The Development of Molecular Tools to Enhance Understanding of Antiviral RNAi in Mosquitoes

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Abstract

Mosquito-borne arboviruses are a considerable threat to human and animal health across the world. Many of them are classed as emerging or re-emerging pathogens and the incidence of disease for a number of serious viral infections has increased as they expand their geographical and host ranges. As with other invertebrates, mosquitoes lack the adaptive immune response present in vertebrates and instead rely on their innate immune defences to modulate viral infections. Nevertheless, in contrast to vertebrates, arboviral infections in their arthropod vector are non-pathogenic and have no cytopathic effect or detrimental impact on their survival. The response considered to be the most important for antiviral defence in mosquitoes is RNA interference (RNAi) which is a sequence-specific RNA silencing mechanism. Most of what is known about antiviral RNAi in arthropods has been established in *Drosophila* as the model insect organism. These studies have benefited from an extensive range of genetic mutants, molecular tools, reporter assays and genetic profiling. The absence of these tools for use in mosquito research is a substantial deficit for arboviral studies in their natural vector system and must be rectified in order to fully understand the influence vector immunity has on virus transmission. This thesis discusses the development of a 'molecular tool-box' for advancing the acquisition of knowledge in this area.

Efficient RNAi gene silencing and its effect on the antiviral RNAi response was established *in vitro* using Semliki Forest virus (SFV) as a model arbovirus. This assay determined that knockdown of Argonaute-2 had the most substantial impact on virus replication compared to the knockdown of other RNAi proteins. In addition, the limited detection of virus-derived small RNAs, key molecules of the antiviral RNAi response by Northern blot analysis provides further support to previous evidence that SFV may circumvent the antiviral response. It is believed to sequester its genomic RNA, resulting in restricted access by the RNAi machinery and preventing the generation of large quantities of virus-derived small RNAs. However, some SFV-derived small RNAs are known to be produced and these have been shown to generate a pattern of 'hot' and 'cold' spots along the full-length coding sequences. This thesis has determined that this pattern is not exclusive to viral-derived double stranded RNA (dsRNA) trigger molecules, but is also exhibited following the treatment of mosquito cells in culture with non-viral dsRNA. This implies that all exogenous dsRNA is processed by RNAi in a similar manner.

This study has also characterised the presence of an RNA-dependent RNA polymerase (RdRP) encoded by *Aedes aegypti* mosquitoes. RdRPs are important for the amplification and spread of the RNAi signal in other organisms such as plants and worms; however, only one study suggested the existence of an RdRP in *Drosophila*. Although, this project proposed the presence and transcription of a homologue of the *Drosophila* RdRP in the *Aedes aegypti*-derived Aag2 cell line, protein knockdown assays revealed that it had no effect on virus replication *in vitro*; suggesting that it does not function as an RdRP in the antiviral RNAi process.

Due to the lack of antibodies against the major RNAi proteins (Dicer-1, Dicer-2, Argonaute-1 and Argonaute-2) in mosquitoes, these antibodies were designed and screened which allowed the identification of several candidates for the detection of the proteins in mosquito cells in culture. Further to this, recombinant forms of the RNAi initiator protein Dicer-2 and the slicer protein Argonaute-2 were successfully generated and tested *in vitro* using different promoters to establish their use for future temporal and spatial kinetic studies. It was concluded that of the promoters tested the most successful for the expression of these reporter constructs was the subgenomic promoter of SFV. However, a second promoter, the *Aedes aegypti* polyubiquitin promoter, may prove more suitable in the future.

Finally, this project studied the antiviral capabilities of a non-haematophagous mosquito cell line which would not come across an arboviral infection by traditional blood- feeding routes. Instead the mosquito larvae sustain their adult life stages by feeding on the larvae of other species which may be vertically infected. A cell line derived from *Toxorhynchites amboinensis* was characterised and was shown to carry out RNAi if induced by dsRNA suggesting that they are able to mount an antiviral response to acquired infections. This study also determined that the cell line contains an endogenous insect specific virus and, although the source of this is unknown, it adds an interesting new dimension to mosquito antiviral immunity.

This thesis enhances RNAi research in *Aedes* mosquitoes by presenting novel molecular tools and reporter assays which will be highly valuable for facilitating future investigations. The studies performed also add to what is already understood regarding the interaction between SFV and mosquito antiviral immunity through the RNAi response and pinpoint the central role of Argonaute-2 in this pathway.

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Author's Declaration

I declare that, except where explicit reference is made to the contribution of others, that this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Claire Donald

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Abbreviations and Units of Measure

µ	Micro (10^{-6})
^{32}P	Radioisotope of phosphorus
^{35}S	Radioisotope of sulfur
aa	Amino Acid
Ae. aegypti	*Aedes aegypti*
Ae. albopictus	*Aedes albopictus*
Ago-1	Argonaute - 1
Ago-2	Argonaute - 2
Ago-3	Argonaute - 3
Amp	Ampicillin
amp	Ampere
AMPs	Antimicrobial peptides
Aub	Aubergine
BHK-21	Baby hamster kidney cells
bp	Base pair(s)
BSA	Bovine serum albumin
BTV	Blue-tongue virus
-C	Carboxy- terminus
CCHF	Crimean-Congo haemorrhagic fever virus
cDNA	Complementary DNA
CFAV	Cell fusion agent Virus
CHIKV	Chikungunya virus
Ci	Curie
CO_2	Carbon dioxide
CPE	Cytopathic effect
cps	Counts per second
CrPV	Cricket paralysis virus
CYV	Culex Y virus
DAPI	4’,6-diamidino-2-phenylindole dihydrochloride
Dcr-1	Dicer-1
Dcr-2	Dicer-2
DCV	Drosophila C virus
DENV	Dengue virus
DIG	Digoxigenin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxy-nucleoside triphosphate
dpi	Days post infection
dsDNA	Double stranded deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
DTT	Dithiothreitol

DXV	Drosophila X virus
E.coli	*Escherichia coli*
ECL	Enhanced chemiluminescence
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EDTA	Ethylenediaminetetra-acetic acid
EEEV	Eastern equine encephalitis virus
eGFP	Enhanced green fluorescent protein
EIP	Extrinsic incubation period
ER	Endoplasmic reticulum
EtBr	Ethidium bromide
FCS	Foetal calf serum
FHV	Flock house virus
Fluc	Firefly luciferase
g	Gram
GMEM	Glasgow's minimal essential media
h	Hour(s)
H_2O	Water
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hpi	Hours post infection
HRP	Horse radish peroxidase
icDNA	Infectious complementary deoxyribonucleic acid
IFN	Interferon
IgG	Immunoglobulin G
IIV6	Invertebrate iridescent virus 6
IMD	Immune deficiency
ISF	Insect-specific Flavivirus
JAK/STAT	Janus kinase/signal transducer activator of transcription
JEV	Japanese encephalitis virus
kb	Kilobase pair(s)
kDa	Kilodalton(s)
l	Litre
L-15	Leibovitz medium
LACV	La Crosse virus
LB	Luria-Bertani
LGTV	Langat virus
Loq-PD	Loquacious isoform PD
m	Milli (10^{-3})
M	Molar
mAb	Monoclonal antibody
MCS	Multiple cloning site
MEB	Midgut escape barrier
MIB	Midgut infection barrier
min	Minute(s)
miRNA	microRNA

MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
n	Nano (10^{-9})
N-	Amino- terminus
NaCl	Sodium chloride
NBCS	Newborn calf serum
NF-κB	Nuclear factor- kappa B
NoV	Nodamura virus
nsP	Non-structural protein
nt	Nucleotide(s)
ONNV	O'nyong-nyong virus
ORF	Open reading frame
p	Pico (10^{-12})
p.i	Post infection
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PBSA	Phosphate buffered saline albumin
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PFU	Plaque forming unit
pH	Potential of hydrogen
piRNA	Piwi-interacting Ribonucleic acid
PMI	Personal molecular imager
PO	Phenoloxidase
pol	Polymerase
Poly(A)	Polyadenylated
PPO	Prophenoloxidase
PRR	Pattern recognition receptors
PTGS	Post-transciptional gene silencing
PUb	Polyubiquitin
RdRP	RNA-dependent RNA polymerase
REL	Relish
RISC	RNA-induced silencing complex
Rluc	*Renilla* luciferase
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RNase	Ribonuclease
rNTP	ribonucleoside tri-phosphate
rpm	Revolutions per minute
RRV	Ross River virus
RT	Reverse transcriptase
RT-PCR	Reverse transcription PCR
RVFV	Rift Valley fever virus
SA	Streptavidin
SBV	Schmallenberg virus

SDS	Sodium dodecyl sulphate
SEB	Salivary gland escape barrier
sec	Second(s)
sfRNA	Subgenomic flavivirus RNA
SFTSV	Severe fever with thrombocytopenia syndrome virus
SFV1	Semliki Forest virus replicating particles
SFV4	Semliki Forest virus Clone 4
SIB	Salivary gland infection barrier
SINV	Sindbis virus
siRNA	Small interfering Ribonucleic acid
SLEV	St. Louis encephalitis virus
SOC	Super optimal carbolite
sPBS	Sterile phosphate buffered saline
sPBSA	Sterile phosphate buffered saline albumin
SSC	Saline sodium citrate
ssRNA	Single stranded ribonucleic acid
TBEV	Tick- bourne encephalitis virus
TE	Transposable element
TEMED	N'N'N'N'-tetramethylethylene-diamine
TLR	Toll-like receptor
TPB	Tryptone phosphate broth
Tris	Tris(hydroxymethyl)aminomethane
TSN	Tudor staphylococcal nuclease
Tx. amboinensis	*Toxorhynchites amboinensis*
U	Units
UTR	Untranslated region
UV	Ultraviolet
V	Volts
v/v	Volume/Volume ratio
VEEV	Venezuelan equine encephalitis virus
viRNA	Virus-derived small interfering Ribonucleic acid
VRP	Virus replicon particles
VSR	Viral suppressor of RNAi
VSV	Vesicular stomatitis virus
w/v	Weight/Volume ratio
WEEV	Western equine encephalitis virus
WNV	West Nile virus
YFV	Yellow fever virus
Zuc	Zucchini

Amino Acid Abbreviations

Amino Acid	Three Letter Code	Single Letter Code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic Acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Chapter 1: Introduction

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1.1 Arboviruses

1.1.1 Overview

Arboviruses are **ar**thropod-**bo**rne **viruses** which are transmitted by haematophagous (blood feeding) arthropod vectors including mosquitoes, ticks, midges and sandflies and can be transmitted to susceptible vertebrates when the vector takes a blood meal. A unique feature of arboviruses is that in order to complete their transmission cycle they are required to replicate in both vertebrate and invertebrate organisms and successfully bypass the immune barriers exhibited by both vector and host. Infections are a major medical, veterinary and ecological burden worldwide. The predominant diseases resulting from infection are encephalitis, haemorrhagic fever and febrile illnesses (Meltzer, 2012). Although some human and animal vaccines against certain arboviral infections exist, for many this form of treatment has yet to be developed (Metz and Pijlman, 2011). Moreover, arboviruses are an increasing global threat. A rise in human travel and trade combined with climate change, greater urbanisation and population densities, as well as insect habitat adaptation, has allowed the incidence of arboviral diseases to become more widespread. The capacity for these viruses to cause disease and reach epidemic status is controlled by several key factors and it is widely accepted that both genetic and environmental influences will affect vector competency. These aspects will be discussed fully in **Chapter 1.2.3** and **Chapter 1.2.4**.

Taxonomically, arboviruses are classed on morphology, antigenic properties, replicative mechanisms and genetic strategies. The majority fall into the families of *Togaviridae*, *Flaviviridae*, *Bunyaviridae*, *Reoviridae* and *Rhabdoviridae* (**Table 1.1**). Only one example of a DNA arbovirus is known, the African swine fever virus (ASFV, *Asfarviridae*) which is spread by ticks (Tulman et al., 2009). The natural enzootic transmission cycle, illustrated in **Figure 1.1**, begins when a vector takes a blood meal from an infected vertebrate host. Animal hosts are often rodents, birds and sub-human primates. The pathogen is then delivered to the midgut where it must cross the epithelium, escape the midgut and cross into the haemocoel (**Chapter 1.2.3**). It must then replicate within a number of tissues and disseminate to the salivary glands. High viral tires are released into the saliva and can be transmitted to a suitable new naive host when the arthropod takes a subsequent meal. Transmission to humans can take place; although, in most cases they are considered to be dead-end hosts as the resulting viraemia is usually too low to allow further transmission to occur. However, efficient transmission of chikungunya (CHIKV), yellow fever virus

(YFV) and dengue (DENV) viruses has been observed where the enzootic cycle has been lost and these viruses are capable of being past between mosquito vectors and human hosts without the need for an animal intermediate (Weaver, 2006, Weaver and Reisen, 2010, Durbin et al., 2013, Weaver, 2014). In this way, extensive epidemics can be efficiently maintained in urban areas. In addition to classical horizontal transmission, other routes of transmission such as vertical (from an infected adult to its offspring) and venereal transmission (from a vertically infected male to a female) are documented. These are less common and are discussed in more detail in **Chapter 6**.

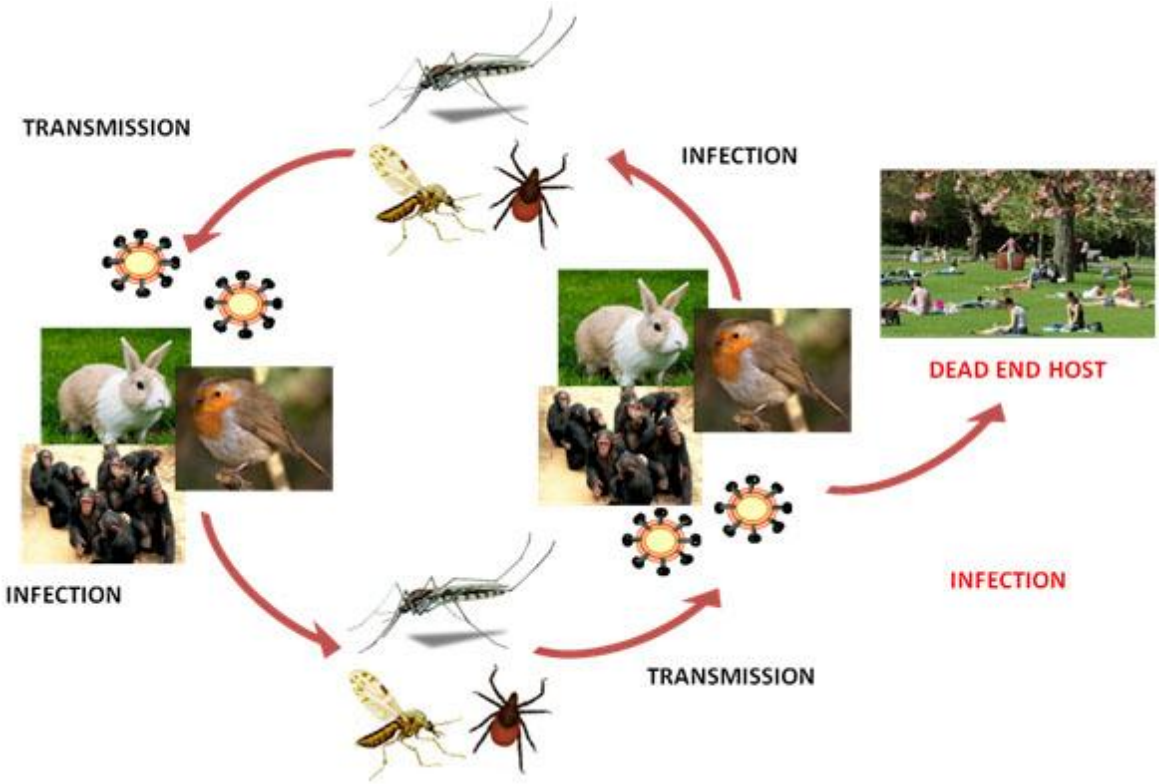

Figure 1.1: Enzootic cycle of arbovirus transmission. Arboviruses cycle between arthropod vectors, such as mosquitoes, midges and ticks, to susceptible vertebrate hosts. Hosts include birds, mammals and sub-human primates. Human infections occur but viraemia is often too low to permit onward transmission and they are therefore considered to be dead end hosts.

Table 1.1: Arbovirus families. Example family members, their genetic structure and vector species.

Genome	Family	Genus	Virus (Abbr.)	Segments	Vector	Main Species	Reference
(+)ssRNA	*Togaviridae*	Alphavirus	Sindbis virus (SINV)	One	Mosquito	*Aedes* spp.	(Griffin, 2013, Kuhn, 2013)
			Chikungunya virus (CHIKV)			*Aedes* spp.	
			Venezuelan equine encepalitis virus (VEEV)			*Aedes* spp.	
			Semliki Forest virus (SFV)			*Aedes* spp.	
			O'nyong nyong virus (ONNV)			*Anopheles* spp.	
			Eastern equine encepalitis virus (EEEV)			*Culiseta* spp.	
			Western equine encepalitis virus (WEEV)			*Culex* spp.	
			Ross River virus (RRV)			*Culex* spp.	
	Flaviviridae	Flavivirus	West Nile virus (WNV)	One	Mosquito	*Culex* spp.	(Gubler et al., 2007, Lindenbach et al., 2007)
			Dengue virus (DENV)		Mosquito	*Ae. aegypti*	
			Yellow Fever virus (YFV)		Mosquito	*Aedes* spp.	
			Japanese encephalitis virus (JEV)		Mosquito	*Aedes* spp.	
			Tick-borne encephalitis virus (TBEV)		Tick	*Ixodes* spp	
			Powassan virus (POWV)		Tick	*Ixodes* spp	
(-)ssRNA	*Bunyaviridae*	Phlebovirus	Rift Valley fever virus (RVFV)	Three	Mosquito	*Aedes* spp.	(Schmaljohn and Nichol, 2007, Beer et al., 2013)
		Orthobunyavirus	La Crosse virus (LACV)		Mosquito	*Ae. triseriatus*	
			Schmallenberg virus (SBV)		Midge	*Culicoides* spp.	
		Nairovirus	Crimean-Congo haemorrhagic fever virus (CCHFV)		Tick	*Hyalomma* spp.	
	Rhabdoviridae	Vesiculovirus	Vesticular stomatitis virus (VSV)	One	Sand fly	*Phlebotominae*	(Lyles et al., 2013)
			Chandipura virus (CHPV)				
dsRNA	*Reoviridae*	Orbivirus	Bluetongue virus (BTV)	Ten	Midge	*Culicoides* spp.	(Roy, 2013)
			African horse sickness virus (AHSV)		Midge	*Culicoides* spp.	
			Colorado tick fever virus (CTFV)		Tick	*Dermacentor andersoni*	
dsDNA	*Asfaviridae*	Asfivirus	African swine fever virus (ASFV)	One	Tick	*Ornithodoros* spp.	(Dixon et al., 2013)

1.1.2 Medically and Veterinary Important Arboviral Families

1.1.2.1 *Bunyaviridae*

The *Bunyaviridae* family is the largest group of RNA viruses and contains >350 viral species, most of which are arthropod-borne and some which are classed as containment level 3/4 pathogens. Given the number of prominent malignant human and animal pathogens this family contains, such as Rift Valley fever virus (RVFV), La Crosse virus (LACV), Crimean-Congo haemorrhagic fever virus (CCFHV) the recently emerged Schmallenberg virus (SBV) and severe fever with thrombocytopenia syndrome virus (SFTSV), bunyaviruses are a significant threat to human health and animal welfare across a number of continents.

Bunyaviruses are divided into five genera: Orthobunyavirus, Phlebovirus, Hantavirus, Nairovirus and Tospovirus (Elliott, 1990, Schmaljohn and Nichol, 2007, Walter and Barr, 2011). Orthobunyaviruses, phleboviruses and nairoviruses are spread by midges, mosquitoes, ticks and sandflies (Tesh, 1988, Labuda, 1991), whereas tospoviruses, the only pathogenic plant genus in the *Bunyaviridae* family, are transmitted by thrip vectors (Whitfield et al., 2005). Hantaviruses are the exception as they are not classed as arboviruses due to the absence of a known vector and are spread by rodents (Schmaljohn and Hjelle, 1997, Zhang, 2014). All member viruses share certain features. They are enveloped and contain a negative sense single stranded RNA (ss(-)RNA) genome which is divided into three segments, named according to their size (Elliott, 1990, Schmaljohn and Nichol, 2007). The L (Large) segment encodes the L protein which acts as the viral RNA-dependent RNA polymerase (RdRp) (Endres et al., 1989). The M (Medium) segment encodes the precursor for the viral glycoproteins which are produced following post-translational processing (Lees et al., 1986). The S (Small) segment encodes the multifunctional nucleocapsid (N) protein (Eifan and Elliott, 2009). Some phleboviruses have an ambisense S segment which allows bi-directional coding during replication (Shope, 1996). As with other multi-segmented genome viruses, bunyaviruses are known to undergo genomic re-assortment during mixed infections (Beaty et al., 1985, Borucki et al., 1999, Reese et al., 2008).

The spherical virions are approximately 100 nm in diameter and are composed of four structural proteins; the two external glycoproteins (Gn and Gc), the N and the L protein. It is understood that the N protein binds and coats the viral RNA and interacts with the L protein to form ribonucleoprotein (RNP) complexes with a specific ‘panhandle’ structure,

linking them to the envelope proteins (Kaukinen et al., 2005). Certain viruses have also been shown to encode non-structural protein(s) on their M (NSm) and/or S (NSs) segments which are hypothesised to interact with the immune system and be involved in viral pathogenesis; although, their functions are still largely unknown (Eifan et al., 2013).

Many bunyaviruses are classed as emerging or re-emerging agents due to the expansion of their host and geographical ranges. In particular, there has been a great increase in the geographic distribution of RVFV over the past 17 years and outbreaks have been reported in East Africa (1997/98 and 2006/07) (Anyamba et al., 2010), Kenya (1997/98 and 2006/07) (Woods et al., 2002, Nguku et al., 2010), Sudan (2007) (Hassan et al., 2011), Southern Africa (2008-2010) (Anyamba et al., 2010) and Saudi Arabia and Yemen (2000, 2010) (Balkhy and Memish, 2003, Aradaib et al., 2013). SFTSV is a newly identified member of the phlebovirus genus and has so far been recognised as a human pathogen in China, Japan and South Korea (Feldmann, 2011, Zhang et al., 2013b). It emerged in 2007 from the Huaiyangshan mountain range in China and is believed to be carried by a tick vector, specifically *Haemaphysalis longicornis* (Xu et al., 2011, Yu et al., 2011, Zhang et al., 2011, Zhang et al., 2012). However, direct human-human transmission has also been documented (Gai et al., 2012, Liu et al., 2012). As the name suggests, symptoms include fever, leukopenia, thrombocytopenia and it has a high case fatality rate of between 12 - 30 %. More recently, the emergence of SBV in 2011 had a devastating agricultural and economic impact following its spread across Germany, the Netherlands, Belgium, France, Italy, Luxembourg, Spain and the United Kingdom (Steukers et al., 2012, Beer et al., 2013, Doceul et al., 2013, Balenghien et al., 2014). The virus, classed as an orthobunyavirus, is transmitted by a midge vector and causes fever, diarrhoea, milk drop, loss of appetite and abortion in sheep, cattle and goat herds. Offspring of infected individuals can be still born and live births were often found to have congenital malformations and physical abnormalities.

1.1.2.2 *Flaviviridae*

There are >70 members of the *Flaviviridae* family which belong to one of three genera, Flavivirus, Hepacivirus and Pestivirus. The Flavivirus genus is the largest and is the only one to contain arboviruses transmitted by either mosquitoes; such as YFV, DENV, West Nile virus (WNV) and Japanese encephalitis virus (JEV), or ticks; such as tick-borne encephalitis virus (TBEV) (Gubler et al., 2007, Schweitzer et al., 2009, Rust, 2012). In addition to arthropod-borne viruses, the flaviviruses also contain a group with no known

vector (Kuno et al., 1998, Cook and Holmes, 2006), as well as insect specific flaviviruses (ISF) which have been shown to be incapable of infecting vertebrates (Cammisa-Parks et al., 1992) (discussed in detail in **Chapter 6**). As a result, flaviviruses are of particular interest to evolutionary virologists as a model for understanding the evolution of vector-borne diseases and may provide some insight into important features of emerging and re-emerging viruses (Holmes, 1998).

Flaviviruses are enveloped and contain a single stranded, un-segmented positive sense (ss(+)RNA) genome which is approximately 10.7 kb long (Rice et al., 1985, Lindenbach and Rice, 2003, Harris et al., 2006, Lindenbach et al., 2007). The genomic RNA has a 5' cap but unlike cellular mRNAs they lack a polyadenylated (poly(A)) tail at the 3' end. It is translated as a single polyprotein flanked by 5' and 3' untranslated regions (UTRs). These regions form complex secondary structures and play important roles in the regulation of translation, RNA synthesis and virus replication (Charlier et al., 2002, Markoff, 2003, Gritsun et al., 2006). The polyprotein is co- and post- translationally cleaved by a combination of viral and host proteases into three structural proteins (the capsid protein [C], the membrane glycoprotein precursor [prM] and the envelope glycoprotein [E]) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). NS5 is the most highly conserved protein within the flaviviruses and codes for the RdRP (Mandl et al., 1989). The NS proteins, including NS5, and additional host factors form the replication complex to facilitate the synthesis of new viral RNA (Lindenbach et al., 2007). The C protein is important for the formation of the icosahedral nucleocapsid which encapsidates the genome (Mukhopadhyay et al., 2005). The nucleocapsid is surrounded by a host-derived lipid bilayer in which is embedded the E protein, the chief structural protein documented to be the principle target for neutralising antibodies (Sanchez et al., 2005, VanBlargan et al., 2013). In immature virions, the prM protein protects the E protein from degradation. During the exit of the virus from the cell, the prM protein is cleaved into the mature M protein and the 'pr' segment so only mature virons present the M protein embedded in the bilayer. The resulting mature virons are spherical and are about 50 nm in diameter (Lindenbach et al., 2007).

Mosquito-borne flaviviruses represent some of the most serious emerging and re-emerging global pathogens (Mackenzie et al., 2004). They can be divided into those transmitted primarily by *Culex* spp. vectors (WNV, JEV) and those from *Aedes* spp. vectors (DENV, YFV). Interestingly, this is also split into those resulting in encephalitic disease being associated with *Culex* transmission and those viruses resulting in haemorrhagic diseases associating with *Aedes* spp. (Gaunt et al., 2001). DENV is responsible for the greatest

incidence of disease and mortality within the genus and there is currently no effective vaccine or specific treatment available. It is the pathogen behind dengue fever as well as the life threatening dengue haemorrhagic fever and dengue toxic shock syndrome. Over 2.5 billion people (> 40 % of the population) are said to live in dengue-endemic areas in more than 100 countries worldwide (Guzman and Kouri, 2002, Bhatt et al., 2013). It results in between 50 - 100 million cases with approximately 22,000 deaths worldwide annually (Gubler, 2012) compared to an estimated 200,000 cases/40,000 deaths from YFV (Monath, 2001) and 50,000 cases/15,000 deaths from JEV worldwide (Solomon and Vaughn, 2002, Fischer et al., 2008). The emergence and recurrence of epidemic dengue has become more frequent with larger epidemics and more severe symptoms (Halstead, 1992, Gubler and Clark, 1995, Gubler, 1998b, Gubler, 2002, Gubler, 2011, Gubler, 2012, Messina et al., 2014, Schaffner and Mathis, 2014). The most recent outbreak occurring in Japan in September 2014, the first since 1945 (Torres, 2014). It should also be noted that recently a fifth dengue virus serotype was identified from an outbreak in Malaysia in 2007 (Normile, 2013), in addition to the four (DENV-1, DENV-2, DENV-3, DENV-4) previously characterised serotypes (Zanotto et al., 1996). This discovery remains controversial, but if confirmed it will further complicate future vaccine developments and has implications for disease control.

1.1.2.3 *Togaviridae*

Viruses belonging to the *Togaviridae* family are divided between two genera: Alphaviruses and Rubiviruses (Kuhn, 2013). The Rubivirus genus contains a single virus, rubella virus which is a common disease in children but with no arthropod vector. This is unlike the Alphavirus genus which contains a number of arboviruses. There are approximately 30 acknowledged arthropod-borne alphaviruses consisting of both human and animal pathogens (Weaver et al., 2000). They are subdivided into those which infect terrestrial hosts, usually mammals or birds, and those infecting fish. Salmonid fish, like trout and salmon, are hosts to aquatic alphaviruses such as salmon pancreatic disease virus and sleeping disease virus. Although sea lice are known to be infected, they have not been conclusively verified as a vector for these viruses (McLoughlin and Graham, 2007, Forrester et al., 2012b, Kuhn, 2013). In addition, a novel ‘mosquito-specific’ alphavirus named Eilat virus, was recently described which is speculated to be incapable of replicating in vertebrate cells (Nasar et al., 2012). The alphavirus transmission cycle generally involves mosquito vectors; although ticks and lice are able to transmit some alphaviruses. Most infections are known to cause a range of symptoms including: fever,

rashes, arthritis, arthralgia and, most prominently, encephalitis (Zacks and Paessler, 2010). Although rubella virus shares several properties with alphaviruses, such as genomic organisation and virion structure, as it is not classed as an arbovirus only the alphavirus genus shall be described in detail.

1.1.2.4 Genus Alphavirus

1.1.2.4.1 Spread and Geographical Distribution

As most alphaviruses are transmitted by arthropod vectors this is likely to impact their geographic dispersal; however, they have been shown to have a worldwide distribution. They can be defined as either Old World or New World viruses depending on the geographical origin of isolation. Old World alphaviruses, including CHIKV, Semliki Forest virus (SFV), Sindbis virus (SINV) and o'nyong-nyong virus (ONNV) originating in Africa and Asia, in addition to Ross River virus (RRV) identified in Australia, are responsible for causing rashes, myalgia and arthralgia. New World alphaviruses consist of those identified in the Americas which generally cause encephalitis (Zacks and Paessler, 2010). These include Eastern equine encephalitis virus (EEEV), Western equine encephalitis virus (WEEV) and Venezuelan equine encephalitis virus (VEEV).

Although historically classed as a virus of Africa, CHIKV has spread extensively throughout the Old World and the New. It was first isolated in 1952 in Tanzania (Robinson, 1955) and was named chikungunya which translates as 'that which bends up' from the native Makonde language to describe the painful, contorted posture of those infected by it. CHIKV was not traditionally believed to be life-threatening; although, in contrast to other arboviral infections only 5 - 25 % of infections are asymptomatic and patients are known to exhibit symptoms including headaches, vomiting, rashes, fevers, myalgia and severe arthralgia. The disease can be highly debilitating and the pain can last for several months to years. As the symptoms and geographic spread are similar to that of DENV it is likely that the diseases were often confused and that CHIKV was under-diagnosed (Carey, 1971). However, more recently, CHIKV has become recognised as one of the most dangerous re-emerging pathogens, largely due to two substantial epidemics infecting naive populations. Unusually, these outbreaks have reported a considerable number of human deaths which may suggest that newly emerged strains of CHIKV are more virulent in humans (Mavalankar et al., 2008, Robin et al., 2008). The first apparently appeared from Kenya in 2004 and spread to islands of the Indian Ocean, most notably to

La Reunion (discussed in **Chapter 1.2.1**). The second was a closely related strain that appeared in India from East Africa and has caused in the region of 1 - 6 million cases. A major fear was the spread of CHIKV to Europe and the Americas, both of which have become a reality as the virus arrived in Italy in 2007 (Rezza et al., 2007) and in the Caribbean at the end of 2013 (Cauchemez et al., 2014, Leparc-Goffart et al., 2014, Noel and Rizzo, 2014, Powers, 2014, Weaver, 2014). Cases of the virus, including autochthonous transmissions, later appeared in the continental United States, South and Central America from June 2014 and according to the Centres for Disease Control and Prevention (CDC) as of the 12th September 2014 there were over 706,000 suspected cases, 8,650 confirmed cases and 113 fatalities; although, these were restricted to Latin Caribbean. The disease is spread by *Ae. aegypti* and *Ae. albopicus* both of which are highly established in these areas (discussed further in **Chapter 1.2**). This, coupled with a naive resident population, high level of travel into and out of these areas and the lack of an effective vaccine means that these numbers may only continue to increase in the future unless appropriate prevention strategies can be implemented.

1.1.2.4.2 Genome Organisation

Alphaviruses consist of a ss(+)RNA genome surrounded by an envelope. The viral genome is approximately 11.5 kb which is capped at the 5' end and polyadenylated (poly(A) tail) at the 3' end. This allows it to function like a cellular messenger RNA (mRNA) and be directly translated by the host cell translation machinery whilst protecting it from degradation (Strauss et al., 1983). It is separated into two open reading frames (ORFs) which encode the nine viral proteins (Strauss and Strauss, 1994). The larger ORF at the 5' end encompasses approximately two-thirds of the genome and produces the four non-structural proteins (nsP1, nsP2, nsP3 and nsP4) from the nsP1234 polyprotein (**Figure 1.3**). The remaining third encodes a separate polyprotein from which the five structural proteins (the capsid protein [C], the 6 kDa protein [6K] and three envelope glycoproteins [E1, E2 and E3]) are generated under the control of the internal subgenomic (26S) promoter (Kaariainen et al., 1987) (**Figure 1.4**). E2 and E3 are produced from a precursor termed p62. The nsPs are known to be multi-functional (Strauss and Strauss, 1994, Kaariainen and Ahola, 2002) and **Table 1.2** summarises their known actions.

Alphavirus particles are in the region of 70 nm in diameter and have a spherical appearance. The surrounding envelope is composed of a lipid bilayer derived from the host cell plasma membrane. On the surface, three E1-E2 heterodimers form each of the eighty

spikes embedded in the envelope surrounding the nucleocapsid (Ziemiecki and Garofff, 1978, Vaux et al., 1988, Metsikko and Garoff, 1990, Owen and Kuhn, 1997). The nucleocapsid is arranged in a T = 4 icosahedral arrangement of 240 copies of the C proteins (Coombs and Brown, 1987, Paredes et al., 1993). The known role that each of the structural proteins plays in the virus life cycle is described in **Table 1.2**.

Table 1.2: The function of each of the nine alphavirus proteins.

	Protein	Size (aa)	Function	Reference
Non-Structural Proteins	nsP1	537	Anchors the replication complex to the host cell membrane.	(Kujala et al., 2001, Spuul et al., 2007)
			Instigates the production of (-) sense RNA from the (+) sense genome RNA.	(Hahn et al., 1989, Wang et al., 1991)
			Part of the RNA capping complex.	(Ahola et al., 1997, Vasiljeva et al., 2000)
	nsP2	799	Acts as a nucleoside triphosphatase and an RNA helicase	(Rikkonen et al., 1994, Gomez de Cedrón et al., 1999, Vasiljeva et al., 2001)
			Directs nuclear localisation	(Peranen et al., 1990, Rikkonen et al., 1992, Rikkonen, 1996)
			Possesses protease activity to cleave the non-structural polyprotein	(Hardy and Strauss, 1989, Takkinen et al., 1991, Merits et al., 2001)
			Regulates subgenomic RNA synthesis	(Suopanki et al., 1998)
	nsP3	482	Regulates RNA synthesis	(Hahn et al., 1989, Li et al., 1990, LaStarza et al., 1994, Vihinen et al., 2001)
	nsP4	614	An RNA-dependent RNA polymerase	(Kamer and Argos, 1984, Hahn et al., 1989, Tomar et al., 2006)
			Possesses protease activity and processes nsP3 from nsP4 from within the polyprotein	(Takkinen et al., 1990, Takkinen et al., 1991)
Structural Proteins	C	267	Forms nucleocapsid core by encapsidating the genomic RNA	(Weiss et al., 1989, Owen and Kuhn, 1996)
			Carboxyl domain is an autocatalytic serine protease	(Aliperti and Schlesinger, 1978, Melancon and Garoff, 1987)
	E3	66	Undefined	(Garoff et al., 1974, Simizu et al., 1984)
	E2	422	Involved in receptor binding	(Garoff et al., 1980, Liu and Brown, 1993, Smith et al., 1995)
			Major epitope for neutralizing antibodies	(Roehrig et al., 1990, Hunt et al., 2010, Hunt et al., 2011, Kam et al., 2012)
			Interacts with nucleocapsid	(Metsikko and Garoff, 1990, Owen and Kuhn, 1997)
	6K	60	E1 leader peptide	(Lusa et al., 1991, Sanz and Carrasco, 2001)
			Putative Ion Chanel	(Sanz et al., 1994, Sanz et al., 2003, Melton et al., 2002)
			Essential for new virion assembly	(Gaedigk-Nitschko and Schlesinger, 1990, Ivanova et al., 1995)
	E1	438	Responsible for membrane fusion	(Omar and Koblet, 1988, Boggs et al., 1989)

1.1.2.4.3 Replication

Alphavirus replication occurs in the cytoplasm (**Figure 1.2**) (Leung et al., 2011). They enter the cell via receptor mediated endocytosis through the action of the E2 glycoprotein binding to the cellular receptor. The exact receptor involved is not known but due to the wide host range that alphaviruses can infect it is suggested that 1) the receptor is highly conserved across vertebrate and invertebrate host species or 2) the E2 protein has multiple receptor binding sites and can enter via a number of cellular molecules (Kuhn, 2013). Following attachment, the virus is taken up through clathrin-mediated endocytosis forming a vesicle which fuses first with an endosome and then with a lysosome to permit the breakdown of the outer viral components (Helenius et al., 1980, Marsh and Helenius, 1980, Marsh et al., 1983, DeTulleo and Kirchhausen, 1998, Strous and Govers, 1999). This occurs due to the decreasing pH within the maturing vesicles creating an acidic environment. As a result, the glycoprotein spike complexes undergo a conformational change promoting E1-mediated fusion of the virus envelope with the lysosome membrane (Wahlberg et al., 1989, Wahlberg and Garoff, 1992, Gibbons et al., 2004). This produces a fusion pore through which the nucleocapsid core is ejected into the cytoplasm, disassociates and releases the viral genome (Fuller et al., 1995).

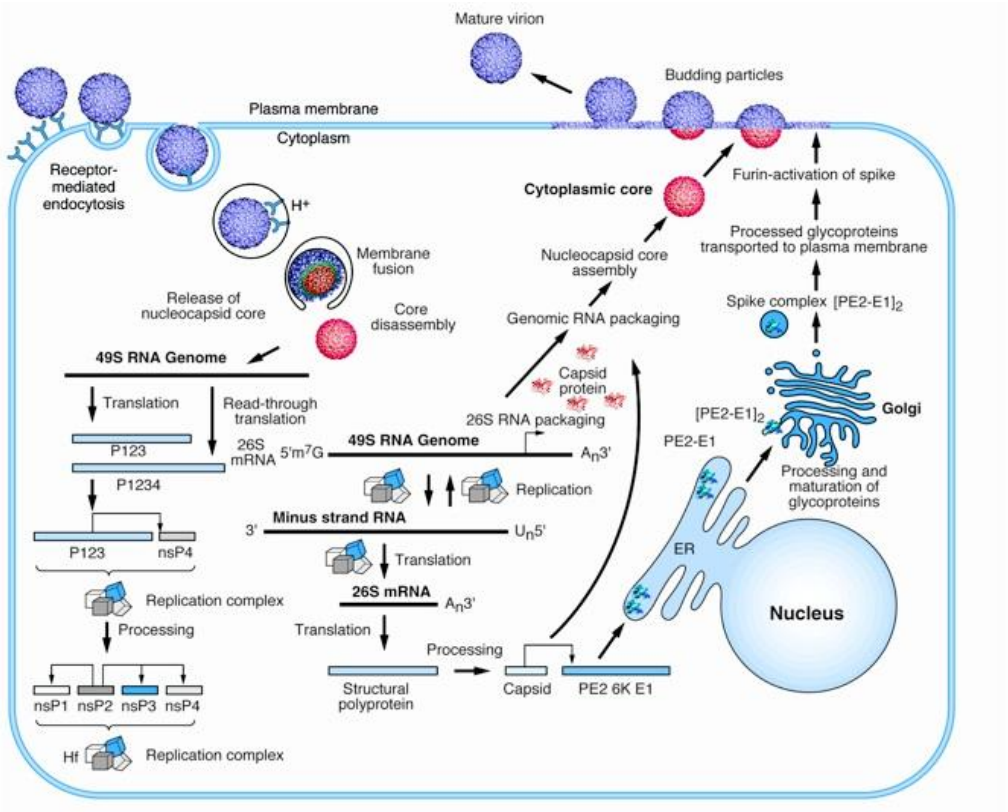

Figure 1.2: The Alphavirus life cycle. Starting at the top left, the virus enters the cell by attaching to the cellular receptor. It is taken up by clathrin-mediated endocytosis before the membranes fuse causing the disassembly of the viral coat. The genome replicates in cytoplasmic replication complexes (bottom left) before associating with the capsid proteins. These come together with the processed glycoproteins and bud as mature virions from the plasma membrane (top right). Taken from Kuhn, 2013.

Translation is initiated at the 5' end and so produces the non-structural P1234 polyprotein first (Takkinen, 1986) (**Figure 1.3** and **Figure 1.4**). The presence of an opal stop codon (UAG) has been determined between the nsP3 and nsP4 genes in a number of alphaviruses including SINV. The result of this is the generation of a greater concentration of P123 polyprotein compared to P1234. However, some P1234 polyprotein is produced as this codon is slightly 'leaky' and allows read-through to occur at an occurrence of 5 – 20 % (Strauss et al., 1983). However, in other alphaviruses, such as SFV and ONNV, this termination codon has been replaced with an arginine codon (CGA) allowing only P1234 to be produced (Strauss et al., 1983, Takkinen, 1986, Tuittila et al., 2000, Myles et al., 2006). The individual proteins are cleaved from the polyprotein through the action of the nsP2 protease which has a papain-like protease domain at its C-terminal (Merits et al., 2001). The proteolytic process always occurs in the same order with nsP4 being released almost immediately followed after a lag by nsP1 and finally nsP3 (**Figure 1.3**) (Kim et al.,

2004). The cleaved non-structural proteins align with each other to form a replication complex which associates with cellular membranes (Friedman et al., 1972, Grimley et al., 1972, Kujala et al., 2001, Salonen et al., 2005, Spuul et al., 2007). A number of inward invaginations termed spherules form at the plasma membrane and it is here that negative sense full length antigenome is transcribed from the template positive sense genome mediated by the viral RdRP, nsP4 and P123 (Spuul et al., 2010) (**Figure 1.4**). Following the cleavage of P123 into its constituent parts, less antigenome is produced and instead new viral positive sense RNA (42S RNA) is generated from the antigenome template, modulated by the cleaved non-structural proteins.

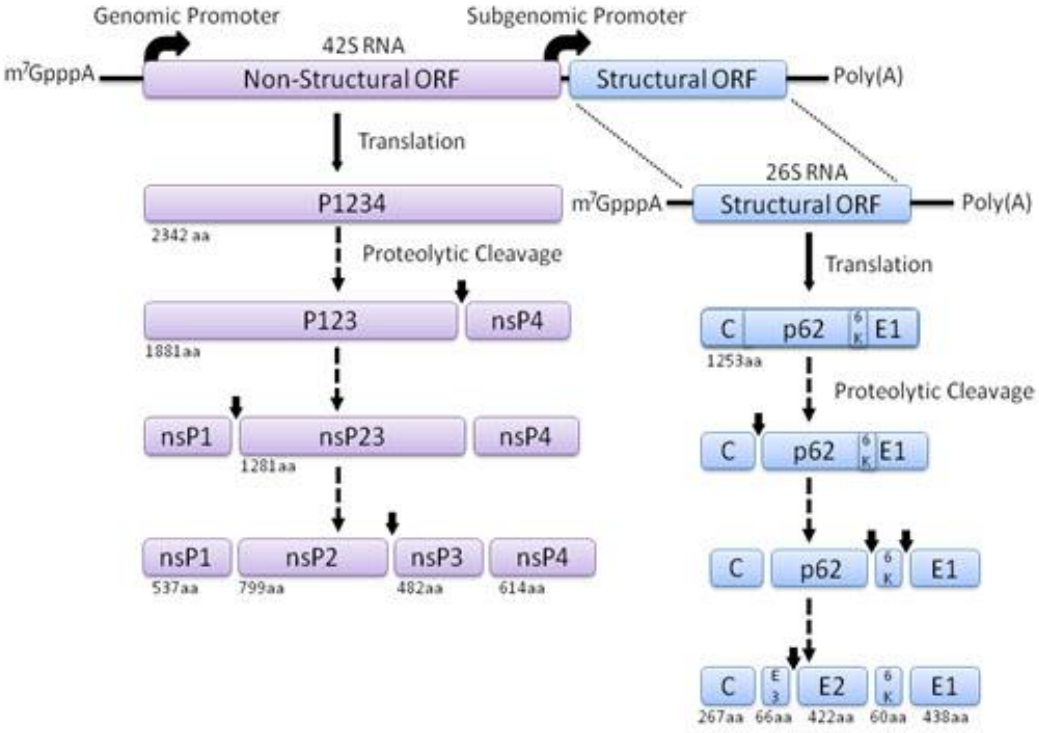

Figure 1.3: Alphavirus polyprotein processing. The non-structural proteins (nsP1, nsP2, nsP3 and nsP4, shown in purple) are translated first and proteolytically cleaved into the individual replicase proteins. The structural proteins (shown in blue) are translated from the subgenomic RNA under the control of the subgenomic promoter and subsequently cleaved into the capsid (C), 6K and glycoproteins (E1, E2, E3). Cleavage sites are indicated with small black arrows.

The structural genes are translated late in the infection cycle from the subgenomic (26S) RNA) which is produced following transcription from the internal subgenomic promoter (Kaariainen et al., 1987, Kim et al., 2004) (**Figure 1.4**). The C protein is autoproteolytic and is rapidly cleaved from the remaining polyprotein, ready to associate with newly

synthesised viral genomes by interacting with the packaging signal (Aliperti and Schlesinger, 1978, Melancon and Garoff, 1986, Weiss et al., 1989, Owen and Kuhn, 1996). Its cleavage exposes the N-terminal signal of p62 which results in the transport of the remaining polyprotein to the endoplasmic reticulum (ER) where host cellular signalases cleave the remaining polyprotein, with the exception of E2 which is processed later in the secretory pathway by furin-like proteases (Bonatti et al., 1984, de Curtis and Simons, 1988, Garoff et al., 1990, Sariola et al., 1995, Zhang et al., 2003). Both p62 and E1 become embedded in the ER membrane due to their transmembrane domains and are modified by the ER and Golgi through the addition of fatty acids, carbohydrate chains and side chains (Melancon and Garoff, 1986). The processed E1 and E2 glycoproteins interact to form heterodimers which are transported to the cell surface via the Golgi apparatus (**Figure 1.2**) (Ziemiecki et al., 1980, Green et al., 1981, de Curtis and Simons, 1988, Sariola et al., 1995). In some alphaviruses, such as SFV, E3 interacts with the viral spikes; however, in others, like SINV, it remains in the cell cytoplasm (Strauss and Strauss, 1994). The nucleocapsid, formed of the C protein and viral genome, associates with the cytoplasmic tail of the E2 glycoprotein which stimulates budding (Vaux et al., 1988, Metsikko and Garoff, 1990, Strauss and Strauss, 1994, Owen and Kuhn, 1997). Release by budding allows the new viral progeny to acquire a membrane derived from the host cell plasma membrane (Acheson and Tamm, 1967, Laine et al., 1973, Vogel et al., 1986, Fuller, 1987).

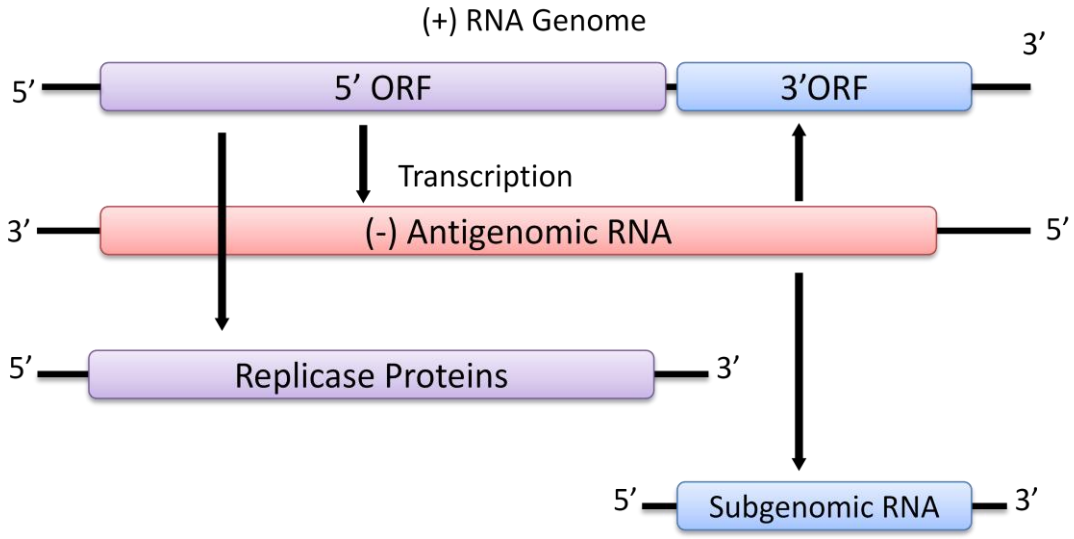

Figure 1.4: Replication of an alphavirus genome. The 5' Open Reading Frame (ORF) encodes the non-structural polyprotein and is translated directly from the positive sense genomic RNA strand. The complete viral genome is transcribed into a full-length, negative sense RNA copy, termed the antigenome which is used to generate further copies of the positive sense genome, as well as producing subgenomic RNA. The structural genes encoded in the 3' ORF of the genome are translated directly from the subgenomic mRNA under the control of an internal subgenomic promoter located in the antigenome.

1.1.2.4.4 Semliki Forest Virus (SFV) as a Model Alphavirus

SFV originates from sub-Saharan Africa and is transmitted by *Aedes* spp. mosquitoes (Fazakerley, 2002). It was first isolated in 1942 in female *Ae. abnormalis* from Semliki Forest in Uganda (Smithburn and Haddow, 1944). Infected patients in the Central African Republic exhibited mild febrile illnesses with symptoms such as fever, myalgia, arthralgia and severe, persistent headaches (Mathiot et al., 1990). Despite being detected in humans, equines, small mammals and primates, the natural host of SFV has yet to be determined.

Along with SINV, SFV is one of the most commonly studied viruses used for alphavirus research for a number of reasons. Firstly, its genome is manipulated easily which allows mutations or foreign reporter genes, such as those encoding fluorescent proteins or luciferase, to be inserted. SFV can also be used as a vector for the delivery of genes for vaccine and therapeutic treatments (Atkins et al., 1996, Atkins et al., 1999, Hoffmann et al., 2001, Lundstrom, 2003, Lundstrom and Ehrengruber, 2003). These genes can be inserted into a number of regions within the genome as required, with varying degrees of expression and stability. For instance, insertion within the replicase acts as an indirect marker of replication, whereas if inserted before, into or after the structural ORF, the

protein will be expressed by the viral progeny (Fragkoudis et al., 2007, Tamberg et al., 2007, Kiiver et al., 2008, Rodriguez-Andres et al., 2012). If required, the subgenomic promoter can also be duplicated to allow a greater quantity of foreign genes to be expressed (Tamberg et al., 2007). Alternatively the structural genes can be removed entirely and replaced with the foreign gene of interest resulting in the production of a replicon. These can be packed into virus replicon particles (VRPs) which have an increased level of biosafety (Liljestrom and Garoff, 1991, Berglund et al., 1993, Smerdou and Liljestrom, 1999). Several examples of reporter strains of SFV and SFV VRPs are used throughout this thesis and are described in **Table 2.4** and **Figure 2.1**. The use of SFV VRPs is also discussed in detail in **Chapter 5**.

Secondly, a number of SFV strains have been produced for laboratory use including the molecular clone of the prototype strain, SFV4. These are either virulent or avirulent in adult mice (Seamer et al., 1967, Bradish et al., 1971, Pusztai et al., 1971, Atkins, 1983, Fazakerley, 2002, Fazakerley, 2004) and these have been used to successfully infect multiple animal models in addition to mice, such as rats, rabbits, guinea pigs and voles, as well as several vertebrate and invertebrate cell lines (Seamer et al., 1967, Bradish et al., 1971, Peleg, 1971, Leake et al., 1980, Atkins et al., 1990). However, none of these strains are considered to result in disease following human infection, with the exception of one fatal case of an immunocompromised scientist who died of encephalitis in 1978 from a strain that has since been removed from use (Willems et al., 1979). As SFV is closely related to CHIKV but lacks the same level of pathogenicity, it can be used as a safer alternative to accurately model its biology and infectivity. Moreover, its rapid replication cycle and easy propagation further add to its appeal for use in arboviral studies.

SFV infections of vertebrate and invertebrate cells differ substantially. Generally, infected mammalian cells exhibit a rapid cytotoxity or cytopathic effect (CPE); although this is not observed in mosquito or tick cells (Peleg, 1968, Leake et al., 1980, Brown, 1984). Infection in mosquito cells is characterised by an initial peak of virus production subsequently followed by a steady decrease into a low level, persistent infection (Fragkoudis et al., 2008). SFV has been previously proven to be an excellent model for studying the innate immune responses in mosquitoes (Fragkoudis et al., 2008, Rodriguez-Andres et al., 2012) and is used exclusively for the analysis of mosquito antiviral RNAi responses in this thesis.

1.2 Mosquito Vectors

1.2.1 Important Vectors of Disease Transmission

Mosquitoes belong to the family *Culicidae* in the order *Diptera*. The *Culicidae* is divided into two subfamilies which contain over 3,500 species. As the work described as part of this thesis focuses on *Ae. aegypti* and *Ae. albopictus* derived cell lines only these two species shall be discussed.

Aedes along with *Culex* spp. are among the small number of animals that are distributed globally. The yellow fever mosquito or *Aedes* (*Stegomyia*) *aegypti* (**Figure 1.5A**) originated in Africa and is involved in the transmission of prominent arboviruses to humans, particularly DENV and YFV. Human transport, mainly the slave trade, is believed to be the cause of its dispersal out of Africa and it now inhabits many temperate, subtropical and tropical regions across the planet (Tabachnick, 1991, Soumahoro et al., 2010, Powell and Tabachnick, 2013). It is well known to be highly anthropogenic (i.e. feeding on humans) and has adapted to human-inhabited areas to become highly domesticated, laying its eggs in artificial containers. Its propensity for feeding on humans and rarely flying beyond 100 meters enhances its ability to maintain urban cycles of arboviruses (Powell and Tabachnick, 2013). As a result of this capacity to maintain epidemics of DENV and YFV around urbanised areas, many control measures have focused on the eradication of this species. Mosquito control measures are of particular importance given the lack of vaccines against many arboviral infections. Eradication campaigns saw the complete removal of *Ae. aegypti* from the Americas (Soper, 1967, Kouri et al., 1989) and control strategies in southern Asia greatly reduced the incidence of disease (Gratz, 1967, Lofgren et al., 1970, Pant et al., 1971, Bang and Pant, 1972, Ooi et al., 2006). Unfortunately, these programmes were unsustainable and since they ended *Ae. aegypti* have returned to these countries (Gubler, 1998a) and over 50 % of homes in many endemic areas are found to harbour their larvae (Nathan and Knudsen, 1991, WHO, 2000). In addition, *Ae. aegypti* has largely developed resistance to multiple insecticides making alternative control strategies necessary (Hemingway et al., 2004).

Aedes (*Stegomyia*) *albopictus* (**Figure 1.5B**), commonly known as the Asian tiger mosquito (Skuse, 1894), is a highly invasive species that has spread outside its origins in the forests of Southeast Asia (Smith, 1956) and is now found additionally in Africa (Savage et al., 1992, Fontenille and Toto, 2001, Coffinet et al., 2007), the Americas (Forattini, 1986, Hawley et al., 1987, Nawrocki and Hawley, 1987, O'Meara et al., 1995,

Moore, 1999, Braks et al., 2003), Europe (Dalla Pozza and Majori, 1992, Knudsen et al., 1996, Adhami and Reiter, 1998, Romi et al., 1999, Schaffner and Karch, 2000, Schaffner et al., 2004, Aranda et al., 2006, Klobucar et al., 2006, Romi et al., 2006, Roiz et al., 2008, Lambrechts et al., 2010, Medlock et al., 2012, Werner et al., 2012) and several locations in the Indian and Pacific Oceans (Elliott, 1980, Laille et al., 1990, Delatte et al., 2008, Bagny et al., 2009, Guillaumot et al., 2012). *Ae. albopictus* has been shown to be more adaptable to temperate climates than *Ae. aegypti* and is found to survive in much more northern regions, where their eggs enter dormancy to survive low temperatures (Nawrocki and Hawley, 1987, Hanson and Craig, 1994, Tran et al., 2013, Capinha et al., 2014, Liu-Helmersson et al., 2014). Its rapid and widespread dispersal is mainly attributed to the international trade of used tires and bamboo harbouring dormant eggs in trapped rain water (Reiter, 1998, Linthicum, 2001, Madon et al., 2002, Benedict et al., 2007). *Ae. albopictus* also demonstrates a high degree of ecological plasticity and is likely to have adapted from being zoophilic (i.e. feeding on wild animals) in its original forest habitat to anthropogenic in human-dominated habitats (rural, urban and suburban areas) through the process of 'domestication' (Paupy et al., 2009). Although it was initially suggested to be a 'bridge vector' and be involved in the transmission of emerging viruses between animals and humans, it is now understood to be a significant vector in its own right (Moore and Mitchell, 1997, Gratz, 2004, Paupy et al., 2009).

As these species continue to spread, they are subjected to interspecies competition which may result in the displacement of the native species. This has implications for the incidence of disease in that area through the disruption of the vector: virus system (Chevillon et al., 2008). *Ae. aegypti* and *Ae. albopictus* are major public health problems, most notably as vectors for both DENV and CHIKV (Gratz, 2004, Manore et al., 2014). Although DENV is primarily transmitted by *Ae. aegypti*, which is present in most areas were DENV is endemic, *Ae. albopictus* is an important secondary vector and is responsible for DENV epidemics in Japan in 1942-1945 (Hotta, 1998, Kuno, 2007) and for outbreaks in Hawaii in 2001-2002 (Effler et al., 2005) and La Reunion and other Indian Ocean islands in 1977-1978 (Coulanges et al., 1979, Michault, 1998, Paupy et al., 2001). Similarly, it was responsible for outbreaks of CHIKV in Central Africa in 2007 (Peyrefitte et al., 2007, Leroy et al., 2009, Paupy et al., 2010). The emergence of CHIKV in 2005-2006 on La Reunion produced clinical cases in almost 40 % of the population (Enserink, 2007b). This epidemic was unusual as the virus was shown to have undergone a point-mutation which altered a single amino acid (A226V) in the E1 glycoprotein (**Page xix**). This facilitated a selective advantage and a greater degree of infectivity for *Ae. albopictus*

over the more common *Ae. aegypti*, making it the sole vector during the epidemic (Reiter et al., 2006, Tsetsarkin et al., 2007, Vazeille et al., 2007, de Lamballerie et al., 2008, Arias-Goeta et al., 2013, Arias-Goeta et al., 2014). Following this mutation dissemination rates were shown to be enhanced from 7 - 15 days to 2 - 6 days and transmission was also improved as viral concentrations were one hundred-fold greater in the salivary glands. The geographical range of the virus could thus be expanded across the Indian Ocean to other areas inhabited by *Ae. albopictus* (Paupy et al., 2012). The first outbreak of CHIKV in Europe occurred in Italy in 2007 and is understood to have been imported from a vireamic individual returning from India and introducing the virus to local *Ae. albopictus* mosquitoes (Enserink, 2007a, Rezza et al., 2007, Angelini et al., 2008). *Ae. albopictus* have also been reported to play a prominent role in the spread of CHIKV and DENV in southern France in 2010 (Grandadam et al., 2011, Vega-Rua et al., 2013), including autochthonous cases of both infections in France (Gould et al., 2010, Grandadam et al., 2011) and emerging cases in Central Africa (Paupy et al., 2010).

Changes in vector distribution and the dominant local vector populations are likely to affect the characteristics of arbovirus emergence. Therefore, there is a growing need to discern the factors involved in the transmission and spread of these important pathogens and develop effective methods of control and prevention.

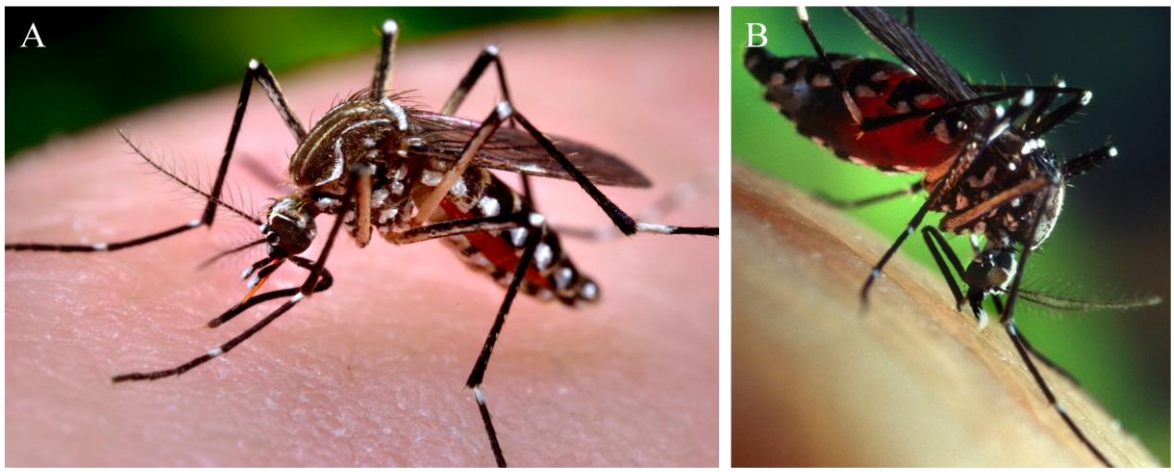

Figure 1.5: Adult female *Ae. aegypti* (A) and *Ae. albopictus* (B) (taken from www.vectorbase.org)

1.2.2 Mosquito Cell Lines

The use of mosquito-derived cell lines has been instrumental in uncovering some of the basic molecular mechanisms involved in understanding arbovirus: mosquito interactions.

To date, over 20 mosquito-derived cell lines have been generated from 13 species of mosquito and are successfully used as an efficient alternative to *in vivo* models for immunity studies (Walker et al., 2014). In contrast to work with whole mosquitoes, *in vitro* models provide a cheaper, safe and convenient system in which to test virus:vector relationships. In some cases they have proved to be more sensitive and reproducible than *in vivo* studies. The cell lines used as part of this work are described in **Table 2.1** and below in **Table 1.3**.

Table 1.3: *Aedes* spp. mosquito cell lines used during this project.

Cell type	Species	Source	Exogenous RNAi activity?	References
Aag2	*Aedes aegypti*	Embryos	Yes	(Peleg, 1968, Lan and Fallon, 1990, Sanchez-Vargas et al., 2009, Siu et al., 2011, Barletta et al., 2012)
U4.4	*Aedes albopictus*	Larvae	Yes	(Sarver and Stollar, 1977, Igarashi, 1978, Condreay and Brown, 1986, Fragkoudis et al., 2008, Attarzadeh-Yazdi et al., 2009, Brackney et al., 2010, Fallon and Gerenday, 2010)
C6/36	*Aedes albopictus*	Larvae	No	
C7-10	*Aedes albopictus*	Larvae	No	

Each of these cell lines were first characterised by Singh in the 1960s (Singh, 1967) and have been used extensively for arboviral research with a number of different viruses. The *Ae. albopictus* cell lines are subclones from the original Singh cultures. Due to the fact that they have arisen from homogenised larvae, the specific tissues from which they derive are unknown. The *Ae. aegypti*-derived Aag2 cell line is non-clonal and is expected to be an amalgamation of the various embryonic tissues.

Both the Aag2 and the U4.4 cell lines have been proven to be immunocompetent and have a functional antiviral response (Riedel and Brown, 1979, Condreay and Brown, 1986, Condreay and Brown, 1988, Miller and Brown, 1992, Attarzadeh-Yazdi et al., 2009, Sanchez-Vargas et al., 2009, Scott et al., 2010, Siu et al., 2011, Barletta et al., 2012, Morazzani et al., 2012, Vodovar et al., 2012, Leger et al., 2013, Schnettler et al., 2013a, McFarlane et al., 2014). They are therefore considered to be an accurate representation for

antiviral responses in their respective species. On the other hand, both the C6/36 and the C7-10 cell lines are described as having a defective RNAi pathway due to a truncation of the Dicer-2 (Dcr-2) protein, a property which is discussed further in **Chapter 5** (Brackney et al., 2010, Scott et al., 2010, Morazzani et al., 2012).

1.2.3 Vector Competence: Intrinsic Factors

The ability of arboviruses to replicate within the mosquito is governed by a suite of factors which alter the insect's competence or capacity to transmit arthropod-borne viruses. These determinants include both genetic and environmental influences and the complexity of the interplay between them is only just beginning to be unravelled.

Following the ingestion of a vireamic blood meal by an immune competent mosquito, the virus is passed to the midgut where the blood meal is digested. The virus must then traverse into the lining epithelial cells and replicate (Myles et al., 2004, Khoo et al., 2010). Once it reaches sufficient titres, the infection disseminates to secondary tissues and organs such as nerve tissue, fat bodies, haemocytes, malphighian tubules and the muscles surrounding the alimentary tract (Beerntsen et al., 2000, Black et al., 2002). When it finally reaches the salivary glands several days later the virus can be transmitted to susceptible vertebrates by the mosquito when it takes its next blood meal. The time interval between ingestion of the virus and its transmission is known as the extrinsic incubation period (EIP) and in order to complete it the virus must first overcome a number of restriction barriers (**Figure 1.6**).

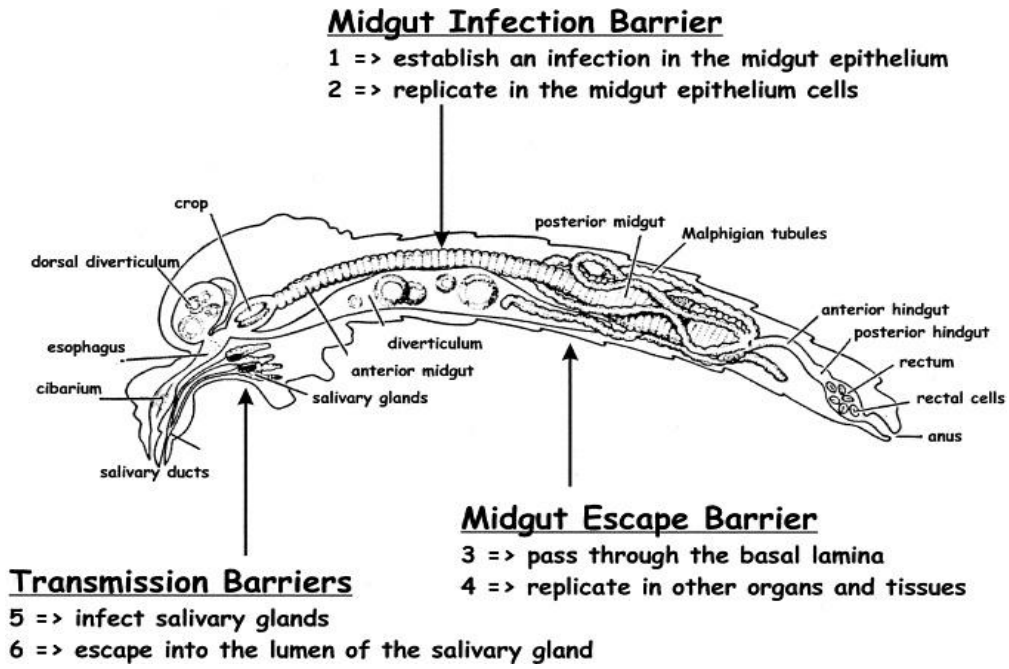

Figure 1.6: Schematic representation of virus transmission barriers in mosquitoes. Taken from Black et al., 2002.

Several alphaviruses and flaviviruses are known to be affected by the midgut escape barrier (MEB) and the midgut infection barrier (MIB) (Hardy et al., 1983, Mellor, 2000, Khoo et al., 2010, Arias-Goeta et al., 2013). Firstly, the MIB impedes virus access to the mesenteronal cells and prevents replication while the MEB restricts the movement of virus particles from the midgut to surrounding tissues in the haemocoel (Hillyer, 2010). The success of these barriers has been shown to be dose-dependent as the virus must pass the required threshold in order to disseminate and high virus titres are known to overcome the MIB (Kramer et al., 1981, Weaver et al., 1984, Miller and Mitchell, 1991, Bosio et al., 1998, Seabaugh et al., 1998, Myles et al., 2004, Mahmood et al., 2006, Pierro et al., 2007). There is therefore an inverse correlation between the infecting dose and EIP. The initial viraemia present in the blood meal varies depending on the vertebrate and the virus; however, it can be around 10^7 PFU/ml of blood (Weaver, 1997).

How the virus is able to evade the midgut barrier is largely unknown but it is understood that the virus population is subjected to two bottlenecks; the first in the midgut and the second upon reaching the salivary glands (Forrester et al., 2012a). Other digestive, intracellular and immunological factors influencing vector competence and pathogen infectivity are innate immune pathways, up-regulation of proteolytic enzymes, the formation of the peritrophic matrix and an influx of antimicrobial molecules (Shahabuddin et al., 1996, Brackney et al., 2008, Kato et al., 2008, Khoo et al., 2010). This is in addition

to the natural microflora present in the midgut and the physical barrier of the midgut lining (Lindh et al., 2008). Further barriers then await the virus progeny that makes it to the salivary glands as they are subjected to the salivary gland infection barrier (SIB) and the salivary gland escape barrier (SEB) which also affects transmission (Sriurairatna and Bhamarapravati, 1977, Takahashi and Suzuki, 1979, Paulson et al., 1989). If successful the virus will replicate in the salivary glands to higher titres than in any other tissue in preparation for exportation in the saliva (Valenzuela et al., 2002, Arca et al., 2007). In relation to the differences between *Ae. aegypti* and *Ae. albopictus* to transmit either DENV or CHIKV, studies have indicated that both share a similar level of competency in specific regions (with the notable exception of the La Reunion outbreak of CHIKV discussed in **Chapter 1.2.1**) (Paupy et al., 2010, Richards et al., 2012). In addition, meta-analysis of the susceptibility of both species suggests that *Ae. albopictus* is more disposed to midgut infections compared to *Ae. aegypti*, whereas viral infections in *Ae. aegypti* are better able to disseminate which may aid their onward transmission (Lambrechts et al., 2010).

There is a substantial degree of variation between vector species and their competency for arboviruses and several phenotypic variations have been documented both inter- and intra-specifically (Gubler and Rosen, 1976, Hardy et al., 1976, Tesh et al., 1976, Hardy et al., 1978, Gubler et al., 1979, Tabachnick et al., 1985, Boromisa et al., 1987, Tardieux et al., 1990, Armstrong and Rico-Hesse, 2001, Paupy et al., 2001, Bennett et al., 2002, Paupy et al., 2003, van den Hurk et al., 2010). Specific MEB, MIB, SEB and SIB have been observed which are refined for specific pathogens and influence an individual's competency (Reisen et al., 1996, Dohm et al., 2002, Mahmood et al., 2006, Alto et al., 2008, Behura and Severson, 2012, Richards et al., 2012). The genetic make-up of both virus and vector are important factors in determining the relationship formed between them and variations within the phenotype of arboviral isolates/genotypes to circumvent barriers and disseminate have been well documented (Reisen et al., 1996, Dohm et al., 2002, Mahmood et al., 2006, Alto et al., 2008, Lambrechts et al., 2009, Fansiri et al., 2013, Lambrechts et al., 2013). The mechanisms which prejudice these differences between populations of the same species are vast and largely undefined. However, it is likely that the genetics of the indiviual has a considerable influence in tandem with environmental factors (Tabachnick, 2013). Implementing genomic analysis, transcriptomic and proteomic studies is expected to identify candidate genes which influence vector competency and may benefit future arboviral control studies (Bosio et al., 1998, Bosio et al., 2000, Bennett et al., 2005b, Bennett et al., 2005a, Chen et al., 2008, Girard et al., 2010, Tchankouo-

Nguetcheu et al., 2010, Behura et al., 2011, Colpitts et al., 2011, Bonizzoni et al., 2012, Campbell et al., 2014, Esquivel et al., 2014).

1.2.4 Vector Competence: Extrinsic Factors

Arboviral infections are dynamic and are constantly expanding their geographical boundaries. This has been largely facilitated by the increase of vector ranges as areas that were once free of these significant pests have now seen an influx of these often invasive species (Gratz, 2004, Benedict et al., 2007, Enserink, 2007a, Paupy et al., 2009, Medlock et al., 2012). Human travel and transport patterns have substantially changed resulting in an enhanced level of international traffic, most crucially from viral endemic to naive areas.

Temperature is considered to be one of the most prominent factors affecting vector permissiveness and it is well known that the earth is warming. A slight rise in temperature allows vector populations to migrate and establish new colonies in immunologically naive, more northern regions, potentially bringing disease with them (Githeko et al., 2000). Indeed, climatic influences and virus outbreaks are so closely linked, analysis of weather patterns allows disease incidences to be predicted, for instance with RVFV (Davies et al., 1985, Linthicum et al., 1999). Alterations in weather patterns are also likely to lengthen transmission seasons, increase breeding grounds and influence vector activity further enhancing the threat of disease spread. A positive correlation between temperature and infection rates in mosquitoes exists – mainly the lower the temperature the longer it takes for the virus to replicate to high enough titres to permit transmission, whereas at higher temperatures the virus is able to replicate proficiently and the extrinsic incubation time may decrease in some cases (Watts et al., 1987, Reisen et al., 1993, Turell, 1993, Brubaker and Turell, 1998, Thu et al., 1998, Dohm et al., 2002, Reisen et al., 2006, Rohani et al., 2009, Lambrechts et al., 2011, Carrington et al., 2013b). There are upper and lower temperature limits which vary depending on the species involved. If the temperature exceeds the upper limit, the mosquitoes lifespan is reduced and they demonstrate a decrease in flight activity (Carrington et al., 2013a). Extremes of temperatures are also deleterious to virus replication and when temperatures are too high, virus replication will accelerate too rapidly, whereas if they are too low the infection will remain dormant (Hurlbut, 1973). Extrinsic temperature variations may also influence the vectors capacity to modulate infections. Only recently has variation in vector competence over a range of temperatures been investigated to reflect more natural conditions (Lambrechts et al., 2011, Xiao et al., 2014).

Larval nutrition and population density are also believed to affect vector competency of the adult insects. Studies on *Culex* and *Aedes* spp. demonstrated that larvae fed on poorer quality diets were more likely to generate higher viral titres (Takahashi, 1976, Baqar et al., 1980, Grimstad and Haramis, 1984, Grimstad and Walker, 1991). In addition, larval density can impact at both the individual and population level affecting development, growth, fecundity and survival into adulthood (Ho et al., 1989, Juliano, 1998, Reiskind and Lounibos, 2009). It is also believed to affect infectivity as female *Ae. aegypti* subjected to a high-density environment at the larvae stage demonstrated a higher susceptibility to SINV than those from low density environments (Muturi et al., 2011b). Just as temperature impacts upon the adults so too does it affect the physiology of mosquito larval stages (Mourya et al., 2004, Muturi and Alto, 2011) and alters the extrinsic incubation time (Kay et al., 1989, Kay and Jennings, 2002). Exposure to insecticides at both the larval and adult stages has also been shown to be important (Mourya et al., 2004, Yadav et al., 2005, Muturi and Alto, 2011, Muturi et al., 2011a). Combined, these factors may intensify adult insect susceptibility to some infections (Takahashi, 1980, Alto et al., 2005, Alto et al., 2008, Bevins, 2008, Westbrook et al., 2010, Muturi and Alto, 2011, Muturi et al., 2011b, Muturi et al., 2012).

Another aspect which is currently lacking understanding is host seeking behaviour. A fundamental part of virus transmission is that it is ultimately passed on to a suitable naive host completing the virus life cycle. It is known that mated females exhibit increased host seeking behaviour over unmated females due to the need to develop eggs (Macdonald, 1956, Jones, 1981, Rossignol et al., 1985, Lima-Camara et al., 2014). Similarly, some pathogens are able to manipulate the biology and behaviour of their vector in order to infect a host (Werren et al., 2008, Qualls et al., 2011). In addition to CO_2 production and certain chemicals released in the breath, mosquitoes are known to be attracted to body temperature. It may be hypothesised that uninfected mosquitoes are attracted to a high body temperature caused by a fever as a result of a virus infection while an infected insect may be manipulated to be more attracted to people with a lower body temperature (i.e. those which are not infected). Infected mosquitoes may also be lead to feed more frequently thus increasing the number of hosts infected, a trait which is observed in LACV infected *Ae. triseriatus* females (Jackson et al., 2012). Further studies are required to determine if this can be extrapolated to other serious arboviruses such as DENV or CHIKV.

The findings described come together to confirm that there are a plethora of influences on mosquito competency which make natural infections almost impossible to accurately replicate artificially in laboratory conditions. The complexity of factors, both intrinsic and extrinsic, interact in non-linear ways and different populations respond differently. As such great care must be taken when interpreting competency observations.

1.3 Insect Innate Immunity and Immune Signalling Pathways

1.3.1 Overview of the Classical Innate Immune Pathways

We are only starting to understand the interplay between arboviruses and arthropod immunity and how this interaction influences vector competence and virus transmissibility. Our understanding of the mechanisms and processes involved in mediating viral infections in the vector is substantially reduced compared to what is known to occur during a host infection. The immune system of vertebrates can be divided into two branches: innate and adaptive. However, the adaptive response is lacking from arthropods which rely solely on their innate immune responses to control bacterial, fungal, protozoa and viral infections. Several pathogen recognition signalling pathways have been identified in *Drosophila*, many of which are highly conserved across both vertebrates and invertebrates; including mosquitoes.

The first step of any signalling pathway is the recognition of the pathogen via pattern recognition receptors (PRRs) which identify the alien entity by its pathogen associated molecular patterns (PAMPs). PAMPs are conserved components of different infectious agents such as: viral nucleic acids or debris; bacterial cell wall components, such as peptidoglycan and lipopolysaccharides (Michel et al., 2001, Ramet et al., 2003); and fungal complex carbohydrates in the cell wall including beta-glucans and possibly chitin (Sorrell and Chen, 2009). Vertebrates are known to encode distinct classes of PRRs such as those that act on the cellular membrane (including Toll-like receptors [TLRs] and C-type lectin receptors [CLRs]) or cytoplasmic receptors (including Retinoic acid-inducible gene (RIG)-I like receptors [RLRs] and NOD-like receptors [NLRs]) (Takeuchi and Akira, 2010). Following pathogen recognition, the host signalling pathway relays the information to stimulate an effector response through the transcriptional activation of certain genes. In mammals these genes mostly constitute those which are interferon-dependent and encode for molecules such as type 1 interferons (IFNs), chemokines, pro-inflammatory cytokines and antimicrobial peptides (AMPs). AMPs, such as defensins, attacin, drosocin, drosomycin and diptericin, are produced in the fat body, which functions as the equivalent to the mammalian liver, and are secreted into the haemolymph in high concentrations to facilitate immunomodulation against the invading pathogen (Hoffmann and Reichhart, 2002). Genetic studies indicate that insects do not encode an interferon response; however, they do encode PRRs homologous to TLRs. The Toll, immunodeficiency (IMD) and the Janus kinase/signal transducer activator of transcription (JAK/STAT) are canonical and

share similarities with their counterparts in mammals. The Toll and IMD pathways are homologues to the nuclear factor-κB (NF-κB) transcription factor found in mammals, whereas JAK/STAT is an important part of the mammalian IFN response. Each of the three pathways are known to be involved in development and immune responses in *Drosophila* where they have been intensively studied. Less work has been performed to establish their action in mosquitoes and other arthropods and many details are still to be ascertained. However, several reviews have also been published recently which discuss what is currently known about their roles in insect immunity and antiviral defence (Fragkoudis et al., 2009, Kingsolver et al., 2013, Merkling and van Rij, 2013, Rueckert et al., 2014, Xu and Cherry, 2014). These signal transduction pathways have been shown to be affected by virus infections in mosquitoes and other invertebrates and a number of interesting differences between the pathways, and their roles in other invertebrates, have emerged (Lin et al., 2004, Dostert et al., 2005, Waterhouse et al., 2007). It is widely believed that their involvement in antiviral defence against arboviruses is highly complex and may be vector and virus specific.

Due to the lack of data generated in mosquitoes, the mechanisms of the signalling pathways described are based upon what is understood from *Drosophila*. Any distinctions, where known, are indicated.

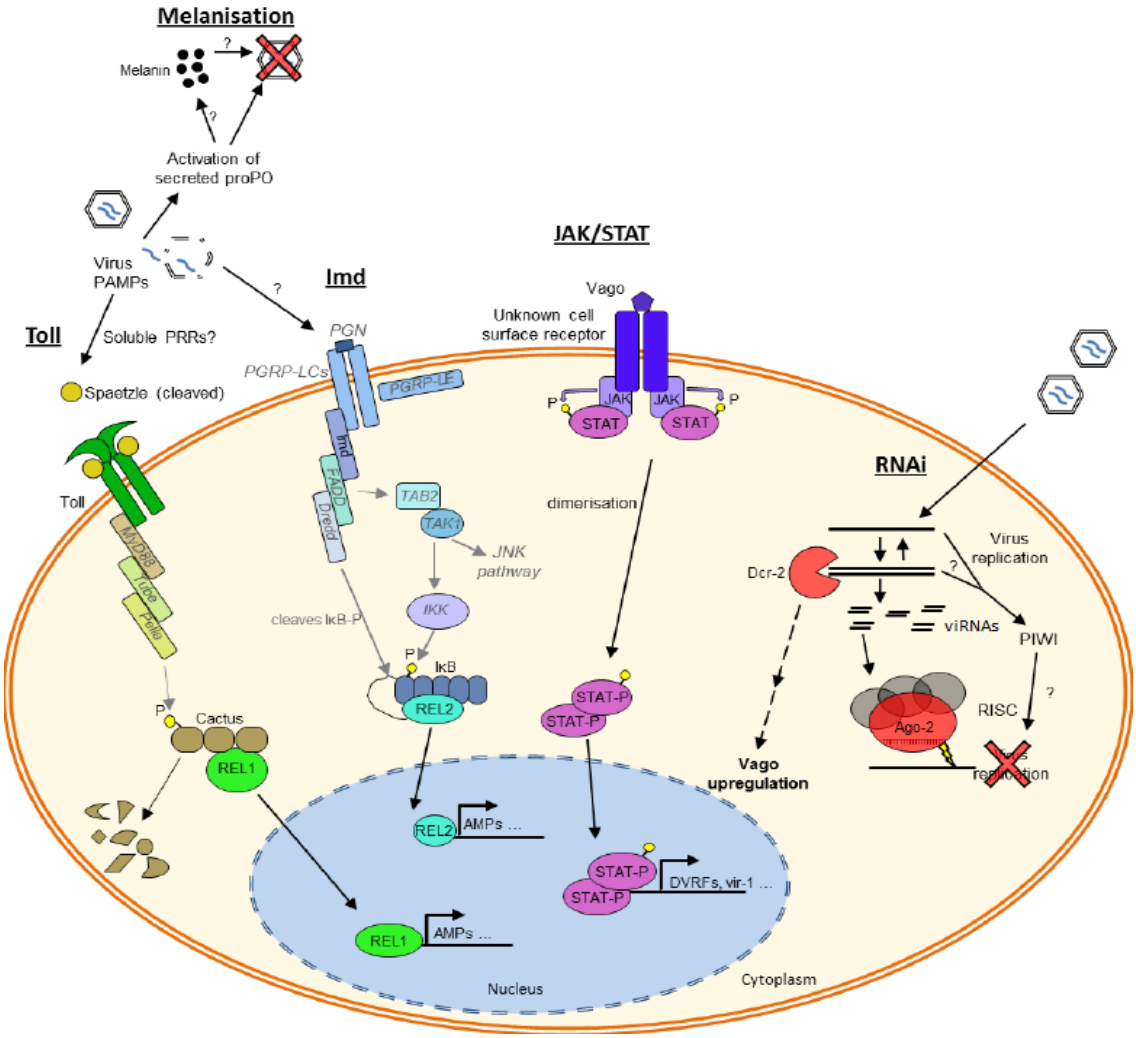

Figure 1.7: Model of innate immune signalling pathways in mosquitoes extrapolated from what is known in *Drosophila*. Toll pathway: The detection of PAMPs initiate the proteolytic signalling cascade resulting in the cleavage of the precursor form of Spätzle, a dimeric cytokine. Active spätzle binds to the toll receptor, induces dimerisation and triggers the recruitment of downstream intracellular proteins. This leads to the phosphorylation of the negative regulator cactus signalling for its degradation. Following this, Relish 1 (REL1) is able to translocate to the nucleus and activates the transcription of effector genes. **IMD pathway**: Detection of diaminopimelic (DAP)- type peptidoglycan from Gram negative bacteria by peptidoglycan recognition proteins (PGRP)-like receptors triggers the IMD pathway and leads to the cleavage of REL2 which translocates to the nucleus and initiates immune gene transcription. **JAK/STAT pathway**: The cytokine Vago binds to an unknown receptor activating the Janus kinase (JAK) which in turn phosphorylates signal transducers and activators of transcription (STAT). STAT is then able to dimerise and translocate to the nucleus and transcribes target genes. **Exogenous RNAi**: Viral-derived dsRNA is recognised by Dicer-2 (Dcr-2) which cleaves it into 21 nt viral derived small RNAs (viRNAs) which are transported to the RNA induced silencing complex (RISC). Argonaute-2 (Ago-2) uses one strand of the viRNA to cleave sequence specific mRNA. Vago production is upregulated following virus infections and is mediated by Dcr-2. Adapted with permission from Rückert et al., 2014.

1.3.2 The Toll Pathway

The most ancient and conserved innate immune response in both animals and plants is thought to be the Toll pathway and associated signalling via NF-κB related proteins. The Toll receptor gives its name to TLRs in mammals as they were initially discovered in *Drosophila* (Lemaitre et al., 1996, Fitzgerald and Chen, 2006, Lemaitre and Hoffmann, 2007). *Drosophila* are known to encode nine Toll receptors; however, unlike mammalian TLRs, arthropod Toll receptors do not act as PRR and are not activated directly through PAMP interactions. Instead they are indirectly stimulated by an endogenous cytokine known as Spätzle (Spz) (**Figure 1.7**). This extracellular ligand is cleaved into its active form following the detection of a pathogen and interacts with the Toll receptor causing the cross linking of two Toll ectodomains (Arnot et al., 2010). This triggers the intracellular association of a receptor-adaptor complex composed of the myeloid differentiation primary-response gene 88 (MyD88), which interacts with the Toll receptor through its Toll/Interleukin-1 receptor domains (Tauszig-Delamasure et al., 2002) and recruits Tube and the protein kinase Pelle, a homologue of IL-1R-associated kinase (IRAK) (Towb et al., 2001). As a result, the downstream IκB orthologue Cactus becomes phosphorylated which targets it for degradation by the proteasome. The NF-κB orthologues Relish 1 (REL1), Dorsal and Dorsal-related immunity factor (Dif) then become activated and translocate to the nucleus causing the transcription of AMPs to be initiated (Lemaitre et al., 1996, Shin et al., 2005, Ferrandon et al., 2007, Lemaitre and Hoffmann, 2007).

Important vector mosquito species have been shown to encode orthologues to a number of *Drosophila* NF-κB proteins. Both *Anopheles gambiae* and *Ae. aegypti* express orthologues to Dorsal, Rel1 and Rel1A/Rel1B respectively, as well as Relish orthologue, Rel2 (Christophides et al., 2002, Shin et al., 2002, Waterhouse et al., 2007). These are known to be involved in innate immunity in these mosquito species and therefore; although, the effector genes involved may be slightly different, the signalling pathways operate in a similar fashion.

The Toll pathway is historically triggered by Gram-positive bacteria and fungi (Lemaitre et al., 1996, Rutschmann et al., 2002, Ferrandon et al., 2007, Kemp and Imler, 2009). In addition to this, recent evidence has been accumulating to indicate that it is also involved in protection against some viral infections in insects; although, our understanding of the processes and mechanisms involved are not yet well established. The Toll pathway has been shown to be activated following Drosophila X virus (DXV) infections in *Drosophila* and is involved in the antiviral response against DENV infection of *Ae. aegypti* mosquitoes (Zambon et al., 2005, Xi et al., 2008, Ramirez and Dimopoulos, 2010). Despite this,

transcriptional induction of NF-κB dependent genes is not observed following infection and is impaired following bacterial stimulation (Sim and Dimopoulos, 2010). This suggests that its antiviral mechanism functions differently from the classical control exhibited over other invading microbes. It also implies that DENV actively restricts rather than evades the Toll immune response in mosquitoes. Certainly, autophagy was stimulated following the detection of vesicular stomatitis virus (VSV) in *Drosophila* which is independent of NF-κB (Shelly et al., 2009, Nakamoto et al., 2012). However, DENV has also been shown to cause an up-regulation of immune genes, including Toll pathway components Toll and Cactus, following infection of the *Ae. aegypti*-derived Aag2 cell line, further highlighting its role in mediating DENV replication (Sim and Dimopoulos, 2010).

On the other hand, studies on alphaviruses have indicated that they are not mediated by the Toll pathway. Replication of SFV was not inhibited by the expression of a constitutively expressed active Toll receptor in the *Ae. albopictus*-derived U4.4 cell line and signalling was found to be reduced (Fragkoudis et al., 2008). Similarly, prior stimulation of Toll before CHIKV infection of the *Ae. aegypti*-derived Aag2 cell line failed to act antivirally but was actively repressed by virus-mediated host cell shut off (McFarlane et al., 2014). SINV is also hypothesised to inhibit Toll signalling following an initial activation stage in *in vivo Ae. aegypti* systems (Sanders et al., 2005). Furthermore, ONNV infection of *An. gambiae* did not show an up-regulation of Toll; although, other immune genes were stimulated and acted antivirally against the infection (Waldock et al., 2012). Interestingly, silencing of Cactus, a negative regulator of Toll, permitted an increase in viral replication compared to controls, although the opposite would have been expected if Toll was involved in viral repression. These findings may be because Toll does not act antivirally against alphavirus infections or may be as a result of viral inhibition of any antiviral activity overriding these effects and future work is required to unravel these complexities.

1.3.3 The IMD Pathway

The second NF-κB-related pathway is the IMD pathway which is essential for the activation of the NF-κB orthologue Relish 2 (REL2). IMD signalling is stimulated by diaminopimelic (DAP)- containing peptidoglycan on the cell wall of Gram negative bacteria in addition to some Gram positive Bacilli spp. (Kaneko et al., 2004). Pathogen recognition is mediated by two receptors: the external peptidoglycan recognition protein (PGRP)-LC and the internal PGRP-LE protein (Takehana et al., 2002, Choe et al., 2005, Kaneko et al., 2006) (**Figure 1.7**). This is subsequently followed by the activation of IMD

and the initiation of the two branches of the signalling pathway which act synchronously (Choe et al., 2002, Gottar et al., 2002, Ramet et al., 2002). The first branch involves IMD binding to the *Drosophila* FAS-associated death-domain protein (dFADD) which in turn recruits the caspase DREDD, a homologue of caspase-8 in mammals (Leulier et al., 2000). DREDD cleaves IMD allowing it to interact with the *Drosophila* inhibitor of apoptosis-2 (IAP-2) resulting in the ubiquitination of IMD (Paquette et al., 2010). IMD is then able to act as a scaffold for association with downstream components. The second pathway involves D*rosophila* transforming growth factor activated kinase-1 (dTAK1) activating the IκB-kinase (IKK) for the phosphorylation of REL2 (Erturk-Hasdemir et al., 2009). Following phosphorylation REL2 is cleaved by DREDD separating the N-terminal DNA binding domain which translocates to the nucleus and regulates effector gene transcription (Leulier et al., 2000, Silverman et al., 2000, Leulier et al., 2002, Naitza et al., 2002, Stoven et al., 2003, Erturk-Hasdemir et al., 2009).

As with the Toll pathway, IMD signalling is involved in antiviral immunity against both arboviruses and pathogenic viruses. In *Drosophila*, IMD mutants showed an increase in viral RNA and lower survival rates following cricket paralysis virus (CrPV) infection (Costa et al., 2009). Similarly, transcripts of IMD pathway components and IMD stimulated AMPs increased following Sigma virus (SIGMAV) infection; although, this appeared to be dependent on the strain of flies involved (Tsai et al., 2008, Carpenter et al., 2009). Nora virus also affected gene expression of an alternative IMD signalling branch (Boutros et al., 2002, Cordes et al., 2013). In contrast to Toll signalling, stimulation of IMD was observed following alphavirus infections. Transgenic *Drosophila* expressing a SINV replicon generated greater levels of genomic viral RNA loads when IMD components were silenced (Avadhanula et al., 2009). Viral replication resulted in an increase in IMD component expression and downstream gene transcription while knockdown of the IMD associated AMP, DptB, generated developmental defects in the transgenic flies and increased virus replication and titres in mutant flies infected with SINV (Huang et al., 2013). In mosquitoes, *An. gambiae* demonstrated that ONNV infection affected IMD component expression; however, silencing of Rel2 did not significantly alter virus replication suggesting another pathway was contributing to virus control (Avadhanula et al., 2009, Waldock et al., 2012, Huang et al., 2013). Certainly, another study indicated that there was no significant immune gene induction 14 days post ONNV infection (Sim et al., 2005). Pre-stimulation of the IMD pathway in *Ae. albopictus*-derived U4.4 cells through heat-inactivated *E.coli* allowed a reduced level of SFV replication and the infection did not successfully activate the pathway (Fragkoudis et al.,

2008). This result is mirrored in CHIKV infection of *Ae. aegypti*-derived Aag2 cells which similarly did not result in any pathway activation (McFarlane et al., 2014).

1.3.4 The JAK/STAT Pathway

The JAK/STAT pathway was initially characterised in insects for its role in development, embryonic segmentation and haemocyte proliferation (Binari and Perrimon, 1994, Zeidler et al., 2000, Luo and Dearolf, 2001, Arbouzova and Zeidler, 2006) but is also known for responding to pathogenic infections (Barillas-Mury et al., 1999). The *Drosophila* pathway relies on four key components; the ligands, their receptors, a single Janus kinase and a single STAT transcription factor. As with the Toll pathway, JAK/STAT pathway signalling is activated by the binding of a secreted ligand to its receptor. Three unpaired-related (Upd1-3) ligands are known to be involved and interact with their receptor, Domeless (Dome), inducing its dimerization and allowing transphosphorylation of the Janus kinase, Hopscotch (Hop), which is homologous to the human JAK2 protein (Binari and Perrimon, 1994, Yan et al., 1996, Harrison et al., 1998, Brown et al., 2001, Hombria et al., 2005). This action subsequently allows the recruitment and transphosphorylation of the transcription factor STAT (STAT92E/Marelle) on the conserved C-terminal tyrosine residue. The phosophorylated STATs dimerize and translocate the nucleus to regulate transcription of downstream effector genes.

Orthologues of proteins which attenuate the pathway in mammals are known in *Drosophila* including SOCS proteins (Socs44F, Scos36E and Socs16D), the phosphatase protein PTP61F and a PIAS orthologue (Su(var)2-10) (Baeg et al., 2005, Waterhouse et al., 2007, Souza-Neto et al., 2009). Comparative genomic studies have also revealed that key mosquito vectors *Ae. aegypti*, *Cx. tritaeniorhynchus* and *An. gambiae* share some major homologues of the JAK/STAT pathway, including dome, hop, STAT, PIAS and SOCS (Barillas-Mury et al., 1999, Lin et al., 2004, Waterhouse et al., 2007, Souza-Neto et al., 2009, Zou et al., 2011). However, the Upd ligands have only been found in the *Drosophila* genome and as a result the JAK/STAT pathway in *Aedes*, *Culex* and *Anopheles* mosquitoes is believed to be initiated by Vago, a novel ligand (discussed in **Chapter 1.3.5.2**) (**Figure 1.7**). It is interesting to note that Vago does not bind to the dome receptor and its true receptor is as yet unknown which contributes to the large number of questions still unanswered regarding mosquito antiviral immunity.

Several studies have demonstrated the involvement of JAK/STAT signalling in antiviral defence in *Drosophila* and mosquitoes (Dostert et al., 2005, Souza-Neto et al., 2009,

Paradkar et al., 2012, Kemp et al., 2013). Secreted Vago has been shown to activate the JAK/STAT pathway following WNV infection of the *Cx. quinquefasciatus*-derived Hsu cell line (Paradkar et al., 2012). Furthermore, only WNV-related dsRNA generated stimulation, unlike bluetongue virus (BTV)-related dsRNA or poly I:C (a structurally similar synthetic analogue of dsRNA). This implicates a degree of virus specificity and a tailored immune system mirrored in the other classical immune signalling pathways (Paradkar et al., 2012). In addition, DENV infection of *Ae. aegypti* mosquitoes stimulates the signalling response resulting in the production of JAK/STAT associated genes as well as DENV restriction factors (DVRF1 and DVRF2) (Souza-Neto et al., 2009, Behura et al., 2011). RNAi knockdown of the major pathway components also resulted in an increase in virus titres (Souza-Neto et al., 2009). As observed with the Toll pathway, neither SFV infection of *Ae. albopictus*-derived U4.4 or CHIKV infection of *Ae. aegypti*-derived Aag2 cells resulted in pathway activation (Fragkoudis et al., 2008, McFarlane et al., 2014). This is in contrast to what has been shown for a further alphavirus infection in *Drosophila* were SINV appears to activate JAK/STAT signalling resulting in an increase in expression of STAT associated genes; although, the survival of hop mutant flies is not significantly different to wildtype flies (Avadhanula et al., 2009, Huang et al., 2013, Kemp et al., 2013). In the *Ae. aegypti*-derived Aag2 cell line, SINV infection resulted in an increase in the transcription factor STAT but no other genes from any of the pathways investigated (Barletta et al., 2012).

1.3.5 Other Immune Mechanisms in Arthropods

There are a number of alternative immune responses which contribute to antiviral defence in arthropods (Kingsolver et al., 2013, Merkling and van Rij, 2013, Rueckert et al., 2014). For instance, the autophagy pathway is responsible for the degradation of cellular components through the lysosome. It is evolutionarily conserved in eukaryotic organisms and is activated in response to cellular stress. Several studies have shown that it plays a role in inhibiting the replication of a number of intracellular pathogens, including bacteria and viruses (Yano et al., 2008, Seay et al., 2009, Shelly et al., 2009, Dreux and Chisari, 2010, Orvedahl et al., 2010, Yordy and Iwasaki, 2011, Nakamoto et al., 2012). The heat-shock response is also believed to be involved as induction of heat shock proteins following a pathogenic attack may trigger an immune response (Matzinger, 2002, Sim et al., 2007, Santoro et al., 2010). However, its exact involvement requires further characterisation. Other factors which pertain to innate immunity in mosquitoes are discussed below.

1.3.5.1 Phenoloxidase Activity

The extracellular phenoloxidase (PO) cascade is a complex humoral immune component in insects and is involved in the production of melanin, a brown-black pigment. The process of melanisation involves a number of sequential enzymatic and non-enzymatic reactions (Christensen et al., 2005, Cerenius et al., 2008). It is initiated through cuticular damage or the recognition of PAMPs; such as lipopolysaccharides (LPS) and peptidoglycans on microbial cell walls. These PAMPs stimulate the serine protease cascade which involves multiple clip-domain serine proteases (cSPs) which cleave phenoloxidase activating proteins (Pro-PAPs) to their active form (PAPs), allowing them to generate PO by proteolysis of the zymogen prophenoloxidase (proPO/PPO). PO catalyses the conversion of mono- and di- phenolic substrates available in the haemolymph to dopamine or 5,6-dihydroxyindole (DHI) that are further processed into melanin (**Figure 1.8**). The melanin builds up at cuticular wound sites and around invading pathogens which aids healing. Other cytotoxic products are also produced such as reactive oxygen species (ROS) which are antimicrobial (Cerenius and Soderhall, 2004, Nappi and Christensen, 2005). The pathway is regulated by the action of serpins acting as serine protease inhibitors to prevent sustained, deleterious effects caused by the production of toxic intermediates (Jiang and Kanost, 2000, Gorman and Paskewitz, 2001).

The role of the PO cascade in defence against fungal, parasitic and bacterial assaults in arthropods has been well documented (Ashida and Brey, 1997, Soderhall and Cerenius, 1998, Hillyer et al., 2003a, Hillyer et al., 2003b, Infanger et al., 2004, Christensen et al., 2005, Hillyer et al., 2005, Leclerc et al., 2006, Yassine et al., 2012). Antiviral activity was determined by Popham and colleagues as haemolymph derived from *Heliothis virescens* (tobacco budworm) was shown to be virucidal against *Helicoverpa zea* single capsid nucleopolyhedrovirus (HzSNPV; *Baculoviridae*) and that there was an increase in virus titres following the inhibition of PO (Ourth and Renis, 1993, Popham et al., 2004, Shelby and Popham, 2006). Similarly, *Autographa californica* multicapsid nucleopolyhedrosis virus (AcMNPV; *Baculoviridae*) was inactivated by DHI (Zhao et al., 2011). The involvement of the PO cascade in the control of arboviruses has been highlighted as suppression of PPO in *Armigeres subalbatus* mosquitoes during infection with a recombinant strain of SINV expressing dsRNA against PPO resulted in a decrease in PO activity and enhanced SINV replication (Tamang et al., 2004). This effect was also observed following the infection of *Ae. aegypti* mosquitoes and the *Ae. albopictus*-derived U4.4 cell line with a recombinant SFV expressing the PO cascade inhibiter, Egf1.0 which

allowed an increase in SFV titres (Rodriguez-Andres et al., 2012). It will be of interest to determine if this phenomenon is observed for other vector: virus interactions.

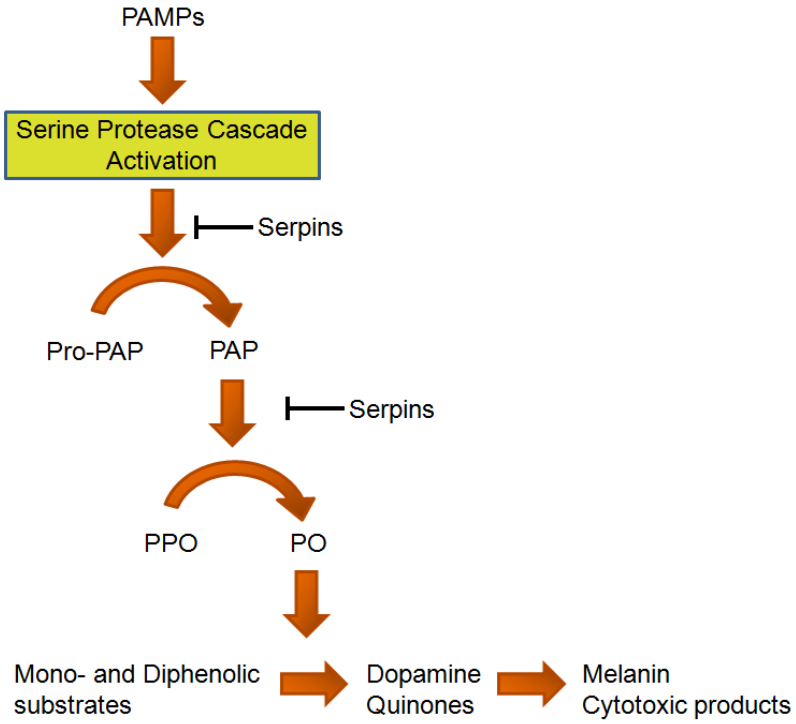

Figure 1.8: The Serine Protease Cascade. PAMPs, such as lipopolysaccharides (LPS) and peptidoglycans on bacterial cell walls stimulate the pathway to produce active phenoloxidase activating proteins (PAPs). PAPs are required to cleave and activate phenoloxidase (PO) from prophenoloxidase (PPO) to catalyse the conversion of mono- and di- phenolic substrates to dopamine and quinines. These products are eventually converted to melanin, produce cytotoxic products and facilitate the encapsulation of pathogens.

1.3.5.2 Vago

In *Drosophila*, the *vago* gene encodes an 18 kDa cysteine-rich polypeptide which is induced following a viral infection. Its induction has been shown to be dependent on Dcr-2 but none of the other RNAi components (Deddouche et al., 2008). Furthermore, it was shown to be up-regulated following infection by Drosophila C virus (DCV) and SINV in *Drosophila*. It has since been determined to have an antiviral role as it controls the viral load in *Drosophila* fat bodies and *vago* null mutants permit a higher level of virus replication (Dostert et al., 2005, Deddouche et al., 2008). In mosquitoes, Vago has been shown to act as a secreted signalling molecule to trigger STAT transcription and stimulate the up-regulation of STAT-dependent virus inducible genes (**Chapter 1.3.4**). Although Vago is not believed to be highly conserved in other insects, recent work in mosquitoes has shown that an orthologue is present in the genome of *Culex* and *Aedes* mosquitoes which is up-regulated following WNV or DENV infection in *Culex* and *Aedes*-derived cell lines (Paradkar et al., 2012, Paradkar et al., 2014). These studies also suggest that dsRNA recognition and cleavage by the DExD/H-box helicase domain of the RNAi initiator protein Dcr-2 activates tumour necrosis factor (TNF) receptor-associated factor (TRAF) which then causes cleavage and release of the amino terminus of REL2, an orthologue of NF-κB. It is then suggested that REL2 induces Vago production by binding to the NF-κB binding site which is conserved on the promoter domain of *vago* (Paradkar et al., 2014).

Vago was described as providing a protective paracrine response as it is able to trigger a signalling cascade and induce an antiviral state in uninfected cells following secretion from infected cells. The identification of a cytokine function is reminiscent of IFN signalling in mammals which also involves the JAK/STAT pathway (Platanias and Fish, 1999, Subramaniam et al., 2001). Vago activation in insects is also analogous with PAMP recognition by RIG-I and MDA-5. This induces TRAF/NF-κB stimulated IFN pathway and is involved in viral control in mammalian systems (Saha et al., 2006, Brass et al., 2009). These findings imply a level of communication between RNAi and JAK/STAT pathways in insects and, although Vago activation appears to be pathogen specific, responding only to replicating viral infections, further work will establish if assaults by other infectious agents result in the stimulation of multiple pathways.

1.3.5.3 *Wolbachia* Endosymbiotic Bacteria

The α-proteobacteria *Wolbachia* is predicted to be found within 60 % of all insect species around the globe. It is inherited by maternal transmission and has been documented as

being capable of manipulating the gene expression of its host by a mechanism termed cytoplasmic incompatibility (CI). CI occurs during the mating of infected males with females. *Wolbachia* modifies the sperm and prevents efficient embryogenesis unless the egg also contains the bacterium (Werren et al., 2008, Rainey et al., 2014). This places infected females at an evolutionary advantage as they may mate with all available males, unlike uninfected females which can only mate successfully with uninfected males and any matings with infected males will produce few or no progeny (Yen and Barr, 1971, Turelli and Hoffmann, 1991, Turelli and Hoffmann, 1995).

It was serendipitously discovered that *Wolbachia* infections reduce vector competency and present a number of advantages towards a new biological application for the control of mosquito-borne diseases (Hancock et al., 2011). Certain strains of *Wolbachia* are known to reduce the life span of their insect host, indirectly inhibiting transmission of the pathogen (Min and Benzer, 1997, Evans et al., 2009, Kambris et al., 2009, McMeniman et al., 2009, Yeap et al., 2011). *Wolbachia* has been shown to interfere with the expression of ferritin (involved in the storage and regulation of iron homeostasis) and iron metabolism in insects (Kremer et al., 2009). This protects the cell from oxidative stress and apoptosis and reduces resources available to invading pathogens. Furthermore, a link has been established between *Wolbachia* and the Toll and melanisation pathways (Pan et al., 2012, Rances et al., 2012). It has been documented that *Wolbachia* infections in mosquitoes generate a reduced susceptibility to mosquito-borne pathogens, such as filarial nematode parasites and plasmodium malaria parasites in both *Ae. aegypti* and *An. gambiae* (Kambris et al., 2009, Moreira et al., 2009). In addition, a number of studies have demonstrated the induction of DENV resistance following *Wolbachia* infection (Hedges et al., 2008, Moreira et al., 2009, Bian et al., 2010, Hoffmann et al., 2011, Walker et al., 2011, Rances et al., 2012, Zhang et al., 2013a). During a *Wolbachia* infection of *Ae. aegypti*, the bacterium has been shown to utilise miRNAs to regulate host methyltransferases which may impact DENV replication (Zhang et al., 2013a, Zhang et al., 2014). This is in contrast to DENV which induces methyltransferase expression. *Wolbachia* infections were also shown to inhibit WNV replication in *Cx. quinquefasciatus* and CHIKV in *Ae. aegypti*, the major vectors of these pathogens (Moreira et al., 2009, Glaser and Meola, 2010).

It is surprising that *Wolbachia* is not known to naturally infect the key arbovirus vector *Ae. aegypti*, although other important vectors, *Ae. albopictus*, *Ae. bromeliae* and *Cx. pipiens*, do harbour some strains (Kittayapong et al., 2000, Armbruster et al., 2003, Osei-Poku et al., 2012) and a recent publication has identified a strain in the malarial vector *An. gambiae* (Baldini et al., 2014). However, laboratory strains can be successfully transferred from

Drosophila (Rainey et al., 2014). For instance the *Drosophila* strain wMel reduces the transmission of DENV (Blagrove et al., 2012). As a result of intense research in recent years, several cage field trials have been successfully used to maintain *Ae. aegypti* populations demonstrating a lower susceptibility to DENV infections and no evident negative impact to the environment or public health (Hoffmann et al., 2011, Walker et al., 2011).

1.4 RNA interference: Small RNA Mediated Regulation of Gene Expression

1.4.1 History and Discovery of RNA interference (RNAi)

Invertebrates have been shown to be deficient in an adaptive immune response which is well characterised in vertebrates. However, their innate immune responses are capable of efficiently defending against viral infections. The mechanism which has been recognised as playing a significant role in antiviral defence is RNA interference (RNAi). The RNAi response was first described in plants in the 1990s when transgenic tobacco plants were found to 'recover' from a viral infection and also developed resistance against that specific pathogen (Lindbo et al., 1993). A relationship between this antiviral activity and its involvement in endogenous gene expression was identified (Ratcliff et al., 1997) and so it was termed 'post-transcriptional gene silencing' when involved in transcriptional control of aberrant genes and 'virus-induced gene silencing' in viral defence (Angell and Baulcombe, 1997, Ruiz et al., 1998). Since its discovery, it has been shown to be highly conserved and exists in a wide range of eukaryotic organisms including plants, fungi, worms, insects and mammals, where it is involved in modulating gene expression, epigenetic control and pathogen defence.

Fire and colleagues are credited with the first report of the inducer of the pathway being long double stranded RNA (dsRNA) molecules through their work in *C. elegans* (Fire et al., 1998). The injection of dsRNA into *Drosophila* embryos recognised a corresponding effect and much of our understanding of insect antiviral RNAi stems from initial work carried out in *Drosophila* systems (Kennerdell and Carthew, 1998, Galiana-Arnoux et al., 2006, van Rij et al., 2006, Wang et al., 2006, Zambon et al., 2006). As mosquitoes transmit a number of both medically and veterinary important viral pathogens, understanding their antiviral immune responses has been paramount to disease control. RNAi in mosquitoes

was identified through the use of recombinant strains of SINV expressing sequences from DENV-2. The infected mosquitoes were refractory to subsequent infection by DENV-2 due to RNA-dependent processing (Gaines et al., 1996, Olson et al., 1996, Adelman et al., 2001). This effect was also evident when mosquitoes were infected with a further recombinant SINV expressing sequences derived from RVFV; although, this was not clearly understood to be due to RNAi at the time (Billecocq et al., 2000). *Drosophila* studies recognised the principal proteins in the RNAi pathway in particular Dicer (Dcr) and Argonaute (Ago) which have been shown to be highly conserved in a wide range of eukaryotes including plants, mammals and invertebrates.

Recently, the use of RNAi has become commonplace in many research areas as a biological tool for the study of gene expression and function. The consequences of gene knockdown on elucidating the role of particular genes in a variety of systems has vastly accelerated understanding in a number of fields.

1.4.2 Introduction to RNAi in Insects

In terms of anti-arbovirus immunity, mosquitoes are the best studied organism. Although it has been demonstrated that arboviruses are able to replicate efficiently within their mosquito vectors, they display minor pathology and fitness costs (Lambrechts and Scott, 2009). This implies that the viral infection is successfully controlled by the antiviral response. RNAi is a highly conserved process in multicellular organisms and is regarded as the preeminent antiviral mechanism in plants (termed post-transciptional gene silencing [PTGS]), fungi (termed quelling) and *C. elegans*; however, its involvement in insect immunity has only been established over the past decade. Recently several reviews have highlighted the essential role of RNAi in the mosquito innate immune responses and it is suggested that the mechanisms are similar to that described in *Drosophila* (Blair, 2011, Donald, 2012, Vodovar and Saleh, 2012, Lucas and Raikhel, 2013, Vijayendran et al., 2013, Bronkhorst and van Rij, 2014, Rueckert et al., 2014). It has four known branches with vital roles in controlling normal growth and development, restricting the mobility of transposable elements and mediating viral infections. These pathways are characterised by the production of small RNA molecules which can be distinguished based on the template they originate from, their size, biochemistry and targets, as well as their distinctive functions in their respective pathways. There are three key small RNA molecules which have been recognised in eukaryotes to date; small interfering RNAs (siRNAs), microRNAs (miRNAs) and PIWI-interacting RNAs (piRNAs) (**Figure 1.9**). These pathways have been

implicated in antiviral defence as evidenced by small RNAs found to match and be derived from viral genomes (Aliyari et al., 2008, Myles et al., 2008, Brackney et al., 2009, Brackney et al., 2010, Flynt et al., 2009, Scott et al., 2010, Hess et al., 2011, Siu et al., 2011, Morazzani et al., 2012, Vodovar et al., 2012, Leger et al., 2013, Schnettler et al., 2013a, Schnettler et al., 2013b).

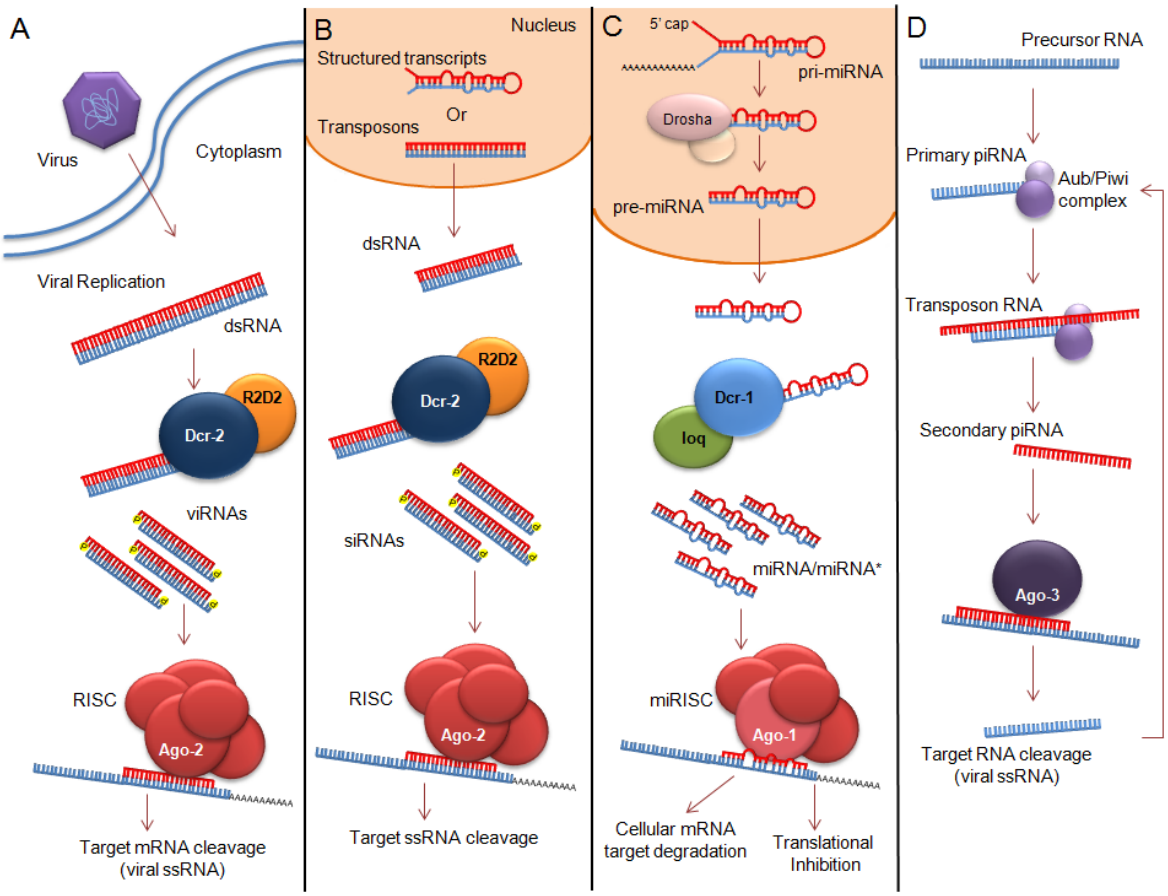

Figure 1.9: Schematic representation of the small RNA pathways present in insects as described for *Drosophila melanogaster*. A) Exogenous siRNA, **B)** Endogenous siRNA, **C)** microRNA (miRNA) and **D)** PIWI-interacting RNA (piRNA). dsRNA, double stranded RNA; ssRNA, single stranded RNA; siRNA, small interfering RNAs; viRNAs, virus-derived small interfering RNAs; piRNA, PIWI-interacting RNA; Dcr, Dicer, RISC, RNA induced silencing complex; Ago, Argonaute; Aub, Aubergine; loq, loquacious. Figure adapted from Donald et al., 2012.

1.4.3 siRNA Pathway

Small interfering RNAs can be separated into two classes; endogenous (endo-siRNAs) or exogenous (exo-siRNAs) based on their template of origin (**Figure 1.9**). The production of endo-siRNAs results from the detection of retrotransposons, sites of convergent

transcription, overlapping transcripts or structured genomic RNA, such as inverted repeats or hairpin structures (differentiated from miRNA targets due to their restricted stem lengths). Their role is the protection of the genome by repressing transposons and other aberrant mobile transcripts and maintaining heterochromatin (Chung et al., 2008, Czech et al., 2008, Ghildiyal et al., 2008, Watanabe et al., 2008, Fagegaltier et al., 2009, Lucchetta et al., 2009, Lim et al., 2011). Exogenous RNAi is provoked by the presence of long, non-cellular dsRNA molecules produced as a result of RNA virus replication or convergent overlapping transcriptional complexes of DNA viruses (Bronkhorst et al., 2012). These dsRNA triggers are discriminated from 'self' entities and recognised as PAMPs which are then cleaved into shorter duplex fragments known as exo-siRNAs or virus-derived small interfering RNAs (viRNAs) which restrict the incoming viral infection. The resulting viRNAs are not biased towards a particular base at position one, suggesting that Dcr-2 cleaves the dsRNA molecules in a non-sequence specific manner. Both the endo- and the exo-siRNA pathways are believed to function in a similar manner in *Drosophila* and utilise the same proteins, with the exception of Loquacious isoform PD (Loqs-PD) which is only known to be involved in the endo-siRNA pathway (Hartig et al., 2009, Marques et al., 2013).

The detection of the dsRNA trigger molecules is performed by the RNase III enzyme, Dcr-2 (Bernstein et al., 2001, Kim et al., 2006, Aliyari and Ding, 2009, Kemp and Imler, 2009), associated with the dsRNA binding protein R2D2 (so named due to the presence of two dsRNA binding domains [R2] and its interaction with Dcr-2 [D2]). Dcr-2 therefore functions as a cellular PRR (Takeuchi and Akira, 2008). The siRNAs/viRNAs are fed into the RNA induced silencing complex (RISC), a ribonucleoprotein complex, containing several components including the second Argonaute protein, Ago-2 (Hammond et al., 2001, Meister et al., 2004, Okamura et al., 2004, Kawamura et al., 2008). Ago-2 has catalytic slicer activity and directly moderates gene silencing. In *Drosophila*, other associated proteins are VIG (Vasa intronic gene) and dFXR (*Drosophila* ortholog of fragile X mental retardation protein) (Caudy et al., 2002). Further components are involved in the assembly of the active RISC which include Component 3 Promoter of RISC (C3PO) (a multimeric complex of Trax and Translin) (Liu et al., 2009, Tian et al., 2011), aubergine (Specchia et al., 2008) and heat-shock protein 90 (Hsp90) (Miyoshi et al., 2010).

Once incorporated into the active RISC, the siRNA/viRNA duplex is unwound and one strand (termed the passenger strand) is discarded while the other (the guide strand) is used by Ago-2 to detect and cleave target mRNAs. This causes the degradation of any mRNA present in the cytoplasm with a high sequence homology to the siRNA loaded into RISC

making RNAi highly sequence specific and siRNA dependent (Ender and Meister, 2010). For the virus this means the inhibition of gene expression and a reduction in progeny produced.

Dcr-2 is fundamental in the antiviral immune response in insects and, in principle, its action alone is sufficient to restrict viral infections. Incidentally it is a member of the same DExD/H box helicase family to which the mammalian PRR RIG-I –like receptors belong (Deddouche et al., 2008). The structure of Dcr-2 has been shown to include in order from the N- to the C- terminus; 1) a DExH/D (DEAD) box helicase ATPase domain, 2) a dsRNA-binding domain, 3) a Piwi/Argonaute/Zwille (PAZ) domain and 4) two tandem RNaseIII domains (Aliyari et al., 2008, Welker et al., 2011, Morazzani et al., 2012) (**Chapter 5**). The PAZ domain is responsible for interacting with the dsRNA molecule while the two RNaseIII domains are known to be implicated in its processing into siRNAs/viRNAs (Blaszczyk et al., 2001, Lee et al., 2004, Flynt et al., 2009). This action generates the characteristic 5' monophosphate and 2 nt 3' overhang viRNA ends and immediately reduces the quantity of RNA encoding viral proteins available for the creation of new viral genomes. It is the distance between the PAZ and RNaseIII domains that determines the 21 nt length of the siRNA molecules which is characteristic of Dcr-2 processing (Zhang et al., 2004, Macrae et al., 2006). The ATPase domain provides the energy necessary for dicing. In *Drosophila*, further molecules implicated in effective dsRNA cleavage are Loqs-PD and Arsenic resistance protein 2 (Ars2) which are thought to augment the affinity Dcr-2 has for dsRNA (Sabin et al., 2009, Zhou et al., 2009a, Marques et al., 2010). Dcr-2 has also been implemented in downstream signalling as its action generates increased expression of the mosquito cytokine, Vago (discussed in **Chapter 1.3.5.2**) (Deddouche et al., 2008, Paradkar et al., 2012) and other genes are also expected to be induced.

Ago-2 in *Drosophila* was the first pathway protein identified as being antiviral in higher eukaryotes (Li et al., 2002). As with Dcr-2, it also contains a PAZ domain, in addition to a Piwi_ago-like domain (Lingel et al., 2003, Kim et al., 2009). The PAZ domain secures the guide strand of the siRNA molecule by the 2 nt 3' overhang within its hydrophobic cleft while the Piwi_ago-like domain possess endonucleolytic activity and is able to cleave the phosphodiester bond of the passenger strand between the 9^{th} and 10^{th} nt from the 5' end (Lingel et al., 2003, Lingel et al., 2004, Ma et al., 2004, Okamura et al., 2004, Rand et al., 2004, Rand et al., 2005, Matranga et al., 2005, Miyoshi et al., 2005). The resulting molecules of 9 and 12 nt are believed to be degraded by C3PO which has exonuclease activity (Liu et al., 2009). Selection of the guide strand depends on the internal

thermodynamic stability of the siRNA duplex, with the strand with the lowest 5' stability being irreversibly incorporated into the RISC (Khvorova et al., 2003, Schwarz et al., 2003). The rapid evolution of antiviral RNAi genes (*dcr2*, *r2d2* and *ago2*) compared to non-immune linked genes (*dcr1*, *r3d1* and *ago1*) in *Drosophila* indicates a constant arms race between the virus and the host (Obbard et al., 2006, Obbard et al., 2009). However, a study in *Ae. aegypti* indicates that unlike *Drosophila*, both miRNA and exo-siRNA pathway genes undergo accelerated evolution highlighting the differences between the two insect species (Bernhardt et al., 2012).

1.4.4 miRNA Pathway

MicroRNAs (miRNAs) have been shown to be conserved in many organisms, such as plants, mammals and invertebrates, and are important for the regulation of gene expression in different tissues and during different stages of development (Jones and Newbury, 2010, Schnall-Levin et al., 2010, Chawla and Sokol, 2011, Asgari, 2013). They are confirmed to be involved in a number of biological processes such as fat metabolism, cardiogenesis, stress regulation, muscle growth, neurogenesis and immune responses (Jin et al., 2004, Xu et al., 2004, Kwon et al., 2005, Sokol and Ambros, 2005, Liu et al., 2007, Parrish et al., 2009, Choi and Hyun, 2012, Hussain et al., 2013). miRNAs are produced from cellular transcripts and modulate expression at the translational level by controlling the availability of messenger RNAs (mRNAs) for translation to proteins (**Figure 1.9**). In general the effect of miRNA function is the down-regulation of their target gene; although, some positive interactions have been described (Lagos-Quintana et al., 2001, Bushati and Cohen, 2007, Henke et al., 2008, Voinnet, 2009, Berezikov, 2011, Choi and Hyun, 2012, Hussain et al., 2012).

Their biogenesis is understood to be conserved within insect species and bioinformatic studies have indicated the existence of genes involved in the miRNA pathway between *Drosophila* and several mosquito species such as *Ae. aegypti*, *An. gambiae*, and *Cx. pipiens* (Campbell et al., 2008). Some of the mechanistic features also share some similarity to the siRNA pathways; for instance, the miRNA pathway is also instigated by the cleavage of dsRNA molecules; however, this is through Dcr-1 rather than Dcr-2 processing. Dcr-1 lacks a DExH helicase domain and so cannot produce siRNAs (Lee et al., 2004). The source of the dsRNA also differs between the two systems as unlike the siRNA pathways which are concentrated in the cytoplasm, the miRNA pathway has phases in both the cytoplasm and nucleus. Primary miRNAs (pri-miRNAs) result from transcripts produced

in the nucleus by the cellular RNA polymerase II. These transcripts fold back on themselves forming an incomplete dsRNA stem-loop structure. The pri-miRNAs are then further processed to precursor miRNAs (pre-miRNAs) of around 70 nt by the nuclease Drosha in combination with Pasha (or DGCR8 in mammals) before being transported to the cytoplasm. Following cleavage by Dcr-1 in association with loq (or the dsRNA binding protein TRBP in mammals) the mature miRNA/miRNA* duplex which is ~21 - 22 nt long is produced. The guide strand of the duplex is finally loaded into RISC (also referred to as miRISC), which contains Ago-1 rather than Ago-2, and can be used to target near-perfect complementarily cellular mRNAs (Okamura et al., 2004). Although, the passenger miRNA* strand is usually degraded, it has been shown to also be incorporated in some situations (Lagos-Quintana et al., 2001; Zeng et al., 2003; Schwarz et al., 2004). The result of Ago-1 targeting is either translational suppression, mRNA degradation or both. The defining properties dictating which of these outcomes occurs are not yet understood; although, it is believed to be related to the degree of complementary between the two strands (Hutvagner and Zamore, 2002, Forstemann et al., 2007, Pillai et al., 2007, Brodersen et al., 2008, Iwasaki et al., 2009, Iwasaki and Tomari, 2009). miRNAs are also the only small RNAs not to be 2'-*O*-methylated which affects their biological stability and endurance, vital for tightly regulated gene control during development. This method of gene regulation is highly energy efficient as the expression of a small RNA in a specific tissue is able to sufficiently shut down unnecessary gene expression and can act systemically (Choi and Hyun, 2012).

Several miRNAs have been identified in a number of mosquito and tick species and their derived cell lines; although, they have still to be confirmed in midges (Winter et al., 2007, Mead and Tu, 2008, Li et al., 2009, Mendes et al., 2010, Skalsky et al., 2010, Barrero et al., 2011, Hussain et al., 2011, Gu et al., 2013, Zhang et al., 2013a, Zhou et al., 2013). Some of these miRNAs are species specific while some have been shown to be conserved between insects and mammals. The involvement of miRNAs during a viral infection has been better studied in mammalian systems where it has been demonstrated as being highly complex with many factors influencing the cellular response and driving the outcome of the infection. These factors include host miRNAs modulating viral transcripts, viral miRNAs modulating host transcripts, in addition to viral miRNAs regulating viral transcripts using host miRNA mechanisms (Pfeffer et al., 2004, Jopling et al., 2006, Ouellet and Provost, 2010, Cullen, 2011, Grundhoff and Sullivan, 2011). Much less is currently known about the influence the miRNA pathway has on a viral infection in mosquitoes and other invertebrates as only a small number of studies have been performed

to date (Vijayendran et al., 2013, Hussain and Asgari, 2014, Asgari, 2014). WNV infection of *Cx. quinquefasciatus* resulted in the up-regulation of one host miRNA (miR-989) but the down regulation of another (miR-92); although, the specific targets of the miRNAs are not yet known (Skalsky et al., 2010). These results were not observed in the *Ae. albopictus* derived C7-10 cell line suggesting that there is a level of pathogen: host specificity involved in the interactions which have been previously determined in other systems (Zhou et al., 2009b, Zeiner et al., 2010). DENV infection of *Ae. aegypti* altered the expression of 35 separate miRNAs up to nine days post infection, four of which were up-regulated (Campbell et al., 2014). Similarly, CHIKV infection of *Ae. albopictus* significantly affected 41 miRNAs and again four of these were up-regulated (Shrinet et al., 2014). Both miR-2b and miR-1000 have been shown to be suppressed by both DENV and CHIKV in *Aedes* mosquitoes signifying that they may be common to viral control in these insects. In the *Ae. albopictus*-derived C6/36 cell line, DENV infection causes a greater than 3-fold increase in miR-252 which is thought to be involved in the antiviral response (Yan et al., 2014). Although, this effect was not observed in whole mosquitoes.

Viral suppressors of RNAi (VSR) proteins are known to interfere with miRNA pathway components, such as the Cucumber mosaic virus-encoded VSR protein 2b which physically inhibits Ago-1 slicing and causes a build up of cellular miRNA targets in plants (Zhang et al., 2006, Gonzalez et al., 2010). However, in *Drosophila* engineered to express a VSR, there were no changes observed in the pattern of miRNAs detected (Berry et al., 2009). Similarly, knockdown of Ago-1 did not enhance the replication of ONNV in *An. gambiae* (Keene et al., 2004) or SFV and CHIKV in *Ae. aegypti*-derived Aag2 cells (Schnettler et al., 2013a, McFarlane et al., 2014). An interaction between viral proteins and host miRNAs has been suggested for the non-structural protein 3 (NS3) of rice hoja blanca tenuivirus which is known to bind to plant-derived miRNAs and results in the suppression of an RNAi knockdown in *Drosophila*-derived cells (Hemmes et al., 2007). This may implicate NS3 as being able to bind insect miRNAs.

DENV-2 infection of both *Ae. aegypti*-derived Aag2 and *Ae. albopictus*-derived RML12 cell lines results in the production of six miRNA-like small RNAs, one from the 5' UTR and the rest from the 3' UTR (Hussain and Asgari, 2014). When these were silenced only one, designated vsRNA-5, resulted in a significant increase in viral replication. It is believed to regulate DENV infection though its association with its target sequence found in NS1. However, this sequence is currently only known for DENV-2 and no other serotypes (Finol, 2014). Likewise, it has been shown that WNV expresses a miRNA molecule from the subgenomic RNA corresponding to the 3' UTR in the *Ae. aegypti*

derived Aag2 and *Ae. albopictus* derived C6/36 cell lines (Hussain et al., 2012). By up-regulating the synthesis of the host GATA4 transcription factor mRNA, the virus induced a pro-viral cellular state and GATA4 was shown to be necessary for its replication. Whether these functions are related to the RNAi suppressor activity of the subgenomic flavivirus RNA (sfRNA) (discussed in **Chapter 1.4.6**, **Chapter 3** and **Chapter 5**) is yet to be determined.

1.4.5 piRNA Pathway

Piwi-interacting RNA (piRNA) molecules have a size range of between 25 - 30 nt and interact with PIWI clade proteins consisting of PIWI, Argonaute 3 (Ago-3) and Aubergine (Aub) (Sarot et al., 2004; Siomi et al., 2011; Luteijin and Ketting, 2013). Although they were originally believed to be specific to germ line and surrounding cells, they have recently been identified in the somatic cells of mosquitoes and vertebrates.

Their biogenesis is poorly understood and the source elements responsible, in addition to the location of the process (nuclear or cytoplasmic) are largely unknown. The mechanism is believed to be Dicer independent (Vagin et al., 2006; Houwing et al., 2007; Zamore, 2007) (**Figure 1.9**). Primary piRNAs are believed to be asymmetrical and generated in antisense from (ss)RNA molecules transcribed from a specific chromosomal loci containing inactive transposable elements (TE) within a region of the genome called a piRNA cluster (Brennecke et al., 2007; Ghildiyal and Zamore, 2009; Senti and Bennecke, 2010; Kawaoka et al., 2013; Mohn et al., 2014; Sapetschnig and Miska, 2014; Zhang et al., 2014). The ss-specific nuclease Zucchini (Zuc) cleaves the ssRNA precursors into primary piRNAs (Ipsaro et al., 2012; Nishimasu et al., 2012) which are then 2'-*O*-methylated at the 3'-terminal (Horwich et al., 2007; Kawaoka et al., 2011; Vodovar et al., 2012) and incorporated into RISC, associated with Aub and PIWI proteins (Brennecke et al., 2007; Gunawardane et al., 2007). The production of secondary piRNAs occurs following the cleavage that occurs when these primary molecules combine with sense RNAs derived from transposons (van Rij and Berezikov, 2009; Saito and Siomi, 2010; Senti and Bennecke, 2010; Siomi et al., 2010 and 2011). Ago-3 is understood to interact with the secondary molecules and use them as a guide for recognising complementary antisense strands of RNA which are cleaved to become new primary piRNAs. As a result, the proceeding ten nucleotides of the primary molecule correspond to the complementary sequence of the Ago-3-associated secondary molecule and this in turn determines the sequence of the succeeding secondary piRNAs (Saito et al., 2006; Brennecke et al., 2007;

Gunawardane et al., 2007). Therefore, the production of primary piRNAs drives the creation of secondary piRNAs and vice versa. This production pathway results in the characteristic 'ping-pong' piRNA signature corresponding to a Uridine at position 1 (U_1) on the antisense primary molecules and an Adenine at position 10 (A_{10}) on sense secondary piRNA molecules (Brennecke et al., 2007; Gunawardane et al., 2007; van Rij and Berezikov, 2009; Haase et al., 2010; Saito and Siomi, 2010; Senti and Bennecke, 2010; Siomi et al., 2010 and 2011).

A number of differences between *Drosophila* and mosquito piRNA pathways are evident. Ago-3 and Aub are exclusively found in the *Drosophila* germline, while PIWI is only additionally found in the adjacent follicular cells (Brennecke et al., 2007, Gunawardane et al., 2007; Nishida et al., 2007; Lau et al., 2009; Saito et al., 2009). Some studies report that piRNA molecules may be present more widely in somatic tissues of *Drosophila* (Yan et al., 2011). However, detection of these molecules in the soma surrounding the ovary indicated their biogenesis was via a more simplistic pathway which was Aub/Ago-3 independent but PIWI dependent. This was referred to as the primary pathway (Li et al., 2009; Malone et al 2009). Phylogenetic analysis indicates that in the *Aedes* and *Culex* genomes there is an expansion of the PIWI family genes compared to what is known in *Drosophila* (*Ae. aegypti*- seven Piwi proteins and one Ago-3, *Cx. pipiens*- six Piwi proteins and Ago-3, *Drosophila*- one Piwi, Ago-3 and Aub only) (Campbell et al., 2008a). Furthermore, these proteins are more widespread and are not limited to the germline as PIWI transcripts and piRNAs have been shown in the head and thorax somatic tissues of *Aedes* mosquitoes (Morazzani et al., 2012). Bioinformatic analysis has also indicated that fewer piRNAs map to TEs in *Ae. aegypti* compared to those in *Drosophila* (Arensburger et al., 2011) suggesting there may be an alternative role in these insects.

The first suggestion that piRNAs may be involved in processing viral RNAs occurred following the observation that PIWI and Aub null mutants were more sensitive to DXV infection (Zambon et al., 2006). Viral RNAs of the expected size of piRNAs were later discovered in *Drosophila* ovarian somatic sheet (OSS) cells (Wu et al., 2010; van Mierlo et al., 2010). Similar molecules of the expected size have since been observed in *Ae. aegypti* mosquitoes infected with DENV (Scott et al., 2010; Hess et al., 2011) and the *Ae. albopictus* –derived C6/36 cell line infected with WNV, SINV and LACV (Brackney et al., 2010). Deep sequencing analysis of *Aedes* mosquitoes and their derived cell lines revealed that following infection by viruses of the *Togaviridae* and *Bunyaviridae* families viral-derived piRNAs (vpiRNAs) were produced displaying an enriched A_{10} (positive polarity vpiRNAs) or U_1 (negative polarity vpiRNAs) signature (Morazzani et al., 2012; Vodovar

et al., 2012; Leger et al., 2013; Schnettler et al., 2013a and b). This indicates vpiRNAs are produced from important arboviruses through the ping-pong biogenesis pathway. Indeed, expression of the VSR protein, B2, in CHIKV was shown to target the piRNA machinery and restrict antiviral activity (Morazzani et al., 2012). Furthermore, the knockdown of piRNA-related proteins, like Ago-3 in the case of ONNV in *An. gambiae* (Keene et al., 2004) and PIWI 4 for SFV in *Ae. aegypti*-derived Aag2 cells (Schnettler et al., 2013a) result in an increase in viral titres. This further emphasises the role that these small RNA molecules play in the antiviral response of mosquitoes.

The production of antiviral small RNA populations has been documented to change during the course of an infection. It has been shown that subsequent to RVFV infection of *Ae. aegypti*-derived Aag2 cells it was observed that while the overall number of small RNAs generated against the infection increased, the initial dominant population of 21 nt viRNA molecules decreased as the infection progressed into persistence and the vpiRNA molecules become preponderant (Léger et al., 2013). This is in contrast to the *Ae. albopictus*-derived C6/36 cell line which is known to be RNAi deficient and allows high levels of virus replication to occur (Brackney et al., 2010; Morazzani et al., 2012) (**Chapter 5**). This cell line does not produce Dcr-2 siRNA products but immediately results in the generation of vpiRNAs. As a result, the lesser antiviral response exhibited by these cells suggests that although the piRNA pathway may act antivirally, it may not be as robust as the exo-siRNA in modulating viral replication and may be redundant in viRNA competent tissues.

1.4.6 Viral RNAi Countermeasures

Some viruses have evolved specific countermeasures to evade or antagonise the RNAi response. By encoding viral suppressors of RNAi the antiviral response can be targeted at specific steps of the pathway as demonstrated by both plant and insect viruses (reviewed Li and Ding, 2006; Ding and Voinnet, 2007; Kemp and Imler, 2009; Blair, 2011; van Mierlo et al., 2011; Donald et al., 2012; Bronkhorst and van Rij, 2014). Many of them act on two levels: targeting dsRNA or siRNA molecules which instigate and determine the sequence specific target of the defence response respectively; and interacting with an RNAi component, often Ago-2 (**Figure 1.10**). These are believed to have co-evolved along with the antiviral responses of the host and a recent publication suggests that in some cases, suppressor activity may be limited to the natural virus vector (van Mierlo et al., 2014).

Examples of VSRs include that of DCV (*Dicistroviridae*), a natural pathogen affecting flies, which encodes a VSR termed protein 1A. It functions to prevent dsRNA cleavage by Dcr-2 (van Rij et al., 2006) and may also affect siRNA loading into RISC (Bonning and Miller, 2010; Nayak et al., 2010). The 1A protein encoded by the closely related CrPV (*Dicistroviridae*) has a slightly different mode of function and acts by directly interacting with Ago-2 resulting in its inhibition (Nayak et al., 2010). This action of Ago-2 inhibition is also observed for viral protein 1 (VP1) encoded by the unrelated *Drosophila* pathogen Nora virus (a novel virus within the order *Picornavirales*) (van Mierlo et al., 2012). The B2 suppressor protein is encoded by members of the *Alphaodavirus* genus, such as flock house virus (FHV) or Wuhan nodavirus (WhNV). Not only does B2 bind dsRNA but it also interacts with siRNA molecules and therefore inhibits Dcr-2 cleavage and siRNA incorporation into RISC (Li et al., 2002; Chao et al., 2005; Lingel et al., 2005; Aliyari et al., 2008; Qi et al., 2012).

Arboviruses were not believed to encode RNAi suppressor proteins (Li and Ding, 2006; Blakqori et al., 2007; Attarzadeh-Yazdi et al., 2009; Fragkoudis et al., 2009; Donald et al., 2012). It was hypothesised that suppression of the antiviral defences would be detrimental to the survival of the vector and would therefore be counterintuitive to the replicative fitness of the virus (Myles et al., 2008; Cirimotich et al., 2009). However, the putative arbovirus Nodamura virus (NoV, *Nodaviridae*) encodes a B2 protein which binds dsRNA and siRNA molecules and cannot replicate without it (Li and Ding, 2006; Aliyari et al., 2008; Myles et al., 2008; Han et al., 2011). Furthermore, a recent published paper by van Rij and colleagues suggests that the mosquito specific Culex Y virus (CYV, *Entomobirnavirus*) viral protein 3 (VP3) acts as an RNAi antagonist protein and binds both dsRNA and siRNA molecules. Despite this, CYV is able to establish a non-pathogenic, persistent infection implicating the possibility of undiscovered VSRs in other non-lethal viruses (van Cleef et al., 2014).

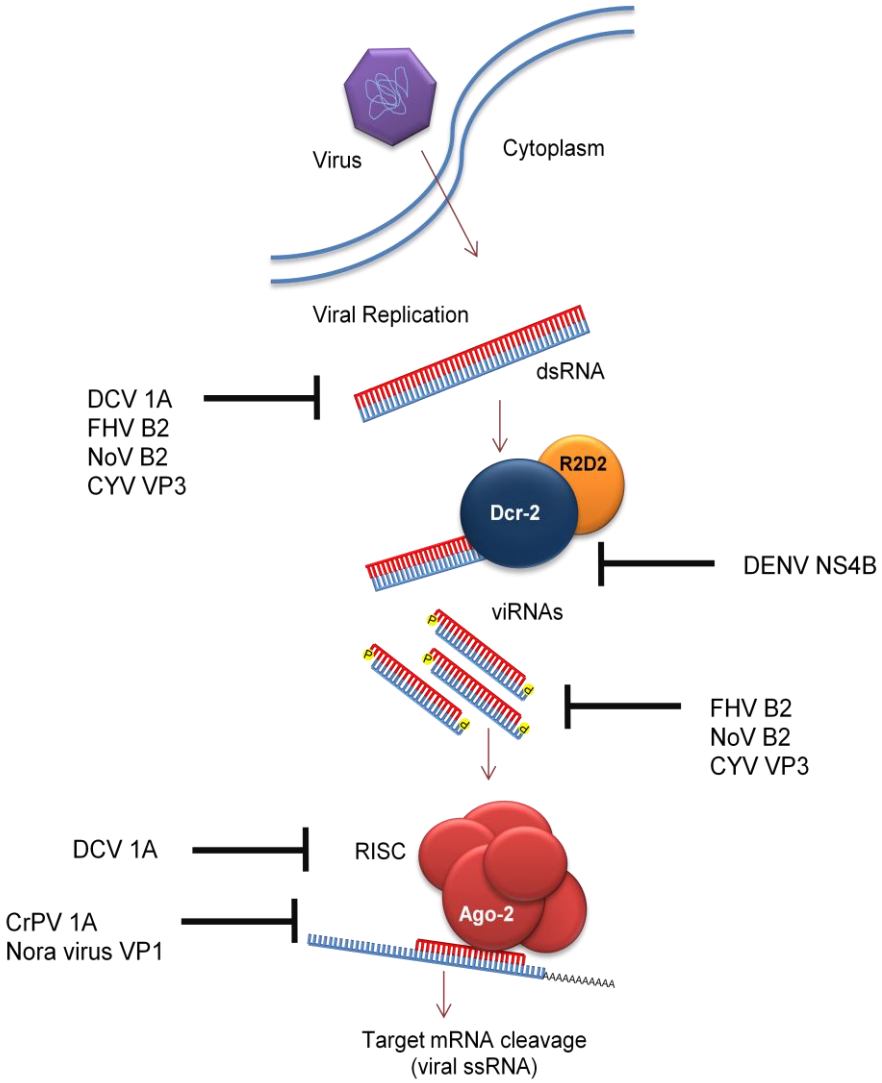

Figure 1.10: Schematic overview of the RNAi pathway in insects and the stages that RNAi suppressor proteins inhibit. Drosophila C virus (DCV 1A), flock house virus (FHV B2), Cricket paralysis virus (CrPV 1A), Culex Y virus (CYV VP3), Nodamura virus (NoV B2), dengue virus (DENV NS4B).

To date, flaviviruses are the only arboviruses known to express an RNAi antagonist in the form of sfRNA molecules. These are produced from the 3' UTR-derived RNA molecule of DENV and WNV (Pijlman et al., 2008; Schnettler et al., 2012). These molecules inhibit RNAi through an RNA decoy mechanism whereby high levels of non-coding RNAs are produced to restrict efficient silencing by occupying the active site of Dcr-2 and preventing the incorporation of coding sequences. sfRNAs are not specific to mosquito-borne flavivirus infections as they are also generated during Langat virus (LGTV) and TBEV infections of tick-derived cell lines (Schnettler et al., 2014). Moreover, the non-structural protein 4B (NS4B) of DENV is also suggested to be an inhibitor of Dcr-2; although, the mechanism is currently unknown (Kakumani et al., 2013). The relative strength of these

inhibitors compared to those described previously is not known. However, it is clear from previous work that overcoming RNAi completely inevitably leads to the death of the vector.

In the absence of a VSR arboviruses are understood to circumvent the antiviral response rather than directly impede it (Uchil et al., 2003; Geiss et al., 2005; Siu et al., 2011; Sabin et al., 2013). This evasion or decoy strategy may indirectly decrease the potency of the antiviral response by restricting or delaying access to the viral genome or by offering regions of the genome that would be less destructive to the virus. The genomes of most dsRNA viruses replicate behind the protection of the viral core which encompasses the genome during replication. Similarly positive stranded RNA viruses, including members of the *Togaviridae* and *Flaviviridae,* are known to replicate within cytoplasmic vacuoles in mammalian cells preventing access to dsRNA replication intermediates which would trigger the immune pathway (Diamond et al., 2003; Geiss et al., 2005; Campbell et al., 2008a; Sanchez-Vargas et al., 2009; Spuul et al., 2010 and 2011). Decoy viRNAs have been shown to be produced against specific regions of the genome following SFV infection of mosquito cells (Siu et al., 2011) (discussed further in **Chapter 3**) as well as for the hairpin structure of the S segment of RVFV and the defective interfering particle of VSV (*Rhabdoviridae*) (Sabin et al., 2013). The introduction of VSRs into viruses not believed to harbour them naturally can be detrimental to the vector. For instance, the outcome of infection by alphaviruses SINV and ONNV engineered to express the B2 VSR was an increase in virus replication, dissemination and viral titre as well as higher mortality rates in *Ae. aegypti*, *Ae albopictus* and *C. tritaeniorhynchus* following *in vivo* injection (Cirimotich et al., 2009; Myles et al., 2008). These findings emphasise the delicate balance that must be established between vector survival and virus transmission.

1.5 Aims and Objectives

Aim

The aim of this project is to expand our understanding of the innate antiviral immune responses of mosquitoes, in particular the RNAi response. Due to the increasing understanding that data collected from the model insect organism, *Drosophila melanogaster*, does not always directly correlate with that obtained from mosquitoes, the natural vectors for major arboviruses, it is important to establish functional assays and biological tools to allow the correct responses of these animals to be ascertained.

Objectives

1. To establish an efficient assay for the silencing of the major proteins involved in the antiviral RNAi response of *Aedes aegypti* –derived cell lines.
2. To investigate the production of viral-derived small RNAs in *Aedes aegypti*-derived Aag2 and *Aedes albopictus*-derived U4.4 cell lines.
3. To investigate the function of an orthologue of *Drosophila* Elp-1 as a potential RNA dependent RNA polymerase in the *Aedes aegypti*-derived Aag2 cell line.
4. To design and develop molecular tools for the study of *Aedes* mosquito RNAi proteins.
5. To characterise the antiviral defences of the *Toxorhychites amboinensis*-derived cell line TRA-171 following SFV infection and determine the antiviral activity of a non-haematophagous mosquito which may potentially serve as safe model for arboviral studies.

Chapter 2: Materials and Methods

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2.1 Bacterial Techniques

2.1.1 Culturing

Two laboratory strains of *Escherichia coli* (*E. coli*) were used for the growth and amplification of DNA plasmids. Amplification of toxic virus plasmids was achieved by transforming SURE 2 competent cells (Stratagene). All other plasmids were amplified using DH5α chemically competent cells (Invitrogen). Both strains of bacteria were grown in sterile Luria-Bertani (LB) broth (E&O Laboratories Ltd) containing the appropriate plasmid selection antibiotic; 100 µg/ml ampicillin (Amp) (Melford Laboratories Ltd.), 50 µg/ml kanamycin (Kana) (Sigma Aldrich) or 20 µg/ml zeocin (Life Technologies). Agar plates were made by pouring melted sterile LB agar (E&O Laboratoriess Ltd) supplemented with the appropriate antibiotic into 10 cm^2 Petri dishes (Greiner Bio One). Following transformation, bacteria were streaked onto the surface of the cooled agar with a plastic spreader (VWR) and allowed to dry before the plate was inverted and incubated overnight (approximately 16 h) at 37 °C. Single colonies were selected and inoculated into LB broth containing the appropriate antibiotic. Cultures were again incubated overnight at 37 °C with orbital shaking at 225 rpm for approximately 16 h. Amplified DNA plasmids were purified from the bacteria by Miniprep (**Chapter 2.3.1.1**) or Maxiprep (**Chapter 2.3.1.2**).

2.1.2 Transformation

Transformation of both strains of bacteria was done according to the respective manufacturer's instructions. Briefly, 50 µl of DH5α bacteria were thawed on ice and transferred to a sterile 1.5 ml tube (Axygen). Approximately 10 ng/µl of plasmid DNA was added to the bacteria suspension and mixed by gently flicking the tube. The bacteria were left on ice for 30 min in advance of heat shocking at 42 °C for 20 sec. Cultures were then returned to ice for a further 2 min to induce plasmid uptake after which 950 µl LB broth pre-warmed to 37 °C was added and the tubes were incubated for 60 min at 37 °C with orbital shaking at 225 rpm. Following incubation, 70 µl of the culture was applied to the surface of the LB agar plates containing the appropriate antibiotic. The remaining culture was then spun at 2,000 rpm for 2 min and 80 % of the supernatant removed. The pellet was resuspended in the remaining supernatant and plated on a second LB agar plate. Transformation efficiency was verified using the pUC19 control DNA (Invitrogen). Once dry, plates were inverted and incubated at 37 °C overnight.

SURE 2 Supercompetent bacteria were first mixed with 2 µl of β-mercaptoethanol (Sigma Aldrich) and incubated on ice for 10 min with agitation every 2 min. After the incubation was complete, 1 µl of plasmid DNA (0.1- 50 ng/µl) was added to the cells and incubated on ice for a further 30 min. Cells were heat shocked for at 42 °C for 20 sec before returning to ice for 2 min. Following this the bacteria were mixed with 900 µl pre-warmed super optimal culture (SOC) media (Invitrogen) and incubated for 60 min at 37 °C with orbital shaking at 225 rpm. The bacteria were plated out onto LB agar plates as described in **Chapter 2.1.1**.

2.1.3 Glycerol Stock Preparation

A single colony expressing the desired plasmid was selected from an agar plate and used to inoculate 5 ml LB broth supplemented with the appropriate selection antibiotic. The culture was incubated overnight at 37 °C with orbital shaking at 225 rpm for approximately 16 h. Subsequently, the culture was spun for 2 min at 2,000 rpm and the bacteria pellet resuspended in 1 ml of 50 % sterile glycerol (Sigma Aldrich) (1 glycerol: 1 H_2O). The suspended bacteria were transferred to a sterile 1.5 ml tube, snap frozen on dry ice and transferred to long term storage at -80 °C. When required, the stock could be used to inoculate sterile LB broth without allowing the bacteria to thaw.

2.2 Eukaryotic Cell Culture

2.2.1 Cell Line Maintenance

Each cell line was maintained in sterile plastic flasks (Nunc) and is described in **Table 2.1**. All tissue culture was carried out under sterile conditions in a Class II biological safety cabinet to avoid microbiological contamination.

2.2.1.1 Insect Cells

Aedes aegypti-derived Aag2, *Aedes albopictus*-derived U4.4, C6/36 and C7-10 mosquito cells were grown in L-15 (Leibovitz) growth culture medium (Life Technologies) supplemented with 10 % (v/v) foetal calf serum (FCS), 10 % tryptose phosphate broth (TPB) (Sigma-Aldrich) and antibiotics (100 U/ml penicillin/100 µg/ml streptomycin (Pen/Strep) (Life Technologies)). *Toxorhynchites amboinensis*-derived TRA-171 mosquito cells were grown in L-15: Mitsuhashi and Maramorosch insect growth medium

(Promocell) (1:1) supplemented with 10 % FCS, 0.05 % Bovine Serum Albumin (BSA) (Sigma-Aldrich), 1 % non essential amino acids and Pen/Strep. Towards the end of this project this media was required to be made in-house using the formulation found in **Chapter 2.8**. Schneider (S2) drosophila cells were grown in Schneider's media (Life Technologies) with added 10 % FCS and Pen/Strep. *Spodoptera frugiperda*-derived Sf21 cells were maintained in TC100 (Life Technologies) media plus 5 % FCS and Pen/Strep. All insect cells were maintained at 28 °C with no additional CO_2.

When cells were approximately 80 % confluent, the old growth media was removed and the cell monolayer was covered with an appropriate volume of fresh media. Cells were detached from the plastic using a cell scraper (Falcon) and transferred to a sterile 15 ml universal tube (Corning). The cells were thoroughly resuspended and 10 µl removed and diluted in 90 µl with sterile phosphate buffered saline (sPBS) (Sigma-Aldrich) and counted using a haemocytometer. The mean number of cells was calculated by the following formula:

$$Mean\ number\ of\ cells\ in\ 1\ ml = mean\ number\ of cells\ per\ quadrant\ of\ the\ Haemocytometer\ x\ 100000$$

The required number of cells were then transferred to a fresh flask with the appropriate growth medium and returned to the incubator. Cells were passaged for approximately 40-50 passages before their growth was observed to slow and they were replaced with fresh stocks from liquid nitrogen storage.

2.2.1.2 Mammalian Cells

Baby hamster kidney (BHK-21) cells were maintained for the propagation and titration of viruses. They were grown at 37 °C in a humid environment with 5 % CO_2 in Glasgow Minimum Essential Medium (GMEM) (Life Technologies) enriched with 10 % Newborn Calf serum (NBCS), 10 % TPB and Pen/Strep.

When cells were approximately 80 % confluent, the old growth media was removed and the monolayer was washed once with 5 ml versene in PBS (E&O Laboratories Ltd) which was replaced with an appropriate volume of 1 x trypsin/EDTA (Invitrogen). Cells were

incubated at 37 °C until the monolayer detached from the plastic before the trypsin reaction was neutralised by the addition of 10 ml fresh GMEM. The cells were then transferred to a 15 ml universal tube and pelleted by centrifugation at 1,500 rpm for 5 min. The supernatant was discarded and the cells thoroughly resuspended in 10 ml fresh growth media. A 10 µl aliquot was removed and diluted in 90 µl sPBS and the cells counted using a glass haemocytometer. The mean number of cells was calculated by the formula described in **Chapter 2.2.1.1**. The required number of cells were transferred to a fresh 175 cm^2 flask with fresh growth medium and returned to the 37 °C incubator. Cells were passaged for approximately 30 - 40 passages before they were replaced with fresh stocks from liquid nitrogen storage.

Table 2.1: Cell lines used in this project

Cell Name	Organism of origin	Growth Media	Growth Conditions	Reference
Aag2	*Aedes aegypti*	L-15 (Leibovitz) growth culture medium supplemented with 10 % (v/v) foetal calf serum (FCS) 8 % tryptose phosphate broth (TPB) and antibiotics (100 U/ml penicillin/100 µg/ml streptomycin [Pen/Strep])	28 °C	Peleg, 1968
U4.4	*Aedes Albopictus*			Condreay and Brown, 1986
C6/36				
C7-10				Received from Prof. R. Hardy
TRA-171	*Toxorhynchites amboinensis*	L-15: Mitsuhashi and Maramorosch insect growth medium (1:1) supplemented with 10 % FCS, 0.05 % BSA, 1 % non essential amino acids and Pen/Strep.		Kuno, 1980
$S2^+$ (infected with Flock house virus (FHV))	*Drosophila melanogaster*	Schneider's media with added 10 % FCS and Pen/Strep		Czech et al., 2008
$S2^-$ (uninfected)				Schneider, 1972
Sf21	*Spodoptera frugiperda*	TC100 media plus 5 % FCS and Pen/Strep		Vaughn et al., 1977
BHK-21	*Mesocricetus auratus*	Glasgow Minimum Essential Medium (GMEM) with added 10 % Newborn Calf serum (NBCS), 10 % TPB and Pen/Strep	37 °C with 5 % CO_2	Macpherson and Stocker, 1962

2.2.2 Freezing and Recovery of Eukaryotic Cell Lines

Cells were frozen to ensure availability of all cell lines. When they were in an active growth stage and < 80 % confluency they were harvested and counted as described prior to being pelleted by centrifugation and the supernatant discarded. The cell pellet was resuspended thoroughly in freezing media (90 % FCS and 10 % dimethylsulphide (DMSO) (BDH Prolabo), a cryoprotective agent) to give approximately 8 x 10^6 /ml insect cells and 5 x 10^6 /ml mammalian cells. Aliquots of 1 ml of the cell suspension were transferred to 1.5 ml cyrovials (Greiner Bio One) which were placed in a freezing canister (Mr. Frosty, Nalgene) containing isopropanol (VWR). This was placed at -80 °C overnight were the combination of the cyroprotective agent and the isopropanol allowed gradual freezing of the cells at approximately 1 °C/min, reducing the formation of damaging ice crystals which would cause cell damage. Finally the cyrovials were placed in the vapour phase above liquid nitrogen at -130 °C for long term storage.

Resurrection of cell lines was achieved by removing a cryovial from liquid nitrogen storage and placing it on dry ice to avoid gradual defrosting. The lid of the cryovial was slightly loosened to allow the escape of any gases and the cells were rapidly thawed by submerging the cryovial in a water bath at 37 °C. Once completely defrosted, 1 ml of the appropriate pre-warmed growth media was added. The total volume of the cryovial was transferred into a 25 cm^2 flask containing 15 ml warm growth media. The flask was placed in the appropriate incubator for 24 h after which the media was replaced to remove the freezing media. When the cells reached confluency they were then passaged as described.

2.3 Nucleic Acid Techniques

2.3.1 Plasmid DNA Extraction from Transformed Bacteria

2.3.1.1 Mini-Preparation of Plasmid DNA

Bacteria cultures were grown as described to allow amplification of plasmid DNA. Small scale isolation of plasmid DNA was achieved by using the Isolate Plasmid mini kit (Bioline) following the manufacturer's guidelines. Briefly, 1.5 ml of the 5 ml culture was transferred to a clean RNase/DNase 1.5 ml tube and pelleted by centrifuging at full speed for 1 min at room temperature. This was then repeated with a further 1.5 ml aliquot so that the final pellet was equivalent to 3 ml of bacterial culture. Any remaining supernatant was removed and replaced with 250 µl re-suspension buffer. This was mixed thoroughly by pipetting to ensure a complete removal of any cell clumps prior to the addition of 250 µl

Lysis buffer P to cause SDS/alkaline lysis of the bacteria cells. The sample was mixed again by inverting the tube six times. After ≤ 5 min, 350 µl Neutralisation buffer was added to halt the reaction and the tube was again inverted six times. The sample was then centrifuged for 10 min at maximum speed at room temperature to clear the lysate of cellular debris. The supernatant was transferred to the silica membrane of a spin column P assembled in a 2 ml collection tube and centrifuged at 12,000 rpm for 1 min. The filtrate was discarded and the spin column returned to the collection tube before 500 µl Wash buffer AP was added. The sample was then centrifuged as before. The filtrate was disposed off and replaced with 700 µl Wash buffer BP supplemented with 100 % ethanol (EtOH) (Fisher Scientific) as directed before the sample was centrifuged as before. To dry the membrane and remove any remaining traces of EtOH, the sample was centrifuged for a further 2 min at maximum speed. Finally, the spin column was transferred to a clean 1.5 ml tube and 30 - 100 µl Elution buffer added, incubated for 1 min at room temperature and spun at 12,000 rpm for 1 min. The eluted DNA was recovered and quantified (as described in **Chapter 2.3.23**) prior to storage at -20 °C.

2.3.1.2 Maxi-Preparation of Plasmid DNA

Large scale isolation of plasmid DNA from bacteria cultures was achieved by using a plasmid Maxi kit (Qiagen) following the manufacturer's guidelines with a slight deviation. Briefly, one colony of transformed bacteria was used to inoculate 100 ml LB broth in a 2 L conical flask. After incubating overnight for approximately 18 h at 37 °C with shaking at 250 rpm, the bacteria were harvested by centrifuging at 6,000 rpm for 20 min at 4 °C. The pellet was completely re-suspended in 10 ml chilled buffer P1 and transferred to a 50 ml universal tube (Corning). The bacteria were lysed by adding 10 ml of buffer P2 and vigorously inverting the tube six times. Following incubation at room temperature for 5 min, 10 ml of chilled buffer P3 was added and mixed by inverting the tube a further six times. The tube was placed on ice and incubated for 20 min. To purify the lysate, a coffee filter was placed in a QIAGEN-tip 500 and equilibrated by passing 11 ml buffer QBT through. The lysate was transferred from the universal into the coffee filter. The cleared supernatant containing the DNA passed through the coffee filter into the QIAGEN-tip while the cell debris remained in the coffee filter. Once all the supernatant has passed through the resin of the QIAGEN-tip, it was washed with 60 ml buffer QC. The bound DNA was eluted with 15 ml buffer QF and collected in a clean 50 ml universal tube. To this, 10.5 ml room temperature isopropanol was added to precipitate the DNA and the

sample was centrifuged at 5,000 rpm for 60 min at 4 °C. The pellet was washed with 5 ml 70 % EtOH to remove traces of the isopropanol and centrifuged as before. After this, the supernatant was carefully decanted and the pellet air -dried for approximately 10 min. The pellet was re-suspended in 100 - 120 µl RNase/DNase free H_2O (Sigma Aldrich), measured as described and stored until required at -20 °C.

2.3.2 DNA Digestion by Restriction Endonucleases

Restriction enzyme digests were performed by mixing approximately 1 µg DNA with one unit (U) of enzyme. One U of enzyme signifies the concentration of a specific enzyme that will cleave 1 µg DNA at its optimum temperature in 1 h. Various restriction endonucleases were used throughout this project. Digestion reactions contained the appropriate volume of 10 x restriction enzyme buffer (New England Biolabs), 10 x acetylated bovine serum albumin (BSA) (New England Biolabs), 1 U/µl restriction enzyme (New England Biolabs) and the desired concentration of DNA. The reaction was made up to an appropriate volume of RNase/DNase free H_2O. For example, a digest in a final volume of 20 µl would contain 2 µl 10 x digestion buffer, 2 µl 10 x BSA, 1 µl enzyme, X µl DNA and made up to 20 µl with H_2O. Reactions were incubated for 2 - 4 h or overnight at the optimum cleavage temperature for each endonuclease. Digestion products were analysed by agarose gel electrophoresis and purified for further use as described (**Chapter 2.3.5**).

Potentially three separate DNA topologies could result from an incomplete restriction digest and these would migrate at different rates depending on the efficiency of the digestion. In order from the furthest – nearest migration rate these are; uncut supercoiled plasmid, linearised plasmid and nicked plasmid. For completely digested DNA a single band would be present for the linearised DNA, or more depending on the number of restriction sites present.

2.3.3 Agarose DNA Gel Electrophoresis

Separation of DNA fragments was achieved by gel electrophoresis on an agarose gel. Gels were prepared by adding agarose (Promega) to Tris-acetate-EDTA (TAE) buffer (Severn Biotech Ltd) to give a final concentration of 0.8 - 2 % agarose depending on the fragment sizes to be separated. The preparation was heated to allow the agarose to dissolve and then allowed to cool till just warm to the touch before the addition of Ethidium Bromide (EtBr) (Promega) to a final concentration of approximately 0.5 µg/ml. EtBr binds to DNA and

allows the visualisation of the nucleic acids under UV light. The preparation was swirled to ensure equal distribution of EtBr and poured into a gel tray with a comb and any bubbles removed with a pipette tip before it was allowed to solidify. The gel tray was then placed into a horizontal gel electrophoresis tank (Bio-Rad), submerged in TAE buffer and the comb removed. DNA samples were mixed with 6 x loading dye (New England Biolabs) and loaded into the wells alongside a DNA ladder of the appropriate size (New England Biolabs) either 100 bp or 1 kb. An electric current was applied to the gel to separate the different nucleic acid fragments. Typically gels were run at 100 V for 30 - 40 min depending on gel percentage, size of the gel tray and size of fragments to be distinguished. Nucleic acid products were visualised using a UV transilluminator (Bio-Rad).

2.3.4 DNA Purification from Solution

DNA products from PCR reactions or enzymatic digestions were purified using the Illustra GFX PCR DNA and Gel Band purification Kit (GE Healthcare) following the recommended protocol. Briefly, 500 µl Capture buffer 3 was mixed gently with ≤ 100 µl sample before the total volume was loaded onto a microspin column. The microspin column was placed in a collection tube and spun at 14,000 rpm for 60 sec. The flow-through was then discarded and replaced with 500 µl Wash buffer type 1. The spin column was again spun as before. To increase the purity of the sample, the wash step was repeated twice. The spin column was transferred to a clean 1.5 ml tube. Between 10 - 50 µl Elution buffer was applied and incubated on the spin column at room temperature for 1 min. Samples were either eluted in RNase/DNase free H_2O (for sequencing only) or in Elution buffer type 4 (10 mM Tris-HCl [pH 8]) for all other applications. The column was then spun as before and the eluted DNA recovered, quantified as described and stored until required at -20 °C.

Large linearised DNA fragments were purified for *in vitro* transcription using high pure PCR product purification kit (Roche) following an adapted procedure to the manufacturer's recommended protocol. Approximately 7 µg viral plasmid DNA was digested in a 100 µl digestion reaction as described and successful linearisation confirmed by loading a small volume onto an agarose gel. Purification of DNA from the remaining reaction was carried out by mixing 500 µl binding buffer with the sample and loading it onto the membrane of a high pure filter tube assembled in a collection tube. Tubes were spun for 1 min at 10,000 rpm. The flow-through was returned to the filter and the tube spun a second time as before. The flow-through was now discarded and the filter washed

twice with Wash buffer. The first application was 500 µl and the second was 200 µl. The tube was spun as before after each application. Following the second wash step the filter was dried by centrifuging for 5 min at 10,000 rpm. The DNA was eluted by transferring the filter to a clean 1.5 ml tube and applying 40 µl Elution buffer pre-warmed to 70 °C. This was allowed to incubate on the column for 2 min before it was centrifuged at maximum speed for 2 min. The elution step was repeated and the purified DNA quantified as described and stored until required at -20 °C.

2.3.5 DNA Extraction and Purification from Agarose Gel

DNA extracted from agarose gel slices was purified using the Illustra GFX PCR DNA and Gel Band purification Kit (GE Healthcare) following the recommended protocol. Briefly, following separation of the DNA products by agarose gel electrophoresis as described the results were visualised using a long wavelength (365 nm) UV transilluminator. The band of interest was excised from the gel using a clean scalpel. This was done with minimal exposure time to reduce the risk of DNA damage. The gel slice was transferred to a clean 1.5 ml tube and weighed. The required volume of Capture buffer was applied at the ratio of 10 µl/10 mg of gel slice for a minimum of 300 mg. The tube was mixed by inversion and heated to 60 °C for 15 - 30 min with further mixing at 3 min intervals. Once the agarose had melted 800 µl of the gel/capture mix was transferred to a microspin column placed in a collection tube. The gel/capture mix was allowed to incubate for 1 min at room temperature before centrifuging at 14,000 rpm for 1 min. This was repeated until the total volume of gel/capture mix had passed though the column. The samples were now treated as described, from the addition of the Wash buffer.

2.3.6 Acrylamide RNA Gel Electrophoresis

A 0.75 mm 12 % denaturing acrylamide gel was prepared by dissolving 4.8 g urea (Sigma Aldrich) in 0.5 ml 10 x Tris-Borate-EDTA Buffer (TBE) (Life Technologies), 3 ml acrylamide/bis-acrylamide (40 %) (Sigma Aldrich) and 1 ml RNase/DNase free H_2O to 10 ml. This was incubated at 37 °C with shaking at 225 rpm until all the urea had dissolved before it was placed on ice for 5 - 10 min. This was then transferred and allowed to polymerise between two glass plates pre-washed overnight with 1 % sodium dodecyl sulfate (SDS) (VWR) and wiped with isopropanol to remove protein and nucleic acid contaminants. Polymerisation occurred following the addition of 100 µl 10 % ammonium

persulfate (APS) (Bio-Rad), 7.5 µl tetramethylethylenediamine (TEMED) (Sigma Aldrich) and RNase/DNase free H_2O to a final volume of 10 ml. Gels were assembled in a tank which had also been pre-washed. Gel slots were rinsed by syringe with 0.5 x TBE running buffer and the gel pre-run for 10 min at 40 V. The slots were then rinsed a second time prior to loading the samples. Each sample was diluted 1:1 with 2 x denaturing RNA loading dye (Fermentas) and boiled for 5 min at 65 °C followed by incubating for 1 min on ice. The samples were then loaded into the gel slots and a current of 150 V passed through the gel until the bromophenol blue dye front almost ran off the bottom of the gel.

2.3.7 Polymerase Chain Reaction (PCR)

Specific regions of DNA were amplified by polymerase chain reaction (PCR). Different sequences required specific primers (described in **Chapter 2.9)** and reaction cycles as. All primers were manufactured by Sigma Aldrich. Furthermore, two separate DNA polymerases, Go Taq® (Promega) or KOD (Novagen), were utilised for this project. Due to its capability for proof reading resulting in a low DNA mutation rate, KOD DNA polymerase was used to generate accurate DNA sequences for cloning and sequencing purposes while Go Taq DNA polymerase, which has no proof reading ability, was used for diagnostic PCRs. The reaction volume for both enzymes was 50 µl prepared on ice in 0.2 ml thin walled PCR tubes (Axygen).

The reaction mix for KOD DNA polymerase consisted of 5 µl 10 x enzyme buffer, 3 µl $MgSO_4$ (25 mM), 5 µl dNTP mix (2 mM each), 1.5 µl sense primer (10 µM), 1.5 µl antisense primer (10 µM), 1 µl KOD DNA polymerase (1 U/µl), X µl DNA template (~ 10 ng) made up to a final volume of 50 µl. Reactions were incubated in a PCR thermal cycler (Veriti®, Applied Biosystems) with the following protocol: 1) an initial denaturation step for 2 min at 95 °C, 2) denaturing at 95 °C for 20 sec, 3) annealing at 5 °C below the lowest primer melting temperature for 10 sec, 4) extension step at 70 °C, the duration of which was target length dependent and 5) a final extension step of 7 min at 70 °C. Steps 2 - 4 were repeated over 25 - 35 cycles depending on the template. Where required, β-actin was used as a house keeping control to indicate successful transcription of cDNA from RNA and to demonstrate relative gene expression.

The reaction mix for Go Taq DNA polymerase contained the following; 10 µl 10 x PCR buffer (Promega), 3 µl $MgCl_2$ (25 mM) (Promega), 1 µl dNTPs (10mM) (Bioline), 1.5 µl sense primer (10 µM), 1.5 µl antisense primer (10 µM), 1 µl Taq DNA polymerase (1

U/µl), X µl DNA template (~ 10 ng) made up to a final volume of 50 µl. Reactions were incubated in a PCR thermal cycler with the following protocol: 1) an initial denaturation step for 2 min at 95 °C, 2) denaturing at 95 °C for 30 sec, 3) annealing at 5 °C below the lowest primer melting temperature for 30 sec, 4) extension step at 72 °C, the duration of which was target length dependent and 5) a final extension step of 7 min at 72 °C. Steps 2-4 were repeated over 25 - 35 cycles depending on the template.

A non-template (H_2O) negative control reaction was run alongside samples for each PCR to test for contamination. All PCR products were analysed by agarose gel electrophoresis (**Chapter 2.3.3**) and temporarily stored at 4 °C prior to downstream applications.

2.3.8 Reverse Transcription PCR (RT-PCR)

Reverse transcription Polymerase Chain Reaction (RT-PCR) was performed to synthesise complimentary DNA (cDNA) from messenger RNA (mRNA) templates using the SuperScript III kit (Invitrogen) according to the manufacturer's instructions. Each reaction was performed in 0.2 ml thin walled PCR tubes and contained; 1 µl Oligo(dT) (50 µM) (Promega), 1 µl dNTP (10mM), maximum 5 µg total RNA (**Chapter 2.3.18**) and sterile, RNase/DNase free H_2O to 13 µl. Reactions were incubated for 5 min at 65 °C and further incubated on ice for a minimum of 1 min. Following a brief centrifugation the following was added; 4 µl 5 x First Strand buffer (Invitrogen), 1 µl dithiothreitol (DTT, 0.1 M) (Invitrogen), 1 µl RNase Inhibitor (Promega), 1 µl Superscript III reverse transcriptase (RT, 200 U/ml) (Invitrogen). The reaction was mixed and incubated at 50 °C for 60 min, in-activated at 70 °C for 15 min and stored at -20 °C.

2.3.9 PCR using Dig-dNTPs

PCR was also carried out using Digoxigenin- labelled dNTPs (Dig-dNTPs) (Roche) to generate probes for Northern blot analysis (**Chapter 2.4.2.4.1**). The reaction was prepared for a reaction using Go Taq® DNA Polymerase as described in **Chapter 2.3.7**; however, normal dNTPs were substituted with 5 µl Dig-dNTPs.

2.3.10 Production of Long dsRNA

DNA for the production of dsRNA molecules was generated by PCR using KOD polymerase (Novagen) using either plasmid template for *Rluc*, *Fluc* or eGFP dsRNA or 2

µl of the cDNA reaction from isolated Aag2 RNA (**Chapter 2.3.8**). Primers contained T7 polymerase recognition sites (described **Chapter 2.9**). The resulting PCR products were cloned into the pJet1.2 plasmid (**Chapter 2.3.22.1**) and sent for sequencing. Correct sequences were used to produce dsRNA using a T7 DNA dependent RNA polymerase as part of the MegaScript RNAi kit (Ambion) as described in the manufacturer's guidelines. Briefly, the reaction was prepared at room temperature in a RNase/DNase free 1.5 ml tube combining the following; 2 µl of each ribonucleotide solution (ATP, CTP, GTP and UTP), 2 µl 10 x reaction buffer, 2 µl T7 enzyme mix, 1 - 2 µg DNA template and RNase/DNase free H_2O to final volume of 20 µl. The reaction was mixed by gently flicking the tube and placing at 37 °C for 2 - 6 h. Annealing of the sense and antisense RNA strands was achieved by incubating the reaction at 75 °C for 5 min before allowing it to gradually cool to room temperature (approximately 3 h). Following this, the digestion of contaminant DNA and ssRNA was catalysed by DNase I and RNase A digestion. The tube was placed on ice and 2 µl of DNase I, 2 µl of RNase, 5 µl of 10 x Digestion buffer and 21 µl RNase/DNase free H_2O was added. The reaction was mixed again before a further incubation step at 37 °C for 60 min. Purification of the dsRNA was carried out by adding 50 µl 10 x Binding buffer, 150 µl RNase/DNase free H_2O and 250 µl 100 % EtOH to the dsRNA solution. The total volume was mixed gently by pipetting and applied to the membrane of a filter cartridge placed in a collection tube. It was allowed to pass through the membrane by centrifuging at maximum speed for 2 min and the resulting filtrate disposed of. The membrane was then washed twice with 500 µl Wash solution containing the required volume of EtOH. Centrifugation was carried out as before to allow both wash steps to pass through the filter. A final centrifugation step was completed following the second wash step to dry the membrane. The filter cartridge was transferred to a clean 1.5 ml tube and between 50 - 100 µl Elution solution pre-warmed to 95 °C was applied directly to the membrane. Following centrifugation as before, the flow through was collected and a second volume applied and centrifuged a final time. Quantification was carried out as described (**Chapter 2.3.23**) and the dsRNA stored at -20 °C.

2.3.11 Production of Long, Fluorescently Labelled dsRNA

DNA for the production of fluorescent dsRNA molecules was again generated by a PCR reaction using KOD polymerase (Novagen), a plasmid template encoding eGFP and eGFP specific primers encoding T7 polymerase recognition sites (described in **Chapter 2.9**). Correct sequences generated by PCR were used to produce dsRNA using a T7 DNA

dependent RNA polymerase (Invitrogen). This involved combining the following in a 1.5 ml RNase/DNase free microcentrifuge tube: 5 µl DNA template (0.1 – 1 µg), 4 µl 5 x T7 Reaction buffer (Invitrogen), 2 µl DTT, 1 µl RNase inhibitor, 2 µl Fluorescein RNA labelling mix (Roche), 1 µl T7 RNA polymerase and made to a total volume of 20 µl with RNase/DNase free H_2O. The reaction was incubated in the dark at 37 °C for 4 h followed by heating to 70 °C for 10 min and then allowing it to gradually cool to room temperature over a period of approximately 2 - 4 h. Finally, 1 µl of DNase (1 U/ml) (Ambion) and 1 µl RNase (Ambion) were added to remove contaminating DNA and ssRNA and the reaction was incubated for a further 30 min at 37 °C.

Purification was carried out by ethanol precipitation. The total volume of dsRNA was mixed with 20 µl RNase/DNase free H_2O, 4 µl 5 M Ammonium Acetate (NH_4Ac) (Sigma Aldrich) and 100 µl 96 % chilled EtOH. This was incubated for 30 min at -70 °C before the reaction was centrifuged for 15 min at 14,000 rpm at 4 °C. The resulting pellet was washed with 500 µl 70 % EtOH and centrifuged again for 5 min as before. Following this the pellet was air dried and re-suspended in 25 µl RNase/DNase free H_2O and stored in the dark at -20 °C.

2.3.12 Production of Long, Radio-labelled dsRNA

Molecules of dsRNA incorporating a radio-labelled dNTP were produced for the *in vitro* dicer cleavage assay. All work involving radioactive material was performed behind Perspex shields in the designated radiation area wearing appropriate personal protective equipment. The reaction was prepared at room temperature in a RNase/DNase free 1.5 ml tube combining the following; 5 µl of 114 nt eGFP PCR product (0.1 - 1 µg) with T7 polymerase sites, 4 µl 5 x Transcription buffer, 2 µl DTT (0.1 M), 1 µl rNTPs (10 mM of each ATP, GTP and UTP and 0.1 mM CTP) (Promega), 3 µl α-^{32}P rCTP (Perkin Elmer), 1 µl T7 RNA polymerase, 1 µl RNase inhibitor and 3 µl RNase/DNase free H_2O. The reaction was mixed by flicking the tube gently and placed at 37 °C for 1 - 3 h. Denaturing and annealing of both the sense and antisense strands was achieved by incubating the reaction at 65 °C for 5 min before allowing it to gradually cool to room temperature (approximately 3 h). Following this, the digestion of contaminant DNA and ssRNA was catalysed by RNase digestion. The tube was placed on ice and 2 µl of DNase I and 1 µl of RNase A were added. The reaction was mixed again preceding a further incubation step at 37 °C for 30 min.

The total volume was then loaded onto a 0.75 mm 8 % native acrylamide gel which was prepared as described in **Chapter 2.3.6**. Each sample was diluted with 6 x loading dye (0.25 % bromophenol blue, 40 % glycerol and RNase/DNase free H_2O to final volume) and loaded into the gel slots before a current of 100 V was passed through the gel for approximately 3 h at 4 °C. The dsRNA molecules could then be purified from the gel as described (**Chapter 2.3.15**).

2.3.13 Production of Radio-labelled siRNAs

Molecules of 21 nt siRNA incorporating a radio-labelled dNTP were produced as a size marker for the *in vitro* dicer cleavage assay. The reaction was prepared at room temperature in a RNase/DNase free 1.5 ml tube combining the following in a 20 µl reaction; 1.7 µl of 21 nt *Fluc* siRNA (500 ng) (Qiagen), 4 µl 5 x kinase exchange buffer (Invitrogen), 2 µl γ-^{32}P ATP, 1 µl T4 polynucleotide kinase (PNK) (Invitrogen), 0.5 µl RNase inhibitor and 10.8 µl RNase/DNase free H_2O. The reaction was mixed by gently flicking the tube and placed at 37 °C for ≥ 1 h. The total volume was then loaded onto a 0.75 mm 12 % native acrylamide gel. Each sample was diluted with 6 x loading dye and loaded into the gel which was run at 4 °C at 100 V for approximately 3 h. The siRNA molecules could then be purified from the gel as described.

2.3.14 Production of Radio-labelled DNA Oligonucleotides

DNA oligonucleotide probes for RNA detection by Northern blotting were radio-labelled with γ-^{32}P ATP by assembling the following components: 1 µl DNA oligonucleotide (5 pmol), 4 µl 5 x kinase forward buffer, 2 µl γ-^{32}P ATP, 1 µl PNK made to a final volume of 20 µl with 12 µl H_2O. The reaction was incubated for > 1 h at 37 °C before the kinase was deactivated by heating to 70 °C for 10 min. Probes were column purified to remove any unincorporated nucleotides using Mini Quick Spin Oligo columns (Roche) following the manufacturer's instructions. Briefly the column was gently flicked to collect the Sephadex matrix in the base of the column and ensure it was evenly re-suspended. The cap was removed followed by the tip and the column then placed in a sterile 1.5 ml microcentrifuge tube. Following centrifugation at 5,500 rpm for 1 min to pull the matrix away from the sides of the column and to remove residual buffer, it was transferred to a fresh 1.5 ml microcentrifuge tube and the total volume of sample (20 µl) applied to the matrix in the middle of the column. The tube was centrifuged for 4 min at 5,500 rpm and the eluate

containing the labelled oligonucleotides was collected. Radioactivity was measured by exposing 1 µl to a Geiger counter (approximately 100 - 200 counts per second (cps)/µl) and 5 µl added to each hybridization reaction. Samples were stored in a Perspex box at -20 °C.

2.3.15 RNA Extraction and Purification from Acrylamide Gel

After the radio-labelled RNA had successfully passed into the acrylamide gel, the gel was dissembled from one of the glass plates and wrapped securely in cling film and placed in a tin tray on absorbent paper. In the dark room, the gel was exposed to photosensitive film (Kodak) for approximately 1 min. Following exposure of the film to the gel, the areas where the radio-labelled RNA molecules were detected were excised from the film to allow it to be used as a template for their removal from the gel. The gel fragments containing the RNA molecules were placed into clean RNase/DNase free 1.5 ml tubes and each ground using micro-pestles (Sigma Aldrich) in 100 µl 2 x PK buffer (200 mM Tris (pH 7.5) (Fisher Scientific), 300 mM NaCl (VWR), 5 mM Ethylenediaminetetra-acetic acid (EDTA) (VWR) and 2 % SDS). A further 900 µl 2 x PK buffer was added and the tube was tightly secured and incubated at room temperature with shaking for approximately 16 h. Samples were then centrifuged for 10 min at 10,000 rpm at room temperature and the supernatant transferred to a fresh 1.5 ml tube. The centrifugation step was repeated and the supernatant was distributed between two fresh 1.5 ml tubes, each contained a final volume of approximately 400 µl. To each tube, 1 µl glycogen (10 µg/µl) and 400 µl phenol/chloroform (Ambion) were added followed by vortexing for 30 sec. Samples were then centrifuged once more as before to allow phase separation. Subsequently, 75 % of the upper aqueous phase was transferred to a clean 1.5 ml tube, 2.5 x volume (1 ml) 96 % EtOH added and samples incubated at -80 °C for $\geq$ 30 min. Following the incubation, tubes were centrifuged for 10 min at 14,000 rpm at room temperature. The supernatant was disposed of and the pellet washed with 2 x volume (800 µl) 70 % EtOH prior to repeating the centrifugation step. On completion, the supernatant was carefully discarded and the pellet dried in a flow cabinet for approximately 10 min. Finally the pellets were re-suspended in 20 µl RNase/DNase free H_2O. Radioactivity was measured by exposing 1 µl to a Geiger counter and the sample stored in a Perspex box at -20 °C.

2.3.16 *In vitro* Transcription of Capped Virus Replicon RNA

Approximately 1 µg of linearised infectious DNA plasmid was used in the reaction. All Semliki Forest virus (SFV) plasmids were linearised by digestion with *Spe*I restriction endonuclease (New England Biolabs), with the exception of the modified SFV1 plasmids which were cut with *Pac*I (New England Biolabs). All capped SFV RNAs were produced by the SP6 RNA polymerase kit (Ambion). The RNA was synthesised at 37 °C for at least 4 h, using cap analogue m7G(5')ppp(5')G (Ambion) to produce capped viral genomic RNA transcripts. The composition of the reaction prepared at room temperature in a 1.5 ml tube was: X µl Enzyme-digested plasmid (approximately 1 µg), 3.0 µl 10 x SP6 Reaction buffer, 2.0 µl 10 mM m7G(5')ppp(5')G (cap), 8.0 µl rNTP mix (10 mM ATP, CTP and UTP, 2 mM GTP), 2 µl SP6 RNA Polymerase (50 U/µl) and X µl H_2O (RNase/DNase free) to a total volume of 30 µl. RNA transcripts were typically used immediately; however, when kept they were stored at -80 °C.

2.3.17 Transfection of Nucleic Acids by Liposome Uptake

Transfection procedure was carried out as previously described (Attarzadeh-Yazdi et al., 2009) Briefly, approximately 0.8 x 10^5 BHK-21 cells, 1.8 x 10^5 Aag2 cells, 2 x 10^5 TRA-171 cells, 2.7 x 10^5 S2 cells or 1.6 x 10^5 U4.4 or C6/36 cells/well were seeded in 24-well plates and incubated for approximately 16 h. Prior to transfection the growth medium was replaced with 500 µl fresh medium. The transfection reagent, Lipofectamine 2000™ (Invitrogen), was prepared in 5 ml round bottom tubes (BD Falcon) by adding 1 µl to 49 µl/well Opti-MEM serum free medium (Life Technologies) for dsRNA and siRNAs or 2 µl to 48 µl/well Opti-MEM for plasmid DNA and gently mixed. This was allowed to incubate at room temperature for 5 min. In a separate tube the required concentration of nucleic acids was prepared and added to 50 µl/well Opti-MEM and mixed gently by flicking. To this 50 µl/well of the Lipofectamine/Opti-MEM preparation was added, mixed gently by flicking and incubated at room temperature for 20 - 30 min as per the manufacturer's guidelines. On completion, the preparation was gently mixed a final time and a total of 100 µl/well applied drop wise to each appropriate well. The plate was then returned to the incubator at the appropriate temperature for 5 h following which the medium was removed and replaced with fresh complete growth medium.

2.3.18 Extraction of Total Cellular RNA- Trizol®

Isolation of cellular RNA was achieved using Trizol® reagent (Life Technologies) using the manufacturer's instructions. Cell monolayers were grown in 24-well plates and were treated by transfection of nucleic acids as appropriate. Following incubation the growth media was removed and an appropriate volume of Trizol was added. Monolayers were allowed to homogenise for 5 min to allow the dissociation of nucleic acid and protein complexes. Lysates were mixed by pipetting and transferred to a sterile RNase/DNase free 1.5 ml tube. Following this, 200 µl/1 ml Trizol chloroform was added to the lysates and the tubes shaken vigorously by hand for 15 sec. Samples were then incubated for 2 - 3 min at room temperature ahead of centrifugation at 14,000 rpm for 15 min at 4 °C. Phase separation was achieved whereby high weight DNA, fat, polysaccharides, proteins and extracellular membranes collected in an organic phase in the lower portion of the tube separated from the RNA present in the upper aqueous phase by a thin interphase. The aqueous phase was collected and transferred to a new RNase/DNase free 1.5 ml tube. Precipitation of the RNA was completed by the addition 500 µl/1 ml Trizol molecular grade isopropanol and 0.5 µl RNase free glycogen (10 mg/ml) followed by an incubation at room temperature for 10 min and centrifugation at 14,000 rpm for 10 min at 4°C. The supernatant was removed and the RNA pellet washed with 1 ml 75 % EtOH/1 ml Trizol. The tube was then vortexed and spun a final time at 14,000 rpm at 4 °C. The resulting pellet was air dried for approximately 10 min and re-suspended in 30 µl sterile RNase/DNase free H_2O.

2.3.19 Separation of Small and Large RNA Molecules from Total RNA- PEG

The total volume of RNA which had been previously isolated by Trizol as described in 20 µl RNase/DNase free H_2O were mixed with 20 µl EDTA (0.2M, pH8) and 250 µl PEG (10 % PEG 8000 (Promega), 1 M NaCl) and incubated for 60 min on ice. This was then centrifuged for 20 min at 4 °C maximum speed to pellet large RNAs and leave the small RNAs in the supernatant. Small RNAs were isolated by transferring the supernatant to a clean 1.5 ml RNase/DNase free tube and filled with 100 % EtOH. This was incubated overnight at -20 °C prior to centrifuging at maximum speed for 30 min at 4 °C. The pellet was washed twice with 500 µl 70 % EtOH and centrifuged for 10 min at 10,500 rpm at 4 °C between each one. The resulting pellet of small RNAs was finally air dried and re-suspended in 15 µl RNase/DNase free H_2O. The PEG pellet, containing the large RNAs, was washed twice with 500 µl EtOH and centrifuged for 10 min at 10,500 rpm at 4 °C

between each one. As before the resulting pellet of large RNAs was air dried and re-suspended in 15 µl RNase/DNase free H_2O. Isolated RNAs were stored at -80 °C.

2.3.20 Separation of Small and Large RNA molecules from Total RNA- mirVana™ miRNA Isolation Kit

Small RNA molecules were also isolated from total RNA samples using the mirVana™ miRNA Isolation Kit (Ambion) following the manufacturer's instructions. Briefly, 50 - 100 µg isolated total RNA was mixed with 5 x volumes lysis/binding buffer and 1/10 x volume miRNA homogenate additive by vortexing in a 1.5 ml RNase/DNase free microcentrifuge tube. This was then incubated on ice for 10 min before 1/3 x volume of 100 % EtOH was added and mixed thoroughly. The total volume was then transferred to a filter cartridge assembled in a collection tube. Following a centrifugation step at 5,000 rpm for 1 min, the eluate containing the small RNA molecules was collected in a fresh 1.5 ml tube and mixed with a further 2/3 x volume 100 % EtOH. The mixture was passed through a second filter cartridge as before and the eluate from this stage was discarded. The filter cartridge was returned to the collection tube and washed with 700 µl miRNA Wash solution 1. The cartridge was centrifuged as before and eluate discarded. The collection tube was reused for two sequential wash steps with 2 x 500 µl miRNA Wash solution 2/3 and centrifuged as before. After disposing the flow-through the cartridge was returned to the collection tube and centrifuged a final time as before to remove any residual Wash solution. The cartridge was then placed in a fresh 1.5 ml tube and 50 µl elution solution (pre-heated to 95 °C) applied, incubated at room temperature for 2 min and centrifuged 10,000 rpm for 1 min. The eluate containing the small RNAs was retained and stored at -80 °C for Northern blot analysis.

2.3.21 Isolation of Small RNAs for Deep Sequencing

Total cellular RNA was isolated by Trizol following the manufacturer's instructions as described. Approximately 6 x 10^5 Aag2 and 8 x 10^5 U4.4 cells/well were seeded in 6-well plates and were either transfected with 1 µg specific dsRNA against eGFP (720 nt) or mock transfected. The purity and concentration were obtained as described. The total RNA was loaded onto an acrylamide urea denaturing gel (15 %) as described in **Chapter 2.3.6** and RNA molecules between 18 - 37 nt were gel purified, linked to adapters and reverse transcribed prior to sequencing. Illumina Solexa deep sequencing was carried out at ARK

Genomics, University of Edinburgh (http://www.ark-genomics.org/) following manufacturer's protocol (Illumina Inc.) as previously described (Siu et al., 2011).

2.3.22 Plasmid Cloning

2.3.22.1 pJet1.2/blunt Vector Cloning

pJet1.2/blunt (Thermo Scientific) was used for blunt end cloning PCR products for sequencing or for subcloning of DNA sequences of interest. The reaction was carried out according to the manufacturer's instructions using 10 µl 2 x reaction buffer, 1 µl pJet1.2/blunt cloning vector (50 ng/µl), 1 µl DNA, 1 µl T4 DNA Ligase and made to a final volume of 20 µl. The reaction was spun briefly and incubated at room temperature for 5 min or ≤ 30 min for DNA products of > 3 kb. The ligation reaction was transformed into DH5α bacteria as described in **Chapter 2.1.2** using 2.5 µl of the reaction.

2.3.22.2 Plasmid Cloning by Restriction Digest

Cloning into plasmid backbones (pIB/V5-His (Invitrogen) or pSP64 Poly A (Promega)) was done by digesting both the plasmids and the DNA inserts with appropriate restriction enzymes (New England Biolabs). Reactions were carried as described (**Chapter 2.3.2**). Following digestion, the samples were run on an agarose gel, extracted and purified as described (**Chapter 2.3.3** and **Chapter 2.3.5**). Ligation of the insert into the new plasmid backbone was performed by T4 DNA ligase (Promega) in a reaction containing 1 µl T4 DNA ligase, 2 µl buffer, and H_2O to a final volume of 20 µl. DNA insert and plasmid were mixed together at different ratios; either 3 insert: 1 plasmid or 2 insert: 1 plasmid. The reactions were incubated for 30 min at room temperature before immediately transforming 2.5 µl of the reaction into DH5α bacteria as described.

2.3.22.3 Viral Plasmid Cloning by Restriction Digest

The original pSFV1 plasmid received from Professor Peter Liljeström (Karolinska Institute, Sweden) was kindly modified by Margit Ool (Institute of Technology, University of Tartu, Estonia) for the insertion of recombinant *Ae. aegypti* Argonuate-2 (Ago-2) and Dicer-2 (Dcr-2) proteins. While SFV1-Ago-2-zsGreen and SFV1-Dcr-2-mCherry were received along with the modified pSFV1, SFV1-Ago-2-V5 and SFV1-Dcr-2-V5 were cloned in house. This was achieved by digesting the modified pSFV1 with *Nru*I and *Spe*I

(New England Biolabs). Ago-2-V5 and Dcr-2-V5 were previously subcloned into pJet1.2/blunt as described and cut with either *Nru*I and *Xba*I or *Hind*III and *Xba*I (New England Biolabs) respectively. Ligation of Ago-2-V5 could be carried out directly as the *Xba*I site is complementary to the *Spe*I site. The Dcr-2-V5 cDNA was first treated with Klenow (New England Biolabs) which has 3'→5' exonuclease activity. The reaction was performed according the manufacturer's guidelines where Dcr-2-V5 inserted into pJet1.2/blunt was digested with *Hind*III prior to blunting by Klenow (1 U/µg DNA). The reaction was incubated for 15 min at 25 °C before the enzyme was inactivated at 75 °C for 20 min. Finally pJet1.2/blunt-Dcr-2-V5 was digested with *Xba*I. Ligation of both tagged proteins into the modified SFV1 plasmid was performed by T4 DNA ligase as described before immediately transforming 2.5 µl of the reaction into DH5α bacteria as described.

2.3.22.4 pGL3-PUb Vector Modification

pGL3-PUb was gratefully received from Professor Zach Adelman (Fralin Life Science Institute and Department of Entomology, Virginia Polytechnic Institute and State University, United States) and has been described previously (Anderson et al., 2010). The vector did not contain a multiple cloning site (MSC) for the insertion of foreign genes and therefore one was designed containing the following sites: 5' *Nhe*I, *Xho*I, *Bgl*II, *Sac*II, *Pac*I, *Xba*I 3'. These sites were inserted after the PUb promoter by performing a mutational PCR using the primers described in **Chapter 2.9**. Following PCR the products were run, extracted and purified from an agarose gel as described in **Chapter 2.3.5**. The purified DNA was treated with *Dpn*I (New England Biolabs) to remove methylated DNA. Ligation of the PCR products was achieved by T4 ligase as described in **Chapter 2.3.22.2**. Following ligation, 2.5 µl of the reaction were transformed into DH5α bacteria as described.

2.3.23 Quantification of Nucleic Acids

The yields of all nucleic acids (dsRNA, ssRNA and dsDNA) were quantified using a NanoDrop ND-1,000 spectrophotometer (Thermal Fisher Scientific). An aliquot of 2 µl from each sample was placed on the measurement pedestal and a reading made to obtain purity and concentration. Samples were considered 'pure' if the 260/280 ratio was ~1.8 for DNA and ~2.0 for RNA.

2.3.24 Sequencing of Plasmid DNA and PCR Products

Verification of successful amplification and cloning of the gene of interest was achieved by sending DNA plasmid or PCR samples, along with appropriate primers, to DNA Sequencing & Services at the University of Dundee (http://www.dnaseq.co.uk/home.html). The resulting sequences were analysed using BioEdit software and Basic Local Alignment search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Further details of the recipes of common solutions can be found in **Chapter 2.8**.

2.4 Molecular Techniques

2.4.1 Western Blot

2.4.1.1 Sample Preparation

The appropriate cell lines were seeded in 24-well plates and infected or transfected as required. They were incubated as appropriate following which they were lysed by removing the growth media and directly adding 30 µl/well of either Laemmli buffer (Bio-Rad) with 5 % β-mercaptoethanol or 2 x loading buffer (0.125 M TRIS HCl (pH 6.8), 4 % SDS, 20 % glycerol, 10 % β-mercaptoethanol, 10 mg bromophenol blue). The lysates were transferred to a 1.5 ml tube, boiled for 10 min at 100 °C before centrifugation for 5 min at 14,000 rpm. If necessary, samples were placed in temporary storage at -20 °C until all those required were prepared.

2.4.1.2 Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis

Unless stated, all gels used were NuPAGE® 4 - 12 % Bis-Tris Mini gels (Life Technologies). When SDS-PAGE gels were prepared in house a 10 % gel was set up using Bio-Rad's Mini-Protean II apparatus. These consisted of two parts; the upper stacking gel and the lower resolving gel. The resolving gel was prepared by combining 2.43 ml distilled H_2O, 1.25 ml acrylamide/bis-acrylamide (40 %), 1.25 ml 1.5 M Tris-HCl (pH 8.8), 50 µl 10 % SDS per gel. Just prior to use polymerisation was initiated by adding 50 µl 10 % APS and 5 µl TEMED. The solution was transferred between two glass plates approximately 0.75 mm apart. A gap of approximately 2 cm was left between the top of the gel and the top of the plates. This was filled with H_2O to ensure the gel set level. Once the resolving

gel had set (approximately 20 min) the H_2O layer was discarded and replaced with the stacking gel. This consisted of 1.598 ml distilled H_2O, 252 µl acrylamide/bis-acrylamide (40 %), 625 µl 0.5 M Tris-HCl (pH 6.8), 25 µl 10 % SDS per gel. As with the resolving gel, polymerisation was initiated by adding 75 µl 10 % APS and 5 µl TEMED shortly before transferring to the two gel plates on top of the resolving gel. A 10 well comb was immediately inserted to create the lanes and the gel was allowed to polymerise for approximately 20 min.

The gel was placed into an electrophoresis tank and covered with the appropriate running buffer (for in house gels 1 x SDS-PAGE running buffer (25 mM Tris, 0.25 M Glycine (Calbiochem), 0.1 % SDS) was used, while NuPAGE gels were run in 1 x NuPAGE MOPS SDS running buffer (Life Technologies)). The samples were prepared as described and loaded into the gel (30 µl/well) alongside 5 µl Hyperpage Pre-stained Protein Marker (Bioline). The voltage was initially set to 100 V until the samples past into the gel where after it was increased to 120 V until the bromophenol blue dye front had run off the resolving gel.

2.4.1.3 Protein Transfer

The gel was removed from between the two glass plates and the stacking gel was disposed of. Two protein transfer techniques were used; wet transfer for large proteins > 100 kDa or semi-dry for smaller proteins. Unless otherwise stated wet transfer was carried out. This involved assembling the transfer cassette submerged in a tray flooded with 1 x wet transfer buffer (50 mM Tris, 375 mM Glycine, 20 % Methanol (VWR)). The transfer cassette contained, in order from the cathode to the anode; a sponge, an extra thick Whatman paper (Bio-Rad), the gel, a Hybond ECL nitrocellulose membrane (GE Healthcare) (previously activated in distilled H_2O), a second Whatman paper and a second sponge. Once assembled the transfer cassette was orientated within the electrophoresis tank (Bio-Rad) to allow the proteins to transfer from the gel to the membrane. An ice pack was added and the tank filled with 1 x wet transfer buffer. A current of 250 mA was applied for 3 h.

Semi-dry transfer was performed by soaking 2 extra thick whatman papers, cut to the size of the gel, in semi dry transfer buffer (48 mM Tris, 39 mM Glycine, 0.0375 % SDS, 20 % Methanol). One was placed on the blotting plate of the semi-dry blotting machine (Bio-Rad) followed by the layer of Hybond ECL nitrocellulose membrane, cut to the appropriate

size and soaked in distilled water, the gel and finally the second whatman paper. A steady voltage of 15 V with a maximum current of 0.31 A was applied for 30 min.

2.4.1.4 Immuno-Detection

Following transfer, the blotted nitrocellulose membranes were washed (3 x 5 min in PBS + 0.1 % Tween®20 (Sigma Aldrich) (PBST) with rocking to remove residual transfer buffer prior to blocking in 2 % blocking buffer (2 % (w/v) milk powder (Sigma Aldrich) in PBST) with rocking at 4 °C overnight or at room temperature for 1 h. The membrane was washed as before prior to covering with an appropriate volume of primary antibody diluted in blocking buffer. The antibodies used are described in **Table 2.2**. All primary antibodies were incubated overnight at 4 °C. Following incubation, the membranes were washed as before (3 x 5 min) and covered with secondary antibody diluted in blocking buffer. This was allowed to incubate at room temperature for approximately 1 h. Following this, the membrane was washed a final time as described. The secondary antibody used was conjugated to horse radish peroxidise (HRP) which could be detected by using either Pierce ECL Western Blotting Detection kit (Thermo Scientific) or Pierce West Pico Western Blot kit ECL (Thermo Scientific). Due to the increased sensitivity of the Pierce West Pico kit it was used to detect the endogenous RNAi proteins where as the ECL Advance kit was used for detection of transfected V5 protein or SFV-nsP3. With both kits the membranes were removed from the PBST and excess buffer carefully removed before covering the membrane in an appropriate volume of ECL solution (reagent one 1: 1 reagent two). After an incubation of 5 min any excess detection reagents were removed before the membrane was placed between two pieces of Melinex® polyester film (PSG Group Ltd). In the dark, a sheet of photosensitive film was placed over the membrane and allowed to incubate before developing with a Konica Minolta SRX-101-A film processor.

Table 2.2: Description of antibodies used for Western Blot

Target	Obtained	Host	Isotype	Label	Dilution	Incubation time
V5	Sigma	Mouse	Monoclonal IgG	-	1 in 2,000	4 °C overnight
Ago-1	Abmart				1 in 100	
Ago-2						
Dcr-1						
Dcr-2						
mCherry	Abcam				1 in 2,000	
ZsGreen	CloneTech	Rabbit	Polyclonal		1 in 1,000	
SFV-nsP3	Dr. Tero Ahola, University of Helsinki, Finland				1 in 2,000	
Rabbit IgG	Abcam	Goat	Monoclonal IgG	Horse Radish Peroxidase	1 in 5,000	Room temperature for 1 h
Mouse IgG	Sigma	Rabbit	Polyclonal		1 in 3,000	

2.4.2 Northern Blot

All the procedures described involve RNase free conditions.

2.4.2.1 Sample Preparation

Tissue culture samples were prepared as appropriate and following a sufficient incubation period the cells were detached by scraping and centrifuged at 2,500 rpm for 6 min at room temperature. The supernatant was removed and 2 ml of Trizol reagent added to the pellet. This was then divided between two 1.5 ml RNase/DNase free microcentrifuge tubes. Total RNAs were isolated and purified as described in **Chapter 2.3.18**. Equal concentrations and volumes of isolated cellular RNA samples were prepared by diluting 1:1 with 2 x RNA loading dye and were loaded onto a 12 % denaturing acrylamide RNA gel and separated as described in **Chapter 2.3.6**. The gel was removed from between the two glass plates and the presence of RNA molecules were visualized with EtBr staining prior to transferring to membrane. The gel was placed in approximately 70 ml 0.5 x TBE with 4 µl EtBr and incubated for 20 min with rocking at room temperature.

2.4.2.2 Blotting of Acrylamide RNA Gel

The RNA was transferred by semi-dry blotting to a nitrocellulose (Hybond™-N+) membrane (GE Healthcare) placed on top of three Whatman papers soaked in 0.5 x TBE. The acrylamide RNA gel was placed on top of the membrane followed by a further three soaked whatman papers. A steady voltage of 10 V with a maximum current of 0.33 A was applied for 1 h.

2.4.2.3 Cross linking RNA to Nitrocellulose Membrane

Transferred RNA was either UV cross linked (by placing it on a UV screen for 1 min each side of the membrane) or chemically cross- linked with carbodiimide as described by (Pall et al., 2007). Briefly, 122.5 µl Methlimidazole (Sigma Aldrich) and 10 ml RNase/DNase free H_2O were combined in a 50 ml falcon tube and the pH adjusted to 7.5 - 8.2 with HCl. To this, 0.373 g 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Sigma Aldrich) was added and the final volume of the solution was adjusted to 12 ml with RNase/DNase free H_2O. The entire volume of solution was applied to a piece of Whatman paper, cut to slightly larger than the membrane, placed on a sheet of cling film. The membrane was added on top of the Whatman paper with the RNA side facing upwards. The orientation of the gel on the membrane was marked with a pencil and the area between the two dye fronts was denoted. The membrane and the paper were quickly wrapped in cling fim to prevent the membrane drying and placed on a tray inside the hybridisation oven for approximately 1 h (< 2 h) at 60 °C. Once this was completed the membrane was washed twice with RNase/DNase free H_2O for 5 min per wash and allowed to dry fully. The membrane was then wrapped in cling film and stored at -20 °C until hybridisation was carried out.

2.4.2.4.1 DIG-labelled DNA Probe Hybridization

Membranes were pre-hybridised for > 5 min at 68 °C in a small hybridisation tube containing 7 ml of PerfectHyb™ Plus hybridization buffer (Sigma Aldrich). Probes were denatured by heating to 100 °C for 10 min followed by placing on ice for 2 min. In this instance a mix of five probes were added per membrane (SFV-nsP1, -nsP2, -nsP3, E1, C/E3). A total volume of 25 µl DIG-labelled probes (5 µl of each) was directly added into the hybridization buffer and incubated at 68 °C overnight. Following this, the buffer and probe were discarded and the membrane washed once with a low stringency wash buffer (2 x saline- sodium citrate (SSC) (Life Technologies) and 0.1 % SDS) at room temperature

for 5 min. Blots were then transferred to small plastic boxes to be washed with approximately 10 ml Washing buffer (Maleic acid buffer (100 mM Maleic acid (Sigma Aldrich), 150 mM NaCl, adjusted to pH 7- 8 with NaOH pellets (Fisher Scientific)) with 0.3 % Tween) for 15 min at room temperature with rocking. Following this detection was performed by pre-incubating membrane in approximately 25 ml 1 % blocking buffer (maleic acid buffer with blocking reagent (Roche)) for 1 h. The blocking buffer was then discarded and replaced with fresh 1 % blocking buffer containing anti-DIG antibody conjugated to alkaline phosphatase (AP) (1: 20,000) (Roche) for 1 h at room temperature. The membrane was then washed four times in washing buffer as described followed by a final wash in AP buffer (100 mM NaCl, 5 mM MgCl (VWR), 100 mM Tris- HCl) for 5 min. Detection was performed by applying 10 ml AP buffer containing 200 µl NBT-BCIP detection agent (Roche). This was allowed to incubate in the dark with rocking until a signal was detected. Development was halted by washing the membrane in distilled H_2O. Images were acquired using the gel doc system (Bio-Rad).

2.4.2.4.2 Radio-labelled DNA Probe Hybridisation

Hybridisation with a radio-labelled oligonucleotide probe by first pre-hybridizing the membrane for > 5 min at 42 °C in a small hybridization tube containing 7 ml of PerfectHyb™ Plus hybridisation buffer. The radio-labelled oligonucleotide probe was then directly added to the hybridization buffer and incubated at the appropriate temperature overnight. Following this, the buffer and probe were discarded and the membrane washed as described for DIG probed membranes. Following this step, blots probed with radio-labelled oligonucleotides were dried before being wrapped in cling film, placed in a development cassette and exposed in the dark to photosensitive film. The cassette was then placed at -70 °C for 4 days to allow signal detection to develop.

2.4.3 *In vitro* Translation by TNT Coupled Reticulocyte Lysate

A coupled transcription/translation approach to protein production was attempted using the TNT® SP6 coupled Rabbit Reticulocyte Lysate System (Promega) following the manufacturer's protocol. Briefly, the following components were added and mixed together in a RNase/DNase free 1.5 ml tube; 25 µl TNT Rabbit reticulocyte lysate, 2 µl TNT Reaction buffer, 1 µl TNT SP6 RNA polymerase, 1 µl amino acid mixture minus methionine (1 mM), 2 µl ^{35}S methionine (1,000 Ci/mmol at 10 mCi/ml, Perkin Elmer), 1 µl

RNasin ribonuclease inhibitor (40 U/µl), 2 µl DNA template (0.5 µg/µl) and RNase/DNase free H_2O to a final volume of 50 µl. The reaction was incubated at 30 °C for 90 min. The translation products were diluted in 2 x loading dye (0.125 M TRIS HCl (pH 6.8), 4 % SDS, 20 % glycerol, 10 % β-mercaptoethanol, 10 mg bromophenol blue), boiled for 3 min at 90 °C then 5 µl run on a NuPAGE® NOVEX® Bis-Tris denaturing protein gel (Invitrogen). Gel electorphoresis was carried out at 30 mA until the bromophenol blue dye front reached the bottom of the gel. Subsequently, the gel was disengaged from the supporting plates and placed in a plastic box with fixing solution (50 % methanol, 10 % acetic acid, to final volume with H_2O) to cover it. This was agitated slowly on an orbital shaker for 30 min before replacing the fixing solution with drying solution (7 % acetic acid, 7 % methanol, 1 % glycerol, to final volume with H_2O) which was applied for 5 min to prevent the gel from cracking during drying. The gel was placed on three sheets of whatman paper, covered in a plastic film and placed on gel dryer (Bio- Rad) to dry at 80 °C for 2 h. The gel was not removed from the gel dryer until it had completely cooled, after a further 1 h. Detection was performed by exposing the fixed gel to a phosphor imaging screen (Bio-Rad) for ≥ 16 h.

2.4.4 *In vitro* Dicer Cleavage Assay

Tissue culture samples were prepared as necessary. Following the required incubation time, the media was removed, the cells re-suspended in sPBS and centrifuged at 1,500 rpm for 5 min. The supernatant was removed and the pellet washed and centrifuged as before. The resulting pellet was re-suspended in 200 µl 1 x lysis buffer (10 mM Magnesium acetate (MgAc) (Sigma Aldrich), 150 mM Hepes-KOH (pH 7.5) and homogenised with a micro-pestle. Cell debris was removed by a further centrifugating at 14,000 rpm at 4 °C for 20 min. The supernatant was transferred to a clean tube and the samples were then stored with minimal freeze thawing at -80 °C. The following were combined and mixed gently in an RNase/DNase free 1.5 ml tube using RNase/DNase free tips; 0.3 µl 20 mg/ml creatine phosphate kinase in 1 x storage buffer (40 mg/ml lyophilized creatine kinase (Calbiochem) in 2 x storage buffer (ice-cold 40 mM tris-acetate (pH 6.8), 200 mM EDTA, 20 mM β-mercaptoethanol) diluted 1:1 in an equal volume of 100 % ice-cold glycerol), 1 µl DTT (1 M), 10 µl creatine phosphate (12 mg/100 µl) (Calbiochem), 20 µl 5 x lysis buffer, 20 µl glycerol, 2 µl RNase Inhibitor, 2 µl ATP (100 mM) (Thermo Scientific) and 4.7 µl RNase/DNase free H_2O. In separate clean 1.5 ml tubes the following were assembled and mixed gently; 5 µl cell extract, 3 µl creatine mix, 3 µl ^{32}P labelled dsRNA (~16.2 pCi), 1 µl

H_2O. A reaction was also prepared with the cell extract component omitted and the remaining volume made up with H_2O for the purification of dsRNA alone. The reactions were incubated overnight at 28 °C. Following this 200 µl 2 x PK buffer (200 mM Tris (pH 7.5), 300 mM NaCl, 5 mM EDTA, 2 % SDS), 1 µl glycogen (10 mg/ml) (Roche) and 0.3 µl proteinase K (10 mg/ml) (Sigma Aldrich) were added and reactions further incubated for 10 min at 65 °C. To this, 200 µl phenol/chloroform/isoamylalcohl (25:24:1) (Ambion) were added prior to the tubes being vortexed for 15 sec and centrifuged 10,000 rpm for 10 min. The water phase was transferred to a new 1.5 ml tube containing 450 µl ice-cold 96 % EtOH and the tube centrifuged for 10 min at 13,000 rpm. Subsequently, the supernatant was removed and replaced with 400 µl ice cold 70 % EtOH. Samples were centrifuged as before and the supernatant removed. Pellets were air dried in a fume cabinet for approximately 5 min, resuspended in 15 µl 1 x FDE loading buffer and boiled for 5 min at 65 °C before placing on ice for ≥ 2 min. Samples were loaded onto a 0.75 mm 12 % acrylamide denaturing gel as described (**Chapter 2.3.6**). Electrophoresis of the RNA was performed at 200 V while the gel was chilled by submerging the tank in ice cold water. The gel was placed on three sheets of whatman paper, covered in Melinex® polyester film and placed on gel dryer to dry at 80 °C for 2 h. Detection was performed by exposing the gel to a phosphor imaging screen for ≥ 16 h and viewed using a personal molecular imager (PMI) (Bio-Rad).

2.4.5 Immunostaining

Cells were either seeded in glass bottom 24-well plates (Greiner Bio-One) or on 13 mm diameter glass coverslips (VWR) in plastic 24-well plates. These were left overnight after which they were either infected or transfected and incubated as required. Neutral buffered 10 % formalin solution (Sigma Aldrich) was gently applied to each well and incubated for 1 h for BHK-21 or 30 min for insect cells. The formaldehyde was discarded and the monolayer was washed three times with PBS (3 x 5 min) with rocking at room temperature. Cell membranes were permeabilised by applying 0.3 % Triton®X-100 (Sigma Aldrich)/PBS for 20 min with rocking. Monolayers were washed as before and 700 µl of blocking agent (either Cas-block (Invitrogen) or 5 % FCS/PBS) applied for 30 min with rocking at room temperature. The blocking agent was removed and replaced with 700 µl primary antibody diluted in the blocking agent to the appropriate dilution (**Table 2.3**). The primary antibody was allowed to incubate with rocking for 2 h at room temperature or overnight at 4 °C. Cells were washed with PBS (3 x 10 min) and the appropriate Alexa

Fluor® labelled secondary antibody applied, diluted appropriately in the blocking agent, for 1.5 h with rocking at room temperature protected from the light. This was discarded and the cells washed a final time with PBS (3 x 10 min). Coverslips in plastic 24-well plates were removed, dried briefly and mounted on glass slides using VECTASHIELD® Mounting media plus DAPI (4',6-diamidino-2-phenylindole) (Vectro Laboratories) to allow visualisation of the cell nuclei. These were then permanently sealed with clear nail polish around the perimeter. Monolayers grown directly in glass well dishes were covered with a drop of mounting media and a coverslip applied. Samples were stored at 4 °C in the dark until the results were viewed with the Zeiss LSM 710 or LSM 510 confocal microscopes using the appropriate lasers as described (**Chapter 2.6**).

Table 2.3: Description of antibodies used for Immunostaining.

<table>
<tr><th>Antibody</th><th>Target</th><th>Obtained</th><th>Host</th><th>Isotype</th><th>Label</th><th>Dilution</th><th>Incubated</th></tr>
<tr><td rowspan="2">Primary</td><td>SFV-nsP3</td><td>Dr. Tero Ahola, University of Helsinki, Finland</td><td>Rabbit</td><td>Polyclonal</td><td>-</td><td>1/800</td><td rowspan="2">2 h or over night</td></tr>
<tr><td>dsRNA (J2)</td><td>Scicons</td><td>Mouse</td><td>Monoclonal IgG1</td><td>-</td><td>1/500</td></tr>
<tr><td rowspan="4">Secondary</td><td>Rabbit</td><td rowspan="4">Molecular Probes, Life Technologies</td><td>Goat</td><td rowspan="4">Polyclonal IgG</td><td>Alexa Fluor 594</td><td rowspan="4">1/1,000</td><td rowspan="4">1.5 h</td></tr>
<tr><td>Mouse</td><td rowspan="3">Donkey</td><td rowspan="3">Alexa Fluor 488</td></tr>
<tr><td>Rabbit</td></tr>
<tr><td>Mouse</td></tr>
</table>

2.5 Virus Techniques

2.5.1 Semliki Forest virus (SFV) and Virus Replicon Particles (VRPs)

Both Semliki Forest virus (SFV) and virus replicon particles (VRPs) were used as part of this project. All recombinant strains of SFV were based on the SFV4 backbone (Liljeström and Garoff, 1991). These were prepared from cDNA plasmids gratefully provided by Prof. Andres Merits (University of Tartu, Institute of Technology, Estonia). Details can be found in **Table 2.4** and **Figure 2.1**. Amplification of the plasmid DNA was achieved by transforming into chemically competent DH5α *E.coli* (**Chapter 2.1.2**), preparing growth cultures and purifying the DNA (described in **Chapter 2.3.1.2**). All plasmids, with the exception of one, were linearised with an appropriate endoribonucleases and used as a template for *in vitro* transcription to produce capped RNA (described in **Chapter 2.3.16**).

Plasmid DNA or *in vitro* transcribed RNA was electroporated in BHK-21 cells as described. As SFV4(3H)-*Fluc* contains a Cytomegalovirus (CMV) promoter the infectious mRNA transcript can be transcribed directly following electroporation into BHK-21 cells so no cap is required.

Table 2.4: Description of viruses and VRPs used as part of this project

Virus / VRP	Feature
SFV4(3H)-*Rluc*	Renilla luciferase gene (*Rluc*) is encoded at the 3' end of the SFV nsP3 gene. *Rluc* is cleaved following translation and replication of the SFV4 genome. Therefore the expression of *Rluc* is an indirect measure of SFV4 replication.
SFV4(3H)-*Fluc*	Firefly luciferase gene (*Fluc*) is encoded at the 3' end of the SFV nsP3 gene. *Fluc* is cleaved following translation and replication of the SFV4 genome and is therefore an indirect measure of SFV4 replication. It is transcribed from the CMV promoter.
SFV4(3H)-*Rluc*-p19	As SFV4(3H)-*Rluc* with the addition of the tombusvirus p19 protein inserted after a duplicated subgenomic promoter following the structural proteins.
SFV4-steGFP	The gene encoding enhanced Green Florescent Protein (eGFP) is inserted between the capsid and p62 (E2/E3) coding regions in the structural ORF.
SFV4(3F)-ZsGreen	The gene which encodes zsGreen is fused to the nsP3 gene at the 3' terminus. During viral replication nsP3 is translated fused to zsGreen and can be used to indicate the location of nsP3.
SFV1-*Rluc*	SFV1 replicon expressing *Rluc* under the control of the subgenomic promoter.
SFV1-ZsGreen	SFV1 replicon expressing zsGreen fluorescent protein under the control of the subgenomic promoter.
SFV1-Dcr-2-mCherry	SFV1 replicon expressing *Ae. aegypti* Dcr-2 fused to mCherry under the control of the subgenomic promoter. See **Chapter 5**.
SFV1-Ago-2-ZsGreen	SFV1 replicon expressing *Ae. aegypti* Ago-2 fused to zsGreen under the control of the subgenomic promoter. See **Chapter 5**.
SFV1-Ago-2-V5/N	SFV1 replicon expressing *Ae. aegypti* Ago-2 fused to V5 tag on the N terminus under the control of the subgenomic promoter. See **Chapter 5**.
SFV1-Ago-2-V5/C	SFV1 replicon expressing *Ae. aegypti* Ago-2 fused to V5 tag on the C terminus under the control of the subgenomic promoter. See **Chapter 5**.

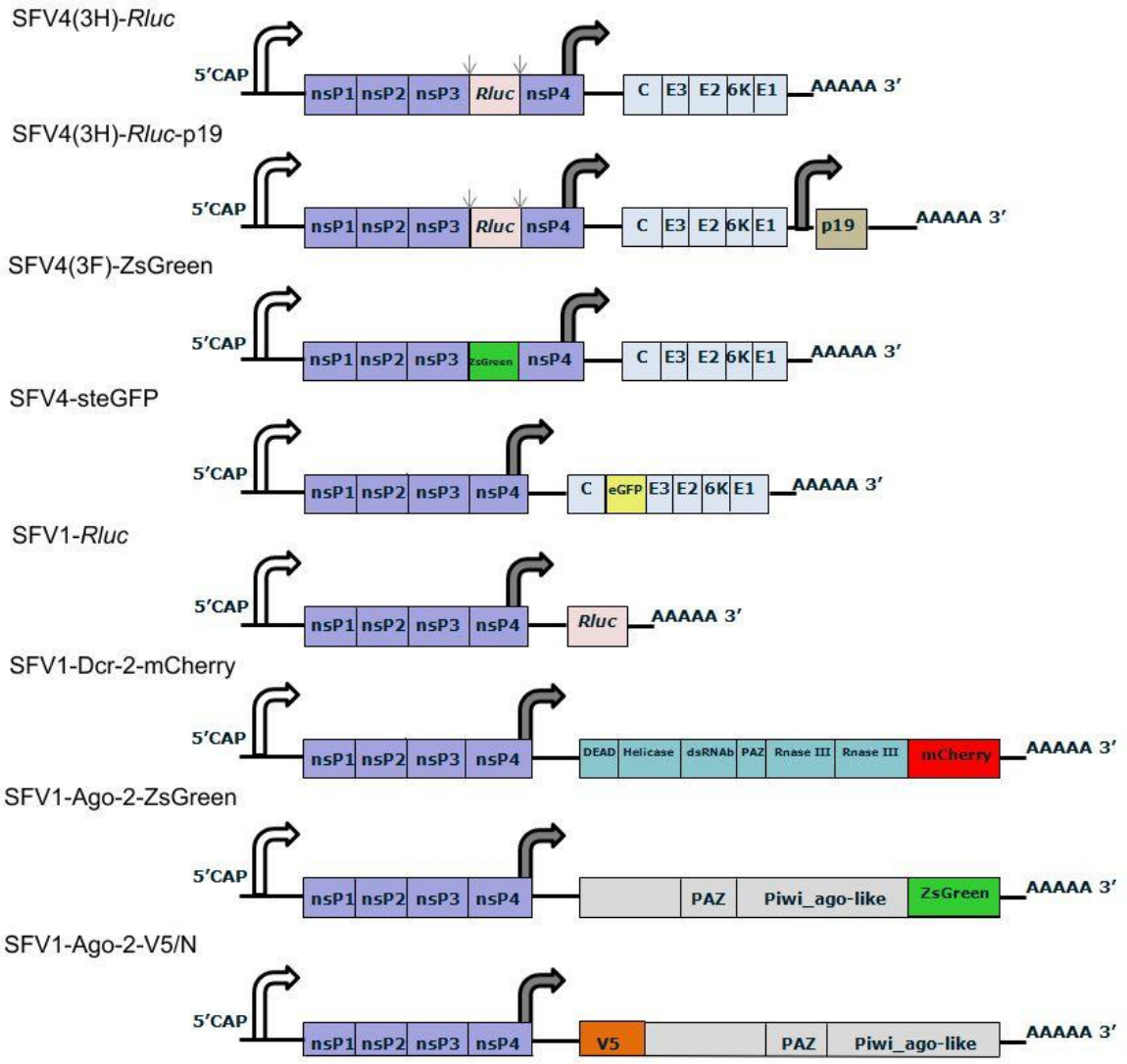

Figure 2.1: Schematic illustration of some of the viruses and VRPs used as part of this project. Viral non-structural proteins (nsP1- 4) are shown in purple and the structural proteins (capsid, C; the envelope glycoproteins, E1-3; and the 6K protein) are in pale blue. nsP2 cleavage sites for the release of *Rluc* during replication of SFV4(3H)-*Rluc* and SFV4(3H)-*Rluc*-p19 are indicated (↓). Example structures are given. SFV4(3H)-*Rluc* and SFV4(3H)-*Fluc* are similar with the appropriate reporter gene substitution as are SFV1-*Rluc*, SFV1-ZsGreen and SFV1-Ago-2-ZsGreen/SFV1-Ago-2-V5/C.

2.5.2 Propagation of Recombinant SFV strains and Production of VRPs

BHK-21 cells were grown until approximately 80 % confluent in an appropriate number of 175 cm^2 tissue culture flask. Once confluent the BHK-21 cells were trypsinized and counted as described (**Chapter 2.2.1.2**). A density of 1 x 10^7 cells/800 µl was prepared per flask and thoroughly resuspended in ice cold PBS. The *in vitro* transcribed RNA (prepared as described in **Chapter 2.3.16**) or DNA (in the case of the CMV based plasmid) was mixed with 800 µl of the cell suspension in sterile 1.5 ml tubes. Viral RNA was electroporated as a single plasmid. However, the VRP production required the combination of two capped RNAs using the split helper system; the first RNA contained the SFV4 non-structural proteins and the gene insert of interest while the second RNA encoded the SFV4 structural proteins. There were two options available for delivery of the structural proteins; either by the helper system (using the Helper 1 plasmid providing all of the structural proteins; C-E3-E2-6K-E1) (Berglund et al., 1993), or the split-helper system (were the capsid and envelope glycoproteins are separately encoded by different plasmids) (Smerdou and Liljeström, 1999). The helper system was used to generate SFV1-Dcr-2-mCherry and SFV1-Ago-2-ZsGreen, while all other VRPs were produced by the split-helper system. The capped RNAs from the *in vitro* transcription of these two plasmids were mixed together before the cell suspension was added. The RNA/cell preparation was allowed to incubate on ice for 2 - 3 min. A 400 µl aliquot of the mixture was then transferred to a 0.4 cm electroporation cuvette (Flowgen Bioscience) and pulsed using a Bio-Rad Gene Pulser Xcell electroporator. Electroporation consisted of two pulses of a square wave of 850 V for 0.4 ms with an interval of 5 sec between pulses. The electroporated BHK-21 cells were carefully transferred to a fresh 175 cm^2 tissue culture flask containing 20 ml pre-warmed GMEM with 10 % NBCS. The process was repeated with the remaining 400 µl aliquot which could then be transferred to the same flask. Each flask was returned to the 37 °C incubator and the supernatant containing the viral particles was harvested after 24, 48 and 72 h post electroporation. Cytopathic effect (CPE) was expected after 24 - 48 h post virus electroporation but no pathogenic effect was observed during the production of SFV VRPs. The supernatant was stored temporarily at 4 °C prior to purification.

2.5.3 Purification of SFV Recombinant viruses and VRPs

BHK-21 supernatant containing virus or VRPs was clarified by centrifuging at 2,000 rpm for 30 min at 4 °C. The supernatant was then transferred into a sterile bottle of appropriate volume containing 23 g/l NaCl and 70 g/l PEG 8000 and incubated overnight at 4 °C with

stirring. Following this it was centrifuged again at 8,000 rpm for 30 min at 4 °C. The supernatant was then discarded and the precipitate containing the PEG-virus pull down was re-suspended in 10 ml GMEM with no additional supplements. The PEG-virus suspension could then be transferred to an ultracentrifuge tube (Beckman Coulter) and 24 ml 20 % sucrose cushion was added below it. The sucrose cushion consisted of 20 % (w/v) sucrose (Sigma Aldrich) in sterile 1 x TNE buffer (pH 7.5) (50 mM Tris (pH 7.5), 140 mM NaCl and 5 mM EDTA (pH 8)). This was balanced and ultracentrifuged at 25,000 rpm for 1.5 h at 4 °C. Following this the supernatant was decanted and the pellet at the bottom of the tube was re-suspended in an appropriate volume of sterile 1 x TNE buffer. For virus this was 400 µl but for VRPs this was 200 µl. Tubes were covered with lab film (Sigma Aldrich) and incubated on ice with rocking at 4 °C for at least 18 h. Afterwards the re-suspended viruses or VRPs were transferred to a sterile 1.5 ml tube and the ultracentrifuge tube rinsed with 50 µl 1 x TNE buffer to collect any residual virus which was then pooled in the 1.5 ml tube. All stocks were snap frozen on dry ice and stored in aliquots of 20 µl in sterile 1.5 ml tubes at -80 °C. When required one aliquot was removed and fast thawed at 37 °C and any remaining stock frozen as described.

2.5.4 Titration of Virus by Standard Plaque Assay

SFV titres were determined by standard plaque assay using BHK-21 cells. Cells were seeded at a density of 3 x 10^5 cell/well of a 6-well plate or 0.8 x 10^5 cell/well of a 24-well plate and incubated at 37 °C. The virus was serially diluted in 10- fold dilutions in PBS with bovine serum albumin (BSA) (PBSA, 0.75 % (w/v) BSA in PBS). The albumin inhibits virus binding to the charged plastic surface of the tissue culture plasticware. The growth media was removed from each well and replaced with an appropriate volume of each dilution in duplicate, starting with the most dilute sample. The plate was incubated at 37 °C for 1 h with gentle rotation approximately every 10 min to ensure an equal distribution of virus.

A choice of two overlies were used during this project, Avicel or molten agar. The Avicel overlay was used with 24-well plates and 1.2 % (w/v) Avicel (FMC BioPolymer) in distilled H_2O (sterilised by autoclaving) was mixed 1:1 with 2 x MEM (Life Technologies) (supplemented with 4 % FCS). To each well 1 ml was gently applied and the plates were carefully returned to 37 °C for three days. As the plates were sensitive to motion they were placed at the back of the incubator and not moved prior to fixing. Molten agar was only used with 6-well plates. The agar was prepared by mixing 4 g of Bacto-agar/100 ml PBS

(sterilised by autoclave) and heated to 90 °C, cooled to below 55 °C prior to adding on top of the cell monolayer. The agar was mixed 10 agar: 3 GMEM with pre-warmed GMEM supplemented with 2 % NBCS (v/v). The agar/GMEM solution was carefully applied to the cell monlayer in a drop-wise manner and was allowed to solidify before the plates were returned to the 37 °C incubator for 3 days.

Following the incubation with both overlays, the plates were fixed by carefully applying 10 % formalin solution and incubated at room temperature for 1 h. The overlay and the fixative were discarded and 0.1 % Toluidine blue stain (Sigma Aldrich) applied to each well. The cells were rocked for approximately 30 min before the stain was removed and each well washed gently with tap water. Round areas without stain were considered to be plaques. These were counted and virus plaque forming units (PFU)/ml calculated by the following formula:

$$Plaque\ Forming\ units\ (PFU)/\text{ml} = \left(\frac{\text{Average number of plaques}}{amount\ of\ inoculum\ (ml)}\right) \times \text{dilution factor}$$

Viral MOI was calculated based on the virus titre in BHK-21 cells.

2.5.5 Titration of VRPs

As VRP do not produce progeny virus particles no plaques (regions of cell death) are formed in the cell monolayer. Therefore a standard plaque assay cannot be used and VRP titration was done by immunostaining. Where no florescent marker was expressed, visualisation was achieved through immunostaining with anti-SFV-nsP3 antibody following the protocol described in **Chapter 2.4.5**. However, some VRPs expressed a florescent protein marker, for example ZsGreen or mCherry, which could be used for direct visualisation. BHKs were seeded at a density of 8 x 10^4 cells/well of a 24-well glass plate and incubated overnight. As with the standard plaque assay, the growth media was removed and replaced with 200 µl of the VRP stock diluted in ten-fold serial dilutions in PBSA. Each dilution was done in duplicate. The cells were then returned to the 37 °C incubator for 1 hr with rocking every 10 min to ensure an equal distribution of VRPs. Following this incubation, the PBSA/VRP solution was removed, 1 ml fresh growth media applied gently to the monolayer and the cells were returned to 37 °C incubator for 20 h.

The cells were then fixed by carefully applying 10 % formalin solution and incubated at room temperature for 1 h. Immunostaining was then performed if required or the results viewed immediately if the VRPs expressed a fluorescent marker gene. Coverslips were mounted using VECTASHIELD® Mounting media plus DAPI, and allowed to dry before being sealed with clear nail polish. Using a Zeiss 710 confocal microscope, the average number of fluorescent (infected) cells in fifteen fields were counted and the VRP titre calculated with the following formula:

$$\begin{aligned}\text{VRP concentration per ml} &= \text{Mean number of VRP infected cells} \\ &\times (\text{proportion of a ml of inoculum} \times \text{dilution factor}) \\ &\times \text{microscope constant}\end{aligned}$$

Where the microscope constant is calculated as:

$$Microscope\ Constant = \frac{Total\ growth\ area}{Area\ Viewed}$$

Where:

$$Area\ viewed = \pi(diameter/2)^2$$

2.5.6 Infection of Cell Monolayers with Virus or VRPs

The appropriate MOI of virus or VRPs was prepared by diluting in the correct volume of PBSA. The cell growth media was removed and replaced with either 400 µl virus in PBSA for 6-well plates or 200 µl for 24-well plates. This was added carefully to each well so as not to disrupt the cell monolayer. The cells were then incubated at the appropriate temperature for the cell line (either 37 °C for BHK-21 cells or 28 °C for insect cells) for 1 h with rocking approximately every 10 min to ensure an equal distribution of virus. Following the incubation the virus/PBSA was discarded and the correct volume of fresh cell-specific growth media applied. Cells were then returned to the appropriate growth conditions for the required incubation time.

2.6 Confocal Microscopy

Visualisation of infected or transfected cells expressing fluorescent constructs was achieved through confocal microscopy. Fixed cells were mounted with VECTASHIELD® Mounting media with DAPI for nuclear staining, however, live cells could also be viewed with no DAPI staining. Fluorescence was viewed using either the Zeiss LSM 710 or LSM 510 confocal microscopes using the appropriate lasers. Fluorophores used during this project are shown below in **Table 2.5**.

Table 2.5: Fluorophores detected by confocal microscopy during this project

Fluorophores	Colour	Excitation (nm)	Emission (nm)	Laser (nm)
DAPI	Blue	358	463	405 Diode
mCherry	Red	587	610	561
eGFP	Green	488	509	488 Argon
ZsGreen	Green	496	506	488 Argon
Alexa 488	Green	498	520	488 Argon
Alexa 594	Red	590	619	561

2.7 Dual Luciferase Assay

Cells seeded in a 24-well plate were lysed by replacing the growth media with 100 µl/well passive lysis buffer (Promega) and incubated on a shaker at room temperature for at least 30 min to allow disruption of the cellular membranes. Relative luciferase light units of both Renilla (*Rluc*) and Firefly (*Fluc*) luciferase expression were measured by dual luciferase kit (Promega) using a GloMax®-Multi+ Microplate Multimode Reader (Promega). To each well of a 96-well plate 25 µl of lysate was added. The luminometer then added 70 µl *Fluc* detection reagent followed by 70 µl Stop and Glo *Rluc* detection reagent with a 2 sec delay, at 200 µl/sec with 10 sec integrals. Both detection reagents were diluted ten times. If non diluted reagents were used only 10 µl of lysate was required.

2.8 Commonly Used Solutions

Cell Culture

Mitsuhashi and Maramorosch 1 x media (without L-glutamine)

Calcium Chloride [$CaCl_2$ $2H_2O$] Dihydrate	250 mg/lt
Magnesium Chloride [$MgCl_2$ $6H_2O$]Hexahydrate	125 mg/lt
Potassium Chloride [KCl]	250 mg/lt
Sodium Bicarbonate [$NaHCO_3$]	150 mg/lt
Sodium Chloride [NaCl]	8750 mg/lt
Sodium Phosphate monobasic [$NaH_2PO_4H_2O$] Monohydrate	250 mg/lt
D-glucose	5000 mg/lt
Lactalbumin Hydrolysate	8125 mg/lt
Yeastolate	6250 mg/lt

Western Blot

2 x Loading Buffer

0.125 M TRIS HCl (pH 6.8), 4 % SDS, 20 % glycerol, 10 % β-mercaptoethanol, 10 mg bromophenol blue

Resolving Gel

2.43 ml distilled H_2O, 1.25 ml acrylamide/bis-acrylamide (40 %), 1.25 ml 1.5 M Tris-HCl (pH 8.8), 50 µl 10 % SDS, 50 µl 10 % APS and 5 µl TEMED

Stacking Gel

1.598 ml distilled H_2O, 252 µl acrylamide/bis-acrylamide (40 %), 625 µl 0.5 M Tris-HCl (pH 6.8), 25 µl 10 % SDS, 75 µl 10 % APS and 5 µl TEMED

1 x SDS-PAGE Running Buffer

25 mM Tris, 0.25 M Glycine, 0.1 % SDS

1 x Wet Transfer Buffer

50 mM Tris, 375 mM Glycine, 20 % Methanol

Semi- Dry Transfer Buffer

48 mM Tris, 39 mM Glycine, 0.0375 % SDS, 20 % Methanol

Antibody Reconstitution Buffer

0.05 % NaN_3 in H_2O

Stripping Buffer

3 ml 1 M TRIS HCl (pH7), 5 ml 20 % SDS, 350 µl β-mercaptoethanol, to final volume 50 ml with H_2O

Northern Blot

2 x FDE Loading Buffer

10 ml de-ionised formamide, 10 mg xylene cyanol, 10 mg bromophenol blue, 200 µl 0.5 mM EDTA (pH 8)

12 % Denaturing Acrylamide RNA Gel

4.8 g urea, 0.5 ml 10 x TBE, 3 ml acrylamide/bis-acrylamide (40 %) and 1 ml RNase/DNase free H_2O to 10 ml

Low Stringency Northern Blot Wash Buffer

2 x SSC and 0.1 % SDS

Maleic Acid Buffer

100 mM Maleic acid, 150 mM NaCl, adjusted to pH 7- 8 with NaOH pellets

Washing Buffer

Maleic acid buffer, 0.3 % Tween

AP Buffer

100 mM NaCl, 5 mM MgCl, 100 mM Tris- HCl

TNT Coupled Reticulocyte

Gel Fixing Solution

50 % methanol, 10 % acetic acid, to final volume with H_2O

Gel Drying Solution

7 % acetic acid, 7 % methanol, 1 % glycerol, to final volume with H_2O

In vitro Dicer assay

5 x Lysis Buffer

10 mM MgAc, 150 mM Hepes-KOH (pH 7.5)

2 x PK Buffer

200 mM Tris (pH 7.5), 300 mM NaCl, 5 mM EDTA, 2 % SDS

2 x Storage Buffer

Ice-cold 40 mM tris-acetate (pH 6.8), 200 mM EDTA, 20 mM β-mercaptoethanol

Virus Purification

1 x TNE Buffer (pH 7.5)

50 mM Tris (pH 7.5), 140 mM NaCl and 5 mM EDTA (pH 8)

2.9 Primer List

NAME	Details	PRIMER SEQUENCE 5'→3'	Fragment size (bp)
Ae. aegypti-Elp1-1 FWD *Ae. aegypti*-Elp1-1 RV	Recognise the first unique site of Elp1 from the Aag2 genome	GTAATACGACTCACTATAGGGAGGACGACACTTTCGGTGATCG GTAATACGACTCACTATAGGGCGCGCTGCACTCTTCCCC	94
Ae. aegypti-Elp1-2 FWD *Ae. aegypti*-Elp1-2 RV	Recognise the second unique site of Elp1 from the Aag2 genome	GTAATACGACTCACTATAGGGCTCAGATATTGAAGGACAA GTAATACGACTCACTATAGGGCCAGCACTTCCGAATCAT	138
Ae. aegypti-Elp1-3 FWD *Ae. aegypti*-Elp1-3 RV	Recognise the third unique site of Elp1 from the Aag2 genome	GTAATACGACTCACTATAGGGAGCTTCTTTAAACCTGACTTCACA GTAATACGACTCACTATAGGGTTGATTGGTTCTATGCAACCA	257
Rluc FWD *Rluc* RV	Primers for *Rluc* with T7 promoter	TAATACGACTCACTATAGGGATGACTTCGAAAGTTTATGATCCAG TAATACGACTCACTATAGGGCTGCAAATTCTTCTGGTTCTAACTTTC	700
eGFP-720nt FWD eGFP-720nt RV	Primers for eGFP with T7 promoter	GTAATACGACTCACTATAGGGATGGTGAGCAAGGGCGAGGAGCTGTTC GTAATACGACTCACTATAGGGCTGGGTGCTCAGGTAGTGGTTGTCGGGC	720
eGFP-400nt FWD eGFP-400nt RV	Primers recognising eGFP with T7 promoter	GTAATACGACTCACTATAGGGGGCGTGCAGTGCTTCAGCCGC GTAATACGACTCACTATAGGGGTGGTTGTCGGGGCAGCAGCAC	400
eGFP-114nt FWD eGFP-114nt RV	Recognise a shorter fragment of eGFP with T7 promoter	GTAATACGACTCACTATAGGGGGCGTGCAGTGCTTCAGCCGC GTAATACGACTCACTATAGGGGCCGTCCTTGAAGAAGATGG	114
Fluc FWD *Fluc* RV	Primers for *Fluc* with T7 promoter	GTAATACGACTCACTATAGGGACTTACGCTGAGTACTTC GTAATACGACTCACTATAGGGGAAATCCCTGGTAATCCG	330
SFV-nsP1 FWD SFV- nsP1 RV	Recognise the nsP1 gene of SFV	GGGAAGGTGCTGGATAGAGA CAAACATAAACGGGGTGGTG	244

SFV-nsP2 FWD SFV-nsP2 RV	Recognise the nsP2 gene of SFV	AGCTTGACGTCCTGCGACTAGGCCGCGCGGGTGCAG AATTCGCCAGTGTCCGAGGAGAAAATACCTGCACCC	220
SFV-nsP3 FWD SFV-nsP3 RV	Recognise the nsP3 gene of SFV	GCAAGAGGCAAACGAACAGA GGGAAAAGATGAGCAAACCA	205
SFV-E1 FWD SFV-E1 RV	Recognise the E1 gene of SFV	CGCATCACCTTCTTTTGTG CCAGACCACCCGAGATTTT	173
SFV-C/E3 FWD SFV-C/E3 RV	Overlap the C/E1 genes of SFV	TGACAACAAGGGGAGGGTAG CTGGAAGCACGGGAAGGTAG	190
Ae. aegypti-Ago-1 FWD *Ae. aegypti*-Ago-1 RV	Recognise Ago-1 from the Aag2 genome	GTAATACGACTCACTATAGGGACAGGTTTCACTGTTCAACCT GTAATACGACTCACTATAGGGGGTTTGACCGTTTTCTAGCTGC	2844
Ae. aegypti-Ago-2 FWD *Ae. aegypti*-Ago-2 RV	Recognise Ago-2 from the Aag2 genome	GTAATACGACTCACTATAGGGGCCCTCAACAAGAAACACC GTAATACGACTCACTATAGGGGGCGTTGATCTTGAGCCA	3255
Ae. aegypti-Dcr-1 FWD *Ae. aegypti*-Dcr-1 RV	Recognise Dcr-1 from the Aag2 genome	GTAATACGACTCACTATAGGGCCACGCCTAAAGTGCTGCGATTAC GTAATACGACTCACTATAGGGGCACTTCCTCCTGTTTCCTGTGTTC	6887
Ae. aegypti-Dcr-2 FWD *Ae. aegypti*-Dcr-2 RV	Recognise Dcr-2 from the Aag2 genome	GTAATACGACTCACTATAGGGATGCTAAAAATTCACAAATTCGATC GTAATACGACTCACTATAGGGAGCCGAGCGCAGATCTGTTAACTG	5924
Ae. aegypti-Actin FWD *Ae. aegypti*-Actin RV	Recognise actin in the Aag2 genome	AACACCCAGTCCTGCTGACAGA TTCGTAGATTGGGACTGTGTGCGA	217
Ae. albopictus-Actin FWD *Ae. albopictus*-Actin RV	Recognise actin in *Ae. albopictus*-derived cell lines	AGAGCACCCAGTTCTCCTGA CAGGGCATAACCCTCGTAGA	216

Ae. aegypti-Dcr-2-V5/N FWD	Recognises Dcr-2 contains *Hind*III	AAGCGCAAGCTTATGGGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCT ACGATGGATATGATTATGCCACAGC	5966
Ae. aegypti-Dcr-2-V5/N RV1	Recognises Dcr-2 contains *Xho*I for cloning into pIB	AACCGGCTCGAGTTACTTAGCACTGCGGTAGTGCT	
Ae. aegypti-Dcr-2-V5/N RV2	Recognises Dcr-2 contains *Xba*I for cloning into modified pSFV1	AACCGGTCTAGATTACTTAGCACTGCGGTAGTGCT	
Ae. aegypti-Dcr-2-V5/C FWD	Recognises Dcr-2 contains *Hind*III	AAGCGCAAGCTTATGGATATGATTATGCCACAGC	
Ae. aegypti-Dcr-2-V5/C RV1	Recognises Dcr-2 contains *Xho*I for cloning into pIB	AACCGGCTCGAGTTACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCT TACCCTTAGCACTGCGGTAGTGCT	
Ae. aegypti-Dcr-2-V5/C RV2	Recognises Dcr-2 contains *Xba*I for cloning into modified pSFV1	AACCGGTCTAGATTACGTAGAATCGAGACCGAGGAG	
Ae. aegypti-Ago-2-V5/N FWD	Recognises Ago-2 contains *Nru*I for cloning into modified pSFV1	AAGCGCTCGCGAATGGGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCT ACGATGATACTAAACGCGAGATATC	3297
Ae. aegypti-Ago-2-V5/N RV	Recognises Ago-2 contains *Xba*I for cloning into modified pSFV1	AACCGGTCTAGATTAAACAAAGAACATCGGGTGAC	
Ae. aegypti-Ago-2-V5/C FWD	Recognises Ago-2 contains *Nru*I for cloning into modified pSFV1	AAGCGCTCGCGAATGATACTAAACGCGAGATATC	
Ae. aegypti-Ago-2-V5/C RV	Recognises Ago-2 contains *Xba*I for cloning into modified pSFV1	AACCGGTCTAGATTACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCT TACCAACAAAGAACATCGGGTGAC	
SFV Hot Spot 7305-7326bp	Recognises SFV genome position 7305-7326	TGGCGAGGGACATTAAGGCGT	
SFV Hot Spot 1268-1289bp	Recognises SFV genome position 1268-1289	TGGGCGAGGGAATACAAGGCA	
SFV Hot Spot 3549-3570bp	Recognises SFV genome position 3549-3570	CGGTTAAAGGCAGTAGGGTTG	
CFAV-3UTR-FW (CFAV Set 1)	Recognise a unique region of CFAV 3'UTR	TAGACGTGATCGAATAGAGCCG	559
CFAV-3UTR-RV (CFAV Set 1)		GCGCATCTATGGTATAGAAAAGATAAT	
CFAV-FW-3359 (CFAV Set 2)	Recognise a unique region of CFAV	GTTGACGACATATTGAAGAGATACG	701
CFAV-RV-4060 (CFAV Set 2)		GCCAAGGATACAGTCCAAAAC	

CFAV-3UTR-FW-100 (CFAV Set 3) CFAV-3UTR-RV-400 (CFAV Set 3)	Recognise a unique region of CFAV 3'UTR	CCTGGCAACAGGGTGTGT CTGCCATTTTATAAGAGCACTGG	330
pGL3-PUb new MCS FWD pGL3-PUb new MCS RV	Inserts a MCS into pGL3-PUb	GGGACAGATCTACCCGCGGACTTAATTAAACTTCTAGAGTCGGGGCGGC GGGGTCTCGAGGTGCTAGCGTGGTTGAAATCTCTGTTGAGCAG	

Chapter 3: RNAi-mediated Antiviral Defence in *Ae. aegypti*-derived cells

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3.1 Introduction

In insects, RNA interference (RNAi) is known to be integral in modulating the replication for a number of important viruses. It is induced through the detection of dsRNA generated during virus accumulation. Although *Drosophila* are the traditionally established model for the study of innate immunity in insects, more recently the focus has been to expand our understanding of the mechanisms involved in the antiviral defence of arboviral vectors and this had lead to increasing momentum in this area. Thanks to the evolution of whole-genome sequencing, the genomes of some of the most significant vector species, such as *Ae. aegypti*, *An. gambiae* and *Cx. pipiens quinquefasciatus*, have been unravelled (Christophides et al., 2002; Holt et al., 2002; Nene et al., 2007; Waterhouse et al., 2007; Arensburger et al., 2010; Bartholomay et al., 2010). Thanks to this, bioinformatic analysis has revealed that orthologues of the focal RNAi proteins are found in the genomes of these vectors (Hoa et al., 2003; Campbell et al., 2008a) suggesting that key features of the pathway may be conserved between *Drosophila*, aedine, anopheline and culex mosquitoes.

Recent findings are contributing to an already extensive body of evidence that the interactions between the invading virus and the arthropod's immune responses are highly complex. The biology of arboviruses has been shown to be different from pathogenic insect viruses as they are modulated by the antiviral response but are still able to establish a low- level, non-pathogenic persistent infection. Any pathogenicity induced through infection in the vector decreases the potential for transmission and so it is vital for the virus to incite minimal fitness costs. RNAi is believed to be the principle virus-specific innate immune response in arthropods. The exogenous branch of the RNAi response is characterised by the production of virus-derived small interfering RNAs, or viRNAs, which are pivotal to its sequence-dependent targeting mechanism. These are believed to be generated by a Dicer-2 (Dcr-2) dependent mechanism as Dcr-2 recognises alien, base-perfect, long dsRNA molecules and cleaves them into shorter duplex 21 nt fragments. The production of dsRNA molecules has been observed during the course of replication for members of all major RNA arbovirus families, with the exception of negative strand RNA viruses (Weber et al., 2006). It has been suggested that these dsRNA molecules are the substrates for Dcr-2 cleavage and the production of viRNAs following infection by members of the *Flaviviridae* (Stollar et al., 1967; Stollar and Stollar, 1970; Westaway et al., 1997 and 1999; Sánchez-Vargas et al., 2009; Brackney et al., 2009 and 2010; Scott et al., 2010; Hess et al., 2011), *Bunyaviridae* (Patterson et al., 1983; Brackney et al., 2010;

Léger et al., 2013; Sabin et al., 2013; Schnettler et al., 2013b), *Togaviridae* (Pfefferkorn et al., 1967; Stollar and Stollar, 1970; Stollar et al., 1972; Myles et al., 2008 and 2009; Brackney et al., 2010; Siu et al., 2011; Morazzani et al., 2012), and *Reoviridae* (Schnettler et al., 2013b).

In the case of ssRNA viruses, these dsRNA molecules were speculated to arise from three origins; 1) secondary structures within the viral genome or transcripts, 2) through complementary binding of RNA molecules to the single stranded genome, or 3) duplexes of replication intermediates (Molnar et al., 2005; Ho et al., 2006; Myles et al., 2008 and 2009). As described in **Chapter 1.1.2.4.3,** replication of single stranded (ss) (+) sense RNA viruses consists of the (+) sense RNA viral genome serving as a template for the production of a (-) sense antigenome. In turn this (-) sense strand acts as a template for further (+) sense genomes (Westaway, 1987; Strauss and Strauss, 1994). The reverse is true during the replication of (-) ssRNA viruses where the (-) sense genome is a template for the (+) sense antigenome (Guu et al., 2012). Although in both cases concentrations of genomic RNA outweigh antigenomic RNA, recent findings have shown that viRNA populations exist in approximately equal quantities of (+) and (–) polarities for both (+) and (-) strand RNA viruses. In addition, the lack of any correlation between viRNA production and genomic secondary structure or region specificity, has strengthened the hypothesis that the source of the dsRNA during (+) and (-) ssRNA virus infections in insects is replication intermediates (Aliyari et al., 2008; Brackney et al., 2009 and 2010; Myles et al., 2008 and 2009; Sánchez-Vargas et al., 2009; Mueller et al., 2010; Scott et al., 2010; Siu et al., 2011; Marques et al., 2013; Schnettler et al., 2013a; reviewed in Bronkhorst and van Rij, 2014) (**Chapter 5**). For alphaviruses this is consistent with the understanding that their genomes incorporate few areas of secondary structure and is therefore unlikely to be the source of dsRNA structures (Ou et al., 1983; Davis et al., 2008; Logue et al., 2008; Nickens and Hardy, 2008; Kulasegaran-Shylini et al., 2009; Siu et al., 2011).

The alphavirus Semliki Forest virus (SFV) of the *Togaviridae* family has previously been shown to be a good model for arbovirus control by insect innate immunity (Garcia et al., 2005; Fragkoudis et al., 2008; Attarzadeh-Yazdi et al., 2009; Siu et al., 2011; Barry et al., 2013). As with other alphaviruses, SFV infection is known to stimulate the production of small RNA molecules from the exogenous RNAi pathway (Myles et al., 2008; Siu et al., 2011), as well as piRNA molecules (Schnettler et al., 2013a). Production of these molecules confirms that the virus is targeted by an active immune response as both these

mechanisms have been implemented in antiviral regulation. Alphaviruses are not believed to express an active RNAi antagonist protein and so replication intermediates would be readily available as a source of viRNA biogenesis (Myles et al., 2009). Deep sequencing analysis has allowed several interesting aspects of these small RNAs to be determined. For instance, the viRNA molecules produced following SFV infection of *Ae. aegypti*-derived Aag2 and *Ae. albopictus*-derived U4.4 cell lines were primarily 21 nt long as expected for Dcr-2 processing (Aliyari et al., 2008). Moreover, compared to the global number of small RNAs isolated from SFV infected mosquito-derived cell lines, these viral-derived small RNAs make up a tenuous proportion (Sui et al., 2011). This has also been documented following infection by other alphaviruses and flaviviruses (Campbell et al., 2008b; Myles et al., 2008 and 2009; Brackney et al., 2009; Scott et al., 2010; Morazzani et al., 2012). Furthermore, the viRNAs are produced across both the genome and antigenome of SFV and are not found to be biased towards a particular base at position one suggesting that Dcr-2 cleaves the dsRNA molecules in a non-specific manner (Siu et al., 2011). The distribution of these molecules is not random and regions of high production frequency or 'hot spots' compared to regions of no/few viRNA reads or 'cold spots' are evident. These 'cold spot' viRNAs were found to be significantly more efficient at targeting the virus for degradation compared to 'hot spot' viRNA molecules. The determining factors influencing the different biology exhibited between 'hot' and 'cold' spot alphavirus-derived viRNAs and their ability to mediate RNAi is currently not known and there is no evident correlation to sequence content, base pairing or their genomic location. Future research is required to understand if the consistent pattern of 'hot'/'cold' spots is due to a difference in availability, either spatially or temporally, where some regions are more easily accessed by Dcr-2 than others during the stages of virus replication. On the other hand it may be suggested that the viral genome is under selective pressure from RNAi activity to generate a high number of viRNAs against 'decoy' regions to restrict detrimental effects on virus replication and thereby evade the immune response. This method of RNAi avoidance has previously been shown for *Drosophila* cells infected with flock house virus (FHV) where most of the 21 nt viRNAs mapped to the 5' region of both RNA segments. In addition, the majority of viRNAs were not loaded into RISC and therefore did not result in silencing (Aliyari et al., 2008; Flynt et al., 2009; van Rij and Berezikov, 2009). Restricted loading of RISC has also been noted for other arboviral infections in mosquitoes and it is believed to reduce the capacity for silencing as the majority of viRNA complementary sequences would not be targeted. A decoy strategy has also been observed following *Arabidopsis thaliana* infection with Cauliflower mosaic pararetrovirus (CaMV) as small RNAs of 21,

22 and 24 nt are generated by each Dicer-like (DCL) protein from both strands of a non-coding viral region (Blevins et al., 2011). Although high numbers of viRNAs are produced, they do not result in virus inhibition suggesting this strategy is not specific to insect infections.

The 'hot'/'cold' spot viRNA production pattern has been shown to be a key feature of many viral infections in insects (Myles et al., 2008 and 2009; Brackney et al., 2010; Scott et al., 2010; Hess et al., 2011; Morazzani et al., 2012). Unlike other arboviruses, flaviviruses are the only ones known to express an RNAi antagonist which is produced from the 3' UTR-derived RNA molecule of dengue virus (DENV) and West Nile virus (WNV) and is known as subgenomic flavivirus RNA (sfRNA) (Pijlman et al., 2008; Schnettler et al., 2012). These molecules have been described as inhibiting RNAi through an RNA decoy mechanism similar to that of FHV and CaMV discussed previously, where high levels of non-coding RNAs inhibit efficient silencing. In addition, the non-structural protein 4B (NS4B) of DENV is also suggested to be an inhibitor of Dcr-2 through an unknown mechanism (Kakumani et al., 2013). However, RNAi and viRNA distribution have not been shown to particularly prejudice these regions (Brackney et al., 2009 and 2010; Scott et al., 2010). Conversely, following Langat virus (LGTV) and tick-borne encephalitis virus (TBEV) infection in tick-derived cell lines, viRNA production was highly biased towards the 5' and 3' terminals, although these similarly do not impact virus replication (Schnettler et al., 2014). A second key feature of insect RNAi is that, as mentioned, the predominant size of viRNAs produced are 21 nt long, and this has been a consistent finding through deep sequencing analysis following a number of RNA viral infections of both *Drosophila* and mosquitoes (Brackney et al., 2009 and 2010; Flynt et al., 2009; Scott et al., 2010; Sui et al., 2011; Morazzani et al., 2012; Vodovar et al., 2012; Schnettler et al., 2013a). Indeed, 21 nt viRNAs are the major size detected following the infection of *Drosophila* with the DNA virus invertebrate iridescent virus 6 (IIV6), evidence that DNA viruses are also actively targeted by the RNAi pathway (Bronkhorst et al., 2012; Kemp et al., 2013).

Following Dcr-2 cleavage of dsRNA, the resulting viRNA molecules have a 5' monophosphate and an overhang of 2 nt on the 3' hydroxyl end necessary for functional RNAi (Zamore et al., 2000; Bernstein et al., 2001; Elbashir et al., 2001a and b; Nykanen et al., 2001; Tang et al., 2003; Zang et al., 2004; Okamura and Lai, 2008; van Rij and Berezikov, 2009). The phosphate group is a characteristic of Dcr-2 processing and is required for enhanced binding to the Dcr-2/R2D2 heterodimer and for subsequent loading

into RISC (Liu et al., 2003; Tomari et al., 2004; Marques et al., 2010). It is understood that R2D2 binds to the most thermodynamically stable end of the siRNA molecule which dictates its orientation (Tomari et al., 2004). The siRNAs have a functional asymmetry and the strand selected to perform as the guide strand is determined by the stability of the 5' end. Therefore, the strand that is bonded less tightly to its complement at this end is incorporated into RISC and becomes the guide strand which is then bound by the PAZ domain of Ago-2 and retained (Khvorova et al., 2003; Lingel et al., 2003 and 2004; Schwarz et al., 2003; Ma et al., 2004). In *Drosophila* RISC, the guide strand is 2'-*O*-methylated at its 3' terminus through the action of the S-adenosylmethionine-dependent methyltransferase Hen-1 (DmHen-1) which protects that strand from degradation (Hammond et al., 2001; Horwich et al., 2007; Saito et al., 2007). The mature, active RISC is now able to target base-perfect mRNAs for degradation through Ago-2 slicing. As with endo-siRNAs, it has been shown that some viRNAs are resistant to Beta (β) elimination treatment which degrades RNAs presenting unmodified 2' and 3' hydroxyl groups at the 3' terminus. This implies that a proportion of the viRNA population must also be associated with Ago-2 and have been 2'-*O*-methylated at their 3' end (Aliyari et al., 2008; Czech et al., 2009). Methylation of small RNAs has been documented in plants and some plant viruses have been confirmed as actively inhibiting this methylation or removing the methyl group from their siRNAs through the action of an encoded viral suppressor protein (Ebhardt et al., 2005; Blevins et al., 2006; Csorba et al., 2007; Vogler et al., 2007). In this way they prevent siRNA stabilisation within RISC and the resulting sequence targeted silencing. This chemical modification can therefore be considered to be a key factor in effective RNAi processing. During the persistent infection of *Drosophila* cells with FHV, it has been shown that the majority of its viRNAs are not methylated (Flynt et al., 2009); however, it is not fully understood if the methyl group is actively removed or if the viRNAs are not taken up by RISC and are therefore not methylated originally. Moreover, there are still a large number of open questions relating to the methylation and phosphorylation of viRNAs/siRNAs during mosquito RNAi which are required to be answered in order to improve our awareness of the biochemical nature of the RNAi response.

The establishment of a number of continuous mosquito-derived cell lines has provided valuable research tools for the study of innate immunity in mosquitoes. They present an easily handled, homogeneous system which is often more sensitive and reproducible than the whole organism. Through work mainly carried out on alphaviruses and flaviviruses, the

Ae. aegypti-derived Aag2 and *Ae. albopictus*-derived U4.4 cell lines have been determined to be RNAi competent and are able to mount an RNAi response against a number of significant arboviruses (Riedel and Brown, 1979; Condreay and Brown, 1986 and 1988; Miller and Brown, 1992; Attarzadeh-Yazdi et al., 2009; Sánchez-Vargas et al., 2009; Scott et al., 2010; Siu et al., 2011; Barletta et al., 2012; Morazzani et al., 2012; Vodovar et al., 2012; McFarlane et al., 2014). *Ae. aegypti* cell lines in particular have become of substantial importance since the landmark publication of their complete genome sequence seven years ago (Nene et al., 2007) and this has vastly contributed to the expansion of knowledge concerning virus:vector interactions in this important vector species. This project aims to utilise these cell lines in order to establish a robust system of RNAi protein knockdown and to determine the effect this - and subsequently the RNAi machinery - really has on SFV replication. Additionally, the combination of existing techniques, as well as establishing new methods was used to further our understanding of siRNA biology during SFV infection.

3.2 Objectives

1. Establish an efficient silencing assay for the knockdown Dicer-1, Dicer-2, Argonaute-1 and Argonaute-2 proteins in the *Ae. aegypti*-derived Aag2 cell line.
2. Determine the role these proteins have on the replication of SFV and SFV replicon.
3. Investigate the production of SFV-derived viRNAs in both the *Ae. aegypti*-derived Aag2 cell line and the *Ae. albopictus*-derived U4.4 cell line by Northern blot analysis.
4. Analyse siRNA production patterns from dsRNA transfection in the *Ae. aegypti*-derived Aag2 cell line and the *Ae. albopictus*-derived U4.4 cell line by deep sequencing.
5. Determine if the production of the 'hot spot' and 'cold spot' siRNA pattern results from viral or cellular processing.

3.3 Results

3.3.1 The Effect of Silencing Key RNAi Proteins on the Replication of SFV in Aag2 Cells

To understand the extent that the key RNAi proteins play in the control of alphavirus infection it was necessary to develop a transient silencing assay. The difficulties faced in using the silencing pathway to knockdown its own components are evident and complete knockdown of these key proteins is never achieved, as by its own nature it is self-limiting. Furthermore, genetic mutants do not exist for mosquitoes and so transient silencing assays were developed based on those already established for *Drosophila*-derived cell lines (Caplen et al., 2000; Hammond et al., 2000). Genetic knockouts of Ago-1 and Dcr-1 proteins in *Drosophila* can reduce their viability while knockouts of Ago-2 and Dcr-2 enhance their susceptibility to infection highlighting the vital role these proteins play in the life of the organism (Galiana-Arnoux et al., 2006; van Rij 2006; Wang et al., 2006; Mueller et al., 2010; Han et al., 2011; Marques et al., 2013). The RNAi response is triggered when Dcr-2 detects dsRNA molecules present in the cell cytoplasm and injection of dsRNA into insects has been shown to artificially stimulate the RNAi response. It has been documented to be an effective method of gene silencing *in vivo* and is capable of transiently silencing RNAi pathway proteins (Keene et al., 2004; Franz et al., 2006; Sánchez-Vargas et al., 2009). However, although *in vitro* silencing assays were developed for *Drosophila* (Bernstein et al., 2001; Li et al., 2002; Hoa et al., 2003; Chotkowski et al., 2008; Mukherjee and Hanley, 2010) this was not the case for mosquito systems which were not well established until recently (Schnettler et al., 2012 and 2013; McFarlane et al., 2014; Paradkar et al., 2014). This project aimed to expand this technique for use with the *Ae. aegypti*-derived Aag2 cell line.

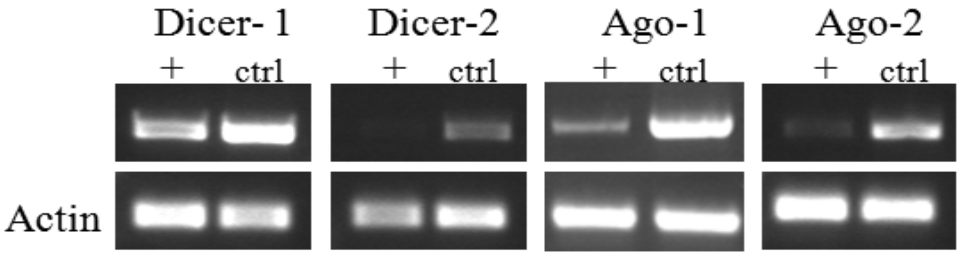
Silencing cellular protein expression was carried out using specific dsRNA molecules produced by *in vitro* transcription against the exogenous RNAi components Dcr-2 and Ago-2, as well as the miRNA pathway proteins Dcr-1 and Ago-1. The primers used to generate the dsRNAs against these regions are described in **Chapter 2.9**. *Ae. aegypti*-derived Aag2 cells were seeded at a density of 1.8 x 10^5 cells/well in 24-well plates and transfected 24 h later with 500 ng/well of dsRNA against the appropriate protein. These results were compared to cells which received 500 ng/well dsRNA specific to eGFP as a control. Efficient silencing of the target transcripts was established by RT-PCR 24 h post transfection. Transcript knockdown efficiency in three repeats was normalised to actin and

quantified by densitometry using the ImageJ software (**Figure 3.1A** and **3.1B**). Values recorded for control eGFP dsRNA treated samples were set to 100 % and the results for the other dsRNA treatments viewed as a percentage of this. The transcripts for Dcr-1, Dcr-2, Ago-1 and Ago-2 were shown to be reduced compared to control eGFP dsRNA by 40 %, 58 %, 32 % and 67 % respectively.

Once successful knockdowns of these transcripts had been achieved the effect that this had on SFV replication was established. This was determined by treating the cells with dsRNA as described prior to infecting with a reporter strain of SFV, SFV4(3H)-*Rluc*, which expresses *Renilla* luciferase (*Rluc*) after the nsP3 gene as a marker of virus replication. The infection was performed at either a high (10) or a low (0.05) multiplicity of infection (MOI) 24 h post transfection to achieve the maximum level of silencing prior to infection. Following a further 24 h incubation, the cells were lysed and luciferase activity determined by luciferase assay. The results are presented as a correlation between eGFP control treated cells (100 %) and *Rluc* expression. Luciferase expression for both MOI 10 (**Figure 3.2A**) and 0.05 (**Figure 3.2B**) indicate there is only a slight, non-significant increase in SFV replication when Dcr-1, Dcr-2 and Ago-1 were silenced. However, when cells were treated with Ago-2 specific dsRNA there was a significant increase in luciferase expression at a high MOI.

A similar experiment was performed substituting SFV with SFV VRPs also expressing *Rluc* (SFV1-*Rluc*) at an MOI of 10. As discussed in **Chapter 2.5.2** and **Chapter 5.3.4**, during the production of VRPs the replicon RNA is encoded separately from the structural proteins and as a result VRPs are unable to produce viral progeny which could be released and spread throughout the culture. Their use is therefore beneficial to study the effects of infection at an individual cellular level. The Aag2 cells were seeded and infected with the SFV replicons packaged into VRPs as described. Again the luciferase results for cells treated with control eGFP dsRNA were set to 100 % and the effect of transcript silencing shown as a percentage of eGFP expression. **Figure 3.3** shows that there was no effect on VRP replication when Dcr-1, Dcr-2 and Ago-1 where silenced. However, when cells were treated with Ago-2 specific dsRNA there was an increase in replication; although, this was less efficient than was observed following SFV infection at an MOI of 10.

A



B

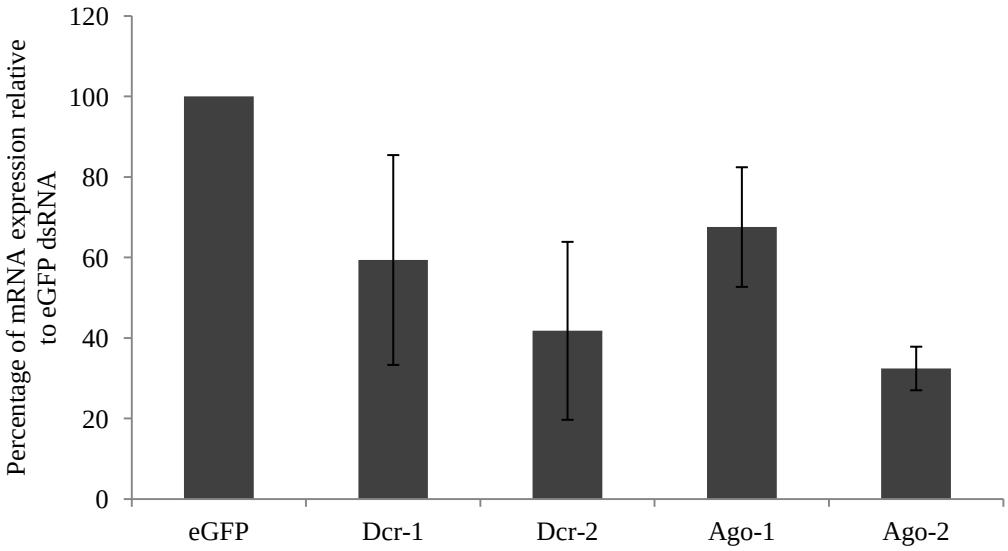
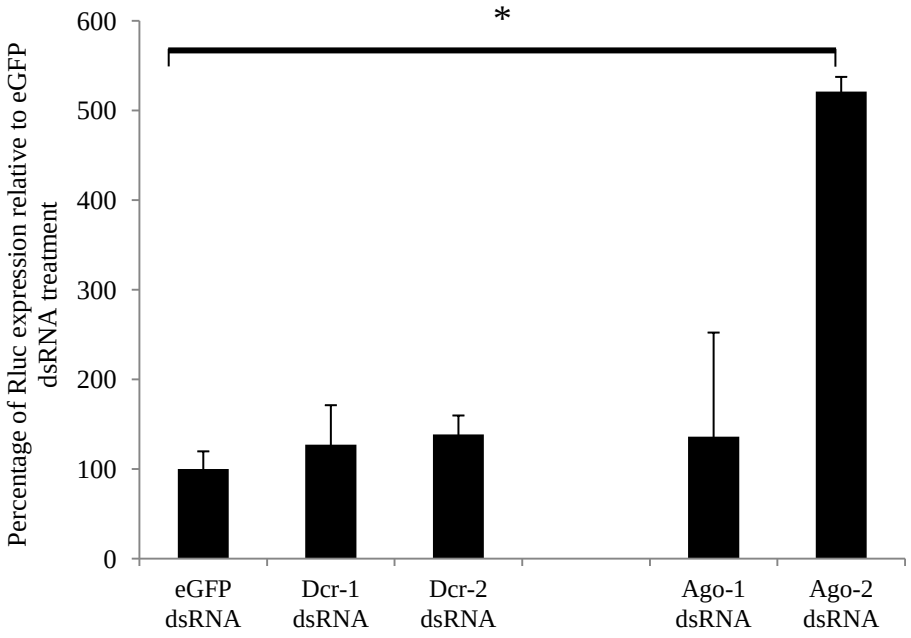


Figure 3.1: Silencing of key RNAi component proteins in Aag2 cells by dsRNA treatment. *Ae. aegypti*-derived Aag2 cells treated with specific dsRNA against Dicer-1 (Dcr-1), Dicer-2 (Dcr-2), Argonaute-1 (Ago-1) and Argonaute-2 (Ago-2) (+) were compared to cells which received a control eGFP (ctrl) dsRNA. **A**) Knockdown was confirmed by RT-PCR using gene specific primers with actin as a loading control. Representative images of the three independent experiments are shown. **B**) dsRNA treated samples were normalised against actin and the pixel density measured using Image J software. Error bars show the standard deviation of three independent experiments performed in triplicate.

A



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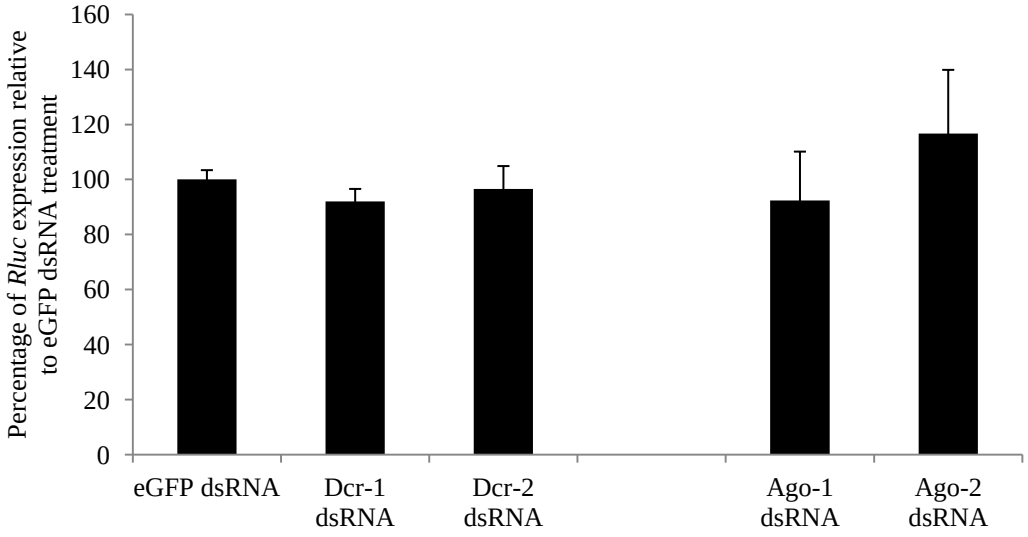

Figure 3.2: Knockdown of exogenous RNAi and miRNA pathway proteins and the subsequent effect on SFV(3H)-*Rluc* replication in *Ae. aegypti*-derived Aag2 cells. Cells were transfected with specific dsRNA against the RNAi proteins Dicer-2 (Dcr-2) and Argonaute-2 (Ago-2) or the miRNA proteins Dicer-1 (Dcr-1) and Argonaute-1 (Ago-1). Cells which received dsRNA specific to eGFP were used as a control. Each condition was then infected with a recombinant strain of SFV expressing *Renilla* (*Rluc*) luciferase (SFV(3H)-*Rluc*) at a **A**) high (10) or **B**) low (0.05) MOI. *Rluc* expression was detected by luciferase assay and measured in relative luciferase light units. Error bars represent the standard deviation of three independent experiments performed in triplicate. * represents $p < 0.05$, Student's t-test.

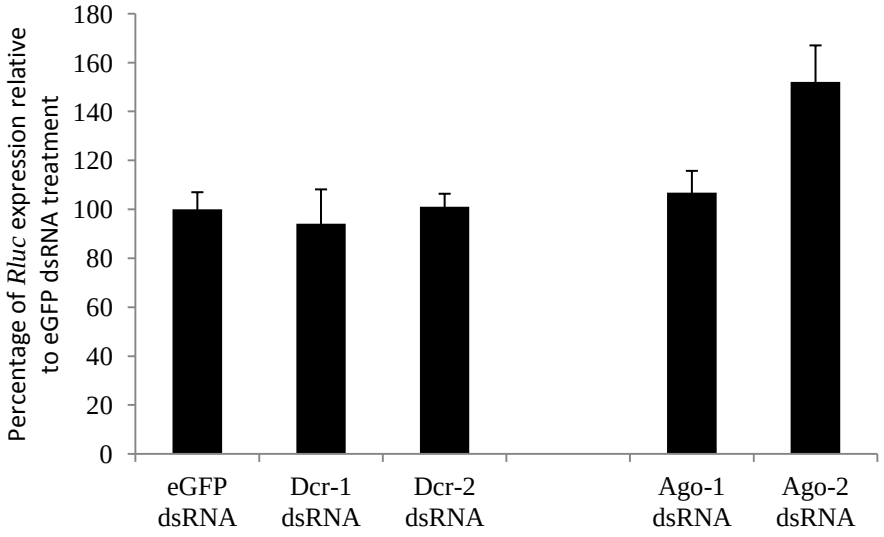

Figure 3.3: Knockdown of exogenous RNAi and miRNA pathway proteins and the subsequent effect on SFV VRP replication in *Ae. aegypti*-derived Aag2 cells. Cells were transfected with specific dsRNA against the RNAi proteins Dicer-2 (Dcr-2) and Argonaute-2 (Ago-2) or the miRNA proteins Dicer-1 (Dcr-1) and Argonaute-1 (Ago-1). Cells which received dsRNA specific to eGFP were used as a control. Each condition was then infected with SFV VRPs expressing *Renilla* (*Rluc*) luciferase (SFV1-*Rluc*) at a high (10) MOI. *Rluc* expression was detected by luciferase assay and measured in relative luciferase light units. Error bars represent the standard deviation of three independent experiments performed in triplicate.

3.3.2 Detection of SFV-derived viRNAs Produced in Aag2 and U4.4 Cells by Northern Blot Analysis

Previously, the characterisation of viRNA molecules produced following SFV infection of the *Ae. aegypti*-derived Aag2 and *Ae. albopictus*-derived U4.4 cell lines was carried out by deep sequencing analysis which allowed an analytical examination to be performed (Siu et al., 2011). A further method not utilised prior to this project was Northern blot analysis. This method offers many benefits over deep sequencing as it allows identification of the size and quantity of small RNAs of a specific sequence, as well as providing the ability to distinguish certain viRNA features such as the presence of a mono- or tri-phosphate or their methylation state. This technique is also cheap, comparable, reliable and reproducible and provides immediate results. In addition, deep sequencing is known to be biased towards certain small RNAs due to their ligation capacity (Jayaprakash et al., 2011; Jones et al., 2012; Sorefan et al., 2012; Zhuang et al., 2012; Raabe et al., 2014) and comparisons between deep sequencing and other methods of analysis commonly result in discrepancies (Baker, 2010; Git et al., 2010). For instance, detection of WNV-derived viRNAs in *Drosophila* S2 cells by Northern blotting indicted they were predominantly 25 nt (Chotkowski et al., 2008); although, deep sequencing analysis determined they were 21 nt (Brackney et al., 2010). Currently, no literature exists for the use of Northern blotting for the detection of arboviral or mosquito-infecting viral small RNAs for mosquito cells in culture, nor was this technique established in the laboratory yet. Therefore, optimisation was required for this technique to be adequately applied.

Both U4.4 and Aag2 cell lines were seeded at a density of 5.1 x 10^6 or 5.3 x 10^6 cells/well respectively. They were subsequently infected with SFV(3H)-*Rluc* at an MOI of 5 and the total RNA was isolated by Trizol extraction after 24, 48 and 72 hours post infection (hpi). Small RNAs were separated from the total isolated RNAs by PEG precipitation and run on an acrylamide gel alongside a small RNA ladder. Prior to blotting and detection by DIG-labelled SFV specific probes the acrylamide gel was stained with Ethidium Bromide (Et Br) to confirm equal quantities of all RNA samples were loaded (**Figure 3.4A**). A band can be detected at the approximate weight for small RNAs of 21 nt in length.

The RNAs from the gel were then transferred to a positively charged nitrocellulose membrane and stained with five probes for specific independent regions of the SFV genome to increase the sensitivity of detection. Previous attempts to establish detection with a single probe had failed (data not shown) and so a combination of probes were used which recognised regions of SFV- nsP1, nsP2, nsP3, E1 and C-E3 proteins. The primers

designed against these regions are described in **Chapter 2.9**. It was observed that after detection with the SFV specific probes the band detected at 21 nt with Et Br was no longer recognised, indicating that these small RNAs are not produced against these viral regions (**Figure 3.4B**). It is therefore suggested that the previously detected 21 nt small RNAs are cellular-derived RNAs, for example endo-siRNAs produced by the endogenous RNA pathway. Another possibility is that they are miRNAs as it is impossible to distinguish between 21 nt and 22 nt small RNAs on this kind of gel.

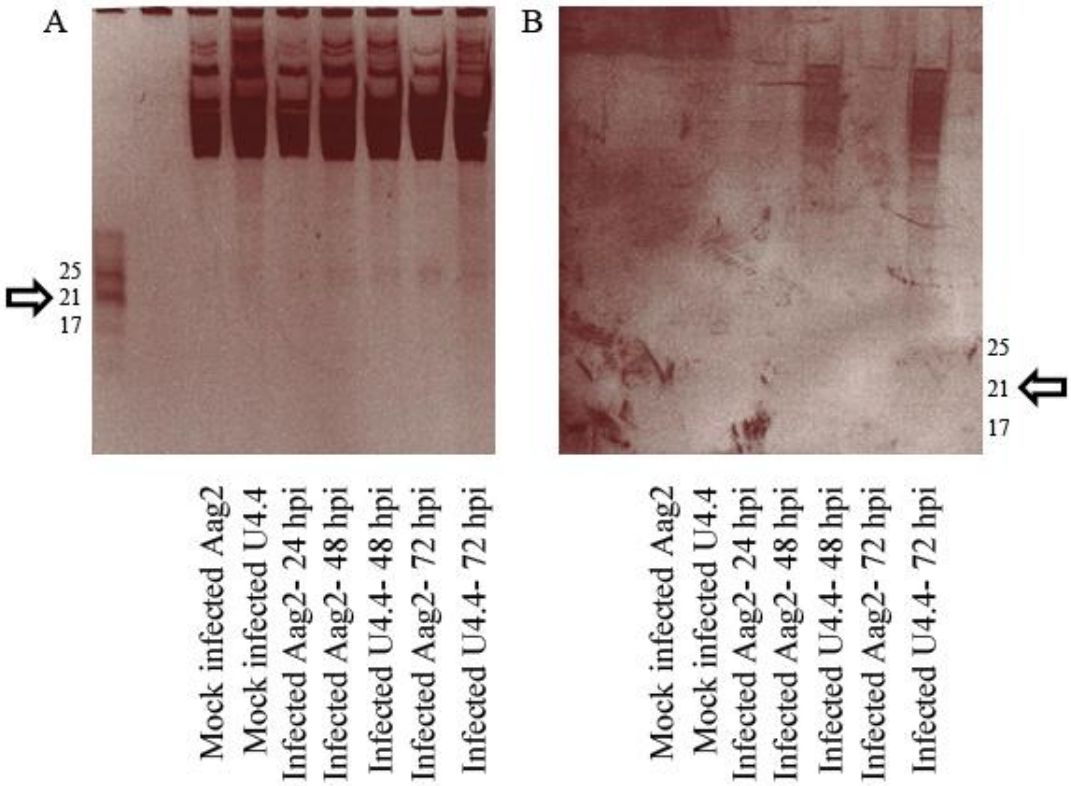

Figure 3.4: Detection of viRNAs derived from SFV infected Aag2 and U4.4 cells by Northern blot analysis. Small RNAs were isolated from SFV infected *Ae. aegypti*-derived Aag2 or *Ae. albopictus*-derived U4.4 cells and separated by gel electrophoresis. **A**) Prior to blotting the gel was stained with Ethidium Bromide to detect all RNAs present in each sample. **B**) The RNAs from this gel were transferred to a membrane and hybridised with a combination of SFV-specific, DIG labelled DNA oligonucleotide probes. Anti-DIG antibodies conjugated to alkaline phosphatase were used to detect the probes and the signal developed using NBT-BCIP detection reagent. The weight of the small RNA fragments is indicated by arrows.

3.3.3 Production and Mapping of siRNAs in Aedine Cell Lines Against Non-Viral Exogenous dsRNA

As previously mentioned the pattern of 'hot' and 'cold' spot RNAs has been well characterised following viral infection of insect cells (Myles et al., 2008 and 2009, Brackney et al., 2009; Sánchez-Vargas et al., 2009; Siu et al., 2011). However, it has not been shown that this arrangement occurs following the introduction of non-viral exogenous dsRNA molecules. To discover if transfected dsRNA alone is capable of inducing the RNAi response and generating 'hot' and 'cold' spot siRNAs, both U4.4 and Aag2 cells were seeded in a 6-well plate at 5 $x10^5$ and 6 $x10^5$ cells/well respectively. Each well was then transfected with 1 µg specific dsRNA generated against a 720 nt eGFP sequence and incubated at 28 °C for 24 h. Following this, the RNA was isolated by Trizol extraction and small RNAs of < 40 nt were analysed by Illumina Solexa deep sequencing technology to determine the frequency of reads at each genetic loci (**Figure 3.5A** and **B**). The results generated indicate that dsRNA resulting from a non-viral exogenous template also produce 21 nt siRNAs as the predominant length in both cell types consistent with Dcr-2 processing. These siRNAs also mapped to both the sense and antisense original eGFP template stands and where distributed across the full length of both strands with evident 'hot' and 'cold' spots.

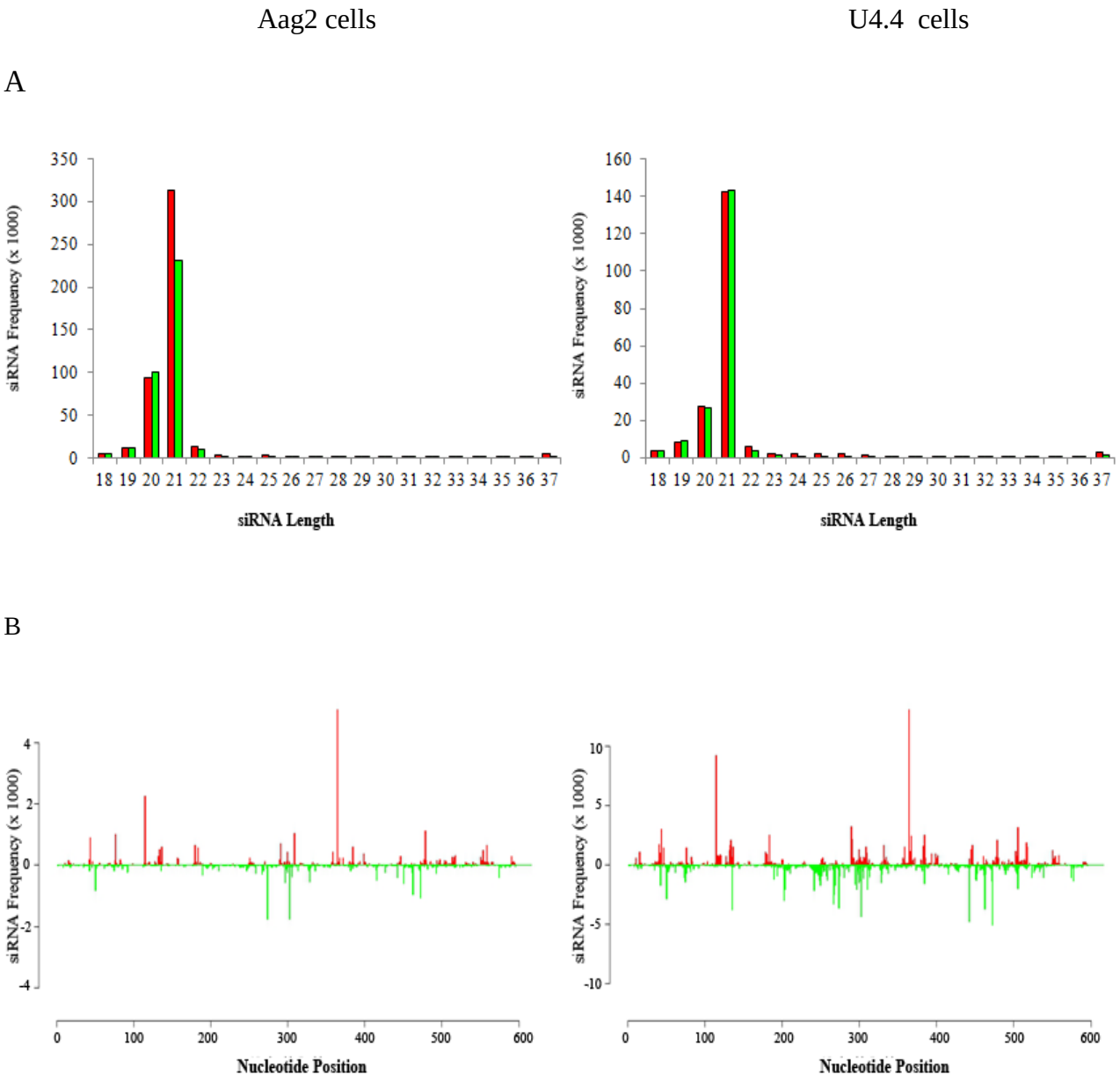

Figure 3.5: Mapping of siRNAs produced against non-cell specific dsRNA in *Ae. aegypti*-derived Aag2 cells and *Ae. albopictus*-derived U4.4 cells. A) The size distribution of small RNA molecules produced between 18 - 37 nt which map to an eGFP sequence (720 nt) following transfection of Aag2 cells with eGFP dsRNA. Red indicates siRNAs arising from the coding strand of the input dsRNA, while green denotes those which map to the non-coding strand. **B**) The abundance distribution of 21 nt small RNAs generated against either the coding strand (red, positive numbers, 5'→3' direction) or the non-coding strand (green, negative numbers, 3'→5' direction) of the eGFP input sequence in Aag2 or U4.4 cells. The x-axis denotes the nucleotide position of the eGFP sequence for each 21 nt siRNA. The frequency of reads for siRNAs at each position is shown on the y-axis.

3.4 Discussion

The innate immune responses in mosquitoes are known to be important in the control and regulation of arbovirus infections. While the involvement of the Toll, IMD and JAK/STAT pathways have been shown to be virus-dependent (Fragkoudis et al., 2008; Xi et al., 2008; Avadhanula et al., 2009; Souza-Neto et al., 2009; Sabin et al., 2010; Kingsolver et al., 2013; Merkling and van Rij, 2013; McFarlane et al., 2014), the exogenous RNAi response has been recognised as the pivotal antiviral defence against a number of different arboviruses and has become the focus of concentrated research (Keene et al., 2004; Attarzadeh-Yazdi et al., 2008; Campbell et al., 2008b; Myles et al., 2008; Sánchez-Vargas et al., 2009; Scott et al., 2010; Hess et al., 2011; Siu et al., 2011; Lambrechts et al., 2012; Léger et al., 2012; Vodovar et al., 2012; Schnettler et al., 2013a; McFarlane et al., 2014).

Initial experiments in *Drosophila* indicated that silencing of the RNAi proteins resulted in hypersensitivity of *Drosophila*-derived cells and null mutant lines to insect-specific infections; such as FHV, Drosophila C virus (DCV), Drosophila X virus (DXV) (Li et al., 2002; Galiana-Arnoux et al., 2006; van Rij et al., 2006; Wang et al., 2006; Zambon et al., 2006; Lim do et al., 2008; Flynt et al., 2009; Han et al., 2011) and arboviral infections (Chotkowski et al., 2008; Mueller et al., 2010; Mukherjee and Hanley, 2010). These results were later found to translate into mosquitoes, and Ago-2 in particular was recognised as being essential for the regulation of both alphaviruses *in vivo* (Keene et al., 2004; Campbell et al., 2008b; McFarlane et al., 2014) and *in vitro* (Li et al., 2004; Schnettler et al., 2013a; McFarlane et al., 2014) as well as *in vivo* (Franz et al., 2006; Sánchez-Vargas et al., 2009) and *in vitro* (Sánchez-Vargas et al., 2009) control of flaviviruses replication. This project has shown that approximately equal levels of gene silencing could be achieved for both Ago-2 and Dcr-2 RNAi genes in Aag2 cells. However, it was also observed that the greatest increase in viral replication was detected following the knockdown of Ago-2. These results suggest that even reduced levels of Dcr-2 are sufficient to detect and cleave an adequate level of dsRNA to initiate the RNAi pathway to a certain degree. Therefore, it can be implied that Dcr-2 cleavage of viral genome molecules alone is not sufficient to prevent virus replication; although, Ago-2 activity is required and plays a crucial and central role in the antiviral immune response. This finding is in agreement with previous results which showed that Dcr-2 silencing in *Drosophila*-derived S2 cells continued to restrict the replication of recombinant FHV deficient in B2 despite it being achieved in *dcr-2* null mutant *Drosophila* embryos (Wang et al., 2006). Similarly, silencing of Dcr-2 in

the midgut of transgenic *Ae. aegypti* facilitated an enhanced Sindbis virus (SINV) infection and an increase in viral dissemination, although mortality rates were not affected (Campbell et al., 2008b; Khoo et al., 2010).

It was necessary to determine the effect knockdown has on SFV replication following infection at two MOIs. Studying the silencing of the RNAi proteins with a high MOI certifies the effect this has on an individual level as every cell in the culture should be infected synchronously. In contrast, at a low MOI the virus undergoes multiple rounds of replication as only a few cells are infected initially and we can ascertain the effect that silencing these key RNAi proteins has on the spread of the virus from an infected cell to its neighbours. However, the MOI is based on that established in BHK-21 mammalian cells and does not translate effectively to what is observed in insect cells in culture (unpublished observations). Although a high proportion of cells can be infected, as a result of the MOI equivalent in insect cells being slightly less, global infection is not achieved without some degree of spread. A substantial increase in viral replication was only observed following the knockdown of Ago-2 during a viral infection at a high MOI (**Figure 3.2A**). Work carried out following these experiments has established that an Ago-2 knockdown in Aag2 cells is capable of allowing significantly high levels of virus replication following a low MOI viral infection (Schnettler et al., 2013a) as well as infection with SFV VRPs (personal communication with Dr. Esther Schnettler). Suggested reasons why this was not the case during this project may include some of the common technical hurdles encountered during these experiments. The age of the cells and the age, composition and batch quality of the transfection reagent can all impact the success of these highly sensitive assays. In addition the weak inhibitory effect at the low MOI is due to the fact that many of the cells will not have been transfected with dsRNA which only reaches ~28 % of Aag2 cells in culture (Schnettler et al., 2013a). However, the consistent lack of enhanced virus replication following the knockdown of miRNA pathway proteins Ago-1 and Dcr-1 suggests that the miRNA pathway is not involved in the control of SFV replication and is consistent with what has been shown for other arbovirus infections (Hoa et al., 2003; Keene et al., 2004; Li et al., 2004; Schnettler et al., 2013a; McFarlane et al., 2014).

Numerous reports have stressed the importance of RNAi and its influence on the interaction between the virus and its vector altering the outcome of the infection. Following the knockdown of RNAi proteins in *Ae. aegypti*, DENV-2 replication was enhanced by a greater than four-fold increase and the extrinsic incubation period before the vector could transmit the virus to a susceptible vertebrate was decreased from 10 - 12 days

to as few as 7 days (Sánchez-Vargas et al., 2009). This in turn allows a greater number of individuals to become infected over the course of the lifetime of the mosquito. The population size of viRNAs has been shown to increase as viral infections progress in both *Drosophila* and mosquitoes. This indicates continued detection and cleavage of viral RNAs by Dcr-2 and highlights that there is maintained control during persistent infections (Li et al., 2002; Sánchez-Vargas et al., 2004; Aliyari et al., 2008; Myles et al., 2008; Brackney et al., 2009; Flynt et al., 2009; Wu et al., 2010). In an RNAi deficient situation, such as loss-of-function mutants, vector viability may be compromised due to unrestricted virus replication (van Rij et al., 2006). As a result, antiviral RNAi is integral to the control of viral infections and protects the arthropod from excessive replication and the associated pathology, while on the other hand the virus also benefits as it is maintained and can complete its transmission cycle when the insect feeds.

Previous findings have suggested it would be counterintuitive to the replicative fitness of the virus to interfere with the immune pathway and may likely explain the lack of RNAi suppressor proteins encoded by the majority of arboviruses (Li and Ding, 2006; Blakqori et al., 2007; Attarzadeh- Yazdi et al., 2009; Fragkoudis et al., 2009; Donald et al., 2012). Unlike true pathogenic viruses, such as DCV, which are known to encode proteins to enhance their virulence, most arboviruses are understood to circumvent the antiviral response rather than directly impede it (Uchil et al., 2003; Geiss et al., 2005; Siu et al., 2011). The seclusion of viral-derived RNAs was eluded too following the results generated by Northern blot analysis. The lack of detection of viRNAs by SFV-specific probes indicated that the virus was able to sequester its dsRNA and restrict access to the RNAi mechanism. As a result of the lack of detection it was suggested that a potential pit fall was the level of sensitivity. Therefore, it was decided to use radio-labelled oligonucleotide probes to enhance any potential signal. The probes were designed based on the three SFV sequences which gave the highest reads during previous deep sequencing analysis (Siu et al., 2011, **Chapter 2.9**). The use of these probes was to further strengthen the chance of detection as they should be present in the highest quantities following viral replication. Unfortunately, this method also failed to generate a result and no signal was detected (data not shown). This is in contrast to what has been shown in plants where this method of detection is highly successful due to the vast quantity of viral-derived small RNAs generated following RdRP amplification (Dalmay et al., 2000; Lakatos et al., 2004; Molnar et al., 2005; Sunpapao et al., 2009; Miozzi et al., 2013). Northern blot analysis has also been used to successfully to identify viRNAs produced in *Drosophila* and their derived cell

lines following infections by FHV (Aliyari et al., 2008; Han et al., 2011), WNV (Chotkowski et al., 2008) and Rift Valley fever virus (RVFV) (Sabin et al., 2013). The findings described here contribute to the suggested hypothesis that alphavirus replication in invertebrate cells occurs in membrane-bound vesicles away from the antiviral response mechanism. In this way it is theorised that Dcr-2 access to its substrate dsRNA would be restricted and therefore the generation of viral-derived viRNA molecules would be impeded. This form of evasion defence strategy has been observed following alphavirus infection in both vertebrate and invertebrate-derived cell lines (Friedman et al., 1972; Salonen et al., 2005; Spuul et al., 2007; Frolova et al., 2010) and similar replication compartments are also evident following flavivirus infection of mammalian and tick cell lines (Senigl et al., 2006; Offerdahl et al., 2012). Such a strategy could explain why viRNA molecules consist of minor proportion of the total small RNA population isolated from infected mosquito cell lines as established by several deep sequencing experiments using a number of different viruses (Campbell et al., 2008b; Myles et al., 2008 and 2009; Brackney et al., 2009; Scott et al., 2010; Siu et al., 2011; Léger et al., 2012; Morazzani et al., 2012).

A decoy strategy as a method for RNAi evasion was also suggested when deep sequencing analysis revealed that although viRNAs are produced across both the genome and anti-genome of SFV, certain regions were more intensely targeted than others. The viRNAs produced in high numbers were shown to be highly ineffective at restricting SFV replication in contrast to those producing lower reads. Therefore, viRNAs which cannot be used for virus repression are suggested to saturate RISC and restrict access to functional viRNAs. This ‘hot’/‘cold’ spot pattern is in line with that observed with other arboviruses such as RVFV (Léger et al., 2013), WNV (Brackney et al., 2009) and chikungunya (CHIKV) (Morazzani et al., 2012). However, it is yet to be established if ‘cold’ spot viRNAs are more successful at targeting the viral sequences than ‘hot’ spot viRNAs derived from these viruses. As expected, this pattern was also observed following transfection of dsRNA specifically targeting the eGFP sequence consistent with processing via the exo-siRNA pathway. Therefore, the exogenous RNAi response is triggered regardless of the source of dsRNA. This indicates that the pattern of high and low siRNA production from different loci is as a result of endogenous cellular processing and is not due to factors involved in virus replication or secondary structures in the viral genome. Why some sequences are targeted more than others is not yet understood and a number of hypotheses have been suggested which require further study. Currently, Dcr-2 is believed to attach to the end of a nucleic acid and cut 21 nt from the start and so on until reaching

the end of the sequence. If this was indeed the case then no siRNA bias would be observed. Therefore, it can be suggested that Dcr-2 may function in a different manner resulting in a variation in the number of siRNAs against specific sequences. The stability of some siRNA molecules may be different due to factors which have not yet been fully explored. Certainly, as described previously, deep sequencing has been shown to display some degree of ligation bias which may alter the outcome from its natural state. It is also of note that piRNAs are not detected following the treatment of eGFP-specific dsRNA which is unlike samples infected with virus and so it can be suggested that the generation of piRNAs requires another stimulant other than the detection of dsRNA (Schnettler et al., 2013a).

The advent of advanced, high through-put techniques such as deep sequencing and micro array expression analysis has greatly contributed to the field of arbovirology. In addition, by combining the viRNA molecules generated in invertebrates, virtually entire virus genomes can be compiled which opens up new avenues for virus discovery (Wu et al., 2010). These results, together with what is already known in the literature, has proven that viRNAs are paramount to RNAi and therefore to the life and wellbeing of vector mosquitoes. Further research is required to fully assess their biochemistry and mode of production. In addition, the rapid technical advances in the field have greatly accelerated our understanding of the antiviral genes involved in blocking virus dissemination and transmission. Thanks to a number of recent key findings our knowledge of how mosquitoes handle arboviral infections has substantially improved; although, many outstanding questions still remain to be answered.

3.5 Future Work

The function of the antiviral proteins Ago-2 and Dcr-2, as well as miRNA- pathway proteins in mosquitoes have been determined by *in vivo* injections and transfections *in vitro*. However, these types of knockdowns are only transient and often inconsistent. It would be ideal to have a stable mosquito line, such as those developed for *Drosophila*, which are genetic null mutants or where there is at least a stable protein knockdown. These would allow the direct experiments which verify their action and are still missing to be completed.

Further modifications could still be made to the Northern blot protocol. In particular, the use of a recombinant strain of FHV which did not encode the B2 RNAi silencing suppressor protein could provide a valuable insight into siRNA production during infection. As this protein acts to inhibit siRNA biogenesis, its removal would increase the number of siRNA available for detection and so would act as a positive control for the experimental set up. However, due to the genetically modified nature of the virus, this could not have been completed during this project due to licensing restrictions. An alternative strategy for the detection of viral RNA that has been successful with Bunyamwera virus (BUNV) requires the *in vitro* transcription of a plasmid containing the viral genome sequence and incorporating radio-labelled nucleotides for detection (Dr. Agnieszka Szemiel, Personal communication). This would result in the creation of a probe recognising the entirety of the viral genome and would detect each viral viRNA rather than a selected few. A further difference is that this probe would be an RNA probe compared to the previously tested DNA probes.

Little has been determined with regards to the involvement of immunity molecules in persistence and transmission of mosquito-borne infections. Further biochemical analysis of small RNAs is required to answer many unknown factors, in particular those relating to their methylation and phosphorylation. Therefore, it is necessary to ascertain the nature of viRNAs derived from SFV during infection in Aag2 cells and to establish if there are any viral proteins which affect their function and therefore the timing and kinetics of the antiviral response. The use of β-elimination assays, Northern blot analysis and deep sequencing would distinguish between methylated and unmethylated viRNAs through changes in molecular weight and would determine if this has any biological relevance to the biochemical differences observed between ‘hot’ and ‘cold’ spot viRNAs. Further

analysis would then be necessary to establish if this is as a result of active demethylation by arboviruses. In addition, the biological relevance of 'hot' and 'cold' spots is still unknown as is the significance of the specific biases across both (+) and (-) strands and therefore further work is necessary to fully characterise them.

There is also very little information available regarding the relationship between the RNAi response and negative strand RNA viruses, such as bunyaviruses and rhabdoviruses, or dsRNA viruses, such as reoviruses. Therefore, the integral differences in their replication kinetics and the strategies used by these viruses is not fully understood and further work is required to investigate this alternative avenue of interest.

3.6 Principle Findings

1. Major *Ae. aegypti* RNAi proteins Dicer-1, Dicer-2, Argonaute-1 and Argonaute-2 can be efficiently silenced by treating cells with transcript-specific dsRNA molecules.

2. The knockdown of Argonaute-2 in *Ae. aegypti*-derived Aag2 cells results in a strong and significant increase in SFV replication, defining this as the key protein in antiviral RNAi.

3. Despite the presence of small RNAs of 21 nt in *Ae. aegypti*-derived Aag2 cells, these are not detected with SFV specific probes by Northern blot analysis.

4. Small RNAs of 21 nt are the majority population produced following transfection of non-viral dsRNA in the *Ae. aegypti*-derived Aag2 and *Ae. albopictus*-derived U4.4 cell lines.

5. These 21 nt small RNAs produced from an artificial dsRNA Dcr-2 substrate emulate the distribution of viral-derived small RNAs with the variation in ‘hot’ and ‘cold’ spots along the coding sequence.

Chapter 4: A Study of the Role of an Orthologue of D-elp1 in *Ae. aegypti* RNAi

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4.1 Introduction

Several studies have shown that intracellular RNA-dependent RNA Polymerases (RdRPs) are required for RNA silencing in fungi, yeast, nematodes and plants (Cogoni and Macino, 1999; Dalmay et al., 2000; Smardon et al., 2000, Mourrain et al., 2000; Sijen et al., 2001; Makeyev and Bamford; 2002; Wassenegger et al., 2006). The conservation of RdRP gene sequences in these organisms is thought to be central in maintaining the integrity of the genome from transposon mobilisation and viral infection. There is also an integral interaction between RdRPs and the RNA interference (RNAi) response necessary for effective gene regulation in at least some of these organisms. RdRPs facilitate the spread of the RNAi response by catalysing the generation of new RNA molecules from existing RNA template strands (**Figure 4.1**). RdRP action has been shown in lower eukaryotes to be linked to the systemic spread of RNAi signalling molecules (siRNAs/dsRNAs) to prepare uninfected neighbouring cells against the invading infection (Smardon et al., 2000; Sijen et al., 2001; Voinnet, 2005). In plants, amplification occurs by two known mechanisms. Firstly, RdRPs are involved in the physical amplification of aberrant single stranded (ss) RNA populations generating full-length dsRNA molecules which can be further targeted by Dicer for cleavage into more siRNA molecules. Aberrant RNAs may result from a viral infection, sense transgenes or from mobile genetic elements. A second model which has been described is *de novo* synthesis of dsRNA by primer extension of siRNAs which are perfectly complementary to the target RNA sequence (Tang et al., 2003). The siRNA-primed dsRNA molecules produced are later cleaved by Dicer into siRNA molecules. These two mechanisms are not mutually exclusive from each other and demonstrate that RdRPs can act in either a primed or un-primed manner (Schiebel et al., 1993; Makeyev and Bamford; 2002). Conversely in *C. elegans*, RdRPs are Dicer independent and can synthesize siRNAs directly from the mRNA target (Smardon et al., 2000; Sijen et al., 2001 and 2007; Pak and Fire; 2007). Targeted processing of the Dicer-catalysed siRNAs by the Argonaute protein, RDE-1, recruits and directs the action of the RdRP complex for the biogenesis of siRNAs by un-primed *de novo* synthesis.

To date, no canonical RdRP homolog has been detected in the genome of insects or higher eukaryotic organisms (Gordon and Waterhouse, 2007; Zong et al., 2009); although, this has not appeared to handicap the RNAi mechanism. On the contrary, studies have suggested that the silencing mechanism is less efficacious against virus control in plants compared to insects. Mutant plant viruses deficient for a functional RNAi suppressor

protein are incapable of causing a systemic infection, although localised cellular infections are not affected (Voinnet et al., 2000; Silhavy et al., 2002). This is not the case in fruit flies infected with flock house virus (FHV) lacking a functional B2 suppressor protein as the virus is rapidly cleared from infected cells unless the RNAi response is compromised (Li et al., 2002). This may suggest that there is a greater reliance on an efficient RISC in these organisms.

Recently, RdRP-like activity was described in *Drosophila* (Lipardi et al., 2001, 2003, 2005 and 2009) and other vertebrates (Sam et al., 1998; Maida et al., 2009; Pelczar et al., 2010). In *Drosophila*, transgene co-suppression assays identified a correlation between the emergence of alcohol dehydrogenase (*Adh*) specific siRNAs and a decrease in mRNA transcript levels once transgene copies surpassed a threshold (Pal-Bhadra et al., 2002). The reputed RdRP-substitute enzyme was identified through an RNAi inhibition screen by Lipardi and Paterson (2009) as elongator subunit 1 (D-elp1), or iκb kinase complex associated protein (IKAP), which is the largest subunit of the *Drosophila* polymerase (pol) II core elongator complex. In contrast to the canonical RdRP, this gene is highly conserved in all eukaryotes, including those which are known to express putative RdRP enzymes and RNA pol II has been independently shown to possess intrinsic RdRP activity (Lehmann et al., 2007). Evidence was established through *in vitro* RNA silencing experiments carried out in *Drosophila* embryo cell-free extracts that siRNAs stimulated a silencing response in addition to initiating template dependent *de novo* dsRNA synthesis (Lipardi et al., 2001 and 2005). D-elp1 was hypothesised to generate new dsRNA molecules as substrates for Dicer-2 (Dcr-2) using either siRNA molecules as primers or by a primer-independent mechanism to allow the propagation of the siRNA population and enhancing targeted silencing in an action similar to that of known RdRPs in plants, fungi and worms (Hutvagner and Zamore, 2002) (**Figure 4.1**). An additional role for RdRPs, such as EGO-1 in *C. elegans* (Smardon et al., 2000; Maniar and Fire, 2011) is their involvement in the control of the germline stability, defending against 'selfish' nucleic acids or transposons. Deep sequencing analysis of endogenous siRNAs (endo-siRNAs) indicated a high proportion were derived from transposons and were dependent on Dcr-2 and Argonaute-2 (Ago-2) processing. These were derived from both the sense and antisense strands of transposon RNA (Chung et al., 2008; Ghildiyal et al., 2008) which could indicate the manufacture of dsRNA from transposable elements. Transposons may be controlled by an association between the aberrant RNAs and the RNA silencing components and the knockdown of D-elp1 had a similar effect as that seen with Dcr-2 on increasing a

population of retrotransposons (Lipardi and Paterson, 2009). Levels of antisense siRNAs were seen to decrease only when D-elp1 was silenced strongly suggesting that D-elp1 is acting as an RdRP by producing the complementary antisense molecule.

Unfortunately, following the initiation of this work, Lipardi and Paterson published a retraction to their 2009 publication (Lipardi and Paterson, 2011) and the interpretations remain controversial. The authors revoked their statement that D-elp1 was an RdRP, although they maintain that it is involved in RNAi, endogenous siRNA production and the suppression of transposable elements.

Due to its widespread nature throughout the eukaryotic kingdom it was interesting to determine if a homologue of D-elp1 existed within the genome of *Ae. aegypti*. This species of mosquito is a major vector for a number of pathogenic arboviruses and their complete genome has been published (Nene *et al*., 2007). They are also known to have a competent RNAi response, which can successfully limit viral titres within the insect (Blair, 2011; Donald et al., 2012). As it was shown that D-elp1 localised in the cytoplasm (Svejstrup, 2007; Johansen et al 2008), the site of replication of important arboviruses and the site of RNA processing, it could potentially form an interaction with Dcr-2. Lipardi stated (2009) that when both Dcr-2 and D-elp1 were over-expressed they were able to interact and form a complex and that recombinant Dcr-2 was capable of using dsRNA molecules generated from both purified and recombinant D-elp1 as substrates for cleavage. This association was maintained following the retraction of the article. RdRP amplification of dsRNAs would be beneficial to mosquitoes to enhance the RNAi signal and its vital role in cellular interactions with arboviruses. In addition to augmenting the quantity of siRNAs available for uptake into RISC, dissemination of an amplified signal to adjacent cells would be permitted to limit arbovirus spread (Attarzadeh-Yazdi et al., 2009). As the movement of siRNAs over short distances has been demonstrated for mosquitoes, RdRP action may be advantageous but not essential for spread of antiviral activity within the organism.

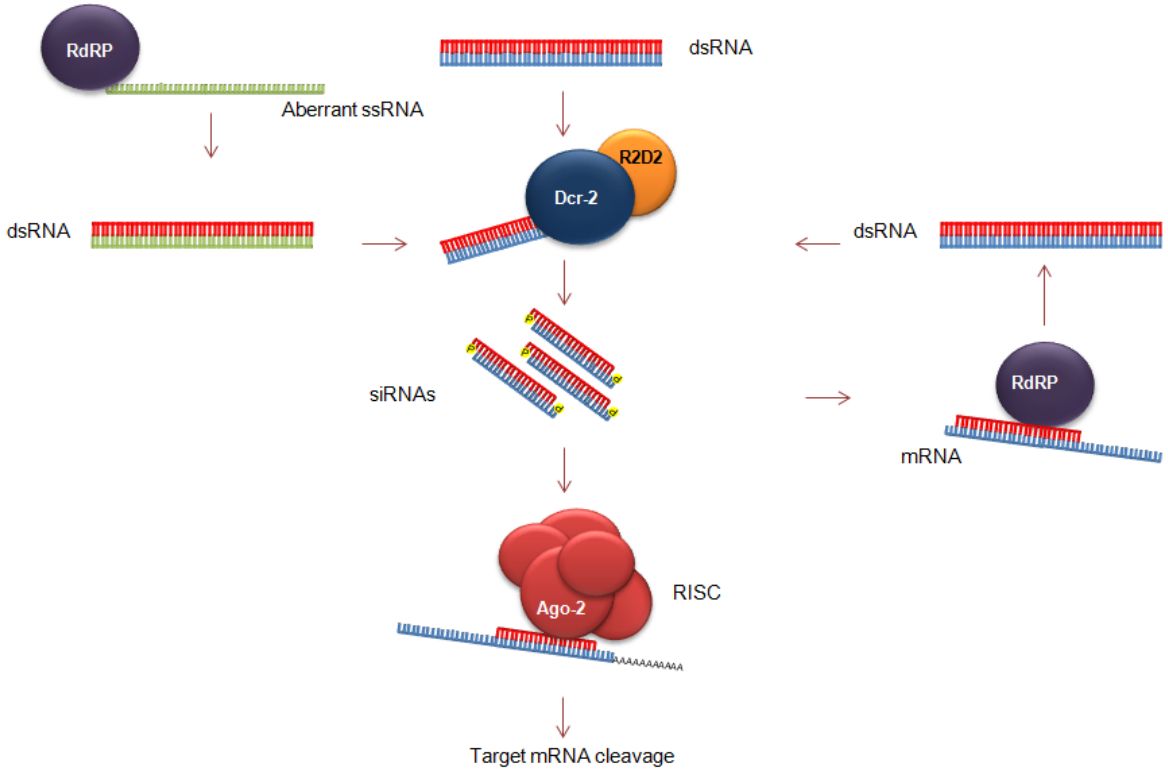


Figure 4.1: Schematic illustration of the involvement of an RNA dependent RNA polymerase (RdRP) in the RNAi pathway. Amplification of substrate dsRNA by the RdRP generates more targets for Dicer-2 and subsequently increases the siRNA population available for loading into RISC. dsRNA, double stranded RNA; Dcr-2, Dicer-2; RISC, RNA induced silencing complex; Ago-2, Argonaute-2; siRNA, small interfering RNA; ssRNA, single-stranded RNA; mRNA, messenger RNA.

4.2 Objectives

1. Identify the presence of a homolog of D-elp1 in the *Ae. aegypti* derived Aag2 cell line.

2. Investigate the effect this homolog has on SFV infection and dsRNA mediated silencing in the *Ae. aegypti* derived Aag2 cells.

4.3 Results

4.3.1 The Presence of an RNA-dependent RNA Polymerase Homologue in *Ae. aegypti* Aag2 Cells

In order to establish the existence of a homologue of D-elp1 within the genome of *Ae. aegypti* a search of the National Center for Biotechnology Information (NCBI) protein database was carried out using the Basic Local Alignment Search Tool (BLAST) online tool with the D-elp1 sequence (AAF54670) as the query against the *Ae. aegypti* reference genome. This generated eight potential matches with the dominant hit, identified as the iκb kinase complex associated protein (AAEL001036-PA/XP_001657869), showing 38 % homology with 97 % coverage. The two sequences have been aligned using EMBOSS Needle6.6.0 (**Figure 4.2**). This was designated as the most likely homologue for D-elp1 in comparison to the other identified hits and shall hereafter be referred to as Aa-elp1.

To determine if Aa-elp1 is expressed in the *Ae. aegypti*-derived Aag2 cell line, it was detected using specific primers to amplify regions of the gene by RT-PCR. These regions were selected as they appeared to be unique within the *Ae. aegypti* genome and could later be used for the production of multiple Aa-elp1-specific dsRNAs. Distinction of novel sequences is vital as this restricts the number of potential dsRNA recognition sites and therefore minimises off-target effects. This approach revealed three unique regions of varying lengths for amplification by PCR (Region 1: 94 bp, Region 2: 138 bp, Region 3: 257 bp long, **Figure 4.2**). Primers were designed (**Chapter 2.9**) which contained T7 polymerase promoter sites to later allow the generation of specific long dsRNAs using the MegaScript RNAi kit. Total cellular RNA was isolated from untreated Aag2 cells and cDNA generated by first strand cDNA synthesis by reverse transcription for partial gene amplification by the specific primers in the PCR. DNA products from the PCR were analysed by agarose gel electrophoresis to confirm specificity, fragment size and gene expression levels (**Figure 4.3**). The size of the fragment was expected to be 42 bp larger than the region described due to the addition of the T7 promoter region. Each primer set successfully amplified fragments of the desired size from the Aag2 cDNA and did not detect anything in the RNA (No RT) control samples confirming that Aa-elp1 is expressed in *Ae. aegypti*-derived Aag2 cells.

Prior to the production of dsRNA, each fragment was cloned into the pJet1.2/blunt cloning vector and sent for sequencing to validate the sequence was correct. Although dsRNA molecules were successfully generated against both regions 2 and 3, this was not possible for region 1 due to its failure to clone successfully. Therefore, only dsRNA molecules against regions 2 and 3 were used for Aa-elp1 silencing experiments.

Figure 4.2: Alignment of elp1 in *Drosophila melanogaster* (D-elp 1) to ikappab kinase complex-associated protein from *Ae. aegypti* (Aa-elp1). A comparison between the sequence of D-elp1 to the genome of *Ae. aegypti* revealed a single protein with 97 % coverage and an identity score of 38 %. Purple underlined sequences indicate regions of primer design. Single letters (amino acids, see **Page xix**), identical amino acid match (|), conserved substitution (:), semi-conserved substitution (.).

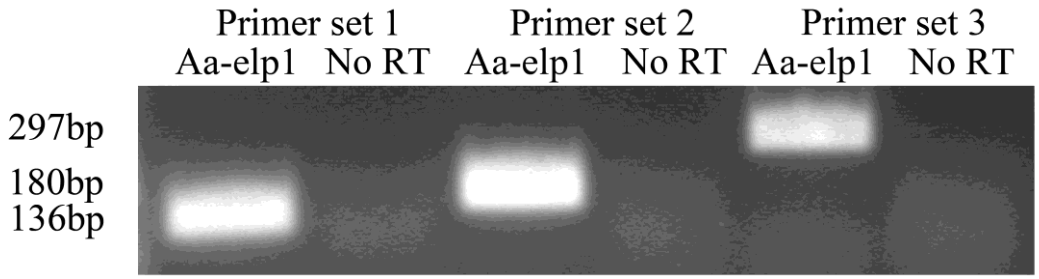


Figure 4.3: Detection of Aa-elp1 gene transcripts in Aag2 cells. Expression of Aa-elp1 in *Ae. aegypti*-derived Aag2 cells was determined by RT-PCR. Cellular mRNA was reverse transcribed to cDNA using oligo-dT primers and the PCR was carried out using three different sets of Aa-elp1 specific primers. No RT control samples were prepared by omitting the superscript III enzyme from the reaction.

4.3.2 The Effect of Aa-elp1 Knockdown on dsRNA Induced Silencing in Aag2 Cells

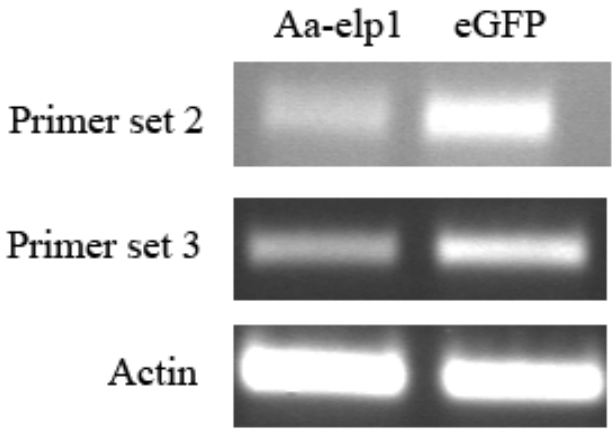
Prior to the characterisation of Aa-elp1 it was first necessary to establish an efficient knockdown in Aag2 cells. Aag2 cells were seeded in 24-well plates at a density of 1.8 x 10^5 cells/well and transfected 24 h later with 50 ng/well of dsRNA, either both specific dsRNA to Aa-elp1 together (50 ng of dsRNA against region 2 plus 50 ng dsRNA against region 3), or control dsRNA (eGFP). Successful silencing of Aa-elp1 transcripts was established by RT-PCR 24 h post transfection and quantified by densitometry using the ImageJ software (**Figure 4.4A** and **B**). Both sets of primers designed against regions 2 and 3 showed a reduction in the level of Aa-elp1 transcripts detected compared to control samples treated with eGFP dsRNA (23.3 % and 20.8 % reduction respectively).

The involvement of Aa-elp1 in dsRNA induced silencing was first determined using plasmids expressing luciferase reporter genes in a more controllable experimental setup compared to a viral infection. As the transfected dsRNA molecules are recognised and processed by the exogenous RNAi pathway, this mimics the detection of a viral infection and is a useful technnique. Aag2 cells were seeded in 24-well plates as before and transfected 24 h later with 50 ng/well of dsRNA, either both specific dsRNA against Aa-elp1 together, control eGFP dsRNA or were mock transfected. Cells were incubated for 24 h post transfection prior to a second treatment where all conditions received reporter plasmid co-transfected with specific dsRNA against that reporter sequence. In these experiments each well received 5 ng/well plasmid pRL-CMV (Promega) that expresses *Renilla* luciferase (*Rluc*) under the control of the cytomegalovirus immediate-early promoter as an internal control and 30 ng/well plasmid pEGFP/VDV-1_s/*Fluc* expressing firefly luciferase (*Fluc*) under the baculovirus OpIE2 promoter which has been described previously (Ongus et al., 2006). Further to this, half the samples in each condition were then treated with 0.01 ng/well *Fluc*-specific dsRNA and the other half with 0.01 ng/well control eGFP-specific dsRNA (**Figure 4.5**).

Aag2 cells have been shown to have a transfection efficiency of 28.6 % for dsRNA (Schnettler et al., 2013a) and 23.4 % for plasmid (unpublished observations). Cells were lysed 24 h post transfection and the ratio of *Fluc*:*Rluc* expression measured by luciferase assay. As expected, samples treated with *Fluc*-specific dsRNA indicated a decrease in luciferase light units compared to control eGFP dsRNA treated samples (**Figure 4.6**). Nevertheless, although dsRNA knockdown was successful, cells treated with Aa-elp1-specific dual dsRNAs showed similar levels of *Fluc* silencing compared to non-silenced

cells. This suggests that Aa-elp1 is not important in dsRNA induced transient gene silencing.

A



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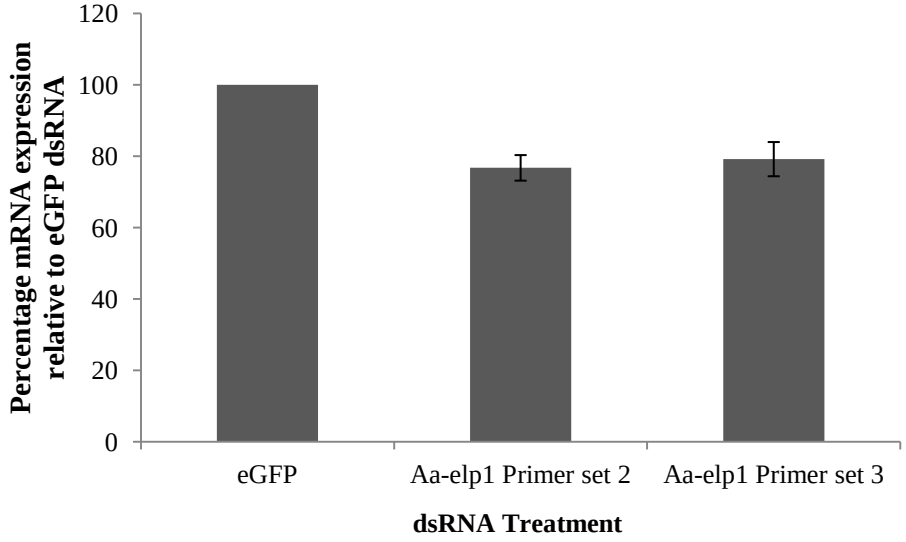


Figure 4.4: Silencing of Aa-elp1 transcripts in Aag2 cells by treating them with Aa-elp1-specific dsRNAs. *Ae. aegypti*-derived Aag2 cells were treated with dsRNA to specifically target Aa-elp1 at two unique regions and these results were compared to control eGFP dsRNA treated cells. **A**) Transcript knockdown was confirmed by RT-PCR using primer sets for each region which are compared to actin primers as an internal control. A representative image is shown. **B**) Three independent experiments were performed and the average pixel density measured using Image J software. The graph shows the results relative to control eGFP dsRNA treated cells. Error bars show standard deviation of the mean.

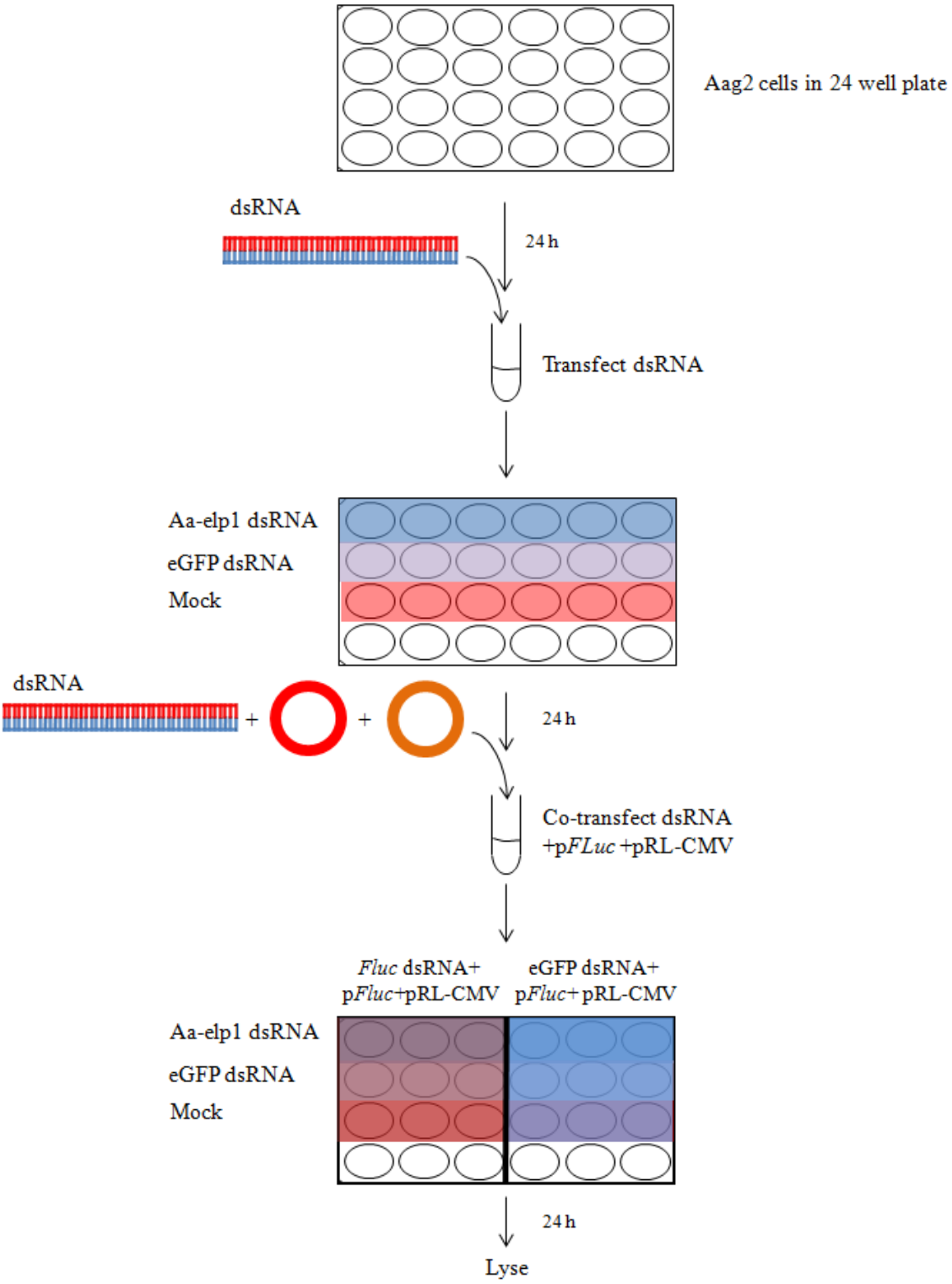


Figure 4.5: A schematic illustration of the experimental set up for the results described in Figure 4.6. *Ae. aeguypi*-derived Aag2 cells seeded in 24-well plates were transfected 24 h post seeding with either dsRNA (Aa-elp1 or eGFP) or were mock transfected. Further to this, 24 h later cells received reporter plasmids (p*Fluc* and pRL-CMV) co-transfected with either *Fluc*-specific dsRNA against that reporter sequence or eGFP-specific control dsRNA.

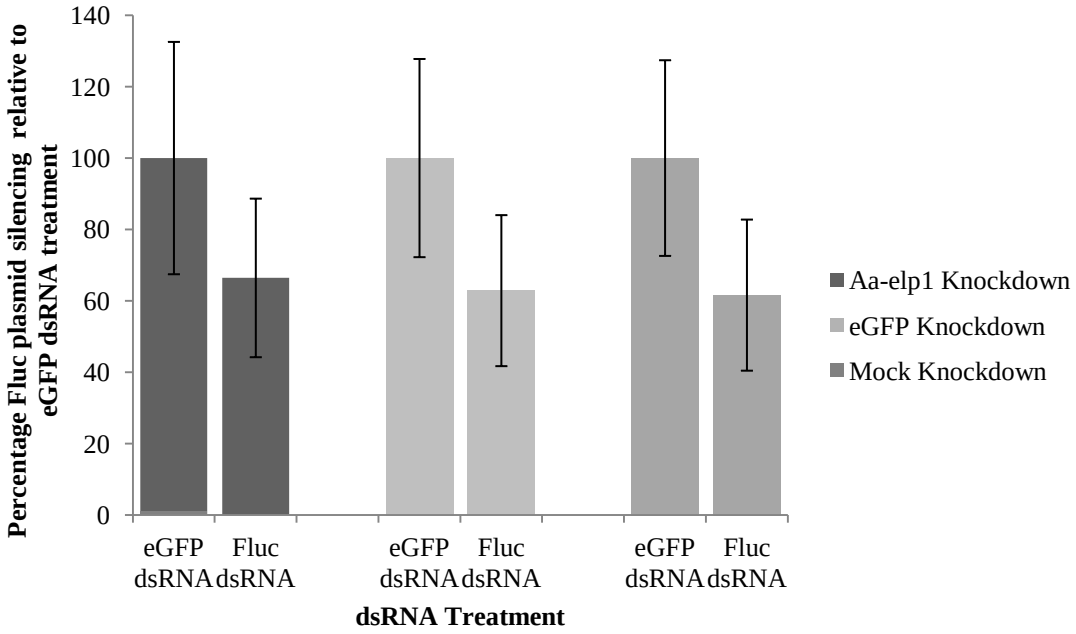


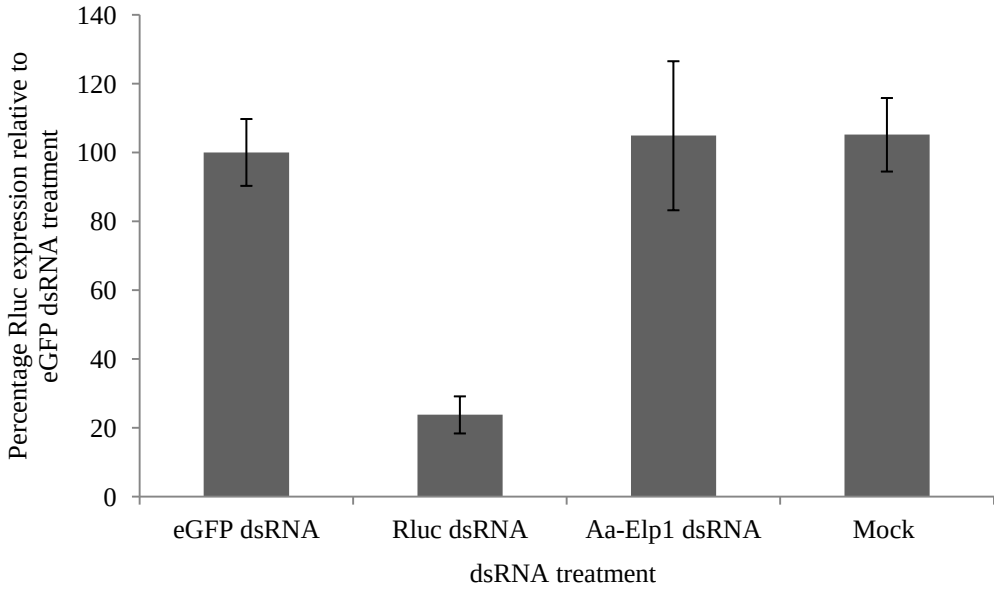
Figure 4.6: The effect of silencing Aa-elp1 in Aag2 cells on dsRNA induced silencing. *Ae. aegypti*-derived Aag2 cells were treated with either the combination of dsRNA against Aa-elp1 (dark grey), eGFP control dsRNA (pale grey) or were mock transfected (light grey). Following an incubation of 24 h, the cells were transfected in parallel with two plasmids (p*Fluc* and pRL-CMV as an internal control), and either specific dsRNA against *Fluc* or control eGFP dsRNA. Cells were incubated for a further 24 h were after the ratio of *Fluc*:*Rluc* expression was measured in relative luciferase light units. Values recorded for control eGFP dsRNA were set to 100 % and the specific *Fluc* dsRNA viewed as a percentage of this. Error bars represent the standard deviation of three independent experiments performed in triplicate.

4.3.3 The Effect of Aa-elp1 Silencing on SFV Replication in Aag2 Cells

As D-elp1 was reputed to be involved in the antiviral RNAi response, the potential role of Aa-elp1 in the control of an SFV infection in Aag2 cells in culture was examined. SFV, as with other alphaviruses and flaviviruses is effectively targeted by the RNAi machinery resulting in the production of virus-derived small RNAs (viRNAs) (Keene et al., 2004; Campbell et al., 2008a and b; Myles et al., 2008 and 2009; Attarzadeh- Yazdi et al., 2009; Sanchez-Vargas et al., 2009; Siu et al., 2011). If Aa-elp1 does display RdRP activity in Aag2 cells and is able to amplify biologically active viRNAs targeting SFV, it would be expected that Aa-elp1 knockdown would allow an increase in virus replication to occur.

To determine the contribution that Aa-elp1 has on the regulation of virus replication, Aag2 cells were seeded in 24-well plates and Aa-elp1 silenced as described. Furthermore, *Rluc*-specific dsRNA was included to confirm the ability of the RNAi machinery in Aag2 cells to target a reporter strain of SFV expressing *Rluc* as a marker of replication (SFV4(3H)-*Rluc*) (Fragkoudis et al., 2008; Attarzadeh-Yazdi et al., 2009). Cells were infected with SFV4(3H)-*Rluc* at either a high multiplicity of infection (MOI, 10) or a low MOI (0.005) 24 h post transfection. Following a 48 h incubation the cells were lysed and luciferase activity determined by luciferase assay. As expected, cells treated with *Rluc*-specific dsRNA showed a decrease in *Rluc* activity at both the high and low MOIs (**Figure 4.7A and B**) validating the efficiency of the assay. However, where Aa-elp1 transcripts had been knocked-down the level of *Rluc* expression closely resembled that detected in control treated cells (eGFP dsRNA or mock transfected). Again this was observed at both MOIs used and demonstrates that there was no effect on virus replication or spread. Details of the effect of silencing key RNAi proteins which do affect SFV replication in Aag2 cells are discussed previously in **Chapter 3**.

A



B

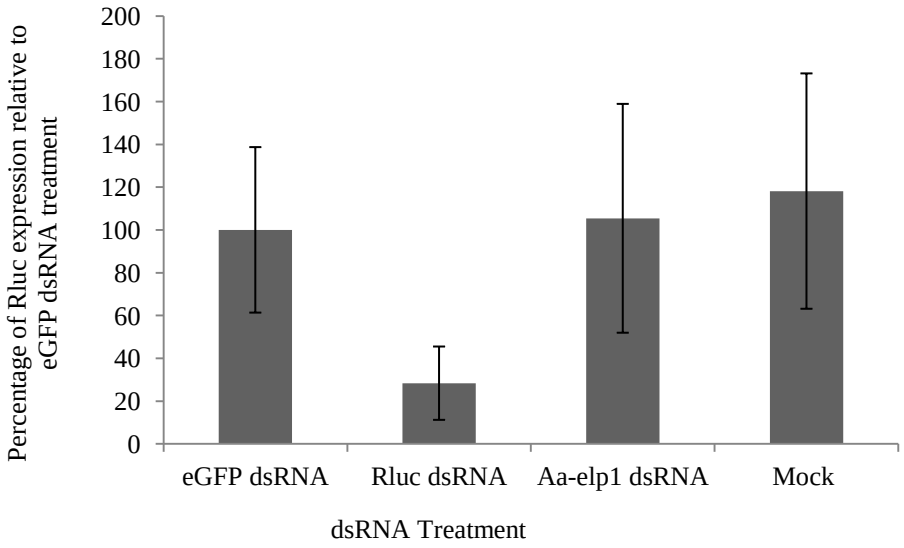


Figure 4.7: The effect of silencing Aa-elp1 in Aag2 cells on SFV replication. *Ae. aegypti*-derived Aag2 cells were treated with either dsRNA (Aa-elp1, Renilla luciferase (*Rluc*) or eGFP specific) or were mock transfected. Following an incubation of 24 h, the cells were infected with SFV4(3H)-*Rluc* at either MOI 10 (**A**) or MOI 0.005 (**B**). Cells were incubated for a further 24 h were after they were lysed and *Rluc* expression was measured in relative luciferase light units. Values recorded for control eGFP dsRNA were set to 100 % and the results for the other dsRNA treatments were viewed as a percentage of this. Error bars represent the standard deviation of two independent experiments performed in triplicate.

4.4 Discussion

It has been determined that in lower eukaryotes RdRPs are required for a robust RNAi response through the amplification and spread of silencing molecules within organisms such as worms and plants (Cogoni and Macino, 1999, Smardon et al., 2000; Dalmay et al., 2000; Mourrain et al., 2000; Wassenegger et al., 2006). The action of an RdRP in those organisms may in part account for the potency of the silencing response and presence of a large population of siRNAs when only a few molecules are needed to induce the response (Fire et al., 1998; Kennerdell and Cathew, 1998; Yang et al., 2000). Furthermore, the generation of secondary siRNAs by RdRP activity is responsible for the spread of the silencing signal from infected to non-infected cells, resulting in a systemic antiviral RNAi response which inhibits an incoming virus infection (Fire et al., 1998; Himber et al., 2003; Voinnet, 2005).

Studies have indicated that there is a lack of efficient spread of RNAi between tissues in insects and higher eukaryotes. It was believed that RNAi was cell autonomous in higher eukaryotes and viral dissemination could not be limited systemically (Kennerdell and Cathew, 1998; Roignant et al., 2003). However, more recently it has been demonstrated that a degree of systemic gene silencing does occur (Tomoyasu et al., 2008; Karlikow et al., 2014) through the translocation of dsRNA molecules *in vivo* in *Drosophila* (Saleh et al., 2009) and siRNA molecules *in vitro* in *Ae. albopictus*-derived cells (Attarzadeh-Yazdi et al., 2009). Systemic RNAi is also evident in some tick species and they have been shown to be capable of dsRNA uptake both *in vitro* and *in vivo* (Nijhof et al., 2007; Kocan et al., 2007). However, despite the existence of an RdRP homologue in their genome its involvement in RNAi and systemic spread has not yet been established (Kurscheid et al., 2009). This stresses the level of divergence within the Arthropoda lineages. Non-cell autonomous RNAi has also been demonstrated in mammalian cells in culture through both cell-to-cell contact and contact-independent means (Rechavi et al., 2009; Cohen and Xiong, 2011). Therefore, with the exception of ticks where an RdRP has been suggested, functional silencing molecules can be passed from infected cells to their neighbours in higher eukaryotes and can propagate the antiviral response within the local vicinity of the infection resulting in systemic RNAi without the known assistance from a definitive RdRP (Hoa et al., 2003).

Although amplification of the RNAi signal may be beneficial to these organisms, these findings are suggestive that RdRp action may be superfluous or play a reduced role, in the spread of RNAi molecules over short distances between cells of higher animals. Indeed, although RdRP amplification is obligatory for RNAi in *C. elegans,* it is not required for short distance movement of silencing molecules in plants although wider transport was affected (Himber et al., 2003). It has also been suggested that RdRp action may not be necessary for dissemination in organisms with efficient circulatory systems where fluids, such as the haemolymph and coelomic fluids, are in contact with the majority of body cells and are able to provide a prolonged, stable signal to various tissues within the body (Blandin et al., 2002; Tijsterman et al., 2004). Certainly an *in silico* analysis between both D-elp1 and Aa-elp1 sequences revealed that there was no significant similarly to other recognised RdRPs detected, something which is stated in Lipardi and colleagues original publication. The lack of identifiable domains or catalytic residues important for RdRP activity, such as the DXDGD motif, further detracts from the statement that D-elp1 functions as an RdRP.

Further to the role of D-elp1 in the primer-dependent or primer-independent dsRNA production model of RNAi suggested by Lipardi and colleagues, alternative proposals by Zamore and colleagues suggested that RNAi may succeed in *Drosophila* without the action of an RdRP (Zamore et al., 2000; Hammond et al., 2000; Nykänen et al., 2001; Schwarz et al., 2002; Roignant et al., 2003). Despite the absence of a determined RdRP orthologue, mosquitoes and *Drosophila* encode the key enzymes involved in RNAi and have demonstrated a strong silencing capacity (Kennderdell and Carthew, 1998; Hammond et al., 2000; Yang et al., 2000; Zamore et al., 2000). From the observations made in this chapter it can be inferred that although a homologue of D-elp1 exists within the *Ae. aegypti* genome, there is no evidence to suggest that it has an active role in the RNAi response. If Aa-elp1 possessed RdRP activity to enhance exogenous siRNA based RNAi in the Aag2 cell line then it would be expected that RNA silencing would be inhibited when Aa-elp1 was knocked-down. As a result expression levels of *Rluc* from SFV(3H)-*Rluc* virus infection would be expected to increase due to the reduction in the population of viRNAs presented to Ago-2. However, results demonstrated that there was no evident inhibition of the exogenous RNAi pathway following the knockdown of Aa-elp1 transcripts *in vitro*. These are inconsistent with Lipardi's findings that RNAi was impaired following D-elp1 knockdown which is upheld despite the retraction. It could be argued that the knockdown efficiency of ~20 % was not sufficient to remove an adequate level of the protein to

produce an effect and that the outstanding protein remaining could function unaffected. Alternatively, if as suggested, Aa-elp1 plays a minor role or has become completely dispensable as RNAi evolved in these organisms, the principle population of siRNAs following the initial infection may be sufficient without amplification to efficiently silence SFV replication. It should therefore be noted that the abundance of siRNA molecules has been shown to differ in certain systems and that siRNAs are more prevalent in *Drosophila* and plants than in *C. elegans* in spite of the activity of their confirmed RdRP (Parrish et al., 2000, Yang et al., 2000; Li et al., 2002). On the other hand, experiments with an alternative virus may generate a different result due to variations in the manner in which they circumvent the antiviral response. Although these results suggested that Aa-elp1 does not affect receptivity of Aag2 cells to SFV infection or a reporter system based on exogenous RNAi induction, a comparison between cell lines and cell-free extracts should also be examined to gain a more complete insight into the functions of Aa-elp1. In particular, it should be noted that cell-to-cell movement of silencing molecules has not been studied in the Aag2 cell line and therefore it has not been shown to exist in these cells despite spread being observed in the *Ae. albopictus*-derived U4.4 cell line (Attarzadeh-Yazdi et al., 2009).

Lipardi stated that when both Dcr-2 and D-elp1 were over-expressed they were able to interact and form a complex, an association which was maintained after this article was retracted. This is in agreement with other canonical RdRPs in lower eukaryotes that have been shown to associate with Dicer enzymes in the cytoplasm (Lee and Collins, 2007). There may therefore be an alternative role for Aa-elp1 in a Dcr-2 dependent mechanism which is yet to be established and would merit further studies. Moreover, the involvement of an insect RdRP in endo-siRNA production and transposon suppression where not investigated and more research is required to further understand these avenues of interest.

Further evidence for the lack of an active RdRP in insects and higher eukaryotes is that the RNAi response in *C. elegans* and plants is not only systemic but transitive. Transitivity results from the production of RdRP-directed dsRNA synthesis which is driven by primary siRNA molecules. The dsRNA molecules are processed into further siRNAs, termed secondary siRNAs which are produced from a different, non-overlapping region of the transcript. In this way the silencing of a gene which was not present in the original dsRNA sequence can be achieved. This is useful in antiviral defence as regions of the viral genome which do not produce/produce low numbers of viRNAs can be targeted. In *C. elegans* transitive RNAi requires an RdRP to amplify the silencing signal through the generation of siRNAs derived against regions further upstream towards the 5' terminus of the original

dsRNA target sequence. These act as primers for primer-dependent *de novo* dsRNA synthesis which is extended from the siRNA binding site on the sense RNA into the flanking sequences upstream in the transcript, although only over a limited distance (Sijen et al., 2001; Alder et al 2003). Further siRNAs, termed secondary siRNAs, that recognise regions upstream of the original target are then produced and accumulate to greater numbers than primary siRNAs; although, their population numbers remain lower than those against the original trigger sequence and decrease in quantity with increasing distance from that sequence (Sijen et al., 2001**;** Alder et al 2003). These secondary siRNAs will also target other RNA transcripts to which they are complementary. Primary and secondary siRNA molecules can be distinguished from each other in *C. elegans* by the chemical structure of their 5' terminus. Primary siRNAs are generated by Dicer and so have a monophosphate on their 5' terminus. RdRPs add ribonucleotides to the 3' terminus of an RNA molecule and their 5' triphosphate has been preserved which gives the secondary siRNAs a recognisable characteristic polarity (5'→3' on the antisense strand) (Ruby et al., 2006; Pak and Fire, 2007; Sijen et al., 2007). In addition, primary siRNAs are predominantly 22 nt while secondary siRNAs are largely 26 nt.

In plants, transitive RNAi is bidirectional and can spread larger distances from the dsRNA inducer sequence towards either the 5' or 3' ends, suggesting a primer-independent mechanism or the use of full-length antisense RNA as a template (Vaistij et al., 2002; Petersen and Albrechtsen, 2005). Secondary siRNAs are not able to be differentiated from primary siRNAs based on structure as both populations are Dicer dependent and so have a 5' monophosphate in contrast to *C. elegans* secondary siRNAs. However, siRNAs are different lengths depending on which of the four dicer-like proteins (DCL) generates them. siRNAs are either 21, 22 or 24 nt long when produced from DCL4, DCL2 or DCL3 respectively, while DCL1 produces microRNAs in a range of sizes (Voinnet, 2008). DCL1 and DCL3 are exclusively located in the cell nuclease, unlike DCL2 and DCL4 which are found in the cytoplasm. The major population of secondary siRNAs are believed to be as a result of DCL4 and DCL2 processing downstream of RdRP action and these are involved in the systemic spread of the silencing signal (Himber et al., 2003; Mlotshwa et al., 2008; Wang et al., 2011). However, definitive clarification of each mechanism is not possible due to the large degree of cross-talk between the pathways and dual protein functions. The knockdown of one protein is easily compensated by a second and therefore the two pathways are not clearly distinguished from each other.

Transitive RNAi is considered to be a distinguishing feature of RdRP function and is thought to be lacking in organisms where no canonical RdRP is present. Certainly transitive RNAi has not been documented in mammals or *Drosophila* either *in vitro* and *in vivo* (Pal-Bhadra et al., 1999 and 2002; Zamore et al., 2000, Nykänen et al., 2001; Celotto and Graveley, 2002; Hutvagner and Zamore, 2002; Schwarz et al., 2002; Roignant et al., 2003; Adelman et al., 2008). An *in vitro* assay showed that *Drosophila* were able to digest dsRNAs into functional siRNAs; however, these were generated against the original dsRNA molecule and not for regions upstream as would be expected for transitive RNAi (Zamore et al., 2000). Lipardi and colleagues suggested that *de novo* dsRNA synthesis was observed in *Drosophila* extracts (Lipardi et al., 2001); however, the authors rescinded this finding (Lipardi and Paterson, 2011). A possible explanation for these controversial findings could lie with the viruses that infect the cells. RNA viruses are known to encode their own RdRP which could account for the detection of signal amplification in some experiments and not in others. In addition, endogenous viruses which 'contaminate' some cultures are only now being unravelled and may provide the answers to erroneous discrepancies (**Chapter 6**). However, transitive RNAi has not been observed in plants and *C. elegans in vitro* either and therefore current lysate preparation methods may not be adequate to represent the mechanism in the whole organism system. Although with this in mind, to date no siRNAs have been identified in *Drosophila* which present with a biochemical signature consistent with the suggestion that they are secondary siRNAs. Much less has been determined for mosquitoes; although, infection of mosquitoes with SINV was shown to produce a new class of endo-siRNAs (Adelman et al., 2012). Therefore much more work is required to definitively state transitive RNAi does not exist in these organisms.

As it is implied that at a cellular level the RNAi response alone, without additional input from an RdRP, provides adequate protection against infection pathology in these organisms compared to those with a canonical RdRP it could be suggested that evolution has exonerated the requirement of an RdRP. *Drosophila* are able to defend against an infection from a virus lacking their virus-encoded RNAi suppressor (VSR) protein while plants remain susceptible to it (Voinnet et al., 2000; Li et al., 2002; Silhavy et al., 2002; Ryabov et al., 2004). In these organisms the enzymatic properties of the RISC may negate the need for amplification to achieve sufficient silencing. However, unlike many viruses which infect plants and *Drosophila*, arboviruses are not understood to express an efficient VSR protein which interferes with RNAi components to diminish the silencing response

(Attarzadeh-Yazdi et al., 2009, Kemp and Imler, 2009; Murray et al., 2013). In this case RNA silencing may not require amplification to contain the infection. A recombinant strain of SFV expressing tombusvirus siRNA-binding protein, p19, which selectively binds to the siRNA duplexes and prevents them being loaded into RISC for targeted silencing was also investigated. This has been previously shown to enhance virus spread in mosquito cell lines (Scholthof, 2006; Attarzadeh-Yazdi et al., 2009). A recombinant strain of SFV encoding p19 (SFV(3H)-*Rluc*-p19) was used in parallel experiments alongside SFV(3H)-*Rluc* to establish the extent a reduction in siRNA availability to Aa-elp1 affected RNA silencing. You would expect that in the absence of an RdRP amplifying siRNAs, SFV(3H)-*Rluc*-p19 would do much better as there would be a great reduction in the number of siRNA molecules available to RISC for silencing. This was indeed the case; however, the outcome was extensive cell death under all conditions infected with the p19 SFV mutant (results not included). Therefore, it can be suggested that the addition of a VSR in arboviruses is selected against due to its deleterious effects. It would have been beneficial to test the effect of the B2 dsRNA binding protein to determine the effects that it has on the RNAi in mosquito cells. However, the creation of a B2 mutant was not permitted due to licensing and could not be completed.

Drosophila melanogaster was one of the first organisms to have its genome sequenced and is arguably the most genetically well known eukaryote. Despite its wide use in research, the work of Lipardi and colleagues is unique in its description of the presence of an RdRP in the *Drosophila* genome. As a result, it generated some scepticism over its disputed findings (Birchler, 2009; Förstemann, 2010). Despite the controversy surrounding the existence of an RdRP in insects, it is possible that alternative mechanisms involved in sustaining and amplifying the RNAi signal occur but are as yet unresolved. This study goes some way to add to the growing evidence that information generated for *Drosophila*, the model insect organism, is not easily translated to mosquitoes. The lineages to which *Drosophila* and mosquitoes belong are evolutionarily divergent and there is strong degree of variability of expression within their range of immune genes (Adams et al., 2000; Waterhouse et al., 2007). In particular, work carried out by our group have shown that important genes identified by screens in *Drosophila* do not translate, at least efficiently, to functional RNAi proteins in mosquitoes (Zhou et al., 2008; McFarlane et al., In preparation). The development of tools to accurately study immunity within the mosquito organism are necessary for investigations into antiviral defence in the natural virus/vector model and will reduce our reliance on the *Drosophila* system while compensating for the

number of immune mutants available for the *Drosophila* model (**Chapter 5**). The results described suggest that despite overlaps in some functions and proteins, such as Dcr-2 and Ago-2, other components of the RNAi pathway may not play identical roles.

4.5 Future Work

The challenges present highlight the undefined nature of many aspects of RNAi in different organisms. Due to the conserved nature of elp-1 in all eukaryotes, further work is necessary to fully determine its function within this wide range of organisms. The work described in this chapter focused on its activity in the exogenous siRNA pathway in *Ae. aegypti*-derived Aag2 cells and investigated its role in the RNAi antiviral immune response against SFV infection. The data generated indicated that there was no obvious disadvantage following SFV infection; however, it would be of interest to confirm this result with another virus to compare the responses. Similarly, different systems should also be investigated such as *in vivo* studies, cell-free extracts or an *in vitro* system derived from an alternative mosquito species as variations in the system may prove more suitable to establishing the potential role of elp1 in the mosquito RNAi response.

Furthermore, Lipardi stated that there was an association between Dcr-2 and D-elp1 which they maintained after this article was retracted. Further advances with the detection of Dcr-2, in addition to other important RNAi proteins (**Chapter 5**) would be useful to employ to define the other proteins and co-factors which are involved in RNAi complexes. There may be an alternative role for elp1 in a Dcr-2 dependent mechanism which could yet be established.

Other aspects of D-elp1 which were not refuted by Lipardi and colleagues were not investigated and merit further studies. The potential role of elp-1 in endo-siRNA production, transposon suppression or the piRNA pathway, in addition to any RdRP function, may reveal some activity in other aspects of gene control.

4.6 Principle Findings

1. A sequence similar to that known in *Drosophila* (D-elp1), a potential RdRP, was identified in the *Ae. aegypti*-derived Aag2 cell line (Aa-elp1) and could be silenced by treating the cells with specific dsRNA molecules.

2. Despite the suggestion that Aa-elp1 could have a role in accentuating the exogenous siRNA signal, evidence of this was not observed in the experiments performed. No difference in luciferase activity was detected from virus replication or plasmid expression when Aa-elp1 was silenced.

Chapter 5: The Development of Novel Tools to Enhance Understanding of the RNAi Response in Mosquitoes

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5.1 Introduction

The fruit fly, *Drosophila melanogaster,* has recently celebrated 100 years as a model organism. It is a well established model for the study of innate immune responses, small RNA biology and antiviral responses in insects and as such there are a considerable number of sophisticated genetic tools available which have allowed a detailed understanding of these processes to be attained (Adams et al., 2000; Huszar and Imler, 2008; Kemp and Imler, 2009; Ding, 2010). As a result, the basis of RNAi research in insects has been founded on the findings generated in *Drosophila* and their derived cell lines (Galiana-Arnoux et al., 2006; van Rij et al., 2006; Wang et al., 2006; Zambon et al., 2006). In the field of arbovirology, the need to further our understanding of mosquito immunity in order to discern the crucial role RNAi plays in the control of arboviral infections has lead to a necessary shift from *Drosophila* towards the natural vectors of such pathogens. The extensive advantages of genetic mutants in *Drosophila* are sadly lacking in mosquitoes and a growing body of evidence has suggested that a certain degree of information obtained in *Drosophila* is not necessarily able to be translated to the mosquito system. As *Drosophila* and mosquitoes belong to evolutionarily distinct phylogenetic lineages and show a high degree of variability in the level of immune genes expression, it is not entirely surprising that this is the case (Adams et al., 2000; Myers et al., 2000; Waterhouse et al., 2007). It is therefore essential that similar tools are developed and implemented to accurately appreciate immunity and the antiviral defence in the natural virus/vector model. These advancements will reduce our current reliance on the *Drosophila* model system and facilitate the generation of veracious results in the mosquito model. Recent findings have emphasised the importance of performing RNAi analysis in an applicable host species by demonstrating that the RNAi response and viral counter measures can be species specific and that the outcome of an infection by a particular pathogen can differ, even between closely related species (van Mierlo et al., 2014).

A turning point in enhancing our awareness of the fundamentals of innate immunity in mosquitoes was the generation of complete genetic sequences for some of the most significant vector species such as *An. gambiae*, *Ae. aegypti* and *Cx. quinquefasciatus* (Christophides et al., 2002; Holt et al., 2002; Nene et al., 2007; Waterhouse et al., 2007; Arensburger et al., 2010; Bartholomay et al., 2010). Further to this, the development of high-throughput sequencing technology has facilitated the characterisation of small RNAs from samples infected with several important viruses; including those from the

Bunyaviridae, *Togaviridae* and *Flaviviridae* families, which have rapidly advanced the RNAi field (Aliyari et al., 2008; Myles et al., 2008 and 2009; Brackney et al., 2009; van Rij and Berezikov, 2009; Parameswaran et al., 2010; Wu et al., 2010; Blair et al., 2011; Hess et al., 2011; Siu et al., 2011; Donald et al., 2012; Morazzani et al., 2012; Vodovar et al., 2012; Schnettler et al., 2013a; Vijayendran et al., 2013; Bronkhorst and van Rij, 2014; Karlikow et al., 2014). The detection of viral specific small RNAs have confirmed the importance of Dicer-2 (Dcr-2) mediated processing in the antiviral response and has allowed its substrate virus sequences to be elucidated through mapping them back to the genome (Scott et al., 2010; Wu et al., 2010).

Undoubtedly one of the greatest questions still to be answered in insect systems is when and where the RNAi pathway is initiated. Dcr-2 is vital for the launch of the antiviral RNAi mechanism through its action as a pathogen recognition receptor (PRR) and the recognition of dsRNA molecules following infection before the RNAi response can be employed. Most studies have focused on (+) RNA virus infections during which it is believed that this dsRNA substrate is due to replication intermediates formed during the amplification of the viral genome (**Chapter 3**) (reviewed Blair, 2011 and Donald et al., 2012). Little is known about the RNAi target following infections by (-) RNA, dsRNA or DNA viruses. Low quantities of dsRNA have been observed in vesicular stomatitis virus (VSV) infections of *Drosophila* cells (Weber et al., 2006; Mueller et al., 2010). However, functional 21 nt small RNAs are detected matching both the full-length of the genome and antigenome in a similar fashion to the pattern generated by (+) RNA virus infection suggesting replication intermediates might again be the target for Dcr-2 cleavage (Brackney et al., 2010; Mueller et al., 2010; Leger et al., 2013; Sabin et al., 2013; Schnettler et al., 2013a; Marques et al., 2013). More research is required in this area to understand the source of Dcr-2 substrates during a (-) RNA virus infection. The recognised scope of the RNAi defence has recently been broadened as it has also been shown to target DNA viruses. *Drosophila* RNAi has been shown to target the dsDNA virus Invertebrate iridescent virus 6 (IIV-6) and the authors suggest that dsRNA is produced by overlapping transcripts which act as Dcr-2 substrates (Bronkhorst et al., 2012; Kemp et al., 2013). In wild-caught *Cx. pipiens molestus* a novel ssDNA densovirus was detected through deep sequencing of viRNAs. Although their biogenesis and function is unknown, it suggests mosquito Dcr-2 is also able to target ssDNA viruses (Ma et al., 2011). Similarly, RNAi targeting, specifically Dcr-2 action, acts antivirally against the dsDNA virus white spot syndrome virus (WSSV) in the economically important shrimp species *Marsupenaeus*

japonicus (Huang and Zhang, 2013). These reports emphasise how sophisticated and powerful the RNAi response is and how little we currently understand of this mechanism of defence.

Despite the theory that functional Dcr-2 processing is sufficient to impede a viral infection alone by reducing the number of RNA strands capable of forming new viral progeny, this has been shown to not be entirely true. The essential part that Argonaute-2 (Ago-2) plays in the antiviral response in *Drosophila* was demonstrated through the increased sensitivity of Ago-2 mutant flies and cell lines (Zambon et al., 2006; Chotkowski et al., 2008; Mueller et al., 2010; Han et al., 2001). Dcr-2 failed to inhibit infection by flock house virus (FHV) in Ago-2 mutant *Drosophila* embryos (Wang et al., 2006), while Ago-2 mutants had increased sensitivity to Drosophila C virus (DCV) and Drosophila X virus (DXV) (van Rij et al., 2006; Zambon et al., 2006). These findings have been reiterated successfully in Ago-2 knockdown mosquitoes and their derived cell lines (both in the literature and presented as part of this thesis) where similar conclusions have been generated highlighting the importance of Ago-2 in the antiviral control responses of these organisms (Hoa et al., 2003; Keene et al., 2004; Franz et al., 2006; Campbell et al., 2008a; Sanchez-Vargas et al., 2009; Schnettler et al., 2013a; McDonald et al., 2014).

Accelerated evolution of antiviral *dcr2, r2d2* and *ago2* RNAi genes in comparison to non-immune genes (*dcr1, r3d1* and *ago1*) in *Drosophila* signifies a sustained battle between the virus and its vector (Obbard et al., 2006 and 2009); although, this may not be the case in mosquitoes as a study in *Ae. aegypti* indicates that unlike *Drosophila*, both miRNA and exo-siRNA pathway genes undergo accelerated evolution highlighting the differences between the two insect species (Bernhardt et al., 2012). True insect viruses are commonly known to encode viral suppressors of RNAi (VSR) as a counter defence against RNAi antiviral activity (reviewed Li and Ding, 2006; Ding and Voinnet, 2007; Kemp and Imler, 2009; van Mierlo et al., 2011) (**Chapter 1.4.6**). Many of them target dsRNA as the pathway initiator or siRNA molecules which determine the sequence specific target. Examples of these include the DCV (*Dicistroviridae*) protein 1A which prevents dsRNA cleavage by Dcr-2 (van Rij et al., 2006) and may also affect siRNA loading into RISC (Bonning and Miller, 2010; Nayak et al., 2010). The 1A protein encoded by Cricket paralysis virus (CrPV) on the other hand acts by directly interacting with Ago-2 and inhibiting its function (Nayak et al., 2010). Members of the *Alphaodavirus* genus, such as FHV or Nodamura virus (NoV) express the B2 protein which not only binds dsRNA but siRNAs as well and therefore inhibits Dcr-2 cleavage in addition to preventing siRNA

incorporation into RISC (Li et al., 2002; Chao et al., 2005; Lingel et al., 2005; Aliyari et al., 2008; Qi et al., 2012). Other RNAi suppressor proteins such as p19 from tombusviruses (*Tombusviridae*), p21 from beet yellow virus (BYV) (*Closteroviridae*) and HC-Pro from potyviruses (*Potyviridae*) all selectively bind duplex siRNA molecules (Voinnet et al., 1999; Lakatos et al., 2006). Moreover, evidence has shown the independent evolution of Ago-2 suppressor proteins within two unrelated pathogenic insect viruses emphasising the importance of Ago-2 in the RNAi pathway (Nayak et al., 2010; van Mierlo et al., 2012). This myriad of countermeasures with different mechanistic actions indicates the whole pathway is involved in viral control. In some cases, however, RNAi suppressor activity may be limited to the natural virus vector. This was identified through the action of viral protein 1 (VP1) that is encoded by the *Drosophila* Nora virus and suppresses Ago-2 cleavage (van Mierlo et al., 2014). A degree of species specificity was observed between different Nora-like viruses suggesting specific co-evolution between the virus and its host.

Recent publications have characterised the activity of RNAi suppressors in a broader range of viruses than previously thought. The first description of an RNAi inhibitor in a mosquito-specific virus was shown in Culex Y virus (CYV) which encodes its own VSR capable of binding both long dsRNA and siRNA duplex molecules (van Cleef et al., 2014). This is also the first example of a link between viral persistence and RNAi antagonism which requires further investigations in the future. Among arboviruses, few are known to encode RNAi inhibitors. This lead to the theory that their absence allowed arboviruses to progress to a persistent, low level infection necessary for their continued transmission. However, two members of mosquito-borne flaviviruses, West Nile virus (WNV) and dengue (DENV) have been shown to suppress RNAi through the production of non-coding subgenomic flavivirus RNAs (sfRNAs) (Pijlman et al., 2008; Schnettler et al., 2012). These are generously produced during viral replication and act as competitive substrates for Dcr-2 and so inhibit processing of coding RNAs. The action of non-structural protein 4B (NS4B) of DENV is also thought to inhibit Dcr-2, although the mechanism is unclear (Kakumani et al., 2013). Moreover, a further study by Schnettler and colleagues has demonstrated that the RNAi silencing suppressor activity of sfRNA is also present for the tick-borne flaviviruses Langat virus (LGTV) and tick-borne encephalitis virus (TBEV) (Schnettler et al., 2014).

The strategy that is traditionally believed to be employed by arboviruses is passive evasion rather than an active attack on the RNAi mechanism. Host cell membranes have been shown to have a major role in the replication of (+) RNA viruses (Tao and Ye, 2010). The

majority of all eukaryotic single stranded, (+) sense RNA viruses, including members of the *Flaviviridae* and *Togaviridae* families, have been shown to replicate within membrane-bound vesicles within mammalian host cells (Grimly, 1968; Strauss and Strauss, 1994; Westaway et al., 1997; Mackenzie et al., 1998; Diamond, 2003; Uchil and Stchidanandam, 2003; Geiss et al., 2005; Spuul et al., 2007, 2010 and 2011; Campbell et al., 2008a; Sanchez-Vargas et al., 2009). These cytoplasmic replication complexes are surrounded by a double membrane derived from the host cell itself and provide the optimal environment for virus replication (Friedman et al., 1972; Grimley et al., 1972; Peranen et al., 1995; Welsch et al., 2009; Gillespie et al., 2010; Chatel-Chaix and Bartenschlager, 2014). The current belief is that the replicating viral genome is protected, hidden behind a 'cell-self' barrier, making it less accessible, to the RNAi machinery. This aids evasion by restricting or delaying the immune response, at least during a vertebrate infection (Keene et al., 2004; Geiss et al., 2005, Frolova et al., 2010). Studies with TBEV and WNV have indicated that this method also functions to delay the stimulation of interferon induction (Hoenen et al., 2007; Overby et al., 2010). There is also evidence of replication complexes observed in a tick cell line for TBEV (Senigl et al., 2006) and LGTV (Offerdahl et al., 2012; personal communication with Dr. Lesley Bell-Sakyi and Dr. Claudia Rückert, Pirbright Institute). However, although there is currently little evidence for these structures in the case of alphavirus infection of mosquitoes and their derived cell lines, a recent paper has demonstrated DENV- induced membrane remodelling in C6/36 cells (Junjhon et al., 2014).

Previous work has shown that punctate co-localised staining was observed between SFV replication complexes and anti-dsRNA antibody detection suggesting a primary association between the two during the early acute stages of infection in *Ae. albopictus*-derived U4.4 cells (Siu et al., 2011). This is in agreement with results for other alphaviruses, in addition to SFV, in both vertebrate and invertebrate cells (Salonen et al., 2005; Spuul et al., 2007; Fragkoudis et al., 2008; Frolova et al., 2010). Viral dsRNA is therefore concentrated in specific loci throughout the cytoplasm; although whether this is within cytoplasmic vacuoles in mosquito cells has yet to be confirmed. Currently little is known about the localisation of the RNAi proteins during infection in mosquitoes. It is hypothesized that Dcr-2 will locate at these sites in order to detect and interact with its substrate viral dsRNA produced during viral replication. Studies have shown that Dcr-2 and Ago-2 can be co-immunoprecipitated from *Drosophila*-derived S2 cells but that they are also biologically separate (Hammond et al., 2001). Therefore, the biochemical properties of their interactions need to be fully investigated in order to acquire an insight into the dynamic

association between these proteins as well as any additional protein: protein/ protein: molecule interactions. Unfortunately, unlike the *Drosophila* system, very few tools exist to allow thorough investigations into the interactions between arboviruses and their natural vectors to be carried out. The provision of tools and assays designed around the mosquito vector are necessary to answer some fundamental questions of arbovirology.

RNAi studies have so far benefited from the wide range of *in vivo* and *in vitro* tools in *Drosophila*. *Drosophila* research has provided a catalogue of readily available genetic mutants and conditional drivers for *in vivo* host-virus interaction analysis. These are currently lacking for mosquitoes although new methods such as 'Clustered Regularly Interspaced Short Palindromic Repeats' (CRISPR) and 'Transcription Activator-like Effector Nucleases' (TALENs) may provide a valuable resource for the creation of transgenic knockdown/knockout mosquitoes in the future (Windbichler et al., 2011; Aryan et al., 2013 and 2014; DeGennaro et al., 2013; Joung and Sander, 2013; Smidler et al., 2013; Bassett and Liu, 2014; Esvelt et al., 2014; Franz et al., 2014; Galizi et al., 2014; Oye et al., 2014; Reeves et al., 2014). *Drosophila* studies further benefit from genome wide microarray analysis for global transcriptional profiling as well as proteomic analysis to monitor protein-protein/ virus-host interactions, protein abundance and post-translational modifications. However, many of these technologies are yet to be fully implemented in mosquito research. In addition to advances in modern methods, progress in traditional molecular cell culture techniques (Fallon and Sun, 2001), as well as the adaptation of *Drosophila* cell-free systems for studying mosquito RNAi *in vitro* (Tuschl et al., 1999; Haley et al., 2003; Yang and Li, 2011; Vodovar et al.,2012) has initiated work into defining the complexities of mosquito immunity within controlled environments.

Cell culture systems have been invaluable in the advancement of arboviral research and the study of host-virus interactions. Cell culture-based RNAi screening is widely used and many *Drosophila* cell lines are available (embryonic, larval, neuronal and haemocyte). They are easy to maintain and many are considered to be an accurate mimic of the whole organism environment. One cell culture system which has proved to be invaluable for mosquito RNAi research is the *Ae. albopictus*-derived C6/36 cell line generated from homogenised mosquito larvae (Igarashi, 1978). It was selected based on its ability to propagate chikungunya and dengue viruses to high titres. Since then it has been shown to be highly permissive to many virus infections and this has been linked to a deficiency in their RNAi pathway (Brackney et al., 2010; Scott et al., 2010; Morazzani et al., 2012). Studies into the biochemical nature of the Dcr-2 protein present in the C6/36 cell line

revealed that the *dcr2* gene which encodes Dcr-2 is 79 % identical to that found in *Ae. aegypti* and is expressed at a reduced level in C6/36 cells compared to the *Ae. aegypti*-derived Aag2 cell line; although, not significantly enough to explain the reduced immune response (Scott et al., 2010). Furthermore, *dcr2* encoded by the C6/36 cell line is shown to contain a homozygous frameshift mutation due to the deletion of a single nucleotide at position 1508 (Scott et al., 2010). This results in a premature termination sequence and produces a truncated protein devoid of a fragment of the PAZ domain and both RNase III domains (**Figure 5.1**). However, even with this nonsense mutation, the full-length mRNA is detected in these cells suggesting that it has not been targeted for degradation as would be expected in the case of early translation termination (Harigaya and Parker, 2010). For this reason, the C6/36 cell line is not considered to be an accurate model for vector/virus interactions unlike a second *Ae. albopictus*-derived cell line, U4.4, which are known to be a suitable, true to life *in vitro* model which exhibit functional Dcr-2 activity and are RNAi competent (Davey and Dalgarno, 1974; Fragkoudis et al., 2008; Attarzadeh-Yazdi et al., 2009; Vodovar et al., 2012; Léger et al., 2013). As such, the C6/36 and U4.4 cell lines can be used in parallel and provide an opportunity to investigate the role and importance of Dcr-2 in lieu of a complete Dcr-2 mosquito knockout; although, difficulties in comparing the results still exist.

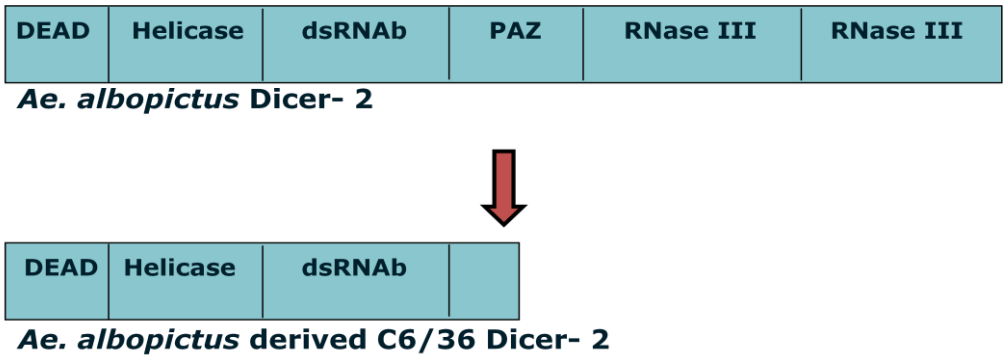


Figure 5.1: Schematic illustrations of the comparison between the Dicer-2 proteins from *Ae. albopictus* mosquitoes and the C6/36 derived cell line. The arrow indicates the point of mutation. DEAD, DExH/D protein family domain; Helicase, helicase domain; dsRNAb, dsRNA binding domain; PAZ, Piwi/Argonaute/Zwille domain; and two RNaseIII domains (Morazzani et al., 2012).

Although antibodies have been developed against the key RNAi proteins in *Drosophila* (Liu et al., 2003; Miyoshi et al., 2005; Hammond et al., 2001) these have been lacking in

mosquito systems. Therefore, to add a further dimension to what is already understood from *Drosophila*, a major aim of this project was to generate a number of tools with which to answer many of the questions still to be solved in mosquitoes. In particular, a substantial number of unknowns regarding the kinetics and dynamics of Dcr-2 and Ago-2 exist. Therefore, the creation of antibodies against these proteins, as well as the production of recombinant- tagged enzymes, should facilitate a considerable contribution towards enhancing our awareness of the key principles involved in the antiviral innate immune response in mosquitoes. The currently employed plasmid expression systems are still weak and often unstable, resulting in the required use of viral vectors for protein expression; although, the development of sufficient plasmid vectors would be preferable. Advances in the capacities of these molecular tools will aid the characterisation of the mechanistic action leading to the degradation of viral RNAs. Understanding how these important processes are modulated should go some way to clarifying these and many other mysteries still surrounding the RNAi response in vectors of important human pathogens.

5.2 Objectives

1. Generate and test antibodies designed to recognise *Ae. aegypti* Dicer-1, Dicer-2, Argonaute-1 and Argonaute-2 proteins.
2. To generate recombinant *Ae. aegypti* Dicer-2 and Argonaute-2 proteins fused to molecular tags.
    2.1. Determine if these tagged constructs are expressed by *Ae. aegypti* and *Ae. albopictus*-derived cell lines and can be detected using an appropriate assay.
    2.2. Perform an *in vitro* dicer cleavage assay to ascertain if the Dicer-2 construct is functional.
    2.3. Establish the kinetics of Dicer-2 during detection of viral dsRNA and determine when and how this occurs.
3. Compare the efficiency of the *Ae. aegypti* polyubiquitin promoter with prevailing insect expression promoters.

5.3 Results

5.3.1 Development of Antibodies Against Major RNAi Proteins

It is commonly accepted that alien dsRNA is the trigger for the initiation of RNAi and that these molecules are a substrate for Dcr-2. However, there is a void in the information available regarding how, when and where Dcr-2 interacts with the dsRNA molecules, where the dsRNA comes from and how is it recognised. Furthermore, the kinetics of the subsequent protein-protein/protein-molecule interactions which complete the pathway are also currently unknown. To go some way towards answering these questions antibodies targeting the endogenous RNAi enzymes Ago-2 and Dcr-2 and the endogenous miRNA pathway proteins Ago-1 and Dcr-1 were designed and developed. Prior to this, no antibodies against these proteins in *Ae. aegypti* were available and their production would be a major advantage to the work carried out by the lab and the wider RNAi field by generating vital tools which would allow the properties of these poorly understood key enzymes to be elucidated. This work was gratefully funded by a Medical Research Council Centenary Award grant.

Production of these antibodies was performed by Abmart (http://www.ab-mart.com) using their monoclonal SEAL™ (Protein Surface Epitopes Targeted by Monoclonal Antibody Library) technology. The library was specially designed against Ago-1 (XP_001662664), Ago-2 (ACR56327), Dcr-1 (AAW48724) and Dcr-2 (AAW48725) sequences from the *Ae. aegypti* genome. Six independent linear peptide antigens were selected based on algorithmic predictions from Abmart's antigen design software. These were recommended as they were in agreement with a number of configuration and property criteria (**Table 5.1**), in addition to the caveat that they could not recognise any of the other target proteins.

Table 5.1: Criteria used in Abmart validation of epitope selection.

Parameter	Specific Aspects
Secondary Structure	Loops Helixes Sheets
Specific Regions	N-Terminal C-Terminal Signal Peptide Trans-membrane domains Disordered Regions Solvent Accessibility
BLAST	Query species compared to mouse
Amino Acid Properties	Antigenic Enhancement Amino Acids Flexibility
Evolution	Positive Selection Discrimination
Requests	Protein Specificity Region Specificity

The epitopes selected were all 10 amino acids long (see **Page xix**). Abmart cloned each into an expression vector that was transformed into bacteria and purified by Nickle-affinity chromatography by means of a polyhistidine (6 x His) affinity tag (**Figure 5.2**). Following purification the antigens were used to immunise three 8 - 12 week old female Balb/c mice. The mouse which presented the greatest immune response was sacrificed and its spleen cells isolated and fused with murine myeloma SP2/O cells to produce hybridomas. The cells were diluted and clones grown from single parent cells in hypoxanthine-aminopterin-thymidine (HAT) medium in microtitre plates. Secreted antibodies were collected from each clone and analysed by enzyme-linked immunosorbent assay (ELISA) to determine their ability to recognise the target antigen. Clones were selected based on efficient antibody production and stability. Finally, the hybridomas were placed back into a mouse host by injection into the peritoneal cavity. The resulting tumours that developed 10 - 14 days post injection contained antibody rich ascites fluid which was collected. The final antibodies were shipped to the UK as lyophilized ascite fluids along with the lyopholized decamer peptides. These were all restored in reconstitution buffer upon arrival in the lab (**Table 5.2**).

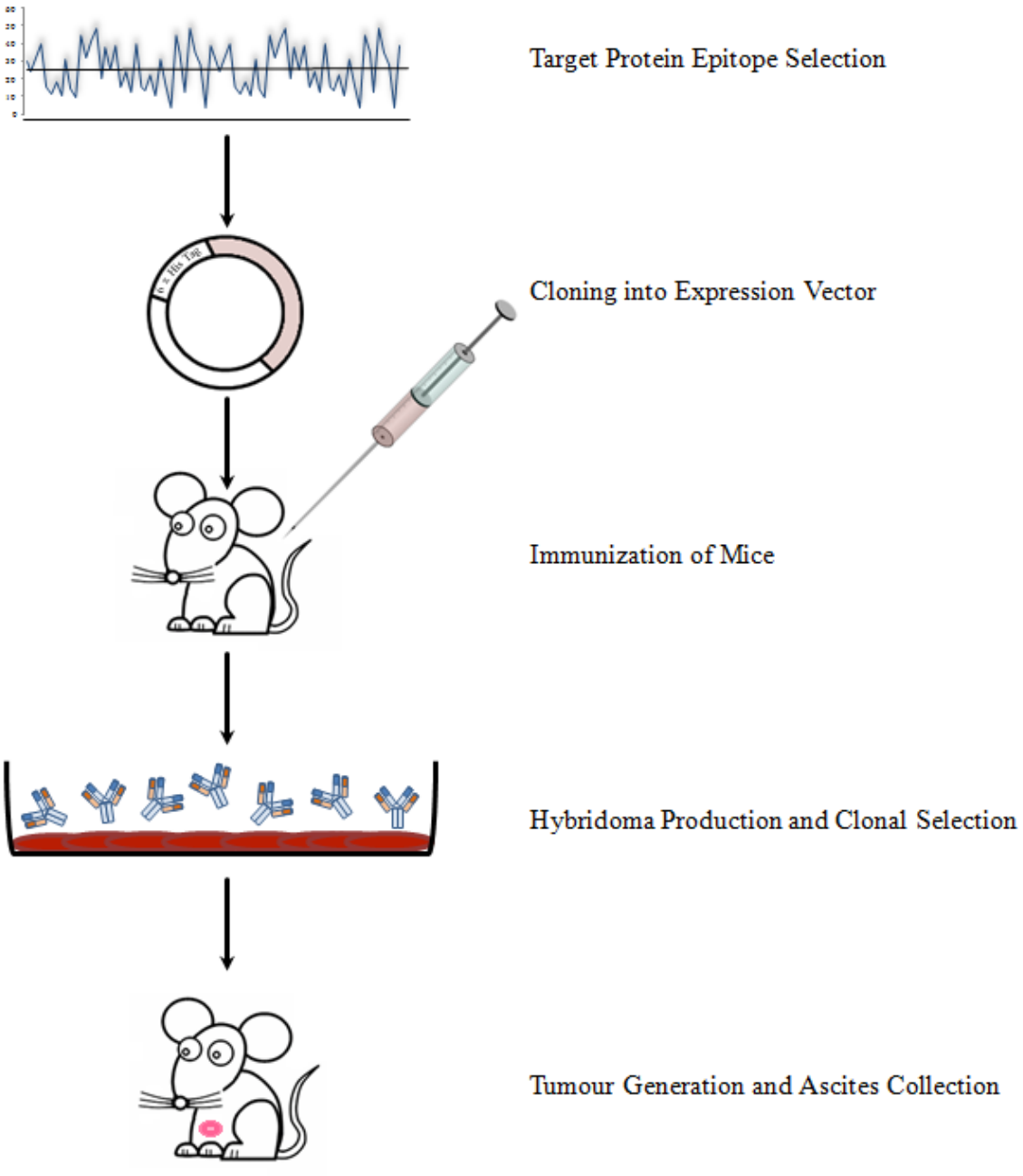


Figure 5.2: Schematic representation of the procedures involved in the production of the anti-RNAi antibodies carried out by Abmart. Target epitopes were selected based on computational analysis and cloned into an expression vector containing a 6 x His tag. This allows peptide purification by Ni-affinity chromatography. Purified peptides were injected into mice hosts which were then sacrificed and their spleen cells harvested. Clonal hybridomas were generated and those which secreted the most antibodies were placed back into a mouse host. The fluid surrounding the resulting tumour was collected and delivered for testing.

Table 5.2: Selected epitopes for the production of antibodies against RNAi proteins. For each RNAi protein; Argonaute-1, Argonaute-2, Dicer-1 and Dicer-2, six target epitopes were selected for the immunisation of mice. Their sequence and position within the protein is noted. Antibodies capable of generating an immune response are indicated and these were collected for testing.

Target Protein	Accession Number	Index	Target Epitope	Start amino acid	End amino acid	Successful production
Argonaute-1	XP_001662554	1	EHDSGEGSHQ	909	918	✓
		2	KKEQSGKSGN	808	817	✓
		3	PVSTTAGAQN	66	75	✓
		4	PPSPTQSQTS	15	24	✓
		5	SNPRTDAKRT	38	47	✓
		6	GVVPATPPAP	82	91	
Argonaute-2	ACR56327	1	QIRTEIQDGH	978	987	
		2	IKIDKQKIHS	156	165	
		3	KIHSSALLPV	162	171	✓
		4	GQSWRPQSHD	117	126	
		5	KDRPLNMNNL	961	970	
		6	SHDPSPASGS	124	133	
Dicer-1	AAW48724	1	PIKDPKEAEI	538	547	✓
		2	KKMGLINKRR	2183	2192	
		3	EMPPSETNKI	1258	1267	
		4	KESDKSAAIE	1277	1286	✓
		5	KPEKLADGRR	2138	2147	✓
		6	QIVIENSEPR	871	880	
Dicer-2	AAW48725	1	DDGKSTRSKH	939	948	✓
		2	GKTINRPDPL	529	538	✓
		3	KNDWQPPLAT	1255	1264	
		4	RAAGSPKREP	1132	1141	✓
		5	GQNKDDAKRA	1632	1641	
		6	DVEYKERKGK	1014	1023	✓

5.3.2 Dot Blot Analysis of Anti-Argonaute-1 Antibodies

As the antigen peptides had also been received along with the antibodies designed to recognise them, a dot blot assay was performed. This is an efficient method for screening a large number of antibodies quickly. Only Ago-1 antibodies were screened using this method . The dot blot involved repeatedly spotting a small volume of sample (3 x 10 µl) onto a piece of membrane lining the bottom of a 96-well plate. The samples used for the dot blot screening were Aag2 cell lysates treated with either dsRNA against 1) Ago-1 or 2) eGFP (negative control). The results obtained from the cellular samples were compared to the supplied peptides used to generate each antibody. The cellular extracts were prepared by seeding Aag2 cells at a density of 1.8 $x10^5$ cells/well in a 24-well plate. These were incubated for 24 h at 28 °C prior to transfection with 500 ng/well dsRNA specific to the appropriate sequence (Ago-1 or eGFP). Samples were incubated for a further 24 h before the cells were lysed with 100 µl/well passive lysis buffer and denatured prior to applying to the membrane. The peptide samples were expected to generate the strongest signal of the three reactions as they should be recognised explicitly. Each of the anti-Ago-1 antibodies tested were diluted as described for a Western blot procedure with identical incubation times. This was followed by primary antibody recognition with anti-mouse secondary antibodies and detection by chemiluminescence. Following antibody detection it was noted that there was no clear pattern of recognition and often the antibodies inconsistently gave a stronger signal in the dsRNA treated samples and a poor response to the peptide (**Figure 5.3**). Following these results it was decided to forgo dot blot testing of the other supplied RNAi enzyme antibodies and each group of antibodies was analysed by Western blot analysis only.

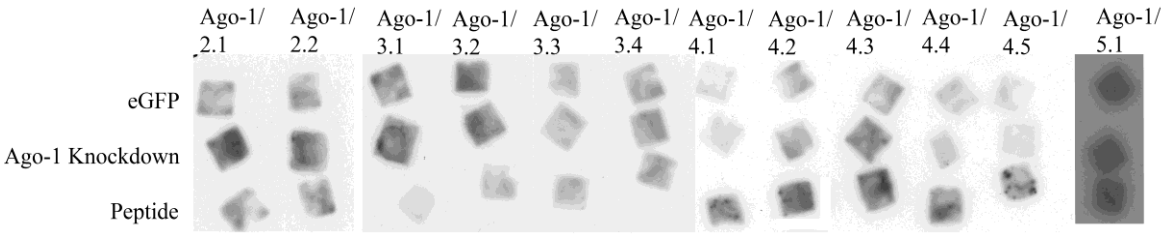


Figure 5.3: Dot blot assay of Argonaute-1 antibodies. *Ae. aegypti*-derived Aag2 cells were treated with either specific dsRNA against eGFP (eGFP) or Ago-1 (Ago-1 knockdown). The cells were lysed and a small volume dropped onto squares of membrane and tested against the provided peptide (Peptide). Each antibody was applied to the membrane and detection was determined using anti-mouse secondary antibody and chemiluminescence.

5.3.3 RNAi Antibody Screening by Western Blotting

All antibodies generated against Ago-1, Ago-2, Dcr-1 and Dcr-2 were tested to determine the degree of specificity against their target protein. Due to the large expected sizes of Ago-1, Ago-2, Dcr-1 and Dcr-2 (93, 114, 250, and 190 kDa respectively) pre-cast gradient (4 - 12 %) gels were used (**Chapter 2.4.1**). Protein electrophoresis was carried out to permit the separation of molecules of different sizes by initially passing a current of 100 V into the gel until samples left the wells whereafter it was increased to 120 V until the bromophenol blue dye front had run off the resolving gel. Proteins were transferred to a nitrocellulose membrane by wet transfer and blotted with antibodies diluted 1:100 to ensure maximum detection proficiency.

To establish successful target recognition by anti-Ago-1 and anti-Dcr-1 antibodies Aag2 cells were treated with dsRNA specific to the appropriate protein (Ago-1 or Dcr-1). The dsRNA used for the protein knockdowns has previously been shown to effectively target their specific transcripts (**Chapter 3**). Proficient detection could be determined by the disappearance of a band(s) at the expected weight in the knockdown samples compared to the controls. Aag2 cell samples were prepared as described previously (**Chapter 5.3.2**) with the exception that the cells were lysed with 100 µl/well 2 x Laemmli sample buffer and denatured prior to gel electrophoresis. Detection of Ago-1 by the 17 supplied antibodies revealed a potential specific band in five of the candidate antibodies; although, it was slightly higher (125 kDa) than the approximate size expected of 93 kDa (**Figure 5.4A**). Many other non-specific bands were also observed and there was often no difference between the Ago-1 knockdown sample and the control sample, with the exception of some bands present in antibodies candidates ‘Ago-1/4.3’ and ‘Ago-1/4.4’.

Of the eight antibodies received targeting Dcr-1 only one candidate was identified that produced a pronounced band at a compatible size (**Figure 5.4B**). However, this band was identified to be approximately 180 kDa, much smaller than the expected 250 kDa. As was observed following Ago-1 detection, the Dcr-1 blots produced a number of aspecific bands and no difference between the Dcr-1 knockdown and the control sample was evident.

Antibodies specific to Dcr-2 and Ago-2 were tested by infecting BHK-21 cells with SFV1-Dcr-2-mCherry and SFV1-Ago-2-ZsGreen VRPs (**Chapter 5.3.4**). BHK-21 cells were used as their genome does not present any putative regions corresponding to any of the antibody recognition sequences (**Table 5.2**) and so successful detection should only be evident in cells expressing the tagged proteins induced through infection. In addition,

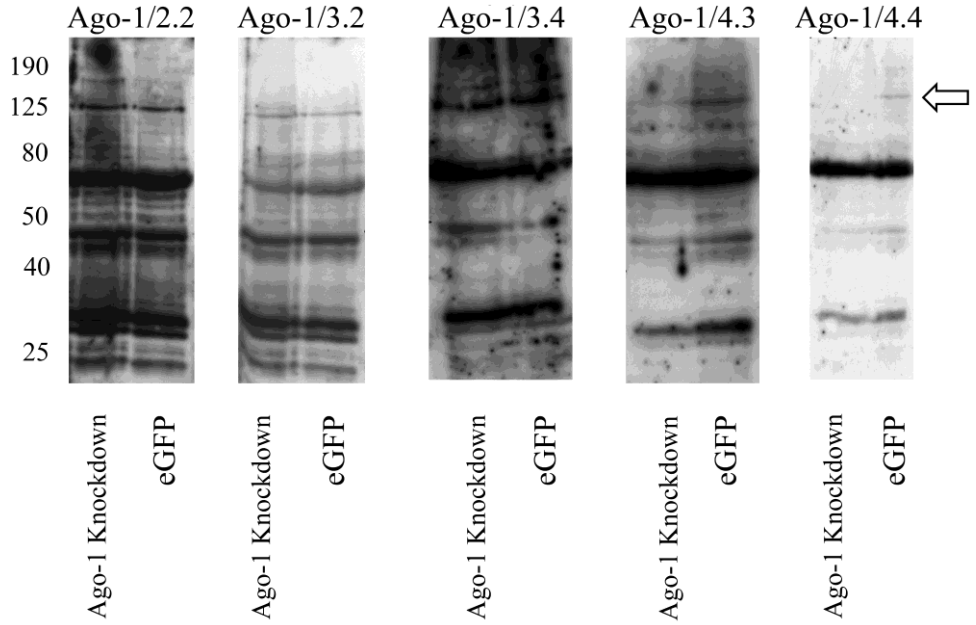
expression of fluorescent proteins in both VRPs has also been demonstrated (**Figure 5.8**). BHK-21 cells were seeded at a density of 8 x10^4cells/well in a 24-well plate and incubated for 24 h at 37 °C prior to infection with either SFV1-Dcr-2-mCherry or SFV1-Ago-2-ZsGreen VRPs at an approximate MOI of 1 as described. Samples were incubated for a further 24 h before protein expression was confirmed by fluorescent microscopy. The cells were then lysed with 100 µl/well 2 x Laemmli sample buffer, denatured and run on a Bis-Tris pre-cast gel. Regrettably, Abmart reported difficulties with the production of the Ago-2 antibody library as many of the antigens selected failed to generate an immune response in the mice. As a consequence, only one of the six epitopes selected rendered any antibodies. Seven antibodies to this epitope were received and tested by Western blot as described (**Figure 5.5A**). Unfortunately, no evidence of purported recognition was evident for any of them.

Confirmation of this result was investigated with the use of anti-ZsGreen antibodies. BHK-21 samples infected with SFV1-Ago-2-ZsGreen were compared with those infected with SFV4-ZsGreen (virus), SFV1-ZsGreen (VRP) as positive controls. BHK-21 samples which had been mock infected were used as a negative control (**Figure 5.5B**). Detection with anti-ZsGreen antibodies is known to be lamentable and as a result is not commonly used for Western blot analysis. However, the results demonstrate that the anti-ZsGreen antibody produces a strong signal at a size consistent with that of ZsGreen (approximately 26 kDa) in the SFV4-ZsGreen and SFV1-ZsGreen samples. Although no band as vivid as those in the virus and VRP samples is detected there is a faint signal at that approximate expected weight of 140 kDa in the SFV1-Ago-2-ZsGreen, in addition to others at 30, 60, 70 and 190 kDa, which are not seen in any of the other samples.

Recognition of Dcr-2 by anti-Dcr-2 antibodies was tested by comparing the results with an anti-mCherry antibody (**Figure 5.6**). Although 10 antibodies were produced only two detected a pattern similar to that seen with the anti-mCherry control antibody. The SFV1-Dcr-2-mCherry infected samples were also different from non-treated BHK samples, which is as expected as BHKs do not contain a corresponding sequence.

From these results it was suggested that there were a total of eight antibodies which merited further examination and that there were potential candidates for the detection of each protein with the exception of Ago-2 (**Table 5.3**).

A



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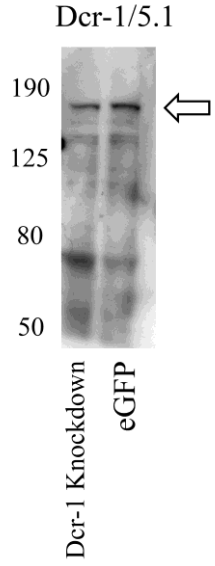
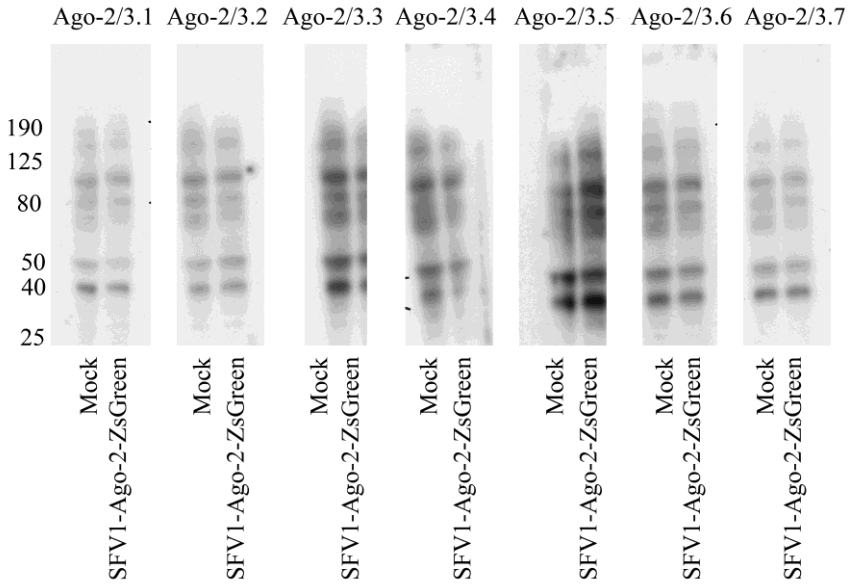


Figure 5.4: Detection of endogenously expressed *Ae. aegypti* Ago-1 and Dcr-1 in *Ae. aegypti*-derived Aag2 cells. *Ae. aegypti*-derived Aag2 cells samples were either transfected with dsRNA against **A**) Ago- 1 (Ago-1 knockdown), **B**) Dcr-1 (Dcr-1 knockdown) or control eGFP (eGFP). Ago-1 protein size was expected to be approximately 93 kDa while Dcr-1 protein size was expected to be approximately 250 kDa. Potential target recognition was achieved by antibodies designated Ago-1/2.2, Ago-1/3.2, Ago-1/3.4, Ago-1/4.3, Ago-1/4.4 and Dcr-1/5.1. Unsuccessful antibody detections are not shown. Protein antibodies were recognised by an anti-mouse secondary antibody and detected by chemiluminescence. Arrows indicate potential protein band weights.

A



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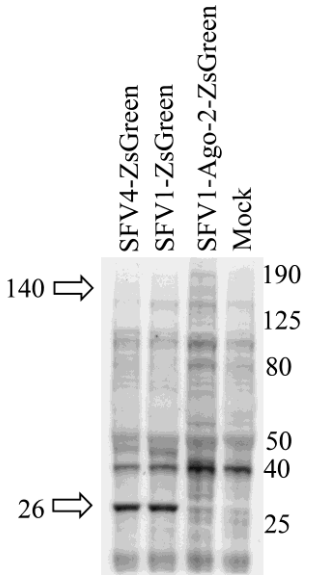


Figure 5.5: Detection of over-expressed *Ae. aegypti* Ago-2 in BHK-21 cells. A) BHK-21 cells were either transfected with SFV1-Ago-2-ZsGreen RNA or were mock transfected (Mock). The protein size was expected to be approximately 140 kDa. No potential target recognition was achieved by any of the anti-Ago-2 antibodies tested. **B**) Detection of ZsGreen in SFV infected BHK-21 cells. BHK-21 cells were either infected with SFV4-ZsGreen (virus) or SFV1-ZsGreen (VRP). These were compared to cells infected with SFV1-Ago-2-Zsgreen VRPs and a mock infected BHK cell sample (Mock) was used as a negative control. Cleaved ZsGreen was expected to be 26 kDa and was detected by an anti-ZsGreen antibody. All primary antibodies were recognised by an anti-mouse secondary antibody and detected by chemiluminescence. Arrows indicate protein band weights.

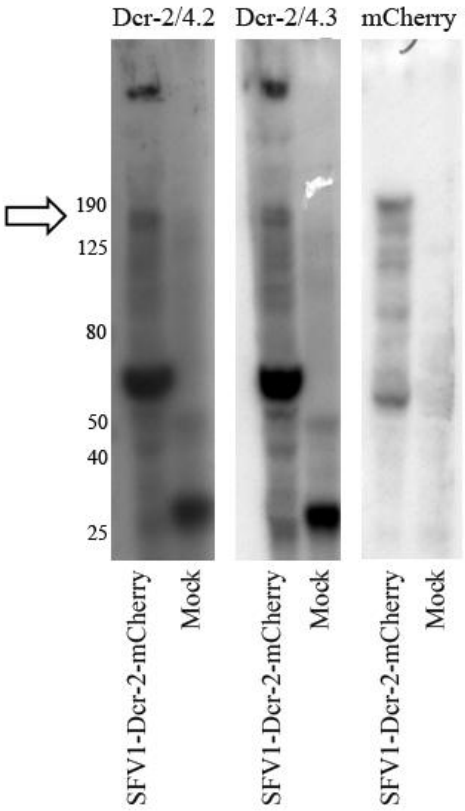


Figure 5.6: Detection of over-expressed *Ae. aegypti* Dicer 2 in BHK cells by Western blot analysis. BHK-21 cells were either transfected with SFV1-Dcr-2-mCherry RNA or were mock transfected (Mock). The size expected for Dcr-2-mCherry was approximately 218 kDa. Potential target recognition was achieved by antibodies designated Dcr-2/4.2 and Dcr-2/4.3. Unsuccessful antibody detections are not shown. A band was detected with both anti-Dcr-2 antibodies at a similar weight for that detected using the anti-mCherry antibody. However, this was lower than expected at approximately 160 kDa. All primary antibodies were recognised by an anti-mouse secondary antibody and detected by chemiluminescence. An arrow indicates the potential protein band weight.

Table 5.3: Successful antibody candidates as determined by Western blot analysis.

Target protein (predicted weight)	Epitope target	Epitope Index	Antibody designation	Successful detection
Argonaute-1 (93 kDa)	EHDSGEGSHQ	1	Ago-1/1.1	
			Ago-1/1.2	
	KKEQSGKSGN	2	Ago-1/2.1	
			Ago-1/2.2	✓
			Ago-1/2.3	
	PVSTTAGAQN	3	Ago-1/3.1	
			Ago-1/3.2	✓
			Ago-1/3.3	
			Ago-1/3.4	✓
	PPSPTQSQTS	4	Ago-1/4.1	
			Ago-1/4.2	
			Ago-1/4.3	✓
			Ago-1/4.4	✓
			Ago-1/4.5	
			Ago-1/4.6	
	SNPRTDAKRT	5	Ago-1/5.1	
			Ago-1/5.2	
Argonaute-2 (114 kDa or 140 kDa fused to ZsGreen)	KIHSSALLPV	3	Ago-2/3.1	
			Ago-2/3.2	
			Ago-2/3.3	
			Ago-2/3.4	
			Ago-2/3.5	
			Ago-2/3.6	
			Ago-2/3.7	
Dicer-1 (250 kDa)	PIKDPKEAEI	1	Dcr-1/1.1	
			Dcr-1/1.2	
	KESDKSAAIE	4	Dcr-1/4	
	KPEKLADGRR	5	Dcr-1/5.1	✓
			Dcr-1/5.2	
			Dcr-1/5.3	
			Dcr-1/5.4	
			Dcr-1/5.5	
Dicer-2 (190 kDa or 218 kDa fused to mCherry)	DDGKSTRSKH	1	Dcr-2/1	
	GKTINRPDPL	2	Dcr-2/2.2	
			Dcr-2/2.1	
	RAAGSPKREP	4	Dcr-2/4.1	
			Dcr-2/4.2	✓
			Dcr-2/4.3	✓
			Dcr-2/4.4	
			Dcr-2/4.5	
			Dcr-2/4.6	
	DVEYKERKGK	6	Dcr-2/6	

5.3.4 Recombinant Dicer-2 and Argonaute-2 for *in vitro* Visualisation

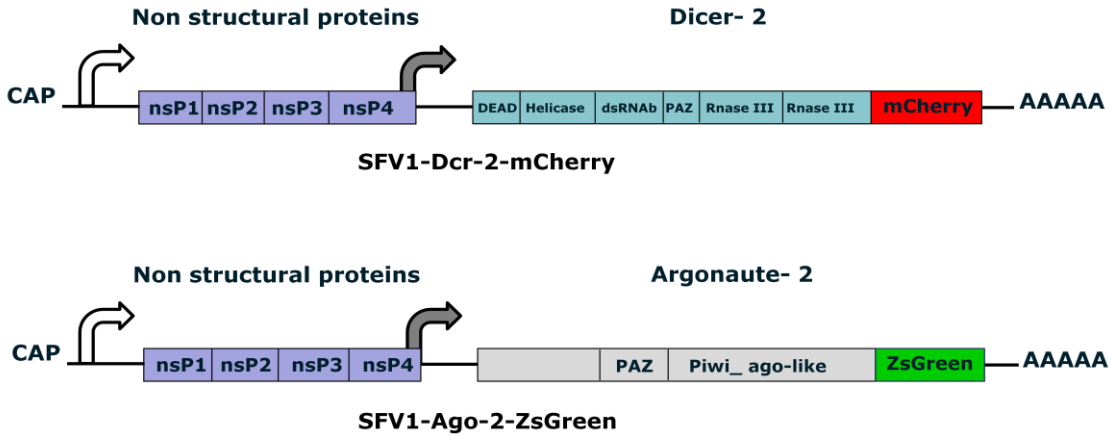
As the experiments described previously suggest that the antibodies designed against endogenous RNAi proteins were largely unsuccessful, an alternative strategy was devised to produce recombinant forms of Dicer-2 (Dcr-2) and Argonaute-2 (Ago-2). These were first produced fused to fluorescent tags to allow the spatial and temporal properties of both proteins following a viral infection to be investigated. In this way, the stage of acute infection that Dcr-2 is able to interact with its substrate within the viral replication complexes and the involvement of the silencer protein Ago-2 could be studied. This was achieved by using the known *Ae. aegypti* Dcr-2 (AAW48725) and Ago-2 (ACR56327) sequences.

Dcr-2 was acquired from ShineGene Molecular Biotech Inc. (Shanghai, China) fused to the mCherry sequence (AY678264) on the carboxyl (C-) terminus. The mCherry protein is known to be monomeric and photostable. It was selected as it had been shown to be unobtrusive and did not interfere with protein folding (Graewe et al., 2009; Kümmerer et al., 2012). The Dcr-2-mCherry fusion was inserted into the pIB/V5-His vector under the control of the OpIE2 promoter. This immediate-early (IE) promoter is derived from the baculovirus *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus (*Op*MNPV) which naturally infects the Douglas fir tussock moth and is known to be an efficient promoter for gene expression in insect cells, including mosquito cell cultures (Pfeifer et al., 1997; Massotte et al., 2003; Xu et al., 2008; Paradkar et al., 2014). Early experiments performed with this construct did not result in any detectable expression of the mCherry tag in either *Ae. aegypti*-derived Aag2 cells, *Ae. albopictus*-derived U4.4 cells or the mammalian BHK-21 cell line (Donald, 2010). It was suggested that due to the extensive size of Dcr-2 a lack of expression could be explained by incomplete transcription/translation, erroneous protein folding or reduced stability. Consequently, it was decided to use a stronger expression system to establish if this enhanced protein expression levels. One suggested to be a suitable alternative was to place the tagged protein behind the subgenomic promoter within the SFV genome and use the virus infection to express the proteins. For this to be achieved the pIB-Dcr-2-mCherry construct was sent to the lab of Prof. Andres Merits (Institute of Technology, University of Tartu, Estonia) for cloning into the backbone of SFV replicating particles (VRPs) (**Figure 5.7A**). The *Ae. aegypti* sequence for Ago-2 was also provided for fusion to the ZsGreen sequence (AFD54300), again on the C- terminus. ZsGreen was preferred over eGFP due to its superior brightness (Bell et al., 2007; Nakamura et al., 2013). Unlike Dcr-2-mCherry, Ago-

2-ZsGreen was not cloned into pIB/V5-His but directly inserted into SFV1. It was first necessary to modify the original SFV1 plasmid to permit the insertion of both RNAi enzymes. This was achieved by inserting two restriction endonuclease recognition sites (*Nru*I and *Spe*I) subsequent to the subgenomic promoter. In addition, as the *Spe*I cleavage sequence was previously used to linearise the plasmid after the poly (A) tail for *in vitro* transcription, this was replaced by the *Pac*I recognition sequence. The constructs fused to their respective fluorescent tags were inserted in place of the viral structural proteins, although, the non-structural proteins remain *in situ*. This cloning was gratefully carried out by Margit Ool (Institute of Technology, University of Tartu, Estonia) (**Figure 5.7B**).

Following the receipt of both SFV1-Dcr-2-mCherry and SFV1-Ago-2-ZsGreen it was first necessary to establish if the recombinant RNAi enzymes could be expressed under the SFV subgenomic promoter in BHK-21 cells. Cells were seeded at a density of 8 x 10^4 cells/well in a 24-well plate and incubated for 24 h prior to transfection with approximately 4 µg *in vitro* transcribed RNA for either SFV1-Dcr-2-mCherry or SFV1-Ago-2-ZsGreen. Cells were incubated for a further 24 h period before they were fixed and fluorescence observed with the Zeiss 710 confocal microscope (**Figure 5.8**). Successful expression was observed following transfection with both constructs suggesting that the SFV subgenomic promoter was able to express both complete protein sequences in BHK-21 cells. SFV1-Dcr-2-mCherry and SFV1-Ago-2-ZsGreen could then be used to induce protein expression following VRP infection.

A



B

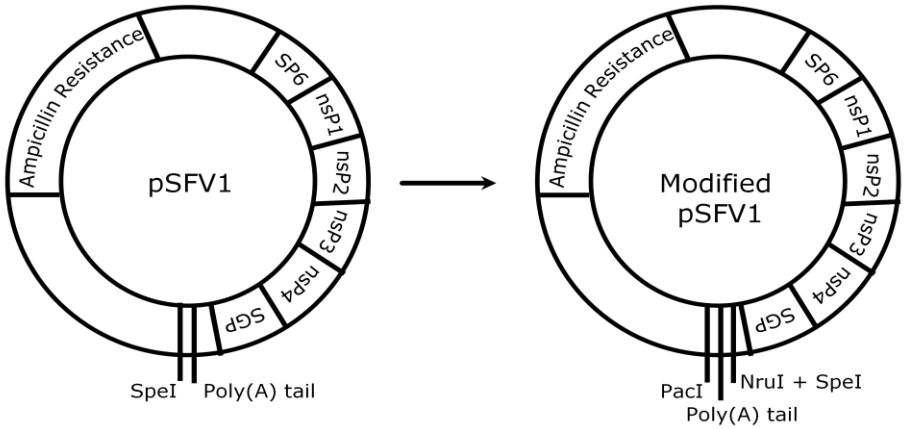
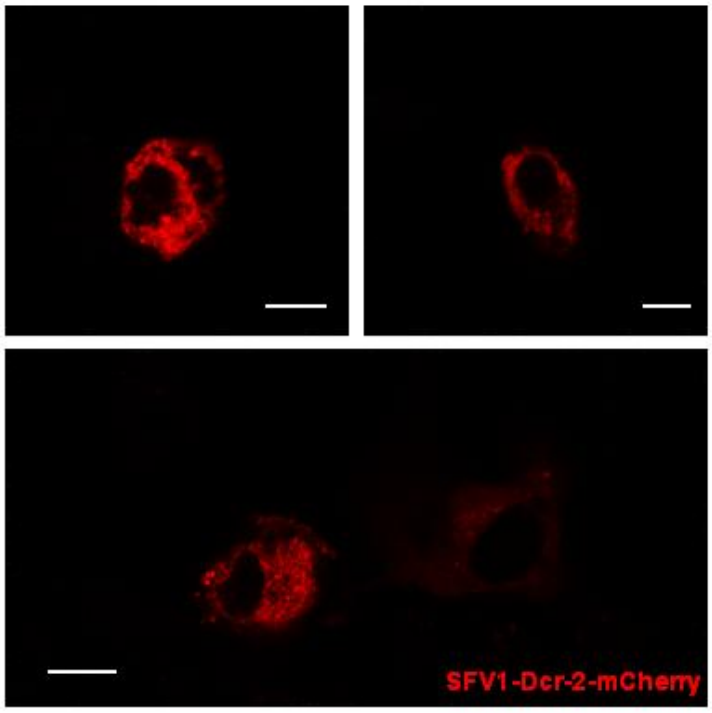


Figure 5.7: SFV replicons expressing *Aedes aegypti* RNAi genes, Dicer-2 and Argonaute-2 after the subgenomic promoter. A) Each protein is further labelled with a fluorophore, mCherry or ZsGreen respectively on the C terminus, for protein detection by confocal microscopy. The white arrow represents the genomic promoter while the grey arrow represents the subgenomic promoter. **B**) The original SFV plasmid was modified to allow insertion of the recombinant proteins. SP6, SP6 promoter; nsP1-4 non-structural proteins 1-4; SGP, subgenomic promoter.

A



B

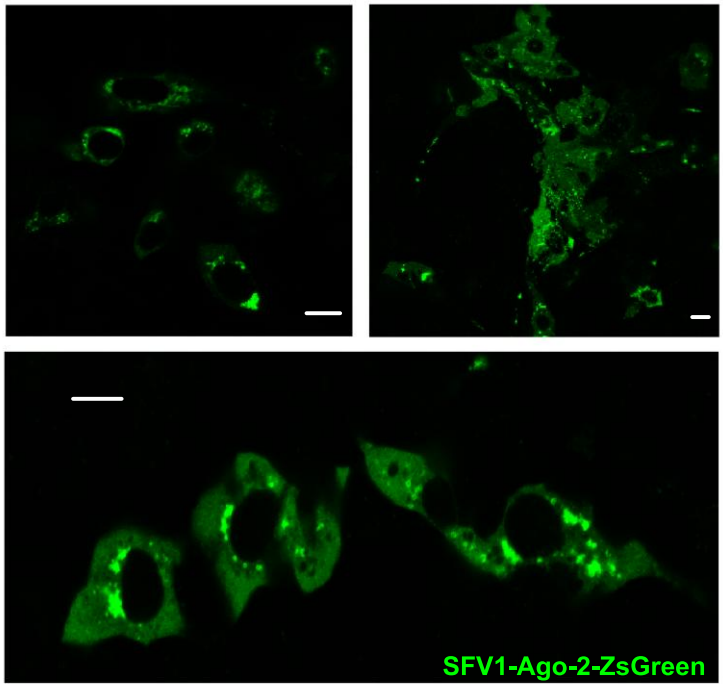


Figure 5.8: BHK-21 cells expressing tagged *Ae. aegypti* RNAi proteins. BHK-21 cells were transfected with either *in vitro* transcribed **A**) SFV1-Dcr-2-mCherry or **B**) SFV1-Ago-2-ZsGreen capped RNA. Fluorophore expression is detected with a Zeiss 710 confocal microscope. Red, SFV1-Dcr-2-mCherry; Green SFV1-Ago-2-ZsGreen. Scale bars indicate 10 μm.

Production of the SFV VRPs required the VRP RNA to be combined with RNA encoding only the viral structural proteins (as described in **Chapter 2.5.2** and **Figure 5.9**). There were two options available for delivery of the structural proteins; either by the helper system (using the Helper 1 plasmid providing all of the structural proteins; C-E3-E2-6K-E1) (Berglund et al., 1993), or the split helper system (were the capsid and envelope glycoproteins are separately encoded by different plasmids) (Smerdou and Liljestrom et al., 1999). As the replicon RNA is encoded separately from the structural proteins VRPs are unable to produce viral progeny which could be released and cause a productive infection. Although the split helper system reduces the risk of viral recombination and the potential production of infectious viral particles observed when using the Helper 1 system (Berglund et al., 1993), this was not deemed to be important for the expression of the recombinant proteins and single helper system (the Helper 1 plasmid) was used for SFV1-Dcr-2-mCherry and SFV1-Ago-2-ZsGreen production. Both plasmids were linearised with the appropriate restriction endonuclease, *in vitro* transcribed and capped prior to combining the RNAs for joint electroporation into BHK-21 cells (**Figure 5.9**).

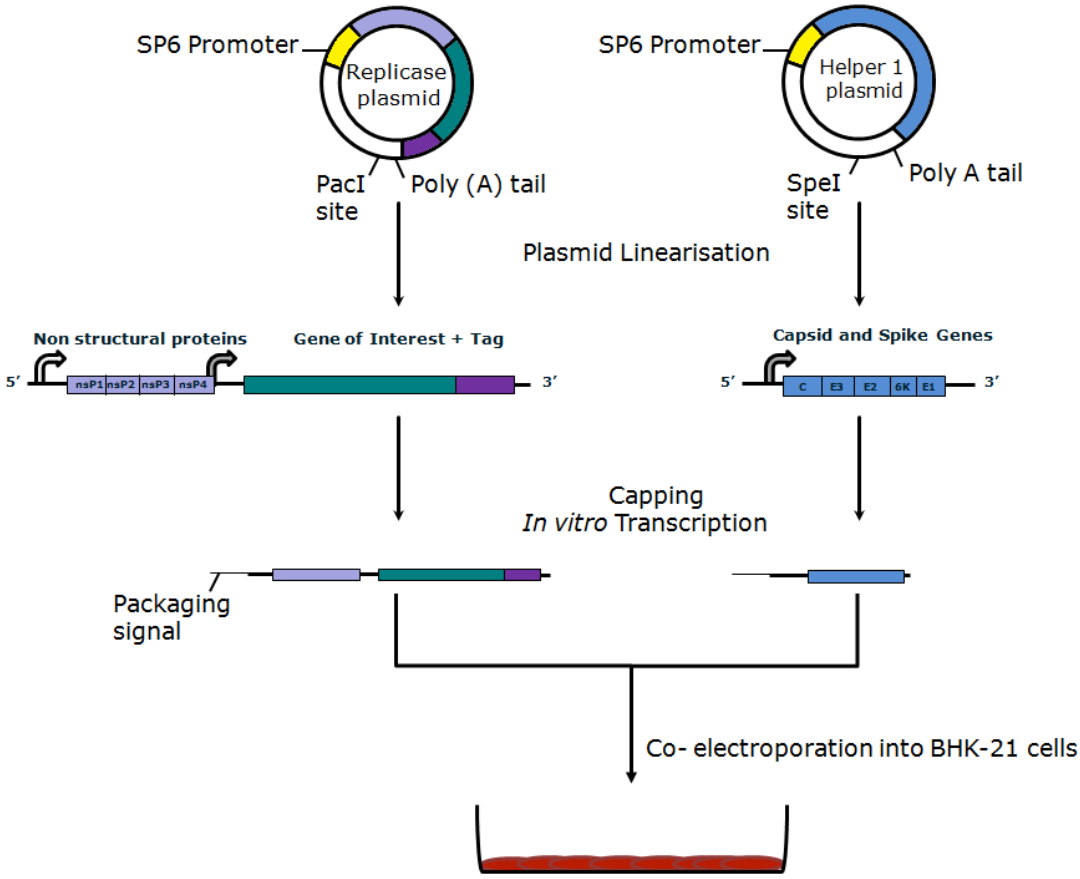


Figure 5.9: Schematic representation of the production of Virus Replicating Particles (VRPs) using the Helper 1 plasmid system. The Helper 1 plasmid encodes the capsid and glycoproteins (C, E3, E2, 6K and E1) and is first linearised prior to capping and *in vitro* transcription from the SP6 promoter alongside the replicase plasmid which contains the non-structural proteins as well as the required foreign gene of interest. The two RNAs are combined and electroporated into BHK-21 cells and VRPs are produced into the supernatant.

SFV1-Dcr-2-mCherry and SFV1-Ago-2-ZsGreen VRPs were produced and used to infect both Aag2 and C6/36 mosquito cells. As both protein sequences were taken from the *Ae. aegypti* genome, it was important to confirm that expression was observed in an *Ae. aegypti*-derived cell line. Although there are several *Ae. aegypti*-derived cell lines, the lab has a substantial level of experience in the handling and maintenance of the Aag2 line and so this cell line was selected. In addition, as previously discussed the C6/36 cell line provides an ideal system for determining the function of the recombinant Dcr-2 construct as a result of its Dcr-2/RNAi null phenotype (Brackney et al., 2010; Scott et al., 2010; Morazzani et al., 2012). The production of the VRPs consistently resulted in a very low titre (approximately 2×10^6 - 1.5×10^8 pfu/ml) (calculated as described in **Chapter 2.5.4**). Moreover, as the TNE buffer used to re-suspend the VRPs was toxic to the cells when applied directly, the VRP suspension was required to be diluted in sPBSA to protect cell viability. Therefore, the final MOI used for each experiment was approximately 1 although a higher MOI would have been preferred to target the greatest number of cells.

Aag2 and C6/36 cells were seeded at a density of 1.8×10^5 and 1.2×10^5 cells/well respectively in a 24-well glass bottom plate 24 h prior to infection with either SFV1-Dcr-2-mCherry or SFV1-Ago-2-ZsGreen VRPs. Cells were incubated at 28 °C for a further 24 h post infection before they were fixed and immunostained with an antibody against SFV-nsP3. As preliminary results suggested that the titre of the VRPs was lower than anticipated, immunostaining with SFV-nsP3 was done to establish if there was a difference between the number of cells which had been infected with the SFV VRPs compared to the number expressing either of the recombinant proteins. Detection of SFV-nsP3 was done using an anti-mouse secondary antibody conjugated to a fluorophore of a different wavelength from the fluorophore tag. In this way when Dcr-2-mCherry was observed (594 nm), nsP3 was identified with a secondary antibody detected at 488 nm and vice versa when Ago-2-ZsGreen (488 nm) was visualised. Finally, a coverslip was mounted using DAPI mounting media to allow the cell nucleus to be discerned. The results indicate that for both Aag2 and C6/36 cell lines (**Figure 5.10** and **5.11**) expression of both fluorescently tagged constructs is evident. However, in both cases the staining for SFV-nsP3 was more wide spread than the signal for the recombinant protein for both cell types (**Table 5.4**).

Table 5.4: Percentage of expression of labelled RNAi protein in comparison to SFV nsP3 staining in Aag2 and C6/36 cell types.

	SFV1-Dcr-2-mCherry		**SFV1-Ago-2-ZsGreen**	
Cell type	Anti-nsP3	Dcr-2-mCherry	Anti-nsP3	Ago-2-ZsGreen
Aag2	80 %	16 %	87 %	5 %
C6/36	99 %	33 %	96 %	17 %

With the success of Dcr-2-mCherry and Ago-2-ZsGreen expression from SFV1 VRPs, this system could be further optimised and used to establish adequate protein function.

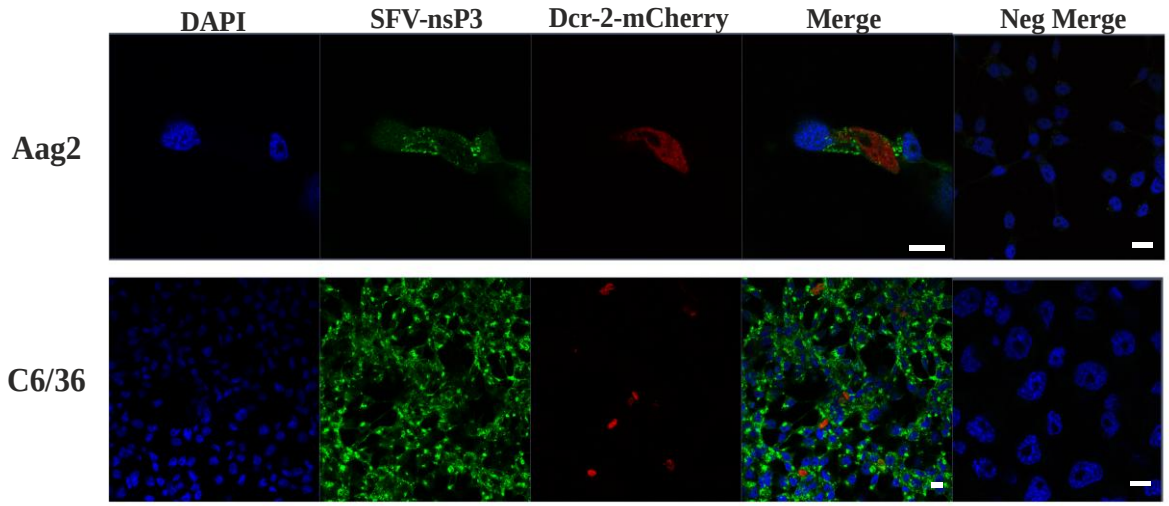


Figure 5.10: Aag2 and C6/36 cell lines expressing Dicer-2-mCherry. Confocal microscopy images of the *Ae. aegypti*–derived Aag2 and *Ae. albopictus*-derived C6/36 cell lines infected with SFV1-Dcr-2-mCherry VRPs. Mock infected samples (Neg Merge) were included as a negative control. Immunostaining was also carried out to detect SFV-nsP3 and nuclear staining was recognised by DAPI expression. Blue, DAPI; Green, SFV-nsP3; red, Dcr-2-mCherry. Scale bars indicate 10 μm.

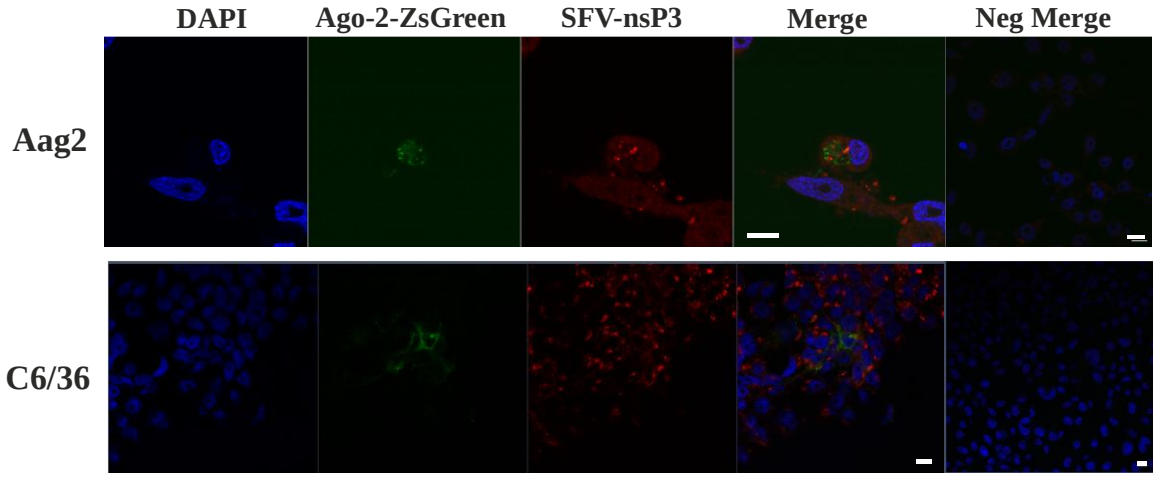


Figure 5.11: Aag2 and C6/36 cell lines expressing Argonaute-2-ZsGreen. Confocal microscopy images of the *Ae. aegypti*–derived Aag2 and *Ae. albopictus*-derived C6/36 cell lines infected with SFV1-Ago-2-ZsGreen VRPs. Mock infected samples (Neg Merge) were included as a negative control. Immunostaining was also carried out to detect SFV-nsP3 expression and nuclear staining was recongised by DAPI expression. Blue, DAPI; Green, SFV-nsP3; red, Dcr-2-mCherry. Scale bars indicate 10 μm.

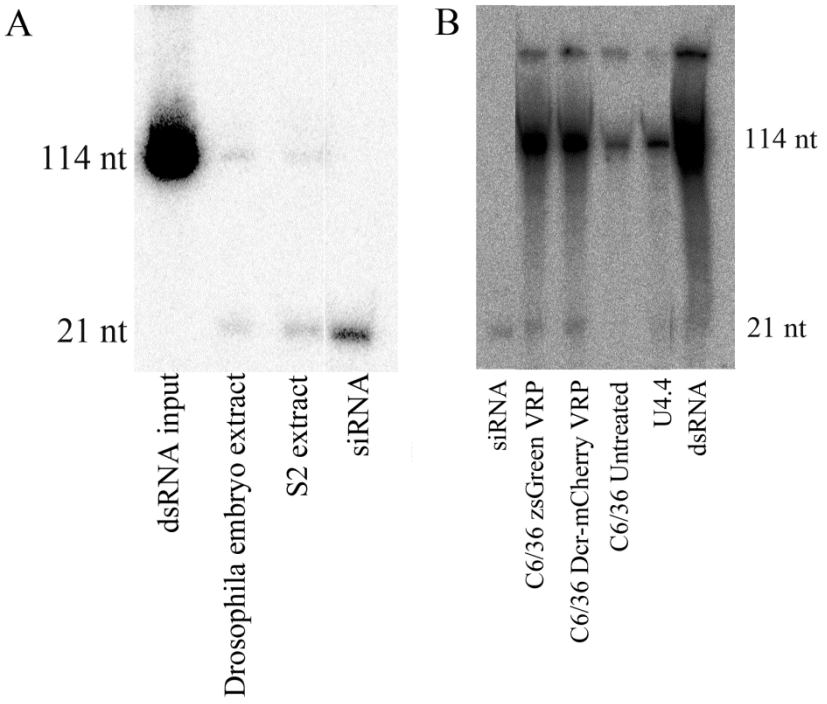
5.3.5 Assessing the Function of Recombinant Dicer-2 in C6/36 Cells

To establish the functionality of the recombinant Dcr-2-mCherry construct the C6/36 cell line was used. Natural function of the recombinant Dcr-2 would be confirmed if the cell line's ability to cleave long dsRNA molecules into shorter 21 nt siRNA molecules could be recovered. To establish if expression of the Dcr-2-mCherry fusion in the C6/36 cell line was able to restore active Dcr-2 processing an *in vitro* dicer cleavage assay was performed. This assay is well established in the literature (Matranga et al., 2005; Vermeulen et al., 2005; Iwasaki et al., 2010; Yoda et al., 2010; Nayak and Andino, 2011; Yang et al., 2011; van Mierlo et al., 2012; Vodovar et al., 2012) but has not previously been used in the lab. To first optimise the assay it was carried out using *Drosophila* embryo extracts gratefully provided by Joël T van Mierlo (Department of Medical Microbiology, Nijmegen Center for Molecular Life Sciences, University Medical Center Nijmegen, The Netherlands). The results were compared to those of *Drosophila*-derived S2 cells which are also known to efficiently process dsRNA in this assay. Confirmation of dsRNA cleavage was achieved using internally [^{32}P] UTP-labelled dsRNA molecules. The S2 cellular extracts were prepared by seeding the cells in a 6-well plate at a density of 1 x 10^6/well. Following an incubation of 24 h at 28 °C, the cells were lysed with 1 x lysis buffer to disrupt the cell membranes. Embryo extracts were prepared as previously described (Haley et al., 2003; van Mierlo et al., 2012). The samples received 16.2 pCi of radio-labelled dsRNA and were incubated overnight at 28 °C before the RNA was isolated, purified and loaded onto a denaturing gel. (**Figure 5.12A**). The size of the fragments generated was determined by a comparison to size markers; input dsRNA (114 nt) and siRNAs (21 nt). The input dsRNA was purified alongside the samples in a cell-free reaction. From the images generated it can be concluded that the assay was successful as both the cell and embryo extracts showed efficient cleavage of dsRNA into shorter RNAs of approximately 21 nt as determined by the siRNA size marker.

Once the assay was successfully established it was subsequently repeated involving extracts derived from both C6/36 and U4.4 *Ae. albopictus* cell lines. U4.4 cells are known to be RNAi competent and produce 21 nt siRNAs which can be detected by this assay (Vodovar et al., 2012). Extracts from these cells were left untreated and were used as a positive control alongside samples from C6/36 cells which were either infected with SFV1-Dcr-2-mCherry or SFV1-ZsGreen VRP (as a negative control) or were untreated. If the Dcr-2-mCherry fusion was functional then a difference would be expected to be observed between the C3/36 sample which obtained Dcr-2-mCherry compared to those were only

the natural truncated form was present. Both cell lines were seeded at a density of 8 x 10^5/well in a 6-well plate and the lysate prepared 24 hpi as described previously. Image J analysis was used to quantify the total of dsRNA and siRNAs present (**Figure 5.12C**). The total quantity of nucleic acids in each lane from three experiments was measured and set to 100 %. The band density of the cleaved dsRNA was then measured as a percentage of this.

It was observed that as expected no strong band was detected in the C6/36 untreated sample (**Figure 5.12B**). Although there was a discernible band detected for the C6/36 sample infected with SFV1-Dcr-2-mCherry, this also appeared in the sample infected with the control SFV1-ZsGreen VRP. Therefore, these results do not allow us to conclude that the recombinant Dcr-2 is functional. The presence of the tag could be disrupting the ability of the enzyme to function correctly suggesting further adjustments are required. In addition, only a very faint band was detected for the U4.4 sample signifying it was not as successful as had been anticipated.



C

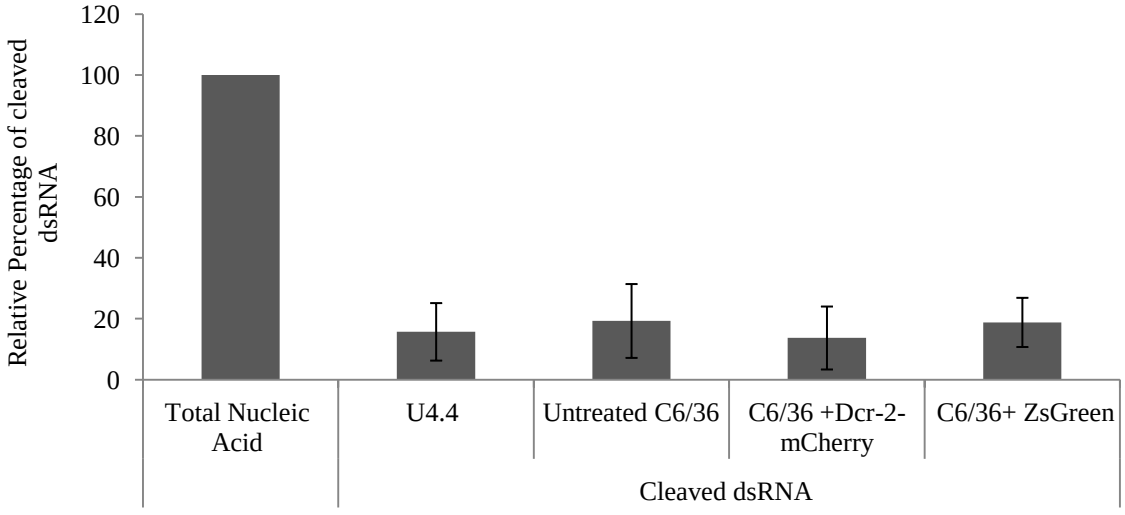


Figure 5.12: Processing of long dsRNA molecules into 21 nt siRNAs detected by an *in vitro* dicer cleavage assay. Cellular extracts were prepared from **A**) *Drosophila* embryos and *Drosophila* S2 cells or **B**) *Ae. albopictus*-derived U4.4 cells, in addition to extracts from C6/36 cells which were either untreated or infected with SFV1-Dcr-2-mCherry or SFV1-ZsGreen. Extracts were incubated with [^{32}P] internally radio-labelled 114 nt dsRNA. A size marker of [^{32}P] radio-labelled 21 nt siRNA was also loaded. This image is a representative of three experiments. **C**) Image J analysis quantifying the percentage of cleaved dsRNAs. Error bars show the standard deviation of three independent experiments performed in triplicate.

5.3.6 Expression of Dicer-2 Using a Cell-Free System

In addition to functional protein production through plasmid amplification in bacteria and expression in cells in culture, cell-free systems are an alternative opportunity to produce and purify proteins for characterisation. The advantages of cell-free systems include; 1) the capacity to produce large quantities of functional protein in a short space of time, reduced from days to hours; 2) coupled lysate reactions incorporating both transcription and translation stages into one reaction greatly simplifying production; 3) proteins can be produced directly from linear PCR products reducing the time and challenges associated with sub-cloning; and 4) detection can be performed immediately due to direct co-translational labelling during synthesis (**Figure 5.13**). This system was not yet established in the lab but was chosen to assist with Dcr-2-mCherry expression and for the development of a new strategy for protein production in the future. If successful it would allow complications with SFV1-Dcr-2-mCherry to be bypassed by removing the difficulty of expressing an antiviral protein within a viral backbone.

Although several systems are available for protein production (*E.coli*, Wheat Germ and *Spodoptera frugiperda*), the rabbit reticulocyte method was selected. This native mammalian set up is recommended for its ability to efficiently produce full-length protein sequences downstream from either T7, T3 or SP6 RNA polymerase promoter sequence sites. A study of the literature and company websites showed that there was no difference between the three promoters. On that basis the SP6 promoter was selected as it is well used in the lab for the *in vitro* transcription of SFV plasmids. In addition, cloning Dcr-2-mCherry between the SP6 promoter sequence and a poly(A) tail of the pSP64 Poly(A) vector has been reported to improve stability and enhance translation (Jackson and Standart, 1990). The cloning strategy used is illustrated in **Figure 5.14** and the correct plasmid sequence was confirmed by both restriction digestion and sequencing. Protein translation was performed by combining the required components into a single tube as described in **Chapter 2.4.3** and incubating at 30 °C for 90 min. A positive control SP6 plasmid encoding the *Fluc* gene was supplied in the Promega kit and was also included to confirm the functionality of the assay. Detection of successful gene expression was permitted by the incorporation of [^{35}S] methionine into the protein products. The lysate was diluted to three different concentrations prior to loading onto a 4 - 12 % Bis-Tris gel to prevent the large protein aggregations collecting in the wells of the gel and restricting movement into the gel. Although strong bands are clearly distinguished in all of the control plasmid dilutions, there is no signal at any of the concentrations of the SP6-Dcr-2-mCherry

sample (**Figure 5.15**). Unfortunately, it can be concluded that this assay requires further optimisation which was not able to be completed within the timescale of this project.

As a result of the findings so far it can be concluded that expression of Dcr-2-mCherry, as well as Ago-2-ZsGreen, is only achieved when the proteins are present within the backbone of SFV VRPs. However, the *in vitro* dicer cleavage assay has shown that the Dcr-2-mCherry appears not to be functional. Therefore, further adaptations are required to optimise successful production of functional RNAi enzymes for investigative studies.

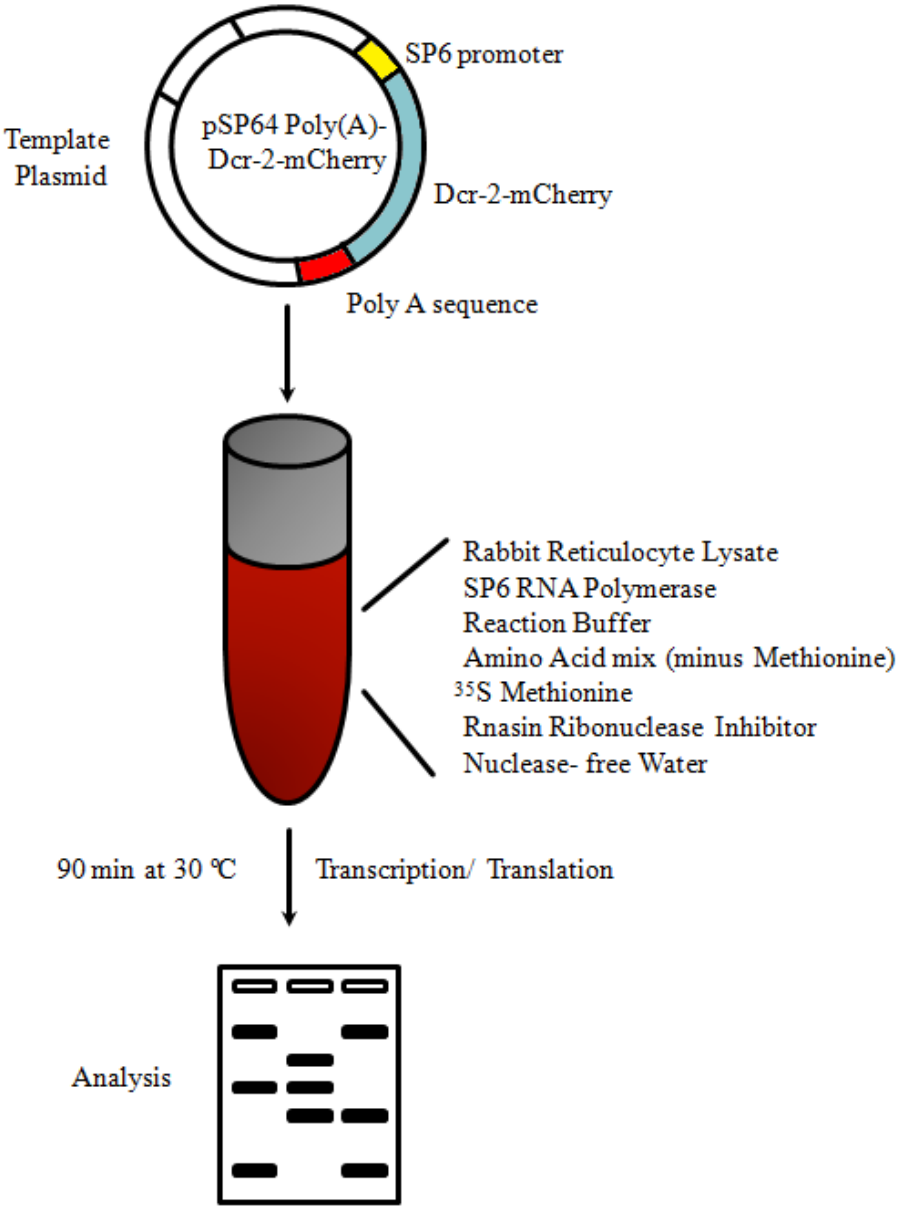


Figure 5.13: Schematic representation of the TNT rabbit reticulocyte lysate system. The *Ae. aegypti* Dcr-2-mCherry recombinant protein was inserted into the pSP64 Poly (A) vector after the SP6 gene promoter and ahead of the Poly(A) tail sequence. All the required components are combined in a single tube, including [^{35}S] methionine, and incubated for 90 min at 30 °C. The samples can then be analysed by gel electrophoresis and exposed to a phosphorimaging screen.

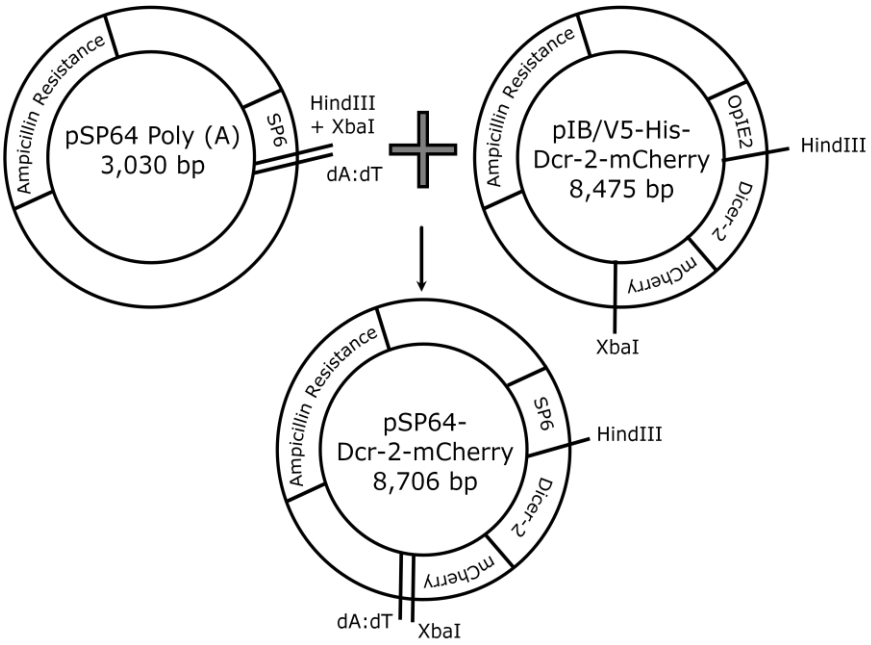


Figure 5.14: Schematic illustration of the insertion of Dcr-2-mCherry from the pIB/V5-His vector into the pSP64 Poly (A) vector. This was done after the SP6 gene promoter sequence for expression in the TNT rabbit reticulocyte lysate protein production system.

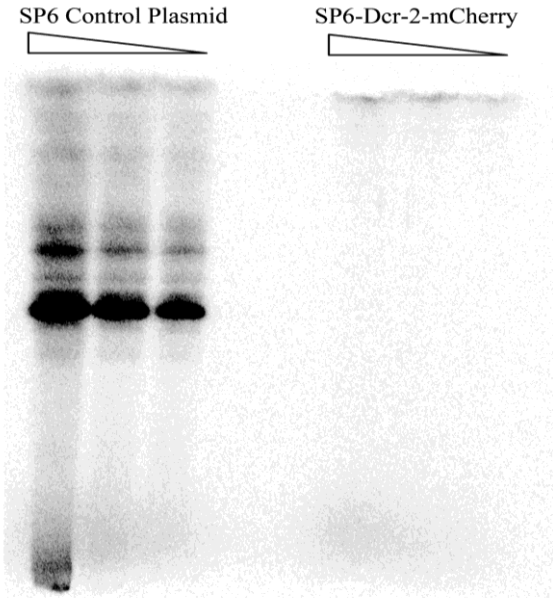


Figure 5.15: Production of the mCherry tagged *Ae. aegypti* Dcr-2 protein by TNT rabbit reticulocyte lysate expression. The SP64-Dcr-2-mCherry plasmid was mixed with the TNT coupled rabbit reticulocyte lysate system, including radio-labelled [^{35}S] methionine. A control SP6 plasmid expressing *Fluc* was used for comparison. Both samples were tested at decreasing concentrations and detected by exposing to a phosphoimaging screen.

5.3.6 Further Developments in Dicer-2 Cloning

To circumvent some of the challenges encountered when designing a fluorescent Dcr-2 reporter protein, a second approach was investigated. A potential pitfall was identified that the fluorescent tags were not sufficiently discrete as to allow for correct protein folding and that a smaller tag may bypass this issue. Therefore, the V5 epitope tag was selected (**Table 5.5**). This 14 amino acid peptide is derived from an epitope of the paramyxovirus simian virus 5 (SV5) located on the P and V proteins and is a widely used tag for immunostaining, immunoprecipitation and Western blotting (Cornwell and Kirkpatrick, 2001; Horng and Medzhitov, 2001; Kaneko et al., 2006; Lipardi and Paterson, 2009; van Cleef et al., 2014; van den Beek et al., 2014; van Mierlo et al., 2014).

Table 5.5: Nucleotide and corresponding amino acid sequence for the V5 tag. See **Page xix** for amino acid codes.

	V5 epitope tag sequence
Single Letter Code	N-GKPIPNPLLGLDST-C
Triple Letter Code	N-Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr-C
Nucleotide Sequence	5'GGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCTACG 3'

As V5 is approximately 5 kDa, it is substantially smaller than both ZsGreen (26 kDa) and mCherry (28 kDa). Primers were designed which allowed the removal of the fluorescent tag and its replacment with the V5 tag either on the amino (N-) or C- terminals of Dcr-2 within the pIB/V5-His vector (**Figure 5.16** and **Chapter 2.9**). Sequencing the cloning products confirmed the sequences were correct. Initial experiments were performed using Aag2 and C6/36 cell lines seeded at a density of 1.8 x 10^5 and 1.2 x 10^5 cells/well respectively in 24-well plates 24 h prior to transfection with 500 ng/well of either pIB-Dcr-2-V5/C or pIB-Dcr-2-V5/N. Cells were incubated at 28 °C for 24 h before they were lysed directly in 100 µl/well 2 x Laemmli sample buffer. Samples were denatured by heating to 100 °C for 10 min prior to loading 30 µl into a self-made 10 % acrylamide gel for Western blot analysis. A positive control was also included courtesy of Dr. Ben Hale (CVR, University of Glasgow) consisting of a sample of MDCK cells which stably expressed the SV5 V protein (Hale et al., 2010). Protein detection was achieved using an anti-V5 antibody. This interaction was then recognised with an anti-mouse secondary antibody and

detected by chemiluminesence. Following anti-V5 antibody detection, a large band is present at 25 kDa in the MDCK V5+ sample in agreement with the expected size of the complete V5 protein (**Figure 5.17**). Although the predicted size of Dcr-2-V5 is 195 kDa, no band is apparent in any of the samples at this weight and all samples resembled non-transfected controls. The only band detected was present at the expected size for bovine serum albumin (BSA).

To further confirm the suggestion that this finding is due to the insufficient strength of the OpIE2 promoter for expression of the tagged Dcr-2 to a sufficient concentration for Western blot analysis, the two Dcr-2-V5 sequences were transfected into *Spodoptera frugiperda*-derived Sf21 cells. These cells are known to be a good expression system for baculovirus promoters and would be the best candidate for successful expression of the recombinant proteins. Sf21 cells were seeded at a density of 2 x 10^5 cells/well in a 24-well plate and transfected using Fectofly™ (Polyplus Transfection), a DNA specific transfection reagent, following the manufacturer's instructions. Briefly, cells were allowed to settle for 2 h at 28 °C prior to preparing 2 µg of DNA with 50 µl 150 mM NaCl and 2 µl of Fectofly with 50 µl 150 mM NaCl. Four plasmids were used for this experiment; pIB-Dcr-2-V5/N, pIB-Dcr-2-V5/C, pIB-eGFP and pIB-Dcr-2-mCherry. Both V5 tagged proteins were included for detection by Western blot analysis while the fluorescent plasmids were used for visualisation by fluorescent microscopy. The total volume of Fectofly/NaCl mix was added to the total volume of the DNA/NaCl mix, vortexed for 10 sec and incubated for 30 min at room temperature. The total volume of 100 µl/well was added dropwise to the cell monolayer and the plates rocked gently to ensure an even distribution of the DNA complexes. The plates were then incubated for 4 h ahead of replacing the media with 1 ml of fresh growth media. Finally, the cells were incubated for a further 48 h and expression of pIB-eGFP and pIB-Dcr-2-mCherry was determined by visualising with a Zeiss Axiovert S100 microscope.

Expression of the pIB-eGFP could be observed; although, pIB-Dcr-2-mCherry could not be detected (data not shown). Western blot analysis was carried out by lysing the pIB-Dcr-2-V5/N and pIB-Dcr-2-V5/C transfected samples in 100 µl/well 6 x loading buffer and denaturing them prior to loading onto a home-made acrylamide gel. As before protein electrophoresis was carried out until the 25 kDa band marker approached the end of the gel before wet transfer was performed. Detection by anti-V5 antibody was carried out as described previously. The anticipated size for Dcr-2-V5 is 195 kDa; although, this was not observed for either pIB-Dcr-2-V5/C and pIB-Dcr-2-V5/N (**Figure 5.18**). A faint band was

observed for the sample transfected with pIB-Dcr-2-V5/C; however, this appears at approximately 90 kDa, much lower than the predicted size of 195 kDa. This strongly suggests that the OpIE2 promoter is not adequate to produce the tagged Dcr-2.

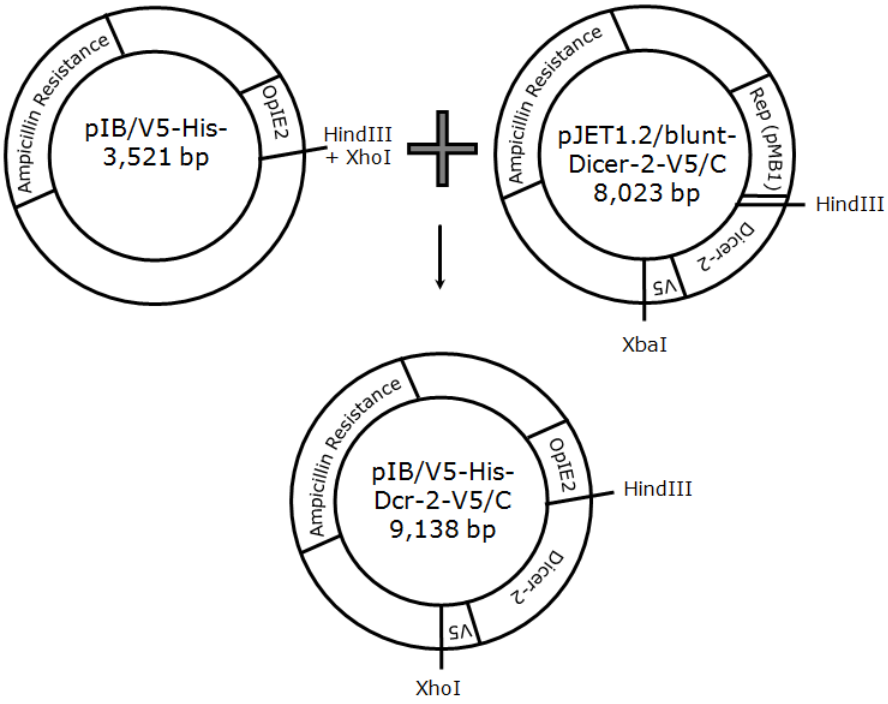
As a result the V5 tagged proteins were further cloned into the modified SFV1 plasmid received from Prof. Andres Merits (Institute of Technology, University of Tartu, Estonia), described in **Chapter 5.3.4**. The Ago-2-ZsGreen was modified using the same protocol as Dcr-2-mCherry to generate Ago-2-V5/N and Ago-2-V5/C. Insertion of both Ago-2-V5 and Dcr-2-V5 proteins was achieved by digesting the modified pSFV1 with *Nru*I and *Spe*I. Due to the presence of one or both of these restriction endonuclease sites within the Ago-2-V5 and Dcr-2-V5 sequences, they were digested from the pJet1.2/blunt vector, into which they had been previously subcloned, with either *Nru*I and *Xba*I or *Hind*III and *Xba*I respectively (**Figure 5.19** and **5.20**). Ligation of Ago-2-V5 could be carried out directly as the *Xba*I site is compatible to the *Spe*I site. Dcr-2-V5 was first required to be treated with Klenow which has 3'→5' exonuclease activity and removes the overhanging nucleotides from the 3' end and generates a blunt end which is able to pair with the blunt end produced by digestion with *Nru*I. Unfortunately, successful cloning of SFV1- Dcr-2-V5/N or SFV1- Dcr-2-V5/C was not completed within the timeframe of the project. Conversely, both SFV1-Ago-2-V5/N and SFV1-Ago-2-V5/C were produced and their sequences confirmed by restriction digestion and sequencing before being carried forward for Western blot analysis. The size of Ago-2-V5 was predicted to be 119 kDa.

To establish if the Ago-2-V5 constructs could be detected with anti-V5 antibody, BHK-21 cells were seeded at a density of 8 x 10^4 cells/well in a 24-well plate and transfected with the *in vitro* transcribed, capped RNA of either SFV1-Ago-2-V5/N, SFV1-Ago-2-V5/C, SFV1-Ago-2-ZsGreen (negative control) or were mock transfected. The cells were incubated at 37 °C for 24 h before they were lysed by the addition of 100 µl/well 2 x Laemmli sample buffer, denatured and run on a pre-cast Bis-Tris gradient gel. Wet transfer was again implemented for transport of the protein molecules to the membrane. As before, MDCK control samples were used to confirm successful antibody detection of the V5 epitope. As predicted the MDCK V5+ sample gave a very strong band at the approximate size of the V5 protein which was not present in the V5- control sample (**Figure 5.21**). Samples transfected with SFV1-Ago-2-V5/C showed a very strong signal which was slightly higher than the expected size of 119 kDa. However, several other bands were also present and these are most likely to be degradation products. In contrast, the sample which received SFV1-Ago-2-V5/N produced one specific band which, although fainter than that

seen in the SFV1-Ago-2-V5/C sample, was unique to that sample. Samples which had received SFV1-Ago-2-ZsGreen or were mock transfected showed no strong bands although a faint, non-specific signal can be perceived at approximately 30 kDa in the SFV1-Ago-2-ZsGreen sample. These results indicate that expression of the tagged Ago-2 is achieved when placed behind the subgenomic promoter of SFV.

Successful completion of both SFV1-Dcr-2-V5 and SFV1-Ago-2-V5 cloning may permit correct folding and functional expression to be achieved; however, the issue of expressing an antiviral protein within a viral backbone still exists which is required to be addressed.

A



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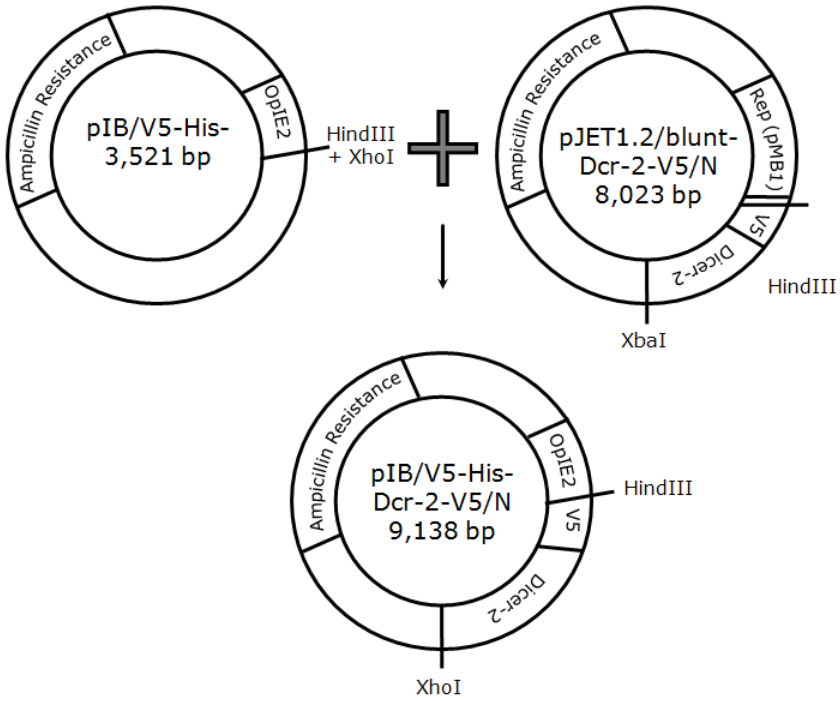

Figure 5.16: Schematic illustration of Dcr-2-V5 cloning into pIB/V5-His. The insertion of **A**) Dcr-2-V5/C or **B**) Dcr-2-V5/N from the sub-cloning vector pJet1.2/blunt into pIB/V5-His after the OPIE2 gene promoter sequence.

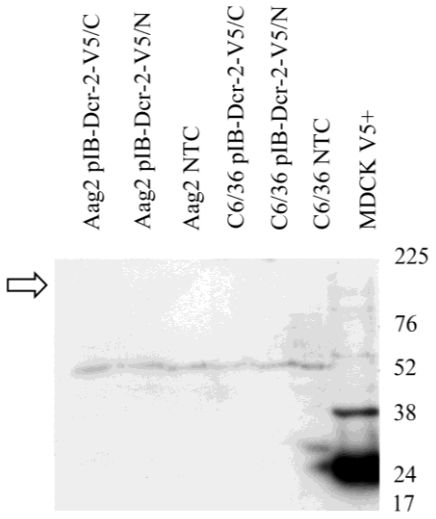

Figure 5.17: Expression of V5 tagged *Ae. aegypti* Dcr-2 protein from pIB/V5-His plasmid in C6/36 and Aag2 cells. *Ae. aegypti*-derived Aag2 cells and *Ae. albopictus*-derived C6/36 cells were transfected with either plasmid encoding Dcr-2 with the V5 tag on the C- (V5/C) or the N-terminus (V5/N). Mock transfected cells (NTC) were used as a negative control while MDCK cell samples constituently expressing the V5 protein were used as a positive control. Protein expression was detected with an anti-V5 antibody by Western blot analysis and chemiluminescence. Arrow indicates the expected protein weight.

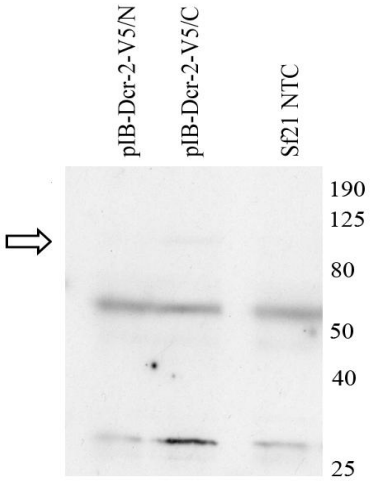

Figure 5.18: Expression of V5 tagged *Ae. aegypti* Dcr-2 protein from pIB/V5-His plasmid in Sf21 *Spodoptera frugiperda* cells. Sf21 cells were transfected with either the pIB/V5-His plasmid expressing Dcr-2 with the V5 tag on the C- (V5/C) or the N- terminus (V5/N). Mock transfected cells (NTC) were used as a negative control while MDCK cell samples constituently expressing the V5 protein were used as a positive control. Protein expression was detected with an anti-V5 antibody by Western blot analysis and chemiluminescence. Arrow indicates a potential protein band.

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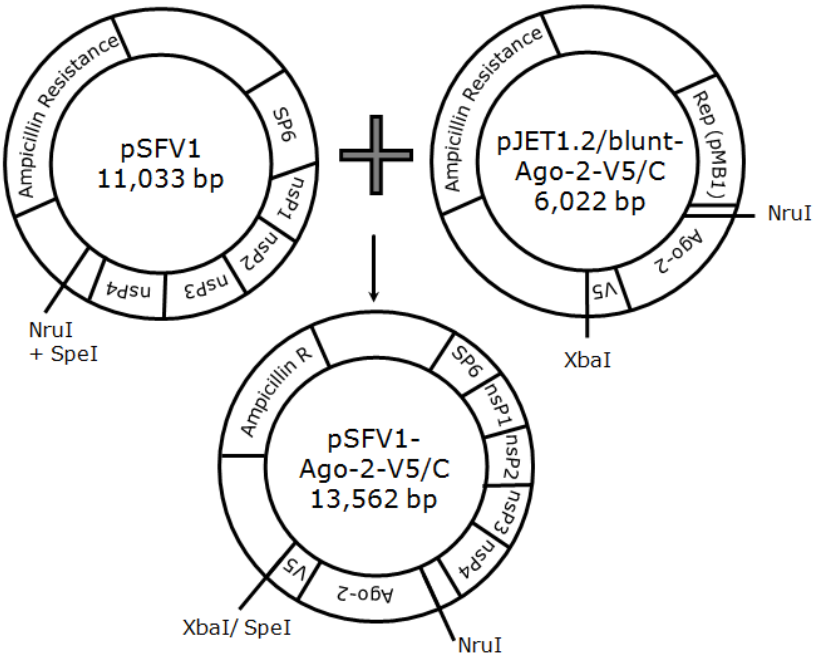

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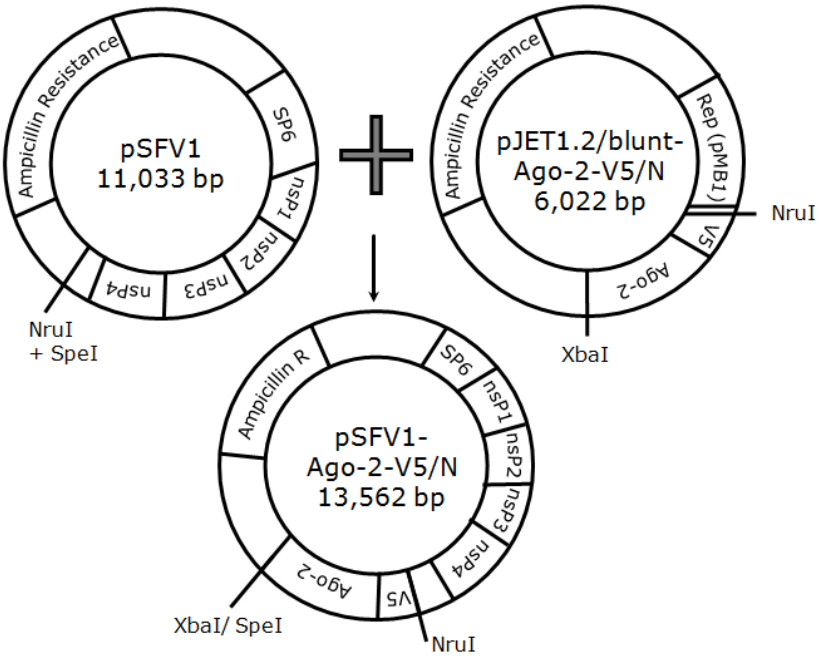
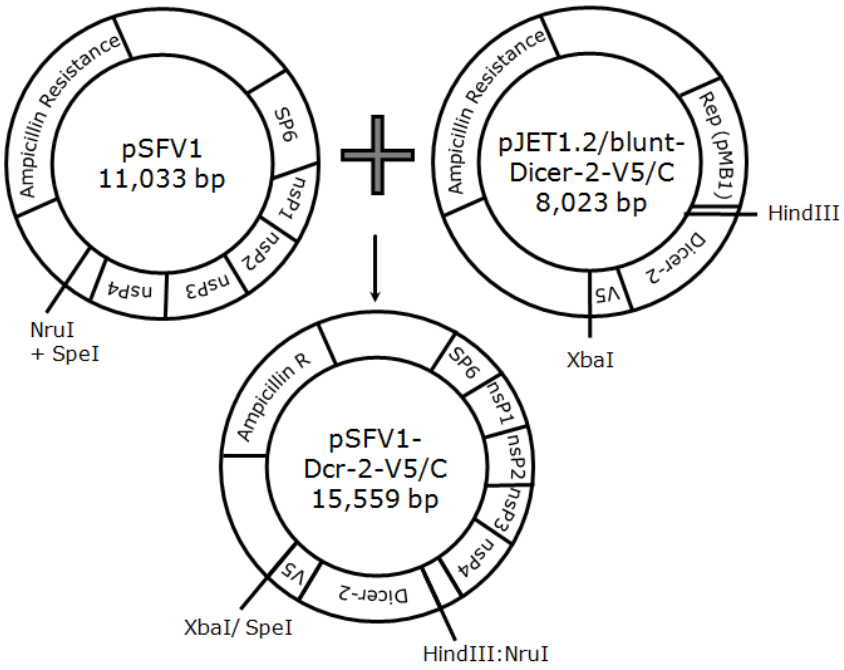


Figure 5.19: Schematic illustration of the cloning of Ago-2-V5 into pSFV1. The insertion of **A**) Ago-2-V5/C or **B**) Ago-2-V5/N from the sub-cloning vector pJet1.2/blunt into SFV1 replicase plasmid after the SP6 gene promoter sequence.

A



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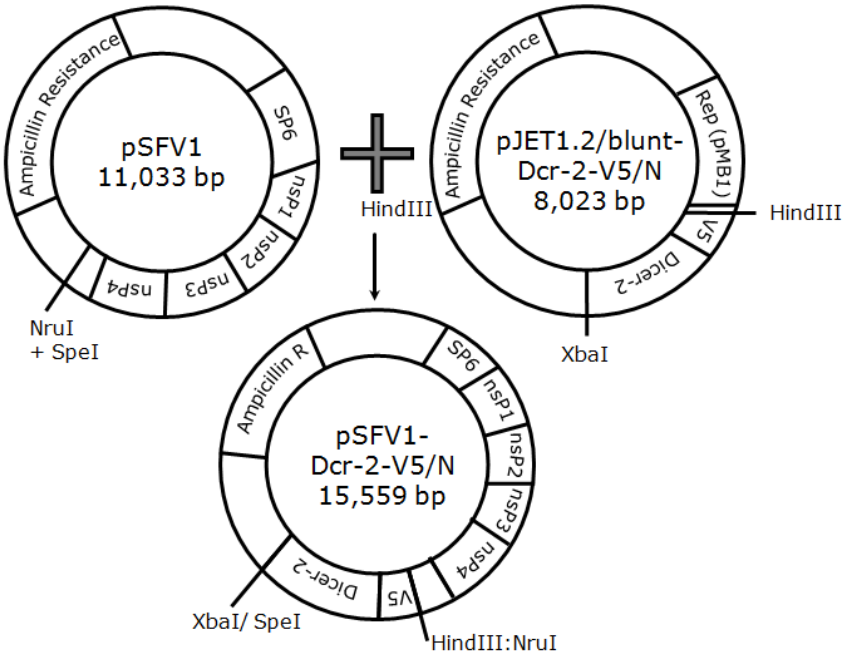

Figure 5.20: Schematic illustration of the cloning of Dcr-2-V5 into pSFV1. The insertion of **A**) Dcr-2-V5/C or **B**) Dcr-2-V5/N from the sub-vector pJet1.2/blunt into SFV1 replicase plasmid after the SP6 gene promoter sequence.

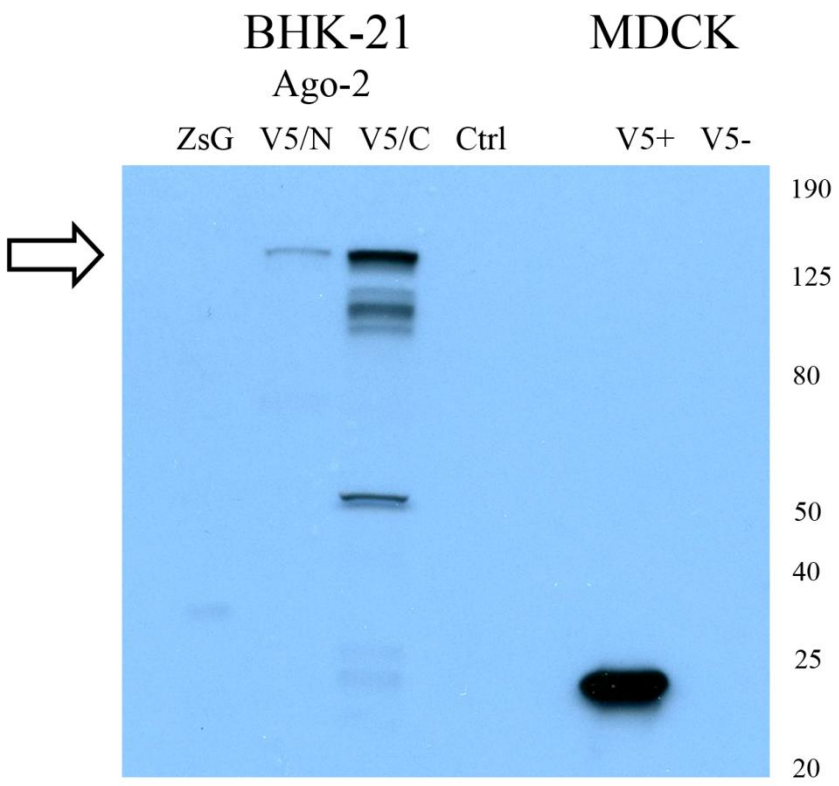

Figure 5.21: Expression of V5 tagged *Ae. aegypti* Ago-2 protein in BHK-21 cells. BHK-21 cells were transfected with SFV1-Ago-2 *in vitro* transcribed capped RNA. Ago-2 was labelled with either a V5 tag on the C (V5/C) or N (V5/N) terminus or a ZsGreen tag (ZsG) as a negative control in addition to mock infected BHK-21 cells (Ctrl). MDCK cell samples either constituently expressing (V5+) or negative for (V5-) the V5 epitope were also included as further controls. Protein expression was detected with an anti-V5 antibody by Western blot analysis and chemiluminescence. An arrow indicates the weight of potential protein bands.

5.3.8 Characterisation and Development of a Highly Active *in vitro* Insect Expression System

One particular challenge encountered when working with mosquito cells in culture is transgene expression from plasmid-based systems. Currently the most efficient and commonly used plasmid promoter is the OpIE2 promoter (described **Chapter 5.3.4** and **5.3.7**). However, a recent publication from Adelman and colleagues described the properties of the *Ae. aegypti* polyubiquitin (PUb) gene promoter for the successful expression of gene products in insect *in vitro* systems (Anderson et al., 2010). This promoter has been studied in *Drosophila* (Davis et al., 1995; Handler and Harrell, 1999) and has recently been shown to generate potent gene expression in both C6/36 and Aag2 mosquito cells. The pGL3-PUb plasmid described in this paper was gratefully received from Professor Zach Adelman (Department of Entomology, College of Agriculture and Life Sciences, Virginia Tech, Virginia, United States of America). To extend the experiments to additional mosquito-derived cell lines, a comparison between the PUb promoter (accession number GU179018) driving expressing from pGL3-PUb and the OpIE2 promoter in the pIZ-*Fluc* plasmid was performed. Both plasmids contain the *Fluc* GL3 gene downstream from their promoter which allows relative luciferase activity to be determined by luciferase assay.

Each cell line to be tested was seeded in 24-well plates at the appropriate cell density required per well (1.8 x10^5 Aag2, 1.2 x10^5 C6/36 and U4.4 and 2 x10^5 TRA-171) and were incubated at 28 °C preceding transfection 24 h later with either: 500 ng/well of pGL3-PUb or pIZ-*Fluc*. Each well was co-transfected with 25 ng/well pRL-CMV (also containing the OpIE2 promoter sequence) as an internal control to account for discrepancies in transfection efficiency. Following a 24 h incubation period each well was lysed and luciferase expression quantified. The level of pIZ-*Fluc* expression was set to 1 and the level of pGL3-PUb expression measured as fold increase relative to pIZ-*Fluc* expression for each cell line (**Figure 5.22**). The results demonstrated that for all of the cell lines tested the level of *Fluc* expression was consistently greater from the pGL3-PUb plasmid than from the currently preferred pIZ-*Fluc* expression plasmid (Aag2 138- fold, C6/36 206-fold, U4.4 45- fold, TRA-171 104- fold increases). This suggests that the PUb promoter is more successful in insect cell lines than the OpIE2 promoter, which is in agreement with what was shown previously in C6/36 and Aag2 cell lines where pGL3-PUb was consistently more actively expressed (Adelman et al., 2010).

As a result of this success it was decided to adapt the plasmid for use in future experiments to permit required genes of interest to be expressed to high levels. In order for this to be implemented, it was first necessary to insert a multiple cloning site (MCS) into the pGL3-PUb plasmid. This was achieved through the design of oligonucleotides which recognised regions of the plasmid backbone but contained restriction endonuclease recognition sites unique to the completed plasmid. These primers are described in **Chapter 2.9**. Due to the strategy which was used to create the pGL3-PUb plasmid, the insertion of a new MCS required the *Fluc* luciferase gene to be removed (**Figure 5.23A**). Following the PCR the DNA was treated with *Dpn*I which only cleaves its recognition site when it is methylated and thus permits the removal of methylated DNA sequences (i.e. the original plasmid grown in *E.coli*) while the plasmid generated by PCR remained intact. The DNA was then treated with T4 ligase which ligates the phosphorylated compatible sites at the end of each primer. The resulting DNA, now referred to as pGL3-PUb/MSC, was transformed into DH5α *E.coli*, purified and the correct sequence confirmed by digestion and sequencing (**Figure 5.23B**). All restriction enzymes cut efficiently in the modified plasmid and not in the original pGL3-PUb. It is hoped that this can be used as an alternative expression strategy for the recombinant Dcr-2 and Ago-2 sequences; however, completion of this was not possible within the timescale of the project.

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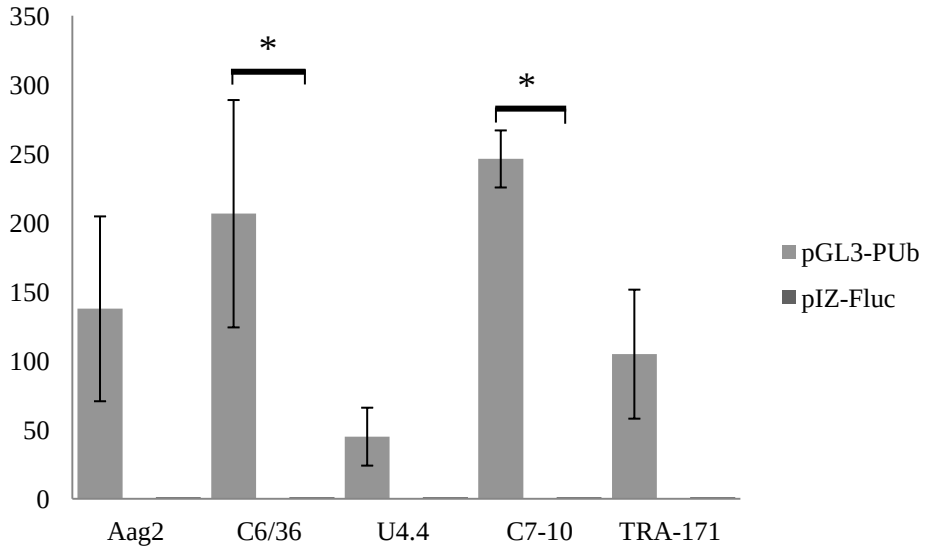

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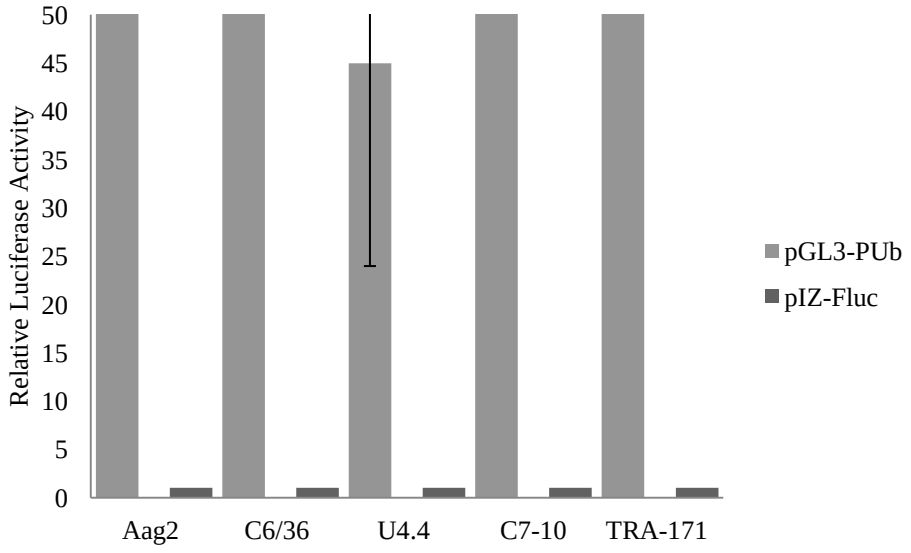

Figure 5.22: Expression of pGL3-PUb and pIZ-*Fluc* plasmids in a number of different mosquito-derived cell lines. The efficiency between two mosquito expression systems was compared in *Ae. aegypti*-derived Aag2 cells, *Ae. albopictus*-derived C6/36, U4.4 and C7-10 cell lines and *Toxorhynchites amboinensis*-derived TRA-171 cells. Cells were transfected with either the pGL3-PUb or pIZ-*Fluc* plasmids and co- transfected with pRL-CMV as an internal control. Following a 24 h incubation, the ratio of *Fluc*:*Rluc* expression was determined by luciferase assay. **A**) Expression of pIZ-*Fluc* was set to 1 and pGL3-PUb measured against it. Panel **B**) shows the pIZ-*Fluc* results in detail. pGL3-PUb expression is shown in (light grey) compared to pIZ-*Fluc* expression which is indicated in (dark grey). Error bars show the standard deviation of three independent experiments performed in triplicate. * represents $p < 0.05$, Student's t-test.

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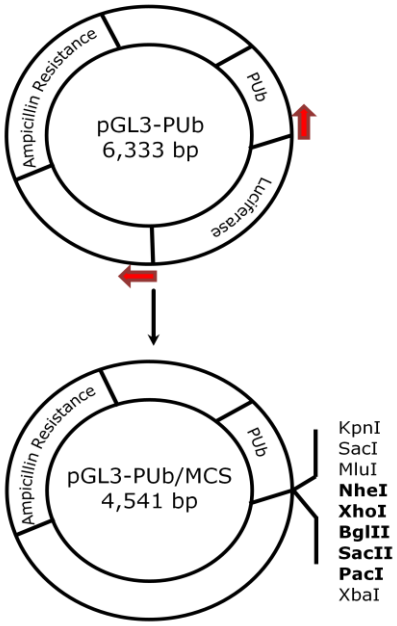

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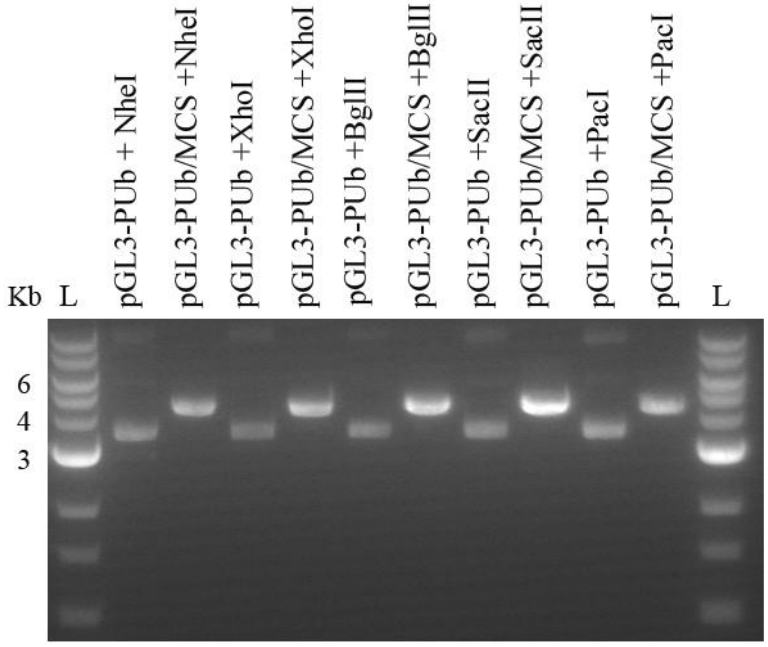

Figure 5.23: Design and creation of pGL3-PUb encoding a MCS. A) Schematic illustration of the removal of the luciferase gene from pGL3-PUb and the insertion of the new MCS. The red arrows indicate the sites where the PCR primers bound and their direction. **B**) Agarose gel picture demonstrating digestion with each restriction endonuclease included in the new MCS of pGL3-PUb/MCS. L, 1 Kb ladder; Kb, kilobase pairs.

5.4 Discussion

As RNAi is the major antiviral defence mechanism in mosquitoes it is important to grasp the intricate details involved in its ability to process a viral assault. Although it has been well studied, there are still a large number of unanswered questions regarding the action of Dcr-2 and Ago-2 and their faculty for dsRNA and siRNA-mediated silencing. For instance what exactly does Dcr-2 bind too and how does it recognise viral replication complexes? Although arboviral replication is known to occur in the cytoplasm, the exact location of where the virus dsRNA meets the antiviral components has yet to be determined. It is known that Dcr-2 does not function independently and is not biologically distinct from co-factors; although, with the exception of R2D2 (Liu et al., 2003) and Ars-2 (Sabin et al., 2009), the interactions between it and other proteins which may regulate its activity in both *Drosophila* and mosquitoes are largely unknown. Moreover, as the infection progresses from acute to persistence, do these interactions remain or are the replaced with associations with different protein(s)/molecule(s) (Flynt et al., 2009; Goic et al., 2013)? Is this also the case with protein(s)/molecule(s) interacting with RISC? Do viral proteins interact directly with Dcr-2/Ago-2 or both? The biological relevance of many of the interactions made by Dcr-2 and RISC/Ago-2 are yet to be fully investigated and this project intended to expand the limited number of biological tools available to answer many of these questions within the mosquito system.

The most ideal situation would be to create antibodies against the endogenous mosquito RNAi proteins. It is known that the development of antibodies against these proteins in mosquitoes has proved to be particularly challenging (personal communication with Dr. Ronald van Rij, Nijmegen University). Although there are some against RNAi components in *Drosophila* and humans, these are not effective when used for cross detection of the same proteins in mosquitoes and their derived cell lines (personal communication with Dr. Esther Schnettler, University of Glasgow). These findings are echoed in the production of the antibodies described in this project, as although an experienced company was tasked with their production, they also reported some difficulties. This is particularly evident in the lack of antibodies against the preferred epitopes of Ago-2. Furthermore, the dot blot which was hoped to be utilised for screening the large number of antibodies was unsuccessful, implying failings with some of the peptides themselves. However, the Western blot experiments described were all performed with the aid of a reducing agent and therefore any antibodies recognising the native conformation of the epitope rather than

the linear structure would be missed. With more time these Western blot experiments should be repeated without the presence of a reducing agent to determine if this improves the number of successful endogenous antibody interactions. However, it should be noted that the expected running pattern of the proteins on the gel is still unknown. Further work with the potential candidate antibodies identified is also required to optimise their use. On completion of this, successful antibodies could be used to facilitate immunoprecipitation and immunofluorescence assays and allow other associated proteins and molecules to be identified, as has previously been shown for other systems (Meister et al., 2005; Peters and Meister, 2007). Understanding the relationship that these proteins have with other enzymes and molecules is essential to give researchers the complete picture of their roles within the cell. In particular, it would be beneficial to understand the distinguishing characteristics between siRNAs which are taken up by RISC and incorporated into Ago-2, compared to those which are not and as a result are unable to target the virus during infection (Aliyari et al., 2008; Myles et al., 2008 and 2009, Brackney et al., 2009; Flynt et al., 2009; Sanchez-vargas et al., 2009; Mueller et al., 2010; Siu et al., 2011). Are all the siRNAs produced functional? Of those viRNAs which are free and not bound to Ago-2, how many are stable? Are they stabilised elsewhere? In addition, it is also not yet known exactly what determines Dcr-2 binding to dsRNA. Does it bind to the whole genome or recognise particular parts? Further studies are required to establish if, like plant RNA viruses, genetic conformation plays a role in inducer recognition and interactions (Siu et al., 2011). These areas of interest can then be extended beyond the cell to further our understanding of the interactions between the cell and the virus and how these associated proteins shape virus/RNAi connections.

Due to the disappointing results obtained for the endogenous antibody trials the alternative method was to over-express recombinant forms of Dcr-2 and Ago-2 fused to specific tags. The initial preference was to use a fluorescent tag which would allow the temporal and spatial kinetics of these proteins to be visualised during the stages of SFV infection of mosquito cells. A significant challenge to completing this aim was to obtain sufficient levels of protein expression. No expression was obtained after cloning into the insect pIB/V5-His vector; although, efficient expression of both Ago-2 and Dcr-2 was achieved following their insertion into SFV VRPs. However, there were issues with the titre of VRPs which could be produced. To date, there is no evidence that large proteins, such as Dcr-2 and Ago-2 are able to be expressed within SFV particles. Indeed, the structural ORF of SFV is approximately 1253 amino acids long compared to the size of Dcr-2-mCherry

(1894 amino acids) and Ago-2-ZsGreen (1223 amino acids). The findings demonstrated here suggest that the presence of large proteins within the virus genome may make it unstable and most likely results in the incorrect folding and the failed formation of the infectious body. Therefore, there is a high probability that the proteins are ejected. This would explain why there is a high degree of SFV-nsP3 detection but very little expression of each tagged protein, in particular Dcr-2 which is the larger of the two. Additionally, such large proteins may have difficulty folding correctly on their own if the environmental conditions are not optimal. It is also likely that the expression of both Dcr-2 and Ago-2 is highly regulated by the cell preventing naturally high levels of protein accumulation which would contribute to the difficulties encountered. However, although the VRPs are difficult to obtain the RNA is sound and further experiments are merited.

Although there is currently no convenient assay available to test the function of Ago-2, the C6/36 cell line provides an invaluable system in which to test the function of the reporter Dcr-2-mCherry construct. This is because it exhibits a Dcr-2/RNAi null phenotype within the context of RNAi and is unable to produce 21 nt siRNA molecules as a result of the lack of dicing activity (Brackney et al., 2010; Scott et al., 2010; Morazzani et al., 2012). The Dcr-2-mCherry construct would be shown to be functional if it was capable of restoring the ability of the C6/36 cell line to digest long dsRNA molecules into 21 nt siRNAs, the hallmark of Dcr-2 activity. Once shown to be functional, it could then be used in a relevant *Ae. aegypti* expression system. This has been previously achieved through the addition of a recombinant human Dicer protein to C6/36 cell lysates (Scott et al., 2010). The successful function of this exogenously applied enzyme indicated that there was no inhibitory affects upon its action. Furthermore, the direct transfection of siRNAs permitted targeted plasmid silencing signifying that the other constituent components of the RNAi pathway are both present and functional (Scott et al., 2010). Unfortunately, despite clear levels of protein expression within the C6/36 cell line, Dcr-2 function could not be confirmed by performing either an *in vitro* dicer cleavage assay or by plasmid silencing in the C6/36 cell line (data not shown). This may be due to insufficient levels of protein expression, subcellular compartmentalisation away from SFV replication complexes or the resulting protein may be incorrectly folded or unstable and unable to recognise or process the dsRNA. The band observed for the C3/36 cell samples in the dicer assay could be larger siRNAs produced by Dcr-1, although this is difficult to confirm due to the poor band produced from the U4.4 sample. It was therefore evident that further work was required to optimise its production.

Of course it is disadvantageous to test the function of antiviral proteins within the confines of a viral infection. Firstly, the cells are not in their natural state but are already fighting an infection. Secondly, high levels of viral replication would be expected to vastly over express the protein above its normal cellular concentrations. However, expression of Dcr-2 from a replicating virus could immediately initiate digestion of the viral RNA and decrease the number of genomes produced. With this in mind, the SP6 promoter was used under a different context in the TNT rabbit reticulocyte lysate system. Unfortunately, this proved unsuccessful and it failed to generate expression of Dcr-2 despite the confirmation that the cloning and protocol were correct. In addition to the restrictions encountered when testing Dcr-2 activity described previously, there may also be inhibitors present in the lysate preventing Dcr-2 activity. A suitable alternative would be to use the native insect cell system. Although, this is a logical choice as it is a natural system and is known to produce highly-active full-length proteins, it requires the use of a baculovirus expression promoter which has been shown to be unsuccessful at producing detectable levels of Dcr-2-mCherry.

A second avenue of interest was explored using an alternative tag inserted onto the end of the RNAi enzymes. Although the fluorophore tags are beneficial for localisation studies, they are known not to be successful with other procedures which would be beneficial to this project and for that reason a second molecular tag was employed. The tag selected was the V5 epitope tag which has been commonly used as a target for protein detection due to its compact size and recognition by reliable, high-affinity antibodies (Southern et al., 1991; Olczak et al., 2005; Mourez et al., 2007; Grimberg et al., 2011). It has been shown to produce robust results when used for immunoprecipitation and protein-protein interaction experiments; as well as Western blotting, immunohistochemistry and protein microarrays which may be useful for future studies. It was decided to place the tag on either the N-terminus or the C- terminus of Dcr-2 and Ago-2 to determine which orientation was more successful to facilitating correct protein folding. It was unfortunate that the Dcr-2-V5 cloning could not be completed during the designated time; however, similar cloning has been successful in other systems. Cloning a FLAG tag onto the N- terminus of the functional Dcr-2 derived from *Bombyx mori* (silkmoth) has recently been achieved (Liu et al., 2012) although the addition of dual polyhistidine tags (one of each terminus) onto *Drosophila* Dcr-2 has been documented as improving expression levels using the baculovirus expression system without affecting functionality (Jiang et al., 2005; Ye and Liu, 2008). It is hoped that if this cloning is successful in the future, the smaller V5 tag may solve some of the potential folding issues encountered with the use of the mCherry

tag. As neither the structure nor folding of *Ae. aegypti* Dcr-2 and Ago-2 are known it was determined that the V5 tag would be placed on either the N- or the C- terminal ends. It was hoped this would provide information relating to the geography of the terminus which would affect ease of folding and accessibility to antibody detection. There were promising initial results for both Ago-2-V5 constructs which indicated strong bands at approximately the weight expected (119 kDa). Given the apparent stability of Ago-2-V5/N compared to Ago-2-V5/C, it is suggested that this would be the more convenient and reliable cloning and expression strategy to use in the future.

Although baculovirus gene promoters has been shown to be functional in a number of insect cell lines (Pfeifer et al., 1997; Massotte et al., 2003; Xu et al., 2008; Paradkar et al., 2014) and are commonly used by groups studying insect biology (Li et al., 2001; Yang et al., 2008; Mon et al., 2009), this project has found that there was a substantial increase in the level of gene expression obtained following gene regulation from the *Ae. aegypti* polyubiquitin promoter over the currently used OpIE2 baculovirus promoter in each of the cell lines tested. This is consistent with results generated previously where there was at least a ten-fold increase in both C6/36 and Aag2 cell lines compared to a second baculovirus IE promoter (Anderson et al., 2010). The PUb promoter has also been shown to maintain enhanced expression levels in transgenic mosquitoes. As a result of these findings it can be suggested that the PUb gene promoter is highly efficient and produces stable expression in both *in vitro* and *in vivo* mosquito systems. Therefore, it will be of exceptional use in the future for gene regulation and function studies in this important disease vector over traditionally used baculovirus promoters (Khoo et al., 2013). It may also be beneficial to combine its use with the insect cell expression system to determine if it is also possible to achieve high levels of functional proteins with the PUb gene promoter in this cell-free system.

A notable lack of genetic and biological tools has been a great impediment to studies into mosquito immunity and it has been a major aim of this PhD project to improve this. The use of the potential endogenous antibodies would negate the need to treat the cells and would allow investigations into the natural intracellular environment prior to infection, without the disadvantages associated with protein over-expression, to be carried out. However, the use of the developed recombinant proteins will greatly facilitate investigations into answering some long standing queries. The successful use of V5 tags on Ago-2, as well as the powerful expression of the reporter genes via the PUb promoter, indicate that there are realistic possibilities available for taking this work forward.

Fundamental questions such as how rapidly post infection the RNAi defence is activated and at what stage during the replication cycle this occurs are currently not known and so it is important to determine the stages of acute infection in which Dcr-2 and RISC play their most predominant role. These crucial factors may also be connected to the rate of viral replication and so may vary depending on the specific pathogenic infection (Grimm et al., 2007). The ability to detect, isolate and purify the main RNAi enzymes will strongly aid the study into their spatial and temporal activities and will considerably reduce the gaps in our awareness of these important processes. Further insights into the mechanism of RNAi will ultimately support public health policies for the development of novel strategies for the control of arthropod transmitted diseases through understanding the mechanisms of transmission regulation.

5.5 Future Work

Although the C6/36 cell line has a Dcr-2 null phenotype they are not a Dcr-2 null cell line as parts of the Dcr-2 protein are still translated. However, we do not know if the other parts of the enzyme which are expressed are important for other responses in addition to the siRNA pathway and this requires further investigation. Although the C6/36 were used to establish if the Dcr-2-mCherry reporter was functional, this could not be concluded. Further to the continued optimisation suggested previously for expression of Dcr-2, an additional *in vitro* dicer cleavage assay would be required. If this was successful the isolation of small RNAs derived from C6/36 cells treated with Dcr-2-mCherry should be collected and analysed by deep sequencing to determine the production of 21 nt siRNAs achieved following this treatment. The identification of an Ago-2 loss-of-function cell line would allow validation of the function of the Ago-2 construct. In addition, due to the low levels of Dcr-2 and Ago-2 expression following SFV infection of mosquito cell lines it would have been beneficial to separate those cells by fluorescence-activated cell sorting (FACS) to generate a homogenous population. However, as the cells were infected with SFV this was not possible. Following the success of the suggested cloning strategies out with the virus background this may be a viable technique available in the future.

Following the success of gene expression under the PUb promoter it would be advantageous to clone the recombinant Dcr-2 and Ago-2 sequences into the pGL3-PUb/MCS. This may allow sufficient expression of both proteins outwith the virus expression system. Alternatively, the insect expression cell system should be tested as a further substitute system. This study should be performed with both the PUb and baculovirus gene promoters.

The construction of Ago-2 with a V5 tag should be completed to confirm that it maintains the correct conformation and activity. The V5 tag will allow RISC immunoprecipiations (RISC-IP) to be performed as well as other protein:protein interactions to be identified and analysed by mass spectrometry which should be accomplished in due course. The completion of the V5 tag cloning onto Dcr-2 would also facilitate further studies of the enzymes kinetics and discovery of associated proteins and molecules involved in the instigation of the RNAi pathway. Similarly, following further optimisation of the antibodies created against the endogenous RNAi proteins these could be used for future applications including Western blotting, cellular localisation and interaction studies as well

as deep sequencing of small RNAs following RISC-IP. A combination approach with both the PUb promoter plasmid and V5 tag would allow us to advance experiments involving immunoprecipitation and immunofluorescence beyond what can be achieved at present.

Mosquito cells expressing the recombinant Dcr-2 and Ago-2 proteins can be infected with a strain of SFV expressing an nsP3-fusion (such as nsP3-ZsGreen) which will highlight replication complexes. Cells can be examined through the early stages of infection to pinpoint subcellular co-localisation. Furthermore, combining this experimental set up with immunostaining with the anti-dsRNA J2 antibody will allow the relationship between the three major elements of the RNAi response- Dcr-2, viral dsRNA and viral replication complexes- to be monitored, especially now that successful protein tagging is within reach.

A further objective which could be achieved on completion of the recombinant RNAi protein expression would be to analyse the important protein domains involved in their activity. To do this, a deletion analysis should be carried out to mutate/delete each known region. Following this, a more accurate analysis can be performed through sequence specific mutations to determine the exact sequences required for their function. In this way the activity and function of the different domains of these proteins could be elucidated as well as determining any additional involvement in other pathways.

5.6 Principle Findings

1. *Ae. aegypti*-derived Dcr-2 and Ago-2 were successful cloned into SFV replicons and VRPs produced. Expression was detected in *Ae. aegypti*-derived Aag2 cells, *Ae. albopictus*-derived C6/36 and mammalian BHK-21 cells.

2. An *in vitro* dicer cleavage assay protocol was successfully implemented. However, it did not allow the efficient function of the recombinant Dcr-2 to be concluded.

3. Recombinant tagged Dcr-2 and Ago-2 failed to be expressed under the control of the OpEI2 promoter suggesting it is too weak.

4. Ago-2 tagged with the V5 epitope was successfully cloned into the SFV replicon and Ago-2 was detected at the expected size by Western blotting with an anti-V5 antibody.

5. Expression of genes inserted downstream of the PUb promoter was shown to be greater in all cell lines tested compared to currently used OpEI2 gene promoter.

Chapter 6: Antiviral Capabilities of *Toxorhychites amboinensis*- derived Cells

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6.1 Introduction

The genus *Toxorhynchites* (Diptera, Culicidae) contains 70 species of morphologically similar members. Although predominantly tropical, they are distributed from the far north of Russia and Canada to the south of Africa (Steffan, 1968; reviewed Focks, 2007). The adults are exceptionally large comparative to other genera (several centimetres long) and are brightly coloured with a long, downward curving proboscis. As they are non-biting, non-haematophagous insects and do not take a blood meal, they are not natural vectors of arboviruses. All instars of *Toxorhynchites* larvae are predatory against the larvae of other mosquito species, in addition to other available aquatic and surface trapped invertebrates. This early accumulation of nutrients sustains the adult females through the remainder of their life cycle and negates the need to take a blood meal to provide the nourishment required for egg development. Instead both sexes consume nectar and other plant derived sugars (Focks, 2007). The larvae feed opportunistically and rely on the eggs being oviposited in an area with an adequate food source. However, it has been shown in laboratory conditions that they will feed on whatever is available, both living and non-living food sources, provided it is of similar size or smaller. Furthermore, cannibalism has been documented as a common behaviour (reviewed Steffan and Evenhuis, 1981; Annis et al., 1990a and b; reviewed Focks, 2007). Female *Toxorhynchites* will lay their eggs in both natural and artificial water containers with no apparent bias between an urban or rural distribution. As such, there is a substantial degree of overlap between *Toxorhynchites* and its predominant prey of the larvae of major arboviral vectors, such as *Aedes* spp. mosquitoes.

Research involving *Toxorhynchites* mosquitoes has concentrated on their potential as an alternative to chemical insecticides for biological pest control of vector mosquitoes (Board of Sciences and Technology for International Development, 1973; reviewed Steffan and Evenhuis, 1981). For instance *Tx. amboinensis* (miss identified as *Tx. splendens*) and *Tx. brevipalpis* were introduced to the Hawaiian Islands from the Philippines in 1953 to control *Ae. albopictus* (Steffan, 1975; Nakagawa, 1963) and *Tx. amboinensis* were released onto Java in 1987 for the joint control of *Ae. aegypti* and *Ae. albopictus* (Annis et al., 1990a). Although it is logical to use vector mosquitoes for arbovirus research, few studies have investigated the advantages of *Toxorhynchites* in this area and the number of major benefits which make these a convenient and adept system for *in vivo* studies. Firstly, as these mosquitoes do not take a blood meal, there is a reduced health risk associated with

the use of females in this field of research since they cannot transmit the virus to their handlers. Secondly, their exceptional size and stability compared to *Aedes* and *Culex* species, which are predominantly used for current *in vivo* studies, would improve the ease of handling, injection accuracy, allow a greater volume of inoculum to be used and provide a greater quantity of tissue samples for detection (e.g. by immunofluorescence and head squashes). Thirdly, numerous studies have shown that they efficiently propagate all four dengue virus serotypes (DENV- 1 - 4), Japanese encephalitis virus (JEV) and St. Louis encephalitis virus (SLEV) to high titres when artificially infected by intrathoracic injection (Rosen et al., 1978; Tesh, 1979; Kuno, 1981; Rosen, 1981). DENV titres in particular are as high as 100 - 1,000 times greater than observed in *Ae. albopictus*–derived C6/36 and *Ae. pseudoscutellaris*–derived AP-61 cell lines in culture typically used for DENV propagation (Tesh, 1979). While the majority of arbovirus studies performed have focused on their ability to propagate dengue and other flaviviruses, *Tx. amboinensis* have also been identified as an artificial host for other arboviruses, including alphaviruses (Ross River (RRV, Tesh et al., 1981), chikungunya (CHIKV) and Venezuelan encephalitis virus (VEV, Scherer and Chin, 1981)), bunyaviruses (La Crosse, San Angelo and Keystone viruses (Rosen, 1981)), as well demonstrating a susceptibility to Rhabdoviruses (vesicular stomatitis virus (VSV, Rosen, 1980a) and the true insect virus Nodamura virus (NoV, Tesh, 1980a). Zeller and Mitchell (1989) also showed the replication of several Reoviruses, Rhabdoviruses and other Bunyaviruses isolated from mosquitoes, birds and mammals. Therefore, the use of *Tx. amboinensis* for arbovirus research may uncover as yet unknown factors related to the interplay between cellular control and the replication of a number of arboviruses from different families.

Continuous insect cells lines have been a vital research tool to investigate different features of insect immunity. They provide a controlled system which is sensitive, reproducible and where subtle changes can be detected following the application of various stimuli. Often *in vitro* findings mimic those from *in vivo* studies in the whole insect organism, validating the cell culture results as true to nature (Riedel and Brown, 1979; Condreay and Brown, 1986 and 1988; Miller and Brown, 1992; Barletta et al., 2012). Following the success of *Tx. amboinensis* as a host for the propagation of dengue viruses, an *in vitro* system was established from them for the recovery and isolation of certain arboviruses from human sera, as well as for the study of virus replication in non-haematophagous mosquito cells in culture. Several cell lines have since been derived from *Tx. amboinensis* (Tesh, 1980a; Kuno, 1980, 1981b and 1982a; Munderloh et al., 1982). TRA-171 cells were created from

live, immotile *Tx. amboinensis* larvae (Kuno, 1980 and 1981b) and, unlike some of the other derived cell lines (TA-42 and TA-9) they exhibit similar levels of sensitivity to dengue observed in the adult insects (Tesh, 1980a, Kuno, 1982a). A subline from a further *Tx. amboinensis*-derived cell line, TRA-284, could be grown in serum free media (TRA-284-SF) and the isolation of DENV serotypes 1, 2 and 4 from human sera was found to be similar to the level detected in the *Ae. albopictus*-derived C6/36 cell line, while exceeding isolation of DENV-3 isolates (Kuno, 1982b). In addition, the adaptation of the cells to serum-free media did not alter their sensitivity to DENV compared to those where bovine sera was included. The aim of cells cultivated in serum-free media was to further reduce material costs and the complexity of detecting clinical viral infections in the field. At the same time it also minimised contamination by antibodies, other viruses, endotoxins and *Mycoplasma* that the bovine serum may contain and which could potentially interfere with arbovirus detection (Kuno, 1983; Nawa et al., 1987). TRA-171 cells have also been shown to allow the replication of all four dengue virus serotypes to high titres (Kuno, 1981a) comparable to those observed from the C6/36 (Igarashi, 1978) and AP-61 cell lines (Varma et al., 1974) which are known to be highly susceptible to DENV infection. Furthermore, DENV infection resulted in evident cytopathic effect (CPE) presenting as syncytial development. This is also described for *Aedes* cells in culture following DENV infection and is a characteristic unobserved in other non-haematophagous insect cell lines (Singh, 1967; Kuno, 1980 and 1981a; Legrand and Hotta, 1983). DENV infection also results in plaque formation in TRA-171 monolayers and the cells were shown to be as equally sensitive as the C6/36 and AP-61 cell lines. This made TRA-171 cells appeal as a candidate to replace previous methods involving vertebrate cells and live adult mosquito systems for the isolation and identification of dengue viruses from clinical human sera samples collected in the field (Tesh, 1979; Kuno, 1981a). Further to dengue, replication of other flaviviruses: Yellow Fever virus (YFV) and SLEV, the rhabdovirus VSV, as well as NoV, have been reported in this cell line reflecting similar results observed in the adult insects (Kuno, 1981a and 1987; Johnson et al., 2004). Legrand and Hotta (1983) also observed CHIKV replication at titres similar to those measured for DENV-2 and DENV-4 serotypes in TRA-171 cells and close to titres expected in infected C6/36 cells (Igarashi, 1978). This demonstrates that TRA-171 cells may be a useful *in vitro* system for studying the replication of different groups of arboviruses.

The natural maintenance of some of the major human pathogens known to replicate within *Toxorhynchites* and their derived cell lines has been demonstrated to involve vertical or

transovarial transmission of the virus to the progeny of infected female mosquitoes. This allows the virus to survive in unfavourable conditions (e.g. absence of susceptible vertebrates, adverse climate conditions for mosquito activity). Vertical transmission of flaviviruses is believed to occur when the fully developed egg passes through the oviduct (Rosen, 1980b, 1987a and b). This has a lower rate of infection compared to true transovarial transmission (direct infection of the developing egg) seen with bunyaviruses (Tesh, 1980c; Rosen, 1987a and b). In contrast to cases of vertical transmission of alphaviruses which are not widely known and can be inconsistent (Kay, 1982; Mourya, 1987; Lindsay et al., 1993; Dhileepan et al., 1996; Vazeille et al., 2009; Bellini et al., 2012), descriptions of this route of infection have been well documented for the majority of mosquito-borne flaviviruses. These include; dengue viruses in *Ae. aegypti* (Khin and Than, 1983; Chen et al., 1990; Joshi et al., 1996), *Ae. albopictus* (Rosen et al., 1983), *Ae. malayensis*, *Ae. polynesiensis*, *Ae. pseudoscutellaris* (Rosen et al., 1985); DENV 1, SLEV and JEV in *Ae. albopictus* (Rosen, 1988); JEV in *Culex tritaeniorhynchus* (Rosen et al., 1980b); YFV in *Ae. aegypti* (Aitken et al., 1979; Fontenille et al.,1997), SLEV in *Ae. atropalpus* (Pelz and Freier, 1990), *Ae. dorsalis*, *Cx. peus*, *Tx. amboinensis* (Hardy et al., 1984) and *Cx. pipens* (Francy et al., 1981), Kunjin virus in *Ae. albopictus* (Tesh, 1980) and West Nile virus (WNV) in *Ae. aegypti* and *Ae. albopictus* (Baqar et al., 1993) as well as *Ae. triseriatus* (Unlu et al., 2010).

In addition, a new branch of the *Flaviviridae* family has recently become the focus of intensive research. The first member of this new group was identified in 1975 by a 'chance observation'. It was termed cell fusion agent virus (CFAV) and was first detected in the medium of endogenously infected *Ae. aegypti* cells (Stollar and Thomas, 1975); although CFAV-related viruses have since been isolated from both male and female mature field-collected *Ae. aegypti*, *Ae. albopictus* and *Culex* species in Puerto Rico (Cook et al., 2006). This suggested that CFAV originated from the adult mosquito and was not a lab strain contamination of the isolated cell line. The RNA genome of CFAV had the characteristic organisation of a flavivirus as it was found to consist of a single open reading frame (ORF) which encoded three structural and seven non-structural proteins (nsPs). Conservation homology with other flaviviruses was greatest in the nsPs and lowest with the structural proteins. Furthermore, antigenic detection with anti-flavivirus antibodies was unsuccessful. CFAV was originally classified as an ungrouped member of the *Togaviridae* family, along with flaviviruses and pestiviruses due to its biophysical and biochemical properties (Porterfield et al., 1978). However, its genome was not sequenced until 17 years later

(Cammisa-Parks et al., 1992) and it was regrouped as a distant member of the *Flaviviridae* family, flavivirus genus (Heinz et al., 2000; Cook et al., 2006). Despite the shared genome motifs and organisation with classical flaviviruses, CFAV was demonstrated to be part of a distinct group referred to as insect-specific flaviviruses (ISF) as it failed to replicate in tick or vertebrate-derived cell lines, chicken embryo fibroblasts or mice (Stollar and Thomas, 1975; Cammisa-Parks et al., 1992).

CFAV is by no means the only ISF to have been discovered. Over the past decade there has been a surge in the number of isolated ISF strains from distinct species which have been identified in various wild mosquito populations across the globe. Most of these are found in *Aedes* and *Culex* species. In addition to CFAV, other tentative members which are believed to belong in this group include Kamiti River virus (KRV) (Crabtree et al., 2003; Sang et al., 2003), *Culex* flavivirus (CxFV) (Hoshino et al., 2007; Morales-Betoulle et al., 2008; Farfan-Ale et al., 2009 and 2010; Kim et al., 2009; Blitvich et al., 2009; Saiyasombat et al., 2010; Huanyu et al., 2012), *Aedes* flavivirus (AEFV) (Hoshino et al., 2009; Calzolari et al., 2012), Quang Binh virus (QBV) (Crabtree et al., 2009), Nounané virus (NOUV) (Junglen et al., 2009), Lammi virus (LAMV) (Huhtamo et al., 2009), Nakiwogo virus (NAKV) (Cook et al., 2009), Calbertado virus (CLBOV) (Bolling et al., 2011; Tyler et al., 2011), *Culex* theileri flavivirus (CTFV) (Parreira et al., 2012), Hanko virus (HANKV) (Huhtamo et al., 2012), Palm Creek virus (PCV) (Hobson-Peters et al., 2013), Nanay virus (NANV) (Evangelista et al., 2013) and Ochlerotatus *Caspius* flavivirus ($OCFV_{PT}$) (Ferreira et al., 2013). The detection of many of these viruses in both male and female adult mosquitoes indicates passage of the virus by vertical transmission (Cook et al., 2006; Lutomiah et al., 2007; Farfan-Ale et al., 2009; Saiyasombat et al., 2011; Haddow et al., 2013). These findings indicate that this new group of viruses is more highly prevalent and geographically more widespread than previously anticipated. This group of newly described viruses are classified into the insect flavivirus clade which diverged as a basal lineage of the genus and may represent predecessors of true flavi- arboviruses (Cook et al., 2012). As not all replicate in insect cells asymptomatically, this cannot be classed as a distinguishing characteristic of the group. However, due to their lifecycle lacking transmission to vertebrates they are not classed as arboviruses. It is of note, therefore, that the flavivirus genus contains members which are conventional arboviruses (mosquito-borne and tick-borne), vertebrate only pathogens (no known vector) and insect only pathogens (Kuno et al., 1998; Cook and Holmes, 2006; Kuno, 2007; Cook et al., 2012).

It is therefore interesting to know if non-vector *Toxorhynchites* mosquitoes have developed an antiviral response against infections most likely acquired by ingestion of larvae which have become infected through vertical transmission of arboviruses or ISFs. Yet the antiviral capacity of these mosquitoes and their derived cell lines has not yet been established. With the absence of an annotated genome to allow the identification of orthologues of *Drosophila* and *Ae. aegypti* RNAi genes, the antiviral defence of TRA-171 cells has been established here based on reporter- gene based assays.

6.2 Objectives

1. To investigate if SFV is capable of establishing an infection in *Toxorhynchites amboinensis* derived TRA-171 cells.
2. To determine if TRA-171 cells possess a functional antiviral immune response capable of mediating SFV infection.
3. If an antiviral response is present, is it mediated by dsRNA molecules?
4. Does this response result in the production of small RNAs of 21 nt length?
5. To investigate if TRA-171 cells are infected by the endogenous insect-specific flavivirus, cell fusion agent virus (CFAV).

6.3 Results

6.3.1 SFV Infection of *Toxorhynchites amboinensis*-derived TRA-171 Cells

Although TRA-171 cells are known to be susceptible to infection with CHKV it has not been demonstrated if they can be infected with the related alphavirus, Semliki Forest virus (SFV). To establish this, and for all further experiments, TRA-171 cells were obtained from the European Collection of Cell Cultures (ECACC). They were seeded at a density of 2.2 x 10^5 cells/ well in a 24 well plate and infected 24 h later with a reporter strain of SFV expressing *Renilla* luciferase (*Rluc*) after the nsP3 gene as a marker of replication (SFV4(3H)-*Rluc*) (**Table 2.4**). The infection was performed at either a high (10), mid (0.01) or a low (0.001) multiplicity of infection (MOI). Following a 24 h incubation at 28 °C the cells were lysed and luciferase activity determined by luciferase assay. As anticipated, SFV replication was detected following infection at each MOI (**Figure 6.1A** and **B**) with levels of *Rluc* expression decreasing proportionally.

The replication kinetics of SFV in TRA-171 were determined by infecting the cells with a second reporter strain of SFV, SFV4(3H)-*Fluc*. This was due to the availability of virus stocks prepared in the lab and, as both strains are structurally identical to each other (with the exception of the reporter gene substitution) the two viruses behave in the same fashion and there is no difference to the experimental outcome. TRA-171 cells were seeded in 6-well plates at a density of 9 x 10^5 cells/well. They were either infected with SFV4(3H)-*Fluc* at an MOI of 10 or mock infected 24 h post seeding and returned to the incubator until the required time point. The medium from three wells was collected for virus production curves which were performed by plaque assay on BHK-21 cells. These wells were washed twice gently with sPBS and recovered with fresh media and returned to the incubator. Growth media was removed from a further three wells for each condition and these cells were scraped and counted to compare the growth rate of SFV infected and uninfected cells. Once a count had been determined the cells were then lysed in passive lysis buffer and virus replication determined by luciferase assay. SFV infection did not appear to have any detrimental effects on cell viability as infected cells grew at the same rate as those which had been mock infected and no visible CPE was evident (**Figure 6.2**). Virus production was measured in plaque forming units (PFU/ml) and shown to increase up to 48 hours post infection (hpi) followed by a decrease until 96 hpi where the beginning of a plateau is evident (**Figure 6.3**). The rate of virus replication was seen to rapidly

increase up to 48 hpi where after there was steady decrease until the final time point at 120 hpi (**Figure 6.4**).

SFV, like other arboviruses, has been shown to be capable of establishing persistent infection in insect cells (Brown, 1984) characterised by low virus production. A persistent infection is suggested for SFV in TRA-171 cells as observed in **Figure 6.3**. Dengue virus was also previously documented to progress into a latent infection in TRA-171 cells. To determine if SFV can establish a long term persistent infection in TRA-171 cells, infected cells were passaged up to 50 times post infection and were seeded at intervals in glass bottom 24-well plates at a density of 2.2 x 10^5 cells/well. Cells were immunostained as described with anti SFV-nsP3 antibodies and viewed with the Zeiss LSM 710 confocal microscope. The results were compared to non-infected TRA-171 cells as well as both infected and non-infected U4.4 cells as a control as it is known that SFV can establish a persistent infection in that cell line (Davey and Dalgarno, 1974; Fragkoudis et al., 2008). It can be seen that SFV-nsP3 can be detected in up to 50 passages post infection (**Figure 6.5** and **Table 6.1**). This is comparable to persistent SFV infection in U4.4 cells.

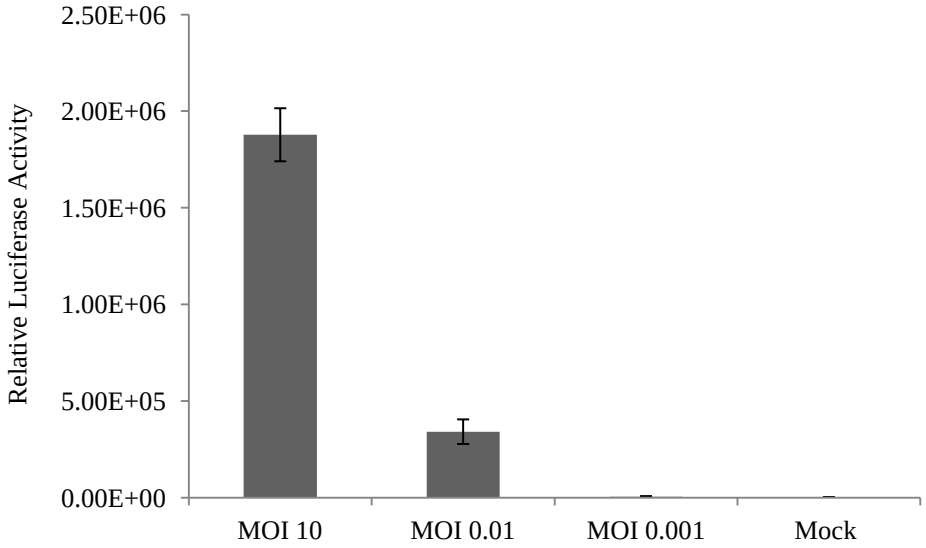
Table 6.1: Percentage of TRA-171 and U4.4 cells cultures infected with SFV after set passages post infection.

Passage Number (post infection)	**Percentage of Culture Infected (%)**	
	U4.4	TRA-171
20	98	47
30	81	35
40	66	28
50	57	25

6.3.2 Processing of dsRNA Generates Antiviral siRNAs in TRA-171 Cells

A key feature of the RNAi response is the production of siRNAs which are loaded into RISC for targeted gene silencing by the Argonaute proteins. In insects, a hallmark of these small RNAs is that they are specifically 21 nt in length as a result of processing by Dcr-2 (Aliyari et al., 2008; Siu et al., 2011; Vodovar et al., 2012). The ability of TRA-171 cells to generate siRNA molecules was determined by an *in vitro* dicer cleavage assay. TRA-171 cells were seeded in a 6-well plate at a density of 9 x 10^5 cells/well. U4.4 cells were also seeded at 8 x 10^5 cells/well as these have been shown to be RNAi competent and produce 21 nt siRNAs which can be detected by this assay (Vodovar et al., 2012). Following a 24 h incubation, the cells were lysed with 1 x lysis buffer and the cell membranes disrupted. Sample extracts were prepared as described (**Chapter 2.4.4**) and incubated with ^{32}P internally radio-labelled dsRNA overnight at 28 °C before the small RNAs were purified, denatured and loaded onto an acrylamide gel. Following electrophoresis, the results were determined by exposing the dried gel to a phosphoimaging screen and viewing with a personal molecular imager (PMI) (**Figure 6.6**). The size of the fragments generated was determined by comparison to size markers; input dsRNA (114 nt) and siRNAs (21 nt) (**Figure 6.6A**). There is a faint band corresponding to small RNAs which can been seen to match the marker size of 21 nt in the U4.4 sample lane used as a positive control. However, there is a much more intense band of cleaved siRNAs produced in the TRA-171 sample lane and this can be seen to be the approximate size of the marker siRNA band. This implies that a mechanism is active in TRA-171 cells causing the cleavage of dsRNA into siRNAs of approximately 21 nt in size. Image J analysis was used to quantify the total of dsRNA and siRNAs present (**Figure 6.6B**). The total quantity of nucleic acids from each sample in the three experiments was measured and set to 100 %. The band density of the cleaved dsRNA was then measured as a percentage of this.

A



B

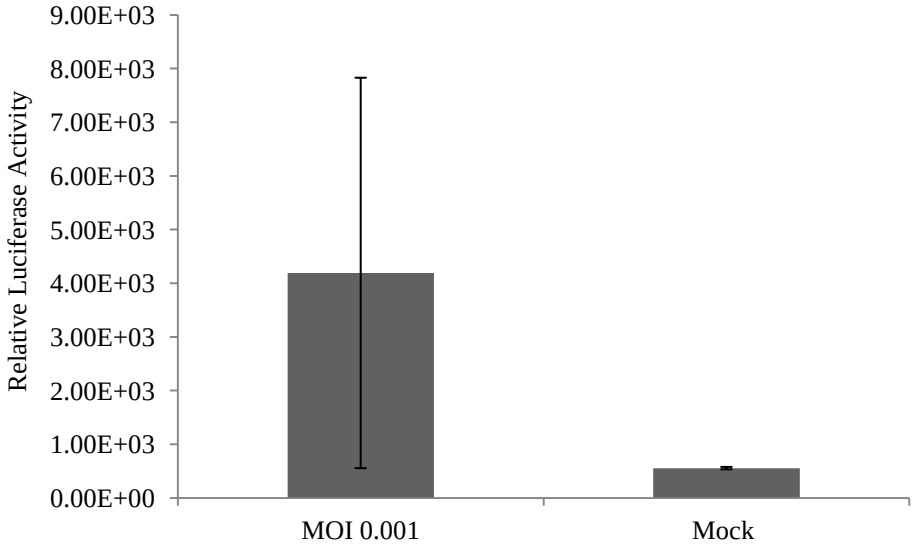


Figure 6.1: Replication of SFV(3H)-*Rluc* in *Tx. amboinensis*-derived TRA-171 cells. A) Cells were infected with a recombinant strain of SFV expressing *Renilla* (*Rluc*) luciferase (SFV(3H)-*Rluc*) at high (10), mid (0.01) and low (0.001) MOIs. *Rluc* expression was detected by luciferase assay and measured in relative luciferase light units. Results were compared to those which had been mock infected. Error bars represent the standard deviation of three independent experiments performed in triplicate. Panel **B**) shows the MOI 0.001 and mock results from Panel A in more detail.

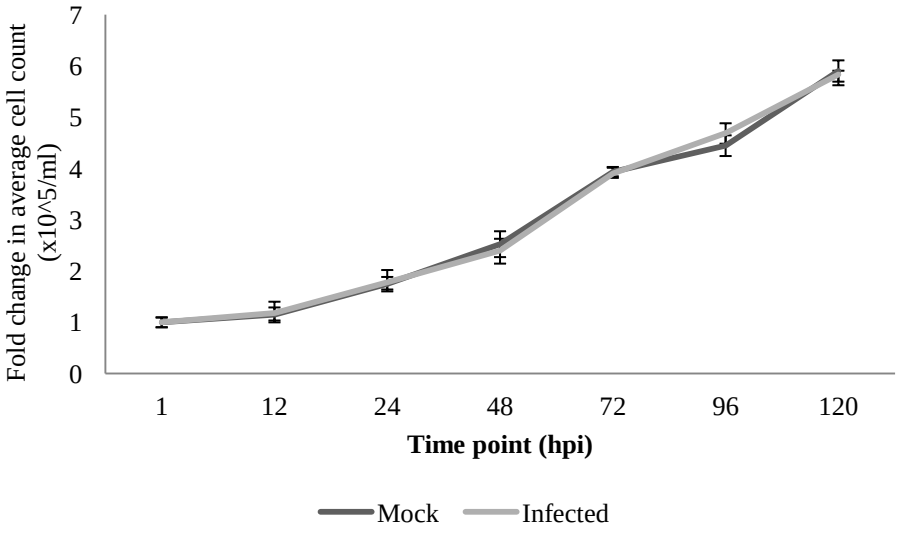


Figure 6.2: Growth curve of SFV4(3H)-*Fluc* infected TRA-171 cells. Growth of TRA-171 cells infected with a recombinant strain of SFV expressing Firefly (*Fluc*) luciferase (SFV4(3H)-*Fluc*) at an MOI of 10 (light grey) or mock infected (dark grey). Error bars represent the standard deviation of two independent experiments performed in triplicate.

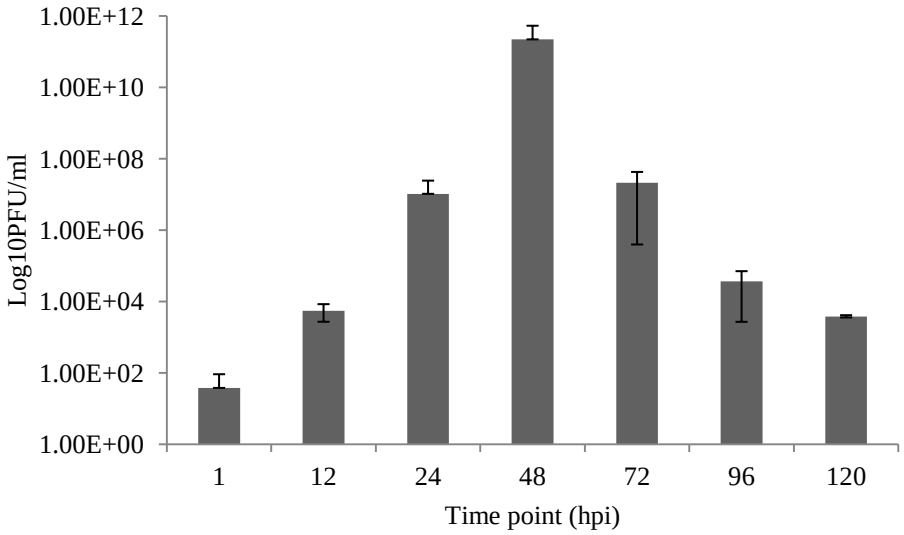


Figure 6.3: The production of SFV4(3H)-*Fluc* in PFU/ml at increasing time points following infection of TRA-171 cells. TRA-171 cells were infected with a recombinant strain of SFV expressing Firefly (*Fluc*) luciferase (SFV4(3H)-*Fluc*) at an MOI of 10, the supernatant was collected at given time points post infection (hours post infection, hpi) and viral titres determined in plaque forming units (PFU/ml) by plaque assay. Error bars represent the standard deviation of two independent experiments performed in triplicate.

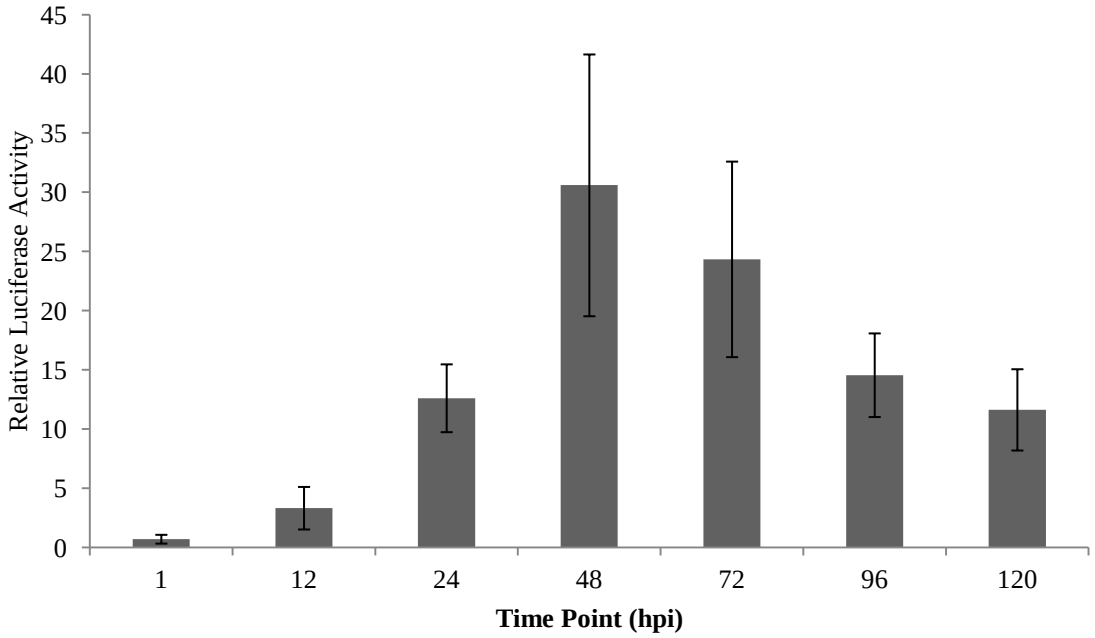


Figure 6.4: The replication kinetics of SFV4(3H)-*Fluc* following infection of TRA-171 cells. TRA-171 cells were infected with a recombinant strain of SFV expressing Firefly (*Fluc*) luciferase (SFV4(3H)-*Fluc*) at a MOI of 10 and relative light units detected by luciferase assay as a representative of virus replication at specific hours post infection (hpi). Error bars represent the standard deviation of two independent experiments performed in triplicate.

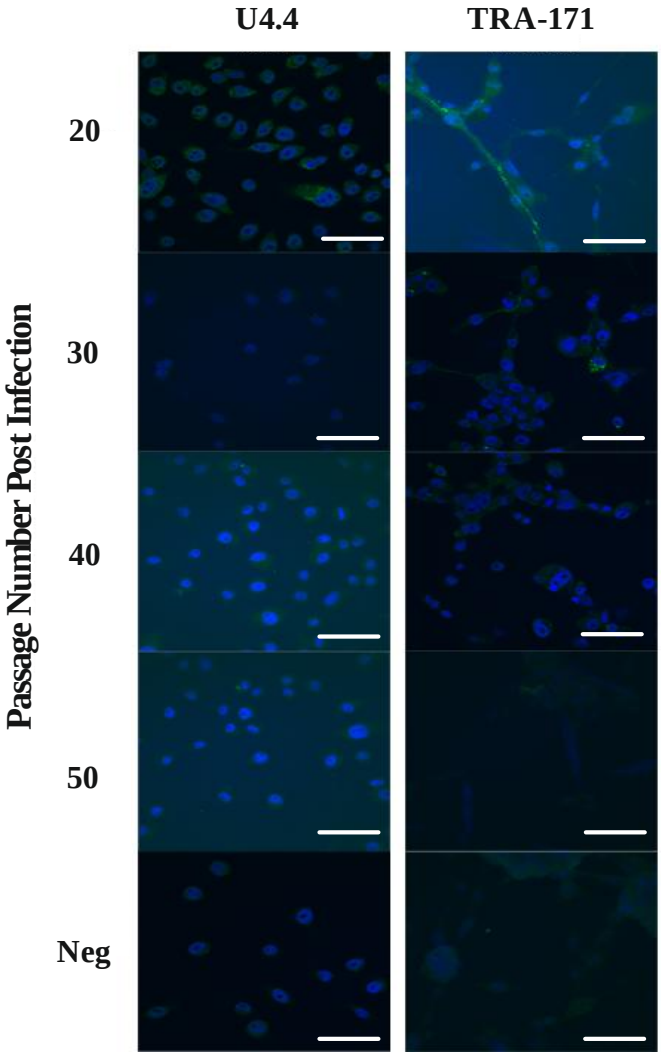


Figure 6.5: Persistence of SFV4(3H)-*Fluc* infection in TRA-171 cells. TRA-171 and *Ae. albopictus* derived U4.4 cells were infected with a recombinant strain of SFV expressing Firefly (*Fluc*) luciferase (SFV4(3H)-*Fluc*) at an MOI of 10 and maintained for 50 passages. At designated time points the cells were seeded and immunostained with anti-SFV nsP3 antibody. Detection was determined with a Zeiss LSM 710 confocal microscope. Results were compared to those which had been mock infected (Neg). Representative images are shown. Blue, DAPI nuclear staining; green, anti-SFV-nsP3 staining. Scale bars indicate 50 μM.

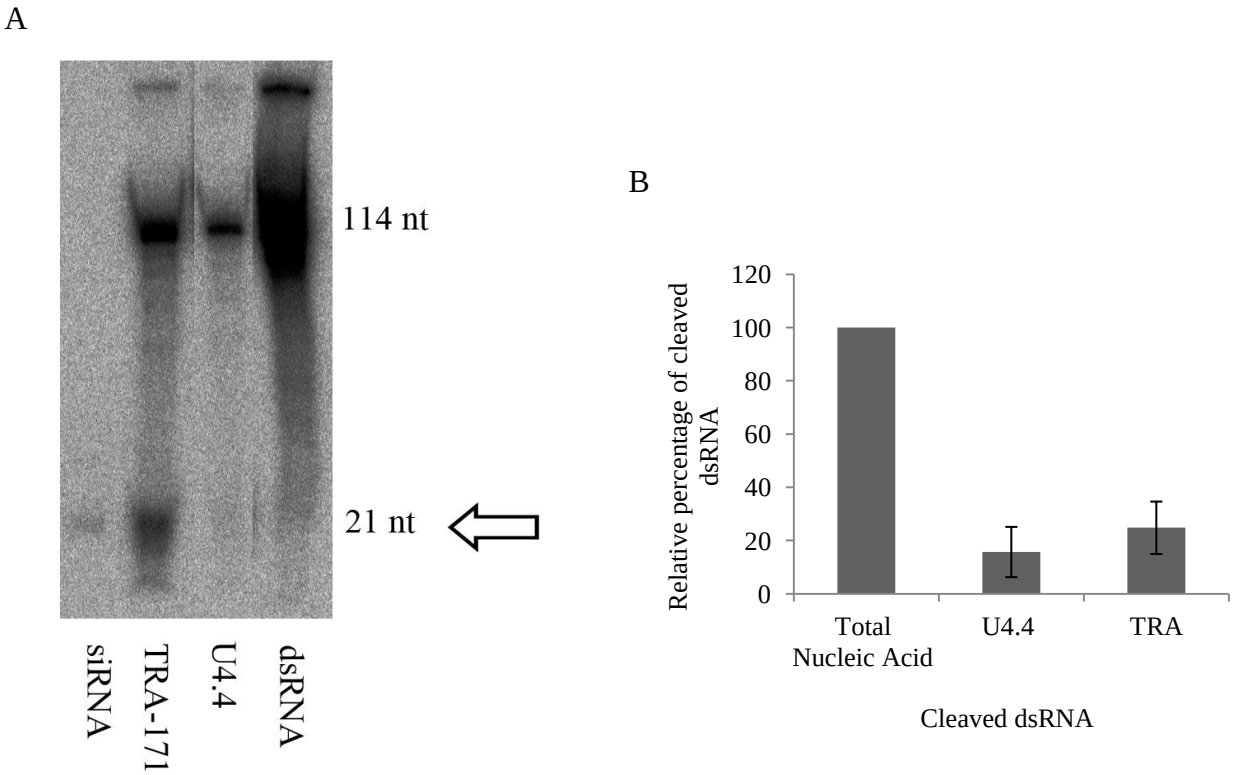


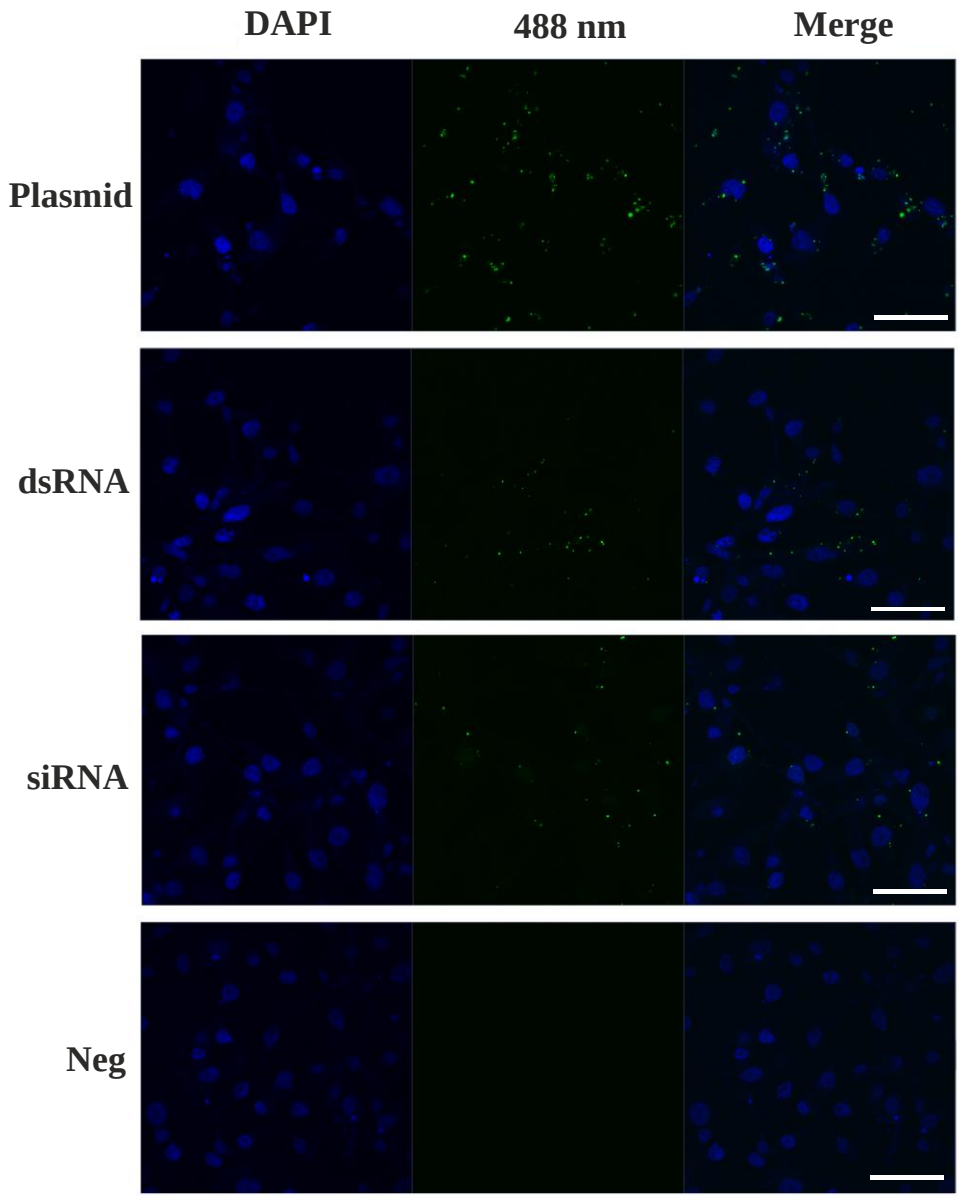
Figure 6.6: Processing of long dsRNA molecules into 21 nt siRNAs by *in vitro* Dicer cleavage assay. **A**) Cellular extracts were prepared from *Ae. albopictus derived* U4.4 and *Tx. amboinensis* derived TRA-171 cells infected with a recombinant strain of SFV expressing Firefly (*Fluc*) luciferase (SFV4(3H)-*Fluc*) at an MOI of 10. Extracts were incubated with ^{32}P labelled 114 nt dsRNA and the RNAs generated detected by loading onto an acrylamide gel and exposing to a photosensitive film. Size markers of ^{32}P labelled 21 nt siRNA and 114 nt dsRNA were also loaded to permit a size comparison. This was repeated three times and a representative image is shown. The arrow indicates the size expected for viral-derived small RNAs (21 nt) **B**) Image J software was used to quantify the percentage of cleaved dsRNAs. Error bars show standard deviation of the mean from the three independent experiments.

6.3.3 dsRNA-mediated Interference in TRA-171 Cells

Prior to performing experiments to ascertain the existence of an inducible RNAi response in TRA-171 cells, they must first be characterised in detail to establish their transfection efficiency in addition to their ability to transact RNAi. To determine the capacity of nucleic acid (plasmid, dsRNA and siRNA) uptake by liposomal transfection, fluorescently labelled plasmid (Fluorescein Label IT®, Mirus), dsRNA (**Chapter 2.3.11**), and siRNA molecules (BLOCK iT™ Fluorescent Oligo, Invitrogen), were transfected into TRA-171 cells seeded in 24-well glass bottom plates at a density of 2.2 x 10^5 cells/well. After a 24 h incubation each well either received: 500 ng plasmid, 500 ng dsRNA or 50 nM siRNAs or were mock transfected with Lipofectamine 2000. Following a further incubation of 24 h the cells were fixed and a coverslip mounted using DAPI mounting media. Images of each condition were taken using the Zeiss LSM 710 confocal microscope (**Figure 6.7**). The images reveal that all forms of nucleic acids were taken up by the TRA-171 cells with a transfection efficiency of 92 % plasmid, 38 % dsRNA and 35 % siRNAs.

The expression efficiency of plasmid encoding *Renilla* luciferase (*Rluc*) was also determined by transfecting cells with pRL-CMV (Promega) that expresses *Rluc* under the control of the cytomegalovirus immediate-early promoter. TRA-171 cells were seeded in 24-well glass bottom plates as previously and 24 h post seeding were transfected with either: 300 ng, 30 ng or 3 ng/well. The cells were incubated for a further 24 h before being lysed with passive lysis buffer and the expression of *Rluc* quantified by luciferase assay. The results of plasmid expression were compared to cells which had been mock transfected (**Figure 6.8**). The levels of expression were variable and so one representative experiment is shown. In each repetition a proportional level of expression was detected for each concentration of plasmid treatment the cells received. Expression via an alternative plasmid promoter is discussed previously in **Chapter 5**.

A



B

	Condition		
	Plasmid	dsRNA	siRNA
Percentage Uptake (%)	92	38	35

Figure 6.7: Uptake of fluorescent plasmid, dsRNA and siRNA in TRA-171 cells. A) Cells were transfected with appropriate concentrations of each nucleic acid and expression was viewed with a Zeiss LSM 710 confocal microscope. Results were compared to those which had been mock transfected (Neg). Representative images are shown. Blue, DAPI nuclear staining; green, fluorescent nucleic acid staining. Scale bars indicate 50 μM. **B**) Cell positively expressing each nucleic acid were counted and averaged for each experiment.

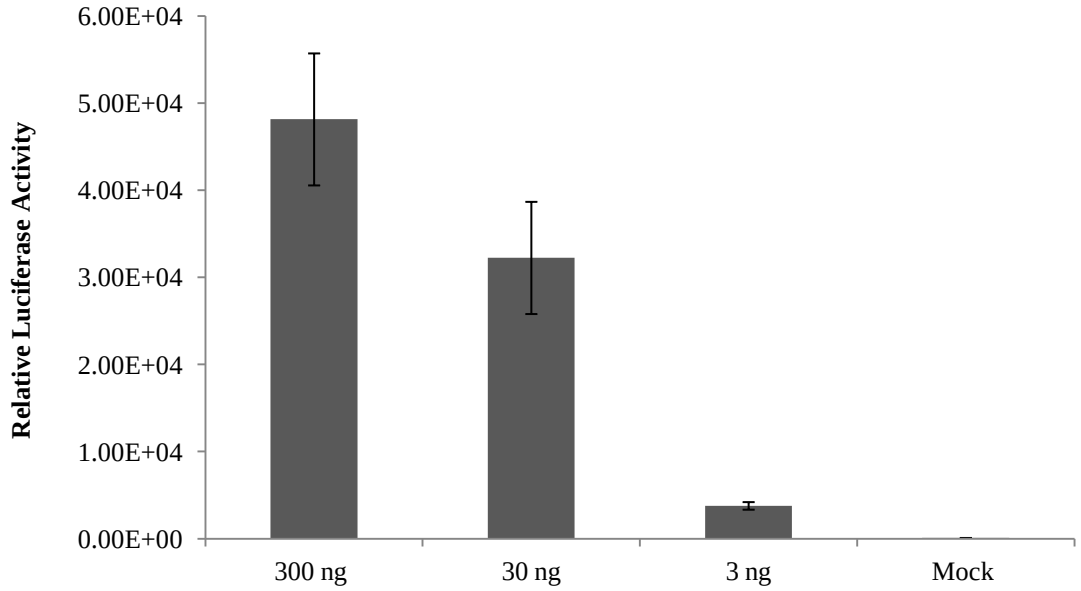


Figure 6.8: Expression of plasmid encoding *Rluc* in TRA-171 cells. Cells were transfected with serial 10- fold dilutions of pRL-CMV. *Rluc* expression was detected by luciferase assay and measured in relative luciferase light units. Results were compared to those which had been mock transfected. A representative of three independent experiments done in triplicate is shown. Error bars represent the standard deviation of three representative replicates performed during a single experiment.

6.3.4 Is There a dsRNA Induced Antiviral Response in TRA-171 Cells?

The presence of a dsRNA inducible RNAi response in TRA-171 cells was first determined using plasmids expressing luciferase reporter genes as this is a more controllable experimental set up than with a virus infection. The cells were seeded in 24-well plates at a density of 2.2 x 10^5 cells/well and co-transfected 24 h later with 30 ng pIZ-*Fluc* plasmid (Ongus et al., 2006), 5 ng pRL-CMV plasmid as an internal control and 0.1 ng of either *Fluc* specific dsRNA, control (eGFP) dsRNA or no dsRNA. RNAi has been shown to be dependent on the concentration of dsRNA (Fire et al., 1998; Kennerdell and Cathew, 1998; Yang et al., 2000). Different concentrations of specific dsRNA were tested to determine the optimal concentration for the experiment. Concentrations > 0.1 ng resulted in complete 100 % targeted silencing which lead to the selection of 0.1 ng/well (results not included). Cells were lysed 24 h post transfection and luciferase expression determined. The results are presented as a correlation between eGFP control treated cells (100 %) and *Fluc* expression (**Figure 6.9**). Cells which received *Fluc* specific dsRNA showed a decrease of 82.4 % in luciferase light units compared to control cells suggesting that a dsRNA induced silencing mechanism is present in TRA-171 cells.

It has been shown that establishing a virus-specific RNAi response before infection can inhibit virus replication (Caplen et al., 2002). To establish if an antiviral pathway exists in TRA-171 cells which is stimulated by the presence of specific, long dsRNA, cells were first treated with *Rluc* specific dsRNA prior to infection with the reporter strain of SFV SFV4(3H)-*Rluc*. The cells were seeded in 24-well plates as described and transfected 24 h later with 50 ng of dsRNA which was either specific for *Rluc* or control dsRNA (eGFP). A further mock transfected control was also included. The cells were incubated for 24 h to allow for the incorporation of the specific *Rluc* siRNAs into RISC ahead of infection with SFV4(3H)-*Rluc* at either a high MOI (10) or a low MOI (0.005). Following a further 24 h incubation the cells were lysed and luciferase activity determined by luciferase assay. The results are presented as a correlation between eGFP control treated cells (100 %) and *Rluc* expression. Luciferase expression for both MOI 10 (**Figure 6.10A**) and 0.05 (**Figure 6.10B**) were reduced in cells treated with *Rluc* specific dsRNA compared to control treated cells (41 and 85 % respectively).

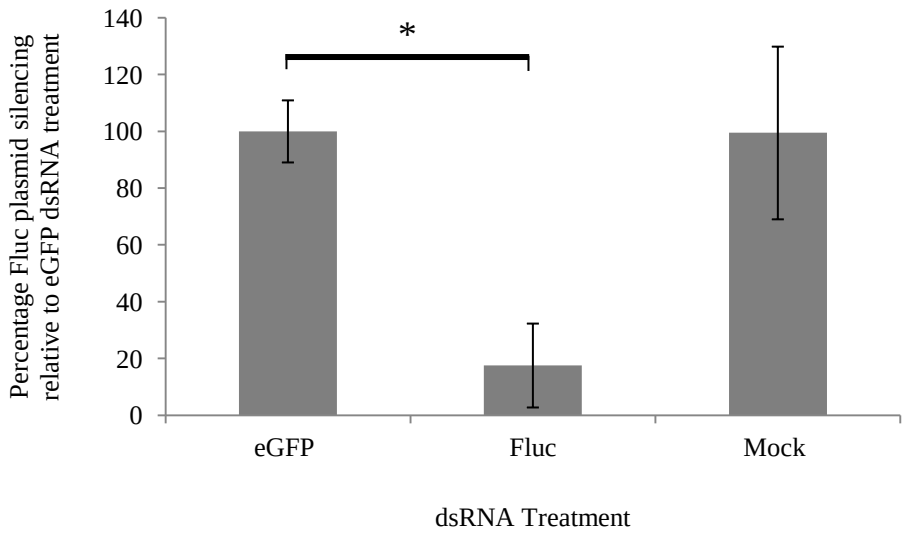
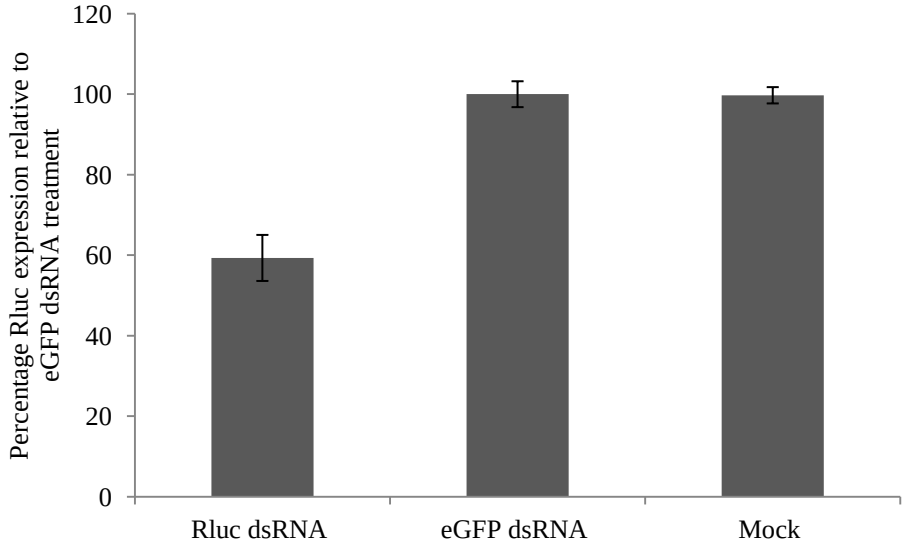


Figure 6.9: The effect of dsRNA induced silencing of *Fluc* encoding plasmid in TRA-171 cells. Cells were co-transfected in parallel with both pIZ-*Fluc* and pRL-CMV (internal control) plasmids and either specific *Rluc* dsRNA, control eGFP dsRNA or no dsRNA. Following this, cells were incubated for 24 h where after they were lysed and the ratio of *Fluc: Rluc* expression was measured in relative luciferase light units. Values recorded for control eGFP dsRNA were set to 100 % and the specific *Rluc* dsRNA viewed as a percentage of this. Error bars represent the standard deviation of three independent experiments performed in triplicate. * represents $p < 0.05$, Student's t-test.

A



B

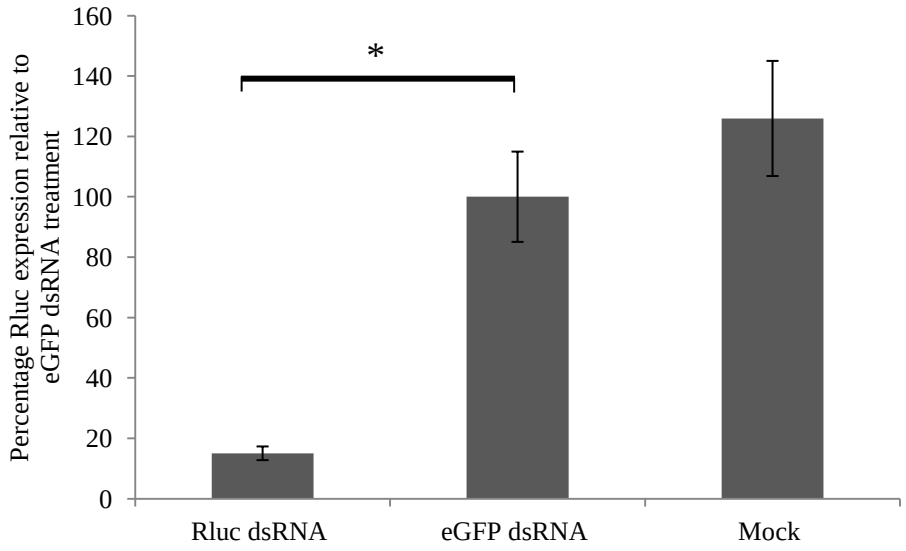


Figure 6.10: The effect of dsRNA induced silencing on SFV(3H)-*Rluc* infection in TRA-171 cells. Cells were transfected with specific dsRNA against *Renilla* (*Rluc*) or control dsRNA (eGFP). Following an incubation of 24 h, the cells were infected with SFV4(3H)-*Rluc* at either a high **A**) (MOI 10) or **B**) a low MOI (MOI 0.005). Cells were incubated for a further 24 h after which they were lysed and *Rluc* expression measured in relative luciferase light units. Values recorded for control eGFP dsRNA treated cells were set to 100 % and the cells treated with specific *Rluc* dsRNA viewed as a percentage of this. Error bars represent the standard deviation of three independent experiments performed in triplicate. * represents $p < 0.05$, Student's t-test.

6.3.5 Detecting Cell Fusion Agent Virus (CFAV) Infection in TRA-171 Cells

Observations between the monolayers of the *Ae. aegypti*-derived Aag2 and TRA-171 cell line revealed that they share morphological similarities which clearly distinguish them from the *Ae. albopictus*-derived cell lines used as part of this project. **Figure 6.11** illustrates representative images obtained with the Zeiss LSM 510-Meta confocal microscope with differential interference contrast (DIC) of the four mosquito cell lines. Both the U4.4 (Panel C) and C6/36 (Panel D) appear as individual, round cells with small protruding filaments. In contrast, Aag2 cells (Panel A) and TRA-171 cells (Panel B) are elongated, fibroblast-like with the cytoplasm stretching away from the nucleus. Often, in the case of the Aag2 cells, large aggregations can be observed (indicated with arrows).

Aag2 cells have been demonstrated to be persistently infected with CFAV (Scott et al., 2010). As a result of this observation it was interesting to investigate if CFAV was detected in our TRA-171 cell line. Primer sets were designed against three regions of CFAV using the Genbank accession number M91671 as a reference sequence (**Chapter 2.9**). Total RNA was isolated from each of the untreated cell lines by Trizol extraction as described (**Chapter 2.3.18**). First strand cDNA synthesis was performed by reverse transcription to generate cDNA for gene detection by PCR. The DNA products from each PCR were analysed by agarose gel electrophoresis (**Figure 6.12**). None of the primer sets showed positive detection in either the U4.4 or C6/36-derived cDNA. Conversely, all three primer sets strongly recognised sequences of the correct size (Primer set 1:559 nt, Primer set 2:701 nt, Primer set 3: 330 nt) in the Aag2 derived cDNA. Similar detection was also observed in the TRA-171 cDNA samples, although the intensity was less than observed in the Aag2 samples. Sequencing of the PCR products generated from each primer set confirmed them to be the expected CFAV sequence for each case. There was no detection by any of the primer sets in the RNA (No RT) control samples. Actin was used as an internal housekeeping control in the *Ae. albopictus* samples to demonstrate the presence of cDNA.

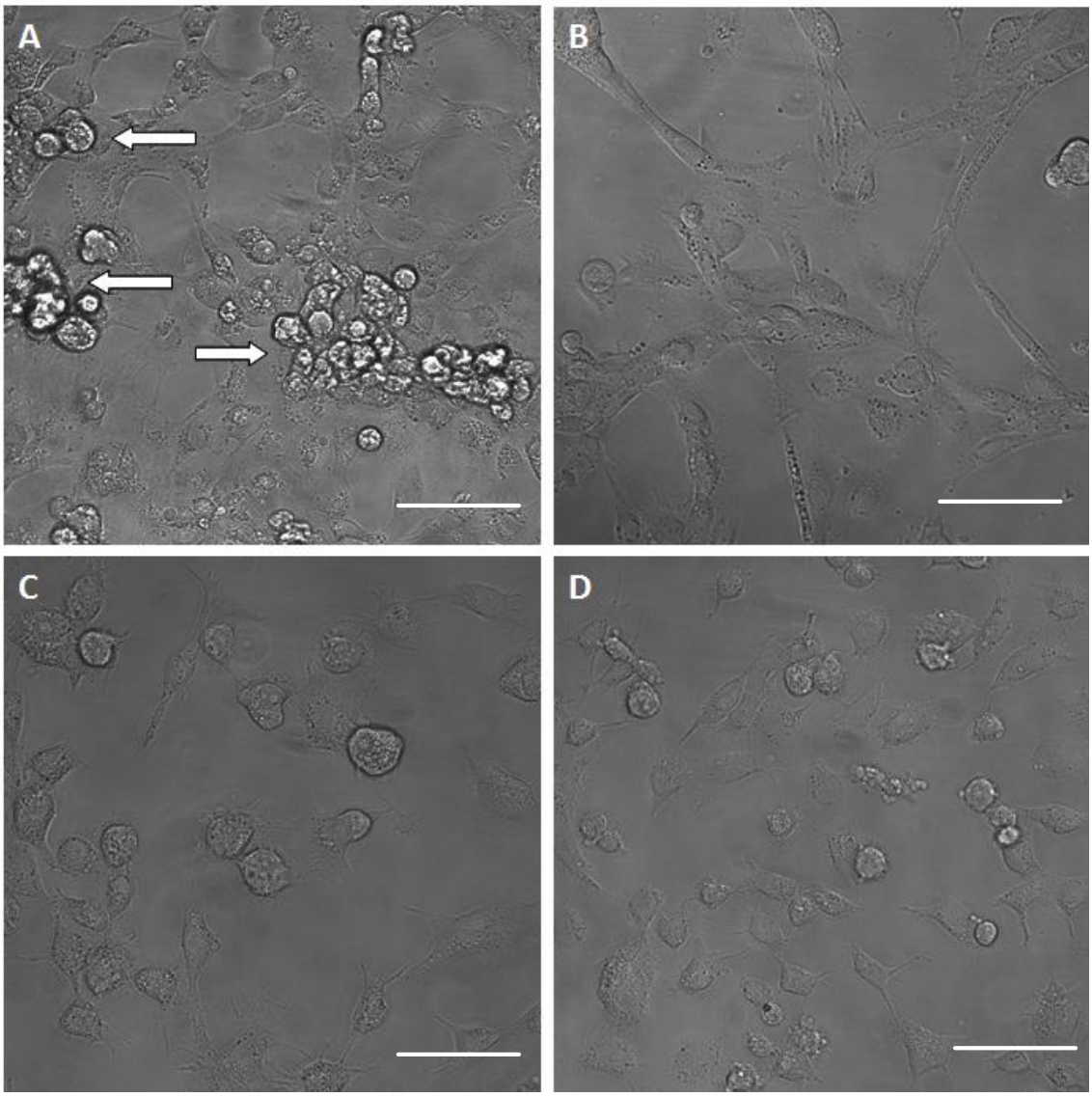


Figure 6.11: Morphology of different mosquito derived cells. DIC confocal images of **A**) *Ae. aegypti*-derived Aag2 cells, **B**) *Tx. amboinensis*-derived TRA-171 cells, **C**) *Ae. albopictus*-derived U4.4 cells and **D**) *Ae. albopictus*-derived C6/36 cells. Aggregates of Aag2 cells are indicated with arrows. Scale bars indicate 50 μM.

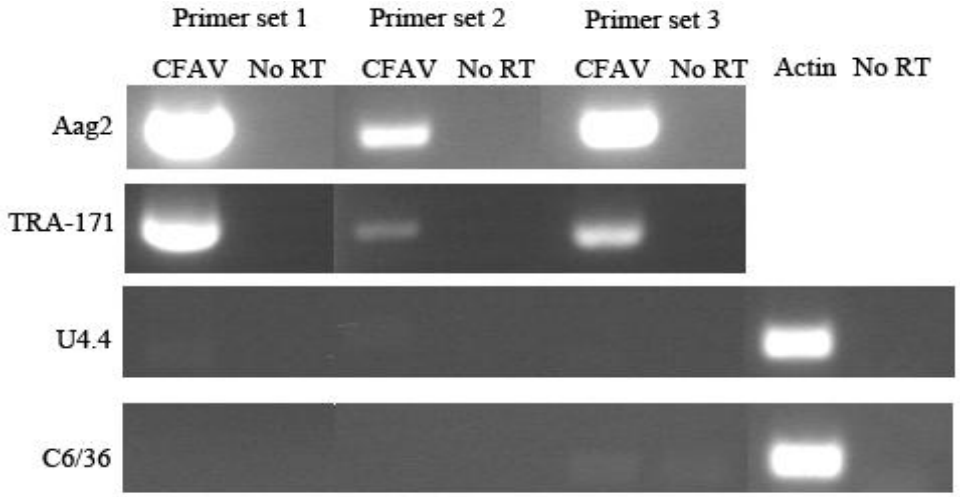


Figure 6.12: Detection of CFAV in different mosquito-derived cell lines. The expression of the insect specific cell fusion agent virus (CFAV) was detected using three primer sets recognising different regions of the genome. RNA was extracted from the *Ae. aegypti* derived Aag2 cell line, *Tx. amboinensis*-derived TRA-171 cells and *Ae. albopictus*-derived U4.4 and C6/36 cell lines. RT-PCR was carried out using random hexamers and PCR was performed using each primer set. No RT indicates samples where the Superscript III enzyme was omitted. Primers recognising *Ae. albopictus* actin were used as a control.

6.4 Discussion

Toxorhynchites mosquitoes may potentially offer an alternative system for arbovirus research with several advantages over current *in vivo* models. However, the interaction between cellular and arboviral factors during infection has been overlooked indubitably due to their non-haematophagous biology. This is despite several studies demonstrating that a number of important human pathogens can replicate and can be readily propagated within these mosquitoes (Rosen et al., 1978 and 1981; Tesh, 1979; Kuno, 1981a; Scherer and Chin, 1981). These findings invoke the need to establish if there is a natural antiviral defence(s) involved in the control of these infections.

It was first established that TRA-171 cells derived from *Toxorhynchites amboinensis* are efficiently infected by the alphavirus SFV at multiple MOIs as expected due to their permissibility to infection by the related CHIKV (Legrand and Hotta, 1983). The appearance and growth characteristics of SFV infected TRA-171 cells were not dissimilar to uninfected cells. This is expected following a natural viral infection as SFV, in addition to other arboviruses, does not cause any insect pathology and allows the survival of the vector ensuring its own transmission to susceptible vertebrates when its vector takes a blood meal. A further characteristic of mosquito-borne alphaviruses is that they have a distinctive growth cycle where an initial rapid increase in viral load during the acute stages of infection progresses through to a steady decline and eventual plateau at a prolonged low level infection which is maintained for the remainder of the insect's lifespan. This latent or persistent stage generates titres of infective virus which are understandably lower than during the acute stage of infection. Following SFV infection in TRA-171 cells this distinctive growth pattern was evident. However, the peak of both virus production and replication was after 48 h rather than after 24 h as observed in U4.4 cells which are known to be a good model for representing infection in the whole organism (Davey and Dalgarno, 1974; Fragkoudis et al., 2008; Attarzadeh-Yazdi et al., 2009). The growth pattern of SFV also differs from observations with CHIKV which did not peak until eight days post infection (dpi) and only reached 10^9 PFU/ml (Legrand and Hotta, 1983). TRA-171 cell growth was not observed to be affected by SFV infection and population doubling time was approximately 40 h for both infected and non-infected cells. It was also observed that cells could be maintained without passaging for two months with no apparent detrimental affects to the monolayer while actively growing cells were passaged nine times during this period. Cell to cell contact was beneficial to the growth rate of TRA-171 cells as a sparse

cell layer resulted in a decrease in growth rate and so cells were maintained at a high density to encourage growth. The characteristics displayed by SFV in the TRA-171 cell line are similar to those observed during infection of the immunocompetent U4.4 cell line. TRA-171 cells appear to inhibit infection and allow SFV to enter persistence in a manner similar to U4.4 cells impling that TRA-171 cells could be used as a suitable alternative model for SFV:vector interactions.

The major antiviral defence in insects is known to be based on RNA silencing pathways. It was unclear if *Toxorhynchites* mosquitoes utilised this response against acquired infections as nothing has been published to date relating to their antiviral defences. In this study TRA-171 cells were shown to be immunocompetent by 1) cleaving dsRNA into small RNA molecules of approximately 21 nt and 2) data produced by plasmid and viral reporter-gene based silencing assays.

The results observed from the *in vitro* dicer cleavage assay indicate that TRA-171 cells can cleave dsRNA molecules into siRNA molecules of approximately 21 nts. It can therefore be proposed that a protein, such as Dicer or similar, with endonuclease activity is acting in these cells. It has been shown that Dicer cleavage of viral genomes in insects generates siRNAs of specifically 21 nt in length (Aliyari et al., 2008; Myles et al., 2008 and 2009; Siu et al., 2011; Vodovar et al., 2012). Due to comparison with the 21 nt size marker also loaded on the gel it can be estimated that these small RNAs are approximately what would be anticipated in RNAi competent insects; however, definitive quantification of the size of these molecules produced by TRA-171 cells would need to be determined by deep sequencing analysis. As the TRA-171 natural antiviral response is most likely required for handling low level infections, functional siRNA molecules against the disseminating virus may be heavily relied upon to spread to neighbouring cells and curtail the incoming infection.

TRA-171 cells efficiently expressed luciferase genes encoded by plasmids under the control of the OpIE2 promoter, known to be functional in other insect cells (See also **Chapter 5.3.8**); although further testing of other promoter types would also be interesting to assess their function in this cell type. Nucleic acid uptake was shown to be proficient in TRA-171 cells, a necessary element for their use as an *in vitro* system for arbovirus research. Natural uptake of dsRNA was also tested by adding the molecules directly to the media, without the addition of a transfection reagent (data not shown). Although dsRNA molecules can be readily taken up from the media by *Drosophila*-derived cells (Saleh et

al., 2009), this is not known for any mosquito-derived cells lines. The findings of this project are consistent with what is already known for mosquitoes cells in culture as dsRNA molecules added directly to the media without the use of a transfection reagent proved to be unsuccessful and no expression or functional response was detected above background. However, siRNAs may play a more important role in non-cell autonomous control of impeding virus dissemination in mosquito cells (Attarzadeh-Yazdi et al., 2009) and should be further investigated to understand the exact mechanism(s) involved.

The existence of an endogenous siRNA pathway induced by dsRNA was determined through silencing of plasmid and viral reporter-gene expression and was comparable to results seen in other insect cell lines such as the U4.4 and Aag2 cell lines which are known to be immunocompetent (Schnettler et al., 2012; Vodovar et al., 2012). Viral silencing in TRA-171 cells was more successful at a low MOI when only a small proportion of the cells are infected and the virus undergoes multiple rounds of replication to disseminate throughout the culture. This suggests that as documented in other cell lines, the TRA-171 cell's immune system is overwhelmed by the high viral load and is less able to control the infection effectively. This is anticipated as the viral titre in vertically infected larvae is less than in the adult female and so the natural response would not encounter such high viral load. As it is known that alphaviruses cannot inhibit the RNAi response due to the lack of an encoded suppressor protein, it can be suggested that TRA-171 cells would be more adept at controlling an alphavirus infection compared to a flavivirus infection which have been shown to express silencing suppressing molecules (Schnettler et al., 2012; Kakumani et al., 2013). However, as both alphaviruses and flaviviruses are capable of establishing a persistent infection this indicates that the cellular defences are able to interfere with the infection (Davey and Dalgarno, 1974; Kuno, 1982a; Brown, 1984; Randolph and Hardy, 1988; Chen et al., 1994; Bowers et al., 1995; Bugrysheva et al., 2001; Mlera et al., 2014). Future research is required to properly understand the mechanisms involved in regulating persistence and understanding when, where and to what extent the immune response is engaged in this aspect of infection.

Taken together these results suggest that TRA-171 cells have a functional RNAi response that can be used to successfully target SFV infection. Further work is required to fully characterise the antiviral response in these mosquitoes and validate their use as a model. This should include small RNA sequencing, viral sensor plasmids assays and knockdown of pathway proteins.

It has previously been demonstrated that the Aag2 cell line is persistently infected with CFAV which is controlled by the RNAi response (Scott et al., 2010). Initial CFAV infections of the *Ae. albopictus* – derived C6/36 cell line resulted in the normally individually distributed cells coming together to form multinucleated cells (Stollar and Thomas, 1975). However, these clumped cells had low viability and were eventually replaced by the few remaining scattered cells. The virus then passed into a persistent stage of infection where few virus particles were produced and the cell monolayer resembled an uninfected culture. Further infection of endogenously infected cells with CFAV does not result in any CPE presenting as syncytia formation and no additional cell fusion is exhibited. This is of particular importance for two reasons. Firstly, Aag2 cells are historically documented as an adherent monolayer which is not uniform and with many clusters of cells (Lan and Fallon, 1990). This suggests that CFAV interacts with Aag2 cells in a different fashion to cells which are more spread within the culture and require fewer cell-to-cell contacts. A similar mechanism appears to be involved with the interaction in TRA-171 cells. Initial tests to investigate viral contamination in the TRA-171 cell line proved to be negative for any endogenous infections (Kuno, 1980), therefore any contamination has occurred following the original generation of line. Although the TRA-171 cell line was maintained in the same environment as the Aag2 cell line it is possible that cross contamination occurred in my hands. However, due to the different growth media of the two cell lines this is unlikely. Certainly no morphological differences were observed in the TRA-171 cells during passaging and they appear to be of similar appearance to those initially described. Secondly, this information indicates that an apparently healthy cell line may be harbouring an unknown agent which presents no obvious symptoms other than initial syncytium formation immediately following infection. This emphasises the need for accurate detection methods to be developed to identify the extent of contamination within cultures and the potential implications this has for their use in arbovirus studies and the effect that endogenous viruses have on further viral infections.

ISFs don't have any known medical impact but are of interest due to the potential for interactions with pathogenic flaviviruses and in advancing the understanding of flavivirus evolution. In addition to ISFs, long flavivirus-related sequences have been found to be integrated into the *Ae. albopictus* and *Ae. aegypti* genomes, although complete putative genomic structures have not been described. One such example is the 'cell silent agent' (CSA, Crochu et al., 2004) which is related to the nsP1- nsP4 genes of CFAV and KRV, as well as to other ISF sequences (Roiz et al., 2009). This could represent a novel mechanism

of horizontal gene transfer in eukaryotic cells from a non-retroviral RNA virus. This unique integration into the genome suggests that insect-only viruses are highly specialised to their insect hosts and it may be postulated that ISFs are an early adaptation of true arthropod transmitted flaviviruses. These integration events are only known for *Aedes* so it will be of interest to uncover if such events are also described in other vector populations.

As *Aedes* and *Culex* mosquitoes are major flavivirus vectors, further research into these viruses may uncover essential information regarding the evolution of arboviruses and vector/host interactions. The presence of ISFs in wild populations of mosquitoes also asks the question of how endogenous insect viruses interact with contracted arbovirus pathogens and if they are capable of 'super-infection exclusion'. In such an instance, an ISF would allow its host to be refractory to a further infection with a related virus thus preventing further transmission. This is becoming of increasing importance due to recent emerging cases of certain arthropod-borne flavivirus infections across the globe. In particular, West Nile virus (WNV) and CxFV have been documented to co-infect. There is conflicting evidence regarding the nature of the association between CxFV and WNV and it is not yet fully understood if the interaction between the two is beneficial or restrictive to WNV transmission (Kent et al., 2010; Newman et al., 2011; Bolling et al., 2012). Certainly super-infection exclusion is virus/vector association dependent and has yet to be fully investigated in the context of persistent ISFs infection; although, genetic diversity, as well as ecological factors, are considered to be critically involved. *Tx. amboinensis* would be a useful model in which to study co-infections due to the low level of risk associated with this species. These experiments are of particular importance to establish if endogenous viruses such as ISFs are naturally capable of/or could be manipulated to restrict arboviral infections in their host.

6.5 Future Work

Further work should be performed to completely characterise SFV infection of TRA-171 cells by determining the viral titres produced during an established persistent infection by plaque assay analysis or by qPCR. Additionally, it would be interesting to compare titres of SFV produced from TRA-171 with those from C6/36 cells. As C6/36 cells are commonly used to produce high titres of mosquito passaged alphaviruses, it would be beneficial to determine if TRA-171 cells may be used for similar purposes and produce high titres of other arboviruses in addition to dengue and other flaviviruses. The success of siRNA production in these cells also suggests that orthologues of key RNAi proteins may be present in their genome and transcriptome analysis would help to identify these. Moreover, to investigate the antiviral RNAi response in these cells deep sequencing analysis of the small RNAs would ascertain if they are 21 nt in length, as documented in RNAi competent insects, or if they are longer and include characteristics of piRNAs that are the major viral specific small RNA population produced in C6/36 cells, which have a Dcr-2 null phenotype (Brackney et al., 2010). Northern blot and deep sequencing analysis of both SFV and the endogenous CFAV infections would determine if these small RNAs are viral-specific and if they are generated from specific regions of the viral genome. If, as is noted in the Aag2 cell line, CFAV activates the antiviral pathway in TRA-171 cells, then you would expect a proportion of the small RNA population to match the CFAV genome. This avenue of analysis would be necessary to determine if the endonuclease active observed in the *in vitro* dicer assay was acting antivirally. In addition, knockdowns of implied RNAi proteins in this cell line followed by viral infection or treatment with viral sensor constructs would also be required to fully understand their function during a viral infection.

As discussed in **Chapter 4**, the spread of silencing molecules is important for effective dissemination of the targeted signal. Potential spread of siRNAs observed within TRA-171 cells should be further investigated to establish if biologically functional siRNAs are able to pass to neighbouring cells and restrict impending infection within the culture. Furthermore, if the viral specific siRNA population plays an important role in viral targeting there would be a substantial effect on viral spread following infection by the SFV reporter strain encoding the tombusvirus siRNA binding protein p19 and these experiments should be carried out to determine if this is the case. If the greatest risk of acquiring arboviral infections is through the consumption of vertically infected larvae then it would also be beneficial to investigate the antiviral defence of TRA-171 cells against flaviviruses

that are well known to be transmitted in this way and is in contrast to alphaviruses which are not. Unfortunately, these experiments could not be performed due to licensing constraints.

6.6 Principle Findings

1. Determined that SFV is capable of establishing an infection which progresses to persistence in the *Toxorhynchites amboinensis* derived TRA-171 cell line.
2. This infection does not result in any apparent CPE and reaches the peak of infection 48 hpi.
3. The TRA-171 cell line successfully expressed plasmid encoded *Rluc* and *Fluc* genes under the control of promoters known to be expressed in insect cells.
4. A functional RNA silencing response initiated by dsRNA was demonstrated against both plasmid DNA and SFV infection in these cells.
5. Small RNA molecules that are approximately 21 nt in length are produced following SFV infection.
6. In our hands, the TRA-171 cell line is endogenously infected by the insect specific flavivirus CFAV.

Chapter 7: Concluding Remarks

Antiviral RNAi in insects is employed by cells to detect long dsRNA molecules generated following a viral infection. Through their recognition by Dcr-2 they are digested into smaller fragments which are then utilised by Ago-2 to recognise complementary homologous coding sequences for degradation. Previously, the most comprehensive studies focusing on the involvement of RNAi in insect antiviral activities were carried out using *Drosophila*. However, the importance of understanding the fine workings of these important RNAi proteins in mosquitoes has become increasing recognised over the past number of years, in particular for the control of mosquito-borne arboviruses. Many of these viruses are highly pathogenic to both humans and animals and pose a substantial risk to their welfare, as well as to the economic and environmental health of infected areas. Conventional mosquito control methods, such as insecticides, breeding site reduction programmes and trapping, have proved to be valuble but difficult to implement and unsustainable. Certainly many arbovirus vectors are rancorous daytime biters and require the implementation of more complex protective apparatus compared to night-time feeders which may be efficiently controlled with properly installed bed nets. Therefore, alternative approaches are sought to stem the growing burden that arboviral infections place on populations across the globe. Understanding the intricacies of the complex interactions between arboviruses and the antiviral immune response will allow new insights into the mechanisms involved in viral limitation at the infection or replication level before transmission is able to occur to be determined. This can, for example lead to the generation of genetically modified mosquitoes or those with defined refractory alleles.

The aim of this project was to extend the scope of our understanding of the RNAi response in *Aedes* mosquitoes through the development of a molecular ‘tool-box’. Although RNAi studies in *Drosophila* benefit from an array of genetic and biological tools, these are conspicuously absent from mosquito research. Moreover, given the growing understanding that the little data already determined for mosquitoes does not always equate with what is known in *Drosophila*, it has been crucial to establish appropriate assays and biological tools in order to authenticate the RNAi response in mosquitoes. Indeed it has recently been brought to light that species specificities may exist, further highlighting the vital need for these tools (van Mierlo et al., 2014).

In **Chapter 3** an efficient gene silencing assay was developed for the knockdown of key RNAi proteins (Ago-1, Ago-2, Dcr-1 and Dcr-2) within the *Ae. aegypti*-derived Aag2 cell line. The treatment of cells with transcript specific dsRNA successfully reduced expression of each of the protein mRNAs after 24 h and revealed that the most significant influence on SFV infection was Ago-2. These findings are therefore in line with other literature in live mosquitoes which also suggests that Ago-2 knockdown enhances alphavirus and flavivirus replication and is a major effector in the antiviral RNAi defences (Keene et al., 2004, Li et al., 2004, Franz et al., 2006, Campbell et al., 2008, Sanchez-Vargas et al., 2009, Schnettler et al., 2013a, McFarlane et al., 2014). Due to the difficulties in generating stable knockout cell lines, establishing effective silencing assays was necessary. These have proved to be important for characterising the hierarchy of each protein and uncovering their roles within the antiviral response in mosquito-derived cell lines. It is known that the competence of different vector populations for disease transmission is influenced by the natural degree of variation in the expression of the RNAi genes (Lambrechts et al., 2013). Therefore, understanding those which play the most prominent role in immunity can drive the development of transgenic mosquitoes to present resistance to arboviral infections through engineering enhanced activity of the RNAi pathway (Adelman et al., 2002, Olson et al., 2002, Franz et al., 2006, Lambrechts et al., 2013). In addition, screening of certain variations in RNAi genes has the potential to aid disease monitoring by identifying vector populations either at risk of enhanced arbovirus transmission or those which are refractory. Certainly, it is important to understand the mechanism(s) involved in vector competency in order to develop novel control strategies and altering the competency of arboviral vector by genetic manipulation has been proposed as a viable approach (Crampton et al., 1990, Travanty et al., 2004).

Furthermore, the production of viral-derived small RNAs from the *Ae. aegypti*-derived Aag2 and *Ae. albopictus*-derived U4.4 cell lines was investigated through the use of Northern blot analysis and deep sequencing technology. Flaviviruses are the only arboviruses known to express inhibitors of RNAi (Pijlman et al., 2008, Schnettler et al., 2012, Kakumani et al., 2013) while SFV, as with other alphaviruses, is believed to evade the RNAi response rather than directly suppress it (Siu et al., 2011). Previously performed deep sequencing analysis determined that 21 nt viRNAs are produced following SFV infection in both cell lines (Siu et al., 2011); however, these were present in such small quantities that Northern blot detection could not be achieved, even against those known to be generated in the greatest concentrations. Many unanswered questions still remain regarding alphavirus replication within invertebrate cells and the kinetics of RNAi

detection and interaction with viral-derived dsRNAs, which is something this thesis aimed to go some way towards addressing. Certainly work described in **Chapter 3** determined that the small RNAs pattern of 'hot' and 'cold' spots is not limited to viral-derived dsRNA. Interestingly, non-target specific external dsRNA also exhibited this pattern of distribution across the coding sequence and that 21 nt was the predominant size produced implementing Dcr-2 processing. Therefore, it can be suggested that the RNAi response is activated by perfect non-viral dsRNA sequences and the 'hot' and 'cold' patterns are due to cellular processing and not as a result of a viral mechanism. Questions as to why some sequences appear to be targeted more than others remain to be clarified and it will be necessary to investigate these in depth in order to fully understand RNAi processing during viral infections. It may be suggested that this is a technical artefact produced due to the detection of both double-stranded siRNAs and single-stranded, incorporated siRNA molecules during the deep sequencing analysis. The 'hot' spot siRNAs may simply be those which are incorporated into RISC and used for silencing, therefore generating a greater number of reads compared to those which are double-stranded and have not been taken up. Alternatively there may be a degradation system in place which rapidly removes the passenger strand which is yet to be characterised. Validation of this hypothesis may only be achieved through RISC immunopreciptations and a method for establishing these in *Ae. aegypti*-derived cells was attempted in **Chapter 5**.

Chapter 4 discusses the interesting hypothesis that an RdRP exists within the genome of *Ae. aegypti*. Although RdRps are crucial for the amplification and spread of the RNAi signal in organisms such as plants and worms, only one group suggested they existed within the *Drosophila* genome (Lipardi et al., 2001, Lipardi et al., 2003, Lipardi et al., 2005, Lipardi and Paterson, 2009). However, since the initiation of this area of my project the suggestion was retracted; although, the authors still maintain that there is an association between the protein, D-elp1 and the RNAi mechanism through an interaction with Dcr-2 (Lipardi and Paterson, 2011). Therefore, it was interesting to understand if an orthologue of D-elp1 existed within the *Ae. aegypti* genome and if it did, was it expressed by the *Ae. aegypti*-derived Aag2 cell line and exhibit any discernible antiviral function. The data obtained from the investigations carried out during this study determined that although a homologue is encoded by *Ae. aegypti* (Aa-elp1) and is translated, the protein does not appear to act antivirally following SFV infection as established by transcript knockdown assays. There was also no evidence of increased virus replication detected following Aa-elp1 silencing. These findings are therefore in agreement with the majority of literature that *Drosophila* and mosquitoes do not express a functional RdRP and so, unlike plants and *C.*

elegans, do not appear to require the amplification of the RNAi signal in order to establish the robust antiviral RNAi response exhibited by these insects (Hammond et al., 2000, Zamore et al., 2000, Nykanen et al., 2001, Schwarz et al., 2002, Roignant et al., 2003).

The development and generation of biological tools for the study of *Ae. aegypti* RNAi proteins is presented in **Chapter 5**. In order to reduce our reliance on *Drosophila* systems, a number of strategies for monitoring the key RNAi proteins were investigated, with varying degrees of success. A major void in the field is the absence of efficient antibodies against the mosquito Ago-1, Ago-2, Dcr-1 and Dcr-2 proteins. Therefore, a number of antibodies were commissioned as part of this project and screened to determine their efficiency to recognise their targets. As a result of this work a number of candidate antibodies were selected against *Ae. aegypti* Ago-1, Dcr-1 and Dcr-2 proteins; however, unfortunately no successful antibodies were identified for Ago-2, which, as previously discussed, has been identified as the most important antiviral protein (Keene et al., 2004, Li et al., 2004, Franz et al., 2006, Campbell et al., 2008, Sanchez-Vargas et al., 2009, Schnettler et al., 2013a, McFarlane et al., 2014). Further work is required to optimise those potential successes for use in future experiments involving detection and tracking of the endogenous proteins during the activation of their respective pathways, as well as protein immunoprecipitations to determine bound small RNAs and associating proteins.

The second approach tackled was to produce fluorescently tagged versions of Ago-2 and Dcr-2 which could be monitored within the cell using confocal microscopy. Initial production of the proteins was problematic due to the lack of expression achieved using conventional insect OpEI2 promoters. Although an inconvenient expression system for the study of antiviral processes, protein production was achieved using the SFV subgenomic promoter. Both Ago-2 and Dcr-2 fused to their respective tags were cloned into the SFV VRPs and used to successfully infect *Ae. aegypti*-derived Aag2 cells and *Ae. albopictus*-derived U4.4 cells as well as mammalian BHK-21 cells. Unfortunately, this resulted in extensive over-expression of both proteins meaning that detailed co-localisation and kinetic studies would not be able to be performed accurately. In addition, performing an *in vitro* Dicer cleavage assay using Dcr-2 null phenotype *Ae. albopictus*-derived C6/36 cells did not allow the successful function of the Dcr-2 construct to be ascertained. Similarly, an alternative expression system using a cell-free, rabbit reticulocyte extract failed to generate any fluorescently tagged Dcr-2. Taken together these results highlight the difficulties faced when developing new means of studying mosquito systems.

However, there were two significant advances produced during this part of my project. Firstly, the creation of Ago-2 constructs with a V5 tag on either the C- or N- terminals was achieved and these were successfully cloned into the SFV replicon. Detection of the expressed V5 tag from BHK-21 cell extracts was accomplished by Western blot analysis using an anti-V5 tag. In the future these constructs will allow immunoprecipitation studies to be performed and permit analysis of the small RNAs incorporated into the active RISCs, in addition to protein-protein interaction studies to investigate co-factors associated with RISC. Secondly, the development of a plasmid containing the *Ae. aegypti* polyubiquitin (PUb) promoter for the control of gene expression within insect cells was accomplished. This promoter was shown to generate significantly higher levels of expression compared to the OpEI2 gene promoter. The adaptation of a promoter which is capable of providing a maintained level of gene expression within both mosquitoes *in vivo* and their derived cell lines would greatly augment studies into gene regulation, expression and function, in particular for arboviral research into immune genes and those related to virus replication and transmission (Anderson et al., 2010). Both of these developments will greatly benefit studies of the small RNA pathways in mosquitoes in the future.

The results discussed in **Chapter 6** indicate that, as with other arboviruses determined in the literature, SFV is able to replicate within the *Tx. amboinensis*-derived TRA-171 cell line. This was important to learn in order to establish that this cell line is a viable model for arbovirus research given the ample number of advantages associated with this large, non-haematophagous mosquito. Moreover, during the course of this research area two crucial findings were also made which further enhances the need to study this mosquito more closely. Firstly, the cell line was shown to perform RNAi if induced by dsRNA. Whether this is truly an active antiviral RNAi response or the adaptation of another small RNA pathway, for example the miRNA pathway, to act antivirally following the detection of exogenous dsRNA has yet to be confirmed. As with other mosquito species it will be necessary to determine if viral-derived small RNAs are produced to ascertain if there is an active exogenous RNAi response defending against a viral infection. It will also be necessary to determine if these insects are able to be infected by insect-specific viruses and if so does this evoke an antiviral reaction? This project also determined that the TRA-171 cells contained an endogenous insect-specific flavivirus, CFAV. Although the origin of this case of infection is unknown and may have occurred through contamination, it is important to understand how these infections affect mosquito immunity. Certainly, the interplay between endogenous viruses and arboviruses may have a significant impact on arbovirus transmission and/or vector fitness and fecundity in the field. It has been shown

that endogenous viruses may decrease the replication of subsequent/co-infecting arboviral infections by the 'super-infection exclusion' principle which is similar to the impact some strains of *Wolbachia* have on a mosquitoes ability to transmit some infections (Bolling et al., 2012, Hobson-Peters et al., 2013, Rainey et al., 2014). The discovery of endogenous viruses adds further complexity to vector: virus interactions and we are only just beginning to elucidate their involvement in modulating simultaneous arbovirus infections. However, it has been suggested that they may be utilised in the future as a potential control method of arboviral transmission and further work will be required to understand this preventative mechanism.

Overall the work presented as part of this thesis goes some way towards filling the void of tools available for researching RNAi in *Ae. aegypti*. It has been established that data obtained in *Drosophila* focused immunity experiments does not always translate well to mosquito based set-ups and these tools are necessary to perform appropriate experiments and achieve accurate results for viral infections in the natural vector system. In the long term these applications will be highly valuable in controlling the transmission, spread and emergence of highly important arboviruses through the use of transgenic mosquitoes or biological control mechanisms.

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Appendix 1- Publications

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