RESEARCH ARTICLE

Integrative transcriptomic and proteomic study of Zika viral infection reveals potential mechanisms for oncolytic therapy in neuroblastoma [version 1; peer review: 1 approved with reservations, 1 not approved]

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Abstract

Background: Paediatric neuroblastoma and brain tumours account for a third of all childhood cancer-related mortality. High-risk neuroblastoma is highly aggressive and survival is poor despite intensive multi-modal therapies with significant toxicity. Novel therapies are desperately needed. The Zika virus (ZIKV) is neurotropic and there is growing interest in employing ZIKV as a potential therapy against paediatric nervous system tumours, including neuroblastoma.
Methods: Here, we perform extensive analysis of ZIKV infection studies to identify molecular mechanisms that may govern the oncolytic response in neuroblastoma cells. We summarise the neuroblastoma cell lines and ZIKV strains utilised and re-evaluate the infection data to deduce the susceptibility of neuroblastoma to the ZIKV oncolytic response. Integrating transcriptomics, interaction proteomics, dependency factor and compound datasets we show the involvement of multiple host systems during ZIKV infection.
Results: We identified that most paediatric neuroblastoma cell lines are highly susceptible to ZIKV infection and that the PRVABC59 ZIKV strain is the most promising candidate for neuroblastoma oncolytic virotherapy. ZIKV induces TNF signalling, lipid metabolism, the Unfolded Protein Response (UPR), and downregulates cell cycle and DNA replication processes. ZIKV is dependent on sterol regulatory element binding protein (SREBP)-regulated lipid metabolism and three protein complexes; V-ATPase, ER Membrane Protein Complex (EMC) and mammalian translocon. We propose ZIKV non-structural protein

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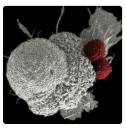
4B (NS4B) as a likely mediator of ZIKVs interaction with IRE1-mediated UPR, lipid metabolism and mammalian translocon.
Conclusions: Our work provides a significant understanding of ZIKV infection in neuroblastoma cells, which will facilitate the progression of ZIKV-based oncolytic virotherapy through pre-clinical research and clinical trials.

Keywords
Neuroblastoma, oncolytic virotherapy, Zika virus, transcriptomics, proteomics



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Keypoints

- The Zika virus may provide the basis for an oncolytic virotherapy against Neuroblastoma
- Most paediatric neuroblastoma cell lines are susceptible to Zika viral infection
- We identified molecular mechanisms that may contribute to the oncolytic response in Neuroblastoma

Contribution to the field

The ability to both induce direct oncolysis and provoke an anti-tumoral immune response makes oncolytic virotherapy an attractive candidate to combat aggressive and heterogenous cancers, such as high-risk neuroblastoma. To progress oncolytic virotherapy to clinical trial it is essential to understand the host mechanisms the virus manipulates to kill cancer cells, alongside any pathology as a consequence of infection of normal cells. Here, we show that ZIKV efficiently infects and induces oncolysis of paediatric neuroblastoma cells and propose a potential TNF pathway-driven immune response. ZIKV's specificity for infection of nervous system cancer cells, while rarely causing nervous system-related pathology in young children, addresses many of its safety concerns. The inclusion of more effective and less toxic novel therapies, such as a potential ZIKV-based therapeutic, in multimodal treatment regimens will pave the way for improving patient long-term health and overall survival.

Introduction

Neuroblastoma is the most common extracranial solid cancer in children, accounting for 6–10% of all paediatric cancers and disproportionately causing 12–15% of paediatric cancer-related deaths.[1] It is an embryonal tumour originating from transformed cells of neural crest lineage and predominately forms tumours in the adrenal medulla and paraspinal sympathetic ganglia. Whilst the majority of patients are diagnosed by the age of 5 years, the median age of patients is 18 months. Prognosis is highly heterogenous and can be predicted by a number of factors, including the presence of metastatic disease, age, chromosomal aberrations and molecular signatures, such as MYC-N amplification.[2] Patients are categorised according to internationally agreed risk groups (INRG), and treatment is stratified accordingly.

Outcome for low- and intermediate-risk neuroblastoma is good, with some patients requiring little or no treatment. However, approximately 50% of patients have high-risk disease, for which prognosis is poor, with overall survival of less than 60%.[3] Current high-risk neuroblastoma treatment regimens are aggressive. These include multiple rounds of induction chemotherapy, surgical resection, myeloablative chemotherapy, autologous stem cell transplantation and post-consolidation therapy such as immunotherapy.[4] The aggressive nature of this regimen carries significant treatment-related mortality and frequently results in long-term toxicities and sequelae impacting the quality of life for surviving patients. Consequently, there is a clear and unmet need for safer and less toxic treatment regimens to combat high-risk neuroblastoma.

Oncolytic virotherapy exploits viruses that preferentially infect and destroy cancer cells *via* two distinct routes of therapeutic action. Following infection, intense viral replication induces oncolysis, releasing virions into the tumour microenvironment to infect neighbouring tumour cells. Induction of a tumour-specific immune response is a crucial secondary mechanism employed by oncolytic virotherapy that can address highly heterogeneous tumours such as high-risk neuroblastoma and central nervous system (CNS) tumours. There is significant interest in combining immuno-modulating cancer therapies with oncolytic virotherapy to augment the anti-tumoral immune response. Oncolytic virotherapy clinical studies have in general reported low toxicity and minimal adverse effects in patients, mainly low-grade constitutional symptoms.[5]

Zika virus (ZIKV) is a mosquito-borne flavivirus consisting of historical African and epidemic-associated Asian lineages. The latter is neurotropic and can cause microcephaly in the developing foetus through infection of neural stem and progenitor cells, causing cell death and growth reduction.[6,7] By contrast, ZIKV rarely causes adverse effects in children and adults, with the majority of cases (50–80%) being asymptomatic.[8] In symptomatic children, ZIKV may cause short-term side effects, namely rash, fever and gastrointestinal symptoms, and in rare instances in adults can cause more severe conditions, such as Guillain-Barré Syndrome, meningitis and encephalitis.[8,9]

Since 2017, the concept of employing the ZIKV as oncolytic virotherapy against brain tumours has gained momentum. ZIKV induces an oncolytic event in infected paediatric brain tumour cells *in vitro* and *in vivo* assays and induces an immune response against spontaneous canine brain tumours.[10–12] Paediatric neuroblastoma, like paediatric brain tumours, are predominantly tumours of the nervous system consisting of cancerous cells with neural characteristics. An initial study assessing ZIKV infection in multiple neuroblastoma cell lines demonstrated ZIKV's potential as a novel neuroblastoma oncolytic virotherapy.[13] Here, we survey over 35 studies that have used neuroblastoma cell lines to model

ZIKV infection. These studies focused on understanding ZIKV pathology and assessing anti-viral compounds. Through re-analysis and integration of the transcriptomics, proteomics and dependency factor screens from these studies, we identify multiple molecular mechanisms implicated in ZIKV infection of neuroblastoma and aim to determine its potential as oncolytic virotherapy.

Methods

RNA-Seq data processing

RNA-Seq data files (.fastq.gz paired-end) of ZIKV-infected paediatric neuroblastoma SH-SY5Y cells were acquired from the European Nucleotide Archive (ENA) (accession: PRJNA630088). Our RNA-Seq processing pipeline consisted of FastQC (V0.11.9-0) (RRID:SCR_014583),[14,15] Trim Galore (V0.6.6-0) (RRID:SCR_011847), HISAT2 (V2.2.0) (RRID:SCR_015530),[16] SAMTOOLS (V1.11) (RRID:SCR_002105)[17] and Subread (V2.0.1) (RRID:SCR_009803).[18] Reads were aligned against the *Homo sapiens* GRCh38 genome.

Differential gene expression and pathway analysis

Differential gene expression analysis was performed using DESeq2 (RRID:SCR_015687)[19] to compare the triplicate of ZIKV-infected SH-SY5Y cells *versus* the triplicate of non-infected SH-SY5Y control cells. Differentially expressed genes (DEGs) were plotted on bar charts, volcano and scatter plots using GraphPad Prism (9.2.0) (RRID:SCR_002798). DEGs (padj < 0.05, fold change > 1.5) were submitted to Database for Annotation, Visualization and Integrated Discovery (DAVID) (DAVID 2021 (Dec. 2021)) (RRID:SCR_001881)[20,21] as official gene symbols for Gene Ontology (GO) (RRID:SCR_002811) (Biological Process Direct),[22,23] Kyoto Encyclopedia of Genes and Genomes (KEGG) (RRID:SCR_012773)[24–26] and Reactome (RRID:SCR_003485) pathway analysis. ZIKV-induced DEGs were mapped onto Pathview (RRID:SCR_002732).[27] ZIKV NS4B host interaction partners were also submitted to DAVID to identify the interaction between NS4B and host pathways. Significance values of DEG and pathway analysis were corrected for multiple testing using the Benjamini and Hochberg method (padj < 0.05).

Interactome and ZIKV dependency factor analysis

Overall, 130 high-confidence ZIKV NS4B interactors in SK-N-BE[2] paediatric neuroblastoma cells (determined by Bayesian statistical modelling) were sourced from IMEx - The International Molecular Exchange Consortium (RRID: SCR_002805) (IM-26452)[28] and mapped in Cytoscape (3.9.1) (RRID:SCR_003032).[29] The viral-host interactome was submitted to STRING (RRID:SCR_005223)[30] for high confidence (0.7) evidence-based physical subnetwork analysis to identify host-host interactions. The viral-host and STRING derived host-host interactions were integrated to identify the interaction of ZIKV NS4B with host protein complexes. Additional data incorporated into the Cytoscape map include known ZIKV dependency factors and the interaction of ZIKV NS2B-3 with the Mammalian Translocon. ZIKV dependency factors were sourced for paediatric SK-N-BE[2] neuroblastoma,[31] glioma stem cells (GSCs),[32] hiPSC-NPC,[33] HEK293FT[32] and HeLa cells.[34]

Results and Discussion

ZIKV displays strong oncolytic properties against neuroblastoma cells

ZIKV infects and significantly reduces the cell viability of a multitude of neuroblastoma cell lines from both primary tumour and metastatic sites (Table 1). ZIKV can significantly reduce neuroblastoma cell viability at multiplicity of infection (MOI) as low as 0.001.[35] The cell viability of 10/15 neuroblastoma cell lines is significantly reduced to below 20% following ZIKV infection and these observations are apparent despite the differences in the cell line, ZIKV strain, viral MOI and the type of assay performed (Table 1). SK-N-Be[1] and SK-N-Be[2] cells are from bone marrow metastasis from the same patient before and after treatment, respectively, and are both highly susceptible to ZIKV. Thus, Table 1 identifies that ZIKV can target neuroblastoma cells originating from the primary tumour, metastatic sites and metastatic sites that are resistant to standard neuroblastoma therapy. SK-N-AS, T-268 and JFEN are highly resistant (cell viability >80%) to ZIKV infection. Susceptibility is independent of patient sex, cell line origin, morphology and MYC-N status (Table 1). The non-sympathetic nervous system and non-paediatric origin of the T-268 and JFEN cells likely explain their resistance to ZIKV infection, as ZIKV has a tropism for paediatric nervous system cancer cells. The resistance of the paediatric SK-N-AS cell line is governed by CD24 expression, which regulates the basal antiviral state of these cells.[13,36] In conclusion, our analysis demonstrates that ZIKV is a strong candidate to employ against paediatric neuroblastoma as oncolytic virotherapy.

ZIKV strains possess different therapeutic potential against neuroblastoma cells

The neurotropism of the Asian lineage makes it the clear choice, over the African lineage, for developing ZIKV oncolytic virotherapy against brain tumours. However, the situation for neuroblastoma is not so apparent. Independent studies have demonstrated inherent differences in the ability of varying ZIKV strains to infect, replicate, and kill neuroblastoma cells.[37,38] Here, we assess all published data concerning ZIKV infection of neuroblastoma cells and ranked the viral

Table 1. ZIKV infects and reduces cell viability in a multitude of paediatric neuroblastoma cell lines. Cell lines are ranked by the degree to which ZIKV infection reduces their cell viability. Ranking was determined by taking the percentage to which ZIKV either reduced cell viability or induced cell death (depending on the in vitro assay used in the study) and applying a ranking system from 1 to 5, with higher numbers denoting a strong ability of ZIKV to reduce cell viability. *Note:* 1, >80%; 2, 60-80%; 3, 40-60%; 4, 20-40%; 5, <20%. ZIKV, Zika virus; MYCN, MYCN Proto-Oncogene; NA, not applicable.

Cell line	Cell viability	Age (years)	Sex	Cancer type	Cell line origin	Morphology	MYCN status	References
SH-SY5Y	5	4	Female	Neuroblastoma	Bone marrow metastasis (thorax)	Epithelial	non-amplified	66,67
SK-N-SH	5	4	Female	Neuroblastoma	Bone marrow metastasis (thorax)	Epithelial	non-amplified	38,68
SK-N-BE(2)	5	2	Male	Neuroblastoma	Bone marrow metastasis	Neuroblast	amplified	66,67
SK-N-BE(2)-M17	5	2	Male	Neuroblastoma	Bone marrow metastasis	Neuroblast	amplified	67
SK-N-DZ	5	2	Female	Neuroblastoma	Bone marrow metastasis	Epithelial	amplified	66,67
IMR-32	5	1	Male	Neuroblastoma	Abdominal mass primary tumour	Neuroblast, Fibroblast	amplified	13,66,67
SMS-KAN	5	3	Female	Neuroblastoma	Pelvic primary tumour	Neuroblast	amplified	13
SMS-KCNR	5	1	Male	Neuroblastoma	Bone marrow metastasis (adrenal)	Neuroblast	amplified	66
SK-N-FI	5	11	Male	Neuroblastoma	Bone marrow metastasis	Epithelial	non-amplified	66
CHLA-42	5	1	NA	Neuroblastoma	Bone marrow metastasis	Epithelial	non-amplified	13
SK-N-BE(1)	4	2	Male	Neuroblastoma	Bone marrow metastasis	Neuroblast	amplified	13
LA-N-6	3	5	Male	Neuroblastoma	Bone marrow metastasis (adrenal)	Neuroblast	non-amplified	13
SK-N-AS	1	6	Female	Neuroblastoma	Bone marrow metastasis (adrenal)	Epithelial	non-amplified	13
T-268	1	22	Female	Olfactory neuroblastoma	Metastasis (paraspinal mass)	NA	NA	66
JFEN	1	22	Male	Olfactory neuroblastoma	Metastasis (chest wall)	NA	NA	66

Table 2. Different ZIKV strains demonstrate varying therapeutic potential against paediatric neuroblastoma cells. ZIKV strains are ranked by their ability to infect (Degree of Infection), replicate within (Viral Titer) and significantly reduce the cell viability (Cell Viability) of a multitude of neuroblastoma cells. Data accordance denotes the degree of similarity of the results between publications that performed cell viability of cell death assays in neuroblastoma cells using the same ZIKV strain. Data accordance of five denotes that the findings of one publication closely support the findings from another, a data accordance of one denotes publications reporting vastly contrasting results. When a viral strain is published in only one paper, it is allocated a data accordance of NA. ZIKV, Zika virus; NA, not applicable.

ZIKV strain	Lineage	Cell viability	Degree of infection	Viral titer	Data accordance	References
PRVABC59	Asian	<20%	>80%	>10^7 per ml	5	13,66,69
Uganda #976	African	<20%	>60%	10^6-10^7 per ml	4	37,38,50
Brazil PE/243	Asian	20-40%	>60%	>10^7 per ml	4	45,70,71
MR766	African	20-40%	>60%	>10^7 per ml	3	37,54,61,68,69,72–75
SZ01/2016/China	Asian	<20%	NA	>10^7 per ml	NA	61
HS-2015-BA-01	Asian	20-40%	NA	>10^7 per ml	4	35,76
French Polynesia/2013	Asian	20-40%	>20%	10^6-10^7 per ml	4	37,38,50,77
Paraiba/2015	Asian	20-40%	NA	10^6-10^7 per ml	2	68,75,78
BR/800/16 Brazil 2016	Asian	40-60%	>20%	10^6-10^7 per ml	NA	38
PLCal_ZV	Asian	>80%	NA	<10^4 per ml	NA	76

strains based on their ability to infect neuroblastoma cells, produce fresh viral progeny and reduce cell viability (Table 2). We identify the PRVABC59 Asian, Uganda #976 African and MR766 African strains as the top three candidates (Table 2). Notably, the PRVABC59 Asian strain induces significantly more DEGs and splice events in SH-SY5Y cells compared to the African MR766 strain; including more immune and inflammatory response genes.[39] Brain metastases develop in 5–11% of patients with neuroblastoma and are correlated with poor prognosis.[40] The neurotropism of the Asian lineage may enhance the therapeutic potential of ZIKV by targeting these brain metastases. The multiple ZIKV pandemics identified that infection by an Asian strain is generally well accommodated by children, thus providing evidence for the safety of employing an Asian strain. Consequently, from those tested to date, we identify that the PRVABC59 strain demonstrates the greatest promise for development as oncolytic virotherapy against paediatric neuroblastoma.

ZIKV infection of neuroblastoma cells induces changes at the transcriptome level

Differential gene expression analysis identifies 453 and 256 significantly upregulated and downregulated genes (fold change > 1.5), respectively, in ZIKV-infected paediatric neuroblastoma SH-SY5Y cells (Figure 1A). GO, Reactome and KEGG pathway analysis identifies nine significantly upregulated and 12 significantly downregulated terms (Figure 1B-C). Upregulated processes include "TNF signalling pathway", lipid metabolism ("Cholesterol biosynthesis", "Cholesterol biosynthetic process", "Activation of gene expression by SREBF (SREBP)"), endoplasmic reticulum (ER) stress ("Response to endoplasmic reticulum stress", "XBP1(S) activates chaperone genes") and transcription ("BMAL1:CLOCK, NPAS2 activates circadian gene expression", "Positive regulation of transcription from RNA polymerase II promoter"). The downregulated terms are predominantly cell cycle- and DNA replication-related processes and this downregulation is apparent when the "Cell Cycle" KEGG pathway is plotted for all DEGs (fold change > 0) (Figure 1D). A potential explanation for this observation is that ZIKV can disrupt the cell cycle by targeting the centrioles in neuroblastoma cells.[41]

ZIKV induces TNF signalling in neuroblastoma cells

Of the top 10 upregulated DEGs in SH-SY5Y cells, four (BIRC3, TNFAIP3, ICAM1 and BCL3) are components of the TNF signalling pathway (Figure 1A). The TNF pathway is particularly noteworthy to consider for oncolytic virotherapy since it may play a role in both oncolysis (direct cell death) and the anti-tumoral immune response. Here, mapping the "TNF Signalling" KEGG pathway for ZIKV-infected SH-SY5Y cannot deduce if ZIKV may activate CASP-mediated apoptosis or CASP-independent necroptosis (Figure 2A). However, ZIKV-infected SH-SY5Y cells clearly show significant upregulation of transcription factors (AP-1, cEBPβ and CREB), leukocyte recruitment and activation

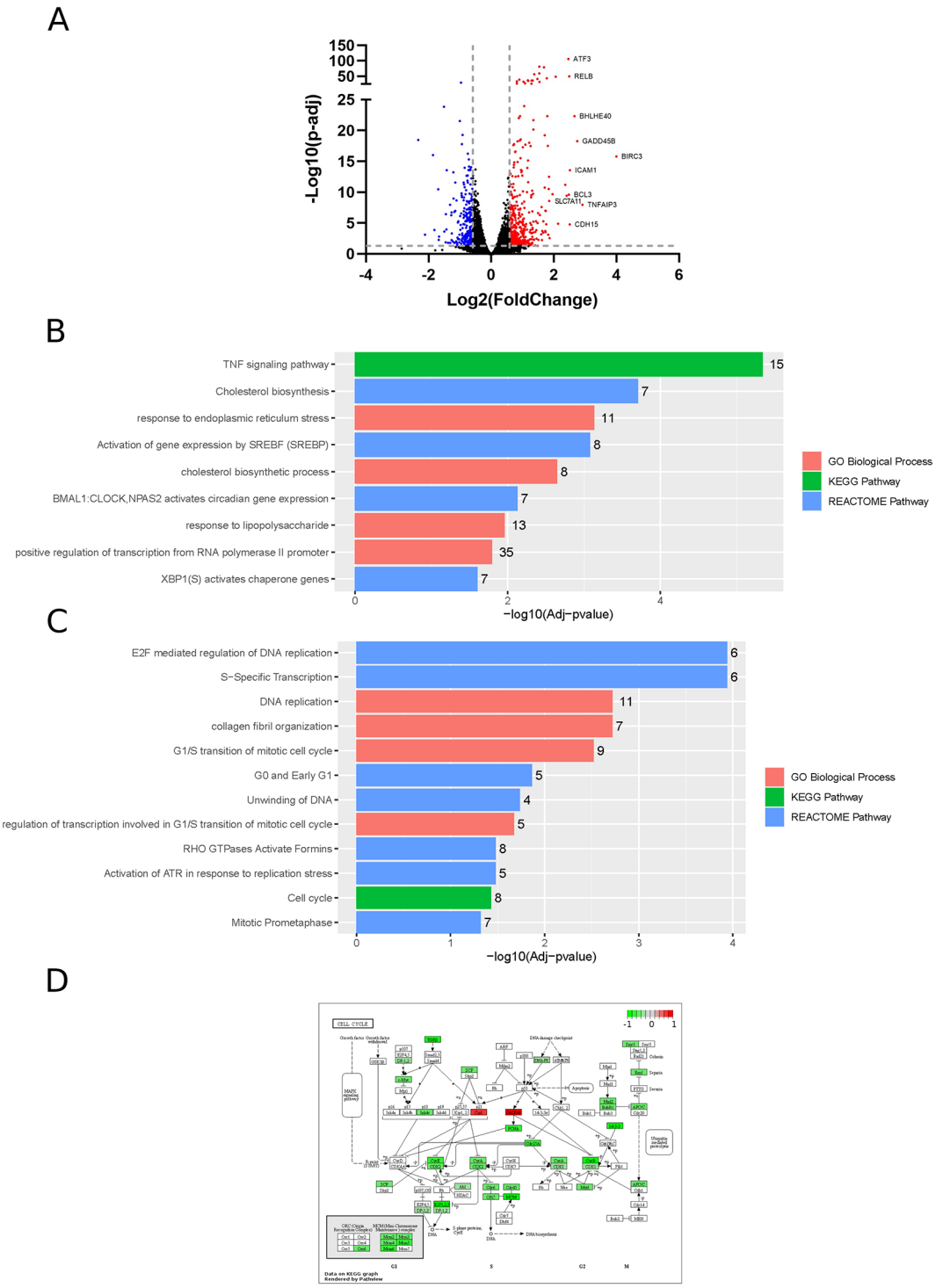


Figure 1. Differential gene expression, GO and pathway analysis of ZIKV infection in SH-SY5Y cells. Volcano plot of genes differentially expressed in response to ZIKV infection of SH-SY5Y cells, with the top 10 upregulated genes labelled (A). Significantly upregulated (B) and downregulated (C) GO Biological Processes, KEGG and Reactome pathways in response to ZIKV infection in SH-SY5Y neuroblastoma cells. KEGG map of the cell cycle, plotted using all DEGs (fold change > 0) (D). Significance values are corrected for multiple testing using the Benjamini and Hochberg method (padj < 0.05). GO, Gene Ontology; ZIKV, Zika virus; KEGG, Kyoto Encyclopedia of Genes and Genomes; DEG, differentially expressed gene.

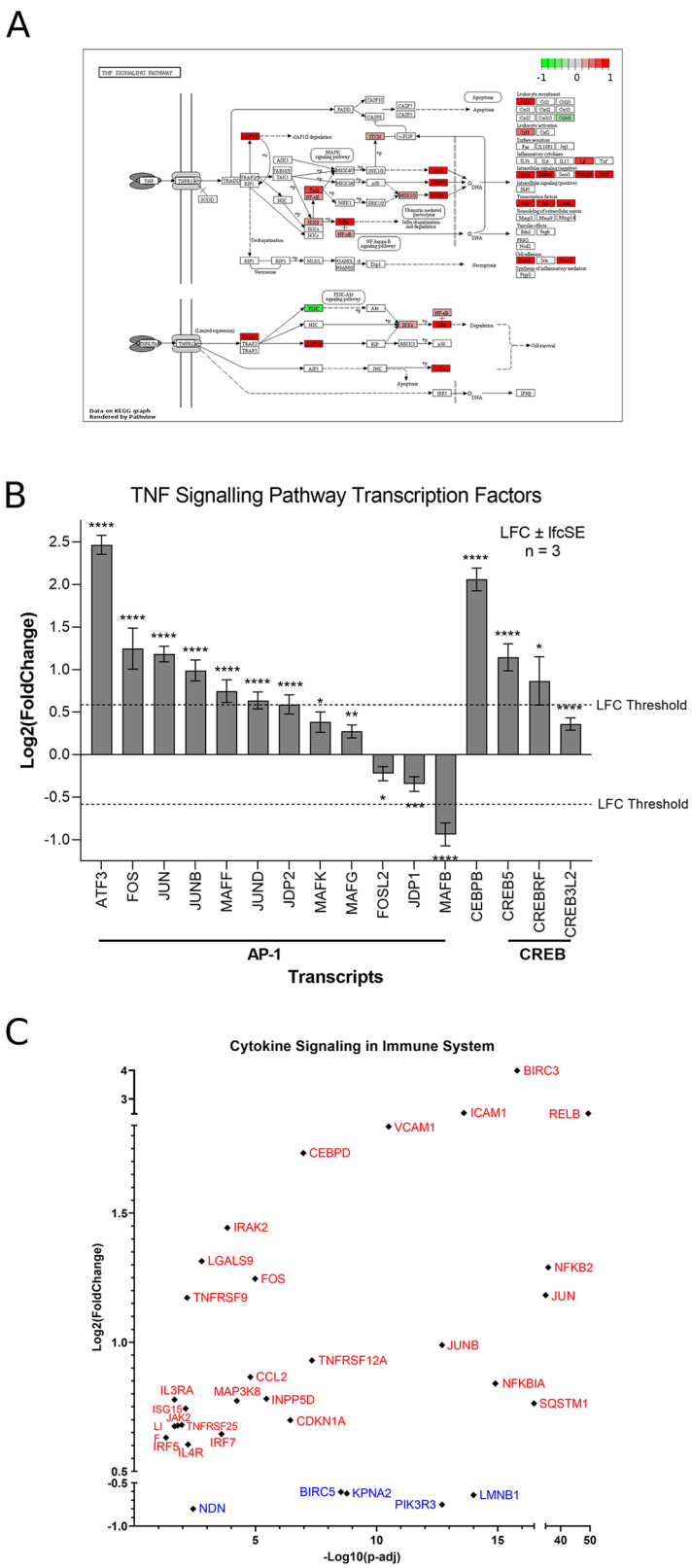


Figure 2. ZIKV infection upregulates the TNF signalling pathway in neuroblastoma cells. KEGG Map plotted for the TNF Signalling Pathway, plotted using all DEGs (fold change > 0) (A). Expression levels of TNF Signalling Transcription Factors in SH-SY5Y cells in response to ZIKV infection (B). Expression levels of cytokine signalling in immune system genes in SH-SY5Y cells in response to ZIKV infection (C). Significance values are corrected for multiple testing using the Benjamini and Hochberg method (padj < 0.05). A threshold line of Log_2(1.5 Fold Change) has been applied for the expression values. Log_2FoldChange (LFC) ± standard error of the LFC estimate (lfcSE), n = 3. ZIKV, Zika virus; KEGG, Kyoto Encyclopedia of Genes and Genomes; DEG, differentially expressed gene; AP-1, activator protein 1; CREB, cAMP response element-binding protein.

(CCL2 and CSF1), intracellular signalling (BCL3, NFKBIA, TNFAIP3 and TRAF1) and cell adhesion genes (Icam1 and Vcam1) (Figure 2A). ZIKV significantly upregulates the expression of multiple Activator protein 1 (AP-1) transcription factors, including members from all four AP-1 subfamilies (ATF, JUN, FOS and MAF) in SH-SY5Y cells (Figure 2B). AP-1 can regulate the expression of a diverse set of genes in response to nutrients, cytokines, stress or pathogen infection, and is involved in innate and adaptive immunity, differentiation, proliferation, survival and apoptosis.[42] AP-1 transcription factors can regulate the immune response of tumours, and significant AP-1 upregulation by ZIKV infection potentially identifies AP-1 as a mechanism through which ZIKV could yield an anti-tumoral immune response against neuroblastoma *in vivo*.[43]

Here, we identify CCL2 (MCP-1) to be significantly upregulated by ZIKV infection, and two independent studies have shown CCL2 to be secreted by ZIKV-infected SH-SY5Y cells.[44,45] CCL2 is a pro-inflammatory mediator that recruits leukocytes *via* chemotaxis to infiltrate tissues, including the CNS, to stimulate inflammation.[46] A non-neurotoxic herpes simplex virus (HSV)-based oncolytic virotherapy, engineered to express physiologically relevant levels of CCL2 (M010), significantly reduced Neuro-2a neuroblastoma growth in the flank of immune-competent mice and recruited CD4+ and CD8+ T-cells to infiltrate the tumour.[46] Additionally, CCL2 is secreted by ZIKV-infected cultured canine glioblastoma cells when in the presence of monocytes and is detected in serum and CSF samples of canines bearing spontaneous brain tumours following ZIKV infection.[12] We propose here that CCL2 may be capable of inducing an anti-tumoral immune response against paediatric neuroblastoma during ZIKV infection. Supporting the notion of a ZIKV-induced inflammatory response, 32 genes implicated in cytokine signalling in the immune system are significantly differentially expressed in ZIKV-infected SH-SY5Y cells: 27 upregulated and five downregulated (Figure 2C).

ZIKV induces lipid metabolism in neuroblastoma cells

ZIKV infection significantly upregulates lipid metabolism-related terms in SH-SY5Y cells; specifically, "Cholesterol biosynthesis" and "Activation of gene expression by SREBF (SREBP)" (Figure 1B). Cholesterol and lipids are essential cellular components and there are complex systems that function to regulate their intracellular abundance and localisation. These systems include regulation of cholesterol biosynthesis by the sterol regulatory element binding protein (SREBP) pathway, intracellular cholesterol trafficking, and cholesterol efflux by the liver X receptor (LXR) pathway. Cholesterol and fatty acids are required for multiple stages of the flavivirus life cycle, including regulating viral entry, the formation of viral replication complexes in the ER membrane and viral egress.[47] ZIKV elevates lipogenesis and remodels the composition of the lipid classes in infected SK-N-SH cells.[48] Here, we identify several approaches to regulate ZIKV infection of neuroblastoma cells through modification of intracellular lipid levels (Table 3). These include supplementation with pathway regulators (PF-429242, fenofibrate, lovastatin, U18666A and LXR 623) or exogenous lipids (oleic acid, docosahexaenoic acid (DHA) and cholesterol).

Table 3. ZIKV infection in neuroblastoma cells can be regulated through modifying lipid abundance, composition and localisation. List of compounds that regulate lipid homeostasis and are capable of restricting or enhancing ZIKV infection in paediatric neuroblastoma cells. ZIKV, Zika virus; LXR, liver X receptor; SREBP, sterol regulatory element binding protein; DHA, docosahexaenoic acid.

Compound	Cell line	Mechanism of action	Effect on ZIKV infection	References
Bafilomycin A1 (V-ATPase inhibitor)	SH-SY5Y	Impairs acidification of endosomal-lysosomal compartments	Restrict	50
U18666A	SH-SY5Y	Cholesterol accumulation impairs late endosomes & lysosomes	Restrict	50
LXR 623 (LXR pathway agonist)	SK-N-SH	Induces cholesterol efflux	Restrict	51
PF-429242 (SREBP pathway inhibitor)	SK-N-SH	Reduces intracellular lipid levels	Restrict	48
Fenofibrate (SREBP pathway inhibitor)	SK-N-SH	Reduces intracellular lipid levels	Restrict	48
Lovastatin (SREBP pathway inhibitor)	SK-N-SH	Reduces intracellular lipid levels	Restrict	48
Oleic Acid	SK-N-SH	Increases lipid droplet abundance	Enhance	48
Cholesterol	SK-N-SH	Increases lipid droplet abundance	Restrict	48
DHA	SH-SY5Y	Anti-inflammatory and neuroprotective effects against ZIKV infection	Restrict	45

The SREBP pathway is a principal regulator of fatty acid and cholesterol biosynthesis. The SREBF1 and SREBF2 transcription factors control this pathway, and although they share a small degree of redundancy, they primarily regulate the expression of fatty acid biosynthesis and cholesterol biosynthesis target genes, respectively.[49] Both SREBF2 and SREBF2-AS1 are significantly upregulated in ZIKV-infected SH-SY5Y cells (Figure 3A) and pathway analysis

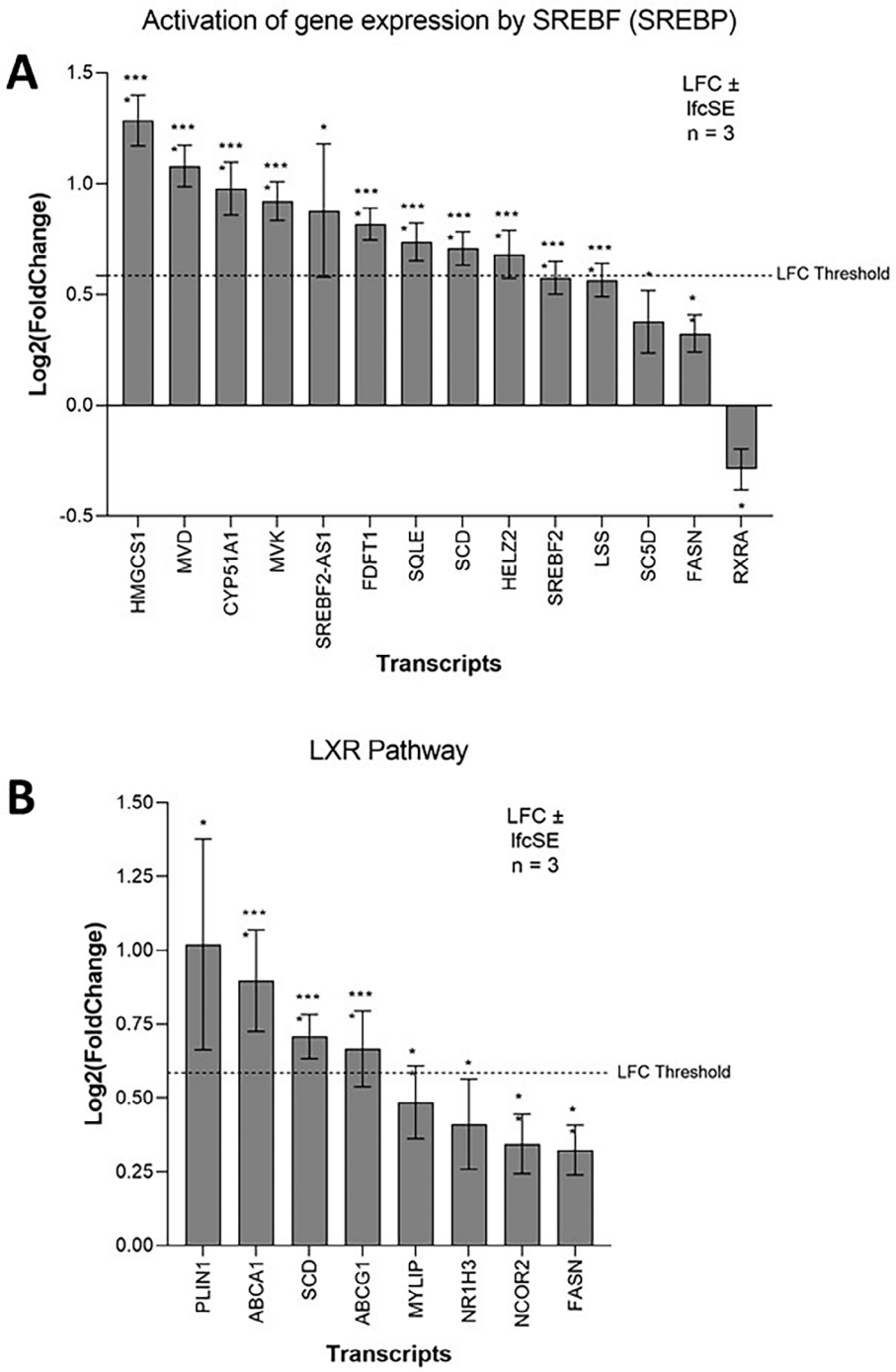


Figure 3. ZIKV infection upregulates lipid metabolism in neuroblastoma cells. Expression of SREBP pathway (A) and LXR pathway (B) genes in neuroblastoma cells in response to ZIKV infection. Significance values are corrected for multiple testing using the Benjamini and Hochberg method (padj < 0.05). A threshold line of Log_2(1.5 Fold Change) has been applied for the expression values. Log_2FoldChange (LFC) ± standard error of the LFC estimate (lfcSE), n = 3. ZIKV, Zika virus; SREBP, sterol regulatory element binding protein; LXR, liver X receptor.

identifies significant upregulation of "Cholesterol biosynthesis" (Figure 1B). Three SREBP pathway inhibitors (PF-429242, fenofibrate and lovastatin) reduce the capability of ZIKV to infect SK-N-SH neuroblastoma cells (Table 3). We identify here that the SREBP pathway is essential for ZIKV infection and propose that it may upregulate cholesterol biosynthesis through the SREBF2 transcriptional pathway in neuroblastoma cells.

Both U18666A and exogenous cholesterol restricts ZIKV infection of neuroblastoma cells (Table 3). U18666A is an intracellular cholesterol transport inhibitor that causes the accumulation of cholesterol in lysosomes to hinder the endosomal-lysosomal system.[50] Exogenous cholesterol also leads to the inactivation of the late endosomal-lysosomal compartments through a build-up of cholesterol.[50] Collectively, this identifies a dependence of the ZIKV life cycle in neuroblastoma cells on intracellular cholesterol for the correct functioning of the endosomal-lysosomal system.

The LXR pathway agonist (LXR 623) promotes cholesterol efflux (Table 3). Flaviviruses require cholesterol for the restructuring of host membranes, and LXR 623 demonstrates this dependence of ZIKV in neuroblastoma by preventing ZIKV-induced vesicle production and ER expansion in SK-N-SH cells.[51] The LXR pathway and expression of its downstream lipid homeostasis genes are regulated by the transcription factors LXR-α (NR1H3) and LXR-β (NR1H2). LXR-α protein is significantly increased by ZIKV infection of SK-N-SH neuroblastoma cells from 48 hr.[51] Although LXR-α mRNA is only marginally upregulated in our study, two major cholesterol efflux factors that are downstream of the LXR pathway, ATP-Binding Cassette A1 and G1 (ABCA1 and ABCG1), are significantly upregulated (Figure 3B). This identifies a dependence of ZIKV on the LXR pathway and suggests that ZIKV may manipulate this pathway in neuroblastoma cells to upregulate cholesterol efflux.

We identify here that lipid abundance, localisation, trafficking and metabolism regulate ZIKV infection of neuroblastoma cells and that ZIKV likely remodels the cellular lipid composition to help produce a favourable environment for efficient replication.

ZIKV induces and is dependent on the ER stress response in neuroblastoma cells

ZIKV upregulates ER-stress-related terms in SH-SY5Y cells, principally "Response to endoplasmic reticulum stress" and "XBP1(S) activates chaperone genes" (Figure 1B). The Unfolded Protein Response (UPR) dictates the ER-stress response. The UPR is normally inactive due to the ER chaperone binding immunoglobulin protein (BIP) sequestering three ER stress sensors (IRE1, PERK and ATF6). Under stress conditions BIP releases IRE1, PERK and ATF6 to assist protein folding, allowing them to activate their respective UPR-mediated ER-stress pathways.

Activation of the IRE1-mediated UPR leads to IRE1 splicing a 26 bp region from the ubiquitously expressed XBP1 mRNA. The active transcription factor XBP1(S) then drives the expression of genes to help alleviate ER stress, primarily chaperone and ER-associated protein degradation (ERAD) genes. ZIKV infection significantly upregulates 15 genes of the IRE1-mediated "XBP1(S) activates chaperone genes" Reactome pathway in SH-SY5Y cells (Figure 4A). XBP1 is the most highly upregulated gene and others include the endoplasmic-reticulum-associated protein degradation (ERAD) gene SYVN1 and the chaperones DNAJC3 and DNAJB9 (Figure 4A). Multiple IRE1-mediated UPR genes (EDEM1, SYVN1, SSR1, SRPRB, ATP6V0D1, and EXTL3) are ZIKV dependency factors in hiPSC-NPCs (Table 4). Chemical inhibition of IRE1 by 4μ8C impairs ZIKV infection *in vivo*.[52] Here, we identify that ZIKV significantly upregulates the IRE1-mediated UPR in SH-SY5Y cells and that ZIKV is dependent on this for efficient infection, likely as a means to regulate and combat viral replication-induced ER stress.

PERK-mediated UPR regulates the expression of genes involved in apoptosis, redox, amino acid transport and autophagy through eIF2 phosphorylation and the transcription factor ATF4. ZIKV infection significantly upregulates seven genes of the Reactome pathway "ATF4 activates genes in response to endoplasmic reticulum stress" (Figure 4B), including the ERAD gene HERPUD1 and the transcription factors ATF3, CEBPB and CEBPG. GADD34 (PPP1R15A), which usually dephosphorylates eIF2α in a negative feedback loop, is significantly upregulated here by ZIKV infection, and ZIKV induces eIF2 phosphorylation in SK-N-SH cells.[53] ZIKV likely upregulates GADD34 to combat ER stress-induced translational repression, as fresh virions require *de novo* protein synthesis. C/EBP homologous protein (CHOP) (DDIT3) is a pro-apoptotic protein downstream of the PERK UPR pathway that others have observed to be significantly upregulated in SH-SY5Y and SK-N-SH cells in response to ZIKV infection.[39,53,54] CHOP induces apoptotic markers, including Caspase 3, leading to cell death. Notably, multiple PERK-mediated UPR genes (ATF4, EIF2AK1, EIF2AK2, EIF2AK3 and EIF2AK4) are ZIKV dependency factors in hiPSC-NPCs (Table 4).

We conclude that ZIKV specifically upregulates and is dependent on the IRE1 and PERK branches of the UPR ER stress response in SH-SY5Y cells; conclusions that are supported by others.[53,54]

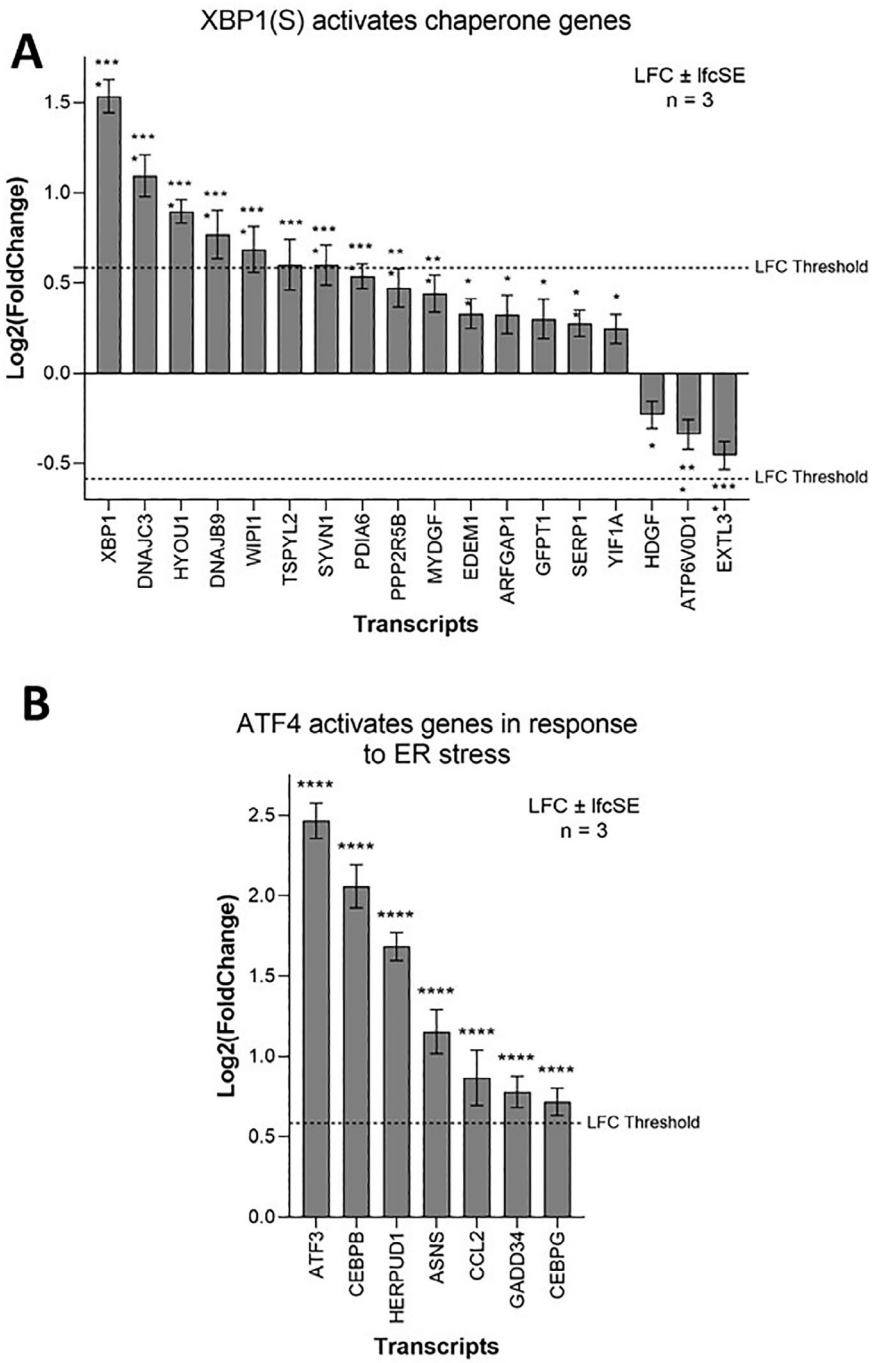


Figure 4. ZIKV infection activates the UPR in neuroblastoma cells. Expression levels of the XBP1(S) activates chaperone genes (A) and ATF4 activates genes in response to endoplasmic reticulum stress (B) genes in neuroblastoma cells in response to ZIKV infection. Significance values are corrected for multiple testing using the Benjamini and Hochberg method (padj < 0.05). A threshold line of Log_2(1.5 Fold Change) has been applied for the expression values. Log_2FoldChange (LFC) ± standard error of the LFC estimate (lfcSE), n = 3. ZIKV, Zika virus; UPR, Unfolded Protein Response; ER, endoplasmic reticulum.

ZIKV is dependent on the EMC in neuroblastoma cells

To determine which host mechanisms ZIKV may be dependent on, we cross-referenced the 22 known proteins that ZIKV requires to infect neuroblastoma cells with ZIKV dependency factors from four cell lines (Figure 5A and Table 4). Between 72–94% of the dependency factors identified across the five different cell lines are cell-specific, highlighting

Table 4. ZIKV infection is dependent on multiple host factors. Lists of known ZIKV dependency factors across SH-SY5Y (NB), GSC, hiPSC-NPC (NPC), HEK293FT and HeLa cells. ZIKV, Zika virus; GSC, glioma stem cell; NB, neuroblastoma.

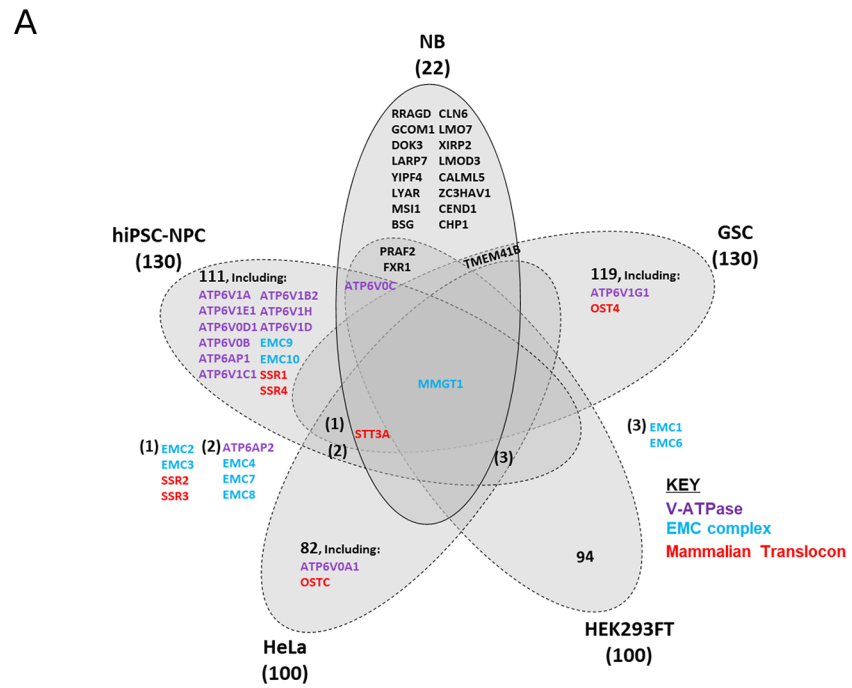
SH-SY5Y	GSC		hiPSC-NPC		HEK293FT		HeLa	
ATP6V0C	AKR1B10	MORF4L1	ANKAR	FBXL21	ALDH1L2	NR6A1	ABI2	MAPKAPK3
BSG	AKT1	MROH2B	AP2B1	FBXO45	AQP5	ORC4	AGAP1	MEN1
CALML5	ANKRD54	MRPS14	ARMCX3	FTH1P18	ARTN	P2RX7	ARF3	MMGT1
CEND1	ANXA2R	MSANTD3	ASCC3	HECTD4	ATP5F1	PADI1	ARHGEF6	MPI
CHP1	ARHGEF10L	MTRNR2L2	ATF4	HS6ST1	ATP6V0C	PBRM1	ATP6AP2	NEK2
CLN6	ARHGEF40	MYO18A	ATP6AP1	HSPA5	AURKAIP1	PDILT	ATP6V0A1	NIPAL2
DOK3	ATG3	NIF3L1	ATP6AP2	IFT27	BAK1	PDZK1	AXL	NIT1
FXR1	ATP6V1G1	NKX1-1	ATP6V0B	ISG15	BMP8B	PHIP	BET1	NPC2
GCOM1	BAALC	NMS	ATP6V0C	JAG2	C2orf16	PMPCA	BPY2	OR4K13
LARP7	C10orf35	NRSN1	ATP6V0D1	KIAA0040	CALCOCO2	POLR3C	BPY2B	OSTC
LMO7	C11orf52	NUDT19	ATP6V1A	KRTAP19-8	CERS1	PPARGC1B	C1ORF227	PER3
LMOD3	C14orf119	NUGGC	ATP6V1B2	MIDN	CLECL1	PPP2R3C	CCDC171	PLA2G16
LYAR	C16orf70	ODF3L1	ATP6V1C1	MMGT1	CNKSR1	PRAF2	CPO	PPP2R5A
MMGT1	C19orf57	OR10AG1	ATP6V1D	MSMO1	COMMD7	PRPS2	CT47A4	PRAC1
MSI1	C1orf116	OR10T2	ATP6V1E1	NBPF9	COPB2	PSMC3	CTTNBP2NL	RAB5C
PRAF2	C21orf91	OR5AS1	ATP6V1H	NDST1	COPS2	PSMD4	CXORF22	RABEP1
RRAGD	CENPH	OST4	ATP8B4	NGB	CTSF	PTPRT	DACT2	RABGEF1
STT3A	CFAP47	OXGR1	B3GALT6	NPVF	DNAJC24	RNF115	DCTPP1	RAI14
TMEM41B	CHD9	PARP9	B4GALT7	NUDT18	DNM2	RPL23	DPM1	RFX4
XIRP2	CLDN20	PLAC8L1	C14orf169	OS9	EDC4	RPL37A	E4F1	RTKN
YIPF4	CLSTN2	PLEKHM3	C3orf58	PAPSS1	EFCAB4B	RPP21	ECM2	SAA2
ZC3HAV1	CPVL	PPAN	CA4	PHPT1	EHHADH	RPS15A	EMC1	SH3GLB2
	CSDC2	PRB2	CD302	PLAC4	ELOVL7	RSL24D1	EMC2	SIPA1L3
	CSMD3	PRRT3	CKMT2	PRAC2	EMC1	SCGB1D1	EMC3	SLC9A3
	CYB561A3	PRY2	CLK2	PTPN2	EMC6	SCYL1	EMC4	SLCO4C1
	CYP26B1	QRICH1	COG1	RNASEK	EPHB3	SDAD1	EMC6	SND1

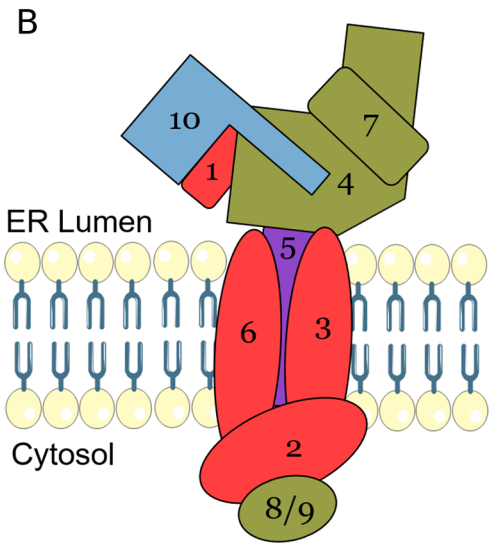
Table 4. *Continued*

SH-SY5Y	GSC		hiPSC-NPC		HEK293FT		HeLa	
	DCDC5	RAB42	COG2	RYBP	ESM 1	SLC25A3	EMC7	SPATA31C2
	DCP1B	RASSF3	COG3	SCARB1	FAM178A	SMPD4	EMC8	SPTBN2
	DCUN1D3	RHOU	COG4	SEL1L	FAM200B	SNRPB	EXT1	SSR2
	DERL2	RIMKLA	COG5	SLC22A20	FBXO4	SPATA16	EXTL3	SSR3
	DNAH7	RNF152	COG6	SLC28A3	FUNDC1	SPCS3	FAM179B	STARD10
	DNAJB8	SBK3	COG7	SLC35B2	FXR1	STOM	FAM43B	STMN4
	ELK3	SCAMP5	COG8	SLC39A9	GHRHR	SV2C	GAD1	STT3A
	EMC2	SIAH3	CSAG3	SOCS3	GK5	TBX2	GLP2R	TAF7L
	EMC3	SNX30	CTAG2	SPATA31C1	GNB2L1	TGFB3	GRIN3A	TGFBRAP1
	EPHA10	SRGAP2B	DCAF7	SPATA8	HEATR1	THAP2	HDLBP	THUMPD2
	FAM78A	SSR2	DDX3X	SPON1	IMPDH2	TMEM108	HEBP2	TMEM2
	FGFBP2	SSR3	DERL1	SPTLC1	IPO9	TMTC3	HHIP	TRAM1
	GABBR2	STK33	DERL2	SRPRB	IQGAP3	TRIM35	HIBCH	TRIM16
	GATA5	STRN3	DERL3	SSR1	LSM2	TRNT1	MIR-4429	TSNAX
	GCNT7	STT3A	DNAJB3	SSR2	LSM5	TROVE2	MIR-451A	TSPY2
	GJD2	SULT1C4	DNAJC10	SSR3	MATK	TSR2	MIR-451B	TSPY4
	GLTPD2	SV2A	EDDM3A	SSR4	MED6	TUBA1B	MIR-944	VCX2
	GNS	TET3	EDDM3B	STAT1	MIA	UBQLN1	HSF5	VPS45
	GPR33	THUMPD2	EDEM1	STAT2	MMGT1	WDR77	IFRD2	WDR7
	GPX6	TLR9	EDEM2	STAT3	MTA2	YWHAH	IQCB1	ZFYVE20
	GTF2F1	TMC7	EDEM3	STT3A	NKX2-8	ZNF584	ISG15	ZNF540
	IL17F	TMEM150B	EHMT2	SUDS3	NOL8	ZNF705D	KIAA1147	ZNF567
	IL27	TMEM176A	EIF2AK1	SYVN1	NPFF	ZNF845	KRTAP20-2	ZNF71
	IRGQ	TMEM41B	EIF2AK2	TM2D3	NR1H3	ZSWIM4	LRRC29	ZNF844
	ITGB5	TMPRSS11F	EIF2AK3	TM9SF2				
	KATNAL1	TNFAIP8L2	EIF2AK4	TMEM165				
	KIAA1522	TOR4A	EMC1	TMEM199				

Table 4. *Continued*

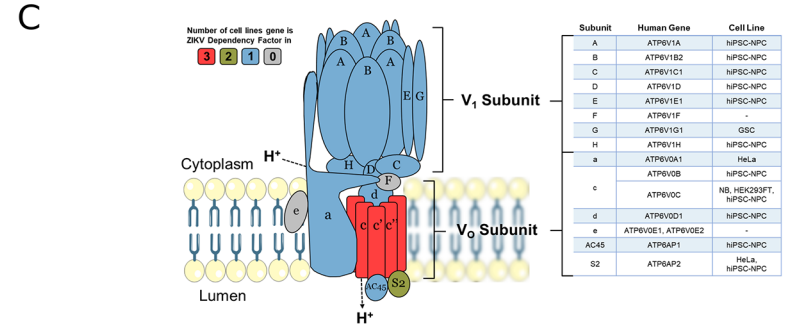
SH-SY5Y	GSC		hiPSC-NPC		HEK293FT		HeLa	
	LACC1	TPT1	EMC10	TP53				
	LDLRAD1	TRAM1	EMC2	TXNRD3				
	LHX9	UBE2G2	EMC3	UBE2G2				
	LOXL2	UBE2J1	EMC4	UBE2J2				
	LRRC61	URAD	EMC6	UGDH				
	LY6K	USP43	EMC7	UHRF1				
	LYPD8	VIPAS39	EMC8	USP17L7				
	LYRM2	WFDC12	EMC9	UXS1				
	MAGEL2	WIPF3	ERLEC1	VMA21				
	MGAM	XYLT2	EXT1	WDR7				
	MMGT1	YDJC	EXT2	ZBED5				
	MORC2	ZNF805	EXTL3	ZNF761				





Number of cell lines gene is ZIKV Dependency Factor in: 5 3 2 1 0

Subunit	Human Gene	Cell Line
1	EMC1	hiPSC-NPC, HeLa, HEK293FT
2	EMC2	hiPSC-NPC, HeLa, GSC
3	EMC3	hiPSC-NPC, HeLa, GSC
4	EMC4	hiPSC-NPC, HeLa
5	MMGT1 (EMC5)	NB, hiPSC-NPC, HeLa, GSC, HEK293FT
6	EMC6	hiPSC-NPC, HeLa, HEK293FT
7	EMC7	hiPSC-NPC, HeLa
8	EMC8	hiPSC-NPC, HeLa
9	EMC9	hiPSC-NPC
10	EMC10	hiPSC-NPC



Subunit	Human Gene	Cell Line
A	ATP6V1A	hiPSC-NPC
B	ATP6V1B2	hiPSC-NPC
C	ATP6V1C1	hiPSC-NPC
D	ATP6V1D	hiPSC-NPC
E	ATP6V1E1	hiPSC-NPC
F	ATP6V1F	-
G	ATP6V1G1	GSC
H	ATP6V1H	hiPSC-NPC
a	ATP6V0A1	HeLa
c	ATP6V0B	hiPSC-NPC
	ATP6V0C	NB, HEK293FT, hiPSC-NPC
d	ATP6V0D1	hiPSC-NPC
e	ATP6V0E1, ATP6V0E2	-
AC45	ATP6AP1	hiPSC-NPC
S2	ATP6AP2	HeLa, hiPSC-NPC

Figure 5. ZIKV dependency factors. Venn diagram of known ZIKV dependency factors across NB, GSC, hiPSC-NPC (NPC), HEK293FT and HeLa cells, to identify shared and cell-specific factors and protein complexes that ZIKV is dependent on for infection (A). Diagram of the EMC (B) and V-ATPase (C), based on their crystal structures. For the subunits in B and C, colours are allocated based on the number of cell types in which they act as ZIKV dependency factors (cell types stated in the adjacent tables). ZIKV, Zika virus; NB, neuroblastoma; GSC, glioma stem cell; EMC, endoplasmic reticulum membrane protein complex.

how ZIKV utilises differing host factors across different cell types for its life cycle. The sparse overlap of ZIKV dependency factors identifies only one factor common to all five cell types. MMGT1 (EMC5) is a key component of the Endoplasmic Reticulum (ER) Membrane Protein Complex (EMC). The EMC is a hetero-oligomer composed of 10 subunits, has chaperone properties by assisting multi-transmembrane protein folding, and is implicated in ER stress, flavivirus infection and lipid trafficking.[55] To assess if ZIKV is dependent on additional EMC subunits during infection, we searched for them in our ZIKV dependency factor dataset. We identify that ZIKV has a strong dependence on the EMC independent of cell type; all 10 EMC proteins are ZIKV-dependency factors in hiPSC-NPC, eight are in HeLa and three in GSC and HEK293FT cells (Figure 5B). The EMC facilitates the expression of ZIKV transmembrane proteins (NS2B, NS4A and NS4B), ZIKV NS4B interacts with EMC subunits, and disrupting the EMC impedes infection by ZIKV and other flaviviruses.[56,57] We propose that the EMC stabilises ZIKV proteins through integration into the ER membrane, thus permitting efficient infection in neuroblastoma cells. If investigated, we predict that additional EMC subunits would present as ZIKV dependency factors in neuroblastoma cells.

ZIKV is dependent on the V-ATPase in neuroblastoma cells

Acidification of the endosomal-lysosomal system by the V-ATPase is a property that viruses can utilise to drive the release of their nucleocapsid into the cytosol. ATP6V0C, a central component of the V-ATPase, is a ZIKV-dependency factor in neuroblastoma, hiPSC-NPC and HEK293FT cells (Figure 5A). A total of 12 additional V-ATPase subunits are ZIKV dependency factors across GSC, hiPSC-NPC and HeLa cells (Figure 5C). These 13 genes consist of multiple subunits from the Vo proton translocation and V1 ATP hydrolytic domains, identifying a functional dependence of ZIKV on the entire V-ATPase complex. The V-ATPase inhibitor Bafilomycin A1 specifically binds ATP6V0C and through V-ATPase inhibition prevents lysosomal acidification and the autophagy-lysosome pathway.[58] Bafilomycin A1 inhibits ZIKV infection of SH-SY5Y cells, supporting our observation (Table 3). siRNA silencing of the V-ATPase significantly impairs ZIKV infection of T98G glioblastoma cells, collaborating its requirement for infection of nervous system tumour cells.[59] We propose that loss of V-ATPase function impairs ZIKV infection in SH-SY5Y cells due to a perturbed pH gradient in the endosomal system. This likely prevents fusion of the viral envelope with the endosomal membrane for release of the nucleocapsid, therefore, trapping ZIKV for degradation in the lysosome, as observed in Vero cells.[60]

ZIKV NS4B possesses oncolytic capability against neuroblastoma cells

ZIKV NS4B protein is principally responsible for the oncolytic effect in SH-SY5Y cells, *via* activating the mitochondrial apoptotic pathway.[61] Determining the interactions and mechanisms underpinning this may yield opportunities to develop a paediatric neuroblastoma therapy based on ZIKV NS4B. ZIKV NS4B has 130 known host interaction partners in SK-N-BE[2] neuroblastoma cells.[31] Here, we analyse this interactome and identify multiple pathways that we, and others, have implicated during ZIKV infection of neuroblastoma cells: including mitochondrial-, lipid metabolism- and ER-associated processes (Figure 6). ZIKV NS4B interacts with 10 lipid biosynthesis proteins, three (SCD, SC5D and DHCR7) of which are expressed in response to SREBP pathway activation. This identifies a direct interaction between ZIKV and host lipid metabolism and the SREBP pathway, supporting our previous observations at the transcriptome level.

ZIKV NS4B recruits BAX to the mitochondria, triggers its activation, and releases Cytochrome c from mitochondria to induce mitochondrial cell death in SH-SY5Y cells.[61] ZIKV NS4B interacts with a multitude of mitochondrial genes (Figure 6). Including, electron transport chain proteins (TIMMDC1, MT-CO2, COX15 and OXA1L), mitochondrial translocases that import proteins into the mitochondrial matrix (TOMM22, TIMM23, TIMM50 and TIMM17B) and Solute Carrier Family 25 members for transport of solutes across the mitochondrial membrane (SLC25A1, SLC25A3, SLC25A6, SLC25A11, SLC25A12, SLC25A13 and SLC25A22). Specifically, MT-CO2, COX15 and OXA1L are conserved catalytic core, assembly and accessory subunits of the Cytochrome c oxidase complex, respectively. The Cytochrome c oxidase complex tightly couples Cytochrome c to the inner mitochondrial membrane. We propose that NS4B interacts with Cytochrome c oxidase to uncouple it from Cytochrome c, causing Cytochrome c release through the BAX pore into the cytosol to drive the mitochondrial cell death pathway in neuroblastoma cells.

ZIKV NS4B interacts with the Mammalian Translocon in neuroblastoma cells

ZIKV NS4B interacts with and is dependent on multiple proteins of the Mammalian Translocon (Figures 5A and 6). The mammalian translocon is primarily composed of the Oligosaccharyl Transferase (OST) complex, the Sec61 complex and the translocon-associated protein (TRAP) complex.[62] The multimeric OST complex co-translationally N-glycosylates proteins within the ER to assist protein folding, stability and trafficking. The Sec61 complex, a heterotrimer of Sec61α, Sec61β and Sec61γ, co-translationally translocates newly synthesised proteins across the ER and during ER stress can regulate IRE1α activity. TRAP is a heterotetramer of SSR1, SSR2, SSR3 and SSR4 that assists co-translational translocation of proteins into the ER and can prevent aberrant N-linked glycosylation during ER stress.

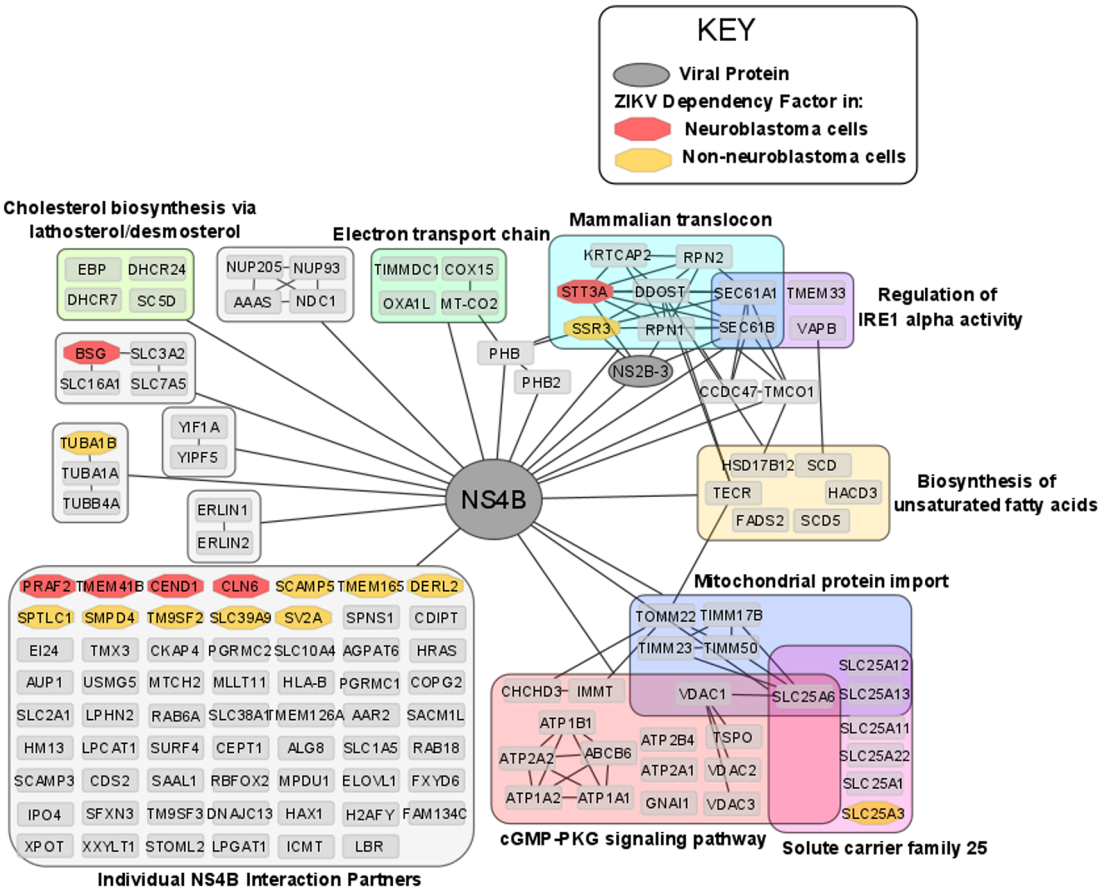


Figure 6. The ZIKV NS4B interactome in neuroblastoma cells and its interaction with ZIKV dependency factors. Nodes are grouped and labelled according to any sets of high-confidence interactions between the host proteins, and by pathways they are involved in. Cross-referencing the interactome with the ZIKV dependency factor datasets identifies the interaction of NS4B with dependency factors in neuroblastoma cells (red) and in GSC, hiPSC-NPC, HeLa and HEK293FT cells (collectively termed non-neuroblastoma cells, orange). To aid visualisation, any nodes possessing no high confidence interactions, other than their interaction with NS4B, have been grouped. All nodes within Figure 6 interact with NS4B, thus, to aid visualisation all edges between NS4B and nodes within a group have been condensed into a single edge between NS4B and the grouped set of nodes. ZIKV, Zika virus; NS4B, non-structural protein 4B; GSC, glioma stem cell.

STT3A, a principal component of the OST complex, interacts with ZIKV NS4B and is a ZIKV dependency factor in neuroblastoma, GSC, hiPSC-NPC and HeLa cells (Figures 5A and 6). Regarding the additional OST subunits, ZIKV NS4B interacts with DDOST, RPN1, RPN2 and KRTCAP2, and OSTC and OST4 are ZIKV dependency factors in HeLa cells and GSCs, respectively (Figures 5A and 6). Two forms of the OST complex exist, the STT3A and STT3B OST paralogs. KRTCAP2 and OSTC are STT3A-specific OST factors, which permit interaction of STT3A with the translocon, whilst TUSC3 and MAGT1 are STT3B-specific OST factors.[62] STT3A and both STT3A-specific OST factors are ZIKV interaction partners and/or dependency factors, but neither STT3B nor the STT3B-specific OST factors are. In addition to the OST factors, multiple N-linked glycosylation-related proteins (DPM1, DERL3, SYVN1, UBE2G2 and UBE2J1) are also ZIKV dependency factors in non-neuroblastoma cells (Table 4). The OST complex inhibitor NGI-1 blocks ZIKV infection of Huh7 cells, and disrupting ZIKV prM and E protein N-glycosylation impairs the release of infectious ZIKV particles from Vero cells.[63,64] Here, we identify that STT3A functions as a bonafide ZIKV dependency factor in multiple cell types, and conclude that efficient infection of neuroblastoma cells by ZIKV is likely dependent on the STT3A OST paralog for N-glycosylation of its viral proteins.

ZIKV NS4B interacts with SEC61A1 and SEC61B of the Sec61 complex in neuroblastoma cells and the Sec61α inhibitor Mycolactone impedes ZIKV infection of HeLa cells.[65] ZIKV NS4B interacts with SSR3 of the TRAP complex in neuroblastoma cells and ZIKV is dependent on at least two of the four TRAP complex subunits for infection of GSC,

hiPSC-NPC and HeLa cells (Figure 5A). Further supporting our observation of ZIKV interacting and being dependent on the mammalian translocon is its dependence on SRPRB, SPCS3 and TRAM1; subunits of the Signal Recognition Particle (SRP), the Signal Peptidase Complex (SPCS) and the Translocating chain-associated membrane protein (TRAM), respectively. Notably, the viral protease NS2B-3 also interacts with subunits of the OST complex (STT3A, RPN1), Sec61 complex (SEC61B) and TRAP complex (SSR3) (Figure 6). These interactions likely facilitate the co-translational cleavage of the viral polypeptide by NS2B-3 into its individual viral proteins.

Here, we identify that ZIKV NS4B and NS2B-3 directly interact with the core complexes of the mammalian translocon and propose that these interactions are essential for the ZIKV life cycle in neuroblastoma cells. The dependency of ZIKV likely stems from the mammalian translocon facilitating viral polyprotein co-translational translocation, viral polyprotein cleavage, viral membrane protein insertion and/or viral protein N-glycosylation. Additionally, ZIKV may utilise its protein interactions with the Sec61 complex, TMEM33 and VAPB, to regulate the IRE1- and PERK-mediated UPR ER stress responses, that we identify to be significantly upregulated at the transcriptome level in ZIKV infected-neuroblastoma cells.

Conclusions

Our study highlights the strong therapeutic potential of ZIKV, specifically the PRVABC59 strain, against multiple neuroblastoma cell-lines. We identify ZIKV to interact with, and be dependent on, multiple host protein complexes and pathways for its life cycle in paediatric neuroblastoma cells and for inducing oncolysis (Figure 7). Although this area of research is still at an early stage, our extensive survey of neuroblastoma ZIKV infection studies clearly demonstrates the potential of a ZIKV-based therapeutic. There are a few avenues that need to be addressed to progress this area of research, including; (1) assessing ZIKV's oncolytic effect against neuroblastoma in xenograft mouse models, (2) assessing ZIKV's capability to induce an anti-tumoral immune response against neuroblastoma in immune-competent *in vivo* models, and (3) considering the effectiveness and safety of employing different forms of ZIKV-based therapeutics against neuroblastoma. Examples of the latter may include live attenuated ZIKV strains or the construction of a virotherapy that collectively expresses ZIKV NS4B and CCL2, which we show here to hold elements of ZIKV's oncolytic and immune activation potential, respectively.

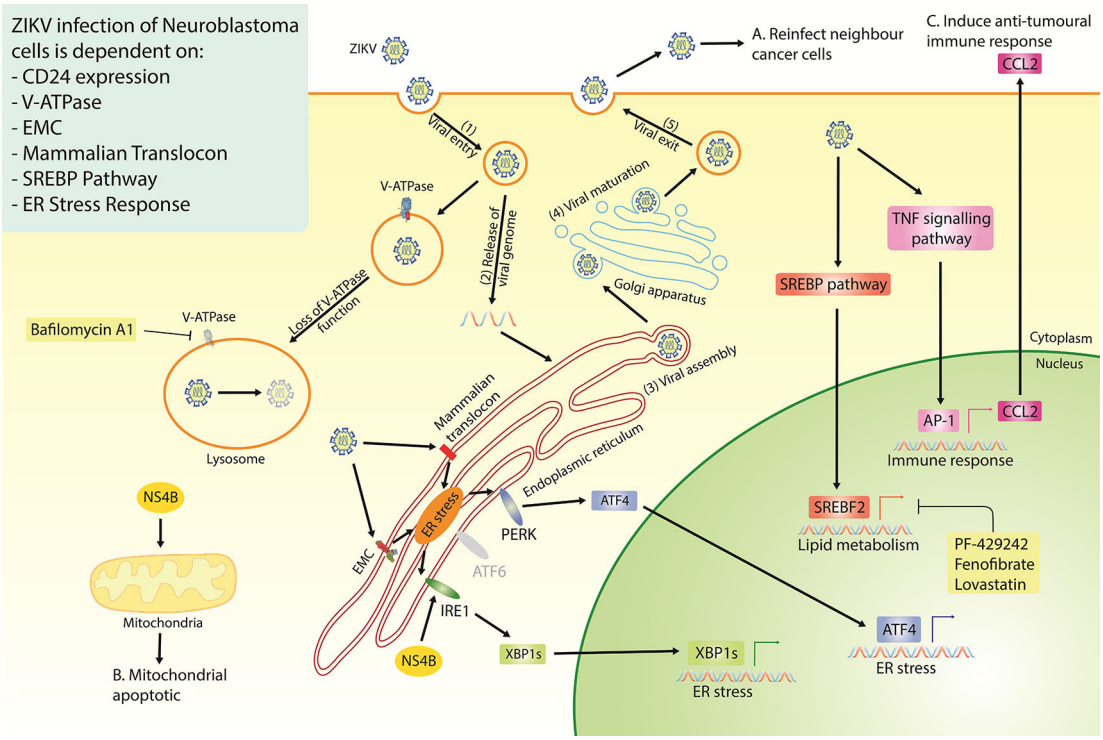


Figure 7. Diagram of the proposed ZIKV life cycle in neuroblastoma cells (Step 1-5), with a summary of all the currently known dependencies that the virus has for infection of neuroblastoma cells. Highlighted are the three essential properties of an oncolytic virus; the production of fresh viral particles to infect additional cancer cells (A), the ability to induce cancer cell death (B) and a mechanism through which ZIKV may induce an anti-tumoral immune response (C). ZIKV, Zika virus; EMC, endoplasmic reticulum membrane protein complex; SREBP, sterol regulatory element binding protein; ER, endoplasmic reticulum; NS4B, non-structural protein 4B.

Data availability

Underlying data

European Nucleotide Archive: Asian Zika virus isolate significantly changes the transcriptional profile and alternative RNA splicing events in a neuroblastoma cell line. Accession number PRJNA630088 (https://identifiers.org/ena.embl/PRJNA630088).[79]

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✗

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Integrative transcriptomic and proteomic study of Zika viral infection reveals potential mechanisms for oncolytic therapy in neuroblastoma.

The article presents an integration of transcriptomics and proteomics of several previous studies where is used the Zika virus (ZIKV) in neuroblastoma cells as a potential therapy in high-risk neuroblastoma. This pediatric cancer, characterized by its aggressive nature and limited treatment options in state-of-the-art non-responder patients, presents a pressing challenge in pediatric oncology. The authors focus on the explanation of the unique properties of ZIKV, its tropism in neuroblastoma cancer cells, and an extensive review of the host immune response in a large-scale analysis referring to previous studies.

The study focuses on different aspects regarding ZIKV-induced cell response, trying to integrate published data to propose possible targets that are characteristic of ZIKV in neuroblastoma. However, all the hypotheses and conclusions made in the study need to be validated in normalized conditions without the bias of putting together many different studies performed in different conditions. Moreover, the article should be rewritten excluding the expressions where the authors attribute the actual empirical work of the assays. They should also state and point out that the work is collecting data from previous studies and better describe the integration methodology putting all together to offer an easier view of unequivocal unique conclusions beyond previous studies.

Overall, the information provided in this article would be enlightening for having a general descriptive view of the different relevant stages in ZIKV infection that can elucidate an integrative understanding of strategies to improve the virotherapy but lacks scientific value since no validation of the the hypothesis is performed.

MAJOR POINTS

- Authors should experimentally validate their bioinformatic-based hypothesis.

MINOR POINTS

- At the end of the introduction, the sentence: “Pediatric neuroblastoma, like pediatric brain tumors, are predominantly tumors of the nervous system consisting of cancerous cells with neural characteristics.” Is redundant and not necessary.

- Authors should better describe the original data used for bioinformatic analysis. Papers that study the topic are presented, but the source of the data should be better described.

- “The non-sympathetic nervous system and non-pediatric origin of the T-268 and JFEN cells likely explain their resistance to ZIKV infection, as ZIKV has a tropism for pediatric nervous system cancer cells” needs citation.

- “The neurotropism of the Asian lineage makes it the clear choice, over the African lineage, for developing ZIKV oncolytic virotherapy against brain tumors” needs citation.

- In Table 1 and Table 2, it is necessary to say in which *in vitro* viability test is based on the assay (metabolic, death markers, etc.)

- In Table 2, the degree of infection, viral titer, and cell viability, is it normalized with the same MOI, and cancer cell type? Which cancer cell type? Would be better to explain how the authors establish the Data accordance value.

- “The multiple ZIKV pandemics identified that infection by an Asian strain is generally well accommodated by children, thus providing evidence for the safety of employing an Asian strain” needs citation.

- In Figure 3A, why authors propose that SREBF2 transcriptional pathway is responsible for the upregulation of cholesterol biosynthesis when there are other pathways with more statistical difference and fold-change.

- In the sentence “ZIKV likely remodels the cellular lipid composition to help produce a favorable environment for efficient replication”, it should be taken into account that ZIKV cannot be the one producing that, but the host viral response be responsible for those effects, and that has not been proved. It could be changed by “ZIKV infection induces all those changes…”

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

No

If applicable, is the statistical analysis and its interpretation appropriate?
I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?
No

Are the conclusions drawn adequately supported by the results?
No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Innate immunity and oncolytic virus research.

We confirm that we have read this submission and believe that we have an appropriate level of expertise to state that we do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 08 Nov 2023

Rob Ewing

We thank the reviewer for the constructive critique of the manuscript. The intention of our study is to integrate and repurpose the multiple omics datasets that have been published in the literature investigating how Zika infects mammalian cells. Many of these studies have used neuroblastoma cells as a model, and we therefore see this as an opportunity to study the potential oncolytic activity of the virus in neuroblastoma, which the original aforementioned studies did not. The reviewer points out that the analysis will be strengthened by validating the hypotheses with further lab-based studies – and we agree with this. However, our intention in this paper is to layout potential molecular mechanisms that are important in the oncolytic response that can be investigated by us and others going forward. We see our paper as an important first step in establishing potential mechanisms using data mining techniques that should stand alone in a field in which there are as yet relatively few studies.

MAJOR POINTS
• Authors should experimentally validate their bioinformatic-based hypothesis.
- **The intent of the paper was to use in silico methods to identify molecular mechanisms involved in ZIKV infection of neuroblastoma cells. For many of the components that are highlighted by our integration and re-analysis of published datasets we have sourced and referenced functional experiments that support any propositions/conclusions which we make. Two representative examples of these include:**
- **We re-analyse RNA-Seq data of ZIKV-infected neuroblastoma cells and propose the SREBP pathway to be upregulated at the transcriptome level. We link this to data that regulation of this pathway through three different inhibitors hinders ZIKV infection in neuroblastoma cells, thus supporting the involvement of this pathway during infection.**
- **We use ZIKV dependency factor screens to propose that ZIKV infection is**

dependent on the ATPase for infection in neuroblastoma cells. We back this hypothesis up with data that shows the ATPase inhibitor Bafilomycin A1 inhibits ZIKV infection in neuroblastoma

MINOR POINTS

• At the end of the introduction, the sentence: "Pediatric neuroblastoma, like pediatric brain tumors, are predominantly tumors of the nervous system consisting of cancerous cells with neural characteristics." Is redundant and not necessary.

- **This sentence has been removed**

• Authors should better describe the original data used for bioinformatic analysis. Papers that study the topic are presented, but the source of the data should be better described.

- **Additional details have been added throughout the methods section to better describe the original data. A representative example is "Scaturro et al., produced this interactome through stable expression of HA-tagged ZIKV proteins in SK-N-BE2 cells, isolation of ZIKV-host protein complexes through HA-affinity purifications, then sample preparation and run for LC–MS/MS (n = 4). 31 Raw data was processed using MaxQuant with Andromeda search engine. High-confidence interactions were determined by Bayesian statistical modelling, with log2(fold change) ≥ 2.5; unadjusted one-sided P ≤ 0.05."**

• "The non-sympathetic nervous system and non-pediatric origin of the T-268 and JFEN cells likely explain their resistance to ZIKV infection, as ZIKV has a tropism for pediatric nervous system cancer cells" needs citation.

- **Citation added**

• "The neurotropism of the Asian lineage makes it the clear choice, over the African lineage, for developing ZIKV oncolytic virotherapy against brain tumors" needs citation.

- **Text has been removed**

• In Table 1 and Table 2, it is necessary to say in which in vitro viability test is based on the assay (metabolic, death markers, etc.)

- **The details of the assays have now been added to both tables**

• In Table 2, the degree of infection, viral titer, and cell viability, is it normalized with the same MOI, and cancer cell type? Which cancer cell type? Would be better to explain how the authors establish the Data accordance value.

- **No the degree of infection, viral titer, and cell viability are not normalised by MOI or cell type. We have added columns to specify the MOI range and cell lines used. With different studies from different laboratories, it would be difficult to normalize the data by these features. Hence we prefer to present the experimental parameters as provided in the papers themselves which we used as a guide to interpretation. We also added this text to explain the 'Data accordance' value:**
- **"Data accordance is a qualitative measure which we employed to describedenotes the degree of similarity of the results between publications that performed ZIKV cell viability of cell death assaysinfection assays in of neuroblastoma cells using the same ZIKV strain. Data accordance of five denotes that the findings of one publication closely support the findings from another, a data accordance of one denotes publications reporting vastly contrasting results. When a viral strain is published in only one paper, it is**

allocated a data accordance of NA"

"The multiple ZIKV pandemics identified that infection by an Asian strain is generally well accommodated by children, thus providing evidence for the safety of employing an Asian strain" needs citation.

- **Citation added**

• In Figure 3A, why authors propose that SREBF2 transcriptional pathway is responsible for the upregulation of cholesterol biosynthesis when there are other pathways with more statistical difference and fold-change.

- **Text has been added to the manuscript to help clarify why we believe the SREBF2 transcriptional pathway may contribute to the upregulation of cholesterol biosynthesis in ZIKV-infected neuroblastoma cells.**

• In the sentence "ZIKV likely remodels the cellular lipid composition to help produce a favorable environment for efficient replication", it should be taken into account that ZIKV cannot be the one producing that, but the host viral response be responsible for those effects, and that has not been proved. It could be changed by "ZIKV infection induces all those changes..."

- **Sentence has been changed to "We propose that lipid abundance, localisation, trafficking and metabolism regulate ZIKV infection of neuroblastoma cells, and that remodelling of the cellular lipid composition within the host cell may produce a favourable environment for efficient replication."**

Competing Interests: No competing interests were disclosed.

Reviewer Report 17 August 2023

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Griffith D Parks
Burnett School of Biological Sciences, University of Central Florida, Orlando, Florida, USA

There is very high interest in mechanisms for improving the potency and specificity of oncolytic viruses, and much of this interest is in large scale analysis of host cell responses. Thus, there is potential significance for the work described here, in the analysis of transcriptomic changes that occur with Zika virus (ZIKV) infection of neuroblastoma cells in culture. An unfortunate weakness is that the work is not presented as what it is: a review of the literature and not a primary study of ZIKV infection.

Strengths of the work include the importance of understanding host factors that modulate RNA virus infections such as those by ZIKV, the global omics approach to the study using prior data sets and literature searches, the survey of published results to conclude best virus and cell type,

and the identification of distinct pathways. The work is largely well done, with interpretations which are consistent with the data. Figure 7 is particularly useful.

The main weakness of the manuscript is the presentation of findings as new and "identified" by the authors. For example, for nearly all the sections there are phrases such as "Here, we identify ZIKV NS4B....directly interact with core complexes...." The authors have not identified a direct interaction, but rather have used prior databases to develop a hypothesis that this may occur. This is a misleading representation of the data presented in this manuscript and there should be a re-write of the text to state that this is from analysis of the literature and data mining.

The authors should make it clear in the abstract in title that this is not a new study, but rather an evaluation of the literature and mining of prior data. In fact, the title is misleading by stating it is a study of ZIKV infection, when it is in reality an analysis of the published data (still useful). In reality, there is a major strength in this large amount of work and the authors are to be commended for it. There needs to be a clear statement that this is a review manuscript.

Other comments.

1. The text states that ZIKV is neurotropic. This should be modified, as this virus appears to have a lot of different host cell types it can infect. Disease is manifested when there is infection of neuro-cells, but this is not tropism.

2. In the results of abstract – please clarify "ZIKV is dependent on..." What part of ZIKV is dependent?

3. The text does not come to a conclusion on data from Table I other than to say lots of cells can be infected. What is the reason behind the graded difference between the 5 and 4 and 3. The authors speculate on the reason for grade 1, but not the others.

4. Page 4 text – "the Asian lineage.....is the clear choice for oncolytic therapy." Not clear where this comes from, and no citation was given.

5. Table 2 is useful, but has the caveat that it does not list the cells that were tested. It would be very useful to include a column with "shared cell lines tested in publications"

6. Fig 1D and 2A are entirely too small for anyone to read. These should be expanded and used a separate figures, so as to allow a reader to see the results.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?

I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: RNA Viruses

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 08 Nov 2023

Rob Ewing

We thank Dr Parks for his review of the paper, and his recognition of the significance of identifying mechanisms that may underlie the oncolytic response to the Zika virus in cancer cells. Our intention has been to integrate and re-analyze many of the omics datasets that have studied Zika viral infection of Neuroblastoma cells rather than review the literature. We believe that re-analysis and integration of the available datasets provides many new insights into the Zika virus – neuroblastoma interaction which are not yet available through the papers published to date. Additionally, we believe that re-interpreting and repurposing the non-omics data (infection assays and functional data) from these studies and drawing conclusions on a subject different to that of the original paper (ie ZIKV as an oncolytic therapy rather than as a pathogenic virus infecting neuro-cells) holds great worth. We recognize that we have not in places provided enough clarity of this intention and have now addressed this point throughout the manuscript.

The main weakness of the manuscript is the presentation of findings as new and "identified" by the authors. For example, for nearly all the sections there are phrases such as "Here, we identify ZIKV NS4B….directly interact with core complexes…." The authors have not identified a direct interaction, but rather have used prior databases to develop a hypothesis that this may occur. This is a misleading representation of the data presented in this manuscript and there should be a re-write of the text to state that this is from analysis of the literature and data mining.

We have now re-phrased these sections to provide clarification. A representative example is "From our re-analysis of a published ZIKV interactome we propose ZIKV NS4B and NS2B-3 to interact with the core complexes of the mammalian translocon, and hypothesise that ZIKV infection in neuroblastoma cells may be dependent on these interactions".

The authors should make it clear in the abstract in title that this is not a new study, but rather an evaluation of the literature and mining of prior data. In fact, the title is misleading by stating it is a study of ZIKV infection, when it is in reality an analysis of the published data (still useful). In reality, there is a major strength in this large amount of work and the authors are to be commended for it. There needs to be a clear statement that this is a review manuscript.

We have now clarified this point throughout the abstract and also through an amended title:
"Integrated re-analysis of transcriptomic and proteomic datasets reveals potential mechanisms for Zika viral-based oncolytic therapy in neuroblastoma"

Other comments.

1. The text states that ZIKV is neurotropic. This should be modified, as this virus appears to have a lot of different host cell types it can infect. Disease is manifested when there is infection of neuro-cells, but this is not tropism.
   - **The references to ZIKV being neurotropic have been changed**
1. In the results of abstract – please clarify "ZIKV is dependent on…" What part of ZIKV is dependent?
   - **Has been corrected to "ZIKV infection is dependent on sterol regulatory element binding protein (SREBP)-regulated lipid metabolism and three protein complexes; V-ATPase, ER Membrane Protein Complex (EMC) and mammalian translocon.".**
1. The text does not come to a conclusion on data from Table I other than to say lots of cells can be infected. What is the reason behind the graded difference between the 5 and 4 and 3. The authors speculate on the reason for grade 1, but not the others.
   - **We have replaced the rankings with the actual values/ranges to make this easier to interpret.**
   - **We have updated the values of LA-N-6 and SK-N-Be(1).**
   - **We have added the following text: "Whilst LA-N-6 shows partial resistance to ZIKV infection (Table 1), analysis of bulk mRNA and protein show cell LA-N-6 to express CD24. 13 Potential reasoning for this partial resistance is that subpopulations within LA-N-6 may be CD24- or a CD24-independent mechanism may be employed to infer resistance. From Table 1 we conclude ZIKV to be a promising oncolytic virotherapy candidate to employ against paediatric neuroblastoma since it can target neuroblastoma cells originating from the primary tumour, metastatic sites, and metastatic sites that are resistant to standard neuroblastoma therapy."**
1. Page 4 text – "the Asian lineage…..is the clear choice for oncolytic therapy." Not clear where this comes from, and no citation was given.
   - **This text has been removed**
1. Table 2 is useful, but has the caveat that it does not list the cells that were tested. It would be very useful to include a column with "shared cell lines tested in publications"

- **The cell lines have now been added to Table 2, including the reference for the publication from which the data has come from**

1. Fig 1D and 2A are entirely too small for anyone to read. These should be expanded and used a separate figures, so as to allow a reader to see the results.

- **Both Fig 1D and 2A (the KEGG pathway diagrams) have been pulled out and put into a new figure (Figure 2). Figures have been renumbered accordingly and there are now 8 Figures in total.**

Competing Interests: No competing interests were disclosed.