INHIBITION OF RIFT VALLEY FEVER VIRUS USING RNA INTERFERENCE TECHNOLOGY

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DECLARATION

I, Tristan Alexander Scott, declare that this thesis is my own, unaided work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other university.

Signature_____ Date_____

Publications and presentations

The work below is a list of publications and conference presentations:

Publications

Scott T, Paweska JT, Arbuthnot P, Weinberg MS. (2012). Pathogenic effects of Rift Valley fever virus NSs gene are alleviated in cultured cells by expressed antiviral short hairpin RNAs. *Antivir Ther.* **17** (4):643-56.

Presentations

2008:

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2010:

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Scott, T. A., Paweska J. T., Arbuthnot, P. and Weinberg, M. S. RNA interference targeted to the S segment of Rift Valley fever virus inhibits viral replication and alleviates pathogenic effects *in vitro.* University of the Witwatersrand, Biennial Health Sciences Research Day, 22nd September, Johannesburg, South Africa.

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Scott, T. A., Paweska J. T., Arbuthnot, P. and Weinberg, M.S. RNA interference targeted to the S segment of Rift Valley fever virus inhibits viral replication and alleviates pathogenic effects *in vitro.* The Molecular Biosciences Research Thrust, University of the Witwatersrand Joint Faculty of Science and Health Science Seminar, 8th December, Johannesburg, South Africa.

2011:

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Scott, T. A., Paweska J. T., Arbuthnot, P. and Weinberg, M.S. RNA interference targeted to the S segment of Rift Valley fever virus inhibits viral replication and alleviates pathogenic effects *in vitro.* Virology Africa, 29th November-2nd December, Cape Town, South Africa.

Scott, T. A., Paweska J. T., Arbuthnot, P. and Weinberg, M.S. Pathogenic effects of Rift Valley fever virus's *NSs* gene are alleviated in cultured cells by expressed antiviral short hairpin RNAs. The Molecular Biosciences Research Thrust, University of the Witwatersrand Joint Faculty of Science and Health Science seminar, 7th December, Johannesburg, South Africa.

2012:

Scott, T. A., Paweska J. T., Arbuthnot, P. and Weinberg, M.S. Inhibition of Rift Valley fever virus replication using antiviral short hairpin RNAs. South African Society of Biochemistry and Molecular Biology (SASBMB), 29th January-1st February, Drakensburg, South Africa.

Scott, T. A., Paweska J. T., Arbuthnot, P. and Weinberg, M. S. Inhibition of Rift Valley fever virus replication using antiviral short hairpin RNAs. University of the Witwatersrand, Biennial Health Sciences Research Day, 19th September, Johannesburg, South Africa.

Scott, T. A., Paweska J. T., Arbuthnot, P. and Weinberg, M. S. Short hairpin RNAs targeted to the S segment of Rift Valley fever virus inhibits viral replication and alleviates NSs-induced pathogenic effects. University of the Witwatersrand, Cross-Faculty Postgraduate Symposium, 19th and 22nd October, Johannesburg, South Africa.

Abstract

Rift Valley fever (RVF) is a disease endemic to Africa, which has recently spread outside of Africa to the Arabian Peninsula. Rift Valley fever virus (RVFV) is the causative agent of RVF and manifests as severe hepatitis, encephalitis and haemorrhagic fever, resulting in mortality in approximately 1% of human cases. RVFV also affects agriculture as it causes high mortality rates in young ruminants (>90% in new-born lambs) and is associated with high levels of abortions, which results in devastating economic losses. RVFV is a single-stranded RNA virus with a genome comprising of three separate genetic elements referred to as the Large (L), Medium (M) and Small (S) segments. The negative sense L segment encodes an RNA-dependent RNA polymerase (RdRp) while the M segment encodes two glycoproteins, Gn and Gc, and two non-structural proteins, NSm1 and NSm2. The glycoproteins are important for viral entry, genome packaging and mature virion formation as well as being the main antigen for the elicitation of neutralising antibodies by humoral immunity. The NSm proteins are required for mosquito vector transmission and preventing viral-induced apoptosis in host cells. The ambisense S segment encodes in the positive orientation a non-structural (*NSs*) gene, and in the negative orientation the nucleocapsid (*N*) gene. NSs is an important virulence factor involved in subverting host defences and the loss of NSs results in a highly attenuated RVFV infection. N is required for RNA synthesis and encapsidation of viral genomes. There are currently very few treatments in the early stages of development and vaccines for RVFV are not readily available. The overall lack of therapeutic strategies for RVFV urges novel therapeutic development such as RNA interference (RNAi). Endogenous RNAi is triggered by dsRNA and is involved in gene regulation through sequence specific suppression of target mRNA. Therapeutic RNAi exploits the RNAi pathway to facilitate targeted degradation of viral genes and has been applied effectively to the inhibition of a number of viruses that cause chronic and acute infections. There are fewer studies that have used RNAi to inhibit highly pathogenic viruses. Efficacy has been demonstrated against Ebola virus, Lassa virus and Dengue fever virus, which suggests applicability to the inhibition of RVFV. In this thesis, short hairpin RNAs (shRNAs) were generated to target the *NSs, N* and *M* genes of RVFV, which are important proteins in the viral life cycle. To determine the knockdown efficacy of the shRNAs, HEK293 cells were transiently transfected with the shRNAs and a vector expressing the respective shRNA gene target fused to a luciferase reporter. The reporter levels were assessed using a dual-luciferase assay and several shRNAs were selected for further characterisation as a result of effective target knockdown. Consequently, the shRNAs reduced the levels of expressed FLAG-tagged NSs, N and M encoded proteins, which were detected using western blot analysis. ShRNAs directed against *NSs* were shown to disrupt this protein's function to result in alleviation of pathogenic

properties. Specifically, NSs was shown to suppress the transcription levels of a luciferase reporter as well as prevent the activation of an IFN-β promoter. When the shRNAs were transiently transfected into HEK293 cells, they were able to reverse NSs-induced suppression in the reporter assays. Furthermore, NSs is cytotoxic as determined by observing cell morphology under transmitted light microscopy, which was quantified using a MTT viability assay and cells that subsequently received anti-*NSs* shRNAs had improved viability. This class of anti-pathogenic shRNAs should be able to down-regulate NSs *in vivo* and attenuate RVFV virulence. However, NSs is not essential for viral replication and as a result of the aggressive pathology of haemorrhagic RVF, essential structural genes were targeted to investigate shRNAs with anti-replicative properties. ShRNAs directed against *N* were transfected 24 hrs prior to infection with RVFV. The inhibition of viral replication was determined by collecting supernatant over 3 days and measuring the levels of N antigen using an ELISA. The shRNAs demonstrated effective suppression of RVFV but N antigen was detected at 72 hrs post-infection, which suggested that the shRNAs were overwhelmed by the virus. A series of shRNAs against *M* were subsequently tested and the anti-*M* shRNAs effectively suppressed viral replication in cultured cells over an extended 96 hr experiment, demonstrating that *M* is a good target for RNAi-mediated inhibition of RVFV. In this thesis, the potential of RNAi-based therapeutics against RVFV was demonstrated and these data contribute to the growing knowledge that RNAi should be developed further as a potential treatment for haemorrhagic fever viruses. Finally, some DNA viruses such as HBV form cellular reservoirs from which new virus can be produced and the DNA is resistant to RNAi-mediated inhibition. RVFV is an RNA virus with an acute infection, which makes it more susceptible to RNAi and an excellent target for this particular therapeutic modality.

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List of Abbreviations

AAV Adeno-associated viral vector

Adv Adenoviral vector

Ago Argonaute

bp Base pair

BSL3 Biological safety level 3

C Cytosine

C13 Clone 13

CCHFV Crimean-Congo haemorrhagic fever virus

cDNA Complementary DNA

CMV Cytomegalovirus

DC Dendritic cells

DGCR8 DiGeorge syndrome critical region gene 8

DENV Dengue virus

DIC Disseminated intravascular coagulation

DMEM Dulbecco's modified eagle's medium

DNA Deoxyribonucleic acid

dNTP Deoxyribonucleotide triphosphate

dsRNA Double-stranded RNA

EBOV Ebola virus

EDTA Ethylenediaminetetraacetic acid

EMEM Essential minimal eagle's media

Exp5 Exportin-5

FLuc Firefly luciferase

G Guanine

Gag Group specific antigen

Gc C-terminal glycoprotein

GFP Green fluorescent protein

Gn N-terminal glycoprotein

Gp Glycoprotein

GTP Guanosine triphosphate

HBV Hepatitis B virus

HCV Hepatitis C virus

HDAC Histone deacetylases

HIV Human immunodeficiency virus

RLuc *Renilla* luciferase

IGR Intergenic region

IFN Interferon

IPTG Isopropyl-β-D-1-thiogalactopyranoside

kb Kilobase

L Large (segment) or L polymerase (protein)

LACV La Crosse encephalitis virus

LASV Lassa virus

LB Luria bertani (broth)

LCMV Lymphocytic choriomeningitis virus

LhRNA Long hairpin RNA

LV Lentiviral vector

M Medium (segment)

MARV Marburg virus

MAPK Mitogen-activated protein kinase

MDA5 Melanoma differentiation-associated protein 5

miRNA MicroRNA

mRNA Messenger RNA

N Nucleocapsid protein

NCoR Nuclear receptor co-repressor 1

Nef Negative effector

NF-kβ Nuclear factor kβ

NPC1 Niemann-pick C1

NSs Non-structural protein on the S segment

nt Nucleotide

OAS Oligoadenylate synthetase

ORF Open reading frame

P-body Processing body

PACT Protein activator of PKR

PAGE Polyacrylamide gel electrophoresis

PBS Phosphate buffered saline

PCR Polymerase chain reaction

PKR Protein kinase R

PMO Phosphorodiamidate morpholinos oligonucleotide

PNK Polynucleotide kinase

Pre-miRNA Precursor microRNA

Pri-miRNA Primary microRNA

PSE Proximal sequence element

RIG-1 Retinoic acid inducible gene 1

RISC RNA-induced silencing complex

RNA Ribonucleic acid

RNP Ribonucleoprotein complex

RNAi RNA interference

RSV Respiratory syncytial virus

RVFV Rift Valley fever virus

S Small (segment)

SAP 30 Sin-3A associated protein

ScAAV Self-complementary AAV

SDS Sodium dodecyl sulphate

shRNA Short hairpin RNA

siRNA Small interfering RNA

ssRNA Single-stranded RNA

TBE Tris-borate-EDTA

$TCID_{50}$ 50% tissue culture infective dose

TFIIH Transcription factor II H

TGS Transcriptional gene silencing

TLR Toll-like receptor

TRAF TNF receptor associated factors

TRBP TAR RNA binding protein

TRIF TIR-domain-containing adapter-inducing interferon-β

tRNA Transfer RNA

U Uridine

UTR Untranslated region

VLP Virus-like particle

VSV Vesicular stomatitis virus

X-Gal 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside

XPB Xeroderma pigmentosum group B

XPD Xeroderma pigmentosum group D

YFV Yellow fever virus

YY1 Yin-yang-1

Chapter 1

Introduction

1.1 Overview

Haemorrhagic fever viruses represent a prevalent global disease burden covering vast geographic areas of Africa, Asia and South America. The severe pathology of this unique group of viruses is marked by rapid disease progression and high mortality, which in some cases can reach as high as 90% (Bowen et al., 1977). Although some of these viruses have effective vaccines, most do not have reliable intervention strategies. A great amount of effort has been placed into understanding viruses that cause chronic infections such as human immunodeficiency virus (HIV), with numerous approaches towards diagnoses and therapies in development. Generally, however, highly pathogenic viruses such as haemorrhagic fever viruses are comparatively neglected, which is reflected in the overall lack of therapeutics. A novel strategy to combat highly pathogenic viruses will therefore form the focus of this thesis.

Rift Valley fever virus (RVFV) is a significant haemorrhagic fever viral pathogen, which causes disease in human and livestock throughout Africa. In this chapter, the biology and pathology of RVFV will be addressed, with specific mention of host innate immunity as an important hurdle to viral infections. The mechanisms by which haemorrhagic viruses subvert innate immunity through virulence factors and their contribution to pathogenesis will be explored. The current state of therapeutics for highly pathogenic viruses in development is addressed with specific focus on virulence factors as attractive therapeutic targets. This will be related to RVFV's virulence factors and their potential as targets for therapeutic intervention.

Since the seminal discovery of RNA interference (RNAi), much effort has been placed into its application as a therapeutic modality. The possibility that RNAi may be an antiviral pathway will be discussed along with its association to a known host defence pathway, the interferon response. The novel nature of RNAi for the treatment of RVFV will be addressed with possible promises and pitfalls in relation to other therapeutic strategies. Furthermore, studies using RNAi-based therapeutics to inhibit other haemorrhagic fever viruses will be explored as well as the potential of RNAi as a treatment for haemorrhagic fever viruses in a clinical setting. Finally, the safety of RNAi-based therapeutics will be addressed with possible methods to prevent unwanted toxicity.

1.2 Rift Valley fever virus

1.2.1 General pathology and epidemiology

Viral haemorrhagic fever syndrome is caused by a unique group of viruses spanning a number of families, namely *Filoviridae, Flaviviridae, Bunyaviridae* and *Arenaviridae*. Haemorrhagic fever viruses are enveloped, single-stranded RNA viruses that are genetically and structurally diverse. They are often transmitted through arthropod or rodent vectors. A viral haemorrhagic fever has a variety of disease presentations ranging from asymptomatic to a haemorrhagic fever, which is characteristic of uncontrolled viral replication resulting in a 'cytokine storm' leading to increased vascular permeability, coagulation defects and fatal hypovolemic shock [Reviewed in (Paessler and Walker, 2012)].

Rift Valley fever virus (RVFV) is an arbovirus of the family *Bunyaviridae* (genus: *Phlebovirus*) and is primarily transmitted by mosquitoes (Linthicum et al., 1985), but infections in humans mostly occur through aerosol contamination by contact with infected blood and tissue (Abu-Elyazeed et al., 1996, Chambers and Swanepoel, 1980). RVFV has spread geographically from its initial site of identification, the Rift Valley in Kenya (Daubney et al., 1931), through long-distance translocation to Mauritania, South Africa, Zimbabwe, Egypt, Madagascar and even crossing to the Arabian Peninsula (Yemen and Saudi Arabia) as a result of the transportation of infected animals or migration of viral vectors [Reviewed in (Ikegami, 2012)]. There is a possibility that rising temperatures as a result of climate change could make regions favourable to mosquito vectors, allowing the establishment of endemic RVFV. Furthermore, RVFV-competent mosquito vectors have been identified in the Mediterranean (Moutailler et al., 2008) and USA (Gargan et al., 1988, Turell et al., 2008), which opens up the possibility of established RVFV in these areas. RVFV has also been identified as a potential bio-terrorism agent (Sidwell and Smee, 2003).

Outbreak in ruminants results in massive economic loss in countries that have agriculture-dependent economies. RVFV is often identified from other epidemics by substantial mortality in young animals and 'abortion storms' (>20%) representative of large-scale miscarriages and foetus deformities (Daubney et al., 1931). In humans, most cases are asymptomatic but a number of infections cause flu-like symptoms that can lead to a more severe form of infection resulting in hepatitis, retinitis, encephalitis and an associated haemorrhagic fever (Madani et al., 2003). Virus can resurge in the brain post-recovery resulting in severe neurological complications. RVFV normally has severe pathology in less than 1% of cases. RVFV is a significant emerging pathogen, which stresses the development of novel therapeutics such as RNAi (section 1.6). Understanding RVFV

biology can give insight into the vital components required for a productive infection, which would assist in the identification of potential targets for RNAi-mediated inhibition.

1.2.2 Viral genome composition

RVFV has a tri-segmented, single-stranded RNA (ssRNA) genome consisting of a Large (L), Medium (M) and Small (S) segment (Figure 1.1). The L and M segments are encoded in the negative sense while the S segment is ambisense. The L segment produces an RNA-dependent RNA-polymerase (RdRp) (Muller et al., 1994), which is a large protein (>200 kda) that forms a biologically active oligomer (Zamoto-Niikura et al., 2009) with conserved motifs essential for viral RNA synthesis (Lopez et al., 1995). The M segment produces a poly-translated mRNA which uses five translation start codons to give rise to precursor proteins that are proteolytically processed by host signal peptidase (Collett et al., 1985, Collett, 1986). This will produce two glycoproteins, Gn and Gc, and two non-structural proteins, NSm1 and NSm2 (Suzich et al., 1990). NSm1 is expressed from the first start codon and consists of the NSm and Gn coding region (Kakach et al., 1989). NSm2 inhibits virus-induced apoptosis (Won et al., 2007), which may be mediated through its association with mitochondria (Terasaki et al., 2012). Although the distinct functions of either protein have not been elucidated, the NSm proteins are involved in vector transmission (Crabtree et al., 2012) and pathogenicity (Bird et al., 2007b). In the positive orientation the ambisense S segment encodes a non-structural protein (NSs) involved in subversion of host antiviral defences (Bouloy et al., 2001). In the negative orientation a nucleocapsid (N) protein is encoded, which is essential for viral replication, transcription, packaging and mature virion architecture (Giorgi et al., 1991). Although RVFV replicates in the cytoplasm, NSs is present in the nucleus and forms large filamentous structures (Struthers et al., 1984) through C-terminal interactions required for NSs self-association (Yadani et al., 1999). *NSm* (Gerrard et al., 2007) and *NSs* (Ikegami et al., 2006) are dispensable for viral replication.

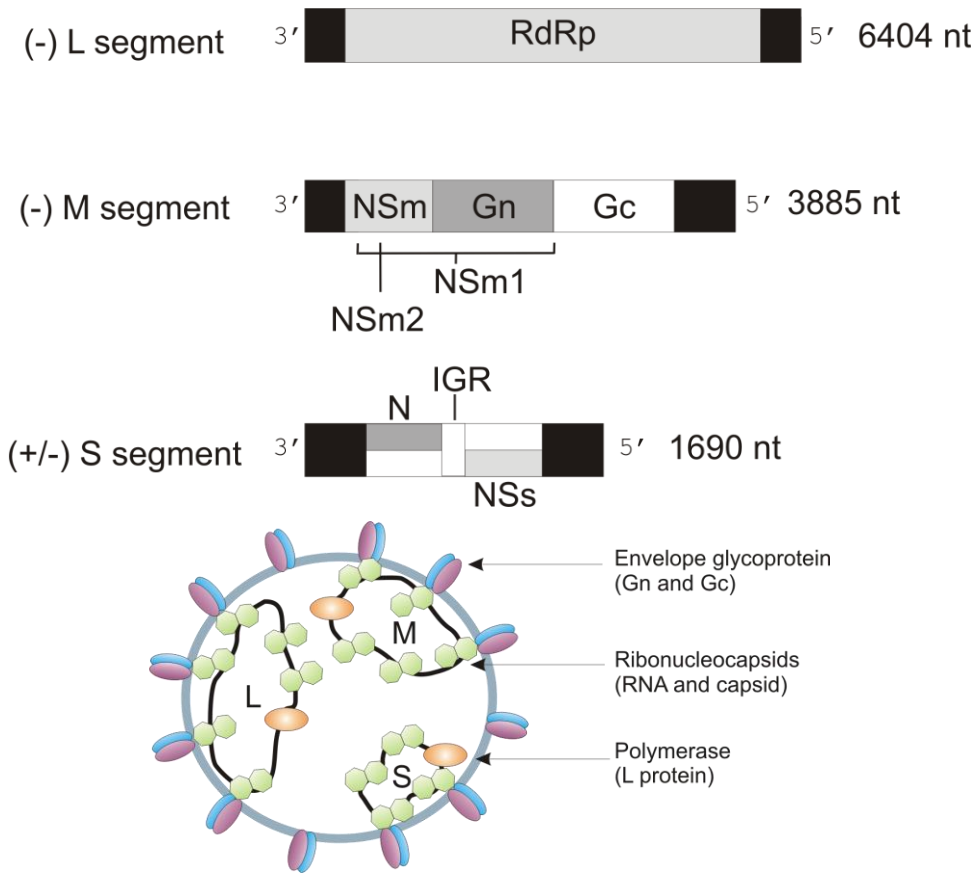

Figure 1.1: Schematic of the RVFV genome. RVFV is a tripartite ssRNA virus consisting of a Large (L), Medium (M) and S (small) segment. The L and M segments are negative sense while the S segment is ambisense. The L segment encodes an RNA-dependent RNA-polymerase (RdRp). The M segment encodes a poly-translated mRNA. Four proteins are produced through host peptidase processing namely two glycoproteins, Gn (56 kda) and Gc (65 kda), and two non-structural proteins, NSm1 (78 kda) and NSm2 (14 kda). The S segment in the positive orientation (5'-3') encodes a non-structural protein, NSs (34 kda), and in the negative orientation (3'-5') encodes a nucleocapsid protein, N (25 kda). The 5' and 3' non-coding regions of the segments act as promoters and transcription terminators, respectively. The region between NSs and N, known as the intergenic region (IGR), acts as a stop site for ambisense transcription. The genomes do not have caps or polyadenylated tails. Below the schematic of the genomes is a structural representation of a virion containing the encapsidated RNA with the L polymerase.

1.2.3 Replication and transcription

RVFV RNA synthesis is confined to the cytoplasm and each segment is a template for replication or transcription (Elliott, 1990). The viral sense (negative) genome involved in replication generates an exact copy antisense genome (positive), whereas transcribed mRNAs are slightly shorter, terminating in the non-coding regions. At the 5' and 3' non-coding regions of most phleboviruses are conserved complementary termini (3' UGUGUUUC/GAAACACA 5') resulting in the segments forming "pan handle" structures that serve as promoters for RNA synthesis (Prehaud et al., 1997). The M segment terminates at a 3'-CGUCGUCG-5' motif (Ikegami et al., 2007) while the L segment is disputed and may produce a L mRNA that is identical to the antisense viral genome

(Albariño et al., 2007) or use a stem-loop terminator (Ikegami et al., 2007) or a 5'-CGAUG-3' motif (Lara et al., 2011). The S segment's transcription termination signals are between the *NSs* and *N* open reading frames (ORFs), which falls within a 82 nt intergenic region (IGR) (Giorgi et al., 1991). The signals for termination within the IGR are 3'-CGUCG-5' motifs in combination with poly-G/C tracts (Albariño et al., 2007). Both L and N proteins are required for replication and transcription (Lopez et al., 1995). *Bunyaviridae* employ a method of host mRNA "cap snatching" for RNA synthesis within the cytoplasm. Bunyaviral N proteins bind host mRNA caps to protect them from degradation (Mir et al., 2008). N preferably uses substrates intended for nonsense mRNA decay and that have high sequence complementarity to the viral RNA (Cheng and Mir, 2012). N then stabilises a cap to the 3' end of viral RNA (Mir et al., 2010) while the cap-dependent endonuclease activity of L (Reguera et al., 2010) possibly cleaves the caps into smaller primer fragments. *Bunyaviridae* then use a "prime-and-realign" mechanism to ensure that the complete viral RNA is synthesised (Garcin et al., 1995). RVFV mRNAs do not possess a polyadenylated tail (Ikegami et al., 2007). Poly-homodimer formation of N is essential for RNA synthesis (Raymond et al., 2010). Different levels of proteins are expressed from each segment, which suggests varying promoter strengths of the non-coding regions (Gauliard et al., 2006).

1.2.4 Viral entry, packaging and release

The viral life cycle of RVFV is incompletely understood (Figure 1.2). Virions have an icosahedral symmetry with a spherical shell in a T=12 lattice (Freiberg et al., 2008, Sherman et al., 2009). There are 122 capsomers consisting of hexamers and pentamers of Gn-Gc heterodimers (Huiskonen et al., 2009), which comprise a Gn head and Gc base (Rusu et al., 2012). Gn is most likely the binding partner of the surface receptor while Gc facilitates membrane fusion through acid-triggered type II fusion (Garry and Garry, 2004). The entry receptor for RVFV has yet to be identified but RVFV can infect multiple tissue types, which suggests it is ubiquitously present on the surface of different cell types.

Bunyaviridae entry is mediated by clathrin-dependent endocytosis (Schudel et al., 2013) and RNP are released by endosomal acidification (Lozach et al., 2010). The L protein is packaged into a mature virion and is required for transcription upon viral entry (Piper et al., 2011). A unique characteristic of Bunyaviral replication is the formation of viral tubes associated with the Golgi apparatus, which concentrates L for RNA synthesis at viral 'factories' (Fontana et al., 2008). When RVFV Gc is expressed alone it associates with the endoplasmic reticulum (ER) because of an ER

retention signal, and upon forming a heterodimer complex with Gn, which has Golgi localisation signal, the complex is trafficked to the Golgi apparatus (Gerrard and Nichol, 2002).

The N protein forms oligomeric rings when associated with RNA (Ferron et al., 2011) through N-terminal arms, which facilitate multimerisation (Le May et al., 2005). These multimers can bind RNA in a conserved binding slot, which interacts with a RNA backbone to encapsidate the genome (Raymond et al., 2012). The newly formed ribonucleoprotein (RNP) complex is trafficked to the Gn-Gc heterodimers. N probably binds Gn's cytoplasmic tail as this would be essential for the virion's stability in the absence of a matrix protein (Piper et al., 2011). The complementary termini binds the Gn cytoplasmic tails acting as a signal for viral release (Piper et al., 2011). The termini are important for RNP incorporation into mature virions and ensures that a large proportion of the viral particles released from the host cell are infectious (Piper et al., 2011). RVFV utilises a co-ordinated packaging system, where the S and M segments are packaged efficiently and possibly through segment-mediated interactions, the L segment is then incorporated into the virions (Terasaki et al., 2011). The 5' non-coding regions are important for genome packaging (Murakami et al., 2012).

Gn, Gc and N are required for the formation of virus, and when co-expressed form virus-like particles (VLPs) that are similar in architecture to a mature RVFV virion (Habjan et al., 2009a). When Gc and N are co-expressed, the VLPs are more diverse in structure, which reinforces the notion that both glycoproteins are required (Liu et al., 2008). As a result of the Golgi retention motif on Gn, there is an accumulation of glycoproteins at the Golgi membrane surface (Gerrard and Nichol, 2002). Upon interaction with the RNP, the immature virus will bud into the lumen of the Golgi apparatus, deriving its envelope from the Golgi membrane and virus maturation is completed through further glycoprotein modification (Gerrard and Nichol, 2007). RVFV will traffick through the cell within vacuoles and when the virus-containing vacuoles fuse with the host cell membrane, the mature virions are released. However, this is not a well understood process and RVFV can also bud directly from the host membrane but this differential budding might be cell-type specific (Anderson Jr and Smith, 1987).

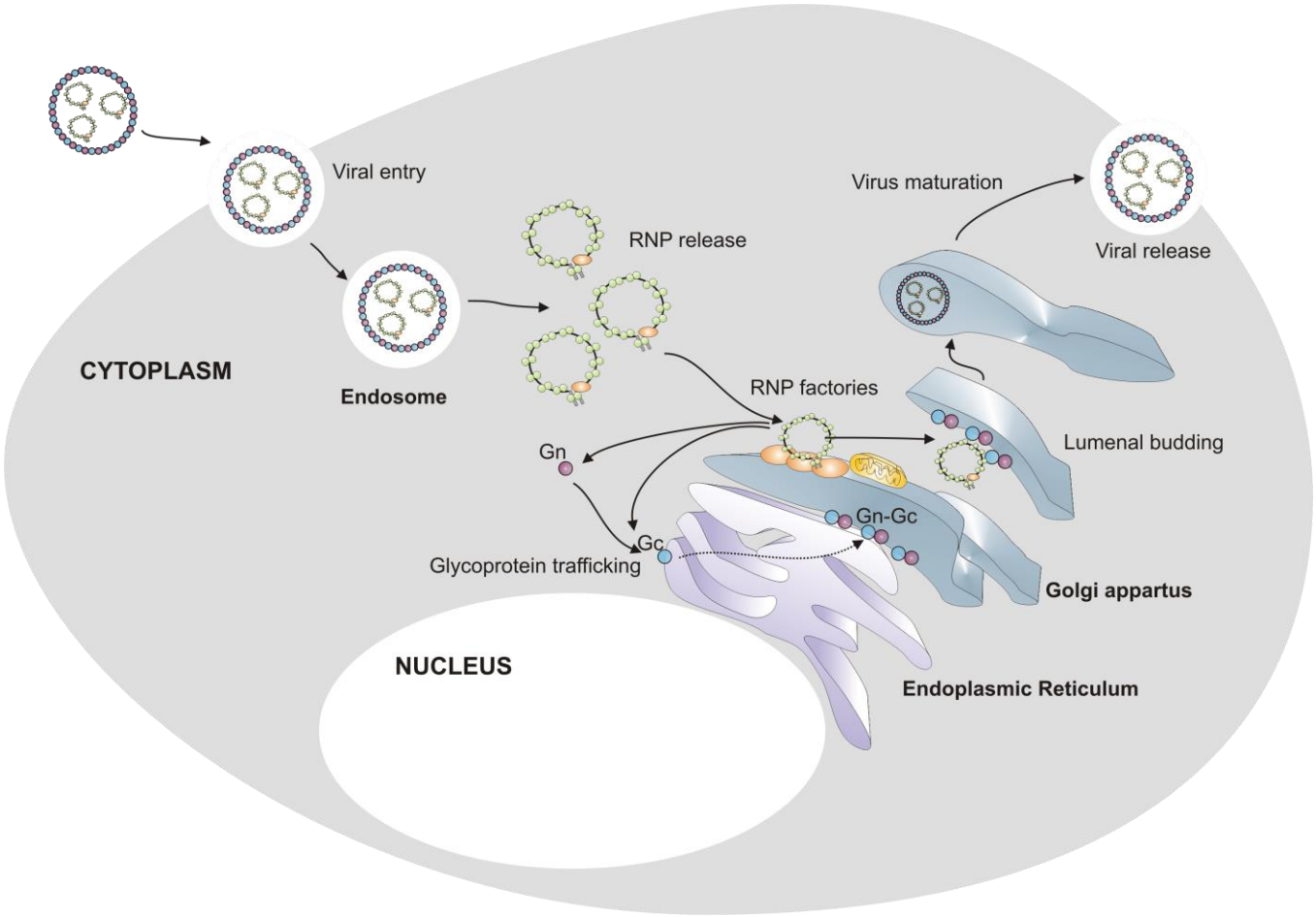

Figure 1.2: Diagrammatic presentation of RVFV entry, maturation and budding. A pentamer Gn-Gc heterodimer will interact with an unknown surface receptor through Gn and fused with the cell membrane using a Gc trimer type II fusion mechanism. Viral entry occurs by clathrin-dependent endocytosis, which will release the RNP at low pH and an incoming L polymerase will subsequently perform primary transcription of RVFV genes. At viral tubes associated with the Golgi apparatus, an L polymerase replicates and transcribes new RNP. The Gc glycoprotein will associate with the endoplasmic reticulum (ER) and only with its association with Gn will the complex traffic to the Golgi apparatus. The segments non-coding regions associate with Gn's cytoplasmic tail as a signal for genome packaging. The immature complex buds into the Golgi apparatus lumen and is trafficked to the host cell membrane by means of vacuoles for viral release.

1.3 Host-virus interactions

The host cell has developed mechanisms that survey for markers of viral genetic material. Upon identification, surveillance proteins will activate signal cascades, resulting in the production of antiviral proteins that will suppress viral replication [Reviewed in (Liu et al., 2011)] and signal adaptive immune response [Reviewed in (Meyer, 2009)]. In a biological arms race, viruses have developed means with which to subvert host defences to establish an infective state. This includes genomes that are not recognised as foreign as they do not produce markers that will be detected by the surveillance proteins (Habjan et al., 2008). Viruses also produce virulence factors, proteins that have elaborate mechanisms with which to circumvent innate immunity to prevent activation of antiviral responses. There is a complex interplay between the two competing genomes, which can result in disease prevention or a spectrum of pathogenesis, ranging from mild symptoms to host death. Understanding the molecular basis of host defence pathways and how viruses circumvent these pathways, can lead to a better understanding of pathogenesis as well as assist in the identification of important viral genes for RNAi targeting.

1.3.1 Interferon pathway

1.3.1.1 Viral recognition and interferon activation

An important antiviral pathway is the type I interferon (IFN) pathway, which has been well described in the literature (Figure 1.3B) [Reviewed in (Liu et al., 2011, Taylor and Mossman, 2012)]. Type II and type III IFN pathways have been identified, which possess unique receptors and signalling molecules [Reviewed in (Donnelly and Kotenko, 2010)]. For the purposes of this section, the type I IFN response will be focused on as a result of its extensive characterisation and importance in antiviral signalling.

Invading disease-causing genetic elements are recognised by pathogen recognition receptors (PRR) that survey the host cell for virus markers in the form of pathogen-associated molecular patterns (PAMP). PRRs consist of the Retinoic acid-inducible gene 1 (RIG-1) and Melanoma differentiation-associated protein 5 (MDA5), which recognise viral RNA genomes (Yoneyama et al., 2005, Andrejeva et al., 2004). The PRRs will then activate the mitochondrial-associated IPS-1 (Kawai et al., 2005), which further activates downstream IκB kinase (IKK)-related kinases, IKKε and TANK-binding kinase-1 (TBK-1) (Fitzgerald, 2003). IKKε and TBK-1 activate Interferon regulator factor 3 (IRF-3), which forms a homodimer (Yoneyama et al., 2002, Lin et al., 1998) that will enter into the nucleus and is critical for IFN-β promoter activation (Yoneyama, 1998). Another PRR is protein kinase R

(PKR), which is auto-activated when interacting with dsRNA, and forms a homodimer that stimulates the IFN-β promoter through NF-κβ (Nanduri et al., 1998). DExD/H-box helicase proteins can also recoginse dsRNA and through IPS-1 activate IRF-3 (Zhang et al., 2011). Finally, upon entry the viral genomes are exposed to Toll-like receptors (TLR) within endosomes that will also activate IRF-3 through IKKε and TBK-1 [reviewed in (Kawai and Akira, 2011)]. Ultimately, RIG-1/MDA5, PKR, DExD/H-box helicases and TLRs recognise viral RNA, which leads to IFN-β activation.

1.3.1.2 Jak/STAT signalling and IFN-stimulated genes

When IFN-β is expressed, it is secreted from the cells to activate adjacent cells through the IFN receptor (IFNAR). Inactivated Janus kinases, Jak1 and Tyk2, bound to IFNAR are activated, which in turn activate STAT proteins [Reviewed in (Stark et al., 1998)]. STAT1 and STAT2 form heterodimers that recruit IRF-9, which translocates to the nucleus and binds to an IFN-stimulated response element (ISRE) leading to expression of interferon stimulated genes (ISGs). Interferon regulatory factor 7 (IRF-7) is expressed and forms a heterodimer with IRF-3, creating a positive feedback mechanism to elicit a "full" IFN response (Sato, 2000).

1.3.1.3 ISG - restriction factors

ISGs encode many proteins but several have been identified as antiviral proteins known as restriction factors. PKR phosphorylates elongation initiation factor 2α (eIF2α), which leads to general down-regulation of translation (Williams, 1999). An 2'-5'-oligoadenylate synthase (OAS) phosphorylates the 5' end at the 2' and 5' linked oligoadenylates known as 2-5A, which activates RNAse L causing degradation of both viral and host mRNA (Silverman, 1994). MxA proteins are large guanosine triphosphates (GTPases) functioning like membrane-associated dynamin-like proteins and have potent antiviral activity at the early stages of the viral life cycle (Haller and Kochs, 2002). The Interferon-induced transmembrane (IFITM) protein family inhibit viral fusion or create inhospitable endosomal compartments [Reviewed in (Diamond and Farzan, 2013)]. Cholesterol-25-Hydroxylase (CH25H) generates an oxidation derivative of cholesterol, which modifies the cellular membrane to prevent viral entry (Liu et al., 2013). These restriction factors work collectively to limit viral replication and spread.

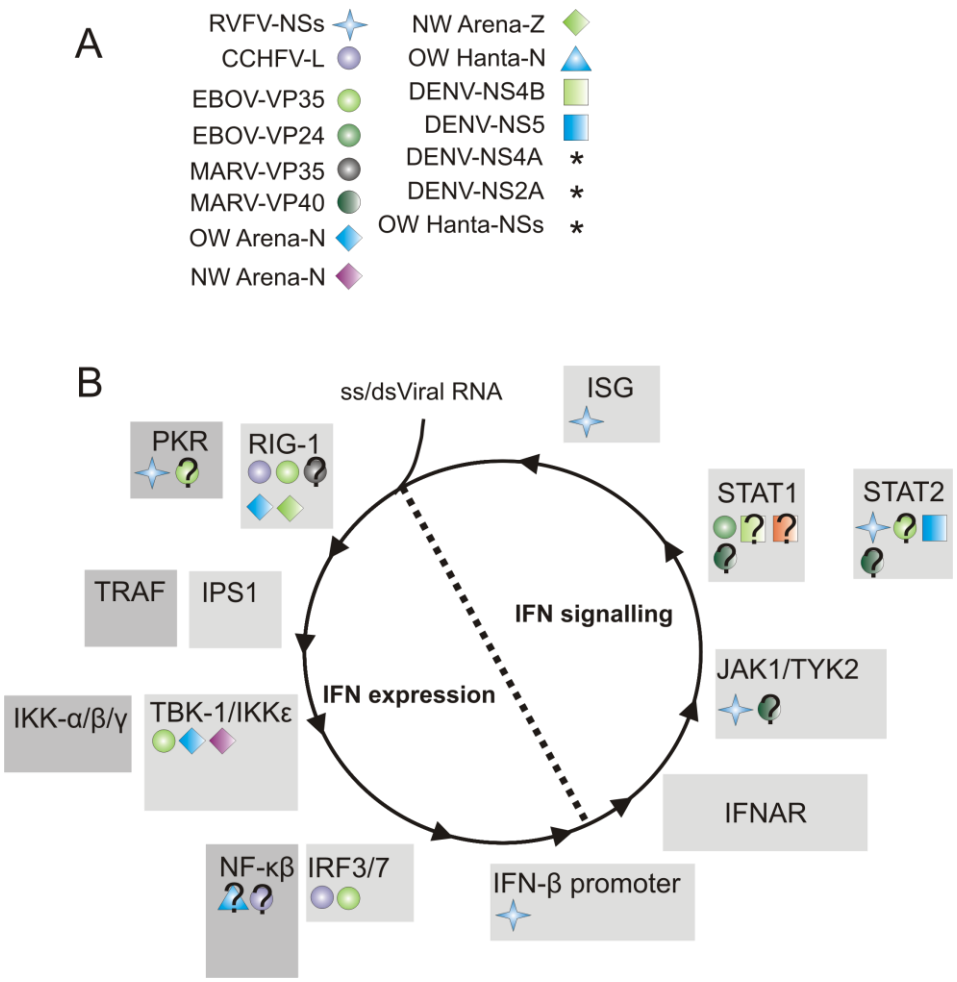

Figure 1.3: Schematic of viral protein interactions with the type I IFN pathway. (A) Viral haemorrhagic fevers and their respective virulence factors. Virulence factors with (*) denote suppressors with an unknown target in the IFN pathway. RVFV-NSs is represented by a blue star. (B) Schematics of the IFN expression and signalling pathways as well as the interactions of virulence factors with its various components. Virus-host interactions that have been described but have unknown mechanisms of action are denoted by (?). RVFV-NSs (blue star) can degrade PKR as well as suppress activation of the IFN-β promoter and various IFN related proteins including STAT2, Tyk2 and OAS. CCHFV=Crimean-Congo haemorrhagic fever virus, DENV=Dengue fever virus, EBOV=Ebola virus, MARV=Marburg virus, NW=New World, OW=Old World, RVFV=Rift Valley fever virus, N=Nucleocapsid, NS=Non-structural protein, NSs=Non-structural protein on the S segment, L=L polymerase, VP=Viral protein, Z=Z matrix protein.

1.3.1.4 IFN response and pathogenicity

The IFN pathway is vital to controlling pathogen invasion. IFN's importance has been demonstrated in numerous IFN-deficient animal models, specifically with regards to haemorrhagic fever viruses. Ebola virus (EBOV) does not produce a significant disease state in mice that are type I IFN-competent (Bray, 2001) but STAT1 knockout mice infected with EBOV resulted in significant pathology leading to lethality (Raymond et al., 2011). IFN-deficient mice that were vaccinated with EBOV VLPs and generated anti-EBOV antibodies were still highly susceptible to EBOV infection, demonstrating the importance of the IFN pathway even in the presence of an adaptive immune

response (Raymond et al., 2011). Crimean-Congo haemorrhagic fever virus (CCHFV) did not cause disease in adult mice (Smirnova, 1979), but in mice lacking the IFN receptor (IFNAR -/-) (Bereczky et al., 2010) or STAT1 (Bente et al., 2010) resulted in hepatic pathology and 100% mortality. Viraemia levels in IFN-deficient mice were similar to severe human cases (Bente et al., 2010) and haematological changes were comparable to predicted fatal outcomes (Swanepoel et al., 1989). In Lassa virus (LASV) infections, a functional IFN response is required for viral clearance (Yun et al., 2012) and IFN-deficient mice had enhanced pathology and dissemination to various organs. A New World (NW) arenavirus, Junin virus, displayed mortality and wide-spread tissue dissemination in IFNAR -/- mice compared to IFN-competent mice (Kolokoltsova et al., 2010). In dengue virus (DENV) infections, STAT1 signalling was determined as critical for controlling DENV infections (Shresta et al., 2005) and STAT1:STAT2 or STAT1:INFAR double knockout mice succumbed to infection quicker than wild-type mice (Perry et al., 2011). Even non-pathogenic forms of RVFV can become pathogenic in IFN-deficient animal models. An attenuated MP12 vaccine strain (Boshra et al., 2011) or a non-lethal RVFV clone 13, (Bouloy et al., 2001) were lethal in IFNAR -/- mice. Overall, the above mentioned studies underscore the importance of the IFN response in controlling haemorrhagic fever viruses and preventing pathogenesis. It is therefore not surprising that many viruses have evolved mechanisms to subvert this response. Haemorrhagic fever viruses potently inhibit host innate immune through IFN antagonists referred to as virulence factors. This may imply that the clinical manifestations of a haemorrhagic fever are, at least in part, as a result of an induced IFN defective state.

1.3.2 Virulence factors and pathogenesis

Haemorrhagic fever viruses have developed extensive and redundant mechanisms to inhibit the IFN pathway. At the heart of these mechanisms are virulence factors that potently suppress IFN-β activation and subsequent Jak/STAT signalling. The mechanisms that haemorrhagic fever viruses employ to subvert innate immunity through their respective virulence factors is summarised in Table 1.1 and Figure 1.3. The importance of virulence factors has been demonstrated in several *in vivo* studies. A point mutation in EBOV's major virulence factor, VP35, affected its ability to bind viral dsRNA and prevent detection by RIG-1, resulting in a avirulent infection in guinea pigs (Prins et al., 2010). EBOV that was previously avirulent in a guinea pig became lethal because of the conversion of a single amino acid within EBOV's VP24, a factor involved in suppressing Jak/STAT signalling (Mateo et al., 2011). Marburg virus (MARV) became infectious as result of changes in the virulence factor, VP40, in both mouse (Warfield et al., 2009) and guinea pig models (Lofts et al., 2007). Adaption of VP40 was linked to species-specific inhibition of the IFN pathway, suggesting IFN

suppression is required for pathogenesis in a new host (Valmas and Basler, 2011). STAT1 and STAT2 can effectively control DENV *in vivo*, even in the absence of one of the STAT proteins (Perry et al., 2011). DENV employs a dual mechanism to inhibit both STAT1 and STAT2 through two virulence factors, NS4B and NS5, respectively. The suppression of both STAT proteins by multiple virulence factors would ensure a productive DENV infection and further illustrates the targeted suppression of innate immunity by virulence factors as a feature of their involvement in viral pathogenesis. As a result of the importance of virulence factors to a haemorrhagic fever virus' pathogenesis makes them attractive targets for therapeutic intervention. A more detailed insight into the function of RVFV's virulence factors will assist in understanding their contribution to infection as well as the benefit of their targeted down-regulation using RNAi.

Table 1.1: Virulence factors of haemorrhagic fever viruses

Virus[1]	Virulence factor[2]	IFN protein target	Mechanism of suppression	Reference
EBOV	VP35	RIG-1	1) Prevents RIG-1 detection through binding viral dsRNA	(Cárdenas et al., 2006) (Hartman et al., 2006) (Kimberlin et al., 2010) (Leung et al., 2010) (Luthra et al., 2013)
		PKR and eIF2α	1) Prevents activation but mechanism is unknown	(Feng et al., 2007) (Schümann et al., 2009)
		IKKε and TBK-1	1) Binds and prevents interactions with IRF-3 and IPS-1	(Prins et al., 2009)
		IRF-3 and IRF-7	1) Modifies SUMOylation markers to prevent nuclear localisation	(Basler et al., 2000) (Basler et al., 2003) (Chang et al., 2009)
	VP24	STAT1	1) Interacts with NPI-1 Karyopherin α protein subfamily to prevent STAT1 nuclear import	(Reid et al., 2006) (Reid et al., 2007) (Mateo et al., 2009) (Zhang et al., 2012a)
		STAT2	1) Prevents STAT2 nuclear import but mechanism is unknown	(Valmas et al., 2010)

MARV	VP35	RIG-1	1) Weakly binds viral dsRNA and might prevent RIG-1 detection	(Bale et al., 2012) (Ramanan et al., 2012)
	VP40	STAT1, STAT2, and Jak1	1) Prevents activation but mechanism is unknown	(Valmas et al., 2010)
CCHFV	L	RIG-1 and NF-κβ	1) Decouples ubiquitination markers to prevent activation	(Frias-Staheli et al., 2007) (James et al., 2011) (van Kasteren et al., 2012)
OW Hantavirus	NSs	?	Unknown	(Jääskeläinen et al., 2007) (Jääskeläinen et al., 2008)
	N	NF-κβ	1) Prevent nuclear accumulation of TNF-α stimulated NF-κβ	(Taylor et al., 2009).
LASV (OW arenavirus)	N	RIG-1	1) Degrades viral dsRNA through 3'-5' exonuclease activity, which prevents detection by RIG-1	(Qi et al., 2010) (Hastie et al., 2011) (Hastie et al., 2012) (Jiang et al., 2013)
		IKKε	1) Binds IKKε and prevents activation of IRF-3	(Martínez-Sobrido et al., 2007) (Pythoud et al., 2012)
NW arenavirus	Z	RIG-1	1) Binds RIG-1 and prevents activation of IPS-1	(Fan et al., 2010)
	N	IKKε	1) Binds IKKε and prevents activation of IRF-3	(Martínez-Sobrido et al., 2007) (Pythoud et al., 2012)
DENV	NS2A and NS4A	?	Unknown	(Muñoz-Jordán et al., 2003)
	NS4B[3]	STAT1	1) Prevents activation but mechanism is unknown	(Muñoz-Jordán et al., 2003) (Muñoz-Jordán et al., 2005).
	NS5	STAT2	1) Promotes degradation of STAT2 through UBR4 ubiquitin ligase recruitment 2) Prevents STAT2 activation but mechanism is unknown	(Jones et al., 2005) (Ashour et al., 2009) (Mazzon et al., 2009) (Morrison et al., 2013)

1) Abbreviation of virus names: CCHFV=Crimean-Congo haemorrhagic fever virus, DENV=Dengue fever virus, LASV= Lassa virus, EBOV=Ebola virus, MARV=Marburg virus, NW=New World, OW=Old World.

2) Abbreviation of virulence factors: N=Nucleocapsid, NS=Non-structural protein, NSs=Non-structural protein on the S segment, L=L polymerase, VP=Viral protein, Z=Z matrix protein.

3) Yellow fever virus (YFV) has also been shown to suppress IFN through NS4B.

1.4 RVFV's virulence factors

1.4.1 RVFV's NSs protein

The NSs protein is an important virulence factor for RVFV (Vialat et al., 2000). The loss of NSs results is a highly attenuated virus, as observed by the naturally occurring strain clone 13 (C13), which has a large deletion (69%) in the *NSs* gene (Muller et al., 1995). C13 has been developed as a possible vaccine candidate (von Teichman et al., 2011) and demonstrates the importance of the NSs protein in RVFV pathogenesis. NSs was identified as an IFN antagonist (Bouloy et al., 2001) and facilitates this through several mechanisms, which will be addressed below (Figure 1.4).

1.4.1.1 Suppression of global mRNA transcription

NSs can interfere with antiviral defence through its ability to down-regulate global host transcription (Billecocq et al., 2004). XPD, XPB, p62 and p44 are important factors of a functional transcription factor II H (TFIIH) [Reviewed in (Zurita and Merino, 2003)]. NSs enters into the nucleus by sequestering p44 and then binds XPB through p44 (Le May et al., 2004), which are incorporated into the filamentous structures of NSs (Struthers et al., 1984). As p44 is used by XPD for nuclear entry, the presence of NSs results in the cytoplasmic accumulation of XPD. NSs can also down-regulate the levels of TFIIH subunit p62 through proteasomal degradation (Kalveram et al., 2011). Sequestering components prevent the formation of new TFIIH complexes, while p62 degradation down-regulates mature TFIIH and therefore, NSs inhibits IFN through active down-regulation of global mRNA transcription (Billecocq et al., 2004).

1.4.1.2 Suppression of the IFN-β promoter and other host genes

A more direct approach to IFN pathway suppression is through NSs's interaction with the IFN-β promoter. NSs interacts with Sin-3A associated protein (SAP30), which associates with the Sin3A/NCoR/HDAC repressor complex (Le May et al., 2008). NSs-SAP30 directs a repressor complex to Yin-Yang-1 (YY1) transcription factor (Huang et al., 2003), which is a known regulatory element of

the IFN-β promoter (Klar and Bode, 2005). The targeting of a repressor complex by NSs to the IFN-β promoter prevented its activation. The deletion of NSs's SAP30 interaction domain resulted in an avirulent RVFV, stressing the importance of directed IFN-β promoter repression.

Large-scale ChIP-on-chip experiments showed that other gene targets could be suppressed by NSs (Benferhat et al., 2012). Factors involved in Jak/STAT signalling pathway were identified for NSs-mediated down-regulation such as Tyk2 and STAT2. STAT2-dependent stimulation of genes is enhanced by a mediator complex, Med14 (Lau et al., 2003), and was also a factor subject to NSs-mediated down-regulation (Benferhat et al., 2012). Restriction factors from the OAS protein family would also be negativity affected by NSs, which will prevent the antiviral response through OAS-mediated activation of RNAse L (Silverman, 1994). Finally, NSs can deregulate several factors in the coagulation cascade by interacting with genomic regulatory elements (Benferhat et al., 2012), which might explain features of severe RVFV cases such as the heamorraghic pathology as result of ineffective clotting as well as the presentation of disseminated intravascular coagulation (DIC) [Reviewed in (Ikegami and Makino, 2011)]. NSs can interact with DNA in regions outside of regulatory elements of genes that contribute to pathogenesis. NSs binds pericentromeric γ-satellites, which has been linked to chromosomal cohesion and segregation defects (Mansuroglu et al., 2010), and is possibly the cause of the high abortion rates in RVFV infections (Daubney et al., 1931).

1.4.1.3 PKR degradation

RVFV can prevent detection of its viral dsRNA and subsequent viral restriction through its interaction with PKR. NSs can down-regulate PKR through proteasomal degradation (Habjan et al., 2009b), a feature which has been recently described for another *Phelobvirus*, Toscana virus (Kalveram and Ikegami, 2013). NSs binds PKR and this interaction is essential to NSs-mediated PKR degradation (Kalveram et al., 2013). Furthermore, NSs can prevent eIF2α phosphorylation through PKR down-regulation (Ikegami et al., 2009a) and would hinder eIF2α suppression of host translation, which could inhibit viral protein synthesis.

1.4.1.4 Regulation of RVFV Life cycle

There are several studies that suggest that NSs may regulate RVFV's life cycle. Firstly, DNA damage response pathways are often used by viruses as a means to enhance pathogenicity [Reviewed in (Li and Hayward, 2011)]. NSs-dependent DNA-damage signalling causes Go/G1 or S phase cell cycle arrest through activation of the ataxia-telangiectasia mutated (ATM) pathway (Baer et al., 2012). S phase arrest enhances viral production of RVFV (Narayanan et al., 2012) and

Curcumin, a compound that decreases S phase arrest, inhibits RVFV replication (Narayanan et al., 2012). This suggests that NSs induces cycle cell arrest as a means of enhancing viral production.

Several viruses manipulate p53 as a result of the role of this protein in host transcription regulation, cell cycle arrest and apoptosis [Reviewed in (Lazo and Santos, 2011)]. NSs-dependent p53 phosphorylation has been implicated in inducing late-stage apoptosis, which may assist in viral production of RVFV (Austin et al., 2012). NSs may also facilitate viral release through transcription suppression, which would eventually lead to late stage cell death (Le May et al., 2004). *Bunyaviridae* use host mRNA "cap snatching" to replicate RNA (Patterson et al., 1984) and NSs-mediated transcriptional suppression will deplete host caps, which could be detrimental to the virus (Le May et al., 2004). Replication of RVFV expressing NSs is unaffected in the presence of a chemical suppressor of transcription, suggesting that NSs has a compensatory mechanism to enable viral replication in the absence of host transcription (Ikegami et al., 2009b). Overall, NSs has been implicated as a possible regulator of RVFV life cycle by enhancing viral production and facilitating viral release.

1.4.2 RVFV's NSm proteins

NSm1 and NSm2 proteins are produced from the first two translation start codons in the M segment mRNA (Gerrard and Nichol, 2007) and there is a complete conservation of the starts codons, which suggests an important evolutionally role of *NSm* within the viral life cycle (Bird et al., 2007a). A RVFV strain lacking *NSm* was slightly attenuated suggesting a role in pathogenesis (Bird et al., 2007b). Many viruses exploit the cellular apoptotic pathways to enhance infectivity [Reviewed in (Everett and McFadden, 1999)]. RVFV's NSm proteins were identified as an antagonist to apoptosis through a deletion mutant and the smaller NSm2 protein was implicated in suppressing viral-induced apoptosis by preventing caspase activity (Figure 1.4) (Won et al., 2007). The C-terminal domain of NSm2 inserts into the outer membrane of mitochondria, which may mediate apoptosis through this interaction but the mechanism has yet to be elucidated (Terasaki et al., 2012). RVFV-NSm may also regulate apoptosis induced by oxidative stress. RVFV induced massive oxidative stress during a viral infection (Narayanan et al., 2011). The NSm proteins were implicated in regulating mitogen-activated protein kinase (MAPK) p38 (Narayanan et al., 2011) and may enhance pro-survival pathways, which would counteract RVFV-induced oxidative stress. Collectively, these studies suggest that NSm prevents early stage apoptosis induced by viral replication and oxidative stress allowing for efficient viral replication.

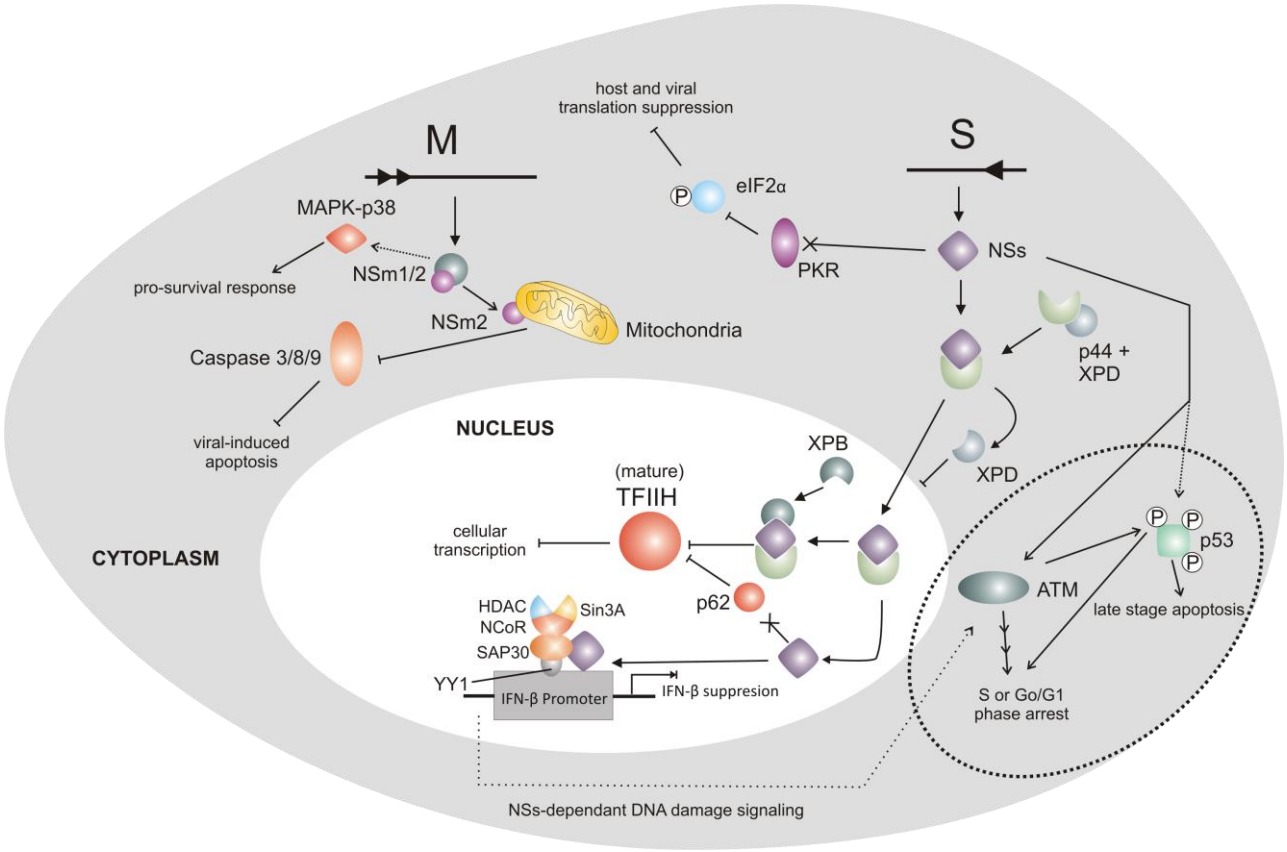

Figure 1.4: Diagrammatic presentation of RVFV's virulence factors. NSs has several functions within the host cell. Firstly, NSs inhibits host mRNA transcription. It achieves this by sequestering p44, which it uses to enter into the nucleus resulting in the cytoplasmic accumulation of XPD. Within the nucleus it binds XPB as well as degrading p62. The p44, p62, XPB and XPD components are essential for the formation of a functional TFIIH. Secondly, NSs interacts with the IFN-β promoter through SAP30 and a Sin3A/NCoR/HDAC repressor complex, which prevents IFN-β promoter activation. NSs also reduces expression of other IFN related genes such as STAT2 and Tyk2 as well as various factors of the coagulation cascade. Thirdly, NSs down-regulates PKR and prevents phosphorylation of eIF2α. Fourthly, NSs interacts with pericentromeric γ-satellite sequences, which results in chromosome cohesion and segregation defects. Fifthly, NSs activates ATM-dependent DNA damage response pathways causing Go/G1 or S phase cell cycle arrest. Finally, NSs-dependent phosphorylation of p53 induces apoptosis and enhances viral release. NSm2 suppress viral-induced apoptosis by preventing activation of caspase 3/8/9, which may be through its interaction with mitochondria. The NSm proteins are possibly involved in regulating MAPK-p38, which could help counteract viral-induced oxidative stress. The circle dotted line donates that signalling and modification may occur in the cytoplasm and nucleus.

1.5 Therapeutics for haemorrhagic fever viruses

A comprehensive understanding of the molecular biology of haemorrhagic fever viruses can facilitate the development of intervention strategies. This is reflected in a RVFV that has been engineered to lack the *NSs* and *NSm* genes, which is being developed as a safe, effective vaccine (Bird et al., 2008). The understanding of basic biology can also be applied to the development of targeted therapeutics. The loss of NSs's interaction with the IFN-β promoter resulted in avirulence (Le May et al., 2008), hinting at a possible site for a targeted therapeutic. However, even with this knowledge there are only a small number of therapeutics in the early stages of development. Understanding the current therapeutics in development puts into perspective the unique application of RNAi.

1.5.1 Ribavirin and Favipiravir

Ribavirin has been explored as a possible haemorrhagic fever virus drug. It is a synthetic purine nucleotide analogue and has a broad-spectrum antiviral effect by inhibiting viral mRNA synthesis [Reviewed in (Graci and Cameron, 2006)]. The therapeutic effect of Ribavirin has been previously explored for RVFV and the IFN inducer poly-L-Lysine and carboxymethylcellulose (Poly[ICLC]) prior to infection, which prevented RVFV symptoms in mice, hamsters and non-human primates (Peters et al., 1986). Ribavirin is associated with toxicity causing reversible haemolytic anaemia (Russmann et al., 2006) as well as teratogenicity (Ferm et al., 1978) and embryotoxicity (Kochhar et al., 1980). For this reason, a pyrazinecarboxamide called T-705 (Favipiravir) was developed, which is processed within the cell to produce a purine nucleotide analogue and is an RdRp inhibitor (Furuta et al., 2009). T-705 can inhibit several RNA viruses, including arenaviruses and bunyaviruses (Furuta et al., 2009) and has inhibitory potential against RVFV *in vitro* (Gowen et al., 2007). Oral administration post-infection of T-705 offered protection *in vivo* with a related bunyavirus, Punta Toro virus, at lower doses than Ribavirin. In a clinically relevant study using a haemorrhagic guinea pig model, T-705 therapy was effective against an arenavirus, Pichinde virus, when oral therapy was started four days post-infection at the point of fever symptoms and protected against lethality (Mendenhall et al., 2011). This suggests that T-705 should be explored further as a potential candidate against RVFV.

1.5.2 Small molecule inhibitors

Small molecule inhibitors have been assessed as potential therapeutics for haemorrhagic fever viruses and several have broad-spectrum antiviral activity. An anti-oxidant NSC62914, which is a scavenger for reactive oxygen species, had antiviral properties and was able to inhibit EBOV, MARV, LASV and RVFV in cultured cells (Panchal et al., 2012). Furthermore, it could protect mice from a lethal filovirus challenge when administered 24 hrs post-infection. Employing high-throughput screening, broad-spectrum antiviral compounds termed LJ001 and FG1-106, were identified. LJ001 targets the host-derived viral membrane of enveloped viruses and irreversibly intercalates to prevent viral fusion (Wolf et al., 2010). LJ001 has broad antiviral activity against enveloped virus including RVFV and a number of haemorrhagic fever viruses *in vitro*. FGI-106 had antiviral activity against EBOV, RVFV and DENV although the mechanism has yet to be determined (Aman et al., 2009). Imino sugar derivatives are competitive inhibitors for cellular α-glucosidases I and II [Reviewed in (Dwek et al., 2002)], the enzymes involved in maturation of viral glycoproteins. The imino sugar derivatives were able to inhibit several haemorrhagic fever viruses including RVFV *in vitro* and protect against EBOV lethality in mice (Chang et al., 2013). Finally, RVFV induces cell cycle arrest, which has been associated with improving viral replication (Baer et al., 2012). Curcumin prevents cell cycle arrest in RVFV infected cells, which inhibits viral replication and improves survival in a mouse model (Narayanan et al., 2012). Overall, there is a variety of potential small molecule inhibitors being developed, which have antiviral activity against RVFV.

1.5.3 Restriction factors

RVFV N is important for viral RNA synthesis (Lopez et al., 1995) and a structural component of the virion (section 1.2.4). MxA sequesters N into perinuclear complexes (Kochs et al., 2002) and prevents primary transcription (Habjan et al., 2009a). Expressed MxA in transgenic INFAR -/- mice increased protection when challenged with a related *Bunyaviridae*, La Crosse encephalitis virus (LACV) (Hefti et al., 1999) and suggests that MxA could be expressed in RVFV infected tissue to facilitate viral suppression. Another restriction factor, the IFITM family is able to inhibit numerous haemorrhagic fever viruses [Reviewed in (Diamond and Farzan, 2013)] including RVFV by preventing viral entry (Mudhasani et al., 2013). CH25H was identified as a viral entry inhibitor and is particularly prominent in the liver, the initial site of RVFV replication (Liu et al., 2013). CH25H converts cholesterol to the 25-hydroxycholesterol (25HC), which prevents viral membrane fusion through modification of the cellular membrane and has broad antiviral activity against membrane viruses

including EBOV and RVFV *in vitro*. 25HC is a soluble molecule that could be artificially synthesised and opens up a novel avenue for the development of broadly active antiviral compounds.

1.5.4 Nucleic acid-based therapeutics

Several nucleic acids-based therapeutics have been employed as potential inhibitors of haemorrhagic fever viruses. Aptamers are highly structured nucleic acids that interact specifically with protein or nucleic acid targets and can have a therapeutic effect by disrupting essential interactions with viral/host components [Reviewed in (Zhou et al., 2012)]. The N of *Bunyaviridae* interacts with the complementary ends of the genomic segments (Mir et al., 2006). Recently, aptamers that interact with RVFV-N were developed (Ellenbecker et al., 2012) and act as competitive substrates for N binding, which may be able to inhibit RVFV replication by the sequestration of N.

Aptamers are generated from a random library of nucleic acids, which selects a structured RNA or DNA that binds to the intended input target [Reviewed in (Guo et al., 2008)] and it must then be confirmed whether the aptamer exerts an inhibitory effect. As a result of the specificity of this interaction, viruses may be able to generate mutations that confer resistance to aptamer-mediated inhibition (Fisher et al., 2005). Several aptamers would need to be created to compensate for escape mutants, but the random process of creating novel aptamers may select for an aptamer that has been identified or aptamers that do not exert an inhibitory affect. This inability to design novel aptamers rationally through a relatively cumbersome selection process limits the application of aptamers to mutable targets like viruses. However, aptamers can be joined to synthetic therapeutic RNAi effectors called small interfering RNAs (siRNAs) (section 1.6.3.1) to prevent viral escape and enhance their applicability when suppressing evolving targets (Zhou et al., 2008).

Morpholino oligomers have been investigated as potential therapeutics for highly pathogenic viruses. Phosphorodiamidate Morpholinos oligonucleotides (PMO) have the traditional DNA nucleotides but with phosphorodiamidate linkages with morpholinos rings [Reviewed at (Warren et al., 2012)]. PMOs are chemically synthesised single-stranded antisense agents, which can be modified to have poly-arginine (Moulton et al., 2004) or a positively charged piperazine to enhance cellular uptake (Swenson et al., 2009). The inhibitory effect of PMOs when binding to complementary mRNA is through steric hindrance and blocking of the translation machinery.

EBOV has been targeted extensively with PMOs to determine its *in vivo* inhibitory affects. The earliest PMO studies comprehensively demonstrated inhibition of EBOV *in vitro* as well as in mouse and non-human primate models (Warfield et al., 2006). The ability of PMOs to function after EBOV infection was evaluated and a single dose of PMO administered post-infection in mice (Enterlein et al., 2006) or guinea pigs (Warfield et al., 2006) protected against EBOV lethality. PMOs

targeting the virulence factors, VP24 and VP35 (Table 1.1), were more effective at preventing lethality in rodents than PMOs targeted to RdRp (Warfield et al., 2006) or N (Iversen et al., 2012). A PMO targeted to VP35 in non-human primates did not protect against EBOV lethality (Warfield et al., 2006) but combined with VP24 was a potent inhibitor (Iversen et al., 2012). PMOs also protected mice against MARV but a PMO against N was more effective than VP35 and VP24 (Iversen et al., 2012), which fits with previous observations that they may not be important virulence factors for MARV but rather VP40 (Table 1.1) (Valmas et al., 2010) (1.3.2.2). Overall, this underscores the rational identification of good therapeutic targets such as virulence factors for antisense technology. PMOs have also been used to inhibit other viruses such as DENV (Kinney et al., 2005, Stein et al., 2008), arenaviruses (Neuman et al., 2011), West Nile virus (Deas et al., 2005) and influenza (Lupfer et al., 2008), which demonstrates its applicability to a broad range of pathogens.

PMOs have the potential to induce severe toxicity, which occurs mostly through sequence-dependent off target effects of the PMOs suppressing host mRNAs. In Zebra fish studies, PMO off targeting can result in a "catastrophe" phenotype arising from genetic instability (Coffman et al., 2004) or a "monster" phenotype (Ekker and Larson, 2001). PMOs can also activate the pro-apoptotic protein, p53 (Robu et al., 2007) and could have severe toxic effects as a result of p53-mediated apoptosis (Gerety and Wilkinson, 2011). Systemically administered peptide conjugated PMOs result in non-specific uptake into numerous tissues (Amantana et al., 2007) and the broad delivery of a PMO combined with the possibility of off target effects could reduce its safety profile.

1.6 RNA interference (RNAi)

Since the discovery of RNAi in *C. elegans* (Fire et al., 1998), RNAi's importance in mammalian host regulation has been established, which has given rise to an RNA universe that exists outside of the protein coding function of RNA. The essential nature of the RNAi pathway is illustrated by the fact that when deregulated, results in disease properties associated with cancer [Reviewed in (Fang and Gao, 2013)]. An understanding of the RNAi pathway has not only expanded our knowledge of gene regulation, but offered a tool for biological study and therapeutic intervention. A basic insight into the mechanism of the RNAi pathway is integral to its exploitation as well as appreciating its novel nature as a therapeutic for RVFV and other haemorrhagic fever viruses.

1.6.1 Human miRNA biogenesis

The endogenous RNAi pathway starts with the expression of an evolutionary conserved primary miRNA (pri-miRNA) (Figure 1.5). These pri-miRNAs are most often produced from Pol II promoters (Lee et al., 2004) but Pol III promoters have been described (Borchert et al., 2006). Pri-miRNAs consist of partially complementary stems, a loop sequence with 5' and 3' flanking extensions (Cai et al., 2004). In canonical miRNA processing, miRNAs are processed by RNAse III Drosha enzyme (Lee et al., 2003) and a DGCR8 co-factor in a microprocessor complex, which cleaves off the single-stranded flanking regions to form a precursor miRNA (pre-miRNA) (Gregory et al., 2004). The cleavage by Drosha results in a 2 nt 3' overhang in the pre-miRNA, which is important for nuclear export (Zeng and Cullen, 2004). A pre-miRNA hairpin is transferred out of the nucleus into the cytoplasm by the nuclear export protein, exportin-5 (Yi et al., 2003, Lund et al., 2004). In the cytoplasm, the pre-miRNA is processed by a RNAse III endonuclease, Dicer, that cleaves off the loop region of the hairpin into ~21 nt mature microRNA (miRNA) duplex fragments (Hutvagner et al., 2001). Dicer also recognises the 2 nt 3' overhang (Ma et al., 2004), which helps with the efficient and uniform processing of the pre-miRNA (Park et al., 2011). The mature duplex miRNAs are the mediators of RNA interference (Elbashir et al., 2001).

The TAR RNA-binding protein (TRBP) (Chendrimada et al., 2005) and a protein activator of the interferon-induced protein kinase (PACT) (Lee et al., 2006) interact with Dicer (Gregory et al., 2005) and may facilitate its recruitment to the Argonaute (Ago) proteins. This large ribonucleoprotein assembly is referred to as the RNA induced silencing complex (RISC) (Martinez et al., 2002). One strand of the miRNA duplex referred to as the guide stand, is selected for incorporation into one of four Ago proteins (1-4) (Meister et al., 2004). Strand selection is based on thermodynamic properties of the miRNAs, where the stand with the least 5' stability is favoured for RISC incorporation (Khvorova et al., 2003, Schwarz et al., 2003). RISC identifies its target mRNA through the complementarity of the guide sequence and facilitates either mRNA repression or degradation.

Even though guide strands can associate with each Ago, Ago2 is the only protein that can catalytically cleave target mRNAs and is often referred to as 'Slicer' (Liu et al., 2004). Extensive complementarity between the guide strand and the mRNA is required to facilitate cleavage (Hutvagner and Zamore, 2002). Furthermore, Ago2 binds perfectly complementary duplexes with higher affinity (Wang et al., 2009). Most miRNAs are only partially complementary to their target sites and facilitate suppression through mRNA destabilisation (Braun et al., 2011) or translation suppression (Zeng et al., 2003). Ago 1,3 and 4 cooperate to facilitate silencing, whereas Ago2

functions independently (Broderick et al., 2011). As a result of the unique features of perfect complementary duplexes, they are referred to as small interfering RNAs (siRNA).

MiRNA base pairing with the target mRNA of the 5' nucleotides from position 2 to 7 or 8, is known as the seed region and is important to facilitate suppression (Lewis et al., 2003). However, increased complementarity at the 3' end enhances suppression (Broderick et al., 2011), which has been shown to stabilise miRNA binding with the target mRNA (Hur et al., 2013). As there are only a few nucleotides that determine target mRNA suppression, miRNAs can regulate a vast number of mRNAs (Lewis et al., 2005) and cellular proteins (Baek et al., 2008). Dicer processing can lead to several miRNA products from one pre-miRNA, which have different seed regions (Park et al., 2011). This heterogeneous population of miRNAs could have different mRNA targets, adding a level of complexity in gene regulation (Starega-Roslan et al., 2011) and underscores the importance of miRNAs as broad regulators of gene expression. It is generally perceived that the strand not incorporated into RISC (passenger strand) is cleaved and rapidly degraded by Ago2 (Leuschner et al., 2006), but there is evidence that the anti-guides can be incorporated into RISC and have active silencing roles (Wei et al., 2009). Although the canonical miRNA biogenesis pathway is the most pronounced, other non-canonical miRNAs have been described [Reviewed in (Yang and Lai, 2011)] as well as a RNAi-mediated gene silencing mechanism that occurs at the level of DNA through epigenetic changes [Reviewed in (Green et al., 2011)], but reviewing these topics is beyond the scope of this work.

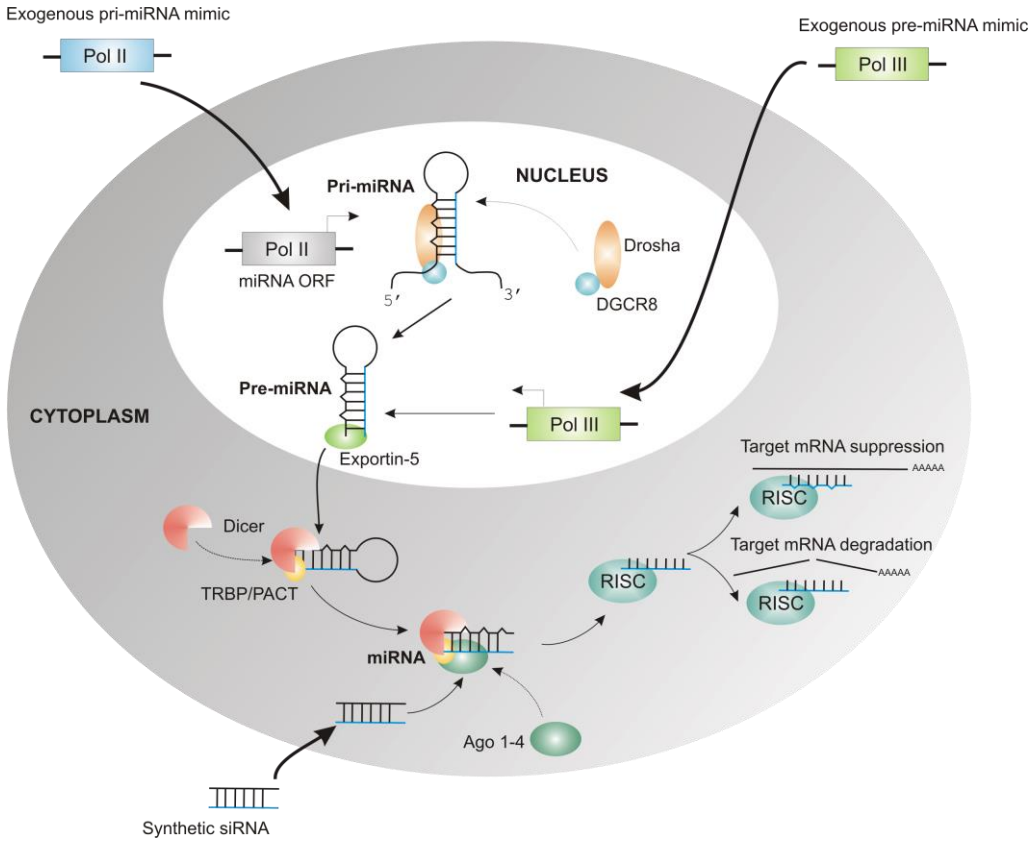

Figure 1.5: Diagrammatic presentation of the endogenous RNAi pathway and therapeutic manipulation. MiRNAs are evolutionary conserved RNAs, generally produced from Pol II transcription and are involved in mRNA regulation. Expressed miRNAs comprise of partially complementary stems, a loop sequence with 5' and 3' flanking extensions and are referred to as primary miRNAs (pri-miRNA). Pri-miRNAs are processed by the Drosha/DGCR8 microprocessor, which cleaves off the single-stranded flanking regions to form a precursor miRNA (pre-miRNA). Pre-miRNAs are transferred out of the nucleus into the cytoplasm by exportin-5 where it is processed by Dicer, which removes the loop resulting in a ~21 nt microRNA (miRNA) duplex. The strand with favourable thermodynamic properties is selected as the guide stand for incorporation into the RNA induced silencing complex (RISC). The guide strand is associated with one of the Ago proteins (1-4) and targets RISC to complementary mRNA to facilitate degradation or suppression. There are several points for therapeutic exploitation of the RNAi pathway through the use of RNAi mimics, namely exogenous siRNAs, expressed pri- and pre-miRNA mimics. Pri-miRNA mimics are endogenous miRNA scaffolds with replaced guide sequences and are often expressed from Pol II promoters. Pre-miRNA mimics, also known as short hairpins (shRNAs), are expressed from Pol III promoters as a result of the specific start and stop transcription termination points. SiRNAs are ~21 nt chemically synthesised RNA duplexes.

1.6.2 RNAi and host defence

1.6.2.1 Crosstalk between IFN and RNAi.

Endogenous RNAi has been implicated as a host defence pathway as there are links between the RNAi pathway and the known antiviral IFN response. DsRNA can be recognised by PRRs to activate IFN signalling, which is also a trigger for the RNAi pathway (section 1.3.1.1 and 1.6.1). There are also several proteins that are common to both IFN and RNAi. This includes the RISC-associated proteins, TRBP and PACT. TRBP is associated with Dicer (Chendrimada et al., 2005), which is involved in RISC loading (MacRae et al., 2008) and is required for function silencing by RNAi (Haase et al., 2005). PACT binds Dicer and enhances miRNA processing (Lee et al., 2006). Both TRBP and PACT stabilise Dicer (Koscianska et al., 2011) and function together to generate siRNAs (Kok et al., 2007). TRBP and PACT are also regulators of the IFN related protein, PKR (Williams, 1999). PACT is a activator of PKR during cellular stress (Patel and Sen, 1998), while TRBP is a negative regulator (Park et al., 1994) and prevents PACT activation of PKR in the absence of stress (Daher et al., 2009). PACT is also associated with RIG-1 and cooperates to potently activate an antiviral response (Kok et al., 2011). Furthermore, PACT activation of RIG-1 is independent of its interaction with Dicer, which suggests that IFN and RNAi are connected pathways but functionally distinct. Furthermore, miRNAs were up-regulated in response to IFN that could target HCV and the overexpression of the HCV specific miRNA mimics inhibited viral replication (Pedersen et al., 2007). MiRNA-122 has been shown to contribute to HCV replication (Jopling et al., 2005) and was down-regulated is response to IFN stimulation, further suggesting important crosstalk between the IFN response and RNAi.

1.6.2.2 Cellular and viral-derived small RNAs

The host may produce endogenous miRNAs that target a viral genome as demonstrated when virus specific miRNAs were identified for primate foamy virus (Lecellier et al., 2005) and HCV (Pedersen et al., 2007). A host-derived small RNA was identified that was up-regulated during HIV infection targeting the HIV primer binding site to inhibit viral replication (Yeung et al., 2009) but this has been contested recently and may be a tRNA degradation product used by HIV to assist reverse transcription (Schopman et al., 2012). Nevertheless, there is evidence to suggest that the host encodes miRNAs that can potentially restrict viral replication by targeting viral genes. Futhermore, one antiviral miRNA can have multiple predicted viral targets (Swaminathan et al., 2013), which suggests broad antiviral activity. However, as a result of the vast number of viruses, the targeted down-regulation by endogenous miRNAs may be too costly to maintain. Another study has shown that host miRNAs down-regulated essential host proteins during viral infection, which could inhibit

several unrelated viruses (Santhakumar et al., 2010). MiRNAs that target host pathways required for viral replication would seem more plausible as a defence strategy.

The host cell could also derive small RNAs from the virus' genome through Dicer processing and retarget the siRNAs to suppress viral genes through RISC-mediated cleavage. It was originally assumed that this did not occur, as virus-derived small RNAs were not detected during viral infection (Pfeffer et al., 2005). However, recently it was revealed that small viral RNAs do accumulate in infected cells and were associated with Ago proteins, which suggests an active role in silencing (Parameswaran et al., 2010). Virus-derived siRNAs were identified during HIV replication (Schopman et al., 2012) as well as Ago-associated small RNAs from a number of other viruses including DENV, West Nile virus and HCV (Parameswaran et al., 2010). Artificially generated mimics based on the HIV viral-derived RNAs were able to inhibit viral replication (Schopman et al., 2012), suggesting that they are functional silencers within infected cells.

Whether cellular or viral-derived miRNAs that target viruses play a role in silencing under physiological conditions has yet to be determined. Host (Pedersen et al., 2007) or virus-derived miRNAs (Schopman et al., 2012) are often "proven" as antiviral by artificially expressed or synthesised mimics that results in viral inhibition. The exogenous introduction of mimics would produce higher levels of miRNAs than the endogenously expressed counterparts, and this disparity in miRNA numbers may reflect a bias in interpreting their true potential as inhibitors. A further concern is whether the manipulation of miRNAs could have wide-ranging effects on host gene regulation as a single endogenous miRNA can regulate dozens of gene targets [Reviewed in (Bartel, 2009)]. The overexpression of miRNAs may not reflect the direct targeting of a viral genome but rather miRNA regulation of other host genes, which indirectly impacts the virus. Overall, the problem with the current studies investigating RNAi as an antiviral pathway is seperating RNAi's involvement in defence from gene regulation, which would affect interpretation of the data. Although there is evidence to warrant an investigation into RNAi as a defence pathway, future studies need to confirm its contribution through careful and precise experimentation.

1.6.3 RNAi-based therapeutics

The endogenous miRNA biogenesis pathway can be potentially exploited for therapeutic purposes. This is by using miRNA mimics that contain the sequences of the desire target mRNA, which can enter into the miRNA biogenesis pathway and be processed by the RNAi machinery. These miRNA mimics have perfect complementarity to their targets, and therefore the suppressive effect is mediated by the catalytic cleavage of target mRNA by Ago2 (Liu et al., 2004). Hence, the therapeutic

mechanism is by triggering the endogenous RNAi pathway with dsRNA mimics that inhibited viral protein expression by Ago2-mediated sequence specific cleavage of viral mRNA. There are three main types of miRNA mimics namely chemically synthesised siRNAs, expressed pri- or pre-miRNA mimics (Figure 1.5).

1.6.3.1 Small interfering RNAs (siRNAs)

Exogenous introduced siRNAs are ~21 nt RNA duplexes that mimic the mature processed miRNA (Elbashir et al., 2001). SiRNAs have 2 nt overhangs for Dicer and Ago2 recognition (Ma et al., 2004). Similar to mature miRNA duplexes, the siRNA guide strands are loaded into RISC to facilitate target mRNA suppression. SiRNAs can be easily produced through chemical synthesis and purchased commercially. Although siRNAs can be potent gene inhibitors, the effects are transient and require repeat administrations (Bartlett and Davis, 2006). For sustained inhibition, an expression system using either pri- or pre-miRNA mimics is required.

1.6.3.2 Pri-miRNA mimics

Pri-miRNA mimics use endogenous miRNA scaffolds, where the guide sequence of a natural miRNA is replaced with the desired target guide sequence. Pri-miRNA mimics have been used extensively against many viruses, especially in chronic infections such HIV (Aagaard et al., 2008) and HBV (Ely et al., 2009). These studies often express the pri-miRNA mimics from Pol II promoters, which create the 5' cap and 3' Poly-A tail flanking regions required for Drosha-dependent processing. Pol II promoters can also be manipulated to control miRNA expression using either inducible (Unwalla et al., 2006) or tissue-specific promoters (Nielsen et al., 2009). Possibly as a result of the "coupling" of all RNAi processing steps from Drosha to RISC, miRNAs are found to be more potent than other RNAi effectors (Borel et al., 2011). For these reasons, pri-miRNA mimics are considered a more 'natural' version of RNAi-based therapeutics.

However, the requirement for an endogenous miRNA backbone may also limit the use of pri-miRNA mimics. Some endogenous miRNA backbones are not amenable to manipulation and the desired guide strands are not always efficiently processed (Aagaard et al., 2008). Flanking sequences of the pri-miRNA are important for processing (Zeng and Cullen, 2005) and manipulation of these structures can reduce (Zhang et al., 2012c) or abolish miRNA generation (Aagaard et al., 2008). The loop sequence and an asymmetrical distortion near the Drosha cleavage site of a miRNA is important for processing, which will need to be maintained in the artificial miRNA (Quarles et al., 2013). Furthermore, bulge regions within the RNA structure of the pre-miRNA are required for accurate miRNA products and removal of these structures leads to alterative miRNAs with potential for off

target effects (Starega-Roslan et al., 2011). When designing a pri-miRNA mimic these structures will need to be maintained and this may not always be possible with the intended artificial guide sequence. Overall, these physical constraints can limit the flexible usage of pri-miRNA mimics.

1.6.3.3 Pre-miRNA mimics

Taking the above points into consideration (section 1.6.3.2), Pre-miRNA mimics, also known as short hairpins (shRNAs), can be employed for more consistent and efficient processing to form mature siRNAs products. ShRNAs are generally expressed off Pol III promoters, which allows for the stable, constitutive expression of small RNAs [Reviewed in (Dieci et al., 2007)]. Several Pol III promoters have been used to express shRNAs as a result of their defined transcription initiation and termination sites. The small nuclear RNA (snRNA) H1-Pol III promoter initiates RNA transcription with either a guanosine or adenosine directly after the promoter and terminates within a tract of five thymidines (Myslinski et al., 2001). This allows for defined expression of a pre-miRNA mimic after the start nucleotide, which will consist of a sense sequence, a loop and antisense sequence that will terminate with a two uridine 3' overhang (Brummelkamp et al., 2002). The 3' overhangs are required for recognition by exportin-5 and downstream RNAi machinery (Zeng and Cullen, 2004). Furthermore, two 3' uridines are necessary for uniform and efficient cleavage by Dicer, which will result in accurate siRNA products and reduces the likelihood of off target effects (Park et al., 2011). Similar to the H1-Pol III promoter, U6+1-Pol III (Miyagishi and Taira, 2002) and 7SK-Pol III promoters (Czauderna et al., 2003) can also be used to express shRNAs.

A Pol II promoter can also be used when designing a shRNA. A U1 snRNA Pol II promoter starts transcription directly after the U1-pol II promoter with a guanosine or adenosine (Lescure et al., 1992). U1-pol II promoters that lack some of the additional 3' sequence upstream of the box terminator can produce shRNAs with a defined hairpin formation (Denti et al., 2004). Therefore, the U1-Pol II promoter has no specific sequence constraints expect the initiation nucleotide. As a result of the defined termination point, unlike the Pol III promoters, the 3' two nucleotide overhang must be included into the shRNA design. A transfer RNA (tRNA) promoter can be used to express a tRNA sequence hybridised with a shRNA that can facilitate inhibition of a target gene. Several tRNA promoters have been used, namely a $tRNA^{lys3}$ (Scherer et al., 2007) and a modified $tRNA^{met}$ (Boden et al., 2003). The $tRNA^{lys3}$ expressed shRNAs have also demonstrated silencing *in vivo* (Dyer et al., 2010). Unlike Pol III promoters where the promoter sequence lies completely upstream of the shRNA, tRNA sequences are expressed with the shRNA and a tRNAse Z^{L} enzyme cleaves the shRNA off the tRNA sequence within the nucleus to enter into the RNAi pathway at exportin-5 (Scherer et al., 2007).

ShRNAs can be designed by a set of guidelines to increase the possibility of efficient processing and RISC loading [Reviewed in (Chen and Xie, 2012)]. Insertion of G:U wobble bases and 5' guide strand instability allows for favourable thermodynamic properties, which facilitates guide sequence selection into RISC (Khvorova et al., 2003, Schwarz et al., 2003). The GC content of the duplex should be between 30% and 50% (Reynolds et al., 2004), and highly stable secondary structures should be avoided (Patzel et al., 2005). Whether the shRNA stem length has an effect on silencing capabilities is contested, though recent evidence suggests there is no correlation between stem length and a shRNA's silencing capability (McIntyre et al., 2011b). A variable siRNA population was associated with longer stem lengths (>21 nt) (Gu et al., 2012), which will result in siRNAs with different seed regions and increase the probability of off target effects. Furthermore, longer stem lengths increased silencing by the passenger strand as a result of varied thermodynamics of the siRNA products, favouring passenger strand incorporation into RISC.

Dicer processing of pre-miRNA is a highly regulated process, which can help facilitate the design of specifically processed shRNAs. Dicer recognises both the 5' (Park et al., 2011) and 3' end (MacRae et al., 2007) of the shRNA to assist accurate processing. A high level of accuracy can be obtained by insuring that a shRNA has a 2 nt 3' overhang and no 5' overhang. Furthermore, Dicer also recognises the loop region of a shRNA, and a loop that is 2 nt from the Dicer cleavage site results in a near homologous population of siRNA products (Gu et al., 2012). Therefore, exploiting the mechanisms of Dicer shRNA processing by incorporating specific 5', 3' and loop requirements can result in the generation of specific siRNA products. The specific design features of a shRNA for efficient siRNA production makes them an attractive RNAi mimic for the suppression of RVFV genes.

1.6.3.4 RNA interference as a novel strategy to inhibit RVFV

RNA interference (RNAi) employs a unique mechanism of viral inhibition, which may have benefits over the above mentioned therapeutics (section 1.5):

1) RNAi silencing is triggered in the presence of dsRNA that will be processed by the endogenous RNAi machinery, resulting in cleavage and degradation of a target mRNA. This is different from other nucleic acid therapeutics, which inhibit viruses by blocking interactions (aptamers) or through steric hindrance (PMO).

2) Similar to PMOs, RNAi effectors are sequence specific and can target a selected gene.

3) Unlike PMOs or aptamers that have to remain bound to their targets to exert an inhibitory effect, RNAi silencers can perform multiple cleavage reactions of target RNAs, potentially enhancing their potency by a repeatable suppressive effect (Gregory et al., 2005). Furthermore, bound nucleic

acids can be displaced, which will diminish suppression. RNAi effectors degrade the target mRNA, which will result in permanent silencing and an inability of the target to reverse suppression.

4) PMOs (Stein et al., 2008) or small molecule therapeutics (Panchal et al., 2012) require numerous repeat administrations *in vivo* to ensure target inhibition. RNAi silencers can overcome this limitation is by using expressed pri- or pre-miRNA mimics, which would require a single administration with a prolonged therapeutic effect.

5) RNAi can be easily multiplexed with other gene therapies, such as restriction factors or aptamers to enhance its inhibitory potential (section 5.7). This could not only enhance suppression of the intended virus but also potentially broaden the effect to include other viral groups.

6) PMOs are non-specifically taken up into multiple tissues during systemic administration (Amantana et al., 2007). RNAi effectors can be targeted to specific tissues by using delivery vectors (section 5.8.1) and would enhance the safety of RNAi-based therapeutics by delivering effectors only to an organ of interest, preventing off target effects in unaffected tissues.

7) There are no sequence targeted therapeutics for RVFV. Furthermore, apart from Curcumin that may inhibit RVFV through NSs (Narayanan et al., 2012), there is no other therapeutic that targets RVFV's virulence factor, an important determinant of pathogenesis.

1.7 Haemorrhagic fever viruses and therapeutic RNAi

1.7.1 RNAi-based inhibitors of haemorrhagic fever viruses

RNAi-based therapeutics have been developed and extensively tested against many acute and chronic viral infections [Reviewed in (Arbuthnot, 2010)]. Comparatively fewer studies have demonstrated the effectiveness of RNAi against highly pathogenic viral infections and the majority of studies have only determined effectiveness *in vitro* (Table 1.2). Understanding the RNAi effectors that have been used against haemorrhagic fever viruses will put into perspective their current state of development.

1.7.1.1 Arenaviridae

Two studies have demonstrated that RNAi effectors can inhibit arenaviruses, which exemplified the specificity of RNAi-based therapeutics. LASV has a conserved region in the 3' termini that act as promoters for transcription for its bi-segmented ambisense genome [Reviewed in (Emonet et al., 2011)]. SiRNAs were designed to suppress expression of LASV genes by targeting the termini and inhibited several strains in cultured cells as a result of the conserved nature of the target

sites (Muller and Gunther, 2007). A NW arenavirus, Junín virus, was also inhibited with siRNAs targeted to the virulence factor, Z, and effectively reduced viral replication *in vitro* (Artuso et al., 2009) but only a few nucleotide differences in the target site decreased siRNA silencing of related arenaviruses. Overall, siRNAs were able to inhibit arenaviruses and only conserved target sites were subject to RNAi inhibition across strains (Muller and Gunther, 2007).

1.7.1.2 Flaviviridae

Two haemorrhagic fever viruses of the family *Flaviviridae*, YFV and DENV, have been inhibited using RNAi. Firstly, H1-Pol III expressed shRNAs were developed to target regions within YFV's genome and investigated its inhibitory potential in a cerebral infection mouse model, but only a mild therapeutic effect was observed (Pacca et al., 2009). However, the primary target of YFV replication is the liver and meningoencephalitis symptoms are self-limiting [Reviewed in (Monath, 2008)]. Further experimentation will determine the true efficacy of YFV inhibition by RNAi in a relevant context.

DENV has been more broadly characterised with RNAi technologies. A H1-Pol III expressed shRNA targeting a conserved 5' non-coding region (Korrapati et al., 2012) or U6-Pol III expressed shRNA targeting the 3' non-coding region of DENV (Zhang et al., 2004) inhibited replication effectively *in vitro*. Host-dependency factors (HDFs) are host proteins required by the virus to complete its life cycle and can be an alternative RNAi target to facilitate viral inhibition (Brass et al., 2008). Surface receptors and clathrin-dependent endocytosis play an essential role in DENV entry into the host cell. Studies have targeted these HDFs in two primary cells types, hepatocytes (Alhoot et al., 2012) and monocytes (Alhoot et al., 2011), tissues that have been implicated in severe DENV infections. In these tissues, siRNAs were shown to inhibit viral entry, replication and release *in vitro*. Other studies have inhibited DENV replication by siRNA-mediated down-regulation of virus-induced heat shock protein 60 (Hsp60) (Padwad et al., 2009) and various components of endosome trafficking and cytoskeleton network (Ang et al., 2010), increasing the number of potential HDF targets. One of the problems with targeting HDFs is identifying host proteins that are not essential for cellular function. The clathrin-dependent endocytosis targets used in the DENV studies did not have obvious detrimental effects on cultured cells (Alhoot et al., 2012). However, Niemann-Pick C1 (NPC1) has been identified as an important entry receptor for EBOV (Carette et al., 2011) but NPC1 mutations are associated with a fatal metabolic disorder (Carstea et al., 1997), which stresses the importance of identifying non-vital HDFs for RNAi-mediated down-regulation.

There have been investigations into whether RNAi effectors can inhibit DENV *in vivo*. SiRNAs that targeted the 5' conserved non-coding region reduced DENV viral loads and prolong survival of

IFNAR -/- mice. (Stein et al., 2011). TNF-α has been linked to plasma leakage and is a marker of haemorrhagic fever in DENV infections (Chen et al., 2007). Subramanya et al. (2010), targeted both DENV's envelope protein and TNF-α with siRNAs, which reduced viral loads and altered DENV-associated cytokine expression *in vitro* (Subramanya et al., 2010). Furthermore, mice treated with the siRNAs were able to reduce artificially induced TNF-α serum levels but further work will be required to determine the potential benefit of targeting TNF-α in alleviating DENV pathogenesis (Subramanya et al., 2010).

1.7.1.3 Bunyaviridae

Hazara virus in a member of the *Bunyaviridae* family and is a model virus for CCHFV. SiRNAs designed to target the S, M and L segments demonstrated a reduction in target protein levels as well as inhibition of viral replication in a cell culture assay (Flusin et al., 2011). Of note, siRNAs targeted to the S segment were able to inhibit viral replication up to 24 hrs post-infection. Hazara virus is non-pathogenic to humans, so further work will be required to determine whether these results can be recapitulated with CCHFV.

1.7.1.4 Filoviridae

In the *Filoviridae* group, both EBOV and MARV have been targeted with siRNAs but unlike EBOV, the work on MARV has been limited to *in vitro* experimentation. In a cell culture assay MARV's *N*, *VP35* and *VP30* were targeted with siRNAs and resulted in a reduction in detected viral protein markers during MARV replication (Fowler et al, 2005). The most extensive investigation of a potential RNAi-based therapeutic to inhibit a haemorrhagic fever virus is with regards to EBOV. Inhibition of EBOV was confirmed *in vitro* with a pool of siRNAs targeted to different regions in the *L* gene (Geisbert et al., 2006). SiRNAs were then encapsulated in liposomes for use in a guinea pig model. The siRNA complex was administered 1 hr post-infection and repeated daily for six days. Substantial amounts of siRNA accumulated in the liver, an important organ for EBOV infection. The levels of viraemia that normally peak on day 7 were not detectable in the siRNA treated groups and guinea pigs were protected against lethality.

Several additional investigations were performed on the siRNAs before testing in a non-human primate model. This included the selection of alternative siRNA target sites, safety profiling and confirmation of siRNA delivery to EBOV relevant tissues. Alternative siRNAs were identified that down-regulated EBOV's virulence factors, VP24 and VP35 (Table 1.1), and a cocktail of siRNAs targeting *VP24, VP35* and *L* reduced EBOV replication in cultured cells (Geisbert et al., 2010). 2'-*O*-methyl groups were included in both strands of the siRNA duplex (Judge et al., 2006), which

prevented the non-specific immune response observed in the previous study (Geisbert et al., 2006). Furthermore, a high dose in mice did not cause any liver toxicity or affect other blood cells such as white blood cells, neutrophils or lymphocytes. Finally, delivery of the siRNAs to myeloid and liver cells was confirmed in mice, as these cell types are important for EBOV pathogenesis.

Rhesus macaques were infected with the estimated infective dose of accidental exposure of a laboratory worker (Geisbert et al., 2010). Treatment was initiated 30 min post-infection and two regimens were used either four treatments on day 1, 3 and 5, or seven treatments given daily for 7 days. One of the three macaques that received 3 treatments died, whereas all four of the non-human primates treated daily survived. All control animals succumbed to infection. Animals that survived had negligible levels of EBOV replication ($<1x10^{2.2}$ pfu/ml) but the treated animal and control animals that died, demonstrated uncontrolled, high levels of viraemia exceeding $1x10^{6.7}$ pfu/ml. This demonstrated that a high treatment regimen of siRNAs controlled EBOV administered post-infection in a non-human primate model. This study is the only non-human primate experiment that has controlled EBOV infection post-exposure using antisense technology and demonstrated the potential for RNAi to treat highly pathogenic viruses.

Table 1.2: RNAi effectors targeted to haemorrhagic fever viruses

Virus[1]	RNAi effector	Target gene[2]	Model	Reference
LASV	siRNA	GPC, N, L and Z	*In vitro* (Vero cells)	(Muller and Gunther, 2007)
NW arenavirus	siRNA	Z	*In vitro* (Vero cells)	(Artuso et al., 2009)
DENV	shRNA	3' and 5' non-coding regions	*In vitro* (Vero and dendritic cells)	(Korrapati et al., 2012) (Zhang et al., 2004)
DENV	siRNA	Host dependency factors	*In vitro* (Monocyte, HepG2, Huh7, U937 cells)	(Alhoot et al., 2012) (Alhoot et al., 2011) (Padwad et al., 2009) (Ang et al., 2010)
DENV	siRNA	5' non-coding region, E and TNF-α	*In vivo* (Mouse)	(Stein et al., 2011) (Subramanya et al., 2010)
YFV	shRNA	NS1 and E	*In vivo* (Mouse)	(Pacca et al., 2009)
Hazara	siRNA	L, glycoproteins and N	*In vitro* (A549 cells)	(Flusin et al., 2011)
MARV	siRNA	N, VP35 and VP30	*In vitro* (Vero cells)	(Fowler et al, 2005)
EBOV	siRNA	L, VP24 and VP35	*In vivo* (Mouse, guinea pigs, Rhesus macaques)	(Geisbert et al., 2006) (Geisbert et al., 2010)

1) Abbreviation of virus names: DENV=Dengue fever virus, LASV=Lassa virus, EBOV=Ebola virus, MARV=Marburg virus, NW=New World, OW=Old World, YFV=Yellow fever virus.
2) Abbreviation of virus genes: E=Envelope, GPC=Glycoprotein, N=Nucleocapsid, L=L polymerase, TNF-α=Tumor necrosis factor α, VP=Viral protein, Z=Z matrix protein.

1.7.2 RNAi as a treatment for highly pathogenic viruses

Although the EBOV studies mentioned above (section 1.7.1.4) have demonstrated a 'proof-of-concept' that RNAi effectors can inhibit haemorrhagic fever viruses *in vivo*, there are still many questions whether this will function within a clinically relevant setting. EBOV was inhibited within 30 min post-infection in non-human primates and 100% protection was achieved when administered daily (Geisbert et al., 2010). Although this may present an intervention strategy in a controlled setting, like accidental exposure to a laboratory worker, it will not be practical in the clinic. Several features of highly pathogenic viruses present hurdles to the feasibility of therapeutic RNAi.

1.7.2.1 Treatment window

In a clinical setting, therapeutic intervention will be applied at the point of diagnosis, which would generally occur at symptom presentation. With haemorrhagic fever viruses, there is an incubation period of between 3-14 days [Reviewed in (Paessler and Walker, 2012)]. Once symptoms of illness are present, they can progress to more complicated forms such as a haemorrhagic fever. Upon onset of a haemorrhagic fever, time to death is generally rapid occurring within 3-9 days. In the case of RVFV, most deaths in humans occur between 3-6 days after onset of severe symptoms, however longer than 12 days has been described (Kahlon et al., 2010). Importantly, the manifestation of haemorrhagic fever in RVF is clinically important as it is associated with the highest incidence of lethality and is the most likely scenario for the application of treatment.

RVF disease progression has been better characterised in controlled studies using animal models. Rhesus macaques have similar susceptibility as humans to RVFV infection (Peters et al., 1989). In severe lethal cases, symptoms were present on day 2-4 with lethality noted as soon as day 8 (Morrill et al., 1990). RVFV infection in a more susceptible primate model using marmosets, presented with disease symptoms similar to a severe haemorrhagic fever infection in humans (Smith et al., 2011). When infected intranasally, high levels of detectable viraemia appeared on day 2 with deaths as soon as day 9 and intranasal exposure is a potential route in humans when working with infected blood and tissues of slaughtered ruminants.

The rapid progress to death upon presentation of severe infections offers a small window for therapeutic intervention. Unlike small molecule therapies that can diffuse readily into the cell and

exert an inhibitory effect, RNAi effectors require processing through the RNAi pathway to down-regulate a target. For expressed RNAi effectors, such as shRNAs, the appearance of inhibitory effects can take longer with down-regulation of a protein occurring within a two week period (Grimm et al., 2006). Furthermore, RNAi does not affect matured proteins and there is a lag before these proteins are degraded through protein turnover. Mature viral proteins may be able to still facilitate viral replication for a period before their down-regulation. Therefore, in clinically relevant complicated cases, the therapeutic window is small and the 'delay' in the RNAi therapeutic effect may not be quick enough to prevent mortality.

1.7.2.2 High viral loads

Symptom presentation often correlates with high viral loads. In lethal cases of RVFV in a rhesus macaque model, viral loads can reach as high as $>1x10^{6.7}$ pfu/ml in serum on day 2 and are maintained at high levels until death (Morner et al., 1999). In the severe disease marmoset model, viral loads can reach as high as $1x10^{7}$ pfu/ml by day 2 when infected intranasally (Smith et al., 2011). As symptom presentation is the most likely point of therapeutic intervention, the high viral load in severe RVFV cases pose a challenge to treatment and may overwhelm the therapeutic effect of RNAi.

There is a clear difference in animals that succumb to lethality during RVFV infections versus animals that survive. In rodents, lethal cases have uncontrolled viral levels of $>1.0x10^{7}$ pfu eq/ml, whereas non-lethal cases remain at $<2.7x10^{4}$ pfu eq/ml until cleared (Bird et al., 2008). In the EBOV studies, siRNA treated non-human primates that survived had viral loads of $\sim1x10^{2.2}$ pfu/ml but in the treated lethal cases rose to around $1x10^{6.7}$ pfu/ml (Geisbert et al., 2010). In the animals that do not progress to lethality, there is a gradual decrease in viraemia with the appearance of anti-RVFV antibodies (Morrill et al., 1990). This suggests that controlling viraemia by a therapeutic is essential to the prevention of RVFV lethality, until induction of a significant adaptive immune response. This differs from the treatment of chronic infections, which are geared towards complete viral suppression. Therefore, in the case of highly pathogenic viruses, treatment would be required to keep viral loads below a "therapeutic threshold". This may play in favour of RNAi therapeutics, which would only be required to "manage" high viral loads, until the host can facilitate viral clearance.

1.7.2.3 Viral tissue dissemination

An important point of concern is tissue dissemination of haemorrhagic fever viruses during infection. Treatment of chronic infections generally requires the targeting of a specific tissue type,

such as T-cells in HIV or hepatocytes in HBV infections. Haemorrhagic fever viruses generally have an initial site of replication that later disseminates to numerous organs [Reviewed in (Paessler and Walker, 2012)]. The liver is an important organ in RVFV infection but the presence of virus in brain tissue is associated with severe neural disease in non-human primates (Morrill et al., 1990). This can manifest in humans as neural symptoms such as confusion and delirium (Kahlon et al., 2010) and viral resurgence in the brain can cause a loss of vision (Madani et al., 2003). The incidence of neural complications may also be higher through aerosol contamination, which has been demonstrated in non-human primates (Smith et al., 2011) and mice (Reed et al., 2013). In mice infected with RVFV, dissemination to other organs, such as the brain, lung and kidney, can occur as early as day 2 (Gowen et al., 2013) with similar observations with an arenavirus in guinea pigs by day 4 (Mendenhall et al., 2011) and suggests that several organs will be infected at the point of treatment.

RNAi effectors are generally targeted to specific organs using delivery vectors. In the EBOV studies, siRNA uptake was present at high levels in the liver, spleen and lungs but low levels in the brain (Geisbert et al., 2006). Therefore, RNAi therapeutics targeted to the liver may not be able to prevent pathologies in other organs. In the case of RVFV, the overall selective delivery of RNAi to the liver may be able to facilitate patient recovery, but poorer delivery to the brain may not be able to prevent delayed neurological resurgence. The above concerns need to be addressed in a series of extensive post-exposure assays to determine whether RNAi can be used as a therapeutic strategy for viral haemorrhagic fevers in clinically relevant scenarios.

1.8 Safety of RNAi-based therapeutics

1.8.1 Sequence-dependent off target effects

As previously mentioned, the seed region of a miRNA is important for its ability to suppress numerous mRNA targets (Lewis et al., 2003) and as a result of the limited number of nucleotide interactions, miRNAs can have a wide range of inhibitory effects on host cell mRNAs (Lewis et al., 2005). Therefore, therapeutic RNAi mimics can affect unintended host mRNA targets through non-specific seed region interactions, which should be reduced prior to clinical application. Design features can be included to enhance RNAi specificity, which includes modification of the seed region with G:U wobble bases (Ui-Tei et al., 2012). This will only be effective if consistent siRNA products with define seed regions are produced through accurate RNAi processing.

As discussed above (section 1.6.3.3), Dicer can recognise the 5' (Park et al., 2011), 3' (MacRae et al., 2007) and loop regions (Gu et al., 2012) of a shRNA to facilitate accurate processing and obtain a near homologous population of siRNAs (97%). Long stem lengths (>21nt) should be

avoided because of variable Dicer processing as well as increased silencing by the passenger strand (Gu et al., 2012). The selection of the passenger strand into RISC can be reduced by increasing its 5' thermodynamic stability (Khvorova et al., 2003, Schwarz et al., 2003). Inserting multiple internal mismatches within the passenger strand can also reduce its activity (Wu et al., 2011b) or using perfect complementary strands instead results in Ago2-mediated cleavage and degradation of the passenger strand preventing its incorporation into RISC (Gu et al., 2011). Furthermore, perfectly complementary siRNAs were inactive when incorporated in other Ago (1,3 and 4) proteins (Gu et al., 2011). Overall, this suggests that complementary duplexes will load guide strands specifically into Ago2, preventing off targeting through the passenger strand and other Ago proteins.

To ensure that the loaded guide sequence does not target host mRNAs, several bioinformatic approaches have been developed. Software programs screen guide sequences with genome-wide filters to prevent host targeting (Taxman et al., 2013) and some have validated the selection criteria using *in vitro* experimentation (Das et al., 2012). A Basic Local Alignment Search Tool (BLAST) can also be performed to ensure that there is no obvious high complementarity of the guide strand to host mRNAs that could potentially result in potent suppression through Ago2-mediated cleavage (Altschul et al., 1990). This increased stringency would help prevent off target effects of the intended guide strand. Furthermore, some intrinsic features of miRNA targeting that would help prevent non-specific effects. The seed region requires ≥2 target sites to effectively suppress a mRNA and these targets sites have to be positioned close to one another (<19bp) (Broderick et al., 2011). As an RNAi mimic would generally be imperfectly bound to host mRNAs, these "requirements" could reduce the likelihood of sequence-dependent off target effects.

1.8.2 Non-specific immune activation

DsRNA is a potent stimulator of innate cellular immunity [Reviewed in (Haller et al., 2006)]. SiRNA duplexes can elicit a broad non-specific IFN response, which would result in unwanted toxicity (Sledz et al., 2003). This was as a result of RIG-1 recognition of blunt siRNAs (Marques et al., 2006) and siRNAs with the characteristic 3' 2 nt overhang, which is recognised by Dicer and Ago, should be employed to prevent non-specific innate responses (Ma et al., 2004). Non-specific immunity can also be avoided by chemical modification of the siRNAs with 2'-*O*-methyl groups (Judge et al., 2006), which prevent immune activation *in vivo* (Geisbert et al., 2010). ShRNAs do not elicit non-specific immunity compared to siRNAs (Robbins et al., 2006), which has been noted by others (Aarbiou et al., 2012). Expression of shRNAs from the nucleus may avoid recognition by endosomal molecules like TLRs (section 1.3.1.1), which can detect siRNAs (Cho et al., 2009). The coupling of shRNA processing

by the RNAi machinery may mask immune activation markers from cytoplasmic RNA detectors. Ultimately, the potential immune stimulatory potential of therapeutic RNAi effectors needs to be address prior to clinical application.

1.8.3 Endogenous miRNA disruption

In recent years, overexpression of RNAi therapeutics has observable toxic effects in the liver (Suhy et al., 2012), neuron cells (Martin et al., 2011) and cardiac cells (Bish et al., 2011) even resulting in lethality in mice (Grimm et al., 2006). Further investigation identified two main points of saturation, namely exportin-5 and Ago2 (Grimm et al., 2010). ShRNAs are particularly susceptible to oversaturation of the endogenous RNAi pathway. Studies have demonstrated that constitutive expression of shRNAs caused a build-up of pre-miRNA mimics, which eventually resulted in toxicity (Ahn et al., 2011). Toxicity would be because of the disrupted processing of endogenous miRNAs, which would be unable to continue through the RNAi pathway to regulate their respective mRNAs.

ShRNAs can have a deleterious effect on the miRNA pathways as a result of overexpression from potent Pol III promoters (i.e. U6-Pol III). Lowering the expression levels of a shRNA can help prevent unwanted toxic effects. This has been achieved by re-engineering a potent U6 promoter to express shRNAs at lower levels (Suhy et al., 2012) or using weaker Pol III promoters such as H1 or 7SK (Grimm et al., 2010). A U1-pol II promoter can also be used, which expresses shRNAs at 25% the level of a U6-Pol III promoter (Denti et al., 2004). ShRNAs can also be engineered to be inducible, which will only express the shRNA in the presence of the exogenously administered drug, doxycycline (van de Wetering et al., 2003) and can temporally control expression *in vivo* for the required therapeutic period (Czauderna et al., 2003). Other studies have used a liver-specific Pol II promoter, which prevented the toxicity observed when using a U6-Pol III promoter (Giering et al., 2008).

1.9 Thesis objectives

It has been demonstrated that RNAi-based therapeutics hold potential for the treatment of haemorrhagic fever viruses. As a result of the lack of available therapeutics or vaccines, the ability of RNAi-based therapeutics to inhibit genes of RVFV needs to be assessed. The objective of this thesis was to develop expressed shRNAs to target RVFV's genome, which would provide a potential RNAi-based therapeutic platform against RVFV. The investigation sought to show that the shRNAs could reduce viral protein levels through RNAi-mediated cleavage of target mRNAs. Furthermore, it was determined whether this translated into the inhibition of RVFV replication in cultured cells as well as

the alleviation of the pathogenic effects of the virulence factor, NSs. Ultimately, this work intends to validate a novel method for RVF disease intervention and further the knowledge that RNAi-based therapeutics hold potential to treat highly pathogenic viruses.

Specific objectives of this thesis were:

1. Design a series of shRNAs targeted to conserved regions within the RVFV genome.
2. Assess the ability of the shRNAs to knockdown target genes of RVFV.
3. Determine whether the shRNAs decrease target protein levels.
4. Investigate the shRNA's ability to alleviate the pathogenic effects of NSs.
5. Determine the ability of the shRNAs to inhibit RVFV replication.

Chapter 2

Materials and methods

2.1 Generation of Pol III expressed shRNA constructs

U6-Pol III expressed shRNAs were constructed using a one-step PCR (Figure 2.1). The template used in the PCR was pTZ-U6+1, a generous gift from Daniela Castanotto (Castanotto et al., 2002), which contains a U6 small nuclear RNA (snRNA) Pol III promoter (Kunkel and Pederson, 1988). A universal U6 forward (U6-F) was used in all U6+1 promoter reactions and binds to the 5' end of the U6-Pol III promoter (Table 2.1). The reverse oligonucleotide primers have a 22 nt region complementary to the 3' end of a U6-Pol III promoter. The reverse oligonucleotides contained a 19 nt sense, a loop region and a complementary 19 nt antisense region (Table 2.1). Six thymidine nucleotides were inserted at the terminus of the oligonucleotide to act as a Pol III promoter termination signal. PCR sequences were amplified in a Mastercycler® (BioRad, CA, USA) using Promega Master Mix [100 ng of pTZ-U6+1 template, master mix buffer (pH 8.5), 200 µM dNTPs, 1.5 mM $MgCl_2$, 25 U/ml of *Taq* polymerase] (Promega, WI, USA). Thermocycling conditions were as follows: initial denaturation at 94°C for 5 minutes (min), denaturation 94°C for 30 seconds (sec), annealing 55°C for 30 sec, extension 72°C for 30 sec for 34 cycles. A final extension for 10 min at 72°C for one cycle was included to ensure addition of adenosine overhangs for subsequent cloning steps. PCR products were loaded into a 1% agarose gel and resolved using electrophoresis run in TAE buffer (Tris-acetate-EDTA; 40 mM Tris-base, 1.74 M glacial acetic acid, 1 mM EDTA), then visualised using ethidium bromide (0.2% w/v) staining and UV light.

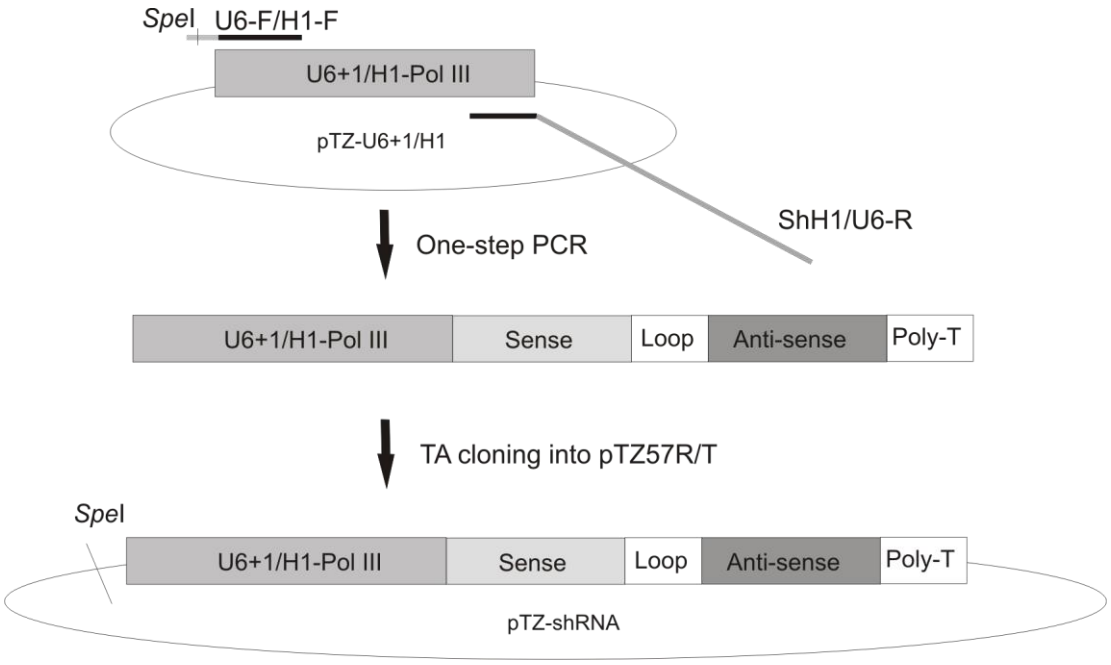

Figure 2.1: Diagram of the PCR cloning strategy for generating Pol III shRNA cassettes. ShRNAs were amplified from a pTZ-Pol III promoter template using a universal Pol III forward primer and a shRNA reverse primer in a once-step PCR. PCR products were subsequently ligated into a pTZ57R/T backbone. To multimerise the shRNAs, a *Spe*I site was included in the H1-Pol III forward primer.

Table 2.1: Oligonucleotide primers used to generate the U6-pol III expressed shRNAs

Primer	Sequence (5'-3')[1]	Length (nt)	Target region (nt)
U6-F	GATCGGGCCCATCGACAAGGTCGGGCAGGAAGAGGCCCT	39	N/A
ShU6-NSs1	AAAAAAGCGGACATTTCTAATGCTG*TCTCTTGAA*CAGCATT AGAAATGTCCT**CGGTGTTTCGTCCTTTCCACAA**	74	506-525
ShU6-NSs2	AAAAAAGGCTTACACAGGATGATAG*TCTCTTGAA*CTATCA TCCTGTGTAAGC**CGGTGTTTCGTCCTTTCCACAA**	74	481-500
ShU6-NSs3	AAAAAAGCCACTCTAGCAATGAGGA*TCTCTTGAA*TCCTCAT TGCTAGAGTGG**CGGTGTTTCGTCCTTTCCACAA**	74	424-443
ShU6-NSs4	AAAAAAGTCCTAGTCACGAGGTTCG*TCTCTTGAA*CGAACC TCGTGACTAGGA**CGGTGTTTCGTCCTTTCCACAA**	74	140-159
ShU6-NSs5	AAAAAAGGATACCTTATTCTATGGT*TCTCTTGAA*ACCATAG AATAAGGTATC**CGGTGTTTCGTCCTTTCCACAA**	74	89-108

ShU6-NSs6	AAAAAAGATGGTCCTCCCAGGATAC*TCTCTTGAA*GCATCC TGGGAGGACCAT**CGGTGTTTCGTCCTTTCCACAA**	74	76-95
ShU6-NSs7	AAAAAAGTTGTGTCAGTGGAGTACT*ACTCTTGAT*AGTACT CCACTGACACAA**CGGTGTTTCGTCCTTTCCACAA**	74	49-68
ShU6-NSs8	AAAAAAGCACCATCGTCCTAGTCAC*TCTCTTGAA*GTGACTA GGACGATGGTG**CGGTGTTTCGTCCTTTCCACAA**	74	132-151
ShU6-NSs9	AAAAAAGCCATATCCTGGCCTCTTG*TCTCTTGAA*CAAGAG GCCAGGATATGG**CGGTGTTTCGTCCTTTCCACAA**	74	328-347
ShU6-NSs10	AAAAAAGTCGGAGAATTCCCATACC*TCTCTTGAA*GATATG GGAATTCTCCGA**CGGTGTTTCGTCCTTTCCACAA**	74	184-203
ShU6-N1	AAAAAAGTCAAGCAGTGGACCGCAA*TCTCTTGG*ACTACAG TCCACTGCTTGA**CGGTGTTTCGTCCTTTCCACAA**	74	38-57
ShU6-N2	AAAAAAGCAGCCAATGAATGCAGCA*TCTCTTG*AATACCGC ATTCATTGGCTG**CGGTGTTTCGTCCTTTCCACAA**	74	591-610
ShU6-N3	AAAAAAGTTATAAGCCATGAGAAGAG*TCTCTTG*AACCCTC CTCATGGCTTATAA**CGGTGTTTCGTCCTTTCCACAA**	76	622-641
ShU6-N4	AAAAAAGCTCTCTGTATCTGCTGCAG*TCTCTT*GAACCACAA CAGATACAGAGAG**CGGTGTTTCGTCCTTTCCACAA**	76	506-525
M13-F	GTAAAACGACGGCCAG	16	-
M13-R	CAGGAAACAGCTATGAC	17	-

[1]Bold sequences indicate regions complementary to the 3' region of the U6-Pol III promoter. Italised sequences represent the loop region. In the U6-F primer, the grey region is an *Apa*I restriction site.

Bands corresponding to the correct molecular weight were cloned into pTZ57R/T through an InsTAclone™ PCR cloning kit protocol (Fermentas, WI, USA). A 3:1 molar ratio of insert:backbone was used in the ligation reaction [5 U of T4 DNA ligase in ligase buffer, 40 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP (pH 7.8)]. Eight microliters of the ligation was mixed with chemically-

competent DH5α *E.coli* (DH5α) (Appendices A1.1), incubated on ice for 30 min then heat-shocked at 42°C for 90 sec (Appendices A1.2). Transformed cells were then spread onto Luria Bertani (LB) broth agar plates containing 1 mg/ml ampicillin, X-gal [5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside] (Sigma, MO, USA) and IPTG [Isopropyl-β-D-1-thiogalactopyranoside; Appendices A1.2] (Sigma, MO, USA). The plates were incubated overnight at 37°C.

White bacterial colonies were selected to be screened for shRNA inserts. Colonies were cultured in 5 ml of LB broth overnight at 37°C. The plasmid DNA was extracted using a standard miniprep protocol (Appendices A1.3). Plasmids were screened for correct inserts through a *Kpn*I and *Hind*III digest (Fermentas, WI, USA). If the molecular weight of the band corresponded to the expected size, a further *Apa*I digest (Fermentas, WI, USA) was performed to determine the shRNA's orientation. The inserts in the reverse orientation were selected for use in further studies, as this was required for insertion into multiple expressed shRNA cassettes. ShRNAs were confirmed using automated sequencing with M13 forward (M13-F) and M13 reverse (M13-R) sequencing primers (Inqaba Biotech, South Africa; Table 2.1).

H1-Pol III expressed shRNA constructs were generated with the same one-step PCR protocol used to generate the U6-Pol III shRNAs (Figure 2.1). The template used in the PCR was a pTZ-H1 containing a human snRNA H1-Pol III promoter (Myslinski et al., 2001). A universal H1 forward (H1-F) primer that binds to the 5' end of a H1-Pol III promoter was used in all H1 template reactions (Table 2.2). The reverse oligonucleotides primers overlap by 20 nt at the 3' end of H1-Pol III promoter and contained a shRNA sequence with the six thymidine nucleotides required for Pol III termination. The PCR conditions, subsequent cloning steps and confirmation of inserts were the same as described for the U6-Pol III expressed shRNAs.

Table 2.2: Oligonucleotide primers used to generate the H1-pol III expressed shRNAs

Primer	Sequence (5'-3')[1]	Length (nt)	Target regions (nt)
H1-F	GATCGAATTCACTAGTGAACGCTGACGTCATCAA	34	N/A
ShH1-NSs4	AAAAAAGTCCTAGTCACGAGGTTCG*TCTCTTG*AACGAA CCTCGTGACTAGGAC**GGATCCGAGTGGTCTCATAC**	73	See Table 2.1
ShH1-NSs6	AAAAAAGATGGTCCTCCCAGGATAC*TCTCTTG*AAGCAT CCTGGGAGGACCATC**GGATCCGAGTGGTCTCATAC**	73	See Table 2.1
ShH1-NSs7	AAAAAAGTTGTGTCAGTGGAGTACT*ACTCTTGA*TAGTA	73	See Table 2.1

	CTCCACTGACACAAC**GGATCCGAGTGGTCTCATAC**		
ShH1-N1	AAAAAAGTCAAGCAGTGGACCGCAA*TCTCTTGG*ACTAC AGTCCACTGCTTGAC**GGATCCGAGTGGTCTCATAC**	73	See Table 2.1
ShH1-N3	AAAAAATTATAAGCCATGAGAAGAG*TCTCTTGC*TCCCTC CTCATGGCTTATAAC**GGATCCGAGTGGTCTCATAC**	74	See Table 2.1
ShH1-N4	AAAAAAGCTCTCTGTATCTGCTGCAG*TCTCTTGA*ACCAC AACAGATACAGAGAGC**GGATCCGAGTGGTCTCATAC**	75	See Table 2.1
ShH1-N5	AAAAAATGGCTGGACATGCCAGGCT*TCTCTTGCT*AACC TAGCATGTCCAGCCAC**GGATCCGAGTGGTCTCATAC**	74	335-354
ShH1-N6	AAAAAATTGACTCTATCACGAGTTGC*TCTCTTGCA*ACAA CTCATGATAGAGTCAAC**GGATCCGAGTGGTCTCATAC**	76	303-322
ShH1-N7	AAAAAAGCAATGAGATTGAACAGTG*TCTCTTGCA*CACT ATTCAATCTCATTGCC**GGATCCGAGTGGTCTCATAC**	74	52-71
ShH1-N8	AAAAAAGCTGCAACGTTCACGCAGC*TCTCTTGCA*ACTA CGTGAACGTTGCAGCC**GGATCCGAGTGGTCTCATAC**	74	576-795
ShH1-M1	AAAAAAGGCTGATCCACCTAGCTGT*TCTCTTGCAA*CAG CTAGGTGGATCAGCCC**GGATCCGAGTGGTCTCATAC**	74	669-688
ShH1-M2	AAAAAAGACTACCAGTCAGCTCATT*AGTCTTGCA*ATGA GCTGACTGGTAGTCC**GGATCCGAGTGGTCTCATAC**	73	757-775
ShH1-M3	AAAAAAGTCAAGTGAGGATGATGGA*TCTCTTGAA*TCCA TCATCCTCACTTGACC**GGATCCGAGTGGTCTCATAC**	74	916-935
ShH1-M4	AAAAAACCTATTGTTCACATGCTAA*TCTCTTGAA*TTAGC ATGTGAACAATAGGC**GGATCCGAGTGGTCTCATAC**	74	1295-1314
ShH1-M5	AAAAAAGAGATTACACTCAAGTATC*TCTCTTGAA*GATA CTTGAGTGTAATCTCC**GGATCCGAGTGGTCTCATAC**	74	1561-1580

[1]Bold sequences indicate regions complementary to the 3' region of the H1-Pol III promoter. Italised sequences represent the loop region. In the H1-F primer, the underline and greyed sequences are *Eco*RI and *Spe*I restriction sites, respectively.

The generation of multimers is illustrated in the appendices (Figure A.2). To generate a multiple expressed shRNA, a *Spe*I site was included in the H1-F primer (Figure 2.1). A shRNA insert was generated when a shRNA cassette was digested with *Sac*I and *Spe*I [2µg of shRNA plasmid with 5 U of each restriction enzyme in Tango™ buffer, 33 mM Tris-acetate (pH 7.9), 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/ml BSA] and incubated for 1 hr at 37°C (Fermentas, WI, USA). A backbone shRNA cassette was generated through digestion with *Sac*I and *Xba*I (Fermentas, WI, USA; according to conditions used for the *Sac*I and *Spe*I digest described above). Both backbone and insert was resolved using agarose gel (1%) electrophoresis and extracted using a phenol:chloroform method (Appendices A1.5). The *Spe*I and *Xba*I digestions generate complementary overhangs, which will allow for a shRNA insert to be ligated into the shRNA backbone, then inserted into the DH5αs (Appendices A1.1 and A1.2) and spread onto LB plates containing ampicillin. The DNA was extracted and confirmed through a *Kpn*I and *Hind*III digest (Appendices A1.3). The process was repeated with shRNA cassettes containing two shRNAs, to generate multimers that contained four shRNAs in a single construct. Molecular weights of the multimers were confirmed through a *Kpn*I and *Hind*III digest (Appendices Figure A.2) and automated sequencing (Inqaba Biotech, South Africa). To generate trimers, a single shRNA insert was ligated into a backbone with two shRNAs. Single and multimeric shRNA constructs that were used in further tissue culture experiments were grown in 100 ml of LB broth containing ampicillin overnight at 37°C and extracted using a Qiagen Plasmid Midiprep kit (Qiagen, CA, USA; Appendices A1.4)

2.2 Cloning of full length and minimal dual-luciferase reporter targets

To generate reporter targets, dual-luciferase vectors were constructed containing *NSs, N* and *M* genes. Target genes were inserted into a pSI-CHECK 2.2 vector (Promega, WI, USA) within the 3' untranslated region (UTR) of a SV40 expressed *Renilla* luciferase (Figure 2.2). This will result in expression of a single fusion mRNA species containing both a *Renilla* luciferase and the RVFV target gene of interest. In the presence of an effective anti-RVFV shRNA, the guide strand will cleave the target mRNA resulting in its degradation, which will cause a reduction in *Renilla* luciferase expression. *Renilla* luciferase activity was normalised to a constitutively HSV TK expressed Firefly luciferase.

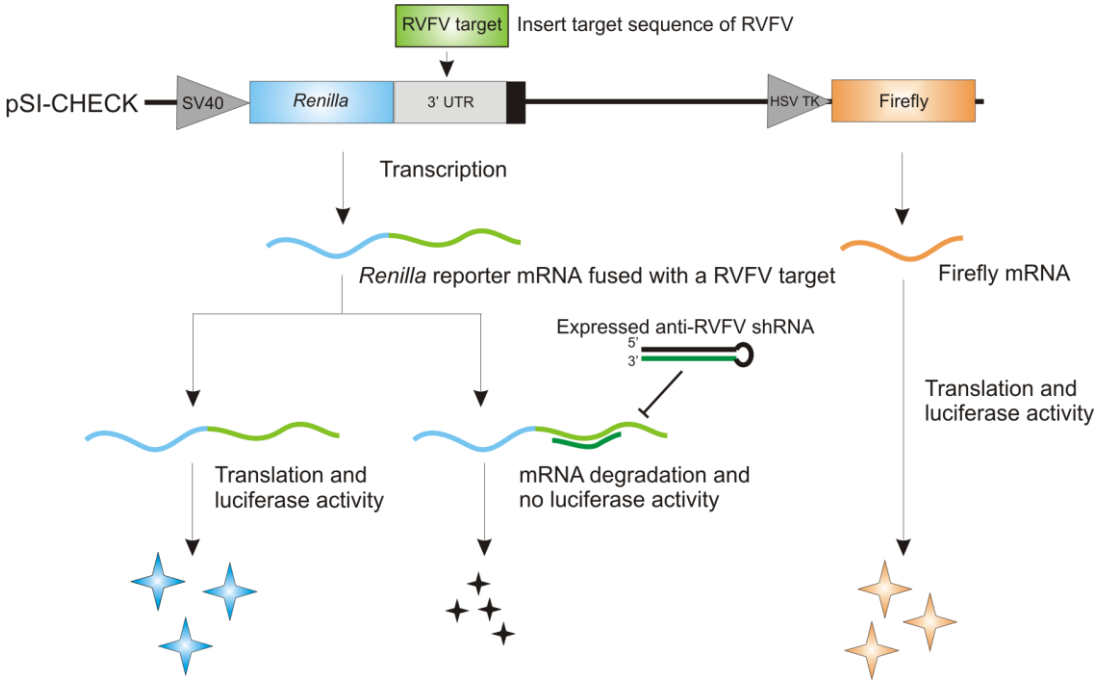

Figure 2.2: Diagram of the dual-luciferase fusion reporter assay. A RVFV target sequence was inserted into the 3' UTR of *Renilla* luciferase and expressed as a fusion *Renilla*:target mRNA. When an anti-RVFV shRNA was expressed in the presence of a fusion reporter, the target RVFV mRNA was degraded resulting in reduced *Renilla* luciferase activity. *Renilla* luciferase activity was normalised to a constitutively expressed Firefly luciferase background control.

Several strains of RVFV were employed in this study and used as described in the results (Table 2.3). RVFV genes were amplified from M and S segments of RVFV cDNA, kindly provided for by Prof. Janusz Paweska (NICD, South Africa). Briefly, an S segment cDNA was generated from RNA extracted from supernatant of RVFV-infected Vero cells. M segment RNA was extracted from reconstituted freeze-dried mouse brain infected with RVFV. The RNA extraction was performed using a QIAamp viral RNA kit according to the manufacturers' instructions (Qiagen, CA, USA). Viral RNA was converted into cDNA using SuperScript® III first-strand synthesis system for RT-PCR (Invitrogen, CA, USA).

Table 2.3: Strains of RVFV genes

Strain	Origin	Year of identification	Accession number
RVFV-825/79	Zimbabwe	1979	EU312131
RVFV-Ar21229	Saudi Arabia	2000	EU312115
RVFV-Tambul	Egypt	1994	EU312110
RVFV-B1143	Kenya	1977	EU312119
RVFV-CAR1662	Central African Republic	1985	EU312122
RVFV-52/99/1	South Africa	1999	EU312127
RVFV-Smithburn	Uganda (vaccine strain)	1955	EU312129
RVFV-Lunyo	Uganda	1955	EU312121
RVFV-ZH548	Egypt	1977	EU312138
RVFV-ZH501	Egypt	1977	EU312137

NSs and *N* genes were PCR amplified in a Mastercycler® (BioRad, CA, USA) using Promega Master Mix [100 ng of S segment cDNA template, master mix buffer (pH 8.5), 200 µM dNTPs, 1.5 mM $MgCl_2$, 25 U/ml of *Taq* polymerase] (Promega, WI, USA). S segment cDNA was used as a template in the PCR. Either NSs forward and reverse primers (NSs-F and NSs-R) or N forward and reverse primers (N-F and N-R) were used to amplify *NSs* and *N,* respectively (Table 2.4). Thermocycling conditions were as follows: initial denaturation at 94°C for 5 min, denaturation 94°C for 4 sec, annealing 60°C for 30 sec, extension 72°C for 30 sec for 34 cycles, with a final extension of 10 min at 72°C for 2 cycles.

M genes were PCR amplified in a Mastercycler® (BioRad, CA, USA) using high fidelity *Taq* polymerase [100 ng of M segment cDNA template, with high fidelity PCR buffer, 1.5 mM $MgCl_2$, 200 mM dNTPs, 2.5 U of high fidelity *Taq* polymerase] (Fermentas, WI, USA). M segment cDNA was used as a template in the PCR. The M forward (M-F) or M reverse (M-R) primers were included in the reaction mix (Table 2.4). Thermocycling conditions for *M* amplification was as follows: initial

denaturation at 94°C for 5 min, denaturation 94°C for 30 sec, annealing 60°C for 1 min, extension 72°C for 1 min for 34 cycles, with a final extension of 10 min at 72°C for 2 cycles.

The PCR amplified *NSs*, *N and M* genes were sub-cloned into pTZ57R/T using a InsTAclone™ PCR cloning kit protocol according to the manufacturers' instructions (Fermentas, WI, USA). The ligated constructs were inserted into DH5αs and the transformed bacteria were grown overnight on LB agar plates containing 1 mg/ml ampicillin (Appendices A1.1 and A1.2). PTZ-*NSs* and pTZ-*N* DH5αs were grown at 37°C. The pTZ-*M* DH5αs were incubated at 30°C. Plasmid DNA was extracted and confirmed using automated sequencing (Appendices A1.1) (Inqaba Biotech, South Africa).

NSs, *N* and *M* genes were prepared for cloning into pSI-CHECK 2.2 by digestion with *Xho*I and *Not*I [2 µg of pTZ-*NSs/N/M* with 5 U of each restriction enzyme in Buffer O, 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM NaCl, 0.1 mg/ml BSA]. The digests were separated using agarose gel (1%) electrophoresis and bands corresponding to the correct molecular weight were gel extracted (Appendices A1.5).

To prepare a pSI-CHECK 2.2 vector for insertion of the target genes, a pSI-CHECK 2.2 vector was digested with *Xho*I and *Not*I and incubated for 1 hr at 37°C (Fermentas, WI, USA). The digested vector was resolved using agarose gel (1%) electrophoresis and gel extracted (Appendices A1.5). *NSs*, *N* and *M* genes were ligated in the pSI-CHECK 2.2 vector at a 3:1 molar ratio [5 U of T4 DNA ligase in ligase buffer, 40 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP (pH 7.8)] (Fermentas, WI, USA) and incubated for 1hr at 22°C. The ligation mix was inserted into DH5αs, spread on LB plates containing ampicillin and incubated overnight at 37°C (Appendices A1.1 and A1.2). The pSI-CHECK vector containing the *M* gene was incubated overnight at 30°C. The extracted DNA was screened for inserts through *Xho*I and *Not*I digestion (Fermentas, WI, USA) and resolved using agarose gel (1%) electrophoresis (Appendices A1.3). The reporter vectors were confirmed using automated sequencing (Inqaba Biotech, South Africa). The sequencing primer used was specific for the 3' region of *Renilla* luciferase (R-Luc; Table 2.4).

To generate pSI-CHECK 2.2 targets containing only a shRNA target sequence (minimal targets), partially complementary oligonucleotides were treated with T4 polynucleotide kinase (PNK) [10 µM of oligonucleotide with 5 U of PNK enzyme in kinase buffer, 40 mM Tris-HCl (pH7.5), 10 mM $MgCl_2$, 5 mM DTT] and incubated for 1 hr at 37°C (Promega, WI, USA) (Table 2.4). The complementary oligonucleotides were then mixed together in equal proportions and PNK was inactivated at 75°C for 10 min. The oligonucleotides were annealed by slowly cooling the mixture to room temperature. The dsDNA mixture was then diluted down to 200 nM and ligated into a *Xho*I and *Not*I digested pSI-CHECK 2.2 [50 ng of pSI-CHECK 2.2 with 5 U of T4 DNA ligase in ligase buffer, 40 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP (pH 7.8)] . The ligations were inserted into

DH5αs and incubated overnight at 37°C (Appendices A1.1 and A1.2). Plasmid DNA was extracted and verified using automated sequencing (Inqaba Biotech, South Africa) (Appendices A1.3). The pSI-CHECK reporter vectors used in tissue culture experiments were prepared and extracted using a Qiagen Plasmid Midiprep kit (Qiagen, CA, USA; Appendices A1.4)

Table 2.4: Oligonucleotides used to generate full length and minimal *NSs*, *N* and *M* reporter targets

Primers	Sequence (5'-3')[1,2]	Length (nt)
NSs-F	GATC**CTCGAG**TATCATGGATTACTTTCCTGT	31
NSs-R	GATCGCGGCCGCCTAATCAACCTCAACAAATCC	33
N-F	GATC**CTCGAG**ACCATGGACAACTATCAAGAGCT	33
N-R	GATCGCGGCCGCTCTGGCTGCTGTCTTGTAAGC	33
M-F	GATC**CTCGAG**ATGTATGTTTTATTAACAATTCTAACCTCGGT TCTGGTGTGTGAAG	56
M-R	GATCGCGGCCGCCTATGAGGCCTTCTTAGTGG	32
NSs4 target (+)	**TCGAG**ATATCGTCCTAGTCACGAGGTTCGGC	31
NSs4 target (-)	GGCCGCCGAACCTCGTGACTAGGACGATATC	31
NSs6 target (+)	**TCGAG**ATATCGATGGTCCTCCCAGGATACGC	31
NSs6 target (-)	GGCCGCGTATCCTGGGAGGACCATCGATATC	31
NSs7 target (+)	**TCGAG**ATATCGTTGTGTCAGTGGAGTACTGC	31
NSs7 target (-)	GGCCGCAGTACTCCACTGACACAACGATATC	31
N1 target (+)	**TCGAG**ATATCTCAAGCAGTGGACCGCAATGAGC	33
N1 target (-)	GGCCGCTCATTGCGGTCCACTGCTTGAGATATC	33
N3 target (+)	**TCGAG**TTATAAGCCATGAGAAGAG	24

N3 target (-)	GGCCGCCTCTTCTCATGGCTTATAA	25
M1 target (+)	**TCGAG**GGCTGATCCACCTAGCTGT	24
M1 target (-)	GGCCGCACAGCTAGGTGGATCAGCC	25
M2 target (+)	**TCGAG**GACTACCAGTCAGCTCATC	24
M2 target (-)	GGCCGCGATGAGCTGACTGGTAGTC	25
M4 target (+)	**TCGAG**CCTATTGTTCACATGCTAA	24
M4 target (-)	GGCCGCTTAGCATGTGAACAATAGG	25
M5 target (+)	**TCGAG**GAGATTACACTCAAGTATC	24
M5 target (-)	GGCCGCGATACTTGAGTGTAATCTC	25
R-Luc	GAGGACGCTCCAGATGAAATG	21

[1]Bold and underlined sequences represent *Xho*I and *Not*I sites, respectively.

[2]The target coordinates of the oligonucleotides within their respective genes are the same as the target regions of their corresponding shRNA as depicted in Table 2.1 and 2.2.

2.3 Cloning of RVFV mammalian expression vectors

To express the NSs and N derived proteins, gene sequences were cloned into a pCI-Neo mammalian expression vector. The pCI-Neo vector expresses proteins from a cytomegalovirus (CMV) Pol II promoter. The pTZ-*NSs*, pTZ-*N* and pCI-Neo backbone was prepared using *Xho*I and *Not*I according to the protocol described above (section 2.2). *NSs* and *N* were ligated into pCI-Neo and inserted into DH5αs (Appendices A1.1 and A1.2). The subsequent steps of DNA extraction and insert confirmation were the same as described in 2.2 (Appendices A1.3).

To detect the expressed proteins, RVFV derived genes were fused with a commercial epitope, a FLAG tag. A FLAG tag is an octapeptide DYKDDDDK that can be recognised by an anti-FLAG antibody (Sigma, WI, USA). A 3xFLAG tag sequence was attached to the 3' end of the *NSs* and *N* genes through a one-step PCR (Figure 2.3). *NSs* and *N* genes were PCR amplified in a Mastercycler® (BioRad, CA, USA) using high fidelity *Taq* polymerase (Fermentas, WI, USA). S segment cDNA was used as a template and NSs-F and N-F primers were used in their respective PCRs (Table 2.4). The

reverse oligonucleotide primer was complementary to the 3' terminus of the *NSs* and *N* genes, which contained an additional 3xFLAG tag sequence (Table 2.5). The stop codon of *NSs* (TAG) and *N* (TAA) was mutated to AGA to allow for read-through into the 3xFLAG tag sequence. The *NSs*-3xFLAG and *N*-3xFLAG PCR products were sub-cloned and inserted into the pCI-Neo expression vector as described above.

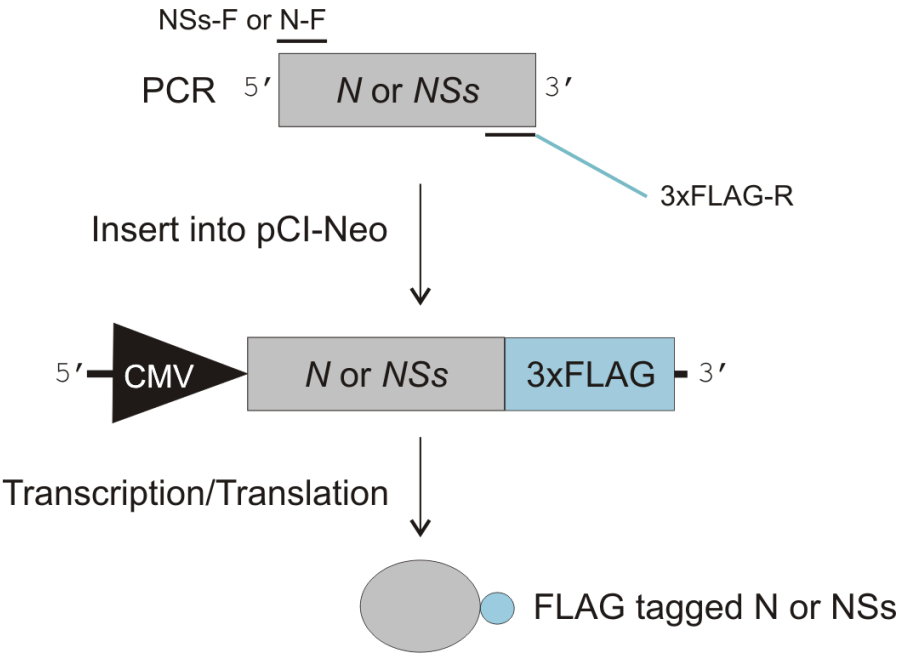

Figure 2.3: Schematic of the *N* and *NSs*-3xFLAG tagged expression vectors. *NSs and N* were fused with a 3xFLAG tag sequence using PCR amplification and inserted into a CMV-Pol II mammalian expression vector. After mRNA translation, a FLAG tag epitope will be present at the C-terminus for detection using anti-FLAG antibodies.

Table 2.5: Reverse oligonucleotide primers used to generate *NSs* and *N* with a 3xFLAG tag

Primer	Sequence (5'-3')[1]	Length (nt)
NSs-3xFLAG-R	GATCGCGGCCGCCTACTTGTCATCGTCATCCTTGTAGTCGAT GTCATGATCTTTATAATCACCGTCATGGTCTTTGTAGTCAGC CCGGGATCTATCAACCTCAACAAATCC	111
N-3xFLAG-R	GATCGCGGCCGCCTACTTGTCATCGTCATCCTTGTAGTCGAT GTCATGATCTTTATAATCACCGTCATGGTCTTTGTAGTCAGC CCGGGATCTGCTGCTGTCTTGTAAGCC	111

[1]Underlined sequences represent *Not*I sites. The greyed sequence represents the 3xFLAG tag sequence.

The *M* expression vector was cloned from a pSP64-ΔNSm construct provided by Dr Sonja Gerrard (Gerrard and Nichol, 2007). A pSP64-ΔNSm vector contains a M sequence, which lacks the NSm region. The pSP64-ΔNSm vector starts expression from the 4th translation start codon, expressing only the Gn and Gc glycoproteins. The sequence has also been engineered to contain a FLAG tag inserted at the N-terminus of the Gc glycoprotein. The pSP64-ΔNSm construct was digested with *Sal*I and *Eco*RI [2 µg of pSP64-ΔNSm with 5 U of each restriction enzyme in Buffer O, 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM NaCl, 0.1 mg/ml BSA] and incubated for 1 hr at 37°C (Fermentas, WI, USA). Digests were resolved using agarose gel (1%) electrophoresis and a band corresponding to the correct size was extracted (Appendices A1.5). A pCI-Neo vector was digested sequentially, firstly with *Xho*I for 1 hr at 37°C, and then the reaction was spiked with *Eco*RI for a further 1 hr digestion at 37°C [2 µg of pCI-Neo with 5 U of each restriction enzyme in buffer R, 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM NaCl, 0.1 mg/ml BSA] (Fermentas, WI, USA). The digested pCI-Neo vector was resolved using agarose gel (1%) electrophoresis and extracted as previously described (Appendices A1.5). Gel extracts were ligated together, inserted into DH5αs (Appendices A1.1 and A1.2) and were incubated overnight at 30°C. The extracted plasmid was confirmed through an *Nde*I digestion and automated sequencing (Inqaba Biotech, South Africa) (Appendices A1.3). The pCI-Neo vector containing a M segment with the deleted NSm region was referred to as the ΔNSm expression vector.

Mammalian expression vectors containing mutant NSs proteins, namely PP1, PP2 and clone 13 (C13) were kindly donated by Prof. Michèle Bouloy and generated as described elsewhere (Billecocq et al., 2004). The 3xFLAG tag NSs PP1 and PP2 mutants were generated using the same procedures as described above. The expression vectors used in tissue culture experiments were prepared using a Qiagen Plasmid Midiprep kit (Qiagen, CA, USA; Appendices A1.4)

2.4 Mammalian tissue culture

Human embryonic kidney (HEK293) and hepatocellular carcinoma (Huh7) cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; BioWhittaker, MD, USA). The media was supplemented with 10% fetal calf serum (FCS) (Biochrom AG, BE, DE) and cultured at a temperature of 37°C with 5% CO_2 (Appendices A1.6). Cells were propagated in 25 cm^2 flasks and seeded into a appropriate size plate for the required experiment.

2.5 Transfections

Transfections were performed using lipofectamine™ 2000 according to the manufacturers' instructions (Invitrogen, CA, USA; Appendices A1.7). Transfections were performed at a ratio of 1 µl of lipofectamine™ 2000: 1 µg of DNA mixed in Opti-MEM® reduced-serum medium (Gibco, BRL, UK). Individual mixtures of DNA or lipofectamine™ 2000 in Opti-MEM® were prepared and incubated for 10 min at room temperature. The solutions were then mixed and incubated for a further 10 min at room temperature prior to addition onto the cells. The total volume of the lipofectamine:DNA mix was 1/5th the cell culture media volume. The media was replaced 24 hrs post-transfection with fresh media.

2.6 Assessment of target knockdown by an shRNA in a reporter assay

To determine the effectiveness of the shRNAs in a dual-luciferase assay, 24 hrs prior to transfection HEK293 cells were seeded at a confluence of 70% in a 24-well culture dish (Appendices A1.6). Nine hundred nanograms of a shRNA was co-transfected with 90 ng of pSI-CHECK containing a full length or minimal target (Appendices A1.7). One hundred nanograms of a vector that expressed a green fluorescent protein (pCI-GFP) was included to visually determine transfection efficiency (Passman et al., 2000). At 48 hrs post-transfection, a dual-luciferase reporter assay was performed (section 2.13).

2.7 Assessment of shRNA expression and processing using northern blot analysis

To assess the expression and processing of the shRNAs, 24 hrs prior to transfection HEK293 cells were seeded at a confluence of 70% into a 10 cm Cellstar® culture dish (Greiner Bio-one, BW, DE; Appendices A1.6). Nineteen micrograms of a shRNA was transfected with 1 µg of pCI-GFP (Appendices A1.7). Forty-eight hours post-transfection RNA was extracted using TRI Reagent® (Sigma, MO, USA) according to the manufacturers' instructions (Appendices A1.8).

Following extraction, 30 µg of each sample of total RNA was loaded per well and resolved using polyacrylamide gel electrophoresis (PAGE). A 15% PAGE was used at a ratio of 1:19 *bis*-acrylamide:acrylamide and 8 M urea in TBE buffer [0.83 M Tris-base, 0.89 M boric acid and 2 mM EDTA (pH 8.0), sterilised by autoclaving]. To determine the molecular weight of the shRNA and processed siRNA, a Decade^{TM} Marker (Ambion®, CA, USA) was included and prepared according to the manufacturers' instructions (Appendices A1.9).

After resolution of the loaded RNA, gels were stained with 10 mg/ml ethidium bromide (Sigma, MO, USA) and visualised using UV light (Kodak Gel Logic 200 Imaging System) to assess the

integrity of the RNA prior to transfer. The RNA was then transferred to a Hybond-N+ (GE Healthcare, NJ, USA) positive charged nylon membrane using semi-dry blotting (Model SV20-SDB, Sigma, MO, USA) at 3.3 mA/cm^2 for 1 hr. The RNA was UV cross-linked at 2000x100 µJ/cm^2 (UVP, CA, USA) and subsequently baked for 1 hr at 80°C (Hybaid, Thermo Fisher Scientific, MA, USA).

DNA oligonucleotide probes (Table 2.1; (+) oligonucleotides) that were complementary to the antisense shRNA/siRNA guides were labelled with γ-32P-ATP (6000 Ci/mmol; Perkin Elmer, MA, USA). Probes were labelled in a PNK reaction [50 mM imidazole-HCl (pH 6.6), 10 mM $MgCl_2$, 5 mM DTT; 0.1 mM spermidine, 0.1 mM EDTA and 5 U PNK enzyme] (Promega, WI, USA) and incubated at 37°C for 20 min. Probes were purified by centrifuging the probe mixture at 800 x g for 2 min through a G-25 sephadex® column (Sigma, MO, USA). Purified probe was added to 10 ml of rapid-hyb buffer with pre-hybrised membrane (GE Healthcare, NJ, USA). The hybridisation reaction was incubated overnight at 42°C in a rotating oven (Hybaid, Thermo Fisher Scientific, MA, USA).

Non-specific probe binding was washed off with 5% SSC (Sigma, MO, USA) and 0.1% SDS (sodium dodecyl sulphate) wash buffer for 20 min at room temperature. Two additional wash steps were performed in 0.1% SDS and 1% SSC at 42°C for 15 min. Following the wash steps, the membrane was exposed to Fuji X-ray film overnight at -70°C. The membranes were then stripped using a 1% SDS solution for 30 min at 80°C prior to a repeat hybridisation with a different probe. U6 snRNA probe was used as a loading control to ensure similar amounts of RNA were loaded in each well. The U6 snRNA probe sequence was: 5'-TAGTATATGTGCTGCCGAAGCGAGCA-3'

2.8 Assessment of the shRNA's ability to reduce RVFV protein levels

To evaluate the effect of the shRNAs on target protein levels, 24 hrs prior to transfection HEK293 cells were seeded at a confluence of 70% in a 6-well culture dish (Appendices A1.6). Two-hundred and fifty nanograms of the *NSs*-3xFLAG or *N*-3xFLAG tag expression vector was co-transfected with 1650 ng of the shRNAs and 100 ng of pCI-GFP (Appendices A1.7). One microgram of the ΔNSm expression vector was transfected with 1 µg of a shRNA construct. Forty-eight hours post-transfection, whole cell lysates were extracted and subjected to western blot analysis.

2.9 Western blot analysis

Two hundred microliters of RIPA buffer (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS; Thermo Fisher Scientific, MA, USA) was added to each well and the cells were resuspended in the lysis buffer. The lysate was transferred to a 1.7 ml Eppendorf tube,

incubated on ice for 5 min and then stored at -20°C. When ready to be used, the lysate was thawed on ice and sonicated (35 kHz; Bandelin Sonorex) for 1 min intervals, with 30 sec interims on ice, until all chromosomal DNA was shredded. The lysate was centrifuged at 16,100 x g for 10 min at 4°C. The supernatant was transferred to a 1.7 ml Eppendorf tube and kept on ice until a Pierce® BCA protein assay could be performed according to manufacturers' instructions (Thermo Fisher Scientific, MA, USA; Appendices A1.10).

One-hundred microliters of supernatant was transferred to a 1.7 ml Eppendorf tube and an equal volume of 2x sample buffer was added [130 mM Tris-HCl (pH 8.0), 20% glyercol, 4.6% SDS, 0.02% Bromophenol Blue, 2% DTT]. The lysate was then boiled at 95°C for 5 min and then cooled to room temperature. The sample was subjected to SDS-PAGE [29:1 acrylamide:*bis*-acrylamide, resolving buffer: 3 M Tris-base (pH 8.8), 0.8% SDS; stacking buffer: 0.5 M Tris-base (pH 6.8), 0.4% SDS]. An amount of 20-40 µg of whole-cell protein lysate was loaded per well onto a 10% gel and resolved for 1.5 hrs at 2 mA/gel within running buffer [0.25 M Tris-base, 1.93 M glycine, 1% SDS]. A Spectra™ multicolour broad range protein ladder was used to determine the molecular weight of the detected proteins (Fermentas, WI, USA). Protein from the gels was then transferred onto Polyvinylidene fluoride membrane (PVDF) (Immunoblot-P, Millipore) using wet-blotting. The transfer was performed for 1 hr at 100 V at 4°C in transfer buffer [25 mM Tris-base, 191 mM glycine, 10% methanol].

The membrane was submerged in blocking buffer [Tris-buffered saline (TBS), 5 mM Tris-base (pH 8.0), 0.138 M NaCl, 2.7 mM KCl with 5% skim-milk] for 1 hr at room temperature. The membrane was sealed within a plastic jacket and a 1 ml mixture of primary antibodies was added. The mixture contained a mouse monoclonal anti-FLAG antibody (1:1000, Sigma, WI, USA) and anti-α-tubulin antibody (1:1000, Sigma, WI, USA) diluted in blocking buffer. The membrane was incubated for 1 hour at room temperature. The membrane was washed with TBS and 0.05% Tween-20 (TBS-T) for 2 min at room temperature. The membrane was sealed in a plastic jacket and 1 ml of secondary polyclonal rabbit anti-mouse antibody (1:1000, Dako, Denmark) diluted in blocking buffer was added. The secondary antibody was coupled to a horseradish peroxidase (HRPO). The membrane was incubated for 1 hr at room temperature, washed six times for 4 min in TBS-T and exposed with either: SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, MA, USA), or DAB staining (Diaminobenzidine; Roche, IN, USA). Chemiluminescent substrate was prepared by adding equal volumes of Luminol enhancer solution to stable peroxide solution (Diaminobenzidine; Roche, IN, USA). A DAB stain was prepared by diluting DAB solution into hydrogen peroxide solution (1:10). One of the prepared substrates was added onto the blot and incubated for 5 min at room temperature. The exposed signal was captured using Syngene G:BOX Chemi Gel imaging equipment

(Syngene, MD, USA) and the density of the bands were quantified using GeneTools software (version 4.00, Syngene, MD, USA).

2.10 Assessment of the shRNA's effect on NSs-induced pathogenic effects

2.10.1 Evaluation of the shRNA's effect on NSs-induced transcription suppression

Twenty four hours prior to transfection HEK293 cells were seeded at 70% confluence in a 24-well culture dish (Appendices A1.6) and a dual-luciferase assay was performed at 48 hrs post-transfection. One-hundred nanograms of pRL-CMV, a Pol II expressed *Renilla* luciferase vector, was included in each experiment. The amount of shRNA transfected was maintained at a ratio of 1:10 (target to shRNA). To evaluate the effects of NSs on transcriptional suppression, HEK293 cells were co-transfected with 20 ng of the expression vectors (Promega, WI, USA; Appendices A1.7). For the dose-responsive transcription suppression by NSs, HEK293 cells were transfected with 20 ng, 10 ng, 5 ng, 2 ng, 1 ng and 0.5 ng of the expression vectors. To evaluate the effects of the shRNAs on NSs transcriptional suppression, HEK293 cells were co-transfected with 20 ng of the expression vectors with a shRNA. To assess the effect of the shRNAs on increasing amounts of NSs, HEK293 cells were transfected with 80 ng, 60 ng, 20 ng and 10 ng of NSs expression vector with a shRNA.

2.10.2 Evaluation of the shRNA's effect on NSs-induced cytotoxic effects

To observe NSs cytotoxicity, 24 hrs prior to transfection HEK293 cells were seeded at a confluence of 70% in a 24-well culture dish (Appendices A1.6). HEK293 cells were transfected with 100 ng of the expression vectors and 100 ng of pCI-GFP (Appendices A1.7). At 48 hr post-transfection, the cells were observed using fluorescence microscopy (Axiovert 100M microscope; Zeiss, Germany).

For the MTT assays, 24 hrs prior to transfection HEK293 cells were seeded at a confluence of 40% in a 96-well culture dish (Appendices A1.6). HEK293 cells were transfected with 20 ng of the expression vectors and 80 ng of the shRNA constructs (Appendices A1.7). Trichostatin A (TSA) was included as a positive control for toxicity at concentrations of 1000 nM, 500 nM, 200 nM and 100 nM. At 48 hrs post-transfection an MTT cell viability assay was performed. As an indicator of cell viability, MTT (Sigma, MO, USA) was added to the cells and incubated for 30 min at 37°C. The media was removed and the precipitated formazan was resuspened in 100 µl of dimethyl sulfoxide (DMSO). The plate was read on a Biorad Model 680 microplate reader (Biorad laboratories, USA) at a wave-length of 570 nm. The OD readings were normalised to 655 nm reference wave-length.

2.10.3 Evaluation of the shRNA's effect on a NSs-suppressed IFN-β promoter

Twenty four hours prior to transfection HEK293 cells were seeded at a confluence of 70% in a 24-well culture dish (Appendices A1.6). HEK293 cells were transfected with 10 ng of the expression vectors with 50 ng of a pIFΔ[-125]lucter (pIF-125luc) reporter vector, kindly donated by Stephen Goodbourn (King and Goodbourn, 1994) (Appendices A1.7). pIF-125luc is a vector with a IFN-β promoter expressed Firefly luciferase reporter. Fifty nanograms of pRL-CMV was included as a background control, to which Firefly luciferase levels were normalised. To evaluate the effects of the shRNAs to reestablish IFN-β promoter activity, HEK293 cells were co-transfected with 2.5 ng of the expression vectors and 200 ng of the shRNA constructs (Appendices A1.7). At 24 hrs post-transfection cells were exposed to 50 ng of Poly (I:C) (Sigma, MO, USA) and incubated for 16 hrs before a dual-luciferase assay was performed. The values were normalised to transfected samples that did not receive Poly (I:C).

2.11 Evaluation of a non-specific IFN response

Twenty four hours prior to transfection HEK293 cells were seeded at a confluence of 70% in a 24-well culture dish (Appendices A1.6). HEK293 cells were co-transfected with a pIF-125luc reporter vector with 100 ng of the shRNA constructs and 100 ng of pRL-CMV (Appendices A1.7). HEK293 cells transfected with Poly (I:C) were included as a positive control for immune stimulation. At 16 hrs post-transfection, a dual-luciferase assay was performed.

2.12 Evaluation of shRNA-mediated saturation of endogenous RNAi

Twenty four hours prior to transfection Huh7 cells were seeded at a confluence of 80% in a 24-well culture dish (Appendices A1.6). Huh7 cells were co-transfected with 20 ng of a pSI-CHECK 2.2 vector containing miRNA target sites in the 3' UTR (Ely et al., 2009) and 900 ng of the shRNA constructs (Appendices A1.7). At 48 hrs post-transfection, a dual-luciferase assay was performed.

2.13 Dual-luciferase reporter assay

Luciferase reporter assays were carried out using a Dual-Luciferase® Reporter assay system according to the manufacturers' instructions (Promega, WI, USA). The media was removed from the cells and 100 µl of passive lysis buffer was added to each well. Lysates were incubated for 20 min at room temperature, resuspended by aspiration and 12 µl of each sample was added to a 96-well plate. Firefly and *Renilla* luciferase activities were measured sequentially by the addition of 50 µl of Luciferase reagent II (LAR) Firefly substrate and then 50 µl of Stop & Glo® *Renilla* substrate. Luciferase activity was measured using a Veritas™ Microplate Luminometer (Turner Biosystems, CA, USA). In experiments where only Firefly or *Renilla* luciferase activity was measured, only LAR or Stop and Glo® were added, respectively.

2.14 RVFV challenge assay

Twenty four hours prior to transfection Huh7 or HEK293 cells were seeded at a confluence of 80% in a 24-well culture dish (Appendices A1.6). Cells were transfected with 900 ng of the shRNA constructs and 100 ng of pCI-GFP (Appendices A1.7). At 24 hrs post-transfection, the media was removed and cells were infected with 100 µl of RVFV at doses described in the results (section 4.2.1, 4.2.2 and 4.2.6). Virus was incubated with the cells for 1 hr. The virus suspension was removed and replaced with 1 ml of stasis media consisting of Essential Minimal Eagle's Media [EMEM, BioWhittaker, MD, USA; with 2% FCS, 50 mg/mL of Penicillin/Streptomycin, 1% non-essential amino acids (NEAA); 1% L-glutamine] (Sigma, MO, USA) and incubated at 37°C with 5% CO_2. Aliquots of 250 µl were taken from each well, every 24 hrs for the described amount of time results (section 4.2.1, 4.2.2 and 4.2.6). An equal amount of 250 µl of stasis media was replaced after each aliquot was taken. Samples were thermo-chemically inactivated by the addition of 250 µl of 0.5% Tween 20 and incubated at 56°C for 1 hr. The N antigen of RVFV was detected in the supernatant as a marker of viral replication using a sandwich ELISA as described elsewhere (Jansen van Vuren and Paweska, 2009) (Appendices A1.11, Figure A.1). All work with infectious virus was performed under biological safety level 3 (BSL3) conditions using specialised facilities at the Special Pathogens Unit (SPU) under the expertise and guidance of Prof. Janusz Paweska.

2.15 Statistical analysis

Statistical calculations were performed using a GraphPad Prism software package (GraphPad, Software, CA, USA). Statistical differences were significant when $p<0.05$ and were determined using an unpaired Student's t-test.

Chapter 3

Expressed short hairpin RNAs alleviate RVFV NSs-induced pathogenic effects in cultured cells

3.1 Introduction

Haemorrhagic fever viruses are important emerging zoonotic pathogens. RVFV causes a haemorrhagic fever, which is characterised by high levels of viral replication and rapid onset of severe illness (section 1.7.2) as observed with other haemorrhagic fever viruses [Reviewed in (Paessler and Walker, 2012)]. An early IFN response and the emergence of adaptive immunity is important for RVFV clearance (Morrill et al., 1990). RVFV imposes a significant public health and socio-economic burden. Even though there are numerous vaccines currently in development [Reviewed in (Bouloy and Flick, 2009)], it will be difficult to justify the wide implementation of RVFV vaccination as a result of the sporadic occurrence of the disease outbreaks. This shifts the focus towards development of therapeutics that can be used as an intervention strategy when required. Very few therapies are currently in development (section 1.5) and innovative novel therapeutic strategies are desired.

The endogenous RNAi pathway can be exploited using miRNA mimics to facilitate sequence specific degradation of target RNA, which can be used to down-regulate viral mRNA to hinder disease progression (section 1.6). Studies have successfully applied RNAi mimics to inhibit haemorrhagic fever viruses, which was demonstrated when siRNAs prevented lethality in non-human primates infected with EBOV (Geisbert et al., 2010) (section 1.7.1.4). Alternatively, pre-miRNA mimics or shRNAs are procesed by Dicer into siRNAs to suppress a target gene (section 1.6.3.3). Expressed shRNAs have been used to control DENV infections *in vitro* (Zhang et al., 2004) and could be used as a targeted inhibitor for RVFV. Furthermore, shRNAs have several rules that can to assist construction under control of a Pol III promoter with defined transcriptional regulatory signals that allow for consistent shRNA expression and silencing (section 1.6.3.3). Expressed shRNAs can inhibit a target gene for extended periods, which could facilitate suppression of a virus throughout the duration of infection. Haemorrhagic fever viruses have a short interval before mortality and the application of a constant suppressive effect during the limited treatment period may better manage the disease (section 1.7.2.1). The relatively high levels of shRNA expression and potent target gene down-regulation may better suppress the high viral loads associated with haemorrhagic fever viruses (section 1.7.2.2). ShRNAs can be easily inserted into viral delivery vectors for targeting to organs that are important in RVFV pathogenesis (section 5.8.1), which could

counteract the problem of tissue dissemination observed during infection (section 1.7.2.3). Finally, RNAi has only been used to inhibit the non-pathogenic Hazara virus of the *Bunyaviridae* family (section 1.7.1.3), and its efficacy against haemorrhagic bunyaviruses still needs to be determined.

Virulence factors are important for the establishment of a disease state, which is demonstrated when mutations that abrogate EBOV-VP35's ability to subvert host immunity resulted in an avirulent infection (section 1.3.2). As a result of their importance in pathogenesis, studies have targeted virulence factors like EBOV's VP35 and VP24 as therapeutic targets (section 1.5.4 and 1.7.1.4). RVFV encodes a *NSs* gene that is involved in subverting host defences through transcriptional suppression (Le May et al., 2004) and direct interaction with the IFN-β promoter (Le May et al., 2008) (section 1.4). Furthermore, suppression of host transcription as well as its interaction with host DNA, which causes genomic defects (Mansuroglu et al., 2010), could have additional cellular toxicity. A RVFV lacking a functional *NSs* gene produces a highly attenuated infection (Muller et al., 1995) resulting in a potent adaptive (Bird et al., 2008) and IFN response (Lihoradova et al., 2012). Therefore, targeted down-regulation of the NSs protein using RNAi effectors could disrupt NSs's function, which would have a significant impact on RVFV pathology.

ShRNAs were designed to target RVFV's *NSs* gene and it was assessed whether the shRNAs could affect its pathogenic properties. The shRNAs were able to suppress *NSs* reporter levels as well as reduce NSs protein concentrations. The anti-*NSs* shRNAs alleviated its pathogenic effects, specifically with regards to transcriptional suppression, IFN-β promoter activity and cytotoxicity. This represents an important development in shRNAs that could attenuate RVFV pathology, which would enhance important cellular and immune responses to help the host facilitate viral clearance. Furthermore, this signifies a novel strategy for the treatment of RVFV, which could be implemented as a control measure during its sporadic outbreaks.

3.2 Results

3.2.1 Inhibition of the NSs *gene in a reporter assay by shRNAs*

A series of shRNAs were designed to target the *NSs* gene of RVFV. The shRNAs were expressed off a U6-pol III promoter and contained a 19 nt sense, 9 nt loop and 19 nt antisense sequence (Figure 3.1) (section 1.6.3.3). A thymidine tract was included as a U6-Pol III termination signal, which would produce a shRNA with a 2 nt 3' overhang, an important feature for downstream RNAi processing (section 1.6.1). Furthermore, there were several additional features that were applied in the selection and design of shRNA candidates. This included shRNAs with a low G:C content, G:U mismatches and favourable guide strand thermodynamics to ensure effective guide

strand incorporation into RISC (1.6.3.3). Lastly, the guide sequences were aligned against the human genome using BLAST to exclude any shRNAs with obvious host targets, reducing the likelihood of off target effects (section 1.8.1). Based on these criteria, shRNAs were designed to target various conserved regions within *NSs*. However, RNAi silencing between shRNAs can still vary and so ten shRNAs were designed to increase the probability of obtaining an effective shRNA.

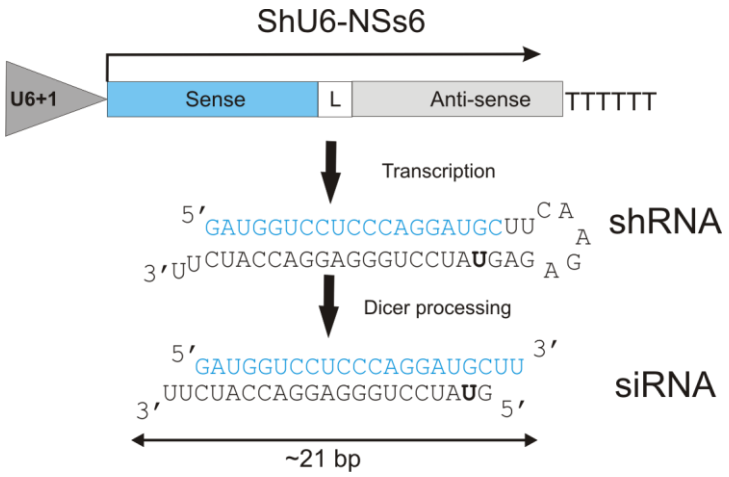

Figure 3.1: Schematic presentation of a shRNA expression cassette. Short hairpin RNAs were designed to have a 19 bp stem, a loop (L) region and a poly-T terminator signal. ShRNAs were expressed off a U6-Pol III promoter upstream of the shRNA sequence that will generate a hairpin RNA with a two nucleotide uridine overhang. Transcribed shRNAs are processed by Dicer into an approximately 21 nt mature siRNAs. G:U wobble bases are indicated in bold.

It was determined whether the shRNAs could effectively inhibit a *NSs* target. To assess the effect shRNAs would have on NSs, a dual-luciferase reporter assay was performed in cultured cells. HEK293 cells were used as a model for suppression by the shRNAs and would be representative of knockdown in other tissue types (Graham et al., 1977). To generate the reporters, a full length *NSs* target sequence was cloned downstream of *Renilla* luciferase in a pSI-CHECK 2.2 reporter vector (Figure 2.2). This vector will express a single fusion *Renilla*:*NSs* mRNA and knockdown of *NSs* will result in a reduction of *Renilla* luciferase activity. Knockdown was determined as a ratio of *Renilla* to background Firefly luciferase activity. A shRNA targeting HIV (shHIV) was included as a negative control (Barichievy et al., 2007). Following transfection of the shRNAs and reporter vectors, shRNAs targeted to *NSs* demonstrated between 20%-90% knockdown of *NSs* reporter levels from sequences derived from the ZH548 and ZH501 strains (Figure 3.2A). The two strains were chosen as they were associated with early severe outbreaks of RVF [Reviewed in (Ahmed Kamal, 2011)] and have been used as model RVFV strains in numerous *in vitro* and *in vivo* studies [Reviewed in (Bouloy and Flick,

2009)]. ShU6-NSs2, ShU6-NSs4, ShU6-NSs6, ShU6-NSs7 and ShU6-NSs8 demonstrated >80% knockdown against one or both *NSs* gene targets. ShU6-NSs1 was the least effective with only ~20% knockdown against *NSs* ZH548 and no effect on *NSs* ZH501, which was the same as shU6-HIV control.

Similar to the *NSs* gene, a series of shRNAs were designed to target the *N* gene of RVFV. ShRNAs against *N* were created to generate multimers that contained shRNAs against *NSs* and *N* to target both genes simultaneously (see below section 3.2.2). The shRNAs targeting *N* were tested against a full length *N* sequence in a luciferase reporter assay and demonstrated knockdown between 50-80% (Figure 3.2B). The most effective shRNA was shU6-N1 with ~80% knockdown of *N* from both the ZH548 and ZH501 strains.

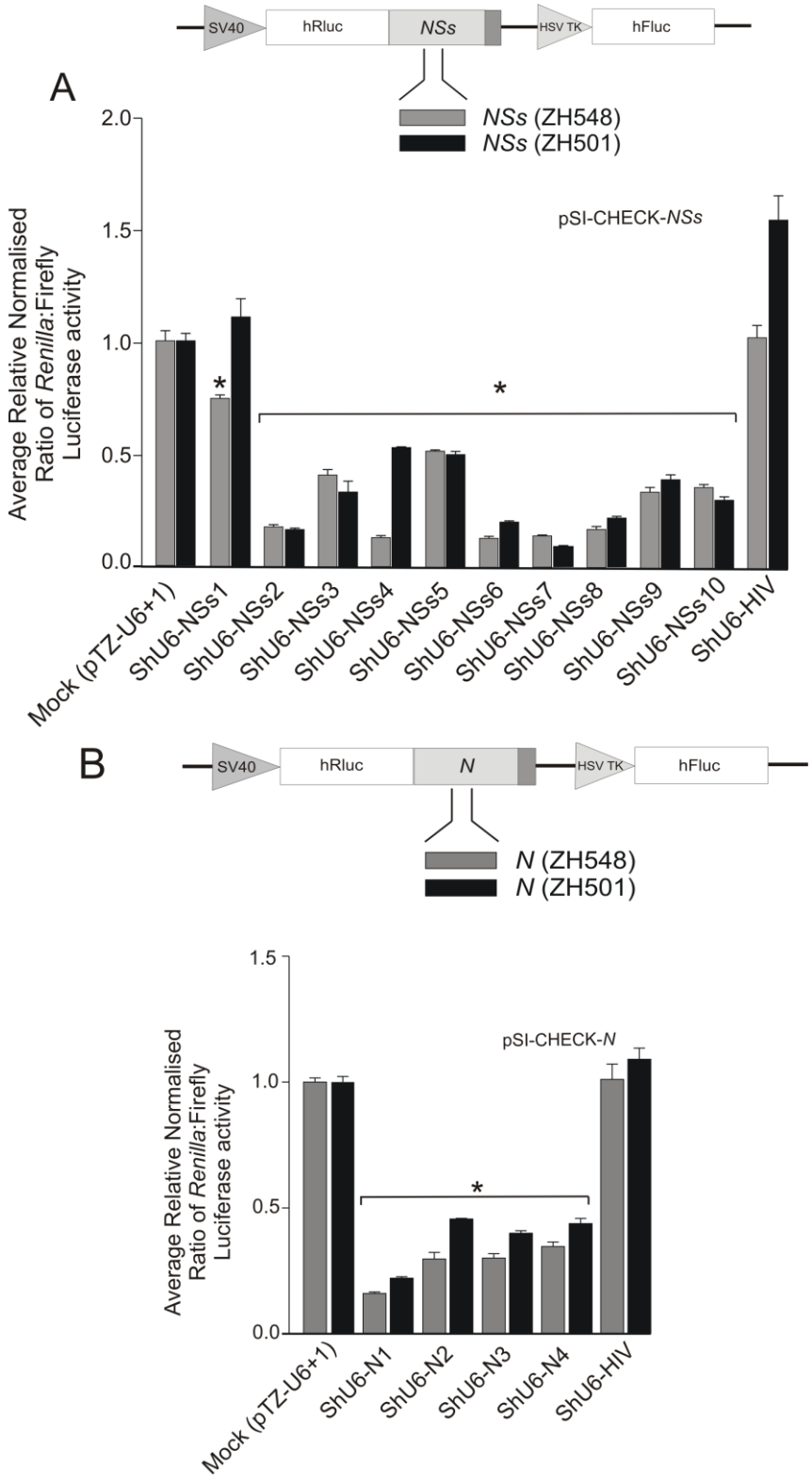

Figure 3.2: Inhibition efficiency of the U6-Pol III expressed shRNAs. HEK293 cells were co-transfected with the shRNAs and a full length (A) pSI-CHECK-*NSs* or (B) pSI-CHECK-*N* reporter vector from the ZH548 and ZH501 strains of RVFV. The values represent an average ratio of *Renilla*:Firefly, which have been made relative to the mock (pTZ-U6+1) control. A shRNA targeted to HIV (shU6-HIV) was used as a negative control. Experiments were performed in technical triplicate with error bars indicating standard deviation (*p<0.05 Unpaired Student's t test compared to the mock control).

To determine whether the target sites were conserved within RVFV, the shRNAs were tested against *NSs* genes derived from other strains of RVFV. The various strains were kindly provided for by Prof. Janusz Paweska (NICD, South Africa) and represent RVFV isolates from major outbreaks of RVF (information about the strains is provided for in Table 2.3). *NSs* was chosen as its variability is greater than *N* and more likely to have reduced conservation within the target regions (Bird et al., 2007a). ShRNAs that demonstrated >80% knockdown against one or both of the *NSs* ZH548 and ZH501 derived strains (Figure 3.2A), were tested against *NSs* genes from eight other strains of RVFV. Apart from shU6-NSs2 that was ineffective against CAR 1662 and Lunyo, the selected shRNAs were able to knockdown the *NSs* of all strains of RVFV (Figure 3.3A). The reason for ineffective silencing by shU6-NSs2 was because of mismatches in the target site, with two and three nucleotide changes in the CAR 1662 and Lunyo strains, respectively (Figure 3.3B). As a result of this loss of sequence conservation, shU6-NSs2 was not used in further studies. Overall the shRNAs demonstrated excellent target conservation and silencing across multiple strains of RVFV, suggesting that the shRNAs targeting other regions would be as conserved. Furthermore, the variability of *N* is less than that of *NSs* and suggests that the target conservation within *N* would be similarly conserved.

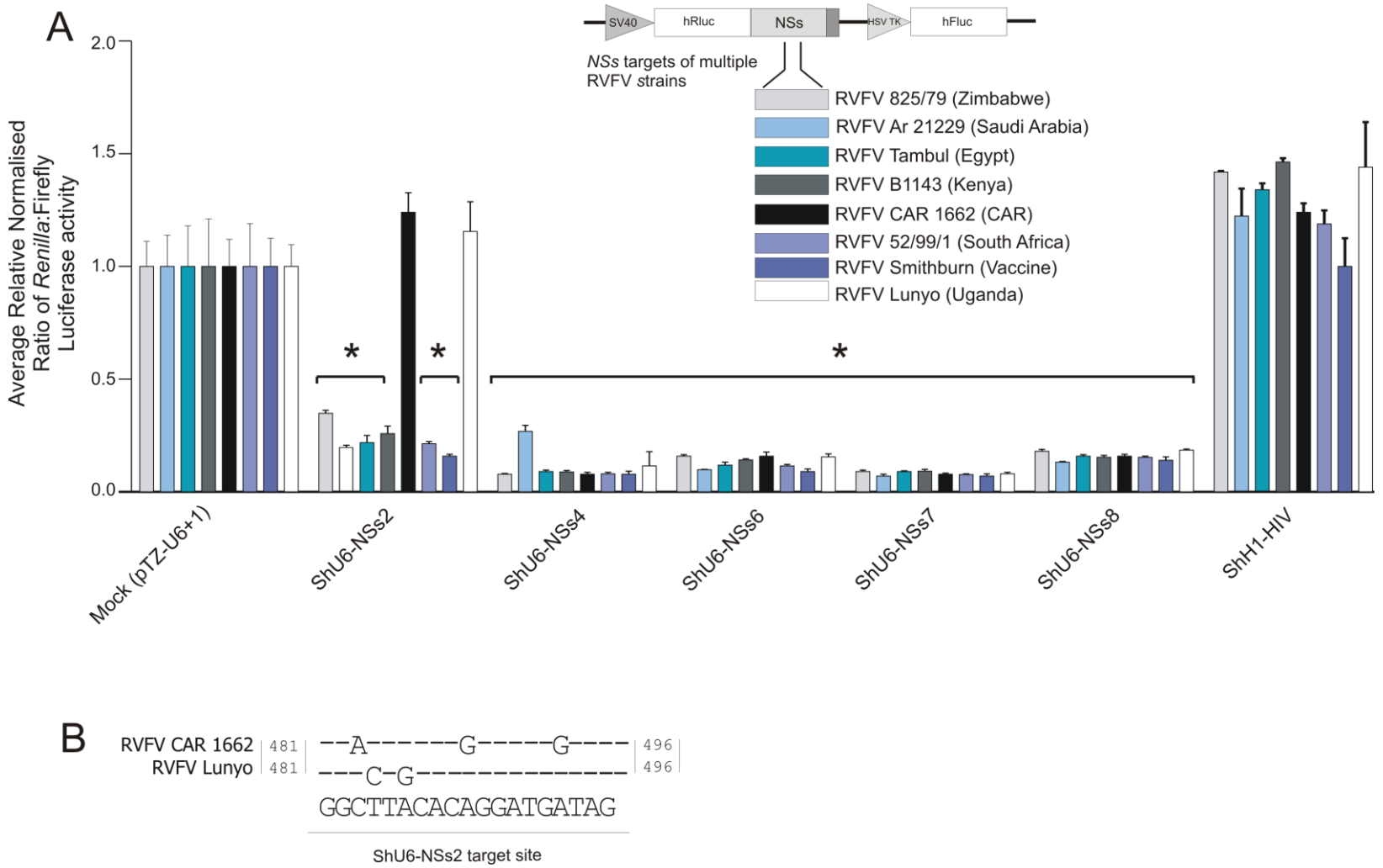

Figure 3.3: Inhibition of multiple strains of RVFV with U6-Pol III expressed shRNAs targeted to *NSs*. (A) HEK293 cells were co-transfected with the shRNAs and a full length pSI-CHECK-*NSs* from eight strains of RVFV. The values represent an average ratio of *Renilla*:Firefly, which have been made relative to the mock (pTZ-U6+1) control. A shU6-HIV was used as a negative control. Experiments were performed in technical triplicate with error bars indicating standard deviation (*p<0.05 Unpaired Student's t test compared to the mock control). (B) Indicated mismatches in the target site of shU6-NSs2 aligned with the CAR 1662 and Lunyo strains of RVFV.

U6-Pol III promoters transcribe high levels of RNA and are more susceptible to disruption of the endogenous RNAi pathway (Grimm et al., 2010). To reduce the likelihood that an expressed shRNA would affect normal miRNA processing, they were redesigned to be expressed off a weaker H1-Pol III promoter. Effective shRNAs in the previous reporter assays (shU6-NSs4, shU6-NSs6, shU6-NSs7 and shU6-N1) were selected to be taken forward (Figure 3.2 and 3.3). H1-Pol III expressed shRNAs were retested against full length reporter targets and knockdown efficacy was comparable to their U6-Pol III counterparts (Figure 3.4). An shRNA targeted to hepatitis B virus (shH1-HBV) was included as a negative control (Carmona et al., 2006).

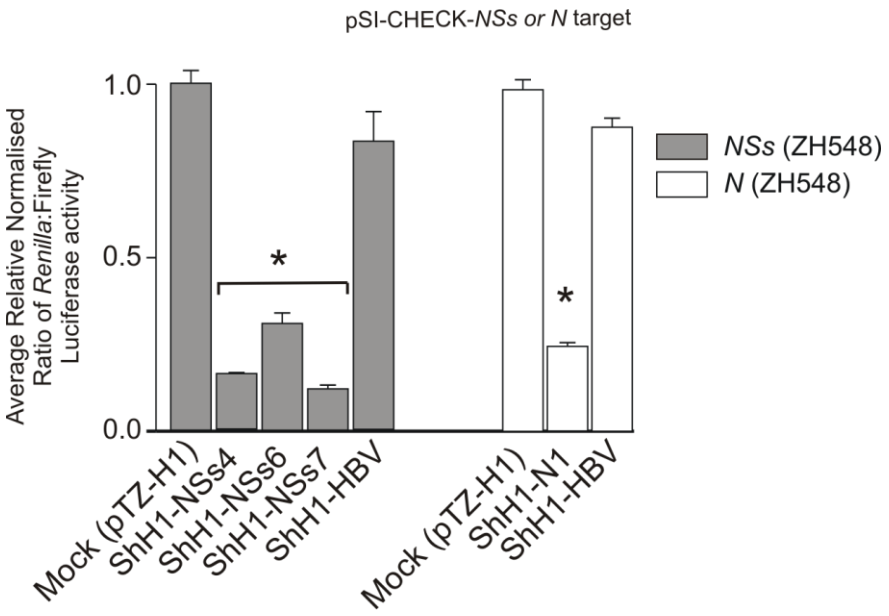

Figure 3.4: Inhibition efficiency of the H1-Pol III expressed shRNAs. HEK293 cells were co-transfected with shRNAs and a full length pSI-CHECK-*NSs* (ZH548) and *N* (ZH548) reporter vector. The values represent an average ratio of *Renilla*:Firefly, which have been made relative to the mock (pTZ-H1) control. A shRNA targeted to hepatitis B virus (shH1-HBV) was used as a negative control. Experiments were performed in technical triplicate with error bars indicating standard deviation (*p<0.05 Unpaired Student's t test compared to the mock control).

3.2.2 Generation and characterisation of multimeric shRNAs

There are benefits to targeting multiple regions simultaneously as this could interfere with the viral life cycle on several levels. In EBOV studies, VP24 and VP35 were inhibited simultaneously using antisense technology and resulted in enhanced protection against lethality *in vivo* compared to targeting the genes individually (section 1.5.4). Multiple RNAi effectors would contribute to enhancing the potency of RNAi, which may assist in rapidly reducing the high viral loads associated with RVFV haemorrhagic cases (section 1.7.2.2). Furthermore, shRNAs can be easily combined into

tandem arrays referred to as multiple expressed shRNAs with protocols that have been developed to simplify expansion of the number of shRNAs within the arrays (McIntyre et al., 2008) (section 2.1, Appendices Figure A.2). There are other potential RNAi platforms to target several viral genes concurrently, firsly, poly-cistronic miRNAs that consist of several miRNAs within a single RNA species, which will be processed by Drosha and subsequent RNAi machinery to form multiple miRNAs targeted to different regions within a virus (Aagaard et al., 2008). Poly-cistrons suffer from the same technical constraints as individual pri-miRNA mimics, which are not always amenable to manipulation (section 1.6.3.2). Secondly, long hairpin RNAs (lhRNAs) are similar in structure to shRNAs except are >40 nt in length and are sequentially processed by Dicer into several siRNA species (Barichievy et al., 2007) but because of it extended stem length would result in a heterogeneous population of siRNAs as well as passenger strand activity, enhancing off target effects (section 1.6.3.3). Therefore, multiple expressed shRNA cassettes offer an attractive system for simultaneously targeting of both the *NSs* and *N* genes of RVFV.

In the multiple expressed shRNAs, H1-Pol III shRNAs were inserted in a 'head-to-tail' fashion within a vector to allow for expression of several shRNAs from a single construct (Figure 3.5). Each shRNA was expressed from an individual H1-Pol III promoter resulting in expression of four shRNAs targeted to RVFV. ShH1-NSs4, shH1-NSs6, shH1-NSs7 and shH1-N1 was placed in each position within the multimeric cassettes. Some shRNAs in the downstream positions (sh3 and sh4) can suffer attenuated silencing more than other shRNAs, although the reason for this remains unclear (McIntyre et al., 2011a). For this reason, eight different cassettes were generated in the hopes of obtaining a multimer with effective shRNAs in each position. Multimers were named according to the position of the shRNAs within the cassette (e.g A multimer that contained shH1-NSs1:shH1-NSs2:shH1-NSs3:shH1-N1 or shH1-NSs2:shH1-NSs1:shH1-NSs3:shH1-N1 would be abbreviated as shNSs1,2,3-N1) and shNSs2,1,3-N1, respectively).

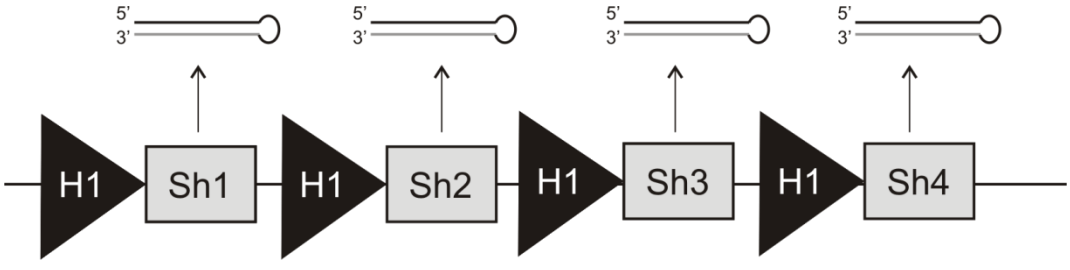

Figure 3.5. Schematic of the multimer cassette. Four shRNAs were placed in series in a 'head-to-tail' organisation within a single construct. Each shRNA was expressed from an individual H1-Pol III promoter, which allowed for four shRNAs to be processed simultaneously, targeting multiple sites within the RVFV genome.

It was assessed whether the shRNAs within the multimers were being efficiently processed by the RNAi machinery and could inhibit their respective cognate targets. To assess target inhibition, pSI-CHECK reporter vectors containing only the target sequence (minimal target) of each shRNA, downstream of *Renilla* luciferase were generated (Figure 3.6A) and knockdown was an indication that the shRNA was functioning within the multimer. When multimers were transfected with their respective reporters, four of the multimers had two shRNAs that lost the ability to silence their targets and four multimers were able to inhibit all the minimal targets (Figure 3.6B). However, even the multimers that could inhibit their respective targets, there was a slight reduction in reporter levels compared to the individual shRNAs, as in the case of shH1-NSs4 (~10%), shH1-NSs6 (~20%) and shH1-N1 (~10%) (Figure 3.6B). ShH1-NSs7 was able to maintain comparable knockdown to the single shRNAs. The reduced target knockdown suggests that there was a slight loss of the shRNAs' ability to silence their respective targets when inserted into multimers, which has been noted by others (ter Brake et al., 2008). Nevertheless, the multimers inhibited effectively their reporter targets and as there was little difference between the four functional multimers, shNSs4,6,7-N1 was selected to be investigated further.

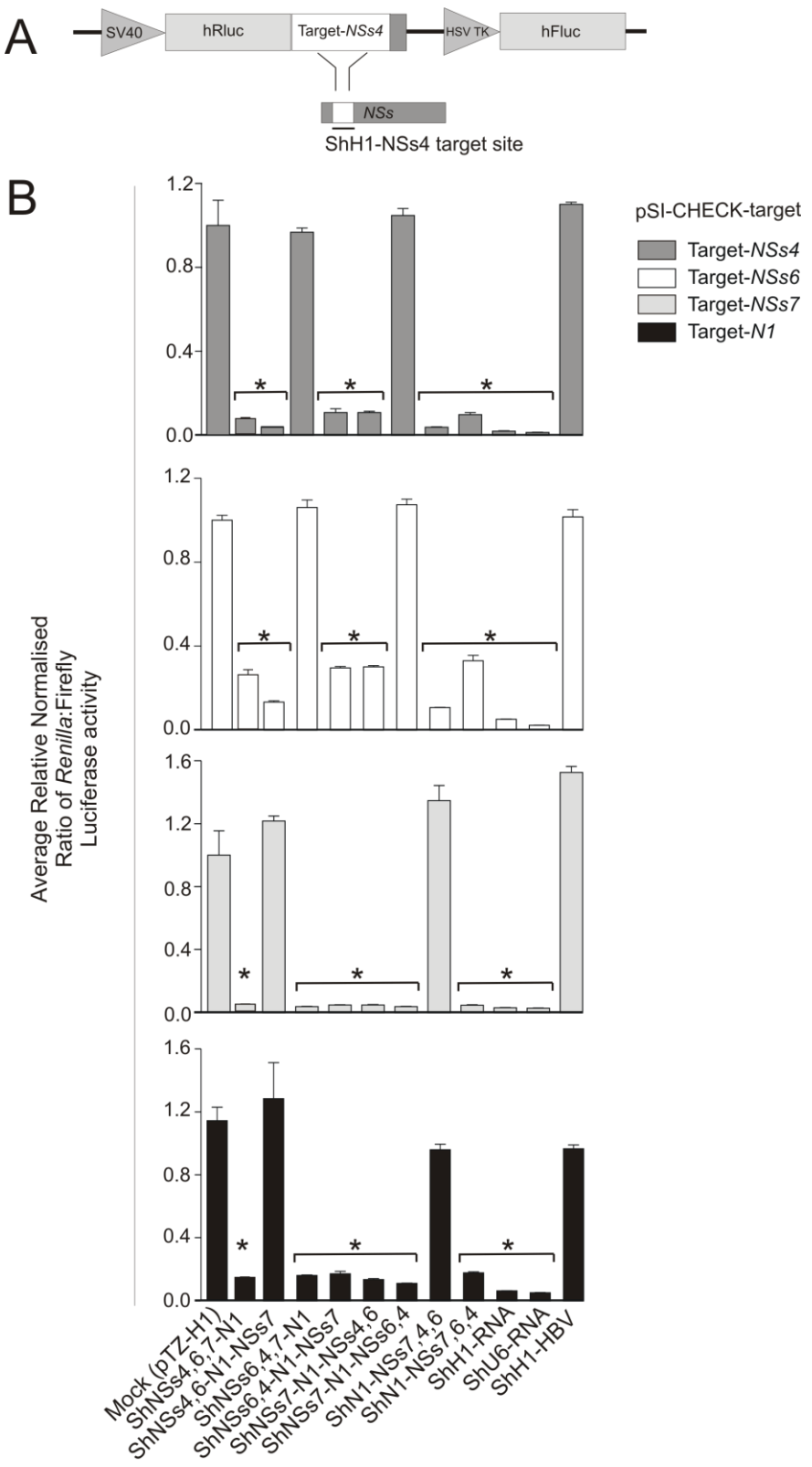

Figure 3.6: Silencing efficiency of the shRNAs from multimer cassettes. (A) Schematic of a minimal target inserted into the 3' UTR of *Renilla* luciferase in a pSI-CHECK reporter vector (B) HEK293 cells were co-transfected with the multimers and a pSI-CHECK reporter vector containing the target site of the shRNA (Target-*NSs4, NSs6, NSs7, N1*). The values represent an average ratio of *Renilla*:Firefly, which have been made relative to the mock (pTZ-H1) control. A H1-Pol III and U6-Pol III shRNA was included as a positive control. ShH1-HBV was included as a negative control. Experiments were performed in technical triplicate with error bars indicating standard deviation (*p<0.05 Unpaired Student's t test compared to the mock control).

3.2.3 ShRNA expression profiling

To determine whether the precursor shRNA was being processed into a mature siRNA, a small RNA northern blot analysis was performed. Total RNA was extracted from cells transfected with the shRNA cassettes and RNAi intermediates detected by hybridising a complementary radiolabeled DNA probe to the guide strand. A mock (pTZ-H1) and shRNA not complementary to the probe was used as a specificity control. ShRNAs (approximately 60 nt in length) and the ~21 nt mature siRNAs were detectable for the single shRNA constructs (Figure 3.7), demonstrating that the shRNAs were expressed and processed by the RNAi pathway. As expected, there was no detectable signal for the mock or the non-complementary shRNAs. Multimeric cassettes produced RNAi intermediates at much lower levels than the single shRNAs, with only detectable concentrations of the 21 nt guide sequences for probes directed to shH1-NSs7 and shH1-N1. This observation, taken with the dual-luciferase minimal target data (Figure 3.6B), suggests that the shRNAs within the multimers were functional, as they could knockdown their respective reporter targets, but were expressed and processed at substantially reduced levels.

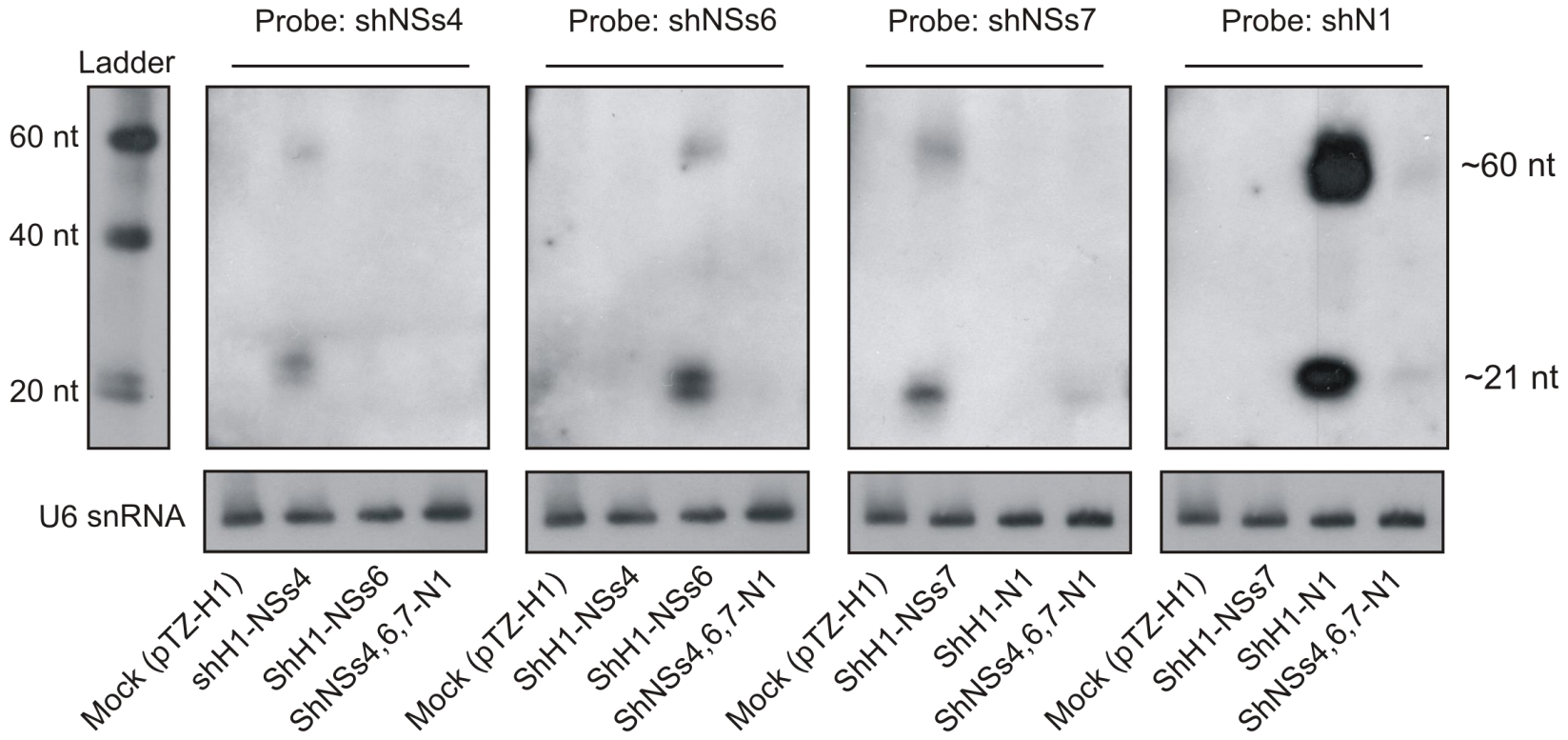

Figure 3.7: Expression and processing of the shRNA constructs. Small RNA northern blot analysis was performed on total RNA extracted from HEK293 cells transfected with the single or multimeric cassettes. A complementary DNA probe to shNSs4, shNSs6, shNSs7 and shN1 guide strand was used to detect the 60 nt precursor shRNA and 21 nt processed siRNA. U6 small nuclear RNA (snRNA) was detected to verify equal loading (nucleotide, nt).

3.2.4 Evaluation of the shRNA's ability to reduce protein levels of expressed NSs

It was assessed whether the shRNAs could have an effect on RVFV's protein levels. To detect the expressed RVFV proteins, a DNA sequence encoding a 3xFLAG tag was attached to the 3' end of a *NSs* and *N* gene (Figure 2.3). The CMV-Pol II expressed FLAG tagged proteins were detected using western blot analysis. When a *NSs*-3xFLAG tag expression vector was transfected with a shRNA, there was a reduction in tagged NSs protein levels for both the ZH548 (~70%) and ZH501 (~60%) strains (Figure 3.8A). Importantly, shH1-N1 did not diminish the levels of NSs, which demonstrates the specificity of the shRNAs targeted to NSs.

As NSs can suppress Pol II transcription (section 1.4.1.1), there may be an unintended bias introduced when detecting the expressed NSs protein. To rule out this possibility, shRNA constructs were transfected with *NSs*-3xFLAG tagged mutants, PP1 and PP2. The NSs mutants have alanine substitutions within the nuclear localisation proline motif (PXXP) rendering them unable to traffic to the nucleus, attenuating their transcription suppressive effects (Billecocq et al., 2004). The reduction in NSs mutant levels by a shRNA was similar to a wild-type protein (Figure 3.8B). However, the FLAG tag itself may interfere with the normal function of NSs, and it needed to be determined whether the FLAG tagged NSs was still able to suppress transcription. A *NSs*-3xFLAG tagged vector was transfected with a *Renilla* luciferase reporter construct and compared to untagged NSs for its ability to suppress *Renilla* luciferase activity. The FLAG tag NSs proteins suppressed *Renilla* luciferase expression similar to that of a NSs without a FLAG tag (Figure 3.8C), demonstrating that the 3xFLAG was not affecting the function of NSs. Overall, the reduction of NSs by a shRNA was independent of NSs-induced global mRNA transcription suppression.

Similarly, it was assessed whether the shRNAs targeted to *NSs* from the shNSs4,6,7-N1 multimer could reduce NSs levels. When the multimer cassette was transfected with a *NSs*-3xFLAG tag expression vector, the reduction of NSs was comparable to that of shH1-NSs7 (Figure 3.8A). Furthermore, shH1-N1 does not affect NSs protein levels and the observed reduction by the multimer was as a result of the anti-*NSs* shRNAs. Therefore, shRNAs targeted to *NSs* within the multimer were able to reduce NSs protein levels, although there was no accumulative benefit in depleting NSs with more than one shRNA.

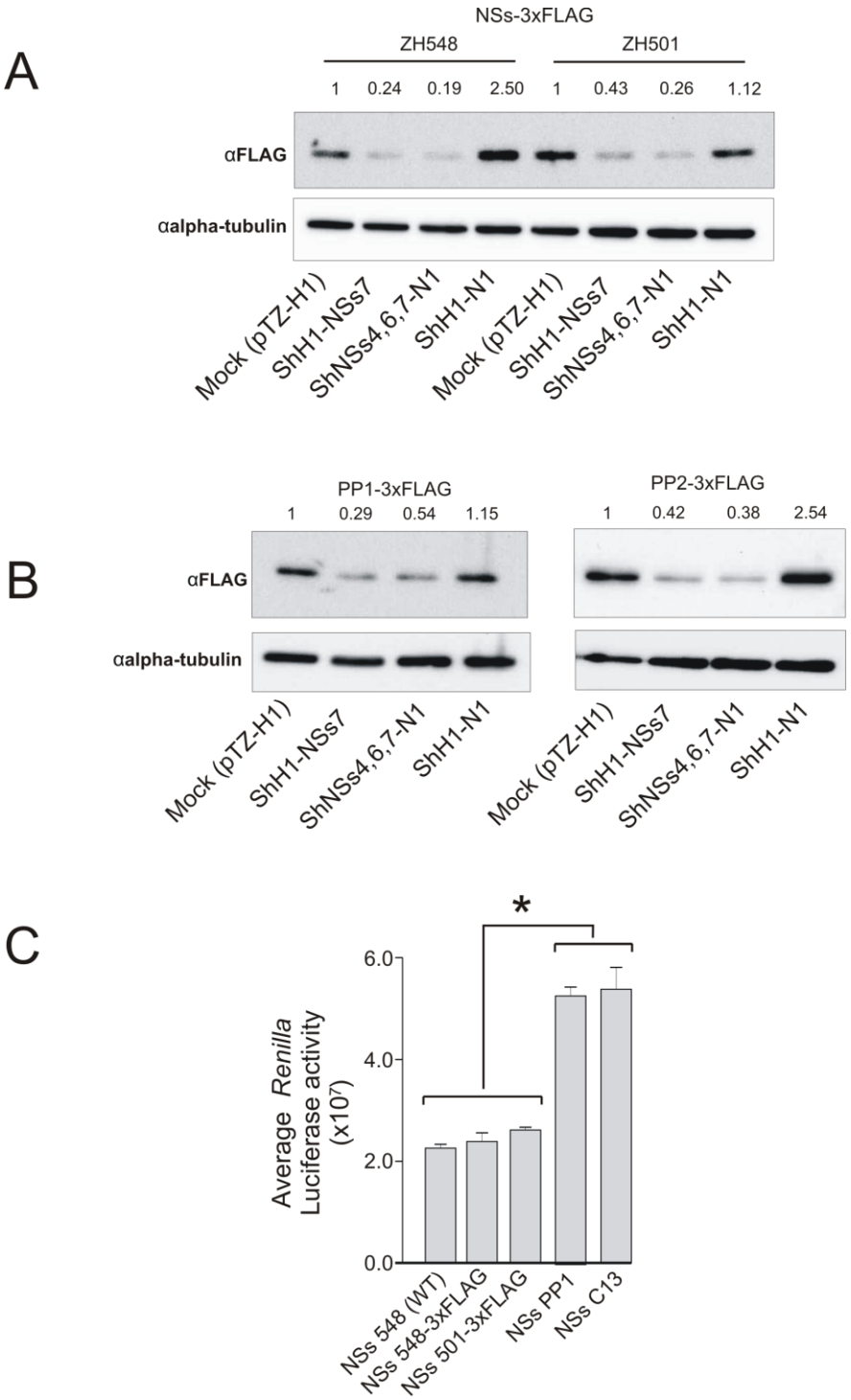

Figure 3.8: Reduction of NSs protein levels by shRNAs. Western blot analysis was performed on HEK293 cells co-transfected with the shRNAs and a vector expressing (A) ZH548 and ZH501 *NSs*-3xFLAG or (B) mutant *NSs*-3xFLAG, PP1 and PP2. A mock (pTZ-H1) and shH1-N1 was included as a negative control. Anti-FLAG antibodies were used to detect tagged NSs. An antibody to α-tubulin was used to verify equal loading. The band densities were quantified using GeneTools software and the values above the lanes are a ratio of FLAG to α-tubulin densities, made relative to the mock control set at 100%. (C) Average *Renilla* luciferase activity was determined in HEK293 cells co-transfected with untagged *NSs* and *NSs*-3xFLAG tagged (ZH548 and ZH501) expression vectors with a *Renilla* luciferase reporter. A nuclear entry NSs mutant, PP1, and truncated NSs, C13, was included as a negative control. Experiments were performed in technical triplicate with error bars indicating standard deviation (*p<0.05 Unpaired Student's t test).

The ability of the shRNAs to reduce 3xFLAG tagged N levels was evaluated. ShH1-N1 reduced N of the ZH501 strain by ~60%, but seemed to have no effect on the ZH548 strain of N (Figure 3.9A). This was unexpected as there was no sequence variation within the target site of the ZH548 that could account for the diminished silencing (data not shown). Nevertheless, this demonstrated that the shRNAs can inhibit N protein levels but in a strain-specific manner. The multimer cassette did not reduce N from either the ZH548 or ZH501 strains (Figure 3.9A). It was determined whether the reduced silencing of shH1-N1 from the multimer observed when knocking down the minimal targets (Figure 3.6B) could account for shNSs4,6,7-N1 inability to reduce N levels. The multimer was transfected with a full length *N* reporter vector to assess whether a clearer distinction of attenuated silencing could be observed. When the multimer was transfected with a full length *NSs* and *N* luciferase reporter target, knockdown against *NSs* was maintained but the knockdown of the *N* reporter target was reduced to 50% (Figure 3.9B). Taken with the northern blot data (Figure 3.7), this suggests that the reduction of shH1-N1 expression and processing from the multimer caused a diminished capacity to reduce N protein expression.

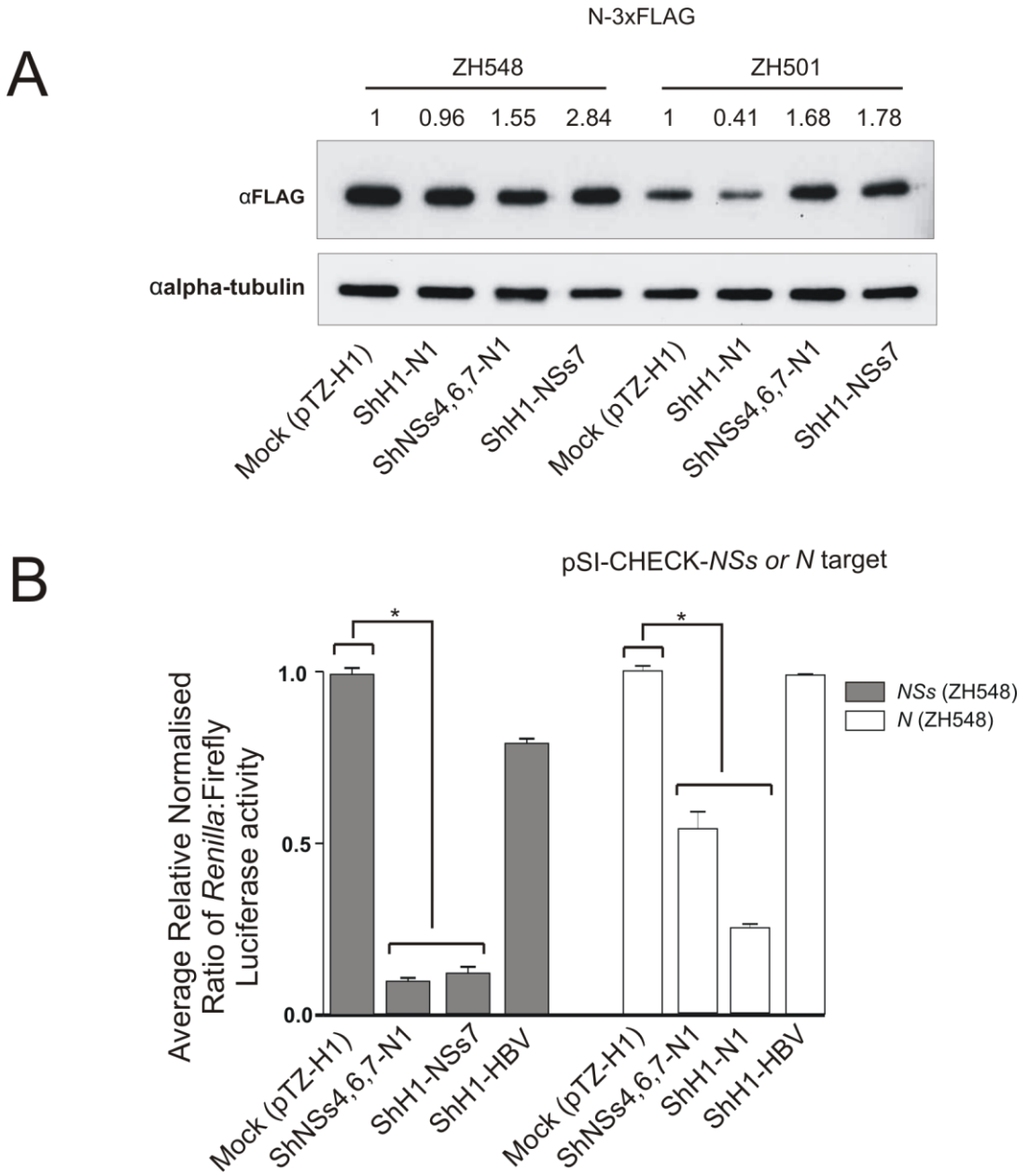

Figure 3.9: Reduction of N protein levels by shRNAs. (A) Western blot analysis was performed on HEK293 cells co-transfected with the shRNAs and a vector expressing *N*-3xFLAG (ZH548 and ZH501). A mock (pTZ-H1) and shH1-NSs7 was included as a negative control. Anti-FLAG antibodies were used to detect FLAG tagged N. An antibody to α-tubulin was used to verify equal loading. The band densities were quantified using GeneTools software and the values above the lanes are a ratio of FLAG to α-tubulin densities, made relative to the mock control set at 100%. (B) HEK293 cells were co-transfected with the shNSs4,6,7-N1 cassette and a full length pSI-CHECK-*NSs* and *N* reporter vector. The values represent an average ratio of *Renilla*:Firefly, which have been made relative to the mock (pTZ-H1) control. A shH1-NSs7 and shH1-N1 was included as a positive control. ShH1-HBV was used as a negative control. Experiments were performed in technical triplicate with error bars indicating standard deviation (*p<0.05 Unpaired Student's t test compared to the mock control).

3.2.5 Evaluation of the shRNA's ability to alleviate pathogenic effects of NSs

3.2.5.1 Assessment of the shRNA's ability to reverse NSs-induced transcription suppression

NSs inhibits host transcription (Le May et al., 2004) and this feature of NSs has been linked to its pathogenic potential, resulting in the inability of the host cell to mount an IFN response (Billecocq et al., 2004). Reversal of this effect by a shRNA would be beneficial to the host during a RVFV infection. Firstly, it was assessed whether NSs could inhibit Pol II transcription. A *Renilla* luciferase reporter was transfected with the expression vectors and luciferase activity was detected as an indicator of the levels of *Renilla* expression. Several NSs mutants that are incapable of suppressing host transcription were used as negative controls, which included the NSs nuclear entry mutants, PP1 and PP2 (Billecocq et al., 2004), and C13 that has a truncated *NSs* gene (Muller et al., 1995). There was a clear reduction (>99%) in the levels of *Renilla* luciferase activity in the presence of the expressed NSs ZH548 (Figure 3.10A) compared to the empty vector (pCI-Neo). Furthermore, a dose-response effect was observed when reducing the amount of NSs expression vector (Figure 3.10B), which suggests that NSs was the determining factor in mediating suppression.

It was then assessed whether shRNAs targeted to NSs could reverse transcriptional suppression. When a shRNA was transfected in the presence of a NSs expression vector, the amount of *Renilla* luciferase was increased by 52% (Figure 3.10C). This effect was maintained even in the presence of increasing amounts of a NSs expression vector (Figure 3.10D). It was then determined whether the reversal of suppression was specific to the NSs ZH548 wild type protein. A shRNA was transfected with PP1, PP2, C13, N, or an empty vector. Only when a shH1-NSs construct was transfected against NSs wild-type was there a ~1-fold increase in *Renilla* luciferase levels (Figure 3.10E). The mock (pTZ-H1), shH1-HBV or shH1-N1 had no effect on luciferase activity. There was a slight increase of ~0.3-fold in *Renilla* luciferase activity when the shRNAs targeted PP1, which correlates with its partial suppression of luciferase activity (~30%) (compared to a empty vector control) (Figure 3.10A). However, there was no observed partial suppressive effect by PP2 (Figure 3.10A), which resulted in no increase in *Renilla* luciferase activity when a shRNA was present (Figure 3.10E), further demonstrating that the reversal by the shRNAs was specifically linked to the suppressive activity of NSs. The multimer cassette was also able to increase the amount of *Renilla* luciferase activity, which was similar to that of a single shRNA (Figure 3.10C and E). Importantly, as shH1-N1 does not affect *Renilla* activity when targeted to NSs (Figure 3.10E), this demonstrates that the shRNAs targeted to NSs within the multimer were the contributing factors in increasing the levels of luciferase. Overall, the above data demonstrate that shRNAs targeted to NSs can reverse NSs-induced transcription suppression.

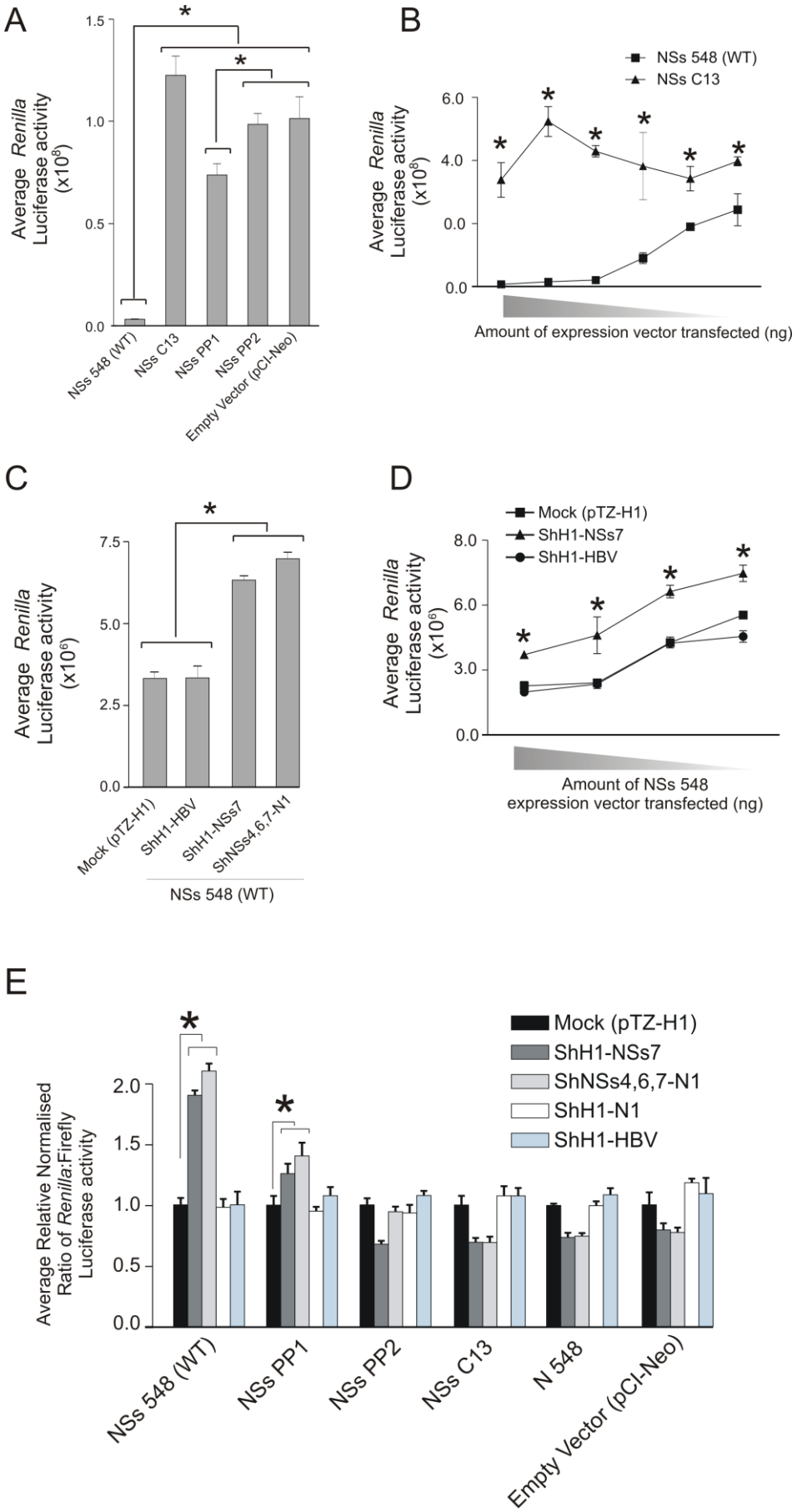
A
Average Renilla Luciferase activity (x10^8)
1.5
1.0
0.5
0.0
*
*
NSs 548 (WT)
NSs C13
NSs PP1
NSs PP2
Empty Vector (pCI-Neo)
B
NSs 548 (WT)
NSs C13
Average Renilla Luciferase activity (x10^8)
6.0
4.0
0.0
0.0
Amount of expression vector transfected (ng)
C
Average Renilla Luciferase activity (x10^6)
7.5
5.0
2.5
0.0
Mock (pTZ-H1)
ShH1-HBV
ShH1-NSs7
ShNSs4,6,7-N1
NSs 548 (WT)
D
Mock (pTZ-H1)
ShH1-NSs7
ShH1-HBV
Average Renilla Luciferase activity (x10^6)
8.0
6.0
3.0
0.0
Amount of NSs 548 expression vector transfected (ng)
E
Mock (pTZ-H1)
ShH1-NSs7
ShNSs4,6,7-N1
ShH1-N1
ShH1-HBV
Average Relative Normalised Ratio of Renilla:Firefly Luciferase activity
2.0
1.5
1.0
0.5
0.0
NSs 548 (WT)
NSs PP1
NSs PP2
NSs C13
N 548
Empty Vector (pCI-Neo)

Figure 3.10: Reversal of NSs-induced transcription suppression by shRNAs. (A) Average *Renilla* luciferase activity was determined when HEK293 cells were co-transfected with *NSs* (ZH548) expression vector and a *Renilla* luciferase reporter. The nuclear entry NSs mutants, PP1 and PP2, a truncated NSs, C13, and empty vector (pCI-Neo) were included as negative controls. (B) Average *Renilla* luciferase activity was determined when HEK293 cells were co-transfected with 20 ng, 10 ng, 5 ng, 2 ng, 1 ng and 0.5 ng of a *NSs* (ZH548) expression vector and a *Renilla* luciferase reporter. A truncated NSs, C13, was included as a negative control. (C) Average *Renilla* luciferase activity was determined when HEK293 cells were co-transfected with a *NSs* (ZH548) expression vector with the shRNAs and a *Renilla* luciferase reporter. A mock (pTZ-H1) and shH1-HBV was included as a negative control. (D) Average *Renilla* luciferase activity was determined when HEK293 cells were co-transfected with 80 ng, 60 ng, 20 ng and 10 ng of a *NSs* (ZH548) expression vector with a shRNA and *Renilla* luciferase reporter. A mock (pTZ-H1) and shH1-HBV was included as a negative control. (E) Average relative *Renilla* luciferase activity was determined when HEK293 cells were co-transfected with expression vectors and the shRNA cassettes with a *Renilla* luciferase reporter. HEK293 cells were transfected with *NSs* 548 WT, the nuclear entry mutants, PP1 and PP2, truncated NSs, C13, *N* expression vector or an empty vector (pCI-Neo). A mock (pTZ-H1), shH1-HBV and shH1-N1 was included as a negative control. All the above experiments were performed in technical triplicate with error bars indicating standard deviation (*$p<0.05$ Unpaired Student's t test compared to the mock control).

3.2.5.2 Assessment of the alleviation of NSs-induced cytotoxic effects by shRNAs

Global host mRNA transcription suppression by NSs, may result in pronounced adverse effects on the host cell. To determine whether NSs could negativity affect the host cell, a NSs expression vector was transfected into cells and a cytotoxic effect was observed using fluorescence microscopy. Cells that received NSs exhibited morphological characteristics of cytotoxicity (rounding, clumping, detached cells) as well as a disrupted monolayer (Figure 3.11A). Cells transfected with the NSs mutants, PP1 or C13, did not visibly affect cell morphology. When observed under a fluorescence microscope, GFP visibility was reduced in cells that received a NSs expression vector, which supports the relationship between cytotoxicity and transcription suppression (Figure 3.11A). The toxicity was then quantified using a MTT assay, which measures mitochondrial reductase activity as an indicator of cell viability. When the *NSs* expression vector was transfected into HEK293 cells, cell viability was ~50% less than that of the control expression vectors (Figure 3.11B). Trichostatin A (TSA) is a chemical compound that causes mammalian cell cycle arrest (Yoshida et al., 1990) and was used as a positive toxic control. Toxicity in the NSs expressing cells was similar to the high-dose (1000 nM) of TSA. It was subsequently determined whether the shRNAs could alleviate the cytotoxicity displayed by NSs expressing cells. When a shRNA was transfected in the presence of a NSs expression vector, there was a 23% increase in cell viability compared to a mock (pTZ-H1) control (Figure 3.11C), which was not observed with the mutant NSs expression vectors, PP1 or C13. Additionally, the multimer cassette was also able to increase cell viability and was similar to the levels observed by shH1-NSs7 (Figure 3.11C). Overall, these data demonstrate that the presence of NSs has adverse effects on the host cell and that shRNAs can alleviate NSs-induced cytotoxicity.

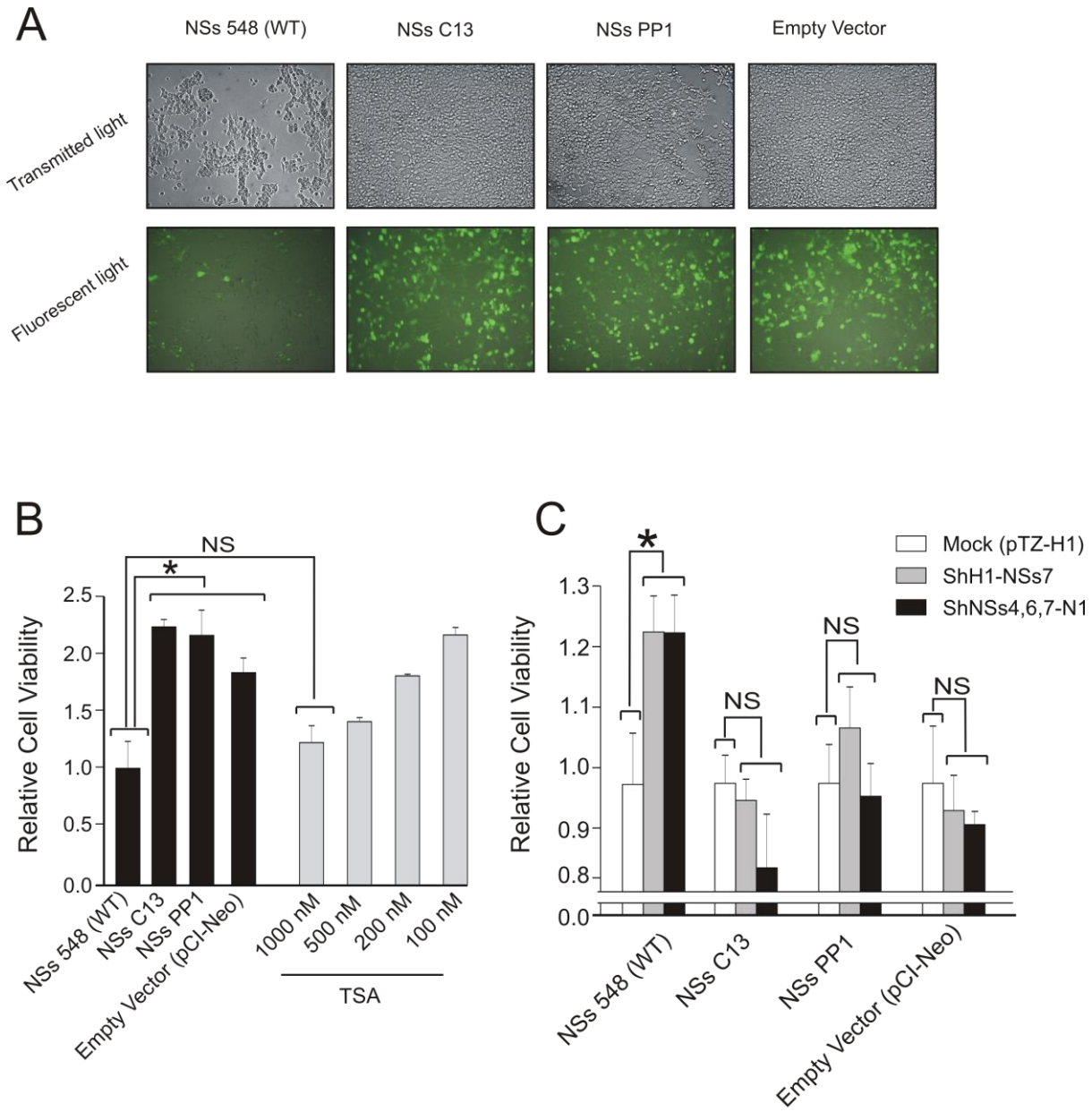

Figure 3.11: Alleviation of NSs-induced cytotoxicity by shRNAs. (A) Transmitted and fluorescence microscopy images were capture of HEK293 cells transfected with a *NSs* (ZH548) expression vector and pCI-GFP reporter. Truncated NSs, C13, a nuclear entry mutant, PP1, and an empty vector was included as a negative control. (B) A MTT cell viability assay was performed on HEK293 cells transfected with a *NSs* (ZH548) expression vector, C13, PP1 and an empty vector. Trichostatin A (TSA) was included at final concentrations of 1000 nM, 500 nM, 200 nM, 100 nM and 10 nM as a toxicity control. (C) A MTT cell viability assay was performed on HEK293 cells co-transfected with the expression vectors, *NSs* 548, C13, PP1 and an empty vector (pCI-neo) with the shRNAs. The experiments were made relative to the mock (pTZ-H1) control. The experiments in (B) and (C) were performed in technical triplicate with error bars indicating standard deviation (*p<0.05 Unpaired Student's t test). Samples were read at a wave-length of 570 nm and normalised to a reference wave-length of 655 nm.

3.2.5.3 Assessment of the shRNA's ability to reverse a NSs-suppressed IFN-β promoter

Previous studies have demonstrated that NSs can direct a repressor complex to the IFN-β promoter to prevent its activation (Le May et al., 2008). To confirm this in a reporter assay, a NSs expression vector was transfected with a Firefly luciferase reporter expressed from an IFN-β promoter (pIF-125luc). In the presence of a potent IFN stimulator, a synthetic dsRNA Poly (I:C) (Stark et al., 1998), Firefly luciferase activity was reduced by NSs (70%) compared to C13 (Figure 3.12A), demonstrating that NSs can suppress IFN-β promoter activity. When the multimeric cassette was transfected with a NSs expression vector, there was a 55% increase in Firefly luciferase activity, which was comparable to C13 (Figure 3.12B). As shH1-N1 did not affect NSs protein levels (Figure 3.8A) or reverse transcriptional suppression (Figure 3.10E), this suggests that the shRNAs targeted to NSs were releasing a NSs-suppressed IFN-β promoter.

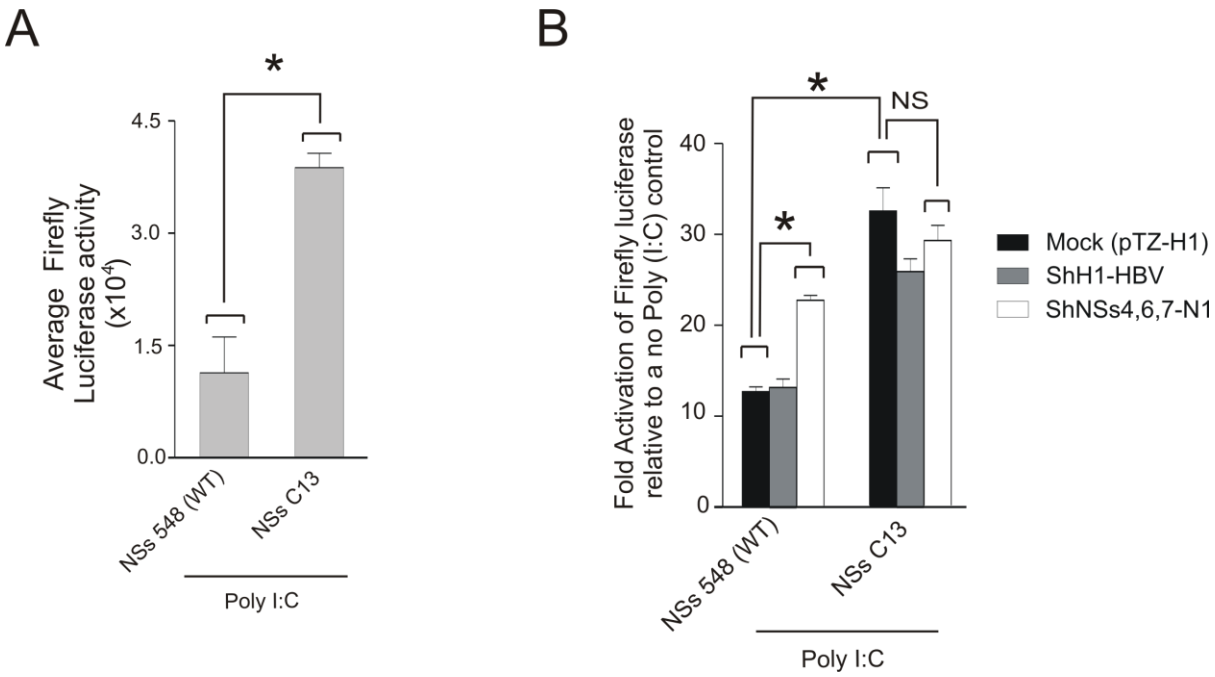

Figure 3.12: Reversing NSs suppression of the IFN-β promoter by shRNAs. (A) Average Firefly luciferase activity was determined when HEK293 cells were co-transfected with the *NSs* (ZH548) or a C13 expression vector and pIF-125luc reporter (a Firefly luciferase reporter expressed from an IFN-β promoter). Cells were treated with Poly (I:C) before quantification of Firefly luciferase activity. (B) Average Firefly luciferase activity was determined when HEK293 cells were co-transfected with a *NSs* (ZH548) or C13 and a multimeric shRNA cassette in the presence of a pIF-125luc reporter. Cells were treated with Poly (I:C) before quantification of Firefly luciferase activity. These data represent a fold-activation of Firefly luciferase activity normalised to a no-Poly (I:C) control. The experiment was made relative to the mock (pTZ-H1). ShH1-HBV was included as a negative control. The above experiments were performed in technical triplicate with error bars indicating standard deviation (*p<0.05 Unpaired Student's t test).

3.3 Discussion

ShRNAs were designed to target the *NSs* gene of RVFV. It was demonstrated that a shRNA targeted to NSs could reverse transcription suppression (Figure 3.10), alleviate NSs-associated cytotoxicity (Figure 3.11) and release the IFN-β promoter suppression (Figure 3.12). Furthermore, a shRNA could reduce NSs protein levels (Figure 3.8A) and demonstrated that down-regulation of NSs by shRNAs was affecting the function of NSs. The removal of NSs results in an attenuated infection (Muller et al., 1995) with enhanced IFN (Lihoradova et al., 2012) and antibody responses (Bird et al., 2008). These data suggest that the shRNAs should recapitulate a NSs-deficient state, which would attenuate RVFV infection pathology.

NSs is suspected to gain nuclear entry through its interaction with p44 to suppress host transcription (Le May et al., 2004). The NSs mutants, PP1 and PP2, have their nuclear import motifs altered that prevents nucleus localisation of NSs (Billecocq et al., 2004). However, unlike the PP2 mutant, PP1 had a partial suppressive effect on reporter levels (Figure 3.10A). This contrasts with the observations of others, who demonstrated that a CMV expressed β-galactosidase reporter was partially suppressed by both mutants with equal effect (Billecocq et al., 2004). This may reflect intrinsic differences in the sensitivity of the assays, where a CMV expressed *Renilla* reporter may be able to discern more subtle difference between mutants. Based on the data reported here, the second proline motif might be more important for nuclear entry, whereas mutation of the first proline motif, although effective, does not completely abrogate its effects in the nucleus and may highlight significant differences in the motifs interaction with NSs's nuclear entry protein, p44 (Le May et al., 2004). Interestingly, PP1 did not demonstrate the same cytotoxic effects as WT NSs, which strongly suggests that nuclear entry was required for toxicity (Figure 3.11). A reduction in GFP reporter expression was observed with NSs-induced morphological changes, which implies that the toxicity was as a result of transcription suppression (Figure 3.11A). Toxicity may also be because of NSs's interaction with YY1 at pericentromeric γ-satellites sequences, which has been linked to segregation and cohesion defects (Mansuroglu et al., 2010). Nevertheless, the data demonstrate that NSs requires nuclear entry to exert a toxic effect and highlights important differences between the entry motifs.

Multiple expressed shRNA cassettes were developed, which targeted three shRNAs to *NSs* and one shRNA to *N* (Figure 3.6). The tetramer demonstrated reduced shRNA expression and guide strand processing in northern blot analysis (Figure 3.7), and reduced silencing (Figure 3.6B) that fits with other studies showing that increasing the number of tandem shRNAs expressed from H1-Pol III promoters results in a general reduction of shRNA's expression and knockdown of their cognate targets (McIntyre et al., 2011a, ter Brake et al., 2008). ShH1-N1 within the tetramer had reduced

silencing activity against a full length *N* reporter and was unable to reduce viral protein levels (Figure 3.9A and B). Extensive studies have shown that the farther downstream the shRNA is placed in tandem in the multimer (position 3 onwards), the greater the deleterious effect on a shRNA (McIntyre et al., 2011a) and the placement of shH1-N1 in the fourth position may be the reason for its attenuated silencing. The multimer targeting NSs had similar levels of knockdown of NSs as an individual shRNA (Figure 3.8A) even though each shRNA had reduced expression from the multimer (Figure 3.7). This may be as a result of an additive effect of each shRNA working in synergy to facilitate target inhibition. The additive effect of shRNAs has not been observed by others (McIntyre et al., 2011a). Sh1-NSs7 within the multimer was also able to maintain silencing in a reporter assay compared to its individual shRNA, whereas shH1-NSs4's and shH1-NSs6's knockdown was slightly decreased (Figure 3.6B) and the data suggest that the most active shRNA within a multimer determines the overall knockdown efficacy.

Several virulence factors of other viruses that cause acute infections have been targeted using RNAi, such as EBOV's VP35 and VP24 (Geisbert et al., 2010), influenza's non-structural gene 1 (NS1), severe acute respiratory syndrome (SARS) coronavirus's non-structural protein 1 (Ni et al., 2005), NW arenavirus's Z (Artuso et al., 2009) and LASV's N (Muller and Gunther, 2007). Most of these studies have demonstrated effective down-regulation of gene expression but only a few have investigated the reversal of pathogenic properties. SiRNAs targeted to NS1 of influenza (Rajput et al., 2012) and NS2 of respiratory syncytial virus (RSV) (Ramaswamy et al., 2006) were able to restablish IFN markers for each virus. An extensive investigation of siRNAs against a IFN antagonist of RSV, NS1, demonstrated an increase in IFN and IFN related genes, such as STAT and restriction factors, *in vitro* (Zhang et al., 2005). Furthermore, higher levels of *IFN-β* mRNA were present in anti-*NS1* siRNA treated mice demonstrating that IFN suppression was reversed *in vivo.* Protection against lethality in mice was attributed to an IFN mechanism rather than direct inhibition of viral replication by the siRNAs and suggests that the observed alleviation of NSs's pathogenic properties in this study would translate into an animal model.

Overall, it was demonstrated that expressed shRNAs could disrupt RVFV pathogenic effects. This will assist in attenuating features of haemorrhagic RVFV cases, through interference of NSs-associated enhancement of infection. Even though NSs is important for subverting host defences, it is not an essential component of the RNA synthesis machinery and not required for viral replication. The removal of NSs by shRNAs would indirectly inhibit viral replication through activation of the IFN response but shRNAs that target structural proteins would directly hinder RVFV. These shRNAs would function by down-regulating essential proteins from the RVFV life cycle and may yield shRNAs with potent anti-replicative properties.

Chapter 4

Inhibition of RVFV replication in cultured cells using expressed shRNAs

4.1 Introduction

Expressed shRNAs were able to alleviate NSs-associated pathogenic effects of RVFV. Although removal of NSs highly attenuates infection (Muller et al., 1995), NSs is not essential for viral replication (Ikegami et al., 2006) and other proteins within RVFV's life cycle should be considered as targets of RNAi. As a result of the high amounts of viraemia that are maintained throughout lethal infections of RVFV (Morrill et al., 1990), the virus would require continuous protein expression to produce new infectious particles. Proteins that are vital for RVFV replication such as structural components of the virus could be targeted with shRNAs. Diminishing the supply of structural proteins by down-regulation of mRNA by RNAi-mediated cleavage, could hinder the ability of the virus to sustain the high levels of replication required in severe cases of RVF, which would improve disease outcome.

There are two genes of RVFV that encode structural proteins, namely *N* and *M*. The N protein is required for RNA synthesis (Lopez et al., 1995), viral assembly (Habjan et al., 2009a) and possibly involved in structural stability of the mature virion (Piper et al., 2011) (section 1.2.3 and 1.2.4). Furthermore, several studies have targeted *N* from other viruses using RNAi such in the case of LASV (Muller and Gunther, 2007) and MARV (Fowler et al., 2005) (section 1.7), demonstrating successful hindrance of viral replication. The *M* gene encodes two glycoproteins, Gn and Gc (section 1.2.2), which are involved in genome packaging (Piper et al., 2011), the formation of uniform, mature viral particles (Habjan et al., 2009a) and are essential components of the receptor recognition complex (Garry and Garry, 2004) (section 1.2.4). The M segment produces a single mRNA encoding both glycoproteins (section 1.2.2), which suggests that a shRNA could effectively down-regulate both glycoproteins simultaneously, potentially enhancing its disruptive capacity. The importance of *N* and *M* within RVFV's life cycle makes them attractive targets for inhibition by expressed shRNAs.

ShRNAs targeted to *N* were able to inhibit RVFV replication in a live-viral challenge assay. Following this assessment, shRNAs against *M* were developed and demonstrated effective knockdown of *M* reporter levels as well as down-regulation of *M* encoded glycoproteins. Finally, shRNAs to *M* were able to potently inhibit RVFV replication in cultured cells. It was demonstrated that shRNAs targeted to structural proteins could effectively inhibit RVFV replication and may better

control the high levels of RVFV replication associated with haemorrhagic fever cases, which in turn could “manage” the disease until host immunity can facilitate viral clearance.

4.2 Results

4.2.1 Inhibitory effects of shRNAs on RVFV replication targeted to N

4.2.1.1 Challenge assay

ShRNAs directed to *N* were previously generated (Figure 3.2B) with shH1-N1 able to reduce N protein levels (Figure 3.9). It was determined whether a reduction in protein levels translated into the inhibition of RVFV replication. To assess whether a shRNA could reduce RVFV replication in cell culture, a challenge assay was performed. Huh7 cells were used in this experiment as they are a liver derived cell line, a target organ of RVFV *in vivo* and can support replication (Nakabayashi et al., 1982). ShRNAs were transfected into Huh7 cells and 24 hrs post-transfection the cells were infected with a South African (SA) isolate 1981 of RVFV. The SA isolate was used as there have been recurring, recent outbreaks of RVFV in South Africa (Grobbelaar et al., 2011). As a result of the low genetic diversity this isolate would be a model RVFV strain from the more recent outbreaks. Supernatant was collected every 24 hrs for three days and the N antigen detected using an ELISA as an indicator of viral replication (Jansen van Vuren and Paweska, 2009) (Appendices A1.11 and Figure A.1). The RVFV work was performed under BSL3 conditions and to reduce exposure to RVFV during this initial assessment, the experiments were performed in technical duplicate.

In the cells that received a shH1-N1 construct, there was a clear reduction in viral replication, resulting in 24.4% and 15.5% reduction at 48 hrs and 72 hrs, respectively (relative to the shH1-HBV control) (Figure 4.1). Cells that received only virus or cells that were transfected with a shH1-HBV control resulted in significant detection of the N antigen. Cells that expressed the shH1-NSs7 did not result in a reduction in viral replication. As NSs is dispensable for viral replication in cell culture, it serves as a RVFV target that does not have anti-replicative properties (Ikegami et al., 2006). The multimer cassette did not inhibit viral replication (Figure 4.1). As it can be ruled out that the shRNAs directed against *NSs* would have an effect on viral replication, this suggests that shH1-N1 had lost its capacity to silence RVFV. This fits with previous data where the multimer was unable to reduce N levels (Figure 3.9A) as a result of shH1-N1’s reduced knockdown, which was below the threshold required to affect protein expression (Figure 3.9B). Overall, this was the first demonstration that expressed RNAi directed against RVFV inhibited viral replication in cultured cells.

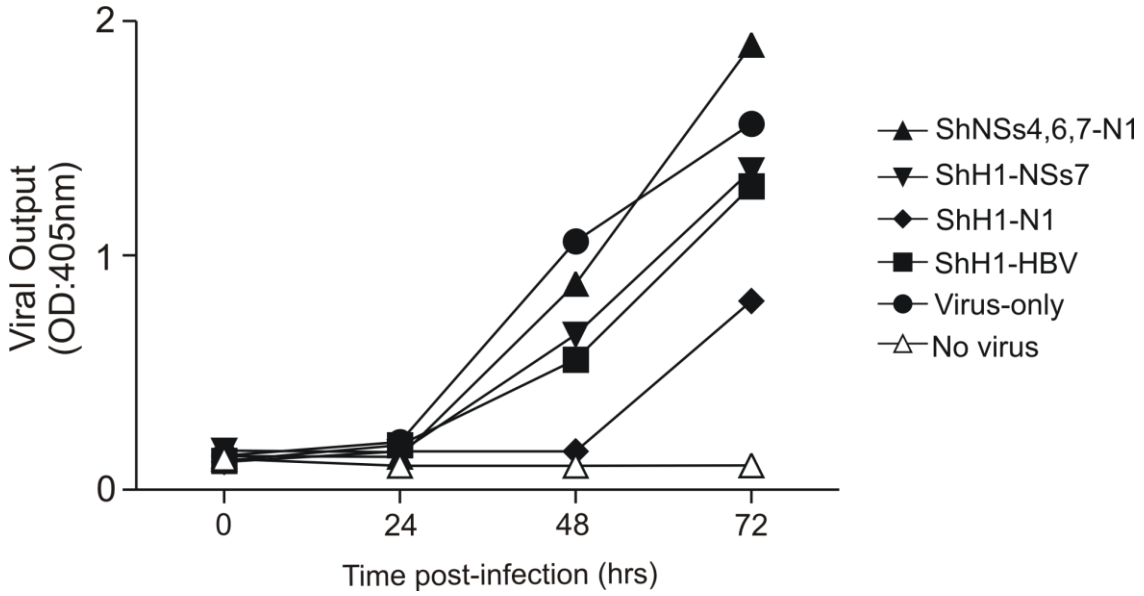

Figure 4.1: Inhibition of viral replication by shRNAs targeted to *N*. Huh7 cells were transfected with the shRNAs, 24 hrs prior to infection with the SA1981 isolate of RVFV at a $TCID_{50}$ $1x10^{3.8}$/ml. Every 24 hrs for 3 days, supernatant was collected and the N antigen was detected using an ELISA. Cells that did not receive a shRNA (Virus-only) or shH1-HBV was included as a negative control. Uninfected (no virus) cells were included to obtain a baseline measurement. Samples were measured at a wave-length of 405 nm. Means were obtained by samples measured in duplicate.

4.2.1.2 Evaluation of alternative shRNAs directed to N

Although shH1-N1 demonstrated a capacity to inhibit viral replication in a challenge assay (Figure 4.1), further alternate shRNAs were investigated to assess whether inhibition could be improved. To this end, four more shRNAs directed to *N* were designed as previously described and expressed from H1-Pol III promoters (Figure 3.1). To determine the knockdown efficiency of the new shRNAs, a dual-luciferase assay was performed. ShRNAs were transfected with a pSI-CHECK-*N* (ZH548 or ZH501) reporter vector and demonstrated a knockdown efficiency of between 20-80% (Figure 4.2A). ShH1-N6 and ShH1-N8 were the most effective with ~60% and ~80% reduction, respectively. It was then determined whether the shRNAs could decrease the levels of N using the western blot analysis protocol that has been previously described (section 3.2.4). To screen a broader range of shRNA candidates, all of new shRNAs (shH1-N5-N8) and two previously described shRNAs, shH1-N3 and shH1-N4, were included for further testing. ShRNAs were transfected into cells with a 3xFLAG tagged *N* (ZH548 or ZH501) expression vector and unlike shH1-N1, whose inhibitory effect was strain specific (Figure 3.9A), shH1-N3 and shH1-N4 reduced N from both strains (Figure 4.2B). However, similar to shH1-N1, the reduction of N was more pronounced against the ZH501

strain, which was between 80-90%, than the ZH548 strain at ~40% (Figure 4.2B). This bias was also observed with shH1-N6, which was only effective at reducing ZH501 levels (~70%). ShH1-N8 did not affect N, even though it was the most effective in the luciferase reporter assay (Figure 4.2A and B).

Based on the western blot analysis, it was assessed whether the two most effective shRNAs, shH1-N3 and shH1-N4, could inhibit viral replication, but the challenge assay was slightly modified from the protocol described above (4.2.1.1). Firstly, HEK293 cells were used rather than Huh7 cells, as it was determined that HEK293 cells could sustain RVFV replication (Appendices Figure A.3). Secondly, a GFP expression vector was included to determine transfection efficiency but not all of the Huh7 cells had visible fluorescence as a result of an incomplete transfection (data not shown), which suggests that there were cells that had not received a shRNA. HEK293 cells have a high transfection efficiency (~99%)[1], which would reduce the possibility that RVFV replication was as a result of cells lacking shRNA expression. Thirdly, a lower infectious dose of RVFV was used at a $TCID_{50}$ $1x10^{2.8}$/ml, as linear replication kinetics were observed at this dose, which could better discern important differences within the experiments (Appendices Figure A.3). Fourthly, supernatants were taken from 24-72 hrs, as there was no difference in virus detected between time points 0 hrs and 24 hrs. Finally, having confirmed an inhibitory effect of a shRNA against RVFV in the initial challenge assay (Figure 4.1), experiments were performed in technical triplicate. All other procedures were carried out as previously described (4.2.1.1, Appendices A1.11). ShH1-N1 was still the most effective shRNA with a reduction in viral replication of 79.8% at 72 hrs (made relative to the shH1-HBV control), but was only slightly better than shH1-N3 of 69.9% (Figure 4.2C). ShH1-N4 did not have any effect on viral replication and had similar detectable N levels to the controls. There was improved inhibition by shH1-N1 using the HEK293 cells (Figure 4.2C) compared to the Huh7 cells (Figure 4.1) and this may be attributed to the better transfection efficiency. Nevertheless, shH1-N3 was identified as a possible alternative candidate for the suppression of RVFV replication.

[1] http://tools.invitrogen.com/Content/SFS/ProductNotes/F_Lipofectamine%202000b-040923-RD-MKT-TL-HL050602.pdf

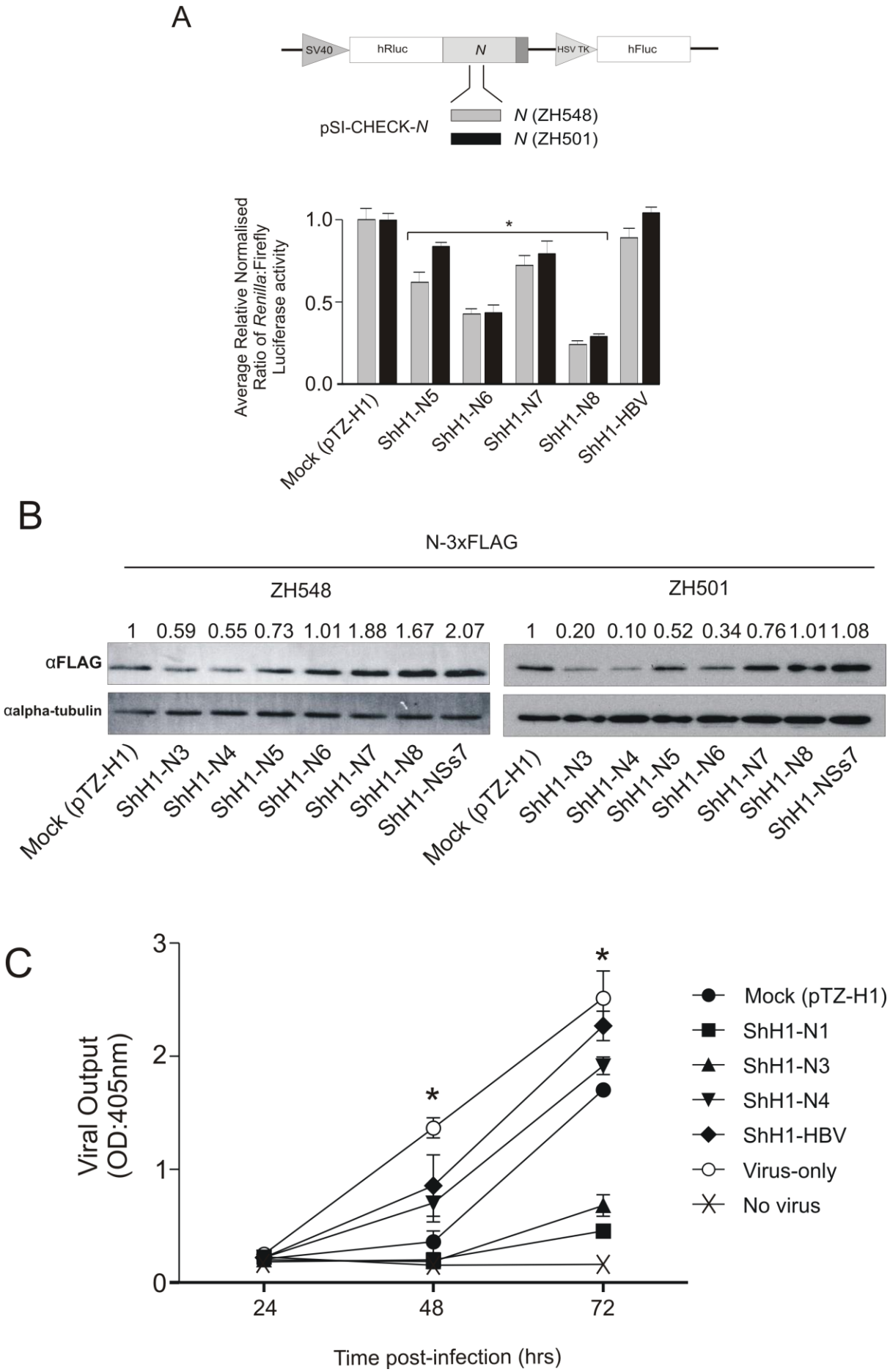
A
SV40
hRluc
N
HSV TK
hFluc
pSI-CHECK-N
N (ZH548)
N (ZH501)
Average Relative Normalised Ratio of Renilla:Firefly Luciferase activity
1.0
0.5
0.0
*
Mock (pTZ-H1)
ShH1-N5
ShH1-N6
ShH1-N7
ShH1-N8
ShH1-HBV
B
N-3xFLAG
ZH548
ZH501
1 0.59 0.55 0.73 1.01 1.88 1.67 2.07
1 0.20 0.10 0.52 0.34 0.76 1.01 1.08
αFLAG
αalpha-tubulin
Mock (pTZ-H1)
ShH1-N3
ShH1-N4
ShH1-N5
ShH1-N6
ShH1-N7
ShH1-N8
ShH1-NSs7
C
Viral Output (OD:405nm)
3
2
1
0
*
*
24
48
72
Time post-infection (hrs)
Mock (pTZ-H1)
ShH1-N1
ShH1-N3
ShH1-N4
ShH1-HBV
Virus-only
No virus

Figure 4.2: Evaluation of alternative shRNAs targeting *N*. (A) HEK293 cells were co-transfected with the shRNAs and a full length pSI-CHECK-*N* (ZH548 or ZH501) reporter vector. The values represent an average ratio of *Renilla*:Firefly, which have been made relative to the mock (pTZ-H1) control. A shH1-HBV was used as a negative control. Experiments were performed in technical triplicate with error bars indicating standard deviation (*p<0.05 Unpaired Student's t test compared to the mock control). (B) Western blot analysis was performed on HEK293 cells co-transfected with the shRNAs and an *N*-3xFLAG tag (ZH548 or ZH501) expression vector. A mock (pTZ-H1) and shH1-NSs7 was included as a negative control. Anti-FLAG antibodies were used to detect tagged N. Antibodies to α-tubulin were used to verify equal loading. The band densities were quantified using GeneTools software and the values above the lanes are a ratio of FLAG to α-tubulin densities, made relative to the mock control set at 100%. (C) ShRNA constructs were transfected into HEK293 cells, 24 hrs prior to infection with the SA1981 isolate of RVFV at a $TCID_{50}$ $1x10^{2.8}$/ml. Every 24 hrs for 3 days, supernatant was collected and the N antigen was detected using an ELISA. Cells that did not receive a shRNA (Virus-only), mock (pTZ-H1) and shH1-HBV were included as negative controls. Uninfected (no virus) cells were included to obtain a baseline. Samples were measured at a wave-length of 405 nm. Experiments were performed in technical triplicate and the samples were measured twice. Error bars indicate standard deviation (*p<0.05 Unpaired Student's t test compared to a shH1-HBV control).

4.2.2 Inhibition of a M *gene reporter by shRNAs*

Although improved inhibition of RVFV was observed in HEK293 cells using the anti-*N* shRNAs (4.2.1) there was still the appearance of viral replication at 72 hrs post-infection (Figure 4.2C), which suggests that the virus was overwhelming the shRNAs. The M segment was subsequently selected as an alternative target as it encodes two other structural proteins, Gn and Gc. Five H1-Pol III expressed shRNAs were designed to target the *M* gene (Figure 3.1) and their ability to inhibit *M* was determined using a dual-luciferase reporter assay. *M* from the ZH501 strain of RVFV was inserted into the 3' UTR of *Renilla* luciferase in a pSI-CHECK 2.2 reporter vector and knockdown was determined as previously described (section 3.2.1). Only the ZH501 strain was used in this study, as it has been previously shown that the chosen shRNA target sites were highly conserved (Figure 3.3) and because of the remarkably high level of sequence conservation of *M* across strains (Bird et al., 2007a). Focus was rather placed on determining the effectiveness of the shRNA's anti-replicative properties. When the shRNAs were transfected with a pSI-CHECK-*M* reporter vector, knockdown was between 50%-80% (Figure 4.3), which was generally better than the observed knockdown of the anti-*N* shRNAs (Figure 4.2A).

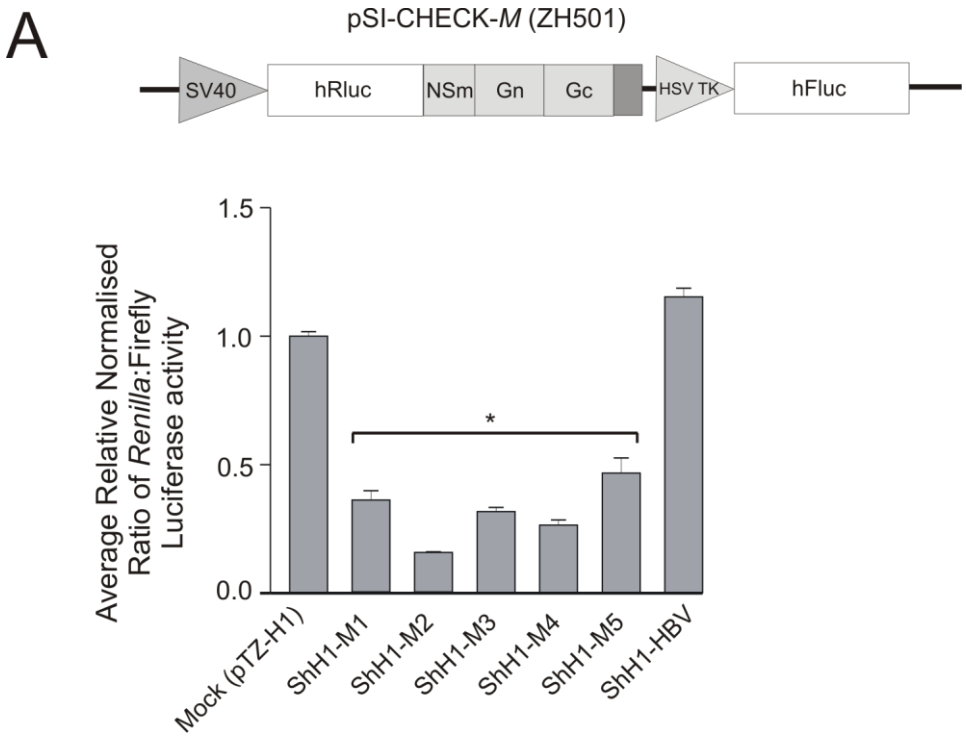

Figure 4.3: Inhibition efficiency of shRNAs targeting *M*. HEK293 cells were co-transfected with shRNAs and a full length pSI-CHECK-*M* (ZH501) reporter vector. The values represent an average ratio of *Renilla*:Firefly, which have been made relative to the mock (pTZ-H1) control. A shH1-HBV was used as a negative control. Experiments were performed in technical triplicate with error bars indicating standard deviation (*p<0.05 Unpaired Student's t test compared to the mock control).

4.2.3 Assessment of the shRNA's ability to decrease M encoded proteins

It was determined whether the shRNAs could decrease the levels of protein expressed from the *M* sequence. An *M* that includes the NSm region cannot be expressed off a Pol II promoter for currently unknown reasons (Personal communication, Sonja Gerrard). A smaller M expression vector consisting of *M* sequence from the 4th translation start codon can be generated, which will express the glycoproteins, Gn and Gc, but lacks NSm1 and NSm2 (ΔNSm) (Figure 4.4). The glycoprotein's sequence was inserted into a mammalian expression vector downstream of a CMV-Pol II promoter. A FLAG sequence was present at the 5' end of Gc allowing for the detection of an N-terminal FLAG tagged Gc (Gerrard and Nichol, 2007). The shRNA target sites were within the Gn region and if a decrease in Gc was observed, it was an indicator that both glycoproteins were being down-regulated by a shRNA.

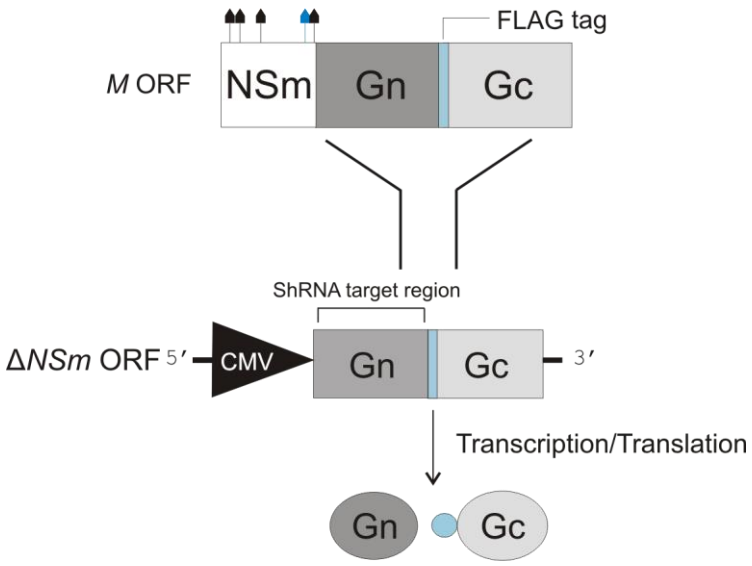

Figure 4.4: Schematic of the ΔNSm expression vector. *M* was cloned from the 4th translation start codon (blue marker) and inserted into a CMV-Pol II mammalian expression vector. A FLAG tag was present at the 5' end of the Gc glycoprotein. After mRNA translation, the Gn-Gc heterodimer will be proteolytically processed into individual Gn and Gc. The Gc has a N-terminal FLAG tag epitope for detection using anti-FLAG antibodies. All shRNA target sites were within the Gn region.

When the shRNAs were transfected with a ΔNSm expression vector, the concentration of Gc was reduced (Figure 4.5). ShH1-M1, shH1-M2, shH1-M4 and shH1-M5 reduced the levels of tagged Gc protein by 73%, 79%, 77% and 85%, respectively, whereas shH1-M3 reduced Gc concentrations to 59%. ShRNAs targeted to *M* were specific as anti-*NSs* and anti-*N* shRNAs did not affect Gc protein levels. Furthermore, the anti-*M* shRNAs appeared to reduce Gc more effectively than the anti-*N* shRNAs reduced N (Figure 3.9A and Figure 4.2B). As the shRNAs targeted the Gn region, these data suggest that the entire ΔNSm mRNA was being degraded, effectively down-regulating both encoded proteins, Gn and Gc.

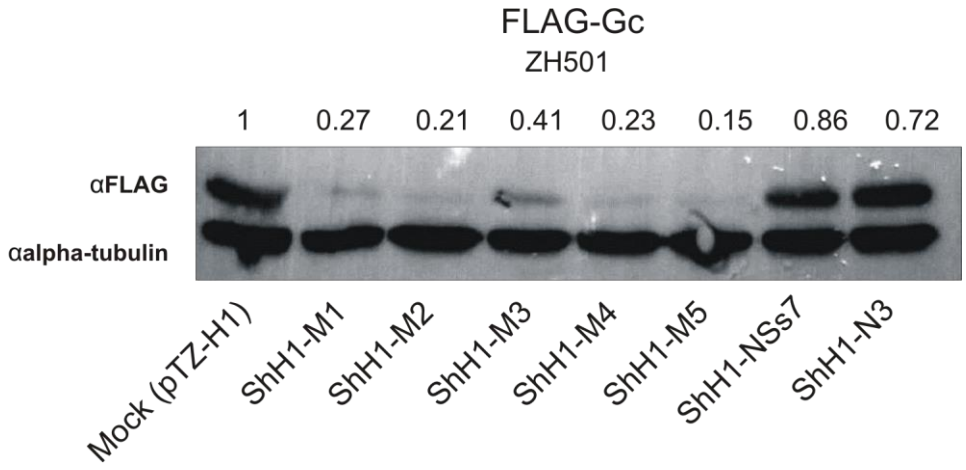

Figure 4.5: Inhibition of *M* encoded proteins. A western blot analysis was performed on HEK293 cells co-transfected with the shRNAs and a FLAG tagged ΔNSm expression vector (ZH501). A mock (pTZ-H1) and shRNAs targeted to the *NSs* and *N* genes of RVFV (shH1-NSs7 and shH1-N3) were included as negative controls. Anti-FLAG antibodies were used to detect tagged Gc glycoprotein. Antibodies specific for α-tubulin were used to verify equal loading. The band densities were quantified using GeneTools software and the values above the lanes are a ratio of FLAG to α-tubulin densities, made relative to the mock control set at 100%.

4.2.4 Inhibitory effects of shRNAs on RVFV replication targeted to M

The anti-replicative effects of the shRNAs targeted to *M* were subsequently assessed in a challenge assay. *M* was more sensitive to RNAi-mediated down-regulation as each shRNA demonstrated a high level of effectiveness based on the above luciferase (Figure 4.3) and protein quantification data (Figure 4.5). This yielded a greater cohort of potentially effective shRNAs against RVFV, as opposed to the fewer effective shRNAs targeted to *N* (Figure 4.2A and B). As a result of the better inhibition demonstrated by the anti-*M* shRNAs, all shRNAs were included in a challenge assay. The challenge assay was performed as previously described, except it was extended to 96 hrs (section 4.2.1.2) to observe whether viral replication could overwhelm the shRNAs in an extended assay. The shH1-M2, shH1-M3 and shH1-M5 constructs resulted in undetectable levels of RVFV over the 96 hr study (Figure 4.6) and there was no statistically significant difference compared to the baseline levels of a no virus control. Although not as potent, shH1-M1 was still able to inhibit viral replication at 78.9% and 74.6% at 72 hrs and 96 hrs, respectively. ShH1-M4 did not affect viral replication. These shRNAs were more effective at suppressing RVFV compared to the shRNAs targeted to *N* (Figure 4.1 and 4.2C) and demonstrated that anti-*M* shRNAs were potent inhibitors of RVFV replication in cultured cells.

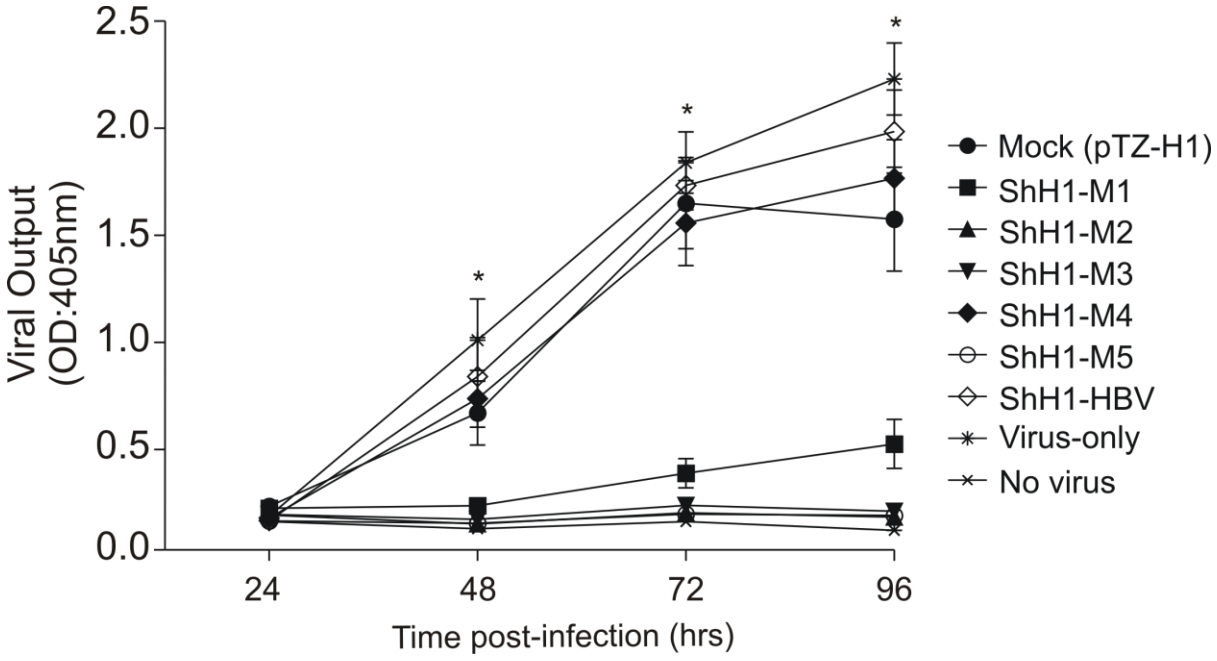

Figure 4.6: Inhibition of RVFV replication by shRNAs targeted to *M*. HEK293 cells were transfected with the shRNAs, 24 hrs prior to infection with the SA1981 isolate of RVFV at a $TCID_{50}$ $1x10^{2.8}$/ml. Every 24 hrs for 4 days, supernatant was collected and the N antigen detected using an ELISA. Cells that did not receive a shRNA (Virus-only), mock (pTZ-H1) and shH1-HBV were included as negative controls. Uninfected (no virus) cells were included to obtain a baseline measurement. Samples were measured at a wave-length of 405 nm. Experiments were performed in technical triplicate and the samples were measured twice. Error bars indicate standard deviation (*$p<0.05$ Unpaired Student's t test compared to the shH1-HBV control).

4.2.5 Evaluation of a functional multimer

Previously it was demonstrated that within the multimeric constructs that contained four shRNAs (tetramers), there was reduced silencing by the shRNAs (Figure 3.6B). ShNSs4,7,8-N1 could not inhibit viral replication (Figure 4.1) as a result of the attenuated silencing by shH1-N1 (Figure 3.9A and B). To determine whether a functional multimer could be obtained that would inhibit RVFV replication, additional multimers were generated. The number of shRNAs were reduced to three shRNAs (trimers) as recent studies have demonstrated increasing the number of shRNAs in a multimer can affect their silencing capacity (McIntyre et al., 2011a). The position of the shRNAs was also assessed as a factor in determining functionality, to increase the potential likelihood of obtaining an effective multimer.

A shRNA targeted to *NSs*, *N* and *M* was included in a trimer, as this would target three factors within RVFV's life cycle and could enhance suppression. Similar to the previous studies, multimers were generated that contained shRNAs expressed off H1-Pol III promoters placed in a "head-to-tail" fashion within a single construct (Figure 4.7A). Constructs were named according to

the position of the shRNA, which were either in the first, second or third position. Four trimer cassettes were generated with shH1-N3, shH1-M1 and shH1-M5 in the second or third position, while shH1-NSs7 was always in the first position. The ability of shH1-N3 and shH1-NSs7 to knockdown their respective reporter targets has been previously described (Figure 3.2A and B). ShH1-N3 was chosen, as unlike shH1-N1 (Figure 3.9A), it could effectively reduce N protein levels of both the ZH548 and ZH501 strains (Figure 4.2B) but was able to maintain comparable inhibition of RVFV replication (Figure 4.2C). ShH1-M5 was chosen as it could potently inhibit protein levels (Figure 4.5) and replication (Figure 4.6). However, it is possible that potent shRNAs in multimers can outcompete other shRNAs as a result of more efficient interaction with the RNAi machinery. ShH1-M1, a slightly weaker inhibitor of RVFV replication (Figure 4.6), was included to determine whether shRNA potency could affect the functionality of other shRNAs within a multimer.

As previously described (section 3.2.2), minimal targets were generated within pSI-CHECK reporter vectors for each shRNA (Figure 3.6A) and knockdown of a reporter was indicative of a functional shRNA within the multimer. When trimers were transfected with the reporter targets, shH1-M1 and shH1-M6 were able to knockdown their respective targets in both the second and the third position, which was comparable to an individual shRNA (Figure 4.7B). ShH1-N3 in all four cassettes lost the ability to silence its target reporter in both positions (Figure 4.7C), even with the weak shH1-M1 or potent shH1-M5. This suggests that shH1-N3 does not tolerate multimerisation and was excluded from future multimer cassettes. However, the shRNAs against *M* were effective in trimers and further investigation of the anti-*M* shRNAs in multimers was explored.

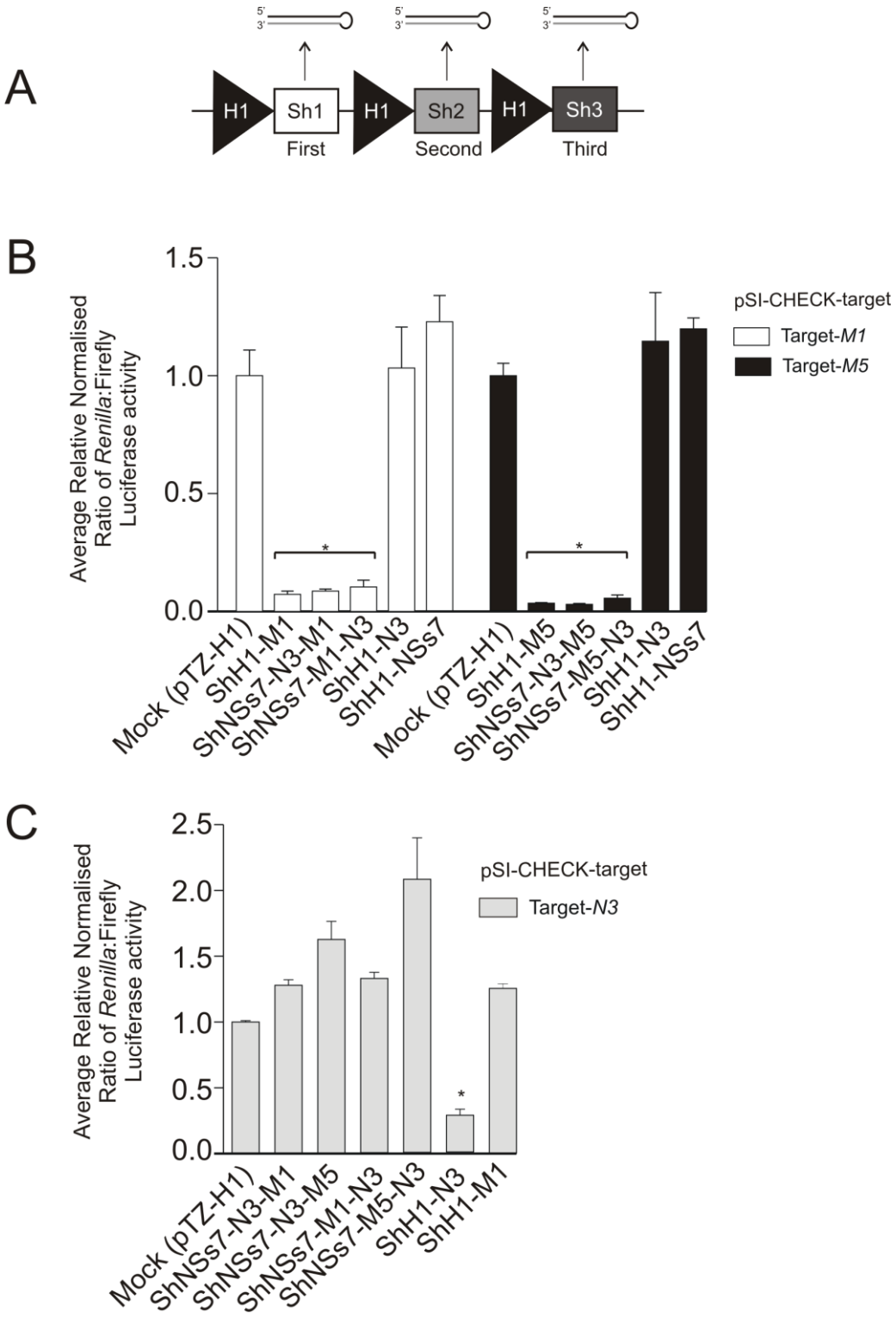

Figure 4.7: Evaluation of trimers with shRNAs targeting *NSs*, *N* and *M*. (A) Schematic of three shRNAs placed in series in a 'head-to-tail' organisation within a single construct. Each shRNA was expressed from an individual H1-Pol III promoter. (B) A pSI-CHECK (*M1* and *M5*) or (C) pSI-CHECK-*N3* reporter vector was transfected in HEK293 cells with the shRNAs. The values represent an average ratio of *Renilla*:Firefly, which have been made relative to the mock (pTZ-H1) control. An H1-Pol III shRNA was included as a positive control. A shH1-NSs7, shH1-N3 or shH1-M1 was included as a negative control where appropriate. Experiments were performed in technical triplicate with error bars indicating standard deviation (*p<0.05 Unpaired Student's t test compared to the mock control).

To determine whether the shRNAs against *M* could better tolerate multimerisation, several more trimer cassettes were generated containing shH1-NSs7, shH1-M1, shH1-M2, shH1-M4 and shH1-M5. A variety of shRNAs were used, potent shH1-M2 and shH1-M5, the weaker shH1-M1 and an ineffective shH1-M4 to determine whether shRNA efficacy was a possible factor in multimer functionality. Again, the position of the shRNAs was varied in the second and third position while shH1-NSs7 was maintained in the first position. Trimer cassettes were transfected with the pSI-CHECK minimal targets and the ability to knockdown their cognate targets was assessed. With the exception of shH1-M4, all the trimers demonstrated complete knockdown of their reporters (Figure 4.8). ShH1-M4 suffered deleterious effects only in the second position and the attenuation of silencing was partial (~30%). However, there does not seem to be a correlation between the potency of a shRNA and its ability to tolerate insertion into a multimer or affect overall multimer functionality. The shRNAs against *M* tolerated multimerisation better than the shRNAs against *N* and each shRNA within shNSs7-M2,5 cassette was able to inhibit its respective targets (>90%) (Figure 4.8). Furthermore, the trimer was able to knockdown its minimal targets with comparable knockdown efficiency to the individual shRNAs (Figure 4.8), and did not lose silencing as was observed with shH1-N1 (~10%) within the tetramer (Figure 3.6B). ShH1-M2 and shH1-M5 were both potent inhibitors of RVFV (Figure 4.6) and therefore, the multimer should be able to inhibit replication in cell culture.

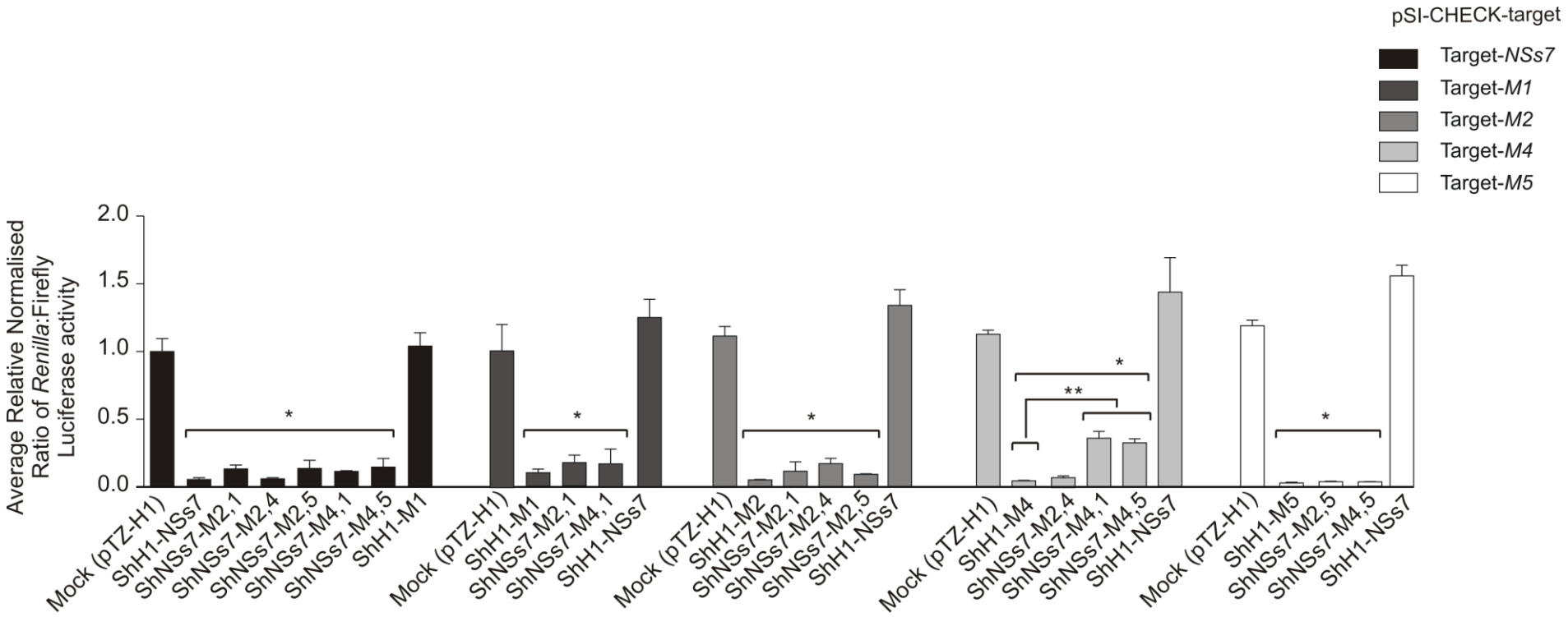

Figure 4.8: Evaluation of trimers containing shRNAs targeting *NSs* and *M*. HEK293 cells were co-transfected with the multimers and a pSI-CHECK (*NSs7*, *M1*, *M2*, *M4* and *M5*) reporter vector. The values represent an average ratio of *Renilla:*Firefly, which have been made relative to the mock (pTZ-H1) control. An H1-Pol III shRNA was included as a positive control. A shH1-NSs7 and shH1-M1 was included as a negative control where appropriate. Experiments were performed in technical triplicate with error bars indicating standard deviation (*p<0.05 Unpaired Student's t test compared to the mock control,**p<0.05 Unpaired Student's t test compared to shH1-M4).

4.2.6 Inhibition of RVFV replication using a multimer cassette

It was determined whether the shNSs7-M2,5 trimer was able to inhibit RVFV in a challenge assay using the same protocol as described above (4.2.4). HEK293 cells transfected with shNSs7-M2,5 were able to completely suppress RVFV replication over the 96 hr study, similar to shH1-M5 (Figure 4.9). As it has been demonstrated that shH1-NSs7 does not affect viral replication (Figure 4.1) or other RVFV structural protein levels (Figure 4.2B and 4.5), this suggests that the shRNAs against *M* were the determining factors in the inhibition of replication. Therefore, a trimer was generated that can target both *NSs* and *M* of RVFV and was functionally superior to the tetramer. This opens up the possibility that multimers could be used to diminish several gene targets within RVFV *in vivo,* to enhance the therapeutic effectiveness of RNAi.

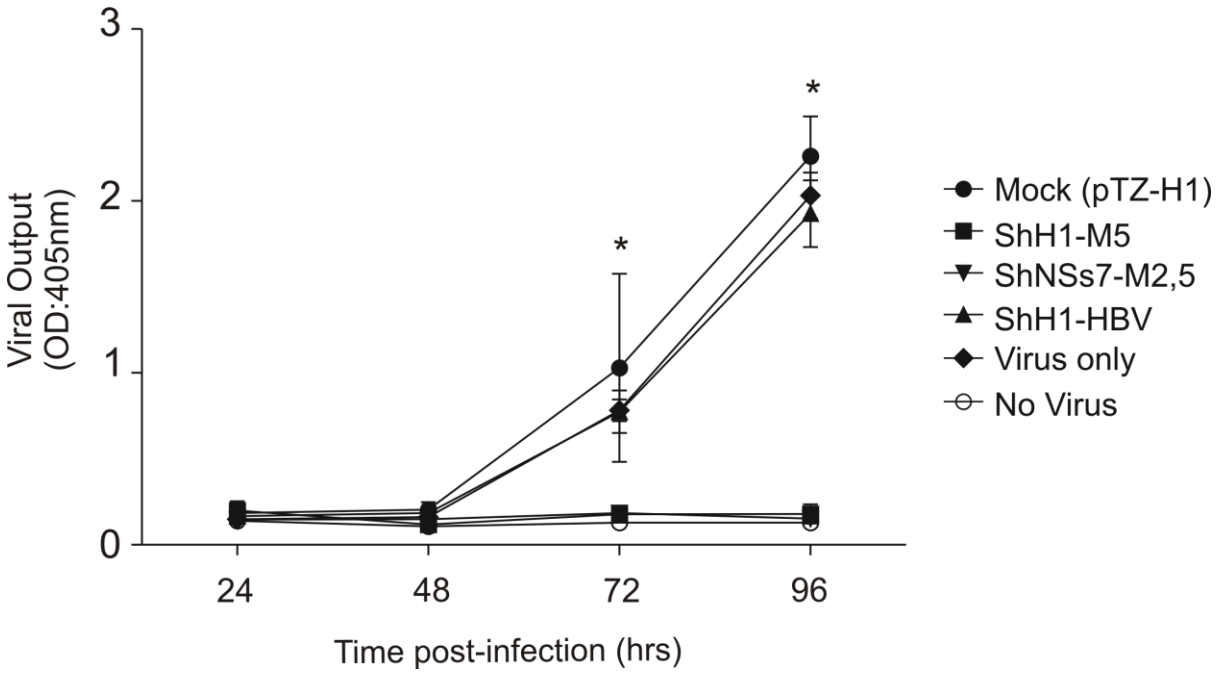

Figure 4.9: Inhibition of RVFV replication using a multimer. HEK293 cells were transfected with the shNSs7-M2,5 cassette, 24 hrs prior to infection with the SA1981 isolate of RVFV at a $TCID_{50}$ $1x10^{2.8}$/ml. Every 24 hrs for 4 days, supernatant was collected and the N antigen detected using an ELISA. Cells that did not receive a shRNA (Virus-only), mock (pTZ-H1) and shH1-HBV were included as negative controls. Uninfected (no virus) cells were included to obtain a baseline measurement. Samples were measured at a wave-length of 405 nm. Experiments were performed in technical triplicate and the samples were measured twice. Error bars indicate standard deviation (*$p<0.05$ Unpaired Student's t test compared to the shH1-HBV control).

4.2.7 Assessment of the shRNA's ability to stimulate a non-specific IFN response

DsRNA can be detected by PRRs that result in activation of the IFN response (section 1.3.1.1). This will cause an innate immune response, which could have an indirect therapeutic effect (Kleinman et al., 2008). Furthermore, a potent IFN inducer, Poly-L-Lysine and Carboxymethylcellulose (Poly(ICLC)), has been used to treat RVFV infections *in vivo* (section 1.5.1) (Peters et al., 1986). As shRNAs are dsRNA, they could potentially activate the IFN response, which would undermine the true potential of the shRNAs to inhibit RVFV replication through an RNAi-mediated mechanism (section 1.8.2). HEK293 cells are IFN-competent (Spiegel et al., 2005) and it needed to be assessed whether the inhibitory effects by the shRNAs were not as a result of indirect innate immune stimulation. ShRNAs were transfected with a pIF-125luc reporter (IFN-β promoter expressed Firefly luciferase) but none of the shRNAs caused an increase in luciferase activity, and were comparable to cells that were not treated with the IFN stimulator, Poly (I:C) (Figure 4.10). These data demonstrate that the shRNAs or multimer cassettes do not elicit a non-specific host immune response and inhibition of RVFV in cultured cells was as a result of an RNAi effect.

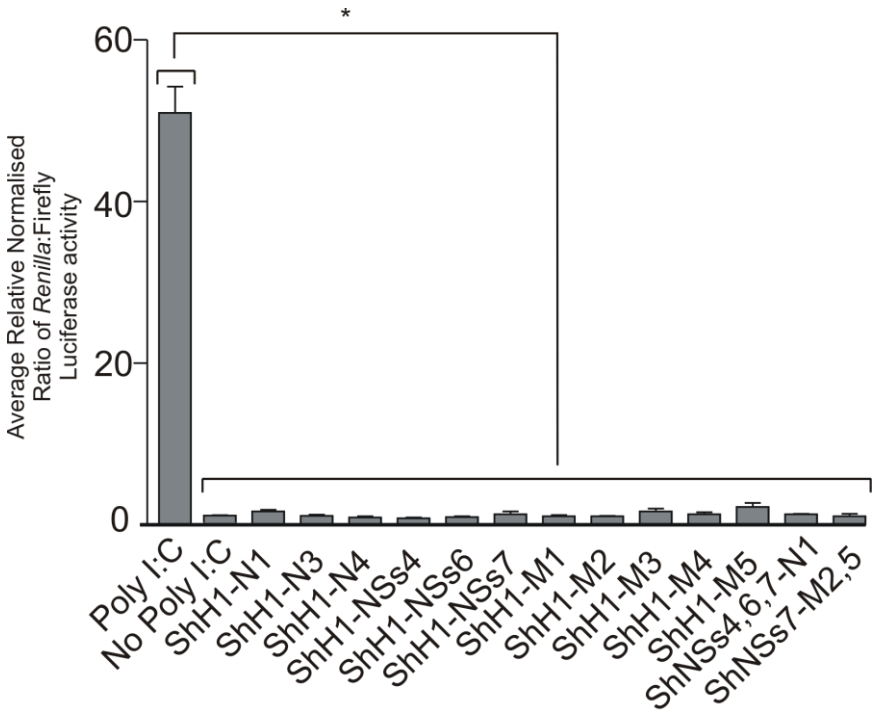

Figure 4.10: Activation of an IFN response by the shRNAs. Average relative Firefly:*Renilla* luciferase activity was determined when HEK293 cells were co-transfected with the shRNAs and a pIF-125luc reporter (IFN-β promoter expressed Firefly luciferase). Poly (I:C) was included as a positive control and untreated cells (No Poly I:C) was included as a negative control. Experiments were performed in technical triplicate with error bars indicating standard deviation (*p<0.05 Unpaired Student's t test compared to the Poly (I:C)).

4.2.8 Assessment of the shRNA's ability to disrupt the endogenous RNAi pathway

Exogenously introduced RNAi effectors may over-saturate the endogenous RNAi pathway, which can result in cellular toxicity (section 1.8.3). Specifically, expressed shRNAs can result in the accumulation of pre-miRNA mimics (Ahn et al., 2011), which disrupts the processing of endogenous miRNAs at the level of exportin-5 and Ago2 (Grimm et al., 2010). With this in mind, the ability of several of the shRNAs used in this study to saturate the RNAi pathway was assessed. To determine whether the shRNAs have the potential to disrupt endogenous RNAi, a sensor for host miRNA activity was employed. The sensor is a pSI-CHECK vector that has seven target sites of a host miRNA in the 3' UTR of *Renilla* luciferase (pSI-CHECK-mIR) (Ely et al., 2009). As RNAi-based therapeutics for RVFV would be targeted to the liver, the target sites were derived from liver-expressed miRNAs namely, miR-122 (Chang et al., 2004) and miR-16 (Wu et al., 2011a). When a sensor was transfected into a liver cell line, the activity of the miRNAs would reduce *Renilla* luciferase activity. If the expressed shRNAs have an adverse effect on the natural miRNA pathway, there will be an increase in *Renilla* luciferase activity as a result of disrupted miRNA processing and an inability to facilitate target suppression.

To determine whether the reporter was sensing endogenous activity, a positive control for disruption was required. A screening of several U6-Pol III expressed shRNAs was performed as U6-Pol III promoters produce shRNAs at high levels and are more likely to disrupt host RNAi (Grimm et al., 2010) (data not shown). A U6-Pol III shRNA directed to HIV's *Nef* gene was found to have a derepression effect (~20%) on liver-expressed miR-122 and miR-16 levels (Figure 4.11A). A U6-Pol III shRNA against *Gag* had no effect of luciferase levels and so shU6-HIV-*Nef* and shU6-HIV-*Gag* were subsequently used as positive and negative controls for disruption, respectively. To ensure that the effect on *Renilla* luciferase by shU6-HIV-*Nef* was not as a result of the shRNA affecting the pSI-CHECK reporter vector, shU6-HIV-*Nef* was transfected with an unmodified pSI-CHECK-2.2 and the shRNA had no effect on the reporter vector (Figure 4.11B). To verify that the miRNA target sites were functional within a pSI-CHECK reporter vector, the reporters were transfected with their respective miR-122 or miR-16 sponges (Ely et al., 2009). Expressed sponges will bind and interfere with the endogenous miRNAs, which will block suppression of the reporter vector. The pSI-miR-122 and pSI-miR-16 reporter vectors had increased *Renilla* luciferase activity in the presence of expressed sponges, demonstrating that the miRNA targets within the reporter vectors were functioning correctly (Figure 4.11C).

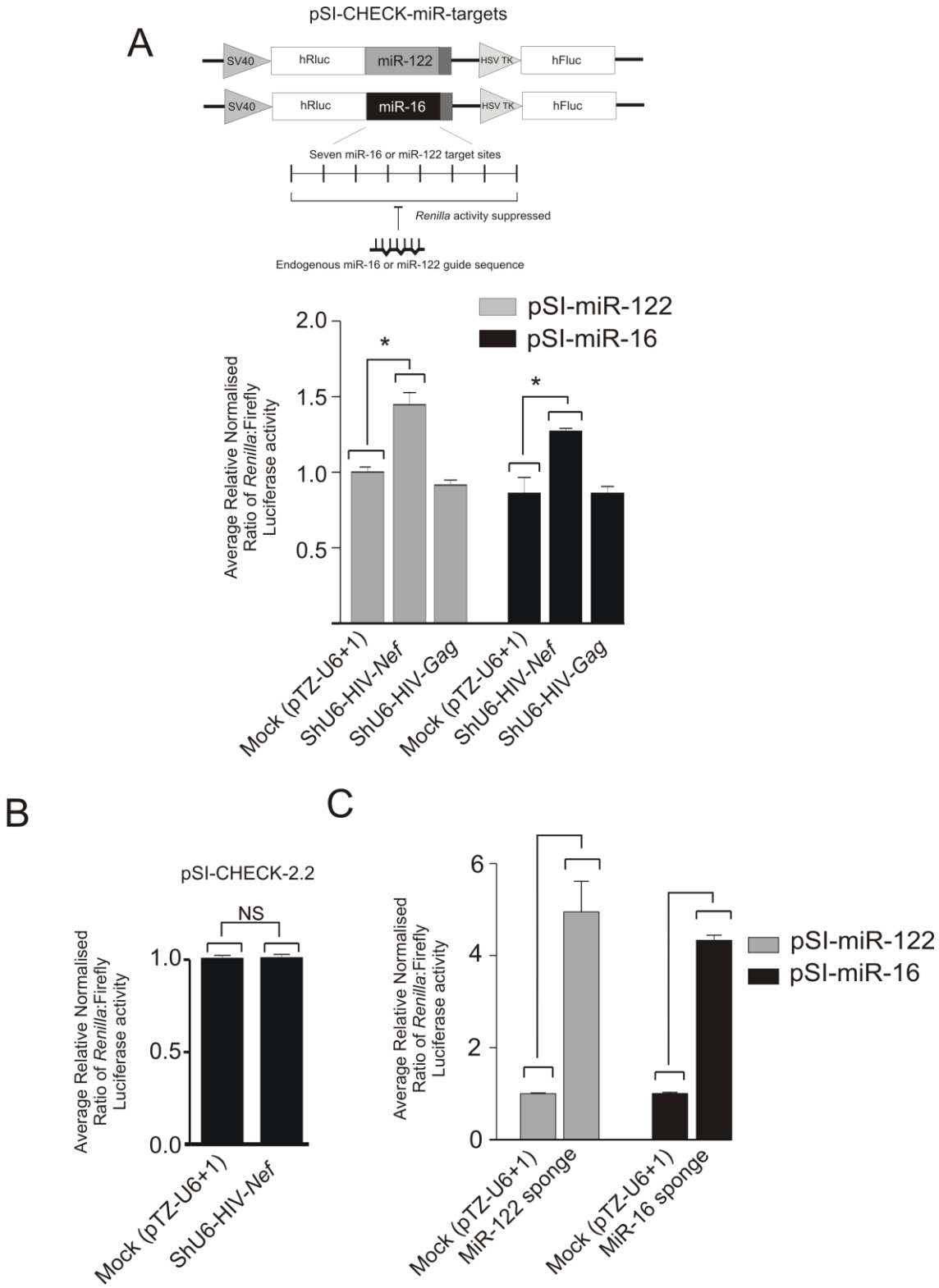

Figure 4.11: Evaluation of a sensor for disruption of the endogenous miRNA pathway. (A) Seven miR-122 or miR-16 target sites were inserted into the 3' UTR of *Renilla* luciferase in a pSI-CHECK 2.2 reporter vector that will be down-regulated by endogenous miRNAs. Huh7 cells were co-transfected with a shU6-HIV-*Nef* or shU6-HIV-*Gag* and a pSI-miRNA reporter vector. (B) Huh7 cells were co-transfected with shU6-HIV-*Nef* with an unmodified pSI-CHECK 2.2 reporter vector. (C) Huh7 cells were co-transfected with the miRNA sponges for miR-122 or miR-16 and their respective pSI-miRNA reporter vectors. Experiments were performed in technical triplicate with error bars indicating standard deviation (*p<0.05 Unpaired Student's t test compared to the mock control).

Having demonstrated that the reporter is functioning correctly, it was determined whether H1-PoI III expressed shRNAs targeted to RVFV could disrupt the endogenous RNAi pathway. The shRNAs selected for testing in this assay were the most likely candidates to be taken into future *in vivo* studies. The anti-pathogenic shH1-NSs7 was included as a result of its extensive characterisation to affect NSs pathology (section 3.2.5), along with two other potential candidates, shH1-NSs4 and shH1-NSs6. The anti-replicative shH1-M2 and shH1-M5 were selected, as they were the most potent inhibitors of M protein expression (Figure 4.5) and viral replication (Figure 4.6). ShRNAs against *N* were excluded as they were unable to completely inhibit viral replication (Figure 4.2C) and could not function within multimers (Figure 3.9 and 4.7C). Further screening and optimisation of anti-*N* shRNAs will be required to obtain an effective candidate. Finally, as an additional investigation it was determined whether the larger cohort of shRNAs within the multimers could enhance their potential for disruption of endogenous RNAi and so shNSs4,7,8-N1 and shNSs7-M2,5 were assessed in the reporter assay.

When individual shRNAs were transfected in the presence of a pSI-miR-122 reporter vector, there was no increase in *Renilla* luciferase levels above that of the controls (Figure 4.12). Although shH1-M2 and shH1-M5 *Renilla* levels were higher than the other shRNAs, these levels were not above that of the pTZ-U6+1 mock control. The multimers had no adverse effects on the endogenous miRNA's ability to suppress *Renilla* luciferase activity and was likely as a result of the shRNAs expressing at lower levels from the multimers (Figure 3.7) (ter Brake et al., 2008). Although these data cannot rule out the possibly of finer perturbations to the miRNA pathway, it does suggest that the shRNA cassettes were not causing any gross disruptions to endogenous RNAi.

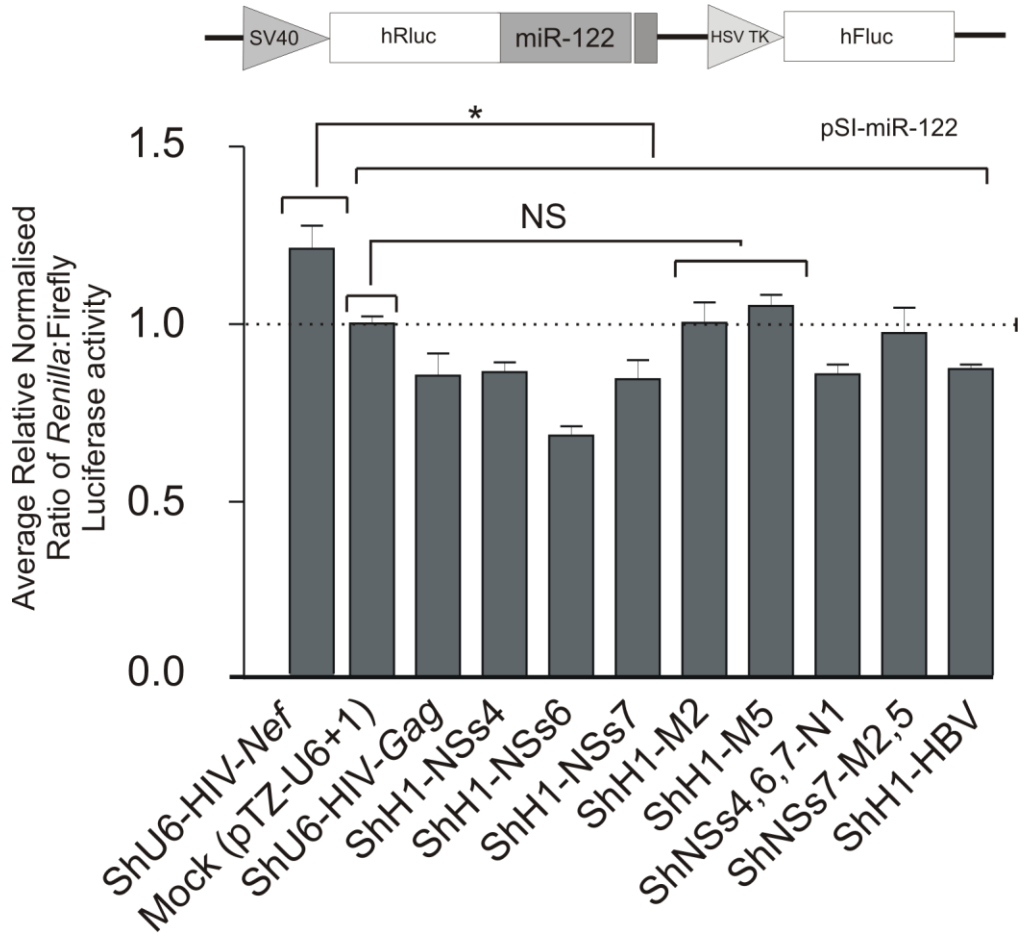

Figure 4.12: ShRNA constructs do not disrupt the endogenous miRNA pathway. (D) Huh7 cells were co-transfected with the shRNAs or multimer cassettes and a pSI-miR-122 reporter vector. The values represent an average ratio of *Renilla*:Firefly, which have been made relative to the mock (pTZ-U6+1) control. Experiments were performed in technical triplicate with error bars indicating standard deviation (*p<0.05 Unpaired Student's t test compared to the mock control).

4.3 Discussion

The use of RNAi mimics to inhibit RVFV *in vitro* was investigated. ShRNAs targeted to *N* demonstrated they could reduce *N* reporter and protein levels (Figure 3.2B, 3.9A, 4.2A and B), which translated into the suppression of viral replication in a challenge assay (Figure 4.1 and 4.2C). As N is involved in RNA synthesis and RNP complex formation (section 1.2.3 and 1.2.4), this suggests that the early stages of RVFV's life cycle were being inhibited by the shRNAs.

Highly active shRNAs in the reporter assays were consistently ineffective at reducing viral protein levels (Figure 3.9A and 4.2B). A fusion mRNA of *Renilla:N* produced by the luciferase reporter construct does not express N protein, which suggests that the presence of N in the western blots may be the factor affecting shRNA-mediated silencing. N of RVFV can form ring oligomers and viral

RNA can 'string' through a central cavity (Ferron et al., 2011). Furthermore, N is a promiscuous binder of RNA and can bind both viral and host mRNA (Raymond et al., 2010). This may suggest that the expressed N was encircling N mRNA, which could prevent access of RISC to facilitate mRNA cleavage. However, this reduced silencing is likely limited to the overexpression studies and not a feature during viral replication as the anti-*M* shRNAs significantly suppressed replication without any attenuating effects suggesting viral mRNA would still be effectively targeted during RVFV infection (Figure 4.6).

It was also consistently observed that the ZH548 N protein levels were reduced less by the shRNAs than the ZH501 N (Figure 3.9A and 4.2B). This was curious as there was no sequence difference within the target site of the two strains (data not shown). Sequence differences outside of a target site can alter the accessibility of RISC, which can reduce silencing (Westerhout et al., 2005) but the sequence variation around the target was maintained and cannot account from this kind of silencing bias. VP35 of EBOV can bind RNA with higher affinity than MARV-VP35 and these binding differences might explain the reduced potential of MARV-VP35 to block recognition by the IFN pathway (Table 1.1) (Bale et al., 2012). Similarly, N from ZH548 may have differences from a ZH501 N that allows for greater dsRNA binding affinity, which could enhance its capacity to disrupt the access of RISC. Sequencing of the two strains used in this study revealed an amino acid substitution at position 61 (alanine to valine), which falls within a region required for the multimerisation of N (Ferron et al., 2011). This substitution may represent a difference in RNA binding by disrupting oligomerisation of RVFV's N, which would enhance RISC access to the mRNA and facilitate silencing. Further work will be required to determine whether this mutation was the reason for the observed differences in the overexpression studies.

ShRNAs were constructed that target the *M* genes of RVFV and demonstrated potent Gc protein (Figure 4.5) and viral replication suppression (Figure 4.6). The M segment is a good therapeutic target as it has surprisingly low sequence variation between strains (Bird et al., 2007a), even though it encodes glycoproteins that would be under selective pressure from the adaptive host immune response. ShRNAs were designed to target conserved regions within *M* and the results obtained could presumably be recapitulated against various other strains of RVFV. ShRNAs were designed to target within the Gn region and resulted in reduced levels of detected Gc (Figure 4.5), which suggests that the entire *M* mRNA was being down-regulated by RNAi-mediated cleavage. The removal of both glycoproteins will result in reduced glycoprotein trafficking, packaging of RNP complexes and subsequent virion maturation, inhibiting late stages of RVFV's life cycle (section 1.2.4). Furthermore, as the M segment has a large coding region (~3500 bp) (section 1.2.2), several

more shRNAs could be designed to target within the Gc or NSm regions, which leaves open the possibility of expanding the cohort of therapeutic shRNAs directed to *M*.

As the mRNA encoding both glycoproteins was degraded (Figure 4.5), this implies that the full M sequence that includes the NSm component will also be down-regulated. The reduction of NSm may have beneficial effects as its removal resulted in reduce lethality in mice (Bird et al., 2007b) but work by others has demonstrated that RVFV lacking NSm has increased incidence of neuropathology (Bird et al., 2007b). A RVFV with both *NSs* and *NSm* coding regions removed elicited a adaptive immune response with no apparent additional pathologies (Bird et al., 2008). This possible pathological concern will need to be taken into consideration when targeting *M* with shRNAs *in vivo* and may require the dual down-regulation of *M* and *NSs* to avoid possible complications.

Besides viral mRNA, the RNA genome of RVFV should also be a target for RNAi-mediated cleavage. However, the shRNAs against NSs should also target the S segment but did not affect viral replication, which suggests that the genomic viral RNA was unaffected by the shRNAs (Figure 4.1). The resistance of genomic RNA has been noted by others with HCV anti-genomes not targetable with shRNAs (Lisowski et al., 2013). The resistance could be as a result of reduced RISC access through, 1) the compartmentalisation of genomic RNA during RVFV RNA synthesis, 2) the formation of dsRNA with the opposite sense RNA, 3) or the preferential encapsidation of genomic RNA. Nevertheless, RVFV mRNAs and not genomic RNA would most likely be the target of RNA-mediated degradation.

Understanding the M segment's biology assisted in its selection as a potential therapeutic target and implies that targets of RNAi could be chosen based on their coding potential. In RNAi studies targeting Hazara virus, it was demonstrated that the L polymerase or the M segment were less effective targets than *N* (Flusin et al., 2011). This does not appear to be the case in this study, as the shRNAs against *M* (Figure 4.5 and 4.6) were far superior suppressors than the shRNAs targeted to *N* (Figure 3.9A, 4.1 and 4.2). This is surprising, as CCHFV, for which Hazara virus is a model, has an M segment that has a similar compact coding strategy to that of RVFV-M segment. CCHFV-M (5'-Mucin/GP38/Gn/NSm/Gc-3') produces a single translated polyprotein, which is proteolytically processed to give rise to numerous viral proteins (Sanchez et al., 2006). It would be expected that similar compounding inhibition would be observed when targeted with RNAi, much like RVFV. This shows that potent RNAi targets can be rationally chosen based on their coding potential, but these targets have to be verified by experimentation.

ShNSs7-M2,5 was able to knockdown its minimal targets (Figure 4.8) and suppress RVFV replication in a challenge assay (Figure 4.9). The dual targeting of a *NSs* and *M* would have the

combined benefit of having both anti-pathogenic and anti-replicative effects in a single construct, potentially enhancing its potency against RVFV, which would be favourable in combating the pathological features of haemorrhagic cases (section 1.7.2). Anti-pathogenic (Zhang et al., 2005) and anti-replicative siRNAs (Bitko et al., 2005) have been investigated against RSV and function through defined mechanisms of action. The anti-pathogenic siRNAs targeting RSV's IFN antagonist NS1 resulted in viral suppression through the activation of IFN, while anti-replicative siRNAs suppressed viral replication by targeting an essential component of the polymerase. However, preventing polymerase activity could reduce the viral dsRNA signals required for IFN stimulation. This poses an interesting conundrum for a multimer with both properties as the anti-replicative shRNAs targeted to RVFV would reduce the signals required for IFN activation, which could render the shRNAs against *NSs* inert. However, in the shNSs7-M2,5 multimer, shRNAs against *M* do not interfere with RNA synthesis (section 1.2.3) but only prevents virus maturation (section 1.2.4), which still allows for the accumulation of viral dsRNA and hence the activation of IFN in NSs-depleted cells. A complete assessment of a multimer with both anti-replicative and anti-pathogenic properties will be required to determine the accumulative benefit *in vivo.*

RNAi effectors have been shown to non-specifically activate the IFN response (Marques et al., 2006) but these data demonstrate that expressed shRNAs were not activators of IFN (Figure 4.10), which has been noted by others (Robbins et al., 2006). The presence of an IFN response is an important difference between lethal and non-lethal infections of RVFV in non-human primates, which correlates with the appearance of neutralising antibodies and reduced viraemia (Morrill et al., 1990). Using expressed shRNAs to reduce viral burden in combination with IFN inducers to activate a host immune response, may be a potent combination to facilitate viral clearance. SiRNAs have even been designed to have both RNAi and IFN stimulatory properties to treat acute viral infections (Ahn et al., 2012). However, unlike siRNAs that are rapidly cleared (Thompson et al., 2012), viral delivered shRNAs can have persistent expression for several months (Grimm et al., 2010). Sustained IFN activation for this period would not be desirable and the overall lack of IFN stimulation by the shRNAs was a favourable safety feature of the shRNAs.

Expressed Pol III shRNAs can disrupt the endogenous RNAi pathway (Grimm et al., 2010). Initially, shRNAs were expressed off a potent U6-Pol III promoter, but the promoter has been implicated in the disruption of endogenous RNAi (section 1.8.3) and reducing expression of the shRNAs would help alleviate toxicity. The reengineering a potent U6-Pol III promoter by exchanging the proximal sequence element (PSE) regions of different U6-Pol III promoters resulted in reduced expression of the shRNAs and absence of toxicity (Suhy et al., 2012). Using a weaker H1-Pol III promoter can also prevent toxicity (An et al., 2006). In this study, gross disruption of the endogenous

RNAi pathway was not observed in a reporter assay from any of the tested H1-Pol III constructs compared to a U6-Pol III expressed shRNA (Figure 4.12). Overall, reducing the expression of a shRNA seems to be effective at preventing saturation of RNAi.

In conclusion, these data demonstrate that expressed shRNAs can suppress the replication of RVFV in cultured cells when targeting structural proteins of RVFV. The most effective candidates were the shRNAs targeted to *M* of RVFV, which significantly reduced detectable levels of viral antigen in a challenge assay. This is an invaluable addition to the repertoire of anti-RVFV shRNAs, which may be able to better "manage" highly pathogenic viruses by potently suppressing essential proteins within the viral life cycle. This work is an important foundation from which to develop anti-replicative RNAi effectors as a novel therapeutic for the treatment of RVFV.

Chapter 5

Discussion and Conclusion

5.1 Summary of results

The studies within this thesis focused on the application of RNAi effectors, specifically expressed shRNAs, as a novel therapeutic strategy for the inhibition of RVFV. Several targets were selected for RNAi targeting within RVFV's genome, namely *NSs*, *N* and *M*. The shRNAs were able to reduce reporter and protein levels of their respective target genes. Anti-*NSs* shRNAs alleviated the pathogenic effects of NSs, reversing NSs-induced transcription and IFN-β promoter suppression as well as increasing cell viability. The shRNAs against *N* inhibited RVFV replication *in vitro,* although the virus was able to overcome suppression. Following this observation, anti-*M* shRNAs were investigated and resulted in significant suppression of viral replication in a challenge assay. Furthermore, shRNAs were incorporated into tetramers and trimers to generate multiple expressed shRNA cassettes that targeted several genes within RVFV. A functional trimer containing anti-*NSs* and anti-*M* shRNAs was obtained, which could inhibit RVFV in a challenge assay. The shRNAs did not elicit a non-specific IFN response demonstrating that the inhibition was specific to an RNAi-mediated mechanism. Finally, there was not a noticeable disruption of the endogenous RNAi pathway caused by the presence of the shRNAs. Future studies will focus on determining the effectiveness of the shRNAs in an animal model.

5.2 Additional potential benefits of targeting RVFV virulence factors with shRNAs

The anti-*NSs* shRNAs would negatively affect the expression of NSs *in vivo* and create a 'NSs-deficient' state during viral replication that could highly attenuate RVFV infection (Muller et al., 1995). Mice infected with a RVFV lacking NSs had enhanced serum IFN (Lihoradova et al., 2012) and an early IFN response correlates with a positive clinical outcome (Morrill et al., 1990). The shRNAs should increase IFN-β promoter activity (Figure 3.12B) and the expression of other IFN related genes suppressed by NSs, such as OAS, Tyk2 and STAT2 (Benferhat et al., 2012) (section 1.4.1.2). Furthermore, an NSs-deficient RVFV has been used to elicit a potent adaptive immune response and has been developed as a potential vaccine candidate (von Teichman et al., 2011) and opens up the possibility that anti-*NSs* shRNAs could result in sustained adaptive protection against RVFV well after the RNAi therapeutic effect has dissipated. RVFV lacking NSs also has a dominant negative

phenotype over wild-type RVFV. When a MP12 vaccine strain was engineered with a truncated NSs it resulted in protection against lethality in mice when administered post-exposure, but a MP12 with a intact *NSs* gene had no effect on survival (Gowen et al., 2013). The removal of NSs by a shRNA could have the added benefit of recapitulating this dominant negative phenotype, which would enhance its overall therapeutic effect.

The nuclear localisation of NSs was required for its cytotoxicity (Figure 3.11) and may be caused by NSs's interaction with pericentromeric γ-satellite sequences, which results in genomic defects (section 1.4.1.2). It has been suggested that the high levels of abortions and teratogenesis seen in pregnant ruminants (section 1.2.1) is caused by this NSs:DNA interaction and as the shRNAs alleviate NSs-induced cytotoxicity (Figure 3.11C), they may be able to also prevent NSs-induced genetic instability. The application of anti-*NSs* shRNAs as a treatment in pregnant ruminants *in utero* could help prevent the 'abortion storms' associated with RVFV infections. Another significant pathology of RVFV infections is coagulation defects, which are possibly caused by NSs's interaction with regulatory elements of clotting factors (section 1.4.1.2) (Benferhat et al., 2012). This deregulation may result in impaired clotting or the presentaion of DIC, features of severe infections [reviewed in (Ikegami and Makino, 2011)]. The removal of NSs by shRNAs, especially in the liver where several clotting factors are synthesised, would help improve disease outcome through alleviation of coagulation defects.

The NSm proteins prevent viral-induced apoptosis (Won et al., 2007) and possibly activate pro-survival pathways through MAPK-p38 to prevent stress-induced cell death (Narayanan et al., 2011) (section 1.4.2). It has been speculated that NSm prevents unwanted early stage apoptosis to allow for the establishment of viral replication. Whether this was happening in the expression studies could not be investigated as the ΔNSm expression vector does not express the non-structural proteins (Figure 4.4). Gc was down-regulated even when the target region of the shRNAs was within Gn (Figure 4.5) and suggests NSm would also be reduced, disrupting its function. Furthermore, the transient activation of pro-survival pathways correlates with the detection of RVFV replication (Popova et al., 2010). ShRNAs against *M* suppressed detectable viral replication in cultured cells (Figure 4.6), suggesting that activation of pro-survival pathways was not occuring in the challenge assays. Nevertheless, reduction of NSm by the shRNAs would enhance induction of apoptosis in virally infected cells, contributing to RVFV inhibition.

To assist vaccine development, methods have been established that can discern between RVFV infections based on the presence of NSs. Individuals that received a recombinant vaccine lacking NSs could be differentiated from wild-type infected individuals as a result of the absence of NSs-specific antibodies in the serum (Bird et al., 2008). A similar methodology could be applied to

individuals who receive the anti-*NSs* shRNAs. The shRNAs could be applied at random to a population during an outbreak and treated individuals can be easily differentiated between infected and uninfected. If the shRNAs are successful in protecting against RVFV, those individuals would be positive for the presence of RVFV antibodies excluding those specific to NSs and could be compared to an untreated, infected population, which would give valuable insight into the effectiveness of the shRNAs as a prophylaxis during outbreaks. However, the shRNAs would have to reduce NSs levels enough to abrogate any form of humoral response and the candidate selected for the trial must be RVFV naive to avoid previous serological conversion. Furthermore, the application of a gene therapy as a prophylaxis does raise ethical concerns. Administration of a shRNA prior to infection may be difficult to justify as a result of the risk associated with a gene therapy combined with the fact that RVFV has sporadic and unpredictable outbreaks.

Understanding the molecular biology of a virus can help in selecting potential targets. This has been demonstrated in that several therapeutics target virulence factors or block essential interactions of protein or RNA structures (section 1.5). The selection of rational targets was demonstrated by choosing *NSs* and *M* genes based on the understanding of RVFV biology (section 1.2.2 and 1.4.1). Unfortunately, there is a complete lack of insight into the basic biology of some haemorrhagic fever viruses such as Omsk haemorrhagic fever virus or Kyasanur forest disease of the *Flaviviridae* family and more effort needs to be placed into researching haemorrhagic viruses to enhance the potential for developing therapeutic platforms.

5.3 Second generation multimers

To improve the design features of multimers that have more consistent and reliable silencing properties, two determining factors can be taken into consideration such as the shRNAs themselves as well as the promoters used to drive their expression. In this study, shH1-N3 and shH1-M4 suffered greater deleterious effects than other shRNAs (Figure 4.7C and 4.8) and the loss of functionality of specific shRNAs has been noted by others (McIntyre et al., 2011a). The sequence of a shRNA that has optimal parameters for RNAi processing (i.e. thermodynamic properties) may outcompete other shRNAs with less favourable parameters. Although this still needs to be determined, selecting shRNAs with comparable properties may be required to enhance the function of the multimers. ShH1-N3 was refractory to multimerisation in all positions (Figure 4.7C) and shH1-M4 lost functionality only in the middle position (Figure 4.8). This was contrary to other studies that have shown that the first and second position maintain silencing and only from the third position onwards

was silencing affected (McIntyre et al., 2011a). The position of a shRNA within a multimer should be investigated further as a feature in determining its functionality.

In the tetramers, the observed attenuation of silencing was with random shRNAs in various positions (Figure 3.6B) and suggests that the shRNAs themselves were not a factor contributing to multimer function but rather the number of shRNAs. This has been noted by others that the larger the shRNA cohort in the multimers, the greater the attenuating effect on the individual shRNAs (McIntyre et al., 2011a) and lowering the number of shRNAs can improve its functionality, as was demonstrated by a trimer that could inhibit RVFV replication (Figure 4.9). Further experimentation will be required to determine the parameters of an optimal shRNA sequence and its relationship to position, to improve multiple expressed shRNA design. As it currently stands, it appears that validation through experimentation of a large panel of diverse multimers is the only true method to obtain a functional multiple expressed shRNA.

In a recent comprehensive study it was observed that the use of repeat promoters can have deleterious effects on shRNA silencing, as a shRNA that was imbedded in an increasing number of H1-Pol III promoters had reduce silencing (McIntyre et al., 2011a), which removed the possible influence of competing shRNAs as a factor. This could be as a result of competition for transcription factors used by identical promoters and exploiting a variety of promoters may overcome the competitive inhibition to enhance shRNA expression. Several promoters can be used to express shRNAs such as 7SK-Pol III (Czauderna et al., 2003), U1-Pol II (Denti et al., 2004) or tRNA promoters (Scherer et al., 2007) (section 1.6.3.3) but a commonly used transcription factor may become a limiting feature, as promoter-diverse multimers still had reduced shRNA expression (ter Brake et al., 2008). However, a multimer containing four shRNAs expressed from diverse promoters was able to maintain silencing compared to individual shRNAs, unlike the multimers in this study (Figure 3.6B). This suggests that using different promoters could be beneficial and a more comprehensive study will be required to determine their advantage in multimer cassettes.

5.4 The applicability of multimers to target RVFV

RVFV has an elegant method to ensure that only infectious particles are generated upon viral release. The complementary ends of an encapsidated genome are recognised by the cytoplasmic tail of Gn, which is the signal for viral release and results in high levels of viral particles with packaged genomes that could be potentially infectious (section 1.2.4). This has been demonstrated with other bunyaviruses that have a viral particle-to-plaque ratio close to 1, which suggests that almost every viral particle produced is virulent (Lowen and Elliott, 2005). This high infectious rate implies that not only does RVFV produce overwhelming levels of virions during a haemorrhagic fever (section 1.7.2.1)

but that each virion can possibly infect another host cell, stressing the importance of trying to reduce the production of viral particles. Multimers offer a platform with which to hinder RVFV on several levels to enhance the potency of RNAi. In this study, multimers were generated that target *NSs* and *M* (Figure 4.8) and could inhibit RVFV replication (Figure 4.9), but a future construct suppressing *NSs*, *N* and *M* simultaneously would be ideal. This was not investigated as it proved difficult to obtain an anti-*N* shRNA that could function within a multimer (Figure 4.7C) but hopefully further investigations that define the parameters of a functional multimer may yield viable candidates (section 5.3).

Anti-*NSs* shRNAs were able to reverse NSs-induced suppression of the IFN-β promoter (Figure 3.12), which suggests the shRNAs will restore immune activity. However, if a cell is infected with RVFV prior to delivery of the shRNA, there will be high levels of NSs already present. As the shRNAs will take time to decrease the levels of NSs, there will be an interval before the IFN response is functional and able to assist in the control of RVFV replication (section 1.3.1), while in the interim allowing for the continuation of viral production. Therefore, anti-*NSs* shRNAs can be combined with other RVFV gene targets that concurrently reduce the supply of structural proteins required for viral production. The dual targeting of *N* and *M* will impact on several stages of viral production. Firstly, the depletion of N will prevent the replication of new genomes and reduce the signal for viral release (section 1.2.3). There will also be less N available for genome encapsidation, which is required for packaging (section 1.2.4). Secondly, Gn is required for trafficking of the heterodimer Gn-Gc to the Golgi apparatus (section 1.2.4) and Gc is required for the optimal expression of Gn (Piper et al., 2011), creating a possible knock-on effect by the shRNA's down-regulation of M encoded proteins. Finally, Gn is required to bind the genomes as well as stabilise the virion through its interaction with N (section 1.2.4). As several protein functions would be disrupted by the combination of anti-*M* and anti-*N* shRNAs, production of infectious viral particles will be significantly hindered. Furthermore, the shRNAs against *N* were overwhelmed by RVFV replication (Figure 4.1 and 4.2C). The shRNAs in the multimers will cooperate to act as a 'layered' therapeutic, so that even if RVFV can overcome one shRNA, there are several others to compensate. Overall, multiple expressed shRNAs offer a potential avenue for further development to enhance the potency of RNAi, which can control the high levels of viraemia associated with haemorrhagic RVF until the host immunity can facilitate viral clearance (section 1.7.2.1).

Viruses can be highly variable genomically and often finding a shRNA that can target different strains of a virus can prove difficult, which was demonstrated when only one RNAi effector was effective against all four serotypes of DENV (Korrapati et al., 2012). Another bunyavirus, CCHFV, has genomic variability at approximately 30% (Deyde et al., 2006) but RVFV has very low genetic

diversity, which is ~4% (Bird et al., 2007a). It has been proposed that this is as a result of either, 1) the early emergence of a RVFV common ancestor, or 2) that the RVFV genome has a very low tolerance for mutation [Reviewed in (Pepin et al., 2010)]. The latter may be true as recent studies have shown that the overall structure of RVFV's NSs was critical to its function (Head et al., 2012), which may prevent the introduction of changes that could affect NSs's architecture. Nevertheless, this means that RVFV is an ideal candidate for RNAi as a broad range of RVFV strains could be inhibited with a single shRNA, which was demonstrated by the conservation of knockdown across multiple strains of *NSs* (Figure 3.3). The careful design of a multimer may not only be able to vigorously down-regulate multiple proteins but also target all strains of RVFV, which would further enhance the applicability of RNAi-based therapeutics for the treatment of RVFV.

5.5 Host dependency factors as potential shRNA targets

In this study, RVFV was the target of the RNAi effectors but there are alternative targets beyond the scope of directly targeting the viral genome. Viruses require essential interactions with HDFs at all stages of the viral life cycle including entry, replication, packaging and release. A genome-wide screen of HDFs identified dozens of potential host proteins that assist influenza (Karlas et al., 2010), vesicular stomatitis virus (VSV) (Panda et al., 2011), West Nile virus and DENV (Krishnan et al., 2008) during infection. Several of these HDFs were down-regulated using RNAi to validate their importance during their respective viruses' life cycle, offering a wealth of alternative targets to suppress viral replication.

Recently, an small RNAi screen revealed a few HDFs that assist RVFV replication (Schudel et al., 2013). Dynamin 2, a protein involved in clathrin-dependent endocytosis (Henley et al., 1998), was identified and a shRNA targeted to this HDF could be an additional target to inhibit RVFV. HDFs may not only be alternative targets to inhibit a particular virus, but offer a broader inhibitory effect against other unrelated viruses, such as dynamin 2, which was also targeted with siRNAs to inhibit DENV (Alhoot et al., 2011) (section 1.7.1.2). This phenomena has been noted by others where lymphocytic choriomeningitis, VSV and human parainfluenza virus type 3 were inhibited when common HDFs were reduced (Panda et al., 2011). Further investigations could exploit HDFs as potential targets of the expressed shRNAs to inhibit RVFV.

5.6 Viral manipulation of endogenous RNAi

Many viruses have the ability to manipulate the RNAi pathway, which must be taken into consideration when developing RNAi-based therapeutics. The proteins involved in suppressing RNAi are generally the same virulence factors that antagonise IFN. Vaccinia and influenza express a NS1 protein, which has dual antagonism for RNAi and IFN (Li et al., 2004). EBOV-VP35 is a IFN antagonist (Table 1.1) (Basler et al., 2000) and a suppressor of RNAi silencing (Haasnoot et al., 2007), which may be facilitated through its binding of siRNAs (Zhu et al., 2012). Of importance, the Bunyavirus LACV encodes an RNAi antagonist, NSs, (Soldan et al., 2005) which can down-regulate transcription through activation of DNA damage response pathways (Verbruggen et al., 2010) and is a suppressor of IFN (Blakqori et al., 2007), which has similarities to the function of RVFV-NSs (section 1.4.1). Although RNAi antagonism has not been identified for RVFV, it is conceivable that various proteins required in the RNAi pathway may be suppressed by NSs-induced transcription suppression (Le May et al., 2004) (section 1.4.1.1). Another possible mode of RNAi antagonism by RVFV may be through induced oxidative stress. Recent studies have demonstrated that under oxidative stress, RNAi components can shift from processing bodies (P-bodies), an important site for target mRNA suppression, to stress granules that attenuates RNAi-mediated silencing (Detzer et al., 2011). Furthermore, when the stress related protein MAPK-p38 is activated, it phosphorylates Ago2, which causes its localisation to P-bodies (Zeng et al., 2008). RVFV causes massive cellular oxidative stress (Narayanan et al., 2011) and modulates MAPK-p38 activation during infection in cultured cells (Narayanan et al., 2011, Popova et al., 2010) (section 1.4.2) suggesting RVFV could indirectly regulate RNAi through stress pathways.

Viruses can also manipulate host miRNAs to enhance viral pathogenesis. HCV exploits the liver-expressed miR-122 to facilitate its replication (Jopling et al., 2005). Hantaviruses up-regulate miRNAs linked to vascular permeability (Pepini et al., 2010), which could explain the plasma leakage of the endothelium, a pathological feature of Hantavirus infections [Reviewed in (Schmaljohn and Hjelle, 1997). West Nile virus induces apoptosis, which contributes to pathogenesis (Samuel et al., 2007) and may occur through the manipulation of miRNAs targeted to anti-apoptotic proteins (Smith et al., 2012). Epstein-Barr virus expresses EBNA2 that modulates two host miRNAs, which contribute to viral-induced tumorigenesis (Rosato et al., 2012). Viruses can also regulate miRNAs targeted to restriction factors as observed in HCV infections, which enhances the expression of a miRNA directed to IFITM1 (section 1.3.1.4) (Bhanja Chowdhury et al., 2012). Overall, the above studies demonstrate that viruses can parasitise the host miRNA pathway.

Nevertheless, the viruses' ability to suppress or manipulate the RNAi pathway needs to be addressed as it could negativity impact the efficacy of an RNAi-based therapeutic. This was

demonstrated in studies where siRNAs that potently reduced EBOV protein levels, were comparatively limited in inhibiting EBOV replication (Fabozzi et al., 2011). Studies targeting influenza did not demonstrate any difference in siRNA knockdown of a target gene in the presence or absence of an RNAi antagonist (Rajput 2012) and seems to hold true for our current study where the shRNAs were able to effectively suppress RVFV replication in cultured cells (Figure 4.1, 4.2C and 4.6). Although, the influence of RVFV-mediated RNAi antagonism (through transcription suppression or oxidative stress) to attenuate the RNAi effect may have gone unnoticed with this particular repertoire of experiments and would require further investigation.

During RVFV infection there will be IFN activation and there are important connections between RNAi and IFN (section 1.6.2.1). These links may represent a possible unknown factor influencing the miRNA pathway, which could indirectly affect the anti-RVFV shRNAs. This is compounded by the fact that viruses target essential factors common to both pathways as EBOV-VP35 interacts with TRBP and PACT (Fabozzi et al., 2011), which prevents activation of RIG-1 (Luthra et al., 2013) and may be a mechanism to regulate PKR (Feng et al., 2007) and RNAi (Haasnoot et al., 2007). EBOV-VP35 can cap dsRNA to prevent detection of viral RNA by RIG-1 (Table 1.1) (Kimberlin et al., 2010) and this "capping" may also prevent Dicer processing of viral dsRNA, which would generate viral-derived siRNAs that hinder EBOV replication (section below 1.6.2.2). The crosstalk between IFN and RNAi, with the additional influence of virulence factors, would need to be taken into consideration, with the effects on an expressed shRNA evaluated to ensure effective therapeutic silencing.

5.*7 ShRNAs combined with alterative inhibitory platforms*

A significant feature of RNAi is that it can be multiplexed with other inhibitory platforms to expand its therapeutic potential and can help alleviate the pressure placed on the endogenous RNAi pathway to process multiple shRNAs (section 1.8.3). Similar to using multiple shRNAs, combining shRNAs with other inhibitors could enhance suppression by targeting multiple factors of the viral life cycle (section 5.4). Dominant negative mutants of viral proteins can sequester essential components of a virus' life cycle into inactive mutant forms to inhibit viral replication. The L polymerase of RVFV forms an active oligomer to facilitate RNA synthesis (section 1.2.2 and 1.2.3) and a mutant that was inactive for polymerase activity inhibited RVFV replication *in vitro* (Zamoto-Niikura et al., 2009). Other inhibitory proteins can be used such as the host restriction factor, MxA, which can inhibit RVFV when exogenously introduced by preventing primary transcription (Habjan et al., 2009a) and sequestering N (Kochs et al., 2002) (section 1.5.3). These inhibitory RVFV proteins could be expressed in conjunction with the shRNAs designed in this study as an additional means to inhibit

RVFV. RNA decoys can be exploited to inhibit viral targets and have been used to sequester proteins of HIV to facilitate inhibition (Michienzi et al., 2002). The complementary sequences at the end of RVFV's genomes are bound by N (Mir et al., 2006) and the cytoplasmic tail of Gn (Piper et al., 2011) (section 1.2.4). A decoy RNA that mimics the complementary sequence could potentially have a dual inhibitory effect on RVFV replication by acting as a competitive binding substrate for Gn and N. An aptamer has also been recently developed for RVFV, which acts as a decoy substrate for N (Ellenbecker et al., 2012) (section 1.5.4) and could be expressed in combination with the shRNAs to suppress RVFV.

5.8 Outlook: in vivo *testing*

5.8.1 Delivery vectors *for* in vivo *experimentation*

One of the greatest hurdles of RNAi-based therapies is the delivery of the constructs to their target organs. Unlike small molecular therapies that can readily move through the cell membrane to elicit its intended effect on a viral target, RNAi effectors do not efficiently cross cellular barriers. Non-viral delivery vectors such as liposomes have been used to encapsulate siRNAs for tissue delivery [Reviewed in (Zhang et al., 2012b)] and have been used in non-human primate studies to deliver siRNAs targeted to EBOV (Geisbert et al., 2010) (section 1.7.1.4). Non-viral vectors have reduced delivery efficiency *in vivo* and are not ideal for delivering expression cassettes such as shRNAs [Reviewed in (Gao et al., 2007)].

There are promising delivery methods for shRNAs using viral delivery vectors, which can be modified to have the desired shRNA sequence inserted into a packaged viral genome for delivery to an intended target tissue. Adeno-associated viral vectors (AAV) offer a simple, versatile and potent delivery vector for the expression of shRNAs [Reviewed in (Grieger et al., 2012)]. Anti-RVFV shRNAs can be inserted easily between two internal terminal repeats (ITRs) within the AAV genome to package the RNAi effector into the delivery system. AAVs also lack the viral elements for integration into the host genome and persist episomally avoiding toxicity associated with insertional mutagenesis improving the safety of the vectors. Originally, AAVs had to go through second strand synthesis to make a double-stranded genome, which resulted in delayed transgene expression (Ferrari et al., 1996). Self-complementary AAVs (scAAVs) have been developed that immediately form into dsDNA upon cell entry, which allows for rapid and potent expression of the anti-RVFV shRNAs (Wang et al., 2003). The quick expression of the shRNAs would be desirable when managing the characteristics of haemorrhagic RVF, such as high viral loads that appear within the early stages

of infection (section 1.7.2.2). AAV-shRNAs would also need to exert a quick therapeutic effect within a few days after haemorrhagic symptom presentation to prevent lethality, making rapid expression favourable (section 1.7.2.1).

The presence of siRNAs delivered using liposomes can be detected as soon as 0.5 hrs in target organs and significant knockdown of mRNA can occur 2 hrs post-transduction (Shi et al., 2011) but lipid-delivered siRNAs to EBOV had to be administered daily to obtain protection against lethality (section 1.7.1.4). An AAV-shRNA can decrease target reporter levels by day 2 in cultured cells (Suhy et al., 2012) and can take a period of 7 days to reduce serum protein levels in mice (Chen et al., 2006). An AAV-delivered anti-RVFV shRNA would require a single administration with a prolonged reduction of a target viral protein in the liver (Grimm et al., 2006), which would last throughout the period of a RVFV infection. Unlike the EBOV siRNAs, which required repeat treatments, the constant application of a suppressive effect by an AAV-shRNA may be able to better control the high levels of viraemia associated with haemorrhagic RVF (section 1.7.2.2). However, the delay in suppression by the AAV-shRNAs may have implications for haemorrhagic RVFV that has a small window of treatment as death can occur at day 3 post-infection (section 1.7.2.1).

Haemorrhagic fever viruses also disseminate to a number of other tissues as early as day 2 post-infection (section 1.7.2.3) and treatment would likely occur when the virus has already began to infect other organs. The systemically administered siRNAs against EBOV were delivered to a variety of tissues important for EBOV pathogenesis (section 1.7.1.4), but lipid-delivered siRNAs do not transduce the brain efficiently (Shi et al., 2011). In the case of RVFV, the major tissues are the liver and brain and the overall selective targeting of RNAi effectors to the liver may not be able to prevent neural symptoms during infections such as encephalitis (section 1.2.1) or resurgence of neural pathology after recovery (section 1.7.2.3). There are numerous serotypes of AAVs that have different tropisms for a variety of tissues but specifically AAVs have been used to deliver shRNAs to the liver (Chen et al., 2006) and neurons (McBride et al., 2008). Importantly, specific AAV serotypes can cross the blood brain barrier when administered systemically and result in diffuse transduction of neurons (Foust et al., 2009, Iida et al., 2013), which would allow for expression of an shRNA to counter RVFV in the brain (Smith et al., 2010). AAVs have also been used in human trials delivered through intracranial injections (Kaplitt et al., 2007). Furthermore, serotype AAV9 used in delivery to the brain, also broadly transduces the liver (Chen et al., 2008), which opens up the possibly of a single serotype to target both organs relevant to RVFV. However, this would also result in transduction of other organs such as the heart and kidneys, which raises additional safety concerns. Nevertheless, a combination strategy that involves the targeting of AAVs with anti-RVFV shRNAs to the liver and brain may be required to combat the tissue dissemination observed during RVFV

infections, which could be accomplished using AAVs. Furthermore, even a high dose of lipid-delivered siRNAs does not completely transduce all hepatocytes and only reaches levels of ~80% (Shi et al., 2011), which could render the siRNAs less effective as a result of viral replication in untreated cells. AAV delivered shRNAs transduce hepatocytes at levels of nearly 100% (Suhy et al., 2012), which would be an asset in eliciting a uniform therapeutic effect during RVFV infections. These promises and pitfalls of AAV-mediated delivery of anti-RVFV shRNAs will need to be assessed in post-exposure experiments to determine the potential advantage of using this particular delivery system in treating RVFV, over the lipid-delivered siRNAs used in the EBOV studies.

Exposure to an AAV will result in the elicitation of a humoral response (Chirmule et al., 1999) and in the advent of a subsequent outbreak of RVF, a patient that received an AAV-shRNA previously will have antibodies, which could neutralise the vector before it can deliver its therapeutic payload. A recent method to subvert immunity was investigated involving the fragmentation of DNA sequences of different AAV serotype capsid proteins and randomly ligating them back together to form a novel AAV serotype (Grimm et al., 2008). These artificial AAVs have reduced reactivity with pre-existing immunity (Li et al., 2008) and would improve the continual applicability of the AAV delivered shRNAs by allowing repeat administrations. “Shuffled” capsids can also increase tissue specificity (Yang et al., 2009), which would enhance its safety profile by ensuring tissue specific expression of an shRNA. Finally, AAVs have demonstrated potential in early clinical trials [Reviewed in (Grard et al., 2012)] and recently was the viral vector used in the first gene therapy drug approved by the Europe Union (Yla-Herttuala, 2012). This sanctioning of AAVs as a gene therapy vector, asserts confidence in its selection as a means of delivery for the anti-RVFV shRNAs.

An additional viral vector candidate to deliver anti-RVFV shRNAs is an Adeno-viral vector (Adv) [Reviewed in (Rauschhuber et al., 2012, Arnberg, 2012)]. Similar to the AAVs, there are several serotypes of which Adv5 has been used for liver-specific delivery and would be the serotype of choice for delivery of anti-RVFV shRNAs. A helper-dependent Adv (Hd-Adv) can have large transgenes inserted into its genome (Parks et al., 1996), which would allow for an expansive cohort of shRNAs to be packaged into Advs. However, Advs are ‘mopped up’ by red blood cells (Seiradake et al., 2009, Stone et al., 2007) as well as other non-hepatocyte liver cells (Tao et al., 2001), which prevents efficient transduction of hepatocytes and would reduce the overall effectiveness of the Adv-shRNAs to control haemorrhagic RVF. The neutralisation of Advs has been tackled with various procedures such as surgical, recombinant and chemically-modifying methods [Reviewed in (Khare et al., 2011)]. The addition of polyethylene glycol (PEG) residues to the surface of Advs can “mask” the virus from most of the immune and cellular obstacles, enhancing the applicability of Advs for anti-RVFV shRNA delivery to the liver. These obstacles will need to be fully addressed as efficient

transduction of the liver would be essential to inhibiting RVFV. Finally, a lentiviral (LV) vector, which is a genetically modified HIV-1, can be used for the stable insertion of an expressed shRNA into the host genome [Reviewed in (Sakuma et al., 2012)]. For the purposes of chronic infections, such as HIV or HBV, integrating LVs serve to have life-long expression of a therapeutic shRNA but have an increased risk associated with insertional mutagenesis. As a result of the acute nature of RVFV infections (section 1.7.2.1), integrating, life-long expression of an shRNA is not ideal.

5.8.2 Additional safety of shRNAs in viral delivery

Although it was demonstrated that the shRNAs tested in this study expressed off a weaker H1-Pol III promoter did not have any gross effects on the endogenous RNAi pathway (Figure 4.12), other studies have shown that the potency of the promoter was not the only factor responsible for toxicity. The host RNAi pathway could still be deregulated when a H1-Pol III expressed shRNA was delivered using a potent delivery vector (Borel et al., 2011) and in these scenarios several other efforts can be made to enhance safety. Lowering the delivery vector dose can be effective at reducing cellular toxicity (Suhy et al., 2012). A less potent delivery vector can also be used, where AAV1 can be substituted with an inferior AAV5 serotype, which will result in reduced expression of the shRNAs with no visible toxicity (Ehlert et al., 2010). The potent liver-specific AAV8 delivery vector expressing an shRNA can cause hepatotoxicity (Borel et al., 2011), but AAV7 or AAV9 deliver fewer copies of the AAVs per cell producing fewer guide strands than AAV8 (Chen et al., 2008) and could be an alternative delivery vector to prevent unwanted toxicity. Some studies have noted a reduction in cognate target inhibition when using an inferior vector (Ehlert et al., 2010) and a similar loss of silencing was observed when lowering the vector dose (McBride et al., 2008). This stresses the importance of designing and selecting potent guide strands, which can still be effective at lower expression levels. Overall, several considerations with regards to the delivery vectors can be taken into account to improve safety of the expressed shRNAs *in vivo.*

5.8.3 RVFV animal models

There are several animal models for RVFV, which could be used to test the shRNAs *in vivo*. Mice are highly susceptible to RVFV with 100% mortality, which would give a clear distinction of the therapeutic effectiveness of the shRNAs [Reviewed in (Ross et al., 2012)]. Mice also manifest hepatic necrosis and neural complications similar to human infections, making them an important model for observing other human-associated pathologies (section 1.2.1). Mice also have been used extensively

in studies that delivered shRNAs using AAVs [Reviewed in (Grimm, 2011)] and are an excellent small rodent model for determining effectiveness of the shRNAs against RVFV in the next stage of experimental investigation.

Future development of the therapeutic shRNAs for use in humans will require a non-human primate model. Rhesus macaques have similar susceptibility and disease progression to humans, although the disease was not uniformly lethal, which can make determining therapeutic effectiveness of the shRNAs problematic (Morrill et al., 1990). A marmoset model was recently developed, which has near uniform lethality depending on the route of exposure (Smith et al., 2011). Marmosets mimic severe cases of RVFV and are an ideal model for investigating the shRNA's ability to manage aggressive cases of RVF. Several routes of infection were investigated with regards to disease progression such as aerosol exposure (intranasal) or mosquito vector (subcutaneous). This would allow for a broader investigation of the therapeutic effectiveness of the shRNAs when controlling RVFV through different exposure routes, as intranasal exposure presented with increased neurological symptoms (section 1.7.2.3), which could have implications for shRNAs targeted specifically to the liver (section 5.8.1). Overall, there are two important models for the development of RNAi against RVFV: a small rodent model to confirm the preliminary effectiveness of the anti-RVFV shRNAs *in vivo*, while later studies would utilise a non-human primate marmoset model to evaluate the shRNA's ability to control haemorrhagic cases of RVFV.

5.9 Conclusion

Haemorrhagic fever viruses are an emerging group of infectious agents that are a significant disease burden. Particularly disturbing was the recent identification of a haemorrhagic fever virus outside of the "classic" families, belonging to the *Rhabdoviridae* family (Grard et al., 2012). The emergence of this novel haemorrhagic virus, combined with the knowledge that a limited number of amino acid substitutions were required for a current haemorrhagic fever virus to become virulent in a previously resistant host (section 1.3.2), suggests that the scope of these viruses could be much larger than previously thought. Furthermore, the "classic" families are genetically very different in terms of structure and encoded genes, so the emergence of a *Rhabdoviridae* haemorrhagic fever implies that any group of viruses, given the correct genetic diversification, could produce a haemorrhagic fever virus strain. This exemplifies the necessity to explore novel inhibitors for the treatment of highly pathogenic viruses.

RVFV represents a significant emerging pathogen and has an incredible ability to spread geographically (section 1.2.1), but there is little in the way of therapeutic development (section 1.5). This thesis focused on a particular strategy of expressed shRNAs as a novel method to inhibit RVFV.

Importantly, two main types of anti-RVFV shRNAs were identified, anti-pathogenic and anti-replicative, which were extensively characterised for their ability to affect RVFV's cytotoxic effects and replication. The shRNAs would have differing properties when inhibiting a RVFV infection, which could complement each other and further assist RNAi-based therapeutic development. Furthermore, this expands the idea that RNAi can be effective against highly pathogenic pathogens such as haemorrhagic fever viruses and should be developed not only as a modality for the treatment of RVFV, but explored as a novel therapeutic for haemorrhagic fevers as a whole. However, expressed shRNAs still need to be assessed *in vivo*, and in particular post-exposure experiments, to ascertain the true potential of the shRNAs to control RVFV. These data in this thesis is the first example of a sequence specific, catalytic therapeutic to inhibit RVFV and further work will be required to determine how the mechanism of RNAi compares to other RVFV therapeutics currently in development. The ultimate goal will be the use of shRNAs in humans with a possible additional application in livestock. The work described in this thesis has substantially contributed to the development of RNAi-based therapeutics as a novel intervention strategy for RVFV.

References

Aagaard, L. A., Zhang, J., Von Eije, K. J., Li, H., Saetrom, P., Amarzguioui, M. & Rossi, J. J. (2008) Engineering and optimization of the miR-106b cluster for ectopic expression of multiplexed anti-HIV RNAs. *Gene Therapy,* 15**,** 1536-1549.

Aarbiou, J., Copreni, E., Buijs-Offerman, R. M., Van Der Wegen, P., Castellani, S., Carbone, A., Tilesi, F., Fradiani, P., Hiemstra, P. S., Yueksekdag, G., Diana, A., Rosenecker, J., Ascenzioni, F., Conese, M. & Scholte, B. J. (2012) Lentiviral small hairpin RNA delivery reduces apical sodium channel activity in differentiated human airway epithelial cells. *The Journal of Gene Medicine,* 14**,** 733-745.

Abu-Elyazeed, R., El-Sharkawy, S., Olson, J., Botros, B., Soliman, A., Salib, A., Cummings, C. & Arthur, R. (1996) Prevalence of anti-Rift-Valley-fever IgM antibody in abattoir workers in the Nile delta during the 1993 outbreak in Egypt. *Bulletin of the World Health Organization,* 74**,** 155–158.

Ahmed Kamal, S. (2011) Observations on rift valley fever virus and vaccines in Egypt. *Virology Journal,* 8**,** 532.

Ahn, J., Ko, A., Jun, E. J., Won, M., Kim, Y. K., Ju, E. S., Jeon, E. S. & Lee, H. (2012) Antiviral Effects of Small Interfering RNA Simultaneously Inducing RNA Interference and Type 1 Interferon in Coxsackievirus Myocarditis. *Antimicrobial Agents and Chemotherapy,* 56**,** 3516-3523.

Ahn, M., Witting, S. R., Ruiz, R., Saxena, R. & Morral, N. (2011) Constitutive Expression of Short Hairpin RNA in Vivo Triggers Buildup of Mature Hairpin Molecules. *Human Gene Therapy,* 22**,** 1483-1497.

Albariño, C. G., Bird, B. H. & Nichol, S. T. (2007) A Shared Transcription Termination Signal on Negative and Ambisense RNA Genome Segments of Rift Valley Fever, Sandfly Fever Sicilian, and Toscana Viruses. *Journal of Virology,* 81**,** 5246-5256.

Alhoot, M. A., Wang, S. M. & Sekaran, S. D. (2011) Inhibition of Dengue Virus Entry and Multiplication into Monocytes Using RNA Interference. *PLoS Neglected Tropical Diseases,* 5**,** e1410.

Alhoot, M. A., Wang, S. M. & Sekaran, S. D. (2012) RNA Interference Mediated Inhibition of Dengue Virus Multiplication and Entry in HepG2 Cells. *PLoS ONE,* 7**,** e34060.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990) Basic local alignment search tool. *Journal of Molecular Biology,* 215**,** 403-410.

Aman, M. J., Kinch, M. S., Warfield, K., Warren, T., Yunus, A., Enterlein, S., Stavale, E., Wang, P., Chang, S., Tang, Q., Porter, K., Goldblatt, M. & Bavari, S. (2009) Development of a broad-spectrum antiviral with activity against Ebola virus. *Antiviral Research,* 83**,** 245-251.

Amantana, A., Moulton, H. M., Cate, M. L., Reddy, M. T., Whitehead, T., Hassinger, J. N., Youngblood, D. S. & Iversen, P. L. (2007) Pharmacokinetics, Biodistribution, Stability and Toxicity of a Cell-Penetrating Peptide-Morpholino Oligomer Conjugate. *Bioconjugate Chemistry,* 18**,** 1325-1331.

An, D., Qin, F., Auyeung, V., Mao, S., Kung, S., Baltimore, D. & Chen, I. (2006) Optimization and functional effects of stable short hairpin RNA expression in primary human lymphocytes via lentiviral vectors. *Molecular Therapy,* 14**,** 494-504.

Anderson Jr, G. W. & Smith, J. F. (1987) Immunoelectron microscopy of Rift Valley fever viral morphogenesis in primary rat hepatocytes. *Virology,* 161**,** 91-100.

Andrejeva, J., Childs, K. S., Young, D. F., Carlos, T. S., Stock, N., Goodbourn, S. & Randall, R. E. (2004) The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN-β promoter. *Proceedings of the National Academy of Sciences,* 101**,** 17264-17269.

Ang, F., Wong, A., Ng, M. & Chu, J. (2010) Small interference RNA profiling reveals the essential role of human membrane trafficking genes in mediating the infectious entry of dengue virus. *Virology Journal,* 7**,** 24.

Arbuthnot, P. (2010) Harnessing RNA interference for the treatment of viral infections. *Drug News Perspect,* 23**,** 341-350.

Arnberg, N. (2012) Adenovirus receptors: implications for targeting of viral vectors. *Trends in Pharmacological Sciences,* 33**,** 442-448.

Artuso, M. C., Ellenberg, P. C., Scolaro, L. A., Damonte, E. B. & García, C. C. (2009) Inhibition of Junín virus replication by small interfering RNAs. *Antiviral Research,* 84**,** 31-37.

Ashour, J., Laurent-Rolle, M., Shi, P. & García-Sastre, A. (2009) NS5 of Dengue Virus Mediates STAT2 Binding and Degradation. *Journal of Virology,* 83**,** 5408-5418.

Austin, D., Baer, A., Lundberg, L., Shafagati, N., Schoonmaker, A., Narayanan, A., Popova, T., Panthier, J. J., Kashanchi, F., Bailey, C. & Kehn-Hall, K. (2012) p53 Activation following Rift Valley Fever Virus Infection Contributes to Cell Death and Viral Production. *PLoS ONE,* 7**,** e36327.

Baek, D., Villen, J., Shin, C., Camargo, F. D., Gygi, S. P. & Bartel, D. P. (2008) The impact of microRNAs on protein output. *Nature,* 455**,** 64-71.

Baer, A., Austin, D., Narayanan, A., Popova, T., Kainulainen, M., Bailey, C., Kashanchi, F., Weber, F. & Kehn-Hall, K. (2012) Induction of DNA Damage Signaling upon Rift Valley Fever Virus Infection Results in Cell Cycle Arrest and Increased Viral Replication. *Journal of Biological Chemistry,* 287**,** 7399-7410.

Bale, S., Julien, J., Bornholdt, Z. A., Kimberlin, C. R., Halfmann, P., Zandonatti, M. A., Kunert, J., Kroon, G. J. A., Kawaoka, Y., Macrae, I. J., Wilson, I. A. & Saphire, E. O. (2012) Marburg Virus VP35 Can Both Fully Coat the Backbone and Cap the Ends of dsRNA for Interferon Antagonism. *PLoS Pathogens,* 8**,** e1002916.

Barichievy, S., Saayman, S., Von Eije, K. J., Morris, K. V., Arbuthnot, P. & Weinberg, M. S. (2007) The Inhibitory Efficacy of RNA POL III-Expressed Long Hairpin RNAs Targeted to Untranslated Regions of the HIV-1 5′ Long Terminal Repeat. *Oligonucleotides,* 17**,** 419-432.

Bartel, D. P. (2009) MicroRNAs: Target Recognition and Regulatory Functions. *Cell,* 136**,** 215-233.

Bartlett, D. W. & Davis, M. E. (2006) Insights into the kinetics of siRNA-mediated gene silencing from live-cell and live-animal bioluminescent imaging. *Nucleic Acids Research,* 34**,** 322-333.

Basler, C. F., Mikulasova, A., Martinez-Sobrido, L., Paragas, J., Mühlberger, E., Bray, M., Klenk, H., Palese, P. & García-Sastre, A. (2003) The Ebola Virus VP35 Protein Inhibits Activation of Interferon Regulatory Factor 3. *Journal of Virology,* 77**,** 7945-7956.

Basler, C. F., Wang, X., Mühlberger, E., Volchkov, V., Paragas, J., Klenk, H., García-Sastre, A. & Palese, P. (2000) The Ebola virus VP35 protein functions as a type I IFN antagonist. *Proceedings of the National Academy of Sciences,* 97**,** 12289-12294.

Benferhat, R., Josse, T., Albaud, B., Gentien, D., Mansuroglu, Z., Marcato, V., Le Bonniec, B., Bouloy, M. L. & Bonnefoy, E. (2012) Large-scale ChIP-on-chip analysis of the interaction of the NSs protein of Rift Valley Fever virus with regulatory DNA regions of the host genome. *Journal of Virology,* 86**,** 11333-11344.

Bente, D. A., Alimonti, J. B., Shieh, W., Camus, G., Ströher, U., Zaki, S. & Jones, S. M. (2010) Pathogenesis and Immune Response of Crimean-Congo Hemorrhagic Fever Virus in a STAT-1 Knockout Mouse Model. *Journal of Virology,* 84**,** 11089-11100.

Bereczky, S., Lindegren, G., Karlberg, H., Åkerström, S., Klingström, J. & Mirazimi, A. (2010) Crimean-Congo hemorrhagic fever virus infection is lethal for adult type I interferon receptor-knockout mice. *Journal of General Virology,* 91**,** 1473-1477.

Bhanja Chowdhury, J., Shrivastava, S., Steele, R., Di Bisceglie, A. M., Ray, R. & Ray, R. B. (2012) Hepatitis C Virus Infection Modulates Expression of Interferon Stimulatory Gene IFITM1 by Upregulating miR-130A. *Journal of Virology,* 86**,** 10221-10225.

Billecocq, A., Spiegel, M., Vialat, P., Kohl, A., Weber, F., Bouloy, M. & Haller, O. (2004) NSs protein of Rift Valley fever virus blocks interferon production by inhibiting host gene transcription. *Journal of Virology,* 78**,** 9798-9806.

Bird, B., Khristova, M., Rollin, P., Ksiazek, T. & Nichol, S. (2007a) Complete genome analysis of 33 ecologically and biologically diverse Rift Valley fever virus strains reveals widespread virus movement and low genetic diversity due to recent common ancestry. *Journal of Virology,* 81**,** 2805-2816.

Bird, B. H., Albariño, C. G., Hartman, A. L., Erickson, B. R., Ksiazek, T. G. & Nichol, S. T. (2008) Rift Valley Fever Virus Lacking the NSs and NSm Genes Is Highly Attenuated, Confers Protective Immunity from Virulent Virus Challenge, and Allows for Differential Identification of Infected and Vaccinated Animals. *Journal of Virology,* 82**,** 2681-2691.

Bird, B. H., Albariño, C. G. & Nichol, S. T. (2007b) Rift Valley fever virus lacking NSm proteins retains high virulence in vivo and may provide a model of human delayed onset neurologic disease. *Virology,* 362**,** 10-15.

Bish, L. T., Sleeper, M. M., Reynolds, C., Gazzara, J., Withnall, E., Singletary, G. E., Buchlis, G., Hui, D., High, K. A., Gao, G., Wilson, J. M. & Sweeney, H. L. (2011) Cardiac Gene Transfer of Short Hairpin RNA Directed Against Phospholamban Effectively Knocks Down Gene Expression but Causes Cellular Toxicity in Canines. *Human Gene Therapy,* 22**,** 969-977.

Bitko, V., Musiyenko, A., Shulyayeva, O. & Barik, S. (2005) Inhibition of respiratory viruses by nasally administered siRNA. *Nature Medicine,* 11**,** 50-55.

Blakqori, G., Delhaye, S., Habjan, M., Blair, C. D., Sanchez-Vargas, I., Olson, K. E., Attarzadeh-Yazdi, G., Fragkoudis, R., Kohl, A., Kalinke, U., Weiss, S., Michiels, T., Staeheli, P. & Weber, F. (2007) La Crosse Bunyavirus Nonstructural Protein NSs Serves To Suppress the Type I Interferon System of Mammalian Hosts. *Journal of Virology,* 81**,** 4991-4999.

Boden, D., Pusch, O., Lee, F., Tucker, L., Shank, P. R. & Ramratnam, B. (2003) Promoter choice affects the potency of HIV-1 specific RNA interference. *Nucleic Acids Research,* 31**,** 5033-5038.

Borchert, G. M., Lanier, W. & Davidson, B. L. (2006) RNA polymerase III transcribes human microRNAs. *Nature Structural & Molecular Biology,* 13**,** 1097-1101.

Borel, F., Van Logtenstein, R., Koornneef, A., Maczuga, P., Ritsema, T., Petry, H., Van Deventer, S. J. H., Jansen, P. L. M. & Konstantinova, P. (2011) In vivo knock-down of

multidrug resistance transporters ABCC1 and ABCC2 by AAV-delivered shRNAs and by artificial miRNAs. *Journal of RNAi and Gene Silencing,* 7**,** 434-442.

Boshra, H., Lorenzo, G., Rodriguez, F. & Brun, A. (2011) A DNA vaccine encoding ubiquitinated Rift Valley fever virus nucleoprotein provides consistent immunity and protects IFNAR−/− mice upon lethal virus challenge. *Vaccine,* 29**,** 4469-4475.

Bouloy, M. & Flick, R. (2009) Reverse genetics technology for Rift Valley fever virus: Current and future applications for the development of therapeutics and vaccines. *Antiviral Research,* 84**,** 101-118.

Bouloy, M., Janzen, C., Vialat, P., Khun, H., Pavlovic, J., Huerre, M. & Haller, O. (2001) Genetic evidence for an interferon-antagonistic function of rift valley fever virus nonstructural protein NSs. *Journal of Virology,* 75**,** 1371-1377.

Bowen, E. T. W., Lloyd, G., Harris, W. J., Platt, G. S., Baskerville, A. & Vella, E. E. (1977) Viral haemorrhagic fever in southern Sudan and northern Zaire. Preliminary studies on the aetiological agent. *Lancet,* 309**,** 571-573.

Brass, A. L., Dykxhoorn, D. M., Benita, Y., Yan, N., Engelman, A., Xavier, R. J., Lieberman, J. & Elledge, S. J. (2008) Identification of Host Proteins Required for HIV Infection Through a Functional Genomic Screen. *Science,* 319**,** 921-926.

Braun, J., Huntzinger, E., Fauser, M. & Izaurralde, E. (2011) GW182 Proteins Directly Recruit Cytoplasmic Deadenylase Complexes to miRNA Targets. *Molecular Cell,* 44**,** 120-133.

Bray, M. (2001) The role of the Type I interferon response in the resistance of mice to filovirus infection. *Journal of General Virology,* 82**,** 1365-1373.

Broderick, J. A., Salomon, W. E., Ryder, S. P., Aronin, N. & Zamore, P. D. (2011) Argonaute protein identity and pairing geometry determine cooperativity in mammalian RNA silencing. *RNA,* 17**,** 1858-1869.

Brummelkamp, T., Bernards, R. & Agami, R. (2002) A system for stable expression of short interfering RNAs in mammalian cells. *Science,* 296**,** 550-553.

Cai, X., Hagedorn, C. H. & Cullen, B. R. (2004) Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. *RNA,* 10**,** 1957-1966.

Cárdenas, W. B., Loo, Y., Gale, M., Hartman, A. L., Kimberlin, C. R., Martínez-Sobrido, L., Saphire, E. O. & Basler, C. F. (2006) Ebola Virus VP35 Protein Binds Double-Stranded RNA and Inhibits Alpha/Beta Interferon Production Induced by RIG-I Signaling. *Journal of Virology,* 80**,** 5168-5178.

Carette, J. E., Raaben, M., Wong, A. C., Herbert, A. S., Obernosterer, G., Mulherkar, N., Kuehne, A. I., Kranzusch, P. J., Griffin, A. M., Ruthel, G., Cin, P. D., Dye, J. M., Whelan, S. P., Chandran, K. & Brummelkamp, T. R. (2011) Ebola virus entry requires the cholesterol transporter Niemann-Pick C1. *Nature,* 477**,** 340-343.

Carmona, S., Ely, A., Crowther, C., Moolla, N., Salazar, F. H., Marion, P. L., Ferry, N., Weinberg, M. S. & Arbuthnot, P. (2006) Effective Inhibition of HBV Replication in Vivo by Anti-HBx Short Hairpin RNAs. *Molecular Therapy,* 13**,** 411-421.

Carstea, E. D., Morris, J. A., Coleman, K. G., Loftus, S. K., Zhang, D., Cummings, C., Gu, J., Rosenfeld, M. A., Pavan, W. J., Krizman, D. B., Nagle, J., Polymeropoulos, M. H., Sturley, S. L., Ioannou, Y. A., Higgins, M. E., Comly, M., Cooney, A., Brown, A., Kaneski, C. R., Blanchette-Mackie, E. J., Dwyer, N. K., Neufeld, E. B., Chang, T., Liscum, L., Strauss, J. F., Ohno, K., Zeigler, M., Carmi, R., Sokol, J., Markie, D., O'neill, R. R., Van Diggelen, O. P., Elleder, M., Patterson, M. C., Brady, R. O., Vanier, M. T., Pentchev, P. G. & Tagle, D. A. (1997) Niemann-Pick C1 Disease Gene: Homology to Mediators of Cholesterol Homeostasis. *Science,* 277**,** 228-231.

Castanotto, D., Li, H. & Rossi, J. (2002) Functional siRNA expression from transfected PCR products. *RNA,* 8**,** 1454-1460.

Chambers, P. G. & Swanepoel, R. (1980) Rift Valley fever in abattoir workers. *Central African Journal of Medicine,* 26**,** 122-126.

Chang, J., Nicolas, E., Marks, D., Sander, C., Lerro, A., Buendia, M. A., Xu, C., Mason, W. S., Moloshok, T., Bort, R., Zaret, K. S. & Taylor, J. M. (2004) miR-122, a Mammalian Liver-Specific microRNA, is Processed from hcr mRNA and May Downregulate the High Affinity Cationic Amino Acid Transporter CAT-1. *RNA Biology,* 1**,** 106-113.

Chang, J., Warren, T. K., Zhao, X., Gill, T., Guo, F., Wang, L., Comunale, M. A., Du, Y., Alonzi, D. S., Yu, W., Ye, H., Liu, F., Guo, J., Mehta, A., Cuconati, A., Butters, T. D., Bavari, S., Xu, X. & Block, T. M. (2013) Small molecule inhibitors of ER α-glucosidases are active against multiple hemorrhagic fever viruses. *Antiviral Research,* 98**,** 432-440.

Chang, T., Kubota, T., Matsuoka, M., Jones, S., Bradfute, S. B., Bray, M. & Ozato, K. (2009) Ebola Zaire Virus Blocks Type I Interferon Production by Exploiting the Host SUMO Modification Machinery. *PLoS Pathogens,* 5**,** e1000493.

Chen, C., Sun, C., Ma, H., Fang, C., Wu, P., Xiao, X. & Tao, M. (2008) Comparative Study of Anti-hepatitis B Virus RNA Interference by Double-stranded Adeno-associated Virus Serotypes 7, 8, and 9. *Molecular Therapy,* 17**,** 352-359.

Chen, C. C., Ko, T. M., Ma, H. I., Wu, H. L., Xiao, X., Li, J., Chang, C. M., Wu, P. Y., Chen, C. H., Han, J. M., Yu, C. P., Jeng, K. S., Hu, C. P. & Tao, M. H. (2006) Long-term inhibition of hepatitis B virus in transgenic mice by double-stranded adeno-associated virus 8-delivered short hairpin RNA. *Gene Therapy,* 14**,** 11-19.

Chen, H., Hofman, F. M., Kung, J. T., Lin, Y. & Wu-Hsieh, B. A. (2007) Both Virus and Tumor Necrosis Factor Alpha Are Critical for Endothelium Damage in a Mouse Model of Dengue Virus-Induced Hemorrhage. *Journal of Virology,* 81**,** 5518-5526.

Chen, J. & Xie, J. (2012) Progress on RNAi-based molecular medicines. *International Journal of Nanomedicine,* 7**,** 3971-3980.

Chendrimada, T. P., Gregory, R. I., Kumaraswamy, E., Norman, J., Cooch, N., Nishikura, K. & Shiekhattar, R. (2005) TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature,* 436**,** 740-744.

Cheng, E. & Mir, M. A. (2012) Signatures of Host mRNA 5' Terminus for Efficient Hantavirus Cap Snatching. *Journal of Virology,* 86**,** 10173-10185.

Chirmule, N., Propert, K., Magosin, S., Qian, Y., Qian, R. & Wilson, J. (1999) Immune responses to adenovirus and adeno-associated virus in humans. *Gene Therapy,* 6**,** 1574-1583.

Cho, W. G., Albuquerque, R. J. C., Kleinman, M. E., Tarallo, V., Greco, A., Nozaki, M., Green, M. G., Baffi, J. Z., Ambati, B. K., De Falco, M., Alexander, J. S., Brunetti, A., De Falco, S. & Ambati, J. (2009) Small interfering RNA-induced TLR3 activation inhibits blood and lymphatic vessel growth. *Proceedings of the National Academy of Sciences,* 106**,** 7137-7142.

Coffman, J., Dickey-Sims, C., Haug, J., Mccarthy, J. & Robertson, A. (2004) Evaluation of developmental phenotypes produced by morpholino antisense targeting of a sea urchin Runx gene. *BMC Biology,* 2**,** 6.

Collett, M. (1986) Messenger RNA of the M segment RNA of Rift Valley fever virus. *Virology,* 151**,** 151-156.

Collett, M. S., Purchio, A. F., Keegan, K., Frazier, S., Hays, W., Anderson, D. K., Parker, M. D., Schmaljohn, C., Schmidt, J. & Dalrymple, J. M. (1985) Complete nucleotide sequence of the M RNA segment of rift valley fever virus. *Virology,* 144**,** 228-245.

Crabtree, M. B., Kent Crockett, R. J., Bird, B. H., Nichol, S. T., Erickson, B. R., Biggerstaff, B. J., Horiuchi, K. & Miller, B. R. (2012) Infection and Transmission of Rift Valley Fever Viruses Lacking the NSs and/or NSm Genes in Mosquitoes: Potential Role for NSm in Mosquito Infection. *PLoS Neglected Tropical Diseases,* 6**,** e1639.

Czauderna, F., Santel, A., Hinz, M., Fechtner, M., Durieux, B., Fisch, G., Leenders, F., Arnold, W., Giese, K., Klippel, A. & Kaufmann, J. (2003) Inducible shRNA expression for application in a prostate cancer mouse model. *Nucleic Acids Research,* 31**,** e127.

Daher, A., Laraki, G., Singh, M., Melendez-Pena, C. E., Bannwarth, S., Peters, A. H., Meurs, E. F., Braun, R. E., Patel, R. C. & Gatignol, A. (2009) TRBP control of PACT-induced phosphorylation of PKR is reversed by stress. *Molecular and Cellular Biology,* 29**,** 254-265.

Das, S., Ghosal, S., Kozak, K. & Chakrabarti, J. (2012) An siRNA designing tool with a unique functional off-target filtering approach. *Journal of Biomolecular Structure and Dynamics,* DOI:10.1080/07391102.2012.736758.

Daubney, R., Hudson, J. R. & Garnham, P. C. (1931) Enzootic hepatitis or rift valley fever. An undescribed virus disease of sheep cattle and man from east africa. *The Journal of Pathology and Bacteriology,* 34**,** 545-579.

Deas, T. S., Binduga-Gajewska, I., Tilgner, M., Ren, P., Stein, D. A., Moulton, H. M., Iversen, P. L., Kauffman, E. B., Kramer, L. D. & Shi, P. (2005) Inhibition of Flavivirus Infections by Antisense Oligomers Specifically Suppressing Viral Translation and RNA Replication. *Journal of Virology,* 79**,** 4599-4609.

Denti, M. A., Rosa, A., Sthandier, O., De Angelis, F. G. & Bozzoni, I. (2004) A New Vector, Based on the PolII Promoter of the U1 snRNA Gene, for the Expression of siRNAs in Mammalian Cells. *Molecular Therapy,* 10**,** 191-199.

Detzer, A., Engel, C., Wünsche, W. & Sczakiel, G. (2011) Cell stress is related to re-localization of Argonaute 2 and to decreased RNA interference in human cells. *Nucleic Acids Research,* 39**,** 2727-2741.

Deyde, V. M., Khristova, M. L., Rollin, P. E., Ksiazek, T. G. & Nichol, S. T. (2006) Crimean-Congo Hemorrhagic Fever Virus Genomics and Global Diversity. *Journal of Virology,* 80**,** 8834-8842.

Diamond, M. S. & Farzan, M. (2013) The broad-spectrum antiviral functions of IFIT and IFITM proteins. *Nature Reviews Immunology,* 13**,** 46-57.

Dieci, G., Fiorino, G., Castelnuovo, M., Teichmann, M. & Pagano, A. (2007) The expanding RNA polymerase III transcriptome. *Trends in Genetics,* 23**,** 614-622.

Donnelly, R. P. & Kotenko, S. V. (2010) Interferon-Lambda: A New Addition to an Old Family. *Journal of Interferon & Cytokine Research,* 30**,** 555-564.

Dwek, R. A., Butters, T. D., Platt, F. M. & Zitzmann, N. (2002) Targeting glycosylation as a therapeutic approach. *Nature Reviews Drug Discovery,* 1**,** 65-75.

Dyer, V., Ely, A., Bloom, K., Weinberg, M. & Arbuthnot, P. (2010) tRNALys3 promoter cassettes that efficiently express RNAi-activating antihepatitis B virus short hairpin RNAs. *Biochemical and Biophysical Research Communications,* 398**,** 640-646.

Ehlert, E., Eggers, R., Niclou, S. & Verhaagen, J. (2010) Cellular toxicity following application of adeno-associated viral vector-mediated RNA interference in the nervous system. *BMC Neuroscience,* 11**,** 20.

Ekker, S. C. & Larson, J. D. (2001) Morphant technology in model developmental systems. *genesis,* 30**,** 89-93.

Elbashir, S., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. & Tuschl, T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature,* 411**,** 494-498.

Ellenbecker, M., Sears, L., Li, P., Lanchy, J. & Stephen Lodmell, J. (2012) Characterization of RNA aptamers directed against the nucleocapsid protein of Rift Valley fever virus. *Antiviral Research,* 93**,** 330-339.

Elliott, R. M. (1990) Molecular biology of the Bunyaviridae. *Journal of General Virology,* 71**,** 501-522.

Ely, A., Naidoo, T. & Arbuthnot, P. (2009) Efficient silencing of gene expression with modular trimeric Pol II expression cassettes comprising microRNA shuttles. *Nucleic Acids Research,* 37**,** e91.

Emonet, S. E., Urata, S. & De La Torre, J. C. (2011) Arenavirus reverse genetics: New approaches for the investigation of arenavirus biology and development of antiviral strategies. *Virology,* 411**,** 416-425.

Enterlein, S., Warfield, K. L., Swenson, D. L., Stein, D. A., Smith, J. L., Gamble, C. S., Kroeker, A. D., Iversen, P. L., Bavari, S. & Mühlberger, E. (2006) VP35 Knockdown Inhibits Ebola Virus Amplification and Protects against Lethal Infection in Mice. *Antimicrobial Agents and Chemotherapy,* 50**,** 984-993.

Everett, H. & Mcfadden, G. (1999) Apoptosis: an innate immune response to virus infection. *Trends in Microbiology,* 7**,** 160-165.

Fabozzi, G., Nabel, C. S., Dolan, M. A. & Sullivan, N. J. (2011) Ebolavirus Proteins Suppress the Effects of Small Interfering RNA by Direct Interaction with the Mammalian RNA Interference Pathway. *Journal of Virology,* 85**,** 2512-2523.

Fan, L., Briese, T. & Lipkin, W. I. (2010) Z Proteins of New World Arenaviruses Bind RIG-I and Interfere with Type I Interferon Induction. *Journal of Virology,* 84**,** 1785-1791.

Fang, Y. X. & Gao, W. Q. (2013) Roles of microRNAs during prostatic tumorigenesis and tumor progression. *Oncogene,* doi:10.1038/onc.2013.54.

Feng, Z., Cerveny, M., Yan, Z. & He, B. (2007) The VP35 Protein of Ebola Virus Inhibits the Antiviral Effect Mediated by Double-Stranded RNA-Dependent Protein Kinase PKR. *Journal of Virology,* 81**,** 182-192.

Ferm, V. H., Willhite, C. & Kilham, L. (1978) Teratogenic effects of ribavirin on hamster and rat embryos. *Teratology,* 17**,** 93-101.

Ferrari, F. K., Samulski, T., Shenk, T. & Samulski, R. J. (1996) Second-strand synthesis is a rate-limiting step for efficient transduction by recombinant adeno-associated virus vectors. *Journal of Virology,* 70**,** 3227-3234.

Ferron, F., Li, Z., Danek, E. I., Luo, D., Wong, Y., Coutard, B., Lantez, V., Charrel, R., Canard, B., Walz, T. & Lescar, J. (2011) The Hexamer Structure of the Rift Valley Fever Virus Nucleoprotein Suggests a Mechanism for its Assembly into Ribonucleoprotein Complexes. *PLoS Pathogens,* 7**,** e1002030.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. & Mello, C. C. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature,* 391**,** 806-811.

Fisher, T., Joshi, P. & Prasad, V. (2005) HIV-1 reverse transcriptase mutations that confer decreased in vitro susceptibility to anti-RT DNA aptamer RT1t49 confer cross

resistance to other anti-RT aptamers but not to standard RT inhibitors. *AIDS Research and Therapy,* 2**,** 8.

Fitzgerald, K. A. (2003) IKKε and TBK1 are essential components of the IRF3 signaling pathway. *Nature Immunology,* 4**,** 491-496.

Flusin, O., Vigne, S., Peyrefitte, C., Bouloy, M., Crance, J. & Iseni, F. (2011) Inhibition of Hazara nairovirus replication by small interfering RNAs and their combination with ribavirin. *Virology Journal,* 8**,** 249.

Fontana, J., López-Montero, N., Elliott, R. M., Fernández, J. J. & Risco, C. (2008) The unique architecture of Bunyamwera virus factories around the Golgi complex. *Cellular Microbiology,* 10**,** 2012-2028.

Foust, K. D., Nurre, E., Montgomery, C. L., Hernandez, A., Chan, C. M. & Kaspar, B. K. (2009) Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes. *Nature Biotechnology,* 27**,** 59-65.

Fowler, T., Bamberg, S., Moller, P., Klenk, H., Meyer, T. F., Becker, S. & Rudel, T. (2005) Inhibition of Marburg virus protein expression and viral release by RNA interference. *Journal of General Virology,* 86**,** 1181-1188.

Freiberg, A. N., Sherman, M. B., Morais, M. C., Holbrook, M. R. & Watowich, S. J. (2008) Three-Dimensional Organization of Rift Valley Fever Virus Revealed by Cryoelectron Tomography. *Journal of Virology,* 82**,** 10341-10348.

Frias-Staheli, N., Giannakopoulos, N. V., Kikkert, M., Taylor, S. L., Bridgen, A., Paragas, J., Richt, J. A., Rowland, R. R., Schmaljohn, C. S., Lenschow, D. J., Snijder, E. J., García-Sastre, A. & Virgin Iv, H. W. (2007) Ovarian Tumor Domain-Containing Viral Proteases Evade Ubiquitin- and ISG15-Dependent Innate Immune Responses. *Cell Host & Microbe,* 2**,** 404-416.

Furuta, Y., Takahashi, K., Shiraki, K., Sakamoto, K., Smee, D. F., Barnard, D. L., Gowen, B. B., Julander, J. G. & Morrey, J. D. (2009) T-705 (favipiravir) and related compounds: Novel broad-spectrum inhibitors of RNA viral infections. *Antiviral Research,* 82**,** 95-102.

Gao, X., Kim, K. & Liu, D. (2007) Nonviral gene delivery: What we know and what is next. *The AAPS Journal,* 9**,** E92-E104.

Garcin, D., Lezzi, M., Dobbs, M., Elliott, R. M., Schmaljohn, C., Kang, C. Y. & Kolakofsky, D. (1995) The 5' ends of Hantaan virus (Bunyaviridae) RNAs suggest a prime-and-realign mechanism for the initiation of RNA synthesis. *Journal of Virology,* 69**,** 5754-5762.

Gargan, T. P., Clark, G. G., Dohm, D. J., Turell, M. J. & Bailey, C. L. (1988) Vector potential of selected North American mosquito species for Rift Valley fever virus. *The American Journal of Tropical Medicine and Hygiene,* 38**,** 440-446.

Garry, C. & Garry, R. (2004) Proteomics computational analyses suggest that the carboxyl terminal glycoproteins of Bunyaviruses are class II viral fusion protein (beta-penetrenes). *Theoretical Biology and Medical Modelling,* 1**,** 10.

Gauliard, N., Billecocq, A. S., Flick, R. & Bouloy, M. L. (2006) Rift Valley fever virus noncoding regions of L, M and S segments regulate RNA synthesis. *Virology,* 351**,** 170-179.

Geisbert, T. W., Hensley, L. E., Kagan, E., Yu, E. Z., Geisbert, J. B., Daddario-Dicaprio, K., Fritz, E. A., Jahrling, P. B., Mcclintock, K., Phelps, J. R., Lee, A. C. H., Judge, A., Jeffs, L. B. & Maclachlan, I. (2006) Postexposure Protection of Guinea Pigs against a Lethal Ebola Virus Challenge Is Conferred by RNA Interference. *The Journal of Infectious Diseases,* 193**,** 1650-1657.

Geisbert, T. W., Lee, A. C., Robbins, M., Geisbert, J. B., Honko, A. N., Sood, V., Johnson, J. C., De Jong, S., Tavakoli, I., Judge, A., Hensley, L. E. & Maclachlan, I. (2010) Postexposure protection of non-human primates against a lethal Ebola virus challenge with RNA interference: a proof-of-concept study. *Lancet,* 375**,** 1896-1905.

Gerety, S. S. & Wilkinson, D. G. (2011) Morpholino artifacts provide pitfalls and reveal a novel role for pro-apoptotic genes in hindbrain boundary development. *Developmental Biology,* 350**,** 279-289.

Gerrard, S. R., Bird, B. H., Albariño, C. G. & Nichol, S. T. (2007) The NSm proteins of Rift Valley fever virus are dispensable for maturation, replication and infection. *Virology,* 359**,** 459-465.

Gerrard, S. R. & Nichol, S. T. (2002) Characterization of the Golgi retention motif of Rift Valley fever virus G(N) glycoprotein. *Journal of Virology,* 76**,** 12200-12210.

Gerrard, S. R. & Nichol, S. T. (2007) Synthesis, proteolytic processing and complex formation of N-terminally nested precursor proteins of the Rift Valley fever virus glycoproteins. *Virology,* 357**,** 124-133.

Giering, J. C., Grimm, D., Storm, T. A. & Kay, M. A. (2008) Expression of shRNA From a Tissue-specific pol II Promoter Is an Effective and Safe RNAi Therapeutic. *Molecular Therapy,* 16**,** 1630-1636.

Giorgi, C., Accardi, L., Nicoletti, L., Gro, M., Takehara, K., Hilditch, C., Morikawa, S. & Bishop, D. (1991) Sequences and coding strategies of the S RNAs of Toscana and Rift Valley fever viruses compared to those of Punta Toro, Sicilian Sandfly fever, and Uukuniemi viruses. *Virology,* 180**,** 738-753.

Gowen, B. B., Bailey, K. W., Scharton, D., Vest, Z., Westover, J. B., Skirpstunas, R. & Ikegami, T. (2013) Post-exposure vaccination with MP-12 lacking NSs protects mice against lethal Rift Valley fever virus challenge. *Antiviral Research,* 98**,** 135-143.

Gowen, B. B., Wong, M., Jung, K., Sanders, A. B., Mendenhall, M., Bailey, K. W., Furuta, Y. & Sidwell, R. W. (2007) In Vitro and In Vivo Activities of T-705 against Arenavirus and Bunyavirus Infections. *Antimicrobial Agents and Chemotherapy,* 51**,** 3168-3176.

Graci, J. D. & Cameron, C. E. (2006) Mechanisms of action of ribavirin against distinct viruses. *Reviews in Medical Virology,* 16**,** 37-48.

Graham, F. L., Smiley, J., Russell, W. C. & Nairn, R. (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *Journal of General Virology,* 36**,** 59-74.

Grard, G., Fair, J. N., Lee, D., Slikas, E., Steffen, I., Muyembe, J., Sittler, T., Veeraraghavan, N., Ruby, J. G., Wang, C., Makuwa, M., Mulembakani, P., Tesh, R. B., Mazet, J., Rimoin, A. W., Taylor, T., Schneider, B. S., Simmons, G., Delwart, E., Wolfe, N. D., Chiu, C. Y. & Leroy, E. M. (2012) A Novel Rhabdovirus Associated with Acute Hemorrhagic Fever in Central Africa. *PLoS Pathogens,* 8**,** e1002924.

Green, V. A., Weinberg, M. S. & Dirk, G. (2011) Small RNA-Induced Transcriptional Gene Regulation in Mammals: Mechanisms, Therapeutic Applications, and Scope Within the Genome. *Progress in Molecular Biology and Translational Science.* Academic Press.

Gregory, R. I., Chendrimada, T. P., Cooch, N. & Shiekhattar, R. (2005) Human RISC Couples MicroRNA Biogenesis and Posttranscriptional Gene Silencing. *Cell,* 123**,** 631-640.

Gregory, R. I., Yan, K. P., Amuthan, G., Chendrimada, T., Doratotaj, B., Cooch, N. & Shiekhattar, R. (2004) The Microprocessor complex mediates the genesis of microRNAs. *Nature,* 432**,** 235-240.

Grieger, J. C., Samulski, R. J. & Theodore, F. (2012) Chapter twelve - Adeno-Associated Virus Vectorology, Manufacturing, and Clinical Applications. *Methods in Enzymology.* Academic Press.

Grimm, D. (2011) The dose can make the poison: lessons learned from adverse in vivo toxicities caused by RNAi overexpression. *Silence,* 2**,** doi: 10.1186/1758-907X-2-8.

Grimm, D., Lee, J. S., Wang, L., Desai, T., Akache, B., Storm, T. A. & Kay, M. A. (2008) In Vitro and In Vivo Gene Therapy Vector Evolution via Multispecies Interbreeding and Retargeting of Adeno-Associated Viruses. *Journal of Virology,* 82**,** 5887-5911.

Grimm, D., Streetz, K. L., Jopling, C. L., Storm, T. A., Pandey, K. & Davis, C. R. (2006) Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature,* 441**,** 537-541.

Grimm, D., Wang, L., Lee, J. S., Schürmann, N., Gu, S., Börner, K., Storm, T. A. & Kay, M. A. (2010) Argonaute proteins are key determinants of RNAi efficacy, toxicity, and persistence in the adult mouse liver. *The Journal of Clinical Investigation,* 120**,** 3106-3119.

Grobbelaar, A., Weyer, J., Leman, P., Kemp, A., Paweska, J. & Swanepoel, R. (2011) Molecular epidemiology of Rift Valley fever virus. *Emerging Infectious Diseases,* 17**,** 2270-2276.

Gu, S., Jin, L., Zhang, F., Huang, Y., Grimm, D., Rossi, J. J. & Kay, M. A. (2011) Thermodynamic stability of small hairpin RNAs highly influences the loading process of different mammalian Argonautes. *Proceedings of the National Academy of Sciences,* 108**,** 9208-9213.

Gu, S., Jin, L., Zhang, Y., Huang, Y., Zhang, F., Valdmanis, P. & Kay, M. (2012) The Loop Position of shRNAs and Pre-miRNAs Is Critical for the Accuracy of Dicer Processing In Vivo. *Cell,* 151**,** 900-911.

Guo, K. T., Paul, A., Schichor, C., Ziemer, G. & Wendel, H. P. (2008) CELL-SELEX: Novel Perspectives of Aptamer-Based Therapeutics. *International Journal of Molecular Sciences,* 9**,** 668-678.

Haase, A. D., Jaskiewicz, L., Zhang, H., Laine, S., Sack, R. & Gatignol, A. (2005) TRBP, a regulator of cellular PKR and HIV-1 virus expression, interacts with Dicer and functions in RNA silencing. *EMBO reports,* 6**,** 961-967.

Haasnoot, J., De Vries, W., Geutjes, E. J., Prins, M., De Haan, P. & Berkhout, B. (2007) The Ebola virus VP35 protein is a suppressor of RNA silencing. *PLoS Pathogens,* 3**,** e86.

Habjan, M., Andersson, I., Klingström, J., Schümann, M., Martin, A., Zimmermann, P., Wagner, V., Pichlmair, A., Schneider, U., Mühlberger, E., Mirazimi, A. & Weber, F. (2008) Processing of Genome 5' Termini as a Strategy of Negative-Strand RNA Viruses to Avoid RIG-I-Dependent Interferon Induction. *PLoS ONE,* 3**,** e2032.

Habjan, M., Penski, N., Wagner, V., Spiegel, M., Överby, A. K., Kochs, G., Huiskonen, J. T. & Weber, F. (2009a) Efficient production of Rift Valley fever virus-like particles: The antiviral protein MxA can inhibit primary transcription of bunyaviruses. *Virology,* 385**,** 400-408.

Habjan, M., Pichlmair, A., Elliott, R. M., Överby, A. K., Glatter, T., Gstaiger, M., Superti-Furga, G., Unger, H. & Weber, F. (2009b) NSs Protein of Rift Valley Fever Virus Induces the Specific Degradation of the Double-Stranded RNA-Dependent Protein Kinase. *Journal of Virology,* 83**,** 4365-4375.

Haller, O. & Kochs, G. (2002) Interferon-Induced Mx Proteins: Dynamin-Like GTPases with Antiviral Activity. *Traffic,* 3**,** 710-717.

Haller, O., Kochs, G. & Weber, F. (2006) The interferon response circuit: Induction and suppression by pathogenic viruses. *Virology,* 344**,** 119-130.

Hartman, A. L., Dover, J. E., Towner, J. S. & Nichol, S. T. (2006) Reverse Genetic Generation of Recombinant Zaire Ebola Viruses Containing Disrupted IRF-3 Inhibitory Domains Results in Attenuated Virus Growth In Vitro and Higher Levels of IRF-3 Activation without Inhibiting Viral Transcription or Replication. *Journal of Virology,* 80**,** 6430-6440.

Hastie, K. M., Kimberlin, C. R., Zandonatti, M. A., Macrae, I. J. & Saphire, E. O. (2011) Structure of the Lassa virus nucleoprotein reveals a dsRNA-specific 3' to 5' exonuclease activity essential for immune suppression. *Proceedings of the National Academy of Sciences,* 108**,** 2396-2401.

Hastie, K. M., King, L. B., Zandonatti, M. A. & Saphire, E. O. (2012) Structural Basis for the dsRNA Specificity of the Lassa Virus NP Exonuclease. *PLoS ONE,* 7**,** e44211.

Head, J. A., Kalveram, B. & Ikegami, T. (2012) Functional Analysis of Rift Valley Fever Virus NSs Encoding a Partial Truncation. *PLoS ONE,* 7**,** e45730.

Hefti, H. P., Frese, M., Landis, H., Di Paolo, C., Aguzzi, A., Haller, O. & Pavlovic, J. (1999) Human MxA Protein Protects Mice Lacking a Functional Alpha/Beta Interferon System against La Crosse Virus and Other Lethal Viral Infections. *Journal of Virology,* 73**,** 6984-6991.

Henley, J. R., Krueger, E. W. A., Oswald, B. J. & Mcniven, M. A. (1998) Dynamin-mediated Internalization of Caveolae. *The Journal of Cell Biology,* 141**,** 85-99.

Huang, N. E., Lin, C., Lin, Y. & Yu, W. C. Y. (2003) Modulation of YY1 activity by SAP30. *Biochemical and Biophysical Research Communications,* 306**,** 267-275.

Huiskonen, J. T., Överby, A. K., Weber, F. & Grünewald, K. (2009) Electron Cryo-Microscopy and Single-Particle Averaging of Rift Valley Fever Virus: Evidence for GN-GC Glycoprotein Heterodimers. *Journal of Virology,* 83**,** 3762-3769.

Hur, J. K., Zinchenko, M. K., Djuranovic, S. & Green, R. (2013) Regulation of Argonaute slicer activity by guide RNA 3' end interactions with the N-terminal lobe. *Journal of Biological Chemistry,* 288**,** 7829-7840.

Hutvagner, G., Mclachlan, J., Pasquinelli, A. E., Balint, E., Tuschl, T. & Zamore, P. D. (2001) A Cellular Function for the RNA-Interference Enzyme Dicer in the Maturation of the let-7 Small Temporal RNA. *Science,* 293**,** 834-838.

Hutvagner, G. & Zamore, P. (2002) A microRNA in a Multiple-Turnover RNAi Enzyme Complex. *Science,* 297**,** 2056-2060.

Iida, A., Takino, N., Miyauchi, H., Shimazaki, K. & Muramatsu, S. (2013) Systemic Delivery of Tyrosine-Mutant AAV Vectors Results in Robust Transduction of Neurons in Adult Mice. *BioMed Research International,* 2013**,** 8.

Ikegami, T. (2012) Molecular biology and genetic diversity of Rift Valley fever virus. *Antiviral Research,* 95**,** 293-310.

Ikegami, T. & Makino, S. (2011) The Pathogenesis of Rift Valley Fever. *Viruses,* 3**,** 493-519.

Ikegami, T., Narayanan, K., Won, S., Kamitani, W., Peters, C. J. & Makino, S. (2009a) Dual Functions of Rift Valley Fever Virus NSs Protein: Inhibition of Host mRNA Transcription and Post-transcriptional Downregulation of Protein Kinase PKR. *Annals of the New York Academy of Sciences,* 1171**,** E75-E85.

Ikegami, T., Narayanan, K., Won, S., Kamitani, W., Peters, C. J. & Makino, S. (2009b) Rift Valley Fever Virus NSs Protein Promotes Post-Transcriptional Downregulation of

Protein Kinase PKR and Inhibits eIF2α Phosphorylation. *PLoS Pathogens,* 5**,** e1000287.

Ikegami, T., Won, S., Peters, C. J. & Makino, S. (2006) Rescue of Infectious Rift Valley Fever Virus Entirely from cDNA, Analysis of Virus Lacking the NSs Gene, and Expression of a Foreign Gene. *Journal of Virology,* 80**,** 2933-2940.

Ikegami, T., Won, S., Peters, C. J. & Makino, S. (2007) Characterization of Rift Valley Fever Virus Transcriptional Terminations. *Journal of Virology,* 81**,** 8421-8438.

Iversen, P., Warren, T., Wells, J., Garza, N., Mourich, D., Welch, L., Panchal, R. & Bavari, S. (2012) Discovery and Early Development of AVI-7537 and AVI-7288 for the Treatment of Ebola Virus and Marburg Virus Infections. *Viruses,* 4**,** 2806-2830.

Jääskeläinen, K., Plyusnina, A., Lundkvist, A., Vaheri, A. & Plyusnin, A. (2008) Tula hantavirus isolate with the full-length ORF for nonstructural protein NSs survives for more consequent passages in interferon-competent cells than the isolate having truncated NSs ORF. *Virology Journal,* 5**,** 3.

Jääskeläinen, K. M., Kaukinen, P., Minskaya, E. S., Plyusnina, A., Vapalahti, O., Elliott, R. M., Weber, F., Vaheri, A. & Plyusnin, A. (2007) Tula and Puumala hantavirus NSs ORFs are functional and the products inhibit activation of the interferon-beta promoter. *Journal of Medical Virology,* 79**,** 1527-1536.

James, T. W., Frias-Staheli, N., Bacik, J., Levingston Macleod, J. M., Khajehpour, M., García-Sastre, A. & Mark, B. L. (2011) Structural basis for the removal of ubiquitin and interferon-stimulated gene 15 by a viral ovarian tumor domain-containing protease. *Proceedings of the National Academy of Sciences,* 108**,** 2222-2227.

Jansen Van Vuren, P. & Paweska, J. T. (2009) Laboratory safe detection of nucleocapsid protein of Rift Valley fever virus in human and animal specimens by a sandwich ELISA. *Journal of Virological Methods,* 157**,** 15-24.

Jiang, X., Huang, Q., Wang, W., Dong, H., Ly, H., Liang, Y. & Dong, C. (2013) Structures of arenaviral nucleoproteins with triphosphate dsRNA reveal a unique mechanism of immune suppression. *Journal of Biological Chemistry,* 288**,** 16949-16959.

Jones, M., Davidson, A., Hibbert, L., Gruenwald, P., Schlaak, J., Ball, S., Foster, G. R. & Jacobs, M. (2005) Dengue Virus Inhibits Alpha Interferon Signaling by Reducing STAT2 Expression. *Journal of Virology,* 79**,** 5414-5420.

Jopling, C. L., Yi, M., Lancaster, A. M., Lemon, S. M. & Sarnow, P. (2005) Modulation of Hepatitis C Virus RNA Abundance by a Liver-Specific MicroRNA. *Science,* 309**,** 1577-1581.

Judge, A. D., Bola, G., Lee, A. C. H. & Maclachlan, I. (2006) Design of Noninflammatory Synthetic siRNA Mediating Potent Gene Silencing in Vivo. *Molecular Therapy,* 13**,** 494-505.

Kahlon, S. S., Peters, C. J., Leduc, J., Muchiri, E. M., Muiruri, S., Njenga, M. K., Breiman, R. F., Clinton White, A. & King, C. H. (2010) Severe Rift Valley Fever May Present with a Characteristic Clinical Syndrome. *The American Journal of Tropical Medicine and Hygiene,* 82**,** 371-375.

Kakach, L. T., Suzich, J. A. & Collett, M. S. (1989) Rift Valley fever virus M segment: phlebovirus expression strategy and protein glycosylation. *Virology,* 170**,** 505-510.

Kalveram, B. & Ikegami, T. (2013) Toscana Virus NSs Protein Promotes Degradation of Double-Stranded RNA-Dependent Protein Kinase. *Journal of Virology,* 87**,** 3710-3718.

Kalveram, B., Lihoradova, O. & Ikegami, T. (2011) NSs Protein of Rift Valley Fever Virus Promotes Post-Translational Downregulation of the TFIIH Subunit p62. *Journal of Virology,* 85**,** 6234-6243.

Kalveram, B., Lihoradova, O., Indran, S. V., Lokugamage, N., Head, J. A. & Ikegami, T. (2013) Rift Valley fever virus NSs inhibits host transcription independently of the degradation of dsRNA-dependent protein kinase PKR. *Virology,* 435**,** 415-424.

Kaplitt, M. G., Feigin, A., Tang, C., Fitzsimons, H. L., Mattis, P., Lawlor, P. A., Bland, R. J., Young, D., Strybing, K., Eidelberg, D. & During, M. J. (2007) Safety and tolerability of gene therapy with an adeno-associated virus (AAV) borne GAD gene for Parkinson's disease: an open label, phase I trial. *Lancet,* 369**,** 2097-2105.

Karlas, A., Machuy, N., Shin, Y., Pleissner, K., Artarini, A., Heuer, D., Becker, D., Khalil, H., Ogilvie, L. A., Hess, S., Maurer, A. P., Muller, E., Wolff, T., Rudel, T. & Meyer, T. F. (2010) Genome-wide RNAi screen identifies human host factors crucial for influenza virus replication. *Nature,* 463**,** 818-822.

Kawai, T. & Akira, S. (2011) Toll-like Receptors and Their Crosstalk with Other Innate Receptors in Infection and Immunity. *Immunity,* 34**,** 637-650.

Kawai, T., Takahashi, K., Sato, S., Coban, C., Kumar, H., Kato, H., Ishii, K. J., Takeuchi, O. & Akira, S. (2005) IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nature Immunology,* 6**,** 981-988.

Khare, R., Chen, C. Y., Weaver, E. A. & Barry, M. A. (2011) Advances and Future Challenges in Adenoviral Vector Pharmacology and Targeting. *Current Gene Therapy,* 11**,** 241-258.

Khvorova, A., Reynolds, A. & Jayasena, S. D. (2003) Functional siRNAs and miRNAs Exhibit Strand Bias. *Cell,* 115**,** 209-216.

Kimberlin, C. R., Bornholdt, Z. A., Li, S., Woods, V. L., Macrae, I. J. & Saphire, E. O. (2010) Ebolavirus VP35 uses a bimodal strategy to bind dsRNA for innate immune suppression. *Proceedings of the National Academy of Sciences,* 107**,** 314-319.

King, P. & Goodbourn, S. (1994) The beta-interferon promoter responds to priming through multiple independent regulatory elements. *Journal of Biological Chemistry,* 269**,** 30609-30615.

Kinney, R. M., Huang, C. Y. H., Rose, B. C., Kroeker, A. D., Dreher, T. W., Iversen, P. L. & Stein, D. A. (2005) Inhibition of Dengue Virus Serotypes 1 to 4 in Vero Cell Cultures with Morpholino Oligomers. *Journal of Virology,* 79**,** 5116-5128.

Klar, M. & Bode, J. (2005) Enhanceosome Formation over the Beta Interferon Promoter Underlies a Remote-Control Mechanism Mediated by YY1 and YY2. *Molecular and Cellular Biology,* 25**,** 10159-10170.

Kleinman, M. E., Yamada, K., Takeda, A., Chandrasekaran, V., Nozaki, M., Baffi, J. Z., Albuquerque, R. J. C., Yamasaki, S., Itaya, M., Pan, Y., Appukuttan, B., Gibbs, D., Yang, Z., Kariko, K., Ambati, B. K., Wilgus, T. A., Dipietro, L. A., Sakurai, E., Zhang, K., Smith, J. R., Taylor, E. W. & Ambati, J. (2008) Sequence- and target-independent angiogenesis suppression by siRNA via TLR3. *Nature,* 452**,** 591-597.

Kochhar, D. M., Penner, J. D. & Knudsen, T. B. (1980) Embryotoxic, teratogenic, and metabolic effects of ribavirin in mice. *Toxicology and Applied Pharmacology,* 52**,** 99-112.

Kochs, G., Janzen, C., Hohenberg, H. & Haller, O. (2002) Antivirally active MxA protein sequesters La Crosse virus nucleocapsid protein into perinuclear complexes. *Proceedings of the National Academy of Sciences,* 99**,** 3153-3158.

Kok, K., Lui, P., Ng, M., Siu, K., Au, S. & Jin, D. (2011) The Double-Stranded RNA-Binding Protein PACT Functions as a Cellular Activator of RIG-I to Facilitate Innate Antiviral Response. *Cell Host & Microbe,* 9**,** 299-309.

Kok, K. H., Ng, M. H., Ching, Y. P. & Jin, D. Y. (2007) Human TRBP and PACT directly interact with each other and associate with dicer to facilitate the production of small interfering RNA. *The Journal of Biological Chemistry,* 282**,** 17649-17657.

Kolokoltsova, O. A., Yun, N. E., Poussard, A. L., Smith, J. K., Smith, J. N., Salazar, M., Walker, A., Tseng, C., Aronson, J. F. & Paessler, S. (2010) Mice Lacking Alpha/Beta and Gamma Interferon Receptors Are Susceptible to Junin Virus Infection. *Journal of Virology,* 84**,** 13063-13067.

Korrapati, A. B., Swaminathan, G., Singh, A., Khanna, N. & Swaminathan, S. (2012) Adenovirus Delivered Short Hairpin RNA Targeting a Conserved Site in the 5' Non-Translated Region Inhibits All Four Serotypes of Dengue Viruses. *PLoS Neglected Tropical Diseases,* 6**,** e1735.

Koscianska, E., Starega-Roslan, J. & Krzyzosiak, W. J. (2011) The Role of Dicer Protein Partners in the Processing of MicroRNA Precursors. *PLoS ONE,* 6**,** e28548.

Krishnan, M. N., Ng, A., Sukumaran, B., Gilfoy, F. D., Uchil, P. D., Sultana, H., Brass, A. L., Adametz, R., Tsui, M. & Qian, F. (2008) RNA interference screen for human genes associated with West Nile virus infection. *Nature,* 455**,** 242-245.

Kunkel, G. R. & Pederson, T. (1988) Upstream elements required for efficient transcription of a human U6 RNA gene resemble those of U1 and U2 genes even though a different polymerase is used. *Genes & Development,* 2**,** 196-204.

Lara, E., Billecocq, A., Leger, P. & Bouloy, M. (2011) Characterization of Wild-Type and Alternate Transcription Termination Signals in the Rift Valley Fever Virus Genome. *Journal of Virology,* 85**,** 12134-12145.

Lau, J. F., Nusinzon, I., Burakov, D., Freedman, L. P. & Horvath, C. M. (2003) Role of Metazoan Mediator Proteins in Interferon-Responsive Transcription. *Molecular and Cellular Biology,* 23**,** 620-628.

Lazo, P. A. & Santos, C. R. (2011) Interference with p53 functions in human viral infections, a target for novel antiviral strategies? *Reviews in Medical Virology,* 21**,** 285-300.

Le May, N., Dubaele, S., De Santis, L. P., Billecocq, A., Bouloy, M. & Egly, J. (2004) TFIIH Transcription Factor, a Target for the Rift Valley Hemorrhagic Fever Virus. *Cell,* 116**,** 541-550.

Le May, N., Gauliard, N., Billecocq, A. & Bouloy, M. (2005) The N terminus of Rift Valley fever virus nucleoprotein is essential for dimerization. *Journal of Virology,* 79**,** 11974-11980.

Le May, N., Mansuroglu, Z., Léger, P., Josse, T., Blot, G., Billecocq, A. S., Flick, R., Jacob, Y., Bonnefoy, E. & Bouloy, M. (2008) A SAP30 Complex Inhibits IFN-β Expression in Rift Valley Fever Virus Infected Cells. *PLoS Pathogens,* 4**,** e13.

Lecellier, C., Dunoyer, P., Arar, K., Lehmann-Che, J., Eyquem, S., Himber, C., Saib, A. & Voinnet, O. (2005) A Cellular MicroRNA Mediates Antiviral Defense in Human Cells. *Science,* 308**,** 557-560.

Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Radmark, O., Kim, S. & Kim, V. N. (2003) The nuclear RNase III Drosha initiates microRNA processing. *Nature,* 425**,** 415-419.

Lee, Y., Hur, I., Park, S. Y., Kim, Y. K., Suh, M. R. & Kim, V. N. (2006) The role of PACT in the RNA silencing pathway. *The EMBO Journal,* 25**,** 522-532.

Lee, Y., Kim, M., Han, J., Yeom, K., Lee, S., Baek, S. H. & Kim, V. N. (2004) MicroRNA genes are transcribed by RNA polymerase II. *The EMBO Journal,* 23**,** 4051-4060.

Lescure, A., Murgo, S., Carbon, P. & Krol, A. (1992) The proximal promoter and the start site cooperate to specify correct U1 snRNA transcription initiation by RNA polymerase II. *Nucleic Acids Research,* 20**,** 1573-1578.

Leung, D. W., Prins, K. C., Borek, D. M., Farahbakhsh, M., Tufariello, J. M., Ramanan, P., Nix, J. C., Helgeson, L. A., Otwinowski, Z., Honzatko, R. B., Basler, C. F. & Amarasinghe, G. K. (2010) Structural basis for dsRNA recognition and interferon antagonism by Ebola VP35. *Nature Structural & Molecular Biology,* 17**,** 165-172.

Leuschner, P. J. F., Ameres, S. L., Kueng, S. & Martinez, J. (2006) Cleavage of the siRNA passenger strand during RISC assembly in human cells. *EMBO reports,* 7**,** 314-320.

Lewis, B. P., Burge, C. B. & Bartel, D. P. (2005) Conserved Seed Pairing, Often Flanked by Adenosines, Indicates that Thousands of Human Genes are MicroRNA Targets. *Cell,* 120**,** 15-20.

Lewis, B. P., Shih, I. H., Jones-Rhoades, M. W., Bartel, D. P. & Burge, C. B. (2003) Prediction of Mammalian MicroRNA Targets. *Cell,* 115**,** 787-798.

Li, R. & Hayward, S. D. (2011) The Ying-Yang of the virus-host interaction: control of the DNA damage response. *Future Microbiology,* 6**,** 379-383.

Li, W., Asokan, A., Wu, Z., Van Dyke, T., Diprimio, N., S Johnson, J., Govindaswamy, L., Agbandje-Mckenna, M., Leichtle, S., Eugene Redmond Jr, D., Mccown, T. J., Petermann, K. B., Sharpless, N. E. & Samulski, R. J. (2008) Engineering and Selection of Shuffled AAV Genomes: A New Strategy for Producing Targeted Biological Nanoparticles. *Molecular Therapy,* 16**,** 1252-1260.

Li, W., Li, H., Lu, R., Li, F., Dus, M., Atkinson, P., Brydon, E. W. A., Johnson, K. L., García-Sastre, A., Ball, L. A., Palese, P. & Ding, S. (2004) Interferon antagonist proteins of influenza and vaccinia viruses are suppressors of RNA silencing. *Proceedings of the National Academy of Sciences,* 101**,** 1350-1355.

Lihoradova, O., Kalveram, B., Indran, S. V., Lokugamage, N., Juelich, T. L., Hill, T. E., Tseng, C., Gong, B., Fukushi, S., Morikawa, S., Freiberg, A. N. & Ikegami, T. (2012) The Dominant-Negative Inhibition of Double-Stranded RNA-Dependent Protein Kinase PKR Increases the Efficacy of Rift Valley Fever Virus MP-12 Vaccine. *Journal of Virology,* 86**,** 7650-7661.

Lin, R., Heylbroeck, C., Pitha, P. M. & Hiscott, J. (1998) Virus-dependent phosphorylation of the IRF-3 transcription factor regulates nuclear translocation, transactivation potential, and proteasome-mediated degradation. *Molecular and Cellular Biology,* 18**,** 2986-2996.

Linthicum, K. J., Davies, F. G., Kairo, A. & Bailey, C. L. (1985) Rift Valley fever virus (family Bunyaviridae, genus Phlebovirus). Isolations from Diptera collected during an inter-epizootic period in Kenya. *Epidemiology & Infection,* 95**,** 197-209.

Lisowski, L., Elazar, M., Chu, K., Glenn, J. S. & Kay, M. A. (2013) The anti-genomic (negative) strand of Hepatitis C Virus is not targetable by shRNA. *Nucleic Acids Research,* 41**,** 3688-3698.

Liu, J., Carmell, M. A., Rivas, F. V., Marsden, C. G., Thomson, J. M., Song, J., Hammond, S. M., Joshua-Tor, L. & Hannon, G. J. (2004) Argonaute2 Is the Catalytic Engine of Mammalian RNAi. *Science,* 305**,** 1437-1441.

Liu, L., Celma, C. & Roy, P. (2008) Rift Valley fever virus structural proteins: expression, characterization and assembly of recombinant proteins. *Virology Journal,* 5**,** 82.

Liu, S., Aliyari, R., Chikere, K., Li, G., Marsden, M., Smith, J., Pernet, O., Guo, H., Nusbaum, R., Zack, J., Freiberg, A., Su, L., Lee, B. & Cheng, G. (2013) Interferon-Inducible Cholesterol-25-Hydroxylase Broadly Inhibits Viral Entry by Production of 25-Hydroxycholesterol. *Immunity,* 38**,** 92-105.

Liu, S., Sanchez, D. J. & Cheng, G. (2011) New developments in the induction and antiviral effectors of type I interferon. *Current Opinion in Immunology,* 23**,** 57-64.

Lofts, L. L., Ibrahim, M. S., Negley, D. L., Hevey, M. C. & Schmaljohn, A. L. (2007) Genomic Differences between Guinea Pig Lethal and Nonlethal Marburg Virus Variants. *Journal of Infectious Diseases,* 196**,** S305-S312.

Lopez, N., Muller, R., Prehaud, C. & Bouloy, M. (1995) The L protein of Rift Valley fever virus can rescue viral ribonucleoproteins and transcribe synthetic genome-like RNA molecules. *Journal of Virology,* 69**,** 3972-3979.

Lowen, A. C. & Elliott, R. M. (2005) Mutational Analyses of the Nonconserved Sequences in the Bunyamwera Orthobunyavirus S Segment Untranslated Regions. *Journal of Virology,* 79**,** 12861-12870.

Lozach, P., Mancini, R., Bitto, D., Meier, R., Oestereich, L., Överby, A. K., Pettersson, R. F. & Helenius, A. (2010) Entry of Bunyaviruses into Mammalian Cells. *Cell Host & Microbe,* 7**,** 488-499.

Lund, E., Guttinger, S., Calado, A., Dahlberg, J. E. & Kutay, U. (2004) Nuclear export of microRNA precursors. *Science,* 303**,** 95-98.

Lupfer, C., Stein, D., Mourich, D., Tepper, S., Iversen, P. & Pastey, M. (2008) Inhibition of influenza A H3N8 virus infections in mice by morpholino oligomers. *Archives of Virology,* 153**,** 929-937.

Luthra, P., Ramanan, P., Mire, C., Weisend, C., Tsuda, Y., Yen, B., Liu, G., Leung, D., Geisbert, T., Ebihara, H., Amarasinghe, G. & Basler, C. (2013) Mutual Antagonism between the Ebola Virus VP35 Protein and the RIG-I Activator PACT Determines Infection Outcome. *Cell Host & Microbe,* 14**,** 74-84.

Ma, J. B., Ye, K. & Patel, D. J. (2004) Structural basis for overhang-specific small interfering RNA recognition by the PAZ domain. *Nature,* 429**,** 318-322.

Macrae, I. J., Ma, E., Zhou, M., Robinson, C. V. & Doudna, J. A. (2008) In vitro reconstitution of the human RISC-loading complex. *Proceedings of the National Academy of Sciences,* 105**,** 512-517.

Macrae, I. J., Zhou, K. & Doudna, J. A. (2007) Structural determinants of RNA recognition and cleavage by Dicer. *Nature Structural & Molecular Biology,* 14**,** 934-940.

Madani, T. A., Al-Mazrou, Y. Y., Al-Jeffri, M. H., Mishkhas, A. A., Al-Rabeah, A. M., Turkistani, A. M., Al-Sayed, M. O., Abodahish, A. A., Khan, A. S., Ksiazek, T. G. & Shobokshi, O. (2003) Rift Valley Fever Epidemic in Saudi Arabia: Epidemiological, Clinical, and Laboratory Characteristics. *Clinical Infectious Diseases,* 37**,** 1084-1092.

Mansuroglu, Z., Josse, T., Gilleron, J., Billecocq, A., Leger, P., Bouloy, M. & Bonnefoy, E. (2010) Nonstructural NSs Protein of Rift Valley Fever Virus Interacts with Pericentromeric DNA Sequences of the Host Cell, Inducing Chromosome Cohesion and Segregation Defects. *Journal of Virology,* 84**,** 928-939.

Marques, J. T., Devosse, T., Wang, D., Zamanian-Daryoush, M., Serbinowski, P., Hartmann, R., Fujita, T., Behlke, M. A. & Williams, B. R. (2006) A structural basis for discriminating between self and nonself double-stranded RNAs in mammalian cells. *Nature Biotechnology,* 24**,** 559-565.

Martin, J. N., Wolken, N., Brown, T., Dauer, W. T., Ehrlich, M. E. & Gonzalez-Alegre, P. (2011) Lethal toxicity caused by expression of shRNA in the mouse striatum: implications for therapeutic design. *Gene Therapy,* 18**,** 666-673.

Martínez-Sobrido, L., Giannakas, P., Cubitt, B., García-Sastre, A. & De La Torre, J. C. (2007) Differential Inhibition of Type I Interferon Induction by Arenavirus Nucleoproteins. *Journal of Virology,* 81**,** 12696-12703.

Martinez, J., Patkaniowska, A., Urlaub, H., Luhrmann, R. & Tuschl, T. (2002) Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. *Cell,* 110**,** 563-574.

Mateo, M., Carbonnelle, C., Reynard, O., Kolesnikova, L., Nemirov, K., Page, A., Volchkova, V. A. & Volchkov, V. E. (2011) VP24 Is a Molecular Determinant of Ebola Virus Virulence in Guinea Pigs. *Journal of Infectious Diseases,* 204**,** S1011-S1020.

Mateo, M., Reid, S. P., Leung, L. W., Basler, C. F. & Volchkov, V. E. (2009) Ebolavirus VP24 Binding to Karyopherins Is Required for Inhibition of Interferon Signaling. *Journal of Virology,* 84**,** 1169-1175.

Mazzon, M., Jones, M., Davidson, A., Chain, B. & Jacobs, M. (2009) Dengue Virus NS5 Inhibits Interferon-α Signaling by Blocking Signal Transducer and Activator of Transcription 2 Phosphorylation. *Journal of Infectious Diseases,* 200**,** 1261-1270.

Mcbride, J. L., Boudreau, R. L., Harper, S. Q., Staber, P. D., Monteys, A. M. & Martins, I. (2008) Artificial miRNAs mitigate shRNA-mediated toxicity in the brain: implications for the therapeutic development of RNAi. *Proceedings of the National Academy of Sciences,* 105**,** 5868-5873.

Mcintyre, G., Groneman, J., Tran, A. & Applegate, T. (2008) An Infinitely Expandable Cloning Strategy plus Repeat-Proof PCR for Working with Multiple shRNA. *PLoS ONE,* 3**,** e3827.

Mcintyre, G., Groneman, J., Yu, Y., Tran, A. & Applegate, T. (2011a) Multiple shRNA combinations for near-complete coverage of all HIV-1 strains. *AIDS Research and Therapy,* 8**,** 1.

Mcintyre, G., Yu, Y., Lomas, M. & Fanning, G. (2011b) The effects of stem length and core placement on shRNA activity. *BMC Molecular Biology,* 12**,** 34.

Meister, G., Landthaler, M., Patkaniowska, A., Dorsett, Y., Teng, G. & Tuschl, T. (2004) Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. *Molecular Cell,* 15**,** 185-197.

Mendenhall, M., Russell, A., Juelich, T., Messina, E. L., Smee, D. F., Freiberg, A. N., Holbrook, M. R., Furuta, Y., De La Torre, J., Nunberg, J. H. & Gowen, B. B. (2011) T-705 (Favipiravir) Inhibition of Arenavirus Replication in Cell Culture. *Antimicrobial Agents and Chemotherapy,* 55**,** 782-787.

Meyer, O. (2009) Interferons and autoimmune disorders. *Joint Bone Spine,* 76**,** 464-473.

Michienzi, A., Li, S., Zaia, J. A. & Rossi, J. (2002) A nucleolar TAR decoy inhibitor of HIV-1 replication. *Proceedings of the National Academy of Sciences,* 99**,** 14047-14052.

Mir, M. A., Brown, B., Hjelle, B., Duran, W. A. & Panganiban, A. T. (2006) Hantavirus N Protein Exhibits Genus-Specific Recognition of the Viral RNA Panhandle. *Journal of Virology,* 80**,** 11283-11292.

Mir, M. A., Duran, W. A., Hjelle, B. L., Ye, C. & Panganiban, A. T. (2008) Storage of cellular 5' mRNA caps in P bodies for viral cap-snatching. *Proceedings of the National Academy of Sciences,* 105**,** 19294-19299.

Mir, M. A., Sheema, S., Haseeb, A. & Haque, A. (2010) Hantavirus Nucleocapsid Protein Has Distinct m7G Cap- and RNA-binding Sites. *Journal of Biological Chemistry,* 285**,** 11357-11368.

Miyagishi, M. & Taira, K. (2002) U6 promoter-driven siRNAs with four uridine 3' overhangs efficiently suppress targeted gene expression in mammalian cells. *Nature Biotechnology,* 20**,** 497-500.

Monath, T. P. (2008) Treatment of yellow fever. *Antiviral Research,* 78**,** 116-124.

Morner, A., Bjorndal, A., Kewalramani, V., Littman, D. R., Inoue, R., Thorstensson, R., Fenyo, E. M. & Bjorling, E. (1999) Primary human immunodeficiency virus type 2 (HIV-2) isolates, like HIV-1 isolates, frequently use CCR5 but show promiscuity in coreceptor usage. *Journal of Virology,* 73**,** 2343-2349.

Morrill, J. C., Jennings, G. B., Johnson, A. J., Cosgriff, T. M., Gibbs, P. H. & Peters, C. J. (1990) Pathogenesis of Rift Valley fever in rhesus monkeys: role of interferon response. *Archives of Virology,* 110**,** 195-212.

Morrison, J., Laurent-Rolle, M., Maestre, A. M., Rajsbaum, R., Pisanelli, G., Simon, V., Mulder, L. C. F., Fernandez-Sesma, A. & García-Sastre, A. (2013) Dengue Virus Co-opts UBR4 to Degrade STAT2 and Antagonize Type I Interferon Signaling. *PLoS Pathogens,* 9**,** e1003265.

Moulton, H. M., Nelson, M. H., Hatlevig, S. A., Reddy, M. T. & Iversen, P. L. (2004) Cellular Uptake of Antisense Morpholino Oligomers Conjugated to Arginine-Rich Peptides. *Bioconjugate Chemistry,* 15**,** 290-299.

Moutailler, S., Krida, G., Schaffner, F., Vazeille, M. & Failloux, A. B. (2008) Potential vectors of Rift Valley fever virus in the Mediterranean region. *Vector-Borne and Zoonotic Diseases,* 8**,** 749-753.

Mudhasani, R., Tran, J. P., Retterer, C., Radoshitzky, S. R., Kota, K., Altamura, L. A., Smith, J. M., Packard, B. Z., Kuhn, J. H., Costantino, J., Garrison, A. R., Schmaljohn, C. S., Huang, I. C., Farzan, M. & Bavari, S. (2013) IFITM-2 and IFITM-3 but not IFITM-1 restrict Rift Vallet Fever Virus. *Journal of Virology,* 87**,** 8451-8464.

Muller, R., Poch, O., Delarue, M., Bishop, D. H. L. & Bouloy, M. (1994) Rift valley fever virus L segment: correction of the sequence and possible functional role of newly identified regions conserved in RNA-dependent polymerases. *Journal of General Virology,* 75**,** 1345-1352.

Muller, R., Saluzzo, J., Lopez, N., Dreier, T., Turell, M., Smith, J. & Bouloy, M. (1995) Characterization of clone 13, a naturally attenuated avirulent isolate of Rift Valley fever virus, which is altered in the small segment. *The American Journal of Tropical Medicine and Hygiene,* 53**,** 405-411.

Muller, S. & Gunther, S. (2007) Broad-Spectrum Antiviral Activity of Small Interfering RNA Targeting the Conserved RNA Termini of Lassa Virus. *Antimicrobial Agents and Chemotherapy,* 51**,** 2215-2218.

Muñoz-Jordán, J. L., Laurent-Rolle, M., Ashour, J., Martínez-Sobrido, L., Ashok, M., Lipkin, W. I. & García-Sastre, A. (2005) Inhibition of Alpha/Beta Interferon Signaling by the NS4B Protein of Flaviviruses. *Journal of Virology,* 79**,** 8004-8013.

Muñoz-Jordán, J. L., Sánchez-Burgos, G. G., Laurent-Rolle, M. & García-Sastre, A. (2003) Inhibition of interferon signaling by dengue virus. *Proceedings of the National Academy of Sciences,* 100**,** 14333-14338.

Murakami, S., Terasaki, K., Narayanan, K. & Makino, S. (2012) Roles of the Coding and Noncoding Regions of Rift Valley Fever Virus RNA Genome Segments in Viral RNA Packaging. *Journal of Virology,* 86**,** 4034-4039.

Myslinski, E., Amé, J., Krol, A. & Carbon, P. (2001) An unusually compact external promoter for RNA polymerase III transcription of the human H1RNA gene. *Nucleic Acids Research,* 29**,** 2502-2509.

Nakabayashi, H., Taketa, K., Miyano, K., Yamane, T. & Sato, J. (1982) Growth of Human Hepatoma Cell Lines with Differentiated Functions in Chemically Defined Medium. *Cancer Research,* 42**,** 3858-3863.

Nanduri, S., Carpick, B. W., Yang, Y., Williams, B. R. G. & Qin, J. (1998) Structure of the double-stranded RNA-binding domain of the protein kinase PKR reveals the molecular basis of its dsRNA-mediated activation. *The EMBO Journal,* 17**,** 5458-5465.

Narayanan, A., Kehn-Hall, K., Senina, S., Hill, L., Van Duyne, R., Guendel, I., Das, R., Baer, A., Bethel, L., Turrell, M., Hartman, A. L., Das, B., Bailey, C. & Kashanchi, F. (2012) Curcumin Inhibits Rift Valley Fever Virus Replication in Human Cells. *Journal of Biological Chemistry,* 287**,** 33198-33214.

Narayanan, A., Popova, T., Turell, M., Kidd, J., Chertow, J., Popov, S. G., Bailey, C., Kashanchi, F. & Kehn-Hall, K. (2011) Alteration in Superoxide Dismutase 1 Causes Oxidative Stress and p38 MAPK Activation Following RVFV Infection. *PLoS ONE,* 6**,** e20354.

Neuman, B. W., Bederka, L. H., Stein, D. A., Ting, J. P. C., Moulton, H. M. & Buchmeier, M. J. (2011) Development of Peptide-Conjugated Morpholino Oligomers as Pan-Arenavirus Inhibitors. *Antimicrobial Agents and Chemotherapy,* 55**,** 4631-4638.

Ni, B., Shi, X., Li, Y., Gao, W., Wang, X. & Wu, Y. (2005) Inhibition of replication and infection of severe acute respiratory syndrome-associated coronavirus with plasmid-mediated interference RNA. *Antiviral Therapy,* 10**,** 527-533.

Nielsen, T. T., Marion, I. V., Hasholt, L. & Lundberg, C. (2009) Neuron-specific RNA interference using lentiviral vectors. *The Journal of Gene Medicine,* 11**,** 559-569.

Pacca, C. C., Severino, A. A., Mondini, A., Rahal, P., D'avila, S. G. P., Cordeiro, J. A., Nogueira, M. L., Bronzoni, R. V. M. & Nogueira, M. L. (2009) RNA interference inhibits yellow fever virus replication in vitro and in vivo. *Virus Genes,* 38**,** 224-231.

Padwad, Y. S., Mishra, K. P., Jain, M., Chanda, S., Karan, D. & Ganju, L. (2009) RNA interference mediated silencing of Hsp60 gene in human monocytic myeloma cell line U937 revealed decreased dengue virus multiplication. *Immunobiology,* 214**,** 422-429.

Paessler, S. & Walker, D. H. (2012) Pathogenesis of the Viral Hemorrhagic Fevers. *Annual Review of Pathology: Mechanisms of Disease,* 8**,** 411-40.

Panchal, R. G., Reid, S. P., Tran, J. P., Bergeron, A. A., Wells, J., Kota, K. P., Aman, J. & Bavari, S. (2012) Identification of an antioxidant small-molecule with broad-spectrum antiviral activity. *Antiviral Research,* 93**,** 23-29.

Panda, D., Das, A., Dinh, P. X., Subramaniam, S., Nayak, D., Barrows, N. J., Pearson, J. L., Thompson, J., Kelly, D. L., Ladunga, I. & Pattnaik, A. K. (2011) RNAi screening reveals requirement for host cell secretory pathway in infection by diverse families of negative-strand RNA viruses. *Proceedings of the National Academy of Sciences,* 108**,** 19036-19041.

Parameswaran, P., Sklan, E., Wilkins, C., Burgon, T., Samuel, M. A., Lu, R., Ansel, K. M., Heissmeyer, V., Einav, S., Jackson, W., Doukas, T., Paranjape, S., Polacek, C., Dos Santos, F. B., Jalili, R., Babrzadeh, F., Gharizadeh, B., Grimm, D., Kay, M., Koike, S.,

Sarnow, P., Ronaghi, M., Ding, S., Harris, E., Chow, M., Diamond, M. S., Kirkegaard, K., Glenn, J. S. & Fire, A. Z. (2010) Six RNA Viruses and Forty-One Hosts: Viral Small RNAs and Modulation of Small RNA Repertoires in Vertebrate and Invertebrate Systems. *PLoS Pathogens,* 6**,** e1000764.

Park, H., Davies, M. V., Langland, J. O., Chang, H. W., Nam, Y. S., Tartaglia, J., Paoletti, E., Jacobs, B. L., Kaufman, R. J. & Venkatesan, S. (1994) TAR RNA-binding protein is an inhibitor of the interferon-induced protein kinase PKR. *Proceedings of the National Academy of Sciences,* 91**,** 4713-4717.

Park, J., Heo, I., Tian, Y., Simanshu, D. K., Chang, H., Jee, D., Patel, D. J. & Kim, V. N. (2011) Dicer recognizes the 5' end of RNA for efficient and accurate processing. *Nature,* 475**,** 201-205.

Parks, R. J., Chen, L., Anton, M., Sankar, U., Rudnicki, M. A. & Graham, F. L. (1996) A helper-dependent adenovirus vector system: Removal of helper virus by Cre-mediated excision of the viral packaging signal. *Proceedings of the National Academy of Sciences,* 93**,** 13565-13570.

Passman, M., Weinberg, M., Kew, M. & Arbuthnot, P. (2000) In Situ Demonstration of Inhibitory Effects of Hammerhead Ribozymes That Are Targeted to the Hepatitis Bx Sequence in Cultured Cells. *Biochemical and Biophysical Research Communications,* 268**,** 728-733.

Patel, R. C. & Sen, G. C. (1998) PACT, a protein activator of the interferon-induced protein kinase, PKR. *The EMBO Journal,* 17**,** 4379-4390.

Patterson, J. L., Holloway, B. & Kolakofsky, D. (1984) La Crosse virions contain a primer-stimulated RNA polymerase and a methylated cap-dependent endonuclease. *Journal of Virology,* 52**,** 215-222.

Patzel, V., Rutz, S., Dietrich, I., Koberle, C., Scheffold, A. & Kaufmann, S. H. E. (2005) Design of siRNAs producing unstructured guide-RNAs results in improved RNA interference efficiency. *Nature Biotechnology,* 23**,** 1440-1444.

Pedersen, I. M., Cheng, G., Wieland, S., Volinia, S., Croce, C. M., Chisari, F. V. & David, M. (2007) Interferon modulation of cellular microRNAs as an antiviral mechanism. *Nature,* 449**,** 919-922.

Pepin, M., Bouloy, M., Bird, B. H., Kemp, A. & Paweska, J. (2010) Rift Valley fever virus (Bunyaviridae: Phlebovirus): an update on pathogenesis, molecular epidemiology, vectors, diagnostics and prevention. *Veterinary Research,* 41**,** 61.

Pepini, T., Gorbunova, E. E., Gavrilovskaya, I. N., Mackow, J. E. & Mackow, E. R. (2010) Andes Virus Regulation of Cellular MicroRNAs Contributes to Hantavirus-Induced Endothelial Cell Permeability. *Journal of Virology,* 84**,** 11929-11936.

Perry, S. T., Buck, M. D., Lada, S. M., Schindler, C. & Shresta, S. (2011) STAT2 Mediates Innate Immunity to Dengue Virus in the Absence of STAT1 via the Type I Interferon Receptor. *PLoS Pathogens,* 7**,** e1001297.

Peters, C., Liu, C., Anderson, G. J., Morrill, J. & Jahrling, P. (1989) Pathogenesis of viral hemorrhagic fevers: Rift Valley fever and Lassa fever contrasted. *Reviews of Infectious Diseases,* 11**,** 743-749.

Peters, C. J., Reynolds, J. A., Slone, T. W., Jones, D. E. & Stephen, E. L. (1986) Prophylaxis of Rift Valley fever with antiviral drugs, immune serum, an interferon inducer, and a macrophage activator. *Antiviral Research,* 6**,** 285-297.

Pfeffer, S., Sewer, A., Lagos-Quintana, M., Sheridan, R., Sander, C., Grasser, F. A., Van Dyk, L. F., Ho, C. K., Shuman, S. & Chien, M. (2005) Identification of microRNAs of the herpesvirus family. *Nature Methods,* 2**,** 269-276.

Piper, M. E., Sorenson, D. R. & Gerrard, S. R. (2011) Efficient Cellular Release of Rift Valley Fever Virus Requires Genomic RNA. *PLoS ONE,* 6**,** e18070.

Popova, T. G., Turell, M. J., Espina, V., Kehn-Hall, K., Kidd, J., Narayanan, A., Liotta, L., Petricoin, E. F., Iii, Kashanchi, F., Bailey, C. & Popov, S. G. (2010) Reverse-Phase Phosphoproteome Analysis of Signaling Pathways Induced by Rift Valley Fever Virus in Human Small Airway Epithelial Cells. *PLoS ONE,* 5**,** e13805.

Prehaud, C., Lopez, N., Blok, M. J., Obry, V. R. & Bouloy, M. L. (1997) Analysis of the 3' Terminal Sequence Recognized by the Rift Valley Fever Virus Transcription Complex in Its Ambisense S Segment. *Virology,* 227**,** 189-197.

Prins, K. C., Cárdenas, W. B. & Basler, C. F. (2009) Ebola Virus Protein VP35 Impairs the Function of Interferon Regulatory Factor-Activating Kinases IKKε and TBK-1. *Journal of Virology,* 83**,** 3069-3077.

Prins, K. C., Delpeut, S., Leung, D. W., Reynard, O., Volchkova, V. A., Reid, S. P., Ramanan, P., Cárdenas, W. B., Amarasinghe, G. K., Volchkov, V. E. & Basler, C. F. (2010) Mutations Abrogating VP35 Interaction with Double-Stranded RNA Render Ebola Virus Avirulent in Guinea Pigs. *Journal of Virology,* 84**,** 3004-3015.

Pythoud, C., Rodrigo, W. W. S. I., Pasqual, G., Rothenberger, S., Martínez-Sobrido, L., De La Torre, J. C. & Kunz, S. (2012) Arenavirus Nucleoprotein Targets Interferon Regulatory Factor-Activating Kinase IKKε. *Journal of Virology,* 86**,** 7728-7738.

Qi, X., Lan, S., Wang, W., Schelde, L. M., Dong, H., Wallat, G. D., Ly, H., Liang, Y. & Dong, C. (2010) Cap binding and immune evasion revealed by Lassa nucleoprotein structure. *Nature,* 468**,** 779-783.

Quarles, K. A., Sahu, D., Havens, M. A., Forsyth, E. R., Wostenberg, C., Hastings, M. L. & Showalter, S. A. (2013) Ensemble Analysis of Primary MicroRNA Structure Reveals an Extensive Capacity To Deform near the Drosha Cleavage Site. *Biochemistry,* 52**,** 795-807.

Rajput, R., Khanna, M., Kumar, P., Kumar, B., Sharma, S., Gupta, N. & Saxena, L. (2012) Small Interfering RNA Targeting the Nonstructural Gene 1 Transcript Inhibits Influenza A Virus Replication in Experimental Mice. *Nucleic Acid Therapeutics,* 22**,** 414-422.

Ramanan, P., Edwards, M. R., Shabman, R. S., Leung, D. W., Endlich-Frazier, A. C., Borek, D. M., Otwinowski, Z., Liu, G., Huh, J., Basler, C. F. & Amarasinghe, G. K. (2012) Structural basis for Marburg virus VP35-mediated immune evasion mechanisms. *Proceedings of the National Academy of Sciences,* 109**,** 20661-20666.

Ramaswamy, M., Shi, L., Varga, S. M., Barik, S., Behlke, M. A. & Look, D. C. (2006) Respiratory syncytial virus nonstructural protein 2 specifically inhibits type I interferon signal transduction. *Virology,* 344**,** 328-339.

Rauschhuber, C., Noske, N. & Ehrhardt, A. (2012) New insights into stability of recombinant adenovirus vector genomes in mammalian cells. *European Journal of Cell Biology,* 91**,** 2-9.

Raymond, D. D., Piper, M. E., Gerrard, S. R., Skiniotis, G. & Smith, J. L. (2012) Phleboviruses encapsidate their genomes by sequestering RNA bases. *Proceedings of the National Academy of Sciences,* 109**,** 19208-19213.

Raymond, D. D., Piper, M. E., Gerrard, S. R. & Smith, J. L. (2010) Structure of the Rift Valley fever virus nucleocapsid protein reveals another architecture for RNA encapsidation. *Proceedings of the National Academy of Sciences,* 107**,** 11769-11774.

Raymond, J., Bradfute, S. & Bray, M. (2011) Filovirus Infection of STAT-1 Knockout Mice. *Journal of Infectious Diseases,* 204**,** S986-S990.

Reed, C., Lin, K., Wilhelmsen, C., Friedrich, B., Nalca, A., Keeney, A., Donnelly, G., Shamblin, J., Hensley, L. E., Olinger, G. & Smith, D. R. (2013) Aerosol Exposure to Rift Valley Fever Virus Causes Earlier and More Severe Neuropathology in the Murine Model, which Has Important Implications for Therapeutic Development. *PLoS Neglected Tropical Diseases,* 7**,** e2156.

Reguera, J., Weber, F. & Cusack, S. (2010) *Bunyaviridae* RNA Polymerases (L-Protein) Have an N-Terminal, Influenza-Like Endonuclease Domain, Essential for Viral Cap-Dependent Transcription. *PLoS Pathogens,* 6**,** e1001101.

Reid, S. P., Leung, L. W., Hartman, A. L., Martinez, O., Shaw, M. L., Carbonnelle, C., Volchkov, V. E., Nichol, S. T. & Basler, C. F. (2006) Ebola Virus VP24 Binds Karyopherin α1 and Blocks STAT1 Nuclear Accumulation. *Journal of Virology,* 80**,** 5156-5167.

Reid, S. P., Valmas, C., Martinez, O., Sanchez, F. M. & Basler, C. F. (2007) Ebola Virus VP24 Proteins Inhibit the Interaction of NPI-1 Subfamily Karyopherin α1 Proteins with Activated STAT1. *Journal of Virology,* 81**,** 13469-13477.

Reynolds, A., Leake, D., Boese, Q., Scaringe, S., Marshall, W. S. & Khvorova, A. (2004) Rational siRNA design for RNA interference. *Nature Biotechnology,* 22**,** 326-330.

Robbins, M. A., Li, M., Leung, I., Li, H., Boyer, D. V., Song, Y., Behlke, M. A. & Rossi, J. J. (2006) Stable expression of shRNAs in human CD34+ progenitor cells can avoid induction of interferon responses to siRNAs *in vitro*. *Nature Biotechnology,* 24**,** 566-571.

Robu, M. E., Larson, J. D., Nasevicius, A., Beiraghi, S., Brenner, C., Farber, S. A. & Ekker, S. C. (2007) p53 Activation by Knockdown Technologies. *PLoS Genetics,* 3**,** e78.

Rosato, P., Anastasiadou, E., Garg, N., Lenze, D., Boccellato, F., Vincenti, S., Severa, M., Coccia, E. M., Bigi, R., Cirone, M., Ferretti, E., Campese, A. F., Hummel, M., Frati, L., Presutti, C., Faggioni, A. & Trivedi, P. (2012) Differential regulation of miR-21 and miR-146a by Epstein-Barr virus-encoded EBNA2. *Leukemia,* 26**,** 2343-2352.

Ross, T. M., Bhardwaj, N., Bissel, S. J., Hartman, A. L. & Smith, D. R. (2012) Animal models of Rift Valley fever virus infection. *Virus Research,* 163**,** 417-423.

Russmann, S., Grattagliano, I., Portincasa, P., Palmieri, V. O. & Palasciano, G. (2006) Ribavirin-Induced Anemia: Mechanisms, Risk Factors and Related Targets for Future Research. *Current Medicinal Chemistry,* 13**,** 3351-3357.

Rusu, M., Bonneau, R., Holbrook, M. R., Watowich, S. J., Birmanns, S., Wriggers, W. & Freiberg, A. N. (2012) An assembly model of Rift Valley fever virus. *Frontiers in Microbiology,* 3**,** doi: 10.3389/fmicb.2012.00254.

Sakuma, T., Barry, M. A. & Ikeda, Y. (2012) Lentiviral vectors: basic to translational. *J Biochemical Journal,* 443**,** 603-618.

Samuel, M. A., Morrey, J. D. & Diamond, M. S. (2007) Caspase 3-Dependent Cell Death of Neurons Contributes to the Pathogenesis of West Nile Virus Encephalitis. *Journal of Virology,* 81**,** 2614-2623.

Sanchez, A. J., Vincent, M. J., Erickson, B. R. & Nichol, S. T. (2006) Crimean-Congo Hemorrhagic Fever Virus Glycoprotein Precursor Is Cleaved by Furin-Like and SKI-1

Proteases To Generate a Novel 38-Kilodalton Glycoprotein. *Journal of Virology,* 80**,** 514-525.

Santhakumar, D., Forster, T., Laqtom, N. N., Fragkoudis, R., Dickinson, P., Abreu-Goodger, C., Manakov, S. A., Choudhury, N. R., Griffiths, S. J., Vermeulen, A., Enright, A. J., Dutia, B., Kohl, A., Ghazal, P. & Buck, A. H. (2010) Combined agonist-antagonist genome-wide functional screening identifies broadly active antiviral microRNAs. *Proceedings of the National Academy of Sciences,* 107**,** 13830-13835.

Sato, M. (2000) Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-α/β gene induction. *Immunity,* 13**,** 539-548.

Scherer, L. J., Frank, R. & Rossi, J. J. (2007) Optimization and characterization of tRNA-shRNA expression constructs. *Nucleic Acids Research,* 35**,** 2620-2628.

Schmaljohn, C. & Hjelle, B. (1997) Hantaviruses: a global disease problem. *Emerging Infectious Diseases,* 3**,** 95–104.

Schopman, N. C. T., Willemsen, M., Liu, Y. P., Bradley, T., Van Kampen, A., Baas, F., Berkhout, B. & Haasnoot, J. (2012) Deep sequencing of virus-infected cells reveals HIV-encoded small RNAs. *Nucleic Acids Research,* 40**,** 414-427.

Schudel, B. R., Harmon, B., Abhyankar, V. V., Pruitt, B. W., Negrete, O. A. & Singh, A. K. (2013) Microfluidic platforms for RNA interference screening of virus–host interactions. *Lab Chip,* 13**,** 811-817.

Schümann, M., Gantke, T. & Mühlberger, E. (2009) Ebola Virus VP35 Antagonizes PKR Activity through Its C-Terminal Interferon Inhibitory Domain. *Journal of Virology,* 83**,** 8993-8997.

Schwarz, D., Hutvagner, G., Du, T., Xu, Z., Aronin, N. & Zamore, P. (2003) Asymmetry in the assembly of the RNAi enzyme complex. *Cell,* 115**,** 199-208.

Seiradake, E., Henaff, D., Wodrich, H., Billet, O., Perreau, M., Hippert, C., Mennechet, F., Schoehn, G., Lortat-Jacob, H., Dreja, H., Ibanes, S., Kalatzis, V., Wang, J. P., Finberg, R. W., Cusack, S. & Kremer, E. J. (2009) The Cell Adhesion Molecule "CAR" and Sialic Acid on Human Erythrocytes Influence Adenovirus *In Vivo* Biodistribution. *PLoS Pathogens,* 5**,** e1000277.

Sherman, M. B., Freiberg, A. N., Holbrook, M. R. & Watowich, S. J. (2009) Single-particle cryo-electron microscopy of Rift Valley fever virus. *Virology,* 387**,** 11-15.

Shi, B., Keough, E., Matter, A., Leander, K., Young, S., Carlini, E., Sachs, A. B., Tao, W., Abrams, M., Howell, B. & Sepp-Lorenzino, L. (2011) Biodistribution of Small Interfering RNA at the Organ and Cellular Levels after Lipid Nanoparticle-mediated Delivery. *Journal of Histochemistry & Cytochemistry,* 59**,** 727-740.

Shresta, S., Sharar, K. L., Prigozhin, D. M., Snider, H. M., Beatty, P. R. & Harris, E. (2005) Critical Roles for Both STAT1-Dependent and STAT1-Independent Pathways in the Control of Primary Dengue Virus Infection in Mice. *The Journal of Immunology,* 175**,** 3946-3954.

Sidwell, R. W. & Smee, D. F. (2003) Viruses of the Bunya- and Togaviridae families: potential as bioterrorism agents and means of control. *Antiviral Research,* 57**,** 101-111.

Silverman, R. H. (1994) Fascination with 2-5A-Dependent RNase: A Unique Enzyme That Functions in Interferon Action. *Journal of Interferon Research,* 14**,** 101-104.

Sledz, C. A., Holko, M., De Veer, M. J., Silverman, R. H. & Williams, B. R. (2003) Activation of the interferon system by short-interfering RNAs. *Nature Cell Biology,* 5**,** 834-839.

Smirnova, S. E. (1979) A comparative study of the Crimean hemorrhagic fever-Congo group of viruses. *Archives of Virology,* 62**,** 137-143.

Smith, D. R., Bird, B. H., Lewis, B., Johnston, S. C., Mccarthy, S., Keeney, A., Botto, M., Donnelly, G., Shamblin, J., Albariño, C. G., Nichol, S. T. & Hensley, L. E. (2011) Development of a Novel Nonhuman Primate Model for Rift Valley Fever. *Journal of Virology,* 86**,** 2109-2120.

Smith, D. R., Steele, K. E., Shamblin, J., Honko, A., Johnson, J., Reed, C., Kennedy, M., Chapman, J. L. & Hensley, L. E. (2010) The pathogenesis of Rift Valley fever virus in the mouse model. *Virology,* 407**,** 256-267.

Smith, J. L., Grey, F. E., Uhrlaub, J. L., Nikolich-Zugich, J. & Hirsch, A. J. (2012) Induction of the Cellular MicroRNA, Hs_154, by West Nile Virus Contributes to Virus-Mediated Apoptosis through Repression of Antiapoptotic Factors. *Journal of Virology,* 86**,** 5278-5287.

Soldan, S. S., Plassmeyer, M. L., Matukonis, M. K. & Gonzalez-Scarano, F. (2005) La Crosse virus nonstructural protein NSs counteracts the effects of short interfering RNA. *Journal of Virology,* 79**,** 234-244.

Spiegel, M., Pichlmair, A., Martinez-Sobrido, L., Cros, J., Garcia-Sastre, A., Haller, O. & Weber, F. (2005) Inhibition of Beta interferon induction by severe acute respiratory syndrome coronavirus suggests a two-step model for activation of interferon regulatory factor 3. *Journal of Virology,* 79**,** 2079-2086.

Starega-Roslan, J., Krol, J., Koscianska, E., Kozlowski, P., Szlachcic, W. J., Sobczak, K. & Krzyzosiak, W. J. (2011) Structural basis of microRNA length variety. *Nucleic Acids Research,* 39**,** 257-268.

Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H. & Schreiber, R. D. (1998) How cells respond to interferons. *Annual Review of Biochemistry,* 67**,** 227-264.

Stein, D. A., Huang, C. Y. H., Silengo, S., Amantana, A., Crumley, S., Blouch, R. E., Iversen, P. L. & Kinney, R. M. (2008) Treatment of AG129 mice with antisense morpholino oligomers increases survival time following challenge with dengue 2 virus. *Journal of Antimicrobial Chemotherapy,* 62**,** 555-565.

Stein, D. A., Perry, S. T., Buck, M. D., Oehmen, C. S., Fischer, M. A., Poore, E., Smith, J. L., Lancaster, A. M., Hirsch, A. J., Slifka, M. K., Nelson, J. A., Shresta, S. & Früh, K. (2011) Inhibition of Dengue Virus Infections in Cell Cultures and in AG129 Mice by a Small Interfering RNA Targeting a Highly Conserved Sequence. *Journal of Virology,* 85**,** 10154-10166.

Stone, D., Liu, Y., Shayakhmetov, D., Li, Z., Ni, S. & Lieber, A. (2007) Adenovirus-Platelet Interaction in Blood Causes Virus Sequestration to the Reticuloendothelial System of the Liver. *Journal of Virology,* 81**,** 4866-4871.

Struthers, J. K., Swanepoel, R. & Shepherd, S. P. (1984) Protein synthesis in Rift Valley fever virus-infected cells. *Virology,* 134**,** 118-124.

Subramanya, S., Kim, S., Abraham, S., Yao, J., Kumar, M., Kumar, P., Haridas, V., Lee, S., Shultz, L. D., Greiner, D., N, M. & Shankar, P. (2010) Targeted Delivery of Small Interfering RNA to Human Dendritic Cells To Suppress Dengue Virus Infection and Associated Proinflammatory Cytokine Production. *Journal of Virology,* 84**,** 2490-2501.

Suhy, D. A., Kao, S., Mao, T., Whiteley, L., Denise, H., Souberbielle, B., Burdick, A. D., Hayes, K., Wright, J. F., Lavender, H., Roelvink, P., Kolykhalov, A., Brady, K., Moschos, S. A., Hauck, B., Zelenaia, O., Zhou, S., Scribner, C., High, K. A., Renison, S. H. & Corbau, R. (2012) Safe, Long-term Hepatic Expression of Anti-HCV shRNA in a Nonhuman Primate Model. *Molecular Therapy,* 20**,** 1737-1749.

Suzich, J. A., Kakach, L. T. & Collett, M. S. (1990) Expression strategy of a phlebovirus: biogenesis of proteins from the Rift Valley fever virus M segment. *Journal of Virology,* 64**,** 1549-1555.

Swaminathan, S., Hu, X., Zheng, X., Kriga, Y., Shetty, J., Zhao, Y., Stephens, R., Tran, B., Baseler, M. W., Yang, J., Lempicki, R. A., Huang, D., Clifford Lane, H. & Imamichi, T. (2013) Interleukin-27 treated human macrophages induce the expression of novel microRNAs which may mediate anti-viral properties. *Biochemical and Biophysical Research Communications,* 434**,** 228-34.

Swanepoel, R., Gill, D. E., Shepherd, A. J., Leman, P. A., Mynhardt, J. H. & Harvey, S. (1989) The Clinical Pathology of Crimean-Congo Hemorrhagic Fever. *Review of Infectious Diseases,* 11**,** S794-S800.

Swenson, D. L., Warfield, K. L., Warren, T. K., Lovejoy, C., Hassinger, J. N., Ruthel, G., Blouch, R. E., Moulton, H. M., Weller, D. D., Iversen, P. L. & Bavari, S. (2009) Chemical Modifications of Antisense Morpholino Oligomers Enhance Their Efficacy against Ebola Virus Infection. *Antimicrobial Agents and Chemotherapy,* 53**,** 2089-2099.

Tao, N., Gao, G., Parr, M., Johnston, J., Baradet, T., Wilson, J. M., Barsoum, J. & Fawell, S. E. (2001) Sequestration of Adenoviral Vector by Kupffer Cells Leads to a Nonlinear Dose Response of Transduction in Liver. *Molecular Therapy,* 3**,** 28-35.

Taxman, D. J., Naito, Y. & Ui-Tei, K. (2013) Designing Functional siRNA with Reduced Off-Target Effects. *siRNA Design.* Humana Press.

Taylor, K. E. & Mossman, K. L. (2012) Recent advances in understanding viral evasion of type I interferon. *Immunology,* 138**,** 190-197.

Taylor, S. L., Frias-Staheli, N., García-Sastre, A. & Schmaljohn, C. S. (2009) Hantaan Virus Nucleocapsid Protein Binds to Importin-α Proteins and Inhibits Tumor Necrosis Factor Alpha-Induced Activation of Nuclear Factor Kappa B. *Journal of Virology,* 83**,** 1271-1279.

Ter Brake, O., T' Hooft, K., Liu, Y. P., Centlivre, M., Von Eije, K. J. & Berkhout, B. (2008) Lentiviral vector design for multiple shRNA expression and durable HIV-1 inhibition. *Molecular Therapy,* 16**,** 557-564.

Terasaki, K., Murakami, S., Lokugamage, K. G. & Makino, S. (2011) Mechanism of tripartite RNA genome packaging in Rift Valley fever virus. *Proceedings of the National Academy of Sciences,* 108**,** 804-809.

Terasaki, K., Won, S. & Makino, S. (2012) The C-Terminal Region of Rift Valley Fever Virus NSm Protein Targets the Protein to the Mitochondrial Outer Membrane and Exerts Antiapoptotic Function. *Journal of Virology,* 87**,** 676-682.

Thompson, J. D., Kornbrust, D. J., Foy, J. W., Solano, E. C. R., Schneider, D. J., Feinstein, E., Molitoris, B. A. & Erlich, S. (2012) Toxicological and Pharmacokinetic Properties of Chemically Modified siRNAs Targeting p53 RNA Following Intravenous Administration. *Nucleic Acid Therapeutics,* 22**,** 255-264.

Turell, M. J., Dohm, D. J., Mores, C. N., Terracina, L., Wallette, D. L. J., Hribar, L. J., Pecor, J. E. & Blow, J. A. (2008) Potential for North American mosquitoes to transmit Rift Valley fever virus. *Journal of the American Mosquito Control Association,* 24**,** 502-507.

Ui-Tei, K., Nishi, K., Takahashi, T. & Nagasawa, T. (2012) Thermodynamic control of small RNA-mediated gene silencing. *Frontiers in Genetics,* 3**,** doi: 10.3389/fgene.2012.00101.

Unwalla, H. J., Li, H. T., Bahner, I., Li, M. J., Kohn, D. & Rossi, J. J. (2006) Novel Pol II fusion promoter directs human immunodeficiency virus type 1-inducible coexpression of a short hairpin RNA and protein. *Journal of Virology,* 80**,** 1863-1873.

Valmas, C. & Basler, C. F. (2011) Marburg Virus VP40 Antagonizes Interferon Signaling in a Species-Specific Manner. *Journal of Virology,* 85**,** 4309-4317.

Valmas, C., Grosch, M. N., Schümann, M., Olejnik, J., Martinez, O., Best, S. M., Krähling, V., Basler, C. F. & Mühlberger, E. (2010) Marburg Virus Evades Interferon Responses by a Mechanism Distinct from Ebola Virus. *PLoS Pathogens,* 6**,** e1000721.

Van De Wetering, M., Oving, I., Muncan, V., Pon Fong, M. T., Brantjes, H., Van Leenen, D., Holstege, F. C., Brummelkamp, T. R., Agami, R. & Clevers, H. (2003) Specific inhibition of gene expression using a stably integrated, inducible small-interfering-RNA vector. *EMBO reports,* 4**,** 609-615.

Van Kasteren, P. B., Beugeling, C., Ninaber, D. K., Frias-Staheli, N., Van Boheemen, S., García-Sastre, A., Snijder, E. J. & Kikkert, M. (2012) Arterivirus and Nairovirus Ovarian Tumor Domain-Containing Deubiquitinases Target Activated RIG-I To Control Innate Immune Signaling. *Journal of Virology,* 86**,** 773-785.

Verbruggen, P., Ruf, M., Blakqori, G., Överby, A. K., Heidemann, M., Eick, D. & Weber, F. (2010) Interferon Antagonist NSs of La Crosse Virus Triggers a DNA Damage Response-like Degradation of Transcribing RNA Polymerase II. *Journal of Biological Chemistry,* 286**,** 3681-3692.

Vialat, P., Billecocq, A., Kohl, A. & Bouloy, M. (2000) The S Segment of Rift Valley Fever Phlebovirus (Bunyaviridae) Carries Determinants for Attenuation and Virulence in Mice. *Journal of Virology,* 74**,** 1538-1543.

Von Teichman, B., Engelbrecht, A., Zulu, G., Dungu, B., Pardini, A. & Bouloy, M. (2011) Safety and efficacy of Rift Valley fever Smithburn and Clone 13 vaccines in calves. *Vaccine,* 29**,** 5771-5777.

Wang, B., Li, S., Qi, H. H., Chowdhury, D., Shi, Y. & Novina, C. D. (2009) Distinct passenger strand and mRNA cleavage activities of human Argonaute proteins. *Nature Structural & Molecular Biology,* 16**,** 1259-1266.

Wang, Z., Ma, H., Li, J., Sun, L., Zhang, J. & Xiao, X. (2003) Rapid and highly efficient transduction by double-stranded adeno-associated virus vectors in vitro and in vivo. *Gene Therapy,* 10**,** 2105-2111.

Warfield, K. L., Bradfute, S. B., Wells, J., Lofts, L., Cooper, M. T., Alves, D. A., Reed, D. K., Vantongeren, S. A., Mech, C. A. & Bavari, S. (2009) Development and Characterization of a Mouse Model for Marburg Hemorrhagic Fever. *Journal of Virology,* 83**,** 6404-6415.

Warfield, K. L., Swenson, D. L., Olinger, G. G., Nichols, D. K., Pratt, W. D., Blouch, R., Stein, D. A., Aman, M. J., Iversen, P. L. & Bavari, S. (2006) Gene-Specific Countermeasures against Ebola Virus Based on Antisense Phosphorodiamidate Morpholino Oligomers. *PLoS Pathogens,* 2**,** e1.

Warren, T. K., Shurtleff, A. C. & Bavari, S. (2012) Advanced morpholino oligomers: A novel approach to antiviral therapy. *Antiviral Research,* 94**,** 80-88.

Wei, J., Yang, J., Sun, J., Jia, L., Zhang, Y., Zhang, H., Li, X., Meng, Y., Yao, L. & Yang, A. (2009) Both Strands of siRNA Have Potential to Guide Posttranscriptional Gene Silencing in Mammalian Cells. *PLoS ONE,* 4**,** e5382.

Westerhout, E. M., Ooms, M., Vink, M., Das, A. T. & Berkhout, B. (2005) HIV-1 can escape from RNA interference by evolving an alternative structure in its RNA genome. *Nucleic Acids Research,* 33**,** 796-804.

Williams, B. R. (1999) PKR; a sentinel kinase for cellular stress. *Oncogene,* 18**,** 6112-6120.

Wolf, M. C., Freiberg, A. N., Zhang, T., Akyol-Ataman, Z., Grock, A., Hong, P. W., Li, J., Watson, N. F., Fang, A. Q., Aguilar, H. C., Porotto, M., Honko, A. N., Damoiseaux, R., Miller, J. P., Woodson, S. E., Chantasirivisal, S., Fontanes, V., Negrete, O. A., Krogstad, P., Dasgupta, A., Moscona, A., Hensley, L. E., Whelan, S. P., Faull, K. F., Holbrook, M. R., Jung, M. E. & Lee, B. (2010) A broad-spectrum antiviral targeting entry of enveloped viruses. *Proceedings of the National Academy of Sciences,* 107**,** 3157-3162.

Won, S., Ikegami, T., Peters, C. J. & Makino, S. (2007) NSm Protein of Rift Valley Fever Virus Suppresses Virus-Induced Apoptosis. *Journal of Virology,* 81**,** 13335-13345.

Wu, G., Yu, F., Xiao, Z., Xu, K., Xu, J., Tang, W., Wang, J. & Song, E. (2011a) Hepatitis B virus X protein downregulates expression of the miR-16 family in malignant hepatocytes in vitro. *British Journal of Cancer,* 105**,** 146-153.

Wu, H., Ma, H., Ye, C., Ramirez, D., Chen, S., Montoya, J., Shankar, P., Wang, X. A. & Manjunath, N. (2011b) Improved siRNA/shRNA Functionality by Mismatched Duplex. *PLoS ONE,* 6**,** e28580.

Yadani, F., Kohl, A., Préhaud, C., Billecocq, A. & Bouloy, M. (1999) The Carboxy-Terminal Acidic Domain of Rift Valley Fever Virus NSs Protein Is Essential for the Formation of Filamentous Structures but Not for the Nuclear Localization of the Protein. *Journal of Virology,* 73**,** 5018-5025.

Yang, J. & Lai, Eric c. (2011) Alternative miRNA Biogenesis Pathways and the Interpretation of Core miRNA Pathway Mutants. *Molecular Cell,* 43**,** 892-903.

Yang, L., Jiang, J., Drouin, L. M., Agbandje-Mckenna, M., Chen, C., Qiao, C., Pu, D., Hu, X., Wang, D., Li, J. & Xiao, X. (2009) A myocardium tropic adeno-associated virus (AAV) evolved by DNA shuffling and in vivo selection. *Proceedings of the National Academy of Sciences,* 106**,** 3946-3951.

Yeung, M. L., Bennasser, Y., Watashi, K., Le, S., Houzet, L. & Jeang, K. (2009) Pyrosequencing of small non-coding RNAs in HIV-1 infected cells: evidence for the processing of a viral-cellular double-stranded RNA hybrid. *Nucleic Acids Research,* 37**,** 6575-6586.

Yi, R., Qin, Y., Macara, I. G. & Cullen, B. R. (2003) Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes & Development,* 17**,** 3011-3016.

Yla-Herttuala, S. (2012) Endgame: Glybera Finally Recommended for Approval as the First Gene Therapy Drug in the European Union. *Molecular Therapy,* 20**,** 1831-1832.

Yoneyama, M. (1998) Direct triggering of the type I interferon system by virus infection: activation of a transcription factor complex containing IRF-3 and CBP/p300. *The EMBO Journal,* 17**,** 1087-1095.

Yoneyama, M., Kikuchi, M., Matsumoto, K., Imaizumi, T., Miyagishi, M., Taira, K., Foy, E., Loo, Y., Gale, M., Akira, S., Yonehara, S., Kato, A. & Fujita, T. (2005) Shared and Unique Functions of the DExD/H-Box Helicases RIG-I, MDA5, and LGP2 in Antiviral Innate Immunity. *The Journal of Immunology,* 175**,** 2851-2858.

Yoneyama, M., Suhara, W. & Fujita, T. (2002) Control of IRF-3 Activation by Phosphorylation. *Journal of Interferon & Cytokine Research,* 22**,** 73-76.

Yoshida, M., Kijima, M., Akita, M. & Beppu, T. (1990) Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. *Journal of Biological Chemistry,* 265**,** 17174-17179.

Yun, N. E., Poussard, A. L., Seregin, A. V., Walker, A. G., Smith, J. K., Aronson, J. F., Smith, J. N., Soong, L. & Paessler, S. (2012) Functional Interferon System Is Required for Clearance of Lassa Virus. *Journal of Virology,* 86**,** 3389-3392.

Zamoto-Niikura, A., Terasaki, K., Ikegami, T., Peters, C. J. & Makino, S. (2009) Rift Valley Fever Virus L Protein Forms a Biologically Active Oligomer. *Journal of Virology,* 83**,** 12779-12789.

Zeng, Y. & Cullen, B. R. (2004) Structural requirements for pre-microRNA binding and nuclear export by Exportin 5. *Nucleic Acids Research,* 32**,** 4776-4785.

Zeng, Y. & Cullen, B. R. (2005) Efficient processing of primary microRNA hairpins by Drosha requires flanking non-structured RNA sequences. *The Journal of Biological Chemistry,* 280**,** 27595-27603.

Zeng, Y., Sankala, H., Zhang, X. & Graves, P. R. (2008) Phosphorylation of Argonaute 2 at serine-387 facilitates its localization to processing bodies. *Biochemical Journal,* 413**,** 429-436.

Zeng, Y., Yi, R. & Cullen, B. (2003) MicroRNAs and small interfering RNAs can inhibit mRNA expression by similar mechanisms. *Proceedings of the National Academy of Sciences,* 100**,** 9779-9784.

Zhang, A. P. P., Bornholdt, Z. A., Liu, T., Abelson, D. M., Lee, D. E., Li, S., Woods, V. L., Jr. & Saphire, E. O. (2012a) The Ebola Virus Interferon Antagonist VP24 Directly Binds STAT1 and Has a Novel, Pyramidal Fold. *PLoS Pathogens,* 8**,** e1002550.

Zhang, S., Zhao, Y., Zhi, D. & Zhang, S. (2012b) Non-viral vectors for the mediation of RNAi. *Bioorganic Chemistry,* 40**,** 10-18.

Zhang, T., Cheng, T., Wei, L., Cai, Y., Yeo, A., Han, J., Yuan, Y., Zhang, J. & Xia, N. (2012c) Efficient inhibition of HIV-1 replication by an artificial polycistronic miRNA construct. *Virology Journal,* 9**,** 118.

Zhang, W., Singam, R., Hellermann, G., Kong, X., Juan, H., Lockey, R., Wu, S., Porter, K. & Mohapatra, S. (2004) Attenuation of dengue virus infection by adeno-associated virus-mediated siRNA delivery. *Genetic Vaccines and Therapy,* 2**,** 8.

Zhang, W., Yang, H., Kong, X., Mohapatra, S., Juan-Vergara, H. S., Hellermann, G., Behera, S., Singam, R., Lockey, R. F. & Mohapatra, S. S. (2005) Inhibition of respiratory syncytial virus infection with intranasal siRNA nanoparticles targeting the viral NS1 gene. *Nature Medicine,* 11**,** 56-62.

Zhang, Z., Kim, T., Bao, M., Facchinetti, V., Jung, S., Ghaffari, A., Qin, J., Cheng, G. & Liu, Y. (2011) DDX1, DDX21, and DHX36 Helicases Form a Complex with the Adaptor Molecule TRIF to Sense dsRNA in Dendritic Cells. *Immunity,* 34**,** 866-878.

Zhou, J., Bobbin, M., Burnett, J. C. & Rossi, J. J. (2012) Current progress of RNA aptamer-based therapeutics. *Frontiers in Genetics,* 3**,** doi: 10.3389/fgene.2012.00234.

Zhou, J., Li, H., Li, S., Zaia, J. & Rossi, J. J. (2008) Novel dual inhibitory function aptamer-siRNA delivery system for HIV-1 therapy. *Molecular Therapy,* 16**,** 1481-1489.

Zhu, Y., Cherukuri, N. C., Jackel, J. N., Wu, Z., Crary, M., Buckley, K. J., Bisaro, D. M. & Parris, D. S. (2012) Characterization of the RNA Silencing Suppression Activity of the Ebola Virus VP35 Protein in Plants and Mammalian Cells. *Journal of Virology,* 86**,** 3038-3049.

Zurita, M. & Merino, C. (2003) The transcriptional complexity of the TFIIH complex. *Trends in genetics,* 19**,** 578-584.

Appendices

A1 Standard laboratory protocols

A1.1 Chemically-competent DH5α *E.coli* bacteria

DH5α *E.coli* cells were grown to a cell count OD (600 nm) reading of between 0.4 and 0.6 in Luria Bertani (LB) broth [10% Bacto-Tryptone (Oxoid, Hampshire, UK); 5% Bacto-Yeast extract (Oxoid, Hampshire, UK); 10% sodium chloride (NaCl)]. 50 ml of cells were collected by centrifugation at 200 x g for 10 min (low acceleration, low brake) and resuspended in 5 ml PIPES transformation buffer [15% glycerol, 100 mM $CaCl_2$, 10 mM PIPES (pH 7.0), autoclave sterilised] and incubated for 20 min on ice. Cells were collected by centrifugation at 200 x g for 10 min, resuspended in 2 ml PIPES transformation and dispensed into 100 µl aliquots. The competent DH5αs were stored at - 80°C.

A1.2 Transformation of DH5α *E.coli* bacteria

An aliquot of chemically-competent DH5αs were thawed on ice and 8 µl of ligation reaction was added. The DH5α ligation mix was incubated on ice for 30 min and then heat-shocked at 42°C for 90 sec and spread on LB agar plates [LB with 12% bacteriological agar containing 1 mg/ml ampicillin]. To differentiate clones by Blue-White screening, the plates also contained 40 µl of X-Gal [20 mg/ml in dimethylformamide] and 8 µl of IPTG [100 mg/ml in dH_20]. Plates were air-dried at 37°C for 20 min prior to the addition of heat-shocked mixture. After the addition of the mixture, the plates were incubated at 37°C overnight.

A1.3 MiniPrep plasmid purification

A colony was inoculated into 5 ml of LB broth with 1 mg/ml of ampicillin and incubated overnight at 37°C. The cultures were pelleted by centrifugation at 3200 x g for 15 min and resuspended in 250 µl of P1 suspension buffer [50 mM Tris-HCl, 10 mM EDTA, 2.5 mg RNase A, (pH 8.5)]. An equal volume of P2 lysis buffer [0.2 M NaOH, 1% SDS] was added, incubated at room temperature for 5 min then neutralised by the addition of 250 µl of P3 neutralisation buffer [4 M guanidine hydrochloride, 0.5 M potassium acetate, (pH 4.2)] and incubated a further 5 min on ice. The lysate was centrifuged at 16,100 x g for 10 min and the supernatant was transferred to a fresh 1.7 ml Eppendorf tube. Seven hundred microliters of propan-2-ol (ISOH) was added and the DNA

precipitated at -80°C for 1 hr then centrifuged at 16,100 x g for 30 min at 4°C. The ISOH was discarded and the pellet was washed by the addition of 70% ethanol and centrifuged at 16,100 x g for 5 min at 4°C. The ethanol was discarded and the pellet air-dry then resuspended in sterile, nuclease-free H_20. The concentration of all DNA preparations was determined using spectrophotometry (NanoDrop, Themo Fisher Scientific, MA, USA).

A1.4 Midiprep plasmid purification

Midiprep plasmid DNA was extracted using the Qiagen Plasmid Midi Kit (Qiagen, CA, USA). One hundred milliliters of LB broth was inoculated from a plate stock of the desired clone and incubated at 37°C overnight in a shaking incubate at 250 rpm. Fifty milliliters of culture was centrifuged at 3200 x g for 25 min and the pellets were resuspended in 6 ml of P1 suspension buffer. After resuspension, 6 ml of P2 lysis buffer was added and incubated for 5 min at room temperature. The reaction was neutralised by adding 6 ml of P3 neutralisation buffer and left on ice for 5 min. The lysate was then centrifuge at 3200 x g for 25 min at 4°C (low acceleration, low brake) and the supernatant was transferred to a High Speed™ Midi column. Prior to the addition of the supernatant, the High Speed™ columns were equilibrated by applying 4 ml of QBT Buffer [750 mM NaCl, 50 mM MOPS, (pH 7.0), 15% ISOH, 0.15% Triton® X-100]. Once the supernatant had flown through, the column was washed twice with 20 ml of QC Buffer [1 M NaCl, 50 mM MOPS, (pH 7.0), 15% ISOH]. The plasmid DNA was eluted with 5 ml of QF Buffer [1.25 M NaCl, 50 mM Tris-HCl, (pH 7.0), 15% ISOH] and precipitated with 3.5 ml of ISOH at -80°C for 1 hr. The DNA was pelleted by centrifugation at 3200 x g for 90 min at 4°C. Two millilitres of 70% ethanol was added to the pellet and centrifuged at 3200 x g for 5 min at 4°C. The pellet was air-dried and resuspended in 200 µl sterile, nuclease-free H_20.

A1.5 Phenol:chloroform agarose gel extraction

Prior to DNA excision, gel filter tubes were prepared. Using a flame heated needle a small hole was created in the bottom of a 500 µl Eppendorf tube. The tube was then packed tightly with fish tank filter and the 500 µl tube was inserted into a 1.7 ml Eppendorf tube. The DNA band was excised from the agarose gel and put into the 500 µl filter Eppendorf tube and centrifuged at 16,100 x g for 1 min. The 500 µl filter tube was discarded and 1/10 the volume of 3 M sodium acetate (NaAc) was added to an equal volume of 1:1 salt-saturated phenol [2M Tris-base (pH 7.4)]:chloroform. The mixture was centrifuged at 16,100 x g for 1 min. The top-phase was removed

and put into a fresh 1.7 ml Eppendorf tube. An equal volume of chloroform was added and centrifuged at 16,100 x g for 1 min. The top-phase was transferred to a fresh 1.7 ml Eppendorf tube and 2.5x the volume of ISOH was added. The DNA was precipitated at -80°C for 1 hr and then centrifuged at 16,100 x g for 30 min at 4°C. Two-hundred microliters of 70% ethanol was added to the pellet and centrifuged at 16,100 x g for 5 min at 4°C. The pellet was air-dried and resuspended in 20 µl of sterile, nuclease-free H_20.

A1.6 Mammalian tissue culture

HEK293 and Huh7 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM), (BioWhittaker, MD, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Biochrom AG, BE, DE) and cultured at a temperature of 37°C with 5% CO_2. Cells were cultured in 25 cm^2 tissue culture flasks and were passaged upon reaching 90% confluence. HEK293 cells were subcultured using 1×TrypLE™ solution (Gibco, BRL, UK) and incubated for 2 min at 37°C and 5% CO_2. Huh7 cells were subcultured in phosphate buffered saline (PBS) (Gibco, BRL, UK) containing 0.4% EDTA for 5 min at 37°C and 5% CO_2. The PBS on the Huh7s was removed and 1×TrypLE™ (Gibco, BRL, UK) was added and incubated for a further 3 min. Cells were re-suspended in an equal volume of DMEM-FCS medium to inactivate the trypsin. HEK293 and Huh7 cells were split into 25 cm^2 flasks at a ratio 1:10 or 1:5, respectively. Prior to transfection, cells were stained with Trypan blue and counted using a haemocytometer. The number of viable cells was determined and seeded according to the experimental requirements.

A1.7 Transfections

Twenty-four hours after seeding the cells, they were transfected using lipofectamine™ 2000 (Invitrogen, CA, USA). For every microgram of DNA transfected, 1 µl of lipofectamine™ 2000 was used. The DNA and lipofectamine™ 2000 were first diluted separately in Opti-MEM® reduced-serum medium (Gibco, BRL, UK) in half of $1/5^{th}$ the volume of the well. The solutions were incubated for 10 min at room temperature. The DNA and lipofectamine™ 2000 solutions were mixed together and incubated for a further 20 min at room temperature. The DNA:lipofectamine™ 2000 mixture was added to the cells and 24 hrs later the media was replaced. Transfection efficiency was determined by including a vector expressing green fluorescent protein (pCI-GFP) (Passman et al., 2000), which was observed under fluorescence microscopy (Axiovert 100M microscope; Zeiss, Germany).

A1.8 RNA extraction from mammalian cells

The medium was removed from the cells and 1 ml TRI Reagent® was added to a 10 cm culture dish (Sigma, MO, USA). The cells were lysed for 5 min at room temperature. The lysates were then transferred to a 1.7 ml Eppendorf tube and 200 µl of chloroform was added. The solution was vortexed and centrifuged at 16,100 x g for 30 min at 4°C. The top-phase was transferred to a fresh 1.7 ml Eppendorf tube and an equal volume of ISOH was added. The mixture was vortexed and subsequently centrifuged at 16,100 x g for 30 min at 4°C. The pellet was air-dried and resuspended in 100 µl of dH_2O. The concentration of the RNA was determined using spectrophotometry (NanoDrop; Themo Fisher Scientific, MA, USA).

A1.9 Ambion® Decade™ Marker System

One-hundred nanograms of Decade Marker RNA was added to kinase reaction buffer with 1 µl [γ-32P]ATP and 10 U of T4 polynucleotide kinase and incubated at 37°C for 1 hr. Following incubation, cleavage reagent was added to the reaction and incubated for 5 min at room temperature. Gel Loading Buffer II was added to the mixture and heated at 95° for 5 min before loading.

A1.10 BCA protein quantification assay

Bovine serum albumin fraction V (Roche, IN, USA) was used to obtain a standard curve with known concentrations of 2 µg, 1.5 µg, 0.75 µg, 0.5 µg, 0.25 µg, 0.125 µg, and 0.025 µg in dH_2O. Distilled water was used as a blank control. Ten microliters of the standard or sample was transferred to a 96-well plate and 200 µl of BCA working reagent (50:1 ratio of BCA Reagent A and B) was added. The plate was incubated at 37°C for 30 min, cooled to room temperature and read at 570 nm in a plate reader (Biorad laboratories, USA).

A1.11 Rift Valley fever virus N detection ELISA

The top half of a 96-well ELISA plate (rows A-D, lanes 1-12) were coated with a 100 µl/well of sheep polyclonal anti-RVFV N serum, while the bottom half (rows E-H, lanes 1-12) were coated with 100 µl/well of sheep RVFV-negative serum, diluted 1:400 in phosphate buffered saline [0.01 M PBS (pH 7.4)] and incubated at 4°C overnight (Figure A.1). Plates were washed 3 times with 300 µl/well of

PBS and 0.1% Tween-20 (PBS-T) and then blocked with 200 µl/well of blocking buffer (PBS with 10% skim milk), which was incubated for 1 hr at 37°C within a moist container. The plates were washed 3 times with 300 µl/well of PBS-T, and incubated with 100 µl of sample. One-hundred microliters of either purified RVFV N at high or low concentrations (1:3000 and 1:25000) or a RVFV negative antigen (1:3000) were included as an internal ELISA control for sensitivity and specificity. The samples were incubated for 1 hr at 37°C within a moist container. The plates were washed 3 times with 300 µl/well of PBS-T and 100 µl/well of rabbit anti-RVFV N antibody (1:3000) in dilution buffer (PBS and 2% skim milk) was added and incubated for 1 hr at 37°C within a moist container. The plates were washed 3 times with 300 µl/well of PBS-T and 100 µl/well of goat anti-rabbit IgG-HRPO was added (KPL, MA, USA) (1:8000) in dilution buffer and incubated for 1 hr at 37°C within a moist container. The plates were washed 3 times with 300 µl/well of PBS-T. One-hundred microlitres of ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic) acid] (Themo Fisher Scientific, MA, USA) was added per well and incubated in the dark at room temperature for 30 min. The reaction was stopped by adding 100 µl of 1% SDS solution and the plate was read at 405 nm.

	1	2	3	4	5	6	7	8	9	10	11	12
A	++	++	A									
B	++	++	A									
C	+	+	B									
D	-	-	B									
E	++	++	A									
F	++	++	A									
G	+	+	B									
H	-	-	B									

Figure A.1: Schematic of the layout of the RVFV sandwich ELISA. The greyed squares represent the wells coated with sheep polyclonal anti-RVFV N serum, whereas the white squares represent wells coated with sheep RVFV-negative serum. A high concentration of purified N (++), low concentration of purified N (+) and RVFV negative control antigen (-) were included as ELISA internal controls. Each sample was measured in duplicate on both the anti-RVFV N and sheep RVFV-negative serum (as shown by A and B).

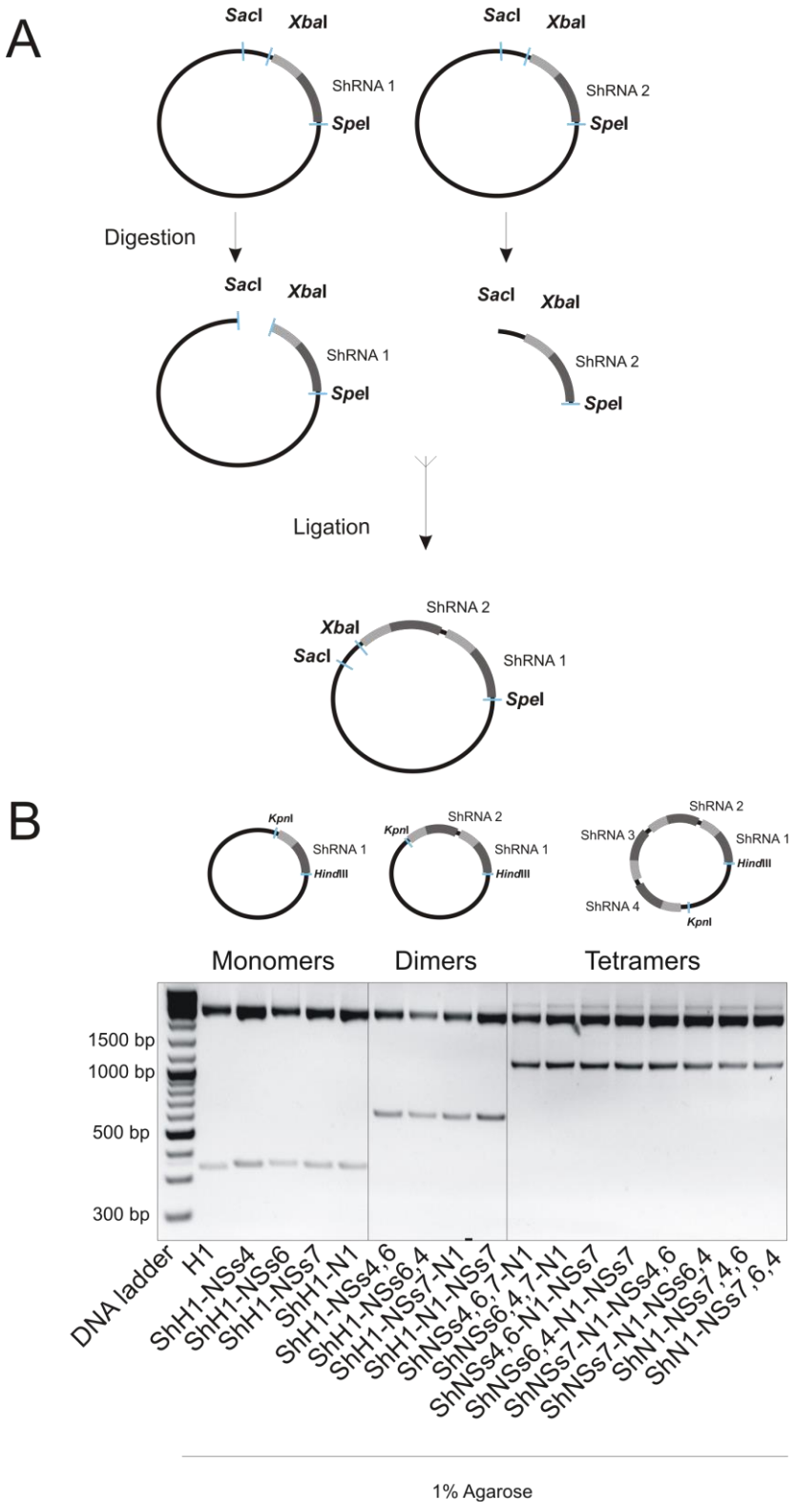

Figure A.2: Schematic of the cloning strategy for generating multimer shRNA cassettes. (A) A shRNA insert was generated through a *Sac*I and *Spe*I digest and ligated into a shRNA backbone digested with *Sac*I and *Xba*I. The process was repeated with shRNA constructs containing two shRNAs to generate multimers containing four shRNAs. (B) To confirm multimerisation; monomer, dimer and tetramer shRNA cassettes were digested with *Kpn*I and *Hind*III. The H1 lane was a construct containing only the H1-Pol III promoter.

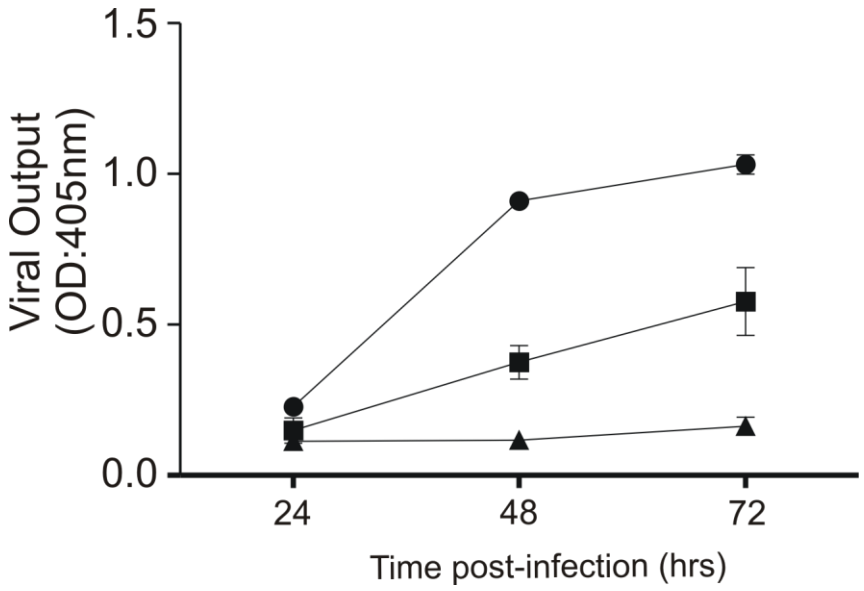

Figure A.3: RVFV replication in HEK293 cells. HEK293 cells were infected with SA1981 isolate of RVFV at a $TCID_{50}$ $1x10^{2.8}$/ml (–■–) and $1x10^{3.8}$/ml (–●–). Every 24 hrs for 3 days, supernatant was collected and the N antigen was detected using an ELISA. Uninfected cells (–▲–) were included to obtain a baseline measurement. The samples were measured at a wave-length of 405 nm. Experiments were performed in technical duplicate and the samples were measured twice. The error bars indicate standard deviation.