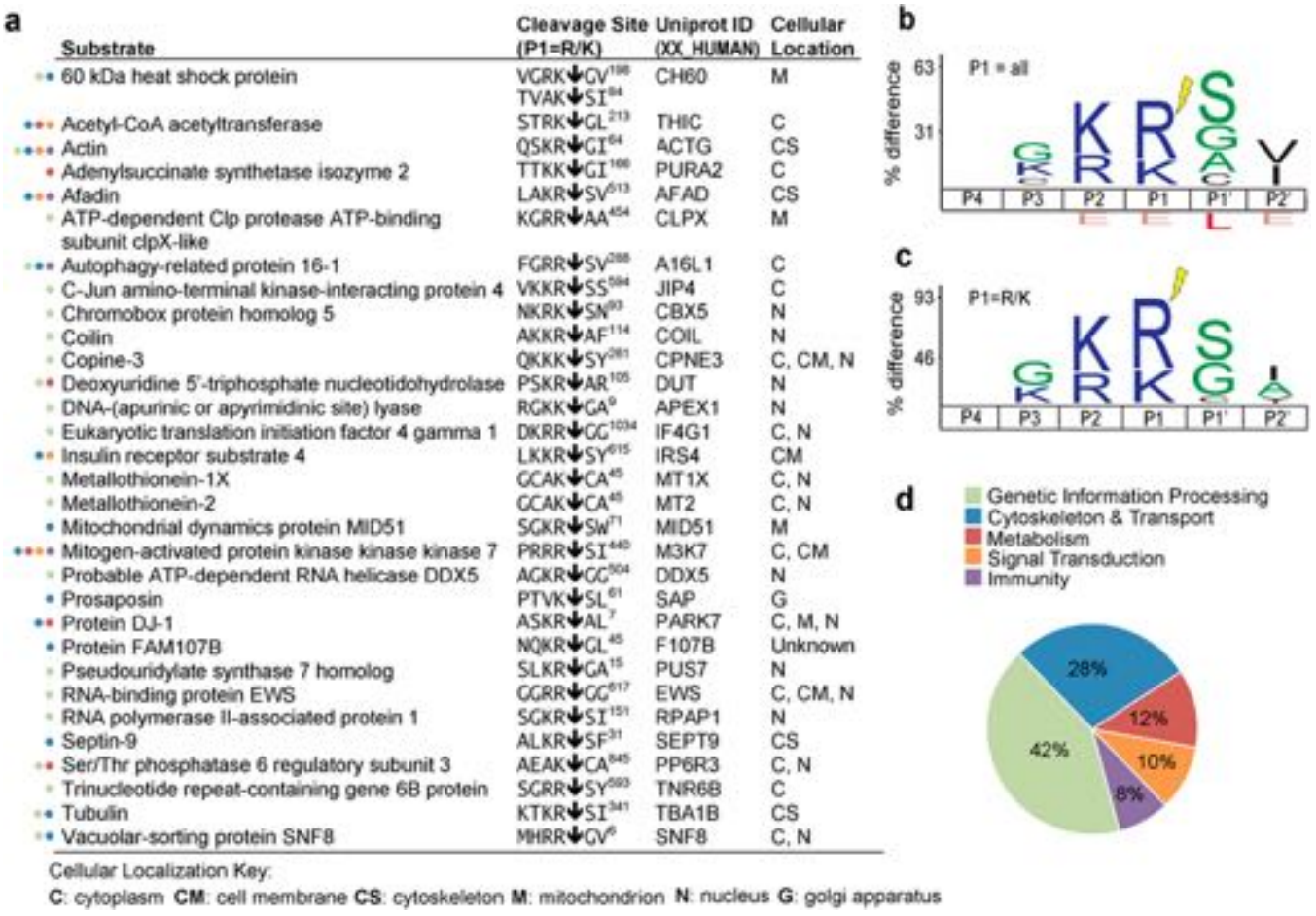

Figure 2. Protein substrates of ZVP revealed using N-terminomics assessment profiling the entire human proteome. (a) Table of 31 putative ZVP substrates identified using N-terminomics indicating the precise cleavage sites. Cellular location and dots representing the functional family colored as in (d) are also shown. (b,c) The sequence (IceLogo[28]) representation of the P4-P2' specificity of ZVP for human host protein substrates discovered using N-terminomics for P1 = all (b) and P1 = R/K (c). A p-value of 0.05 was used as a cut-off to calculate the sequence logo. (d) KEGG analysis[29] of ZVP protein substrates reveals the biological processes that may be affected upon cleavage of host proteins.

proteins by ZVP, HEK 293 cells were grown in light ([^{12}C,^{14}N]-Lys/Arg) or heavy ([^{13}C,^{15}N]-Lys/Arg) medium,[21] and cell lysates were treated with either ZVP (light) or buffer (heavy) (Figure S2 and Supporting Information xlsx file). Newly generated N-termini were tagged by subtiligase with a biotinylated peptide, which allowed isolation of newly cleaved sequences and identification by LC-MS/MS (Figure S2). As in the prior reports of N-terminomics,[22−25] we have no evidence that significant numbers of cellular proteins in the lysates were denatured during analysis. Denaturing conditions would have led to a tremendously increased number of substrates being observed and would have resulted in the loss of function to both ZVP and subtiligase, which must be functional for the N-terminomics to proceed.

To avoid bias and minimize false positives during our analysis, in addition to subtracting substrates observed in the heavy condition, we also excluded any of the 2144 cleavage sites that had been previously observed in untreated samples using subtiligase N-terminomics[26] and any sites wherein P1 was not R/K. The only previously reported ZVP host protein substrate, FAM134B,[27] was not observed in our analysis. We know that N-terminomics, like any MS-based method, will miss targets if the tryptic peptides generated are too small or large or in low abundance. In total, 31 putative human ZVP substrates were identified (Figure 2a, Supporting Information xlsx file 1).

Conventional nomenclature to describe protease recognition sequences comes from Schechter and Berger,[13] wherein P denotes the substrate (peptide) position and S denotes the protease specificity subsite (Figure 1b). Prior peptide substrate analysis suggested that ZVP strongly preferred a positively charged residue (Arg or Lys) at the P1 position.[30,31] This was likewise true for intact protein substrates in our analysis (Figure 2b,c) wherein the overall substrate specificity (Figure 2b) showed a similar preference for Arg or Lys at the P1 position. Focusing the substrate list to those containing R/K at P1 indicated a preference for a positively charged residue in the P2 position also, with less stringent specificity in the P3 position (favoring either Gly or Lys) and no requirements in the P4 position (Figure 2c). ZVP preferentially cleaves sequences containing a small amino acid residue, Gly or Ser, at the P1′ with more tolerance in the P2′ position. This specificity profile matches very well to the cleavage sites of the ZIKV polyprotein (capsid, VTRR↓GS; NS2A/NS2B, SGKR↓SW; NS2B/NS3, TGKR↓SG; NS3/NS4A, AGKR↓GA; and NS4B/NS5, VKRR↓GG), all featuring positively charged residues at P1 and P2, and a small residue at P1′. These data are well in agreement with the specificity attained from profiling peptides, other than for the P3 position, wherein a

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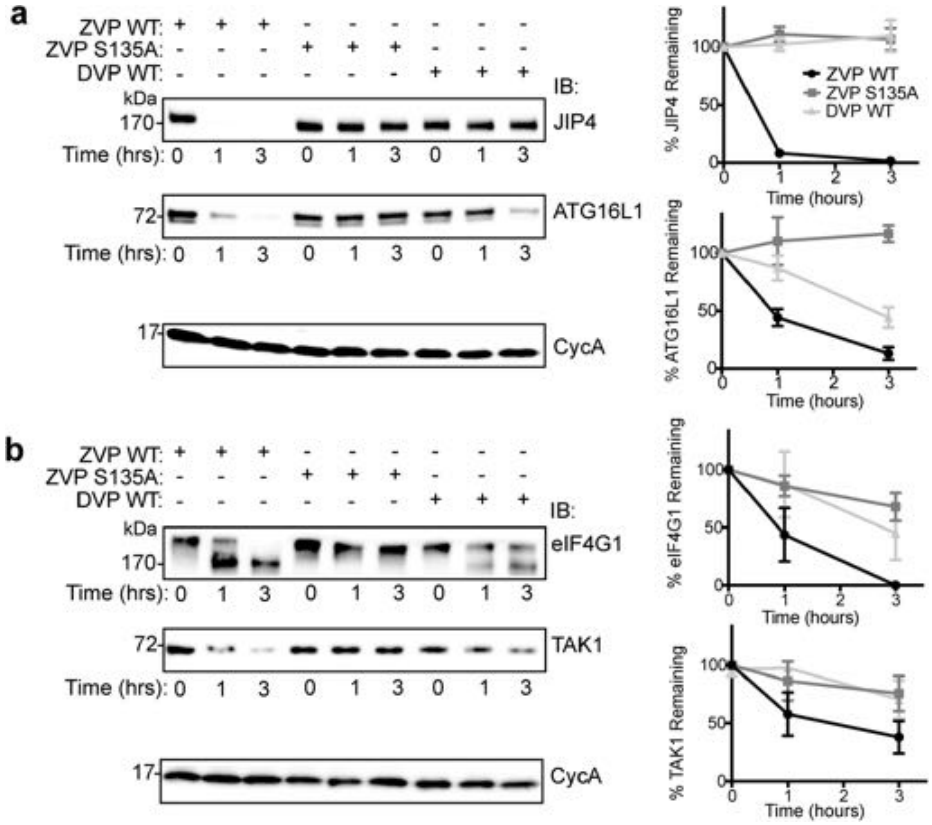

Figure 3. ZVP cleaves host proteins more efficiently than DVP. (a) Immunoblot analyses of SK-N-AS neuroblastoma cell lysates show that JIP4 and ATG16L1 are both robustly cleaved by added WT ZVP but not by the inactive S135A variant in which the catalytic serine has been replaced. DVP also cleaves ATG16L1 but not JIP4 despite the VKKR↓SS cleavage sequence. (b) eIF4G1 is cleaved by ZVP and DVP, whereas TAK1 appears to be cleaved only by ZVP. Quantifications of protein levels were normalized to levels of cyclophilin A.

strong selection for a positively charged residue was observed.[31] These results are in contrast with another study that reported a preference for Val at the P4 position, perhaps influenced by a bulky N-terminal protecting group close to the cleavage site.[30] This demonstrates that N-terminomics is a more robust strategy for identification of both host cell protein substrates of viral proteases and *bona fide* cellular substrate preferences.

Of the 31 host proteins cleaved by ZVP (Figure 2a), 42% are involved in genetic information processing, 28% in transport and cytoskeleton, 12% in metabolism, 10% in signal transduction, and 8% in immunity as assessed by KEGG mapper[29] (Figure 2d), and they localize in subcellular locations typical of other N-terminomics data sets (Figure S3). Some of this may be due to the fact that these lysate-based studies allow mixing of cellular substructure. In three of the 15 substrates annotated as residents of the nucleus (DJ-1, SNF8, and DNA lyase), cleavage of a short positively charged N-terminal sequence was observed, suggesting that cleavage of noncytosolic substrates by ZVP might also occur following cytosolic translation en route to the nucleus or other subcellular compartment.

Of the substrates involved in genetic information processing, 37% are involved in translation, 26% in replication and repair, 19% in transcription, and 18% in folding, sorting, and degradation. Of the 31 substrates, 25 are reported to be expressed in physiologically relevant cell types infected by Zika virus, including brain (astrocytes), testes (Sertoli cells), bone marrow (macrophages), and placenta.[32,33] This profile suggests that the ZVP complex affects a wide range of host cell processes and pathways, some of which might contribute to infectivity or disease pathology.

To assess the extent of cleavage of human ZVP substrates and to independently confirm our proteomics results, purified ZVP was added to cell lysates and cleavage of four key substrates was monitored as a function of time (Figure 3a,b). In parallel, cleavage by DVP was also monitored to determine which of these substrates are specific to ZIKV infection. First, autophagy-related protein 16-1 (ATG16L1) was of considerable interest because it has been shown to be important for antiviral activity of IFN-γ in mice[34] and vertical transmission of Zika.[35] Second, ZIKV NS3 has been shown to block translation of host cell proteins, a critical aspect of the antiviral response.[36] For this reason, eukaryotic translation initiation factor 4 gamma 1 (eIF4G1), was selected for further study. Indeed, eIF4G1 is an established viral protease target that is cleaved by three picornaviral proteases (poliovirus, coxsackievirus, and rhinovirus), which all cleave at a different site, R681.[37,38] During poliovirus infection, eIF4G1 cleavage prevents stress granule assembly, thereby benefiting viral replication.[39,40] Third, c-Jun amino-terminal kinase-interacting protein 4 (JIP4) was investigated because it activates the p38 mitogen-activated protein kinase (MAPK) pathway,[41] which has been shown to be involved in the interferon (IFN)-α-mediated antiviral response against another virus in the Flaviviridae family, hepatitis C virus.[42] Finally, TAK1 (mitogen-activated protein kinase kinase kinase 7 or MAP3K7) was investigated in more detail because of its role in the MAPK cascade. Specifically, its interaction with c-Jun-

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NH_2-terminal kinase (JNK) is required for the function of IFN regulatory factor 3, an important transcription factor that has antiviral activity.[43]

We observed that all four selected substrates identified by N-terminomics, JIP4 and ATG16L1 (Figure 3a) and eIF4G1 and TAK1 (Figure 3b), were efficiently cleaved by ZVP in lysates from SK-N-AS, a neuroblastoma cell line. DVP only cleaved ATG16L1 and eIF4G1 and was notably less efficient than ZVP. JIP4 (VKRR↓SS) appears not to be a good substrate for DVP, likely because although flavivirus specificity is generally recognized as R/K/Q-R/K↓G/S/A,[44−46] DVP has a preference for Arg over Lys at P2.[15] Alternatively, lack of JIP4 cleavage may suggest that factors other than simple consensus may influence substrate selection. Overall, these data show close agreement between N-terminomics results and ZVP cleavage in human cell lysates.

Some viruses selectively infect certain cell types, likely due to differential protein expression patterns. To study how widely observed Zika substrate cleavage events are, we additionally assessed ZVP cleavage events in lysates from A549, a human lung cancer cell line. In this case, we elected to monitor both depletion of substrate and formation of the cleaved products of the three most rapidly cleaved substrates. Cleavage of JIP4, ATG16L1, and eIF4G1 by ZVP and DVP were unchanged from SK-N-AS cells (Figure 4a) suggesting that these are *bona fide* and commonly expressed substrates of ZVP. Hybrid $ZVP^{DVP48^*-100^*}$ was also analyzed to assess the role of the cofactor in substrate recognition. While $ZVP^{DVP48^*-100^*}$ efficiently cleaved eIF4G1 and ATG16L1, JIP4 levels were not as affected (Figure 4a, Figure S4). The lack of cleavage of JIP4 cannot entirely be explained by differences in the catalytic rate of the $ZVP^{DVP48^*-100^*}$ hybrid. The cleavage rates for JIP4 are much more dramatically impacted than the cleavage rates for eIF4G1 and ATG16L1 by the $ZVP^{DVP48^*-100^*}$ hybrid, suggesting that JIP4 cleavage may rely on other factors such as an exosite for recognition, which we hypothesized might reside in the cofactor region. In contrast, $DVP^{ZVP48^*-100^*}$ hybrid, consisting of the DVP protease core with the ZVP NS2B cofactor, gained the ability to cleave JIP4 (Figure 4a). This suggests that the ability to recognize Zika specific substrates relies on the NS2B region. Thus, the substrate differences between ZVP and DVP significantly depend on the cofactor, which may impact specificity for substrate residues at P2/P3 or function as an exosite contributing to enhanced substrate selection.

To explore the contributions of the cofactor region, we generated and tested a fluorogenic peptide substrate representing the JIP4 recognition sequence (VKKR-AMC). Proteins that contain the ZVP cofactor cleave the VKKR-AMC peptide substrates more effectively than the GRR-AMC substrate, whereas those with the DVP cofactor cleave the GRR-AMC peptide more effectively (VKKR/GRR ratio, Table S1b,c), suggesting that the ZVP cofactor improves recognition of the JIP4 VKKR cleavage site. The molecular basis of this phenomenon has been previously investigated. In particular, cofactor residue 83* (Figure 4b) is consistently either polar or negatively charged in the flaviviral proteases. Interestingly, a correlation between the identity of residue 83* and the preference for either Lys or Arg at P2 in the native polyprotein cleavage sequence has been suggested.[15] All four serotypes of DVP contain a Ser or Thr at position 83*, which correlates with their preferences for recognizing Arg in the P2 sites of substrates. ZVP, with a negatively charged Asp at position 83*, better recognizes Lys in the P2 sites of substrates. The JIP4 cleavage sequence (VKKR) has Lys in the P2 position, which would appear to be preferred by ZVP over DVP using this rationale. Nevertheless, both hybrids can cleave isolated VKKR peptide and do so with similar overall catalytic efficiency (Figure 4c, Table S1b). Given that both DVP and $ZVP^{DVP48^*-100^*}$ hybrid cannot cleave intact JIP4 substrate but can cleave VKKR peptide strongly indicates that the ZVP cofactor region could be functioning as an exosite that serves to recognize regions of the substrate outside of the cleavage motif.

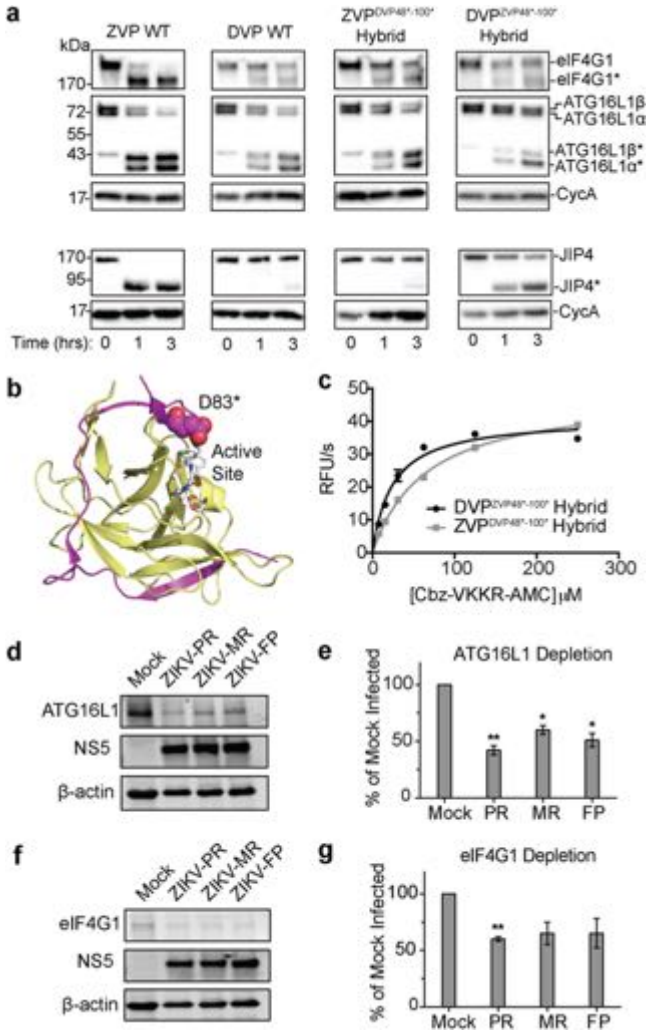

Figure 4. ZVP NS2B cofactor guides substrate recognition, while ZIKV infection reduces levels of ATG16L1 and eIF4G1. (a) $ZVP^{DVP48^*-100^*}$ hybrid cleaves host substrates eIF4G1, ATG16L1, and JIP4 similarly to DVP rather than ZVP in A459 cells. Conversely, $DVP^{ZVP48^*-100^*}$ mimics ZVP by gaining the ability to cleave JIP4. *Indicates cleavage products of identified substrates. (b) ZVP structure (pdb 5LC0) highlighting the NS2B residue D83* in spheres. (c) Both $DVP^{ZVP48^*-100^*}$ hybrid and $ZVP^{DVP48^*-100^*}$ hybrid cleave the JIP4 cleavage sequence similarly using the fluorogenic peptide substrate Cbz-VKKR-AMC. (d,e) ATG16L1 and (f,g) eIF4G1 levels decrease in infected A549 cells using ZIKV American (PR), French Polynesian (FP), and African (MR) strains. Levels of ZIKV NS5 and β-actin are shown as infection and loading control, respectively. Quantifications of protein levels were normalized to levels of β-actin. *P*-values were estimated by a two-tailed paired Student's *t* test. $^*P < 0.05$, $^{**}P < 0.01$.

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In a biological context, ZVP functions in conjunction with the other ZIKV NS proteins that form the viral replication complex. We therefore investigated whether levels of two putative ZVP substrates were decreased during infection. A549 cells were infected with three different strains of ZIKV, including two recent outbreak strains (American [PR] and French Polynesian [FP]) and a preoutbreak strain (African [MR]). Forty-eight hours post-infection, levels of ATG16L1 and eIF4G1 proteins were reduced by approximately 50% (Figure 4d,e) and 40% (Figure 4f,g), respectively, compared to the mock infection while no reduction was observed in their mRNA levels (Figure S5a,b). Importantly, these results support the suggestion that cleavage of ATG16L1 and eIF4G1, identified by N-terminomics analysis, are indeed impacted by ZIKV infection.

ZIKV employs multiple strategies to subvert antiviral defense pathways in host cells. For example, several ZIKV proteins block interferon production and signaling pathways[47] and the cellular stress response.[36] Evidence suggests that flavivirus proteases also target cellular viral restriction factors. The DENV protease inhibits type I interferon production by targeting STING protein[48] whereas ZIKV and other flavivirus proteases cleave FAM134B, a host cell restriction factor associated with ER-localized reticulophagy.[27] As ATG16L1 is a known viral restriction factor that is important for IFN-γ-mediated suppression of murine norovirus,[34] its depletion during ZIKV infection would be expected to impair type II IFN signaling and complement the NS5-mediated impairment of type I IFN signaling. We recently reported that ZIKV infection induces phosphorylation of eIF2α, resulting in reduction of cellular translation.[36] Data in the present study suggest that this virus may also use another mechanism to interfere with host cell translation. Reduction of eIF4G1 in ZIKV-infected cells may provide a discriminating mechanism that allows the virus to selectively target cellular mRNA translation, which is dependent on eIF4G1-mediated RNA circularization. Flavivirus RNAs have complementary "cyclization sequences" at both 5′ UTRs and 3′ UTRs thus enabling them to bypass eIF4G1-mediated RNA circularization and channel more cellular resources for viral translation.

In summary, these data demonstrate that ZVP has enhanced catalytic abilities compared to other flaviviruses including DVP and an extended substrate clientele. This increased activity may expand the range of host cell substrates. Altogether, 31 novel ZVP substrates were identified. Notably, ZVP cleaved some protein substrates (JIP4 and TAK1) that DVP did not. Cleavage of Zika specific substrates was dependent on the presence of the NS2B cofactor, which is responsible for substrate selection. Thus, we predict that the substrate pool for ZVP may underlie some of the unique aspects of ZIKV pathology. Furthermore, ATG16L1 and eIF4G1 levels in ZIKV infected cells were considerably lower than in uninfected cells, consistent with them being host substrates of ZVP. Given the key roles of proteins cleaved by ZVP, it seems clear that alteration of their activities by ZIKV plays important roles in viral infectivity and pathogenesis.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.8b00508.

Full list of mass spectrometry data including peptides, cleavage sites and substrates identified (XLSX)

Methods and alignment of NS2B cofacter regions of ZVP and DVP, K_m, k_{cat}, and k_{cat}/K_M for the hybrid and WT enzymes on different substrates, experimental workflow of subtiligase N-terminomics in combination with SILAC, subcellular localization of ZVP human host substrates, quantification of substrate cleavage in A549 cell lysates, and ZIKV infection effect on mRNA levels of ATG16L and eIF4G1 (PDF)

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Notes

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