Abstract

Zika virus gained notoriety in recent years due to the outbreak in the Americas in 2015-2016 that caused congenital microcephaly in unborn children of mothers infected during pregnancy. The mechanism is not fully understood as of now. Proteomics research has shown that viral protein NS4A interacts with host protein ANKLE2 which is linked to microcephaly. Further research in *Drosophila* has confirmed that neural cell development is impaired in an ANKLE2 dependent manner when subjected to ZIKV NS4A, showing that this interaction is a likely culprit for microcephaly. Here we attempt to elucidate whether the interaction between NS4A and ANKLE2 is beneficial for the virus as we hypothesized that ZIKV uses this interaction to facilitate its own replication. In our experiments we found a large reduction of ZIKV replication in ANKLE2 KO Huh7, JEG-3 and A549 cells. We also saw that other Asian lineage ZIKV reciprocated this reduction in replication and that African lineage MR766 replication was unaffected by the knockout of ANKLE2. Additionally, we discovered that the role of ANKLE2 in viral replication is somewhat conserved in related flaviviruses. We also found a slight restoration of viral replication when ANKLE2 was reintroduced in one knockout clone, but an increase in another. All in all, our data shows that ANKLE2 facilitates the replication of ZIKV and that a role of ANKLE2 in viral replication is conserved across several different flaviviruses.

Introduction

Zika virus (ZIKV) is an arthropod-borne virus (arbovirus) in the genus flavivirus and the family *Flaviviridae*. The genus flavivirus contains some other well-known viruses such as dengue virus (DENV), Yellow fever virus (YFV) and West Nile virus (WNV). ZIKV gained notoriety after an outbreak in the Americas during 2015-2016. This enveloped positive sense single stranded RNA virus is spread primarily through the bite of infected mosquitos of the *Aedes* genus, with infections in humans being caused mostly by *Aedes aegypti* while other *Aedes* species are responsible for infections in other mammals. Human to human infection is also possible but this route of infection is only responsible for a small percentage of total infections. Non-human primates can act as a reservoir to sustain ZIKV in the wild when no outbreak is active in humans[1].

The Flavivirus genome consists of 11 kb of positive-sense ssRNA that encodes for one polypeptide that is subsequently cleaved in 10 different proteins. Capsid (C), membrane (M) and envelope (E) function as structural proteins. NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 are non-structural proteins that are responsible for replication or immune evasion. The non-structural proteins also hijack host machinery to facilitate viral replication[2]. Interactions between viral and host proteins are essential for successful virus replication due to the low amount of unique viral proteins in the ZIKV genome[3].

Human infections with ZIKV usually manifest either asymptomatically or with mild symptoms. In very rare cases it can result in Guillain-Barré syndrome, a neurological condition that mostly affect the extremities[4]. During the epidemic in 2015-2016 it

became apparent that ZIKV could also cause neurological defects in unborn children from mothers who were infected with ZIKV during pregnancy. These conditions are referred to as Congenital Zika syndrome (CZS)[5]. CZS consist of a spectrum of different clinical manifestations including congenital contractions, cortical calcifications, ocular anomalies, and microcephaly in severe cases. Microcephaly is an affliction where children are born with significantly reduced head and brain size. This is associated with various complications which can persist even later in life such as delays in development, intellectual disabilities and a predisposition to seizures[6]. Aside from ZIKV, microcephaly can also be caused by infections with other pathogens during pregnancy, genetic mutations, and through exposures of to certain toxins during pregnancy[7].

The mechanism by which ZIKV causes microcephaly is not fully understood. It is known that ZIKV is capable of infecting placental tissue, potentially allowing ZIKV to access the fetal compartment[8,9]. Additionally, ZIKV has the capability to breach the blood-brain barrier allowing infection of neuronal tissues[10,11]. Infection leads to tissue damage through cytopathogenic effect (CPE) and dysregulation of developmental pathways[12].

Proteomics research has been done with ZIKV proteins to establish protein-protein interactions between host and viral proteins using affinity purification and mass spectrometry. This revealed many different host virus interactions of which the interaction between viral NS4A and host ANKLE2 was the most interesting for future research[13]. Genetic defects in ANKLE2 were already linked to congenital microcephaly in previous human population studies[14]. Further studies on Ankle2 have shown that brain development of *Drosophila* gets perturbed when *ankle2* is mutated. Reintroduction of Ankle2 rescued normal development, this phenotype was also rescued when human ANKLE2 was introduced[14].

ANKLE2, also referred to as LEM4 or MCPH16, is named after the ankyrin-repeat domain and LEM domain it contains. The LEM domain is shared by several proteins with widely varying sequences outside of the LEM domain. The LEM domain allows interaction with barrier-to-autointegration factor (BAF); this protein's main role is generally believed to link other proteins to chromatin. ANKLE2 and ANKLE1 are unique among these proteins with them containing an ankyrin-repeat motif. But both their function and sequence are widely different from each other. This motif is found within many structural and regulatory proteins and is used to mediate protein-protein interactions. ANKLE2 is involved in post mitotic nuclear reassembly[15].

When ZIKV NS4A was ubiquitously expressed in *Drosophila* larva it caused perturbation in an ANKLE2 associated manner at the 3rd larval instar. When either human or fly ANKLE2 was expressed into the same system, neural development returned to normal[13]. This showed that ZIKV NS4A is capable of perturbing neural development in flies and marking it as a likely culprit for microcephaly in unborn children.

Viruses need to use a plethora of host proteins for their replication and ZIKV is no exception. This leaves the question whether NS4A purposefully hijacks ANKLE2 to

facilitate viral replication, or if this is just a random interaction between proteins that unfortunately results in CZS.

In this project we attempted to elucidate whether ANKLE2 benefits the replication of ZIKV or if the interaction between ZIKV and ANKLE2 is a random interaction that serves no benefit for the virus. Various human cell lines were used where we knocked out ANKLE2 with CRISPR-Cas9 mutagenesis. These cells were infected with ZIKV strains at various MOIs, and supernatant was harvested at set time intervals. This supernatant was subsequently tittered via plaque assay to determine whether the deletion of ANKLE2 had an impact on viral replication. Additionally, DENV and YFV, two relatives of ZIKV, were also tested to see if the effect of ANKLE2 knockout would be preserved in other flaviviruses.

Layman's summary

Zika virus became well known in recent years due to an outbreak in the Americas in 2015-2016. This virus spreads though the bites of infected yellow fever mosquitos (*Aedes aegypti*) that are found in the warmer regions of Africa, Asia, and the Americas. Zika virus typically leads only to minor disease symptoms in healthy adults. However, when a pregnant woman is infected, the virus can pass to her fetus and lead to birth defects. Children being born with a condition called microcephaly, where the head and brain are abnormally small, is the most well-known of these birth defects. Microcephaly leads to many mental handicaps and developmental problems throughout life. How Zika virus infection leads to microcephaly is not fully understood at this point of time.

Zika virus infects the cells of its host and multiplies using the biological machinery that is present within these cells. To do this the virus hijacks various host proteins within these cells to utilize their functions to help the virus achieve things the virus cannot do with its own proteins. Earlier research has found a possible mechanism that could be behind microcephaly. This research found that Zika virus interacts with a host protein called ANKLE2. Naturally occurring mutations in this protein have been seen in children with microcephaly.

In this project we wanted to find out if Zika virus purposefully hijacks ANKLE2 to facilitate its own replication or if this is just an accidental interaction that has no benefit for Zika virus.

To do this we made cells that could no longer produce the ANKLE2 protein. We then infected these cells with Zika virus and measured the number of infectious viruses present at specific time points after adding the virus. We found that Zika viruses could not multiply as well when the ANKLE2 protein was no longer present within the cell. We tested this for liver, lung, and placental cells to ensure this was not specific to a particular cell type. In addition, we also tested this with different strains of Zika virus to ensure it was not strain specific. These results show that the presence of the ANKLE2 benefits the multiplication of Zika virus in some way.

We were also curious if the ANKLE2 protein was used by relatives of Zika virus. So, we infected cells without ANKLE2 with either dengue virus or yellow fever virus and measured the number of infectious viruses after specific timepoints. We found that the

multiplication of dengue virus was altered when the ANKLE2 protein was no longer present. The effect on yellow fever virus multiplication was very small though.
It is important to further understand how Zika virus causes microcephaly and how these interactions might benefit the virus. As, the mosquito species that transmits Zika virus is spreading to more countries due to global warming it is becoming more important to understand microcephaly and how to manage it as a future Zika virus outbreak is very possible. And this information could help us develop possible treatments for Zika virus disease related issues.

Materials and Methods

Cell culture

Huh7 (gift of Dr. Raul Andino), Vero (ATCC), A549 (ATCC, CCL-185), Jeg-3 (ATCC) were cultured in Dulbecco modified Eagle's medium (DMEM, Gibco ThermoFisher) supplemented with 9% fetal bovine serum (FBS, Gibco ThermoFisher). Cells were kept at 37˚C and 5% CO_2 inside an incubator. Cells were washed with Dulbeccos's phosphate buffered saline (D-PBS, Life technologies) and dissociated with 0.05% Trypsin-EDTA (Life Technologies). Cells were tested for *mycoplasma spp.* via MycoStrip™ (InvivoGen)

ANKLE2 knockout cell-lines and Rescue cell-lines

Knockouts were made from Huh7, A549 and Jeg-3. These knockout cell-lines were made with CRISPR-CAS9 by our lab members. The gRNA sequence of AGTTCCTCCGCCAAGCGCGGC was obtained from the MIT library. These were ligated into the BsmBI site of a plasmid backbone. Plasmids were packaged into lentivirus us a 4-way transfection with RSV-Rev, GagPol and VSV-g in HEK293T cells. Lentivirus was harvested after 48-hour incubation at 37˚C by collecting and filtering supernatant. Huh7, A549 and Jeg-3 cells were transduced with lentivirus for 48 hours and selected with puromycin and validated using western blot. Knockouts were then clonally selected in a 96 wells plates. These clones were screened via western blot. ncgRNA control cell-lines were made with the same method minus the clonal selection. Huh7 Knockout clone sequence was verified by harvesting RNA, this was converted into cDNA using reverse transcriptase and used as a template for PCR amplification of the *ANKLE2* target site. This product was purified and sent to Azenta for AmpliconEZ sequencing.
Huh7-ANKLE2 rescue cells were made using the same lentiviral transduction method with a pFUGW plasmid with a Zeomycin resistance gene. These plasmids also encode for either ANKLE2-3xFLAG or GFP-3xFLAG. Cells were selected with 400µg/ml of Zeomycin and validated with Western Blot.

Virus and stock virus preparation

All virus stocks were propagated in Vero cells. Supernatant was harvested after CPE occurred at multiple timepoints. Cell debris was removed by centrifugation (Eppendorf centrifuge 5810 R, Rotor S-4-104, 211g, 5 minutes, 20˚C). 1mL aliquots were transferred in Eppendorf tubes and subsequently stored at -80 ˚C. These aliquots were

tittered by plaque assay as described below. Aliquots were only used once to prevent multiple freeze thaw cycles. Strains used were ZIKV PLCal2013 (Gift of Dr. Richard Wozniak), ZIKV PRVABC59 (Gift of Dr. Lark Coffey), ZIKV MR766 (BEI), DENV-2 16881 (Isolated by *Kinney et all*[16]), and YFV-17D (Gift of Dr. Lark Coffey).

Viral Infection of cell-lines

Huh7, A549 and Jeg-3 Ankle2 KO cells were seeded in a 6 wells plate at a density of 4e5 cells per well and left to incubate overnight at 37 ˚C. The following day the virus was thawed slowly in ice over the course of an hour and the media was removed from the cells via aspiration. 2-3 mL of DMEM was added to each well via pipetting to ensure equal volumes in each well. Virus was added at either MOI 0.1, 1 or 10. After 0-, 18-, 24-, 48- and 72-hours aliquots of supernatant were harvested and stored at -80 ˚C for future use. For ZIKV and YFV 80 µl aliquots were taken and for DENV 120 µl aliquots were taken. Aliquots were used only once to prevent multiple cycles of freeze thawing. After the 72 hpi aliquot has been taken the media and virus were removed from the cells and the cells were washed 1x with d-PBS. The cells were subsequently lysed for 5 minutes with RIPA buffer (150 mM NaCl, 50 mM Tris Base, 1% Triton X-100, 0.5% Sodium deoxycholate) supplemented with protease inhibitors. Lysates were removed from the well and gently spun at 4˚C for 30 minutes and stored at -20 ˚C until further use.

Western Blot

Protein contents of lysates were normalized via a Pierce™ BCA protein assay kit (Thermo scientific) performed according to manufacturer's protocol. Protein samples were resuspended in NuPAGE LDS sample buffer supplemented with TCEP and boiled for 10 minutes. Samples were run on 12% polyacrylamide gel for ~ 1 hour. Samples were subsequently transferred to PVDF membranes (VWR) for 1 hour at 330mA in ice or in a refrigerator. Membranes were blocked with 5% Milk for 1 hour before being incubated with primary antibody **(Table S1)** overnight. Membranes were washed 3 times in Tris-buffered saline with Tween-20 (TBS-T) (150 mM NaCl, 20mM Tris Base, 0,1% Tween-20, Fisher) and incubated with secondary antibody conjugated with HRP for 1 hour in 5% milk at room temperature. Membranes were washed 3x in TBS-T and 1x in TBS without Tween-20. Membranes were activated with Pierce™ ECL (Fisher) and imaged with an Amersham imager 600(GE). Images were analyzed with Fiji.

Plaque assays

Vero cells were plated with a density of 1e6 cells per well in a 6 wells plate and incubated overnight at 37 ˚C in an incubator. Viral harvest aliquots were thawed in ice and subjected to 10-fold serial dilutions in a deep well plate. Media was removed from the Vero cells, and the monolayer was washed once with 1 mL D-PBS. For ZIKV and YFV 500 µl of virus dilution was added on top of the monolayer and incubated for 1 hour with periodic rocking. For DENV 800 µl virus dilution was added and was incubated for 2 hours with periodic rocking. After incubation, the virus was removed and cells were overlayed with 3mL DMEM with 0.8% methylcellulose (Sigma), 1% FBS and 1% penicillin/streptomycin (Thomas Scientific). Cells were incubated at 37 ˚C for 4 days if infected with ZIKV, 7 days for YFV and 8 days for DENV. Cells were subsequently fixed

with 4% formaldehyde (Fisher) for 30 minutes at room temperature. Media and formaldehyde were removed, and the cells were washed with water and 1 mL of 0.23% Crystal violet (Fisher) was added. After 30 minutes the solution was removed, the cells were washed, dried and the plaques were counted manually using Fiji.

Statistical analysis

Statistical analysis was performed via Microsoft Excel (Microsoft office 365) and data plotting was done with GraphPad Prism 6 software. (GraphPad Prism 6.0; GraphPad Software Inc., La Jolla, CA, USA). Error bars represent standard deviation and data was considered statistically significant if a p value of below 0.05 was determined by using a Student's T-test.

Results

Replication of ZIKV PLCal is reduced in Huh7-ANKLE2 knockouts at 3 different MOIs

First, we tested Huh7 ANKLE2 KO clones for the impact ANKLE2 knockout has on viral replication. This hepatic cancer cell line was chosen as a model for its ability to effectively replicate ZIKV, and the liver is a site of natural infection of ZIKV in mammals. Huh7 ANKLE2 KO clone 1A and 1C were seeded in a 6 wells plate together with a ncgRNA control. These control cells underwent the CRISPR-Cas9 procedure with scrambled gRNA instead of gRNA targeting *ANKLE2.* The KO and control cells were infected with the ZIKV PLCal (Thailand, 2013) strain at a multiplicity of infection (MOI) 1. Clinically the Asian lineages strains PRVABC59 and PLCal are more relevant as they are the culprit of the recent outbreaks, including the outbreak in the Americas[17]. Supernatant was harvested at 0-, 48- and 72-hours post infection (hpi) and was tittered via plaque assays to determine viral replication. The results show an about 1~2 $\log_{10}$ decrease in viral titers in Huh7 ANKLE-2 KO clone 1C compared to the ncgRNA control in both 48 and 72 hpi. Clone 1A also showed a decrease in viral replication compared to the ncgRNA control although at a much lesser extend of around 0.5~1 $\log_{10}$ difference ***(Figure 1A)***. This data shows that the deletion of ANKLE2 inhibits viral replication, establishing that ANKLE2 has a role in the replication of ZIKV. We also tested whether this reduction in viral replication is preserved at different MOI and to test which MOI is the most efficient to test in future experiments. Huh7 knockout cells were infected with PLCal at either MOI 0.1 or 10. The data shows that the reduction in viral replication is reciprocated in both MOI 0.1 and 10. However, MOI 10 shows a much smaller reduction than the other MOIs and there is no reduction in replication for 1A after 72hpi ***(Figure 1A-C)***. This is most likely caused by the fact that a plateau of virus titers has been reached. The difference between clone 1A and 1C is also preserved between all MOIs. This data shows that there is a very small difference between MOI 0.1 and 1. However, MOI 0.1 shows a slightly larger difference in phenotype and will be used for most future experiments. MOI 10 seems to plateau after only 48 hpi and will therefore not be used for any future experiments.

A western blot was done on lysates harvested 72 hpi of Huh7 ANKLE2 KO clones and ncgRNA controls infected with PLCal at MOI 0.1 and 1. Host ANKLE2 was stained to show viral NS4A and capsid were stained to show the measure of viral infection and GAPDH served as a loading control. The results show an effective knockout of ANKLE2 in both KO clones. Similar to the plaque assay results there is a reduction of both viral proteins in the ANKLE2 KO clones and the decrease in capsid is larger in clone 1C than in clone 1A ***(Figure 1D)***. Sequencing data reveals that the KO in clone 1C is more effective than clone 1A potentially explaining why the reduction in viral replication is larger in clone 1C.

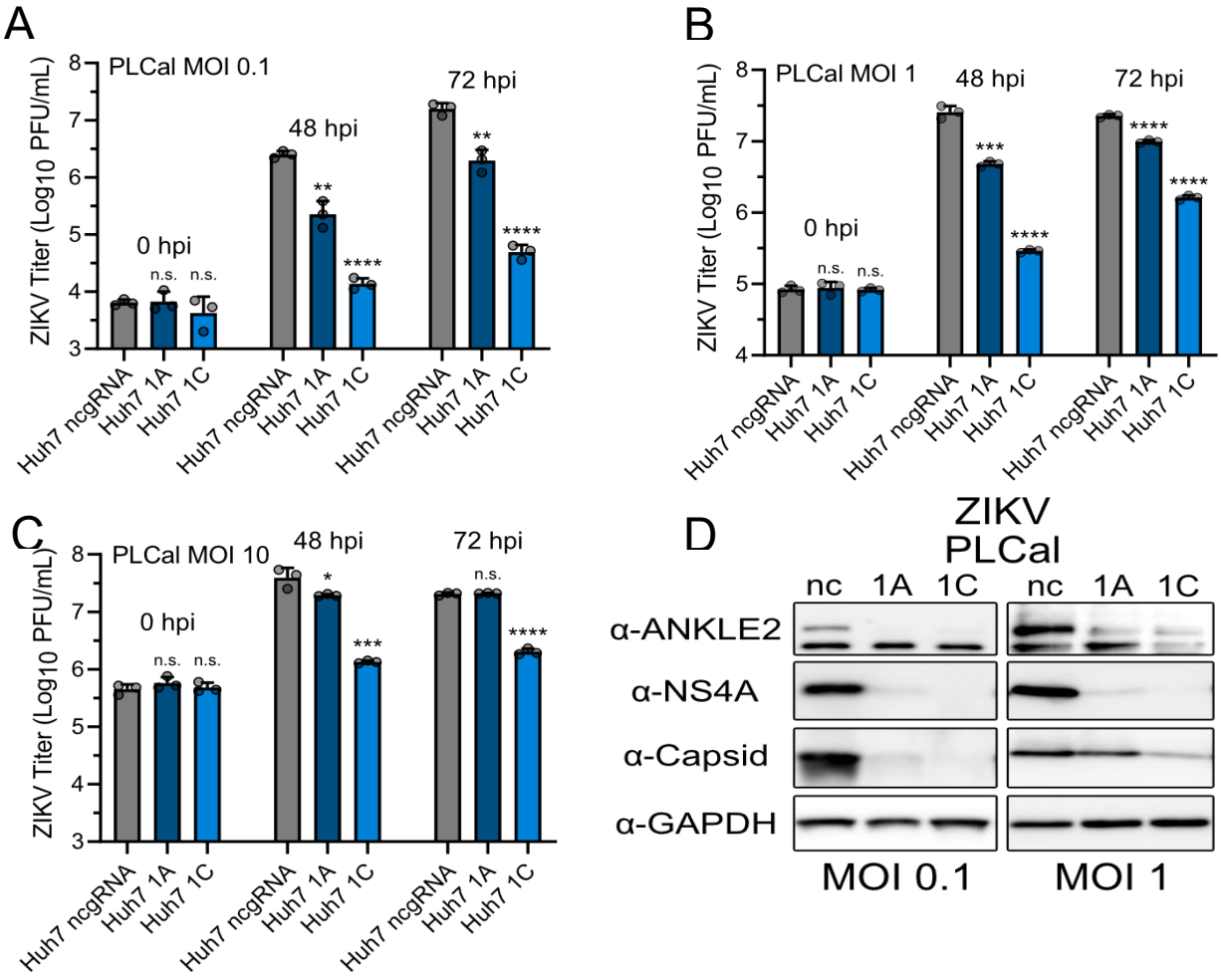

Figure 1: Reduction of viral replication in Huh7 ANKLE2 KO cell-lines. (A,B,C) *PLCal replication in Huh7 ANKLE2 KO (clone1A and 1C) and ncgRNA control cells were measured over a 72h time period tittered via plaque assay expressed PFU/mL in log_{10} scale. * Represent the P value of the statistical analysis (* = 0.05, ** = 0.01, *** = 0.001 and **** = 0.0001). All conditions were done in triplicate with each dot representing a replicate. Cells were infected at a MOI of **(A)** 0.1, **(B)** 1 and **(C)** 10. At both MOI 0.1 **(A)** and 1 **(B)** there was a significant reduction of viral replication in both KO clones. The knockout was stronger in 1C. For MOI 10 **(C)** there was a significant reduction for 1C but the reduction for 1A was negligible. **(D)** shows a western blot of lysates of both MOI 0.1 and 1 after 72 hpi stained for host ANKLE2, GAPDH, viral NS4A and viral capsid. Viral proteins visualize the scale of viral infection. ANKLE2 stain visualizes the effectivity of the knockout.*

Reduction of replication in ANKLE2 KO Huh7 is not shared between Asian and African lineage ZIKV

Next, we wanted to see if this phenotype was preserved in other ZIKV strains, so we infected Huh7 ANKLE2 knockout cells with either the Asian lineage PRVABC59 (Puerto Rico, 2015) at MOI 0.1 or the African lineage MR766 (Uganda, 1947) at MOI 0.01. We tested only clone 1C for PRVABC59 since it showed the largest reduction in the last experiment. We chose MOI 0.01 for MR766 due to preliminary experiments in the lab resulted in high titers reaching a plateau phase at an early timepoint possibly obfuscating a phenotype. Here we found that PRVABC59 also had a 1 log_{10} reduction in replication in clone 1C showing that a reduction in replication also occurs for PRVABC59 confirming that this phenotype is preserved across different Asian lineage ZIKV strains ***(Figure 2A)***. On the other hand, MR766 showed no decrease in viral replication in either clone ***(Figure 2B)***. However, MR766 was isolated in 1947 and cultured in mouse brains before stable cell lines were established and was passaged many times in stable cell-lines once available. As a result, this virus has genetically adapted to stable cell lines and a lab environment which may result behavior different from clinically relevant strains[18]. Therefore, this data cannot exclude whether this phenotype is not present in all African lineage ZIKV or if only the MR766 strain itself is unaffected by the knockout of ANKLE2.

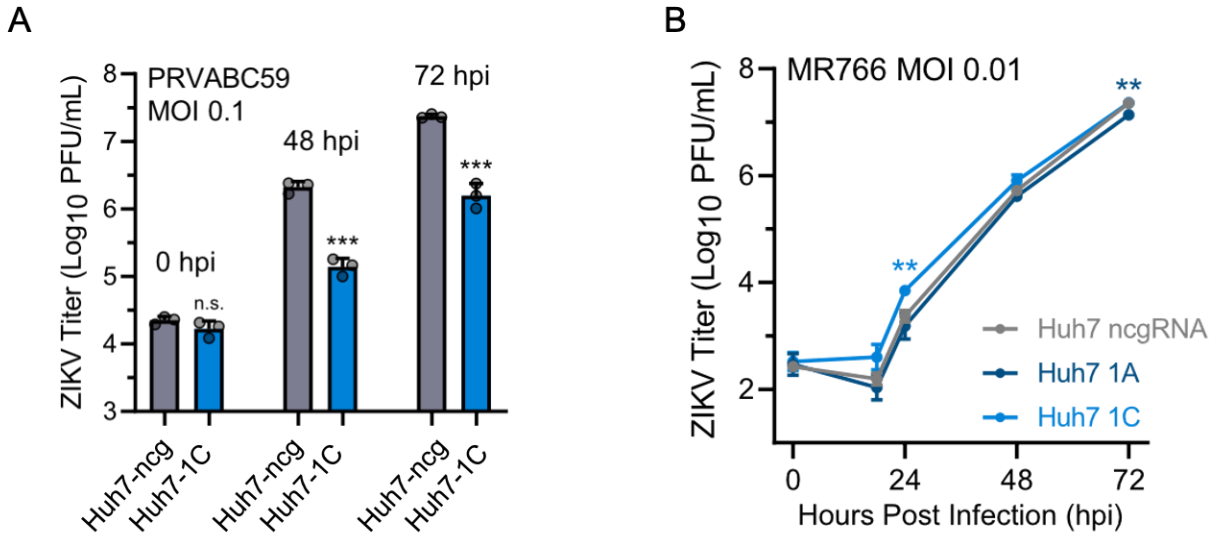

Figure 2: Reduction of viral replication of ZIKV strain PRVABC59 and MR766. *Huh7 ANKLE2 KO clones and ncgRNA control were infected with either* ***(A)*** *Asian lineage PRVABC59 at MOI 0.1 or* ***(B)*** *African lineage MR766 at MOI 0.01.* ***(A)*** *Supernatant was harvested at 0, 48 and 72 hpi for PRVABC59.* ***(B)*** *Five timepoints were harvested for at 0, 18, 24, 48 and 72 hpi for MR766. Supernatant was tittered via plaque assay expressed in PFU/ml in log_{10} scale. * Represents the measure of significance. A large decrease in viral replication was observed for PRVABC59 but no meaningful change in replication was observed for MR766.*

Reduction in viral replication occurs in multiple different ANKLE2 KO cell lines

We have confirmed that knocking ANKLE2 in Huh7 cells leads to a reduction of viral replication for Asian lineage ZIKV at multiple different MOIs. While Huh7 are a relevant model for natural replication it is not the only cell type ZIKV infects naturally,

additionally, Huh7 is a cancer cell line which might warp the results. To resolve this, we also tested the human choriocarcinoma (placental cancer) cell line JEG-3 and the human lung carcinoma cell line A549. JEG-3 being a placental cell line makes it a relevant model to study since ability of ZIKV to cross the placental barrier is an important cause for CZS and microcephaly. A549 were used since an ANKLE2 KO A549 cell line was already available in the lab but the lung is not one of ZIKVs primary sites of replication *in vivo*. For both cell lines 2 knockout clones and a ncgRNA control were infected with PLCal at MOI 0.1 and at 0, 48 and 72 hpi supernatant was harvest and was tittered via plaque assays.

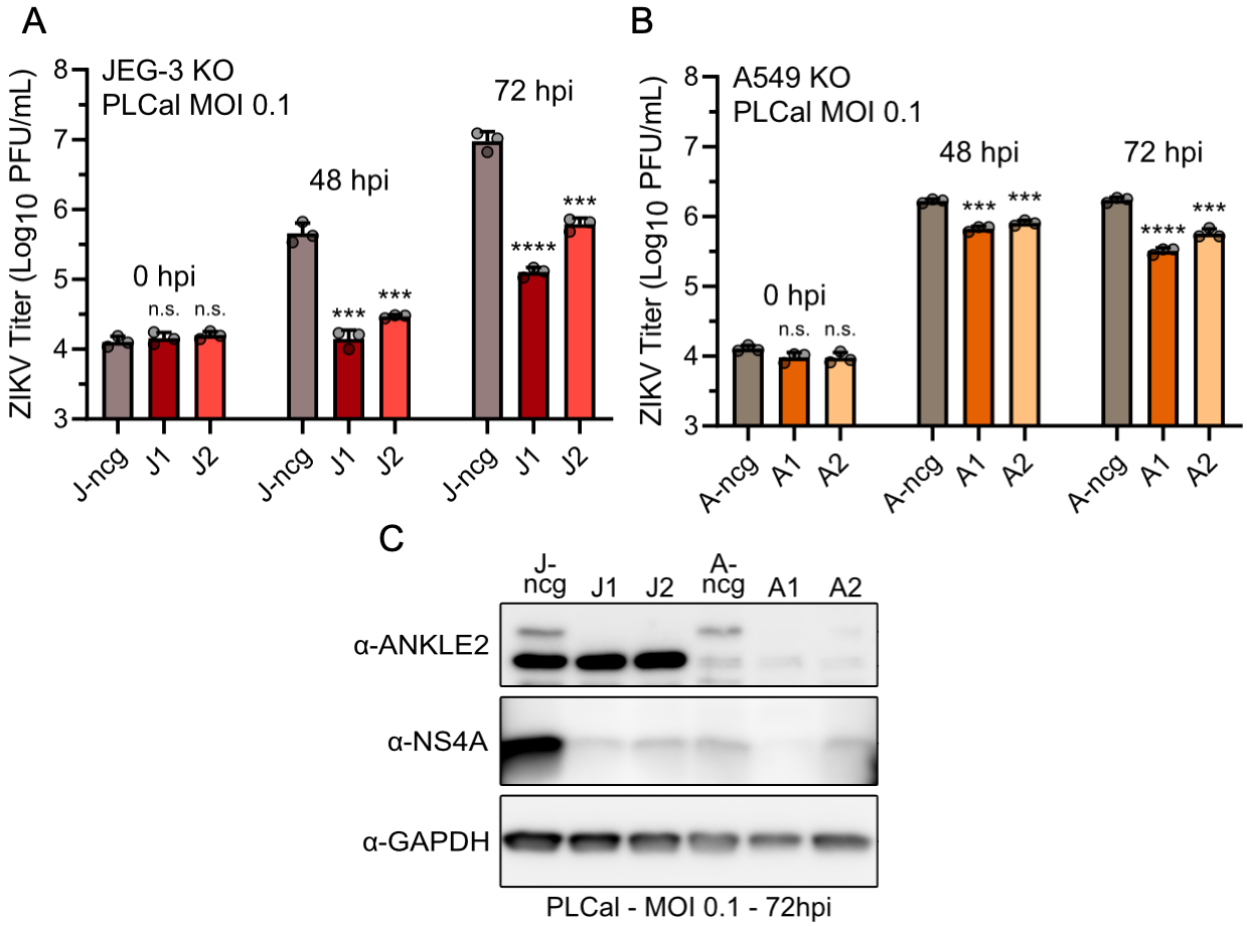

Figure 3: Replication of PLCal in Placental Jeg-3 and lung A549 cells. (A,B) *JEG-3 and A549 ANKLE2 KO clones and ncgRNA clones were infected with PLCal at MOI 0.1. Supernatant was harvested over a 72-hour time course at 0, 48 and 72 hpi and tittered via plaque assay expressed in PFU/ml in log_{10} scale. * Represent the level of significance* ***(A)*** *Shows a substantial reduction in viral replication in the KO clones compared to the control.* ***(B)*** *The reduction was still present in the A549 cells but to a much smaller degree.* ***(C)*** *Shows a western blot of lysates taken from cells infected with PLCal at MOI 0.1 at 72 hpi and stained for host ANKLE2, virus NS4A and GAPDH.*

We found that replication of PLCal is statistically significantly reduced in both cell lines. For both Jeg-3 knockout clones the reduction of viral replication is similar to the phenotype found in Huh7 clone 1C (***Figure 3A)***. Conversely, the decrease in virus replication for PLCal in A549 knockout clones is much smaller than in either Jeg-3 or Huh7 but remains statistically significant (***Figure 3B)***. Altogether, this data shows that the decrease in replication is conserved across multiple different cell-lines. We also

observed that the decrease seems to be higher in cell lines that are more relevant to natural ZIKV tropism, but this should be tested with more cell lines to make a proper conclusion on this. The knockout of ANKLE2 was verified with a western Blot showing a very effective knockout of ANKLE2 for all knockout clones. This western blot data also shows a large reduction of total viral protein NS4A in JEG-3 J1 and J2 and A549 clone A1 recapitulating the results found by plaque assay (***Figure 3C)***.

The reduction of viral replication is somewhat conserved in other flaviviruses

After this, we wondered whether the role of ANKLE2 in viral replication would be conserved in other flaviviruses. Earlier proteomics works have shown that host ANKLE2 can interact strongly with DENV NS4A and weakly with YFV NS4A. Affinity purification experiments by our lab have confirmed the ability of DENV and YFV NS4A to bind host ANKLE2. To test whether the role of ANKLE2 was conserved, we infected Huh7 ANKLE2 KO clones with either DENV-2 16881 or YFV-17D and tittered replication after 0, 18, 24, 48 and 72 hpi via plaque assays.

We found that DENV experienced a reduction in viral titers in early timepoints when ANKLE2 was knocked out in both tested clones. The replication did catch up to the ncgRNA control after 48 hours when a plateau in titers was reached ***(Figure 4A)***. For YFV we found that there only was a small reduction for 48 hpi for clone 1C but still statistically significant ***(Figure 4B)***. A western blot was performed on lysates taken from cells 72 hours after being infected with DENV staining for host ANKLE2 to show effectivity of ANKLE2 KO, viral NS5 and capsid to show measure of infection and GAPDH as loading control. Only a small reduction in capsid was found in 1C and no visible decrease in NS5 ***(Figure 4C)***. This is consistent with the plaque assay results showing that viral titers were similar after 72 hours across both KO clones and the ncgRNA. ANKLE2 deletion impacts DENV replication slightly different than ZIKV replication as it initially slows down replication instead of reducing it. The differences between DENV and YFV are somewhat expected since DENV is a much closer relative to ZIKV than YFV is. Also, the interaction between viral NS4A and ANKLE2 was much weaker for YFV. But this data shows that the role of ANKLE2 in replication is conserved in flaviviruses but becomes weaker the further the familial distance is to ZIKV.

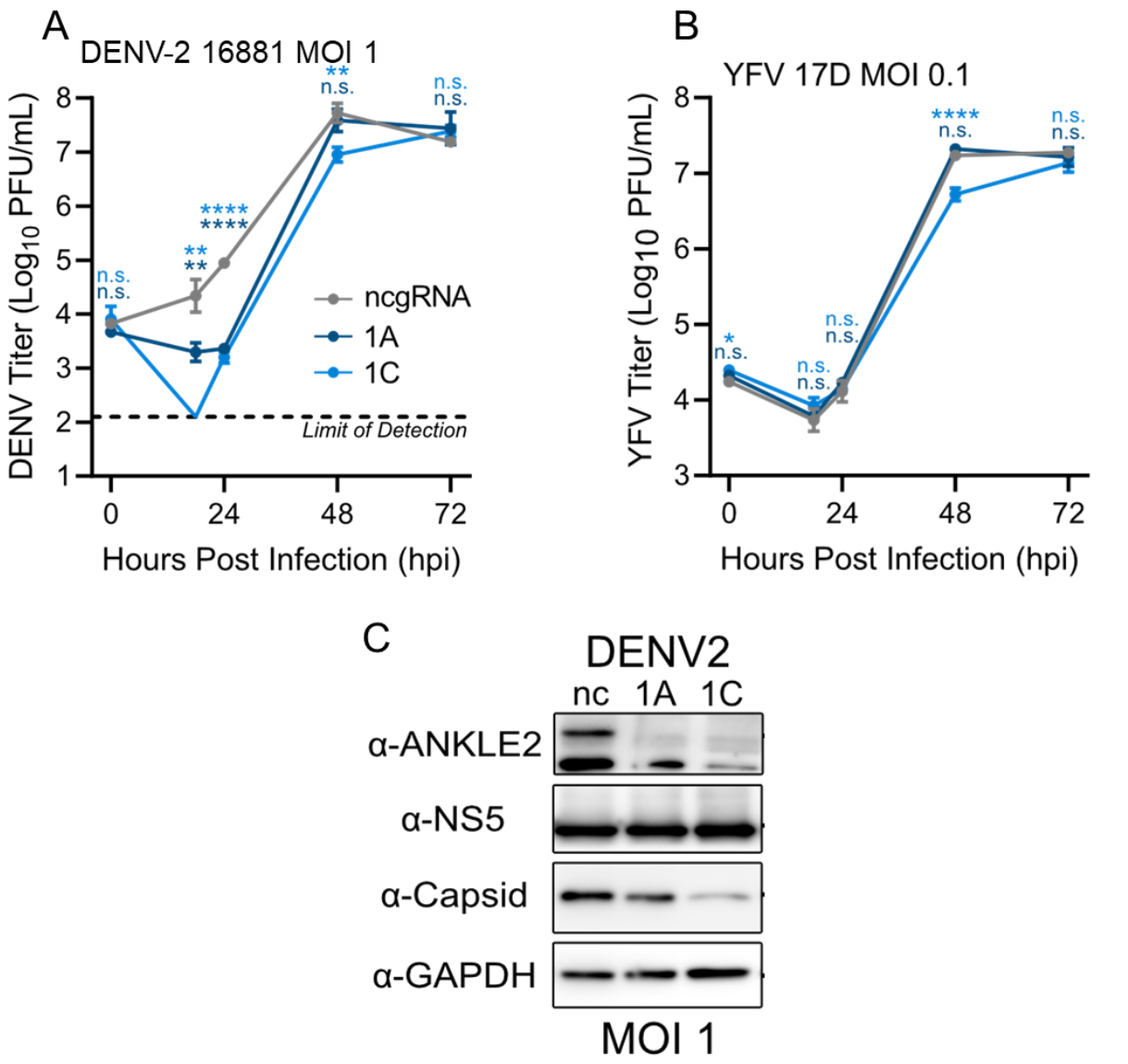

Figure 4: Reduction of flavivirus replication in Huh7 ANKLE2 KO clones. *Huh7 ANKLE2 KO clones and ncgRNA cells were infected with* ***(A)*** *DENV-2 16881 at MOI 1 or* ***(B)*** *YFV-17D at MOI 0.1. Supernatant was harvest at 0, 18, 24, 48 and 72 hpi and tittered via plaque assay. Titers are expressed in PFU/ML in log$_{10}$ scale. * Represent the measure of statistical significance. For* ***(A)*** *DENV a bottom limit of detection of the Plaque assay is shown. A large reduction of viral titers was found in both Huh7 KO clones that were infected with DENV with 1C 18hpi even being below the limit of detection. Titers returned to similar levels as the ncgRNA control at later timepoints.* ***(B)*** *Only a small decrease in YFV viral replication for 1C 48 hpi.* ***(C)*** *A Western blot was performed on lysate harvest from cells infected with DENV 72 hpi. Host ANKLE2 was stained to visualize KO efficiency, viral NS5 and capsid were stained to show the scale of viral infection. GAPDH served as a loading control.*

ANKLE2 rescues in Huh7 knockouts did not return viral replication to normal levels

To cement the role ANKLE2 has in viral replication of ZIKV we attempted to make Huh7 ANKLE2 KO cell-lines that stably express ANKLE2 via a plasmid insert. GFP insert cell lines were made to act as a control. These cells were infected with PLCal at MOI 0.1 and were tittered via plaque assay. The data from this experiment was somewhat

disappointing and very inconclusive. We found an increase in viral replication in Huh7 clone 1C with inserted ANKLE2 compared to clone 1C with inserted GFP, but replication was still significantly below the ncgRNA control, however, this was juxtaposed by clone 1A having reduced viral replication when ANKLE2 was inserted **(Figure 5A)**. As a result, it is difficult to make any conclusions since the results are very contradictory making it difficult to claim whether a partial rescue occurred in clone 1C when ANKLE2 was inserted. Western blot data shows that ANKLE2 was successfully inserted in the KO cell-lines excluding it as a cause. However, the western blot also shows that the rescue cells have odd artefacts such as a band above α-FLAG (ANK) for the 1C clone **(Figure 5B)**. As a result, it is difficult to make any conclusion from this data.

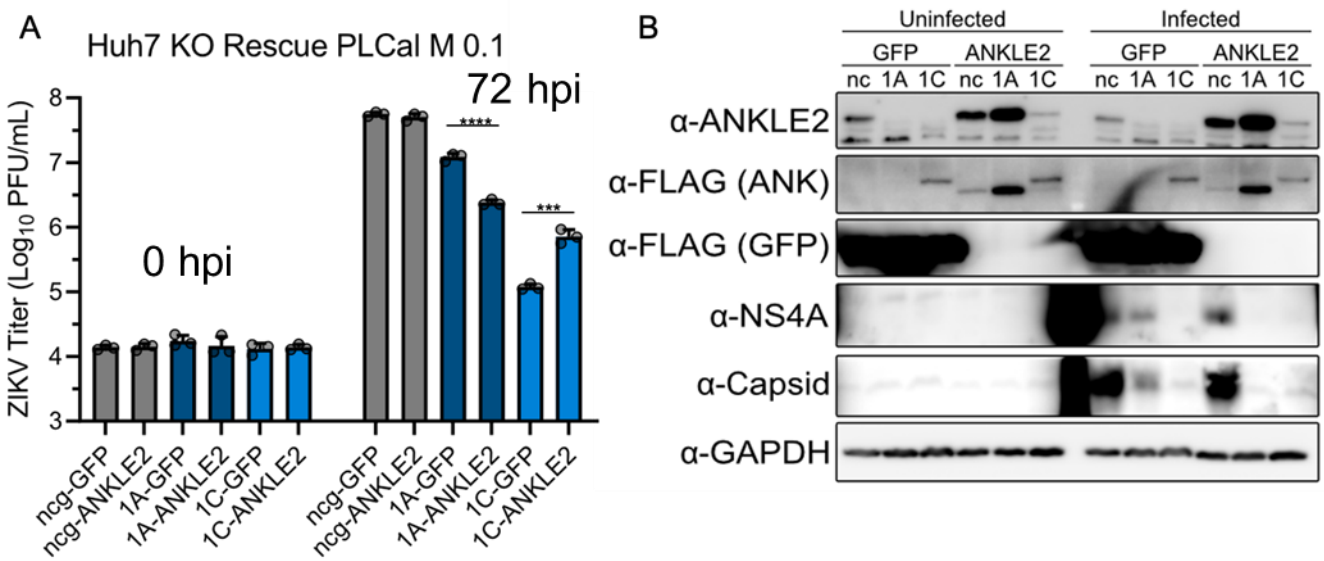

Figure 5: Impact PLCal replication for ANKLE2 rescues in Huh7 ANKLE2 KO clones. *Huh7 ANKLE2 KO clones and ncgRNA controls were rescued by stably expressing ANKLE2 via lentiviral plasmid insertion, GFP insertion acted as a control.* ***(A)*** *ANKLE2 rescue cells were infected with PLCal at MOI 0.1. Supernatant was harvested after 0 and 72 hours and tittered via plaque assay expressed in PFU/ml in log_{10} scale. * Represent the level of significance. For clone 1A the insertion of ANKLE2 led to a further reduction of viral replication while for clone 1C the insertion of ANKLE2 led to an increase in viral replication.* ***(B)*** *A western blot was performed on lysates obtained from ANKLE2 rescue cell-lines infected with PLCal at MOI 0.1 at 72hpi. Stained for ANKLE2, α-FLAG for ANKLE2 and GFP, GAPDH as a loading control and viral proteins NS4A and Capsid.*

Discussion

In this project we aimed to show whether host protein ANKLE2 has a role in the replication of ZIKV in human host cells. The interaction between this host protein and viral NS4A is very likely to be a key player in causing CZS and microcephaly. We tried to elucidate whether this interaction facilitates viral replication in some manner or is just a random interaction that serves no benefit to the virus. We did this by infecting various ANKLE2 knockout cell-lines with ZIKV and tittering supernatant via plaque assay to determine whether replication was reduced by the deletion of ANKLE2. First, we found that PLCal replication was significantly reduced when ANKLE2 was knocked out of the liver carcinoma cell line Huh7 across 3 different MOIs. This confirmed that ANKLE2 has a beneficial role in the replication of ZIKV in human host cells. We then tested whether

this phenotype was reciprocated with different strains of ZIKV to ensure that this phenotype is not PLCal specific. We used both the Asian lineage PRVABC59 and the African lineage MR766. We found that PRVABC59 showed a similar phenotype for clone 1C. No reduction in replication was observed for the African lineage MR766. This is likely a result from MR766 extensive history of lab passage and its initial culture in mouse brains modifying it to be more resilient to changes[18]. Therefore, the behavior MR766 is not very indicative of how clinical strains of ZIKV behave. We postulate that the lack of a distinct phenotype in knockout cell lines is caused by these lab adaptations. Another possibility is that MR766 unlike the Asian lineage strains is capable of using alternative isoforms of ANKLE2 to facilitate its replication as only the primary ANKLE2 isoform is knocked out in our KO cell lines. Regardless, Asian lineage ZIKV constitute the vast majority of human infections and caused the recent ZIKV outbreak in the Americas[19].

After finding that replication is reduced in both tested Asian lineages ZIKV we showed that viral replication also went down in the lung carcinoma A549 and the choriocarcinoma JEG-3 ANKLE2 KO cell-lines. This confirms that this phenotype is present in multiple different cell-lines and not an artifact created due to cancer mutations. Though all 3 cell-lines are cancer cells it is very unlikely that the observed phenotype being an anomaly caused by cancer. JEG-3 KO clones showed the greatest reduction in viral replication whereas in A549 KO clones the reduction was much smaller. JEG-3 in particular is very interesting due to it being a placental cell line and ZIKV unique ability compared to other flaviviruses to infect the placenta and pass the placental barrier. A neural cell-line such as SK-N-SH would be a very interesting candidate for these types of experiments as the interaction with ANKLE2 and NS4A causes perturbation of neuronal development. However, we were unable to finish creating stable knockout clones of a neural cell line as of the time of writing due to their slow growth.

Next, we found that the alteration in viral replication when ANKLE2 was deleted was conserved to varying degrees in both DENV-2 16881 and YFV-17D. Dengue appeared to have a delayed start of replication instead of a large decrease in titers in late timepoints that was found for ZIKV. The difference in replication was larger for DENV than for YFD-17D where the decrease was relatively minimal. This difference is somewhat expected as DENV is a closer relative to ZIKV than YFV. From these results it is likely to assume that ANKLE2's ability to facilitate viral replication is somewhat preserved across other Aedes vector flaviviruses. WNV is an interesting candidate to test in the future since it is transmitted by *Culex* mosquito species but is still closely related to ZIKV phylogenetically. The fact that only ZIKV causes CZS and microcephaly despite other flaviviruses interacting with ANKLE2 can be explained by ZIKVs exclusive ability to cross the placental barrier[20]. It is very possible that infections with other flaviviruses could cause CZS like symptoms and microcephaly if they were able to cross the placental barrier.

Lasty, we attempted to rescue normal viral replication rates by re-inserting ANKLE2 in the Huh7 ANKLE2 KO cell lines. This, unfortunately, led to mixed results where we

found that there was a slight increase in viral replication but still far below the ncgRNA control in clone 1C when ANKLE2 was reinserted compared to the normal KO clone. But, for clone 1A we instead found a decrease in replication when we reinserted ANKLE2. The western blot data showed that the cells were suboptimal with an unknown FLAG protein showing up for 1C. This makes it difficult to determine whether the increase we found for clone 1C is caused by the insertion of ANKLE2 or if the increases or decreases in the rescue experiment are caused by artifacts in the cell line. While we detected a large increase of ANKLE2 in the insert cells via western blot this also does not confirm whether the insert leads to ANKLE2 that can perform its natural function inside the cells. The worst-case explanation is that the reduction of viral replication we have observed in the KO cell-lines is not caused by the deletion of ANKLE2 but by a random off target deletion or mutation that occurred during the CRISPR-Cas9 procedure. This, however, seems somewhat unlikely since we made multiple different KO clones that all saw a reduction in viral replication.

All in all, our data shows that the host protein ANKLE2 plays a role in the replication of ZIKV in mammalian host cells. Our collaborators have also found that viral replication is also reduced in *Aedes* cell lines. This shows that ANKLE2 plays a role in replication in all ZIKV hosts. Very little is known about the method how this perturbation occurs or what role ANKLE2 has in viral replication. Future experiments should be done to further elucidate this. The most likely mechanism could be that ANKLE2 acts as a scaffolding protein that binds and localizes proteins that benefit viral replication. Another possibility is that ANKLE2 enhances NS4A's role in folding of the ER membrane to create the viral replication compartment. Mass spectrometry proteomics is a good first step to test the possible role as a scaffold protein by identifying possible binding partners that facilitate viral replication. Potential binding partners can be further tested by inhibiting binding or inhibition of expression to confirm a possible culprit.

Another future direction is using mouse models to further study if the reduction of viral replication is also present in vivo and help study how the interaction between NS4A and ANKLE2 cause microcephaly and other CZS symptoms. This is, however, somewhat difficult by the fact that homozygous deletion of *ankle2* is fatal in unborn mice and heterozygous mice might produce enough Ankle2 to obfuscate a possible phenotype. Further mouse studies can also help to identify if there are certain genetic dispositions that are more vulnerable to microcephaly. Hopefully, this knowledge can then be used to determine mothers whose children are at risk of developing microcephaly after infection with ZIKV during pregnancy. This allows for treatment options or abortion if available.

Future epidemics are very possible, since *Aedes aegypti* and *Aedes albopictus* will spread to more countries due to climate change possibly leading to an increase of ZIKV prevalence in the world. A possible estimation is that 1.3 billion people will be exposed to ZIKV by 2050[21]. Therefore, it is imperative to understand how microcephaly is caused and which groups are most vulnerable to it to create a response to future outbreaks.

Supplementary table 1: Antibodies

Antibody	Host Species	Dilution used	Supplier (catalog #)	RRID
GAPDH	Mouse	1:1000	Fisher (PIMA515738)	AB_2537652
ANKLE2	Rabbit	1:1000	Bethyl Labs (A302-966A-M)	AB_2780882
FLAG-M2	Mouse	1:1000	MilliporeSigma (F1804)	AB_262044
ZIKV NS4A	Rabbit	1:1000	GeneTex (GTX133704)	AB_2887067
ZIKV Capsid	Rabbit	1:1000	GeneTex (GTX133317)	AB_2755861
DENV NS5	Rabbit	1:1000	GeneTex (GTX103350)	AB_1240701
DENV Capsid	Rabbit	1:1000	GeneTex (GTX103343)	AB_1240697
Anti-mouse IgG-HRP	Rabbit	1:5000	SouthernBiotech (6170-05)	AB_2796243
Anti-Rabbit IgG-HRP	Goat	1:5000	SouthernBiotech (4030-05)	AB_2687438

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