Zika virus NS4A hijacks host ANKLE2 to promote viral replication

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Abstract

Zika virus (ZIKV) is infamous among flaviviruses for its unique association with congenital birth defects, notably microcephaly. We previously mapped ZIKV-host protein interactions and identified the interaction between ZIKV NS4A and host ANKLE2, which itself has established ties to congenital microcephaly. In fruit flies, NS4A induces microcephaly phenotypes in an ANKLE2-dependent manner. This suggests that NS4A interacts with ANKLE2 to dysregulate cell behavior and contributes to abnormal host neurodevelopment. Here, we explore the role of ANKLE2 in ZIKV replication to understand the biological significance of the interaction from the viral perspective. We show that knockdown of ANKLE2 reduces replication of two ZIKV strains, across multiple MOIs. We observe that localization of ANKLE2 is drastically shifted to sites of NS4A and viral replication. We investigate which domains of ANKLE2 mediate this behavior and the interaction with NS4A. Using co-immunoprecipitation, we show that deletion of either the transmembrane or LEM domain has little impact on the interaction, but deletion of both significantly reduces interaction with NS4A. Finally, we show that the C-terminal transmembrane domains of NS4A stabilize the interaction with ANKLE2. Together, these results suggest NS4A interacts with ANKLE2 through a combination of its

transmembrane and LEM domains, bringing it to sites of ZIKV replication to promote replication through an unknown mechanism. Taken together with our previous results, our findings indicate that, in the process of hijacking ANKLE2 for replication, ZIKV disrupts its physiological function to cause disease.

Introduction

Zika virus (ZIKV) is a positive sense RNA virus belonging to the genus *Flavivirus* and can cause severe disease. ZIKV infection in otherwise healthy adults typically leads to mild symptoms and very rarely Guillain-Barré Syndrome (1). The primary concern surrounding ZIKV arises from the occurrence of Congenital Zika Syndrome (CZS) in individuals born from mothers infected during pregnancy (2). CZS is a spectrum of disease and can be clinically characterized by multiple hallmark features, including congenital contractures, ocular anomalies, cortical calcifications, and in the most severe cases, microcephaly (3, 4).

Microcephaly is a condition in which the head and brain size are significantly reduced at birth and is associated with a wide range of complications, including developmental delays, intellectual disability, and predisposition to seizures (5). Beyond CZS, microcephaly can arise from genetic mutations (6), exposure to toxins during pregnancy (7–9), and congenital infections by pathogens such as Rubella virus (10), cytomegalovirus (11), and *Toxoplasma gondii* (12).

The specific molecular mechanisms by which ZIKV contributes to abnormal neurodevelopment and microcephaly are not entirely understood. It is well established that ZIKV can infect placental tissue and gain access to the fetal compartment by compromising barrier integrity (13–15). Gestational timing and type I interferon responses *in utero* are also critical in ZIKV susceptibility (16, 17). Upon fetal infection, ZIKV also breaches the blood-brain barrier and infects susceptible neuronal tissue (18–22). Infection of these neuronal tissues is associated with direct tissue damage through the cytopathic effect (23–25), molecular level pathogenesis (26), disruption of cell division (23, 27–29), and dysregulation of developmental pathways (30–36).

Previously, we used affinity-purification and mass-spectrometry to identify ZIKV-host protein-protein interactions that may contribute to the development of microcephaly in CZS (37). By searching for host proteins with known roles in neurodevelopment or associations with microcephaly, we identified the interaction between the ZIKV non-structural protein 4A (NS4A) and host Ankyrin-repeat and LEM domain containing protein 2 (ANKLE2). ANKLE2 has well established roles in human congenital microcephaly, which has been replicated *in vivo* using *Drosophila* (38, 39). Mutations in *ankle2* result in small brain phenotypes and increased apoptosis in 3rd instar larvae. These phenotypes are rescued by the expression of human ANKLE2, suggesting that human ANKLE2 and *Drosophila* Ankle2 are functionally conserved in brain development (38). Using this *Drosophila* model, we showed that transgenic expression of ZIKV NS4A induces identical microcephaly phenotypes which are also rescued by the expression of human ANKLE2. Overall, this suggests NS4A induces microcephaly *in vivo* in an ANKLE2-dependent manner (37, 39).

Flaviviruses are well known to physically hijack host factors for their own replication and utilize many virus-host protein interactions to reshape the host cell into a niche which maximizes virus replication (40). Whether NS4A inhibition of ANKLE2 function during development is simply an unfortunate coincidence, or if there is a functional role for this virus-host protein interaction in ZIKV replication is unknown. ANKLE2 is primarily considered a scaffolding protein, facilitating protein-protein interactions between kinases, phosphatases, and their substrates. ANKLE2 localizes to the ER and inner nuclear membrane where it mediates interactions with proteins, including BANF1, VRK1, and PP2A, to control nuclear membrane disassembly during cell division (41, 42). NS4A itself is integral to the formation of flavivirus replication compartments in the ER (43, 44) and has been found to facilitate other aspects of flavivirus replication through interactions with host proteins (45–48). We hypothesize that NS4A interacts with ANKLE2 to hijack its scaffolding function in a way beneficial to virus replication.

In this study we explore the role of ANKLE2 in ZIKV replication and what structural domains mediate the biophysical interaction between ANKLE2 and NS4A. We show that knockdown of ANKLE2 reduces ZIKV replication across multiple ZIKV strains and MOIs. We find ANKLE2 is concentrated at

sites of ZIKV replication during infection. Finally, we identify that the transmembrane (TM) domain of ANKLE2 localizes it to the ER, but both the TM and LEM domains are necessary for its physical interaction with NS4A. Together, we report the novel function of ANKLE2 in promoting ZIKV infection, providing support that NS4A disruption of neurodevelopment through ANKLE2 may arise from an underlying virus replication mechanism.

Results

Knockdown of ANKLE2 reduces ZIKV replication

To explore the role of ANKLE2 in ZIKV replication, we used a gene perturbation approach. We depleted ANKLE2 using CRISPRi with two synthetic guide RNAs (gRNAs) (Supplementary Figure 1A). Huh7 cells stably expressing dCas9 were generated using lentiviral transduction (Supplementary Figure 1B). We chose Huh7 cells, derived from a hepatocarcinoma, since they are readily infected by ZIKV *in vitro* (49, 50), and the liver is an established site of ZIKV replication *in vivo* (51–53). CRISPRi gRNAs were delivered by reverse transfection. At 72 hours post transfection (hpt) the expression of ANKLE2 was clearly decreased compared to the non-coding RNA (ncRNA) control (Supplementary Figure 1C-D). *ANKLE2* mRNA was also significantly reduced by both gRNAs compared to ncRNA (Supplementary Figure 1E). We confirmed that cell viability of ANKLE2 knockdown cells was comparable to that of cells transfected with ncRNA (Supplementary Figure 1F).

To determine if ANKLE2 plays a role in ZIKV replication, we infected Huh7-dCas9 cells with ZIKV after 72 hours of CRISPRi knockdown. Supernatant was harvested immediately after addition of virus (time 0) and then at 18, 24, 48, and 72 hours post infection (hpi) and titered by plaque assay (Figure 1A). Western blot analysis of uninfected cells showed that ANKLE2 knockdown persists for 6 days post transfection, which would span the entire experimental time course (Figure 1B). Infection with ZIKV PLCal (Thailand, 2013) revealed an initial drop in titers at 18 hpi for all conditions tested. This was followed by steady increases in titers at 24, 48, and 72 hpi. There were modest but statistically

significant differences between ncRNA and *ANKLE2* gRNA treated conditions at 24 hpi (Figure 1C). Furthermore, we found that *ANKLE2* knockdown led to consistent decreases in viral replication across multiple MOIs (Figure 1D, Supplementary Figure 2A-C). To further support the role of ANKLE2 in ZIKV replication we repeated this experiment using the ZIKV strain MR766 (Uganda, 1947). This showed similar decreases in viral replication across two MOIs (Figure 1E-F, Supplementary Figure 2D). These modest phenotypes (maximum effect ~8-fold) are expected given CRISPRi does not completely eliminate ANKLE2 protein expression. Together, this shows knockdown of *ANKLE2* reduces ZIKV replication in Huh7 cells.

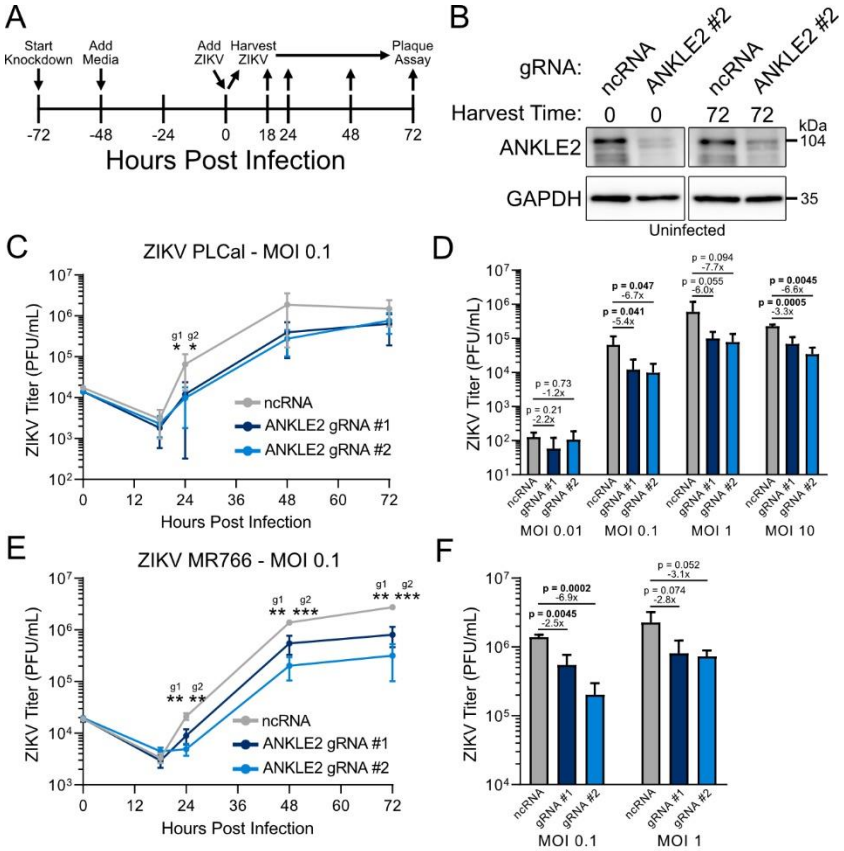

Figure 1: Knockdown of *ANKLE2* reduces ZIKV replication across multiple MOIs. A) Experimental setup to evaluate ZIKV replication after CRISPRi knockdown of *ANKLE2*. Following knockdown with gRNAs for 72 hours, media is replaced to initiate ZIKV infection. Virus supernatant was harvested at listed times and titered by plaque assay. **B)** Validation that ANKLE2 knockdown persists over six days. **C)** Quantification of ZIKV PLCal kinetics at MOI 0.1 across 72 hours. **D)** ZIKV PLCal titers at 24 hpi

across four MOIs. Average fold decrease compared to matched ncRNA is shown above each bar. **E)** Quantification of ZIKV MR766 kinetics at MOI 0.1 across 72 hours. **F)** ZIKV MR766 titers at 48 hpi across two MOIs. Error bars represent standard deviation. Average fold decrease compared to matched ncRNA is shown above each bar. Assessment of ZIKV PLCal at MOI 0.1 and 1 were done with five biological replicates. The remaining conditions were done with three technical replicates. Student's two-tailed T-test, paired for PLCal MOI 0.1 and 1, unpaired for remaining conditions. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

ANKLE2 colocalizes with NS4A and sites of ZIKV replication

ANKLE2 is known to localize to the ER and inner nuclear membrane where it facilitates host protein-protein interactions (41). NS4A is essential for remodeling the host ER into a niche suited for flavivirus replication. NS4A alone can induce membrane curvature which promotes the formation of virus replication compartments (44). It can also hijack host proteins to further facilitate ER remodeling (48). Given our previous proteomics that established the interaction between ANKLE2 and NS4A (37) we speculated that the scaffolding function of ANKLE2 may be hijacked by NS4A at sites of virus replication. We therefore evaluated ANKLE2 subcellular localization dynamics during ZIKV infection. Due to a lack of compatible immunofluorescence-grade antibodies we generated HEK293T cell lines that express FLAG affinity tagged fusions of ANKLE2 (ANKLE2-FLAG). As controls, we also generated cell lines that express GFP-FLAG as a general non-specific control and ANKLE1-FLAG to distinguish between intrinsic localization dynamics in this family of proteins, and dynamics unique to ANKLE2. We infected these cells with several ZIKV strains (PLCal, MR766, and PRVABC59) and evaluated the localization of FLAG and NS4A. Strikingly, we found that ANKLE2 distribution was concentrated to clusters that had near perfect co-localization with clusters of NS4A during infection (Figure 2A). Conversely, we found that the localization and behavior of GFP and ANKLE1 did not change during ZIKV infection (Figure 2B-C). We measured colocalization between FLAG and NS4A signal using Pearson's correlation and consistently found very high levels of colocalization between ANKLE2 and

NS4A (Pearson's R-value = 0.93 ± 0.04 across all 3 strains), which was significantly higher than GFP or ANKLE1 with NS4A (R-value = 0.43 ± 0.10, 0.46 ± 0.15, respectively) (Figure 2D).

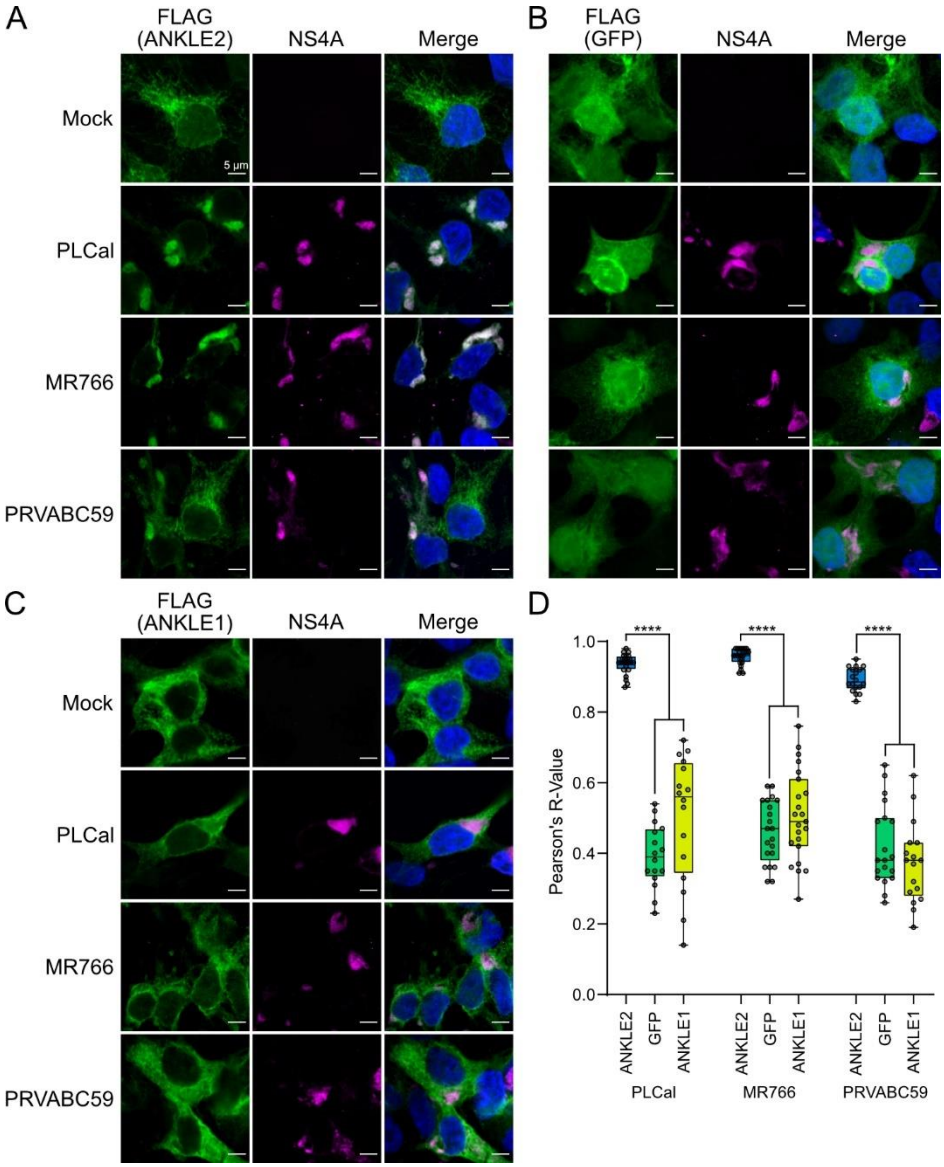

Figure 2: ANKLE2 localization pattern is altered during ZIKV infection and colocalizes with NS4A. A-C) HEK293T cells expressing ANKLE2, GFP, or ANKLE1 were infected with noted ZIKV strain for 48 hours. Proteins were visualized by immunofluorescence and confocal microscopy: FLAG (green) and NS4A (magenta) and Hoechst (blue). All scale bars = 5 µm. **D)** Pearson's correlation was measured between the FLAG and NS4A signal in infected cells. Five images per cover slip, n = 16-24 cells measured per condition. Student's two-tailed T-test. **** $p < 0.0001$.

Given the colocalization of ANKLE2 and NS4A during infection, the positive role of ANKLE2 in the ZIKV replication cycle, and the known role of NS4A in forming ZIKV replication compartment, we next explored if ANKLE2 localizes to sites of active ZIKV replication in both HEK293T and Huh7 cells. We observed that ZIKV MR766 had the highest replicative efficiency in HEK293T cells (Supplementary Figure 3) and therefore used it for our remaining immunofluorescence experiments. To evaluate the colocalization of ANKLE2 with sites of ZIKV replication we used antibodies against endogenous ANKLE2 and flavivirus envelope (Env) protein (4G2) or dsRNA (rJ2) (Figure 3A-B). We observed high colocalization between ANKLE2 and Env (R-value = 0.89 ± 0.05) that clustered similarly in cells to FLAG-tagged ANKLE2 when infected with the same ZIKV MR766 strain (Figure 3G). We observed that clusters of ANKLE2 signal in ZIKV infected cells also visually correlated with sites of dsRNA signal, although quantitative measurement revealed only modest colocalization (R-value = 0.44 ± 0.10). To confirm these were indeed sites of ZIKV replication we performed the same experiment evaluating the colocalization between NS4A and Env or dsRNA, since NS4A is inherently involved in ZIKV replication (Figure 3C-D). This revealed very high correlation between NS4A and Env (R-value = 0.96 ± 0.02). Colocalization between NS4A and dsRNA was lower (R-value = 0.42 ± 0.12) and similar to that of ANKLE2 with dsRNA. In fact, there was no statistically significant difference between them (Figure 3H). As an additional control we compared Env and dsRNA colocalization with GFP, which had significantly lower colocalization than ANKLE2 or NS4A (Figure 3E-H). We performed the same analysis in Huh7 cells and observed similar clustering by ANKLE2 in infected cells, although quantitative measurements of colocalization were lower in these cells (Supplementary Figure 4). Collectively, these results suggest that during ZIKV infection, ANKLE2 concentrates at sites of active virus replication.

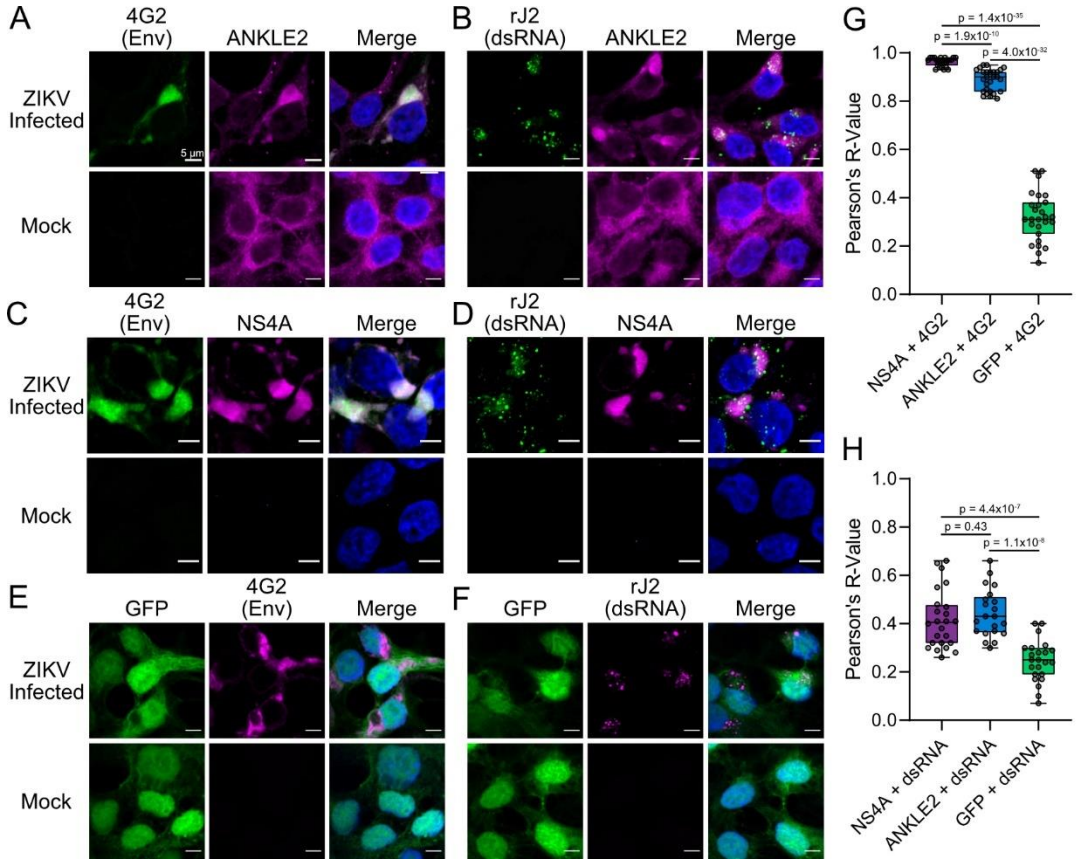

Figure 3: ANKLE2 colocalizes with ZIKV proteins and dsRNA. **A-F)** HEK293T cells were infected with ZIKV MR766 (MOI 5) for 48 hours prior to fixation and antibody staining. All scale bars = 5 µm. **G-H)** Pearson's R-value measured using ImageJ colocalization tool of n = 21-27 cells. P-values were calculated using a Student's unpaired two-tailed T-test.

NS4A interacts with ANKLE2 through its TM and LEM domains

To further understand the biophysical interaction between NS4A and ANKLE2, we sought to determine which domains of ANKLE2 were necessary for the interaction. The structure of ANKLE2 has not yet been resolved. Thus, we employed the structural prediction provided by AlphaFold2 (54, 55). This revealed high-confidence structured regions corresponding to the known TM, LEM, and ankyrin repeat (ANK) domains. Surprisingly, this also revealed three previously uncharacterized high-confidence structures (Supplementary Figure 5A). Using this predicted structure as a template, we then generated seven C-terminal truncation mutants with progressively fewer of these domains or

structures, each with FLAG affinity tags (3xF) (Figure 4A). Following co-transfection with C-terminally Strep-tagged NS4A-2K plasmid we performed FLAG co-immunoprecipitation (co-IP) to evaluate interaction of truncations with NS4A-2K. Intriguingly, FLAG co-IP revealed that each truncation maintained its interaction with NS4A-2K, although Δ54-938 only very weakly interacted (Figure 4B).

This result indicated that the TM domain was important for the interaction with NS4A. Thus, we generated additional mutants with either N-terminal deletions of the TM domain only (Δ2-53), the TM and LEM domains (Δ2-158), the LEM domain only (Δ54-158), or the LEM through ANK domains (Δ54-474) (Figure 5A). Expression and FLAG co-IP of these ANKLE2 mutants revealed that only deletion of both the TM and LEM domains (Δ2-157) ablated interaction with NS4A-2K (Figure 5B). To corroborate this biochemical finding, we visualized the colocalization of these ANKLE2 truncations with NS4A during infection. Here, we clearly observed similar clusters of NS4A that colocalized with wild-type ANKLE2, but neither the Δ2-53 and Δ2-158 mutants. Deletion of the TM region (Δ1-64) has been shown to alter the subcellular localization of ANKLE2 in HeLa cells from the ER to the cytoplasm (56). Consistent with this published result, we observed our Δ2-53 and Δ2-158 ANKLE2 mutants appeared to be distinctly separated from sites of virus replication within the ER (Figure 5C-D, white arrows). Altogether, these results suggest that while the TM domain controls ER localization of ANKLE2, both the TM and LEM regions together contribute to the interaction with NS4A, with presence of at least one being sufficient for the biochemical interaction.

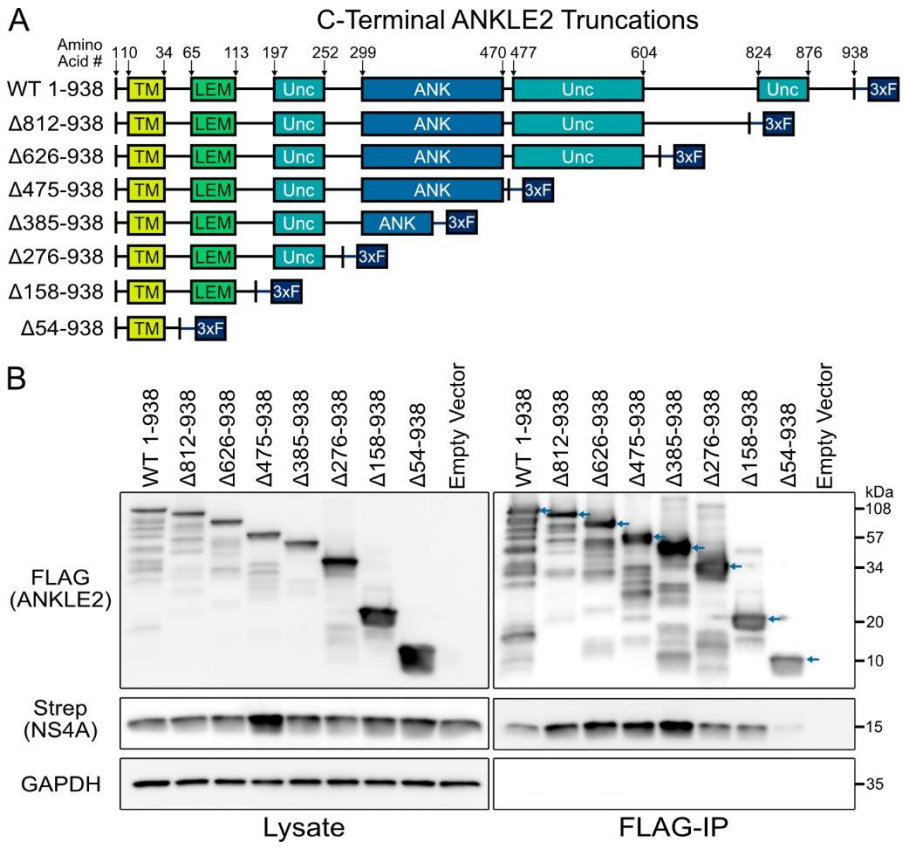

Figure 4: C-terminal ANKLE2 truncations retain interaction with NS4A-2K. A) C-terminal ANKLE2 truncations were designed based on a structural prediction by AlphaFold2. TM = transmembrane domain; LEM = LAP2, emerin, MAN1 domain; Unc = uncharacterized structure predicted by AlphaFold2; ANK = ankyrin repeat domain; 3xF = 3x FLAG affinity tag. **B)** ANKLE2-3xFLAG truncations and NS4A-2K-2xStrep were co-transfected into HEK293T cells to evaluate their interaction with NS4A. Empty vector condition denotes transfection with NS4A-2xStrep plasmid and an empty Strep plasmid to account for potential FLAG-IP background. Primary bands denoted with blue arrows.

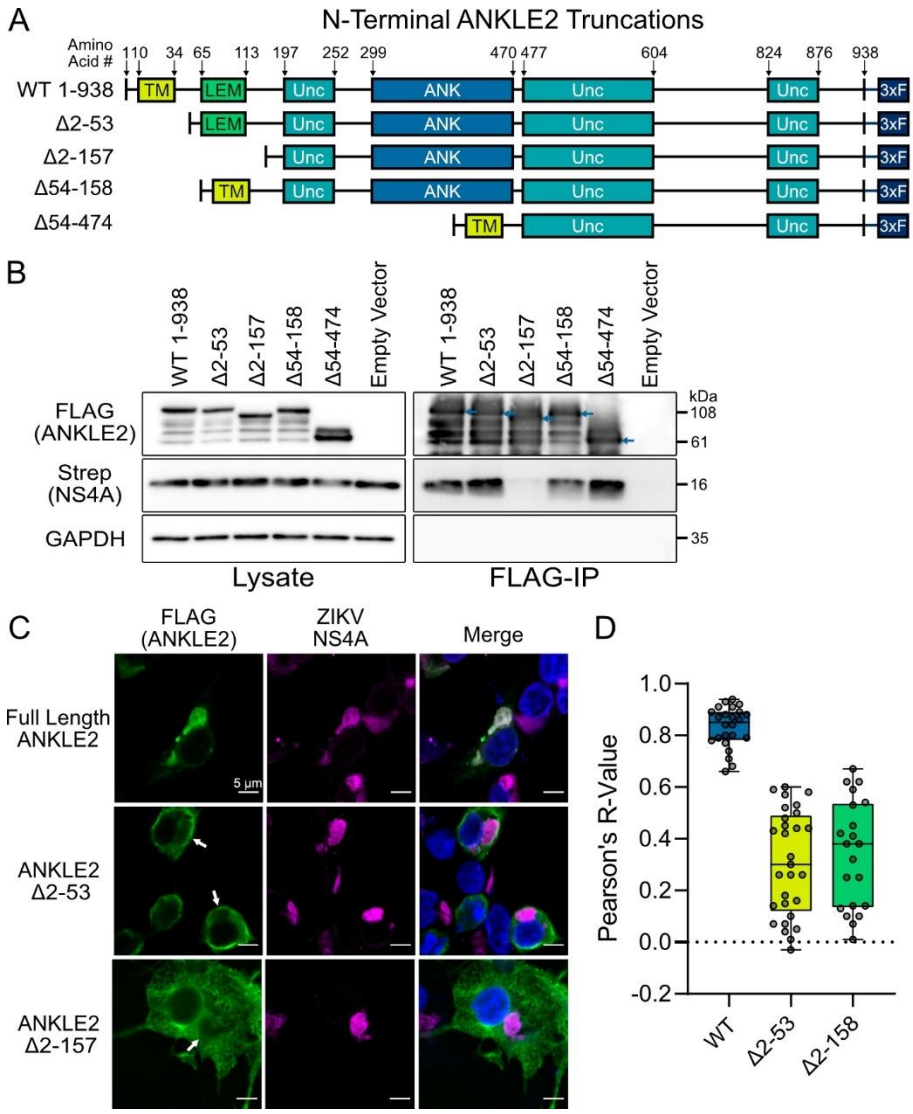

Figure 5: ANKLE2 truncations reveal that ER localization, and the LEM domain are important for interaction with NS4A-2K. A) N-terminal ANKLE2 truncation design. TM = transmembrane domain, LEM = LAP2, emerin, MAN1 domain, Unc = uncharacterized structure predicted by AlphaFold2, ANK = ankyrin repeat domain, 3xF = 3x FLAG affinity tag. **B)** Western blotting of lysate and FLAG-IP samples. Empty vector condition denotes transfection with NS4A-2K-2xStrep plasmid and an empty plasmid instead of one harboring an ANKLE2-3xF sequence to account for potential FLAG-IP background. Primary bands denoted with blue arrows. **C)** HEK293T cells expressing indicated ANKLE2 construct and infected with ZIKV were analyzed by immunofluorescence microscopy for ANKLE2-3xFLAG (green) and NS4A (magenta) localization, and Hoescht nuclear stain (blue). White arrows denote regions where truncated ANKLE2 is excluded from clusters of NS4A. **D)** Pearson's correlation

was quantified from n = 21-29 cells across 5 images per condition. All scale bars = 5 µm. P-values were calculated with a Student's two-tailed T-test.

NS4A-ANKLE2 interaction is dependent on NS4A C-terminal TM domains

To further explore the determinants of the NS4A-ANKLE2 interaction, we sought to generate NS4A truncation mutants. Using a previous evaluation of dengue virus (DENV) NS4A as a template (44), we initially generated plasmids encoding one of three C-terminal truncations, each removing an additional TM domain (Figure 6A). We then expressed these plasmids in HEK293T cells and performed co-IP against the Strep affinity tag to determine interaction with endogenous ANKLE2 (Figure 6B). The first of these truncations, NS4A 1-127, had better expression and interaction with ANKLE2 than our NS4A-2K. This also represents a biologically relevant form of NS4A during ZIKV infection, since the 2K-peptide cleaved to separate NS4A from NS4B (57). The other truncations, NS4A 1-96 and 1-72, had significantly reduced to no visible interaction with ANKLE2, suggesting that at least TM3 is crucial for stabilizing the interaction with ANKLE2. To validate this result, as well as our previous results (Figure 5B), we co-transfected our ANKLE2-3xFLAG and NS4A-2xStrep truncation constructs and performed FLAG Co-IP (Figure 6C). Here, to display the importance of NS4A TM domains, we additionally generated a fourth NS4A truncation that does not express any TM domains (NS4A 1-54). In this experiment we observed that NS4A 1-96 had detectable, but dramatically reduced, interaction with ANKLE2. However, neither NS4A 1-72 nor 1-54 had any detectable interaction, supporting the essential nature of TM2 and TM3. Finally, we evaluated the effect of the 2K-peptide on the interaction between NS4A and ANKLE2 Δ2-158, which had strongly reduced interaction with NS4A-2K previously (Figure 5B). We observed faint and reduced interaction between ANKLE2 Δ2-158 and NS4A-2K, but no detectable interaction between ANKLE2 Δ2-158 and NS4A 1-127, suggesting that the 2K-peptide may stabilize the interaction outside of the TM and LEM domains, but that the processed form of NS4A requires these ANKLE2 domains for interaction.

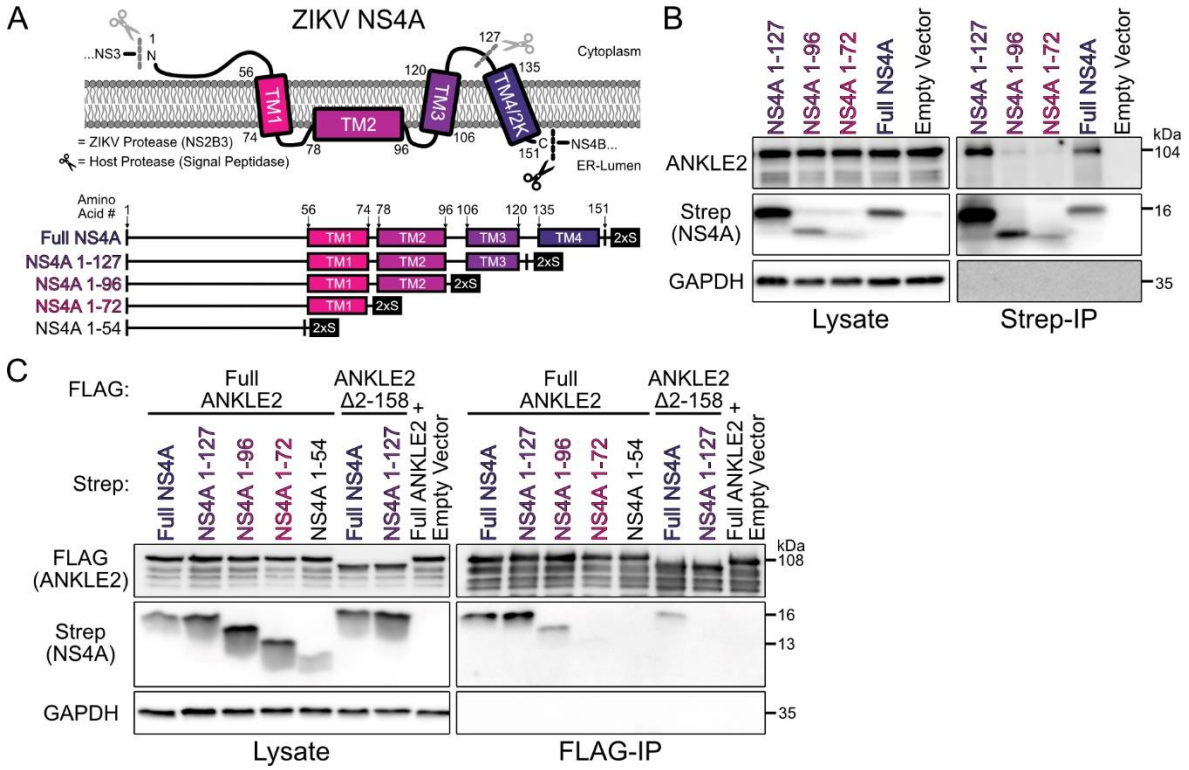

Figure 6: C-terminal TM domains of NS4A mediate interaction with ANKLE2 *in vitro*. A) Schematic of NS4A with annotated amino acid number for predicted transmembrane domains (above). C-terminal truncations mutants were generated based on these domains, each with C-terminal 2xStrep affinity tags (below). TM = transmembrane domain, 2xS = 2xStrep affinity tag. **B)** NS4A truncations were expressed in HEK293T cells and interaction with endogenous ANKLE2 was determined using Strep co-IP. **C)** NS4A truncations and WT ANKLE2-3xFLAG or ANKLE2 Δ2-158 were co-transfected and interaction with NS4A was determined using FLAG Co-IP. ANKLE2-3xFLAG co-transfected with an empty Strep plasmid was used as a control.

Discussion

In this study we explore the relationship between ZIKV and host ANKLE2, with a focus on virus replication. Knockdown of ANKLE2 led to reductions in ZIKV replication at multiple timepoints across a range of MOIs and was present for multiple ZIKV strains. The observed decreases in ZIKV replication

are modest (maximum effect ~8-fold), likely due to the incomplete nature of our knockdown. It is possible that full genetic knockout of ANKLE2 would yield more dramatic virus replication defects.

The supportive role of ANKLE2 in ZIKV replication raises exciting possibilities about ANKLE2 function at sites of replication. Beyond BANF1, PP2A, and VRK-1 (41, 42), ANKLE2 also interacts with many other host proteins. For example, ANKLE2 also influences the cell cycle by interacting with Aurora-A and ERα to mediate ERα phosphorylation (58). Plentiful other ANKLE2-host protein interactions have been found in large proteomic screens (59–62). Presumably the bulk of these interactions are mediated through its ANK domain, which facilitates scaffolding functions in many proteins (63, 64), or by the LEM domain, which is known to mediate interaction with the inner nuclear membrane lamina and BANF1 (65–67). It is particularly appealing to speculate that ZIKV leverages the protein interaction/scaffolding function of ANKLE2 to facilitate protein interactions within replication compartments. Future mechanistic studies exploring ANKLE2 protein interactions in replication compartments will be valuable.

To our knowledge, the NS4A-ANKLE2 interaction is a rare example of a ZIKV-host protein interaction that dysregulates the host function to cause neuropathogenesis (37, 39) in the process of promoting ZIKV replication. Several other protein interactions impact aspects of neurodevelopment but appear distinct from virus replication. ZIKV NS3 cleaves host BMP2, inducing osteogenesis and intracranial calcification commonly seen in CZS (26). Expression of NS2A *in vivo* impacts neurodevelopment by disrupting adherens junctions in radial glial cells (30). Expression of NS4A and NS4B impairs the growth of neural stem cells *in vitro* and perturbs autophagy (28). Yet the host factors involved in pathogenesis were not linked to virus replication directly in these studies. A notable exception is the interaction between ZIKV Capsid and Dicer. Capsid interacts with Dicer and inhibits its antiviral activity to promote ZIKV replication while simultaneously inducing neurodevelopmental defects (34). These types of virus-host interactions that result in compounding losses for the host represent an exciting model system to simultaneously study virus replication and pathogenesis.

Our work provides molecular-level insight into the biophysical nature of the NS4A-ANKLE2 interaction. Co-IP studies revealed that both the TM and LEM domains of ANKLE2 together mediate the interaction. Interestingly, loss of the TM alone is sufficient to disrupt the concentration of ANKLE2 to sites of NS4A during infection, though the biochemical interaction is still possible following deletion of just the TM domain. The TM anchors ANKLE2 to the ER and nuclear envelope, and its loss relocalizes ANKLE2 to the cytoplasm (56). This deletion separates ANKLE2 from sites of replication and away from NS4A, which would otherwise interact with the LEM domain. This does not imply an inherent necessity of the TM domain for the interaction with NS4A. The presence of this domain varies between organisms with no clear phylogenetic separation. Mammalian (human, primate, mouse, etc.) ANKLE2 and the *C. elegans* homolog LEM-4L all contain a N-terminal TM. *Drosophila* Ankle2 lacks a clear TM but does have structured elements at the N-terminus (Supplementary Figure 6). Interestingly, *Drosophila* Ankle2 still localizes to the ER and is inhibited by NS4A (39), suggesting this structured region may serve as a previously uncharacterized TM, or it has another mechanism of ER localization. Moreover, the interaction with NS4A could still be detected biochemically by co-IP for the TM-deleted ANKLE2. This is likely due to mixing of cytoplasmic and ER compartments during lysis, which enables the interaction through the LEM domain even in the absence of correct ER localization. Thus, ER localization and the LEM domain, rather than the TM specifically, are the major contributors to the interaction. Dissecting this interaction with amino acid resolution in the future has the potential to identify ANKLE2 mutants that do not interact with NS4A (or vice versa). This would enable direct testing of the importance of this protein interaction for ZIKV replication. Ultimately it could reveal protective ANKLE2 variants which are functional in brain development, but do not interact with NS4A and thus do not support replication. Thus, this compounded loss scenario for the host could be turned into a compounded win

In summary, we report that knockdown of *ANKLE2* reduces ZIKV replication. During infection, ANKLE2 becomes concentrated at sites of virus replication within the ER. The NS4A-ANKLE2 physical interaction is mediated by the N-terminal TM and LEM domains of ANKLE2, whereas NS4A requires

its C-terminal TM domains. Future studies exploring how ANKLE2 function is hijacked by NS4A in virus replication compartments and which amino acids mediate this interaction will provide molecular mechanistic insight into this virus-host protein interaction involved in replication and pathogenesis.

Methods

Cells. HEK293T (gift of Dr. Sam Díaz-Muñoz), Huh7 (gift of Dr. Raul Andino), and Vero (ATCC) cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco ThermoFisher) supplemented with 10 % fetal bovine serum (FBS, Gibco ThermoFisher) at 37ºC, 5% CO_2. Cells we washed with Dulbecco's phosphate buffered saline (D-PBS, Life Technologies) and dissociated with 0.05% Trypsin-EDTA (Life Technologies). Cells were tested for *Mycoplasma spp.* monthly by PCR.

Plasmids. Lentiviral plasmid (pHR-UCOE-EF1α-KRAB-dCas9-P2A-Bls) encoding a catalytically dead Cas9 (dCas9) with a C-terminal hemagglutinin (HA) tag was a gift from Dr. Sean Collins. For stable, inducible expression in HEK293T cells, ANKLE2 and GFP sequences were amplified by PCR and cloned into pLVX-TetOne-Puro, cut with EcoRI, using Gibson assembly. ANKLE1 (NM_152363.6) sequence was acquired from Twist BioSciences and similarly inserted into pLVX-TetOne-Puro, cut with EcoRI, with C-terminal APEX2 and 3xFLAG affinity tags. ANKLE2 truncations were designed based on AlphaFold2 structural prediction (54, 55) using NM_015114.3 accession sequence (searched August 8th, 2021). Codon optimized DNA fragments with C-terminal 3xFLAG affinity-tags were acquired from Twist Bioscience and inserted into the pcDNA4_TO, cut with KpnI and ApaI, using Gibson assembly. A full length pcDNA4_TO ZIKV NS4A-2xStrep plasmid was previously generated (37), and used as a template for generation of NS4A truncation sequences using PCR amplification. Products were inserted into pcDNA4_TO cut with BamHI and EcoRI upstream of 2xStrep using Gibson assembly. All plasmids were prepared in Stbl3 or DH5α using MiniPrep (Sigma-Aldrich) or MidiPrep (Macherey-Nagel) kits and

verified using sequencing services provided by GeneWiz. Primer sequences used for the generation of all our constructs are available in Table S2.

Lentiviral packaging, transduction, and cell selection. Lentiviral packaging and transduction were performed as previously described (68) using the calcium phosphate protocol (69). In short, 3.5 µg of cloning product plasmid were transfected into HEK293T with lentiviral packaging plasmids including 1.8 µg pMDLg/p-RRE, 1.25 µg pCMV-VSV-g and 1.5 µg pRSV-Rev. After 48 hours lentivirus particles were collected, and cell debris was removed by centrifugation (Eppendorf centrifuge 5810 R, Rotor S-4-104, 94 g, 5 minutes) and filtration through a 0.45 µm filter. The resulting lentiviral stocks were used to transduce HEK293T cells. Transduced cells were bulk selected for puromycin resistance (10 µg/ml, ThermoFisher). A control lentiviral plasmid encoding GFP without a selection marker was used in tandem as a control to ensure efficient packaging, transduction, and selection.

Viruses and stock preparation. All ZIKV stocks were propagated in Vero cells and monitored for CPE. Supernatant was then harvested, and cell debris was removed by centrifugation (Eppendorf centrifuge 5810 R, Rotor S-4-104, 211 g, 5 minutes, 4°C) Cleared supernatant was then distributed into 500 µL aliquots and frozen at -80°C. Each aliquot was only used once to prevent repetitive freeze-thaw. Aliquots were titered by plaque assay (method below). Strains used were ZIKV PLCal/2013 (gift of Dr. Richard Wozniak), ZIKV PRVABC59 (gift of Dr. Lark Coffey), and ZIKV MR766 (BEI Resources, NIAID, NIH, as part of the WRCEVA program: Zika Virus, MR 766, NR-50065).

***ANKLE2* CRISPRi knockdown and ZIKV Infection.** Custom synthetic guide RNAs (gRNA) were acquired from Sigma and resuspended to 3 µM in TE buffer (10 mM Tris Base, 1 mM EDTA, pH 8.0). For Huh7-dCas9 knockdown in 12-well dishes 30 µL of each gRNA was combined with 7 µL TransIT-CRISPR transfection reagent (Sigma) in 363 µL OPTI-MEM (Life Technologies) and complexed at

room-temperature for 20 minutes prior to being added to each well (90nM final gRNA concentration). A total of $1.2x10^5$ Huh7-dCas9 cells were then added and grown overnight at 37ºC. Additional DMEM was then added 24 hpt. Viability experiments were done 72 hpt with ZombieGreen dye (BioLegend, gift of Dr. Scott Dawson) diluted 1:100 in D-PBS and incubated on live cells for 5 minutes. Ten images were taken for each condition and total and dead cells were then counted to determine viability. ZIKV replication after *ANKLE2* knockdown was done by removing media from each well 72 hpt. 2 mL of fresh DMEM and appropriate volume of ZIKV stock was then added to each well. Aliquots of supernatant were harvested at 0, 18, 24, 48, and 72 hpi and frozen at –80ºC.

Plaque assay. Vero cells were grown as a monolayer in 6-well dishes overnight. Virus aliquots were thawed on ice then subjected to 10-fold serial dilution. Media was removed from Vero cells and the monolayer was washed once with 2mL D-PBS. 500µL of each virus dilution was then added and incubated for 1 hour at 37ºC with periodic rocking. Virus was then removed and cells were overlayed with 3 mL of DMEM with 0.8% methylcellulose (Sigma), 1% FBS, 1% penicillin/streptomycin (Thomas Scientific) and incubated at 37ºC for 4 days. Cells were then fixed with 4% formaldehyde (Fisher) for 30 minutes at room temperature. Formaldehyde and media were then removed, and cells were stained with 0.23% crystal violet solution (Fisher) for 30 minutes. Solution was then removed, and plaques were counted.

Quantitative RT-PCR. RNA was harvested using *Quick*-RNA Miniprep kit per manufacturer's instructions (Zymo). Purified RNA (500ng) was then used to make cDNA using iScript™ cDNA synthesis kit (Bio-Rad). After cDNA synthesis each sample was resuspended to a total volume of 100µL using RNase-Free water. A total of 2 µL of cDNA was then used for each qPCR reaction (done in technical triplicates) using LightCycler 480 SYBR Master Mix (Fisher). Samples were run in a Roche LightCycler® 480 II Instrument. Changes in gene expression were calculated using the Livak method ($2^{\Delta\Delta Ct}$) (70).

Confocal immunofluorescence microscopy. HEK293T or Huh7 cells cultured on #1.5 coverslips were fixed with 4% paraformaldehyde (Fisher) for 15 minutes at room temperature. Cells were permeabilized with 0.1% Triton X-100 (Integra) for 10 minutes and blocked with 5% goat serum (Sigma) in PBS-Tween (0.1% Tween-20, Fisher). Coverslips were incubated with primary antibodies overnight at 4°C. Coverslips were then washed in PBS-Tween and incubated in secondary antibody at room temperature for 1 hour. Nuclei were visualized with Hoechst (Invitrogen). Confocal imaging used an Olympus FV1000 Spectral Scan point-scanning confocal fitted to an Olympus IX-81 inverted microscope. Images were acquired using a PlanApo 60x/NA1.40 oil immersion lens. Laser lines at 405, 488, and 543nm were employed sequentially for each image using optics and detector stock settings in the "Dye List" portion of the FluoView microscope-controlling software. All antibodies and dilutions are listed in Supplementary Table S1. Confocal images were analyzed using ImageJ (Fiji) software (71). Signal colocalization was quantified using Pearson's correlation coefficient (R-value) determined with the "Colocalization 2" analysis tool within Fiji after masking individual cells across at least 5 images.

ANKLE2 and NS4A co-Transfection and FLAG affinity-purification. For transfection $5x10^6$ HEK293T cells were plated in 10 cm dishes and grown overnight. Transfection was performed by combining 3.5 µg of each corresponding plasmid DNA with 700 µL of serum-free DMEM. Next, 21 µL of PolyJet transfection reagent (SignaGen) was combined with 700 µL serum-free DMEM and added to each plasmid DNA tube. Samples were mixed and incubated at room temperature for 15 minutes prior to addition to cells. Cells were then grown for an additional 24 hours. Transfection efficiency was confirmed using a GFP encoding plasmid. Media was then removed from each plate. To dissociate cells, 5 mL of D-PBS supplemented with 10 mM EDTA was added and allowed to incubate for several minutes. Cells were resuspended in 5 mL of D-PBS and transferred to 15 mL conical tubes prior to centrifugation 94 g, 4ºC for 5 minutes (Eppendorf centrifuge 5810 R, Rotor S-4-104). Cell pellets were washed with 5 mL D-PBS and centrifugation was repeated. Supernatant was removed and pellets were

then resuspended in 1 mL IP buffer (50 mM Tris Base, 150 mM NaCl, 0.5 M EDTA, pH 7.4) with Pierce™ protease (Thermo Scientific) and Pierce™ phosphatase (Fisher) inhibitor tablets supplemented with 0.5% NP-40 Substitute (Igepal™ CA-630, Affymetrix). Cells were lysed for 30 minutes at 4ºC, and lysate was then centrifugated at 845 g, 4ºC, for 20 minutes (Eppendorf centrifuge 5424 R, Rotor FA-45-24-11). A portion of each lysate (60-100 µL) was collected, normalized by BCA assay (Thermo Scientific), and saved for western blot analysis. Remaining lysate was added to 40 µL of magnetic FLAG beads (Sigma) and incubated overnight at 4ºC with gentle rotation. Beads were then washed 4 times with 1 mL IP buffer with 0.05% NP-40 and once with 1 mL IP buffer without NP-40. Beads were then incubated in 40 µL of 100 ng/mL 3x FLAG peptide (APExBIO) at 211 g for 1 hour at room temperature (Eppendorf ThermoMixerC). Eluate was then removed. Eluate and lysate were resuspended in NuPAGE LDS sample buffer and bond-breaker TCEP (Thermo Scientific) according to manufacturer's recommendation. Samples were boiled for 10 minutes at 95ºC prior to evaluation by western blot (below).

Western blot. For whole cell lysates, cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris Base, 1% Triton X-100, 0.5% sodium deoxycholate) supplemented with protease inhibitors for 5 minutes at room temperature. Cell lysate was incubated on ice for 30 minutes prior to centrifugation (Eppendorf centrifuge 5424 R, Rotor FA-45-24-11, 13,500 g, 4ºC, 20 min). When possible, the total protein concentration of each sample was normalized by BCA assay. Protein samples (lysates or IP eluates) were resuspended in NuPAGE LDS sample buffer supplemented with TCEP and boiled at 95ºC for 10 minutes. Samples were run on 7.5-12% polyacrylamide gels for ~1 hour at 150V and transferred to PVDF membranes (VWR) for 1 hour at 330 mA on ice. Membranes were then blocked in 5% milk solution for 1 hour prior to overnight incubation in primary antibodies (Table S1) at 4ºC. Membranes were washed 3x in Tris-buffered saline with Tween-20 (TBS-T) (150mM NaCl, 20 mM Tris Base, 0.1% Tween-20, Fisher) and incubated with HRP-conjugated secondary antibodies in 5% milk for 1 hour at room temperature. Membranes were again washed 3x in TBS-T and 1x in TBS (without Tween-20)

prior to Pierce™ ECL activation (Fisher). Membranes were imaged using Amersham Imager 600 (GE). Western blot images were analyzed using Fiji. Densitometry was calculated by measuring the band intensity ratio of the experimental band to the loading control band.

Statistical analysis.

Statistical analysis and plotting were performed using GraphPad Prism 6 software (GraphPad Prism 6.0; GraphPad Software Inc., La Jolla, CA, USA). Error bars represent standard deviations. Data were considered statistically significant when a $p < 0.05$ was determined by Student's T-test.

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Author Contributions

ATF and PSS conceived of work and designed experiments. ATF performed and analyzed all experiments. VH, NL, TNS, and STH assisted with experiments. STH, NL, TNS, and SK performed cloning and plasmid preparation. ATF and PSS wrote the manuscript. All authors revised the manuscript. PSS secured funding for work. All authors read and approved the final manuscript.

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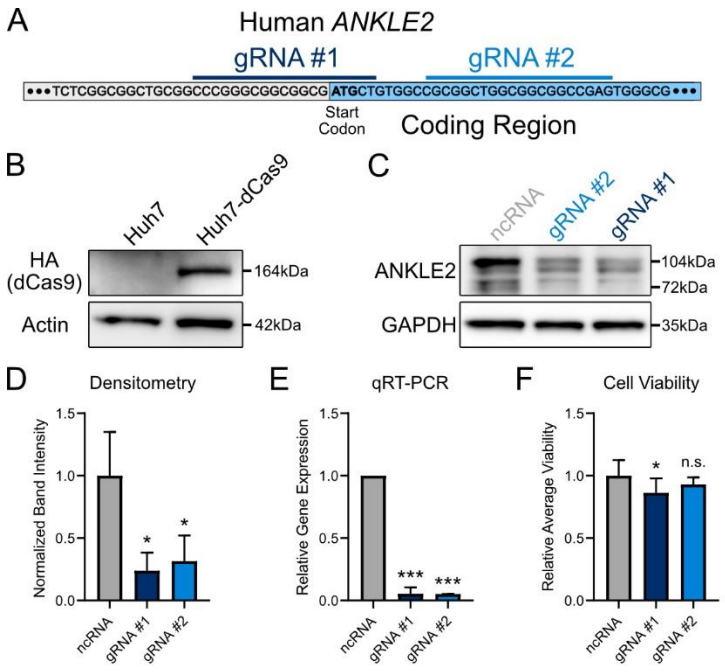
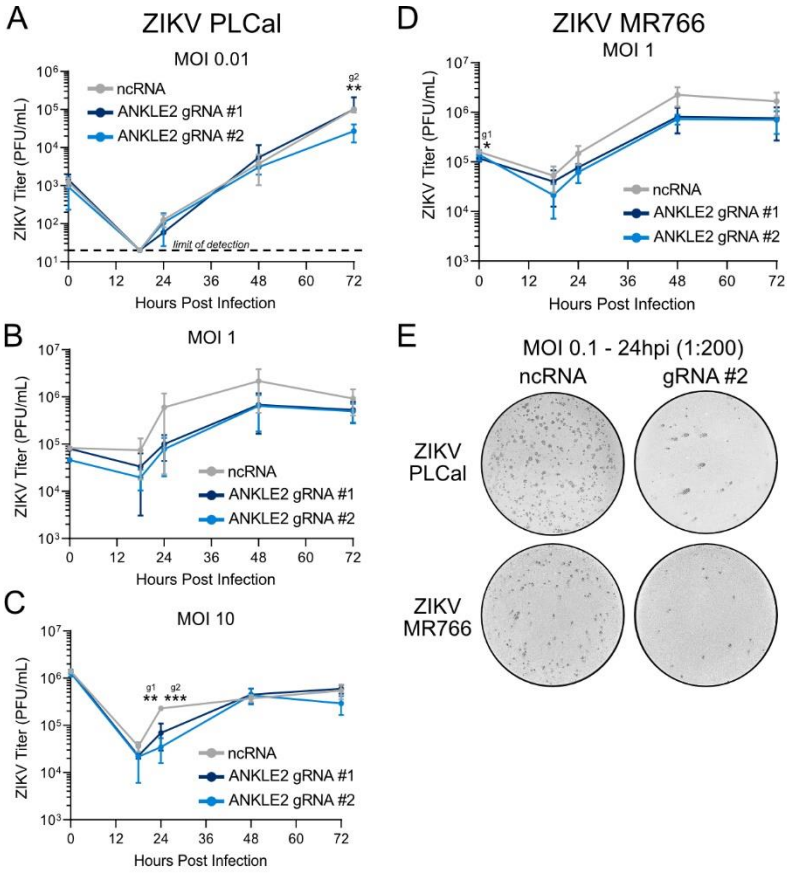
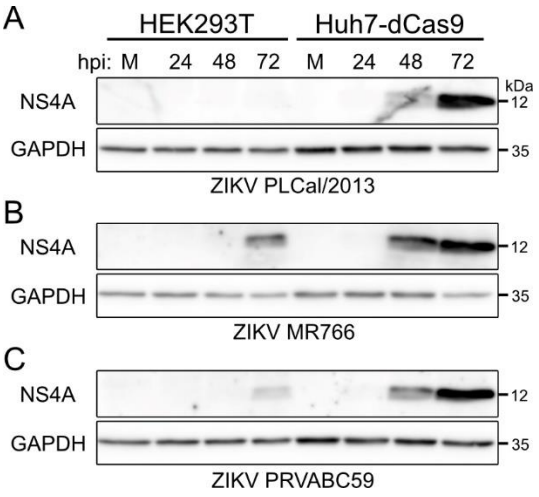
Supplementary Figures



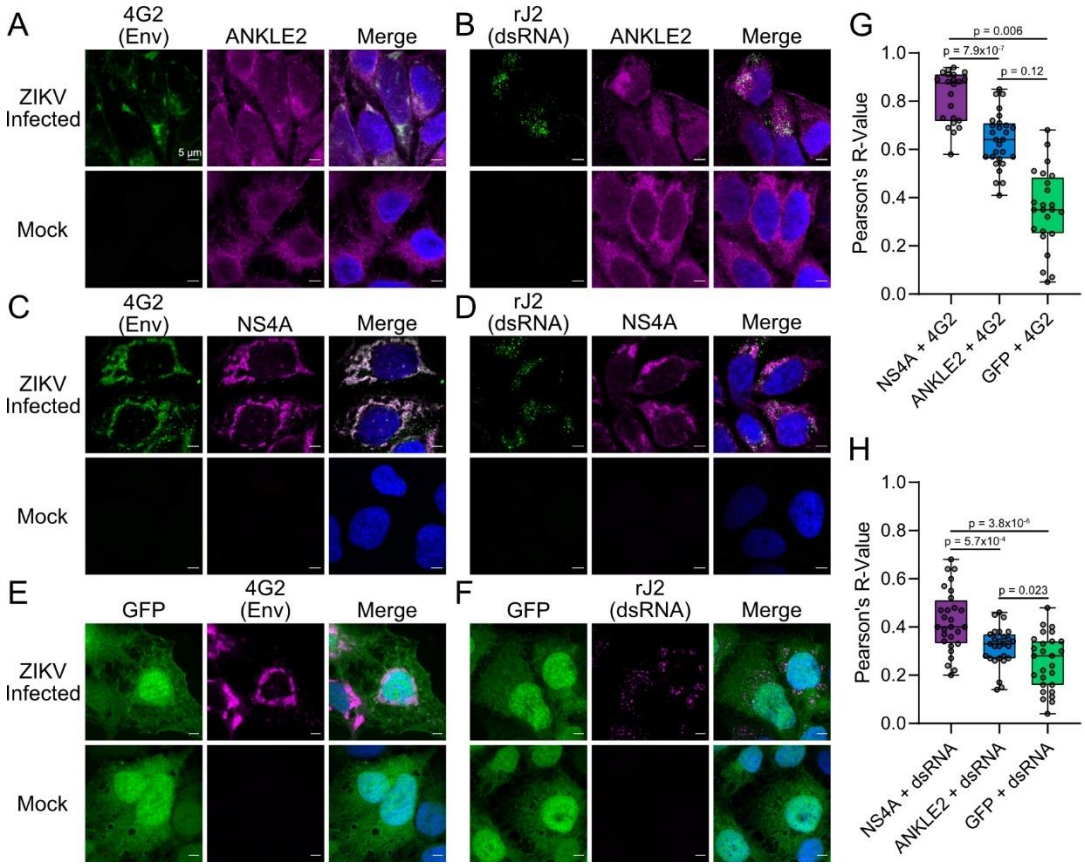
Supplementary Figure 1: Knockdown of *ANKLE2* in Huh7-dCas9 cells using CRISPRi. **A)** Schematic of *ANKLE2* and the targeting guide RNAs (gRNA) that were used to knockdown *ANKLE2* expression. **B)** Western blot to evaluate the expression of dCas9-HA in Huh7 cells that were used for CRISPRi experiments. **C)** Western blot to evaluate ANKLE2 expression after CRIPSRi knockdown with three different gRNAs. The primary isoform is shown at 104kDa, and the knockdown of various other isoforms can be observed at 76 and 72kdA. **D)** Densitometry analysis to quantify ANKLE2 band intensity. ANKLE2 band intensity was normalized to its respective loading control and then normalized to ncRNA. Error bars represent the standard deviation between 3 biological replicates. **E)** *ANKLE2* gene expression after knockdown was quantified by qRT-PCR. Error bars represent the standard deviation between 3 biological replicates. **F)** Cell viability was evaluated using ZombieGreen dye. Data represent the average cell viability across 10 images for each condition. **D-F)** Error bars represent the standard deviation. Student's unpaired two-tailed t-test, compared to ncRNA. n.s., not significant * $p > 0.05$, *** $p > 0.001$.



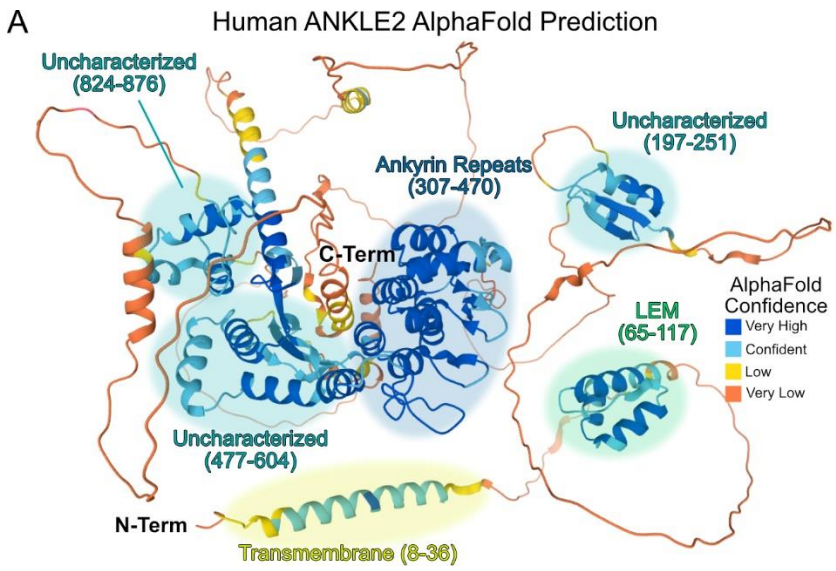
Supplementary Figure 2: Expanded ZIKV infection kinetics in Huh7-dCas9 cells after *ANKLE2* knockdown. A-D) Huh7-dCas9 cells were transfected with noted gRNA for 72 hours then infected with ZIKV PLCal (**A-C**) or MR766 (**D**) at noted MOI for 72 hours. Virus supernatant was harvested at denoted timepoints and titered by plaque assay. Data represent five biological replicates (**B**) or three technical replicates (**A, C-D**). Error bars denote standard deviation between replicates, some error bars not visualized due to small size. Student's two-tailed T-test compared to ncRNA, either paired (**B**) or unpaired (**A, C-D**). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Any other comparisons to ncRNA are not significant. **E)** Representative plaque assay images from matched timepoints, dilution, and replicate for ncRNA and *ANKLE2* gRNA #2. Image artificially changed to grayscale for visual clarity.



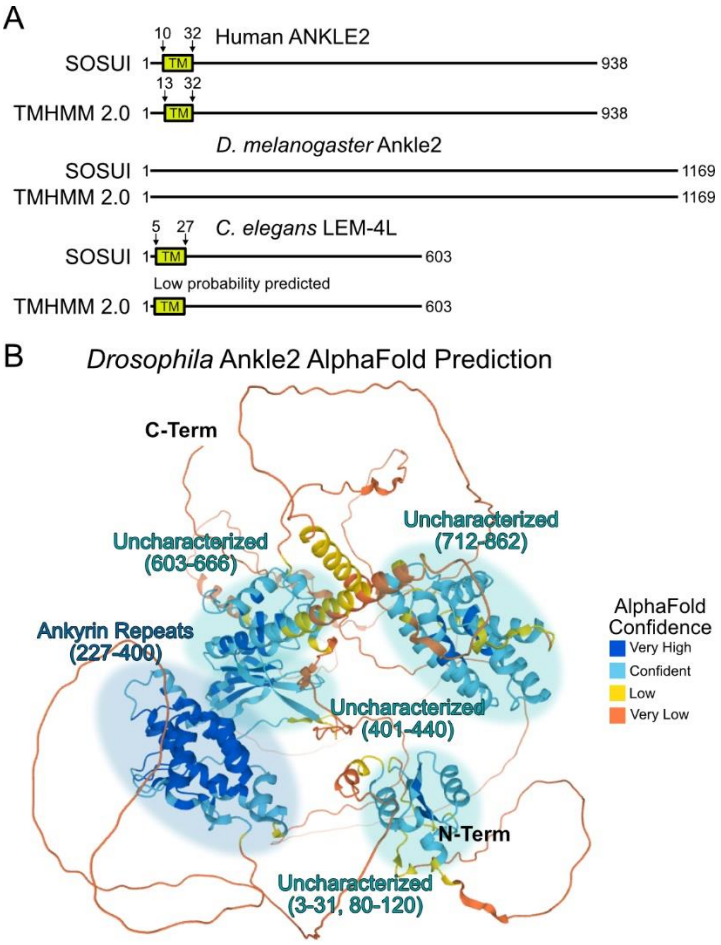
Supplementary Figure 3: Differing ZIKV strain infectivity in HEK293T and Huh7-dCas9 cells. A-C) HEK293T or Huh7-dCas9 cells were infected ZIKV PLCal (**A**), MR766 (**B**), or PRVABC59 (**C**) at MOI 1 for denoted number of hours. Cell lysate was harvested and evaluated by western blot. M = mock infected. All conditions were protein-concentration matched, then probed and imaged in tandem.



Supplementary Figure 4: ANKLE2 colocalizes with sites of ZIKV genome replication in Huh7-dCas9 cells. **A-F)** Huh7-dCas9 cells plated on cover slips were infected with ZIKV for 24-48 hours and subsequently immunostained for with noted antibodies and their target (in the case of 4G2/Env and rJ2/dsRNA). Cover slips were imaged by confocal microscopy and analyzed using ImageJ. All scale bars = 5 µm **G-H)** Pearson's R-value measured using ImageJ colocalization tool. N = 22 to 28 cells across 5 images per condition.



Supplementary Figure 5: AlphaFold2 protein structure prediction reveals uncharacterized structural regions in human ANKLE2. AlphaFold2 was searched for human ANKLE2 and predicted structures were compared against known domains. AlphaFold2 confidence is shown as a gradient from very high (blue) to very low (orange). Known domains are highlighted, ankyrin repeats = dark blue, LEM = green, TM = yellow, uncharacterized = light blue.



Supplementary Figure 6: Transmembrane domain prediction of ANKLE2 homologs and structural prediction of *Drosophila* Ankle2. A) Transmembrane domain prediction of human ANKLE2, *Drosophila melanogaster* Ankle2 (NM_001298446.1), and *C. elegans* LEM-4L. TM prediction was determined using SOSUI and TMHMM 2.0 modeling (72, 73). **B)** AlphaFold2 was searched for *Drosophila* Ankle2 and predicted structures were compared against known domains. AlphaFold2 confidence is shown as a gradient from very high (blue) to very low (orange). Known domains are highlighted, ankyrin repeats = dark blue, uncharacterized = light blue.

Supplementary Table 1: Antibodies

Antibody	Host Species	Dilution Used	Supplier (Catalog #)	RRID
GAPDH	Mouse	1:1000 (WB)	Fisher (PIMA515738)	AB_2537652
FLAG-M2	Mouse	1:200 (IF), 1:1000 (WB)	MilliporeSigma (F1804)	AB_262044
Strep	Mouse	1:1000 (IF, WB)	Qiagen (34850)	AB_2810987
4G2 (Flavivirus Env)	Mouse	1:250 (IF)	ATCC (HB-112)	CVCL_J890
rJ2 (dsRNA)	Mouse	1:40 (IF)	MilliporeSigma (MABE1134)	AB_2819101
ANKLE2	Rabbit	1:80 (IF)	Atlas Antibodies (HPA074838)	N/A
ANKLE2	Rabbit	1:1000 (WB)	Sigma (SAB2701489)	N/A
ZIKV NS4A	Rabbit	1:1000 (IF, WB)	GeneTex (GTX133704)	AB_2887067
Actin	Rabbit	1:5000 (WB)	Sigma (A2066)	AB_476693
Anti-Mouse IgG-HRP	Rabbit	1:5000 (WB)	SouthernBiotech (6170-05)	AB_2796243
HA	Rat	1:2000 (WB)	MilliporeSigma (3F10) Gift from Dr. Joanna Chiu	AB_2314622
Anti-Rabbit IgG-HRP	Goat	1:5000 (WB)	SouthernBiotech (4030-05)	AB_2687483
Anti-Mouse AlexaFlour-488	Goat	1:1000 (IF)	Invitrogen (A28175)	AB_2536161
Anti-Rabbit AlexaFlour-555	Goat	1:1000 (IF)	Invitrogen (A27039)	AB_2536100
Anti-Mouse AlexaFlour-680	Goat	1:1000 (IF)	Invitrogen (A21057)	AB_2535723

WB = Western blot, IF = Immunofluorescence

Supplementary Table 2: Oligonucleotides

Oligo Name	Sequence	Application
GAPDH Forward	ACATCGCTCAGACACCATG	qPCR Control
GAPDH Reverse	TGTAGTTGAGGTCAATGAAGGG	qPCR Control
ANKLE2 Forward	AAAGAGAACCAGGCTTCCATC	qPCR Analysis
ANKLE2 Reverse	CACGTTGACTACATCTGCATTTC	qPCR Analysis
NS4A Forward	CTTGGTACCGAGCTCGGATCCGCCACCATGGGAGCCG	Gibson Assembly of ZIKVpr NS4A C-terminal truncations
NS4A 1-54 Reverse	CATCCACCGCCTCCCTCGAGCGTTTCGAGAGTTTCCGGGAG	Gibson Assembly of ZIKVpr NS4A 1-54
NS4A 1-72 Reverse	GTGCTGGATATCTGCAGAATTCAAGGACGAAAAATATACCGAGAGAGACG	Gibson Assembly of ZIKVpr NS4A 1-72
NS4A 1-96 Reverse	GTGCTGGATATCTGCAGAATTCCCACATCAGCCATGCGC	Gibson Assembly of ZIKVpr NS4A 1-96
NS4A 1-127 Reverse	GTGCTGGATATCTGCAGAATTCGCGTTGCTTCTCCGGCT	Gibson Assembly of ZIKVpr NS4A 1-127
GFP Forward #1	TAAAGGCCGCCATGGTGAGCAA	Gibson Assembly preparation of GFP-APEX2-3xFLAG
GFP Reverse #1	CGCTCTTGTACAGCTCGTCCAT	Gibson Assembly preparation of GFP-APEX2-3xFLAG
GFP Forward #2	CTTATACCAACTTTCCGTACCACTTCCTACCCTCGTAAAGGCCGCCATGGTGAGCA	Gibson Assembly of GFP-APEX2-3xFLAG
GFP Reverse #2	CAGATCCACCTCCTGAACCACCTCCGCTACCGCCACCGCTCTTGTACAGCTCGTCCATGC	Gibson Assembly of GFP-APEX2-3xFLAG
ANKLE2 Forward #1	TAAAGGCCGCCATGCTGTGGCCGCGG	Gibson Assembly preparation of ANKLE2-APEX2-3xFLAG
ANKLE2 Reverse #1	GCTCAGGGCGGCAAGCTCAGCCAGG	Gibson Assembly preparation of ANKLE2-APEX2-3xFLAG
ANKLE2 Forward #2	ACTTCCTACCCTCGTAAAGGCCGCCATGCTG	Gibson Assembly of ANKLE2-APEX2-3xFLAG
ANKLE2 Reverse #2	CCGCTACCGCCACCGCTCAGGGCGGCAAG	Gibson Assembly of ANKLE2-APEX2-3xFLAG
ANKLE1 Forward #1	GCCGCCATGTGTTCCGAAGCCC	Gibson Assembly preparation of ANKLE1-APEX2-3xFLAG
ANKLE1 Reverse #1	CGCTTCCACGCGCTTGAATATCT	Gibson Assembly preparation of ANKLE1-APEX2-3xFLAG
ANKLE1 Forward #2	ACTTCCTACCCTCGTAAAGGCCGCCATGTGT	Gibson Assembly of ANKLE1-APEX2-3xFLAG
ANKLE1 Reverse #2	TCCGCTACCGCCACCGCTTCCACGCG	Gibson Assembly of ANKLE1-APEX2-3xFLAG
Mycoplasma Forward #1	TGCACCATCTGTCACTCTGTTAACCTC	For *Mycoplasma sp.* detection
Mycoplasma Reverse #1	GGGAGCAAACAGGATTAGATACCCT	For *Mycoplasma sp.* detection
Mycoplasma Forward #2	GGCGAATGGGTGAGTAACACG	For *Mycoplasma sp.* detection
Mycoplasma Reverse #2	CGGATAACGCTTGCGACCTATG	For *Mycoplasma sp.* detection