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Research Article

A novel IFNbeta-induced long non-coding RNA ZAP-IT1 interrupts Zika virus replication in A549 cells

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ABSTRACT

Zika virus (ZIKV) infection can cause severe neurological diseases including neonatal microcephaly and Guillain-Barre syndrome. Long noncoding RNAs (lncRNAs) are the by-products of the transcription process, which are considered to affect viral infection. However, it remains largely unexplored whether host lncRNAs play a role in ZIKV infection. Here, we identified a group of human lncRNAs that were up-regulated upon ZIKV infection and were dependent on the type I interferon (IFN) signaling. Overexpression of lncRNA ZAP-IT1 leads to an impairment of ZIKV infection. Correspondently, deficiency of ZAP-IT1 led to an enhancement of ZIKV infection. We further confirmed that ZAP-IT1, an intronic lncRNA with total 551 nt in length, is mainly located in the nuclear upon ZIKV infection. Knockout of ZAP-IT1 also led to the increase of dengue virus (DENV), Japanese encephalitis virus (JEV), or vesicular stomatitis virus (VSV) infection. Mechanically, we found that the antiviral effect of ZAP-IT1 was independent of the type I IFN signaling pathway. Therefore, our data unveiled that host lncRNA ZAP-IT1 induced by the type I IFN signaling, showed robust restriction on ZIKV infection, and even on DENV, JEV, and VSV infection, which may benefit the development of antiviral therapeutics.

1. Introduction

Zika virus (ZIKV) is an emerging arthropod-borne virus that belongs to the *Flavivirus* genus of the *Flaviviridae* family (Pierson and Diamond, 2018), which includes dengue virus (DENV), yellow fever virus (YFV), West Nile virus (WNV), and Japanese encephalitis virus (JEV) (Pierson and Diamond, 2020). ZIKV has been reported that can lead to a devastating congenital syndrome in the fetuses of pregnant mothers and cause the Guillain-Barre syndrome in adults (Zhang et al., 2021). Nonetheless, there are no approved vaccines or therapeutic countermeasures.

Long non-coding RNAs (lncRNAs) are a class of RNA transcripts exceeding 200 nucleotides (nt) in length as well known that they do not code for proteins (Batista and Chang, 2013; Zhang et al., 2021). LncRNAs play an important role in modulating the expression of the gene at the epigenetic, transcription, and post-transcriptional processes (Qiu et al., 2018; Liu et al., 2019; Wang et al., 2019a). Some lncRNAs participate in the response after virus infection, such as lincRNA-Cox2 (Carpenter et al., 2013; Xue et al., 2019), NRAV (Ouyang et al., 2014; Li et al., 2020), and NEAT1 (Zhang et al., 2013; Imamura et al., 2014; Ma et al., 2017), which can inhibit virus replication. Other lncRNAs, such as lncRNA-ACOD1 (Wang et al., 2017), NeST (Gomez et al., 2013), and lncRNA-CMPK2 (Kambara et al., 2014), are hijacked by viruses to promote virus replication. Lnc-ITPRIP-1 (Xie et al., 2018), NEAT1 (Zhang et al., 2013; Ma et al., 2017), lnc-Lsm3b (Jiang et al., 2018), and lncRHOXF1 (Penkala et al., 2016) have been reported to be involved in the regulation of innate immunity.

RNA-seq analysis in human neural progenitor cells (hNPCs) by Hu et al. has shown that 149 lncRNAs are differentially expressed in response to ZIKV infection (Hu et al., 2017), although the function of the differentially expressed lncRNAs has not been determined. Recently, our group has demonstrated that 79 lncRNAs and 140 mRNAs screened by lncRNA-microarray assay were differentially expressed in ZIKV-infected human lung epithelial A549 cell line (Wang et al., 2021). Three lncRNAs RPL27-OT1, Rec8-OT3, and OASL-IT1 exhibited a potent

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restriction effect on ZIKV infection. Importantly, OASL-IT1 exerted a significant antiviral activity, and OASL-IT1 and IFNs regulated each other in a positive feedback loop. However, only a few hundred lncRNAs have been annotated and reported to participate in the response of viral infection, and the functions of lncRNAs in ZIKV infection still remained to be known.

In order to explore whether more lncRNAs might be involved in ZIKV replication, we analyzed the microarray assay (GSE124094) in NCBI's Gene Expression Omnibus (GEO) database. We found that lncRNAs including LAP1-AS1, LINC21762, VAMP1-AS1, ZAP-IT1 were differentially expressed after ZIKV infection. In this study, we demonstrated that ZIKV enhanced the expression of these lncRNAs through the type I IFN pathway. And we found that lncRNA ZAP-IT1 could inhibit the replication of ZIKV, as well as DENV, JEV and vesicular stomatitis virus (VSV). In addition, we found that the antiviral effect of ZAP-IT1 was independent of the type I IFN signaling. Taken together, our work identified that lncRNA ZAP-IT1 induced by IFN-β might interrupt viral replication, which could be served as a potential molecular target for anti-ZIKV agents.

2. Materials and methods

2.1. Cell culture

Human lung epithelial cell A549 (ATCC CCL-185), human glioblastoma cells LN229 (ATCC CRL-2611), human embryonic kidney cells 293T (ATCC CRL-3216), and African green monkey kidney cells Vero (ATCC CCL-81) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% or 10% (v/v) fetal bovine serum (FBS) (Gibco, New York, USA). The mosquito cell line C6/36 (ATCC, CRL-1660) was maintained in RPMI-1640 medium (Invitrogen, Carlsbad, USA) supplemented with 10% FBS, 1% sodium pyruvate. All cells were supplemented with 100 units/mL of streptomycin and penicillin (Invitrogen), cultured at 37 °C with 5% CO_2. Human monocyte-differentiated macrophages were obtained as previous study (Wang et al., 2021) and used for subsequent experiments.

2.2. Virus, virus infection, and titration

ZIKV strain H/PF/2013 (GenBank accession number KJ776791), Dengue-2 virus New Guinea C (DENV2 NGC, GenBank accession number AF038403), and JEV (14-14-2 vaccine strain) were provided by the Guangzhou Centres for Disease Control. The Vesicular Stomatitis Virus (VSV, Indiana Strain) and the herpes simplex virus 1 (HSV-1, Kos strain) were kindly provided by Prof. Dongyan Jin (University of Hong Kong).

ZIKV and DENV2 NGC were amplified by C6/36 or Vero cells. VSV and HSV-1 were propagated and titrated on Vero cells. Viral concentrations were titrated on Vero cells and viral stocks were stored at −80 °C.

Cells were infected with ZIKV, DENV2, JEV, VSV, or HSV-1 at indicated multiplicity of infection (MOI). The supernatants were harvested at indicated hours post-infection (h.p.i.) for virus titration by standard plaque-forming assay (PFA) as previously described (Huang et al., 2020).

2.3. LncRNA microarray analysis

The lncRNA microarray data are available on the NCBI GEO database, (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE124094). The GEO accession number is GSE124094 (GPL25961).

2.4. Quantitative real-time PCR (qRT-PCR)

Total RNAs were extracted using Trizol reagent (Invitrogen). Before reverse transcribing, the RNase-Free DNase (Promega, Madison, USA) was used to degrade the genome DNA. Then the reverse was transcribed using M-MLV reverse transcriptase (Promega) according to the manufacturer's instructions. qRT-PCR analysis was performed using the LightCycler 480 SYBR Green I Master (Roche, Basle, Switzerland) on a CFX96 Real-Time System (Bio-Rad, Basle, Switzerland). Differences of gene expression from qRT-PCR data were analyzed using $^{\Delta\Delta}$CT values as described previously (Huang et al., 2020). Human *U6* or *β-actin* level was measured as an internal control. The primer sequences used in qRT-PCR were listed in Supplementary Table S1.

2.5. Western blotting

Cells were harvested at indicated time and treated with RIPA lysis buffer (pH 7.4) (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.5% NP-40, 1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L PMSF, 1% protease inhibitor cocktails, 1 mmol/L Na_3VO_4, and 1 mmol/L NaF). Western blotting was then performed as previously described (Wang et al., 2019b).

The primary antibodies used in this study were shown as follows: rabbit polyclonal antibodies against ZIKV E (GeneTex, GTX133314), rabbit polyclonal antibodies against ZAP (Proteintech, 16820-1-AP), and rabbit polyclonal antibodies against GAPDH (Proteintech, I0494-I-AP). Secondary antibodies included IRDye 800 CW-conjugated goat anti-rabbit IgG (LI-COR, 926-32211, Lincoln, USA). Immunoreactive bands were analyzed with the Odyssey infrared imaging system (LI-COR). Quantity One program (Bio-rad) was used to quantify the Western blotting results.

2.6. Plasmid construction

5′- and 3′-rapid amplification of cDNA ends (RACE) PCR assays were performed to amplify lncRNAs using the SMARTer RACE cDNA amplification kit (TaKaRa, 6106, Osaka, Japan) and cloned into the pcDNA3.1(+) vector. The primer sequences used in cloning were listed in Supplementary Table S2. The sequences of plasmids were verified by sequencing.

2.7. Generation of lncRNA expressing cells

The pcDNA3.1(+)-lncRNA plasmids were transfected into A549 cells by Lipofectamine 2000 (Invitrogen). The lncRNA-expressing cells were selected by G418 (Invivogen) for 7–10 days.

2.8. Generation of knockout cells by CRISPR/Cas9 gene editing

CRISPR/Cas9 system was utilized to generate knockout cell clones as previously described (Ran et al., 2013; Ma et al., 2019). The single-guide RNAs (sgRNAs) targeting ZAP-IT1 were inserted into the lentiCRISPR v2 vector (Addgene, #52961), and the sequences of sgRNAs were listed in Supplementary Table S3. 293T cells were transfected with lentiCRISPR carrying ZAP-IT1 sgRNA together with pSPAX2 and pMD2.G using FuGENE® HD Transfection Reagent (Promega). And lentiCRISPR carrying negative control sgRNA was transfected into 293T cells to select the control cell. Supernatants were collected at 48 h post-transfection (h.p.t.) and applied to transduce A549 cells. $MAVS^{Ko}$ cells, $IFNAR1^{Ko}$ cells, and $STAT1^{Ko}$ cells were generated by CRISPR/Cas9-based gene editing previously (Zhou et al., 2019; Wang et al., 2021). Knockout cells were selected by puromycin and limiting dilution cloning.

2.9. IFN-β treatment

A549 cells were treated with 500 units/mL of IFN-β (Sigma-Aldrich, I9032, Germany) for 24 h to harvest the total cellular RNA. Cells that had not received IFN-β treatment were served as control, and total RNA was extracted.

2.10. Cytoplasm and nuclear RNA fraction assay

Cells were washed, trypsinized and collected as a cell pellet prior to lysis. RLN buffer (50 mmol/L Tris-HCl, pH 8.0, 140 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.5% (v/v) NP-40) were added to lyse cell plasma membrane on ice for 5 min. After centrifugation at 300×*g* at 4 °C, the supernatant containing cytoplasmic extract was collected into a new

centrifuge tube. Cytoplasmic RNA was extracted by RNeasy Mini Kit (QIAGEN, RY25, Germany). The pellet contained nuclei washed with RLT (a guanidine-thiocyanate-containing lysis buffer supplied by the RNeasy Mini Kit) buffer and was applied to extract nucleic RNA using TRIzol reagent (Invitrogen). qRT-PCR was performed to measure the levels of β-actin, U6, and ZAP-IT1 (primer sequences were listed in Supplementary Table S1).

2.11. RNA interference

The sequences of siRNAs targeting human *ZAP* mRNA are GCAGTCAAGAGGACCTCTT. A control siRNA with the scrambled sequence was used as a negative control (siNC). Transfection was carried out with 16 nmol of siRNAs by using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's instructions. At 48 h.p.t., cells were harvested for further analysis.

2.12. Statistical analysis

All statistical analyses of viral RNA levels or viral titers were performed with an unpaired, two-tailed Student's *t*-test. Data were presented as mean ± standard deviation (SD).

3. Results

3.1. Differential expression of lncRNAs is induced by ZIKV

In order to explore whether more lncRNAs may be involved in ZIKV replication, we analyzed the microarray assay (GSE124094) in NCBI's GEO database precisely. We found that the abundance of lncRNAs including NONHSAT095710, ENST00000621762, ENST00000619166, and lnc-ZC3HAV1L-1:1 was increased more than two folds in the microarray assay (Table 1 and Supplementary Table S4). To confirm the differential expression of lncRNAs from the microarray assay, A549 cells were infected with ZIKV, and the total RNA was collected at 24 h.p.i. to detect the expression of lncRNA by qRT-PCR. These lncRNAs were increased 10 folds or more than 20 folds upon ZIKV infection compared to uninfected cells (control) (Fig. 1A). According to the HUGO Gene Nomenclature Committee (HGNC, www.genenames.org) decision tree for naming lncRNA genes with unknown functions (Seal et al., 2020), NONHSAT095710, ENST00000621762, ENST00000619166, and lnc-ZC3HAV1L-1:1 were named into LAP3-AS1, LINC21762, VAMP1-AS1, and ZAP-IT1, respectively.

To find out whether the differential expression of these four lncRNAs is cell type-specific, monocyte-derived macrophages cells isolated from

Table 1
The information of selected lncRNAs.

LncRNA ID	Database	Chr	Classification	Style	Name
NONHSAT095710	NONCODE	Chr4	Exonic_antisense	Up	LAP3-AS1
ENST00000621762	Ensembl	Chr22	Intergenic	Up	LINC21762
ENST00000619166	Ensembl	Chr12	Intronic_antisense	Up	VAMP1-AS1
lnc-ZC3HAV1L-1:1	LNCipedia	Chr7	Intronic_sense	Up	ZAP-IT1

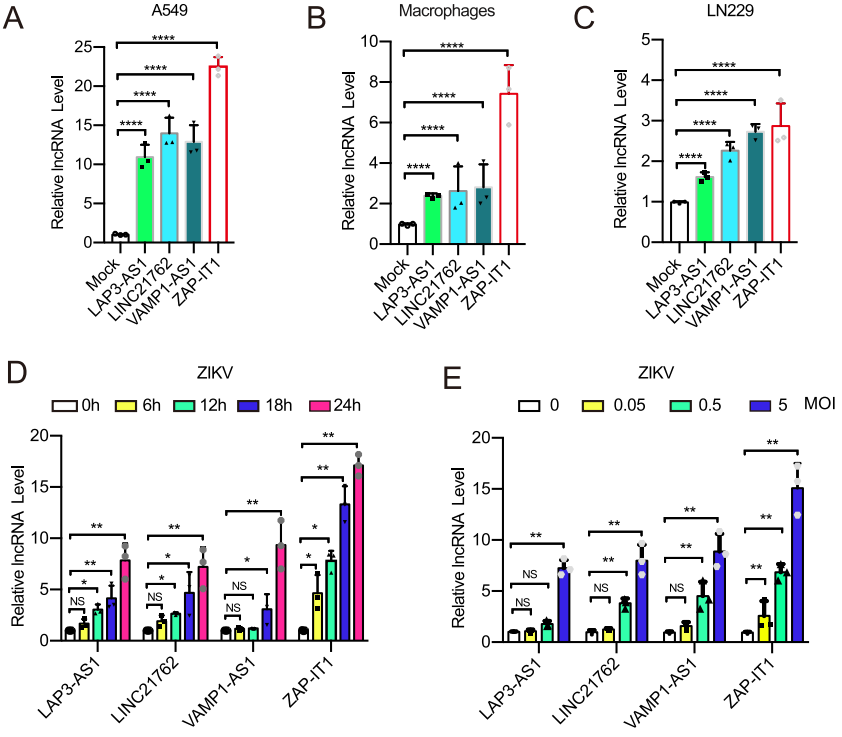

Fig. 1. Differential expression of lncRNAs induced by ZIKV. A549 cells (**A**), monocyte-differentiated macrophages (**B**) or LN229 cells (**C**) were infected with ZIKV (MOI = 3) and harvested for total RNAs extraction at 24 h.p.i. qRT-PCR was performed to detect the RNA level of LAP3-AS1, LINC21762, VAMP1-AS1, and ZAP-IT1. **D** A549 cells were infected with ZIKV (MOI = 3) and total RNAs were harvested at indicated time points (0, 6, 12, 18, and 24 h.p.i.) to detect the lncRNA level by qRT-PCR. **E** A549 cells were infected with ZIKV at indicated MOI (0, 0.05, 0.5, 5) and total RNAs were harvested at 24 h.p.i. to detect the lncRNA level by qRT-PCR. Human *U6* level was measured as an internal control and normalized to uninfected cell (mock, 0 h or MOI = 0). All experiments were independently repeated for three times. Data were shown as means ± standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$. NS, not significant; h.p.i., hours post-infection.

peripheral blood mononuclear cell (PBMC) were infected with ZIKV and the expression of these four lncRNAs was detected by qRT-PCR. As expectedly, LAP3-AS1, LINC21762, VAMP1-AS1, and ZAP-IT1 were induced to increase about two to eight folds after ZIKV infection (Fig. 1B). These results demonstrated that the differential expression of the selected lncRNAs was not cell type-specific. We examined the expression of these four lncRNAs in human glioma cells (LN229) as well. Up-regulation of lncRNAs was also shown in LN229 after ZIKV infection (Fig. 1C). This suggested that lncRNAs might play a role in ZIKV infected nervous system.

The kinetic assays were also performed in A549 cells. And the results demonstrated that the differential expression of these lncRNAs triggered by ZIKV was in a time- and dose-dependent manner (Fig. 1D and E).

3.2. Differential expression of lncRNAs induced by different viruses

To find out whether other viruses in *Flavivirus* genus like DENV and JEV have impact on the expression of the selected lncRNAs, A549 cells were infected with DENV2 or JEV, respectively. And the total RNAs were extracted for qRT-PCR. Increased expression level of all four lncRNAs was observed after DENV2 or JEV infection (Fig. 2A and B). To test whether negative single-stranded RNA virus or DNA virus could affect the expression of these lncRNAs, A549 cells were infected with VSV or HSV-1 for 12 h.p.i. Surprisingly, LAP3-AS1 could not be induced by VSV, while LINC21762, VAMP1-AS1, and ZAP-IT1 were significantly up-regulated after VSV infection (Fig. 2C). As for HSV-1, the expression level of LAP3-AS1 and LINC21762 increased more than 10 folds after HSV-1 infection, while only 5 folds of increase was observed in that of VAMP1-AS1 and ZAP-IT1. This was different from the RNA virus (Fig. 2D). The above observations indicated that the selected lncRNAs were responsive to other viral infections as well.

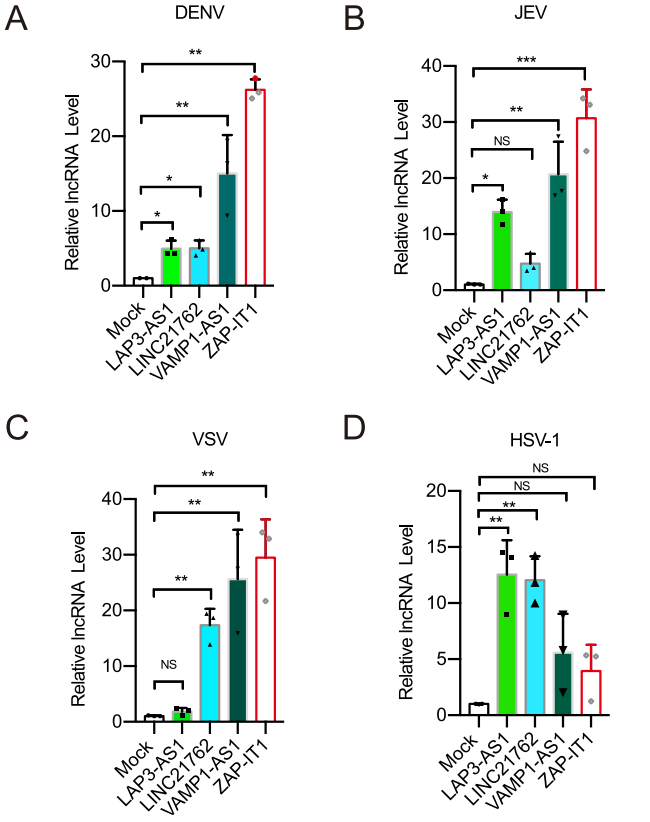

Fig. 2. Differential expression of lncRNAs induced by different viruses. A549 cells were infected with DENV2 NGC (MOI = 5) (**A**) or JEV (MOI = 5) (**B**), and total RNAs were harvested at 24 h.p.i. to detect the lncRNA level by qRT-PCR. A549 cells were infected with VSV (MOI = 1) (**C**) or HSV-1 (MOI = 1) (**D**), and total RNAs were harvested at 12 h.p.i. to detect the lncRNA level by qRT-PCR. Human *U6* level was measured as an internal control and normalized to uninfected cell (mock). All experiments were independently repeated for three times. Data were shown as means ± standard deviation. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. NS, not significant; h.p.i., hours post-infection.

3.3. The expression of lncRNAs is dependent on the production of IFN and the activation of IFN signaling pathway

To elucidate the mechanism of the up-regulated expression of lncRNAs induced by ZIKV infection, we carried out bioinformatics analysis to predict potential binding sites of transcription factors on the promoter region by analyzing ENCODE ChIP-seq data. As shown in Fig. 3A, putative

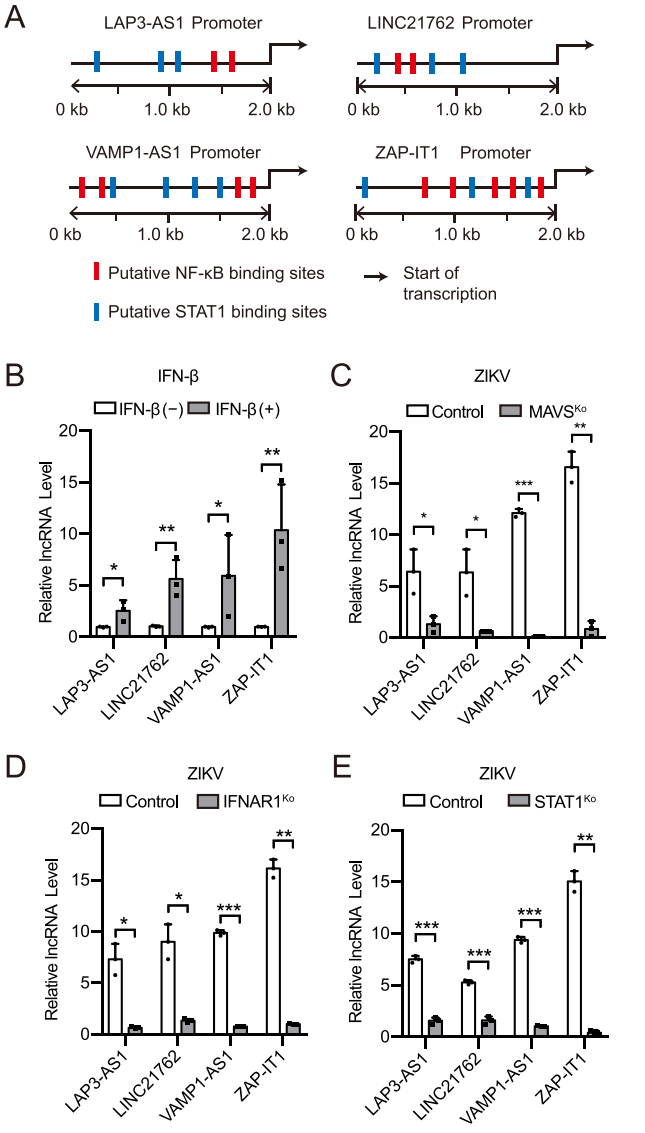

Fig. 3. IFN pathway is involved in the induction of lncRNAs. **A** Putative transcription factor binding sites on the promoter region of LAP3-AS1, LINC21762, VAMP1-AS1, and ZAP-IT1, respectively. **B** A549 cells were treated with 500 units/mL of IFN-β for 24 h and total RNAs were harvested to detect the lncRNA level by qRT-PCR. Human *U6* level was measured as an internal control and normalized to IFN-β (−) cell. **C–E** Control cells and MAVS^{Ko} cells (**C**), IFNAR1^{Ko} cells (**D**), or STAT1^{Ko} cells (**E**) were infected with ZIKV (MOI = 3). Total RNAs were harvested at 24 h.p.i. to detect the lncRNA level by qRT-PCR. Human *U6* level was measured as an internal control and normalized to uninfected cell (mock, 0 h or MOI 0). All experiments were independently repeated for three times. Data were shown as means ± standard deviation. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Ko, knockout.

binding sites of NF-κB and STAT1 were identified, implying a potential association between lncRNAs and NF-κB or STAT1. We hypothesized that ZIKV might induce the expression of these lncRNAs through activating the IFN signaling pathway. To verify this hypothesis, A549 cells were treated with IFN-β. The expression of four lncRNAs was significantly enhanced after the IFN-β treatment (Fig. 3B). To further confirm this result, we employed the MAVS^{Ko}, IFNAR1^{Ko}, or STAT1^{Ko} A549 cells, which blocked the production of IFN and the activation of IFN signaling pathway. We found that the enhanced expression of lncRNAs was blocked after knockout of MAVS, IFNAR1, or STAT1 compared to the control cells (Fig. 3C and E). Taken together, our results indicated that LAP3-AS1, LINC21762, VAMP1-AS1, and ZAP-IT1 probably belonged to interferon-stimulated genes (ISGs) and were specifically induced by IFN and the activation of IFN pathway.

3.4. Overexpression of ZAP-IT1 restricts the replication of ZIKV

Next, we investigated the role of LAP3-AS1, LINC21762, VAMP1-AS1, and ZAP-IT1 in ZIKV infection. Firstly, the natural abundance of lncRNAs in uninfected cells was detected by qRT-PCR (Supplementary Fig. S1 and Supplementary Table S5). Next, A549 cells stably overexpressing LAP3-AS1, LINC21762, VAMP1-AS1 or ZAP-IT1 were successfully established, with 5–8 folds of expression level of that in control cells (empty vector) (Fig. 4A), and were then infected with ZIKV. No significant change of ZIKV RNA was detected after overexpressing LAP3-AS1, LINC21762, and VAMP1-AS1, while overexpression of ZAP-IT1 led to a 70% decrease of ZIKV RNA (Fig. 4B). The expression of ZIKV E protein was validated by Western blotting. And the result showed that overexpression of these four lncRNAs, especially ZAP-IT1, led to a decreased ZIKV E protein level (Fig. 4C). Consistently, overexpression of ZAP-IT1 significantly decreased the infectious level of progeny ZIKV (Fig. 4D). These results suggested that ZAP-IT1 conferred the most robust antiviral activity. In view of this and the above results that the highest level of ZAP-IT1 was observed after ZIKV infection among these four lncRNAs (Fig. 1), ZAP-IT1 was chosen for further analysis.

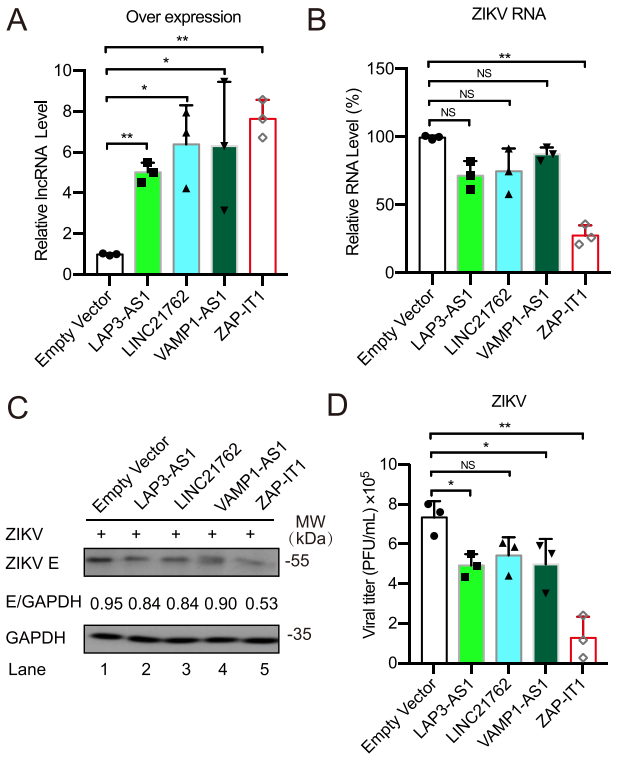

Fig. 4. Overexpression of ZAP-IT1 impairs the replication of ZIKV in A549 cells. **A** Overexpression of lncRNAs in A549 cells was validated by qRT- PCR. **B–D** The effect of overexpression of lncRNAs on ZIKV RNA level (**B**), E protein level (**C**), and viral titer (**D**). The empty vector-transfected cells and lncRNAs-overexpressing A549 cells were infected with ZIKV (MOI = 3) for 24 h. The viral RNA level was measured by qRT-PCR using primers specific to ZIKV NS1. The data were normalized with the vector cells. Western blotting was performed to detect ZIKV E protein at 24 h.p.i. And the viral titers were determined by PFA. Human *U6* level was measured as an internal control and normalized to emtpty vector cell. All experiments were independently repeated for three times. Data were shown as means ± standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. NS, not significant; h.p.i., hours post-infection.

3.5. Identification of ZAP-IT1 as a nuclear non-coding RNA

5′ and 3′ rapid amplification of cDNA ends (RACE) of total RNA from A549 cells was carried out, and one transcript from Zap-it loci was identified (Fig. 5A). Human ZAP-IT1 is located on the reverse strand of chromosome 7, at position of 139,049,456–139,050,006 (551 nt) and is in the intron 12 of ZAP. The nucleotide sequence of human ZAP-IT1 shared 92.3% identity with that of rhesus, but didn't show any homology with mouse, dog, and elephant (USCS genome browser, hg19, Supplementary Fig. S2A), suggesting that ZAP-IT1 was highly species-specific.

To define if ZAP-IT1 had any protein-coding potential, we used Coding Potential Assessment Tool (CPAT) to explore the coding possibility of ZAP-IT1. The CPAT analysis predicted that the ZAP-IT1 possessed an extremely low coding potential to encode protein as other lncRNAs, NEAT1 and OASL-IT1, compared to the protein-coding genes *ACTB* and *GAPDH* (Fig. 5B). To further validate these predictions, the sequence of ZAP-IT1 fused with HA tag was inserted into pcDNA3.1 vector (pcDNA3.1-ZAP-IT1-HA). The pcDNA3.1 (empty vector), pcDNA3.1-ZAP-IT1-HA, and the positive control pcDNA3.1-GFP-HA were transfected into 293T cells. A specific HA band was shown in the pcDNA3.1-GFP-HA-transfected cells, while no band was detectable in the cells transfected with pcDNA3.1-ZAP-IT1-HA (Fig. 5C). These data demonstrated that ZAP-IT1 was indeed a non-protein-coding RNA.

To examine the subcellular localization of ZAP-IT1, a cytoplasm and nuclear RNA fraction assay were performed. β-actin and U6 mainly presented in the cytoplasm or nuclei as the control, respectively (Fig. 5D and E). ZAP-IT1 was rarely detectable in uninfected A549 cells but mainly presented in the nuclei fraction upon ZIKV infection (Fig. 5F). These results demonstrated that ZAP-IT1 was a nuclear non-coding transcript upon ZIKV infection.

3.6. Depletion of ZAP-IT1 promotes ZIKV replication

To find out whether deficiency of ZAP-IT1 may affect the replication of ZIKV, CRISPR/Cas9 gene editing-based knockout technology was used to construct ZAP-IT1 knockout (ZAP-IT1^{Ko}) A549 cells. To generate large fragment deletions of ZAP-IT1, two sgRNAs were designed that targeted both the N and C terminal of ZAP-IT1 (Fig. 6A). About 372 nt was removed out of ZAP-IT1 in the knockout cells (Fig. 6B and Supplementary Fig. S2B). And the expression of ZAP-IT1 in the ZAP-IT1^{Ko} cells would not be induced by ZIKV infection compared to control cells (Fig. 6C).

A significant enhancement in the level of ZIKV genomic RNA and ZIKV E protein was observed in ZAP-IT1^{Ko} cells compared to control cells after ZIKV infection (Fig. 6D and E). Furthermore, the increased viral yield of ZIKV was also shown in ZAP-IT1^{Ko} cells (Fig. 6F). Since ZAP-IT1 could be induced by DENV, JEV, VSV, and HSV-1 at different levels, we further examined whether ZAP-IT1 affected the replication of these viruses. Intriguingly, the viral yields of DENV, JEV and VSV were significantly increased in ZAP-IT1^{Ko} cells compared to the control cells after infection (Supplementary Figs. S3A–C), while the titer of HSV-1 did not show remarkable change (Supplementary Fig. S3D). Together, these results suggested that ZAP-IT1 might play a restriction role in ZIKV, DENV, JEV and VSV infection, but not in HSV-1.

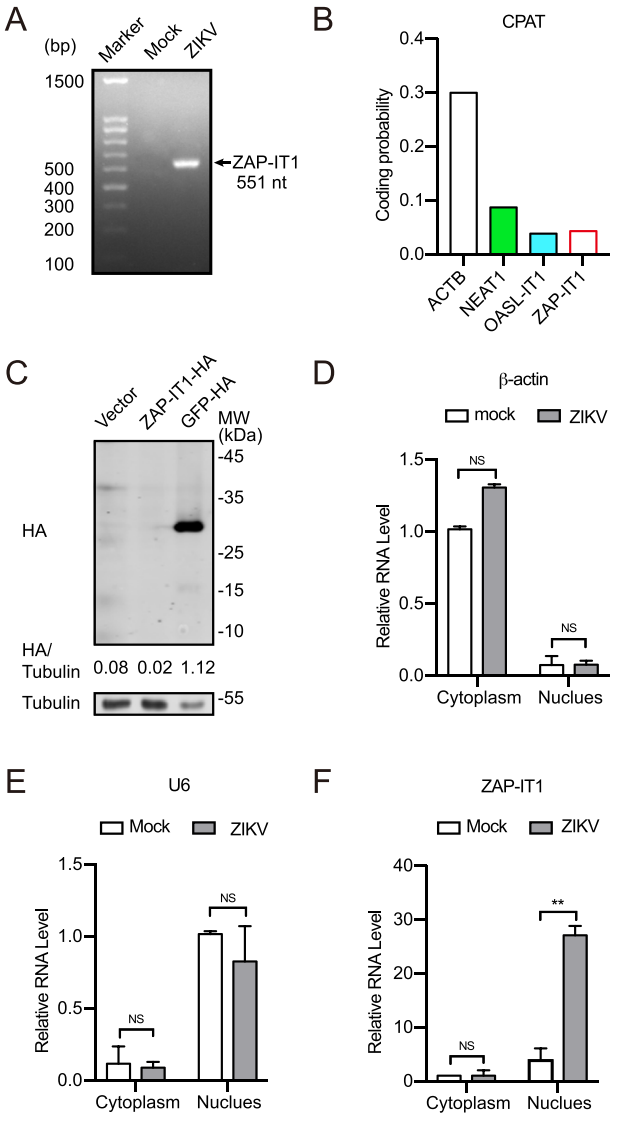

Fig. 5. ZAP-IT1 is a nuclear non-protein-coding RNA. **A** 5′- and 3′- RACE to determine the full length of ZAP-IT1. A549 cells were infected with ZIKV (MOI = 3) and total RNA were collect at 24 h.p.i. The lncRNA was reverse transcripted and amplified by the SMARTer RACE cDNA amplification kit. The RACE products were detected by nucleic acid electrophoresis. Arrow points to the only band that shows differential amplification between mock and ZIKV infected samples. **B** CPAT was employed to predict the coding probability of ZAP-IT1. *ACTB* and *GAPDH* served as control coding genes, and *NEAT1* and *OASL-IT1* served as control noncoding genes. **C** pcDNA3.1 (empty vector), pcDNA3.1-ZAP-IT1-HA, or pcDNA3.1-GFP-HA (positive control) were transfected into 293T cells. Western blotting was performed to detect anti-HA bands. **D–F** A549 cells were infected with ZIKV (MOI = 3) and harvested at 24 h.p.i. for the cytoplasm and nuclear RNA fraction. qRT-PCR was performed to measure the levels of β-actin (**D**), U6 (**E**), and ZAP-IT1 (**F**), respectively. Human *U6* or *β-actin* level was measured as an internal control and normalized to uninfected cell (mock). All experiments were independently repeated for three times. Data were shown as means ± standard deviation. $^{**}P < 0.01$. NS, not significant; h.p.i., hours post-infection; RACE, rapid-amplification of cDNA ends.

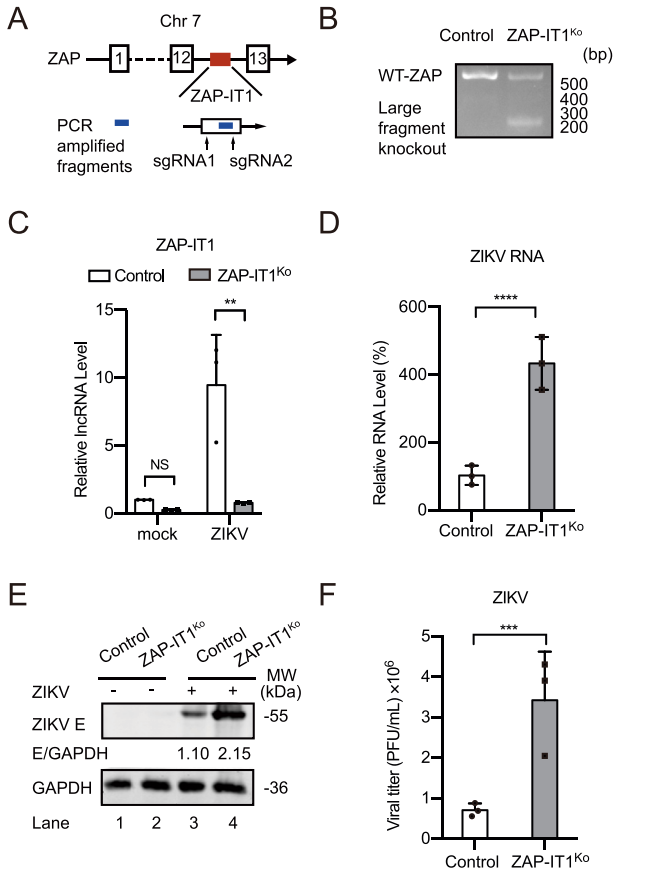

Fig. 6. Knockout of ZAP-IT1 enhances ZIKV replication. **A** Scheme diagram of sgRNA targeting ZAP-IT1. **B** PCR fragments amplified from the genomic DNA of control cells and ZAP-IT1^{Ko} cells were detected by agarose gel electrophoresis. **C** Control cells and ZAP-IT1^{Ko} cells were infected with ZIKV (MOI = 3) and total RNAs were extracted to detect the level of ZAP-IT1 by qRT-PCR. **D–F** Control cells and ZAP-IT1^{Ko} cells were infected with ZIKV (MOI = 3) and the total RNA, protein, and supernatant were collected at 24 h.p.i. The viral RNA level was measured by qRT-PCR using primers specific to ZIKV NS1. The data were normalized with the control cells. Western blotting was performed to detect ZIKV E protein. And the viral titers were determined by PFA. Human *U6* level was measured as an internal control and normalized to uninfected cell (mock). All experiments were independently repeated for three times. Data were shown as means ± standard deviation. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. NS, not significant; h.p.i., hours post-infection; Ko, knockout; sgRNA, single-guide RNA.

3.7. The antiviral effect of ZAP-IT1 is independent of the type I IFN signaling pathway and ZAP

To determine if the impact of ZAP-IT1 deficiency on ZIKV infection was mediated by the production of IFN or the IFN response directly, we evaluated the transcriptional level of IFN-β, and Mx1, known as a protein-coding ISG with antiviral activity against ZIKV. The results showed that the mRNA level of IFN-β and Mx1 didn't be affected by knockout of ZAP-IT1 compared to control after ZIKV infection (Fig. 7A and B).

To test whether the knockout of ZAP-IT1 affects the expression of *ZAP*, the protein-coding gene sharing the same sequence of its intron 12 with ZAP-IT1, the mRNA and protein level of ZAP were detected. Intriguingly, the mRNA and protein level of ZAP-IT1 did not show a remarkable change in ZAP-IT1^{Ko} cells compared to control (Fig. 7C and D), indicating that the knockout of ZAP-IT1 had no effects on the expression of ZAP. To find out whether ZAP plays a role in ZIKV infection, siRNA targeting ZAP (siZAP) or siNC was transfected into A549 cells. Neither the level of ZIKV E protein nor the viral titer showed the obvious change in siZAP cells compared to siNC cells (Fig. 7E and F). These results were consistent with the findings in Chiu's study (Chiu et al., 2018). Taken together, these results suggested that the antiviral effect of ZAP-IT1 was independent of the type I IFN signaling pathway and ZAP.

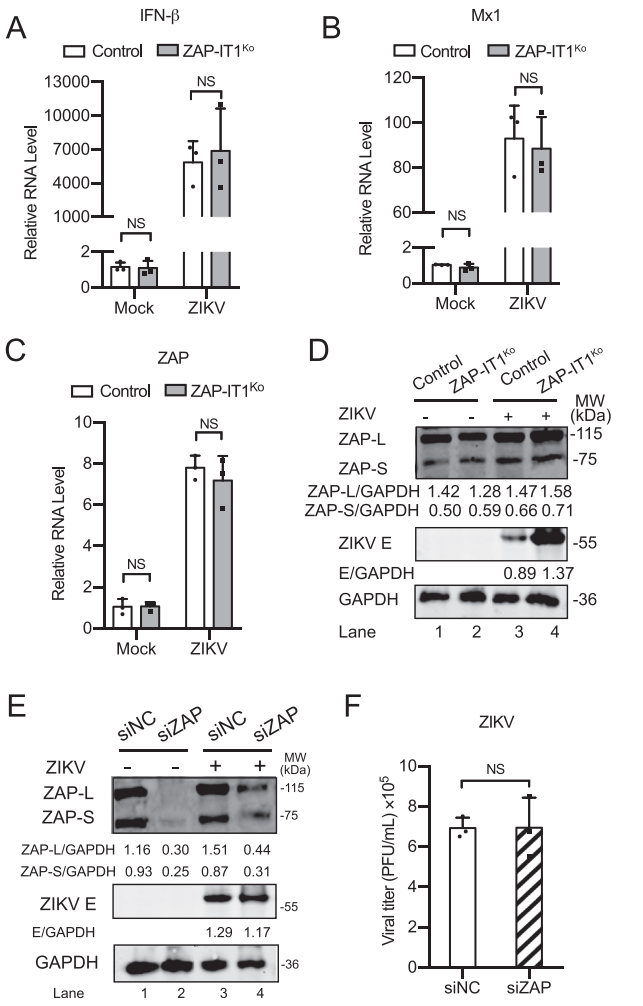

Fig. 7. The antiviral effect of ZAP-IT1 was independent of IFN signaling pathway. **A–C** Control cells and ZAP-IT1^{Ko} cells were infected with ZIKV (MOI = 3) and total RNAs were harvested at 24 h.p.i. to detect the mRNA level of IFN-β (**A**), Mx1 (**B**), or ZAP (**C**) by qRT-PCR. Human *U6* or *β-actin* level was measured as an internal control and normalized to uninfected cell (mock). **D** Control cells and ZAP-IT1^{Ko} cells were infected with ZIKV (MOI = 3). Western blotting was performed to detect ZAP-L and ZAP-S (isoform ZAP-large and ZAP-small), and ZIKV E protein at 24 h.p.i. **E, F** A549 cells were transfected with siRNA targeting ZAP or siNC as a negative control for 48 h. Cells were then infected with ZIKV (MOI = 3). Western blotting was performed to detect the protein level of ZAP-L, ZAP-S, and ZIKV E at 24 h.p.i. And the supernatant was also harvested at 24 h.p.i. to determine the viral titers by PFA. All experiments were independently repeated for three times. Data were shown as means ± standard deviation. NS, not significant; h.p.i., hours post-infection.

4. Discussion

ZIKV is an emerging arbovirus and can cause severe congenital abnormalities in the fetuses and Guillain-Barre Syndrome in adults (Pierson and Diamond, 2018, 2020; Zhang et al., 2021). Long non-coding RNAs (lncRNAs) constitute for approximate 95% of the human genome transcripts and possess low coding possibility (Djebali et al., 2012; Qiu et al., 2018; Liu et al., 2019; Li et al., 2020; Wang et al., 2021). LncRNAs are considered to be dysregulated during ZIKV infection. ZIKV infection can cause differential expression of lncRNAs profile in *Aedes aegypti* Mosquitoes cells and human neural progenitor cells (hNPCs) (Etebari et al., 2017; Hu et al., 2017). Our previous study also found that ZIKV infection could cause differential expression of 149 lncRNAs in A549 cells and confirmed the lncRNA OASL-IT1 was a defense against viral replication via regulating innate immune response (Wang et al., 2021). Current study revealed that a group of lncRNAs differentially expressed after virus infection and confirmed that a novel lncRNA, ZAP-IT1, played an antiviral role in ZIKV, DENV, JEV, and VSV replication in a type I IFN signaling independent way.

LncRNAs are found to be up- or down-regulated following TNF-α, LPS, IL-1β, IFN-α/β/γ treatment or after viral infection (Carnero et al., 2016; Covarrubias et al., 2017; Liu et al., 2019; Barriocanal et al., 2022; Chen et al., 2022; Ghafouri-Fard et al., 2022). In our study, we predicted multiple putative NF-κB and STAT1 binding sites on the promoter region of the selected lncRNAs (LAP1-AS1, LINC21762, VAMP1-AS1, and ZAP-IT1) and experimentally demonstrated that both NF-κB and STAT1 were important for the up-regulation of the selected lncRNAs induced by IFN-β and ZIKV. Therefore, the differential expression of host lncRNAs is closely related to viral infection or cytokines treatment.

Intriguingly, we found that the selected lncRNAs also significantly increased in ZIKV-infected macrophages. Recently, An's group indicated that macrophages could carry ZIKV into testis (Yang et al., 2020). During ZIKV infection, dendritic cells and macrophages detected the foreign antigens followed by the activation of the innate immune system (Lee et al., 2021; Schneider et al., 2014). Therefore, whether lncRNAs characterized in our study might play a role in any process of ZIKV infected macrophages deserves further discussion in the future.

It's reported that lncRNAs are employed by virus to promote viral susceptibility or infection (Carpenter et al., 2013; Gomez et al., 2013; Rapicavoli et al., 2013; Kambara et al., 2014; Li et al., 2016), while some other lncRNAs act as a restriction factor against viral replication (Ma et al., 2017; Xue et al., 2019; Li et al., 2020; Wang et al., 2021). In our study, LAP1-AS1, LINC21762, AND VAMP1-AS1 showed a moderate restriction effect on ZIKV infection, while ZAP-IT1 showed the strongest effect among the four lncRNAs. ZAP-IT1 also played similar role in DENV, JEV and VSV infection but not in HSV-1. It seemed that the expression of ZAP-IT1 was not significantly induced after HSV-1 infection, implying that ZAP-IT1 may not participate in DNA virus infection. As for the obvious antiviral effect of ZAP-IT1, our study provided evidence that a large fragment of ZAP-IT1 was removed, which was predicted to form the majority stem-loop of ZAP-IT1. Stem-loop is the structural basis for lncRNAs functional properties. The stem-loop of lncRNAs is reported to function via interacting with proteins (Kino et al., 2010; Wang et al., 2017, 2018; Jiang et al., 2018; Zheng et al., 2021; Yin et al., 2022). Future studies including ZAP-IT1 minimum free energy (MFE) prediction and individual nucleotide resolution CLIP (Huppertz et al., 2014) to examine the interaction between ZAP-IT1 stem-loop and ZIKV are still needed.

ZAP-IT1 is an intronic lncRNA and located at the nuclei upon ZIKV infection. LncRNAs distributed in the nucleus, regulating the production of the gene at the transcriptional level through cis or trans-acting mechanisms (Batista and Chang, 2013; Kambara et al., 2014; Qiu et al., 2018). Surprisingly, ZAP-IT1 didn't affect the expression of its neighboring gene *ZAP*. Of note, since the induction of ZAP-IT1 is associated with the type I IFN signaling pathway, we further examined whether the antiviral function of ZAP-IT1 depends on this pathway. Our data demonstrated that the antiviral effect of ZAP-IT1 on ZIKV was independent of the type I IFN signaling pathway.

Taken together, our work provides the first evidence to show a relationship between a novel lncRNA ZAP-IT1 and virus infection. The expression of ZAP-IT1 could be significantly induced by IFN-β and in turn ZAP-IT1 interrupted ZIKV infection. Moreover, the antiviral effect of ZAP-IT1 in ZIKV replication was not dependent on the type I IFN signaling pathway. Further investigation including the screen of ZAP-IT1 interacting proteins through RNA pull-down and proteomics is carrying out to reveal the mechanism of ZAP-IT1 restricts ZIKV replication.

5. Conclusions

Overall, our study investigated the role of lncRNAs in ZIKV infection, and unveiled that ZAP-IT1 induced by IFN-β interrupted ZIKV replication

independent of IFN signaling pathway, which might serve as a novel molecular target for anti-ZIKV therapeutics.

Data availability

The datasets generated for this study can be found in the NCBI GEO database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE124094). The accession number is GSE124094.

Ethics statement

Experiments involving human monocyte-differentiated macrophages were reviewed and approved by the local Ethics Committee of the Sun Yat-sen University, and were conducted ethically in accordance with the World Medical Association Declaration of Helsinki. All donors provided written informed consent. All samples were anonymized before use.

Author contributions

Yanxia Huang: conceptualization, formal analysis, investigation, methodology, software, writing-original draft, writing-review & editing. Yu Su: conceptualization, data curation, investigation, methodology, writing-original draft, writing-review & editing. Li Shen: investigation, methodology, software, validation. Zhiting Huo: investigation, methodology,. Cancan Chen: methodology, funding acquisition. Tao Sun: investigation. Xu Tian: investigation, software. Ning Li: investigation. Chao Yang: funding acquisition, project administration, supervision, writing-review & editing.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2022.08.003.

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