RNA VIRUS MODULATION OF IFN, PI3K AND APOPTOSIS

Marian J. Killip

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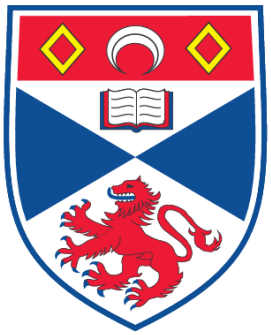
University of St. Andrews



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RNA virus modulation of IFN, PI3K and apoptosis

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ABSTRACT

Interferon (IFN) and phosphatidylinositol 3-kinase (PI3K) are apoptosis regulators that are targeted by viruses to promote survival of infected cells. Significant crosstalk exists between IFN and PI3K, and this study sought to investigate the relationships between IFN, PI3K and apoptosis during virus infection. Parainfluenza virus 5 (PIV5) and influenza A virus (IAV) are both negative-sense single-stranded RNA viruses that encode multifunctional proteins in order to maximise their genome coding capacity. The PIV5 V and IAV NS1 proteins are well-studied as IFN antagonists and, in addition, both are reported to modulate PI3K signalling. Less well-studied is the role of these proteins in apoptosis regulation; the ability of V and NS1 to inhibit apoptosis was therefore investigated. PIV5/V was found to limit cell death in response to a number of apoptosis inducers in a manner that required its STAT1-degradative activity and also inhibited activation of the PI3K downstream target, Akt. IAV/NS1 binds directly to PI3K to stimulate its activity, and this is reported to mediate anti-apoptotic signalling during IAV infection. However, a virus expressing an NS1 unable to bind PI3K did not induce more apoptosis than wt virus. NS1 expression, either in a stable cell-line or during virus infection, was also unable to protect cells from pro-apoptotic stimuli. NS1-mediated PI3K activation similarly had no effect on IFN production or ISG expression in infected cells. In contrast, other NS1 mutant viruses induced large amounts of apoptosis. These viruses also induced significant levels of IFN and were unable to cause apoptosis in IFN-deficient cells, indicating that NS1 limits apoptosis induction through its IFN antagonist functions. The implications of this work for anti-cancer and anti-viral therapies are discussed.

DECLARATIONS

I, **Marian J. Killip**, hereby certify that this thesis (which is approximately 51,000 words in length, excluding bibliography) has been written by me, that it is the record of work carried out by me, and that it has not been submitted in any previous application for a higher degree.

Date ……..... Signature of candidate …….....

I was admitted as a research student in September 2005 as a candidate for the degree of **Doctor of Philosophy (PhD) in Molecular Virology**; the higher study for which this is a record and was carried out at the University of St. Andrews between 2005 and 2009.

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I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of Doctor of Philosophy at the University of St. Andrews, and that the candidate is qualified to submit this thesis in application for that degree.

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Prof. R.E. Randall

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Prof. R.E. Randall

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ABBREVIATIONS

%	Percentage
°C	Degrees celsius
4E-BP	Eukaryotic initiation factor 4E-binding protein
Ab	Antibody
Abs	Absorbance
ADP	Adenosine diphosphate
AdV	Adenovirus
Akt	Protein kinase B (PKB)
AP	Alkaline phosphatase
AP-1	Activator protein 1
APAF	Apoptotic protease activating factor
ASFV	African swine fever virus
ASK	Apoptosis signal-regulating kinase
ATP	Adenosine triphosphate
Bcl-2	B cell lymphoma 2
BDV	Borna disease virus
BH	Bcl-2 homology
BH	B-cell receptor homology
BHV	Bovine herpesvirus
BIR	baculoviral IAP repeat
bNIP	BCL2/adenovirus E1B 19 kDa protein-interacting protein
BSA	Bovine serum albumin
BVDV	Bovine viral diarrhoea virus
Ca2+	Calcium ion
CAD	Caspase-activated DNase
CaMK	Calcium/calmodulin-dependent protein kinase
CARD	Caspase recruitment domain
CASP8AP	Caspase-8 associated protein
CBP	CREB-binding protein
CDK	Cyclin-dependent kinase
CED	Cell death protein
cFLIP	Cellular FLICE (caspase-8)-inhibitory protein
$cFLIP_S$	cFLIP (short splice variant)
$cFLIP_L$	cFLIP (long splice variant)
CHX	Cycloheximide
cIAP	Cellular inhibitor of apoptosis
CPE	Cytopathic effect
CpG	Cytosine followed by guanine in a DNA sequence
CPSF	Cleavage and polyadenylation factor
CREB	cAMP-responsive-element-binding protein
CrkL	Crk-like
CSFV	Classical swine fever virus
CTL	Cytotoxic T-lymphocyte
CXCL	C-X-C motif chemokine
DAK	Dihydroxyacetone kinase
DAPI	4', 6-diamidino-2-phenylindole
dATP	deoxyadenosine triphosphate
DAXX	Death domain associated protein
DC	Dendritic cell
DD	Death domain
DDB1	Damage-specific DNA-binding protein
DDX3	DEAD box protein 3, X-chromosomal

DED	Death effector domain
DISC	Death-inducing signalling complex
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	2' deoxyribonucleic acid
dpi	Days post-infection
DR	Death receptor
DRAF	dsRNA-activated factor
ds	Double-stranded
DTT	Dithiothreitol
EBOV	Ebola virus
EBV	Epstein-Barr virus
ECACC	European Collection of Cell Cultures
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
eIF	Eukaryotic initiation factor
EDTA	Ethylene diamine tetra-acetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbant assay
EMCV	Encephalomyocarditis virus
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
F	Fusion
FADD	Fas-associated death domain-containing protein
FasL	Fas ligand
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FOXO	Forkhead box protein O
g	Gram
GAF	Gamma-activated factor
GAS	Gamma-activated sequence
GEF	Guanine-nucleotide-exchange factor
GM-SCF	Granulocyte-macrophage colony stimulating factor
GSK	Glycogen synthase kinase
GTP	Guanosine triphosphate
HA	Haemagglutinin
HAT	Histone acetyltransferases
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HDAC	Histone deacetylases
HeV	Hendra virus
HHV	Human herpesvirus
HIV	Human immunodeficiency virus
HMG	High mobility group
HN	Haemagglutinin-neuraminidase
hpi	Hours post-infection
hPIV3	Parainfluenza virus 3
HPV	Human papillomavirus
HRP	Horseradish peroxidase
HSV	Herpes simplex virus
HTLV	Human T-cell leukaemia virus
HTNV	Hantavirus
IAV	Influenza A virus
ICAD	Inhibitor of CAD
IFN	Interferon

IFNAR	IFN-α/β receptor
IFNGR	IFN-γ receptor
IGF	Insulin-like growth factor
IgG	Immunoglobulin G
IkB	Inhibitor of NF-kB
IKK	IkB kinase
IL	Interleukin
IP3	Inositol 1,4,5-trisphosphate
IRAK	IL-1 receptor associated kinases
IRF	IFN regulatory factor
IRS	Insulin receptor substrate
ISG	IFN-stimulated gene
ISGF3	ISG factor 3
ISRE	IFN-stimulated response element
JAK	Janus kinase
JEV	Japanese encephalitis virus
JH1	JAK homology 1
JNK	c-Jun N-terminal kinase
kDa	Kilodalton
LCMV	Lymphocytic choriomeningitis virus
LGP2	Protein D11Lgp2 homolog
LGTV	Langat virus
LPS	Lipopolysaccharide
M	Molar
MAPK	MAP kinase
MAPKKK	MAPK kinase kinase
MPRV	Mapuera virus
MAVS	Mitochondrial antiviral signalling protein
MEK	MAPK/ERK kinase
MeV	Measles virus
Mcl	Induced myeloid leukaemia cell differentiation protein
MCM	Minichromosome maintenance protein
mda-5	melanoma differentiation-associated gene-*5*
Mdm2	p53-binding protein Mdm2
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
MPyV	Murine polyoma virus
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
mTORC	mTOR complex
MuV	Mumps virus
MyD88	Myeloid differentiation primary-response protein 88
NA	Neuraminidase
NAT	N-acetyl trypsin
NB	Nuclear body
NDV	Newcastle disease virus
NEMO	NF-kappa-B essential modulator; IKKγ
NEP	Nuclear export protein
NES	Nuclear export signal
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIK	NF-kB-inducing kinase
NiV	Nipah virus
NK	Natural killer
NLRX1	NLR family member X1
NLS	Nuclear localisation signal

NP	Nucleocapsid protein
NPro	N-terminal autoprotease
NS	Non-structural protein
OAS	2'-5'-oligoadenylate synthase
ORF	Open reading frame
P	Phosphoprotein
p53	Cellular tumour antigen p53
PABP	poly(A) binding protein
PACT	PKR activator
PARP	Poly(ADP) ribose polymerase
PBS	Phosphate-buffered saline
pDC	Plasmacytoid dendritic cell
PDK	phosphoinositide-dependent kinase
PFU	Plaque-forming units
pH	$-\log_{10}[H^+]$
PH	Pleckstrin homology
PI	Propidium iodide
PI3K	Phosphotidylinositol 3-kinase
PIAS	Protein inhibitors of activated STAT
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PIV5	Parainfluenza virus 5
PKB	Protein kinase B; Akt
PKC	Protein kinase C
PKR	Interferon-induced, double-stranded RNA-activated protein kinase
PML	Promyelocytic leukaemia
poly(I:C)	Polyinosinic: polycytidylic acid
PP2A	Protein phosphatase 2A
PR8	IAV strain A/Puerto Rico/8/34
pRB	Retinoblastoma-like protein
PRMT	Protein arginine methyl-transferase
PTEN	Phosphatase and tensin homologue deleted on chromosome ten
PTK	Protein tyrosine kinase
PVDF	Polyvinylidene difluoride
RABV	Rabies virus
RBD	RNA-binding domain
RIG-I	Retinoic acid-inducible gene product I
RIP	Receptor-interacting protein
RLU	Relative light units
RNA	Ribonucleic acid
RNF	RING finger protein
RPV	Rinderpest virus
RSV	Respiratory syncytial virus
RT-PCR	Reverse transcriptase-polymerase chain reaction
RZF	Ring zinc finger
S6K	Ribosomal protein S6 kinase
SARS-CoV	Severe acute respiratory syndrome coronavirus
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
siRNA	Small inhibitory RNA
SeV	Sendai virus
SFFV	Spleen focus forming virus
SGK	Serum- and glucocorticoid-inducible kinase
SH	Small hydrophobic
SH2	Src-homology 2
SH3	Src-homology 3
SHIP	SH2 domain-containing inositol 5'-phosphatases

SHP	SH2 domain-containing protein tyrosine phosphatases
SLIM	STAT1-interacting LIM protein
SOCS	Suppressor of cytokine signalling
ss	Single-stranded
STAT	Signal transducer and activator of transcription
STING	stimulator of interferon genes
STS	Staurosporine
SUMO	Small ubiquitin-related modifier
SV	Simian virus
TAB	TAK-1 binding proteins
TAK	Transforming growth factor β-associated kinase
TANK	TRAF-associated NF-kB activator
TBK	TANK-binding kinase
Th	Helper T cell
THD	TNF homology domain
THOV	Thogoto virus
TIR	Toll/Interleukin-1resistance
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFR	TNF receptor
TRADD	TNFR-associated death domain protein
TRAF	TNF receptor-associated factor
TRAIL	TNF-related apoptosis inducing ligand
TRIF	TIR domain-containing adaptor inducing IFN-β
TRIM	Tripartite motif-containing protein
Tris	Tris-hydroxymethyl-aminomethane
Tyk	Tyrosine protein kinase
UBP	Ubiquitin carboxyl-terminal hydrolase
Ud	IAV strain A/Udorn/72
UV	Ultraviolet
V	Volts
V5	Epitope tag derived from a small 14-amino acid region of PIV5 V
v/v	Volume per volume
VACV	Vaccinia virus
VPg	Picornavirus 3B protein
VSV	Vesicular stomatitis virus
w/v	Weight per volume
WSN	Influenza A virus strain A/WSN/33
wt	Wildtype
XIAP	X-linked inhibitor of apoptosis
ZBP	Z-DNA-binding protein

1 INTRODUCTION

Limiting virus replication and spread is of major importance to the host cell. Prior to activation of the adaptive immune response, innate antiviral mechanisms can severely impair virus replication and are often sufficient to clear a viral infection. Virus adsorption and entry to the host cell can be prevented by circulating neutralising antibodies which bind to the viral capsid or envelope. These include 'natural' or spontaneous antibodies present in the sera of non-immunised humans which significantly reduce viral titre in peripheral organs (Ochsenbein et al., 1999, Ochsenbein and Zinkernagel, 2000, Gobet et al., 1988). In addition, the complement cascade can result in the direct coating of virions with complement components, thereby preventing virus entry and promoting phagocytosis or even lysis of the virus (Lachmann and Davies, 1997). If these first defenses fail (or the virus is able to overcome them) and the virus enters the cell, a whole host of intracellular and humoral pathways are activated that restrict virus replication. Detection of viral components by the cell rapidly stimulates the interferon (IFN) response and induction of apoptosis; viral replication is subsequently inhibited and virus-infected cells are effectively cleared by phagocytic immune cells, limiting virus spread. Both IFN and apoptosis are so efficient at hampering virus infections that many (if not all) viruses express viral inhibitors of these responses. In the following sections, induction of IFN in response to infection, IFN signal transduction and the establishment of the IFN-induced antiviral state will be examined. The role of apoptosis as an essential arm of the antiviral response and apoptosis regulation by IFN and phosphatidylinositol 3-kinase (PI3K) will be discussed. Finally the mechanisms by which viruses (in particular parainfluenza virus 5 [PIV5] and influenza A virus [IAV]) inhibit IFN and apoptosis are described.

1.1 The IFN response

The IFN family of cytokines are secreted in response to virus infection and elicit an 'antiviral state' in cells by upregulating expression of many genes, including those involved in inhibiting protein synthesis, inhibiting cell growth and promoting apoptosis. As a result, the host cell becomes a highly unfavourable environment for virus replication. Three groups of IFNs exist. In humans, type I IFNs are a large group of molecules that are further split into five classes: IFN-α (of which there are twelve different subtypes), IFN-β, and the more recently characterised IFN-ε, IFN-κ and IFN-ω. Type II IFNs comprise IFN-γ only, and type III IFNs include IFN-λ1 (IL-

29), IFN-λ2 (IL-28A) and IFN-λ3 (IL-28B). Virus infection induces expression of IFN-α, IFN-β and IFN-ω, with the ratio and absolute levels of production of these classes varying with cell type and viral stimuli (Pestka et al., 2004), while IFN-γ is produced by activated T-lymphocytes and natural killer (NK) cells.

1.1.1 Induction of the IFN-β gene

Given the huge variety in virus modes of entry and replication, there exist a number of different ways by which the host cell can detect a virus infection. IFN can be induced in response to a number of viral stimuli, including viral proteins and nucleic acids. Production of IFN-β in fibroblasts following virus infection or treatment with synthetic double-stranded RNA (dsRNA) has been intensively studied. This is considered to be the best model of IFN-induction and will be described here. The different virus detection pathways co-ordinate assembly of several transcription factors at the IFN-β promoter to stimulate IFN-β transcription; IFN-β induction therefore occurs without the need for *de novo* protein synthesis. The minimal events required for IFN-β transcription are activation of the IFN regulatory factor 3 (IRF3) and nuclear factor kappa B (NF-kB) transcription factors, recognition sites for which exist in the IFN-β promoter. Latent IRF3 exists in the cytoplasm as a monomer but dimerises and translocates to the nucleus upon its activation by virus infection (Lin et al., 1998b, Dragan et al., 2007). Similarly, inactive NF-kB is retained in the cytoplasm by its inhibitor, inhibitor of NF-kB (IkB). Following the appropriate signal, IkB becomes phosphorylated by IKK (IkB kinase), leading to its ubiquitination and subsequent degradation. Removal of IkB-mediated inhibition allows NF-kB to translocate to the nucleus and regulate transcription from NF-kB-responsive promoters. An additional transcription factor, activator protein 1 (AP-1), is required for maximal induction of the IFN-β gene (Wathelet et al., 1998). IRF3, NF-kB and AP-1, in the presence of high mobility group (HMG1) proteins, together form the 'enhanceosome' (Thanos and Maniatis, 1995). The enhanceosome recruits the co-activator cAMP-responsive-element binding protein (CREB)-binding protein (CBP)/p300 to initiate IFN-β transcription (Merika et al., 1998).

Type I IFNs have been divided into two classes ('primary' and 'secondary') according to their temporal expression during virus infection. As detailed above, the IFN-β gene is a primary IFN gene that requires no new protein synthesis for its expression in response to viral stimuli. IFN-β stimulates expression of IRF7, a transcription factor that is essential for transcription of secondary IFN-α genes, in the continual presence

of viral stimuli (Sato et al., 1998, Marie et al., 1998). In most cell-types, IRF7 is expressed at very low or undetectable levels; as a result, in these cells, IFN-β is required for induction of IRF7 and IFN-α (Erlandsson et al., 1998, Yeow et al., 2000). IRF7 can additionally bind to the IFN-β promoter to upregulate transcription in a positive feedback mechanism (Wathelet et al., 1998, Panne et al., 2007). In plasmocytoid dendritic cells (pDCs) however, massive amounts of IFN-α are produced early in virus infection: this is due to constitutive IRF7 expression in these cells (Prakash et al., 2005).

1.1.2 Detection of virus infection by the host cell

1.1.2.1 The Toll-like receptors

Toll-like receptors (TLRs) are membrane-bound glycoproteins: they comprise extracellular leucine-rich repeats that mediate recognition of pathogen-associated molecular patterns (PAMPs) and an intracellular TIR (Toll/Interleukin-1 resistance) domain that mediates recruitment of downstream signalling proteins (Akira and Takeda, 2004). Binding of PAMPs to the TLR promotes receptor dimerisation and subsequent changes in receptor conformation. This permits the TIR domain to recruit adaptor proteins (the nature of which varies with the TLR) and initiate downstream signalling.

Detection of extracellular or endosomal viral dsRNA

dsRNA, produced during replication of positive-stranded RNA viruses and DNA viruses (Weber et al., 2006), is exceptionally efficient at inducing IFN (Marcus, 1983). Cells can rapidly respond to extracellular dsRNA (present in the culture medium) or dsRNA internalised by the cell via the endosomal pathway. The cellular dsRNA receptor responsible for this was identified as TLR3 (Alexopoulou et al., 2001), which is essential for defence against certain viruses (Tabeta et al., 2004, Zhang et al., 2007). TLR3 is expressed in endosomes (and also at the cell surface in fibroblasts) (Matsumoto et al., 2003, Johnsen et al., 2006); presence of TLR3 in these compartments allows detection of extracellular viral dsRNA or endosomal dsRNA (formed following virus uncoating) without the requirement for virus replication.

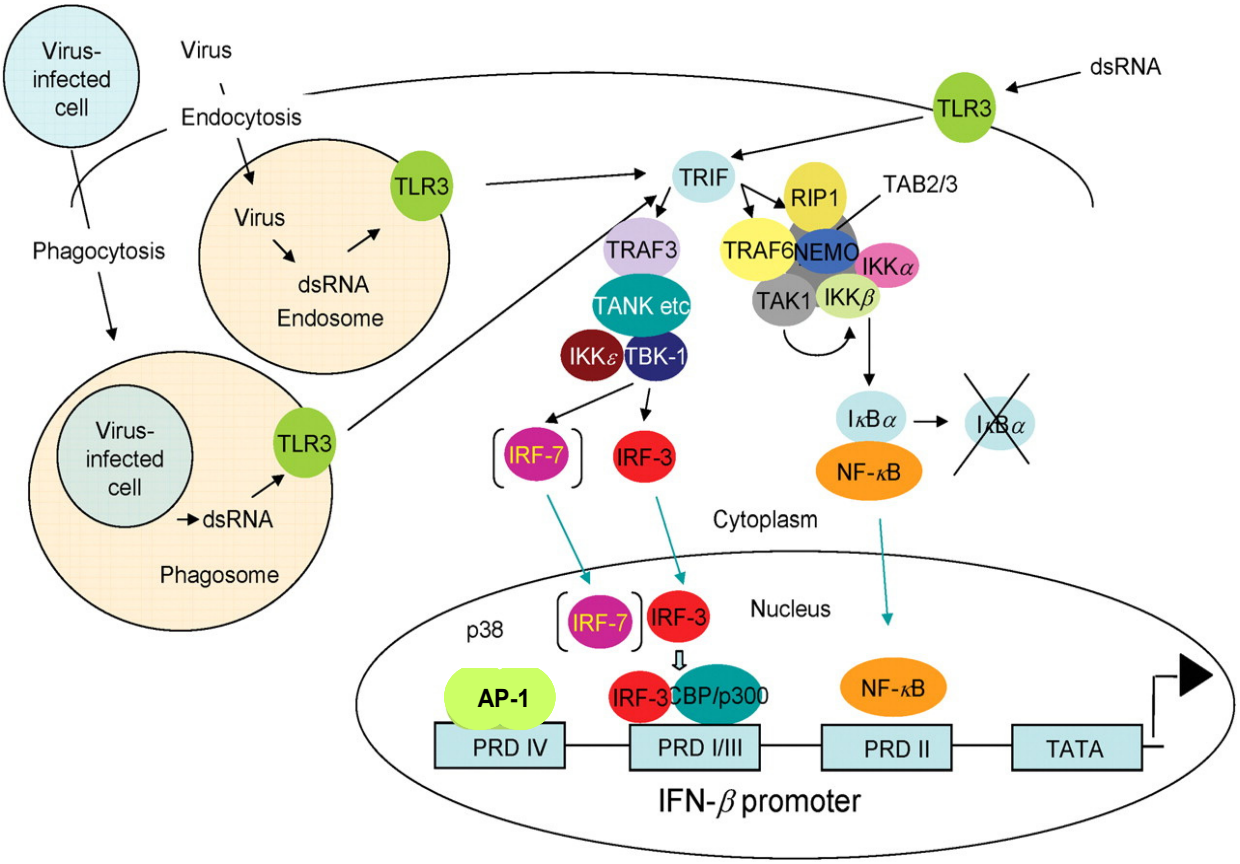
Downstream of TLR3 stimulation, NF-kB and IRF3 activation occurs via the TRIF (TIR domain-containing adaptor inducing IFN-β) adaptor protein and members of the

TNF receptor-associated factor (TRAF) family **(Figure 1.1A**, Yamamoto et al., 2002, Yamamoto et al., 2003, Hoebe et al., 2003, Jiang et al., 2004, Hacker et al., 2006). TRIF binds TLR3 and recruits the E3 ligase TRAF6 and the receptor-interacting protein RIP1 (Sato et al., 2003, Cusson-Hermance et al., 2005, Meylan et al., 2004); TRAF6 subsequently catalyses the Lys63 polyubiquitination of itself and RIP1. These polyubiquitin chains mediate association of TRAF6 and RIP1 with the TAK1 binding proteins TAB2 and TAB3 (Kanayama et al., 2004, Chen, 2005, Deng et al., 2000). TAK1 (transforming growth factor β-associated kinase 1) and IKK are recruited to this protein complex through their interactions with TAB2/TAB3 and polyubiquitinated RIP1 respectively (Wang et al., 2001a, Ea et al., 2006, Li et al., 2006a, Wu et al., 2006). IKK is heterotrimeric and comprises the catalytic subunits IKKα and IKKβ, and the regulatory subunit IKKγ/NEMO (DiDonato et al., 1997, Mercurio et al., 1997, Zandi et al., 1998, Rothwarf et al., 1998). The IKK complex is activated through TAK1-dependent phosphorylation of IKKβ (Wang et al., 2001a) which results indirectly in NF-kB activation through inactivation of IkB. TRIF activates IRF3 through its association with TRAF3 (Hacker et al., 2006, Oganesyan et al., 2006). A TRAF3-dependent protein complex forms, comprising the TRAF-associated NF-kB activator, TANK (Li et al., 2002), TANK-binding kinase 1 (TBK1) and IKKε (Sharma et al., 2003, Fitzgerald et al., 2003, Hemmi et al., 2004). TBK1 and IKKε catalyse IRF3 phosphorylation to promote its nuclear translocation, and in this respect these two kinases appear to be functionally redundant (Hemmi et al., 2004). In addition to their effects on IRF3, TBK-1 and IKKε can also phosphorylate and activate IRF7 (Sharma et al., 2003).

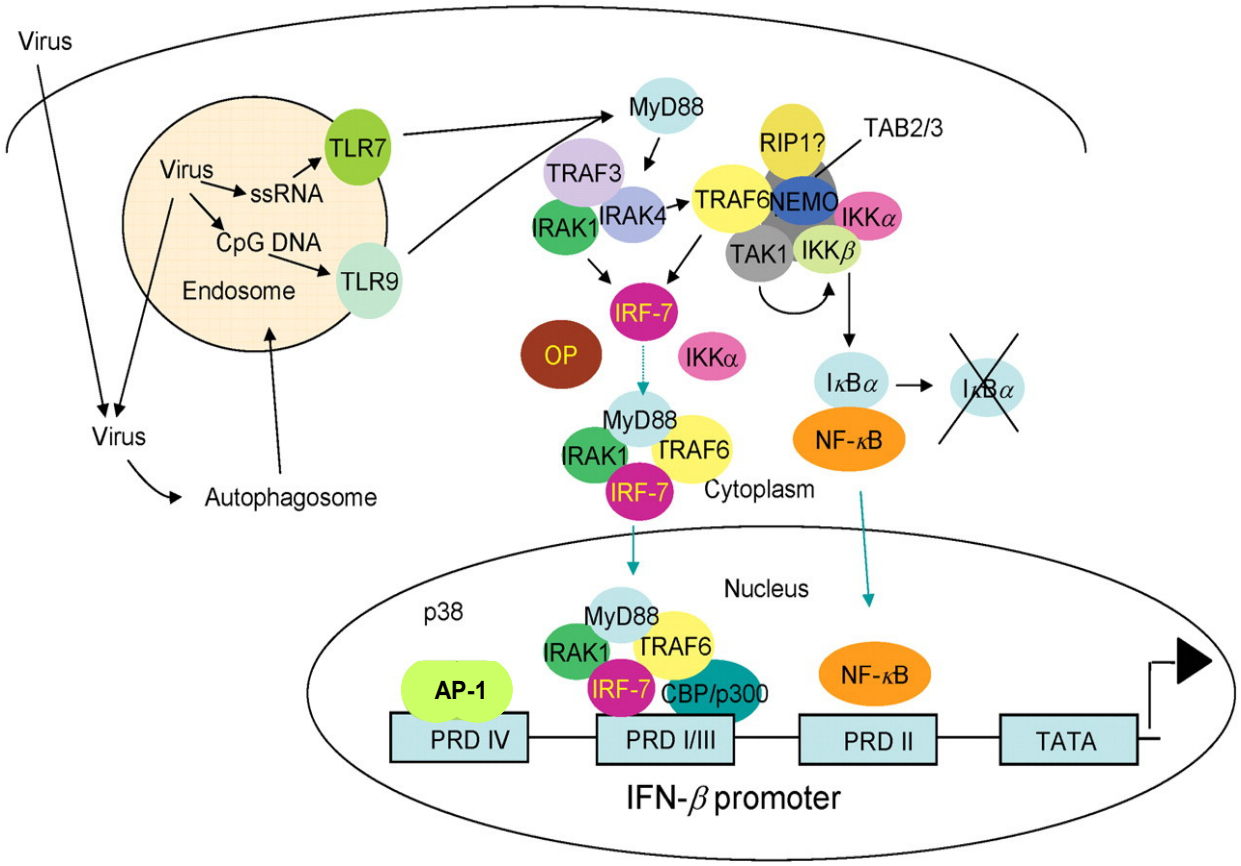
Detection of endosomal viral ssRNA and DNA

pDCs secrete large amounts of IFN in response to viral nucleic acids (Colonna et al., 2004), but they do not express TLR3 (Kadowaki et al., 2001). They do, however, express an abundance of TLR7 and TLR9 in their endosomal compartments (Diebold et al., 2004, Kadowaki et al., 2001, Jarrossay et al., 2001). Studies involving virus-derived or synthetic single-stranded RNA (ssRNA) have revealed that TLR7 is required for ssRNA-induced IFN production (Heil et al., 2004, Diebold et al., 2004) and for recognition of ssRNA viruses such as IAV and vesicular stomatitis virus (VSV) (Lund et al., 2004). In contrast, TLR9 recognises unmethylated CpG motifs present in viral DNA (Hemmi et al., 2000, Rutz et al., 2004, Abe et al., 2005) and is required for recognition of DNA viruses including herpes simplex virus-1 (HSV-1) and human cytomegalovirus (HCMV) (Krug et al., 2004a, Krug et al., 2004b).

(A)


Virus-infected cell
Virus
Endocytosis
dsRNA
TLR3
Phagocytosis
Virus
dsRNA Endosome
TLR3
TRIF
RIP1
TAB2/3
TRAF3
TRAF6
NEMO
IKKα
TANK etc
TAK1
IKKβ
IKKε
TBK-1
Virus-infected cell
TLR3
dsRNA
Phagosome
IRF-7
IRF-3
IκBα
NF-κB
Cytoplasm
p38
IRF-7
IRF-3
Nucleus
AP-1
IRF-3
CBP/p300
NF-κB
PRD IV
PRD I/III
PRD II
TATA
IFN-β promoter

(B)


Virus
Virus
ssRNA
CpG DNA
TLR7
TLR9
Endosome
MyD88
RIP1?
TAB2/3
TRAF3
IRAK1
IRAK4
TRAF6
NEMO
IKKα
TAK1
IKKβ
IRF-7
OP
IKKα
IκBα
NF-κB
Virus
Autophagosome
MyD88
IRAK1
TRAF6
IRF-7
Cytoplasm
Nucleus
p38
MyD88
IRAK1
TRAF6
IRF-7
CBP/p300
AP-1
NF-κB
PRD IV
PRD I/III
PRD II
TATA
IFN-β promoter

(C)

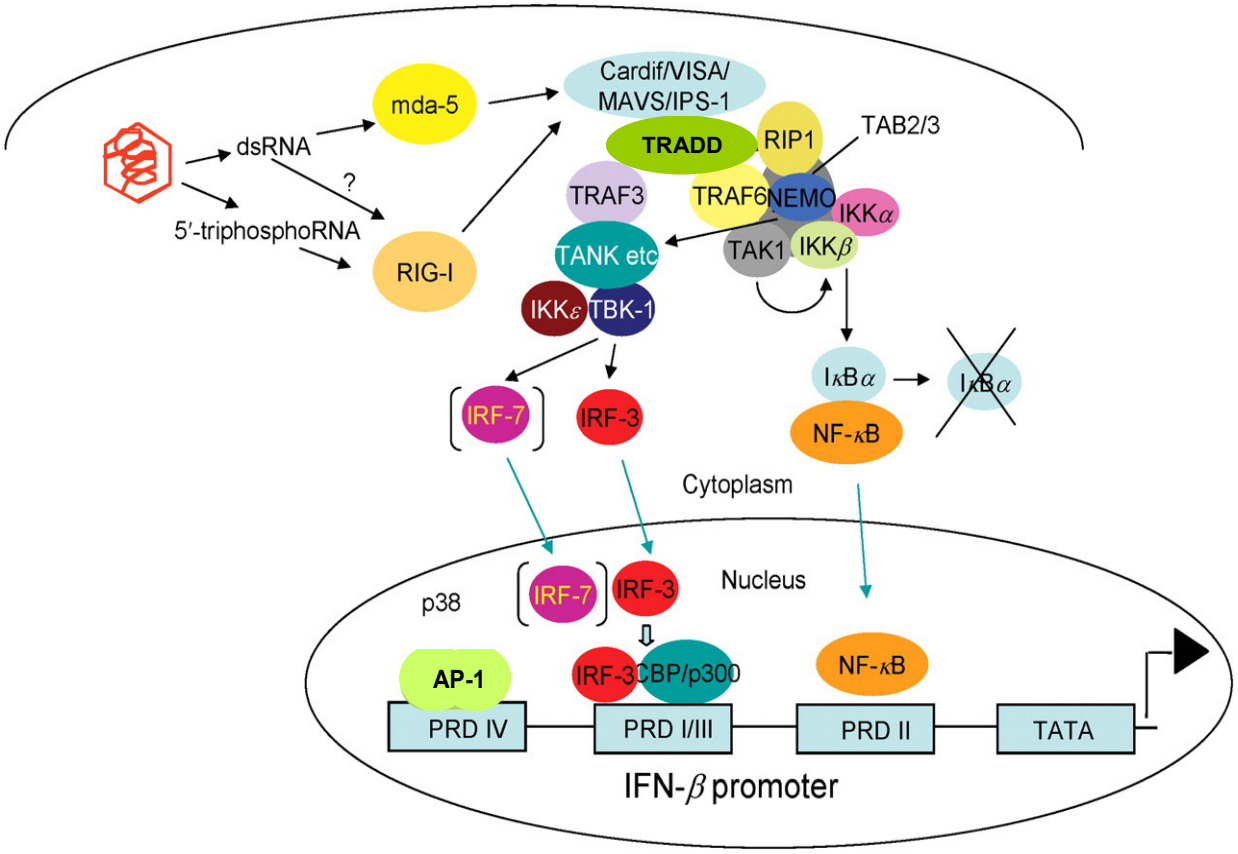


Figure 1.1: Induction of IFN-β by viral nucleic acid.

Pathways of TLR3 (**A**), TLR 7 and 9 (**B**), and RIG-I/mda-5 (**C**) signalling in response to viral nucleic acids are shown. Downstream signalling from these receptors requires TRIF, MAVS or MAVS adaptor proteins respectively. TRAFs bind these adaptors to orchestrate the assembly of multi-protein complexes that activate the IRF3 (or IRF7) and NF-kB transcription factors. Once activated, IRFs and NF-kB translocate to the nucleus where they stimulate transcription of the IFN-β gene. *Modified from Randall and Goodbourn, 2008.*

Observations that TLR7 and TLR9 are absent from the pDC cell surface (Kawai and Akira, 2006) and that intact endocytic pathways are required for TLR7-mediated IFN-induction (Lund et al., 2004, Lund et al., 2003) indicate that virus internalisation is required before viral nucleic acid can be detected by TLR7/9. Viral genomes possess characteristics, for example the CpG motifs recognised by TLR9, that distinguish them from cellular nucleic acids. Even so, host RNA and DNA have been reported to activate TLRs (Diebold et al., 2004, Kariko et al., 2004). Consequently, it is thought that nucleoside modifications and/or subcellular localisation play a critical role in distinguishing host and viral nucleic acids (Kariko et al., 2005, Crozat and Beutler, 2004, Ishii and Akira, 2005).

TLR7 and TLR9 activate downstream signalling through the adaptor myeloid differentiation primary-response protein 88 (MyD88; **Figure 1.1B**) (Krug et al., 2004b, Lund et al., 2004). MyD88 recruits a protein complex consisting of IRAK1 and IRAK4 (IL-1 receptor-associated kinases) and TRAF6 to the TLR. TRAF6 mediates activation of IKK by a TAB2/3 and TAK1-dependent mechanism similar to signalling downstream of TLR3. In pDCs, IRF7 is the predominant IRF activated early in infection (Prakash et al., 2005); IRF7, not IRF3, is activated by TLR7 and TLR9 in these cells. The MyD88/IRAK1/IRAK4/TRAF6 complex directly binds to IRF7 (Honda et al., 2004, Uematsu et al., 2005) through a process that may also be dependent on ubiquitinated RIP1 (Huye et al., 2007). IRF7 becomes ubiquitinated by the E3 ligase activity of TRAF6 (Kawai et al., 2004); subsequent phosphorylation by IRAK1 allows IRF7 to translocate to the nucleus to stimulate transcription (Uematsu et al., 2005).

1.1.2.2 Cytoplasmic nucleic acid receptors

Detection of cytoplasmic RNA

TLR3 appears to play a dispensable role in the antiviral response to certain dsRNA, ssRNA and DNA viruses (Edelmann et al., 2004) and mice lacking either TLR9 or MyD88 are still able to control local HSV-1 infection (Krug et al., 2004b). These data indicate that virus infection can be detected by additional, TLR-independent mechanisms. The IFN-inducible RNA helicases RIG-I (retinoic acid-inducible gene product I) and mda-5 (melanoma differentiation-associated gene-5) have been identified as two such TLR-independent viral nucleic acid detectors (Yoneyama et al., 2004, Andrejeva et al., 2004), and disruption of RIG-I or mda-5 genes prevents virus-induced IFN expression (Yoneyama et al., 2005). These proteins carry a C-terminal

helicase domain with ATPase activity and two caspase-recruitment domains (CARDs) at their N-terminus. Activation of RIG-I and mda-5 by viral nucleic acid induces a conformational change that exposes the CARDs so they can mediate downstream protein-protein interactions (Yoneyama et al., 2004). The CARDs of both proteins bind the mitochondrial antiviral signalling protein, MAVS. MAVS, which is anchored to the outer mitochondrial membrane, acts as an adaptor to recruit signalling molecules that lead to NF-kB and IRF3 activation and resultant IFN-β gene transcription (**Figure 1.1C**, Seth et al., 2005, Kawai et al., 2005, Meylan et al., 2005). NF-kB activation occurs through an association between MAVS and tumour necrosis factor receptor (TNFR1)-associated death domain protein (TRADD). TRADD orchestrates formation of a complex containing TRAF3, TRAF6, TANK, RIP1 and FADD (Balachandran et al., 2004, Kawai et al., 2005, Michallet et al., 2008, Xu et al., 2005). IKKγ/NEMO is recruited to this complex to activate IRF3 by a TBK1/IKKε-dependent pathway and NF-kB through activation of IKK (Zhao et al., 2007). MAVS can also stimulate a non-canonical pathway of NF-kB activation (see later, **Section 1.1.3.4**): the RIG-I:MAVS complex interacts with IKKα and NF-kB-inducing kinase (NIK) to stimulate the processing of p100/ NF-kB2 to p52 (Liu et al., 2008b).

The RIG-I:MAVS interaction requires Lys63-ubiquitination of the RIG-I CARD by the E3 ligase TRIM25; TRIM25 knockout cells are subsequently unable to produce IFN-β in response to Sendai virus (SeV) infection (Gack et al., 2007). The same modification is not required for signalling downstream of mda-5 (Gack et al., 2007), however mda-5 may be subject to negative regulation by dihydroxyacetone kinase (DAK) in quiescent cells (Diao et al., 2007). Signalling from both RIG-I and mda-5 is also negatively regulated by the RIG-I-related IFN-inducible helicase LGP2. This protein lacks the CARDs required for downstream signalling so it was suggested that LGP2 antagonism of IFN-induction occurred through RNA sequestration (Yoneyama et al., 2005, Rothenfusser et al., 2005). However, LGP2-mediated inhibition may be more complex, as LGP2-deficient mice are more sensitive to viruses that activate mda-5 than those that activate RIG-I signalling (Venkataraman et al., 2007). Ubiquitination by the E3 ligase RNF125 and subsequent degradation of RIG-I, mda-5 and MAVS has also been demonstrated, resulting in suppression of IFN-induction in response to dsRNA or SeV infection (Arimoto et al., 2007). RNF125 is itself an interferon-stimulated gene (ISG) product, indicating that this ubiquitination represents a negative feedback regulation loop. MAVS activity is negatively regulated by NLRX1 which co-localises with MAVS at the mitochondrial membrane and prevents its interaction with RIG-I and mda-5 (Moore et al., 2008).

While both RIG-I and mda-5 are activated by synthetic dsRNA [poly(I:C)] (Andrejeva et al., 2004, Yoneyama et al., 2004), their activities are not redundant. Studies using mda-5-knockout mice revealed that mda-5 recognises poly(I:C) while RIG-I detects *in vitro* transcribed dsRNAs (Kato et al., 2006), suggesting RIG-I and mda-5 may recognise different dsRNA structures. It has since been shown that RIG-I recognises viral genomic RNA bearing uncapped 5'-triphosphates (Hornung et al., 2006, Pichlmair et al., 2006). Since cellular RNAs are 5'-capped during post-transcriptional processing, they are not recognised by RIG-I whereas *in vitro* transcribed RNAs do carry a 5'-phosphate, and therefore activate RIG-I signalling (Hornung et al., 2006). In support of their differential roles in viral RNA detection, RIG-I and mda-5 mediate detection of different types of viruses to different extents. For instance, RIG-I, but not mda-5, signalling is activated following infection with paramyxoviruses and IAV while mda-5, but not RIG-I, is required for detection of picornavirus infection (Gitlin et al., 2006, Loo et al., 2008, Pichlmair et al., 2006, Berghall et al., 2006, Kato et al., 2006). Viruses recognised by RIG-I transiently produce uncapped 5'-triphosphorylated mRNAs during their replication (Hornung et al., 2006), while picornavirus infection does not activate RIG-I as the 5' ends of picornavirus RNAs are covalently linked to the VPg protein throughout the virus life cycle (Lee et al., 1977). Infection with reoviruses and flaviviruses however, trigger both RIG-I and mda-5-mediated innate immune responses (Loo et al., 2008), indicating either a degree of overlap in recognised structures or the production of different RNA species during the life cycle of these viruses.

Recently, an additional pathway of NF-kB and IRF3 activation has been identified; this pathway requires stimulator of interferon genes (STING) which localises to the ER membrane and associates with the translocon. STING is required for induction of type I IFN genes in response to the negative-stranded RNA virus VSV and co-precipitates with RIG-I (Ishikawa and Barber, 2008). RIG-I and STING may therefore function to detect viral RNAs that are being translated by the host translational machinery. The signalling molecules downstream of STING that lead to NF-kB and IRF3 activation have yet to be identified.

Detection of cytoplasmic DNA

Evidence is mounting that a cytoplasmic DNA sensor exists to activate IFN expression by a TLR9-independent pathway: direct introduction of dsDNA into the cytoplasm triggers expression of IFN in a TBK-1/IKKε/IRF3-dependent manner (Ishii

et al., 2006, Stetson and Medzhitov, 2006). The IFN-inducible Z-DNA-binding protein 1 (ZBP1) is a candidate for this DNA sensor. ZBP1 binding to dsDNA promotes its dimerisation and enhances its interactions with TBK1 and IRF3 to activate IFN-β transcription (Takaoka et al., 2007, Wang et al., 2008). Additionally, siRNA-mediated knockdown of ZBP1 impairs antiviral responses to the DNA virus HSV-1 but not the RNA virus Newcastle disease virus (NDV) (Takaoka et al., 2007). The downstream signalling molecules involved in the activation of IFN expression by DNA remain to be fully elucidated, but it has been suggested that dsDNA activates antiviral signalling through a RIG-I/MAVS-dependent pathway (Cheng et al., 2007). Indeed, STING (see above) has also been implicated in the recognition of intracellular DNA and DNA virus infection (Ishikawa and Barber, 2008).

1.1.3 Signalling pathways activated by IFN

Cytokines, growth factors and hormones exert their intracellular effects through interaction with, and activation of, membrane-bound receptors. Following binding of the appropriate ligand, these receptors undergo rapid tyrosine phosphorylation. While growth factor receptors contain intrinsic tyrosine kinase activity, most cytokine receptors do not and require receptor-associated protein tyrosine kinases (PTKs) for their phosphorylation. The Janus kinase (JAK) family of PTKs are responsible for downstream signalling from a wide variety of cytokines, including IFNs and interleukins (Burfoot et al., 1997, Johnston et al., 1995, Platanias et al., 1996, Darnell et al., 1994). In quiescent cells, JAKs are associated with cytokine receptors in an enzymatically inactive form. Regions in the N-terminus of JAKs are essential for both receptor association and downstream signalling (Chen et al., 1997, Gauzzi et al., 1997, Frank et al., 1995). Several features of JAKs distinguish them from other classes of PTKs. Firstly, they possess two kinase-like domains at their C-terminus, JAK homology 1 (JH1) and JH2. Only the JH1 domain appears to have functional tyrosine kinase activity, while JH2, which lacks certain residues critical for enzymatic activity, negatively regulates JAK activity and prevents its activation in the absence of ligand stimulation (Luo et al., 1997, Saharinen et al., 2000). Following ligand-binding, oligomerisation of receptors causes local aggregation of JAKs at the intracellular membrane surface, and results in their activation through auto- or trans-phosphorylation (Rane and Reddy, 2000). Active JAKs subsequently phosphorylate the receptors at specific tyrosine residues; these phosphotyrosines recruit downstream Src-homology 2 (SH2) domain-containing signalling molecules, such as the signal transducers and activators of transcription (STATs) and

phosphatidylinositol 3-kinase (PI3K). IFN stimulation can therefore trigger a number of intracellular signalling pathways through its activation of JAKs (reviewed in Takaoka and Yanai, 2006).

1.1.3.1 STAT signalling

The STATs

In humans, the STAT family comprises seven members, STAT1-4, STAT5a, STAT5b and STAT6, which can homo- or heterodimerise and activate transcription in response to cytokine stimulation (reviewed by Schindler and Plumlee, 2008). Each STAT protein contains seven conserved domains, including a DNA-binding domain, a coiled-coil domain that mediates interactions with regulatory proteins, an SH2 domain that mediates receptor association and dimerisation, a tyrosine activation motif and a transcriptional activation domain containing serine phosphorylation sites that facilitate coactivator recruitment. The STATs most critical for IFN signalling are STAT1 and STAT2.

STAT activation by type I IFNs

STATs are the best characterised JAK substrates and JAK/STAT signalling is known as the 'classical' pathway of IFN signal transduction (Du et al., 2007). Activation of classical STAT signalling is depicted in **Figure 1.2**. Type I IFNs (IFN-α and IFN-β) bind to the IFN-α/β receptor (IFNAR), which comprises two subunits, IFNAR1 and IFNAR2 (Novick et al., 1994). Prior to receptor stimulation, the intracellular domains of IFNAR1 and IFNAR2 are physically associated with the cytoplasmic JAKs Tyk2 and JAK1 respectively (Novick et al., 1994, Abramovich et al., 1994, Muller et al., 1993, Colamonici et al., 1994). IFNAR2 additionally associates with STAT2 prior to activation by IFN, and STAT1 is also recruited to IFNAR through a weak interaction with STAT2 (Stancato et al., 1996, Precious et al., 2005a). IFN-α/β binding to IFNAR induces receptor dimerisation and conformational change that results in tyrosine phosphorylation of IFNAR1 (at Tyr466) by Tyk2. This phosphotyrosine enables a strong association between IFNAR1 and STAT2, and STAT2 is subsequently phosphorylated by Tyk2 at Tyr690. STAT2 phosphorylation is required for subsequent STAT1 phosphorylation by JAK1 at Tyr701 (Leung et al., 1995). STAT1 and STAT2 are now able to form stable heterodimers and expose a non-classical nuclear localisation signal (NLS) that promotes their nuclear import (Melen et al.,

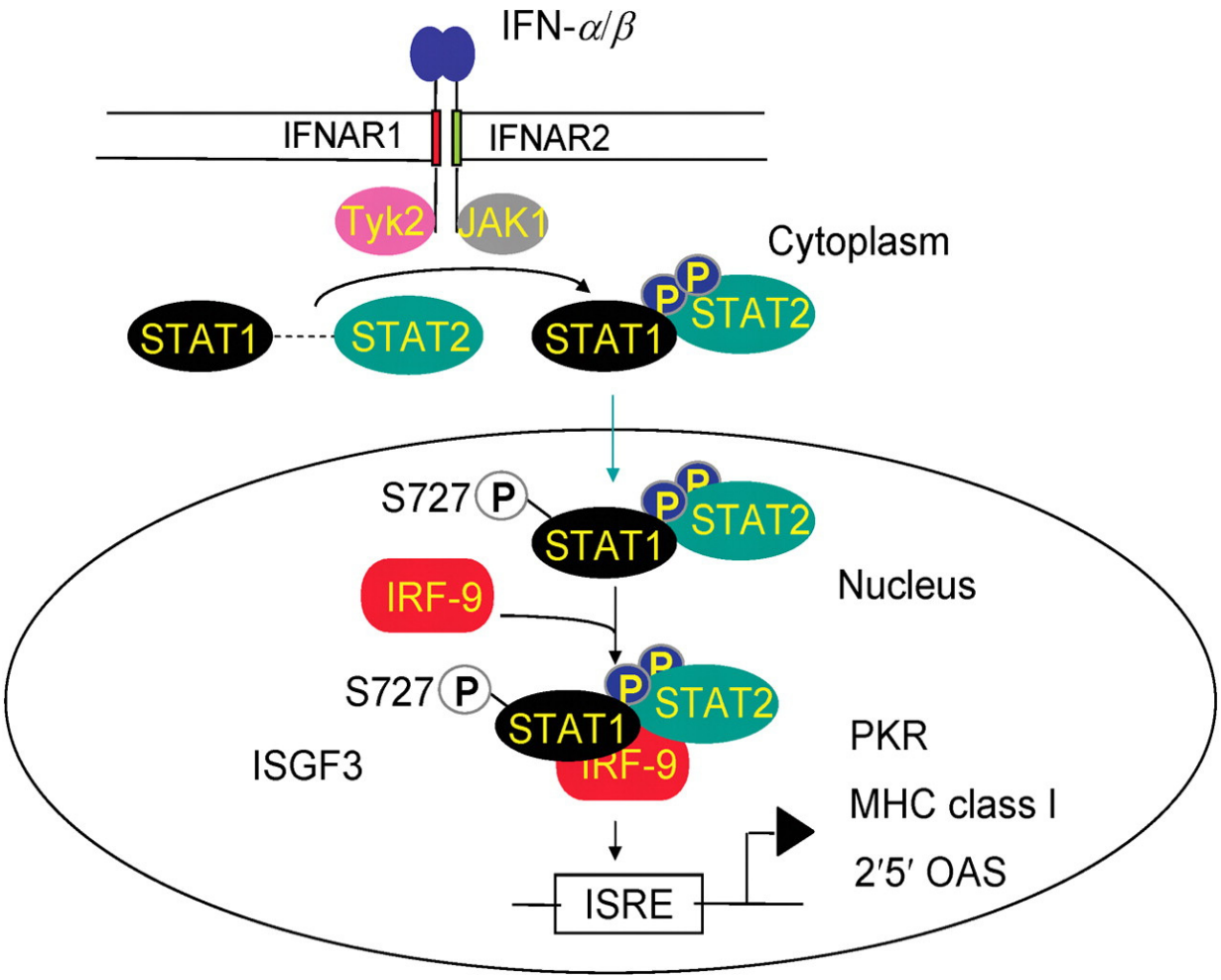


Figure 1.2: Classical JAK/STAT signalling activated by type I IFN.

Binding of IFN-α/β to IFNAR activates the tyrosine kinases JAK1 and Tyk2, which phosphorylate tyrosine residues in STAT1 and STAT2. STAT1 and STAT2 then strongly associate through their SH2 domains and associate with IRF9 to form the ISGF3 transcription factor. In the nucleus, ISGF3 stimulates transcription from ISRE elements in the promoters of IFN-stimulated genes.
From Randall and Goodbourn, 2008.

2003, Fagerlund et al., 2002). STAT1 and STAT2 remain in the nucleus until their dephosphorylation leads to the exposure of nuclear export signals (NESs) and their export to the cytoplasm (McBride et al., 2000, Banninger and Reich, 2004). Through a direct interaction with STAT2, IRF9 is recruited to the STAT1/STAT2 heterodimer to form the interferon-stimulated gene factor 3 (ISGF3) (Martinez-Moczygemba et al., 1997, Veals et al., 1992). Reports that IRF9 can directly bind to IFNAR2 and that STAT2 and IRF9 associate even in the absence of IFN-stimulation indicate that ISGF3 formation may occur at the IFNAR (Tang et al., 2007b, Martinez-Moczygemba et al., 1997). Once in the nucleus, ISGF3 binds to, and activates transcription from, interferon-stimulated response elements (ISREs) that occur upstream of IFN-α/β-responsive genes (Kessler et al., 1990, Levy et al., 1989).

The formation of additional STAT dimers in response to type I IFN, such as STAT1, STAT3, STAT4 or STAT6 homodimers, or STAT1/3, STAT1/4, STAT1/5, STAT2/3 or STAT5/6 heterodimers, has also been described (Darnell, 1997, Stark et al., 1998, Meinke et al., 1996, Platanias, 2005, Farrar et al., 2000, Torpey et al., 2004, Matikainen et al., 1999, Fasler-Kan et al., 1998). These dimers bind to gamma-activated sequence (GAS) elements in the promoters of ISGs to activate their transcription (**Figure 1.3**). Formation of these different STAT dimers allows for differential regulation of ISG subsets. Some ISG promoters contain ISREs, some contain GAS elements and some contain both; as a result, combinations of STAT dimers may be required for the maximal expression of an ISG (Platanias, 2005). Different STAT dimers appear to display divergent biological activities which may account for the wide range of biological activities induced by IFN. For example, during virus infection both *in vitro* and *in vivo*, IFN-α-induced secretion of IFN-γ is negatively regulated by STAT1 but activated by STAT4. (Nguyen et al., 2002b).

STAT activation by type II IFNs

Type II IFN (IFN-γ) induction of JAK/STAT signalling shares many common features with type I IFN stimulation. Like IFNAR, the IFN-γ receptor (IFNGR) is composed of two distinct subunits, IFNGR1 and IFNGR2. Similarly, the cytoplasmic domains of the receptor associate with JAKs: IFNGR1 and IFNGR2 bind to JAK1 and JAK2 respectively (Igarashi et al., 1994). IFN-γ stimulation causes receptor dimerisation which results in the sequential activation of JAK2 and JAK1. The JAKs mediate phosphorylation of IFNGR1 to form two binding sites for STAT1, which subsequently recruit two molecules of STAT1 through their SH2 domains. STAT1 is activated by

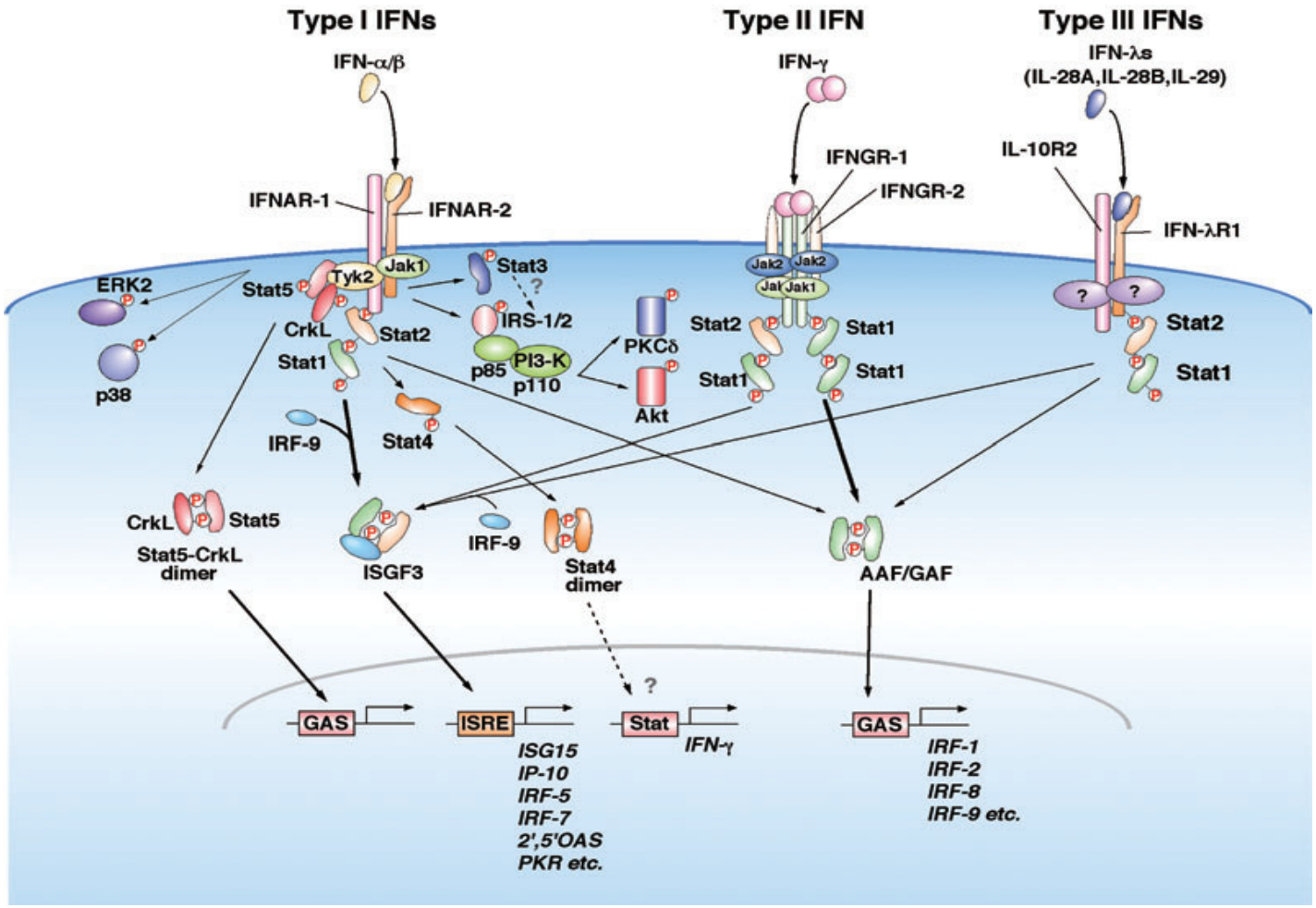


Figure 1.3: Signalling downstream of the IFN receptors.

Signalling pathways activated downstream of the type I IFN (IFN α/β), type II IFN (IFN-γ) and type III IFN (IFN-λ) receptors are shown. All of them activate 'classical' STAT signalling but additional 'non-classical' pathways can also be activated. Stimulation with type I IFN recruits Crk/CrkL to the IFN receptor, where it becomes phosphorylated by Tyk2 and forms a heterodimer with phosphorylated STAT5. STAT5/Crk translocates to the nucleus to stimulate GAS-dependent gene transcription. Additinal STAT dimers can also form; for example, STAT4 homodimers are induced by certain subtypes of IFN-α in T-cells. PI3K signalling is activated downstream of the type I IFN receptor through phosphorylated IRS proteins (with a possible role for STAT3; see text for details). Active PI3K stimulates Akt and PKC-δ activity. In addition, ERK2 and p38 MAPK signalling can be activated, promoting STAT1 serine phosphorylation; MAPK signalling is not discussed in this thesis, but is reviewed in Li *et al*., 2007.
From Takaoka and Yanai, 2006.

phosphorylation at Tyr701, resulting in its dissociation from the receptor. SH2 domains then mediate STAT1 homodimerisation and STAT1/STAT1 dimers (also called GAF; gamma-activated factor) translocate to the nucleus and activate transcription from GAS elements in the promoters of IFN-γ-responsive genes.

STAT regulation

IFN-induced tyrosine phosphorylation of STATs is required for their homo- and heterodimerisation and subsequent nuclear translocation. However, for maximal transcriptional activity, STATs also require additional biochemical modifications and recruitment of additional cellular proteins. The STATs are also negatively regulated through the activities of protein phosphatases and the suppressor of cytokine signalling (SOCS) family.

Serine phosphorylation

Treatment with both type I and type II IFNs induces the serine phosphorylation of STAT1 at Ser727, near its C-terminus (Wen et al., 1995). Serine phosphorylation of STAT1 has been attributed to several kinases, including PKC-δ, PKC-ε, calcium/calmodulin-dependent protein kinase II (CaMKII) and the ERK2 and p38 MAP kinases (MAPKs), depending on the cell-type (Uddin et al., 2002, Deb et al., 2003, Choudhury, 2004, Nair et al., 2002, David et al., 1995, Gollob et al., 1999). This modification is independent of tyrosine phosphorylation and has no effect on STAT1 dimerisation, nuclear translocation or DNA binding affinity, but enhances transcriptional activity at a subsequent stage (Wen and Darnell, 1997, Decker and Kovarik, 2000). Ser727 phosphorylation allows STAT1 to more efficiently recruit the transcriptional coactivators p300, CBP, and minichromosome maintenance protein 5 (MCM5) (Varinou et al., 2003, Zhang et al., 1998). p300/CBP enhances transcription from ISGs by causing local chromatin remodelling (Zhang et al., 1998) while MCM5 possesses helicase activity (Shuai, 2000). Expression of IFN-γ-induced genes is strongly reduced in macrophages expressing a STAT1 mutant that is unable to undergo Ser727 phosphorylation; this suggests serine phosphorylation is required for IFN-γ-dependent innate immunity (Varinou et al., 2003). A recent study revealed that Ser727 phosphorylation is required for the subsequent SUMOylation of STAT1 and therefore plays a role in the co-ordination of STAT1 cellular responses (Vanhatupa et al., 2008).

ISGylation

STAT1 can be conjugated to ISG15, a small, ubiquitin-like protein that is upregulated by IFN (Malakhov et al., 2003). Stabilising ISGylation by knocking out UBP43, the protease responsible for removing ISG15 from ISGylated proteins, renders mice hypersensitive to IFN-β. This was found to be due to prolonged STAT1 tyrosine phosphorylation and enhanced ISG expression, indicating that STAT1 ISGylation is required for regulation of JAK/STAT signalling (Malakhova et al., 2003). However, more recent studies have concluded that STAT1 ISGylation is not essential for STAT1 signalling responses to IFN or virus infection. The IFN hypersensitivity of UBP43 (-/-) mice was shown to be ISG15-independent and ISG15 (-/-) mice displayed no obvious alterations in phenotype (Osiak et al., 2005, Knobeloch et al., 2005, Kim et al., 2006). The biological implications of STAT1 ISGylation therefore remain to be fully elucidated.

SUMOylation

SUMOylation of STAT1 at Lys703 by members of the protein inhibitors of activated STAT (PIAS) family, which are known to inhibit STAT1 activity, has also been reported (Ungureanu et al., 2003, Rogers et al., 2003). PIAS1 interacts with STAT1 to inhibit STAT1-dependent gene expression (Liu et al., 1998). Ungureanu et al. reported that IFN-γ-treatment induces STAT1 SUMO conjugation through PIAS1 and that mutation of Lys703 enhances transcription from GAS elements, implicating SUMO modification in the negative regulation of STAT signalling (Ungureanu et al., 2003, Ungureanu et al., 2005). However, Rogers et al. implicate PIAS-α, not PIAS1, STAT1-SUMO conjugation, and demonstrate that SUMOylation has no effect on the ability of STAT1 to activate transcription (Rogers et al., 2003). Further studies are therefore required to clarify the role of SUMO modification in STAT signalling.

Ubiquitination

Polyubiquitination of STAT1 has been reported in HeLa cells (Kim and Maniatis, 1996) and leads to its degradation by the proteasome. Ubiquitinated STAT1 is targeted to the proteasome by the E3 ligase SLIM (STAT1-interacting LIM protein), the overexpression of which leads to a reduction in STAT1 activity due to enhanced STAT1 degradation (Tanaka et al., 2005b). However, STAT1 ubiquitination is not thought to play a significant role in IFN signalling.

Methylation

STAT1 can be methylated by the protein arginine methyl-transferase PRMT1 at Arg31 and methylation at this site enhances the DNA binding activity of STAT1 (Mowen et al., 2001). This has, however, been refuted by subsequent studies and the validity of the experimental methods utilised by Mowen et al. brought into question (Meissner et al., 2004, Komyod et al., 2005).

Acetylation

Acetylation/deacetylation of histones by p300/CBP enhances IFN-dependent transcription by STATs (see above). Additionally, histone acetyltransferases (HATs) can directly acetylate STAT1 at Lys410 and Lys413 (Kramer et al., 2006). This modification functions as a molecular switch that promotes the association of STAT1 with the p65 subunit of NF-kB to inhibit NF-kB nuclear translocation, DNA binding and the expression of NF-kB-responsive genes. In the absence of deacetylase activity, innate antiviral responses are impaired, indicating that acetylation/deacetylation, either of histones or of the STAT1 protein, plays a pivotal role in the IFN response (Nusinzon and Horvath, 2003); indeed, IFN-α has been reported to induce STAT1 acetylation (Kramer et al., 2006). Inhibition of histone deacetylases (HDACs) reduces IFN-α-induced transcription without affecting STAT1 tyrosine phosphorylation, ISGF3 formation, nuclear translocation or DNA binding activity (Nusinzon and Horvath, 2003). Similarly, HDAC inhibitors inhibit IFN-β expression in response to virus infection or dsRNA (Nusinzon and Horvath, 2006). The role of HDAC in type II IFN signalling may differ to type I IFN signalling: while HDAC activity is also required for gene expression in response to type II IFN, HDAC inhibitors prevented JAK1 activation and subsequent STAT1 tyrosine phosphorylation and nuclear translocation (Klampfer et al., 2004).

A degree of crosstalk exists between the different modifications of STAT1. For example, methylation at Arg31 inhibits association of STAT1 with PIAS1, which may inhibit its SUMOylation (Mowen et al., 2001). Similarly, Ser727 phosphorylation of STAT1 has been shown to promote its SUMOylation (Vanhatupa et al., 2008).

Interaction with Crk proteins

Crk proteins are a family of adapters that are homologous to an oncogene encoded by the CT10 avian sarcoma virus (Mayer et al., 1988). These proteins contain both SH2 and Src-homology 3 (SH3) domains that mediate associations with a wide range of signalling components (Feller, 2001). Crk proteins were first implicated in the regulation of IFN signalling in a study by Ahmad et al., which demonstrated that type I IFN treatment induced an association between CrkL (Crk-like protein) and Tyk2, resulting in the rapid tyrosine phosphorylation of CrkL (Ahmad et al., 1997). A later study reported a similar phosphorylation event following IFN-γ treatment (Alsayed et al., 2000). CrkL couples the IFN receptors to the guanine-nucleotide-exchange factor (GEF) C3G, with which it interacts through its SH3 domain (Ingham et al., 1996, Sawasdikosol et al., 1995, Smit et al., 1996). C3G is a GEF for Rap1, a G-protein that possesses tumour suppressor activity (Gotoh et al., 1995, Kitayama et al., 1989, Hattori and Minato, 2003). IFN-stimulation can therefore activate growth inhibitory pathways through CrkL-dependent activation of Rap1 (Lekmine et al., 2002, Platanias et al., 1999, Alsayed et al., 2000).

CrkL also has Rap1-independent effects on IFN-α/β-signalling. Type I IFN treatment induces formation of a complex between CrkL and tyrosine-phosphorylated STAT5, a complex which translocates to the nucleus, binds to GAS elements and enhances ISG expression (**Figure 1.3**, Lekmine et al., 2002, Fish et al., 1999); indeed, activation of STAT5 has been shown to be necessary for maximal transcription of GAS-regulated genes (Uddin et al., 2003). One such gene is the promyelocytic leukaemia (PML) protein that is involved in IFN-induced growth inhibition (Grumbach et al., 2001, Takahashi et al., 2004).

SOCS

The SOCS proteins antagonise STAT function through direct inhibition of JAK activity (Yasukawa et al., 1999, Sasaki et al., 1999) or by targeting signalling proteins, such as JAKs or insulin receptor substrate (IRS) proteins, for degradation by the proteasome (Frantsve et al., 2001, Kamizono et al., 2001, Rui et al., 2002). Their expression is induced by IFNs; SOCS regulation of IFN therefore constitutes a negative feedback loop.

Protein tyrosine phosphatases

Since tyrosine phosphorylation of STATs plays such a critical role in their ability to activate transcription, it comes as no surprise that protein tyrosine phosphatases can negatively regulate STAT signalling by dephosphorylating tyrosine residues. The SH2 domain-containing protein tyrosine phosphatases SHP1 and SHP2, among others, have been implicated in returning both STATs and cytokine receptors to their basal, unphosphorylated state (reviewed by Mustelin et al., 2005).

1.1.3.2 PI3K signalling

The PI3Ks

Although the JAK/STAT pathway has become synonymous with signalling downstream of IFNs, it is now becoming clear that PI3K also plays an important role in the IFN response. PI3Ks are involved in signal transduction from many cell surface receptors, and consequently regulate many intracellular events including cell survival, cell proliferation, metabolism, and cell movement (reviewed by Hawkins et al., 2006). PI3K activation by IFN appears to counter many functions of STAT signalling; for example JAK/STAT signalling upregulates many genes involved in apoptosis while PI3K signalling promotes cell survival. The way in which cells respond to IFN therefore appears to be subject to complex regulation, with PI3K and STAT pathways (and perhaps other signalling pathways) competing to dictate the outcome of the IFN response.

Three classes of PI3Ks exist which are further divided into subclasses, each with different tissue distributions, substrate specificity and modes of activation (Fruman et al., 1998). The primary function of the class IA PI3Ks is to catalyse phosphorylation of the inositol ring (at position D3) of phosphatidylinositol lipids in response to cytokines, growth factors and certain hormones (Cantley, 2002). The phosphatidylinositol-3,4,5-trisphosphate (PIP3) product of this reaction functions as an important second messenger that regulates multiple effector proteins (reviewed by Hawkins et al., 2006, Rameh and Cantley, 1999, Cantley, 2002). Class I PI3Ks are also reported to possess serine/threonine kinase activity and regulate downstream proteins directly (Rameh and Cantley, 1999, Platanias et al., 1996, Vanhaesebroeck et al., 1999, Stoyanova et al., 1997, Dhand et al., 1994). While this protein kinase activity was initially thought to be a distinct kinase tightly associated

with PI3K (Carpenter et al., 1993b), subsequent studies have shown it may be an intrinsic activity to PI3K (Foukas et al., 2004, Dhand et al., 1994).

Regulation of PI3K activity

Class 1A PI3Ks are heterodimers made up of a p110 catalytic subunit and a regulatory subunit (Wymann and Pirola, 1998). Three isoforms of the catalytic subunit have been identified; p110α, p110β, and p110δ. The regulatory subunit is encoded by one of three genes, p85α, p85β and p55γ, but splice variants of these gene products also exist. The regulatory subunit contains a B-cell receptor homology (BH) domain, an SH3 domain and two SH2 domains that promote interactions with signalling proteins and the catalytic subunit of PI3K. The p85 regulatory subunit inhibits the lipid kinase activity of the catalytic subunit in quiescent cells and plays a role in promoting conformational stability (Yu et al., 1998b). The importance of this negative regulation is demonstrated by studies that show mutations in the p85-binding site of p110 result in potent activation of PI3K and high oncogenicity (Zhao et al., 2005b, Kang et al., 2005). Binding of p85 SH2 domains to phosphotyrosines (in a consensus YXXM motif) within activated receptors or receptor-associated adaptor proteins relieves its restraint on p110 catalytic activity (Carpenter et al., 1993a, Rordorf-Nikolic et al., 1995, Yu et al., 1998a). p85-mediated recruitment of PI3K to cell membrane receptors may itself affect p110 activation by increasing the effective concentration of p110 phosphatidylinositol substrates (Vanhaesebroeck et al., 2005). It has also been suggested that monomeric p85 can sequester SH2-binding protein away from PI3K: free p85 competes with p85/p110 heterodimer for IRS-1 binding, preventing PI3K activation downstream of the insulin receptor (Luo and Cantley, 2005). However, other reports indicate PI3K exists as an obligate heterodimer and that monomeric p85 is unstable (Geering et al., 2007a, Geering et al., 2007b), so p110 inhibition through sequestration may not occur *in vivo*.

p110 catalytic activity can also be modulated through post-translational modification of p85. Src kinase mediates phosphorylation of p85 at Tyr688 which relieves its inhibitory effect on p110 and activates PI3K (Cuevas et al., 2001). In contrast, serine phosphorylation of p85 negatively regulates p110 catalytic activity (Foukas et al., 2004). Furthermore, p110 is reported to mediate PI3K autoregulation by directly phosphorylating p85 at Ser608 (Dhand et al., 1994, Vanhaesebroeck et al., 1999). Ser608 phosphorylation is observed both *in vitro* and *in vivo*, and mutation of Ser608 affects PI3K heterodimerisation and enzymatic activity (Foukas et al., 2004).

Moreover, defective p85 Ser608 phosphorylation, which leads to constitutive PI3K activation, may be involved in the development of human colon and ovarian tumours (Foukas et al., 2004, Philp et al., 2001).

Protein interactions that occur independently of the SH2 domains can also modulate PI3K activity. Rho GTPases such as Rac1 and Cdc42 can bind p85 through its BH domain to activate p110 (Tolias et al., 1995, Zheng et al., 1994b). Also important for PI3K activation is binding of the active form (GTP-bound) of the Ras GTPase to a Ras-binding domain in p110 (Rodriguez-Viciana et al., 1994). This interaction elevates basal PI3K activity, indicating that Ras-binding has a direct effect on catalytic activity (Rodriguez-Viciana et al., 1996).

The PTEN (phosphatase and tensin homologue deleted on chromosome ten) and SHIP (SH2 domain-containing inositol 5'-phosphatases) phosphatases oppose PI3K action by dephosphorylating PIP3 at the 3-position and the 5-position respectively. The PIP2 products of this reaction can themselves signal to downstream pathways to regulate cellular processes (reviewed by Hawkins et al., 2006). PTEN is well-characterised as a tumour-suppressor and its mutation or silencing is implicated in a number of human cancers (Li et al., 1997, Sansal and Sellers, 2004). In addition to its negative regulation of the p110 catalytic subunit, p85α has been shown to upregulate PTEN activity, thus negatively regulating the PI3K pathway (Taniguchi et al., 2006).

PI3K activation by IFN

IRS-dependent activation

Like IFNAR and IFNGR-mediated activation of STATs, IFN receptors use PTKs as mediators to signal to PI3K (**Figure 1.3**). Following type I IFN treatment, IFNAR-associated Tyk2 recruits IRS-1 and IRS-2 through their pleckstrin homology (PH) domains, resulting in their rapid phosphorylation at certain tyrosine residues (Platanias et al., 1996, Uddin et al., 1995). Phosphotyrosines on IRS-1 and IRS-2 form docking sites for the SH2 domains of the p85 regulatory subunit of PI3K, allowing for activation of p110 (Platanias et al., 1996, Uddin et al., 1995). Both protein and phospholipid kinase activities of PI3K are activated by IFN-α treatment (Uddin et al., 1995, Uddin et al., 1997).

IRS-independent activation

IRS-independent activation of PI3K by IFN has also been reported. Type I IFN-stimulated tyrosine phosphorylation of IFNAR1 was found to recruit STAT3 to the IFN receptor. STAT3 acts as an adaptor that, through two phosphotyrosine residues, provides a docking site for p85. Once bound to the receptor through STAT3, p85 itself undergoes tyrosine phosphorylation (Pfeffer et al., 1997), a modification that activates PI3K catalytic activity (Cuevas et al., 2001). However, the involvement of STAT3 in PI3K activation has been disputed by subsequent studies: PI3K has been reported to associate with IFNAR1 in a ligand- and STAT3-independent manner (Uddin et al., 2000, Rani et al., 1999). IFN-γ treatment also activates PI3K independently of IRS proteins (Platanias et al., 1996, Uddin et al., 2000) indicating that other adaptor proteins must recruit PI3K to the type II IFN receptor. The proto-oncogene product CBL is a candidate for such an adaptor since it is activated by IFN-γ and can bind p85 through its SH3 domain (Panchamoorthy et al., 1996, Soltoff and Cantley, 1996). Several pathways of PI3K activation by IFN may therefore exist, and the contribution of each pathway to activation of PI3K may be cell-type, and/or IFN-type-specific.

Downstream effectors of PI3K

Membrane-associated PIP3 is thought to be the major signal generated in response to PI3K activation. PIP3 recruits a large number (at least 20, Krugmann et al., 2002) of PH domain-containing effector proteins to the cell membrane. Through PIP3 therefore, PI3K can regulate a large number of signalling pathways (reviewed by Hawkins et al., 2006). The way these effector proteins become activated by PIP3 binding is unclear, but several mechanisms have been suggested and these may contribute to different extents in the activation of different proteins. Recruitment of PH-domain-containing proteins to the cell membrane alters the subcellular localisation of effector proteins and may itself be considered ‘activating’ due to an increase in the effective substrate/binding partner concentration. Additionally, the effector may be brought into close proximity with activating kinases (Hawkins et al., 2006). In some proteins, the PH domain mediates inhibition of other domains. Binding of PIP3 to the PH domain relieves this inhibition, increasing catalytic activity or altering affinity for binding partners. For example, a PH domain restricts the catalytic activity of the GEF Dbl domain: PIP3 binding to the PH domain relieves inhibition of the Dbl domain and restores GEF catalytic activity (Das et al., 2000).

There is emerging evidence that the serine/threonine kinase activity of PI3K may also activate downstream protein kinase cascades. Wymann and colleagues demonstrated that a PI3K with disrupted lipid kinase activity but intact protein kinase activity activated MAPK signalling (Bondeva et al., 1998).

Perhaps the best-studied of the PH-domain-containing effector proteins is the serine/threonine kinase Akt, also called protein kinase B (Brazil et al., 2004); the mechanism of Akt activation by PI3K is shown in **Figure 1.4**. Akt is recruited to the cell membrane through PIP3:PH domain interactions where it undergoes phosphorylation at Thr308 and Ser473 by PDK1 (phosphoinositide-dependent-kinase 1) and mammalian target of rapamycin (mTOR) complex 2 (mTORC2) respectively (Sarbassov et al., 2005, Alessi et al., 1997). Although PDK1 is constitutively active, its phosphorylation of Akt is PI3K-dependent as PIP3 binding to the Akt PH domain is required to make the Thr308 residue available for PDK1-dependent phosphorylation (McManus et al., 2004). In addition, PDK1 recruitment to the membrane is dependent on PIP3:PH domain interaction. Phosphorylation of Ser473 is also PI3K-dependent (Bayascas and Alessi, 2005) but the mechanism of mTORC2 activation by PI3K has not yet been determined. Phosphorylation at both sites leads to an increase in the catalytic activity of Akt, resulting in phosphorylation of a variety of downstream effectors (**Figure 1.5**, reviewed by Manning and Cantley, 2007).

PI3K and regulation of the IFN response

PI3K can regulate signalling from TLR3 in response to dsRNA stimulation. TLR3 and PI3K directly associate, resulting in PI3K and Akt activation (Sarkar et al., 2004). Although active PI3K does not affect IRF3 dimerisation and nuclear translocation, PI3K is required for complete IRF3 phosphorylation and therefore its maximal DNA binding activity (Ehrhardt et al., 2006, Sarkar et al., 2004). PI3K can also regulate NF-kB signalling downstream of TLR3 in response to poly(I:C). In DCs, the PI3K inhibitors wortmannin and LY294002 enhance IFN-β expression: PI3K physically interacts with TRIF to selectively inhibit NF-kB (but not IRF3) activation and IFN-β production (Aksoy et al., 2005).

Since serine phosphorylation of STATs is required for their maximal activity and PI3K serine kinase activity is activated by type I IFN treatment (Platanias et al., 1996), it was postulated that PI3K activity may be required for maximal transcription from ISRE/GAS elements (Platanias and Fish, 1999). While PI3K inhibitors are reported to

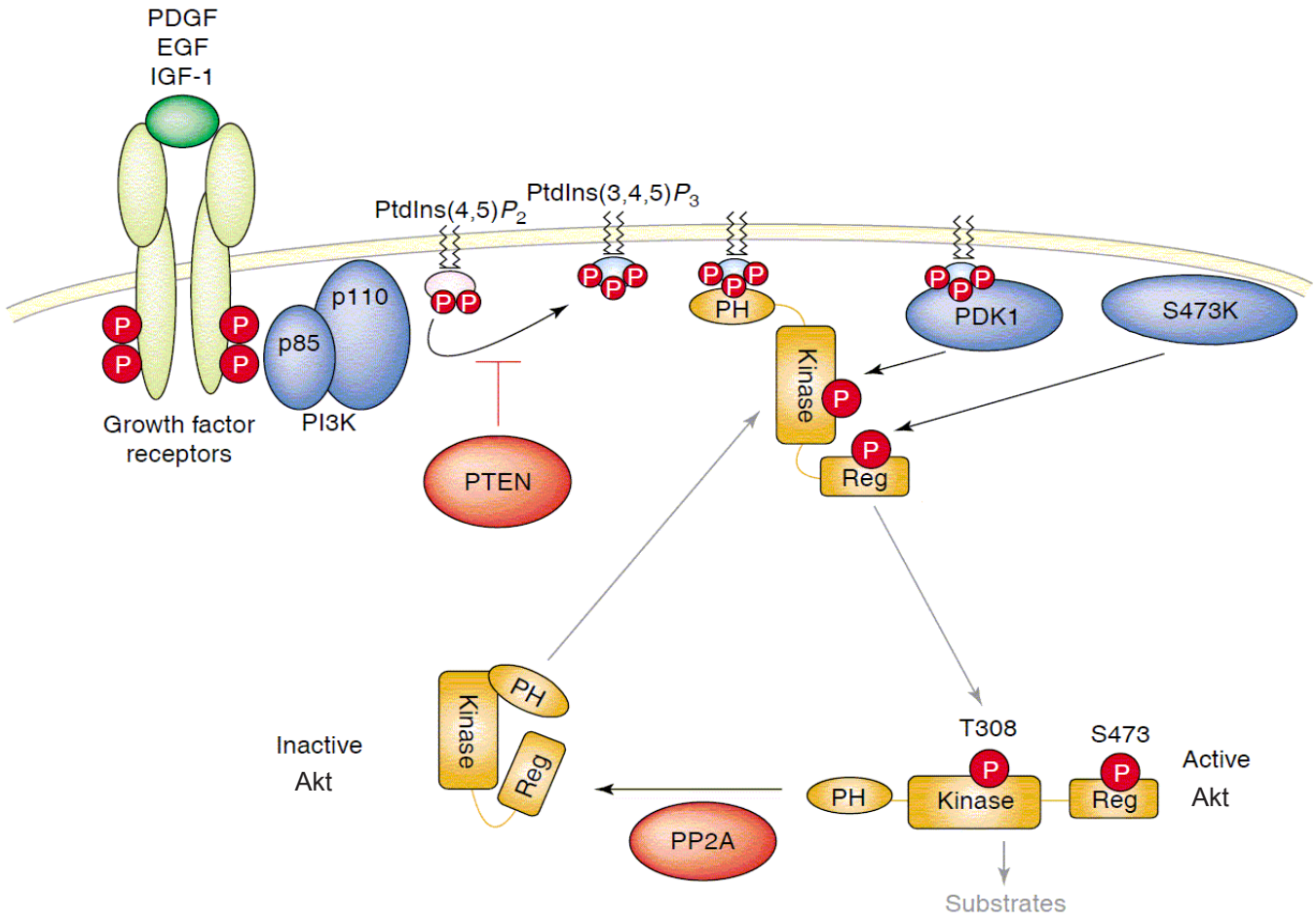


Figure: 1.4: Activation of PI3K and Akt downstream of growth factor receptors.

PI3K is recruited to the intracellular domains of growth factor receptors through receptor phosphotyrosine residues. Active PI3K generates phosphatidylinositol (3,4,5) triphosphate which recruits inactive Akt to the plasma membrane. Ser473 (in the regualatory loop) and Thr308 (in the kinase domain) of Akt are subsequently phosphorylated by mTORC2 (labelled as S473K in the diagram) and PDK1 respectively. Active Akt can then phosphorylate its downstream substrates. PI3K activation is regulated by PTEN, while Akt is inactivated by protein phosphatase 2A (PP2A).
Modified from Brazil et al., 2004.

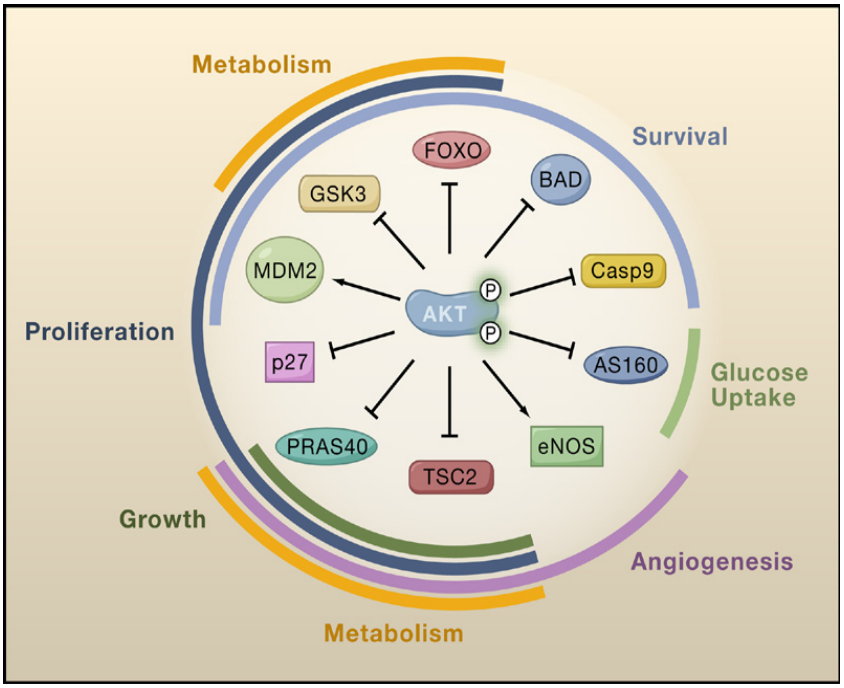


Figure: 1.5: Cellular functions of Akt.

Phosphorylation of target proteins by Akt leads to their activation (arrows) or inhibition (blocked arrows). Through modulation of the activity of these substrates, Akt can regulate a number of cellular process, including cell survival, cell growth and proliferation, metabolism, glucose uptake and angiogenesis. Some of these substrates exert multiple functions; for example, GSK3 has a role in cell survival, proliferation and metabolism regulation. *From Manning and Cantley, 2007.*

have no effect on type I IFN-induced gene transcription (Thyrell et al., 2004, Hjortsberg et al., 2007, Uddin et al., 1997), a recent study demonstrated that ISG transcription is impaired in PI3K knockout cells (Kaur et al., 2008b). The same results were not observed in Akt knockout cells (Kaur et al., 2008a), indicating that PI3K exerted its effects on ISG transcription by an Akt-independent mechanism. The PI3K substrates responsible for this effect have yet to be identified, but may include the PKC or Tec protein families, both of which are PI3K targets and are known to regulate gene transcription by phosphorylating STATs (Uddin et al., 2002, Deb et al., 2003, Choudhury, 2004, Saharinen et al., 1997, Jiang et al., 2007). However, STAT1 serine phosphorylation and STAT1-dependent transcription following type II IFN treatment appears to require both PI3K and Akt activation (Nguyen et al., 2001): PKC-δ is activated by IFN-γ in a PI3K-dependent manner and its inhibition blocks STAT1 Ser727 phosphorylation (Deb et al., 2003, Uddin et al., 2002).

Akt is activated downstream of PI3K in response to IFN in a range of cell lines (Yang et al., 2001, Nguyen et al., 2001, Lei et al., 2005, Kaur et al., 2008a). PI3K/Akt activation appears to play a key role in regulation of ISG translation. Treatment with type I and type II IFNs results in activation of p70 S6 kinase (p70 S6K), which causes downstream phosphorylation of the S6 protein of the ribosomal 40s subunit to stimulate mRNA translation. This pathway is dependent on PI3K, Akt and the mTOR complex 1 (mTORC1) (Kaur et al., 2008b, Lekmine et al., 2003, Kaur et al., 2008a). Additionally, PI3K activation leads to phosphorylation and negative regulation of translational repressor eukaryotic initiation factor 4E-binding protein (4E-BP1) and subsequent activation of the translation initiation factor eIF4E (Lekmine et al., 2003, Kaur et al., 2008b). Expression of the ISGs ISG15 and CXCL10 in response to IFN-α and IFN-γ is significantly reduced in p85 and Akt knockout cells; these cells are subsequently much less sensitive to the antiviral effects of IFN-α than wildtype cells (Kaur et al., 2008a). This indicates that PI3K selectively increases ISG translation to generate an antiviral state.

1.1.3.4 NF-kB signalling

NF-kB binding sites are present in the promoters of certain ISGs (Du et al., 2007) and type I IFN activates NF-kB DNA-binding and transcriptional activity in a wide range of cell types (**Figure 1.6**). Type I IFNs promote phosphorylation and degradation of the NF-kB inhibitor IkB (Yang et al., 2000) through a pathway that requires STAT3, PI3K and Akt (Yang et al., 2001). PI3K-dependent NF-kB activation

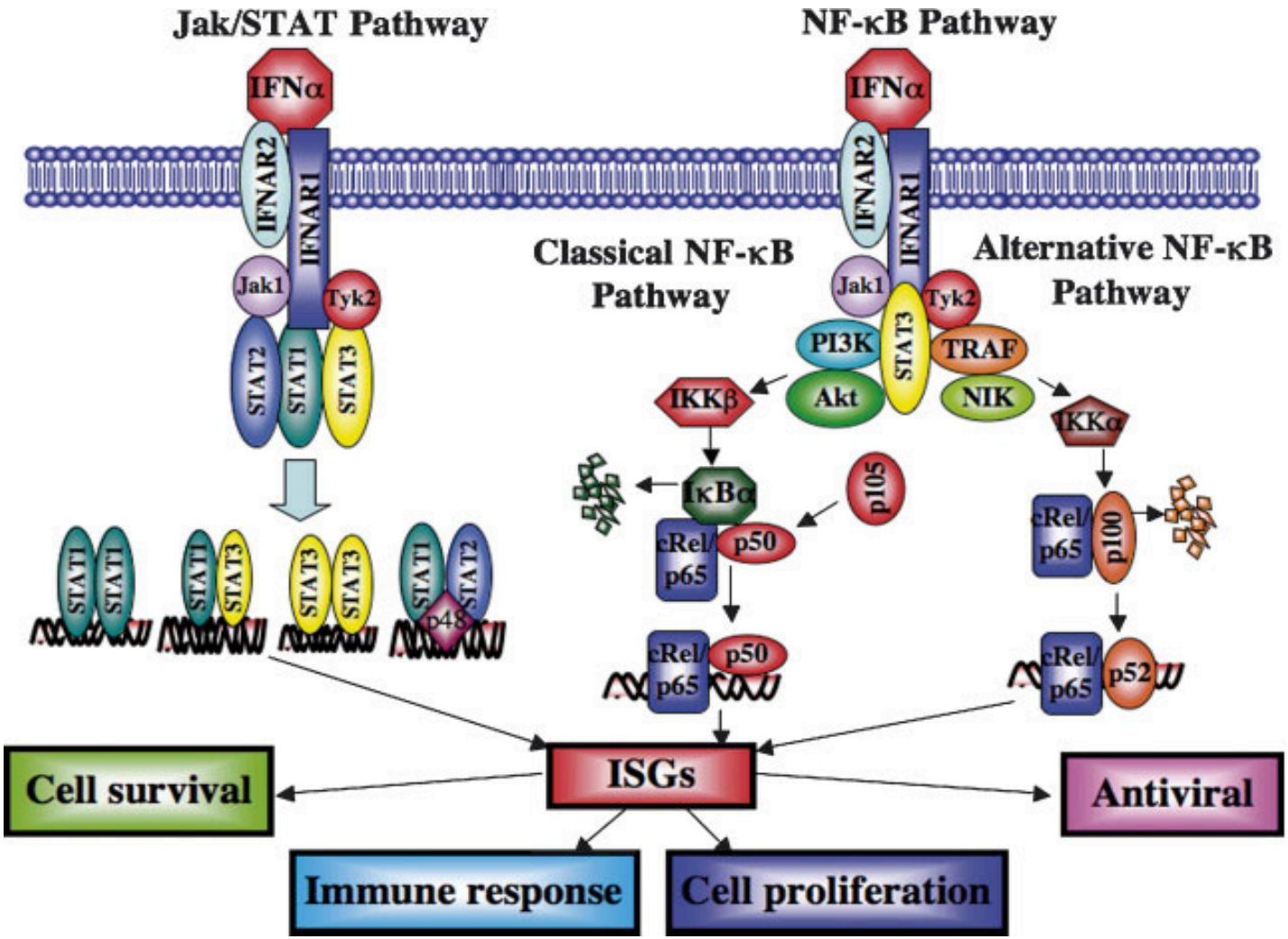


Figure 1.6: Activation of classical and alternative NF-kB signalling downstream of the type I IFN receptor.

The JAK/STAT pathway activated by type IFNs is shown. Type I IFNs also activate classical NF-kB signalling through recruitment of STAT3, PI3K and Akt to IFNAR. This pathway promotes degradation of IkB to release NF-kB, which subsequently undergoes nuclear translocation to regulate gene expression. In addition, an alternative, non-canonical pathway of NF-kB activation occurs through TRAF2 and NIK: NIK processes the NF-kB2 p100 precursor to form p52, which translocates to the nucleus and regulates ISG transcription.
From Du et al., 2007.

has no effect on transcription from the ISRE, indicating that NF-kB activation may represent a distinct mechanism by which IFN can regulate gene expression (Du et al., 2007). The CXCL11 chemokine gene, an ISG that is selectively induced by IFN-β but not IFN-α (Rani et al., 1996), requires both ISGF3 and NF-kB for its expression (Rani et al., 2001). CXCL11 induction is also STAT3- and PI3K/Akt-dependent (Yang et al., 2007a, Rani et al., 2002), providing further evidence that type I IFN activates NF-kB in a STAT3/PI3K/Akt-dependent manner to stimulate ISG transcription.

An alternative, non-canonical pathway of IFN-induced NF-kB activation also exists that occurs independently of PI3K activation and IkB proteolysis (**Figure 1.6**, Pomerantz and Baltimore, 2002, Du et al., 2007). This pathway requires TRAF2 and its downstream effector NIK (Xiao et al., 2001, Yang et al., 2005, Senftleben et al., 2001). NIK processes the NF-kB2/p100 precursor to remove its IkB-like C-terminus; the resulting p52 product then heterodimerises, translocates to the nucleus and regulates gene transcription (Pomerantz and Baltimore, 2002). p52 can be detected in IFN-α/β-treated cells, and generation of this product is not affected by PI3K inhibitors (Yang et al., 2005). Type I IFN can therefore stimulate both classical, PI3K-dependent, and non-canonical, TRAF2/NIK-dependent pathways of NF-kB activation; activation of both of these pathways generates survival signals that protect cells from the pro-apoptotic effects of IFN (Yang et al., 2001, Yang et al., 2005). Studies using NF-kB knockout cells revealed that there is a subset of ISGs that are either positively or negatively regulated by NF-kB, and these gene products include ISG15 and components of antigen-presentation machinery (Pfeffer et al., 2004, Wei et al., 2006).

1.1.4 The IFN-induced antiviral state

IFNs induce an ‘antiviral state’ in cells through induction of ISGs. More than 300 ISGs have been identified; they perform roles in a vast range of biological processes including transcriptional and translational regulation of gene expression, cell cycle modulation, apoptosis, and stimulation of immune cells (de Veer et al., 2001). The combined effects of these ISGs make the cell an exceptionally hostile environment for virus replication and spread; consequently, recombinant IFNs are frequently used to clinically treat infections with certain viruses (for example, chronic hepatitis C virus [HCV] infection). The critical role of IFNs in defending the cell against virus infection has been demonstrated by studies in mice with targeted disruption of the IFNAR or IFNGR genes. These mice are highly sensitive to virus infection, despite being able

to mount normal T-cell responses, and virus titres in internal organs are much higher than in wildtype mice (Hwang et al., 1995b, Huang et al., 1993, Muller et al., 1994). The roles of the best-studied ISGs in the antiviral state are described below.

1.1.4.1 Antiviral ISGs and their functions

PKR

The IFN-inducible double-stranded RNA-activated protein kinase (PKR) is constitutively expressed at low levels in quiescent cells. PKR exists in an inactive form through steric interference between its N-terminus and its catalytic domain (Gelev et al., 2006). Binding of viral dsRNA to binding sites in the N-terminus induces a conformational change that relieves this inhibition of the kinase domain (Nanduri et al., 2000). Catalytic activity requires PKR homodimerisation and subsequent autophosphorylation at a number of residues (Dar et al., 2005, Dey et al., 2005, Romano et al., 1998, Taylor et al., 1996). Active PKR phosphorylates eIF2α to halt cellular translation. PKR additionally plays a role in promoting apoptosis, and this will be discussed in a later, in **Section 1.2.5.1**.

OAS/RNaseL

The IFN-inducible 2'-5'-oligoadenylate synthase (OAS) proteins become activated by dsRNA and catalyse the conversion of ATP into 2'-5'-linked adenosine oligomers (Rebouillat and Hovanessian, 1999). These oligomers subsequently activate RNaseL (itself IFN-upregulated), an endonuclease that is constitutively expressed in an inactive form (Zhou et al., 1993, Slattery et al., 1979). Active RNaseL degrades both viral and cellular RNAs into short fragments, a process that inhibits virus replication by inhibiting protein synthesis (Zhou et al., 1998, Scherbik et al., 2006, Silverman, 2007). RNaseL overexpression has been shown to suppress the replication of encephalomyocarditis virus (EMCV), parainfluenza virus 3 (hPIV3), VSV and vaccinia virus (VACV) (Zhou et al., 1998). Nucleotide fragments produced by RNaseL can also enhance IFN-β expression by binding to RIG-I and mda-5 to activate MAVS-dependent signalling (Malathi et al., 2007). Effects of OAS/RNaseL on apoptosis induction will be discussed in **Section 1.2.5.1**.

ISG15

ISG15 is a ubiquitin homologue that is conjugated to cellular proteins (Loeb and Haas, 1992). This process (ISGylation), like ubiquitination, requires the activities of an E1 activating enzyme, an E2 conjugating enzyme and an E3 ligase (Takeuchi et al., 2005, Yuan and Krug, 2001, Zhao et al., 2004, Kim et al., 2004, Wong et al., 2006, Zou and Zhang, 2006); these enzymes are also upregulated by IFN (Kang et al., 2001a, Nyman et al., 2000). While ubiquitination of proteins generally leads to their degradation by the proteasome, ISGylation results in protein translocation (Loeb and Haas, 1994), stabilisation (Lu et al., 2006b) or non-degradative negative regulation (Zou et al., 2007b, Kim et al., 2008). ISG15-deficient mice are hyper-sensitive to infection with a number of viruses, including IAV and HSV-1 (Lenschow et al., 2007), and in mice unable to respond to IFN, ISG15 expression offered protection against Sindbis virus (Lenschow et al., 2005). Knockout of UBP43, the protein required for ISG15-deconjugation, renders mice more sensitive to IFN and poly(I:C) treatment than wildtype mice (Malakhova et al., 2003, Zou et al., 2007a). These mice are also resistant to infection with VSV and lymphocytic choriomeningitis virus (LCMV) (Ritchie et al., 2004). Similarly, siRNA knockdown of UBP43 potentiates the inhibitory effect of IFN on HCV replication (Randall et al., 2006), indicating that ISG15-conjugation may be required to mount an efficient IFN response. Indeed, many proteins involved in IFN pathways, including STAT1, Jak1, Erk1, PKR and MxA, undergo ISG-conjugation (Zhao et al., 2005a, Malakhov et al., 2003): the role of ISGylation in STAT1 regulation is described in **Section 1.1.3.1**. The ISG15-conjugation system may also perform a negative regulatory role in the IFN response, as ISGylation of RIG-I has been reported to inhibit stimulation of the IFN-β promoter during infection with NDV (Kim et al., 2008).

Mx GTPases

The Mx group of guanine-hydrolysing enzymes were first identified as antiviral during studies which revealed mice carrying mutations in the Mx gene are sensitive to orthomyxovirus infection (Lindenmann, 1962). The human genome encodes two Mx proteins, MxA and MxB, both IFN-inducible and cytoplasmic (Aebi et al., 1989), of which only MxA demonstrates antiviral activity. Mx proteins contain an N-terminal GTPase domain and a C-terminal leucine zipper domain, both of which are required to bind virus structures (Kochs and Haller, 1999). By recognising virus nucleocapsids, MxA can inhibit replication of Thogoto virus (THOV) by preventing

nuclear import of the virus genome (Kochs and Haller, 1999, Weber et al., 2000). Additionally, MxA that is artificially given a nuclear localisation can directly bind and inhibit viral polymerase activity in THOV and IAV-infected cells (Turan et al., 2004, Weber et al., 2000, Pavlovic et al., 1992). Replication of a wide range of viruses is inhibited by MxA expression, including paramyxoviruses, orthomyxoviruses, bunyaviruses, rhabdoviruses and picornaviruses (Sadler and Williams, 2008).

PML

The IFN-inducible PML protein displays antiviral activity against a number of viruses including VSV and IAV (Chelbi-Alix et al., 1998, Djavani et al., 2001, Everett, 2001, Regad et al., 2001). PML localises to nuclear bodies (NBs) that also contain many other proteins; some, like Sp100 and PA28, are also IFN-inducible. Many NB components are involved in chromatin remodelling, transcription, DNA repair or elimination of mis-folded protein aggregates (reviewed by Everett and Chelbi-Alix, 2007). PML NBs may also play a key role in IFN-induced apoptosis, and the evidence for this will be presented in **Section 1.2.5.1**.

Immunomodulation

Both type I and type II IFNs upregulate a number of components involved in class I antigen presentation to cytotoxic T-lymphocytes (CTLs) (Der et al., 1998a). These include MHC class I molecules, proteasome subunits and transporters required for MHC expression at the cell surface (Der et al., 1998a, Schroder et al., 2004, Epperson et al., 1992). Type I IFNs can additionally promote DC maturation (Le Bon et al., 2003), stimulation of NK cell cytotoxicity (Nguyen et al., 2002a) and CTL proliferation (Tough et al., 1996, Marrack et al., 1999, Kolumam et al., 2005).

IFN-γ is secreted by activated type I helper T (Th1) cells and, in addition to promoting an antiviral state in non-immune cells, performs a number of roles to co-ordinate an antiviral response from immune cells (reviewed by Schroder et al., 2004). Briefly, these include upregulation of MHC class II components and proteases involved in production of antigenic peptides in macrophages and DCs, promotion of NK cell activity and enhancement of lysosome activity in macrophages.

Apoptosis

Induction of a cellular pro-apoptotic state is an important function of IFNs, and the IFN-inducible PKR, PML and the OAS/RNaseL systems all play roles in the establishment of such a state. Similarly, apoptosis regulation is a major function of PI3K/Akt activation. The role of apoptosis in the IFN-induced antiviral state and the mechanisms by which PI3K/Akt regulates apoptosis will be dealt with in **Sections 1.2.5 and 1.2.6**.

Cell cycle regulation

Treatment with type I IFNs causes cells to spend prolonged periods in the G1, S and G2 phases of the cell cycle (Balkwill and Taylor-Papadimitriou, 1978), which may lead to induction of apoptosis. IFN modulates cell-cycle progression through upregulation of a number of cyclin-dependent kinase (CDK) inhibitors (Sangfelt et al., 1999, Sangfelt et al., 1997b, Mandal et al., 1998) and the p200 family of proteins (Asefa et al., 2004). For instance, the CDK inhibitor p21Waf1 is upregulated by type I and II IFNs and is linked with cell cycle arrest and apoptosis (Subramaniam et al., 1998, Mandal et al., 1998, Urashima et al., 1997, Chin et al., 1996).

1.1.4.2 PI3K regulation of the IFN-induced antiviral state

A small subset of IFN-α-induced genes that are regulated by PI3K have been identified by microarray (Hjortsberg et al., 2007). PI3K and Akt activation by IFN may function to negatively regulate the antiviral state, since PI3K/Akt activates many pathways that IFN inhibits, and vice versa. Seemingly in contradiction to the pro-apoptotic state induced by IFN, a major function of PI3K/Akt activation is the prevention of apoptosis and promotion of cell survival. The mechanisms by which PI3K/Akt regulate apoptosis will be dealt with in **Section 1.2.6**. Inhibition of 4E-BP1 and p70 S6K by Akt promotes mRNA translation (see above), in contrast to PKR-mediated translation inhibition stimulated by IFN. In addition, Akt may enhance membrane biosynthesis (a requirement for cell growth and proliferation) through regulation of ATP citrate lyase, an enzyme that catalyses formation of acetyl-CoA required for fatty acid synthesis (Berwick et al., 2002). PI3K/Akt promotes cell growth through its effects on protein and membrane synthesis. The IFN-stimulated CDK inhibitors p21Waf1 and p27Kip1 are also Akt target proteins. Akt-mediated phosphorylation of these proteins inhibits their nuclear translocation, thereby

promoting cell proliferation (Shin et al., 2002, Viglietto et al., 2002, Zhou et al., 2001, Liang et al., 2002). Expression of p21Waf1 and p27Kip1 is also reduced through the effects of Akt on p53 and FOXO transcription factors (see **Section 1.2.6**) (Collado et al., 2000, Dijkers et al., 2000b, Medema et al., 2000). In addition, the downstream Akt effector glycogen synthase kinase 3β (GSK3β) usually promotes rapid turnover of the G1 cyclins D and E, and the transcription factors c-jun and c-myc (Diehl et al., 1998), (Wei et al., 2005, Welcker et al., 2003, Yeh et al., 2004). Inhibition of GSK3β by Akt elevates expression of these proteins, which are all important for G1/S phase transition.

1.2 Apoptosis

Apoptosis is a tightly controlled process whereby cells activate pathways leading to their self-destruction in response to a wide range of stimuli, including virus infection, DNA damage and signals from immune cells. These death pathways culminate in engulfment of apoptotic cell fragments by neighbouring and phagocytic cells, preventing leakage of cell contents into the extracellular environment. This is in contrast to necrosis, where cells swell and burst, leading to activation of the inflammatory response. Apoptotic cells exhibit characteristic morphological and biochemical features, including chromosome condensation, DNA fragmentation, membrane 'blebbing', collapse of the mitochondrial membrane potential and loss of membrane phospholipid asymmetry. Different pathways of apoptosis induction are activated depending on the nature of the apoptotic stimulus. However, these pathways converge at the point of the protease effectors of apoptosis that are responsible for dismantling the cell.

1.2.1 Protease effectors of apoptosis

Caspases

The role of the caspase family of cysteine proteases in apoptosis has been extensively researched, so much so that current models of apoptosis induction have been described as 'caspase-centric' (Hail et al., 2006). Caspases are highly conserved among multicellular organisms and cleave a large number of cellular proteins in response to apoptotic stimuli. They are synthesised as inactive precursors, procaspases, that require activation allosterically or by proteolytic cleavage (Saikumar et al., 2007, Ayala et al., 1994). Caspases-8 and -9 are activated by the death-receptor pathway and the mitochondrial pathways of apoptosis respectively (**Figure 1.7**). These caspases are often called 'initiator' caspases because once activated, they cleave and activate a downstream 'caspase cascade'. The cascade culminates in activation of 'effector' caspases-3 and -7: effector caspases represent a point of convergence between the mitochondrial and death receptor pathways. Caspases cleave an array of downstream targets including nuclear lamins and cytoskeletal proteins, cleavage of which brings about the morphological changes associated with apoptosis. Caspase cleavage inactivates the inhibitor of caspase-activated DNase (ICAD), releasing the DNase responsible for

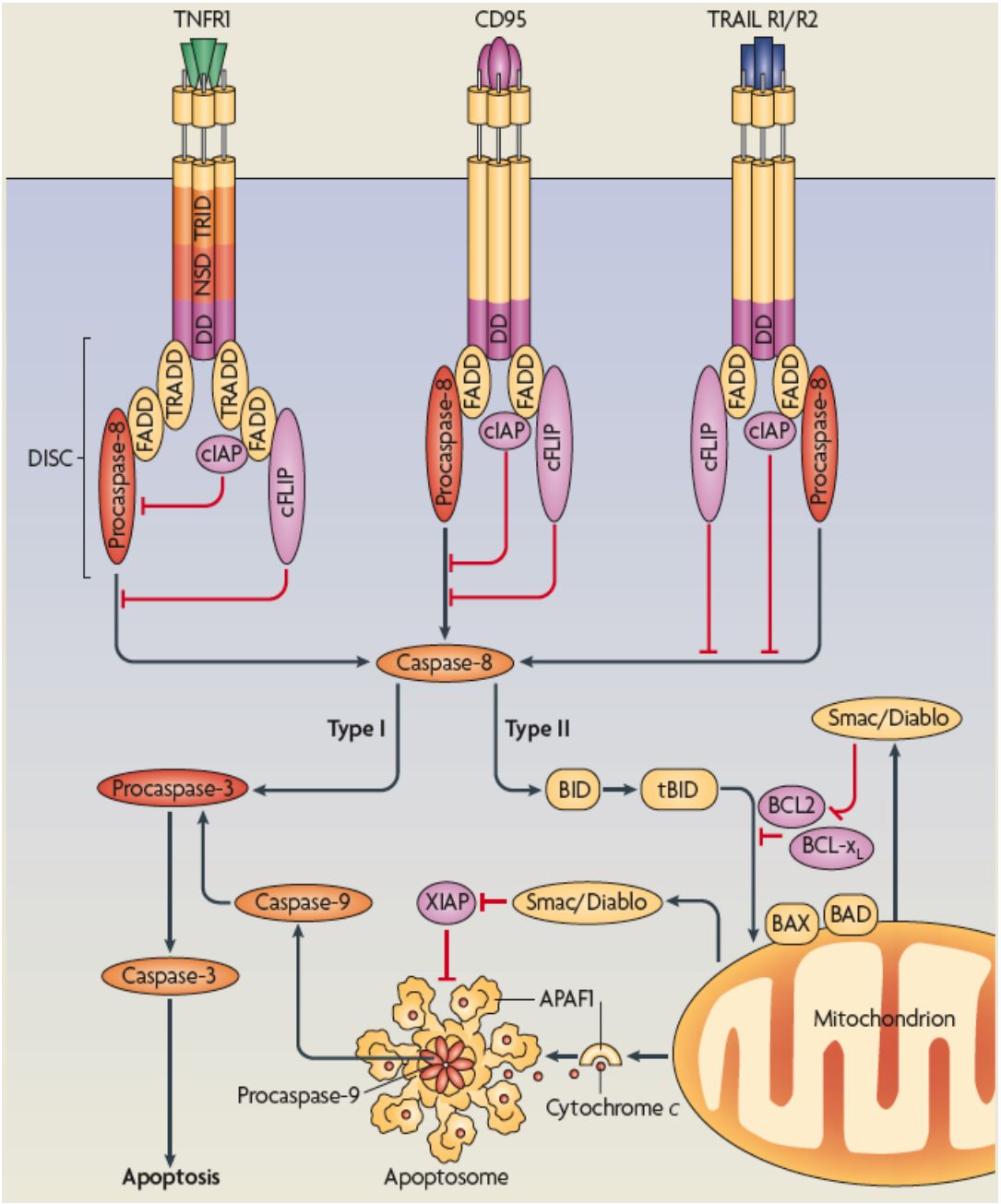


Figure 1.7: The death receptor and mitochodrial pathways of apoptosis.

In response to ligand binding, the TNFR, Fas and TRAILR death receptors recruit specific adaptor proteins to form the DISC (FADD and procaspase-8) via their intracellular death domains. TNFR1 requires TRADD to recruit FADD and procaspase-8, while Fas and TRAILR directly bind these DISC components. The DISC cleaves procaspase-8 to form active caspase-8, which subsequently activates downstream caspases. Caspase-8 can also signal to the mitochondrial pathway of apoptosis by promoting BID cleavage. Truncated tBID translocates to the mitochondria and interacts with BAX and BAD results to stimulate cytochrome c and APAF-1 release from the mitochondria. APAF1, cytochrome c and procaspase-9 form the apoptosome, which activates caspase-9 and the caspase cascade. These pathways are negatively regulated at the level of the DISC by cIAP and cFLIP and at the mitochondrial level by BCL-2 and BCL-XL. These inhibitory proteins themselves are counteracted by mitochondrial proteins such as Smac/Diablo.
From Schutze et al., 2008.

nucleosomal DNA fragmentation, CAD (Sakahira et al., 1998). Additionally, caspases cleave and inactivate a number of cell signalling proteins, including STAT1 (King and Goodbourn, 1998), IkB (Barkett et al., 1997) and NF-kB (Kang et al., 2001b) and the anti-apoptotic Bcl-2 family members Bcl-2 (Cheng et al., 1997) and Bcl-X_L (Clem et al., 1998). Conversely, cleavage of Bid by caspase-8 augments its pro-apoptotic properties, resulting in transduction of the apoptotic signal from the cytoplasm to the mitochondria (Li et al., 1998a).

Cathepsins

The cathepsin family can be divided into cysteine, aspartate and serine proteases, and, like the caspases, are synthesised as inactive precursors that require proteolysis for activation. The roles of the cysteine proteases cathepsins B and L, and of cathepsin D, an aspartate protease, in apoptosis have been the most studied. These cathepsins are found in lysosomes and endosomes, but are secreted into the cytoplasm during apoptosis (Li et al., 1998b, Roberg and Ollinger, 1998). Here, they cleave and activate pro-apoptotic Bid and procaspases and degrade anti-apoptotic Bcl-2, Bcl-X_L, XIAP (X-linked inhibitor of apoptosis) and Mcl-1 (myeloid leukaemia cell differentiation protein) (Stoka et al., 2001, Droga-Mazovec et al., 2008, Ishisaka et al., 1999, Zhou and Salvesen, 1997, Vancompernolle et al., 1998). Cathepsins are additionally involved in p53-dependent apoptosis, as two p53 binding sites exist in the cathepsin D promoter and cathepsin D inhibitors suppress p53-induced cell death (Wu et al., 1998).

Calpains

Calpains are a group of cytosolic cysteine proteases that are activated by increases in the levels of intracellular free Ca2+. Studies have shown calpains to become activated during apoptosis in a variety of cell types (Squier et al., 1994, Debiasi et al., 1999, Knepper-Nicolai et al., 1998), and calpain inhibitors have an inhibitory effect on cell death in response to some stimuli but not others (Squier et al., 1999, Gressner et al., 1997). A large number of calpain substrates have been identified, including p53 (Kubbutat and Vousden, 1997), Bax (Wood et al., 1998), and procaspases-3 and -9 (Wolf et al., 1999). Calpain also cleaves several elements of the cytoskeleton, suggesting it may play a role in destruction of the cellular architecture during apoptosis (Knepper-Nicolai et al., 1998). The nature of crosstalk between calpain and caspases remains to be elucidated (and may be dependent on cell type and the

nature of the stimulus), with some studies placing calpain upstream of caspase activation (Waterhouse et al., 1998), some placing it downstream (Wood and Newcomb, 1999, Porn-Ares et al., 1998), and some indicating that caspases are not required for calpain activation (Wolf et al., 1999).

Granzymes

CTLs and NK cells induce apoptosis in target cells by expressing death receptor ligands on their surface (Montel et al., 1995, Suda et al., 1995) to activate the death receptor pathway of apoptosis in target cells. CTLs and NK cells can also induce apoptosis through 'granule exocytosis' which involves secretion of perforins and granzymes into the extracellular environment. Perforins insert into the target cell membrane and form pores, leading to cell permeabilisation and possibly aiding entry of granzymes (Masson and Tschopp, 1985, Young et al., 1986). Granzyme B cleaves and activates pro-caspases-3, -7, and -9 through its serine protease activity (Gu et al., 1996, Quan et al., 1996, Duan et al., 1996). In addition, granzyme B regulates Bcl-2 proteins through cleavage of pro-apoptotic Bid (Heibein et al., 2000) and by degrading anti-apoptotic Mcl-1 (Han et al., 2004). Caspase-independent cleavage of ICAD by granzyme B has also been reported, triggering apoptotic DNA fragmentation (Thomas et al., 2000, Sharif-Askari et al., 2001).

The proteasome

An increase in the abundance of proteasomal subunits and proteasomal activity have been observed during apoptosis (Dawson et al., 1995, Jones et al., 1995), and proteasome inhibitors can inhibit or activate apoptosis depending on the cell type and apoptosis inducer (Grimm et al., 1996, Meriin et al., 1998, Imajoh-Ohmi et al., 1995). Proteasome inhibitors can increase caspase-3 activity, suggesting that the proteasome protects cells from apoptosis by degrading caspase-3 or its upstream proteases (Fujita et al., 1996a, Drexler, 1997). The proteasome also plays an important role in p53 and NF-kB signalling pathways. Mdm2 and IkB, which negatively regulate p53 and NF-kB respectively, are degraded by the proteasome following their ubiquitination (Chang et al., 1998, Chen et al., 1995). Bax, the proapoptotic Bcl-2 protein, is also degraded by the proteasome (Chang et al., 1998).

1.2.2 Death receptor-mediated apoptosis

1.2.2.1 Death receptors and their ligands

The tumour necrosis factor (TNF) superfamily of death receptor (DR) ligands contains 19 proteins that are involved in the control of the immune and inflammatory responses (reviewed in Bodmer et al., 2002). They are expressed on the surfaces of cells of the immune system, while their receptors are widely expressed across different cell types. Activation of the TNF receptors therefore occurs during contact between immune cells and other cell types. Activation can also occur in the absence of cell to cell contact, as soluble ligands (particularly TNF-α) can be formed following their cleavage from the cell surface (McGeehan et al., 1994, Mohler et al., 1994). The best characterised of these ligands are the prototypical member TNF-α, Fas ligand (FasL) and TNF-related apoptosis inducing ligand (TRAIL). TNF-α is secreted by activated T cells and macrophages in response to infection, while FasL is expressed on the surface of CTLs and NK cells to mediate destruction of virus-infected cells. TNF-α, FasL and TRAIL all contain a conserved TNF homology domain (THD) which is responsible for binding the appropriate DR (TNFR1, Fas [or CD95] and DR4/DR5 respectively). The receptors are required to oligomerise prior to ligand binding in order to function (Chan et al., 2000); their cytoplasmic domains can then form platforms for the binding of one of two classes of signalling molecules; the TRAFs or the 'death domain' (DD) containing proteins (Locksley et al., 2001).

1.2.2.2 Signalling downstream of death receptors

Following ligand-binding, DRs promote apoptosis through the formation of the multi-protein death-inducing signalling complex (DISC), which comprises Fas-associated DD-containing protein (FADD) and procaspase-8 (Figure 1.7, reviewed by Peter and Krammer, 2003). The receptors recruit FADD through its DD: while Fas and DR4/DR5 can associate directly with FADD, TNFR1 requires TRADD as an intermediate adaptor for FADD recruitment. Procaspase-8 is activated by its association with the DISC (Kischkel et al., 1995, Medema et al., 1997) and activates the caspase cascade through downstream cleavage of caspase-3 and other pro-apoptotic proteins (Li et al., 1998a, Scaffidi et al., 1998). Caspase-10 can also be recruited to the DISC and can initiate apoptosis, even in the absence of caspase-8 (Kischkel et al., 2001, Wang et al., 2001b). While one study indicates caspase-10 cannot substitute functionally for caspase-8 (Sprick et al., 2002), caspase-10 still

appears to play an important role in apoptosis since defective caspase-10 has been implicated in apoptosis abnormalities seen in certain autoimmune diseases (Wang et al., 1999b).

In addition to inducing apoptosis, DR stimulation can also activate anti-apoptotic NF-kB signalling. TNF-α and FasL promote NF-kB activation by distinct mechanisms: TNFR1-mediated activation requires TRADD and RIP1, while Fas-mediated activation requires FADD and caspase-8 (Imamura et al., 2004). TNFR1 is thought to promote sequential assembly of two distinct complexes at its cytoplasmic tail (Micheau and Tschopp, 2003). Immediately following TNF-α-binding, TNFR1 recruits TRADD, RIP1, and TRAF2 (**Figure 1.8A**): TRAF2 ubiquitinates RIP1, a modification that recruits the NEMO subunit of the IKK complex, leading to NF-kB activation. It is worth noting that this complex exhibits many similarities to the NF-kB-activating complex formed downstream of RIG-I/mda-5 activation by viral RNA (**Section 1.1.2.2**). At later times (at least 2h (Muppidi et al., 2004)) following receptor activation, TRADD, RIP1 and TRAF2 dissociate from TNFR1, and recruit FADD and procaspase-8 to form the DISC. The temporal delay between the formation of these complexes allows sufficient time for the expression of NF-kB-induced anti-apoptotic proteins cFLIP (cellular FLICE inhibitory protein), cellular inhibitor of apoptosis 1 (cIAP-1), cIAP-2, TRAF 1 and TRAF2 that prevent induction of apoptosis by the DISC (Wang et al., 1998a). If NF-kB signals are absent, these anti-apoptotic proteins are not upregulated and DISC formation results in cell death. The sequential formation of these two complexes is thought to be achieved through receptor internalisation (**Figure 1.8**, reviewed by Schutze et al., 2008). While TNFR1 is present at the cell-surface, it mediates anti-apoptotic NF-kB signalling; following clathrin-dependent endocytosis of the receptor however, RIP1 is degraded and TRADD recruits DISC components to mediate pro-apoptotic signalling (**Figure 1.8A**). Fas is unable to bind TRADD, so Fas-activation of NF-kB occurs in a TRADD-independent manner. Following Fas activation, low amounts of FADD and procaspase-8 are recruited to the receptor and activate NF-kB; however, following clathrin-dependent receptor internalisation, higher levels of these DISC components bind the receptor to promote apoptosis (**Figure 1.8B**).

1.2.2.3 Regulation of death receptor-mediated apoptosis

Caspase-8 activity is negatively regulated at the level of the DISC by cFLIP which is recruited to these complexes through its death effector domains (DEDs) (Irmler et al.,

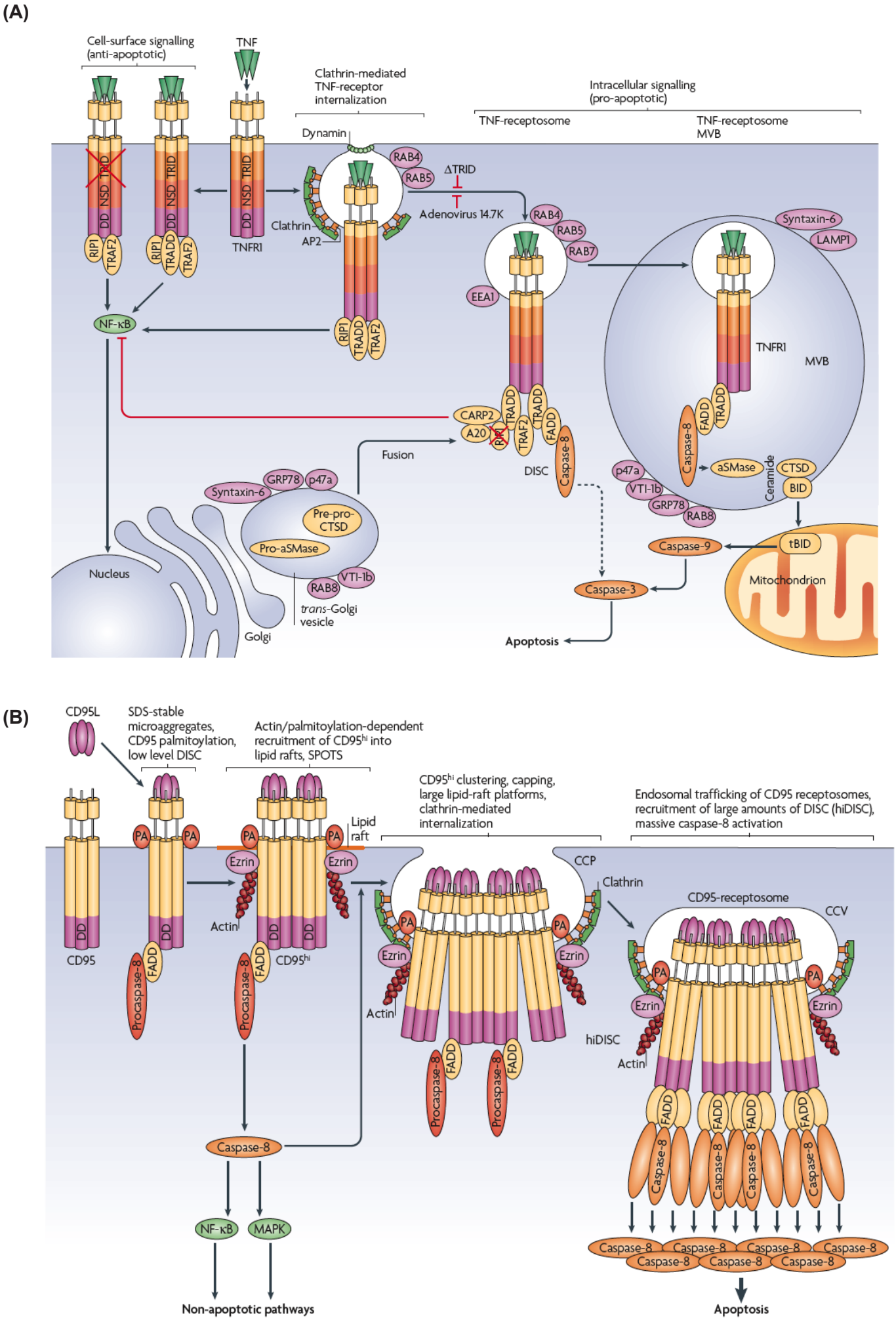

(A)
Cell-surface signalling (anti-apoptotic)
TNF
Clathrin-mediated TNF-receptor internalization
Intracellular signalling (pro-apoptotic)
TNF-receptosome
TNF-receptosome MVB
Dynamin
RAB4
RAB5
ΔTRID
Adenovirus 14.7K
Clathrin
AP2
TNFR1
RIP1
TRADD
TRAF2
NF-κB
EEA1
RAB7
Syntaxin-6
LAMP1
MVB
CARP2
A20
FADD
Caspase-8
DISC
Fusion
aSMase
CTSD
BID
Ceramide
p47a
VTI-1b
GRP78
RAB8
Syntaxin-6
Pre-pro-CTSD
Pro-aSMase
trans-Golgi vesicle
Golgi
Nucleus
Caspase-9
tBID
Mitochondrion
Caspase-3
Apoptosis
(B)
CD95L
SDS-stable microaggregates, CD95 palmitoylation, low level DISC
Actin/palmitoylation-dependent recruitment of CD95hi into lipid rafts, SPOTS
CD95hi clustering, capping, large lipid-raft platforms, clathrin-mediated internalization
Endosomal trafficking of CD95 receptosomes, recruitment of large amounts of DISC (hiDISC), massive caspase-8 activation
Lipid raft
PA
Ezrin
Actin
CD95
FADD
Procaspase-8
CD95hi
CCP
Clathrin
CD95-receptosome
CCV
hiDISC
Caspase-8
NF-κB
MAPK
Non-apoptotic pathways
Apoptosis

Figure 1.8: Activation of cell survival and apoptotic pathways downstream of death receptors.

(**A**) TNFR1 activates NF-kB through recruitment of TRADD, RIP1 and TRAF2. Soon after ligand binding, TNFR1 is internalized via clathrin-dependent endocytosis. NF-kB signalling is terminated by the recruitment of CARP2 and A20, leading to RIP1 degradation. TRADD then recruits FADD and procaspase-8 to TNFR1 to form the DISC. Activated caspase-8 is subsequently released, resulting in activation of the caspase cascade and induction of apoptosis. Further along the endocytic pathway, TNF-receptosomes form multivesicular bodies that can mediate apoptosis through cleavage of BID and activation of caspase-9.
(**B**) Soon after activation of Fas/CD95, low levels of FADD and procaspase-8 are recruited to the receptor. Signalling protein oligomerization transduction structures (SPOTS) are formed, resulting in Fas/CD95 clustering (a process known as 'capping') which depends on active caspase-8. This leads to the formation of large lipid-raft platforms. At this stage, Fas/CD95 can promotes cell survival through NF-kB and MAPK activation, but not apoptosis. 5-15 minutes after receptor activation, Fas/CD95 is internalized by clathrin-mediated endocytosis. During endosomal trafficking, high levels of DISC components FADD and caspase-8 are recruited, resulting in significant caspase-8 activation and apoptosis. *From Schutze et al., 2008.*

1997, Sharp et al., 2005, Micheau and Tschopp, 2003). Several mRNA splicing variants of cFLIP exist, however, only two have been detected at the protein level; $cFLIP_L$ and the shorter $cFLIP_S$. $cFLIP_S$ is an enzymatically inactive homologue of caspase-8 and is well-characterised as a caspase-8 inhibitor (Krueger et al., 2001, Scaffidi et al., 1999). Although the $cFLIP_L$ variant is also regarded as a caspase-8 inhibitor (Irmler et al., 1997, Sharp et al., 2005), it has been suggested that $cFLIP_L$ can act as either an inhibitor or an activator depending on its expression levels (Chang et al., 2002). cFLIP can also activate both NF-kB and ERK signalling pathways in human T lymphocytes through its interaction with components of these pathways (Kataoka et al., 2000). cIAP-1 negatively regulates apoptosis by forming part of an anti-apoptotic protein complex that assembles at DRs and prevents caspase-8 activation (**Figure 1.7**, Sun et al., 2008b). The IAPs can also antagonise DR signalling by inhibiting effector caspase activity (see below).

1.2.3 Mitochondria-mediated apoptosis

Intracellular stresses such as DNA damage, reactive oxygen species and treatment with certain drugs affect the integrity of the mitochondria. If these stresses are sufficient, permeabilisation of the mitochondrial membrane occurs. Cytochrome c is subsequently released from the mitochondrial intermembrane space into the cytosol (Liu et al., 1996). Here, cytochrome c combines with APAF-1 (apoptotic protease activating factor-1), procaspase-9 and dATP to form a pro-apoptotic complex, the 'apoptosome' (**Figure 1.7**). Active caspase-9 is released from this complex and can activate downstream caspases (Zou et al., 1999).

1.2.3.1 Regulation of mitochondria-mediated apoptosis

The Bcl-2 proteins

The apoptosome is regulated by the B cell lymphoma 2 (Bcl-2) family of proteins. There are over 20 different Bcl-2 proteins, each containing a conserved Bcl-2 homology (BH) domain, which have either pro- or anti-apoptotic properties. Anti-apoptotic family members include Bcl-2 proper, $Bcl\text{-}X_L$, Bcl-w, A1 and Mcl1, while the pro-apoptotic members can be further subdivided into the multi-domain Bax family and the BH3-only proteins (Cory and Adams, 2002).

Anti-apoptotic Bcl-2 proteins are associated with intracellular membranes, including those of the mitochondria, nucleus and endoplasmic reticulum (ER). Bcl-2 is an integral membrane protein (Janiak et al., 1994), while Bcl-w and Bcl-X_L only associate with the membrane in response to apoptotic signals (Cory and Adams, 2002). Studies in *Caenorhabditis elegans* have shown that in normal cells, CED-9, a Bcl-2 homologue, sequesters CED-4 (the APAF-1 homologue), anchors it to the mitochondrial membrane and prevents it from activating the caspase CED-3 (Spector et al., 1997). In apoptotic cells however, CED-4 is displaced from CED-9 by the BH3-only protein EGL-1, and CED-4 adopts a perinuclear localisation (Chen et al., 2000).

The Bax family of pro-apoptotic Bcl2 proteins include the widely expressed Bak and Bax. Disruption of Bak or Bax alone has a negligible effect on apoptosis, but disruption of both proteins dramatically impairs apoptosis (Lindsten et al., 2000). During apoptosis, Bax, normally present as a monomer in the cytosol, becomes inserted in the outer mitochondrial membrane, where it oligomerises (Nechushtan et al., 2001, Antonsson et al., 2001). In quiescent cells, Bak exists in the mitochondrial membrane as an oligomer, but during apoptosis it changes conformation (Griffiths et al., 1999). These Bax and Bak oligomers affect mitochondrial membrane permeability and promote cytochrome c release (Pastorino et al., 1998, Gross et al., 1998). This is thought to occur in one of two ways; either by forming pores themselves (Antonsson et al., 2001, Saito et al., 2000, Pavlov et al., 2001) or by interacting with and enlarging existing mitochondrial permeability transition pores (Tsujimoto and Shimizu, 2000).

Eight or more BH3-only proteins exist, each containing a BH3 domain which is responsible for the ability of these proteins to bind to and inactivate anti-apoptotic proteins such as Bcl-2 (Chittenden et al., 1995). In addition, the BH3 domain of Bid is able to bind and activate pro-apoptotic Bax and Bak (Wang et al., 1996). It has been suggested that Bid acts by promoting Bax and Bak oligmerisation, forming pores in the mitochondrial membrane and releasing cytochrome c into the cytosol (Korsmeyer et al., 2000).

IAPs

The IAP family of apoptosis regulators inhibit apoptosis in a number of distinct ways (reviewed by Liston et al.). The IAP BIR (baculoviral IAP repeat) domain mediates associations with the initiator caspase-9 and the effector caspases-3 and -7 (Roy et

al., 1997, Takahashi et al., 1998, Maier et al., 2002) and inhibits caspase activity by either precluding substrate entry to the caspase active site (Huang et al., 2001, Chai et al., 2001) or by stabilising the inactive form of the enzyme (Shiozaki et al., 2003). The ring zinc finger (RZF) motif of IAPs has also been implicated in apoptosis inhibition: the RZFs of cIAP2 and XIAP promote ubiquitination and degradation of caspase-3 and caspase-7 (Huang et al., 2000, Suzuki et al., 2001).

1.2.4 Endoplasmic reticulum-mediated apoptosis

The presence of Bcl-2 family members in ER membranes (Krajewski et al., 1993) led to the suggestion that they may modulate Ca2+ release from the ER (Baffy et al., 1993, Lim et al., 2008). Ca2+ is a versatile second messenger that mediates both cell survival and cell death (Rong and Distelhorst, 2008). Proapoptotic Ca2+ signals are enhanced by Bax, Bak and the BH3-only proteins. Bax and Bak stimulate the rearrangement of calcium stores from the ER to the mitochondria (Nutt et al., 2002). The BH3-only protein Bik enhances Ca2+ release from the ER (Mathai et al., 2005), while Bid increases the uptake of Ca2+ by the mitochondria (Csordas et al., 2002). In contrast, the anti-apoptotic Bcl-2 proteins inhibit calcium release from the ER (Distelhorst et al., 1996, Pinton et al., 2001, Der et al., 1998b) through interaction with IP3 receptor calcium channels to prevent their opening (Chen et al., 2004). Interestingly, Bcl-2 also inhibits the uptake of Ca2+ by the nucleus, suggesting a role for the nucleus in apoptosis induction (Marin et al., 1996).

1.2.5 Regulation of apoptosis by IFN and STAT1

Apoptosis plays a critical role in the IFN-induced antiviral state. Induction of cell death during the early stages of virus infection significantly limits viral replication, and reduces or eliminates the spread of progeny virus (Clem and Miller, 1993). Type I and type II IFNs can induce apoptosis in a wide range of cell-lines (Chawla-Sarkar et al., 2001, Morrison et al., 2001, Chen et al., 2001a, Dai and Krantz, 1999, Thyrell et al., 2002, Sangfelt et al., 1997a) and as a consequence, treatment with IFN is used in the clinic to treat certain cancers (Pestka et al., 2004). Selective induction of apoptosis in virus-infected cells (while leaving uninfected cells viable) is IFN-dependent (Tanaka et al., 1998, Sedger et al., 1999). The mechanism by which IFN induces apoptosis is similar to the death-receptor-mediated pathway and involves FADD and caspase-8: in the presence of caspase-8 inhibitors or knockdown of FADD, IFN treatment has no effect on cell viability (Balachandran et al., 2000). Many

cell-lines are resistant to apoptosis induced by IFN alone, but IFN pre-treatment sensitises these cells to apoptosis in response to other stimuli, such as Fas agonist antibody (Ahn et al., 2002, Kurdi and Booz, 2007), TNF-α (Kulkarni et al., 2006), dsRNA (Stewart et al., 1973, Stewart et al., 1972) or IAV infection (Balachandran et al., 2000). Additionally, EMCV, VSV and HSV-induced apoptosis can be inhibited by making cells unable to respond to IFN (Tanaka et al., 1998). Together, these studies indicate that the IFN-induced antiviral state can stimulate apoptosis in the presence of additional stimuli (Barber, 2001). While IFN can promote apoptosis in response to infection with IAV, IFNs inhibit apoptosis induced by VSV and Sindbis virus (Balachandran et al., 2000), presumably through inhibition of viral protein synthesis and replication early in infection (Harrell et al., 1982, Julkunen et al., 1982, Despres et al., 1995). The antiviral state can therefore promote either cell survival or apoptosis depending on the viral stimulus (Balachandran et al., 2000).

As a component of the ISGF3 and GAF transcription factors, STAT1 plays an integral role in IFN-dependent ISG induction. Many of these ISGs are involved in apoptosis regulation; as a result, constitutive STAT1 activation enhances IFN-induced pro-apoptotic signalling (Sironi and Ouchi, 2004). At least 15 ISGs with pro-apoptotic functions have been identified by microarray (Der et al., 1998b, de Veer et al., 2001), including STAT1 itself (Der et al., 1998b), procaspases (Refaeli et al., 2002, Chin et al., 1997), DRs (Meng and El-Deiry, 2001, Lee et al., 2000b, Xu et al., 1998) and DR ligands (Xu et al., 1998). In addition, STAT1 has been implicated in regulation of apoptosis in response to DNA damage or stress stimuli such as heat or ischaemia (Janjua et al., 2002, Stephanou et al., 2000b, Stephanou et al., 2001, Townsend et al., 2004). STAT1-null cells are resistant to TNF-α-induced apoptosis (Kumar et al., 1997); interestingly, apoptosis sensitivity could be restored by expression of a STAT1 variant that is unable to undergo tyrosine phosphorylation and dimerisation. Similarly, STAT1 promotion of IFN-γ-induced apoptosis in lymphocytes is only partially dependent on IFN-γ signalling (Lee et al., 2000a). These data indicate STAT1 can additionally regulate transcription of a number of genes independently of ISGF3/GAF formation. STAT1 can also interact with a number of cellular signalling proteins to modulate apoptosis by transcription-independent mechanisms. The different mechanisms by which STAT1 promotes apoptosis are summarised in **Figure 1.9**.

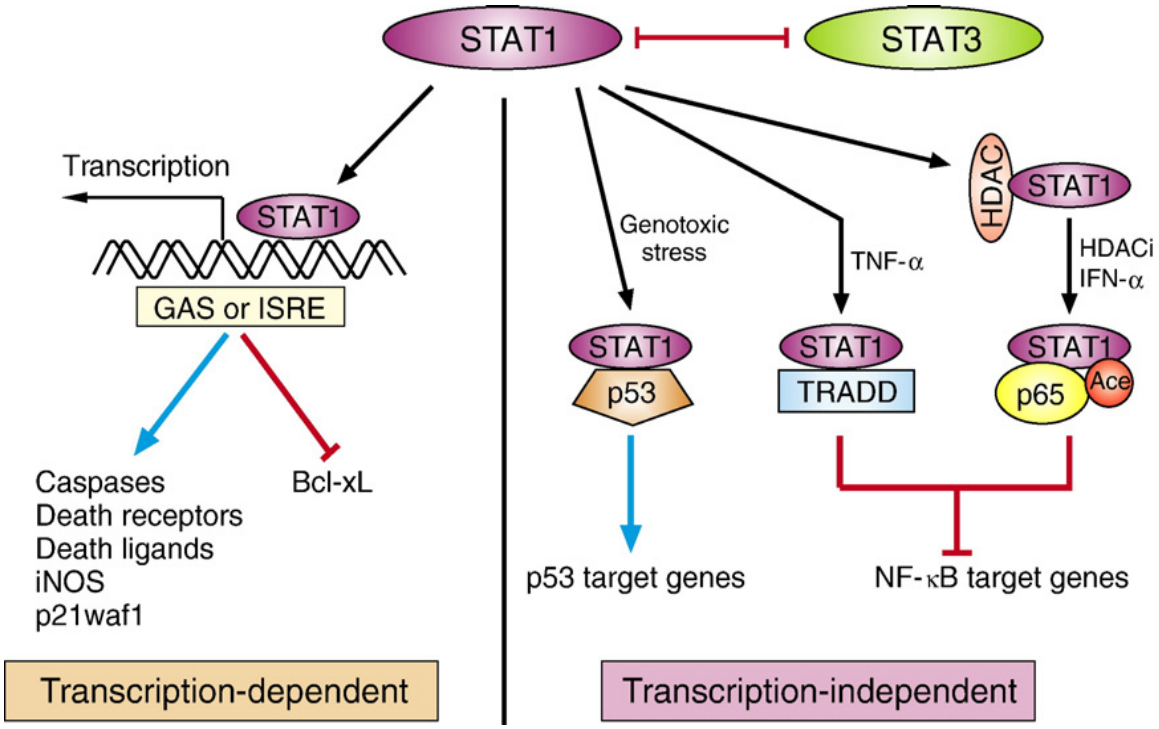


Figure 1.9: Regulation of cell death by STAT1.

STAT1 activates or suppresses transcription of a number of genes encoding apoptosis regulators, including procaspases, death receptors and their ligands, and Bcl-2 proteins. In addition, STAT1 can modulate apoptosis through transcription-independent mechanisms: STAT1 interacts with TRADD and p53 to enhance apoptosis. Inhibition of the interaction between HDAC and STAT1 by HDAC inhibitor or IFN-α promotes STAT1 acetylation and a direct interaction with the p65 subunit of NF-kB to inhibit NF-kB signalling. *From Kim and Lee, 2007.*

1.2.5.1 Pro-apoptotic ISGs regulated by ISGF3/GAF

Death receptors and their ligands

Pre-treatment with type I or type II IFNs sensitises a variety of cell types to Fas-mediated apoptosis by enhancing Fas expression (Xu et al., 1998, Spets et al., 1998, Ahn et al., 2002). However, increasing Fas expression alone is not always sufficient to sensitise cells to Fas-mediated apoptosis, as Fas expression can be detected on the surface of resistant cells (Ugurel et al., 1999). It is likely then that the sensitisation effect of IFN is due to the combined upregulation of pro-apoptotic ISGs and downregulation of anti-apoptotic genes. In addition to upregulating the expression of Fas, IFN-γ has also been reported to stimulate relocation of the Fas receptor from intracellular vesicles to the surface of vascular smooth muscle cells by a PI3K/Akt-dependent mechanism (Rosner et al., 2006). TNFR expression on the surface of certain cancer cells is similarly elevated following treatment with IFN-γ (Ossina et al., 1997, Aggarwal et al., 1985). This regulation is indirect as production of TNFR mRNA requires protein synthesis, and likely requires the expression of an IFN-γ-responsive transcription factor, such as IRF1 (Ossina et al., 1997). The role of IFN in regulation of TNFR expression is unclear however, as both IFN-α and IFN-γ inhibit lipopolysaccharide (LPS)-induced transcription of the TNFR gene (Tannenbaum et al., 1993). Enhancement of DR expression by IFNs presumably increases the sensitivity of infected target cells to TNF-α and FasL-mediated CTL and NK cell cytotoxicity.

IFNs upregulate TRAIL expression to induce apoptosis (Chen et al., 2001a, Kayagaki et al., 1999). Expression of dominant-negative TRAIL receptors, or neutralising antibody to TRAIL therefore inhibits the pro-apoptotic effect of IFN (Chawla-Sarkar et al., 2001, Chen et al., 2001a). FasL expression can also be enhanced by IFN-γ in colon adenocarcinoma cells (Xu et al., 1998) while IFN-α stimulates FasL transcription and surface expression in peripheral blood mononuclear cells (Kirou et al., 2000, Kaser et al., 1999).

Procaspases

Expression of procaspases-4 and -8 is increased by type I and type II IFNs (de Veer et al., 2001); IFN-γ additionally induces transcription of procaspase-1, -3 and -12 genes, and may also modulate procaspase-7 and procaspase-10 expression (Kalai

et al., 2003, Chin et al., 1997, Tamura et al., 1996, Dai and Krantz, 1999, Ossina et al., 1997).

Bcl-2 proteins

The pro-apoptotic Bcl-2 family members Bak and Bax are upregulated by IFN-γ (Soond et al., 2007, Der et al., 1998b, Ossina et al., 1997) while anti-apoptotic Bcl-2 is down regulated (Zhou et al., 2008).

PKR

In addition to its inhibitory effects on translation (see **Section 1.1.4.1**), PKR also plays an important role in apoptosis regulation. Studies into the effects of PKR on translation demonstrated a growth suppressive phenotype in cells overexpressing PKR which was reversed by the expression of PKR with a defective catalytic domain (Barber et al., 1993, Chong et al., 1992). In other studies, expression of a dominant-negative mutant of PKR caused malignant transformation of murine fibroblasts (Balachandran et al., 1998, Koromilas et al., 1992, Meurs et al., 1993). The tumour-suppressive effect of PKR was later shown to be due to the induction of apoptosis (Lee and Esteban, 1994). Knockout of PKR or expression of kinase inactive variants inhibited apoptosis induction by dsRNA (Balachandran et al., 1998, Der et al., 1997), TNF-α (Der et al., 1997, Yeung et al., 1996) and IAV (Takizawa et al., 1996). The mechanisms by which PKR causes apoptosis have not been fully elucidated, but it appears PKR may perform multiple roles. It has been suggested that inhibition of protein synthesis may be important for PKR-induced apoptosis: inhibition of the translational machinery by PKR may suppress expression of anti-apoptotic proteins. In support of this, PKR-induced apoptosis was blocked by expression of eIF2α that cannot undergo PKR-mediated phosphorylation (Gil et al., 1999), and expression of an eIF2α variant that mimics phosphorylated eIF2α was sufficient to induce apoptosis (Srivastava et al., 1998). Inducible expression of a dominant-negative PKR also reduced transcription of several pro-apoptotic genes, including Fas, TNFR-1, FADD, caspase-8, Bax and Bad, in response to dsRNA treatment (Balachandran et al., 1998). A substrate of PKR is the NF-kB inhibitor IkB, which is degraded following PKR-mediated phosphorylation to activate NF-kB (Kumar et al., 1994). Expression of an unphosphorylatable IkB variant suppresses activation of NF-kB by PKR and inhibits PKR-induced apoptosis (Gil et al., 1999). Several pro-apoptotic proteins are NF-kB-responsive, including Fas (Chan et al., 1999, Kuhnel et al., 2000), the TNFR-

related DR6 (Kasof et al., 2001) and the TRAIL receptor (Ravi et al., 2001), so PKR could exert its pro-apoptotic effects through enhanced DR expression. However, NF-kB additionally up-regulates a number of anti-apoptotic proteins including cIAPs (Hong et al., 2000, Wang et al., 1998b) and cFLIP (Kreuz et al., 2001). p53 may also be required for PKR-induced apoptosis. PKR and p53 can directly associate to mediate p53 activation (Cuddihy et al., 1999b) and p53 activity is inhibited in PKR knockout cells as evidenced by a reduction in p21Waf1 and Mdm2 expression (Cuddihy et al., 1999a). PKR may therefore function as a dsRNA receptor that triggers induction of pro-apoptotic genes and apoptosis (see **Section 1.2.7**).

2'-5'-OAS/RNaseL

Transient transfection of OAS into cancer cell-lines reduces colony growth by inducing apoptosis and IFN-γ-induced apoptosis requires OAS induction (Mullan et al., 2005). Pro-apoptotic OAS activity appears to be limited to one splice variant of OAS1, termed the 9-2 isozyme, which promotes apoptosis in mammalian cells, possibly through interactions with Bcl-2 and Bcl-X_L (Ghosh et al., 2001). In addition, activation of RNase L by OAS-synthesised oligoadenylates induced apoptosis in fibroblasts, while expression of a dominant-negative form of RNaseL rendered cells resistant to apoptosis induced by dsRNA, staurosporine or poliovirus infection (Castelli et al., 1997, Castelli et al., 1998). Additionally, spontaneous apoptosis occurs at a reduced rate in the thymus and spleen of RNaseL-null mice (Zhou et al., 1997) and mutations in the RNaseL gene have been linked to the development of prostate cancer in several studies (Carpten et al., 2002, Rokman et al., 2002, Wang et al., 2002b, Rennert et al., 2002, Casey et al., 2002).

PML

Overexpression of PML leads to rapid induction of cell death (Quignon et al., 1998) and PML expression is essential for apoptotic responses to agonist Fas antibody, TNF-α, ceramide and type I and II IFNs (Wang et al., 1998c). More recent studies implicate p53 in PML-induced apoptosis. p53 is recruited to PML NBs where it undergoes PML-mediated acetylation, which is essential for p53 activation and p53-induced apoptosis (Pearson et al., 2000, Fogal et al., 2000, Guo et al., 2000, Bischof et al., 2002). Additionally, PML bodies recruit a number of proteins involved in p53 activation or stabilisation, leading to the suggestion that PML NBs act as a scaffold for p53 recruitment and regulation (Takahashi et al., 2004). PML NBs have also been

linked to regulation of DR-mediated apoptosis through recruitment of CASP8AP2 (caspase-8 associated protein 2) and Daxx (reviewed in Krieghoff-Henning and Hofmann, 2008). A caspase-independent pathway of PML-mediated apoptosis activation has additionally been described that is independent of *de novo* transcription (Quignon et al., 1998).

XAF-1

The IFN-stimulated XIAP-associated factor (XAF-1) is an interactor of XIAP (Leaman et al., 2002, Liston et al., 2001). One of the human IAPs, XIAP directly binds and inhibits caspases-3, 7 and 9 (Deveraux et al., 1998, Deveraux et al., 1997). Additionally, XIAP promotes cell-survival through activation of NF-kB and JNK1 (Hofer-Warbinek et al., 2000). XAF-1 overexpression prevents inhibition of caspase activity by XIAP (Liston et al., 2001), and the XAF-1/XIAP complex promotes the degradation of an additional IAP family member, survivin, by a ubiquitin/proteasome-mediated pathway (Arora et al., 2007).

IRFs

The role of IRF3 in apoptosis induction will be discussed in **Section 1.2.7**. Additional members of the IRF family have been linked to apoptosis regulation. Inactivation of IRF1 leads to accelerated cell transformation (Taniguchi et al., 1998) while overexpression of IRF1 leads to caspase-1 activation and sensitises cells to radiation-induced apoptosis in T lymphocytes (Tamura et al., 1995). IRF1 may additionally be required for IFN-induced upregulation of TNFR (Ossina et al., 1997). IFN-γ-inducible IRF8 is expressed only in lymphoid cells and macrophages and appears to be required for DNA-damage and IFN-γ-induced apoptosis, possibly through upregulation of Fas expression (Yang et al., 2007b) and downregulation of Bcl-X_L (Gabriele et al., 1999).

p53

Expression of p53 is enhanced by type I IFNs (Takaoka et al., 2003). While IFN treatment does not activate p53, subsequent infection with VSV, NDV or HSV does, and induces an apoptotic response that is independent of IFN (Takaoka et al., 2003). Although the mechanism of p53 activation by virus infection is unknown, current

evidence indicates an involvement for PKR, which can directly bind p53 to enhance its transcriptional activity (Cuddihy et al., 1999a, Cuddihy et al., 1999b).

1.2.5.2 ISGF3/GAF-independent STAT1 transcription

In addition to its role in ISG expression in response to IFN, STAT1 can also regulate ISG transcription independently of IFN. For example, while several procaspases are upregulated by IFN, procaspase expression can also be stimulated by STAT1 in an IFN-independent manner. STAT1 is required for constitutive expression of procaspases-1, 2 and 3 in human fibroblasts (Kumar et al., 1997) and expression of procaspases-1 and 11 in murine lymphocytes (Lee et al., 2000a): tyrosine phosphorylation and dimerisation is not required for this role of STAT1 (Kumar et al., 1997). Instead, constitutive procaspase expression and induction of cell death by STAT1 appears to require Ser727 phosphorylation (Kumar et al., 1997), suggesting a role for serine-phosphorylated, STAT1 monomers in pro-apoptotic gene transcription. Indeed, serine-phosphorylated STAT1 plays a critical role in the pathogenesis of the kidney cancer Wilms' tumour (Timofeeva et al., 2006). PKCδ, the enzyme thought to mediate STAT1 serine phosphorylation (Uddin et al., 2002), is required for mitochondria-mediated apoptosis (Matassa et al., 2001); DNA-damage induces apoptosis through PKCδ-dependent STAT1 serine phosphorylation and nuclear translocation (DeVries et al., 2004). Non-tyrosine phosphorylated, serine-phosphorylated STAT1 can also up- or downregulate constitutive expression of a number of additional ISGs: upregulated genes include those involved in MHC-mediated antigen presentation and the CDK inhibitor p21Waf1, while downregulated genes include Bcl-2, Bcl-X_L and cell cycle regulators (Chatterjee-Kishore et al., 2000, Agrawal et al., 2002, Chin et al., 1996, Stephanou et al., 2000a). Additionally, constitutive expression of the 20S proteasome subunit LMP2 is regulated by binding of a complex consisting of non-tyrosine-phosphorylated STAT1 and IRF1 to a GAS element (Chatterjee-Kishore et al., 2000).

1.2.5.3 Transcription-independent apoptosis regulation by STAT1

STAT1 can also modulate apoptosis independently of transcription, through its interactions with signalling proteins. STAT1 directly binds both TNFR1 and TRADD to inhibit formation of the TRADD/RIP/TRAF2 complex and suppress NF-kB activation. Although STAT1 appears to interact with TRADD in quiescent cells (Wesemann and Benveniste, 2003, Pajak and Orzechowski, 2007), treatment of

HeLa cells with TNF-α increases the STAT1:TRADD association, leading to inhibition of NF-kB activity and TNF-α-induced apoptosis (Wang et al., 2000b). In contrast, IFN-α treatment diminishes the STAT1:TRADD interaction by shunting STAT1 into the JAK/STAT signalling pathway; consequently, in cells which are resistant to the cytotoxic effects of IFNs, IFN-α activates NF-kB and cell survival (Pajak and Orzechowski, 2007). In support of this, TNF-α-treatment of STAT1-deficient cells leads to enhanced NF-kB activation (Wesemann and Benveniste, 2003). Similarly, IFN-γ treatment abrogates recruitment of STAT1 to TNFR in macrophages by promoting STAT1 translocation to the nucleus. It appears then that IFN and TNF-α receptors compete for STAT1 to regulate NF-kB activation.

Acetylation of STAT1 at Lys410 and Lys413 is also implicated in regulation of NF-kB and cell death. Acetylated STAT1 interacts with the p65 subunit of NF-kB to inhibit NF-kB nuclear localisation and DNA binding activity (**Figure 1.9**, Kramer et al., 2006). Consequently, STAT1 acetylation diminishes expression of anti-apoptotic NF-kB-responsive genes to enhance the rate of apoptosis (Kramer et al., 2006). Thus, inhibition of HDAC activity sensitises colon cancer cells to IFN-γ-induced apoptosis, at least in part due to down-regulation of anti-apoptotic Bcl-X_L and survivin (Klampfer et al., 2004, Kramer et al., 2006).

STAT1 also appears to play an important role in p53 regulation. STAT1 directly interacts with p53, an association that is enhanced in response to DNA damage, and functions as a p53 coactivator to stimulate transcription of p53-responsive genes such as Bax, Noxa and Fas (Townsend et al., 2004). In addition, STAT1 negatively regulates expression of the p53 inhibitor Mdm2 (Townsend et al., 2004). The importance of STAT1 in p53-mediated apoptosis is demonstrated by a decreased susceptibility to apoptosis following DNA damage in STAT1-deficient cells (Townsend et al., 2004). p53 has also been implicated in the activation of STAT1: in response to genotoxic drugs, STAT1 becomes activated by p53 by a mechanism that does not require p53-transcriptional activity but that is dependent on association of p53:STAT1 with the proto-oncogene product c-Abl1 (Youlyouz-Marfak et al., 2008). Interestingly, PIAS1, an E3 ligase that binds and SUMOylates STAT1 (Liu et al., 1998, Ungureanu et al., 2003), also catalyses SUMO-conjugation of p53 (Kahyo et al., 2001, Schmidt and Muller, 2002). These data suggest that STAT1:p53 complexes may associate with different cellular proteins (PIAS1 or c-Abl1) to regulate either p53 or STAT1 activity, depending on other cellular signals.

1.2.6 Regulation of apoptosis by PI3K

While IFN upregulates many pro-apoptotic genes through activation of STATs, IFN also activates PI3K signalling, which promotes cell survival. Indeed, PI3K activation by IFN is able to protect against virus-induced cell death (Prejean et al., 2001). The activation of both pro- and anti-apoptotic pathways by IFN indicates that IFN-induced apoptosis is subject to complex regulation: IFN may cause either cell death or cell survival, depending on the extent of STAT and PI3K activation.

Many of the anti-apoptotic effects of PI3K are mediated by Akt, which phosphorylates a number of known apoptosis regulators (**Figure 1.5**). Indeed, Akt was first identified as an oncogene expressed by the mouse leukaemia virus (Bellacosa et al., 1991). Akt activation in response to IFN is thought to determine the sensitivity of cells to IFN-induced apoptosis. Colorectal cancer cells that are most resistant to IFN-induced apoptosis displayed induction of Akt activity following IFN-β treatment, while Akt activity was reduced in the most sensitive cells (Lei et al., 2005). PI3K/Akt is involved in NF-kB activation downstream of the type I IFN receptor (Section 1.1.3.4, Du et al., 2007, Yang et al., 2001), so in cells resistant to IFN-induced apoptosis, IFN presumably stimulates NF-kB survival pathways rather than apoptosis.

Akt can negatively regulate a number of pro-apoptotic BH3-only proteins at both the transcriptional level, through its effects on the transcription factors p53 and Forkhead box protein O (FOXO), and post-transcriptionally through their direct phosphorylation. Expression of constitutively active Akt stabilises Mdm2 by mediating its phosphorylation at two sites (Feng et al., 2004) and promotes its nuclear translocation, where it targets p53 for degradation and reduces its transcriptional activity (Mayo and Donner, 2001, Ogawara et al., 2002). Downstream transcriptional targets of p53 are involved in cell senescence, cell-cycle arrest and apoptosis. Transcription of two BH3-only proteins, PUMA and Noxa, is regulated by p53 and these proteins are important mediators of p53-regulated apoptosis in response to DNA damage (Villunger et al., 2003). Inhibition of PI3K by LY294002 upregulates the number of p53 and PUMA mRNA transcripts, leading to apoptosis in gastric cancer cells (Chun-gen Xing, 2008). Additionally, it has been shown that PI3K prevents p53 activation by inhibiting its phosphorylation at Ser6 (Filiz et al., 2008). Akt also negatively regulates the FOXO family of transcription factors (Tang et al., 1999) by promoting their phosphorylation at three sites. This impairs their DNA-binding activity and causes their nuclear export (Kops et al., 1999, Rena et al., 1999, Biggs et al.,

1999, Brownawell et al., 2001). Expression of FOXO-dependent genes, including the pro-apoptotic BH3-only proteins BIM and bNIP3 (Behzad et al., 2007, Tran et al., 2002, Dijkers et al., 2000a, Stahl et al., 2002) and the death-receptor ligands FasL and TRAIL (Modur et al., 2002, Ciechomska et al., 2003, Behzad et al., 2007), is subsequently inhibited. Transcription of anti-apoptotic Bcl-X_L is also indirectly suppressed by FOXO4 through its upregulation of the transcriptional repressor Bcl-6 (Tang et al., 2002). In addition, another PI3K-activated serine/threonine kinase, serum- and glucocorticoid-inducible kinase (SGK), phosphorylates FOXO3a and prevents apoptosis (Brunet et al., 2001).

In the absence of cell survival signals, pro-apoptotic BAD is found in association with the anti-apoptotic Bcl-X_L at the outer mitochondrial membrane. In response to cell survival signals, Akt directly phosphorylates BAD at Ser136 (Datta et al., 1997), resulting in the release of Bcl-X_L and activation of cell survival pathways in a number of cell types (Datta et al., 2000, Datta et al., 2002). Akt phosphorylates and inhibits GSK3, a serine-threonine kinase that regulates many physiological processes, including glucose and protein synthesis (Cross et al., 1995). A GSK3 substrate is the anti-apoptotic Bcl-2 protein Mcl-1, which becomes ubiquitinated and subsequently degraded following GSK3 phosphorylation, leading to apoptosis (Maurer et al., 2006). PI3K/Akt can also upregulate Bcl-2 and Mcl-1 at the transcriptional level to inhibit the mitochondrial apoptosis pathway. This is mediated by CREB (Skorski et al., 1997, Pugazhenthi et al., 2000, Wang et al., 1999c), which becomes directly phosphorylated by Akt. The ability of CREB to recruit its coactivator CBP is enhanced (Du and Montminy, 1998), enabling the activation of cAMP-responsive-elements (CREs) in the Bcl-2 promoter. In addition, Akt can directly phosphorylate caspase-9 at Ser196, inactivating it and offering partial resistance to mitochondrial apoptosis (Cardone et al., 1998).

The three main MAPK cascades, JNK, p38 and ERK, have all been implicated in apoptosis induction. Akt inhibits apoptosis activated by JNK and p38 through direct interaction with the apoptosis signal-regulating kinase (ASK) 1. ASK1 is a MAPK kinase kinase (MAPKKK) that acts upstream of JNK and p38 and interaction with Akt leads its phosphorylation and negative regulation (Kim et al., 2001). Akt also inhibits Raf/MEK/ERK signalling through direct interaction with, and phosphorylation of, Raf, which results in cell survival (Zimmermann and Moelling, 1999).

Activation of Akt protects cells from apoptosis in response to a number of stimuli, including TRAIL and TNF-α through activation of the NF-κB pathway (Bortul et al.,

2003, Suzuki et al., 2008, Reddy et al., 2000). Conversely, inhibition of PI3K/Akt by PTEN inhibits TNF-α-induced NF-κB activation and enhances apoptosis (Koul et al., 2006). A proposed mechanism of NF-κB activation is the direct phosphorylation of IKKα by Akt at Thr23 (Ozes et al., 1999). By activating NF-κB, Akt upregulates NF-κB-responsive genes, including anti-apoptotic cFLIP (Micheau et al., 2001, Bortul et al., 2003), TRAF1 and 2, A1/Bfl-1 (Wang et al., 1999a), cIAP1 and cIAP2 (Wang et al., 1998a, Deveraux et al., 1998) and A20, a negative regulator of NF-κB that has also been implicated in apoptosis inhibition (Sarma et al., 1995, Beyaert et al., 2000).

PI3K/Akt may also be able to act pro-apoptotically. Inhibition of PI3K/Akt can protect epidermal cells from FasL-mediated apoptosis (Lu et al., 2006a), and IFN-α-induced and IFN-γ-induced apoptosis have been shown to require PI3K and mTOR (Thyrell et al., 2004, Fang et al., 2006, Lekmine et al., 2003, Nguyen et al., 2001, Wen et al., 1995). It has recently been reported that GSK3 forms an anti-apoptotic protein complex DDX3 and cIAP1. This complex associates with DRs and inhibits DISC formation and caspase-8 activation; inhibitors of GSK3 (including PI3K/Akt) can therefore potentiate DR-stimulated apoptosis (Sun et al., 2008b). PI3K/Akt also promotes Fas receptor trafficking to the cell membrane (Rosner et al., 2006). Interestingly, in TNF-α-treated neutrophils, PI3K directly binds the TNFR and triggers the secretion of microbicidal oxygen radicals (Korchak and Kilpatrick, 2001).

1.2.7 Viral RNA induces apoptosis independently of IFN

Viral dsRNA exhibits potent cytotoxic activity that is independent of *de novo* protein synthesis (Cordell-Stewart and Taylor, 1973), indicating that dsRNA can cause apoptosis independently of IFN production. Indeed, virus infection induces apoptosis even in cells that are non-responsive to IFN (Weaver et al., 2001). Detection of cytoplasmic dsRNA or IAV infection by RIG-I in human keratinocytes and macrophages induces caspase-1 and -3 activation and subsequent apoptosis in a pathway that is dependent on MAVS (Rintahaka et al., 2008). Pro-apoptotic signalling by RIG-I/MAVS requires downstream activation of IRF3 (McAllister and Samuel, 2008, Holm et al., 2007) to stimulate transcription from a distinct subset of proapoptotic ISGs (Heylbroeck et al., 2000, Weaver et al., 2001, Weaver et al., 1998, McAllister and Samuel, 2008, Holm et al., 2007). Consequently, IRF3 is an absolute requirement for apoptosis induction of during infection with reovirus (Holm et al., 2007), Bunyamwera virus (Kohl et al., 2003), NDV (Weaver et al., 2001), and SeV (Heylbroeck et al., 2000, Peters et al., 2008). Constitutive expression of IRF3 is toxic

to cells and its inducible expression and activation is sufficient to cause apoptosis in a number of cell types (Heylbroeck et al., 2000, Weaver et al., 2001). IRF3-mediated apoptosis requires formation of the dsRNA activated factor DRAF-1 (composed of IRF3 and CBP/p300) to mediate gene transcription (Heylbroeck et al., 2000, Weaver et al., 2001, Weaver et al., 1998): DRAF-1 binds to promoter sequences containing the ISRE and several flanking adenine residues (Daly and Reich, 1993, Daly and Reich, 1995) to regulate expression of a subset of ISGs. Caspase-8 appears to be the predominant caspase involved in IRF3-mediated death, indicating the involvement of extrinsic apoptosis pathways (Heylbroeck et al., 2000). PKR also appears to be involved in this pathway: PKR activation by 5'-triphosphate RNA leads to apoptosis induction, and RNA-induced apoptosis is significantly reduced in PKR knockdown cells (McAllister and Samuel, 2008). Removal of 5'-triphosphate from the RNA stimulus decreases apoptosis without affecting PKR activation, while knockdown of MAVS further inhibits apoptosis in PKR-deficient cells (Zhang and Samuel, 2008, McAllister and Samuel, 2008), perhaps suggesting that PKR acts downstream of RIG-I but upstream of MAVS. Although the role of PKR in RIG-I/MAVS-mediated IRF3 activation is unclear, the reported interaction between TRAF3 and PKR (Oganesyan et al., 2006) makes it easy to speculate that PKR regulates IRF3 activation downstream of RIG-I through TRAF3 (Zhang and Samuel, 2008). In conclusion, viral nucleic acid stimulates both an IFN-mediated antiviral state and an IRF3-dependent pro-apoptotic program; these two pathways appear to involve many of the same molecules.

1.3 Viral inhibition of intracellular antiviral responses

The IFN and apoptosis responses are incredibly efficient at limiting virus replication. In order to establish infection *in vivo* therefore, viruses must evade these responses; consequently, many (if not all) viruses encode factors that antagonise IFN and/or apoptosis. The ways in which viruses antagonise IFN and apoptosis will be outlined in the following section.

1.3.1 IFN antagonism

The IFN response can be counteracted at a number of different stages, and the mechanisms by which viruses antagonise IFN have been the subject of several comprehensive reviews (Randall and Goodbourn, 2008, Goodbourn et al., 2000, Weber et al., 2004). Many viruses interfere with induction of IFN in response to viral stimuli (**Figure 1.10**), IFN signalling (**Figure 1.11**) or the functions of IFN-induced antiviral proteins. Additionally, global inhibition of transcription or translation is a common viral strategy for preventing IFN and ISG expression. The mechanisms by which viral IFN antagonists function vary extensively, and viruses often encode more than one antagonist activity to allow more efficient evasion of the IFN response. In some cases, these distinct inhibitory activities may be encoded by the same viral protein, particularly in the case of small RNA viruses where many proteins are multi-functional in order to maximise the coding capacity of the genome.

1.3.1.1 Inhibition of IFN induction

The mechanisms by which viruses interfere with IFN-α/β induction are summarised in **Figure 1.10**. Restricting the production of PAMPs or preventing their recognition by the IFN-induction machinery is of primary importance to the virus *in vivo*. Many viruses encode dsRNA-binding proteins that sequester dsRNA away from viral nucleic acid sensors. In addition, these proteins also prevent dsRNA-mediated activation of PKR and OAS. The dsRNA-binding affinity of the ς3 protein of reoviruses has been linked to strain-specific differences in IFN production (Bergeron et al., 1998). The vaccinia virus (VACV) E3L (Deng et al., 2008, Xiang et al., 2002) and Ebola virus (EBOV) VP35 (Cardenas et al., 2006, Hartman et al., 2006) proteins also bind intracellular dsRNA to antagonise IFN production. An alternative method for preventing IFN induction by dsRNA is employed by bovine viral diarrhoea virus (BVDV). The BVDV E^{rns} glycoprotein is secreted out of the cell, where it binds and

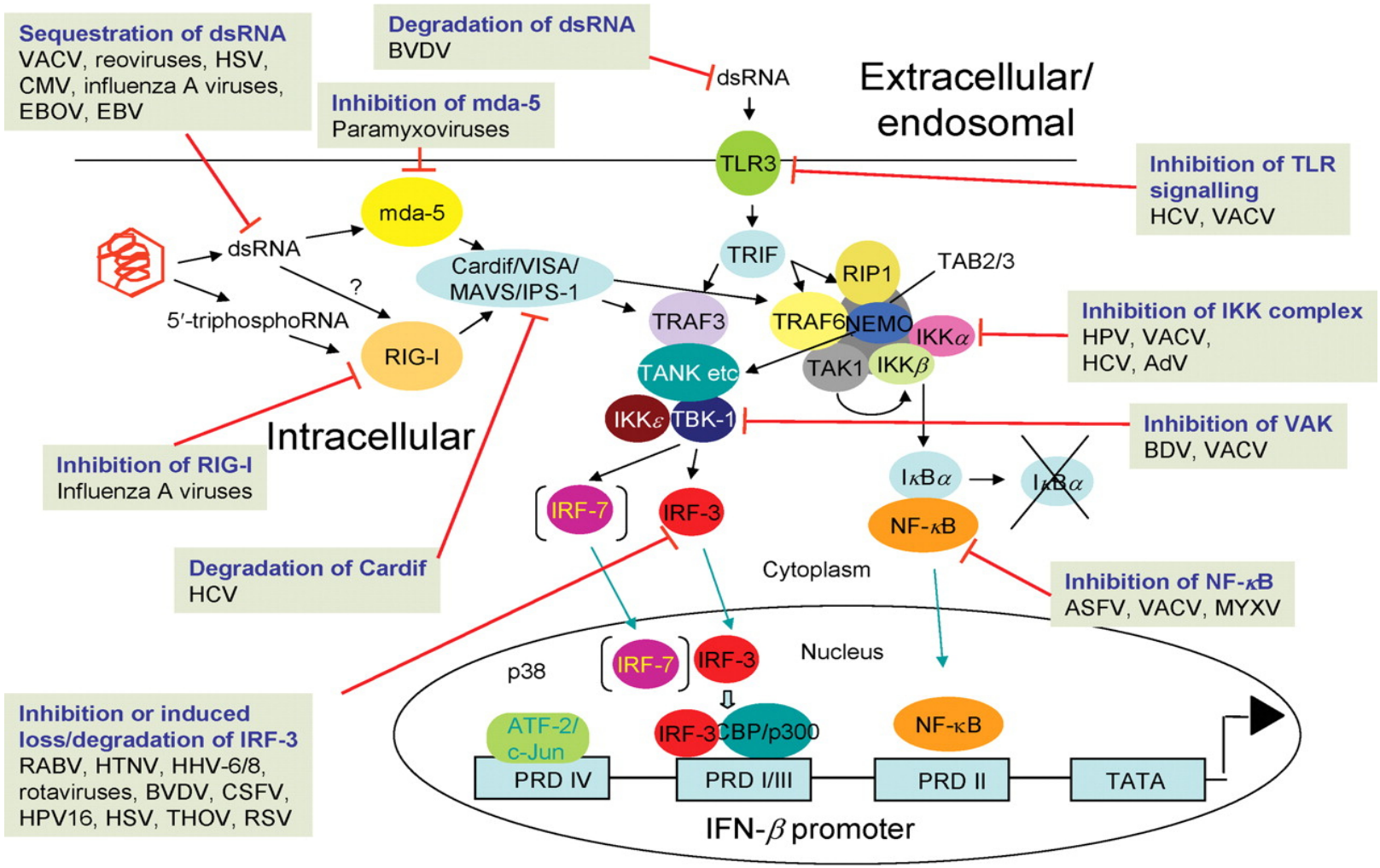


Figure 1.10: Viral antagonists of IFN-α/β induction.

The signalling scheme presented shows IFN-β induction by both TLR3- and RIG-I/mda-5-dependent pathways. The sites of intervention by several antagonists are indicated. Note that some antagonists, such as BVDV/NPro, are extremely effective at blocking IFN-α/β induction from a variety of PAMPs, because they target signalling molecules (IRF3) that are common to TLR3 and RIG-I/mda-5 pathways of IFN-induction. Others, such as the V proteins of paramyxoviruses, may block only one arm of the induction pathway (mda-5). Also note that other IFN antagonists, such as HCV/NS3/4A, have more than one cellular target. *From Randall and Goodbourn, 2008.*

degrades dsRNA through its dsRNA-binding and RNase activity respectively (Iqbal et al., 2004); as a result, extracellular dsRNA is unable to bind TLR3 and IFN is not induced.

Some viruses interfere with TLR and RIG-I/mda-5/ZBP-1 signalling. The V proteins of most paramyxoviruses bind and inhibit mda-5 (Andrejeva et al., 2004, Childs et al., 2007), while RIG-I activity is inhibited by the NS1 protein of IAV (discussed in **Section 1.3.4**). The NS3/4A protease of HCV prevents signalling downstream of TLR3 through cleavage of TRIF (Li et al., 2005a), while the VACV A46R and A52R proteins prevent signalling downstream of multiple TLR pathways through inhibition of TRIF and MyD88 (Stack et al., 2005, Harte et al., 2003). HCV NS3/4A, in addition to its effects on TRIF, also targets MAVS, the mda-5/RIG-I adaptor protein. NS3/4A mediates MAVS cleavage away from the mitochondrion, preventing its activation of TBK1 and IKKε (Li et al., 2005b, Meylan et al., 2005, Cheng et al., 2006, Kaukinen et al., 2006, Lin et al., 2006a, Loo et al., 2006).

Downstream of MAVS and TRIF, viruses can inhibit NF-kB activation in a number of ways. The human papillomavirus (HPV) E7 (Spitkovsky et al., 2002), HCV core (Joo et al., 2005), adenovirus (AdV) E3 (Friedman and Horwitz, 2002) and VACV N1 (DiPerna et al., 2004) proteins associate directly with the IKK complex to inhibit its kinase activity, impairing IkB phosphorylation and degradation. VACV can additionally inhibit IkB degradation through its K1 protein (Shisler and Jin, 2004). The A238L gene product of African swine fever virus (ASFV) is an IkB homologue that interacts with NF-kB to prevent its transcriptional activity (Powell et al., 1996, Revilla et al., 1998, Tait et al., 2000); MNF from myxoma virus may also function in a similar manner since it co-localises with NF-kB and inhibits NF-kB signalling (Camus-Bouclainville et al., 2004).

Inhibition of IRF3 expression or activation is also a common viral strategy. Rotavirus NSP1, bovine herpesvirus-1 (BHV-1) bICP0 and the NPro proteases of BVDV and classical swine fever virus (CSFV) target IRF3 for degradation by the proteasome (Saira et al., 2007, Ruggli et al., 2005, Bauhofer et al., 2005, Bauhofer et al., 2007, La Rocca et al., 2005, Hilton et al., 2006, Graff et al., 2007). The E6 protein of HPV-16 binds and sequesters IRF3 (Ronco et al., 1998), while HSV-1 ICP0 recruits IRF3 and CBP/p300 to nuclear structures, away from host chromatin (Melroe et al., 2004, Lin et al., 2004a, Melroe et al., 2007). IRF3 dimerisation and activity are inhibited by the E1 protein of human herpesvirus 6 (HHV-6)(Jaworska et al., 2007) and the Ml

protein of THOV (Hagmaier et al., 2004, Jennings et al., 2005) while IRF3 phosphorylation by TBK-1 is inhibited by rabies virus (RABV) P (Brzozka et al., 2005), hantavirus (HTNV) G1 (Alff et al., 2006), Borna disease virus (BDV) P (Unterstab et al., 2005) and VACV N1 proteins (DiPerna et al., 2004). Both bovine and human respiratory syncytial virus (RSV) NS1 and NS2 proteins interfere with IRF3 function by uncharacterised mechanisms (Spann et al., 2005, Bossert et al., 2003).

1.3.1.2 Inhibition of IFN signalling

The mechanisms by which viruses interfere with IFN signalling are summarised in **Figure 1.11**. Like viral inhibition of IFN induction, viruses antagonise IFN signalling at different stages by a diverse range of mechanisms. Transduction of type I and IFN signals can be inhibited prior to IFN:receptor binding. Poxviruses secrete soluble IFN-α- and IFN-γ-binding proteins that sequester extracellular IFN and prevents its activation of the receptor (Colamonici et al., 1995, Symons et al., 1995, Upton et al., 1992, Alcami and Smith, 1995, Mossman et al., 1995). Additionally, these proteins can bind the surface of neighbouring cells and prevent them responding to IFNs (Alcami et al., 2000). The K3 and K5 proteins of Kaposi's sarcoma-associated herpesvirus downregulate expression of IFNGR1 subunit of the IFN-γ receptor by targeting it for endocytosis and degradation (Li et al., 2007).

Activation of the IFN receptor and associated JAKs can also be inhibited. Dengue virus infection, HPV-18 E6 protein and the Japanese encephalitis virus (JEV) NS5 protein block Tyk2 activation (Li et al., 1999, Ho et al., 2005, Lin et al., 2004b, Lin et al., 2006b). Several viruses target JAK1, a component of both type I and type II IFN signalling, thereby inhibiting both pathways. HCMV enhances JAK1 degradation by the proteasome (Miller et al., 1998, Miller et al., 1999) and HSV-1 infection also decreases JAK1 expression (Chee and Roizman, 2004). The large T antigen of murine polyoma virus (MPyV) binds to JAK1 to inactivate signalling from the IFN receptors (Weihua et al., 1998) while the paramyxoviruses mumps virus (MuV) and measles virus (MeV) prevent JAK1 phosphorylation (Yokota et al., 2003, Kubota et al., 2002). The NS5 of Langat virus (LGTV) antagonises both type I and type II IFN signalling through association with IFNAR and IFNGR and preventing JAK1 and Tyk2 phosphorylation (Best et al., 2005).

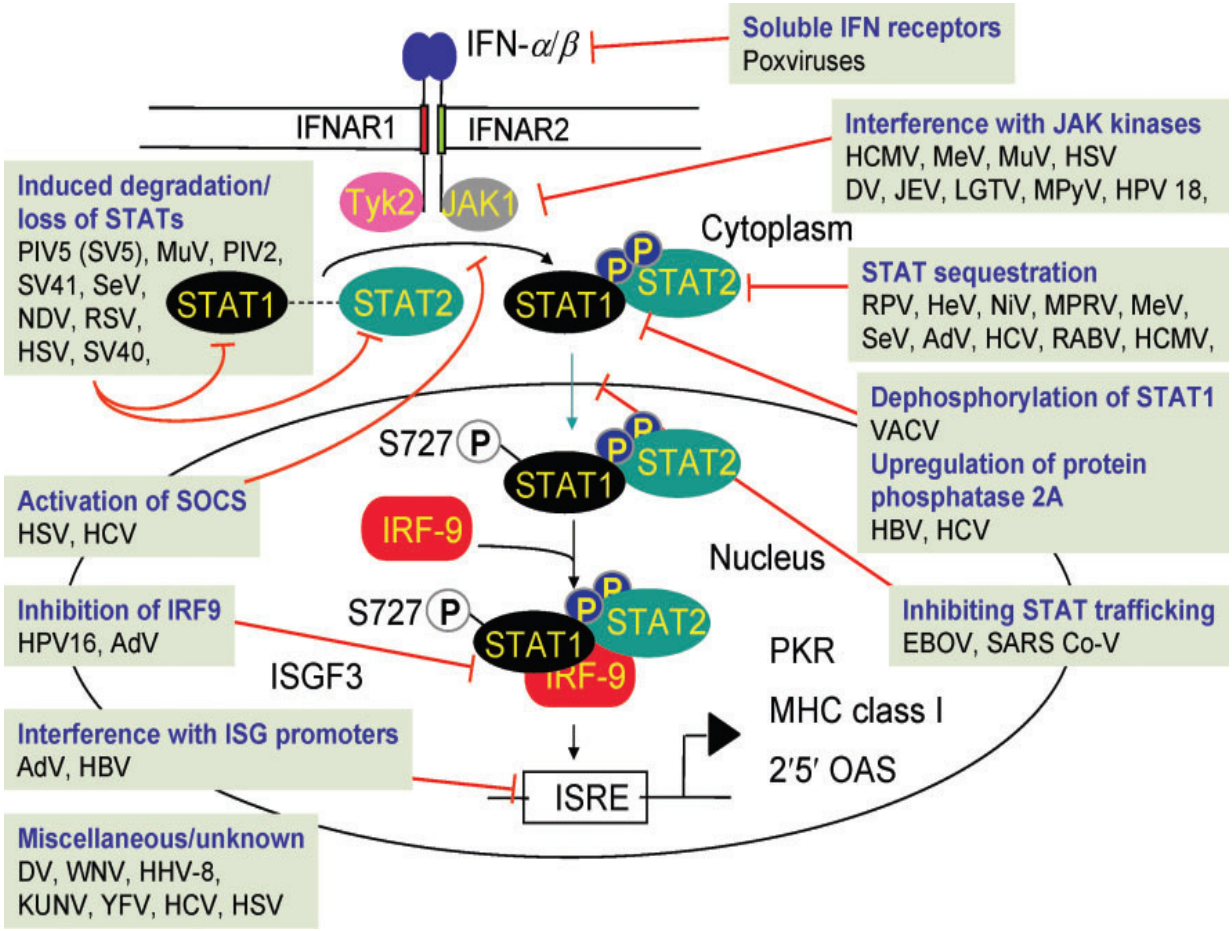


Figure 1.11: Viral antagonists of IFN-α/β signalling.

The 'classical' JAK/STAT signalling pathway activated by type I IFNs is shown. Sites of intervention of several viral antagonists are indicated. Note that many, but not all, of these IFN antagonists also inhibit IFN-γ signalling. For example, the V protein of PIV5 targets STAT1 for degradation and thus blocks IFN-α/β, IFN-γ and type III IFN signalling, whilst the V protein of hPIV2 targets STAT2 for degradation (in human cells) and thus does not block IFN-γ signalling. See text for details.
From Randall and Goodbourn, 2008.

Given the critical role of STATs in IFN signalling, it comes as no surprise that they are targeted by many viral IFN antagonists. The V proteins of some paramyxoviruses target STATs for proteasomal degradation: MuV targets STAT1 and STAT3, NDV, PIV5 and simian virus 41 (SV41) target STAT1 while parainfluenza virus type 2 (PIV2) targets STAT2 (Ulane et al., 2003, Kubota et al., 2001, Nishio et al., 2001, Didcock et al., 1999b, Huang et al., 2003, Young et al., 2000, Andrejeva et al., 2002b, Precious et al., 2005b, Parisien et al., 2001). The STAT-degradative function of SeV is attributed to the C protein, which promotes STAT1 ubiquitination (Garcin et al., 2002, Garcin et al., 1999). RSV targets STAT2 through the activities of the NS1 and NS2 proteins (Ramaswamy et al., 2006, Ramaswamy et al., 2004, Elliott et al., 2007). Viruses from other families also increase STAT turnover: STAT2 levels decrease significantly during HSV-1 infection (Chee and Roizman, 2004). Viruses can also target STAT proteins without enhancing their degradation. For example, the paramyxovirus MeV, Mapuera virus (MPRV), Rinderpest virus (RPV), Hendra virus (HeV) and Nipah virus (NiV) V proteins sequester STATs to prevent their translocation to the nucleus (Nanda and Baron, 2006, Rodriguez et al., 2002, Rodriguez et al., 2003, Palosaari et al., 2003, Hagmaier et al., 2007). STAT nuclear translocation can be actively inhibited by EBOV VP24 and severe acute respiratory syndrome coronavirus (SARS Co-V) ORF6 proteins which bind the karyopherin nuclear import proteins to inhibit STAT nuclear accumulation (Reid et al., 2006, Frieman et al., 2007). The P protein of RABV binds to tyrosine-phosphorylated STAT1 and STAT2, inhibiting both their nuclear translocation and DNA binding activity (Vidy et al., 2007, Brzozka et al., 2006). The HCMV IE1 protein associates with STAT1 and STAT2 in the nuclei of infected cells and prevents association of ISGF3 with the ISRE (Paulus et al., 2006). HBV precore/core proteins interact directly with the ISRE in the MxA promoter, downregulating MxA transcription (Fernandez et al., 2003). Viruses can also affect the phosphorylation states of STATs. SeV C inhibits STAT1 phosphorylation in addition to its effects on total STAT1 levels (Garcin et al., 2003, Saito et al., 2002, Komatsu et al., 2002, Young et al., 2000). HCV core protein also reduces STAT1 phosphorylation to prevent its association with STAT2 and inhibit ISGF3 formation (Lin et al., 2006c). VACV encodes a viral phosphatase, VH1, responsible for dephosphorylation and deactivation of STAT1 (Najarro et al., 2001). HBV and HCV upregulate protein phosphatase 2A (PP2A)(Christen et al., 2007, Duong et al., 2004) which leads to a decrease in STAT1 transcriptional activity due to STAT1 hypomethylation and an increased association between STAT1 and PIAS1, the protein responsible for SUMO-modification of STAT1 (Duong et al., 2004, Duong et al., 2005, Christen et al.,

2007). SOCS, a cellular inhibitor of STAT signalling, is upregulated by HCV core protein (Bode et al., 2003) and by HSV-1 infection (Yokota et al., 2004). In addition to STATs, IRF9 is also a viral target due to its role in the ISGF3 transcription factor. HPV-16 E7 binds IRF9 directly, preventing ISGF3 formation (Barnard and McMillan, 1999), while AdV E1A decreases IRF9 expression to inhibit IFN-α signalling (Leonard and Sen, 1997).

1.3.1.3 Inhibition of ISG products

Viral dsRNA-binding proteins, in addition to preventing induction of IFN, sequester dsRNA away from the antiviral proteins that require dsRNA for their activation. Consequently, the dsRNA-binding activities of VACV E3L, Epstein-Barr virus (EBV) SM and HSV-1 Us11 are required to inhibit PKR and OAS activation (Sanchez and Mohr, 2007, Poppers et al., 2003, Chang et al., 1992, Rivas et al., 1998, Poppers et al., 2000). In addition, some viruses encode RNA species, such as the AdV virus associated RNA I (VAI RNA), that complex with PKR to inhibit its autophosphorylation and activation of downstream signalling (Katze et al., 1987). Because dsRNA-independent activation of PKR can also occur through PKR activator PACT, viruses have evolved additional mechanisms of PKR inhibition. HCV NS5A directly binds PKR to inhibit its activity (Gale et al., 1997). A similar function has been attributed to the HSV-1 Us11 protein (Peters et al., 2002) indicating that this viral protein inhibits PKR activation by two distinct mechanisms. An as yet unidentified protease encoded by poliovirus specifically degrades PKR (Black et al., 1989, Black et al., 1993) while VACV K3L shares a number of conserved residues with the PKR substrate eIF2α and acts as a pseudosubstrate PKR inhibitor (Kawagishi-Kobayashi et al., 1997). dsRNA-independent viral inhibition of the OAS/RNaseL system has also been reported: EMCV and human immunodeficiency virus HIV-1 upregulate expression of a cellular RNaseL inhibitor (Martinand et al., 1998, Martinand et al., 1999) while HCV NS5A directly binds OAS to inhibit its activity (Taguchi et al., 2004).

1.3.1.4 Inhibition of global protein synthesis and secretion

A large number of viruses affect cellular protein expression through inhibition of global transcription or mRNA processing, nuclear export or translation. A major function of host protein synthesis shut-off is thought to be the reduction of IFN and ISG expression. In support of this, the NSs protein of Bunyamwera virus, which

interferes with mRNA transcription (Thomas et al., 2004), prevents IFN induction. An NSs deletion mutant is a much stronger inducer of IFN than wt and displays attenuated growth characteristics (Weber et al., 2002); a phenotype similar to wt virus can be restored in cells that are unresponsive to IFN (Young et al., 2003). Viral inhibition of global protein secretion, for instance by the 3A protein of several picornaviruses (Choe et al., 2005), may also play a role in preventing IFN release into the extracellular fluid.

1.3.2 Viral inhibition of apoptosis

One of the first studies into the effects of apoptosis on virus replication demonstrated that apoptosis decreased multiplication of an insect virus by about 50-fold (Clem and Miller, 1993). Subsequent studies, however, have shown that viruses (including HSV-1, VSV and poliovirus) can replicate considerably in apoptotic cells and that apoptosis induction only slightly reduces viral titres (Koyama et al., 1998). It has been suggested that apoptosis may play a more predominant role in limiting *in vivo* infections, as phagocytosis of virus-infected, apoptotic cells completely abrogates virus multiplication (Fujimoto et al., 2000, Nakanishi et al., 2008). The primary reason that many viruses block apoptosis then may be to prevent apoptotic cell clearance by macrophages (Koyama et al., 2000). The benefits to the virus of limiting phagocytosis *in vivo* may be two-fold: to increase virus multiplication and to suppress cell-mediated immunity.

Apoptosis inhibition by DNA and RNA tumour viruses has been studied in depth due to its implications for cancer research. These studies dominate the literature and will only be summarised here as they have been comprehensively reviewed elsewhere (Benedict, 2003, Benedict et al., 2002, White, 2006). Since DNA viruses and retroviruses hijack host transcriptional and translational machinery, they require continued cell survival for efficient virus replication. Consequently, these viruses encode viral apoptosis inhibitors to prolong the life of the cell; these inhibitors can act at a number of different stages of apoptosis and often are homologous to cellular inhibitors. Many viruses antagonise mitochondrial apoptosis by encoding Bcl-2 mimics or through post-translational modification of the Bcl-2 proteins. For example, the AdV E1B-19K protein is similar in both sequence and function to the Bcl-2 family members and binds to Bax, preventing its oligomerisation and subsequent cytochrome c release (Sundararajan et al., 2001, Sundararajan and White, 2001). The HIV-1 NEF protein stimulates Bad phosphorylation, abrogating its pro-apoptotic

activity and suppressing apoptosis (Wolf et al., 2001) while the human T-cell leukaemia virus type I (HTLV-1) Tax protein increases transcription from the Bcl-X_L promoter (Tsukahara et al., 1999). The DR-mediated pathway of apoptosis is also targeted by viral proteins. In a similar manner to their antagonism of the IFN response, poxviruses secret soluble TNF-α receptors (Smith et al., 1990, Reading et al., 2002). In contrast, AdV E3 proteins decrease Fas and TRAIL receptor expression on the cell surface, reducing cell sensitivity to DR ligands (Shisler et al., 1997, Tollefson et al., 1998, Benedict et al., 2001). The EBV LMP1 engages TRAFs and TRADD in a similar manner to TNFR and subsequently activates anti-apoptotic NF-kB signalling (Uchida et al., 1999, Cahir McFarland et al., 1999, Izumi et al., 1999). Modulation of caspase activity is also a function of many viral apoptosis inhibitors: the viral caspase-8 inhibitors, vFLIPs, encoded by herpesviruses and poxviruses, block caspase-8 activation by the DISC (Bertin et al., 1997) whilst baculoviruses encode IAP orthologues that inhibit caspase activity (Seshagiri and Miller, 1997, Clem and Miller, 1994). Viruses can additionally disrupt apoptosis through inactivation of p53. The large T antigen of SV40 sequesters p53 into an inactive complex (Linzer and Levine, 1979, Zheng et al., 1994a, Yanai and Obinata, 1994), and the AdV E1B-55K and E4orf6 proteins promote p53 ubiquitination and degradation (Steegenga et al., 1998, Querido et al., 2001).

Given the importance of the IFN response in promoting a pro-apoptotic state in cells, viral IFN antagonists can also antagonise apoptosis through inhibition of pro-apoptotic ISG expression and/or function. In addition, dsRNA can induce apoptosis through RIG-I/MAVS and PKR to activate IRF3-dependent transcription from a distinct subset of pro-apoptotic ISGs (see **Section 1.2.7**). Viruses that encode dsRNA-binding proteins or inhibitors of RIG-I, PKR, MAVS or IRF3 can therefore prevent both the induction of IFN and the induction of apoptosis. In support of this, several viral dsRNA-binding proteins, including VACV E3L and IAV NS1 (see below), are oncogenic (Garcia et al., 2002) and the PKR-binding function of NS5A has been linked to the disruption of apoptosis (Gale et al., 1999). Degradation of IRF3 by CSFV NPro limits both IFN and apoptosis induction (Ruggli et al., 2005, La Rocca et al., 2005, Bauhofer et al., 2007). BVDV infection inhibits dsRNA-induced IFN production and apoptosis (Schweizer and Peterhans, 2001), and although inhibition of apoptosis by BVDV has not yet been attributed to NPro activity, CSFV and BVDV NPro target IRF3 by a conserved mechanism (Hilton et al., 2006, Seago et al., 2007). Similarly, the NSs of Bunyamwera virus inhibits IFN and apoptosis by interfering with IRF3 transcription (Kohl et al., 2003, Leonard et al., 2006, Weber et al., 2001). Some

viruses limit apoptosis through activation of NF-kB pro-survival pathways: HCV core protein inhibits TNF-α-induced apoptosis (Ray et al., 1998) through enhancement of NF-kB activation and upregulation of cFLIP (Saito et al., 2006, You et al., 1999, Marusawa et al., 1999).

Many viruses have evolved ways of activating PI3K or Akt in order to block or delay virus-induced apoptosis. For some viruses, PI3K/Akt activation contributes to latent infection. For example, EBV LMP1 binds the p85 subunit of PI3K to mediate the activation of PI3K and downstream Akt phosphorylation (Dawson et al., 2003). Inhibition of EBV-induced PI3K activity decreases cell viability, suggesting PI3K/Akt is essential for promoting the survival of infected cells (Dawson et al., 2003). HPV-16, also a virus associated with prolonged periods of latency, encodes the E5 protein which associates with the epidermal growth factor receptor (EGFR) resulting in its stimulation (Hwang et al., 1995a, Crusius et al., 1998). PI3K is subsequently recruited to the receptor and PI3K/Akt cell survival pathways are activated (Zhang et al., 2002). PI3K/Akt also plays a role during chronic viral infection, particularly with hepatitis B virus (HBV) and HCV. The HBx protein of HBV blocks apoptosis by activating a PI3K/Akt/Bad-dependent pathway that has not yet been fully characterised (Lee et al., 2001) while HCV NS5A associates directly with p85 to enhance its tyrosine phosphorylation, promoting Akt activation and blocking apoptosis induction by reducing FOXO1 transcriptional activity (He et al., 2002b, Street et al., 2005, Street et al., 2004). Transient activation of PI3K/Akt also occurs early on in infection with several small RNA viruses in order to mediate short-term cell survival. RSV prevents premature apoptosis induction through PI3K/Akt-dependent activation during the first few hours of infection (Thomas et al., 2002), a function that may be due to the non-structural proteins NS1 and NS2 (Bitko et al., 2007). A number of apoptosis regulators, including Mdm2 (Groskreutz et al., 2007), NF-kB and GSK-3β (Thomas et al., 2002) are activated downstream of PI3K/Akt during RSV infection. This effect on cell death is only transient however, preventing apoptosis just long enough for the virus to complete its replication phase; later in infection, apoptosis is induced, perhaps to facilitate virus spread.

1.3.3 Antagonism of antiviral responses by PIV5 V protein

PIV5 (also known as simian virus 5; SV5) is a prototypical member of the rubulavirus genus of the *Paramyxoviridae* family (paramyxovirus taxonomy and molecular biology are comprehensively reviewed in Lamb and Kolakofsky, 2001). PIV5 is an

enveloped virus with a negative-sense non-segmented ssRNA genome that encodes eight gene products from seven genes. The integral membrane proteins HN (haemagglutinin-neuraminidase) and F (fusion) are present in the viral envelope and are required for cell attachment and fusion respectively. Also in the envelope is the SH (small hydrophobic) protein, which has been implicated in apoptosis inhibition (He et al., 2001, Lin et al., 2003, Wilson et al., 2006). The P (phosphoprotein) and L (large) proteins form the viral polymerase while M (matrix) associates with the inside of the viral envelope and plays a role in virus assembly. Viral genome is encapsidated within helical capsids formed from NP (nucleocapsid protein); also found in association with NP are the viral polymerase components and the V protein.

PIV5 is a poor inducer of IFN, and IFN-α is unable to activate transcription from the ISRE in PIV5-infected cells (Didcock et al., 1999a); PIV5 therefore efficiently circumvents the IFN response through inhibition of both IFN production and IFN signalling. This IFN antagonism function has been attributed to the multifunctional PIV5 V protein (He et al., 2002a, Didcock et al., 1999b, Poole et al., 2002, Andrejeva et al., 2004), which also plays key roles in viral RNA encapsidation (Precious et al., 1995, Randall and Bermingham, 1996), viral RNA synthesis (Lin et al., 2005, Gainey et al., 2008a, Nishio et al., 2008), cell cycle regulation (Lin and Lamb, 2000), inhibition of pro-inflammatory cytokine (IL-6) expression (Lin et al., 2007) and inhibition of apoptosis (Sun et al., 2004). V is a faithful transcript of the P/V gene, while the P protein is generated by a shift in the reading frame caused by a viral polymerase 'stuttering' mechanism (Vidal et al., 1990). Consequently, V shares a 164-residue N-terminus with P, while its cysteine-rich C-terminus is unique. This C-terminal domain is highly conserved among all paramyxoviruses and contains a zinc-finger domain which is thought to be involved in mediating interactions between V and other proteins (Paterson et al., 1995, Thomas et al., 1988).

V inhibits both type I and type II IFN signalling by targeting STAT1 for degradation by the ubiquitin/proteasome system (Andrejeva et al., 2002b, Didcock et al., 1999a, Young et al., 2000, Didcock et al., 1999b). Critical for STAT1 degradation is an interaction between V and DDB1, the damage-specific DNA binding protein (Leupin et al., 2003, Lin et al., 1998a, Andrejeva et al., 2002a). The observations that DDB1 complexes with Cullin 4a to form E3 ligases (Wertz et al., 2004, Shiyanov et al., 1999) and that there is an absolute requirement for STAT2 in V-mediated STAT1 degradation (Parisien et al., 2002) led to the suggestion that a multiprotein complex comprising V, STAT1, STAT2, DDB1 and Cullin 4a was responsible for STAT1

degradation (Precious et al., 2005b, Ulane and Horvath, 2002). In support of this, studies into the molecular interactions between these proteins demonstrated that V is unable to directly bind STAT1, but can bind STAT2 and DDB1 independently; STAT1 is recruited to this complex through its interaction with STAT2 (Precious et al., 2005a). V therefore acts as an adaptor, bringing STAT1/STAT2 heterodimers into close proximity to the DDB1/Cullin 4a ubiquitin ligase complex (Precious et al., 2007, Precious et al., 2005a). Following STAT1 ubiquitination and degradation, the binding affinity of V for STAT2 decreases and STAT2 is released from the complex, allowing for continued rounds of STAT1 ubiquitination (Precious et al., 2007). Naturally-occurring isolates of PIV5 exist that differ in their ability to inhibit IFN signalling (**Figure 3.11**, Chatziandreou et al., 2002). The PIV5-CPI- strain was isolated from a dog that had been experimentally infected with the PIV5-CPI+ virus (Baumgartner et al., 1981). While PIV5-CPI+ can inhibit IFN signalling, three amino acid substitutions in the N-terminus of the V render the PIV5-CPI- virus unable to target STAT1 for degradation (Southern et al., 1991, Chatziandreou et al., 2002).

V can also limit IFN induction by directly binding mda-5, the cytoplasmic RNA sensor, and inhibiting its activity (Poole et al., 2002, He et al., 2002a, Andrejeva et al., 2004). This function of V is specific to mda-5, as RIG-I activity is unaffected by V expression (Childs et al., 2007, Komatsu et al., 2007, Yoneyama et al., 2005). A mechanism of mda-5 inhibition by V has recently been proposed (Childs et al., 2009). Activation of mda-5 by dsRNA requires homo-oligomerisation through its helicase domain. Since the V-binding site of mda-5 has been mapped to a stretch of residues (between aa 676 and 816, although additional contacts are important in the interaction) in its C-terminal helicase domain, V competes with dsRNA for mda-5 binding to inhibit mda-5 oligomerisation (Childs et al., 2007, Childs et al., 2009).

Wildtype strains of PIV5 are relatively uncytopathic in tissue culture and can establish persistent infections (Parks et al., 2002, Sun et al., 2004, Choppin, 1964, Chatziandreou et al., 2002). However, a recombinant virus lacking the conserved C-terminus of V (rPIV5-VΔC) induces large amounts of apoptosis as well as IFN (Sun et al., 2004, He et al., 2002a). This apoptosis does not seem to require IFN-production, since rPIV5-VΔC also caused more cell death than wt virus in Vero (IFN-α/β-deficient) and U3A (STAT1-deficient) cells (Sun et al., 2004, He et al., 2002a). He and colleagues demonstrated activation of caspase-12 during rPIV5-VΔC-induced apoptosis, a caspase associated with ER-mediated apoptosis (Szegezdi et al., 2003, Sun et al., 2004). The significance of this result is open to question

however, since human caspase-12 genes have acquired a premature stop codon that precludes expression of the full-length protein and a loss-of-function mutation in a domain critical for catalytic activity (Fischer et al., 2002, Xue et al., 2006). The P/V-CPI- virus, a recombinant PIV5 virus engineered to express the P/V gene of PIV5-CPI-, generated by Parks and colleagues, also causes more apoptosis than wt in a variety of cell-lines, suggesting that the three amino acids associated with the ability to target STAT1 for degradation may also be involved in apoptosis regulation (Wansley and Parks, 2002).

V can directly bind viral RNA through a series of basic residues in its N-terminus (Lin et al., 1997). V also inhibits replication and translation of the viral RNA (Lin et al., 2005, Gainey et al., 2008a), and it has been suggested that this prevents production of aberrant RNAs that may otherwise trigger IFN and apoptosis (Dillon and Parks, 2007). In support of this, P/V-CPI- virus, a potent inducer of IFN-β and apoptosis, also demonstrated a higher level of viral mRNA synthesis than a P/V-CPI-expressing wild-type V (Wansley and Parks, 2002, Dillon and Parks, 2007). Interestingly, while virus transcription was increased, the overall rate of host and viral protein synthesis was lower in cells infected with P/V-CPI- than wt virus; this effect correlated with PKR activation and eIF2α phosphorylation (Gainey et al., 2008a). V therefore seems to limit induction of antiviral pathways through limiting viral RNA production and PKR activation. The PIV5 P protein, which shares its N-terminal 164 residues with V, also exhibits these effects on RNA synthesis and translation (Gainey et al., 2008a, Dillon and Parks, 2007), indicating the shared RNA-binding domain may be critical for this function. Curbing aberrant viral RNA production to limit antiviral responses may be a mechanism shared by the paramyxoviruses: the measles virus C protein also regulates viral RNA synthesis (Reutter et al., 2001) and its deletion leads to eIF2α phosphorylation and translation inhibition (Nakatsu et al., 2006).

Some evidence exists for the involvement of Akt in the replication of PIV5 (and other paramyxoviruses). Bioinformatic analysis of the V protein revealed an Akt1 binding motif centred around Ser176, a region that appears to be well conserved among paramyxoviruses, and a V:Akt interaction was confirmed by co-immunoprecipitation (Sun et al., 2008a). Moreover, pharmacological inhibition of Akt reduced PIV5 titre by three orders of magnitude, indicating that Akt plays a critical role in virus replication. The biological function of such a V:Akt interaction was not determined, but the authors speculated that V may exert its effects on the cell cycle, apoptosis or IL-6

expression through an effect on Akt. A subsequent report from the same laboratory demonstrated that the PIV5 L protein enhanced Akt phosphorylation to activate NF-kB signalling. The authors speculated further that, due to its ability to inhibit expression of the NF-kB-responsive IFN and IL-6 genes (Poole et al., 2002, Lin et al., 2007), V blocks L-stimulated NF-kB signalling through inhibition of Akt (Luthra et al., 2008), although no evidence was produced to support this.

1.3.4 Antagonism of antiviral responses by IAV NS1 protein

Influenza A viruses, members of the *Orthomyxoviridae* family, are enveloped viruses with a segmented negative-sense, ssRNA genome. Eight genome segments code for up to eleven viral proteins: segments 1,2 and 3 encode the PB2, PB1 and PA components of the viral polymerase; segment 4, the haemagglutinin (HA) glycoprotein; segment 5, the nucleocapsid protein NP; segment 6, neuraminidase (NA); segment 7, the matrix proteins M1 and M2 ; segment 8 encodes the non-structural (NS1) and nuclear export proteins (NEP). Segment 2 additionally encodes PB1-F2 through a shift in the reading frame. The functions of these viral proteins and their roles during the virus life cycle have been reviewed extensively (Lamb and Krug, 2001).

A co-linear mRNA transcript of segment 8 codes for the ~26kDa NS1 protein. It carries an RNA-binding domain (RBD) at its N-terminus (comprising the first 73 amino acid residues) that binds dsRNA and the IAV RNA genome (Hatada and Fukuda, 1992, Chien et al., 2004, Hatada et al., 1992, Qian et al., 1995). The rest of the protein forms the C-terminal ‘effector’ domain, which is required for interactions with cellular proteins and stabilisation of the RBD (Wang et al., 2002c). NS1 appears to exist as a homodimer both *in vitro* and *in vivo*, with both the RBD and the effector domain contributing to dimerisation (Chien et al., 1997, Liu et al., 1997, Hale et al., 2008a, Nemeroff et al., 1995, Wang et al., 2002c). The NS1 protein has been assigned multiple functions (recently reviewed in Hale et al., 2008c). A major function of NS1 appears to be limiting host innate antiviral responses activated during influenza infection (Egorov et al., 1998, Garcia-Sastre et al., 1998, Kochs et al., 2007, Zhirnov et al., 2002). It is an IFN antagonist and suppresses expression of a large number of antiviral genes, including IFN and ISGs (Hayman et al., 2007, Geiss et al., 2002, Hayman et al., 2006, Kochs et al., 2007, Noah et al., 2003). Consequently, delNS1 viruses or viruses with C-terminal truncations in NS1 are excellent inducers of IFN and are attenuated in IFN-competent cells. In Vero cells

however, which lack IFN-α/β genes, these viruses demonstrate similar growth kinetics to wt (Garcia-Sastre et al., 1998, Egorov et al., 1998).

NS1 prevents activation of IRF3, NF-kB and AP-1, and subsequent IFN induction, in response to dsRNA or virus infection (Talon et al., 2000, Ludwig et al., 2002, Wang et al., 2000a). This effect was shown to require an intact RBD: mutation of two residues in this domain, Arg-38 and Lys-41, abrogated RNA binding and a virus expressing these mutations was unable to block IRF3 activation or IFN induction (Donelan et al., 2003, Talon et al., 2000, Wang et al., 1999d). As a result, it was proposed that NS1 sequesters viral RNA away from cytoplasmic sensors such as RIG-I (Talon et al., 2000). More recent studies however indicate that NS1 may interact with RIG-I to inhibit its activity and limit IFN induction (Opitz et al., 2007, Guo et al., 2007, Pichlmair et al., 2006, Mibayashi et al., 2007). Interestingly, an intact RBD (and specifically Arg-38 and Lys-41) was also required for RIG-I binding and inhibition (Guo et al., 2007, Pichlmair et al., 2006), hinting at a role for RNA in the NS1:RIG-I interaction. NS1 also inhibits the function of the IFN-induced proteins PKR and OAS. Since OAS is activated by dsRNA, NS1 is thought to sequester viral RNA through the activity of its RBD, thereby preventing OAS activation (Min and Krug, 2006). Although it was initially thought that NS1 inhibited PKR in a similar manner (Hatada et al., 1999, Lu et al., 1995), it has since been demonstrated that NS1 can inhibit PKR activation by dsRNA or PACT (Li et al., 2006b). Moreover, NS1 binds directly to PKR and residues 123-127, but not the Arg-38 residue required for RNA-binding, are essential for this interaction (Li et al., 2006b, Min et al., 2007, Tan and Katze, 1998). A model has been proposed based on this evidence: NS1 binds to residues in the linker region of PKR and blocks the conformational change required for PKR activation in response to either dsRNA or PACT (Li et al., 2006b).

NS1 can limit the IFN-induced antiviral state post-transcriptionally by blocking pre-mRNA processing and nuclear export (Fortes et al., 1994, Qiu and Krug, 1994). Three distinct mechanisms are utilised by NS1 to achieve this. The first involves an interaction between NS1 and the 30kDa subunit of cleavage and polyadenylation specificity factor (CPSF30) (Nemeroff et al., 1998, Noah et al., 2003, Twu et al., 2006), a host protein that, in concert with poly(A)polymerase, plays a key role in the formation of cellular pre-mRNA 3' ends. The NS1:CPSF30 association is thought to prevent host pre-mRNA cleavage and polyadenylation by sequestering CPSF30 and prevent its binding to RNA substrate (Nemeroff et al., 1998). IFN-β and ISG expression occurs earlier and in much larger amounts in IAV expressing NS1 with a

mutated CPSF30 binding site, illustrating the importance of this function in IFN antagonism (Noah et al., 2003). Secondly, NS1 inhibits function of the poly(A) binding protein II (PABPII), preventing elongation of pre-mRNA 3' poly(A) chains and its nucleo-cytoplasmic shuttling (Chen et al., 1999). As a result of both CSPF30 and PABPII inhibition, processing and nuclear export of cellular mRNAs can be specifically inhibited: since polyadenylation of influenza virus mRNAs is mediated by the viral polymerase (Poon et al., 1998), viral mRNA export is unaffected. There is some evidence for the formation of a ternary complex comprising NS1, CPSF30 and PABPII, indicating that these two binding functions may act in concert to inhibit host mRNA processing (Chen et al., 1999). Additionally, NS1 downregulates expression of the nucleoporin Nup98 and forms an inhibitory complex with NXF1/TAP, p15/NXT, Rae1/mrnp41, and E1B-AP5 (Satterly et al., 2007). These proteins are key components of the mRNA nuclear export machinery that direct mRNAs through the nuclear pore complex.

Influenza A virus infection induces apoptosis in a wide range of cell types both *in vitro* and *in vivo* (Mori et al., 1995, Hinshaw et al., 1994, Fesq et al., 1994, Takizawa et al., 1993, Price et al., 1997). Within 2-3 days of in vivo infection, apoptosis is induced in bronchial, bronchiolar and alveolar epithelial cells of the airways (Mori et al., 1995) although rates of cell death differ with the virus subtype (Price et al., 1997, Mok et al., 2007). While originally thought to be a host defence mechanism to limit replication of the virus (Kurokawa et al., 1999), evidence is mounting that apoptosis may play a more facilitatory role in influenza virus infection (Stray and Air, 2001): IAV-induced caspase-3 and p53 activation, and Fas, FasL and TRAIL expression are all required for efficient virus yield (Takizawa et al., 1999, Fujimoto et al., 1998, Wurzer et al., 2003, Wurzer et al., 2004, Takizawa et al., 1995, Wada et al., 1995, Turpin et al., 2005). This said however, IAV multiplication can be effectively abrogated by phagocytosis of infected apoptotic cells, but not by apoptosis of infected cells alone, indicating that apoptosis limitation may still be of primary importance to the virus (Takizawa et al., 1999, Koyama et al., 2000, Fujimoto et al., 2000, Nakanishi et al., 2008). Further support for the important role of macrophage phagocytosis in clearing IAV infection comes from the observation that the IAV PB1-F2 protein promotes apoptosis more readily in macrophages than in epithelial cells (Coleman, 2007, Chen et al., 2001c, Chanturiya et al., 2004, Zamarin et al., 2005).

NS1 has been implicated in regulation of IAV-induced apoptosis, and has been assigned both pro-apoptotic (Schultz-Cherry et al., 2001, Morris et al., 2002, Lam et

al., 2008) and anti-apoptotic roles (Morris et al., 2002, Zhirnov et al., 2002, Stasakova et al., 2005). Cells infected with a delNS1 virus exhibit apoptosis markers much earlier than wt virus, a difference that was attributed to the effects of NS1 on the IFN response since wt and delNS1 viruses induce apoptosis to similar extents in Vero cells which lack IFN-α/β genes (Zhirnov et al., 2002). The NS1-mediated post-transcriptional block on host gene expression has its implications for apoptosis inhibition as well as IFN. In addition to preventing the induction of pro-apoptotic ISGs (Geiss et al., 2002), NS1 inhibits TNF-α-induced expression of NF-kB-responsive genes (Hayman et al., 2006) and may contribute to TNF-α sensitivity of IAV (Seo et al., 2002). PKR and OAS/RNaseL may also play a key role in the induction of apoptosis during IAV infection, given their pro-apoptotic functions (Section 1.2.5.1, Takizawa et al., 1996, Mullan et al., 2005, Castelli et al., 1997, Castelli et al., 1998): NS1-mediated inhibition of PKR and OAS may therefore function to delay both the induction of IFN and apoptosis.

IAV also activates PI3K, possibly to promote cell survival pathways (Zhirnov and Klenk, 2007, Shin et al., 2007a, Ehrhardt et al., 2007). The NS1 effector domain mediates a direct interaction with the p85 subunit of PI3K to promote its activation (Hale et al., 2006, Shin et al., 2007c, Ehrhardt et al., 2007); this interaction appears to be specific to the p85β isoform (Li et al., 2008, Hale et al., 2006, Hale et al., 2008b). Akt activation has been demonstrated downstream of PI3K activation by NS1, and Akt phosphorylation occurs at an early stage (during the first 6h) of infection (Zhirnov and Klenk, 2007, Hale et al., 2006). NS1 requires a tyrosine at position 89 for interaction with p85: mutation of this residue to a phenylalanine abrogates the NS1:p85 interaction and Akt activation (Hale et al., 2006). Additionally, a recombinant virus expressing NS1-Y89F displays attenuated growth characteristics (Hale et al., 2006) indicating that PI3K activation is required for efficient virus propagation. Other NS1 residues implicated in NS1:PI3K interaction are Met93 (Hale et al., 2006), Pro164/Pro167 (Shin et al., 2007a) and Leu141/Glu142 (Li et al., 2008). NS1 is thought to activate PI3K by masking the p85β residues responsible for regulating p110 catalytic activity (Hale et al., 2008b, Li et al., 2008). During infection with avian IAVs, NS1 hyperactivates the PI3K/Akt pathway through additionally interacting with Crk and CrkL proteins (Heikkinen et al., 2008). Crk/CrkL are involved in a number of signalling pathways that regulate diverse cellular functions through their roles as adaptor proteins. Significantly, associations between Crk/CrkL and p85 have been reported, both through a direct interaction (Gelkop et al., 2001, Gesbert et al., 1998) and indirectly through intermediate linker proteins (Reedquist et al., 1996,

Fukazawa et al., 1996, Husson et al., 1997). Avian NS1 may act as such a linker to bring p85 and Crk/CrkL into close proximity, resulting in more efficient PI3K activation.

1.4 Aims

The overall aim of this study was to explore the relationships between IFN, PI3K signalling and apoptosis. The negative-stranded RNA viruses PIV5 and IAV both encode the IFN antagonist proteins V and NS1 respectively; these proteins are also reported to target PI3K/Akt signalling to enhance virus replication. This project sought to determine whether modulation of IFN and PI3K pathways by PIV5/V and IAV/NS1 could affect host cell apoptosis. To achieve this, a system for the study of apoptosis in suitable (i.e. physiologically relevant) cell-lines first has to be developed. Once this is in place, the effects of PIV5/V and IAV/NS1 protein expression, in the context of both virus infection and stable cell-lines, on evasion of cell death will be explored. More specifically, the effects of PIV5/V-mediated STAT1-degradation on apoptosis will be studied. In addition, the V:Akt association between V and Akt and its implications for modulation of Akt signalling will be examined. With regards to IAV/NS1, reports that NS1-mediated PI3K activation promotes anti-apoptotic signalling will be investigated. Since PI3K also plays a role in the IFN response, effects of PI3K activation by NS1 on IFN production and ISG expression will also be studied. NS1 modulates the activity of a number of other host cell proteins to antagonise the IFN response; the effects of mutations in NS1 reported to abrogate these binding activities on cell death will also be determined.

2 MATERIALS AND METHODS

2.1 Cell-lines and tissue culture

2.1.1 Cells used in this study

1321N1: Human brain astrocytoma cell-line (from Prof. C. P. Downes, University of Dundee).

293T: A highly transfectable derivative of the human embryonic kidney 293 cell-line, constitutively expressing the SV40 large T-antigen (provided by Prof. R. Iggo, University of St Andrews).

A549: A human epithelial cell-line derived from a human lung carcinoma (European Collection of Cell Cultures; ECACC).

HEp2: Human cervix carcinoma epithelial cells derived from the HeLa cell-line (ECACC).

HFFF: Human foetal foreskin fibroblasts. Although HFFFs normally have a limited passage number, this clone expresses telomerase reverse transcriptase in order to increase number of cell passages achievable before senescence (McSharry et al., 2001). Provided by Prof. G. Wilkinson, Cardiff University.

MDCK: Canine kidney cells (ECACC).

MRC5: Untransformed human foetal lung fibroblasts with a finite life span (ECACC).

Neutrophils: Isolated and provided by Prof. I. Dransfield, University of Edinburgh.

Vero: Fibroblast-like cells established from the kidney of an African Green monkey (ICN Pharmaceuticals Ltd.).

In addition to the basic cell-lines mentioned above, the following permanent cell-lines (generated by others) were also used:

A549-ISRE.Luc: A549 cells stably expressing the firefly luciferase gene under the control of an ISRE (provided by M. Valahaus, University of St Andrews).

A549-NFkB.Luc: A549 cells stably expressing the firefly luciferase gene under the control of the NFκB promoter (provided by Prof. Ron Hay, University of Dundee).

MDCK/V: MDCK cells stably expressing the V protein of PIV5 (Precious et al., 2005b).

The following stable cell-lines were also generated using lentivirus vectors and used as part of this study:

A549 and **MRC-5** cells expressing the following proteins:

BVDV/NPro: A549 cells stably expressing the N^{pro} protein of BVDV (Pe515 strain) with an N-terminal V5 tag.

HCV/NS3/4a: A549 cells stably expressing the NS3 and NS4a proteins of HCV, gla1 strain- 4a. NS4a carries a C-terminal V5 tag.

PIV5/V(CPI-): A549 cells stably expressing the V protein of the CPI- strain of PIV5.

PIV5/V(w3): A549 cells stably expressing the V protein of the w3 strain of PIV5.

A549 and **1321N1** cells expressing the following proteins:

PR8/NS1 wt: A549 cells stably expressing the NS1 protein of IAV, PR8 strain, with a C-terminal V5 tag.

PR8/NS1-Y89F: A549 cells stably expressing the PR8/NS1 carrying the Y89F amino acid substitution, with a C-terminal V5 tag.

2.1.2 Cell maintenance

Cell monolayers were maintained in $25cm^2$ or $75cm^2$ tissue culture flasks (Greiner) in DMEM (Dulbecco's modified Eagle's medium; Invitrogen) supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS; Biowest) and incubated at 37^oC/5% CO_2. Cells were routinely passaged using trypsin/EDTA (Becton Dickinson Ltd.), at a frequency dictated by the rate of cell growth.

2.1.3 Cell stock freezing and resuscitation

Adherent cells were trypsinised, resuspended in DMEM/10% FCS, and centrifuged at ~500x*g* for 5mins. Pelleted cells were resuspended in DMEM supplemented with 20% FCS and 10% DMSO and aliquoted into cryovials. Cell stocks were frozen at -70°C before long-term storage in liquid nitrogen. For resuscitation of cells, cryovials were thawed at 37°C before centrifugation at ~500x*g*. Pelleted cells were then resuspended and grown in DMEM/10%FCS at 37°C/5% CO_2. Medium was replaced after 24 hours in order to remove traces of DMSO.

2.1.4 Treatment of cells

IFN treatment

Cells were treated overnight (at least 12 hours, unless where otherwise stated) with media supplemented with Roferon recombinant human IFNα-2a (Roche Diagnostics) or IFN-γ (Invitrogen) at a concentration of 10^3 units/ml. To activate the ISRE promoter for luciferase assay, cells were treated with 10^4 units/ml.

Apoptosis induction

To induce apoptosis, cells were treated with media supplemented with 15ng/ml TNF-α (often in conjunction with 10 µg/ml cycloheximide; CHX, Sigma-Aldrich) or 1µg/ml staurosporine (STS; Sigma-Aldrich). Treatment with FasL (200ng/ml; Alexis Biochemicals) required the presence of anti-FLAG M2 antibody (2µg/ml; Sigma-Aldrich) to promote multimerisation of FasL via its N-terminal FLAG tag.

Treatment with inhibitors

PI3K activity was inhibited by LY294002 (25µM; Calbiochem). Protein synthesis was inhibited using CHX (10µg/ml).

Transfections with plasmid DNA

Transfection of cells with plasmid DNA was carried out using FuGENE 6 transfection reagent (Roche) according to the manufacturer's instructions.

2.2 Virus infections

2.2.1 Viruses used in this study

EMCV: Encephalomyocarditis virus.

PIV5-w3: standard laboratory strain of PIV5 (Choppin, 1964).

PIV5-CPI+: canine isolate of PIV5 (Evermann et al., 1981)

PIV5-CPI-: canine isolate of PIV5, isolated from the brain tissue of a dog infected experimentally with CPI+ (Baumgartner et al., 1987). CPI- virus fails to block IFN signalling due to three amino-acid changes in the N-terminal domain of the V protein (Chatziandreou et al., 2002).

PIV5-VΔC: recombinant PIV5 that lacks the C-terminal cysteine-rich domain of the V protein (He et al., 2002a). This virus is unable to block IFN production or signalling.

SeV: Sendai virus strain Z, a gift from D. Kolakofsky (University of Geneva, Switzerland).

rUd wt: recombinant wild-type IAV (A/Udorn/72; H3N2), provided by D. Jackson, Northwestern University, USA (Hale *et al*., 2006).

rWSN wt: recombinant wild-type IAV (A/WSN/33; H1N1), provided by D. Jackson (Northwestern University, USA).

The following recombinant viruses were generated and provided by D. Jackson and C. Galloway. All viruses were isolated and subsequently grown in MDCK/V cells.

rUd-Y89F: recombinant Udorn virus expressing NS1 with the Y89F amino-acid substitution. Generated and provided by D. Jackson, Northwestern University, USA (Hale *et al*., 2006).

rWSN-Y89F: recombinant WSN virus expressing NS1 with the Y89F amino acid substitution. Generated and provided by D. Jackson Northwestern University, USA (Hale et al., 2006).

rUd-P164/7A: The NS1 protein carries two amino acid substitutions at positions 164 and 167 that are reported to abrogate PI3K binding (Shin et al., 2007a).

rUd-P162/4/7A: The NS1 protein carries three amino acid substitutions at positions 162, 164 and 167. Virus isolation took much longer

than for other mutants, and was initially a mixed population of virus, also containing rUd NS1-Δ99.

rUd-Δ99: This virus encodes an NS1 protein carrying only amino acids 1-98, and was isolated during rescue of the rUd-P162/164/167A virus.

rUd-184-8(L): At positions 184-188, five amino acids, GLEWN have been substituted for RFLRY. This mutation eliminates the binding site for CPSF30 (Noah et al., 2003). This virus took much longer to rescue than other mutants, and isolated virus was initially a mixed population, also containing rUd NS1-184-8(P).

rUd-184-8(P): Isolated during the rescue of rUd-184-8(L). rUd-184-8(P) differs from rUd-184-8(L) at position 186, where it carries a proline instead of a leucine residue.

rUd-103/106: The NS1 protein carries F103L and M106I amino acid substitutions. Residues 103 and 106 are important for stabilising the NS1:CPSF30 interaction ((Kochs et al., 2007, Das et al., 2008).

rUd-123/4: The binding site for PKR (residues 123-127) is mutated in the NS1 protein. Mutation of this site abrogates the ability of NS1 to inhibit PKR during virus infection (Min et al., 2007).

rUd-R38A: The NS1 protein carries an amino acid substitution at position 38. This residue is absolutely required for the dsRNA binding activity of NS1, and replacement of arginine with alanine abrogates this activity (Wang et al., 1999d).

2.2.2 Virus infections

To infect with paramyxovirus isolates, monolayers were inoculated with virus diluted in DMEM supplemented with 2% FCS at an appropriate multiplicity of infection (MOI), or DMEM only (for mock infections). For virus infections in 6-well plates, cells were inoculated in a volume of 1ml per well and placed on a rocking platform at 37°C for an adsorption period of 1-2 hours. Inoculations in 96-well plates were carried out without rocking in a volume of 50µl per well. Virus inoculum was then removed and replaced with DMEM/ 2%FCS. Cells were incubated at 37°C/5% CO_2 until harvested. For investigating PI3K/Akt activation, monolayers were washed in DMEM only prior to inoculation and infections carried out in serum-free DMEM.

IAV infections were carried out in serum-free DMEM. Monolayers were washed in DMEM prior to infection to remove all traces of serum. Cell monolayers were infected with 400µl virus (per well of a 6-well plate) diluted in serum-free DMEM at an appropriate MOI (or DMEM only for mock infections). Cells were incubated for 1hr at 37°C, with gentle agitation at regular intervals. Inoculations in 96-well plate were carried out as for paramyxoviruses (see above). Virus inoculum was removed and replaced with serum-free DMEM. Cells were incubated at 37°C/5% CO_2 until harvested.

2.2.3 Preparation of virus stocks

Stocks of PIV5 isolates, other paramyxoviruses and EMC virus were kindly maintained and provided as required by D. Young (University of St. Andrews).

To prepare stocks of IAV, 90% confluent MDCK of MDCK/V monolayers were washed twice in serum-free DMEM in order to remove traces of serum. Cells were inoculated in serum-free DMEM at an MOI of ~ 0.001 plaque-forming units (PFU)/cell. After a 1-2h adsorption period (rocking, 37°C), virus inoculum was removed and monolayers washed with serum-free DMEM. 12mls (per 75cm^2 flask) of serum-free DMEM supplemented with 2.5µg/ml N-acetyl trypsin (NAT) were added to the monolayers and cells were incubated at 37°C/5% CO_2. Supernatants were harvested when 50-70% CPE (cytopathic effect) was observed (~36h). Cellular debris was removed by centrifugation (~3000x*g* for 10mins) and aliquots were frozen at -70°C. Virus titres were determined by plaque assay as described below.

2.2.3 Virus titration

To titrate PIV5 virus, monolayers of Vero cells were grown in 6-well plates until 80-90% confluent. Preparations of virus were serially diluted 10-fold in DMEM/2% FCS and monolayers were inoculated with 1ml of each virus dilution per well. After adsorption for 1h, rocking at 37°C/5% CO_2, virus inoculum was removed, and 6mls of overlay medium (DMEM supplemented with 2% FCS and 0.5% carboxy methyl cellulose [Methocel; Sigma-Aldrich]) was added to each well. Cells were incubated at 37°C/5% CO_2 for 8-10 days until plaques had formed. Overlay medium was then aspirated from the well and monolayers fixed with PBS/5% formaldehyde/2% sucrose. Plaques were visualised by staining the monolayers with crystal violet (0.1%

crystal violet, 3.6% formaldehyde, 1% methanol, 20% ethanol in H_2O) for 20mins, followed by rinsing in water. Plaques were counted, and titres estimated as PFU/ml virus preparation, taking into account the original dilutions made.

Titration of IAV was carried out on confluent MDCK monolayers in 6-well plates. Cells were washed twice in serum-free DMEM in order to remove all traces of serum. Virus preparations were serially diluted 10-fold in serum-free DMEM, and cells were inoculated with 400μl of each virus dilution per well. Cells were incubated at 37°C/5% CO_2 and plates were agitated every 10 minutes to ensure even adsorption of the virus. During this period, 2x overlay medium (13.4gDMEM/3.7g $NaHCO_3$ per 485ml water supplemented with 4μg/ml NAT) was incubated at 37°C. 2% agarose in water was melted and placed in a 55°C water bath until required. After an adsorption period of 1h, virus inoculum was removed, the 2x overlay medium and the 2% agarose (Biogene Ltd.) were mixed in a 1:1 ratio, and 2ml of this was added to each well. After the overlay had set, plates were inverted and incubated at 37°C/5% CO_2 until distinct plaques had formed (~3 days). Cells were fixed by adding 2ml PBS/5% formaldehyde/2%sucrose on top of the agarose plugs, and the monolayers were left to fix overnight. Agarose plugs were then removed and plaques were stained with crystal violet (0.1% crystal violet, 3.6% formaldehyde, 1% methanol, 20% ethanol in H_2O) or visualised by immunostaining as described below. Titres were estimated as PFU/ml virus preparation, taking into account the original dilutions of virus that were made.

2.2.4 Immunostaining of influenza virus plaques

Fixed MDCK monolayers were permeabilised (0.5% IGEPAL, 10% sucrose/ 0.1% sodium azide in PBS) for 15 min, and then incubated in PBS supplemented with 1% FCS for 1hr. Monolayers were incubated for 1h at room-temperature with 500μl/well of diluted (1/2000) primary antisera (rabbit antisera raised against whole X31 [H3N2]; diluted in PBS/1% FCS). Cells were washed with PBS/0.1%TWEEN, and monolayers were subsequently incubated for 1h at room-temperature with 500μl/well of diluted (1/2000) secondary anti-rabbit IgG alkaline phosphatase (AP)-conjugated antibody. Monolayers were subsequently washed with PBS/0.1%TWEEN, and incubated with 500μl/well of alkaline phosphatase substrate (as per manufacturer's instructions; Sigma-Aldrich) until plaques were easily visualised. Monolayers were rinsed with water in order to stop the reaction.

2.2.5 Virus yield assay

To determine titres of infectious influenza virus over multiple replication cycles, confluent cell monolayers were infected at a MOI of 0.001 PFU/cell in serum-free DMEM as described above. After an adsorption period of 1-2hrs, virus inoculum was removed and cells were washed in serum-free DMEM. Monolayers were subsequently incubated at 37°C/5% CO_2 in serum-free DMEM (supplemented with NAT; 2μg/ml for MDCK and A549, 1μg/ml for MRC-5 cells). Samples of culture supernatants were removed at desired times post-infection, centrifuged to remove cell debris and frozen at -70°C until required. Virus titres were determined by plaque assay on MDCK cells as described above.

2.3 Plasmid DNAs

2.3.1 Plasmids used in this study

Several plasmids were used in this study, either directly, or to produce lentivirus in order to produce cell-lines stably expressing virus proteins.

pCMV5-HA-Akt: Plasmid expressing HA-tagged Akt1; a kind gift from B. Vanhaesebroeck, Queen Mary University, London.

pCMVR8.91: Plasmid expressing the gag/pol, tat and rev genes of HIV-1 (used in lentivirus production, provided by Y.-H. Chen).

pdl'MCS(C'V5): Empty lentivirus vector for expressing proteins with a C-terminal V5-tag, used as a control during transient transfections. Expression is controlled by the constitutive SFFV (spleen focus forming virus) promoter, which produces a single bicistronic mRNA that encodes for the protein of interest with a puromycin resistance product to enable cell selection (modified from the self-inactivating lentivirus vector pHR-SIN-CSGW by Y.-H. Chen, University of St Andrews).

pdl'BVDV/NPro (N'V5): Lentivirus vector expressing the N-terminally V5-tagged BVDV/NPro; provided by M. Galliano, University of St Andrews.

pdl'HCV/NS3/4A (C'V5): Lentivirus vector expressing the NS3/4A protease of HCV. NS4A carries a C-terminal V5-tag; provided by Y.-H. Chen, University of St Andrews.

pdl'MuV/V(C'V5): Lentivirus vector expressing the C-terminally V5-tagged V protein of MuV; provided by Y.-H. Chen, University of St Andrews.

pdl'PIV5/V(CPI-): Lentivirus vector expressing the V protein of PIV5 (CPI- strain); provided by Y.-H. Chen, University of St Andrews.

pdl'PIV5/V(w3): Lentivirus vector expressing the V protein of PIV5 (w3 strain); provided by Y.-H. Chen, University of St Andrews.

pdl'PR8/NS1(C'V5): Lentivirus vector expressing C-terminally V5-tagged PR8/NS1; provided by Y.-H. Chen, University of St Andrews (Hale et al., 2006).

pdl'PR8/NS1-Y89F (C'V5): Lentivirus vector expressing C-terminally V5-tagged PR8/NS1 with the Y89F mutation; provided by B. G. Hale (Hale et al., 2006).

pEF.PIV5/V: Plasmid expressing the V protein of PIV5 (w3 strain); provided by J. Andrejeva, University of St Andrews.

pMD-G: Plasmid expressing the vesicular stomatitis virus glycoprotein (VSV-G) gene (used in lentivirus production, provided by Y.-H.Chen).

2.3.2 Propagation of plasmids

E. coli DH5α cells were grown in Luria-Bertani (LB) medium (10g/l bacto-tryptone, 5g/l yeast extract, 10mM NaCl, pH 7.5), or plated on solid LB medium supplemented with 1.5% (w/v) agar and 10mM $MgSO_4$. As appropriate, media were supplemented with ampicillin (100 μg/ml) for selection.

2.3.2 Transformation of competent cells

1µg plasmid was added directly to 100µl of thawed, competent cells (Invitrogen). After incubation on ice for 1h, cells were transferred to a 42°C water bath for 2mins before being immediately returned to ice for a further 2mins. Cells were resuspended in 1ml LB broth and incubated at 37°C for 1h. The cell suspension was plated out onto LB-agar plates supplemented with ampicillin (90 mm-diameter Petri dishes; Scientific Laboratory Supplies Ltd., U.K.). Plates were inverted and incubated at 37°C overnight. Mini-cultures were prepared from selected colonies.

2.4.4 Preparation of plasmid DNA

For small-scale preparations, bacterial cell cultures of 10 ml (in LB broth containing 100µg/ml ampicillin) were grown overnight in a 37°C shaking incubator. DNA was extracted from cells and purified using the QIAGEN DNA mini-prep kit (QIAGEN Ltd., UK), according to the manufacturer's instructions. To produce larger-scale DNA preparations, 100ml of bacterial culture was grown overnight in a 37°C shaking incubator. DNA was extracted from cells using the QIAfilter Plasmid Maxi Kit (according to the manufacturer's instructions; QIAGEN).

2.3.5 UV spectrophotometry

The concentration of plasmid DNA was quantified by measurement of Abs_{260} using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). DNA purity was estimated by calculating the Abs_{260}/Abs_{280} ratio. Ratios greater then 1.8 were considered acceptable.

2.4 Lentivirus-mediated generation of stable cell-lines

2.4.1 Lentivirus production

To generate recombinant lentivirus, a 75 cm^2 tissue culture flask 293T cells (70% confluent) was co-transfected with three plasmids: 3µg pCMVR8.91, 3µg pMD-G, and 5µg of the pdl vector containing the construct of interest. Supernatants were harvested at 48h and 72h post-transfection and pooled. Cellular debris was removed by centrifugation at 3,000x*g* for 10mins and filtering through 0.45µm Tuffryn membrane filters (Invitrogen). Virus aliquots were stored at -70°C until required.

2.4.2 Transduction of target cells

30% confluent target cell monolayers (25cm^2 flask) were infected with the harvested lentivirus supernatant (estimated MOI of 1PFU/cell) in the presence of polybrene (8µg/ml; Sigma-Aldrich). 48h post-infection, transformed cells were selected by resistance to puromycin (Invivogen); MRC5 cells, 1µg/ml; A549/ 1321N1 cells 2µg/ml. Puromycin-containing medium was replaced every four days until control naïve cells were dead. Cells were kept under continuous selection with puromycin

and stocks were stored in liquid nitrogen. Expression of the required protein was confirmed by immunoblot or immunofluorescence analysis.

2.5 Protein analysis

2.5.1 SDS polyacrylamide gel electrophoresis

Cell lysates were prepared by adding disruption buffer (6M Urea, 2M β-mercaptoethanol, 4% (w/v) SDS, with bromophenol blue colouring) to cells in a 1:1 ratio. Viscosity of samples was reduced by sonication (2 x 20s) or digestion with benzonase (Novagen). Polypeptides were separated through 4-12% NuPAGE polyacrylamide gradient gels (Invitrogen) by electrophoresis (200V for 50 min) in MOPS [3-(*N*-morpholino)propanesulphonic acid] buffer (Invitrogen).

2.5.2 Immunoblotting

Protein lysates were subjected to SDS-PAGE as described above, and transferred to polyvinylidene difluoride (PVDF) membrane using the XCell II Blot Module according to the manufacturer's instructions (Invitrogen, U.K.). After transfer, membranes were blocked for 30 mins in 5% (w/v) skimmed milk powder, 0.1% (v/v) Tween 20 in PBS (blocking buffer), and incubated for either 1h or overnight with primary antibody diluted (in blocking buffer) according to the manufacturer's instructions. After multiple washes with PBS/ 0.1% Tween 20, interactions between protein and antibody were detected by incubating the membrane for 1h with horseradish peroxidase (HRP)-conjugated antibody raised against the appropriate species, diluted appropriately in blocking buffer. Unbound antibody was washed away with PBS/ 0.1% Tween 20, and specific polypeptide bands were visualised using western blotting chemiluminescence luminal reagent (Santa Cruz) according to the manufacturer's instructions. N.B. For probing for phospho-proteins, 5%BSA/0.1%Tween/PBS was used instead of milk as blocking buffer.

2.5.3 Immunofluorescence

On coverslips

Cells grown on 10mm coverslips were fixed for 10mins in 5% formaldehyde/2% sucrose/PBS then permeabilised with 0.5% NP-40/10% sucrose/PBS. Non-specific-binding sites were blocked for at least 30 min with PBS/ 1% FCS/ 0.1% sodium

azide, then monolayers were incubated with appropriately diluted primary antibody for 1h. Unbound antibody was washed away with PBS, and cells were incubated for 1h with Texas Red or fluoroscein isothiocyanate (FITC) conjugated secondary antibody (Oxford Biotechnology Ltd., U.K.). If staining of the nucleus was required, the DNA-binding fluorochrome 4', 6-diamidino-2-phenylindole (DAPI; 0.5 µg/ml; Sigma Aldrich) or acridine orange was also added to this solution. Coverslips were washed in PBS, fixed again in 5% formaldehyde/2% sucrose/PBS and mounted on slides using Citifluor AF-1 mounting solution (Citifluor). All reactions were performed at room temperature in a humidified chamber. Immunofluorescence was visualised using a Nikon Microphot-FXA microscope.

In 96-well plates

For checking for 100% infection of cells in 96-well plates, duplicate wells in the 96-well plate were set-up and infected in the same manner. At desired times post-infection, monolayers were fixed with PBS/ 5% formaldehyde/ 2% sucrose for at least an hour, then permeabilised and stained as above. In between antibody incubations, monolayers were washed multiple times in PBS/ 0.1% Tween 20. Following staining, plates were re-fixed in 5% formaldehyde/2% sucrose/PBS and immunofluorescence was visualized using an Olympus CKX41 fluorescence microscope.

2.5.4 Immunoprecipitations

Cells were washed twice in ice-cold PBS and lysed for at least 10mins on ice in immunoprecipitation buffer (20 mM Tris-HCl (pH 7.8)/5 mM EDTA/ 0.5% NP-40/150mM NaCl freshly supplemented with a protease inhibitor cocktail tablet; Roche Diagnostics); 300µl/2x10^6 cells. Viscosity of lysates was reduced by sonication. Soluble antigen extract was prepared by centrifuging the cell lysate at 12,000x*g* for 50mins to pellet cell debris. 1ml soluble antigen extract was incubated with 15µl cross-linked Ab beads (see below) overnight (with agitation) at 4°C. Siliconised tubes (Sigma-Aldrich) were used to minimize any non-specific protein binding to the tube. After extensive washing in immunoprecipitation buffer, proteins in complex with antibody were dissociated by heating in SDS-PAGE loading buffer at 100°C for 5min. Proteins were analysed by SDS-PAGE.

2.5.5 Production of cross-linked Ab beads

Protein G-Sepharose beads (binding capacity for IgG >20mg/mL; Sigma-Aldrich) were pre-washed several times with ice-cold PBS. An appropriate amount of isolated beads was mixed with anti-V5 mAb or anti-Akt polyclonal Ab (approx. 4mg of mAb per mL of beads), resuspended in binding buffer A (20mM Tris-HCl (pH 7.8); 650mM NaCl), and incubated overnight at 4°C with agitation. Ab-bead complexes were rescued and isolated after four repeated wash cycles of low-speed centrifugation to pellet beads, aspiration of supernatant and resuspension of beads in binding buffer Complexes were then washed twice in buffer B (3M NaCl/1.5M glycine [buffered to pH 8.9 with 3M NaOH]), and twice with buffer C (3M NaCl/50mM Borate [buffered to pH 8.9 with 3M NaOH]). Complexes were incubated with freshly prepared buffer D (40mM di-methyl-pimelimidate/3M NaCl/200mM Borate [buffered to pH 8.9 with 3M NaOH]) for 1h at 4°C with agitation. Beads were pelleted by low-speed centrifugation and incubated on ice for 10 min with buffer E (200mM ethanolamine [buffered to pH 8.0 with HCl]). Complexes were washed twice more with buffer E and then re-incubated for a further 2h at room temperature with buffer E with agitation. Finally, the cross-linked Ab beads were washed four times with buffer A. Efficiency of cross-linking was determined by analysing bead samples by SDS-PAGE at various stages throughout the procedure. Cross-linked beads were produced as required and stored as a 50% (v/v) slurry in buffer A at 4°C.

2.5.6 Antibodies

Antibodies were used in immunoprecipitations, immunoblotting, immunostaining, immunofluorescence and apoptosis induction.

Primary antibodies used are shown in **Table 2.1**.

Secondary antibodies: Anti-mouse, rabbit and sheep IgG Texas Red and FITC conjugated antibodies were from Oxford Biotechnology. HRP-conjugated anti-mouse IgG was from Sigma, anti-rabbit IgG HRP conjugated antibody was from Santa Cruz and anti-sheep IgG HRP conjugates were from Abcam.

Target Protein/ Tag	Manufacturer
β-actin (monoclonal)	Sigma-Aldrich
Akt (polyclonal)	Cell Signaling Technology
Phospho-Akt (Ser 473; monoclonal)	Cell Signaling Technology
Cleaved caspase-3 (Asp 175; polyclonal)	Cell Signaling Technology
FasL (polyclonal)	Abcam
FLAG (M2; monoclonal)	Sigma-Aldrich
IRF3 (polyclonal)	Santa Cruz Biotechnology
ISG15 (polyclonal)	A kind gift from A. L. Haas, Medical College of Wisconsin, USA.
MAVS (polyclonal)	A kind gift from I. Julkunen, National Public Health Institute, Helsinki, Finland.
p53 (DO1; monoclonal)	A kind gift from R. T. Hay, Dundee University
Phospho-p53 (Ser 15; polyclonal)	Cell Signaling Technology
PARP (full-length; monoclonal)	Abcam
PARP (full-length & cleaved; polyclonal)	Roche
PIV5 V (polyclonal)	Serum produced by Diagnostics Scotland. Affinity-purified by B. Precious.
PIV5 V (C-terminus; monoclonal)	A kind gift from R. Lamb, Northwestern University, US.
STAT1α p91 (monoclonal)	Santa Cruz Biotechnology
V5 (also reacts with PIV5 V protein; monoclonal)	Serotec
V5 (HRP-conjugated; monoclonal)	Serotec
X31 (H3N2; polyclonal)	A kind gift from A. Douglas, National Institute for Medical Research, London
NS1 (polyclonal)	Serum produced by Diagnostics Scotland. Affinity-purified by H. Xiao.

Table 2.1: Primary antibodies used in this study.

2.6 Miscellaneous assays

2.6.1 Cell-based IFN production assay

The relative amount of IFN present in cell culture supernatants was estimated using a cell-based EMCV-inhibition assay. Supernatant samples were centrifuged to remove any cell debris and exposed to UV light for 3 min (to inactivate any virus present). Samples were titrated by serial 2-fold dilution in DMEM/2% FCS and transferred to confluent A549/BVDV/NPro cells in 96-well plates. Plates were incubated for 24h at 37°C/5% CO_2 and then infected with EMCV at an MOI of 10. When sufficient CPE was observed (~36-48h later), plates were fixed in PBS/5%formaldehyde/2% sucrose and stained with crystal violet. The "end point" for each sample was taken to be the dilution at which cells showed ~50% CPE.

2.6.2 Luciferase assays

For NF-kB and ISRE activation

Following virus infection and/or desired treatment, cell monolayers (in 6-well plates) were washed once in PBS and lysed for 2mins in 300µl (per well) luciferase buffer A (25mM Tris-phosphate (pH 7.8), 8mM $MgCl_2$, 1mM DTT, 1mM EDTA, 1% TritonX-100). Plates were frozen at -70°C until required. Upon thawing, 300µl of luciferase buffer B (30% glycerol, 0.8mM ATP, 2% BSA in luciferase buffer A) was added to each well. Cell extracts were transferred to a microfuge tube and briefly centrifuged () to remove debris. 200 µl of soluble cell extract was transferred to a cuvette and mixed with 100µl of luciferase substrate (1.5mM luciferin in Tris/EDTA buffer). Light emitted over the first 10s following substrate addition (in relative light units; RLU), was measured using a Lumat LB9501 luminometer (Bertold, Germany).

For caspase activity

Cells were seeded in sterile white-walled 96-well plates suitable for luminometry and infected as required. To measure activation of caspases-3 and -7, Caspase-Glo 3/7 Reagent (Promega) was added to directly to wells according to the manufacturer's instructions. This reagent lyses cells and contains a caspase-3/7 substrate. Caspase cleavage generates a substrate for luciferase; a "glow-type" luminescent signal results that is proportional to caspase activity in the sample. Plates were briefly shaken and incubated for 1h at room temperature. Luminescence was read on a 96-

well microplate reader. Blank (medium only) wells were included on each plate and blank readings were subtracted from experimental values.

2.6.3 Cell viability assay

Cells were seeded in 96-well plates and infected and/or treated and incubated at 37°C/5% CO_2. At desired times post-infection/treatment, wst-1 cell-viability reagent (Roche) was added to each well according to the manufacturer's instructions and plates were briefly shaken to mix culture medium and reagent. Plates were incubated at 37°C until significant colour had developed in the wells containing untreated/uninfected cells. Plates were then read at 450nm on an ELISA microplate reader using 690nm as a reference wavelength. Assays were carried out in triplicate and averaged. Percentage cell viability for each condition was calculated relative to uninfected and/or untreated cells.

2.6.4 Flow cytometry

Following desired treatment, cells were labeled with annexin V and propidium iodide (PI). 2µl FITC-conjugated annexin V (final concentration 5µM) was added to 1ml annexin V binding buffer (HBBS, 5mM $CaCl_2$). 280µl annexin V and buffer was then added to a tube containing 20µl of neutrophils at $4x10^6$ cells /ml and incubated on ice for 10 min. Adherent A549 cells were trypsinised and regularly agitated to keep them in suspension, and were used at a similar cell number/ml as neutrophils. Immediately prior to analysing the sample, 1µl of 1mg/ml PI was added to the cells. Annexin V and PI were measured using FL1 and FL2 detectors respectively on a FACSCalibur flow cytometer (BD Biosciences). Apoptotic cells were those that stained positive for annexin V but negative for PI, and were calculated as a percentage of the total cell population. Data were analysed using CellQuest software.

3 RESULTS

The first part of this section concerns the setting up of a system for the study of apoptosis in subsequent sections. Secondly, modulation of apoptosis by the PIV5 V protein is explored, while the third section concerns activation of PI3K signalling by IAV NS1 and its implications for host IFN and apoptotic responses. In addition, the contributions of other cellular-protein-binding functions of NS1 to apoptosis inhibition and IFN antagonism are examined.

3.1 Sensitivity of cell-lines to apoptosis inducers

Human cell-lines differ wildly in their sensitivity to apoptosis (Shima et al., 1995, Smets et al., 1994, Shafer and Williams, 2003). For the study of virus-induced apoptosis *in vitro* then, it is desirable to use cells similar to the genuine *in vivo* target tissue of the virus. By their very definition, immortalised (or continuous) cell-lines are transformed in order to allow indefinite passage. Although immortalised cells are not necessarily neoplastically or malignantly transformed, many are derived from tumours, and as such, possess mutations in genes encoding apoptosis and cell proliferation regulators (Williams et al., 1993); this adds a degree of complexity to *in vitro* apoptosis studies carried out in these cell-lines. Untransformed cells are considered to be more suitable for the study of apoptosis since they cannot be propagated indefinitely and undergo senescence after a finite number of passages. However, untransformed cells are not without their caveats. Their expression profiles alter with increased population doublings (McSharry et al., 2001), so differences in cell passage number between experiments may lead to inconsistency in results. Additionally, untransformed cell-lines (particularly those that have undergone a number of passages) may comprise a mixed population of replicating and senescent cells: senescent cells differ from replicating cells in their morphology, gene-expression profiles and, potentially, in their sensitivity to virus infection (McSharry et al., 2001, Faragher and Kipling, 1998). When carrying out apoptosis studies *in vitro* therefore, it is very important to consider the limitations of the particular cell-lines used. This section will examine apoptosis induction in a number of different cell-lines, including tumour-derived and untransformed cells, in order to select suitable cells for subsequent experiments.

3.1.1 Response of A549 cells to apoptosis inducers

The main criteria for choosing suitable cell-lines for this study are that cells should be representative of IAV and PIV5 target tissue and that both viruses should be able to replicate efficiently in the chosen cells. Although A549 cells are derived from a human lung carcinoma, they display properties similar to type II alveolar epithelial cells (Standiford et al., 1990, Smith, 1977, Lieber et al., 1976, Garofalo et al., 1996); studies using *ex vivo* lung tissue revealed these cells are the primary site of IAV infection (Nicholls et al., 2007, van Riel et al., 2006). They are therefore considered to be biologically relevant to IAV infection and are used widely used in influenza research. A549 cells are also routinely used to study paramyxovirus infection of the respiratory tract (Zhang et al., 2001, Young et al., 2003, Garofalo et al., 1996, Watanabe et al., 2005a), making them an ideal choice of cell-line for this study.

A549 cell sensitivity to the apoptosis inducers FasL, TNF-α and staurosporine (STS) was investigated: FasL and TNF-α are DR ligands, while STS causes apoptosis in a wide range of cell-lines through inhibition of protein kinase C (Jarvis et al., 1994, Bertrand et al., 1994, Tamaoki et al., 1986). Cell viability assays following treatment with these apoptosis inducers revealed that A549 cells were sensitive to STS but resistant TNF-α- or FasL-induced cell death, even after extended periods of treatment (**Figure 3.1**).

3.1.2 Cycloheximide sensitises A549 cells to DR-mediated apoptosis

Binding of DR ligands to their receptors activates anti-apoptotic NF-kB signalling in addition to apoptosis (Qin et al., 2002, Mitsiades et al., 2006, Imamura et al., 2004, Laegreid et al., 1994, Meichle et al., 1990). Protein synthesis inhibitors such as cycloheximide (CHX) are reported to sensitise cells to TNF-α- and FasL-induced apoptosis by preventing synthesis of anti-apoptotic proteins (Kreuz et al., 2001, Ogura et al., 2008, Micheau et al., 2001, Fulda et al., 2000, Mitsiades et al., 2000, Arscott et al., 1997). In agreement with these studies, treatment of A549 cells with TNF-α or FasL in combination with CHX caused markedly more cell death than TNF-α, FasL or CHX treatment alone; TNF-α/CHX or FasL/CHX treatment killed ~80% of cells within 24h (**Figure 3.2**).

To determine whether the sensitising effect of CHX on TNF-α- and FasL-treatment was due to inhibition of NF-kB signalling, NF-kB activity in A549 cells expressing an

NF-kB-responsive luciferase gene was measured following treatment with apoptosis inducers. Untreated cells exhibited a basal level of NF-kB activity that was CHX-sensitive (**Figure 3.3**). Inhibition of this basal activity had no effect on cell death, since CHX treatment alone caused no reduction in cell viability (**Figure 3.2**). As expected, TNF-α treatment resulted in a large increase in NF-kB activity which is completely blocked by CHX treatment (**Figure 3.3**). However, FasL treatment did not activate NF-kB: NF-kB was activated to a similar extent to mock- and FasL-treated cells both in the presence and absence of CHX. STS treatment alone inhibited NF-kB activity and STS/CHX was unable to reduce NF-kB activity any further.

3.1.3 Cytokines can act synergistically to induce apoptosis

Many factors affect the biological effects of a particular cytokine on a target cell, including the target cell type, the cytokine concentration and the abundance of complementary receptors on the target cell surface. In addition, cytokines can modulate sensitivity of target cells to the activity of other cytokines. For instance, cytokines, including IFNs, induced during virus infection can render infected cells more prone to FasL and TNF-α-mediated NK cell and CTL cytotoxicity. Pretreatment of a number of cell-lines, including A549, with IFN sensitises them to Fas-mediated apoptosis induced by agonist anti-Fas antibody (Tamura et al., 1996, Kim et al., 2002, Wen et al., 1997, Kurdi and Booz, 2007). To determine whether IFN/FasL treatment was also able to induce apoptosis, A549 cells were pretreated with IFN (-α or -γ) overnight, then treated with FasL. No significant cell death was apparent following FasL or IFN-α treatment alone (**Figure 3.4A**). While IFN-γ treatment induced a significant amount of cell death (~25%), treatment with IFN-γ and FasL in concert caused considerably more; IFN-γ/FasL caused ~75% reduction in cell viability. Immunoblot analyses revealed that this cell death was apoptotic, as cleaved PARP (a marker for apoptosis) was detected in IFN-γ/FasL-treated cells, but not cells treated with IFN-γ or FasL alone (**Figure 3.4C**). IFN-α also seemed to potentiate FasL-induced apoptosis (albeit less efficiently than IFN-γ), since IFN-α/FasL (~20%) caused 20% more cell death than either IFN-α or FasL (**Figure 3.4A**).

IFN-γ is reported to sensitise vascular smooth muscle cells to Fas-mediated apoptosis by stimulating trafficking of the Fas receptor to the cell surface in a PI3K/Akt-dependent manner (Rosner et al., 2006). To determine whether a similar PI3K-dependent mechanism of IFN-γ/FasL synergy exists in A549 cells, it was first determined whether IFN-γ could activate PI3K/Akt signalling. A549 monolayers were

treated with IFN-α or IFN-γ and cell lysates were probed for phospho-Akt (the activated form of Akt). Foetal calf serum (FCS) used in cell culture contains growth factors that activate PI3K/Akt (Hale et al., 2006); as a result, cells were IFN-treated in both serum-free medium and medium supplemented with 10% FCS. In the absence of serum, Akt was not activated in untreated cells, while IFN-α or IFN-γ treatment slightly increased Akt activation (**Figure 3.5A**). In the presence of serum, Akt was activated while IFN-γ treatment, but not IFN-α, significantly activated Akt in the presence of serum (**Figure 3.5A**). Since IFN-γ could activate PI3K/Akt signalling, the effects of the PI3K inhibitor LY294002 (LY) on IFN-γ/FasL synergy were investigated. Cells were treated with LY or mock-treated with DMSO, then treated with IFN-γ/FasL. IFN-γ/FasL treatment activated Akt and induced apoptosis in DMSO-treated cells (as demonstrated by the presence of phospho-Akt and cleaved PARP; **Figure 3.5B**). It is worth noting that the phospho-Akt band in IFN-γ/FasL-treated cells appeared to have a slightly higher mobility than Akt in untreated cells. This smaller Akt fragment is likely to be a caspase-cleavage product, as cleavage and inactivation of Akt by caspases during apoptosis has been reported (Rokudai et al., 2000, Asselin et al., 2001, Jahani-Asl et al., 2007). LY treatment alone did not cause any apoptotic cell death and reduced the basal level of phospho-Akt, consistent with PI3K inhibition (**Figures 3.5B,C**). However, LY did not prevent IFN-γ/FasL-induced apoptosis; LY/IFN-γ/FasL-treated cells displayed similar apoptotic morphologies (**Figure 3.5C**) and levels of PARP cleavage (**Figure 3.5B**) to those treated with DMSO/IFN-γ/FasL. Interestingly though, LY did not prevent Akt activation by IFN-γ/FasL treatment, perhaps indicating that Akt becomes activated during apoptosis in a PI3K-independent manner. These data demonstrate that IFN-γ/FasL synergy does not require PI3K activity. Furthermore, studies into the effects of PIV5/V on IFN-γ/FasL-induced apoptosis revealed that IFN-γ acts via a STAT1-dependent pathway to promote FasL-induced cell death (**see Section 3.2.3**).

In some cells types, IFN-α or IFN-γ can also sensitise cells to TNF-α-induced apoptosis (Kulkarni et al., 2006, Frankel et al., 2006, Kimura et al., 2003, Manna et al., 2000, Suk et al., 2001, Sasagawa et al., 2000). This does not appear to be the case in A549 cells however, since these cells were resistant to TNF-α-induced apoptosis irrespective of IFN-α or IFN-γ pre-treatment (**Figure 3.4B**).

It has been reported that agonist anti-Fas antibody and TNF-α can act synergistically to induce apoptosis (Kimura and Gelmann, 2000, Frankel et al., 2006), so it was investigated whether A549 cells could be sensitised to FasL- or TNF-α-induced

apoptosis through treatment with FasL and TNF-α simultaneously. This was found not to be the case: FasL/TNF-α treatment did not cause any more cell death than FasL or TNF-α treatment alone (**Figure 3.2**), indicating that concurrent activation of more than one type of death receptor is insufficient to cause death in A549 cells.

3.1.4 Comparison of rates of apoptosis induction in different cell-lines

A549 cells are derived from a malignant tumour; they consequently exhibit defects in tumour-suppressor expression and may express higher levels of anti-apoptotic proteins than untransformed cells (Uno et al., 2004, Ji et al., 1999, Roz et al., 2002, Leech et al., 2000, Reeve et al., 1996). To investigate whether A549 cells were more resistant to apoptosis than untransformed cells, cell death responses of A549 and MRC-5 cell-lines were compared. MRC-5 cells are diploid lung fibroblasts that are untransformed and subsequently undergo senescence following continued passage (Jacobs et al., 1970). As an additional comparison, apoptosis induction in cervix carcinoma-derived Hep2 cells, which are routinely used in virus research, was investigated. Like A549 cells, Hep2 cells were FasL-resistant (**Figure 3.6A**) but were sensitive to TNF-α/CHX and STS (**Figures 3.6B,C**). A549 cells were more resistant to STS than Hep2 cells while Hep2 cells displayed greater resistance to TNF-α/CHX, probably due to cell-type-specific or tumour-type-specific differences in expression of apoptosis regulators. In contrast to A549 and Hep2 cells, MRC-5 cells were sensitive to FasL alone (in the absence of CHX or IFN; **Figure 3.6A**) and exhibited considerable cell death within 6 hours of FasL treatment (**Figures 3.6A,D**). MRC-5 cells also died rapidly following treatment with TNF-α/CHX and STS: while prolonged treatment (up to 24 hours) was required to induce 50% cell death in A549 and Hep2 cells, the majority of MRC-5 cells died within 10 hours. Like A549 cells however, MRC-5 cells still require protein synthesis inhibition by CHX to undergo TNF-α-induced apoptosis, as they were resistant to TNF-α treatment alone (**data not shown**). MRC-5 cell death induced by FasL, TNF-α/CHX and STS was apoptotic, as cleaved PARP was detectable by immunoblotting from as early as 4hrs post-treatment (**Figure 3.6E**). It should also be noted that even untransformed cells show considerable variation in their response to apoptosis inducers. Like MRC-5, HFFF cells are untransformed and undergo rapid apoptosis following FasL or STS treatment. Unlike MRC-5 however, these cells are resistant to TNF-α/CHX-induced apoptosis (**data not shown**).

As stated previously, the criteria for choosing suitable cell-lines for this study were that cells should be representative of IAV and PIV5 target tissue and should be able to support replication of these viruses. Hep2 cells do not fulfil this criteria as, in addition to being persistently infected with oncogenic papillomavirus (Abdulkarim et al., 2002, Pater and Pater, 1985), IAV cannot replicate efficiently in these cells (Ludwig, 2006). A549 cells however are ideal for the study of human respiratory viruses as they represent the *in vivo* target tissue of IAV and PIV5 and have previously been used to study these viruses (Standiford et al., 1990, Smith, 1977, Lieber et al., 1976, Garofalo et al., 1996, Nicholls et al., 2007, van Riel et al., 2006, Zhang et al., 2001, Young et al., 2003, Watanabe et al., 2005a). The sensitivity of MRC-5 cells to apoptosis also makes them an excellent choice for the study of virus-induced cell death. These cells have also been used previously to study paramyxoviruses, including PIV5 (Young et al., 2003), and while they are not routinely used in IAV research, MRC-5 cells support productive IAV infection (Herrero-Uribe et al., 1983, de Ona et al., 1995). Since both tumour-derived and untransformed cells have their disadvantages (discussed above), and MRC-5 cells are difficult to handle in the laboratory and have slow doubling rates, it was decided that A549 and MRC-5 cells should both be used in this study.

Conclusions

A549 cells were sensitive to STS, but required treatment with the protein synthesis inhibitor CHX to undergo TNF-α- or FasL-induced cell death. NF-kB activity contributed to TNF-α but not FasL resistance. IFN-γ pre-treatment sensitised cells to FasL-induced apoptosis in a manner that was independent of PI3K/Akt signalling, but was unable to promote TNF-α-induced cell death. MRC-5 cells underwent rapid apoptosis in response to TNF-α/CHX and STS; they were also sensitive to FasL without the need for CHX or IFN-γ treatment.

3.2 The PIV5 V protein, IFN and apoptosis

The V protein is essential for PIV5 evasion of host antiviral responses. In addition to its effects on IFN production and signalling (Poole et al., 2002, Didcock et al., 1999b), V has been implicated in inhibition of host cell apoptosis (Wansley and Parks, 2002, Wansley et al., 2003, Sun et al., 2004, Dillon et al., 2006): this section examines the role of PIV5/V in apoptosis regulation.

3.2.1 Characterisation of cell-lines expressing viral IFN antagonists

To determine the effects of PIV5/V ectopic expression on apoptosis, A549 and MRC-5 cell-lines stably expressing V (from the w3 strain of PIV5) were created using lentivirus vectors. In parallel, cells expressing the Npro protease of BVDV and the NS3/4A serine protease of hepatitis C virus (HCV) were produced. Both proteins block IFN production much more efficiently than PIV5 V (Foy et al., 2005, Li et al., 2005a, Li et al., 2005b, Seago et al., 2007, Gil et al., 2006): BVDV/NPro targets IRF3 for degradation (Hilton et al., 2006, Chen et al., 2007), while HCV/NS3/4A cleaves MAVS to inhibit RIG-I/mda-5 signalling (Li et al., 2005a, Li et al., 2005b). IRF3 and MAVS have been implicated in apoptosis induction as well as IFN production (see **Section 1.2.7**); BVDV/NPro and HCV/NS3/4A were therefore included to determine whether IRF3- and MAVS-deficient cells are more resistant to apoptosis.

BVDV/NPro and HCV/NS3/4A carry the V5 epitope tag which is derived from a small 14-amino acid region of PIV5/V (Hanke et al., 1992). Immunoblot analyses showed that expression of all three viral proteins could be detected in the A549 and MRC-5 cell-lines using antibody to the V5 tag (**Figures 3.7A,B**). To confirm proper functioning of these proteins, cell lysates were also probed for their cellular targets. As described above, PIV5/V targets STAT1 for degradation. STAT1 is normally present in cells at low levels, but its expression is increased by IFN (Der et al., 1998a). As expected, in naïve A549 and MRC-5 cells, STAT1 was upregulated by IFN-α treatment, while STAT1 was not detectable in A549/PIV5/V or MRC-5/PIV5/V cells even following IFN-α treatment (**Figures 3.7A,B**). STAT1 expression was not affected by expression of BVDV/NPro (**Figures 3.7A,B**) or HCV/NS3/4A (**data not shown**). IRF3 was undetectable in A549 and MRC-5 cells expressing BVDV/NPro, irrespective of IFN-α treatment. Interestingly though, an IRF3 fragment (of ~35kDa; the band is indicated by an asterisk in **Figure 3.7B**) was present in considerable amounts in MRC-5/BVDV/NPro cells. This fragment disappeared following IFN-α

treatment and was absent from A549/BVDV/NPro cells, indicating that its generation was cell-type specific. HCV/NS3/4A clips MAVS away from the mitochondrion and in accordance with this, no full-length MAVS could be detected in HCV/NS3/4A-expressing cells; MAVS appeared as a slightly smaller fragment corresponding to its cleavage by HCV/NS3/4A at its C-terminus (**Figure 3.7A,B**, Li et al., 2005b).

To confirm that these viral proteins could inhibit IFN production, levels of IFN secretion from A549/PIV5/V, A549/BVDV/NPro and A549/HCV/NS3/4A cells in response to an IFN-inducing virus (rPIV5-VΔC) were quantified using a cell-based IFN assay. A549/PIV5/V produced a similar level of IFN to naïve cells while A549/BVDV/NPro and A549/HCV/NS3/4A did not produce detectable amounts of IFN (**Figure 3.7C**), indicating that BVDV/NPro and HCV/NS3/4A were blocking IFN induction efficiently. PIV5/V expression does not significantly limit IFN induction in response to rPIV5-VΔC; reasons for this will be described in the **Discussion**.

In addition, effects of PIV5/V, BVDV/NPro and HCV/NS3/4A on activation of the ISRE were investigated. The same lentivirus vectors used to infect cells in Figure 3.10A were used to express these viral proteins in an A549 ISRE-reporter cell-line. Luciferase activity in these cells was measured following mock-treatment or treatment with IFN-α. As expected, IFN-α induced significant ISRE activation in naïve cells due to activation of JAK/STAT signalling (**Figure 3.7D**). This induction was completely abrogated by expression of PIV5/V. In comparison, expression of the V protein of PIV5-CPI- was unable to block ISRE activation by IFN-α due to its inability to target STAT1 for degradation. Similarly, BVDV/NPro and HCV/NS3/4A, which are not reported to block IFN signalling, did not abrogate ISRE activation by IFN-α. The viral IFN antagonists expressed in A549 and MRC-5 cells are therefore functioning correctly.

3.2.2 Effect of viral IFN antagonists on apoptosis

Given that IFN is an important regulator of apoptosis, viral IFN antagonists may affect the sensitivity of cells to apoptosis inducers. Additionally, PIV5/V, BVDV/NPro and HCV/NS3/4A are multifunctional proteins and may play a role in apoptosis regulation that is independent of their ability to antagonise the IFN response. It was therefore of interest to determine whether ectopic expression of these proteins rendered cells more or less resistant to apoptosis. A549 and MRC-5 cells expressing PIV5/V, BVDV/NPro or HCV/NS3/4A were treated with FasL alone (MRC-5 only),

FasL/CHX (A549 only), TNF-α/CHX or STS, and cell viability was measured at several times post-treatment. FasL/CHX induced a similar cell death curve in A549 naïve, A549/BVDV/NPro and A549/HCV/NS3/4A cells (**Figure 3.8A; left panel**). In A549/PIV5/V cells however, induction of cell death was delayed, and these cells exhibited a higher basal survival level (the proportion of cells still viable following prolonged treatment) than naïve cells. Similarly, expression of PIV5/V limited apoptosis induction by TNF-α/CHX or STS in A549 cells (**Figures 3.8B,C; left panel**). Interestingly, following TNF-α/CHX treatment, apoptosis was also reduced in A549/HCV/NS3/4A cells. These data indicate that, in A549 cells, PIV5/V delays apoptosis induced by FasL, TNF-α/CHX and STS. In addition, HCV/NS3/4A may inhibit TNF-α/CHX in A549 cells, while BVDV/NPro expression has no effect on cell death.

The same experiment in MRC-5 cells was much less conclusive. MRC-5/PIV5/V cells exhibited higher cell viability levels at 4h post-treatment with FasL and STS, but there was no elevation of the basal survival level (**Figures 3.8A,C; right panel**). Additionally, neither PIV5/V nor HCV/NS3/4A seemed to offer protection from TNF-α/CHX in MRC-5 cells (**Figure 3.8B; right panel**).

STAT1 promotes apoptosis by stimulating transcription of pro-apoptotic genes and through direct interactions with apoptotic regulators (see **Section 1.2.5**). It was therefore determined whether the delay of apoptosis exhibited in PIV5/V-expressing cells was due to knockdown of STAT1 levels. Cell viability following treatment with apoptosis inducers was compared in A549 and MRC-5 cells expressing PIV5/V(w3), which are STAT1-deficient, and PIV5/V(CPI-), which express STAT1. PIV5/V(w3) expression protected A549 cells from treatment with FasL/CHX- and TNF-α/CHX-induced apoptosis while V(CPI-) expression did not (**Figures 3.9A,B; left panel**). PIV5/V(w3) appears then to limit apoptosis induced by DR ligands in A549 cells by promoting STAT1 degradation. Results obtained in MRC-5 cells were more difficult to interpret. V(w3), but not V(CPI-), limited FasL-induced apoptosis, but neither V(w3) nor V(CPI-) delayed apoptosis induced by TNF-α/CHX (**Figures 3.9A,B; right panel**). V(w3) and V(CPI-) both protected A549 cells from STS treatment (although V(w3) was more efficient in this respect; **Figure 3.9C; left panel**). In MRC-5 cells however, V(CPI-) is unable to limit STS-induced apoptosis (**Figure 3.9C; right panel**), perhaps indicating that the involvement of STAT1 in apoptosis is cell-type-specific.

3.2.3 IFN-γ/FasL synergy is inhibited by PIV5/V

Since PIV5/V antagonises IFN signalling by targeting STAT1 for degradation, it was investigated whether IFN-γ/FasL synergism was inhibited in A549/PIV5/V cells. As demonstrated above, A549/PIV5/V(w3) cells express no STAT1, even following IFN treatment (**Figure 3.7A**). Significantly, A549/PIV5/V(w3) cells did not die following 24hrs treatment with IFN-γ/FasL, compared to the extensive (~70%) cell death observed in naïve cells (**Figure 3.10A**). Since PIV5/V is multifunctional and was found to delay apoptosis in the previous section, it was necessary to confirm that PIV5/V inhibition of IFN-γ/FasL-induced apoptosis was dependent on its ability to promote STAT1 degradation. A549/PIV5/V(CPI-) cells, in which IFN signalling pathways are intact, were sensitive to IFN-γ/FasL, exhibiting over 50% cell death within 24hrs (**Figure 3.10A**). Interestingly though, the level of cell death in A549/PIV5/V(CPI-) was lower than in naïve cells, perhaps suggesting that PIV5/V also inhibits apoptosis independently of its effects on STAT1. Additional evidence for PIV5/V(w3)-mediated inhibition of IFN-γ/FasL synergy was obtained by serial titration of FasL on IFN-γ-pre-treated A549 cells. Even at the highest concentration of FasL (200ng/ml), V(w3)-expressing cell monolayers remained intact, while naïve and PIV5/V(CPI)-expressing cells exhibited cell death down to 25ng/ml FasL (**Figure 3.10B**). In support of these data, immunoblot analyses showed that both naïve and PIV5/V(CPI-) expressing cells, but not A549/PIV5/V(w3), exhibited significant PARP cleavage in response to IFN-γ/FasL (**Figure 3.10C**). An additional observation was that in A549 naïve and A549/PIV5/V (CPI-) cells, STAT1 expression decreased following treatment with IFN-γ/FasL. This was probably due to caspase-dependent cleavage and inactivation of STAT1 during apoptosis (King and Goodbourn, 1998).

To determine whether expression of other viral IFN antagonists could block IFN-γ/FasL-induced apoptosis, A549 cells stably expressing PIV5/V(w3), HCV/NS3/4A, BVDV/NPro, the V protein of mumps virus (MuV/V) or IAV/NS1 were treated with IFN-γ/FasL. HCV/NS3/4A, BVDV/NPro and IAV/NS1-expressing monolayers exhibited considerable cell death (**Figure 3.10D**). These viral proteins did not block IFN-γ/FasL-induced apoptosis because, while they block activation of the IFN promoter, they do not affect transduction of the IFN signal so are unable to block the pro-apoptotic effects of IFN-γ. In contrast, there was no evidence of cell death in MuV/V-expressing cells; expression of MuV/V therefore conferred resistance to IFN-γ/FasL-induced apoptosis. This is because, like PIV5/V, MuV/V targets STAT1 for degradation (Kubota et al., 2001, Yokosawa et al., 2002). In conclusion, IFN-γ/FasL

synergy was dependent on STAT1 expression, and as such could be inhibited by PIV5 and MuV V proteins.

3.2.4 IFN and apoptosis induction by different PIV5 strains

The previous section reported that the STAT1-degradative function of V was responsible for inhibiting apoptosis caused by non-viral inducers. It was next determined whether V contributed to apoptosis regulation during PIV5 infection. The PIV5-w3, PIV5-CPI+, PIV5-CPI- and rPIV5-VΔC strains of PIV5 all express V proteins with different biological properties, including their ability to target STAT1 for degradation. The amino acid differences in V between these strains and the consequences for STAT1 and mda-5 inhibition are depicted in **Figure 3.11**. The w3 strain of PIV5 is generally considered to be a PIV5 prototype virus. As described above, its V protein is an IFN antagonist; it targets STAT1 for proteasomal degradation and limits IFN induction by inhibiting mda-5 activity (Didcock et al., 1999b, Andrejeva et al., 2004). The V protein of PIV5-CPI+ behaves much like V(w3), even though these proteins differ by three amino acids in their N-termini (Chatziandreou et al., 2002); its V protein is also able to bind mda-5 and target STAT1 for degradation. The PIV5-CPI- virus was isolated from a dog experimentally infected with PIV5-CPI+ (Baumgartner et al., 1987). V(CPI-) is unable to target STAT1 for degradation due to a further three amino acid substitutions in its N-terminus, but is still able to bind mda-5 to limit IFN induction (Poole et al., 2002, Chatziandreou et al., 2002, Southern et al., 1991). The recombinant rPIV5-VΔC virus lacks the conserved C-terminus of V; as a result, this virus is unable to block either IFN production or JAK/STAT signalling (He et al., 2002a, Poole et al., 2002). PIV5-w3, PIV5-CPI+, PIV5-CPI- and rPIV5-VΔC are reported to vary in their ability to induce IFN (Poole et al., 2002). Since an important function of the IFN response is to establish a pro-apoptotic state in cells (Barber, 2000), induction of large amounts of IFN by a virus may increase cell death. Indeed, rPIV5-VΔC is reported to induce significant amounts apoptosis as well as IFN (Sun et al., 2004). It was therefore investigated whether differences in V altered IFN and apoptosis induction by these viruses.

IFN production by A549 cells infected with PIV5-w3, PIV5-CPI+, PIV5-CPI- and rPIV5-VΔC was estimated in a cell-based IFN assay. As expected, these viruses induced different levels of IFN. rPIV5-VΔC was the best inducer; infected cells produced at least 5-fold more IFN than any of the other strains at 48 hours post-

infection (hpi) (**Figure 3.12**). In contrast, cells infected with PIV5-w3 produced no IFN until very late in infection and even at 48hpi, only small amounts of IFN were detectable. PIV5-CPI+ and PIV5-CPI- induced similar levels of IFN, with both strains inducing significantly more IFN than PIV5-w3. Naturally-occurring and experimentally-introduced mutations in the V protein therefore affect the ability of PIV5 to induce IFN.

To determine whether these PIV5 strains also varied in their ability to induce apoptosis, A549 cell viability following PIV5 infection was measured by wst-1 assay. While PIV5-w3 did not cause significant cell death, even as late as 7 days post-infection (dpi), infection with PIV5-CPI- virus did (~60% reduction in viability at 7 dpi; **Figure 3.13A**). Infection with PIV5-CPI+ caused less cell death than PIV5-CPI-, despite both viruses being similar inducers of IFN. Surprisingly, rPIV5-VΔC, which is reported to be apoptogenic, did not cause any cell death.

To investigate whether induction of cell death by PIV5-CPI- was due to its inability to target STAT1 for degradation, A549 cells defective in the IFN response (IRF3-deficient A549/BVDV/NPro and STAT1-deficient A549/PIV5/V) were infected with all four strains of PIV5. Despite induction of considerable cell death in naïve A549 cells, PIV5-CPI- caused no cell death in A549/BVDV/NPro cells (**Figure 3.13B**). PIV5-CPI-induced cell death was also reduced in A549/PIV5/V cells, to levels similar to PIV5-CPI+ (**Figure 3.13C**). Notably, rPIV5-VΔC, which did not induce cell death in naïve A549 cells, caused large amounts of apoptosis in A549/BVDV/NPro and A549/PIV5/V (~80% at 4 days post-infection; **Figures 3.13B,C**). These results suggest that rPIV5-VΔC was unable to replicate in naïve cells due to the establishment of an IFN-induced antiviral state. In A549/BVDV/NPro and A549/PIV5/V cells however, IFN is either not produced or cannot signal effectively, so is virus replication is not inhibited and virus induces significant cell death.

3.2.5 PIV5 infection and apoptosis induction by non-viral inducers

It was next determined whether V expression in the context of PIV5 infection was able to inhibit apoptosis in response to non-viral inducers. A549 cells were infected with PIV5-w3, rPIV5-VΔC, PIV5-CPI+ and PIV5-CPI- at high MOI, then treated with the DR ligands TNF-α or FasL (in the presence of CHX), or STS. Surprisingly, given the inhibitory effects of PIV5/V ectopic expression on apoptosis, the response of cells

to TNF-α/CHX, FasL/CHX or STS treatment was not significantly affected by infection with any of the PIV5 strains (**Figures 3.14**).

Conclusions

Depletion of IRF3 by BVDV/NPro expression had no effect on cell death, while NS3/4A expression was able to limit TNF-α/CHX-induced apoptosis. PIV5/V(w3) ectopic expression limited apoptosis induction by FasL/CHX, TNF-α/CHX and STS in A549 cells. In addition, PIV5/V(w3) could block the facilitatory effect of IFN-γ on FasL-induced apoptosis. In MRC-5 cells, PIV5/V(w3) expression inhibited FasL and STS, but not TNF-α/CHX-induced apoptosis. These effects were dependent on the ability of V to promote STAT1-degradation. Expression of V in the context of PIV5 infection was unable to block apoptosis in response to the same inducers however. Naturally occurring and experimentally introduced substitutions in V affected the ability of PIV5 to induce IFN and apoptosis. Compromising the IFN response by knocking out STAT1 or IRF3 altered the apoptosis induction profiles of viruses expressing these V proteins.

3.2.6 Modulation of PI3K/Akt signalling by PIV5/V

The ability of PIV5/V (CPI-) to partially block STS-induced apoptosis in A549 cells suggests PIV5/V may regulate host-cell apoptosis independently of STAT1; V may be therefore be modulating additional signalling pathways in the cell. A role of Akt in PIV5 infection has been proposed: V is reported to interact with Akt to enhance virus replication (Sun et al., 2008a). Many viruses activate PI3K/Akt to prevent or delay virus-induced apoptosis (Peters et al., 2008, Lee et al., 2001, Dawson et al., 2003, Zhang et al., 2002, Bitko et al., 2007), so the interaction between V and Akt may also play a role in limiting apoptosis inhibition. However, modulation of Akt signalling by V has not yet been reported. This section seeks to elucidate the effects of V expression on Akt activation.

3.2.6.1 Akt activation is inhibited by PIV5/V

To determine whether ectopic PIV5/V expression could affect Akt activation, naïve A549 and A549/PIV5/V(w3) cells were probed for levels of phospho-Akt in the presence of serum. While PIV5/V(w3) expression did not activate Akt, it was surprising to note that phospho-Akt levels were lower in A549/PIV5/V(w3) than naïve

cells (**Figure 3.15**). Since IFN activates Akt (**Figure 3.5A**) and PIV5/V blocks IFN signalling by promoting STAT1 degradation, it follows that V may be able to block IFN-stimulated Akt activation. Indeed, Akt activation by IFN-α or IFN-γ was inhibited in A549 cells expressing PIV5/V(w3) (**Figure 3.15**). PIV5/V(w3) can therefore block Akt activation by both serum and IFN.

The V protein is reported to directly interact with Akt (Sun et al., 2008a); this interaction may be important for PIV5/V inhibition of Akt. To determine whether a V:Akt interaction could be detected, 293T cells were transfected with plasmids expressing PIV5/V(w3), Akt or V and Akt and lysed 24hrs later. V and Akt were immunoprecipitated using anti-V5 and anti-Akt antibodies under low-salt conditions then immunoprecipitated V and Akt were detected by immunoblotting. The data shown in **Figure 3.16** is representative of three independent experiments. No Akt was detected in lysates immunoprecipitated with antibody to V and conversely, V could not be detected in lysates immunoprecipitated with anti-Akt antibody. No evidence of V:Akt interaction was therefore obtained in this experiment.

To determine whether the ability of PIV5/V(w3) to inhibit Akt activation was dependent on its ability to target STAT1 for degradation, A549 cells expressing PIV5/V(CPI-) were treated with IFN and probed for active Akt. Following IFN-α and IFN-γ treatment or in the presence of serum only, A549/PIV5/V(CPI-) cells showed a similar level of Akt activation to naïve cells (**Figure 3.15**). This suggests that the ability of serum and IFN to activate Akt is dependent on an intact STAT signalling pathway and that PIV5/V inhibits Akt activation through its effects on STAT1 expression.

3.2.6.2 Akt activation during PIV5 infection

It has been suggested previously that the V protein may inhibit PIV5-induced Akt activation (Luthra et al., 2008). To investigate Akt activation during PIV5 infection, A549 cells were infected with PIV5-w3, SeV and IAV. SeV and IAV were included as positive controls as both viruses are reported to activate PI3K/Akt (Peters et al., 2008, Hale et al., 2006). As expected, IAV infection strongly activated Akt: large amounts of phospho-Akt could be detected from 4 to 24 hpi (**Figure 3.17A**). Transient Akt activation was also induced by SeV but this was much weaker than during IAV infection; phospho-Akt was detectable between 8 and 12 hpi, but had completely disappeared by 24 hpi. PIV5-w3 infection, on the other hand, did not

activate Akt significantly, as even at 24 hpi, phospho-Akt levels were only slightly higher than background.

As demonstrated in the previous section, IFN treatment activates Akt and this can be inhibited by PIV5/V. PIV5-w3-infected cells may be unable to activate Akt either because PIV5-w3 induces only minimal amounts of IFN or because its V protein blocks IFN signalling and Akt activation. Strains of PIV5 that are better IFN inducers than w3 or that are unable to block IFN signalling may therefore activate Akt more efficiently. To investigate whether this was the case, A549 cells were infected with PIV5-w3, PIV5-CPI+, PIV5-CPI- and rPIV5-VΔC at equal MOI and cell lysates probed for Akt activation and viral protein expression. The polyclonal anti-V antibody used to detect V also reacts with the PIV5 P protein due to their shared N-termini (**Figure 3.17B**). Although rPIV5-VΔC does not express a full-length V protein, a protein with a similar mobility to full-length V was detected in rPIV5-VΔC-infected cells. This band corresponds to a degradation product of the P protein (Thomas et al., 1988, He et al., 2002a), and is labelled P* in **Figure 3.17B**. Lysates were therefore also probed with antibody specific to the C-terminus of V: no V expression was detectable in rPIV5-VΔC-infected cells. In PIV5-w3 and PIV5-CPI+ infected cells, phospho-Akt could only be detected late in infection (36 hpi; **Figure 3.17B**). In contrast, PIV5-CPI- infection activated Akt from 24hrs with even more potent activation at 36hpi, while rPIV5-VΔC activated Akt strongly from 12 hpi onwards. Activation of Akt by PIV5 therefore appears to be related to both the levels of IFN induction and the functionality of STAT signalling in infected cells.

Conclusions

PIV5/V inhibits Akt activation by serum and IFN in A549 cells in a manner that requires its ability to degrade STAT1. In support of this, PIV5 strains that are unable to degrade STAT1 (PIV5-CPI- and rPIV5-VΔC) activate Akt much more strongly than those that do (PIV5-w3 and PIV5-CPI+).

3.3 IAV NS1, PI3K activation and innate antiviral responses

The NS1 protein of IAV is reported to interact with a number of host cell proteins to interfere with the IFN system and apoptosis. One of these interactors is PI3K: a direct interaction between the IAV NS1 protein and the p85 regulatory subunit of PI3K results in activation PI3K/Akt signalling (Hale et al., 2006, Shin et al., 2007c). As discussed in the **Introduction**, PI3K and its downstream target Akt play a pivotal role in the regulation of apoptosis and the IFN response. This section explores the implications of this p85:NS1 interaction for host cell apoptosis and the IFN system. In addition, the biological consequences of NS1 mutations that prevent its interaction with other host cell proteins are investigated.

3.3.1 IAV NS1 activates PI3K

3.3.1.1 The kinetics of PI3K activation by IAV are cell-type specific

The ability of IAV to activate PI3K in a wide range of cell-lines was investigated. Lysates of cells infected with the Udorn strain of IAV were prepared at various times post-infection, then probed for PI3K activation using Akt phosphorylation at Ser473 as a marker. In MDCK (canine kidney epithelial) cells, PI3K activation was extremely transient (**Figure 3.18**); phospho-Akt was present in large amounts at 8h, but reduced to just above background level from 12h onwards, despite significant expression of NS1. In contrast, A549 cells displayed considerable Akt activation until 24hrs, after which phospho-Akt levels decreased to background levels at 48 hrs. In the fibroblast cell-lines MRC-5 and HFFF, Akt activation was much more prolonged, with significant phospho-Akt still detectable at 48 hrs post-infection. Similarly, 1321N1 (human brain astrocytoma) cells displayed considerable amounts of phospho-Akt as late as 60 hrs post-infection. Akt was also activated by IAV infection (albeit transiently) in Vero (monkey kidney) cells, which are unable to produce IFN due to deletion of IFN-α/β genes (Desmyter et al., 1968), and in Hep2 cells in which IAV cannot replicate productively (Ludwig, 2006). These data demonstrate that the kinetics of Akt activation by IAV differ significantly with cell-type.

3.3.1.2 rUd-Y89F is unable to activate PI3K

The introduction of a Y89F substitution into NS1 abrogates the NS1:PI3K interaction; consequently, a recombinant virus expressing this mutant NS1 is unable to activate

PI3K in MDCK cells (Hale et al., 2006). For reasons described in **Section 3.1.4**, A549 and MRC-5 were considered the most suitable cell-lines for this study. To investigate the biological implications for PI3K activation by NS1 then, it was important to ensure that rUd-Y89F was also unable to activate PI3K/Akt in these cell types. A significant amount of previous work on the PI3K:NS1 interaction has been carried out in MDCK cells, so these cells were also included to enable direct comparison of results with these studies. In accordance with previous work (Hale et al., 2006), rUd wt infection of MDCK cells transiently activated Akt while no phospho-Akt was detectable in cells infected with the rUd-Y89F virus (**Figure 3.19**). Similarly, A549 and MRC-5 cells exhibited significant Akt activation following infection with rUd wt but not rUd-Y89F.

3.3.1.3 rUd-Y89F is attenuated in A549 and MRC-5 cells

In MDCK cells, rUd-Y89F displays a small-plaque phenotype and grows to a titre ~10-fold lower than rUd wt in a single-step growth curve (Hale et al., 2006). To establish whether rUd-Y89F was also attenuated in A549 and MRC-5 cells, the growth rates of rUd wt and rUd-Y89F were determined in a multi-step growth curve. In A549 cells, rUd-Y89F virus yield was ~2 orders of magnitude lower than for rUd wt (**Figure 3.20A**). rUd-Y89F was also attenuated in MRC-5 cells, but the difference was less pronounced than in A549: rUd-Y89F grew to a titre ~10-fold lower than rUd wt (**Figure 3.20B**).

Conclusions

rUd wt activates PI3K/Akt in a wide range of cells, but the kinetics of Akt activation differ according to the cell-type. The rUd-Y89F virus is unable to activate PI3K/Akt signalling and is attenuated in A549 and MRC-5 cells.

3.3.2 Effect of PI3K activation by NS1 on apoptosis

3.3.2.1 IAV induces apoptosis at high multiplicity

IAV infection causes apoptosis *in vitro* and *in vivo* (Mori et al., 1995, Hinshaw et al., 1994, Fesq et al., 1994, Takizawa et al., 1993, Price et al., 1997). Phase-contrast time-lapse microscopy of IAV-infected A549 cells (**Figure 3.21; supplied disc**) revealed that a proportion of infected cells went into apoptosis within a 24hr period;

these cells exhibited membrane blebbing and detached from the cell monolayer. However, apoptosis was only seen at very high MOI (~50 PFU/cell) and most cells did not go into apoptosis, suggesting that IAV may be able to inhibit cellular apoptosis.

3.3.2.2 NS1 expression does not protect cells from apoptosis

Numerous studies have attributed NS1-mediated PI3K activation an anti-apoptotic role in the host cell (Zhirnov and Klenk, 2007, Ehrhardt et al., 2007, Shin et al., 2007c). It was therefore determined whether NS1-mediated PI3K activation could protect cells from DR ligand and STS-induced apoptosis. A549 cell lines stably expressing the NS1 or NS1-Y89F proteins from the PR8 strain of IAV were produced using lentivirus vectors. Both NS1 proteins were expressed at similar levels, and expression of NS1, but not NS1-Y98F, in A549 cells activated Akt in the absence of serum (**Figure 3.22F**). Cell viability was measured in these cells following treatment with TNF-α (+/- CHX), FasL (+/- CHX) and STS. Surprisingly, there was no significant difference in apoptosis induction in NS1 wt and NS1-Y89F-expressing cells following treatment with any of these inducers (**Figures 3.22**).

3.3.2.3 rUd-Y89F does not cause more apoptosis than rUd wt in epithelial cells and fibroblasts

The results obtained in the previous section contradicted a previous report that NS1-mediated PI3K activation could prevent STS-induced apoptosis (Ehrhardt et al., 2007). The role of PI3K activation in IAV-induced apoptosis was therefore investigated further: it was determined whether viruses expressing NS1-Y89F induced apoptosis more readily than wt virus. MDCK, A549 and MRC-5 cells were infected with wt or -Y89F rUd or rWSN viruses and cell viability was measured at various times post-infection. Neither rUd-Y89F nor rWSN-Y89F, also unable to bind and activate PI3K (Hale, 2007), caused any more cell death than wt virus in A549 and MDCK cells (**Figures 3.23A,B**). In MRC-5 cells, rUd-Y89F and rWSN-Y89F seemed to cause slightly (~15%) more cell death than wt virus (**Figure 3.23C**) at very late times in infection (at least 30 hpi). However, as PI3K/Akt is activated from 8hpi in MRC-5 cells (**Figure 3.19**), this difference in cell death late in infection is unlikely to be a direct effect of PI3K activation on apoptosis. In addition, given the sensitivity of MRC-5 cells to apoptosis (**Figure 3.6**), it was surprising to note that MRC-5 cells did

not readily die following infection with either wt or -Y89F virus; ~60% of cells are still alive up to 50 hpi.

To further compare onset of apoptosis following rUd wt and rUd-Y89F infection, lysates from infected cells were probed for caspase activation. The activation of caspase-3 or cleavage of PARP, both hallmarks of apoptosis, in rUd-Y89F-infected MDCK, A549 and MRC-5 cells was not any higher than in rUd wt-infected cells (**Figure 3.24**).

3.3.2.4 rUd wt infection does not protect cells from non-viral apoptosis inducers

A similar experiment to the one in **Figure 2.22** was carried out to determine whether PI3K activation during rUd infection could protect cells from non-viral apoptosis inducers. A549 and MRC-5 cells were infected with rUd wt and rUd-Y89F for a period of 12-14hrs, then treated with FasL, FasL/CHX (A549 only), TNF-α, TNF-α/CHX and STS. Notably, there was no difference between the cell death responses of rUd wt and rUd-Y89F-infected cells in response to any of these treatments (**Figure 3.25**). However, IAV infection (either rUd wt or rUd-Y89F) did appear to sensitise cells to apoptosis. FasL-resistant A549 cells (**Figure 3.1**) were sensitised to FasL-mediated apoptosis (**Figure 3.25A; left panel**) following IAV infection, and infected MRC-5 cells, which are already FasL sensitive (**Figure 3.6**), went into FasL-induced apoptosis more rapidly than uninfected cells (**Figure 3.25A; right panel**). In addition, prior infection of A549 cells with rUd accelerated FasL/CHX-induced cell death compare to either infection or treatment alone (**Figure 3.25B**). MRC-5 cells are normally resistant to TNF-α treatment, but were sensitised to TNF-α following IAV infection, but infection did not have the same facilitatory effect on TNF-α treatment in A549 cells for unknown reasons (**Figure 3.25C**). Virus infection offered slight protection from TNF-α/CHX-induced apoptosis, particularly in MRC-5 cells (**Figure 3.25D**), while there was no significant difference in the rate of STS-induced apoptosis between infected and mock-infected A549 and MRC-5 cells (**Figure 3.25E**).

3.3.2.5 The PI3K inhibitor LY294002 can give misleading results

Previous studies into the effect of PI3K activation by NS1 on apoptosis relied on the pharmacological PI3K inhibitor LY294002 (LY) to inhibit PI3K activation (Ehrhardt et al., 2007, Zhirnov and Klenk, 2007). However, LY is also a potent inhibitor of several other kinases and may inhibit virus entry to cells (Ehrhardt et al., 2006, Bain et al.,

2007, Davies et al., 2000, Gharbi et al., 2007). To investigate whether use of LY in previous studies was the reason for contradictions with data obtained in this study, cell viability was measured during infection with rUd wt or rUd-Y89F in the presence of DMSO or LY. LY treatment alone caused significant cell death in both A549 and MRC-5 cells (**Figure 3.26**). rUd infection in the presence of LY caused more cell death than either LY or rUd alone: this result could be interpreted to mean that inhibition of PI3K activation by NS1 causes significant cell death. An alternative explanation is that the dual assault of drug treatment and virus infection sensitises cells to apoptosis independently of PI3K inhibition. Indeed, combining LY treatment and rUd-Y89F infection also caused more cell death than either LY or rUd-Y89F alone in A549 cells (**Figure 3.26A**). In MRC-5 cells, which are very sensitive to apoptosis, the difference between rUd-Y89F-induced cell death with/without LY was particularly marked (~25%; **Figure 3.26B**). The effect of LY on IAV-induced apoptosis is therefore independent of its ability to inhibit PI3K activation by NS1, and results obtained using LY should be interpreted with caution.

3.3.2.6 PI3K activation by NS1 reduces p53 expression and activation

p53 is activated during IAV infection but is not a major factor in promoting IAV-induced apoptosis (Technau-Ihling et al., 2001, Turpin et al., 2005, Zhirnov and Klenk, 2007). Since PI3K activation reduces p53 expression and activation (Mayo and Donner, 2001, Chun-gen Xing, 2008, Filiz et al., 2008), the effects of PI3K activation by NS1 on p53 were investigated. In both rUd wt and rUd-Y89F-infected cells, p53 was phosphorylated at Ser-15 from 10hrs post-infection onwards (**Figure 3.27A**). However, the level of active p53 was greater in cells infected with rUd-Y89F. Total p53 also seemed to increase during rUd-Y89F, but not rUd wt, infection despite similar levels of NS1. In support of these observations, 1321N1 cell-lines expressing PR8/NS1-Y89F exhibited higher phospho-p53 levels than 1321N1 naïve and 1321N1/NS1 wt (**Figure 3.27B**). In addition, cells expressing NS1 wt had decreased total p53 levels compared to NS1-Y89F.

3.3.2.7 PI3K activation by NS1 does not affect FasL expression

Akt negatively regulates FasL expression through inhibition of FOXO transcription factors (Suhara et al., 2002, Uriarte et al., 2005, Tang et al., 1999, Ciechomska et al., 2003). In addition, p53 activation can promote FasL expression (Hsu et al., 2004b). While data obtained thus far indicates that PI3K does not mediate-anti-apoptotic

signalling during IAV infection, it may still be able to modulate FasL expression. Although FasL is predominantly expressed on the surfaces of NK cells, it is also expressed on the surface of various tumour cell-lines (Kawasaki et al., 2000, Strand et al., 1996, Hahne et al., 1996, Shurin et al., 1998, Muschen et al., 2000, O'Connell et al., 1996, Niehans et al., 1997). To investigate whether PI3K/Akt activation by NS1 could affect FasL expression, A549 cells which express FasL (Hsu et al., 2004b, Du et al., 2006, Kawasaki et al., 2000), were infected with rUd wt or rUd-Y89F and probed for FasL expression. The levels of FasL were similar in rUd wt and rUd-Y89F-infected cells however, despite differences in PI3K/Akt activation (**Figure 3.28**).

3.3.2.8 PI3K activation by NS1 prevents apoptosis in neutrophils

Neutrophils, mobile phagocytes that are rapidly recruited to the sites of virus infections to promote virus clearance (Grundy et al., 1998, Tate et al., 2008), are reported to function as a vehicle for IAV systemic spread (Zhao et al., 2008, Ratcliffe et al., 1992). These cells have a short-half life of about 24hrs and undergo apoptotic cell death after this period (Savill et al., 1989). PI3K/Akt promotes neutrophil survival (Webb et al., 2000, Francois et al., 2005, Yasui et al., 2002), so a preliminary experiment was carried out to determine whether PI3K activation by NS1 could inhibit neutrophil apoptosis. Freshly isolated neutrophils or A549 cells (as a negative control) were infected with rUd wt and rUd-Y89F and the proportion of apoptotic cells quantified at 24hpi by annexin V/propidium iodide (PI) staining and flow cytometry. In support of previously obtained data, rUd-Y89F did not cause any more apoptosis than rUd wt in A549 cells (**Figure 3.29A**). In neutrophils however, infection with rUd-Y89F caused over 15% more apoptosis than wt (**Figure 3.29B**).

Conclusions

PI3K activation by NS1 down-regulates p53 expression and activity, but this does not correlate with apoptosis induction: IAVs expressing NS1-Y89F did not cause any more apoptosis than wt virus in MDCK, A549 and MRC-5 cells. In addition, PI3K activation by IAV infection or ectopic NS1 expression was unable to protect cells from non-viral apoptosis inducers. However, PI3K activation by IAV may play a role in prolonging neutrophil survival.

3.3.3 Effect of PI3K activation by NS1 on the IFN response

3.3.3.1 PI3K activation by NS1 has no effect on IFN production

As described in the **Introduction**, PI3K activation limits induction of the IFN promoter by inhibiting NF-kB (Aksoy et al., 2005). Additionally, p53 contributes to IFN release from virus-infected cells (Munoz-Fontela et al., 2008). It follows then that PI3K/Akt activation and/or the decrease in p53 expression and activation seen during rUd wt infection (**Figure 3.27**) may affect IFN induction in infected cells. The amount of IFN secreted from A549 cells infected with rUd wt or rUd-Y89F viruses was quantified at several points post-infection by cell-based IFN assay. However, there was no difference in IFN induction by rUd wt or rUd-Y89F viruses (**Figure 3.30**).

3.3.3.2 PI3K activation by NS1 has no effect on NF-kB activation

While PI3K activation can interfere with IFN production by inhibiting NF-kB activation in response to dsRNA (Aksoy et al., 2005), PI3K/Akt can also activate NF-kB signalling in response to IFN treatment (Du et al., 2007). It was therefore of interest to determine whether PI3K activation by NS1 had any effect on NF-kB transcriptional activity. A549 cells stably expressing luciferase under the control of an NF-kB-responsive promoter were infected with rUd wt or rUd-Y89F. TNF-α treatment served as a positive control and increased NF-kB activity over two-fold in these cells (**Figure 3.31A**). Basal levels of NF-kB activity in uninfected cells were relatively high and were significantly reduced by rUd infection (from 2hpi), indicating that rUd can shut-off host protein expression from a very early point in infection. However, there was no difference in NF-kB activation between cells infected with rUd wt and rUd-Y89F, despite differences in Akt activation (**Figure 3.31**).

3.3.3.3 PI3K activation by NS1 has no effect on ISRE activation and ISG expression

IFN treatment induces Ser727 phosphorylation of STAT1 and enhances its transcriptional activity through a kinase downstream of PI3K/Akt (Nguyen et al., 2001, Wen et al., 1995). PI3K can also increase mRNA translation of ISGs through an mTOR-mediated pathway (Kaur et al., 2008a, Lekmine et al., 2003). Additionally, p53 has been reported to enhance IFN signalling (Munoz-Fontela et al., 2008). Activation of PI3K and/or the subsequent reduction of p53 activity may therefore affect transcription from the ISRE. ISRE activation during rUd wt and rUd-Y89F

infection was examined using A549 cells expressing luciferase under the control of an ISRE. Treatment of these cells with IFN-α (the positive control) increased luciferase activity ~10-fold (**Figure 3.32A**). The ISRE was also activated (to a much smaller extent) following rUd infection, although ISRE activation declined as the infection proceeded. However, there was not a significant difference between ISRE induction with rUd wt and rUd-Y89F viruses. Similarly, PI3K activation by NS1 did not affect IFN-α signalling to the ISRE activation either, as determined by IFN-α treatment of rUd-infected cells (**Figure 3.32B**). The ability of rUd wt and rUd-Y89F infection to affect ISG15 expression was also investigated. In accordance with its role as an ISG, ISG15 levels in A549 cells were upregulated by IFN-α treatment (**Figure 3.33**), and rUd infection significantly reduced ISG15 expression levels due to shut-off of cellular protein synthesis. However, there was not a significant difference in ISG15 expression between rUd wt and rUd-Y89F-infected cells, either with or without IFN-α treatment.

Conclusions

rUd wt and rUd-Y89F did not differ in their induction of IFN or activation of NF-kB. Similarly, no difference in ISRE activation and ISG15 expression was observed in infected cells. However, IAV infection (either rUd wt or rUd-Y89F) inhibited ISG15 expression in response to IFN-α, and prevented expression of luciferase in NF-kB and ISRE reporter systems due to shut-off of host protein synthesis.

3.3.4 Mutations in NS1 affect apoptosis and IFN induction

NS1 is reported to interact with a significant number of cellular and viral factors, including dsRNA, PKR and CPSF30, to modulate host antiviral responses. The residues of NS1 responsible for these interactions have been mapped and introduced into recombinant viruses to investigate the biological consequences of each interaction. The relative contribution of the dsRNA, PKR, CPSF30 and PI3K interactions to evasion of antiviral responses has not yet been established, as direct comparison between these studies is difficult due to the different virus backgrounds used for the production of mutant viruses. As part of ongoing influenza research at the University of St Andrews, a series of recombinant Udorn viruses have been produced (by D. Jackson and C. Galloway). Since these NS1 mutations are expressed in the same virus background, comparative study of the contribution of each mutation to virus replication and pathogenesis is enabled. The generated

mutants are shown in **Table 3.1.** They comprise those reported to interfere with PI3K binding and activation (rUd-Y89F, rUd-P164/7A and rUd-P162/4/7A), dsRNA binding (rUd-R38A), PKR binding (rUd-123/4), and interaction with CPSF30 (rUd-184-8(L) and rUd-103/106). During the rescue of these viruses, a virus expressing NS1 with a C-terminal truncation (rUd-Δ99) and a virus related to rUd-184-8(L), rUd-184-8(P), were also isolated. The positions of these mutations in the NS1 protein are displayed in **Figure 3.34**.

3.3.4.1 Effects of NS1 mutations on PI3K activation

As part of the characterisation of these mutant viruses, NS1 expression and PI3K activation in response to virus infection was investigated. By 24 hpi, NS1 expression was detected following infection with all viruses except rUd-Δ99 in both A549 and MRC-5 cells (**Figure 3.35**). However, the level of NS1 in rUd-184-8(P)-infected cells was significantly lower than for rUd wt. As expected, Akt was strongly activated in rUd wt-infected A549 and MRC-5 cells and similar levels of phospho-Akt were detected in cells infected with rUd-R38A, rUd-123/4 and rUd-103/106 (**Figures 3.35**). In comparison, cells infected with rUd-Y89F, rUd-164/7A, rUd-162/4/7A, rUd-Δ99 and rUd-184-8(P) did not express more phospho-Akt than background.

3.3.4.2 Mutations in NS1 affect IAV-induced apoptosis

The panel of recombinant viruses was used to determine whether any of these NS1 mutations rendered IAV more apoptogenic. Cell viability was measured in MDCK and A549 cells following infection with these viruses. rUd-Y89F, rUd-P164/7A, rUd-123/4 and rUd-103/106 caused no more cell death than infection with wt in both cell-types (**Figures 3.36A,B**). In contrast, rUd-162/4/7A, rUd-Δ99 and rUd-184-8(P) caused significantly more cell death than wt virus. Cell death caused by the remaining mutants appears to be cell-type specific: rUd-R38A induced cell death in MDCK but not A549 cells, while rUd-184-8(L) induced apoptosis in A549 but not MDCK cells (**Figures 3.36A,B**).

MRC-5 cells, which are extremely sensitive to apoptosis (**Figure 3.6**), were infected with a selection of mutant viruses: rUd-Y89F, rUd-P164/7A, rUd-P162/4/7A and rUd-184-8(P). While rUd-Y89F and rUd-P164/7A did not induce any more cell death than rUd wt, both rUd-P162/4/7A and rUd-184-8(P) did (~20% and ~55% respectively; **Figure 3.36C**).

Caspase activity in infected cells was assessed qualitatively and quantitatively. At 24 hpi, cleaved PARP fragments could be detected by immunoblotting in rUd-184-8(P)-infected A549 and MRC-5 cells (**Figures 3.35A,B bottom panel**). PARP cleavage was also detectable in A549 cells infected with rUd-P162/4/7A. Caspase-3 and -7 activity was measured quantitatively by luciferase assay using a commercially available kit. In both A549 and MRC-5 cells, caspase activity was higher in wt infected than in mock cells (36- and 3-fold respectively; **Figures 3.37**) consistent with IAV infection inducing a small amount of apoptosis. Significantly more caspase activity was observed following infection with rUd-184-8(P) (3.2-fold increase in A549 cells; 6.8-fold increase in MRC-5 cells) and rUd-P162/4/7A (2.1-fold increase in A549 cells; 3.9-fold increase in MRC-5 cells). Interestingly, caspase activity in rUd-Δ99-infected A549 cells was no higher than wt (**Figure 3.37A**); the reasons for this are unknown.

Changes in nuclear morphology are characteristic of apoptosis: apoptotic cells exhibit highly condensed and fragmented nuclear material (Toné et al., 2007). MDCK cells infected with selected mutants were fixed and their nucleic acid stained with acridine orange; cells were subsequently visualised by fluorescence microscopy. No abnormal nuclear morphologies were detected in cells infected with rUd wt, rUd-Y89F or rU-P164/7A (**Figure 3.37C**). In contrast, rUd-P162/4/7A and rUd-184-8(P)-infected cells clearly displayed apoptotic nuclear condensation.

rUd-184-88(P) did not activate PI3K/Akt in infected cells (**Figure 3.35**). To demonstrate that this inability to activate PI3K was not responsible for rUd-184-8(P)-induced apoptosis, it was determined whether PI3K activation by serum could prevent rUd-184-8(P)-induced cell death. A549 cells were infected with rUd-184-8(P) then incubated in either serum-free medium or medium containing 10% FCS. Cell lysates were prepared at 24 hpi, while cell viability was measured at 36 hpi. These time-points were chosen as caspase activation can be detected in rUd-184-8(P)-infected cells by immunoblotting at 24hpi, but there is no detectable reduction in cell viability at this time; for this reason, cell viability was measured at 36hpi, when a significant reduction in cell viability can be detected. rUd-184-8(P) induced the same level of cell death and PARP cleavage in the absence or presence of serum (**Figure 3.38**). Consequently, NS1-independent PI3K activation does not prevent apoptosis caused rUd-184-8(P)-induced apoptosis.

3.3.4.3 IFN induction by NS1 mutant viruses correlates with apoptosis induction

Since NS1 is an IFN antagonist, NS1 mutant viruses that induce a large amount of apoptosis may also be inducing significant levels of IFN. To investigate whether this was the case, IFN induction by the panel of mutants was quantified by cell-based IFN assay. In A549 cells, rUd-184-8(P) induced a large amount of IFN; ~250-fold higher than wt at 24 hpi (**Figures 3.39B**). rUd-184-8(L) and rUd-Δ99 were also better IFN inducers than wt virus. In fact, all mutants, with the exception of rUd-Y89F, induced more IFN than wt in A549 cells. rUd-184-8(P), rUd-184-8(L) and rUd-Δ99 also induced more IFN than wt virus in MRC-5 cells (**data not shown**).

To determine whether apoptosis induction by the different mutant viruses correlated with their ability to induce IFN, cell viability was measured following infection A549/BVDV/NPro cells, which are unable to produce IFN due to IRF3 depletion (characterised in **Section 3.2.1**). Viruses that caused extensive cell death in naïve A549 cells (rUd-P162/4/7A, rUd-Δ99 and rUd-184-8(P)) were unable to cause significant cell death in A549/BVDV/NPro (**Figure 3.40A**). Similar results were also observed in STAT1-deficient A549/PIV5/V cells (**data not shown**).

Conclusions

Recombinant Ud viruses expressing NS1 mutations differed in their ability to activate PI3K/Akt, induce apoptosis and induce IFN. A number of mutants (rUd-184-8(P), rUd-Δ99 and rUd-P162/4/7A) caused more cell death than rUd wt virus. rUd-184-8(P) caused significant caspase activation and nuclear fragmentation in infected cells. While this virus was unable to activate PI3K/Akt, this was not responsible for its ability to induce apoptosis. Significant induction of apoptosis by rUd-184-8(P) was linked to its ability to induce large amounts of IFN; this virus was unable to cause cell death in IFN-deficient cells.

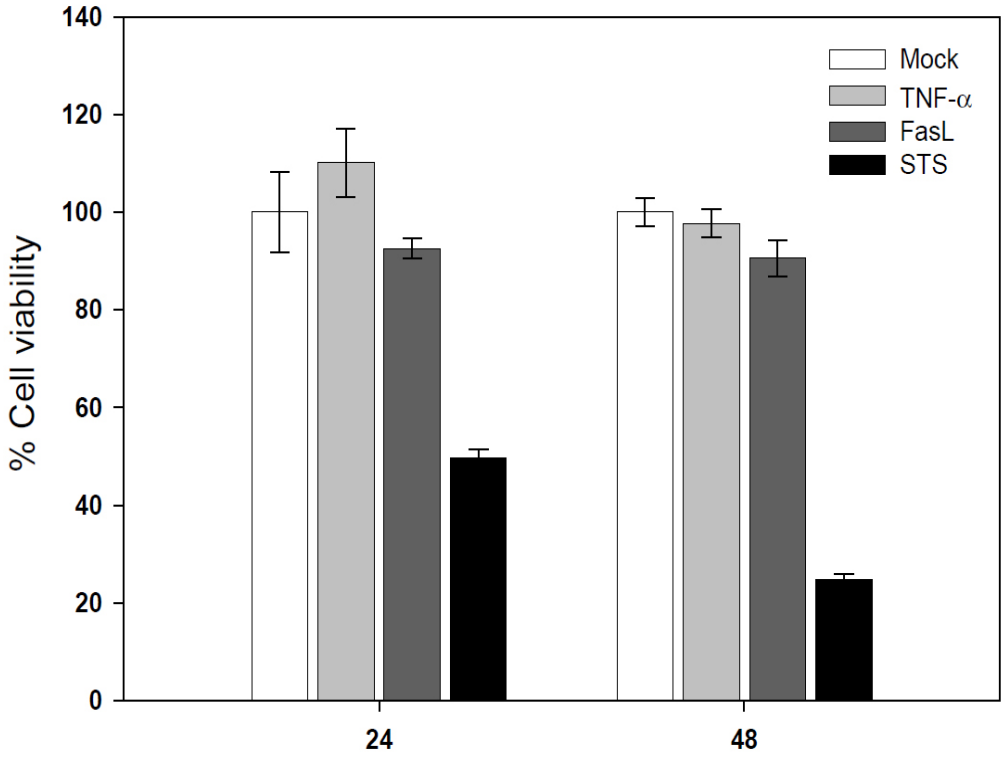


Figure 3.1: A549 cells are sensitive to staurosporine, but not TNF-α– or FasL-induced apoptosis.

A549 cell monoloayers were mock-treated or treated with TNF-α, FasL or staurosporine (STS). 24h and 48h later, cell viability was measured in triplicate by wst-1 assay. Mean viability is plotted as a percentage of mock-treated cells. Error bars represent the standard deviation of each triplicate set.

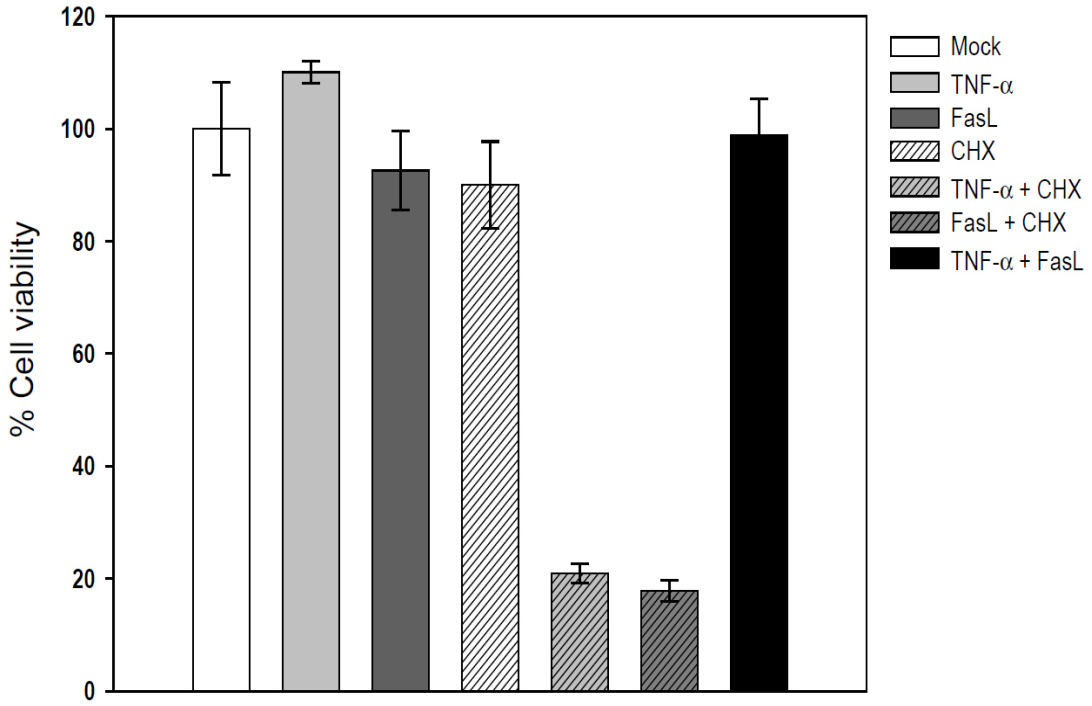


Figure 3.2: Cycloheximide sensitises A549 cells to TNF-α- and FasL- induced apoptosis.

A549 cell monolayers were mock-treated or treated with cycloheximide (CHX), TNF-α, FasL, or combinations of these treatments. After 24 hrs, cell viability was measured in triplicate by wst-1 assay. Mean viability is plotted as a percentage of mock-treated cells. Error bars represent the standard deviation of each triplicate set.

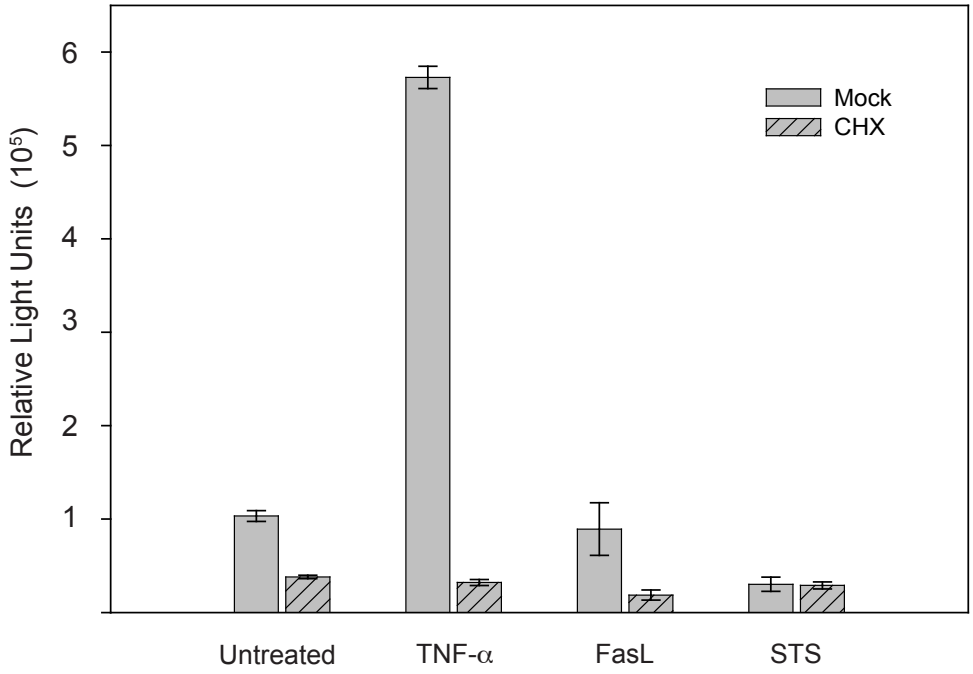


Figure 3.3: TNF-α, but not FasL, activates NF-kB in a CHX-sensitive manner.

A549 cells expressing a luciferase gene under the control of an NF-kB-responsive promoter were treated for 6h with TNF-α, FasL or STS in the presence or absence of CHX. Duplicate measurements of luciferase activity were made. Mean activity is plotted and error bars represent the standard deviation of each duplicate set.

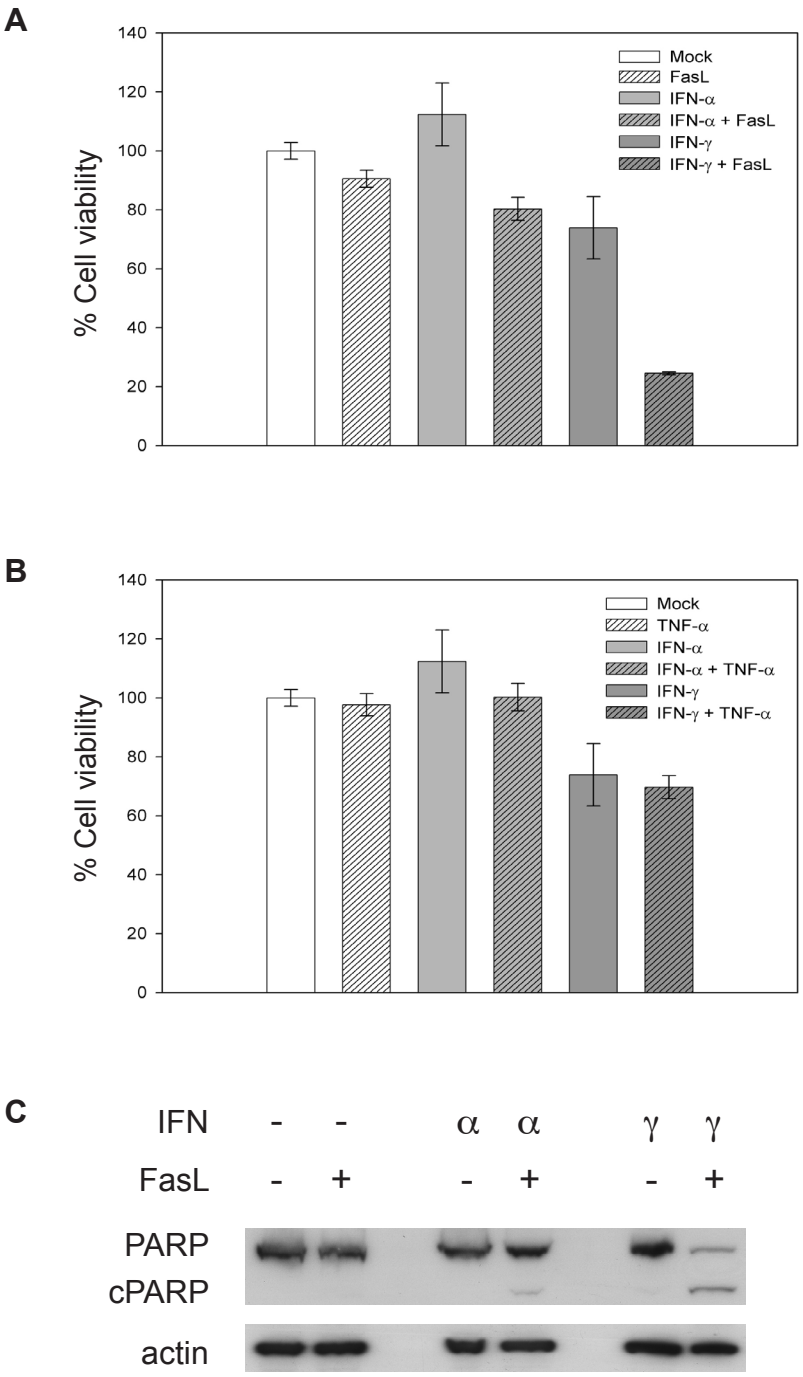


Figure 3.4: IFN-γ sensitises A549 cells to FasL-induced, but not TNF-α-induced apoptosis.

A549 cell monolayers were mock-treated or pre-treated with IFN-α or IFN-γ. 16h later, cells were treated with FasL (**A**) or TNF-α (**B**) for 48h. Cell viability was measured in triplicate using wst-1. Mean viability is plotted as a percentage of mock-treated cells and error bars represent the standard deviation of each triplicate set. (**C**) A549 cells were pre-treated for 16h with IFN-α or IFN-γ and treated with FasL for 24h. Cell lysates were prepared, subjected to SDS-PAGE and immunoblotted.

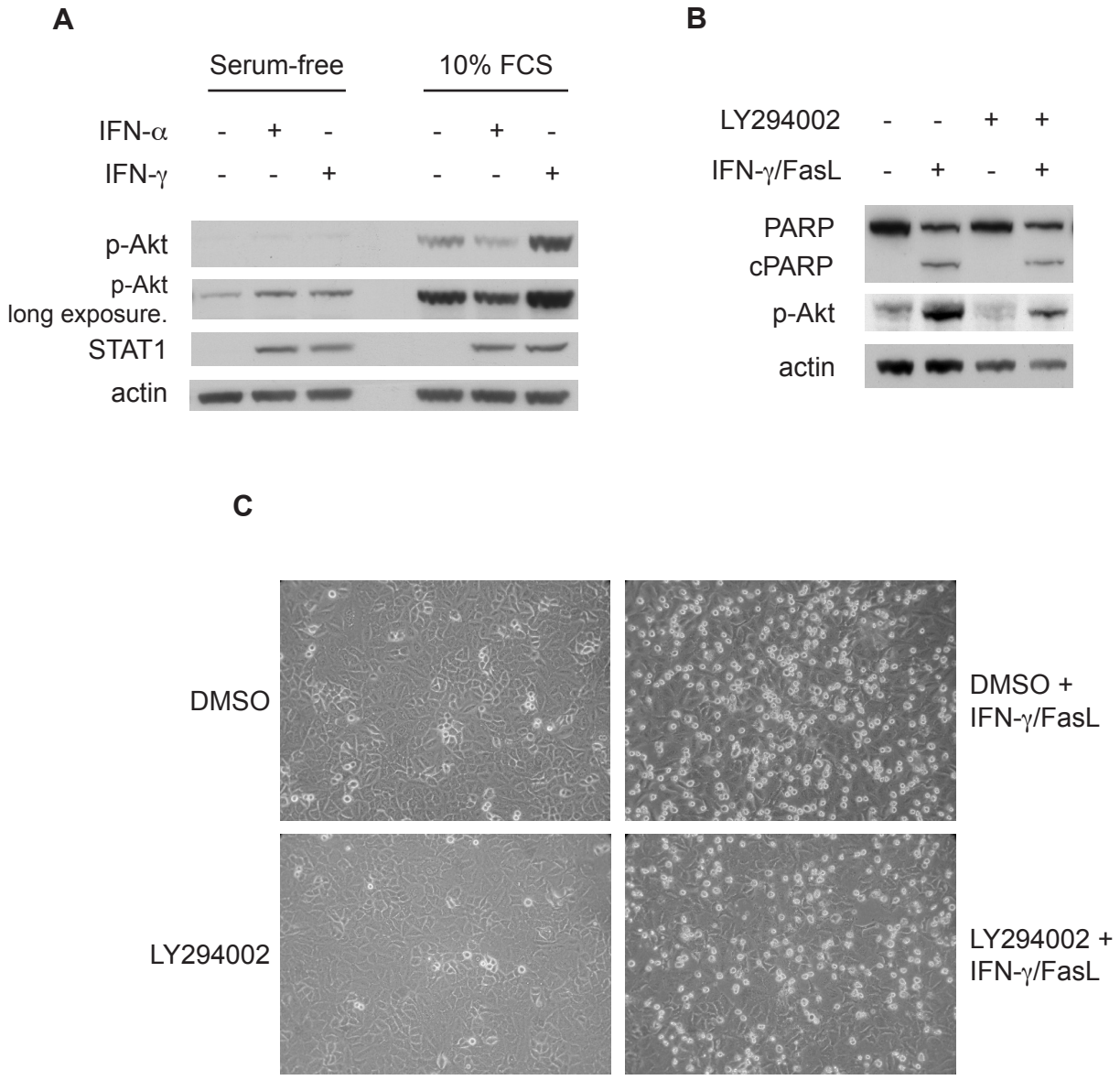
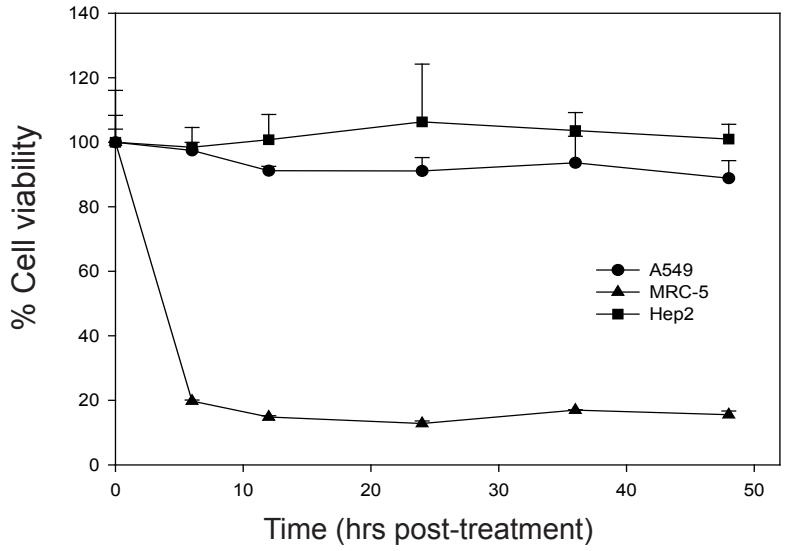


Figure 3.5: IFN-γ/FasL synergy is independent of PI3K activation.

(**A**) A549 monolayers were mock-treated of treated with IFN-α or IFN-γ in the presence or absence of 10% FCS. 16hrs later, cell lysates were prepared, subjected to SDS-PAGE and immunoblotted. (**B**) Cells were mock-treated with DMSO or treated with the PI3K inhibitor LY294002 for 1hr. IFN-γ was added to culture medium overnight, then cells were treated with FasL for 12hrs (all in the presence of 10% serum and DMSO or LY294002). Cells were harvested, subjected to SDS-PAGE and immunoblotted. (**C**) Cells were treated as in (B) and photographed by phase contrast microscopy.

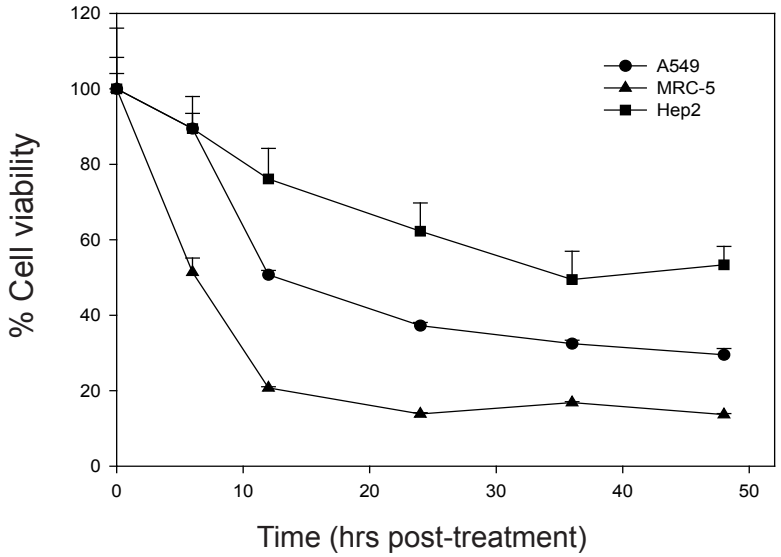
A

FasL



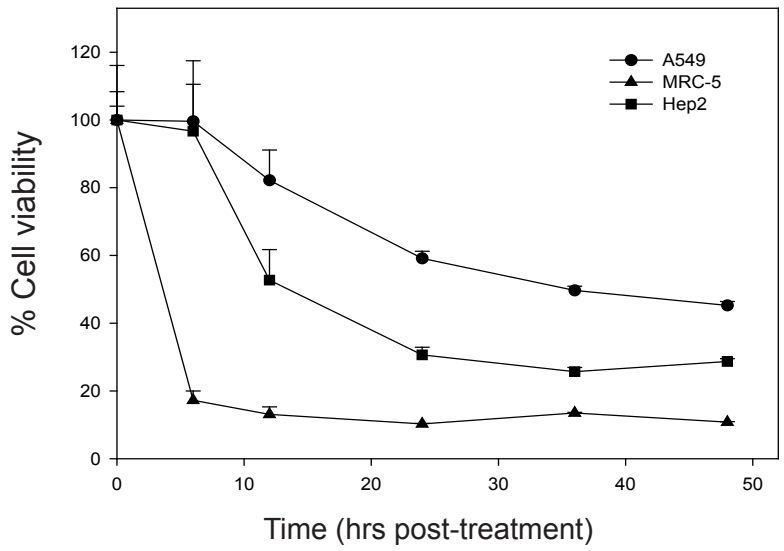
B

TNF-α/CHX



C

STS



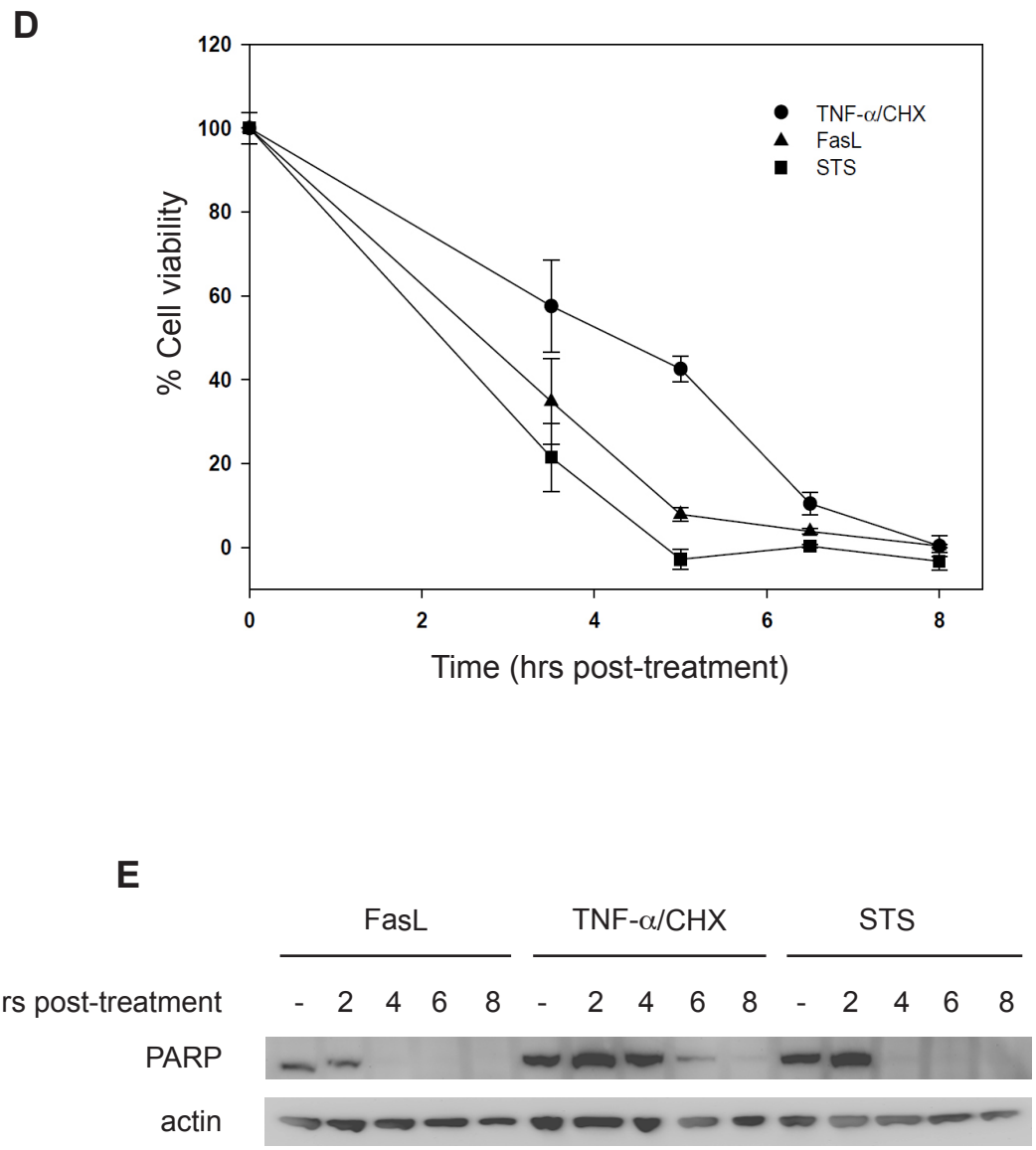


Figure 3.6 : MRC-5 cells are much more sensitive to apoptosis inducers than other cell-types.

A549, MRC-5 and Hep2 cell monolayers were treated with FasL (**A**), TNF-α/CHX (**B**) or STS (**C**). At various times post-treatment, cell viability was measured in triplicate by wst-1 assay. Mean viability is plotted as a percentage of untreated cells and error bars represent the standard deviation of each triplicate set. **(D)** MRC-5 cells were treated with FasL, TNF-α/CHX or STS. At various times post-treatment, cell viability was measured as above. **(E)** MRC-5 cells were treated with FasL, TNF-α/CHX or STS and harvested at various times post-treatment. Lysates were subjected to SDS-PAGE and immunoblotted.

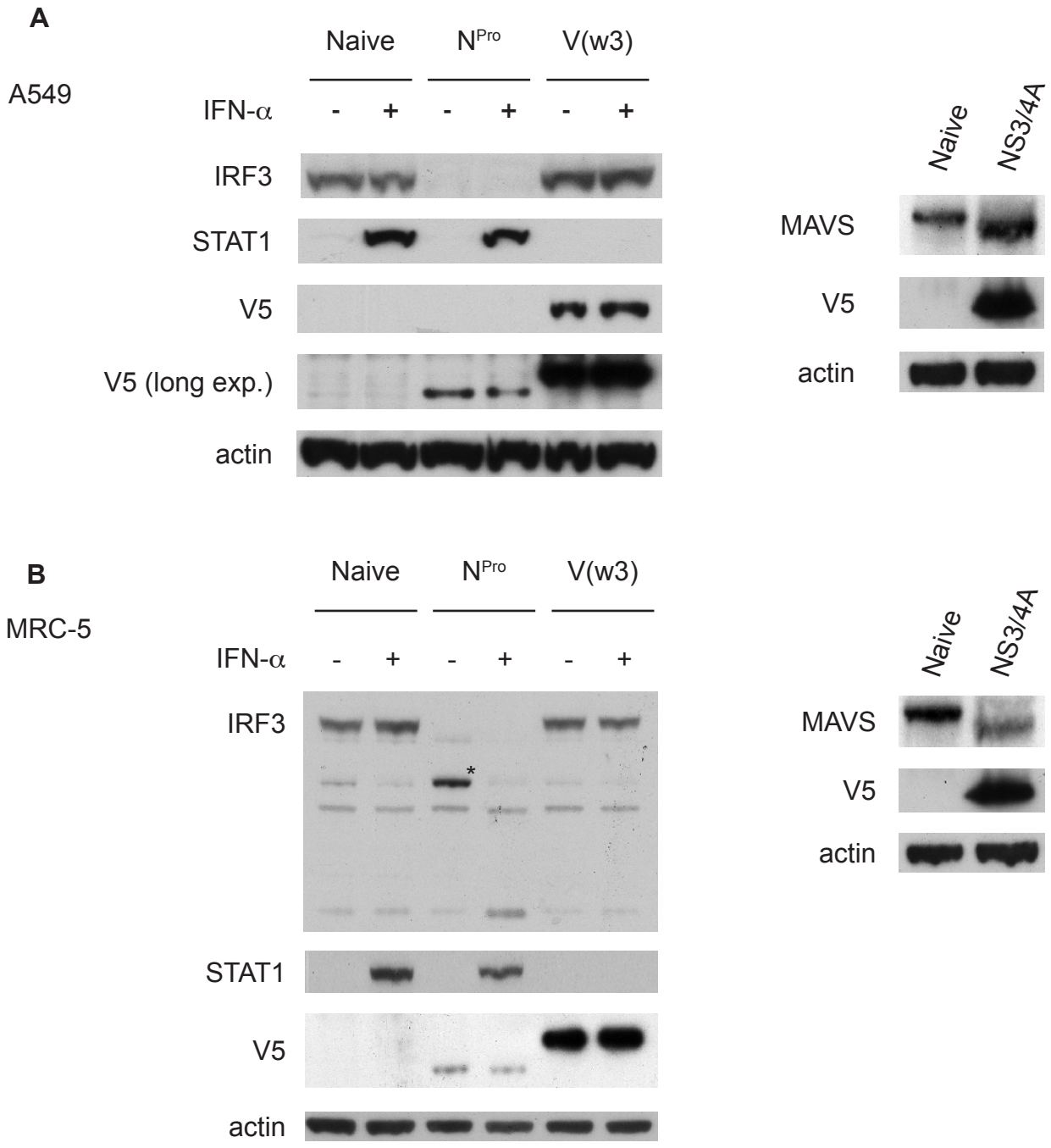
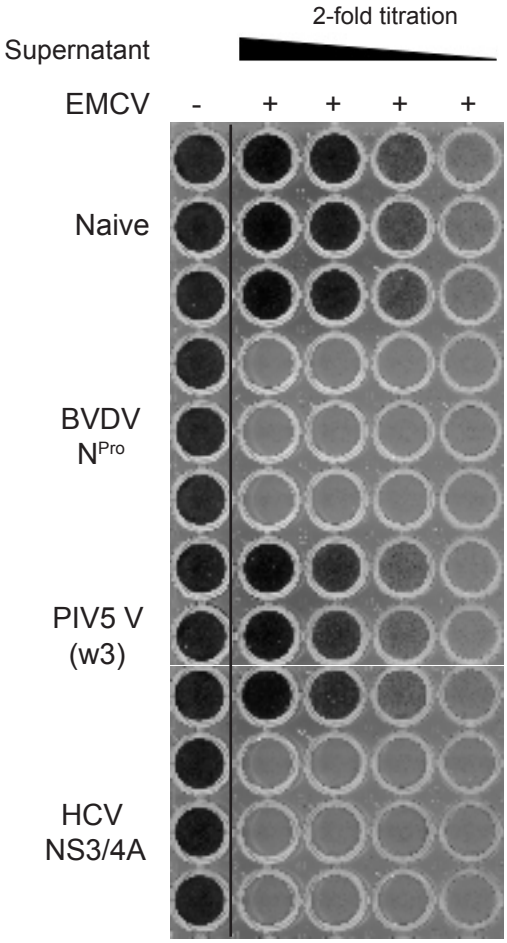


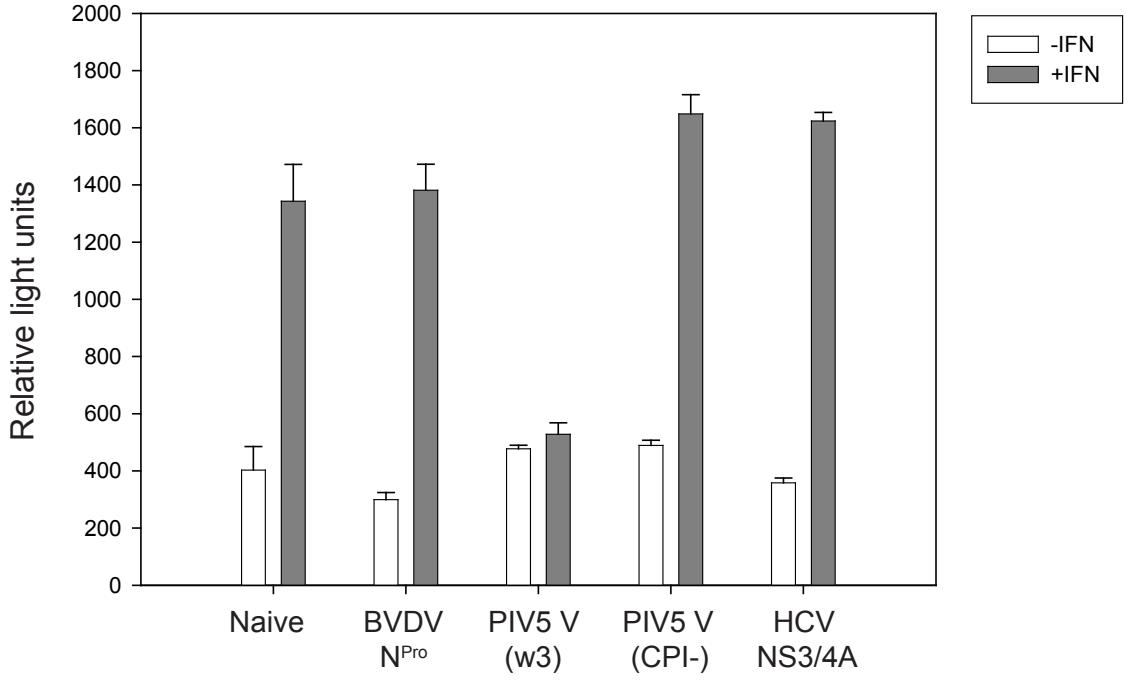
Figure 3.7: PIV5/V, BVDV/NPro and HCV/NS3/4a antagonise the IFN response.

A549 (**A**) or MRC-5 (**B**) cells stably expressing PIV5/V(w3) or BVDV/NPro were mock-treated or treated with IFN-α overnight. Cells were then harvested and immunoblotted. Naive cells or cells stably expressing HCV/NS3/4A were harvested and immunoblotted in the absence of IFN treatment. An IRF3 fragment seen in MRC-5/NPro cells is marked with an asterisk. (**C**) Monolayers of naive A549 cells or cells epressing PIV5 V, BVDV NPro or HCV NS3/4A were infected with PIV5 VΔC at an MOI of 10. 24hrs later, culture supernatant was collected and the amount of secreted IFN was quantified in triplicate using a cell-based IFN assay. Briefly, supernatants were serially diluted 2-fold and incubated A549/BVDV/NPro cells. 24hrs later, cells were infected with EMC virus; IFN present in the supernatant protects cells from EMCV-induced cell death. Plates were fixed and cell monolayers were stained with coomassie blue. (**D**) A549 cells expressing a firefly luciferase gene under the control of an ISRE were made to stably express BVDV NPro, PIV5 V (w3 and CPI- strains) or HCV NS3/4A. Triplicate monolayers were mock-treated or treated with 10^3 units/ml IFN-α overnight and luciferase activity was measured. Mean relative light units are plotted, and error bars show the standard deviation of each set of triplicates.

C


2-fold titration
Supernatant
EMCV - + + + +
Naive
BVDV NPro
PIV5 V (w3)
HCV NS3/4A

D

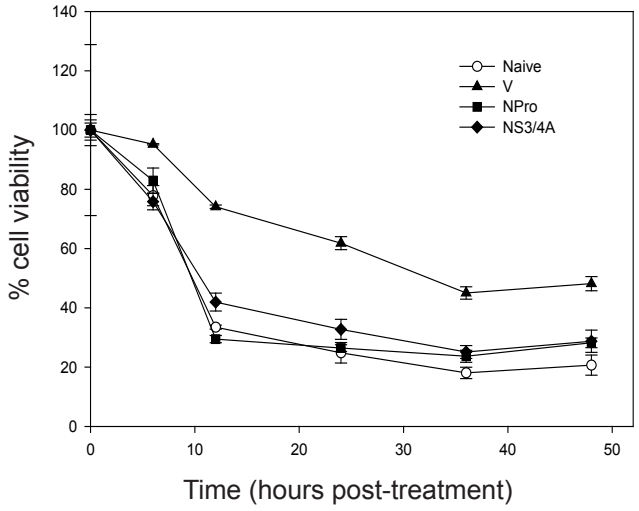

Relative light units
2000
1800
1600
1400
1200
1000
800
600
400
200
0
-IFN
+IFN
Naive
BVDV NPro
PIV5 V (w3)
PIV5 V (CPI-)
HCV NS3/4A

A549

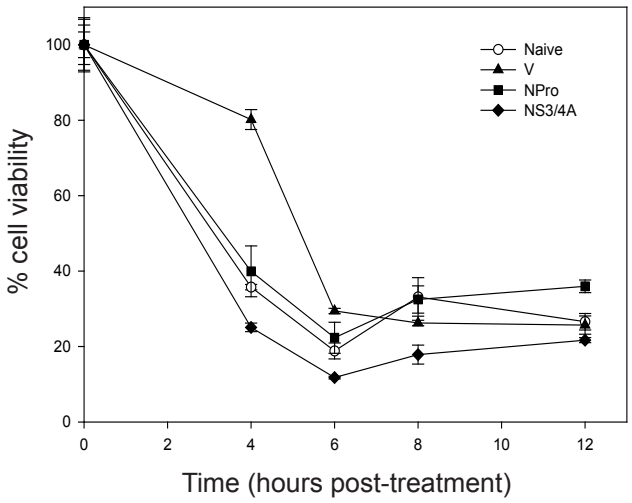
MRC-5

A

FasL/CHX

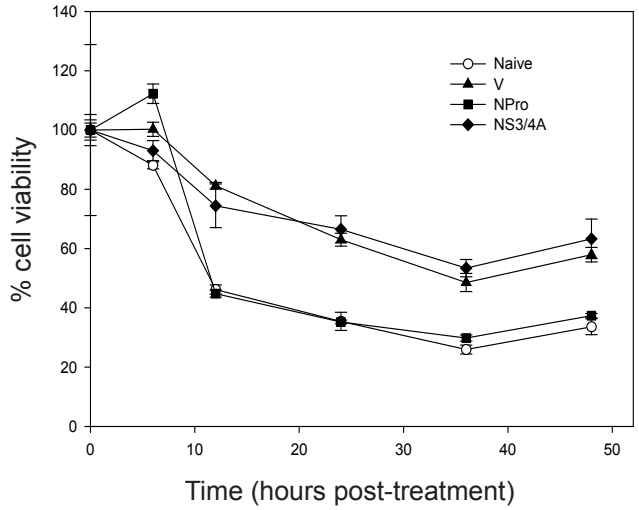


FasL

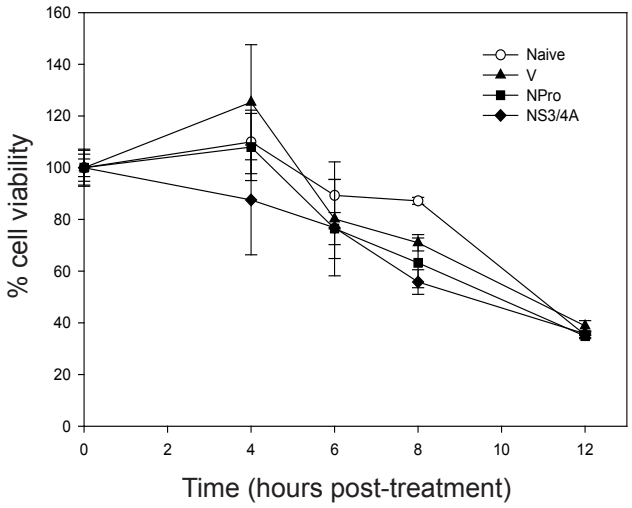


B

TNF/CHX

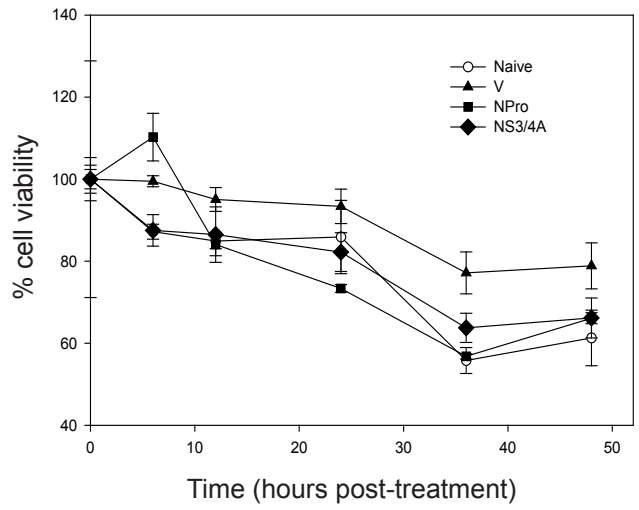


TNF/CHX



C

STS



STS

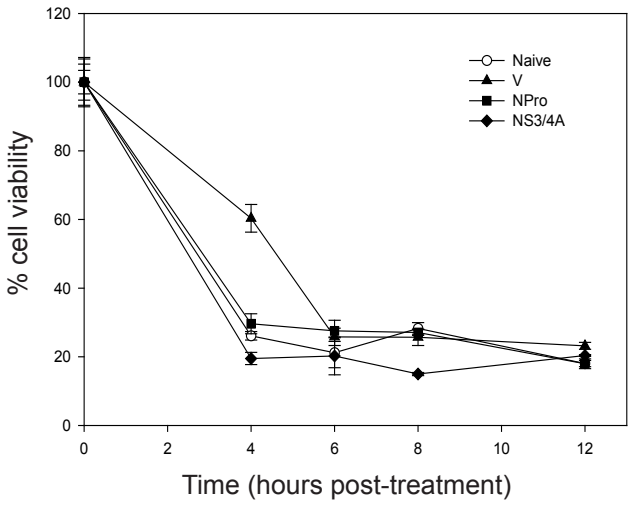


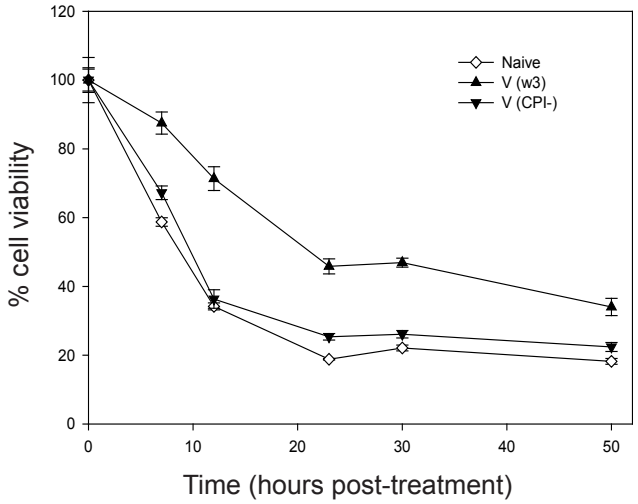
Figure 3.8: The effect of viral IFN antagonists on apoptosis.

Naive A549 and MRC-5 cells or cells expressing PIV5, BVDV NPro or HCV NS3/4A were treated with FasL/CHX (**A**), TNF-α/CHX (**B**) or STS (**C**). At various times post-treatment, cell viability was measured in triplicate by wst-1 assay. Mean viability is plotted as a percentage of untreated cells and error bars represent the standard deviation of each triplicate set.

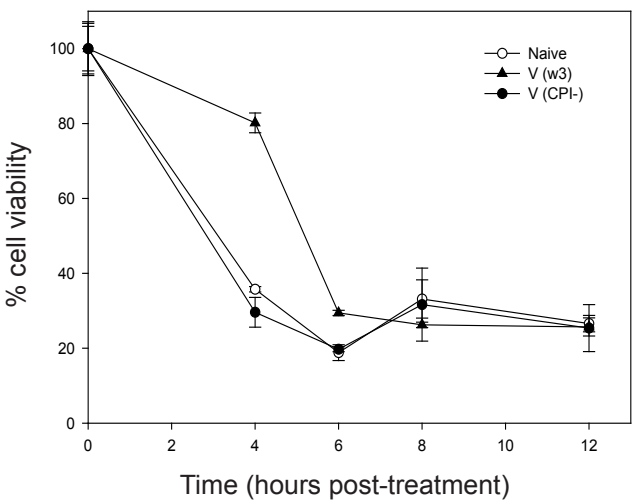
A549 MRC-5

A

FasL/CHX

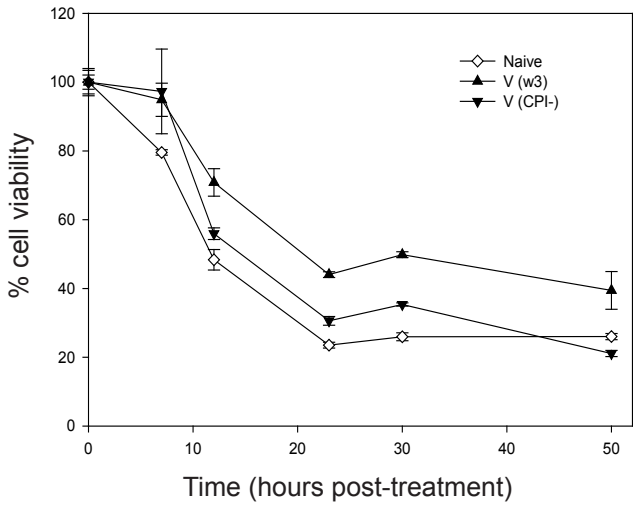


FasL

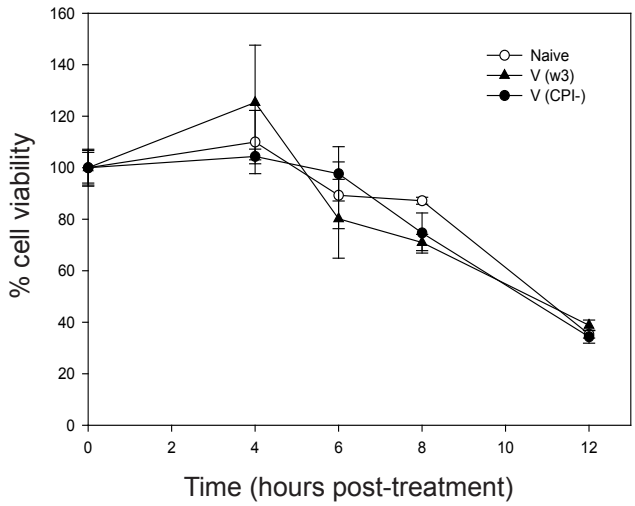


B

TNF/CHX

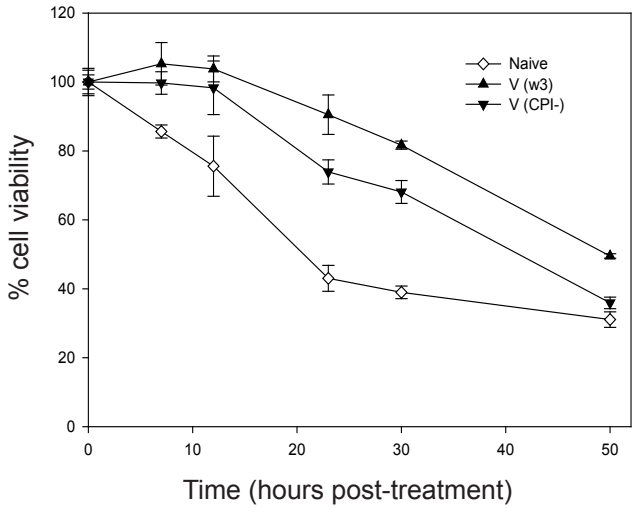


TNF/CHX



C

STS



STS

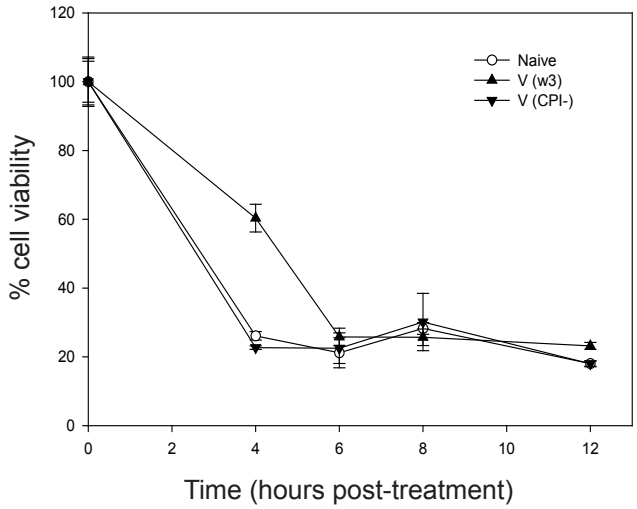
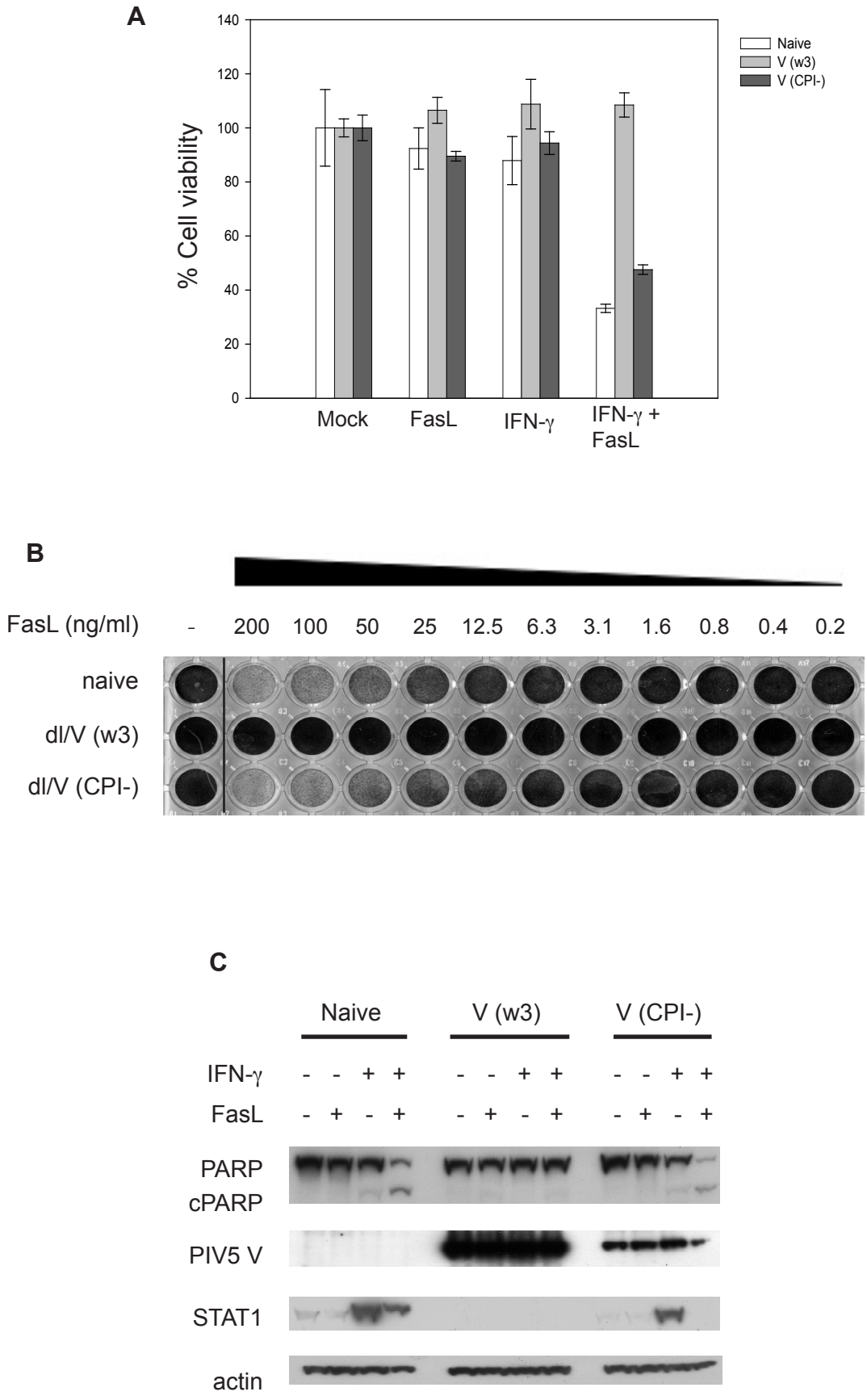


Figure 3.9: Contribution of STAT1 degradation to the effects of PIV5 V on apoptosis.

Naive A549 and MRC-5 cells or cells expressing PIV5 V (w3) or V (CPI-) were treated with FasL/CHX (**A**), TNF-α/CHX (**B**) or STS (**C**). At various times post-treatment, cell viability was measured in triplicate by wst-1 assay. Mean viability is plotted as a percentage of untreated cells and error bars represent the standard deviation of each triplicate set.


A
% Cell viability
140
120
100
80
60
40
20
0
Mock
FasL
IFN-γ
IFN-γ + FasL
Naive
V (w3)
V (CPI-)
B
FasL (ng/ml)
- 200 100 50 25 12.5 6.3 3.1 1.6 0.8 0.4 0.2
naive
dl/V (w3)
dl/V (CPI-)
C
Naive
V (w3)
V (CPI-)
IFN-γ - - + + - - + + - - + +
FasL - + - + - + - + - + - +
PARP
cPARP
PIV5 V
STAT1
actin

D

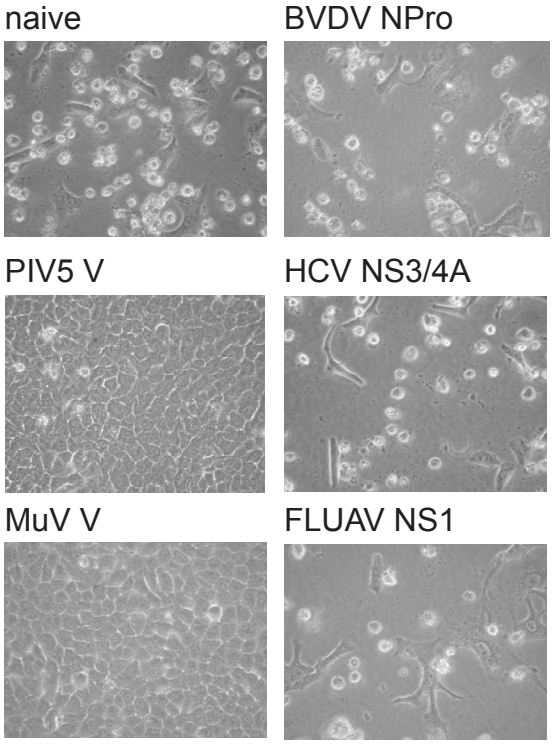
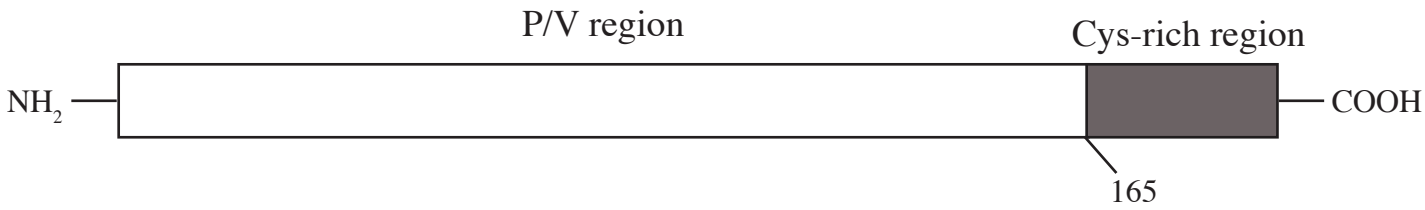


Figure 3.10: IFN-γ/FasL synergy is STAT1 dependent and is inhibited by PIV5 V.

(**A**) A549 cells stable expressing the V protein of PIV5 w3 and CPI- strains were mock-treated or pre-treated with IFN-α or IFN-γ for 16h, then mock-treated or treated with FasL for 48hrs. Cell viability was then measured in triplicate by wst-1 assay. Mean viability is plotted as a percentage of mock-treated cells and error bars represent the standard deviation of each triplicate set. (**B**) Naive A549 cells or cells expressing V (w3) or V (CPI-) were seeded in a 96-well plate and were pre-treated with IFN-γ for 16h. FasL was titrated by serial 2-fold dilution (starting at 200ng/ml). Dilutions were transferred to the plate of cells. 48h later, cells were fixed and stained with crystal violet. (**C**) Duplicate samples from (**A**) were subjected to SDS-PAGE and immunoblotted. (**D**) A549 cells expressing PIV5/V, MuV/V, BVDV/NPro, HCV/NS3/4A or IAV/NS1 were treated with IFN-γ/FasL. 48h later, cells were photographed by phase-contrast microscopy.



VIRUS	26	32	33	50	102	157	166	170	172	STAT1	mda-5
PIV5-w3	Y	V	T	L	L	S	D	F	R	Degraded	Inhibited
PIV5-CPI+	Y	I	I	L	L	F	D	F	R	Degraded	Inhibited
PIV5-CPI-	H	I	I	P	P	F	D	F	R	Stable	Inhibited
rPIV5-VΔC	Y	V	T	L	L	S	Y	*	*	Stable	Unaffected

Figure 3.11: Strain-specific differences in the PIV5 V protein.

The positions of naturally occuring or experimentally introduced amino acid substitutions in PIV5-w3, PIV5-CPI+, PIV5-CPI-, PIV5-VΔC are shown. The ability of each virus to target STAT1 for degradation and inhibit mda-5 is also indicated and their subsequent effects on STAT1 degradation and mda-5 inhibition are shown. Asterisks above the table denote the positions of the three amino acids required to target STAT1 for degradation. *Adapted from Wansley and Parks, 2002.*

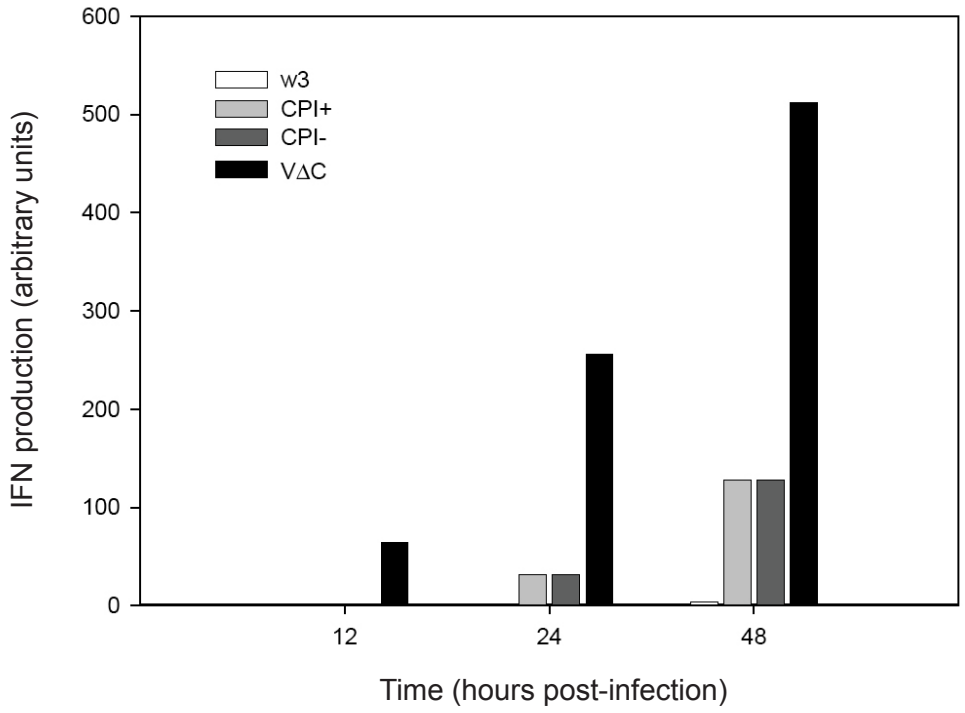
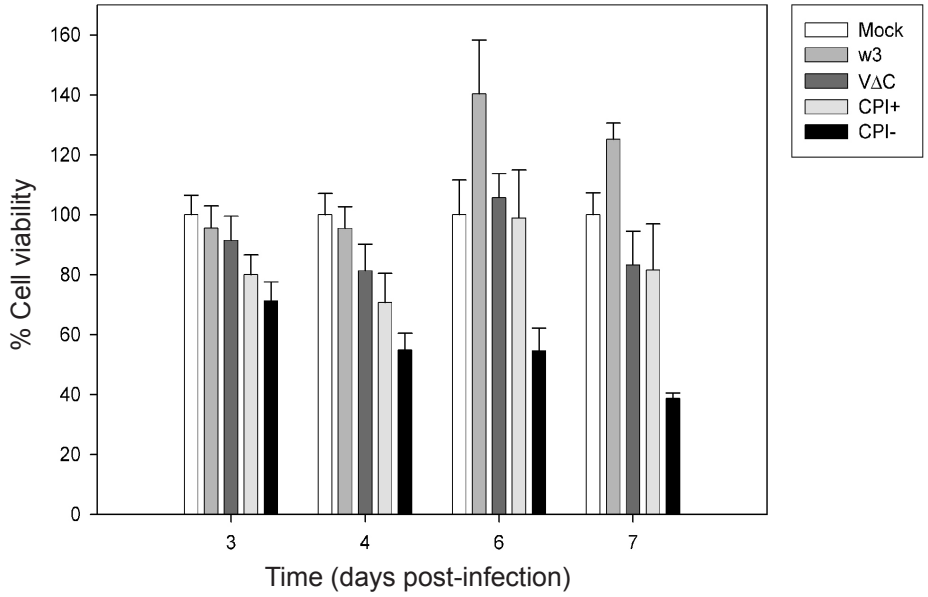


Figure 3.12: PIV5-w3, PIV5-CPI+, PIV5-CPI- and rPIV5-VΔC vary in their ability to induce IFN.

A549 cells infected with PIV5-w3, PIV5-CPI+. PIV5-CPI- and rPIV5-VΔC at an MOI of 10. At 12h, 24h and 48h post-infection, culture supernatants were collected and the amount of IFN present was quantified in triplicate by cell-based IFN assay. Mean IFN production is plotted and error bars represent the standard deviation of each triplicate set.

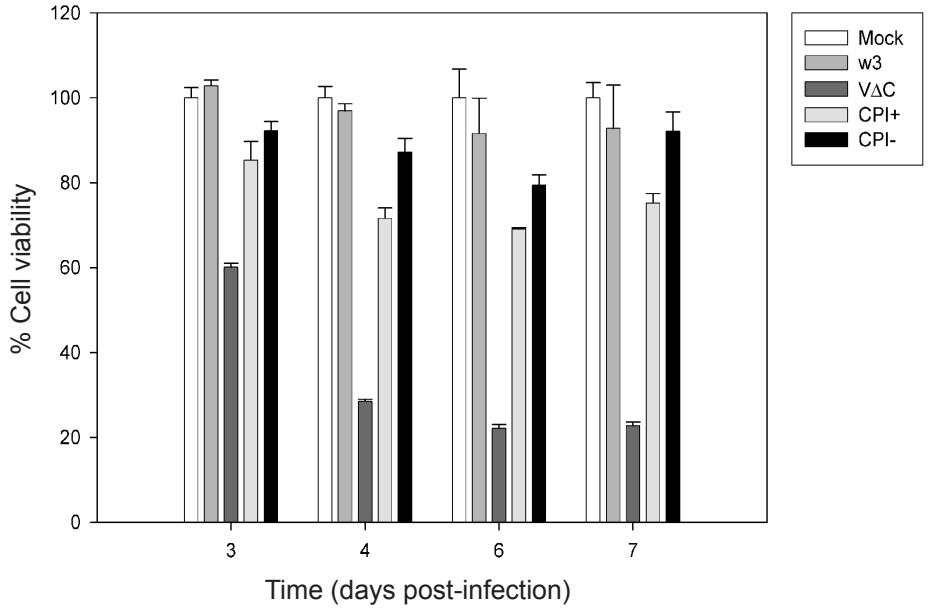
A

A549 naive



B

A549/BVDV/NPro



C

A549/PIV5/V

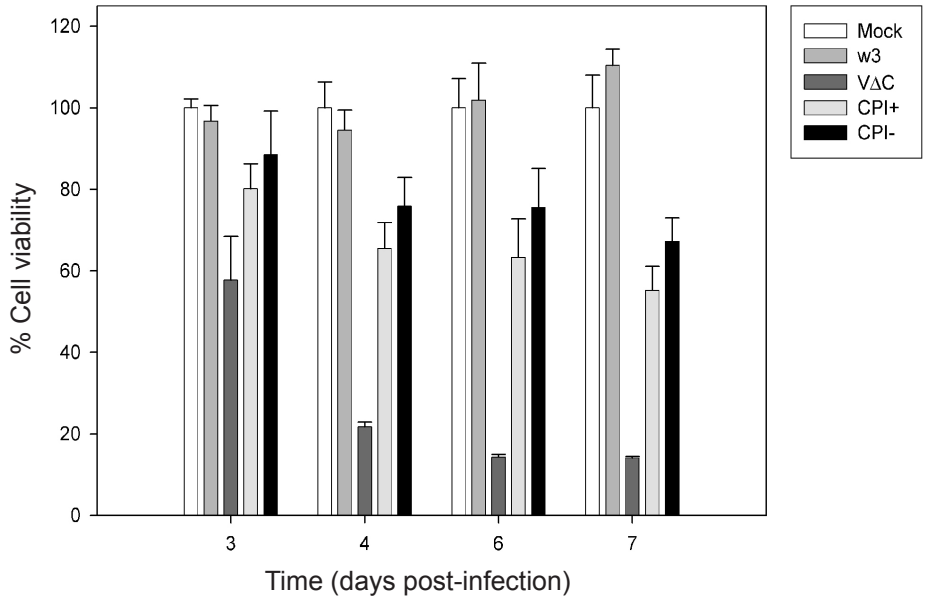


Figure 3.13: PIV5-w3, PIV5-CPI+, PIV5-CPI- and rPIV5-VΔC induce diferent amounts of cell death.

Naive A549 cells (**A**), A549/BVDV/NPro (**B**) or A549/PIV5/V (**C**) were infected with PIV5-w3, PIV5-CPI+. PIV5-CPI- and rPIV5-VΔC at an MOI of 50. At various times post-infection, cell viability was measured in triplicate by wst-1 assay. Mean cell viability is plotted as a percentage of mock-infected cells, and error bars represent the standard deviation of each triplcate set. N.B. 100% of cells were infected, as determined by immunofluorescence.

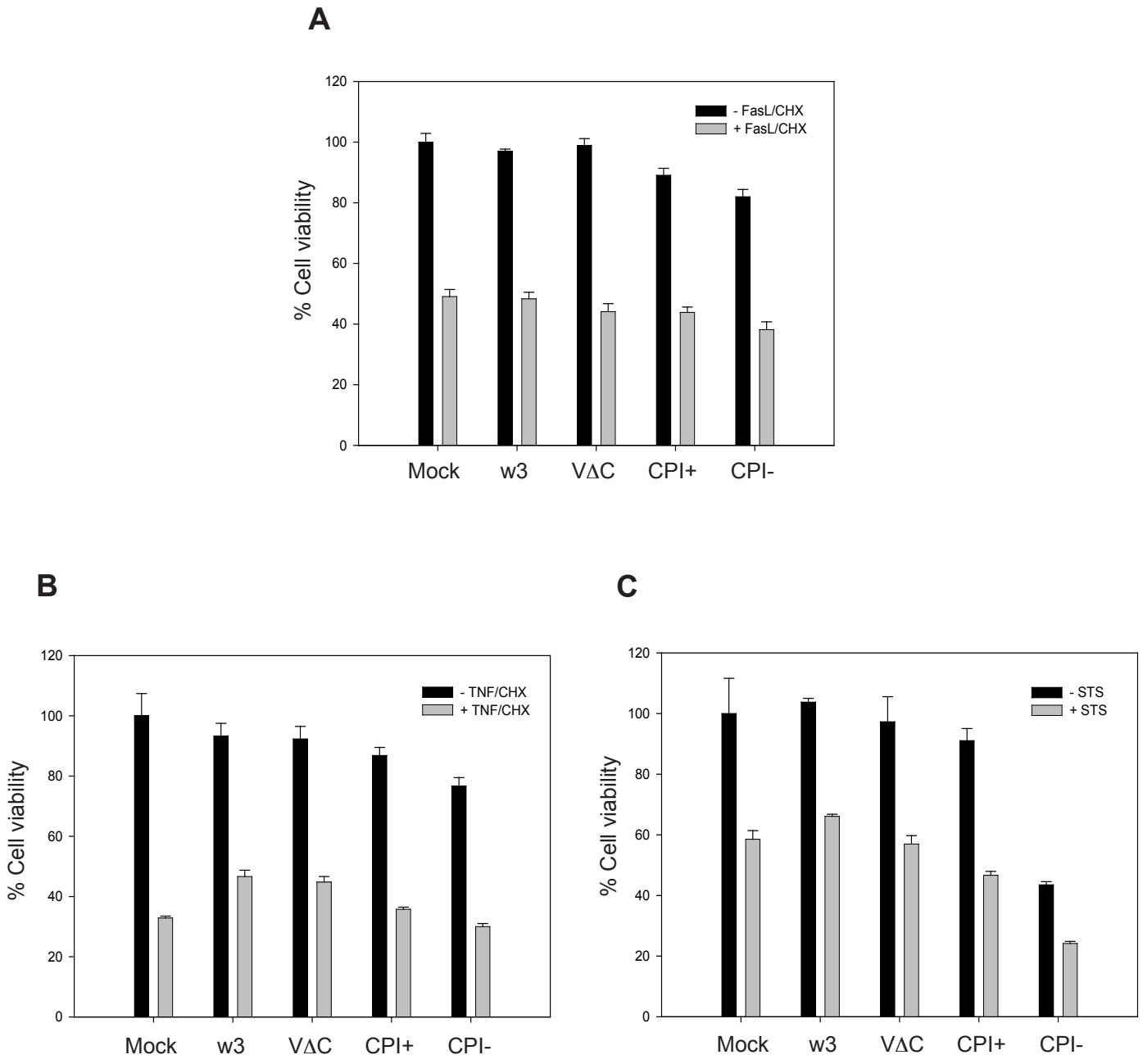


Figure 3.14: The response of w3, CPI+, CPI- and VΔC-infected cells to non-viral apoptosis inducers.

A549 cells were infected with PIV5-w3, PIV5-CPI+. PIV5-CPI- and rPIV5-VΔC at an MOI of 50. 18h later (or 36h later for STS treatment), cells were treated with FasL/CHX (A), TNF-α/CHX (B) or STS (C). At various times post-treatment, cell viability was measured in triplicate by wst-1 assay. Mean viability is plotted as a percentage of mock-infected, untreated cells and error bars represent the standard deviation of each triplicate set.

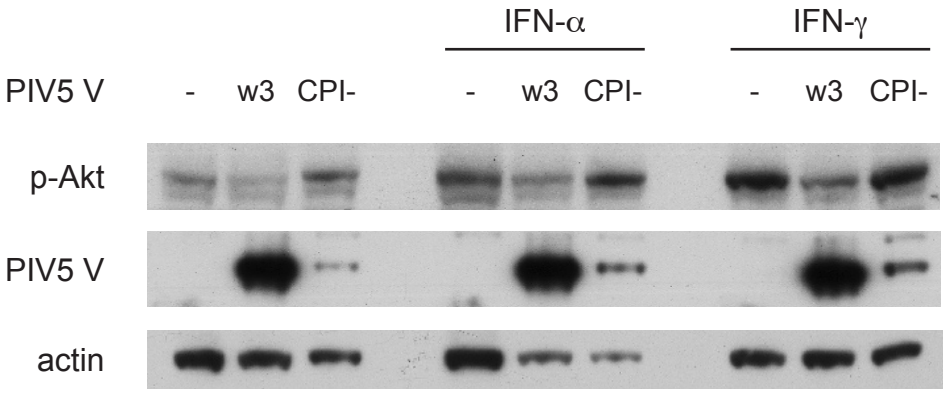


Figure 3.15: Expression of PIV5/V (w3) inhibits Akt activation.

Naive A549 cells or cells expressing PIV5/V (w3) or PIV5/V (CPI-) were treated with IFN-α or IFN-γ overnight. Cells were harvested, subjected to SDS-PAGE and immunoblotted.

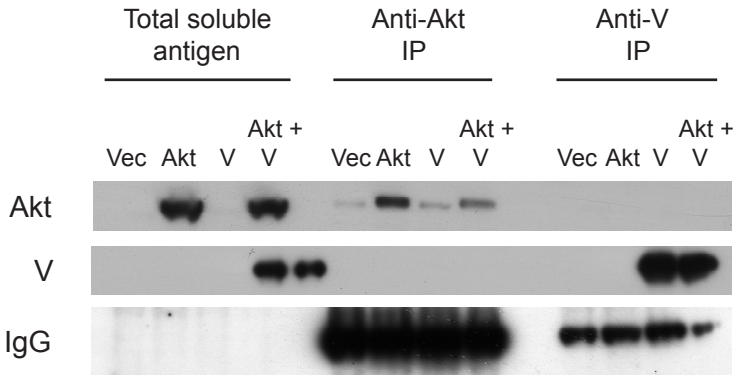


Figure 3.16: PIV5/V and Akt do not co-immunoprecipitate.

293T cells were transfected with expression plasmids for PIV5/V (w3), Akt or PIV5/V plus Akt. 24hrs later, soluble antigen extracts were prepared, and V and Akt were immunoprecipitated using antibodies to Akt or V5. Immunoprecipitated protein complexes were subjected to SDS-PAGE and immunoblotted for the presence of V and Akt.

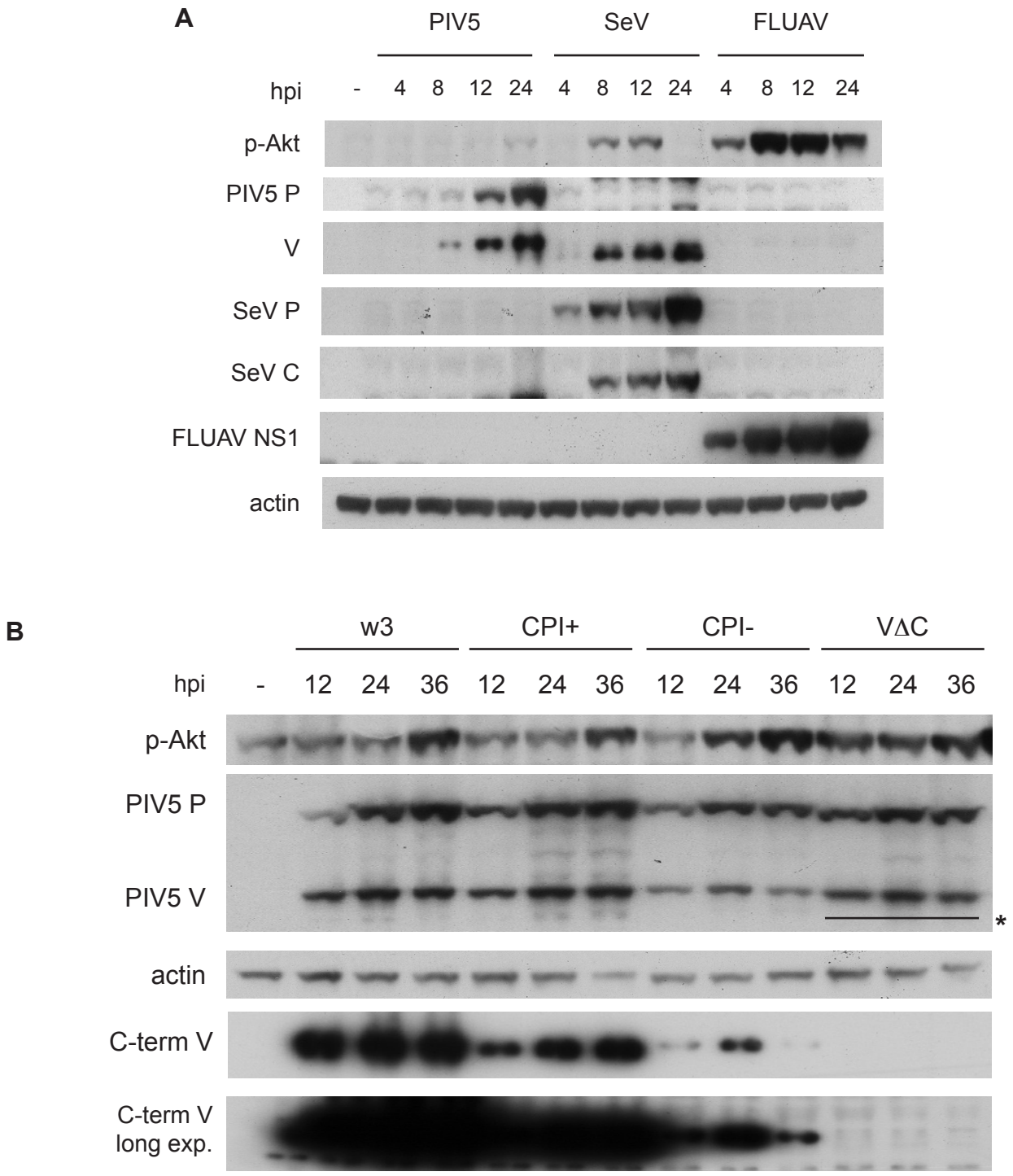
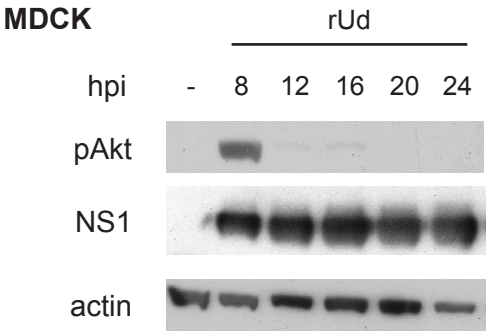


Figure 3.17: Activation of Akt in PIV5 infected cells.

(**A**) A549 monolayers were infected with FLUAV (rUd), SeV (strain Z) and PIV5-w3, in the absence of serum, at an MOI of 10. Cells were harvested at various times post-infection, subjected to SDS-PAGE and immunoblotted for phospho-Akt, viral proteins and actin. (**B**) A549 cells were infected with PIV5-w3, PIV5-CPI+, PIV5-CPI- or rPIV5-VΔC at an MOI of 10 in the absence of serum. 12h, 24h and 36h later, cells were harvested, subjected to SDS-PAGE and immunoblotted for phospho-Akt, viral proteins and actin. The P degradation product, P*, is marked with an asterisk.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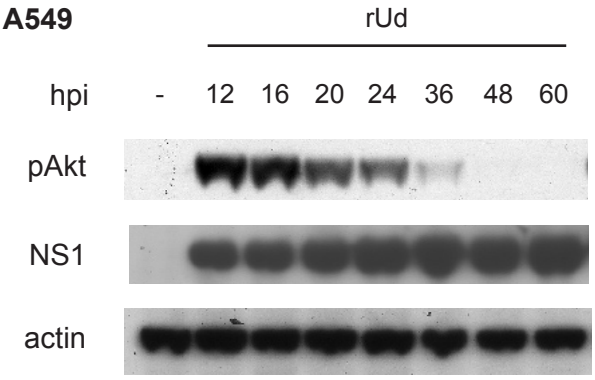
hpi
-
8
12
16
20
24
pAkt
NS1
actin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hpi
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12
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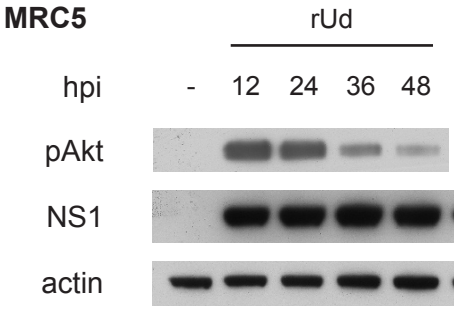
20
24
36
48
60
pAkt
NS1
actin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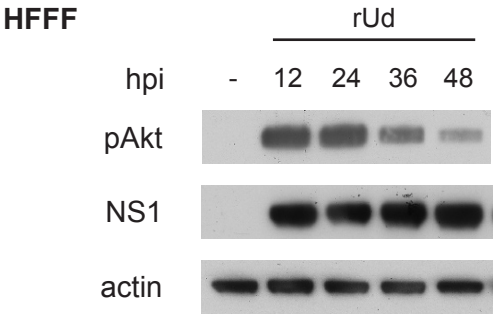
-
12
24
36
48
pAkt
NS1
actin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-
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24
36
48
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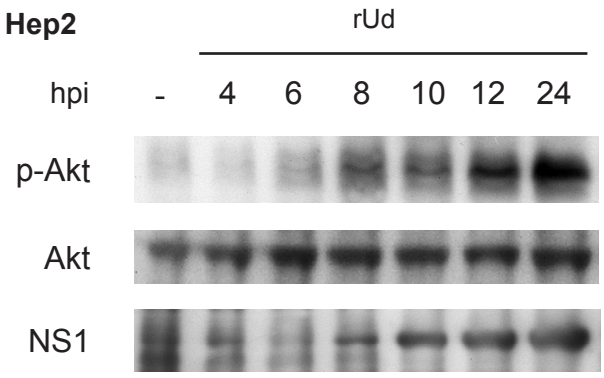
NS1
actin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4
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10
12
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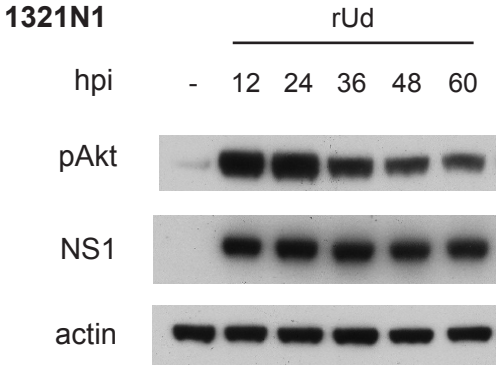
p-Akt
Akt
NS1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hpi
-
12
24
36
48
60
pAkt
NS1
actin

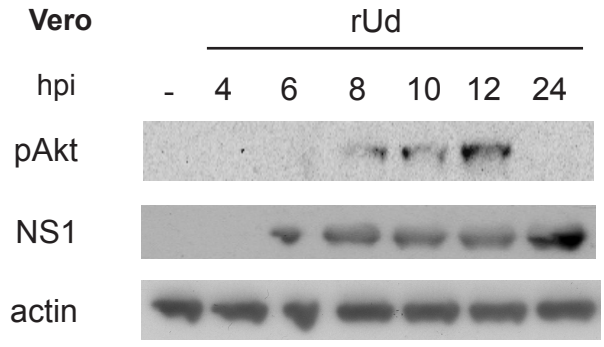

Vero
rUd
hpi
-
4
6
8
10
12
24
pAkt
NS1
actin

Figure 3.18: The kinetics of PI3K/Akt activation by FLUAV are cell-type dependent.

MDCK, A549, MRC-5, HFFF, Hep2, 1321N1 or Vero cells were mock-infected or infected with rUd wt FLUAV virus at an MOI of 5. At various times post-infection (hours post-infection; hpi), cells were harvested, subjected to SDS-PAGE and immunoblotted for Akt activation and NS1 expression.

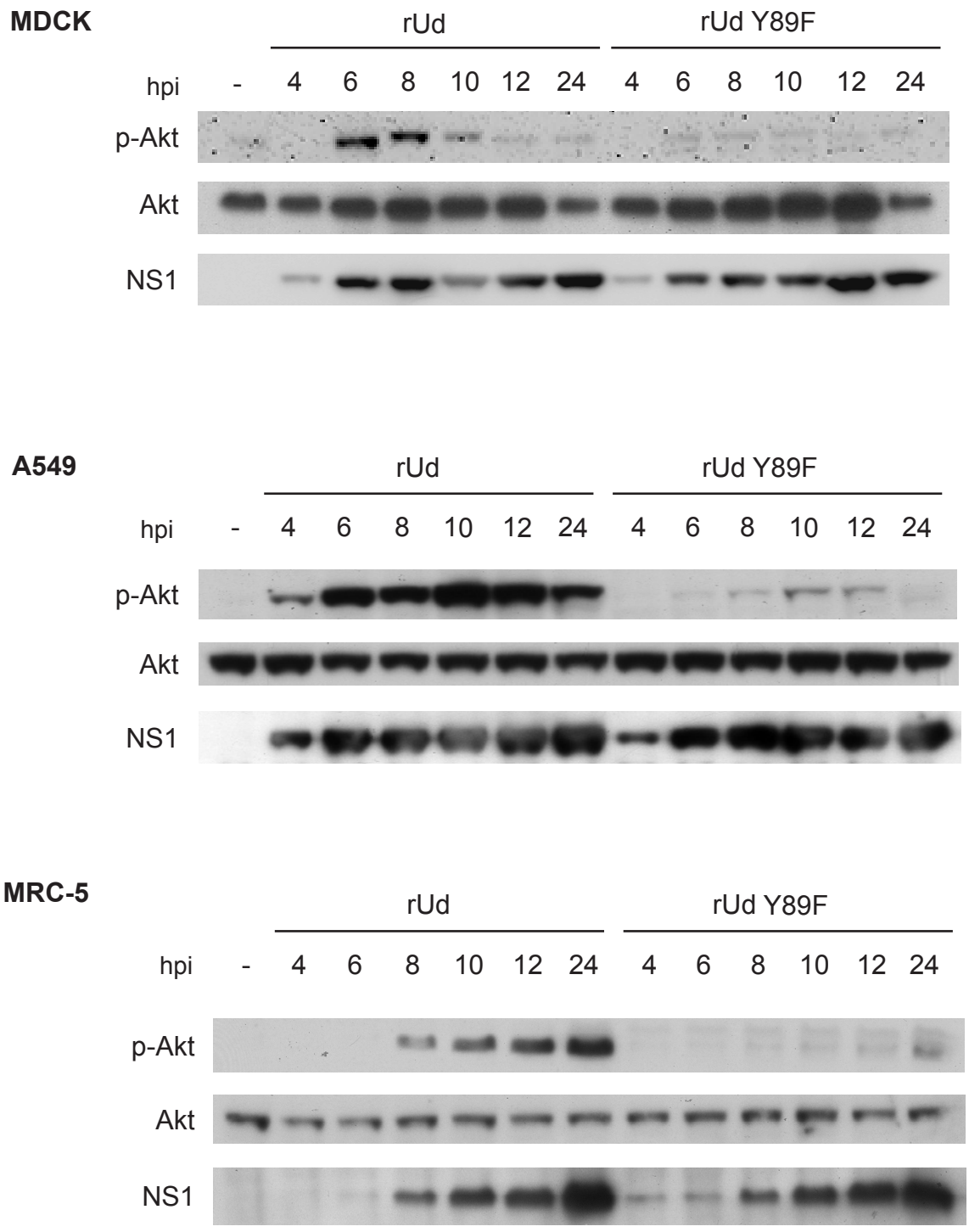


Figure 3.19: rUd-Y89F is unable to activate PI3K.

MDCK, A549 or MRC-5 cells were mock-infected or infected with rUd wt or rUd-Y89F virus at an MOI of 10. At various times post-infection, cells were harvested, subjected to SDS-PAGE and immunoblotted for Akt activation and NS1 expression.

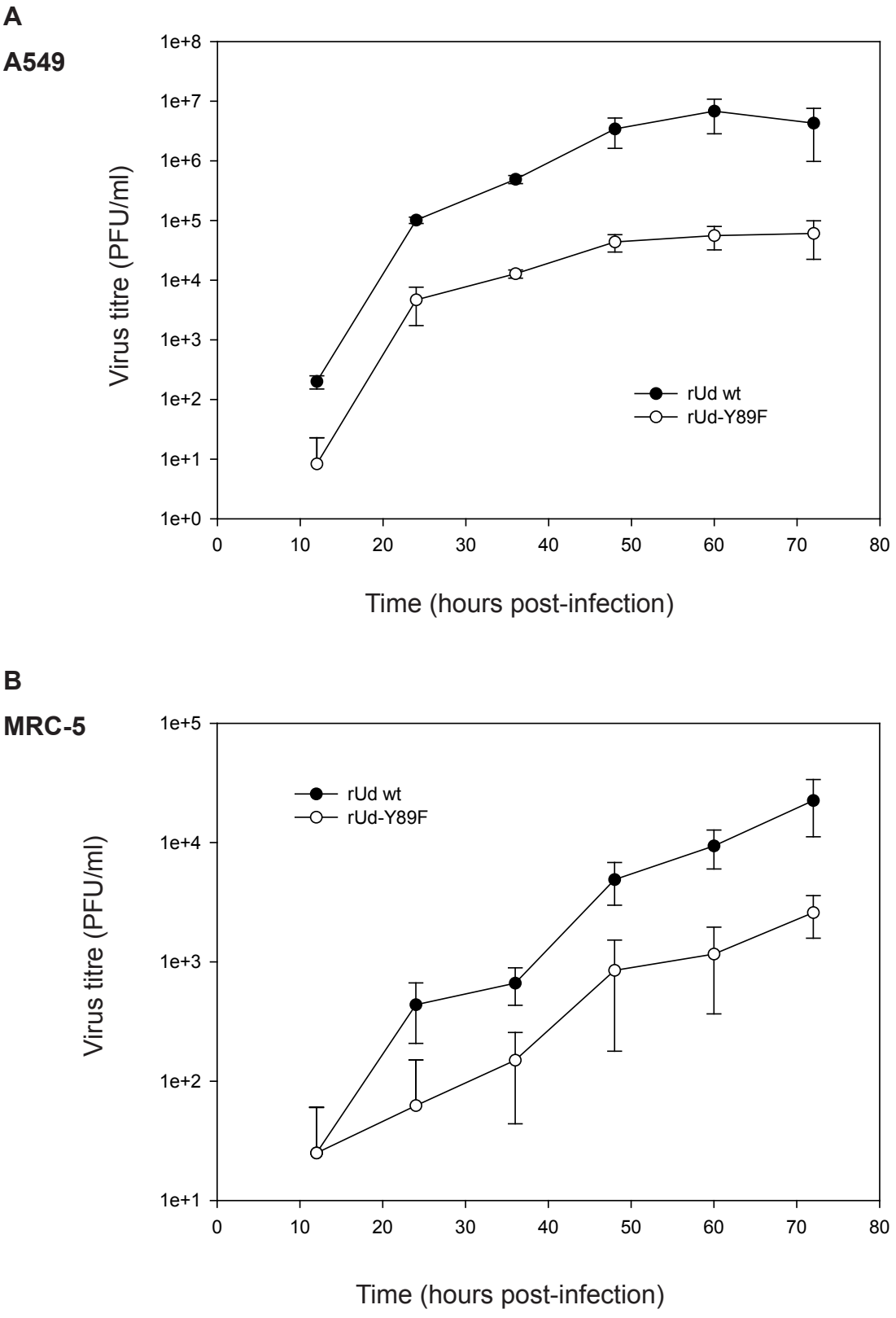


Figure 3.20: rUd-Y89F is attenuated in A549 and MRC-5 cells

Triplicate A549 (**A**) or MRC-5 (**B**) monolayers were infected with rUd wt or rUd-Y89F at an MOI of 0.001 in the presence of trypsin. At various times post-infection, the supernatant was removed and titrated by plaque assay on MDCK cells. Error bars represent the standard deviation of each triplicate set.

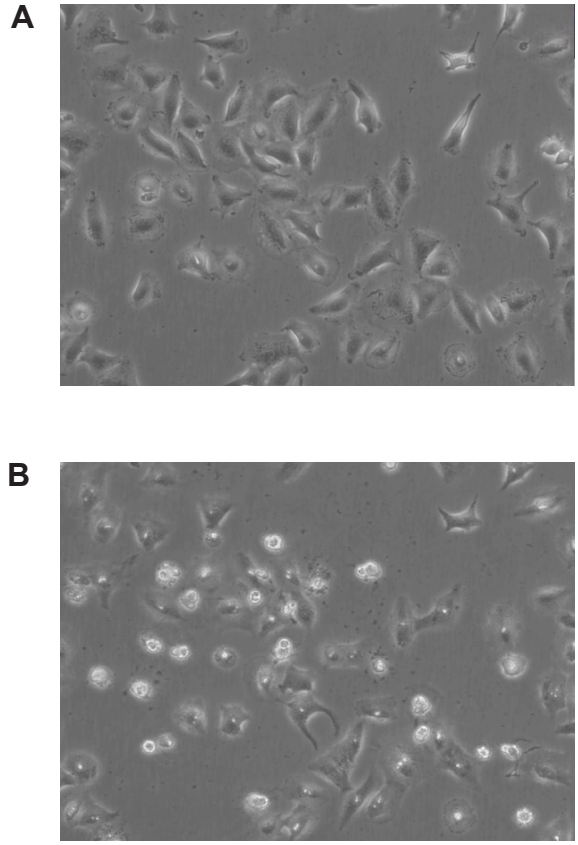
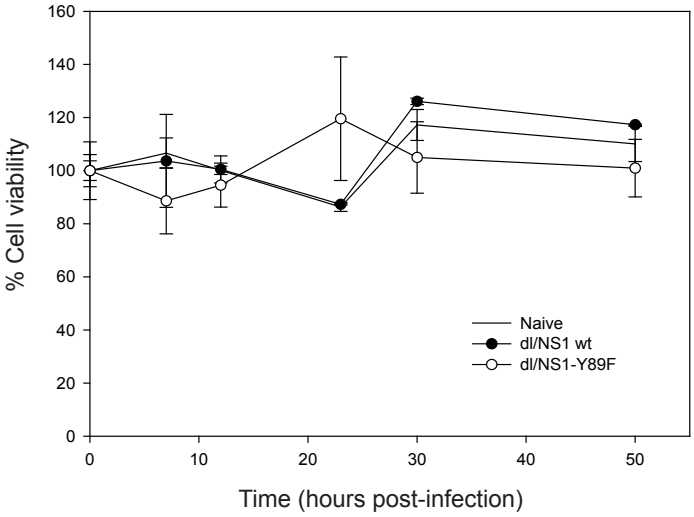


Figure 3.21: FLUAV infection causes apoptosis at high MOI.

A549 monolayers were infected with rUd wt at an MOI of over 100. A time-lapse video of infected cells was produced from 8-24hrs post-infection (supplied on disc). Shown are two stills from the video: (**A**) shows cells at 12h, (**B**) shows the same cells 12h later.

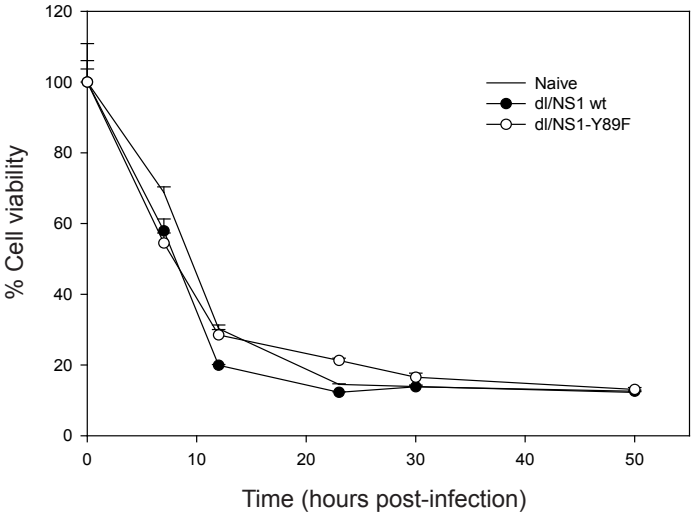
A

FasL



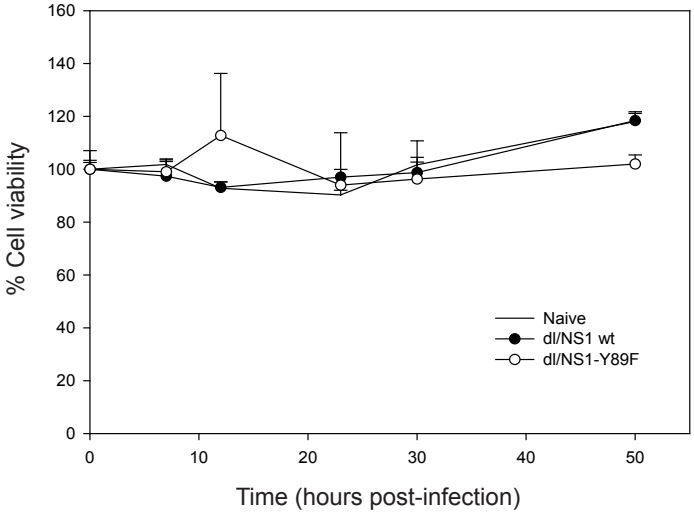
B

FasL/CHX



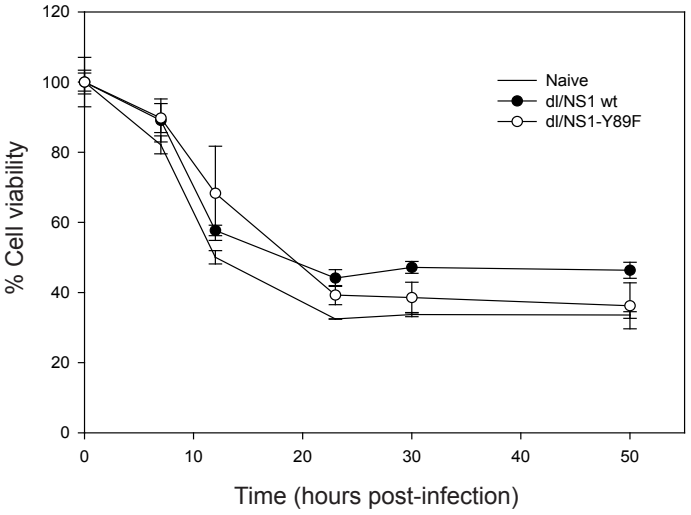
C

TNF-α



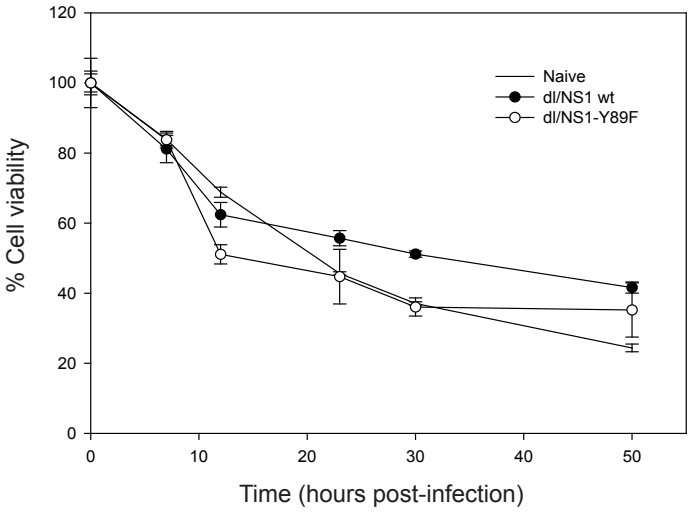
D

TNF-α/CHX



E

STS



F

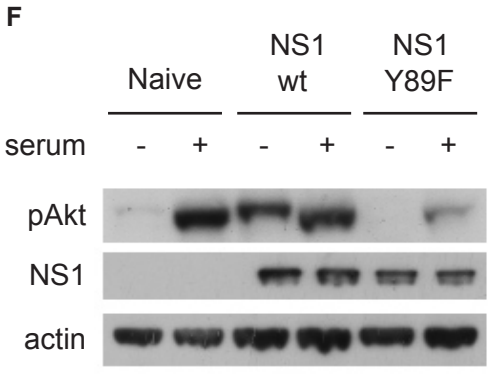
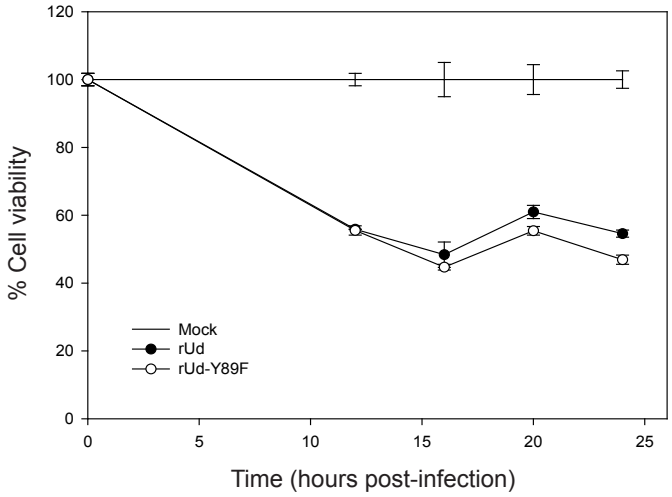
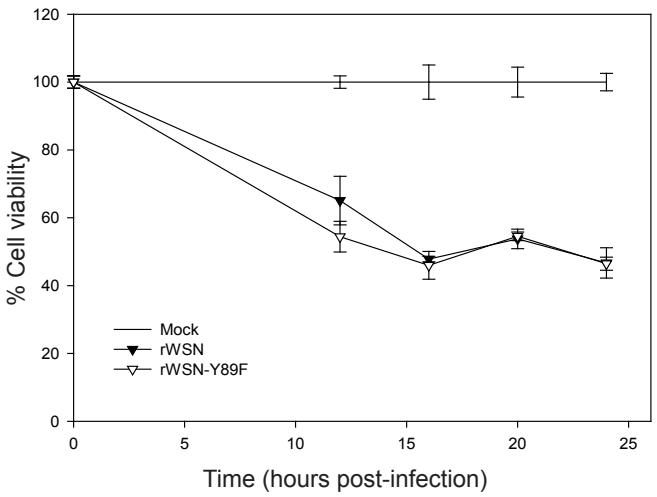


Figure 3.22: PI3K activation in NS1-expressing A549 cells does not protect cells from non-viral apoptosis inducers.

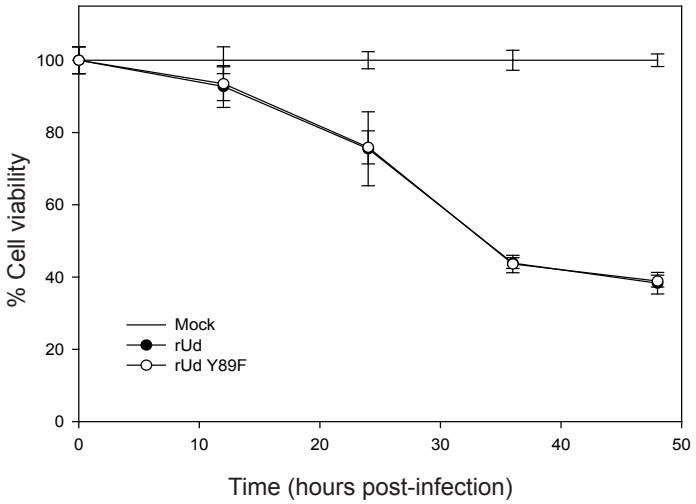
A549 cells stably expressing NS1 wt or NS1-Y89F were mock-treated or treated with FasL (**A**), FasL/CHX (**B**), TNF-α (**C**), TNF-α/CHX (**D**) or STS (**E**). At various times post-treatment, cell viability was measured in triplicate by wst-1 assay. Mean viability is plotted as a percentage of untreated cells and error bars represent the standard deviation of each triplicate set. (**F**) Naive A549 cells or cells stably expressing NS1 wt or NS1-Y89F were cultured in the presence or absence of serum. Lysates were subjected to SDS-PAGE and immunoblotted for Akt activation and NS1 expression.

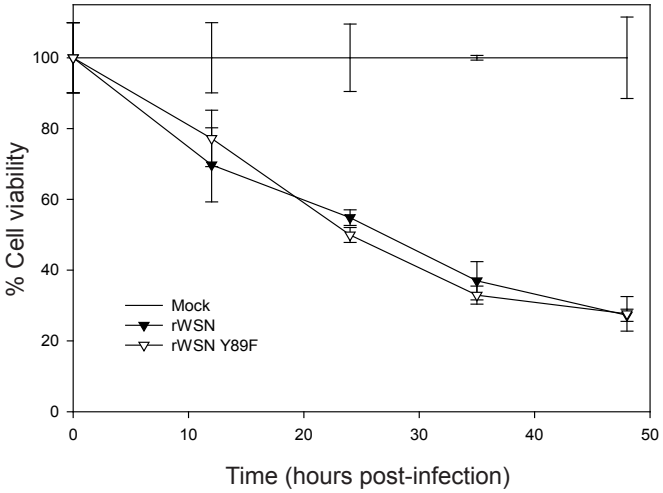
A
MDCK



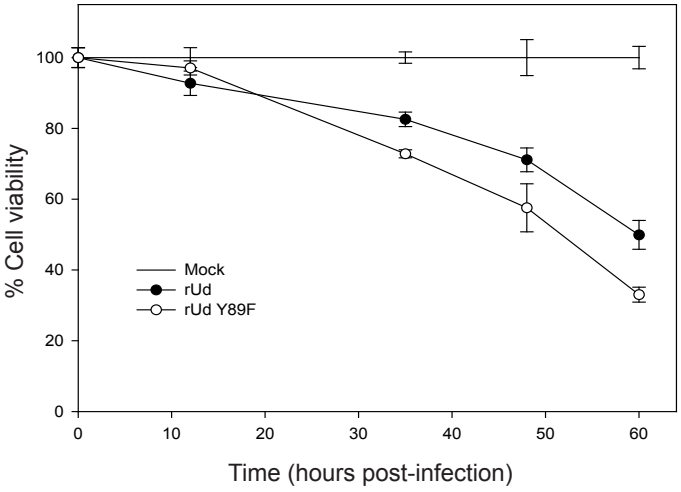


B
A549





C
MRC-5



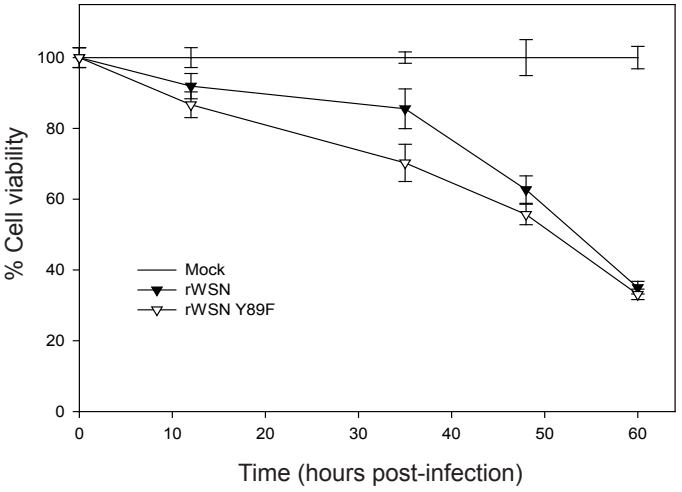


Figure 3.23: Effect of PI3K activation by NS1 on IAV-induced cell death.

Triplicate MDCK (**A**), A549 (**B**) or MRC-5 (**C**) monolayers were infected with either rUd wt and rUd-Y89F, or rWSN wt and rWSN-Y89F at an MOI of 35. Cell viability was measured in triplicate at various times post-infection using wst-1. Mean viability is plotted as a percentage of mock-treated cells and error bars represent the standard deviation for each set of triplicates. N.B. 100% of cells were infected, as determined by immunofluorescence.

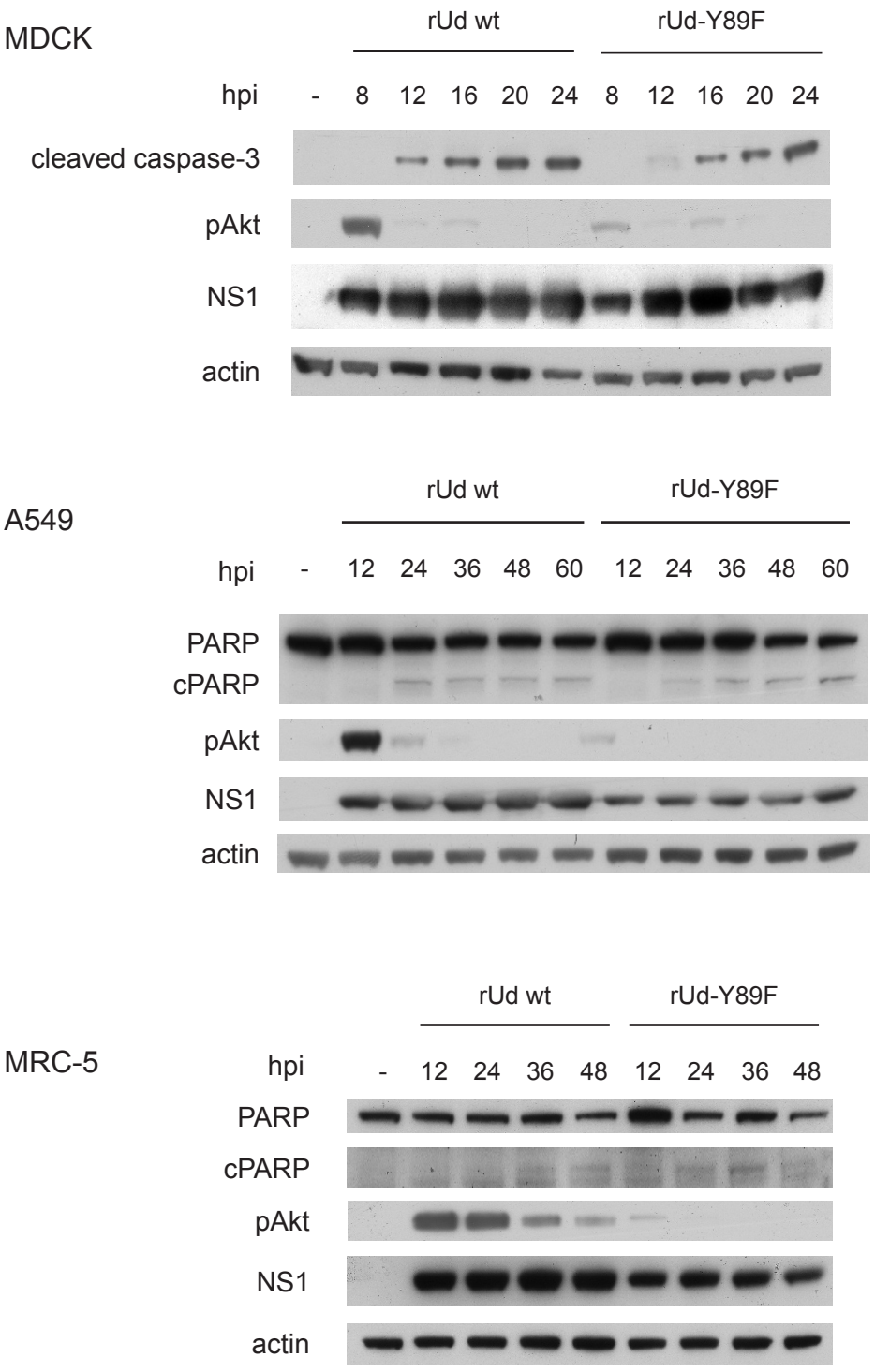
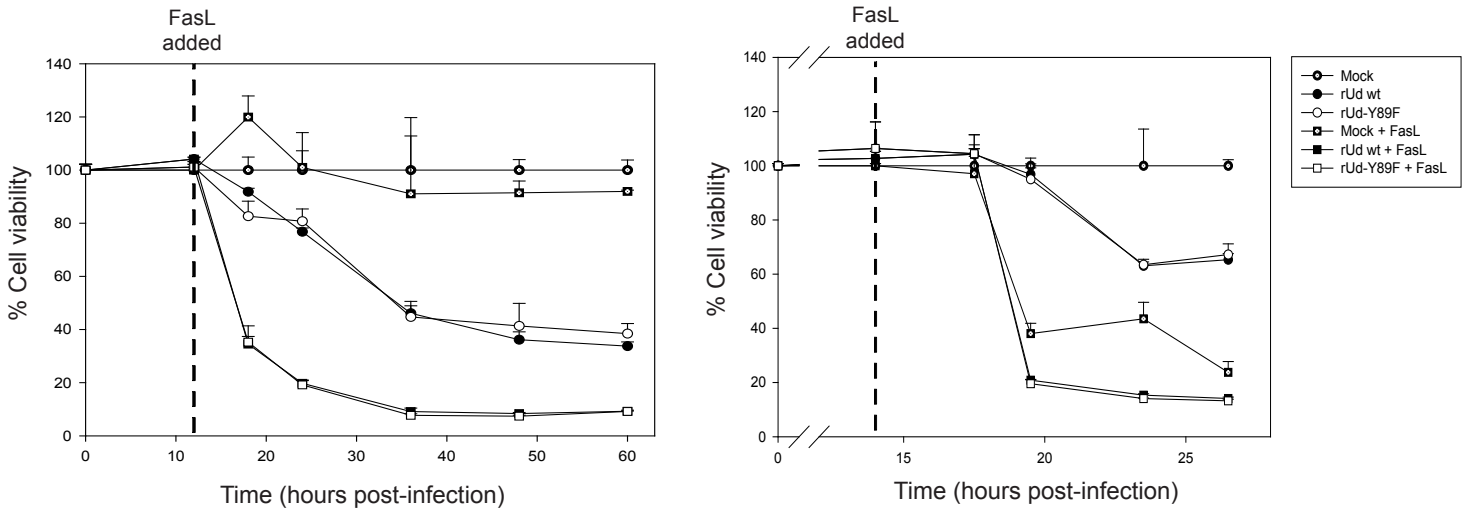


Figure 3.24: rUd-Y89F does not induce more apoptosis than rUd wt.

MDCK, A549 and MRC-5 cell monolayers were infected with rUd wt or rUd-Y89F at an MOI of 5. Cells were harvested at various times post-infection, subjected to SDS-PAGE and immunoblotted.

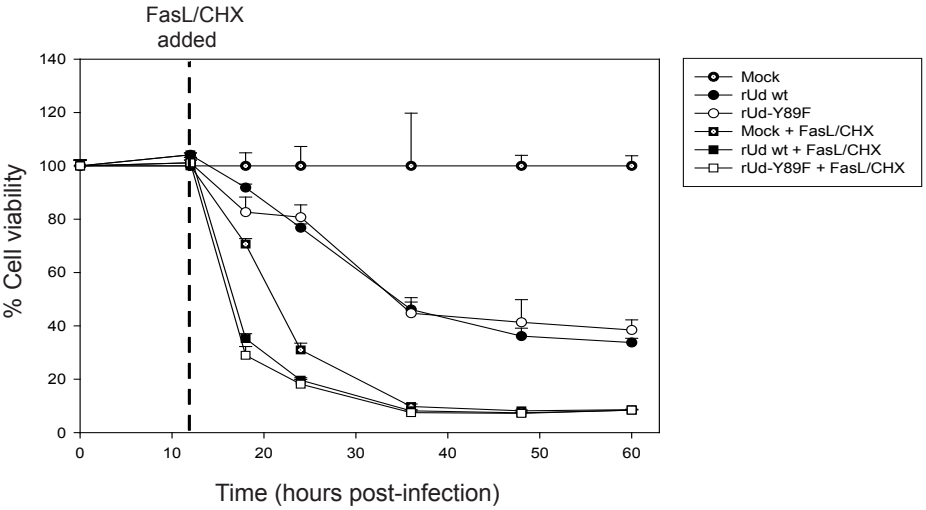
A **A549** **MRC-5**

FasL



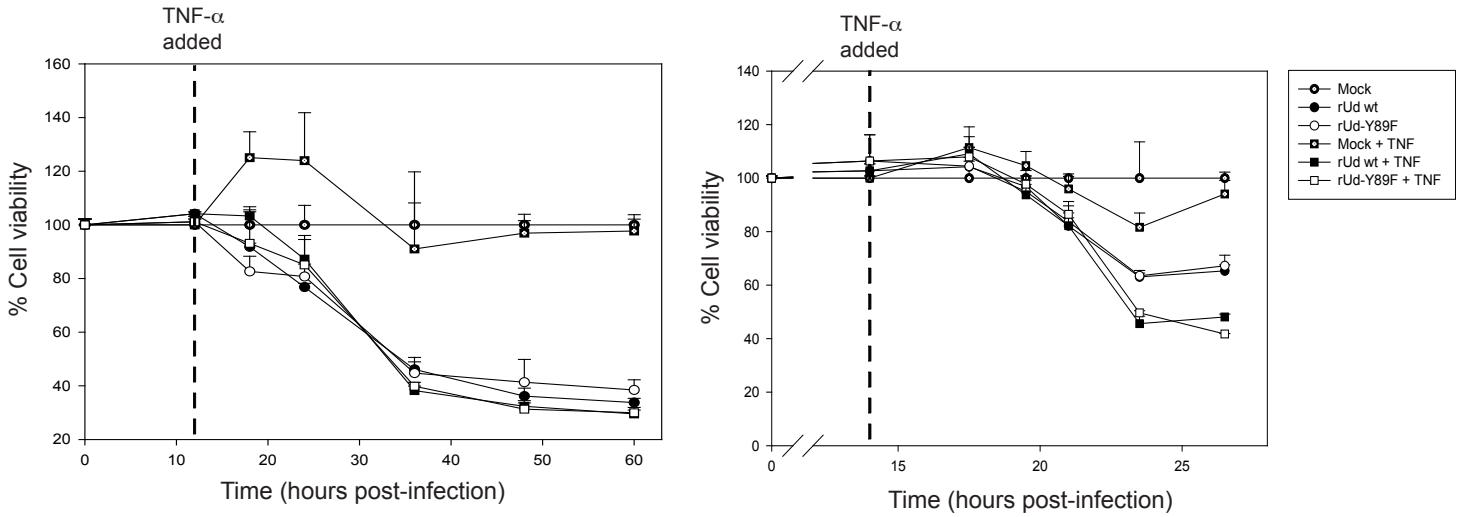
B

FasL/CHX



C

TNF-α



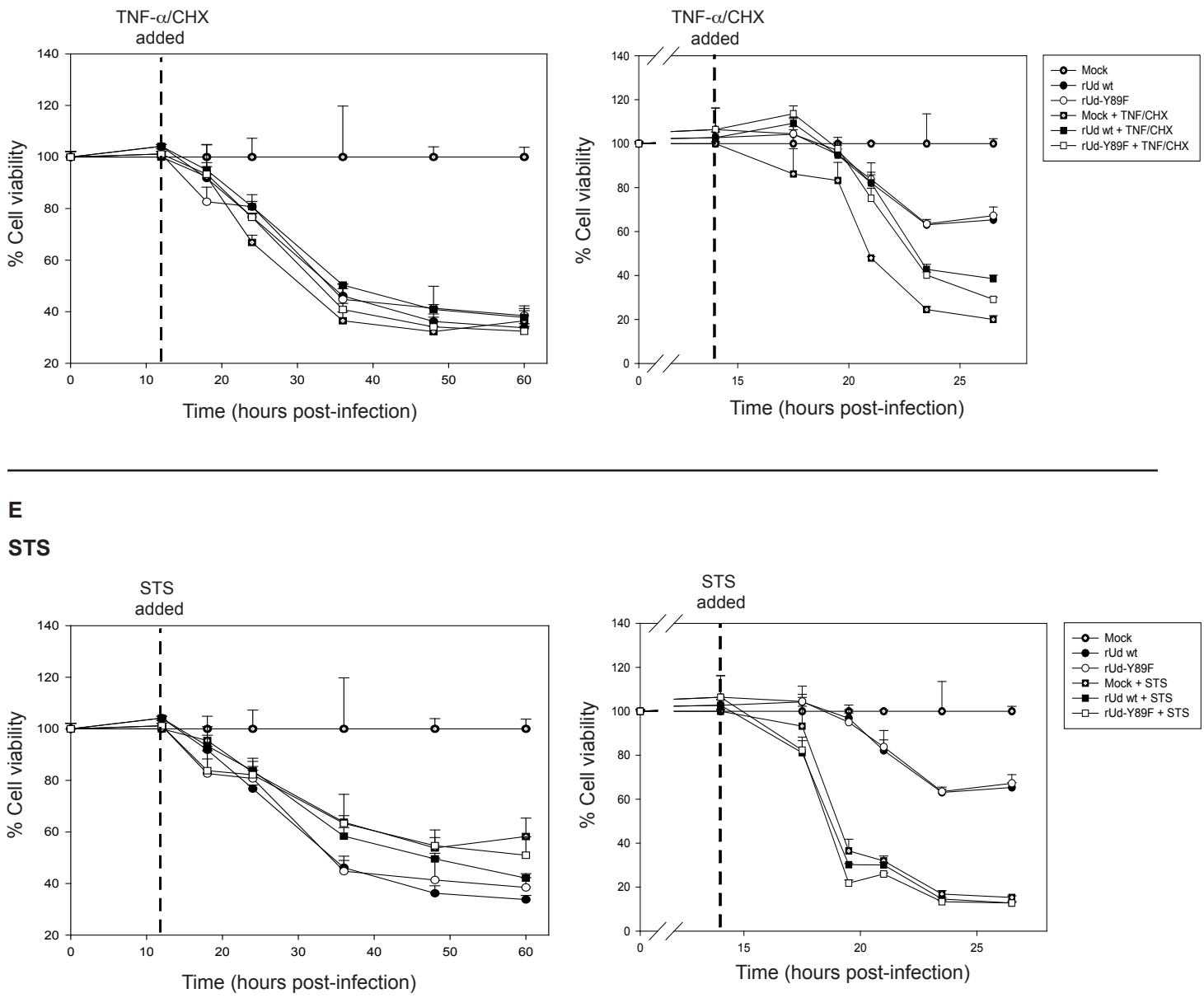


Figure 3.25: PI3K activation by NS1 does not protect cells from non-viral apoptosis inducers.

A549 and MRC-5 cels were mock-infected or infected with rUd wt or rUd-Y89F at an MOI of 35. After 12 hrs, cells were mock-treated or treated with FasL (**A**), FasL/CHX (A549 only)(**B**),TNF-α (**C**), TNF-α/CHX (**D**) or STS (**E**). At various times post-treatment, cell viability was measured in triplicate by wst-1 assay. Mean viability is plotted as a percentage of mock-infected, untreated cells and error bars represent the standard deviation of triplicate sets. N.B. 100% of cells were infected, as determined by immunofluorescence.

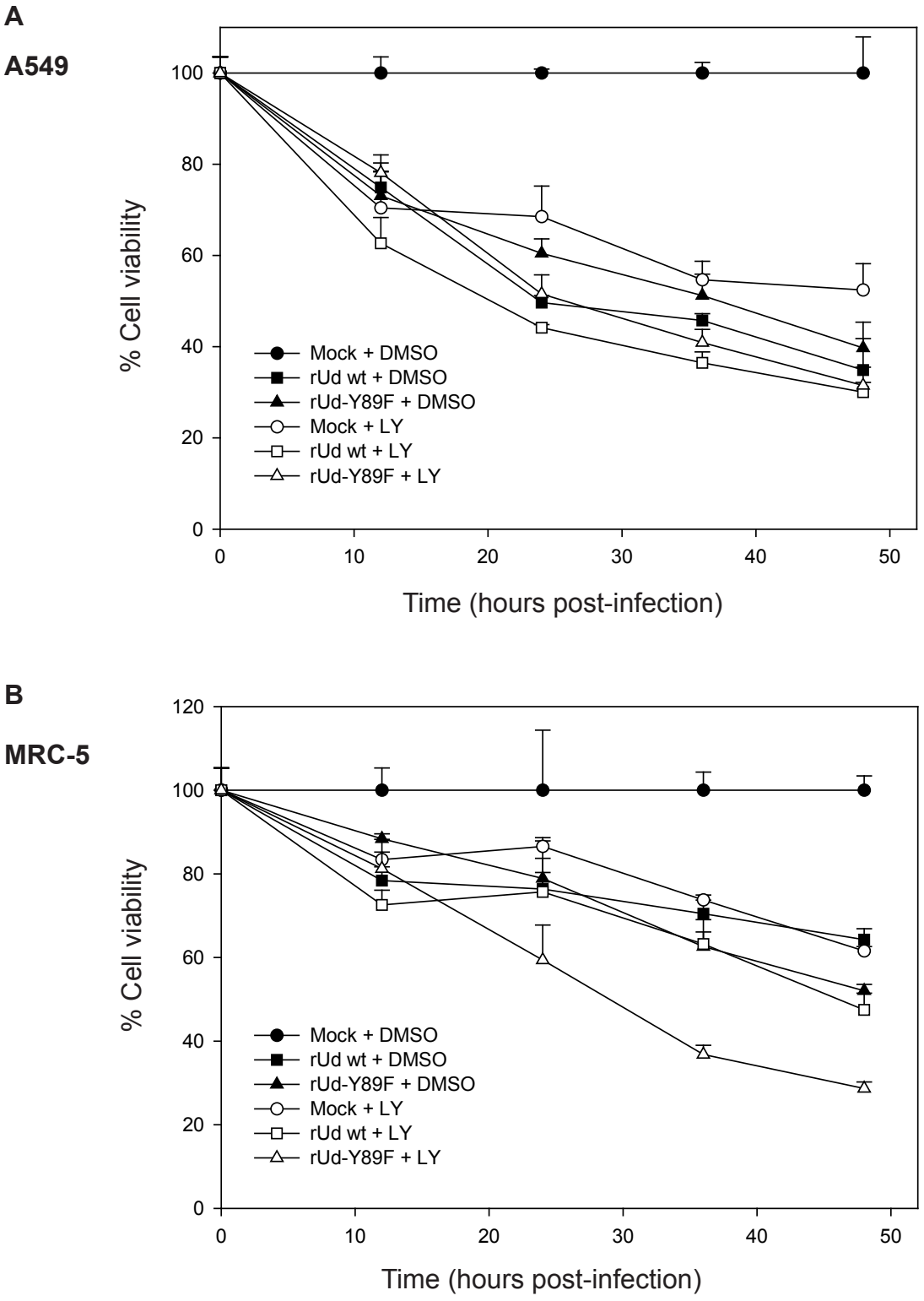


Figure 3.26: Use of LY294002 to inhibit PI3K activation by NS1 may give misleading results.

A549 (**A**) and MRC-5 (**B**) cells were mock-treated with DMSO or treated with 25uM LY294002 for 1h, then mock-infected or infected with rUd wt or rUd-Y89F at an MOI of 35 in the presence of DMSO or LY294002. At various times post-infection, cell viability was measured in triplicate by wst-1 assay. Mean viability is plotted as a percentage of mock-infected/DMSO-treated cells and error bars represnt standard deviation for each triplicate set. N.B. 100% of cells were infected, as determined by immunofluorescence.

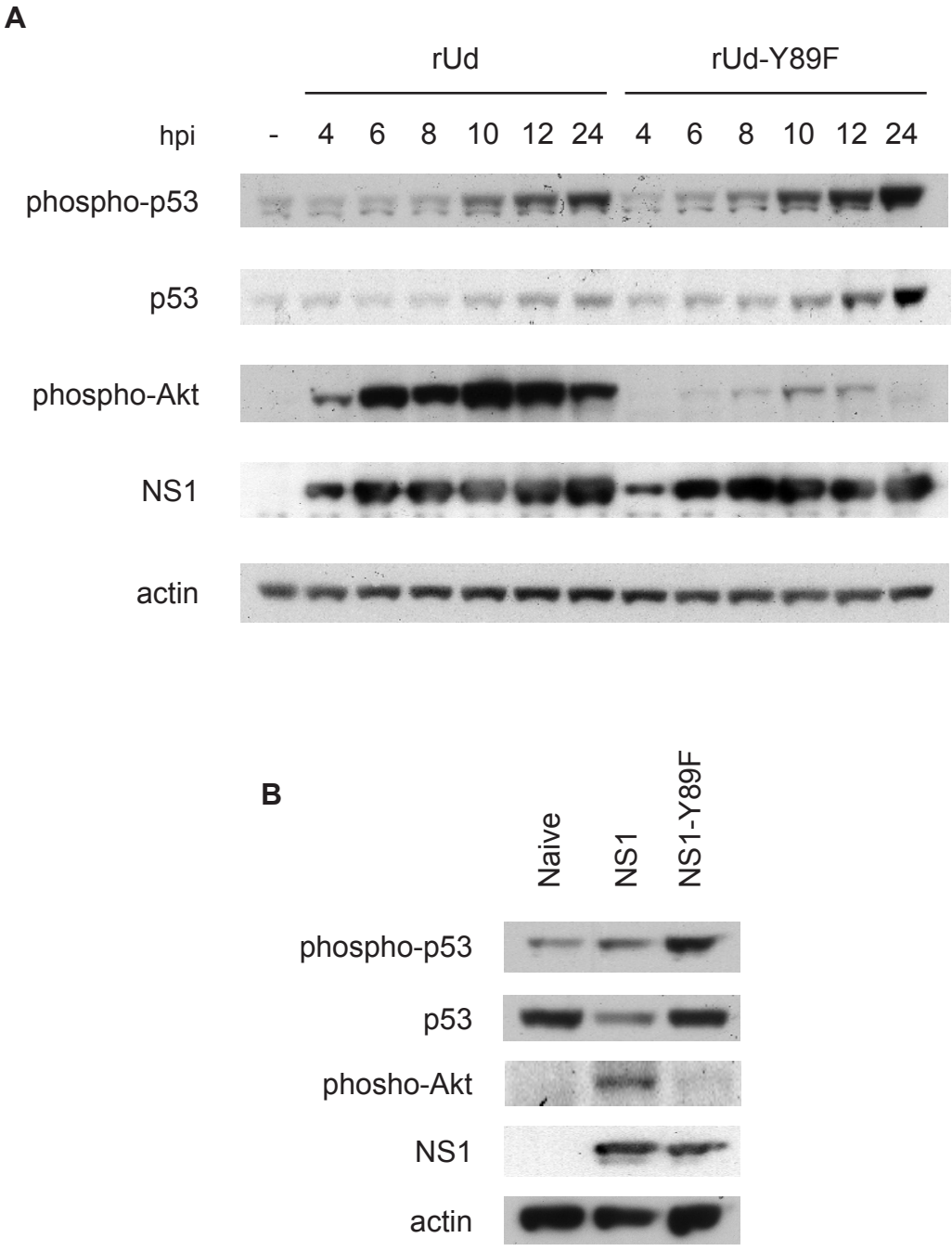


Figure 3.27: Akt activation by NS1 affects p53 expression and activation.

(**A**) A549 monolayers were infected with rUd wt or rUd-Y89F at an MOI of 10. Cells were harvested at various times post-infection and immunoblotted. (**B**) 1321N1 cells stably expressing PR8 NS1or PR8 NS1-Y89F were harvested and immunoblotted.

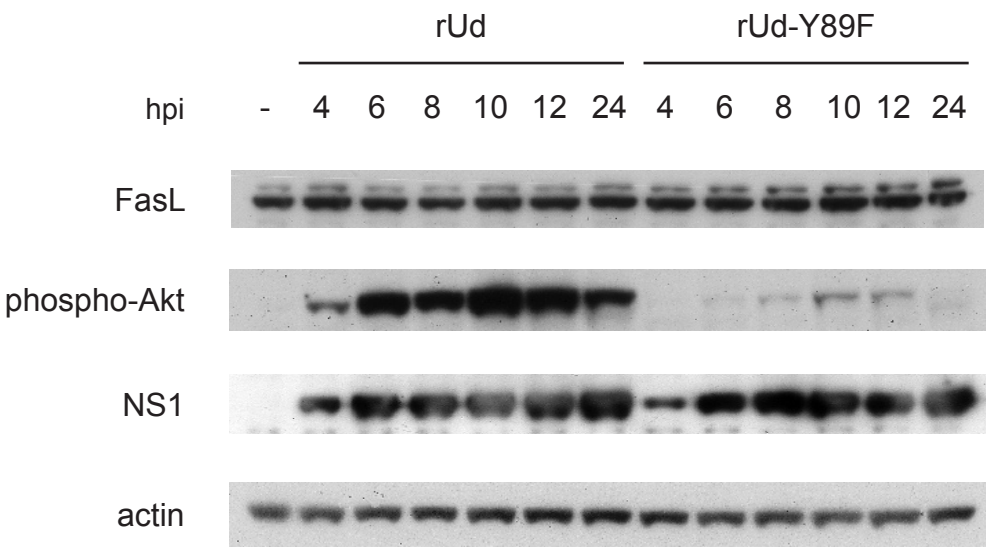


Figure 3.28: PI3K/Akt activation by NS1 has no effect on FasL expression.

A549 monolayers were infected with rUd wt or rUd-Y89F at an MOI of 10. Cells were harvested at various times post-infection and immunoblotted.

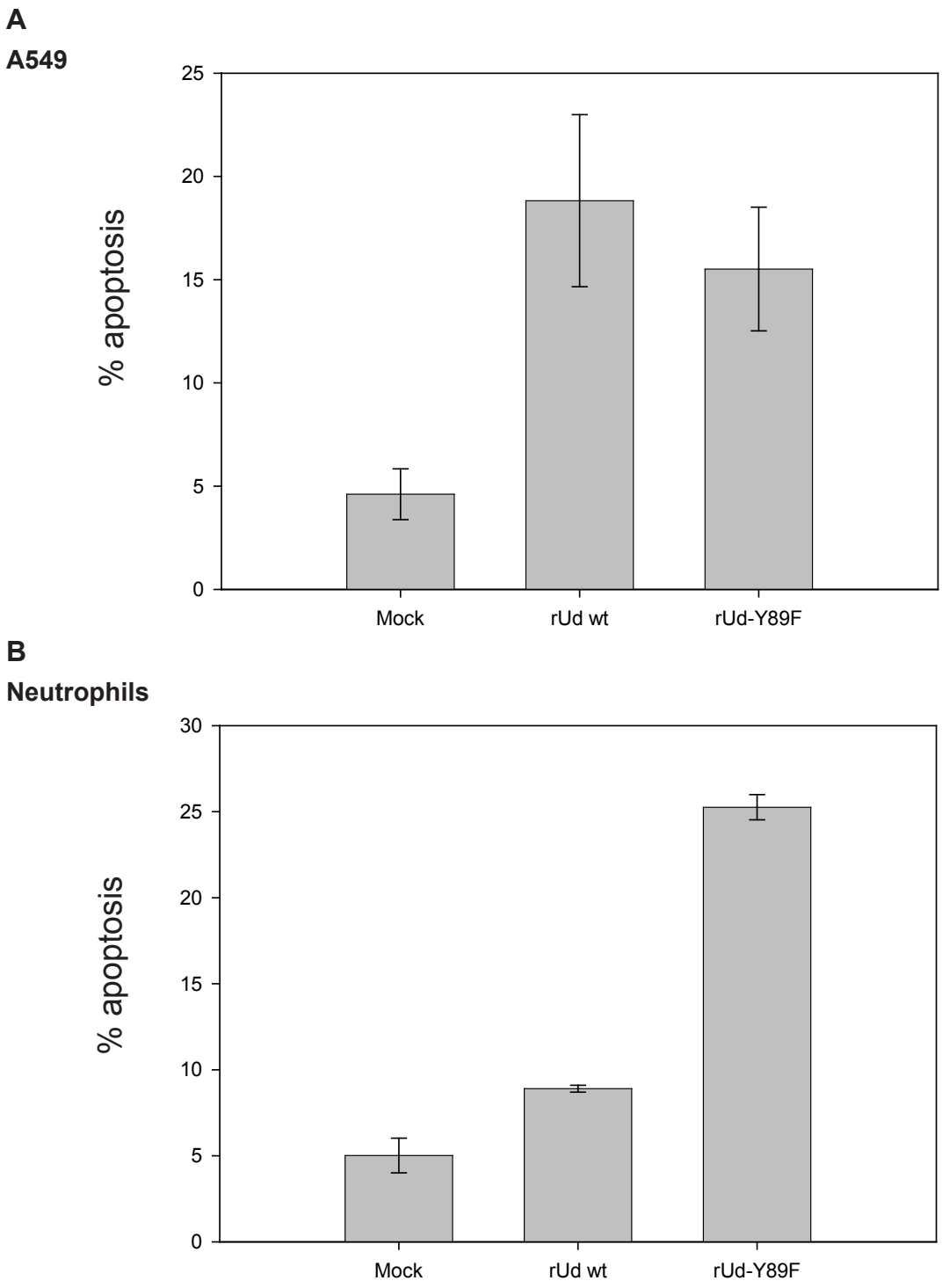


Figure 3.29: rUd-Y89F causes more apoptosis than rUd wt in neutrophils.

Triplicates of A549 cells (A) or freshly-isolated neutrophils (B) were infected with rUd wt or rUd-Y89F at an MOI of 50. Apoptotic cells were quantified by annexin-V/PI staining and flow cytometry, and calculated as a proportion of the cell population.

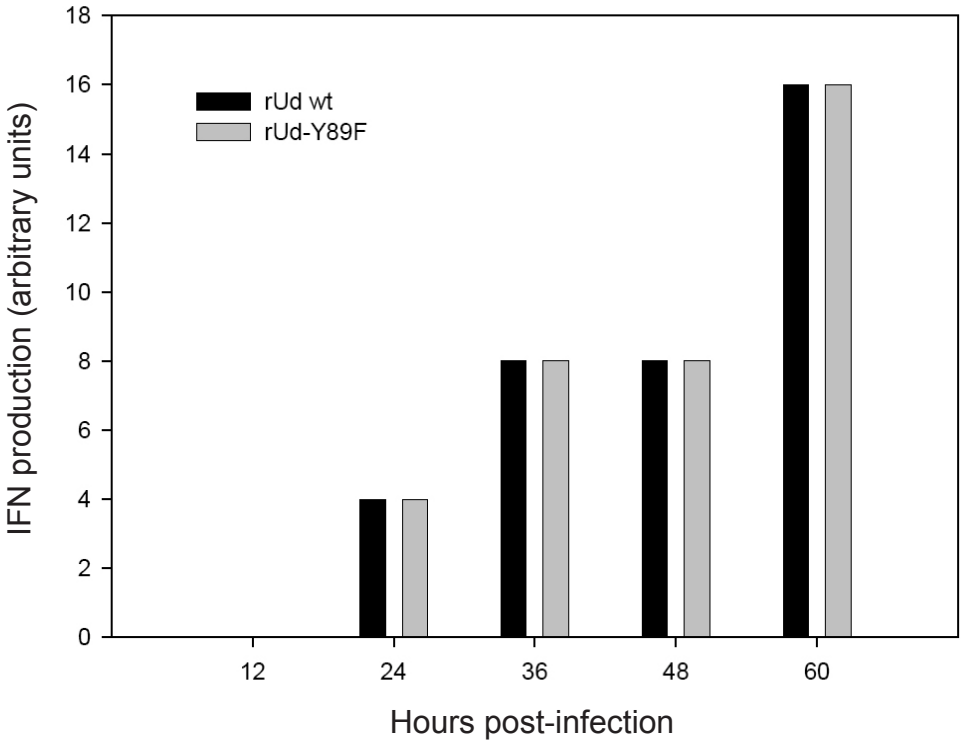
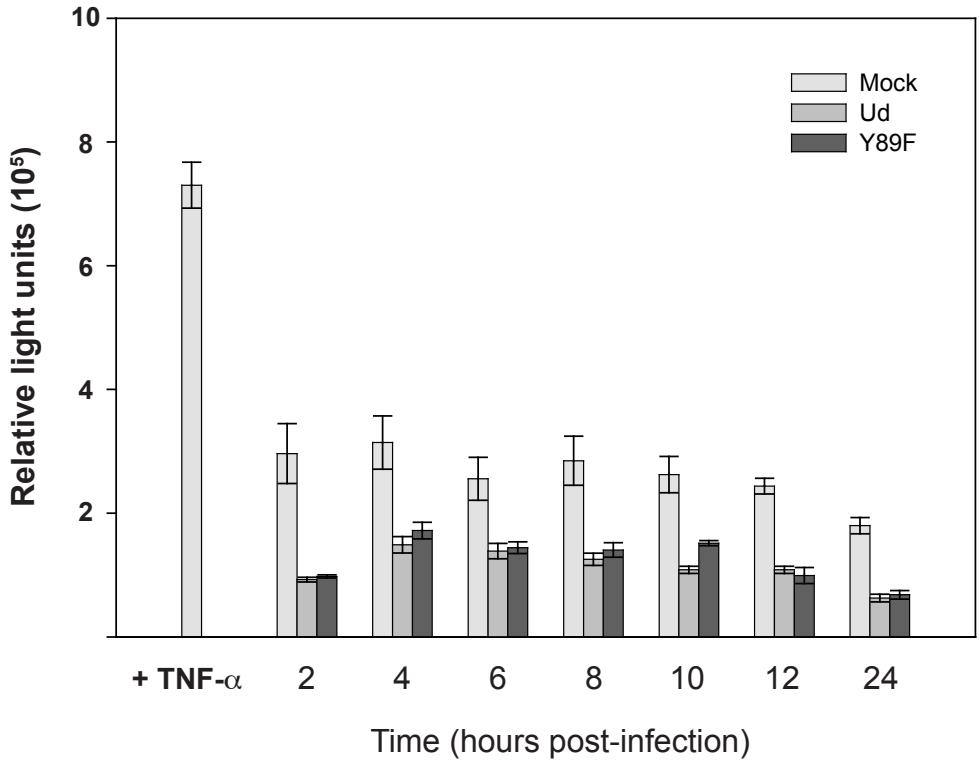


Figure 3.30: PI3K activation by NS1 has no effect on IFN production.

A549 cells were infected with rUd wt or rUd-Y89F at an MOI of 10. At 12, 24, 36, 48 and 60 hrs post-infection, the culture supernatant was collected. The amount of IFN present was estimated using a cell-based IFN assay. Titrations were carried out in triplicate.

A



B

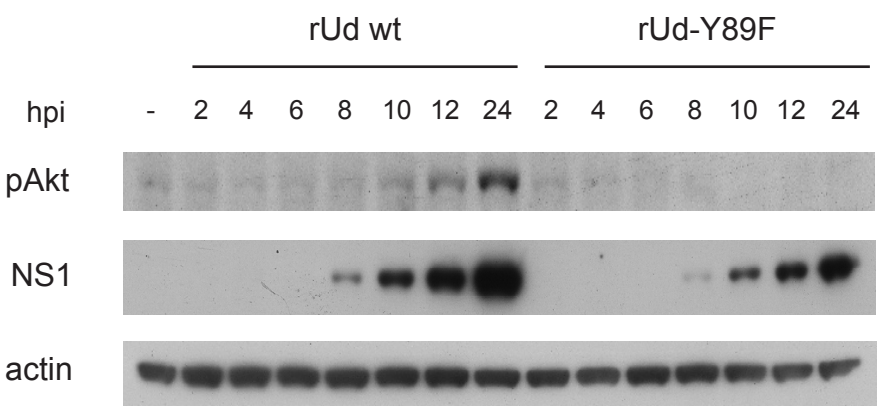
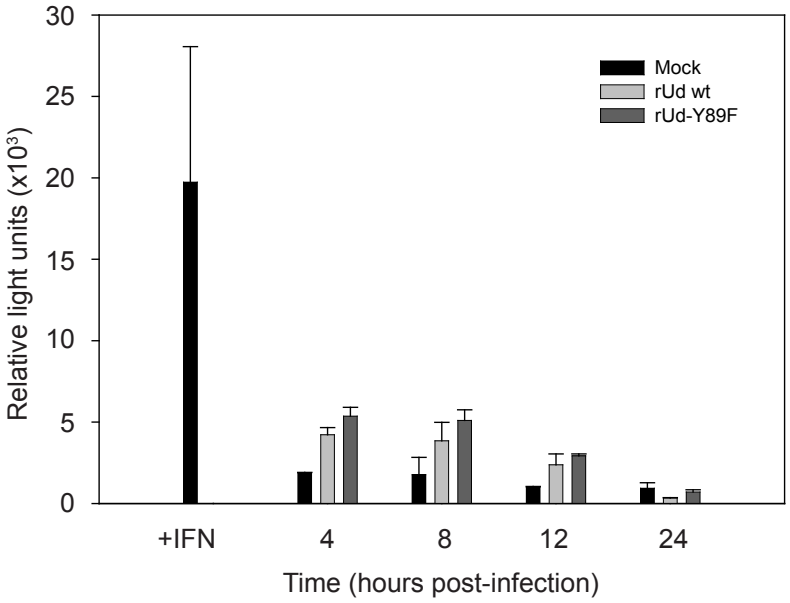
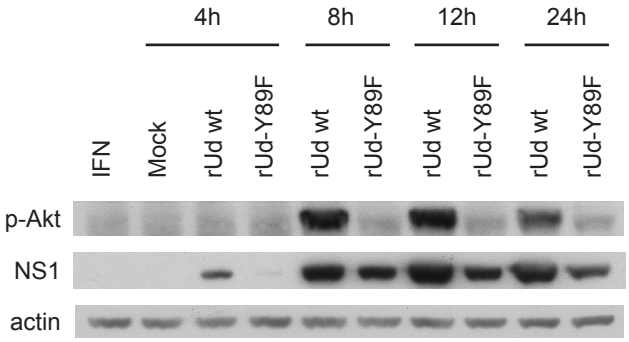


Figure 3.31: PI3K activation by NS1 has no effect on NF-kB activation.

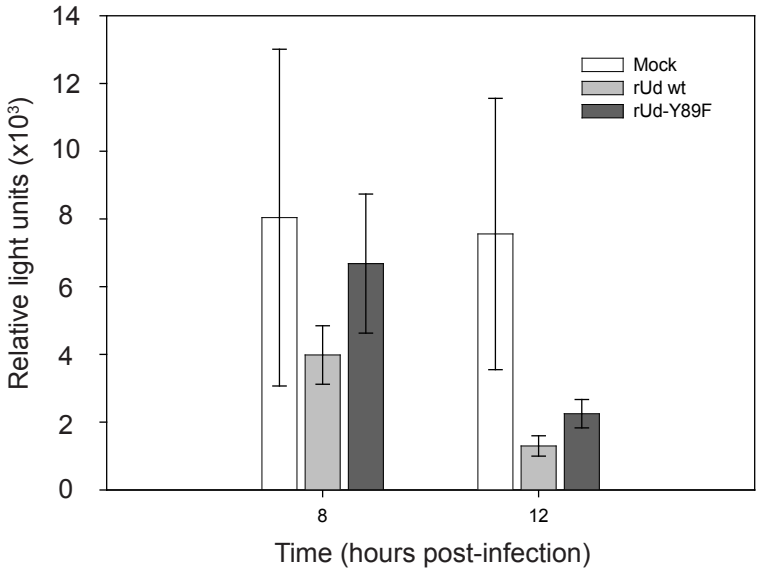
Triplicate monolayers of A549 cells stably expressing firefly luciferase under the control of an NF-κB-responsive promoter were infected with rUd wt or rUd-Y89F at an MOI of 10. Cells were treated with TNF-α for 6hrs as a positive contol. At various times post-infection, cells were harvested and the amount of luciferase present was quantified. Mean luciferase activity is plotted and error bars represent standard deviation of each set of triplicates (**A**). Duplicate lysates were subjected to SDS-PAGE and immunoblotted (**B**).

A


30
25
20
15
10
5
0
Relative light units (x10³)
Mock
rUd wt
rUd-Y89F
+IFN
4
8
12
24
Time (hours post-infection)


4h
8h
12h
24h
IFN
Mock
rUd wt
rUd-Y89F
rUd wt
rUd-Y89F
rUd wt
rUd-Y89F
rUd wt
rUd-Y89F
p-Akt
NS1
actin

B



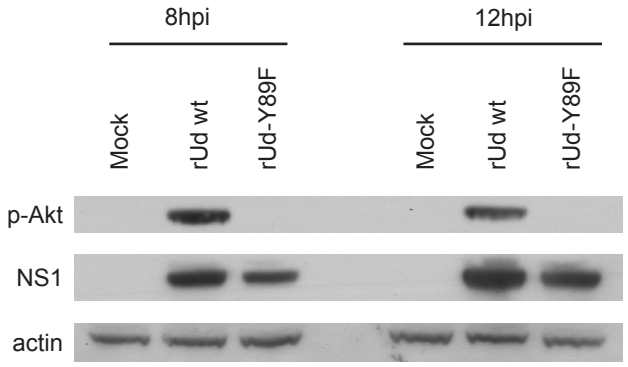


Figure 3.32: PI3K activation by NS1 does not have a significant effect on ISRE activation.

A549 cells stably expressing luciferase under the control of an ISRE were mock-infected or infected with rUd wt or rUd-Y89F at an MOI of 5. (**A**) At various times post-infection, cells were lysed and luciferase activity was measured (in duplicate). Error bars represent standard deviation for duplicate set. Identically treated cells were also harvested at the same times post-infection, subjected to SDS-PAGE and immunoblotted. (**B**) Cells were infected as in (A). 6 hrs prior to harvesting, cells were treated with IFN-α (10^4 units/ml). Cells were harvested at 8 and 12hrs post-infection and assayed for luciferase activity in duplicate.

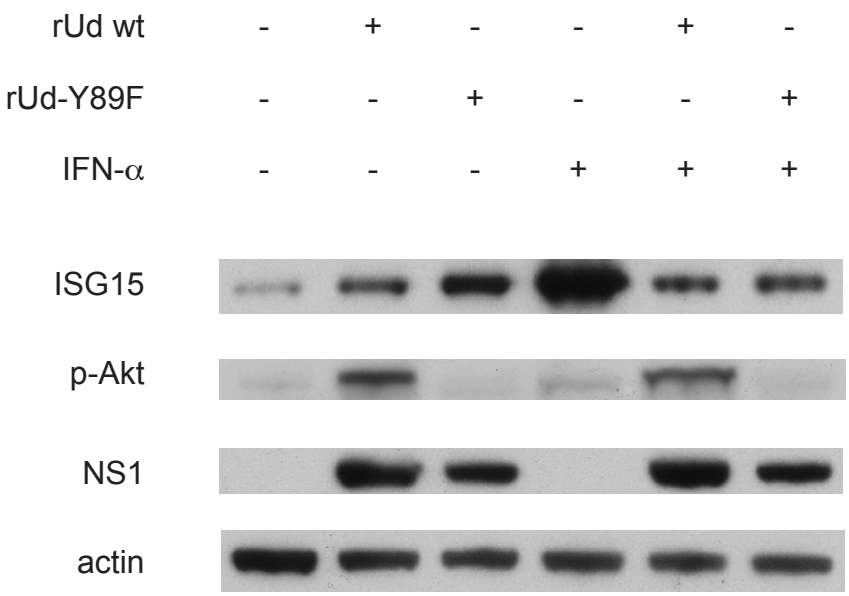


Figure 3.33: PI3K activation by NS1 does not have a significant effect on ISG expression.

A549 cells were mock-infected or infected with rUd wt or rUd-Y89F at an MOI of 5. 8hrs later, cells were mock-treated or treated with IFN-α (10^3units/ml). 16h later, cells were harvested, subjected to SDS-PAGE and immunoblotted.

MUTANT	MUTATION(S)	EFFECT ON NS1 FUNCTION	REFERENCE(S)
rUd-Y89F	Tyr89Phe	Unable to bind and activate PI3K	Hale et al., 2006 Hale., 2007
rUd-P164/7A	Pro164Ala Pro167Ala	Unable to activate PI3K Reported to induce apoptosis	Shin et al., 2007
rUd-P162/4/7A	Pro162Ala Pro164Ala Pro167Ala		
rUd- Δ99	Ser99Stop	C-terminally truncated NS1 of 98 amino acids	-
rUd-R38A	Arg38Ala	Prevents interaction with dsRNA Prevents inhibition of OAS/RNaseL system	Wang et al., 1999
rUd-123/4	Ile123Ala Met124Ala	Impaired PKR binding Leads to increased vRNA production early in infection	Min et al. , 2007
rUd-184-8(P)	Gly184Arg Leu185Phe Glu186Pro Trp187Arg Asn188Tyr	Unknown	-
rUd-184-8(L)	Gly184Arg Leu185Phe Glu186Leu Trp187Arg Asn188Tyr	Prevents interaction with CPSF30	Noah et al., 2007 Kochs et al., 2007 Das et al., 2008
rUd-103/106	Phe103Leu Met106Ile	Destabilises the NS1:CPSF30 interaction	Twu et al., 2007 Kochs et al., 2007

Table 3.1: NS1 mutations introduced into rUd and their biological effects.

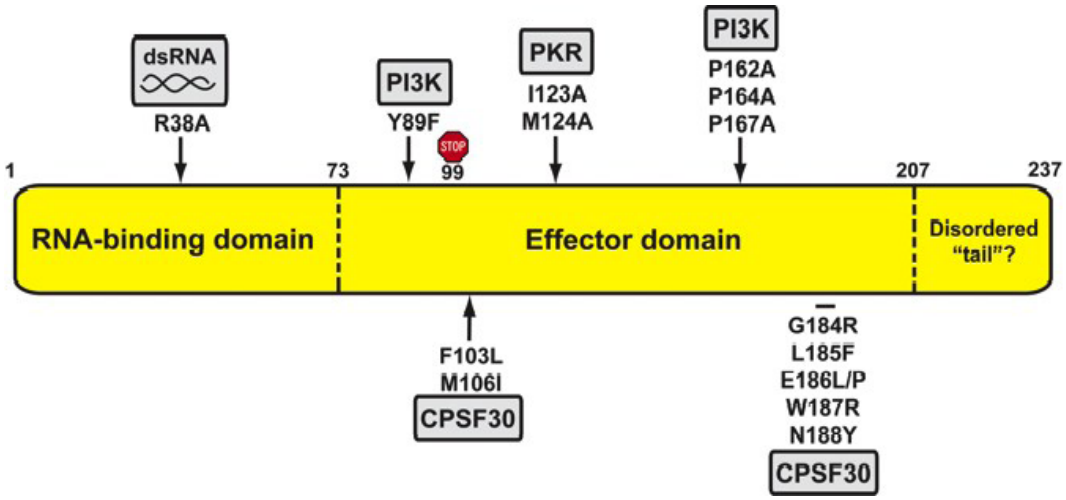


Figure 3.34: NS1 mutations introduced into rUd.

The nature and position of amino acid substitutions resulting from mutation in the NS segment are shown. Cellular interactors of NS1 whose binding is affected by these mutations are shown in grey.
Modified from Hale et al., 2006 by Dr. D. Jackson.

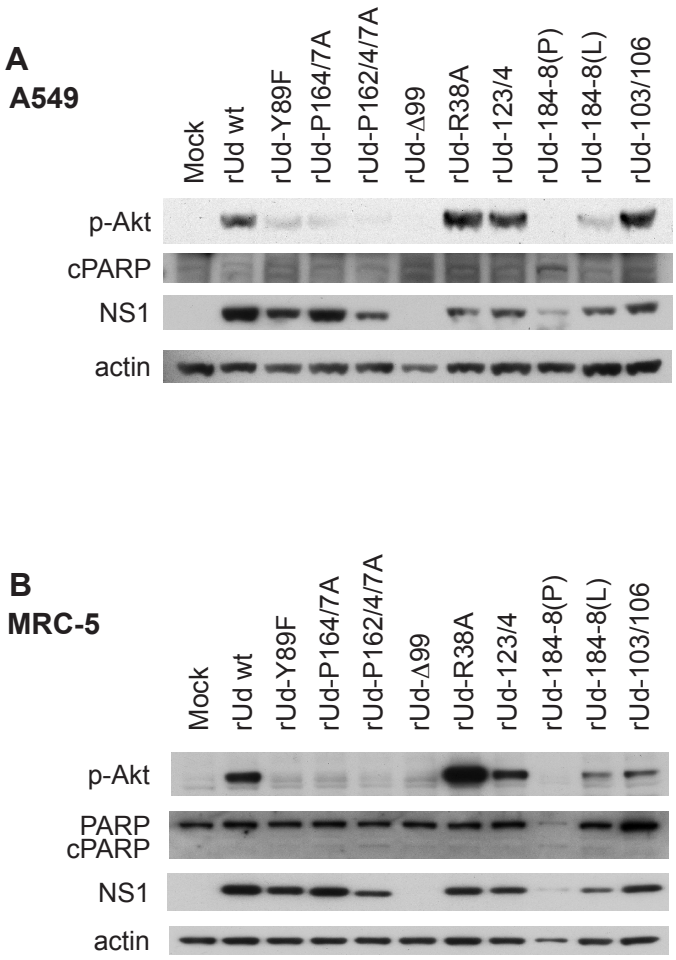
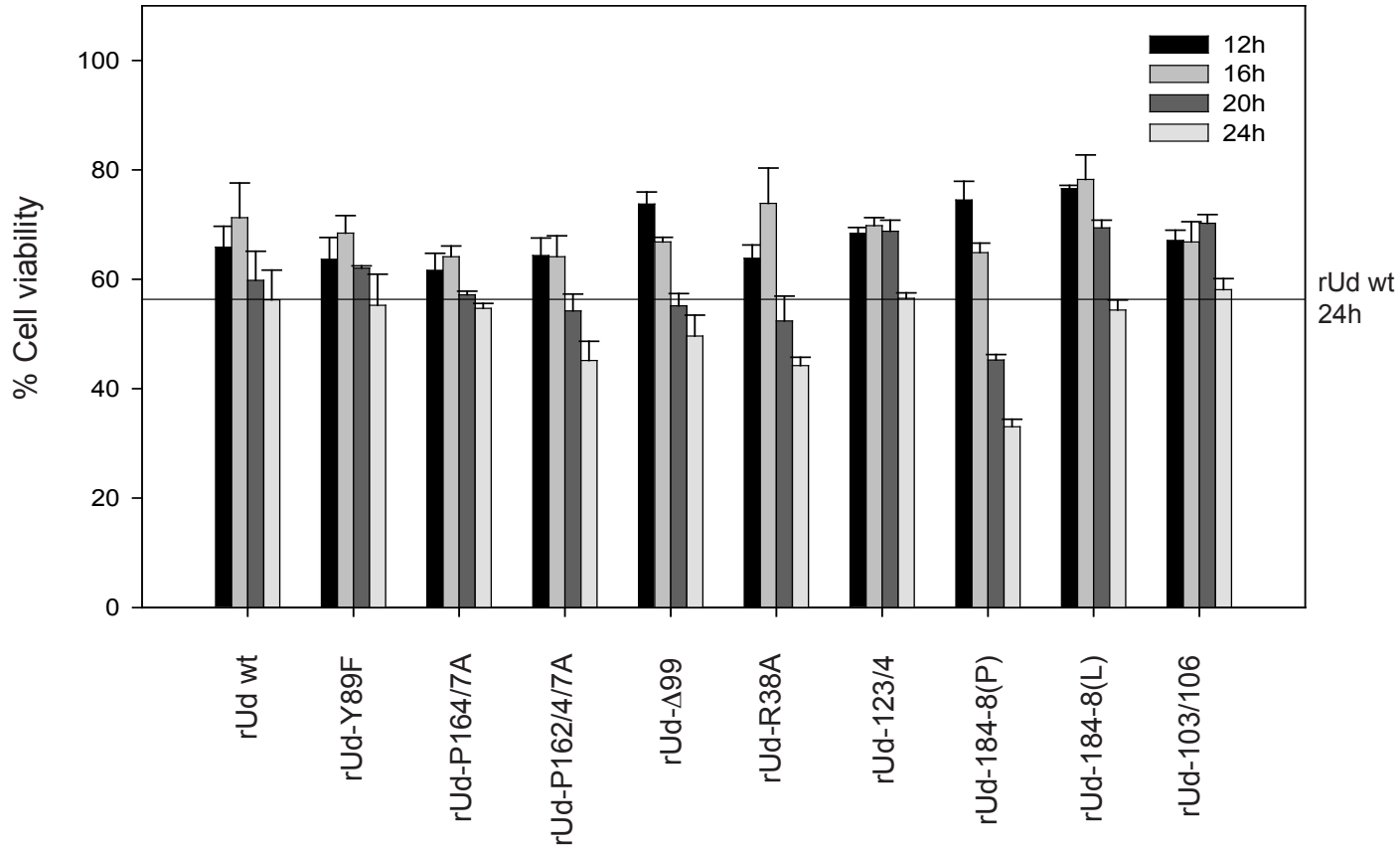


Figure 3.35: PI3K activation, NS1 expression and induction of apoptosis in cells infected with different rUd NS1 mutants.

A549 (**A**) or MRC-5 (**B**) cells were infected with rUd wt, rUd-Y89F, rUd-P164/7A, rUd- P162/4/7A, rUd-Δ99, rUd-R38A, rUd-123/4, rUd-184-8(P), rUd-184-8(L) or rUd-103/106 at an MOI of 5. 24h later, cells were harvested, subjected to SDS-PAGE and immunoblotted.

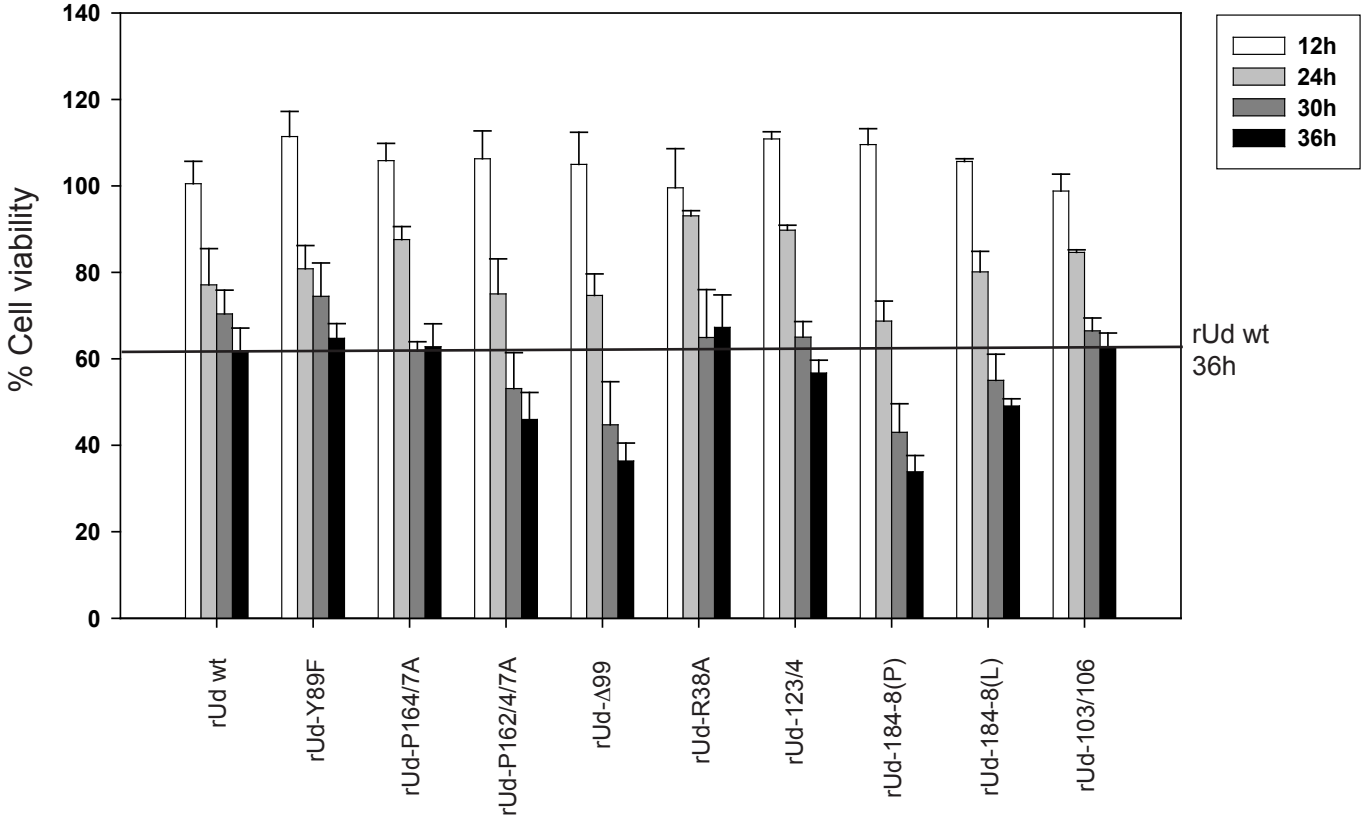
A

MDCK


12h
16h
20h
24h
% Cell viability
100
80
60
40
20
0
rUd wt
24h
rUd wt
rUd-Y89F
rUd-P164/7A
rUd-P162/4/7A
rUd-Δ99
rUd-R38A
rUd-123/4
rUd-184-8(P)
rUd-184-8(L)
rUd-103/106

B

A549


12h
24h
30h
36h
% Cell viability
140
120
100
80
60
40
20
0
rUd wt
36h
rUd wt
rUd-Y89F
rUd-P164/7A
rUd-P162/4/7A
rUd-Δ99
rUd-R38A
rUd-123/4
rUd-184-8(P)
rUd-184-8(L)
rUd-103/106

C

MRC-5

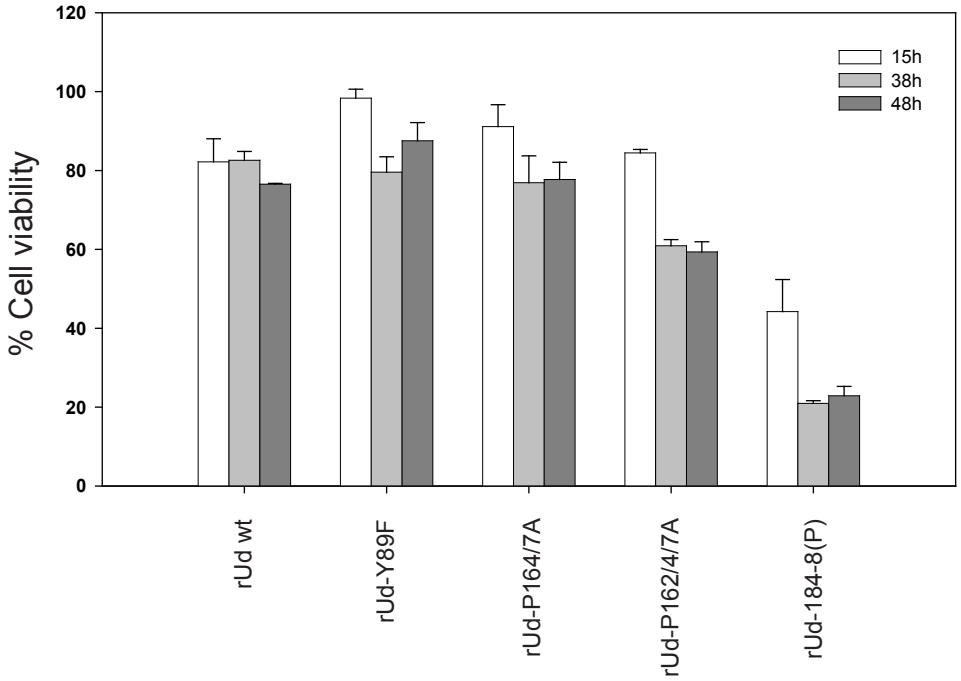
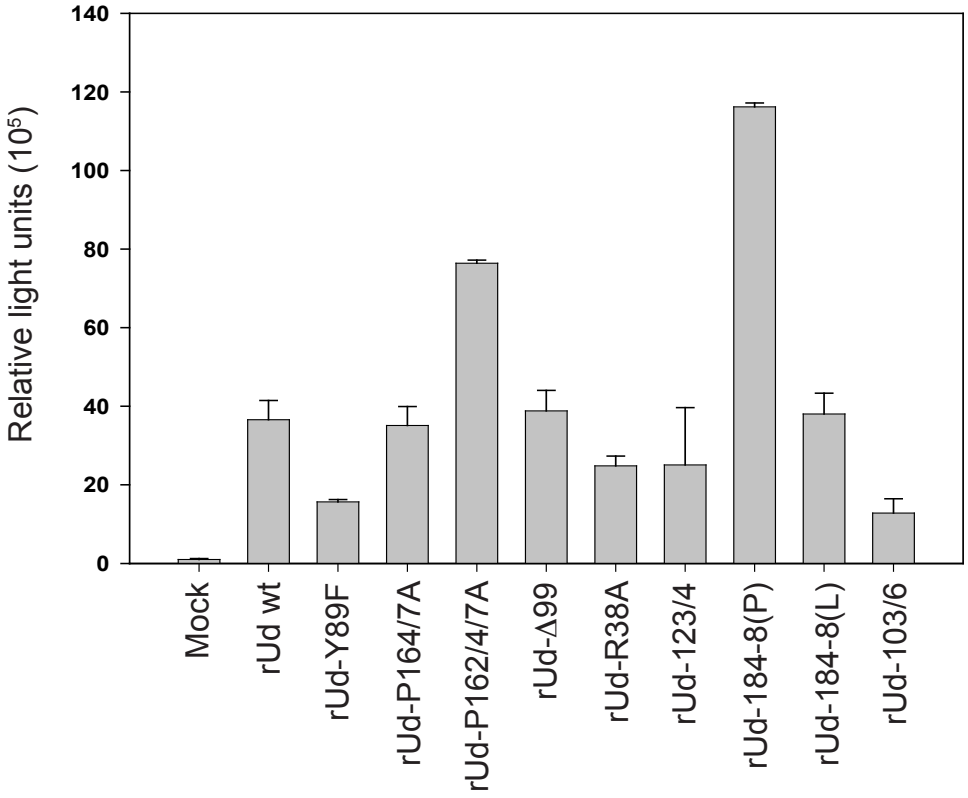


Figure 3.36: Rate of cell death following infection with rUd NS1 mutants.

MDCK (**A**) or A549 cells (**B**) were infected with the full panel of rUd NS1 mutant viruses (rUd wt, rUd-Y89F, rUd-P164/7A, rUd- P162/4/7A, rUd-Δ99, rUd-R38A, rUd-123/4, rUd-184-8(P), rUd-184-8(L) and rUd-103/106) at an MOI of 35. At various times post-infection, cell viability was measured in triplicate by wst-1 assay. Mean viability is plotted and error bars represent standard deviation for each triplicate set. (**C**) MRC-5 cells were infected with a selection of the panel of rUd NS1 mutants (rUd wt, rUd-Y89F, rUd-P164/7A, rUd-P162/4/7A and rUd-184-8(P)) at an MOI of 35. At various times post-infection, cell viability was measured in triplicate by wst-1 assay. Mean viability is plotted and error bars represent standard deviation for each triplicate set. N.B. 100% of cells were infected, as determined by immunofluorescence.

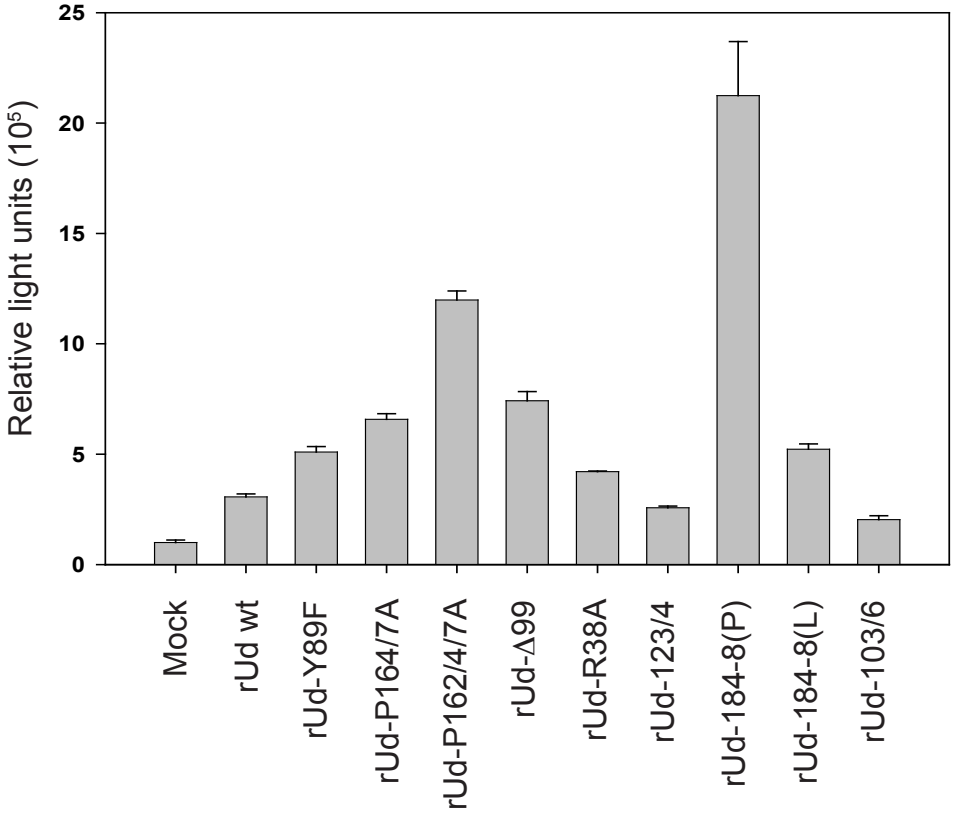
A

A549


Relative light units (10^5)
140
120
100
80
60
40
20
0
Mock
rUd wt
rUd-Y89F
rUd-P164/7A
rUd-P162/4/7A
rUd-Δ99
rUd-R38A
rUd-123/4
rUd-184-8(P)
rUd-184-8(L)
rUd-103/6

B

MRC-5


Relative light units (10^5)
25
20
15
10
5
0
Mock
rUd wt
rUd-Y89F
rUd-P164/7A
rUd-P162/4/7A
rUd-Δ99
rUd-R38A
rUd-123/4
rUd-184-8(P)
rUd-184-8(L)
rUd-103/6

C

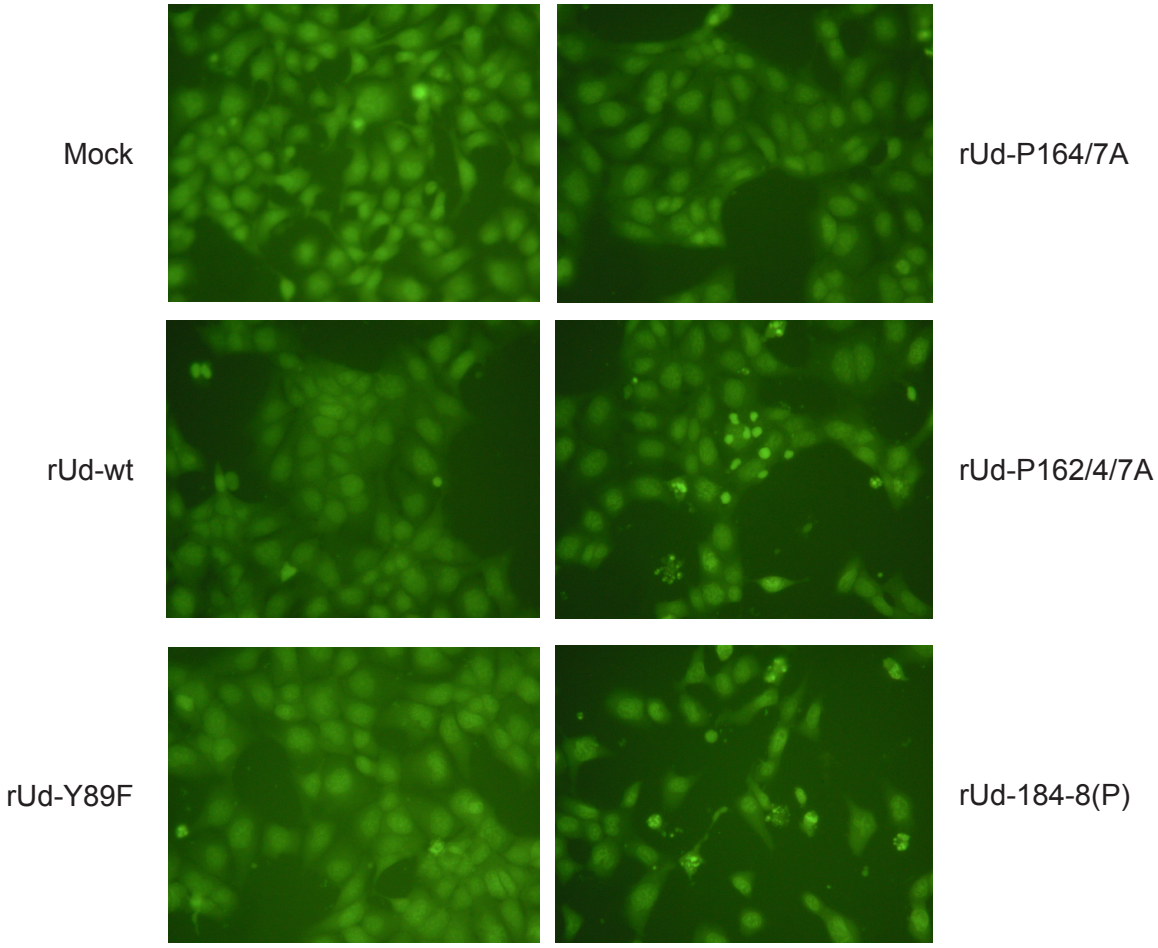
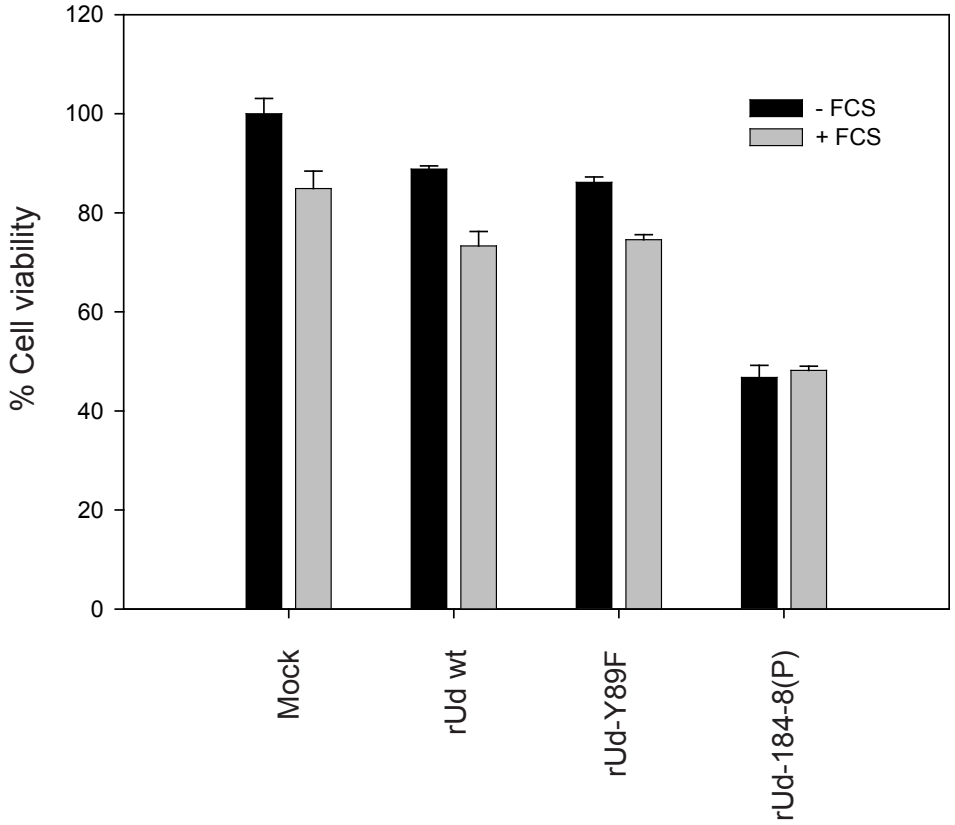


Figure 3.37: Caspase activity and apoptosis in cells infected with rUd NS1 mutant viruses.

A549 (**A**) or MRC-5 (**B**) cells were infected with rUd wt, rUd-Y89F, rUd-P164/7A, rUd- P162/4/7A, rUd-Δ99, rUd-R38A, rUd-123/4, rUd-184-8(P), rUd-184-8(L) or rUd-103/106 at an MOI of 35. Cells were harvested 24h later and caspase-3 and -7 activity was quantified in duplicate by Caspase-Glo 3/7 luciferase assay. Mean luciferase activity is plotted and error bars represent standard deviation for duplicates. (**C**) MDCK cells were mock-infected or infected with rUd wt, rUd-Y89F, rUd-164/7A, rUd-P162/4/7A or rUd-184-8(P) at an MOI of 35. At 20h post-infection, cells were fixed and stained with acridine orange. Apoptotic nuclei were visualised using a fluorescence microscope with a fluorescein filter. N.B. 100% of cells were infected, as determined by immunofluorescence.

A



B

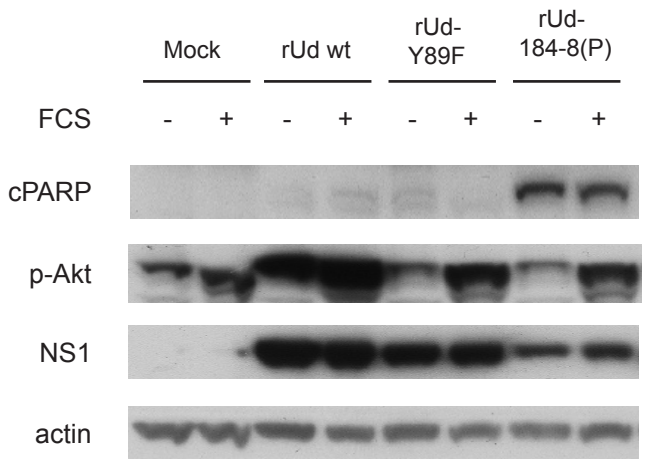


Figure 3.38: PI3K activation by serum does not prevent apoptosis induction by rUd-184-8(P).

A549 cells were mock-infected or infected with rUd wt, rUd-Y89F or rUd-184-8(P) at an MOI of 35. After the inital virus adsorption period (1h), virus inoculum was removed and replaced with serum-free DMEM or DMEM supplemented with 10% foetal calf serum. Cell viability was measured by wst-1 assay 36hpi (**A**) or cell lysates were immunoblotted at 24hpi (**B**). N.B. 100% of cells were infected, as determined by immunofluorescence.

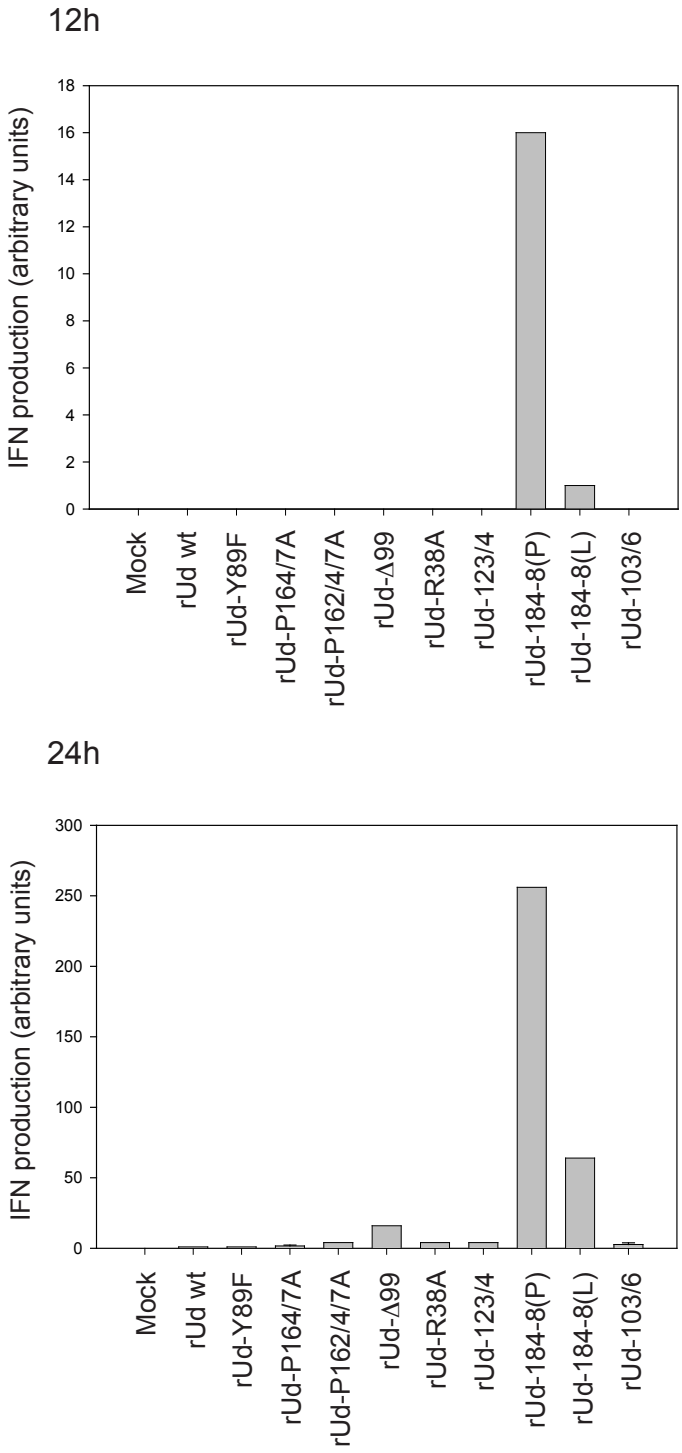


Figure 3.39: rUd NS1 mutants induce different levels of IFN.

A549 cells were infected with rUd wt, rUd-Y89F, rUd-P164/7A, rUd-P162/4/7A, rUd-Δ99, rUd-R38A, rUd-123/4, rUd-184-8(P), rUd-184-8(L) or rUd-103/106 at an MOI of 5. At 12h and 24h post-infection culture supernatants were removed and the amount of IFN present was titrated in triplicate in a cell-based IFN assay. Mean relative IFN production is plotted and with error bars to show standard deviation for each set of triplicates.

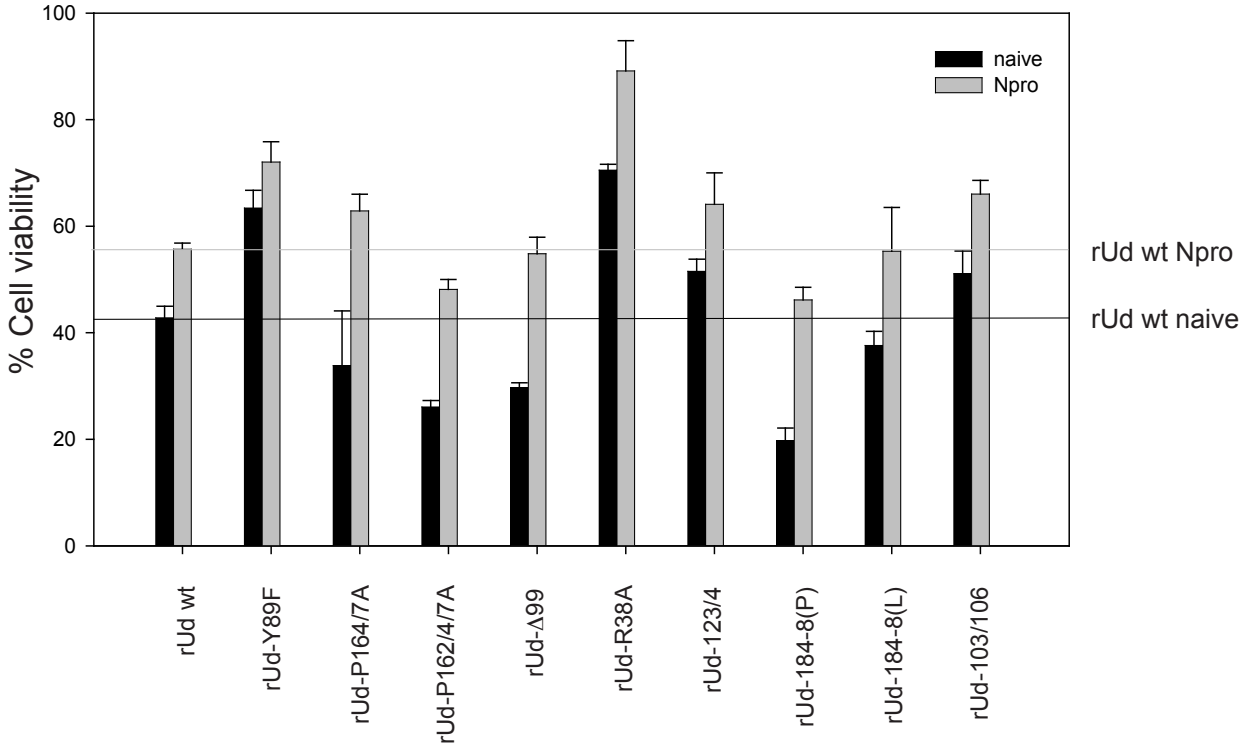


Figure 3.40: Rate of cell death following infection with rUd NS1 mutants in cells deficient in the IFN response.

A549 naive and A549/BVDV/NPro cells were infected with the full panel of rUd NS1 mutant viruses (rUd wt, rUd-Y89F, rUd-P164/7A, rUd- P162/4/7A, rUd-Δ99, rUd-R38A, rUd-123/4, rUd-184-8(P), rUd-184-8(L) and rUd-103/106) at an MOI of 35. At 36hpi, cell viability was measured in triplicate by wst-1 assay. Mean values are plotted and error bars represent standard deviation for each triplicate set. N.B. 100% of cells were infected, as determined by immunofluorescence.

4 DISCUSSION

4.1 Sensitisation of cells to apoptosis inducers

4.1.1 CHX promotes TNF-α and FasL-induced apoptosis

This study examined the sensitivity of A549 and MRC-5 cells to a number of apoptosis inducers. A549 cells were resistant to TNF-α and FasL-induced apoptosis but became sensitised to these treatments in the presence of CHX. This has been reported previously (Kreuz et al., 2001, Micheau et al., 2001, Ogura et al., 2008, Fulda et al., 2000, Mitsiades et al., 2000, Arscott et al., 1997), and indicates that resistance to TNF-α and FasL is mediated by a labile inhibitor. cFLIP, cIAPs and anti-apoptotic Bcl-2 family members have all been implicated in TNF-α and FasL resistance (Schoemaker et al., 2002, Wang et al., 1998a, Wang et al., 1999a, Stehlik et al., 1998, Chu et al., 1997, Ichikawa et al., 2005, Zou et al., 2004, Notarbartolo et al., 2004, Gordon et al., 2007, Yemelyanov et al., 2006, Chang et al., 2002, Irmler et al., 1997, Micheau et al., 2002, Sekine et al., 2008, Ruemmele et al., 2002). In addition, CHX has been reported to downregulate TNFR1 expression in A549 cells by promoting its cleavage from the cell surface (Ogura et al., 2008): it has been suggested that TNF-α sensitivity of a particular cell-type is dictated by the ratio of TNFR1 to cFLIP expression. The ability of FasL to cause apoptosis in MRC-5 cells without the need for CHX treatment suggests that these cells may express low levels of anti-apoptotic proteins.

As described in the **Introduction**, DRs activate NF-kB signalling to upregulate anti-apoptotic genes (Kreuz et al., 2001, Micheau et al., 2001, Ogura et al., 2008). In support of this, sensitisation of A549 cells to TNF-α by CHX was found to correlate with abrogation of NF-kB-dependent gene expression. Unlike TNF-α however, FasL treatment did not activate NF-kB signalling (**Figure 3.3**), so FasL-resistance in A549 cells is unlikely to be mediated by NF-kB-dependent gene expression. The contribution of Fas-mediated NF-kB activation to apoptosis resistance may vary between cell-lines: while NF-kB mediates Fas-resistance in eosinophils and T cells (Qin et al., 2002), resistance in thyroid carcinoma cell-lines is NF-kB-independent (Mitsiades et al., 2006). Since FasL did not activate NF-kB signalling in this study, resistance to FasL in A549 cells must be mediated by an additional anti-apoptotic signalling pathway. Indeed, cFLIP expression can be induced in an NF-kB-

independent manner (Duverger et al., 2004). Furthermore, FasL can activate MAPK signalling, and activation of this pathway prevents Fas-mediated apoptosis in Jurkat T cells (Holmstrom et al., 1998, Barnhart et al., 2004, Ahn et al., 2001).

4.1.2 IFN pre-treatment potentiates FasL-induced apoptosis

Both IFN-α and IFN-γ potentiated FasL-induced apoptosis in A549 cells. IFN treatment is thought to upregulate expression of pro-apoptotic ISGs such as the Fas receptor and procaspases (Tomita et al., 2003, de Veer et al., 2001, Wen et al., 1997, Lee et al., 2000b, Ahn et al., 2002, Spets et al., 1998, Xu et al., 1998). Although IFN-α and IFN-γ upregulate Fas receptor expression to similar extents in human fibrosarcoma cells (Der et al., 1998a), results obtained here indicated that IFN-γ is much more efficient than IFN-α at sensitising A549 cells to FasL-mediated apoptosis. This has been reported previously: Reinhold and colleagues demonstrated that IFN-γ, but not IFN-α, sensitised a range of melanoma cell-lines to anti-Fas agonist antibody (Ugurel et al., 1999).

Non-classical IFN signalling pathways may participate in IFN-γ/FasL synergy in some cell types: IFN-γ sensitises vascular smooth muscle cells to Fas-mediated apoptosis by stimulating Fas receptor trafficking to the cell surface in a PI3K-dependent manner (Rosner et al., 2006). Furthermore, it has been reported that IFN-γ/FasL synergy in A549 cells is JAK-dependent but STAT1-independent (Kurdi and Booz, 2007). In support of a role for PI3K/Akt in IFN-γ/FasL synergy, IFN-γ, but not IFN-α, activated Akt in A549 cells (**Figure 3.5**). However, PI3K inhibition by LY294002 treatment did not affect IFN-γ/FasL-induced apoptosis, indicating that PI3K signalling was not responsible for FasL potentiation by IFN-γ. Moreover, in direct contradiction with Kurdi and Booz, IFN-γ/FasL synergy was found to require STAT1, and as such, could be blocked by PIV5/V (**Figure 3.10**). While this study used FasL to stimulate the Fas receptor, Kurdi and Booz used an anti-Fas antibody used by Kurdi and Booz (Kurdi and Booz, 2007): FasL and anti-Fas agonist antibody have been reported to stimulate different signalling pathways (Thilenius et al., 1997). Using anti-Fas antibody may therefore not give an accurate representation of Fas-mediated apoptosis in some situations. An additional factor contributing to contradictions between this and previous work may be the different methods used to knock-down STAT1; this will be discussed later, in **Section 4.2.2**.

Neither IFN-α nor IFN-γ were able to sensitise A549 cells to TNF-α-induced apoptosis. This is despite reports that IFN and TNF-α can synergistically induce apoptosis through upregulation of the TNF-α receptor in some cell types (Frankel et al., 2006, Kimura et al., 2003, Kulkarni et al., 2006, Manna et al., 2000, Sasagawa et al., 2000, Suk et al., 2001). The ability of IFN-γ to potentiate FasL but not TNF-α-induced apoptosis in A549 cells may be due to the association of STAT1 with TNFR/TRADD, but not the Fas receptor, to inhibit TNF-α-induced NF-kB activation (Wang et al., 2000b). In support of this, IFN-γ has been reported to inhibit TNF-α-induced apoptosis in murine myoblasts by promoting NF-kB-dependent cIAP1 expression (Tolosa et al., 2005). IFN treatment limits STAT1:TRADD association by shunting STAT1 into the JAK/STAT signalling pathway, alleviating NF-kB inhibition (Pajak and Orzechowski, 2007, Wang et al., 2000b). IFN-γ treatment in A549 cells may therefore make cells more resistant to TNF-α by enhancing NF-kB activity.

The resistance of A549 cells to TNF-α and FasL-induced apoptosis, may have implications for their response to NK cell and CTL cytotoxicity during *in vivo* virus infections. Sensitisation of cells to FasL by IFN-γ suggests that IFN produced during virus infection may indirectly aid virus clearance by promoting FasL-mediated NK cell cytotoxicity. *In vivo*, IFNs can also modulate NK cytotoxicity independently of Fas/FasL pathways. NK cell cytotoxicity is augmented by IFN-α treatment through elevated perforin expression (Kaser et al., 1999, Liang et al., 2003) while IFN can render hepatocytes resistant to CTL cytotoxicity by promoting expression of granzyme B inhibitors (Willberg et al., 2007). Cytokine synergism may also be important during disease development as IFN-γ/TNF-α synergy has been implicated in the destruction of pancreatic β-cells *in vitro* and development of autoimmune diabetes *in vivo* (Suk et al., 2001, Kim et al., 2007).

4.2 Apoptosis modulation by PIV5/V

4.2.1 PIV5/V expression limits apoptosis

In A549 cells, expression of PIV5/V(w3) was found to limit apoptosis induced by FasL/CHX and TNF-α/CHX. In accordance with the pro-apoptotic functions of STAT1 (see **Introduction**), this effect was due to STAT1 depletion in A549/PIV5/V cells; PIV5/V(CPI-), which is unable to target STAT1 for proteasome-mediated degradation, was completely unable to inhibit apoptosis by the same inducers. STAT1 can inhibit expression of NF-kB-dependent pro-survival genes through a direct association with the p65 NF-kB subunit (Kramer et al., 2006). In addition, STAT1 interacts with TNFR1/TRADD to inhibit TRADD/RIP/TRAF2 complex formation and prevent downstream NF-kB activation (Wang et al., 2000b, Pajak and Orzechowski, 2007, Wesemann and Benveniste, 2003). STAT1 can also positively regulate apoptosis through enhancement of p53-dependent transcription and inhibition of Mdm2 expression (Townsend et al., 2004). Consequently, it has been suggested that STAT1 levels determine whether TNF-α treatment induces cell death or cell survival in a particular cell-line. Indeed, TNF-α treatment of STAT1-null cells led to enhanced NF-kB activation and subsequent resistance to apoptosis in previous studies (Wesemann and Benveniste, 2003, Kumar et al., 1997). While TRADD is not involved in NF-kB activation downstream of Fas and TRAIL receptors (Imamura et al., 2004), STAT1 and TRADD interact even in quiescent cells (Pajak and Orzechowski, 2007, Wesemann and Benveniste, 2003); STAT1 may therefore inhibit basal NF-kB activity even in cells treated with FasL and TRAIL. However, STAT1-dependent expression of pro-apoptotic genes was not responsible for the anti-apoptotic effect of PIV5/V(w3) here, as new protein synthesis was abrogated in cells treated with TNF-α/CHX and FasL/CHX. The role of STAT1 in FasL/CHX and TNF-α/CHX-induced apoptosis is therefore likely to involve transcription-independent mechanisms (see **Introduction**). Interestingly, a transcription-independent role for NF-kB in cell death may exist. The NF-kB2/p100 precursor contains a death domain that permits its recruitment to death receptors, where it induces apoptosis (Wang et al., 2002d, Hacker and Karin, 2002). NIK, the enzyme responsible for NF-kB2/p100 processing and removal of its death domain, is required for Fas and TNF-α-mediated NF-kB activation (Malinin et al., 1997). It follows then that STAT1 may prevent NIK activation through its interaction with TRADD; in doing so, STAT1 may prevent NF-kB2/p100 processing, resulting in accumulation of NF-kB2/p100 and apoptosis. Conversely STAT1 depletion, as seen in A549/PIV5/V(w3) cells, may increase NIK

activity, reducing NF-kB/p100 levels and preventing NF-kB2/p100-induced apoptosis. The direct association between STAT1 and p53 (Townsend et al., 2004) also hints that STAT1 may modulate p53 expression and activity by promoting its post-translational modification.

TNF-α/CHX induced similar levels of apoptosis in MRC-5 and MRC-5/PIV5/V cells. This may indicate that in MRC-5 cells, PIV5/V(w3) exerts its effects on apoptosis by modulating gene expression, not through transcription-independent mechanisms. In support of this, MRC-5 cells expressing PIV5/V(w3) were less sensitive to FasL-induced apoptosis (in the absence of CHX) than MRC-5 naïve cells. Here, NF-B activity may be increased by STAT1 depletion in MRC-5/PIV5/V(w3) cells, resulting in expression of anti-apoptotic proteins. It would be interesting to determine whether the reverse is true: STAT1 overexpression in MRC-5 cells may sensitise them to TNF-α-induced apoptosis by preventing NF-kB-dependent gene expression.

PIV5/V(w3) also limited STS-induced apoptosis in both A549 and MRC-5 cells. Since CHX treatment is not required for STS-induced cell death, this effect may be due to transcription dependent and/or transcription-independent mechanisms. Interestingly, in A549 cells, PIV5/V(CPI-) expression also reduced STS-induced apoptosis (albeit less efficiently than PIV5/V(w3); the effects of PIV5/V on STS-induced apoptosis therefore appear to be less dependent on STAT1 depletion than TNF-α/CHX and FasL/CHX. This is perhaps not surprising as STAT1 plays a predominant role in DR-mediated apoptosis due to its recruitment to TNFR/TRADD. However, STS treatment may indirectly affect STAT1-dependent transcription through inhibition of PKC-δ (Gschwendt et al., 1995, Seynaeve et al., 1994): PKC-δ is required for STAT1 serine phosphorylation (DeVries et al., 2004, Uddin et al., 2002) and its inhibition by STS may impair STAT1-dependent transcription independently of PIV5/V expression.

4.2.2 PIV5/V expression prevents IFN-γ/FasL synergy

In addition to its effects on TNF-α, FasL and STS-induced apoptosis, PIV5/V expression also inhibited IFN-γ/FasL synergy. STAT1 degradation was required for this effect, since PIV5/V(w3), but not PIV5/V(CPI-), could prevent IFN-γ/FasL-induced cell death. IFN-γ/FasL-induced apoptosis was also inhibited by MuV/V. While MuV/V targets STAT1 for degradation (Kubota et al., 2001, Yokosawa et al., 2002), MuV/V also disrupts association of STAT1 with the IFN receptors (Kubota et al., 2002); the effect of MuV/V on IFN-γ/FasL is presumably a function of these two

properties. In A549/PIV5/V(w3) and A549/MuV/V cells, IFN-γ-treatment may be unable to upregulate pro-apoptotic ISG expression due to STAT1 depletion/inactivity. This is in stark contrast to a report that JAK1 inhibition, but not STAT1 knockdown by siRNA, blocks IFN-γ/Fas-mediated apoptosis in A549 cells (Kurdi and Booz, 2007). The writers of this study proposed that the pro-apoptotic effects of IFN-γ occurs downstream of JAK1 activation via a non-canonical pathway, independent of STAT1. A possible reason for this discrepancy is the inefficiency of siRNA knockdown in the study by Kurdi et al: while siRNA reduced IFN-γ-induced STAT1 levels, basal levels of STAT1 were still detectable and may have been sufficient to activate ISG expression. Since no STAT1 could be detected in A549/PIV5/V(w3) cells following IFN-γ treatment (**Figure 3.10C**), expression of PIV5/V may represent a more efficient method for STAT1 knockdown than siRNA. Another possible reason, mentioned in **section 4.1.2**, is the differential stimulation of signalling pathways by FasL and anti-Fas agonist antibody (Thilenius et al., 1997).

Paramyxoviruses target STATs to antagonise IFN signalling (see **Section 1.3.1.2**). Apart from the observation that MuV/V promotes apoptosis in transformed cells through STAT3 degradation (Ulane et al., 2003), the contribution of STAT degradation by paramyxoviruses to apoptosis regulation has been largely unstudied until now. Results obtained in this study indicate that the ability of PIV5/V to target STAT1 for degradation appears to confer an additional advantage to the virus by limiting apoptosis induction. This occurs either through inhibition of pro-apoptotic ISG expression or through interference with STAT1 interactions with apoptosis regulators. Limiting apoptosis induction may have implications for the ability of PIV5 to establish persistent infections.

4.2.3 PIV5/V expression inhibits Akt activation

Many viruses activate PI3K/Akt signalling to prevent or delay host cell apoptosis (Bitko et al., 2007, Dawson et al., 2003, Lee et al., 2001, Zhang et al., 2002, Peters et al., 2008) and recent reports suggest that PI3K and Akt play important roles during paramyxovirus infection (Peters et al., 2008, Sun et al., 2008a). While the previous section found that PIV5/V expression could inhibit apoptosis through its effects on STAT1, it was also investigated whether PIV5/V could inhibit apoptosis through PI3K/Akt activation. Surprisingly, PIV5/V(w3) expression in A549 cells was actually found to inhibit Akt activation following IFN treatment. This supports a suggestion that V may prevent Akt activation by the L protein during PIV5 infection (Luthra et al.,

2008). In contrast to A549/PIV5/V(w3), A549/PIV5/V(CPI-) exhibited similar levels of Akt activation to naïve cells, suggesting that STAT1 degradation is required for Akt inhibition by PIV5/V. Further evidence for this was obtained during PIV5 infection: PIV5-CPI- and PIV5-VΔC, which do not cause STAT1 degradation, induced Akt activation earlier and to a greater extent than PIV5-w3 and PIV5-CPI+ (**Figure 3.17B**). PIV5/V(w3) may therefore prevent upregulation of an ISG involved in promoting Akt activation. While this study makes no attempt to identify the protein responsible for this effect, several candidate ISGs exist. The chemokine CCL5 and its receptor CCR5 are both upregulated by IFN (Kitaya et al., 2007, Plskova et al., 2006, de Veer et al., 2001): PI3K is activated downstream of CCL5/CCR5 association (Ignatov et al., 2006, Huang et al., 2008a, Tyner et al., 2005). The RhoC GTPase is also upregulated by IFN (Der et al., 1998a) and activates PI3K/Akt (Ruth et al., 2006), presumably through direct interaction with the p85 subunit of PI3K in the same manner as other Rho family members (Tolias et al., 1995, Zheng et al., 1994b).

While no evidence for V:Akt association was obtained in this study, such an interaction has been reported by He and colleagues (Sun et al., 2008a). They proposed an unsubstantiated model whereby V interacts with Akt to limit Akt-dependent NF-kB activation by the PIV5 L protein (**Figure 4.1**, Luthra et al., 2008). V may therefore inhibit Akt activation by two distinct mechanisms: by preventing production of an Akt-activating ISG and through direct association with Akt. Indeed, PIV5/V(w3) expression reduced phospho-Akt levels even in the absence of IFN treatment; while this may represent inhibition of constitutive STAT1-dependent gene expression (see **Introduction**), it could also be due to direct inhibition of Akt. It would be interesting to determine which parts (if any) of V are responsible for Akt binding, and whether a hierarchy of Akt, mda-5 and STAT1 inhibition functions exists: interaction with Akt may render V unable to bind STAT2, DBB1 or mda-5. If a direct interaction is required for Akt inhibition, this interaction presumably requires amino acid residues in its N-terminus since V(w3) and V(CPI-) differ by six amino acids in their N-termini. Further experiments are also required to determine whether V can inhibit Akt-dependent NF-kB activation.

It is interesting to note that inhibition of Akt activation by PIV5/V did not promote apoptosis since PIV5/V expression actually limited apoptosis induced by a range of treatments. This indicates that viruses may modulate PI3K/Akt signalling for apoptosis-independent reasons. For example, IL-6 expression and release, which is regulated by PI3K/Akt, is blocked by PIV5/V (Lee et al., 2006, Qian et al., 2009, Tang

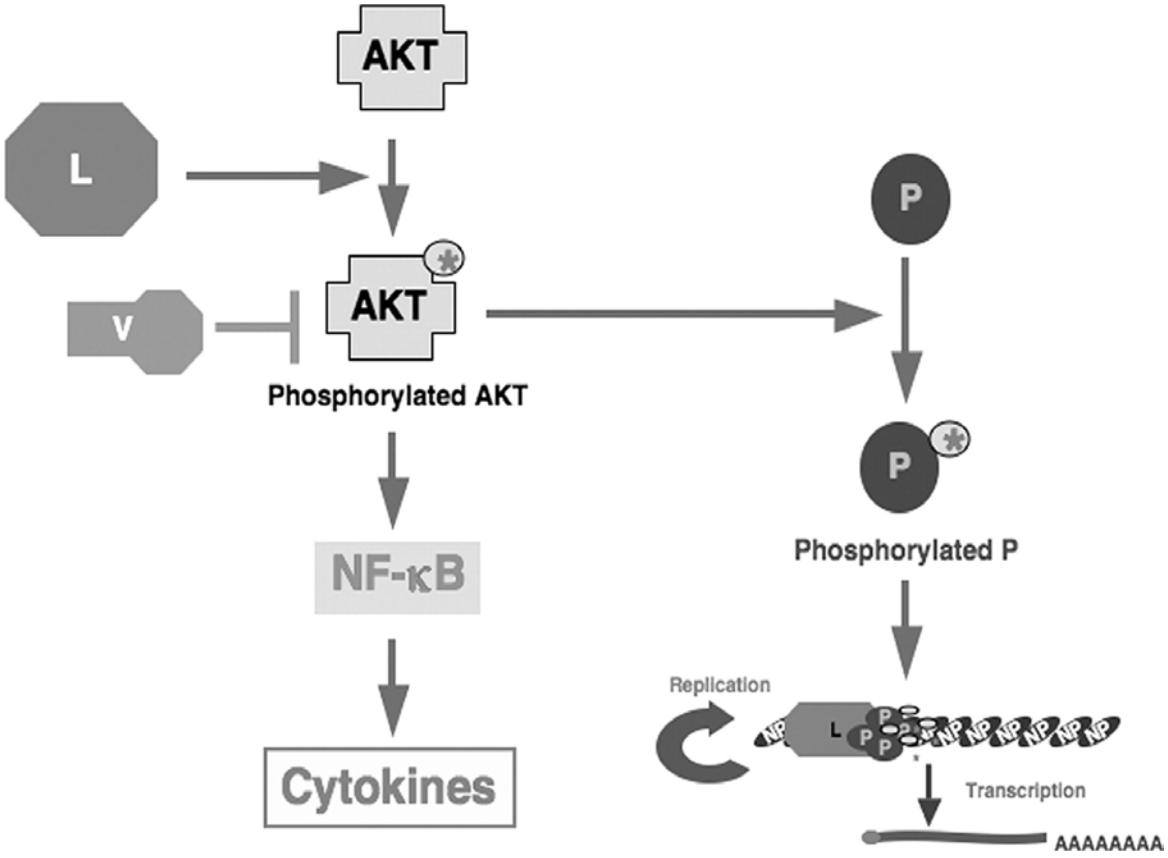


Figure 4.1: Model for activation of AKT in PIV5-infected cells and the roles of AKT in PIV5 replication.

AKT plays a critical role in phosphorylation of P, which is an essential component of the viral polmerase. Luthra et al. proposed that L activates AKT by causing phosphorylation of AKT. Activated AKT contributes to the activation of the expression of cytokines such as IFN-β and IL-6 as well as phosphorylation of P. Since V blocks expression of IFN-β and IL-6 and reportedly interacts with AKT, they speculate that V can block the activation of NF- B through its interaction with AKT. *Modified from Luthra et al., 2008.*

et al., 2007a, Young et al., 2006). V expression also slows the cell cycle (Lin and Lamb, 2000); this may occur through Akt inhibition since Akt promotes cell cycle progression in a number of ways (see **Introduction**). In addition, Akt and downstream mTORC1 are also required for enhancement of ISG translation (Kaur et al., 2008a, Kaur et al., 2008b, Lekmine et al., 2003): while V enhances host and viral protein synthesis by limiting PKR activation (Gainey et al., 2008a), V-mediated Akt inhibition may limit ISG expression to antagonise the IFN response.

4.2.4 Induction of IFN and apoptosis during PIV5 infection

The PIV5-w3, PIV5-CPI+, PIV5-CPI- and rPIV5-VΔC strains of PIV5, which express V proteins with different biological properties, differed in their ability to induce IFN. rPIV5-VΔC was the best inducer while PIV5-w3 was the poorest, in accordance with a previous study (Poole et al., 2002). PIV5-CPI+ and PIV5-CPI- viruses induced significant levels of IFN in infected cells, despite the ability of their V proteins to inhibit mda-5 activity (Childs et al., 2007, Andrejeva et al., 2004, Poole et al., 2002). This may be explained by reports that paramyxoviruses activate RIG-I, but not mda-5 signalling (Kato et al., 2006, Loo et al., 2008); since PIV5 is unable to block RIG-I signalling (Andrejeva et al., 2004, Childs et al., 2007), these viruses will still induce IFN. One obvious question is raised by these observations: why do paramyxoviruses actively inhibit mda-5 activity if paramyxovirus infection predominantly signals through RIG-I? It may be that PIV5 activates both mda-5 and RIG-I, but only RIG-I activity can be detected in infected cells due to mda-5 inhibition by V. In support of this, rPIV5-VΔC, whose V protein is unable to inhibit mda-5 signalling due to a C-terminal truncation (Poole et al., 2002, He et al., 2002a), induces large amounts of IFN: rPIV5-VΔC may be activating both RIG-I and mda-5 signalling. Comparisons of IFN induction by different virus strains may additionally be complicated by the presence of defective interfering (DI) particles in virus stocks. DIs lack one or more functions required for genome replication or synthesis/assembly of virus particles and can therefore replicate only in the presence of a replication-competent (“helper”) virus. DIs are potent inducers of IFN and apoptosis, presumably because they are able to efficiently produce the inducer of IFN/apoptosis but are unable to antagonise these responses (Johnston, 1981, Sekellick and Marcus, 1982, Aoki et al., 2001, Strahle et al., 2006, Fuller and Marcus, 1980b, Fuller and Marcus, 1980a, Strahle et al., 2007). DIs are difficult to correct for, as virus titres obtained by plaque assay are representative of the number of non-defective (plaque-forming) particles, but not DIs. Estimates of DI numbers in a particular virus stock can be obtained from the ratio of

haemagglutinin titre to plaque-forming units; however, in this experiment the levels of DIs in virus stocks were unknown. The large amounts of IFN induced by rPIV5-VΔC could therefore be due to the presence of large amounts of DIs.

PIV5-CPI-, but not PIV5-CPI+, induced significant amounts of apoptosis in A549 cells. While PIV5-CPI- and PIV5-CPI+ induced similar levels of IFN, PIV5-CPI- is unable to block STAT1 signalling, so IFN produced during PIV5-CPI- infection can upregulate pro-apoptotic genes and promote apoptosis. Accordingly, in A549/BVDV/NPro and A549/PIV5/V cells, which are unable to produce or respond to IFN respectively, PIV5-CPI- and PIV5-CPI+ viruses cause a similar amount of cell death. Somewhat surprisingly, rPIV5-VΔC, which is reported to be apoptogenic (Sun et al., 2004), did not cause cell death in A549 naïve cells, despite inducing large amounts of IFN. In IFN-defective A549/BVDV/NPro and A549/PIV5/V cells however, rPIV5-VΔC induced significant apoptosis. These results suggest that the large amount of IFN produced during rPIV5-VΔC infection induced an antiviral state in naïve cells: rPIV5-VΔC is unable to antagonise the IFN response, so the virus is unable to replicate efficiently and is unable to cause cell death. In contrast, the antiviral state is not established in A549/BVDV/NPro and A549/PIV5/V cells so rPIV5-VΔC can replicate efficiently, resulting in apoptosis induction. The ability of PIV5 to induce cell death therefore seems to depend on the balance between limitation of virus replication by the host cell, and the ability of the virus to circumvent host cell responses to enhance its replication. Since A549/BVDV/NPro cells are IRF3-deficient, rPIV5-VΔC-induced cell death did not require IRF3; this is a surprising result since IRF3 is a requirement for apoptosis induced by dsRNA, reovirus, Bunyamwera virus, NDV and SeV (Heylbroeck et al., 2000, Peters et al., 2008, Weaver et al., 2001, McAllister and Samuel, 2008, Kohl et al., 2003, Holm et al., 2007). This indicates the existence of IRF3-independent pathways of apoptosis induction by PIV5. IRF3-independent modes of apoptosis induction may also be activated by infection with other viruses: while Bunyamwera virus is still able to induce significant cell death in cells with low levels of IRF3 expression (Kohl et al., 2003).

PIV5-w3 was unable to induce apoptosis in IFN-competent or IFN-compromised cells; this indicates that this virus may not produce significant amounts of the viral inducer of apoptosis. Work by Parks and colleagues revealed that the 6 amino acid differences between the shared P/V N-terminus of PIV5-w3 and PIV5-CPI- are responsible for differences in viral RNA production and subsequent PKR activation

(Gainey et al., 2008a). During PIV5-w3 infection, Gainey et al. suggest a model by which P/V gene products decrease activity and increase fidelity of the viral polymerase, thereby limiting production of aberrant viral RNA to prevent activation of antiviral responses. In contrast, the P/V gene products from PIV5-CPI- are unable to do this, so PIV5-CPI- induces IFN and apoptosis (Gainey et al., 2008a). In fact, the three amino acid differences between the PIV5-w3 and PIV5-CPI+ P/V proteins are sufficient to affect RNA production by these viruses (Timani et al., 2008). Both PIV5-CPI+ and PIV5-CPI- are therefore unable to limit viral RNA synthesis. This would go some way to explaining why PIV5-CPI+ and PIV5-CPI- induce similar levels of IFN and apoptosis and why both viruses induce more IFN/apoptosis than PIV5-w3 (**Figures 3.12 and 3.13**). Interestingly though, ectopic expression of V(w3) could only partially prevent PIV5-CPI- or PIV5-CPI+ induced cell death in this study. This may indicate that while V(w3) expression levels in A549/PIV5/V are sufficient to deplete STAT1, they are insufficient to limit viral RNA production by PIV5-CPI- and PIV5-CPI+. In conclusion, there may be at least four factors dictating induction of IFN and apoptosis by PIV5 infection: the ability of the V protein to target STAT1 for degradation, the ability of V to bind and inhibit mda-5, the numbers of DIs in virus stocks and the ability of V to limit viral RNA production.

Despite observations that PIV5/V(w3) can inhibit apoptosis when stably expressed in cell-lines, apoptosis induction by FasL/CHX, TNF-α/CHX and STS was not significantly affected in PIV5-infected cells. PIV5-CPI- and PIV5-VΔC-infected cells did not go into apoptosis faster with these inducers than cells infected PIV5-w3 and PIV5-CPI+ (**Figure 3.14**), despite differences in STAT1 expression between these cells. IFN induced during virus infection may be affecting apoptosis induction, so it would be interesting to repeat this experiment in IFN-deficient cells.

4.2.5 An additional role for mda-5 inhibition by PIV5/V?

In addition to limiting IFN induction, inhibition of mda-5 by paramyxovirus V proteins may have additional, as yet uncharacterised effects in the host cell. mda-5 was first identified as a tumour-suppressor and may play an important role in apoptosis regulation (Kovacsovics et al., 2002, Cocude et al., 2003, Kang et al., 2002). During apoptosis, mda-5 is cleaved by caspases; this results in liberation of the C-terminal helicase domain from the N-terminal CARDs (the presence of CARDs may also be an indicator of role for mda-5 in apoptosis). The helicase domain subsequently translocates to the nucleus, where it accelerates apoptosis-induced DNA degradation

(Kovacsovics et al., 2002, Barral et al., 2007). MAVS, the mda-5 downstream interactor (Kawai et al., 2005), is also involved in apoptosis; it acts downstream of RIG-I to promote IRF3-mediated apoptosis during virus infection (Heylbroeck et al., 2000, Weaver et al., 2001, Rintahaka et al., 2008, McAllister and Samuel, 2008, Holm et al., 2007, Zhang and Samuel, 2008). Since mda-5 also activates IRF3, mda-5 and RIG-I may perform similar but non-redundant roles in promoting IRF3-dependent apoptosis in response to infection with different types of viruses. In addition, the mda-5/MAVS complex recruits FADD and RIP1 to induce IFN (Balachandran et al., 2004, Michallet et al., 2008); FADD and RIP1 may activate apoptotic pathways through DISC formation and activation of the caspase cascade. It would be of interest to determine whether mda-5 inhibition by paramyxovirus V proteins also has implications for host cell apoptosis induction. Preliminary experiments reveal that PIV5-CPI-, which inhibits mda-5 but is unable to promote STAT1 degradation, prevents mda-5 cleavage during apoptosis, but this may be due to inhibition of caspase-activation rather than a direct effect of V:mda-5 interaction (**data not shown**). The effects of V expression and mda-5 inhibition on dsRNA/virus-induced apoptosis remain to be investigated.

4.3 Effects of BVDV/NPro and HCV/NS3/4A on apoptosis

In addition to PIV5/V, the effects of the viral IFN antagonists BVDV/NPro and HCV/NS3/4A on apoptosis induction were investigated in this study. Ectopic expression of BVDV/NPro rendered A549 and MRC-5 cells IRF3 deficient but this was found to have no effect on TNF-α, FasL or STS-induced apoptosis. This is somewhat surprising since IRF3 is well-described as a positive regulator of apoptosis (Holm et al., 2007, McAllister and Samuel, 2008, Peters et al., 2008). However, PIV5-CPI- and rUd-184-8(P), viruses that cause significant amounts of apoptosis in naïve A549 cells, are unable to cause apoptosis in A549/BVDV/NPro. IRF3 therefore appears to be required for induction of apoptosis during virus infection: this is supported by previous studies which suggest that IRF3 acts downstream of RIG-I/MAVS to stimulate apoptosis in response to viral RNA (McAllister and Samuel, 2008, Holm et al., 2007, Peters et al., 2008, Weaver et al., 2001, Heylbroeck et al., 2000, Kohl et al., 2003). Expression of BVDV/NPro presumably inhibits dsRNA- and virus-induced apoptosis, but is unable to affect cell death following treatment with other inducers.

HCV/NS3/4A clips MAVS away from the outer mitochondrial membrane and prevents MAVS oligomerisation to inhibit IFN induction (Baril et al., 2009, Li et al., 2005b, Foy et al., 2005). Like IRF3, MAVS is important in IFN-independent induction of apoptosis by virus infection (Heylbroeck et al., 2000, Weaver et al., 2001, Rintahaka et al., 2008, McAllister and Samuel, 2008, Holm et al., 2007, Zhang and Samuel, 2008). NS3/4A has been reported to both promote and inhibit apoptosis (Nomura-Takigawa et al., 2006, Fujita et al., 1996b, Tanaka et al., 2006, Deng et al., 2006, Ishido and Hotta, 1998). In this study, NS3/4A expression neither promoted nor inhibited FasL/CHX or STS-induced apoptosis in A549 cells but did reduce cell-death in response to TNF-α/CHX treatment. This supports a report that hepatocytes stably expressing NS3/4A were resistant to TNF-α, but not Fas-mediated apoptosis (Frelin et al., 2006). Like BVDV/NPro, HCV/NS3/4A may play a more predominant role in inhibiting IRF3-mediated apoptosis in response to dsRNA and virus infection.

4.4 PI3K activation by IAV/NS1

4.4.1 Kinetics of PI3K/Akt activation in IAV-infected cells

The kinetics of PI3K/Akt during IAV infection varied considerably according to cell-type. While IAV infection activated Akt during the first 8-12hpi in all cell-types tested, Akt activation lasted for different lengths of time. In canine kidney MDCK (widely used in IAV research) and Vero (simian kidney) cells, activation of Akt was extremely transient while A549, MRC-5 and HFFF cells displayed prolonged Akt activation. The reasons for these differences are unknown, but may represent varying virus replication rates between cell-types. Alternatively, it may reflect apoptosis induction: apoptosis induces caspase-mediated cleavage and inactivation of Akt (Asselin et al., 2001, Jahani-Asl et al., 2007, Rokudai et al., 2000). MDCK cells go into apoptosis rapidly following IAV infection (destruction of the monolayer is seen by 24hpi) while MRC-5 cells do not die rapidly during IAV infection (**Figure 3.23**). A positive feedback loop may exist: phosphorylated (active) Akt is more resistant to caspase cleavage than unphosphorylated (inactive) Akt (Jahani-Asl et al., 2007) so may be able to prolong cell-survival thus preventing further caspase cleavage. However, a role for PI3K activation in delaying apoptosis is refuted by other data obtained in this study.

Because accurate, direct measurement of PI3K activity is technically demanding (Hawkins et al., 2006), many studies, including this one, rely on phosphorylation of Akt as a marker of PI3K activation. The kinetics of PI3K activation during IAV infection may differ from those indicated by Ser473 phosphorylation of Akt. For instance, there may be a significant temporal delay between PI3K and Akt activation, and additional cellular factors may be involved in prolonging/shortening the period of Akt phosphorylation. In addition, Akt can be activated independently of PI3K following insulin receptor activation (Luan et al., 2009). Besides Ser473 (the marker used in this study), there also appears to be plethora of other modifications that affect Akt catalytic activity. For instance, Akt is phosphorylated at Thr308 and activated in a PI3K-dependent manner (Walker et al., 1998). In addition, tyrosine phosphorylation of Akt, at Tyr474 (and possibly also Tyr315 and Tyr326), is also required for its catalytic activity (Chen et al., 2001b, Conus et al., 2002). These tyrosine modifications are mediated by Src kinases in a manner that may or may not be PI3K-independent (Chen et al., 2001b, Conus et al., 2002). There appears to be crosstalk between these modifications, as mutation of either Ser473 or Tyr474 markedly reduces Thr308 phosphorylation and kinase activity (Conus et al., 2002). While

Thr308 phosphorylation of Akt during IAV infection has previously been shown (Hale, 2007), tyrosine phosphorylation of Akt downstream of the NS1:PI3K interaction has not yet been demonstrated. To further confuse the issue, Akt can also regulate its own activity through autophosphorylation at Thr72 and Ser246: mutation of these sites significantly reduces its kinase activity (Li et al., 2006c). Akt activation may therefore occur during IAV infection independently of NS1-mediated PI3K activation, and Akt catalytic activity can not necessarily be inferred by Ser473 phosphorylation detection alone.

4.4.2 NS1-mediated PI3K activation and host cell apoptosis

Results obtained in this study indicated that PI3K activation during IAV infection does not mediate anti-apoptotic signalling. While introduction of the Y89F mutation into both Udorn and WSN backgrounds rendered IAV unable to bind PI3K and activate Akt (**Figure 3.19**, Hale, 2007, Hale et al., 2006), infection of epithelial and fibroblast cell-lines with these mutant viruses did not cause significantly more apoptosis than wt virus. In fact, neither wt nor Y89F viruses were able to cause rapid apoptosis in A549 and MRC-5 cells: despite being extremely sensitive to apoptosis inducers, a significant proportion of MRC-5 cells were still viable up to 60hpi. Additionally, even though PI3K and Akt are reported to mediate their anti-apoptotic roles, at least in part, through activation of NF-kB (Bortul et al., 2003, Reddy et al., 2000, Suzuki et al., 2008), no difference in activation of an NF-kB reporter system by rUd and rUd-Y89F could be detected (**Figure 3.31**).

These findings contradict several previously-published studies which assign PI3K activation by NS1 an anti-apoptotic role during IAV infection (Ehrhardt et al., 2007, Shin et al., 2007a, Zhirnov and Klenk, 2007). A number of factors may contribute to these differences. Previous work has focused on apoptotic responses of MDCK and CV-1 cells (Ehrhardt et al., 2007, Shin et al., 2007a, Zhirnov and Klenk, 2007), while experiments here were carried out in A459 and MRC-5 cells which are human alveolar epithelial and lung fibroblast cells respectively, and may more accurately reflect IAV infection *in vivo*.

Zhirnov and Klenk demonstrated that infection with a delNS1 virus caused significantly more apoptosis than wt virus and attributed this effect to Akt activation (Zhirnov and Klenk, 2007). However, given the multifunctional nature of NS1, knocking out the entire NS1 protein may have a number of effects on apoptosis that

are independent of its PI3K activation function. In contrast, the rUd-Y89F virus appears to be a much more specific tool for studying the role of PI3K activation during IAV infection since other functions of NS1 appear to be intact (Hale, 2007).

Two studies report that pharmacological inhibition of PI3K in IAV-infected cells using LY294002 correlated with an increase in PARP cleavage and caspase-9 activation (Zhirnov and Klenk, 2007, Ehrhardt et al., 2007). However, LY294002 is widely regarded as a promiscuous inhibitor and directly affects activity of a significant number of other kinases, including the PI3K downstream targets mTOR and GSK-3, at the concentrations required to inhibit PI3K (Bain et al., 2007, Brunn et al., 1996, Gharbi et al., 2007, Davies et al., 2000). Moreover, one study has expressly recommended that LY294002 no longer be used in PI3K cell-based assays (Bain et al., 2007). Additionally, LY294002 and LY303511 (a structural analogue of LY294002 that is unable to inhibit PI3K (Ding et al., 1995, Vlahos et al., 1994)) have been demonstrated to inhibit NF-kB activity, cytokine induction and cell proliferation independently of its effects on PI3K, and its use has previously given misleading results in innate immunity studies (Kim et al., 2005, Hazeki et al., 2006, Kristof et al., 2005). LY294002 and LY303511 can also promote apoptosis induced by chemotherapeutic agents by enhancing H_2O_2 production (Poh and Pervaiz, 2005) and sensitise cells to TRAIL-mediated apoptosis by inducing DR oligomerisation (Poh et al., 2007). Receptor multimerisation is also required for sensitisation to Fas-mediated apoptosis (Stel et al., 2007), so LY294022 may sensitise cells to other DR ligands, independently of its effects on PI3K. In support of this, **Figure 3.26** demonstrated that LY294002 treatment of rUd-Y89F-infected cells (in which PI3K/Akt is not activated) induced more apoptosis than either rUd-Y89F infection or LY294002 alone. Wortmannin and PI-103 are alternative commercially available PI3K inhibitors, but while they are more specific for PI3K than LY294002, these inhibitors also have non-PI3K targets (Bain et al., 2007, Fan et al., 2006).

Pi3K/Akt activation has previously been shown to protect cells from FasL (Ivanov et al., 2002), anti-Fas antibody (Hatano and Brenner, 2001, Moumen et al., 2007, Osaki et al., 2004), TNF-α (Pastorino et al., 1999, Burow et al., 2000, Hatano and Brenner, 2001, Imose et al., 2003) TNF-α/CHX (Suzuki et al.), TRAIL (Oka et al., 2006, Alladina et al., 2005, Lane et al., 2007) and STS treatment (Wan et al., 2002, Goswami et al., 1999, Liu et al., 2008a, Shao et al., 2008, Wang and Damania, 2008). In addition, Ehrhardt et al. reported that transfection of NS1 into MDCK cells limited STS-induced apoptosis. They concluded that PI3K/Akt activation was

responsible for this effect because Akt activation was observed in NS1-expressing cells. However, the multi-functional nature of NS1 means that apoptosis could be affected independently of Akt. In this study, activation of PI3K by NS1 was found not to protect cells from non-viral apoptosis inducers (**Figures 3.22 and 3.25**). In direct contradiction with Ehrhardt et al., stable NS1 expression in A549 cells did not render them more resistant to apoptosis induced by STS. TNF-α/CHX and FasL/CHX-induced apoptosis was similarly unaffected. Since NS1 expression levels were lower in these cells than during IAV infection (**data not shown**), high amounts of NS1 may be required to block apoptosis. However, rUd infection was also unable to protect A549 or MRC-5 cells from FasL, TNF-α or STS-induced apoptosis. Moreover, there was no difference between rUd wt and rUd-Y89F-infected cells in sensitivity to these apoptotic stimuli. These data indicate that the primary function of PI3K activation during IAV infection is not to protect infected cells from apoptosis.

Interestingly, infection with either rUd wt or rUd-Y89F sensitised cells to FasL- and TNF-α-induced apoptosis. This effect is probably due to global inhibition of protein synthesis by IAV; NS1 has been shown to inhibit cellular pre-mRNA processing and nuclear export (Fortes et al., 1994, Qiu and Krug, 1994). The ability of IAV to inhibit translation therefore appears to have disadvantages to the virus: infected cells may become more sensitive to the cytotoxic effects of NK cells and CTLs due to down-regulation of anti-apoptotic proteins. A549 cells stably expressing PR8/NS1 or PR8/NS1-Y89F were not sensitised to FasL and TNF-α however: PR8/NS1 is unable to bind CPSF30 to inhibit cellular mRNA processing (Das et al., 2008, Kochs et al., 2007) In addition, other viral proteins may be more important than NS1 for protein synthesis inhibition; for example, the viral polymerase interacts directly with cellular RNA polymerase II (Engelhardt et al., 2005), possibly to promote its degradation (Rodriguez et al., 2007).

4.4.3 NS1-mediated PI3K activation and p53

p53 activation by Ser15-phosphorylation was observed at late times during rUd wt infection, and appeared to correlate with a decrease in Akt phosphorylation. This is consistent with previous studies that report p53 activation by IAV (Technau-Ihling et al., 2001, Turpin et al., 2005, Zhirnov and Klenk, 2007). Zhirnov and Klenk demonstrated similar kinetics of Akt and p53 activation, and in addition, demonstrated that a delNS1 virus was unable to activate p53 (Zhirnov and Klenk, 2007). This indicated that NS1 is the viral factor responsible for p53 activation and

implied that activation of Akt and p53 were inversely linked. However, in this study, p53 activation was also seen during infection with rUd-Y89F, indicating that this is not the case: NS1 appears to activate PI3K/Akt and p53 independently. Indeed, an interaction between NS1 and p53 has been reported (Luke et al., 2005). This said however, PI3K/Akt activation by NS1 regulates PI3K-independent NS1-mediated p53 expression and activation. rUd-Y89F-infected cells exhibited increased levels of p53 expression than rUd wt-infected cells (**Figure 3.27**), consistent with PI3K/Akt-mediated down-regulation of p53 gene transcription (Chun-gen Xing, 2008) and stabilisation of the p53 inhibitor Mdm2 (Feng et al., 2004, Mayo and Donner, 2001, Ogawara et al., 2002). In addition, p53 phosphorylation at Ser15 was more pronounced at 24hpi following rUd-Y89F infection than with rUd wt. While this effect may be due to an increase in total p53 levels, it may also represent an ability of PI3K/Akt to limit p53 activation. Indeed, PI3K has been shown to inhibit p53 activation by inhibiting its phosphorylation at Ser6 (Filiz et al., 2008). The ability of NS1 to limit p53 activity and expression through PI3K activation does not necessarily correlate with cell survival however. Apoptosis induced by delNS1 virus is not accompanied by significant p53 activation, and experiments in human lung cells with a regulated p53 gene demonstrated that IAV-induced cell death is independent of p53 expression (Zhirnov and Klenk, 2007). The authors suggested that p53 activation observed late in IAV infection is instead a secondary product of p53-independent apoptotic events.

4.4.4 NS1-mediated PI3K activation may prevent apoptosis in neutrophils

IAV-infected cells are susceptible to phagocytosis by neutrophils and macrophages *in vitro* and *in vivo;* phagocytosis inhibits virus replication and aids IAV clearance (Fujimoto et al., 2000, Shiratsuchi et al., 2000, Hashimoto et al., 2007, Watanabe et al., 2002, Watanabe et al., 2004, Tate et al., 2008, Fujisawa et al., 1987, Fujisawa, 2001). Phagocytic responses may be important in determining IAV pathogenicity as inhibition of phagocytosis augments mortality in IAV-infected mice (Watanabe et al., 2005b) and a moderately virulent IAV strain becomes severely virulent in the absence of an efficient neutrophil response (Tate et al., 2008). Moreover, infection with highly pathogenic H5N1 and 1918 pandemic strains is associated with rapid and excessive neutrophil and alveolar macrophage recruitment to the lungs (Tumpey et al., 2005, Perrone et al., 2008). It has been suggested that neutrophils function as a vehicle for IAV replication and systemic spread (Zhao et al., 2008, Ratcliffe et al., 1992). Furthermore, PI3K plays an important role in promoting neutrophil survival in

response to granulocyte-macrophage colony stimulating factor (GM-SCF), TLR ligands and hypoxia, through upregulation of Mcl-1 expression (Leuenroth et al., 2000, Al-Shami et al., 1997, Al-Shami and Naccache, 1999, Yasui et al., 2002, Epling-Burnette et al., 2001, Klein et al., 2000, Francois et al., 2005). IAV may activate PI3K to prolong neutrophil survival, thereby promoting virus replication and dissemination. NS1 from the 1918 strain of IAV hyperactivates PI3K through an interaction with Crk/CrkL (Heikkinen et al., 2008). It is possible then that excessive PI3K activation (and subsequent neutrophil survival and chemotaxis) is responsible for the virulence of these strains and severity of disease symptoms in humans.

While rUd wt infection in neutrophils induced apoptosis (in agreement with previous reports: Engelich et al., 2001, Colamussi et al., 1999), rUd-Y89F induced significantly more apoptosis than rUd wt. These data support a role for NS1-mediated PI3K activation in neutrophil survival. However, these results should be considered preliminary, as PI3K activation by these two viruses has not yet been investigated in neutrophils. In addition, it has been suggested IAV infection in neutrophils is abortive and that these cells are unable to produce infectious virus (Cassidy et al., 1988), so further studies are required to examine IAV replication and PI3K/Akt activation in neutrophils.

4.4.5 NS1-mediated PI3K activation does not modulate the IFN response

PI3K is required for complete IRF3 phosphorylation and maximal IRF3 transcriptional activity (Ehrhardt et al., 2006, Sarkar et al., 2004). In addition, PI3K can negatively regulate IFN induction by inhibiting NF-kB (Aksoy et al., 2005) and p53 activation promotes IFN secretion from virus-infected cells (Munoz-Fontela et al., 2008). However, NS1-mediated PI3K activation does not appear to be involved in modulation of IFN induction in A549 cells since rUd wt and rUd-Y89F-infected cells produced similar levels of IFN in this study. Additionally, there was no difference in NF-kB activation in rUd wt or rUd-Y89F-infected cells. Since PI3K-mediated inhibition of NF-kB activity and IFN-β expression has so far only been observed in DCs (Aksoy et al., 2005), NS1 may preferentially inhibit IFN production in these cells. DCs are potent inducers of IFN-α/β expression during IAV infection (Cella et al., 1999, Feldman et al., 1994) and NS1 could activate PI3K in these cells to efficiently limit initiation of the IFN response *in vivo*.

NS1-mediated PI3K activation was not able to regulate ISRE activation or ISG15 expression (**Figures 3.32 and 3.33**). This is in accordance with previous reports that PI3K inhibition is unable to affect type I IFN induced gene transcription (Thyrell et al., 2004, Hjortsberg et al., 2007, Uddin et al., 1997). Indeed, rUd-Y89F is attenuated in MDCK cells that are unable to respond to IFN (Hale, 2007), so the facilitatory role of PI3K in virus replication is independent of IFN signalling, at least in MDCK cells. However, while PI3K activation by NS1 does not affect 'classical' IFN signalling, it may promote expression of a subset of ISGs independently of the ISRE. It has recently been reported that PI3K/Akt activity is required for STAT1 phosphorylation and STAT1-dependent expression of the chemokine CCL5 in A549 cells infected with IAV (Huang et al., 2008b). The authors did not specify whether tyrosine or serine phosphorylation was affected, but type I IFNs can induce CCL5 gene transcription through a STAT1-dependent, but ISGF3-independent, pathway (Cremer et al., 2002), suggesting that PI3K/Akt may promote STAT1 serine phosphorylation. In support of this, PI3K/Akt promotes PKC-δ-mediated STAT1 serine phosphorylation in response to IFN-γ (Nguyen et al., 2001, Deb et al., 2003, Uddin et al., 2002). Additional work is required to determine whether PI3K activation by NS1 can promote transcription from non-ISRE STAT1-responsive promoters.

4.4.6 Mutations in NS1 alter IFN and apoptosis induction by IAV

While rUd-Y89F did not to cause more apoptosis than wt virus, recombinant IAVs in which the entire NS1 protein has been deleted (delNS1 viruses) are reported to induce large amounts of apoptosis (Zhirnov et al., 2002, Zhirnov and Klenk, 2007, Stasakova et al., 2005). While efforts to recover a rUd-delNS1 virus have been unsuccessful, a range of other rUd NS1 mutants have been produced in our laboratory. These viruses express NS1 mutations reported to interfere with binding to dsRNA and the cellular proteins PI3K, PKR and CPSF30. In addition, a virus expressing NS1 with a C-terminal truncation was isolated; this virus lacks most of the NS1 effector domain. Cell viability studies of cells infected with these mutant viruses revealed that two were notable in their induction of A549 cell death: rUd-Δ99 and rUd-184-8(P) (**Figure 3.36 and 3.37**). Induction of apoptosis appeared to be inversely correlated with NS1 stability: rUd-184-8(P)-infected A549 and MRC-5 cells only express low levels of NS1 protein. Similarly, NS1 was not detected in rUd-Δ99-infected cells. Further studies carried out by D. Jackson revealed expression levels of other virus proteins were similar between rUd-Δ99-, rUd-184-8(P)- and rUd wt-infected cells: NS1 expressed by rUd-Δ99 and rUd-184-8(P) is therefore likely to be

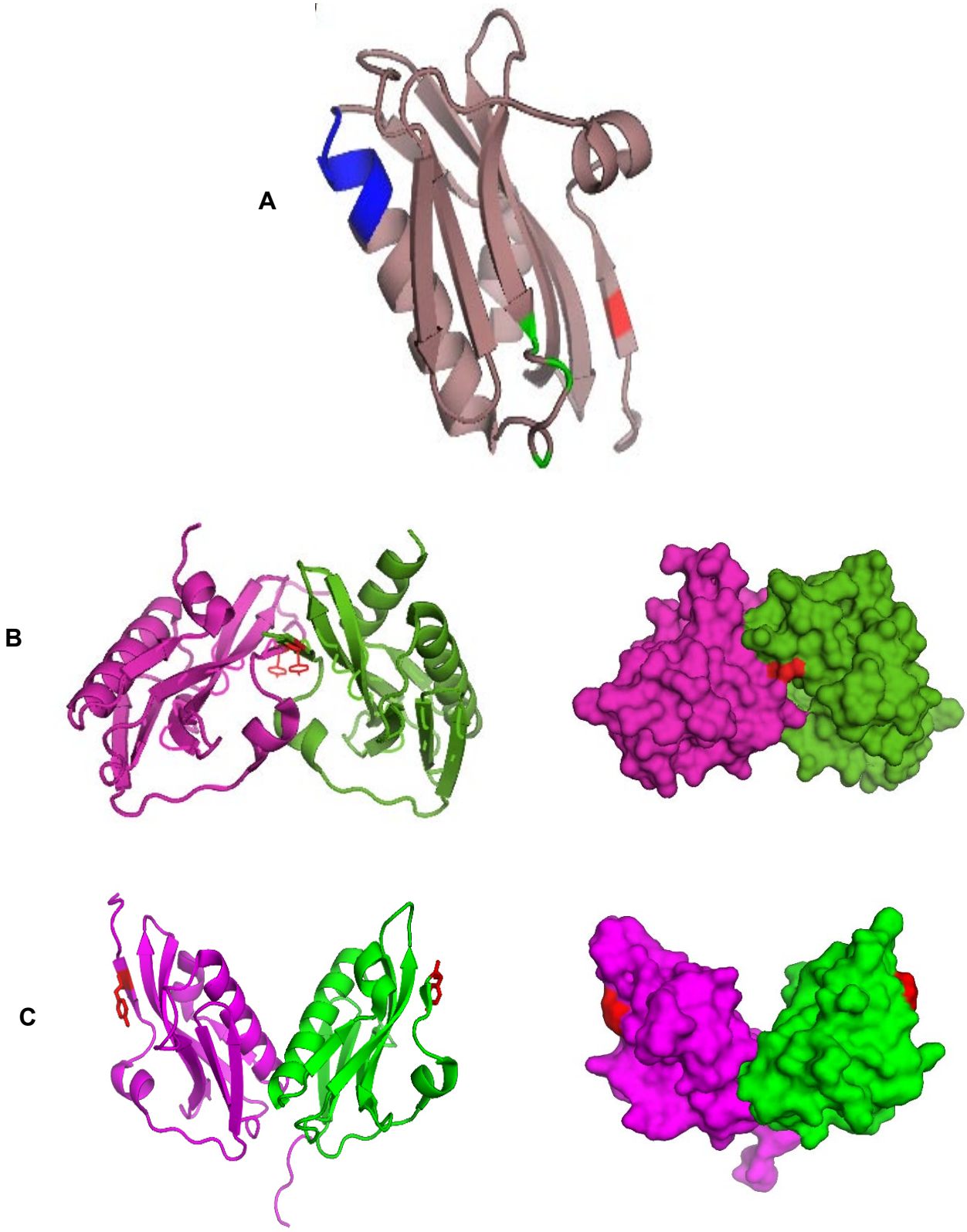


Figure 4.2: The position of mutated residues in the NS1 effector domain monomer and dimer structures.

(A) The monomer structure of the NS1 effector domain. Tyr89 is highlighted in red, Pro162, Pro164 and Pro167 are highlighted in green and residues 184-188 are shown in blue. Two proposed dimer structures of the NS1 effector domain are shown in (B) and (C). The monomers in each dimer are coloured magenta and green. Both cartoon and surface representations are shown, with Tyr89 highlighted in red. *Images were prepared with PyMol (Protein Data Bank files 3D6R (A & C) and 2GX9 (B).*

unstable in the cell. Neither rUd-Δ99 nor rUd-184-8(P) infection activated PI3K/Akt. However, this was not the reason for apoptosis induction by these viruses, as activation of PI3K by serum was unable to prevent apoptosis in rUd-184-8(P)-infected cells (**Figure 3.38**).

The rUd-P164/7A virus, in which residues that are also reported to be important for PI3K binding are mutated (Shin et al., 2007a), did not induce significant cell death. This provides further evidence that NS1-mediated PI3K activation does not perform an anti-apoptotic role. These results contradict an observation by Shin et al. that a PR8 virus carrying the P164/7A mutation was much more apoptogenic than wt (Shin et al., 2007a). While this contradiction could be explained by the different virus backgrounds affecting the P164/7A phenotype, closer analysis of the primer sequences used by this group revealed that they may have generated a with mutations of three (P162/4/7A), rather than two (P164/7A), proline residues. To investigate this hypothesis, a rUd-P162/4/7A virus was also produced: this virus induced more cell death, caspase activation and nuclear fragmentation than both wt and rUd-P164/7A in all three cell-lines tested (**Figures 3.36 and 3.37; data not shown**). In addition, while Shin et al. reported that PR8-P164/7A exhibited attenuated growth characteristics, rUd-P164/7A displays a plaque phenotype very similar to wt virus. In contrast, rUd-P162/4/7A gave a small plaque phenotype when titrated on MDCK cells (D. Jackson, unpublished observations). These data indicate that the apoptogenic PR8 virus produced by Shin et al. likely expressed a P162/4/7A mutation, and not P164/7A as was reported (Shin et al., 2007a). These prolines are found in a turn region in the NS1 monomer (**Figure 4.2A**) and are presumably required for proper NS1 folding and function. The introduction of three (or even two) proline to alanine substitutions may have effects for the whole NS1 structure and may abrogate multiple functions of NS1. Tyr89 does not appear to form a structural role in the structure of the NS1 effector domain (**Figure 4.2A**) and the Y89F mutation is a conservative change so is unlikely to affect the overall structure of NS1. While the effector domain of Udorn NS1 is thought to exist as a monomer (R. Russell, unpublished observations), the effector domains of NS1 from other IAV strains are thought to dimerise. Two structures of the NS1 effector domain homodimer have been proposed (see **Figure 4.2**), with dimer interfaces comprising of either helix-helix (Hale et al., 2008a) or strand-strand interactions (Bornholdt and Prasad, 2006). Analyses of these structures reveal that Tyr89 is exposed for p85 binding in both conformations and does not appear to be involved in dimer formation: the residue is either exposed within a cleft formed at the dimer-interface (**Figure 4.2B**, Bornholdt

and Prasad, 2006) or exposed at the edge of the dimer (**Figure 4.2C**, Hale et al., 2008a). In addition, work carried out by B. Hale has shown that NS1-Y89F displays identical subcellular localisation to NS1 wt and is still able to interact with RIG-I (Hale, 2007), indicating that the overall structure and multiple functions of NS1 are unlikely to be affected by this mutation. Given that pharmacological PI3K inhibitors non-specifically inhibit PI3K activity (**Section 4.4.2**) and the P164/7A and P162/4/7A mutations may not specifically abrogate PI3K binding, recombinant viruses expressing the NS1-Y89F mutation appear to be a much better tool for the study of NS1-mediated PI3K activation.

It was interesting to note that viruses expressing the R38A, 123/4, 184-8(L) and 103/106 mutations did not induce significant amounts of apoptosis in A549 cells (**Figures 3.36 and 3.37**). Introduction of an R38A mutation into a Ud NS1 background completely abrogates the ability of NS1 to bind dsRNA (Wang et al., 1999d): the primary function of dsRNA binding NS1 is reported to be sequestration of dsRNA away from OAS/RNaseL to prevent induction of the antiviral state (Min and Krug, 2006). The inability of the rUd-R38A virus to cause cell death indicates that NS1-mediated OAS/RNaseL regulation does not contribute to apoptosis inhibition by NS1, despite the pro-apoptotic roles of OAS/RNaseL (see **Introduction**). The 123/4 mutation abrogates direct binding to, and inhibition of, PKR (Min et al., 2007). Interestingly, a recombinant rUd virus expressing this mutation was previously shown to exhibit enhanced viral RNA synthesis early in infection (Min et al., 2007), perhaps suggesting that NS1 can prevent induction of apoptosis by limiting aberrant RNA synthesis. The NS1:CPSF30 interaction is required to inhibit processing of cellular mRNAs and is reported to be disrupted by the 184-8(L) and 103/106 mutations (Noah et al., 2003, Kochs et al., 2007, Das et al., 2008, Twu et al., 2006). However, rUd viruses expressing 123/4, 184-8(L) and 103/106 mutations did not induce more apoptosis than wt virus. It should be noted however that binding studies between these mutant rUd NS1 proteins and their cellular binding partners have not yet been carried out. Interactions between each of the mutant NS1s and cellular proteins are currently being investigated to aid the characterisation of these mutant viruses. These studies will identify those mutations that are specific for any one binding function, and identify those mutations that have non-specific effects on cellular protein binding due to disruption of the overall NS1 structure.

delNS1 viruses have been shown to induce more apoptosis than wt viruses in IFN-competent, but not IFN-deficient Vero cells (Zhirnov et al., 2002). Any inhibitory

effects of NS1 on apoptosis are therefore likely to be a direct result of IFN antagonism. Indeed, rUd-184-8(P) is a potent inducer of IFN as well as apoptosis in A549 and MRC-5 cells (**Figure 3.39; data not shown**), and is unable to cause cell death in A549/BVDV/NPro (IRF3-deficient; **Figure 3.40**) or A549/PIV5/V cells (STAT1-deficient; **data not shown**). rUd-184-8(L) also induced significantly more IFN than wt virus, in accordance with a previous report (Noah et al., 2003), but this does not render the virus more apoptogenic. It is likely that other functions of NS1-184-8(L) are still intact and are acting to limit the antiviral state. rUd-184-8(P) may induce more IFN than rUd-184-8(L) either because NS1-184-8(P) is unstable, thus unable to mediate its other functions, or because this virus produces DIs that induce IFN and apoptosis (see **section 4.2.4**).

4.4.7 Potential roles for PI3K in the IAV life cycle

Previous studies have demonstrated an important role for PI3K activation in virus replication. Pharmacological PI3K inhibition inhibits IAV growth (Ehrhardt et al., 2006) while the rUd-Y89F virus has previously been found to be attenuated in MDCK cells (Hale et al., 2006). This study has demonstrated that rUd-Y89F is also attenuated in A549 and MRC-5 cell-lines, indicating that PI3K activation by NS1 is also essential for efficient virus replication in human lung cells. However, as stated above, PI3K activation does not perform an anti-apoptotic role in these cells, so PI3K must enhance virus replication at another stage in the IAV life cycle.

4.4.7.1 PI3K and virus entry

Observations that wortmannin could only reduce IAV titre when administered at early stages (less than 4hpi) in infection (Ehrhardt et al., 2006) suggested a role for PI3K during an early step of the virus replication cycle. Moreover, a reduction in IAV protein synthesis was observed in A549 cells treated with wortmannin, but this effect was not due to global inhibition of cellular transcription or translation and was not due to impaired viral polymerase activity (Ehrhardt et al., 2006). Instead, wortmannin appeared to inhibit virus uptake at a stage prior to endosomal transport (Ehrhardt et al., 2006); the authors suggested that PI3K may regulate interaction with clathrin-coated pits, as virus particles appeared to remain at the cell border in wortmannin-treated cells. It is difficult to see how NS1-mediated PI3K activation could regulate virus entry however, as current dogma states that NS1 is not present in the virus

particle and as such, is only produced following viral RNA transcription and translation (Krug and Etkind, 1973, Lazarowitz et al., 1971).

4.4.7.2 PI3K and selective translation of viral mRNA

Expression of viral proteins is slower during rUd-Y89F infection than rUd wt (D. Jackson, unpublished observations). An additional study has reported reduced viral RNA and protein synthesis following PI3K inhibition (Shin et al., 2007b). While these observations may be consistent with impaired virus entry, they may also indicate a role for PI3K activation by NS1 in promotion of viral translation. As described in the **Introduction**, PI3K/Akt can selectively enhance translation through the activities of mTORC1: PI3K/Akt/mTORC1 activation causes the phosphorylation and inhibition of p70 S6K and 4E-BP1 to stimulate mRNA translation (Kaur et al., 2008a, Kaur et al., 2008b, Lekmine et al., 2003). Some viruses, particularly DNA viruses, activate this pathway to enhance their replication (reviewed in Buchkovich et al., 2008); for example, the HPV E7 protein and adenoviral proteins E4-ORF1 and E4-ORF4 activate mTORC to promote protein translation (Oh et al., 2006, O'Shea et al., 2005). NS1 has already been attributed a role in selectively enhancing viral mRNA translation (de la Luna et al., 1995, Enami et al., 1994, Hatada et al., 1990). However, the effect of NS1 on translation is thought to be mediated by interactions between NS1 and the 5'UTR of viral RNA, eIF4GI (the large subunit of eIF4F), PABP1, and possibly hStaufen (de la Luna et al., 1995, Enami et al., 1994, Aragon et al., 2000, Burgui et al., 2003). Furthermore, it has been demonstrated that viral mRNA translation is insensitive to rapamycin, an mTOR inhibitor, and occurs even when eIF4E, the target of 4E-BP1, has been functionally impaired (Burgui et al., 2007). NS1 may be able to regulate translation in an mTORC-independent manner however: there may be a link between PI3K activation and $p58^{IPK}$, a PKR inhibitor that is activated during IAV infection (Melville et al., 1999, Lee et al., 1990, Lee et al., 1992). $p58^{IPK}$ appears to play an important role in virus replication: IAV mRNA translation is impaired in fibroblasts from $p58^{IPK}$(-/-) mice (Goodman et al., 2007). It would be interesting to determine whether activation of $p58^{IPK}$ during IAV infection occurs downstream of PI3K/Akt.

4.4.7.3 PI3K, cytokine production, cytotoxicity and chemotaxis

NS1 plays an important role in limiting inflammation and establishment of T-cell responses during IAV infection. Upon contact with virus antigen, DCs undergo

maturation; this upregulates cell surface receptors involved in antigen presentation to T-cells (including MHC class II molecules) and a range of proinflammatory cytokines and chemokines. In addition to inhibiting IFN induction, NS1 limits expression of a significant number of other cytokines involved in DC maturation, migration and T-cell activation, independently of its ability to globally inhibit protein synthesis (Fernandez-Sesma et al., 2006). DCs infected with a delNS1 virus secreted significantly higher amounts of IL-6, IL-8, IL-12, MIP-1β, CCL5 and TNF-α than those infected with wt virus (Fernandez-Sesma et al., 2006). PI3K activation may be important for inhibition of cytokine expression: IL-12, a cytokine that enhances NK cell and CTL cytotoxicity (Kobayashi et al., 1989), is negatively regulated by PI3K (Kamda and Singer, 2009, Fukao et al., 2002) while PI3K inhibits IL-6 and TNF-α production in monocytes (Kramer et al., 2009). NS1-mediated PI3K activation may play an additional role in downregulating FasL-mediated NK cell and CTL cytotoxicity. FasL expression is downregulated by PI3K through p53 activation and FOXO inhibition (Ciechomska et al., 2003, Hsu et al., 2004a). While no difference in FasL expression was observed in A549 cells infected with rUd wt or rUd-Y89F (**Figure 3.28**), PI3K may play a more predominant role in regulation of FasL expression in NK cell and CTLs.

The role of PI3K/Akt in chemotaxis is an area of intensive study (reviewed by Van Haastert and Devreotes, 2004, Merlot and Firtel, 2003). Experiments in the *Dictyostelium discoideum* amoeba revealed that, in response to chemoattractants, PI3Ks rapidly collect at the leading edge of the cell while PTEN accumulates at the sides and the rear (Devreotes and Janetopoulos, 2003, Iijima and Devreotes, 2002, Funamoto et al., 2002). The result is a local concentration of PIP3 at the leading membrane followed by recruitment of PH domain-containing proteins and pseudopod formation. While the upstream and downstream proteins involved in PI3K-mediated chemotaxis remain to be determined, Akt appears to be involved in mediating downstream signals since Akt-null cells display aberrant chemotaxis in response to cAMP (Meili et al., 1999). It appears that macrophage and neutrophil chemotaxis is also PI3K/Akt-dependent (Li et al., 2000, Hirsch et al., 2000, Sasaki et al., 2000, Servant et al., 2000, Wang et al., 2002a, Weiner et al., 2002). If PI3K activation were to promote cell chemotaxis during IAV infection, it may have implications for systemic spread of the virus (Zhao et al., 2008, Ratcliffe et al., 1992).

4.4.7.4 PI3K and sodium transport

Recent work has demonstrated a potential role for NS1-mediated PI3K activation in sodium transport in human airway epithelial cells. PI3K can activate the downstream kinase SGK1, which promotes increased expression and activation of epithelial sodium channels (Vasquez et al., 2008, Lang et al., 2003, Lang et al., 2006). Collaborative work between our laboratory and S. Wilson (University of Dundee) has revealed that SGK1 may be activated during IAV infection, and that infection of human airway epithelial cells with rUd wt, but not rUd-Y89F, activates these sodium channels to stimulate sodium transport.

4.5 Outlook

4.5.1 Implications for oncolytic therapy

Although IFN was initially hoped to be a “miracle drug” for cancer treatment due to its pro-apoptotic properties, in practice, its use is far more limited: it is currently used in the treatment of malignant melanoma, renal cell cancer and some types of lymphoma and leukaemia (Bryan, 2008). While loss or down-regulation of JAK/STAT signalling has been implicated in IFN resistance (Wong et al., 1997, Sun et al., 1998, Dron and Tovey, 1993, Sakamoto et al., 1998), it is not always a major factor in the resistance of tumours to IFN treatment (Choi et al., 2003). For example, activation of PI3K/Akt renders some tumour cells insensitive to IFN: a direct correlation between Akt activation and IFN-β sensitivity has been demonstrated in human colorectal cancer cells (Lei et al., 2005). Additionally, in some cell types, IFN-treatment can actually make cells more resistant to apoptosis, presumably through activation of NF-kB cell survival signals (Pajak and Orzechowski, 2007, Tamai et al., 2006, Tanaka et al., 2005a). The significant degree of cross-talk between IFN and PI3K in the induction of apoptosis indicates that treatment with IFN and PI3K/Akt inhibitors may prove an effective form of combination therapy. Further investigation into activation of additional, non-classical pathways by IFN may highlight novel targets for anti-tumour drugs.

The synergistic effect of IFN on pro-apoptotic cytokine action also has implications for anti-cancer therapy. DR ligands represent an area of potential anti-tumour agents currently being investigated (reviewed in Ashkenazi and Herbst, 2008). TNF-α is effective in the treatment of soft tissue sarcomas (Grunhagen et al., 2006), while

FasL has demonstrated significant efficacy against a range of tumour cells. Soluble TRAIL or TRAIL-R agonistic antibodies appear to exhibit potent anti-tumour activity with minimal cytotoxic effects on normal cells (Ashkenazi et al., 1999, Chuntharapai et al., 2001, Ichikawa et al., 2001, Lawrence et al., 2001). Treatment with IFN in conjunction with these DR agonists may vastly improve their efficiency. Alternatively, resistant tumour cells could be specifically sensitised to these DR-mediated therapies by selective induction of IFN in these tumours. A possible mechanism for achieving this would be targeting of IFN-inducing viruses to tumour cells; IFN could then upregulate expression of DRs at the cell surface and render these cells more sensitive to subsequent treatment with DR agonists. In fact, since IFN has been shown to induce apoptosis in established tumour cell-lines, IFN induction alone may be sufficient to induce apoptosis in these cells (Chawla-Sarkar et al., 2001, Chen et al., 2001a, Morrison et al., 2001, Thyrell et al., 2002). Since delNS1 viruses replicate efficiently in IFN-deficient cells (Garcia-Sastre et al., 1998, Egorov et al., 1998), they may be used to target those tumours that are IFN-resistant (Muster et al., 2004). PIV5 with mutations in the P/V gene may have a similar use: a virus carrying the P/V gene of PIV5-CPI- displays oncolytic potential in human prostate cancer cells that are defective in IFN production (Gainey et al., 2008b). The use of paramyxoviruses and influenza viruses as vehicles for the delivery of genetic material has been described (Lorence and Reichard, 2006, Li et al., 1993), so recombinant PIV5 and IAV lacking the V and NS1 genes respectively could be used due to their ability to induce large amounts of IFN and apoptosis (Sun et al., 2004, Zhirnov et al., 2002, He et al., 2002a, Stasakova et al., 2005).

delNS1 viruses may be able to target other tumour types, not just those that are defective in the IFN response. NS1 mutants defective in PI3K activation can be used to specifically target tumour cells with constitutively active PI3K/Akt. PR8delNS1 replicates to high titres in cells overexpressing oncogenic Ras and has significant oncolytic properties in these cells, but not in non-malignant cells (Bergmann et al., 2001). Ras promotes PR8delNS1 replication either through inhibition of PKR (Bergmann et al., 2001, Mundschau and Faller, 1992), or by promoting PI3K activation (Rodriguez-Viciana et al., 1994, Rodriguez-Viciana et al., 1996), both properties are knocked out in the PR8delNS1 virus. In addition, because delNS1virus induces apoptosis independently of p53 (Zhirnov and Klenk, 2007), it may be an effective oncolytic agent for tumour cells that are defective in p53-dependent pathways.

Many tumour cells express FasL at their cell surfaces; this represents a mechanism of immune evasion during tumour development, since these cells can actively induce Fas-mediated apoptosis in FasL-sensitive lymphocytes (Strand et al., 1996, Shurin et al., 1998, Muschen et al., 2000, O'Connell et al., 1996, Hahne et al., 1996, Niehans et al., 1997, Kawasaki et al., 2000). In addition, levels of soluble FasL are elevated in serum from patients suffering from large granular lymphocytic leukaemia and NK cell lymphoma (Tanaka et al., 1996). Since PI3K/Akt activation can negatively regulate FasL expression through inhibition of FOXO transcription factors (Suhara et al., 2002, Tang et al., 1999, Uriarte et al., 2005), activation of PI3K, possibly through NS1 expression, may downregulate FasL expression and secretion. This may prevent induction of lymphocyte apoptosis by tumour cells, or limit FasL serum levels in NK cell lymphoma patients, both of which may alleviate some symptoms of these diseases.

4.5.2 Implications for anti-viral drugs

Replication of both PIV5 and IAV is impaired in the presence of PI3K and Akt inhibitors (Ehrhardt et al., 2006, Hale et al., 2006, Sun et al., 2008a). Given that small molecule inhibitors of PI3K and Akt are being developed for use as anti-inflammatory and anti-cancer drugs (Yap et al., 2008, Collins, 2009), they may also have important uses as anti-viral drugs. While inhibitors of cellular PI3K signalling pathways may be less sensitive to drug resistance than virus-specific targets, due to the wide range of biological functions mediated by PI3K in the cell, PI3K inhibitors may have toxic side-effects. Inhibition of PI3K/Akt activation by IAV and PIV5 may be an attractive approach: preliminary screens have been carried out (in collaboration with N. Westwood, University of St Andrews) to identify potential small molecule inhibitors of the NS1:p85 interaction. If hyperactivation of PI3K by NS1 is responsible for the virulence of 1918 and avian IAV strains, small molecule inhibitors of the NS1:p85 and NS1:Crk/CrkL interactions may be of pivotal importance in the treatment of these infections.

4.6 Conclusions

An important function of the IFN response is to establish a pro-apoptotic state in virus-infected cells. PI3K (and its downstream target, Akt) is an important regulator of both the IFN and apoptosis antiviral responses and this study sought to further investigate the crosstalk between IFN, PI3K and apoptosis. PIV5 and IAV are negative-sense single-stranded RNA viruses that encode multifunctional proteins in order to maximise the coding capacity of their genomes. The PIV5/V and IAV/NS1 proteins are well-studied as antagonists of the IFN response and both proteins are reported to modulate PI3K signalling. Less well-studied is the role of these proteins in apoptosis regulation: the ability of these proteins to inhibit apoptosis induction was investigated. PIV5/V was found to limit cell death in response to a number of apoptosis inducers in a manner that required its STAT1-degradative activity. In addition, PIV5/V expression inhibited Akt activation. Apoptosis induction and Akt activation by PIV5 strains expressing V proteins with different biological properties was also investigated. IAV/NS1 binds directly to PI3K to stimulate its activity, and this is reported to mediate anti-apoptotic signalling during IAV infection. However, a virus expressing an NS1 unable to bind PI3K did not induce more apoptosis than wt virus. NS1 expression and PI3K activation, either in a stable cell-line or during virus infection, was also unable to protect cells from pro-apoptotic stimuli. NS1-mediated PI3K activation similarly had no effect on IFN production or ISG expression in infected cells. In contrast, viruses that express other NS1 mutations were able to induce large amounts of IFN and apoptosis. These viruses were unable to cause apoptosis in cells deficient in the IFN response, indicating that NS1 may limit apoptosis induction through its IFN antagonist function. This work may have implications for anti-cancer and anti-viral therapies.

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