The triggers and mechanism of apoptosis in mammalian cells infected with Semliki Forest virus

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Declaration

I declare that all work included in this thesis is my own except where otherwise stated. No part of this work has been or will be submitted for any other degree of professional qualification

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Abstract

Multicellular organisms employ many tactics to defend themselves against invading pathogens. In an attempt to limit virus replication and spread, both innate and adaptive immune responses are activated. Apoptosis in response to many virus infections may be viewed as an innate immune response; it limits the time a virus has to replicate and produce new virus particles. Many viruses have adapted to cell death by inhibiting or delaying the apoptotic response. In order for a cell to undergo apoptosis in response to virus infection, the cell must first detect the virus. Different stages of the virus life cycle, such as virus entry or genome replication may act as signals. The signal detected and the apoptotic pathway activated may differ between viruses, between cell types and even between cellular differentiation states. Alphaviruses are medically and experimentally important RNA viruses. This thesis uses the alphavirus Semliki Forest virus (SFV) to investigate mechanism(s) of virus induced apoptosis.

Cell death in response to SFV infection is apoptotic, although perhaps not completely caspase dependent. During infection membranes of the mitochondria are disrupted, mitochondrial membrane potential is lost and caspases- 3, -8, and -9 are activated. Fas, RNase-L, ISG-12 and PKR are not required for cell death. PKR is however a strong inhibitor of early virus production; relative to wt mouse embryo fibroblasts (MEFs), MEFs with a disruption of the PKR gene produce SFV more rapidly and die more rapidly. Cellular detection of dsRNA can trigger signal transduction cascades resulting in activation of interferons, pro-inflammatory cytokines and pro-apoptotic pathways. Inhibition of these dsRNA induced cascades delays death of SFV infected cells demonstrating that detection of virus RNA replication is one trigger of apoptotic cell death. Synthesis of virus glycoproteins is not required for, but contributes to, cell death. SFV structural proteins build up in the endoplasmic reticulum triggering the unfolded protein response and activating the pro-apoptotic proteins caspase-12 and CHOP.

In summary, SFV infection of continuously cultured mammalian cells in vitro leads to caspase activation and apoptotic cell death. The apoptosis is triggered by the detection of dsRNA as well as the virus structural proteins building up in the ER; other factors may also play a role.

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Abbreviations

AIF	Apoptosis inducing factor
Apaf-1	Apoptotic protease activating factor 1
APS	Ammonium persulfate
ARE	Antioxidant response element
ATF	Activating transcription factor
ATP	Adenosine triphosphate
Bcl	B-cell lymphoma
BH	Bcl-2 homology
BHK	Baby hamster kidney
BIR	Baculovirus inhibitor of apoptosis protein repeat
BSA	Bovine serum albumin
BVDV	Bovine viral diarrhoea virus
c. *elegans*	*Caenorhabditis elegans*
CAD	Caspase-activated deoxyribonuclease
CARD	Caspase recruitment domain
Cardif	CARD adaptor inducing IFN-β
CD	Complement defence
Ced	Cell death abnormal
CHOP	C/EBP homologous protein
CNS	Central nervous system
CpG	Cytosine-phosphate-guanine
Crm	Cytokine response modifier
d1eGFP	short half-life ($t_{1/2}$= 1 hour), eGFP
DAB	Diaminobenzidine
DapI	4', 6-diamidino-2-phenylindole
DD	Death domain
DED	Death effector domain
dH_2O	Distilled water
DISC	Death-inducing signalling complex
DMEM	Dulbecco's modified Eagle's medium

DMSO	Dimethyl sulfoxide
dsRNA	Double stranded RNA
ECL	Enhanced chemiluminescent
EDTA	Ethylene diamine tetraacetic acid
EEEV	Eastern equine encephalitis virus
eGFP	Enhanced GFP
eIF2α	Eukaryotic translation initiation factor 2α
EndoG	Endonuclease G
ER	Endoplasmic reticulum
FADD	Fas-associated DD protein
FCS	Foetal calf serum
FKBP38	FK506-binding protein
g	Gravity
GCN4	General Control Nonderepressible 4
GFP	Green fluorescent protein
GMEM	Glasgow's minimum essential medium
GTP	Guanosine triphosphate
hsp	Heat shock protein
HSV	Herpes Simplex virus
i.n.	Intranasally
i.p	Intraperitoneally
IAP	Inhibitor of apoptosis protein
ICAD	Inhibitor of CAD
IFN	Interferon
Ig	Immunoglobulin
IMDM	Iscove's modified Dulbecco's medium
IPS-1	IFN-beta promoter stimulator-1
IRAK-2	Interleukin-1 receptor–associated kinase-2
IRE-1	ER-resident transmembrane kinase/endoribonuclease inositol requiring 1
IRF	Interferon regulatory factor
ISG-12	Interferon stimulated gene-12

JAK-STAT	Janus kinase - signal transducers and activators of transcription
KO	Knockout
LAT	latency-associated transcript
LB	Luria Bertani
LPS	Lipopolysaccharide
MAVS	Mitochondrial antiviral signalling
MDA5	Melanoma differentiation-associated gene 5
MDBK	Madin-Darby bovine kidney
MEFs	Mouse embryo fibroblasts
MHV	Murine gammaherpesvirus
mRNA	Messenger RNA
MyD88	Myelin differentiation marker 88
NBCS	New born calf serum
NBF	Neutral buffered formaldehyde
NFκB	Nuclear factor κB
NGS	Normal goat serum
NRF2	Nuclear factor erythroid 2 related factor 2
nsP	Non-structural protein
OAS	Oligoadenylate synthetase
P	Passage
PACT	PKR activator
PAMP	Pathogen associated molecular pattern
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PERK	PKR-like ER kinase
PI(3)KC3	PI(3) kinase class III
PIV	Parainfluenza virus
PKR	Protein kinase R
Poly I:C	Poly(cytidylic-inosinic) acid
PRD	Prion related domains
PS	Phosphotidylserine
PTP	Permeability transition pore

PUMA	p53 upregulated modulator of apoptosis
RIG-I	Retinoic acid-inducible gene-I
RNA	Ribonucleic acid
RNAi	RNA interference
RNase L	Ribonuclease L
ROCK-1	Rho-associated kinase 1
SDS	Sodium dodecyl sulphate
SeV	Sendai virus
SFV	Semliki Forest virus
SG	Stress granule
ssDNA	Single stranded DNA
STS	Staurosporine
SV	Sindbis virus
TBE	Tris-borate
TEMED	Tetramethylethylenediamine
TGF	Transforming growth factor
TIA-1	T-cell internal antigen-1
TIAR	TIA-1-related protein
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAF-6	Tumour necrosis factor receptor–associated factor 6
TRAIL	TNF-related apoptosis inducing ligand
TRIF	Toll/IL-1R domain-containing adapter inducing IFN-beta
tRNA	Transfer RNA
UPR	Unfolded protein response
UV	Ultraviolet
UVRAG	UV irradiation resistance-associated gene
v / v	Volume per volume
VEEV	Venezuelan equine encephalitis virus
VISA	Virus induced signal adaptor
VLP	Virus-like particles
VSV	Vesicular stomatitis virus

WEEV	Western equine encephalitis virus
WNV	West Nile virus
Wt	Wild-type
XBP1	X-box binding protein1
XIAP	X-chromosome-linked inhibitor of apoptosis protein
zVAD	zVAD-fmk

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Chapter 1: Introduction

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Introduction

In a multicellular organism, most virally infected cells have a predilection towards undergoing apoptosis. In response to virus infection, this reaction may be viewed as an arm of the innate immune system. For example, olfactory receptor neurons prevent dissemination of neurovirulent influenza A virus into the brain by undergoing virus-induced apoptosis (Mori *et al.*, 2002). This can be seen as a utilitarian act if the cell succeeds in committing suicide before large numbers of viral particles can be released. The effectiveness of apoptosis in response to virus infections is underlined by the existence of many anti-apoptotic strategies that are employed by different viruses to combat this defence mechanism. For example, cowpox virus expresses cytokine response modifier (Crm) A, which can inhibit specific caspases (Komiyama *et al.*, 1994; Nathaniel *et al.*, 2004), and baculovirus expresses a strong caspase inhibitor, P35 (Bertin *et al.*, 1996). The mechanism by which virus infection triggers the cell apoptotic response may vary between viruses, between cell types and even between cellular differentiation states. The aim of this project is to use a well-characterised model virus, Semliki Forest virus (SFV), to investigate the mechanism(s) by which a cell detects virus infection and undergoes cell death.

Alphaviruses

Alphaviruses are grouped in the family *Togaviridae* along with rubiviruses. There are about 40 members in the A*lphavirus* genus but only one in the *Rubivirus* genus, rubella virus. The *Togaviridae* are a family of positive single-stranded ribonucleic acid (RNA) viruses with a worldwide distribution. The RNA has a methylated nucleotide cap at the 5' end and is polyadenylated at the 3' end, thus resembling cellular mRNA. The genome is surrounded by a layer of capsid protein that is enveloped by a lipid membrane (when the virus buds) that is punctuated with virus glycoprotein spikes (Strauss & Strauss, 1994).

Alphaviruses are arboviruses, transmitted mainly by mosquitoes, although other vectors such as lice and mites are known to carry particular alphaviruses. The vertebrate hosts for each virus are dictated by the arthropod vector but are generally small mammals or birds. Larger mammals and humans are susceptible to infection

but are generally 'dead end' hosts because the viraemia established in these hosts is generally not high enough to allow efficient transmission of the virus (Gibbs, 1976). Symptoms in humans following alphavirus infection vary from asymptomatic to fever, arthralgia and encephalitis. Recently documented alphavirus outbreaks in human populations include, Ross River virus outbreaks in Southern Australia and Chikungunya virus infection on Reunion Island off the east coast of Africa, in India and in Italy and O'nyong nyong virus infections in Western Africa (Borgherini *et al.*, 2007; Horwood & Bi, 2005; Mavalankar *et al.*, 2007; Posey *et al.*, 2005; Rezza *et al.*, 2007). No specific treatments against alphavirus infections exist, but a number of vaccines have been developed against some members of the alphavirus family. Vaccines for horses against Western (W), Eastern (E) and Venezuelan (V) equine encephalitis viruses (EEV) are commercially available, but vaccines for humans are limited (Edelman *et al.*, 1979; Edelman *et al.*, 2003). Due to the high profile outbreaks of Chikungunya virus in recent times, a vaccine for this virus is expected to be developed in the near future.

Alphaviruses are worldwide but individual viruses are geographically restricted in their distribution; they have been found on all continents except Antarctica (Powers *et al.*, 2001). Viruses such as VEEV and WEEV are located in the Americas, O'nyong nyong and Semliki Forest virus (SFV) are both restricted to Africa, Chikungunya is spread throughout Southeast Asia, Southern India, parts of Europe and Africa, while Ross River virus is in Australia (Garmashova *et al.*, 2007b).

Alphaviruses are recognised as useful research tools, because of their comparatively simple genome and structure. Primarily, SFV and Sindbis virus (SV) have been utilised to study various aspects of cellular and virus biology, including viral encephalitis, the innate and adaptive responses of the immune system to virus infection and virus induced cell death. They have also been useful as tools to investigate other central nervous system (CNS) related diseases such as multiple sclerosis (Atkins *et al.*, 1999; Fazakerley, 2002; Fazakerley & Walker, 2003; Sammin *et al.*, 1999).

Alphavirus life cycle and genome

Alphaviruses enter cells by receptor mediated endocytosis (Helenius *et al.*, 1980; Marsh *et al.*, 1983). The receptor that alphaviruses bind to has not yet been identified, but once bound, an active clathrin-dependent endocytic pathway is required for successful infection (DeTulleo & Kirchhausen, 1998). The endocytosed vesicle fuses with an endosome and then a lysosome so that the contents can be broken down (Strous & Govers, 1999). The interior of lysosomes are more acidic than the cytosol. This low pH induces a conformational change in the glycoprotein spike complexes allowing the virus to fuse with the lysosome membrane and release the virus genome into the cytosol (Fuller *et al.*, 1995; Helenius *et al.*, 1982).

The alphavirus genome is relatively short (11.7 kb for SV and 11.5 kb for SFV) but can encode 9 different genes. The genome is split into 2 distinct open reading frames; the 5' two-thirds of the genome contains the genes that encode for the non-structural proteins (nsP1-4). The nsPs are primarily responsible for the replication of the virus genome. The 3' one-third of the genome codes for the structural proteins (capsid protein C, 6K, and the structural glycoproteins E1-E3) required to make new virus particles (Kaariainen *et al.*, 1987).

When the virus genome is released into the cytosol, it is treated as cellular mRNA by cellular translational machinery. The translation begins at the 5' end and the nsPs are made as a polyprotein. In many alphaviruses including SV, an opal termination codon exists between nsp3 and nsp4. This means that a nsP123 polyprotein is made in preference to the nsP1234 polyprotein. Readthrough can occur but only at a low frequency (10-20 %). In SFV this opal codon is generally replaced by a codon for arginine except in SFV A7(74). It is suggested that the opal codon may be responsible for the attenuation of SFV A7(74), because replacement of the opal codon with arginine increases the virulence of SFV A7(74) (Tuittila *et al.*, 2000). Having arginine in place of an opal codon means that a higher proportion of the nsP1234 polyprotein is made (Strauss *et al.*, 1988; Takkinen, 1986). The polyprotein gets processed into its individual proteins by nsp2, which has protease activity (Merits *et al.*, 2001). This processing always occurs in the same order and there is a lag period between each cleavage event. NsP4 is cleaved off almost immediately followed by nsP1 and then nsP2 and nsP3 separate (Kim *et al.*, 2004a).

Once processed the proteins stay together in a complex, capable of replicating the virus genome. The timing of the processing of the polyprotein dictates the type of RNA transcription that takes place. A complex of the nsP123 polyprotein and nsP4 efficiently makes minus strand RNA using the positive strand as a template. Once nsP1 is cleaved off the polyprotein and especially as nsP2 and nsP3 are separated, the complex looses its affinity for making minus strand and concentrates on making positive strand RNA. The transcription of positive strand RNA can start at the 3' end so that the whole strand is transcribed or it can start at a subgenomic promoter located 3' of the structural protein genes. The subgenomic RNA can be translated separately to make the structural proteins (Kaariainen *et al.*, 1987; Kim *et al.*, 2004a).

Each of the nsPs has multiple functions and they are all crucial to virus genome replication. NsP1 has methyl and guanyl-transferase activity, modifying the newly made virus RNA (Mi *et al.*, 1989; Scheidel & Stollar, 1991). NsP1 is also responsible for localising the virus genome (to which it is attached) to endosome-lysosome-like structures called cytoplasmic vacuoles that form during alphavirus infection (Kujala *et al.*, 2001; Spuul *et al.*, 2007). At the cytoplasmic vacuoles, the membranes invaginate inwards to form spherules, in which the replication of virus RNA takes place (Froshauer *et al.*, 1988; Kujala *et al.*, 2001).

NsP2, as explained previously is responsible for the processing of the virus polyprotein; it does have other functions however. NsP2 is known to have NTPase and helicase activity (Gomez de *et al.*, 1999; Rikkonen *et al.*, 1994). NsP2, in association with nsP1, is also responsible for the capping of newly made RNA, through its RNA triphosphatase activity (Vasiljeva *et al.*, 2000). Recently nsP2 has been shown to have an inhibitory affect on cellular interferon (IFN) production. This effect is dependent on the localisation of nsP2. NsP2 localises to the cytoplasm and to the nucleus, however if the nuclear localisation of nsP2 is disrupted then the amount of IFN-α/β released by infected cells increases and neurovirulence of SFV is reduced (Breakwell *et al.*, 2007; Fazakerley *et al.*, 2002). NsP2 has been shown to be cytotoxic when expressed individually from an expression vector. The cytotoxic effect of SV nsP2 is partly attributed to its ability to cause a shutdown of transcription through an unknown mechanism (Garmashova *et al.*, 2006). Mutations

in SFV nsP2 can reduce virus cytotoxicity, although this can quite often be attributed to a reduction in RNA synthesis rather than a direct cytotoxic effect of nsP2 (Tamm *et al.*, 2008)

NsP3 is a phosphoprotein but the function of nsP3 is as yet unclear, although some research suggests it plays a role in minus strand synthesis (De *et al.*, 2003; Peranen *et al.*, 1988; Wang *et al.*, 1994). NsP4 on the other hand acts as the RNA dependent RNA polymerase that is vital for virus genome replication. NsP4 has also been shown to have protease activity as it contributes to the cleavage of nsP4 from nsP3 (Kamer & Argos, 1984; Takkinen *et al.*, 1990).

Once the subgenomic RNA is made, translation of the structural proteins begins. A polyprotein is again made. The capsid protein has autoprotease activity and cleaves itself from the polyprotein very quickly (Aliperti & Schlesinger, 1978). The P62 protein is preceded by a signal sequence, which directs the synthesis of the following polyprotein to the ER (Bonatti *et al.*, 1984). E1 also has a signal sequence and both P62 and E1 have transmembrane domains which anchor them in the endoplasmic reticulum (ER) after synthesis (Melancon & Garoff, 1986). The ER and golgi then process the proteins and these are transported to the cell membrane. The processed glycoproteins then embed into the cell membrane while the capsid proteins assemble. The capsid proteins interact with a packaging signal present on the virus RNA and this interaction initiates assembly of the nucleocapsid core (Geigenmuller-Gnirke *et al.*, 1991; Weiss *et al.*, 1989). The nucleocapsid then interacts with the cytoplasmic tail of the E2 protein and this interaction stimulates budding of the virus particle (Lopez *et al.*, 1994). The 6K protein is important for correct assembly of the budding particles; if it is absent virus particles are made but with incorrect glycoprotein spike structures and reduced infectivity (McInerney *et al.*, 2004).

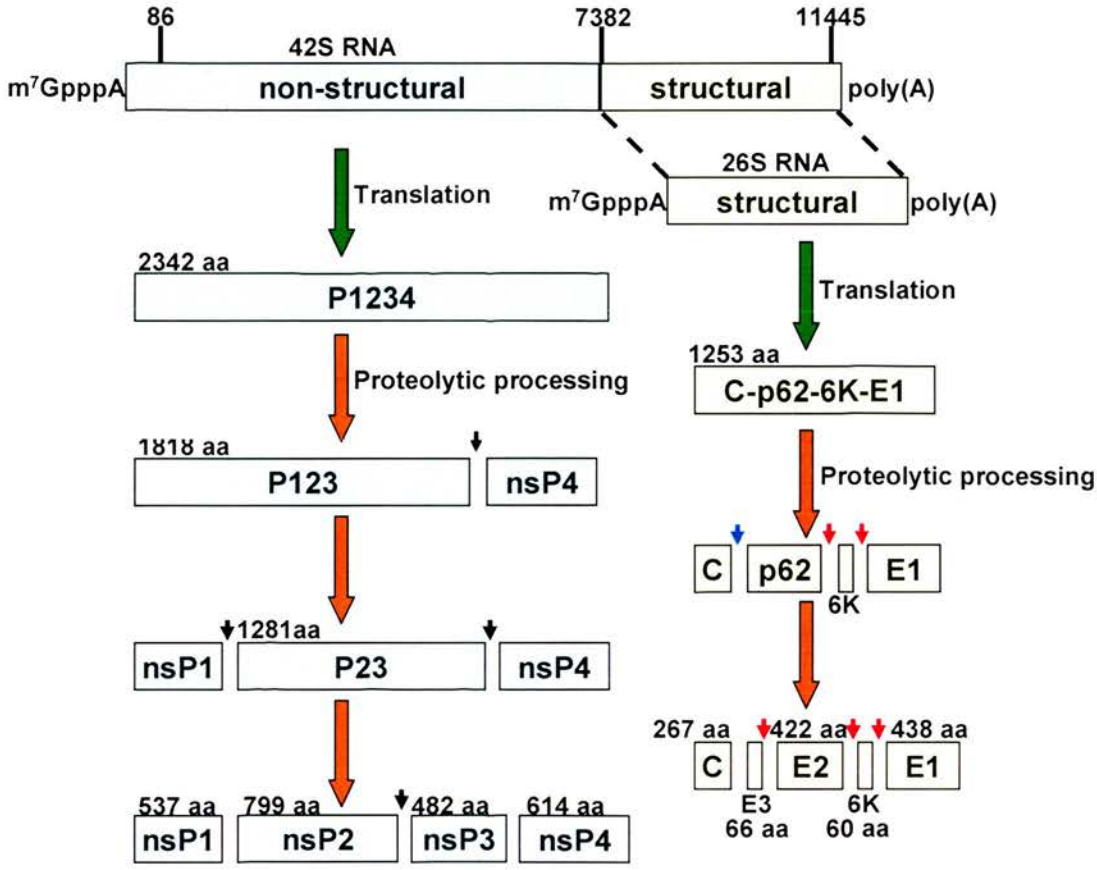

Figure 1.1: An illustration of alphavirus replication and protein production. Alphavirus non-structural proteins are translated first, allowing replication of the genome. The structural proteins are translated from the sub-genomic RNA. (reproduced from the thesis of Dr. Rennos Fragkoudis, University of Edinburgh)

Semliki Forest virus

SFV was first isolated from mosquitos in the Semliki Forest in Uganda in 1942 (Smithburn and Haddow, 1944). The isolated strain was passaged through mice on multiple occasions, causing the virus to adapt to mice and become more virulent. SFV is a neuropathogenic virus capable of causing encephalitis in a wide variety of mammals. The virus is neuroinvasive but can infect a broad range of cells, showing little tropism for a particular cell type *in vivo* or *in vitro* (Fazakerley *et al.*, 2006; Fragkoudis *et al.*, 2007). Only one lethal SFV infection has been documented in humans; a laboratory worker who was probably immunocompromised was infected with a strain of SFV that has now been taken out of laboratory circulation (Willems

et al., 1979). Natural infection with SFV causes a mild febrile illness with joint and muscle pain as was recorded by a study in Central Africa (Mathiot *et al.*, 1990).

In small mammals, the outcome of SFV infection depends on the age of the host and the strain of SFV used. Adult mice infected peripherally with an avirulent strain of SFV (e.g. A7(74)) do not show any symptoms of infection. The virus is detectable in the blood and muscle, and forms small foci of infection surrounding blood vessels in the brain. High titres of infectious virus are detectable in the brain during the first week (Amor *et al.*, 1996; Donnelly *et al.*, 1997; Fazakerley *et al.*, 1993). The type I IFN response to SFV infection is crucial to limit the spread of avirulent strains outside the CNS but is not responsible for the limited spread in the brain (Fragkoudis *et al.*, 2007). In *nu/nu* mice (mice that make immunoglobulin (Ig) M antibodies but cannot class switch to produce IgG antibodies and have no T-cells) SFV A7(74) persists in the brain at low levels for extended periods of time but is cleared from the periphery within 10 days of infection indicating that antibodies and / or T-cells are crucial for the clearance of SFV from the CNS. These *nu/nu* mice also lack demyelinating lesions that are normally present in SFV infected brains. The lesions are restored by reconstitution of the *nu/nu* mice with immune cells from immunocompetent mice suggesting the lesions are immune cell mediated and not directly caused by the virus (Fazakerley *et al.*, 1983). In neonatal mice the infection pattern is different; peripheral infection with SFV A7(74) leads to widespread infection in the periphery and the CNS, and death 2-4 days after infection. In contrast, virulent strains of SFV such as L10 spread rapidly in both adult and neonatal mice, causing death in 2-4 days (Fazakerley, 2002; Fazakerley *et al.*, 2006). Multiple sequence differences between the virulent and avirulent strains of mice have been found. Differences in the replicase genes nsP2 and nsP3 as well as differences in the E2 glycoprotein have all been implicated in affecting the neurovirulence of different SFV strains (Fazakerley *et al.*, 2002; Santagati *et al.*, 1995; Santagati *et al.*, 1998; Tuittila *et al.*, 2000).

The reason for the age-dependent death of mice infected with avirulent strains of SFV is unclear. It has been suggested that it is related to the susceptibility of neurons to undergo apoptosis in response to SFV infection (Allsopp & Fazakerley, 2000). Immature neurons in the neonatal mouse brain undergo programmed cell

death following SFV infection. Developing neurons in areas such as the olfactory bulb and cerebellum rapidly die following infection and exhibit morphological hallmarks of apoptosis. Other areas of the brain where neurons have fully developed show resistance to apoptosis, adopting a morphology reminiscent of necrosis but the cells tend not to die, instead remaining infected for extended periods of time (Allsopp & Fazakerley, 2000; Fazakerley *et al.*, 2006).

Semliki Forest virus: a useful tool

The SFV genome is relatively simple; containing only 9 genes, with a total size of 11.5 Kb. Using SFV complementary deoxyribonucleic acid (cDNA), the genome can be readily manipulated through mutations or the addition of new genetic material. Infectious DNA clones containing the SFV genome have been developed; these can be used to produce infectious virus if transcribed correctly in cells. Foreign genes can be inserted into these clones and then expressed efficiently upon infection of cells. For example SFV has been engineered with an eGFP gene inserted into the whole virus genome. This virus genome encodes both non-structural and structural proteins as well as eGFP. Because the whole virus genome is present, new, infectious virus particles can be made (Tamberg *et al.*, 2007). Given SFV's neurotropism, it can be used to study virus encephalitis and virus neuron interactions, as well as neuronal cell death responses, which are relevant to understanding the pathogenesis of virus encephalitis and to understanding fundamental virus cell interactions. SFV has also been used to study the mechanism of virus induced demyelination diseases such as multiple sclerosis. In the periphery and *in vitro* SFV triggers apoptosis in cells so it is an excellent tool to explore cellular apoptotic responses to virus infection. SFV vectors have been developed as vaccine delivery systems and for therapeutic gene therapy (Atkins *et al.*, 1996; Atkins *et al.*, 1999; Lundstrom *et al.*, 2001).

Biosafety of recombinant SFV systems can be increased by using replication deficient vectors. SFV virus-like particles (VLPs) are structurally identical to normal SFV but the encapsidated genome lacks sequences for the structural genes. A foreign gene sequence can be substituted in its place for example, a sequence coding for a short half-life, enhanced green fluorescent protein (d1eGFP). The inserted foreign gene (d1eGFP) is under the control of the virus subgenomic promoter and is

expressed when the structural proteins would normally be expressed (Smerdou & Liljestrom, 1999).

Apoptosis

Apoptosis is a form of cell death that was first described by Wyllie, Kerr and Currie in 1972 (Kerr *et al.*, 1972). The term was used initially to describe cells that displayed a unique morphology when they died. Kerr et al put forward the idea that apoptosis was crucial for cell number control in development and cancer. A good example to illustrate this is the development of the human hand. Initially a paddle like structure forms at the end of the arm but the cells in between the bones of the forming fingers die by apoptosis and are absorbed by the developing foetus, exposing the fingers (Milligan & Schwartz, 1997). Apoptosis has subsequently been found to be a highly conserved process.

Viewing an apoptotic cell from the outside

A shrinking cell is the first morphological sign that a cell is undergoing apoptosis (Kerr *et al.*, 1972). The cell membrane starts to pull inwards as the cell condenses in on itself. Inside the cell, cytoskeleton proteins such as actin and fodrin are cleaved by activated pro-apoptotic proteins (Cryns *et al.*, 1996; Mashima *et al.*, 1995). Intracellularly, the nucleus also condenses due to lamin cleavage (Kluck *et al.*, 1997). *In vivo* the condensed apoptotic cell will be phagocytosed at this stage (Fadok *et al.*, 1992a; Savill *et al.*, 1989). However, *in vitro* if all the cells are undergoing apoptosis phagocytosis cannot occur so dying cells continue to condense and eventually break up into pieces that are known as apoptotic bodies (Kerr *et al.*, 1972). Apoptotic bodies are membrane bound vesicles containing parts of the cells contents.

When cells undergo apoptosis, specific proteins are expressed on the cell surface, tagging them as apoptotic cells. Phosphotidylserine (PS) is a phospholipid localised to the inner surface of the plasma membrane (Seigneuret & Devaux, 1984). It can be detected on the outer surface of cells undergoing apoptosis (Fadok *et al.*, 1992b). The mechanism of transferring PS from the inner surface to the outer surface of the plasma membrane is still unclear. However, it is known that PS acts as a

phagocytosis marker to other cells when it is on the outer surface. A lack of PS on the surface of apoptotic cells affects phagocytosis (Shiratsuchi *et al.*, 2000). Annexin 1 is another protein that is translocated to the surface of apoptotic cells. This translocation is caspase dependent as it can be blocked by treating cells with a pan-caspase inhibitor. Annexin 1 colocalises with PS on the cell surface and is involved in apoptotic cell phagocytosis as inhibiting annexin exposure on the surface affects engulfment (Arur *et al.*, 2003). Calreticulin is expressed on the surface of apoptotic cells when an immune response is required. Calreticulin, normally located on the surface of the ER, can interact with receptors on the surface of engulfing cells (particularly dendritic cells) to initiate phagocytosis. Apoptotic cells lacking calreticulin or that have calreticulin blocked by an antibody are not phagocytosed efficiently. Calreticulin expression on the surface of apoptotic cells may be linked to immune system upregulation (increases engulfment by dendritic cells) so may only be expressed in certain situations such as apoptosis triggered by virus infection, or particular chemicals (Gardai *et al.*, 2005; Obeid *et al.*, 2007).

Inside an apoptotic cell

Apoptosis is a highly regulated process that can involve numerous protein activation cascades and the upregulation or downregulation of multiple different genes and proteins. The types of expression and activation profiles are dictated by the insult to the cell, but all result in death of the cell. Central to apoptosis are proteins known as caspases. Caspases are zymogens, becoming active only when specifically cleaved. Once active, caspases act as cysteine-dependent aspartate-directed proteases (Lazebnik *et al.*, 1994). Much of the early work on caspases was done in the worm *Caenorhabditis elegans* (C. *elegans*). The hermaphrodite form of the worm C. *elegans* has 959 cells when fully developed but starts off with 1090, losing 131 cells by programmed cell death during development (Sulston *et al.*, 1983; Sulston & Horvitz, 1977). A total of 14 genes are involved in apoptosis in C. *elegans* Hengartner & Horvitz, 1994). Ced (cell death abnormal) -3 and Ced-4 are two genes responsible for executing the cell death. If either of these genes is disrupted, the majority of the cells that normally die, survive (Ellis & Horvitz, 1986). Ced-9 also stops the cells from dying but only if it is over-expressed. If it is not expressed many

cells that would normally survive, as well as cells expected to die, undergo apoptosis (Hengartner *et al.*, 1992; Hengartner & Horvitz, 1994). Ced-9 has subsequently been shown to have homology with the mammalian gene Bcl (B-cell lymphoma) -2 and other Bcl-2 family members while Ced-3 is homologous with mammalian caspases and apoptotic protease activating factor (Apaf) -1 is the mammalian homologue of Ced-4 (Hengartner & Horvitz, 1994; Yuan *et al.*, 1993; Zou *et al.*, 1997). Ced- 9 interacts with Ced-4 preventing Ced-4 from interacting with Ced-3. Ced-4, Ced-3 interaction leads to apoptosis (Chinnaiyan *et al.*, 1997). Other C. *elegans* genes are involved in processes such as the timing of cell death (Ced-8) and engulfment after death (Ced-5, Ced-6, Ced-7, Ced-8 and Ced-10) (Ellis *et al.*, 1991; Stanfield & Horvitz, 2000). The work on programmed cell death in C. *elegans* has led to great advances in the understanding of mammalian apoptosis mechanisms.

The initial pathways of apoptosis

In mammals two reasonably distinct pathways that both lead to apoptosis are the 'intrinsic' and the 'extrinsic' or 'receptor mediated' pathways. The intrinsic pathway centres on the mitochondria. In response to signals that increase the permeability of the mitochondrial outer membrane and the subsequent release of cytochrome c, a complex of apoptotic protease activating factor (Apaf) -1 molecules, pro-caspase-9, cytochrome c and ATP, known as the apoptosome forms (Zou *et al.*, 1999). This mirrors the interaction of ced-4 and ced-3 in C. *elegans* (Wu *et al.*, 1997; Yan *et al.*, 2005). Upon forming this structure, caspase-9 is activated by autoproteolysis (Srinivasula *et al.*, 1998); active caspase-9 cleaves and activates the executioner caspases-3 and -7.

Cytochrome c release from mitochondria is crucial for correct apoptosome formation. Apaf-1 binding to adenosine triphosphate (ATP) causes a conformational change in Apaf-1. This conformation is stabilised by cytochrome c binding to Apaf-1 and allows multiple Apaf-1 proteins to bind together and interact with caspase-9, forming the apoptosome (Zou *et al.*, 1997; Zou *et al.*, 1999). Cytochrome c is synthesised in the cytoplasm as apocytochrome, which is transported to the mitochondria where a Heme group is attached to make it functional (Gonzales & Neupert, 1990). Cytochrome c then attaches to the inner mitochondrial surface and is

primarily involved in the production of ATP through the transport of electrons as part of the electron transport chain (reviewed in Zeviani & Di, 2004). The release of cytochrome c from the mitochondria during apoptosis requires the permeabilisation of the mitochondrial membrane. Bax and Bak, both proteins with homology to Bcl-2 at their Bcl-2 homology (BH) domains, are believed to oligimerise and bind to the surface of mitochondria forming a pore that allows the release of cytochrome c as well as other proteins. Bax and other Bcl-2 related proteins show homology to the T domain of diphtheria toxin. This toxin forms pores in lipid membranes as do Bcl-2 like proteins such as Bid and Bax (Kuwana *et al.*, 2002; Suzuki *et al.*, 2000). Studies using Bax and Bak doubly deficient cells or knockout (KO) mice show that cytochrome c release is impaired when apoptosis is stimulated using various different treatments (Wei *et al.*, 2001).

The activation and activity of Bax and Bak are not completely understood. Other pro-apoptotic factors include tBid, Bim and p53 upregulated modulator of apoptosis (PUMA). Activated T-cells normally die rapidly in culture; however activated T-cells lacking functional Bim and PUMA do not die. Two possible activation models exist to explain this. Firstly Bcl-xl has been shown to bind to Bak, preventing its oligomerisation. Bak is released when other BH3 only proteins (proteins with Bcl-2 homology at domain 3 only; proteins such as PUMA and Bim) bind to the Bcl-xl in preference to Bak (Willis *et al.*, 2005). The second model suggests a direct interaction between Bax, Bak and BH3 only proteins. The BH3 only protein tBid, when activated can bind to Bak and Bax, inducing their oligomerisation (Eskes *et al.*, 2000; Wei *et al.*, 2000). Bcl-2 and Bcl-xl have been shown to prevent oligomersation of Bax and Bak by sequestering BH3 only proteins (Cheng *et al.*, 2001). It is possible that a model incorporating both scenarios exists, whereby the affinity of different BH3 only proteins for anti-apoptotic Bcl-2-like proteins varies. For example, if Bax interaction with Bim is crucial for Bax oligomerisation, Bcl-2 bound to Bax and Bim will prevent their interaction. However if another protein with a greater affinity for Bcl-2 is expressed so that it displaces Bax and Bim then Bax and Bim can interact and Bax can oligomerise. Support for this model comes from studies by Cartron *et al*. These studies show that a physical interaction between Bax and particular BH3 only proteins is essential for Bax activation. The binding site

where this occurs is very similar to the binding site of Bcl-2 proteins, suggesting a competition between Bcl-2 proteins and Bax for BH3 proteins (Cartron *et al.*, 2004). Interestingly, the concentration of Bcl-2 may be crucial to its activity. If Bcl-2 is over-expressed, too much localises to the mitochondria and ER causing the membranes of these organelles to fragment, releasing calcium and cytochrome C. At lower concentrations it plays an anti-apoptotic role (Hanson *et al.*, 2008). Whether Bcl-2 ever reaches the high concentrations required for pro-apoptotic functions in physiological conditions is still unclear; the results may be an artefact of unnatural levels of Bcl-2. However, this is an area of intensive research.

Calcium uptake by mitochondria in a healthy cell is required to maintain the efficient operation of the Krebs cycle. However overload of calcium in mitochondria can lead to permanent opening of the permeability transition pore (PTP), swelling of the inner matrix and eventual bursting of the mitochondria. This enhances the release of cytochrome c as well as other apoptotic proteins that are in mitochondria such as apoptosis inducing factor (AIF) (Andreyev & Fiskum, 1999). Cytochrome c has been shown to induce the release of calcium from the ER by binding to inositol (1,4,5) trisphosphate receptors. This calcium release will then amplify cytochrome c release and increase the chances of apoptosome formation (Boehning *et al.*, 2003).

An alternative to the intrinsic caspase-9 mediated pathway of cell death is the extrinsic pathway initiated by caspase-8. This pathway is cell surface receptor mediated. The predominant family of receptors involved is the tumour necrosis factor (TNF) family, including members such as Fas, TNF-α and TNF-related apoptosis inducing ligand (TRAIL) receptor. These receptors all have an intracellular death domain (DD) (reviewed in Ashkenazi & Dixit, 1998; Itoh & Nagata, 1993). Ligand binding to their respective receptors allows oligomerisation of the receptors. This may induce a conformational change that allows the binding of adaptor proteins such as FADD (Fas-associated DD protein) to the DD of the receptor forming a protein complex known as a DISC (death-inducing signalling complex) (Kischkel *et al.*, 1995; Muzio *et al.*, 1996). This interaction activates FADD and it in turn interacts with caspase-8 through their common death effector domains (DED). Multiple caspase-8 molecules bind to the DISC and their close association allows proteolysis and activation of neighbouring proteins. Both Caspase-8 and FADD play

essential non-redundant roles in most death receptor pathways (Varfolomeev *et al.*, 1998; Yeh *et al.*, 1998). Caspase-8, once activated can directly activate caspase-3 (Enari *et al.*, 1996) and it can also activate the pro-apoptotic molecule Bid (Li *et al.*, 1998a). Bid can then translocate to the mitochondria and trigger cytochrome c release thus amplifying the death signal by triggering the intrinsic death pathway (Luo *et al.*, 1998).

The executioner pathways of cell death

Once caspases-8 and -9 are activated, caspase-3 and -7 are rapidly cleaved and activated. Caspase-3 can cleave a number of different proteins leading to the destruction of the cell. Caspase-3 can stimulate the release of more cytochrome c from the mitochondria through the cleavage of inactive caspase-8, enhancing caspase activation and also ablating the mitochondria's ability to function properly.

Caspase-3 can also target a number of proteins that target DNA. It is known to activate the protein caspase-activated deoxyribonuclease (CAD) by cleaving the inhibitor of CAD (ICAD). CAD can then enter the nucleus and degrade chromosomal DNA (Enari *et al.*, 1998). Another target of caspase-3 is PARP (Poly (ADP-ribose) polymerase). PARP repairs cut DNA, so by inhibiting PARP caspase-3 ensures that the degraded DNA isn't repaired. Caspase-3 also degrades cytoskeleton proteins as well as inhibitors of caspase-3, such as XIAP (X-chromosome-linked inhibitor of apoptosis protein) (Slee *et al.*, 2001). Caspase-3 has also been shown to cleave and activate the protein ROCK-1 (Rho-associated kinase-1). This protein is suggested to be responsible for the contraction of the cell and blebbing as the cell undergoes apoptosis by degrading numerous proteins responsible for the structural integrity of the cell (Coleman *et al.*, 2001).

Caspase independent cell death

Emerging evidence suggests that many pro-apoptotic proteins are involved in cell death independent of caspase activity. AIF and endonuclease G (endoG) are two proteins that are strongly implicated in this type of cell death. Both AIF and endoG are ordinarily located in the mitochondria where they have essential roles in cellular proliferation (endoG) and oxidative phosphorylation (AIF) (Huang *et al.*, 2006;

Vahsen *et al.*, 2004). When released from the mitochondria, it is suggested that both translocate to the nucleus and may be involved in DNA degradation. AIF has been shown to cause PS exposure on the cell surface, chromatin condensation and DNA fragmentation. If AIF is anchored to the mitochondria it can not carry out these functions (Cheung *et al.*, 2006; Daugas *et al.*, 2000). AIF carries out its roles by interacting with other proteins and activating them. It has been shown to interact with an endoG homologue in C. *elegans* and cyclophylin A in mice (Cande *et al.*, 2004; Wang *et al.*, 2002). EndoG has also been implicated in DNA degradation (van *et al.*, 2001).

Inhibition of apoptosis

Inhibitory proteins exist that counteract the activity of pro-apoptotic proteins. The Bcl-2 family has multiple pro- and anti-apoptotic members, all with BH domain homology. Bcl-2, Bcl-xl, Bcl-w, and Mcl-1 are all anti-apoptotic Bcl-2 family members. As mentioned previously, Bcl-2 for example binds to Bax, to prevent its insertion into mitochondrial membranes (Haraguchi *et al.*, 2000). This action can be abrogated by other BH3 only proteins.

Inhibitor of apoptosis proteins (IAPs) have the ability to bind to activated caspases and neutralise their activities. In mammals, the most extensively studied IAP is XIAP. XIAP can bind and inhibit processed caspases-9, -3 and -7 (Chai *et al.*, 2001; Deveraux *et al.*, 1998). DIAP1 plays a similar role in Drosophila (Meier *et al.*, 2000). If the gene for DIAP1 is deleted, widespread apoptosis ensues, resulting in early embryonic lethality (Wang *et al.*, 1999b). Interestingly however, if XIAP is deleted from a mouse, expression of other IAPs (cIAP1 and cIAP2) increase and the mouse develops normally, suggesting redundancy in the mammalian system. In Drosophila, the anti-apoptotic actions of DIAP are counteracted by the proteins Reaper, HID, Grim and Sickle (Chen *et al.*, 1996; Grether *et al.*, 1995; Srinivasula *et al.*, 2002; White *et al.*, 1994). All IAPs possess a 'baculovirus inhibitor of apoptosis protein repeat' (BIR) domain. Reaper, HID, Grim and Sickle can bind to this BIR domain and displace or prevent IAPs from binding to caspases. In mammals a number of proteins have been found to play similar roles to Reaper, HID, Grim and Sickle. Two of those proteins are Smac/Diablo and HtrA2/Omi. (Verhagen *et al.*,

2000; Verhagen *et al.*, 2002). Smac/Diablo and HtrA2/Omi are both ordinarily located in the mitochondria but are released along with other pro-apoptotic proteins. Smac/Diablo has been shown to interact with XIAP preventing it from binding and inhibiting caspase-9 (Ekert *et al.*, 2001). HtrA2/Omi can also bind and inhibit XIAP through a Reaper-like domain (Martins *et al.*, 2002). HtrA2/Omi is also suggested to have protease activity that is important in caspase independent cell death, although its exact role in this process is not completely clear (Blink *et al.*, 2004).

In Drosophila, a protein, Scythe, has been found to interact with Reaper. Depletion of Scythe from Xenopus egg extracts removes Reapers ability to induce apoptosis (Thress *et al.*, 1998). Evidence suggests that Scythe has anti-apoptotic characteristics that are suppressed by Reaper, and Reapers pro-apoptotic IAP inhibition properties may be reliant on its interaction with Scythe. Furthermore, isolated Scythe has no effect on mitochondria, DNA or caspases. Protein binding experiments have shown that Scythe can interact with a number of proteins including the chaperone heat shock protein (hsp) 70; when Reaper binds to Scythe it releases hsp70, which may then induce cytochrome c release from mitochondria by altering the tertiary confirmation and consequently activating pro-apoptotic proteins (Thress *et al.*, 1999; Thress *et al.*, 2001). Scythe has also been shown to be involved in the stabilisation of AIF (Desmots *et al.*, 2008). Further work is necessary to resolve the correct functions of Sythe.

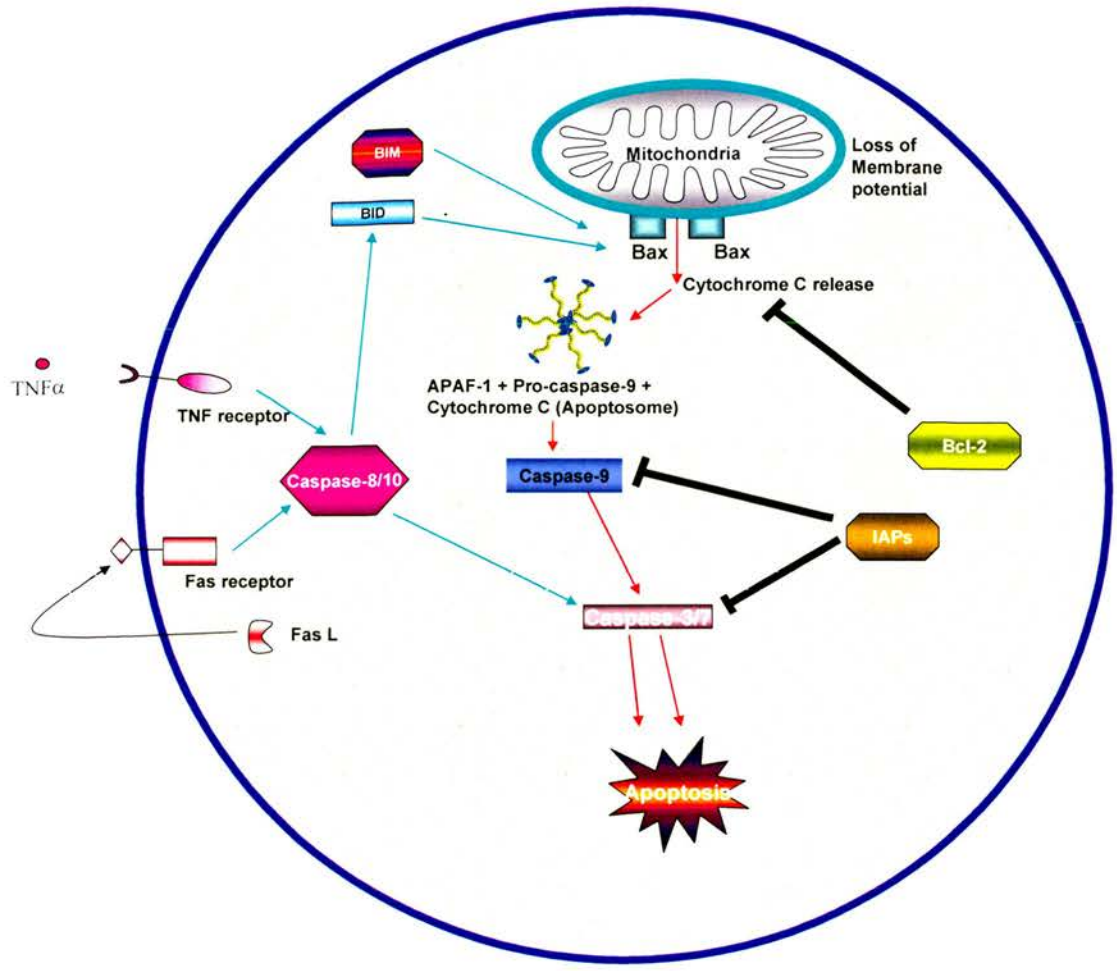

Figure 1.2: Apoptotic pathways involving caspases.
The extrinsic pathway of apoptosis acts primarily through the activation of caspase-8 which can activate caspase-3 directly or can activate the intrinsic pathway by causing cytochrome c release from the mitochondria. Cytochrome c associates with APAF-1, ATP and caspase-9, causing the activation of caspase-9. IAPs and anti-apoptotic Bcl-2 proteins can disrupt the apoptosis at various stages.

Virus infection can cause cells to undergo apoptosis

Apoptosis is an inherent cellular property that can be triggered by a variety of external stimuli including virus infection. Apoptosis as a result of virus infection could be triggered by the virus to aid its spread or it could be an altruistic gesture by an infected cell, stopping the virus from replicating and being released to infect surrounding cells. Numerous viruses have evolved pro- or anti-apoptotic genes to aid their replication and spread.

Many viruses, particularly DNA viruses, have evolved anti-apoptotic genes that express proteins capable of inhibiting apoptosis. The herpesvirus group have a

lytic and a latent phase to their life cycle. Expression of particular anti-apoptotic proteins are important for maintenance of herpesviruses in a latent phase. Murine gammaherpesvirus-68 (MHV-68) for example expresses a protein that is a homologue of mammalian Bcl-2. This protein helps the virus establish a latent infection *in vivo* by blocking mitochondria dependent apoptosis (de Lima *et al.*, 2005; Feng *et al.*, 2007). In fact all known gammaherpesviruses express at least one homologue of Bcl-2 (Hardwick & Bellows, 2003). Herpes Simplex virus (HSV) -1 encodes latency-associated transcript (LAT), a gene that is expressed during latent infection of neurons. The LAT protein has been attributed with anti-apoptotic activity; cells expressing the LAT protein have reduced caspase-3 activation and reduced DNA laddering in response to cold shock induced apoptosis (Carpenter *et al.*, 2007). Cytomegalovirus expresses a number of anti-apoptotic proteins, including M36, a protein that can suppress caspase-8 activation. M36 has been shown to bind directly to the caspase-8 pro-domain preventing it from being cleaved properly (Skaletskaya *et al.*, 2001).

Viruses in the poxvirus family are also well recognised for expressing anti-apoptotic proteins. The poxvirus life cycle does not involve a latent stage like herpesviruses. In contrast poxviruses inhibit apoptosis to allow time to replicate and produce large numbers of virus particles. Fowlpox virus expresses a Bcl-2 homologue that can bind to Bak and inhibit its activity (Banadyga *et al.*, 2007). A cowpox protein, cytokine response modifier A (Crm A), can inhibit caspase-8 and caspase-1 activation (Zhou *et al.*, 1997b). Vaccinia virus expresses a dsRNA binding protein called E3L. Vaccinia virus mutants lacking E3L are impaired; however, this impairment is lost in cells lacking functional protein kinase R (PKR). This implies that E3L is functionally inhibiting the actions of PKR (including it's pro-apoptotic function) in a vaccinia virus infection (Zhang *et al.*, 2008).

Some RNA viruses also express proteins that are homologues of mammalian apoptosis related proteins. Hepatitis C virus for example, expresses the protein NS5a, which has homology to Bcl-2. NS5a interacts with the pro-apoptotic protein Bax to suppress its activity and it also interacts with the anti-apoptotic FK506-binding protein (FKBP38) to promote survival (Chung *et al.*, 2003; Shirane & Nakayama,

2003; Wang *et al.*, 2006). Coxsackievirus B3 may inhibit apoptosis through the downregulation of p53 by an unknown mechanism (Kim *et al.*, 2004b).

Interestingly, some viruses have been shown to express pro-apoptotic homologues. The bunyavirus protein NSs shows sequence similarity to the Drosophila protein Reaper. Reaper as explained previously is a pro-apoptotic protein that binds to and inhibits IAPs. The bunyavirus NSs protein if expressed as an individual protein can induce cytochrome c release and caspase activation and can also contribute to shutdown of host protein translation (Colon-Ramos *et al.*, 2003). This however may not be the natural role of the NSs protein and could be as a result of over-expression of the protein. NSs has also been shown to downregulate pro-apoptotic signals downstream of IRF-3 (Kohl *et al.*, 2003). NSs causes downregulation of transcription through interaction with MED8, a protein that is important for RNA polymerase II mediated transcription (Leonard *et al.*, 2006). If NSs is removed from the bunyavirus genome virus propagation is severely affected (Bridgen *et al.*, 2001). This bunyavirus protein, NSs, may in fact encourage apoptosis, but only at a stage in its replication cycle when apoptosis of the host cell would facilitate the spread of the virus particles. This is similar to influenza A, whereby the activation of apoptosis through the upregulation of Fas and Fas ligand actually benefits the virus. This up-regulation is stimulated by nuclear factor κB (NFκB) activation and if NFκB activation is inhibited virus propagation is also affected (Wurzer *et al.*, 2004)

What stages of the virus life cycle elicit a response from the infected cell?

There are many interactions between viruses and host cells and these depend on the particular virus, the host cell type and probably its differentiation/activation state and also a variety of extracellular influences. The following section looks at virus host cells responses concentrating on SFV and on the mechanism(s) that may lead to cell responses including apoptosis and IFN production.

Virus attachment and entry

The first point at which virus might trigger cellular responses is attachment and entry. The coronavirus, mouse hepatitis virus, triggers cell death at the stage of attachment and entry. Oligodendrocytes infected with ultraviolet (UV) inactivated mouse hepatitis virus die despite lack of virus replication or protein expression (Liu *et al.*, 2006). Virus attachment and entry activates the Fas signalling pathway which triggers caspase-8 activation. Cell death can be inhibited by the prevention of Fas receptor Fas ligand binding (Liu & Zhang, 2007).

Vaccinia virus has also been shown to trigger apoptosis through attachment and entry. UV-inactivated replication deficient vaccinia virus infection of Chinese hamster ovary cells triggered apoptosis. Treatment with a neutralising antibody to the virus L1R protein inhibits attachment and inhibits apoptosis (Ramsey-Ewing & Moss, 1998).

It has been suggested that entry of SV triggers apoptosis by activating acid sphingomyelinase, which leads to the release of ceramide, although this evidence was inconclusive (Jan *et al.*, 2000). In other systems ceramide is known to elicit an apoptotic response, which can be blocked by Bcl-2 over-expression (Geley *et al.*, 1997; Zhang *et al.*, 1996). Ceramide has recently been shown to form pores in mitochondria walls, that are large enough to allow the release of cytochrome c (Siskind *et al.*, 2006). Bcl-2 family proteins can inhibit the formation of these pores (Siskind *et al.*, 2008). Acid sphingomyelinase can hydrolyse the phophodiester bond of sphingomyelin, yielding ceramide as part of the process (Haimovitz-Friedman *et al.*, 1997). The use of mice with a deletion in the acid sphingomyelinase gene has shown that cells lacking this enzyme are resistant to apoptosis, at least as provoked by ionising radiation (Lozano *et al.*, 2001). It is unclear whether SFV entry triggers apoptosis. An important point to note is that if the viral entry step to infection is bypassed and SFV RNA is directly transfected into cells, apoptosis still occurs (Glasgow *et al.*, 1998). This suggests that virus attachment and entry are not required to trigger cell death. However, it does not preclude this as one mechanism amongst others. UV-inactivated SFV, which can attach and enter cells but not replicate, does not trigger apoptosis implying that SFV attachment and entry do not induce cell

death (Personal communication, Dr. Asa Hidmark, Karolinska Institute, Stockholm, Sweden).

The detection of double-stranded (ds) RNA

Positive-strand RNA viruses replicate through a dsRNA intermediate, using their genome as a template. This process is then reciprocated, by using the newly made strands to make multiple copies of the virus genome. DsRNA is only seen in uninfected cells in the form of short interfering RNA and micro RNA. Above a size threshold of approximately 25 nucleotides, dsRNA is foreign to mammalian cells and illicites an immune response; therefore any reference to dsRNA triggering an immune response or apoptosis in mammalian in this thesis is a reference to dsRNA above this size threshold. Multiple dsRNA detection systems exist in mammalian cells with the ability to trigger anti-viral protein activation and expression in response to dsRNA. DsRNA, in the form of Poly (cytidylic-inosinic) acid (Poly I:C), triggers apoptosis in cells *in vitro* (Zhang & Samuel, 2007). Poly I:C also triggers the expression of IFN and other anti-viral proteins (Colby & Morgan, 1971).

Protein Kinase R

Protein kinase R (PKR) binds to and is activated by dsRNA. It was first discovered by treating cells with Poly I:C bound to sepharose and then isolating proteins that bound to the Poly I:C (Hovanessian & Kerr, 1979). Binding to dsRNA allows activation of PKR through homodimerisation (Wu & Kaufman, 1997). When dsRNA is present, PKR autophosphorylates and acts as a serine/threonine kinase to mediate an antiproliferative and proapoptotic response (Feng *et al.*, 1992; Koromilas *et al.*, 1992; Meurs *et al.*, 1993). In an unphosphorylated, dormant state, PKR is omnipresent at low levels and its expression can be upregulated by IFN-α/β. Activated PKR can modulate host cell translation by phosphorylation of the eukaryotic translation initiation factor 2α (eIF2α) (de Haro *et al.*, 1996; Lu *et al.*, 1999; Siekierka *et al.*, 1984; Srivastava *et al.*, 1998). PKR also activates the transcription factor nuclear factor κB (NFκB) which is important in many cell responses including production of IFN and pro-inflammatory cytokines (Kumar *et al.*, 1994). Activation of PKR can also induce apoptosis (Gil & Esteban, 2000).

Phosphorylation of eIF2α leads to a dramatic decrease in the levels of eIF2–guanosine triphosphate (GTP) –transfer (t) RNA^{Met}, the complex that is required for loading the initiator methionine onto the 48S preinitiation complex to begin translation. If eIF2α cannot be de-phosphorylated, Guanosine diphosphate (GDP) cannot be exchanged for GTP and consequently eIF2 cannot bind $tRNA^{Met}$ and translation cannot be initiated (Wek *et al.*, 2006). T-cell internal antigen-1 (TIA-1) and TIA-1-related protein (TIAR) that are normally located in the nucleus enter the cytoplasm upon eIF2α phosphorylation and bind to the 48S preinitiation complex (Kedersha *et al.*, 1999). This binding ensures that translation is strongly repressed. TIA-1 and TIAR both contain prion related domains (PRDs), which mediate aggregation into cytoplasmic granules known as stress granules (SGs) (Kedersha & Anderson, 2002). SGs contain mRNA and a number of eukaryotic initiation factors such as eIF2, eIF4E and eIF4G (Kedersha *et al.*, 2002; Kimball *et al.*, 2003). Up to 50% of the messenger RNA (mRNA) present in a cell can be located in SGs during times of stress (Kedersha *et al.*, 1999). This sequestering of RNA is selective, with the majority of mRNA transcripts from genes such as housekeeping genes brought into the SGs. Conversely, some transcripts are not sequestered; these include for example the transcripts from the hsp 70 gene (Harding *et al.*, 2000). This implies that a bias exists when cells are under stress allowing the continued production of proteins that may help the cell survive the stress.

SFV infection triggers eIF2α phosphorylation, causing shutdown of host cell translation but virus structural protein translation is unaffected. The phosphorylation of eIF2α is not essential for expression of virus structural proteins but when eIF2α is phosphorylated, the virus nsPs cannot be translated properly (McInerney *et al.*, 2005). In contrast the virus structural proteins are expressed normally (or possibly at a higher rate) when eIF2α is phosphorylated. When eIF2α is phosphorylated, the translational machinery probably focuses predominantly on the few mRNA sequences that can be translated correctly, which may lead to an increased rate of translation for the transcripts that are eligible. This avoidance of translational shutdown is due to a loop structure in the virus RNA, located at the 5' end of the capsid gene. This structure causes delay of the ribosome allowing it time to recruit $tRNA^{Met}$ correctly (Ventoso *et al.*, 2006).

PKR regulates NFκB through phosphorylation of its inhibitor, IκB (Kumar *et al.*, 1994). Phosphorylation of IκB targets it for polyubiquitination and subsequent degradation by the proteasome, thereby releasing NFκB. Release of NFκB allows it to act as a transcription factor for pro-inflammatory genes such as IFN (Tergaonkar, 2006). IFNs -α and -β play a pivotal role in the outcome of virus infections; they are crucial for the activation of numerous genes that play a role in innate resistance to virus infection. Mice deficient in receptors for IFN-α/β show a reduced ability to resist many viral infections (Muller *et al.*, 1994). If mice lacking an intact IFN system are infected intraperitoneally (i.p.) with SFV A7(74) and sampled 1 day post-infection, virus can be found throughout the periphery infecting almost all tissues. In contrast an intact IFN system prevents extensive spread of the virus with very few areas of infection detectable. Also mice lacking an intact IFN system die three days post-infection with SFV A7(74) whereas wild-type (wt) mice survive and remain asymptomatic (Fragkoudis *et al.*, 2007). IFN-α/β are generally expressed in response to virus infections and act through the janus kinase - signal transducers and activators of transcription (JAK-STAT) signal transduction pathway to activate and/or upregulate the expression of many anti-viral genes. IFNs can act both in an autocrine and a paracrine fashion, promoting anti-viral states in cells, by for example upregulating viral detection systems such as PKR and TLR3 (Weber & Haller, 2007).

PKR is vital for innate immunity against some virus infections. Infection of PKR deficient mouse embryo fibroblasts (MEFs) with vesicular stomatitis virus (VSV) results in apoptosis 18 hours post-infection; in contrast wt MEFs remain viable for at least 36 hours. However, PKR deficient MEFs remain viable when pre-treated with IFN and then infected with VSV. Furthermore, intranasal inoculation of VSV is lethal in PKR deficient mice whereas, wt mice exhibit no overt clinical signs, even when the level of infection is increased ten-fold compared to that in mutant mice (Balachandran *et al.*, 2000). HSV infection causes activation of NFκB and upregulation of multiple anti-viral genes. Activation of NFκB does not occur in HSV infected PKR KO MEFs implying that PKR is essential for a correct innate immune response to HSV infection (Taddeo *et al.*, 2003). PKR has also been shown to activate caspase-8 and caspase-9. PKR activates caspase-8 independently of Fas or

TNF-α, suggesting an interaction with FADD and caspase-8 (Gil & Esteban, 2000). Caspase-9 activation is downstream of caspase-8 activation suggesting that caspase-9 is activated through the cleavage of Bid by caspase-8 (Gil *et al.*, 2002).

Autophagy is a process that involves the controlled break down of cellular proteins by cellular enzymes. Proteins become sequestered by autophagosomes, which then probably fuse with lysosomes leading to protein degradation. Autophagy helps in the maintenance of homeostasis in a cell but can also be involved in cell death (Lerena *et al.*, 2008). PKR is required to activate autophagy when MEFs are infected with HSV. PKR KO MEFs do not activate an autophagic response when infected with HSV (Talloczy *et al.*, 2002). Beclin 1, in association with PI(3)KC3 (PI(3) kinase class III) and UVRAG (UV irradiation resistance-associated gene), promote autophagosome formation. It has been found that the HSV protein ICP34.5 binds to beclin 1 preventing it from interacting with UVRAG or PI(3)KC3. HSV lacking ICP34.5 is neuroattenuated *in vivo* but is neurovirulent in PKR KO mice (Orvedahl *et al.*, 2007).

Toll-Like receptors

Toll-like receptors (TLRs) are a family of proteins that can detect many different pathogen associated molecular patterns (PAMPs) (Salaun *et al.*, 2007). Activation of TLRs through the detection of specific PAMPS leads to activation of NFκB, and phosphorylation of interferon regulatory factor (IRF) -3 and -7 (Fitzgerald *et al.*, 2003; Salaun *et al.*, 2007). Detection of virus infection by TLRs is a multi-pronged system; TLR 9 can detect cytosine-phosphate-guanine (CpG) rich viral DNA, TLRs 7 and 8 detect single-stranded RNA, rich in guanine and uridine, while TLR3 responds to dsRNA (Alexopoulou *et al.*, 2001; Bauer *et al.*, 2001; Diebold *et al.*, 2004; Diebold *et al.*, 2006). Cellular localisation of TLRs is dependent on cell type. TLRs can be present on the cell surface; TLR 3 function in a human lung fibroblast cell line was inhibited by a monoclonal antibody that bound to it on the cell surface (Matsumoto *et al.*, 2002). In some cell types, TLR 3 localises to intracellular vesicles such as phagosomes and endosomes; here TLR 3 may detect dsRNA early in infection (Matsumoto *et al.*, 2003). TLR 7, 8 and 9 all require endosomal association to mature and TLR 7 and 9 have been shown to predominantly localise to endosomes

rather than the cell surface (Heil *et al.*, 2003; Nishiya *et al.*, 2005; Nishiya & Defranco, 2004). Activated TLR 3 interacts with and activates Toll/IL-1R domain-containing adapter inducing IFN-beta (TRIF) (Kaiser & Offermann, 2005; Ruckdeschel *et al.*, 2004; Salaun *et al.*, 2006). The other virus detection TLRs, TLR 7, 8 and 9, all associate with the adaptor protein Myelin differentiation marker 88 (MyD88) in preference to TRIF (Takeuchi & Akira, 2007).

Mice deficient in TLR 3 have reduced responses to Poly I:C with reduced production of inflammatory cytokines (Alexopoulou *et al.*, 2001). However the importance of TLR 3 in virus infection is less clear cut. TLR 3 deficient mice have the same level of susceptibility to infection and course of virus pathogenesis as wt mice, when infected with VSV or lymphocytic choriomeningitis virus (Edelmann *et al.*, 2004). In contrast to these results, TLR 3 has been shown to play a role in West Nile virus (WNV) infection of mice. TLR 3 KO mice have enhanced viral load and impaired cytokine production in response to peripheral WNV infection (Wang *et al.*, 2004). The importance of TLR 3 in virus infections is emphasised by the existence of a number of viral evasion mechanisms. It has been shown that vaccinia virus expresses two proteins, A46R and A52R, that can target and inhibit the TLR 3 signalling pathway at different stages. A46R can bind to the TLR 3 adaptor protein, TRIF and disrupt its signalling whereas A52R binds to interleukin-1 receptor–associated kinase-2 (IRAK-2) and tumour necrosis factor receptor–associated factor-6 (TRAF-6) to prevent NFκB activation (Harte *et al.*, 2003; Stack *et al.*, 2005). The hepatitis C virus protein NS3/4A also targets and cleaves TRIF, preventing TLR 3 signalling (Li *et al.*, 2005a) .

In certain cell types both TLR 7 and 8 are essential for efficient responses to virus infection. TLR 7 and 8 are not required in fibroblast cell lines infected with Sendai virus (SeV), but are utilised in myeloid cells to detect the same infection (Melchjorsen *et al.*, 2005). Similarly, both influenza and VSV infections are detected in plasmacytoid dendritic cells and B cells by TLR 7 and 8. leading to the upregulation of numerous anti-viral cytokines (Lund *et al.*, 2004). TLR activation can lead to cytokine expression including IFN and also to apoptosis. TRIF can activate caspase-8 (Kaiser & Offermann, 2005). MyD88 activation can activate caspase-8 via an interaction with FADD (Aliprantis *et al.*, 2000).

Ribonuclease L

Ribonuclease (RNase) L is similar to PKR because it responds to dsRNA but in contrast it is not directly activated by dsRNA. 2',5'-oligoadenylate synthetase (OAS) acts as the link between dsRNA and RNase L; 2'-5' OAS is activated by binding to dsRNA (Sarkar & Sen, 1998). Once activated 2'-5' OAS binds to RNase L allowing RNase L homodimerisation and activation (Dong & Silverman, 1995). Activated RNase L can cleave both cellular and virus single-stranded (ss) RNA leading to downregulation of protein production; some evidence suggests that RNase L targets viral RNA in preference to cellular RNA through an unknown mechanism (Floyd-Smith *et al.*, 1981; Li *et al.*, 1998b).

Treatment of L929 cells with a combination of Poly I:C and IFN-α/β results in a 90 % decrease in cell viability. However, when RNase L is inhibited, L929 cells remain viable significantly longer (Castelli *et al.*, 1997). HeLa cells exhibit a similar pattern in their reaction to poliovirus infection; inhibition of RNase L in these cells suppresses virally induced apoptosis (Castelli *et al.*, 1997). Similarily, MEFs deficient in RNase L do not die when infected with SV. SV establishes a persistant infection in these cultures (Sawicki *et al.*, 2003). This however, is not the case with SFV; SFV infections produce 10 fold higher concentrations of RNA than SV and SFV causes apoptosis in MEFs deficient in RNase L (Sawicki *et al.*, 2003). In vivo, mice triply deficient in PKR, RNase L, and Mx-1 (another gene involved with viral immunity) infected with SV, showed no change in the course of infection (Ryman *et al.*, 2002).

Retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5

Retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) are both RNA helicases that bind to dsRNA (Yoneyama *et al.*, 2004). Both proteins also contain two caspase recruitment domains (CARD) at their N-terminal ends (Yoneyama *et al.*, 2005). Binding to dsRNA, stimulates structural changes in RIG-I and MDA5, exposing their CARD domains and allowing interaction with the mitochondrial bound protein virus induced signal adaptor (VISA), which activates NFκB, and IRF-3 (Seth *et al.*, 2005). VISA is also known as

mitochondrial antiviral signalling (MAVS) (Xu *et al.*, 2005), IFN-beta promoter stimulator-1 (IPS-1) (Kawai *et al.*, 2005) and CARD adaptor inducing IFN-β (Cardif) (Meylan *et al.*, 2005). The functions of RIG-I and MDA5 do not compensate for each other. Recent evidence suggests that one of the main ligands for RIG-I is single-stranded RNA with a 5'-triphosphate end; MDA5 is not activated by this (Hornung *et al.*, 2006; Pichlmair *et al.*, 2006). It is also suggested that while both RIG-I and MDA5 can detect certain virus infections, particular infections require one or the other. For example, RIG-I is essential for detection of influenza A virus, influenza B virus, and human respiratory syncytial virus infection. SeV infection generally stimulates high levels of NFκB and IFN-β expression as well as IRF-3 phosphorylation through RIG-I activation but does not in RIG-I KO cells (Loo *et al.*, 2008). If MAVS is depleted from HEK 293 cells, following SeV infection the levels of NFκB and IFN-β expression and IRF-3 phosphorylation do not increase indicating that IRF-3 interaction with RIG-I is mediated by MAVS (Seth *et al.*, 2005). Interestingly MDA5 is not required for immune signalling in response to SeV but is required for SeV defective interfering particles (Yount *et al.*, 2008). Detection of reovirus or SeV infections of mammalian cells leads to apoptosis and this apoptotic response is suppressed in the absence of MAVS indicating that MAVS, probably through IRF-3 activation can contribute to an apoptotic response to virus infection (Holm *et al.*, 2007; Peters *et al.*, 2008).

RIG-I and MDA5 activity can be regulated by cellular inhibitors and by viral inhibitors. LGP2 is a cellular protein that acts as a negative regulator of both RIG-I and MDA5. LGP2 is closely related to MDA5 and RIG-I, has a helicase domain but has no CARD domains. The helicase domain allows LGP2 to bind to RNA, preventing RIG-I or MDA5 from binding to it (Saito *et al.*, 2007). LGP2 is upregulated by IFN and can interact directly with MAVS, preventing RIG-I and MDA5 interaction with MAVS (Komuro & Horvath, 2006). NLRX1, is a recently discovered cellular protein that inhibits MAVS activation. SeV infection of HEK293 cells triggers NFκB activation and IFN expression. If NLRX1 levels are knocked down by RNA interference (RNAi), then NFκB and IFN levels increase dramatically. Also by immunoprecipitation, NLRX1 has been shown to interfere with the binding of RIG-I to MAVS. NLRX1 is located on the outer membrane of mitochondria and it

is suggested that NLRX1 binds to the CARD domain of MAVS, competing with RIG-I (Moore *et al.*, 2008; Tattoli *et al.*, 2008).

Some viruses have developed ways of evading MDA5 or RIG-I mediated detection. The paramyxovirus V protein for example, binds to the helicase domain of MDA5, preventing it from binding to dsRNA (Andrejeva *et al.*, 2004; Childs *et al.*, 2007). The influenza A protein NS1, targets RIG-I and MAVS, binding to both and preventing downstream signalling (Mibayashi *et al.*, 2007). The hepatitis C virus protein NS3/4a cleaves MAVS, releasing it from the mitochondria and preventing it from functioning correctly (Chen *et al.*, 2007; Kaukinen *et al.*, 2006).

Many viruses have evolved to protect their dsRNA rather than / as well as targeting the proteins that detect the dsRNA. Reoviruses express the outer capsid protein Sigma 3, which binds to dsRNA (Imani & Jacobs, 1988). Interestingly different reoviruses induce different levels of IFN from infected cells and these have been attributed to the affinity of the virus Sigma 3 protein for dsRNA (Bergeron *et al.*, 1998). The vaccinia virus protein E3L also binds dsRNA, preventing its detection and reducing IFN production (Chang & Jacobs, 1993; Garcia *et al.*, 2002). The Ebola virus protein, VP35 binds dsRNA and also suppresses RNA silencing (Cardenas *et al.*, 2006; Haasnoot *et al.*, 2007). The influenza A virus protein NS1, as mentioned above, can target RIG-I and MAVS. As well as that however it can bind to dsRNA, enhancing further the inhibition of IFN induction (Lu *et al.*, 1995).

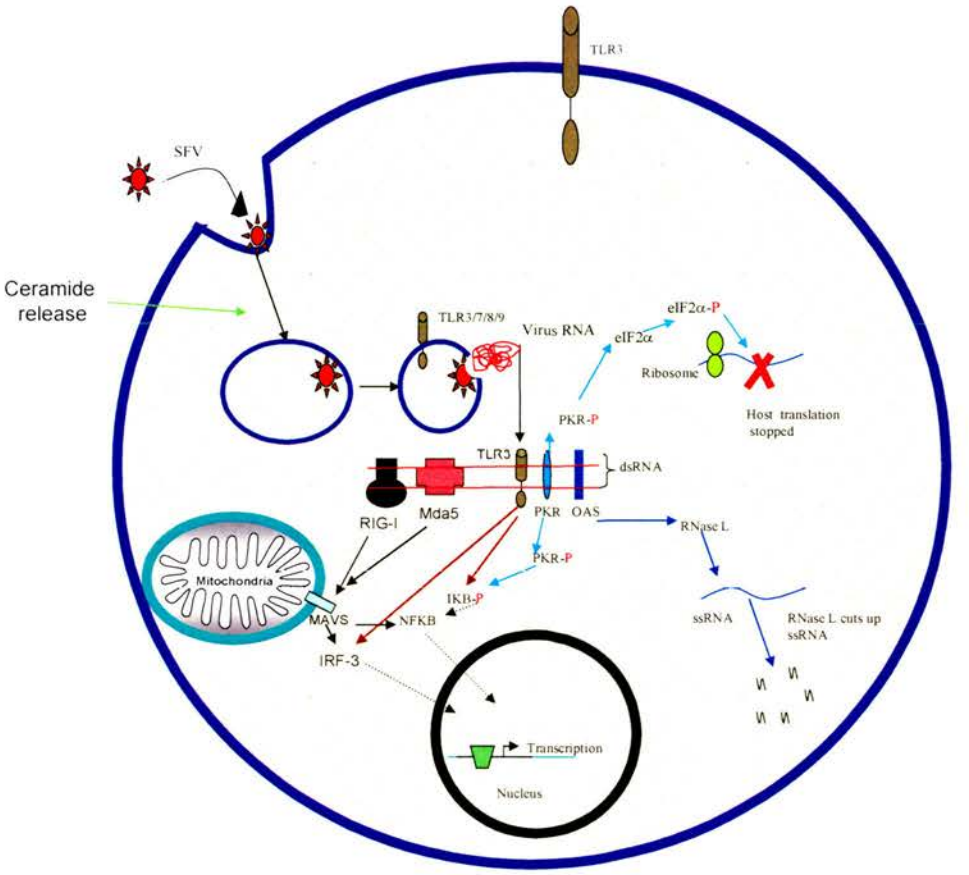

Figure 1.3: Illustration of dsRNA detection mechanisms and the major downstream signalling pathways established as a result of dsRNA detection. PKR, 2'-5' OAS, MDA5, TLR3 and RIG-I all detect dsRNA and activate numerous downstream pathways (see text).

Virus structural proteins may build up in the endoplasmic reticulum:

The unfolded protein response

The SFV genome codes nine distinct proteins. The 26S RNA species encodes for the capsid protein C, 6K and the structural glycoproteins E1, E2 and E3. These viral proteins may act as a trigger for apoptosis. The transmembrane domains of the SV envelope glycoproteins expressed from an inducible plasmid in AT3 cells are sufficient to induce apoptosis (Joe *et al.*, 1998). Transfected SFV RNA, with a deletion of all the structural genes, still induces apoptosis implying that the structural proteins may play a role but are not required for apoptosis (Glasgow *et al.*, 1998).

Virus glycoproteins could trigger apoptosis or other cell responses by activation of the unfolded protein response (UPR). The ER is an organelle consisting of interwoven cisternae, responsible for many of the final modifications of newly synthesised proteins, to ensure they fold correctly and are functional. In the ER and then the Golgi protein disulphide bonds are created, proteins are glycosylated and transported to different areas of the cell according the signal sequences on the protein. The ER also acts as a regulator of protein concentrations in the cell; the ER can up-regulate or down-regulate protein synthesis and folding. Accumulation of proteins in the ER can provoke a stress response, which can lead to up-regulation of protein folding, down-regulation of protein translation and induction of cell death if the protein build up cannot be resolved. This response is known as the unfolded protein response (reviewed in Schroder & Kaufman, 2006). The Ig heavy chain binding protein BiP (GRP78) is an ER chaperone and was the first hsp70 protein discovered (Haas & Wabl, 1983). BiP has a dual function and its localisation reflects this, being found in the ER lumen and also embedded in the ER membrane (Bole *et al.*, 1989). BiP's primary role is as a chaperone, targeting unfolded proteins containing a large proportion of hydrophobic amino acids (Blond-Elguindi *et al.*, 1993). In doing this BiP translocates from the ER membrane into the ER lumen and binds the hydrophobic regions of unfolded proteins. At the ER membrane, BiP is ordinarily bound to a number of different proteins. BiP serves as a negative regulator of the ER stress proteins PKR-like ER kinase (PERK), ER-resident transmembrane kinase/endoribonuclease inositol requiring 1 (IRE1) (Bertolotti *et al.*, 2000) and activating transcription factor (ATF) 6 (Shen *et al.*, 2005). BiP binds to these proteins only releasing from them when it binds unfolded proteins in the ER lumen; over-expression of BiP prevents activation of the UPR (Dorner *et al.*, 1992). Unbound PERK or IRE1 can homodimerise and become activated. Unbound ATF6 does not need to dimerise and instead once released from BiP, a Golgi localisation site is revealed, allowing it to translocate to the Golgi where it is processed further (Chen *et al.*, 2002c; Shen *et al.*, 2002). The transcription factor domain of ATF6, bZIP is cleaved off and translocates to the nucleus where it upregulates a number of stress response elements such as protein chaperones by binding to different promoters (Kokame *et al.*, 2000; Wang *et al.*, 2000). PERK, must oligomerise to

become activated and once activated it has two main targets. PERK, like PKR phosphorylates eIF2α causing protein translation shut-down (Harding *et al.*, 1999). This dramatically reduces the amount of protein that is being made in the cell, giving the ER time to resolve the unfolded protein build-up problem. PERK also phosphorylates the bZIP transcription factor nuclear factor erythroid 2 related factor 2 (NRF2) (Cullinan *et al.*, 2003). When phosphorylated, NRF2 moves from the cytoplasm to the nucleus, associates with other transcription factors and binds to the antioxidant response element (ARE) (Cullinan & Diehl, 2004). This upregulates the expression of proteins involved in reducing reactive oxygen species which can cause oxidative stress in the cell.

Interestingly, certain cell stress proteins can still be made in the presence of eIF2α phosphorylation. ATF4 for example actually requires eIF2α phosphorylation, before it can be translated. For translation to initiate the ribosomal 40S and 60S subunits must bind at a starter sequence and then move along the RNA until a stop codon is reached. At this point, the 60S subunit disassociates but the 40S remains attached to the RNA. For initiation of translation to occur, $tRNA^{Met}$-eIF2-GTP must bind to the ribosome 40S subunit before reaching a start codon. At the start codon this gets converted to GDP, the 60S subunit binds and translation begins. If eIF2α is phosphorylated, $tRNA^{Met}$-eIF2-GDP cannot be converted back to GTP thus limiting the initiation of translation. When a stop codon is reached, the 60S subunit is released but the 40S codon remains attached to the RNA. Translation can only reinitiate if another $tRNA^{Met}$-eIF2-GTP binds. If the concentration of $tRNA^{Met}$-eIF2-GTP is low then the 40S can pass through start codons without reinitiating translation. The open reading frame of ATF4 is downstream of other start codons. If a high concentration of $tRNA^{Met}$-eIF2-GTP is present, translation starts at the first open reading frame and then reinitiates quickly. However if the concentration of, $tRNA^{Met}$-eIF2-GTP is low (when eIF2α is phosphorylated) the 40S subunit reads through until it finally binds with, $tRNA^{Met}$-eIF2-GTP and translation is initiated at the next start codon. This time delay allows the ribosome to reach the ATF4 open reading frame and ATF4 to be translated. To examine this ER stress mediated translation and monitor ATF4 expression level changes *in vitro*, the 5' end of ATF4 was linked with luciferase expression and when ER stress was stimulated, luciferase

expression increased 500-fold (Harding *et al.*, 2000). This type of expression system is highly similar to the way GCN4 is controlled in yeast during nutrient deprivation (Hinnebusch, 2005).

ATF4 is a transcription factor that upregulates a number of different genes including CHOP (C/EBP homologous protein) (Talukder *et al.*, 2002). CHOP has pro-apoptotic activity; it suppresses the expression of the Bcl-2 gene, dramatically downregulating the amount of Bcl-2 RNA and protein made in the cell. This releases pro-apoptotic proteins to trigger apoptosis. The unfolded protein response does not always lead to the death of the cell. Early protein chaperone expression is an attempt to reduce the unfolded protein burden, but if this problem cannot be removed, the cell is likely to trigger cell death (McCullough *et al.*, 2001).

IRE1 is activated in a similar fashion to PERK; release from BiP and dimerisation on the ER membrane. Dimerisation causes autophosphorylation and a conformational change to expose an RNase domain similar to RNase L (Bork & Sander, 1993; Shamu & Walter, 1996). IRE1 detaches from the ER and cleaves specific RNA sequences in the cytoplasm. Two mRNAs it cleaves are that of X-box binding protein (XBP1) and complement defence (CD) 59 (Calfon *et al.*, 2002; Oikawa *et al.*, 2007; Yoshida *et al.*, 2001). The cleavage of CD59 by Ire1 prevents it from being translated, thus downregulating the levels of CD59 protein. CD59 is involved in the complement system, but it is unclear what role it plays in the UPR. The splicing of XBP1 mRNA establishes a sequence that can be translated into a transcription factor that upregulates the expression of ER stress proteins including protein chaperones (Yoshida, 2007). The unspliced mRNA can be translated into a protein that lacks a transcriptional activation domain, but it has been found to bind spliced XBP1 protein, increasing its degradation (Yoshida *et al.*, 2006).

As mentioned previously, caspases are generally integrally involved with programmed cell death, and ER stress induced cell death is no exception. In rodents, pro-caspase-12 is located on the surface of the ER. It is only activated in response to ER stress and no other known cellular insults (Nakagawa *et al.*, 2000). No functional form of caspase-12 exists in humans but human caspase-4 is believed to carry out similar functions to caspase-12 in response to ER stress (Kim *et al.*, 2006a). ER stress can cause calcium release from the ER, which can lead to calcium build up in

the mitochondria, triggering permeabilisation of the mitochondria and release of pro-apoptotic proteins (Deniaud *et al.*, 2008). ER stress induced calcium release into the cytoplasm from the ER can also cause the activation of caspase-12 (Nakagawa *et al.*, 2000). Calcium accumulation, triggers calpain activation in the cytoplasm and calpains are suggested to be responsible for caspase-12 activation; caspase-12 is not activated in calpain-null MEFs (Nakagawa & Yuan, 2000; Tan *et al.*, 2006). Upon activation, fragments of caspase-12 can translocate to the nucleus possibly acting as a transcription factor (Fujita *et al.*, 2002; Rao *et al.*, 2001; Sanges & Marigo, 2006). Caspase-12 can also cleave caspase-9 independent of APAF-1 thus triggering the intrinsic pathway of cell death (Rao *et al.*, 2002). Caspase-12 has also recently been shown to reduce cellular inflammatory responses. Caspase-12 binds to the proinflammatory protein caspase-1, inhibiting its function. Mice lacking caspase-12 are more susceptible to sepsis, but respond more effectively to bacterial infections (Saleh *et al.*, 2006). Infection of Madin-Darby bovine kidney (MBDK) cells with bovine viral diarrhoea virus (BVDV) correlates with activation of PERK, hyperphosphorylation of eIF2α, activation of caspase-12, downregulation of Bcl-2 and apoptosis (Jordan *et al.*, 2002).

Pro-apoptotic cell surface receptors may be activated

The TNF superfamily of receptors are well known regulators and effectors of the innate and adaptive immune systems (Benedict & Ware, 2001; Locksley *et al.*, 2001). As explained previously Fas is a known initiator of an apoptotic pathway when stimulated. Association of Fas molecules by the binding of Fas-L causes phosphorylation of the intracellular domains and leads to the formation of a death-inducing signalling complex, triggering the activation of caspase-8. Activated caspase-8 can cleave and activate caspase-3 and can also activate the intrinsic pathway of cell death by processing the protein Bid. Bid then translocates to mitochondria and interacts with proteins such as Bax and Bad, leading to the permeabilisation of mitochondrial outer membranes and the release of pro-apoptotic proteins (Li *et al.*, 1998a; Logue & Martin, 2008; Luo *et al.*, 1998).

Treatment of cultures of PC-12 cells (a neuronal cell line) with Z-IETD-FMK, a caspase-8 inhibitor, before infection with SV, greatly reduces the number of

cells that undergo apoptosis 48 hours post infection. Similarly, pre-treatment of cultures of PC-12 cells with soluble TNF-α receptor or anti-TNF-α antibody reduces the amount of cell death after infection with SV (Sarid *et al.*, 2001). Adenovirus has developed a mechanism to prevent Fas mediated cell death. The adenovirus E3 protein cleaves TRAIL and Fas receptors from the cell surface and targets them for degradation (Lichtenstein *et al.*, 2004). Influenza virus also triggers TNF receptor mediated cell death. In this case, activation appears to be beneficial to the virus. If Fas or TRAIL activity is inhibited, then propagation of virus is restricted (Wurzer *et al.*, 2004). It is suggested that influenza infection leads to activation of NFκB, which upregulates TRAIL and Fas-L. This is interesting as influenza also activates p58, a protein that can inhibit PKR and thus reduce NFκB activation (Bilgin *et al.*, 2003; Korth *et al.*, 1996). It is possible that initially apoptosis is inhibited to allow the virus time to replicate and produce new virus particles and subsequently apoptosis is induced.

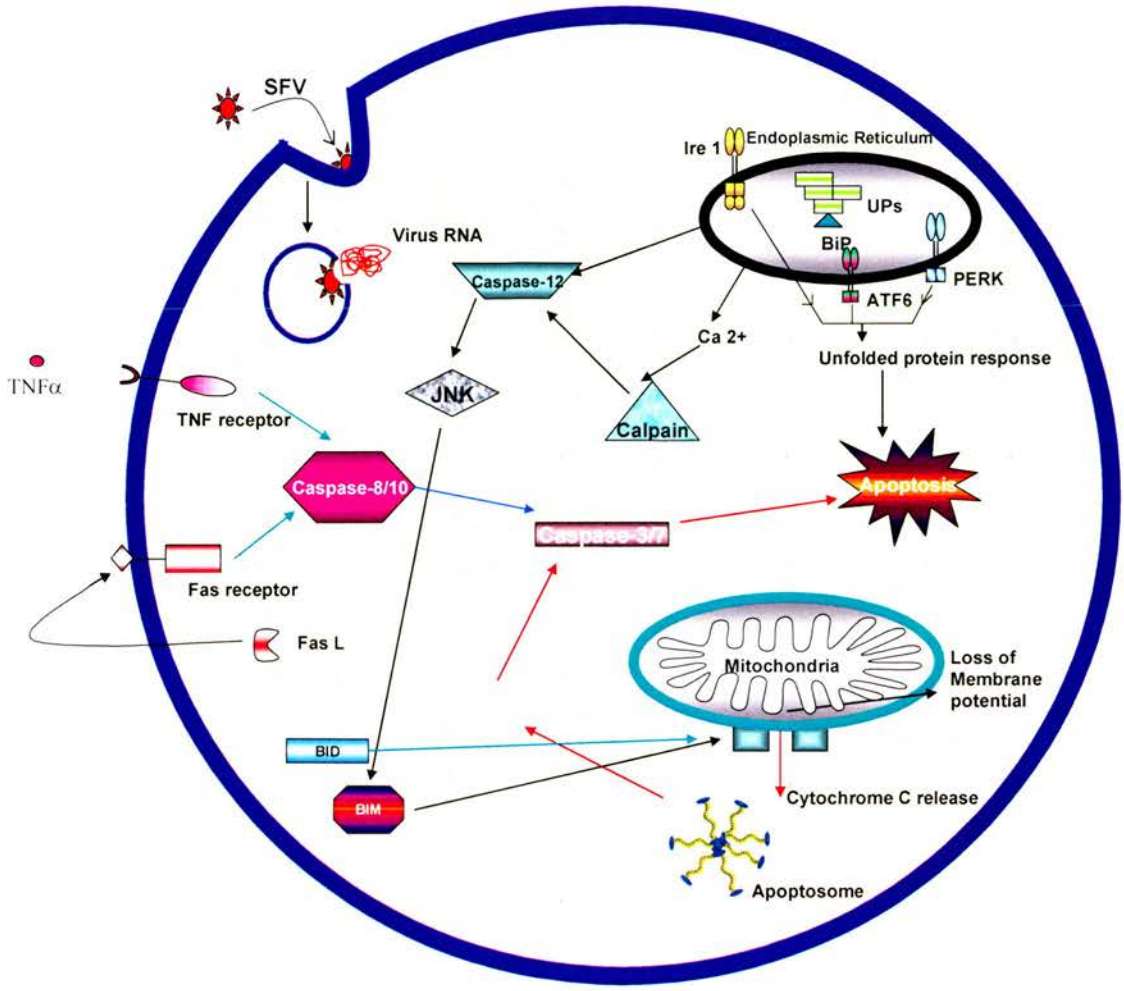

Figure 1.4: An illustration of intrinsic and extrinsic pathways of apoptosis as well as the unfolded protein response, activated as a result of protein build-up in the ER.

Summary

SFV infection can be broken down into four main stages: attachment and entry; genome replication; production of new structural proteins and finally virion assembly and budding.

Selective pressures suggest that it would be an advantage for a cell to evolve mechanisms that detect virus infection as soon as possible. This would imply that attachment and entry should be the first point at which a virus infection is detected and a cellular response triggered. While it has been previously suggested that virus entry causes apoptosis, UV inactivated SFV that can bind to and enter the cell but which cannot replicate its genome, does not trigger apoptosis in mouse embryo fibroblasts (Personal communication, Dr. Asa Hidmark, Karolinska Institute,

Stockholm, Sweden). This suggests that, in the case of SFV, virus attachment and entry into a cell is insufficient to trigger cell death.

The next detection point could be the virus genome. For many viruses the presence of dsRNA is an essential part of their life cycle and should be a clear sign of infection to the cell. Numerous proteins exist, which could recognise SFV dsRNA and trigger an anti-viral response. Virus proteins may also have a role to play; the build-up and folding of structural proteins in the ER may evoke the ER stress response and ultimately apoptosis.

Assembly of virus particles and budding may disrupt the cell membrane to such an extent that it causes cell death. In the case of SFV, VLPs lacking structural proteins still trigger cell death, implying that a build up of structural proteins in the ER, virus particle assembly and budding are not essential but may contribute to the death of an infected mammalian cell. It is likely that the majority of mammalian cell types detect the presence of SFV at a number of these different stages, inducing an anti-viral state and triggering apoptosis. The exception of course is neurons in the mature brain. The reasons for mature neurons resisting apoptosis have yet to be elucidated, although as neurons mature and establish connections with other neurons they are bombarded with pro-survival, anti-apoptotic signals which may help these cells to resist the induction of apoptosis (Frebel & Wiese, 2006).

Aims of the project

1. Determination of the mechanism(s) involved in detection of SFV infection of mammalian cells.
2. Determination of the apoptotic pathways involved in mammalian cell death following SFV infection.

Chapter 2: Materials and Methods

Contents **Page**

Cell culture

- All cell lines were maintained in sterile plasticware (Nunc) at 37°C in a humidified 5 % CO_2 atmosphere.
- Baby hamster kidney (BHK) -21 cells were cultured in Glasgow's minimum essential medium (Gibco), supplemented with 10% (volume / volume (v / v)) new born calf serum (NBCS) (Invitrogen, UK), 10 % (v / v) tryptose phosphate broth (Invitrogen, UK), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 25 mM and L-glutamine (200 mM, Merck).
- MEFs were produced from mice of varying backgrounds. The MEFs were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco), supplemented with 10 % (v / v) foetal calf serum (FCS) (Invitrogen, UK), penicillin (100 U / ml, Merck), streptomycin (100 µg / ml, Merck) and L-glutamine (200 mM, Merck). Hep-2 cells were grown in the same media as MEFs.
- NIH 3T3 cells were cultured in DMEM (Gibco) with 10 % (v / v) NBCS and L-glutamine (200 mM, Merck).
- MCF-7 (Human breast adenocarcinoma cell line) cells and RD (human rhabdomyosarcoma) cells were cultured in the same media as the MEFs.
- Human embryonic kidney (HEK) 293 cells were cultured in Iscove's modified Dulbecco's medium (IMDM) and Glutamax™ (Gibco) supplemented with 10 % (v / v) FCS, blasticydin (5 µg / ml), zeocin (30 µg / ml).
- Neurons were cultured in minimum essential medium without calcium and magnesium. This medium was supplemented with 10 % (v / v) FCS, glucose (3 g / 500 ml), glutamine (0.15 g / 500 ml), potassium chloride (0.7 g / 500 ml), penicillin (100 U / ml, Merck) and streptomycin (100 µg / ml, Merck).

Harvesting and counting of cells

Adherent cells at 80-90 % confluency were removed from flasks by trypsinisation. Media was removed, the cells were briefly washed with PBS and the monolayer was then incubated at 37°C with Trypsin-ethylene diamine tetraacetic acid (EDTA) (0.05 % Trypsin, 0.53 mM EDTA, Invitrogen) until the cells had detached. The trypsin

mixture was diluted in an equal volume of media to inactivate the trypsin. The cells were then concentrated at the bottom of a universal tube by centrifugation at 478 x gravity (g) for 5 min. The pellet was resuspended in 10 ml of medium, 10 µl of this was diluted 1 in 10 with trypan blue and a haemocytometer was used to count the cells. The required number of cells was then seeded into fresh culture flasks.

Deriving MEFs from mouse embryos

Mouse embryos were extracted at 13.5 days of gestation to make MEFs. Embryos were decapitated and their livers were removed. The remaining tissue was ground down using a scalpel and then incubated at 37°C for 10 minutes with 1 ml trypsin-EDTA. Trituration with a 1 ml Gilson pipette was then used to break down the tissue further, a fresh 1 ml of trypsin-EDTA was added to speed up the process and the tissue was incubated for a further 10 minutes. A single cell suspension was eventually prepared by repeated trituration and incubation. This mix was then centrifuged at 478 x g for 5 min. The pellet was plated in a T80 culture flask and cultured or frozen at passage (P) 2. The MEFs were never kept past P8.

Freezing and thawing cells

Cells were frozen when they were healthy and in the middle of a growth phase, but no more than 80 % confluent. The cells were trypsinised and pelleted, resuspended in 10 ml of media and then counted using a haemocytometer. The optimal concentration of cells for freezing was 5 x 10^6 cells per ml. Once counted the cells were pelleted by centrifugation at 478 x g for 5 minutes and then resuspended in freezing media which consisted of 10 % dimethyl sulfoxide (DMSO) and 90 % FCS. 1 ml of this suspension was then put into 1.5 ml Nunc cryovials, the tubes were placed in a freezing box that contained isopropanol, and the box was put into the -80°C freezer. It was important to freeze the cells slowly to allow water to escape from the cells so that they were not damaged. The isopropanol allowed the cells to cool at approximately 1°C per minute. These cells were left at -80°C overnight. The next day, the cells were transferred to a liquid nitrogen storage container. The liquid nitrogen chamber cools the cells to below -130°C, the temperature above which damaging ice crystals can form.

When bringing cells up from liquid nitrogen, it was crucial to warm them as rapidly as possible. Before getting the cells 10 ml of warmed growth media was put into a 25 cm^2 flask, while another 10 ml of media was put into a universal. The cells were then transported from liquid nitrogen on dry ice to ensure they didn't defrost. Before warming the tubes, the caps on the cryovials were loosened slightly to release any gases that may have built up. The tubes were then placed into a water bath heated to 37°C, to ensure the cells were defrosted as rapidly as possible. Once defrosted the freezing media containing the cells was transferred into the universal containing growth media and the universal was centrifuged at 478 x g for 5 minutes. The supernatant was then poured off, the cell pellet was resuspended in growth media and the whole suspension was transferred to the tissue culture flask that had been pre-filled with media. The flask was then placed in the incubator and the cells were allowed to grow normally. A normal 25 cm^2 flask would normally contain no more than a maximum of 2-3 million cells, however because of the high level of death during the freezing and thawing process, a large excess of cells (1 cryovial contained 5 x 10^6 cells) was necessary to ensure survival of the culture.

Linearisation of DNA plasmids

DNA concentration was measured at 260 nm using a Nanodrop spectrophotometer. Once the DNA concentration was known it was possible to calculate the amount of restriction enzyme required for each reaction. 1 unit of enzyme is defined as the ability to cleave 1 μg of DNA at the appropriate temperature in 1 hour. The linearisation mix included restriction enzyme buffer (10x), acetylated bovine serum albumin (BSA) (10x), plasmid DNA, restriction enzyme and nuclease free water. This was put at the appropriate temperature (according to the manufacturer) for 2 hours to allow the reaction to proceed. The results were checked by agarose gel electrophoresis. On an agarose gel DNA plasmids run as three forms. The three forms are supercoiled (which runs quickly), linear (in the middle) and nicked plasmid (highest band).

Making VLPs

DNA plasmids were kindly provided by Andres Merits, Estonian Biocentre, Estonia. The VLPs were produced according to the protocol already described (Smerdou & Liljestrom, 1999). Briefly, two plasmids (1.66 µg of each plasmid), the first containing the genes for the SFV4 non-structural proteins and a foreign gene (e.g. d1 eGFP), and the second plasmid containing the genes for the structural proteins of SFV4 were linearised using Spe I and *in vitro* transcribed to make RNA (see below for *in vitro* transcription reaction mix). The RNA was electroporated into BHK-21 cells and the supernatant was harvested at 24 and 48 hours post-infection, into 30 ml corex tubes, and clarified by centrifugation at 27,200 x g. The VLP sample was then purified and concentrated by centrifugation through a 20 % sucrose cushion at 82,700 x g, resuspended in 100 µl of TNE buffer pH 7.4 (50 mM Tris HCl pH 7.4, 100 mM NaCl and 0.1 mM EDTA pH 8.0, filter sterilized) and then left on ice for two hours. The TNE buffer containing VLPs was collected after two hours and the bottom of the tube was washed with a further 50µl of TNE buffer. All the TNE containing VLPs was pooled, aliquoted, snap frozen in liquid nitrogen and stored at -80°C.

Virus was made and purified in the same fashion using a single plasmid that contained the whole genome of the virus. New stocks of virus were always made from the plasmid rather than propagating from an older generation of virus. This was done to avoid using virus that may have developed mutations over time.

***In vitro* transcription reaction:**

Spe I cut plasmid (1.66µg)	xx.x µl
10X SP6 buffer (GE Healthcare)	5.0 µl
10mM M^7G (5')ppp (5') G (cap) (GE Healthcare)	5.0 µl
50 mM DL Dithiothretiol (Sigma)	5.0 µl
rNTP mix (10 mM ATP, CTP and UTP, 5 mM GTP) (GE Healthcare)	5.0 µl
H_20 (Ambion, nuclease free)	x.x µl
Recombinant RNasin Ribonuclease Inhibitor (60 U/1.5µl) (Promega)	1.5 µl
SP6 RNA Polymerase (50 U/µl) (GE Healthcare)	1.5 µl
Total Volume	**50.0 µl**

This mix was incubated at 37°C for 3 hours and the results were check on an agarose gel.

Strain	Backbone	Modification
SFV4		Prototype strain
SFV A7 (74)		Isolated originally as a naturally occurring strain of SFV.
SFV1-d1eGFP VLP	SFV4	VLP that contains d1eGFP under the control of the subgenomic promoter.
SFV4(3F)-eGFP	SFV4	Virus that has eGFP fused to its nsP3 gene so that the two proteins are expressed as a polyprotein highlighting the location of nsP3

Table 2.1: List of viruses and VLPs used during this project

Infection of cells in vitro with virus or VLPs

For infection of cells in culture, the virus or VLP was suspended in phosphate buffered saline (PBS) and BSA (PBSA, 0.75 g BSA per 100 ml PBS (0.75 %)). The albumin prevented virus or VLPs binding to the eppendorf, pipette tip, or other plastics. To infect cells in a 6-well plate the media was removed from the wells, the required amount of virus or VLPs was diluted in 450 µl of PBSA. 400 µl of this was then added to each well, being careful not to dislodge the monolayer. The cells were then placed on a rocker at room temperature and rocked gently for 1 hour to allow even spread and attachment of the virus or VLPs. After 1 hour the PBSA containing virus or VLPs was removed and 2 ml of fresh media was added to the well. In the case of a 96–well plate, the volume of PBSA and virus or VLPs added was 30 µl. Time zero in all experiments was taken as the time at which virus was added to the cells.

Titrating virus: plaque assay

Infectivity of purified virus stocks was determined by a standard plaque assay in BHK cells. Agar was prepared by mixing 4 g Bacto-agar per 100 ml PBS. This was then autoclaved to ensure it was free from contamination. BHK-21 cells were seeded

in 6-well plates at a density of 3 x 10^5 cells per well and incubated overnight. These monolayers were then infected; serial 1:10 dilutions were made of the test material in PBSA, media was then removed from the wells and 400 µl of each dilution was placed into 2 wells of a 6 - well plate (each dilution was done in duplicate). The plates were then allowed to rock gently for 1 hour. During the hour of rocking the agar was melted in a water bath heated to 100°C and then cooled to 55°C. At this stage BHK-21 media with 2 % NBCS (v / v) was prepared and warmed to 37°C. After 1 hour of rocking the 55°C agar was mixed with the 2 % BHK-21 media in a 1:3 ratio (agar:media) and the agar solution was gently added to the cell monolayers. The agar hardened almost immediately. Once all the wells had been covered, the plates were incubated at 37°C and 5 % CO_2 for 3 days.

After 3 days the cells were fixed with 10 % neutral buffered formaldehyde (NBF) (Surgipath) by pouring the NBF directly on top of the agar and leaving the plates at room temperature for at least 1 hour. The fixative was then poured off and the agar was removed using a metal spatula. Approximately, 1 ml of 0.1 % toludine blue was then poured into each well to stain the cells. The plates were put on a gentle rocker for 1 hour to allow complete distribution of the dye over the monolayer. The dye was then poured off and the wells were washed gently with tap water. Areas of dead cells due to virus infection were left clear by the dye and these were described as plaques. The titre of the virus was calculated using the average number of plaques between two wells of the same dilution.

Count wells with 2-70 plaques

Plaque forming units (pfu) per ml =

$$\frac{\text{Average no. of plaques}}{\text{Amount of inoculum (ml) x dilution factor}}$$

Titrating VLPs

In contrast to virus, VLPs could not be titred by plaque assay because VLPs do not produce new virus particles following infection. To get around this, immunostaining was used to identify how many cells in a culture were infected and the titre was calculated from that. In some cases, if the VLP expressed its own marker gene (e.g.

GFP) immunostaining was not required to identify infected cells. The procedure was as follows. A 6-well plate, with 20 x 20 mm coverslips in all the wells, was seeded with 3 x 10^5 cells per well and left overnight in an incubator. The next day 6 dilutions of the VLP to be titred were made. 10 µl of the stock was mixed with 545 µl of PBSA. 55 µl of this was then added to 500 µl of PBSA in a fresh tube; 55 µl of this new solution was added to 500 µl of fresh PBSA and so on, until all 6 dilutions were made. The media was removed from each of the wells in the 6-well plate and 450 µl from each tube was put into an individually marked well. The plate was then rocked gently for 1 hour before the PBSA virus solution was replaced with media and the plates were placed in the incubator. 20 hours later, the cells were fixed with 10 % NBF and immunostained for infection (or viewed immediately if the cells were GFP positive). The coverslips were mounted onto glass slides using an aqueous mounting medium and then viewed using the Zeiss Axioskop 2 microscope. Under the 40 x objective, 15 separate fields were examined and the number of infected cells was counted. The average number of infected cells was calculated and this number was then used to calculate the titre of the VLP stock. The calculation was:

$$\frac{\text{Mean number of VLPs x Microscope constant}}{\text{Volume of VLPs added to the well}}$$

For example: Mean number of VLPs = 1.37

Microscope Constant = 4365.3

Volume = .009 µl

Answer = 6.7 x 10^8 VLPs/ml

Throughout this work a multiplicity of infection (MOI) of 1 refers to the infectivity of SFV4 in BHK cells. It was found that infecting MEFs using a MOI of 1 only infected a small number of cells (as determined by immunostaining). In order to establish approximately 100 % infection a MOI (BHK cells) of 50 had to be used (as determined by immunostaining). For a low MOI infection, a MOI (BHK-21 cells) of 1 was appropriate.

Mice

The RNase L KO mice were on a C57 BL/6 background. The PKR KO mice were on a C57 BL/6 x 129 background (both strains were kindly obtained from Dr. Jovan Pavlovic, University of Zurich, Switzerland). Mice were bred and maintained in the Centre for Infectious Diseases Animal Unit, College of Medicine & Veterinary Medicine, University of Edinburgh, UK, in specific-pathogen-free and environmentally enriched conditions with food and water supplied *ad libitum*. All breeding and experimental studies were agreed by the University of Edinburgh Ethical Review Committee and were carried out under the authority of a UK Home Office licence.

Infection of mice

Eight day old mice were inoculated intranasally (i.n.) with 1 x 10^5 PFU of virus in PBSA while under a light halothane: oxygen anaesthetic. The virus, contained in PBSA, was dropped onto the nose of each mouse and was taken in with the breath of the mouse. Mice were killed with a lethal dose of CO_2, at 1 or 2 days post-infection and their tissues were sampled. Brains were cut sagitally down the midline, with half being fixed in 4 % neutral buffered paraformaldehyde and the other half being snap frozen on dry ice. The frozen tissue was stored at -80°C. The fixed tissue was sent to the histology department, Easter Bush, Edinburgh where 5 μm paraffin sections were cut.

Immunocytochemistry

Paraffin sections

The sections were first placed in clearene for seven minutes. This was repeated a second time to remove as much paraffin as possible. The slides were then put into 100 % ethanol for five minutes before being immersed in 0.3 % hydrogen peroxide (in 100 % methanol) for ten minutes. The sections were then gradually rehydrated by dipping them into troughs containing different dilutions of ethanol. (95, 70, 50 and 30 %, 1 minute each). The slides were equilibrated in 0.85 % sodium chloride for 5 minutes. For the antigen retrieval step the sections were submerged in a beaker

containing citrate buffer (12 ml 0.1 M citric acid monohydrate, 48 ml 0.1 M trisodium citrate and 540 ml distilled water (dH_2O)), boiled in a microwave for 12 minutes and then allowed to cool for 20 minutes. Once cool, the sections were covered with proteinase K (10 µl of 20 mg / ml stock solution, 9.9 ml tris pH 7.8, 100 µl 0.2 M calcium chloride), and placed at 37°C for 15 minutes. The proteinase K was then inactivated by an EDTA / glycine / PBS (2 g glycine, 10 ml 2.5 M EDTA, 990 ml PBS) solution for five minutes which was subsequently washed off with PBS (2 x 5 minutes).

The slides were blocked for thirty minutes with 10 % normal goat serum (NGS) and then covered with the primary antibody (made up in 2 % NGS) for 3 hours – overnight. The sections were washed with PBS (3 x 5 minutes) to remove residual primary antibody and the biotinylated secondary antibody, again made in 2 % NGS, was added and left for 1 hour. The Vector ABC signal amplification system followed the secondary antibody. This system allows avidin - biotin enzyme complexes (ABC) to bind to each biotin molecule on the secondary antibody used. During the secondary incubation diaminobenzidine (DAB, Sigma) was made up according to the manufacturer's instructions (30 minutes before use). The sections were washed with PBS (3 x 5 minutes) and then incubated with DAB until the sections turned light brown. The nuclei of cells in each section were stained with hematoxylin QS (Vector laboratories). Once the sections were sufficiently stained, they were dehydrated by incubating them in troughs containing different concentrations of ethanol (30, 50, 70, 95, 100 %, 1 minute each). Once dehydration was complete, a few drops of permanent mounting media were placed on the slide and a glass coverslip was laid on top of the media, ensuring no air bubbles were trapped in the process. The media dried within 1 hour and the slide was then viewed using a Zeiss Axioskop 2 microscope or a Nikon Diaphot 200 inverted microscope.

Cells fixed on a glass slide

Cells were fixed with 10 % NBF for at least 1 hour. The slides were then washed with PBS (3 x 5 minutes) to remove the NBF and then incubated with 0.3 % TritonX / PBS for 20 minutes to break down the cell membranes slightly and allow antibodies to enter the cells. The slides were then washed with PBS (3 x 5 minutes) and blocked

with Cas-block (Zymed) for 30 minutes. The primary antibody was then made up in Cas-block and added to the cells for 2 hours. After washing (3 x 12 minutes) the biotinylated secondary antibody was made up and added to the sections for 1 hour. The slides were then washed again with PBS (3 x 12 minutes) and incubated with the tertiary antibody for 45 minutes. The slides were finally washed with PBS (2 x 10 minutes) and covered with a coverslip. The mounting media used in this case was a hard set media from Vector Laboratories designed for fluorescent microscopy. The mounting media used occasionally contained 4', 6-diamidino-2-phenylindole (DapI), which is a nuclear stain as it binds to DNA. The slide were left in the dark to dry for 30 minutes and were then sealed with clear nail polish before being viewed using a Zeiss Axioskop 2 microscope with filters suitable for visualisation of eGFP, Alexa Fluor 594 (red) and DapI or a confocal microscope.

Target	Source	Host	Isotype	Technique	Dilution	Time
SFV	Internal	Rabbit	Polyclonal	Immunostaining	1 in 400	3 hours
Caspase-3	Calbiochem	Rabbit	Polyclonal	Immunostaining	1 in 50	Overnight
dsRNA	Scicons	Mouse	Monoclonal IgG1	immunostaining	1 in 400	2 hours
SFV NSP3	Tero Ahola	Rabbit	Polyclonal	Immunostaining	1 in 1000	2 hours
Activated Caspase-12	Cell signalling	Rabbit	Polyclonal	Western Blot	1 in 1000	Overnight
β-actin	Dr. Ghassem Attarzadeh	Rabbit	Polyclonal	Western Blot	1 in 1000	Overnight
Chop	Santa Cruz	Mouse	Monoclonal IgG1	Western Blot	1 in 1000	Overnight
Rabbit IgG Biotinylated	Vector Labs	Goat	Monoclonal IgG	Immunostaining	1 in 750 cultured cells 1 in 500 in paraffin sections	1 hour
Mouse IgG Biotinylated		Sheep	Monoclonal IgG	Immunostaining	1 in 750 cultured cells	1 hour
SA-Alexa Fluor 594	Invitrogen	Goat	Monoclonal IgG	Immunostaining	1 in 1400	45 minutes
Peroxidase labelled mouse IgG	GE Healthcare	Sheep	Monoclonal	Western Blot	1 in 3000	1 hour
Peroxidase labelled rabbit IgG	GE Healthcare	Donkey	Monoclonal	Western Blot	1 in 3000	1 hour

Table 2.2: List of antibodies used during this project

Isolation of protein for sodium dodecyl sulphate–polyacrylamide gel electrophoresis

The cell lysis buffer consisted of 50 µl of β-mercaptoethanol (Sigma) mixed with 950 µl of Laemmli buffer (Bio-rad). Cells in a well of a 6-well plate were lysed by removing the media from the well and adding 200 µl of lysis buffer. The mix was then collected after 1 minute of incubation at room temperature and boiled for 5 minutes at 100 ^{o} C. The samples were then frozen until required.

Measuring protein concentration

The concentration of protein in the Laemmli buffer solution was measured using an RC DC protein assay kit (Bio-rad). The manufacturer's protocol was followed. This assay is based on the Lowry protocol (Lowry *et al.*, 1951) which takes advantage of the ability of copper ions to bind to peptide bonds and then react with Folin reagent. The product of this reaction was measured spectrophotometrically at 750 nm.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis

A 12 % gel consisted of two parts, a resolving gel and a stacking gel. The resolving gel was made with 30 ml 40 % acrylamide (Sigma), 43 ml dH_2O, 25 ml 1.5 M tris-base pH 8.8 (Sigma), 1 ml 10 % sodium dodecyl sulphate (SDS) (Sigma). To use this 5 µl of tetramethylethylenediamine (TEMED, Sigma) and 50 µl of 10 % ammonium persulfate (APS, Sigma) were added to 5 ml of the solution and it was poured between the glass plates used to make the gel. A space of approximately 2 cm was left at the top for the stacking gel. This space was filled with isobutanol (Sigma) while the gel set, to prevent air bubbles forming. To make the 4 % stacking gel, 10 ml 40 % acrylamide (Sigma) was mixed with 64 ml dH_2O, 25 ml 0.5 M tris-base pH 6.8 (Sigma) and 1 ml 10 % SDS (Sigma). 5 µl of TEMED (Sigma) and 50 µl of APS (Sigma) were again added to a 5 ml aliquot to polymerise and set the gel. The isobutanol was removed once the resolving gel had set and the stacking gel was poured on top. A comb was then inserted to make the wells. Once the gel had set it was placed in an electrophoresis tank which was subsequently filled with running buffer (see recipe below). An equal amount of protein was loaded into each well as well as a rainbow molecular weight marker (GE Healthcare). The gel was then run at

50 V for 30 minutes to allow the protein to enter and pass through the stacking gel slowly and then run at 120 V until the blue dye band reached the bottom of the gel and the protein had separated though the gel.

Staining SDS-polyacrylamide gels with coomassie blue

Coomassie Brilliant Blue R250 (0.25 g) was dissolved in a 45 ml water, 45 ml methanol and 10 ml of acetic acid mix. The gel was immersed in the solution and placed on a slow rocker for at least 4 hours. To remove excess stain the gel was placed in a de-stain solution which consisted of 30 ml methanol, 60 ml H_2O and 10 ml acetic acid. The de-stain solution was changed on a number of occasions until the protein bands were clearly visible.

Transfer of proteins from SDS-polyacrylamide gels to nitrocellulose paper

The gel was removed from the glass plates that were holding it during the electrophoresis and the stacking gel was cut from the resolving gel and discarded. The gel and nitrocellulose paper (GE Healthcare), cut to the same size as the gel were soaked in transfer buffer for 10 minutes prior to the transfer. 3 mm chromotography paper (Whatmann) and sponges were also soaked briefly. The transfer apparatus was assembled starting with a piece of filter paper on top of a sponge. The gel was then placed on top of the filter paper with the nitrocellulose paper going on top of the gel. The 'sandwich' was finished with another piece of 3 mm chromatography paper and a second sponge on top. This was placed in a tank filled with transfer buffer. The proteins in the gel are negatively charged after mixing with the Laemmli buffer so an electrical charge was used to transfer the proteins onto the nitrocellulose paper. The electricity was turned on for 15 minutes at 15 volts and then 50 minutes at 50 volts. The transfer apparatus was then taken apart and the nitrocellulose paper was placed in blocking buffer for 1 hour at room temperature.

Detecting target proteins of interest using antibodies (Western blot)

After blocking, the nitrocellulose paper was incubated with the primary antibody (mixed in blocking buffer) overnight on a rocker at 4°C. It was then washed with PBS + 0.1 % Tween – 20 (PBS-T) (4 x 4 minutes), immersed in PBS-T containing the secondary antibody for 1 hour at room temperature, rewashed (4 x 4 minutes) and then incubated with SuperSignal West Pico Chemiluminescent Substrate according to the manufacturer's protocol for 4 minutes, before exposing to enhanced chemiluminescent (ECL) Hyperfilm (GE Healthcare).

Western blot buffers

Transfer buffer	Running buffer
39 mM glycine	25 mM Tris
48 mM Tris Base	250 mM glycine
0.037 % SDS	0.1 % SDS
20 % Methanol	

All materials required for the transfer and running buffer were from Sigma. Both protocols were taken from Molecular Cloning, A laboratory Manual by Sambrook, Fritsch and Maniatis.

Blocking buffer

5 % nonfat dried milk (Marvel)

PBS (oxoid) + 0.1 % Tween-20 (Sigma)

Measuring mitochondrial membrane potential

Mitochondrial membrane potential can be measured using a chemical called JC-1 (Sigma). JC-1 is cell permeable so can be used on live cells to see whether cells have an intact $\Delta\psi$ or not. JC-1 powder was dissolved in DMSO at a concentration of 1 mg / ml, aliquoted and frozen at – 20°C. A 6-well plate, with 20 x 20 mm coverslips in all the wells, was seeded with 3 x 10^5 cells per well and left overnight in an incubator. When the cells were ready, the JC-1 stock was left at room temperature for at least 30 minutes to allow complete re-dissolving of any JC-1 that had come out of solution. 1 µl of stock was then diluted in 100 µl of medium and 50 µl of this was

then mixed with 1 ml of medium and added to a well of the 6-well plate. The plate was then placed back in the incubator for 10 mins-2 hours (until the positive control showed distinct orange staining (monitored with an inverted microscope, time differed depending on the cell line examined).

When mounting, a clear aqueous mounting medium (Surgipath) was used. To investigate whether virus infection was affecting mitochondrial membrane potential, cells were infected first and then incubated with JC-1 at different times post-infection. The positive control for the loss of mitochondrial membrane potential was to treat the cells with 10 % NBF 1 hour to ablate mitochondrial activity.

Quantitative caspase assay

The caspase-3 and -8 assay kits were purchased from Sigma, while the caspase-9 kit was purchased from Chemicon. Cells were seeded in 6-well plates at a concentration of 3 x 10^5 cells per well. These were allowed to grow overnight before being infected with virus or VLPs and left for a set amount of time. The infection was stopped by lysing the cells and isolating the protein from the cells. To do this, cells were trypsinised, pelletted and incubated with the supplied lysis buffer at 4°C. The protein concentration was measured and equal amounts of protein from infected and uninfected cells were used in the assay. Each assay was carried out in triplicate at least. The assay was set up in a 96-well plate as shown in the table below. Each well was filled according to this table with the amount of sample used varying according to their concentration. The mixtures were incubated in the dark for 12 - 24 hours at 37°C and the colourimetric outputs were measured at 405nm.

	1x Assay Buffer	Caspase positive control 10 ug / ml	Sample	Inhibitor	Caspase substrate
Blank	90 µl	.....	.....	.....	10 µl
Positive control	80 µl	10 µl	.....	.....	10 µl
Positive control + inhibitor	78 µl	10 µl	.....	2 µl	10 µl
Sample	90 µl – x µl	.....	X µl	.....	10 µl
Sample and inhibitor	88 µl – x µl	.....	X µl	2 µl	10 µl

Table 2.3: Caspase assay protocol

Cell viability assay

The cell viability assay used a product called Wst-1 (Roche), a tetrazolium salt that is cleaved to a formazan dye by mitochondrial dehydrogenases that are active in live cells. The colourimetric output from dye production was read by a spectrophotometer. As cells die less formazan dye is made. Cells were seeded at a concentration of 3 x 10^4 cells / well in 96-well plates and incubated overnight. At the required time, 10 µl Wst-1 was added to each well (containing 100 µl media). Spectrophotometric readings were taken after 1 hour using a microplate reader at 420 – 480 nm. The reference wavelength was > 600 nm. For a timecourse of cell viability following infection or treatment with a chemical, replicates of 3-4 wells of cells were used for each timepoint. Background readings were always present using this technique so the graphs never quite reached zero at the timepoints observed.

Radiolabelling cellular protein

To look at translational shut-down in cells infected with SFV newly synthesised proteins were labelled with [^{35}S] labelled methionine and cysteine (GE Healthcare). This work was carried out according to University of Edinburgh radiation safety regulations. MEFs were grown in 6-well plates with three wells per timepoint. The cells were seeded at a density of 3 x 10^5 cells per well and allowed to grow overnight. The cells were infected at time zero (MOI 50) and then placed back in the incubator. 30 minutes before each specified timepoint the media was removed from the wells and 1 ml of starvation media (DMEM (1X), liquid - without sodium pyruvate, methionine or cystine, Invitrogen) was added. At each timepoint, the starvation media was removed and replaced with starvation media containing 25 µCi / ml [^{35}S] methionine and cysteine. This incubation lasted 30 minutes, after which the media was removed and disposed of safely. The cells were lysed using a β-mercaptoethanol / Laemmli buffer lysis buffer, as described previously.

Visualising radiolabelled proteins

In a fumehood, equal amounts of radiolabelled samples were run on a 12 % SDS-polyacrylamide gel as described previously. After electrophoresis the gel was placed on top of a gel sized piece of 3 mm chromatography paper (Whatmann) and dried in

a gel dryer (Model 543, Bio-rad) for 2 hours 30 minutes or until completely dry. Once dry, the gel was placed into a photographic film exposure cassette, a sheet of ECL Hyperfilm (GE Healthcare) was placed on top, and the cassette was put at –80°C for varying exposure times (24 - 48 hours) before development.

RNA extraction

RNA extraction was carried out from mammalian cells in culture using a Qiagen RNeasy mini kit according to the manufacturer's instructions. 1 x 10^6 cells were trypsinised, resuspended in media and pelleted in a universal by centrifugation at 478 x g. Cells were lysed with 350 µl of RLT buffer containing 1 % β-mercaptoethanol. The lysate was then triturated through a 21- guage needle at least 10 times to ensure cells were completely broken down. To this, 350 µl of 70 % ethanol was added and mixed by pipetting. The whole mixture was then transferred to an RNeasy mini column. The column was inserted into an eppendorf and centrifuged for 20 seconds at 8000 x g to force the liquid through the filter in the RNeasy mini column and into the eppendorf. This flow through was disposed of after the centrifugation. The column was washed twice by putting 350 µl and then 500 µl of RW1 buffer on the filter and centrifuging at 8000 x g for 15 seconds. Each time the flow-through was removed. 500 µl of RPE buffer was then added to the column and the column centrifuged at 8000 x g for 5 minutes. The column was then put into a fresh 1.5 ml eppendorf and spun for 2 minutes at 8000 x g to dry the column and remove excess ethanol which would affect the purity of the RNA. To elute the RNA, 30 µl of RNase free water was poured directly onto the filter and left at room temperature for 1 minute before the column was placed in a fresh eppendorf and spun at 8000 x g for 30 seconds. This was then repeated using the same flow-through. The purified samples were frozen at -80°C.

Transforming bacteria

To amplify plasmids they were transformed into a chemically competent Escherichia coli strain, DH5α cells (Invitrogen). 20 ng DNA was mixed with 100 µl of bacteria and placed on ice for 30 minutes. The tube containing the bacteria and DNA was then dipped in water heated to 37°C for 45 seconds. This heat-shock results in uptake

of the DNA. The tube was then placed back on ice for 2 minutes. After this time 900 µl of Luria Bertani (LB) broth was added, the tube was placed in a shaker and left for 1 hour at 37°C.

Petri dishes, previously prepared, each containing 25 ml of an enriched agar (7.5 g agar, 500 ml LB) supplemented with ampicillin (100 µg / ml) were placed in an incubator at 37°C to warm. After 1 hour, 100 µl of the bacteria was poured onto one plate and 10 µl onto another. A glass spreader was used to evenly distribute the bacteria across the plate. To monitor transformation efficiency, a transformation with pUC 19 plasmid was carried out at the same time. All plates were allowed to dry beside a Bunsen burner for a few minutes before being put upside down into a 37°C incubator and left overnight. The next day, the plates were examined for colony growth. The plasmids that were transformed contained an ampicillin resistance gene, so only bacteria that had taken up the plasmid should grow on the plates. A pipette tip was used to transfer bacteria from a colony into 5 ml of LB broth and ampicillin (100µg /ml), contained in a universal. The universal was shaken for 8 hours at 37°C and the whole mixture was then transferred into a large beaker containing 250 ml LB broth and ampicillin (100 µg / ml). This was placed in a shaker at 37°C for approximately 16 hours. At the end of this time the LB was visibly cloudy, due to the high concentration of bacteria present. The LB mixture was centrifuged for 20 minutes at 6000 x g, to pellet the bacteria and the pellet was then frozen at -80°C until required.

Purifying DNA plasmids from bacteria

Mini-prep

DNA plasmids were purified on a small scale using a Qiagen mini prep kit. The procedure was carried out according to manufacturer's instructions. Briefly, amplification of the plasmid was carried out as described above up to the stage of growth in a universal. Instead of freezing the bacterial pellet was resuspended in 250 µl buffer P1. The cells were then lysed using 250 µl buffer P2 which is a detergent based buffer probably containing an alkali such as sodium hydroxide, helping to solubilise the phospholipid membrane of the bacteria and denature the DNA and protein. 350 µl of a neutralisation buffer, N3, was then added to neutralise the buffer.

This precipitates all protein, lipid and DNA, except for the plasmid DNA. This solution was mixed by inversion 4 - 6 times and then centrifuged at approximately 17,900 x g for 10 minutes. A white pellet formed at the bottom of the tube with the plasmid DNA left in the supernatant. The supernatant was then poured onto a qiaprep mini column and centrifuged at 17,900 x g for 1 minute. The flow-through was discarded and the filter was washed by pouring 750 µl PE buffer onto the filter and centrifuging at 17,900 x g for 1 minute. The flow through was again discarded and the filter was centrifuged dry at 17,900 x g for a further 2 minutes. The plasmid DNA was then eluted by adding 30 µl of RNase, DNase-free water to the centre of the column, leaving it at room temperature for 1 minute and then centrifuging at 17,900 x g for 1 minute. The DNA was frozen at - 20°C after purification.

Maxi-prep

All maxi preps were carried out using a Qiagen endotoxin free maxiprep kit. After growing plasmid containing bacteria in 250 ml of LB broth and ampicillin (100 µg / ml) overnight at 37°C the media was centrifuged at 6000 x g for 20 minutes to pellet the bacteria as described above. The pellet was thoroughly resuspended in 10 ml of buffer P1, and then mixed with 10 ml of buffer P2 and allowed to sit at room temperature for 5 minutes to break down cell walls and release the DNA. To neutralise this, the mixture was mixed with 10 ml of buffer P3 and then poured into a qiafilter cartridge. The precipitate formed in the previous stage was allowed to settle at the top of the cartridge over a 10 minute period and the lysate was pushed through the filter into a 50 ml falcon tube. 2.5 ml of ER buffer was then added to the flow through, it was mixed thoroughly by inverting 10 times and the mix was then placed on ice for 30 minutes. While this was incubating the qiagen-tip 500 was equilibrated by allowing 10 ml of buffer QBT to flow through it. After the 30 minute incubation the sample was poured into the qiagen tip-500 and allowed to flow through. The plasmid DNA stuck to the filter at this stage. The filter was washed twice with 15 ml of buffer QC and then the DNA was eluted by passing 15 ml of buffer QN through the filter. To precipitate the DNA, 10.5 ml of isopropanol was added and the mixture was then centrifuged at 15,000 x g for 30 minutes. The DNA precipitate that pelleted at the bottom of the tube was washed with 70 % ethanol and then centrifuged again

at 15,000 x g for 10 minutes to remove all traces of isopropanol. At the end of this centrifugation, the supernatant was poured off and the pellet was allowed to air dry at 37°C. The pellet was then redissolved in 500 µl of endotoxin free buffer TE.

Polymerase chain reaction

Different Polymerase chain reaction (PCR) programs were used during this project. The cycle conditions are listed below along with the primers. PCR was used to amplify specific sections of DNA for diagnostic or investigative purposes. The PCR reaction mix contained 5 µl magnesium free buffer (10x) (Promega), 3 µl $MgCl_2$ (25 mM) (Promega), 1 µl primer 1 and 2 (50 pM/ µl) (MWG), 1 µl dNTPs (10mM) (Promega), dH_2O, DNA and 0.4 µl Taq polymerase (Promega). The results were checked by agarose gel electrophoresis.

PKR

94°C 4 minutes, (94 °C 30 seconds, 55 °C 30 seconds, 72 °C 2 minutes) x 30, 72 °C 15 minutes. The primers used were:

For: 5'-caaagcaggaggcaagaaac-3'; Rev: 5'-gctgactgggaaacaccatt-3'

XBP1

94°C 4 minutes, (94°C 30 seconds, 56 °C 45 seconds, 72 °C 1 minute) x 30, 72 °C 15 minutes. The primers used were:

For: 5'- aaacagagtagcagcgcagactgc -3'; Rev: 5'- ggatctctaaaactagaggcttggtg -3'

B-actin was always used as a sample and PCR control. The primers used were:

Forward: 5'- cgttgacatccgtaaagacc -3'; Reverse: 5' ctggaaggtggacagtgag -3'

Reverse transcription PCR

Reverse transcription (RT) -PCR was used to make multiple cDNA copies of a RNA template using an oligo dT primer. 1 µl of deoxyribonucleotide triphosphate solution (dNTPs, 10 mM) (Promega), 1 µl oligo dT (500 µg / ml) (Promega), ,dH_2O and 5 µg RNA made up to a total volume of 10.5 µl were mixed and put at 65°C for 5 minutes followed by 5 minutes on ice. Meanwhile, a mix of 4 µl 5x first strand buffer (Promega) , 2 µl dithiothreitol (DTT, 0.1 M) (Promega) and 0.5 µl RNasin (60 U /

1.5µl) (Promega) was made up and put on ice. The two solutions were then mixed and incubated at 42°C for 2 minutes. 1 µl of superscript II (Invitrogen) was then added and the mix incubated again at 42°C for a further 1 hour. It was heat inactivated by heating the mixture to 70°C for 10 minutes. The results were checked by agarose gel electrophoresis.

Agarose gel electrophoresis

A 1 % gel was made by mixing 0.6 g agarose (Sigma) with 60 ml of tris-borate (TBE) buffer and microwaving for 1 minute at full power to dissolve the agarose. The gel mix was then allowed to cool before adding ethidium bromide to a final concentration of 0.5 µg / ml. Once mixed, the gel was poured into a plastic frame and a comb placed into it ensuring no bubbles were trapped in the gel; the gel hardened within half an hour. The comb was removed and DNA samples were loaded mixed with loading buffer (Promega). Generally, gels were run at 120 volts for approximately 1 hour. As size markers, a 100 bp and 1 kb DNA ladder (Promega) were run alongside the samples. The DNA bands were visualised using a UV transilluminator.

DNA precipitation

DNA in water was mixed with 2.5 volumes of 100 % ethanol and 0.5 volumes 7.5 M ammonium acetate. The mixture was placed at -80°C for 30 minutes and then centrifuged at approximately16, 000 x g for 30 minutes at 4°C. A pellet formed at the bottom of the tube. The pellet was washed twice with cold 80 % ethanol (centrifuging at 16, 000 x g after each wash). The pellet was then resuspended in a desired volume of DNase free water.

Cell transfection using Lipofectamine 2000

Cells were seeded at a concentration of 3 x 10^5 cells per well in a 6-well plate and grown overnight. For transfection the media in the wells was replaced with 1 ml of antibiotic-free media. In two polystyrene tubes, 50 µl of Optimem (Invitrogen) per well was aliquoted. 1 µl Lipofectamine 2000 per well was put in one tube with Optimem, while the plasmid to be transfected was put in the other with Optimem.

These were left at room temperature for 5 minutes and then mixed together and incubated at room temperature for 30 minutes. 100 µl of the solution was then added to each well and the plates were placed back in the incubator until required.

Luciferase assay

A Dual-Glo Luciferase Assay System (Promega) was used to measure both firefly and Renilla luciferase. Cells were lysed by removing media from the wells of a 6-well plate and adding 200 µl lysis buffer (stock diluted 1 in 5 with water). The cells were agitated for 5 minutes to disrupt the cells fully. 1 µl of the lysate was mixed with 70 ul luciferase assay reagent and measured in a luminometer. 70 µl of stop-glo reagent was added to quench firefly luciferase and react with Renilla luciferase. This was then measured again in the luminometer. For the luciferase assay two plasmids were used. A Renilla luciferase expressing plasmid, pGL4.75 (Promega), under the control of a CMV promoter, was used as a transfection control in all experiments. The firefly luciferase expressing plasmid, p55A2Luc (a kind gift from Takashi Fujita, Department of Tumor Cell Biology, The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan (Yoneyama *et al.*, 1998)) was used to measure NFκB activity.

Caspase inhibition

A pan-caspase inhibitor, carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (zVAD-fmk, Promega) was used to inhibit activated caspases by binding to the catalytic site of caspases. The optimum concentration of zVAD-fmk (zVAD) was determined to be 200 µM as more concentrated solutions led to toxicity induced cell death. The zVAD was mixed with cell culture media and added to cells 2 hours before any apoptotic stimulus. The apoptotic stimulus was then added (eg. SFV) and the cells were again incubated with cell culture media containing zVAD (200 µM).

Chapter 3: Cell death pathways activated following SFV infection

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Introduction

Many viruses have adapted to mammalian cell innate defence mechanisms by blocking signalling pathways such as those that trigger IFN-α/β expression or by inhibiting or delaying apoptosis. The V protein of many paramyxoviruses interferes with IFN-β signalling through an interaction with MDA5 (Andrejeva *et al.*, 2004). MHV-68 expresses a protein, M11, that can inhibit Fas and TNF-α induced apoptosis (Wang *et al.*, 1999a). Apoptosis in response to virus infection can be seen as a tissue defence mechanism, reducing the time available for virus replication in an attempt to minimise virus spread. SFV has a small genome (approximately 11 kb) that does not contain any known anti-apoptotic genes. Most SFV infected mammalian cells produce large titres of virus and then die approximately 20-30 hours after infection.

Apoptosis is a carefully regulated form of cell death controlled by specific proteins which degrade DNA, cleave cellular proteins and eventually cause cell condensation and disintegration. Caspases are inactive proteases that are central to most apoptotic events when activated. Caspase-8 and caspase-9 are both known as initiator caspases that can activate other executioner caspases such as caspase-3 and -7. Caspase-8 can directly activate caspase-3 by cleaving it at a specific aspartic acid residue, or it can indirectly activate caspase-9 by activating proteins that trigger the release of cytochrome c from mitochondria. Caspase-9 is activated when mitochondria lose their membrane integrity and release cytochrome c. Cytochrome c complexes with ATP, APAF-1 and inactive caspase-9, triggering autoproteolysis and activation of caspase-9; caspase-9 cleaves and activates caspase-3 and -7 (reviewed in Creagh *et al.*, 2003).

A number of proteins involved in cellular defence against virus infection have been implicated in caspase activation. PKR activation upregulates pro-apoptotic proteins such as Fas, Fas L (Gil & Esteban, 2000) and p53 (Wu & Lozano, 1994) leading to caspase-8 and -9 activation. PKR can also activate caspase-8 through the activation of FADD (Gil & Esteban, 2000). RNase L is also thought to be proapoptotic; RNase L KO cells are defective in their response to numerous apoptosis inducing agents (Zhou *et al.*, 1997a). The activation of RNase L in mammalian cells by expression of activated 2', 5' OAS causes cells to die by apoptosis. This cell death involves the release of cytochrome c from mitochondria

and is caspase dependent as the death can be blocked by a caspase inhibitor (Rusch *et al.*, 2000).

Virus infections could also trigger a cell to undergo apoptosis through the activation of the ER stress response. Extended activation of this response causes expression of CHOP, which can down regulate Bcl-2 and prime cells for apoptosis (McCullough *et al.*, 2001). ER stress can also lead to the release of calcium from the ER; calcium can translocate to the mitochondria and trigger the release of cytochrome c (Deniaud *et al.*, 2007).

P53, an important anti-tumourigenic protein (Donehower *et al.*, 1992) is also involved in cell defence against many viruses. It is upregulated by IRF-3, an anti-viral protein involved in IFN induction (Kim *et al.*, 2006b). Activated p53 can upregulate PUMA and Bax, which are both proapoptotic proteins capable of permeabilising mitochondria and causing the release of cytochrome c (Jeffers *et al.*, 2003; Miyashita *et al.*, 1994).

Cellular proteins also exist that can kill cells without caspase activation; AIF, HtrA2/omi and endoG are three proteins that can be released when the mitochondrial membrane ruptures. AIF, cleaved from the inner mitochondrial membrane by a cysteine protease translocates to the nucleus and can cause chromatin condensation and fragmentation of DNA (Susin *et al.*, 1999; Yuste *et al.*, 2005). It does not have endonuclease activity but it may interact with endonucleases already present in the nucleus; for example AIF can interact with endoG in *C. elegans* (Mate *et al.*, 2002). HtrA2/omi cleaves a number of substrates including inhibitors of apoptosis and the cytoskeletal proteins such as tubulin and β-actin leading to cell death (Vande *et al.*, 2007). Any / all of these proteins may also be active in a caspase independent manner during a SFV infection.

It is clear that a myriad of systems exist in cells that can potentially cause apoptosis in response to virus infection. This chapter presents data on the caspases activated in SFV infected mammalian cells, and the involvement of caspase independent mechanisms in the death of these infected cells.

Objectives:

1. Determine whether SFV infection triggers caspase activation in mammalian cells.
2. If activated, determine in what order individual caspases become activated.
3. Determine if and when mitochondrial membrane potential is lost as a result of SFV infection.
4. Determine whether SFV induced cell death is caspase dependent.

Results

TUNEL staining has previously shown that nicked DNA is present as cells die following SFV infection (Allsopp *et al.*, 1998). Nicked DNA however can be present in necrotic cells as well as apoptotic cells (Charriaut-Marlangue & Ben Ari, 1995). Morphologically, necrotic cells tend to swell up and rupture whereas cells dying by apoptosis tend to condense, develop pyknotic nuclei and eventually break up into small pieces called apoptotic bodies (Kerr *et al.*, 1972). SFV or SFV1-d1eGFP VLP infected cells display the morphological hallmarks of apoptosis (figure 3.1). The cells condense, bleb on their cell surface and eventually break up into small pieces.

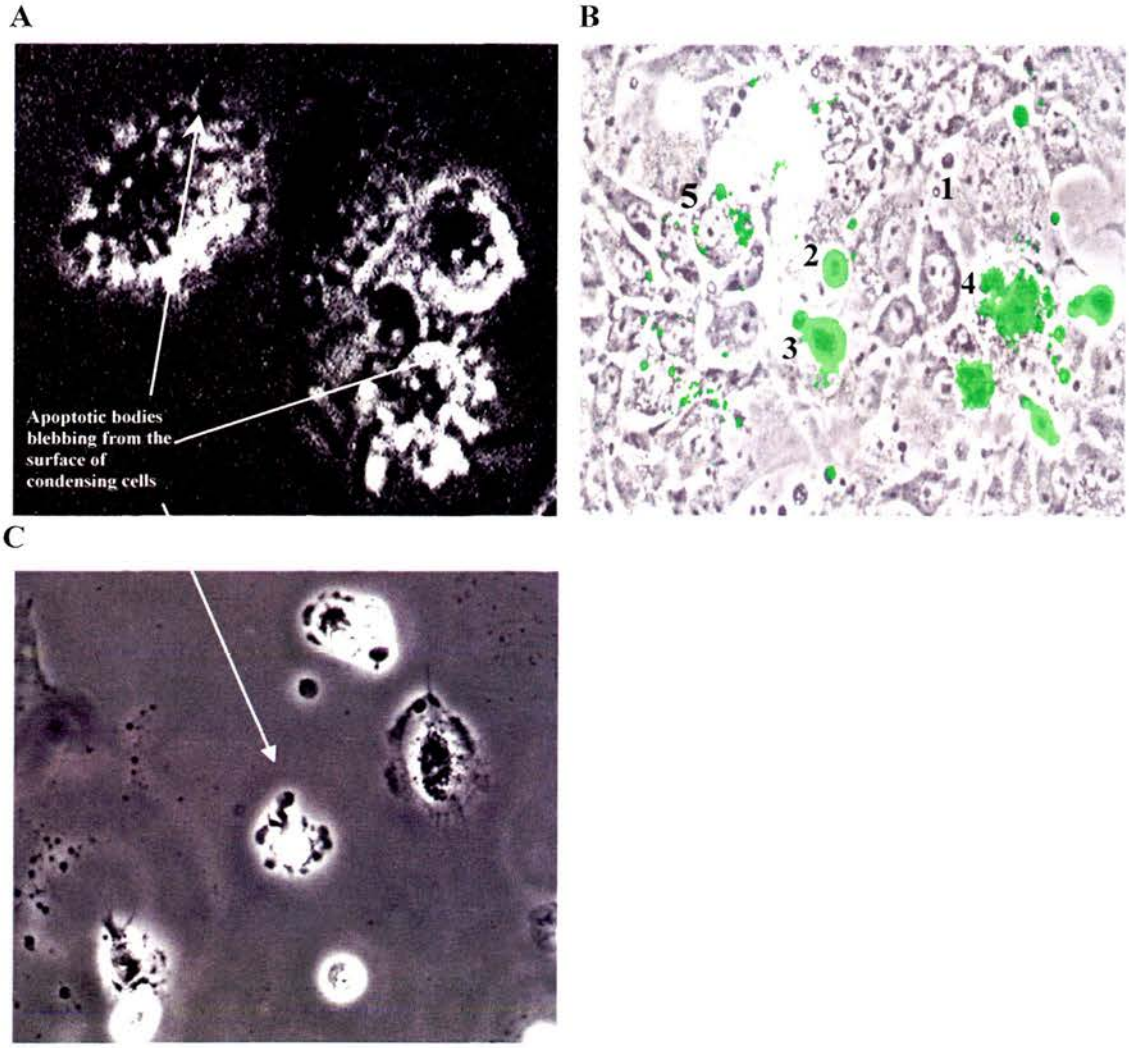

Figure 3.1: SFV or SFV VLP infected cells display the characteristic morphology of apoptotic cells.
(A) 100x magnification, light microscopy. Apoptotic body formation is observed in most SFV infected mammalian cells (BHK-21 cells, 20 hours post-infection, infected with SFV4) (B) 40x magnification, light microscopy. Cells infected with SFV1-d1eGFP VLPs appear green under UV light. (1) Uninfected MEFs, (2 & 3) infected cells start to condense, (4) infected cells start to break up into membrane bound fragments called apoptotic bodies, (5) apoptotic bodies are phagocytosed by healthy cells. (C) 40x magnification, light microscopy. Cells treated with 1 μM staurosporine for 30 hours undergo apoptosis, and display apoptotic body formation. Images were acquired using a Nikon Diaphot 200 inverted light microscope.

The rate of cell death of a population of mammalian cells infected with SFV was analysed by Wst-1 assay. As cells die mitochondria stop functioning and mitochondrial dehydrogenases gradually lose their activity. Wst-1 measures mitochondrial dehydrogenase activity so the assay is a measurement of cell population viability. Cultures of wt MEFs were infected with SFV4 (MOI 50) and then separate quadruplicate cultures of cells were assayed with Wst-1 at different timepoints to determine viability over time. The viability of the infected cultures remained unchanged for approximately 10 hours post-infection and then started to decrease rapidly. Virtually no activity was detectable 30 hours post-infection. The viability values of an uninfected population increased gradually over time.

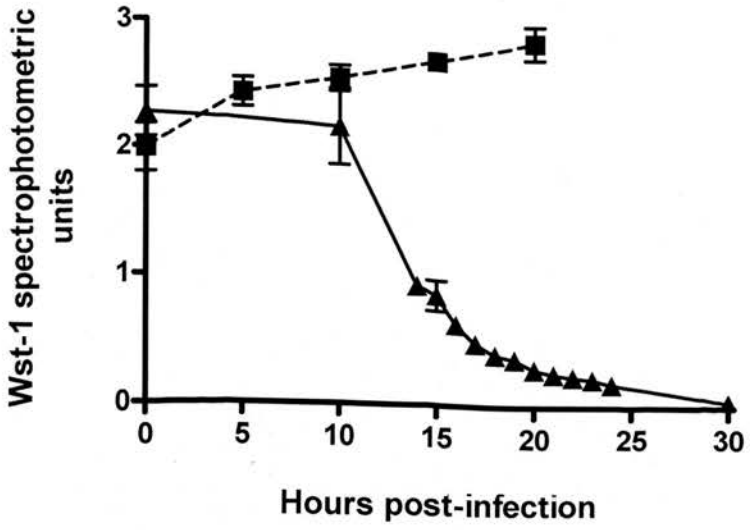

Figure 3.2: Wt MEFs lose viability over approximately 30 hours as a result of SFV infection.

Populations of wt MEFs were infected with SFV4 (MOI 50) (▲) and a Wst-1 assay was used to measure cell viability over time post-infection. An uninfected population (■) was used to show that without virus infection the viability of the population increased over time. The viability of the infected population decreased over approximately 30 hours. Each value represents the average of 4 separate populations and error bars are standard deviations of the mean.

Are caspases activated in mammalian cells in response to SFV infection?

The extrinsic and / or the intrinsic pathways of apoptosis may be activated during an SFV infection. The extrinsic pathway generally starts with the activation of caspase-8 whereas the intrinsic pathway starts with the activation of caspase-9. To see which caspases are activated following SFV infection, wt MEFs were infected with SFV4 (MOI 50) and then lysed at set timepoints. The protein concentrations of the lysates were measured and equal amounts of protein were analysed for caspase activity. Three assays were carried out for active caspase-3, -8 and -9. No activity could be seen at nine hours post-infection but all caspases were detectable by 10 hours post-infection (Figure 3.3). This implies that all caspases were being activated between 9 and 10 hours post-infection and indicates that both the intrinsic and extrinsic pathways are activated with no discernable difference in timing following SFV infection. As a control experiment to look at the specificity of these assays, the caspase-8 positive control protein was mixed with the caspase-9 substrate, while the caspase-9 positive control protein was mixed with the caspase-8 substrate. Both reactions were incubated for 24 hours, and neither reaction gave a reading above the negative control. This suggests that each assay is specific to the specified caspase.

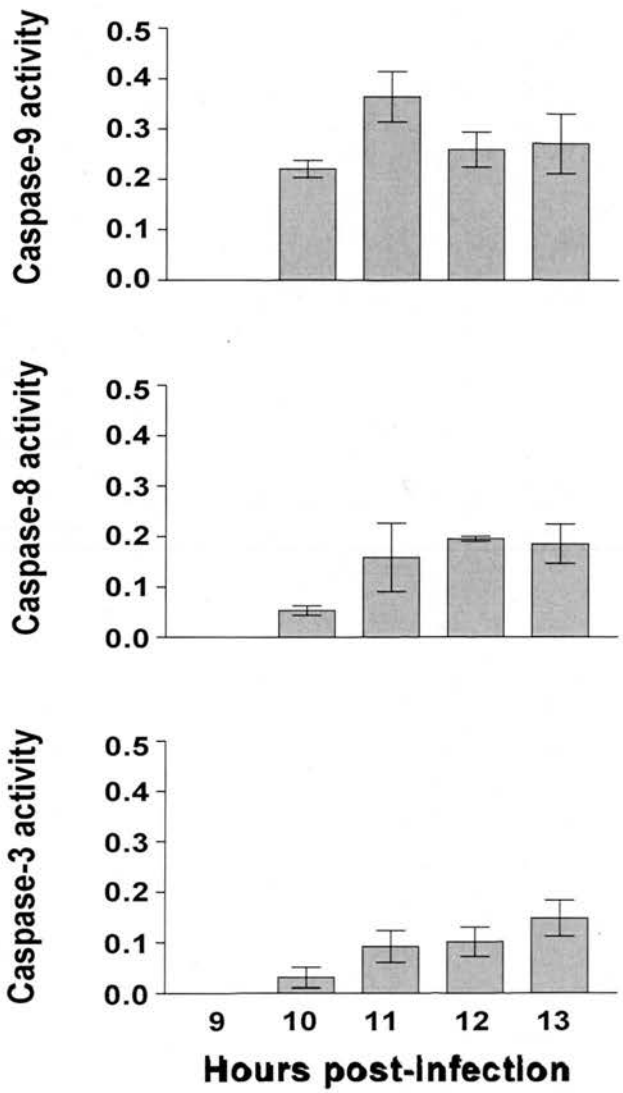

Figure 3.3: Caspases are activated as a result of SFV infection.
Wt MEFs were infected with SFV4 (MOI 50). Activated caspases-3, -8, and -9 were identified in the infected cells as early as 10 hours post-infection. Bars represent the means of triplicate populations of cells at different timepoints. The error bars are standard deviations of the mean. The y-axis units are spectrophotometric values, measurements of the colour produced by the cleaved caspase substrate.

Do mitochondria in infected cells lose their membrane integrity?

Functional mitochondria establish an electrochemical gradient across their intermembrane space as ATP is made along the inner membrane. The loss of mitochondrial membrane potential is a sign that pores have formed in the membrane and have allowed the potential to dissipate. To investigate whether this loss occurs in SFV infected cells, the cationic dye JC-1 was used to monitor the electrochemical

gradient across the membranes of mitochondria. JC-1 aggregates in mitochondria with an intact membrane potential and fluoresces red/orange; when the potential is lost the aggregates dissipate and the JC-1 monomers fluoresce green. When cultures of NIH 3T3 cells were infected with SFV4 (MOI 10), treated with JC-1 and viewed microscopically, both green and red/orange cells were observed under UV light (Figure 3.4). Control uninfected cells were predominantly red/orange. In order to quantify this over time, parallel cultures of NIH 3T3 cells were infected with SFV4 (MOI 50) and then treated with JC-1 at different stages post-infection. Percentages of green cells versus orange/red cells were calculated at the different timepoints (figure 3.4). The percentage of cells with intact mitochondrial membrane potentials was high early in infection but cells with collapsed mitochondrial membrane potential increased over time, suggesting that mitochondrial membranes were being disrupted during infection and that mitochondria play a role in the cell death triggered by SFV.

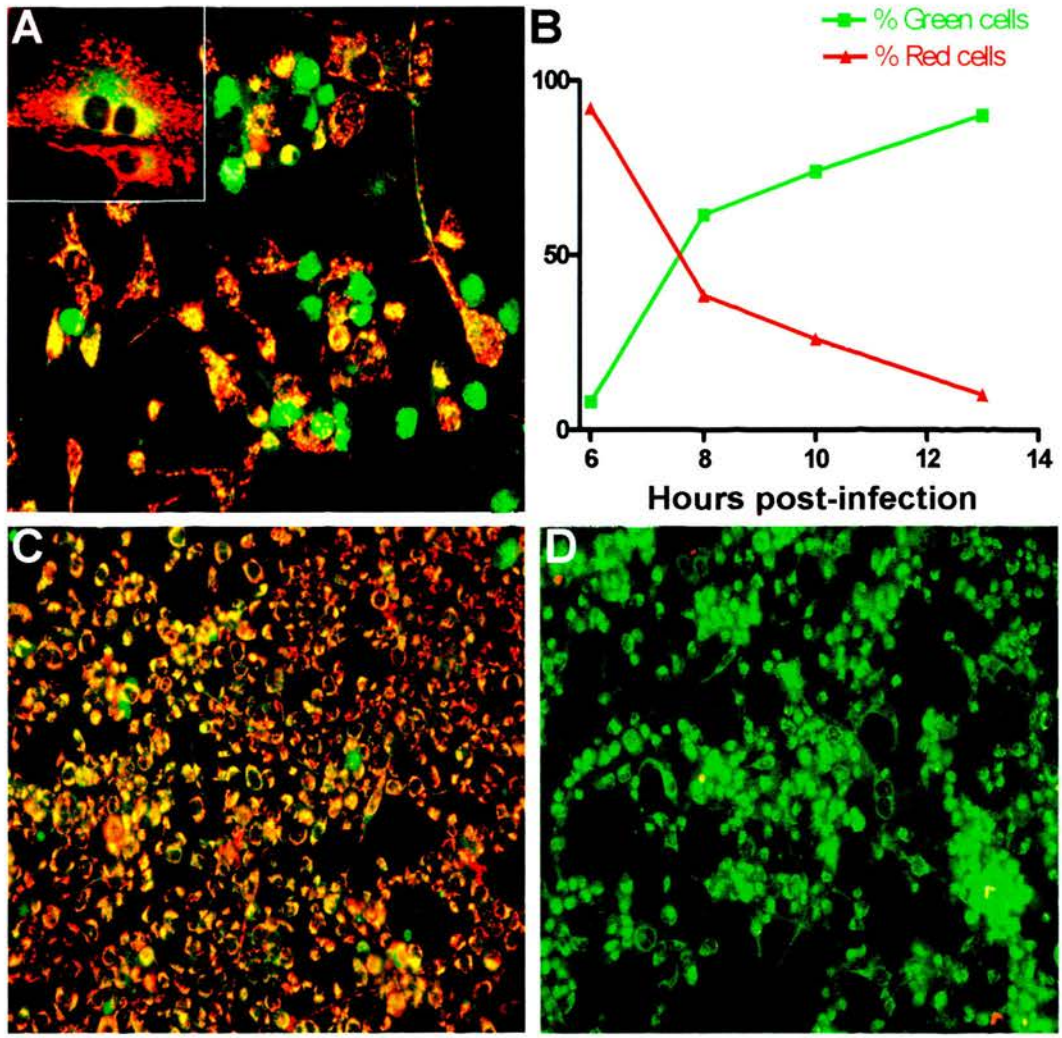

Figure 3.4: The mitochondria of infected cells lose their membrane potential
(A) NIH 3T3 cells infected with SFV4 (MOI 10); the majority of condensed cells have lost their membrane potential (green) and uncondensed cells generally have not lost their mitochondrial membrane potential (red/orange). (B) NIH 3T3 cells infected with SFV4 (MOI 50) lost their mitochondrial membrane potential over time The percentage of green cells increases (green lines) and the percentage of red/orange cells decreases (red lines). (C) Uninfected NIH 3T3 cells acted as a control for intact membrane potential and (D) formaldehyde treated cells (covered with formaldehyde for 1 hour) acted as a control for lost membrane potential. Images were acquired using a Zeiss Axioskop 2 plus microscope at a 10x (C&D) or 40x (A) magnification.

SFV induced cell death is not caspase dependent.

Caspase independent cell death was measured by using the pan-caspase inhibitor, zVAD. To ensure the zVAD was working correctly separate cultures of NIH 3T3 cells were treated with staurosporine (1 μM) in the presence or absence of zVAD (50 μM) for 24 hours. Staurosporine kills mammalian cells by caspase activation. zVAD treated cells were unaffected by staurosporine whereas staurosporine treatment in the absence of zVAD resulted in decreased viability (figure 3.5).

To test zVAD's effect on SFV infection, NIH 3T3 cells were treated with zVAD (200 μM) for 2 hours and then infected with SFV4 (MOI 50). Cells were then re-incubated with zVAD (200 μM) and their culture viability was measured at 30 hours post-infection (figure 3.6). Parallel cultures of zVAD untreated cells were also infected with SFV4 (MOI 50). The viability of the infected culture decreased, even when treated with zVAD. However, at 30 hours post-infection there was a significant difference between the viability of the zVAD treated and untreated cultures; the treated cultures had higher viability (in all infected populations viability eventually reached zero, data not shown). Higher concentrations of zVAD were used to see if the cells could be rescued further but these concentrations were toxic to the cells (data not shown). These results indicate that zVAD had an effect but that this was not sufficient to protect the cells from death.

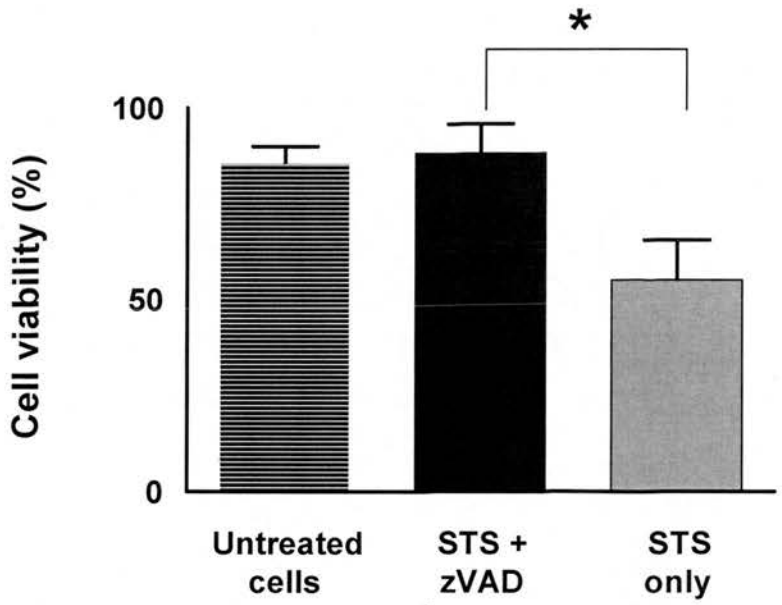

Figure 3.5: A control experiment for zVAD efficacy
NIH 3T3 cells were treated with staurosporine (STS, 50 μM) for 24 hours in the presence or absence of zVAD. The viability of the zVAD treated culture was significantly (* = $p < 0.05$, Mann Whitney) higher than the culture that did not receive zVAD.

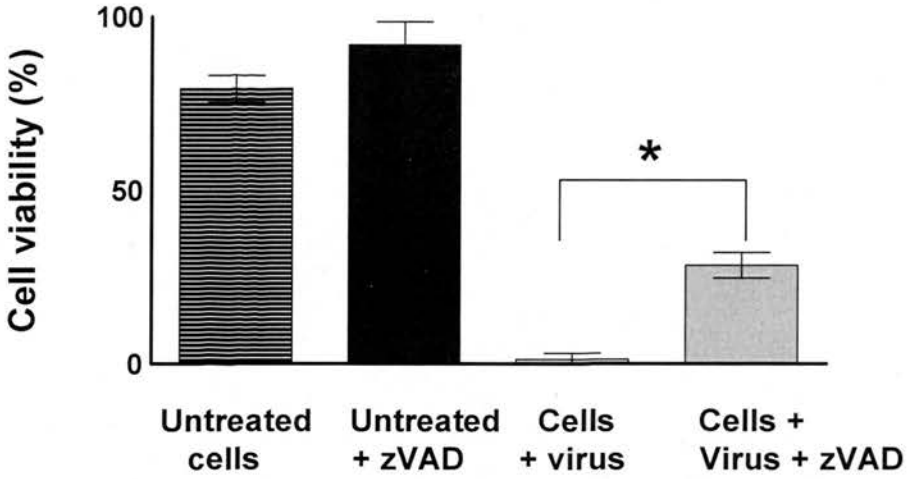

Figure 3.6: The pan-caspase inhibitor, zVAD, reduces viability loss in cultures of SFV infected cells but does not rescue the cells from death.
NIH 3T3 cells were infected with SFV4 (MOI 50) for 30 hours in the presence or absence of zVAD (200 μM), a pan-caspase inhibitor. The infected culture treated with zVAD showed significantly higher viability (* = $p < 0.05$, Mann Whitney) than the zVAD untreated culture.

Discussion

It has been demonstrated in this chapter that infected cells activate caspases-3, -8 and -9 between 9 and 10 hours post-infection. However, an infected population of cells takes over 24 hours to completely lose its viability as measured by the activity of mitochondrial dehydrogenases. During an infection, SFV causes a loss of mitochondrial membrane potential indicating that mitochondria are disrupted as a result of the infection. SFV infected cells dying in the presence of a pan-caspase inhibitor suggests that cells are dying by both caspase dependent and independent mechanisms.

Apoptosis pathways directed through the mitochondria are accompanied by the establishment of pores in the outer membrane of the mitochondria, allowing release of proapoptotic proteins such as cytochrome c and Smac/Diablo into the cytoplasm (Adrain *et al.*, 2001; Heiskanen *et al.*, 1999). The release of cytochrome c from mitochondria can occur before the loss of membrane potential (Bossy-Wetzel *et al.*, 1998) and loss of membrane potential can occur without the loss of cytochrome c (McGill *et al.*, 2005). Therefore, a measurement of membrane potential loss is not a measurement of cytochrome c release from mitochondria. However, membrane potential loss, as shown in this chapter, does signify that the membranes of mitochondria are being disrupted during an SFV infection suggesting that mitochondria are involved in the death of the cell.

According to the JC-1 staining presented in this chapter, all cells do not lose the membrane potential synchronously. This coincides with the Wst-1 assay result showing that a decrease in cell population viability occurs over a period of time after infection and not all at once. The high MOI used in these experiments ensures that virtually all cells are infected at time zero, so the different rates of membrane potential loss may be as a result of the virus replicating at different rates in different cells or more virus particles infecting some cells compared to others. Alternatively, the cells may be responding at different rates to the same level of infection; their response may be linked to the cell cycle. MEFs can be maintained in a contact inhibition induced G_0 / G_1 phase when grown at high densities (Daniel *et al.*, 2005). When MEFs were seeded at lower densities, allowed to grow for 16-20 hours and then infected at a high MOI they lost viability at the same rate as synchronised

populations according to a Wst-1 assay (data not shown). While this does not preclude cell cycle as a factor it suggests that other factors may be more important.

Disruption of the mitochondrial membrane may be caused by a number of factors. SFV may somehow be interacting with mitochondria as part of the virus life cycle causing them to burst. It is known that other viruses, such as flock house virus, replicate on the outer membranes of mitochondria (Miller *et al.*, 2001). Rubella virus, of the *Togaviridae,* associates with mitochondria during its replication cycle; electron microscopy has been used to co-localise the Rubella virus core particles with mitochondria (Lee *et al.*, 1999). No link between this localisation and apoptosis has been established. SFV is known to replicate in close association with endosomal-like vacuoles but no record of SFV proteins interacting with mitochondria has been documented despite immunocytochemical and electron microscopy studies into the location of SFV proteins, replication complexes and particles intracellularly (Kujala *et al.*, 2001). It therefore seems unlikely that SFV directly damages mitochondrial membranes in infected cells.

An alternative is that cellular proteins are causing the breakdown of the mitochondrial membranes as a result of infection. Bax, cleaved into an active form by caspases or other activated Bcl-2 family proteins, can localise to the mitochondrial surface and form pores in association with Bak and Bid (Korsmeyer *et al.*, 2000). This pore stimulates the release of cytochrome c and Smac/Diablo among other proteins. VDAC, a pore ordinarily involved in ion exchange across mitochondria membranes, is suggested to also interact with Bax and or Bak causing the release of pro-apoptotic proteins (Shimizu *et al.*, 1999). Cellular proteins that detect SFV may stimulate Bcl-2 family proteins to disrupt mitochondrial membranes. Alternatively, trafficking of SFV structural proteins through the ER may cause calcium release from the ER as a result of ER stress (Scorrano *et al.*, 2003). This can be taken up by mitochondria and can lead to the formation of pores in mitochondrial membranes (Szalai *et al.*, 1999).

P53 may also be activated in response to SFV infection and this could trigger mitochondrial permeabilisation. It is known that the capsid protein of West Nile virus can activate and stabilise p53. Activated p53 can activate the pro-apoptotic protein Bax (Yang *et al.*, 2007). Hepatitis B virus has also been shown to trigger apoptosis in

mammalian cells as a result of p53 activation. The expression of the hepatitis B protein pX triggers apoptosis in MEFs and p53 is essential to this as p53 KO MEFs do not die when pX is expressed (Chirillo *et al.*, 1997). P53 is not essential to SFV cell death as p53 KO cells still die when infected with SFV (Glasgow *et al.*, 1998). This does not mean however that p53 is not involved in mitochondrial membrane permeabilisation. Glasgow et al did not analyse the cell death closely enough to see if a lack of p53 was having an effect on the rate or mechanism of cell death.

Caspase activation may or may not be a direct result of protein release from the mitochondria. Caspase-3, -8 and -9 were all activated within one hour of each other but measurements were unable to identify the order of activation. The timing of activation would have helped to identify which pathway was the initiator of cell death in SFV infected cells.

It is possible that caspase-8 was activated first and caused cytochrome c release from the mitochondria through the cleavage of the Bcl-2 family protein, Bid. Alternatively, caspase-9 could have been activated first, causing subsequent caspase-3 and caspase-8 activation. It is also possible that both caspase-8 and -9 were activated independently of each other at approximately the same time. The virus may be activating more than one pathway during its replication cycle.

Influenza virus triggers caspase activation and DNA fragmentation in mammalian cells following infection (Lin *et al.*, 2002). It is known that influenza virus upregulates Fas and Fas ligand, leading to caspase-8 activation and cell death (Fujimoto *et al.*, 1998). Flock house virus, a nodavirus, causes apoptosis by downregulating an inhibitor of apoptosis (DIAP), which leads to caspase activation and cell death. PKR activation, triggered by many viruses, can lead to caspase-8 and caspase -9 activation in HeLa cells (Gil *et al.*, 2002). Another common protein activated by many viral infections, RNase L, can trigger cytochrome c release from mitochondria and caspase-9 activation (Domingo-Gil & Esteban, 2006).

It is unclear exactly how SFV activates caspases but the loss of mitochondrial membrane potential in some cells before caspase activation can be detected suggests that mitochondria were disrupted before caspase activation. This implies that the intrinsic pathway of cell death was activated first. Low levels of initially activated caspases, may then amplify the levels of pro-apoptotic proteins released from the

mitochondria, possibly by increasing Bax concentration on the surface of mitochondria (Lakhani *et al.*, 2006).

AIF, HtrA2/omi and endoG can be released from mitochondria when specific pores form in mitochondrial membranes. These proteins can cleave cellular cytoskeleton proteins, proteins that inhibit caspase dependent apoptosis and they can also cause DNA fragmentation. VSV and rabies virus both kill cells by caspase dependent and independent methods; both have been shown to trigger the release of AIF from the mitochondria of mammalian cells. Furthermore, VSV triggered apoptosis can be slowed down but not blocked through the use of a pan-caspase inhibitor such as zVAD (Gadaleta *et al.*, 2005; Sarmento *et al.*, 2006). Similarly, SFV infections could not be inhibited by a pan-caspase inhibitor; the loss of viability in a population of cells was delayed however, indicating that the inhibitor was having an effect. This suggests that AIF and other mitochondria derived proteins play a role in SFV induced caspase independent cell death; other possibilities however cannot be excluded. It is not known whether zVAD is completely effective at blocking caspase activity. The inhibitor works on a competition basis, whereby it acts as a substrate for activated caspases and out-competes other substrates. 100 % inhibition cannot be guaranteed and small undetectable amounts of activated caspases that bind to other substrates may be sufficient to kill the infected cell. The other possibility is that zVAD may be hindering virus replication. zVAD is not toxic to the cells at the concentration used but it may be toxic to the virus, reducing the burden on infected cells and delaying an apoptotic response to the virus. Further work is required to distinguish between these possibilities. In conclusion, these studies demonstrate that in SFV infection caspase-3, -8 and -9 are all activated and the mitochondria lose their membrane potential suggesting that the intrinsic and possibly also the extrinsic pathway of apoptosis is active; caspase independent cell death may also be occurring.

Chapter 4: Does PKR, RNase L, Fas, ISG-12 or neuronal cell differentiation state affect the course of cell death following SFV infection?

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Introduction

Cells have evolved multiple ways of detecting pathogens and triggering an appropriate response. The cell surface receptor, Fas, can activate the extrinsic pathway of cell death in response to some virus infections. DISC formation, incorporating FADD through its DED and caspase-8 at the intracellular domain of Fas, leads to autoproteolysis and activation of caspase-8 (Kischkel *et al.*, 1995; Medema *et al.*, 1997). The role of Fas in alphavirus induced cell death has not been investigated up to now; Fas activation may be important in SFV induced cell death. The role of the IFN-α inducible nuclear membrane protein, interferon stimulated gene (ISG) -12 has been previously implicated in alphavirus induced apoptosis (Labrada *et al.*, 2002; Martensen *et al.*, 2001). When mice less than 14 days old are infected i.n. with SFV A7(74) they die within a few days, however mice over 14 days old survive (Oliver *et al.*, 1997). Labrada *et al.*, showed that ISG-12 may play a role in age related responses to alphavirus infection. ISG-12 is not expressed in neonatal mice but is expressed in adults and enforced expression of ISG-12 in neonatal mice provided significant protection against SV infection (Labrada *et al.*, 2002). This suggests that ISG-12 may play an important role in defence against a virus infection. The roles of Fas and ISG-12 in SFV induced apoptosis are examined in this chapter.

TLRs detect multiple PAMPs and elicit a response according to what is detected. TLR 4, for example, is located on the cell surface and detects bacterial lipopolysaccharide (LPS). LPS is an essential component of the outer membrane of Gram-negative bacteria. TLR 4 detects LPS and triggers interferon expression (Akashi *et al.*, 2003; Okamoto *et al.*, 2003). TLR 9 detects non-methylated CpG dinucleotides. Mammalian cells tend to methylate CpG dinucleotides, whereas bacteria generally do not so this acts as a pathogen marker detected by TLR 9. Once detected, cells generally deal with virus infection by signalling to neighbouring cells, trying to restrict virus replication and / or undergoing apoptosis.

Many viruses go through a dsRNA intermediate stage as they replicate their genome. The main TLR to detect dsRNA is TLR 3. Alexopoulou *et al.* found that when kidney cells only expressing one TLR at a time were stimulated with poly I:C, NFκB activation only occurred when TLR 3 was expressed. TLR 3 KO mice do not

die when given a dose of poly I:C that would normally kill a wt mouse and they also show reduced amounts of IFN-α/β expression (Alexopoulou *et al.*, 2001). When TLR 3 is activated by dsRNA, signal transductions include nuclear localisation of NFκB and IRF-3; these upregulate IFN expression (Doyle *et al.*, 2002).

PKR appears to play multiple roles in the cell. It has been implicated in cell growth, tumour suppression and apoptosis (Lee & Esteban, 1994; Meurs *et al.*, 1993; Zamanian-Daryoush *et al.*, 1999). Many cells have basal levels of PKR, that are upregulated by IFN-α/β expression but PKR only becomes activated when it interacts with dsRNA (ordinarily from a replicating virus) or the PKR activator (PACT) protein (Galabru & Hovanessian, 1987; Garcia *et al.*, 2007; Meurs *et al.*, 1990). Once activated PKR can, among other things, (i) phosphorylate eIF-2α (Meurs *et al.*, 1992) leading to a down-regulation of cellular mRNA translation, (ii) activate NFκB by phosphorylating IκB (Kumar *et al.*, 1994), (iii) activate IRF-1 (Yang *et al.*, 1995).

RNase L is another protein involved in defence against viral infection. IFN induces the expression of 2'-5' OAS. This enzyme is activated by dsRNA and in turn activates RNase L. RNase L has the ability to cleave ssRNA into small pieces thus halting host and viral translation (Silverman, 2003). RNase L activation and degradation of cellular RNA can trigger apoptosis (Zhou *et al.*, 1997a).The roles PKR and RNase L play in SFV infected cells will be investigated in this chapter.

Objectives:

1. Determine whether neuronal cell maturity affects apoptotic cell death.
2. Determine whether Fas or ISG-12 are essential for cell death in response to SFV infection *in vitro*.
3. Determine whether PKR or RNase L is essential for cell death in response to SFV infection *in vitro* and *in vivo*.
4. Compare the time course of cell death between SFV infected wt MEFs and MEFs lacking PKR, RNase L, ISG-12 or Fas.
5. Determine whether PKR affects caspase activation following SFV infection.
6. Determine whether PKR affects replication of SFV.

Results

Does the developmental state of neurons *in vitro* alter their response to SFV infection?

The outcome of SFV and other alphavirus infections in mice is age dependent. *In vitro,* rat embryonic dorsal-root-ganglia explants cultured for a minimal amount of time and then infected with SV die within one to four days, whereas explants allowed to mature in culture for two weeks before infection survive for more than 14 days (Levine *et al.*, 1993).

To see if the same phenotype exists for SFV infected cells *in vitro,* rat hippocampal neurons were isolated from new born rats and seeded onto glass slides in 6 well plates in collaboration with Dr. Colin Rickman (Centre for Integrative Physiology, University of Edinburgh, United Kingdom). These neurons were then incubated for 2 days allowing them to settle and establish growth on the glass slides. After 2 days half the cultures were infected with SFV A7(74) (MOI 10). These were recently seeded neurons and were designated 'immature neurons'. The remaining cultures were kept for a further 2 weeks and then infected with SFV A7(74) at the same MOI (10). These cells were designated 'mature neurons'. The infected neurons were observed microscopically every 24 hours to assess morphological changes. By 24 hours minimal cytopathic effect was observed in the immature neuron cultures; the majority of cells appeared healthy (figure 4.1, A). However, by 48 hours, the majority of the cells had rounded up and detached from the glass slides (figure 4.1, B). The morphology of the cells suggested they had died by apoptosis as a result of the virus infection. In contrast, the mature neurons reacted quite differently to the virus infection. At 2 days post-infection the cells appeared morphologically normal (figure 4.1, B). By 4 days post-infection the culture began to look a little less populated, but not many cells were floating in the media (figure 4.1, C). Many cells remained attached to the glass slides although breaks began to appear in some dendrites. By day 5 the neurons seemed to still be attached to the slides however their morphology suggested they were extremely unhealthy, probably dead. Bubbles had formed in many parts of the cells, multiple breaks were scattered along dendrites and the cell bodies were difficult to distinguish from the rest of the cell (figure 4.1, D). This morphology was in stark contrast to the infected immature neuron cultures.

While the immature neurons quickly condensed and detached from the glass slides, the mature neurons did not condense at all and instead seemed to burst and break down, in a necrotic fashion; this occurred at day 4 post-infection at the earliest. These results correlate closely with the observations made previously *in vivo*; that immature neurons rapidly die by apoptosis whereas mature neurons remain alive and infected for longer periods of time.

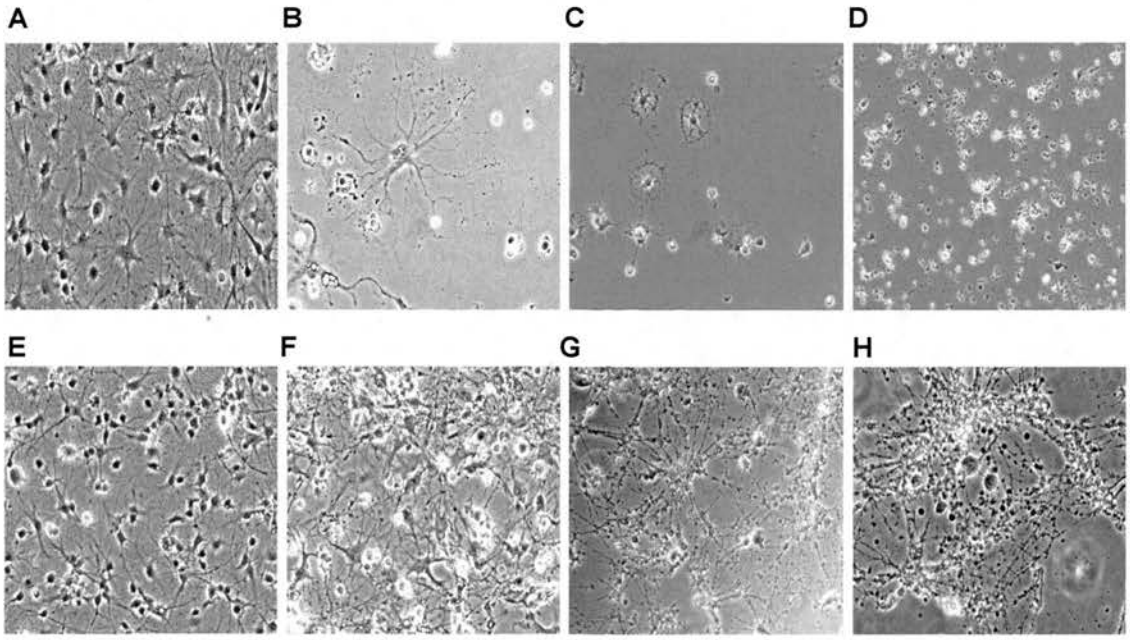

Figure 4.1: Mature neuronal cultures survive longer than immature cultures following SFV infection
Immature and mature hippocampal neuron cultures were infected with SFV A7(74) (MOI 10). Morphology was monitored by light microscopy. This figure is representative of several different cultures. Panels A-D show immature neurons (2 days old); panels E-H show mature neurons (2 weeks old). Images were acquired using an inverted Nikon Diaphot 200 light microscope at a 20x magnification.

Is Fas or ISG-12 required for apoptosis in response to SFV infection?

The cell viability of populations of Fas KO MEFs and ISG-12 KO MEFs were measured 24-hours after infection with SFV4 (MOI 50) and compared to the cell viability of uninfected populations that were grown parallel to the infected

populations. Lacking Fas or ISG-12 did not stop the cells losing viability as a result of virus infection (figure 4.2)

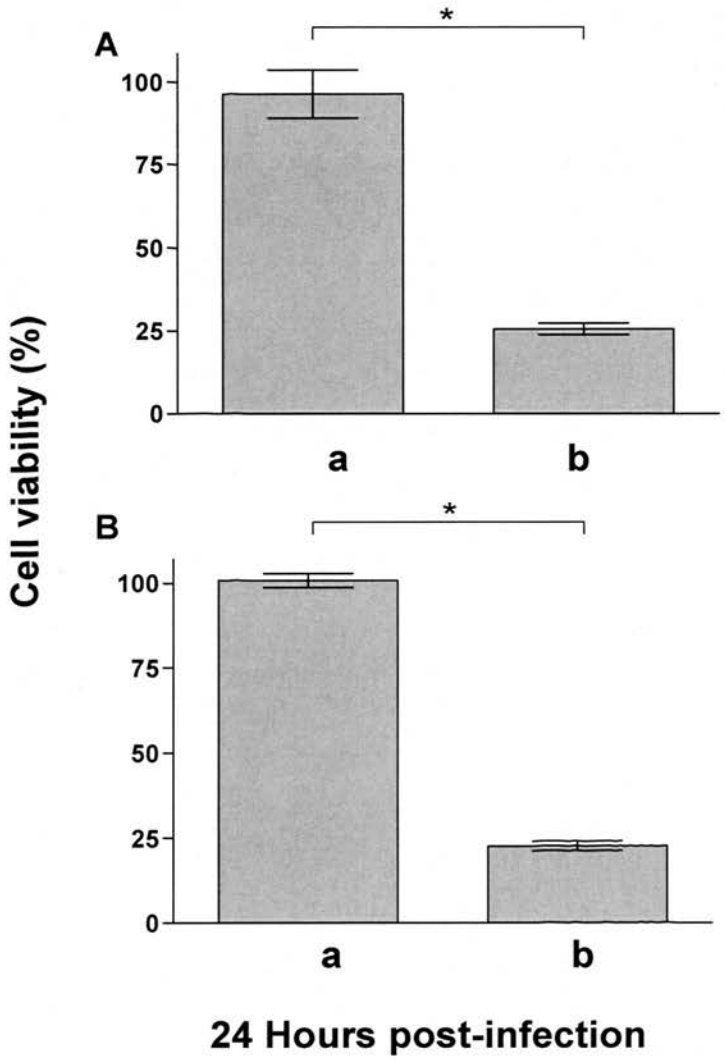

Figure 4.2: Neither Fas nor ISG-12 is required for cell death following SFV infection

A Wst-1 assay was used to measure the loss of cell viability in a population of Fas KO (A) or ISG-12 KO (B) MEFs uninfected (a) or infected (b) with SFV4 (MOI 50). Each bar is the mean of quadruplicates, error bars are standard deviations of the mean. * = significant difference ($P < 0.05$, Mann Whitney test).

Is ISG-12 required to protect adult mice against SFV infection?

A pilot experiment was carried out to investigate the effect lacking ISG-12 would have on the ability of adult mice to deal with a SFV infection. 4 week old wild type (n = 5) and ISG-12 KO mice (n = 4) were infected i.n. with 1 x 10^5 plaque forming

units (PFU) of SFV A7(74). Half the mice were sacrificed on day 3 post-infection and the other half were sacrificed on day 7 post-infection. The brains were frozen at -80°C and virus titres in the brains were determined by plaque assay. Whereas the brain virus titre decreased from day 3 to 7 in wt mice it increased in the ISG-12 KO mice. However, due to the low sample number, no significance can be drawn from this study; further studies are required.

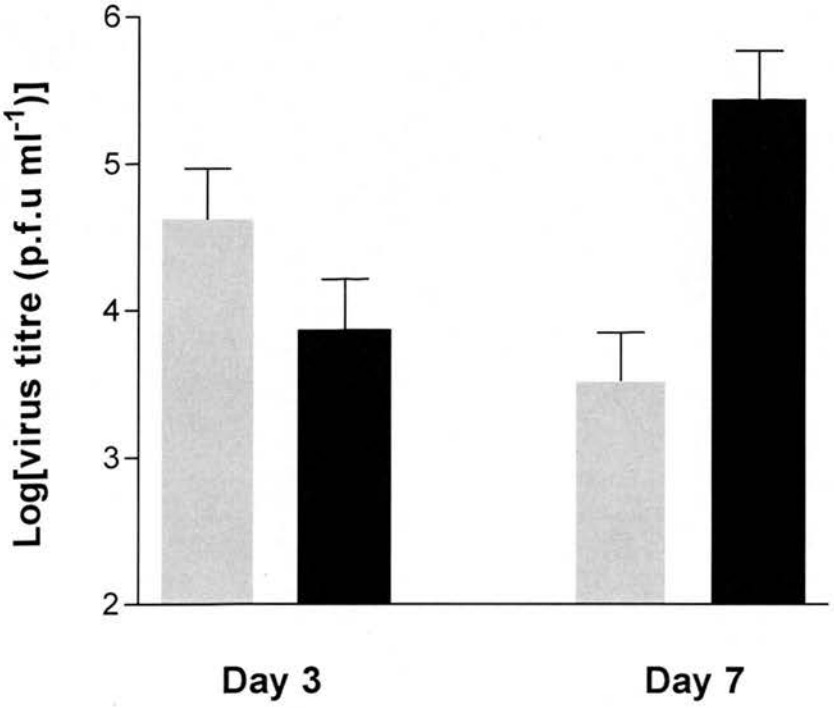

Figure 4.3: Virus titres in the brains of ISG-12 KO and wt mice.
Four week old wt (grey) (n = 5) and ISG-12 KO (black) mice (n = 4) were infected i.n. with SFV A7(74) and brains were sampled at 3 and 7 days post-infection. Virus titres in the brains were determined by plaque assay. Error bars are standard deviations of the mean. Sample numbers were too low to determine significance.

Is PKR or RNase L required for apoptosis in SFV infected cells in culture?

Virus induced apoptosis was also investigated in PKR KO and RNase-L KO MEFs *in vitro*. Parallel cultures of wt or mutant MEFs (PKR KO, RNase L KO) were infected with SFV4 (MOI 50). Cell viability was measured at different timepoints and compared to an uninfected population. Lacking functional PKR or RNase L did not prevent MEFs losing viability following SFV infection indicating that PKR or RNase L was not required for apoptosis in response to SFV infection (figure 4.5).

Morphological analysis of these cells, strongly suggested that they were dying by apoptosis (figure 4.4). The cells rounded up, condensed, blebbed and eventually detached from the plate surface and floated away or were engulfed by neighbouring cells.

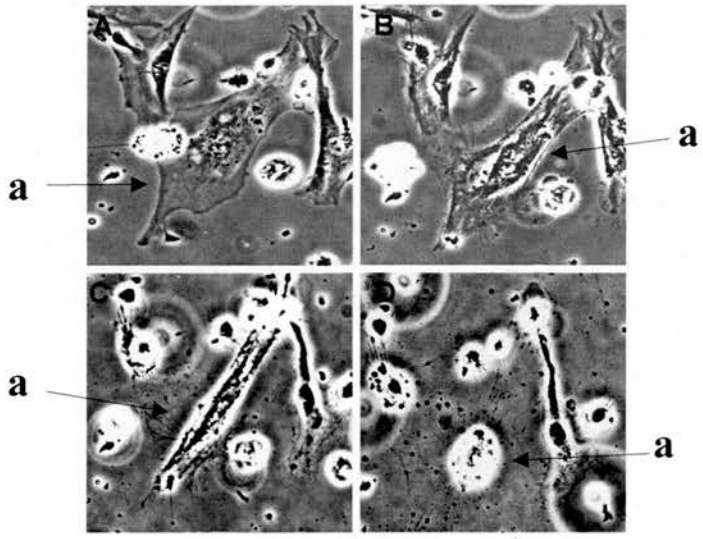

Figure 4.4: MEFs infected with SFV4 display morphological characteristics of apoptosis.
Panels A-D show a time course of death for one cell (a) infected with SFV4. Panel A is taken at 17 hours post-infection (hpi), B 18 hpi, C 20 hpi, D 23 hpi. The cell condensed, blebbed and eventually detached from the plate. The image was acquired using a Nikon Diaphot 200 inverted light microscope at a 20x magnification.

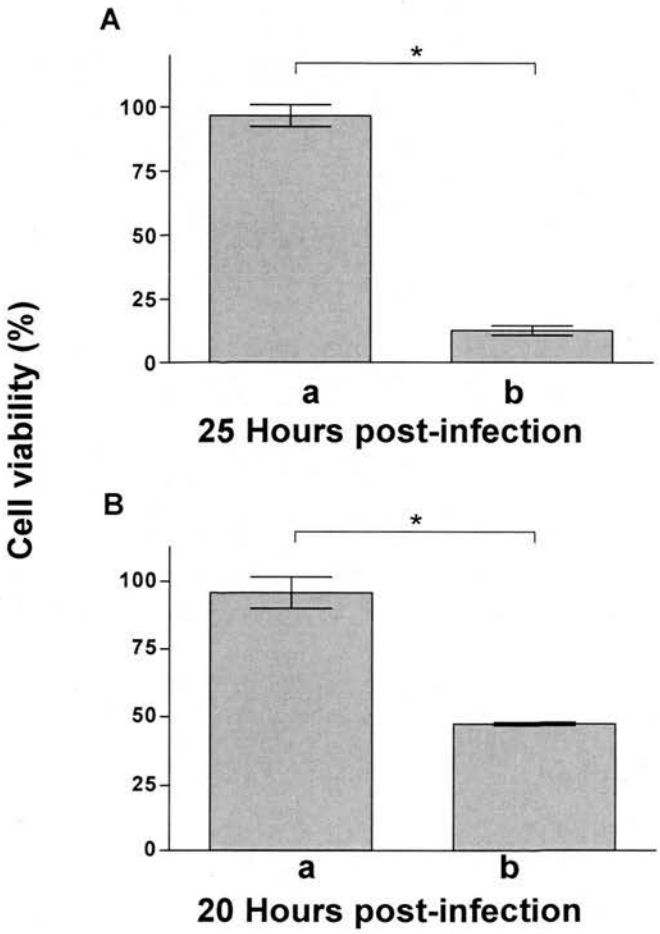

Figure 4.5: Neither PKR nor RNase L is required for cell death following SFV infection
A Wst-1 assay was used to measure the loss of cell viability in a population of PKR KO (A) or RNase L KO (B) MEFs uninfected (a) or infected (b) with SFV4 (MOI 50). Each bar is the mean of quadruplicates, error bars are standard deviations of the mean. * = significant difference ($P < 0.05$, Mann Whitney test).

Is PKR or RNase L required for SFV induced cell death *in vivo*?

Eight day old (P8) mice (11 RNase L KO mice, 13 PKR KO mice, 25 wt mice) were inoculated intranasally with 1 x 10^5 PFU of SFV A7(74) and brain tissue was sampled and fixed at 24 and 48 hours post-infection. Paraffin embedded saggital sections (5 μm) of the brains were immunostained for SFV structural proteins (3 slides per brain, 3 sections per slide). After intranasal infection of neonatal mice, SFV A7(74) moved along neuronal connections into the olfactory bulb. The virus then trafficked through all areas of the brain in a seemingly unrestricted fashion. Infection was widespread by 24 hours post-infection but even more extensive by 48

hours post-infection. Staining for SFV structural proteins was seen in both the grey matter and white matter regions of the brain; it was seen in the cytoplasm but not in the nuclei of infected cells. The staining showed that no difference existed in the spread of virus between RNase L, PKR and wt mice. Figure 4.6 shows typical infections observed in the brains of the P8 mice.

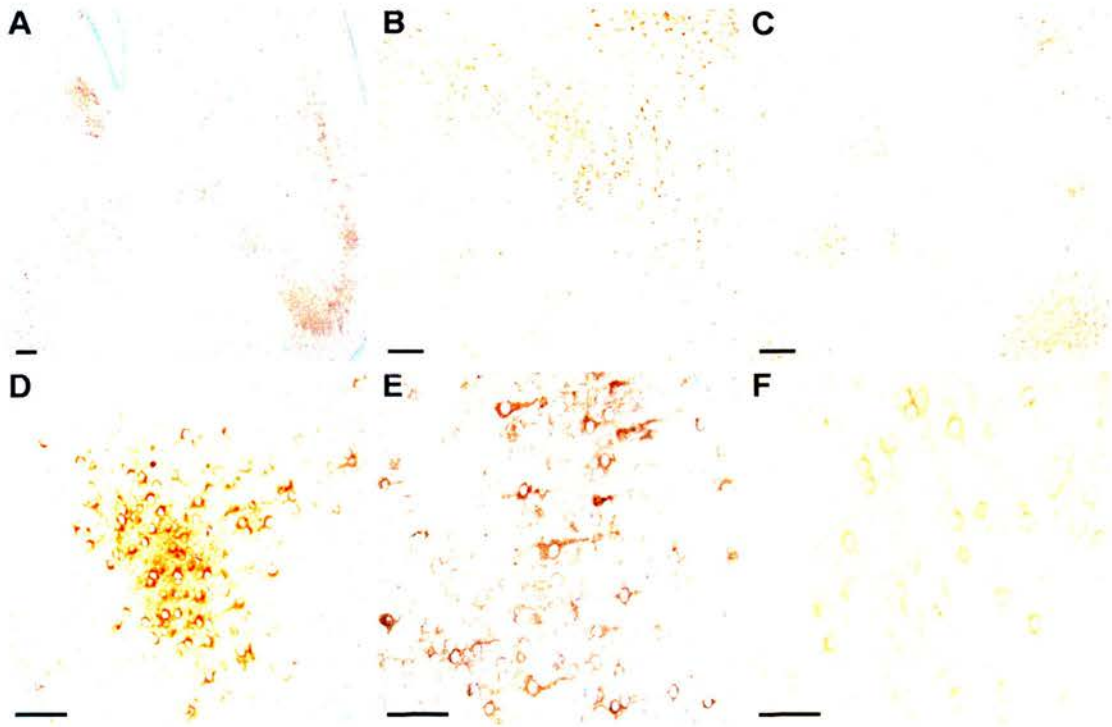

Figure 4.6: Virus spread throughout the brain is unaffected by a lack of functional PKR or RNase L.
Paraffin embedded saggital sections of P8 mouse brains from wt (A, D), PKR KO (B, E) and RNase L KO (C, F) mice were immunostained for SFV (brown). The images are representative of all the samples examined. Bars represent 100 μm.
To assess cell death, paraffin sections were immunostained for caspase-3 activation. Images were acquired using a Zeiss Axioskop 2 plus microscope.

Based on previous work (personal communication Prof. J.K. Fazakerley), it was known that caspase-3 is activated in specific neuronal populations of the wt P8 mouse brain following SFV infection *in vivo*. Areas of relatively immature neurons such as the dentate gyrus, the olfactory bulb and the cerebellum are the areas that exhibit most apoptosis when infected with SFV. In the experiments described here, neurons in most areas of the brain had a swollen appearance, not apoptotic; this was

in contrast to the condensed pyknotic appearance of cells undergoing apoptosis in areas of neurogenesis (for example: the olfactory bulb, dentate gyrus and cerebellum). Many infected cells in these areas were condensed and detached from neighbouring cells. Darkly stained, pyknotic nuclei and activated caspase-3 staining in the cytoplasm were characteristic of cells undergoing apoptosis. These cells were most frequently observed in areas that were known to be highly infected with SFV. This directly linked the SFV infection with the apoptotic response because apoptosis was not observed in uninfected brains. Similar patterns of caspase-3 activation were observed in the wt, PKR KO and RNase L KO mice (figure 4.7).

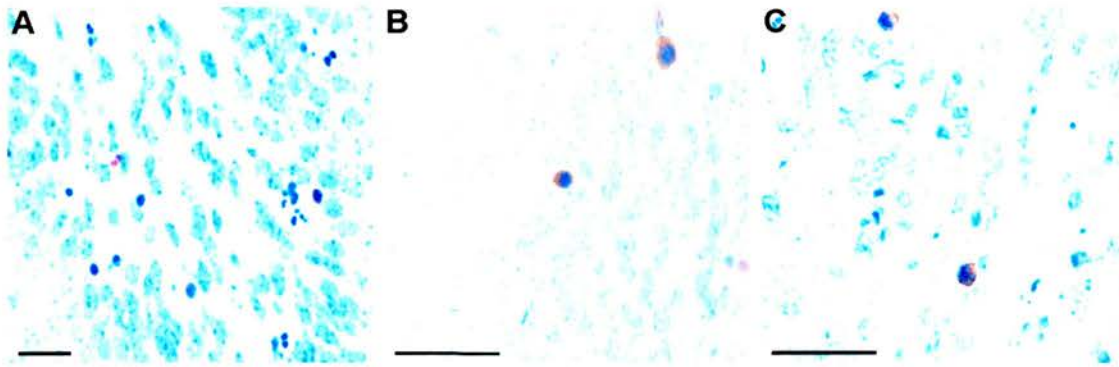

Figure 4.7: Caspase-3 is activated in some neuronal cells following SFV infection.
Paraffin sections of mouse brains from wt (dentate gyrus, A), PKR KO (cerebellum, B) and RNase L KO (cerebellum, C) mice were stained for activated caspase-3 (brown) and the cell nucleus (blue). Highly pyknotic nuclei, highlighted by the dark blue stain, are probably cells in the late stages of apoptosis. Bars represent 100 μm. Images were acquired using a Zeiss Axioskop 2 plus microscope.

Do cells lacking PKR, RNase L, ISG-12 or Fas die at a different rate to wt cells?

There are numerous examples of biological systems that can compensate for each other. Myoglobin is a protein known to be important in oxygen transport; myoglobin KO mice however appear normal (Garry *et al.*, 1998). This indicates that another system can compensate for the loss of the myoglobin gene. A screen of gene deletions at more than 500 loci in yeast highlighted this when less than half showed growth defects (Winzeler *et al.*, 1999). This suggests that even in a relatively simple organism the loss of a protein can often be compensated by another protein.

Biological redundancy is also seen at the level of RNA translation where different codons code for the same amino acid.

Compensation however may not be absolute and small but significant differences may exist when a protein is lacking. If Fas, ISG-12, PKR or RNase L normally plays a role in the cells' apoptotic response to SFV then lack of that protein may affect the rate at which a population of cells dies (for example). Cells may trigger apoptosis quicker so the population dies more rapidly, or the cells may react more slowly as they cannot efficiently or rapidly detect the virus so the population dies at a slower rate. An examination of the kinetics of cell death may reveal roles for proteins that are ordinarily hidden.

In separate experiments the rate of SFV induced death in wt MEFs and MEFs derived from mice lacking functional Fas, ISG-12, PKR or RNase L were determined. Fas KO, ISG-12 KO, PKR KO, RNase L KO and wt MEFs were seeded into wells of a 96-well plate at a concentration of 3×10^4 cells per well. Each timepoint was represented by 4 wells. Cells were incubated overnight and then infected with SFV4 (MOI 50). The high MOI ensured that all cells were infected at time zero. At the allocated timepoints a Wst-1 assay was performed on the pre-assigned wells and measurements were taken. At each subsequent timepoint a fresh set of wells was assayed for both wt and KO cell lines. The data was normalised to uninfected controls (figure 4.8). Experiments were repeated independently on at least one other occasion, generally twice more. In all cultures, viability remained unchanged for approximately 10-12 hours and then began to decrease. The viability decreased rapidly initially, and then slowed. PKR KO MEFs were the only cells that showed a difference to wt. The PKR KO MEF population lost viability more rapidly than the wt population. In contrast, the three other cell lines followed a similar path to the wt populations, suggesting that the loss of PKR somehow affected the cells' ability to respond to infection. The PKR KO cell line was therefore investigated further.

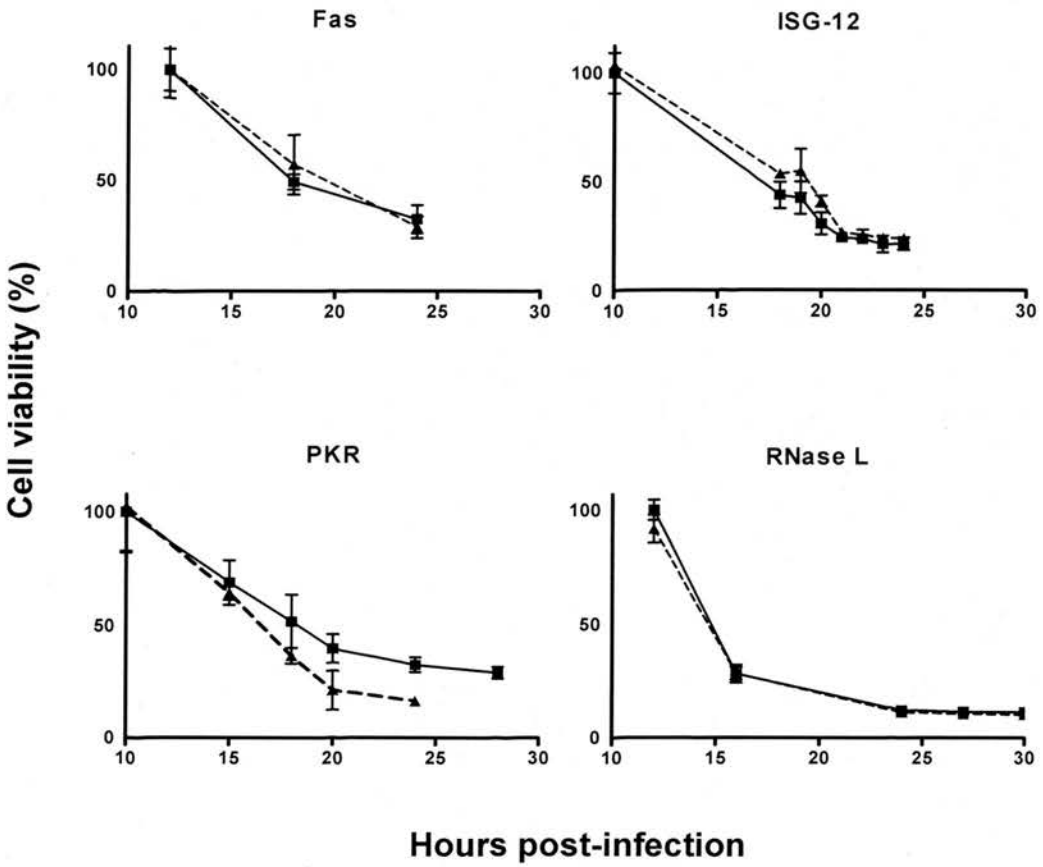

Figure 4.8: Analysing the rate of cell death of a population of cells as a result of SFV infection.
MEFs lacking functional Fas, RNase L, PKR or ISG-12 (broken line, ▲) were infected with SFV4 (MOI 50) alongside populations of wt MEFs (unbroken line, ■). Loss of viability was measured by a WST-1 assay. These assays were carried out in quadruplicate. The error bars are standard deviations of the mean.

Do cells lacking PKR activate caspases as part of their apoptotic response to infection?

Caspases-3, -8 and -9 are activated as a result of SFV infection (Chapter 3). Caspases-8 and -9 are initiator caspases; they are most likely to be the first caspases switched on when the cell is undergoing apoptosis in response to infection. They can activate downstream caspases such as caspase-3 and -7. To compare the time course of caspase activation in wt and PKR KO cells, parallel cultures of wt and PKR KO cells were seeded in triplicate, grown overnight, infected with SFV4 (MOI 50) and lysed at specified times post-infection. The lysates were analysed using caspase assay kits to determine if and when caspase-3, -8 and / or -9 were present in activated

forms (figure 4.9). Caspases-3, -8 and -9 were all activated between 9 and 10 hours post-infection. Activation of these caspases occurred at approximately the same time in PKR KO and wt cells (as seen in chapter 3). There was no significant switch on of caspases-8 or -9 at an earlier timepoint in the PKR KO cells as compared to the wt cells. The levels of caspase-3 activation were equivalent early in infection in PKR KO and wt MEFs. Caspase activation in wt and PKR KO MEFs were not compared at timepoints later than 13 hours.

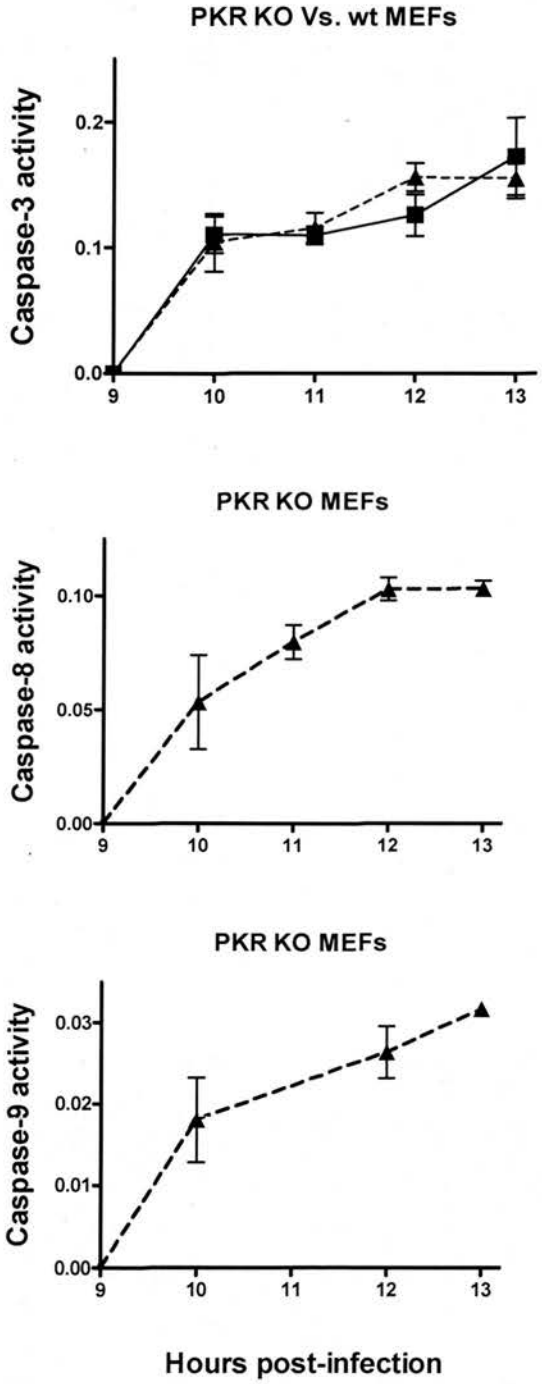

Figure 4.9: The levels of caspase-3 activation in PKR KO and wt MEFs are similar early in infection. Caspase-8 and -9 are activated in PKR KO cells at the same time as in wt cells.
PKR KO and wt MEFs were infected with SFV4 (MOI 50). Caspase-3 was measured in the wt (unbroken line, ■) and PKR KO MEFs (broken line, ▲). Caspase-8 and -9 activation was also measured in PKO KO MEFs. The experiments were carried out in triplicate. The values shown are the mean results. Error bars are standard deviations of the mean.

Does SFV replicate more efficiently in cells lacking functional PKR?

In the absence of PKR, detection of virus may be delayed so the anti-viral response of the cell to limit virus replication may be hindered. Two approaches were taken to investigate this possibility. The first was to measure virus protein production within the cell. The second was to measure infectious virus production.

To investigate protein production SFV1-d1eGFP VLPs were used to infect cells. VLPs cannot produce new virions and provide a single round of infection. SFV1-d1eGFP VLPs have the structural open reading frame (ORF) replaced with a short half-life ($T_{1/2}$ = 1 hour) form of eGFP. EGFP thus accurately reports the timing of translation of the structural ORF. SFV contains a translational enhancer element located in the 5' region of the capsid gene, which allows the structural genes to be translated efficiently when translational shutdown occurs in an infected cell (Ventoso *et al.*, 2006). The SFV1-d1eGFP VLPs do not have a structural enhancer and are therefore not only a good measure of the timing of structural protein translation but also of host translational shut-down.

PKR KO and wt MEFs were seeded in 6-well plates at a concentration of 3 x 10^5 cells per well, incubated overnight and then infected with SFV1-d1eGFP VLPs (MOI 50). Triplicate cultures were observed every 15 minutes post-infection to determine the timing of eGFP expression. A culture was considered GFP-positive if > 10 green cells were observed. This point was reached in the PKR KO populations as early as 3 hours 30 minutes but was not seen in the wt cultures until on average 7 hours post-infection. These times did not vary more than 15 minutes from the average and the experiment was repeated with similar results. This indicates that in the absence of PKR, the rate of virus structural protein synthesis was greatly

enhanced. PKR may be required to inhibit virus genome replication and / or virus protein production.

To look at the timing and rate of translation more closely, protein expression in virus infected cells was examined using radiolabelled (^{35}S) cysteine and methionine. PKR KO and wt MEFs were seeded in 6-well plates at a concentration of 3 x 10^5 cells per well, incubated overnight and then infected with SFV4 (MOI 50). At specified times post-infection the media in selected wells was replaced with 'starvation media' lacking methionine and cysteine. Half an hour later, this media was replaced with 'starvation media' supplemented with radiolabelled cysteine and methionine. The cells were incubated with this media for 30 minutes allowing incorporation of radiolabelled amino acids into newly synthesised proteins. The cells were then lysed and the protein concentration of each sample was measured. Equal amounts of protein were run on an SDS polyacrylamide gel which was then exposed to X-ray film (figure 4.10). Virus proteins started to be made at a detectable level in wt cells about 4 hours post-infection, reaching a high concentration by 5 hours. However, virus proteins were detectable by approximately 3 hours in cells lacking functional PKR, reaching a high concentration by 4 hours. Virus proteins were therefore made more quickly and in greater amounts in cells without functional PKR. In the absence of functional PKR, there was also a delay in the cellular translational shut-down caused by the virus infection. Shut-down began at approximately 3.5 - 4 hours in the wt MEFs but not until approximately 5 hours post-infection in the PKR KO MEFs.

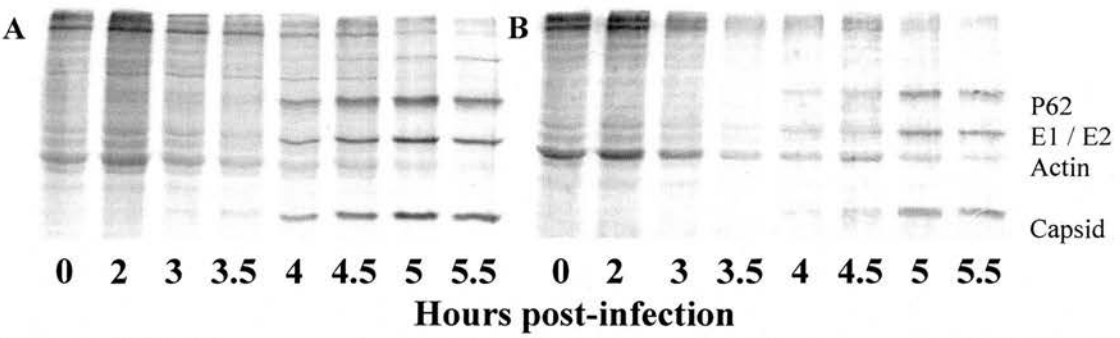

Figure 4.10: Virus proteins are detectable at an earlier stage post-infection in PKR KO cells as compared to wt cells

PKR KO (A) and wt MEFs (B) were infected with SFV4 (MOI 50). Newly synthesised proteins were radiolabelled and equal amounts were run on an SDS-polyacrylamide gel. Virus proteins were visible earlier and at higher levels in the PKR KO cells than in the wt cells.

Do PKR KO MEFs produce more virus particles than wt MEFs?

To determine whether earlier virus protein production in PKR KO cells led to greater virus production, one-step and multi-step growth curves were constructed. Triplicate parallel cultures of PKR KO and wt cells were infected with SFV4 at a MOI of 1 (low MOI) or 50 (high MOI). The high MOI infection allowed measurement of virus production from synchronously infected cells. The low MOI infection meant that only a small percentage of the culture was infected at time zero and virus production would be limited by the ability of virus to spread throughout the culture. PKR KO and wt MEFs were seeded at a concentration of 3 x 10^5 cells per well in a 6-well plate, incubated overnight and then infected at the different MOIs. At specific times post-infection, samples of culture media were collected and frozen at -80°C. Supernatants were titred for virus by plaque assay (figure 4.11). At high and low MOI, virus production occurred earlier in PKR KO cells than in wt cells; titres were approximately 100-fold greater in PKR KO cells than in wt cells at 6 h post-infection at both MOIs. The peak virus titres were approximately one log higher in the PKR KO cells at both MOIs. Taken together with the metabolic labelling studies, these results clearly demonstrate that SFV replicated faster in PKR KO cells.

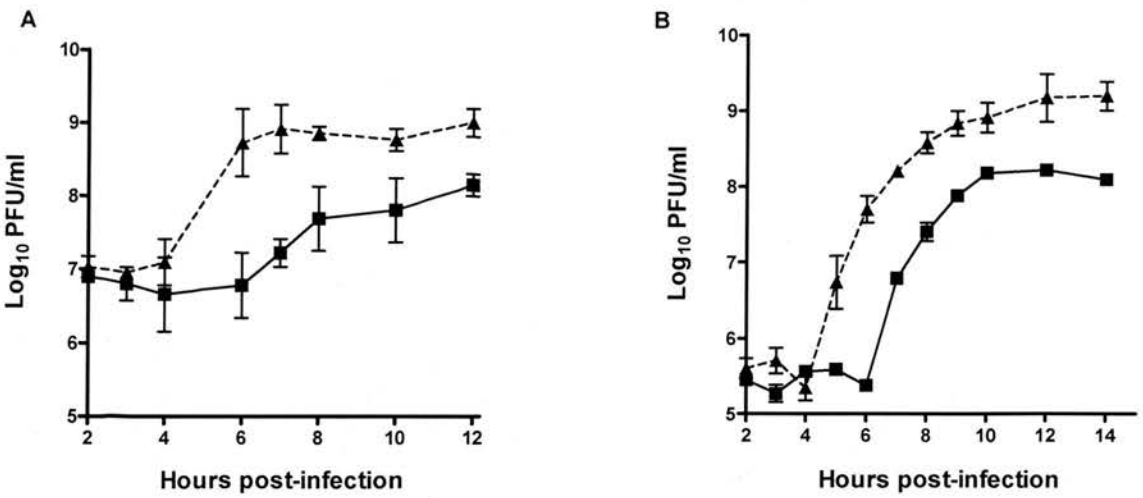

Figure 4.11: SFV4 grows faster in PKR KO cells than in wt cells
PKR KO (broken line, ▲) and wt (unbroken line, ■) MEFs were infected with SFV4 at a MOI 1 (A) or 50 (B). The amount of virus produced was measured over time. Each data point represents the average of three independent cultures. The error bars are standard deviations of the mean.

Is NFκB activity affected following SFV infection if PKR is not functional?

One of the main targets of activated PKR is IκB; phosphorylation of IκB by PKR forces it to release NFκB, allowing NFκB to enter the nucleus and act as a potent transcription factor. To determine whether this signalling is hindered in the absence of functional PKR, wt and PKR KO MEFs were seeded in 6-well plates and then transfected with a plasmid expressing firefly luciferase under the control of a promoter targeted by NFκB (repeated positive regulatory domain II elements of the IFN-β promoter) (Yoneyama *et al.*, 1998). When NFκB is activated, it binds to this promoter and firefly luciferase is expressed. The cells were infected with SFV4 (MOI 50) 24 hours after transfection. Luciferase production was measured from all samples to determine NFκB activity as a result of SFV infection. Levels of luciferase started to increase as early as 2 hours post-infection in the PKR KO MEFs whereas there was no increase measured in the wt populations up to 4 hours post-infection (Timepoints after this were not accurate due to translation shut-down preventing luciferase from being produced) (figure 4.12). This indicates that NFκB was activated very early post-infection in PKR KO MEFs whereas it took longer in wt MEFs.

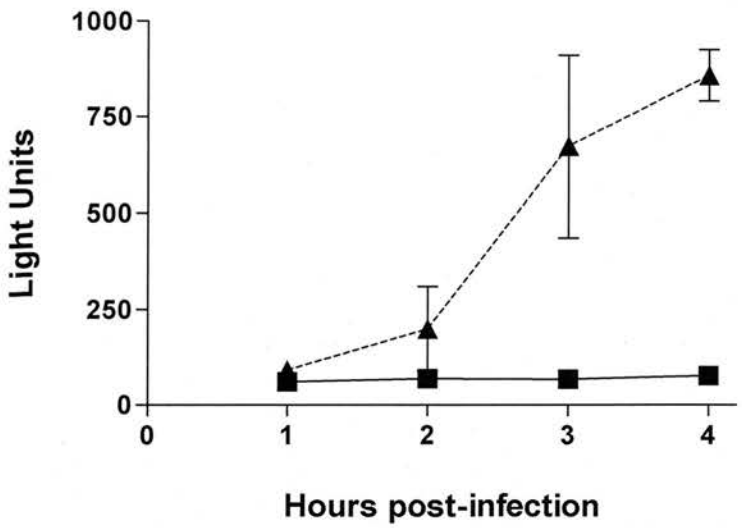

Figure 4.12: NFκB is active earlier in PKR KO MEFs
PKR KO (broken line, ▲) and wt (unbroken line, ■) were transfected with a plasmid that expressed firefly luciferase in response to NFκB activity. Cells were then infected with SFV4 (MOI 50) and NFκB activity (through a luciferase signal) was measured in response to the infection. All measurements are the average of triplicate experiments. The error bars are standard deviations of the mean.

Discussion

Many human virus infections cause more severe problems in the very young compared to adults. Cytomegalovirus infection of children can lead to neurological problems whereas adult infections are generally asymptomatic except in immunocompromised patients (Morgello *et al.*, 1987). This situation is similarly observed in animals. Neonatal mice are extremely susceptible to coxsackievirus B3 infection but that vulnerability disappears once the mouse reaches 7 days of age (Feuer *et al.*, 2003). Previous work has shown that mature post-mitotic neurons *in vivo* can survive for long periods of time when infected with SFV A7(74) whereas immature developing neurons in areas such as the olfactory bulb tend to rapidly undergo apoptosis post-infection (Fazakerley *et al.*, 2006; Oliver & Fazakerley, 1998). The results presented here show that this can be reproduced *in vitro*; neurons matured in culture were more resistant to cell death than immature neurons. While the immature neurons were dead by 48 hours, the mature neurons survived at least double that time. The mature neurons still died but the nature of cell death was distinct from that of the infected immature neurons. The immature neurons condensed and detached, whereas the mature neurons slowly fragmented; leaking their contents into the media, leaving only the outer shell behind. This latter death had none of the morphological features of apoptosis. Mature neurons may not trigger apoptosis in response to SFV infection or alternatively, mature neurons may actively inhibit apoptosis.

Inhibition of apoptosis can lead to necrosis. For example, if cytochrome c is released from mitochondria but cannot form the apoptosome, apoptosis will not take place but the mitochondria will still lose their ability to function properly and the cell will slowly die by necrosis. *In vivo*, post-mitotic neurons become infected but do not die for at least 30 days (Fazakerley *et al.*, 2006). This is much longer than the neurons in culture that lasted for a maximum of 5 days. Clearly the environment that the cells are living in *in vivo* is more suited to their survival and paracrine signals from heterogenous populations of neighbouring cells may be playing a role in cell survival. However, the *in vitro* data illustrates how maturation can also play a role and further maturation *in vitro* may lead to even longer survival after infection. An important question however is how does maturation lead to longer survival after

infection? *In vivo,* as connections are made between neurons, survival factors are released preventing apoptotic death. Immature neurons are primed to die unless they make connections. This may explain why they die so quickly upon virus infection. Most tissues can afford to lose a small percentage of cells through apoptosis following virus infection and with many viruses this may provide a protection which allows the organism to survive the infection. In contrast, losing cells in areas of the brain that are crucial to survival could be severely detrimental to the organism, which may explain why this maturation related suppression of apoptosis may have evolved.

As neurons mature they may express different proteins that control responses to infection; one such protein may be ISG-12. ISG-12, while being less well characterised than other proteins discussed in this section, has previously been shown to be involved in protection against SV induced death *in vivo* (Labrada *et al.*, 2002). ISG-12 is also upregulated during influenza virus infection although its role is unclear (Zhang *et al.*, 2007). Work published at a recent meeting suggests that ISG-12 is responsible for the transport of proteins from the nucleus to the cytoplasm (Papac-Milicevic *et al.*, 2007). It may play a role in the transport of anti-viral proteins from the nucleus to the cytoplasm.

In this study, MEFs lacking functional ISG-12 died when infected with SFV and lost viability at the same rate as wt MEFs infected with SFV4. This indicates that, at least in the case of MEFs, ISG-12 is not required for cell death following SFV infection. A pilot experiment was established to test this *in vivo*. Four-week old mice without functional ISG-12 were infected with SFV A7(74); they survived and remained healthy throughout the infection illustrating that ISG-12 is not required for protection in adult mice. This however was a pilot experiment and larger populations are required to associate any significance with this result. Time constraints did not allow further investigations into this area. ISG-12 is located on the surface of the nucleus and may be responsible for transporting proteins into or out of the nucleus; these proteins may be important during virus infection. In SFV infected adult mice either ISG-12 is not important or its role is compensated for by other proteins. It would be interesting to express ISG-12 in neonatal mice to see if expression protects against SFV infection as suggested for SV infection (Labrada *et al.*, 2002).

A number of virus infections have been shown to cause cell death through activation of Fas. Hepatitis C virus infection of hepatocytes leads to an upregulation of Fas expression and this upregulation correlates with hepatocyte apoptosis suggesting Fas plays a role in hepatitis C virus cell death (Pianko *et al.*, 2000). Similarly, Fas is upregulated during an influenza virus infection. Anti-Fas antibodies, that inhibit the activity of Fas on the surface of infected cells suppress influenza induced cell death (Takizawa *et al.*, 1995). Influenza virus propagation is also hindered when Fas activation is inhibited (Wurzer *et al.*, 2004). When cells lacking Fas were infected with SFV4 they underwent apoptosis and no difference was observed in the rates of cell death between Fas KO MEFs and wt MEFs following SFV infection. This indicates that Fas does not play a significant role in SFV induced cell death. Fas is part of the TNF family of cell surface receptors. Other members include TRAIL and TNFα and the family in total consists of 19 members. Many of these TNF family members can be upregulated during an immune response, and this is likely to be the case during SFV infection. The death domain that Fas possesses is common to a number of the family members and it is possible that other TNF family members are stimulated and trigger caspase-8 activation and subsequent apoptosis. If these receptors are also involved then they may well be compensating for the loss of Fas. Alternatively, or in addition, the intrinsic pathway of apoptosis may be activated, which would again result in death independent of Fas.

Mammalian cells have developed different dsRNA detection proteins including TLR3 (Alexopoulou *et al.*, 2001), MDA5 (Kang *et al.*, 2002), RIG-I (Yoneyama *et al.*, 2004) 2,5-OAS (Hovanessian & Kerr, 1979) and PKR (Meurs *et al.*, 1990). Each of these proteins is activated by dsRNA and activation leads to anti-viral responses. Using MEFs deficient in functional PKR Yang *et al* showed that cells were apparently unresponsive to artificial dsRNA (poly I:C) at concentrations which induced apoptosis in wt MEFs (Yang *et al.*, 1995). Recently, it has been shown that neuronal death in response to treatment with HIV glycoprotein (gp-120) requires PKR activation. Neurons with a PKR KO genotype were significantly protected from gp-120 neurotoxicity (Alirezaei *et al.*, 2007). HeLa cells infected with influenza virus die by apoptosis but if the cells have an inactive form of PKR then

apoptosis does not occur (Takizawa *et al.*, 1996). These studies all indicate that PKR can play a role in the apoptotic response to a virus.

RNase L also has proapoptotic properties. Zhou *et al* showed that cells isolated from RNase L KO mice showed reduced susceptibility to apoptosis inducing chemicals such as staurosporine and TNFα (Zhou *et al.*, 1997a). Hepatitis C virus proteins expressed from a vaccinia virus vector killed wt MEFs. However, expression in RNase L KO MEFs resulted in cell survival suggesting that RNase L was involved in triggering the death (Gomez *et al.*, 2005).

In the current study, both *in vivo* and *in vitro*, PKR KO and RNase L KO MEFs underwent apoptosis after SFV infection. This implies that neither PKR nor RNase L is required for apoptosis in response to SFV infection. The rate of cell viability loss was no different between RNase L KO and wt MEFs, but there was a difference between PKR KO MEFs and wt MEFs. The PKR KO MEFs lost viability faster than wt cultures. This is not consistent with the hypothesis that PKR is required to detect virus and trigger apoptosis. Possible explanations include that PKR directly delays induction of apoptosis or that PKR delays virus replication resulting in delayed induction of apoptosis. The mechanism by which the cell senses infection and triggers apoptosis remains unknown but prime candidates include RIG-I and MDA5.

Caspases are activated in cells dying as a result of SFV infection (chapter 3). Early caspase activation occurs approximately 10 hours post-infection. Because cells without PKR died more quickly than wt cells, it was expected that the apoptosis initiators caspases-8 and -9 would be activated earlier in PKR KO cells. Caspases were activated in PKR KO MEFs, but no time difference was observed relative to wt MEFs; caspases were activated between 9 and 10 hours post-infection in both cell types. Furthermore, at least between 9 and 13 hours post-infection no differences in the levels of caspase-3 were observed between PKR KO and wt MEFs. A difference may emerge later in infection. Caspases while important may not be the only factors involved in SFV induced cell death. Results from chapter 3 suggest that caspase independent factors (e.g. AIF and EndoG) may also play a role in SFV induced cell death and while caspases may be dominant, they may not completely dictate the rate at which cells die after infection.

To understand the role of PKR further, virus replication in the presence and absence of functional PKR was examined. Newly synthesised proteins in infected cells were radiolabelled with ^{35}S methionine and cysteine and then visualised on an SDS polyacrylamide gel. The virus structural proteins were visible earlier and at higher concentrations in the PKR KO MEFs compared to the wt MEFs. There also appeared to be a delay in the shutdown of cellular protein translation in PKR KO MEFs. These results indicate that PKR, directly or indirectly inhibits the production of virus structural proteins in wt cells. The delay may seem small (approximately 1 hour) but when one considers that the whole virus life cycle from entry to budding takes only 4-5 hours, this 1 hour delay can be considered quite substantial. SFV, as far as is known, does not harbour any anti-apoptotic genes in its small genome, unlike a lot of DNA viruses which dedicate substantial amounts of their genome to preventing cell death. Instead the virus relies on speed to escape the apoptotic cell trap. SFV replicates fast and produces large amounts of infectious virus particles before the cell undergoes apoptosis. The rate of cell death is likely to have been an important selective pressure in virus evolution with viruses having to replicate sufficiently fast so as not to be affected by this process or having to delay or suppress this response. Once the cell dies no more virus particles are produced. The virus life cycle is delayed by approximately 15-20 % and this affects the amount of virus particles that are produced. Almost 10-fold more virus was produced from PKR KO MEFs compared to wt MEFs according to the one-step growth curve. A multi-step growth curve showed a similar difference.

Functional PKR may detect dsRNA early in SFV infection and could rapidly switch on anti-viral signalling pathways. Activated PKR would also phosphorylate eIF2-α and initiate translational shut-down in the infected cell. Presumably, this shut-down dramatically reduces translation of virus non-structural proteins and limits virus genome replication. If shut-down is delayed, perhaps more non-structural proteins and therefore more genomic and subgenomic RNA and ultimately more virus particles are made.

The absence of PKR also affects anti-viral signalling; NFκB is activated earlier in the absence of PKR. This suggests that the increased rate of SFV replication is causing earlier detection of the virus. This implies that either PKR

restricts replication of SFV from very early in infection through a mechanism independent of translational shut-down or PKR restricts the activation of other virus detection proteins or the down stream signalling. For example, the interaction of PKR with SFV dsRNA may disrupt or slow down the replication of the virus.

Higher virus replication rates also suggest why MEFs die more rapidly in the absence of PKR. The triggering of the cascade of proteins involved in apoptosis, and the strength of the apoptotic signal may be influenced by virus RNA and protein load within the cell as well as the amount of virus produced from the cell. More dsRNA may be more easily detected by dsRNA detection proteins that can trigger cell death, more proteins may put the cell under more stress, and more budding of virus particles may disrupt the cell by an unknown mechanism that enhances cell death. These factors are greater in PKR KO cells and each or all could contribute to the increased rate of cell death.

One of the main ideas behind this research has been that the cell tries to kill itself as quickly as possible in order to trap the virus, stop it from replicating and stop new virus particles from spreading to neighbouring cells. It was found that cells lose their viability more rapidly in the absence of the presumed anti-viral protein, PKR. This paradoxical result invokes the question, why PKR is necessary at all? Would it not be better for the cell if PKR were not activated, as the cell would die more rapidly without it? The final results in this chapter describing the virus protein production and titres in the absence of PKR help to resolve this apparent contradiction. It is clear the cell must strike a balance between committing suicide and limiting virus spread by other means. While committing suicide is important, early detection of the virus and the establishment of an anti-viral state are also crucial as this allows the limitation of virus production and allows the infected cell to warn neighbouring cells; if the cell dies too quickly it may not have enough time to produce interferon for example.

The *in vitro* data presented here show that the absence of PKR leads to more virus production at earlier timepoints and cells die more rapidly. In the brains of wt and PKR KO mice however, at 1 or 2 days post-infection, virus spread and levels of apoptotic cells were similar. Other studies have shown that virus infections can be lethal in the absence of PKR. VSV for example causes death if inoculated i.n. into

mice lacking functional PKR, whereas it is harmless in wt mice (Balachandran *et al.*, 2000). Similarly Bunyamwera virus kills mice more quickly if the mice lack functional PKR (Streitenfeld *et al.*, 2003). It is possible that the differences seen *in vitro* in this chapter are not significant enough to provoke a major difference *in vivo*. Importantly, the *in vivo* work described earlier was carried out with avirulent SFV A7(74) while the *in vitro* work was done with the virulent strain SFV4. The differences between these two strains of virus may explain why the results documented *in vitro* did not manifest as a difference *in vivo* compared to wt mice.

Chapter 5: The mechanisms of cell death caused by SFV infection

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Introduction

The mechanism(s) by which cell death is triggered during SFV infection has been explored but is not really understood. Generally mammalian cells rapidly die by apoptosis following SFV infection (Chapter 3; Glasgow *et al.*, 1997), but it remains unclear when or how the cell receives the signal to die.

The SFV life cycle can be divided into a number of stages. The first is virus entry, which can itself be divided into binding to the cell membrane, entry by endocytosis, low pH dependent fusion of the viral membrane proteins with the endosome and release of its genome into the cytoplasm (reviewed by Kielian, 1995). The cell may have an ability to detect virus entry at any of these points and initiate a pro-apoptotic and pro-inflammatory response. It has been suggested that SV entry triggers cell death through sphingomyelinase activation and ceramide upregulation in the cytoplasm (Jan *et al.*, 2000). These studies used replication competent SV to infect mouse neuroblastoma cells. Replication competent virus may trigger sphingomyelinase activation through entry and fusion, but SV may also activate sphingomyelinase and subsequent apoptosis by replicating its genome or synthesising new viral proteins; these potential contributing factors were not addressed in this paper.

In contrast to this, work has been carried out with replication incompetent, UV inactivated, SFV; this virus binds to the cell surface and fuses with the endosome as normal but does not replicate its genome or make new virus proteins. Dendritic cells activated IRF-3 and produced IFN-α and -β in response to this virus whereas wt MEFs were unresponsive (Hidmark *et al.*, 2005). Interestingly, UV inactivated SFV did not kill MEFs (unpublished data, personal communication with Dr. Asa Hidmark, Karolinska Institute, Sweden). It would be logical for a cell to detect virus infection at the point of entry so that it could respond as quickly as possible. However, this is unlikely to be the only stage at which cells detect SFV infection.

The second stage of the virus life cycle is release of the genome into the cytoplasm, translation and RNA replication. The virus replicates via a dsRNA intermediate. Cellular proteins such as TLR3, RIG-I, PKR and MDA5 all recognise dsRNA and activate anti-viral pathways. Many viruses have evolved different ways of preventing the cell from detecting or reacting to the dsRNA. The outer σ3 capsid

of reovirus binds dsRNA and variations in the sensitivity of reovirus strains to IFN can be related to the affinity of σ3 for dsRNA (Bergeron *et al.*, 1998). BVDV-infected cells release a viral encoded glycoprotein, Erns, which has RNase activity and degrades dsRNA released from infected cells (Iqbal *et al.*, 2004). This is probably an attempt to stop TLR3 on the surface of neighbouring cells from detecting the RNA. The NS3/4a protein of hepatitis-C virus cleaves MAVS from the surface of mitochondria, disabling the downstream signalling of RIG-I and MDA5 (Li *et al.*, 2005c).

Positive-stranded RNA virus replication occurs on the surface of intracellular membranes; different viruses use different membranes including the ER (Restrepo-Hartwig & Ahlquist, 1996), endosomes (Grimley *et al.*, 1968) or mitochondria (Miller *et al.*, 2001). The virus manipulates these structures causing invaginations called spherules to form on the membrane; the virus RNA is then replicated in these spherules. SFV adapts endosomal-lysosomal compartments that form in the cytoplasm of the infected cells. These cytoplasmic vacuoles have small spherules projecting from the membrane surface into the lumen of the vacuole. It has been shown by electron microscopy that each spherule contains a complex of the non-structural proteins along with RNA that is being replicated (Kujala *et al.*, 2001). These invaginations of vacuole surfaces probably provide both an area for replication complexes to form, and a mechanism to exclude dsRNA detection proteins. The question of whether dsRNA detection takes place and contributes to cell death remains unclear. It is possible that RNA replication begins before the spherules form properly. This would leave small amounts of dsRNA exposed to cellular detection proteins. It is also possible that detection proteins can enter spherules and detect dsRNA; the spherules have a narrow entrance and it is unclear whether cellular detection proteins can enter this space while it is filled with viral proteins.

Following translation of the non-structural proteins and replication of virus RNA, SFV must synthesis new structural proteins and produce new virus particles. The structural virus glycoproteins are glycosylated while they pass through the ER and the Golgi. The overloading of this system could lead to the activation of the UPR and activation of apoptosis.

The UPR is a cellular response to accumulation of unfolded proteins in the lumen of the ER. The response is not specific to virus proteins and indeed occurs in healthy cells as well as infected cells; it does not necessarily lead to cell death. The build up of unfolded proteins is monitored by a protein called BiP. BiP is located in the lumen of the ER and in unstressed cells BiP binds to the luminal domains of three ER membrane bound proteins: PERK, IRE1 and ATF6. BiP is a protein chaperone and part of the heat shock protein 70 family. BiP binds to unfolded proteins that come into the ER lumen and with the help of other protein chaperones, folds them into a functional conformation (Haas, 1994). There is a large excess of BiP compared to IRE1, PERK or ATF6 (Ghaemmaghami *et al.*, 2003) so a high concentration of unfolded protein is required before PERK, ATF6 and IRE1 are unbound from BiP. Release of BiP from these proteins leads to their oligomerisation and activation. PERK can phosphorylate EIF-2α, which allows the translation of ATF4; ATF4 upregulates the transcription of CHOP (Harding *et al.*, 2000). Activated IRE1 splices XBP1 mRNA into a translatable form (Lee *et al.*, 2002) and finally ATF6 travels to the Golgi, gets cleaved and then acts as a transcription factor, upregulating a number of genes including protein chaperones to improve the folding capacity of the ER (Wang *et al.*, 2000). The splicing of XBP1 mRNA occurs early in the UPR and is a clear sign of cell stress. Ordinarily, a cell can control stress by down-regulating protein production and increasing chaperones to fold what is already there. CHOP upregulation on the other hand indicates that the cell has maintained a stressed state for a substantial amount of time. CHOP sensitises the cell to death by altering the expression levels of various pro- and anti-apoptotic genes. The second part of this chapter examines cell translation and activation of the UPR in SFV infected cells.

Objectives

1. Determine whether SFV dsRNA is involved in SFV induced cell death.
2. Determine whether RIG-I, MDA5 or MAVS is involved in SFV induced cell death.
3. Determine whether the ER UPR contributes to the death of SFV infected cells.

Results

To start to investigate early events in infection, virus replication complexes were visualised by infecting NIH 3T3 cells with SFV4(3F)-eGFP. In this virus the nsP3 gene of SFV is translated as a fusion protein with eGFP. Cells were then stained with an antibody against dsRNA. As expected, dsRNA displayed punctate cytoplasmic co-localisation with nsP3 (figure 5.1). To determine whether the dsRNA binding protein MDA5 also co-localised to these complexes, an antibody against MDA5 was used to stain cells that had been infected with SFV4(3F)-eGFP. Unfortunately, the immunostaining was unsuccessful.

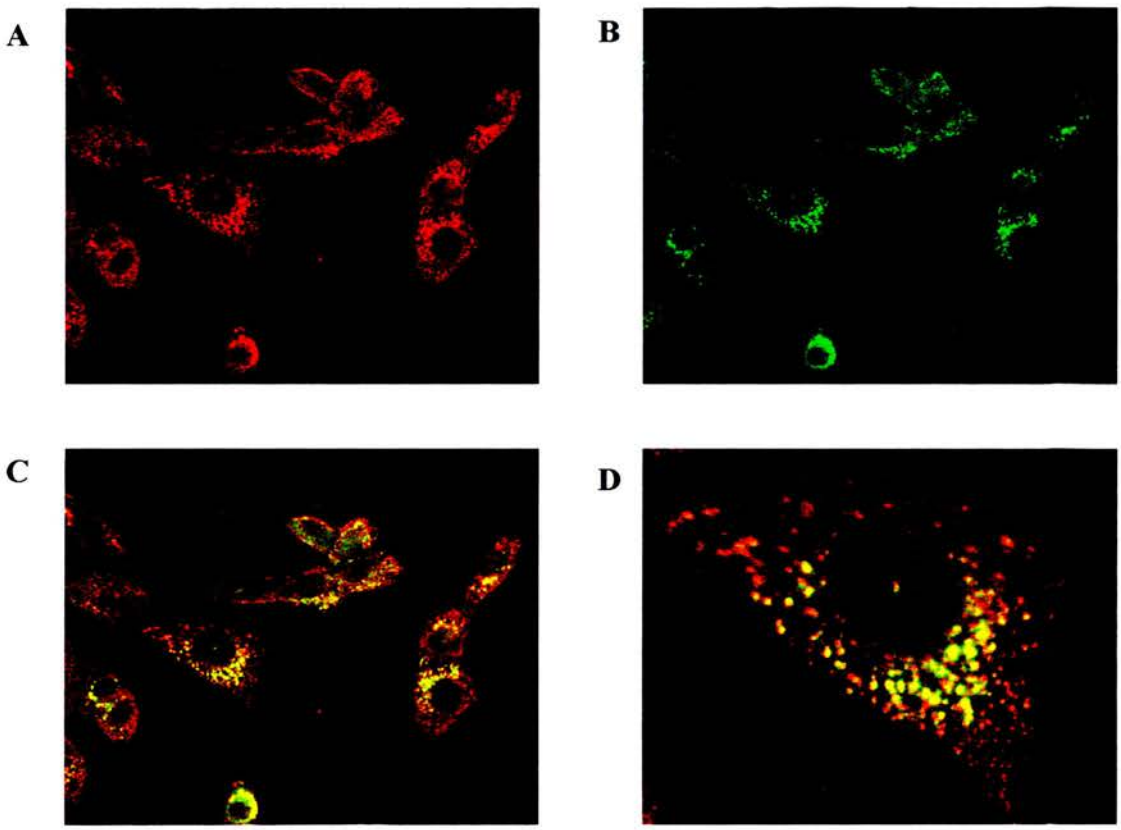

Figure 5.1: DsRNA and SFV nsP3 co-localise
NIH 3T3 cells were infected with SFV (3F) 4-eGFP and immunostained for dsRNA 8 hours post-infection. (A) dsRNA (red), (B) nsP3 (green), (C) merged image, (D) magnified picture of one cell from panel C. Images were acquired using a Zeiss confocal microscope.

Does virus dsRNA contribute to cell death following infection?

It can be hypothesised that detection of virus dsRNA by cellular helicases (PKR, RIG-I, MDA5) triggers not only the interferon response but also cell death. In collaboration with Professor Richard Randall (University of St. Andrews) this hypothesis was tested using a number of Hep-2 cell lines expressing proteins that bind dsRNA or inhibit signalling from these helicases; the proteins expressed are listed below. The proteins were expressed constantly as each gene was under the control of a spleen focus-forming virus promoter (construction explained in Hale *et al.*, 2006).

- **Influenza A protein, NS1:** This protein has various functions including helicase activity. It is reported to bind to dsRNA and protect the RNA from cellular detection proteins (Qian *et al.*, 1995).
- **A mutant form of NS1, Y89F:** This can still bind dsRNA but cannot activate the PI3 kinase signalling pathway (Hale *et al.*, 2006).
- **Vaccinia virus protein, E3L:** This protein also has helicase properties and can bind dsRNA as well as inhibiting IRF-3 (Chang & Jacobs, 1993; Xiang *et al.*, 2002).
- **Hepatitis C protein, NS3/4a:** It can cleave MAVS from the surface of mitochondria (Li *et al.*, 2005c).
- **parainfluenza virus (PIV) 5 V protein:** inhibits MDA5 and IRF-3. (Andrejeva *et al.*, 2004; Kiyotani *et al.*, 2007).
- **SeV, C protein:** inhibits RIG-I and STAT1 (Garcin *et al.*, 2002).
- **SeV, V protein:** inhibits IRF-3 and MDA5 (Komatsu *et al.*, 2004).
- **BVDV, NPro protein:** inhibits IRF-3 (Hilton *et al.*, 2006).

Each of these cell lines and a cell line that contained no plasmid (naive) were seeded in 96-well plates, allowed to grow overnight and then infected with SFV4 (MOI 50). Cell viability over time was then measured by a Wst-1 assay and compared to the naive population (figure 5.2). Each graph represents an individual cell line compared to the naive cell line.

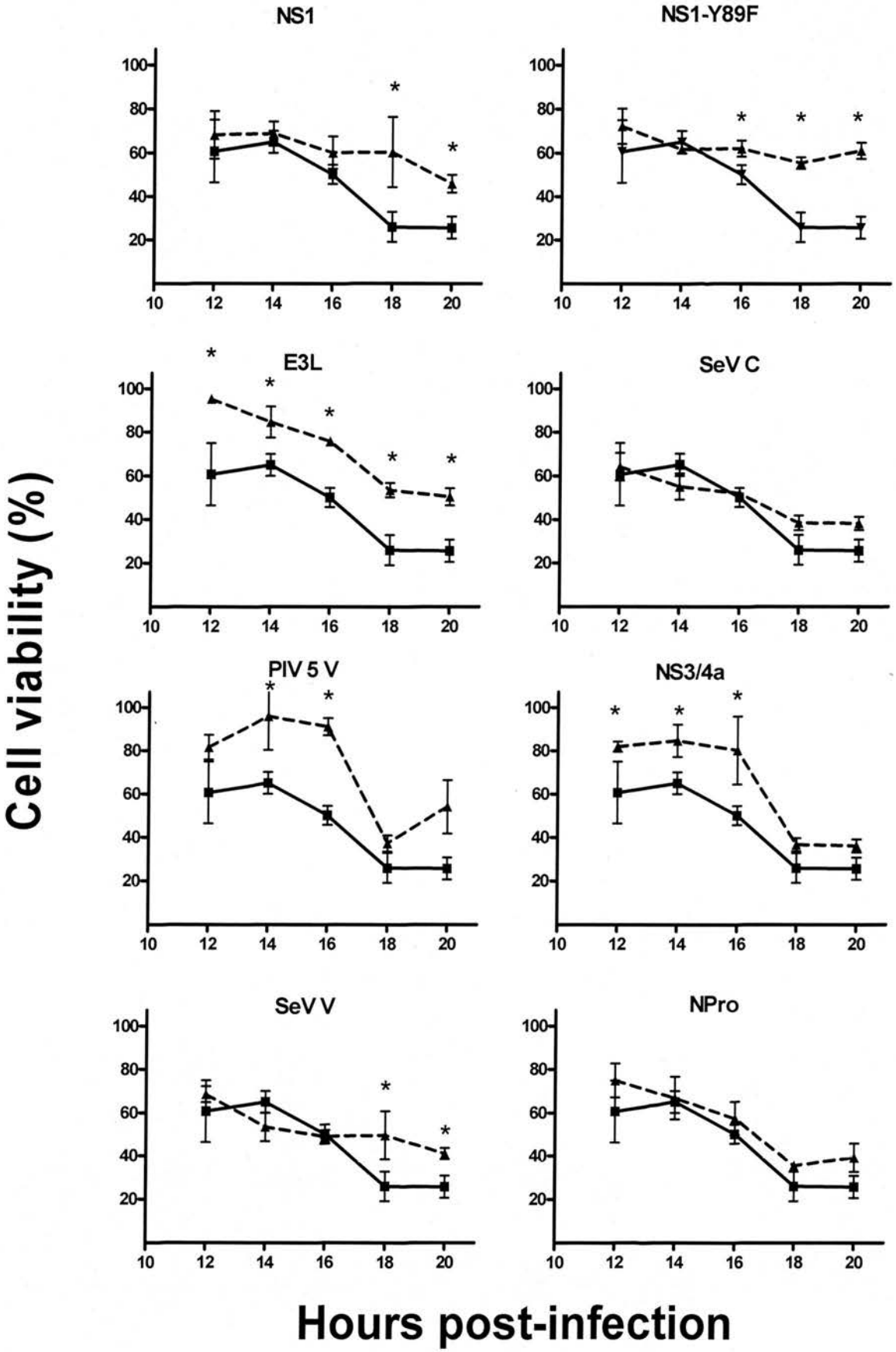
NS1
NS1-Y89F
E3L
SeV C
PIV 5 V
NS3/4a
SeV V
NPro
Cell viability (%)
Hours post-infection

Figure 5.2: Expression of different virus proteins in Hep-2 cells infected with SFV.
Cells expressing different virus proteins were infected with SFV4 (MOI 50). Loss of viability was measured over time (broken line, ▲) and compared to a naïve cell line (no plasmid), infected in parallel (unbroken line, ■). The experiment was carried out in triplicate. The values given are the means. The error bars are standard deviations of the mean. Significant differences according to an unpaired t-test ($P<0.05$) between the test cell line and the naive cell line are signified by a star.

All the cell lines expressing proteins that bind to dsRNA and potentially block its detection within cells (NS1, Y89F and E3L), showed significant reductions in their rate of cell death compared to naive cells. Reduced cell death was also observed in cells expressing NS3/4a and PIV5-V. The former suggests that activation of MAVS by helicases may contribute to cell death. The latter suggests that MDA5, a known target of PIV5-V protein, is involved in cell death. Interestingly, a reduced rate of cell death is also seen in cells expressing SeV-V protein. This protein has similar functions to the PIV5-V and although the difference is measurable later in infection it may suggest a similar pathway is involved in both cases. NPro from BVDV and SeV C protein both antagonise the interferon pathway but neither seems to have an effect on cell death rates. Both proteins target IRF-3, which may not play any significant role in cell death induced by SFV. The high MOI nature of these studies may preclude assessment of protective autocrine mechanisms. Furthermore RIG-I is inhibited by SeV-C; no effect on cell death rates suggests that RIG-I may not be involved in death pathways in cells infected with SFV.

Do events later in the virus life cycle affect cellular apoptosis?

If virus RNA coding for only the non-structural proteins is transfected into cells then the cells undergo apoptosis. This might imply that the structural proteins are not contributing to death, because when they are absent cells still die. However, it is likely that there are several triggers of cell death and these combine to determine the rate of cell death. To determine whether structural protein expression had any effect on cell death, the rate of cell death was compared in cells infected with virus which did (SFV4) or did not (SFV1-d1eGFP VLPs) produce virus structural proteins.

MEFs were seeded in two 96-well plates and allowed to grow overnight. These cells were then infected with SFV4 or SFV1-d1eGFP VLPs (MOI 50). MOI

50 ensured that all cells were infected at time zero to ensure that any observed difference was because of infection and not because of SFV4 ability to infect more cells in a second round of infection. Cell viability was measured using a Wst-1 assay at specific times post-infection. The viability of the cultures infected with SFV4 was compared to cultures infected with VLPs (figure 5.3). VLPs killed the cells as expected, however, the viability at each timepoint was significantly higher compared to the cultures infected with SFV4 ($p < 0.05$, Mann-Whitney test) indicating that the VLP infected cells took longer to die.

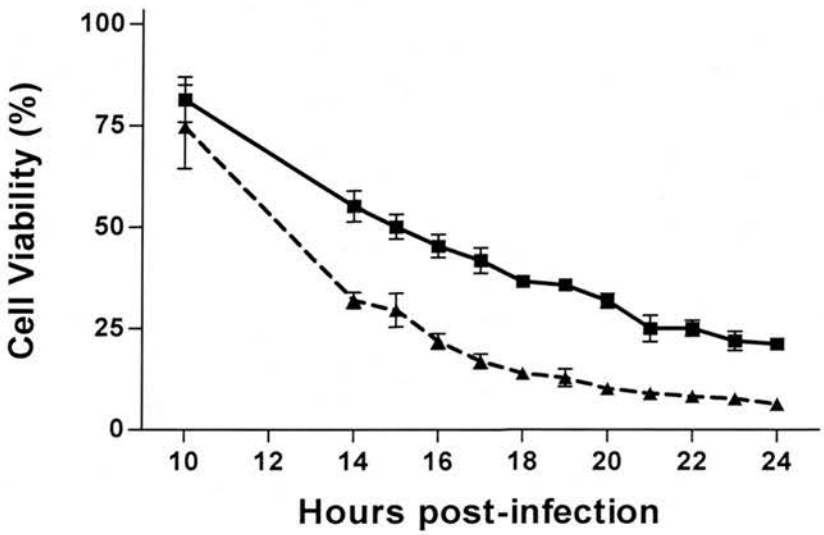

Figure 5.3: Cells infected with SFV1-d1eGFP VLPs died at a slower rate than cells infected with SFV4.
MEFs were infected with SFV4 (broken line, ▲) or SFV1-d1eGFP VLPs (unbroken line, ■). (MOI 50). Wst-1 assay measured the viability of cell populations each hour. The experiments was carried out in quadruplicate and repeated independently three times. The graph shows the results of one experiment. Error bars are standard deviations of the mean.

How do SFV structural proteins contribute to the death of mammalian cells?

It seems likely from the previous result that production of SFV structural proteins accelerates cell death. This suggests that the structural proteins induce a secondary apoptotic signal, which enhances the rate of cell death.

SFV envelope glycoproteins are directed through the ER and these may clog up the ER and trigger the UPR. To investigate this hypothesis, a number of markers of the UPR were analysed to see if activation of the UPR was taking place.

Is XBP1 RNA spliced after cells are infected with SFV?

Early in the UPR, IRE1 becomes activated and moves from the surface of the ER to the cytoplasm. IRE1 splices XBP1 mRNA so that it can be translated into a functional transcription factor, upregulating, among other genes, multiple chaperones (Back *et al.*, 2005). The splicing of XBP1 RNA is an early indicator of UPR activation. To assess XBP1 splicing in SFV infected cells, RNA was isolated from infected NIH 3T3 cells at different times post-infection and reverse transcribed into cDNA. Primers specific for XBP1 were then used to amplify the XBP1 splice variants. The variants differ by only a few base pairs and are therefore difficult to see on an agarose gel so a further step was employed to make the difference more obvious. The amplified cDNA was treated with the restriction enzyme Pst I. The cleavage site for Pst I is only present in the unspliced form. If any spliced RNA is present it will not be cleaved and will appear as a higher band on the agarose gel (see figure 5.4 for explanation). Pst I digested samples from each timepoint were then run on a gel with a negative control sample from uninfected cells and a positive control sample from tunicamycin treated cells; tunicamycin inhibits N-linked glycosylation of proteins and initiates the UPR. Spliced XBP1 was clearly detectable approximately 9 hours post-infection (figure 5.5).

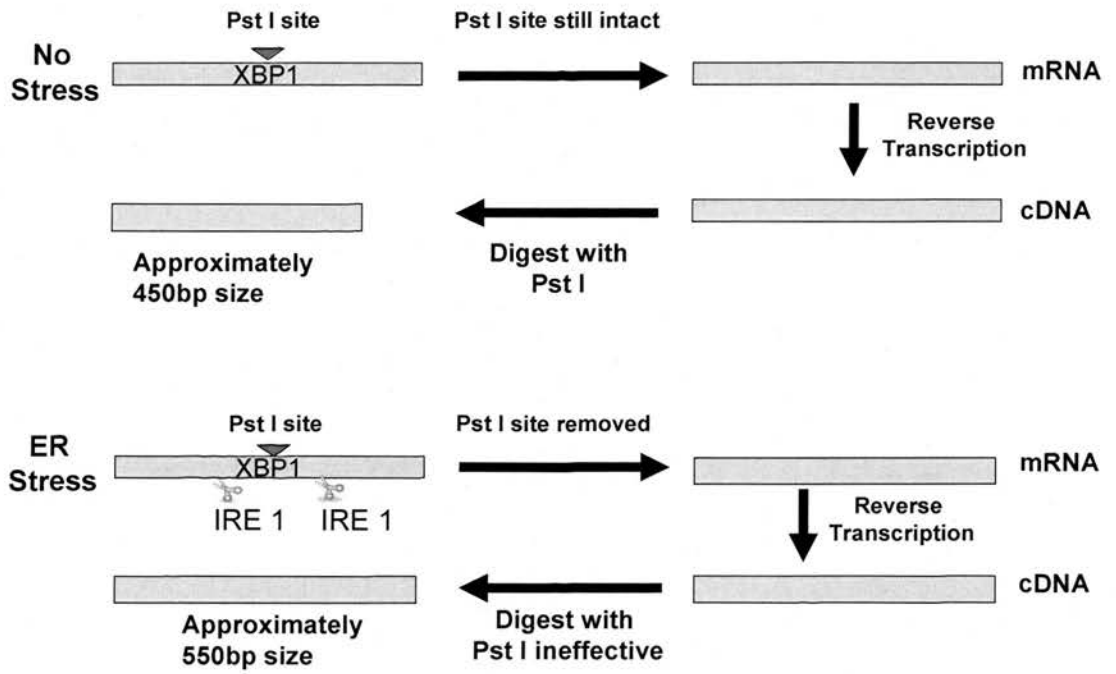

Figure 5.4: A scheme explaining the principle behind XBP1 splicing. In stressed cells XBP1 mRNA is cleaved by IRE1, removing a Pst I site in the process. When cDNA is made and Pst I digestion carried, it is ineffective and therefore a large band of approximately 600 bp is left. When pst I digestion is carried out on XBP1 cDNA from non-stressed cells, the Pst I site is present se it is cleaved leaving a smaller band of approximately 300 bp.

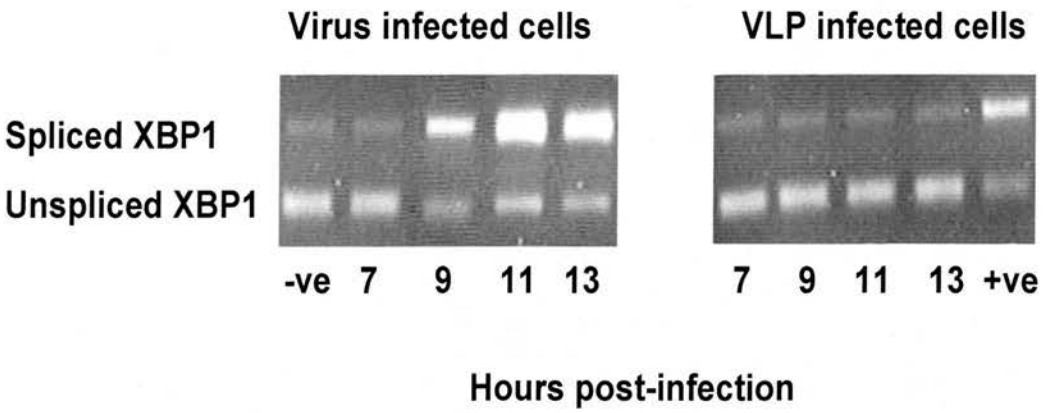

Figure 5.5: SFV infection of NIH 3T3 cells induced splicing of XBP1 mRNA. NIH 3T3 cells were infected with SFV4 or SFV1 d1eGFP VLPs (MOI 50). RNA was isolated from these cells, cDNA was made, XBP1 was amplified and then cleaved with Pst I (only cleaves the unspliced form). The negative (-ve) control was uninfected cells, the positive (+ve) control was tunicamycin (5 μg / ml) treated cells.

Does the UPR activate pro-apoptotic proteins?

The UPR is initially switched on to control a backlog of unfolded proteins in the ER. This can happen from time to time in a healthy cell and does not necessarily lead to cell death. The cell decreases the amount of new proteins coming into the ER by reducing translation, and it upregulates protein chaperones to speed up the folding of proteins. However, if the problem cannot be resolved the UPR upregulates and activates pro-apoptotic proteins that kill the cell. CHOP is one of the main pro-apoptotic proteins upregulated by the UPR. CHOP can inhibit Bcl-2 which in turn promotes apoptosis. In a healthy cell CHOP is expressed at a very low, almost undetectable level and so detection in infected cells implies that the UPR is triggering its upregulation (McCullough *et al.*, 2001). Proteins were isolated from NIH 3T3 cells that had been infected with SFV4 or SFV1-d1eGFP VLPs for 7, 9, 11, and 13 hours. A negative control of proteins from uninfected cells and a positive control of proteins from tunicamycin treated cells were also used. The protein concentrations were measured and equal amounts of protein from each sample were loaded onto an SDS-polyacrylamide gel and CHOP was assayed by western blot (figure 5.6). CHOP expression can be seen as early as 9 hours post infection in both the virus and VLP infected MEFs. The negative control and the 7 hour timepoint showed equivalently low amounts of protein.

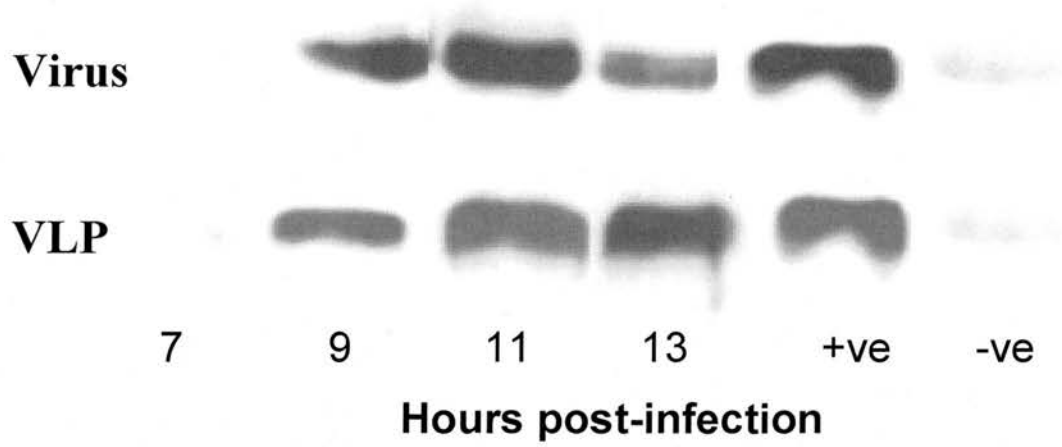

Figure 5.6: The UPR induced protein CHOP was upregulated when NIH 3T3 cells are infected with SFV.
NIH 3T3 cells were infected with SFV4 or SFV1-d1eGFP VLPs (MOI 50). Protein was isolated from these cells at different timepoints post-infection and a western blot for CHOP was carried out. The negative control (-ve) was protein from uninfected cells. The positive control (+ve) was protein from tunicamycin treated cells.

Another protein that is strongly linked to ER stress is caspase-12. Caspase-12 is located on the surface of the ER and when activated can interact with other caspases involved in apoptosis. To determine whether caspase-12 was activated in response to SFV4 or SFV1-d1eGFP VLP infection, protein was isolated from NIH 3T3 cells infected with SFV4 or SFV1-d1eGFP VLPs (MOI 50) for 7, 9, 11 or 13 hours. The protein concentration was equalised with protein from uninfected cells (negative control) and tunicamycin treated cells (positive control) and used for a western blot using an antibody against activated caspase-12. Caspase-12 was activated (figure 5.7) in response to SFV infection by 9 hours post-infection but not activated by 13 hours post-infection in response to SFV1-d1eGFP VLP infection.

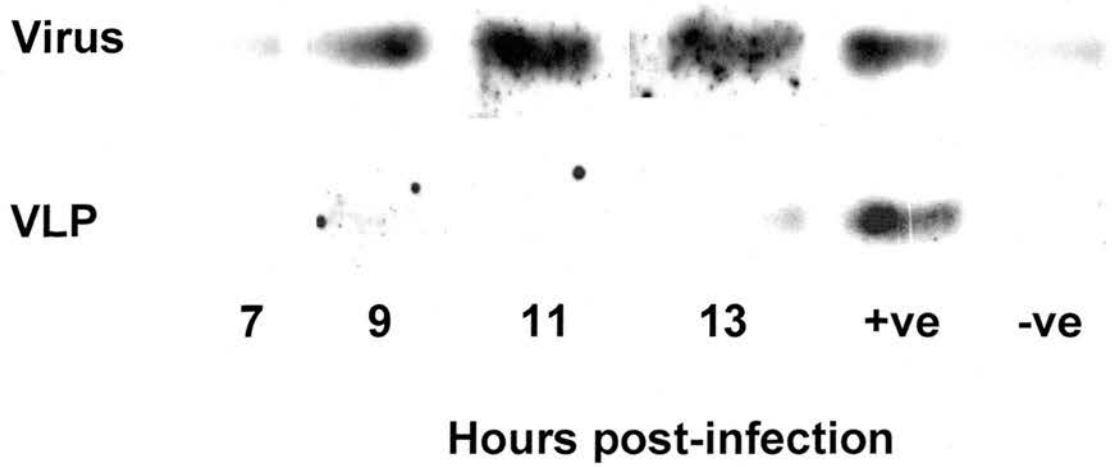

Figure 5.7: Caspase-12 was activated by SFV infection in NIH 3T3 cells.
NIH 3T3 cells were infected with SFV4 at a MOI 50 and lysed at 14 hours post-infection. Protein was isolated and used for a western blot. Equal amounts of protein from an uninfected negative control and a tunicamycin (5 μg /ml) treated positive control were also probed for caspase-12.

To prove that equal amounts of total protein were being loaded in each well for each of the western blots shown above, a western blot was carried out using an antibody against β-actin. Each of the lanes was loaded with the same amount of protein that was loaded for the CHOP and caspase-12 western blots. This blot proved that the BIO-RAD protein assay measuring protein concentrations was accurate as all the bands appeared to have equal intensity (figure 5.8).

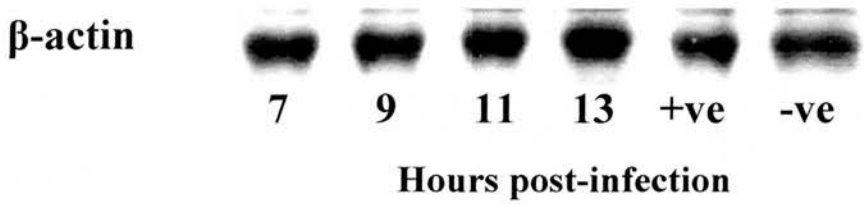

Figure 5.8: To prove that an equal amount of protein was loaded into each well for all of the western blots carried out, a western blot using an antibody against β-actin was carried out.

Discussion

In this chapter, aspects of the virus life cycle that alert the cell to infection and induce the cell to undergo apoptosis were investigated. Replication of virus RNA begins soon after the virus has entered the cell and dsRNA was detectable at 3-4 hours post-infection at the earlies (data not shown). The majority of the dsRNA co-localised with virus replication complexes in a punctate pattern as marked by nsP3-eGFP fusion protein and dsRNA immunostaining, suggesting that it was located within cytoplasmic spherules. One of the purposes of these spherules may be to protect virus dsRNA from cellular detection. It is likely that dsRNA is present earlier than 3 hours post-infection but the immunostaining technique used may not be sensitive enough to detect it. The location of dsRNA earlier than 3 hours post-infection is therefore unclear. Where, when and if virus dsRNA is detected by cellular helicases remains unclear. Detection could occur before or after spherule formation; attempts to determine whether MDA5 co-localised to replication complexes were not successful. It is unclear why this immunostaining was unsuccessful. A number of different concentrations of antibody were tried but other variations in the protocol could be varied in the future to achieve a result.

Many negative-stranded RNA viruses have adopted a different strategy to prevent dsRNA detection. DsRNA intermediates are almost undetectable in cells infected with negative strand RNA viruses (Weber *et al.*, 2006). Many nucleoproteins bind to negative strand RNA and allow only very small sections of dsRNA to form, probably maintaining the length below the size threshold that triggers an immune response. Other viruses express proteins that bind the dsRNA. The influenza A protein NS1, for example, has multiple functions, one of which is to bind dsRNA and prevent or reduce binding of cellular proteins (Qian *et al.*, 1995).

Conflicting evidence suggests that NS1 can be pro- or anti-apoptotic. It is suggested to be pro-apoptotic because individual expression of NS1 in Madin-Darby canine kidney cells or HeLa cells caused the cells to die (Schultz-Cherry *et al.*, 2001). On the other hand a number of papers have suggested that NS1 has anti-apoptotic properties by activating the PI3K pathway (Ehrhardt *et al.*, 2007; Zhirnov & Klenk, 2007). The PI3K pathway is regarded as anti-apoptotic as it can inhibit TRAIL activation and signalling (Larribere *et al.*, 2004). In BVDV infected MEFs detection of dsRNA is a crucial part of the cell detecting virus infection and triggering cell death; BVDV infection of PKR, 2', 5'-OAS double KO cells show very little apoptosis whereas wt MEFs rapidly undergo apoptosis (Yamane *et al.*, 2006). It seemed logical, therefore, to study this by hindering the cells ability to detect SFV dsRNA and then analyse the impact of this on cell death.

Cell lines expressing NS1 from influenza, Y89F (a mutant form of NS1 that still binds dsRNA as normal but does not activate the PI3K pathway) and E3L from vaccinia virus that also binds dsRNA were infected with SFV and loss of viability curves were constructed and compared to cells that were infected but did not express any foreign protein (naïve cells). Cells expressing NS1, Y89F or E3L survived longer than naive cells. This suggests that inhibiting detection of dsRNA slows down activation of cell death. This in turn implies that dsRNA does trigger an anti-viral response which includes apoptosis. It is interesting however that the cells did die. Likely explanations for this include, (i) that the foreign proteins do not completely inhibit dsRNA detection and dsRNA is still detected, perhaps at a lower level so it takes longer to reach the threshold to initiate cell death; (ii) that dsRNA is no longer detected but there are other signals, such as the synthesis of viral proteins, that activate cell death.

Proteins that interfere with the cellular interferon signalling pathway such as the C proteins from SeV that inhibits IRF-3 and interacts with STAT1 (Didcock *et al.*, 1999; Takeuchi *et al.*, 2001) or the BVDV protease NPro, which degrades IRF-3 (Hilton *et al.*, 2006) were expressed in Hep-2 cells and infected with SFV4. No difference was measured between the rate of cell death of these cells and naive cells infected with SFV. The high MOI used in these experiments probably excluded any effect the inhibition of interferon signalling may have had. The paramyxovirus V

proteins have been directly implicated in apoptosis suppression. Cells infected with PIV5, for example, do not undergo apoptosis but when the conserved C-terminal domain of the V protein is deleted infection is cytopathic. This can be prevented by *trans* expression of the V protein (Hilton *et al.*, 2006). Both the SeV and PIV5 V proteins can degrade STAT 1 and also inhibit MDA5 (Andrejeva *et al.*, 2004). Expression of either of these proteins showed an effect; PIV5 V protein expression lead to significantly more cellular viability early in infection compared to naïve cells, whereas SeV-V lead to significant differences late in infection. This suggests that MDA5 signalling may contribute to cell death. It is interesting to note that the protein directly downstream of MDA5, MAVS, is located on the surface of the mitochondria. Mitochondria are central to the intrinsic pathway of cell death and the potential link between MAVs and apoptosis is appealing. MAVS has been suggested to be important in reovirus induced apoptosis. If MAVS is reduced by siRNA there is a significant decrease in caspase-3 activation after reovirus infection (Holm *et al.*, 2007). With this in mind it was important to investigate MAVS activation during SFV infection. The hepatitis C virus protein NS3/4a is known to cleave MAVS from the surface of mitochondria and inhibit its activity (Li *et al.*, 2005c). Similar to the V protein from PIV5, when NS3/4a was expressed in cells and the cells were then infected with SFV the early rate of cell death was significantly different to naive cells infected with SFV although the difference disappeared later in infection. The early difference may exist because RIG-I and MDA5 detection of dsRNA is an early point in infection and therefore activation of MAVS would occur quite early as well. If this host-cell virus interaction was inhibited, then one signal for cell death would be lost. However, this would not stop cells from dying as other signalling pathways are switched on in parallel and subsequently.

The second part of this chapter examined the effect SFV structural proteins have on the infected cells. Flaviviruses are known to utilise the ER as the main site for replication and protein synthesis. Hepatitis C virus proteins activate ER stress when proteins are expressed from a plasmid in osteosarcoma cells. CHOP was upregulated and as a result the anti-apoptotic protein BCL-2 was down-regulated (Ciccaglione *et al.*, 2005). More recently, another flavivirus, West Nile Virus (WNV) has been shown to activate the UPR. WNV infection of human neuroblastoma cells

and primary rat hippocampal neurons led to XBP1 splicing, phosphorylation of eIF-2α and upregulation of CHOP. Interestingly, WNV titres were higher in CHOP KO cells suggesting that CHOP is somehow regulating the replication of WNV, possibly by initiating apoptosis, as is suggested for SFV (Medigeshi *et al.*, 2007).

SFV infection results in splicing of XBP1, upregulation of CHOP and activation of caspase-12. Importantly, SFV-d1eGFP VLPs do not trigger ER stress. The absence of virus structural proteins that would normally traffic through the ER dramatically reduced the stress on the cells ER. This lack of stress may be a reason why VLP infected cells die more slowly than SFV4 infected cells.

When cells were infected with SFV1-d1eGFP VLPs or SFV4 CHOP was expressed between 7 and 9 hours post infection. VLPs do not express structural proteins but still cause CHOP expression. PKR, which is activated by dsRNA, can phosphorylate eIF-2α independent of the UPR. eIF-2α phosphorylation leads to translational downregulation, ATF4 translation and CHOP upregulation (Harding *et al.*, 2000).

Aside from CHOP, caspase-12 was also activated in response to SFV4 infection but not in response to SFV-d1eGFP VLP infection (at least up to 13 hours post infection). This indicates that caspase-12 plays a role in SFV induced cell death. Caspase-12 is located on the cytoplasmic surface of the ER and it is suggested to be activated as a result of calcium ions released from the ER. This calcium release activates calpains which may cleave caspase-12 (Nakagawa & Yuan, 2000) or caspase-7, which is also suggested to interact and cleave caspase-12 (Rao *et al.*, 2001). The Tula virus, a Hantavirus of the *Bunyaviridae*, has been shown to trigger apoptosis through the activation of the ER stress pathway; CHOP upregulation and caspase-12 activation were reported (Li *et al.*, 2005b). Similarly, the rhabdovirus, VSV, activates caspase-12 during its infection of mammalian cells. Both of these viruses use the ER as part of their life cycle as does SFV (Gaddy & Lyles, 2005).

While it is clear that other factors play a role alongside the UPR, the upregulation of CHOP and the activation of caspase-12 probably contribute towards cell death. CHOP is known to cause a downregulation of Bcl-2 (McCullough *et al.*, 2001) and an upregulation of BIM, a proapoptotic protein (McCullough *et al.*, 2001). Caspase-12 interacts directly with the intrinsic pathway of cell death by binding to

caspase-9 and activating it (Rao *et al.*, 2002). Infection with SFV1-d1eGFP VLPs resulted in delayed cell death compared to SFV infection. Importantly the ER does not come under stress and caspase-12 is not activated as a result of VLP infection. This suggests that ER stress and caspase-12 activation are playing a role in the death of the cell. To conclusively prove that caspase-12 is playing a role in cell death, it would be necessary to infect caspase-12 KO cells with SFV to see if there was a delay in cell death. This would show that the delay was as a result of caspase-12 inactivation. Both caspase-12 and CHOP may act as secondary death signals that enhance the initial danger signal given to the cell by dsRNA. Apart from caspase-12 and CHOP, cell stress may also be contributing other pro-death signals to the cell, enhancing the rate of cell death. However if the ER is not stressed as is the case when cells are infected with SFV VLPs, these signals may be taken away or reduced, leading to slow down in cell death rate. The cell will still die but it will not die as rapidly because secondary apoptotic mechanisms which probably boost the rate of cell death will not be expressed.

Final discussion

Alphaviruses are extensively studied at a basic biological level and are also being developed for potential vaccine use and even as a tumour therapeutic. They may be used to target and kill tumour cells for example; SV appears to target tumour cells in preference to non-tumour cells allowing infection of tumour cells without causing harm to the host (Tseng *et al.*, 2004). If it is the case that alphavirus vectors preferentially infect tumour cells, tumours could be reduced or cleared by virus infection due to the strong apoptotic response alphaviruses evoke in infected cells. Alternatively, as a vaccine SFV vectors are being designed to express foreign virus or bacteria proteins; this would help to establish immunity against the particular viruses or bacteria. SFV VLPs are ideal for vaccine use as they infect cells, express proteins to a high level, but never spread because they lack genes for virus structural proteins. They elicit a good immune response and so are useful as adjuvants, but because they do not make structural proteins or produce new virus particles, it is unlikely that the immune system will establish immunity against SFV, thus allowing repeated administrations (Berglund *et al.*, 1999; Chen *et al.*, 2002b; Forsell *et al.*, 2007).

SFV infection of humans is generally asymptomatic, but other alphaviruses can cause disease. Alphavirus infections can cause fever, arthralgia and even encephalitis. Chikungunya virus for example, leads to temporarily debilitating arthralgia and brief febrile illness. Chikungunya is an important emerging virus as it has recently caused widespread illness in areas surrounding the Indian ocean and most recently in Europe (Ligon, 2006). Using SFV as a model, the biology of alphaviruses as a whole can be understood in more detail. This will allow the development of therapies against alphavirus infection or the harnessing of alphaviruses for other beneficial treatments such as vaccines.

SFV infection of mammalian cells generally results in apoptotic cell death. The aims of this project were to uncover the apoptotic pathways triggered as a result of SFV infection and also to identify what stages of the virus life cycle are contributing to the death of the infected cell. A complex picture has emerged, with multiple stages of the virus life cycle playing a role, triggering different apoptotic pathways. Both the extrinsic and intrinsic pathways of apoptosis are activated during

the infection. Mitochondrial membrane potential loss suggests that the intrinsic pathway may be activated first, followed by rapid activation of the extrinsic pathway although this could not be conclusively proven. Caspase-3 if activated by caspase-9 can activate caspase-8 which in turn will amplify the amount of activated caspase-3 and -9, increasing the death signals rapidly. In response to a virus infection it is usually beneficial to the organism if infected cells undergo rapid cell death (except in areas such as the brain where the death of cells could not easily be replaced), reducing the time virus has to replicate and therefore reducing the amount of new virus particles produced from the infected cells. Therefore it is beneficial to the cell to activate every possible apoptotic pathway once SFV has been detected. Both caspase dependent and independent mechanisms are probably involved, to ensure rapid death of the cell. Many viruses, particularly DNA viruses, have evolved ways of blocking the apoptotic responses of infected cells, but SFV seems to have evolved a different strategy. SFV appears to rely on its rapid replication to help it escape. The alphavirus genome is approximately 11 kb in length and the replication cycle of the virus takes approximately 5-6 hours from time of infection to production of virus. On the other hand, evidence of cell death does not noticeably begin until approximately 14 hours post-infection. It is interesting to compare the strategies adopted by other positive strand RNA viruses of similar size to SFV. Picornaviruses and flaviviruses are both examples of viruses that rapidly replicate to avoid being trapped by the apoptotic cell. On top of this however, picornaviruses and flaviviruses, in contrast to SFV, also have anti-apoptotic properties to help extend cell life. Both Dengue virus and JEV activate PI3K which is anti-apoptotic and can inhibit caspase activation (Lee *et al.*, 2005). Hepatitis C can inhibit apoptosis and IFN production by repressing PKR. The hepatitis C protein NS5A binds directly to PKR, inhibiting its activity, while the E2 protein can inhibit TRAIL (Gale, Jr. *et al.*, 1999). This allows flaviviruses extra time to replicate and produce new virus particles; the cells do die by apoptosis eventually. Similarly, coxsackievirus slows down cell death by inhibiting caspase-3 (Salako *et al.*, 2006). Picornaviruses generally trigger apoptosis but manage to replicate fast enough to overcome this (Buenz & Howe, 2006). One exception to this is hepatitis A which can establish a persistent infection *in vitro*. Hepatitis A replicates slowly and inhibits IFN-β-induced anti-viral pro-apoptotic

mechanisms thus inhibiting apoptosis in infected cells *in vitro* (Brack *et al.*, 2002; Goswami *et al.*, 2004). Alphaviruses do not have any known anti-apoptotic properties.

In order for the cell to undergo apoptosis when infected, it must first detect the infection. Entry, genome replication, virus protein expression, assembly and release are all essential stages in the virus life cycle and allow virus detection by the cell. In this study the affects both virus genome replication and virus protein expression have on cell induction of apoptosis were examined. Many negative strand RNA viruses have a nucleoprotein that binds to the virus genome. During replication these proteins only expose small amounts of the virus genome to polymerases, minimising dsRNA exposure (See Fields Virology). Some negative strand viruses also produce proteins that bind to dsRNA (for example the NS1 protein of influenza A); binding to dsRNA may inhibit cellular proteins detecting the dsRNA and triggering an anti-viral response. Positive strand RNA viruses have not adopted this strategy and instead replicate in association with cellular membranes. SFV induces the formation of invaginations in membranous structures of endosomic and lysomic origin and carries out replication in these invaginations (Kujala *et al.*, 2001). It has been suggested that replicating in these invaginations may protect virus dsRNA from detection. When dsRNA binding proteins such as NS1 were expressed in SFV infected cells, the cells survived longer than in the naïve cells; the dsRNA binding proteins provided further protection to the dsRNA, suggesting that replication in these invaginations did not completely guard the dsRNA from detection. Because the cells survived longer it was also clear that dsRNA contributes to the death of the cell. PKR has been associated with apoptosis induction, but RIG-I and MDA5 have not. Inhibition of MDA5 or MAVS lengthened survival also suggesting that these proteins may also have a link to apoptosis induction. The location of MAVS, on the surface of mitochondria would suggest that it is ideally placed to act as a link between virus detection and cell death. Interestingly the C-terminus that anchors MAVS to mitochondria is highly homologous the Bcl-2 family of proteins. It is suggested that MAVS may affect mitochondrial membrane integrity, leading to cytochrome c release but any link to apoptosis has not yet been proven.

Overexpression of MAVS leads to increased IFN-β and NFκB activation but does not increase apoptosis (Meylan *et al.*, 2005).

Interestingly, MEFs with PKR helicase activity inhibited died faster than wt MEFs when infected with SFV. The virus replicated faster and the cells seemed to respond quicker to the infection (NFκB activity was earlier). These results indicate that PKR, as well as detecting virus infection, reduces virus replication. The exact mechanism(s) underlying this inhibition of replication remains unclear however. The physical interaction of PKR with dsRNA may reduce replication rates by inhibiting dsRNA separation. It would be interesting to express the dsRNA binding region of the PKR protein in the PKR KO MEFs to see if a wt phenotype could be recovered. If the dsRNA binding activity was important then reintroducing this interaction might reduce virus replication to levels close to those seen in wt cells. Alternatively, the kinase activity of PKR may be affecting early virus replication. PKR phosphorylation of hepatitis D virus nuclear phosphoproteins inhibits the virus because removing this kinase activity increases virus replication (Chen *et al.*, 2002a). The SFV nsP3 is a known phosphoprotein (Peranen *et al.*, 1988) and it's possible that PKR phosphorylates nsP3 causing nsP3 to function inefficiently. If PKR was not present then nsP3 activity might improve; although the exact role of nsP3 is unknown it is clearly involved in RNA replication, so alterations of phosphorylation patterns might increase replication rates. PKR may also phosphorylate other cellular proteins which in turn could downregulate virus replication. While it is clear PKR inhibits virus replication, further investigations are required to fully elucidate how PKR carries out this function.

Another major stage in the SFV life cycle is structural protein production. The virus glycoproteins E1, E2 and E3 are processed in the ER, and this study has shown that the presence of structural proteins induces ER stress, upregulates CHOP expression and activates caspase-12 all by 9 hours post-infection. In the absence of structural proteins cell stress is not invoked and caspase-12 is not activated (by 13 hours post-infection) but CHOP is still upregulated. CHOP expression is probably as a result of PKR activation triggering eIF2-α phosphorylation, which can cause an upregulation of ATF4 and CHOP. Lack of caspase-12 activation and ER stress inactivation, may be reasons for the delay in cell death following SFV1-d1eGFP

VLP infection, indicating that ER stress activation and particularly caspase-12 is somehow contributing to cell death. The timing of CHOP upregulation is between 7 and 9 hours in the presence or absence of SFV structural proteins and the concentration of CHOP appears roughly similar in both cases (bands looked similar on gel). This suggests that while CHOP may play a role in the death of SFV infected cells it is not directly responsible for the delay seen in SFV VLP induced cell death. WNV activates ER stress during infection and upregulates CHOP; infection of CHOP KO cells led to higher titres of virus but it is unclear whether this was due to decreased apoptosis or upregulated rates of virus replication (Medigeshi *et al.*, 2007). JEV also triggers ER stress and upregulates CHOP expression. CHOP can downregulate Bcl-2 (McCullough *et al.*, 2001) but is not the only pro-apoptotic mechanism associated with ER stress. Calcium release from the ER can cause cytochrome c release from mitochondria and subsequent caspase activation (Deniaud *et al.*, 2008). Hepatitis C virus infection causes ER stress, calcium depletion from the ER, cytochrome c release from mitochondria and apoptosis. It has been reported previously that in SFV infected chicken embryo cells the mitochondria take up increased amounts of calcium (Peterhans *et al.*, 1979). It is not clear however whether the ER was releasing this calcium or whether this calcium was triggering mitochondrial membrane depolarisation. ER stress can also lead to activation of the JNK (c-Jun amino-terminal kinases) pathway through the interaction of IRE1, ASK1 and JNK; the JNK pathway is suggested to be pro-apoptotic (Nishitoh *et al.*, 2002). Expression of SFV structural proteins to a high level independent of the virus nsPs or RNA replication would highlight further the apoptotic affect these proteins have. A DNA plasmid containing the structural protein genes under the control of ubiquitous expression promoter could be transfected into cells, leading to high level structural protein expression. This build up of structural proteins would be predicted to trigger ER stress and eventually apoptosis. Unfortunately, time constraints did not allow such an experiment to be carried out.

It appears that SFV has evolved a straight forward approach to its life cycle. It replicates rapidly, utilises cellular machinery and releases new virus particles with an apparent disregard for cellular apoptotic responses. Evidence has emerged suggesting that SFV nsP2 downregulates IFN production by an unknown mechanism

(Breakwell *et al.*, 2007). NsP2 is also involved in transcription down-regulation in infected cells; the mechanism for this has not yet been established (Garmashova *et al.*, 2007b). The VEEV capsid protein associates with nuclear pore complexes, interfering with transport to and from the nucleus. This allows inhibition of transcription and is suggested to be a feature of new world alphaviruses only and not SFV and SV for example (Garmashova *et al.*, 2007a; Garmashova *et al.*, 2007b). To date, no evidence for alphavirus anti-apoptotic mechanisms exist. SFV triggers multiple apoptotic pathways at different stages of its life cycle, and this apoptosis is probably both caspase dependent and independent. Mutations in SFV have reduced its ability to induce apoptosis but these mutations have also dramatically the replicative ability of the virus, often rendering it unusable. The reduction in cytopathogenicity of these mutated viruses is probably as a result of reduced RNA and virus protein build up in the infected cells. There may be a threshold of RNA or protein concentration below which cellular apoptotic mechanisms are not activated.

Overall the work undertaken in this study has shown that SFV infection is a potent and multi-pronged activator of cell death in mammalian cells. The cell detects infection and induces signalling pathways that lead to cell death as well as other responses such as IFN production. The virus in turn has evolved to avoid the affect of cell death by replicating and producing large amounts of virus particles well before the cell dies.

Reference List

Adrain, C., Creagh, E. M. & Martin, S. J. (2001). Apoptosis-associated release of Smac/DIABLO from mitochondria requires active caspases and is blocked by Bcl-2. *EMBO J* **20**, 6627-6636.

Akashi, S., Saitoh, S., Wakabayashi, Y., Kikuchi, T., Takamura, N., Nagai, Y., Kusumoto, Y., Fukase, K., Kusumoto, S., Adachi, Y., Kosugi, A. & Miyake, K. (2003). Lipopolysaccharide interaction with cell surface Toll-like receptor 4-MD-2: higher affinity than that with MD-2 or CD14. *J Exp Med* **198**, 1035-1042.

Alexopoulou, L., Holt, A. C., Medzhitov, R. & Flavell, R. A. (2001). Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* **413**, 732-738.

Aliperti, G. & Schlesinger, M. J. (1978). Evidence for an autoprotease activity of sindbis virus capsid protein. *Virology* **90**, 366-369.

Aliprantis, A. O., Yang, R. B., Weiss, D. S., Godowski, P. & Zychlinsky, A. (2000). The apoptotic signaling pathway activated by Toll-like receptor-2. *EMBO J* **19**, 3325-3336.

Alirezaei, M., Watry, D. D., Flynn, C. F., Kiosses, W. B., Masliah, E., Williams, B. R., Kaul, M., Lipton, S. A. & Fox, H. S. (2007). Human immunodeficiency virus-1/surface glycoprotein 120 induces apoptosis through RNA-activated protein kinase signaling in neurons. *J Neurosci* **27**, 11047-11055.

Allsopp, T. E. & Fazakerley, J. K. (2000). Altruistic cell suicide and the specialized case of the virus-infected nervous system. *Trends Neurosci* **23**, 284-290.

Allsopp, T. E., Scallan, M. F., Williams, A. & Fazakerley, J. K. (1998). Virus infection induces neuronal apoptosis: A comparison with trophic factor withdrawal. *Cell Death Differ* **5**, 50-59.

Amor, S., Scallan, M. F., Morris, M. M., Dyson, H. & Fazakerley, J. K. (1996). Role of immune responses in protection and pathogenesis during Semliki Forest virus encephalitis. *J Gen Virol* **77 (Pt 2)**, 281-291.

Andrejeva, J., Childs, K. S., Young, D. F., Carlos, T. S., Stock, N., Goodbourn, S. & Randall, R. E. (2004). The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN-beta promoter. *Proc Natl Acad Sci U S A* **101**, 17264-17269.

Andreyev, A. & Fiskum, G. (1999). Calcium induced release of mitochondrial cytochrome c by different mechanisms selective for brain versus liver. *Cell Death Differ* **6**, 825-832.

Arur, S., Uche, U. E., Rezaul, K., Fong, M., Scranton, V., Cowan, A. E., Mohler, W. & Han, D. K. (2003). Annexin I is an endogenous ligand that mediates apoptotic cell engulfment. *Dev Cell* **4**, 587-598.

Ashkenazi, A. & Dixit, V. M. (1998). Death receptors: signaling and modulation. *Science* **281**, 1305-1308.

Atkins, G. J., Sheahan, B. J. & Liljestrom, P. (1996). Manipulation of the Semliki Forest virus genome and its potential for vaccine construction. *Mol Biotechnol* **5**, 33-38.

Atkins, G. J., Sheahan, B. J. & Liljestrom, P. (1999). The molecular pathogenesis of Semliki Forest virus: a model virus made useful? *J Gen Virol* **80 (Pt 9)**, 2287-2297.

Back, S. H., Schroder, M., Lee, K., Zhang, K. & Kaufman, R. J. (2005).ER stress signaling by regulated splicing: IRE1/HAC1/XBP1. *Methods* **35**, 395-416.

Balachandran, S., Roberts, P. C., Brown, L. E., Truong, H., Pattnaik, A. K., Archer, D. R. & Barber, G. N. (2000). Essential role for the dsRNA-dependent protein kinase PKR in innate immunity to viral infection. *Immunity* **13**, 129-141.

Banadyga, L., Gerig, J., Stewart, T. & Barry, M. (2007). Fowlpox virus encodes a Bcl-2 homologue that protects cells from apoptotic death through interaction with the proapoptotic protein Bak. *J Virol* **81**, 11032-11045.

Bauer, S., Kirschning, C. J., Hacker, H., Redecke, V., Hausmann, S., Akira, S., Wagner, H. & Lipford, G. B. (2001). Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proc Natl Acad Sci U S A* **98**, 9237-9242.

Benedict, C. A. & Ware, C. F. (2001). Virus targeting of the tumor necrosis factor superfamily. *Virology* **289**, 1-5.

Bergeron, J., Mabrouk, T., Garzon, S. & Lemay, G. (1998). Characterization of the thermosensitive ts453 reovirus mutant: increased dsRNA binding of sigma 3 protein correlates with interferon resistance. *Virology* **246**, 199-210.

Berglund, P., Fleeton, M. N., Smerdou, C. & Liljestrom, P. (1999). Immunization with recombinant Semliki Forest virus induces protection against influenza challenge in mice. *Vaccine* **17**, 497-507.

Bertin, J., Mendrysa, S. M., LaCount, D. J., Gaur, S., Krebs, J. F., Armstrong, R. C., Tomaselli, K. J. & Friesen, P. D. (1996). Apoptotic suppression by baculovirus P35 involves cleavage by and inhibition of a virus-induced CED-3/ICE-like protease. *J Virol* **70**, 6251-6259.

Bertolotti, A., Zhang, Y., Hendershot, L. M., Harding, H. P. & Ron, D. (2000). Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nat Cell Biol* **2**, 326-332.

Bilgin, D. D., Liu, Y., Schiff, M. & Dinesh-Kumar, S. P. (2003).P58(IPK), a plant ortholog of double-stranded RNA-dependent protein kinase PKR inhibitor, functions in viral pathogenesis. *Dev Cell* **4**, 651-661.

Blink, E., Maianski, N. A., Alnemri, E. S., Zervos, A. S., Roos, D. & Kuijpers, T. W. (2004). Intramitochondrial serine protease activity of Omi/HtrA2 is required for caspase-independent cell death of human neutrophils. *Cell Death Differ* **11**, 937-939.

Blond-Elguindi, S., Cwirla, S. E., Dower, W. J., Lipshutz, R. J., Sprang, S. R., Sambrook, J. F. & Gething, M. J. (1993). Affinity panning of a library of peptides displayed on bacteriophages reveals the binding specificity of BiP. *Cell* **75**, 717-728.

Boehning, D., Patterson, R. L., Sedaghat, L., Glebova, N. O., Kurosaki, T. & Snyder, S. H. (2003). Cytochrome c binds to inositol (1,4,5) trisphosphate receptors, amplifying calcium-dependent apoptosis. *Nat Cell Biol* **5**, 1051-1061.

Bole, D. G., Dowin, R., Doriaux, M. & Jamieson, J. D. (1989). Immunocytochemical localization of BiP to the rough endoplasmic reticulum: evidence for protein sorting by selective retention. *J Histochem Cytochem* **37**, 1817-1823.

Bonatti, S., Migliaccio, G., Blobel, G. & Walter, P. (1984). Role of signal recognition particle in the membrane assembly of Sindbis viral glycoproteins. *Eur J Biochem* **140**, 499-502.

Borgherini, G., Poubeau, P., Staikowsky, F., Lory, M., Le, M. N., Becquart, J. P., Wengling, C., Michault, A. & Paganin, F. (2007). Outbreak of chikungunya on Reunion Island: early clinical and laboratory features in 157 adult patients. *Clin Infect Dis* **44**, 1401-1407.

Bork, P. & Sander, C. (1993).A hybrid protein kinase-RNase in an interferon-induced pathway? *FEBS Lett* **334**, 149-152.

Bossy-Wetzel, E., Newmeyer, D. D. & Green, D. R. (1998). Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. *EMBO J* **17**, 37-49.

Brack, K., Berk, I., Magulski, T., Lederer, J., Dotzauer, A. & Vallbracht, A. (2002). Hepatitis A virus inhibits cellular antiviral defense mechanisms induced by double-stranded RNA. *J Virol* **76**, 11920-11930.

Breakwell, L., Dosenovic, P., Karlsson Hedestam, G. B., D'Amato, M., Liljestrom, P., Fazakerley, J. & McInerney, G. M. (2007). Semliki Forest virus nonstructural protein 2 is involved in suppression of the type I interferon response. *J Virol* **81**, 8677-8684.

Bridgen, A., Weber, F., Fazakerley, J. K. & Elliott, R. M. (2001). Bunyamwera bunyavirus nonstructural protein NSs is a nonessential gene product that contributes to viral pathogenesis. *Proc Natl Acad Sci U S A* **98**, 664-669.

Buenz, E. J. & Howe, C. L. (2006). Picornaviruses and cell death. *Trends Microbiol* **14**, 28-36.

Calfon, M., Zeng, H., Urano, F., Till, J. H., Hubbard, S. R., Harding, H. P., Clark, S. G. & Ron, D. (2002).IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature* **415**, 92-96.

Cande, C., Vahsen, N., Kouranti, I., Schmitt, E., Daugas, E., Spahr, C., Luban, J., Kroemer, R. T., Giordanetto, F., Garrido, C., Penninger, J. M. & Kroemer, G. (2004).AIF and cyclophilin A cooperate in apoptosis-associated chromatinolysis. *Oncogene* **23**, 1514-1521.

Cardenas, W. B., Loo, Y. M., Gale, M., Jr., Hartman, A. L., Kimberlin, C. R., Martinez-Sobrido, L., Saphire, E. O. & Basler, C. F. (2006). Ebola virus VP35 protein binds double-stranded RNA and inhibits alpha/beta interferon production induced by RIG-I signaling. *J Virol* **80**, 5168-5178.

Carpenter, D., Hsiang, C., Brown, D. J., Jin, L., Osorio, N., BenMohamed, L., Jones, C. & Wechsler, S. L. (2007). Stable cell lines expressing high levels of the herpes simplex virus type 1 LAT are refractory to caspase 3 activation and DNA laddering following cold shock induced apoptosis. *Virology* **369**, 12-18.

Cartron, P. F., Gallenne, T., Bougras, G., Gautier, F., Manero, F., Vusio, P., Meflah, K., Vallette, F. M. & Juin, P. (2004). The first alpha helix of Bax plays a necessary role in its ligand-induced activation by the BH3-only proteins Bid and PUMA. *Mol Cell* **16**, 807-818.

Castelli, J. C., Hassel, B. A., Wood, K. A., Li, X. L., Amemiya, K., Dalakas, M. C., Torrence, P. F. & Youle, R. J. (1997).A study of the interferon antiviral mechanism: apoptosis activation by the 2-5A system. *J Exp Med* **186**, 967-972.

Chai, J., Shiozaki, E., Srinivasula, S. M., Wu, Q., Datta, P., Alnemri, E. S. & Shi, Y. (2001). Structural basis of caspase-7 inhibition by XIAP. *Cell* **104**, 769-780.

Chang, H. W. & Jacobs, B. L. (1993). Identification of a conserved motif that is necessary for binding of the vaccinia virus E3L gene products to double-stranded RNA. *Virology* **194**, 537-547.

Charriaut-Marlangue, C. & Ben Ari, Y. (1995).A cautionary note on the use of the TUNEL stain to determine apoptosis. *Neuroreport* **7**, 61-64.

Chen, C. W., Tsay, Y. G., Wu, H. L., Lee, C. H., Chen, D. S. & Chen, P. J. (2002a). The double-stranded RNA-activated kinase, PKR, can phosphorylate hepatitis D virus small delta antigen at functional serine and threonine residues. *J Biol Chem* **277**, 33058-33067.

Chen, M., Hu, K. F., Rozell, B., Orvell, C., Morein, B. & Liljestrom, P. (2002b). Vaccination with recombinant alphavirus or immune-stimulating complex antigen against respiratory syncytial virus. *J Immunol* **169**, 3208-3216.

Chen, P., Nordstrom, W., Gish, B. & Abrams, J. M. (1996).grim, a novel cell death gene in Drosophila. *Genes Dev* **10**, 1773-1782.

Chen, X., Shen, J. & Prywes, R. (2002c). The luminal domain of ATF6 senses endoplasmic reticulum (ER) stress and causes translocation of ATF6 from the ER to the Golgi. *J Biol Chem* **277**, 13045-13052.

Chen, Z., Benureau, Y., Rijnbrand, R., Yi, J., Wang, T., Warter, L., Lanford, R. E., Weinman, S. A., Lemon, S. M., Martin, A. & Li, K. (2007).GB virus B disrupts RIG-I signaling by NS3/4A-mediated cleavage of the adaptor protein MAVS. *J Virol* **81**, 964-976.

Cheng, E. H., Wei, M. C., Weiler, S., Flavell, R. A., Mak, T. W., Lindsten, T. & Korsmeyer, S. J. (2001).BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Mol Cell* **8**, 705-711.

Cheung, E. C., Joza, N., Steenaart, N. A., McClellan, K. A., Neuspiel, M., McNamara, S., MacLaurin, J. G., Rippstein, P., Park, D. S., Shore, G. C., McBride, H. M., Penninger, J. M. & Slack, R. S. (2006). Dissociating the dual roles of apoptosis-inducing factor in maintaining mitochondrial structure and apoptosis. *EMBO J* **25**, 4061-4073.

Childs, K., Stock, N., Ross, C., Andrejeva, J., Hilton, L., Skinner, M., Randall, R. & Goodbourn, S. (2007).mda-5, but not RIG-I, is a common target for paramyxovirus V proteins. *Virology* **359**, 190-200.

Chinnaiyan, A. M., O'Rourke, K., Lane, B. R. & Dixit, V. M. (1997). Interaction of CED-4 with CED-3 and CED-9: a molecular framework for cell death. *Science* **275**, 1122-1126.

Chirillo, P., Pagano, S., Natoli, G., Puri, P. L., Burgio, V. L., Balsano, C. & Levrero, M. (1997). The hepatitis B virus X gene induces p53-mediated programmed cell death. *Proc Natl Acad Sci U S A* **94**, 8162-8167.

Chung, Y. L., Sheu, M. L. & Yen, S. H. (2003). Hepatitis C virus NS5A as a potential viral Bcl-2 homologue interacts with Bax and inhibits apoptosis in hepatocellular carcinoma. *Int J Cancer* **107**, 65-73.

Ciccaglione, A. R., Costantino, A., Tritarelli, E., Marcantonio, C., Equestre, M., Marziliano, N. & Rapicetta, M. (2005). Activation of endoplasmic reticulum stress response by hepatitis C virus proteins. *Arch Virol* **150**, 1339-1356.

Colby, C. & Morgan, M. J. (1971). Interferon induction and action. *Annu Rev Microbiol* **25**, 333-360.

Coleman, M. L., Sahai, E. A., Yeo, M., Bosch, M., Dewar, A. & Olson, M. F. (2001). Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. *Nat Cell Biol* **3**, 339-345.

Colon-Ramos, D. A., Irusta, P. M., Gan, E. C., Olson, M. R., Song, J., Morimoto, R. I., Elliott, R. M., Lombard, M., Hollingsworth, R., Hardwick, J. M., Smith, G. K. & Kornbluth, S. (2003). Inhibition of translation and induction of apoptosis by Bunyaviral nonstructural proteins bearing sequence similarity to reaper. *Mol Biol Cell* **14**, 4162-4172.

Creagh, E. M., Conroy, H. & Martin, S. J. (2003). Caspase-activation pathways in apoptosis and immunity. *Immunol Rev* **193**, 10-21.

Cryns, V. L., Bergeron, L., Zhu, H., Li, H. & Yuan, J. (1996). Specific cleavage of alpha-fodrin during Fas- and tumor necrosis factor-induced apoptosis is mediated by an interleukin-1beta-converting enzyme/Ced-3 protease distinct from the poly(ADP-ribose) polymerase protease. *J Biol Chem* **271**, 31277-31282.

Cullinan, S. B. & Diehl, J. A. (2004).PERK-dependent activation of Nrf2 contributes to redox homeostasis and cell survival following endoplasmic reticulum stress. *J Biol Chem* **279**, 20108-20117.

Cullinan, S. B., Zhang, D., Hannink, M., Arvisais, E., Kaufman, R. J. & Diehl, J. A. (2003). Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival. *Mol Cell Biol* **23**, 7198-7209.

Daniel, R., Marusich, E., Argyris, E., Zhao, R. Y., Skalka, A. M. & Pomerantz, R. J. (2005). Caffeine inhibits human immunodeficiency virus type 1 transduction of nondividing cells. *J Virol* **79**, 2058-2065.

Daugas, E., Susin, S. A., Zamzami, N., Ferri, K. F., Irinopoulou, T., Larochette, N., Prevost, M. C., Leber, B., Andrews, D., Penninger, J. & Kroemer, G. (2000). Mitochondrio-nuclear translocation of AIF in apoptosis and necrosis. *FASEB J* **14**, 729-739.

de Haro, C., Mendez, R. & Santoyo, J. (1996). The eIF-2alpha kinases and the control of protein synthesis. *FASEB J* **10**, 1378-1387.

de Lima, B. D., May, J. S., Marques, S., Simas, J. P. & Stevenson, P. G. (2005). Murine gammaherpesvirus 68 bcl-2 homologue contributes to latency establishment in vivo. *J Gen Virol* **86**, 31-40.

De, I., Fata-Hartley, C., Sawicki, S. G. & Sawicki, D. L. (2003). Functional analysis of nsP3 phosphoprotein mutants of Sindbis virus. *J Virol* **77**, 13106-13116.

Deniaud, A., Sharaf El, D. O., Maillier, E., Poncet, D., Kroemer, G., Lemaire, C. & Brenner, C. (2007). Endoplasmic reticulum stress induces calcium-dependent permeability transition, mitochondrial outer membrane permeabilization and apoptosis. *Oncogene*.

Deniaud, A., Sharaf El, D. O., Maillier, E., Poncet, D., Kroemer, G., Lemaire, C. & Brenner, C. (2008). Endoplasmic reticulum stress induces calcium-dependent permeability transition, mitochondrial outer membrane permeabilization and apoptosis. *Oncogene* **27**, 285-299.

Desmots, F., Russell, H. R., Michel, D. & McKinnon, P. J. (2008). Scythe Regulates Apoptosis-inducing Factor Stability during Endoplasmic Reticulum Stress-induced Apoptosis. *J Biol Chem* **283**, 3264-3271.

DeTulleo, L. & Kirchhausen, T. (1998). The clathrin endocytic pathway in viral infection. *EMBO J* **17**, 4585-4593.

Deveraux, Q. L., Roy, N., Stennicke, H. R., Van Arsdale, T., Zhou, Q., Srinivasula, S. M., Alnemri, E. S., Salvesen, G. S. & Reed, J. C. (1998).IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. *EMBO J* **17**, 2215-2223.

Didcock, L., Young, D. F., Goodbourn, S. & Randall, R. E. (1999). The V protein of simian virus 5 inhibits interferon signalling by targeting STAT1 for proteasome-mediated degradation. *J Virol* **73**, 9928-9933.

Diebold, S. S., Kaisho, T., Hemmi, H., Akira, S. & Reis e Sousa (2004). Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* **303**, 1529-1531.

Diebold, S. S., Massacrier, C., Akira, S., Paturel, C., Morel, Y. & Reis e Sousa (2006). Nucleic acid agonists for Toll-like receptor 7 are defined by the presence of uridine ribonucleotides. *Eur J Immunol* **36**, 3256-3267.

Domingo-Gil, E. & Esteban, M. (2006). Role of mitochondria in apoptosis induced by the 2-5A system and mechanisms involved. *Apoptosis* **11**, 725-738.

Donehower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery, C. A., Jr., Butel, J. S. & Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* **356**, 215-221.

Dong, B. & Silverman, R. H. (1995).2-5A-dependent RNase molecules dimerize during activation by 2-5A. *J Biol Chem* **270**, 4133-4137.

Donnelly, S. M., Sheahan, B. J. & Atkins, G. J. (1997). Long-term effects of Semliki Forest virus infection in the mouse central nervous system. *Neuropathol Appl Neurobiol* **23**, 235-241.

Dorner, A. J., Wasley, L. C. & Kaufman, R. J. (1992). Overexpression of GRP78 mitigates stress induction of glucose regulated proteins and blocks secretion of selective proteins in Chinese hamster ovary cells. *EMBO J* **11**, 1563-1571.

Doyle, S., Vaidya, S., O'Connell, R., Dadgostar, H., Dempsey, P., Wu, T., Rao, G., Sun, R., Haberland, M., Modlin, R. & Cheng, G. (2002).IRF3 mediates a TLR3/TLR4-specific antiviral gene program. *Immunity* **17**, 251-263.

Edelman, R., Ascher, M. S., Oster, C. N., Ramsburg, H. H., Cole, F. E. & Eddy, G. A. (1979). Evaluation in humans of a new, inactivated vaccine for Venezuelan equine encephalitis virus (C-84). *J Infect Dis* **140**, 708-715.

Edelman, R., Wasserman, S. S., Bodison, S. A., Perry, J. G., O'Donnoghue, M. & DeTolla, L. J. (2003). Phase II safety and immunogenicity study of type F botulinum toxoid in adult volunteers. *Vaccine* **21**, 4335-4347.

Edelmann, K. H., Richardson-Burns, S., Alexopoulou, L., Tyler, K. L., Flavell, R. A. & Oldstone, M. B. (2004). Does Toll-like receptor 3 play a biological role in virus infections? *Virology* **322**, 231-238.

Ehrhardt, C., Wolff, T., Pleschka, S., Planz, O., Beermann, W., Bode, J. G., Schmolke, M. & Ludwig, S. (2007). Influenza A virus NS1 protein activates the PI3K/Akt pathway to mediate antiapoptotic signaling responses. *J Virol* **81**, 3058-3067.

Ekert, P. G., Silke, J., Hawkins, C. J., Verhagen, A. M. & Vaux, D. L. (2001).DIABLO promotes apoptosis by removing MIHA/XIAP from processed caspase 9. *J Cell Biol* **152**, 483-490.

Ellis, H. M. & Horvitz, H. R. (1986). Genetic control of programmed cell death in the nematode C. elegans. *Cell* **44**, 817-829.

Ellis, R. E., Jacobson, D. M. & Horvitz, H. R. (1991). Genes required for the engulfment of cell corpses during programmed cell death in Caenorhabditis elegans. *Genetics* **129**, 79-94.

Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A. & Nagata, S. (1998).A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* **391**, 43-50.

Enari, M., Talanian, R. V., Wong, W. W. & Nagata, S. (1996). Sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis. *Nature* **380**, 723-726.

Eskes, R., Desagher, S., Antonsson, B. & Martinou, J. C. (2000). Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. *Mol Cell Biol* **20**, 929-935.

Fadok, V. A., Savill, J. S., Haslett, C., Bratton, D. L., Doherty, D. E., Campbell, P. A. & Henson, P. M. (1992a). Different populations of macrophages use either the vitronectin receptor or the phosphatidylserine receptor to recognize and remove apoptotic cells. *J Immunol* **149**, 4029-4035.

Fadok, V. A., Voelker, D. R., Campbell, P. A., Cohen, J. J., Bratton, D. L. & Henson, P. M. (1992b). Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J Immunol* **148**, 2207-2216.

Fazakerley, J. K. (2002). Pathogenesis of Semliki Forest virus encephalitis. *J Neurovirol* **8 Suppl 2**, 66-74.

Fazakerley, J. K., Amor, S. & Webb, H. E. (1983). Reconstitution of Semliki forest virus infected mice, induces immune mediated pathological changes in the CNS. *Clin Exp Immunol* **52**, 115-120.

Fazakerley, J. K., Boyd, A., Mikkola, M. L. & Kaariainen, L. (2002).A single amino acid change in the nuclear localization sequence of the nsP2 protein affects the neurovirulence of Semliki Forest virus. *J Virol* **76**, 392-396.

Fazakerley, J. K., Cotterill, C. L., Lee, G. & Graham, A. (2006). Virus tropism, distribution, persistence and pathology in the corpus callosum of the Semliki Forest virus-infected mouse brain: a novel system to study virus-oligodendrocyte interactions. *Neuropathol Appl Neurobiol* **32**, 397-409.

Fazakerley, J. K., Pathak, S., Scallan, M., Amor, S. & Dyson, H. (1993). Replication of the A7(74) strain of Semliki Forest virus is restricted in neurons. *Virology* **195**, 627-637.

Fazakerley, J. K. & Walker, R. (2003). Virus demyelination. *J Neurovirol* **9**, 148-164.

Feng, G. S., Chong, K., Kumar, A. & Williams, B. R. (1992). Identification of double-stranded RNA-binding domains in the interferon-induced double-stranded RNA-activated p68 kinase. *Proc Natl Acad Sci U S A* **89**, 5447-5451.

Feng, P., Liang, C., Shin, Y. C., E X, Zhang, W., Gravel, R., Wu, T. T., Sun, R., Usherwood, E. & Jung, J. U. (2007).A Novel Inhibitory Mechanism of Mitochondrion-Dependent Apoptosis by a Herpesviral Protein. *PLoS Pathog* **3**, e174.

Feuer, R., Mena, I., Pagarigan, R. R., Harkins, S., Hassett, D. E. & Whitton, J. L. (2003). Coxsackievirus B3 and the neonatal CNS: the roles of stem cells,

developing neurons, and apoptosis in infection, viral dissemination, and disease. *Am J Pathol* **163**, 1379-1393.

Fitzgerald, K. A., Rowe, D. C., Barnes, B. J., Caffrey, D. R., Visintin, A., Latz, E., Monks, B., Pitha, P. M. & Golenbock, D. T. (2003).LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. *J Exp Med* **198**, 1043-1055.

Floyd-Smith, G., Slattery, E. & Lengyel, P. (1981). Interferon action: RNA cleavage pattern of a (2'-5')oligoadenylate--dependent endonuclease. *Science* **212**, 1030-1032.

Forsell, M. N., McInerney, G. M., Dosenovic, P., Hidmark, A. S., Eriksson, C., Liljestrom, P., Grundner, C. & Karlsson Hedestam, G. B. (2007). Increased human immunodeficiency virus type 1 Env expression and antibody induction using an enhanced alphavirus vector. *J Gen Virol* **88**, 2774-2779.

Fragkoudis, R., Breakwell, L., McKimmie, C., Boyd, A., Barry, G., Kohl, A., Merits, A. & Fazakerley, J. K. (2007). The type I interferon system protects mice from Semliki Forest virus by preventing widespread virus dissemination in extraneural tissues, but does not mediate the restricted replication of avirulent virus in central nervous system neurons. *J Gen Virol* **88**, 3373-3384.

Frebel, K. & Wiese, S. (2006). Signalling molecules essential for neuronal survival and differentiation. *Biochem Soc Trans* **34**, 1287-1290.

Froshauer, S., Kartenbeck, J. & Helenius, A. (1988). Alphavirus RNA replicase is located on the cytoplasmic surface of endosomes and lysosomes. *J Cell Biol* **107**, 2075-2086.

Fujimoto, I., Takizawa, T., Ohba, Y. & Nakanishi, Y. (1998). Co-expression of Fas and Fas-ligand on the surface of influenza virus-infected cells. *Cell Death Differ* **5**, 426-431.

Fujita, E., Kouroku, Y., Jimbo, A., Isoai, A., Maruyama, K. & Momoi, T. (2002). Caspase-12 processing and fragment translocation into nuclei of tunicamycin-treated cells. *Cell Death Differ* **9**, 1108-1114.

Fuller, S. D., Berriman, J. A., Butcher, S. J. & Gowen, B. E. (1995). Low pH induces swiveling of the glycoprotein heterodimers in the Semliki Forest virus spike complex. *Cell* **81**, 715-725.

Gadaleta, P., Perfetti, X., Mersich, S. & Coulombie, F. (2005). Early activation of the mitochondrial apoptotic pathway in Vesicular Stomatitis virus-infected cells. *Virus Res* **109**, 65-69.

Gaddy, D. F. & Lyles, D. S. (2005). Vesicular stomatitis viruses expressing wild-type or mutant M proteins activate apoptosis through distinct pathways. *J Virol* **79**, 4170-4179.

Galabru, J. & Hovanessian, A. (1987). Autophosphorylation of the protein kinase dependent on double-stranded RNA. *J Biol Chem* **262**, 15538-15544.

Gale, M., Jr., Kwieciszewski, B., Dossett, M., Nakao, H. & Katze, M. G. (1999). Antiapoptotic and oncogenic potentials of hepatitis C virus are linked to interferon resistance by viral repression of the PKR protein kinase. *J Virol* **73**, 6506-6516.

Garcia, M. A., Guerra, S., Gil, J., Jimenez, V. & Esteban, M. (2002). Anti-apoptotic and oncogenic properties of the dsRNA-binding protein of vaccinia virus, E3L. *Oncogene* **21**, 8379-8387.

Garcia, M. A., Meurs, E. F. & Esteban, M. (2007). The dsRNA protein kinase PKR: virus and cell control. *Biochimie* **89**, 799-811.

Garcin, D., Marq, J. B., Strahle, L., le, M. P. & Kolakofsky, D. (2002). All four Sendai Virus C proteins bind Stat1, but only the larger forms also induce its mono-ubiquitination and degradation. *Virology* **295**, 256-265.

Gardai, S. J., McPhillips, K. A., Frasch, S. C., Janssen, W. J., Starefeldt, A., Murphy-Ullrich, J. E., Bratton, D. L., Oldenborg, P. A., Michalak, M. & Henson, P. M. (2005). Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte. *Cell* **123**, 321-334.

Garmashova, N., Atasheva, S., Kang, W., Weaver, S. C., Frolova, E. & Frolov, I. (2007a). Analysis of Venezuelan equine encephalitis virus capsid protein function in the inhibition of cellular transcription. *J Virol* **81**, 13552-13565.

Garmashova, N., Gorchakov, R., Frolova, E. & Frolov, I. (2006). Sindbis virus nonstructural protein nsP2 is cytotoxic and inhibits cellular transcription. *J Virol* **80**, 5686-5696.

Garmashova, N., Gorchakov, R., Volkova, E., Paessler, S., Frolova, E. & Frolov, I. (2007b). The Old World and New World alphaviruses use different virus-specific proteins for induction of transcriptional shutoff. *J Virol* **81**, 2472-2484.

Garry, D. J., Ordway, G. A., Lorenz, J. N., Radford, N. B., Chin, E. R., Grange, R. W., Bassel-Duby, R. & Williams, R. S. (1998). Mice without myoglobin. *Nature* **395**, 905-908.

Geigenmuller-Gnirke, U., Weiss, B., Wright, R. & Schlesinger, S. (1991). Complementation between Sindbis viral RNAs produces infectious particles with a bipartite genome. *Proc Natl Acad Sci U S A* **88**, 3253-3257.

Geley, S., Hartmann, B. L. & Kofler, R. (1997). Ceramides induce a form of apoptosis in human acute lymphoblastic leukemia cells that is inhibited by Bcl-2, but not by CrmA. *FEBS Lett* **400**, 15-18.

Ghaemmaghami, S., Huh, W. K., Bower, K., Howson, R. W., Belle, A., Dephoure, N., O'Shea, E. K. & Weissman, J. S. (2003). Global analysis of protein expression in yeast. *Nature* **425**, 737-741.

Gibbs, E. P. (1976). Equine viral encephalitis. *Equine Vet J* **8**, 66-71.

Gil, J. & Esteban, M. (2000). The interferon-induced protein kinase (PKR), triggers apoptosis through FADD-mediated activation of caspase 8 in a manner independent of Fas and TNF-alpha receptors. *Oncogene* **19**, 3665-3674.

Gil, J., Garcia, M. A. & Esteban, M. (2002). Caspase 9 activation by the dsRNA-dependent protein kinase, PKR: molecular mechanism and relevance. *FEBS Lett* **529**, 249-255.

Glasgow, G. M., McGee, M. M., Sheahan, B. J. & Atkins, G. J. (1997). Death mechanisms in cultured cells infected by Semliki Forest virus. *J Gen Virol* **78 (Pt 7)**, 1559-1563.

Glasgow, G. M., McGee, M. M., Tarbatt, C. J., Mooney, D. A., Sheahan, B. J. & Atkins, G. J. (1998). The Semliki Forest virus vector induces p53-independent apoptosis. *J Gen Virol* **79 (Pt 10)**, 2405-2410.

Gomez de, C. M., Ehsani, N., Mikkola, M. L., Garcia, J. A. & Kaariainen, L. (1999).RNA helicase activity of Semliki Forest virus replicase protein NSP2. *FEBS Lett* **448**, 19-22.

Gomez, C. E., Vandermeeren, A. M., Garcia, M. A., Domingo-Gil, E. & Esteban, M. (2005). Involvement of PKR and RNase L in translational control and induction of apoptosis after Hepatitis C polyprotein expression from a vaccinia virus recombinant. *Virol J* **2**, 81.

Gonzales, D. H. & Neupert, W. (1990). Biogenesis of mitochondrial c-type cytochromes. *J Bioenerg Biomembr* **22**, 753-768.

Goswami, B. B., Kulka, M., Ngo, D. & Cebula, T. A. (2004). Apoptosis induced by a cytopathic hepatitis A virus is dependent on caspase activation following ribosomal RNA degradation but occurs in the absence of 2'-5' oligoadenylate synthetase. *Antiviral Res* **63**, 153-166.

Grether, M. E., Abrams, J. M., Agapite, J., White, K. & Steller, H. (1995). The head involution defective gene of Drosophila melanogaster functions in programmed cell death. *Genes Dev* **9**, 1694-1708.

Grimley, P. M., Berezesky, I. K. & Friedman, R. M. (1968). Cytoplasmic structures associated with an arbovirus infection: loci of viral ribonucleic acid synthesis. *J Virol* **2**, 1326-1338.

Haas, I. G. (1994). BiP (GRP78), an essential hsp70 resident protein in the endoplasmic reticulum. *Experientia* **50**, 1012-1020.

Haas, I. G. & Wabl, M. (1983). Immunoglobulin heavy chain binding protein. *Nature* **306**, 387-389.

Haasnoot, J., de, V. W., Geutjes, E. J., Prins, M., de, H. P. & Berkhout, B. (2007). The Ebola virus VP35 protein is a suppressor of RNA silencing. *PLoS Pathog* **3**, e86.

Haimovitz-Friedman, A., Kolesnick, R. N. & Fuks, Z. (1997). Ceramide signaling in apoptosis. *Br Med Bull* **53**, 539-553.

Hale, B. G., Jackson, D., Chen, Y. H., Lamb, R. A. & Randall, R. E. (2006). Influenza A virus NS1 protein binds p85beta and activates phosphatidylinositol-3-kinase signaling. *Proc Natl Acad Sci U S A* **103**, 14194-14199.

Hanson, C. J., Bootman, M. D., Distelhorst, C. W., Maraldi, T. & Roderick, H. L. (2008). The cellular concentration of Bcl-2 determines its pro- or anti-apoptotic effect. *Cell Calcium.*

Haraguchi, M., Torii, S., Matsuzawa, S., Xie, Z., Kitada, S., Krajewski, S., Yoshida, H., Mak, T. W. & Reed, J. C. (2000). Apoptotic protease activating factor 1 (Apaf-1)-independent cell death suppression by Bcl-2. *J Exp Med* **191**, 1709-1720.

Harding, H. P., Novoa, I., Zhang, Y., Zeng, H., Wek, R., Schapira, M. & Ron, D. (2000). Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Mol Cell* **6**, 1099-1108.

Harding, H. P., Zhang, Y. & Ron, D. (1999). Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. *Nature* **397**, 271-274.

Hardwick, J. M. & Bellows, D. S. (2003). Viral versus cellular BCL-2 proteins. *Cell Death Differ* **10 Suppl 1**, S68-S76.

Harte, M. T., Haga, I. R., Maloney, G., Gray, P., Reading, P. C., Bartlett, N. W., Smith, G. L., Bowie, A. & O'Neill, L. A. (2003). The poxvirus protein A52R targets Toll-like receptor signaling complexes to suppress host defense. *J Exp Med* **197**, 343-351.

Heil, F., hmad-Nejad, P., Hemmi, H., Hochrein, H., Ampenberger, F., Gellert, T., Dietrich, H., Lipford, G., Takeda, K., Akira, S., Wagner, H. & Bauer, S. (2003). The Toll-like receptor 7 (TLR7)-specific stimulus loxoribine uncovers a strong relationship within the TLR7, 8 and 9 subfamily. *Eur J Immunol* **33**, 2987-2997.

Heiskanen, K. M., Bhat, M. B., Wang, H. W., Ma, J. & Nieminen, A. L. (1999). Mitochondrial depolarization accompanies cytochrome c release during apoptosis in PC6 cells. *J Biol Chem* **274**, 5654-5658.

Helenius, A., Kartenbeck, J., Simons, K. & Fries, E. (1980). On the entry of Semliki forest virus into BHK-21 cells. *J Cell Biol* **84**, 404-420.

Helenius, A., Marsh, M. & White, J. (1982). Inhibition of Semliki forest virus penetration by lysosomotropic weak bases. *J Gen Virol* **58 Pt 1**, 47-61.

Hengartner, M. O., Ellis, R. E. & Horvitz, H. R. (1992). Caenorhabditis elegans gene ced-9 protects cells from programmed cell death. *Nature* **356**, 494-499.

Hengartner, M. O. & Horvitz, H. R. (1994).C. elegans cell survival gene ced-9 encodes a functional homolog of the mammalian proto-oncogene bcl-2. *Cell* **76**, 665-676.

Hidmark, A. S., McInerney, G. M., Nordstrom, E. K., Douagi, I., Werner, K. M., Liljestrom, P. & Karlsson Hedestam, G. B. (2005). Early alpha/beta interferon production by myeloid dendritic cells in response to UV-inactivated virus requires viral entry and interferon regulatory factor 3 but not MyD88. *J Virol* **79**, 10376-10385.

Hilton, L., Moganeradj, K., Zhang, G., Chen, Y. H., Randall, R. E., McCauley, J. W. & Goodbourn, S. (2006). The NPro product of bovine viral diarrhea virus inhibits DNA binding by interferon regulatory factor 3 and targets it for proteasomal degradation. *J Virol* **80**, 11723-11732.

Hinnebusch, A. G. (2005). Translational regulation of GCN4 and the general amino acid control of yeast. *Annu Rev Microbiol* **59**, 407-450.

Holm, G. H., Zurney, J., Tumilasci, V., Leveille, S., Danthi, P., Hiscott, J., Sherry, B. & Dermody, T. S. (2007). Retinoic acid-inducible gene-I and interferon-beta promoter stimulator-1 augment proapoptotic responses following mammalian reovirus infection via interferon regulatory factor-3. *J Biol Chem* **282**, 21953-21961.

Hornung, V., Ellegast, J., Kim, S., Brzozka, K., Jung, A., Kato, H., Poeck, H., Akira, S., Conzelmann, K. K., Schlee, M., Endres, S. & Hartmann, G. (2006).5'-Triphosphate RNA is the ligand for RIG-I. *Science* **314**, 994-997.

Horwood, C. M. & Bi, P. (2005). The incidence of Ross River virus disease in South Australia, 1992 to 2003. *Commun Dis Intell* **29**, 291-296.

Hovanessian, A. G. & Kerr, I. M. (1979). The (2'-5') oligoadenylate (pppA2'-5'A2'-5'A) synthetase and protein kinase(s) from interferon-treated cells. *Eur J Biochem* **93**, 515-526.

Huang, K. J., Ku, C. C. & Lehman, I. R. (2006). Endonuclease G: a role for the enzyme in recombination and cellular proliferation. *Proc Natl Acad Sci U S A* **103**, 8995-9000.

Imani, F. & Jacobs, B. L. (1988). Inhibitory activity for the interferon-induced protein kinase is associated with the reovirus serotype 1 sigma 3 protein. *Proc Natl Acad Sci U S A* **85**, 7887-7891.

Iqbal, M., Poole, E., Goodbourn, S. & McCauley, J. W. (2004). Role for bovine viral diarrhea virus Erns glycoprotein in the control of activation of beta interferon by double-stranded RNA. *J Virol* **78**, 136-145.

Itoh, N. & Nagata, S. (1993).A novel protein domain required for apoptosis. Mutational analysis of human Fas antigen. *J Biol Chem* **268**, 10932-10937.

Jan, J. T., Chatterjee, S. & Griffin, D. E. (2000). Sindbis virus entry into cells triggers apoptosis by activating sphingomyelinase, leading to the release of ceramide. *J Virol* **74**, 6425-6432.

Jeffers, J. R., Parganas, E., Lee, Y., Yang, C., Wang, J., Brennan, J., MacLean, K. H., Han, J., Chittenden, T., Ihle, J. N., McKinnon, P. J., Cleveland, J. L. & Zambetti, G. P. (2003). Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. *Cancer Cell* **4**, 321-328.

Joe, A. K., Foo, H. H., Kleeman, L. & Levine, B. (1998). The transmembrane domains of Sindbis virus envelope glycoproteins induce cell death. *J Virol* **72**, 3935-3943.

Jordan, R., Wang, L., Graczyk, T. M., Block, T. M. & Romano, P. R. (2002). Replication of a cytopathic strain of bovine viral diarrhea virus activates PERK and induces endoplasmic reticulum stress-mediated apoptosis of MDBK cells. *J Virol* **76**, 9588-9599.

Kaariainen, L., Takkinen, K., Keranen, S. & Soderlund, H. (1987). Replication of the genome of alphaviruses. *J Cell Sci Suppl* **7**, 231-250.

Kaiser, W. J. & Offermann, M. K. (2005). Apoptosis induced by the toll-like receptor adaptor TRIF is dependent on its receptor interacting protein homotypic interaction motif. *J Immunol* **174**, 4942-4952.

Kamer, G. & Argos, P. (1984). Primary structural comparison of RNA-dependent polymerases from plant, animal and bacterial viruses. *Nucleic Acids Res* **12**, 7269-7282.

Kang, D. C., Gopalkrishnan, R. V., Wu, Q., Jankowsky, E., Pyle, A. M. & Fisher, P. B. (2002).mda-5: An interferon-inducible putative RNA helicase with double-stranded RNA-dependent ATPase activity and melanoma growth-suppressive properties. *Proc Natl Acad Sci U S A* **99**, 637-642.

Kaukinen, P., Sillanpaa, M., Kotenko, S., Lin, R., Hiscott, J., Melen, K. & Julkunen, I. (2006). Hepatitis C virus NS2 and NS3/4A proteins are potent inhibitors of host cell cytokine/chemokine gene expression. *Virol J* **3**, 66.

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

Kedersha, N. & Anderson, P. (2002). Stress granules: sites of mRNA triage that regulate mRNA stability and translatability. *Biochem Soc Trans* **30**, 963-969.

Kedersha, N., Chen, S., Gilks, N., Li, W., Miller, I. J., Stahl, J. & Anderson, P. (2002). Evidence that ternary complex (eIF2-GTP-tRNA(i)(Met))-deficient preinitiation complexes are core constituents of mammalian stress granules. *Mol Biol Cell* **13**, 195-210.

Kedersha, N. L., Gupta, M., Li, W., Miller, I. & Anderson, P. (1999).RNA-binding proteins TIA-1 and TIAR link the phosphorylation of eIF-2 alpha to the assembly of mammalian stress granules. *J Cell Biol* **147**, 1431-1442.

Kerr, J. F., Wyllie, A. H. & Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* **26**, 239-257.

Kielian, M. (1995). Membrane fusion and the alphavirus life cycle. *Adv Virus Res* **45**, 113-151.

Kim, K. H., Rumenapf, T., Strauss, E. G. & Strauss, J. H. (2004a). Regulation of Semliki Forest virus RNA replication: a model for the control of alphavirus pathogenesis in invertebrate hosts. *Virology* **323**, 153-163.

Kim, S. J., Zhang, Z., Hitomi, E., Lee, Y. C. & Mukherjee, A. B. (2006a). Endoplasmic reticulum stress-induced caspase-4 activation mediates apoptosis and neurodegeneration in INCL. *Hum Mol Genet* **15**, 1826-1834.

Kim, S. M., Park, J. H., Chung, S. K., Kim, J. Y., Hwang, H. Y., Chung, K. C., Jo, I., Park, S. I. & Nam, J. H. (2004b). Coxsackievirus B3 infection induces cyr61 activation via JNK to mediate cell death. *J Virol* **78**, 13479-13488.

Kim, T. K., Lee, J. S., Jung, J. E., Oh, S. Y., Kwak, S., Jin, X., Lee, S. Y., Lee, J. B., Chung, Y. G., Choi, Y. K., You, S. & Kim, H. (2006b). Interferon regulatory factor 3 activates p53-dependent cell growth inhibition. *Cancer Lett* **242**, 215-221.

Kimball, S. R., Horetsky, R. L., Ron, D., Jefferson, L. S. & Harding, H. P. (2003). Mammalian stress granules represent sites of accumulation of stalled translation initiation complexes. *Am J Physiol Cell Physiol* **284**, C273-C284.

Kischkel, F. C., Hellbardt, S., Behrmann, I., Germer, M., Pawlita, M., Krammer, P. H. & Peter, M. E. (1995). Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *EMBO J* **14**, 5579-5588.

Kiyotani, K., Sakaguchi, T., Kato, A., Nagai, Y. & Yoshida, T. (2007). Paramyxovirus Sendai virus V protein counteracts innate virus clearance through IRF-3 activation, but not via interferon, in mice. *Virology* **359**, 82-91.

Kluck, R. M., Martin, S. J., Hoffman, B. M., Zhou, J. S., Green, D. R. & Newmeyer, D. D. (1997). Cytochrome c activation of CPP32-like proteolysis plays a critical role in a Xenopus cell-free apoptosis system. *EMBO J* **16**, 4639-4649.

Kohl, A., Clayton, R. F., Weber, F., Bridgen, A., Randall, R. E. & Elliott, R. M. (2003). Bunyamwera virus nonstructural protein NSs counteracts interferon regulatory factor 3-mediated induction of early cell death. *J Virol* **77**, 7999-8008.

Kokame, K., Agarwala, K. L., Kato, H. & Miyata, T. (2000). Herp, a new ubiquitin-like membrane protein induced by endoplasmic reticulum stress. *J Biol Chem* **275**, 32846-32853.

Komatsu, T., Takeuchi, K., Yokoo, J. & Gotoh, B. (2004).C and V proteins of Sendai virus target signaling pathways leading to IRF-3 activation for the negative regulation of interferon-beta production. *Virology* **325**, 137-148.

Komiyama, T., Ray, C. A., Pickup, D. J., Howard, A. D., Thornberry, N. A., Peterson, E. P. & Salvesen, G. (1994). Inhibition of interleukin-1 beta converting enzyme by the cowpox virus serpin CrmA. An example of cross-class inhibition. *J Biol Chem* **269**, 19331-19337.

Komuro, A. & Horvath, C. M. (2006).RNA- and virus-independent inhibition of antiviral signaling by RNA helicase LGP2. *J Virol* **80**, 12332-12342.

Koromilas, A. E., Roy, S., Barber, G. N., Katze, M. G. & Sonenberg, N. (1992). Malignant transformation by a mutant of the IFN-inducible dsRNA-dependent protein kinase. *Science* **257**, 1685-1689.

Korsmeyer, S. J., Wei, M. C., Saito, M., Weiler, S., Oh, K. J. & Schlesinger, P. H. (2000). Pro-apoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in the release of cytochrome c. *Cell Death Differ* **7**, 1166-1173.

Korth, M. J., Lyons, C. N., Wambach, M. & Katze, M. G. (1996). Cloning, expression, and cellular localization of the oncogenic 58-kDa inhibitor of the RNA-activated human and mouse protein kinase. *Gene* **170**, 181-188.

Kujala, P., Ikaheimonen, A., Ehsani, N., Vihinen, H., Auvinen, P. & Kaariainen, L. (2001). Biogenesis of the Semliki Forest virus RNA replication complex. *J Virol* **75**, 3873-3884.

Kumar, A., Haque, J., Lacoste, J., Hiscott, J. & Williams, B. R. (1994). Double-stranded RNA-dependent protein kinase activates transcription factor NF-

kappa B by phosphorylating I kappa B. *Proc Natl Acad Sci U S A* **91**, 6288-6292.

Kuwana, T., Mackey, M. R., Perkins, G., Ellisman, M. H., Latterich, M., Schneiter, R., Green, D. R. & Newmeyer, D. D. (2002). Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. *Cell* **111**, 331-342.

Labrada, L., Liang, X. H., Zheng, W., Johnston, C. & Levine, B. (2002). Age-dependent resistance to lethal alphavirus encephalitis in mice: analysis of gene expression in the central nervous system and identification of a novel interferon-inducible protective gene, mouse ISG12. *J Virol* **76**, 11688-11703.

Lakhani, S. A., Masud, A., Kuida, K., Porter, G. A., Jr., Booth, C. J., Mehal, W. Z., Inayat, I. & Flavell, R. A. (2006). Caspases 3 and 7: key mediators of mitochondrial events of apoptosis. *Science* **311**, 847-851.

Larribere, L., Khaled, M., Tartare-Deckert, S., Busca, R., Luciano, F., Bille, K., Valony, G., Eychene, A., Auberger, P., Ortonne, J. P., Ballotti, R. & Bertolotto, C. (2004).PI3K mediates protection against TRAIL-induced apoptosis in primary human melanocytes. *Cell Death Differ* **11**, 1084-1091.

Lazebnik, Y. A., Kaufmann, S. H., Desnoyers, S., Poirier, G. G. & Earnshaw, W. C. (1994). Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* **371**, 346-347.

Lee, C. J., Liao, C. L. & Lin, Y. L. (2005). Flavivirus activates phosphatidylinositol 3-kinase signaling to block caspase-dependent apoptotic cell death at the early stage of virus infection. *J Virol* **79**, 8388-8399.

Lee, J. Y., Marshall, J. A. & Bowden, D. S. (1999). Localization of rubella virus core particles in vero cells. *Virology* **265**, 110-119.

Lee, K., Tirasophon, W., Shen, X., Michalak, M., Prywes, R., Okada, T., Yoshida, H., Mori, K. & Kaufman, R. J. (2002).IRE1-mediated unconventional mRNA splicing and S2P-mediated ATF6 cleavage merge to regulate XBP1 in signaling the unfolded protein response. *Genes Dev* **16**, 452-466.

Lee, S. B. & Esteban, M. (1994). The interferon-induced double-stranded RNA-activated protein kinase induces apoptosis. *Virology* **199**, 491-496.

Leonard, V. H., Kohl, A., Hart, T. J. & Elliott, R. M. (2006). Interaction of Bunyamwera Orthobunyavirus NSs protein with mediator protein MED8: a mechanism for inhibiting the interferon response. *J Virol* **80**, 9667-9675.

Lerena, C., Calligaris, S. D. & Colombo, M. I. (2008). Autophagy: for better or for worse, in good times or in bad times. *Curr Mol Med* **8**, 92-101.

Levine, B., Huang, Q., Isaacs, J. T., Reed, J. C., Griffin, D. E. & Hardwick, J. M. (1993). Conversion of lytic to persistent alphavirus infection by the bcl-2 cellular oncogene. *Nature* **361**, 739-742.

Li, H., Zhu, H., Xu, C. J. & Yuan, J. (1998a). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* **94**, 491-501.

Li, K., Foy, E., Ferreon, J. C., Nakamura, M., Ferreon, A. C., Ikeda, M., Ray, S. C., Gale, M., Jr. & Lemon, S. M. (2005a). Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. *Proc Natl Acad Sci U S A* **102**, 2992-2997.

Li, X. D., Lankinen, H., Putkuri, N., Vapalahti, O. & Vaheri, A. (2005b). Tula hantavirus triggers pro-apoptotic signals of ER stress in Vero E6 cells. *Virology* **333**, 180-189.

Li, X. D., Sun, L., Seth, R. B., Pineda, G. & Chen, Z. J. (2005c). Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. *Proc Natl Acad Sci U S A* **102**, 17717-17722.

Li, X. L., Blackford, J. A. & Hassel, B. A. (1998b).RNase L mediates the antiviral effect of interferon through a selective reduction in viral RNA during encephalomyocarditis virus infection. *J Virol* **72**, 2752-2759.

Lichtenstein, D. L., Toth, K., Doronin, K., Tollefson, A. E. & Wold, W. S. (2004). Functions and mechanisms of action of the adenovirus E3 proteins. *Int Rev Immunol* **23**, 75-111.

Ligon, B. L. (2006). Reemergence of an unusual disease: the chikungunya epidemic. *Semin Pediatr Infect Dis* **17**, 99-104.

Lin, C., Holland, R. E., Jr., Donofrio, J. C., McCoy, M. H., Tudor, L. R. & Chambers, T. M. (2002). Caspase activation in equine influenza virus induced apoptotic cell death. *Vet Microbiol* **84**, 357-365.

Liu, Y., Pu, Y. & Zhang, X. (2006). Role of the mitochondrial signaling pathway in murine coronavirus-induced oligodendrocyte apoptosis. *J Virol* **80**, 395-403.

Liu, Y. & Zhang, X. (2007). Murine coronavirus-induced oligodendrocyte apoptosis is mediated through the activation of the Fas signaling pathway. *Virology* **360**, 364-375.

Locksley, R. M., Killeen, N. & Lenardo, M. J. (2001). The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* **104**, 487-501.

Logue, S. E. & Martin, S. J. (2008). Caspase activation cascades in apoptosis. *Biochem Soc Trans* **36**, 1-9.

Loo, Y. M., Fornek, J., Crochet, N., Bajwa, G., Perwitasari, O., Martinez-Sobrido, L., Akira, S., Gill, M. A., Garcia-Sastre, A., Katze, M. G. & Gale, M., Jr. (2008). Distinct RIG-I and MDA5 signaling by RNA viruses in innate immunity. *J Virol* **82**, 335-345.

Lopez, S., Yao, J. S., Kuhn, R. J., Strauss, E. G. & Strauss, J. H. (1994). Nucleocapsid-glycoprotein interactions required for assembly of alphaviruses. *J Virol* **68**, 1316-1323.

Lozano, J., Morales, A., Cremesti, A., Fuks, Z., Tilly, J. L., Schuchman, E., Gulbins, E. & Kolesnick, R. (2001). Niemann-Pick Disease versus acid sphingomyelinase deficiency. *Cell Death Differ* **8**, 100-103.

Lu, J., O'Hara, E. B., Trieselmann, B. A., Romano, P. R. & Dever, T. E. (1999). The interferon-induced double-stranded RNA-activated protein kinase PKR will phosphorylate serine, threonine, or tyrosine at residue 51 in eukaryotic initiation factor 2alpha. *J Biol Chem* **274**, 32198-32203.

Lu, Y., Wambach, M., Katze, M. G. & Krug, R. M. (1995). Binding of the influenza virus NS1 protein to double-stranded RNA inhibits the activation of the protein kinase that phosphorylates the elF-2 translation initiation factor. *Virology* **214**, 222-228.

Lund, J. M., Alexopoulou, L., Sato, A., Karow, M., Adams, N. C., Gale, N. W., Iwasaki, A. & Flavell, R. A. (2004). Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proc Natl Acad Sci U S A* **101**, 5598-5603.

Lundstrom, K., Schweitzer, C., Rotmann, D., Hermann, D., Schneider, E. M. & Ehrengruber, M. U. (2001). Semliki Forest virus vectors: efficient vehicles for in vitro and in vivo gene delivery. *FEBS Lett* **504**, 99-103.

Luo, X., Budihardjo, I., Zou, H., Slaughter, C. & Wang, X. (1998). Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell* **94**, 481-490.

Marsh, M., Bolzau, E. & Helenius, A. (1983). Penetration of Semliki Forest virus from acidic prelysosomal vacuoles. *Cell* **32**, 931-940.

Martensen, P. M., Sogaard, T. M., Gjermandsen, I. M., Buttenschon, H. N., Rossing, A. B., Bonnevie-Nielsen, V., Rosada, C., Simonsen, J. L. & Justesen, J. (2001). The interferon alpha induced protein ISG12 is localized to the nuclear membrane. *Eur J Biochem* **268**, 5947-5954.

Martins, L. M., Iaccarino, I., Tenev, T., Gschmeissner, S., Totty, N. F., Lemoine, N. R., Savopoulos, J., Gray, C. W., Creasy, C. L., Dingwall, C. & Downward, J. (2002). The serine protease Omi/HtrA2 regulates apoptosis by binding XIAP through a reaper-like motif. *J Biol Chem* **277**, 439-444.

Mashima, T., Naito, M., Fujita, N., Noguchi, K. & Tsuruo, T. (1995). Identification of actin as a substrate of ICE and an ICE-like protease

and involvement of an ICE-like protease but not ICE in VP-16-induced U937 apoptosis. *Biochem Biophys Res Commun* **217**, 1185-1192.

Mate, M. J., Ortiz-Lombardia, M., Boitel, B., Haouz, A., Tello, D., Susin, S. A., Penninger, J., Kroemer, G. & Alzari, P. M. (2002). The crystal structure of the mouse apoptosis-inducing factor AIF. *Nat Struct Biol* **9**, 442-446.

Mathiot, C. C., Grimaud, G., Garry, P., Bouquety, J. C., Mada, A., Daguisy, A. M. & Georges, A. J. (1990). An outbreak of human Semliki Forest virus infections in Central African Republic. *Am J Trop Med Hyg* **42**, 386-393.

Matsumoto, M., Funami, K., Tanabe, M., Oshiumi, H., Shingai, M., Seto, Y., Yamamoto, A. & Seya, T. (2003). Subcellular localization of Toll-like receptor 3 in human dendritic cells. *J Immunol* **171**, 3154-3162.

Matsumoto, M., Kikkawa, S., Kohase, M., Miyake, K. & Seya, T. (2002). Establishment of a monoclonal antibody against human Toll-like receptor 3 that blocks double-stranded RNA-mediated signaling. *Biochem Biophys Res Commun* **293**, 1364-1369.

Mavalankar, D., Shastri, P. & Raman, P. (2007). Chikungunya epidemic in India: a major public-health disaster. *Lancet Infect Dis* **7**, 306-307.

McCullough, K. D., Martindale, J. L., Klotz, L. O., Aw, T. Y. & Holbrook, N. J. (2001). Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. *Mol Cell Biol* **21**, 1249-1259.

McGill, A., Frank, A., Emmett, N., Turnbull, D. M., Birch-Machin, M. A. & Reynolds, N. J. (2005). The anti-psoriatic drug anthralin accumulates in keratinocyte mitochondria, dissipates mitochondrial membrane potential, and induces apoptosis through a pathway dependent on respiratory competent mitochondria. *FASEB J* **19**, 1012-1014.

McInerney, G. M., Kedersha, N. L., Kaufman, R. J., Anderson, P. & Liljestrom, P. (2005). Importance of eIF2alpha phosphorylation and stress granule assembly in alphavirus translation regulation. *Mol Biol Cell* **16**, 3753-3763.

McInerney, G. M., Smit, J. M., Liljestrom, P. & Wilschut, J. (2004). Semliki Forest virus produced in the absence of the 6K protein has an altered spike structure as revealed by decreased membrane fusion capacity. *Virology* **325**, 200-206.

Medema, J. P., Scaffidi, C., Kischkel, F. C., Shevchenko, A., Mann, M., Krammer, P. H. & Peter, M. E. (1997).FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). *EMBO J* **16**, 2794-2804.

Medigeshi, G. R., Lancaster, A. M., Hirsch, A. J., Briese, T., Lipkin, W. I., Defilippis, V., Fruh, K., Mason, P. W., Nikolich-Zugich, J. & Nelson, J.

A. (2007). West Nile virus infection activates the unfolded protein response, leading to CHOP induction and apoptosis. *J Virol* **81**, 10849-10860.

Meier, P., Silke, J., Leevers, S. J. & Evan, G. I. (2000). The Drosophila caspase DRONC is regulated by DIAP1. *EMBO J* **19**, 598-611.

Melancon, P. & Garoff, H. (1986). Reinitiation of translocation in the Semliki Forest virus structural polyprotein: identification of the signal for the E1 glycoprotein. *EMBO J* **5**, 1551-1560.

Melchjorsen, J., Jensen, S. B., Malmgaard, L., Rasmussen, S. B., Weber, F., Bowie, A. G., Matikainen, S. & Paludan, S. R. (2005). Activation of innate defense against a paramyxovirus is mediated by RIG-I and TLR7 and TLR8 in a cell-type-specific manner. *J Virol* **79**, 12944-12951.

Merits, A., Vasiljeva, L., Ahola, T., Kaariainen, L. & Auvinen, P. (2001). Proteolytic processing of Semliki Forest virus-specific non-structural polyprotein by nsP2 protease. *J Gen Virol* **82**, 765-773.

Meurs, E., Chong, K., Galabru, J., Thomas, N. S., Kerr, I. M., Williams, B. R. & Hovanessian, A. G. (1990). Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon. *Cell* **62**, 379-390.

Meurs, E. F., Galabru, J., Barber, G. N., Katze, M. G. & Hovanessian, A. G. (1993). Tumor suppressor function of the interferon-induced double-stranded RNA-activated protein kinase. *Proc Natl Acad Sci U S A* **90**, 232-236.

Meurs, E. F., Watanabe, Y., Kadereit, S., Barber, G. N., Katze, M. G., Chong, K., Williams, B. R. & Hovanessian, A. G. (1992). Constitutive expression of human double-stranded RNA-activated p68 kinase in murine cells mediates phosphorylation of eukaryotic initiation factor 2 and partial resistance to encephalomyocarditis virus growth. *J Virol* **66**, 5805-5814.

Meylan, E., Curran, J., Hofmann, K., Moradpour, D., Binder, M., Bartenschlager, R. & Tschopp, J. (2005). Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* **437**, 1167-1172.

Mi, S., Durbin, R., Huang, H. V., Rice, C. M. & Stollar, V. (1989). Association of the Sindbis virus RNA methyltransferase activity with the nonstructural protein nsP1. *Virology* **170**, 385-391.

Mibayashi, M., Martinez-Sobrido, L., Loo, Y. M., Cardenas, W. B., Gale, M., Jr. & Garcia-Sastre, A. (2007). Inhibition of retinoic acid-inducible gene I-mediated induction of beta interferon by the NS1 protein of influenza A virus. *J Virol* **81**, 514-524.

Miller, D. J., Schwartz, M. D. & Ahlquist, P. (2001). Flock house virus RNA replicates on outer mitochondrial membranes in Drosophila cells. *J Virol* **75**, 11664-11676.

Milligan, C. E. & Schwartz, L. M. (1997). Programmed cell death during animal development. *Br Med Bull* **53**, 570-590.

Miyashita, T., Krajewski, S., Krajewska, M., Wang, H. G., Lin, H. K., Liebermann, D. A., Hoffman, B. & Reed, J. C. (1994). Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. *Oncogene* **9**, 1799-1805.

Moore, C. B., Bergstralh, D. T., Duncan, J. A., Lei, Y., Morrison, T. E., Zimmermann, A. G., ccavitti-Loper, M. A., Madden, V. J., Sun, L., Ye, Z., Lich, J. D., Heise, M. T., Chen, Z. & Ting, J. P. (2008).NLRX1 is a regulator of mitochondrial antiviral immunity. *Nature* **451**, 573-577.

Morgello, S., Cho, E. S., Nielsen, S., Devinsky, O. & Petito, C. K. (1987). Cytomegalovirus encephalitis in patients with acquired immunodeficiency syndrome: an autopsy study of 30 cases and a review of the literature. *Hum Pathol* **18**, 289-297.

Mori, I., Goshima, F., Imai, Y., Kohsaka, S., Sugiyama, T., Yoshida, T., Yokochi, T., Nishiyama, Y. & Kimura, Y. (2002). Olfactory receptor neurons prevent dissemination of neurovirulent influenza A virus into the brain by undergoing virus-induced apoptosis. *J Gen Virol* **83**, 2109-2116.

Muller, U., Steinhoff, U., Reis, L. F., Hemmi, S., Pavlovic, J., Zinkernagel, R. M. & Aguet, M. (1994). Functional role of type I and type II interferons in antiviral defense. *Science* **264**, 1918-1921.

Muzio, M., Chinnaiyan, A. M., Kischkel, F. C., O'Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J. D., Zhang, M., Gentz, R., Mann, M., Krammer, P. H., Peter, M. E. & Dixit, V. M. (1996).FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death--inducing signaling complex. *Cell* **85**, 817-827.

Nakagawa, T. & Yuan, J. (2000). Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. *J Cell Biol* **150**, 887-894.

Nakagawa, T., Zhu, H., Morishima, N., Li, E., Xu, J., Yankner, B. A. & Yuan, J. (2000). Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature* **403**, 98-103.

Nathaniel, R., MacNeill, A. L., Wang, Y. X., Turner, P. C. & Moyer, R. W. (2004). Cowpox virus CrmA, Myxoma virus SERP2 and baculovirus P35 are not functionally interchangeable caspase inhibitors in poxvirus infections. *J Gen Virol* **85**, 1267-1278.

Nishitoh, H., Matsuzawa, A., Tobiume, K., Saegusa, K., Takeda, K., Inoue, K., Hori, S., Kakizuka, A. & Ichijo, H. (2002).ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats. *Genes Dev* **16**, 1345-1355.

Nishiya, T. & Defranco, A. L. (2004). Ligand-regulated chimeric receptor approach reveals distinctive subcellular localization and signaling properties of the Toll-like receptors. *J Biol Chem* **279**, 19008-19017.

Nishiya, T., Kajita, E., Miwa, S. & Defranco, A. L. (2005).TLR3 and TLR7 are targeted to the same intracellular compartments by distinct regulatory elements. *J Biol Chem* **280**, 37107-37117.

Obeid, M., Tesniere, A., Ghiringhelli, F., Fimia, G. M., Apetoh, L., Perfettini, J. L., Castedo, M., Mignot, G., Panaretakis, T., Casares, N., Metivier, D., Larochette, N., van, E. P., Ciccosanti, F., Piacentini, M., Zitvogel, L. & Kroemer, G. (2007). Calreticulin exposure dictates the immunogenicity of cancer cell death. *Nat Med* **13**, 54-61.

Oikawa, D., Tokuda, M. & Iwawaki, T. (2007). Site-specific cleavage of CD59 mRNA by endoplasmic reticulum-localized ribonuclease, IRE1. *Biochem Biophys Res Commun* **360**, 122-127.

Okamoto, M., Oshikawa, T., Tano, T., Ohe, G., Furuichi, S., Nishikawa, H., Ahmed, S. U., Akashi, S., Miyake, K., Takeuchi, O., Akira, S., Moriya, Y., Matsubara, S., Ryoma, Y., Saito, M. & Sato, M. (2003). Involvement of Toll-like receptor 4 signaling in interferon-gamma production and antitumor effect by streptococcal agent OK-432. *J Natl Cancer Inst* **95**, 316-326.

Oliver, K. R. & Fazakerley, J. K. (1998). Transneuronal spread of Semliki Forest virus in the developing mouse olfactory system is determined by neuronal maturity. *Neuroscience* **82**, 867-877.

Oliver, K. R., Scallan, M. F., Dyson, H. & Fazakerley, J. K. (1997). Susceptibility to a neurotropic virus and its changing distribution in the developing brain is a function of CNS maturity. *J Neurovirol* **3**, 38-48.

Orvedahl, A., Alexander, D., Talloczy, Z., Sun, Q., Wei, Y., Zhang, W., Burns, D., Leib, D. A. & Levine, B. (2007).HSV-1 ICP34.5 confers neurovirulence by targeting the Beclin 1 autophagy protein. *Cell Host Microbe* **1**, 23-35.

Peranen, J., Takkinen, K., Kalkkinen, N. & Kaariainen, L. (1988). Semliki Forest virus-specific non-structural protein nsP3 is a phosphoprotein. *J Gen Virol* **69 (Pt 9)**, 2165-2178.

Peterhans, E., Haenggeli, E., Wild, P. & Wyler, R. (1979). Mitochondrial calcium uptake during infection of chicken embryo cells with Semliki Forest virus. *J Virol* **29**, 143-152.

Peters, K., Chattopadhyay, S. & Sen, G. C. (2008).IRF-3 activation by Sendai virus infection is required for cellular apoptosis and avoidance of persistence. *J Virol* **82**, 3500-3508.

Pianko, S., Patella, S. & Sievert, W. (2000). Alcohol consumption induces hepatocyte apoptosis in patients with chronic hepatitis C infection. *J Gastroenterol Hepatol* **15**, 798-805.

Pichlmair, A., Schulz, O., Tan, C. P., Naslund, T. I., Liljestrom, P., Weber, F. & Reis e Sousa (2006).RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Science* **314**, 997-1001.

Posey, D. L., O'rourke, T., Roehrig, J. T., Lanciotti, R. S., Weinberg, M. & Maloney, S. (2005).O'Nyong-nyong fever in West Africa. *Am J Trop Med Hyg* **73**, 32.

Powers, A. M., Brault, A. C., Shirako, Y., Strauss, E. G., Kang, W., Strauss, J. H. & Weaver, S. C. (2001). Evolutionary relationships and systematics of the alphaviruses. *J Virol* **75**, 10118-10131.

Qian, X. Y., Chien, C. Y., Lu, Y., Montelione, G. T. & Krug, R. M. (1995). An amino-terminal polypeptide fragment of the influenza virus NS1 protein possesses specific RNA-binding activity and largely helical backbone structure. *RNA* **1**, 948-956.

Ramsey-Ewing, A. & Moss, B. (1998). Apoptosis induced by a postbinding step of vaccinia virus entry into Chinese hamster ovary cells. *Virology* **242**, 138-149.

Rao, R. V., Castro-Obregon, S., Frankowski, H., Schuler, M., Stoka, V., Del, R. G., Bredesen, D. E. & Ellerby, H. M. (2002). Coupling endoplasmic reticulum stress to the cell death program. An Apaf-1-independent intrinsic pathway. *J Biol Chem* **277**, 21836-21842.

Rao, R. V., Hermel, E., Castro-Obregon, S., del Rio, G., Ellerby, L. M., Ellerby, H. M. & Bredesen, D. E. (2001). Coupling endoplasmic reticulum stress to the cell death program. Mechanism of caspase activation. *J Biol Chem* **276**, 33869-33874.

Restrepo-Hartwig, M. A. & Ahlquist, P. (1996). Brome mosaic virus helicase- and polymerase-like proteins colocalize on the endoplasmic reticulum at sites of viral RNA synthesis. *J Virol* **70**, 8908-8916.

Rezza, G., Nicoletti, L., Angelini, R., Romi, R., Finarelli, A. C., Panning, M., Cordioli, P., Fortuna, C., Boros, S., Magurano, F., Silvi, G., Angelini, P., Dottori, M., Ciufolini, M. G., Majori, G. C. & Cassone, A. (2007). Infection with chikungunya virus in Italy: an outbreak in a temperate region. *Lancet* **370**, 1840-1846.

Rikkonen, M., Peranen, J. & Kaariainen, L. (1994).ATPase and GTPase activities associated with Semliki Forest virus nonstructural protein nsP2. *J Virol* **68**, 5804-5810.

Ruckdeschel, K., Pfaffinger, G., Haase, R., Sing, A., Weighardt, H., Hacker, G., Holzmann, B. & Heesemann, J. (2004). Signaling of apoptosis through TLRs critically involves toll/IL-1 receptor domain-containing adapter inducing IFN-beta, but not MyD88, in bacteria-infected murine macrophages. *J Immunol* **173**, 3320-3328.

Rusch, L., Zhou, A. & Silverman, R. H. (2000). Caspase-dependent apoptosis by 2',5'-oligoadenylate activation of RNase L is enhanced by IFN-beta. *J Interferon Cytokine Res* **20**, 1091-1100.

Ryman, K. D., White, L. J., Johnston, R. E. & Klimstra, W. B. (2002). Effects of PKR/RNase L-dependent and alternative antiviral pathways on alphavirus replication and pathogenesis. *Viral Immunol* **15**, 53-76.

Saito, T., Hirai, R., Loo, Y. M., Owen, D., Johnson, C. L., Sinha, S. C., Akira, S., Fujita, T. & Gale, M., Jr. (2007). Regulation of innate antiviral defenses through a shared repressor domain in RIG-I and LGP2. *Proc Natl Acad Sci U S A* **104**, 582-587.

Salako, M. A., Carter, M. J. & Kass, G. E. (2006). Coxsackievirus protein 2BC blocks host cell apoptosis by inhibiting caspase-3. *J Biol Chem* **281**, 16296-16304.

Salaun, B., Coste, I., Rissoan, M. C., Lebecque, S. J. & Renno, T. (2006).TLR3 can directly trigger apoptosis in human cancer cells. *J Immunol* **176**, 4894-4901.

Salaun, B., Romero, P. & Lebecque, S. (2007). Toll-like receptors' two-edged sword: when immunity meets apoptosis. *Eur J Immunol* **37**, 3311-3318.

Saleh, M., Mathison, J. C., Wolinski, M. K., Bensinger, S. J., Fitzgerald, P., Droin, N., Ulevitch, R. J., Green, D. R. & Nicholson, D. W. (2006). Enhanced bacterial clearance and sepsis resistance in caspase-12-deficient mice. *Nature* **440**, 1064-1068.

Sammin, D. J., Butler, D., Atkins, G. J. & Sheahan, B. J. (1999). Cell death mechanisms in the olfactory bulb of rats infected intranasally with Semliki forest virus. *Neuropathol Appl Neurobiol* **25**, 236-243.

Sanges, D. & Marigo, V. (2006). Cross-talk between two apoptotic pathways activated by endoplasmic reticulum stress: differential contribution of caspase-12 and AIF. *Apoptosis* **11**, 1629-1641.

Santagati, M. G., Maatta, J. A., Itaranta, P. V., Salmi, A. A. & Hinkkanen, A. E. (1995). The Semliki Forest virus E2 gene as a virulence determinant. *J Gen Virol* **76 (Pt 1)**, 47-52.

Santagati, M. G., Maatta, J. A., Roytta, M., Salmi, A. A. & Hinkkanen, A. E. (1998). The significance of the 3'-nontranslated region and E2 amino acid mutations in the virulence of Semliki Forest virus in mice. *Virology* **243**, 66-77.

Sarid, R., Ben Moshe, T., Kazimirsky, G., Weisberg, S., Appel, E., Kobiler, D., Lustig, S. & Brodie, C. (2001).vFLIP protects PC-12 cells from apoptosis induced by Sindbis virus: implications for the role of TNF-alpha. *Cell Death Differ* **8**, 1224-1231.

Sarkar, S. N. & Sen, G. C. (1998). Production, purification, and characterization of recombinant 2', 5'-oligoadenylate synthetases. *Methods* **15**, 233-242.

Sarmento, L., Tseggai, T., Dhingra, V. & Fu, Z. F. (2006). Rabies virus-induced apoptosis involves caspase-dependent and caspase-independent pathways. *Virus Res* **121**, 144-151.

Savill, J. S., Wyllie, A. H., Henson, J. E., Walport, M. J., Henson, P. M. & Haslett, C. (1989). Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. *J Clin Invest* **83**, 865-875.

Sawicki, D. L., Silverman, R. H., Williams, B. R. & Sawicki, S. G. (2003). Alphavirus minus-strand synthesis and persistence in mouse embryo fibroblasts derived from mice lacking RNase L and protein kinase R. *J Virol* **77**, 1801-1811.

Scheidel, L. M. & Stollar, V. (1991). Mutations that confer resistance to mycophenolic acid and ribavirin on Sindbis virus map to the nonstructural protein nsP1. *Virology* **181**, 490-499.

Schroder, M. & Kaufman, R. J. (2006). Divergent roles of IRE1alpha and PERK in the unfolded protein response. *Curr Mol Med* **6**, 5-36.

Schultz-Cherry, S., Dybdahl-Sissoko, N., Neumann, G., Kawaoka, Y. & Hinshaw, V. S. (2001). Influenza virus ns1 protein induces apoptosis in cultured cells. *J Virol* **75**, 7875-7881.

Scorrano, L., Oakes, S. A., Opferman, J. T., Cheng, E. H., Sorcinelli, M. D., Pozzan, T. & Korsmeyer, S. J. (2003).BAX and BAK regulation of endoplasmic reticulum Ca2+: a control point for apoptosis. *Science* **300**, 135-139.

Seigneuret, M. & Devaux, P. F. (1984).ATP-dependent asymmetric distribution of spin-labeled phospholipids in the erythrocyte membrane: relation to shape changes. *Proc Natl Acad Sci U S A* **81**, 3751-3755.

Seth, R. B., Sun, L., Ea, C. K. & Chen, Z. J. (2005). Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell* **122**, 669-682.

Shamu, C. E. & Walter, P. (1996). Oligomerization and phosphorylation of the Ire1p kinase during intracellular signaling from the endoplasmic reticulum to the nucleus. *EMBO J* **15**, 3028-3039.

Shen, J., Chen, X., Hendershot, L. & Prywes, R. (2002).ER stress regulation of ATF6 localization by dissociation of BiP/GRP78 binding and unmasking of Golgi localization signals. *Dev Cell* **3**, 99-111.

Shen, J., Snapp, E. L., Lippincott-Schwartz, J. & Prywes, R. (2005). Stable binding of ATF6 to BiP in the endoplasmic reticulum stress response. *Mol Cell Biol* **25**, 921-932.

Shimizu, S., Narita, M. & Tsujimoto, Y. (1999). Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. *Nature* **399**, 483-487.

Shirane, M. & Nakayama, K. I. (2003). Inherent calcineurin inhibitor FKBP38 targets Bcl-2 to mitochondria and inhibits apoptosis. *Nat Cell Biol* **5**, 28-37.

Shiratsuchi, A., Kaido, M., Takizawa, T. & Nakanishi, Y. (2000). Phosphatidylserine-mediated phagocytosis of influenza A virus-infected cells by mouse peritoneal macrophages. *J Virol* **74**, 9240-9244.

Siekierka, J., Manne, V. & Ochoa, S. (1984). Mechanism of translational control by partial phosphorylation of the alpha subunit of eukaryotic initiation factor 2. *Proc Natl Acad Sci U S A* **81**, 352-356.

Silverman, R. H. (2003). Implications for RNase L in prostate cancer biology. *Biochemistry* **42**, 1805-1812.

Siskind, L. J., Feinstein, L., Yu, T., Davis, J. S., Jones, D., Choi, J., Zuckerman, J. E., Tan, W., Hill, R. B., Hardwick, J. M. & Colombini, M. (2008). Anti-apoptotic Bcl-2 family proteins disassemble ceramide channels. *J Biol Chem.*

Siskind, L. J., Kolesnick, R. N. & Colombini, M. (2006). Ceramide forms channels in mitochondrial outer membranes at physiologically relevant concentrations. *Mitochondrion* **6**, 118-125.

Skaletskaya, A., Bartle, L. M., Chittenden, T., McCormick, A. L., Mocarski, E. S. & Goldmacher, V. S. (2001).A cytomegalovirus-encoded inhibitor of apoptosis that suppresses caspase-8 activation. *Proc Natl Acad Sci U S A* **98**, 7829-7834.

Slee, E. A., Adrain, C. & Martin, S. J. (2001). Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. *J Biol Chem* **276**, 7320-7326.

Smerdou, C. & Liljestrom, P. (1999). Two-helper RNA system for production of recombinant Semliki forest virus particles. *J Virol* **73**, 1092-1098.

Spuul, P., Salonen, A., Merits, A., Jokitalo, E., Kaariainen, L. & Ahola, T. (2007). Role of the amphipathic peptide of Semliki forest virus replicase protein nsP1 in membrane association and virus replication. *J Virol* **81**, 872-883.

Srinivasula, S. M., Ahmad, M., Fernandes-Alnemri, T. & Alnemri, E. S. (1998). Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. *Mol Cell* **1**, 949-957.

Srinivasula, S. M., Datta, P., Kobayashi, M., Wu, J. W., Fujioka, M., Hegde, R., Zhang, Z., Mukattash, R., Fernandes-Alnemri, T., Shi, Y., Jaynes, J. B. & Alnemri, E. S. (2002).sickle, a novel Drosophila death gene in the reaper/hid/grim region, encodes an IAP-inhibitory protein. *Curr Biol* **12**, 125-130.

Srivastava, S. P., Kumar, K. U. & Kaufman, R. J. (1998). Phosphorylation of eukaryotic translation initiation factor 2 mediates apoptosis in response to activation of the double-stranded RNA-dependent protein kinase. *J Biol Chem* **273**, 2416-2423.

Stack, J., Haga, I. R., Schroder, M., Bartlett, N. W., Maloney, G., Reading, P. C., Fitzgerald, K. A., Smith, G. L. & Bowie, A. G. (2005). Vaccinia virus protein A46R targets multiple Toll-like-interleukin-1 receptor adaptors and contributes to virulence. *J Exp Med* **201**, 1007-1018.

Stanfield, G. M. & Horvitz, H. R. (2000). The ced-8 gene controls the timing of programmed cell deaths in C. elegans. *Mol Cell* **5**, 423-433.

Strauss, E. G., Levinson, R., Rice, C. M., Dalrymple, J. & Strauss, J. H. (1988). Nonstructural proteins nsP3 and nsP4 of Ross River and O'Nyong-nyong viruses: sequence and comparison with those of other alphaviruses. *Virology* **164**, 265-274.

Strauss, J. H. & Strauss, E. G. (1994). The alphaviruses: gene expression, replication, and evolution. *Microbiol Rev* **58**, 491-562.

Streitenfeld, H., Boyd, A., Fazakerley, J. K., Bridgen, A., Elliott, R. M. & Weber, F. (2003). Activation of PKR by Bunyamwera virus is independent of the viral interferon antagonist NSs. *J Virol* **77**, 5507-5511.

Strous, G. J. & Govers, R. (1999). The ubiquitin-proteasome system and endocytosis. *J Cell Sci* **112 (Pt 10)**, 1417-1423.

Sulston, J. E. & Horvitz, H. R. (1977). Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. *Dev Biol* **56**, 110-156.

Sulston, J. E., Schierenberg, E., White, J. G. & Thomson, J. N. (1983). The embryonic cell lineage of the nematode Caenorhabditis elegans. *Dev Biol* **100**, 64-119.

Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Snow, B. E., Brothers, G. M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D. R., Aebersold, R., Siderovski, D. P., Penninger, J. M. & Kroemer, G. (1999). Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* **397**, 441-446.

Suzuki, M., Youle, R. J. & Tjandra, N. (2000). Structure of Bax: coregulation of dimer formation and intracellular localization. *Cell* **103**, 645-654.

Szalai, G., Krishnamurthy, R. & Hajnoczky, G. (1999). Apoptosis driven by IP(3)-linked mitochondrial calcium signals. *EMBO J* **18**, 6349-6361.

Taddeo, B., Luo, T. R., Zhang, W. & Roizman, B. (2003). Activation of NF-kappaB in cells productively infected with HSV-1 depends on activated protein kinase R and plays no apparent role in blocking apoptosis. *Proc Natl Acad Sci U S A* **100**, 12408-12413.

Takeuchi, K., Komatsu, T., Yokoo, J., Kato, A., Shioda, T., Nagai, Y. & Gotoh, B. (2001). Sendai virus C protein physically associates with Stat1. *Genes Cells* **6**, 545-557.

Takeuchi, O. & Akira, S. (2007). Recognition of viruses by innate immunity. *Immunol Rev* **220**, 214-224.

Takizawa, T., Fukuda, R., Miyawaki, T., Ohashi, K. & Nakanishi, Y. (1995). Activation of the apoptotic Fas antigen-encoding gene upon influenza virus infection involving spontaneously produced beta-interferon. *Virology* **209**, 288-296.

Takizawa, T., Ohashi, K. & Nakanishi, Y. (1996). Possible involvement of double-stranded RNA-activated protein kinase in cell death by influenza virus infection. *J Virol* **70**, 8128-8132.

Takkinen, K. (1986). Complete nucleotide sequence of the nonstructural protein genes of Semliki Forest virus. *Nucleic Acids Res* **14**, 5667-5682.

Takkinen, K., Peranen, J., Keranen, S., Soderlund, H. & Kaariainen, L. (1990). The Semliki-Forest-virus-specific nonstructural protein nsP4 is an autoproteinase. *Eur J Biochem* **189**, 33-38.

Talloczy, Z., Jiang, W., Virgin, H. W., Leib, D. A., Scheuner, D., Kaufman, R. J., Eskelinen, E. L. & Levine, B. (2002). Regulation of starvation- and virus-induced autophagy by the eIF2alpha kinase signaling pathway. *Proc Natl Acad Sci U S A* **99**, 190-195.

Talukder, A. H., Wang, R. A. & Kumar, R. (2002). Expression and transactivating functions of the bZIP transcription factor GADD153 in mammary epithelial cells. *Oncogene* **21**, 4289-4300.

Tamberg, N., Lulla, V., Fragkoudis, R., Lulla, A., Fazakerley, J. K. & Merits, A. (2007). Insertion of EGFP into the replicase gene of Semliki Forest virus results in a novel, genetically stable marker virus. *J Gen Virol* **88**, 1225-1230.

Tamm, K., Merits, A. & Sarand, I. (2008). Mutations in the nuclear localization signal of nsP2 influencing RNA synthesis, protein expression and cytotoxicity of Semliki Forest virus. *J Gen Virol* **89**, 676-686.

Tan, Y., Dourdin, N., Wu, C., De, V. T., Elce, J. S. & Greer, P. A. (2006). Ubiquitous calpains promote caspase-12 and JNK activation during endoplasmic reticulum stress-induced apoptosis. *J Biol Chem* **281**, 16016-16024.

Tattoli, I., Carneiro, L. A., Jehanno, M., Magalhaes, J. G., Shu, Y., Philpott, D. J., Arnoult, D. & Girardin, S. E. (2008).NLRX1 is a mitochondrial NOD-like receptor that amplifies NF-kappaB and JNK pathways by inducing reactive oxygen species production. *EMBO Rep.*

Tergaonkar, V. (2006).NFkappaB pathway: a good signaling paradigm and therapeutic target. *Int J Biochem Cell Biol* **38**, 1647-1653.

Thress, K., Evans, E. K. & Kornbluth, S. (1999). Reaper-induced dissociation of a Scythe-sequestered cytochrome c-releasing activity. *EMBO J* **18**, 5486-5493.

Thress, K., Henzel, W., Shillinglaw, W. & Kornbluth, S. (1998). Scythe: a novel reaper-binding apoptotic regulator. *EMBO J* **17**, 6135-6143.

Thress, K., Song, J., Morimoto, R. I. & Kornbluth, S. (2001). Reversible inhibition of Hsp70 chaperone function by Scythe and Reaper. *EMBO J* **20**, 1033-1041.

Tseng, J. C., Levin, B., Hurtado, A., Yee, H., Perez, d. C., I, Jimenez, M., Shamamian, P., Jin, R., Novick, R. P., Pellicer, A. & Meruelo, D. (2004). Systemic tumor targeting and killing by Sindbis viral vectors. *Nat Biotechnol* **22**, 70-77.

Tuittila, M. T., Santagati, M. G., Roytta, M., Maatta, J. A. & Hinkkanen, A. E. (2000). Replicase complex genes of Semliki Forest virus confer lethal neurovirulence. *J Virol* **74**, 4579-4589.

Vahsen, N., Cande, C., Briere, J. J., Benit, P., Joza, N., Larochette, N., Mastroberardino, P. G., Pequignot, M. O., Casares, N., Lazar, V., Feraud, O., Debili, N., Wissing, S., Engelhardt, S., Madeo, F., Piacentini, M., Penninger, J. M., Schagger, H., Rustin, P. & Kroemer, G. (2004).AIF deficiency compromises oxidative phosphorylation. *EMBO J* **23**, 4679-4689.

van, L. G., Schotte, P., van, G. M., Demol, H., Hoorelbeke, B., Gevaert, K., Rodriguez, I., Ruiz-Carrillo, A., Vandekerckhove, J., Declercq, W., Beyaert, R. & Vandenabeele, P. (2001). Endonuclease G: a mitochondrial protein released in apoptosis and involved in caspase-independent DNA degradation. *Cell Death Differ* **8**, 1136-1142.

Vande, W. L., Van, D. P., Lamkanfi, M., Saelens, X., Vandekerckhove, J., Gevaert, K. & Vandenabeele, P. (2007). Proteome-wide Identification of HtrA2/Omi Substrates. *J Proteome Res* **6**, 1006-1015.

Varfolomeev, E. E., Schuchmann, M., Luria, V., Chiannilkulchai, N., Beckmann, J. S., Mett, I. L., Rebrikov, D., Brodianski, V. M., Kemper, O. C., Kollet, O., Lapidot, T., Soffer, D., Sobe, T., Avraham, K. B., Goncharov, T., Holtmann, H., Lonai, P. & Wallach, D. (1998). Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. *Immunity* **9**, 267-276.

Vasiljeva, L., Merits, A., Auvinen, P. & Kaariainen, L. (2000). Identification of a novel function of the alphavirus capping apparatus. RNA 5'-triphosphatase activity of Nsp2. *J Biol Chem* **275**, 17281-17287.

Ventoso, I., Sanz, M. A., Molina, S., Berlanga, J. J., Carrasco, L. & Esteban, M. (2006). Translational resistance of late alphavirus mRNA to eIF2alpha phosphorylation: a strategy to overcome the antiviral effect of protein kinase PKR. *Genes Dev* **20**, 87-100.

Verhagen, A. M., Ekert, P. G., Pakusch, M., Silke, J., Connolly, L. M., Reid, G. E., Moritz, R. L., Simpson, R. J. & Vaux, D. L. (2000). Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* **102**, 43-53.

Verhagen, A. M., Silke, J., Ekert, P. G., Pakusch, M., Kaufmann, H., Connolly, L. M., Day, C. L., Tikoo, A., Burke, R., Wrobel, C., Moritz, R. L., Simpson, R. J. & Vaux, D. L. (2002). HtrA2 promotes cell death through its serine protease activity and its ability to antagonize inhibitor of apoptosis proteins. *J Biol Chem* **277**, 445-454.

Wang, G. H., Garvey, T. L. & Cohen, J. I. (1999a). The murine gammaherpesvirus-68 M11 protein inhibits Fas- and TNF-induced apoptosis. *J Gen Virol* **80 (Pt 10)**, 2737-2740.

Wang, J., Tong, W., Zhang, X., Chen, L., Yi, Z., Pan, T., Hu, Y., Xiang, L. & Yuan, Z. (2006). Hepatitis C virus non-structural protein NS5A interacts with FKBP38 and inhibits apoptosis in Huh7 hepatoma cells. *FEBS Lett* **580**, 4392-4400.

Wang, S. L., Hawkins, C. J., Yoo, S. J., Muller, H. A. & Hay, B. A. (1999b). The Drosophila caspase inhibitor DIAP1 is essential for cell survival and is negatively regulated by HID. *Cell* **98**, 453-463.

Wang, T., Town, T., Alexopoulou, L., Anderson, J. F., Fikrig, E. & Flavell, R. A. (2004). Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. *Nat Med* **10**, 1366-1373.

Wang, X., Yang, C., Chai, J., Shi, Y. & Xue, D. (2002). Mechanisms of AIF-mediated apoptotic DNA degradation in Caenorhabditis elegans. *Science* **298**, 1587-1592.

Wang, Y., Shen, J., Arenzana, N., Tirasophon, W., Kaufman, R. J. & Prywes, R. (2000). Activation of ATF6 and an ATF6 DNA binding site by the endoplasmic reticulum stress response. *J Biol Chem* **275**, 27013-27020.

Wang, Y. F., Sawicki, S. G. & Sawicki, D. L. (1994). Alphavirus nsP3 functions to form replication complexes transcribing negative-strand RNA. *J Virol* **68**, 6466-6475.

Weber, F. & Haller, O. (2007). Viral suppression of the interferon system. *Biochimie* **89**, 836-842.

Weber, F., Wagner, V., Rasmussen, S. B., Hartmann, R. & Paludan, S. R. (2006). Double-stranded RNA is produced by positive-strand RNA viruses and DNA viruses but not in detectable amounts by negative-strand RNA viruses. *J Virol* **80**, 5059-5064.

Wei, M. C., Lindsten, T., Mootha, V. K., Weiler, S., Gross, A., Ashiya, M., Thompson, C. B. & Korsmeyer, S. J. (2000).tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. *Genes Dev* **14**, 2060-2071.

Wei, M. C., Zong, W. X., Cheng, E. H., Lindsten, T., Panoutsakopoulou, V., Ross, A. J., Roth, K. A., MacGregor, G. R., Thompson, C. B. & Korsmeyer, S. J. (2001). Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* **292**, 727-730.

Weiss, B., Nitschko, H., Ghattas, I., Wright, R. & Schlesinger, S. (1989). Evidence for specificity in the encapsidation of Sindbis virus RNAs. *J Virol* **63**, 5310-5318.

Wek, R. C., Jiang, H. Y. & Anthony, T. G. (2006). Coping with stress: eIF2 kinases and translational control. *Biochem Soc Trans* **34**, 7-11.

White, K., Grether, M. E., Abrams, J. M., Young, L., Farrell, K. & Steller, H. (1994). Genetic control of programmed cell death in Drosophila. *Science* **264**, 677-683.

Willems, W. R., Kaluza, G., Boschek, C. B., Bauer, H., Hager, H., Schutz, H. J. & Feistner, H. (1979). Semliki forest virus: cause of a fatal case of human encephalitis. *Science* **203**, 1127-1129.

Willis, S. N., Chen, L., Dewson, G., Wei, A., Naik, E., Fletcher, J. I., Adams, J. M. & Huang, D. C. (2005). Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. *Genes Dev* **19**, 1294-1305.

Winzeler, E. A., Shoemaker, D. D., Astromoff, A., Liang, H., Anderson, K., Andre, B., Bangham, R., Benito, R., Boeke, J. D., Bussey, H., Chu, A. M., Connelly, C., Davis, K., Dietrich, F., Dow, S. W., El, B. M., Foury, F., Friend, S. H., Gentalen, E., Giaever, G., Hegemann, J. H., Jones, T., Laub, M., Liao, H., Liebundguth, N., Lockhart, D. J., Lucau-Danila, A., Lussier, M., M'Rabet, N., Menard, P., Mittmann, M., Pai, C., Rebischung, C., Revuelta, J. L., Riles, L., Roberts, C. J., Ross-MacDonald, P., Scherens, B., Snyder, M., Sookhai-Mahadeo, S., Storms, R. K., Veronneau, S., Voet, M., Volckaert, G., Ward, T. R., Wysocki, R., Yen, G. S., Yu, K., Zimmermann, K., Philippsen, P., Johnston, M. & Davis, R. W. (1999). Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis. *Science* **285**, 901-906.

Wu, D., Wallen, H. D., Inohara, N. & Nunez, G. (1997). Interaction and regulation of the Caenorhabditis elegans death protease CED-3 by CED-4 and CED-9. *J Biol Chem* **272**, 21449-21454.

Wu, H. & Lozano, G. (1994).NF-kappa B activation of p53. A potential mechanism for suppressing cell growth in response to stress. *J Biol Chem* **269**, 20067-20074.

Wu, S. & Kaufman, R. J. (1997).A model for the double-stranded RNA (dsRNA)-dependent dimerization and activation of the dsRNA-activated protein kinase PKR. *J Biol Chem* **272**, 1291-1296.

Wurzer, W. J., Ehrhardt, C., Pleschka, S., Berberich-Siebelt, F., Wolff, T., Walczak, H., Planz, O. & Ludwig, S. (2004).NF-kappaB-dependent induction of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas/FasL is crucial for efficient influenza virus propagation. *J Biol Chem* **279**, 30931-30937.

Xiang, Y., Condit, R. C., Vijaysri, S., Jacobs, B., Williams, B. R. & Silverman, R. H. (2002). Blockade of interferon induction and action by the E3L double-stranded RNA binding proteins of vaccinia virus. *J Virol* **76**, 5251-5259.

Xu, L. G., Wang, Y. Y., Han, K. J., Li, L. Y., Zhai, Z. & Shu, H. B. (2005).VISA Is an Adapter Protein Required for Virus-Triggered IFN-beta Signaling. *Mol Cell* **19**, 727-740.

Yamane, D., Kato, K., Tohya, Y. & Akashi, H. (2006). The double-stranded RNA-induced apoptosis pathway is involved in the cytopathogenicity of cytopathogenic Bovine viral diarrhea virus. *J Gen Virol* **87**, 2961-2970.

Yan, N., Chai, J., Lee, E. S., Gu, L., Liu, Q., He, J., Wu, J. W., Kokel, D., Li, H., Hao, Q., Xue, D. & Shi, Y. (2005). Structure of the CED-4-CED-9 complex provides insights into programmed cell death in Caenorhabditis elegans. *Nature* **437**, 831-837.

Yang, M. R., Lee, S. R., Oh, W., Lee, E. W., Yeh, J. Y., Nah, J. J., Joo, Y. S., Shin, J., Lee, H. W., Pyo, S. & Song, J. (2007). West Nile virus capsid protein induces p53-mediated apoptosis via the sequestration of HDM2 to the nucleolus. *Cell Microbiol.*

Yang, Y. L., Reis, L. F., Pavlovic, J., Aguzzi, A., Schafer, R., Kumar, A., Williams, B. R., Aguet, M. & Weissmann, C. (1995). Deficient signaling in mice devoid of double-stranded RNA-dependent protein kinase. *EMBO J* **14**, 6095-6106.

Yeh, W. C., Pompa, J. L., McCurrach, M. E., Shu, H. B., Elia, A. J., Shahinian, A., Ng, M., Wakeham, A., Khoo, W., Mitchell, K., El-Deiry, W. S., Lowe, S. W., Goeddel, D. V. & Mak, T. W. (1998).FADD: essential for embryo development and signaling from some, but not all, inducers of apoptosis. *Science* **279**, 1954-1958.

Yoneyama, M., Kikuchi, M., Matsumoto, K., Imaizumi, T., Miyagishi, M., Taira, K., Foy, E., Loo, Y. M., Gale, M., Jr., Akira, S., Yonehara, S., Kato, A. & Fujita, T. (2005). Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *J Immunol* **175**, 2851-2858.

Yoneyama, M., Kikuchi, M., Natsukawa, T., Shinobu, N., Imaizumi, T., Miyagishi, M., Taira, K., Akira, S. & Fujita, T. (2004). The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol* **5**, 730-737.

Yoneyama, M., Suhara, W., Fukuhara, Y., Fukuda, M., Nishida, E. & Fujita, T. (1998). Direct triggering of the type I interferon system by virus infection: activation of a transcription factor complex containing IRF-3 and CBP/p300. *EMBO J* **17**, 1087-1095.

Yoshida, H. (2007). Unconventional splicing of XBP-1 mRNA in the unfolded protein response. *Antioxid Redox Signal* **9**, 2323-2333.

Yoshida, H., Matsui, T., Yamamoto, A., Okada, T. & Mori, K. (2001).XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell* **107**, 881-891.

Yoshida, H., Oku, M., Suzuki, M. & Mori, K. (2006).pXBP1(U) encoded in XBP1 pre-mRNA negatively regulates unfolded protein response activator pXBP1(S) in mammalian ER stress response. *J Cell Biol* **172**, 565-575.

Yount, J. S., Gitlin, L., Moran, T. M. & Lopez, C. B. (2008).MDA5 Participates in the Detection of Paramyxovirus Infection and Is Essential for the Early Activation of Dendritic Cells in Response to Sendai Virus Defective Interfering Particles. *J Immunol* **180**, 4910-4918.

Yuan, J., Shaham, S., Ledoux, S., Ellis, H. M. & Horvitz, H. R. (1993). The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. *Cell* **75**, 641-652.

Yuste, V. J., Moubarak, R. S., Delettre, C., Bras, M., Sancho, P., Robert, N., d'Alayer, J. & Susin, S. A. (2005). Cysteine protease inhibition prevents mitochondrial apoptosis-inducing factor (AIF) release. *Cell Death Differ* **12**, 1445-1448.

Zamanian-Daryoush, M., Der, S. D. & Williams, B. R. (1999). Cell cycle regulation of the double stranded RNA activated protein kinase, PKR. *Oncogene* **18**, 315-326.

Zeviani, M. & Di, D. S. (2004). Mitochondrial disorders. *Brain* **127**, 2153-2172.

Zhang, J., Alter, N., Reed, J. C., Borner, C., Obeid, L. M. & Hannun, Y. A. (1996). Bcl-2 interrupts the ceramide-mediated pathway of cell death. *Proc Natl Acad Sci U S A* **93**, 5325-5328.

Zhang, P., Jacobs, B. L. & Samuel, C. E. (2008). Loss of protein kinase PKR expression in human HeLa cells complements the vaccinia virus E3L deletion mutant phenotype by restoration of viral protein synthesis. *J Virol* **82**, 840-848.

Zhang, P. & Samuel, C. E. (2007). Protein kinase PKR plays a stimulus- and virus-dependent role in apoptotic death and virus multiplication in human cells. *J Virol* **81**, 8192-8200.

Zhang, W., Li, H., Cheng, G., Hu, S., Li, Z. & Bi, D. (2007). Avian influenza virus infection induces differential expression of genes in chicken kidney. *Res Vet Sci.*

Zhirnov, O. P. & Klenk, H. D. (2007). Control of apoptosis in influenza virus-infected cells by up-regulation of Akt and p53 signaling. *Apoptosis* **12**, 1419-1432.

Zhou, A., Paranjape, J., Brown, T. L., Nie, H., Naik, S., Dong, B., Chang, A., Trapp, B., Fairchild, R., Colmenares, C. & Silverman, R. H. (1997a). Interferon action and apoptosis are defective in mice devoid of 2',5'-oligoadenylate-dependent RNase L. *EMBO J* **16**, 6355-6363.

Zhou, Q., Snipas, S., Orth, K., Muzio, M., Dixit, V. M. & Salvesen, G. S. (1997b). Target protease specificity of the viral serpin CrmA. Analysis of five caspases. *J Biol Chem* **272**, 7797-7800.

Zou, H., Henzel, W. J., Liu, X., Lutschg, A. & Wang, X. (1997). Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell* **90**, 405-413.

Zou, H., Li, Y., Liu, X. & Wang, X. (1999). An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. *J Biol Chem* **274**, 11549-11556.