

INNATE IMMUNE RECOGNITION AND EVASION BY VIRUSES



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Abstract

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Viral infections represent a major share of the world-wide infectious disease burden. Cell-autonomous recognition of viral pathogens, and both local and systemic antiviral cytokine responses, are critically required for limiting infection. Zika virus (ZIKV) and Varicella-Zoster virus (VZV), two viruses with significant associated morbidity, are poorly characterised regarding their interaction with the innate immune system. More specifically, cell-autonomous mechanisms that govern recognition of these viruses are virtually undescribed. In addition, strategies employed by these viruses to evade recognition and clearance by the host require investigation.

ZIKV is a Flavivirus and represents a major public health concern in the Americas. It causes Zika fever in adults and can be detrimental to foetal development if acquired during pregnancy. ZIKV is closely related to other important human pathogens such as Dengue virus (DENV). Flaviviruses are positive-sense ssRNA viruses that are commonly transmitted to humans by mosquitoes or ticks. VZV is an alpha-herpesvirus that causes chickenpox upon primary infection. The virus establishes latency and causes shingles upon reactivation. Herpesviruses are dsDNA viruses that all establish latency in the human host. ZIKV and VZV differ vastly in their virion architecture, life cycle, and associated morbidities, and are both insufficiently characterised. In this study, I elucidated the molecular requirements for cells to mount antiviral type I interferon (IFN) responses to both ZIKV and VZV. I further identified a novel antagonist of cell-autonomous recognition encoded by VZV.

Infection with ZIKV induced potent type I IFN responses in A549 cells. Transcriptional induction of the IFN β response to RNA from ZIKV-infected cells was fully dependent on recognition of immunostimulatory RNA by the RNA sensors RIG-I and MDA5 and their common adaptor protein MAVS. ZIKV genomic RNA bound to RIG-I protein in a cell-free system, indicating that viral RNA can be directly recognised by cellular sensors.

Using monocytic THP1 cells as a model system I identified the cGAS/STING DNA-sensing pathway to be critically required for type I IFN induction in response to VZV infection. Using knockout cell lines, I showed that cGAS, STING, TBK1, and IRF3 were all required for induction of the IFN β transcript. Furthermore, I did not observe phosphorylation of STAT1 and STAT2 and secretion of IP-10 upon VZV infection in cells knocked-out for these factors. Given the importance of the cGAS/STING pathway for VZV recognition, I hypothesised that VZV encodes an antagonist of this pathway. I screened all unique VZV open reading frames (ORFs) and identified the tegument protein ORF9 as a novel antagonist of cGAS. Ectopically expressed ORF9 bound to endogenous cGAS and blocked the type I IFN response to transfected DNA. Confocal microscopy revealed co-localisation of ORF9 and cGAS. I further showed that this interaction occurred in VZV infected cells. ORF9 and cGAS protein also interacted directly in a cell-free system. Additional mechanistic data suggested that ORF9 blocked the production of cGAMP by cGAS.

Taken together, this work contributes to the ongoing molecular characterisation of both ZIKV and VZV. I highlighted the importance of RNA sensors for recognition of viral RNA in the context of ZIKV infection. I further demonstrated the importance of the cGAS/STING DNA sensing pathway for VZV recognition and identified a previously uncharacterised immune antagonist that directly blocks DNA sensing via cGAS.

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Acronyms

AA	amino acid
ADAR1	adenosine deaminase acting on RNA 1
ADE	antibody-dependent enhancement
AdV	Adenovirus
AGS	Aicardi-Goutières syndrome
AIDS	acquired immunodeficiency syndrome
AMP	adenosine monophosphate
APC	antigen presenting cell
ATG5	autophagy related 5
ATP	adenosine triphosphate
BAC	bacterial artificial chromosome
CAA	2-chloroacetamide
CARD	caspase recruitment domain
cGAMP	cyclic guanosine monophosphate–adenosine monophosphate
cGAS	cyclic GMP-AMP (cGAMP) synthase
CMV	Cytomegalovirus
CNS	central nervous system
CRISPR	clustered regularly interspaced short palindromic repeats
CSB	cytoskeleton stabilisation buffer
CVS	congenital varicella syndrome
DAI	DNA-dependent activator of IFN-regulatory factors
DAMP	damage-associated molecular pattern
DAPI	4,6-diamidin-2-phenylindol

DC	dendritic cell
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing Non-integrin
DDX	DEAD-box helicase
DENV	Dengue virus
DHX	DExH-box helicase
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethylsulfoxide
DRG	dorsal root ganglion
DTT	dithiothreitol
EBV	Epstein-Barr virus
ecDHFR	<i>E.coli</i> dihydrofolate reductase
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β -aminoethyl ether)-N,N,N,N-tetraacetic acid
EHV	Equine herpes virus 1
ELISA	enzyme-linked immunosorbent assay
EMCV	Encephalomyocarditis virus
ERCC6L	excision repair cross-complementing 6-like
ERP44	endoplasmic reticulum protein 44
FACS	fluorescence-activated cell sorting
FCS	foetal calf serum
FITC	fluorescein
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GAS	gamma-activated sequence
GATD2A	GATA zinc finger domain containing 2A
GBS	Guillain-Barré syndrome
GFP	green fluorescent protein
GMP	guanosine monophosphate
GST	glutathione S transferase

GTP	guanosine triphosphate
HA	hemagglutinin
HDP-RNP	HEXIM1-DNA-PK-paraspeckle components-ribonucleoprotein
HEK	human embryonic kidney
HEXIM1	hexamethylene bisacetamide inducible protein 1
HIV	Human Immunodeficiency virus
hpi	hours post infection
HPV	Human Papillomavirus
HRP	horseradish peroxidase
HSV	Herpes Simplex virus
IAV	Influenza A virus
IFI16	γ -interferon-inducible protein 16
IFI44	interferon induced protein 44
IFIH1	interferon induced with helicase C domain 1
IFIT1	interferon-induced protein with tetratricopeptide repeats 1
IFITM	IFN-induced transmembrane
IFN	interferon
IFNAR	IFN α receptor
IFNGR	IFN γ receptor
IFNLR	IFN λ receptor
IKK	inhibitor of nuclear factor κ -B kinase
IL	interleukin
iNKT	invariant natural killer T-cell
IP	immunoprecipitation
IPTG	isopropyl β -D-1-thiogalactopyranoside
IRAK	interleukin-1 receptor-associated kinase
IRF	IFN-regulatory factor
ISD	immunostimulatory DNA
ISG	interferon-stimulated gene

ISGF3	IFN-stimulated gene factor 3
ISRE	IFN-stimulated response element
IUGR	intrauterine growth restriction
IVT	<i>in vitro</i> transcribed
JAK	receptor-associated Janus kinase
JEV	Japanese Encephalitis virus
KSHV	Kaposi's sarcoma-associated Herpesvirus
LAT	latency associated transcript
LC-MS/MS	liquid chromatography tandem mass spectrometry
LDS	lithium dodecylsulfate
LGP2	Laboratory of Genetics and Physiology 2
LPS	lipopolysaccharide
LRRFIP1	LRR-binding FLII interacting protein 1
MAPK	mitogen-activated protein kinase
MAVS	mitochondrial antiviral-signalling protein
MBD2	methyl-CpG binding domain protein 2
MDA5	melanoma differentiation-associated protein 5
MEM	Minimal Essential Medium
MHC	major histocompatibility complex
MHV68	Murine Herpesvirus 68
MMR	measles mumps rubella
MOPS	3-morpholinopropane-1-sulfonic acid
MRE11	meiotic recombination 11
mTOR	mammalian target of rapamycin
MyD88	myeloid differentiation primary response gene 88
NCR	non-coding region
NEAT1	nuclear enriched abundant transcript 1
NEMO	NF κ B essential modulator
NFκB	nuclear factor κ -light-chain-enhancer of activated B cells

NHEJ	non-homologous end joining
NHP	non-human primate
NK	natural killer
NONO	non-POU domain-containing octamer-binding protein
NS	non-structural
ORF	open reading frame
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEI	polyethylenimine
PHN	post-herpetic neuralgia
PI3K	phosphoinositide 3-kinase
PKR	protein kinase R
PMA	phorbol-12-myristat-13-acetat
PRR	pattern-recognition receptor
PRV	Pseudorabies virus
PSPC1	paraspeckle component 1
PTFE	polytetrafluoroethylene
PTM	post-translational modification
RIG-I	retinoic acid-inducible gene I
RIPA	radioimmunoprecipitation assay
RLR	RIG-I-like receptor
RPMI	Roswell Park Memorial Institute
RT-qPCR	reverse transcription quantitative PCR
SAMHD1	SAM domain and HD domain-containing protein 1
SCID	severe combined immunodeficiency
SD	standard deviation
SDS	sodium dodecylsulfate

SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEAP	secreted embryonic alkaline phosphatase
SEM	standard error of the mean
SFPQ	splicing factor, proline- and glutamine-rich
sfRNA	subgenomic flaviviral RNA
SLC19A1	solute carrier family 19 member 1
SLE	systemic lupus erythematosus
STAT1	signal transducers and activators of transcription 1
STAT2	signal transducers and activators of transcription 2
STING	stimulator of interferon genes
SVV	Simian Varicella virus
TBEV	Tick-borne Encephalitis virus
TBK1	TANK-binding protein 1
TBS	Tris buffered saline
TCEP	tris(2-carboxyethyl)phosphine
TEG	triethylene glycol
TGN	<i>trans</i> -Golgi network
TIM1	T cell immunoglobulin and mucin domain 1
TK	thymidine kinase
TLR	toll-like receptor
TMP	trimethoprim
TNFα	tumor necrosis factor α
TRAF	tumor necrosis factor receptor-associated family
TREX1	three prime repair exonuclease 1
TRIF	toll-like receptor adaptor molecule 1
TRIM25	tripartite motif-containing protein 25
TRIS	tris(hydroxymethyl)aminomethane
USP7	ubiquitin-specific-processing protease 7
UTR	untranslated region

VACV	Vaccinia virus
vhs	virion host shutoff
VLP	virus-like particle
VSV	Vesicular Stomatitis virus
VZV	Varicella-Zoster virus
WHO	World Health Organization
XRCC	X-Ray repair cross complementing
YFP	yellow fluorescent protein
YFV	Yellow Fever virus
ZBP1	Z-DNA-binding protein
ZIKV	Zika virus

Chapter I

Introduction

The innate immune system is at the forefront of defence against pathogens invading the human body¹. The first contact of bacteria, viruses, fungi, and parasites are most often made at mucosal surfaces. Physical, chemical, and biological barriers, such as the corneated part of the skin, low pH, and antimicrobial peptides, respectively, are all indispensable innate defence mechanisms. In addition, specialised cell types within the human body recognise invading pathogens based on conserved pathogen-associated molecular patterns (PAMPs). Recognition of PAMPs by pattern-recognition receptors (PRRs) expressed in innate immune cells, such as natural killer cells, basophils, eosinophils, mast cells, neutrophils, macrophages, and dendritic cells (DCs), is a key component of the human immune response. When activated by PAMP recognition, these cells fight infections by phagocytosis of pathogens and rapid secretion of cytokines and other soluble mediators. Chemical factors, such as histamine, prostaglandins, and leukotrienes, and inflammatory cytokines, such as interleukin (IL)-1, IL-6, and tumor necrosis factor α (TNF α), can increase the influx of immune cells, trigger systemic responses such as fever, and mediate cross-talk with other arms of the immune system. Furthermore, activation of PRRs can lead to secretion of cytokines called interferons (IFNs), named based on their ability to interfere with viral infection. Specialised cells of the immune system have superior abilities for recognition of PAMPs and cytokine secretion. However, all cells within the human body can become infected with pathogens and have the ability to respond cell-autonomously to invading pathogens. Analogous to immune cells, these recognition events rely on PAMPs and PRRs.

I.1 The Concept of Pattern Recognition

The idea that cells express germ line-encoded receptors to recognise conserved molecular patterns that can be found on or within pathogens, but not host cells, was first conceptualised by Charles Janeway in 1989². While the immunostimulatory properties of certain pathogen-

derived substances, such as lipopolysaccharide (LPS), were known before Janeway's proposals, his ideas were the starting point of discovery for a plethora of PRRs. Initially, pattern recognition was investigated primarily as a mechanism to activate DCs, B cells, and T cells in the context of adaptive immune responses. It became apparent, however, that pattern recognition is fundamental for the activity of innate immune cells and cell-autonomous response to pathogens by non-immune cells.

It is understood today that mammalian cells have evolved to recognise a wide array of substance classes in order to distinguish pathogens from self. These include, but are not limited to, poly- and monosaccharides and other carbohydrates, nucleic acids, modified amino acids, peptides, and lipoproteins. Different classes of receptors are able to bind these substances both inside and outside of cells. In addition to recognition of particular molecular patterns, the (mis)localisation of ligands is an important mechanism in self vs. non-self discrimination by PRRs.

Bacteria, fungi, and parasites are self-sufficient in their life cycle. Within another organism, they will often be encountered by the host's immune cells as extracellular entities. In contrast, viruses rely on cells for replication. By definition, they are intracellular pathogens. Mechanisms that can sense pathogens inside cells are hence indispensable for antiviral responses. As a consequence, mammalian organisms harbour an array of recognition pathways that can elicit both localised and systemic innate immune responses after sensing of PAMPs both inside and outside of host cells.

Because viruses originate from eukaryotic cells, they lack the presence of non-eukaryotic molecular patterns that can be recognised as PAMPs. Viral recognition by mammalian immune mechanisms therefore relies to a large extent on sensing of viral nucleic acids. Nucleic acids are of course part of host cells as well. Within the last decades it has been recognised that endogenous molecular patterns, in particular nucleic acids, can trigger PRR activation during sterile tissue damage and inflammation. The concept of pattern recognition has therefore been extended to include damage-associated molecular patterns (DAMPs) in addition to PAMPs.

The following sections will introduce different nucleic acid recognition mechanisms found in human cells, with a focus on the sensing of double stranded DNAs as PAMPs and DAMPs. In addition, the interferon system will be described as it governs antiviral responses. Lastly, an overview will be given on the pathogens investigated in this study, Zika virus (ZIKV) and Varicella-Zoster virus (VZV).

I.2 PAMP Recognition Through Toll-like Receptors

In 1997, Ruslan Medzhitov and Charles Janeway described that a homologue of the *Drosophila* gene *Toll* can activate NF κ B signalling in human cells³. In the following year, Bruce Beutler's group mapped bacterial LPS responsiveness of mice to the *Tlr4* gene⁴. This was the first toll-like receptor (TLR) discovered in mammals and is today known as TLR4, one of nine TLRs found in human cells (reviewed in REF. 5, see Figure 1). They are primarily expressed by immune cells and bind extracellular or endosomal ligands. TLRs can recognise a wide array of PAMPs, such as lipoproteins, polysaccharides, bacterial and fungal sugars, and nucleic acids (ssRNA, dsRNA, dsDNA, DNA:RNA hybrids). The ligands of each TLR are displayed in Figure 1.

Downstream signalling from TLRs in most cases involves the adaptor molecule MyD88⁵ (Figure 1). TLRs homo- or heterodimerise upon ligand binding which allows recruitment of MyD88. Through a complex signalling cascade involving IRAK1, IRAK2, IRAK4, TRAF6, and IKK β , stimulation of TLRs commonly leads to activation of the transcription factors NF κ B and IRF7. This results in the transcription of inflammatory cytokines, and type I and III IFNs. In addition, the MAPK pathway is activated and contributes to the expression of inflammatory cytokines. Notably, TLR4 activates type I IFN transcription through a pathway in which the adaptor molecule TRIF activates IRF3 by recruitment of TRAF3, TBK1, and IKK ϵ . TLR3 exclusively signals through the TRIF pathway, independently of MyD88.

The first protein to be identified as a sensor of dsDNA was TLR9⁶. TLR9 is preferentially activated by CpG DNA, a type of DNA found predominantly in microbial genomes. It was recognised soon after, however, that expression of TLR9 is restricted to immune cells. Furthermore, given its localisation, TLR9 cannot recognise cytosolic DNA. Many non-immune cells, however, have the ability to respond to exogenous DNA with type I IFNs and inflammatory cytokines. It was clear from these observations that other, TLR-independent DNA sensing mechanisms must exist^{7;8}.

I.3 Sensing of Cytosolic DNA

The potency of cytosolic double-stranded DNA to induce a cytokine response was recognised long before the concept of pattern recognition was introduced and DNA sensors were

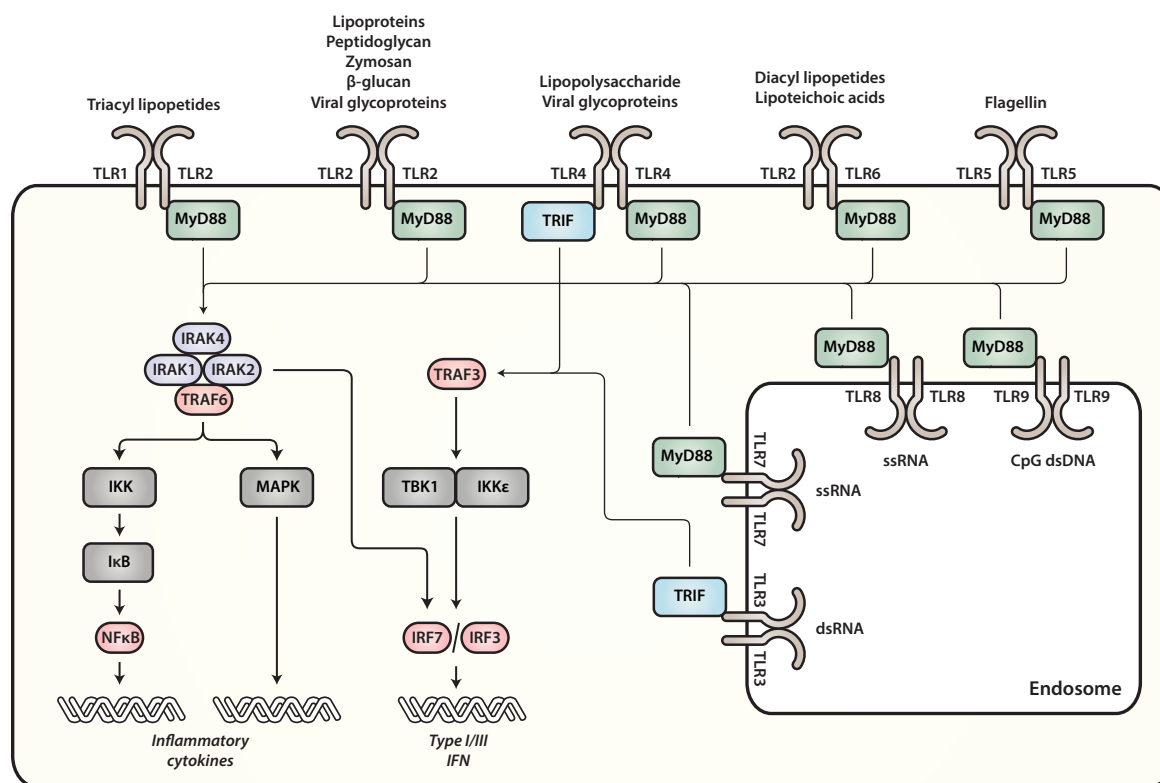


Figure 1: The ligands and intracellular signalling of human TLRs. Nine TLRs recognise a variety of PAMPs as homo- or heterodimers. Signalling via MyD88 activates a cascade involving IRAK1, IRAK2, IRAK4, TRAF6, IKKβ, NFκB, and IRF3/7 which ultimately leads to the expression of inflammatory cytokines and type I and III IFNs. TLR3 and TLR4 activate expression of type I/III IFNs through the adaptor molecule TRIF and TRAF3, TBK1, and IKKε.

identified^{9;10}. As mentioned, while TLR9 had been described as a sensor for dsDNA, it could not explain cytokine responses to cytosolic DNA^{7;8}. In the years after the discovery of TLR9, multiple proteins have been proposed to be responsible for the recognition of dsDNA in the cytosol. The first one was DAI (now called ZBP1)¹¹. ZBP1 can bind DNA and induce type I IFN in response to DNA in certain cell types. However, its absence in mice fails to impede IFN production after DNA virus challenge. It is now increasingly recognised that the primary role of ZBP1 could rather lie in cell death induction after recognition of Z-conformation RNA^{12;13}.

A major discovery was the identification of stimulator of interferon genes (STING) in 2008^{14;15}. STING was identified not as a putative sensor of DNA, but as a protein that serves as a signalling adaptor. Importantly, STING KO mice are severely impaired in their ability to mount cytokine responses to DNA virus challenge¹⁶. In the years after, RNA polymerase III^{17;18}, LRRFIP1¹⁹, DHX9 and DHX36²⁰, IFI16²¹, DDX41²², DNA-PK²³, and MRE11²⁴ were described as cytosolic DNA sensors that induce type I IFNs. In addition, AIM2 was identified to activate the inflammasome in response to DNA^{25–27}. In 2013, the

discovery of cyclic GMP-AMP (cGAMP) synthase (cGAS) and cyclic guanosine monophosphate–adenosine monophosphate (cGAMP) ended the race to find the putative cytosolic DNA sensor^{28–30} (see below). Nevertheless, AIM2, RNA Polymerase III, IFI16, and DNA-PK have non-redundant functions either as sensors in the context of specific pathogens, or as accessory proteins for the canonical cGAS/STING DNA-sensing pathway (see below).

I.3.1 The cGAS/STING Pathway

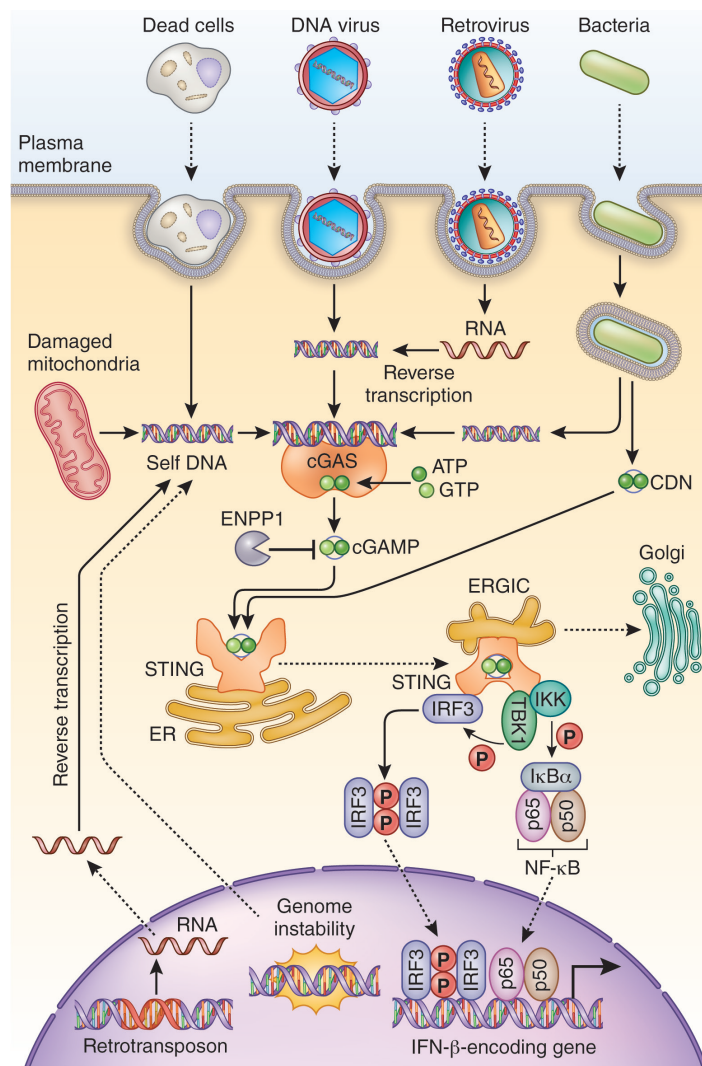


Figure 2: The cGAS/STING DNA-sensing pathway. DNA of exogenous (viruses, bacteria) origin or self-DNA binds cGAS and leads to the production of cGAMP from ATP and GTP. Binding of cGAMP to STING induces trafficking of the ER-resident protein to the Golgi. Recruitment of TBK1 and IKK kinases allows activation of the transcription factors IRF3 and NF-κB. Together they activate transcription of the *IFNβ* gene. Figure taken from REF. 31.

Upon binding of dsDNA, cGAS catalyses the synthesis of cGAMP from ATP and GTP^{28–30} (Figure 2). The protein recognises DNA in a sequence-independent manner through interaction with the sugar-phosphate backbone. Binding of DNA induces a conformational change that allows ATP and GTP to access the catalytic site^{32;33}. The nucleotidyl transferase activity of the catalytic site enables synthesis of 2'-3'-cGAMP. Rearrangement of the catalytic site by agonist binding is essential for cGAS enzymatic activity since dsRNA and ssDNA can also bind cGAS but fail to induce the conformational change³². The enzymatically active cGAS-DNA structure is a complex in which two cGAS molecules bind two DNA molecules^{34;33}. As

a result, cGAS dimers can form

ladder-like structure on stretches of DNA³⁵. This phenomenon is one explanation why long dsDNA activates cGAS more potently than short DNA. Another reason could be that, in co-

operation with Zn^{2+} , cGAS and DNA can oligomerise and induce liquid phase separation³⁶. The longer the DNA, the more efficiently cGAS-DNA complexes separate. These compartments facilitate cGAMP production. Liquid phase separation of cGAS and DNA has also been suggested as a mechanism of controlling cGAS activity since a certain threshold of both cGAS and DNA concentration needs to be reached for this to occur³⁶.

The cGAMP produced after cGAS activation serves as a second messenger by binding to STING^{30;37;38} (Figure 2). In its inactive form, dimeric STING resides in the ER membrane¹⁵. Ligand binding induces conformational changes within the protein and as a result, STING is trafficked to the Golgi³⁹. It is here that TBK1 and IRF3 are recruited to STING. A series of as yet incompletely understood events results in the phosphorylation of STING, TBK1, and IRF3. Phosphorylated IRF3 forms a dimer and acts as a transcription factor. In addition to the phosphorylation of IRF3, stimulation of STING also results in the activation of NF κ B. NF κ B signalling is required to induce transcription together with IRF3. It furthermore induces transcription of inflammatory cytokines. Activation of MAPK pathways downstream of STING has also been reported⁴⁰. The precise molecular mechanisms that govern NF κ B activation by STING are an area of active investigation.

In addition to the transcriptional responses mediated through TBK1 and IRF3, activation of STING can induce autophagy. Stimulation of STING by cyclic dinucleotides induces a ATG5-dependent autophagic response^{39;41–43}. Autophagy in this context is important for clearance of intracellular pathogens. In addition, autophagy is an essential regulatory mechanism of cGAS/STING activity through removal of immunostimulatory DNA^{39;41;44–46} and degradation of signalling proteins, such as STING itself^{43;47}. Lastly, an interesting link between autophagic removal of damaged mitochondria (mitophagy), release of cGAS-stimulatory mitochondrial DNA, and Parkinsons disease has been uncovered recently⁴⁸. Given the increasingly recognised role of immunostimulatory mitochondrial DNA in infectious and non-infectious pathologies, it is likely that autophagic removal of damaged mitochondria is essential for limitation of excessive immune responses in this context.

It is noteworthy that STING-activating ligands do not necessarily originate from cGAS activation directly prior or in the same cell (Figure 2). 2'3'-cGAMP produced by cGAS is transmitted to neighbouring cells *via* gap junctions⁴⁹. It can also be packaged into new virions in infected cells and activate STING upon infection of secondary cells^{50;51}. A role of cGAS in the activation of STING in response to endocytosed extracellular cyclic-dinucleotides has

been suggested recently⁵². Additionally, the membrane transport protein SLC19A1 can import cGAMP from the extracellular space with subsequent stimulation of STING⁵³. Lastly, STING can be activated by bacterial cyclic dinucleotides, such as cyclic-di-AMP, cyclic-di-GMP, and 3'3'-cGAMP^{54;55}. STING consequently can be described as both a PRR and adaptor protein that has functions in innate defence dependent and independent of dsDNA recognition.

I.3.1.1 Recognition of Viruses by cGAS/STING

The cGAS/STING pathway senses a variety of viral pathogens from different families of viruses. The type I IFN response to the DNA virus Herpes Simplex virus (HSV) 1 relies on cGAS and STING both *in vitro* and *in vivo*^{16;30;56–60}. Furthermore, cGAS/STING have been implicated in sensing of other herpesviruses, such as human and murine Cytomegalovirus (CMV), Murine Herpesvirus 68 (MHV68), and Kaposi's sarcoma-associated Herpesvirus (KSHV)^{61–65}. Other DNA viruses, like Vaccinia virus (VACV), Adenovirus (AdV), and Human Papillomavirus (HPV), induce a cGAS/STING dependent type I IFN response^{66–69}. Recognition of dsDNA by cGAS further plays a role in the cell-autonomous antiviral response to Human Immunodeficiency virus (HIV)^{70–74}. Endogenous DNA from mitochondria is sensed by cGAS during infection with the flavivirus Dengue virus (DENV)^{75;76}. The results outlined above highlight a crucial role of the cGAS/STING DNA-sensing pathway in viral pathogen recognition.

I.3.1.2 Endogenous Regulation of cGAS/STING Activity

The activity of DNA sensing by cGAS and STING is tightly controlled to allow efficient recognition of pathogens, while at the same time avoiding pathogenic effects due to exacerbated IFN responses to self-DNA.

Cellular nucleases are essential to avoid accumulation of immunostimulatory DNA as a result of non-pathogenic cellular processes. Cytosolic DNA is degraded at baseline by the exonuclease TREX1^{77;78}. The importance of this mechanism is highlighted by type I IFN mediated immune pathologies in patients with monogenic defects in *TREX1* (see below). Another nuclease, DNase II, is localised in endosomes and is important for degradation of endocytosed DNA^{79;80}. Recent work on cGAS and anti-tumour responses (see below) further points at the possibility of endocytosed DNA gaining access to the cytosol, thus

potentially activating cGAS. Individuals with monogenic defects in the *DNASE2* gene and *Dnase2* knockout mice show clear IFN signatures^{81–84}. Importantly, ablation of cGAS or STING rescues DNase II IFN pathology in mice, suggesting an important role for DNase II in removal of cGAS-stimulatory DNA^{85;86}. As mentioned above, induction of autophagy after cGAS/STING activation also contributes to removal of stimulatory DNA.

The gene encoding cGAS is an interferon-stimulated gene (ISG) thus forming a positive feedback loop for increased sensitivity in the context of infection⁶⁴. While high cGAS concentrations at baseline might cause pathogenic responses to aberrant self-DNA, a low activation threshold is essential in the context of infection. A decreased activation threshold through higher enzyme concentration after IFN stimulation further fits the aforementioned phase separation-induced model of activation. In turn, cancer cells have been shown to epigenetically silence transcription of cGAS and STING to evade DNA damage detection and subsequent elimination by the immune system⁸⁷.

Post-translational modifications (PTMs) represent another layer of activity regulation. Ubiquitination is an essential PTM in the regulation of both cGAS and STING activity. Ubiquitination of cGAS by two distinct mechanisms can enhance its enzymatic activity and inhibit autophagic cGAS degradation^{88;89}. Numerous ubiquitination pathways have been extensively characterised to regulate activity and turnover of STING^{90–95}. The activity of cGAS has further been shown to be both positively and negatively regulated by glutamylation and SUMOylation^{96–98}. Another PTM, palmitoylation, is important for STING activity (see below)^{99;100}. Lastly, phosphorylation of both cGAS and STING have been reported and, in the case of STING, are well known to directly influence activity^{43;101–104}.

From the findings discussed above it is clear that cGAS and STING are part of a complex regulatory network whose characterisation has only just begun. Notably, an additional important aspect of the regulation of cGAS/STING activity is spatial separation of ligands, receptors, and signalling adaptors (see below).

1.3.1.3 The Subcellular Localisation of cGAS

When cGAS was discovered, it was biochemically purified from cytosolic cell extracts²⁹. This localisation conceptually matched the idea of it recognising DNA in the cytoplasm, a place where DNA is not found physiologically. Multiple studies have now observed cGAS protein in the nucleus^{71;105–108}. While the idea of nuclear cGAS initially was discarded by many as

an experimental artefact, it is now being recognised more and more as being physiologically relevant.

A study found a constant nuclear pool of cGAS which associates preferentially with centromeric DNA¹⁰⁸. This pool of nuclear cGAS is enzymatically active and produces cGAMP, albeit at a much lower rate than stimulated cytosolic cGAS. Whether cGAMP produced in the nucleus can access the cytosol and stimulate STING is unknown. Furthermore, the molecular basis for reduced enzymatic activity of nuclear cGAS is not fully elucidated. It has been shown that nucleosomes, i.e. DNA associated with histones, are poor cGAS agonists^{71;109}. Upon DNA damage, cGAS can translocate to the nucleus and inhibit DNA repair¹¹⁰. This in turn can promote tumour development. In addition, liquid phase separation of cGAS DNA complexes, shown to be important for cGAS activity in the cytoplasm³⁶, might be inhibited in the nucleus due to a lack of free Zn²⁺ ions¹⁰⁸. Recently, nuclear cGAS has been shown to promote cell death during mitotic arrest¹⁰⁹.

Interestingly, nuclear cGAS appears to be relevant not only in the context of interaction with self-DNA, but also recognition of DNA as a PAMP. In the nucleus, together with a protein called NONO, cGAS has been shown to sense HIV cDNA⁷¹. In light of the fact that nuclear DNA is part of the life cycle of different types of viruses, the relevance of intranuclear pathogen-derived DNA sensing by cGAS will be interesting to explore in the future.

Recently, tethering of cGAS to cellular membranes by interaction with phosphoinositides was suggested as a mechanism for self/non-self discrimination¹¹¹. The functional implications of this phenomenon remain to be confirmed.

I.3.1.4 cGAS and STING in Non-infectious Pathologies

Initially, recognition of cytosolic DNA was investigated in the context of infection, i.e. sensing of exogenous DNA. However, overwhelming evidence has established a central role of self-DNA responses in a number of non-infectious diseases.

Self-DNA that activates cGAS can have two origins in cells: the nucleus and mitochondria (Figure 2). DNA that originates from both of these cellular compartments has been demonstrated to activate cGAS and STING, both *in vitro* and *in vivo* (reviewed in REF. 40 and 112).

Type I IFNs are well known to play an important part in anti-tumour immune responses¹¹². To a large part, this effect is mediated by type I IFN stimulation of antigen presenting cells to

prime lymphocytes for tumour cell detection and eradication. A widely accepted model is the uptake of dying tumour cells by DCs and cGAS/STING stimulation by tumour cell DNA. While the mechanism through which this DNA gains access to the cytosol of the phagocyte is unknown, the anti-tumour effects of type I IFNs, and cGAMP in particular, are well documented in murine models⁴⁰. STING KO mice are less able to control tumour growth compared to WT animals¹¹³ and intratumoural delivery of cGAMP attenuates tumour growth¹¹⁴.

Given the potent anti-tumour effects of cGAMP, it is not surprising that cancer cells often inhibit cGAS/STING signalling to evade senescence and immune recognition⁸⁷. In some cancer contexts, activation of cGAS and STING can have pro-tumour roles^{110;115;116}. It is clear from these findings that the pro- and antitumour effects of cGAS and STING are still being elucidated.

In addition to cancer, sensing of self-DNA is a major contributing factor to multiple autoimmune and autoinflammatory diseases. Mutations in the genes encoding TREX1, SAMHD1, and RNaseH2, among others, cause Aicardi-Goutières syndrome (AGS) in humans¹¹⁷. AGS is characterised by chronically elevated type I IFNs and thus classified as an interferonopathy¹¹⁷. TREX1 is a cytosolic nuclease that degrades dsDNA, and recognition of self-DNA through cGAS and STING has been demonstrated to be causative for the disease in mouse models of *TREX1* AGS^{85;118;119}. Similar results were obtained for RNase H2 and SAMHD1^{120–122}.

Limited evidence points at a role of cGAS and STING in the pathogenesis of systemic lupus erythematosus (SLE). Elevated serum IFN is a well-known hallmark of patients with SLE¹²³. Furthermore, the presence of antibodies against nuclei and DNA in patients indicates a key role of self-DNA in SLE¹²³. Peripheral blood mononuclear cells (PBMCs) from lupus patients show elevated expression of cGAS, and cGAMP could be detected in sera from lupus but not rheumatoid arthritis patients or healthy controls¹²⁴. Patient sera have been shown to contain apoptosis-derived membrane vesicles that can stimulate IFN production in a STING dependent manner¹²⁵.

I.3.1.5 Exogenous Modulation of DNA Sensing

Given the potency of cGAS and STING to sense and consequently restrict viral infection, both proteins are unsurprisingly targeted by viral proteins. From the growing list of viral antagonists of cGAS and STING it appears that in particular human herpes viruses have

evolved multiple antagonist mechanisms (extensively reviewed in REF. 126). For example, the protein encoded by ORF52 of KSHV, now named kicGAS, can inhibit cGAS enzymatic activity and in its absence, KSHV infection generates elevated type I IFN responses¹²⁷. UL37 and VP22 of HSV-1 inhibits cGAS by deamidation or interaction with the protein, respectively^{128;129}. STING is inhibited by an impressive number of proteins from both human and murine cytomegalovirus^{63;130–133}. In addition to the ones described here, countless other viral proteins from different families of viruses were identified to interfere with cGAS/STING activation¹³⁴. This emphasises the central importance of this pathway for viral restriction and suggests the existence of more, yet unidentified viral antagonists.

As mentioned before, the cGAS/STING pathway is involved in multiple non-infectious pathologies and therefore represents an attractive target for pharmacological modulation. Given the detrimental effects of cGAS and STING activation in certain malignant and autoimmune-inflammatory diseases, considerable effort has been put into the development of specific inhibitors.

A small molecule, termed Ru.521, was described to potently inhibit the activity of murine cGAS¹³⁵. Novel compounds based on the medicinal chemistry of Ru.521 were subsequently described to also potently inhibit activity of recombinant human cGAS protein¹³⁶. Another small molecule compound, PF-06928215, has been reported to inhibit human cGAS enzymatic activity but failed to prevent IFN production in cellular cGAS activity assays¹³⁷. Suramin, an approved organic compound for treatment of river blindness and African sleeping sickness, can inhibit activity of cGAS in THP1 cells by acting as a nucleic acid mimic¹³⁸. Notably, Aspirin (acetylsalicylic acid) could alleviate pathology in AGS patient cells and murine models of AGS through acetylation of the enzyme¹³⁹.

Aside from cGAS, STING has also been actively investigated as a target for inhibitors. Two small molecules, C-176 and H-151, have been described to specifically inhibit murine and human STING, respectively¹⁰⁰. These compounds target a conserved cysteine residue and prevent its palmitoylation. This palmitoylation has been described previously to be essential for STING activity⁹⁹.

Currently, research on pharmacological inhibition of cGAS and STING is being actively pursued by multiple biotechnology enterprises and early phase clinical trials are expected to start in 2020¹⁴⁰.

I.3.2 RNA Polymerase III

Before the discovery of cGAS, RNA Polymerase III was identified to be responsible for the IFN responses to foreign DNA in the cytosol^{17;18}. RNA Polymerase III is one of three RNA polymerases expressed in animal cells and transcribes tRNAs, 5S rRNA, and a variety of other non-coding RNA species in the nucleus¹⁴¹. Interestingly, in the cytosol, the enzyme can also generate RNA transcripts from a poly dA:dT DNA template^{17;18}. Due to cytosolic localisation, the generated 5'-triphosphate-bearing transcripts escape modification by the nuclear capping machinery. Consequently, these transcripts generated by RNA polymerase III potentially activate the cytosolic RNA sensor RIG-I (see section I.4.1), and thus the type I IFN response. Detection of exogenous DNA by RNA polymerase III contributes to the type I IFN responses to bacterial pathogens such as *Legionella pneumophila*, *Shigella flexneri*, and viral infections such as HSV-1, VZV, and Epstein-Barr virus (EBV)^{17;18;142;143}. Given that RNA polymerase III only senses A:T-rich DNA, its roles in type I IFN responses to pathogens are likely to be specialised and/or accessory to other DNA-sensing mechanisms. However, a physiological relevance of RNA polymerase III for pathogen detection was clearly demonstrated by the identification of patients with mutations in RNA polymerase III subunits who suffer from severe central nervous system (CNS) pathology due to disseminated VZV¹⁴³ (see section I.7.7.1).

I.3.3 DNA-PK

DNA-PK is a heterotrimeric complex of proteins consisting of the catalytic subunit DNA-PKc, and the two DNA binding proteins Ku70 and Ku80. DNA-PK has a well-established role in repair of DNA double-strand breaks via non-homologous end joining (NHEJ)¹⁴⁴. In addition, the Ku70 protein was identified to trigger type III IFN rather than type I IFN responses to exogenous DNA¹⁴⁵. Shortly after, the DNA-PK complex as a whole was demonstrated to trigger an IRF3-dependent type I IFN response after VACV infection *in vitro* and *in vivo*²³. This response was further dependent on STING and TBK1. Interestingly, in these experiments, activation of DNA-PK by VACV only marginally triggered NFκB and did not result in inflammatory cytokine secretion. Recently, the initially described role of Ku70 for activation of type III IFNs was also reported to be STING-dependent¹⁴⁶. This reinforces the notion that STING serves as a central signalling hub downstream of multiple PRRs that sense dsDNA.

It will be interesting in future research to delineate in detail the signalling events related to STING that enable the distinct transcriptional outcomes mentioned above. It is nevertheless noteworthy that DNA-PK, cGAS, and TLR9 are the only described dsDNA-recognising PRRs that induce type I IFN for which knockout mouse models show impaired IFN responses and impaired control of viral infections¹⁴⁷.

I.3.4 IFI16

Initially described as a *bona-fide* DNA sensor²¹, IFI16 is now recognised as a protein that has roles as an accessory protein in the cGAS/STING pathway, as well as independent functions for viral restriction. The role of IFI16 for DNA recognition further appears to be cell type specific. IFI16 and cGAS cooperate in human keratinocytes and macrophages to facilitate cGAMP production^{148;149}. In addition, IFI16 was shown to be part of a cGAS-independent DNA-damage response pathway that involves STING¹⁵⁰.

IFI16 is targeted for degradation by HSV-1, although conflicting results regarding the mechanism have been reported^{151–153}. Nevertheless, this phenomenon demonstrates a key role of the protein in herpesviral restriction. In fact, both direct viral restriction and effects on sensing of viral DNA have been reported for IFI16^{60;106;149;154;155}.

I.4 Sensing of Cytosolic RNA

In addition to dsDNA, RNA in different molecular conformations commonly acts as a PAMP during viral infections or DAMP in the context of non-infectious settings¹⁵⁶. In addition to TLRs that recognise RNA (see above), members of the family of RIG-I-like receptors (RLRs) respond to exogenous or self-RNA. These are the proteins RIG-I, MDA5 and LGP2. LGP2 lacks signalling activity and is involved in regulation of RNA recognition by RIG-I and MDA5^{157–161}. It will therefore not be discussed further here. In contrast, RIG-I and MDA5 directly sense certain types of RNA and are critically involved in recognition of viral infection (Figure 3). RIG-I and MDA5 are both DExD/H helicase proteins and share the helicase domain, a C-terminal domain, and two CARD domains in their structure¹⁵⁶. The helicase domain of both proteins can interact with RNA in an ATP-dependent manner. RLRs are ubiquitously expressed but undetectable at steady-state, in particular MDA5^{156;162}. Both RIG-I and MDA5 are strongly inducible by type I and type III IFNs in most cells types, creating a

potent positive-feedback loop. In fact, RIG-I expression is stimulated by IRF3, directly downstream of PRR activation¹⁶³. This stimulation is independent of type I IFN signalling and serves as a rapid response mechanism to RNA virus infection.

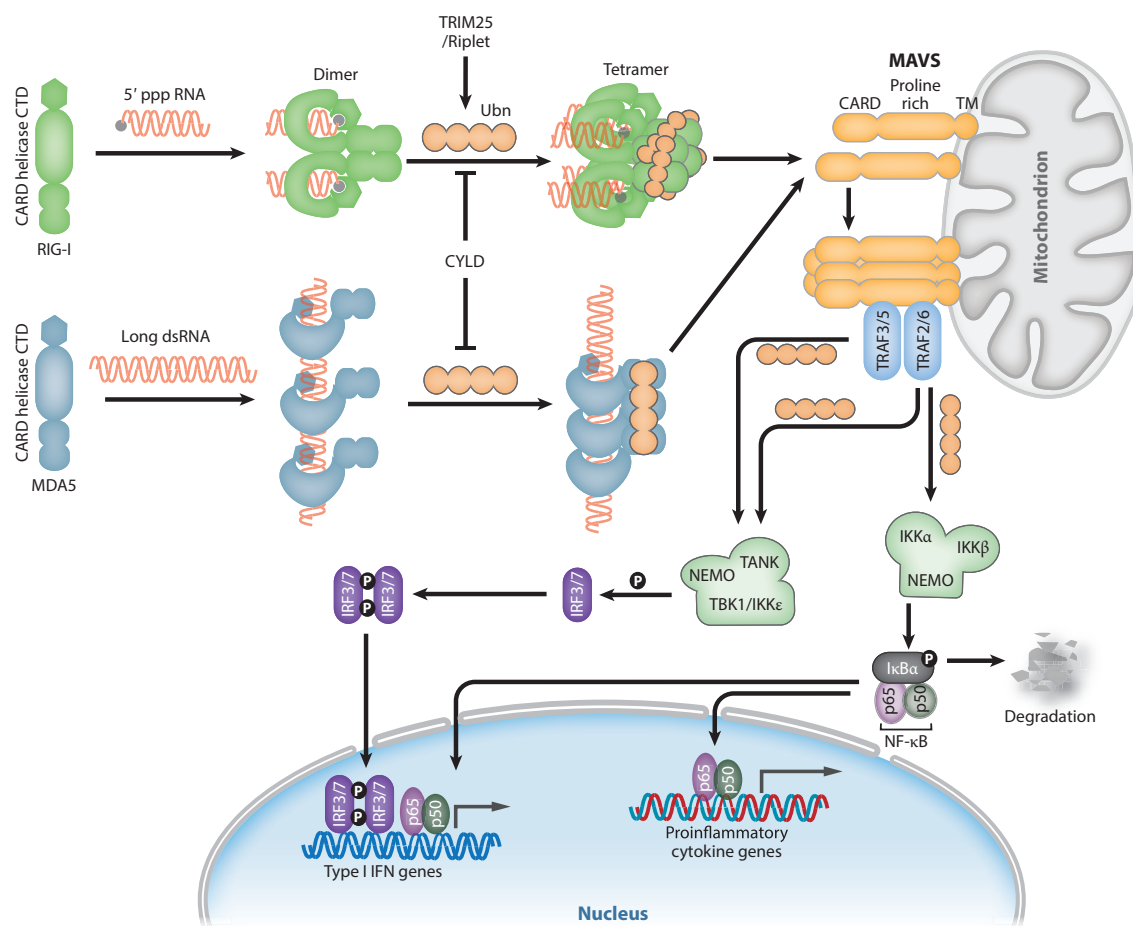


Figure 3: The intracellular signalling of RLRs. MDA5 and RIG-I recognise long dsRNA and 5'-triphosphate RNA, respectively. Conformational changes and ubiquitination events allow interaction of RIG-I and MDA5 with MAVS via CARD domains. MAVS oligomerisation and TRAF binding lead to recruitment of the kinases IKKε and TBK1. They phosphorylate the transcription factors IRF3 and IRF7. Additionally, IK kinases activate NFκB. These transcription factors induce expression of type I IFNs, type III IFNs, and inflammatory cytokines. Figure is adapted from REF. 164

I.4.1 RIG-I

RIG-I recognises short ssRNA or dsRNA through interaction with their ends that have a particular confirmation¹⁵⁶. The presence of a di- or triphosphate moiety and near-end base pairing are required for RIG-I stimulatory activity^{165–171}. Discrimination of self- vs. non-self RNA by RIG-I relies to a large extent on 5'-end RNA cap structures. A 7-methylguanylate cap that is further modified by a 2-O-methyl group has been identified as the molecular signature that prevents activation of RIG-I by endogenous RNAs^{172;173}. Viruses have evolved

to mimic cellular cap structures on their genomes to evade RIG-I recognition (see Chapter III). RIG-I has been shown to be essential for recognition of a number of viral infections. As an example, orthomyxoviruses, such as Influenza A virus (IAV), are primarily sensed by RIG-I¹⁷⁴.

In addition to the recognition of exogenous RNA, RIG-I can be stimulated by modified cellular RNA. This includes non-coding RNAs that are available for RIG-I recognition during viral infection and cleavage products of cellular RNA generated by activated RNase L^{175;176}.

I.4.2 MDA5

Stimulation of both RIG-I and MDA5 by an RNA ligand induce identical downstream signalling events (see below). In contrast to RIG-I, however, the molecular signatures that govern MDA5 activation are not well-described. The notion that long dsRNA activates MDA5 is derived mostly from experiments in cell-free systems, and the observation that high-molecular weight poly(I:C) potently activates MDA5¹⁶². In addition, IFN responses to Encephalomyocarditis virus (EMCV) and other picornaviruses depend entirely on MDA5 in mice^{174;177}. Long dsRNA generated during picornavirus infection has been shown to activate MDA5^{178;179}. However, MDA5 stimulatory potential for ssRNA generated during viral infection has also been reported¹⁸⁰. While multiple RNA species can induce hydrolysis of ATP by MDA5 in cell-free systems and are therefore reported as ligands, the relevance of these molecules for MDA5 activation in cells is often unknown¹⁶².

The precise molecular characterisation of the putative MDA5 ligand is ongoing. Nevertheless, it is clear that MDA5 contributes to the antiviral response to numerous viruses. This is highlighted by increased susceptibility to viral infections in individuals with mutations in the *IFIH1* gene (encodes MDA5)^{181–183}. Furthermore, in addition to the aforementioned essential role of MDA5 in sensing of picornavirus infections, *in vitro* experiments have demonstrated MDA5 activity during Zika virus, Measles virus, Rotavirus, and HSV-1 infection^{184–187}.

Similar to RIG-I, MDA5 can further be activated by cellular RNAs. This includes mitochondrial RNA¹⁸⁸ and RNA that originates from transcription of particular genetic elements. Transcription of repetitive sequences within the human genome, such as endogenous retroviruses and Alu elements, can generate ligands for MDA5^{189–191}. Of note, MDA5 stimulation by the aforementioned transcripts is thought to be prevented through modification of the RNA by the RNA-editing enzyme ADAR1¹⁶².

I.4.3 Signalling Downstream of RLRs

Stimulation of both RIG-I and MDA5 results in the activation of MAVS through the interaction of the CARD domains of RIG-I/MDA5 and MAVS¹⁵⁶ (Figure 3). MAVS aggregates into prion-like structures and recruits TBK1 for the phosphorylation of downstream transcription factors. TRAF2, TRAF3, TRAF5, and TRAF6 are also part of the signalling platform aggregated by MAVS. The transcription factors that are activated by MAVS/TBK1 include IRF3, IRF7, and NFκB. Similar to signalling events downstream of STING, these proteins induce transcription of type I IFNs and inflammatory cytokines. Their activation can further result in transcription of type III IFNs and a subset of ISGs that is stimulated directly by IRF3, such as *DDX58* (RIG-I)¹⁶³.

MAVS is primarily localised on the cytosolic membrane of mitochondria at baseline¹⁹². Additional pools of MAVS proteins can be found on peroxisomes¹⁹³. Studies have suggested that the predominant transcriptional outcome of MAVS activation is dependent on MAVS localisation^{193–195}. Peroxisomal MAVS has been shown to induce transcription of type III IFNs exclusively¹⁹⁴. However, the results shown in REFS. 194 and 195 are conflicting regarding the role of MAVS localisation. Further studies are needed to fully understand the molecular events that precede activation of a particular MAVS pool after RLR-stimulation and decide the signalling outcome.

I.5 The Antiviral Cytokine Response

Downstream of PRR activation, in particular of those that respond to nucleic acids, lies the transcription of antiviral cytokines, most importantly interferons. The group of interferons is comprised of type I, type II, and type III IFNs (Figure 4).

I.5.1 Type I IFNs

Type I IFNs are a family of cytokines that represent the first line of defence against viral pathogens. In humans, type I IFNs encompass 13 subtypes of IFNα, IFNβ, IFNω, IFNκ, and IFNε¹⁹⁶. All of them have antiviral activity, yet differ in their expression in terms of tissue restriction and kinetics. The expression of IFNε and IFNκ are limited to the female reproductive tract and keratinocytes, respectively^{197;198}. IFNω is expressed by immune cells upon PRR stimulation and has antiviral properties similar to IFNα and IFNβ¹⁹⁹. IFNα and

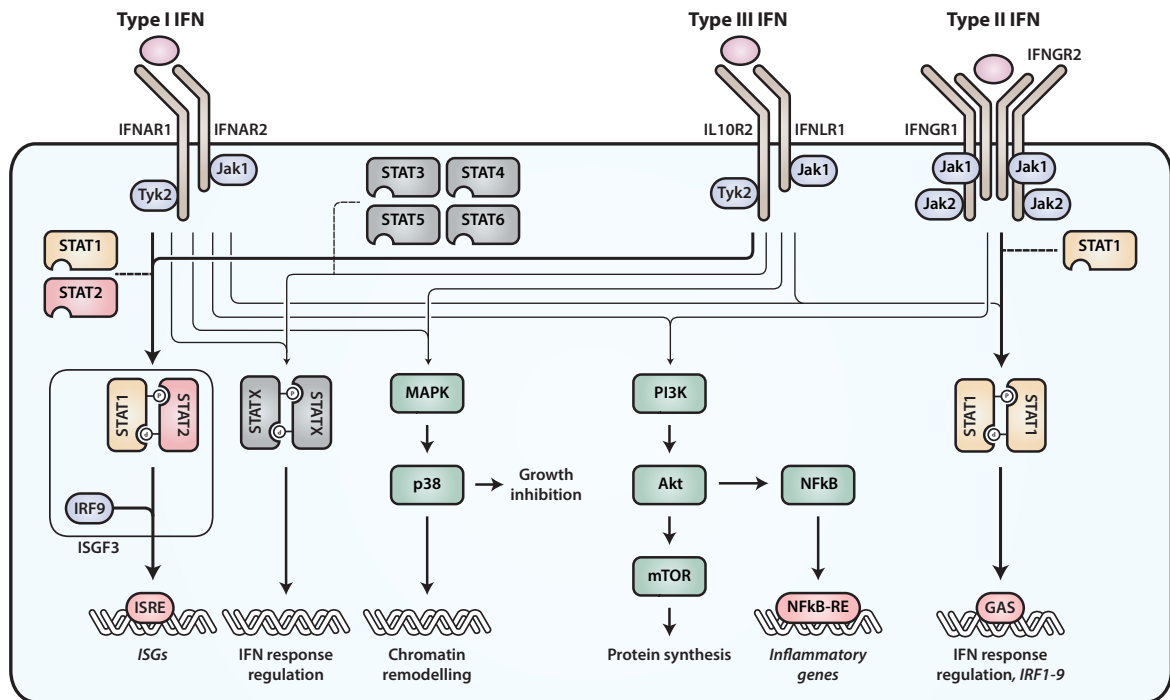


Figure 4: The intracellular signalling of interferons. The IFN group of cytokines consist of type I, type II, and type III IFNs. Each type of IFN binds to its own dedicated receptor. Type I and type III IFN canonical signalling via ISGF3 (pSTAT1-pSTAT2-IRF9) results in expression of ISGs through binding to ISRE. Type II IFN canonical signalling activates GAS in the promoter regions of genes through pSTAT1 homodimers. Downstream signalling from the type I and type III IFN receptor can additionally result in formation of homo- and heterodimers of phosphorylated STAT1, STAT2, STAT3, STAT4, STAT5, and STAT6. It can further activate the MAPK/p38 pathway that leads to cell growth inhibition and chromatin remodelling. Non-canonical signalling from the type I and type II IFN receptor can also result in stimulation of the PI3K/Akt pathway. This facilitates protein synthesis via mTOR signalling and can lead to transcription of inflammatory genes through NFκB. Bold lines indicate the canonical signalling pathway for each receptor.

IFNβ can be expressed by all cells of the human body in response to viral infection and constitute the main antiviral effectors of the type I IFN system¹⁹⁶.

The key transcription factors that govern expression of type I IFNs are IRF3 and IRF7. IRF3 activation lies at the end of PRR downstream signalling cascades (see sections I.3.1 and I.4.3). Homodimers of phosphorylated IRF3 bind to response elements in the *IFNB1* gene promoter. NFκB is additionally required to induce transcription. The promoter sequences of many type I IFNs additionally harbour IFN-stimulated response element (ISRE) sequences, forming a positive feedback loop. In fact, signalling downstream of the type I IFN receptor is required for transcription of IFNα genes. Only IFNβ gene transcription can be induced by IRF3 and NFκB alone²⁰⁰. While IRF3 is constitutively expressed, IRF7 protein levels increase through type I IFN stimulation, forming another layer of the positive feedback loop²⁰⁰. Activated IRF7 can induce the expression of large quantities of IFNα²⁰⁰. This can be observed particularly in pDCs. As mentioned, activated IRF3 can stimulate transcription of certain ISGs, such as *DDX58* (RIG-I), directly without the involvement of type I IFNs.

All type I IFNs bind to a single receptor (IFNAR) consisting of an IFNAR1-IFNAR2 heterodimer¹⁹⁶. The intracellular part of the IFNAR1 and IFNAR2 receptor chains are constitutively bound to the receptor-associated Janus kinases (JAKs) TYK2 and JAK1, respectively (Figure 4). Ligand binding induces a conformational change within the IFNAR1/2 receptor chains and allows *trans*-phosphorylation of the receptor chains by the associated kinases¹⁹⁶. The phosphorylation of tyrosine residues allows recruitment of signal transducers and activators of transcription 1 (STAT1) and STAT2, which are also phosphorylated by the same kinases. Phosphorylated STAT1 and STAT2 dimerise and additionally recruit IRF9¹⁹⁶. This complex, called ISGF3, binds to ISRE in gene promoters and induces transcription. Type I IFNs stimulate the expression of a large number of genes, collectively called ISGs²⁰¹.

In addition to the canonical signalling described above, ligand binding to the type I IFN receptor can lead to activation of multiple non-canonical signalling cascades¹⁹⁶. Firstly, STAT3, STAT4, STAT5, and STAT6 can be phosphorylated by JAK1 and TYK2 instead of STAT1 and STAT2 (Figure 4). Heterodimers of multiple STATs have been observed in a cell-type specific manner¹⁹⁶. The contribution of STAT3-STAT6 homo- and heterodimers to IFN signalling are incompletely characterised. It is believed that these signalling events can constitute the canonical pathway of type I IFN signalling in certain cell types¹⁹⁶. Furthermore, they are suggested to have regulatory functions in the type I IFN response. In addition to signalling *via* STATs, the type I IFN receptor can stimulate the MAPK/p38 and PI3K/Akt pathways¹⁹⁶. These signalling events are partly required for ISGF3-induced ISG transcription. The MAPK pathway further leads to the inhibition of cell growth and globally alters the transcriptional landscape through chromatin remodelling¹⁹⁶. The PI3K/Akt pathway stimulates protein synthesis for efficient ISG transcript translation and leads to expression of inflammatory genes *via* NF κ B.

Type I IFNs have astonishing antiviral activities against many different types of viruses (reviewed in REF. 202). This has been demonstrated extensively *in vitro* and in murine knockout models of the type I IFN receptor or other non-redundant signalling components. Protection against viruses by type I IFNs is mediated through upregulation of ISG expression, inflammatory gene expression, inhibition of cell growth, and apoptosis induction in a subset of cells. The group of ISGs contains genes that encode PRRs, adaptor proteins involved in signalling of the type I IFN axis, and proteins that are direct restriction factors²⁰¹.

Viral restriction factors can directly disturb different steps within the life cycle of viruses. The group of IFITM proteins, for example, facilitate diversion of incoming virions of certain viruses to lysosomes^{203;204}.

As mentioned, all type I IFNs bind to the same receptor. This implies that all of them lead to the activation of essentially the same downstream signalling and poses the question why such diversity in the group of type I IFNs exists. All type I IFNs bind to the type I IFN receptor with vastly different affinities^{205–207}. IFNAR2 binds type I IFNs with high affinity whereas IFNAR1 forms low-affinity interactions with its ligands. A model of IFNAR activation suggests that IFNAR1 and IFNAR2 are monomeric at baseline²⁰⁷. High-affinity ligand interaction of IFNAR2 then leads to recruitment of IFNAR1 and signalling initiation. IFNAR1 has been shown to be able to discriminate different type I IFN subtypes based on affinity, whereas IFNAR2 cannot²⁰⁷. In addition, interaction of IFN β with IFNAR1 can induce downstream signalling independent of IFNAR2²⁰⁸. All these observations point at complex molecular and biophysical circuits that govern the outcome of type I IFN signalling. To date it is poorly understood why higher organisms have evolved and maintained this number of closely related type I IFN genes.

Strikingly, genetic evidence in humans questions an essential role of type I IFNs for defence against viral pathogens *in vivo*. A homozygous loss-of-function mutation in the *STAT2* gene was identified in a child at the age of five²⁰⁹. While this child had disseminated measles disease that required intensive care as a result of routine measles mumps rubella (MMR) vaccination at 18 months of age, no further episodes of abnormal viral disease were reported until genetic evaluation at five years of age. Interestingly, *in vitro* analysis showed impaired ability of patient cells to control different types of viruses compared to healthy controls. Furthermore, an infant with a homozygous loss-of-function mutation in the *IFNAR2* gene has been reported recently²¹⁰. While this child succumbed to fatal encephalitis following MMR vaccination at 13 months of age, no history of viral disease was reported before. Similar to the *STAT2*-deficient patient mentioned above, virus restriction was impaired *in vitro*. These findings pose an interesting conceptual question regarding the role of type I IFNs in antiviral defence in humans *in vivo*. The patients did not show increased susceptibility to natural viral diseases but were unable to control vaccine viruses. It is possible that redundancy between type I and type III IFNs at mucosal surfaces sufficiently prevents natural viral infection. The contribution of different IFNs to antiviral defence *in vivo* is an area of active investigation.

I.5.2 Type II IFN

The term type II IFN is synonymous with IFN γ . It binds to the IFN γ receptor (IFNGR) consisting of two IFNGR1-IFNGR2 heterodimers²¹¹ (Figure 4). IFN γ has crucial functions in the activation and regulation of adaptive immune responses but also has direct antiviral activities (reviewed in REF. 212). Receptor activation leads to the formation of homodimers of phosphorylated STAT1 which bind to gamma-activated sequences (GASs) in the promoter regions of genes (Figure 4). Non-canonical signalling can additionally result in activation of the PI3K/Akt pathway²¹¹.

In contrast to type I and type III IFNs, IFN γ is produced only by T cells, B cells, NK-cells, and APCs²¹³. Its production can be stimulated by PAMP recognition but is additionally triggered by other cytokines, such as IL-12, IL-15, IL-18, and type I IFNs. It is part of an intricate regulatory circuit that governs the interplay between lymphocytes and APCs in the context of adaptive immune responses²¹³.

I.5.3 Type III IFNs

Type III IFNs, also called IFN λ s, are a group of recently discovered cytokines with potent antiviral activity^{214–216}. Three genes in humans encode IFN λ 1, IFN λ 2, and IFN λ 3. An additional gene, *IFNL4*, has been identified but produces a functional protein only in individuals with a certain polymorphism in the coding region²¹⁷.

Conceptually, expression of type III IFNs is induced analogously to type I IFNs, namely downstream of PRR activation. The same transcription factors, IRF3, IRF7, and NF κ B, govern expression of the IFN λ genes. They do, however, show differences in their temporal dynamics of expression. It has been demonstrated in hepatocytes that IFN α and IFN β are expressed first as short bursts²¹⁸. Following that, IFN λ genes are transcribed in a sustained fashion. It has been suggested that IFN λ expression is primarily dependent on NF κ B and to a lesser extent on IRF3/7^{219;220}. In contrast, type I IFN transcription shows the opposite transcription factor requirements with a major contribution from IRF3/7 (see section I.5.1). In the mouse intestine, rotavirus infection is controlled by type III and not type I IFN²²¹. Molecularly this has been attributed to the MAVS adaptor protein being localised primarily on peroxisomes instead of mitochondria¹⁹⁴ (also see section I.4.3).

Furthermore, a DNA sensing pathway orchestrated by the DNA damage protein Ku70

and STING exclusively induces IFN λ without type I IFN transcription in human cells^{145;146}. Lastly, IFN λ expression can additionally be stimulated downstream of the type I IFN receptor²²². The pattern in which type I and type III IFNs are expressed is likely to be dependent on the cell type, upstream stimulus, and input from other signalling cascades.

Type III IFNs are structurally unrelated to type I IFNs and bind to their own receptor (IFNLR) consisting of the IL-10R2 chain and IFNLR1 chain¹⁹⁶ (Figure 4). The IL-10R2 chain is shared with a number of other cytokine receptors but is ubiquitously expressed. Importantly, the expression of the IFNLR1 chain is restricted to selected cell types, mainly epithelial cells²²³. Type III IFNs are therefore tissue restricted in their effect and act primarily on mucosal surfaces. However, an important role of IFN λ in restriction of fungus infection through neutrophils has been uncovered recently²²⁴.

Although type III IFNs bind to their own receptor, the canonical downstream signalling is identical to type I IFNs and results in ISGF3 formation and ISG transcription¹⁹⁶ (Figure 4). In addition, the IFNLR can engage non-canonical signalling through STAT1-STAT6 homo- and heterodimers and the MAPK pathway²¹⁶. However, type I and type III IFN signalling outcomes are not entirely identical. Mechanistic studies have begun to identify molecular determinants of differential signalling outcomes of type I and type III IFNs²²⁵.

Given their relatively recent discovery, the antiviral effects of type III IFNs and their role in infectious and non-infectious settings are still being investigated actively²¹⁶. For example, type I and type III IFNs have, cell type dependent, redundant and non-redundant roles in the defence against intrabronchial IAV infection in mice^{226–228}. Importantly, compared to type I IFN, IFN λ induces ISG expression less potently and does not induce an inflammatory response that can lead to tissue damage^{227;229;230}. A model has been proposed in which type III IFNs govern frontline defence against invading viral pathogens at mucosal surfaces²²⁷. Only if the infection is insufficiently controlled will the type I IFN system be activated. This secondary response is more potent in viral clearance but comes at the cost of potentially extensive tissue damage. Another study showed entirely non-redundant, compartmentalised roles of type I and type III IFNs in the mouse gut by acting on non-epithelial lamina propria cells or gut epithelial cells, respectively^{231;232}. The above highlighted findings demonstrate a complex interplay of these two different groups of IFNs that awaits further exploration.

I.6 Zika Virus

ZIKV is a positive-sense ssRNA virus and is part of the *Flavivirus* genus within the family of *Flaviviridae*²³³. It is related to other Flaviviruses that cause human disease, such as DENV, Yellow Fever virus (YFV), Japanese Encephalitis virus (JEV), and Tick-borne Encephalitis virus (TBEV). Most flaviviruses can be transmitted to humans *via* mosquitoes or ticks and are hence called arboviruses (arthropod-borne viruses). Zika virus is primarily transmitted by mosquitoes of the *Aedes* family.

I.6.1 Virology

All flaviviruses are enveloped viruses with a linear positive-sense RNA genome contained in an icosahedral capsid. The ZIKV genome is 10.7 kb in length and encodes a single open reading frame (ORF) (Figure 5)^{234;235}. Given the positive-stranded nature of the genomic RNA, it serves directly as a template for protein translation. The viral ORF is flanked by 5'- and 3'- non-coding regions (NCRs) which form secondary structures. These secondary structures can stall cellular exonucleases and give rise to subgenomic flaviviral RNAs (sfRNAs)^{236–238}. These sfRNAs are primarily derived from the 3'-NCR. The functional relevance of sfRNAs is generally not well-understood for flaviviruses. However, for WNV, sfRNAs are indispensable for viral replication²³⁶. For DENV, a role in mammalian vs. insect host adaption has been proposed²³⁹. The sfRNAs from an epidemic Zika virus strain have been suggested to antagonise innate immune responses²³⁷.

Flaviviral genomic RNA is capped on the 5'-end with a 7-methylguanylate cap, similar to eukaryotic mRNAs²³³. The polyprotein encoded by the single ORF is co-translationally cleaved by the viral and cellular proteases into three structural, seven non-structural (NS) proteins, and the 2K signal peptide. The structural proteins include Capsid (C), pr-Membrane (prM), and Envelope (E). The C protein forms the nucleocapsid together with the viral RNA. The E and M proteins are found in the viral envelope. The non-structural proteins are NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. NS2 and NS3 together form the viral protease. NS5 is the RNA-dependent-RNA-polymerase and also has a methyl-transferase domain. The 2K signal peptide is believed to regulate activity of cellular proteases and have a role in correct localisation of NS4A^{240;241}.

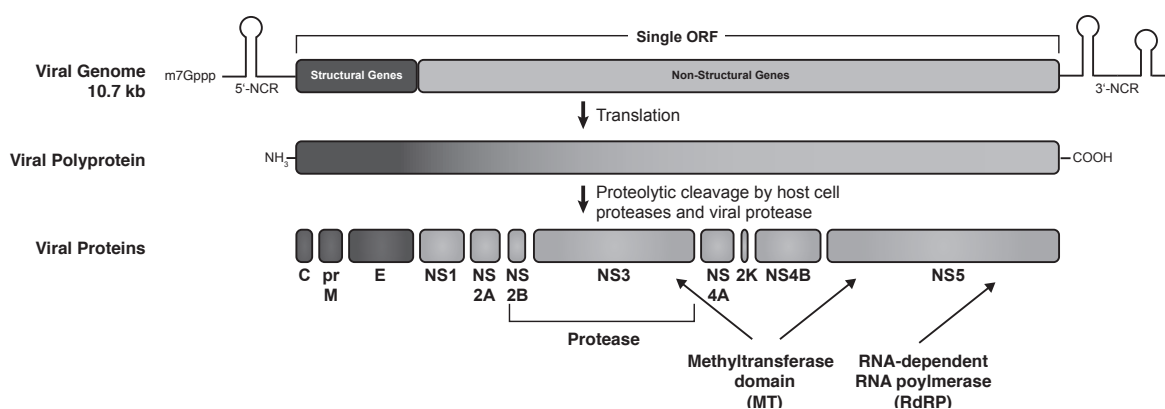


Figure 5: The ZIKV genome. ZIKV has a (+)ssRNA genome that harbours a single ORF. The ORF is flanked by NCRs and the genome is capped with a 7-methyl-guanylate-triphosphate moiety on its 5'-end. The single ORF is translated into a polyprotein that is cleaved into the individual viral proteins Capsid (C), pr-Membrane (prM), and Envelope (E) and non-structural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. The viral protease consists of a NS2B-NS3 dimer. NS3 and NS5 harbour methyltransferase domains. NS5 further has RNA-dependent-RNA-polymerase activity.

I.6.2 Life Cycle

Our knowledge regarding the molecular details of ZIKV intracellular replication are incomplete. Models of Zika virus replication rely to a large extent on results obtained from studying other flaviviruses, such as DENV. However, the precise mechanisms that govern particle assembly within the ER are insufficiently understood for any flavivirus.

The infectious cycle starts with attachment of the Zika virion to the cell surface and subsequent clathrin-mediated endocytosis²⁴³ (Figure 6). Different proteins have been proposed to mediate attachment and entry of virions. On cells of neuronal origin, a protein called Axl has been demonstrated to serve as an entry receptor^{244–247}. On cells of the skin and the placenta, Tyro3 and TIM1, as well as the TLRs 3 and 8 can mediate endocytosis additional to Axl^{243;248–250}. On dendritic cells, virions utilise DC-SIGN for entry^{251;252}. After entry, lower endosomal pH allows the surface proteins of the virion to induce membrane fusion²⁵³. As a result, the viral RNA is released into the cytoplasm. The genomic RNA is used by the ER-associated translational machinery as a template for synthesis of the viral polyprotein. The viral proteins with transmembrane domains (prM, E, NS2A, NS2B, NS4A, 2K, and NS4B) are directly inserted into ER-membranes after co-translational cleavage from the polyprotein by host and viral proteases²⁵⁴. The membrane-associated non-structural proteins form vesicle structures within ER membranes which serve as replication factories, spatially separated from the cytoplasm^{251;255–257}. This spatial separation has interesting implications for innate immune recognition of the virus (see Chapter III).

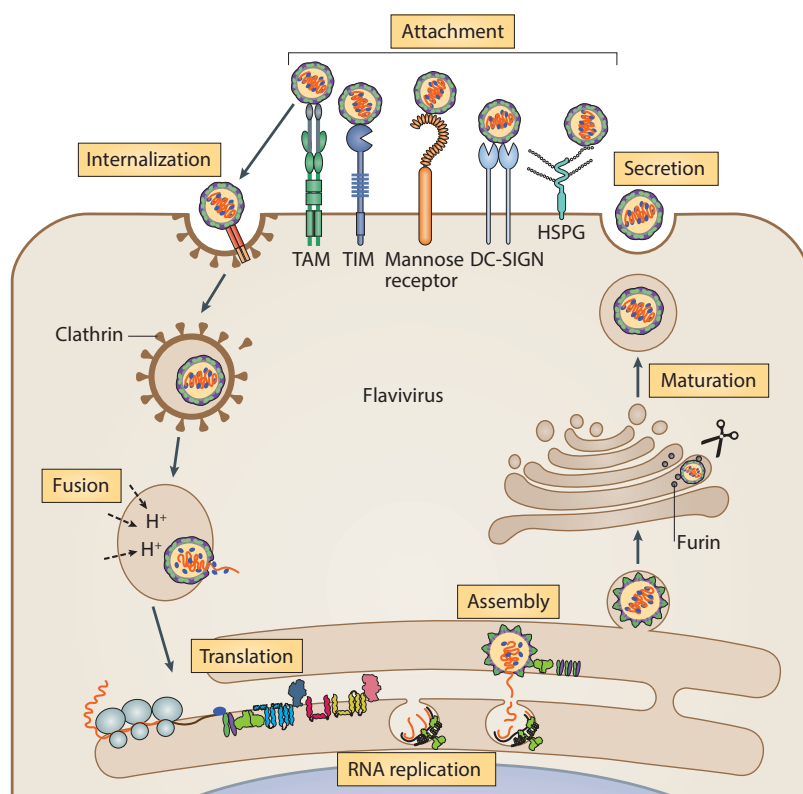


Figure 6: The Flavivirus replication cycle. After internalisation of the virion by endocytosis, membrane fusion is induced by acidification of the vesicle lumen. Viral RNA is released into the cytosol and translated at the ER. The viral polyprotein is co-translationally cleaved into the viral proteins which remain ER associated. Replication of the viral genome and virion assembly occurs in ER invaginations. New particles pass through the Golgi for maturation. This includes cleavage of the Membrane precursor protein by cellular furin proteases and pH-induced conformational changes of the viral envelope surface. Mature particles are then secreted. Figure taken from REF. 242.

The viral genome is replicated by the NS5 protein. Other viral proteins are believed to be involved, however, how the viral RNA replicated through a (-)ssRNA intermediate is poorly understood²⁵³. The ER membrane invaginations serving as replication factories ultimately form new virions by budding into the ER lumen^{258;259}. Immature virions pass through the *trans*-Golgi network (TGN), undergoing a maturation process. This process mainly entails cleavage of the pr moiety from the prM protein by host furin proteases and a pH-dependent conformational change of the Membrane-Envelope protein dimer²⁶⁰. This conformational change leads to formation of the highly ordered surface structure observed on flaviviruses. Virions are then released into the extracellular space. Only virions with a "matured" surface structure are intrinsically infectious (reviewed in REF. 261). It has been demonstrated that maturation of particles in the TGN is incomplete and virions of all maturation stages are released from infected cells. Interestingly, even partly matured virions can be rendered infectious through binding of antibodies (see description of ADE below).

Given that mosquitoes are intermediate hosts for Zika virus, the virus must be able to

replicate in both insect and mammalian cells. Possible differences in terms of replicative cycle between these different species are not characterised molecularly. However, growth kinetics and infectivity of viruses grown in human and insect cell lines were shown to differ *in vitro*²⁶².

I.6.3 Transmission and Pathology

Transmission of Zika virus between human hosts is believed to occur primarily through *Aedes* mosquitoes²³⁴. In addition, sexual transmission has been described²⁶³. Vertical transmission *in utero* is further clinically relevant due to its association with congenital Zika syndrome^{264–268} (see section I.6.4). Upon host entry, the virus can infect skin keratinocytes, dermal fibroblasts, and local DCs²⁵¹. If replication of the virus at the site of infection is insufficiently controlled, generalised viremia will lead to the febrile illness commonly referred to as Zika fever. The virus can be recovered from circulating myeloid DCs and CD14⁺/CD16⁺ monocytes of infected patients^{252;269}.

In pregnant women, the virus can cross the placenta and induce foetal viremia. Zika does not commonly infect the CNS in infected adults, however, meningoencephalitis has been described after Zika infection²⁷⁰. In contrast to adults, the virus is highly neurotropic in the developing foetus. Infection of the foetal brain and inflammation within the developing nervous system is believed to be the cause for intrauterine growth restriction (IUGR), also called congenital Zika syndrome. The precise molecular cause of neurological maldevelopment in affected foetuses is still unknown. A growing body of evidence has nevertheless demonstrated intrauterine neuroinvasion of the virus. Viral RNA and replication competent virus have been found in brain tissue of aborted, microcephalic foetuses^{271;265}. Viral antigens were found associated with micro-calcifications in microcephalic foetal brains²⁶⁴. Mouse models of congenital Zika infections have further been able to recapitulate human disease and to elucidate pathogenic mechanisms^{272–274}. For example, Zika virus induces death of neural progenitor cells in the developing brain²⁷⁵. The viral NS4A and NS4B proteins have further been described to interfere with Akt-mTOR signalling and deregulate neural development²⁷⁶. In addition to direct neuropathic effect of replicating virus, tissue damage induced by type I IFN has been shown to contribute to congenital Zika syndrome in mice²⁷⁷.

In most infected adults, Zika fever is a self-limiting disease that is treated with unspecific

anti-inflammatory agents²⁷⁸. A subset of patients, however, develops Guillain-Barré syndrome (GBS) as a consequence of Zika infection²⁷⁹. GBS is an immune-mediated muscle weakness caused by peripheral nerve fibre damage (reviewed in REF. 280). The disease commonly occurs after infections with a number of different bacteria and viruses. The exact molecular pathophysiology of GBS is diverse; in most cases, however, auto-antibodies induce damage to myelin sheaths of peripheral nerves. For some infectious agents that commonly cause GBS, molecular mimicry of bacterial/viral epitopes and disease causing anti-ganglioside antibodies has been described. For Zika virus, such a molecular link has not been established. GBS is a serious disease that can require intensive care treatment. 5% of patients do not survive GBS.

I.6.4 Zika Virus as an Emerging Pathogen

Zika virus obtained its name from the Zika forest in Uganda where it was first described in 1947 after isolation from rhesus monkeys²⁸¹. A year later, the virus was detected in *Aedes africanus* mosquitoes within the Zika forest²⁸². It was recognised already at the time that humans can be infected and transmission of the virus through mosquitoes was hypothesised^{282;283}. Formal evidence for arthropod transmission of Zika virus was obtained in 1956 from experiments with mice and monkeys²⁸⁴.

Throughout the following decades, the virus was never recognised as a major human pathogen. Sporadically, infections with the virus were reported to have occurred in Africa and Asia (reviewed in REF. 285). It was not until 2007 that the virus first attracted widespread international attention from epidemiologists and public health agencies due to an outbreak of febrile illness on the Yap island in Micronesia²⁸⁶. Zika virus was detected in the serum of patients suffering from rash, arthralgia, and conjunctivitis. This was the first report of Zika infections which, in terms of numbers of affected individuals, was classified as an outbreak. The report further showed that the virus had spread from mainland Africa and Asia, the only regions it was previously detected in. In the following years, outbreaks of multiple mosquito-borne viruses were reported from a number of Pacific Islands (e.g. French Polynesia, New Caledonia)²⁸⁷.

Only a year after the last outbreak of Zika virus on the Cook Islands in 2014, the first human infection was detected in Brazil²⁸⁸. Shortly after, the first reports of an association between Zika virus infection during pregnancy and intrauterine growth restrictions in

foetuses started to emerge^{266;271;289}. This phenomenon was termed congenital Zika virus syndrome and includes arthrogryposis (congenital joint contractures), and neuronal complications, such as microcephaly. As a result, in early 2016 the World Health Organization (WHO) declared the Zika virus outbreak in South America a "Public Health Emergency of International Concern"²⁹⁰. Within the following months, Zika virus infections had been reported from 40 countries in the Americas²⁹¹. Phylogenetic analysis demonstrated that the epidemic strains are part of the Asian Zika virus lineage^{292;234}. They further showed that a virus originating from Pacific Islands with previous outbreaks was introduced into South America prior to 2014. The Zika virus epidemic of 2015/2016 was not only of concern to the international public, it also sparked the interest of researchers worldwide to characterise the virus better. In particular, molecular determinants of pathogenicity in terms of IUGRs were investigated with great efforts.

I.6.5 Immune Response to ZIKV

I.6.5.1 Innate Responses

A study showed that PBMCs isolated from patients acutely infected with Zika virus do not show transcriptional patterns indicative of a type I IFN mediated antiviral response²⁹³. In another study, however, monocytes and dendritic cells isolated from a patient showed a clear type I IFN transcriptional pattern during acute infection²⁶⁹. Whether a transcriptional signature can be detected in blood cells is likely to depend on the time point after primary infection, strain of virus, and host genetics.

In vitro, the virus causes PRR activation and type I IFN secretion. In A549 cells, the virus potently induces a type I IFN response¹⁸⁴. This response is fully MAVS- and primarily RIG-I dependent. Another study confirmed a major role of RIG-I in the type I IFN response to ZIKV²⁹⁴. ZIKV genomic RNA can bind to and activate RIG-I *in vitro*^{184;295}. Interestingly, infection with pre-epidemic strains of ZIKV induced stronger responses when compared to strains from the 2015 Brazil outbreak²⁹⁴. This indicates that differences in innate recognition could explain the virulence of pandemic strains. In skin fibroblasts, ZIKV infection induces a type I IFN response²⁵¹. Placental macrophages and placental syncytiotrophoblasts induce transcription of IFN α , IFN β , and IFN λ 1 in response to ZIKV infection²⁴⁹. In placental trophoblasts, infection results in the secretion of type III IFNs²⁴⁸. Inflammatory cytokines, but no type I IFNs were detected after infection of PBMCs²⁴⁹.

Even though there appear to be differences in IFN induction between different cell types and virus strains, it is very clear that type I IFNs are extremely potent in restricting Zika virus infection. Furthermore, IFN γ appears to potently inhibit Zika virus. Pre-treatment of cells with both type I and II IFNs drastically inhibited viral replication^{251;296}.

The potency of type I IFNs in terms of ZIKV inhibition is further highlighted by murine infection models. Mice with a functioning type I IFN system are refractory to infection with Zika virus. Mouse models of infection rely on, for example, *Ifnar1*^{-/-} knockout, *Ifnar1*^{-/-}/*Ifngr1*^{-/-} double knockout, or *Irf3*^{-/-}/*Irf5*^{-/-}/*Irf7*^{-/-} triple knockout^{297;298}. This, in part, can be explained by the fact that ZIKV NS5 selectively degrades human STAT2 and thus fails to antagonise murine IFN signalling pathways²⁹⁹.

I.6.5.2 Adaptive Responses

Data from DENV and other flaviviruses suggest that adaptive responses to these viruses can be both disease limiting and exacerbating³⁰⁰. Flavivirus infections typically generate antibody responses against the structural proteins E and prM, as well as the NS1 protein (reviewed in REF. 301). This has been extensively characterised in the context of DENV infection. Antibodies against E, prM, and NS1 have also been found in Zika virus infected patients³⁰², indicating a humoral immune response similar to other flaviviruses. A number of antibody clones obtained from patients with ZIKV as the primary flavivirus infection had the ability to neutralise the virus *in vitro*³⁰³. A protective role of antibodies in the context of Zika virus infection has been established through vaccine trials in mice and non-human primates^{304;305}. The immunogenic epitopes on the E protein of different flaviviruses are similar³⁰¹. As a consequence, antibodies against ZIKV or DENV are often cross-reactive. If these cross-reactive antibodies are not neutralising, they are not protective but rather enhance infection and impair control of related viruses. It is believed that the uptake of viral particles into phagocytes is facilitated by non-neutralising antibody opsonisation^{306–309}. This, however, results in infection of the cells rather than viral clearance. This phenomenon, termed antibody-dependent enhancement (ADE), was first observed in patients that were subsequently infected with different DENV serotypes^{306;307;310;311}. It is now recognised that DENV seroconversion prior to Zika infection is associated with worse disease progression due to ADE. The ability of antibodies found in DENV-experienced patients to bind Zika epitopes has been demonstrated extensively^{304;312;313}. *In vitro* data have very convincingly shown the potent effects of ADE in the context of DENV and ZIKV. A causality between DENV prevalence in South America, ADE, and the 2015/2016 ZIKV outbreak with associ-

ated IUGR remains to be confirmed.

The role of T cells in the immune response to Zika virus is only at the beginning of characterisation. One study has reported that in ZIKV infected patients CD4 T cell responses are directed towards non-structural and structural proteins whereas CD8 responses target primarily non-structural proteins³¹⁴. Further evidence comes from a study showing that CD4 T cells from patients with a history of Zika virus infection can be stimulated to proliferate *ex vivo* with E and NS1 protein³⁰². The functional relevance of these responses for protection from disease are unknown³⁰⁰. In mice, CD8 T cell responses to epitopes contained in the prM, E, and NS5 proteins were protective^{315;316}. The relevance of these studies for human disease, however, is limited given that murine Zika virus models rely on the absence of a functional type I IFN system. This will inevitably influence adaptive responses. Furthermore, unless mice are HLA transgenic, T cell responses will differ from humans.

I.6.5.3 Immune Evasion

For pathogens, evasion of host immunity is crucial for replication and survival. Zika virus is no exception to this and a multitude of innate immune evasion mechanisms have been described (Figure 7). A major target of flaviviral antagonism are the mechanisms that induce, regulate, or are a target of type I IFNs. The NS5 protein of ZIKV can interfere with activation of IRF3 and therefore suppress IFN transcription^{184;317;296;318}. The NS4A protein has further been reported to disrupt the interaction of MAVS with RLRs^{319;293}. Interestingly, a mutation in the NS1 protein has been shown to confer IFN antagonist functions to the protein in epidemic strains of the Asian lineage^{318;320}.

Viral RNA recognition by RIG-I is further antagonised by sfRNAs generated during Zika virus infection²³⁷. The NS5 protein of ZIKV, in addition to targeting IRF3, also disrupts IFN signalling through degradation of STAT2^{299;184;321}. Lastly, the viral NS2B-NS3 protease complex can degrade JAK1³²⁰.

Interestingly, antagonism of DNA sensing mechanisms has been suggested for both DENV and ZIKV^{75;322–324} (Figure 7). Viral proteins of both viruses can cleave STING and target cGAS for degradation. For DENV, activation of the cGAS/STING pathway as a result of mitochondrial damage and subsequent leakage of DNA has been experimentally shown^{75;76}. Similarly, an increase in mitochondrial DNA bound to cGAS was observed in ZIKV-infected cells³²⁴.

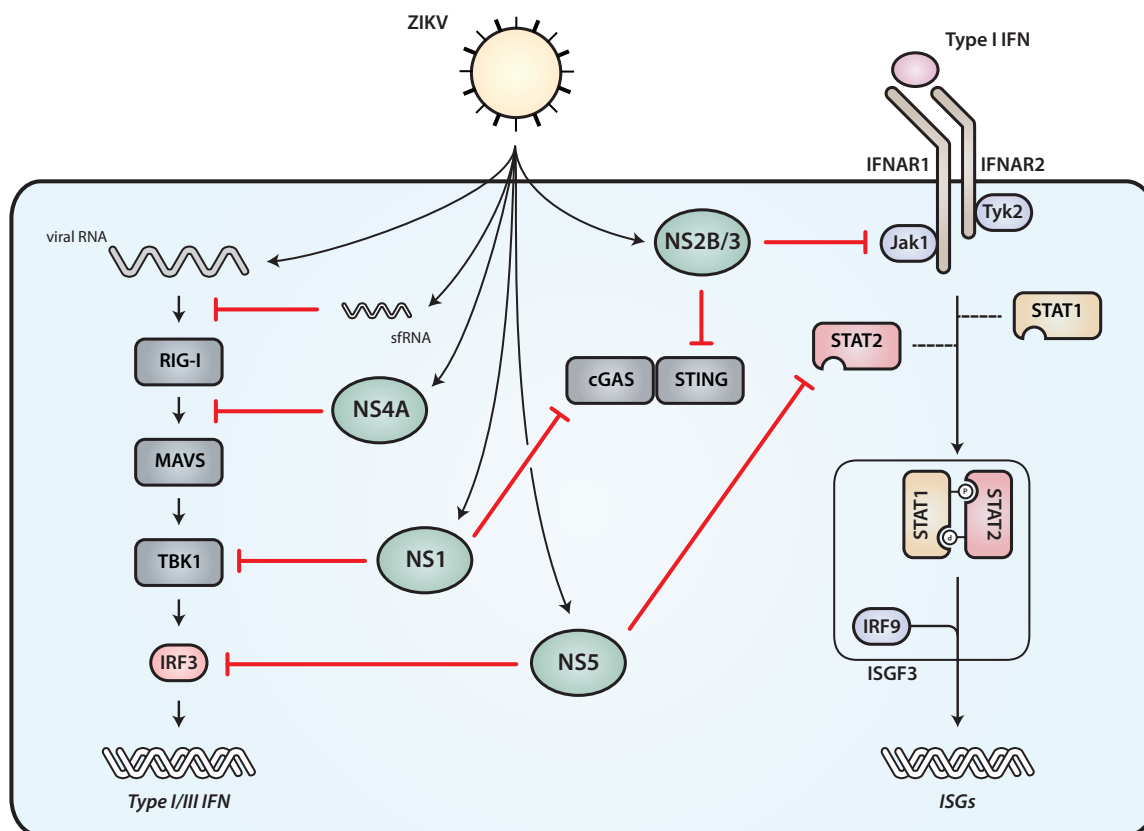


Figure 7: ZIKV innate immune evasion mechanisms. The viral proteins NS4A, TBK1, and NS5 inhibit the RLR signalling cascade by targeting the RIG-I-MAVS interaction, TBK1, and IRF3, respectively. Recognition of viral RNA by RIG-I is additionally antagonised by ZIKV sfRNA. ZIKV NS1 and the viral protease NS2B/NS3 target cGAS and STING, respectively. Type I IFN receptor signalling is inhibited by NS2B/NS3 and NS5 that target Jak1 and STAT2, respectively.

I.7 Varicella-Zoster Virus

Varicella-Zoster virus (VZV) is one of nine herpes viruses that infect humans³²⁵. Primary infection causes chickenpox (*Varicella*). Like all human herpes viruses, VZV establishes life-long latency in an infected host and can reactivate as shingles (*Herpes zoster*). Already in the late 19th century it was postulated that chickenpox and shingles share an infectious aetiology³²⁵. The virus was first isolated and propagated in tissue culture in 1953³²⁶.

I.7.1 Virology

VZV is a member of the *Varicellovirus* genus within the family of α -herpesviruses³²⁵. It is the only varicellovirus that infects humans. Other viruses in this genus include Simian Varicella virus (SVV), Pseudorabies virus (PRV), and Equine herpes virus 1 (EHV). They infect, among others, species like cows, cats, dogs, and horses³²⁵. The most closely related viruses to VZV that infect humans are the two other human α -herpesviruses, HSV 1 and 2.

VZV and HSV are similar in the way that both viruses have tropism for neuronal cells and establish latency in peripheral ganglia. Unlike HSV, VZV additionally infects T lymphocytes as an important step in its life cycle (see section I.7.3). While VZV only infects cells of human or guinea pig origin, HSV is permissive for infection of cells from a multitude of species³²⁵.

Like all herpesviruses, VZV is an enveloped virus with a dsDNA genome³²⁵ (Figure 8A). Mature virions are around 100 nm in diameter. The genome is packaged in the nucleocapsid. To date, the proteins encoded by ORFs 20, 21, 23, 33, 40, and 41 have been identified to be either structural capsid components or associated with the dsDNA genome³²⁵. Between the viral envelope and the nucleocapsid lies the tegument, a layer of viral proteins. So far, 14 viral proteins, namely ORFs 4, 9, 10, 11, 12, 22, 38, 47, 53, 57, 62, 63, 64, and 69, have been demonstrated to be part of the VZV tegument³²⁵. The envelope contains eight viral glycoproteins, gK, gN, gC, gB, gH, gM, gL, gI, and gE³²⁷. Interestingly, VZV does not express a homologue of gD, a glycoprotein that is conserved in all other human herpesviruses, and in those viruses has important functions in cell entry³²⁷. In fact, VZV uses a unique cell-entry pathway (see section I.7.4).

I.7.2 Genome

VZV harbours a 125 kb dsDNA genome, the smallest genome among the human herpesviruses³²⁵. Restriction endonuclease analysis and recombinant DNA technology enabled investigation of the VZV genome with the first reports on partial sequence information and genome organisation in the late 1970s^{328–331}. It was established that the genome consists of two unique regions, unique long (U_L , ca. 100 kb) and unique short (U_S , ca. 5 kb) (Figure 8B). Both regions are neighboured by repeat sequences on either side. U_L is flanked by terminal repeat long (TR_L) and internal repeat long (IR_L), and U_S is flanked by terminal repeat short (TR_S) and internal repeat short (IR_S). The genome has C- and G-overhangs at its 5'- and 3'- ends, respectively. These can be ligated to form the circular confirmation of VZV DNA commonly found in the nucleus of latently infected cells, and very rarely in infectious virions³²⁵. The first complete genomic DNA sequence was established by Davison et al. in 1986³³². This report also included the annotation of 70 predicted ORFs, i.e. viral genes. At present, the VZV genome encompasses 71 conventional ORFs (Figure 8B). All but one viral genes are intronless. The two genome segments previously annotated as ORF42 and ORF45 are now recognised as two exons of the same spliced gene (called ORF42/45). The

genome segment harbouring ORFs 71, 70, and 69 is an inverted duplication of the ORFs 62, 63, and 64.

Two copies of these genes are seemingly required for proper viral replication since deletion of the ORF 69-71 cluster is corrected after replication of the virus *in vivo*^{333,334}. Recently, long-read transcriptome sequencing has identified a plethora of novel transcripts in VZV infected cells³³⁵. These include a number of novel spliced, protein-coding transcripts, non-coding RNA species, and read-through transcripts that encode multiple ORFs in a polycistronic manner. The biological relevance of these newly identified, viral RNA species is mostly unknown.

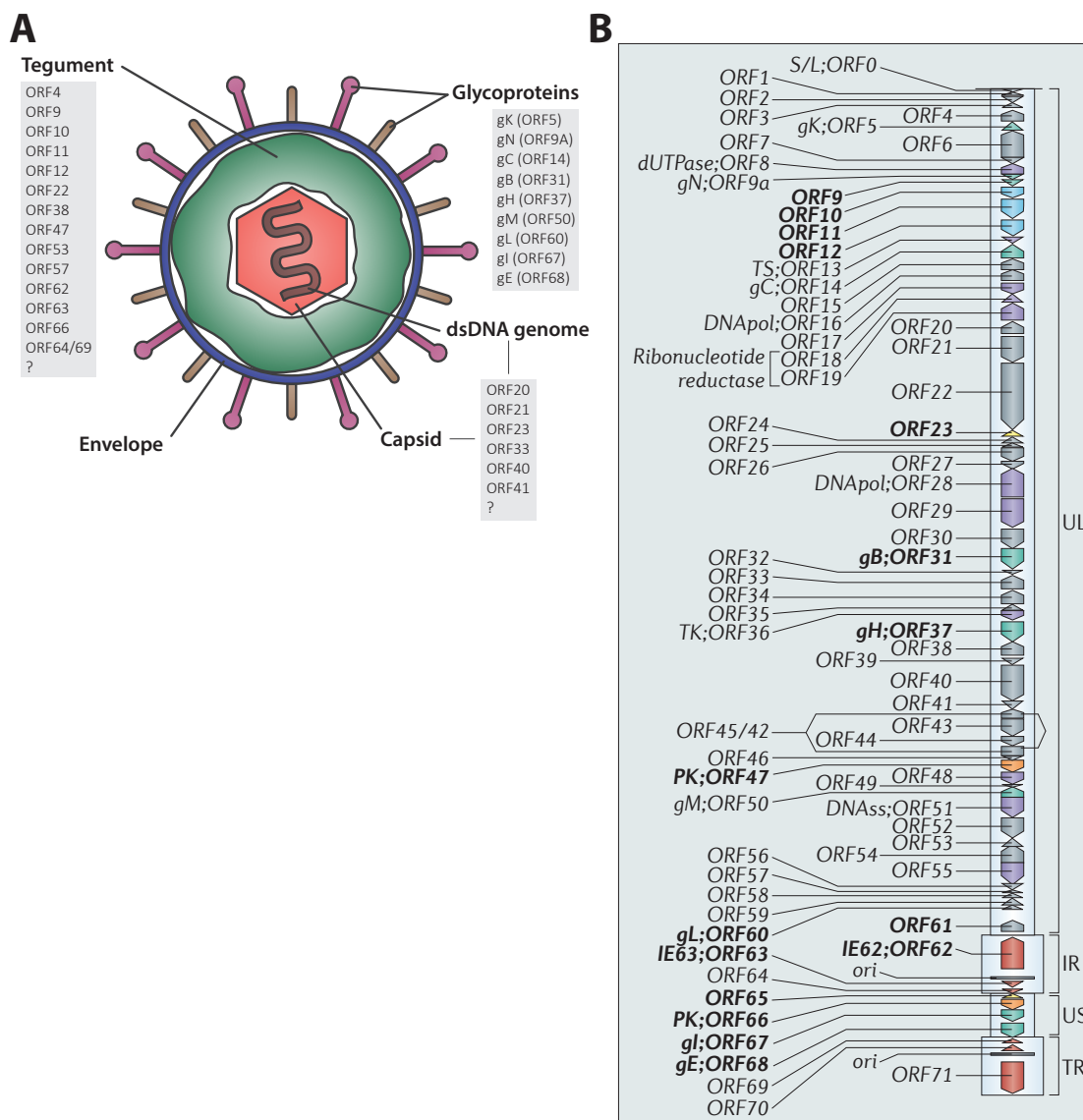


Figure 8: The architecture of the VZV virion and its genome organisation. (A) VZV is an enveloped virus with a dsDNA genome inside a nucleocapsid. Between capsid and envelope lies the tegument layer. Grey boxes indicate viral proteins that constitute the respective structural feature. **(B)** Graphical representation of the VZV linear genome showing the canonical ORFs. Panel (B) is taken from REF. 336

The VZV genome is remarkably stable and different clinical isolates show high sequence identity³²⁵. Five clades of virus have been described, but even viruses with the largest phylogenetic distance show 99.83 % sequence identity³³⁷. One study found that the attenuated VZV vaccine strain vOka harbours 42 nucleotide differences compared to its parental pOka strain³³⁸.

These changes are predicted to cause 21 amino acid differences. The majority of AA changes can be found in the sequence of ORF62, the major transactivator that controls expression of other viral genes³³⁹. To date, however, the precise molecular fundamentals of vOka attenuation are unclear. This is further complicated by the fact that vOka vaccine preparations actually consist of an oligoclonal pool of virions with partly different mutations compared to pOka³⁴⁰.

I.7.3 Transmission and Pathology

Primary infections with VZV are airborne as the virus is transmitted through aerosolised liquid droplets^{325;341}. The first cells to encounter VZV are therefore respiratory epithelial cells (Figure 9). The virus is believed to readily infect the mucosal epithelium to establish a localised infection³²⁵. Infiltration of lymphocytes in response to inflammation allows the virus to infect T cells. It is believed that these T cells then traffic to local lymphoid organs, primarily the Waldeyer's tonsillar ring³³⁶. Alternatively, the virus can infect tissue resident DCs at the site of infection. Infected DCs will also migrate to lymphoid organs and can spread the virus to T cells. The current models of initial viral spread are largely based on *in vitro* studies and SVV infection models in non-human primates (NHPs). It is, to date, not possible to investigate airborne infection with VZV in animal models (see section I.7.6).

Infection of large numbers of T lymphocytes causes generalised T cell viremia (Figure 9). T cells leave the tonsils as a result of viral modulation of skin-homing marker expression on infected cells³⁴². Homing to the skin allows transfer of viruses to keratinocytes and other skin cells. Establishment of infection in the skin manifests as the characteristic *Varicella* blister rash. The blisters contain highly infectious fluid that is responsible for transmission of the virus to uninfected individuals³⁴³. In contrast to historically established models, it is now appreciated that respiratory droplets are irrelevant for viral spread³⁴¹. Acutely VZV-infected individuals without skin blisters are unable to spread the virus³⁴³. Infectious droplets are believed to originate solely from skin blisters³⁴¹.

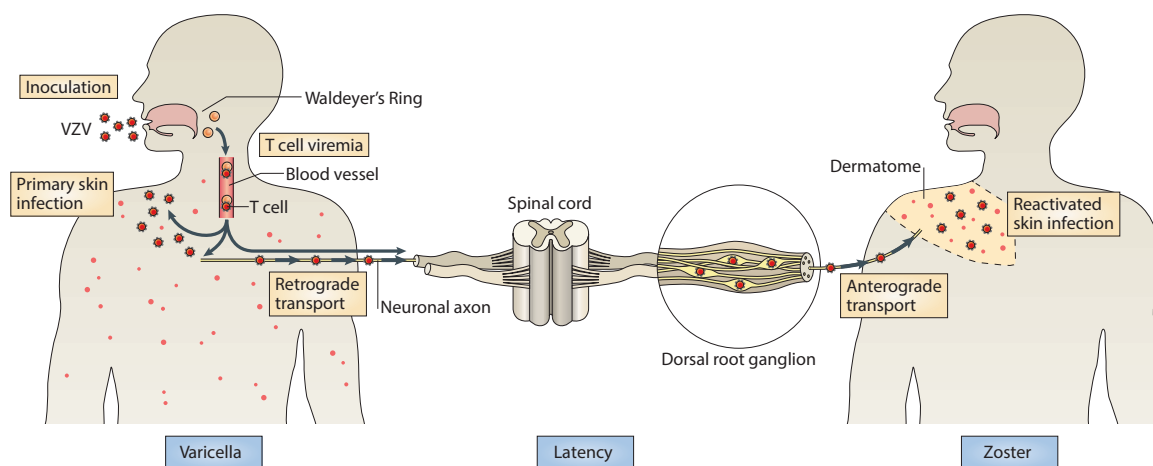


Figure 9: The pathogenesis of VZV. Inoculation of the human host with VZV occurs orally. Infection of respiratory epithelial cells and spread of virus to T cells in local lymphoid organs leads to T cell viremia. Infection of skin cells causes the characteristic chickenpox (*Varicella*) blister rash. During primary skin infection the virus can infect peripheral nerve endings and establish latency in dorsal root or cranial ganglia. Alternatively, ganglia neurons can become infected with VZV through haematogenous spread by T cells. Reactivation of the virus from latency leads to anterograde transport of virions into the innervated dermatome and establishment of the skin infection known as shingles (*Zoster*). Figure taken from REF. 336.

While replicating in the skin, the virus can infect peripheral neurons via their axon endings in the skin (Figure 9). These peripheral neurons, located in dorsal root or cranial ganglia, are the site of latency for VZV^{325;341}. Infection of neurons by direct virus transfer from T cells that have reached ganglia through a haematogenous route has also been suggested since latent VZV was found in ganglia of vaccinated children with no history of skin blister rash^{341;344}. Haematogenous spread of VZV to neurons is further evidenced by the latent infection of enteric ganglia with VZV³⁴⁵. What role infection of these ganglia and possible enteric reactivation of the virus plays physiologically is not clear³⁴¹.

As a result of insufficient control by the immune system, the virus can reactivate from latency³⁴¹ (Figure 9). Lytic replication in affected neuronal cell bodies leads to anterograde axonal spread of infectious virions. Viral replication in the skin manifests as shingles (*Herpes Zoster*). Shingles skin blisters, analogous to chickenpox, are filled with highly infectious fluid and can spread the virus to uninfected individuals. Lytic replication of the virus in the ganglion damages neuronal cell bodies and can lead to fibrosis³³⁶. This tissue damage is believed to be causative for the severe pain that can be associated with shingles and with the occurrence of post-herpetic neuralgia³³⁶.

VZV can disseminate into the CNS and other organs during both *Varicella* and *Zoster* (see section I.7.5). Immune control is important for prevention of disseminated VZV disease given that it is usually associated with immunosuppression. The precise immune cells and

pathways that commonly prevent spread of VZV into the CNS are not well-understood. An interesting link between monogenic defects in the RNA polymerase III DNA-sensing pathway and neuroinvasion of the virus has been uncovered recently (see section I.7.7.1).

Primary infection with VZV during pregnancy can be associated with severe complications for both the mother and the foetus³⁴⁶ (see section I.7.5). Varicella pneumonia is a significant risk factor for pregnant women. Intrauterine infection of the foetus can lead to congenital varicella syndrome (CVS) characterised by, among other complications, neurological maldevelopment and skeletal abnormalities³⁴⁷.

I.7.4 Replication and Life Cycle

The viral life cycle begins with the attachment of virions to the plasma membrane of cells³³⁶ (Figure 10). VZV can infect cells by fusion of the envelope with the plasma membrane or endocytosis of full virions and subsequent fusion of the envelope with endosomal membranes. Membrane fusion is governed by a complex of gB and the gH/gL heterodimer. The B glycoprotein is highly conserved in herpes viruses and constitutes the fusogenic entity after activation by gH/gL³⁴⁸. An integral part of the entry complex in other herpesviruses is gD³²⁷. This protein facilitates entry by binding to heparan sulphates on the surface of cells. VZV, interestingly, lacks a gD homologue³²⁷. Attachment to cells and priming of the gH/gH-gB fusion complex must thus rely on a unique mechanism. Other VZV glycoproteins, such as the gE/gI heterodimer, have been hypothesised to play a role in virion entry³²⁷.

Since the intracellular replication process for VZV has not been sufficiently investigated experimentally, the current model of VZV's life cycle is based largely on studies with HSV (reviewed in REF. 336, see Figure 10). After entry, VZV capsids are shuttled to nuclear pores by the cellular transport machinery. The capsid/tegument proteins and cellular motor proteins required for this process have not been determined for VZV. For HSV it was shown that both kinesin- and dynein-mediated transport along microtubules are required for nuclear targeting of capsids³⁴⁹. Specific tegument proteins are likely required for motor protein attachment to capsids. At the nuclear pores viral DNA is translocated to the nucleus. The tegument proteins ORF62 and ORF4 are believed to be also imported into the nucleus to induce expression of immediate early viral genes. Viral mRNAs are translated in the cytosol and viral proteins re-imported into the nucleus. Capsid assembly and viral DNA packaging occurs inside the nucleus. New capsids egress from the nucleus by budding through the

nuclear membranes in a process that includes primary envelopment and de-envelopment. Naked capsid acquire the tegument and undergo secondary envelopment in a process that requires Golgi-derived membranes. During this process, virions are diverted into late endosomes where some of them lose infectivity and some reach the plasma membrane³⁵⁰.

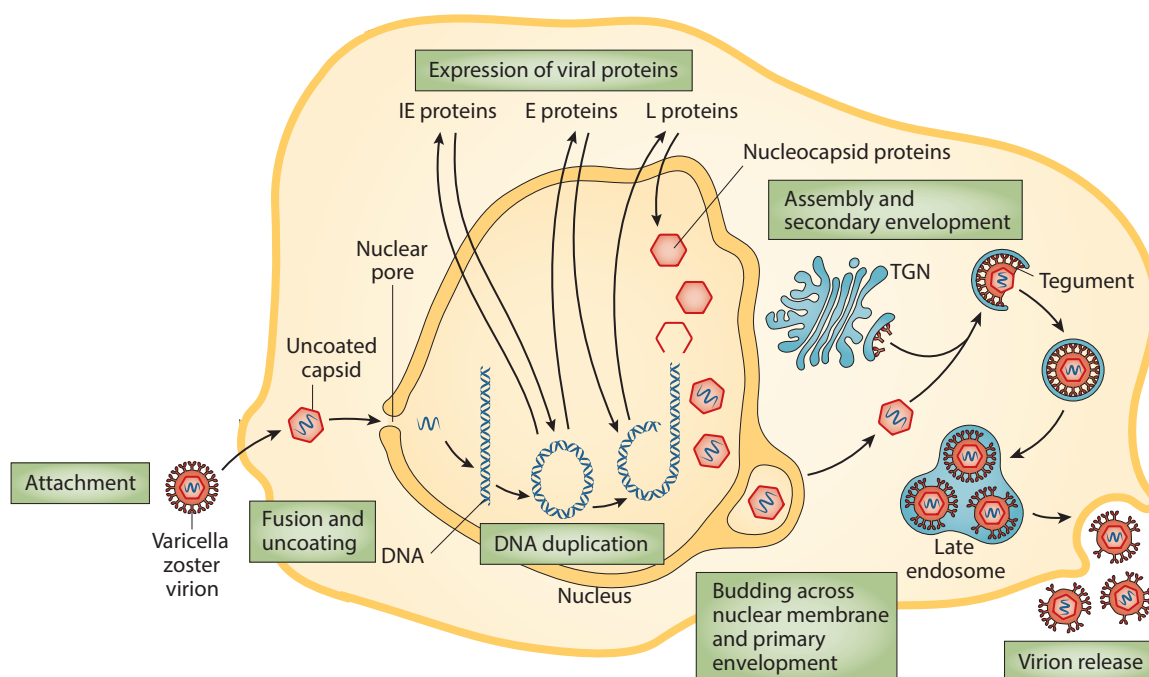


Figure 10: The VZV lytic replication cycle. After attachment of virions to the plasma membrane and membrane fusion, capsids are transported to nuclear pores. The viral DNA is translocated into the nucleus and replicated. At the same time, viral genes are expressed and protein synthesised in the cytoplasm. Viral proteins are re-imported into the nucleus for capsid assembly. DNA-containing capsid bud through the nuclear membranes and undergo secondary envelopment in the cytoplasm. Virions are then released in a process that involves late endosomes. Figure is adapted from REF. 341

In contrast to most other herpesviruses, VZV remains highly cell-associated *in vitro*. Mature viral particles can be observed attached to the plasma membrane both intra- and extracellularly³⁵¹. However, it is clear from the molecular pathology of the virus *in vivo* that VZV can generate highly infectious, cell-free particles. Its replication cycle is likely cell type dependent given that infection of skin and infection of T cells have different phenotypes³³⁶. In skin cells, VZV infection induces membrane fusion and syncytia formation³⁵². *In vivo*, this process is part of skin blister formation and essential for pathogenesis.

VZV latent infection of neurons is characterised even more poorly than its lytic replication. In cells that become latently infected, i.e. neurons, viral DNA is permanently circularised once inside the nucleus and forms an episome³²⁵. Which, if any, viral genes are expressed during latency, and whether viral proteins are synthesised, is highly controversial³⁴¹. Due

to restriction to the human host, studying latent VZV in primary cells is extremely difficult. Few studies have investigated human primary neurons from ganglia cell bodies that were removed immediately *post mortem* and have reported the presence of mRNAs encoding ORFs 4, 21, 29, 62, 63, and 66^{353–359}. Transcripts of the same viral genes were found in enteric neurons removed during surgery³⁴⁵. Recently, a spliced transcript has been identified in latently infected ganglia and was proposed as the latency associated transcript (LAT) of VZV³⁶⁰. However, the data obtained thus far regarding VZV latency are primarily descriptive. Mechanistic insight into molecular determinants of latency establishment and maintenance by both the virus and the host is largely lacking.

I.7.5 Clinical Features of Infection

After the first contact between host and virus, a 10-21 day incubation period will pass before appearance of skin blisters³⁴¹. A fever is typically observed at the same time as appearance of the rash. This rash is usually self-limiting and disappears after 1-2 weeks without leaving scars. *Varicella* disease severity is generally correlated with age at the time of primary infection³⁶¹. Typical VZV infections do not require specialised treatment besides antipyretics for fever control.

Rarely, primary VZV infection can be associated with complications that are caused by dissemination of the virus into the central nervous system³⁶². This manifests in self-limiting cerebellar ataxia, meningitis, meningoencephalitis, and intracerebral vasculopathy. The latter symptom can ultimately cause stroke. Disseminated VZV infection with CNS involvement occasionally requires intensive care treatment and is commonly treated by systemic administration of antiviral agents, such as acyclovir. In rare instances, severe VZV infections can be fatal³⁴¹. Although disseminated VZV disease and CNS invasion of the virus can occur in immunocompetent adults, people with compromised immune function are at particular risk for CNS infection³⁴¹. This includes HIV/AIDS patients, cancer patients, transplant recipients, and individuals that suffer from monogenic immune defects (see section I.7.7.1).

Clinically relevant VZV reactivation from latency, i.e. shingles, is characterised by skin blistering that is confined to a particular dermatome³⁴¹. Shingles commonly affects dermatomes in the chest area as well as the face. While the skin blisters observed during shingles are phenotypically indistinguishable from chickenpox blisters, *Zoster* is commonly associated with significant itching and/or pain³⁴¹. Some individuals experience general signs of

infection, such as fever and fatigue. Shingles pain can precede occurrence of skin manifestations and persist for month after blisters have resolved. The latter phenomenon is referred to as post-herpetic neuralgia (PHN) and has significant impact on the quality of life of affected individuals³⁶³. Notably, occurrence of shingles in the face can affect the eyes or ears. Ocular manifestations of shingles can result in temporary, or in some cases permanent, vision loss³⁶⁴. *Zoster* that affects the ears is referred to as Ramsey-Hunt syndrome and can result in permanent hearing loss and facial paralysis³⁶⁵. Shingles are commonly treated with antiviral agents, such as acyclovir. Acyclovir treatment can accelerate resolution of the disease but no evidence has been found for PHN risk reduction³⁶⁶.

I.7.6 *In Vivo* Models

VZV is regarded as a human-restricted pathogen that does not infect other species³⁶⁷. *In vitro*, the virus replicates only in cells of human and guinea pig origin^{326;368}. For a number of decades, researchers have aimed to model the different aspects of human VZV infection *in vivo*. Infection of classic rodent animal models, such as mice and rats, with VZV results in seroconversion but the animals do not get ill³⁶⁹. To date, no animal model has been identified that recapitulates human clinical disease (both chickenpox and shingles). However, immunisation of guinea pigs and cotton rats results in latency establishment by the virus in peripheral ganglia of the animals^{370;371}. Culture of infected neurons from these animals *ex vivo* has been useful in the study of the latent virus.

For the purpose of studying VZV in the context of a particular human tissue, a xenograft model was developed³⁶⁷. Severe combined immunodeficiency (SCID) mice are transplanted with human tissues, such as patches of skin^{372;373}. The skin xenograft can subsequently be infected with VZV to model primary *Varicella* infection. Pathogenesis of the virus in this model mimics human infection as the characteristic vesicular skin rash can be observed. This model is a valuable tool to study molecular aspects of virus replication in the context of a 3D tissue. Innate immune responses can be studied in the absence of systemic responses due to the immunodeficiency of the host animal. In addition to the skin model, human dorsal root ganglion (DRG) of foetal origin can be xenografted into SCID mice and infected with VZV³⁷³. This model has provided valuable insight particularly in the context of neurotropism of the virus and molecular determinants of replication in neurons.

The full spectrum of VZV disease (primary varicella, reactivation, and shingles) can cur-

rently only be studied experimentally *in vivo* by infection of NHPs with SVV³⁶⁷. SVV is closely related to VZV and causes similar disease in NHPs, for example African green monkeys. Importantly, in these animals SVV can be reactivated by immunosuppression and shingles pathogenesis can be studied *in vivo*. The SVV-NHP model has been indispensable for modelling many aspects of VZV pathogenesis, in particular systemic immune responses, in the context of a living organism.

Despite the development of different model systems, studying VZV *in vivo* remains challenging. The models currently available are resource intensive, require specialised animal facilities, and rely on establishment of elaborate techniques, such as xenografting.

I.7.7 Immune Response to VZV

I.7.7.1 Innate Responses

Innate immune cells contribute to the defence against VZV³⁷⁴. Specifically, natural killer (NK) cells and invariant natural killer T-cell (iNKT) cells are crucial for control of the virus *in vivo*. Two individuals with iNKT cell deficiency are described to have suffered from disseminated *Varicella*^{375;376}. Furthermore, iNKT cell numbers were lower in patients with recurrent shingles compared to controls³⁷⁷. Fatal VZV infection was observed in a child with selective NK cell deficiency³⁷⁸. Significant changes of NK numbers that correlated with disease severity have been reported in adult chickenpox patients³⁷⁹. NK cells have cytotoxic activity against VZV-infected cells *in vitro*^{380–382}.

In addition to cell-mediated mechanisms, innate cytokine responses are important for VZV control. Increased IFN α and IFN γ can be detected in patients with a primary VZV infection³⁸³. This systemic type I IFN response temporally precedes the onset of adaptive responses (see below). The relevance of systemic IFN α in virus restriction is highlighted by improved clinical outcomes of VZV infection in immunocompromised patients treated with recombinant IFN α ³⁸⁴.

The potency of type I, type II, and type III IFNs in restricting VZV replication has been demonstrated *in vitro*^{385–388}. However, what induces expression of these cytokines during VZV infection is less well-characterised. A number of PRRs has been implicated in antiviral cytokine responses to VZV. Plasmacytoid DCs secrete IFN α after VZV infection in a partly TLR9-dependent fashion³⁸⁹. Recognition of viral glycoproteins by TLR2 leads to inflammatory cytokine secretion in macrophages³⁹⁰. In dermal fibroblasts, the type I and type III IFN

response to VZV is mediated by STING³⁸⁸. Infection of PBMCs with VZV results in type I IFN production that is dependent on the RNA polymerase III DNA-sensing pathway³⁹¹. Interestingly, a cohort of both adults and children with severe and/or recurrent CNS VZV infections have monogenic defects in RNA polymerase III subunit genes¹⁴³. It is currently being investigated whether this phenotype is indeed dependent on dysfunctional IFN production and, if yes, which cell types this is relevant in. Overall, the recognition pathways and PAMPs that govern antiviral responses to VZV remain incompletely understood.

I.7.7.2 Adaptive Responses

Adaptive immune responses are essential for the control of primary and reactivating VZV infection^{374;392}. This is highlighted by increased incidence of disseminated, life-threatening *Varicella* in children that are immunocompromised due to cancer treatment.

The incubation period between infection and onset of the characteristic skin blister rash is 10-21 days³²⁵. Interestingly, VZV-specific T cell and antibody responses can be detected only from day three after onset of the rash³⁸³. It appears that VZV viremia and dissemination to the skin is, in regard to adaptive responses, immunologically silent. How the virus achieves this is highlighted below in section I.7.7.3. After rash onset, virus specific T cells can be detected and their frequency increases over time³⁸³. These T cells appear to be important for virus control since the time of T cell response onset correlates with disease severity³⁸³. The fact that T cells are critically important for control of primary VZV infection is further highlighted by increase susceptibility of HIV-infected children to severe *Varicella*^{393–395}.

In contrast to cell-mediated defence mechanisms, humoral immune responses play a minor role in control of primary VZV infection. Viral glycoprotein-specific IgM and IgG antibodies can be readily detected after rash onset^{396–398}. No antibodies are generated during the incubation time, similar to the T cell responses. *In vitro*, VZV-specific antibodies found in patients have neutralising ability^{396;399}. However, individuals with B cell deficiencies are not at higher risk for disseminated VZV at primary infection⁴⁰⁰. Furthermore, there is no correlation between time of onset of the antibody response and disease severity³⁸³. It has been proposed that antibodies against VZV are important to maintain immunity and prevent re-infection with circulating viruses^{374;392}.

It is believed that re-exposure to VZV by circulating viruses in the environment and sub-

clinical reactivation from latency maintains VZV-specific T cells³⁷⁴. The highest levels of those cells can be found in individuals between 30 and 40 years of age⁴⁰¹. In contrast, from the age of 55 onwards, a large proportion of people have no detectable T cells that recognise VZV epitopes⁴⁰¹. This appears causative for occurrence of shingles given that its incidence is correlated with declining VZV-specific immunity^{402–404}. Furthermore, immunosuppressive treatment commonly leads to shingles, in particular in elderly patients⁴⁰⁵.

I.7.7.3 Immune Evasion Mechanisms

VZV employs mechanisms to evade both innate and adaptive responses^{406;336}. To escape NK cell killing, the virus modulates the surface expression of ligands to the NK cell receptor NKG2D⁴⁰⁷. VZV blocks induction of antiviral cytokines downstream of PRR activation by targeting IRF3 with proteins encoded by ORF47, ORF61, and ORF62^{408–410}. Limited evidence has been presented towards antagonism of NF κ B and thus inflammatory cytokine expression^{411;412}. Multiple viral mechanisms interfere with STAT1 and STAT2 functionality and thus broadly antagonise IFN signalling pathways. In infected cells, phosphorylated STAT1 fails to translocate to the nucleus⁴¹³. The viral ORF66 protein interferes with STAT1 phosphorylation⁴¹⁴. Infected cells show reduced STAT2 phosphorylation after type I IFN treatment, although no mechanism has been elucidated⁴¹⁵. The signalling downstream of the type I and type III IFN receptor is additionally antagonised by degradation of IRF9 through the viral ORF63 protein^{415;416}.

In order to prevent recognition by cytotoxic T cells, VZV downregulates MHC-I surface expression. ORF66 of VZV leads to retention of the MHC-I molecules at the Golgi^{417;418}. The aforementioned viral mechanisms that suppress IFN signalling are believed to prevent IFN γ -induced upregulation of MHC-II in infected antigen presenting cells (APCs)⁴¹⁹. In addition to MHC-II, VZV infection of DCs also prevents upregulation of the co-stimulatory molecules CD80 and CD86⁴²⁰.

I.7.8 Epidemiology and Vaccines

VZV is a pathogen that affects humans worldwide⁴²¹. Before introduction of the *Varicella* vaccine, only 5 % of individuals in moderate climate regions were still susceptible to VZV infection by 30 years of age, i.e. have not had chicken pox³²⁵. VZV infection incidence is lower in tropical climates with half of young adults being seronegative for the virus³²⁵.

Climate conditions appear to be important for VZV spread since seasonal waves of VZV infections can be observed. Although chickenpox is commonly a self-limiting disease, primary infection in children requires hospitalisation in 0.3 % of cases in Europe⁴²². This number increases with age at the time of primary infection. Fatal cases of primary VZV infection are rare. However, before vaccine introduction, three out of 100,000 individuals with *Varicella* succumbed to disseminated infection⁴²¹.

The life-time risk for shingles in individuals that experienced childhood chickenpox is around 27 % in the UK⁴²³. The incidence of shingles correlates with age and shows a marked increase after 50 years of age. At age 85, the probability of having had at least one *Zoster* episode rises to 50 %³²⁵. The increase of shingles with age correlates with declining cell-mediated immune responses in elderly individuals. CVS, in which primary infection with VZV during pregnancy leads to birth defects, occurs in approx. 0.003 % of pregnancies³²⁵. Within the group of women that experience primary infection in the first trimester of pregnancy, around 2 % give birth to an infant with CVS. Administration of VZV immunoglobulins to pregnant women after VZV exposure can prevent disease manifestation in the foetus⁴²⁴. Introduction of antibody prophylaxis has reduced CVS incidence substantially⁴²⁴.

VZV is the only human herpesvirus for which a vaccine has been licensed. Varivax and Varilix are both preparations of the attenuated vOka vaccine strain of VZV offered by Merck (US) and GlaxoSmithKline (GSK, UK), respectively⁴²⁵. The vOka strain of VZV was generated by serial passage of the parental Oka strain in tissue culture⁴²⁶. The vaccines are licensed for the prevention of chicken pox in both children and adults. The vaccine virus establishes latency and can cause shingles upon reactivation. The incidence of shingles, however, is lower in individuals immunised with vOka compared to wild-type virus⁴²⁷. The chicken pox vaccine is part of the routine vaccination schedule of many developed countries, such as the US, South Korea, Japan, and Germany⁴²⁵. In other countries, such as the UK, the vaccine is given only to children and adults with increased risk for severe VZV infection.

The vOka virus is further licensed for the prevention of shingles in elderly individuals⁴²⁵. This vaccine, called Zostavax, is a higher titre preparation of vOka compared to the chickenpox vaccine. The vaccine can prevent around 50 % of shingles cases in individuals over 60 but efficacy declines rapidly with increasing age at the time of vaccination⁴²⁸. Recently, a new subunit vaccine for the prevention of shingles in individuals over 50 years of age has been licensed (Shingrix). The vaccine is an adjuvanted preparation of recombinant VZV

gE and shows superior efficacy of >90 % in the prevention of shingles and PNH compared to Zostavax^{429;430}. Importantly, it is safe to use in individuals that could previously not be vaccinated with Zostavax (e.g. immunocompromised patients).

I.8 Objective of The Study

The cells of higher organisms, such as mammals, harbour powerful mechanisms that restrict infection with viral pathogens. These mechanisms are the result of ongoing co-evolution of host and pathogen. At the same time, viruses employ equally powerful mechanisms that counteract host detection and defence.

Understanding how animal cells autonomously recognise invading pathogens and restrict infection is essential for understanding the systemic immune response. The innate immune system represents the first line of defence against invading pathogens and has indispensable roles for the priming of adaptive, secondary responses.

It is surprising how little is known about the interplay between the powerful innate antiviral defence system of humans and important viral pathogens. In this study, I characterise the cell-autonomous immune response to two incompletely characterised viruses, Zika virus and Varicella-Zoster virus. Furthermore, I identify mechanisms that enable VZV to evade host recognition.

I test whether Zika virus infection is recognised by the RLR group of RNA sensors. I further investigate whether it is viral RNA that binds to PRRs and causes an antiviral response. I hypothesise a role of RLRs in Zika virus recognition based on our knowledge about related flaviviral pathogens such as DENV, WNV, and JEV. For these viruses, a crucial role of viral RNA recognition by cytosolic RNA sensors has been demonstrated.

With a similar approach I will investigate which nucleic acid sensing mechanisms govern the type I IFN response to VZV. I hypothesise a crucial role of the cGAS/STING DNA sensing system in cell-autonomous recognition of VZV. This is based on VZV's identity as a dsDNA virus and the established role of cGAS and STING in recognition of HSV-1. Inhibition of type I IFN induction and specifically cGAS and STING has been demonstrated previously for other viral pathogens. I therefore hypothesise that VZV expresses a protein that antagonises DNA sensing directly at the level of cGAS and STING. I screen VZV genes for their ability to interfere with and characterise the mechanism of action of newly identified cGAS/STING antagonists.

While medical research has developed powerful pharmacological and biological tools to fight bacterial infections, effective antivirals are lacking. Molecular characterisation of the way that cells recognise viral infections and fight them, as well as how viruses evade antiviral

mechanisms, will aid efforts to establish novel antiviral strategies. A better understanding of how viral pathogens evade clearance by the immune system can allow us to develop drugs that facilitate recognition and clearance of viruses by the powerful mechanisms that the human body already has.

Chapter II

Materials and Methods

II.1 Materials

II.1.1 Cells

All cells were cultured at 37° C and 5% CO₂ and checked regularly for mycoplasma contamination. Adherent cells were passaged using Trypsin-EDTA (0.25%) dissociation reagent (Gibco) at appropriate confluence. Foetal calf serum (FCS) was obtained from Sigma-Aldrich.

HEK293	Human embryonic kidney (HEK) cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich) containing 4.5 g/L glucose, supplemented with 10% (v/v) FCS and 2 mM L-glutamine (gibco). The cells were a kind gift from Caetano Reis e Sousa (The Francis Crick Institute, UK).
HEK293 FLAG-RIG-I	HEK293 cells ectopically expressing 3xFLAG-tagged RIG-I were described previously (REF. 167) and maintained as parental HEK293 cells.
HEK293p125	HEK293 cells stably transduced with the p125-GreenFire construct (GFP and Firefly luciferase expressed under <i>IFN</i> β -promotor control). Parental, RIG-I-KO, MDA5-KO, and MAVS-KO were used. These cells are all described in REF. 184. Cells were maintained as parental HEK293 cells.
HEK293T	Human embryonic kidney cells expressing the SV40 large T antigen were maintained in DMEM containing 4.5 g/L glucose, supplemented with 10% (v/v) FCS and 2 mM L-glutamine. The cells were a kind gift from Caetano Reis e Sousa (The Francis Crick Institute, UK).

- 3C11** 3C11 cells are a type I IFN Firefly luciferase/GFP reporter cell line derived from HEK293 cells by lentiviral transduction with the pGreenFire-ISRE reporter construct (previously described in REF. 50). Cells were maintained as parental HEK293 cells.
- MeWo** MeWo cells are a melanoma cell line used for the propagation of VZV. Cells were maintained in Minimal Essential Medium (MEM) (gibco) containing 1 g/L glucose supplemented with 10% (v/v) FCS and 2 mM L-glutamine, 10 mM non-essential amino acids (gibco), and 1 mM sodium pyruvate (gibco). Cells were a kind gift from Jeffrey Cohen (NIH, US).
- THP1** THP1 cells are monocytic cell line derived from a patient with acute monocytic leukaemia. The cells used in this study are dual reporter cells (THP1 Dual, invivogen). They secrete Lucia luciferase expressed under control of the ISG54 minimal promotor and secrete secreted embryonic alkaline phosphatase (SEAP) under control of a NF κ B responsive promotor. The cells are resistant to Zeocin and blasticidin. Parental cells and cells which are knockout for *CGAS*, *TMEM173* (STING), *MYD88*, *TBK1*, and *IFNAR2* alleles were a kind gift from Soren Paludan (Aarhus University, Denmark). Knockout cells for *MAVS* and *IRF3* were generated from parental cells as described in section II.2.9.1. Cells were maintained in Roswell Park Memorial Institute (RPMI) medium (Sigma-Aldrich) containing 4.5 g/L glucose supplemented with 10% (v/v) FCS and 2 mM L-glutamine.
- HaCaT** HaCaT cells are spontaneously immortalised keratinocytes and were maintained in DMEM (Sigma-Aldrich) containing 4.5 g/L glucose, supplemented with 10% (v/v) FCS and 2 mM L-glutamine (gibco). The cells were a kind gift from Leonie Unterholzner (Lancaster University, UK).

II.1.2 Viruses

- VZV** VZV rOka (cosmid-derived recombinant pOka) was a gift from Jeffrey Cohen (NIH, Bethesda, USA) and is described in REF. 431. The virus was maintained as infected monolayers of MeWo cells cryopreserved in freezing medium (90% FCS, 10% DMSO) and stored in liquid nitrogen. For generation of infected cell stocks, monolayers of infected cells were monitored for cytopathic effect. Cells that showed high level of infection were detached and infected cells were mixed at appropriate ratios (1:2-1:4) with uninfected cells and re-plated. Aliquots of infected cells were frozen (see above) and thawed for experiments.
- VZV^{ORF9-V5}** Recombinant VZV in which a C-terminal V5 tag was added to the coding sequence of ORF9. The virus was a kind gift from Catherine Sadzot (University of Liege, Belgium) and is described in REF. 432.

II.1.3 Plasmids

Name	Description	Origin
p125-F-Luc	Expresses Firefly luciferase under control of the hIFN β promotor.	Kind gift from T. Fujita (Kyoto University, Japan). Described in REF. 433.
pRL-TK	Expresses Renilla luciferase under control of the HSV-1 thymidine kinase (TK) promotor. Used as transfection control in Dual luciferase assays.	Promega
ISG54-F-Luc	Expresses Firefly luciferase under control of the <i>ISG54</i> promotor.	Clontech
RIG-I-CARD	Expression plasmid for constitutively active variant of RIG-I (exposed CARD domain).	Kind gift from Andreas Pichlmair (Technical University Munich, Germany).
pCMV-VSV-G	VSV-G expressing plasmid for lentiviral pseudotyping.	Kind gift from Bob Weinberg (Addgene plasmid number 8454).
p8.91	Lentiviral packaging plasmid.	Kind gift from Greg Towers (UCL, UK).

Continued on next page

Name	Description	Origin
pX458/Ruby/MAVS	CRISPR/Cas9 plasmid for targeting of MAVS.	Philip Hublitz (Genome Engineering Facility, Weatherall Institute of Molecular Medicine, UK).
pX458/Ruby/IRF3	CRISPR/Cas9 plasmid for targeting of IRF3.	REF. 184
pGag-eGFP	Expression plasmid for HIV-1 gag fused to eGFP	NIH AIDS Reagent Programme (Cat. number 11468)
pLenti6.3/hcGAS-V5	Expression plasmid for human cGAS with C-terminal V5-tag.	From pCR8/hcGAS, see section II.2.1.3. Generated by Lise Chauveau (Rehwinkel Lab).
pcDNA3.2/hcGAS-FLAG	Expression plasmid for human cGAS with C-terminal FLAG-tag.	From pCR8/hcGAS, see section II.2.1.3.
pLenti6.3/hSTING-V5	Expression plasmid for human STING with C-terminal V5-tag.	From pCR8/hSTING, see section II.2.1.3. Generated by Lise Chauveau (Rehwinkel Lab).
pcDNA3.2/STING-HA	Expression plasmid for human cGAS with C-terminal HA-tag.	From pCR8/hcGAS, see section II.2.1.3.
pLenti6.3/lacZ-V5	Expression plasmid for lacZ with C-terminal V5-tag.	Invitrogen
pLenti6.3/EMCV-L-V5	Expression plasmid for L protein of EMCV with C-terminal V5-tag.	REF. 184
pLenti6.3/ORF9-V5	Expression plasmid for VZV ORF9 with C-terminal V5-tag.	See section II.2.1.1.
pLenti6.3/GFP-V5	Expression plasmid for eGFP with C-terminal V5-tag.	From pCR8/GFP, see section II.2.1.3.
pLenti6.3/ZIKV-NS5	Expression plasmid for the NS5 protein of ZIKV with C-terminal V5-tag.	REF. 184
pLenti6.3/puro/FLAG-ORF9	Expression plasmid for VZV ORF9 with N-terminal FLAG-tag.	From pCR8/ORF9-STOP, see section II.2.1.3.
pT-Rex-DEST31/ORF9	Expression plasmid for VZV ORF9 with N-terminal 6xHis-tag.	From pCR8/ORF9-STOP, see section II.2.1.3.
pLenti6.3/puro/FLAG-ORF9-I-STOP	Expression plasmid for VZV ORF9 truncation mutant I with N-terminal FLAG-tag.	From pCR8/ORF9-I-STOP, see section II.2.1.3.
pLenti6.3/puro/FLAG-ORF9-II-STOP	Expression plasmid for VZV ORF9 truncation mutant II with N-terminal FLAG-tag.	From pCR8/ORF9-II-STOP, see section II.2.1.3.

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Name	Description	Origin
pLenti6.3/puro/FLAG-ORF9-III-STOP	Expression plasmid for VZV ORF9 truncation mutant III with N-terminal FLAG-tag.	From pCR8/ORF9-III-STOP, see section II.2.1.3.
pLenti6.3/puro/FLAG-ORF9-IV-STOP	Expression plasmid for VZV ORF9 truncation mutant IV with N-terminal FLAG-tag.	From pCR8/ORF9-IV-STOP, see section II.2.1.3.
pLenti6.3/puro/FLAG-ORF9-V-STOP	Expression plasmid for VZV ORF9 truncation mutant V with N-terminal FLAG-tag.	From pCR8/ORF9-V-STOP, see section II.2.1.3.
pLenti6.3/puro/FLAG-ORF9-VI-STOP	Expression plasmid for VZV ORF9 truncation mutant VI with N-terminal FLAG-tag.	From pCR8/ORF9-VI-STOP, see section II.2.1.3.
pLenti6.3/puro/FLAG-ORF9-VII-STOP	Expression plasmid for VZV ORF9 truncation mutant VII with N-terminal FLAG-tag.	From pCR8/ORF9-VII-STOP, see section II.2.1.3.
pcDNA3.1/eYFP	Expression plasmid for eYFP.	eYFP coding sequence was PCR amplified and TA-ligated into pcDNA3.1/V5-His-TOPO (Invitrogen). Generated by Gregorio Dias (Rehwinkel group).
Gateway entry vectors		
pCR8/hcGAS	hcGAS ORF w/o STOP.	From THP1 cDNA, see section II.2.1.3. Generated by Lise Chauveau (Rehwinkel Lab).
pCR8/hSTING	hSTING-HAQ ORF w/o STOP.	From THP1 cDNA, see section II.2.1.3. Generated by Lise Chauveau (Rehwinkel Lab).
pCR8/eGFP	eGFP ORF w/o STOP.	From pGag-eGFP, see section II.2.1.3.
pCR8/ORF9-STOP	VZV ORF9 with STOP.	From pCR8/ORF9, see section II.2.1.3.
pCR8/ORF9-I-STOP	VZV ORF9 truncation mutant I AA 1-151 with STOP codon.	From pCR8/ORF9, see section II.2.1.3.
pCR8/ORF9-II-STOP	VZV ORF9 truncation mutant II AA 152-302 with STOP codon.	From pCR8/ORF9, see section II.2.1.3.
pCR8/ORF9-III-STOP	VZV ORF9 truncation mutant III AA 81-302 with STOP codon.	From pCR8/ORF9, see section II.2.1.3.
pCR8/ORF9-IV-STOP	VZV ORF9 truncation mutant IV AA 1-240 with STOP codon.	From pCR8/ORF9, see section II.2.1.3.

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Name	Description	Origin
pCR8/ORF9-V-STOP	VZV ORF9 truncation mutant V AA 81-240 with STOP codon.	From pCR8/ORF9, see section II.2.1.3.
pCR8/ORF9-VI-STOP	VZV ORF9 truncation mutant VI AA 152-240 with STOP codon.	From pCR8/ORF9, see section II.2.1.3.
pCR8/ORF9-VII-STOP	VZV ORF9 truncation mutant VII AA 81-151 with STOP codon.	From pCR8/ORF9, see section II.2.1.3.

Gateway destination vectors

pT-Rex-DEST31	Mammalian expression vector that confers a N-terminal 6xHis-tag.	Invitrogen
pLenti6.3/TO/V5-DEST	Lentiviral mammalian expression vector that confers a C-terminal V5-tag.	Invitrogen
pcDNA3.2/FLAG-DEST	Mammalian expression vector that confers a C-terminal FLAG-tag.	See section II.2.1.2.
pcDNA3.2/HA-DEST	Mammalian expression vector that confers a C-terminal HA-tag.	See section II.2.1.2.
pLenti/TO/N-FLAG-DEST	Lentiviral mammalian expression vector that confers a N-terminal FLAG-tag.	See section II.2.1.2.
pLenti/TO/N-FLAG/puro-DEST	Lentiviral mammalian expression vector that confers a N-terminal FLAG-tag. Expresses puromycin instead of blasticidin resistance.	See section II.2.1.2.

Bacterial expression vectors

pGEX6P1	E.coli expression vector that confers N-terminal GST-tag. Used for cloning.	Kind gift from Martin Reijns (University of Edinburgh, UK).
pGEX6P1-DEST-HA	Bacterial expression vector that confers N-terminal GST-tag and C-terminal HA-tag. Gateway destination vector. Used for cloning.	Kind gift from Martin Reijns (University of Edinburgh, UK)
pGEX6P1-FLAG-ORF9	Bacterial expression vector for FLAG-tagged VZV ORF9 protein.	See section II.2.1.4.
pGEX6P1-FLAG-eGFP	Bacterial expression vector for FLAG-tagged eGFP protein.	See section II.2.1.4.
pGEX6P1-hcGAS-HA	Bacterial expression vector for HA-tagged hcGAS protein.	See section II.2.1.4.
pGEX6P1-hcGAS	Bacterial expression vector for hcGAS protein.	Kind gift from Martin Reijns (University of Edinburgh, UK).

II.1.4 Oligonucleotides

All oligonucleotides were purchased from Sigma Aldrich.

II.1.4.1 Primers for VZV ORF PCR

ORF	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
0	gccgccatggcgaccgtgcactactcc	tgtagttagttgggaggttctctcg
1	gccgccatgtccagggtatcggagtatggg	ttctcgcttcagcttgcgc
2	gccgccatgcatgtaatttctgagacac	catcaatacgccctccg
3	gccgccatggatacaacgggagcttccg	tagtccgccgacagccg
4	gccgccatggcctctgctcaattc	gcagttaaagggtactacactaaa
5	gccgccatgcaggcttaggaatca	atgtttctgggagtttcac
6	gccgccatggataaatctccaaacc	actcgaagttaaattggataatt
7	gccgccatgcagacggtgtgtgcc	tacaagcataacatgggatttctga
8	gccgccatgaacgaagcggtaattg	atgttttagtagaaaatcgacat
9	gccgccatggcatcttccgacggtga	tttcgcgcacatcagttctga
9A	gccgccatgggatcaattaccgcttcg	ccacgtgctgcgtaatacagaac
10	gccgccatggagtgaatttaggaaccg	acgcgttaaaaaccaca
11	gccgccatgcagtcgggtcattataa	atattttcgtagtaaatgcatgg
12	gccgccatgtttctcggttgcgcg	atgatgactcttaggcgtattttcct
13	gccgccatgggagacttgcatgttg	aagagccatttccatttttaggg
14	gccgccatgaagcggatacaataaatttaatt	tgaacagcaacggatgca
15	gccgccatggccgtgaatggtgaa	cgatacatatgtaccacatagatagc
16	gccgccatggatttgaggctgcgt	tttaactgtacatattacgtcagattcac
17	gccgccatggggctcttggactga	attccaatatatttgaatacag
18	gccgccatggatcagaaagattgc	taaatcgttatcactgtgc
19	gccgccatggagttcaaaagaattttta	taaagcacaactggtagc
20	gccgccatggggagtcaccaacc	ataataacattcgttccatgtattgt
21	gccgccatggaagaaccaatttgta	agggtcactcccacttg
23	gccgccatgacacaaccgcacg	caccctacgacttctgaagc
24	gccgccatgtcacggagaacgtatg	ttccagaaaagcaccgc
25	gccgccatgtacgaatcggaatg	agcatcctcaatatttcag
26	gccgccatggatcggttagaatcag	gacatacttcgatagggtg
27	gccgccatgcatttaaagcctaccag	ccgaggaggaacaaagt
28	gccgccatggcgatcagaacgggg	actttgatggagaattgctttggaa
29	gccgccatggaaaatactcagaagact	aatcatttccattgtaattgtcc
30	gccgccatggaattggatattaatcgaacattg	tgaaaacgccgggtc
31	gccgccatgtttgttacggcggtt	cacccccgttacattctcg
32	gccgccatggaatcgtctaacttaacg	atcgggtgcagaatcttcac
33	gccgccatggctgctgaagctgac	acaccgccccaccatcat
33.5	gccgccatggcttctgtagcaggtaacgc	acaccgccccaccatcat

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ORF	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
34	gccgccatgacggcgagatatgggtt	cggtgtggaggcaaact
35	gccgccatgtccgctagtcgaattcgg	cccatgggaaaacatcccgg
36	gccgccatgtcaacggataaaaccgat	ggaagtgtgtcctgaacg
37	gccgccatgtttcgctagtttttagc	tgtcagagggtattttattatattct
38	gccgccatggaatttccatatcattcaac	cctttgggttttttccc
39	gccgccatgaaccaccccaagcccg	aaacgaaatagatgttttaacataacacgg
40	gccgccatgacaacggtttcatgt	tcgcggaagaggaaga
41	gccgccatggctatgccatttgagat	cactgaatcacggcc
42	gccgccatgtcattgataatgtttggt	tttaataggcataaacacgg
43	gccgccatggaagcccatttgga	tttatgggggttgggaatagag
44	gccgccatggaattacaacgcataatttccg	ggtggtttaggttccggt
45	gccgccatgtcattgataatgtttggtctacg	tttaataggcataaacacggaatccg
46	gccgccatgtcaggccacactcca	cacatccgtgtgtgggg
47	gccgccatggatgctgacgacacacc	tgctgatcctatccaatccc
48	gccgccatggcacgatcgggattg	aagcaacggtttctccg
49	gccgccatgggacaatcttcatccag	acattttgcgcatttgga
50	gccgccatgggaactcaaaagaagg	ctcccaccactgtt
51	gccgccatgtctccaacaccggg	taaactttcaaaattaccgccccg
52	gccgccatggacgcaacgcagatt	taaaaacaagaagttatatgaagc
53	gccgccatgcagcggattcgacct	ctttacaaccggtggtgaatttt
54	gccgccatggccgaaataacgtct	agatcttcgatcacgtcg
55	gccgccatgaaaagatcaatttctgt	atacacaacgtgtacg
56	gccgccatgaaaaatccgcagaaattagcga	cgcggttgcggcgctccc
57	gccgccatggacgtacgagaacgtaatg	acgttgaggagccttgc
58	gccgccatgttttcggagttgctcc	cgttctctgacgtccatga
59	gccgccatggatgtgtctggggag	tataacactccaatcgatctcg
60	gccgccatggcatcacataaatggttactgc	ttggcatacgcgttgaacaaa
61	gccgccatggataccatattagcg	ggacttcttcatcttg
62/71	gccgccatggatacgccgccgatgc	ccccgactctgcgggg
63/70	gccgccatgttttcacctcaccggcta	cacgccatgggggggc
64/69	gccgccatgaatctctgcggatccc	ggatctctcgtgggttcttg
65	gccgccatggccggacaaaacacc	tccaacaaattgtgacgttat
66	gccgccatgaacgacgttgatgaac	atctccaacttccattggatttg
67	gccgccatgttttaataccaatgtttgat	tttaacaaacgggtttaca
68	gccgccatggggacagttaataaacc	ccgggtcttatctataacaccgtgt

II.1.4.2 General Primers

Name/Purpose	Sequence (5'-3')
IAV M qPCR F	cgaggaacatccagactc
IAV M qPCR R	attggagatcctgaagtcc
ZIKV qPCR F	cttctaaccgaggtcgaaacgta

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Name/Purpose	Sequence (5'-3')
ZIKV qPCR F	ggtgacaggattggtctgtcttta
hcGAS F	gccgccatgcagccttggcacg
hcGAS R	aaattcatcaaaaactggaaactcattg
hSTING F	gccgccatgccccactccagc
hSTING R	agagaaatccgtgcggag
eGFP F	gccgccatggtgagcaagggcgag
eGFP R	ctgtacagctcgtccatgcc
ORF9S F	gccgccatggcatcttccgacggtga
ORF9S R	ctatttcgcgcacatcagttcttgatg
ORF9-IS F	gccgccatggcatcttccgacggtga
ORF9-IS R	ctaggcaattgcgcctgctcc
ORF9-IIS F	gccgccatgagcgggagaccaatttccttcag
ORF9-IIS R	ctatttcgcgcacatcagttcttgatg
ORF9-IIIS F	gccgccatgtccagcggatcggaagatg
ORF9-IIIS R	ctatttcgcgcacatcagttcttgatg
ORF9-IVS F	gccgccatggcatcttccgacggtga
ORF9-IVS R	ctataggtctgcttcattagcggcttg
ORF9-VS F	gccgccatgtccagcggatcggaagatg
ORF9-VS R	ctataggtctgcttcattagcggcttg
ORF9-VIS F	gccgccatgagcgggagaccaatttccttcag
ORF9-VIS R	ctataggtctgcttcattagcggcttg
ORF9-VIIS F	gccgccatgtccagcggatcggaagatg
ORF9-VIIS R	ctaggcaattgcgcctgctcc
eYFP F	atggtgagcaagggcgagg
eYFP R	ttacagctcgtccatgccgag
3xFLAG BbsI	cctccatagaagacaccgggaccgatccgcctccggaccatggactacaaagaccatgacggtg
3xFLAG XhoI	gatgggctcgaggatatacccggtcttgcacatcgtcatccttgtaac
3xFLAG GW XbaI	tcgtcgactagttcgtttagtgaaccgtcag
3xFLAG GW SpeI	taatacgactcactataggg
Puro Acc65I F	ctggcgatggtaccttgcgccttttccaaggcag
Puro Acc65I R	gtcttaaaggtagcgagctcgaattgtgctcaggcaccgggcttgc
3xFLAG ApaI	tgatctagagggcccggttgaagactacaaagaccatgacggtg
3xFLAG AgeI	tttaaactcattactaaccggtagcgttgcacatcgtcatccttgtaac
HA GW F	ggtgaacactatcccatatcaccag
HA GW R	agcgtaatctggaacatcgatgggtattcgaaccgcgggc
HA GW EcoRI	ggtgaacactatcccatatcaccag
HA GW AgeI	aactcattactaaccggtagcgcgaatctggaacatcgatg
VACV-70mer F	ccatcagaaagaggtttaataattttgtgagaccaicgaagagagaaagagataaaactttttacgact
VACV-70mer R	agtcgtaaaaaagttttatctcttctcttcgatgggtcacaaaaatattaaaccttttctgatgg
ISD F	tacagatctactagtgtatgatgactgtatgtacatgatctaca
ISD R	atgtctagatgatcactagatactgactagacatgtactagatgt

II.1.4.3 TaqMan Primer/Probes

All TaqMan primer/probes were purchased from Invitrogen.

Gene	Protein	AssayID	Gene	Protein	AssayID
<i>GAPDH</i>	GAPDH	Hs02758991_g1	<i>TNFA</i>	TNF α	Hs0074128_m1
<i>ACTB</i>	β -actin	Hs99999903_m1	<i>IL6</i>	IL-6	Hs00174131_m1
<i>IFNB1</i>	IFN β	Hs02621180_s1	<i>IL1B</i>	pro-IL-1 β	Hs01555410_m1
<i>IFNL1</i>	IFN λ 1	Hs00601677_g1	<i>DDX58</i>	RIG-I	Hs01061436_m1
<i>IFNA5</i>	IFN α 5	Hs04186137_sH	<i>IFIT1</i>	IFIT1	Hs03027069_s1
<i>IFI44</i>	IFI44	Hs00951349_m1			

II.1.5 Antibodies

Target	Conjugate	Source	Manufacturer	Clone/Cat.#	App.	Dilution
FLAG	HRP	Mouse monoclonal	Sigma Aldrich	M2	WB	1:10000
FLAG	-	Mouse monoclonal	Sigma Aldrich	M2	IP	-
FLAG	-	Rabbit monoclonal	CellSignaling	D6W5B	IF	1:800
V5	-	Mouse monoclonal	Biolegend	680602	IP	-
V5	FITC	Mouse monoclonal	Invitrogen	R963-25	IF	1:500
V5	HRP	Mouse monoclonal	Invitrogen	R961-25	WB	1:5000
HA	-	Mouse monoclonal	Invitrogen	2-2.2.14	IP	-
HA	HRP	Rabbit monoclonal	CellSignaling	C29F4	WB	1:1000
RIG-I	-	Mouse monoclonal	Calteg	Alme1	WB	1:1000
β -actin	HRP	Mouse monoclonal	Sigma Aldrich	AC-15	WB	1:200000
VZV gE/GI	FITC/PE	Mouse monoclonal	GE	MAB8612	FACS	1:500
VZV gE/GI	-	Mouse monoclonal	GE	MAB8612	WB	1:2000

Continued on next page

Target	Conjugate	Source	Manufacturer	Clone/Cat.#	App.	Dilution
VZV ORF62	-	Mouse monoclonal	Meridian Life Science	C05107MA	WB	1:1000
mIgG2b isotype	FITC	Mouse monoclonal	BD	27-35	FACS	-
pSTAT1 (Y701)	-	Rabbit monoclonal	CellSignaling	58D6	WB	1:1000
STAT1	-	Rabbit monoclonal	CellSignaling	42H3	WB	1:1000
pSTAT2 (Y690)	-	Rabbit monoclonal	CellSignaling	D3P2P	WB	1:1000
STAT2	-	Rabbit monoclonal	CellSignaling	D9J7L	WB	1:1000
cGAS	-	Rabbit monoclonal	CellSignaling	D1D3G	WB	1:1000
STING	-	Rabbit monoclonal	CellSignaling	D2P2F	WB	1:1000
MyD88	-	Rabbit monoclonal	CellSignaling	D80F5	WB	1:1000
TBK1	-	Rabbit monoclonal	CellSignaling	D1B4	WB	1:1000
MAVS	-	rabbit polyclonal	ENZO Life Science	ALX-210-929-C100	WB	1:1000
IRF3	-	Rabbit monoclonal	CellSignaling	D6I4C	WB	1:1000
VZV ORF9	-	rabbit polyclonal	REF. 432	-	WB	1:2000
Rabbit IgG	AlexaFluor 647	goat polyclonal	Invitrogen	A21246	IF	1:500

II.2 Methods

II.2.1 Cloning

II.2.1.1 Generation of VZV ORF Expression Library

Primers for polymerase chain reaction (PCR) amplification of individual VZV ORF sequences were designed based on an annotated VZV genome sequence (GenBank accession: AB09-7933.1). The forward primer included a Kozac sequence (GCCGCC) to be added before the start codon of the individual ORF. The reverse primer excludes the Stop codon to allow for addition of a C-terminal tag through the vector (see below). The PCR template was generated by extracting RNA from VZV infected MeWo cells using QIAshredder (Qiagen) and RNeasy Mini Kit (Qiagen). The RNA was reverse transcribed into cDNA using SuperScript II Reverse Transcriptase (Invitrogen). For some ORFs, DNA from infected MeWo cells extracted with DNeasy Blood and Tissue Kit (Qiagen) served as template. Amplicons were generated using Phusion High-Fidelity DNA Polymerase (New England Biolabs) or Herculase II Fusion DNA Polymerase (Agilent Technologies). PCR reactions were analysed by agarose gel electrophoresis and correct amplicons were extracted from the gel using QIAquick Gel Extraction Kit (Qiagen). Fragments were TOPO-ligated into pCR8/TOPO Gateway entry vector (Invitrogen). Plasmid DNA from single clones of transformed *E.coli* (New England Biolabs) was analysed for correct orientation of the insert by Sanger sequencing. Inserts were then shuttled into pLenti6.3/TO/V5-DEST (Invitrogen) using LR Clonase II Enzyme Mix (Invitrogen) for Gateway recombination. All clones with pLenti6.3/TO/V5 backbone were propagated in recombination-deficient Stbl3 bacteria (Invitrogen).

Amplification of the entire coding sequence of ORF22 (8256 bp) was unsuccessful using various amplification and cloning technologies. Therefore, two Gateway entry vectors encoding individual segments within the ORF22 coding sequence (see Table V.1) were obtained from Jurgen Haas (University of Edinburgh, UK), described previously⁴³⁴. Expression vectors with the pLenti6.3/TO/V5 backbone were generated as described above.

II.2.1.2 Generation of Gateway Destination Vectors

The pT-Rex-DEST31 plasmid (Invitrogen) is a Gateway destination vector that confers a N-terminal 6xHis tag to expressed proteins. I created pT-Rex-N-FLAG-DEST, a vector that

confers 3xFLAG instead of 6xHis. To exchange the 6xHis tag for a 3xFLAG tag the coding sequence for 3xFLAG was PCR amplified (Herculase II Fusion DNA Polymerase) from pcDNA3-3FLAG-hRIG-I¹⁶⁷ with BbsI and XhoI restriction site overhangs and gel purified. The pT-Rex-DEST31 vector and purified insert were digested with BbsI and XhoI restriction enzymes (New England Biolabs). The vector was additionally de-phosphorylated using Antarctic Phosphatase (New England Biolabs). All fragments were gel purified and ligated using Instant Sticky-end Ligase Master Mix (New England Biolabs). After transformation and DNA Mini preps (Qiagen) correct clones were identified by Sanger sequencing.

Next, I created pLenti/TO/N-FLAG-DEST, a version of pLenti6.3/TO/V5-DEST that expressed an N-terminal 3xFLAG tag instead of C-terminal V5. The entire Gateway recombination cassette including the N-terminal 3xFLAG tag was PCR amplified from pT-Rex-N-FLAG-DEST (see above) with SpeI and XbaI overhangs. PCR amplicon was gel purified and digested with SpeI and XbaI restriction enzymes (New England Biolabs). Vector pLenti6.3/TO/V5-DEST was similarly digested. Correctly ligated clones were created as described above.

The pLenti constructs express the blasticidin resistance gene for selection after lentiviral transduction. For experiments with THP1 cells I required a different antibiotic resistance given that these cells already carry the blasticidin resistance (see section II.1.1). To create pLenti/TO/N-FLAG/puro the hPGK-puromycin expression cassette was PCR amplified from the pLKO.1-puro vector (Sigma Aldrich) with Acc65I restriction sites. The blasticidin resistance cassette was exchanged for the amplified puromycin cassette using restriction enzyme cloning with Acc65I (New England Biolabs) as described above.

The pcDNA3.2/FLAG-DEST and pcDNA3.2/HA-DEST vectors were created from pcDNA3.2/V5-DEST (Invitrogen) for expression of proteins with a C-terminal FLAG-tag or HA-tag. Through restriction enzyme cloning using ApaI and AgeI (New England Biolabs) the V5-tag sequence was replaced by the 3xFLAG sequence, PCR-amplified from pcDNA3-3FLAG-hRIG-I. Correct clones were identified as described above. To create pcDNA3.2/HA-DEST, the entire Gateway recombination cassette was PCR-amplified from pcDNA3.2/V5 with modified primers that amplify an HA-tag sequence instead of V5 and provide restriction enzyme sites. The recombination cassette was inserted into pcDNA3.2 using EcoRI and AgeI restriction enzymes (New England Biolabs). Correct clones were identified as described above.

II.2.1.3 Generation of Further Expression Vectors

Coding sequences of the desired ORF was PCR-amplified from indicated cDNA or plasmids with Kozac sequence (GCCGCC). Generation of the pCR8 entry vector, gateway recombination and selection of expression clones was performed analogous to the VZV ORF expression library (see section II.2.1.1).

II.2.1.4 Generation of *E.coli* Expression Vectors

To create pGEX6P1-FLAG-ORF9 the FLAG-ORF9 cassette was PCR-amplified from pLenti-6.3/TO/N-FLAG/puro/ORF9 with BamHI restriction sites. The purified PCR amplicon was inserted into pGEX6P1 using restriction enzyme cloning with BamHI (New England Biolabs) as described above. The pGEX6P1-FLAG-eGFP plasmid was created in the same way. To generate pGEX6P1-hcGAS-HA, the coding sequence for human cGAS was shuttled from pCR8-hcGAS into pGEX6P1-DEST-HA using Gateway recombination as described above.

II.2.2 Generation of Immunostimulatory Nucleic Acids

II.2.2.1 IVT-RNA

Neo¹⁻⁹⁹ *in vitro* transcribed (IVT) RNA was generated as previously described¹⁶⁷. In brief, the first 99 nucleotides of the neomycin resistance gene were *in vitro* transcribed using T7 RNA polymerase (T7 MEGAscript kit, Ambion).

II.2.2.2 Infected-cell Total RNA

A549-ZIKV-RNA was generated by infection of A549 cells with ZIKV (isolate ZIKV/H.sapiens-/Brazil/PE243/2015, GenBank accession KX197192.1, described in²³⁷) at an MOI of 5. Unless indicated otherwise, cells were lysed in TRIzol (Invitrogen) 20 h later and total RNA was extracted according to manufacturer's instructions. Infection of A549 cells with ZIKV was performed by Dr. Claire Donald and Dr. Alain Kohl (University of Glasgow, UK) and frozen TRIzol samples were shipped to us. VERO-EMCV-RNA was generated as described previously¹⁸⁰. In brief, Vero cells were infected with EMCV at MOI 0.1 overnight and total RNA was extracted using TRIzol (Invitrogen). HEK293T-IAV-RNA was generated by infection of HEK293T cells with IAV (A/PR/8/34 (H1N1), kind gift from Alain Townsend, Oxford, UK) at an MOI of 5. Total RNA was extracted 24 h later using TRIzol according to manufacturer's instructions.

II.2.2.3 VACV-70mer and ISD dsDNA

Complimentary oligonucleotides (ssDNA) corresponding to the VACV-70mer sequence or immunostimulatory DNA (ISD)-45 (see section II.1.4.2) were obtained from Sigma Aldrich in water and mixed together. The solution was heated to 95°C for 5 min and then left at ambient temperature for ssDNA to anneal. VACV-70mer-Biotin was generated by addition of a TEG-Biotin moiety to the 3'-end of one oligonucleotide by the manufacturer.

II.2.3 HEK293p125 Reporter Assay

HEK293p125 parental, RIG-I KO, MDA5-KO, and MAVS-KO cells were seeded at 2.5×10^4 cells per well in 96-well plates. On the following day, cells were transfected with 50 ng per well A549-ZIKV-RNA (Figure 11C) or 25 ng HEK293T-IAV-RNA, Vero-EMCV-RNA, and A549-ZIKV-RNA (Figure 12D) using Lipofectamine 2000 (Invitrogen). Activation of the *IFN* β promoter was assessed one day later using ONE-Glo luciferase assay (Promega).

II.2.4 HEK293-FLAG-RIG-I Pulldowns

1.2×10^7 parental HEK293 or HEK293-FLAG-RIG-I cells were seeded in 15 cm cell culture dishes. The next day, parental cells were transfected with 24 μ g IVT-RNA and HEK293-FLAG-RIG-I cells were transfected with 24 μ g IVT-RNA, HEK293T-IAV-RNA, or A549-ZIKV-RNA (see section II.2.2). 16 h later, cells were lysed in IP buffer (20 mM TRIS-HCl pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, Protease Inhibitor Cocktail (CellSignaling), 0.05 U/mL RNasin Plus RNase inhibitor (Promega)). Protein concentrations in lysates were determined using Pierce BCA Protein Assay Kit (Thermo Scientific) and equalised by dilution of samples with lysis buffer. Lysate fractions containing 90 μ g and 35 μ g were removed as input fractions for RNA isolation and immunoblot analysis, respectively. The remaining lysates were incubated with 100 μ l α -FLAG Dynabeads suspension. Beads were prepared by coupling Dynabeads M-270 Epoxy (Invitrogen) to α -FLAG antibody (clone M2, Sigma-Aldrich) at 30 μ g antibody per mg beads according to manufacturer's instructions and beads were resuspended at 10 mg/ml. Lysate-bead suspensions were rotated at 4°C for 1 h. Beads were separated from supernatant and lysate fractions containing 90 μ g and 35 μ g were removed from supernatant as unbound samples. Beads were resuspended in IP buffer and split 10-90% for immunoblot and RNA extraction, respectively. For immunoblotting, 4x Laemmli sample buffer (200 mM TRIS-HCl, 8% SDS, 20% β -mercaptoethanol, 40% glycerol,

0.01% bromophenol blue, pH 6.8) was added to input, unbound and bound fractions and samples were incubated at 95°C for 10 min. Beads were removed and samples were subjected to SDS-PAGE and blotting as described in section II.2.20. TRIzol was added to input, unbound and bound fractions and RNA was extracted according to manufacturer's instructions. RNA was resuspended in 30 µL water. RNA concentrations were determined using Qubit RNA HS Assay Kit (Invitrogen). For luciferase assay, HEK293p125 reporter cells were seeded at 2.5×10^4 cells per well in 96-well plates, transfected with 20 ng of RNA samples per well using Lipofectamine 2000 and assayed the next day using ONE-Glo luciferase assay (Promega).

II.2.5 Recombinant RIG-I Pulldowns

1.25 µg recombinant FLAG-RIG-I protein¹⁶⁵ and 5 µg HEK293T-IAV-RNA or A549-ZIKV-RNA (see section II.2.2) were incubated under rotation at 4°C for 1 h in 250 µL IP lysis buffer (20 mM TRIS-HCl pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, Protease Inhibitor (Cell-Signaling), 0.05 U/mL RNasin Plus RNase inhibitor (Promega)). Where indicated, RIG-I protein was denatured by heating to 95°C for 10 min prior to mixing with RNA. As input samples, 5 µL and 40 µL were removed for Western Blot analysis and RNA extraction, respectively. Samples were rotated as previously with 4 µg α-FLAG antibody (clone M2, Sigma-Aldrich) or mIgG1 isotype control antibody (clone MOPC-31C, BD Pharmingen) for 2 h. Samples were added to 50 µL Protein G Dynabeads (Invitrogen) and rotated again for 2 h. Beads were separated from supernatant and 5 µL and 40 µL were removed from supernatant as unbound samples. Beads were washed three times in IP lysis buffer, resuspended in IP lysis buffer and split 10-90% for Western Blot and RNA extraction, respectively. For immunoblotting, 4x Laemmli sample buffer (200 mM TRIS-HCl, 8% SDS, 20% β-mercaptoethanol, 40% glycerol, 0.01% bromophenol blue, pH 6.8) was added to input, unbound and bound fractions and samples were incubated at 95°C for 10 min. Beads were removed and samples were subjected to SDS-PAGE and blotting as described section II.2.20. TRIzol was added to input, unbound and bound fractions and RNA was extracted according to manufacturer's instructions. RNA was resuspended in 20 µL water. For luciferase assay, HEK293p125 reporter cells were seeded at 2.5×10^4 cells per well in 96-well plates, transfected with 1 µL of RNA samples per well using Lipofectamine 2000 and assayed the next day using ONE-Glo luciferase assay (Promega). For RT-qPCR, 4 µL of RNA samples were reverse transcribed into

cDNA using SuperScript II Reverse Transcriptase (Invitrogen) and random hexamer primers (Qiagen) in a 20 μ L reaction. The 10 μ L qPCR reaction containing 1 μ L cDNA, EXPRESS SYBR GreenER qPCR Supermix (Invitrogen) and 10 μ M primers (see section II.1.4.2) was run on QuantStudio 7 Flex Real-Time PCR System (ThermoFischer) with the following cycle conditions: 10 min 95 °C, and 40 cycles of 15 s 95 °C followed by 1 min 60 °C, and melt curve analysis. Abundance was analysed with the Ct method and expressed as 2^{-Ct} .

II.2.6 VZV ORF Library Assays

II.2.6.1 VZV ORF Library Immunoblot

HEK293T were seeded at 5×10^5 cells per well in 12-well plates. The next day, cells were transfected with 600 ng VZV ORF or control expression plasmids using 3 μ L Lipofectamine 3000 (Invitrogen) per well. 24 h later cells were lysed in RIPA buffer (10 mM TRIS-HCl pH 8, 140 mM NaCl, 1 % Triton-X 100, 0.1 % SDS, 0.1 % sodium deoxycholate, 1 mM EDTA, 0.5 % EGTA). Lysates were clarified by centrifugation and 30 μ g protein was subjected to immunoblotting (see section II.2.20).

II.2.6.2 IFN Signalling ISRE Reporter Assay

HEK293 cells were seeded at 4×10^4 cells per well in 96-well plates. On the following day, cells were transfected with 20 ng ISG54-F-Luc plasmid, 5 ng pRL-TK, and 50 ng of VZV ORF expression plasmids using Lipofectamine 2000 (Invitrogen). 24 h later cells were treated with 25 U/ml recombinant hIFN β (R&D Systems). The next day activation of the luciferase constructs was assessed using Dual Luciferase assay system (Promega).

II.2.6.3 IVT-RNA IFN Reporter Assay

HEK293 cells were seeded at 4×10^4 cells per well in 96-well plates. On the following day, cells were transfected with 20 ng p125-F-Luc plasmid, 5 ng pRL-TK, and 50 ng of VZV ORF expression plasmids using Lipofectamine 2000 (Invitrogen). 24 h later media was exchanged and cells were transfected with 40 ng IVT-RNA using Lipofectamine 2000. The next day activation of the luciferase constructs was assessed using Dual Luciferase assay system (Promega).

II.2.6.4 cGAS/STING IFN Reporter Assay

HEK293T cells were seeded at 3.5×10^4 cells per well in 96-well plates. On the following day, cells were transfected with 20 ng p125-F-Luc plasmid, 5 ng pRL-TK, 1 ng hcGAS expression plasmid, 25 ng hSTING expression plasmid, and 50 ng of VZV ORF expression plasmids using Lipofectamine 2000 (Invitrogen). The next day activation of the luciferase constructs was assessed using Dual Luciferase assay system (Promega).

II.2.6.5 RIG-I-CARD IFN Reporter Assay

HEK293T cells were seeded at 3.5×10^4 cells per well in 96-well plates. On the following day, cells were transfected with 20 ng p125-F-Luc plasmid, 5 ng pRL-TK, 10 ng RIG-I-CARD expression plasmid, and 50 ng of VZV ORF expression plasmids using Lipofectamine 2000 (Invitrogen). The next day activation of the luciferase constructs was assessed using Dual Luciferase assay system (Promega).

II.2.7 Lentiviral Particles

II.2.7.1 Lentiviruses for Transduction

HEK293T cells were seeded in 15 cm cell culture dishes at 1.2×10^7 cells in growth medium. The next day cells were transfected with 9 μ g lentiviral expression plasmids harbouring the gene of interest, 9 μ g p8.91 plasmid, and 3 μ g pVSV-G using Lipofectamine 2000. 16 h later, medium was replaced with fresh growth medium. 24 h later, lentivirus containing supernatant was harvested, clarified by centrifugation, and stored at 4°C. Cells were overlaid with fresh growth medium. 8 h later supernatants were harvested again and pooled with previous supernatant. After 16 h, lentivirus containing supernatants were harvested for a third time. Pooled supernatants from three harvests were filtered through a 0.45 μ m filter, aliquoted into cryovials and stored at -80°C. For transduction of cells, Polybrene (Sigma Aldrich) was added to lentiviral supernatants to a final concentration of 8 μ g/ml. THP1 cells were pelleted and resuspended in lentiviral supernatant containing polybrene. The next day, cells were pelleted and resuspended in fresh growth medium. After overnight incubation, cells were once again pelleted and resuspended in growth medium containing 10 μ g/ml blasticidin or 1 μ g/ml puromycin (both Gibco) depending on which vector was used. In parallel, untransduced cells were also treated with antibiotics as control. Surviving cells were used for experiments. HaCaT cells were transduced using the same protocol adapted for adherent cells.

II.2.7.2 cGAMP-Loaded VLPs

Empty virus-like particles (VLPs) or VLPs which incorporated cGAMP were generated using a modified procedure based on a previously described method⁵⁰. HEK293T cells were seeded in 6-well plates at 1.2×10^6 cells. The next day cells were transfected with 2 μ g of plasmids pDEST31/VZV-ORF9 or pcDNA3.1/eYFP, 1 μ g pGag-eGFP (NIH AIDS reagent program), 500 ng pVSV-G, and 1 μ g of pcDNA3.2/hcGAS or pcDNA3.2/empty-vector. VLPs were harvested as described above for transduction lentiviruses.

II.2.8 IFN Bioassay

Type I IFN in cell culture supernatants was measured using 3C11 cells as described previously⁵⁰. 3C11 cells were seeded into 96-well plates at 4×10^4 cells per well in growth medium. The next day, supernatants to be measured were clarified by centrifugation and put onto 3C11 cells seeded before. A standard containing 2-fold dilutions of recombinant IFN-AD (Sigma-Aldrich) was carried along. After overnight incubation reporter activation in 3C11 cells was assessed by measuring luciferase activity using ONE-Glo luciferase assay (Promega).

II.2.9 THP1 Knockout Cells

II.2.9.1 KO Cell Generation *via* CRISPR/Cas

For generation of knockout cells using CRISPR/Cas9 technology the pX458-mRuby plasmid encoding the Cas9 protein, the sgRNA, and mRuby was used¹⁸⁴. For knockout of IRF3 a sgRNA targeting exon 3 of the IRF3 locus was designed using the MIT algorithm (crispr.mit.edu) and cloned into the px458 vector by Philip Hublitz (Genome Engineering Facility, Weatherall Institute of Molecular Medicine, UK). The plasmid to target MAVS was described before¹⁸⁴.

1×10^7 THP1 cells were transfected with 50 μ g of respective pX458 plasmids using Lipofectamine LTX (Invitrogen) and incubated overnight. The next day cells were stained with 10 μ g/ml DAPI in PBS for 10 min. After resuspension of cells in growth medium, live, mRuby-positive cells were FACS-sorted into eppendorf tubes containing growth medium with 20% FCS. Cells were dispensed at 1 or 3 cells per well into 96-well plates by limiting dilution in growth medium with 20% FCS. The cells were incubated for several weeks until clones grew out.

The absence of the targeted protein in each clone was assessed by immunoblot analysis. For functional validation, cells were seeded into 96-well plates at 1.5×10^5 cells per well in growth medium containing 10 ng/ml phorbol-12-myristat-13-acetat (PMA) (Sigma-Aldrich). The next day, cells were transfected with 5 ng IVT RNA¹⁶⁷ or 15 ng *E.coli* DNA (invivogen) using Lipofectamine 2000 (invitrogen). 24 h later IFN in supernatants was measured using the type I IFN bioassay (see section II.2.8). Knockout clones were further analysed for insertions and deletions in the genomic gene loci. MAVS and IRF3 loci were PCR-amplified using Herculase II Fusion DNA Polymerase. PCR amplicons were gel extracted (QIAquick Gel Extraction Kit) and sequenced by Sanger sequencing. Sequencing traces were analysed using the TIDE software (<https://tide.nki.nl>)⁴³⁵.

II.2.9.2 THP1 KO Panel Validation Immunoblot

5×10^5 THP1 WT, cGAS, STING, MyD88, and TBK1 KO cells, or 2×10^6 THP1 WT, IFNAR2, MAVS, and IRF3 KO cells were treated or not with 100 U/ml IFN-A/D (Sigma Aldrich) overnight. Cells were washed three times in PBS, lysed in RIPA buffer, and processed for immunoblotting (see section II.2.20).

II.2.9.3 THP1 KO Panel Validation RT-qPCR

THP1 WT and cGAS, STING, MyD88, TBK1, IFNAR2, MAVS, and IRF3 KO cells were seeded in 6-well plates at 6×10^6 cells per well in growth medium containing 10 ng/ml PMA (Sigma-Aldrich). The next day medium was changed to normal growth medium and cells were mock transfected or transfected with 500 ng VACV-70mer DNA or 200 ng IVT-RNA using Lipofectamine 2000 (Invitrogen). 24 h later cells were lysed in RLT buffer and processed for RT-qPCR (see section II.2.21).

II.2.10 VZV Infections

II.2.10.1 Flow cytometry Infection Assay

Cells were washed in FACS tubes with PBS and incubated with LIVE/DEAD Fixable Violet Dead Cell Stain (Invitrogen) diluted 1:1000 in PBS for 30 min at 4°C. For some experiments, viability staining step was omitted since CellTrace Violet was used as a tracer dye. Cells were washed with PBS and resuspended in 100 µl FACS buffer (1% FCS, 2 mM EDTA in PBS) containing 1:500 FITC- or PE-coupled antibody against VZV gE/gI complex. Cells

were incubated 30 min at 4°C. After washing with PBS cells were resuspended in PBS and an equal volume of 8% methanol-free formaldehyde in PBS was added. Cells were fixed for 15 min at room-temperature. Cells were washed with PBS, resuspended in FACS buffer, and analysed on Attune NxT Acoustic Focusing Cytometer (ThermoFisher).

II.2.10.2 Transwell VZV Infection System

For this assay 6-well inserts with 1 µm pore size were used (Sarsedt Catalogue number 83.3930.101). Importantly, both sides of the insert membrane are treated for cell adherence.

Inserts were placed upside-down in 15 cm cell culture dishes. Aliquots of cryopreserved MeWo cells (uninfected and VZV-infected) were thawed in growth medium. For infection conditions, uninfected and infected cells were mixed at ratios ranging from 2:1 to 4:1 (batch dependent) to yield a final concentration of 3×10^6 cells per ml. For uninfected controls, only uninfected MeWo cells were used. 500 µl of cell suspension were carefully pipetted onto up-facing membrane of inserts to form a liquid dome. Cells were let adhere overnight.

Wells of a 6-well plate were filled with 3 ml PBS. Using forceps, excess liquid was decanted from inserts. Inserts were placed into 6-well plates so the cells on the bottom-side of the membrane were submerged in PBS for washing. PBS was aspirated and replaced with PBS containing 1:1000 CellTrace Violet reagent (Invitrogen). Cells were incubated 20 min at 37°C. Inserts were transferred to 6-well plates filled with 3 ml growth medium and incubated at least 5 min at 37°C.

MeWo and THP1 cells were used as target cells. MeWo cell suspension was prepared at 1×10^6 cells per ml in growth medium. THP1 cell suspension was prepared at 3×10^6 cells per ml in growth medium containing 10 ng/ml PMA. For all cells 1 ml of cell suspension was added into the inserts for cells adhere to the top-side of the insert membrane. Cells were incubated for 48 h. Cells in the insert were washed with PBS and 200 µl trypsin-EDTA was added for detachment. 800 µl growth medium were added and cells were resuspended by pipetting. Cells were analysed by flow cytometry (see section II.2.10.1) and RT-qPCR (see section II.2.21).

II.2.10.3 THP1 VZV Infection

THP1 cells with respective genotypes were seeded in 6-well plates at 6.125×10^6 cells per well in growth medium containing 10 ng/ml PMA (Sigma-Aldrich). As a control, 1.625×10^6

MeWo cells were seeded in MEM. The next day, cells were washed with PBS and overlaid with PBS containing CellTrace Far Red dye (Invitrogen) diluted 1:5000. Cells were labelled for 15 min at 37°C, an excess of growth medium was added and cells were incubated for another 5 min. Meanwhile, aliquots of uninfected and VZV-infected MeWo cells were thawed, washed, and resuspended in MEM. Cells were counted and concentration was adjusted to 6.25×10^5 live cells per ml (for THP1 cells) or 1.25×10^5 live cells per ml (for MeWo cells) with MEM. Medium was removed from labelled cells and cells were overlaid with 2 ml MeWo -/+VZV cell suspension. Cells were incubated at 37°C for 1h. Cell suspension was removed, cells washed with PBS, overlaid with their respective growth medium and incubated for 48h.

Supernatants were removed from cells, clarified by centrifugation and stored at -80°C. IP-10 cytokine levels were determined using Human CXCL10/IP-10 Quantikine ELISA Kit (R&D Systems) according to manufacturer's instructions. Cells were washed with PBS and incubated with Trypsin-EDTA until they started to detach. Growth medium was added, cells were resuspended by gentle pipetting and transferred to FACS tubes on ice. 45% of cell suspension were transferred to eppendorf tubes on ice for extraction of RNA and generation of protein lysates, respectively. The cells in those eppendorf tubes were pelleted, washed with PBS, and then lysed either in RLT buffer and processed for RT-qPCR (see section II.2.21) or lysed in RIPA buffer and processed for immunoblot analysis (see section II.2.20). Remaining cells in FACS tubes were used for analysis by flow cytometry (see section II.2.10.1).

II.2.10.4 HaCaT Time-course VZV Infection

HaCaT cells were seeded in 6-well plates at 1.225×10^6 cells per well. The next day, cells were washed with PBS and overlaid with PBS containing CellTrace Far Red dye (Invitrogen) diluted 1:5000. Cells were labelled for 15min at 37°C, an excess of growth medium was added and cells were incubated for another 5 min. Meanwhile, aliquots of uninfected and VZV-infected MeWo cells were thawed, washed and resuspended in MEM. Cells were counted and concentration was adjusted to 6.25×10^5 live cells per ml with MEM. Medium was removed from labelled cells and cells were overlaid with 2 ml MeWo -/+VZV cell suspension. Cells were incubated at 37°C for 1 h. Cell suspension was removed, cells washed with PBS, overlaid with growth medium and incubated for 8 h, 16 h, 24 h, 32 h, 40 h, and 48 h. At harvest of each time point, supernatants were removed from cells, clarified by centrifugation and stored at -80°C. Cells were scraped into PBS and transferred to FACS tubes on ice.

45% of cell suspension were transferred to eppendorf tubes on ice for extraction of RNA and generation of protein lysates, respectively. The cells in those eppendorf tubes were pelleted, washed with PBS, and then lysed either in RLT buffer and stored at -80°C for later RT-qPCR processing (see section II.2.21) or lysed in RIPA buffer stored at -80°C for later immunoblot analysis (see section II.2.20). Remaining cells in FACS tubes were used for analysis by flow cytometry (see section II.2.10.1). Cells for flow cytometry analysis were stored at 4°C after fixation and all samples were acquired together after the last time point.

II.2.10.5 THP1 VZV Infection for Immunoprecipitation

THP1 cells were seeded at 3.5×10^7 cells per dish in 10 cm cell culture dishes in growth medium containing 10 ng/ml PMA. HaCaT cells were seeded at 4×10^6 per dish. The next day, cells were labelled with CellTrace FarRed and infected by co-culture with VZV- or VZV^{ORF9-V5}-infected MeWo cells as described in section II.2.10.3. 48 h later cells were scraped into PBS and washed twice with PBS. 1 % of cells were taken and processed for flow cytometry analysis using VZV-gE/gI-PE antibody (see section IV.1.1). The remaining cells were lysed in IP lysis buffer (20 mM TRIS-HCl pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, Protease Inhibitor (CellSignaling)) and clarified by centrifugation. Lysates were incubated with 50 μl Dynabeads Protein G suspension (Invitrogen) per sample for 30 min under rotation at 4°C for pre-clearing. Beads were removed and lysates were incubated with 50 μl Dynabeads Protein G that were coated in 5 μg anti-V5 antibody per sample for 1 h under rotation at 4°C . The supernatant was removed and beads were washed three times in lysis buffer. Input and bound fractions were subjected to immunoblotting as described in section II.2.20.

II.2.11 HEK293T ORF9 Pulldowns

1.4×10^7 HEK293T cells were seeded in 15 cm cell culture dishes. The next day, cells were transfected with 12.7 μg of ORF9-V5, cGAS-FLAG, and STING-HA expression plasmids using Lipofectamine 2000. 16 h later, cells were lysed in IP buffer (20 mM TRIS-HCl pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, Protease Inhibitor Cocktail (CellSignaling)). After clarification, an aliquot was removed as input sample and the lysate was split into four equal volumes. Each aliquot was incubated with 50 μl Dynabeads Protein G that were coated in 5 μg anti-V5, anti-FLAG, anti-HA, or control IgG antibody for 1 h under rotation at 4°C . The

supernatant was removed, an aliquot was stored as unbound fraction from each sample, and beads were washed three times in lysis buffer. Input, unbound, and bound fractions were subjected to immunoblotting as described in section II.2.20.

II.2.12 THP1 ORF9 DNA Stimulation

THP1 cells stably expressing ORF9-V5 or GFP-V5 (see section II.2.7.1) were seeded at 2×10^6 per well in 12-well plates in growth medium containing 10 ng/ml PMA. The next day, cells were mock transfected or transfected with 10 ng, 25 ng, 50 ng, 100 ng, or 200 ng *E.coli* (DNA Invivogen) using 3 μ l Lipofectamine 2000. 24 h later, cells were harvested in RLT buffer and processed for RT-qPCR (see section II.2.21).

II.2.13 THP1 ORF9 Pulldowns

THP1 cells stably expressing ORF9-V5 or GFP-V5 (see section II.2.7.1) were seeded at 2×10^7 per dish in 15 cm cell-culture dishes in growth medium containing 10 ng/ml PMA. 16 h later, cells were lysed in IP buffer (20 mM TRIS-HCl pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, Protease Inhibitor (CellSignaling)). After clarification, the lysate was incubated with 50 μ l Dynabeads Protein G suspension (Invitrogen) per sample for 30 min under rotation at 4°C for pre-clearing. Beads were removed, an aliquot was removed as input sample, and lysates were incubated with 50 μ l Dynabeads Protein G that were coated in 5 μ g anti-V5 antibody per sample for 1 h under rotation at 4°C. The supernatant was removed and beads were washed three times in lysis buffer. Input and bound fractions were subjected to immunoblotting as described in section II.2.20.

II.2.14 Recombinant Protein Pulldowns

For each reaction, 3 μ l recombinant cGAS-HA protein was mixed with 1.5 μ l FLAG-ORF9 or FLAG-GFP in IP lysis buffer (20 mM TRIS-HCl pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40). Solutions were incubated with 25 μ l Dynabeads Protein G that were coated in 2.5 μ g anti-HA or anti-FLAG antibody per sample for 1 h under rotation at 4°C. The supernatant was removed, an aliquot was stored as unbound fraction from each sample, and beads were washed three times in lysis buffer. Input, unbound, and bound fractions were subjected to immunoblotting as described in section II.2.20.

II.2.15 Biotin-DNA Pulldowns

3 μ l recombinant cGAS-HA protein, 1.5 μ l FLAG-ORF9 or FLAG-GFP protein, or combinations of these proteins were mixed with 250 pmol VACV-70mer-Biotin dsDNA or not in IP lysis buffer (20 mM TRIS-HCl pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40). Solutions were incubated with 10 μ l Dynabeads M-280 Streptavidin (Invitrogen) per sample for 30 min under rotation at 4°C. The supernatant was removed and beads were washed three times in lysis buffer. Input and bound fractions were subjected to immunoblotting as described in section II.2.20.

II.2.16 Far Western Technique

The experimental procedures for Far Western was based on a protocol described in REF. 436. 3 μ l recombinant cGAS-HA protein, 1.5 μ l FLAG-ORF9, and 1.5 μ l FLAG-GFP protein per lane were subjected to SDS-PAGE and blotting as described in section II.2.20. After transfer, the membrane was incubated for 30 min at RT temperature in AC buffer (6 M Guanidine HCl, 100 mM NaCl, 20 mM TRIS-HCl pH 7.6, 0.5 mM EDTA, 10 % glycerol, 0.1 % Tween-20, 2 % skim milk powder, 1 mM DTT). The membrane was then incubated in AC buffers with decreasing concentrations of Guanidine HCl (3 M, 1 M, 0.1 M) for 30 min each. For the least incubation the membrane was transferred to 4°C and thereafter incubated in AC buffer free of Guanidine HCl overnight. The membrane was blocked for 1 h in 5 % milk powder in PBST (PBS with 0.05 % Tween-20) and then incubated with recombinant proteins as probes in 3 % milk powder in PBST for 1 h. Per 5 ml buffer 5 μ l protein was used of FLAG-ORF9 and FLAG-GFP, and 10 μ l protein was used of cGAS. Membranes were washed for 10 min with PBST thrice and then incubated with antibodies to detect recombinant proteins (anti-cGAS or anti-FLAG) for 1 h. After washing, membranes were incubated with appropriate secondary antibodies, washed again, and imaged. Hereafter membranes were stripped and re-probed as described for conventional immunoblot (see section II.2.20).

II.2.17 Immunofluorescence

1.75x10⁵ HEK293T cells were seeded onto-glass coverslips. The next day, cells were transfected with 250 ng of cGAS-V5 or FLAG-ORF9 expression plasmid using 1.5 μ l Lipofectamine 3000 per well. Additional coverslips were co-transfected with both plasmids. 24 h later

cells were washed with PBS and fixed with 4 % formaldehyde in cytoskeleton stabilisation buffer (CSB; 10 mM KCl, 274 mM NaCl, 8 mM NaHCO₃, 0.8 mM KH₂PO₄, 2.2 mM Na₂HPO₄, 4 mM MgCl₂, 10 mM PIPES, 4 mM EGTA, 11 mM glucose) for 15 min. Cells were washed and permeabilised with 0.1 % Triton X-100 in CSB for 20 min and incubated with 100 mM glycine in CSB for 10 min afterwards. Cells were washed with PBS four times and blocked using 1 % BSA and 5 % normal goat serum in PBS for 1 h. Coverslips were incubated with primary antibodies in blocking solution for 3 h at room temperature. Cells were washed three times with PBS and incubated with secondary antibodies in blocking solution for 1 h. Coverslips were washed with PBS three times and mounted into glass slides using ProLong Diamond Antifade Mountant with DAPI (Invitrogen). Images were acquired using a Zeiss LSM780 confocal microscopy system (Zeiss).

II.2.18 Recombinant Protein Expression

BL21-pLysS-Rosetta *E.coli* (kind gift from Simon Davis, Oxford, UK) were transformed with bacterial expression vectors. A single colony or a glycerol stock from a single colony were used to inoculate a starter culture of 25 ml LB-medium containing 34 µg/ml Chloramphenicol and 100 µg/ml Carbenicillin. A large 400 ml LB culture was inoculated using the starter culture. OD was measured in intervals and recombinant protein expression was induced by adding 0.1 mM IPTG once OD reached 0.6. Bacteria were grown 3 h at 37°C. For expression of recombinant cGAS and cGAS-HA bacteria were grown to OD 0.8 and chilled to 18°C. Protein expression was induced by adding 0.4 mM IPTG and bacteria were incubated for 16 h at 18°C. For all constructs bacteria were pelleted after incubation at 6,000 g for 7 min and the pellet was resuspended in 12 ml lysis buffer (20 mM TRIS pH7.4, 500 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 0.5 % NP40, 1:100 Protease Inhibitor Cocktail (CellSignaling)). The suspension was sonicated on ice three times at 20 % amplitude with 15 sec ON and 30 sec OFF (Branson Sonifier). Insoluble material was removed by centrifugation for 15 min at 24,000 g. In the meantime, 150 µl Glutathione Sepharose beads (GE Healthcare) were prepared according to manufacturer's instructions. The beads were added to the clarified lysate and incubated under rotation for 4 h at 4°C. The beads were pelleted by centrifugation at 500 g and washed five times with lysis buffer. The beads were washed once in PreScission Cleavage buffer (50 mM TRIS pH 7.5, 150 mM, 1 mM freshly added DTT) and resuspended in 500 µl PreScission Cleavage buffer. After addition of 12 µl PreS-

cession Protease (GE Healthcare) suspension was incubated under rotation for 3 h at 4°C. The supernatant containing the recombinant protein was separated from beads and stored at -80°C. Aliquots from the various purification steps were analysed by SDS-PAGE and subsequent staining of the gel with EZBlue Gel Staining Reagent (Sigma Aldrich). Based on band intensities the protein concentrations were estimated to be ca. 1 µg/µl for FLAG-ORF9 and FLAG-GFP, and ca. 0.5 µg/µl for cGAS and cGAS-HA.

II.2.19 AP-MS analysis

HaCaT and THP1 cells were transduced with lentiviruses to generate stable cell lines expressing either FLAG-ORF9 or FLAG-GFP (see section II.2.7.1). For AP-MS experiments, two 15 cm cell-culture dishes of cells were seeded per replicate. For HaCaT cells, confluent dishes were split 1:2 the day before the experiment. For THP1 cells, 6×10^7 cells were seeded per dish in growth medium containing 10 ng/ml PMA. Analysis was performed with quadruplicate samples, i.e. eight dishes were seeded per condition. The day after seeding, ISD was transfected into half the dishes. 1.5 µg ISD was incubated with 4.5 µg PEI in OPTI-MEM medium per dish for 30 min at room temperature and added to cells. 16 h later, cells were scraped into PBS and washed with PBS twice. Cell pellets were flash frozen in liquid nitrogen. Pellets were thawed on ice and lysed in TAP lysis buffer (50 mM TRIS-HCl pH 7.5, 1.5 mM MgCl₂, 100 mM NaCl, 5 % glycerol, 0.2 % NP-40) supplemented with cOmplete EDTA-free Protease Inhibitor (Roche) for 30 min on ice. Samples were sonicated for five cycles of 30 sec ON 30 sec OFF on HIGH using a Bioruptor device (Diagenode). Lysates were clarified for 30 min at 15,000 g. Protein concentration was determined using 280 nm absorbance (Nanodrop, ThermoFisher) and normalised across samples. All samples were diluted 1:1 with lysis buffer free of NP-40. FLAG affinity resin (Sigma Aldrich) was incubated with the samples for 2 h at 4°C. Beads were washed four times 5 min each with TAP wash buffer (50 mM TRIS-HCl pH 7.5, 1.5 mM MgCl₂, 100 mM NaCl, 5 % glycerol, 0.1 % NP-40) and four times 5 min each with TAP wash buffer without NP-40. Pelleted beads were re-suspended in Guanidium chloride buffer (100 mM TRIS-HCl pH 8.0, 6 M guanidium chloride, 10 mM TCEP, 40 mM CAA). 500 ng Trypsin/Lys-C Mix (Promega) were added and incubated at 37°C overnight under rigorous shaking. 5x Stagetip loading buffer (10 % acetonitrile, 3 % trifluoroacetic acid) was added 1:4 and samples were stored at 4°C. Peptides were purified using the Stagetip protocol⁴³⁷. Pipette tips were loaded with C18-PTFE Empore

filter dish material (3M), washed once with methanol and twice with Buffer A (0.5 % acetic acid). Samples were applied to Stagetips and washed afterwards with Buffer A. Peptides were eluted with Buffer B (80 % acetonitrile, 0.5 % acetic acid). The peptides were dried at 45°C in a vacuum centrifuge and resuspended in Buffer C (2 % Acetonitrile, 0.1 % trifluoroacetic acid). Samples were analysed by LC-MS/MS on a LTQ-Orbitrap XL mass spectrometer (ThermoFischer) as previously described⁴³⁸. Raw data files were processed using MaxQuant⁴³⁹ version 1.2.6.10 and its Andromeda search engine⁴⁴⁰ to identify and quantify proteins from the human proteome by label-free quantification. The Perseus software⁴⁴¹ version 1.6.2.2 was used to analyse protein enrichment between samples. AP-MS data from HaCaT cells and THP1 cells were processed separately. Proteins that were identified in at least three out of four replicates in at least one condition per experiment were included in the analysis. Missing values were imputed using a normal distribution (width = 0.3, down shift = 1.8). Differences in measured protein intensities between GFP and ORF9 pulldowns were identified for each cell type and condition using Welch t-test with $S_0 = 2$ for THP1 and $S_0 = 0.5$ for HaCaT. Multiple testing was corrected using permutation-based FDR statistics (FDR = 0.01, 250 permutations). The proteins significantly enriched in ORF9 pulldowns from all cells and conditions were submitted to the STRING database⁴⁴². Interactions were detected based on experimental and database evidence using a minimum interaction score of 0.7 (high confidence). Analysis of the dataset by STRING also included gene set enrichment analysis using Gene Ontology (GO) biological process and GO molecular function annotations. This analysis is described in detail in REF 443. In brief, p-values are calculated using a hypergeometric statistical test and corrected for multiple testing using the Benjamini and Hochberg method. Cytoscape software was used to generate protein network visualisation⁴⁴⁴. AP-MS experiments were performed in the laboratory of Andreas Pichlmair (TU Munich, Germany). Virginie Girault kindly assisted with sample preparation and performed MaxQuant and most of the Perseus analysis.

II.2.20 Immunoblotting

Protein concentrations in lysates were determined using Pierce BCA Protein Assay Kit (Thermo Scientific) and equalised by dilution of samples with lysis buffer. Subsequently, 4x NuPAGE LDS Sample Buffer (Invitrogen) was added and samples were incubated at 95°C for 10 min. Samples were run on NuPAGE Novex 4-12% Bis-TRIS gels (Invitrogen) using

NuPage MOPS-SDS running buffer (Invitrogen). Proteins were subsequently blotted onto PROTRAN Pure nitrocellulose membrane (PerkinElmer) using transfer buffer (25 mM TRIS, 192 mM glycine). Membranes were blocked with 5% skim milk powder (Sigma-Aldrich) in TBS 0.1% Tween-20 (5% milk TBS-T) for 1 h at room temperature and were then incubated with primary antibodies in 5% skim milk TBS-T overnight at 4 °C. Primary antibodies that bind to phosphorylated residues were diluted in 5% BSA in TBS-T. Membranes were washed thrice with TBS-T and incubated with horseradish peroxidase (HRP)-coupled secondary antibodies in 5% skim milk TBS-T for 1 h at room temperature. After three further washes with TBS-T, proteins were detected using Western Lightning Plus-ECL (PerkinElmer) and iBright FL1000 Imaging System (ThermoFisher) or Amersham Hyperfilm MP (GE Healthcare). If needed, antibodies were stripped from the membrane with stripping buffer (200 mM glycine, 0.1% SDS, 1% Tween-20, pH 2.2) for 20 min at room temperature. Membranes were washed with TBS-T, blocked as previously and re-probed.

II.2.21 RT-qPCR

Cells were lysed in RLT Plus buffer and RNA was extracted using RNeasy Plus Mini Kit (Qiagen). Alternatively, total cell RNA was extracted using TRIzol reagent (Invitrogen). The RNA was reverse transcribed using SuperScript IV Reverse Transcriptase (Invitrogen) and oligo-dT primers (Invitrogen). The qPCR reaction containing TaqMan Universal PCR Master Mix (Applied Biosystems) and TaqMan Primer/Probes (see section II.1.4.3) was run on QuantStudio 7 Flex Real-Time PCR System (Thermo Fischer) with standard settings. Gene expression was analysed with the Ct method using *GAPDH* expression for normalisation.

II.2.22 Protein Sequence Analysis

The HSV-1 VP22 (PDB: 4XAL) and MHV68 ORF52 (PDB: 2OA5) crystal structures were used to graphically represent secondary structure features. For VZV ORF9 (UniProt accession Q4JQW6) and KSHV ORF52 (UniProt accession F5HBL8), structural features and sequence disorder was predicted by the PROFphd secondary structure prediction algorithm through submission of protein sequences to the predictprotein.org web interface⁴⁴⁵. The protein sequences of VZV pOka strain ORF9 (UniProt accession Q4JQW6), HSV-1 strain 17 VP22, (UniProt accession Q4JQW6), KSHV ORF52 (UniProt accession F5HBL8), and MHV68 ORF52 (UniProt accession P88989) were aligned using the Clustal Ω algorithm⁴⁴⁶.

The alignment including conserved amino acids and consensus motifs was visualised using the SnapGene software (GSL Biotech).

II.2.23 Data Analysis and Software

General data was analysed using Excel for Mac 16.28 (Microsoft) and GraphPad Prism 8 (GraphPad Software). SnapGene 4.3.8.1 (GSL Biotech) and ApE 2.0.49.10 (M. Wayne Davis, The University of Utah) was utilised to assist cloning. Statistical analysis as detailed in the figure legends was performed using GraphPad Prism 8. Graphs and figures were created using GraphPad Prism 8 and Adobe Illustrator CC 17.1.0 (Adobe Systems). Immunoblot images were processed using web-based iBright Image Analysis software (Thermo Fisher). Flow cytometry data was analysed using FlowJo 10.6.0 (FlowJo, LLC). Fiji 2.0.0 software was used to process confocal microscopy images⁴⁴⁷.

Chapter III

Zika Virus Innate Immune Recognition

ZIKV first started to emerge as an important human pathogen after an outbreak in Micronesia in 2007²⁸⁶. The virus then received widespread international attention by both public health professionals and researchers in light of further outbreaks in South America starting in 2015^{290;291}. However, given its recent emergence, scientific studies on ZIKV regarding molecular pathology and interaction with the human immune system were lacking. Generally, it is well known that the type I IFN system is critically required for the defence against viral pathogens. Before ZIKV started to attract attention of virologists and immunologists, extensive studies had focused on the importance of type I IFNs in the context of DENV infection, a related member of the flavivirus family.

During initial stages of infection with DENV, PBMCs isolated from patients show transcriptional signatures associated with activation of the type I IFN system^{448;449} and elevated serum levels of type IFN can be detected^{450;451}. Type I IFNs can restrict replication of DENV *in vitro*^{452;453} and mouse models of DENV infection have further established the critical importance of type IFNs in control of the virus⁴⁵⁴. Similarly, recent studies have begun to elucidate the role of type I IFNs in control of ZIKV. The relevance of IFN α and IFN β in the context of ZIKV is highlighted by the fact that only mice on an *Ifnar1*^{-/-} background or mice treated with an IFNAR1-blocking antibody are susceptible to lethal ZIKV infection^{273;298;455}. Furthermore, treatment of cells *in vitro* with type I IFN restricts ZIKV infection²⁹⁶.

Given the importance of type I IFNs, a key question is which PRRs trigger expression of IFN α and IFN β during ZIKV infection. For innate recognition of DENV, critical roles of TLR3 and the RLRs RIG-I and MDA5 had been suggested prior to the start of this project^{456;457}. However, the question which protein(s) cell-autonomously recognise infection with ZIKV remained unanswered. This led us to hypothesise a role for RLRs in recognition of ZIKV infection and test this experimentally.

Our findings regarding innate recognition and evasion of ZIKV have been published (REF. 184, full text attached in the Appendix). Some of the data is part of my MSc dissertation (REF. 458) and the DPhil thesis of Dr Antonio Gregorio Dias Junior, a former student in the group (REF. 459). In this Chapter I highlight results that, while being part of our publication, have neither been part of my dissertation nor Dr. Dias' thesis.

III.1 Infection with ZIKV Generates RLR-stimulatory RNA

To investigate whether infection with ZIKV triggers a type I IFN response *in vitro*, our collaborators Dr. Claire Donald and Dr. Alain Kohl (University of Glasgow, UK) infected A549 cells with ZIKV and lysed the cells in TRIzol at different time points after infection. After receiving frozen lysates and extraction of RNA, I utilised RT-qPCR to investigate whether ZIKV triggered a type I IFN response in these cells (Figure 11A). Indeed, starting 24 h after infection the IFN β transcript (*IFNB1*) and the transcripts of multiple ISGs were upregulated compared to mock infected cells (Figure 11B). No upregulation of IFN β could be observed at 4 h and 8 h post infection. Most of the ISG's expression was elevated marginally above mock levels at 4 h and 8 h post infection. These results showed that infection with ZIKV generated an immunostimulatory PAMP in A549 cells. The observation of virtually no stimulation at 4 h and 8 h after infection indicates that viral replication was required.

Given our knowledge about DENV, we hypothesised that the PAMP generated during infection was immunostimulatory RNA. The presence of RLR-stimulatory RNA can be tested by extraction of total RNA from infected cells and transfection of IFN β reporter cells (see schematic in Figure 11). This assay has been used to demonstrate the accumulation of stimulatory RNA during IAV and EMCV infection^{167;180}. Transfection of parental, RIG-I-KO, MDA5-KO, and MAVS-KO cells can further define which RLRs are stimulated¹⁷⁴. Transfection of RNA isolated from A549 cells at different time points after ZIKV infection (same as used above) resulted in a potent reporter induction for RNA from cells infected for 24 h or longer (Figure 11C). This confirms that 24 h after infection immunostimulatory RNA molecules accumulated in infected cells. As also demonstrated by the RT-qPCR results in Figure 11B, no stimulatory RNA was recovered at 4 h and 8 h after infection. MAVS-KO cells failed to respond to transfected RNA at all time-points, indicating that the stimulatory RNA molecules triggered type I IFN expression *via* RLRs. Indeed, the response to transfected RNA was strongly reduced in RIG-I-KO cells. MDA5-KO cells responded similar to parental

cells. Transfection of RNA from uninfected A549 cells did not stimulate reporter expression in this setting (data not shown, experiments performed by Dr Antonio Gregorio Dias Junior^{184;459}). This confirms that the presence of stimulatory RNA species in these cells was caused by ZIKV infection.

These results showed that ZIKV replication in A549 cells generated RIG-I-stimulatory RNA that potently triggered type I IFN expression. A residual response of RIG-I-KO cells to transfected RNA indicated that MDA5-stimulatory RNA was also generated during infection.

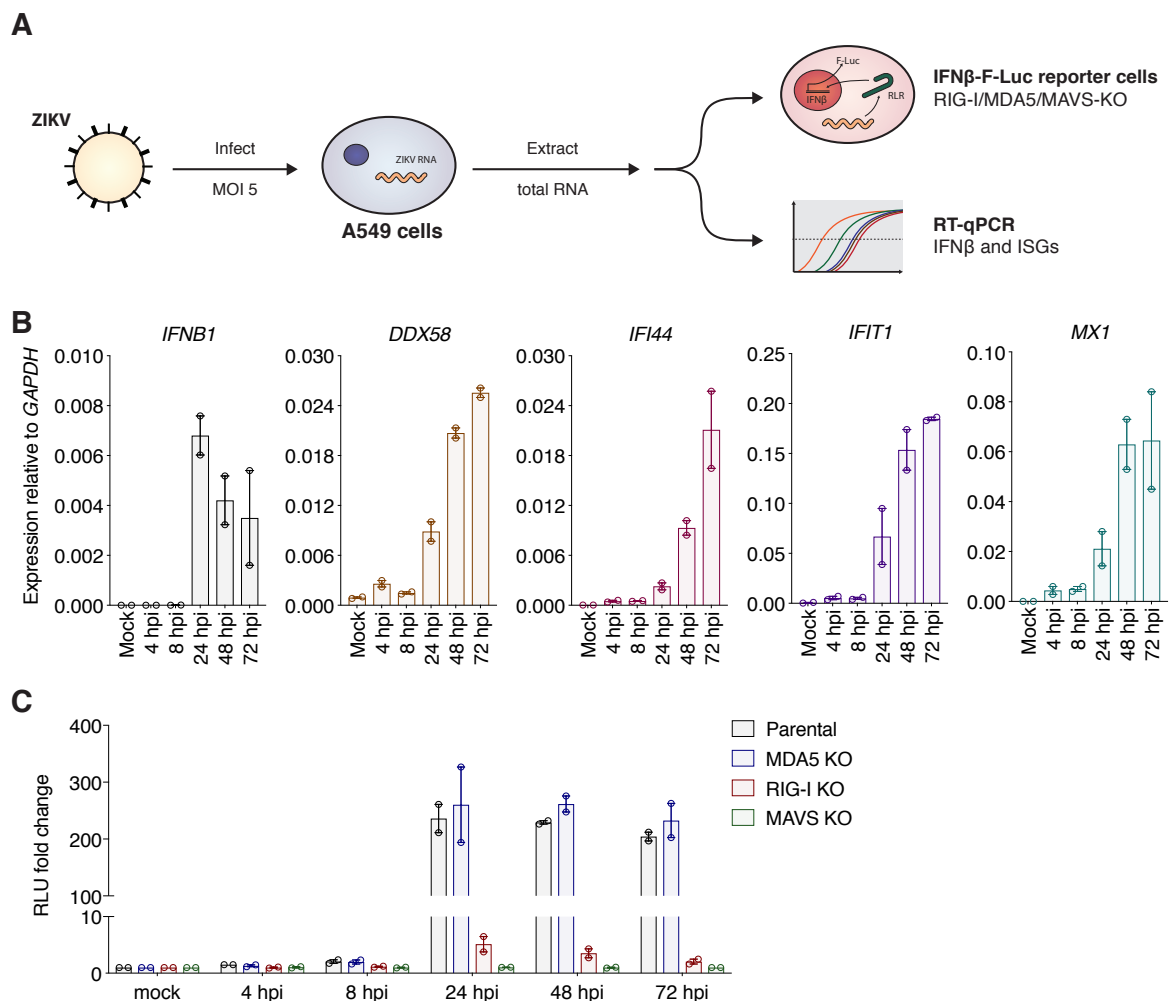


Figure 11: ZIKV infection generates RLR-stimulatory RNA in A549 cells. (A) Schematic detailing the experimental procedures for results shown in (A) and (B). (B) A549 cells were infected with ZIKV at MOI 5 for 4 h, 8 h, 24 h, 48 h, or 72 h. Cells were lysed in TRIzol and RNA was extracted. RT-qPCR was performed for the indicated genes. (C) RNA from A549 cells infected with ZIKV as in (A) was transfected into parental, MDA5-KO, RIG-I-KO, or MAVS-KO HEK293p125 IFNβ-F-Luc reporter cells. 24 h later luciferase activity was measured. RLU, relative light unit. Results shown in (B) and (C) are pooled data from two independent biological repeat experiments (average and SEM (n=2)). The RT-qPCR analysis for one of the repeats of (B) was performed by Dr Antonio Gregorio Dias Junior.

III.2 RIG-I Can Bind ZIKV RNA Directly

After establishing the presence of RIG-I-stimulatory RNA in ZIKV infected cells, we hypothesised that viral RNA can bind directly to RIG-I protein. An elegant way of testing this hypothesis would be RIG-I immunopurification from ZIKV infected cells and subsequent RNA isolation from the precipitated protein. Unfortunately, at that time, our experimental set up did not allow working with infectious ZIKV (see above, infection experiments performed by our collaborators). Instead, I aimed to immunoprecipitate ectopically expressed FLAG-RIG-I-protein from cells that had been transfected with A549-ZIKV-RNA. For this I utilised HEK293 cells that stably express FLAG-RIG-I. As a positive control, cells were transfected with HEK293T-IAV-RNA. It has been demonstrated previously that RIG-I stimulatory RNAs accumulate in IAV infected cells and that these RNAs can be recovered *via* immunoprecipitation of RIG-I protein¹⁶⁷. As a negative control, cells were transfected with Vero-EMCV-RNA, an RNA preparation that primarily stimulates MDA5¹⁸⁰. IP fractions were then analysed by immunoblotting. RNA in input and unbound fractions as well as RNA bound to precipitated RIG-I protein was purified using TRIzol. As previously, the RNA was then transfected into HEK293p125 reporter cells to test stimulatory potential (Figure 12A).

To confirm the anticipated stimulatory potential of the infected cell RNAs used here I first transfected them directly into parental, RIG-I-KO, and MDA5-KO HEK293p125 reporter cells (Figure 12B). The results demonstrate that A549-ZIKV-RNA stimulates primarily RIG-I, but clearly also contains MDA5 stimulatory RNAs (attenuated response in RIG-I KO cells). This confirms results for A549-ZIKV-RNA obtained in experiments described in section III.1 and Figure 11C. Importantly, similar observations can be made for HEK293T-IAV-RNA. These results show that both preparations contain RIG-I stimulatory RNAs. Of note, the same amount of HEK293T-IAV-RNA was more stimulatory than A549-ZIKV-RNA. Vero-EMCV-RNA behaved as expected since RIG-I-KO cells responded like parental cells and MDA5-KO cells failed to be stimulated.

The results of the immunoprecipitation experiments show that RIG-I protein could be enriched successfully from all cell lysates (Figure 12C). Stimulatory RNA could be recovered from HEK293T-IAV-RNA input samples but not from Vero-EMCV-RNA or A549-ZIKV-RNA samples (Figure 12 D). Given their lower stimulatory potential (see above), these transfected RNAs were most likely degraded intracellularly due to the 16 h incubation before lysis. As

expected, RIG-I-stimulatory RNA was depleted in unbound and enriched in bound fractions after IP from HEK293T-IAV-RNA transfected cells (Figure 12D). No stimulatory RNA was present in bound fractions from Vero-EMCV-RNA transfected cells. No bound stimulatory RNA could be recovered from RIG-I protein purified from A549-ZIKV-RNA transfected cells either. Given that direct transfection of A549-ZIKV-RNA into reporter cells was less stimulatory compared to HEK293T-IAV-RNA (Figure 12B), I concluded that this assay was not sensitive enough to demonstrate binding of stimulatory RNA after A549-ZIKV-RNA transfection.

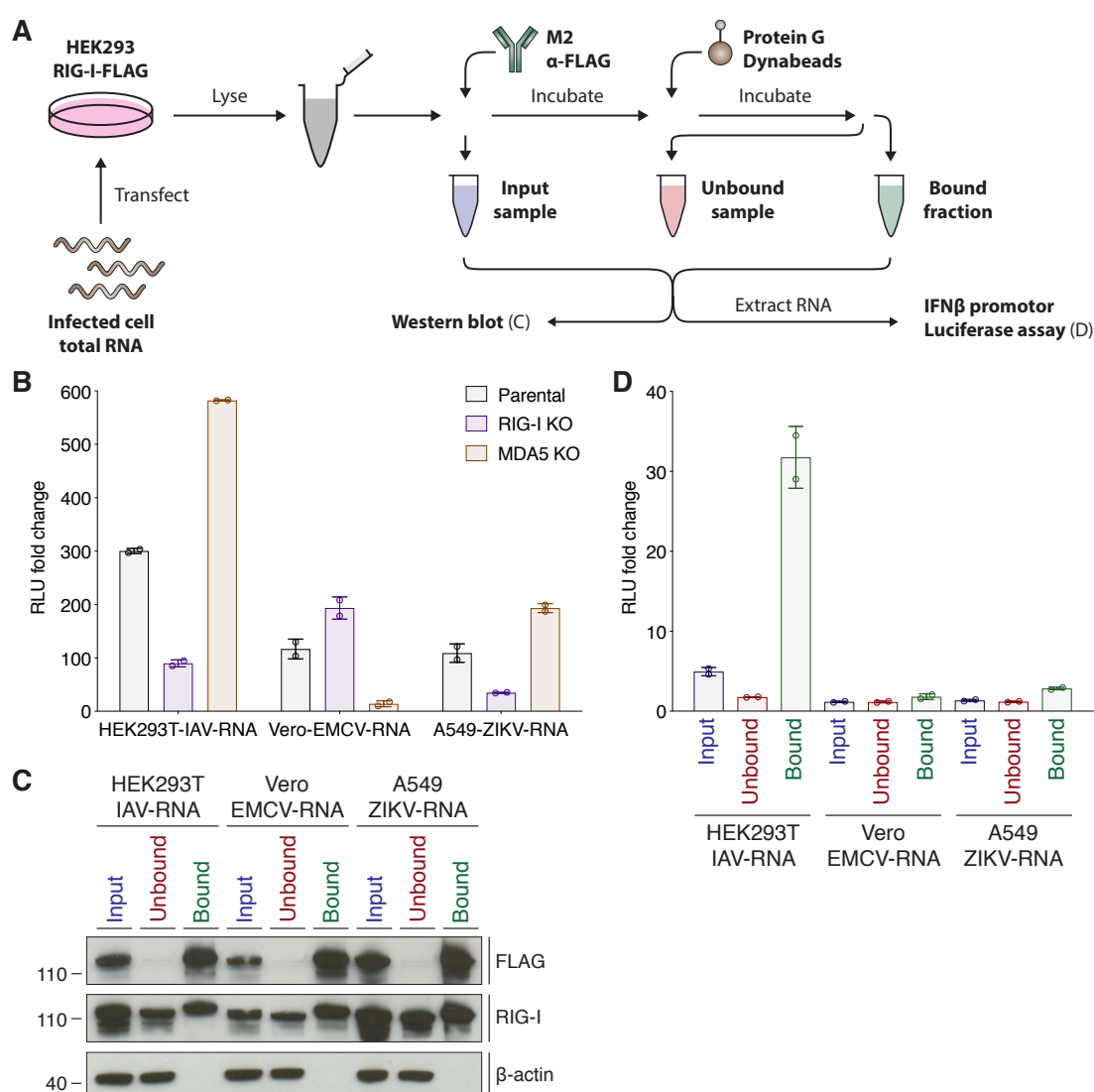


Figure 12: Immunoprecipitation of ectopically expressed RIG-I-FLAG fails to recover stimulatory RNA after transfection of A549-ZIKV-RNA. (A) Schematic of the experimental setup. (B) Parental, RIG-I-KO, and MDA5-KO HEK293p125 reporter cells were transfected with HEK293T-IAV-RNA, Vero-EMCV-RNA, and A549-ZIKV-RNA. The next day luciferase activity was determined and normalised to control samples treated with transfection reagent alone. RLU, relative light unit. (C) RNAs extracted from input, unbound and bound samples were transfected into HEK293p125 cells. After 16 h, luciferase activity was determined and normalised to control samples treated with transfection reagent alone. RLU, relative light unit. (D) Input, unbound and bound samples were analysed by immunoblotting using α-FLAG antibody. Panels (B), (C) and (D) are representative of two independent experiments. Panel (B), and (D) show technical duplicates (average and SD (n=2)).

Due to the limitations of using FLAG-RIG-I-HEK293 cells, I took a more reductionist approach. I investigated RNA bound to recombinant FLAG-RIG-I protein mixed with infected cell RNA in the test tube. A549-ZIKV-RNA or HEK293T-IAV-RNA was incubated with FLAG-RIG-I-protein, which was then immunoprecipitated, and IP fractions were analysed as previously (Figure 13 A). Additionally, I utilised RT-qPCR to determine if RIG-I-bound RNA corresponded to the viral genomes. As a negative control I included RIG-I protein that had been denatured by incubation at 95°C to abrogate RNA binding.

The recombinant protein was successfully immunoprecipitated in all conditions (Figure 13 B). No protein was recovered from control samples without recombinant protein and samples immunoprecipitated with IgG isotype control antibody. IP efficiency was higher if the RIG-I protein was denatured, probably due to better accessibility of the FLAG epitope.

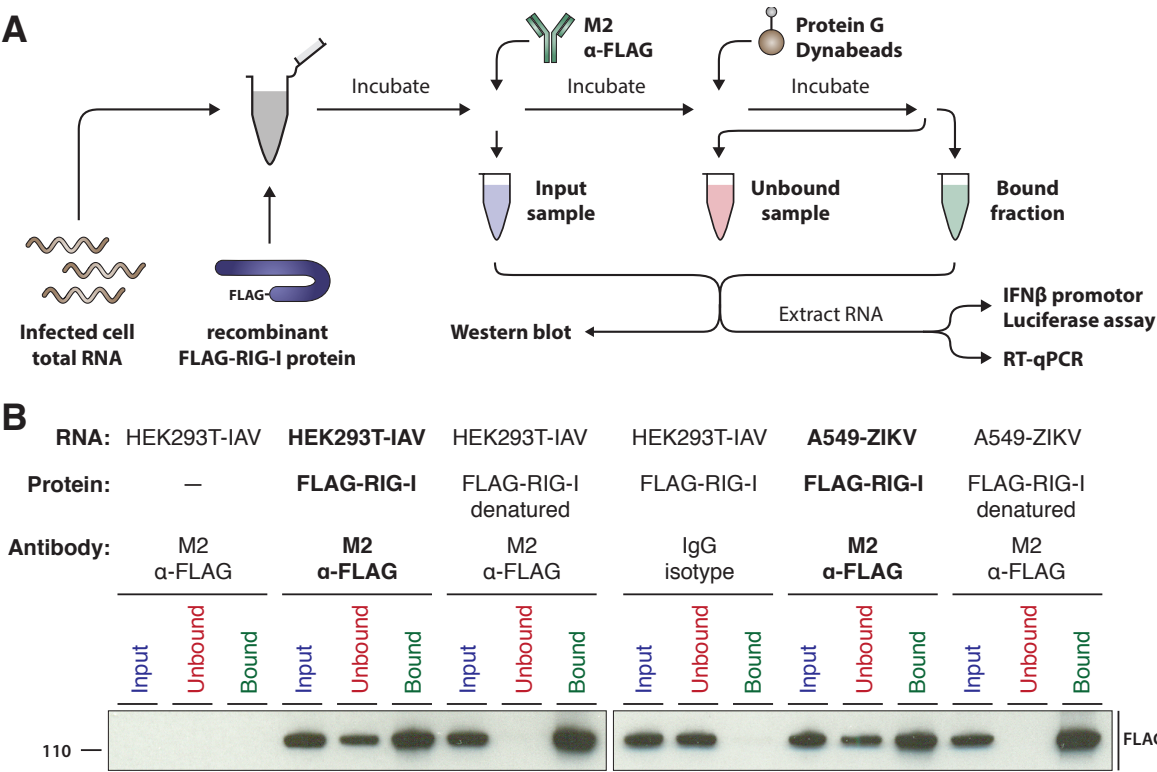


Figure 13: An *in vitro* assay to recover RIG-I-bound RNA. (A) Schematic of the experimental setup. HEK293T-IAV-RNA and A549-ZIKV-RNA were used. **(B)** Input, unbound and bound samples were analysed by immunoblotting using α-FLAG antibody. Panel (B) is representative of two (controls) or three (IAV and ZIKV samples) independent experiments.

Stimulatory RNA could be recovered from input samples in all experimental conditions (Figure 14 A). In control samples (no protein, isotype antibody IP, or denatured protein) RNA recovered from unbound fractions gave results similar to input fraction RNA. No stimulatory RNA was recovered from control sample bound fractions. In contrast, stimulatory RNA was

depleted in unbound fractions and enriched in bound fractions from both samples incubated with HEK293T-IAV-RNA and A549-ZIKV-RNA. This RNA specifically activated RIG-I in the reporter assay since HEK293p125 RIG-I-KO cells failed to respond. As expected, RT-qPCR analysis showed that in HEK293T-IAV-RNA samples RIG-I bound RNA is enriched for RNA species that correspond to IAV RNA (M protein RNA) (Figure 14 B). Similarly, in A549-ZIKV-RNA samples, RIG-I-bound RNA was enriched for RNA corresponding to nucleotides 4930-5029, a 100 nt sequence in the middle of the ZIKV genome. In control samples RNAs corresponding to the respective viral RNA sequences could only be detected in input and unbound but not the bound fractions.

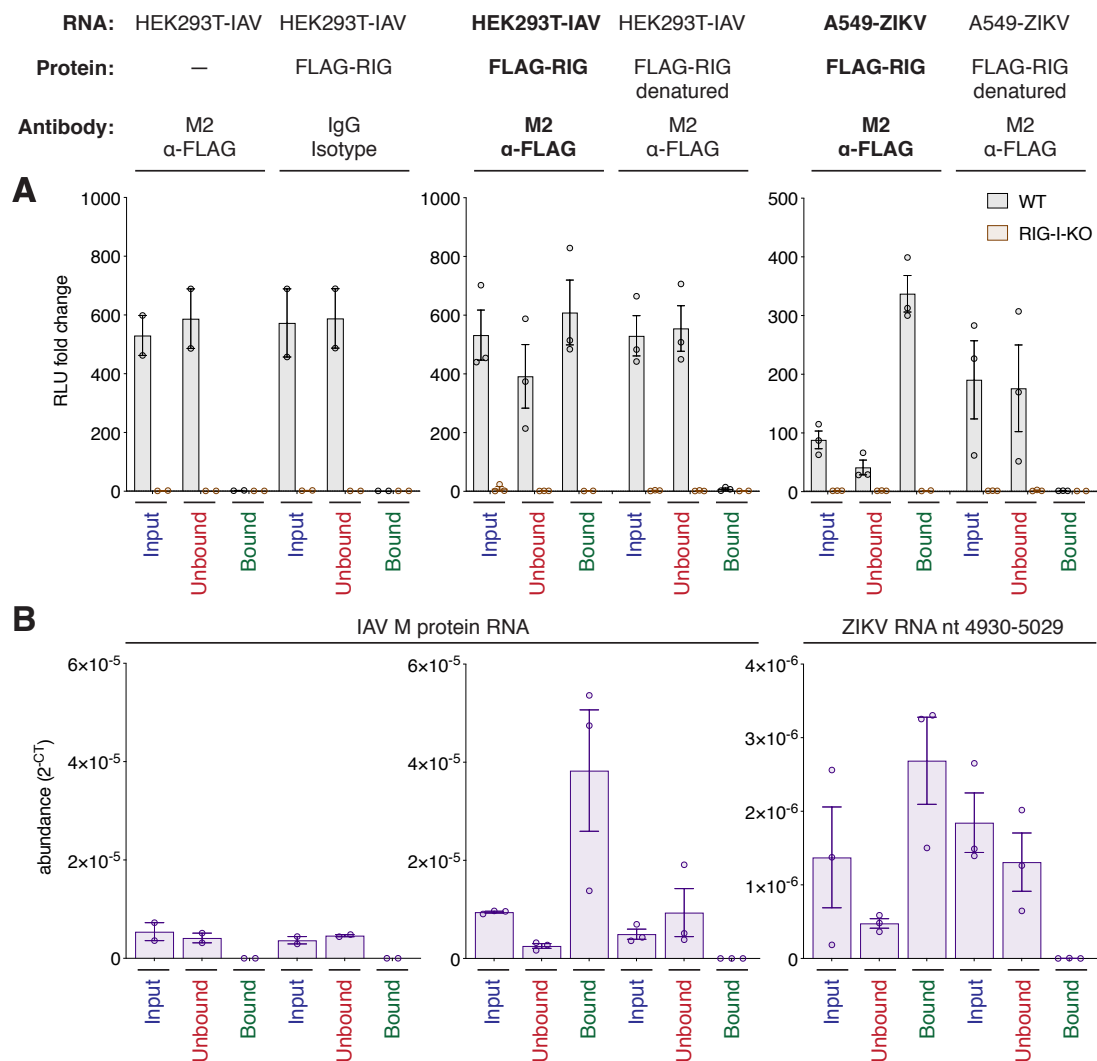


Figure 14: Recombinant RIG-I binds ZIKA RNA. (A) RNAs extracted from input, unbound and bound samples were transfected into parental and RIG-I-KO HEK293p125 cells. After 16 h, luciferase activity was determined and normalised to control samples treated with transfection reagent alone. RLU, relative light unit. (B) RNA samples from (A) were analysed in technical duplicate by RT-qPCR using the indicated probes. Relative viral transcript abundance was analysed by converting Ct data to 2^{-Ct} values. Panels (A) and (B) show pooled data from two (controls) or three (IAV and ZIKV samples) independent experiments (average and SEM (n=2/3)).

The results described in this section demonstrate that during ZIKV infection RNA species are generated that can be recognised by RIG-I *in vitro*. I further showed that some of those RNAs correspond to the viral genome. Re-transfection of bound RNAs stimulated RIG-I specifically indicating that these RNAs have the correct biochemical properties to activate RIG-I downstream signalling upon binding. 5'-triphosphates (5'-PPP) on RNA molecules is the molecular signature that activates RIG-I in cells¹⁶⁷. Indeed, further experiments performed by Dr Gregorio Antonio Dias Junior as part of this project support the hypothesis^{184;459}. Treatment of A549-ZIKV-RNA with alkaline phosphatase to remove 5'-PPP abolished its potential to stimulate RIG-I. MDA5 stimulation was unaffected. We can therefore conclude that during ZIKV infection, viral RNAs with 5'-PPP moieties are generated and that these RNAs activate RIG-I. Whether these RNAs are the viral genomic RNA, replication intermediates, or potentially sfRNA remains to be shown (see Discussion below).

III.3 Discussion

My findings are corroborated by results from other groups regarding the induction of an antiviral state *via* type I IFNs in response to ZIKV. Multiple studies have demonstrated that ZIKV infection induces the expression of type I IFNs in various cell lines, including results similar to ours for A549 cells^{320;460}. Similar findings were also obtained in different primary cell types^{250;251;319;461}.

I further established the presence of RIG-I-stimulatory RNA in infected cells and the ability of recombinant RIG-I protein to bind virus-derived RNA species. A549 cells clearly responded cell-intrinsically to ZIKV infection, indicating that viral RNA directly activates RIG-I in cells. However, my cell-free experimental approach did not formally show this. The use of total RNA from infected cells and recombinant RIG-I protein has the caveat that it excludes any possible compartmentalisation of stimulatory RNAs and/or RIG-I in infected cells.

Flaviviruses are well known to extensively remodel the cellular organelle architecture to form viral replication factories (reviewed in REF. 462). These dedicated replication sites do not only serve to spatially control the viral life cycle but are also believed to shield viral RNAs from recognition and degradation. For DENV, extensive ultrastructural evidence has been provided which clearly demonstrates the presence of ER-bound vesicles serving as replication sites²⁵⁵. A similar phenomenon has been demonstrated to occur in ZIKV infected cells^{251;256;257}. Interestingly, the study on DENV also showed that these vesicles contain

pores for the exchange of molecules required for viral replication (e.g. nucleotides). It is conceivable that viral RNA species gain access to the cytosol via these pores and activate RIG-I. Alternatively, RIG-I could shuttle into viral replication factories.

The question of whether RIG-I actually gains access to and binds viral RNA in ZIKV infected cells remains open. Due to experimental limitations I was unable to characterise RIG-I-bound RNAs in infected cells. However, another group subsequently demonstrated the presence of viral RNA bound to RIG-I protein purified from infected cells²⁹⁵. Interestingly, this study reports enrichment of ZIKV RNA that corresponds to the 5'-end of the viral genome. This is in line with RIG-I recognising the phosphorylated 5'-end of RNA. These findings further strengthen the evidence that activation of RIG-I by viral RNA plays a critical role for type I IFN induction and control of the virus. However, flaviviruses generate a methylated cap on the 5'-end of their genome to prevent RIG-I activation. This poses the question why viral RNA can be recovered from RIG-I given that a 5'-cap would prevent binding. The study mentioned previously sequenced RIG-I-bound RNAs in the context of DENV infection. Their results indicate that RIG-I senses nascent RNA molecules being generated by the viral polymerase which are not yet capped. To date, the RNA species bound to RIG-I in the context of ZIKV infection have not been fully sequenced. This would be important in order to shed light on the question what type of virus-derived RNAs are recognised by RIG-I during ZIKV infection.

During flavivirus replication multiple different RNA species are generated (reviewed for ZIKV in REF. 463). The incoming virion contains the positive-strand RNA genome which is capped at its 5'-end. The viral polymerase generates negative-strand viral RNA as an intermediate for generation of novel positive-strand RNA to be incorporated into new virions. In addition, sfRNAs are generated during flavivirus infection. These RNAs are derived from viral genomic RNA that has been incompletely degraded by cellular nucleases. Flavivirus genomic RNA contains 5'- and 3'-untranslated regions (UTRs) which form extensive secondary structures. Cellular nucleases are stalled at these structures and give rise to sfRNAs. Multiple roles for both DENV and ZIKV sfRNAs in viral evasion of innate defence mechanisms have been demonstrated^{237;464;465}. In contrast, whether sfRNAs can serve as PAMPs and contribute to sensing of flaviviruses remains to be investigated. Total RNA extracted from ZIKV infected cells is likely to contain sfRNA. ZIKV sfRNAs are mostly derived from the 3'-UTR therefore lacking possible 5'-PPP moieties to activate RIG-I. However, 5'-UTR-

derived sfRNAs exist and it is theoretically possible that these RNA species contributed to reporter activation in my experiments. It would be interesting to investigate the contribution of different RNA species to the total RNA stimulatory potential in future experiments.

While the importance of type I IFNs in protection against ZIKV infection by now is well established, the role of type III IFNs (IFN λ) is underexplored. Type III IFNs can protect primary human trophoblasts from ZIKV infection²⁴⁸, highlighting their potentially critical role during ZIKV infection *in vivo* (vertical placental transmission). IFN λ stimulates gene expression patterns similar to type I IFNs but their expression is tissue restricted. So far, one study has found evidence for limited type III IFN activation in monocyte-derived DCs after ZIKV infection⁴⁶⁶. RIG-I activation can lead to transcription and secretion of IFN λ in the context of viral infection⁴⁶⁷. Given my findings it is conceivable that IFN λ transcription is a result of ZIKV-mediated RIG-I activation. Whether cells actually secrete type III IFNs in response to ZIKV infection and their relevance *in vivo* are important questions for future investigations.

The results and discussion above highlight the relevance of RIG-I as a major sensor of flavivirus infection in my experimental setup. Nevertheless, other cellular sensing mechanisms can contribute to a type I IFN response in different contexts. For example, it has been demonstrated that both DENV and ZIKV infections lead to mitochondrial DNA release into the cytoplasm and a subsequent type I IFN response via cGAS/STING^{75;76;324}. In turn, the viral NS2B protein targets cGAS for degradation⁷⁵ and the viral protease NS2B-NS3 cleaves STING³²². A similar cleavage of STING by ZIKV has been demonstrated³²³. Zika virus further antagonises cGAS through a mechanism distinct from DENV³²⁴. The engagement of different cellular sensing systems and their evasion by pathogens highlight the evolutionary pressure exerted by host and pathogen on each other. Cells have evolved a plethora of sensing and restriction pathways as a response to countless evasion mechanisms developed by pathogens and *vice versa*.

Taken together, in this Chapter I presented my findings regarding sensing of ZIKV, an RNA virus. In the next chapters I will highlight my work on innate sensing of VZV, a DNA virus with an entirely different molecular architecture and life cycle.

Chapter IV

Innate Sensing of Varicella-Zoster Virus

In the previous chapter I have detailed my findings on innate sensing of ZIKV, an RNA virus. The remaining time as a DPhil student I spent investigating the interaction of Varicella-Zoster virus (VZV) with the innate immune system.

Even though VZV is an important human pathogen (see Introduction), little is known about the role of the type I IFN system in restriction of the virus. In particular, the cellular proteins that recognise VZV infection are not well characterised. Experimental research with VZV is hampered by cell-association of the virus *in vitro* (see below) and the scarcity of animal models⁴⁶⁸. A close relative of VZV, the α -herpesvirus Herpes-Simplex virus 1 (HSV-1), is much better characterised.

Type I IFN induction after HSV-1 infection has been shown to rely on multiple different PRRs, depending on the model system and the cell type. In mice, DCs can induce IFN expression through recognition of exogenous viral DNA via TLR9^{469–471}. However, animals that lack TLR9 are only mildly compromised in their ability to control the virus. In contrast, mice lacking cGAS or STING are unable to restrict HSV-1 infection^{16;56–59}. In humans, clinical genetics have shown a non-redundant role of TLR3 in protection from HSV-1 CNS infections⁴⁷². *In vitro*, cGAS is required in human cells for activation of IRF3 in response to HSV-1 infection^{473;474}. Lastly, multiple HSV-1 proteins were shown to antagonise cGAS and STING activation^{128;129;475;476}. The above summarised data demonstrate an important role of the cGAS/STING DNA-sensing pathway for the response to HSV-1.

Based on this knowledge about recognition of HSV-1, I hypothesised cGAS and STING to be required for sensing of VZV.

IV.1 VZV Infection in Cell Culture

MeWo cells, a melanoma cell line, are commonly used for propagation of VZV in cell culture⁴⁷⁷. While the virus replicates efficiently in these cells, no cell-free virus is produced. Rather, the virus propagates by inducing fusion of infected cells with adjacent, uninfected cells. Syncytia formation can be observed in an infected cell population by microscopy (yellow arrow in Figure 15). Over the course of the infection syncytia increase in size and formation of characteristic round, dome-like structures can be witnessed (orange and red arrows in Figure 15). The nuclei of fused cells are assembled as a ring surrounding a core of denser material. While these structures are quite striking to observe, the reason why this happens and the relevance for viral replication are unknown. Eventually, viral replication will destroy the cells and a cytopathic effect can be observed. Maintenance of VZV-infected cell cultures relies on continuous re-seeding of infected cells in excess of fresh, uninfected cells.

The fluid within chickenpox and shingles blisters is extremely infectious and contains high titres of virus^{326;478;479}. This highlights that the virus *per se* has the ability to exist as a cell-free, infectious particle. Very limited evidence has been provided regarding the molecular fundamentals of VZV cell-association *in vitro*. Cell-free virus preparations can be generated experimentally by mechanical disruption of infected cells. However, cell-free virus is unstable and virus preparations are of low titre. While more advanced protocols for higher-titre virus preparations have been developed^{480–484}, experimental work with cell-free virus remains largely impractical. Infection of cells *in vitro* is therefore commonly achieved by co-culture of target cells with infected MeWo cells⁴⁷⁷. In this setting, infection is propagated through cell-cell fusion or transfer of virions *via* neighbouring membranes. In order to know whether target cells actually became infected I needed to assess the number of infected cells. For this purpose I established a method to quantify infected cells using flow cytometry.

IV.1.1 VZV Flow Cytometry Infection Assay

Infected cells display a heterodimer of the viral glycoproteins gE and gI on their surface⁴⁸⁵. Cells in which the virus is replicating can consequently be identified by cell-surface staining for gE/gI. Initially, I used a commercially available diagnostic reagent (VZV DFA reagent, Millipore). The reagent is a solution of two FITC-coupled antibodies against VZV gE/gI and VZV ORF62. It is designed for diagnostic detection of VZV in skin biopsies through direct immun-

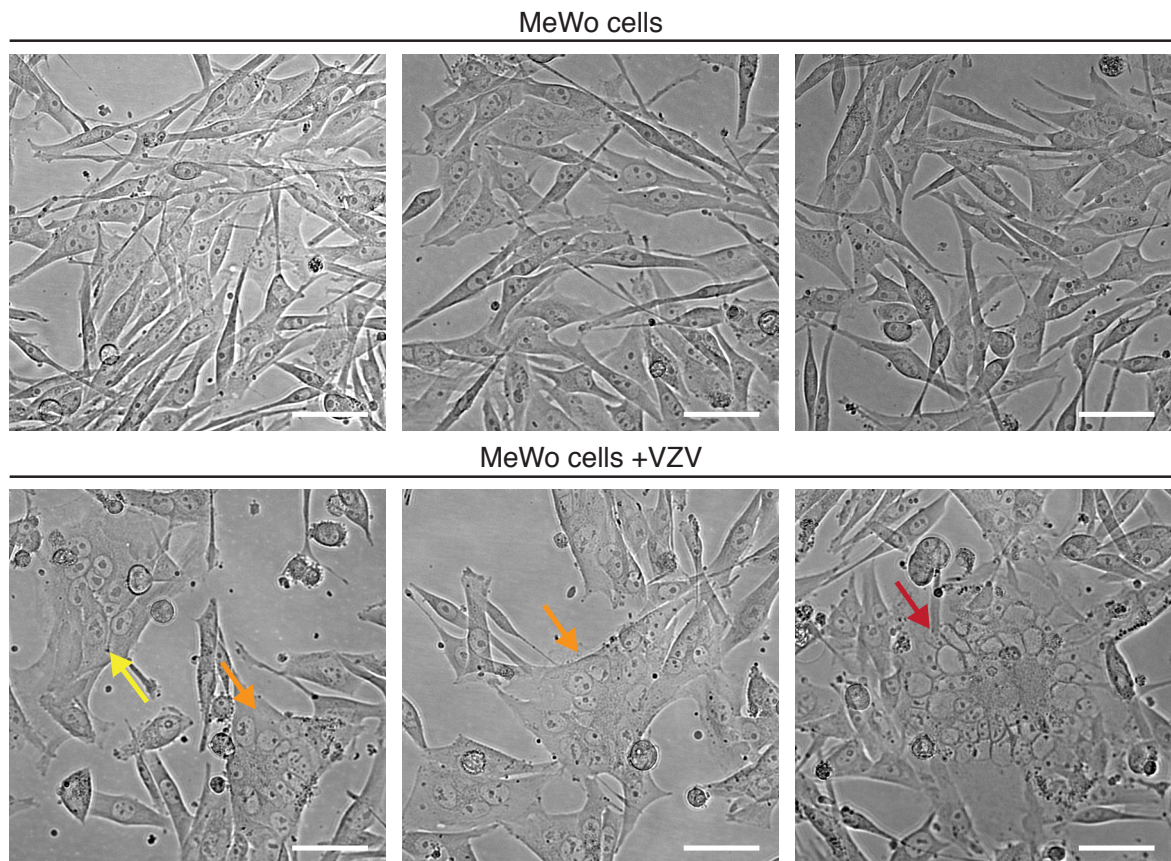


Figure 15: VZV leads to syncytia formation *in vitro*. Pictures of uninfected MeWo cells or VZV-propagating MeWo cells were acquired by brightfield microscopy after 24 h of co-culture. In pictures of infected cells arrows indicate early (yellow), intermediate (orange), or late (red) syncytia formation. Medium and late syncytia show a characteristic ring of nuclei. Scale bar: 50 μ m.

ofluorescence. Staining of uninfected or VZV-infected MeWo cells for flow cytometry gave a FITC-positive signal that could only be observed in infected cells (Figure 16A). Unspecific binding of the antibody was minimal since the fluorescence signal observed in uninfected cells was only marginally higher than the unstained control (Figure 16B). Unfortunately, the DFA reagent also contained Evans blue as a counterstain for immunofluorescence. This dye is cell-permeable and hence leads to a red fluorescence signal in flow cytometry. It was therefore impossible to use this reagent to stain VZV-infected cells in combination with other dyes.

The monoclonal antibody against gE/gI in the DFA reagent is commercially available. I conjugated the purified antibody to FITC using a coupling kit (the antibody is hereafter referred to as VZV-gE/gI-FITC). Testing of the antibody in flow cytometry revealed staining with higher fluorescence compared to the DFA reagent at dilutions 1:100 and 1:200 (Figure 16C). The background staining of a mIgG2b-FITC isotype control antibody was similar to that of the unstained sample in Figure 16B. I tested a higher antibody dilution (1:1000) and

could achieve the same staining with DFA reagent and VZV-gE/gI-FITC (Figure 16D). Based on these experiments I used the VZV-gE/gI-FITC antibody for identification of infected cells *via* flow cytometry for all future experiments.

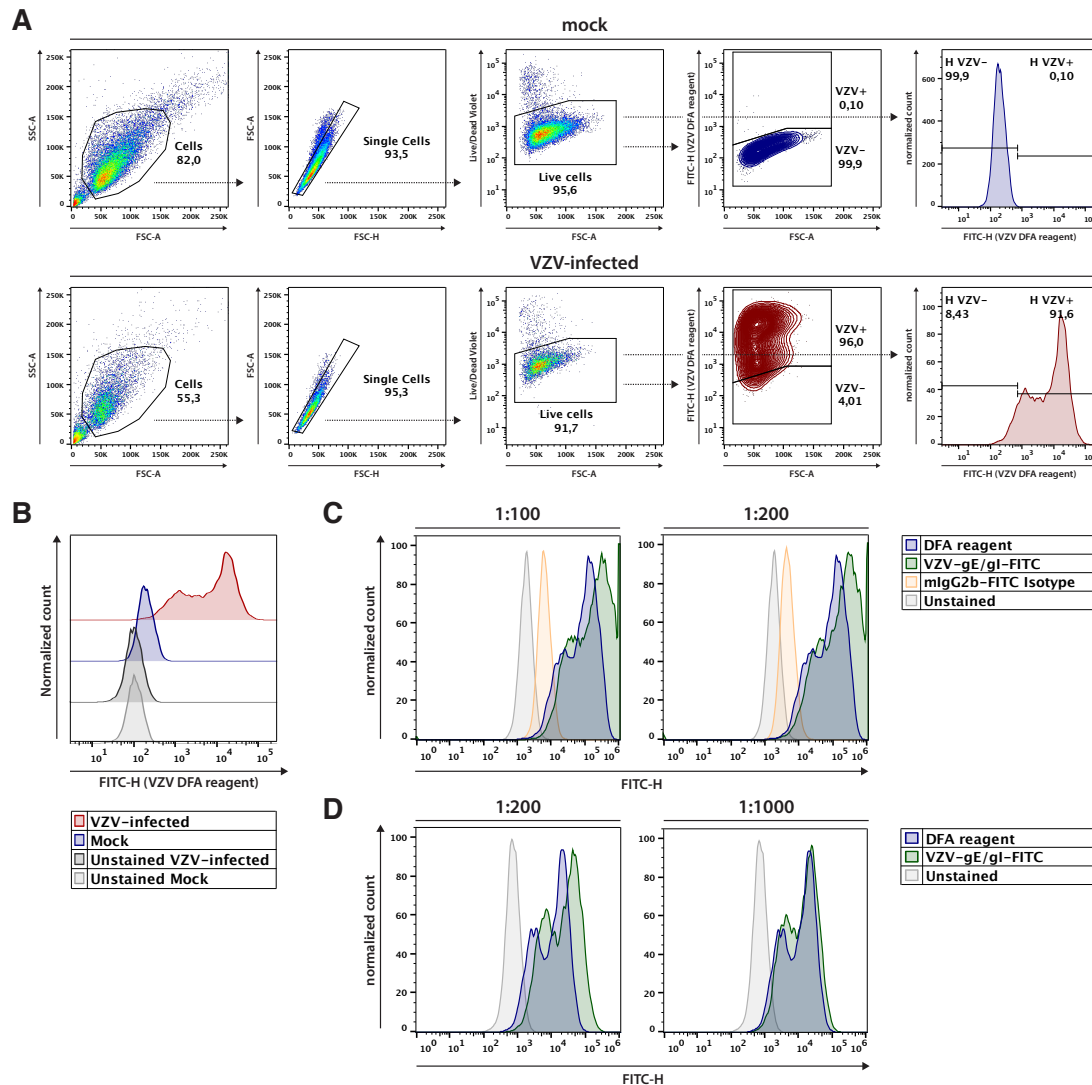


Figure 16: A flow cytometry assay to measure VZV-infection. (A) Uninfected (mock) or VZV-infected MeWo cells were stained with Live/Dead Violet and VZV DFA reagent and processed for flow cytometry. Dotted line arrows show gating hierarchy. (B) FITC channel intensity histogram for mock or VZV-infected cells (samples in (A)) as well as unstained controls. (C) Monoclonal antibody against VZV gE/gI was coupled to FITC (VZV-gE/gI-FITC). VZV-infected cells were stained with VZV-gE/gI-FITC or mlgG2B-FITC isotype control at indicated dilutions. VZV DFA reagent was used as in (A) and (B). (D) Repeat of experiment in (C) with higher antibody dilutions.

IV.1.2 Transwell System for VZV-infection

As mentioned previously, infection of cells with VZV is commonly achieved by co-culture with infected MeWo cells. Consequently, however, the cells that can be harvested will represent a mixture of target cells and MeWo inoculum cells. Cell sorting can be used to yield a pure population of target cells. This, however, complicates the experimental procedure

and makes readouts that require high numbers of cells impractical. I therefore conceived a novel way of infecting cells with VZV. Transwell inserts were used to spatially separate MeWo inoculum cells and target cells (Figure 17A). The inserts used for this technique had a 1 μ m pore size and, importantly, both sides of the transwell membrane were treated for cell adherence. Uninfected or infected MeWo cells were seeded on the bottom side of the membrane (Figure 17A). After cells adhered overnight they were labelled with a tracer dye to investigate later whether cells had migrated through the membrane pores. Target cells, in this case THP1 cells and MeWo cells as control, were seeded on the top side of the membrane. After 48 h the target cells were harvested from the top membrane and VZV infection was assessed by flow cytometry (Figure 17B). Firstly, for uninfected and infected MeWo and THP1 cells virtually all cells were negative for the tracer dye that inoculum cells were labelled with (compare to tracer dye positive inoculum cells shown in the last row of FACS plots in Figure 17B). This indicated that cells harvested from the top membrane indeed represented a pure population of target cells without inoculum cells. Assessment of VZV infection through staining with VZV-gE/gI-FITC revealed that both MeWo and THP1 cells could be successfully infected with VZV using this system. In this setting, virus was transferred from spatially separated inoculum cells to target cells through the transwell membrane.

The goal of these experiments was to eventually investigate innate sensing of VZV using RT-qPCR on a panel of VZV-infected THP1 KO cells. I therefore wanted to compare RT-qPCR analysis of THP1 cells infected through co-culture or through the newly developed transwell system. For co-culture, THP1 cells were PMA differentiated overnight. The cells were labelled with a tracer dye and co-cultured with uninfected or infected MeWo cells for 1 h. After washing, the cells were incubated for 48 h and then harvested for FACS and RT-qPCR. For transwell infections, uninfected or infected MeWo cells were seeded on transwells as described before. The next day THP1 target cells were added in PMA-containing medium. 48 h later the THP1 cells were harvested and processed together with co-cultured cells. In addition, both co-culture and transwell infections were also carried out with MeWo cells as target cells for control purposes. Flow cytometry analysis shows that for both infection methods around 40 % of THP1 cells got infected with VZV (Figure 18A). Due to the nature of the assay, the harvested cells infected by transwell were a pure THP1 population.

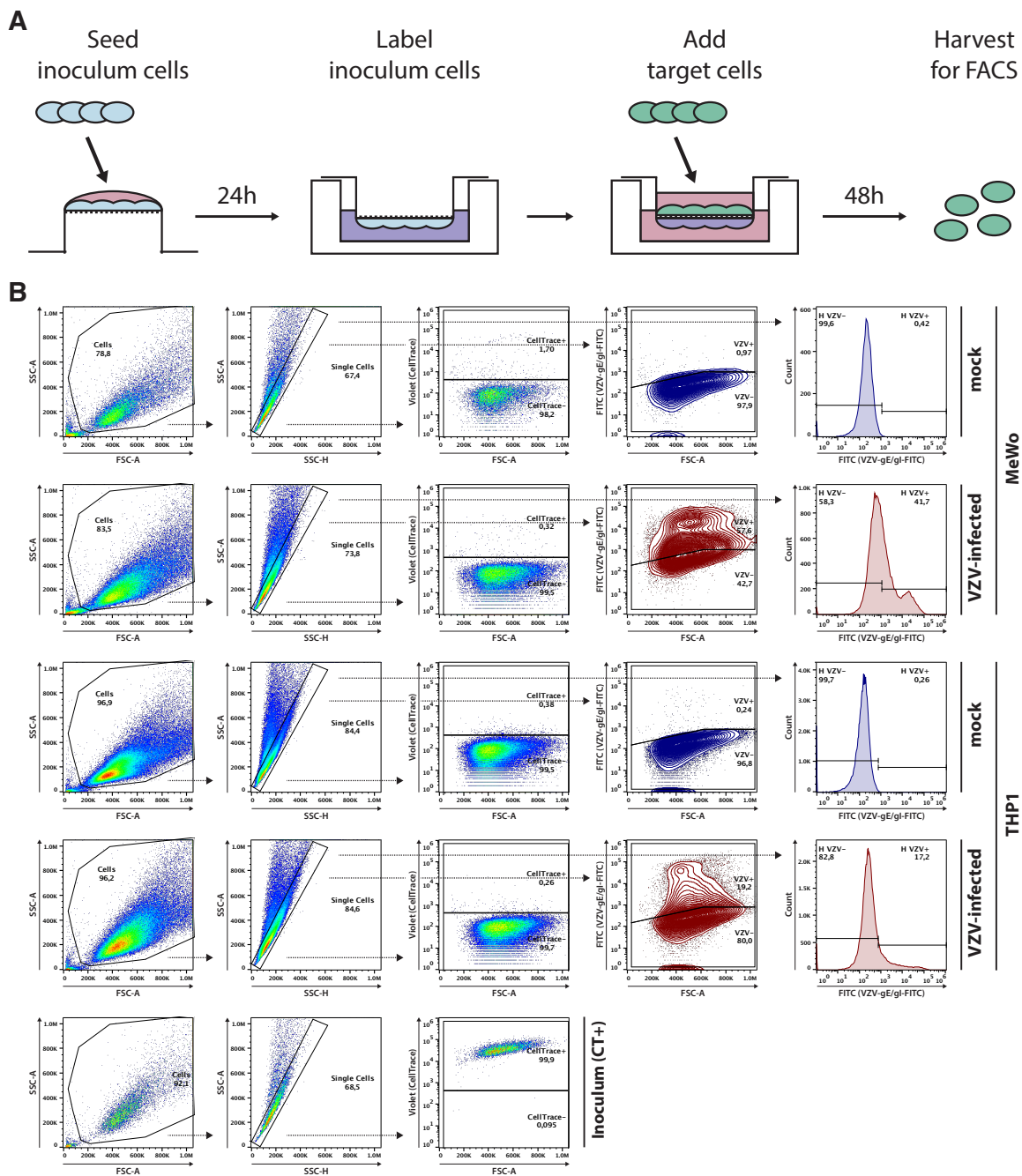


Figure 17: MeWo and THP1 cells can be infected with VZV using a newly developed transwell system. (A) Schematic detailing the experimental procedure. See Results and Methods sections for details. (B) MeWo and PMA-differentiated THP1 cells were mock or VZV-infected using transwell assay detailed in (A). Uninfected or VZV-infected MeWo inoculum cells were seeded on bottom membrane of transwells. The next day, the cells were labelled using CellTrace Violet dye. PMA-differentiated THP1 cells or MeWo cells as control were seeded onto top membrane of transwells and incubated for 48 h. Cells from the top membrane were processed for flow cytometry and infection assessed by staining with VZV-gE/gI-FITC. Cells from the bottom membrane were harvested to demonstrate CellTrace staining (panels in last row). Dotted arrows indicate gating hierarchy. Results in B are representative of seven independent experiments.

In contrast, "THP1" cells harvested from co-culture infections contained around 10% MeWo cells (see quadrant gates in last column of FACS plots in Figure 18A). For uninfected cells, MeWo cells actually represented almost 90 % of cells harvested. This had implications for the RT-qPCR analysis shown in Figure 18B. In the RT-qPCR analysis the results shown

for "THP1" represented the samples for which FACS plots are shown in Figure 18A. Results shown for "MeWo" represented control infections done with MeWo cells as target cells (FACS plots not shown). In both MeWo and THP1 cells, infection with VZV increased the expression of the *IFNB1* gene (Figure 18B). This was independent of the method of infection. The transcript levels were higher in uninfected cells for the transwell samples compared to co-culture samples. This was likely a result of co-culture samples being a mixture of MeWo and THP1 cells and indicated that, at baseline, THP1 cells had higher *IFNB1* transcript levels than MeWos. Similar observations as for *IFNB1* could be made for the ISGs *DDX58* (encodes RIG-I) and *IFI44*, albeit with differing magnitudes.

Generally, the data showed that *IFNB1*, *DDX58*, and *IFI44* transcript levels were higher at baseline in transwell THP1 samples compared to co-culture THP1 samples. After infection, transcript levels in co-culture samples were higher than in transwell samples. At first glance, this indicated that in a co-culture setting, THP1 cells responded more strongly to VZV infection by upregulating *IFNB1* expression. However, drawing these kind of conclusions from this experiment is near impossible given the inherently different nature of the samples. This includes the aforementioned different mixtures of cells but also extends further. Given that PMA-differentiated THP1 cells are macrophage-like cells they will respond to the presence of other cells in a co-culture setting, in particular dying cells, by phagocytosis. Phagocytosis of dead cells and intracellular materials would undoubtedly trigger PRRs and influence type I IFN induction. Another aspect is the differentiation of THP1 cells with PMA. In a co-culture setting, cells were differentiated in plastic tissue culture plates and then infected. For the transwell, the cells were differentiated on a porous membrane already with uninfected and infected MeWo cells present on the other side of the membrane. This could have affected how cells respond to VZV infection.

In light of these limitations, I decided to use conventional co-culture infection for the experiments with the THP1-KO cell panel. Proper comparison of the two methods of infection and validation of the transwell system seemed unrealistic given the scope of this project. Additionally, for the various experimental readouts high cell-numbers were required that were impractical to achieve using the transwell infection method. Nevertheless, I believe the transwell system is a useful resource for future experiments with VZV. I have successfully used the transwell system to infect a neuroblastoma cell line (SKN-BE) for proteomics analysis by a collaborator.

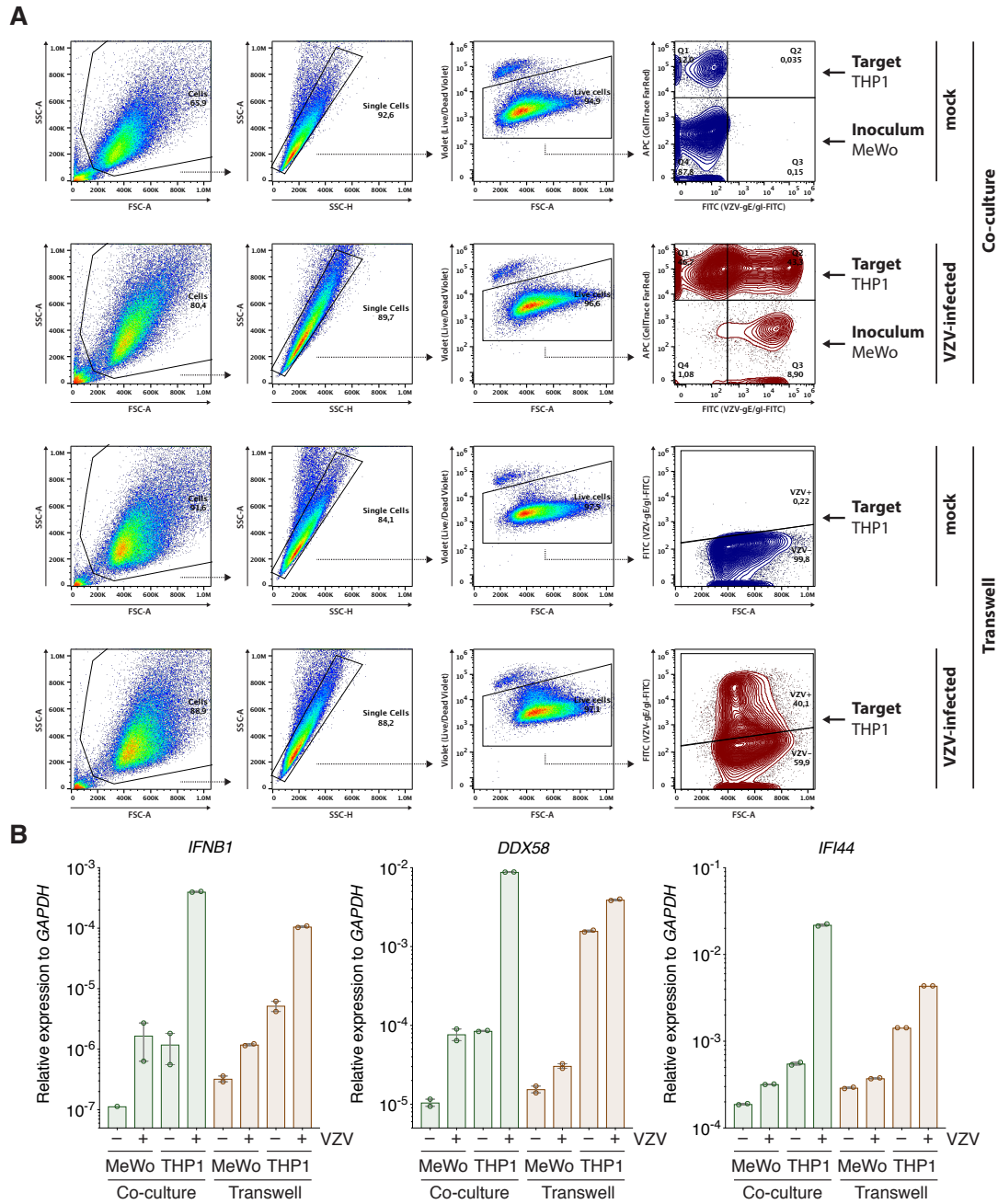


Figure 18: THP1 cells respond by type I IFN transcription to VZV-infection through co-culture and the transwell system. (A) For co-culture, THP1 cells were PMA-differentiated overnight and labelled with CellTrace FarRed. Cells were mock- or VZV-infected through 1 h co-culture with uninfected or infected MeWo cells, respectively. After 48 h, cells were harvested for FACS and RT-qPCR. **(B)** MeWo and THP1 cells were mock- or VZV-infected through both co-culture and the transwell assay as shown in (A) for THP1 cells. RT-qPCR was performed for the indicated genes. Panels (A) and (B) represent one independent experiment. Panel (B) shows technical duplicates (average and range (n=2)).

IV.2 VZV Innate Sensing

After having established the co-culture system as the method of choice for infection, I aimed to investigate the hypothesised role of cGAS and STING in cell-autonomous sensing of VZV. To test this experimentally I decided to use THP1 cells as a model system. These

cells are one of the genetically manipulatable cell lines that respond to DNA with type I IFN via cGAS/STING. To test the role of cGAS/STING in VZV recognition I obtained THP1 cells knocked-out for cGAS, STING, and TBK1. I further generated KO cells for IRF3. To test whether TLR signalling is required I also obtained KO cells for MyD88. The contribution of RLR signalling was tested by generation of MAVS-KO cells. IFNAR2-KO cells were also obtained and tested.

IV.2.1 Generation of THP1 Knockout Cells

The panel of THP1 KO cells available for infection experiments were cGAS-KO, STING-KO, MyD88-KO, TBK1-KO, IFNAR2-KO, MAVS-KO, and IRF3-KO. The MAVS-KO and IRF3-KO cells I generated myself. Generation and testing of single-cell clones is described in Methods section II.2.9.1. Clones I1 and I7 were chosen as IRF3-KO based on the absence of the targeted protein and the lack of response to both IVT-RNA and *E.coli* DNA transfection (Figure 19). Clones M1 and M3 were chosen as MAVS-KO since these cells failed to respond to IVT-RNA, responded normally to transfected DNA, and had no detectable MAVS protein (Figure 19). All four chosen clones were further characterised by Sanger sequencing of the sgRNA-targeted locus. In Clone I1 half the sequencing traces indicate a 1 bp insertion and half a 1 bp deletion. Clone I7 shows 50 % 2 bp deletion and 50 % 5 bp deletion. THP1 cells have been demonstrated to be diploid⁴⁸⁶. These results consequently indicate that both clones are derived from a single cell in which both alleles were edited by the Cas9 nuclease. MAVS-KO clones M1 and M3 show a 1 bp and 7 bp deletion, respectively, in 100 % of sequences, indicating the same editing event occurred on both alleles. In summary I showed that all four clones are clean KOs characterised by the absence of the targeted proteins and expected functional responses. Genomic DNA sequence analysis further indicates that the clones are single-cell derived. For infection experiments with VZV, IRF3 clone I1 and MAVS clone M1 were used.

To validate the panel of KO cells I verified the absence of the respective proteins (Figure 21A,B). THP1 cells of each knockout clone were PMA differentiated, treated or not with recombinant IFN, and lysates were processed for immunoblotting. Probing with antibodies against cGAS, STING, MyD88, and TBK1 clearly showed the complete absence of protein for each of these KO cell lines (Figure 21A).

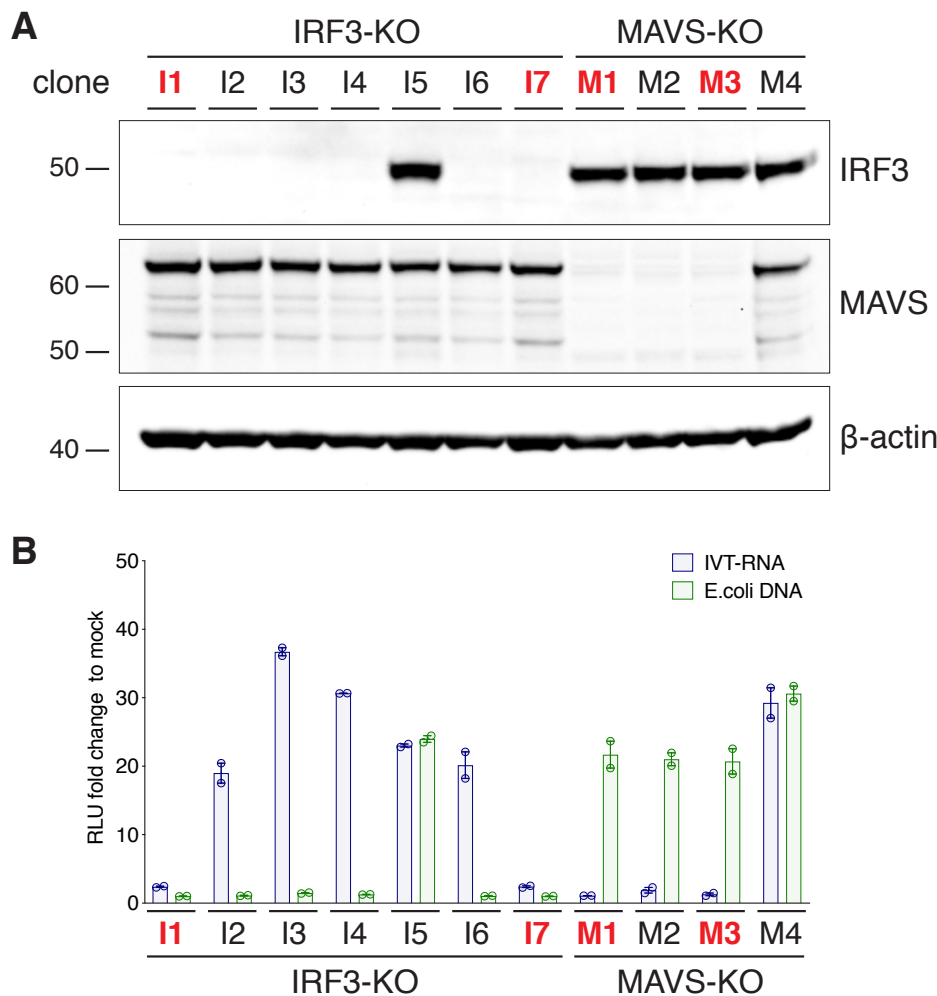


Figure 19: IRF3 and MAVS THP1 knockout clones were identified by functional testing. (A) THP1 cells were transfected with IRF3- and MAVS-targeting CRISPR plasmids and single-cell expanded by limiting dilutions. Cells of indicated clones were processed for immunoblotting. Membranes were probed with indicated antibodies. (B) Cells from the same clones as in (A) were PMA-differentiated and transfected with IVT-RNA or *E.coli* DNA. The next day, supernatants were transferred onto 3C11 cells (IFN bioassay). 24 h later luciferase intensity was measured and fold changes were calculated based on mock-transfected samples. Panel shows one independent experiment. For (B) average and range (technical duplicates) are shown.

RIG-I protein levels were increased in IFN treated cells indicating that ISG expression was stimulated in those cells. In THP1 cells, cGAS, STING, MyD88, and TBK1 do not appear to be ISGs since their expression level remained unchanged upon IFN treatment. Probing for MAVS and IRF3 confirmed the absence of protein in the respective KO cell lines (Figure 21B). For MAVS, both full-length MAVS protein (ca. 60 kDa) and mini-MAVS (ca. 50 kDa) were absent in the MAVS-KO cell line. IFN treatment showed that expression of MAVS and IRF3 was not stimulated by type I IFN in THP1 cells. Lacking an antibody against the IFNAR2 protein I validated the IFNAR2-KO cells functionally by probing for phosphorylated STAT1 and STAT2 after type I IFN stimulation. In WT, MAVS-KO, and IRF3-KO cells treatment with IFN α leads to the presence of phosphorylated STAT1 and STAT2 (Figure 21B).

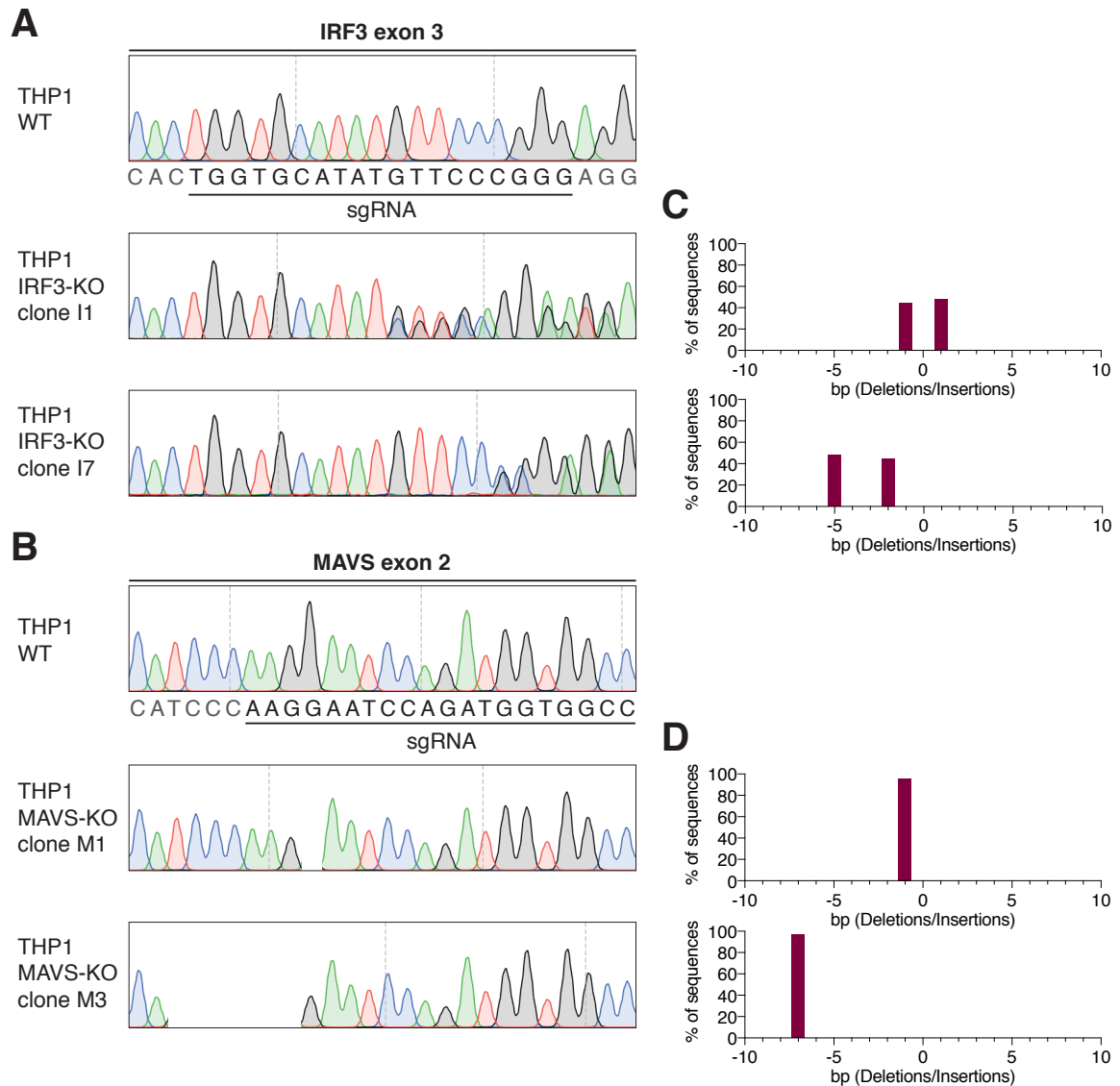


Figure 20: IRF3 and MAVS THP1 knockout clones show defined editing of targeted genomic loci. (A), (B) The IRF3 or MAVS locus was PCR-amplified from genomic DNA and analysed by Sanger sequencing. Panels show electropherogram from WT cells and two knockout clones. **(C), (D)** Sanger sequencing traces from (A) and (B) were analysed using the TIDE algorithm. Panels (A) - (D) show one independent experiment.

Total STAT1 protein levels are increased in these cells since STAT1 is an ISG. In contrast, IFN-treated IFNAR2-KO cells showed a complete absence of the phosphorylated forms of STAT1 and STAT2. Furthermore, no change in expression of total STAT1 protein could be observed. These findings confirmed that IFNAR2-KO cells lack a functional type I IFN receptor.

The panel of THP1 KO cells was further validated functionally. PMA-differentiated cells were transfected with IVT-RNA to stimulate RNA sensing pathways, and dsDNA (VACV 70mer) to stimulate DNA sensing pathways. *IFNB1* expression was analysed by RT-qPCR (Figure 21C). In WT, cGAS-KO, STING-KO, MyD88, and TBK1-KO cells transfection of IVT-

RNA strongly upregulated expression of the *IFN β* gene (ca. 3000-fold vs. mock). In IRF3-KO, IFNAR2-KO, and MAVS-KO cells this increase in expression was reduced to ca. 500-fold, 200-fold, and 30-fold, respectively.

As expected, the absence of MAVS, the adaptor protein downstream of RLR activation, strongly reduced the response to IVT RNA. Similarly, IRF3 was required for a full response to IVT-RNA. However, the effect of IRF3 KO was less pronounced than for MAVS. This indicated that there was some redundancy for IRF3 (e.g. with IRF7). THP1 cells that lack IFNAR2 also showed an attenuated response to IVT-RNA. The type I IFN receptor is not primarily involved in expression of *IFNB1* downstream of RIG-I. However, these cells probably lacked tonic IFN stimulation leading to lower baseline expression of PRRs and associated signalling proteins. They further failed to amplify the activation stimulus through increased ISG expression. The cells therefore showed an attenuated response. Surprisingly, TBK1-KO cells responded like WT cells to IVT-RNA. Given that together with MAVS, TBK1 phosphorylates and activates IRF3, one would expect strongly reduced *IFN β* expression after stimulation. However, IK kinases, such as IKK ϵ , are also part of this signalling cascade. It is likely that there is redundancy in the kinases that mediate IRF3 phosphorylation depending on the upstream stimulus.

In response to transfected VACV 70mer, WT, MyD88-KO, and MAVS-KO cells showed increased *IFN β* gene expression (ca. 5000-fold vs. mock). In cells that lack IRF3, cGAS, IFNAR2, TBK1, and STING upregulation of *IFNB1* was reduced to ca. 800-fold, 400-fold, 300-fold, 100-fold, and 10-fold, respectively. As expected, cells virtually did not respond to transfected DNA in the absence of STING. STING is the central adaptor protein for cytosolic DNA sensing pathways. The canonical DNA sensor cGAS is only partially responsible for the response to VACV 70mer given that these cells responded more strongly than STING-KO cells. This indicates that other, cGAS-independent, DNA-sensing pathways were activated in this setting. A lack of IRF3 or IFNAR2 protein attenuated the response to DNA due to reasons highlighted above for IVT-RNA. While TBK1 was not required for the response to IVT-RNA, absence of TBK1 strongly reduced *IFNB1* transcription after DNA transfection. As mentioned, it appears that redundancy for TBK1 is dependent on the upstream stimulus. The amounts of IVT-RNA and VACV 70mer dsDNA used in this experiment were saturating doses as indicated by the high expression fold changes for *IFNB1*. In future repeat experiments lower doses of stimuli will be used to demonstrate further the distinct functionality of the

different KO cell lines. Despite these limitations the data in Figure 21 shows that each of the KO cells lacks expression of the respective protein and responds to PAMPs as expected.

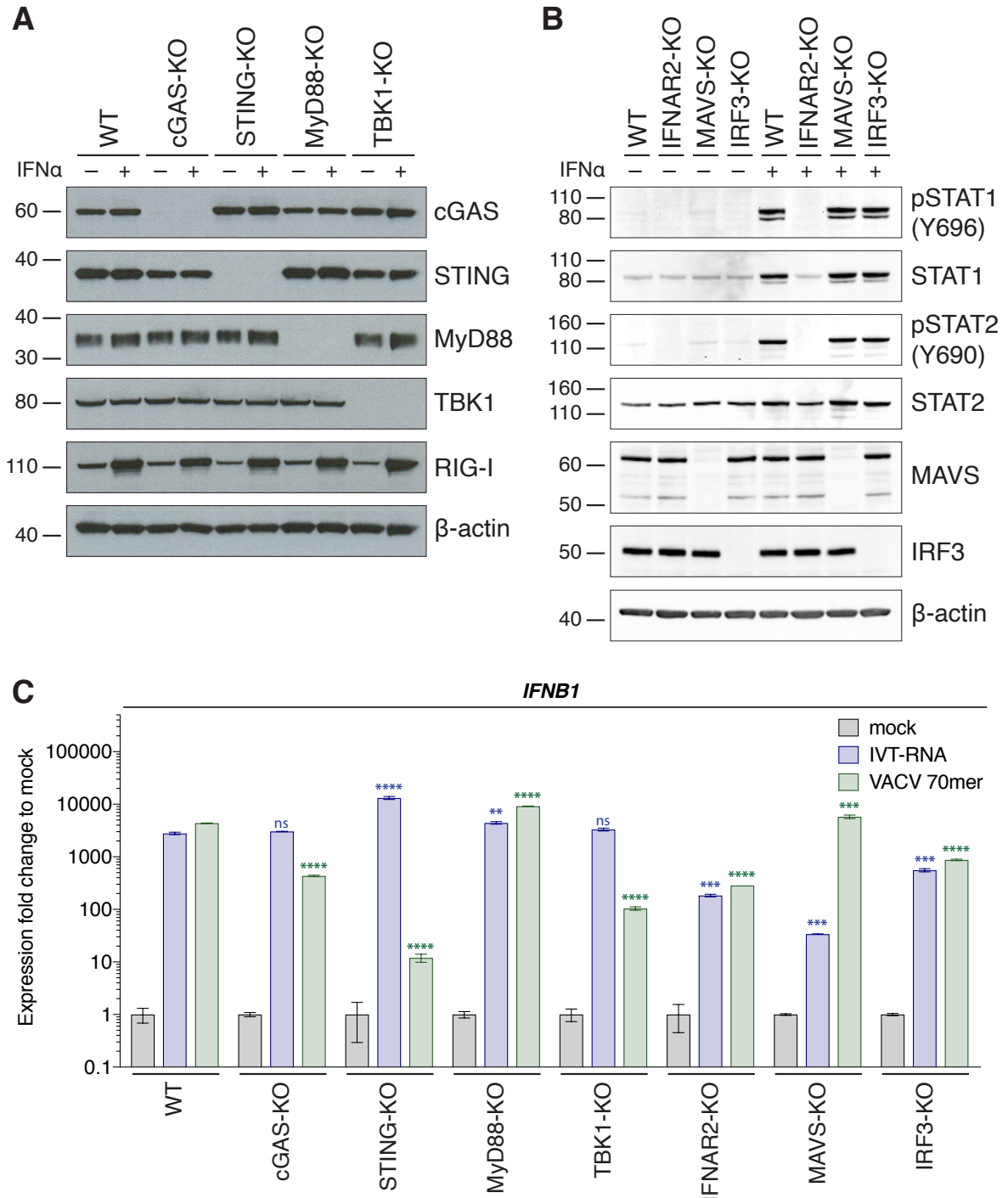


Figure 21: The THP1 knockout cell panel shows absence of relevant proteins and expected functionality. (A) THP1 cells from indicated KO cell lines were PMA differentiated and treated or not with recombinant IFN α . Lysates were processed for immunoblotting and membranes were probed with indicated antibodies. (B) THP1 cells from indicated KO cell lines were processed as in (A). (C) THP1 cells from indicated KO cell lines were PMA differentiated and mock transfected or transfected with 200 ng IVT-RNA or 500 ng VACV 70mer. RNA was extracted and RT-qPCR was performed for the *IFNB1* gene. Panel (A) is representative of three independent experiments. Panel (B) shows one independent experiment. Panel (C) shows one independent experiment (average and range (technical duplicates)). One-way ANOVA with Dunnett's multiple comparisons test was used to compare results for each KO cell line to WT cells as indicated above bars in (C). ****=p<0.0001, ***=p<0.001, **=p<0.01, ns=not significant.

IV.2.2 In THP1 Cells cGAS/STING Is Required for type I IFN Response to VZV Infection

After validating the panel of THP1 KO cells, I tested the innate immune response to VZV infection in these cells. THP1 WT and KO cells were infected with VZV by co-culture. Distribution of inoculum and target cells as well as rate of infection was quantified by flow cytometry (Figure 22). Cells were further analysed by RT-qPCR (Figure 23,24) and immunoblotting (Figure 25). Concentrations of IP-10 in supernatants was determined by ELISA (Figure 26).

Exemplary FACS plots for WT THP1 cells highlighting the gating strategy for analysis are shown in Figure 22A. Flow cytometry data for all repeat experiments is provided in Supplementary Figures S1-S4 in the Appendix. Live, single cells are separated into inoculum cells (black gate) and target cells (green gate) using tracer dye fluorescence. Within the target cell population, cells are further separated into uninfected cells (blue gate) and infected cells (red gate). The distribution of inoculum vs. target cells is shown as pooled data from three independent experiments in Figure 22B. As seen previously in optimisation experiments, inoculum cells represent a greater proportion of total cells in uninfected samples compared to infected. In the different THP1 KO cells they represent between 15 % and 50 % of total cells. In infected samples, MeWo cells only contribute between 5 % and 15 % of cells. Of the THP1 cells in infected samples, between 10 % and 25 % are infected with VZV by average depending on the THP1 genotype (Figure 22C). Variability regarding the proportion of infected cells was particularly high in cGAS and STING KO cells as indicated by the error bars.

The transcript levels of the *IFNB1* gene, and three ISGs (*DDX58*, *IFI44*, *IFIT1*) were analysed by RT-qPCR (Figure 23). Infection with VZV led to strong upregulation of the transcript levels of all four genes analysed in WT, MyD88-KO, and MAVS-KO cells. This was particularly evident when comparing transcript fold inductions between uninfected and infected samples (right column graphs in Figure 23). In all other THP1 KO cell lines the infection with VZV did not appear to induce elevated transcription when comparing fold changes. However, more detailed information could be gathered when analysing relative expression levels (left column graphs in Figure 23). It appeared that the *IFNB1* transcript level was also marginally higher in infected cells for cGAS-KO, STING-KO, IFNAR2-KO, and IRF3-KO. As mentioned previously, however, uninfected and infected samples contained MeWo cells to different extents.

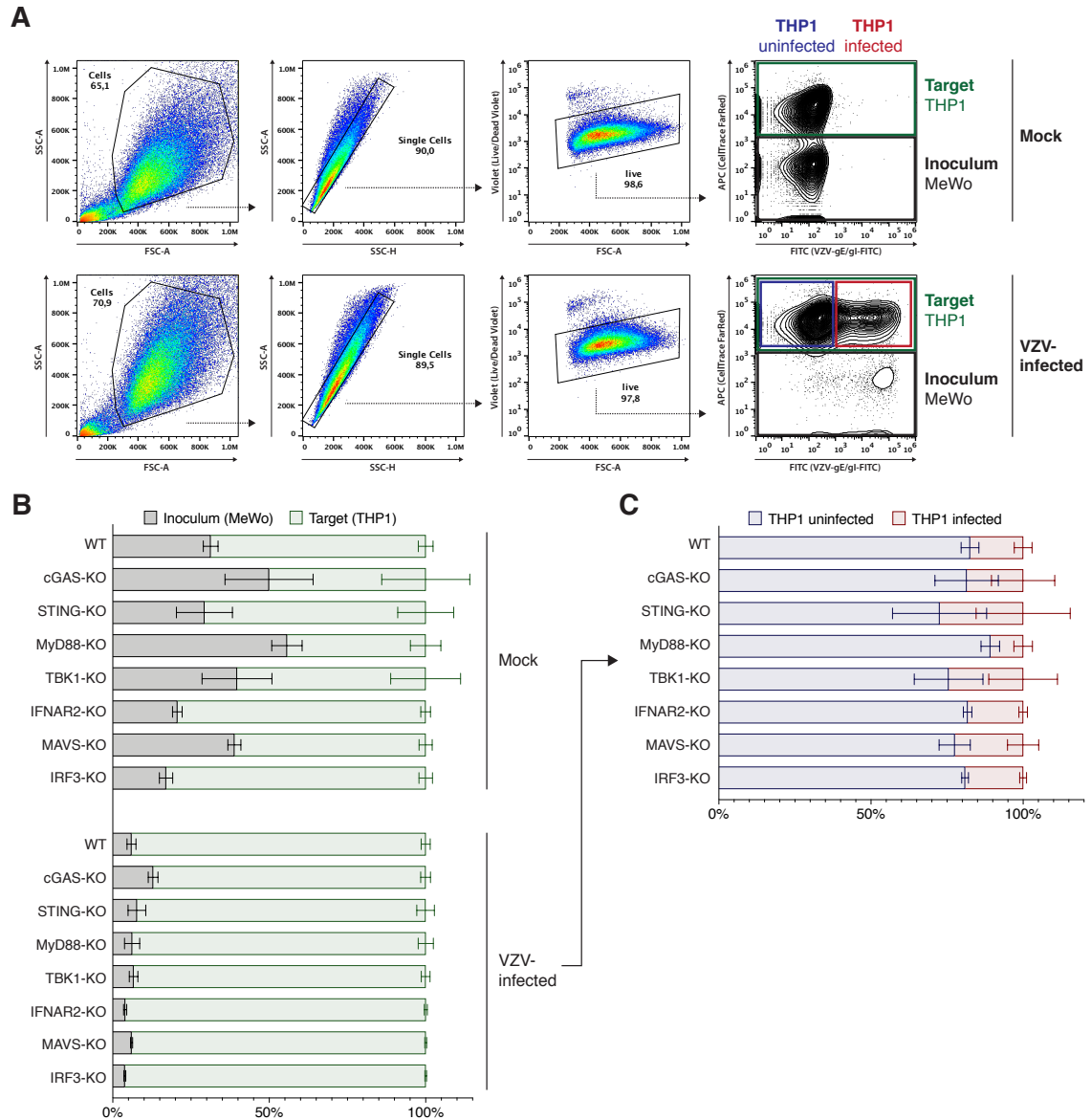


Figure 22: The THP1 knockout cell panel was infected with VZV by co-culture. (A) WT THP1 cells were PMA-differentiated overnight and labelled with CellTrace FarRed. Cells were mock or VZV-infected by co-culture with uninfected or infected MeWo cells, respectively. 48 h later cells were harvested and a fraction of the cells was processed for flow cytometry by staining with VZV-gE/gI-FITC. Coloured boxes indicate gates used for quantification shown in (B) and (C). Dotted arrows indicate gating hierarchy. **(B)** Inoculum cells (MeWo, CellTrace-, black gate) and target cells (THP1, CellTrace+, green gate) from co-culture experiments were quantified as percentage of live cells. **(C)** Rate of infection (blue vs red gate) was quantified in target cells (THP1, green gate). Results in A are representative of six independent experiments. Panels B and C show pooled data from six (WT) or three (KOs) independent experiments (average and SEM (n=3/6)).

THP1 cells had higher *IFNB1* transcript levels than MeWo cells at baseline (see RT-qPCR results of transwell experiments in Figure 18B). Mixtures of THP1 and MeWo cells at different ratios therefore had varying *IFNB1* transcript levels. These artefacts arising from the experimental procedures made further conclusions based on small transcript level differences impossible. Regardless, the RT-qPCR data clearly showed that in response to VZV infection, the cGAS/STING system induced elevated transcription of the *IFN β* gene in this THP1 cell model system.

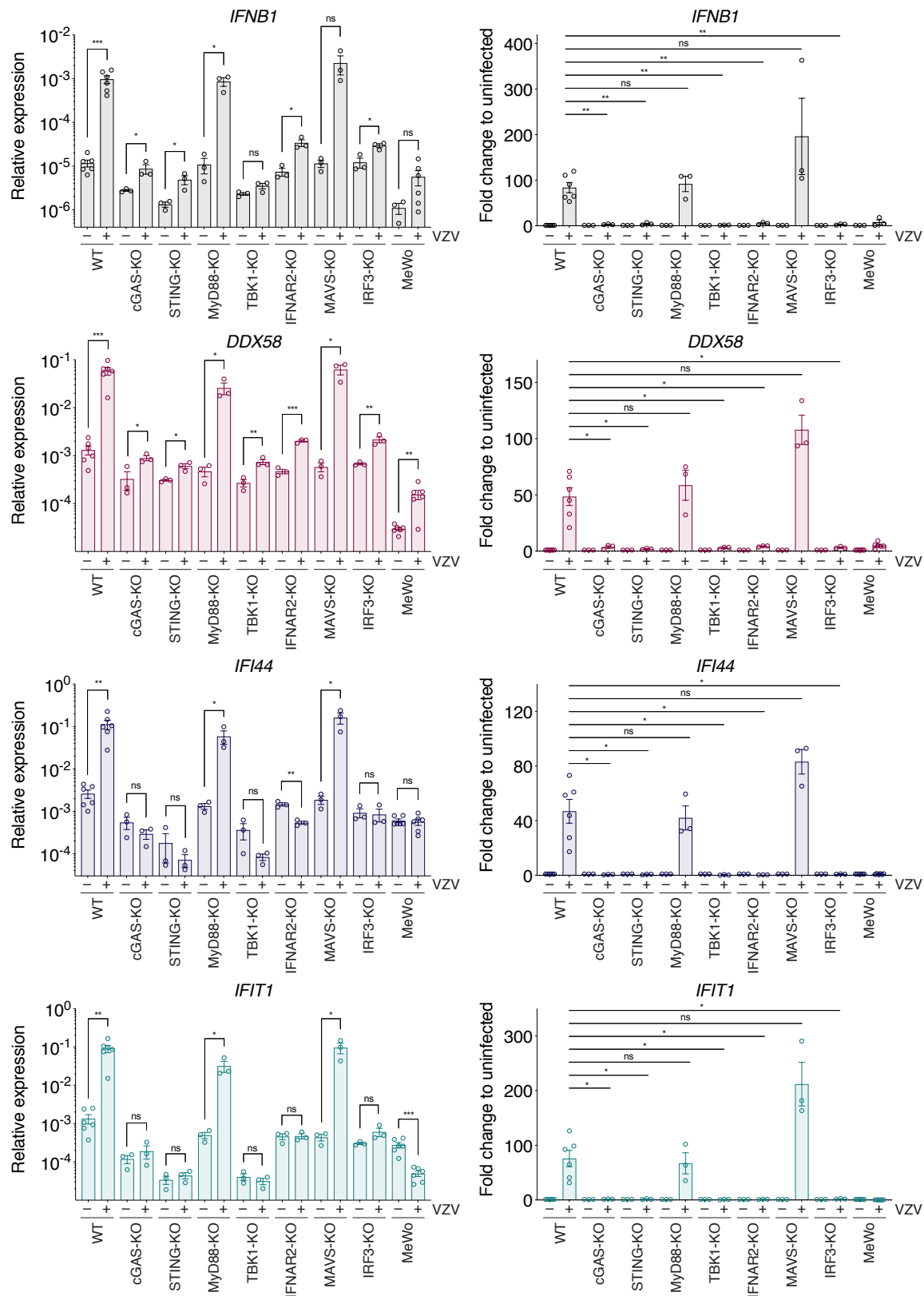


Figure 23: THP1 cGAS KO, STING KO, TBK1 KO, IRF3 KO and IFNAR2 KO cells fail to respond to VZV infection through transcriptional upregulation of *IFNB1* and ISGs. WT and KO THP1 cells were PMA-differentiated overnight. As controls, MeWo cells were seeded. Cells were labelled with CellTrace FarRed and mock or VZV-infected by co-culture with uninfected or infected MeWo cells, respectively. 48 h later cells were harvested and processed for RT-qPCR for indicated genes. Left panels show expression relative to *GAPDH* and right panels show expression fold change to uninfected samples. Panels show pooled data from six (WT, MeWo) or three (KOs) independent experiments (average and SEM (n=3/6)). Left panels: Unpaired, two-tailed t-test. Right panels: Welch One-way ANOVA with Dunnett's multiple comparisons test. ***=p<0.001, **=p<0.01, *=p<0.05, ns=not significant.

In addition to the expression of the *IFN β* gene and ISGs in response to VZV infection, I also analysed transcript levels of the *IL6* and *IL1 β* genes, two inflammatory cytokines (Figure 24). Strikingly, *IL6* transcript levels were lower in infected THP1 samples compared to uninfected cells. This was largely independent of the THP1 cell genotype. In contrast, *IL6* expression was higher after VZV infection in MeWo only control samples. It thus appears that VZV infection reduced *IL6* transcript levels below baseline in THP1 cells but increased in expression in MeWo cells. This is unlikely to have been an effect of THP1/MeWo mixtures (discussed above) given that *IL6* transcript levels in MeWo-only samples are lower than in uninfected THP1 samples. If anything, uninfected THP1 samples contain more MeWo cells than infected samples and would therefore show lower *IL6* transcript levels, not higher ones. Changes in transcript levels of pro-IL1 β (*IL1 β*) in response to VZV infection followed similar trends as seen for *IFNB1* and ISGs (Figure 24).

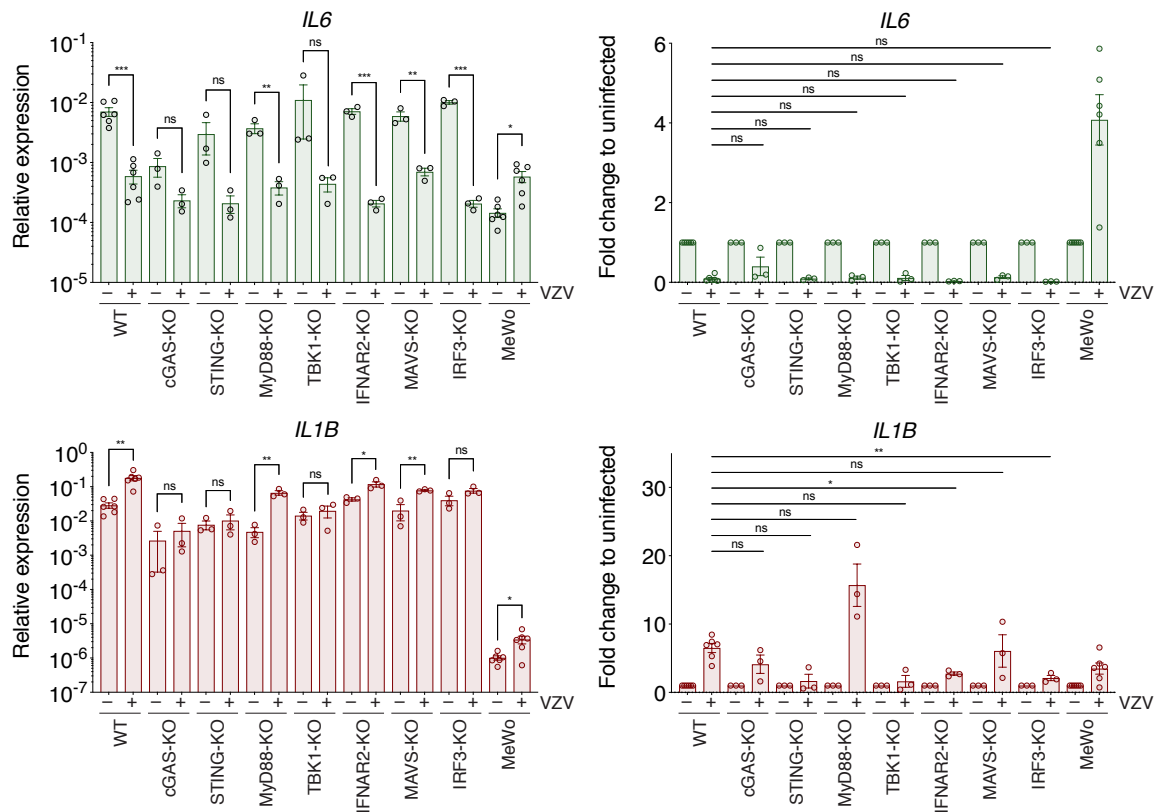


Figure 24: Inflammatory cytokine gene transcription shows distinct patterns in the THP1 knockout cell panel after VZV-infection. WT and KO THP1 cells were PMA-differentiated overnight. As controls, MeWo cells were seeded. Cells were labelled with CellTrace FarRed and mock or VZV-infected by co-culture with uninfected or infected MeWo cells, respectively. 48 h later cells were harvested and processed for RT-qPCR for indicated genes. Left panels show expression relative to *GAPDH* and right panels show expression fold change to uninfected samples of the same genotype. Panels show pooled data from six (WT, MeWo) or three (KOs) independent experiments (average and SEM (n=3/6)). Left panels: Unpaired, two-tailed t-test. Right panels: Welch One-way ANOVA with Dunnett's multiple comparisons test. ***=p<0.001, **=p<0.01, *=p<0.05, ns=not significant.

The *IL1B* gene encodes the pro-IL1 β polypeptide. Pro-IL1 β needs to be processed by activated caspase-1 into the mature IL1 β cytokine in the context of inflammasome activation. In WT, MyD88-KO, and MAVS-KO cells *IL1B* expression increased between 5- and 20-fold after infection. In cGAS-KO, STING-KO, TBK1-KO, IFNAR2-KO, and IRF3-KO cells the observed increase in expression was reduced. In MeWo only samples, a 5-fold induction of *IL1B* could be observed. However, it is noteworthy that *IL1B* transcript levels are generally around four orders of magnitude lower in MeWo cells compared to THP1 cells. This complicates interpretation of the results. With the aforementioned limitations in mind, we can nevertheless conclude that activation of the cGAS/STING pathway contributes to inflammasome activation on the transcriptional level. In order to make a definitive statement about inflammasome activation in THP1 cells after VZV infection, other hallmarks of inflammasome activation needed to be tested. This includes, for example, release of matured IL1 β and other inflammasome components into the supernatant. This was, however, outside the scope of this project.

Analysis of VZV-infected cells by immunoblotting confirmed the findings in RT-qPCR (Figure 25). Phosphorylated STAT1 and STAT2 protein could only be detected in infected WT, MyD88-KO, and MAVS-KO cells. This indicated that only in those samples sufficient type I IFN was secreted to trigger detectable phosphorylation of STAT1 and STAT2. Total STAT1 and STAT2 protein levels were markedly increased in these samples as expression of those proteins is IFN stimulated. Immunoblotting for another ISG, RIG-I, confirmed a similar pattern. Smaller differences in protein levels could be observed for STAT1, STAT2, and RIG-I in cells that did not respond to VZV infection (cGAS-KO, STING-KO, TBK1-KO, IFNAR2-KO, IRF3-KO). However, all samples represented a mixture of MeWo and THP1 cells. The proportion of MeWo cells was higher in uninfected samples (see Figure 22B). As evident in Figure 25, MeWo cells did not express RIG-I protein. Therefore, small differences in protein levels of RIG-I were likely caused by MeWo cell "contamination" rather than upregulation in THP1 cells in response to the virus. Viral proteins are abundant in all infected samples as shown by immunoblotting for VZV proteins gE/gI and ORF62.

MeWo cells do not seem to intrinsically respond to VZV infection as there is no pSTAT1 and pSTAT2 detectable. STAT1 and STAT2 protein levels are not increased. Interestingly, STAT2 protein levels are decreased in infected MeWo cells. Based on the results for MeWo cells we can conclude that, in THP1 samples, phosphorylation of STAT1 and STAT2 in response to VZV infection is indeed dependent on the THP1 genotype and requires cGAS, STING, TBK1, IRF3, and IFNAR2.

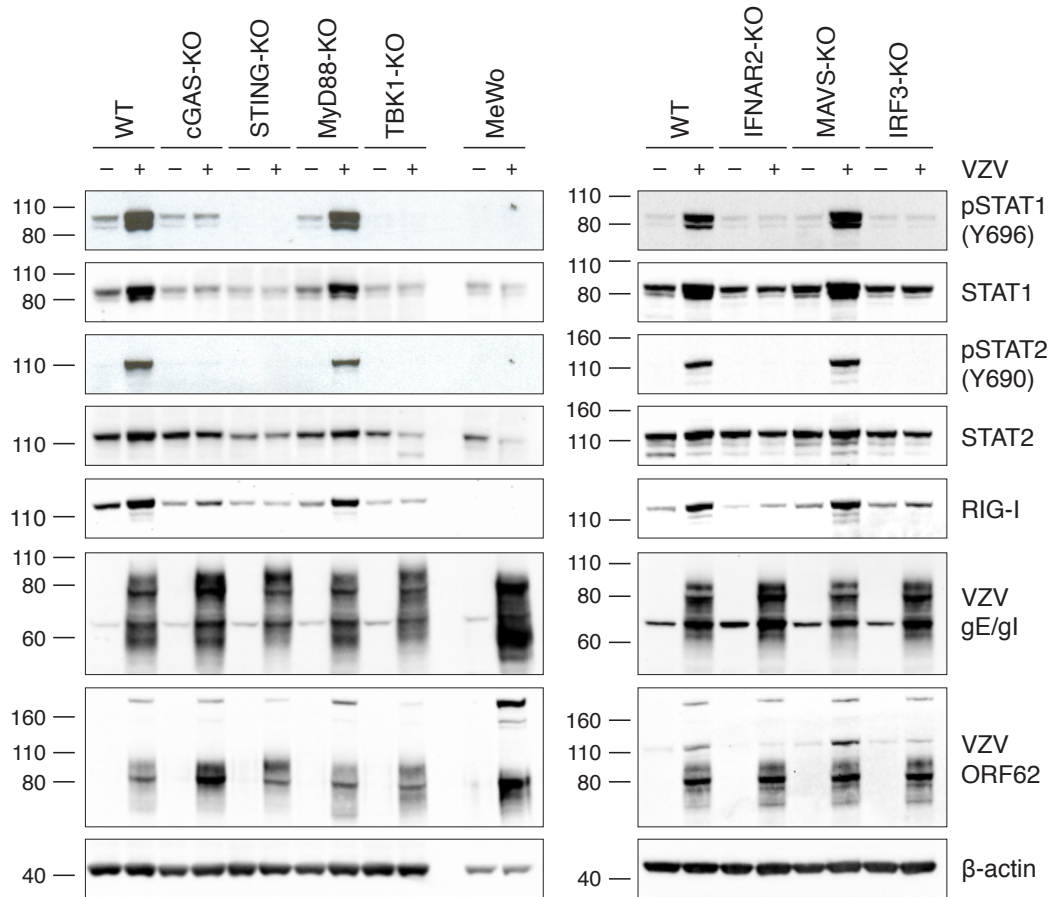


Figure 25: THP1 cGAS KO, STING KO, TBK1 KO, IRF3 KO and IFNAR2 KO cells fail to phosphorylate STAT1 and STAT2 in response to VZV-infection. WT and KO THP1 cells were PMA-differentiated overnight. As controls, MeWo cells were seeded. Cells were labelled with CellTrace FarRed and mock or VZV-infected by co-culture with uninfected or infected MeWo cells, respectively. 48 h later cells were harvested and processed for immunoblotting with indicated antibodies. Size markers indicate molecular weight in kDa. Panel shows representative results of three independent experiments.

Further evidence for a critical role of the cGAS/STING DNA-sensing pathway for the type I IFN response to VZV is provided by analysis of secreted IP-10. IP-10 is a cytokine that is secreted following type I IFN stimulation and is often used as a surrogate for IFN β . In WT, MyD88-KO and MAVS-KO cells IP-10 levels in the supernatant are increased after VZV infection (Figure 26). Low levels of IP-10 can also be detected in the absence of infection in these samples. However, even though the data is somewhat variable, IP-10 levels are higher after infection for each experiment (results for independent experiments represented by data point shapes in Figure 26). No IP-10 is detectable in supernatants of cGAS-KO, STING-KO, TBK1-KO, and IFNAR2-KO cells, neither in infected nor uninfected cells. MeWo cells also fail to secrete IP-10 in response to VZV. Low levels of IP-10 can be detected in IRF3-KO cells after infection. These data confirm the crucial role of the cGAS/STING signalling axis in the type I IFN response to VZV in THP1 cells.

As mentioned, in the absence of infection, low IP-10 levels can be detected only in WT, MyD88-KO and MAVS-KO cells. PMA treatment has been shown to induce DNA damage in primary leukocytes⁴⁸⁷. It is possible that differentiation of THP1 cells with PMA induces low-level DNA damage and a subsequent, cGAS/STING-dependent type I IFN response. Alternatively, THP1 cells may mount such a response to the presence of MeWo cells in the co-culture.

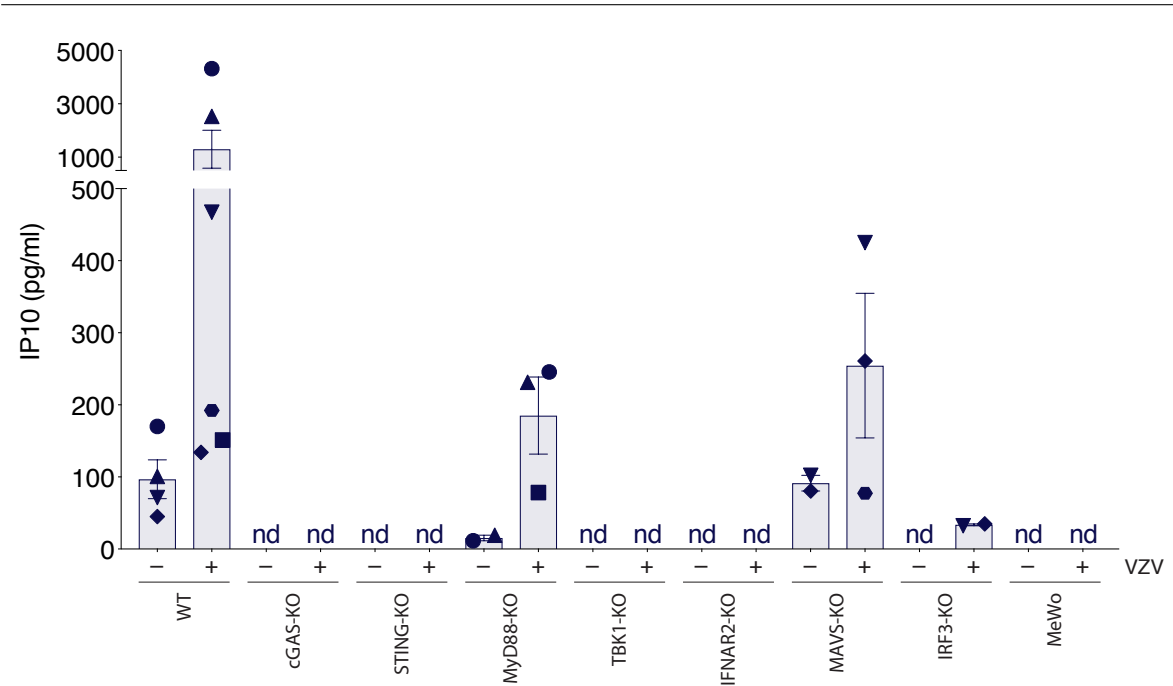


Figure 26: VZV-infection does not induce IP-10 secretion in cGAS KO, STING KO, TBK1 KO, IFNAR2 KO THP1 cells. WT and KO THP1 cells were PMA-differentiated overnight. As controls, MeWo cells were seeded. Cells were labelled with CellTrace FarRed and mock or VZV-infected by co-culture with uninfected or infected MeWo cells, respectively. 48 h later supernatants were harvested and processed for IP-10 ELISA. Panel shows pooled data from six (WT) or three (KOs, MeWo) independent experiments (average and SEM (n=3/6)). Data point shapes correspond to independent experiments. Missing data points or "nd" (not determined) indicate values below the detection limit.

IV.2.3 VZV Evades Sensing in HaCaT Keratinocytes

The data in the previous section clearly showed that THP1 cells mount a type I IFN response to VZV and that this response is dependent on cGAS/STING signalling. However, the tropism of VZV *in vivo* is focused on respiratory epithelial cells, keratinocytes, T-cells, and neurons. Hence, I wanted to test cell-intrinsic recognition of VZV in a more relevant cell type. For this I utilised HaCaT cells, a cell line of spontaneously immortalised human keratinocytes. I used the co-culture infection assay as previously to infect HaCaT cells with VZV. Given that I had no previous data on how VZV replicates in these cells, I performed a time course experiment. Cells were harvested 8 h, 16 h, 24 h, 32 h, 40 h, and 48 h after the

start of co-culture. Infection was assessed by flow cytometry and cells were also harvested for RT-qPCR and immunoblot analysis (Figure 27).

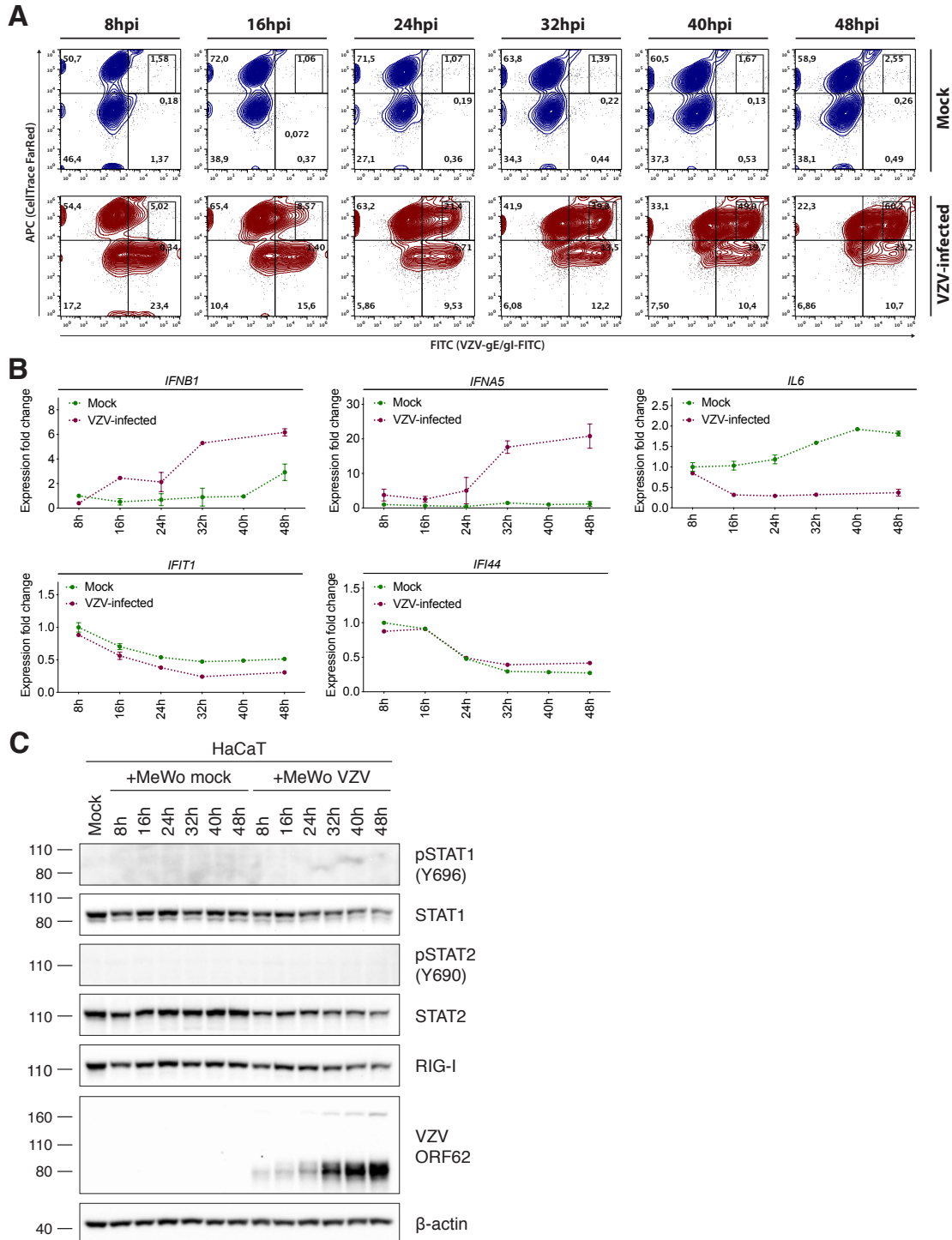


Figure 27: VZV Evades Sensing in HaCaT Keratinocytes. (A) HaCaT cells were labelled with CellTrace FarRed and mock or VZV-infected by co-culture with uninfected or infected MeWo cells, respectively. At the indicated time points after co-culture cells were harvested and processed for flow cytometry by staining with VZV-gE/gI-FITC. Plots show events pre-gated on single cells. (B) Samples shown in (A) were analysed by RT-qPCR for the indicated genes. Graphs show fold changes of expression relative to *ACTB* normalised to the first time point. (C) Samples shown in (A) and a sample of HaCaT cells alone were analysed by immunoblotting using the indicated antibodies. All panels show one independent experiment. Panel (B) shows technical duplicates (average and range (n=2)). hpi: hours post infection

Before co-culture, HaCaT cells were labelled with a tracer dye (analogous to the experiments with THP1 cells). In the flow cytometry analysis shown in Figure 27A, the cells can therefore be found in the top two quadrant gates. The data shows that HaCaT cells indeed became infected with VZV. The proportion of infected cells expanded with increasing incubation time. RT-qPCR revealed that, in response to VZV infection, HaCaT cells only marginally increased *IFNB1* transcription starting at 16 h (Figure 27B). The expression of *IFNA5* increased ca. 20-fold starting after 24 h incubation. *IL6* transcript levels decreased in infected cells, a phenomenon that could already be observed in experiments with THP1 cells (see section IV.2.2 and Figure 24). Expression of the ISGs *IFIT1* and *IFI44* was identical in mock-infected and VZV-infected cells. In both uninfected and infected samples, however, transcript levels for both ISGs decreased over the 48 h incubation period.

No phosphorylated STAT1 or STAT2 could be detected in infected HaCaT cells by immunoblot (Figure 27C). Protein levels of total STAT1, total STAT2, and the ISG RIG-I decreased in infected cells over time. Increasing protein levels of VZV ORF62 over time in infected samples further showed that the virus indeed propagated in HaCaT cells. In this preliminary experiment, a positive control for the phosphorylation of STAT1 and STAT2 was lacking. This would be included in future experiments. Published studies have demonstrated that HaCaT cells phosphorylate STAT1 and STAT2 in response to IFN α treatment⁴⁸⁸.

In summary, HaCaT cells can be aptly infected with VZV. While transcript levels of *IFNB1* and *IFNA5* modestly increased in infected cells, expression of the ISGs *IFIT1* and *IFI44* was unchanged. No phosphorylated STAT1 and STAT2 protein could be detected. These data together indicate that HaCaT cells weakly respond to infection with VZV at the transcriptional level but fail to secrete type I IFN protein. This is in stark contrast to results obtained in THP1 cells.

IV.3 Discussion

In this Chapter, I have provided evidence that the cGAS/STING DNA-sensing pathway is critically required for THP1 cells to mount a type I IFN response to VZV. In the absence of cGAS, STING, TBK1, and IRF3, cells fail to upregulate *IFNB1* as well as ISG transcripts. Immunoblot and ELISA results indicate that cells do not produce type I IFN if cGAS, STING, TBK1, or IRF3 protein was absent. The involvement of cGAS shows that the primary PAMP

driving type I IFN production during VZV infection is DNA. Given that MAVS-KO cells show results similar to WT cells, RLR-stimulation does not appear to be relevant for the IFN response in this setting. Similarly, in the absence of MyD88, THP1 cells respond normally to VZV infection. This showed that endosomal TLR-signalling is also not required in this model.

According to my results, cGAS, STING, TBK1, and IRF3 are responsible for the antiviral cytokine response to VZV infection. Hence, in the absence of these proteins, more cells should become infected with the virus due to the lack of type I IFN. The average infection rate, however, is similar between the different THP1 KO cells (Figure 22C). This average is over three independent experiments I performed. Closer examination of the three experiments reveals an interesting observation, though. In experiment I, ca. 10% and ca. 15% of WT and MyD88-KO cells, respectively, became infected (Figure S1 and S2). In contrast, 30%, 50%, and 40% of cGAS-KO, STING-KO, and TBK1-KO were positive for VZV. In this experiment, cells without cGAS, STING, and TBK1 were clearly impaired in their ability to restrict viral replication. Unfortunately, this phenotype was not reproducible in experiment II and III. It became clear at a later point in this project that with increasing time in culture, THP1 cells become more resistant to virus infection. Increased resistance to virus infection in these cells could be attributable to changes in tonic type I IFN signalling over time. Connected to that, epigenetic changes induced by type I IFN have been shown to influence antiviral signalling and responses⁴⁸⁹. In light of these observations the results obtained in experiment I should be reproducible in freshly cultured cells. I therefore tentatively conclude that cGAS, STING, and TBK1, indeed have the ability to limit the spread of virus in culture.

Multiple previous studies have reported that VZV infection can trigger type I IFN, type II IFN, and inflammatory cytokine responses in different cell types. A number of studies have also implicated specific PRRs in the cell-autonomous recognition of VZV. Infection of human PBMCs with VZV triggers secretion of IFN α ³⁸⁹. This response is largely driven by activation of TLR9 in pDCs. However, TLR9-independent pathways also contributed to type I IFN secretion on this setting. Experimental evidence for a role of STING in the type I IFN response induced by VZV has been provided previously³⁸⁸. In this study, knockdown of STING and IRF3 via siRNAs led to reduced type I IFN expression in response to VZV infection. Furthermore, increased viral titres were observed if expression levels of these proteins was reduced. Interestingly, however, these results were obtained from infections of HaCaT cells, a keratinocyte cell line, and primary dermal fibroblasts. In another study, VZV infection also

lead to increased expression of *IFN* α and *IFN* β in fibroblasts and HaCaT keratinocytes⁴⁹⁰. Notably, in this set of experiments, infection of THP1 macrophages lead only to a marginal upregulation of *IFN* α expression. Transcript levels of *IFN* β actually decreased in THP1 cells after VZV infection. In all three cell types used in this study, VZV infection triggered the secretion of the inflammatory cytokines IL6 and IL8, as well as IP-10.

These results are in contrast to my experiments, in which THP1 cells responded strongly via type I IFNs to VZV infection whereas HaCaT cells did so to a much lesser extent. A number of reasons could explain these contrasting results: Firstly, the experimental protocols for VZV infection through co-culture vary between different studies. The way infected inoculum cells are prepared and duration of co-culture will certainly influence the outcome of infection. As mentioned previously, this includes the presence of inoculum cells and possibly cell fragments which will influence the target cell's response. Secondly, given the cell associated nature of VZV, the number of infectious units, i.e. the dose of virus, is hard to control and almost impossible to compare between experiments in different laboratories.

In any case, in my experiments, HaCaT keratinocytes do not appear to secrete type I IFN in response to VZV infection. Keeping in mind that these are preliminary data based on a single experiment, they nevertheless indicate that recognition of VZV is indeed dependent on the cell type. Keratinocytes are crucially important for VZV's pathogenesis *in vivo*. I would therefore hypothesise that the virus has specifically evolved to replicate in these cells without being recognised by PRRs. This innate immune evasion is presumably achieved through the action of viral antagonist proteins. In future experiments, it will be interesting to delineate whether it is indeed antagonism of cGAS directly that is responsible for the lack of an IFN response in HaCaT cells. Published data indicate that HaCaT cells respond to cytosolic DNA with a type I IFN response that is dependent on cGAS and STING¹⁴⁸. Sensing of VZV in HaCaT cells should be characterised in the context of the novel cGAS antagonist, ORF9, described in Chapter VI.

An aspect of VZV restriction that was outside the scope of this study, is the involvement of type III IFNs in the restriction of VZV infection. Given that type III IFN have antiviral activity primarily in barrier surfaces such as the skin and mucosal tissues, it would only be logical that they are relevant in the context of VZV infection. Considering that infectious VZV particles mainly spread by being aerosolised through the characteristic blister rash, the skin and its cells are fundamentally involved in the life cycle of the virus. Indeed, infection of

HaCaT cells with VZV leads to secretion of IFN λ and in turn, IFN λ has an inhibitory effect on VZV replication similar to type I IFNs³⁸⁸. However, the relevance of type III IFNs in the context of VZV infection is underexplored, in particular *in vivo*. While type I and type III IFNs have overlapping antiviral activity, models of other viral infections have provided insight into their distinct functions. A mouse model of Influenza A virus (IAV) infection has demonstrated an intricate relationship between type I and type III IFNs *in vivo*²²⁷. Intra-bronchial infection with low viral loads triggers secretion of type III IFNs and their antiviral activity is sufficient for viral clearance. Only if type III IFNs are unable to control the infections is the secretion of type I IFN induced. Type I IFNs are more potent than type III IFNs but also have the potential to cause extensive tissue damage. The study proposes an elegant model in which type III IFNs control low-dose infections that remain silent due to the absence of tissue damage and inflammation. Only if absolutely required will the type I IFN system be activated to control the infections, at the cost of damage to affected organs. It is conceivable that the interplay of type I and type III IFNs is critically involved in control of VZV infection. The virus might further specifically antagonise or modulate both IFN pathways for optimal replication. It is poorly understood how, molecularly, activation of PRRs differentially induces expression of type I and type III IFNs. Furthermore, the type I and type III IFN receptors induce the same canonical downstream signalling cascade yet their activation leads to different transcriptional patterns. It would be interesting to explore these mechanisms in detail in the context of VZV infection.

Multiple studies have demonstrated previously that type I IFNs have the ability to inhibit VZV replication *in vitro*^{385–387}. Furthermore, type II IFN (IFN γ) inhibits VZV in a cell-line dependent manner³⁸⁷. The importance of type I IFNs is further highlighted by the fact that an ORF63-deleted VZV clone is hypersensitive to IFN α ⁴¹⁶. ORF63 encodes a type I IFN signalling antagonist. In this chapter, I have demonstrated that cGAS and STING are critically required for cells to induce type I IFNs after VZV infection. Given their potency in restriction of viral infection, we hypothesised that the virus will encode proteins that prevent type I IFN induction and/or signalling. In particular, we wanted to investigate whether VZV interferes with activation of cGAS and STING directly. The results of screens that aimed at identification of novel innate immune antagonists encoded by VZV are described in Chapter V.

Chapter V

Screening of VZV ORFs for Innate Immune Antagonism

The results discussed in Chapter IV show that infection of cells with VZV *in vitro* induces type I IFN. The ability of type I IFNs to limit viral infection has been demonstrated extensively (reviewed in REF. 202), including for VZV^{386;491;492}. Viruses therefore often dedicate significant genetic coding capacity to antagonists of the IFN pathway. Antagonism of the type I IFN system has been described for virtually all types of viruses, highlighting the importance of this system. Viral proteins can attack the type I IFN mediated cellular defence system at numerous points.

Firstly, viruses can target the induction of type I IFN by interference with detection of virus-derived PAMPs. The most important way for cells to detect viruses is through recognition of virus-derived nucleic acids. A quite straight-forward approach employed by viruses is therefore to spatially separate their replication from sensor proteins, i.e. to hide their genome. As mentioned in Chapter III, flaviviruses create replication factories separated from the cytoplasm by membranes. VACV, a dsDNA virus that replicates in the cytoplasm, employs a similar strategy⁴⁹³. In addition, even viruses that replicate in the nucleus use strategies to hide their genome. IAV, for example, has been demonstrated to use viral proteins to shield the 5'-triphosphates on its RNAs to prevent RIG-I activation⁴⁹⁴. Furthermore, viruses express RNA binding proteins to sequester RNA species formed during replication. For instance, the HSV US11 protein prevents protein kinase R (PKR) activation by binding to dsRNA⁴⁹⁵.

Secondly, another strategy used by viruses is to directly target cellular proteins involved in recognition. This includes direct inhibition or degradation of PRRs as well as proteins involved in PRR downstream signalling. For example, the NS1 protein of IAV inhibits activation of RIG-I through disruption of its interaction with TRIM25⁴⁹⁶. HSV-1 specifically inhibits the human cGAS protein by deamidation¹²⁸. Additionally, many viruses target IRF3

since signalling from multiple PRRs converges on this transcription factor. Interestingly, the VZV proteins ORF47, ORF61, and ORF62 have been shown to inhibit IRF3 through distinct mechanisms^{408–410}.

Thirdly, viruses can target the effects of type I IFNs on cells. This often happens through downregulation of the type I IFN receptor, degradation of transcription factors, or prevention of phosphorylation events. As an example, the NS5 proteins of multiple flaviviruses have been shown to employ various such mechanisms. ZIKV and DENV NS5 lead to the degradation of STAT2^{184;299;497} and WNV NS5 reduces IFNAR cell-surface expression levels⁴⁹⁸. The ORF63 protein of VZV has been demonstrated to degrade IRF9, a part of the transcription factor complex that induces the expression of ISGs downstream of the type I IFN receptor⁴¹⁵.

In summary, viruses employ a plethora of viral innate immune evasion strategies and the type I IFN system presents endless points of attack for viruses. The examples above only highlight a small fraction of those. We hypothesised that VZV, in addition to its known immunomodulatory ORFs, encodes further viral proteins that interfere with the type I IFN system. For the purpose of identifying novel innate immune antagonists, I generated an expression library for all unique ORFs encoded by VZV. I then screened the viral ORFs for antagonism of innate signalling using different luciferase reporter based systems.

V.1 Generation of a VZV ORF Library

To create a library of plasmids for the expression of individual full-length ORFs, I PCR-amplified the coding sequences from infected cell cDNA (see Methods section II.2.1.1). In cases where PCR from cDNA was unsuccessful, I amplified the coding sequences from infected cell total DNA (gDNA). VZV ORFs are intron-less genes, therefore amplification from cDNA or gDNA is equivalent. The only exception are ORFs 42 and 45. Initially described as individual ORFs and hence given separate names, these two sequences are now recognised as two exons of one intron-containing viral gene (called ORF42/45). I created two constructs, one called ORF42 which is amplified from cDNA and is therefore intron-less, and one called ORF45, amplified from gDNA, which covers the entire gene sequence. I was unable to create a full-length construct for ORF22 (the largest ORF) and instead obtained two previously published plasmids⁴³⁴.

Construct/ORF	Annotated function	Required <i>In vitro</i> ?	Length (bp)	MW (kDa)	Ectopic Expression	Note
0	RNA Transport	Essential/Impaired	390	19	Yes, correct MW	
1	Membrane protein	Dispensable	327	17	Yes, correct MW	
2	<i>Unknown</i>	Dispensable	717	31	No	
3	<i>Unknown</i>	Impaired	540	24	Yes, correct MW	
4	Transactivator	Essential	1359	57	Yes, correct MW	
5	gK	Essential	1023	44	Yes, incorrect MW	
6	Helicase/primase complex	Essential	3252	128	No	
7	<i>Unknown</i>	Dispensable	780	33	Yes, correct MW	
8	Deoxyuridine triphosphatase	Impaired	1191	50	Yes, correct MW	
9	Complex with gE	Essential	909	38	Yes, correct MW	
9A	gN, syncytia formation	Essential	264	15	Yes, correct MW	
10	Transactivator, efficient virion assembly	Dispensable	1233	52	Yes, correct MW	
11	RNA binding	Dispensable	2460	94	No	
12	Predicted tegument protein	Dispensable	1986	79	Yes, correct MW	
13	Thymidylate synthetase	Dispensable	906	40	Yes, correct MW	
14	gC	Impaired	1683	66	Yes, correct MW	
15	Integral membrane protein	Dispensable	1221	50	No	
16	DNA polymerase processivity factor	Essential	1227	51	Yes, correct MW	
17	RNA cleavage	Essential	1368	56	Yes, correct MW	
18	Ribonucleotide reductase, small subunit	Impaired	921	41	Yes, correct MW	
19	Ribonucleotide reductase, large subunit	Impaired	2328	92	Yes, correct MW	
20	Intercapsomeric triplex component	Essential	1452	59	Yes, correct MW	
21	Nucleocapsid protein	Essential	3117	121	Yes, correct MW	
22A	<i>Unknown</i>	Essential	1809	71	No	<i>a</i>
22B	<i>Unknown</i>	Essential	2103	84	Yes, correct MW	<i>a</i>

Continued on next page

Construct/ORF	Annotated function	Required <i>In vitro</i> ?	Length (bp)	MW (kDa)	Ectopic Expression	Note
23	Small capsid surface protein	Impaired	708	30	Yes, correct MW	
24	<i>Unknown</i>	Essential	810	36	Yes, correct MW	
25	DNA packaging	Essential	471	23	Yes, correct MW	
26	DNA packaging	Essential	1758	71	Yes, correct MW	
27	Nuclear matrix protein	Essential	1002	43	Yes, correct MW	
28	DNA polymerase	Essential	3585	139	Yes, correct MW	
29	ssDNA binding protein	Essential	3615	133	Yes, incorrect MW	
30	DNA packaging	Essential	2313	92	Yes, incorrect MW	
31	gB	Essential	2607	103	Yes, incorrect MW	
32	ORF47 kinase substrate	Dispensable	432	21	Yes, correct MW	
33	Major capsid scaffold protein; protease	Essential	1818	70	Yes, correct MW	
33.5	Minor capsid scaffold protein	Essential	909	38	Yes, correct MW	
34	DNA packaging	Essential	1740	70	Yes, correct MW	
35	Cell-cell fusion	Impaired	774	35	No	
36	Deoxypyrimidine (thymidine) kinase	Dispensable	1026	43	Yes, correct MW	
37	gH; three domains DI-DIII	Essential	2526	99	Yes, correct MW	
38	<i>Unknown</i>	Essential	1626	65	Yes, correct MW	
39	Integral membrane protein	Essential	723	32	No	
40	Major nucleocapsid protein, hexon/penton component	Essential	4191	160	Yes, correct MW	
41	Minor capsid protein	Essential	951	40	Yes, correct MW	
42	DNA packaging	Essential	2241	88	Yes, correct MW	<i>b</i>
43	DNA packaging	Essential	2031	79	Yes, correct MW	
44	<i>Unknown</i>	Essential	1092	45	Yes, correct MW	
45	DNA packaging	Essential	574	40	Yes, correct MW	<i>b</i>
46	<i>Unknown</i>	Essential	600	28	Yes, correct MW	
47	Serine/Threonine kinase (conserved)	Impaired	1533	62	Yes, correct MW	

Continued on next page

Construct/ORF	Annotated function	Required <i>In vitro</i> ?	Length (bp)	MW (kDa)	Ectopic Expression	Note
48	Deoxyribonuclease	Essential	1656	66	Yes, correct MW	
49	<i>Unknown</i>	Impaired	246	14	Yes, correct MW	
50	gM	Impaired	1308	54	Yes, correct MW	
51	Origin binding protein	Essential	2508	100	Yes, incorrect MW	
52	Helicase/primase complex (presumed)	Essential	2316	91	No	
53	<i>Unknown</i>	Essential	996	43	Yes, correct MW	
54	<i>Unknown</i>	Essential	2310	92	Yes, correct MW	
55	Helicase/primase complex	Essential	2646	104	Yes, correct MW	
56	<i>Unknown</i>	Essential	732	32	Yes, correct MW	
57	Virion egress	Dispensable	216	12	Yes, correct MW	
58	<i>Unknown</i>	Dispensable	666	30	Yes, correct MW	
59	Uracil-DNA glycosylase	Dispensable	918	39	Yes, correct MW	
60	gL, chaperone for gH	Essential	480	23	Yes, correct MW	
61	Transactivator/repressor; E3 ligase; IFN inhibition	Essential	1404	56	Yes, correct MW	
62/71	Transactivator; IFN inhibition	Essential	3933	145	Yes, correct MW	c
63/70	Phosphoprotein	Essential	837	36	Yes, correct MW	c
64/69	<i>Unknown</i>	Dispensable	543	25	Yes, correct MW	c
65	Virion protein	Dispensable	309	17	Yes, correct MW	
66	Serine/Threonine kinase	Dispensable	1182	49	Yes, correct MW	
67	gI; binds gE	Impaired	1065	44	Yes, correct MW	
68	gE, large unique N-terminus	Essential	1872	75	Yes, correct MW	

Table V.1: The 72 constructs of the VZV ORF expression library. The library encompasses 72 constructs which cover 70 of 71 unique VZV ORFs in full length and one unique ORF with two partial constructs. For individual ORFs the annotated functions and whether the ORF is required for viral growth *in vitro* are based on data summarised in REF. 325. MW states the predicted molecular weight in kDa based on expression of the construct from the pLenti63/TO/V5 construct (ORF + V5-tag). Data in the Expression column is based on overexpression in HEK293T cells (see Figure 28 and Figure 29). *a*: No full length ORF22 constructed could be cloned. ORF22A and ORF22B constructs were obtained from collaborator (see section II.2.1.1). The ORF22A construct covers nucleotides 2219-4029, the ORF22B construct nucleotides 4012-6114. *b*: The ORF42/45 contains an intron. Spliced cDNA (designated ORF42) and the intron-containing sequence (designated ORF45) was cloned. *c*: ORF69, ORF70, and ORF71 are duplications of ORF64, ORF63, and ORF62, respectively.

These partially cover the ORF22 sequence and are suitable for Gateway recombination. All coding sequences were shuttled into the pLenti6.3/TO/V5 vector for transient or lentiviral expression of the viral proteins with a C-terminal V5-tag. In total, the entire library encompasses 72 constructs which cover 70 of 71 unique VZV ORFs in full length and one unique ORF with two partial constructs. The constructs and the ORFs they encode are summarised in Table V.1.

I wanted to test which of the constructs in the library express stable proteins and whether these have the expected molecular weights. Individual constructs were transfected into HEK293T cells and ectopically expressed proteins were analysed by immunoblotting (Figure 28 and Figure 29).

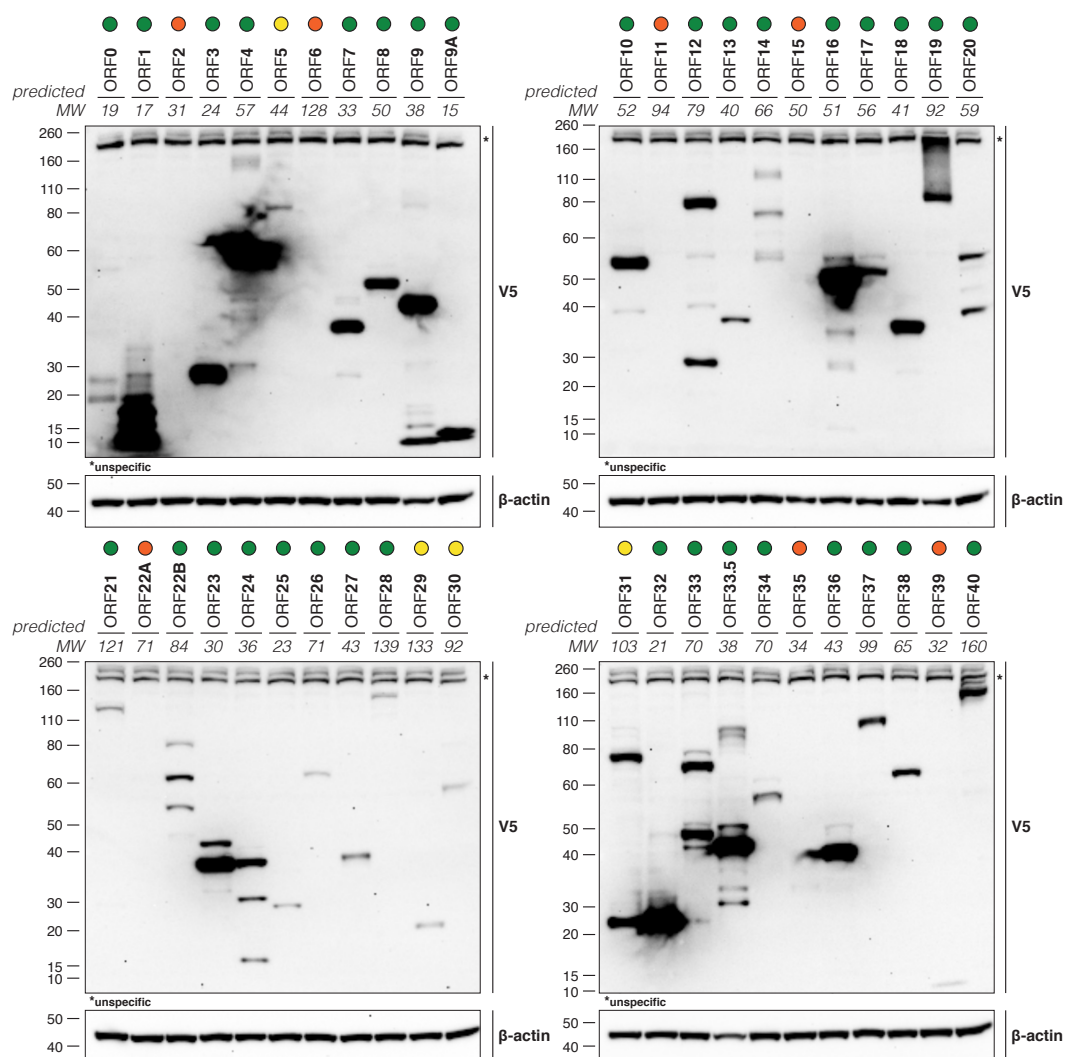


Figure 28: VZV ORF library immunoblot ORF0 - ORF40. HEK293T cells were transfected with indicated expression plasmids. The next day lysates were prepared and subjected to immunoblotting using the indicated antibodies. Circles above immunoblot lanes indicates expression at predicted MW (green), at the wrong MW (yellow), or no expression (orange). Expression data are summarised in Figure 29B. The data are representative of two independent experiments.

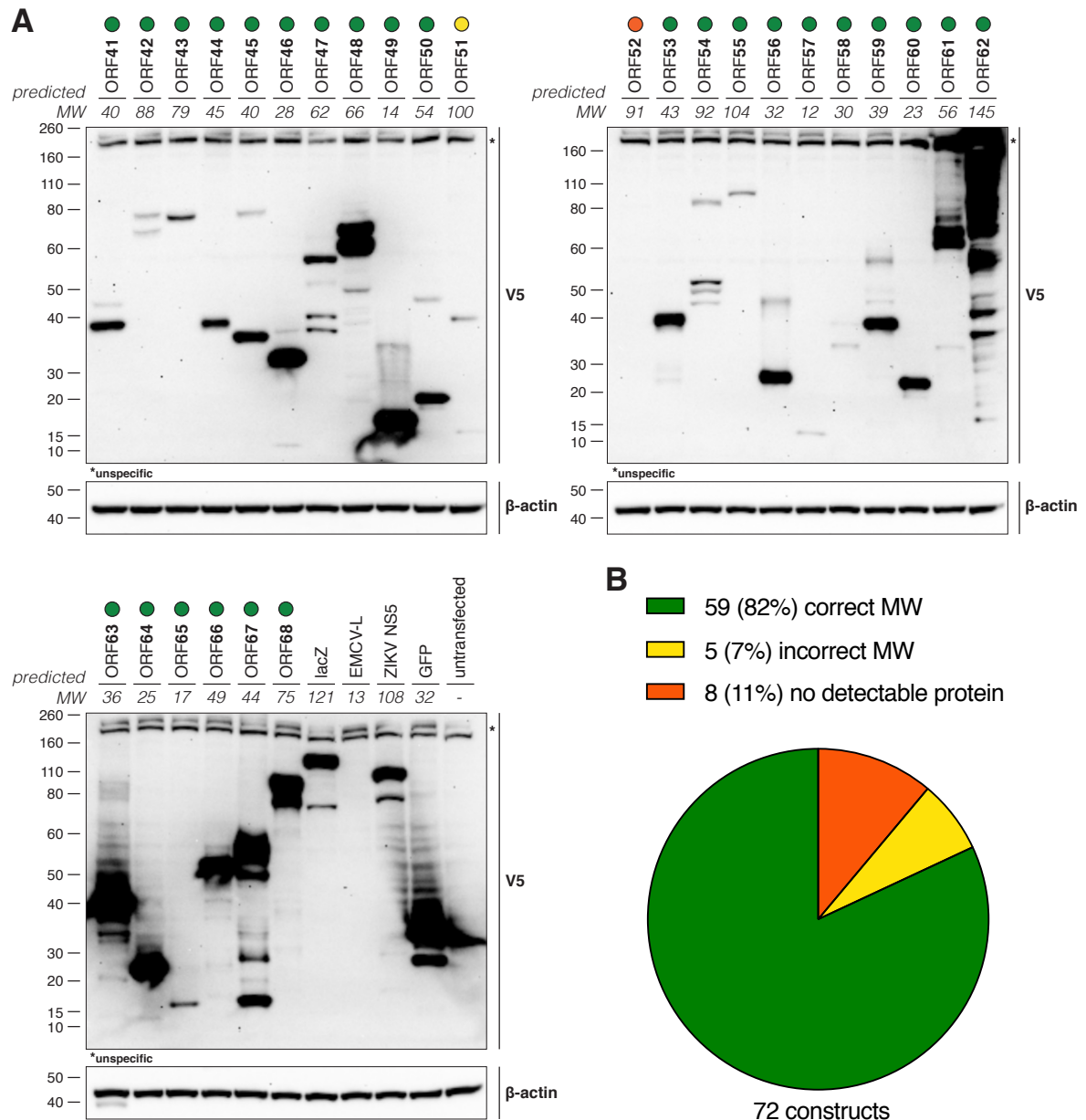


Figure 29: VZV ORF library immunoblot ORF41 - ORF68. (A) HEK293T cells were transfected with indicated expression plasmids. The next day lysates were prepared and subjected to immunoblotting using the indicated antibodies. Circles above immunoblot lanes indicates expression at predicted MW (green), at the wrong MW (yellow), or no expression (orange). (B) Summary of information about expression of the ORF constructs indicated by coloured circles in Figure 28 and this Figure. The data are representative of two independent experiments.

Expression at the expected molecular weight is indicated by green circles. Yellow and orange circles denote expression at the wrong molecular weight or no expression at all, respectively. In summary, 59 out of 72 constructs are expressed at the expected molecular weight. For eight constructs no protein could be detected by immunoblotting (see summary graph in Figure 29B).

V.2 Screening for IFN Signalling Antagonism

After validating the expression of the ORF library I wanted to investigate whether any VZV ORF(s) would interfere with signalling from the type I IFN receptor. HEK293 cells were transfected with expression plasmids for individual ORFs, a reporter plasmid that expressed Firefly luciferase under the control of an *ISG54* promoter, and a Renilla luciferase expressing plasmid as transfection control. The next day, cells were treated with recombinant type I IFN and luciferase activity was measured 24 h later. After IFN stimulation, binding of transcription factors to the ISREs within the *ISG54* promoter drove luciferase expression. This in turn allowed identification of ORFs which prevent this activation. For each ORF, a luciferase fold change to empty vector transfected, untreated control samples was calculated. A z-value was then obtained for each ORF based on the results for all ORFs. A z-value expresses the number of standard deviations a given value is different from the mean of all values. The results of one experiment are shown in Figure 30A with ORFs sorted by descending z-value.

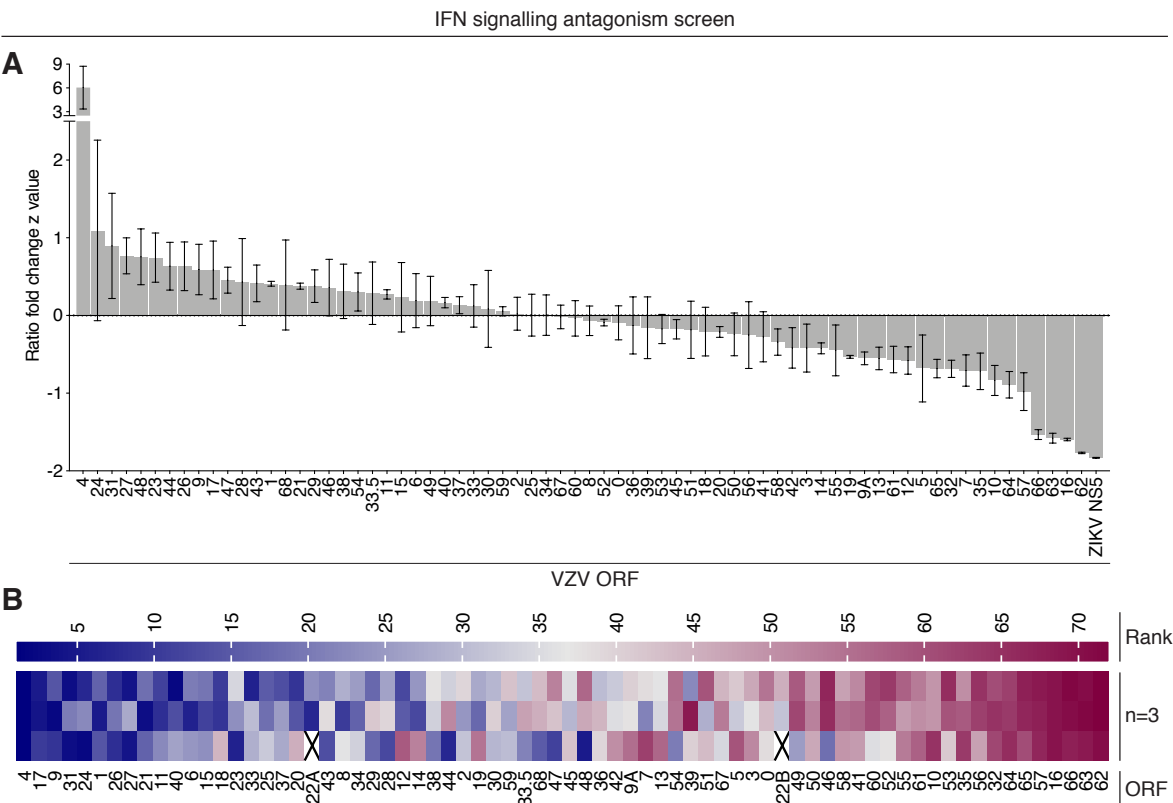


Figure 30: Individual VZV ORFs were screened for IFN signalling antagonism. (A) HEK293 cells were transfected with pRL-TK, ISG54-F-Luc, and expression plasmids for individual ORFs. The next day cells were treated with 25 U/ml recombinant IFN β . 24 h luciferase activity was determined. For each ORF, a z-value was calculated and ORFs sorted by descending values. (B) The experiment shown in panel A was repeated three times and for each ORF in each experiment a rank was determined according to z-value (highest z-value = rank 1). Ranks are displayed as a heatmap. Panel (A) is representative of three independent experiments (average and SD (technical triplicates)). The three independent experiments are summarised in panel (B). Crossed-out cells in (B) indicate that the construct was not used in this experiment.

The purpose of this experiment was to identify ORFs that inhibit signalling. Consequently, ORFs of interest are shown on the right end of the x-axis. The strongest phenotype is shown by ZIKV NS5, a positive control for antagonism of IFNAR signalling¹⁸⁴. The experiment shown in Figure 30A was repeated independently three times. For each experiment, ORFs were ranked based on their z-value (highest z-value corresponds to rank 1). A heatmap summarising the results is shown in Figure 30B. One of the consistently lowest ranking VZV proteins is ORF63, which is in line with previous reports that have characterised ORF63 as an antagonist of IFN signalling through IRF9 degradation. For a number of VZV ORFs (e.g. 16, 32, 57, 62, 64, 65, 66) results similar to those for ZIKV NS5 and ORF63 could be obtained. These viral proteins are therefore candidates for possible novel IFN signalling antagonists.

V.3 Screening for RIG-I Antagonism

After generating a dataset for antagonism of IFN signalling, I tested whether VZV ORFs can prevent the response to IVT-RNA *via* RIG-I. Screening for RIG-I antagonists may seem counter-intuitive given that VZV is a dsDNA virus and RIG-I senses 5'-triphosphate RNA. However, it is being increasingly recognised that RNA sensing mechanisms are able to restrict infection with DNA viruses. Vice-versa, proteins important in the recognition of cytosolic DNA, such as STING, are involved in defence against RNA viruses. Contributing to this phenomenon is the fact that virus infections can lead to the presence of endogenous ligands for DNA- and RNA-sensors^{175;499}. A great example of this is the cellular response to DNA released from damaged mitochondria in the context of DENV infection, an RNA virus^{76;75}. The significance of this particular restriction pathway is highlighted by the fact that DENV actively antagonises cGAS/STING signalling^{75;500}. For HSV, it was demonstrated that infection generates endogenous RIG-I ligands¹⁷⁵ and that RIG-I activation is blocked by viral proteins^{501;502}. For VZV, to have a RIG-I antagonist could be beneficial for an additional reason. RNA polymerase III can transcribe pathogen-derived AT-rich DNA into RNA molecules which then have antiviral activity through RIG-I and type I IFN¹⁷. Clinical evidence points at a crucial role of the RNA polymerase III DNA-sensing pathway for VZV recognition during CNS infections^{503;504;391}. It would therefore be conceivable that VZV encodes a direct antagonist of RIG-I. To test this, I utilised a similar assay system as for the ISRE screen

described before. Instead of the *ISG54* reporter plasmid, I used the p125 reporter plasmid which expressed Firefly luciferase under control of the IFN β gene promoter. HEK293 cells were transfected with ORF expression constructs and luciferase plasmids. The next day, cells were additionally transfected with IVT-RNA to stimulate RIG-I. Luciferase activity was measured 24 h later.

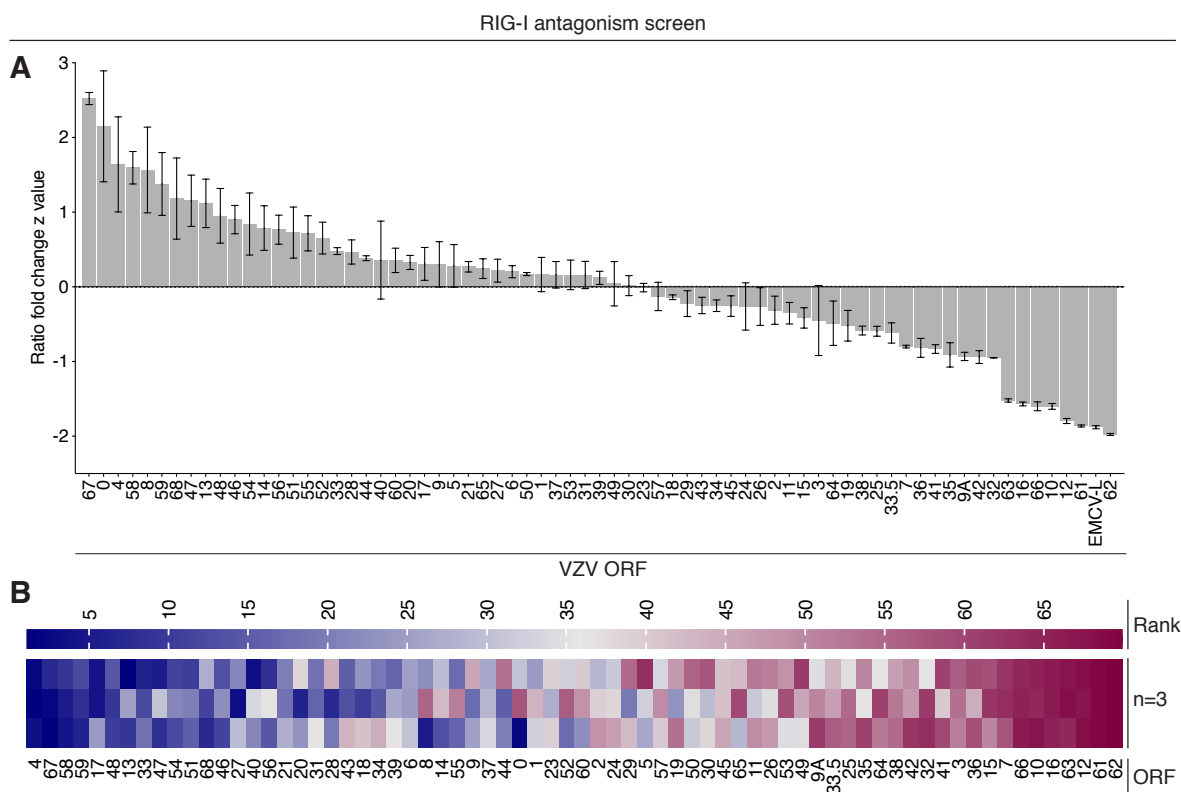


Figure 31: Individual VZV ORFs were screened for RIG-I antagonism. (A) HEK293 cells were transfected with pRL-TK, p125-F-Luc, and expression plasmids for individual ORFs. 24 h later cells were transfected with IVT-RNA. The next day luciferase activity was determined. For each ORF a z-value was calculated and ORFs sorted by descending values. (B) The experiment shown in panel A was repeated three times and for each ORF in each experiment a rank was determined according to z-value (highest z-value = rank 1). Ranks are displayed as a heatmap. Panel (A) is representative of three independent experiments (average and SD (technical triplicates)). The three independent experiments are summarised in panel (B).

As previously, results were analysed by calculating z-values (Figure 31). The L-protein of EMCV, an IRF3 antagonist, served as a positive control in these experiments⁵⁰⁵. Ranking lowest, i.e. showing the strongest inhibition phenotype, are known IRF3 antagonists encoded by VZV (ORF61, ORF62). This validates the assay in that I can identify previously described signalling antagonists. In addition to these known antagonists, the ORFs 12, 16, 10, 66, 7, and 15 reproducibly rank high and therefore represent candidates ORFs for RIG-I antagonism.

V.4 Screening for cGAS/STING Antagonism

In Chapter IV, I presented results that showed a critical role of the cGAS/STING system for the type I IFN response to VZV. Given these data, we specifically wanted to investigate whether the virus expressed a protein that antagonises cGAS directly. For this purpose, I set up a cGAS/STING overexpression assay in HEK293T cells combined with the p125 *IFN* β reporter used previously. HEK293T cells do not endogenously express cGAS and STING⁵⁰. Ectopic expression of both proteins activates transcription of the *IFN* β gene given that, once expressed, cGAS responds to the plasmid DNA present in the cells. Co-transfection of individual ORFs allowed for the identification of viral proteins which interfered with cGAS induced type I IFN activation.

HEK293T cells were co-transfected with the p125 luciferase plasmid, expression plasmids for human cGAS and human STING, and expression plasmids for individual ORFs. Luciferase intensity was measured 24 h later. As previously, I performed this screening assay three times. An exemplary result and a heatmap summary of the three independent experiments are shown in Figure 32 A and B, respectively. Again, the EMCV-L protein and known VZV IRF3 antagonists (VZV ORF61, ORF62) consistently rank lowest, i.e. strongly inhibit reporter luciferase expression. This demonstrates clearly that with this assay I was able to identify ORFs which inhibit signalling downstream of cGAS and STING. To identify viral proteins which act directly on cGAS and/or STING, I sub-screened the 16 highest ranking ORFs from the heatmap analysis shown in Figure 32B. The candidate ORFs were again screened for cGAS/STING antagonism as described above. In parallel, the *IFN* β luciferase reporter was also stimulated by co-expression of RIG-I-CARD with the ORFs. RIG-I-CARD is a constitutively active variant of RIG-I. Upon expression the protein stimulates *IFN* β transcription *via* TBK1 and IRF3. At the point of TBK1, the downstream signalling of RLRs and the cGAS/STING pathway converge. Therefore, if an ORF inhibits reporter activation both by cGAS/STING and RIG-I-CARD, it must act on TBK1 or downstream. However, ORFs that selectively inhibit activation by cGAS/STING must be directly acting on those proteins (or associated regulatory proteins). The results of this mini-screen are shown in Figure 32C. ORF12 was used in this experiment as a negative control since it did not inhibit reporter activation by cGAS/STING in the full screen (Figure 32B). In possible future experiments, an unrelated negative control, such as GFP, would be desirable.

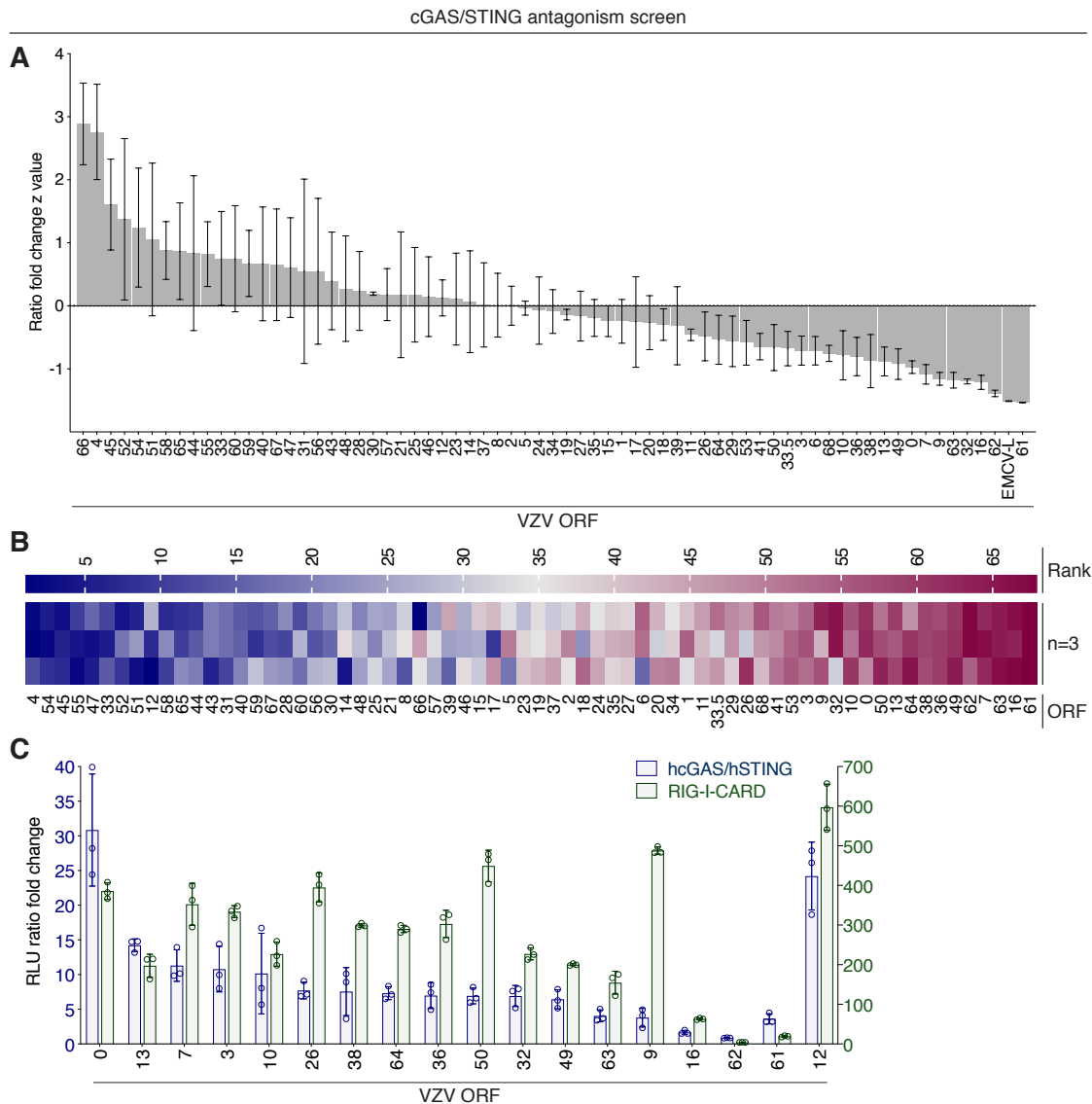


Figure 32: Screening of individual VZV ORFs for direct antagonism of cGAS/STING identifies candidate viral proteins. (A) HEK293T cells were transfected with pRL-TK, p125-F-Luc, expression plasmids for human cGAS and human STING, as well as expression plasmids for individual ORFs. The next day luciferase activity was determined. For each ORF a z-value was calculated and ORFs sorted by descending values. (B) The experiment shown in panel A was repeated three times and for each ORF in each experiment a rank was determined according to z-value (highest z-value = rank 1). Ranks are displayed as a heatmap. (C) For candidate ORFs HEK293 cells were transfected as in (A). In parallel, reporter expression was stimulated by co-transfection of the RIG-I-CARD plasmids instead of hcGAS and hSTING for the same ORFs. A luciferase fold change was calculated to unstimulated cells. Panel (A) is representative of three independent experiments (average and SD (technical triplicates)). The three independent experiments are summarised in panel (B). Panel (C) is representing one independent experiment (average and SD (technical triplicates)). The experiments in this figure were performed in collaboration with Sonja Roll, an undergraduate rotation student I supervised.

The IRF3 antagonists ORF61 and ORF62 potently inhibited both cGAS/STING and RIG-I-CARD (Figure 32C). Interestingly, I could also identify three viral proteins which appear to selectively inhibit cGAS/STING, but not RIG-I-CARD (ORF26, ORF50, ORF9). The strongest phenotype could be observed for ORF9. It inhibited cGAS/STING activation similar to ORF61 but left RIG-I-CARD activation unaffected. I therefore aimed to investigate the mechanism by which ORF9 inhibits cGAS/STING, as described in Chapter VI.

V.5 Discussion

In this chapter, I have presented data describing the creation of an expression library for all VZV ORFs. The library encompasses all unique VZV ORFs. Most of them can be detected on protein level after transfection. The majority (>80 %) of expressed ORFs further appear to have the expected molecular weight of the full-length protein.

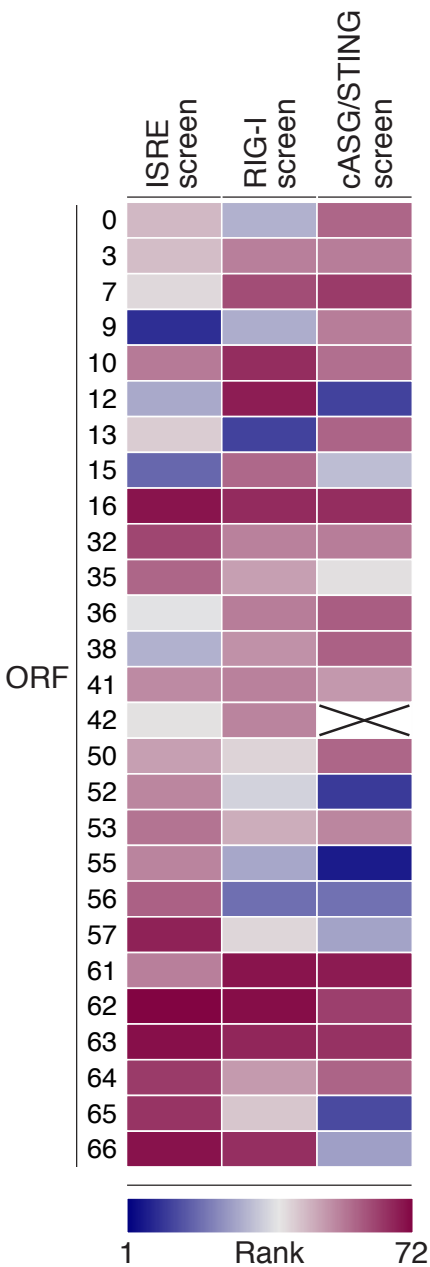


Figure 33: Heatmap comparison of top ranking ORFs from the different screens. The 15 lowest ranking ORFs of each screen are displayed. The colours correspond to the rank each ORF had on average in the respective screen. Crossed-out cells indicate that the construct was not used in these experiments.

I have used the library to screen all ORFs for antagonism of IFN signalling, antagonism of RIG-I activation, and antagonism of cGAS/STING. Each of the screens produced a list of candidate viral antagonist proteins. A number of viral proteins came up as candidates in more than one screen. This is not unexpected given that the intracellular pathways of the different screens share signalling proteins. For further analysis, I have taken from each screen the 15 ORFs with the lowest z-value and compared their ranks in the different screens (Figure 33).

Firstly, it is reassuring to note that only one viral protein, ORF9, appears to be low ranking in the cGAS/STING screen but not the other two assays. This reinforces the hypothesis that ORF9 specifically antagonises cGAS/STING activation but not related signalling pathways. It further justifies our choice to investigate ORF9 as a candidate cGAS/STING antagonist in more detail (see Chapter VI).

Secondly, we can identify viral proteins which only come up as low ranking in the ISRE screen (ORF52, ORF55, ORF56, and ORF57). These ORFs likely represent candidate proteins that directly interfere with type I IFN downstream signalling. ORF15 and ORF12 selectively inhibit reporter activation in the RIG-I screen. Similarly, these proteins are candidates for direct RIG-I

antagonism. Interestingly, ORF12 inhibited reporter activation after IVT-RNA stimulation of RIG-I but did not appear to affect stimulation by RIG-I-CARD expression (compare Figure 31B and 32C). The protein could affect RIG-I activation by sequestration of stimulatory RNA or inhibition of RIG-I conformational changes that expose its CARD domains for signalling. With these potential mechanisms ORF12 would affect MAVS activation after RIG-I stimulation by RNA but not after expression of constitutively active RIG-I-CARD and explain my observations.

Additionally, I could identify a number of viral proteins which appear to be inhibitory in both the ISRE and RIG-I screens (ORF66, and to lesser extents ORF35 and ORF65). These proteins likely inhibit IFN signalling but influence RIG-I activation through a secondary priming effect. Activation of RIG-I by IVT-RNA leads to the secretion of type I IFN which will stimulate expression of ISGs through the type I IFN receptor. RIG-I itself is an ISG and will increase in expression, thereby amplifying the signal from IVT-RNA stimulation. A viral protein that interferes with IFN signalling may therefore show up as low-ranking in the RIG screen as a consequence of the positive-feedback loop disruption. This phenomenon does not affect the cGAS/STING screen given that the sensor and adaptor proteins are ectopically expressed and therefore independent of cellular transcriptional control. For one of the candidates, ORF66, indirect evidence for a possible immunomodulatory function has been provided⁴¹⁴. After treatment of T-cells with IFN γ (type II IFN), reduced levels of phosphorylated STAT1 protein can be observed in VZV-infected cells compared to uninfected cells. However, pSTAT1 levels similar to mock infected cells could be detected in cells infected with an ORF66 deletion mutant of VZV. ORF66 consequently appears to be involved in modulating the phosphorylation levels of STAT1. STAT1 is a key transcription factor in downstream signalling of both the type I and type II IFN receptors. In this study, the absence of ORF66 during VZV infection had profound effects of viral replication in T cells but not MeWo cells⁴¹⁴. This could be explained by the fact that IFN γ is involved in the activation and differentiation of T cells. Alternatively, it is conceivable that in the absence of ORF66, type I IFN signalling is not inhibited and the virus cannot replicate in T-cells. This would not affect MeWo cells as these cells fail to phosphorylate STAT1 in response to WT virus in the first place (see Chapter IV). A direct immunomodulatory role of ORF66 in regard to type I IFNs remains to be confirmed experimentally.

Furthermore, the screens identified viral proteins that inhibit reporter activation in the

RIG-I and cGAS/STING screen but not the ISRE screen (ORF36 and ORF38). These proteins likely interfere with reporter activation at the level of TBK1 and/or IRF3. In addition to IRF3, downstream signalling from PRRs activates the NF κ B pathway. A number of further signalling proteins, such as NEMO and multiple IK kinases, are involved in the signal integration. NF κ B and IRF3 can act as transcription factors independently of each other and in conjunction. Both are required for expression of the *IFN β* gene while NF κ B can activate transcription of pro-inflammatory cytokines alone. In my experiments I did not use NF κ B reporter plasmids to further delineate possible specific signalling modulation and/or antagonism mechanisms. Interestingly, for many different viruses it appears that proteins involved in NF κ B signalling are required for replication and as a whole, the NF κ B pathway can have pro-viral roles (reviewed in REF. 506 and REF. 507). For example, viruses have evolved to hijack NF κ B proteins as transactivators of viral gene expression. This has been demonstrated for HSV-1 and HIV^{508;509}. Another example of herpesviruses specifically modulating the intertwined signalling cascades involving IRF3 and NF κ B is the MCMV protein m152. Downstream of STING m152 selectively blocks IRF3 activation but leaves NF κ B signalling unaffected as this is beneficial for the virus⁶³. Limited experimental evidence suggests that VZV, in contrast to HSV-1 and other herpesviruses, indeed antagonises NF κ B signalling^{411;412}. The role of NF κ B and related upstream and downstream signalling is unclear for VZV at present. ORF36 and ORF38 of VZV should therefore be further investigated for modulation or interference with NF κ B signalling.

Lastly, there are VZV proteins which had very low ranks in all three screens (ORF16, ORF62, ORF63). These proteins likely non-specifically inhibited intracellular signalling and/or suppressed transcription and translation. ORF16 is a viral DNA polymerase subunit that can form high-affinity interactions with DNA (UniProt entry Q4JQV9, protein properties inferred by homology to HSV). It is conceivable that once expressed, ORF16 can bind transfected plasmid DNA and prevent transcription. ORF62 is the major transactivating protein that regulates expression of many VZV genes upon infection³²⁵. ORF62 has further been described as an IRF3 antagonist⁴¹⁰. In my experiments, ORF62 does not only block IRF3-mediated reporter activation (in the cGAS/STING and RIG-I/IVT-RNA screen) but also activation of ISRE, independently of IRF3. ORF62 can bind directly to cellular transcription factors such as Sp1, USF, TBP, and TFIIB^{510–512}. These interactions are required for ORF62's transactivating function during viral replication. However, in an overexpression setting ORF62 alone

might have an global inhibitory effect on transcription due to its interaction with cellular transcription factors. This would explain the results I have obtained and suggests additional undescribed immunomodulatory functions of ORF62. ORF63 is previously described to inhibit IFN signalling through degradation of IRF9⁴¹⁵. In my experiments, it also disrupts IRF3-mediated IFN β reporter activation. The ORF63 protein can disrupt the cellular transcription pre-initiation complex and repress expression of genes controlled by viral and cellular promoters⁵¹³. My results likely represent mixed effects of direct IRF9 inhibition and possible global transcription repression.

In addition to ORF61 and ORF62, the viral protein ORF47 has been described previously to disrupt IRF3-mediated signalling⁴⁰⁸. However, in my experiments I could not recapitulate these findings for ORF47. In the RIG-I and cGAS/STING screens, both relying on IRF3 to activate the IFN β reporter, ORF47 actually enhanced rather than inhibited transcription (Figure 31B and Figure 32B). The published data for ORF47 originates from experiments with mutant virus infections. It is likely that IRF3 antagonism by ORF47 requires additional viral proteins that are not present in my assays.

A viral protein that, somewhat surprisingly, has not come up in any of the screens is ORF17. ORF17 encodes the virion host shutoff (vhs) of VZV. Vhs proteins downregulate cellular protein production by cleavage of cellular mRNAs. In my setting, one would expect ORF17 to degrade reporter mRNA. The protein is a homologue of the HSV1 vhs, UL41. In HSV-infected cells UL41 efficiently suppresses host cell protein biogenesis⁵¹⁴. In addition, ectopic expression of UL41 leads to cleavage of reporter mRNAs⁵¹⁵. However, ORF17 has been shown to only marginally affect cellular protein production⁵¹⁶. Furthermore, it did not cleave reporter mRNAs in previous studies^{517;518}. Host shutoff in the context of VZV infection is believed to be accomplished rather through transcription block by ORF63⁵¹⁸ (see above).

The VZV ORF expression library is an experimental tool for the medium-throughput screening of individual viral proteins for a given function. However, it is important to keep in mind that ectopic expression of individual viral genes without the context of viral infection can introduce artefacts. In the absence of other viral proteins or virus-induced changes in the host cell the expressed proteins might be mis-localised and degraded. Furthermore, ectopic expression in this setting relies on a constitutively active promoter. Viral proteins might not be stable or non-functional if they are not expressed in an infected cell with the correct

kinetics. In reverse, viral proteins might also be overactive if their expression and stability is not regulated by other viral proteins. Another aspect to consider is that all VZV proteins are expressed with a C-terminal V5-tag. The presence and/or location of an epitope tag can disrupt protein function. Lastly, false-negative results could have been obtained with proteins that need to be expressed with other viral proteins to be functional. For example, the ORFs 18 and 19 encode the small and large subunit of the viral ribonucleotide reductase and hence form a functional dimer.

In conclusion, the ORF expression library I created is a valuable tool for functional screens of individual proteins encoded by VZV. This library is a community resource which will be made available to interested researchers. This includes both Gateway-adapted entry clones and mammalian expression vectors, allowing for a wide array of applications.

Using the collection of plasmids I was able to identify a candidate cGAS/STING antagonist. I further present data on two other screens which, in summary, propose a number of VZV proteins as innate immune signalling antagonist for further validation and characterisation. In the next chapter I will present my results that describe ORF9 as a novel cGAS antagonist.

Chapter VI

Antagonism of DNA Sensing by VZV ORF9

The VZV proteins ORF9, ORF26, and ORF50 were identified to selectively antagonise *IFN* β reporter activation after stimulation of cGAS/STING, but not RIG-I (see Chapter V). Out of these three, ORF9 showed the strongest phenotype in terms of selective antagonism. ORF9 was further the only viral protein that did not appear high ranking in my screens for ISRE and RIG-I antagonism. We therefore decided to investigate ORF9 in more detail as a possible selective cGAS/STING antagonist.

The protein encoded by VZV ORF9 is a homologue of the HSV-1 VP22 protein and a member of the α -herpesvirus UL49 gene family³²⁵. ORF9 is one of 12 VZV tegument proteins of VZV⁵¹⁹ and highly expressed in infected cells. In fact, multiple studies have reported the ORF9 mRNA to be the most abundant one during lytic infection^{520;521}. ORF9 is an essential gene since transfection of cells with ORF9-deleted or -mutated VZV-BAC clones fails to produce progeny virus^{522;523}. Previous results have proposed a role of ORF9 in nuclear egress and secondary envelopment^{432;524;525}. ORF9 gets phosphorylated during infection by the viral kinase ORF47, and phosphorylated ORF9 is required for viral replication⁴³². An interaction of ORF9 with the ORF11 protein is necessary for proper tegument assembly during assembly of newly produced viral particles⁵²⁵. ORF9 interacts with the major transactivator of VZV, ORF62⁵²⁶. The functional relevance of this interaction, however, is unknown. Overall, the function of ORF9 during viral replication is incompletely understood. The protein therefore constitutes an interesting candidate for further investigation in the context of innate immune modulation.

VI.1 VZV ORF9 Interacts with cGAS

As a starting point of my investigations, I wanted to test whether the protein encoded by the ORF9 gene potentially interacts with proteins directly involved in sensing of DNA, such as cGAS and STING. V5-tagged ORF9 was expressed in HEK293T cells together with FLAG-tagged cGAS and HA-tagged STING. The ectopically expressed proteins were immunoprecipitated using antibodies against their epitope tags and IP fractions were analysed by immunoblotting (Figure 34A).

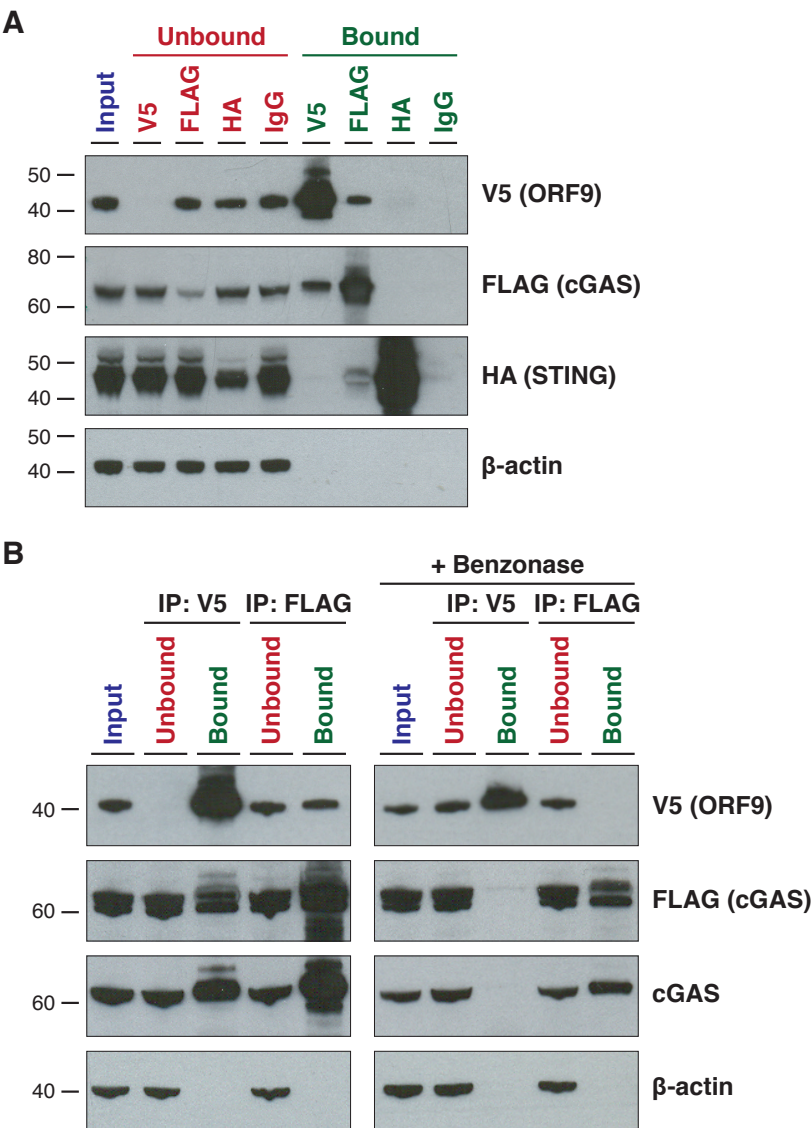


Figure 34: ORF9 interacts with cGAS in a nucleic-acid dependent manner. (A) HEK293T cells were transfected with expression plasmids for ORF9-V5, cGAS-FLAG, and STING-HA. 24 h later, cells were lysed and ectopically expressed proteins were immunoprecipitated using epitope tag antibodies. IP fractions were subjected to immunoblotting using the indicated antibodies. (B) HEK293T cells were transfected with expression plasmids for ORF9-V5 and cGAS-FLAG. 24 h later, cells were lysed and half the lysate was treated with benzonase. Ectopically expressed proteins were immunoprecipitated using epitope tag antibodies. IP fractions were subjected to immunoblotting using the indicated antibodies. Data is representative of one (A) or three (B) independent experiments.

All proteins were enriched in their respective bound fraction. Interestingly, cGAS protein could be detected in the fraction in which ORF9 was precipitated. *Vice-versa*, ORF9 also precipitated together with cGAS. Importantly, ORF9 protein showed no interaction with STING.

Given that cGAS is a DNA-binding protein, I wanted to test whether DNA is required for the interaction with ORF9. I repeated the experiment described before with ORF9 and cGAS only and treated one half of the lysate with benzonase. Benzonase is a nuclease that will degrade any nucleic acid under a wide range of biochemical conditions. The interaction between ORF9 and cGAS could be confirmed in the condition without benzonase (Figure 34B). Intriguingly, binding of the two proteins to each other was lost if the lysate was treated with benzonase. This indicates that a nucleic acid could indeed be involved in the proposed ORF9-cGAS interaction. Of note, the efficiency with which proteins were precipitated in this experiment was clearly lower when benzonase was present in the lysate. It is therefore possible that the observed effect is indirect rather than specifically due to the loss of a nucleic acid species.

To investigate whether it is DNA that is required for this interaction, I attempted immunoprecipitation experiments comparing the presence of DNase and RNase in lysates. However, it became apparent that the activity of recombinant DNase I is difficult to control in a cell lysate. DNase I is strongly inhibited by the presence of monovalent cations at concentrations present in a conventional cell lysis buffer. Furthermore, dsDNA fragments as short as 45bp can bind to human cGAS. Even if the DNase would be active in the lysate, it is likely that the digestion reaction could yield, at least in parts, fragments larger than 45bp. Considering the aforementioned limitations, I decided to investigate the involvement of DNA at a later point in this project using recombinant proteins in a cell-free system.

The results shown in Figure 34 demonstrate that ORF9 and cGAS proteins can bind each other. To confirm that this interaction actually occurs in a living cell, I expressed ORF9, cGAS, or both together in HEK293T cells and investigated their distribution using immunofluorescence analysis (Figure 35). Expression of ORF9 or cGAS alone resulted in exclusively cytoplasmic staining. In some cells, cGAS accumulated in cytoplasmic foci which also show a DAPI signal, i.e. DNA was present in these areas. Interestingly, in cells transfected with ORF9 and cGAS, both proteins appeared together in DNA-positive cytoplasmic foci. Importantly, the accumulation of ORF9 in foci could not be observed if the protein is expressed without cGAS. These results indicate that ORF9 and cGAS indeed interacted in cells.

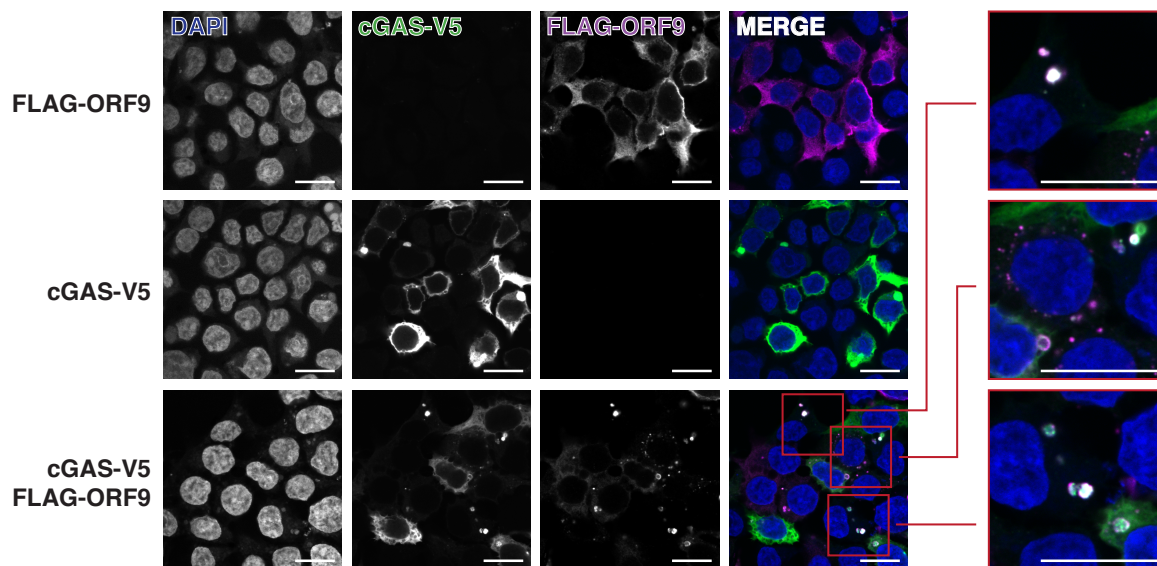


Figure 35: Ectopically expressed cGAS and ORF9 co-localise. HEK293T cells seeded on coverslips were transfected with expression plasmids for FLAG-ORF9, cGAS-V5, or both together. The next day, cells were fixed, permeabilised and stained with primary antibodies against the FLAG- and V5-tags and fluorescent secondary antibodies. Images were acquired using a confocal microscope. Scale bar: 15 μ m. Panel shows representative results of three independent experiments.

VI.2 VZV ORF9 Attenuates DNA Sensing Through cGAS

After confirming that the ORF9 protein has the ability to interact with cGAS, I wanted to investigate whether it can block sensing of DNA through endogenous cGAS. For that, I used THP1 monocytes, the same cell system used to investigate DNA sensing during VZV infection (see Chapter IV). Via lentiviral transduction I generated THP1 cells expressing ORF9 or GFP as a negative control protein. Expression of both proteins was validated as part of subsequent immunoprecipitation experiments (see immunoblot in Figure 36B). DNA-sensing was stimulated in these cells by transfection of increasing doses of DNA. *IFNB1*, *IFNL1*, and *TNFA* ($\text{TNF}\alpha$) transcript levels were used as a readout for activation of cGAS/STING (Figure 36A). As expected, GFP-expressing cells responded to transfected DNA by upregulation of *IFNB1*, *IFNL1*, and *TNFA* transcription in a dose-dependent manner. In contrast, cells that expressed ORF9 failed to respond entirely at low doses of transfected DNA. At higher doses, the response was attenuated when compared to GFP-expressing cells. These results showed that ectopically expressed ORF9 protein indeed had the ability to attenuate sensing of DNA and the downstream activation of the type I IFN system. To confirm the hypothesis that ORF9 attenuates sensing of DNA through interaction with cGAS, I immunoprecipitated ORF9 and GFP from the THP1 cells described before (Figure 36B).

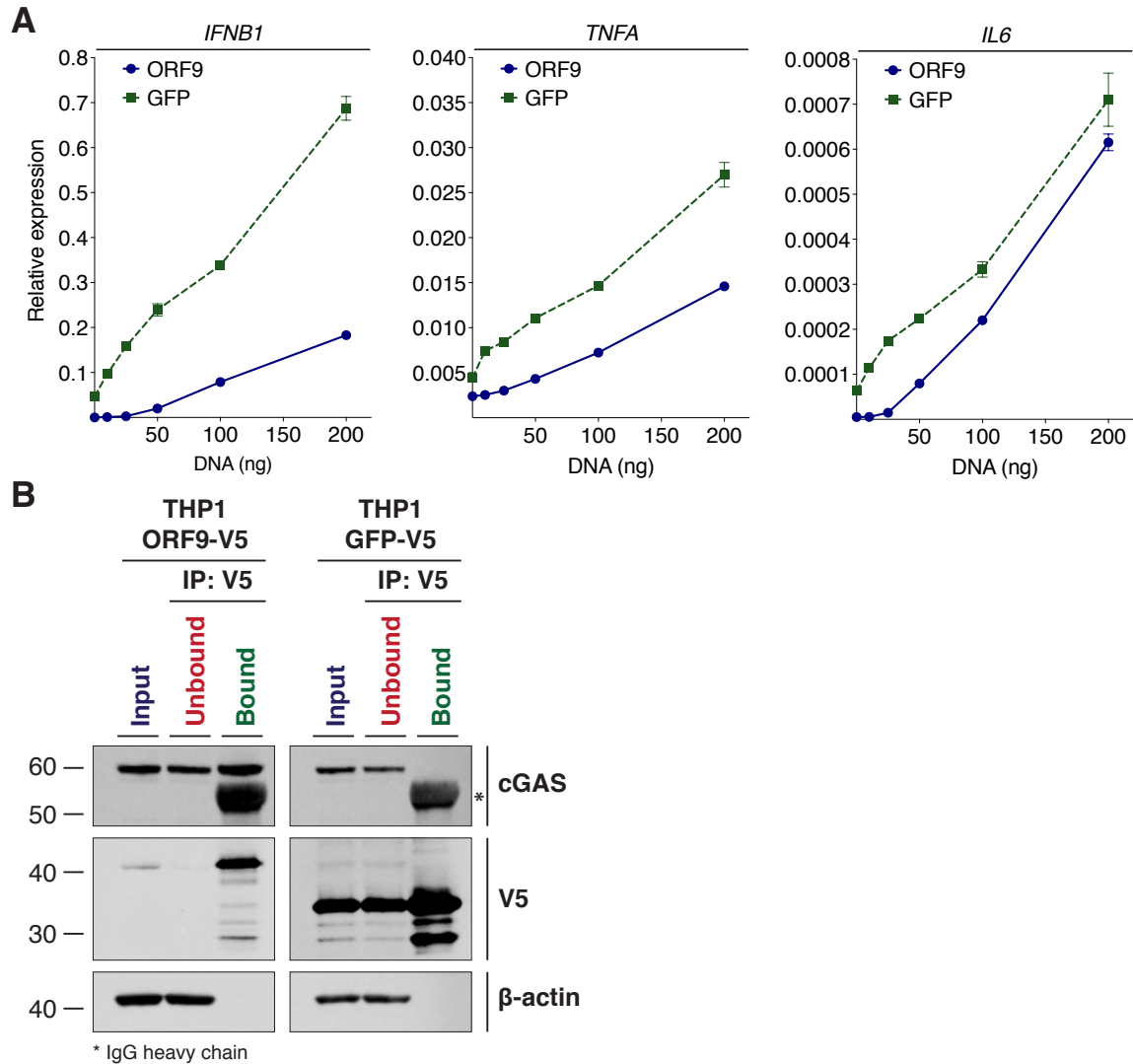


Figure 36: VZV ORF9 interacts with cGAS and blocks DNA sensing in THP1 cells. (A) THP1 cells stably expressing either ORF9-V5 or GFP-V5 were PMA-differentiated and transfected with indicated doses of DNA. The next day RNA was extracted and RT-qPCR was performed for indicated genes. Values show expression relative to *GAPDH*. (B) Cells used in (A) were PMA-differentiated, lysed 24 h later and ectopically expressed proteins were immunoprecipitated through their V5-tag. IP fractions were subjected to immunoblotting using the indicated antibodies. Panel (A) shows technical duplicates (average and range (n=2)) of one independent experiment. Panel (B) is representative of three independent experiments.

Endogenous cGAS protein could be detected in the bound fraction after precipitation of ORF9 but not GFP. This confirms that ectopically expressed ORF9 protein can interact with endogenous cGAS and further indicates that this interaction is relevant for functional antagonism of DNA sensing by ORF9.

VI.3 VZV ORF9 Interacts with cGAS in VZV-infected Cells

Next, I wanted to investigate whether endogenous ORF9 interacts with cGAS in VZV-infected cells. There is no commercial antibody available for the efficient immunoprecipitation of

cGAS. The polyclonal serum against ORF9 we have obtained from a collaborator is also not suitable for IP applications. I therefore made use of a recombinant virus in which a C-terminal V5 epitope tag was added to the endogenous ORF9 coding sequence in the viral genome (hereafter referred to as VZV^{ORF9-V5}). The recombinant virus additionally expresses GFP from the BAC-backbone used for recombination. I infected HaCaT and THP1 cells with VZV or VZV^{ORF9-V5} by co-culture with infected MeWo cells for 48 h. Similar to the infection experiments described in Chapter IV, this also included labelling of HaCaT and THP1 cells with a fluorescent tracer dye. After harvest, a small fraction of cells was retained for flow cytometry analysis of infection (Figure 37A). The remaining cells were lysed and incubated with an antibody against the V5 tag for immunoprecipitation. IP fractions were analysed by immunoblot (Figure 37B). In previous flow cytometry analyses of VZV infection the FITC-coupled antibody against VZV-gE/gI was used (see section IV.1.1 and Figure 16). GFP expression by VZV^{ORF9-V5} was incompatible with detection of FITC fluorescence.

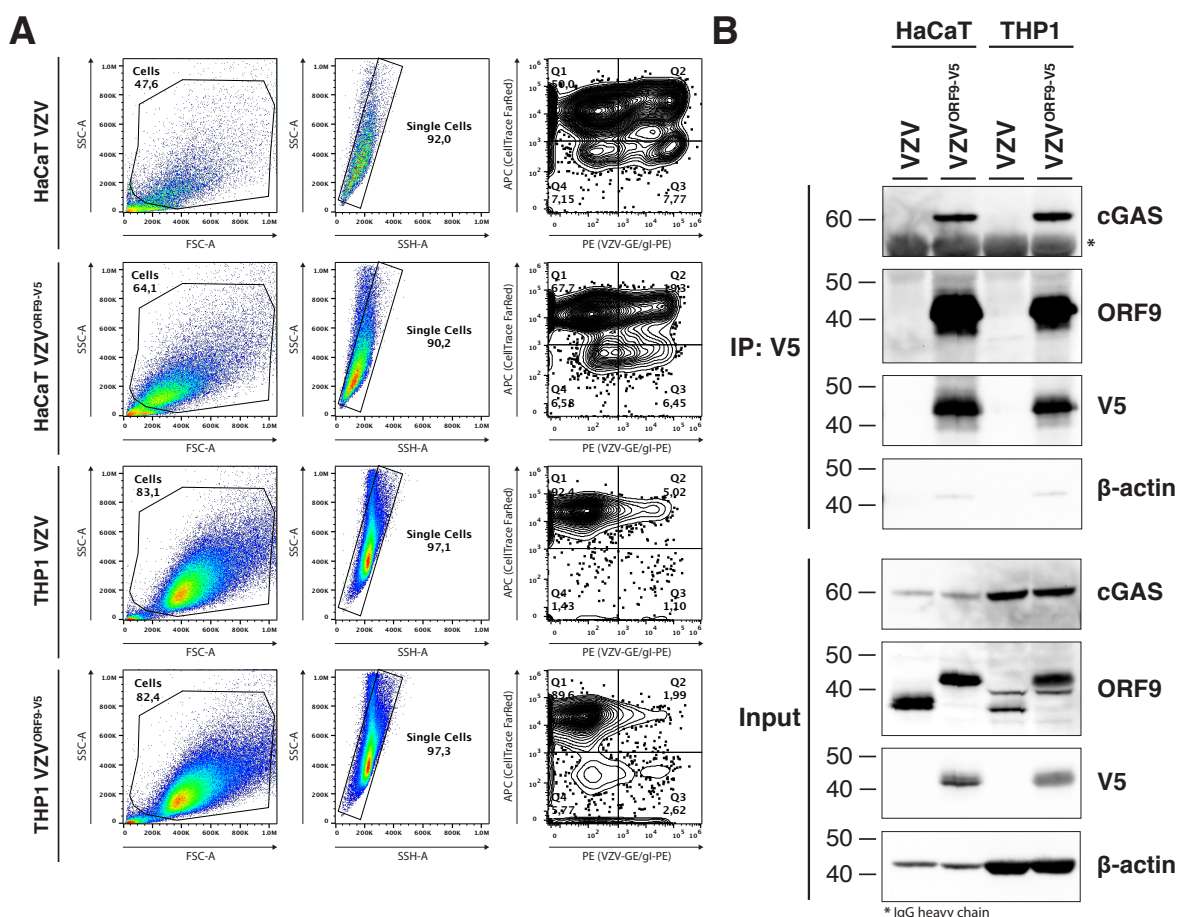


Figure 37: Endogenous ORF9 interacts with cGAS during VZV infection. HaCaT and PMA-differentiated THP1 cells were infected with WT VZV or VZV^{ORF9-V5} by co-culture. 48 h later cells were lysed and immunoprecipitated with antibody against V5. IP fractions were subjected to immunoblotting using the indicated antibodies. Data are representative of three (THP1) and two (HaCaT) independent experiments.

I therefore coupled VZV-gE/gI to PE (referred to as VZV-gE/gI-PE) and used this antibody to quantify infection in this experiment. Infection of HaCaT and THP1 cells with VZV yielded around 30% and 4% infected cells, respectively (Figure 37A). Numbers of infected cells were around half of those for VZV^{ORF9-V5}, with 15% in HaCaT cells and 2% in THP1 cells. Despite different rates of infection, I could identify a population of infected cells in all conditions. Immunoblot analysis of IP fractions further revealed a robust signal for ORF9 protein in the input fractions of all conditions, showing productive infection (Figure 37B). As expected, the V5 epitope could only be detected in cells infected with VZV^{ORF9-V5}. Bound fractions of both HaCaT and THP1 cells infected with VZV^{ORF9-V5} were enriched for ORF9 protein and V5 signal. Untagged ORF9 protein in lysates from cells infected with VZV was not precipitated. Only in bound fractions enriched for ORF9 protein could cGAS be detected. This showed that endogenous ORF9 expressed in infected cells indeed interacted with cGAS and that this interaction was not due to unspecific binding of cGAS to beads.

VI.4 VZV ORF9 Binds cGAS in a Cell-free System

In the previous section I presented results that show the ability of VZV ORF9 to bind to cGAS protein and inhibits its ability to activate type I IFN in response to DNA. To characterise the molecular interaction of ORF9 and cGAS in more detail, I decided to use a reductionist, cell-free approach with recombinant proteins. Coding sequences for N-terminally FLAG-tagged ORF9 and GFP, as well as C-terminally HA-tagged and untagged cGAS were inserted into the pGEX61 vector. Using *E.coli*, the genes of interest were expressed as GST-fusion proteins. From bacterial lysates, the proteins were affinity purified using glutathione beads. The GST-moiety was then removed by on-bead cleavage and the supernatant containing the recombinant protein was used for experiments. The detailed purification procedure is described in section II.2.18. Coomassie-stained protein gels showing samples from the purification steps and final protein preps can be found in Figure 38. For all produced proteins contaminating bands of lower and higher MW than the full-length protein were observed. I, however, decided to use these protein preparations for preliminary experiments. If promising results were to be obtained, I planned to optimise purification conditions.

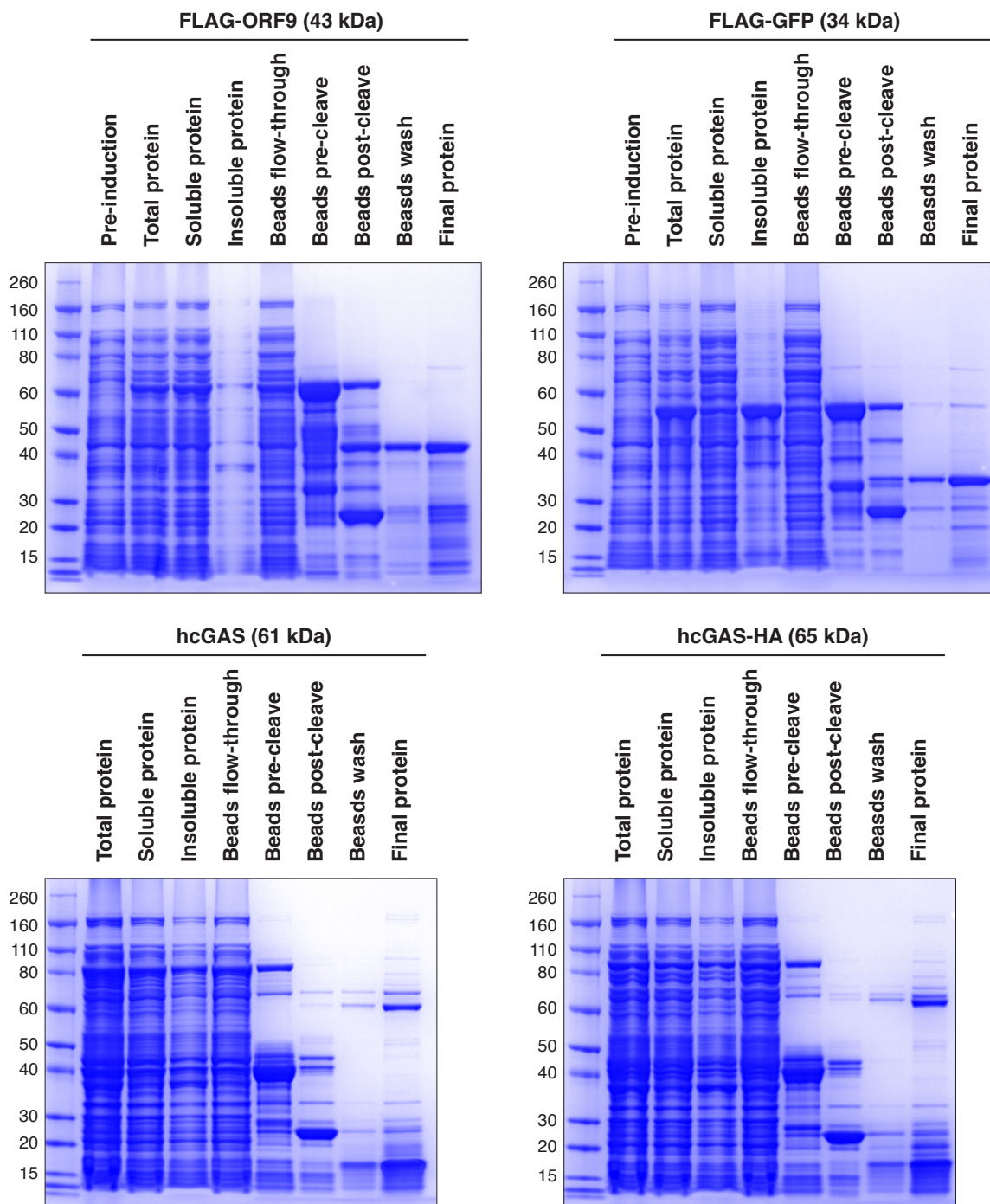


Figure 38: Recombinant protein production was analysed by SDS-PAGE. Images of Coomassie stained gels from production of recombinant proteins described in section II.2.18.

I first wanted to test whether recombinant cGAS and ORF9 can interact in a cell-free system. FLAG-ORF9 or FLAG-GFP were mixed with cGAS-HA protein in lysis buffer. The proteins were then precipitated *via* their tags and IP fractions were analysed by immunoblotting (Figure 39). The cGAS-HA protein was enriched very efficiently in the bound fraction from both FLAG-ORF9 and FLAG-GFP containing mixtures. ORF9 protein co-

immunoprecipitated with cGAS, indicating an interaction between these two proteins. GFP protein could also be detected in the bound fraction after cGAS IP, however, to a much lesser extent compared to ORF9. The inverse IPs, i.e. precipitation of ORF9 and GFP revealed that only ORF9, but not GFP, were able to co-precipitate cGAS protein. Taken together, these data strongly indicate that ORF9 and cGAS protein have the ability to interact specifically in the absence of other cellular proteins.

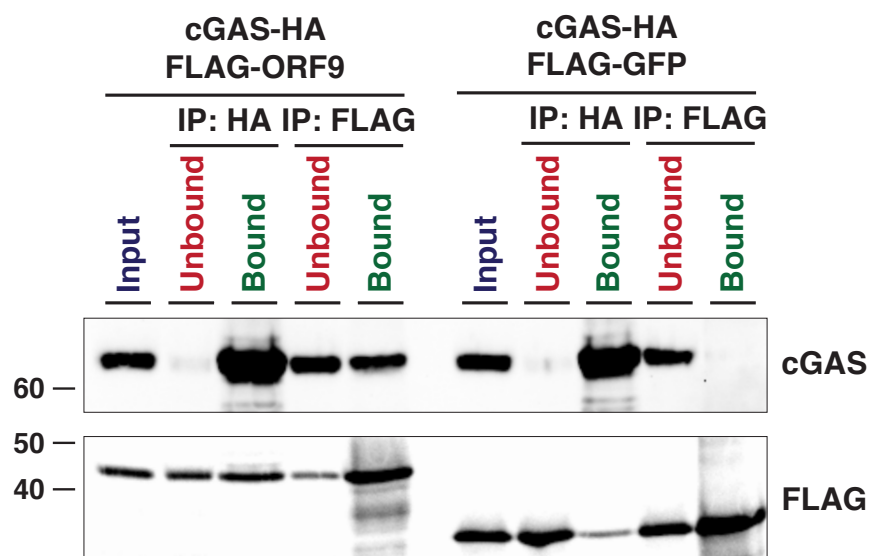


Figure 39: ORF9 and cGAS protein interact in a cell-free system. Recombinant cGAS-HA protein was incubated with recombinant FLAG-ORF9 or FLAG-GFP protein in lysis buffer. Proteins were immunoprecipitated using epitope tag antibodies and IP fractions were subjected to immunoblotting using indicated antibodies. Data are representative of two independent experiments.

To confirm the direct interaction of cGAS and ORF9, I chose to utilise the Far West-ern technique. Protein lysates or purified proteins are separated by denaturing SDS-PAGE and transferred onto nitrocellulose membranes. The proteins on the membrane are then fully denatured and renatured by incubation in decreasing concentrations of guanidine hydrochloride. Recombinant proteins are then used as probes to recognise proteins on the membrane. The probes can be detected with antibodies and visualised just as a conventional immunoblot. The procedure is also summarised in Figure 40A. For my experiments, I separated FLAG-GFP and FLAG-ORF9 on SDS-PAGE and used recombinant cGAS protein and an anti-cGAS mAb for detection (Figure 40B). A band at the size of FLAG-ORF9 but not of FLAG-GFP could be observed after detection of cGAS protein. After stripping, both FLAG-ORF9 and FLAG-GFP could be detected using an antibody against FLAG, since both proteins were initially separated by SDS-PAGE. This result showed clearly that, in this

assay, recombinant cGAS specifically interacted with ORF9 but not GFP. Similarly to the results discussed previously, no cellular proteins were involved here.

I also attempted the reverse experiment and separated cGAS-HA and cGAS protein by SDS-PAGE. FLAG-ORF9 or FLAG-GFP served as probes (Figure 40C). They were detected using an anti-FLAG antibody. The ORF9 protein recognised both cGAS proteins while using GFP as a probe did not yield any detectable signal. This confirms that ORF9 and cGAS indeed interact in this cell-free system.

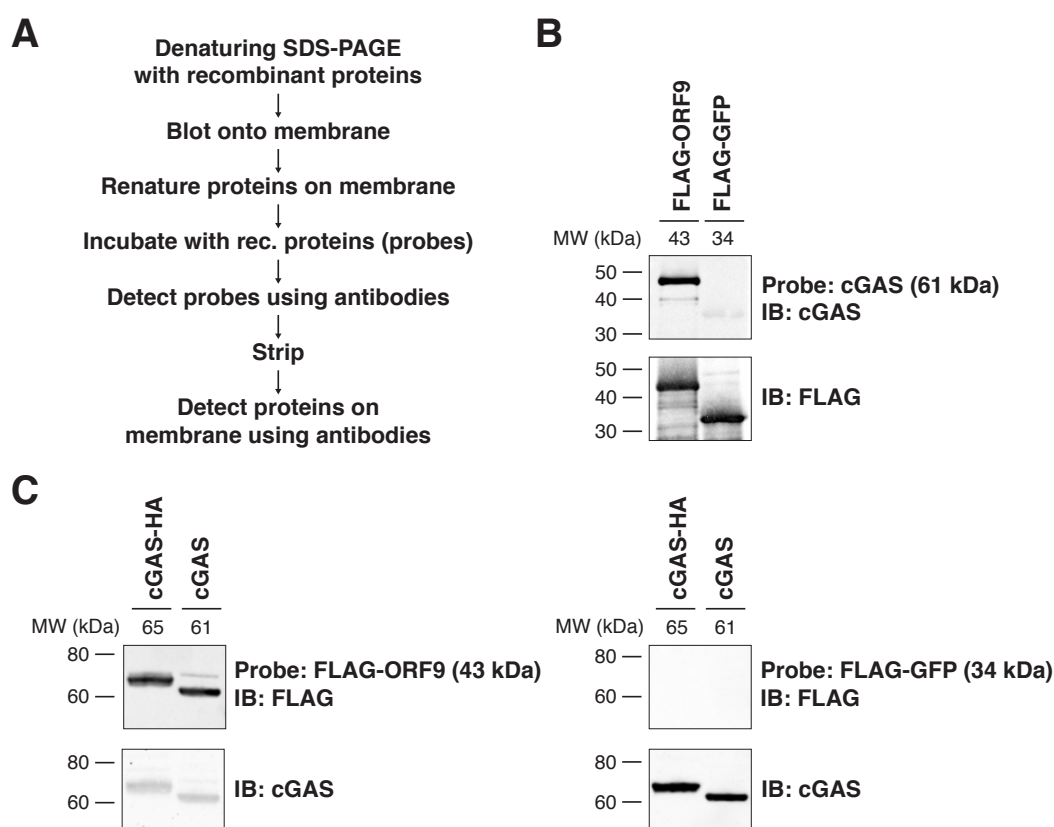


Figure 40: The Far Western technique detects ORF9-cGAS interaction. (A) Step-by-step description of the Far Western technique. (B) Recombinant FLAG-ORF9 and FLAG-GFP protein were subjected to denaturing SDS-PAGE. Recombinant cGAS protein was used as a probe and detected using anti-cGAS antibody. FLAG-tagged proteins on the membrane were detected using anti-FLAG antibody. (C) Recombinant cGAS-HA and cGAS protein were subjected to denaturing SDS-PAGE. Recombinant FLAG-ORF9 or FLAG-GFP protein was used as a probe and detected using anti-FLAG antibody. The cGAS proteins on the membrane were detected using anti-cGAS antibody. Data are representative of two independent experiments.

VI.5 VZV ORF9 Directly Binds to dsDNA

At the beginning of this Chapter, the DNA dependency of the interaction between ORF9 and cGAS was discussed briefly. An interesting question in this context is whether the ORF9 protein itself can bind dsDNA. To test this, I incubated recombinant FLAG-ORF9 protein with a biotinylated version of VACV 70mer, a 70 bp dsDNA sequence from VACV. The nucleic acid

was then precipitated using streptavidin coupled beads. Recombinant cGAS protein was used as a positive control since it is a known dsDNA binding protein. FLAG-GFP served as a negative control. A further control condition included reactions without DNA. Input fractions and proteins bound to beads were analysed by immunoblotting (Figure 41). As expected, cGAS protein could be precipitated with streptavidin beads in the presence of VACV-70mer-Biotin. Interestingly, ORF9 protein could also be precipitated with DNA. This interaction occurred with ORF9 alone, but also if ORF9 was mixed with cGAS or GFP. GFP failed to bind to DNA in all conditions. ORF9 and cGAS protein was not precipitated with beads alone, demonstrating that this was indeed DNA dependent. These results clearly demonstrate that the VZV ORF9 protein has the intrinsic ability to bind to dsDNA.

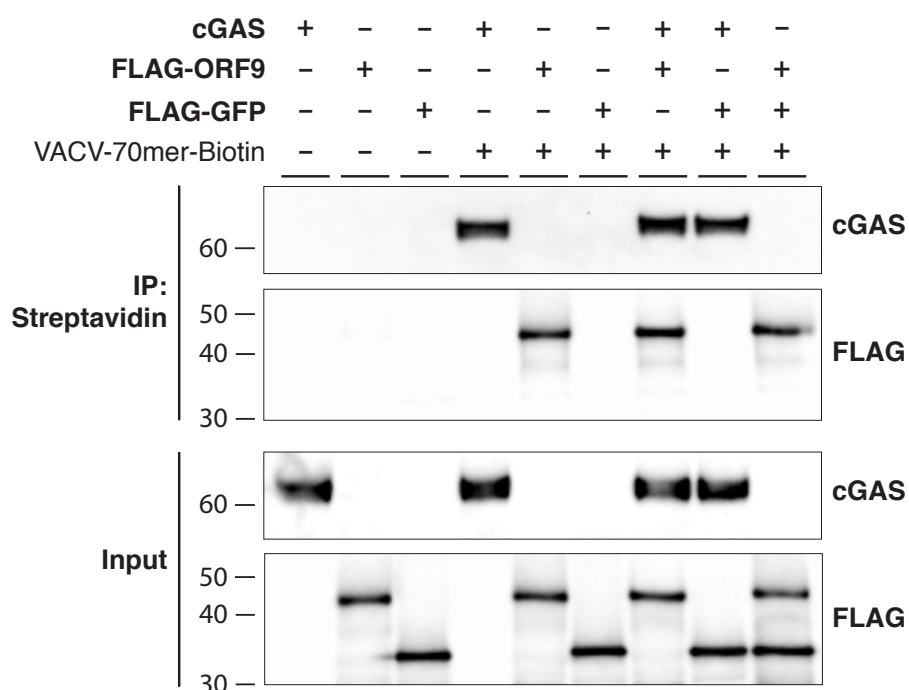


Figure 41: ORF9 protein binds DNA in a cell-free system. Recombinant cGAS, FLAG-ORF, FLAG-GFP, or indicated combinations of these proteins were incubated with or without biotinylated dsDNA (VACV-70mer) in lysis buffer. DNA was precipitated using streptavidin beads. Input and precipitated fractions were subjected to immunoblotting using the indicated antibodies. Panel shows result of one experiment.

VI.6 The N-terminal Half of VZV ORF9 is Dispensable for cGAS Binding

After having characterised binding of ORF9 to cGAS, I was interested which part or domain of the ORF9 protein is responsible for antagonism of cGAS. For this, I sought to construct

truncated versions of ORF9 and test these for cGAS binding. To inform design of the truncation mutants I used an algorithm that can predict whether regions of a protein are ordered or disordered based on the amino acid (AA) sequence. It further predicts secondary structure features, such as α -helices and β -sheets, in ordered regions. Analysis of the ORF9 AA sequence revealed two predicted ordered regions, one in the N-terminal half of the protein sequence and one in the C-terminal half (Figure 42A).

In the N-terminal half there are four successive sheets followed by a helix. Between AA 151 and AA 240, there are further three helices and a sheet predicted. Based on these sequence features, I constructed seven truncation mutants of ORF9 (Figure 42B).

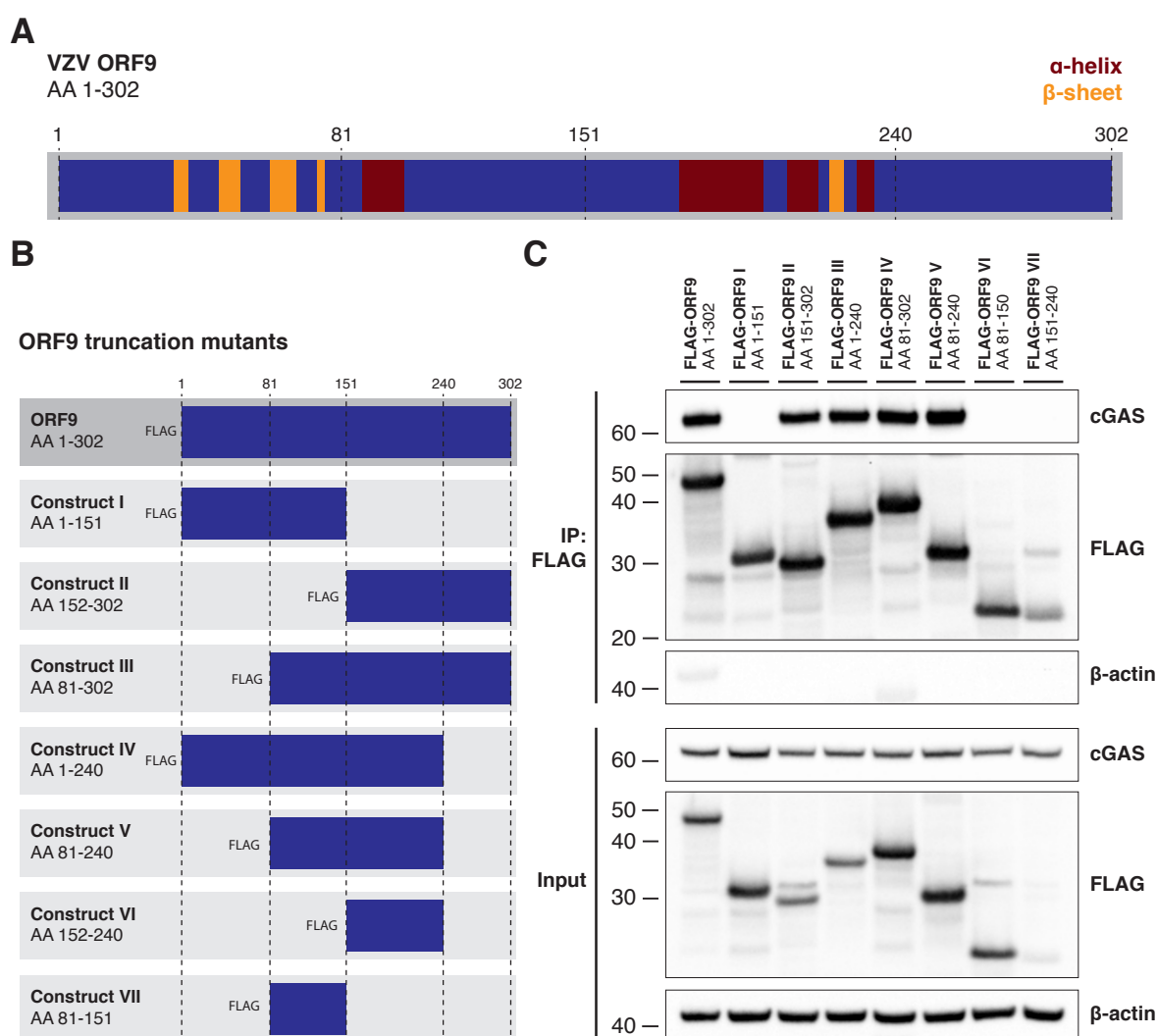


Figure 42: ORF9 AA 1-151 are dispensable for cGAS binding. (A) Schematic detailing the amino acid sequence of VZV ORF9 regarding ordered regions. The presence of α -helices and β -sheets was predicted based on AA sequence using the PHD prediction algorithm (see Methods). (B) Schematic showing the truncation mutants of ORF9 that were constructed and used in experiment shown in (C). (C) HEK293T cells were transfected with an expression plasmid for cGAS-V5 and indicated, FLAG-tagged truncation variants of ORF9. 24 h later cells were lysed and immunoprecipitated with antibody against FLAG. IP fractions were subjected to immunoblotting using the indicated antibodies. Data are representative of two (ORF9 variants I-V) or one (ORF9 variants VI-VII) independent experiments.

These include both halves of the protein (Constructs I, II), the C-terminal end of the protein without the N-terminal sheets (Construct III), the N-terminal end of the protein without the disordered C-terminal sequence (Construct IV), the middle region of the protein including most ordered features (Construct V), and Construct V further split into two (Constructs VI, VII). All truncation mutants were constructed with a N-terminal FLAG tag similar to the full-length protein.

All different variants of ORF9 were then expressed in HEK293T cells together with cGAS and immunoprecipitated *via* their FLAG-tag. Analysis of the IP fractions by immunoblot showed that all truncation mutants of ORF9 were expressed, with the exception of construct VII for which only a faint band could be detected in input lysates (Figure 42C). In all lysates the cGAS could protein be detected. As previously, cGAS robustly co-precipitated with the full-length ORF9 protein. It could further be detected bound to construct II, III, IV, and V, but not construct I and VII. Constructs II-V share AA 151-240 of the full-length ORF9 and this region contains the predicted helix-helix-sheet-helix feature in the second half of the protein. Unfortunately, construct VI, which covers exactly AA 151-240, failed to co-precipitate cGAS. These results nevertheless suggest the AA 151-240 region of ORF9 to be analysed in more detail since this construct might not fold correctly (see discussion). While the exact AAs sufficient for cGAS binding need to be further investigated, this experiment indicates that the motif is located in the C-terminal half of the ORF9 protein.

VI.7 Does VZV ORF9 Affect cGAMP Production by cGAS?

In section VI.2 I presented results showing that in THP1 cells the presence of ORF9 attenuated sensing of DNA *via* cGAS. Upon activation, cGAS synthesises the second messenger cGAMP. Inhibiting cGAS with the result of reduced type I IFN activation hence would imply reduced cGAMP production. To test whether ORF9 can inhibit the enzymatic activity of cGAS, I made use of an experimental system our group has characterised previously⁵⁰. Upon transfection of HEK293T cells with a plasmid expressing the gag protein of HIV and a plasmid expressing glycoprotein G of VSV as an envelope protein, these cells generate virus-like particles (VLPs, described in Figure 43A). If cGAS is co-expressed in VLP-producer cells, cGAMP will be incorporated into newly generated VLPs. This can be tested by transfer of VLP preparations onto HEK293 cells which naturally express STING⁵⁰. Upon infection with

cGAMP-loaded VLPs, the stimulation of STING by cGAMP will lead to secretion of type I IFN by these cells. Since the gag protein used here is fused to GFP, the infectivity of the VLP preparations can be measured by flow cytometry of the infected cells.

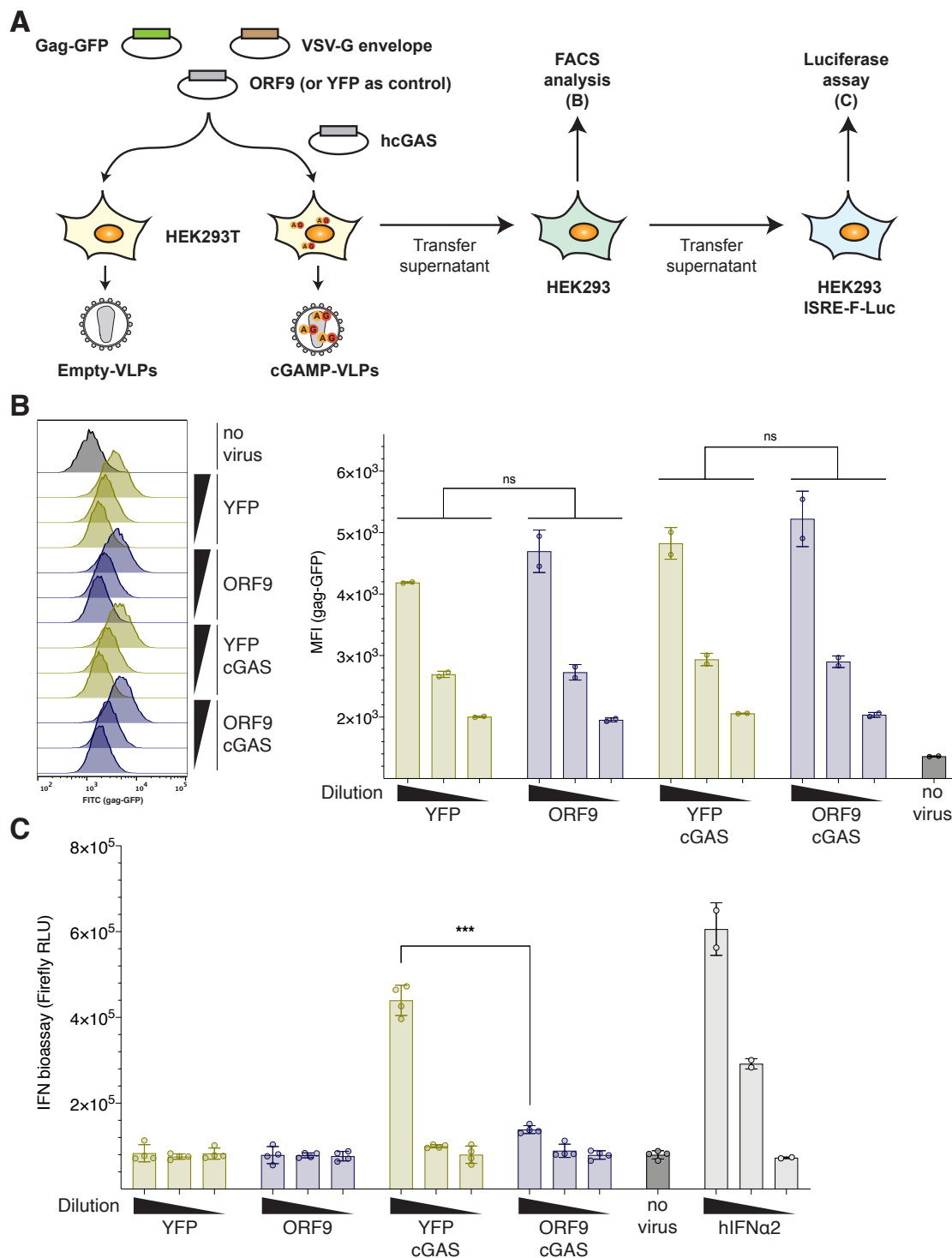


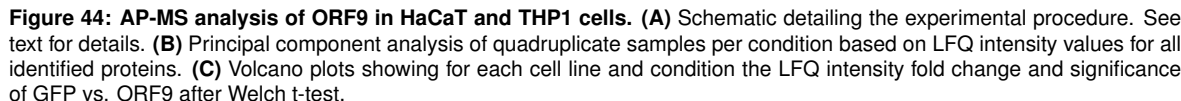
Figure 43: ORF9 inhibits cGAMP production by cGAS. (A) Schematic detailing the experimental procedure. See text for details. (B) Histogram and quantification of gag-GFP fluorescence measured by flow cytometry in cells infected with VLPs. (C) Type I IFN in supernatants of cells infected with VLPs was measured using an IFN bioassay. Recombinant human IFN α 2 was used as control. Panel shows results of one experiment. (B): Paired, two-tailed t-test. (C): Two-tailed t-test. ***= $p < 0.001$, ns = not significant

To measure the type I IFN secreted by the HEK293 cells, the supernatant is transferred onto ISRE luciferase reporter cells. I hypothesised that, if ORF9 was to be expressed in producer cells alongside cGAS, these would generate VLPs but those would not contain cGAMP.

I generated VLPs in the presence of VZV ORF9 or YFP as a negative control, both with and without co-expression of cGAS. Infection of HEK293 cells with VLPs resulted in similar measurable GFP fluorescence in all four conditions (Figure 43B). This showed that VLPs were indeed produced to similar levels, independent of the presence of ORF9. However, only VLPs produced in the presence of cGAS and YFP but not cGAS and ORF9 induced type I IFN secretion after infection (Figure 43C). This indicates that the presence of ORF9 during VLP generation prevented loading of the particles with cGAMP. These results might further suggest that this phenomenon is due to a negative effect of ORF9 on cGAMP production by cGAS.

VI.8 VZV ORF9 AP-MS Analysis Identifies Cellular Binding Partners

To elucidate further the molecular mechanism of cGAS antagonism by ORF9 and identify additional proteins in a possible complex, we decided to perform affinity purification mass spectrometry (AP-MS) analysis. These experiments were performed during a secondment in the laboratory of Andreas Pichlmair at the TU Munich. I generated stable HaCaT and THP1 cell lines that express either FLAG-ORF9 or FLAG-GFP. The ectopically expressed proteins were affinity purified from these cell line under two conditions: cells that were left untreated or cells that were transfected with ISD before harvest (see the schematic in Figure 44A). We decided to include DNA-transfected conditions in our analysis given the previous results on a possible role of DNA in the interaction of ORF9 and cGAS. This gave us four conditions: ORF9 mock, ORF9 DNA, GFP mock, and GFP DNA in both HaCaT and THP1 cells. Affinity purified protein complexes were then digested and peptides were analysed by LC-MS/MS (Figure 44A). Based on peptide counts proteins were identified and quantified in each sample. Each condition was analysed in quadruplicate samples. PCA analysis of the replicates showed that, overall, replicates of the same condition clustered together but different conditions separated (Figure 44B). For HaCaT cells, one replicate of the GFP mock condition clustered with the replicates of GFP DNA, and one replicate of ORF9 DNA clustered with ORF9 mock.



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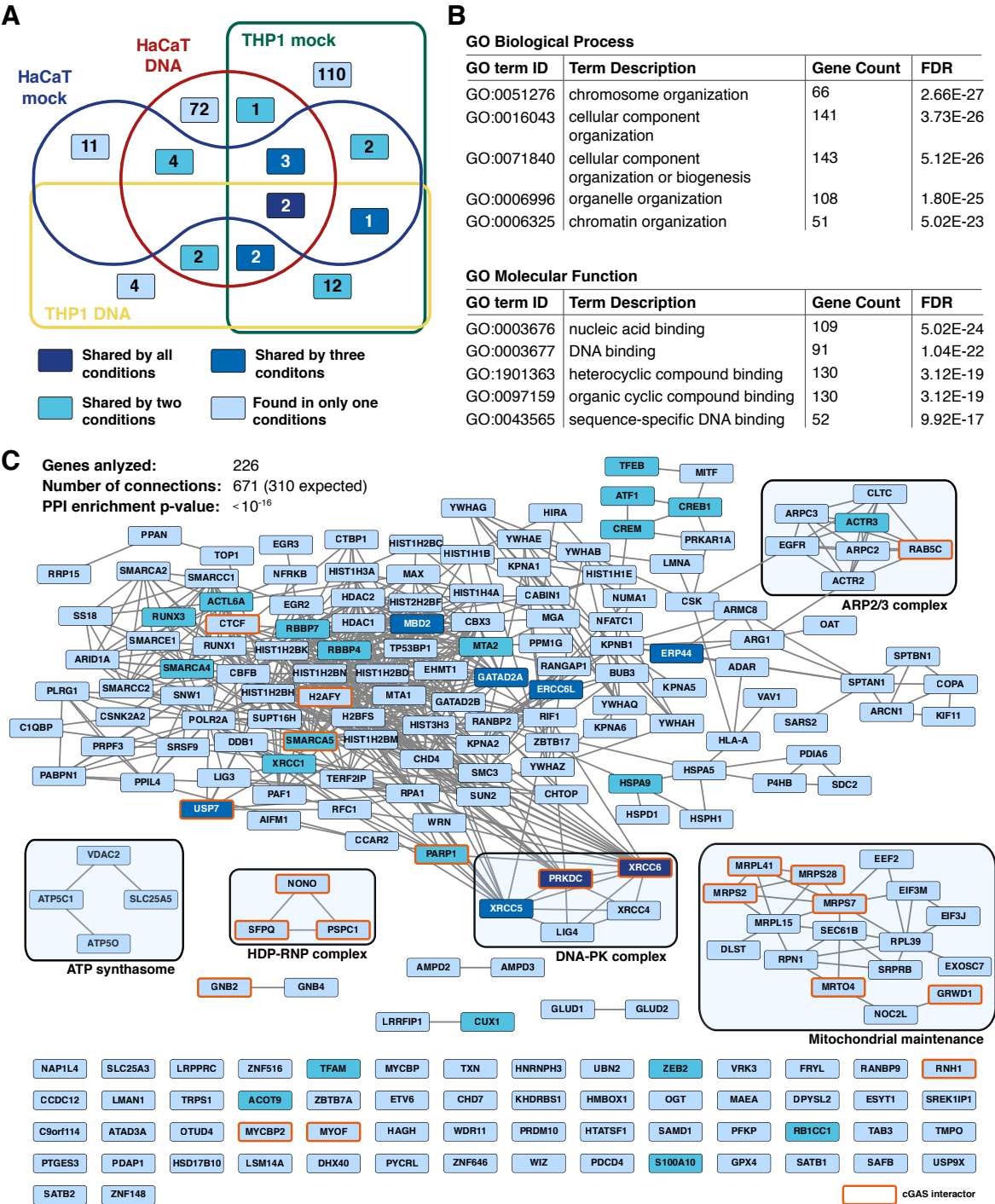
cluster together. This indicates that both proteins indeed precipitate different binding partners. Interestingly, it appears that for both cell lines all four conditions clustered in the PCA analysis with about the same distance from each other. This showed that for both ORF9 and GFP, DNA transfection induced the same degree of difference in immunoprecipitated proteins as between ORF9 and GFP overall.

Proteins that were enriched in ORF9 or GFP pulldowns were identified by statistical testing of ORF9 vs. GFP samples in each conditions (see Method section II.2.19 for details). Results were plotted as volcano plots showing the fold change between ORF9 and GFP, as well as the significance (Figure 44C). 87 and 78 proteins were significantly enriched in pulldowns of ORF9 from mock and DNA-transfected HaCaT cells, respectively. 132 and 21 proteins were significantly enriched the same conditions in THP1 cells. The large difference in the numbers for THP1 cells was likely a result of the fact that THP1 ORF9 DNA samples showed greater variance between each other (see PCA analysis in Figure 44B). As a result, the number of significant proteins was lower, given that significance in this case was largely dependent on reproducibility between replicates. Out of the 318 proteins identified, 226 were unique and mappable to protein databases. A full list of these proteins with data on fold enrichment and significance can be found in Table S1 in the Appendix.

Figure 45A shows a Venn diagram that details the distribution of proteins shared between the samples. Out of 226 proteins, 29 were found in two or more conditions. All 226 proteins were analysed using the STRING database of protein-protein interactions. The five most significantly enriched gene ontology (GO) biological process and molecular function terms are shown in Figure 45B. 109 proteins were identified to be nucleic acid binding, with 91 proteins binding specifically to DNA. In connection to that were 66 and 51 proteins identified to be involved in chromosome and chromatin organisation, respectively. These GO terms were significantly enriched in the group of 226 proteins identified compared to what would be expected by a randomly distributed list of proteins (demonstrated by the FDR values).

STRING database analysis further provides information about experimentally demonstrated interactions between proteins in a given group. A graphical representation of the annotated interactions between the 226 proteins I analysed is provided in Figure 45C. 671 connections were detected, which is significantly more than in a random group of proteins (see PPI enrichment value). This indicates that ORF9 interacted with proteins that are part of larger protein complexes. About half of the proteins were part of a large complex of

proteins that in itself consists of groups of proteins that are involved in chromosome organisation and transcription (top-left in 45C). Furthermore, proteins bound to ORF9 were part of the ARP2/3, DNA-PK, HDP-RNP, and ATP synthasome complexes, as well as a complex of proteins involved in mitochondrial maintenance.



Out of the 29 proteins that were identified in more than one condition, 20 are annotated as DNA binding and involved in biological processes related to chromatin. The proteins PRKDC and XRCC were significantly enriched in all four experimental conditions. MBD2, USP7, GATD2A, ERCC6L, ERP44, and XRCC5 were enriched in three out of four conditions. With the exception of USP7 and ERP44, these proteins are all DNA binding. Interestingly, cGAS was not among the interaction partners of ORF9 in any of the conditions. However, proteins described in previous studies to interact with cGAS were among the ORF9 binding partners and are highlighted with red borders in Figure 45C. The functional relevance of identified ORF9 interaction partners will be discussed below.

VI.9 Discussion

The results presented in this Chapter establish the protein encoded by the ORF9 gene of VZV as a novel antagonist of cGAS. I demonstrated that ORF9 protein interacted with cGAS and can inhibited cGAS activation. This interaction occurred if ORF9 was overexpressed, in VZV-infected cells, and in cell-free systems. Preliminary data indicated that ORF9 negatively affects cGAMP synthesis by cGAS. I further showed that ORF9 directly binds dsDNA.

VZV ORF9 is a homologue of the HSV-1 and HSV-2 VP22 protein (gene name UL49). ORF9 and VP22 belong to the family of VP22 tegument proteins found in α -herpesviruses that infect humans, cows, horses, and chickens. ORF9/VP22 do not have homologous proteins in human β - or γ -herpesviruses. VZV ORF9 and HSV-1 VP22 share 25 % amino acid sequence identity and 36 % similarity⁵²⁶. Within the core UL49 family homology region, which is located approximately between amino acids 130 - 250 of ORF9, VP22 and ORF9 share 43 % identity and 56 % similarity⁵²⁶.

Interestingly, it has been demonstrated previously that HSV-1 VP22 can inhibit activation of cGAS during HSV infection¹²⁹. Another study has found an intriguing structural similarity between VP22 of HSV-1 and a protein encoded by ORF52 of the murine γ -herpesvirus MHV68⁵²⁷. Crystallisation of parts of both proteins revealed a conserved Helix-Helix-Sheet-Helix confirmation (see schematic in Figure 46A). MHV68 ORF52 and its homologue in the human γ -herpesvirus KSHV have been characterised as cGAS antagonists¹²⁷. While no structural data is available for KSHV ORF52 and VZV ORF9, secondary structure prediction indeed revealed that a region of ORF9 and KSHV ORF52 could also fold into a Helix-

Helix-Sheet-Helix (H-H-S-H) confirmation (Figure 46A). Protein sequence alignment of VZV ORF9, HSV-1 VP22, KSHV ORF52, and MHV68 ORF52 further show a number of conserved residues that fall into the regions with the proposed conserved structure (residues highlighted in green in Figure 46B).

Similar to VZV ORF9, KSHV ORF52 has intrinsic DNA binding properties and this DNA binding ability is required for antagonism of cGAS¹²⁷. The lysine residues 68 and 69 were identified to be required for DNA binding. These residues are located within the predicted α_2 -helix of KSHV ORF52 (Figure 46A). K69 of KSHV ORF52 is part of a VxxLxAxxAxK consensus motif identified by sequence alignment of VZV ORF9, HSV-1 VP22, KSHV ORF52, and MHV68 ORF52 (grey box in Figure 46B). This motif falls within the first α -helix of the proposed H-H-S-H shared structural feature. The crystal structure of MHV68 ORF52 revealed that the protein forms a dimer and within that dimer, two α_2 -helices align to form a positively charged groove structure. Given that the K68/69 residues are required for DNA-binding, I hypothesise that this groove acts as a DNA-binding domain shared by VZV ORF9, HSV-1 VP22, KSHV ORF52, and MHV68 ORF52. I further propose a model in which a Helix-Helix-Sheet-Helix structural feature is conserved within tegument proteins of distantly related herpesvirus to exert cGAS antagonist functions.

While correct folding and localisation of ORF9 truncation mutants need to be verified in future experiments, my results indicated that the C-terminal half of the ORF9 protein was responsible for cGAS binding. Constructs lacking the C-terminal 61 AA also bound cGAS, pointing at a role of AA 151 - 240 in the interaction with cGAS. This region also harbours the above mentioned H-H-S-H structural feature. Expression of AA 151-240 alone did not result in cGAS binding. Future experiments with additional truncation and/or deletion mutants are planned to identify the precise region responsible for cGAS binding. Additional experiments will also address regions or residues of ORF9 responsible for DNA binding. It would in this context be interesting to mutate the AA K195 of ORF9 given that it corresponds to K69 of KSHV ORF52. This residue within the aforementioned VxxLxAxxAxK consensus motif is required for DNA binding. In light of the fact that ORF9 is easily expressed in *E.coli* as a soluble protein, it would certainly be interesting to attempt crystallisation of ORF9, potentially in a complex with cGAS and DNA (see Chapter VII).

While the proposed structure conservation between KSHV ORF52 and VZV ORF9 further strengthens the evidence of a novel cGAS antagonist, it does not provide a mechanism.

Identification of novel ORF9 binding partners by AP-MS could yield candidate proteins for further investigation. Surprisingly, cGAS itself was not among the proteins that could be identified in any sample of the AP-MS experiments.

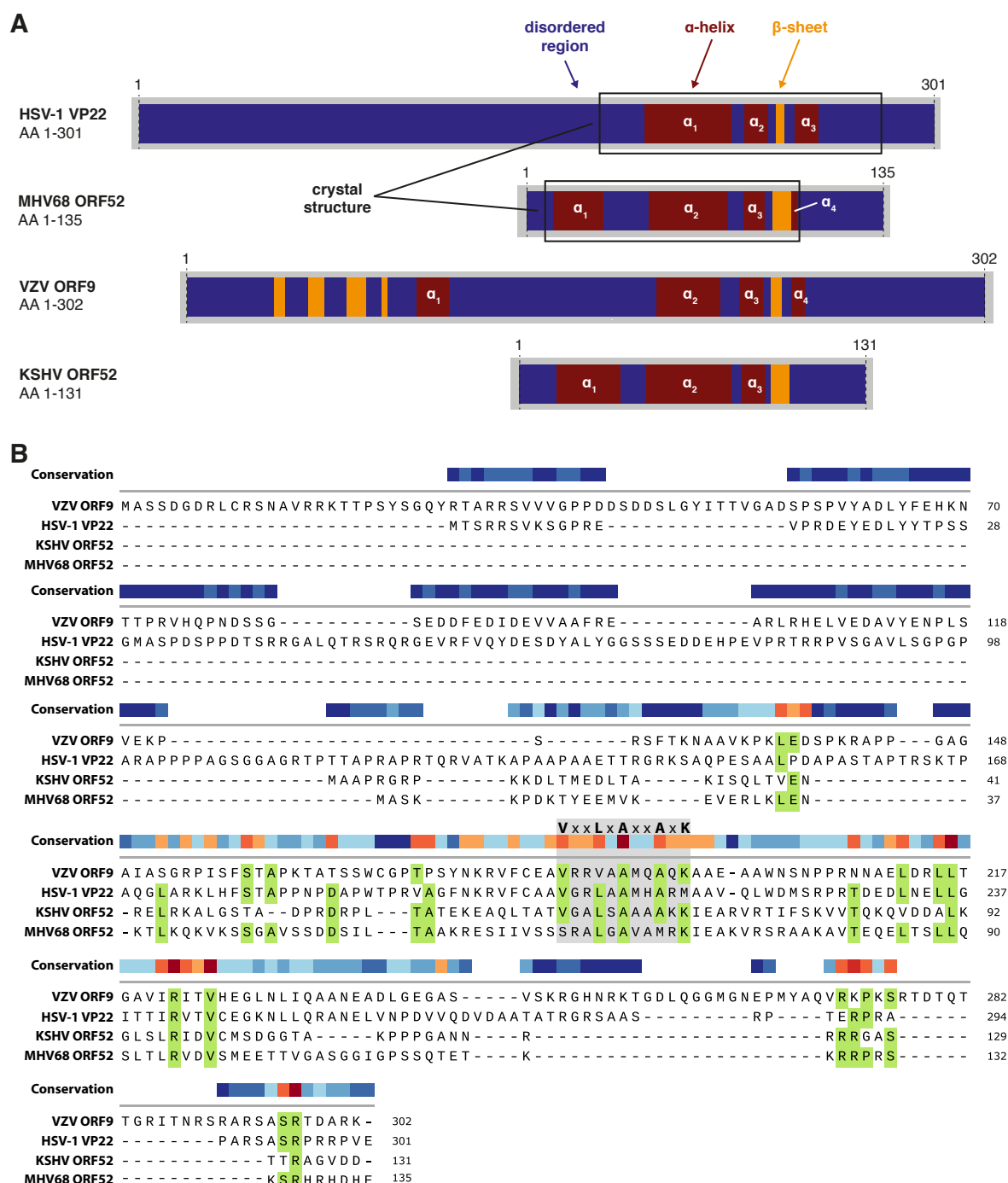


Figure 46: VZV ORF9 and related protein sequence analysis. (A) Schematic of secondary structure features of HSV-1 VP22, MHV68 ORF52, VZV ORF9, and KSHV ORF52 protein sequences. For HSV-1 VP22 and MHV68 ORF52, features were obtained from published structure data as indicated by black boxes. For VZV ORF9, and KSHV ORF52 features were predicted using secondary structure prediction algorithms. (B) Amino acid sequences of VZV ORF9, HSV-1 VP22, KSHV ORF52, and MHV68 ORF52 were aligned using the Clustal Ω algorithm. The conservation of amino acid is visualised by boxes with blue indicating low conservation and shades of red indicating higher conservation. Amino acids that are conserved in at least three sequences are further highlighted in green. See method section II.2.22 for UniProt and PDB accession numbers and details on structure prediction.

It is likely that our experiments were not sensitive enough to detect cGAS protein. We identified a large number of DNA binding proteins interacting with ORF9. Given ORF9's DNA-binding ability, it is likely that a considerable number of detected interactions are DNA-mediated and might be non-specific, i.e. do not involve a direct interaction with ORF9. Compared to other DNA-binding proteins, cGAS has relatively low affinity to DNA³⁴. It is therefore possible that cGAS was simply outcompeted in its binding to ORF9 and DNA. Nevertheless, 21 identified ORF9 interactors have been described previously as cGAS binding partners^{528;529}. Six proteins are part of a complex that is involved in mitochondrial maintenance. Strikingly, none of these proteins are in itself DNA binding, raising the possibility that this is indeed a specific interaction of ORF9, cGAS, and a mitochondrial maintenance complex.

Furthermore, PRKDC, XRCC5, and XRCC6 were identified as ORF9 binding partners. Remarkably, PRKDC and XRCC6 were identified in all four AP-MS conditions, and XRCC5 in three out of four. These three proteins form the hetero-trimeric DNA-PK complex consisting of the catalytic subunit DNA-PK_C (PRKDC), Ku70 (XRCC6), and Ku80 (XRCC5)⁵³⁰. The DNA-PK complex is traditionally involved in NHEJ during DNA double strand break repair⁵³⁰. Data from mouse models and patients have further demonstrated a role of DNA-PK in V(D)J-recombination as *Prkdc* knockout mice and patients with mutations in the *PRKDC* gene have SCID^{531;532}. Intriguingly, DNA-PK has also been implicated in type I IFN responses to dsDNA (see Introduction). Importantly, the IFN response to DNA mediated by DNA-PK is cGAS independent. The relevance of this pathway is highlighted by the fact that VACV encodes multiple antagonists of DNA-PK⁵³³. Recently, a study has proposed a novel role of the DNA-PK complex in regulation of cGAS activity⁵²⁹. Together with the protein HEXIM1, the long-noncoding RNA NEAT1, and the paraspeckle proteins SFPQ, NONO, and PSPC1, DNA-PK forms the newly identified HDP-RNP complex. The HDP-RNP complex is proposed to serve as a platform for IRF3 phosphorylation after cGAS activation. Absence of one of its components inhibited the response to cytosolic DNA. Most interestingly, expression of KSHV ORF52 disrupted the interaction between cGAS and the HDP-RNP complex. In addition to proteins of the DNA-PK complex, SFPQ, NONO, and PSPC1 were also significant binding partners of ORF9 in my AP-MS experiments. This leads to the hypothesis that ORF9 attenuates DNA sensing by cGAS through disruption of the interaction of cGAS and the HDP-RNP. While a crucial role of the HDP-RNP complex in cGAS regulation needs to

be confirmed, a possible role in ORF9 cGAS antagonism will be attractive to test in future experiments.

To strengthen the evidence that antagonism of cGAS by ORF9 is functionally relevant during infection, experiments with a knockout virus would be desirable. One would hypothesise that infection with a virus lacking ORF9 would lead to elevated type I IFN levels. As mentioned, however, ORF9 is an essential gene and ORF9 knockout virus clones are unavailable. An ORF9 deleted VZV BAC clone produces progeny virus if transfected into a ORF9-expressing cell line⁵²². This would generate single round infection viruses that harbour ORF9 protein in their tegument but are unable to produce it during replication. Infection of cells with such viruses and assessment of type I IFN secretion would help answer the question whether tegument ORF9 protein or newly synthesised ORF9 protein is responsible for cGAS antagonism during infection. These investigations could be complemented by experiments in which ORF9 is degraded upon infection. We will obtain from a collaborator a VZV clone that expresses ORF9 with a C-terminal *E.coli* dihydrofolate reductase (ecDHFR) degron motif tag. In the absence of trimethoprim (TMP), an antibiotic that stabilises the degron tag, the tagged protein is targeted for proteasome-dependent degradation in cells⁵³⁴. This recombinant virus can thus be propagated in the presence of TMP and replicate normally. Infection of target cells in the absence of antibiotic should result in the rapid degradation of ORF9 protein once it accesses the cytosol. I hypothesise that this will result in a stronger, cGAS-dependent type I IFN response compared to wild-type virus. In the long-term future, these investigations could even be extended *in vivo*. The group of Ann Arvin has established the human skin xenograft VZV infection model (see section I.7.6). Skin sections could be easily infected with the ORF9-degron virus in the presence or absence of TMP. TMP can be easily administered to mice in the drinking water. The type I IFN response to the virus in the skin can then be assessed by standard techniques.

In summary, I have identified and characterised VZV ORF9 as a novel cGAS antagonist. Future experiments will aim to elucidate further the molecular mechanism based on the different hypotheses presented here and will thus establish the biological relevance of cGAS antagonism by ORF9 during infection with the virus.

Chapter VII

Discussion

This study aimed to characterise the interplay of two viruses, Zika virus and Varicella-Zoster virus, with cell-autonomous pathogen recognition mechanisms.

I showed that infection of A549 cells with ZIKV stimulated increased expression of *IFN β* and ISG transcripts. The recognition of immunostimulatory RNA extracted from ZIKV-infected cells was largely dependent on RIG-I and, to a lesser extent, MDA5. Cells did not respond in the absence of MAVS. RNA from ZIKV infected cells contained RIG-I stimulatory RNA that bound to recombinant RIG-I in a cell-free system. These RNA species included ZIKV viral RNA. The NS5 protein of ZIKV targets STAT2 for degradation and thereby antagonises type I IFN signalling. These results were not discussed in Chapter III but are part of our publication¹⁸⁴ (see Appendix) and were also reported by another group²⁹⁹. We further provide preliminary evidence that the NS5 protein can also interfere with transcription of *IFNB1* downstream of RLR activation. Our results suggest a model in which infection with ZIKV is sensed by cells through RLRs to induce a type I IFN response. This response is antagonised by the virus both at the level of IFN transcriptional induction and signalling by the viral NS5 protein. As discussed in sections I.6.5 and III.3, other viral proteins have been implicated in IFN antagonism by ZIKV. In certain cell types, ZIKV infection fails to induce any detectable IFN transcription and secretion in vitro. This indicates that the virus indeed has the ability to antagonise antiviral responses entirely. It will be interesting to characterise these phenomena more comprehensively in future studies. It will be particularly insightful to set this into context with studies that investigate mechanisms of ZIKV foetal demise. Murine model of ZIKV infections have implicated type I IFNs in neuronal cell death during embryogenesis.

In addition to my studies on ZIKV, I decided to characterise in detail the interactions of another virus, VZV, with cell-autonomous innate recognition mechanisms. My findings regarding recognition of VZV are summarised in Figure 47 at the end of this Chapter. I

could show that the cGAS/STING DNA sensing pathway is indispensable for the type I IFN response to VZV in THP1 cells. The absence of cGAS, STING, TBK1, and IRF3 abrogated increased transcription of *IFNB1* after infection. It further prevented phosphorylation of STAT1 and STAT2 and the increase of ISG protein levels. Lastly, in the absence of the cGAS/STING pathway, cells failed to secrete IP-10 after VZV infection. While VZV infection induced a robust cGAS-dependent type I IFN response in THP1 cells, HaCaT keratinocytes failed to respond strongly to the virus. These very preliminary data indicate that the virus has the ability to sufficiently evade recognition in this cell type. Future experiments will further characterise the response in HaCaT cells. This will include experiments with cGAS and STING KO cells. Additionally, we will use IFI16 KO cells given the previously demonstrated role of IFI16 as a co-factor of cGAS/STING-mediated responses to DNA in keratinocytes¹⁴⁸ (also see below). Finally, we will validate our findings in primary human keratinocytes using siRNAs for knockdown of proteins.

It will be interesting to characterise molecularly the DNA that induces type I IFN via cGAS after VZV infection in THP1 cells (and potentially HaCaT cells). This can be achieved by DNA-protein cross-linking of VZV infected cells, subsequent immunoprecipitation of cGAS-DNA complexes, and sequencing of DNA. Furthermore, labelling of viral genomes *via* Click-chemistry can be used to visualise possible co-localisation of viral DNA and cGAS. Both the sequencing of cGAS-bound DNA and viral genome labelling has been successfully applied to HSV-1 infection models⁴⁷⁴. In these experiments, viral DNA did indeed bind cGAS and contributed to IFN activation during HSV infection. Future experiments will test whether in VZV-infected cells viral DNA directly induces cGAS activation.

The fact that HSV-1 DNA directly stimulates cGAS raises the question how herpesviral DNA gains access to the cytosol. It is well established that after viral entry into cells, capsids are transported by cellular kinesin and dynein motor proteins to nuclear pores³⁴⁹. It is here that viral DNA translocates directly into the nucleoplasm. After nuclear replication of viral DNA and capsid protein synthesis, DNA-containing capsids bud through the nuclear membrane into the cytoplasm. In this model, viral DNA is never available for PRR recognition in the cytoplasm. Consequently, cGAS either senses DNA in the nucleus, or, contrary to the prevailing view in the literature, viral DNA can actually be found in the cytoplasm. Single cell analysis of HSV-1 infected cells has revealed that type I IFN activation in a bulk population of cells originates in a sub-population of abortively infected cells⁵³⁵. These cells show low to

absent viral gene expression but strong transcriptional IFN responses. In addition, immunofluorescence experiments reveal that IRF3 translocation is efficiently blocked in productively infected cells. In contrast, IRF3 can be readily detected in the nuclei of abortively infected cells. The type I IFN response to HSV-1 infection has been shown to be cGAS dependent in mice and *in vitro*^{30;58;60}. It is therefore likely that the IFN produced by the aforementioned sub-population of cells is a result of cGAS activation. It is conceivable that these cells were infected with virions harbouring defective capsids that allowed leakage of viral DNA into the cytoplasm. In addition, cells might express restriction factors that specifically target viral capsids to allow release and recognition of DNA. Release of viral DNA from capsids in the cytosol has indeed been observed for HSV-1⁴⁷⁴. It will be interesting to test whether a similar phenomenon occurs in VZV infected cells, whether the DNA-releasing capsids are defective, and if there are cellular mechanisms that facilitate direct sensing of incoming capsid DNA.

An alternative, but not mutually exclusive, possibility is that herpesviral DNA is actually recognised in the nucleus. This could be achieved by cGAS itself, by nuclear proteins such as IFI16, or a cooperation of them. It has been previously demonstrated that IFI16 restricts HSV-1 gene expression but is dispensable for the type I IFN cytokine response to HSV-1⁶⁰. Other groups have shown that IFI16 is partly required for production of cGAMP by cGAS and for the stimulation of STING^{148;536}. A possible explanation is that IFI16 makes viral DNA accessible for recognition by cGAS, either in the nucleus or the cytoplasm. The interplay of cGAS, STING, IFI16, and possibly other accessory proteins for recognition of DNA during viral infection is incompletely understood and an area of active investigation. The precise molecular requirements for sensing of viral DNA are likely to be cell-type and virus dependent.

The hypothesis of defective capsids being the explanation for cGAS activation is particularly attractive in the context of VZV. VZV has an extremely high particle to PFU ratio of 40.000:1⁵³⁷. This means that a VZV inoculum that produces one plaque contains 40.000 viral particles. The likelihood of defective virions causing IFN activation could therefore be much higher than for HSV-1 (particle:PFU ratio of 10:1⁵³⁸). It is noteworthy that these considerations are complicated by the cell-associated nature of VZV *in vitro*. Infection of cells by co-culture, i.e. cell-to-cell spread, will inevitably differ from infection with preparations of cell-free virus *in vitro*, and natural human-to-human transmission of VZV. The aforementioned particle:PFU ratio was determined in tissue culture and is likely different for virions shed

from *Varicella* and *Zoster* blisters. Whether defective capsids are relevant for the innate responses to naturally acquired infections is unknown but would be interesting to explore.

While it is likely that viral genomic DNA is indeed the cGAS ligand that induces IFN β after VZV infection, self-DNA could also be sensed. Both nuclear DNA and mitochondrial DNA can be sensed by cGAS. HSV-1 infection causes mitochondrial stress that results in recognition of mitochondrial DNA by cGAS⁴⁹⁹. The relevance of this is further highlighted by the fact that HSV-1 encodes a protein that leads to the rapid depletion of mitochondrial DNA upon infection^{539;540}. It is therefore possible that recognition of endogenous DNA is partly responsible for the type I IFN response to VZV we observed in THP1 cells. Sequencing of cGAS-bound DNA (see above) will shed light on the contribution of self-DNA. Further experiments could additionally investigate mitochondrial integrity dynamics during the course of VZV infection.

In my investigations I have used THP1 cells as a model system and plan to extend our experiments to keratinocytes as a relevant cell type for VZV. In addition to cells of the skin, VZV also infects neuronal cells and T-cells as part of its life cycle. The way in which VZV replicates in keratinocytes, neurons, and T-cells, has been shown to be different for each cell type³³⁶. It is therefore conceivable that in each cell type distinct mechanisms govern recognition of VZV. In turn, the virus might have evolved specific ways of antagonism, depending on the host cell type. Future experiments could aim to comprehensively characterise sensing of VZV infection in all cell types for which the virus shows tropism.

Since I demonstrated a key role for cGAS and STING in VZV recognition in THP1 cells, we hypothesised that the virus harbours mechanisms that interfere specifically with cGAS/STING activation. I generated an expression library for all unique VZV ORFs and screened individual viral genes for their ability to block type I IFN induction and signalling. Importantly, I identified VZV ORF9 to attenuate IFN β reporter activation specifically downstream of cGAS/STING stimulation. In addition, screening of VZV ORFs for antagonism of RIG-I-stimulated reporter activation and antagonism of IFN receptor signalling identified a list of candidate viral genes for further characterisation. These data are a valuable resource that can serve as a starting point for future investigations of VZV's interference with antiviral signalling events. Furthermore, the comprehensive library of VZV ORF expression constructs is a valuable resource for the research community.

I showed that the VZV ORF9 protein bound cGAS both if ectopically expressed and dur-

ing viral infection. Cells expressing ORF9 showed attenuated IFN responses to transfected DNA. Recombinant ORF9 and cGAS protein interacted in a cell-free system. Both ORF9 and cGAS were intrinsically DNA binding. Their interaction was abrogated by Benzonase treatment, indicating a role for a nucleic acid in their interaction. I showed that the C-terminal half of the ORF9 protein is sufficient for cGAS binding. Taken together, the findings presented in this thesis establish ORF9 as a novel VZV cGAS antagonist. The precise mechanism of action, however, is yet to be elucidated.

A set of future experiments will aim to shed light on the molecular fundamentals of cGAS inhibition by ORF9. The results I have obtained thus far allow for multiple hypotheses. It is possible that, while ORF9 and cGAS can interact directly, binding of ORF9 to cGAS is insufficient for functional inhibition. ORF9 might reduce the activity of cGAS in cells by disruption of a signalling platform. One such example could be the HDP-RNP complex that ORF9 interacted with in AP-MS experiments. We will validate the interaction of ORF9 with proteins of this complex and test a possible functional relevance for inhibition.

Alternatively, ORF9 could functionally antagonise the activation of cGAS directly. Recombinant ORF9 and cGAS protein interacted in the absence of other proteins. This could be a direct protein-protein interaction, a tri-molecular interaction of cGAS, ORF9, and DNA, or an indirect interaction of cGAS and ORF9 that is entirely based on protein-DNA interactions of both ORF9 and cGAS.

A key to the mechanistic characterisation of ORF9 cGAS inhibition will be the production of purified, nucleic acid-free, and biologically active recombinant cGAS and ORF9 protein. This can be used for cell-free cGAS activity assays in which cGAS protein, if incubated with DNA, ATP, and GTP, will produce cGAMP. Different methods for cGAMP quantification are well described in the literature. For example, radioactive ATP can be utilised to assess cGAMP production in a semi-quantitative way through thin-layer chromatography^{541;68}. Furthermore, fluorescent ATP can be used to monitor its consumption as a proxy for cGAMP production³⁵.

I will produce the proteins and use these activity assays to test whether ORF9 inhibits production of cGAMP by cGAS directly. If the presence of ORF9 protein negatively affects the enzymatic activity of cGAS, this would be strong evidence that ORF9 does not exert its inhibitory function through disruption of a signalling complex or recruitment of a negative regulator. ORF9 could, instead, prevent cGAS activation by direct binding to the protein.

This could, for example, prevent binding of DNA, ATP, or GTP, obstruct the active site of the enzyme, or prevent the conformational change of the cGAS protein that allows cGAMP synthesis upon DNA binding. It has been shown previously that dsRNA can bind cGAS but fails to induce the aforementioned change in protein conformation, thus not activating the enzyme³². ORF9 could act in a similar manner.

Additionally, it is well documented that formation of multimeric complexes of cGAS and DNA facilitates activation of both human and mouse cGAS^{33–36;542}. Both proteins form complexes with DNA in a 2:2 stoichiometry. DNA and cGAS can further form oligomer-like, higher order structures that facilitate cGAMP synthesis. Human cGAS has superior abilities to form these structures and relies on them for activation more than mouse cGAS⁵⁴². This is connected to the lower promiscuity that human cGAS shows regarding DNA length when compared to murine cGAS. Human cGAS requires a minimum of 45 bp in a molecule of dsDNA whereas mouse cGAS can be activated by shorter fragments⁵⁴². Human cGAS has further been demonstrated to form ladder-like protein structures on stretches of DNA³⁵. In this model, the cGAS molecules on a stretch of DNA align in a "head-to-head" and "tail-to-tail" fashion and form filaments. Given that two parallel strands of dsDNA are required to build these structures, their nucleation and formation can be further enhanced by proteins that alter DNA curvature to facilitate complex nucleation³⁵. The requirement of human cGAS for longer DNA and higher order complex formation for its activation is believed to have evolved in humans to prevent activation of cGAS by short fragment of endogenous DNA and thus autoimmunity phenotypes⁵⁴². Additionally, production of cGAMP by cGAS has been demonstrated to be facilitated by liquid phase separation³⁶. In this model, binding of cGAS and DNA leads to condensation of their complexes into phase separated compartments. This separation is facilitated by Zn²⁺ ions. Higher concentrations of both the cGAS enzyme and the DNA ligand in these phase-separated compartments increases higher-order complex formation and thus cGAMP production by cGAS.

ORF9 could inhibit cGAS activation by disruption of the higher-order cGAS:DNA complexes discussed above. Given that ORF9 can bind DNA, it could displace cGAS or other proteins that facilitate complex formation. It is conceivable that ORF9 disrupts the cGAS-DNA interactions during filament formation on long dsDNA by placing itself specifically between cGAS molecules. Alternatively, it could displace cGAS molecules from DNA based simply on higher-affinity binding to dsDNA. It would be interesting to investigate whether

ORF9 binds specifically only to DNA, or whether it has binding sites for both cGAS and DNA separately. It is further possible that ORF9 intercalates into a stretch of dsDNA and prevents cGAS binding by steric hindrance.

ORF9 is a tegument protein and thus delivered to infected cells as soon as virion fusion occurs. This aligns well with a model in which ORF9 is already present during virus entry to prevent activation of cGAS before viral gene synthesis can even occur. ORF9 could elegantly prevent activation of cGAS by DNA that originates from defective capsids (discussed above).

It would be interesting in this context to test ORF9's ability to antagonise mouse cGAS. Mouse cGAS relies less on higher order complex formation than human cGAS for activation and produces cGAMP when stimulated with short dsDNA fragments^{34;35;542}. If ORF9 fails to inhibit mouse cGAS activation, it is likely that ORF9 indeed acts on higher-order cGAS:DNA complexes. These experiments could be extended to include stimulatory DNA molecules of different lengths to decipher in detail whether ORF9 can specifically prevent activation of cGAS after stimulation with long, complex-favouring DNA species. Notably, selective antagonism of human but not mouse cGAS could further explain why VZV fails to infect mouse tissue. Possibly, in mouse cells and thus in the absence of functional ORF9, efficient detection of the virus by cGAS allows viral clearance.

In order to elucidate further the molecular fundamentals of the cGAS-ORF9 interaction, we have started a collaboration with structural biologists. We aim to co-crystallise human cGAS and ORF9 protein, potentially with DNA. These experiments will be exploratory, since at this point we do not know whether an ORF9 structure can be solved at all, and whether both proteins form a direct complex. If successful, however, these investigations are certainly informative regarding a structural basis for cGAS inhibition. This is particularly interesting in light of possible cGAS inhibitor development (see below).

In summary, the precise mechanism of cGAS antagonism by ORF9 is yet to be determined. I have conceptualised above that ORF9 could inhibit cGAS activity directly or dependent on interaction with other proteins. From the evidence obtained up to this point I favour the model in which ORF9 has the intrinsic ability to abrogate cGAS activation. This is largely drawn from the results of cell-free experiments and the concept I put forward in section VI.9. Antagonism of cGAS by KSHV ORF52 required the DNA-binding ability of ORF52 but did not depend on other proteins¹²⁷. The prospective experiments outlined above will together unequivocally solve the molecular fundamentals of cGAS inhibition by ORF9.

Evasion of antiviral type I IFN responses is crucial for replication of viruses. Antagonism of cGAS and STING is therefore a straight-forward strategy employed by DNA and RNA viruses to prevent type I IFN expression. However, many viruses, including VZV, employ mechanisms that prevent IFN activation through antagonism of IRF3 and downstream signalling from the type IFN receptor. Why do viruses then dedicate part of their coding capacity to direct cGAS and STING antagonism? In addition to the transcriptional response that governs type I IFN induction, STING signalling can also stimulate autophagy and cell death pathways⁵⁴³. It has been demonstrated recently that induction of cell death and autophagy are evolutionary conserved functions of STING that exist in species that lack IRF3 and NFκB signalling⁵⁴⁴. Importantly, some of these species do employ a functioning signalling cascade in which cGAS or a cGAS-related enzyme synthesises a cyclic di-nucleotide that stimulates STING⁵⁴⁵. This includes the anemone species *Nematostella vectensis* that diverged evolutionary from humans more than 500 million years ago. Herpesviruses have also co-evolved with the animal kingdom for hundreds of millions of years^{546;547}. It is therefore conceivable that the antagonism of cGAS and STING by viruses is an ancient mechanism of immune evasion that benefited viruses before antiviral cytokines evolved. In section VI.9 I have put forward a hypothesis that proposes a structural feature that enables cGAS antagonism to be conserved across different herpesvirus families. This model could be particularly attractive if antagonism of cGAS and cGAS-like enzymes is indeed relevant in herpesviruses that are much more distantly related to VZV than KSHV and MHV68. In-depth phylogenetic analysis and structural modelling of a wide array of proteins from distantly related herpesviruses could explore this hypothesis systematically.

Inhibition of cGAS is relevant not only in the context of infection. As discussed in section I.3.1.4, stimulation of cGAS by endogenous DNA and the consequential type IFN response contribute to pathologies like auto-inflammatory disorders. A small molecule, Ru.512, has been developed as a cGAS inhibitor¹³⁵. Structural studies have begun to elucidate its mechanism and show allosteric inhibition outside of DNA- and NTP-binding pockets^{135;542}. This compound can successfully inhibit murine cGAS in animal models of autoimmune disease and *in vitro*, but has lower inhibitory capacity with human cGAS⁵⁴². Recently, drug-library screening identified a novel class of compounds that can inhibit human cGAS *in vitro*¹³⁶. However, cGAS inhibitor development has only begun in the recent past and effective, specific, and safe compounds are still lacking. Characterisation of the mechanism of action

through which a viral protein can block cGAS activity, may be informative for the development of novel pharmaceutical inhibitors. In future experiments we might be able to identify a motif of amino acids in the ORF9 protein that mediates cGAS functional inhibition. This could be the basis of the development of peptide drugs for therapeutic application. Furthermore, structural work on cGAS and ORF9 might reveal a part of the cGAS protein that represents an attractive target for inhibitor interaction.

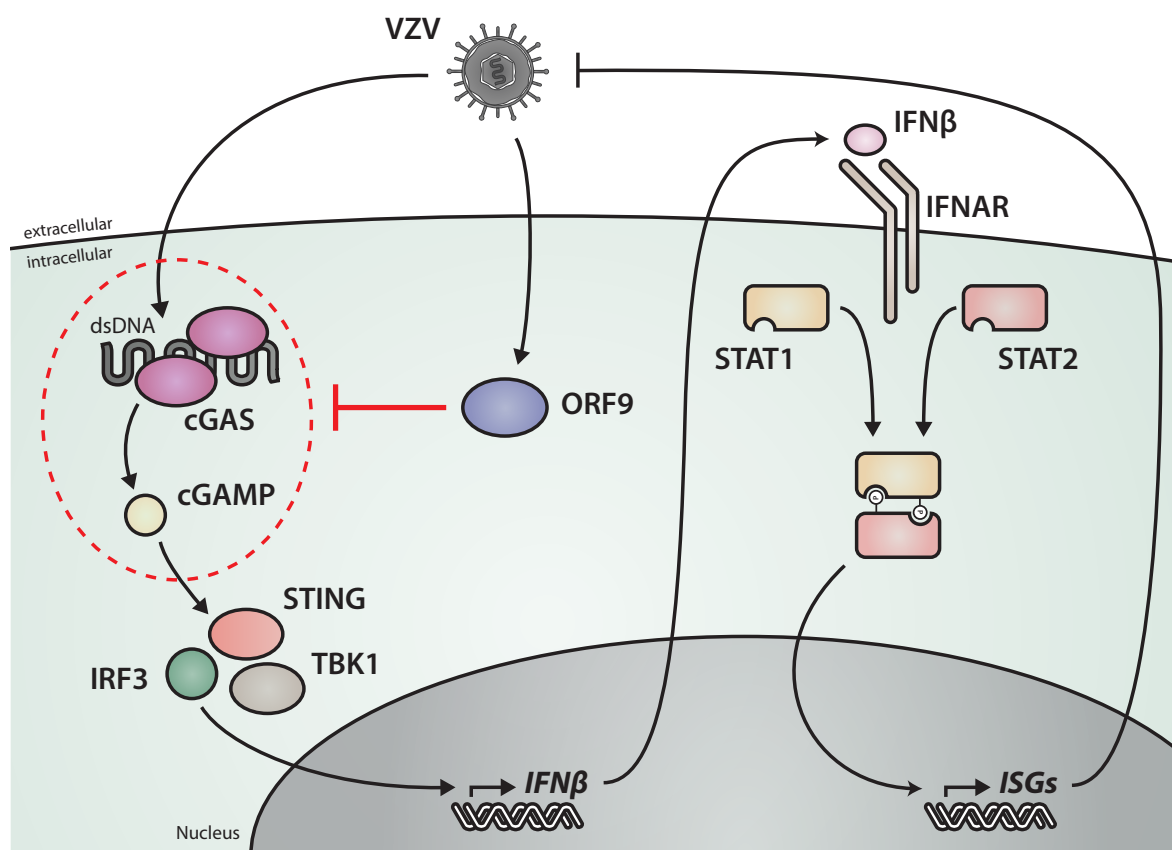


Figure 47: VZV is sensed by cGAS and antagonises recognition through the viral protein ORF9. This schematic summarises the findings of this study. VZV infection is sensed through recognition of dsDNA by cGAS. After binding of the second messenger cGAMP to STING, TBK1 phosphorylates IRF3 and *IFNβ* transcription is induced. *IFNβ* stimulates in an autocrine manner the type I IFN receptor (IFNAR). Ligand binding to IFNAR allows phosphorylation of STAT1 and STAT2 which heterodimerise and induce the transcription of ISGs. Many ISGs have antiviral activity and will inhibit VZV infection. Importantly, the VZV tegument protein ORF9 antagonises activation of cGAS and the production of cGAMP, thus contributing to innate immune evasion of VZV.

In conclusion, the work presented in this thesis feeds into the characterisation of mechanisms that viruses use to inhibit cGAS/STING signalling. This is an area of active research, given that cGAS was only discovered in 2013. A better understanding of such mechanism will advance our understanding of cGAS biology both in the context of infectious and non-infectious pathologies. It might further pave the way for development of novel pharmaceutical interventions that target cGAS.

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List of Figures

- 1 **The ligands and intracellular signalling of human TLRs.** Nine TLRs recognise a variety of PAMPs as homo- or heterodimers. Signalling via MyD88 activates a cascade involving IRAK1, IRAK2, IRAK4, TRAF6, IKK β , NF κ B, and IRF3/7 which ultimately leads to the expression of inflammatory cytokines and type I and III IFNs. TLR3 and TLR4 activate expression of type I/III IFNs through the adaptor molecule TRIF and TRAF3, TBK1, and IKK ϵ 4

- 2 **The cGAS/STING DNA-sensing pathway.** DNA of exogenous (viruses, bacteria) origin or self-DNA binds cGAS and leads to the production of cGAMP from ATP and GTP. Binding of cGAMP to STING induces trafficking of the ER-resident protein to the Golgi. Recruitment of TBK1 and IK kinases allows activation of the transcription factors IRF3 and NF κ B. Together they activate transcription of the *IFN β* gene. Figure taken from REF. 31. 5

- 3 **The intracellular signalling of RLRs.** MDA5 and RIG-I recognise long dsRNA and 5'-triphosphate RNA, respectively. Conformational changes and ubiquitination events allow interaction of RIG-I and MDA5 with MAVS via CARD domains. MAVS oligomerisation and TRAF binding lead to recruitment of the kinases IKK ϵ and TBK1. They phosphorylate the transcription factors IRF3 and IRF7. Additionally, IK kinases activate NF κ B. These transcription factors induce expression of type I IFNs, type III IFNs, and inflammatory cytokines. Figure is adapted from REF. 164 14

- 4 **The intracellular signalling of interferons.** The IFN group of cytokines consist of type I, type II, and type III IFNs. Each type of IFN binds to its own dedicated receptor. Type I and type III IFN canonical signalling via ISGF3 (pSTAT1-pSTAT2-IRF9) results in expression of ISGs through binding to ISRE. Type II IFN canonical signalling activates GAS in the promoter regions of genes through pSTAT1 homodimers. Downstream signalling from the type I and type III IFN receptor can additionally result in formation of homo- and heterodimers of phosphorylated STAT1, STAT2, STAT3, STAT4, STAT5, and STAT6. It can further activate the MAPK/p38 pathway that leads to cell growth inhibition and chromatin remodelling. Non-canonical signalling from the type I and type II IFN receptor can also result in stimulation of the PI3K/Akt pathway. This facilitates protein synthesis via mTOR signalling and can lead to transcription of inflammatory genes through NF κ B. Bold lines indicate the canonical signalling pathway for each receptor. 17

- 5 **The ZIKV genome.** ZIKV has a (+)ssRNA genome that harbours a single ORF. The ORF is flanked by NCRs and the genome is capped with a 7-methyl-guanylate-triphosphate moiety on its 5'-end. The single ORF is translated into a polyprotein that is cleaved into the individual viral proteins Capsid (C), pr-Membrane (prM), and Envelope (E) and non-structural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. The viral protease consists of a NS2B-NS3 dimer. NS3 and NS5 harbour methyltransferase domains. NS5 further has RNA-dependent-RNA-polymerase activity. 23
- 6 **The Flavivirus replication cycle.** After internalisation of the virion by endocytosis, membrane fusion is induced by acidification of the vesicle lumen. Viral RNA is released into the cytosol and translated at the ER. The viral polyprotein is co-translationally cleaved into the viral proteins which remain ER associated. Replication of the viral genome and virion assembly occurs in ER invaginations. New particles pass through the Golgi for maturation. This includes cleavage of the Membrane precursor protein by cellular furin proteases and pH-induced conformational changes of the viral envelope surface. Mature particles are then secreted. Figure taken from REF. 242. 24
- 7 **ZIKV innate immune evasion mechanisms.** The viral proteins NS4A, TBK1, and NS5 inhibit the RLR signalling cascade by targeting the RIG-I-MAVS interaction, TBK1, and IRF3, respectively. Recognition of viral RNA by RIG-I is additionally antagonised by ZIKV sfRNA. ZIKV NS1 and the viral protease NS2B/NS3 target cGAS and STING, respectively. Type I IFN receptor signalling is inhibited by NS2B/NS3 and NS5 that target Jak1 and STAT2, respectively. 30
- 8 **The architecture of the VZV virion and its genome organisation. (A)** VZV is an enveloped virus with a dsDNA genome inside a nucleocapsid. Between capsid and envelope lies the tegument layer. Grey boxes indicate viral proteins that constitute the respective structural feature. **(B)** Graphical representation of the VZV linear genome showing the canonical ORFs. Panel (B) is taken from REF. 336 32
- 9 **The pathogenesis of VZV.** Inoculation of the human host with VZV occurs orally. Infection of respiratory epithelial cells and spread of virus to T cells in local lymphoid organs leads to T cell viremia. Infection of skin cells causes the characteristic chickenpox (*Varicella*) blister rash. During primary skin infection the virus can infect peripheral nerve endings and establish latency in dorsal root or cranial ganglia. Alternatively, ganglia neurons can become infected with VZV through haematogenous spread by T cells. Reactivation of the virus from latency leads to anterograde transport of virions into the innervated dermatome and establishment of the skin infection known as shingles (*Zoster*). Figure taken from REF. 336. 34
- 10 **The VZV lytic replication cycle.** After attachment of virions to the plasma membrane and membrane fusion, capsids are transported to nuclear pores. The viral DNA is translocated into the nucleus and replicated. At the same time, viral genes are expressed and protein synthesised in the cytoplasm. Viral proteins are re-imported into the nucleus for capsid assembly. DNA-containing capsid bud through the nuclear membranes and undergo secondary envelopment in the cytoplasm. Virions are then released in a process that involves late endosomes. Figure is adapted from REF. 341 36

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- 11 **ZIKV infection generates RLR-stimulatory RNA in A549 cells.** (A) Schematic detailing the experimental procedures for results shown in (A) and (B). (B) A549 cells were infected with ZIKV at MOI 5 for 4 h, 8 h, 24 h, 48 h, or 72 h. Cells were lysed in TRIzol and RNA was extracted. RT-qPCR was performed for the indicated genes. (C) RNA from A549 cells infected with ZIKV as in (A) was transfected into parental, MDA5-KO, RIG-I-KO, or MAVS-KO HEK293p125 IFN β -F-Luc reporter cells. 24 h later luciferase activity was measured. RLU, relative light unit. Results shown in (B) and (C) are pooled data from two independent biological repeat experiments (average and SEM (n=2)). The RT-qPCR analysis for one of the repeats of (B) was performed by Dr Antonio Gregorio Dias Junior. 79
- 12 **Immunoprecipitation of ectopically expressed RIG-I-FLAG fails to recover stimulatory RNA after transfection of A549-ZIKV-RNA.** (A) Schematic of the experimental setup. (B) Parental, RIG-I-KO, and MDA5-KO HEK293p125 reporter cells were transfected with HEK293T-IAV-RNA, Vero-EMCV-RNA, and A549-ZIKV-RNA. The next day luciferase activity was determined and normalised to control samples treated with transfection reagent alone. RLU, relative light unit. (C) RNAs extracted from input, unbound and bound samples were transfected into HEK293p125 cells. After 16 h, luciferase activity was determined and normalised to control samples treated with transfection reagent alone. RLU, relative light unit. (D) Input, unbound and bound samples were analysed by immunoblotting using α -FLAG antibody. Panels (B), (C) and (D) are representative of two independent experiments. Panel (B), and (D) show technical duplicates (average and SD (n=2)). 81
- 13 **An *in vitro* assay to recover RIG-I-bound RNA.** (A) Schematic of the experimental setup. HEK293T-IAV-RNA and A549-ZIKV-RNA were used. (B) Input, unbound and bound samples were analysed by immunoblotting using α -FLAG antibody. Panel (B) is representative of two (controls) or three (IAV and ZIKV samples) independent experiments. 82
- 14 **Recombinant RIG-I binds ZIKA RNA.** (A) RNAs extracted from input, unbound and bound samples were transfected into parental and RIG-I-KO HEK293p125 cells. After 16 h, luciferase activity was determined and normalised to control samples treated with transfection reagent alone. RLU, relative light unit. (B) RNA samples from (A) were analysed in technical duplicate by RT-qPCR using the indicated probes. Relative viral transcript abundance was analysed by converting Ct data to 2^{-Ct} values. Panels (A) and (B) show pooled data from two (controls) or three (IAV and ZIKV samples) independent experiments (average and SEM (n=2/3)). 83
- 15 **VZV leads to syncytia formation *in vitro*.** Pictures of uninfected MeWo cells or VZV-propagating MeWo cells were acquired by brightfield microscopy after 24 h of co-culture. In pictures of infected cells arrows indicate early (yellow), intermediate (orange), or late (red) syncytia formation. Medium and late syncytia show a characteristic ring of nuclei. Scale bar: 50 μ m. 89
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- 16 **A flow cytometry assay to measure VZV-infection. (A)** Uninfected (mock) or VZV-infected MeWo cells were stained with Live/Dead Violet and VZV DFA reagent and processed for flow cytometry. Dotted line arrows show gating hierarchy. **(B)** FITC channel intensity histogram for mock or VZV-infected cells (samples in (A)) as well as unstained controls. **(C)** Monoclonal antibody against VZV gE/gI was coupled to FITC (VZV-gE/gI-FITC). VZV-infected cells were stained with VZV-gE/gI-FITC or mIgG2B-FITC isotype control at indicated dilutions. VZV DFA reagent was used as in (A) and (B). **(D)** Repeat of experiment in (C) with higher antibody dilutions. 90
- 17 **MeWo and THP1 cells can be infected with VZV using a newly developed transwell system. (A)** Schematic detailing the experimental procedure. See Results and Methods sections for details. **(B)** MeWo and PMA-differentiated THP1 cells were mock or VZV-infected using transwell assay detailed in (A). Uninfected or VZV-infected MeWo inoculum cells were seeded on bottom membrane of transwells. The next day, the cells were labelled using CellTrace Violet dye. PMA-differentiated THP1 cells or MeWo cells as control were seeded onto top membrane of transwells and incubated for 48 h. Cells from the top membrane were processed for flow cytometry and infection assessed by staining with VZV-gE/gI-FITC. From one transwell cells from the bottom membrane were harvested to demonstrate CellTrace staining (panels in last row). Dotted arrows indicate gating hierarchy. Results in B are representative of seven independent experiments. . . 92
- 18 **THP1 cells respond by type I IFN transcription to VZV-infection through co-culture and the transwell system. (A)** For co-culture, THP1 cells were PMA-differentiated overnight and labelled with CellTrace FarRed. Cells were mock- or VZV-infected through 1 h co-culture with uninfected or infected MeWo cells, respectively. After 48 h, cells were harvested for FACS and RT-qPCR. **(B)** MeWo and THP1 cells were mock- or VZV-infected through both co-culture and the transwell assay as shown in (A) for THP1 cells. RT-qPCR was performed for the indicated genes. Panels (A) and (B) represent one independent experiment. Panel (B) shows technical duplicates (average and range (n=2)). 94
- 19 **IRF3 and MAVS THP1 knockout clones were identified by functional testing. (A)** THP1 cells were transfected with IRF3- and MAVS-targeting CRISPR plasmids and single-cell expanded by limiting dilutions. Cells of indicated clones were processed for immunoblotting. Membranes were probed with indicated antibodies. **(B)** Cells from the same clones as in (A) were PMA-differentiated and transfected with IVT-RNA or *E.coli* DNA. The next day, supernatants were transferred onto 3C11 cells (IFN bioassay). 24 h later luciferase intensity was measured and fold changes were calculated based on mock-transfected samples. Panel shows one independent experiment. For (B) average and range (technical duplicates) are shown. 96
- 20 **IRF3 and MAVS THP1 knockout clones show defined editing of targeted genomic loci. (A), (B)** The IRF3 or MAVS locus was PCR-amplified from genomic DNA and analysed by Sanger sequencing. Panels show electropherogram from WT cells and two knockout clones. **(C), (D)** Sanger sequencing traces from (A) and (B) were analysed using the TIDE algorithm. Panels (A) - (D) show one independent experiment. 97

- 21 **The THP1 knockout cell panel shows absence of relevant proteins and expected functionality.** (A) THP1 cells from indicated KO cell lines were PMA differentiated and treated or not with recombinant IFN α . Lysates were processed for immunoblotting and membranes were probed with indicated antibodies. (B) THP1 cells from indicated KO cell lines were processed as in (A). (C) THP1 cells from indicated KO cell lines were PMA differentiated and mock transfected or transfected with 200 ng IVT-RNA or 500 ng VACV 70mer. RNA was extracted and RT-qPCR was performed for the *IFNB1* gene. Panel (A) is representative of three independent experiments. Panel (B) shows one independent experiment. Panel (C) shows one independent experiment (average and range (technical duplicates)). One-way ANOVA with Dunnett's multiple comparisons test was used to compare results for each KO cell line to WT cells as indicated above bars in (C). ****= $p < 0.0001$, ***= $p < 0.001$, **= $p < 0.01$, ns=not significant. 99
- 22 **The THP1 knockout cell panel was infected with VZV by co-culture.** (A) WT THP1 cells were PMA-differentiated overnight and labelled with CellTrace FarRed. Cells were mock or VZV-infected by co-culture with uninfected or infected MeWo cells, respectively. 48 h later cells were harvested and a fraction of the cells was processed for flow cytometry by staining with VZV-gE/gI-FITC. Coloured boxes indicate gates used for quantification shown in (B) and (C). Dotted arrows indicate gating hierarchy. (B) Inoculum cells (MeWo, CellTrace-, black gate) and target cells (THP1, CellTrace+, green gate) from co-culture experiments were quantified as percentage of live cells. (C) Rate of infection (blue vs red gate) was quantified in target cells (THP1, green gate). Results in A are representative of six independent experiments. Panels B and C show pooled data from six (WT) or three (KOs) independent experiments (average and SEM (n=3/6)). 101
- 23 **THP1 cGAS KO, STING KO, TBK1 KO, IRF3 KO and IFNAR2 KO cells fail to respond to VZV infection through transcriptional upregulation of *IFNB1* and ISGs.** WT and KO THP1 cells were PMA-differentiated overnight. As controls, MeWo cells were seeded. Cells were labelled with CellTrace FarRed and mock or VZV-infected by co-culture with uninfected or infected MeWo cells, respectively. 48 h later cells were harvested and processed for RT-qPCR for indicated genes. Left panels show expression relative to *GAPDH* and right panels show expression fold change to uninfected samples. Panels show pooled data from six (WT, MeWo) or three (KOs) independent experiments (average and SEM (n=3/6)). Left panels: Unpaired, two-tailed t-test. Right panels: Welch One-way ANOVA with Dunnett's multiple comparisons test. ***= $p < 0.001$, **= $p < 0.01$, *= $p < 0.05$, ns=not significant. 102

- 24 **Inflammatory cytokine gene transcription shows distinct patterns in the THP1 knockout cell panel after VZV-infection.** WT and KO THP1 cells were PMA-differentiated overnight. As controls, MeWo cells were seeded. Cells were labelled with CellTrace FarRed and mock or VZV-infected by co-culture with uninfected or infected MeWo cells, respectively. 48 h later cells were harvested and processed for RT-qPCR for indicated genes. Left panels show expression relative to *GAPDH* and right panels show expression fold change to uninfected samples of the same genotype. Panels show pooled data from six (WT, MeWo) or three (KOs) independent experiments (average and SEM (n=3/6)). Left panels: Unpaired, two-tailed t-test. Right panels: Welch One-way ANOVA with Dunnett's multiple comparisons test. ***=p<0.001, **=p<0.01, *=p<0.05, ns=not significant. 103
- 25 **THP1 cGAS KO, STING KO, TBK1 KO, IRF3 KO and IFNAR2 KO cells fail to phosphorylate STAT1 and STAT2 in response to VZV-infection.** WT and KO THP1 cells were PMA-differentiated overnight. As controls, MeWo cells were seeded. Cells were labelled with CellTrace FarRed and mock or VZV-infected by co-culture with uninfected or infected MeWo cells, respectively. 48 h later cells were harvested and processed for immunoblotting with indicated antibodies. Size markers indicate molecular weight in kDa. Panel shows representative results of three independent experiments. 105
- 26 **VZV-infection does not induce IP-10 secretion in cGAS KO, STING KO, TBK1 KO, IFNAR2 KO THP1 cells.** WT and KO THP1 cells were PMA-differentiated overnight. As controls, MeWo cells were seeded. Cells were labelled with CellTrace FarRed and mock or VZV-infected by co-culture with uninfected or infected MeWo cells, respectively. 48 h later supernatants were harvested and processed for IP-10 ELISA. Panel shows pooled data from six (WT) or three (KOs, MeWo) independent experiments (average and SEM (n=3/6)). Data point shapes correspond to independent experiments. Missing data points or "nd" (not determined) indicate values below the detection limit. 106
- 27 **VZV Evades Sensing in HaCaT Keratinocytes. (A)** HaCaT cells were labelled with CellTrace FarRed and mock or VZV-infected by co-culture with uninfected or infected MeWo cells, respectively. At the indicated time points after co-culture cells were harvested and processed for flow cytometry by staining with VZV-gE/gI-FITC. Plots show events pre-gated on single cells. **(B)** Samples shown in (A) were analysed by RT-qPCR for the indicated genes. Graphs show fold changes of expression relative to *ACTB* normalised to the first time point. **(C)** Samples shown in (A) and a sample of HaCaT cells alone were analysed by immunoblotting using the indicated antibodies. All panels show one independent experiment. Panel (B) shows technical duplicates (average and range (n=2)). hpi: hours post infection 107

28	VZV ORF library immunoblot ORF0 - ORF40. HEK293T cells were transfected with indicated expression plasmids. The next day lysates were prepared and subjected to immunoblotting using the indicated antibodies. Circles above immunoblot lanes indicates expression at predicted MW (green), at the wrong MW (yellow), or no expression (orange). Expression data are summarised in Figure 29B. The data are representative of two independent experiments.	118
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- 32 **Screening of individual VZV ORFs for direct antagonism of cGAS/STING identifies candidate viral proteins.** (A) HEK293T cells were transfected with pRL-TK, p125-F-Luc, expression plasmids for human cGAS and human STING, as well as expression plasmids for individual ORFs. The next day luciferase activity was determined. For each ORF a z-value was calculated and ORFs sorted by descending values. (B) The experiment shown in panel A was repeated three times and for each ORF in each experiment a rank was determined according to z-value (highest z-value = rank 1). Ranks are displayed as a heatmap. (C) For candidate ORFs HEK293 cells were transfected as in (A). In parallel, reporter expression was stimulated by co-transfection of the RIG-I-CARD plasmids instead of hcGAS and hSTING for the same ORFs. A luciferase fold change was calculated to unstimulated cells. Panel (A) is representative of three independent experiments (average and SD (technical triplicates)). The three independent experiments are summarised in panel (B). Panel (C) is representing one independent experiment (average and SD (technical triplicates)). The experiments in this figure were performed in collaboration with Sonja Roll, an undergraduate rotation student I supervised. 124
- 33 **Heatmap comparison of top ranking ORFs from the different screens.** The 15 lowest ranking ORFs of each screen are displayed. The colours correspond to the rank each ORF had on average in the respective screen. Crossed-out cells indicate that the construct was not used in these experiments. 125
- 34 **ORF9 interacts with cGAS in a nucleic-acid dependent manner.** (A) HEK293T cells were transfected with expression plasmids for ORF9-V5, cGAS-FLAG, and STING-HA. 24 h later, cells were lysed and ectopically expressed proteins were immunoprecipitated using epitope tag antibodies. IP fractions were subjected to immunoblotting using the indicated antibodies. (B) HEK293T cells were transfected with expression plasmids for ORF9-V5 and cGAS-FLAG. 24 h later, cells were lysed and half the lysate was treated with benzonase. Ectopically expressed proteins were immunoprecipitated using epitope tag antibodies. IP fractions were subjected to immunoblotting using the indicated antibodies. Data is representative of one (A) or three (B) independent experiments. 132
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- 42 **ORF9 AA 1-151 are dispensable for cGAS binding.** (A) Schematic detailing the amino acid sequence of VZV ORF9 regarding ordered regions. The presence of α -helices and β -sheets was predicted based on AA sequence using the PHD prediction algorithm (see Methods). (B) Schematic showing the truncation mutants of ORF9 that were constructed and used in experiment shown in (C). (C) HEK293T cells were transfected with an expression plasmid for cGAS-V5 and indicated, FLAG-tagged truncation variants of ORF9. 24 h later cells were lysed and immunoprecipitated with antibody against FLAG. IP fractions were subjected to immunoblotting using the indicated antibodies. Data are representative of two (ORF9 variants I-V) or one (ORF9 variants VI-VII) independent experiments. 142
- 43 **ORF9 inhibits cGAMP production by cGAS.** (A) Schematic detailing the experimental procedure. See text for details. (B) Histogram and quantification of gag-GFP fluorescence measured by flow cytometry in cells infected with VLPs. (C) Type I IFN in supernatants of cells infected with VLPs was measured using an IFN bioassay. Recombinant human IFN α 2 was used as control. Panel shows results of one experiment. (B): Paired, two-tailed t-test. (C): Two-tailed t-test. ***= $p < 0.001$, ns = not significant 144
- 44 **AP-MS analysis of ORF9 in HaCaT and THP1 cells.** (A) Schematic detailing the experimental procedure. See text for details. (B) Principal component analysis of quadruplicate samples per condition based on LFQ intensity values for all identified proteins. (C) Volcano plots showing for each cell line and condition the LFQ intensity fold change and significance of GFP vs. ORF9 after Welch t-test. 146
- 45 **Protein network analysis of ORF9 AP-MS results.** (A) Venn diagram showing the proteins significantly enriched in ORF9 pulldowns that are shared between conditions. (B) All 226 protein-coding genes summarised in (A) and (C) were submitted to the STRING database and analysed for GO term enrichment. Top-five most significant GO terms for biological process and molecular functions are shown. (C) Diagram showing high-confidence protein-protein interactions as detected by STRING database analysis. Nodes are coloured according to in how many conditions they were significantly enriched (see (A)). Proteins previously identified as cGAS-binding are further displayed with a red border. 148
- 46 **VZV ORF9 and related protein sequence analysis.** (A) Schematic of secondary structure features of HSV-1 VP22, MHV68 ORF52, VZV ORF9, and KSHV ORF52 protein sequences. For HSV-1 VP22 and MHV68 ORF52, features were obtained from published structure data as indicated by black boxes. For VZV ORF9, and KSHV ORF52 features were predicted using secondary structure prediction algorithms. (B) Amino acid sequences of VZV ORF9, HSV-1 VP22, KSHV ORF52, and MHV68 ORF52 were aligned using the Clustal Ω algorithm. The conservation of amino acid is visualised by boxes with blue indicating low conservation and shades of red indicating higher conservation. Amino acids that are conserved in at least three sequences are further highlighted in green. See method section II.2.22 for UniProt and PDB accession numbers and details on structure prediction. 151

47	VZV is sensed by cGAS and antagonises recognition through the viral protein ORF9. This schematic summarises the findings of this study. VZV infection is sensed through recognition of dsDNA by cGAS. After binding of the second messenger cGAMP to STING, TBK1 phosphorylates IRF3 and <i>IFNβ</i> transcription is induced. IFN β stimulates in an autocrine manner the type I IFN receptor (IFNAR). Ligand binding to IFNAR allows phosphorylation of STAT1 and STAT2 which heterodimerise and induce the transcription of ISGs. Many ISGs have antiviral activity and will inhibit VZV infection. Importantly, the VZV tegument protein ORF9 antagonises activation of cGAS and the production of cGAMP, thus contributing to innate immune evasion of VZV.	163
S1	THP1 VZV Infection flow cytometry analysis. Corresponds to results summarised in Figure 22 and discussed in section IV.2.2. WT or cGAS-KO THP1 cells were PMA-differentiated overnight and labelled with CellTrace FarRed. Cells were mock or VZV-infected by co-culture with uninfected or infected MeWo cells, respectively. 48 h later cells were harvested and a fraction of the cells was processed for flow cytometry by staining with VZV-gE/gI-FITC. Panel shows data from six (WT) or three (cGAS-KO) independent experiments. Continued in Figure S2.	II
S2	Continued: THP1 VZV Infection flow cytometry analysis. Corresponds to results summarised in Figure 22 and discussed in section IV.2.2. STING-KO, MyD88-KO, or TBK1-KO THP1 cells were PMA-differentiated overnight and labelled with CellTrace FarRed. Cells were mock or VZV-infected by co-culture with uninfected or infected MeWo cells, respectively. 48 h later cells were harvested and a fraction of the cells was processed for flow cytometry by staining with VZV-gE/gI-FITC. Panel shows data from three independent experiments. Continued in Figure S3.	III
S3	Continued: THP1 VZV Infection flow cytometry analysis. Corresponds to results summarised in Figure 22 and discussed in section IV.2.2. IFNAR2-KO, MAVS-KO, or IRF3-KO THP1 cells were PMA-differentiated overnight and labelled with CellTrace FarRed. Cells were mock or VZV-infected by co-culture with uninfected or infected MeWo cells, respectively. 48 h later cells were harvested and a fraction of the cells was processed for flow cytometry by staining with VZV-gE/gI-FITC. Panel shows data from three independent experiments. Continued in Figure S4.	IV
S4	Continued: MeWo VZV Infection flow cytometry analysis. Corresponds to results summarised in Figure 22 and discussed in section IV.2.2. MeWo cells were let adhere overnight and labelled with CellTrace FarRed. Cells were mock or VZV-infected by co-culture with uninfected or infected MeWo cells, respectively. 48 h later cells were harvested and a fraction of the cells was processed for flow cytometry by staining with VZV-gE/gI-FITC. Panel shows data from six independent experiments.	V

Appendix

Supplementary Figures

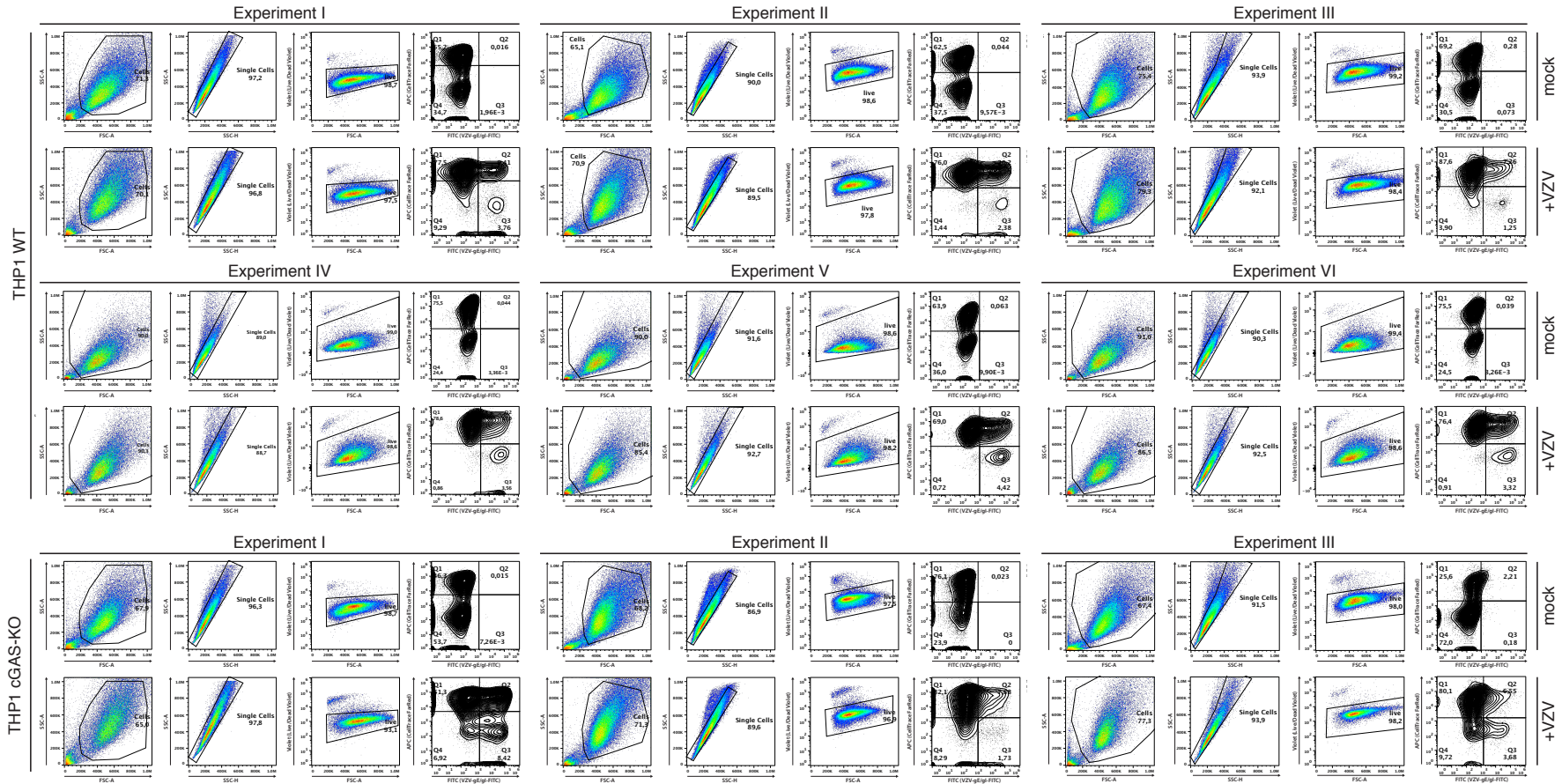


Figure S1: THP1 VZV Infection flow cytometry analysis. Corresponds to results summarised in Figure 22 and discussed in section IV.2.2. WT or cGAS-KO THP1 cells were PMA-differentiated overnight and labelled with CellTrace FarRed. Cells were mock or VZV-infected by co-culture with uninfected or infected MeWo cells, respectively. 48 h later cells were harvested and a fraction of the cells was processed for flow cytometry by staining with VZV-gE/gI-FITC. Panel shows data from six (WT) or three (cGAS-KO) independent experiments. Continued in Figure S2.

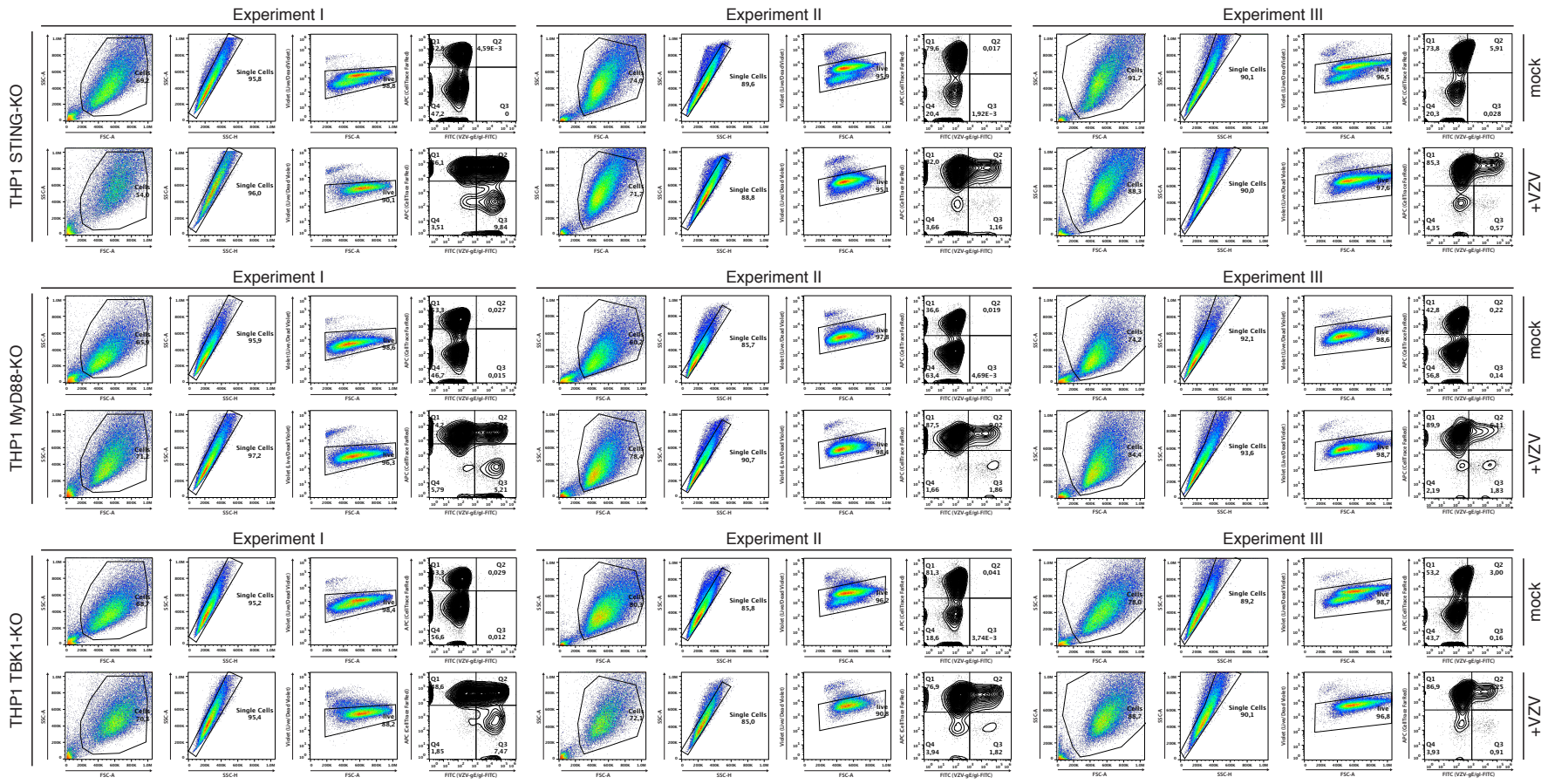


Figure S2: Continued: THP1 VZV Infection flow cytometry analysis. Corresponds to results summarised in Figure 22 and discussed in section IV.2.2. STING-KO, MyD88-KO, or TBK1-KO THP1 cells were PMA-differentiated overnight and labelled with CellTrace FarRed. Cells were mock or VZV-infected by co-culture with uninfected or infected MeWo cells, respectively. 48 h later cells were harvested and a fraction of the cells was processed for flow cytometry by staining with VZV-gE/gI-FITC. Panel shows data from three independent experiments. Continued in Figure S3.

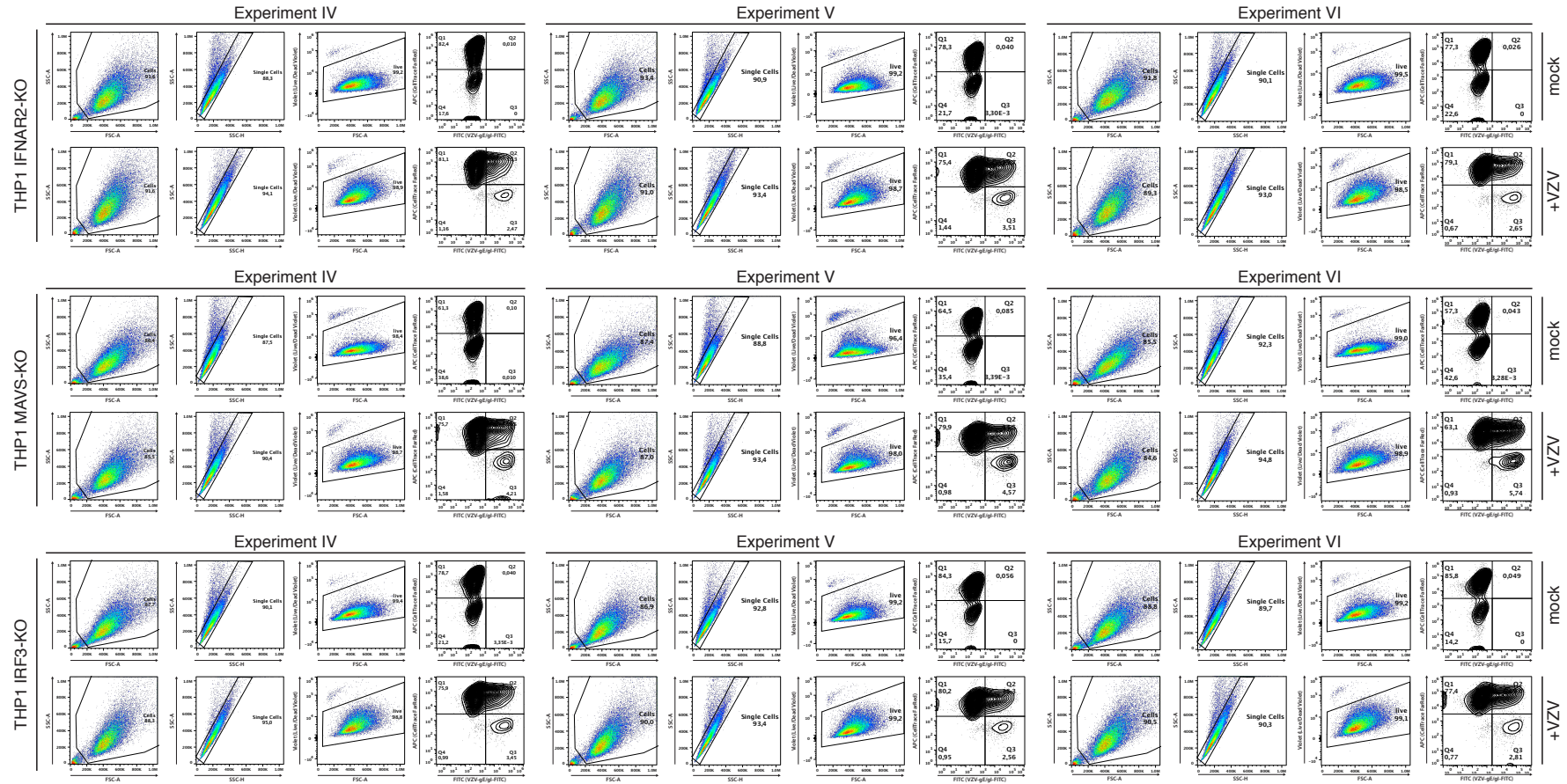


Figure S3: Continued: THP1 VZV Infection flow cytometry analysis. Corresponds to results summarised in Figure 22 and discussed in section IV.2.2. IFNAR2-KO, MAVS-KO, or IRF3-KO THP1 cells were PMA-differentiated overnight and labelled with CellTrace FarRed. Cells were mock or VZV-infected by co-culture with uninfected or infected MeWo cells, respectively. 48 h later cells were harvested and a fraction of the cells was processed for flow cytometry by staining with VZV-gE/gI-FITC. Panel shows data from three independent experiments. Continued in Figure S4.

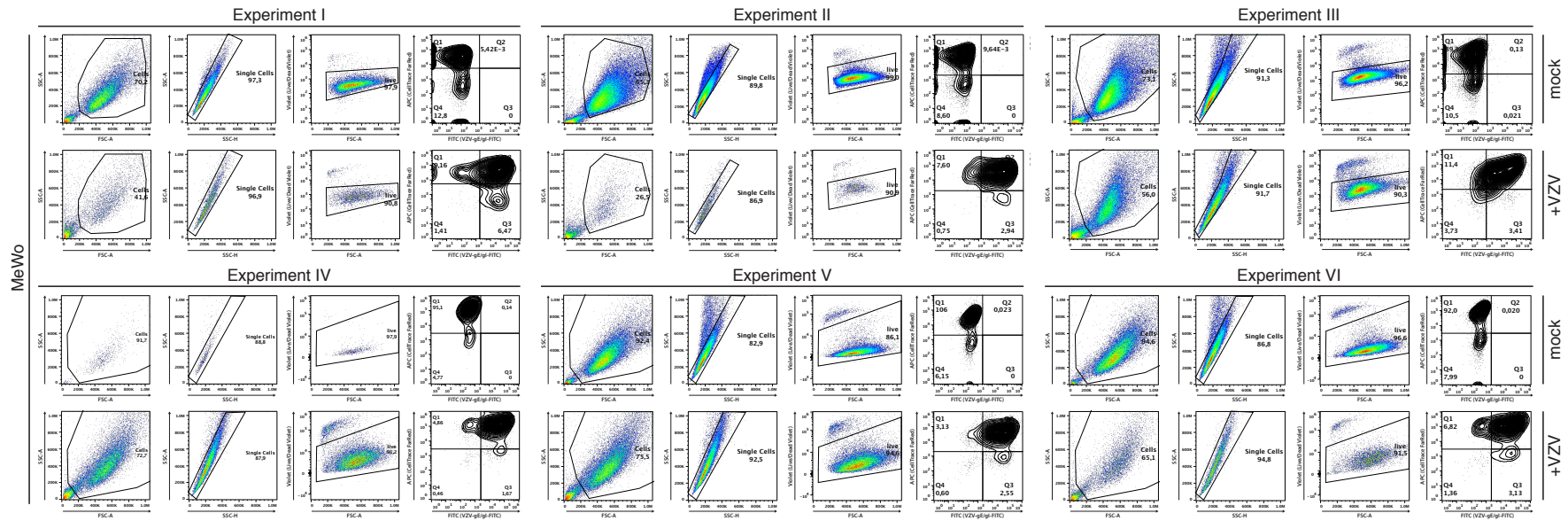


Figure S4: Continued: MeWo VZV Infection flow cytometry analysis. Corresponds to results summarised in Figure 22 and discussed in section IV.2.2. MeWo cells were let adhere overnight and labelled with CellTrace FarRed. Cells were mock or VZV-infected by co-culture with uninfected or infected MeWo cells, respectively. 48 h later cells were harvested and a fraction of the cells was processed for flow cytometry by staining with VZV-g/gi-FITC. Panel shows data from six independent experiments.

Supplementary Tables

Protein	HaCaT mock			HaCaT DNA			THP1 mock			THP1 DNA		
	log2(fold)	-log(p-value)	sign?	log2(fold)	-log(p-value)	sign?	log2(fold)	-log(p-value)	sign?	log2(fold)	-log(p-value)	sign?
XRCC6	2.4810	1.3807	+	2.8979	1.7695	+	3.9412	3.4216	+	3.5114	2.1840	+
PRKDC	2.6983	1.9614	+	1.8307	2.0811	+	4.4201	3.5777	+	3.3898	2.0848	+
ERCC6L	-	-		3.2500	1.8273	+	4.2400	4.2405	+	3.8732	4.5507	+
XRCC5	1.4248	0.7987		2.0405	2.6087	+	3.3703	3.2914	+	3.7292	2.3422	+
PARP1	-0.9198	1.2802		2.5074	1.4441	+	4.5003	2.7397	+	4.4186	3.9987	+
USP7	2.6484	2.5403	+	1.7262	0.9424		2.5784	3.6893	+	3.0032	5.1597	+
ERP44	2.8092	2.4870	+	3.5547	2.2366	+	2.0713	1.4840	+	1.4653	1.0187	
GATAD2A	1.8273	1.7976	+	2.3863	2.0269	+	1.6697	2.4666	+	1.6318	2.0526	
ATF1	-1.4888	1.2979		2.4776	2.0692	+	2.5767	1.9061	+	3.8542	2.9439	+
RUNX3	-1.2538	3.0264	+	-0.2548	0.3404		3.7146	3.0369	+	5.1578	3.1217	+
MBD2	1.8902	1.9844	+	1.6074	3.3852	+	2.3694	3.2860	+	0.7419	1.2994	
SNW1	-2.6469	1.8860	+	2.4507	1.5488	+	-2.5838	1.6003	+	0.9299	0.6526	
CHTOP	-3.9686	1.1585	+	3.6301	1.4002	+	-4.4013	1.7175	+	2.4485	0.9897	
USP9X	2.6484	2.5403	+	1.7262	0.9424		3.5946	4.4471	+	2.5840	2.8117	
TFAM	0.2069	0.4702		0.0300	0.3651		5.5762	3.7496	+	4.5515	2.5120	+
TFEB	0.2069	0.4702		0.0300	0.3651		6.0427	4.8217	+	4.0509	2.5231	+
ACTL6A	1.6870	1.6647		2.4096	1.4555	+	3.5729	2.6557	+	2.6275	2.2499	
ZEB2	-0.2474	0.6258		0.9469	0.9788		4.5536	3.0751	+	4.9389	3.2896	+
CHD7	0.1715	0.2460		2.8098	2.3675	+	4.6752	3.8538	+	2.4620	2.5331	
ACTR3	1.9794	1.0548		-1.1547	1.8894		3.8041	1.7409	+	4.2147	3.6556	+
CUX1	-0.8878	2.6839		0.0933	0.1634		5.2153	2.5314	+	3.5510	2.1088	+
ETV6	-0.9611	1.6217		2.6124	1.9591	+	3.2594	1.9121	+	2.9899	2.4477	
EGR3	0.5833	0.2065		2.3695	1.4215	+	1.9947	0.8613		2.8673	3.8362	+

Continued on next page

Protein	HaCaT mock			HaCaT DNA			THP1 mock			THP1 DNA		
	log2(fold)	-log(p-value)	sign?	log2(fold)	-log(p-value)	sign?	log2(fold)	-log(p-value)	sign?	log2(fold)	-log(p-value)	sign?
EHMT1	0.5833	0.2065		2.3695	1.4215	+	3.3695	3.3719	+	1.2093	1.7501	
ARG1	2.5930	1.5551	+	-0.1683	0.0694		1.1850	1.4179		3.8197	1.7647	+
XRCC1	-1.3564	1.5355		1.1117	0.8997		3.7584	2.9039	+	3.6509	2.3810	+
ACOT9	2.3758	2.5459	+	1.1152	0.8096		2.0586	1.2308	+	1.4980	1.3113	
SMARCA5	-1.2583	1.4261		2.3699	1.5400	+	1.6338	1.5672		3.9677	1.4660	+
MTA2	2.1320	2.4190	+	1.8113	4.2275	+	1.4561	2.3911		1.1592	2.7279	
CREM	-0.8413	1.1662		0.8711	0.9929		2.5767	1.9061	+	3.8542	2.9439	+
CREB1	-0.8413	1.1662		0.8711	0.9929		2.5767	1.9061	+	3.8542	2.9439	+
SMARCA4	0.4625	2.1799		0.7186	1.4457		2.0055	1.8459	+	3.0599	4.4085	+
RBBP4	1.7790	3.6980	+	2.0868	2.5918	+	1.3463	3.9469		1.0260	3.1102	
KIF11	2.2790	0.9963		-2.4197	1.3266	+	5.4635	2.0377	+	0.0917	0.0375	
KPNA5	0.3785	0.3025		1.6738	2.4487	+	2.9944	3.5808	+	0.2915	0.1278	
KPNA6	0.3785	0.3025		1.6738	2.4487	+	2.9944	3.5808	+	0.2915	0.1278	
KPNA2	0.3785	0.3025		1.6738	2.4487	+	2.9944	3.5808	+	-	-	
WDR11	1.3155	1.3005		-2.0145	3.4254	+	2.5272	1.5057	+	2.9614	2.0858	
EGR2	0.5833	0.2065		2.3695	1.4215	+	-1.1187	2.2584		2.8673	3.8362	+
RBBP7	1.9503	3.7165	+	1.7511	2.6420	+	0.2927	0.1049		0.5967	0.4760	
SS18	-1.9647	1.2742		2.4894	2.2450	+	1.9952	1.6206	+	1.9396	1.4702	
RPA1	-0.5303	0.2930		-2.0263	2.5380	+	3.6891	3.3330	+	3.1996	2.1829	
H2AFY	-1.4718	2.1284	+	3.0440	1.3938	+	-0.2637	0.1929		2.9466	0.9734	
SMARCC1	-1.2583	1.4261		2.3699	1.5400	+	1.9822	2.5354	+	1.0258	1.4733	
VRK3	-1.9684	0.9887		3.0043	1.7000	+	3.4508	2.2484	+	-0.4380	0.5148	
HSPA9	1.8807	5.3735	+	1.7505	1.8139	+	0.4201	0.6659		-0.4682	0.8484	
S100A10	-1.7904	1.2077		2.2539	1.6021	+	-0.0746	0.0228		3.0815	2.5523	+

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Protein	HaCaT mock			HaCaT DNA			THP1 mock			THP1 DNA		
	log2(fold)	-log(p-value)	sign?	log2(fold)	-log(p-value)	sign?	log2(fold)	-log(p-value)	sign?	log2(fold)	-log(p-value)	sign?
TP53BP1	-1.2206	1.2642		-1.5679	1.9066	+	3.8667	2.0831	+	2.3124	2.4745	
RB1CC1	2.9534	1.2338	+	-1.7071	1.2287		2.4857	2.3565	+	-0.5523	0.3567	
RUNX1	-1.2538	3.0264	+	-0.2548	0.3404		2.1888	1.4554	+	2.4435	0.9764	
H2BFS	-1.4718	2.1284	+	1.8222	1.8061	+	-0.2637	0.1929		2.9466	0.9734	
KPNA1	2.2790	0.9963		-2.4197	1.3266	+	2.9944	3.5808	+	0.1362	0.2080	
ARID1A	0.1833	0.0794		-1.8350	2.0192	+	2.8080	1.7299	+	1.7452	1.9909	
ARPC2	1.7819	1.4675		-1.8285	2.0758	+	2.2048	1.5136	+	0.4262	0.1957	
SMARCA2	-2.4308	1.5400	+	-0.1952	0.1107		2.0945	1.0790	+	2.7030	2.3813	
MITF	-2.6474	1.3833	+	1.3867	0.9257		3.4656	2.3899	+	-0.3479	0.1664	
MAEA	1.2229	1.0420		-1.7397	1.8374	+	1.8974	1.2597	+	0.3493	0.4803	
PFKP	-1.8826	1.6944	+	1.9566	1.4802	+	1.0297	1.6244		0.5209	0.5657	
OAT	2.5845	2.7443	+	0.2859	0.0774		-1.6908	1.8534	+	0.3367	0.3459	
PTGES3	-2.7619	2.5295	+	0.9263	0.5989		1.9908	2.8262	+	0.7508	0.4559	
SREK1IP1	-2.1751	2.4348	+	-0.3385	0.2727		3.4019	1.3357	+	-0.0067	0.0017	
SDC2	-3.8829	2.5509	+	1.6660	0.6020		2.1548	2.6708	+	0.8658	1.5777	
SAMD1	-3.2636	1.6330	+	1.5507	0.8244		2.5714	1.4675	+	-0.1275	0.0981	
PDCD4	-0.0638	0.0811		-2.1349	2.3461	+	2.6141	3.8940	+	0.1604	0.0903	
PRPF3	-2.0811	2.8696	+	2.4947	1.9537	+	-0.6948	1.6034		0.6828	2.1183	
MYOF	-2.3141	1.4910	+	2.6233	1.2279	+	-0.2599	0.4591		0.3247	0.5919	
SARS2	-3.4180	1.5559	+	1.4564	0.6433		1.9031	3.5116	+	0.3627	0.1476	
SLC25A3	-2.7564	1.4994	+	4.0832	2.2049	+	-2.4005	0.6560		1.3149	0.5239	
CLTC	-0.3111	0.1718		2.0240	1.5442	+	-2.9361	1.6872	+	1.2874	0.6927	
RPN1	-1.7067	1.1126		2.5459	1.5232	+	-2.4090	2.0482	+	1.3336	0.3843	
ESYT1	-0.9611	1.6217		2.6124	1.9591	+	-2.9528	1.8659	+	0.7700	0.5388	

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Protein	HaCaT mock			HaCaT DNA			THP1 mock			THP1 DNA		
	log2(fold)	-log(p-value)	sign?	log2(fold)	-log(p-value)	sign?	log2(fold)	-log(p-value)	sign?	log2(fold)	-log(p-value)	sign?
PABPN1	-1.4628	0.9488		2.6907	1.6404	+	-2.9503	1.9270	+	1.1741	0.9209	
LMAN1	-0.8267	0.7955		1.5491	3.0310	+	-1.9507	1.4621	+	0.4880	0.1796	
CCDC12	-2.1521	1.5622	+	1.6497	2.0169	+	-1.0982	2.1498		0.6258	1.2048	
ATP5C1	-1.3348	0.8424		2.7879	1.6467	+	-3.2562	2.0503	+	0.8275	0.6880	
SEC61B	-1.9059	1.7778	+	2.1758	1.9169	+	-0.4828	0.1900		-0.8738	0.5385	
VDAC2	-1.9684	0.9887		3.0043	1.7000	+	-2.3944	1.1965	+	-0.1360	0.0652	
SRSF9	-1.9647	1.2742		2.4894	2.2450	+	-2.7641	1.6043	+	0.6944	0.3635	
ATP5O	-1.3275	0.9892		2.0947	1.6336	+	-4.0801	2.9093	+	1.4214	0.7126	
GNB2	-2.4920	1.6924	+	1.3893	0.7697		-2.7947	1.5661	+	1.6326	0.7856	
SAFB	-2.2027	1.2436		2.3191	1.4834	+	-4.0271	2.1505	+	1.4001	0.8425	
SLC25A5	-2.5935	1.1626		3.2616	1.1800	+	-4.1598	2.3803	+	0.9378	0.2978	
LIG3	-1.0961	0.8296		0.7374	0.5316		5.1707	2.8347	+	3.3770	1.6833	
CTBP1	1.6202	1.2940		0.6067	0.6114		3.3860	3.2505	+	1.7739	1.9168	
CTCF	1.6202	1.2940		0.6067	0.6114		2.5670	2.3480	+	2.5829	1.4295	
YWHAQ	1.9798	0.8406		-1.7880	1.3258		3.5629	2.6762	+	3.1642	1.5642	
SATB2	-0.3352	0.9797		0.6535	1.2810		3.4980	3.5887	+	2.9698	2.0359	
CSNK2A2	1.6202	1.2940		0.6067	0.6114		2.8366	3.1679	+	1.6207	0.8284	
SUPT16H	0.1895	0.1464		1.1932	1.0684		2.4126	1.2459	+	2.7002	1.2742	
CBX3	0.9070	0.8744		2.0049	2.5040	+	0.5903	0.9604		2.7503	2.0269	
CBFB	-1.0081	1.3482		0.3805	0.7966		3.9423	2.3053	+	2.7866	1.6150	
ZNF516	-1.7984	1.3750		1.9225	1.3286		3.9214	3.1975	+	1.9897	1.6195	
PYCRL	0.1953	0.3565		1.0519	2.5540		2.3185	2.9996	+	2.4363	2.9642	
TRPS1	-1.6164	1.1973		0.4775	0.1993		4.6497	2.2760	+	2.4867	2.1990	
CHD4	0.1715	0.2460		2.8098	2.3675	+	1.0428	1.9971		1.8927	2.1979	

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APPENDIX

Protein	HaCaT mock			HaCaT DNA			THP1 mock			THP1 DNA		
	log2(fold)	-log(p-value)	sign?	log2(fold)	-log(p-value)	sign?	log2(fold)	-log(p-value)	sign?	log2(fold)	-log(p-value)	sign?
ARCN1	-0.1940	0.1460		0.9623	0.8924		1.1850	1.4179		3.8197	1.7647	+
TXN	1.0177	3.3948		1.0181	0.9876		2.3173	6.3569	+	1.3976	2.6559	
YWHAE	1.9798	0.8406		-1.7880	1.3258		3.4554	3.8756	+	2.0980	2.7085	
ZNF148	-0.6490	0.3333		2.3925	1.2090		2.0852	1.7117	+	1.8656	1.6277	
ZBTB7A	1.9798	0.8406		-1.7880	1.3258		4.0087	2.8773	+	1.4623	0.6606	
HIST2H2BF	-0.6770	0.3184		1.8222	1.8061	+	0.9253	0.5154		3.4907	1.0748	
GATAD2B	1.3053	1.8830		1.2203	2.2930		1.7739	3.1053	+	1.1700	3.2969	
MTA1	1.1364	1.2135		2.3219	1.8205	+	0.8852	2.1144		1.0295	2.7125	
MRPS7	1.2167	0.8452		2.1620	1.5220	+	0.0883	0.1758		1.9028	1.2340	
YWHAH	1.9798	0.8406		-1.7880	1.3258		3.2476	3.6043	+	1.8282	4.6171	
HDAC1	1.0972	2.8367		1.3001	3.2865	+	1.3458	2.8981		1.5129	3.4547	
DHX40	-0.7980	0.8212		-0.1017	0.0378		3.4542	4.1067	+	2.5786	2.5640	
DPYSL2	-0.1822	0.1831		-0.5586	0.8505		0.0840	0.0149		5.7356	3.2437	+
NUMA1	0.8403	1.9496		0.2716	0.3796		2.4945	4.5195	+	1.4580	2.0221	
NFATC1	0.2988	0.5545		-0.4838	1.3617		2.3721	2.3021	+	2.8686	2.2686	
HDAC2	1.5525	1.9080		1.4382	2.0432	+	1.1913	1.9019		0.8444	2.7508	
HSPH1	2.1259	1.3813		0.5287	0.3502		2.0825	3.2928	+	0.2770	0.4059	
RFC1	-0.4002	0.3480		-0.9073	2.6972		3.5711	3.6287	+	2.7412	3.2965	
XRCC4	-1.3564	1.5355		1.1117	0.8997		3.3243	3.2090	+	1.7614	1.2552	
ZBTB17	1.9798	0.8406		-1.7880	1.3258		3.5613	3.8144	+	1.0485	0.6421	
ARPC3	1.1194	1.5307		-1.1779	1.3302		2.6233	2.7089	+	2.2341	2.7271	
HIST1H1E	1.1087	0.3485		-1.6416	0.8611		2.4501	0.9705	+	2.8478	0.7329	
HMBOX1	0.2536	0.2487		-0.1881	0.1434		3.2512	3.2713	+	1.4272	0.8486	
HIST3H3	-0.6770	0.3184		2.3194	1.7051	+	1.2929	0.5627		1.8044	0.8218	

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Protein	HaCaT mock			HaCaT DNA			THP1 mock			THP1 DNA		
	log2(fold)	-log(p-value)	sign?	log2(fold)	-log(p-value)	sign?	log2(fold)	-log(p-value)	sign?	log2(fold)	-log(p-value)	sign?
YWHAZ	1.9798	0.8406		-1.7880	1.3258		2.8188	2.8180	+	1.7238	3.7186	
HLA-A	-0.2802	0.2067		1.8890	1.7871	+	1.2929	0.5627		1.8044	0.8218	
RANGAP1	1.6949	1.6386		0.0904	0.0503		2.9177	2.9710	+	-0.1084	0.0473	
DLST	0.9343	1.1192		2.5261	1.6613	+	0.4861	1.1618		0.5907	1.4492	
RANBP9	1.2574	1.2647		-0.6214	0.7676		2.3202	1.7957	+	1.5754	2.7238	
HIRA	0.3105	0.4441		-0.9617	0.7883		2.8726	1.9687	+	2.2745	2.7913	
SMARCE1	0.4597	0.2665		-0.8662	0.9421		2.2208	1.9308	+	2.6572	1.3839	
WIZ	0.6097	0.9737		-1.0836	1.4535		3.0039	3.0460	+	1.9190	3.0207	
MRPS28	-	-		2.2522	1.8339	+	0.2992	0.6248		1.8701	1.7489	
WRN	0.6097	0.9737		-1.0836	1.4535		4.0404	2.0211	+	0.6510	0.4636	
PRDM10	-0.7062	0.4924		0.8006	0.4477		3.0313	2.2425	+	1.0662	0.3792	
MAX	2.0317	1.1180		0.0473	0.0196		3.1189	1.9035	+	-1.1695	0.4007	
DDB1	2.0159	2.5678	+	0.3477	0.6828		1.1699	2.9523		0.4623	0.7025	
HSD17B10	1.3541	2.6448	+	0.3654	0.2487		1.1998	0.6792		0.9305	0.6563	
AMPD2	-2.1109	1.3225		1.0906	0.5740		2.6308	2.1353	+	2.1277	1.6552	
HIST1H3A	-0.7548	0.6150		2.3194	1.7051	+	0.3556	0.4600		1.8031	0.9474	
GRWD1	2.0051	1.0611		-1.1655	0.7744		1.7349	1.6492	+	1.1263	1.9029	
YWHAB	1.9798	0.8406		-1.7880	1.3258		2.4891	2.9369	+	1.0085	3.6534	
HIST1H4A	-0.6770	0.3184		3.0793	1.9147	+	-0.7862	0.8985		2.0616	0.9519	
HSPA5	2.0912	3.5250	+	1.4432	1.4335		0.7600	1.4839		-0.6686	1.7396	
LIG4	-1.0961	0.8296		0.7374	0.5316		4.0713	3.7172	+	-0.0870	0.0233	
POLR2A	0.0905	0.1340		1.7037	1.8118	+	0.4819	0.2131		1.2561	1.4998	
HTATSF1	0.3297	1.8286		0.5508	1.9177		2.2630	1.0317	+	0.3779	0.1172	
SATB1	-0.3352	0.9797		0.6535	1.2810		1.7457	2.0432	+	1.4503	1.0801	

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Protein	HaCaT mock			HaCaT DNA			THP1 mock			THP1 DNA		
	log2(fold)	-log(p-value)	sign?	log2(fold)	-log(p-value)	sign?	log2(fold)	-log(p-value)	sign?	log2(fold)	-log(p-value)	sign?
UBN2	1.0703	1.3902		-0.0926	0.1365		2.7087	1.6826	+	-0.1932	0.1441	
RNH1	1.1295	0.9748		-1.7636	1.3680		1.9367	4.8192	+	2.1602	2.4941	
AMPD3	-2.1109	1.3225		1.0906	0.5740		2.2155	2.5103	+	2.2560	2.2326	
LRPPRC	0.5094	0.5824		1.7620	2.4368	+	-0.8703	0.7892		2.0273	3.1717	
OGT	0.7519	1.5719		0.9679	2.1469		1.8687	2.0188	+	-0.1606	0.1695	
NAP1L4	0.2428	0.6653		0.4656	1.1284		1.7962	2.4622	+	0.9215	2.0006	
FRYL	1.5654	1.3259		0.2282	0.2630		3.0150	1.6234	+	-1.4180	0.8496	
PPM1G	0.7740	0.3596		-0.5111	0.3927		1.9694	1.9934	+	1.1286	2.2455	
GPX4	2.0051	1.0611		-1.1655	0.7744		2.6834	2.5925	+	-0.2150	0.1286	
PPAN	0.6126	0.9054		0.4988	0.7915		1.7011	4.0685	+	0.4881	2.6533	
YWHAG	1.9798	0.8406		-1.7880	1.3258		2.2860	2.2477	+	0.7939	2.8091	
ACTR2	1.3832	1.2745		-1.2734	1.2638		2.5013	1.0854	+	0.6583	2.3939	
KPNB1	0.6577	2.0007		1.6966	2.0014	+	-	-		0.8787	0.4610	
GNB4	0.4727	1.4584		1.7974	1.6443	+	0.6656	2.2643		0.2843	0.8970	
RIF1	0.7187	1.2828		0.2735	0.2936		2.3045	4.7869	+	-0.1011	0.0601	
SMARCC2	0.6420	0.4409		-0.8129	0.5829		2.1867	1.9197	+	1.1537	1.0724	
MRPS2	-0.6923	0.5376		2.1999	1.5133	+	-0.1772	0.3338		1.7863	1.4371	
NONO	-0.2360	0.3608		1.7925	2.4908	+	1.0589	3.8730		0.4523	0.5698	
ARMC8	0.9997	0.5553		-0.9894	1.0059		2.1337	1.9809	+	0.9224	1.3245	
C1QBP	2.2054	2.8143	+	1.0875	0.9986		-0.5531	0.5757		0.2965	0.2089	
COPA	-0.5611	0.6631		2.1324	1.7566	+	-0.2640	0.2470		1.6870	1.7896	
NFRKB	0.2988	0.5545		-0.4838	1.3617		2.0639	2.3132	+	1.0731	0.9104	
RANBP2	0.6635	2.3058		1.1304	3.6313	+	0.8672	3.9979		0.2322	0.6513	
TERF2IP	0.2069	0.4702		0.0300	0.3651		1.8086	1.6275	+	0.8374	0.6808	

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Protein	HaCaT mock			HaCaT DNA			THP1 mock			THP1 DNA		
	log2(fold)	-log(p-value)	sign?	log2(fold)	-log(p-value)	sign?	log2(fold)	-log(p-value)	sign?	log2(fold)	-log(p-value)	sign?
EXOSC7	0.2030	0.4620		0.2762	0.1708		2.1594	1.2700	+	0.2346	0.1016	
MYCBP	0.5206	0.4810		0.1840	0.1774		3.7581	1.6631	+	-1.6205	1.0447	
ZNF646	-1.7984	1.3750		1.9225	1.3286		2.4207	1.7155	+	0.2613	0.1315	
EGFR	0.5833	0.2065		2.3695	1.4215	+	-1.1187	2.2584		0.9668	1.2945	
RPL39	-0.5964	1.8392		0.2682	0.5002		3.0177	0.8739	+	0.0646	0.1428	
BUB3	-0.8160	0.5498		2.4530	1.4482	+	0.3998	0.1633		0.6975	2.8415	
HNRNPH3	-1.7856	1.3301		2.8786	1.8632	+	0.3949	0.1527		1.1431	0.7231	
EIF3M	1.4189	2.2961	+	-1.3790	1.2356		0.7763	0.2682		1.7847	0.8069	
NOC2L	-1.5699	0.9806		1.6348	0.8091		2.3006	2.5279	+	0.1770	0.1342	
SFPQ	0.2684	0.3013		1.5913	2.9700	+	-0.1998	0.2991		0.8443	1.0031	
CSK	-0.8526	0.8002		1.6223	0.8756		1.9107	1.8283	+	-0.2662	0.2853	
PPIL4	-0.7614	0.4633		0.1699	0.4523		2.5559	1.7008	+	0.4384	0.1998	
PRKAR1A	-0.1391	0.0992		0.6835	0.7288		2.6019	2.1350	+	-0.8208	0.6892	
MYCBP2	0.5206	0.4810		0.1840	0.1774		1.9009	3.7544	+	-0.2802	0.3356	
VAV1	-1.1015	1.1362		0.5770	0.7678		1.7351	3.2164	+	1.0839	1.0654	
CABIN1	-1.4254	1.1844		-0.6070	0.4087		3.2600	2.0883	+	1.0658	1.2654	
PSPC1	0.8460	0.8630		-0.6899	0.6912		2.4851	2.5782	+	-0.3631	0.1638	
HSPD1	0.7804	1.3244		1.7464	4.6807	+	-0.6369	1.1982		0.3702	0.4816	
KHDRBS1	0.0979	0.1011		1.8691	2.6526	+	-0.8987	1.4202		1.1153	1.9211	
GLUD2	1.8859	1.7370	+	1.9407	1.2616		-0.7289	0.2194		-1.0808	0.4156	
SUN2	0.1895	0.1464		1.1932	1.0684		1.7430	1.9043	+	-1.1110	1.1221	
MRPL41	-0.2797	0.6078		1.8849	1.6585	+	-0.9941	0.7870		1.3713	1.0097	
MRPL15	-0.2171	0.4605		1.8707	1.5548	+	-0.9564	1.4290		1.1818	1.0523	
SPTAN1	1.9008	1.5392		-1.0577	0.7834		2.7252	1.8577	+	-1.6923	1.1293	

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Protein	HaCaT mock			HaCaT DNA			THP1 mock			THP1 DNA		
	log2(fold)	-log(p-value)	sign?	log2(fold)	-log(p-value)	sign?	log2(fold)	-log(p-value)	sign?	log2(fold)	-log(p-value)	sign?
SPTBN1	1.8684	1.5473		-0.9705	0.7505		2.6788	1.9856	+	-1.7718	0.8969	
TAB3	-0.5012	0.4304		0.2327	0.3484		3.6848	3.5326	+	-1.6339	1.3135	
HIST1H1B	0.4568	0.3699		-1.3547	1.7444		1.7910	3.1987	+	0.8233	0.5292	
MRT04	-1.2588	1.0195		2.0740	1.6995	+	-1.0965	3.1201		1.8597	0.8213	
HAGH	-0.0504	0.0619		-0.0893	0.1395		1.8455	1.4690	+	-0.1471	0.0442	
EEF2	-0.3293	1.3336		1.4668	2.7055	+	0.0367	0.1293		0.3225	1.2976	
MGA	1.3288	2.4397	+	0.4162	0.5886		-0.0199	0.0661		-0.2393	0.6246	
LSM14A	-0.8316	0.6874		-1.3976	1.4224		3.6334	1.7942	+	-0.0714	0.0540	
PDAP1	-1.0479	1.0951		1.4471	1.4299		1.8318	2.4254	+	-0.9122	0.5654	
SMC3	-0.3322	0.2115		1.8040	1.7025	+	-1.6692	1.5582		1.5082	0.9001	
LMNA	-0.1367	0.2285		1.6309	2.6500	+	-0.6486	0.8929		0.4565	0.3187	
OTUD4	0.9691	0.5355		-0.2220	0.1562		2.2736	1.3079	+	-1.7886	1.0683	
CCAR2	0.0668	0.1720		1.6194	1.7771	+	-1.0982	2.1498		0.6258	1.2048	
AIFM1	-0.2998	0.1700		1.9378	2.0857	+	0.3348	0.5064		-0.7614	0.4284	
PLRG1	-1.6617	0.9634		2.2575	1.4924	+	-1.7593	0.7038		2.2897	3.3544	
LRRFIP1	1.4817	1.0215		-2.1323	1.3776		1.9085	2.5804	+	-0.1585	0.2290	
SRPRB	-0.1609	0.3005		2.1101	3.5179	+	-0.6863	3.5967		-0.1873	0.3282	
ATAD3A	-1.4888	1.2979		2.4776	2.0692	+	-0.5819	0.2640		0.6537	0.3595	
HIST1H2BC	-2.5797	0.6611		1.8222	1.8061	+	-0.3979	0.5341		2.1986	0.9417	
HIST1H2BD	-2.5797	0.6611		1.8222	1.8061	+	-0.3979	0.5341		2.1986	0.9417	
HIST1H2BH	-2.5797	0.6611		1.8222	1.8061	+	-0.3979	0.5341		2.1986	0.9417	
HIST1H2BK	-2.5797	0.6611		1.8222	1.8061	+	-0.3979	0.5341		2.1986	0.9417	
HIST1H2BM	-2.5797	0.6611		1.8222	1.8061	+	-0.3979	0.5341		2.1986	0.9417	
HIST1H2BN	-2.5797	0.6611		1.8222	1.8061	+	-0.3979	0.5341		2.1986	0.9417	

Continued on next page

Protein	HaCaT mock			HaCaT DNA			THP1 mock			THP1 DNA		
	log2(fold)	-log(p-value)	sign?	log2(fold)	-log(p-value)	sign?	log2(fold)	-log(p-value)	sign?	log2(fold)	-log(p-value)	sign?
RRP15	-0.6874	0.3524		-1.1604	0.7567		2.2075	5.1892	+	0.6733	0.9562	
PAF1	-0.9198	1.2802		2.5074	1.4441	+	-1.2650	1.0361		0.4878	0.2095	
C9orf114	-1.4254	1.1844		-0.6070	0.4087		2.2887	2.0581	+	0.4650	0.2529	
RAB5C	-0.3568	0.3864		1.8058	2.8002	+	-0.5641	1.3432		-0.4073	0.8658	
TOP1	-0.8919	0.5702		-1.4284	1.1615		2.0875	6.5880	+	0.6128	1.1281	
P4HB	0.6513	0.6208		2.1343	2.0161	+	-1.0302	3.3485		-1.4094	3.0477	
ADAR	-0.5372	0.5734		1.3995	2.2609	+	-1.2716	1.4273		0.7551	0.8570	
PDIA6	-0.4394	0.5240		1.8568	2.8954	+	-1.3851	1.7969		0.2402	0.2415	
EIF3J	1.8226	2.1209	+	-1.8375	1.4829		1.2686	1.2548		-1.0243	1.2829	
GLUD1	1.8859	1.7370	+	-1.9918	0.8985		-0.7289	0.2194		-1.0808	0.4156	

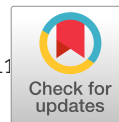
Table S1: Full list of significant hits in AP-MS experiments. Corresponds to AP-MS results discussed in section VI.8. Table shows all proteins that were identified as significantly enriched bound to VZV ORF9 in at least one condition. For each protein the fold enrichment (as log2), p-value after Welch t-test (as -log10), and whether it was a significant hit is shown.

Publications

Full text and supplementary materials for REF. 184:

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Research Article

Infection with a Brazilian isolate of Zika virus generates RIG-I stimulatory RNA and the viral NS5 protein blocks type I IFN induction and signaling

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Zika virus (ZIKV) is a major public health concern in the Americas. We report that ZIKV infection and RNA extracted from ZIKV infected cells potently activated the induction of type I interferons (IFNs). This effect was fully dependent on the mitochondrial antiviral signaling protein (MAVS), implicating RIG-I-like receptors (RLRs) as upstream sensors of viral RNA. Indeed, RIG-I and the related RNA sensor MDA5 contributed to type I IFN induction in response to RNA from infected cells. We found that ZIKV NS5 from a recent Brazilian isolate blocked type I IFN induction downstream of RLRs and also inhibited type I IFN receptor (IFNAR) signaling. We defined the ZIKV NS5 nuclear localization signal and report that NS5 nuclear localization was not required for inhibition of signaling downstream of IFNAR. Mechanistically, NS5 blocked IFNAR signaling by both leading to reduced levels of STAT2 and by blocking phosphorylation of STAT1, two transcription factors activated by type I IFNs. Taken together, our observations suggest that ZIKV infection induces a type I IFN response *via* RLRs and that ZIKV interferes with this response by blocking signaling downstream of RLRs and IFNAR.

Keywords: Interferon · MDA5 · RIG-I · STAT · Zika virus



Additional supporting information may be found in the online version of this article at the publisher's web-site

Introduction

Viruses from the *Flaviviridae* family are enveloped and contain a positive sense, single stranded RNA genome. This virus

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family includes many pathogens important to human health such as hepatitis C virus and mosquito-borne dengue virus (DENV), West Nile virus and Zika virus (ZIKV). ZIKV was initially described in 1947 after isolation from monkeys living in the Zika forest in Uganda [1, 2] but has only received notable attention since the recent epidemic in Brazil and other parts of the Americas. ZIKV can be categorized into viruses of the Asian and African lineage. The 2015/2016 epidemic strain belongs to the Asian lineage and shares a common ancestor with viruses causing outbreaks in Polynesia in 2013/2014 [3, 4]. Infection is often asymptomatic or is characterized by a self-limiting acute febrile illness, including mild fever, maculopapular rash, arthralgia and conjunctivitis [5, 6]. In adults, ZIKV infection has also been suggested to trigger Guillain-Barré syndrome, a rapid-onset muscle weakness caused by an autoimmune response [7]. ZIKV is most commonly transmitted by *Aedes* mosquitoes; however, other routes of transmission include sexual and maternal-fetal during pregnancy [8–10]. In the latter case, *in utero* ZIKV infection may cause developmental defects resulting in microcephaly [11]. Indeed, the recent epidemic coincided and overlapped geographically with an increase in microcephaly cases in newborns [3]. Moreover, ZIKV infects neural progenitor cells and vertical transmission as well as fetal microcephaly have been documented in mouse models [reviewed in: [7]].

Type I interferons (IFNs, including IFN- α and IFN- β) are cytokines which coordinate many aspects of the mammalian immune response to infectious microorganisms [12]. During viral infections, type I IFNs are often crucial to successful immunity. In the course of an infection, their expression is induced at the transcriptional level in different types of cells. This occurs downstream of pathogen sensing by innate immune receptors [13, 14]. Sensors of virus presence often detect nucleic acids as molecular signatures of infection; for example, viral RNA or DNA are potent triggers for type I IFN induction [13, 14]. These sensors include toll-like receptors, which survey the endosomal compartment, as well as cytosolic DNA receptors and RIG-I-like receptors (RLRs) that are localized in the cytosol of cells [13, 14]. RLRs are helicase proteins and include RIG-I and MDA5. RIG-I recognizes viral RNAs that have uncapped 5'-ends marked by tri- or diphosphate groups [15, 16]. Upon binding to viral or unusual RNAs, RIG-I and MDA5 engage the adaptor protein MAVS. Signaling downstream of MAVS activates transcription factors including IRF3 and NF- κ B, which then drive transcription of the genes encoding type I IFNs and other antiviral genes [13, 14]. Once secreted, type I IFNs bind to the dimeric type I IFN receptor (IFNAR) on the same or other cells [12]. This results in activation of JAK1 and TYK2 kinases, which in turn phosphorylate and thereby activate STAT1 and STAT2. These transcription factors then form a complex with IRF9 and induce the expression of hundreds of interferon-stimulated genes (ISGs). The proteins encoded by ISGs have a variety of direct and indirect antiviral effects [17].

ZIKV infection in cultured human cells and mice is controlled by type I IFNs. For example, treatment of human skin fibroblasts or A549 cells with IFN- α or IFN- β prior to infection diminishes ZIKV replication [18, 19]. *In vivo*, ZIKV infection in mice is restricted by type I IFNs. Virus replication, transmission and pathology are

exacerbated in animals lacking IFNAR, STAT2, MAVS or IRF transcription factors and after treatment with an IFNAR blocking antibody [20–26]. Interestingly, in human monocyte-derived dendritic cells and skin fibroblasts, the addition of type I IFN after or concomitantly with infection only modestly reduces ZIKV replication [18, 27], suggesting viral interference with the effects of type I IFNs. Indeed, recent studies have reported that ZIKV NS5 proteins from African and French Polynesian isolates antagonize IFNAR signaling by targeting STAT2 for degradation [19, 28]. However, NS5 and other ZIKV proteins from virus strains associated with the latest outbreak in Brazil have not yet been analyzed in this context, which is important given that Zika virus isolates differ significantly in *in vivo* and *in vitro* infection settings [29, 30]. Additionally, ZIKV infection has been suggested to prevent STAT1 and STAT2 phosphorylation [27].

We addressed the open question of how ZIKV is detected by infected cells to induce type I IFNs. We report that ZIKV infected cells accumulated RNA molecules that potentially activated RIG-I in a 5'-phosphate-dependent manner. MDA5-stimulatory RNAs were also generated during infection, suggesting that ZIKV infection is sensed by RLRs. Based on sequence information from a Brazilian ZIKV isolate, we individually cloned all viral proteins into a mammalian expression vector. We demonstrate that NS5 from a Brazilian 2015/2016 epidemic strain of ZIKV blocks type I IFN induction mediated by RLRs and IFNAR signaling. The latter function of NS5 involved depleting STAT2 levels and preventing phosphorylation of STAT1. Nuclear localization was not required for this function of NS5. We propose a model in which ZIKV induces a type I IFN response via cytosolic RNA sensors of the RLR family and antagonizes this response by blocking signaling downstream of RLRs and IFNAR.

Results

ZIKV infection induces type I IFN and generates RLR-stimulatory RNA

Infection of cells with RNA viruses often results in RIG-I- and/or MDA5-dependent type I IFN production, and infected cells accumulate RLR-stimulatory RNA molecules. Experimentally, this can be tested by the extraction of RNA from infected cells followed by transfection of reporter cells. For example, IAV infected cells accumulate RIG-I-stimulatory RNAs and encephalomyocarditis virus (EMCV) infection results in the generation of extractable RNAs that activate MDA5 [31, 32]. This assay therefore faithfully predicts which RLRs are involved in detecting the virus in the infected cell [33]. To test this in the context of ZIKV, we infected A549 cells with a ZIKV strain from Brazil (PE243, [34]) at a multiplicity of infection (MOI) of 5 and extracted total RNA at different time points post infection. We first tested whether a type I IFN response was induced in this setting. Indeed, using RT-qPCR, we found that *IFN β* mRNA and several transcripts encoded by ISGs were upregulated in ZIKV-infected A549 cells (Fig. 1A). Next, we transfected RNA from A549 cells infected with ZIKV for 20 hours

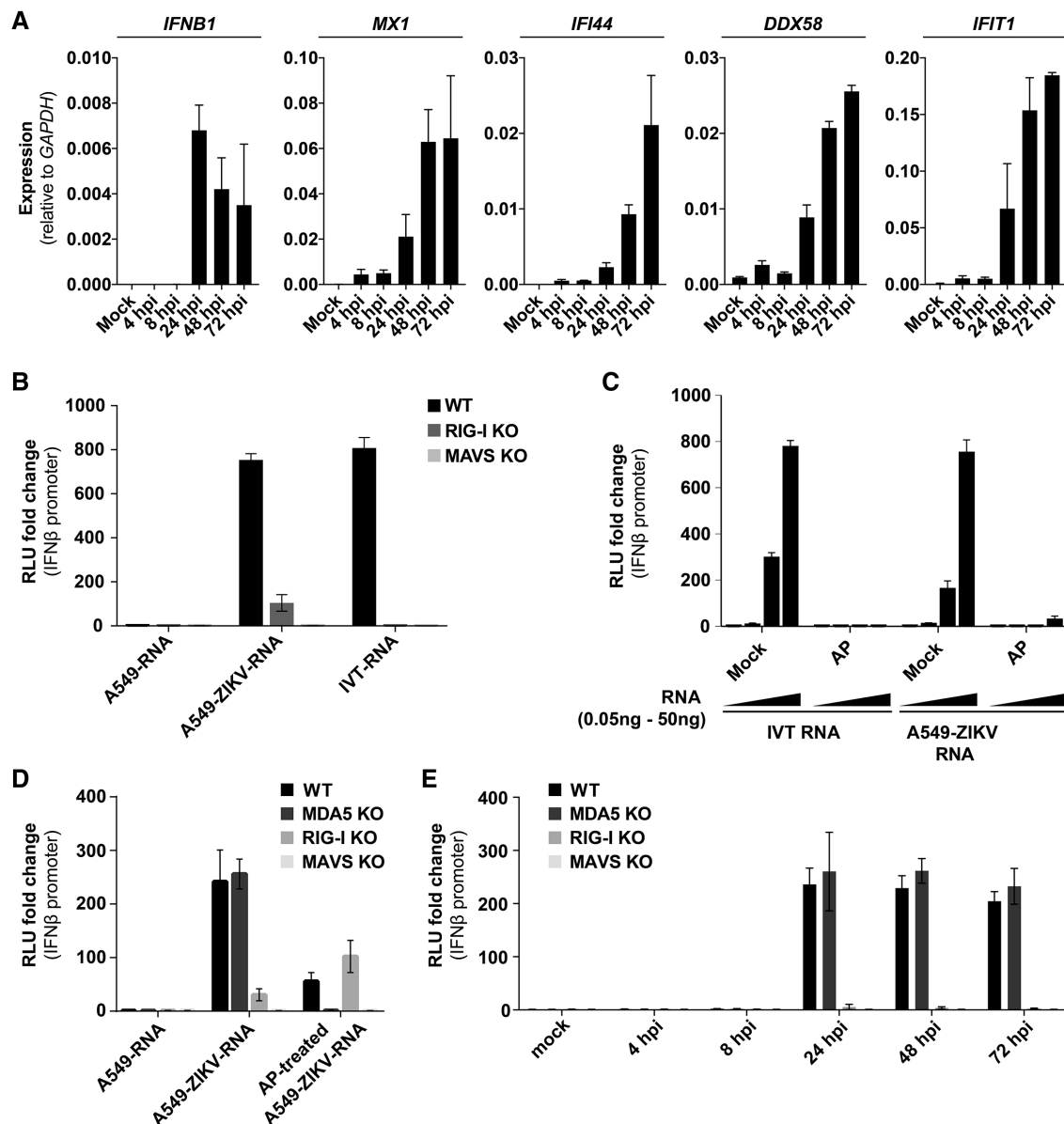


Figure 1. ZIKV infection generates RIG-I- and MDA5-stimulatory RNAs. (A) A549 cells were infected with ZIKV (MOI of 5) and total RNA was extracted at the indicated time points. Expression levels of the indicated mRNAs were determined by RT-qPCR with samples assayed in technical duplicates. Data were analyzed by the comparative C_t method. (B) A549 cells were infected with ZIKV (MOI of 5) and total RNA was extracted after 20 h (A549-ZIKV-RNA). 100 ng A549-ZIKV-RNA or RNA from uninfected cells (A549-RNA) was then transfected into the indicated p125HEK cells (see Supporting Information Fig. 1). After 16 h, luciferase activity was determined. Data are shown as fold change compared to cells treated with transfection reagent only. IVT-RNA, Neo¹⁻⁹⁹ in vitro transcribed RNA (RIG-I agonist); RLU, relative light unit. (C) IVT-RNA and A549-ZIKV-RNA were treated with alkaline phosphatase (AP). Control samples were processed in parallel omitting AP (mock). These RNAs were then analyzed as in (B) using wild-type p125HEK cells and four doses of RNA (0.05, 0.5, 5, and 50 ng). (D) The experiment shown in (B) was repeated using the indicated p125HEK cell lines that were pre-treated with 3 U/ml IFN- α/δ for 16 h and were then transfected with 50 ng of total RNA from uninfected cells (A549-RNA) or with A549-ZIKV-RNA that was or was not treated with AP. (E) The experiment shown in (B) was repeated using A549-ZIKV-RNA isolated at the indicated time points after infection. Panel (A) shows pooled data of two independent experiments (average and SEM ($n = 4$)). Panel (B) is representative of three independent experiments (average and SD ($n = 3$)). Panels (C) and (D) are representative of two independent experiments (average and SD ($n = 3$)). Panel (E) shows pooled data of two independent experiments (average and SEM ($n = 6$)).

(A549-ZIKV-RNA) into HEK293 cells stably expressing firefly luciferase (F-Luc) under control of the IFN β promoter (Supporting Information Fig. S1A-C). A549-ZIKV-RNA induced expression of the IFN β reporter and the magnitude of the response was similar to that triggered by Neo¹⁻⁹⁹ in vitro transcribed (IVT) RNA,

a 5'-triphosphate containing RIG-I agonist [31] (Fig. 1B). RNA from uninfected A549 cells did not induce the IFN β promoter in these settings (Fig. 1B). Next, we analyzed the response in RIG-I or MAVS knockout reporter cells (Supporting Information Fig. S1D-O). As expected, the response to IVT-RNA was fully

RIG-I- and MAVS-dependent (Fig. 1B). Induction of the *IFN β* reporter after A549-ZIKV-RNA transfection was strongly reduced in RIG-I-deficient cells and was completely absent in MAVS knock-out cells (Fig. 1B). To corroborate this finding, we treated A549-ZIKV-RNA and IVT-RNA as a control with alkaline phosphatase (AP), which removes the 5'-phosphates required for RIG-I activation [31]. Indeed, AP treatment abolished IFN induction in response to IVT-RNA, and largely prevented the response to A549-ZIKV-RNA (Fig. 1C). These results show that A549-ZIKV-RNA contains RIG-I-stimulatory RNA molecules. To test whether the remaining response to A549-ZIKV-RNA observed in RIG-I-deficient cells or after AP treatment could be attributable to MDA5, we included MDA5 knockout cells in the analysis (Supporting Information Fig. 1). Indeed, the response to AP-treated A549-ZIKV-RNA was MDA5-dependent (Fig. 1D). We also analyzed the kinetics of RLR-stimulatory RNA accumulation in ZIKV-infected cells and found this had occurred between 8 and 24 h post-infection (Fig. 1E), correlating with the time course of *IFN β* and ISG mRNA induction in infected cells (Fig. 1A). This therefore suggests that viral replication might be required to activate RLRs. Taken together, these observations show that ZIKV infection results in the accumulation of RIG-I- and MDA5-stimulatory RNAs.

We next asked whether RIG-I directly binds viral RNAs. We employed an in vitro assay in which recombinant FLAG-tagged RIG-I protein and total RNA from infected cells were mixed in a test tube [16]. After incubation, RIG-I was immunoprecipitated with α -FLAG antibodies and RNAs that co-purify with the protein were extracted. These RNAs were then analyzed for their potential to induce a response in our *IFN β* promoter reporter cells and for the presence of viral RNAs (Fig. 2A). Negative controls included immunoprecipitation with non-specific control antibody and the use of denatured RIG-I protein. Western blot analysis confirmed that both native and denatured RIG-I were precipitated by α -FLAG but not by control antibody (Fig. 2B, E and data not shown). As RIG-I recognizes the RNA genome of IAV [31], we used total RNA from HEK293T cells infected with this virus as a positive control. As expected, native but not denatured RIG-I bound to RNAs that stimulated reporter expression in a RIG-I-dependent manner (Fig. 2C and data not shown). RT-qPCR analysis confirmed that RNA corresponding to the M segment of IAV was bound by native RIG-I (Fig. 2D). Similarly, pulldown of native RIG-I mixed with A549-ZIKV-RNA retained IFN-stimulatory RNAs (Fig. 2F). Moreover, RT-qPCR using a probe corresponding to nucleotides 4930–5029 of the full length ZIKV RNA genome demonstrated an association of ZIKV RNA with RIG-I (Fig. 2G). This finding is consistent with the accumulation of RIG-I-stimulatory RNAs in ZIKV-infected cells and shows that RIG-I is capable of recognizing ZIKV RNAs.

Construction of ZIKV Brazil protein expression vectors

To further study the interaction between ZIKV and the type I IFN system, we prepared a library of expression constructs for individual ZIKV proteins. Importantly, we based our analysis on the sequence of ZIKV strain Natal RGN, Brazil (KU527068) [11]. In

contrast, an earlier study used sequences derived from a viral strain from French Polynesia [19]. In brief, coding sequences were assembled by gene synthesis, cloned into a gateway entry vector and then shuttled into pcDNA3.2 for expression in mammalian cells with an C-terminal V5 tag. Our library included the three structural proteins (C, prM, E), the seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5), as well as several fusion proteins (NS2B-NS3, NS4A-2K, 2K-NS4B, NS4A-2K-NS4B) (Fig. 3A). Following transient transfection of HEK293 cells, expression of ZIKV proteins was tested by Western blot using an antibody against the V5 tag (Fig. 3B–D). C, NS1, NS3, NS4A, NS4B, NS5 and 2K-NS4B constructs were expressed well and a single band of the predicted molecular weight was detected (Fig. 3B). Other constructs were expressed at lower levels and for some multiple bands were evident, which may reflect post-translational modifications, protein degradation or aggregation (Fig. 3B, C). We also constructed a vector expressing NS5 fused at its C-terminus to enhanced yellow fluorescent protein (eYFP) for localization studies (Fig. 3D).

ZIKV NS5 inhibits the induction of type I IFN downstream of RLRs

Given that ZIKV infection generates RNAs that activate the RLR pathway (Figs. 1 and 2), we asked whether the virus encodes an antagonist of RLR-driven type I IFN induction. To test this idea, we transiently transfected HEK293 cells with ZIKV expression vectors. We included an *IFN β* promoter F-Luc reporter plasmid and, for normalization, a *Renilla* luciferase control plasmid in the transfection mix. After overnight incubation, cells were stimulated with RLR agonists and induction of the reporter was determined (Fig. 4A). Empty vector and an expression plasmid for EMCV-L, a known antagonist of IRF3 [35], served as negative and positive controls, respectively. As expected, *IFN β* promoter activation by the synthetic RIG-I agonist IVT-RNA and by A549-ZIKV-RNA were blunted by EMCV-L expression (Fig. 4B). Interestingly, ZIKA NS5 had similar inhibitory effects, while the other viral proteins had no effect on expression of the *IFN β* promoter reporter (Fig. 4B). NS5 also blocked activation of the reporter by an MDA5 agonist, VERO-EMCV-RNA [32] (Fig. 4C). These data indicate that ZIKV NS5 is a viral inhibitor of type I IFN induction mediated by RLRs.

NS5 blocks type I interferon receptor signaling

We next investigated whether NS5 or other ZIKV proteins block type I interferon receptor signaling. We recently described a stable reporter cell line that contains F-Luc under the control of an interferon-stimulated response element (ISRE) [36]. Exposure to type I IFN induces F-Luc expression in these cells. We tested whether overexpression of ZIKV proteins blocks the induction of the ISRE reporter gene. When ZIKV proteins were expressed individually, only NS5 reduced F-Luc induction in response to recombinant type I IFN (Fig. 5A). Other ZIKV proteins did not reduce

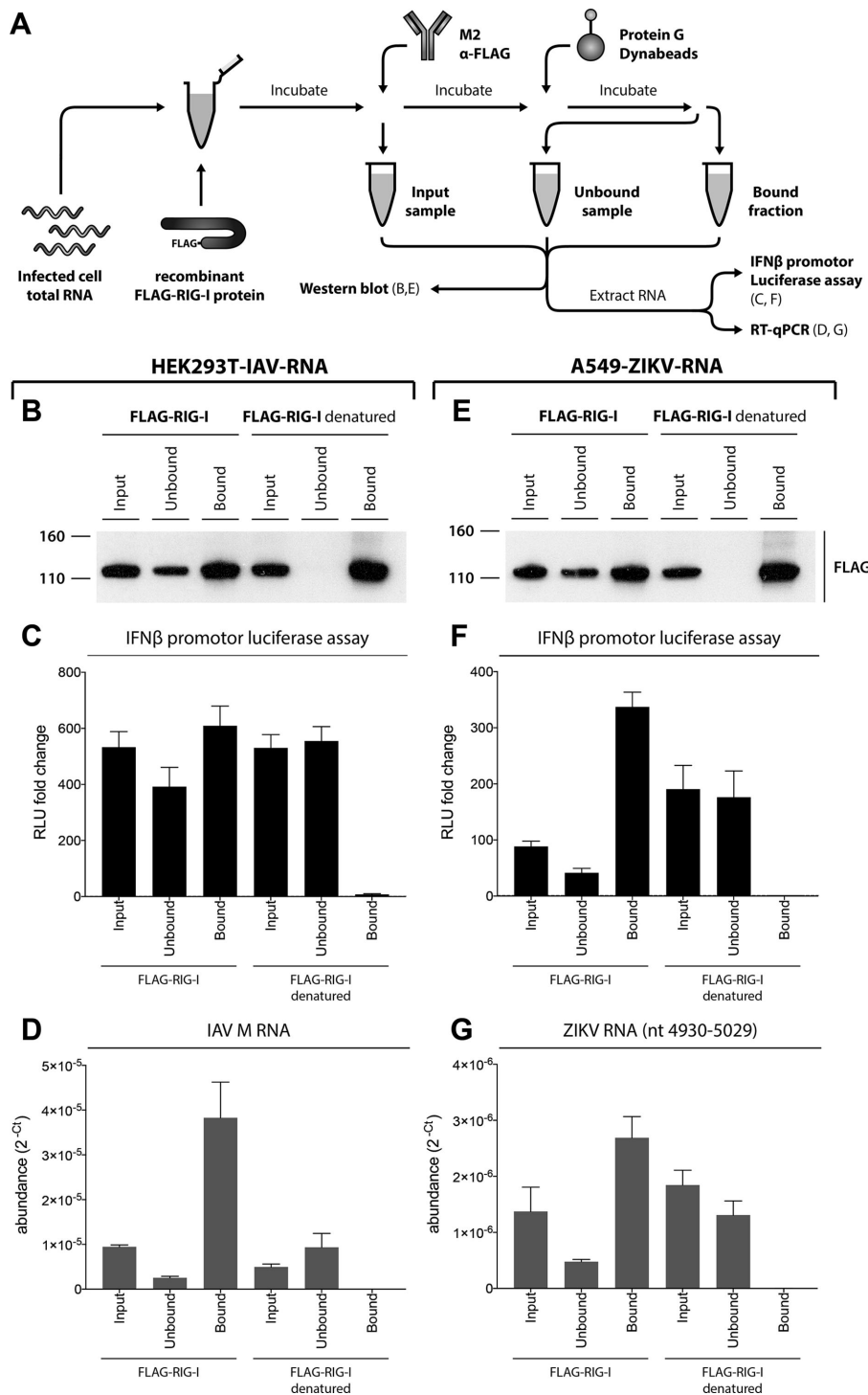


Figure 2. RIG-I binds ZIKA RNA. (A) Schematic of the experimental setup. Please see results for detail. HEK293T-IAV-RNA (panels B–D) and A549-ZIKV-RNA (E–G) were used. (B, E) Input, unbound and bound samples were analyzed by Western blot using α -FLAG antibody. (C, F) RNAs extracted from input, unbound and bound samples were transfected into p125HEK cells (see Supporting Information Fig. 1). After 16 h, luciferase activity was determined and set to 1 for control samples treated with transfection reagent alone. RLU, relative light unit. (D, G) RNA samples from (C) and (F) were analyzed in technical duplicate by RT-qPCR using the indicated probes. Relative viral transcript abundance was analyzed by converting C_t data to 2^{-C_t} values. Panels (B) and (E) are representative of three independent experiments. Panels (C), (D), (F) and (G) show pooled data from three independent experiments (average and SEM ($n = 6$)).

the induction of the ISRE reporter (Fig. 5A). It is possible that different ZIKV proteins may act together to block IFNAR signaling; hence, we repeated the experiment by co-transfecting groups of five ZIKV expression plasmids arranged so that each ZIKV protein was expressed with every other protein at least once. Any plasmid combination containing ZIKV NS5 blocked ISRE induction, while combinations not including NS5 did not interfere with the

response to type I IFN (Fig. 5B). In line with this observation, we found that combinations of three plasmids not including NS5 did not block IFNAR signaling (Fig. 5C).

The effect of NS5 in the stable reporter cells used in Figs. 5A–C was incomplete. This may be due to limited transfection efficiency and responses from non-transfected cells contained in the well. We therefore repeated the experiment by transient transfection

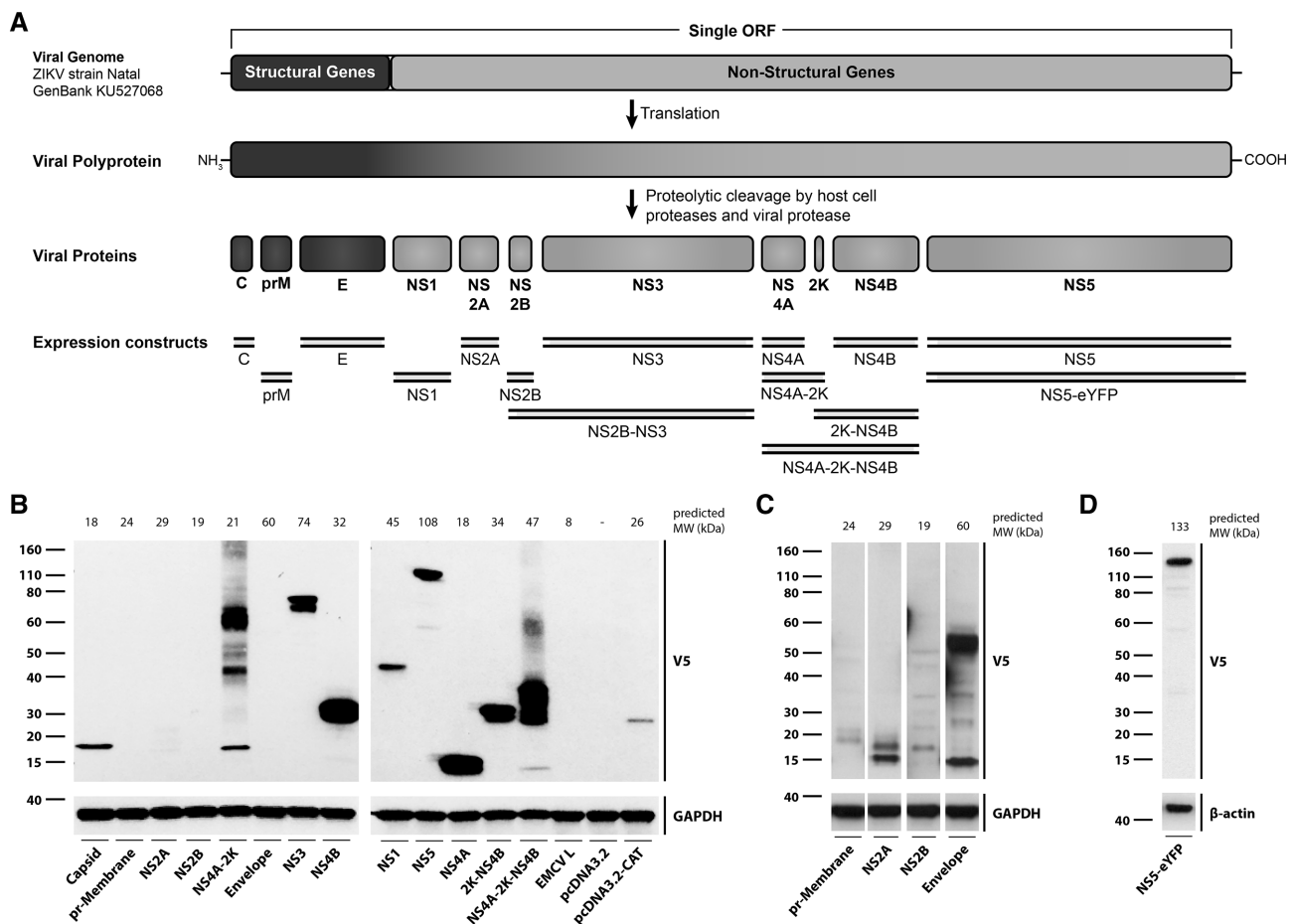


Figure 3. Cloning and expression of ZIKV proteins. (A) Expression constructs. The DNA sequence of the single ZIKV open reading frame encoding all structural and non-structural proteins was derived from a sequenced ZIKV complete genome (GenBank accession KU527068; origin Brazil) recovered from the brain of a microcephalic, aborted foetus. Segments encoding individual proteins were annotated using sequence alignment with a reference genome (GenBank accession NC.012532.1). Expression constructs generated and used in this study are shown as double lines. (B) HEK293 cells were transfected with plasmids expressing the indicated ZIKV proteins. Cell lysates were prepared 24 h after transfection and protein expression was determined by Western blot using the indicated antibodies with GAPDH as loading control. MW, molecular weight. (C, D) The experiment shown in panel (B) was repeated using HEK293T cells and the indicated plasmids. Data are representative of at least three independent experiments.

of an ISRE-F-Luc reporter plasmid together with ZIKV expression constructs and a constitutively expressed *Renilla* luciferase plasmid. In this setting, the response to recombinant type I IFN was completely blocked by NS5 (Fig. 5D). NS4A weakly blocked ISRE induction and all other ZIKV proteins failed to have an effect (Fig. 5D). N-terminally eYFP tagged NS5 also blocked ISRE induction (Fig. 5E).

To test whether NS5 interferes with induction of endogenous ISGs after exposure of cells to type I IFN, we measured the expression of *DDX58* (encoding RIG-I), *MX1*, *IFIT1* and *IFI44* by RT-qPCR. Expression of these ISG mRNAs was induced to different levels after type I IFN treatment and this was blocked by the presence of NS5 (Fig. 6A). Similarly, RIG-I protein levels were induced by type I IFN and this was prevented by NS5 expression (Fig. 6B). Taken together, these data demonstrate that NS5 is the major antagonist of IFNAR signaling and ISG induction encoded by a ZIKV isolate from Brazil.

ZIKV NS5 depletes STAT2 levels and blocks STAT1 phosphorylation

Earlier work suggested that ZIKV NS5 targets STAT2 for degradation [19, 28] and that ZIKV infection blocks STAT1 phosphorylation [27]. Therefore, we investigated the mechanism by which NS5 interferes with IFNAR signaling and tested STAT1 and STAT2 expression and phosphorylation levels by Western blot. NS5 bound to STAT2 and STAT2 levels were reduced by NS5 suggesting that NS5 may target STAT2 for degradation (Fig. 6C and S2). In contrast, STAT1 protein levels were unchanged in NS5 expressing cells that were not treated with IFN (Fig. 6C). *STAT1* is an ISG [17] and, as expected, induction of STAT1 protein by IFN was blocked by NS5 (Fig. 6C). Moreover, STAT1 phosphorylation was readily detectable in control cells treated with type I IFN but was strongly reduced in NS5 expressing cells (Fig. 6C). Analysis of the ratio of total and phosphorylated STAT1 by densitometry showed that NS5

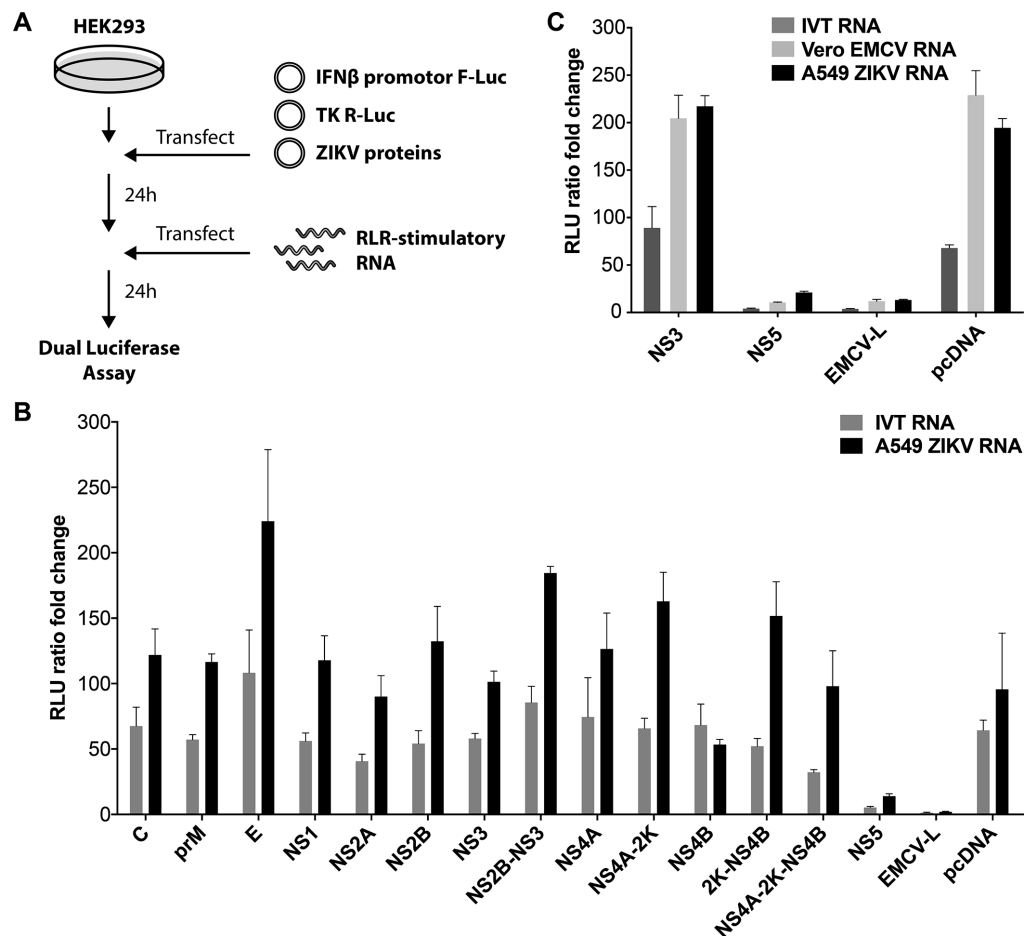


Figure 4. ZIKV NS5 blocks type I IFN induction by RLRs. (A) Schematic of the experimental setup. Please see results for detail. (B, C) HEK293 cells were transiently transfected with the indicated ZIKV plasmids, a plasmid encoding F-Luc under control of the IFN β promoter and a plasmid expressing Renilla luciferase (R-Luc) from the constitutive thymidine kinase (TK) promoter. Cells were transfected with RLR-stimulatory RNAs after 24 h as indicated. F-Luc and R-Luc activities in cell lysates were determined after an additional 24 h and the F-Luc / R-Luc ratio was set to 1 in cells that received only transfection reagent at the RNA transfection step. Data are representative of three independent experiments (average and SD (n = 3)).

expression prevented STAT1 phosphorylation (Fig. 6D) suggesting that NS5 is the viral protein blocking STAT1 phosphorylation during ZIKV infection [27]. Taken together, these data indicate that NS5 blocks IFNAR signaling by multiple mechanisms, including reduced STAT2 levels and inhibition of STAT1 phosphorylation.

NS5 nuclear localization is not required for inhibition of IFNAR signaling

ZIKV NS5 is predominantly localized in the cell nucleus and binds to importin α/β 1 [28, 37–39]. NS5 has an N-terminal methyltransferase (MTase) domain and a C-terminal RNA polymerase domain, which are separated by the interdomain region. In DENV NS5, this region contains nuclear localization signals (NLSs) including the aNLS element that is recognized by importin α/β 1 [40, 41]. The DENV NS5 aNLS is characterized by two clusters of functionally essential basic residues [41], some of which are conserved in ZIKV (Fig. 7A). To test if these residues are required for nuclear

localization of ZIKV NS5, we mutated both clusters individually and together (Fig. 7A). We then expressed wild-type and mutant NS5-eYFP fusion proteins in HEK293T cells and analyzed NS5 localization by fluorescence microscopy. As expected, wild-type NS5 was found in the cell nucleus (Fig. 7B). Mutation of cluster 1 (RQ to AA) or cluster 2 (KHK to AAA) did not change this pattern, while mutation of both clusters together resulted in redistribution of NS5 to the cytoplasm (Fig. 7B). This observation defines the RQ-KHK aNLS motif in ZIKV NS5 as a functional bipartite NLS. Interestingly, we found that the NS5 NLS mutant was capable of blocking ISRE induction and reducing STAT2 levels (Fig. 7C, D).

The MTase activity of NS5 contributes to the formation of cap structures on viral transcripts by guanine N-7 and ribose 2'-OH methylation [42]. The E218A mutation in yellow fever virus NS5 disrupts ribose 2'-OH methylation [43]. To test if MTase activity, in the absence of the canonical viral RNA substrate, is related to the effects of NS5 on IFNAR signaling, we introduced the same mutation into ZIKV NS5. However, overexpression of the E218A

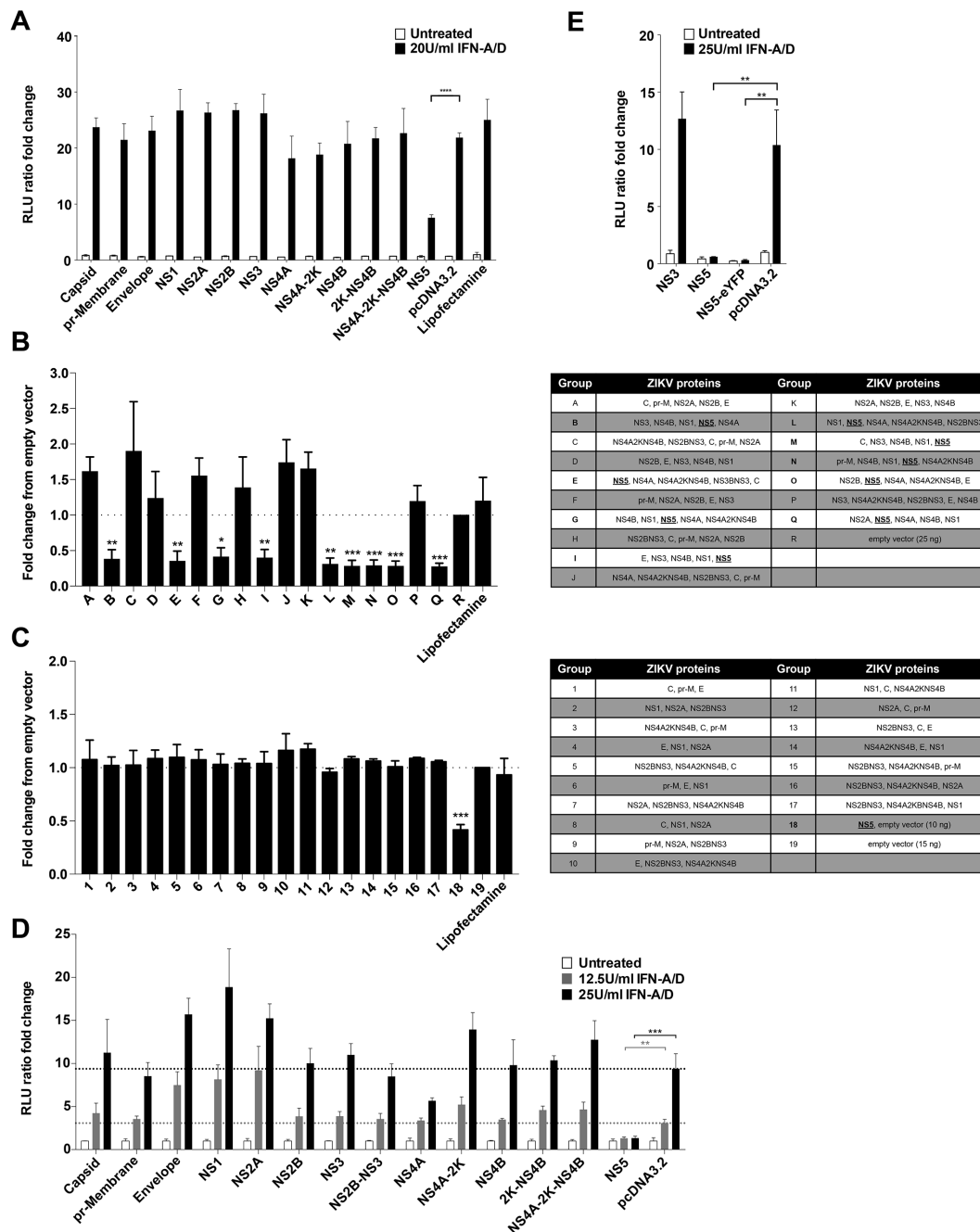


Figure 5. ZIKV NS5 blocks type I IFN signaling. (A) Stable HEK293-ISRE-reporter cells were transfected with ZIKV plasmids as indicated and, 24 h later, were treated or were not with 20U/ml IFN-A/D. Luciferase activity in cell lysates was determined 24 h after transfection and was set to 1 in untreated cells that received empty vector. (B,C) The experiment shown in (A) was repeated using plasmid combinations as indicated on the left. (D,E) HEK293T cells were transiently transfected with the indicated ZIKV plasmids, a plasmid encoding F-Luc under the ISRE control and a plasmid expressing Renilla luciferase (R-Luc) from the TK promoter. Cells were treated with IFN-A/D after 24 h as indicated. F-Luc and R-Luc activities in cell lysates were determined after an additional 24 h and the F-Luc / R-Luc ratio was set to 1 in untreated cells that received empty vector. Panels (A), (D), (E) are representative of three independent experiments (average and SD ($n = 3$)). Panels (B) and (C) show pooled data from three experiments (average and SEM ($n = 9$)). (A), (D), (E): unpaired, two-tailed t-test as indicated. (B), (C): unpaired, two-tailed t-test vs empty vector. ($p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

NS5 mutant in the absence of a viral substrate for methylation neither impaired NS5-mediated inhibition of ISRE induction nor STAT2 depletion (Fig. 7C, D). Comparison of the African MR-766 ZIKV isolate and Brazilian strains revealed two amino acid sub-

stitutions in NS5, M2634V in the MTase domain and M3392V in the RNA polymerase domain [3]. We reverted these two valines at position 2634 and 3392 in our NS5 construct to methionine to test if these residues are involved in antagonism of IFNAR signaling.

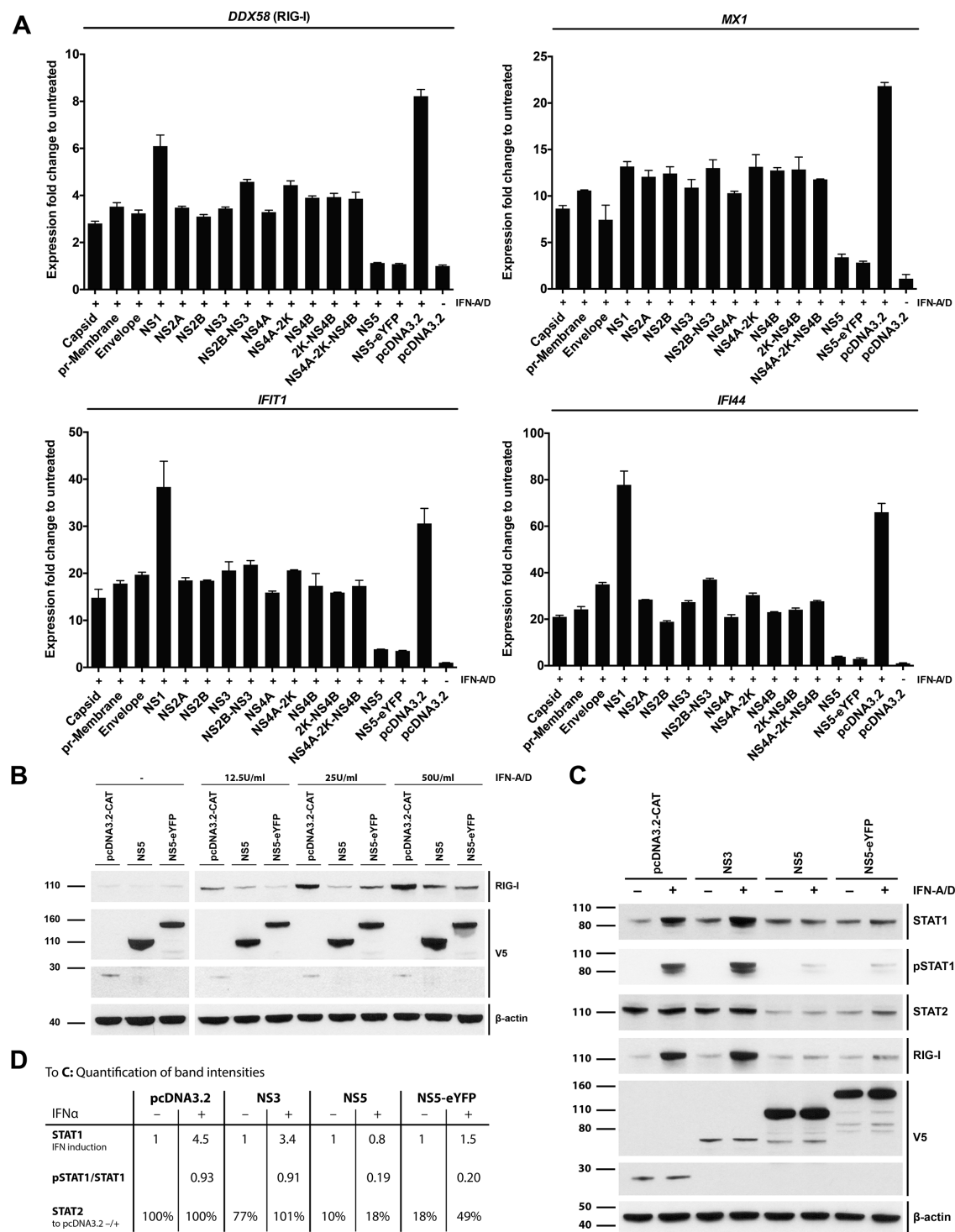


Figure 6. NS5 triggers STAT2 depletion and blocks STAT1 phosphorylation. (A) HEK293T cells were transfected with the indicated ZIKV plasmids and either were or were not treated with 25U/ml of IFN-A/D. RNA was extracted after 24 h and expression levels of the indicated mRNAs were determined by RT-qPCR with samples assayed in technical duplicate. Data were analyzed by the comparative C_t method and set to 1 for untreated cells transfected with empty vector. (B,C) Cell lysates were prepared from HEK293T cells transfected with the indicated plasmids and treated with IFN-A/D for 24 h. Expression of the indicated protein was determined by Western blot with β -actin as loading control. (D) Quantification of signals in (C) using the image densitometry analysis tool of ImageJ. Panels (A) - (C) are representative of three independent experiments. Panel (A) shows average and range ($n = 2$).

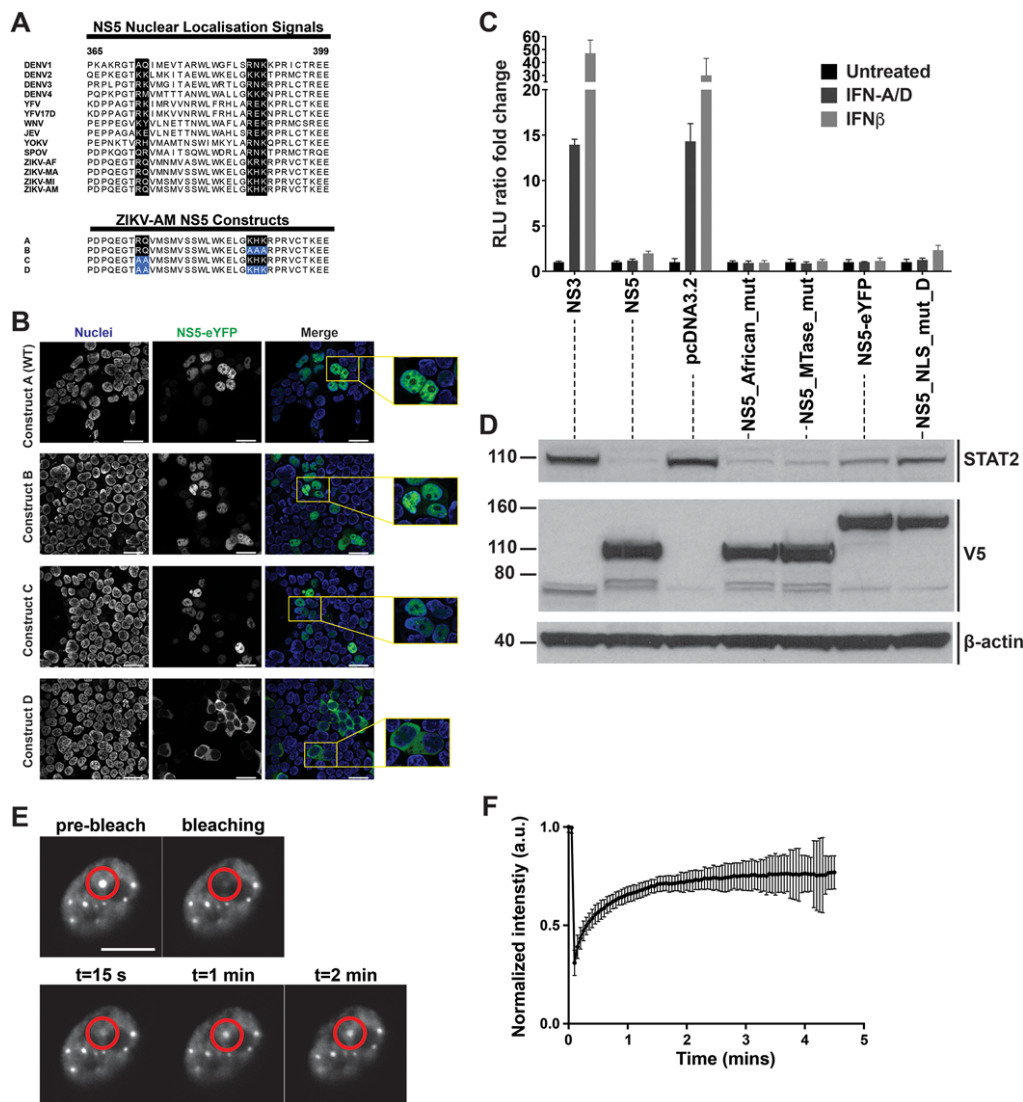


Figure 7. Blockade of type I IFN signaling by NS5 does not require NS5 nuclear localisation. (A) The NS5 interdomain regions from different flaviviruses were aligned and the area around the aNLS is shown. Residues involved in nuclear localization of DENV NS5 are highlighted in black. The aNLS mutations studied in (B) are indicated in blue at the bottom. DENV: Dengue virus; YFV: yellow fever virus; WNV: West Nile virus; JEV: Japanese encephalitis virus; YOKV: Yokose virus; SPOV: Spondweni virus; ZIKV: Zika virus – AF: Africa, MA: Malaysia, MI: Micronesia, AM: America. (B) HEK293T cells were transfected with wild-type NS5-eYFP or NS5 NLS mutant constructs as shown in panel (A). After 24 h, live cells were imaged with a confocal microscope. Nuclei were stained with the membrane permeable dye, NucBlue™. Scale bar is 25 μm. (C, D) HEK293T cells were transfected as in Fig. 5D using the indicated ZIKV plasmids and were treated with 25U/ml of IFN-A/D or IFNβ. 24 h after transfection, luciferase activities were analyzed as in Fig. 5D (C) and cell lysates from untreated samples were analyzed by Western blot with β-actin as loading control (D). (E) HEK293T cells were transfected with ZIKV NS5-eYFP. After 24 h, the mobility of nuclear NS5 speckles was analyzed by fluorescence recovery after photobleaching (FRAP). Representative snapshots of a cell nucleus expressing NS5-eYFP before, during and after bleaching of the speckle indicated by the red circle are shown, indicating that fluorescence of NS5 recovered and that the protein was mobile and in a fluid environment. Scale bar is 2 μm. (F) The intensity of NS5 speckles was normalized to 1 before photobleaching and was analyzed over time after photobleaching. The fluorescence intensity in NS5 speckles dropped to ~25% of the initial value and then recovered back to ~75%. Panels (B)–(F) are representative of at least three independent experiments. Panel C shows average and SD ($n = 3$). Panel (F) shows average of five speckles and SD.

However, this “NS5 African mutant” behaved like the parental NS5 construct based on the Brazilian strain sequence (Fig. 7C and D). Taken together, these data show that NS5 nuclear localization was not required for blocking IFNAR signaling.

Our analysis of ZIKV NS5 localization showed that NS5 is not uniformly distributed across the cell nucleus, but appears

to be enriched in dot-like structures (Fig. 7B). We investigated the dynamics of these NS5 speckles using fluorescence recovery after photobleaching (FRAP). NS5 speckles recovered at the site of bleaching within about 1 min, suggesting that these structures are highly dynamic and presumably “liquid” in nature [44] (Fig. 7E and F).

Discussion

Innate immune responses play an important role in determining the course of disease caused by virus infection. It is therefore important to understand how such responses are initiated and how viruses evade and antagonize them. Here, we studied this question in the context of the emerging pathogen ZIKV, and show that ZIKV infection generates RNA molecules that stimulate RIG-I and MDA5. These sensors of virus invasion induce type I IFNs, which activate an anti-viral gene expression program by signaling through their receptor, IFNAR. We report that ZIKV antagonizes RLR and IFNAR signaling by means of its NS5 protein, and investigate sequences and cellular location responsible.

Our results demonstrate that ZIKV infection results in the accumulation of RIG-I stimulatory RNAs. RIG-I detects RNA molecules bearing 5'-triphosphate groups [31]. NS5 is an RNA-dependent RNA polymerase that replicates the genomes of viruses belonging to the *Flaviviridae* family and employs a primer-independent mechanism of initiation [45]. This results in the presence of triphosphate groups derived from the initiating ribonucleoside triphosphate at the 5'-ends of nascent viral RNAs, which we predict activate RIG-I in ZIKV infected cells. Indeed, we report that RIG-I could directly associate with ZIKV RNA and that phosphatase-treatment renders RNA from ZIKV infected cells unable to stimulate RIG-I. It will be interesting to confirm this by infection experiments in RIG-I-deficient cells and by RIG-I pulldowns from infected cells.

Flaviviral replication complexes assemble on ER membranes and reside in vesicles formed by membrane invaginations, also known as replication factories [45]. How then, does RIG-I—which is localized in the cytosol—gain access to newly replicated viral RNAs? Analysis of ZIKV infected cells by electron microscopy revealed pore-like openings connecting replication factories to the cytosol [46]. It is conceivable that at least some nascent viral RNAs escape through these pores and are then sensed by RIG-I. Indeed, the RIG-I pathway is very sensitive and is triggered by less than 20 RNA molecules per cell [47]. Moreover, replication factories are likely to be dynamic structures.

Interestingly, we find that ZIKV infection generates not only RIG-I agonists but also MDA5-stimulatory RNAs. Dual activation of RIG-I and MDA5 is consistent with the observation that IFN induction downstream of both RIG-I and MDA5 is blocked by sfRNA, a subgenomic, non-coding RNA expressed during ZIKV infection [34]. Furthermore, infection with other members of the *Flaviviridae* family, such as hepatitis C virus, activates both RLRs [48]. Although the molecular properties of RNAs that activate MDA5 are not fully understood, it is clear that MDA5 activation, in contrast to RIG-I, does not require the presence of 5'-triphosphates [13, 14]. NS5 in conjunction with NS3 caps flaviviral RNAs by sequential triphosphatase, guanylyltransferase and methyltransferase activities [45]. It is likely that capping is an evasion strategy that allows viral RNAs to escape detection by RIG-I and other factors such as IFIT proteins [15]. It is possible that MDA5 detects mature ZIKV RNAs and thereby functions as a backup if RIG-I activation is limited by capping.

Many viruses, including flaviviruses, encode inhibitors of the type I IFN system [49–51]. Viral antagonists typically dampen but do not completely shut-down the host response and block type I IFN induction or repress IFNAR signaling. We found that ZIKV NS5 acted at both of these levels and prevented RLR-driven type I IFN induction as well as IFN-mediated ISG expression. The former observation suggests that the reduced type I IFN response reported in ZIKV infected cells transfected with synthetic RLR agonists [19, 27, 52] is due to NS5. The finding that type I IFN responses to both RIG-I and MDA5 stimuli were blocked by NS5 further suggests that NS5 acts at the level of the common adaptor MAVS or downstream in the RLR signaling pathway. This is consistent with a recent study suggesting that NS5 interacts with and blocks IRF3 [30]. Another possibility is that NS5 inhibits RLR signaling indirectly by antagonizing ISG expression, as, in a feed-forward loop, both RIG-I and MDA5 are encoded by ISGs. It will be interesting for future studies to investigate the mechanism by which NS5 blocks RLR signaling.

Recent work using African and French Polynesian ZIKV isolates already demonstrated that ZIKV NS5 interferes with IFNAR signaling by targeting STAT2 for proteasomal degradation [19, 28]. Here, we extend these findings in several ways. First, we show that NS5 from a Brazilian ZIKV strain collected from an aborted, microcephalic fetus blocks the response to type I IFN. This demonstrates that IFNAR signaling is targeted by a ZIKV isolate derived from the recent epidemic. Second, by using our expression constructs for ZIKV proteins alone or in combination, we report that NS5 is likely to be the only potent ZIKV antagonist of IFNAR signaling. It is possible that other viral proteins make a small contribution to blocking IFNAR signaling. For example, we found that NS4A expression slightly dampens the response to type I IFN (Fig. 5D) and Wu et al. as well as Xia et al. recently reported effects of similar magnitude for NS2B-NS3 and other viral proteins [30, 53]. It is likely that this fine-tuning of the response depends on the virus strain, kinetics and precise experimental setup, while it is clear from our data and those of others [19, 28] that NS5 has the most potent and lasting effect. Thirdly, we show that ZIKV NS5 not only reduces STAT2 levels but also blocks STAT1 phosphorylation. This is consistent with a report studying the effects of ZIKV infection in dendritic cells [27] and indicates that ZIKV NS5 has evolved multiple strategies to counteract IFNAR signaling [50, 51]. Fourthly, we define a NLS in ZIKV NS5. Earlier work using the African MR-766 strain found that deletion of four amino acids (KRKR), which overlap with the second half of the bipartite RQ-KHK motif identified here (Fig. 7A), abrogates nuclear localization of overexpressed NS5 [38]. In contrast, we found that the KHK residues in NS5 from a Brazilian isolate were required, but not sufficient, for nuclear localization of the protein, which also required the RQ motif. These observations highlight differences between ZIKV strains. Furthermore, we demonstrate that NS5 nuclear localization was not required for the inhibition of IFNAR signaling. Future studies should address the molecular mechanism by which NS5 targets STAT transcription factors. For example, it will be interesting to identify the E3 ubiquitin ligase required for STAT2 degradation.

It will also be important to delineate the role of NS5 in the cell nucleus. It has been suggested that nuclear NS5 from other flaviviruses blocks IL-8 induction [reviewed in: [54]]. Interestingly, we found that NS5 is organized as speckles in the nucleus. Several recent studies reported similar findings for overexpressed and endogenous NS5 [28, 38, 39]. Using immunofluorescence with α -PML antibody, we found that these structures do not correspond to PML bodies (data not shown). Instead, it is possible that these nuclear NS5 speckles correspond to sites of splicing, given that NS5 co-localizes with SC35, a cellular protein involved in pre-mRNA splicing [38]. Indeed, DENV NS5 has been reported to bind to components of the spliceosome and to modulate splicing of host genes, including *DDX58* (encoding RIG-I) [55]. We further report that the molecules in these speckles were in constant exchange with the surrounding NS5, which suggests that these compartments are “liquid” in nature. This kind of liquid-liquid phase separation has been described for proteins forming membrane-less compartments such as P granules, typically together with nucleic acids or other proteins [56, 57]. This type of compartmentalization plays a role in several processes such as RNA metabolism, biogenesis of the ribosome, DNA damage response and signal transduction [58]. Therefore, it will be an intriguing quest for the future to elucidate the role of this compartmentalization in NS5 function.

Finally, it would be interesting to define mutations in NS5 that selectively disrupt its ability to block RLR or IFNAR signaling while leaving intact its essential functions in the viral life cycle. We predict that such mutant viruses induce elevated IFN responses and only replicate efficiently in cells deficient for components of the type IFN system.

Taken together, we define the interplay between ZIKV and the type I interferon system and identify three points of interaction between the virus and cells: ZIKV infection generates stimuli for the pattern-recognition receptors RIG-I and MDA5, and the viral NS5 protein blocks the ensuing antiviral response by antagonizing RLR signaling and STAT transcription factors that are involved in IFNAR signaling. It is likely that these host-pathogen interactions are important for determining the outcome of disease.

Materials and methods

Cells

Cells were maintained in DMEM (Sigma Aldrich) supplemented with 10% FCS (Sigma-Aldrich) and 2 mM L-Glutamine (Gibco) at 37°C and 5% CO₂. 3C11 cells are HEK293 cells (originally from Caetano Reis e Sousa, The Francis Crick Institute, London, UK) stably transduced with an ISRE-Luc reporter construct [36]. A549 cells were obtained from R. E. Randall, University of St Andrews, UK.

Plasmids

To generate p125-GreenFire, the human *IFN β* promoter region was amplified from the p125-Luc plasmid [59] and cloned into the pGreenFire1-ISRE lentiviral reporter system (System Biosciences, TR016PA-P) using EcoRI and BamHI restriction enzymes to excise the ISRE elements and the minimal CMV promoter. The puromycin N-acetyl-transferase gene was replaced by the blasticidin-S deaminase gene from the pLenti6.3/V5-DEST™ Gateway® Vector (Invitrogen) using the restriction enzyme KpnI.

Plasmids used for luciferase reporter assays were p125-Luc [59], ISG54-Luc (Clontech), and pRL-TK (Promega).

A DNA sequence of the single ZIKV open reading frame encoding for all structural and non-structural proteins was derived from a sequenced ZIKV complete genome (GenBank accession KU527068) recovered from the brain of a microcephalic aborted foetus [11]. The genome was annotated for sequence segments encoding individual proteins using sequence alignment with an annotated reference genome (GenBank accession NC.012532.1) [60]. DNA fragments encoding for the Capsid, Envelope, and pr-Membrane proteins as well as the non-structural proteins NS2A, NS2B, NS3, NS4A-2K, and NS4B were obtained by gene synthesis (GeneArt String linear DNA fragments; Thermo Fisher Scientific) after addition of a start codon (AUG) to the 5' end of DNA sequences. Fragments were cloned into the pCR8/GW/TOPO Gateway entry vector (Invitrogen). After sequence integrity was verified *via* sequencing, inserts were Gateway-cloned into the pcDNA3.2/V5-DEST vector (Invitrogen) using the Gateway LR Clonase II Plus Enzyme Mix (Invitrogen). Fragments encoding the non-structural proteins NS1 and NS5 were generated by gene synthesis into the pDONR221 Gateway donor vector (GeneArt Gene, Thermo Fischer Scientific) and Gateway-cloned into the pcDNA3.2/V5-DEST vector. DNA constructs encoding the other ZIKV proteins utilized in this project (NS4A, 2K-NS4B, NS4A-2K-NS4B, NS2B-NS3, NS5-eYFP) were created by (Overlap)-PCR from existing ZIKV protein encoding plasmids using Phusion High Fidelity DNA Polymerase (New England Biolabs). NS5 mutants were created by mutagenesis PCR and confirmed by sequencing. ZIKV expression plasmids will be made available via Addgene.

A549-ZIKV-RNA, IVT-RNA, VERO-EMCV-RNA and HEK293T-IAV-RNA

A549-ZIKV-RNA was generated by infection of A549 cells with ZIKV (isolate ZIKV/H.sapiens/Brazil/PE243/2015, GenBank accession KX197192.1, described in [34]) at an MOI of 5. Unless indicated otherwise, cells were lysed in TRIzol (Invitrogen) 20 h later and total RNA was extracted according to manufacturer's instructions. Neo¹⁻⁹⁹ in vitro transcribed RNA was generated as previously described [31]. Purified RNAs were incubated with alkaline phosphatase (Roche) using 2 units of enzyme per microgram of RNA. Samples were incubated at 50°C for 1 h and RNAs were purified by phenol-chloroform extraction. Negative control reactions were performed in parallel by omitting the enzyme.

VERO-EMCV-RNA was generated as described previously [32]. VERO EMCV-RNA (CIP) was created by treatment of purified Vero EMCV-RNA with 2 units calf intestinal phosphatase (CIP) (Roche) per μg of RNA for 1 h at 50°C and subsequent purification by phenol-chloroform extraction. HEK293T-IAV-RNA was generated by infection of HEK293T cells with influenza A virus (IAV) (A/PR/8/34 (H1N1), kind gift from Alain Townsend, Oxford, UK) at an MOI of 5. Total RNA was extracted 24 h later using TRIzol according to manufacturer's instructions.

Generation and validation of the p125-HEK reporter cell line

Lentiviruses were produced in HEK293T cells (kind gift from Caetano Reis e Sousa, The Francis Crick Institute, London, UK), using Eugene6 (Promega) to transfect the p125-GreenFire plasmid in combination with the Vira Power packaging mix (Invitrogen). Medium was replaced the day after transfection. Supernatant was collected 48 h post transfection and filtered through a $0.45\ \mu\text{m}$ PES filter. 1.5×10^6 HEK293 cells (kind gift from Caetano Reis e Sousa, The Francis Crick Institute, London, UK) were subsequently infected with 1 mL of viral supernatant in the presence of $8\ \mu\text{g}/\text{mL}$ of polybrene. The following day, the inoculum was removed and fresh medium was added to the cells. One week later, cells were cloned by limiting dilution. After 26 days, several clones were harvested from plates that had initially been seeded with less than 0.4 cells per well. These clones were expanded and tested by transfection of IVT-RNA. Clone 17 was selected for having both, a high amplitude of response and a high sensitivity.

Generation and validation of p125-HEK KO cell lines using CRISPR technology

sgRNAs targeting RIG-I, MDA5 and MAVS were selected based on the MIT algorithm (crispr.mit.edu). Specifically, the RIG-I sgRNA targets exon 2, the MDA5 sgRNA exon 1, and the MAVS sgRNA exon 5, which is the first common exon shared by the full-length protein and Mini-MAVS [61]. All sgRNAs were cloned into a modified version of the pX458 plasmid (Addgene 48138, deposited by Dr. Feng Zhang). We replaced the eGFP moiety of pX458 by insertion of the mRuby2 cDNA and all sgRNAs were cloned using the dual BbsI strategy and the incompatible CACC/AAAC overhangs (oligos used for cloning are listed in Supporting Information Table 1). For RIG-I, an additional G was added at sgRNA position 0 to reconstitute an optimum Pol-III promoter, whereas for MDA5 and MAVS the original protospacers were cloned.

For the generation of the KO cell lines, p125-HEK clone 17 cells were seeded in a 6-well plate at 8.5×10^5 cells per well one day before transfection with $5\ \mu\text{g}$ of plasmid complexed with $5\ \mu\text{L}$ of Lipofectamine LTX (Invitrogen) and $5\ \mu\text{L}$ of Plus reagent per well. The medium was replaced one day later. On the following day, cells were harvested and single Ruby-positive cells

were FACS-sorted into 96-wells. Clones were harvested 27 days later.

In order to identify the mutations at the target loci, sgRNA targeted p125-HEK cells were washed with PBS and re-suspended in QuickExtract solution (Epicentre, catalogue # QE 09050). After 15 seconds of vortexing, lysates were incubated for 6 min at 65°C , vortexed again, then incubated for 2 min at 98°C . 200 ng of DNA was used in a $50\ \mu\text{L}$ PCR reaction using Phusion polymerase in the presence of 3% of DMSO. PCR products were gel purified with QIAquick Gel Extraction Kit and sequenced using the PCR primers listed in Supporting Information Table 1. Sequences were analyzed using the TIDE software (<https://tide.nki.nl>) [62].

To further validate the KO cell lines, cells were seeded into either 24-well (WT, RIG-I KO and MAVS KO) or 6-well (WT and MDA5 KO) plates. Once almost confluent, cells were either treated with $140\text{U}/\text{mL}$ of IFN- α/D overnight before lysis in 1% NP-40 lysis buffer (25 mM Tris-HCl pH7.4, 150 mM NaCl, 2 mM EDTA, 5% glycerol, 1% NP-40, 1:100 Protease inhibitor cocktail) or infected with Sendai virus at an MOI of 0.5 per well for 24 h, before lysis in 0.5% NP-40 lysis buffer (20 mM Tris-HCl pH7.4, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1:100 Protease inhibitor). Lysates were incubated for 30 min on ice, then cellular debris was removed by centrifugation and supernatants were used for SDS-PAGE and blotting as described below.

p125-HEK cell stimulation

p125-HEK clone 17 cells were seeded at 50 000 cells per well in a 96-well plate. On the following day, cells were transfected with different doses of IVT-RNA or A549-ZIKV-RNA complexed with $0.2\ \mu\text{L}$ of Lipofectamine per well. Activation of the IFN β promoter was assessed one day later by OneGlo luciferase assay (Promega).

RIG-I- and MDA5-KO p125-HEK cells were validated by stimulation with IVT-RNA and VERO-EMCV-RNA (CIP), selective RIG-I and MDA5 agonists, respectively (Supporting Information Fig. 1N). To validate that MAVS-KO p125-HEK cells can still activate the IFN β promoter, WT and MAVS-KO p125-HEK cells were seeded at 20 000 cells per well in a 96-well plate. On the next day, cells were treated with $200\text{U}/\text{mL}$ IFN- α . After 24 h, cells were transfected with 100 ng of pcDNA3-MAVS. Luciferase activity was measured 1 day later.

FACS

We adhered to EJI guidelines for FACS. p125-HEK cells were seeded at 200 000 cells per well in a 24-well plate 1 day before transfection with graded doses of IVT-RNA complexed with $1\ \mu\text{L}$ of Lipofectamine. On the next day, cells were trypsinized, resuspended in FACS buffer (PBS, 1% FCS, 2 mM EDTA, 0.02% sodium azide) containing $1\ \mu\text{g}/\text{mL}$ of DAPI and immediately analyzed using a CyAn ADP High-Performance Flow Cytometer (DakoCytometry). Cellular debris and doublets were gated out using Forward Scatter and Side Scatter Channels, and dead cells using DAPI

staining (violet 1 channel). Between 27 000 and 30 000 live single cells were analyzed per sample for GFP expression.

Antibodies

Primary antibodies utilized for immunoblotting were: STAT1 (clone 42H3, Cell Signaling, 1:1000), pSTAT1(Y701) (clone D4A7, Cell Signaling, 1:1000), STAT2 (clone D9J7L, Cell Signaling, 1:1000), RIG-I (clone Alme-1, AdipoGen, 1:1000), MAVS (Enzo Life Sciences #ALX-210-929-C100, 1:500), MDA5 (clone 17, mouse monoclonal antibody raised in-house), V5-HRP (Invitrogen, 1:5000), beta-actin-HRP (clone AC-15, Sigma Aldrich, 1:10 000), GAPDH-HRP (Proteintech, 1:10 000) and FLAG-HRP (clone M2, Sigma Aldrich, 1:10 000). HRP-coupled secondary antibodies include sheep- α -mouse and donkey- α -rabbit (both GE Healthcare, 1:3000).

Western blot analysis

HEK293 or HEK293T cells were seeded at a density of 8×10^5 or 1×10^6 cells/well, respectively, in 6-well plates. 24 h later, cells were transfected with 1500 ng of ZIKV or control plasmids using Lipofectamine 2000 (Invitrogen). Where indicated cells were treated with recombinant human IFN-A/D on day three (Sigma-Aldrich or R&D Systems). After an additional 24 h incubation period, cells were lysed in lysis buffer (50 mM TRIS-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate) containing 1:100 Protease Inhibitor Cocktail (Cell signaling Technology). Phosphatase Inhibitor Cocktail 3 (Sigma-Aldrich) was added for analysis of STAT protein phosphorylation. Supernatants were removed after centrifugation. Protein concentrations were determined using Pierce BCA Protein Assay Kit (Thermo Scientific) and equalized by dilution of samples with lysis buffer. Subsequently, 5 $\times$ Laemmli sample buffer (312.5mM TRIS-HCl, 10% SDS, 25% beta-mercaptoethanol, 50% glycerol, 0.01% bromophenol blue, pH 6.8) was added and samples were incubated at 95°C for 10 min. Samples were run on NuPAGE Novex 4–12% Bis-TRIS gels (Invitrogen) using NuPAGE MOPS-SDS running buffer (Invitrogen). Proteins were subsequently blotted onto PROTRAN Pure nitrocellulose membrane (PerkinElmer). Membranes were blocked with 5% skim milk powder (Sigma-Aldrich) in TBS 0.1% Tween-20 (5% milk TBS-T) for 1 h at room temperature and were then incubated with primary antibodies in 5% milk TBS-T overnight at 4°C. Primary antibodies that bind to phosphorylated residues were diluted in 5% BSA in TBS-T. Membranes were washed thrice with TBS-T and incubated with HRP-coupled secondary antibodies in 5% milk TBS-T for 1 h at room temperature. After three further washes with TBS-T, proteins were detected using Western LightningPlus-ECL (PerkinElmer) and Amersham Hyperfilm MP (GE Healthcare). If needed, antibodies were stripped from the membrane with stripping buffer (200 mM glycine, 0.1% SDS, 1% Tween-20, pH 2.2) for 20 min at

room temperature. Membranes were washed with TBS-T, blocked as previously and re-probed.

Luciferase assay

For luciferase assays, cells either stably transduced with F-Luc reporter constructs (3C11, p125-HEK, p125-HEK-KOs) or transiently transfected with F-Luc reporter and *Renilla* luciferase control plasmids were utilized. For stable cell lines, One-GLO reagent and GLO-Max luminometer (both Promega) were used to measure F-Luc intensity. For cells transfected with firefly and *Renilla* luciferase plasmids, Dual-Luciferase Assay System (Promega) was used according to manufacturer's instructions and F-Luc activity was normalized to R-Luc activity.

RT-qPCR

A549 cells were infected with ZIKV (see above) at an MOI of 5 and total RNA was extracted after 4, 8, 24, 48 and 72 h using TRIzol (Invitrogen) according to manufacturer's instructions. RNA concentration was measured with a Nanodrop system (Thermo Fischer Scientific). RNA was reverse transcribed into cDNA using SuperScript II Reverse Transcriptase (Invitrogen). The PCR reaction containing TaqMan Universal PCR Master Mix (Applied Biosystems) and TaqMan Primer/Probes (see below) was run on 7500 Fast Real time PCR System (Applied Biosystems) or QuantStudio 7 Flex Real-Time PCR System (Thermo Fischer Scientific) with the following cycle conditions: 10 min 95°C, and 40 cycles of 15 s 95°C followed by 1 min 60°C. Gene expression was analyzed with the C_t method using *GAPDH* expression for normalization.

HEK293T cells were seeded at a density of 1.5×10^5 cells/well in 24 well plates. 24 h later, cells were transfected with 300 ng of ZIKV or control plasmids using Lipofectamine 3000 (Invitrogen). On day three of the experiment, cells were treated with recombinant IFN-A/D. After an additional 24-h incubation period, RNA was extracted from cells using the QIAshredder (Qiagen) and RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. RNA was used for RT-qPCR as described above.

TaqMan primer probes used include *GAPDH* (Assay ID: Hs02758991.g1), *DDX58* (RIG-I) (Assay ID: Hs01061436.m1), *IFIT1* (Assay ID: Hs03027069.s1), *MX1* (Assay ID: Hs00895608.m1), *IFI44* (Assay ID: Hs00951349.m1) and *IFNB1* (Assay ID: Hs02621180.s1).

Microscopy

NS5-eYFP was excited with a 488 nm laser and the emission was collected between 520 and 570 nm. For visualization of cellular localization of NS5 NLS mutants in living cells, nuclei were co-stained with the membrane permeable NucBlue® Live ReadyProbes® Reagent (Thermo Fisher). For FRAP analysis, 1%

laser power ($\sim 100 \mu\text{W}$) was used for imaging. A circular region of interest (ROI) was bleached using 75% of the laser power ($\sim 10 \text{ mW}$). 100 frames were recorded after the bleaching with no time interval between the frames. A recovery curve was generated using the Stack T-Function - Intensity vs. Time Monitor plugin of ImageJ. A Zeiss 880 confocal microscope was used in all experiments.

RIG-I pulldowns

For investigation of RIG-I bound RNA, 1.25 μg recombinant FLAG-RIG-I protein [16] and 5 μg infected cell total RNA (see above) were incubated under rotation at 4°C for 1 h in 250 μL IP buffer (20 mM TRIS-HCl pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.05 U/mL RNasin Plus RNase inhibitor (Promega)). Where indicated, RIG-I protein was denatured by heating to 95°C for 10 min prior to mixing with RNA. As input samples, 5 and 40 μL were removed for Western Blot analysis and RNA extraction, respectively. Samples were rotated as previously with 4 μg α -FLAG antibody (clone M2, Sigma-Aldrich) or mIgG1 isotype control antibody (clone MOPC-31C, BD Pharmingen) for 2 h. Samples were added to 50 μL Protein G Dynabeads (Invitrogen) and rotated again for 2 h. Beads were separated from supernatant and 5 and 40 μL were removed from supernatant as unbound samples. Beads were resuspended in IP buffer and split 10–90% for Western Blot and RNA extraction, respectively.

For Western blotting, 4 $\times$ Laemmli sample buffer (200 mM TRIS-HCl, 8% SDS, 20% beta-mercaptoethanol, 40% glycerol, 0.01% bromophenol blue, pH 6.8) was added to input, unbound and bound fractions and samples were incubated at 95°C for 10 min. Beads were removed and samples were subjected to SDS-PAGE and blotting as described above.

TRIzol was added to input, unbound and bound fractions and RNA was extracted according to manufacturer's instructions. RNA was resuspended in 20 μL water. For luciferase assay, p125HEK reporter cells were seeded at 2.5×10^4 cells/well in 96 well plates, transfected with 1 μL of RNA samples per well using Lipofectamine 2000 and assayed as described above. For RT-qPCR, 4 μL of RNA samples were reverse transcribed into cDNA using SuperScript II Reverse Transcriptase (Invitrogen) in a 20 μL reaction. The 10 μL PCR reaction containing 1 μL cDNA, EXPRESS SYBRTM GreenERTM qPCR Supermix (Invitrogen) and 10 μM primers (see Supporting Information Table 1) was run on QuantStudio 7 Flex Real-Time PCR System (Thermo Fischer Scientific) with the following cycle conditions: 10 min 95°C , and 40 cycles of 15 s 95°C followed by 1 min 60°C , and melt curve analysis. Abundance was analyzed with the C_t method and expressed as 2^{-C_t} .

Data analysis and statistics

Primary data were analyzed using Office Excel 2016 (Microsoft) and GraphPad Prism v7.00 (GraphPad Software). SnapGene (GSL Biotech) and ApE (M. Wayne Davis, The University of Utah) were utilized to assist cloning. The TIDE algorithm was used to analyze

KO clone sequences [62]. Quantitative analysis of Western blots was performed using ImageJ [63]. Graphs and figures were created using GraphPad Prism v7.00 and Adobe Illustrator (Adobe Systems). Statistical analysis was performed in GraphPad Prism v7.00 as detailed in the Figure legends.

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Author contributions: A.G.D.J., J.H., R.E.R. and J.R. conceived the study, designed experiments, analyzed data and wrote the manuscript. A.G.D.J., J.H. and R.E.R. performed experiments. A.M. and P.H. generated and validated reporter and knockout cell lines. C.L.D. and A.K. provided RNA from ZIKV infected cells. E.S. performed FRAP experiments under supervision of C.E. C.S. and J.B. raised the MDA5 antibody. All authors read and approved the final manuscript.

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Abbreviations: DENV: Dengue virus · EMCV: Encephalomyocarditis virus · IFNs: Interferons · MAVS: Mitochondrial antiviral signaling protein · MOI: Multiplicity of infection · ZIKV: Zika virus

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**Infection with a Brazilian isolate of Zika virus generates RIG-I stimulatory RNA
and the viral NS5 protein blocks type I IFN induction and signaling**

Supplementary Figure 1

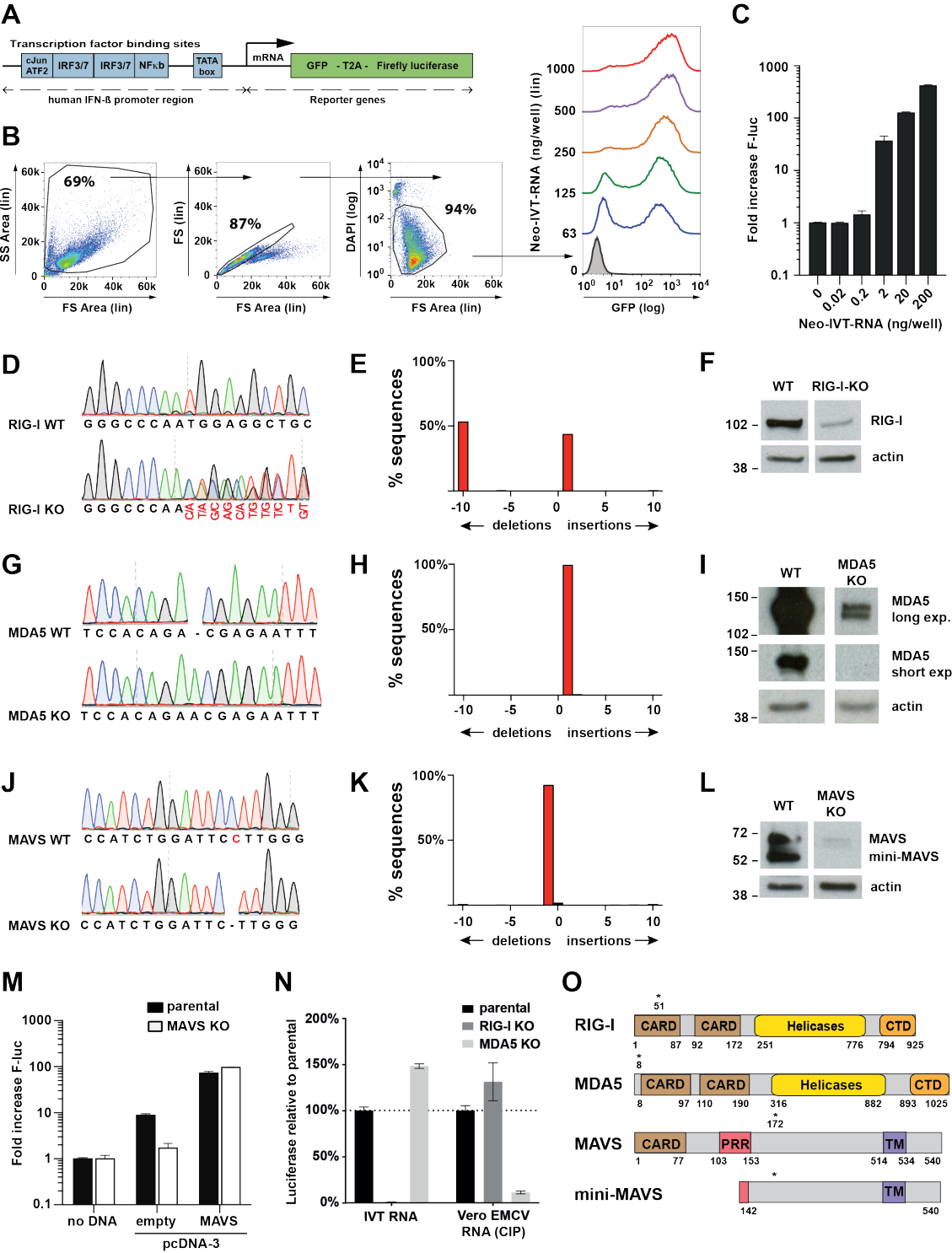


Fig. S1. Generation of RLR reporter cell lines.

(A) *IFN* β reporter construct used to generate the reporter cell line. This construct co-expresses copGFP and F-Luc, linked by a self-cleaving T2A peptide, under the control of the human *IFN* β promoter region.

(B) HEK cells stably transduced with the construct described in (A) - and hereafter referred to as p125-HEK cells - were seeded in a 24-well plate one day before transfection with graded doses of IVT-RNA. Cells were harvested 24 hours post transfection and expression of GFP was assessed by flow cytometry (gated on DAPI negative, single cells).

(C) p125-HEK cells were seeded in 96-well plates one day before transfection with graded doses of Neo¹⁻⁹⁹ IVT-RNA. F-Luc activity in cell lysates was determined 24 hours after transfection. Data are shown as fold change compared to cells treated with transfection reagent only.

(D - O) p125-HEK clones knocked out for RIG-I, MDA-5 and MAVS were generated using CRISPR technology.

(D, G, J) Genomic DNA was extracted from RIG-I KO (D), MDA5 KO (G) and MAVS KO (J) p125-HEK cells and from parental cells. A fragment of DNA surrounding the targeted area was amplified by PCR, gel purified and sequenced. Superposition of peaks in the RIG-I KO clone sequence indicates that different alleles bear different mutations.

(E, H, K) Sequences generated in (D), (G) and (J) were analyzed with TIDE software. This algorithm decomposes the sequencing data to determine the spectrum of indels and their frequencies. The graphs show the number of nucleotides inserted or deleted and percentage of sequences affected. One nucleotide was inserted into one allele of the *RIG-I* gene, inducing a frameshift and therefore a modification of the amino acid sequence from position 51, potentially giving rise to a protein containing 86 aa. In the second allele, 10 nucleotides were deleted, also inducing a modification of the amino acid sequence from position 51 and

potentially giving rise to a protein containing 73 aa. The insertion of one nucleotide into the *IFH11* gene (encoding MDA5) creates a frameshift at position 8, potentially giving rise to a protein containing 58 aa. In the case of MAVS, the deletion of one nucleotide induced a frameshift and therefore a different amino acid sequence from position 172, potentially giving rise to a protein containing 189 aa.

(F,L) p125-HEK cells of the indicated genotype were treated with 140 U/ml of IFN α overnight, then lysed in a buffer containing 1% of NP-40. Protein expression was tested by Western blot using the indicated antibodies.

(I) MDA5 KO and WT p125-HEK cells were infected overnight with Sendai virus at an MOI of 0.5, then lysed in a buffer containing 0.5% of NP40. MDA5 and actin expression were analyzed by Western blot.

(M) MAVS KO and WT p125-HEK cells were treated with 200 U/ml of IFN α overnight, before transfection with 100ng per well of pcDNA3 plasmid expressing human MAVS or an empty pcDNA3 vector as control. F-Luc activity in cell lysates was determined 24 hours after transfection. Data are shown as fold change compared to cells treated with transfection reagent only.

(N) RIG-I KO, MDA5 KO, and parental p125-HEK cells were transfected with 25 ng of IVT RNA or Vero EMCV RNA (CIP). F-Luc activity in cell lysates was determined 24 hours after transfection. Data are shown as luciferase values relative to parental p125-HEK.

(O) Schematic representation of RIG-I, MDA5 and MAVS proteins. The star indicates the position targeted by the sgRNA.

Panels (B) - (M) are representative of two or more independent experiments. Panels (C) and (M) show average and SD (n=3). Panel (N) shows pooled data from two independent experiments (average and SEM (n=4)).

Supplementary Figure 2

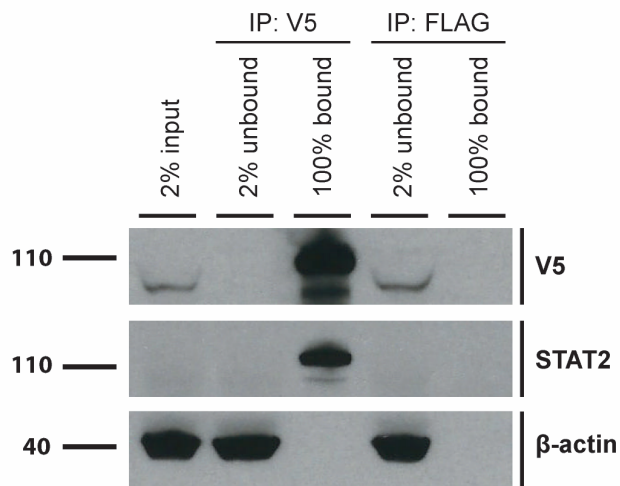


Fig. S2. ZIKV NS5 binds STAT2.

HEK293T cells were transfected with a plasmid expressing ZIKV NS5-V5. 24 hours later, cells were lysed and NS5 was immunoprecipitated with α -V5 antibody. As a negative control, α -FLAG antibody was used. Input, unbound and bound fractions were analyzed by Western blot using the indicated antibodies. Data are representative of three independent experiments.

Supplemental Table 1. Oligonucleotides.

Name	Purpose	Sequence
751_2K-NS4B_f	Cloning of 2K-NS4B	AGGAGGACAGCTATGTCTCCCCAGGACAACCA AATGGCAATCATCATCATGGTAGCAGTAGGTC TTCTGGGCTTGATTACCGCCAATGAACTCGGA TGGTTGGAG
734_2K-NS4B_r	Cloning of 2K-NS4B	ACGTCTCTTGACCAAGCCAG
749_NS4A_f	Cloning of NS4A	AGGAGGACAGCTATGGGAGCGGCTTTTGGAGT G
750_NS4A_r	Cloning of NS4A	TCTTTGCTTTTCTGGCTCAGG
753_NS4A-2K-NS4B_f	Cloning of NS4A-2K-NS4B (overlap PCR)	GGCGGTAATCAAGCCCAGAAG
754_NS4A-2K-NS4B_f2	Cloning of NS4A-2K-NS4B (overlap PCR)	GGCAATCATCATCATGGTAGCAGTAGGTCTTC TGGGCTTGATTACCGCCAATGAACTCGGATGG TTGGAGAG
779_NS2B-NS3_r1	Cloning of NS2B-NS3 (overlap PCR)	CAGGCACATCCCATAGAGCACTCCTTTTT CCAGTCTTCACGTATACGTA
780_NS2B-NS3_f2	Cloning of NS2B-NS3 (overlap PCR)	CGTGGTACGTATACGTGAAGACTGGAAAAAG GAGTGGTGCTCTATGGGATGTGCCTG
781_NS2B-NS3_r2	Cloning of NS2B-NS3 (overlap PCR)	TCTTTTCCAGCGGCAAACTCCTTG
797_NS2B-NS3_f1	Cloning of NS2B-NS3 (overlap PCR)	AGGAGGACAGCTATGAGCTGGCC
783_NS5-eYFP_r1	Cloning of NS5-eYFP (overlap PCR)	GCGGCCGCCACTGTGCTGGATATCAACCACTT TGTACAAGAAAGCTGGGTCGAATTCGCCCTCA GCACTCCAGGTGTAGACCCTTCTTC
784_NS5-eYFP_f2	Cloning of NS5-eYFP (overlap PCR)	TTCTTGTAACAAGTGGTTGATATCCAGCACAG TGGCGGCCGCTCGAGTCTAGAGGGGCCGCGGT TCGAAGTGAGCAAGGGCGAGGAGCTG
785_NS5-eYFP_r2	Cloning of NS5-eYFP (overlap PCR)	CAGCTCGTCCATGCCGAGAGTG
786_NS5-eYFP_f1	Cloning of NS5-eYFP (overlap PCR)	AGGAGGACAGCTATGGGGGGTG
aNLS mutant – Cluster 1 (KHK to AA) Fwd	NS5 NLS mutagenesis PCR	cctggtgtggaagagctaggcgagccgcacggccacgagtctgtacc aaag
aNLS mutant – Cluster 1 (KHK to AA) Rev	NS5 NLS mutagenesis PCR	ctttggtacagactcgtggccgtgcggtcgcgctagctctttccacaaccag g
aNLS mutant – Cluster 2 (RQ to AA) Fwd	NS5 NLS mutagenesis PCR	ccagacccccaagaaggcactgctgcggttatgagcatggtc
aNLS mutant – Cluster 2 (RQ to AA) Rev	NS5 NLS mutagenesis PCR	gaccatgctcataaccgcagcagtgcttcttgggggtctgg
2-O ⁷ methyltransferase mutant - Fwd	NS5 E218A cap1 mutagenesis PCR	cgcgaactctacacatgcgatgtactgggtctctg
2-O ⁷ methyltransferase mutant - Rev	NS5 E218A cap1 mutagenesis PCR	cagagaccagttacatgcgatgttagagttgcgg
NS5 M2634V F	Cloning of NS5 “African mutant”	atagctttgcaccaacatgggttcttcatgaccag
NS5 M2634V R	Cloning of NS5 “African mutant”	ctggctcatgaagaacctgttggtgcaaagctat
NS5 M3392V F	Cloning of NS5 “African mutant”	cacctatgatcctgcgcatcatgttgactgtgtttta
NS5 M3392V R	Cloning of NS5 “African mutant”	taaaaacacagtcaacatgatgcgcaggatcataggtg
RIG-I sgRNA Fwd	Cloning of RIG-I sgRNA	CACCCGgaacaacaagggcccaatgg
RIG-I sgRNA Rev	Cloning of RIG-I sgRNA	AAACccattgggccctgtgttcC
MAVS sgRNA Fwd	Cloning of MAVS sgRNA	CACCGgccaccatctggattcctt
MAVS sgRNA Rev	Cloning of MAVS sgRNA	AAACaaggaatccagatggtggcc

MDA-5 sgRNA Fwd	Cloning of MDA5 sgRNA	CACCGtagcggaaattctcgtctg
MDA-5 sgRNA Rev	Cloning of MDA5 sgRNA	AAACcagacgagaatttccgctaC
RIG-I KO gDNA sequencing Fwd	Validation of RIG-I targeting	ttacattgtctcagactaagaggc
RIG-I KO gDNA sequencing Rev	Validation of RIG-I targeting	Gtgaagaatgggcacagtcggcc
MAVS KO gDNA sequencing Fwd	Validation of MAVS targeting	Ggctgaggcctataggagatgcc
MAVS KO gDNA sequencing Rev	Validation of MAVS targeting	gcctgaccacaggatcatatgc
MDA5 KO gDNA sequencing Fwd	Validation of MDA5 targeting	cgtcattgtcaggcacagag
MDA5 KO gDNA sequencing Rev	Validation of MDA5 targeting	agctctgccactgtttttcc
IAV PR8 M F	RT-qPCR	CTTCTAACCGAGGTCGAAACGTA
IAV PR8 M R	RT-qPCR	GGTGACAGGATTGGTCTTGTCTTTA
ZIKV PE243 F	RT-qPCR	CGAGGAACATCCAGACTC
ZIKV PE243 R	RT-qPCR	ATTGGAGATCCTGAAGTTCC