Characterisation of Lentiviral Vpr Function and Mechanism

Christoffer van Tulleken

University College London

Department of Infection and Immunity

A thesis submitted for the degree of

Doctor of Philosophy

July 2017

Declaration

I confirm that the work presented in this thesis is my own other than where I have indicated that information, images or data come from other sources.

Acknowledgements

As a clinician starting comparatively late in life in a molecular lab I have been given immense support and assistance by everyone in the Towers Laboratory and from colleagues and collaborators in our division at UCL.

I would particularly like to thank Greg who has been an invaluable source of inspiration and advice about both the technical and metaphysical aspects of this PhD (and science in general). He has been a true mentor.

(Choon Ping) Tan rescued me from a failing project and help establish the techniques and procedures that have seen me through to the end of this PhD. Becky Sumner took over from Tan as a post-doctoral mentor with a seemingly limitless supply of positivity and help. Stephane Hue helped me to understand phylogenetics, use the required programs and showed me how to align un-alignable sequences. Hataf Khan helped make the HIV-1 M mutants and has been a great friend and sounding board. Layal Liverpool was my student and friend for almost a year and helped with the CypA over expression and knock down experiments. David Stirling was endlessly generous with his time and help using the Hermes and the multiple failed attempts to visualise stained plasmids. Claire and Yasu were on my thesis committee and provided ongoing advice particularly at upgrade. Elizabetta Gropelli sequenced the initial NFκB promoter and was a great source of inspiration and advice. Petra Mlcochova, Isobel Honeyborne, Jane Turner, Doug Fink, Doug King and Tafima Haider have all helped variously and continuously with advice, friendship and protocols. Matt Tranter helped read the manuscript and gave advice on statistics. Christine my mother-in-law provided work space, company and lots of advice while my mother Kit edited the final manuscript in more detail than I could have ever asked for. My daughter Lyra arrived just in time to force a viva reschedule. I am grateful to my examiners Richard and Leo for allowing me to enjoy my first week with her without pressure.

My brothers and both my parents support me endlessly throughout everything I do and this PhD has been no exception – I am fortunate beyond words to have had their love and support.

The same is true of my wife, Dinah. She has also put up with late nights and failed experiments as much as if it was her own project. If there was any justice in the world this thesis would have her name on the cover.

Abstract

Genetic conflict between viruses and their hosts has driven an 'arms race', forcing the evolution of both immune defenses and multiple viral strategies to counteract and evade them. In the case of HIV many of these processes are well characterised – the virus carries with it a set of accessory proteins which target specific host restriction factors. Of these accessory proteins Vpr is the least well understood, with no described role that adequately explains its conservation across all known primate lentiviruses.

Unpublished data from our lab indicate that Vpr is able to rescue infection in macrophages from addition of cGAMP, a second messenger protein produced by the cytosolic DNA sensor cGAS which activates antiviral immune signalling pathways.

This study first sought to test the hypothesis that Vpr has evolved to counteract cGAS/STING mediated cytosolic DNA sensing using a co-transfection assay to test Vpr proteins from all groups of primate lentiviruses. Initial observations appeared to demonstrate specific degradation of innate immune signalling proteins. It was subsequently shown that HIV-1 M Vpr antagonises expression from all tested co-transfected plasmids. This phenotype was demonstrated to be species specific, and to correlate with both the history of zoonotic transmission and localisation of Vpr to the nuclear rim. Additionally, it was shown that Vpr antagonises NFκB signalling activated by TNFα, independent of an effect on expression from transfected plasmids, but with the same dependence on nuclear localisation, putatively by the same mechanism.

Next, this study characterised an observation that the Vpr from the lentivirus infecting a mona monkey (SIVmon) stimulates NFκB signalling. It was hypothesised that the SIVmon Vpr might have molecular binding partners in common with the HIV-1 M Vpr and conditions were optimised for proteomics studies to determine these binding partners.

This study provides insights into the role of Vpr in antagonising innate immune sensing. Additionally, overexpression assays have been used widely in the literature describing Vpr. The data presented here indicate that observations using these assays, apparently demonstrating specific degradation of host cellular proteins, should be interpreted cautiously.

List of Tables

Abbreviations

6HB	6-helix bundle
Ab	antibody
AIDS	acquired immune deficiency syndrome
APC	antigen presenting cell
APOBEC3	Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3
APS	ammonium persulfate
ART	Antiretroviral therapy
AZT	azidothymidine
BLAST	Basic Local Alignment Search Tool
BSA	bovine serum albumin
CA	capsid
cART	combined ART
CCL	chemokine ligand
CCR5	C-C chemokine receptor 5
CD4	cluster of differentiation 4
CD8	cluster of differentiation 8
CDK	cyclin-dependent kinase
cDNA	Complementary DNA
cGAMP	cyclic GMP-AMP or cyclic [G(2'-5')pA(3'-5')p]
cGAS	cyclic GMP-AMP synthase
CHR	C-terminal helical region
CLR	C-type lectin receptors
CMV	Cytomegalovirus
cPPT	central PPT
CPSF6	cleavage and polyadenylation specificity factor 6
CsA	cyclosporine A
Ct	cycle threshold
CTD	C-terminal domain
CTL	cytotoxic T lymphocyte
CXCR4	C-X-C chemokine receptor 4
CypA	cyclophilin A
DBP	DNA binding protein
DC	dendritic cell
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulphoxide

EDTA	ethylenediaminetetraacetic acid
EIAV	Equine Infectious Anaemia Virus
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
Env	envelope
ER	endoplasmic reticulum
ESCRT	endosomal sorting complexes required for transport
FBS	foetal bovine serum
FISH	fluorescent *in situ* hybridisation
FIV	Feline Immunodeficiency Virus
FoC	fate of capsid
FRAP	fluorescence recovery after photobleaching
FSC-H	forward scatter height
Gag	Group specific antigen
GALT	gut associated lymphoid tissue
GFP	green fluorescent protein
gp120	glycoprotein 120kDa
gp160	glycoprotein 160kDa
gp41	glycoprotein 41kDa
GPI	glycophosphatidylinositol
GWAS	Genome-wide association study
HA	hemagglutinin
HAART	highly active ART
HIV	Human Immunodeficiency Virus
HLA	human leukocyte antigen
HTLV	Human T-Lymphotropic Virus
ICAM-1	intracellular adhesion molecule 1
IF	immunofluorescence
IFN	interferon
IFNAR2	IFNWα/β receptor 2
IL-2	interleukin 2
IN	integrase
ISG	IFN-stimulated gene
IU	infectious unit
Jun	Jun proto-oncogene
kb	Kilobase
kDa	Kilodalton
LB	lysogeny broth

LEDGF/p75	lens epithelium-derived growth factor
LTNP	Long-term non-progressor
LTR	long terminal repeat
MA	matrix
MDM	Monocyte-derived macrophage
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MLV	Murine Leukaemia Virus
MOI	multiplicity of infection
MxB	Myxovirus resistance protein B
NC	nucleocapsid
Nef	negative regulatory factor
NES	nuclear export signal
NFAT	nuclear factor of activated Tcells
NFκB	nuclear factor κB
NHEJ	Non-homologous end joining
NHR	N-terminal helical region
NLS	nuclear localisation signal
NMR	nuclear magnetic resonance
NPC	nuclear pore complex
NTD	N-terminal domain
Nup	nucleoporin
ORF	Open reading frame
OWM	Old world monkey
p-TEFb	positive transcription elongation factor b
PAGE	polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular pattern
PBMC	peripherial blood mononucleocytes
PBS	phosphate buffered saline
PBS	primer binding site
PCP	Pneumocystis carinii pneumonia
PEG	polyethylene glycol
PFA	paraformaldehyde
PHA	phytohemagglutinin
PI	PR inhibitor
PIC	pre-integration complex
POD	peroxidase
PPIase	peptidyl-prolyl isomerase

PPT	polypurine tract
PR	protease
PRR	pattern recognition receptor
PVDF	polyvinylidene difluoride
qPCR	quantitative/real time PCR
RANBP2	Ran binding protein 2, a.k.a. Nup358
Rev	regulator of expression of virion proteins
RING	Really Interesting New Gene
RLU	relative light units
RNAPII	RNA polymerase II
RPMI	Roswell Park Memorial Institute Medium
RRE	Rev responsive element
RRM	RNA recognition motif
RS	arginine/serine-rich
RT	reverse transcriptase
RTC	reverse transcription complex
SAMHD1	sterile α motif and HD domain-containing protein 1
SDM	site directed mutagenesis
SDS	sodium dodecyl sulphate
SGA	single genome analysis
SIV	Simian Immunodeficiency Virus
SP	spacer peptide
SR	serine/arginine-rich
SSC-H	side scatter height
STING	stimulator of IFN genes
SV40	Simian Virus 40
TAE	Tris acetate EDTA
TAK-1	tumour growth factor-β activated kinase 1
TAR	transactivation responsive region
Tat	trans-activator of transcription
TCR	T cell receptor
TE	Tris EDTA
TEMED	tetramethylethylenediamine
Tm	melting temperature
TNFα	tumour necrosis factor α
TNPO3	transportin 3, a.k.a. TRN-SR2
TRIM	tripartite motif
UTR	untranslated region

V3	variable loop 3
Vif	viral infectivity factor
VLP	Virus-like particle
Vpr	viral protein r
Vpu	Viral protein u
Vpx	Viral protein x
VS	Virological synapse
VSV	Vesicular stomatitis virus
VSV-G	VSV G envelope protein
WHO	World Health Organisation
WT	Wild type
Ψ	HIV-1 packaging signal

1 Chapter 1: Introduction

1.1 HIV history and Origins

1.1.1 The history of retroviruses

Agents of disease that we now know to be retroviruses were discovered at the beginning of the 20th century. Equine infectious anaemia virus (EIAV) and avian leukaemia virus (ALV) were partially described in 1904 and 1908, respectively, using extracts from infected animals, filtered so that they were free from cells or bacteria (1,2). The pathology caused by these viruses was poorly understood at the time, so neither of these discoveries had the same impact as another series of experiments performed by a young pathologist at the Rockefeller Institute called Peyton Rous. A farmer's wife asked him to investigate a Plymouth Barred Rock hen which had a large tumour and, after transplanting the tumour to other hens, he successfully demonstrated that further tumours could be induced with a cell free filtrate (3). Rous's discovery showed clearly for the first time that a tumour could be caused by infection, although it would be 53 years until the discovery of the first human oncovirus: Epstein-Barr (EBV). The isolation of these first retroviruses set in motion a series of immense scientific paradigm shifts, but these did not happen quickly; it would be 55 years before the significance of Rous's discovery was recognised with the award of the Nobel Prize.

Our modern understanding of the life cycle of retroviruses, requiring the conversion of an RNA genome into a DNA provirus, was proposed by Howard Temin in 1964 with a seemingly heretical challenge to the "Central Dogma" of molecular biology: that genetic information is transferred in a hierarchy from DNA to RNA to protein. By the mid 1970's the discovery of reverse transcriptase confirmed this hypothesis (4,5) and this paved the way for the huge advances in the understanding of cellular biology, cancer biology and human retroviruses that continue to this day.

1.1.2 Classification of retroviruses

The family Retroviridae of enveloped viruses is divided into two families: Spumavirinae (a poorly characterised group which cause foaming of cultured cells); and Orthoretrovirinae, which includes the genus Lentivirus from "lente" in Latin, so called because of the prolonged incubation period between infection and disease (Freed and Martin, 2001). The lentiviral genome is carried as two non-identical, single strands of positive sense RNA and its replication has two defining hallmarks – the genomic RNA must be reverse transcribed into a linear

double stranded DNA copy and this DNA must be subsequently integrated into the host cell genome.

Lentiviruses cause chronic persistent infection in bovines, equines, ovines, felines and primates (6). Most are exogenous, transmitting horizontally between hosts, whilst some have infected germ line cells and are transmitted vertically as genomic loci. Examples include prosimian endogenous lentiviruses, which infected germ-line cells of lemurs approximately four million years ago (8,9). These “viral fossils” allow direct inferences about the timescales of lentiviral evolution (10).

1.1.3 Discovery of HIV-1

In June 1981, the CDC published a case series of five young men who were treated for a rare Pneumocystis carinii (now jiroveci) pneumonia in Los Angeles, California (11). They were the first cases of HIV-1 infection to be recorded. The causative agent was unknown but immunodeficiency and subsequent infection with opportunistic pathogens lead to individuals being classified as having acquired immune deficiency syndrome or AIDS. The discovery of the first human retrovirus human T-cell lymphotropic virus type I (HTLV-I) by Robert Gallo had required the development of techniques both for culturing T lymphocytes and for the sensitive detection of reverse transcriptase that would prove essential in the isolation of HIV-1. In 1983, in the laboratory of Luc Montagnier at the Pasteur Institute in Paris, Barré-Sinoussi et al described their isolation of a new human retrovirus from a lymph node of a patient with lymphadenopathy (12). In early 1984 Robert Gallo’s group published four papers describing in more detail the new human retrovirus as the probable cause of AIDS and several more labs isolated what we now know as HIV in quick succession, each giving it a different name but by 1986 the International Committee on Taxonomy of Viruses recommended the current name of Human Immunodeficiency Virus or HIV. A second similar virus, also a simian zoonosis, was discovered in 1986; HIV was renamed HIV-1 and the new virus named HIV-2.

Over three decades later HIV has become, by almost any metric, the most studied virus on earth and basic science research has transformed the clinical picture. What was previously an almost universally fatal disease can now be treated so that in the developed world, life expectancy is approaching that of non-infected people in some patient cohorts (13,14).

1.1.4 The Origins of HIV

The majority of emerging infectious diseases have a zoonotic origin and HIV is no exception. But among primate lentiviruses the Human Immundeficiency Virus type 1 (HIV-1) is unique in terms of pathogenicity and pandemic spread. Lentiviruses are known to infect over 40 different

species of primate (figure 1.1A) but, despite the wide distribution across species and geography, there are only 12 known zoonotic transmission events to human beings (figure 1.1 B). HIV-1 has four groups (M, N, O and P), each from a separate transmission event. M and N originated in chimpanzees (*Pan troglodytes troglodytes*) whilst O and P were likely transmitted from Gorillas (*Gorilla gorilla gorilla*) (15–18).

1.1.4.1 HIV-2 origins

HIV-2 is known to have had at least eight independent host transfers from sooty mangabeys (*Cercocebus atys),* each one creating a distinct lineage, although in most cases only a single sequence has been isolated (18,19). HIV-2 remains largely confined to west Africa and whilst it causes an illness similar to HIV-1, disease progression is slower with a larger number of patients failing to progress to end stage infection. It also appears to be less transmissible (20).

1.1.4.2 HIV-1 origins

Like HIV-2, HIV-1 groups N, O and P are found almost exclusively in west Africa. Only the HIV-1 M (for Main group) has spread to any appreciable degree. The pandemic has infected 60 million people and killed over half of them originated from a single transmission event from a chimpanzee most probably during the early part of the 20th century (21–23). There are nine subtypes of HIV-1 M: A-D, F-H, J and K. Subtype C is predominant in Africa and the Indian sub-continent accounting for almost half of cases world-wide, whilst subtype B is the dominant subtype in the Americas, Australia, and western Europe recombinant subtypes are being detected with increasing frequency (24).

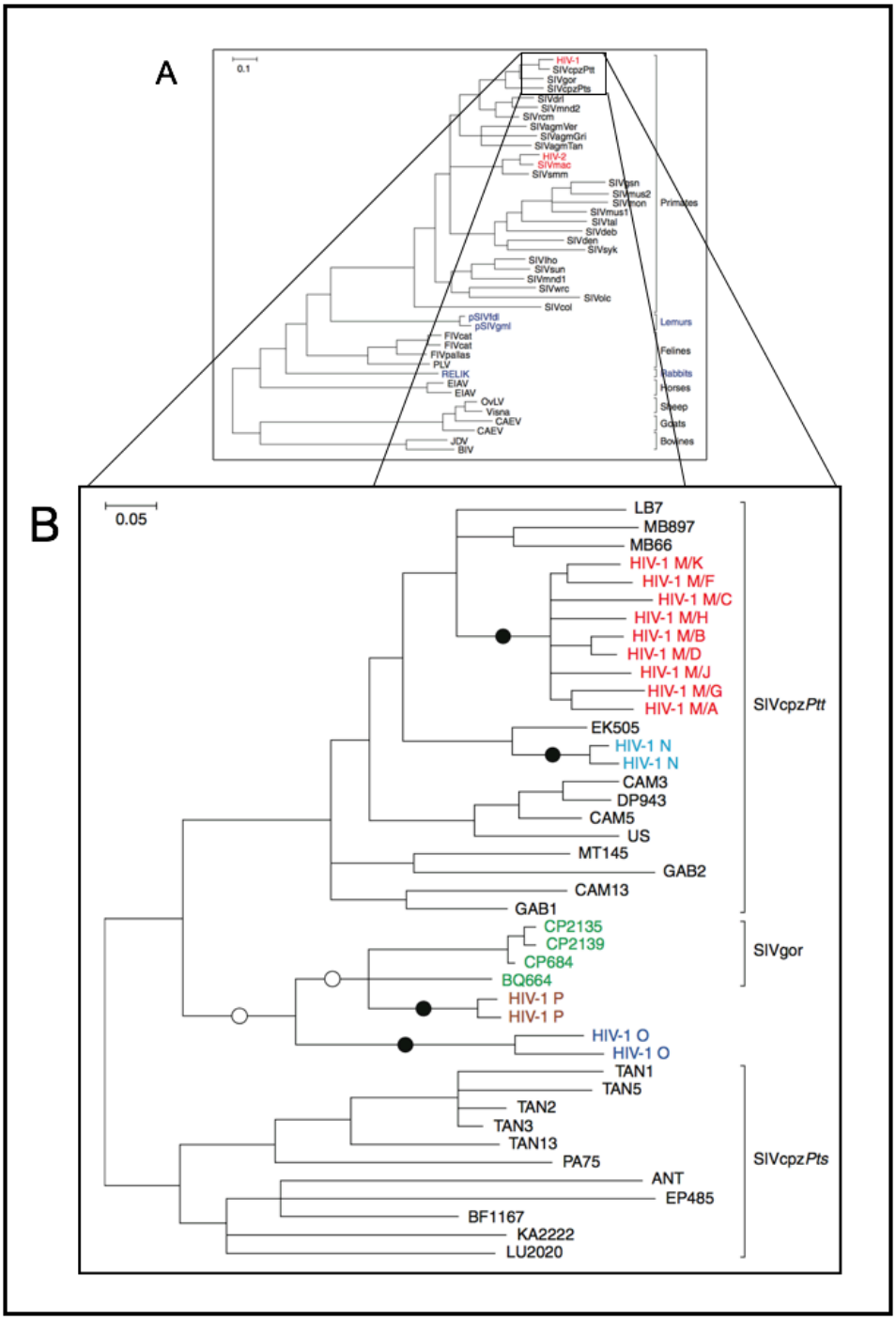

Figure 1.1 Phylogeny of lentiviruses (A) and SIVcpz/HIV-1 (B). (A) Mammalian lentiviruses phylogenetic relationships of *Pol*; host species are shown at the right. HIV-1, HIV-2, and SIVmac are in red; endogenous viruses in purple, exogenous viruses are in black. Horizontal branch lengths are shown to scale. Scale bar represents 0.10 aa replacements per site. (B) Phylogenetic relationships of representative SIVcpz, HIV-1, and SIVgor strains shown for a region of Pol. SIVcpz in black, SIVgor in green. The four clades of HIV-1 are shown in different colours. Black circles show zoonotic transmission to humans. White circles show branch possibilities for chimpanzee-to-gorilla transmission. Scale bar represents 0.05 nt substitutions per site. Both trees estimated using maximum likelihood methods (25). Adapted from Sharp, P.M., and Hahn, B.H. (2011). Origins of HIV and the AIDS pandemic. Cold Spring Harb. Perspect.

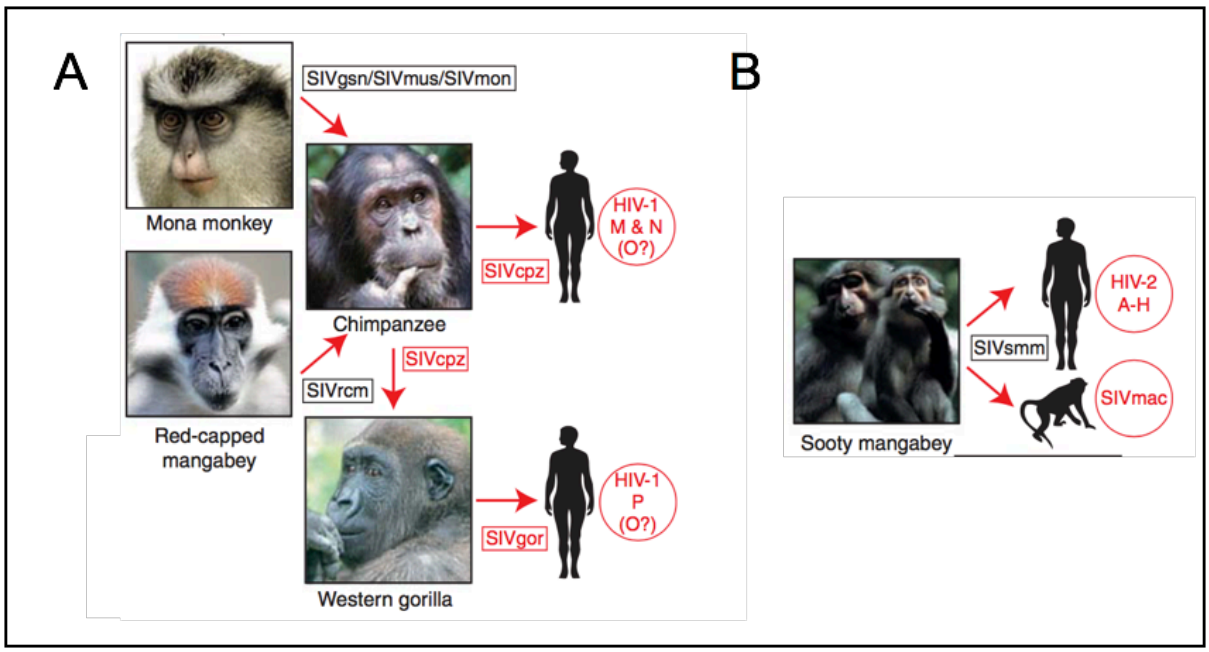

Figure 1.2 Origins of HIV. 40 different lentiviruses infect old world monkeys and African apes. Known examples of cross species transmission are highlighted in red with tramissions of HIV-1 from Gorillas and Chimpanzees (A) and HIV-2 from Sooty mangabeys (B). Adapted from Sharp, P.M., and Hahn, B.H. (2011). Origins of HIV and the AIDS pandemic. Cold Spring Harb. Perspect (18).

1.1.4.3 Simian Immunodeficiency Viruses

SIVs are each named with a three letter designation derived from the common name of the corresponding non-human primate species; e.g. SIVrcm for SIVs from red-capped mangabey, SIVgsn for greater spot nosed monkeys, etc. Most primate species harbour a single lineage of SIV which clusters in a phylogenetic group with viral sequences from the same species indicating that the vast majority of spread is within not between species. There have been recombination events between each of the different major lineages which complicate the SIV phylogeny. The position a virus takes in an evolutionary tree varies depends on the genomic region which is analysed. SIVrcm, SIVmnd2 and SIVgsn all show discordant phylogenies depending on the region analysed indicating that both cross species transmission and subsequent co-infection with divergent strains have occured (26–29). There have been some significant cross species transmissions however with substantial secondary spread including SIVgor and most notably HIV-1 and 2 (figure 1.2A and B). There are also SIVs that have emerged as a result of recombination including SIVcpz (26,30,31). Chimpanzees hunt other monkeys and through this predation may have acquired SIV. Phylogenetic analysis has shown that SIVcpz is a complex mosaic generated from recombination (see figure 1.3 A and B). The 3' LTR, the 5' half and the *nef* gene align most closely with SIVrcm (red capped mangabey - *Cercocebus torquatus*) while the *vpu, tat, rev* and *env* genes align most closely with a clade of SIV's from Cercopithecus species (the greater spot nosed, moustached and mona

monkeys) (17,32). The current range of the central *P.t.trogodytes* overlaps with these two species and this recombination most likely occurred in a single chimp and became the ancestor of today's SIVcpz*Ptt* (figure 2 A). No other SIV has been observed in a chimpanzee despite frequent exposure from hunting other small primates (33).

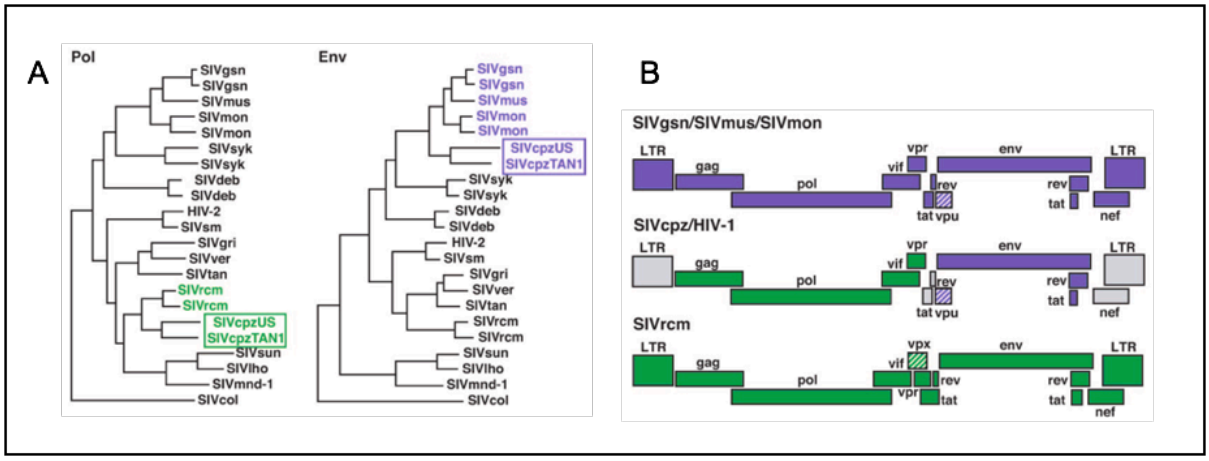

Figure 1.3 Recombination of SIVcpz. (A) Maximum-likelihood phylogenies of primate lentivirus Pol and Env sequences. The close alignment of SIVcpz to SIVrcm in Pol, and of SIVcpz to SIVgsn, SIVmus, and SIVmon in Env, are highlighted in green and purple, respectively. (B) Schematic of the genome of SIVcpz. Genomic regions are colored as in (A) according to their alignment with SIVrcm (green) or the SIVgsn/SIVmus/SIVmon lineage (purple). Grey areas are of unknown origin. Vpu and vpx genes are highlighted. Adapted from from Sharp, P.M., Shaw, G.M., and Hahn, B.H. (2005). Simian immunodeficiency virus infection of chimpanzees. J. Virol. 79, 3891–3902

Mitochondrial DNA sequencing has been used to classify chimpanzees into four subspecies: *Pan troglodytes troglodytes, Pan troglodytes schweinfurtheii, Pan troglodytes verus and Pan troglodytes ellioti*. These subspecies are geographically separated from each other across western and central equatorial Africa. Of these four subspecies only two are known to harbour SIV: *Pan troglodytes troglodytes* in west/central Africa and *Pan troglodytes schweinfurtheii* in central/east Africa. SIV has not been detected in either *P.t. verus* or *P.t. ellioti* suggesting that chimpanzees acquired SIVcpz after the geographical isolation of West African and Nigerian subspecies (34).

Sequence analysis of SIVcpz strains reveals two separate lineages SIVcpz*Ptt* and SIVcpz*Pts* (17), which show only 50-70% homology in their Gag, Pol and Env protein sequences. Initial studies of SIV prevalence were undertaken on animals captured as infants and so under represented the rates of infection in the wild (35). Subsequent development of non-invasive methods for the detection and characterisation of SIVcpz in faecal and urine samples has meant that many thousands of samples from almost 100 field sites are available for analysis. Wild seroprevalence appears highly variable. Some chimpanzee troops in both east and west

Africa have up to 50% prevalence whilst in other areas cases are rare or absent (21,36). It was initially believed that SIVs were non-pathogenic in their natural hosts but sequential faecal sampling in habituated populations of eastern chimpanzees showed that SIV infection is associated with a 10-16 fold increase in risk of death at a given age as well as reduced fertility (37). Additionally, AIDS like illness and pathology following infection has been described in wild P.t.schweinfurtheii chimps at post mortem and in a captive P.t.troglodytes with low CD4+ counts and infection with unusual and diverse pathogens (38).

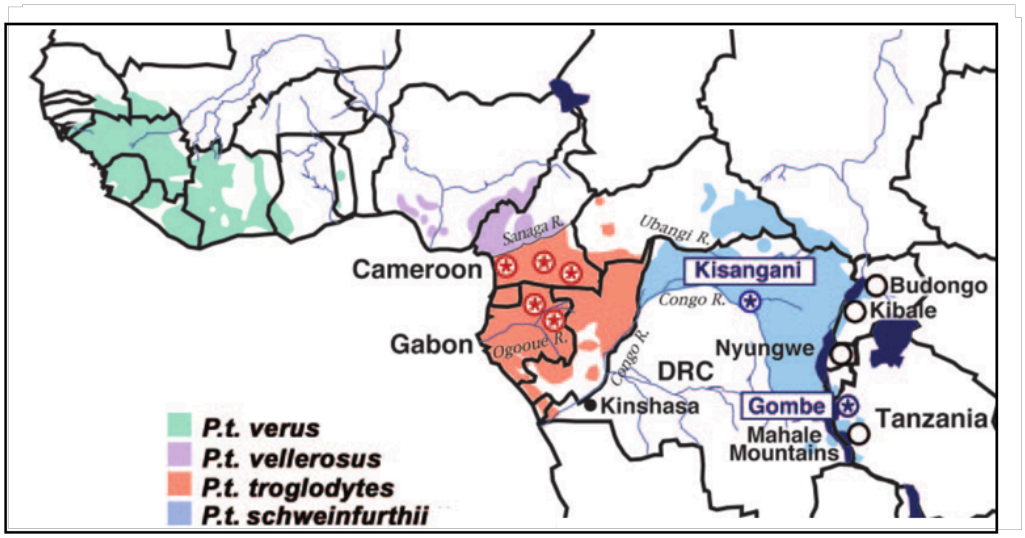

Figure 1.4 Chimpanzee Reservoirs of Pandemic and Non-pandemic HIV-1. Study sites in Uganda, Rwanda, Tanzania and Democratic Republic of Congo are indicated. Asterisks indicate the presence of natural SIVcpz infection. Adapted from Sharp, P.M., Shaw, G.M., and Hahn, B.H. (2005). Simian immunodeficiency virus infection of chimpanzees. J. Virol. 79, 3891–3902 (39).

1.1.4.4 Zoonotic transmission of SIV

The same faecal sampling studies also traced the ancestor of HIV-1M and N to particular *P. t. troglodytes* communities in south eastern and central Cameroon (40). HIV-1 group N is most closely related to SIVcpz*Ptt* from groups in the Dja forest of South Central Cameroon and group M aligns best with chimpanzee viruses from the confluence of the Sangha and Ngoko rivers on the border of Cameroon and Central African Republic (figure 1.4). The details of the transmission are unknown, and possibly unknowable, but must have involved blood or mucous membrane exposure to blood or bodily fluids from an infected ape: hunting and butchering bushmeat offers the most likely explanation. The secondary forests of west-central Africa where HIV-1 M crossed the species barrier have dense thorny foliage meaning hunters acquire multiple skin breaches and so butchery of bushmeat offers ample opportunity for blood to blood contact (figure 1.5). Although the first case reports of HIV were only published in 1981 the earliest record of HIV-1 infection is from 1959. The virus was found a serum sample from

a Bantu male living in Leopoldville, Belgian Congo (now Kinshasa in the DRC) and molecular clock analyses date the start of the group M epidemic to the beginning of the 20th century (23,36,41).

Figure 1.5 Bayaka hunter butchering a mona monkey. Photo C. van Tulleken.

Whilst SIV cpz*Ptt* is known to have transmitted to humans at least twice there is no known counterpart of SIVcpz*Pts* in humans (41). Since SIVcpz*Ptt* strains have transmitted to humans and gorillas a minimum of five times the lack of evidence of similar transmission from *Pts* chimpanzees from the east is striking and infections rates determined by field sampling are estimated to match those found in *P. t. troglodytes*. Differences in interactions between humans and apes in central and east Africa may explain the lack of observed transmissions or it may be that transmissions have gone unrecognised because of a lack of human testing using lineage specific serological tests. But it may also be that there is a molecular species barrier. SIVcpz*Pts* has been found to replicate in CD4 cells *in vitro* but these assays are unlikely to recapitulate the necessary conditions for *in vivo* transmission and replication (27).

This study identifies SIV and HIV Vpr proteins which, when ectopically expressed, differentially antagonise innate immune signalling pathways and expression from transfected DNA. This

may be a determinant of cross species spread since all the viral lineages that have spread to humans do antagonise sensing and many of those that have not spread are much less able to antagonise sensing including SIVcpz*Pts Vpr.* A sequence alignment of all available HIV-1, SIVcpz and SIVgor Vprs was constructed which shows an 11-18 residue deletion at the C-terminal end of the protein. Additionally, an SIV Vpr from a mona monkey has been identified as stimulating NFKB signalling in the absence of co-transfected immune protein and may prove to be a useful tool for dissection of NFkB signalling pathways.

1.1.4.5 Origins of Vpr and Vpx

Two of the six major lineages of primate lentiviruses encode Vpx: HIV-2/SIVsm (sooty mangabey) related viruses and a lineage represented by SIVrcm (from red capped mangabeys). However all extant lineages of SIV and HIV encode a paralog of Vpx: Vpr (42,43) (figure 1.3A and B). *Vpr* and *vpx* share sequence similarity and thus a common ancestry. It was previously believed that the additional Vpx gene in SIVsm resulted from a gene duplication event after the divergence of this lineage but this would require that the *vpx* and *vpr* from SIVsm are more closely related to each other than to *vpr* from any other lineage and, as can been seen from the phylogenetic trees generated for this thesis as well as those published previously (43,44), this is not the case. *Vpr* from SIVsm is more closely related to *vpr* from HIV-1/SIVcpz but *vpx* is more closely related to the *vpr* of SIVagm. This is inconsistent with a duplication event after divergence. A duplication before divergence of the different lineages seems unlikely as it would mean that vpx had been deleted from multiple separate lineages. Thus, the most parsimonious explanation seems to be that SIVsm acquired vpz by recombination rather than duplication, presumably in a monkey co-infected with both viruses. There is other evidence of recombination between these lineages (SIVagm has had its own 3' gag and 5' pol genes replaced with those of SIVsm) and these African green monkeys and sooty mangabeys share habitats in West Africa.

1.2 Clinical HIV and HIV Epidemiology

Over 70% of the estimated 35 million people living with HIV are in sub-Saharan Africa (45). Prevalence is increasing due to improved access to antiretroviral therapy increasing the life expectance of infected individuals: in 2015 over 17 million people were estimated to be on antiretroviral therapy compared with just 7.5 million in 2010. In summer 2016 United Nations member states committed to ending the AIDS epidemic by 2030 setting out a Political Declaration calling for access to comprehensive sexuality education, harm reduction services and focused outreach to at risk groups (young women, men who have sex with men, commercial sex workers, injecting drug users, transgender, incarcerated and displaced people) (46). Whilst efforts globally have reduced heterosexual transmission, regressive attitudes toward harm reduction policies in many countries, especially in the former Soviet bloc and eastern Europe, have limited the implementation of demonstrably effective opioid substitution and needle exchange programs (47).

In western Europe, Australasia and the Americas the primary route of transmission is between men who have sex with men and incidence has remained constant (48) owing to a complex and poorly understood combination of “therapeutic optimism” (an increase in risk taking behaviour following the availability of effective treatment), the increased risk of transmission associated with receptive anal intercourse and limited access to care determined by societal factors like stigma (49,50).

Globally HIV is the fifth leading cause of loss of life calculated by disability adjusted life years (51) but deaths have decreased from 2.3 million in 2005 to just over 1 million in 2015 (52).

1.2.1 Clinical HIV infection

HIV targets cells expressing CD4 and chemokine receptors CCR5 and/or CXCR4 including T cells, monocytes, macrophages and dendritic cells. It has been reported that cells lacking these receptors, including renal epithelial cells and astrocytes, may also be infected (53,54) but these studies have not been replicated. Transmission appears to be of a single or small number of founder viruses and is followed by a period of rapid viral replication accompanied by a large systemic inflammatory response (55,56). Viral load then stabilises at a set point level that may have orders-of-magnitude variation ranging from <20 virions/ul of plasma up to over 1 million for reasons involving both host genetics, adaptive and innate immune responses and virus genotype (57,58). Estimates vary but some studies report that over 30% of the variation in set point viral load (SPVL) is heritable (58). Understanding this variation is

important since SPVL appears to be the most robust surrogate marker of virulence or disease progression as well as being the major risk factor for transmission (57).

The initial infection may be asymptomatic or in around 70% of people followed by a brief “flu-like” seroconversion illness or acute retroviral syndrome characterised by a rapid increase in markers of acute inflammation, the production of non-neutralising antibodies and HIV specific CD4 and CD8 T cells (59). HIV RNA in the blood falls from an initial peak at around 6 weeks post infection and the SPVL is established between weeks 9 and 12. This is typically followed by a prolonged period of clinical latency. CD4 T cells are lost progressively in the blood but gastrointestinal CD4 cells are lost early on. For example, individuals with the *HLA-B27* allele infected with clade B can mount an effective T cell mediated immune response but the eventual progression to immunodeficiency with loss of CD4 T cells is almost universal. There is both death of cells and loss of effector function but surprisingly the mechanisms underlying these processes remain unclear (60). It is not known, for example, if it is the infected cells themselves that die (61)or if it is an effect on uninfected bystander cells that is the predominant cause of cell loss (62).

The WHO classifies adult HIV patients in four clinical stages, from asymptomatic (stage 1) to AIDS (stage 4). At approximately three months following infection patients develop neutralising antibodies and this in turn selects for viral escape mutants (63). Even in the roughly 20% of patients that produce broadly neutralising antibodies, escape mutants mean that they confer little benefit to the patient (64,65).

Progressive, catastrophic immune dysfunction due to DC4 T cell depletion is the clinical hallmark of HIV-1 infection with the largest observed depletion happening early in the gastrointestinal tract. Unlike the early loss of CD4 cells in the blood, gastrointestinal T cells recover only minimally even with treatment (66). As well as loss of T cells there are effects on T cell subsets with loss of Th17 cells and the mucosal associated cells required for bacterial defense. In both humans and rhesus macaques, a combination of loss of gastrointestinal lymphocytes, endothelial apoptosis and increase gut permeability raises plasma levels of bacterial components like lipopolysaccharide which activates TLR4 leading to the production of IL-6 and TNFα (67). Other factors increasing inflammation include production of IFNα secondary to TLR7 and TLR8 activation(68), co infection with CMV (69) and dysregulation of T cell homeostasis with altered ratios of T helper 17 and regulatory T cells (70). Inflammatory markers remain increased even when CD4+ T cell counts are restored with antiretroviral therapy whilst the fact that intensification of treatment with integrase inhibitors has been observed as reducing T cell activation may indicate that ongoing viral replication on treatment may contribute to persistent elevated inflammatory markers (71).

Following the latent or asymptomatic phase often lasting up to a decade, without treatment the progression to stage 2, the symptomatic stage of infection, begins. It is characterised by unexplained weight loss (<10% of total body weight), persistent generalised lymphadenopathy, oral lesions such as thrush and oral hairy leukoplakia, hypoproliferative anaemia and thrombocytopenia and reactivation of varicella-zoster (shingles).

Stage 3, the moderately symptomatic stage, has additional clinical manifestations including greater weight loss, unexplained diarrhoea, bacterial infections including pulmonary tuberculosis, pyelonephritis, pneumonia, empyema meningitis and osteomyelitis. Mucocutaneous conditions worsen.

Stage 4 is the severely symptomatic stage. There is a move away from use of the term AIDS (Acquired Immune Deficiency Syndrome) although it is still widespread(72). Clinical conditions that allow a diagnosis of AIDS in the absence of other biological information include Penumocystis pneumonia, extrapulmonary TB, HIV encephalopathy, CNS toxoplasmosis, chronic orolabial herpes simplex, oesophageal candidiasis and Kaposi's sarcoma (73).

1.2.2 Treatment and prevention of HIV infection

The advent of highly effective and relatively side effect free treatment in the developed world has meant that for people with HIV the major causes of death in high income countries are very similar to people without the virus – cancer and cardiovascular disease (14).

Since the development of combination antiretroviral therapy in the mid 1990s there are now more than 25 drugs licenced for treatment and effective single dose regimens with minimal side effects profiles are now widely available in the developed world. Life expectancy is now estimated to be approaching normal for patients with restored CD4 counts and suppressed viral loads on treatment (74). Typically, treatment will combine two nucleoside reverse transcriptase inhibitors (NRTIs) with a non-nucleoside reverse transcriptase inhibitor (NNRTIs), protease inhibitor (PI) or integrase inhibitor. Whilst these regimens reduce the plasma viral load to below the limit of detection in most people, CD4 T cell number increases are more variable and individuals with limited CD4 T cell recovery have elevated chances of poor outcomes (75). Debate continues over the CD4 threshold for starting treatment and most international guidelines have raised the required CD4 level to 500/ul. This may have a benefit of reducing transmission but may increase exposure to toxic effects of drugs and increase the chance of resistance (76). In the UK current guidelines recommend all people infected with HIV start antiretroviral treatment (77). Poor adherence increases the risk that viral replication will take place in the presence of therapy resulting in the selection of resistant viruses and it

has been shown that these viruses can transmit; there is >10% prevalence of drug resistant virus in antiretroviral naïve patients in high income countries (78).

There is robust evidence to support treatment as a prevention strategy since viral load levels, which are dramatically reduced by effective treatment, are the main factor influencing transmissibility. A 2011 study demonstrated that early initiation of treatment reduced HIV transmission in discordant couples by 96% (79) and this has been confirmed with several subsequent studies (80,81) but the implementation of “Test and Treat” strategies that use treatment as prevention has proved challenging in underdeveloped and rural settings (82). Treatment as prevention has been used for more than two decades to prevent mother to child transmission and studies conducted in developed setting indicate that only 14 days of treatment is required to reduce risk of transmission to less than 1% (83,84). Pre-exposure prophylaxis has also been shown to be extremely effective (85–87) but despite the population wide benefits as well as the economic and moral arguments (85) it is still not widely available in developed settings where state funded health care is available, a surprising deficiency in settings where more progressive policies might be expected (88).

1.3 Overview: HIV-1 structure

1.3.1 The HIV genome

All retroviruses share three common genes: *gag* (for “group specific antigen”) which codes for the proteins which form the matrix, capsid and the nucleocapsid; *pol* (for DNA polymerase) which encodes the reverse transcriptase, integrase and protease enzymes (PRO, RT and IN); and *env* (for envelope proteins) which encodes the surface and transmembrane proteins of the viral envelope (figure 1.6). Of the nine genera of retroviruses, five have a “simple genome” characterised by having not more than one coding region in addition to *gag, pro, pol* and *env*. Complex retroviruses, including all simian and human immunodeficiency viruses, have multiple splice donors in the genome giving rise to additional regulatory proteins derived from multiply spliced mRNAs. HIV is a complex retrovirus with six additional reading frames: *vif*, *vpr*, *vpu*, *tat*, *rev* and *nef,* all of which are conserved in almost all clinical isolates (figure 1.6).

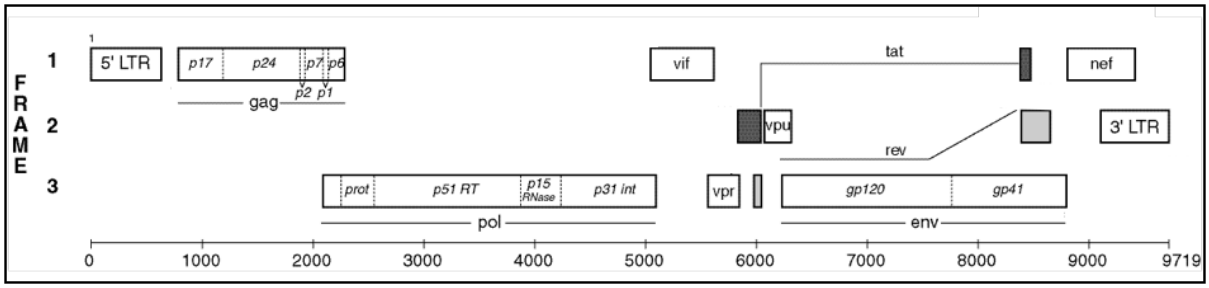

Figure 1.6 Landmarks of the HIV-1 genome, HXB2 (K03455). The layout of the HIV-1 genome. Boxes show the open reading frames and the non-coding, regulatory 5’ and 3’ LTRs. Reading frames are labelled 1,2,3 on the left and box position indicates the reading frame of each gene. Dashed lines indicate maturation cleavage sites for *gag, pol* and *env* polyproteins. The tat and rev spliced exons are shown as shaded rectangles. Adapted from Los Alamos National Laboratory HIV-1 Gene Map 2014.

The integrated genome of HIV-1, or provirus, is 9.7kb in length (89). Repeated sequences called Long Terminal Repeats (LTRs) flank each end of the provirus. The LTRs contain important regulatory regions for initiation of transcription and polyadenylation (90). Adjacent to these are unique untranslated regions U5 and U3. The U5 region contains the “kissing loop” required for genome dimerization and packaging. Between the LTRs are three classes of genes: i) structural proteins (Gag, Pol and Env) ii) regulatory proteins (Tat and Rev) and iii) accessory proteins (Vpu, Vpr, Vif and Nef) (91).

1.3.1.1 Structure of the mRNA genome

There are a number of important structural elements of the transcribed mRNA. TAR is the target sequence for viral transactivation comprising first 45 nucleotides of the mRNA which

form a hairpin stem loop. This sequence provides the binding site for the viral Tat protein which activates transcription elongation. The RRE is the Rev responsive element and consists of 200nt between gp120 and gp41 which provide a binding site for Rev (92). The Psi elements are stem loop structures overlapping the Gag start codon which allow binding of unspliced genomic transcripts by Gag p7 (NC) protein and packaging of genomes (93,94). Finally SLIP is a TTTTTT slippery site which regulates the ribosomal frameshift out of Gag and into Pol allowing production of up to 20 times more Gag than Gag-Pol (as discussed below in 1.7.2.2 and 1.8.9.4) (95).

1.3.2 Overview of Transcription, Translation and gene products

1.3.2.1 mRNA splicing

The HIV-1 primary transcript contains multiple 5' splice donors and 3' splice site acceptors and generates more than 25 mRNAs (96). While many of these mRNAs are polycistronic, they generally only express a single gene product. Initiation codon efficiency and proximity to the 5’ end of the mRNA determines the reading frame used.

1.3.2.2 Viral mRNA products

There are three size classes of mRNA produced. First the 9.7-kb full length, unspliced RNA produces the Gag and the Gag-Pol precursor proteins or is incorporated into the virion as the RNA genome. It produces two proteins from one initiation codon due to translational frameshifting discussed above in 1.7.1.1 and below in 1.8.9.4). Second, the incompletely spliced 4kb class of RNAs use the splice donor site closest to the 5’ end of the genome together with any of the central region splice acceptors. These RNAs encode Vif, Vpr, Vpu and Env and the single exon form of Tat. Finally, the doubly spliced 2kb class of mRNAs from which both introns of HIV have been spliced out. They express the two exon form of Tat, along with Rev and Nef. In each class of spliced RNA there are multiple species generated by the presence of several different 3’ and 5’ splice sites.

1.3.2.3 Gag and Gag-Pol fusion protein

The *gag* gene produces an unspliced mRNA which encodes the 55kDa Gag myristoylated precursor protein (p55). After budding during viral maturation this polypeptide is cleaved by the virally encoded protease (PR) into the structural proteins matrix (MA, p17), capsid (CA, p24) and nucleocapsid (NC, p7), as well as p6 and the spacer peptides 1 and 2 (SP1/2) (reviewed in Freed, 2015). During translation a ribosomal frameshift event, triggered by a specific cis-acting RNA motif, occurs around 5% of the time. This means that the ribosomes shift to the pol reading frame without translation being interrupted, producing a Gag-Pol fusion

protein (98). This Gag-Pol fusion protein (p160) is the precursor for the viral enzymes. Pol encodes the enzymes protease (PR, p10), reverse transcriptase (RT, p51/66) and integrase (IN, p31). Finally, during maturation the Pol polypeptide is cleaved away from Gag by the viral protease which then digests it to produce further protease (PR, p10), RT (p66) and the integrase (detailed in section 1.8.11).

1.3.2.4 Env

The *env* gene encodes the envelope glycoprotein of 160kDa (gp160) which is produced as a precursor and then cleaved by the host protease furin into two glycoproteins of 120 and 41kDa: the external gp120 and the internal gp41. These form the stable Env heterodimers which are secreted to the cell surface forming trimers. Both binding sites for the CD4 receptor and the chemokine receptors, which act as co-receptors for HIV-1, are found on gp120 (97,99).

1.3.3 HIV-1 mature virion structure

Viral particles are assemblies of both viral and host cellular macromolecules. All exogenous viruses form particles which have a common function – the protection and transmission of the viral genome, particularly from environmental challenges (like temperature, desiccation, chemicals, enzymes and pH). Whilst HIV *in vitro* has been shown to be quite hardy outside the body persisting for up to a week on a wet surface (100) in real terms, transmission requires sexual or blood contact. It may be that much of viral structure in the case of HIV has evolved to avoid the challenges of the intra and extracellular immunity; thermal and chemical challenges to transmission and replication may be less significant *in vivo*.

1.3.3.1 The viral envelope

The mature virion is roughly spherical with a lipid bilayer membrane derived from the host cell and embedded with viral Env spikes consisting of gp120/gp41 trimers as well as host cell surface proteins such as intracellular adhesion molecule 1 (ICAM-1) (99,101).

Gp41 comprises a cytoplasmic C terminal tail, a transmembrane domain and an ectodomain with two large helical regions (the C- and N- terminal helical regions or CHR and NHR). Gp120 is a globular protein that lies on the cell surface and consists of five conserved loops and five highly variable loops (C1-5 and V1-5). Env timers are incorporated onto the surface of new HIV-1 virions during budding so that each virion has 10-15 Env spikes on the surface (Kwong et al., 1998; Wilen et al., 2012a). Env is the only viral protein exposed on the surface of the virions and is thus the major target for antibodies. It is thought that only one or two Env spikes are needed for viral fusion and maintaining a low number of surface viral proteins may contribute to antibody evasion (104).

1.3.3.2 The capsid core

Under the lipid bilayer of the mature virion is a shell of MA (105). Beneath MA is the conical viral core of CA protein. This mature capsid assumes the geometric configuration of a 'fullerene cone', named after the architect and systems theorist, Buckminster Fuller, who popularised the architectural use of geodesic domes where combinations of hexamers and pentamers are used to create 3 dimensional curvature in surfaces. In capsid cores hexamers of the capsid protein form a lattice which incorporates capsid-protein pentamers so that it closes. A structure consisting of only hexamers forms only tubes whilst the introduction of pentamers allows for curvature in an additional dimension allowing a closed structure to form. Estimates of the total number of subunits varies with the most recent atomic level model suggesting a lattice of 216 hexamers and 5-7 pentamers at each end of the capsid. Capsid-protein structure has been determined by X-ray crystallography (106) and cryo-electron microscopy (107).

The amino-terminal domain (NTD) of HIV-1 capsid protein comprises seven α-helices and a β-hairpin. The carboxy-terminal domain (CTD) comprises four α-helices and a flexible linker with a 3_{10}-helix connecting the two structural domains (108–110). A 3_{10}-helix is a protein secondary structure with three amino acid residues per turn and 10 atoms in the ring formed by making the hydrogen bond. Interactions between the N-terminal domains form the hexamers and pentamers but it is interactions between the CTDs that form the final shape of the viral core. Zhao et al showed with cryo-electron microscopy and mutagenesis studies that the structure of a tubular assembly of hexamers has a three helix bundle at the interface between three hexamers with critical hydrophobic interactions required for capsid assembly, stability and to produce infectious virus (111).

1.3.3.3 The viral genome

Inside the capsid core is the dimeric single stranded RNA genome. Genome RNAs are packed as a non-covalent dimer or duplex held together by the interaction of sequences in their 5' UTR which form a "kissing loop" structure. Unspliced viral RNA is selectively packaged owing to a requirement for elements located downstream of the first splice donor (112,113). It may be that in fact the genome is more than simply "packaged". Unpublished data from our lab show that changes in packaged genome length alter viral titre and recent work in RNA phages shows that an RNA genome may play a significant role in capsid assembly (114).

The viral mRNA genomes are capped at their 5' ends and poly-adenylated at the 3' ends like eukaryotic mRNAs. They are associated with the nucleocapsid and Vif as well as the integrase

and reverse transcriptase enzymes required for the early stages of infection. RNA dimerization is required for it to be packages and for virions to be infectious (115,116).

HIV-1 particles also package a specific host transfer RNA (tRNA). RT needs both a primer and a template: the plus stranded genomic RNA forms the template and the primer is the host tRNA Lys3 which has a 3' end is complementary to a sequence at the 5' end of the viral genome called the primer binding sequence (PBS) just downstream of U5 and 18 nucleotides at the 3' end of the tRNA (117).

1.4 The Viral Life Cycle

The retrovirus lifecycle is, by convention, divided into two phases. The first or early phase involves entry to the cell after binding and fusion, followed by reverse transcription of the RNA genome to DNA and the subsequent integration of the viral DNA into the host genome. The late phase consists of viral gene expression and particle formation through transcription, nuclear export, assembly, budding and maturation. Transmission follows maturation and precedes progression to the early phase of the next life cycle.

1.4.1 Receptor Binding

The first phase of the viral replication cycle is viral entry and is a highly-orchestrated process involving a series of protein-protein interactions and the hijacking of various components of the cellular machinery (figure 1.7). CD4 and a co-receptor engagement by Env are necessary events but there are other processes (viral membrane surfing, endocytic entry, synaptic transmission, and intracellular signalling pathways activated through receptors) which may have roles in overcoming host restrictions and enhancing infection efficiency. Viral entry starts following virus host cell adhesion and ends following fusion of the cell and viral membranes and arrival of the viral capsid in the cytoplasm.

Both viral Env protein and/or host cell membrane proteins incorporated into the virion enable host cell to virus binding. Env mediated attachment can be non-specific (Env is able to interact with cell surface heparan sulfate proteoglycans (118)) or results from specific interactions between Env and α4β7 integrin (119), or pattern recognition receptors such as DC-SIGN (Arrighi et al., 2004). Binding to these attachment factors allows Env to come into close proximity with the viral receptor, CD4, and co-receptors and has been shown to augment infection *in vitro* and *in vivo*.

Binding of Env to the cell surface receptor CD4, its primary receptor, is the first step of entry which is known to be required for infection (121). Env comprises a glycosylated trimer of gp120 and gp41 heterodimers. There are five conserved domains (C1–C5) in the gp120 subunit along with and five variable loops (V1–V5), which show wide genetic heterogeneity between them. The variable loops are located on the surface of gp120 and their position and variability, especially the V3 loop, have important roles in immune evasion and co-receptor binding (122). The gp120 subunit binds CD4, a member of the immunoglobulin superfamily that enhances T cell receptor signalling (121,123). Direct interaction between the highly conserved CD4 binding site of gp120 and CD4 is the first irreversible step in HIV-1 entry (103,124).

Env binding to CD4 leads to conformational changes in V1 and V2 which cause the exposure of the third variable loop and formation of a 4 stranded β-sheet called the bridging sheet. These are both co-receptor binding sites and this initiates the third step in viral entry: co-receptor engagement (125,126).

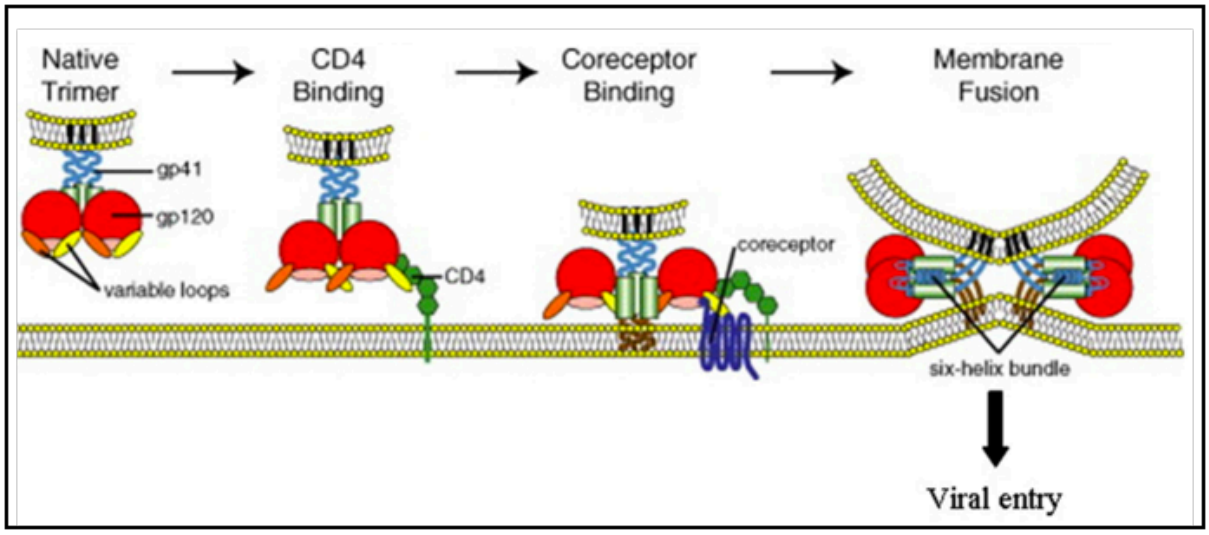

Figure 1.7 HIV-1 entry. The gp120 trimer (red) undergoes structural changes following binding to CD4 (green). These open variable loops V1/V2 and V3 (orange and yellow), exposing the CD4 "bridging sheet" that will be recognised by the co-receptor (CCR5 and/or CXCR4) and trigger the insertion of the gp41 fusion peptide into the cell membrane. Adapted from Connell, B.J., and Lortat-Jacob, H. (2013). Human Immunodeficiency Virus and Heparan Sulfate: From Attachment to Entry Inhibition. Front. Immunol. 4, 385

1.4.2 Co-receptor engagement

HIV-1 can use two different co-receptors, CXCR4 and CCR5 (127–132). Both CXCR4 and CCR5 are seven transmembrane domain G coupled chemokine receptors. Early studies found that CD4 was insufficient for infection by HIV and this lead to the search for additional receptors (121,133). In 1995, CXCR4 was identified as a major HIV co-receptor (134). CXCR4 was found to function as a co-receptor for what was previously called T-cell line tropic strains of HIV but not for macrophage tropic viral strains unable to enter T-cell lines. The natural ligands for the CCR5 receptor (MIP-1a, MIP-1B and RANTES) were found to block entry into CD4+ T-cells and this lead to the discovery of CCR5 as a co-receptor for macrophage tropic virus strains (130,135). The importance of CCR5 was demonstrated when a 32 base-pair deletion in *ccr5*, termed *ccr5Δ32* was discovered to cause retention of CCR5 in the endoplasmic reticulum. Homozygosity confers a high level of resistance to HIV-1 while heterozygosity confers partial resistance to infection and disease progression (136).

Chemokine receptor CCR5 using viruses are termed R5 HIV. Viruses that use CXCR4 are termed X4 HIV. Viruses that can use both co-receptors are called dual or R5X4 (137). With

few exceptions only R5 and R5X4 viruses are transmitted between infected individuals(138). This is poorly understood but likely relates to available, infectable cells at the anatomical sites of infection i.e. predominantly mucous membranes. Viral chemokine use or tropism is generally related to immune status. In early stage HIV infection, almost all HIV is CCR5-tropic, sometimes referred to as R5 virus. In patients with more advanced HIV infection, about half will have X4 virus (HIV that uses the CXCR4 co-receptor) or dual-/mixed-tropic virus (139,140). Co-receptor switching is poorly understood. X4 viruses may be more recognisable to the immune system and so do not arise until dysregulation has already occurred or it may be that the virus is forced to switch tropism after the body becomes depleted of CCR5 expressing cells (141).

Whilst the structure of CD4 alone and in complex with HIV has been solved less is known about the structure of Env with the HIV co-receptors (103,124) and the precise interactions of Env and co-receptor have yet to be elucidated.

HIV may hijack host cell machinery to gain access to sites with greater membrane fusion efficiency. If the site of attachment is distal, on filopodia or microvilli for instance, HIV has been shown on cell lines to use a “surfing” process to move to a more proximal part of the cell. This may facilitate endocytic uptake, allowing the virus to reach regions with high co-receptor expression levels enabling fusion to take place close to the nucleus, the ultimate target of the virus. It is not known if this is an important process *in vivo* (142).

1.4.3 Membrane fusion

The final stage of viral entry requires further conformational change in gp120/gp41 induced by Env binding. Gp41 has a hydrophobic fusion peptide which is inserted into the host cell membrane and then folds at a hinge region bringing together the amino-terminal and carboxy-terminal helical regions of each gp41 subunit, forming a bundle of six-helices (6HB)(102). The fusion peptide brings the amino-terminal region domain close to the host cell membrane, while the gp41 transmembrane domain holds the carboxy-terminal helical region domain close to the viral membrane, so the 6HB brings the two membranes into apposition, forming a fusion pore (135).

1.4.3.1 Cell to cell transfer

The efficiency of viral infection in cell culture systems is limited by attachment of the virus to a target cell and can be improved by simple methods such as spinning the virus down in culture medium to increase the number of cellular contacts virions (spinoculation). But, in an infected person most virus is in $CD4^+$ T cells in lymphoid tissue where uninfected potential

target cells mix closely with infected potential donor cells so cell-to-cell transmission between $CD4^+$ T cells in contact with each other may represent the most frequent mechanism of HIV-1 spread (126,143,144). This cell to cell spread is estimated to be 10-100 fold more efficient than cell free spread *in vitro* and may play a role in antibody evasion *in vivo* (145,146).

Understanding of immunological synapses enabled the identification of retroviral transmission between T cells via polarized virological synapses (VS) between T cells (147). Transmission across a synapse describes virus exiting the donor cell into a synaptic cleft between the donor and the target cells. This process is separate from virus transmission facilitated by contacts between cell cytoplasm (145,148). The cellular events underpinning T cell-T cell HIV-1 virological synapses are poorly characterised but transmission depends on extensive cytoskeletal modification in donor and target cells(146,149).

The CD4-gp120 interaction is essential for the formation of a VS but it is thought to be stabilised by integrin-ICAM-1 interactions (143) and also to require lipid raft integrity, cell surface adhesion molecules, tetraspanins and tyrosine kinase signalling (143). It has also been demonstrated that HIV-1 hijacks $CD4^+$ T cell regulated secretory pathways to enable cell-to-cell transmission (119,150,151).

Dendritic cells (DCs) can cause HIV infection of co-cultured CD4+ T cells without becoming infected (152). DCs are professional antigen-presenting cells (APCs) which roam peripheral tissues, sampling antigens as they do so. They are frequently located in the mucosa where exposure to foreign antigens may be more likely and may thus be among the first cells that an HIV virion encounters on transmission to a new individual. DCs migrate to the lymph nodes following binding of antigen, where they present the antigen to T cells. This initiates an adaptive immune response. Low CD4 and co-receptor expression, constitutive expression of restriction factors and blocks to transcription mean that DCs are relatively resistant to productive infection (153). Nonetheless, DC can bind HIV virions, most likely via a C-type lectin. Structural studies of the infectious synapse show that DCs produce membranous protrusions which form surface-accessible invaginations that trap virions. On contact with DCs, CD4+ T cells extend filopodia, which have dense clusters of CD4 and coreceptor, into these invaginated compartments where the virus is transferred (154,155) thus enabling evasion of intracellular detection or restriction in DC.

1.4.3.2 Location of Membrane Fusion

Other mechanistic details of viral entry are less clear, especially with respect to viral particle endocytosis by target cells involved in virological synapses. It is not fully resolved whether HIV entry occurs at the cell surface or is endosomal or both. Unlike many viruses HIV does not

require entry into a low pH, intracellular compartment in order to trigger membrane fusion. It was believed that HIV fused at the surface of the cell since 1) Env expression at the surface allows cell - cell fusion 2) increasing endosomal pH does not inhibit HIV fusion and 3) preventing endocytosis of CD4 does not affect HIV infection. But a series of studies have provided inconsistent results about whether endocytosis may be necessary *in vivo* (reviewed in Uchil and Mothes, 2009). Experiments using single virion fluorescent imaging combined with lipid and content mixing assays in HeLa cells indicate that entry takes place in perinuclear compartments and therefore that fusion requires endocytosis (157). Green fluorescent protein (GFP)-fused HIV-1 Gag traffics from the surface to endosomal structures in the target cell following transmission from the donor using live microscopy (158). The generalizability of these experiments is difficult to determine since HIV is diverse both in terms of genetics and cell types infected. Allowing the fusion event to take place closer to the nucleus may improve efficiency and could help to avoid innate immune detection (reviewed in Sloan et al., 2013; Wilen et al., 2012b) and it could be the case that while HIV entry is pH independent it may still occur in endocytic vesicles in certain circumstances.

1.4.4 Post-entry events

The virus loses its envelope during entry and the remaining viral components must traffic to the nucleus, and convert the viral RNA to double stranded DNA ready for integration. While the biochemistry of reverse transcription is well understood the timing, location and cofactors for uncoating, genome replication, and nuclear trafficking have proved more challenging to investigate. It may be the case that these processes differ in different cell types especially in the cell lines that are often used to study HIV. It may also be the case that what happens to the majority of individual virions, and is easily detectable with biochemical assays is not the same as the comparatively rarer events that actually lead to infection.

1.4.4.1 The constituents of the RTC or PIC

Incoming cytoplasmic viral complexes have been referred to as either reverse transcription complexes (RTCs) or pre-integration complexes (PICs) betraying the uncertainty about the configuration of their components. Indeed, there is now evidence that the RTC comprises an intact core for much of the journey to the nucleus. Attempts to define the components of these have produced varying results probably because the composition changes, accumulating co-factors and then uncoating. As the RTC traffics and the host proteins and structure alter with location. The trigger of initiation of RT is not known and the site of RT has also been the focus of some controversy. It may be that the entire contents of an intact capsid are present including NC, a nucleic acid chaperone that coats HIV-1 genomic RNA which has been implicated in

genome dimerization and packaging, tRNA unfolding and annealing, facilitating strand transfers and destabilising secondary structures in RNA such that RT can progress. (159). IN is also present in the RTC and curiously many IN mutations inhibit RT but not those which alter its enzymatic activity. Thus IN is thought to play a structural role in the RTC. (160). Vpr, Vif and Tat may also be constituents of the RTC but their roles have not yet been elucidated. Vpr localises to the nuclear rim or nucleus and there are various models in the literature where the function of Vpr is to act as a nuclear localisation signal. This PhD proposes that Vpr is largely contained within an intact capsid core and functions to interrupt innate immune signalling through interactions at the nuclear rim only once the core is partially, or completely, disassembled.

1.4.4.2 Site of uncoating

Evidence suggests that the core traffics to the nucleus using a large number of cytoskeletal components including microtubules and actin as well as perinuclear actin filaments called microfilaments (161) but examining the relationships between HIV-1 and the cytoskeleton has proved difficult due to redundancy and toxicity of inhibiting or depleting the proteins involved. Various models have been proposed for genome uncoating and the loss of the viral capsid including i) break down after membrane fusion followed by reverse transcription within a nucleoprotein complex; (ii) gradual break down during RT regulated by host factors; (iii) RT takes place in an intact core which breaks down at the nuclear pore complex (162–167).

1.4.4.3 The early uncoating hypothesis

Experiments with transmission electron microscopy and fractionation of infected cell lysates provided some evidence that the viral core spontaneously uncoats soon after entry into the cytoplasm. However viral cores are hard to detect using visual techniques because in cross-section they are indistinguishable from cellular structures and the fractionation of infected cell lysates uses strong detergents which disrupt important interactions. These experiments therefore produced quite variable results (reviewed in Arhel, 2010). It has also been proposed that uncoating could happen gradually and *in vitro* uncoating assays suggest it is a biphasic process. When virions are stripped of their membrane and MA with detergent the resulting cores can be centrifuged through sucrose to separate the dense cores from monomeric CA and the relative proportions can then be assessed by immunoblot. (168). But there are clear differences between this assay and the uncoating processes in the cellular environment and, since a number of cellular factors are now implicated in core uncoating, the physiological relevance of these *in vitro* biochemical studies may be limited.

1.4.4.4 The late uncoating hypothesis

The variation in the location and composition of cytoplasmic viral complexes may be due to attrition at each stage of viral entry - it has been calculated that only 1 in 8 virions which reverse transcribes will successfully integrate (169) but these experiments were done in various cell lines and may not closely relate to what happens during the course of infection. In primary T cells for example, it is possible that only the virions which reach the nuclear pore complex with the core intact can integrate. There is now increasingly compelling evidence that for infection to be successful the HIV-1 capsid remains intact until contact with the nuclear pore and RT takes place within an intact capsid (165,170). This hypothesis is consistent with observations of other viruses: Herpesviruses, Adenoviruses, Hepadnaviruses and Parvoviruses are all known to dock at the NPC prior to delivering their genomes to the nucleus (171).

There are multiple pieces of evidence for RT taking place within an intact capsid core. Capsid has been detected in RTCs using incorporation of fluorescent dNTPs and furthermore these intact cores could be detected in perinuclear regions as early as 30 minutes post infection (161). Intact capsids have been observed at the nuclear envelope. Cryo scanning em studies by Arhel, et al. using immunogold p24 labelling and DNA flurorescent in situ hybridization (FISH) showed that intact cores containing DNA can dock at NPCs. They also showed that the disappearance of perinuclear p24 correlates with the appearance of nuclear DNA implying that core disassembly and nuclear entry of genome are simultaneous (172). Additionally, studies suing 6aa tetra-cysteine tags, which fluoresce when in complex with fluorescein arsenical binder (FlAsH) and EM has allowed comparison of the shapes of IN containing complexes both in extracellular virions and inside cells. Many cytoplasmic IN containing complexes appear as identical 100nm long conical structures and importantly were often seen in perinuclear regions. Together with the mutational and function studies these imply that intact conical cores can be found and likely have evolved to dock at the nuclear pore prior to disassembly (173).

More compelling evidence comes from observations that assembled capsid proteins interact with several nucleoporins and that these interactions are required for HIV-1 nuclear entry indicating that at least some CA must remain associated with the virus until it reaches the NPC (174–177). Nuclear pore protein Nup153 has been shown to bind hexameric CA by isothermal titration calorimetry (ITC) but not monomeric capsid strongly arguing for the presence of intact capsid at the nuclear pore (178)

It is now hypothesised that prematurely uncoated viruses may trigger innate immune sensing and cellular cofactors which bind CA are required to prevent HIV-1 DNA from being detected by cytoplasmic innate immune sensors so maintaining the integrity of the capsid until arrival at the nuclear pore may be a strategy to prevent innate immune detection, concealing viral DNA from these sensors (165,170).

1.4.4.5 The relationship between Reverse Transcription and uncoating

Despite increasing evidence that RT takes place within an intact core until recently it had been unclear how HIV imported dNTPs into the capsid. By comparing all known CA NTD crystal structures Jacques et al. showed that there is a dynamic electrostatic channel in the center of each capsid hexamer enabling the electrostatic transport of dNTPs. Previous structures suggested that the amino-terminal β-hairpins of each capsid monomer blocked the opening of such a potential pore, but it was demonstrated by comparing X-ray structures that this β-hairpin is flexible and can tilt up to 15 Å away from the axis of symmetry depending on the conditions of the crystal in the X-ray. When the constituent monomers adopt this 'open' β-hairpin conformation, a central channel is formed. Structures that were obtained at a high crystallization pH adopted a closed conformation, whereas structures that were obtained at a low pH adopted an open conformation suggesting that conformational change is possibly dependent on the protonation status of a histidine residue at the base of the β-hairpin. At physiological pH, the β-hairpin assumes an intermediate position, potentially indicating high flexibility. Thus, the β-hairpin may function as a 'molecular iris' opening and closing a central pore in the capsid hexamer. The authors also observed efficient strong-stop reverse transcription (the first DNA synthesis step) when cores were incubated with dNTPs but addition of DNase I, RNase A or benzonase did not prevent reverse transcription, supporting the idea of a size-selective pore which allows dNTPs into the interior of the capsid but not larger nucleases (178).

As well as protecting viral nucleic acid species from innate cellular immune receptors, undertaking RT in an intact capsid may help to maintain the high local concentration of RT and dNTP needed around the template for completion of RT. There is also evidence that RT may itself drive uncoating (Arhel, 2010). If RT is inhibited with NNRTIs (nevirapine) the core is stabilised and uncoating is delayed. This has been assessed using the Fate of Capsid (FoC) biochemical assay involving ultracentrifugation of virus infected cell extracts through a sucrose gradient pelleting intact cores but not monomeric capsid. The RT inhibitor, NVP, increases the proportion of intact capsid (166,179).

1.4.4.6 Host proteins that influence uncoating

Lentiviruses are unusual among the retroviridae as they can infect non-dividing cells. This requires the crossing of an intact nuclear envelope via the Nuclear Pore Complex (NPC). The NPC is fundamental to a vast array of cellular processes from cell division to gene expression. It comprises a 112MDa protein complex of over 500 molecules and 30 different proteins which forms a 39nm diameter channel. The constituent proteins are called Nups followed by number denoting nuclear mass. Nups can be classified into 6 categories depending on position and role: pore membrane proteins (POMs), coat nucleoporins, adaptor nucleoporins, channel nucleoporins, cytoplasmic filaments and nuclear basket nucleoporins. 40kDa molecules can diffuse through the pore but larger molecules require translocation using transportins or karyopherins (180) although it is likely more complex than this simply size related view. Extensive FG repeats line the channel acting as the pore's permeability barrier (181) and all transportins/karyopherins are mildly hydrophic which may be an essential quality to pass through this channel (182). Karyopherins interact with their cargo via nuclear localisation/export signals (NLS/NESs) and direction of travel is determined by a gradient of RanGTP across the nuclear envelope. While classical NLSs have been reported in MA, IN, Vpr and in the cPPT mutations in these regions do not appear to affect nuclear entry in HeLa cells (183) and it is CA that is likely to orchestrate events prior to nuclear entry. MLV is a gamma retrovirus which is unable to infect non dividing cells and chimeric HIV-1 with MLV CA retains the MLV phenotype and certain mutations in CA (A92E and G94D) make HIV-1 cell cycle dependent (184). CA is known to interact with several host cell cofactors include CypA, TNP03, Nups358 and Nup153 and CPSF6 (discussed in section 1.10.1).

1.4.5 Reverse Transcription

1.4.5.1 Reverse Transcriptase

Whilst the exact triggers of RT are not known it is possible that exposure to dNTPs inside the target cell provide a sufficient trigger (163). Reverse Transcriptase comprises a heterodimer of p66 and p50. P66 comprises an RNA/DNA dependent DNA polymerase domain and an RNase H domain. It initially forms homodimers but the RNase H domain is cleaved from one monomer creating the functional p66/51 heterodimers often referred to simply as RT (185). The p66 monomer contains both active sites (the polymerase and the RNase) whilst p51 has a structural role. The polymerase domain assumes a hand shaped configuration. Without a ligand the thumb is opposed but moves back to reveal the nucleic acid binding cleft and the fingers move forward to form a dNTP binding pocket. (162).

1.4.6 The mechanism of Reverse Transcription (figure 1.8)

Minus strand DNA priming and synthesis 1: The viral reverse transcriptase exists as a dimer and has an RNA/DNA dependent DNA polymerase activity (enabling the production of a dsDNA product from a +ssRNA viral genome) and a ribonuclease H activity (which selectively hydrolyses the RNA part of an RNA DNA hybrid but does not degrade unhybridised RNA). Like many other DNA polymerases, RT needs both a template (in this case the plus stranded RNA genome) and a primer.

All retroviruses use tRNAs as primers for their RT enzymes. HIV-1 uses tRNA (Lys,3) which is packaged into HIV-1 virions via interactions with Gag and Pol (186). The 3' sequence of tRNA is complementary to an 18nt sequence within the HIV-1 genome called the PBS (primer binding site) which lies immediately 3' to the 5' LTR. Priming from this site reverse transcription synthesises the U5 and repeat minus strand DNA from the plus strand RNA template.

Minus Strand transfer: The DNA copy of the R sequence at the 5' end of the viral genome hybridises with the R sequence at the 3' end. This is called first strand transfer since the strong stop DNA minus strand is transferred from the 5' end to the 3' end of the genome RNA. The exact mechanism by which this takes place is not clear. Conventionally the complex is drawn linearly but the molecules may remain circular. In the case of in-core RT the CA and NC proteins and their organization of nucleic acid likely organise the RT process (114).

Minus strand DNA synthesis 2: The strong stop DNA is extended by reverse transcriptase to copy the entire remaining part of the genome RNA up through the primer binding site making a full length minus strand copy of the genome. Because of the strand transfer this DNA

molecule contains a copy of the R sequence between the U3 sequence of the viral RNA and the transferred U5 sequence. This U3 R U5 sequence is the long terminal repeat.

Plus strand DNA priming and synthesis 1: The distance between the polymerase and the RNase H domain of RT can accommodate 17-18 nucleotides and thus RNase H mediated degradation begins 17-18 nts behind DNA synthesis. (186). As it is copied into DNA most of the RNA genome is digested by RNAse H apart from a resistant polypurine tract (ppt) at the 5' end of the u3 sequence. This residual RNA serves as a primer of the subsequent synthesis of plus strand strong stop DNA by reverse transcriptase. After initiation at the ppt this is extended through the U3 R U5 LTR and then the 18 nucleotides of the tRNA that were initially hybridised to the PBS on the genome RNA. This creates the short DNA intermediate "plus strand strong stop DNA".

DNA plus strand transfer and synthesis 2: RNase digestion of the 3' end of the tRNA removes it from the minus strand DNA copy and exposes the primer binding site on the plus strand strong stop DNA which can then hybridise with its complimentary PBS sequence at the other end of the newly synthesised minus strand. This is the second strand transfer and again while it is drawn in a linear fashion the plus strand strong stop may act as a bridge enabling the two ends of the minus strand to circularise. Finally, the minus and plus strands are extended by reverse transcriptase to the ends of their respective template strands creating the proviral DNA.

1.4.6.1 Generation of diversity through reverse transcription

Cellular DNA polymerases proofread using a 3' to 5' exonuclease activity which enables them to remove regions containing mis-paired bases and allowing their repair but RT lacks this ability so between 1-10 nucleotide errors are introduced during synthesis of each provirus. Reverse transcription further contributes to viral diversity by allowing recombination between the two viral genomes to occur. Each HIV-1 virion contains two +ssRNA genomes and RT readily moves between them particularly during long pauses, the strand transfers or when it encounters nicks between the genomic RNA (reviewed in Acheson, 2011; Fields et al., 2013; Hu and Hughes, 2012). This is important for generation of viral diversity through proviral recombination. The high frequency of recombination events perhaps also supports the hypothesis of in core RT as it is unclear how the RT molecule could swap between strands of the strands were floating free in the cytoplasm after uncoating.

1.4.6.2 Drugs which inhibit RT

There are two classes of ARVs which inhibit RT: nucleoside and non-nucleoside reverse transcriptase inhibitors (NRTIs and NNRTIs). There are 11 licenced NRTIs which mimic dNTPs and are incorporated into viral DNA by RT. Since they lack the 3' OH group required for incorporation of the next dNTP they act as chain terminators. The 5 licenced NNRTIs are allosteric inhibitors that cause significant conformational changes to both the polymerase active site and the primer binding site of RT (104,189).

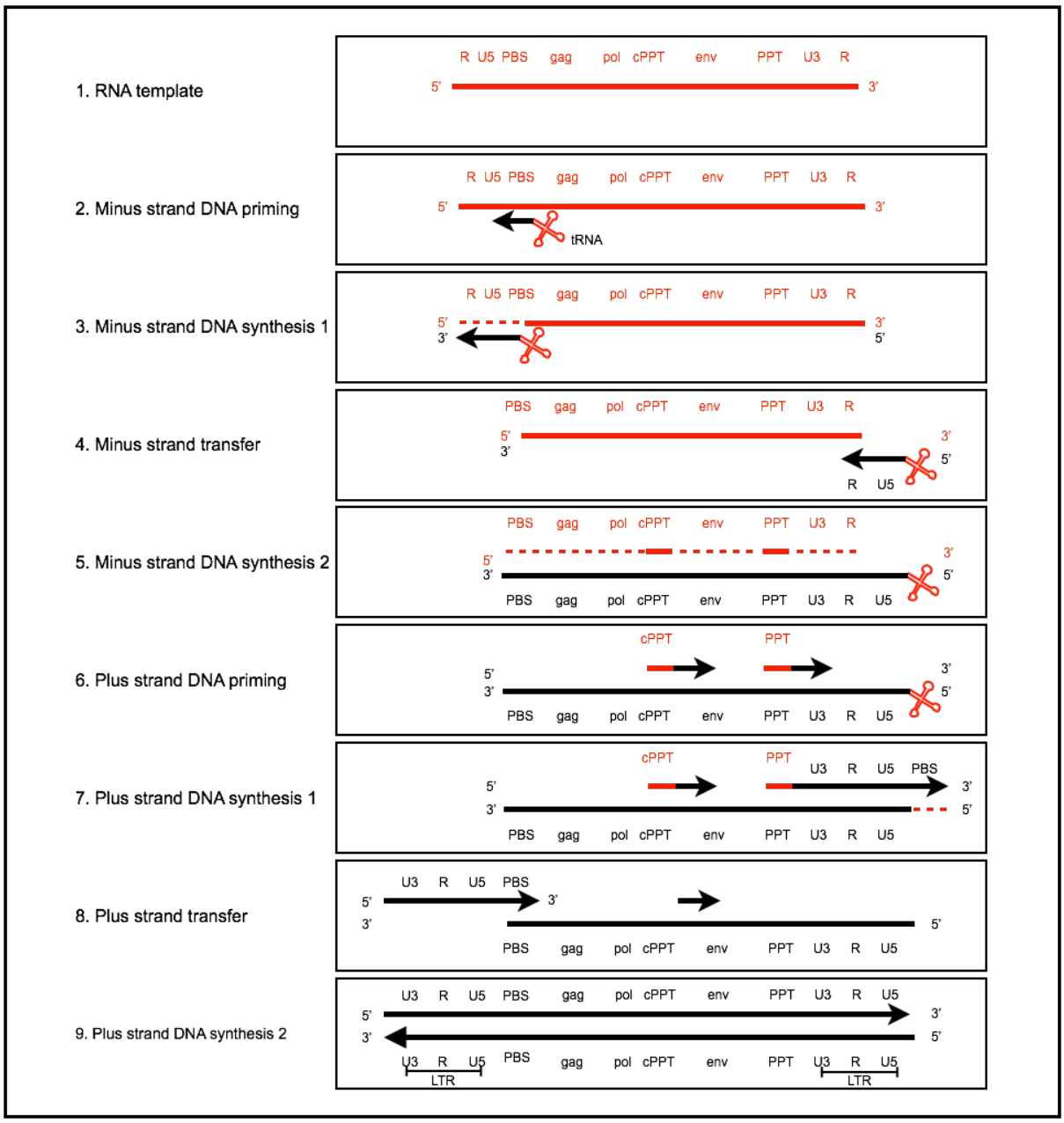

Figure 1.8 The stages of HIV reverse transcription. RNA is coloured red and dotted following degradation by RNase H activity. DNA is coloured black. U3 is the 3' UTR, U5' UTR, R is the repeat region - all are regions of the ITR. PBS is the primer binding site. PPT is the polypurine tract. cPPT is the central polypurine tract. Arrows show direction of polymerisation.

1.4.7 Integration

Once inside the nucleus the HIV-1 DNA integrates into the host genome becoming a permanent part of the target cells genome, copied to any daughter cells during replication (187).

1.4.7.1 Integrase

The viral integrase enzyme (IN) mediates integration. It is a polynucleotidyl transferase with three domains. The structure of the complex of DNA and integrase has not been solved. The blunt ends of the viral dsDNA are bound by a homotetramer of viral IN forming a complex called the intasome. The bound DNA is distorted in the intasome so that each of the two active sites binds a single end of the viral genome. One catalytic site enables 3' processing and the strand transfer reaction for the end of DNA it binds. The distance between these two active sites dictates the 5nt distance between the two phosphodietser bonds attacked during integration. In the first step (3′ end processing), IN catalyses the removal of the two 3' nucleotides from each 3′ end of the viral DNA creating a 2nt 5' overhang at each end. It then catalyses the next stage, called DNA strand transfer, where the free 3' ends target two phosphodiester bonds on each side of the target DNA backbone five nucleotides apart. The 3' ends of the viral DNA are joined to the target DNA covalently. Cellular enzymes repair the two nucleotide overhang on the viral DNA at the 5' end completing integration (190).

Raltegravir is the only IN inhibitor currently licenced. It binds to the intasome interacting with the DNA and with the Mg2+ ions within the active site (104).

1.4.7.2 Circle formation

Viral DNA that enters the nucleus but does not successfully integrate is circularised by the host cell DNA repair enzymes forming one of several dead end viral products. An alternative way of thinking about this may be that whatever DNA is not circularized defensively by the cell, is then integrated. Linear DNA ends may be joined by non-homologous end joining (NHEJ) making a 2-LTR circle. Non-homologous end joining describes nuclear DNA repair events which act as a as a protective host response to double stranded DNA. The result is the ligation of viral LTRs (191). This only occurs in the nucleus so measurement of 2 LTR circles is often used as a measure of nuclear entry. Circles with a single LTR may form from homologous recombination between the LTRs in a 2-LTR circle, or from stalled RT products. Additionally viral DNA ends may autointegrate into pre-existing HIV DNA circles resulting a series of complex circular DNA structures depending on the site of integration and subsequent recombinations (162).

1.4.7.3 Integration site selection - genomic features associated with integration sites

Early analysis of the DNA sequences at integration sites showed a lack of dependence on sequence specificity for the integration reaction: different proviruses have been shown to integrate at different site sequences. But after the sequence map of the human genome was completed in 2001 (192,193) it became possible to map integration sites on this and to measure the genomic features surrounding them in more detail. Schroder et al reported that in SupT1 cells HIV-1 preferentially integrates inside genes and into transcriptionally active regions of the genome (194) and all subsequent measurements of gene density in all cell lines and primary cells have shown strong preference for integration into gene rich regions, with the largest study by Wang examining over 40,000 integration sites of two different HIV1 strains (195–197).

The correlation of many genomic features with each other complicates differentiating causality from mere association with respect to the identification of those features that determine integration targeting. Active transcription units are characterised by high G/C content, gene density, CpG island density and frequencies of Alu repeats, as well as short introns, low frequencies of LINE repeats, and particular epigenetic modifications. These same features correlate with integration sites and many groups have reported correlation with transcriptionally active chromain (H3K4 trimethylation, H3K9 and H3K14 acetylation and H4 acetylation) and negative correlation with markers of transcriptional repression like H3K27 trimethylation (194,197,198).

This favouring of active transcription units by HIV contrasts with the targeting preferences of other genomic parasites which have perhaps evolved to optimise persistence. When cells with a short half life are infected, integration within transcription units is likely to be more favourable for efficient transcription in a short time period. Integration site selection seems to be independent of route of entry; this has been demonstrated by comparing VSV-G pseudotyped virus with HIV R5 env (195).

1.4.7.4 Integration site selection - host factors involved in integration

Host cell factors are important for efficient integration and target site selection. Lens Epithelium-derived growth factor p75 (LEDGF/p75) is a transcriptional co-activator which has a chromatin binding domain and is also able to form a complex with IN and is hypothesised to tether IN to genomic DNA directing integration to LEDGF binding sites. So LEDGF targets the viral genome into genes but not specific genes. Knock out or dominant negative expression causes post entry block to HIV-1 infection (199,200). LEDGF also dictates the location of

integration and fusion of the IN binding domain to other DNA binding domains retargets the viral DNA (201).

LEDGF on its own is not sufficient for integration in gene rich areas; Genome structure can also determine integration site selection; DNA distortion for example can promote integration. Very densely packaged DNA is not accessible so regions of heterochromatin such as centromeres and telomeres are cold spots for HIV-1 integration (202). In euchromatin integration is favoured within nucleosomes (203) putatively because reaction cycle completion requires distortion of DNA so DNA kinked on the nucleosome may have lower activation energy. This may also explain preference for AT rich DNA which has increased flexibility.

1.4.7.5 The role of capsid in integration

CA mutations or depletion of co-factors can also lead to reduction in gene density surrounding integrated proviruses (174,204) and HIV-1/MLV capsid chimeras have been used to show the influence of CA on integration sites (205). It has been reported that CA protein mutant N74D abrogates sensitivity to TNPO3 (a nuclear import receptor) but becomes sensitive to knockdown of other nuclear pore proteins (206,207). Although these studies need further confirmation it was this data that first suggested that specific nuclear pore protein interactions were required for PIC nuclear transit and that multiple redundant pathways may be used for nuclear entry. We now know that capsid dictates interaction with HIV1 cofactor such as CypA, TNP03 and Nup358 whose depletion also changes the average gene density surrounding HIV-1 integration sites (204). Currently the most accurate model for predicting integration sites still shows regions of unexpectedly high and low density so there may be as yet undiscovered co factors (197).

Whilst integration into a transcriptionally active region would seem to be obviously beneficial for the virus it may come with some penalties. If a provirus is located near a highly transcribed gene in the same orientation then transcriptional read through may occur (208) and if it's in an opposite direction then convergent transcription may occur.

And while microarrays of infected and uninfected cells show that the median level of transcription for viral genes is 1.5-3x higher than host genes (194) the protein Tat enhances viral transcription up to 75 x suggesting that while a local environment can alter proviral transcription tat will enhance activity of all proviruses (209).

1.4.8 Latency

HIV-1 can persist as a transcriptionally inactive provirus following integration in a resting memory CD4+ T cell. This is the basis for the latent reservoir (LR) with a long half-life (210). The reservoir seems to be created in the early stages of infection and has been shown to be both quantitatively and qualitatively stable. Siliciano et al. showed the half-life for latently infected cells which could produce virus in a quantitative virus outgrowth assay was 44-months (211). They estimated that eradication of the latent reservoir would therefore require over 70 years of treatment. These studies imply that the latent reservoir in resting memory $CD4^+$ T cells ensures persistence of the virus for the lifetime of the patient. For patients on long-term suppressive anti-retroviral therapy both HIV-1 DNA levels and genetic compositions seem to be stable with little evolution of the virus (212,213).

Since HIV-1 in the periphery has not been observed to establish productive infection efficiently in resting $CD4^+$ T cells (214,215) the LR may be established when activated CD4+ T cells become infected and revert back to a resting memory state. HIV-1 efficiently infects activated $CD4^+$ T cells many of which are hypothesised to die quickly as a result of the cytopathic effects of the virus or host immune responses (Ho et al. 1995). However it may be that a small number of these activated infected cells survive and are able to revert back to a resting state creating the latent reservoir. What determines which T cells die and which survive remains contentious (61,62) and the anatomical location of the reservoir is also unclear. Measurments to date have focused on peripheral blood but it may well be cells in lymphnodes that play a more important role.

HIV latency was not considered important until after the advent of Highly Active Antiretroviral Therapy (HAART) in the 1990s. Prior to this RT-PCR assays for viremia (216) showed active replication through all stages of infection. The primary strategy evolved for immune response evasion is not latency but rapid generation of escape mutations which prevent recognition by neutralizing antibodies and cytotoxic T lymphocytes (217). Latency became an issue when it was clear that populations of infected cells could survive prolonged treatment courses and by as early as 1997 persistence of latently infected cells in patients, despite responses to HAART, had been demonstrated (210).

1.4.8.1 Maintenance of latency

Even in latently infected cells proviruses are frequently integrated in highly expressed genes suggesting that transcriptional interference plays a larger role in latency that integration into non-active heterochromatin (205). Because HIV-1 gene expression depends on host transcription factors which are induced after antigen exposure, it is possible for the

transcriptionally silent provirus to persist in cells whose very purpose is to survive for prolonged periods in a resting state.

While heterochomatin may not play a major role, other aspects of chromatin biology and structure may be important. Nucleosomes consist of 147bp of DNA wrapped around an octet of histone proteins. Post-translational modification of the histone tails regulates the nucleosomes and governs gene expression. There are two nucleosomes which consistently form in the 5' LTR of HIV even when it is integrated in transcriptionally active euchromatin. Because they overlap with transcription factor binding sites for tat these nucelosomes regulate 5'LTR transcriptional activity and therefore promote HIV gene expression (218).

In resting CD4+ T cells, therefore, the absence of the necessary activated cellular transcription factors, together with the absence of Tat and its cellular cofactors limit viral transcriptional initiation and elongation. DNA methylation and repressive histone modifications may further repress proviral transcription.

1.4.8.2 Ongoing replication

Persistent virus replication and rapid evolution in lymphoid tissue reservoirs has been demonstrated by some studies and may contribute to reservoir maintenance (71,219,220). Most studies however indicate that it is the expansion and contraction of individual CD4 cell clones that contributes to reservoir maintenance(212,221). A recent study compared HIV-1 DNA from patients after many years of suppressive ART with the viral sequences from before ART in the same patients and found that the viral populations remained genetically stable for almost 2 decades (222). The disagreement between studies may be because in the shorter duration studies, death of short lived cells in the first few months of treatment results in sequence changes which look like backwards evolution or it may be that there is ongoing viral replication but all the studies to date have measured total HIV-1 DNA and replication is occurring below the detection limit.

1.4.8.3 Latency and cure

The long half life of resting T cells has been postulated to prevent cure by ART alone and one of the main challenges in the search of an HIV cure is to develop strategies that can eliminate the persistent viral reservoir. In the latent state, the virus is unaffected by antiretroviral drugs or immune responses but if the host cell encounters antigen, or other activating stimuli, then latency may be reversed, with the subsequent initiation of virus production. This idea provides a major approach to curing HIV: infected cells in which latency is reversed may die from either direct viral cytopathic effects or be killed by specific cytotoxic T lymphocytes. Novel cellular

infections caused by virus released in this process would in theory be prevented by HAART. This approach, called shock-and-kill(223,224), comes from the early discovery that histone deacetylase (HDAC) inhibitors can reactivate HIV-1 from latency(225). Putting this approach into practice has proved challenging: while a single dose of the HDAC inhibitor vorinostat, has been shown to induce HIV Gag RNA associated with cells in some patients, it proved to be due to read through transcription of host genes, not functional *de novo* proviral transcription (226).

Compounds which are unable to activate T cells, such as HDAC inhibitors, have been the focus of drug discovery and clinical trials. Use of novel viral outgrowth assay to test drugs that do in fact activate T cells (PMA + ionomycin) showed that these agents may be necessary for viral transcription (227). This presents the likelihood of significant off target effects. Our current understanding of the mechanism of HIV latency is rudimentary and identifying small molecules capable of reactivating all HIV-1, with a tolerable side effect profile, will be a significant challenge.

1.4.9 Transcription of HIV

A single promoter in the 5' LTR mediates transcripton. The viral proteins come from a single primary transcript. This happens three ways: (i) alternative splicing (where ambiguous splicing signals generate unspliced, singly spliced or double spliced mRNA), (ii) leaky scanning of the initiation codon (which happens in the case of the mRNAs encoding vpu/env and rev/nef where the first initiation codon is occasionally missed allowing the second protein to be made), and (iii) ribosomal frameshifting (the mechanism by which gag and gag-pol are produced from the full length unspliced mRNA – see below).

1.4.9.1 Initiation of transcription

Initiation of transcription requires inducible host transcription factors (TFs) including NFkB, KFAT and AP-1 all of which are activated by extracellular stimuli including T-cell receptor ligation and a range of cytokines (228). Binding sites for NFKB and NFAT are present in the U3 of the LTR which also contains binding sites for other transcription factors such as Sp1, LEF and ets-1. In resting cells, both NF-κB and NFAT are cytoplasmic and inactive, and thus are not available to promote transcription. Sp1 also binds three sites in the core promoter and may act, through association with TATA-box-binding protein (TBP), TBP-associated factor (TAF) 250, and TAF55 (229). Sp1 and κB site proximity has been shown to be crucial for full LTR activation (230).

The NF-κB/Rel family of transcription factors both activate and inhibit the HIV-1 LTR. Once bound the p50 homodimer recruits HDAC1 and leads to chromatin condensation and transcriptional inhibition (231). p50/RelA heterodimers (the prototypical NF-κB complex) are able to displace p50 homodimers and induce strong transcriptional activation by recruiting p300/CREB-binding protein (CBP). CBP causes acetylation of histone tails and increased RNA Pol II and TFIIH/CDK7 complex binding. This complex mediates promoter clearance and activation of the RNA Pol II (232).

RelA has shown to enable recruitment of P-TEFb complex to the HIV LTR. This complex, comprises cyclin T1 and CDK9 and also phosphorylates the CTD of RNA Pol II. This promotes effective Pol II elongation. The complex is released from an inactive 7SK snRNP complex by HIV Tat causing increased Pol II elongation (see below) (233). HIV-1 proviral transcription is initiated when transcription factors bind the promoter in the 5′ long terminal repeat (LTR). These include NF-κB, Sp1, the TATA box binding protein, and RNA polymerase II. The host RNApolymerase II (RNAPII) starts transcription and enables production of a small number of viral transcripts, which can then be spliced and translated.

1.4.9.2 The role of Tat

Trans-activator of transcription (or Tat) is an early, basic 85 AA protein produced by doubly spliced HIV-1 mRNA and localised to the nucleus. Tat is not required for the initiation of transcription by RNAPII but it increases the efficiency of elongation which allows the production of more Tat in a positive feedback loop. Tat activity requires a regulatory element found downstream of the transcription start site: the transactivation – responsive region (TAR). TAR forms a stem loop structure in viral RNA with two functional regions. The first is a conserved 3-nucleotide (nt) pyrimidine bulge that binds the Tat protein. The second is a 6-nt loop which shows Tat-dependent binding to pTEFb. pTEFb has a kinase component, which, after TAR binding, causes phosphorylation of the C-terminal domain of RNA polymerase II, thus enhancing its processivity (233–236).

1.4.9.3 Nuclear export of mRNA

More than 40 different species of mRNA are produced from provirus due to splicing between 4 donor 5’ splice sites and 8 acceptor 3’ splice sites. These fall into three groups: 1) unspliced (9Kbp encoding Gag and Gag-Pol and are also the viral genome. 2) incompletely spliced (~4Kbp species encoding Vif, Vpr or ENv and Vpu) and 3) completely spliced ~1.8Kbp species that encode Tat, Rev or Nef (6). Only cellular messenger mRNAs which are completely spliced are transported to the cytoplasm (237). Other mRNAs produce non-functional proteins

so initially only the fully spliced viral mRNAs encoding Tat, Rev or Nef can be exported and translated.

But for viral replication retroviruses require transport of unspliced mRNA to the cytoplasm because they produce the required mRNAs from full-length transcript which has been alternatively spliced, and because their viral genomic RNAs (gRNAs) are unspliced. Accordingly they have evolved the ability to transport their intron-containing RNAs from nucleus to cytoplasm. The viral mRNAs exit the nucleus using a viral protein Rev via a pathway that normally exports small RNA species. Rev is needed to export of 9- and 4-kb unspliced and incompletely spliced mRNAs encoding viral proteins from the nucleus, but not the 2kb RNAs(238). So when Rev expression is low the 2kb class (including those encoding Rev, Tat and Nef) are expressed. As levels of Rev rise the remaining mRNAs are expressed.

Rev is an RNA binding protein which binds to the Rev responsive element, a ~250nt sequence that lies within an intron in env and is present in all incompletely spliced or unspliced viral mRNAs. Like TAR the RRE forms a stable multiple loop stem structure in the viral RNA and one of the loops has a binding site for the arginine rich RNA binding region of Rev. Like Tat, Rev binding to the RRE causes conformational changes in the RNA structure (98) and also dimerization of Rev itself. Two Rev monomers form a V shape interacting with adjacent sites in the RRE RNA and creating an interface for more Rev dimers to bind cooperatively. On the surface of the complex of Rev oligomers on the RNA there is a leucine rich nuclear export signal. Exportin-1 (Crm-1) binds to the nuclear export signal and to the GTP bound form of Ran and acts as the receptor for Rev dependent export of the RNAs bound to it. Rev itself functions as an adapter directing the viral mRNAs to a pre-existing cellular export receptor. Translocation of the mRNA, REV and exporting complex requires specific nuceloporins. Once in the cytoplasm hydrolysis of GTP bound to Ran by a protein only present in the cytoplasm induces dissociation of the export complex. Rev then shuttles back to the nucleus to pick up another RNA cargo.

1.4.9.4 HIV-1 translation and frameshifting

All other retroviruses including HIV-1 have evolved frameshifting mechanisms where sequence and structural signals in the mRNA specify a translational mRNA reading frame shift(239). All viral mRNAs are translated on cytosolic polysomes with Env and Vpu as the excpetions. They are translated on the rough ER (240). The longest viral mRNA encodes Gag and Gag-Pol. 1 in 20 times this is translated there is a + 1 frameshift at the end of Gag causing read through into Pol and synthesis of the Gag Pol Polyprotein. This frameshift is due to a hexanucleotide “slippery” sequence (UUUUUUA) and a stem-loop pseudoknot structure

located just 3′ to the slippery sequence. This increases the time the ribosome is associated with the slippery sequence and allows a the resultant frameshift to occur (98).

1.4.10 Assembly, maturation and budding.

Virion morphogenesis can be subdivided into assembly, budding and maturation (figure 1.9). During assembly, the virion components are packaged together. This is followed by budding, where the virion acquires a lipid envelope. Maturation is the process by which the virion develops its final infectious structure. The Gag polyprotein coordinates these stages a in a process that requires the coordinated synthesis of all viral proteins as domains of the Gag and Gag-Pro-Pol polyproteins. This ensures that components are made in correct proportions (95).

1.4.10.1 Contents of a mature virion

An assembled virion must contain 2 copies of the +ve sense viral RNA, cellular tRNA (Lys,3) which act to prime synthesis of cDNA, Env, the Gag polyproteins as well as the viral enzymes. Assembly of all components takes place on the plasma membrane (95).

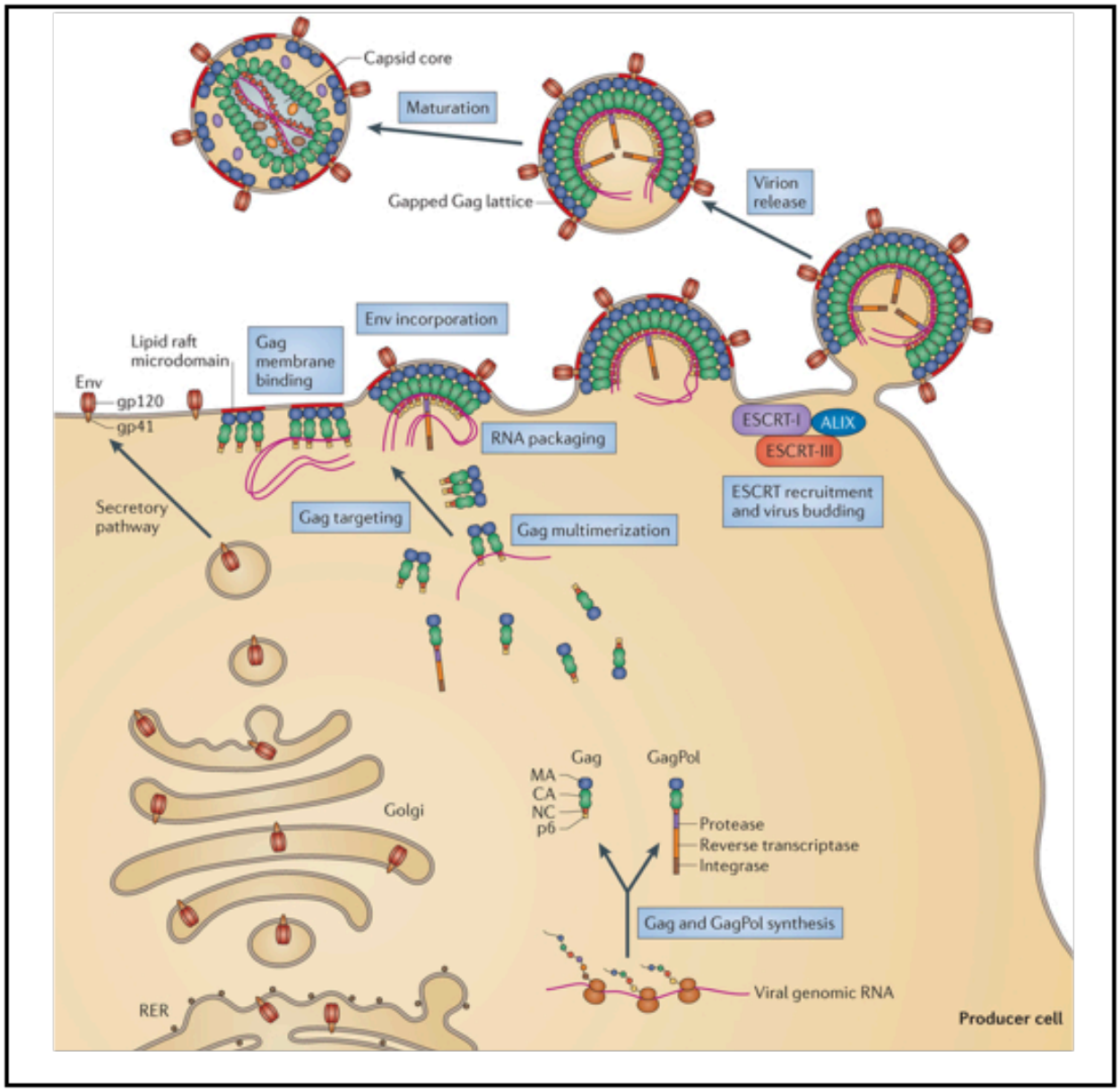

Figure 1.9 The late stages of the HIV-1 life cycle Assembly, budding and maturation stages are coordinated by the Gag polyprotein. Left side: Env glycoproteins are synthesised and glycosylated on the RER, and move to the membrane via the secretory pathway. Right side: Gag is synthesized in the cytosol from full-length viral RNA. 5% of the time a Gag-Pol fusion protein is produced. Gag recruits viral genomic RNA, and forms multimers. At the membrane Gag induces the recruitment and coalescence of lipid rafts which serve as platforms for virus assembly. Following Env incorporation the virion recruits ESCRT-I and the ESCRT-associated factor ALIX. ESCRT-III and VPS4 complexes are then recruited to enable virion release. During maturation the viral protease cleaves Gag and GagPol polyprotein precursors. Adapted from Freed, E.O. (2015). HIV-1 assembly, release and maturation. Nat. Rev. Microbiol. 13, 484–496 (97)

1.4.10.2 The role of Gag and Gag-Pro-Pol

The Gag precursor comprises matrix (MA), capsid (CA), nucleocapsid (NC) and p6 domains, and the spacer peptides, SP1 and SP2. It was previously believed that Gag acted merely as a scaffold, forming the core of the virus. Gag has now been shown to recruit genomic RNA together with viral and host proteins on route to the plasma membrane. It binds other virion components directly and acting as a single targeting signal for assembly of all components (241,242). Gag and Gag-Pro-Pol traffic to the plasma membrane without extensive polymerisation and localize to microdomains (243) (113). This may enable the incorporation of Env glycoproteins into virions (243) and may enhance cell–cell viral transfer at virological synapses (244).

Gag polyprotein coordinates virion assembly, binding the plasma membrane, making the interactions necessary for the creation of spherical particles as well as packaging the viral genomic RNA through interactions with its packaging sequence Ψ. Cytoplasmic Gag is soluble and either monomeric or in low order multimers. Folded domains within Gag are joined by flexible linking regions. These contain the protease cleavage sites. Once at the membrane, Gag extends into a rod and polymerizes at nucleation sites composed of Gag–RNA complexes (113,245).

At the amino terminal of Gag is Matrix (MA) which binds the cell membrane recruiting Env. The amino terminus is also modified during translation by the covalent attachment of a 14-carbon fatty acid. Binding of MA to the plasma membrane specific lipid pohsphatidyl insositl (4,5) bisphosphate (PI(4,5)P2) exposes the myristoyl group and anchors it to the cell membrane (246). The mechanism of action of Gag targeting to the cell membrane remains unclear (247).

The central Gag domain is Capsid (CA) which enables immature virion assembly and then forms the conical core of the virus. Genome recognition and recruiting of viral RNA to the Gag complex is mediated by nucleocapsid (NC) which contains two zinc-finger-like domains. The C-terminal p6 domain is unstructured, and recruits the host ESCRT machinery that causes budding. CA also contains the binding sites for Vpr. The spacer peptides control the conformational changes needed for the maturation phase (97,187,248) .

1.4.10.3 Env incorporation

Env is inserted into ER membranes during translation and is an integral membrane protein. It becomes glycosylated during trafficking and assembles into trimers. A cellular protease (furin) processes these trimers into transmembrane (TM) gp41 and surface (SU) gp120 subunits and

which are taken to the plasma membrane by vesicular transport. It is not understood if Env is incorporated passively into new virions, or through co targeting of Gag and Env to the same specific PM site or if the MA of Gag and Env interact directly (97). Genetic and biochemical studies have shown that the long intracellular tail of gp41 helps coordinate the arrangement of Env into “raft”-like domains (249).

1.4.10.4 RNA encapsidation

RNAs traffic to the cytoplasm, following nuclear synthesis, where they are translated or packaged into new virions. Like all retroviruses, HIV-1 packages two copies of its RNA genome into the virion (115,250). The two RNA genomes in each particle enables recombination and provides some degree of redundancy if one RNA copy is damaged (162). The two RNA strands are dimerized through formation of a “kissing-loop” structure and this acts at the signal for the genome to be incorporated into virions (251).

The zinc finger like domains of the NC domain of Gag bind to a packaging signal in the 5′ region and enable the genomic RNA to be incorporated. This signal spans the major splice donor and the Gag initiation codon (112). Only unspliced viral RNA is packaged precisely because packaging requires signals downstream from the first splice donor. Two copies of tRNALys3 are packaged along with the viral genome through annealing to the mRNA at an 18 base-pair sequence termed the primer binding sites (PBS), found in the 5′LTR (117).

1.4.10.5 Budding

The Gag protein and the full-length genomic RNA assemble into a curved lattice at the cell membrane. This, with the GagPol precursor, begins the formation of the virion. The Gag-RNA contacts initiate assembly and multimerization of Gag is largely determined by CA. The Gag molecules in the immature virion assume a radial orientation around the growing partial spheroid of the nascent particle – like spokes in a three-dimensional wheel. The immature lattice of Gag is stabilized through protein interactions involving the CA-SP1 region (252) and is a continuous sheet with gaps creating the curvature that will form the viral particle (253,254). Cryoelectron microscopy (cryo-EM) and cryoelectron tomography (cryo-ET) techniques indicate the arragment of Gag into hexamers with 8 nm spacing. Gag assembly and budding may be competitive, such that virions bud prior to the final multimerisation of Gag into shells. A virion of roughly 130 nm diameter is estimated to comprise roughly 2500 Gag molecules (241).

Membrane fission releases the particle from the cell membrane. Gag levels increase at the assembly site until budding. Gag is coordinates virion assembly, but the virus additionally co-

opts the host ESCRT pathway which ends Gag polymerization and starts membrane fission (255). HIV-1, in common with other enveloped viruses, has evolved to use the same pathways that cause the membrane fission events involved in vesicle release into endosomes or that separate cells during the final stages of cytokinesis. These events are all catalysed by ESCRT factors which tighten cytoplasm-filled membrane tubules closing them off at the neck (256).

There are three short peptide motifs, or late domains, that drive release through interactions with cellular machinery. The HIV-1 p6Gag PTAP late domain mimics a cellular ESCRT-I recruiting motif, and interacts with tumour susceptibility gene 101 protein (TSG101), a part of ESCRT-I. The second p6Gag late domain, also bears a YPXL-type binding site (Tyr-Pro-X-Leu, where "X" can vary in sequence and length) for the ESCRT factor ALG2-interacting protein X (ALIX) (257).

The interactions with HIV-1 that lead to virus budding are not entirely elucidated but current evidence suggests the following sequence of events. Late domain binding to ALIX and TSG101 recruit ESCRT proteins CHMP1, CHMP2 and CHMP4 which polymerise into filaments forming a dome which starts to constrict the membrane neck of the nascent virion.. The CHMP filaments then recruit VPS4 ATPases which may allow formation of the ESCRT-III dome or removal of ESCRT-III subunits from the dome, reducing its stability and enabling the completion of fission (97,256,258).

Using fluorescently tagged Gag and TIRF microscopy, fluorescence resonance energy transfer (FRET) and fluorescence recovery after photobleaching (FRAP), the assembly time for individual particles has been estimated at 5–9 minutes. Assembling Gag molecules seem to be derived from the rapidly diffusing cytoplasmic pool rather than diffusing laterally in the membrane or entering from vesicular transport (245,259).

Innate immune processes have been selected to restrict these mechanisms. Tetherin is an antiviral protein which tethers nascent viral particles to the plasma membrane thus preventing budding. It in turn is antagonised by the Vpu accessory protein(260).

1.4.10.6 Virion maturation

Maturation is initiated simultaneous with (or immediately following) budding. This process creates the capsid core and is mediated by cleavage of the Gag and Gag-Pro-Pol polyproteins at ten different sites by viral protease (PR). This produces fully processed MA, CA, NC, p6, PR, RT, and IN proteins (6,261). The viral protease efficiency for cleavage of the target sites is variable, leading to a stepwise processing cascade. There are three categories of cleavage site: rapid, intermediate and slow . MA/CA cleavage disassembles the immature lattice leaving

a membrane bound MA layer. CA/SP1 cleavage produces free CA that can form the mature conical core and NC Sp2 cleavage is required for release of NC and formation of the NC/RNA ribonucleoprotein complex (97).

The HIV-1 protease works as a dimer. A “fireman’s grip” of two side chains activate a water molecule to break the peptide bond. During Gag assembly and budding PR becomes active through an undefined mechanism. For PR to be active it must form a dimer, so within the Gag-Pro-Pol polyprotein it is inactive. Before processing PR constructs dimerize only very weakly but transient dimeric “encounter” complexes may have enough enzymatic activity to allow the amino-terminal cleavage site to be inserted into the substrate-binding cleft. This auto-processing will cause exponential growth of levels of stable PR dimers with full catalytic activity (262). Trafficking of Gag may have evolved to prevent premature PR dimerization prior to virion budding and release.

Maturation can be inhibited using 2 classes of ARVs PIs and Maturation inhibitors. All of the 10 licenced PIs interact with the active site of the PR dimer and directly occlude substrate binding. The unlicensed maturation inhibitor Berivimat was discovered to inhibit HIV-1 by binding to the CA-SP1 site, rather than to PR, preventing cleavage.

1.5 HIV and the Innate Immune System

1.5.1 The adaptive and innate immune systems – history and overview

The human body provides a desirable ecological niche for microbial life providing a stable environment in terms of warmth, moisture, pH and nutrition. An elaborate system of defences has therefore evolved to protect against the microbial threats to the body including retroviruses. In vertebrates, the immune system has two branches, an ancient innate system and a more recent adaptive system. Innate immunity comprises physical barriers (skin and chemical defences like acid and mucus), cell intrinsic responses and a set of proteins and cells (including professional phagocytic cells like neutrophils, macrophages and natural killer cells). These innate immune defences do not depend on previous exposure to a pathogen and help to induce and direct the pathogen specific adaptive immune responses.

Charles Janeway was the first to describe the idea of these two interrelated systems (263). He was convinced that antigen could not be the sole determinant of whether a lymphocyte would proliferate and attack – how could a receptor with a randomly generated ligand binding site determine a suitable substrate for the initiation of an immune response? The requirement for adjuvants to elicit T and B cell adaptive responses offered experimental evidence that the adaptive immune response must rely on receptors that have been selected over evolutionary time but which are not randomly generated.

We now know that there is indeed a set of germline encoded receptors which are not randomly generated but which detect conserved molecular patterns in microbes and vaccine adjuvants called pattern recognition receptors or PRRs. They are the molecular trip wires of the cell-autonomous innate immune system and sense pathogen-associated molecular patterns (PAMPs), conserved molecular structures most of which are unique to pathogens and distinct from self. They initiate signalling cascades resulting in the production of type I interferons, pro-inflammatory cytokines and interleukin-1β. Type I IFNs then induce a second round of gene transcription including a large array of antipathogen genes. The IFNs signal through the through the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway (264). These processes trigger early mechanisms of host defence as well as priming and orchestrating the antigen-specific adaptive immune responses (265,266).

1.5.1.1 The role of effector cells

PRR detection of HIV activates both bystander cells and infected cells as well as activating innate immune cells such as Natural Killer (NK) cells and monocytic cells. NK cells comprise

a set antiviral effector cells. Individuals have heterogeneous activation and inhibitory receptor expression on their NK cell populations which determine the effectiveness of the NK response to infection (267). Various receptors are expressed: the polymorphic activating and inhibitory killer immunoglobulin-like receptors (KIRs) interact with MHC I families (268) which act as ligands for inhibitory KIRs. Virally mediated down-regulation of MHC I thus removes the inhibition of killing. NK cells can also recognize and kill HIV-1 infected cells through direct NK receptor recognition of viral proteins or virus-induced stress ligands. In HIV-1–infected individuals viruses are frequently selected with KIR-escape mutations, but the mechanisms remain to be resolved (269,270).

1.5.1.2 The modulation of adaptive immunity

NK cells modulate DC and T cell function, and so inform an adaptive immune response and while the cross talk between these two arms of the immune system is poorly understood in the context of HIV it is plausible that the NK response may influence subsequent antiviral T cell function. Several studies provide evidence that the clinical course of HIV infection may depend on early immune events in the first stages of infection: the severity and duration of the initial seroconversion illness are associated with the rate of CD4+ T cell depletion, AIDS related mortalilty (271) and certain MHC class I alleles (HLA-B27) offer protection against rapid disease progression (272) while early induction of cytokines is associated with delayed disease progression (273).

1.5.1.3 Pattern Recognition Receptors

PRRs can be divided into two groups by localisation. The extrinsic Toll-like receptors (TLRs), (located on the plasma membrane or the endosomal compartment) and the internal intracellular cytosolic nucleic acid sensors. Several families of PRRs have been characterized by structure including TLRs, nucleotide-binding oligomerization domain (Nod)-, leucine-rich repeat–containing receptors (NLRs), RIG-I-like receptors (RLRs), C-type lectin receptors (CLRs) and AIM-2 like receptors. There are also enzymes which sense intracellular nucleic acid. These include the family of OAS proteins and cGAS (274–276).

1.5.1.4 External PRRs

The first extrinsic receptors were discovered in the mid-1990s and include members of the TLR family (277). Cell extrinsic receptors are found in cells specialised in pathogen detection including epithelial and myeloid cells and are able to detect extracellular pathogens. Their ligand-binding domains project extracellularly or into the lumen of the endosome, and they have a cytosolic signalling domain (278). TLR1, TLR2, TLR4, TLR5 and TLR6, and CLRs, dectin-1, dectin-2 and mincle, are specialized in the recognition of common components of

bacterial and fungal cell walls while viral recognition outside the cell is mediated by TLR3, TLR7, TLR8, TLR9 and TLR13 (279). These are able to sense nucleic acid on the endosomal membrane of immune cells. They signal using two pathways: TLR3 activates TRIF whereas TLRs 7, 8, 9 and 13 activate MyD88. Both these adaptors activate NFKB but IRF3 is activated by TRIF alone and IRF7 by the MyD88 pathway. The TLRs show varying ligand specificity for ssRNA, dsRNA or other RNA motifs. (274).

1.5.1.5 Internal PRRs

Cell intrinsic receptors require intracellular localisation of the pathogen and detect viral RNA (RLRs), viral DNA (cytosolic DNA sensors) and bacterial cyclic di-guanylate monophosphate (STING). Nucleic acid species in the cytosol are powerful PAMPs indicating viral infection or DNA damage to the cell and unlike most PAMPs they are not unique to pathogens and so use of nucleic acids as a PAMP is a two edged sword. One the one hand, because all microbes must use nucleic acid, it enables detection of a wider range of pathogens, but on the other it creates the risk of recognising host nucleic acids which is the basis for several autoimmune diseases.

1.5.2 Cytosolic RNA sensing - RLRs

All three RLR receptors (RIG-I, MDA5 and LGP2) share conserved domains including: two N-terminal caspase activation and recruitment domains (CARDs) that mediate signalling to downstream adaptor proteins (275,280). They have preferences for varying RNA ligands, though the ligand for MDA-5 is least well understood. Signalling downstream of RIG-I is best understood. It depends on MAVS (mitochondrial antiviral signalling; or IPS-1, CARDIF, VISA), an adaptor protein. Binding of viral dsRNA releases the CARD and induces MAVS aggregation on the mitochondrial membrane (281). (275). CARD domains that have been released bind to the CARD domain of MAVS, creating prion like aggregations of MAVS molecules. These function as “seeds” binding more MAVS molecules into ever larger aggregates which rapidly amplify RIG-I signalling, such that small amounts of viral RNA can trigger massive IRF3 activation. MAVS aggregates recruit multiple TRAF proteins which in turn induce ubiquitination. These recruit NEMO to the MAVS signalling complex (282,283). NEMO is a subunit of IKK and TBK1 with regulatory functions and contains two ubiquitin binding domains, functioning as a ubiquitin sensor. It is crucial in viral infection for IKK and TBK1 activation and subsequent activation of NF-κB and IRF3(282).

1.5.2.1 Cytosolic RNA sensing - OAS

The IFN-inducible protein 2'5'-oligoadenylate synthetase (OAS) and dsRNA-dependent protein kinase R (PKR) are also able to recognize cytoplasmic dsRNA. Following dsRNA binding OAS catalyzes the activation of the latent ribonuclease RNase L which then degrades viral and cellular ssRNAs - the cleaved products induce more type I IFN production via the RIG-I pathway (275,284,285). PKR is activated by binding to dsRNA and once active it inhibits translation initiation thus inhibiting viral replication and inducing type I IFN (275).

1.5.3 Cytosolic DNA sensing

DNA was known to be a potent Immune stimulant long before it was discovered to be genetic material. In his Nobel Prize acceptance speech in 1908, Mechnikov, the founding father of innate immunity, stated that nucleic acids could recruit protective phagocytes.

"There are already surgeons in France and in Germany, who introduce into the abdominal cavity nucleic acid or other substance, with the object of bringing to the scene a protective army of phagocytes to ward the microbes off. The results achieved are so encouraging that it is possible to predict new progress in the approach to the dressing of wounds."

Despite the intervening 100 years, it has taken until the second decade of the 21st century to understand how DNA trigger innate immune signalling. DNA is typically confined to eukaryotic nuclei and mitochondria, so cytosolic DNA indicates either infections, cellular damage or inappropriate cell division, and triggers robust immune responses including inflammasome activation and type I IFN induction.

1.5.3.1 The cGAS–STING pathway of cytosolic DNA sensing

Various receptors have been identified as putative DNA sensors for detecting viral DNA (including DDX41, DNA-PK, DAI, RNA Polymerase III, IFI-16 and several other DNA helicases) but none has been as universally accepted as Cyclic GMP-AMP (cGAMP) synthase (cGAS) (figure 1.10) (286). The role of these other proteins in cytosolic DNA sensing is uncertain as genetic studies have failed to confirm their involvement. Mouse strains and human cells knocked out or depleted of these proteins using CRISPR show no apparent defect in the induction of interferons in response to cytosolic DNA or DNA viruses. By contrast depletion or deletion of cGAS completely abolishes IFN induction by cytosolic DNA or DNA viruses. (287,288).

PQBP1 has been reported to facilitate the sensing of HIV-1 DNA by cGAS and KD has been shown in to inhibit the induction of interferon by HIV but not induction achieved by infection with DNA viruses but the relationship between HIV, PQBP1 and cGAS remains unclear (289).

cGAS activates innate immune responses mediated by the second messenger cGAMP. This activates the endoplasmic reticulum protein STING (Stimulator of Interferon inducible Genes) (290). Genetic approaches using cGAS deficient mice have shown that cGAS is a sensor for a variety of pathogens. Mice deficient in cGAS or STING do not produce interferons in response to infection by DNA viruses (e.g. HSV-1 or vaccinia virus) have high viral titres and mortality (291). Interestingly these mice are also more sensitive to infection with RNA viruses, despite the fact that they have normal interferon production in response to RNA viruses. It may be that the cGAS pathway produces basal amounts of interferons important for arresting early viral infection *in vivo*. It is also possible that infection with RNA viruses induces cellular damage with 'leakage' of cellular DNA, and that this stimulates the cGAS pathway to help the immune defense against infection with RNA viruses (292).

1.5.3.2 cGAS Structure and regulation by DNA binding

cGAS is a nucleotidyltransferase (NTase) and shows structural and sequence homology to the catalytic domain of OAS. It contains a nucleotidyltransferase domain and two major DNA-binding domains (293). cGAS exists in an autoinhibited state (294) but is activated by all dsDNA independent of structure and length. This DNA-sequence-independent activation is due to cGAS binding to the sugar-phosphate backbone of dsDNA but not to any of the bases as shown by crystal structures. Single-stranded DNA with internal duplex structures can also activate cGAS and short dsDNA (~15 base pairs in length) is sufficient to bind and activate cGAS *in vitro.* Longer DNA is needed to activate the cGAS pathway in cells, putatively because of cytosolic nucleases which degrade short strands (294).

1.5.3.3 cGAMP functions as a second messenger

DNA binding induces a conformational change which initiates the synthesis of cyclic GMP-AMP (cGAMP) from ATP and GTP. This cGAMP contains two phosphodiester bonds and functions as a second messenger by activating STING. (293). cGAMP can also spread from cell to cell through gap junctions enabling rapid responses by surrounding cells. This is important as some viruses have mechanisms to antagonise the production of type I IFNs in host cells which are infected (295). It may also have evolved to be incorporated into virions and some viruses incorporate cGAMP enabling a rapid response to viral infection (296).

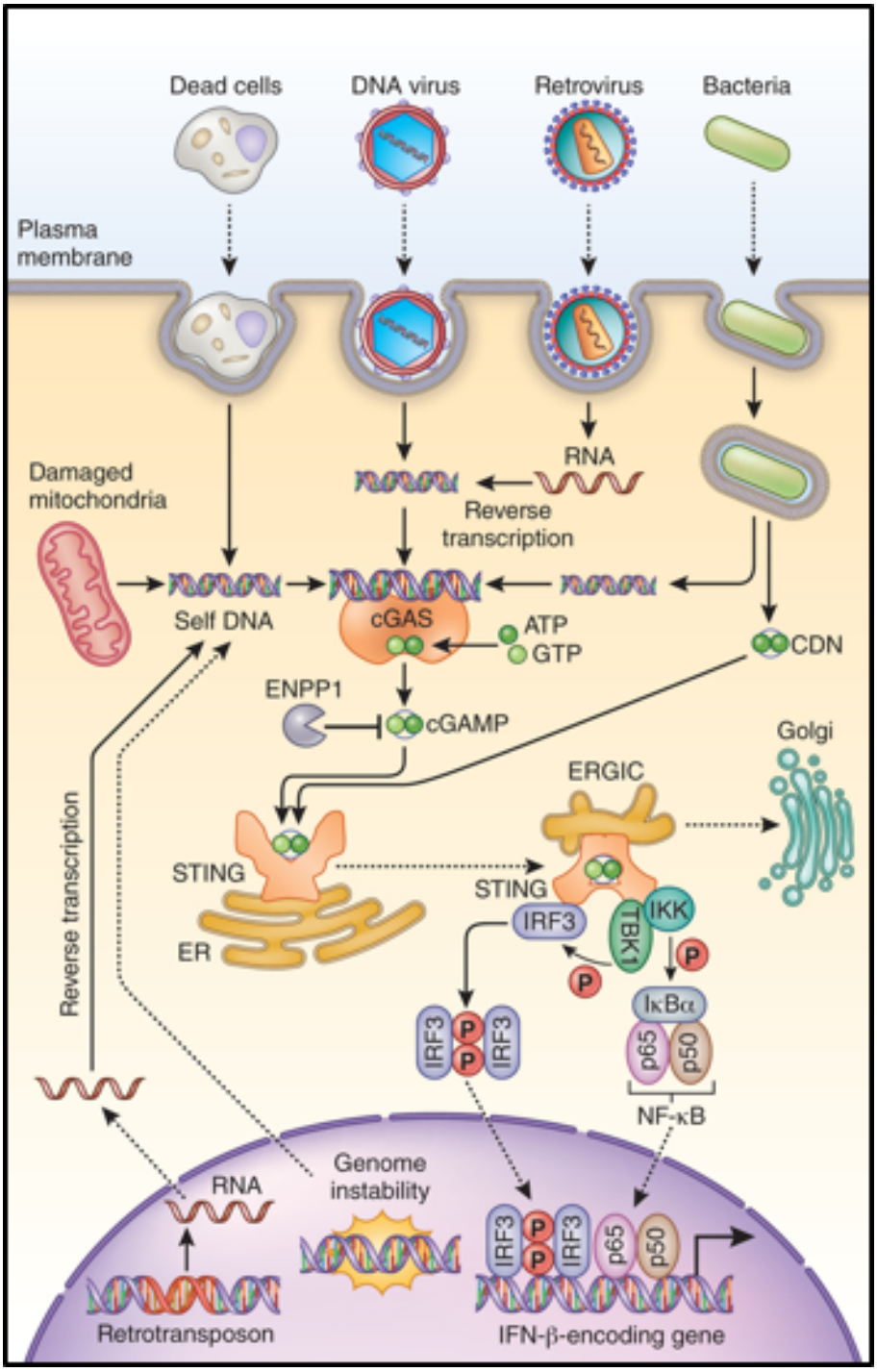

Figure 1.10 The cGAS–STING cytosolic DNA sensing pathway. Cytosolic DNA binds to and activates cGAS, stimulating the production of 2′3′-cGAMP. This binds to STING which once activated moves to the ER-Golgi intermediate compartment and the Golgi apparatus where it activates IKK and TBK1. TBK1 phosphorylates STING, enabling it to recruit IRF3 for phosphorylation. IRF3 then forms dimers and enters the nucleus, where, together with NF-kB it activates expression of type I IFNs and other immunomodulatory molecules. From Chen Q, et al Regulation and function of the cGAS–STING pathway of cytosolic DNA sensingrom Nature Immunology. 13, 484–496 (292)

1.5.3.4 Activation of STING by cGAMP and bacterial cyclic dinucleotides

Stimulator of interferon genes (STING /MITA, MYS, ERIS and TMEM173) was identified as an adaptor of type I IFN production using cDNA expression screens for genes that can activate the IFNβ promoter. STING has a wide tissue distribution (297). It contains four trans-membrane domains at the amino terminus that anchor the protein to the ER membrane, with the large carboxy-terminal domain projecting into the cytoplasm. (298) STING forms a butterfly shaped dimer on the ER membrane with or without a ligand (299–301).

cGAMP is the most potent activator of STING and it can also be activated by cyclic-dinucleotides (CDNs) secreted directly from bacteria; the CDN gene expression profile is indistinguishable cell stimulated by DNA (275). cGAMP binds to STING more tightly than other cGAMP isomers or bacterial CDNs, including cyclic di-GMP and cyclic di-AMP (294), and induces a more potent IFN-β response (302,303).

cGAMP binds to a central crevice of the butterfly shaped STING dimer (294) and induces conformational change moving the “wings of the butterfly” closer together by about 20Å releasing the carboxy-terminal tail (CTT) which recruits and activates TANK-binding kinase 1 (TBK1) (304). This CTT, which has just 39 amino acid residues, is all that is required to activate TBK1 and recruit IRF3 to TBK1, but is not, as yet, visible in crystal structures of STING. (206,294).

1.5.3.5 STING trafficking and signalling

On activation by cGAMP, STING rapidly traffics from the ER to an ER-Golgi intermediate compartment and the Golgi apparatus and forms large punctate structures (305). Inhibition of this trafficking blocks activation of the downstream pathway suggesting that the trafficking is important for activation (306). During this process the CTT recruits and activates TBK1 which in turn phosphorylates STING at several serine and threonine residues. This phosphorylation of STING enables the subsequent phosphorylation of IRF3 by TBK1. Phosphorylated STING binds to a positively charged region of IRF3 and thereby recruits IRF3 for phosphorylation by TBK1. The phosphorylated IRF3 dimerizes and then enters the nucleus. This elaborate process of “back phosphorylation” of STING by TBK1 allows a fine degree of control of downstream signalling. While three adaptors of innate immunity, MAVS, TRIF and STING, can activate TBK1 via their respective RIG-I–MAVS, cGAS-STING, and TLR3/4-TRIF pathways, there are other pathways that also activate TBK1. However IRF3 phosphorylation

and subsequent IFN production by TBK1 is only seen in the same pathways that use MAVS, STING, or TRIF as adaptors (275).

These all share a highly conserved motif that is phosphorylated in response to stimulation with their cognate ligands and this provides a 'licensing' mechanism for the phosphorylation of IRF3 by TBK1 ensuring only a few agents that can activate TBK (e.g. viral infection) lead to IRF3 activation and IFN induction. This specification of TBK1-mediated IRF3 activation allows careful regulation of IFN production, avoiding autoimmune disease.

STING also activates the kinase IKK, which phosphorylates the IkB family of inhibitors of the transcription factor NF-kB. (305). These phosphorylated IkB proteins are subsequently degraded by the ubiquitin-proteasome pathway releasing NF-kB. This translocates to the nucleus, where it functions together with IRF3 and other transcription factors to induce the expression of interferons and inflammatory cytokines such as TNF, IL-1b and IL-6 as well as numerous innate immune genes, including IFN and members of the IFIT family (Abe and Barber, 2014).

Classically TBK-1 is linked to IRF-3 release and IKK phosphorylates the NFKB inhibitor IkB causing release of NFKB subunits but there is evidence that TBK-1 can act directly to stimulate NFKB signalling (307)

STING has also been reported to recruit and phosphorylate STAT6 using TBK1 which causes chemokine induction (308).

1.5.3.6 Reconstitution of cGAS and STING signalling in HEK293T cells

cGAS is induced by interferon providing positive feedback and amplification of the pathway (309). Many tumor cell lines, including the cells used in the majority of the assays in this thesis, HEK293T cells, have silenced expression of cGAS and STING and do not activate an innate immune signalling response following stimulation by DNA or DNA viruses (310). That the cGAS–STING pathway is disabled in cancer cells implies that a functional pathway might prevent transformation putatively by assisting the identification of cancer cells by the immune system.

Human Embryonic Kidney 293 cells (HEK293) and their derivatives are among the most commonly used cells in laboratories. HEK293 cells were derived in 1973 from an aborted embryo. Primary embryonic kidney cells were exposed to the sheared DNA of adenovirus type 5 (AD5) and transformed (311). Despite their widespread use in cell biology, details of their phenotype, karyotype instability, and tumorigenicity are still unclear. The chromosome number

and aberrations vary even between cells from the same culture and from different cell banks and labs. The fact that 293 or other cell lines are easily transfected is poorly understood but the utility of 293 cells in transfection experiments would imply that that they lack intact DNA sensing pathways. It has been shown that transfection of DNA into cells with intact DNA sensing pathways leads to innate immune responses (312). HEK293T cells have no endogenous detectable STING, but overexpression activates IRF3 and NFκB and stimulates IFN-β production (313).

cGAS regulation is poorly understood, but there are likely to be mechanisms to ensure a sensitive and specific response to foreign DNA while remaining unresponsive to self DNA. Studies have suggested that post-translational modifications of cGAS regulate its enzymatic activity their influence on overall cGAS activity is not clear (314).

Understanding of regulation of STING is also in its infancy: after STING has signalled, it is rapidly degraded, possibly by the autophagy kinase ULK1-ATG1 but the mechanism remains to be elucidated. (292,315).

1.5.4 PRRs and HIV-1

Protein and nucleic acid components of HIV are detected by a range of PRRs. The Env glycoprotein gp120 is recognised by surface TLR2 and TLR4 on epithelial cells (316). The HIV-1 genome is recognized by endosomal TLR7 and TLR8 in plasmacytoid DCs and other myeloid cells. (317). RNA helicase RIG-I31 is a cytosolic PRR which may recognise HIV genomic RNA and induce signalling in HIV target cells (318,319). The ISG product Tetherin is expressed on the surface of cells, and 'tethers' virions to the plasma membrane to antagonise their release (320,321). It is also a PRR, inducing an signalling cascade which activates NFκB (322).

Interferon inducible protein 16 (IFI16) and cyclic GMP-AMP synthase (cGAS) sense infection early, by detecting viral reverse transcriptase products. Various other receptors have been identified as putative DNA sensors for detecting viral DNA: DNA-PK, DAI, RNA Polymerase III, and several other DNA helicases have been suggested but none have been as universally accepted as cGAS and IFI16 (Keating et al., 2011).

1.5.5 Evasion of DNA sensing by microbial pathogens and HIV

Viruses and bacteria have numerous strategies to antagonise or avoid the cGAS STING pathway which may include 'hiding' their DNA from the cytoplasm and using cellular co-factors.

1.5.5.1 The role of Capsid, CypA and CPSF6

Retroviruses and many DNA viruses may have evolved to conceal DNA in the viral core while they traverse the cytoplasm and then deliver their viral DNA into the nucleus. HIV-1 viral capsid in the cytoplasm recruits the host factors CPSF6 and CypA. CypA is a prolyl isomerase enzyme that binds to the HIV-1 capsid and isomerizes the peptide bond between residues G89 and P90 between cis and trans conformation. CPSF6 predominantly localizes to the nucleus, and enables in 3′-end mRNA processing. This may help to target HIV-1 to an advantageous nuclear entry pathway (165). Together, these host factors help coordinate reverse transcription, capsid uncoating and entry of the viral pre-integration complex into the nucleus, such that viral DNA is not exposed to the cytoplasm. HIV-1 replication in primary human monocyte-derived macrophages (MDM) is dependent on these co-factors (323). Depletion of these factors, or genetic disruption of their interaction with CA, triggers DNA sensors and activates a type 1 IFN response in macrophages infected with wild-type HIV-, suppressing replication. Blockade of IFN signalling using an IFN receptor antibody largely rescues this replication defect (165,177,323,324). Importantly, CypA and CPSF6 have been shown to be unnecessary for replication in cell lines with defective DNA sensing pathways (323,325) however experiments in our lab have shown that co-factors are required for replication in T cells, which appear to lack DNA sensing pathways. Additionally, co-factors are not required in THPs which appear to have intact pathways. Production of cGAMP is diminished after treatment with RT inhibitors but not with inhibitors of later stages in the replication cycle, indicating that reverse transcription products act as PAMPs for cGAS. The exact ligands have yet to be identified (326).

HIV-2, but not by HIV-1, can infect and activate dendritic cells (327) because HIV-2 encodes Vpx, which degrades SAMHD1. Subsequent recognition of HIV-2 PAMPs by PRRs leads to activation of dendritic cells (328,329). The HIV-1 and 2 capsids have different CypA affinity. They both form a complex with CypA but in HIV-1 CypA –capsid binding masks viral nucleic acid from the PRRs. By contrast, some studies have shown that HIV-2 capsid-CypA binding allows the viral nucleic acid in the cytosol to be sensed by cGAS. So disruption of capsid HIV-1 CypA binding in the presence of vpx leads to PRR activation in DCs. KD of cGAS prevents this activation. KD of IFI16 does not. Some studies have shown that HIV-1 with vpx alone doesn't activate sensing, i.e. in DCs SAMHD1 antagonises replication of HIV-1, but if you add vpx then the capsid interaction with CypA allows evasion of sensing by cGAS. These studies offer the possibility of a vaccine through 'recovery' of the DC innate immune response against HIV-1 through activation of cGAS using PAMPs (327,330).

It may also be that the proteins involved in nuclear import act as co-factors. Various studies describe functional interactions between capsid and components of the nuclear pore including Nup358, Nup153 and the karyopherin TNPO3 (164,167,204,331–334). Nup 153 has been shown to bind capsid at the same interface as CPSF6 and this makes it plausible that Nup153 displaces CPSF6 at the nucleus to enable uncoating, allowing DNA to enter the nucleus without exposure to cytoplasmic PRRs. These processes may enable HIV-1 to evade innate immune activation through hiding viral DNA, and viral DNA ends, from cytoplasmic PRRs (164,175,204,334).

1.5.5.2 The role of TREX1

One of the first cellular co-factors identified as being used by HIV-1 is the DNase TREX1 (335). It is perhaps an unexpected co-factor since it is an ER associated nuclease which causes HIV-1 DNA to be degraded. It may work by degrading, not genomic DNA ready for integration, but cytoplasmic DNA products of mistimed RT or DNA exposed by premature uncoating. Neither of these would be able to establish infection in any case, but both of which could provide a substrate for DNA sensors and a subsequent immune response. Depletion of the DNase TREX1 causes HIV-1 DNA to accumulate in the cytoplasm, which triggers the induction of interferon via cGAS. This sugests that HIV-1 co-opts host proteins to minimize the exposure and accumulation of viral DNA in the cytoplasm, which would otherwise trigger the cGAS–STING pathway (336–338). It is not clear why in the event of premature uncoating, or DNA synthesis, TREX1 does not degrade viral DNA universally protecting HIV-1 from activating an immune response.

1.5.5.3 Specific antagonism of DNA sensing by accessory proteins

Specific viral antagonists of cGAS/STING signalling have been noted in other viruses but not discovered in HIV. The γ-herpesvirus KSHV encodes ORF52, a tegument protein that inhibits cGAS activity through direct binding to cGAS as well as through binding to DNA. Herpes simplex virus type 1 encodes the regulatory protein ICP27, which interacts with STING and TBK1 and thereby prevents the phosphorylation of IRF3 by TBK1. The E1A protein of adenovirus and E7 protein of human papilloma virus inhibit STING signalling by binding to STING through a LXCXE motif (Leu-X-Cys-X-Glu, where ‘X’ is any amino acid) of these viral proteins (292).

1.5.5.4 IFI16 and HIV

IFI16 is an ISG and is expressed both in the nucleus and the cytosol. It has a PYHIN domain mediating protein-protein interactions and can recognise and physically bind to DNA products of RT through a HIN domain which recognises specific PAMPs (including a segment of the

LTR). Much of the literature on IFI16 is conflicting and uncompelling. It has been shown in transfected cells to co-localise with HIV-1 DNA. It signals via the adaptor STING (339). Knockdown of IFI16 has been shown to increase permissiveness to HIV-1 and improve replication although these experiments have not been replicated (340). In CD4+ T cell 'bystander cells' (i.e. non-permissive to infection), IFI16 activates the inflammasome and causes inflammatory cell death or pyroptosis to infection but these data appear, at present, uncompelling (341). It is unclear what determines interaction with STING vs activation of the inflammasome. It is hypothesised to be one of the proximate causes of CD4 helper cell loss in clinical HIV infection. HIV has been shown to induce the inflammasome in different cell types. The inflammasome is a protein complex which activates caspase-1 and stimulates the processing of pro–interleukin 1β into mature IL-1β, an inflammatory cytokine. Non-activated CD4+ T cells are not permissive to HIV infection. HIV-1 can bind and enter the cells because of SAMHD1 depletion of nucleoside pools reverse transcription is limited. These abortively infected cells are known as bystander cells. These cells die by pyroptosis, characterized by the activation of caspase-1. IFI16 is proposed to initiate this inflammasome signalling and T cell pyroptosis by detecting reverse transcriptase products in the cytoplasm. So IFI16 may drive two differential responses controlled by PAMP signalling and interactions with signalling proteins. In activated CD4+ T cells it induces an antiviral state, compared to resting T cells where infection induces inflammasome activation and pyroptosis response (342,343).

In summary, it is not clear why there would be two DNA sensors, both of which signal via STING in HIV infection. They may increase sensitivity to host DNA ligands in the cytosol. TREX1 is an exonuclease which removes DNA from the cell cytoplasm: inactivating mutations in TREX1 lead to severe pathology (Aicardi-Goutieres syndrome, a neuroinflammatory condition). Depletion of TREX1 increases detection of HIV-1 dsDNA in CD4 cells and stimulates an interferon response that abrogates infection (336). So while the primary function if TREX1 is to avoid an inflammatory response to self dsDNA it also acts as a co-factor for HIV infection by limiting recognition of cytosolic DNA PAMPs. Differential regulation of TREX1, by both host and virus, may determine innate immune responses to viral dsDNA.

1.6 HIV accessory proteins, restriction factors and co-factors

With only nine open reading frames HIV-1 carries with it a relatively small set of tools for evasion of innate immune detection. As well as specifically antagonising restriction factors, the IFN-stimulated factors that restrict HIV, with accessory genes (Vif, Vpu, Nef and Vpr), HIV uses cellular co-factors which may provide “cloaking” functions that enable evasion of innate immune pattern recognition receptors (PRRs).

PRR ligand binding initiates signalling cascades which activate transcription factors including IRF3, IRF7 and NFkB which in turn induce antiviral and inflammatory genes including those encoding interferon. This then causes a second wave of transcriptional changes and the induction of hundreds of ISGs. The antiviral genes that are activated include restriction factors APOBEC3, TRIM5a, SAMHD1, tetherin, Schlafen11, IFITM and MX2 all of which can limit HIV infection *in vitro*. In resting cells these proteins may be found at low levels or not at all (344–347).

The accessory proteins were so called because they were not necessary for replication in various cell types *in vitro*. As their roles became clear, it became apparent that they counteract host antiviral defense mechanisms and that there were common themes to their mechanisms for doing so. HIV-1 Vif and Vpu and Vpr lack enzymatic activity, instead serving as substrate adaptors for cellular ubiquitin ligases to antagonise immune responses. Ubiquitination consists of post-translational protein modification which enables regulation of protein degradation and trafficking. E3 ubiquitin ligases transfer ubiquitin from E2 ubiquitin-conjugating enzymes to target proteins. They are multi-protein complexes consisting of a scaffold, a specific adaptor, and a substrate that is the target protein. Each accessory protein thus targets different cellular factors using related mechanisms.

1.6.1 Tetherin and Vpu

The transmembrane protein tetherin acts at the plasma membrane of infected cells, physically tethering budding HIV-1 virions (321). HIV-1 has evolved the accessory protein Vpu to antagonise this restriction. Vpu is also a transmembrane protein which localises to the ER. Interestingly the parental SIVcpt*Ptt* uses Nef to antagonise tetherin. Species specificity studies have provided evidence that its antagonism was required for the pandemicity of HIV-1M. Zoonotic transmission of SIVcpz required the virus to evolve another way to antagonise tetherin. A small number of amino acid changes enable SIVcpz/HIV-1 M Vpu to target human

tetherin (348). HIV-2 antagonises tetherin with its envelope glycoprotein protein illustrating immense adaptability in viral strategies to avoid and antagonise host cell restriction.

Δvpu HIV-1 virions cluster on the plasma membrane without budding (349,350). Additionally, they have been shown to have fewer glycoprotein spikes. Vpu relieves the block to virus release by sequestering tetherin near the nucleus and leading to its endocytosis and degradation in the proteasome.

Tetherin also acts as a PRR. As it tethers budding virions it detaches from the actin cytoskeleton at the cortex of the cell. It is subsequently phosphorylated and recruits the signalling adaptors TRAF2, TRAF6 and the mitogen-activated protein kinase TAK-1 and induces NFκB mediated interferon production (351,352).

1.6.2 TRIM5α

The existence of a restriction factor for the capsids of HIV-1, SIV, and other retroviruses was predicted when restricted infection was observed in a various mammalian cell lines (353). TRIM5α was identified in a screen of rhesus macaque genes and was demonstrated to restrict HIV-1 infection when expressed in human cells. TRIM5α is a ~500 amino acid cytoplasmic protein and it restricts early in the life cycle. TRIM5α restriction is characterised by inhibition of viral cDNA sythesis (179). TRIM5α is part of the large tripartite motif family. In humans there are around 100 genes in this family and all, including TRIM5α, comprise a RING E3 ubiquitin ligase domain, one or two B box domains, and an alpha-helical domain. The carboxy-terminal domain is not highly conserved - in the case of TRIM5α it is a PRYSPRY domain determines capsid recognition species specificity.

Different species TRIM5α inhibit different retroviruses. The human TRIM5α protein restricts N-MLV, as well as equine infectious anaemia virus (EIAV) but is has no effect against HIV-1. Conversely, TRIM5α proteins from Old World monkey species generally inhibit HIV-1. Since TRIM5α proteins often only weakly inhibit retroviruses in their host species, but are active against retroviruses found in other species they pose a barrier to cross-species transmission infection (179). The mechanisms by which TRIM5 proteins act to block retroviral infection are not completely understood. Retrotransposition events in owl monkeys and rhesus macaques resulted in chimeric TRIM5-CypA fusion proteins (TRIMCyp) with a CypA protein domain replacing the PRYSPRY domain (179,354). These proteins are potent inhibitors of lentiviruses like HIV-1 whose capsids bind CypA. TRIM5α and TRIMCyp bind directly to HIV-1 capsids. Recent studies have shown that, as well as being a retroviral restriction factor, TRIM5α acts as a PRR specific for capsid, and initiates innate immune AP-1 and NFκB signalling (355).

1.6.3 APOBEC3G and Vif

All primate lentiviruses have the accessory gene, virion infectivity factor (Vif). It antagonises the APOBEC3 family of cytidine deaminase restriction factors which restrict HIV by causing hypermutation, cytidine to uracil deamination of the viral DNA minus strand, and suppressing DNA synthesis (356). It is expressed in many cell types, including $CD4^+$ T cells and MDMs. Incorporation into assembling virions is needed for infectivity (357). Packaged APOBEC3 may also prevent primer-binding site binding of tRNA and thus terminate synthesis of minus-strand DNA (358).

Vif induces ubiquitin mediated degradation of APOBEC prior to its incorporation into the virion. It binds to APOBEC3 molecules and recruits a cullin 5–based E3 ubiquitin ligase complex. The bound APOBEC3 proteins are ubiquitinated and degraded at the proteasome (359,360). Paradoxically, by generating sequence diversity and accelerating evolution through non-lethal mutagenesis, the packaging of small quantities of APOBEC3 proteins may provide a selective advantage to the virus, and there is experimental evidence that APOBEC3 may increase the rate of drug resistant virus and escape from cytotoxic T lymphocytes (361,362).

1.6.4 SAMHD1 and Vpx

SAM domain–and HD domain–containing protein 1 (SAMHD1) is a deoxynucleoside triphosphate triphosphohydrolase expressed in myeloid cells and resting T cells (363,364). It consists of an N-terminal SAM (sterile alpha motif) domain and a C-terminal HD domain characteristic of enzymes with phosphodiesterase, phosphatase and nuclease activities. It contains an NLS and localises to the nucleus. It restricts HIV-1 infection by reducing the concentration of dNTPs to a level below the threshold required for reverse transcription. Addition of exogenous deoxynucleosides (dN) removes restriction in DCs (365). It may also have an RNA and single strand exonuclease activity which helps in restricting virus replication (366).

SAMHD1 is antagonised by virion packaged Vpx an accessory protein found only in HIV-2 and some SIVs (from both red-capped mangabeys, SIVrcm, and sooty mangabeys, SIVsm). Infection of MDMs with Vpx-containing virus-like particles (VLPs) increases their susceptibility to HIV-1 (which lacks Vpx) by two orders of magnitude. HIV-1 with packaged Vpx shows a similarly increased infection titre in DCs and MDMs (Sunseri et al., 2011). The differential binding of the HIV-1 and 2 capsids to MDM co-factors may provide an explanation as to why HIV-1 does not need to encode Vpx and similarly why HIV-2 is less pathogenic (see below).

Similar to Vif, Vpx complexes with a culin 4A–based E3 ubiquitin ligase, which includes DCAF1 and DDB1 among its constituents. This E3 ligase is involved in the regulation and degradation of various cellular DNA repair proteins, replication enzymes and transcription factors. It was shown, using mass spectrometry–pull-down, that it is hijacked by Vpx to degrade a restriction factor identified as SAMHD1 (364,370). SAMHD1 is rapidly degraded following infection levels remain depleted for 2-3 days. SAMHD1 NLS deletion localises it to the cytoplasm and renders it refractory to degradation by Vpx suggesting that Vpx specifically degrades nuclear or perinuclear SAMHD1, consistent with the idea that uncoating and RT happen very close to, or at, the nuclear pore (371,372). It may also be that, in the case of HIV-1, SAMHD1 acts as a TREX1 like co-factor degrading RT products in the cytoplasm and serving to protect the virus from innate immune recognition.

Whilst SAMHD1 degradation is mediated by Vpx it may have been Vpr that initially evolved for this purpose. The Vpr encoded by the SIV of African green monkeys (SIVagm) degrades SAMHD1. Sequence analysis of primate SAMHD1 indicates that amino acids bound by Vpx are under strong selection pressure. Theories can only be developed from extant viral sequences but there is evidence that during primate evolution, *SAMHD1* gene mutations that enabled escape from Vpx were selected. Lentiviral accessory genes often have multiple functions. During SIV evolution the Vpr open reading frame was duplicated which enable functional specialization of the two gene products. Vpx could then rapidly adapt to SAMHD1 sequence changes. Modern SIVcpz lacks Vpx, which was likely deleted during the transmission from Old World monkeys (373,374). Vif, which has an overlapping reading frame, was subsequently free to evolve to counteract APOBEC3 in chimpanzees.

It is not entirely clear how HIV-1 replicates without Vpx. It may be that the RT of HIV-1 has a higher dNTP affinity so it can synthesize DNA at lower concentrations or it may be that replication in myeloid cells is unnecessary due to T cell to T cell transmission. HIV-2 and SIV by contrast may require myeloid to T cell transmission which may be important if there are fewer activated T cells.

HIV-2, unlike HIV-1, can activate and infect dendritic cells *in vitro*. (327) This is likely because HIV-2 encodes Vpx, which degrades SAMHD1, allowing innate immune sensing of PAMPs the subsequent activation of dendritic cells (328,329). Paradoxically this degradation of a restriction factor may explain the reduced pathogenicity of HIV-2: it stimulates an earlier protective immune response by activating dendritic cells. It may be that the restriction in DCs prevents proper activation.

1.6.5 SERINC and Nef

Like all accessory proteins, Nef performs multiple functions, including enhancing the infectivity of virions. It has been recently demonstrated that Nef targets serine incorporator 3 (SERINC3) and SERINC5, causing them to be redirected to endosomes from the cell membrane. Usami et al. identified SERINC3 using proteomic analysis of virions from producer cells infected with wild-type versus Nef-defective HIV-1. SERINC3 was found in Nef-defective virus but not in wild-type HIV-1 (375). Rosa et al. used a global transcriptome analysis following infection of cell lines with either wild-type or Nef-defective HIV-1 and were able to correlate SERINC5 expression with Nef-dependent changes in viral infectivity (376). It was subsequently demonstrated, using tagged SERINC3 and SERINC5, that without Nef, both proteins remain in the membrane and are virion incorporated. Nef, disrupts this incorporation. The SERINC proteins may stop the fusion pore enlarging following binding, thus preventing cytoplasmic entry of the capsid core and reducing infectivity but the antiviral mechanism is generally unclear (375,376).

1.6.6 MX2 and Capsid

The GTPase MX2 or MXB was recently revealed as being able to restrict HIV-1 infection in IFN-treated cells (345,347). It localises to the nuclear membrane and suppresses infectivity following reverse transcription prior to nuclear entry. CypA capsid binding mutants are not sensitive to Mx2 restriction suggesting the possibility of direct interaction between capsid, CypA and Mx2. These mutants remain sensitive to IFN treatment, which blocks reverse transcription not nuclear entry, despite their escaping Mx2 (377). This may be evidence of further host restriction factors for HIV-1 that restrict infection before MxB gets to restrict viral nuclear entry.

1.6.7 Schlafen11

SLFN11 has structural similarity to the family of RNA helicases. It acts late in the replication cycle suppressing viral protein production by binding to tRNA and thus antagonising viral-directed changes which aim to increase the amount of tRNAs available for viral protein synthesis. Increased expression levels of SLF11 in CD4+ T cells blocks protein synthesis so effectively it confers elite controller status of chronic HIV-1 infection (344,378).

1.6.8 IFITM1–3

IFITM1–3 restrict early in the HIV replication cycle by inhibiting entry of a range of viruses likely by antagonising viral fusion (379). If overexpressed in cells where HIV-1 is actively

replicating they colocalise with the HIV-1 proteins Env and Gag. They have been shown to be incorporated into new virions, and restrict viral entry into new target cells (380,381).

1.7 Viral Protein R - overview

The literature on Vpr catalogues a series of often unrelated or inconsistent findings rather than a coherent narrative of discovery that explains its universal conservation among lentiviruses. The protein is variously described as enigmatic, multitasking, multifaceted and multifunctional. Vpr has been proposed have evolved to cause G2 cell cycle arrest, mediate nuclear import of the pre-integration complex, enable macrophage infection, activate the HIV-1 LTR as well as having wide ranging clinical effects causing dementia, nephropathy, immune dysfunction and metabolic dysregulation but despite the many functions and interactions attributed to the protein, some of which have been characterised in detail, there is no single phenotype that convincingly illuminates its conservation. Critically, while it is absolutely required for replication *in vivo,* Vpr has proven to be dispensable in most cell culture systems and this is possibly why its function has remained elusive.

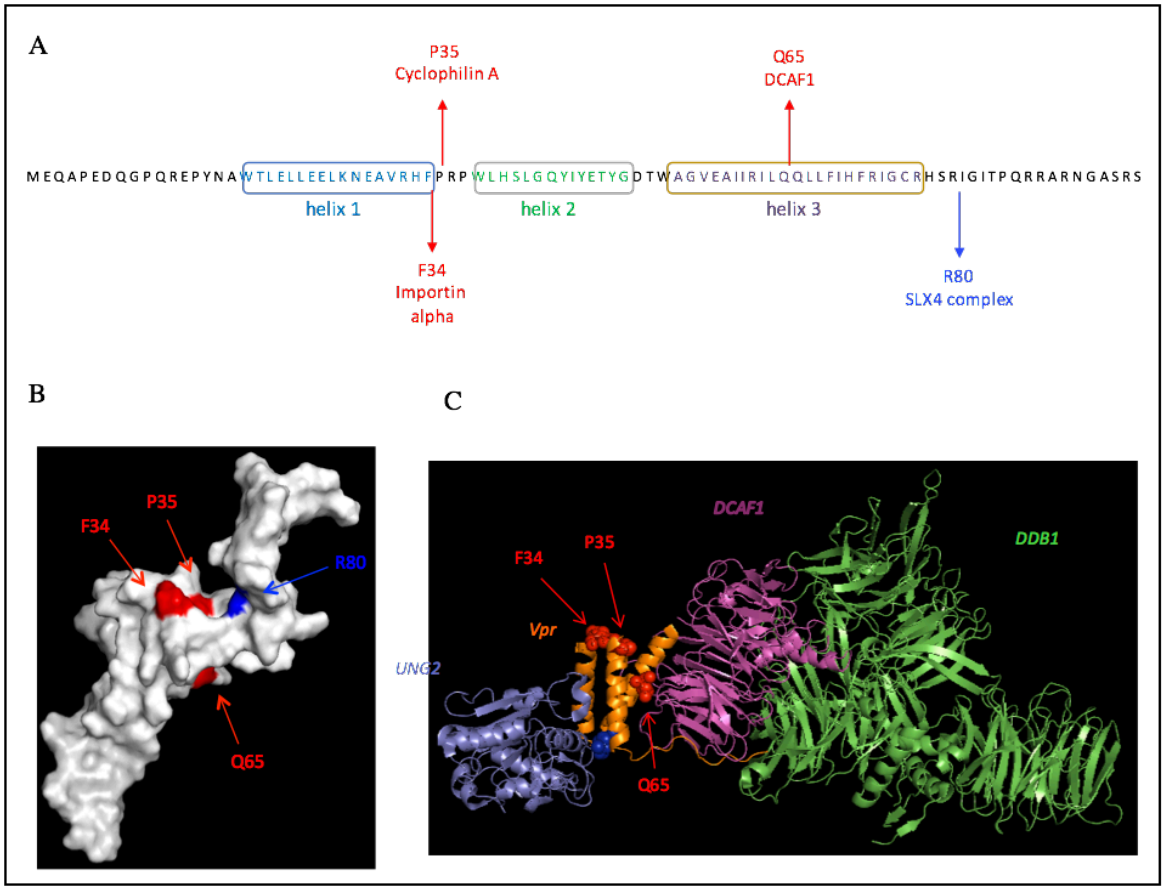

Figure 1.11 Vpr structure and functional mutations (A) HIV-1 M Vpr SUMA FJ496150 amino acid sequence with helices and significant mutants annotated. (B) Space filling three-dimensional cartoon structures of Vpr. (C) A representation of the DDB1–DCAF1–Vpr–UNG2 complex in ribbon representation showing how Vpr may function as an adaptor molecule. Individual proteins are colored: green, DDB1; purple, DCAF1; orange, Vpr; blue, UNG2. Images were generated using Pymol (382) Adapted with help from Hataf Khan from (383,384).

Much of the published research of Vpr derives from the findings that the other HIV-1 and 2 accessory proteins, Vpu, Vif and Vpx, have evolved to commandeer ubiquitin ligase complexes, targeting cellular restriction factors for degradation at the proteasome. HIV-1 and 2 counteract tetherin, APOBEC3G and SAMHD1 by this mechanism (385). Vpr has also been shown to have a role in ubiquitin ligase recruitment, and several putative cellular targets have been identified; UNG2 (386,387), MCM10 (388) and MUS81 (389) are downregulated in a Vpr and DCAF dependent manner. The structural basis of SAMHD1 recruitment to the DCAF–Vpx complex has recently been solved (390) but there are no similar data to explain the interaction between DDB1 and DCAF1, or DCAF1 and Vpr. Little is known about how Vpr and Vpx proteins confer substrate specificity to the same CRL4–DCAF1 E3 ligase and the precise advantages gained from degradation of these molecules remains unclear: the degradation of these molecules does not explain all the observed *in vivo* Vpr phenotypes.

Unpublished data from our lab has shown that Vpr can rescue HIV-1 infection in macrophages following stimulation with the DNA sensing pathway second messenger molecule cGAMP (see chapter 3) indicating that it may have a role in protecting HIV from innate immunity but this has not been discussed in the published literature. This observation may also explain the inconsistent finding that Vpr defective viruses are unable to replicate in macrophages. Stimulation of macrophages may be dependent on several factors (the infecting supernatant, the immunological condition of the person they were taken from etc) and Vpr may rescue only when innate sensing pathways are activated.

Additionally, although various functions of HIV-1 Vpr have been shown *in vitro*, many of the putative roles for Vpr have been studied using over expression assays but have failed to recognize the main discovery of this PhD: that Vpr antagonises expression from all tested co-transfected plasmids. The finding that Vpr suppresses expression is consistent but is often interpreted as degradation of a specific protein product (391–393). Additionally, the co-immunoprecipitation assays in many studies are performed in a single direction with tagged protein, meaning that Vpr may be precipitating non-specifically.

1.7.1 Structure

Vpr is a small, basic protein (14KDa) of 96 amino acids. Among primate lentiviruses it is believed to be universally conserved (394). It was first described over 2 decades ago (395,396) and was originally described as an accessory protein because it was dispensable *in vitro* for HIV-1 replication and cytopathogenicity in lymphoid cells *in vitro*.

Vpr is virion packaged so may have a role early in infection. It is incorporated via a direct interaction with a leucine-rich (LR) motif (397,398) in the C-terminal p6 region of the Gag

precursor (399,400). Structurally Vpr comprises a flexible N-terminal region, three alpha-helical domains and C-terminal region which is believed to be unstructured (figure 1.11). Recruitment of Vpr oligomers to the plasma membrane is mediated by the HIV-1 Pr55Gag precursor. It is recruited into the virus core following assembly and cleavage of the Pr55Gag precursor. It is tightly associated with viral RNA in the mature virion (401).

Vpr binding to Pr55Gag is crucial for Vpr oligomerization and localisation at the cell membrane during Pr55Gag assembly. Oligomer function is unclear (402). Vpr incorporation has also been used to target molecules (cellular or viral proteins or drugs) into viral particles. HIV particles can be labelled with Vpr fused to GFP in order to localise intracellular virus during early infection (402).

Efficient packaging requires the integrity of the α-helices and the p6 region of the Gag precursor which has a leucine-rich motif (403–405). It is not clear how many particles of Vpr are incorporated. Experiments with cysteine labelled MT-4 cells determined that Vpr is present in a molar ratio of approx. 1:7 (406) but monomeric or oligomeric Vpr may interact with multimeric Gag. Interactions with other cellular proteins may reduce the amount of Vpr available for Gag interations so the true value may differ significantly from this estimate (407).

NMR, fluorescence spectroscopy and circular dichroism spectroscopy have all been used to investigate Vpr structures (the latter technique uses the differential absorption of left or right handed circularly polarised light that occurs when a molecule contains one or more chiral chromophores; it allows the analysis of the secondary structure and conformation of macromolecules and can be used to study how secondary structure changes with environment) (383,408–410). Structural studies of the full-length molecule have proved technically challenging due to difficulties in crystallization. Use of NMR techniques is complicated by the self-association properties of Vpr and the fact that the structure of Vpr depends critically on the solution conditions.

Morellet and colleagues determined the tertiary structure of full length Vpr using nuclear magnetic resonance analysis. The polypeptide was dissolved in acidic aqueous-organic solvents (383). There are three amphipathic α-helical domains α-H1 (17–33), α-H2 (38–50) and α-H3 (55–77). These are linked and surround a hydrophobic core. Conserved prolines in positions 5, 10, 14 and 35 in the N-terminal domain show cis/trans isomerization and a CypA interaction between 14 and 35 may determine correct folding (411,412).

Between residues 73 and 96 of the C-terminal domain there are six arginines, resembling an arginine-rich protein transduction domain. This may explain why Vpr can be used for transduction and how it crosses lipid bilayer membranes (413–415).

Effects on the cell cycle (apoptosis, cell-cycle arrest, defects in mitosis) have been observed to be C-terminal domain dependent (416). The three α-helices may have a role in nuclear localisation mediated by Vpr (417). The leucine rich third helix of Vpr presents a stretch of hydrophobic side chains on one side which forms a leucine-zipper like motif (418). This region may enable oligomer formation. (419,420).

Vpr forms dimers, trimers, tetramers and higher order multimers (420) but the relationship between multimerization and function remains unclear. Flow cytometry fluorescence resonance energy transfer (FRET) studies have shown that Vpr multimers self-assemble *in vivo* in cells and that this is dependent on a hydrophobic patch on the third α-helix. G2 arrest was unaffected my mutations here. Arginine mutations such as R80A and R87/88A prevented Vpr induced G2 arrest but left oligomerization unaffected suggesting that this function is independent of oligomerization (421).

1.7.2 Functions

1.7.2.1 Cell cycle arrest

G2 cell cycle arrest is possibly the best studied effect of Vpr. Packaged Vpr induces cell cycle arrest (422). It was first observed in 1995 (423,424) when Vpr was seen to block proliferation of newly infected T lymphocytes which accumulated at G2 M phase with a 4N DNA content. Following cell division the cell undergoes a growth phase (G1) prior to DNA duplication (S phase) and then a second growth phase (G2). This comprises interphase and prepares the cell for mitosis. During G2 the DNA is checked for errors and mitosis is blocked by the G2 checkpoint mechanism if there is evidence of DNA damage. The 4 phases of mitosis or cell division follow G2: prophase, metaphase, anaphase and telophase.

Cell cycle arrest by Vpr is conserved (425) but it is unclear how this advantages the virus. Unexpectedly, in view of its effect on the cell cycle, several studies have described Vpr as enhancing replication in fully differentiated non-dividing macrophages but having no effect on replication in proliferating T cells (426–429). It may inhibit early steps in immune response activation and create a favorable cellular environment for viral replication since proviral transcription is higher during G2 (430,431). As this is more active in the G2 phase it may be through epigenetic control of the HIV-1 LTR promoter. Consistent with this, G2 arrest caused by Vpr correlates with efficient replication in primary human T cells (430,432).

Vpr has been shown to induce G2 cell cycle arrest and apoptosis in proliferating CCR5+ CD4+ T cells using a human hematopoietic stem cell-transplanted humanized mouse model. Since these cells are mainly CD4+ T cells it may explain their depletion in infected patients (433).

The mechanism by which Vpr induces G2 arrest has not been entirely elucidated but hypotheses have been advanced regarding nuclear envelope integrity, and induction of a DNA damage response either through degradation of a specific repair factor or through direct effects of DNA or chromosomal structure. Vpr localises to the nuclear envelope and has been shown to induce herniations of the nuclear membrane (434). This could redistribute nuclear proteins to the cytoplasm including the cyclins involved in the cell cycle regulation, thus contributing to arrest at G2. Studies using video fluorescence microscopy to monitor the movement of the proteins that regulate CDC-2 showed prominent transient nuclear envelope herniations in the presence of Vpr that were not formed by cell cycle arrest mutants (434) but this work has not been repeated or confirmed.

Vpr has been shown to activate DNA damage response pathways. It has multiple interactions with complexes that sense and repair damaged DNA, activating a major signalling pathway of the DNA damage response: ATR (ataxia-telangiectasia and Rad3-related) protein and its downstream target Chk1 (435–437). This in turn activates DNA damage response markers, such as replication protein A (RPA), γH2AX and FANCD2 at nuclear foci and blocks the cell cycle at G2 (438). The interaction of Vpr with the host DCAF1 adaptor of the Cul4 E3 Ub ligase complex is required for these interactions and pathway activations (439) but the mechanism by which Vpr causes replication stress is unclear.

Vpr has epigenetic effects and can displace heterochromatin protein 1-α (HP1-α) through acetylation of the histone H3 (440). This has been hypothesised to cause premature chromatid separation and G2 arrest through Vpr-induced activation of the DNA damage surveillance proteins ataxia-telangiectasia-mutated kinase (ATM) and ATM and Rad3-related kinase (ATR). These sense DNA damage and activate signalling pathways (422,436,441). Consistent with this, inhibition of ATR has been observed to disrupt Vpr dependent G2 arrest. ATR and ATM induce G2 arrest following DNA lesions but whether Vpr specifically causes DNA damage is not known (437,442).

Vpr expression correlates with hypo-phosphorylation and inactivation of the p34/CDC2 CDK1 kinase associated with cyclin B and a range of *in vitro* studies have examined proteins which regulate the cell cycle and found interactions with Vpr but detailed mechanistic insight is still lacking. The most promising recent evidence is that there is a target of Vpr caused to be degraded by the host proteasome machinery.

X-ray crystallography and increased understanding of the Vpr related protein, Vpx, provided an insight into the potential molecular mechanisms used by Vpr to recruit host cell ubiquitination machinery in order to inhibit host restriction factors or innate immune proteins. Like Vpr, Vpx comprises a zinc ion stabilised three-helix bundle. It recruits the E3 ligase substrate adaptor DCAF1 and SAMHD1 (discussed in detail in section 1.11.4). Studies of Vpx suggested how Vpr could hijack the proteosomal protein degradation pathway to inactivate innate immune components and several groups have shown that like Vif and Vpx, Vpr orchestrates a E3 Ub ligase complex composed of DCAF1, DDB1, Cul4 and Rbx1 (DDB1-Cullin4-associated factor 1; damage-specific DNA binding protein 1, cullin 4 and ring box protein 1 respectively) also named as the CRL4 complex. However the specific targets of Vpr have remained elusive (364,390). Vpr is proposed to interact with the DCAF1 adaptor of the Cul4 E3 Ub ligase. This is hypothesized to cause cell cycle arrest by causing the degradation of an unknown protein. It has been proposed that Vpr itself escapes proteosomal degradation by association with the Cul4-DDB1 ubiquitin ligase (443). It has been unclear whether it is the degradation of the host protein or the resulting G2 arrest that is the evolved purpose of Vpr. Belzile et al. have proposed a model where nuclear Vpr and specifically Vpr inside nuclear foci associates with DCAF1 and the DDB1-CuL4-E3 ubiquitin ligase. These co-localise with DNA repair foci which contain γH2AX and RPA2. This may cause the K48-linked poly-ubiquitination induced degradation of a target bound to nuclear DNA. This in turn causes G2 arrest through ATR activation (439).

Interactions between Vpr and Cul 1 & 4 have been shown (444). DCAF1 was identifiedas a substrate specificity module in Cul4 and DDB1 based ubiquitin ligase E3 complexes (445,446). The DDB1-CuL4-E3 ubiquitin ligase complex is able to recruit various DCAFs and is hypothesized to have a role in maintenance of genome stability, DNA replication and cell cycle check point control (447). In 2007 DCAF1 was recognised as a host factor critical for Vpr induced cell cycle arrest (431,448). Vpr is proposed to use two separate interfaces for binding: the LR motif found between amino acids 60 and 68 binds to VprBP/DCAF1 while the flexible C-terminal region binds an unknown target for ubiquitination and degradation (390).

Laguette et al used tandem affinity purification, immunoprecipitation and mass spectrometry to identify the structure-specific endonuclease (SSE) regulator SLX4 complex (SLX4com) as a target of Vpr using proteomic analysis and showed that it is crucial for the G2-arrest activity. Glycerol gradient sedimentation and co-immunoprecipitation studies suggested that SLX4 subunits, DCAF1 and Vpr assemble into a single complex.

The SLX4 complex is comprised of 12 proteins that together resolve DNA lesions formed during DNA replication stress and/or homologous recombination repair. SLX4 itself acts as

molecular platform for a SLX4complex comprising several structure specific endonucleases (SSEs) including ERCC1-ERCC4/XPF, MUS81-EME1 and SLX1. The SLX4 complex is suggested to repair the structures resulting from homologous recombination repair: double strand breaks and collapsed replication forks. (449,450). MUS81-EME1 and SLX1 repair X-Holliday Junctions, caused by replication, during homologous recombination (451,452). They are strictly regulated during the cell cycle to ensure removal of inadequate DNA recombination intermediates before chromosome segregation. Laguette et al. showed a slight Vpr dependent reduction in expression of EME1 and MUS81. Levels of polo-like kinase 1 (PLK1) and phospho-PLK1 which activates the endonuclease activity of EME1 were shown to increase. They also observed Vpr dependent remodelling of SLX4com following binding to phospho-EME1 and the DCAF1 ubiquitin ligase. Following time course experiments they concluded that activation of SLX4com by Vpr occurs prior to G2/M arrest rather than being a consequence of it. Additionally they suggested that silencing of subunits of the SLX4com (SLX4, EME1 or MUS81) prevented induction of G2 arrest by Vpr . This was confirmed by Berger et al. (44). Berger et al also showed that Vpr proteins from lentiviruses infecting African green monkeys and related primates also interact with African green monkey SLX4, suggesting that this is a conserved function for Vpr(453). Laguette *et al* also hypothesise that the active SLX4com being targeted by Vpr may enable evasion of innate immune detection of DNA damage though do not elaborate on the details of how this could work (389). This work could be combined with the model proposed by Belzile so that the activation of SLX4com would cause replication stress leading to creation of FANCD2 foci and cleavage of DNA replication intermediates. This would induce G2 arrest.

Contrasting with these reports a 2016 study from the Emerman lab expressed Vprs from HIV-1 and HIV-2 using an adeno-associated virus vector system in U2OS cells and found that while DNA damage response is conserved, the interaction of Vpr and SLX4 is variable among HIV-1 and HIV-2 isolates. Using clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 to knockout U2OS and 293T cells for SLX4 they observed that the DNA damage response and cell cycle arrest induced by Vpr are not dependent on the interactions of Vpr and SLX4 and argue that the mechanisms by which Vpr induces a DNA damage response have yet to be elucidated.

1.7.2.2 Vpr induced apoptosis

The role of Vpr in apoptosis is unclear. Vpr has been observed to induce apoptosis in many cell types, however, the proposed mechanisms vary and both the phenotype and mechanisms are often cell-type specific and may be a feature of overexpression.

Apoptosis is a cell death mechanism which avoids damage to nearby cells and inflammation through the controlled degradation of cellular components. Upstream signalling events induce dimerization and activation of initiator caspases 8 and 9 which activate executioner caspases-3, -6, and -7. These orchestrate the degradation structural proteins and the activation of apoptotic enzymes. There are intrinsic and extrinsic pathways. The extrinsic pathway is activated through death receptor ligand binding. Caspase-8 is then recruited and activated and subsequently initiates apoptosis directly, by activating executioner caspases, or indirectly, through activation of the intrinsic pathway.

The intrinsic or mitochondrial apoptosis pathway is initiated via a range of cellular stresses all of which cause cytochrome *c* release from mitochondria and subsequent formation of the apoptosome. This is a complex composed of APAF1, cytochrome *c*, ATP, and caspase-9. Caspase-9 is activated and then cleaves and activates executioner caspases instigating apoptosis (454).

Various studies have suggested that treatment of cells with soluble Vpr induces caspase-dependent apoptosis in CD4+ T cell lines, neurons, hepatocytes, fibroblasts, and primary peripheral blood lymphocytes with both caspase-8 and caspase-9 mediated mechanisms having been implicated (455–457). Vpr has been observed to be a cell-penetrating protein and is also detected in the fluids of HIV-infected patients. It is therefore a potential cause of bystander cell death (414) though it is unclear also how these observations fit in with the mechanism of cell death for CD4 T cells proposed by Doitsch et al (62) who showed that most quiescent lymphoid CD4 cells die by caspase-1-mediated pyroptosis initiated through inflammatory signals.

Other models of Vpr induced apoptosis have been proposed. Jacotot et al determined that the apoptotic functions of Vpr appear to localise to a C-terminal domain which binds to the adenine nucleotide translocator (ANT) component of the mitochondrial permeability transition pore complex (PTPC). Mutations in this domain of Vpr (e.g., R73A, R77A, and R80A) diminish the ability of Vpr to induce apoptosis in T lymphocytes (458,459).

Some studies have proposed that apoptosis results from G2 arrest (460) whilst others have shown these effects to be independent (461).

Apoptosis may be linked to HIV related neurocognitive disorders. Jones et al determined that neuronal Vpr could cause apoptosis and suggested that Vpr may mediate neuron death through the release of Il-6 by astrocytes (462).

1.7.2.3 Vpr effects on reverse transcription

Vpr was used as bait in a yeast two-hybrid screen of a human cDNA library. This identified cellular uracil DNA glycosylase (UDG) UNG as a possible binding partner suggesting that Vpr might enable UNG incorporation into virions. (386).

The subsequent finding that the cytidine deaminase APOBEC3Gs generates cytidine to uracil changes in viral genomes which are degraded before being integrated, lead to the hypothesis that Vpr might have evolved to deliberately encapsidate UNG. There are two forms of UNG with different amino terminals: UNG1 is mitochondrial while UNG2 localises to the nucleus. Both are base excision repair system enzymes that remove uracil bases from DNA thus preventing mutagenesis (463). These initial findings have lead to a series of contradictory studies some showing that UNG is incorporated and others showing that UNG is degraded by Vpr. It is possible to imagine UNG2 advantaging the virus by repairing uracil sites induced by APOBEC3 mediated deamination of cytidine.

Mansky et al. demonstrated that Vpr reduces the rate of mutation during reverse transcription by recruiting UNG. They measured *in vivo* rate of forward virus mutations per replication cycle and showed a 4-fold increase in mutation rate without Vpr expression (464). A 16-fold increase was seen in primary MDMs (465).

The association of Vpr with UNG2 may allow Vpr to decrease the mutation rate but other studies have shown Vpr can induce the proteasomal degradation of UNG2. This may prove to be an artefact of suppression of expression from overexpression assays (444,466). Other studies indicate that endogenous UNG2 being reduced by Vpr is not mediated by degradation at the proteasome (467,468). A decrease in replication was shown with virus produced from cells which had been depleted for UNG2, assessed by demonstrating a reduced number of viral transcripts measured (469). It may be that incorporated UNG2 reduces uracil accumulation in the viral genome when virions infect non-dividing cells as these usually have reduced amounts of UNG and with increases in dUTP levels (470). This however is at odds with the studies that show that Vpr causes degradation of UNG (444). That UNG2 has a role in HIV-1 infection seems likely but whether that role is as a co –factor (469,471) or as a restriction factor (472) is not clear.

Wu et al determined the crystal structure of HIV-1 Vpr in complex with human UNG2 and the CRL4 acceptor-receptor proteins DDB1-DCAF1 demnstrating that Vpr and Vpx both bind DCAF at a similar site through the N-terminal tail and a-helix a3. Vpr interacts with the DNA-binding region of UNG2 by mimicking DNA using a cleft between helices a1 and a2 (384). This serves to further illustrate the similarities between Vpr and Vpx which have similar helices

for binding DCAF but which are able to adapt other molecular regions to determine substrate specificity. It also illustrates a central problem of our understanding of the biology of Vpr – that even detailed structural information can fail to shed light on biological functions and the advantages conferred through interaction with particular proteins.

1.7.2.4 Vpr effects on nuclear import

Vpr has been identified as promoting nuclear localisation of the pre-integration complex or the delivery of capsid to the nuclear pore. Additionally, nuclear localisation of Vpr has been reported as increasing infection of primary CD4 cells (473,474). Other findings however show that Vpr is not required for nuclear transport of the PIC (184). Given the intra-capsid localisation of Vpr and the increasingly prevailing hypothesis that capsid remains intact until nuclear docking it may prove that Vpr acts at the nuclear pore but only once capsid disassembly starts.

Vpr localises both to the nuclear rim and inside the nucleus but there is no canonical or M9-dependant nuclear localisation signal (475–477). Several arginine residues in the C-terminus of Vpr resemble a basic NLS but do not appear to function as one; instead it is the N-terminus that is required for protein–protein interactions (416,478). Vpr seems to use a non-classical pathway for nuclear entry through direct binding to importin-α (475,479). It has been hypothesised that Vpr may transport viral DNA to the nucleus (480,481). Vpr has been described as being able to bind to nuclear pore structures such as p54 and p58 and the hCG1 nucleoporin and this may cause Vpr accumulation at the nuclear envelope (482,483).

There are two classical pathways enabling transport of proteins across the nuclear pore complex (NPC): The NLS and M9-dependent pathways (484). The NLS pathway requires binding of an NLS to importin-α. This then binds importin-β and this protein complex then binds the NPC. Both systems depend on the GTPase Ran/TC4 (485,486). Vpr localisation seems to be independent of either of these pathways and is unaffected by peptides which block the NLS, dominant negative importin-α or dominant negative Ran or Ran inhibitors.

Nuclear localisation of Vpr has been shown to involved all three helices (416). There have been conflicting studies about Vpr importin-α binding with some groups finding only a weak interaction (487) while others found stronger interactions (488). Miyatake et al made a complex crystal of Vpr C-terminal residues 85–96 (Vpr85–96) with a mouse importin-α2 protein lacking the IBB domain (ΔIBB–m-importin-α2). They used X-ray crystallography to investigate the interaction between the Vpr C terminus and importin-α and suggested that Vpr may modify the aggregation state of importin-α. They also suggested that Vpr binds the minor NLS-binding site of importin-α as well as the major site but with lower affinity. The biological

significance of these studies conducted on peptide fragments from different species remains to be determined.

1.7.2.5 Modulation of gene expression by Vpr

There is evidence suggesting that Vpr may regulate transcription from the HIV-1 LTR as well as other host genes. Candidates include *ung2*, *nlbp2* and *nhe1* which may increase viral replication *in vivo* (467,489–491).

Langevin et al 2009 re-examined the influence of Vpr on the amount of endogenous UNG2 and found that it did weakly reduce the UNG2 isoform by regulating transcription but used overexpression reporter assays to confirm this phenotype. As with the studies reported in this PhD they determined that this was independent of proteosomal degradation and cell cycle arrest , since it was observed mutants unable to induce G2 arrest, including the VprQ65R mutant (467).

Liu et al (2015) found that HIV-1 M Vpr suppressed transcription from the promoter of cytomegalovirus (CMV) (492) They showed that Vpr-mediated inhibition of the transcription of both CMV and the host gene *ung2* was dependent on sites in helix 1 of Vpr. Surprisingly they hypothesised that suppression of CMV promoter activation would provide a survival benefit to the host (and thus the virus) through reduction of opportunistic infections. This study lacked robust negative controls to demonstrate specificity at the promoter.

Weak transactivation activity at the HIV-1 LTR, as well as other heterologous promoter elements has been ascribed to Vpr (493). Transcription may be mediated via both *cis* elements and *trans*-acting factors. The U3 region of the LTR contains known *cis* elements for HIV-1 expression including NF-AT, glucocorticoid response element (GRE), NRF, NFκB, and Sp1 binding sites, the TATA box and the tat-responsive RNA element, TAR. Agostini et al mapped this Vpr phenotype with truncated HIV-1 LTR-CAT constructs locating it to a region (2278 to 2176) containing the GRE sequences in the HIV-1 LTR.

Hogan et al used electrophoretic mobility shift analysis (EMSA) to show that Vpr and HIV-1 LTR sequences that span the adjacent C/EBP site I, the NFκB site II, and the ATF/CREB binding site associate. They also showed that over 90% of LTRs isolated from peripheral blood contained C/EBP site I variants with a high Vpr binding affinity suggesting that cis-acting elements with high affinity for Vpr are maintained and provide some evidence that Vpr may act to regulate C/EBP transcription factor/LTR interactions.

Additionally, Vpr is found in sera and CSF of AIDS patients likely as a result of breakdown of viral particles. Levy et al found that purified serum HIV-1 M Vpr activated HIV expression from latently infected cell lines and from PBMCs of infected individuals and hypothesised this was due to direct, or indirect, activation of proviral transcription (494).

Kichler et al, reasoning that Vpr could interact with nucleic acids and enter the nucleus, either independently or in the context of a pre-integration complex, tested whether Vpr could be used as a plasmid DNA delivery system in mammalian cells (415). They used synthetic Vpr and reported that the C-terminal domain of Vpr (52-96) interacts with plasmid DNA and enables efficient transfection comparable to the best transfection reagents. They used SMD2 Luc and PCIneo-Luc which both have CMV promoters and a GFP also with a CMV promoter. Follow up studies by the same group showed using electron microscopy and transfection experiments that a Vpr C-terminal peptide can transfect plasmid DNA and that the entry pathway is endocytic. Vpr has not been pursued as a potential transfection reagent in the last decade or so since these studies (415,495).

1.7.2.6 Clinical effects of Vpr

A number of animal studies have demonstrated clinical effects of Vpr expression which appear to mimic observed effects in humans.

Whether they express the Vpr transgene or are infused with synthetic Vpr, mice recapitulate the adipose dysfunction, altered metabolic parameters and hepatic defects found in HIV patients. These include increased lipolysis, diminished fat and insulin resistance mass (496–498). Vpr was seen to block pre-adipocyte differentiation via cell cycle arrest, whilst increasing lipolysis in mature adipocytes and reducing peroxisome proliferator-activated receptor γ (PPARγ) and glucocorticoid receptors (GR). This is presumed to be the same mechanisms which underlie the adipose tissue dysfunction characteristic of HIV-1 patients (499).

Hoch et al. (1995) showed that infection of rhesus macaques with SIVmac without the Vpr gene has diminished viral loads and disease progression (500).

Goh and colleagues (1998), analysed Vpr sequence changes over time in an individual accidentally infected during laboratory work with a virus containing mutations in *vpr.* These encoded an unstable and non-functional Vpr because of the insertion of a single thymidine resulting in a premature truncation of the protein. They found positive selection for Vpr function *in vivo* in both human and chimpanzees: within 2 years the protein lost the thymidine insertion and reverted back to an intact Vpr open reading-frame (501).

Additionally, Vpr mutations are associated with the clinical phenotype of long- term non-progression (LTNPs). Some HIV patients have detectable HIV replication yet do not progress to immunosuppression even without treatment. There is a higher frequency of R77Q *Vpr* mutations in patients with LTNP than in patients with progressive disease (457).

Jacquot et al sequenced Vpr from a long term non-progressor (LTNP) patient. Vpr proteins were observed to accumulate at the nuclear envelope and showed cytostatic and pro-apoptotic activities. An LTNP *Vpr* allele, harbouring the Q65R substitution, was unable to bind DCAF1 and no longer exhibited nuclear envelope localisation, possibly indicating a functional link between localisation and cytostatic activity. Contradicting other published results, the R77Q substitution, found in LTNP alleles, was not observed to affect pro-apoptotic activity (429).

1.7.2.7 Vpr and cyclophilin

While the interaction of Vpr with the peptidyl prolyl isomerase cyclophilin A (CypA) (502–504) has been characterised the *in vivo* relevance of the interaction remains unclear.

CypA is a dispensable cellular protein (505) but HIV-1 replication is inhibited by CypA inhibitors which has made it a potential drug target (506,507). Capsid (CA) mutants N74D and P90A, which are unable to bind cofactors Cleavage and Polyadenylation Specificity Factor subunit 6 (CPSF6) and cyclophilins (Nup358 and CypA) respectively, are defective for replication in human monocyte derived macrophages (MDM) putatively due to triggering of cytosolic DNA sensors and subsequent induction of an interferon response and antiviral state (165,174,325).

CypA has been shown to catalyse prolyl cis/trans isomerization of conserved Vpr proline residues 5, 10, 14, 35 at low relative concentrations of both CypA and the Vpr (508). Only N-terminal peptides containing Pro-35 (which has shown to be involved in multiple functions of Vpr) are able to bind to CypA as measured by surface plasmon resonance (SPR) implying that P35 is important for CypA binding (508).

There is consensus in the literature that N-terminal residues from 30-34, which overlap the N-terminal binding region of Vpr to CypA, are required for Vpr induced G2 arrest (478,509). There is also consensus that Pro-35 mutations disrupt the interaction with CypA (411,502) but Ardon et al also observed that an Arg-80 Ala mutant inhibited co-immunoprecipitation with CypA. Solbak et al studied the interaction of Vpr with CypA and suggest the presence of a novel specific non-Pro-containing C-terminal binding region comprising the 16 residues ^{75}GCRHSRIGVTRQRRAR^{90}. They found a dissociation constant of Vpr to CypA was of 320 nM. This may indicate stronger binding than that of the well described interaction of

CypA capsid interaction (503). Whether the inhibition of HIV-1 replication by cyclosporine and SDZ-NIM811, specific inhibitors of the isomerase activity of CypA, is due to the disruption of interactions with Vpr seems unlikely since the P90A capsid mutant is known to be sensitive to cyclosporine.

1.7.2.8 Degradation of DICER by Vpr

The endoribonuclease Dicer has also been identified as a target for Vpr-mediated degradation via DDB1-CuL4-E3 ubiquitin ligase. Klocklow et al identified human Dicer as assembling in a complex with HIV-1 Vpr by immunoprecipitation of Myc-tagged Dicer which resulted in the co-purification of HIV-1 Vpr. They did not perform the appropriate reverse immunoprecipitation of Vpr to look for Dicer co-purification though and, while they consistently observed lower Dicer levels in lysates from cells co-expressing Vpr their observation that Dicer is “degraded by Vpr” is consistent with the expression defects observed from co-transfected plasmids that form the core of this PhD. Since there was a lack of adequate negative control plasmids it is hard to conclude that they demonstrated a specific degradation of DICER.

2 Chapter 2: Materials and Methods

2.1 DNA preparation and manipulation

2.1.1 Preparation of plasmid DNA - Small scale

Following transformation, plasmid DNA was purified from amplified bacterial clones by alkaline lysis using the QIAprep Spin Miniprep Kit (Qiagen). For small-scale preps, single colonies were picked from agar plates and used to inoculate 5ml of ampicillin-containing LB media which were grown in a shaker at 250rpm for 16-18 h at 37 °C. Plasmid DNA concentration was measured using Nanodrop NDW1000 (Thermo Fischer Scientific Inc.).

2.1.2 Preparation of plasmid DNA - Large Scale

5ml LB was inoculated with a single colony as above and agitated and incubated at 37°C for 6-8 hours. 200µl of this starter culture was then used to inoculate 200ml LB, which was incubated, shaking, at 37°C for approximately 18 hours. Cultures were centrifuged at 4000g, at 4°C, for 20mins, and the supernatant was removed. Plasmid DNA was extracted using QIAfilter Plasmid Midi Kit (Qiagen).

2.1.3 Agarose gel electrophoresis and purification of DNA

Electrophoretic separation in 0.8 % agarose gel at 80-125mV in TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM ethylenediaminetetraacetic acid (EDTA)) was used to separate and analyse PCR products and DNA from ligation or restriction digest reactions. SYBR® Safe (Invitrogen) and long wave UV illumination were used to visualise DNA. DNA products were excised in gel slices and purified using the QiaQuick Gel Extraction Kit (Qiagen) according to the manufacturer's protocol.

2.1.4 Restriction enzyme digestion

Plasmid vectors and PCR products were digested using restriction enzymes. Digestion mixes comprised 1.5µg of DNA, 0.5µl of the necessary restriction enzymes (New England Biolabs or Promega) and 1ul of the appropriate 10 x restriction enzyme buffer (New England Biolabs or Promega), and were made up to 10ul with water. Digests were incubated for 2 h at 37 °C and products were analysed by agarose gel electrophoresis and purified from the agarose gel as described above.

Each digestion contained 1.5 µg DNA, 1 µl appropriate 10 x restriction enzyme buffer (Roche), 0.5 µl of each restriction enzyme (Roche), and made up to 10 µl with water. Restriction digests

were incubated at 37 °C for 2 h. Digestion products were analysed by agarose gel electrophoresis (see 2.1.2) and the DNA was purified from the gel (see 2.1.3).

2.1.5 DNA ligation

Ligation of digested PCR products or digestion products with desired digested plasmid backbones was performed at a 2:1 molar ratio of insert:vector and this ratio was varied depending on transformation efficiency. Each ligation reaction contained 100 ng digested vector, the appropriate ng of digested insert, 10 x T4 DNA ligase buffer (Promega) and 1 U T4 DNA ligase (Promega), made up to 10 µl with water. Reactions were incubated for 3-24hrs at room temperature.

2.1.6 Production of competent *E. coli* bacteria

Chemically competent HB101 (F- mcrB mrr hsdS20(rB- mB-) recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20(SmR) glnV44 +-, described in (510)): A single starter colony was cultured overnight in 3 ml LB broth without antibiotic and then inoculated into 200ml lysogeny broth (LB - tryptone 10 g/l, yeast extract 5 g/l, NaCl 5 g/l) which had been previously autoclaved at 121°C for 15 min. This suspension was grown at 30°C until OD550nm = 0.45-0.55, chilled at 0°C for 10 min, divided into 4x50 ml aliquots, and centrifuged 3500 rpm for 10 min at 4 °C (Sorvall Legend RT Centrifuge). Supernatant was discarded and pellets kept on ice at 0°C throughout further manipulations. Pellets were gently resuspended in 20 ml TFB1 buffer (30 mM KAc, 100 mM RbCl, 10 mM CaCl, 50 mM MnCl, 15% glycerol in dH2O, sterilised through a 0.45 µm filter), incubated at 0°C for 5 min, centrifuged at 3500 rpm for 10 min, the supernatant was discarded and the remaining pellets resuspended in 2 ml TFB2 buffer (10 mM PIPES, 75 mM CaCl2, 10 mM RbCl, 15% glycerol in dH2O, sterilised through a 0.45 µm filter). Bacteria were aliquoted at 50µl, and snap frozen in liquid nitrogen, stored at -80 °C. Competency was assessed by transforming 50µl HB101 with 10pg pUC19 >100 colonies indicated high transformation efficiency.

2.1.7 Transformation of *E. coli*

50µl of chemically competent HB101 *Escherichia (E.) coli* cells were transformed with 5µl of ligation reaction mixture and incubated on ice for 30 min. Bacteria were heat shocked at 42 °C for 1 min and returned to ice for a further 5 min. Transformed bacteria were then spread on Luria Broth (LB) agar plates containing ampicillin (100 µg/ml) using aseptic technique, Plates were incubated overnight at 37 °C to allow bacterial growth.

2.1.8 Bacterial colony screening

Screening of the transformed bacterial colonies was used to identify bacteria harbouring the desired plasmid. Individual colonies were transferred to 2 ml LB with ampicillin (100 µg/ml) and incubated for 1 h at 37 °C with shaking. 1 µl of this bacterial culture was then used in a PCR containing 10 x GoTaq buffer (Promega), 25 mM MgCl2, 10 mM dNTPs, 0.2 µM of the primers originally used to amplify the desired gene, 1 U GoTaq polymerase (Promega) and water up to a volume of 20 µl. DNA amplification was then performed in a thermocycler using the following programme. Following PCR amplification, DNA products were analysed by agarose gel electrophoresis.

Temperature (°C)	Time (min)	Repeat
95	5	X1
95	0.5	X5
50	0.5	
72	1 per kbp	
95	0.5	X 25
55	0.5	
72	1 per kbp	
72	5 per kbp	X1

Table 2.1 Bacterial colony screening

2.1.9 Vpr Plasmid construction

Codon optimised Vpr cDNA was synthesised by GeneArt® and designed to include BamHI and No1I sites. These were manufactured in a PMA vector and subsequently digested and ligated to pcDNA3.1, which contains a FLAG-epitope tag appended to the Vpr N-terminus. Correct insertion was checked through sequencing (Beckman Coulter Genomics). FLAG was excised with BamHI and XhoI.

2.1.10 Site directed mutagenesis (SDM)

Point mutations were introduced into plasmids using site directed mutagenesis with overlapping forward and reverse primers, each bearing the mutation. SDM primers were designed to be 30-45bp long, to begin and end in a G/C, to have 20-30bp overlap, 40-70% GC content, similar melting temperatures (TM) of 55 - 80°C, a maximum of 3 mismatches, and mutation/s were located toward the 3' end of the primer. The reaction mixture is described in table 2.2 Cycling parameters are described in table 2.3. A negative control consisted of the

same reaction mixture but without *PfuTurbo*. 5 µl of the Control and SDM reaction products were separated by 1% agarose gel electrophoresis to confirm amplification. 45 µl of the remaining reaction sample was mixed with 1 µl DpnI (NEB) and 5µl Buffer 4 (NEB) and incubated at 37°C for 2hrs to digest any original methylated plasmid produced by bacteria whilst leaving the PCR-produced DNA intact. The QIAquick PCR purification kit (Qiagen) was used to clean up DpnI digested PCR product and 5 µl was used to transform 50 µl HB101. Lack of negative control PCR colonies indicated successful DpnI digest. Plasmids were then prepared and sent for sequencing (Beckman).

Component	Conc/Amt
Pfu Turbo DNA Polymerase 2.5U/µl Stratagene	2.5U / 1µl
Pfu Turbo 10x Buffer	1x / 5µl
dNTPs (Promega) 25mM each	250µM / 5µl
Primers (Sigma) 100µM	2µM / 1µl
Plasmid Template	50- 100ng
dH2O	To total
Final volume	50µl

Table 2.2 SDM PCR components

Step	Plasmid DNA <10kb		
	Temp (°C)	Time (secs)	No. of Cycles
Initial denaturing	95	30	1
Denaturing	95	30	12/16 for 1/2 mutations
Annealing	55	60	
Extension	72	60/kb	
Final extension	72	120/kb	1

Table 2.3 Cycling parameters

2.2 Cell culture

2.2.1 Maintenance of cells

HEK293T (human embryonic kidney cell lines) cells, BSC-1 (African green monkey epithelial cell line of kidney origin), CV-1 and Vero cells (both African green monkey kidney fibroblast cell lines) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10 % (v/v) foetal calf serum (FCS) and penicillin/streptomycin (P/S) (50 µg/ml). FCS was treated for 1 h at 56 °C to inactivate complement. HEK293T Luc cells (with an integrated NFκB sensitive reporter) were maintained in the same way with additional puromycin (50 µg/ml).

2.2.2 Fugene-6 transfection

Cells were seeded a day prior to transfection such that they were 70 % confluent when transfected. Transfection mixes were prepared by combining DNA plasmids with OPTIMEM in a ratio of 50 µl per 1 µg of DNA. Fugene-6 reagent (Roche) was then added (2 µl per 1 µg DNA), vortexed briefly and incubated at room temperature for 20 min. DMEM (as above) was added to the transfection mixture at an appropriate volume (according to the cell culture plate being used) and this was used to replace the growth medium previously on the cells. Media was once more replaced at 24 hrs and cells were harvested after 24-48 h. See table appendix for a list of plasmids used in this study.

2.2.3 Generation of cell line with integrated NFκB sensitive reporter

Ready-to-transduce, replication incompetent, HIV-based, VSV-G pseudotyped lentiviral particles (NFkB-luc lentivirus) at $2x10^7$ TU/ml was purchased from Qiagen. The NFκB-responsive luciferase construct encodes the firefly luciferase gene under the control of a minimal (m)CMV promoter and tandem repeats of the NFκB transcriptional response element. 50,000 cells were plated onto in 6 well plate in 2ml DMEM. 150ul of virus was added and the media was changed at 24hrs. Spinoculation was not performed. At 48 hrs the media was replaced with media containing 2.5ug/ml puromycin. When cells were stable at 2.5 µg/ml puromycin, the drug concentration was increased to 7.5 µg/ml. Single cell clones were prepared by limiting dilution and stored in liquid nitrogen.

2.3 Reporter gene assays

HEK293T cells (50,000 cells/well) were seeded into 48-well plates 24 h prior to transfection. Cells were transfected with 2.5ng of the indicated firefly luciferase reporter plasmids, 5ng of GL3-renilla luciferase (TK- renilla) plasmid, and titration series or single doses of expression

plasmids for the proteins of interest as indicated in figures. For the reporter gene assays, to determine the effect on varying promoters only the firefly reporter plasmids and Vpr expression plasmids were transfected. For the reporter gene assays testing transfected vs integrated NFκB reporter, a titration series of transfected reporter was performed to establish the dose of transfected reporter with the same baseline luciferase expression as the integrated reporter. This was found to be 55ng of the NFκB firefly luciferase plasmid. The total DNA in all wells was always made up to the same amount using EV. Transfection was performed using Fugene-6. Cells were 70 % confluent when transfected. Following transfection media was once more replaced at 24 hrs and cells were harvested after 24-48 h. See table appendix for a list of plasmids used in this study. Following the transfections and treatments described in figure legends, cells were washed once with PBS (150 µl per well) and harvested in passive lysis buffer (Promega, 100 µl/well) followed by freeze-thawing of cells to aid cell lysis and firefly and renilla luciferase activity measurement of 20 µl in a 96Wwell GloMax Luminometer Light Plate (Promega). 30µl of Dual-Glo® Luciferase Assay Reagent was then added to each well and incubated for 10 minutes. The number of relative light units (RLU) were read 3 times using a GloMax 96 Microplate Luminometer (Promega) and a mean was taken. Except where indicated the same procedure was then followed after the addition of 30ul Dual-Glo Stop & Glo reagent and renilla luminescence was measured. The firefly-luciferase readings of each sample were normalised to the renilla-luciferase readings to account for cell number and transfection efficiency and fold inductions were calculated relative to the mock-stimulated controls as explained in figure legends.

2.4 RNA extraction and cDNA synthesis

The QIAgen RNeasy RNA extraction kit was used to extract RNA from cells using the manufacturer's protocols. For 24- and 6-well plates the cells were harvested in 350 µl buffer RNeasy lysis buffer (RLT). For reverse transcription to synthesize complementary DNA (cDNA), each reaction contained 1µg RNA, 2.5µM oligo dT, 500µM dNTP, made up to 13µl with nuclease-free water. These reactions were incubated at 65°C for 5 min and then transferred directly to ice for 1 min. To each reaction 4 µl 5x First-strand buffer (Invitrogen), 5 mM DTT, 40 U RNase OUT (Invitrogen) and 50 U Superscript III reverse transcriptase (Invitrogen) was added, made up to a total of 20 µl with nuclease-free water. The reactions were incubated at 50°C for 1 h, followed by 70°C for 15 min. cDNA was diluted 1:2 in nuclease-free water before quantitative reverse transcription-polymerase chain reaction (qRT- PCR) analysis or array.

2.5 qRT-PCR

Each qRT-PCR, performed in 96-well plates, in duplicate, contained 2 µl cDNA, 10 µl 2x SYBR® Green PCR master mix (Applied Biosystems), 0.5 µM of each primer, made up to 20 µl with water per well. A ΔΔCt relative quantity assay was then performed where the amplification of genes of interest was normalised to an endogenous control (GAPDH) on a 7900HT Real-Time PCR machine (Applied Biosystems) using the following programme: 2 min at 50°C; 10 min at 95°C; 15 s 95°C; 1 min at 60°C X 40 cycles. Data were analysed using the RQ Manager 1.2 software (Applied Biosystems).

2.6 Preparing short hairpin RNA (shRNA)-expressing MLV vectors

Potential 19mer RNAi sequences were selected using online Clontech RNAi algorithms (http://bioinfo.clontech.com/rnaidesigner/sirnaSequenceDesignInit.do). BLAST was used to check target sequences in cyclophilin A and Nup358 for complementarity to human genome transcripts. Three target oligonucleotides incorporating the 19mer per for each of CypA and Nup358 were designed using online Clontech designer tools. Oligonucleotides were annealed by mixing 10 µl of each stock (previously made to 1 µg/ml in water). Then 2.5 µl of 2 M NaCl was added, and the reaction mixture was heated at 98 °C for 5 min and cooled slowly (~0.1 °C/sec), to 4 °C. 350 µl DNase-free water, 40 µl of 3 M NaAc (to reduce DNA interaction with dH2O) and 1.1 ml 100% EtOH were then added, mixed and stored at -80 °C overnight. Centrifugation at 13,000 rpm at 4 °C for 45 min was used to pellet DNA. The EtOH was removed and the pellet was air dried and resuspended in 50 µl DNase-free water. 3 µl annealed oligonucleotides were ligated with 1 µl pSIREN-RetroQ vector (~50 ng) digested *EcoRI*/*BamHI*. Colonies were screened by *MluI* digest as inserts (annealed oligonucleotides) contain unique *MluI* sites. Pseudotyped, retroviral-based vectors incorporating shRNA-encoding transcripts were produced by three plasmid transfection of HEK293T cells. MoMLV GagPol particles were produced by co-transfection of: i) pSIREN-RetroQ encoding shRNA transcripts (the vector without viral genes, encoding a transgene under an internal constitutive CMV promoter, flanked by the *cis*-acting viral elements required for RNA packaging, reverse transcription and integration in target cells); ii) pCMVi packaging plasmid (contains retroviral genes required for enzymatic activity (*pol*) and particle formation (*gag*) under control of a CMV promoter, such that they are constitutively expressed in HEK293T cells); and iii) a VSV-G expression plasmid, pMD-G (encoding the envelope glycoprotein) as described by Naldini et al in 1996 (511). A 10 cm^2 dish of the same HEK293T cells used in transfection experiments was co-transfected just before at 80% confluency with 1µg GagPol expressor plasmid, 1 µg

VSV-G expressing plasmid and 1.5µg retroviral vector was made to ~15µl with water. 12µl Fugene 6 (Roche) was then added to 200µl Opti-Mem without allowing Fugene 6 to touch the sides of the tube and mixed. The DNA mix was then added to this mixture, spun briefly to remove drops from the sides of the tube and incubated at room temperature for 20 min. Cell culture media was replaced with 8 ml fresh DMEM, 15% FCS, Penicillin and Streptomycin (as in previously) and the transfection mixture was added drop by drop to the replaced media. The media was replaced at 24hrs and vector containing supernatant was harvested at 48 and 72 hrs, filtered through a 0.45µm filter, aliquoted in 1.5 ml tubes and stored at -80 °C. 1 ml viral supernatant was used to transduce ~2 x 10^5 cells. At 48hrs cells were transferred to a 10 cm^2 dish in DMEM with 2.5µg/ml puromycin. As a control, non-transduced cells were also treated with puromycin. Transduced cells were plated for infection assays or frozen for storage. When cells were stable at 2.5µg/ml puromycin, the drug concentration was increased to 7.5µg/ml. Single cell clones were prepared by limiting dilution. 100 cells were suspended in 50 ml of DMEM (10% FCS, 100 U/ml penicillin, 100µg/ml streptomycin) with 10µg/ml puromycin and plated at 500µl/well in 4 x 48 well plates. Wells containing multiple colonies were discarded. Once single colonies had reached 50% confluency they were transferred to 6 well plates.

2.7 Protein analysis

2.7.1 SDS-PAGE

For sodium dodecyl sulphate (SDS) – polyacrylamide gel electrophoresis (SDS-PAGE) analysis cells were collected in 80ul Passive Lysis Buffer (Promega) and 30ul was used for luminometry analysis. The remaining 50 was centrifuged at 4°C, the cellular debris discarded, the supernatant removed and heated at 100°C for 5 min in 6x protein loading buffer (β-mercaptoethanol (BME) (50 mM Tris-HCl (pH 6.8), 2 % (w/v) SDS, 10% (v/v) glycerol, 0.1% (w/v) bromophenol blue, 100 mM BME) prior to loading on a 10 % or 12 % polyacrylamide gel. The proteins were separated by electrophoresis at 120 V in SDS running buffer (25 mM Tris-HCl, 250 mM glycine, 0.1 % (w/v) SDS).

2.7.2 Nu-PAGE

For the analysis of proteins using the Invitrogen Nu-PAGE system, proteins were heated at 100°C for 5 min in 4x Nu-PAGE protein loading buffer (Invitrogen). The proteins were then separated on a 4-12 % Bis-Tris polyacrylamide gradient gel (Invitrogen) at 120 V in MES buffer (Invitrogen).

2.8 Immunoblotting

After PAGE, proteins were transferred to a Hybond nitrocellulose membrane (Amersham biosciences) in transfer buffer (25 mM Tris-HCl, 250 mM glycine, 20 % (v/v) methanol) using a semi-dry transfer system (Biorad) and blocked by incubation for 1 h at room temperature in 5 % (w/v) milk proteins + 0.01 % (v/v) Tween-20 in PBS (PBST). The membranes were then incubated overnight at 4 ^{o}C with the primary antibody (Ab, see below) diluted in 5 % (w/v) milk proteins in PBST. Membranes were washed three times for 5 min in PBST and probed with IR-dye labeled secondary anti-rabbit or anti-mouse antibodies labelled for detection in the 700 or 800nm channel (LiCor, 1:10 000) in 5% (w/v) milk proteins in PBST for 1 h at room temperature. The membranes were rinsed three times for 15 min with PBST and briefly with deionized water. Images were made with the Odyssey Infrared Imaging System (LI-COR Biosciences). Blots were imaged at 169 µm. Quantification was performed with the analysis software provided. Tubulin, Beta-actin or Valosin-containing protein (VCP) were used as loading controls. VCP is a 97 kDa ATPase involved in the ubiquitin-proteasome degradation pathway, membrane fusion, transcription activation, cell cycle control, apoptosis, and molecular choning (522).

2.9 Silver staining of proteins

Proteins separated by PAGE were silver stained using the Invitrogen SilverQuest silver staining kit according to the manufacturer's protocol.

2.10 Immunofluorescence and Confocal Microscopy

For immunofluorescence assays, glass coverslips were placed in a 24 well plate and incubated with poly-L-lysine (Sigma) for 12hrs at 37°C. Coverslips were washed 3 time with PBS and cells were seeded the day before transfection at 50,000 cells per well. Cells were transfected as previously described and at 72hrs cells were washed three times with cold PBS and fixed in 4 % (v/v) paraformaldehyde in PBS containing 250 mM HEPES (pH 7.4) by incubation on ice for 5 min and then at room temperature for 15 min. The cells were then washed three times with PBS, incubated in 50 mM ammonium chloride in PBS for 5 min to quench free aldehyde autofluorescence, washed three times in PBS, and permeabilised with 0.1 % (v/v) Triton X-100 in PBS for 5 min. The cells were again washed three times in PBS then blocked in 5 % (v/v) FBS in PBS (blocking buffer) for 30 min at room temperature. The coverslips were then inverted and incubated on a 70 µl drop of primary anti-FLAG Ab diluted

in blocking buffer for 90 min. The coverslips were washed three times in blocking buffer followed by incubation on a 70 µl drop of secondary Ab (see below) diluted in blocking buffer for 90 min. The coverslips were then washed twice with blocking buffer and once with PBS. Finally, the coverslips were dipped in distilled water to prevent the formation of PBS crystals, placed on to a slide prepared with a 30 µl drop of mounting medium (Mowiol 4-88, containing 4',6-diamidino-2-phenylindole (DAPI)) and allowed to set before storing at 4°C. The cells were visualised using a Leica SPE1 Confocal Microscope at 63X magnification with an oil immersion lens and images were collected using Leica Image Browser.

2.11 Immunoprecipitation assays

Prior to harvesting, the cells were washed twice on ice with ice-cold PBS followed by scraping in an appropriate volume of immunoprecipitation (IP) buffer (NP40 0.5% in PBS, supplemented with protease inhibitors (Roche)). The cells were collected in Eppendorf tubes, slowly rotated at 4°C to allow lysis, and then the cell debris was collected by centrifugation at 14,000 x *g* for 20 min, 4°C. 50µl of the lysate supernatant was transferred to a tube (input) and the remainder transferred to another tube for the IP assay. 30µl of anti-FLAG M2 agarose beads (Sigma-Aldrich) were prepared per IP protocol by washing once with 1 ml water and twice with 1 ml IP buffer (centrifuging at 600 x *g* for 1 min per wash). The beads were resuspended in an appropriate volume of IP buffer and added directly to the cell lysates. The lysates were incubated with the beads by rotation at 4°C for 2 h and then washed three times in pre-chilled IP buffer (centrifuging at 600 x *g* for 1 min per wash). Finally, they were resuspended in 20 µl 6x protein sample buffer for analysis by SDS-PAGE and immunoblotting.

2.12 Transcriptional Profiling

12 samples were prepared for array analysis: 3 replicates each of 4 conditions (SIVmon, SIVgsn, SIVmonS40N and empty vector). RNA was extracted as described previously using the QIAgen RNeasy RNA extraction kit with the manufacturer's protocols. Genomic DNA was removed with the TURBO DNA-free kit (Ambion). RNeasy MinElute Cleanup kit (QIAGEN) was used to concentrate the RNA. RNA quality control was assessed with the Agilent 2100 Bioanalyzer (Agilent Technologies). Range of RNA was determined to be 56-179 ng/u with RNA integrity numbers (RIN) of: 7.2-10. The sample with the lowest integrity number of 7.2 was discarded. cRNA from all samples was fluorophore-labelled. with the Low Input QuickAmp labelling kit, and hybridized to SurePrint G3 Human Gene Expression v3 8 × 60K or Human Gene Expression v2 4 × 44K whole-genome microarrays (Agilent Technologies). Array images were acquired with Agilent's dual-laser microarray scanner G2565BA and analyzed with Agilent Feature Extraction software (v9.5.1). This extracts raw data from the scanner

output files, log2-transforms median Cy3 and Cy5 signal intensities and normalises these intensities using LOESS local linear regression to the mean signal of all the samples using the R package agilp (512)(513). Analysis of all microarray data was conducted on log2-transformed data and restricted to gene symbol–annotated probes expressed above background negative-control levels in at least one sample. Significant gene expression differences between datasets were identified using Mann-Whitney *U* tests for nonparametric data in MultiExperiment Viewer v4.9 (http://www.tm4.org/mev.html) with a false discovery rate of 0.05 and a filter for greater than 2-fold differences in median normalised expression values. Gene ontology and pathway analyses were performed in InnateDB, which identifies biological pathways that are statistically over-represented in a given gene list. Network graphics of gene and pathway association were generated using Gephi (http://gephi.github.io/). A principle component analysis (PCA) was performed in R and one SIVgsn sample did not cluster with other replicates of the same condition and was excluded from further analysis. Significant gene expression differences between datasets were identified. There were 30 transcripts with increased expression and 29 with reduced expression in the SIVmon samples. Gene ontology (GO) and pathway analyses were performed in innateDB (514). InnateDB incorporates pathway annotation (all pathways not just immune relevant ones) from major public databases including KEGG, Reactome, NetPath, INOH, BioCarta and PID. GO over-representation analysis (ORA) examines a gene/protein list for the occurrence of GO annotation terms, which are more prevalent in the dataset than expected by chance. Annotations that occur more frequently than expected in a gene list can be identified and may point towards a biological process or pathway that is being differentially regulated in the condition of interest, in this case SIVmon. GO ORA is restricted to biological processes. A second analysis was performed restricted to the “reactome”. Then, a transcription factor binding site analysis was performed using online software oPOSSUM to detect over-represented conserved transcription factor binding sites and binding site combinations in sets of genes or sequences but there were no results with a Z score of >10, which would indicate significance. These analyses were performed comparing SIVmon Vpr to all controls and an Ingenuity pathway analysis was performed using IPA (http://www.ingenuity.com), which applies causal networks that integrate previously observed cause–effect relationships reported in the literature. IPA® is a web-based software application that enables researchers to analyze data derived from expression and SNP microarrays, RNA-sequencing, proteomics and metabolomics experiments, and small-scale experiments (such as PCR) that generate gene or protein lists and enables understanding of the significance of data or targets of interest in relation to larger biological or chemical systems. The causal analysis is derived from a structured repository of biological and chemical “findings” curated from various sources including the literature.

2.13 Phylogenetic analysis

Vpr sequences obtained from the Los Alamos database were manually aligned using the Se-Al sequence editor (http: //tree.bio.ed.ac.uk/software/seal/). Whilst Vpr and Vpx are extremely diverse proteins and not amenable to consistent alignment, multiple strategies consistently give the clades of interest strong support. A maximum-likelihood phylogeny structure was reconstructed by using the General Time-Reversible model of nucleotide substitutions and adjusting evolution rates across sites (GTR?CAT) and FastTree v2.1.5 software (515). Branch support was calculated by Shimodaira-Hasegawa-like local branch support (SH-like test), as implemented in FastTree. The phylogeny was edited with the program FigTree (http://tree.bio.ed.ac.uk/software/seal/). The tree was artificially rooted and branch lengths indicate the number of nucleotide substitutions per site.

2.14 Structural alignments

To guide a mutagenesis strategy, a structural alignment was made using the online T-Coffee multiple alignment software. This uses an algorithm combining progressive and consistency-based alignment, and is available for online usage and is considered to be useful for alignments of more divergent sequences and which also incorporates three-dimensional structural information for the alignment of protein sequences (http://www.ch.embnet.org/software/TCoffee.html) (516,517). The ESPript (*Easy Sequencing in PostScript)* online software was then used, a program that renders sequence similarities and secondary structure information from aligned sequences (518).

3 Chapter 3 Antagonism of reconstituted innate immune sensing in 293 cells with lentiviral Vprs

3.1 HIV-1 M Vpr is able to rescue a spreading infection in monocyte derived macrophages from addition of cGAMP

Unpublished data from our lab (Jane Rasaiyaah) show that HIV-1 M Vpr is able to rescue a spreading infection in monocyte derived macrophages from inhibition by addition of cGAMP, the second messenger released by cGAS following activation by a dsDNA ligand, which activates STING (figure 3.1). This initiates a signalling cascade culminating in the production of type I interferon (IFN) and pro-inflammatory genes via IRF3 and NF-κB transcription factors. We hypothesised that Vpr has evolved to antagonise these signalling pathways to protect viral replication from antiviral responses induced by DNA sensing.

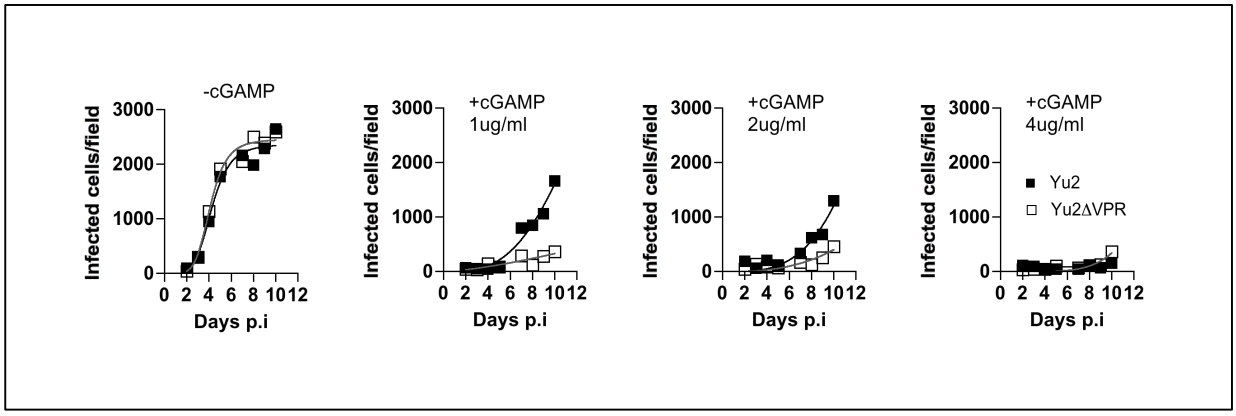

Figure 3.1HIV-1 M Vpr is able to rescue a spreading infection in monocyte derived macrophages from addition of cGAMP. Replication of HIV-1 YU2 WT or a Vpr-null mutant measured over 11 days with 0ng, 1ng, 2ng or 4ug/ml (L to R) of cGAMP added on day 1 post infection.

3.2 Co-expression of cGAS/STING activates an NFκB-sensitive promoter

In order to investigate the effect of HIV-1 M Vpr, this signalling pathway was reconstituted in HEK293T cells using transfected expression plasmids. HEK293T cells have been reported to deficient in cGAS and STING signalling (326) and indeed this may underlie their utility as transfectable cells. Being unable to detect and respond appropriately to cytosolic DNA may be central to the poorly understood mechanisms of transfection. Absence of these proteins was demonstrated on immunoblot (figure 3.2) and a transfection assay to recapitulate cGAS/STING activation using a NFκB-promoter firefly luciferase reporter was optimised.

HEK293T cells were transfected in 48-well plates with 0, 1 or 3ng of expression vectors for cGAS and/or STING to activate NFκB signalling (figure 3.2A). Simultaneously, a firefly luciferase reporter plasmid was transfected, where the expression of firefly luciferase is under the control of a synthetic NFκB sensitive promoter. This promoter contains five copies of an NF-κB response element (NF-κB-RE) that drives transcription of the luciferase reporter (Promega).

Early experiments were duplicated with a reporter plasmid under control of the natural promoter of the *immunoglobulin kappa light chain* gene which is also sensitive to NFκB subunits p50 and p65 and contains three repeat κB sequences. The binding sequence is 5'-GGGRNYYYCC-3', where R is a purine, Y is a pyrimidine, and N is an unspecified base (personal communication Andrew McDonalds and 523–525). No significant differences were seen in either NFκB activation or antagonism between the synthetic and natural promoters. Where NFκB activity is shown it is from the synthetic promoter. This was chosen as it is identical to the integrated reporter in figures 4.7, 4.10 and 5.3.

7.5ng of a transfection control reporter plasmid was also co-transfected to confirm consistency between transfections and lack of toxicity (figure 3.2B). This plasmid contained a minimal promoter fragment from the HSV thymidine kinase (TK) promoter adjacent to a reporter gene, the secreted luciferase from the anthozoan coelenterate *Renilla reniformis* (Promega). At 48 hours post transfection cells were harvested for immunoblot. Firefly and renilla luciferase activity were measured 48 hours after transferction by luminometry (Fig 2 A & B). The firefly luciferase activity was then normalised to renilla luciferase activity (Fig 3.2 C).

Whereas ectopic cGAS expression alone failed to activate NFκB signalling, in support of the notion that there is no endogenous STING in these cells, transfection of STING alone was able induce NFκB promoter activity possibly because of low levels of endogenous cGAS able to sense the transfected DNA (figure 3.2A&C)). This would be consistent with previous published data reporting very low levels of cGAS RNA in HEK293T cells (293).

STING induced NFκB promoter activity is augmented ~10 fold by simultaneous transfection of cGAS in a 1:1 ratio and further augmentation of signal was achieved by increasing the dose of each molecule and adding 100ng more of empty expression vector (pcDNA.3) (figure 3.2 A&C). This may act as a substrate for cGAS, triggering the production of cyclic GMP-AMP (cGAMP). This observation supports the notion that cGAS/STING mediated NFκB activation may be mediated by cGAS sensing the transfected DNA itself as well as being a feature of overexpression.

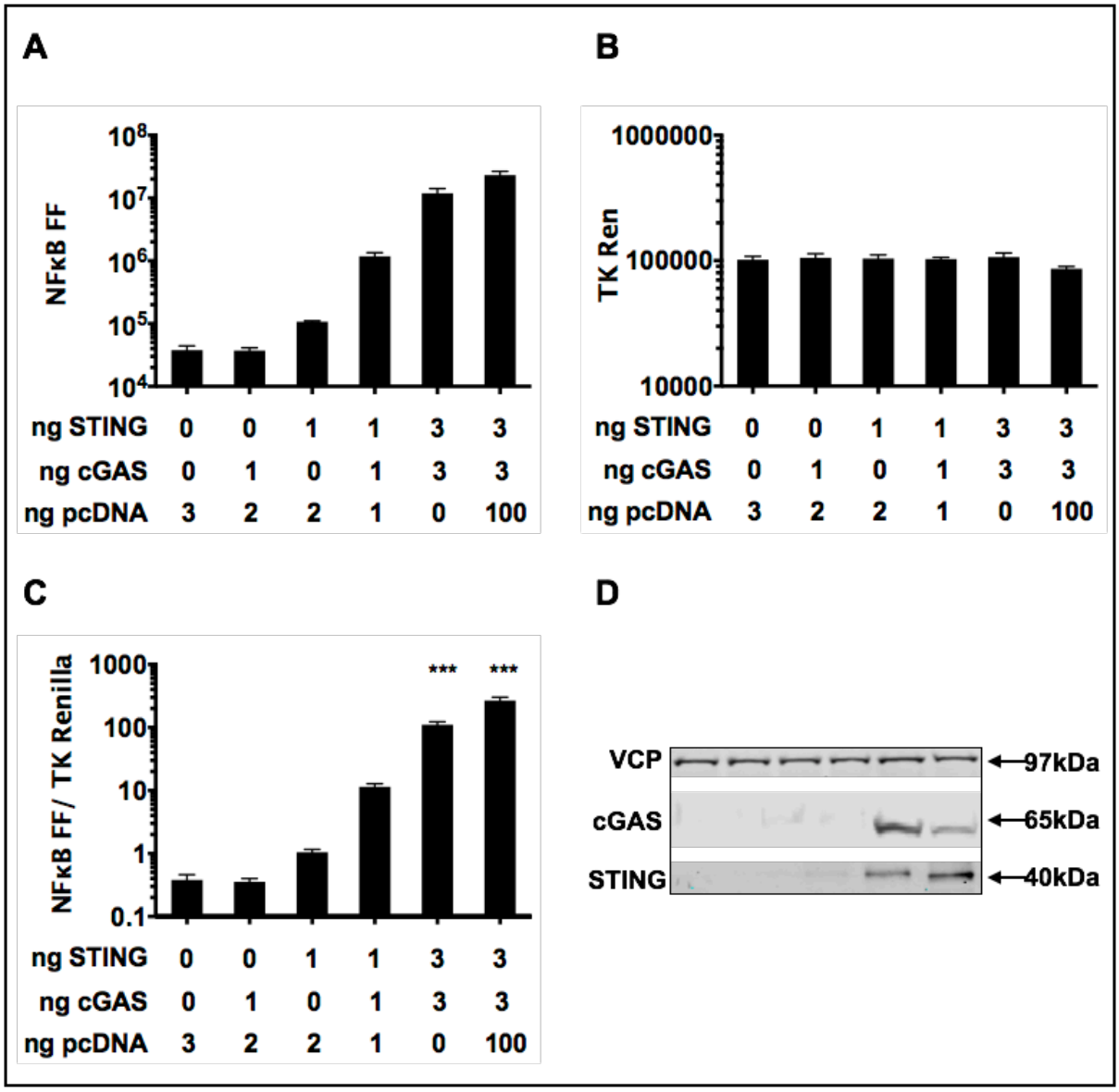

Figure 3.2 Co-expression of cGAS/STING activates NFκB-sensitive promoter driven luciferase expression. Firefly (FF) and renilla (Ren) luciferase signals were quantified with luminometry (A & B, respectively), with renilla fluorescence acting as a transfection control. Firefly luciferase activity was normalised to renilla luciferase activity (C). Data are from three independent experiments and represented as mean ± SEM. (D) A representative immunoblot shows relative expression of HA-cGAS and HA-STING with VCP as a loading control. ***: P<0.001 compared to 0ng STING, 0ng cGAS, one-way ANOVA with Dunnett's post-hoc test. Valosin-containing protein (VCP) is a 97 kDa protein used as a loading control. VCP is an ATPase involved in the ubiquitin-proteasome degradation pathway, membrane fusion, transcription activation, cell cycle control, apoptosis, and molecular choning (522).

3.3 Co-expression of cGAS/STING induces expression of endogenous NFκB genes

To confirm stimulation of endogenous gene expression following ectopic expression of cGAS and STING, mRNA levels for three different genes, all sensitive to NFκB, were quantified with qRT-PCR: ISG56, CXCL-10 and IFIT-2 (figure 3.3A&B).

ISG56: IFNAR (IFN-α/β receptor) is common surface receptor for all type I IFNs. It activates the JAK-STAT signalling pathway. This leads to transcription of more than 200 IFN-stimulated genes (ISGs) (309). IFN-stimulated gene 56 (ISG56) was one of the first interferon (IFN)-inducible genes to be cloned almost 3 decades ago (523). It is one of a family of viral stress-inducible genes (VSIG) with high sensitivity to IFN treatment and activation of innate immune signalling pathways. VSIGs are induced by activation of IFN regulatory factors (IRFs) which initiate transcription following recognition of the IFN-stimulated response elements or ISREs - cis-acting elements found approximately 200 bp upstream of the TATA box in the VSIG promoters. IRF-3, is activated by dsRNA (via TLR3 or RIG-I/MDA-5), by lipopolysaccharides (via TLR4) or by dsDNA (via cGAS and STING), potently induces ISG56 family genes, and ISG56 expression is frequently used as the readout for IRF-3 transcriptional activity (524).

CXCL10 is a CXC chemokine which chemo-attracts activated T cells and myeloid cells through CXCR3 receptor binding. Its expression is controlled by multiple transcription factors including IRF3 and NFκB (525).

IFIT2 is part of the *Ifit* family of ISGs which are strongly induced by type I IFNs and by many signalling pathways that activate IRF-3 or IRF-7 (526).

RNA was extracted from HEK293T cells 48 hrs post transfection with a 5 point titration series of cGAS and STING in a 1:1 ratio and used for qRT-PCR analysis to examine the expression of ISG mRNAs. cGAS and STING robustly stimulated the tested genes in a dose dependent manner (figure 3.3). STING levels were measured by immunoblot (figure 3.3A)

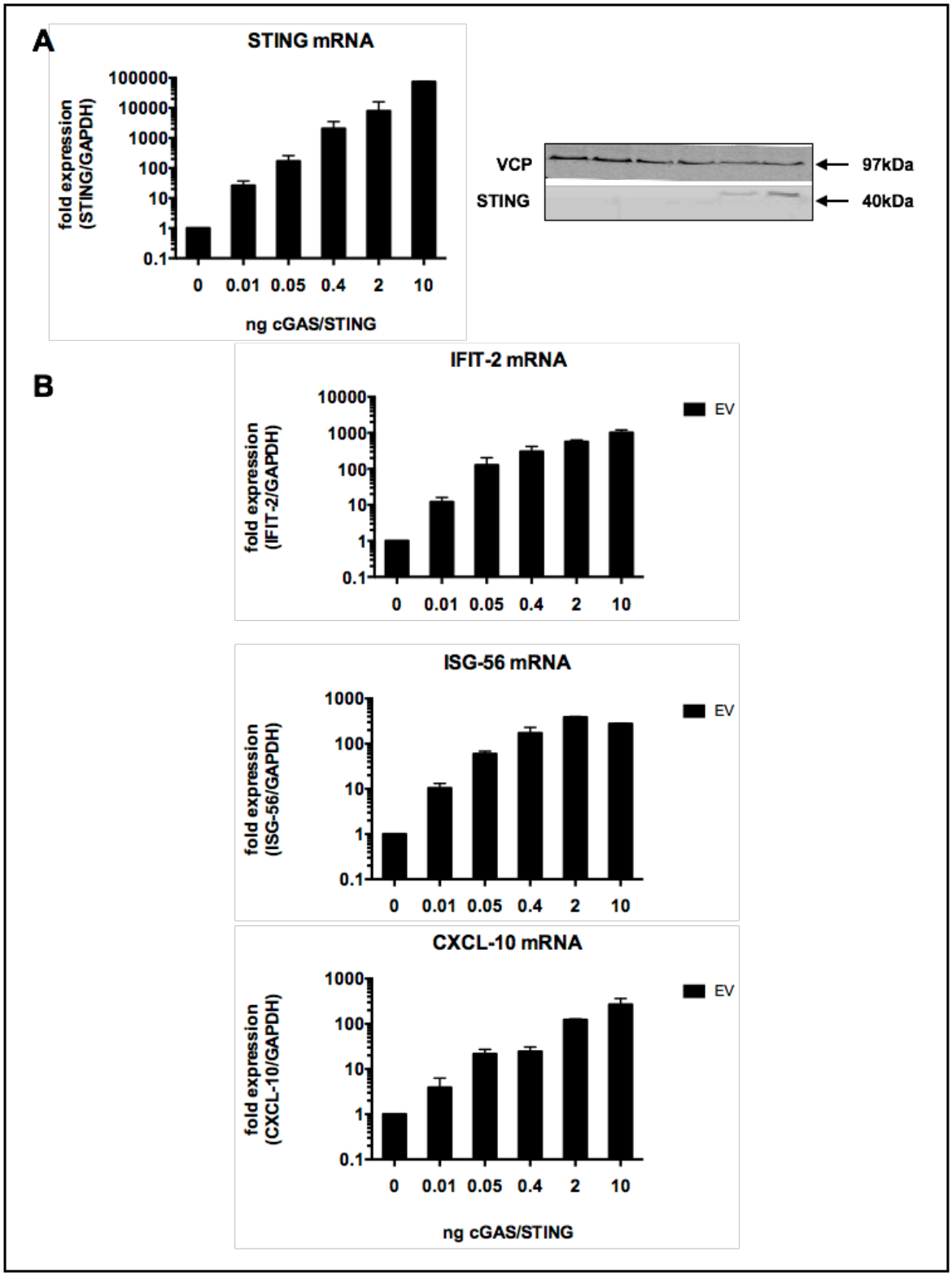

Figure 3.3 Co-expression of cGAS/STING activates endogenous NFκB sensitive genes. qRT-PCR analysis of ISG expression for presence of STING mRNA (3.3 A) and induction of ISG56, CXCL-10 and IFIT-2 (3.3 B). Data are presented as the mean (of three separate experiments) fold induction relative to the unstimulated control of each cell line ± SEM. The whole cell lysates were separated by SDS-PAGE and analysed by immunoblot with anti-HA antibodies for detection of STING. Protein molecular mass is indicated (kDa) (A).

3.4 Co-expression of HIV-1 YU2 Vpr selectively antagonises NF-κB induced expression of luciferase

To test the effect of HIV-1 M Vpr on the dsDNA sensing pathway a plasmid expressing HIV-1 YU2 Vpr was transfected at 0, 50 or 150ng with the cGAS, STING and reporter plasmids. The plasmid used in this experiment was the first Vpr construct tested and was codon optimised at Blue Heron Bio. Subsequent Vprs were codon optimised by another proprietary algorithm (Geneart) which caused them to have far greater expression levels. At 48hrs post transfection luminometry and immunoblots were performed on cell lysates. The HIV-1 M YU2 Vpr was found to antagonise luciferase expression from the NFκB sensitive promoter, and from the promoter of the IFNβ gene which contains binding sites for the NF-κB, IRF3 and AP-1 transcription factors (527,528) (figure 3.4 A and B).

An ISG56-promoter firefly luciferase reporter was used to test the effect of Vpr on IRF3 signalling (figure 3.4C). Transcription of this gene is highly dependent on, and specific for the IRF3 transcription factor (529) and was unaffected by transfection of even high doses of Vpr. The constitutively expressed TK renilla control reporter was not significantly affected.

Interestingly there was a reduction in levels of STING detected on immunoblot observed in all three experimental conditions (Fig 3.4 A, B & C). This was unexpected: luciferase expression from the IRF3 sensitive reporter was unaffected by HIV-1 M Vpr, despite the loss of the STING which was stimulating activation at this promoter, presumably by nuclear translocation of IRF3. I hypothesised that Vpr was causing STING to be degraded but that there were potentially two pools of STING, one causing IRF3 activation and one causing NFκB activation. If this was the case and one of these pools (leading to NFκB signalling) was selectively degraded then the other (leading to IFR3) might remain unaffected. There could also be a distal block to signalling in the pathway specific for NFκB but which left IFR3 signals unaffected. This is in contrast to a study by Vermeire et al which found a weakly stimulatory effect of Vpr on NFκB signalling (530). Given the centrality of NFκB signalling in the up regulation of antiviral genes and the known sensitivity of HIV-1 to interferon a selective advantage to antagonising NFκB signalling in early infection is biologically plausible. It is subsequently shown in this study that the apparently specific effect on NFκB signalling is in fact due to the weak codon optimization of this Vpr gene causing it to be relatively poorly expressed combined with the greater sensitivity of the ISG56 promoter to low levels of IRF3 (see figure 3.6).

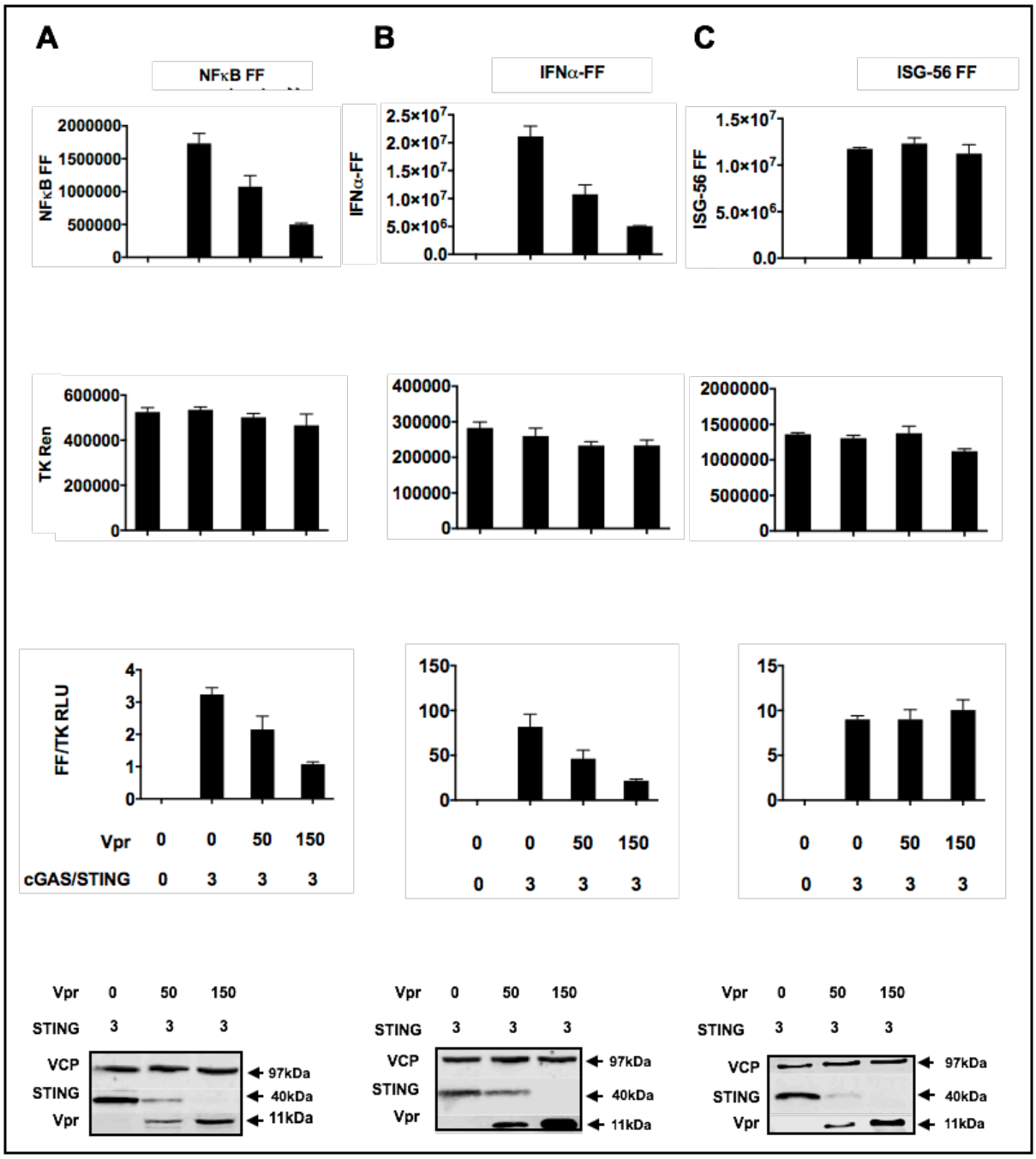

Figure 3.4 Co-expression of HIV-1 YU2 Vpr antagonises expression from NFkB sensitive promoters but not IRF3 sensitive promoters. HA epitope tagged cGAS and STING expression plasmids were transfected into HEK293T cells at a constant dose of 3ng simultaneously with transfection of firefly luciferase reporters and 7.5ng of TK renilla luciferase and with either an NFκB sensitive promoter (A), the IFNβ promoter (B) or the ISG56 promoter. HIV-1 M YU2 Vpr was co-transfected at 150 or 50ng. Cells lysates were assessed with luminometry. Firefly luciferase (top row) and renilla luciferase (middle row) are presented. Firefly luciferase was normalised to the renilla (bottom row). Data are from three separate experiments and are represented as mean ± SEM. Whole cell lysates were separated by SDS-PAGE and immunoblotted with anti-HA antibodies and anti-Vpr antibodies. The location of protein molecular mass markers is indicated (kDa).

3.5 Expression of HIV-1 M Vpr does not cause an increase in LIVE/DEAD cell staining

Vpr has been widely reported to cause apoptosis in a number of different cell lines and types (460,462,531,532). To ensure that the phenotype of antagonism of NFκB signal was could not be attributed to cell death cells were transfected with Vpr up to a dose of 300ng/50,000 cells, a far higher dose than was used in any other experiment reported. Cells were then stained with a viability dye (LIVE/DEAD Aqua, Invitrogen) at 48hrs post transfection then fixed and analyzed on a BD FACSCalibur (Figure 3.5 A&B). Cells were gated by structure, size, and LIVE/DEAD stain. Representative plots at 48hrs are shown.

A number of additional checks were made to ascertain that cell viability was not compromised by the transfection. pH indicators in the DMEM culture medium showed identical colour change across wells with increasing doses of Vpr. Subjective observations of cell viability as well as Trypan Blue exclusion dye counting gave no indication of cell death, changes in adherence or morphological changes. Finally, the consistency of loading controls on immunoblot (figure 3.5B), the maintenance of expression of renilla luciferase from the control plasmid and the specificity of the phenotype for NFκB inhibition all support the notion that the effect of Vpr expression is not an artifact of toxicity.

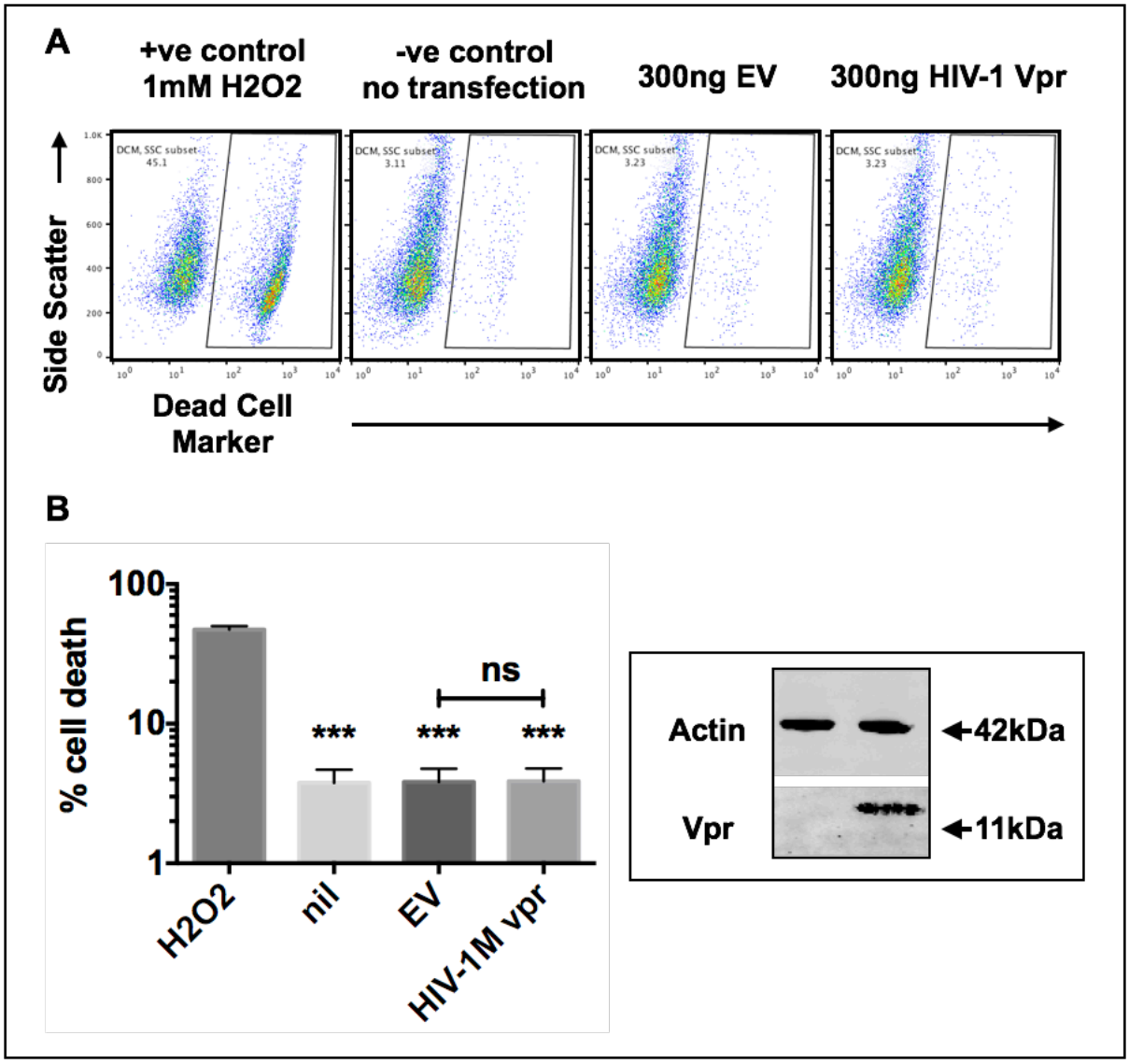

Figure 3.5 Ectopic expression of Vpr does not cause an increase in LIVE/DEAD cell staining (A) Cells were transfected as previously with either Vpr empty vector (EV). For a negative control cells were left untransfected (nil). Hydrogen Peroxide treatment (H2O2 1mM for 3hrs at 37°C) was used to kill cells as a positive control. Cells were harvested at 48hrs and stained with a viability dye (LIVE/DEAD Aqua, Invitrogen), then fixed and analyzed on a BD FACSCalibur. Cells were gated by structure, size, and LIVE/DEAD. Representative plots at 48hrs are shown for the highest dose of Vpr. Cell death induced by hydrogen peroxide was used as a positive control and untransfected cells as a negative control. (B) Presence of Vpr was confirmed on immunoblot. Statistical analysis was performed using a paired t-test. ***= $p<0.001$.

3.6 Primate Vprs exhibit differential antagonism of cGAS/STING induced NF-κB promoter driven expression of luciferase

Previous studies of cellular restriction factors for HIV and their antagonism by viral accessory proteins have provided evolutionary and mechanistic insight into host virus interactions and transmission events. The species specific degradation of tetherin by either Nef or Vpu being prototypical examples (533,534).

To determine whether the observed signalling antagonism is species specific, or conserved, a panel of primate lentiviral Vprs were tested against NFκB activity stimulated by cGAS/STING transfection (as in the assay described in figure 3.4A). This panel represented HIV-1 M group (YU2 primary isolate) and HIV-2 A, two SIVcpz (both from central chimpanzees, *Pan troglodites troglodites*,), SIVsmm (Sooty mangabey *Chlorocebus pygerythrus*) SIVtal (Talapoin, *Miopithecus ogouensis*) and SIVver (Vervet monkey *Chlorocebus pygerythrus*). The HIV-1 YU2 construct was codon optimized with a proprietary algorithm for protein expression optimization from Blue Heron Bio whereas the other constructs were designed and codon optimized for these experiments by Geneart. FLAG-epitope tags were cloned into the open reading frame at the N terminus for detection on Western blot. HEK293T cells were co-transfected with the Vprs as in figure 3.4A and expression was normalised to a control transfected with empty vector (Figure 3.6 A).

Antagonism of cGAS/STING signalling, compared to transfection of an empty vector, was observed with Vpr from both human viruses and the parental primate lineages from which they originated. Interestingly the most effective antagonists were the SIVcpz*ptt* Vprs from the western Chimpanzee subspecies *pan troglodytes troglodytes*. It was hypothesised that this was due to poor codon optimisation of the HIV-1 M which had undetectable expression levels and yet was still able to significantly antagonise.

Importantly this antagonism matched the reduction in STING intensity on western blot with an R^2 of 0.73. (Figure 3.6 B&C). Vif, Vpu, and Vpx all link to cullin-RING finger ubiquitin ligases to induce the polyubiquitination and proteasomal degradation of their cellular targets and it was hypothesised that the reduction of STING on immunoblot by Vpr could be enabled by a similar mechanism. With the exception of the comparison of SIVcpz Vprs and HIV-1 M Vpr the degree of antagonism was independent of levels of expression of Vpr indicating this phenotype may be due to structural differences between Vprs rather than as a product of expression levels (Figure 3.6D).

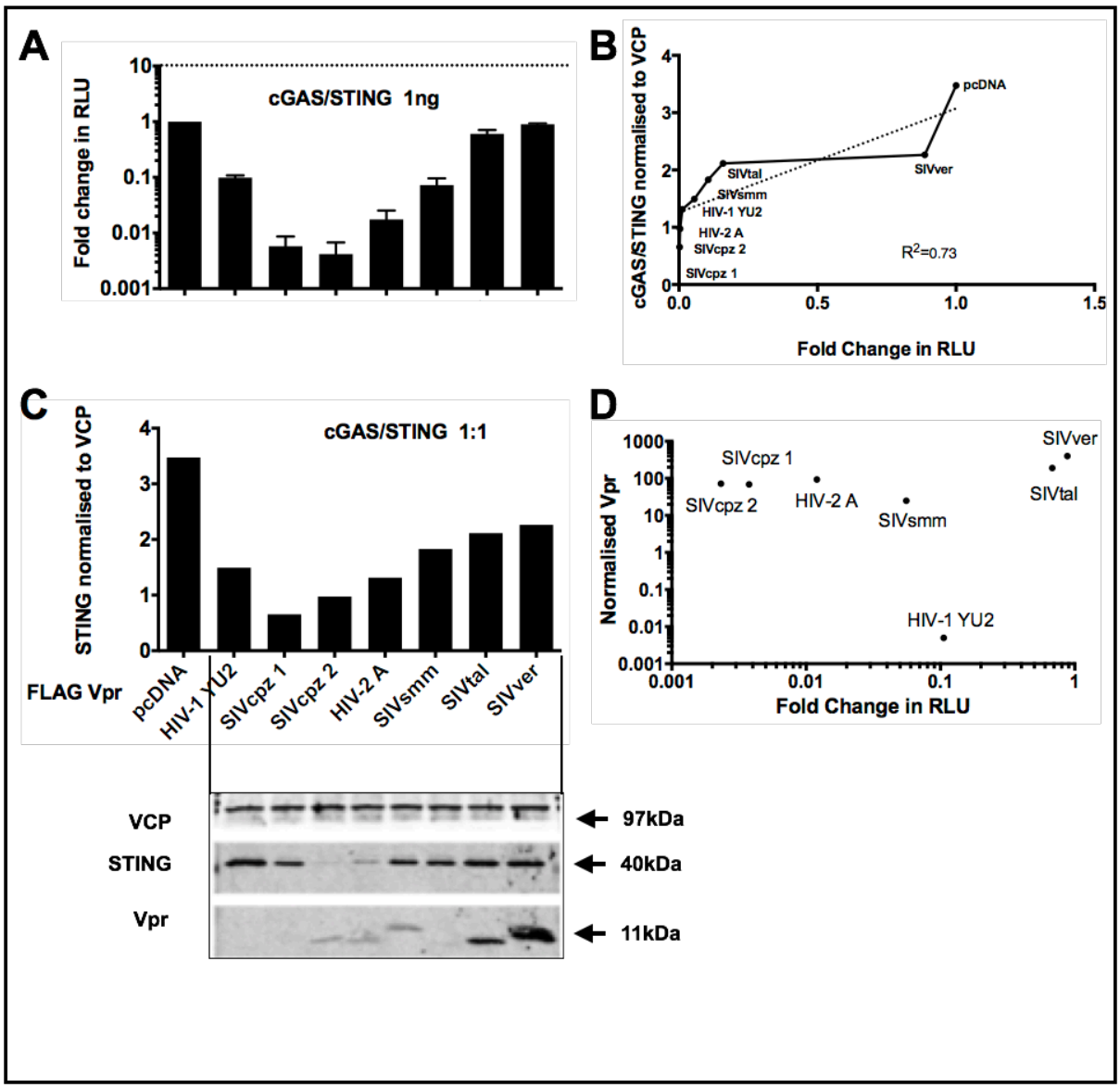

Figure 3.6 Differential antagonism of NF-kB activity by primate lentiviral Vpr proteins. (A) HEK293T cells were transfected as previously described with reporter plasmids, cGAS and STING and HIV or SIV Vprs or an empty vector control. NFkB activation was determined as previously described (by luminometry) 48 h post-transfection. Data are presented as the fold induction relative to an identical dose of cGAS/STING transfected with an empty expression vector. The mean values of three transfections ±SEM are shown. (C) Immunoblotting was used to confirm expression of proteins with anti-HA and anti FLAG antibodies and anti VCP as a loading control. Molecular mass markers in kDa are shown. (B) relationship between loss of STING on immunoblot and RLU. (D) differential expression levels of Vpr plotted against RLU.

3.7 Codon optimised HIV-1 M Vpr shows greater antagonism of cGAS/STING mediated NFκB promoter activation than HIV-2

To investigate whether HIV-1 M is able to antagonise NFκB signalling more effectively than other Vprs at a given level of expression, a panel of HIV-1 and HIV-2 Vpr expression vectors was constructed.

Vpr sequences were chosen from viruses isolated within the first weeks or months following clinical infection which may have unique properties compared to viruses derived from cell culture amplification (535). Many HIV-1 clones come from virus produced in cell culture. While this process selects for strains with an *in vitro* growth advantage, these strains may not represent the virus present *in vivo*. For this reason clinical sample clones were used including HIV-1 YU2, SUMA, WITO and CH77.

HIV-1 M YU2 was cloned directly from uncultured brain tissue of a patient with AIDS dementia complex (536,537). HIV-1 M CH77, SUMA and WITO are infectious molecular clones (IMCs) representing transmitted/founder (T/F) viruses. The sequence of the complete T/F virus genomes used here were inferred from subjects with early/acute clade B infection, the dominant HIV subtype in the Americas, Western Europe and Australasia, using viral RNA (vRNA) obtained early during infection, as well as sequencing of single-genome amplicons (SG amplicons) and phylogenetic inference based on a model of random virus evolution (535). These techniques have been used to show that in nearly 80% of the cases of HIV-1 sexual transmission, viral sequences came from a single ancestral sequence, the T/F sequence (538,539).

All constructs in this experiment were codon-optimised using the Geneart proprietary algorithm. This increased the expression levels of the HIV-1 M YU2. As in figure 3.6, a directly proportional relationship between loss of STING and loss of NFκB driven luciferase expression was seen (figure 3.7A&B). However, whereas previously HIV-2 was seen to be a more effective antagonist of cGAS/STING mediated signalling than HIV-1 M, in this panel the HIV-1 constructs were up to 10 fold more effective (figure 3.7A). The SUMA Vpr T/F clone was the most effective. This was probably attributable to small differences in expression or transfection as detected on immunoblot (figure 3.7B).

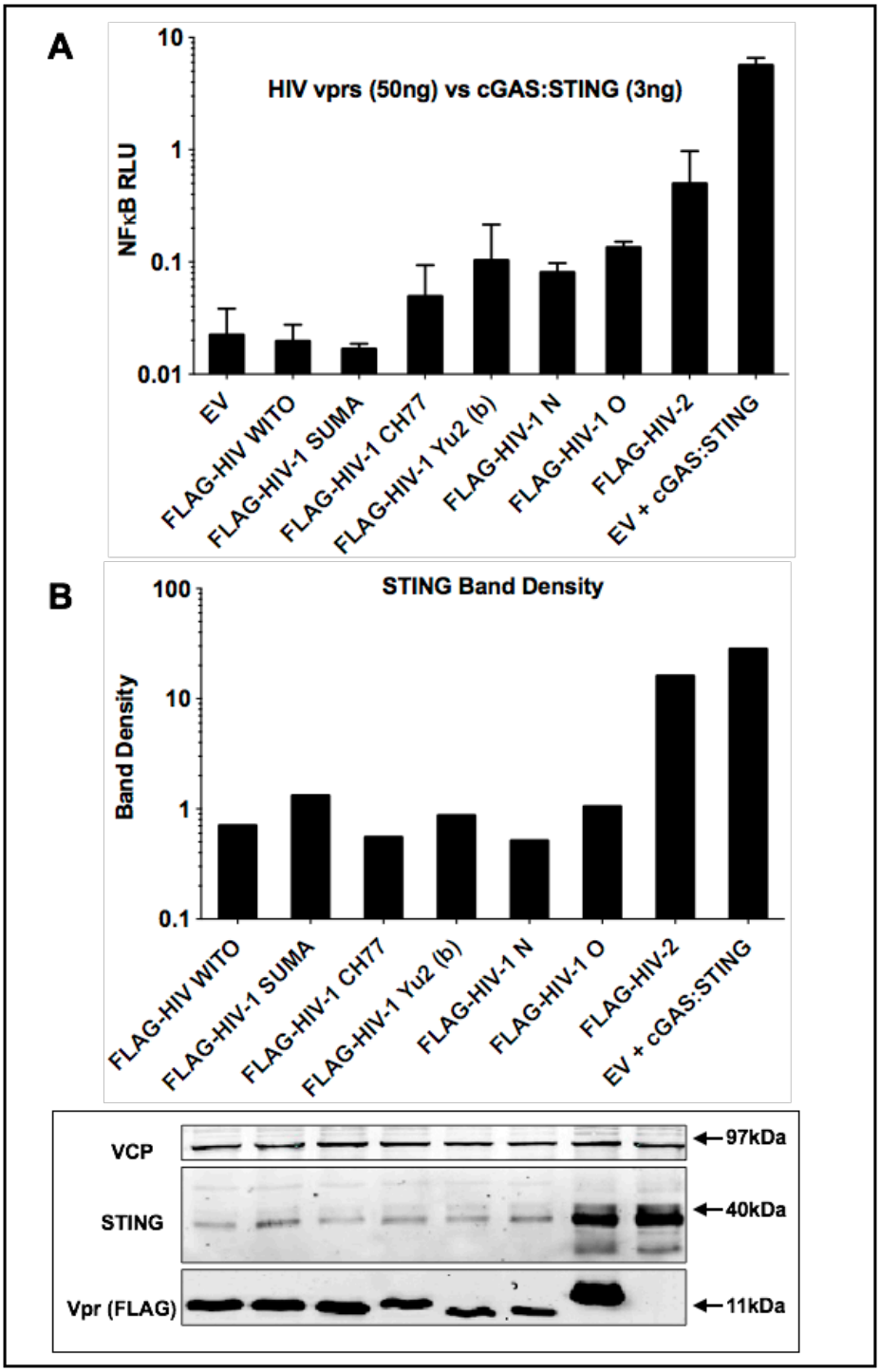

Figure 3.7 Transmitted/founder isolates of HIV-1 M Vpr antagonise cGAS/STING induced NF-κB activation more effectively than HIV-2 Vpr. (A) HEK293T cells were transfected as previously with reporter plasmids, cGAS and STING and HIV Vprs or an empty vector control. NFkB activation was determined as previously described (by luminometry) 48 h post-transfection. Data are presented as the fold induction relative to an identical dose of cGAS/STING transfected with an empty expression vector. The mean values of three transfections ±SEM are shown. (B) Immunoblotting from the same 48-well plates was used to confirm expression of proteins. Molecular mass markers in kDa are shown.

3.8 NFκB signal inhibition by Vpr is unaffected by FLAG epitope tags at the Vpr N-terminal

To confirm that the phenotype was not influenced by the FLAG epitope tag a panel of Vpr expression vectors were cloned without the tag and then tested in parallel with a wider panel of HIV and SIV Vprs (figure 3.8). This panel represents viruses from all families of human and non-human primates infected with lentiviruses including the African apes and the old world monkeys (Cercopithecidae). *Cercopithecidae* monkeys are divided into two subfamilies named *Colobinae* (which include the African colobus monkeys and the Asian genera which do not naturally carry lentiviruses and have only forced zoonotic infections) and *Cercopithecinae* (mainly African, but includes the diverse genus of macaques, which are Asian and North African). *Cercopithecinae* monkeys, from which most non-human primate lentiviruses have been isolated, are categorized in two tribes (Cercopithecini and Papionini) as well as many species and subspecies found across sub-Saharan Africa (540).

There are six approximately equidistant groups of primate lentiviruses, classified, using phylogenetic analysis. The tested range of Vprs represent all of six (541,542).

(i) SIVcpz/HIV-1 lineage from chimpanzees (*Pan troglodytes*) and humans human immunodeficiency virus type 1 (HIV-1) and SIVgor (*Gorilla gorilla gorilla*).

(ii) SIVsm lineage from sooty mangabey (*Cercocebus atys*) together with HIV-2.

(iii) SIVagm lineage from the four main species of African green monkeys, i.e., grivet, sabeus, vervet, and tantalus (members of the genus Chlorocebus).

(iv) SIVsyk lineage from Sykes' monkeys (*Cercopithecus mitis albogularis*), SIVmon from mona monkeys (*Cercopithecus mona*), SIVgsn from greater spot-nosed guenons (*Cercopithecus nictitans*), SIVmus from mustached monkeys (*Cercopithecus cephus*), and SIVdeb from De Brazza's monkeys (*Cercopithecus neglectus*).

(v) The SIVlhoest lineage, composed of SIVlhoest from L'Hoest monkeys (*Cercopithecus lhoesti*), SIVsun from sun-tailed monkeys (*Cercopithecus solatus*), and SIVmnd-1 from mandrills (*Mandrillus sphinx*);

(vi) SIVcol lineage from black, white and olive colobus (*Colobus guereza*).

As more sequences have been analyzed the phylogeny has been complicated with discordant viruses from SIVrcm, gsn and mnd2 which change phylogeny according to the gene region analyzed (26–28) and SIVrcm and gsn sequences were included. The panel included the original sequences in figures 3.6 and 3.7 with further sequences from the from the eastern chimpanzee (*Pan troglodytes schweinfurthii*), the western gorilla (*Gorilla gorilla gorilla*) the SIVagm group, SIVtan (Tantalus *Cercopithecus tantalus*), SIVrcm (Red Capped Mangabey, *Cercocebus torquatus)*, SIVdeb (De Brazza's monkeys, *Cercopithecus neglectus*), SIVsyk (Sykes' monkey, *Cercopithecus mitis*), 2 Vprs from SIVmon(Mona Monkey *Cercopithecus mona*), SIVmus, mustached monkey (*Cercopithecus cephus*); greater spot-nosed monkey (*Cercopithecus nictitans*); SIVolc (Olive Colobus, *Procolobus verus*) and SIVlst (L'Hoest's Guenon, *Cercopithecus lhoesti*). In no case did the tag significantly affect the phenotype antagonism of sensing.

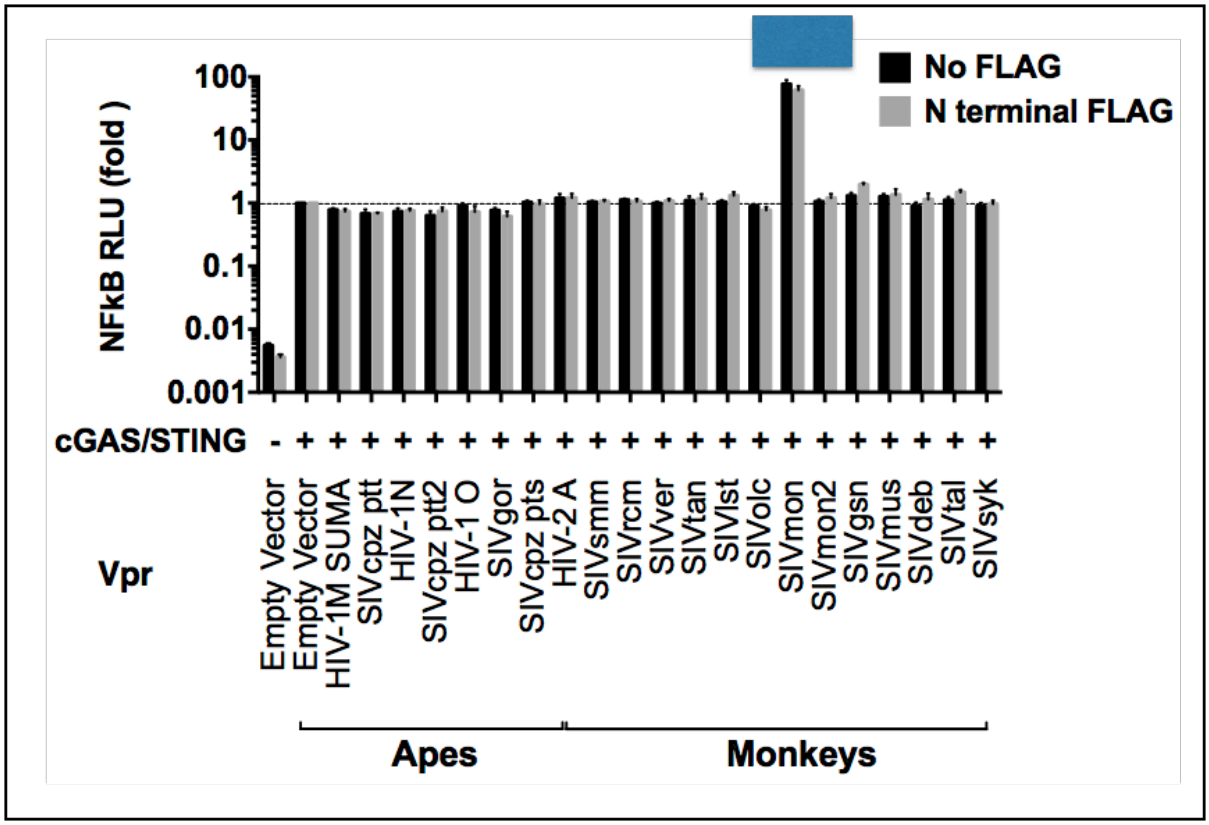

Figure 3.8 Inhibition of cGAS/STING induced NFκB signalling is unaffected by a FLAG epitope tag at the N-terminal of the Vpr. HEK293T cells were transfected as previously with an NFκB sensitive reporter, cGAS and STING (3ng) and HIV or SIV Vprs or an empty vector control. NFκB activation was determined by luminometry 48 h post-transfection. The mean fold change in RLU (relative light units – firefly luciferase normalised to renilla) relative to a control with no Vpr (empty vector + cGAS:STING) is displayed. Data are presented as the fold induction relative to an identical dose of cGAS/STING transfected with an empty Vpr expression vector. The mean values of three transfections ±SEM are shown. Data were analysed for the effect of the FLAG epitope tag using a two way ANOVA (analysis of variance) test and no statistically significant difference was found between tagged and untagged Vprs. Western blot data for this experiment are shown in figure 3.9)

3.9 Inhibition of NFκB induced signalling is not conserved across all Vprs from different primate lineages.

When the effects on NFκB sensitive promoter expression are compared with immunoblot data from the full panel of Vprs there is an overall correlation between loss of STING and loss of NFκB sensitive luciferase expression (figure 3.9A&B). The luminometry data represent 4 separate experiments whereas the immunoblot data is from a single representative experiment. The Vpr effect is not binary but there is a clear pattern of variable antagonism across species. HIV-1 viruses and their parent species inhibit >10X. HIV-2 and its parent species along with other mangabeys show reduced inhibition. The African Green Monkey SIVs (SIVver, SIVtan from the Chlorocebus Genus), the guenon SIVs (L'Hoest's, De Brazza's, Moustached, Great-spot nosed and 2 sequences of Mona), the Talapoin and the Colobus (Olive) showed variably reduced inhibition of luciferase activation and loss of STING (figure 3.9B).

Of particular interest, I noted that the SIVcpz*pts* showed diminished ability to antagonise signalling compared to other ape viruses. Only 3 lineages of SIV have transmitted to humans from non-human primates. SIVcpz, SIVgor and SIVmm. Statistically significant antagonism of sensing was observed in all lineages which have transmitted to human beings but the parent species of HIV-1 were able to antagonise more robustly than either HIV-2 or SIVsmm. No lineage assessed that failed to antagonise has transmitted to humans suggesting that the observed effect (subsequently shown in chapter 4 to be inhibition of cGAS and STING expression) may be necessary for zoonotic transmission to humans.

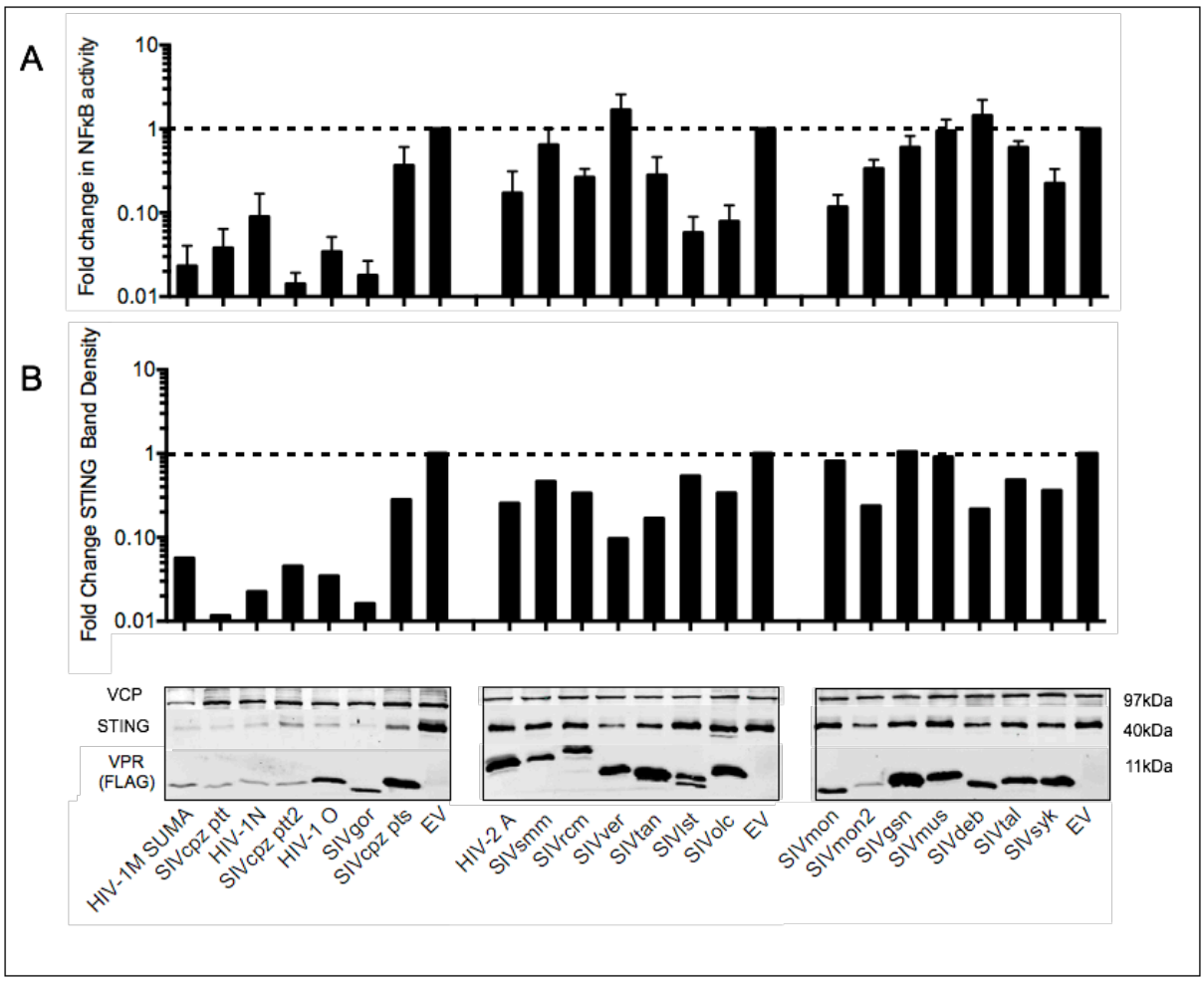

Figure 3.9 Inhibition of NFκB induced signalling is not conserved across Vprs from different primate lineages. (A) HEK293T-cells were transfected as previously described. NFκB induced firefly luciferase expression was normalised to a thymidine kinase promoter-driven renilla luciferase reporter. Data are presented as the fold induction relative to an identical dose of cGAS/STING transfected with an empty expression vector -/+ SEM.(B) Immunoblotting from the same plates was used to confirm expression of proteins and calculate relative quantities of STING. Molecular mass markers in kDa are shown.

3.10 NFκB signal inhibition and Vpr phylogeny

To better understand the relationships between NFκB signal inhibition and host species a phylogenetic tree of Vpx and Vpr alignments was constructed using sequences from the Los Alamos database including the sequences cloned into expression vectors. Sequences were manually aligned and validated using PRANK online software. An 'approximate maximum likelihood' algorithm was used to generate phylogeny structure with online software FastTree (515). Branch support was calculated by Shimodaira-Hasegawa-like local branch support (SH-like test) and the nodes with SH-test p-values < 0.1 noted with a black rectangle (figure 3.10A). The tree is mid-point rooted and branch lengths indicate the number of nucleotide substitutions per site. The scale bars represent 0.2 substitutions per site.

This tree enabled examination of the relationship between antagonism and cross species transmission of SIV to humans: clades with transmission to humans have appreciably greater inhibition of signalling with the clade including HIV-1 M Vpr, the only pandemic primate lentivirus, inhibiting most strongly (figure 3.10B).

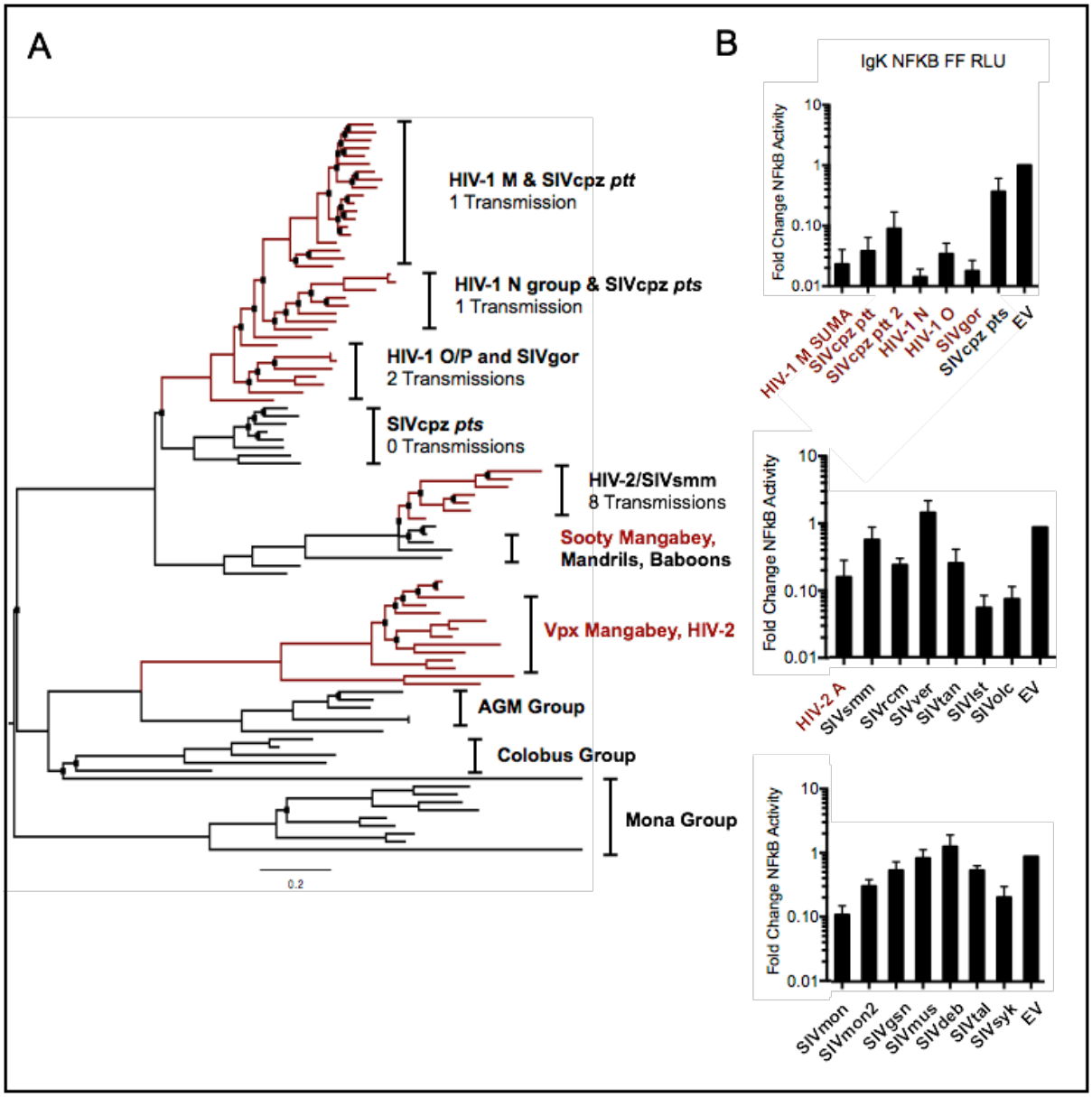

Figure 3.10 NFκB signal inhibition and Vpr phylogeny. (A) Phylogenetic analysis of Vpr sequences from SIV and HIV. The lentiviral Vpr from viruses that have had zoonotic transmission to humans species are coloured red. Sequences were manually aligned and validated using PRANK online software. An 'approximate maximum likelihood' algorithm was used to generate phylogeny structure with online software FastTree (515). Branch support was calculated by Shimodaira-Hasegawa-like local branch support (SH-like test). and the nodes with SH-test p-values < 0.1 noted with a black rectangle. The tree is artificially rooted and branch lengths indicate the number of nucleotide substitutions per site. The scale bars represent 0.2 substitutions per site. (B) NFκB activation data from figure 3.9 are presented next each clade.

3.11 SIVcpz Vpr from the eastern chimpanzee does not antagonise cGAS/STING stimulated NFκB signalling.

The only chimpanzee subspecies with a lentivirus that has failed to transmit between species, the eastern chimpanzee (*Pan troglodytes schweinfurthii*) (543), was noted to lack significant inhibition despite >5 fold increased expression levels compared to HIV-1 or SIVcpz*ptt* Vprs (see immunoblot figure 3.9B). A phylogenetic analysisof Vpr sequences from HIV-1 and its ancestor viruses was constructed using the same methods as the tree in figure 3.10A. A further sequence was cloned and compared with HIV-1 M and SIVcpz*ptt* sequences and this finding was confirmed (figure 3.11B). Given that SIVcpz*ptt* strains have been transmitted to humans and gorillas at least 5 times the lack of evidence of similar transmission SIVcpz*pts* is striking especially since infections rates determined by field sampling seem to be very similar to those found in P. t. troglodytes (544–546). Whilst it is possible that there are social and cultural determinants of cross species transmission between humans and great apes in east and west Africa I hypothesised that the apparent inability of Vpr to antagonise NFκB signalling (in fact the inability to inhibit expression of cGAS/STING – see chapter 4) may provide an extra barrier.

A sequence alignment of all available HIV-1, SIVcpz and SIVgor Vprs was constructed which shows an 11-18 residue deletion at the C-terminal end (figure 3.11C). The only other uniquely different residue in SIVcpz*pts* Vpr is a tryptophan at position 102 which aligns with tyrosines, phenylalanines or leucines in HIV-1, SIVgor or SIVcpz*ptt* Vprs all of which have hydrophobic side chains and may, therefore, not be significant substitutions.

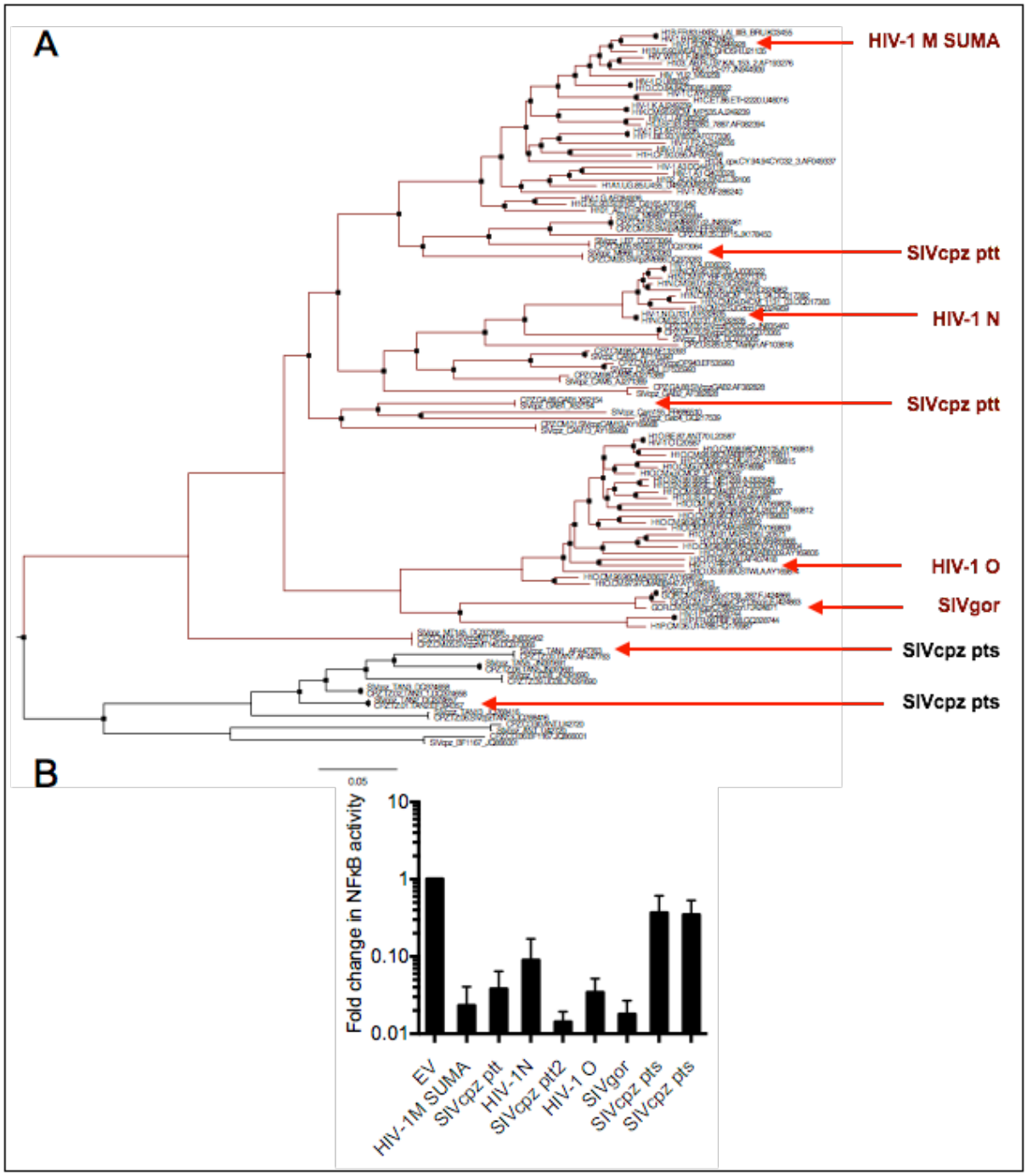

Figure 3.11 A&B SIVcpz Vprs from eastern and western Chimpanzees differentially antagonise NFκB mediated signalling (A) Phylogenetic analysis of Vpr sequences from HIV-1 and its ancestor viruses SIVcpz and SIVgor. Tree branches from viruses that have had zoonotic transmission to humans species are coloured red. Sequences were aligned and a phylogeny was generated as in figure 3.10. The tree is artificially rooted and branch lengths indicate the number of nucleotide substitutions per site. The scale bars represent 0.2 substitutions per site. (B) Fold change in NFκB induced firefly luciferase expression. Cells were transfected as before with cGAS and STING and one of HIV-1 M SUMA, 2 different SIVcpz*ptt*, HIV-1 N, SIVgor, HIV-1 O and 2 different SIVcpz*pts* Vprs. Data are presented as the fold induction relative cGAS/STING transfected with an empty expression vector. The mean values of three transfections ±SEM are shown.

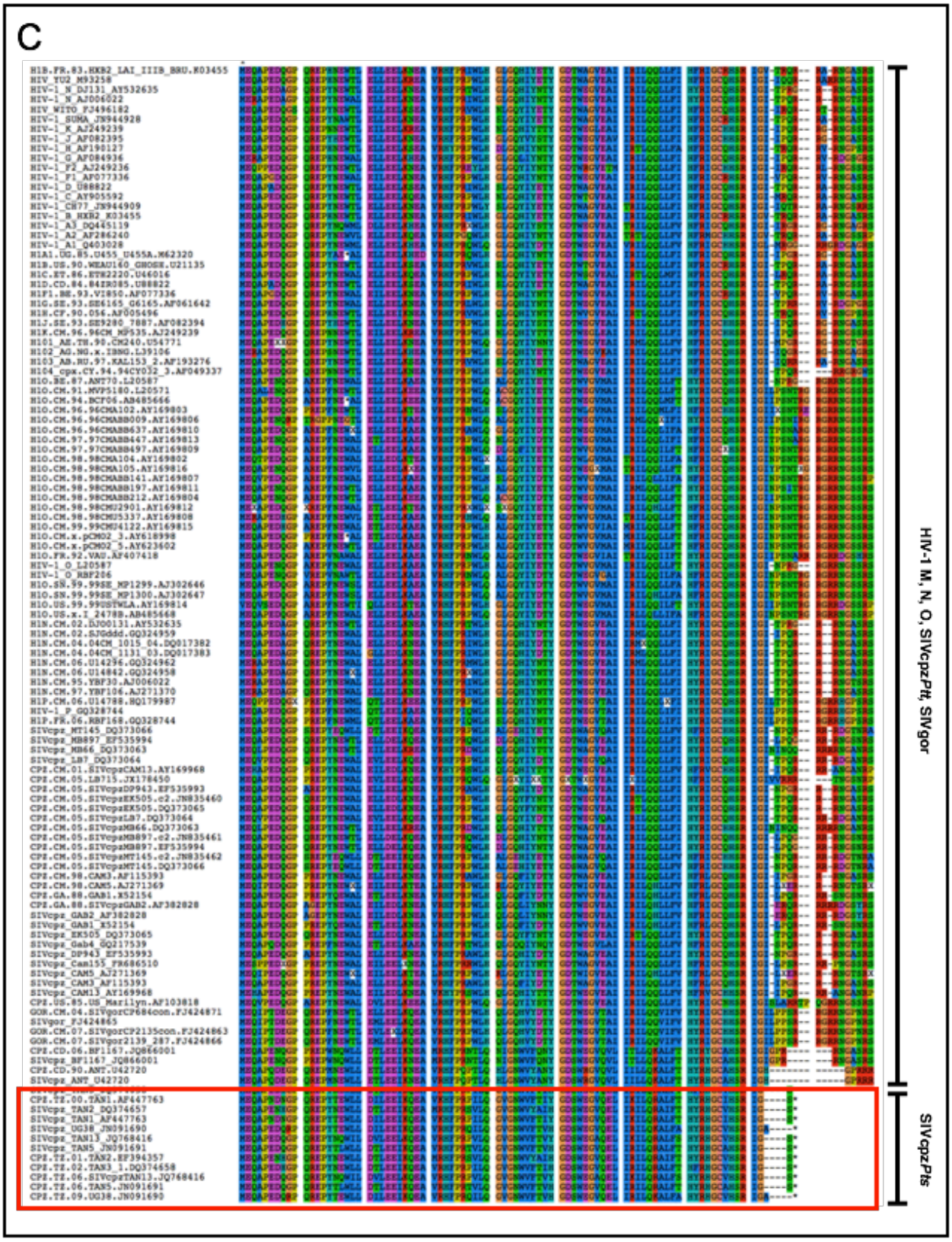

Figure 3.11 C Vpr alignment 160 full-length Vpr nucleotide sequences derived from 24 HIV/SIV strains from the Los Alamos HIVSequence Database (http://www.hiv.lanl.gov) manually aligned using the Se-Al sequence editor (http: //tree.bio.ed.ac.uk/software/seal/) showing the deletion in the strains derived from the eastern *Pan troglodytes Schweinfurtheii* subsepecies

3.12 Inhibition of differentially activated NFκB signalling by Vpr

In order to investigate the level at which Vpr is inhibiting the pathway, innate immune molecules capable of stimulating NFκB signalling were ectopically expressed with HIV-1 M YU2. Five different stimuli were used: cGAS/STING as previously; a constitutively active mutant of the canonical IKK, IKKβ; the non-canonical IKK, TBK1 (known also to activate IRF3 signalling); MAVS (mitochondrial antiviral signalling adaptor) and TNFα. Significant inhibition of signalling by Vpr was seen with signalling mediated by IKKβ and TBK1 but not with MAVS or TNFα (figure 3.12). Additionally IKKβ and TBK1 showed reduced expression on immunoblot as had been shown previously with STING (figure 3.12B&C). Considering the convergence of signalling pathways on TBK1 and IKKβ, and the loss of both IKKβ and TBK1 on immunoblot the lack of effect on TNFα and MAVS mediated signalling was unexpected.

NF-κB is activated downstream of the TLRs, RLRs, some NLRs and DNA sensors, as well as the TNFα receptor (TNFR) and IL-1 receptor (IL-1R). NF-κB-activating signalling converges on the IKK complex, consisting of the canonical IKKs (IKKα and IKKβ and NF-κB essential modulator (NEMO) (547)) or IKKγ, a non-enzymatic regulatory component (548). The IKKs phosphorylate members of the inhibitor of κB (IκB) family directly inducing proteosomal degradation (549) and NF-κB is then free to translocate to the nucleus.

Signalling pathways leading to IRF3 activation downstream of TLRs, RLRs and cytoplasmic DNA sensors converge on the non-canonical IKKs; These PRRs utilise various adaptors like MAVS but generally require TRAF3 to co-ordinate upstream signalling events and activate TBK1 and IKKε which phosphorylate IRF3 causing it to dimerise and translocate to the nucleus (282,550,551).

I hypothesised that TNFα and MAVS mediated NFκB signalling in HEK293T cells is mediated via unknown pathway members unaffected by YU2 Vpr. I also hypothesized that IKKβ and TBK1 were bing degraded in complex with STING since these molecules are known to associate(290). It became clear after completeing the experiments presented in chapter 4 that the HIV-1 M YU2 tested was insufficiently expressed to show an effect on MAVS mediated signalling and the lack of effect on TNFα signalling was because the molecule was added to the transfected cells rather than being transfected as a plasmid (see chapter 4).

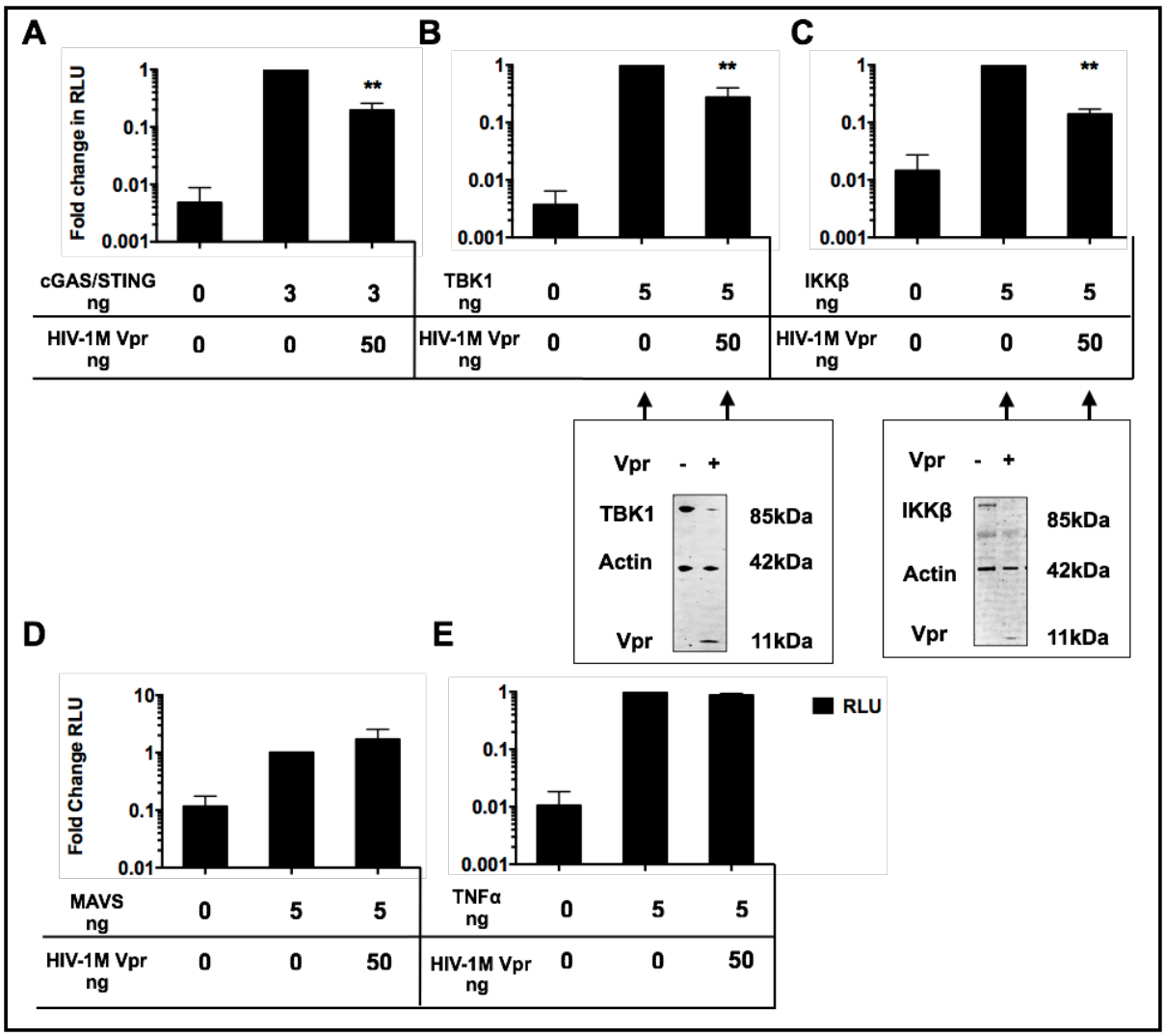

Figure 3.12 Differential antagonism of NFκB signalling when activated by different stimuli. HEK293T cells were transfected as previously with an NFκB sensitive reporter, and HIV-1 M YU2 Vpr or an empty vector control. NFκB signalling was activated by transfection of one of HA epitope tagged cGAS and STING (A) IKKβ (B), TBK1 (C), or untagged MAVS (D) or addition of TNFα (E). NFκB activation was determined by luminometry, as previously described, 48 h post-transfection. The mean fold change in RLU (relative light units – firefly luciferase normalised to renilla) relative to a control with no Vpr (empty vector + cGAS:STING) is displayed. The mean values of three transfections ±SEM are shown. The whole cell lysates for (B) IKKβ and (C) TBK1 were separated by SDS-PAGE and analysed by immunoblotting with anti-HA antibodies. The location of protein molecular mass markers is indicated (kDa). **=P<0.01 one-way ANOVA with Dunnett's post-hoc test.

3.13 The pattern of species specific antagonism by primate lentiviral Vprs is preserved across different NFκB activating stimuli

A similar pattern of species specific activity is observed with a panel of different species' Vprs co-transfected with different NFκB activating stimuli. The panel of Vprs used in figures 3.8 & 3.9 was tested against these different NFκB activating proteins (figure 3.13 A-D). This revealed a conserved differential effect against MAVS, IKKβ and TBK1 across all Vprs (figure 3.13 A-D).

The clinical isolates with improved expression over YU2 all antagonised MAVS-mediated signalling robustly >10 fold (figure 3.13 D). Expression of Vprs following addition of TNFα was confirmed with immunoblot. It was hypothesised that Vpr was acting distally in an NFκB pathway common to TBK1, IKK, cGAS/STING and MAVS but that the TNFα activated pathway converged on a different point. It was also hypothesised that Vpr caused the degradation of a putative complex containing the activating molecules. Conceivably, given the viral LTR's sensitivity to NFκB and the role of NFκB in an inflammatory response, the virus could have evolved to modulate NFκB signalling with great sensitivity. At this point it was not considered that the observed effects could be explained by inhibition of activator molecule (TBK1, IKKβ, cGAS/STING) expression since from the data in figure 3.4 IRF3 signalling appeared to be unaffected.

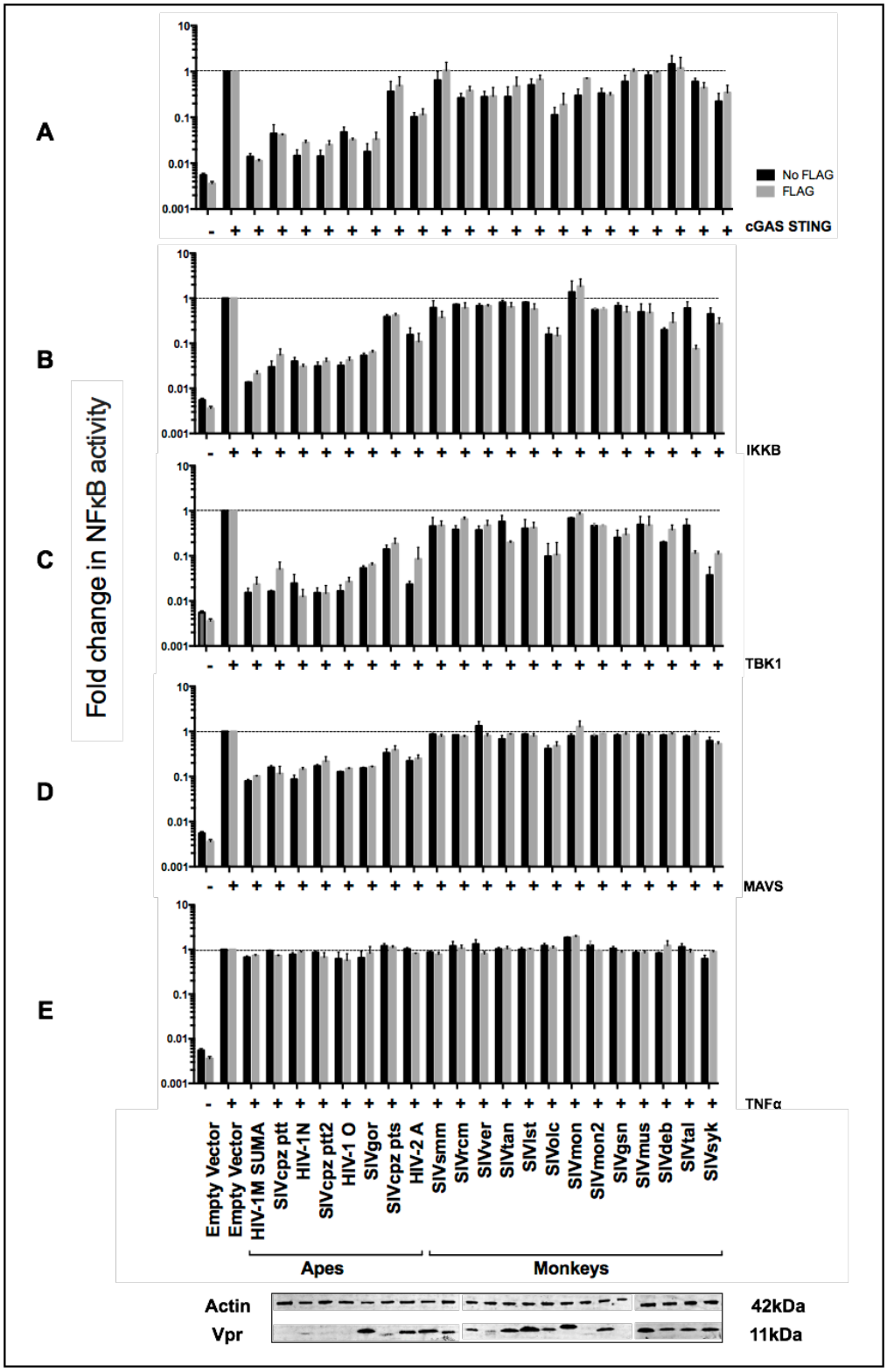

Figure 3.13 Preserved pattern of antagonism across a panel of primate lentiviral Vprs co-transfected with different NFκB activating stimuli HEK293T cells were transfected as in figure 3.8. NFκB signalling was activated by transfection of one of HA epitope tagged cGAS and STING (A) IKKβ (B), TBK1 (C), or untagged MAVS (D) or addition of TNFα (E). The mean fold change in RLU relative to a control with no Vpr is displayed. The mean values of three transfections ±SEM are shown. The cell lysates were analysed by immunoblotting to confirm expression of Vpr from the TNFα experiment (E).

3.14 HIV-1 M Vpr causes loss of TRAF2 & TRAF6 on immunoblot

Co-transfection of Tumor necrosis factor receptor-associated factors (TRAFs) 3 and 6 also activated NFκB signalling and unexpectedly the Vpr also caused a dose dependent loss of TRAF3 and TRAF6 expression measured by immunoblot and a proportional loss of NFκB signalling (figure 3.14 B&C). cGAS and STING were co-transfected as a positive control (figure 3.14A).

With the exception of TLR3, all TLRs recruit myeloid differentiation factor 99 (MyD88) to activate a signalling complex that interacts with TNF receptor-associated factor 6 (TRAF6) which then promotes the downstream activation of the IKK complex (550,552). TLR3 recruits the non-canonical IKKs via an adaptor molecule TRAF3 (551). TRAF3 also links signalling downstream of the RLRs to the non-canonical IKKs by interacting with MAVS.

From the published literature on NFκB signalling pathways it was hard to conceive of a mechanism by which Vpr could specifically antagonise NFκB signalling when stimulated by STING/TBK/IKK/MAVS/TRAF3 and TRAF6 whilst leaving signalling stimulated via TNFα unaffected and IRF3 signalling unaffected.

Additionally, whilst the putative degradation of multiple signalling molecules as part of a complex is in theory possible, the range of molecules that showed reduced expression on immunoblot are not known to associate. Subsequent experiments detailed in chapter 4 demonstrated that Vpr was not causing a complex to be degraded but was in fact inhibiting expression from the transfected plasmids.

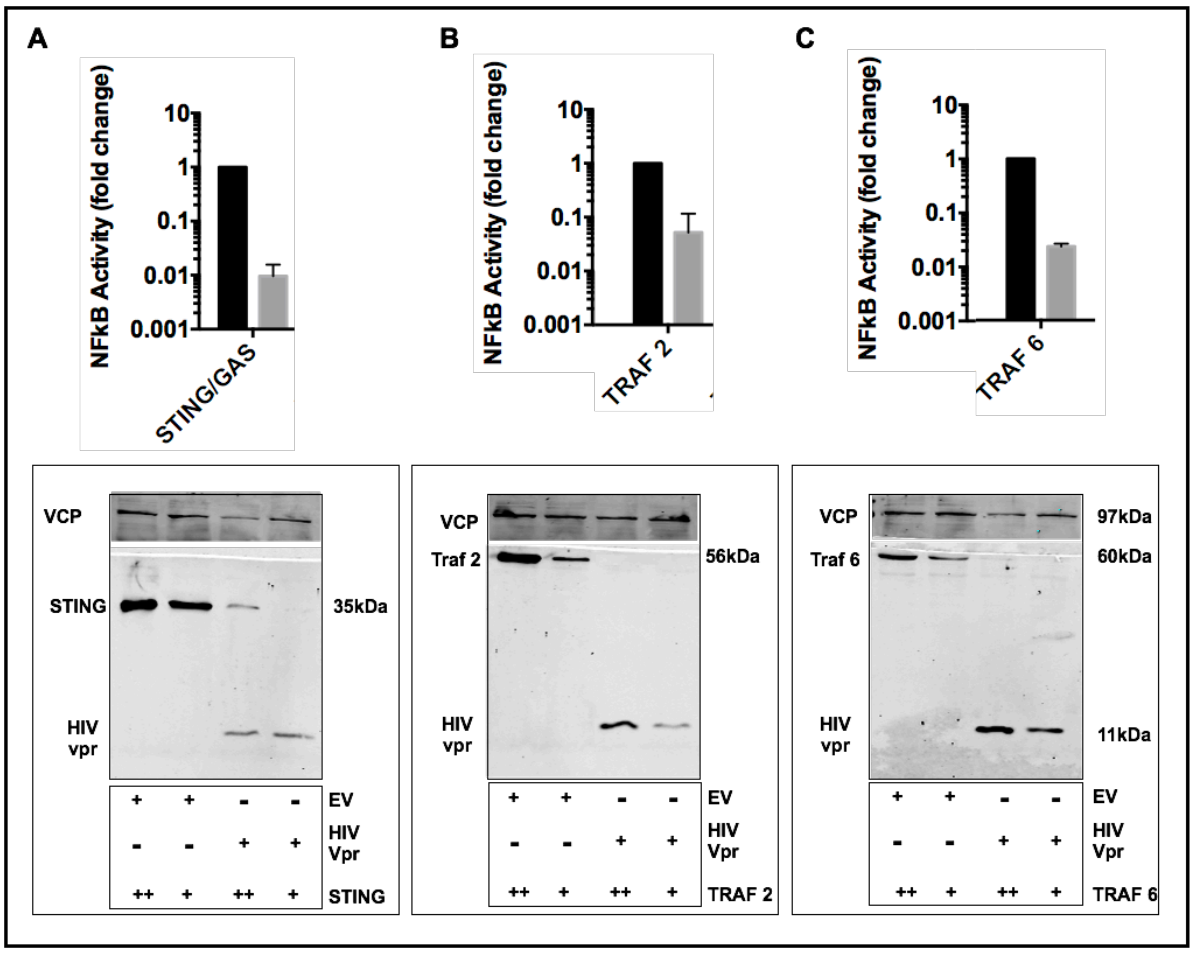

Figure 3.14 Co-transfected HIV-1 M Vpr causes loss of HA-TRAF2 & HA-TRAF6 on immunoblot Two doses of expression plasmids encoding HA epitope tagged TRAF 2 and 6 were transfected into HEK293T cells together with reporter plasmids and either HIV-1 M Vpr as previously. 48 hours post transfection the cells were harvested for luminometry. Firefly luciferase activity was measured and normalised to the renilla luciferase activity. Data from three experiments are represented as the fold induction relative to an identical dose of activating molecule with an empty expression vector -/+ SEM. The whole cell lysates were separated by SDS- PAGE and analysed by immunoblotting with anti-FLAG and anti-HA antibodies.

4 Chapter 4 - HIV Vpr non-specifically suppresses expression from co-transfected plasmids

It was noticed in untransfected experimental controls that even without co-transfection of an NFκB signal activating molecule (e.g. IKKβ, TBK1 etc) one of the two Vprs from SIVmon (infecting the mona monkey species) specifically stimulated NFκB signalling ~100fold both with and without a FLAG epitope tag compared to empty vector (see chapter 5, figure 5.1). It was hypothesised that SIVmon Vpr could provide a useful reagent to determine putative interaction partners for HIV-1 M Vpr and the stimulation of NFκB signalling by SIVmon Vpr was characterized (see chapter 5). In the course of characterizing the SIVmon Vpr activation of NFκB it was determined that HIV-1 M Vpr was able to inhibit this NFκB signal and caused loss of SIVmon Vpr on immunoblot.

However whilst conducting these experiments it was noted that HIV-1 M Vpr additionally caused loss on immunoblot of two other Vprs, inactive for NFκB signalling: SIVgsn Vpr and SIVmon2 Vpr (figure 4.1A). Whilst loss of SIVmon Vpr could be hypothesised to be due to the degradation of a large signalling complex that it had joined, it seemed less probable that these inactive Vprs were part of the same signalling complex. This observation needed to be reconciled with the observation that HIV-1 M Vpr seemed able to specifically antagonise NFκB signalling from all co-transfected molecules. Indeed, the only signalling stimulus that had been tried which was not inhibited was TNFα, but this cytokine had been added to cells post transfection rather than being a cDNA transfected simultaneously with Vpr (figure 3.13 & 4.1B).

4.1 HIV-1 M Vpr antagonism of NFκB signalling requires transfection of the activating stimulus

To test the hypothesis that the inhibition of the NFκB signal was a transfection dependent phenotype HEK293T cells were transfected with a plasmid encoding TNFα (figure 4.1B). This recapitulated the same pattern of inhibition across the panel of FLAG epitope tagged and untagged Vprs that was seen in figure 3.13. Thus, inhibition of signal appeared to be dependent on a property of the transfected plasmid rather than being dependent on the molecule encoded.

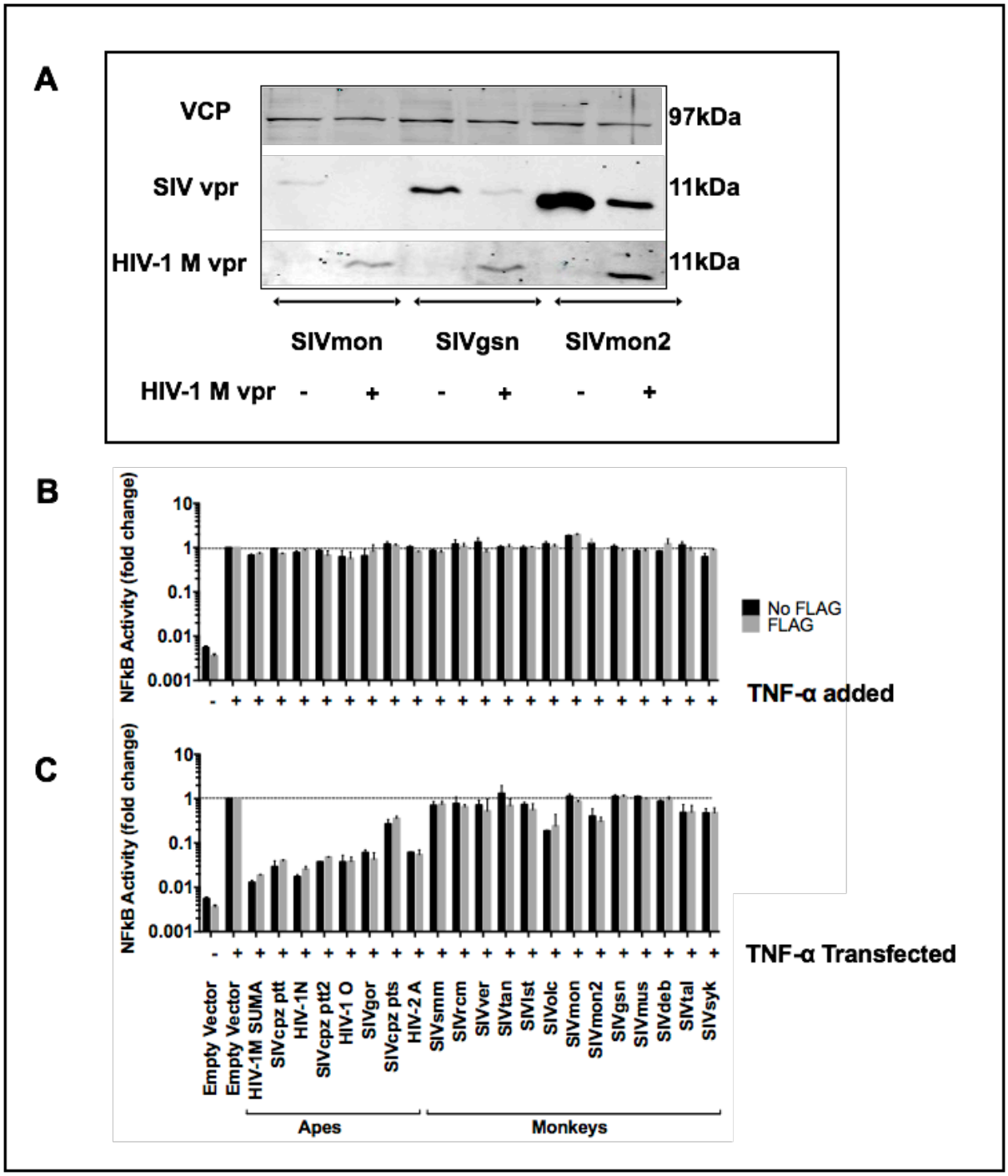

Figure 4.1 Transfected HIV-1 M Vpr antagonises NFκB signalling depending on whether the activating molecule is added or transfected.(A) FLAG tagged SIVmon group SIV Vprs were transfected with untagged HIV-1 M Vpr or empty vector. Cell lysates analysed by immunoblot with anti-FLAG anti-HIV-Vpr, for loading, anti-VCP. (B) NFκB signalling was stimulated as previously with added TNFα or with an expression plasmid for TNFα under the control of a CMV promoter and tested against the panel of tagged and untagged Vprs as used previously. The mean fold change in RLU (relative light units – firefly luciferase normalised to renilla) relative to a control with no Vpr (empty vector + cGAS:STING) is displayed. The mean values of three transfections ±SEM are shown.

4.2 HIV-1 M Vpr inhibits expression from a co-transfected GFP encoding plasmid

To test whether only transfected molecules involved in innate immune signalling pathways showed reduced expression when co-transfected with HIV-1 Vpr, a pcDNA.3 expression vector (identical to the vector used for the Vprs) encoding GFP under the control of a CMV promoter was transfected. Both GFP positive cell number and mean fluorescence intensity were both significantly reduced at 48 hr post transfection (figure 4.2).

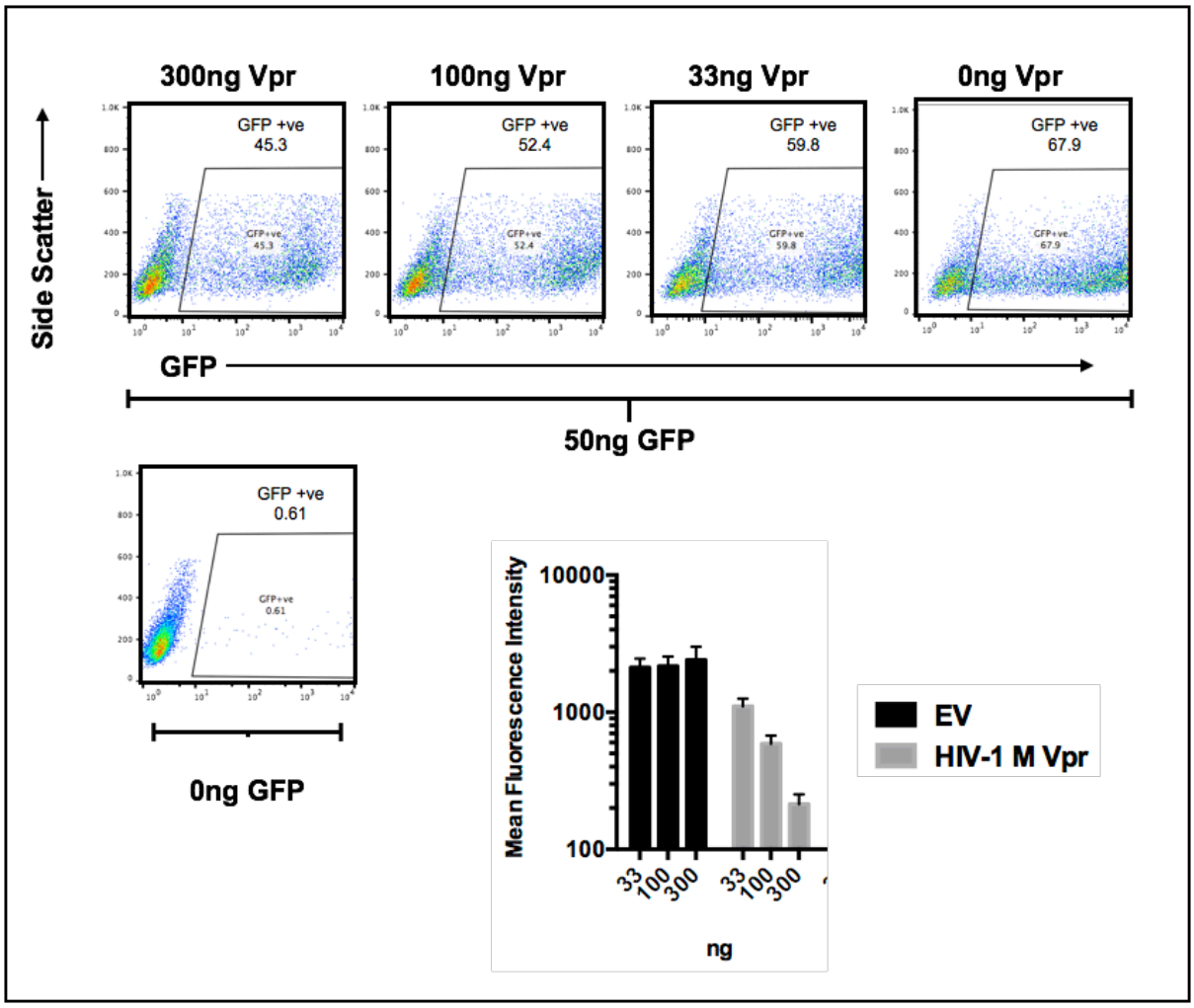

Figure 4.2 HIV-1 M Vpr inhibits expression from a co-transfected plasmid encoding GFP driven by a CMV promoter. HIV-1 M Vpr or empty vector was transfected from 0-300ng with a pcDNA.3 plasmid encoding GFP. 48 hours after transfection the cells were collected for and analysed by FACS. Data are from one representative experiment of two.

4.3 HIV-1 M Vpr inhibits mRNA expression from co-transfected cGAS and STING encoding plasmids.

I hypothesised that rather than specifically inhibiting NFκB signalling pathways HIV-1M Vpr inhibited expression from the co-transfected activating plasmids possibly in a promoter dependent fashion. To confirm this effect of HIV-1 M Vpr on cGAS and STING mRNA levels was tested by qRT-PCR. HEK293T cells were transfected in triplicate in 48 well plates with a 5 fold dilution series of cGAS and STING plasmids in a 1:1 ratio between 0.01ng and 10ng and simultaneously transfected with either HIV-1M Vpr at a constant dose of 50ng or the same dose of an empty expression vector. For qRT-PCR analysis the cells were harvested 48hr post transfection, the RNA was extracted, cDNA was synthesized and used for qRT-PCR analysis using primers specific for human cGAS and STING. HIV-1 M Vpr reduced mRNA levels of cGAS and STING by >10 fold (figure 4.3 A and B).

To investigate the previously unexplained specificity for NFκB signalling (figure 3.12) qRT-PCR was also performed to measure transcription of ISG-56 mRNA. It was observed that maximal expression of ISG56 could be induced with between 0.4 and 2ng of cGAS/STING. Co-transfection of HIV-1M Vpr with 10ng of cGAS/STING reduced the cGAS and STING expression levels to the equivalent of 2ng of transfected plasmid i.e. still enough to maximally induce ISG56 (figure 4.3C). So even though the activating molecules (STING and cGAS) had reduced expression when co-expressed with Vpr, this was not detected by RT PCR for ISG mRNA or by the IRF3 sensitive ISG56 luciferase reporter construct.

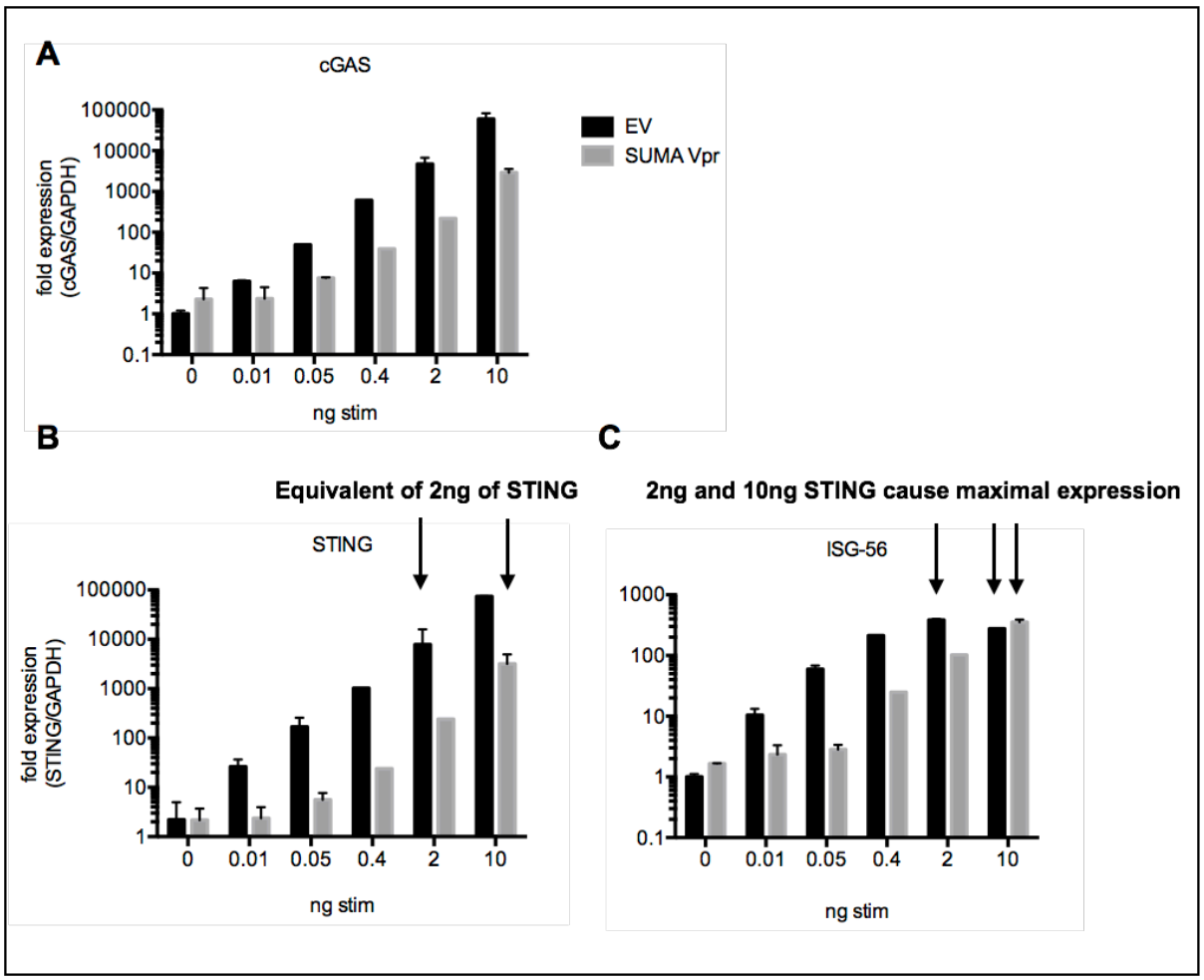

Figure 4.3 HIV-1 M Vpr inhibits mRNA expression from co-transfected cGAS and STING. HEK293T cells were transfected in triplicate in 48-well plates with HIV-1 M Vpr at 50ng or empty vector control (EV) expression plasmid per well and increasing doses of cGAS and STING in a 1:1 ratio between 0.01ng and 10ng After 48 hrs the cells were lysed and RNA was extracted from the cells, cDNA was synthesized and used for qRT-PCR analysis in duplicate using primers specific for human *cGAS, STING* and *ISG56.* Data are represented as mean ± SD stimulation relative to sample one of the mock-treated EV control. Arrows indicate left to right i) mRNA level of 2ng transfected STING without Vpr ii) the level of STING mRNA detected when Vpr is transfected with STING iii) and iv) that 2ng and 10ng STING respectively stimulate ISG56 mRNA transcription to the same maximal level.

4.4 HIV-1 M Vpr inhibits luciferase expression from different promoter constructs

To further investigate the effect of Vpr on co-transfected plasmids, site directed mutagenesis was used to create Q65R and F34I/P35N mutants of HIV-1M Vpr which have been reported previously as DCAF and cyclophilin binding mutants respectively. Neither have any effect on Vpr induced cell cycle arrest (431). Together with HIV-1 M Vpr (from the SUMA clonal isolate) these were tested against a variety of reporter constructs with differing promoters.

All the pathway activating proteins in these studies were expressed from a pcDNA.3 expression vector i.e. under the control of a CMV promoter. A report by Liu et al in 2015 indicated that Vpr could have a slight inhibitory effect on expression of GFP from a CMV promoter (492). They found that there was no defect in this inhibition using the Q65R binding mutant or in cells depleted of DCAF using RNAi. They also found that the proteasome inhibitor MG132 did not alter the efficiency of Vpr inhibition. They did not test transfected plasmids with expression from other promoters.

I confirmed dose dependent inhibition of expression of luciferase from CMV promoters with constructs expressing both firefly and renilla luciferase over a titration series (figure 4.4 A&B). The CMV promoter contains multiple transcription factor binding sites; two AP2, AP1, four NF-κB, and five CREB binding sites, SP1 (Promega) (553).

The Q65R mutant did retain some ability to antagonise signalling but also demonstrated greater expression on immunoblot at a lower dosage – putatively due to reduced degradation. If normalised to expression level the DCAF mutant is a less effective antagonist. The F34i, P35N mutant showed markedly reduced inhibition.

Immunoblot confirmed greater levels of the expressed mutant Vprs. This is likely due to reduced auto-inhibition of the transfected plasmid (figure 4.4C). The greater the antagonism of the reporter the greater the antagonism of expression of Vpr itself.

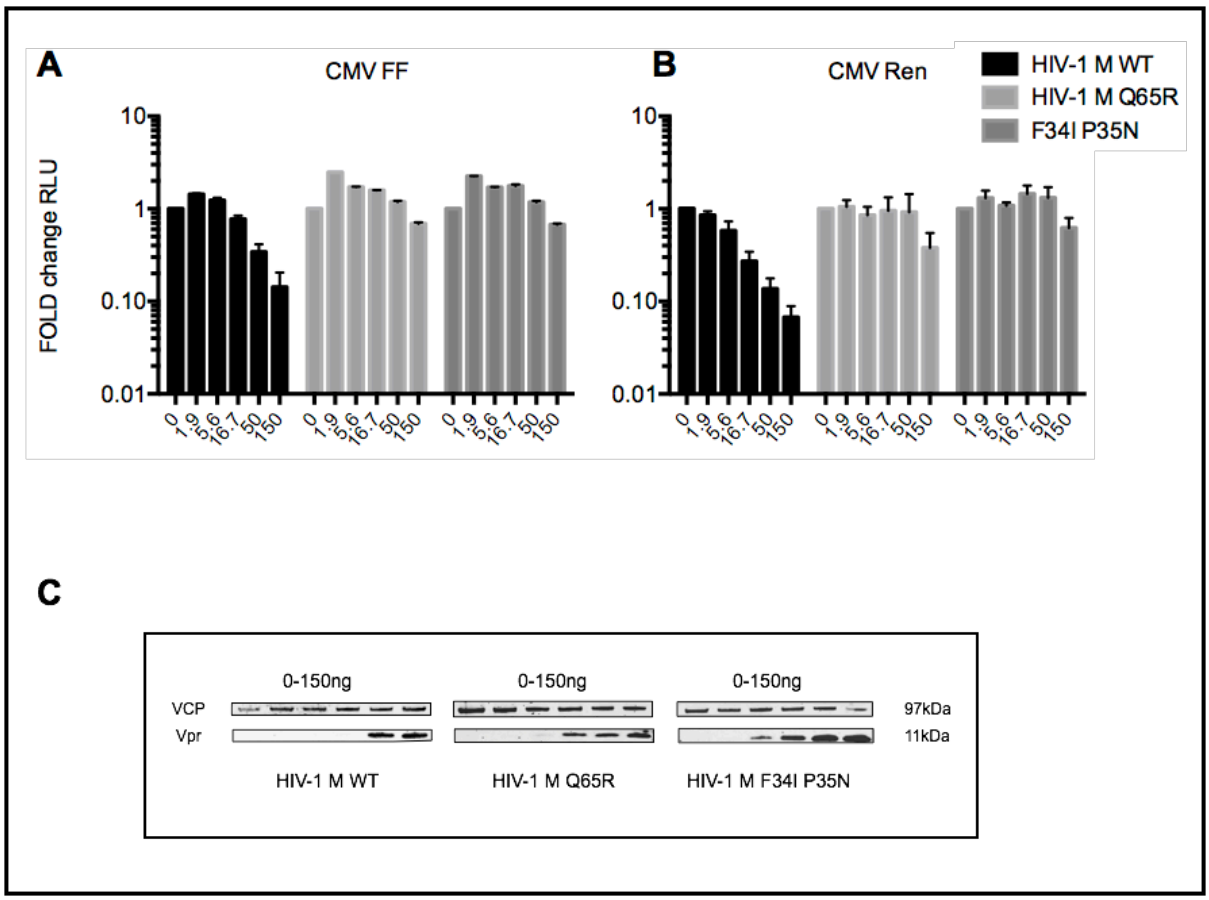

Figure 4.4 HIV-1 M Vpr inhibits expression from the CMV promoter. HEK293T cells were transfected with increasing doses of HIV-1 M Vpr WT or Q65R or F34I/P35N mutants together with 2.5ng of (A) CMV firefly or (B) renilla reporter constructs. Cell lysates were analysed by luminometry at 48hrs. Data are represented as mean ± SEM stimulation relative to sample one of the mock-treated EV control. (C) 48 hours after transfection cell lysates were analysed by immunoblotting with anti-FLAG anti-HIV-Vpr, and for the loading control anti-VCP.

4.5 HIV-1 M Vpr inhibits expression from a panel of viral promoters

Further viral promoter constructs were then tested including the HSV TK (Herpes Simplex Virus Thymidine Kinase) promoter; the M5P LTR (from Murine Leukaemia virus); the SFFV (spleen focus forming virus) promoter and the SV40 (Simian vacuolating virus 40) promoters (figure 4.5 A, B, C&D respectively). The same patterns of inhibition were seen as with the CMV promoters. The HSV-1 *thymidine kinase* promoter binds Sp1, CCAAT-enhancer-binding proteins CCAAT-TF (C/EBPs) (554,555). The Simian vacuolating virus 40 (SV40) promoter binds SP1, AP1 and AP2 (Promgea) (553,556). The Spleen focus-forming virus (SFFV) promoter and the M5P promoter are both from murine leukaemia viruses; since gene transfer vectors derived from the Moloney murine leukemia virus (MLV) have been used in gene therapy clinical trials since 1991 (557) these promoters are well characterized. Multiple members of the Ets family of transcription factors bind to two sites and the core-binding factor (CBF) is a heterodimeric binding protein that recognizes the enhancer core region (558,559).I confirmed dose dependent inhibition of expression of luciferase from all these plasmids including the TK renilla plasmid used previously as a control. I had not observed an effect on the renilla previously as the control dose used (7.5ng) was much higher than the dose tested here (2.5ng) and so the effect was minimalized. Also intital experiments optimizing the assay had used the poorly expressed Blue Heron Bio codon optimized HIV-1M YU2 Vpr.

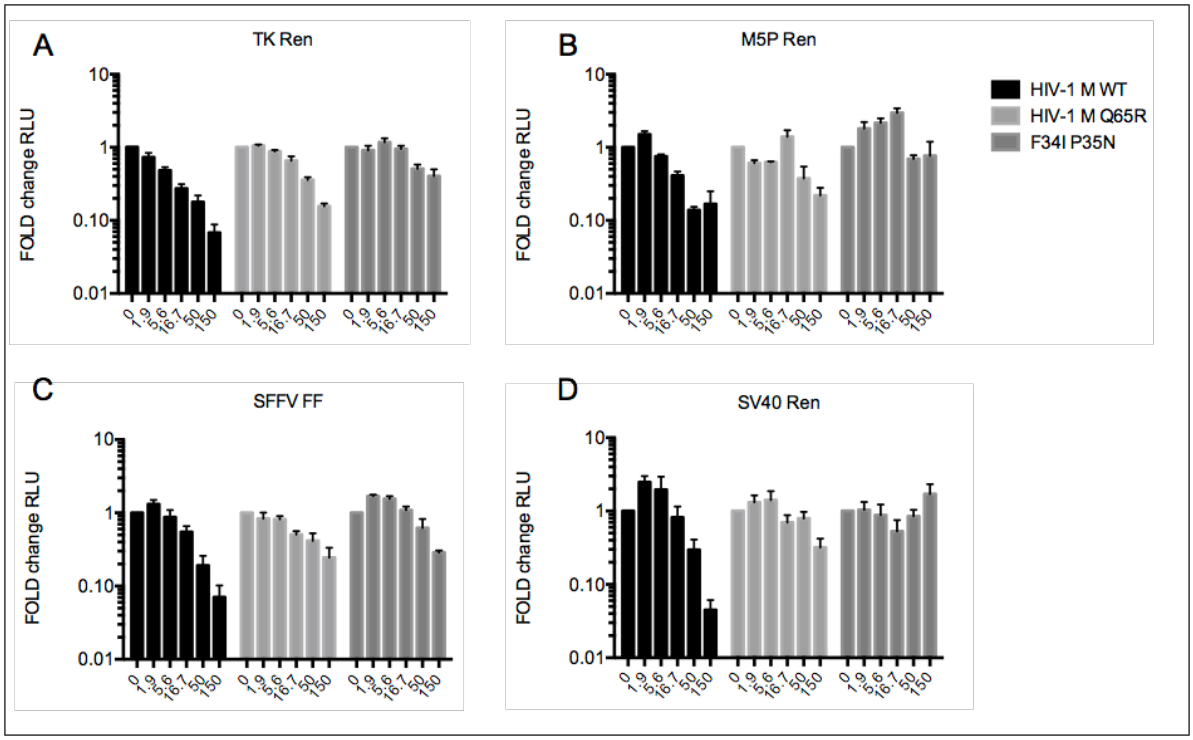

Figure 4.5HIV-1 M Vpr inhibits expression from a panel of viral promoters. HEK293T cells were transfected increasing doses of HIV-1 M Vpr, its Q65R mutant or the F34I/P35N double mutant with firefly or renilla reporter constructs with 2.5ng of (A) HSV TK, (B) MLV LTR (M5P), (C) SFFV or (D) SV40 promoters. Data are represented as mean ± SEM stimulation relative to sample one of the mock-treated EV control.

4.6 HIV-1 M Vpr inhibits expression from two human promoters

To test whether viral promoters were specifically inhibited human promoters for IgK and Ubiquitin B were tested and the same pattern of inhibition was observed (figure 4.6 A&B). The natural promoter of the *immunoglobulin kappa light chain* gene sensitive to NFκB subunits p50 and p65 contains three repeat kB sequences. The binding sequence is 5'-GGGRNYYYCC-3' (where R is a purine, Y is a pyrimidine, and N is an unspecified base). (personal communication Andrew McDonalds, (519–521). The SFXU construct with a Ubiquitin B promoter has binding sites for HSF, NFκB, AP-1(c-jun), NF-IL6 and Sp1 CEBP/B, CREB, GATA-2 and Sp1(560,561).

With such a diverse range of transcriptional activators required across these promoters I hypothesised that this indicated that inhibition of expression is a feature of transfection rather than being promoter dependent.

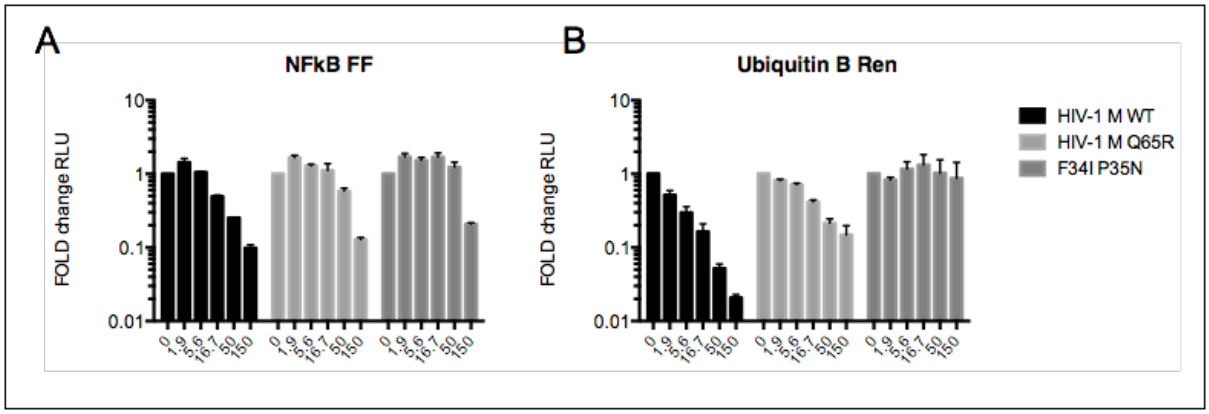

Figure 4.6 HIV-1 M Vpr inhibits expression from two human promoters. HEK293T cells were transfected with a 5 point dilution series of HIV-1 M Vpr, its Q65R mutant or the F34I/P35N double mutant together with constant doses of with firefly or renilla reporter constructs with either the human (A) IgK or (B) Ubiquitin B promoters. Data are represented as mean ± SEM stimulation relative to sample one of the mock-treated EV control.

4.7 HIV-1 M Vpr inhibits expression from a transfected NFκB promoter but not from the same promoter when it is integrated into the cell genome using a retrovirus

Background luciferase expression from an integrated reporter in HEK293Ts is unaffected by HIV-1 M Vpr. However, when the NFκB sensitive reporter plasmid with the same promoter region is co-transfected with HIV-1 Vpr in the absence of any NFκB activating stimulus the background expression of luciferase is inhibited >10fold providing further evidence of a transfection specific inhibition (figure 4.7). The dose of the reporter plasmid was adjusted so that the basal expression level was the same as the integrated reporter. This provides evidence that there is not a detectable effect of Vpr on basal NFkB activity but rather the effect is on transfected plasmids irrespective of promoter or expressed gene.

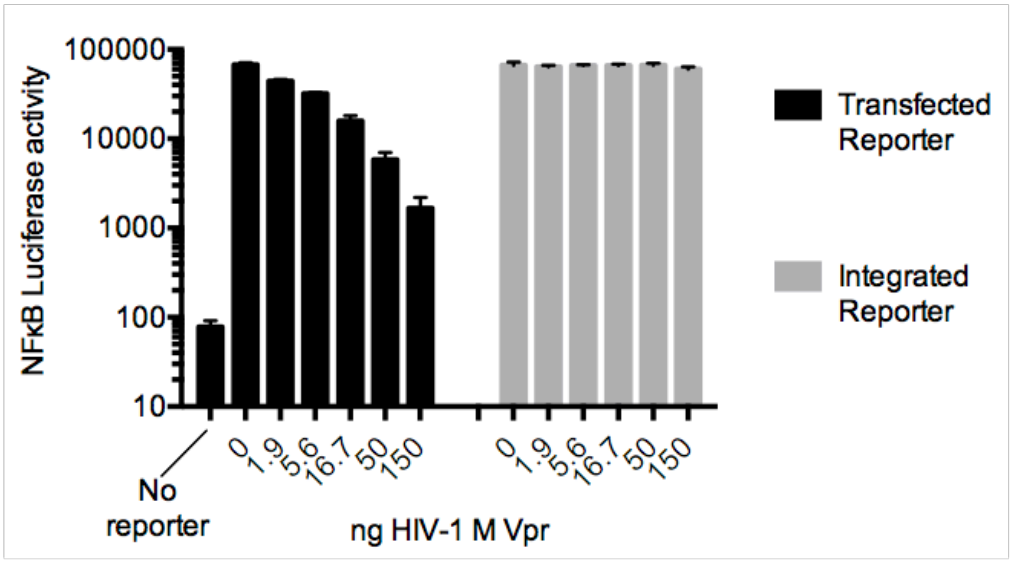

Figure 4.7 HIV-1 M Vpr inhibits unactivated expression of a transfected NFκB promoter but not from the same integrated promoter HEK293T cells or HEK293T-Luc cells (with an integrated NFκB sensitive luciferase reporter) were transfected with a 5 point dilution series of HIV-1 M Vpr. The cells without an integrated reporter were also transfected with a dose of NFκB sensitive luciferase reporter plasmid. Cells were harvested for luminometry at 48hrs. Data are represented as mean ± SEM stimulation relative to sample one of the mock-treated EV control

4.8 Nuclear localisation and inhibition of expression

Previous observations have shown that Vpr localizes to the nuclear rim although the details of the pathway and interacting partners for this localisation have not been described (476,478,562). In order to investigate the relationship between intracellular localisation and the inhibition of expression from co-transfected plasmids immunofluorescence confocal microscopy was used to measure nuclear rim localisation of HIV-1 M Vpr. I found minimal cytoplasmic or intranuclear expression (figure 4.8A). HEK293T cells were fixed and stained for imaging at 48hrs post transfection. While the wild type HIV-1M Vpr localized to the nuclear rim, by contrast the Q65R and F34I/P35N mutants showed expression in both nuclear and cytoplasmic regions on cross-sectional images indicating that Vpr induced inhibition of expression from transfected plasmids may be dependent on nuclear rim localisation (figure 4.8 B&C). Further evidence for this was provided by work done with a collaborator in our lab (Hataf Khan – PhD student) who showed that the phenotype of inhibition of expression from co-transfected plasmids correlated exactly with nuclear rim localisation (table 4.1). A table of phenotypes associated with Vpr mutants is provided on page 150 (table 4.2).

Vpr residue	Nuclear rim localisation	Inhibition
WT	Yes	Yes
A30S	Yes	Yes
V31S	Yes	Yes
A30S+V31S	Yes	Yes
F34I	No	No
P35N	No	No
F34I+P35N	No	No
W54R	Yes	Yes
Q65R	No	No
H71R	No	No
R73S	Yes	Yes
S79A	Yes	Yes
R80A	Yes	Yes
R90A	Yes	Yes

Table 4.1 Correlation between rim localisation and inhibition of expression – work on mutants other than HIV-1 M Vpr WT, and the two mutants Q65R and F34I/P35N was undertaken by H. Khan.

Vpr residue	Biological Function	Inhibition
F34	Importin alpha interaction (563)	No
P35	Cyclophilin interaction (411)	No
W54	UNG2 interaction (509)	Not tested
Q65	DCAF1 complex recruitment (370)	No
H71	Dimer formation (563)	No
R73	LTR transcription(564)	Yes
S79	TAK1 interaction (565)	Yes
R80	Cell cycle mutant (431)	Yes
R90	Vpr suppression of IL-12 in DC (566)	Yes
WT		Yes

Table 4.2 Vpr mutant phenotypes

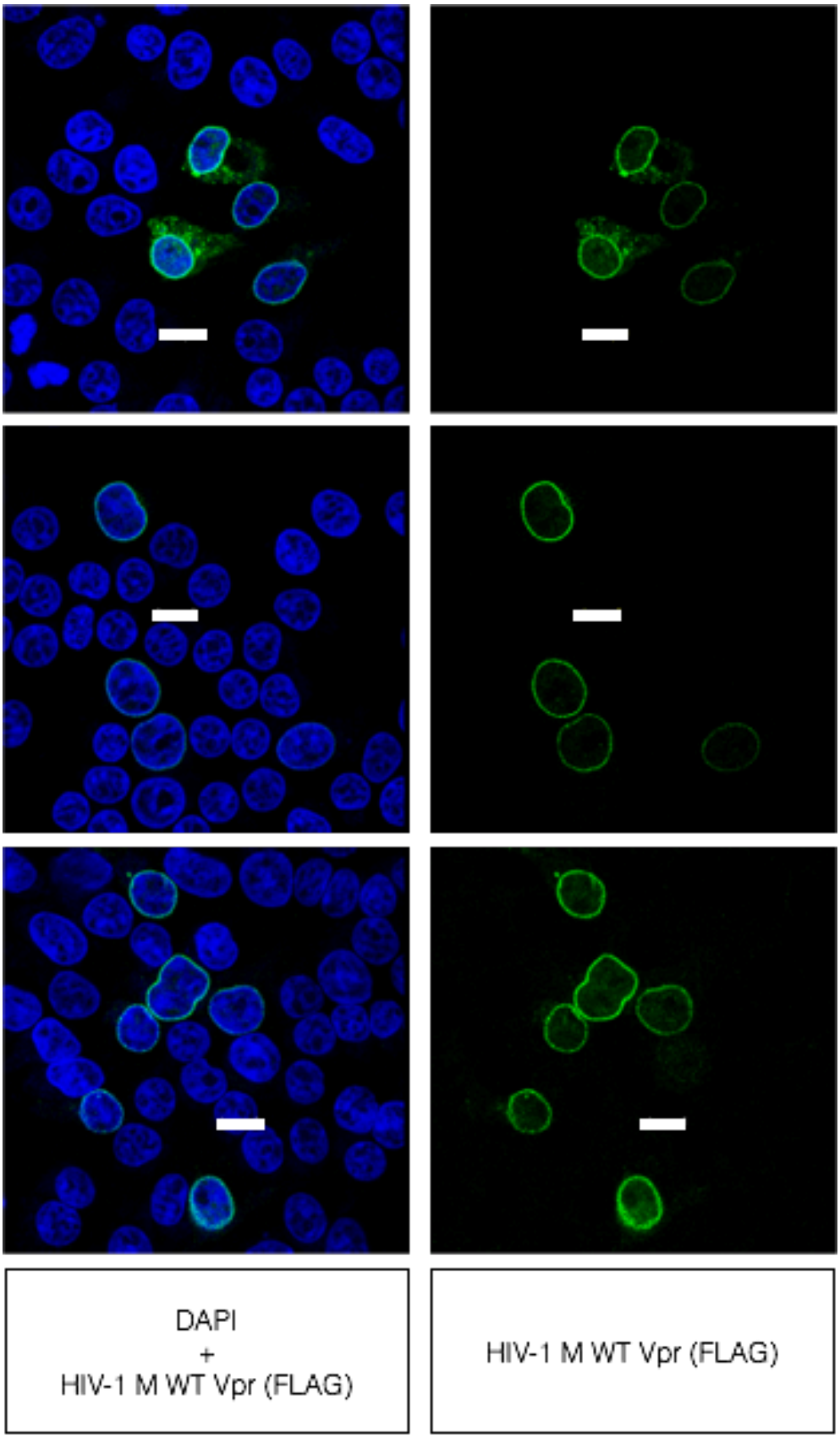

Fig 4.8 A Transfected HIV-1 M Vpr localises to the nuclear rim in HEK293T cells

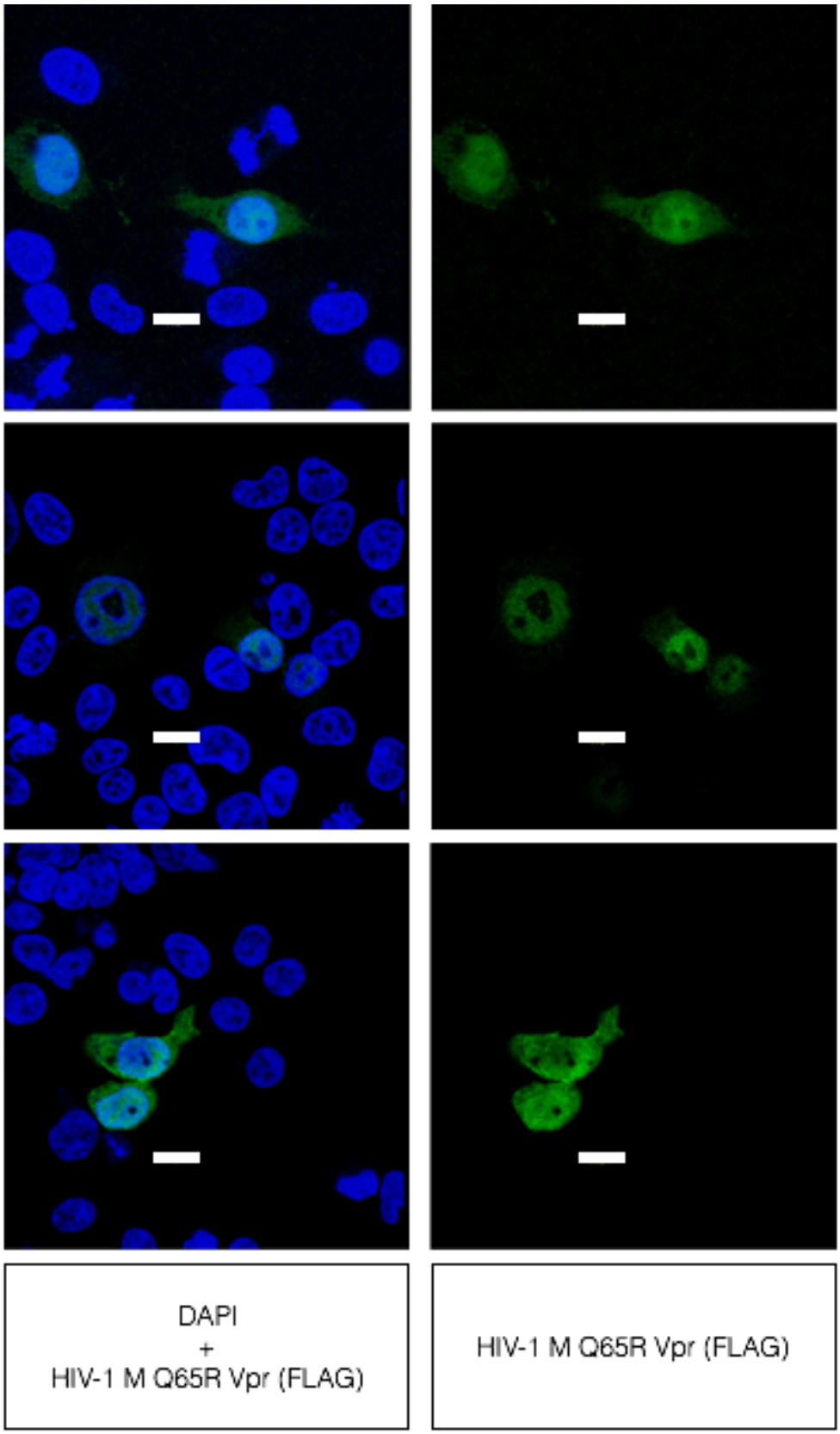

Fig 4.8 B Transfected HIV-1 M Vpr Q65R mutant does not localise to the nuclear rim in HEK293T cells

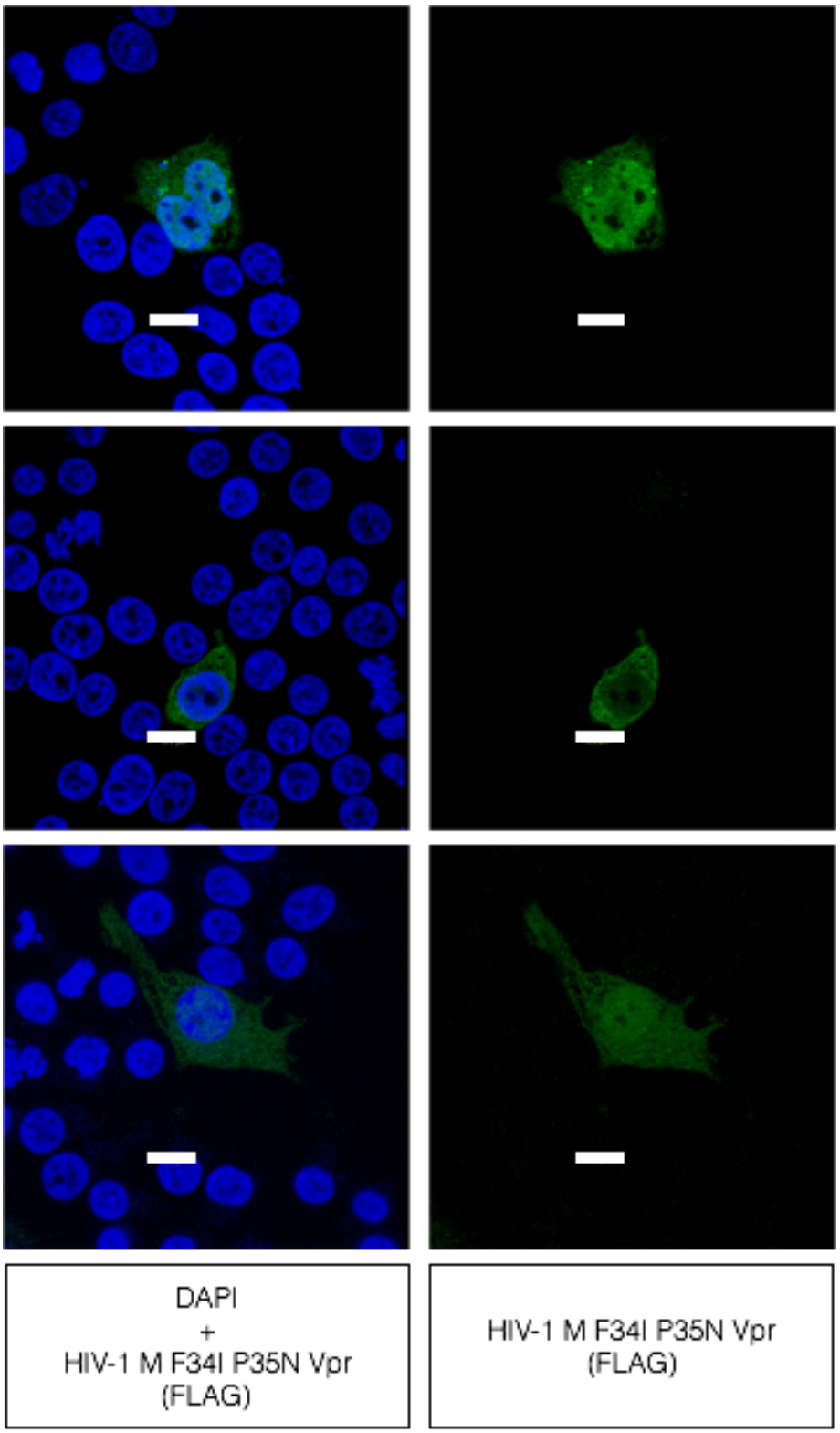

Figure 4.8 C Localisation of Vpr and mutants HEK293T cells were transfected with HIV-1 M Vpr (A), its Q65R mutant (B) or the F34I/P35N double mutant (C). Cells were then fixed, permeabilised and stained for FLAG epitope tags and with DAPI nuclear stain. Confocal microscopy was used to analyse the localisation of Vpr in these cells. White lines indicate scale (10 μM).

4.9 Visualisation of co-transfected stained expression vector

Following these observations, I hypothesised that Vpr may be creating a block to nuclear entry by either 1) binding the cyclophilin domain of Nup358 through its cyclophilin binding domain or 2) binding another region of the nuclear pore complex or 3) binding the nuclear envelope. To investigate whether expression of Vpr altered the intracellular localisation of transfected plasmids a GFP encoding pcDNA.3 plasmid was labelled with LabelIT intracellular nucleic acid tracker Cy5 and was co transfected into HEK293T cells with HIV-1M Vpr or empty vector. Stained plasmid localisation and GFP expression were visualised using the automated WiScan Hermes high resolution microscopy platform.

The cells expressing Vpr showed a reduction in GFP expression as expected: both the number of green cells and the mean fluorescence intensity of those cells were reduced at 48hrs in cells. Stained plasmid was clearly visualized (figure 4.9). The images show that whilst every cell contains stained plasmid DNA not every cell is expressing GFP. This is consistent with the notion that the rate limiting step in expression from transfected plasmids is nuclear entry and raises the possibility that Vpr is manipulating this step.

It was not possible to quantitatively detect changes in cytoplasmic or nuclear plasmid localisation due to lack of resolution – plasmid at the edge of the nucleus could not be distinguished from plasmid inside the nucleus. Additionally, since the automated platform is not confocal and cross-sectional imaging is not possible. Since the cells used are more disc-like than sphere-like it was assumed that stained plasmid over lying the nucleus would be inside it but this would need to be confirmed using confocal microscopy.

The quantity of plasmid DNA required to express visible GFP may be invisible to detection using this microscope and there may be more appropriate techniques to visualize specific sequences (like fluorescence in-situ hybridization (FISH)) that could be combined with immunofluorescence and depletion of nuclear rim proteins to test the hypothesis that Vpr prevents nuclear entry of transfected plasmid DNA (see discussion chapter 6.1.1).

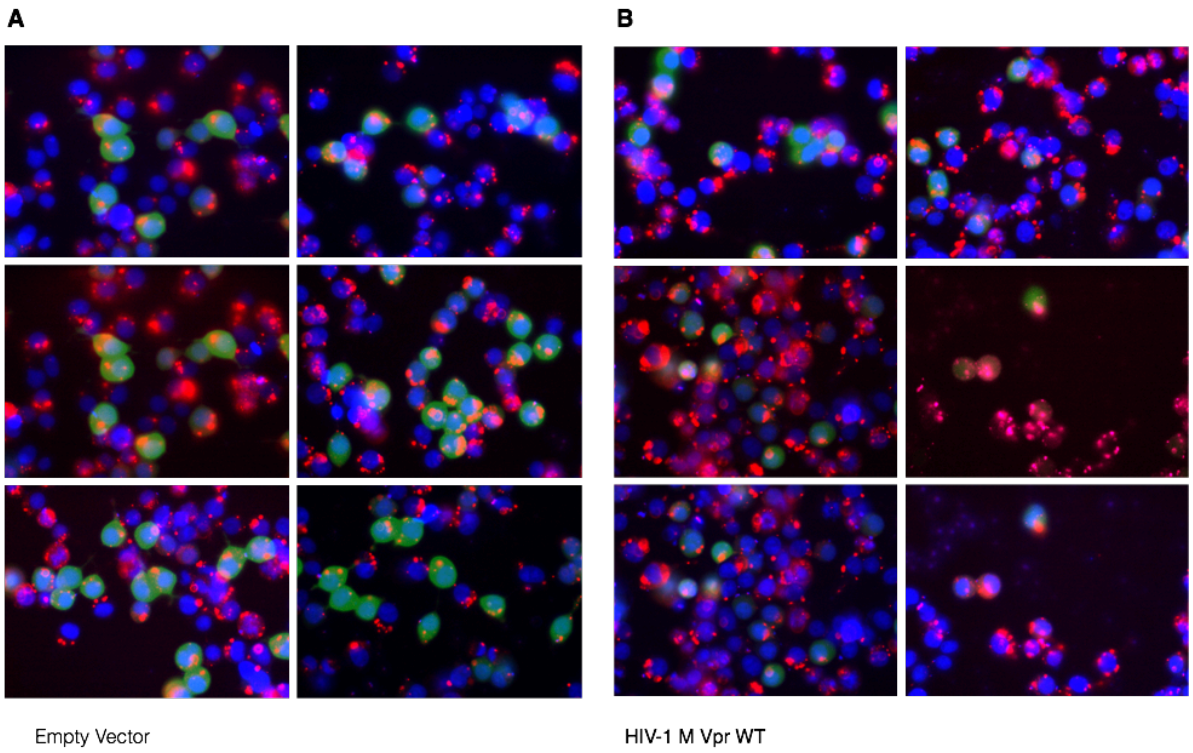

Figure 4.9 Transfected HIV-1 M Vpr with Label-IT stained GFP expression vector. HEK293T cells were transfected overnight with a plasmid expressing empty vector (A) or HIV-1 M Vpr (B) at 150ng/well. 50ng of a plasmid encoding GFP, labelled with Rhodamine Cx, was simultaneously transfected. At 48hrs post transfection cells were then fixed and stained with DAPI nuclear dye. The localisation of GFP (green), nuclear DNA (blue) and stained plasmids (red) is shown.

4.10 Transfected HIV-1 M Vpr antagonises NFκB luciferase expression stimulated by added TNFα at an integrated reporter

Intriguingly, a final experiment did show HIV-1 M Vpr inhibition of expression from an integrated NFκB sensitive luciferase reporter. Soluble TNFα (rather than transfected plasmid encoding TNFα) was used to activate the same integrated NFκB sensitive promoter as used figure 4.7. Vpr was transfected 24hrs prior to addition of TNFα.

Luciferase expression stimulated by TNFα was antagonised up to 5 fold over a titration series and this inhibition of signal was diminished with the Q65R DCAF mutant and the F34I/P35N cyclophilin mutant (figure 4.10). This antagonism was not observed previously (in figure 3.13 E) most likely because the doses of both soluble TNFα stimulation and NFκB reporter were too high to detect any antagonism.

This assay, measuring the effect of Vpr on an NFκB signaling induced by a soluble cytokine at an integrated reporter may be more physiologically relevant than the assays measuring the effect on expression from co-transfected plasmids - in the body soluble cytokines initiate signaling cascades but there is no known physiological process closely equivalent to transfection. However, since the effect of Vpr on integrated NFκB reporter activity stimulated by soluble TNFα is abrogated in the same way by the Q65R and F34i/P35N mutants as the effect on expression from co-transfected plasmids, I hypothesise that both effects are mediated by the same mechanism – putatively through the regulation of nuclear import by Vpr (see discussion chapter 6).

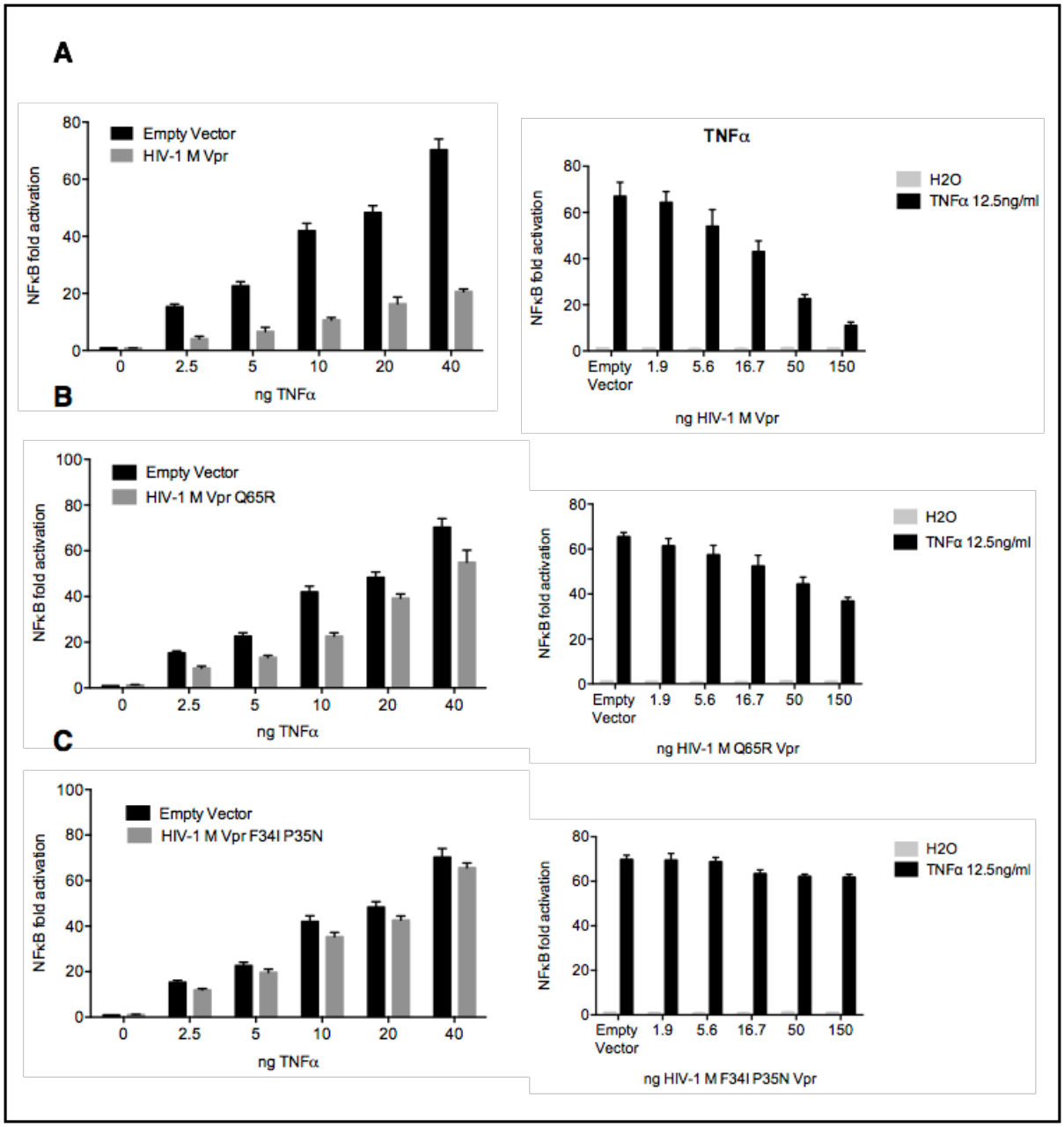

Figure 4.10 Transfected HIV-1 M Vpr antagonises NFκB luciferase expression stimulated by added TNFα at an integrated reporter. NFκB signalling in HEK293T-Luc cells (with an integrated NFκB sensitive luciferase reporter) was activated with a dilution series of added TNFα with a constant dose of Vpr (left column) or a constant dose of TNFα, with a dilution series of Vpr (right column). HIV-1 M WT (A), Q65R (B) and (C) F34i/P35N were tested. Data are represented as mean ± SEM stimulation relative to sample one of the mock-treated EV control.

5 Chapter 5: Characterisation of SIVmon (mona monkey) Vpr to identify determinants of block to sensing

5.1 SIVmon Vpr activates NFκB signalling

In experiments using the panel of primate species' Vprs it was noticed in the untransfected experimental control wells that even without co-transfection of an NFκB signal activating molecule (e.g. cGAS/STING, IKKB, TBK1 etc) one of the two Vprs from the mona monkey stimulated luciferase expression driven by an NFκB sensitive promoter ~100 fold compared to empty vector. This stimulation was unaffected by am N-terminal FLAG epitope tag (figure 5.1).

Prior to the characterization of HIV-1 M Vpr's inhibitory effects on transcription from all tested transfected plasmids I hypothesised that SIVmon Vpr may be activating the NFκB signalling pathway by joining the same cellular signalling complex as the HIV-1 M Vpr but activating it rather than inhibiting it. I sought to characterize this stimulatory activity of SIVmon Vpr to see if it could provide a useful experimental reagent to better understand NFκB signalling and Vpr antagonism, and to help identify binding partners.

These studies on SIVmon Vpr were started prior to determining the effect of HIV-1 M Vpr on transcription. Their relevance to illuminating our understanding of the function and mechanism of HIV Vpr is perhaps less clear but since there seems to be a true effect on NFκB signalling as well as suppression of transfected NFκB activation molecule expression, SIVmon Vpr may yet prove useful as an experimental reagent both for understanding HIV-1 M Vpr and for understanding NFκB signalling.

Previous studies have used tandem affinity purification to identify SAMHD1 and SLX4 as a binding partner for Vpr but since the HEK293T cells seem to constitutively lack many of the putative binding partners involved in the inhibition of NFκB signalling (i.e. the co-transfected molecules that show reduced expression on immunoblot when co-transfected with HIV-1 M Vpr) this technique seemed unlikely to be successful in this cell line (438).

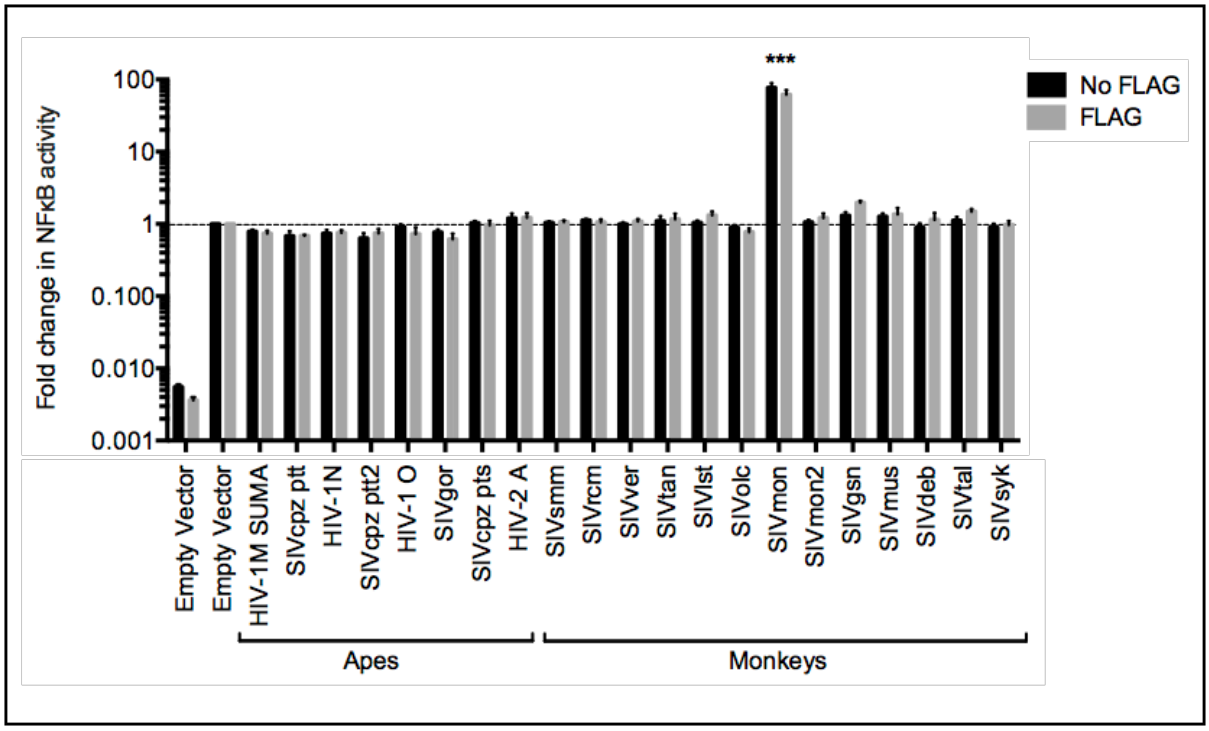

Figure 5.1 SIVmon Vpr stimulates an NFκB sensitive promoter luciferase construct in the absence of transfected innate immune molecules HEK293T cells were transfected as previously with expression plasmids for Vpr and two reporter plasmids (sensitive to NFκB and constitutively expressed respectively). Cells were harvested for luminometry at 48hrs. Firefly luciferase activity was measured and normalised to the renilla luciferase activity. Data are presented as the fold induction relative to an identical dose an empty expression vector. The mean values of three transfections ±SEM are shown. ***=P<0.001 one-way ANOVA with Dunnett's post-hoc test.

5.2 SIVmon Vpr expression activates a co-transfected NFκB reporter and SIVmon Vpr expression is suppressed by co-transfection of HIV-1M Vpr

A three-fold dilution series of SIVmon Vpr with only reporter plasmids indicated SIVmon Vpr robustly stimulated signalling from the NFκB sensitive reporter but in a non dose dependent fashion with 1.9ng activating the pathway more than 150ng (figure 5.2A). This is not unusual for signalling activation and may imply that over-expression titrates a signalling molecules out of a complex leading to reduced activation (567,568). As with other stimulatory molecules SIVmon Vpr showed reduced expression on immunoblot and inhibition of the NFκB signal with addition of increasing doses of HIV-1 M Vpr (figure 5.2B). Prior to the realization that HIV-1 M Vpr inhibits expression from all co-transfected plasmids this was interpreted as a potential indication of an effect on a common pathway. In fact, expression of SIVmon Vpr was being inhibited in the same way as the plasmids in chapters 3 and 4.

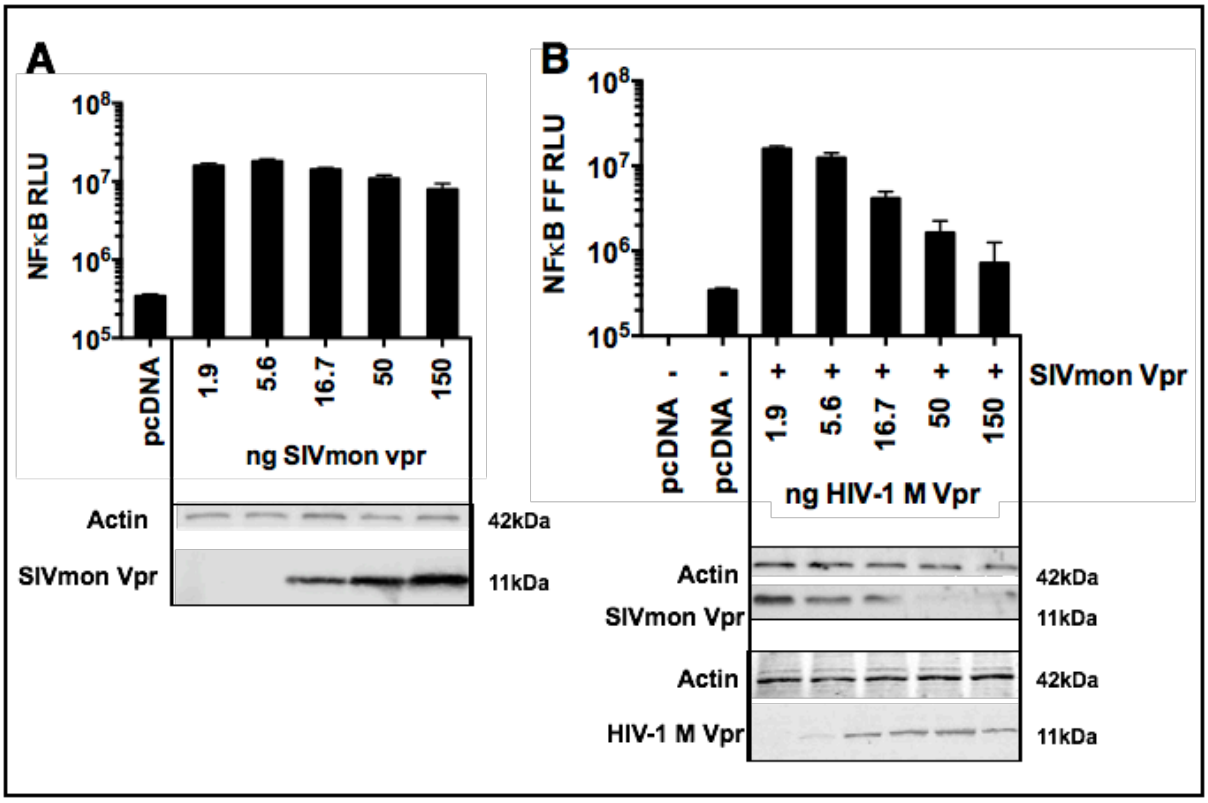

Figure 5.2 Ectopic expression of SIVmon Vpr activates a co-transfected NFκB reporter and is inhibited by co-transfection of HIV-1M Vpr. (A) SIVmon Vpr was transfected in a five point dilution series together with luciferase reporters. (B) SIVmon Vpr was transfected at a constant dose of 5.6ng and HIV-1M Vpr was added in a five point three fold dilution series. 48 hours after transfection the cells were harvested for luminometry. Firefly luciferase activity was measured and normalised to the renilla luciferase activity. Data from three experiments are represented as the fold induction relative to an identical dose of activating molecule with an empty expression vector -/+ SEM. Cell lysates were analysed by immunoblot with anti-FLAG antibodies and actin as a loading control.

5.3 SIVmon Vpr activates NFKB signalling from an integrated reporter

To investigate whether the stimulatory activity of SIVmon Vpr was an effect confined to transfected reporter plasmids a HEK293T cell line was transduced with VSV-G pseudotyped lentiviral particles with a reporter encoding for a mammalian codon-optimized, non-secreted form of the firefly luciferase under the control of a minimal CMV promoter and tandem repeats of the NFκB transcriptional response element (TRE) i.e. the same promoter used in the transfected reporter plasmids. Here the activation of NFκB signalling by SIVmon Vpr was linearly dose dependent compared to the transfection where an attenuation of signal was seen at higher doses indicating possible antagonism of expression from a co-transfected plasmid by SIVmon Vpr. HIV-1 M Vpr did not affect basal NFκB signal whereas co-transfected cGAS and STING acting as a positive control activated the pathway as in figure 4.7.

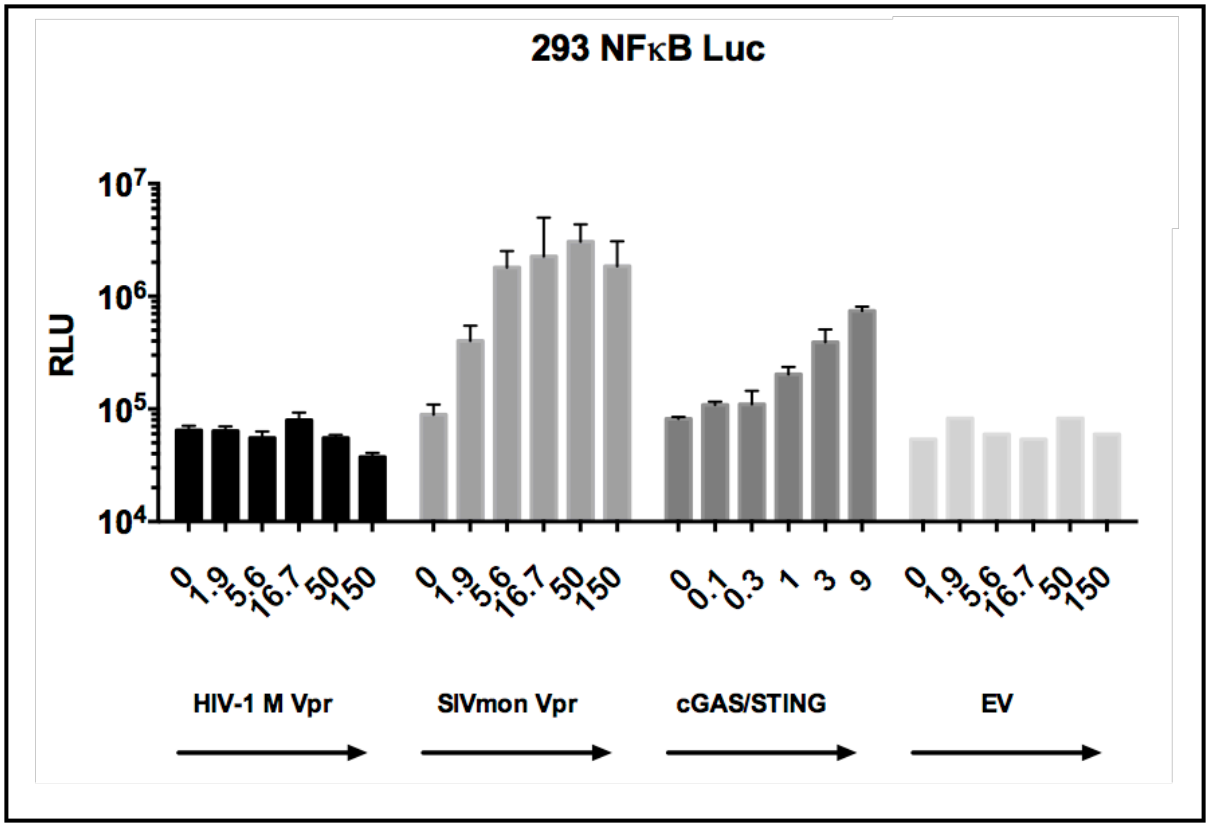

Figure 5.3 SIVmon Vpr activates expression from an integrated NFκB sensitive luciferase reporter HEK293T cells with an integrated luciferase gene under the control of the NFkB promoter were transfected in a titration series with SIVmon Vpr, HIV-1 M Vpr, cGAS /STING or an empty expression plasmid. Luciferase activity was measured at 48hrs post transfection. Data from three independent experiments are represented as the fold induction relative to an identical dose of empty expression vector -/+ SEM.

5.4 Mutagenesis of SIVmon Vpr

Experiments on the HIV-1 M Vpr had shown that a cyclophilin binding mutant P35N is defective for nuclear localisation (fig 4.8B) and the phenotype of signal antagonism. To guide a mutagenesis strategy for testing the SIVmon Vpr, a structural alignment was generated with T-Coffee multiple alignment software which can incorporate three-dimensional structural information for the alignment of protein sequences (516,517). The ESPript (*Easy Sequencing in PostScript)* online software was then used, a program which renders sequence similarities and secondary structure information from aligned sequences (518) (figure 5.4).

The Serine at position 40 aligned consistently with the Proline and was mutated to Asparagine using site directed mutagenesis (SDM). We hypothesised that this might disrupt the same binding site and allow us to infer potential binding partners.

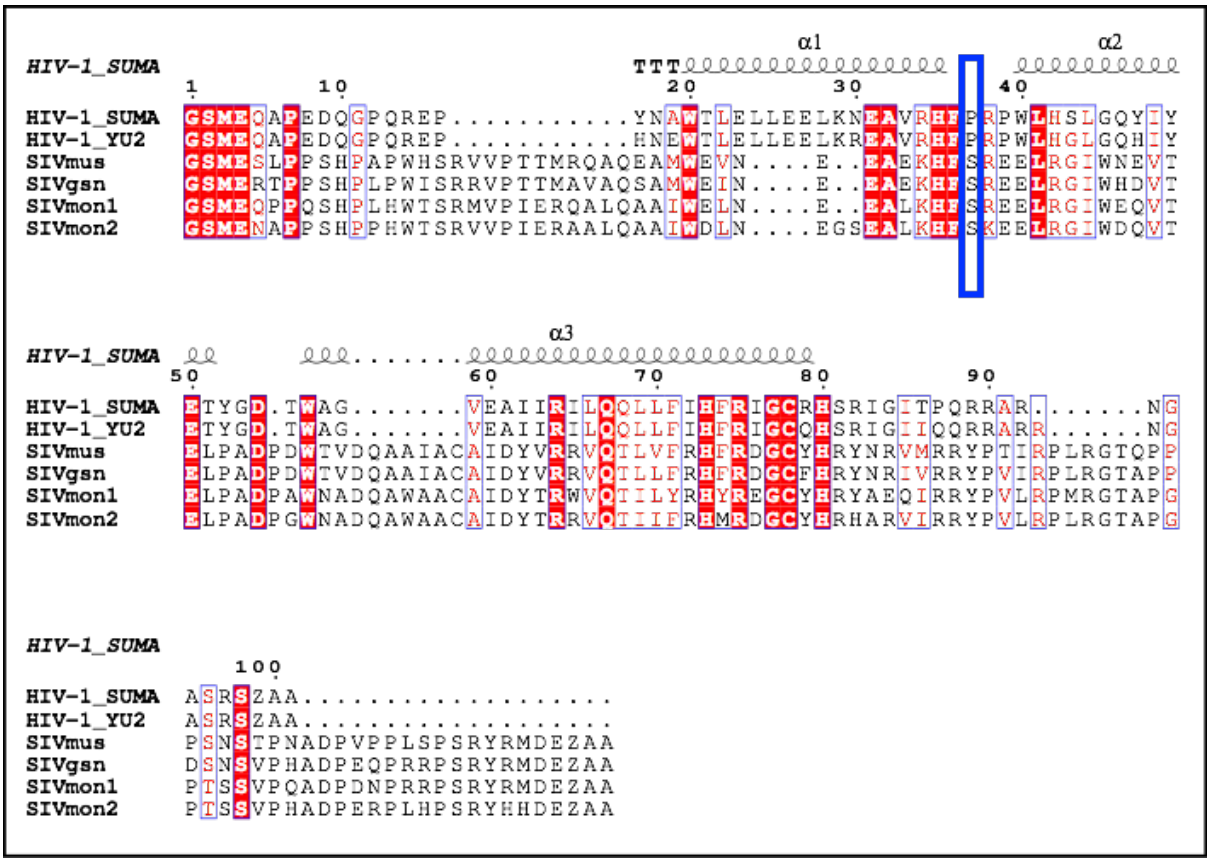

Figure 5.4 Alignment of Mona monkey group SIV, HIV_YU2 and SIVcpz Vprs. Primary and secondary structures and sequence data were aligned using the webserver program ESPript3.0

5.5 SIVmon Vpr specifically activates NFκB signalling

To characterize the ability of SIVmon Vpr, its S40N mutant and the SIVgsn Vpr from the same group, the effect of ectopic expression of these proteins on transcriptional reporters in HEK293T cells was examined across a range of immune related promoters with cGAS and STING acting as a positive control (figure 5.5 A-F). They were tested over a three fold dilution series against six separate luciferase promoter constructs including the IFNβ promoter which contains binding sites for the NF-κB, IRF3 and AP-1 transcription factors (527,528); the ISG56 promoter sensitive to IRF3 (524,529); an Interferon Sensitive Response Element (ISRE) sensitive to the ISGF3 complex and IRF1&3 (569,570); an AP-1 sensitive promoter encoding the firefly luciferase reporter gene under the control of a minimal (m)CMV promoter and tandem repeats of the TPA-induced transcriptional response element (TRE) (QIAGEN); the synthetic NFκB sensitive promoter as used as the reporter throughout chapter 3; and the HSV Thymdine Kinase (TK) promoter Renilla luciferase construct with binding sites described for TFIID, Spl, and CCAAT-binding proteins (Promega) (555). This was the same construct used as a transfection control throughout chapter 3.

Data in figure 5.5 are presented as the raw values from the luminomter of relative light units (RLU) for the firefly luciferase expression to show the basal activation level of each promoter. Both the S40N mutant and the SIVgsn Vpr showed little or no effect on luciferase expression from any plasmid. However the wild type SIVmon Vpr increased transcription from the NFκB promoter whilst dramatically suppressing expression from the ISG56 and TK promoters >10fold (figure 5.5).

This is hard to explain in terms of known transcription factors but there is “cross talk” between the NFκB and IRF3 signalling pathways; NF-κB p65 and IRF3 can form stable complexes that can be recruited through an interferon-response element (IRE) or a κB site (571). Additionally NF-κB target genes can be IRF dependent and this is determined by an IRE in close proximity to the κB site (572). Glucocorticoid receptors inhibit a subset of NF-κB-dependent transcriptional responses following ligand binding and can directly displace IRF3 from p65 (573). Assembly of NF-κB, IRF and ATF–c-Jun transcription factors at the enhancer of the gene encoding interferon-β, occurs after viral infection (574). Additionally p50 homodimers repress a subset of interferon-inducible genes through direct binding to guanine-rich IRE sequences and probably through direct competition with IRF3 (575).

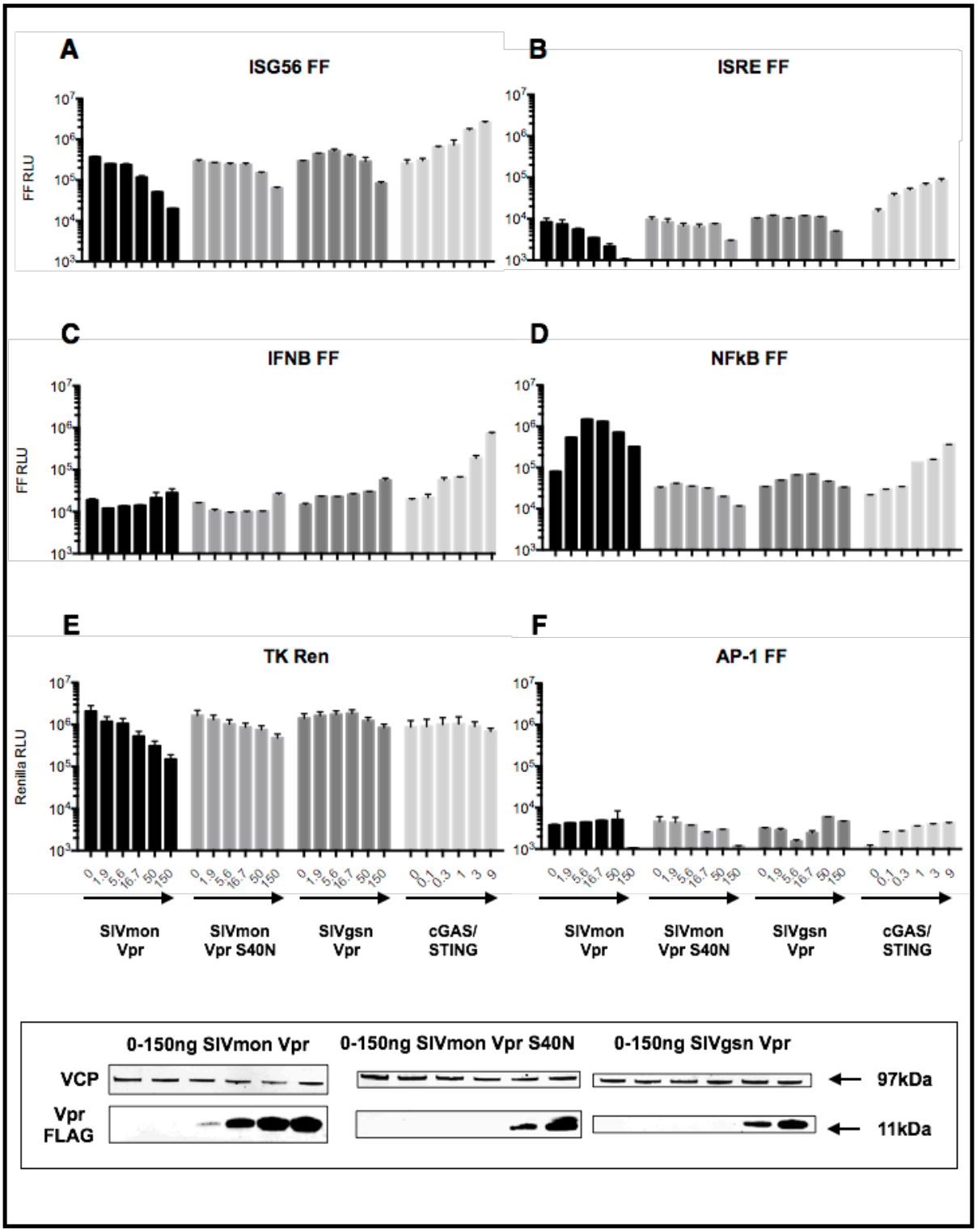

Figure 5.5 SIVmon Vpr specifically activates NFκB signalling HEK293T cells were transfected with luciferase reporter plasmids with the indicated promoters and then a dilution series of either SIVmon WT Vpr, SIVmon S40N Vpr, SIVgsn Vpr, or cGAS/STING. Cells were harvested for luminometry and immunoblot at 48hrs. Data are presented as the mean raw relative light unit (RLU) value for the firefly luciferase substrate from three experiments +/- SEM.

5.6 Depletion of Nup358 reduces SIVmon Vpr NFκB signalling

HIV-1 M, like Nup385, localises to the nuclear rim (see figure 4.8) and is known to have a cyclophilin (cyp) binding domain previously described by numerous previous studies (408,502,503,508,576,577). Nup358 has a cyclophilin homology domain and has been described as a co-factor for HIV-1 M nuclear entry (164,165,167,331,332). While the cyclophilin binding domain of SIVmon Vpr has not been studied the putative S40N mutant with diminished function aligns with the P35 in HIV-1 M Vpr believed to be central to binding of cyclophilin.

I hypothesised that SIVmon and HIV-1 M Vprs could be binding to cyclophilin A and the cyclophilin homology domain of Nup358 through their putative cyclophilin binding domains and through this binding could be modulating nuclear entry of NFκB subunits or transfected DNA.

To test this hypothesis initially, Nup358 was depleted with short hairpin RNA (shNup358) in HEK293T cells and these cells were transfected with either empty vector (with and without HIV-1 M Vpr) or cGAS and STING in a 1:1 ratio (with and without HIV-1 M Vpr) (figure 5.6A). As expected HIV-1 M Vpr reduced cGAS/STING induced NFκB driven luciferase expression in both the shControl and the shNup358 cells (figure 5.6A). But determining the effect of Nup358 depletion on HIV-1 M Vpr inhibition of NFκB activation was complicated by the fact that depletion of Nup358 reduced the NFκB activation induced by cGAS and STING transfection (figure 5.6A). This makes it difficult to determine any further effect of Nup358 depletion on HIV-1 M Vpr on signal reduction. This difficulty was one of the main reasons for investigation and characterization of the SIVmon Vpr: to identify binding partners of HIV-1 M Vpr that could be further investigated.

NFκB activation was then tested in the same shNup358 or shControl HEK293T cells using either transfected SIVmon Vpr or soluble TNFα. TNFα induced NFκB signalling was not significantly affected but there was a small reduction in SIVmon Vpr induced NFκB driven luciferase expression. Nup358 depletion left TNFα mediated NFκB signalling unaffected, but signalling activated by SIV mon was significantly antagonised potentially indicating an interaction between these proteins or a dependency on Nup358 for SIVmon Vpr, but not TNFα, mediated NFκB signalling (figure 5.6B) although the observed inhibition of the SIVmon Vpr NFκB signal was not large.

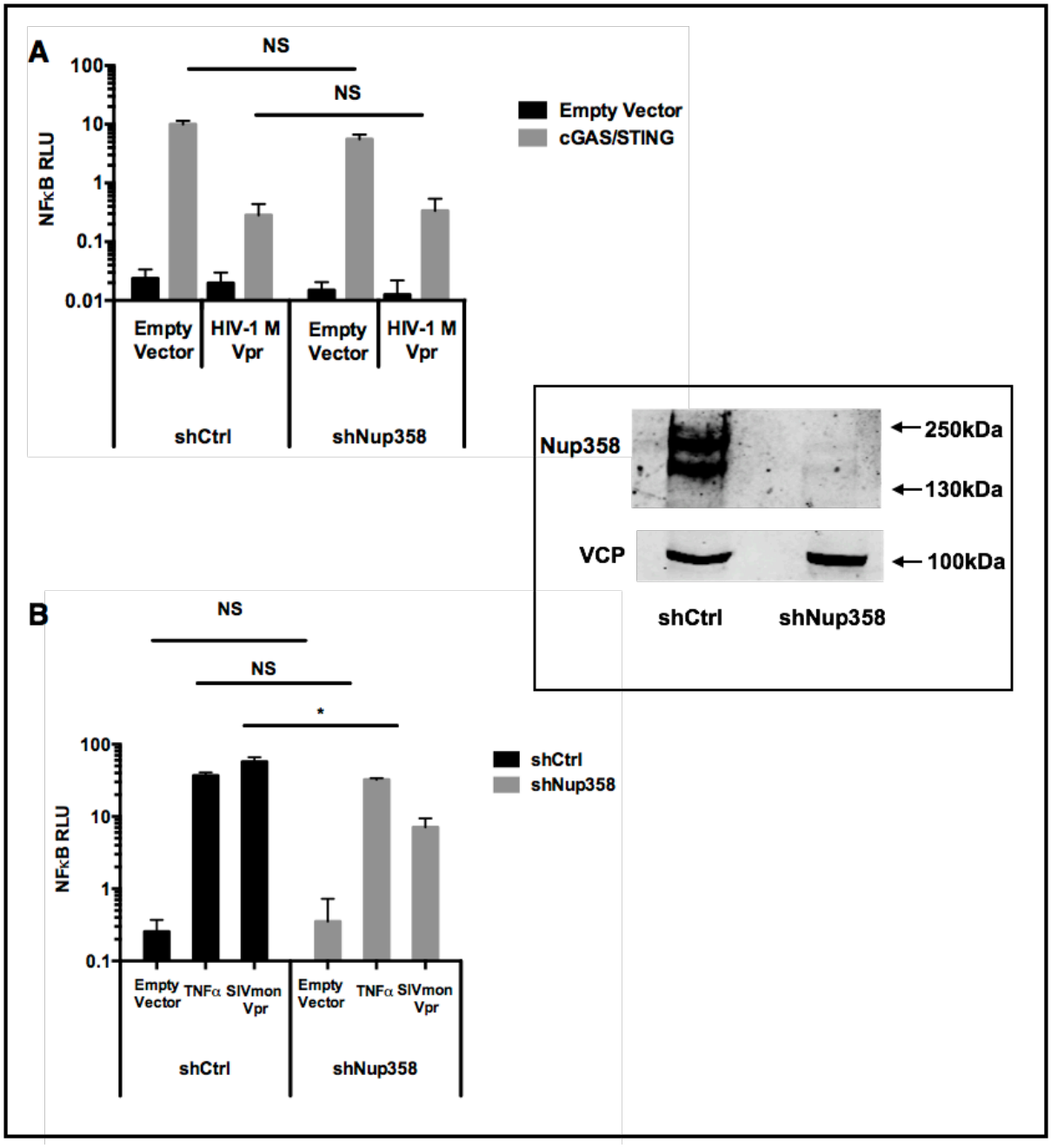

Figure 5.6 Depletion of Nup358 reduces SIVmon Vpr NFκB signalling. Depletion of Nup358 in HEK293T cells using shRNA lentiviral vector transduction followed by transfection with (A) cGAS/STING +/- HIV-1M Vpr and an NFκB luciferase reporter. (B) Depleted cells were also either transfected with SIVmon Vpr or TNFα was added. Luminometry was performed at 48hrs. Data from three experiments are represented as the fold induction relative to an identical dose of empty expression vector -/+ SEM. Cell lysates were then analyzed by immunoblotting with anti-Nup358. *=P<0.05 one-way ANOVA with Dunnett's post-hoc test.

5.7 Depletion of cyclophilin A reduces SIVmon Vpr NFκB signalling

In order to determine whether the observed SIVmon Vpr activation of an NFκB reporter was dependent on cyclophilin A, short hairpin RNA was used to deplete cyclophilin A in HEK293T cells. These cells were then transfected with empty vector, SIVmon Vpr or cGAS and STING in a 1:1 ratio in order to stimulate NFκB signalling and NFκB promoter activity was measured at 48 hours post transfection by luminometry. Cyp A depletion was confirmed using on an immunoblot. Since it has overlap with SIVmon Vpr FLAG on immunoblot the empty vector lysate was tested for depletion (figure 5.7 B).

Cyp A depletion inhibited SIVmon Vpr induced expression of luciferase driven by an NFκB promoter approximately 2 fold whilst cGAS/STING induced reporter expression was left unaffected (figure 5.7A). While the effect is not large it is statistically significant potentially indicating cyp A as a co-factor for SIVmon Vpr NFκB activation.

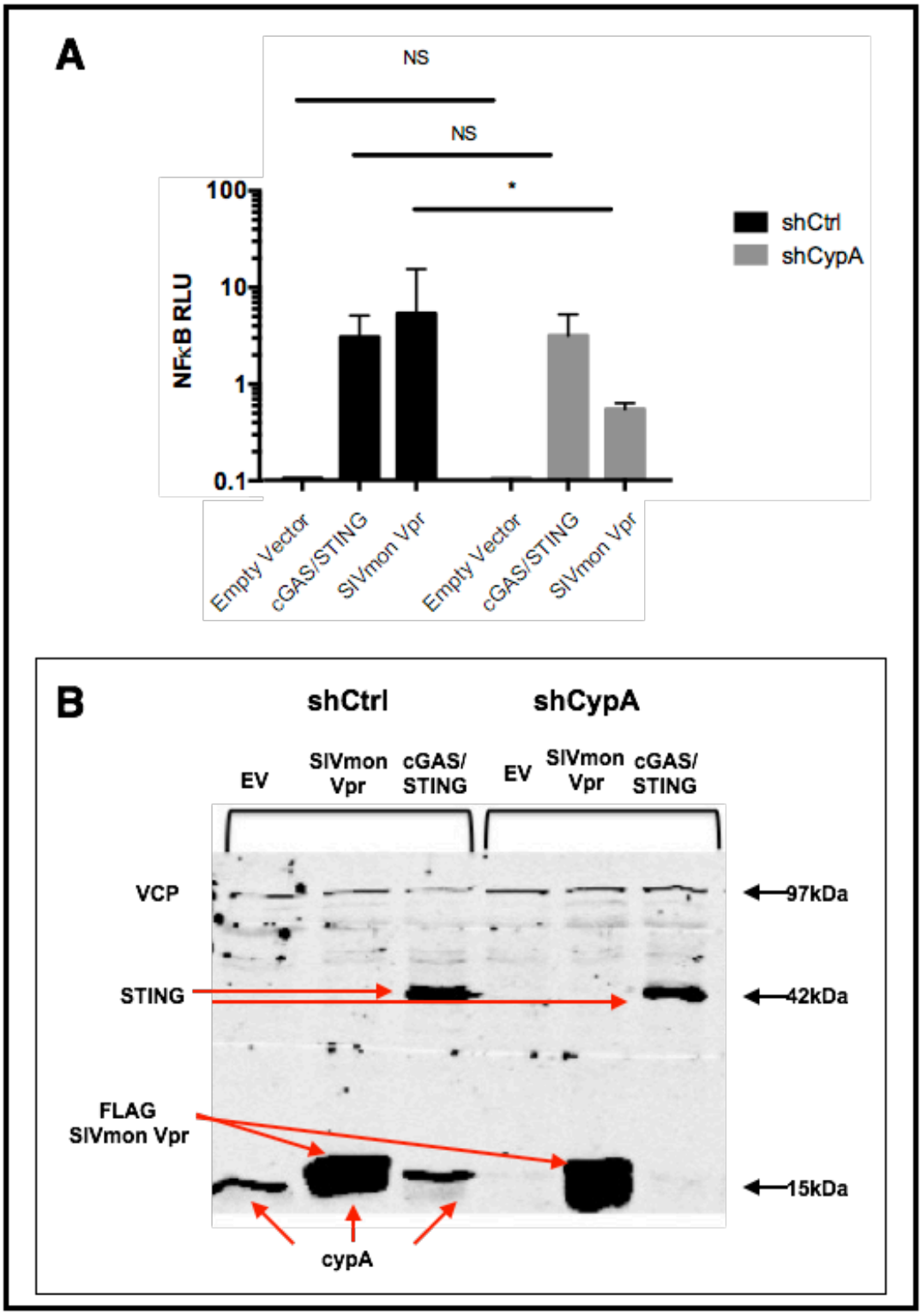

Figure 5.7 Depletion of CypA reduces activation of an NFκB reporter by SIVmon Vpr CypA was depleted in HEK293T cells using shRNA lentiviral vector transduction followed by transfection with cGAS/STING or SIVmon Vpr and an NFkB luciferase reporter. Data from three experiments are represented as the fold induction relative to an identical dose of empty expression vector ± SEM. Cell lysates were analyzed by Western blotting with anti FLAG, anti CypA and, as a loading control, anti-VCP. *=P<0.05 one-way ANOVA with Dunnett's post-hoc test.

5.8 Overexpression of CypA or Nup358cyp leaves SIVmon Vpr activated NFκB signalling unaffected.

To test whether overexpression of CypA or the cyclophilin homology domain of Nup358 has any effect on SIVmon Vpr induced NFκB sensitive reporter activation both molecules were expressed in HEK293T cells with co-transfection of cGAS and STING in a 1:1 ration or SIVmon Vpr (figure 5.8 A for CypA and B for 358cyp). NFκB signalling was left unaffected by either molecule. Additionally, unpublished data from our lab indicate that overexpression of CypA and Nup358 also leave NFκB signal antagonism by HIV-1 M Vpr unaffected (personal communication, Lucy Thorne).

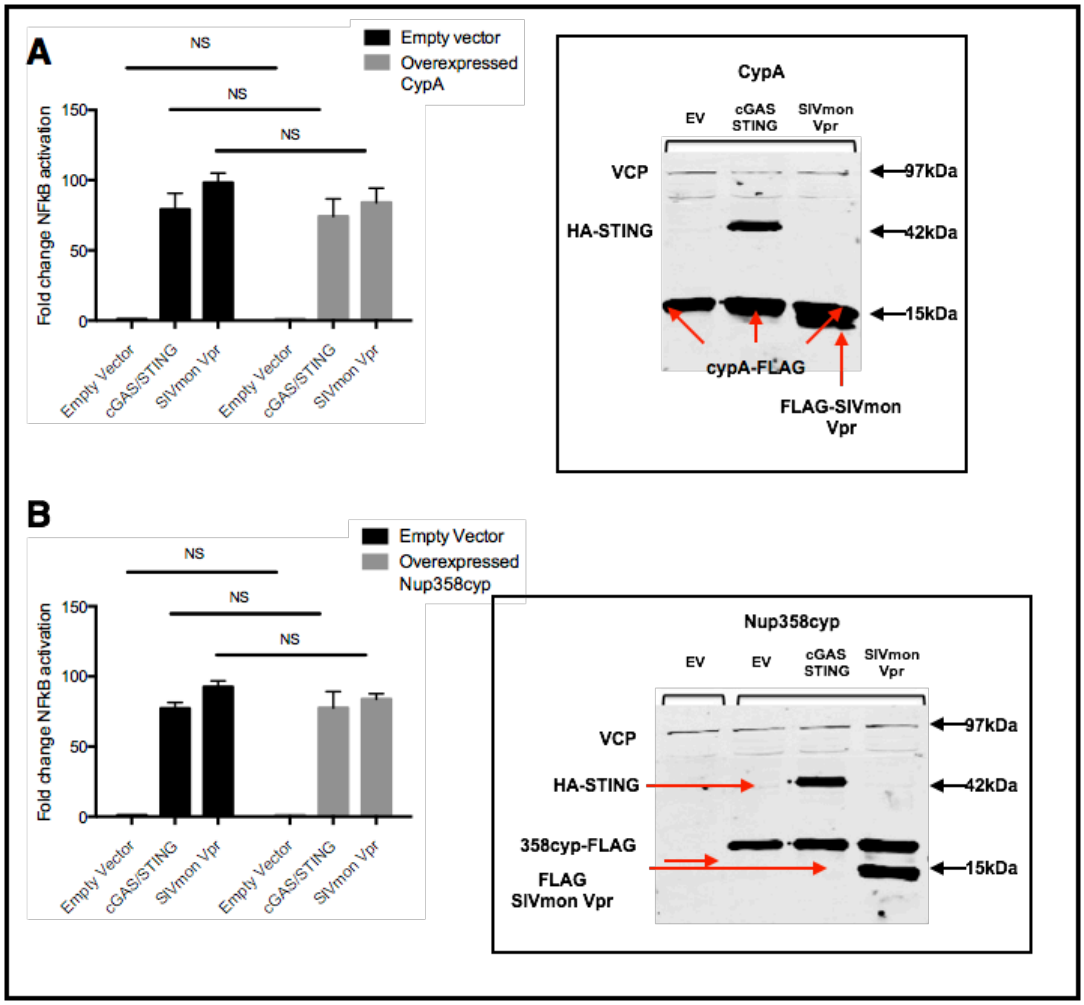

Figure 5.8 Overexpression of CypA or Nup358cyp leaves SIVmon Vpr activated NFκB signalling unaffected. CypA (A) and 358cyp (B) were overexpressed in HEK293T cells with either empty vector, cGAS/STING or SIVmon Vpr and an NFkB luciferase reporter. Data from three experiments are represented as the fold induction relative to an identical dose of empty expression vector -/+ SEM. Cell lysates were analysed by Western blotting with anti FLAG, anti HA and as a loading control anti-VCP. NS=non-significant, one-way ANOVA with Dunnett's post-hoc test.

5.9 SIVmon Vpr does not co-immunoprecipitate with co-transfected members of the pathway

As well as being a useful reagent for understanding of NFκB signaling I hypothesised that the SIVmon Vpr may help to identify the binding partner of HIV-1 Vpr since it was able to activate signalling without co-transfection of any signalling moecules (e.g. cGAS/STING). To investigate whether if SIVmon Vpr binds specific members of the NFκB signalling pathway, I attempted to immunoprecipitate FLAG-tagged SIVmon Vpr from the cleared cell lysates of cells expressing HA tagged IKK, TBK, cGAS, STING, p50, p65 or 358cy using a mouse-anti-HA Ab coupled to protein agarose beads following co-transfection with FLAG SIVmon Vpr (figure 5.9). The transfected molecules were detected in the whole cell lysates but the SIVmon Vpr was not found to co-immunoprecipitate, indicating that as had been hypothesised for HIV-1 M the binding site was not in any transfected molecule.

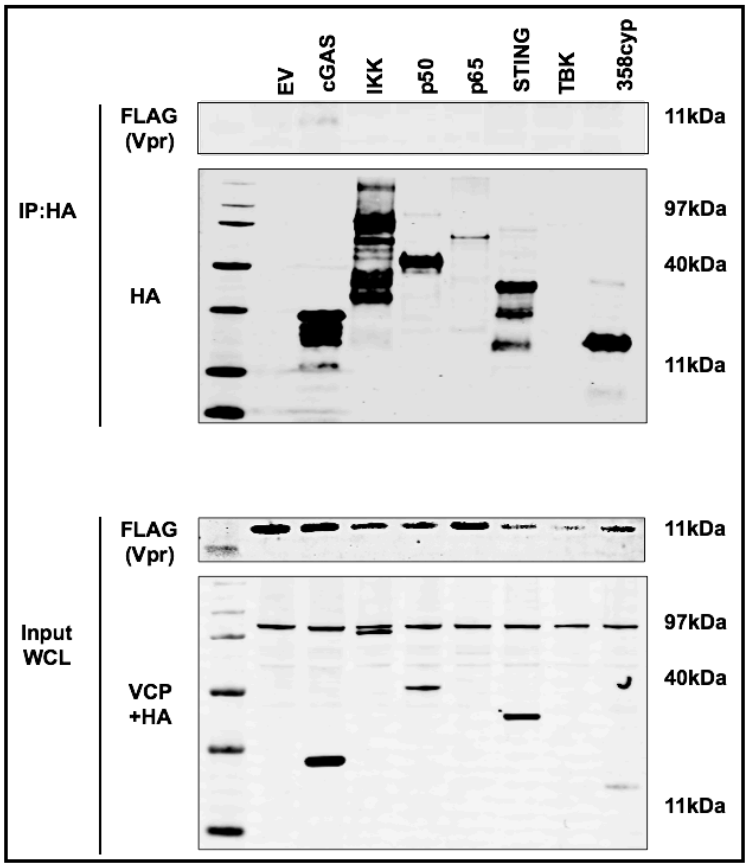

Figure 5.9 SIVmon Vpr does not co-immunoprecipitate with co-transfected members of the pathway. HEK293T cells were transfected with empty vector, HA tagged IKK, TBK, cGAS, STING, p50, p65 and 358cyp together with FLAG SIVmon Vpr. At 48hrs cell lysates were incubated with mouse-anti-HA Ab coupled to agarose beads. Whole cell lysates (Input WCL) and immunoprecipitated proteins (IP) were separated by SDS-PAGE and analysed by immunoblotting with mouse-anti-HA, mouse-anti-FLAG and rabbit-anti-VCP antibodies. The locations of protein molecular mass markers are indicated (kDa).

5.10 SIVmon localises to the nucleus

I hypothesised that if SIVmon Vpr was acting at the same binding site in the pathway as HIV-1 M Vpr then it might show the same pattern of intracellular localisation. Cellular localisation of SIVmon Vpr, SIVgsn Vpr and SIVmon S40N was studied by immunofluorescence in HEK293T cells 48hrs post transfection all three molecules being detected using a rabbit-anti-FLAG polyclonal antibody and DAPI nuclear stain. All three localised to the nucleus but with differing distributions (figure 5.10A, B&C).

SIVmon Vpr localised to discrete puncta or “dots” within the nucleus (figure 5.10A). The localisation pattern resembled that of the promyelocytic leukaemia (PML) nuclear bodies first identified in the nuclei of malignant cells in the early 1960s (578). PML bodies measure 0.1-1.0um and are found in the nuclear matrix a poorly defined structural component of the nucleus believed to regulate many nuclear functions including transcription and epigenetic modification (579). They sequester an expanding number of partner proteins but many details of their function remain unclear. PML bodies have been linked to regulation of apoptosis and senescence but a mechanism by which SIVmon Vpr might bind to these subnuclear structures and active NFκB signalling is not obvious from previous studies of PML nuclear bodies. Indeed, the mere similarity between PML body appearance and SIVmon Vpr localisation does not confirm a link between these proteins and there are other nuclear dots which have a similar appearance (580).

The SIVmon Vpr S40N mutant, putatively analogous to the P35N cyclophilin binding mutant in HIV-1 M had a markedly different distribution to the wild type SIVmon Vpr (figure 5.10B). It was evenly distributed in the nucleus with some cytoplasmic expression. The SIVgsn Vpr from the other Mona Group monkey which is defective for NFκB signal activation localised inside the nucleus but with a tendency to collect around the inside of the nuclear envelope (figure 5.10C). None of these Vpr proteins contain a classic nuclear localisation signal or a known DNA binding region was assessed using NucPred (581) and PredictProtein (582).

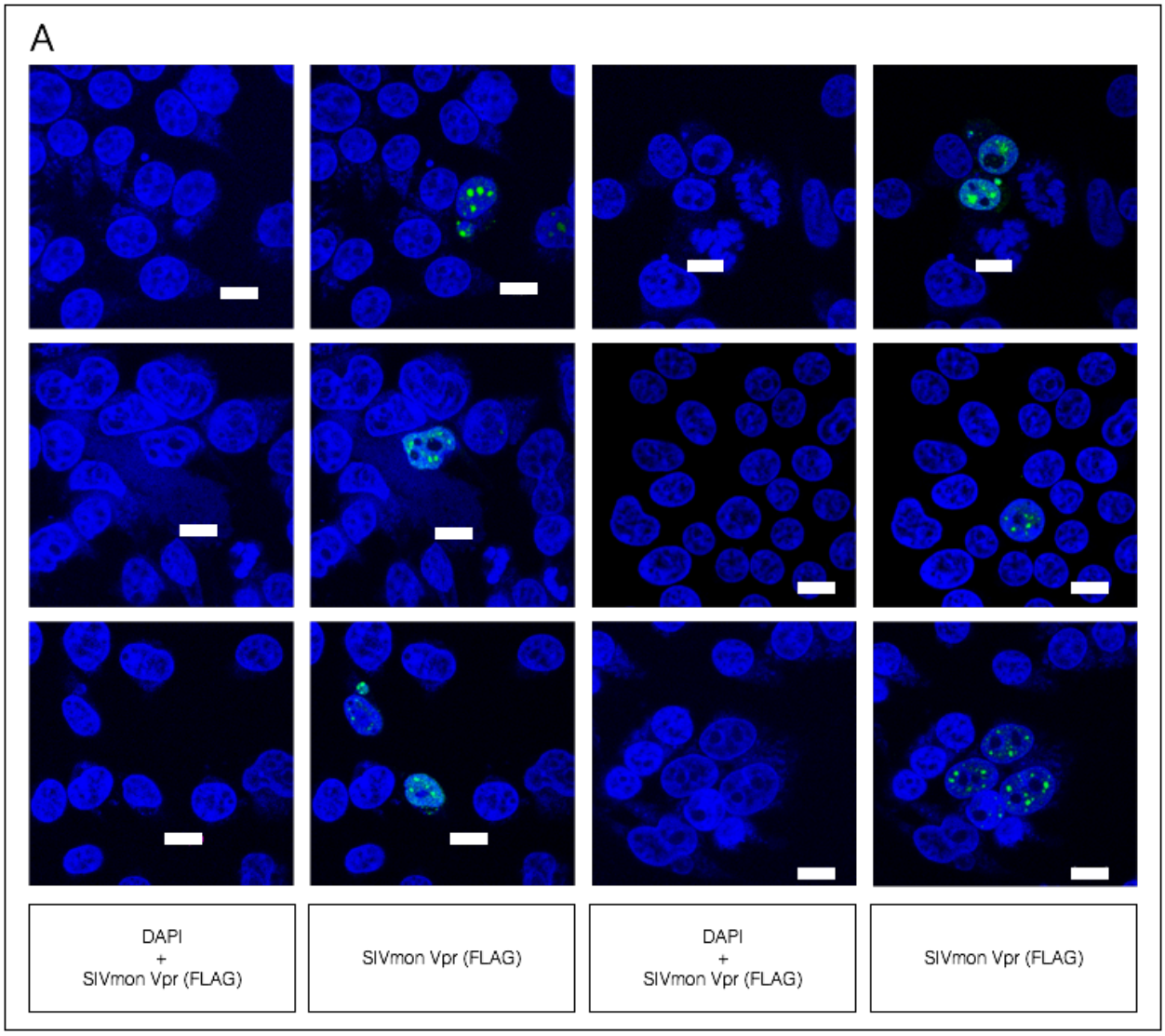

Figure 5.10 A Localisation of SIVmon

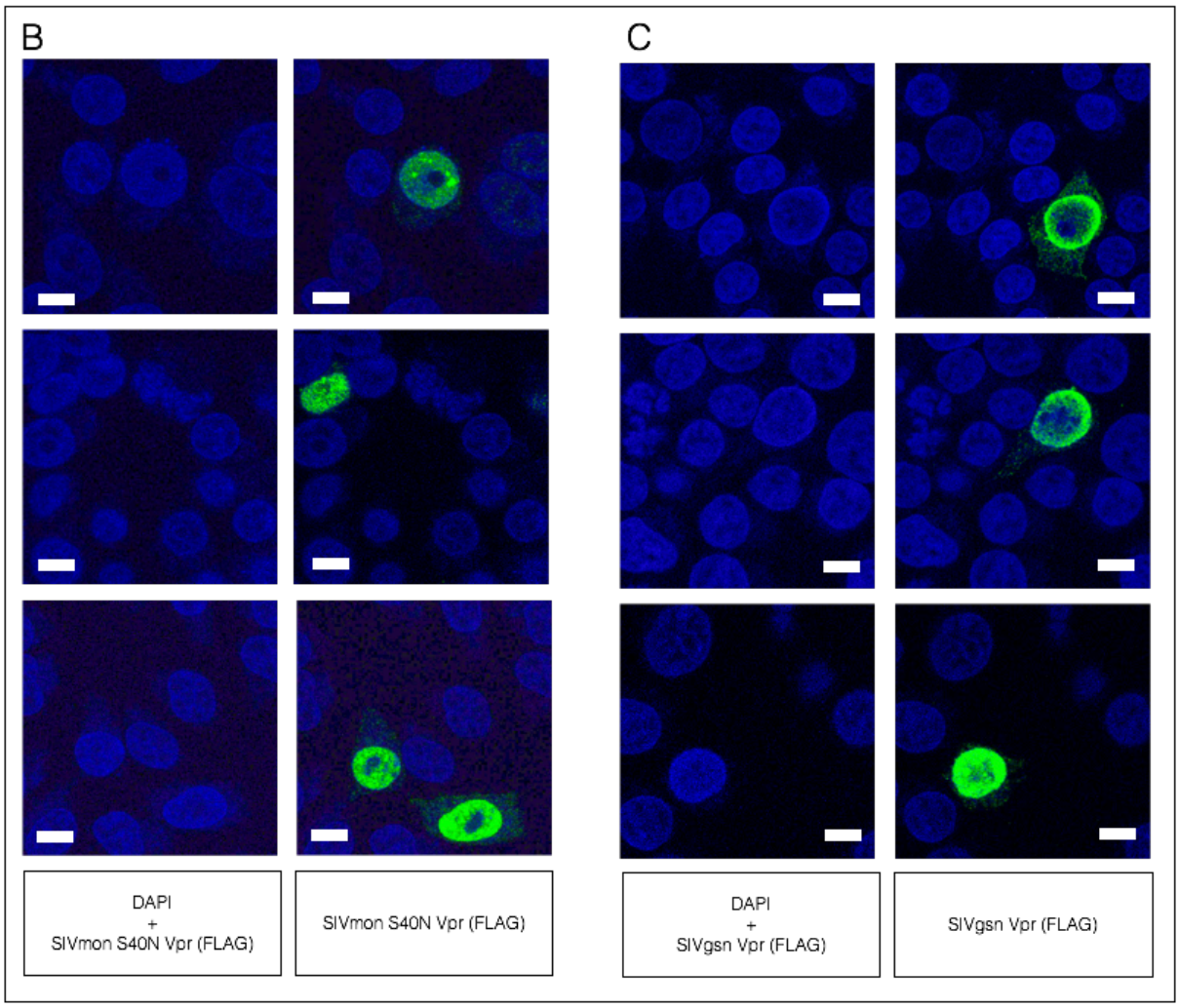

Figure 5.10 B and C Localisation of SIVmon Vpr by immunofluorescence. HEK293T cells were transfected overnight with a plasmid expressing FLAG epitope tagged SIVmon Vpr (A), its S40N mutant (B) or the SIVgsn Vpr (C). Cells were then fixed and stained with an anti-FLAG antibody. The localisation of Vpr (green), nuclear DNA stained with DAPI (blue), and merged images are shown.

5.11 Optimization of SIVmon Vpr expression for determination of binding partner by SILAC

Since SIVmon Vpr is observed to differ markedly in localisation compared to HIV-1 M Vpr, and the evidence for a common binding partner seems inconclusive, its utility as a reagent for understanding the function and mechanism of HIV-1 M Vpr may be through its use in dissecting NFκB signaling pathways: since it has a specific effect on NFκB signalling it may prove to be a useful reagent to further characterise this signalling pathway. With this in mind preparations were made to identify relevant interacting proteins using affinity purification followed by quantitative mass spectrometry using stable isotope labeling of amino acids in cell culture (SILAC) (583) (figure 5.11A&B are replicates).

In the SILAC experiment, three cell populations are generated in media with light, medium and heavy isotopes conditions. This allows for direct comparison of protein expression levels by mixing the non-labeled “light” and the labeled “medium” and “heavy” cell populations. Each peptide appears as a pair in MS analysis with a difference in mass, and the relative peak intensities reflect the abundance ratios (584).

HEK293T cells were transfected overnight with plasmids expressing either empty vector, FLAG epitope tagged SIVmon Vpr, FLAG epitope tagged SIVmon Vpr S40N mutant or FLAG epitope tagged SIVgsn Vpr. The expressed proteins were then immunoprecipitated using mouse-anti-FLAG antibody coupled agarose beads and detected in both the input lysate samples and the immunoprecipitate using mouse anti-FLAG antibodies. The light and heavy chains from the mouse anti FLAG immunoglobulin on the beads are also visible on the immunoblot since the secondary antibody used for detection of the anti-FLAG antibody was a fluorophore coupled to an anti-mouse antibody (figure 5.11 A&B).

The main aims of the optimization experiment were to ensure that the protein of interest could be expressed and would immunoprecipitate. Coomassie staining was used as an indicator to determine that enough protein and putative interacting partners immunoprecipitate for mass spectrometry analysis. Additionally, the amount of each Vpr expressed and immunoprecipitated was optimized to have them as similar as possible in order to enable a comparative mass spectrometry analysis and ensure that if a cellular binding partner is found it is not an artifact of better expression and immunoprecipitation. FLAG immunoprecipitation was performed and proteins were stained by coomassie blue after SDS-PAGE but cell populations were not labeled at this stage.

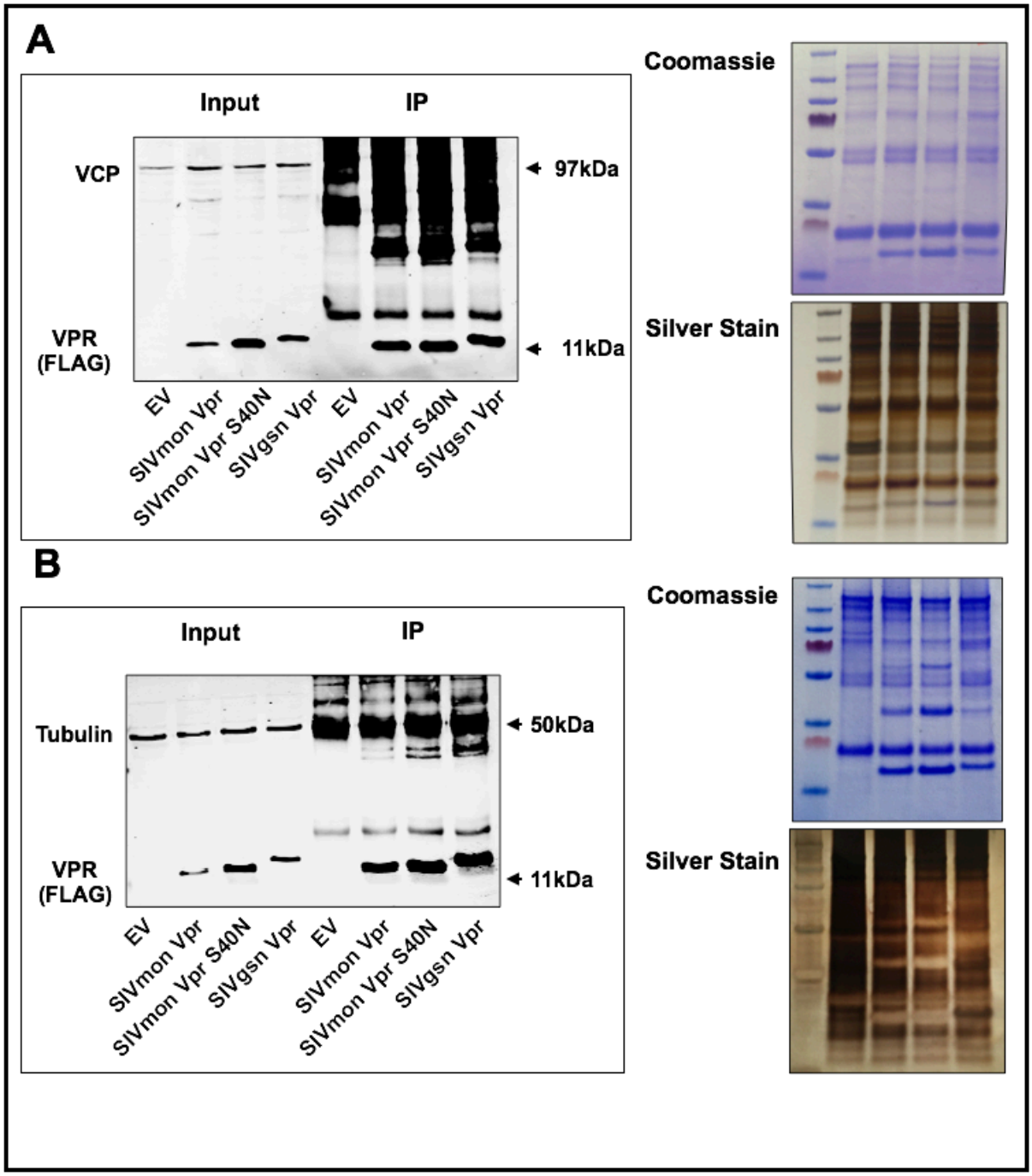

Figure 5.11 Optimization of expression and immunoprecipitation for SILAC. (A&B are replicates) HEK293T cells were transfected overnight with a plasmid expressing FLAG epitope tagged SIVmon Vpr, its S40N mutant or the SIVgsn Vpr or an empty vector control. At 48 hrs the cells were harvested in IP buffer (see chapter 2) and the cleared lysates were incubated with mouse-anti-FLAG antibody coupled to agarose beads to immunoprecipiatate the epitope-tagged proteins. Whole cell lysates (INPUT) and immunoprecipitated proteins (IP) were separated by SDS-PAGE and analysed by immunoblotting with mouse-anti-FLAG, and rabbit-anti-tubulin antibodies as a loading control. The location of protein molecular mass markers is indicated (kDa). IP samples were run on a pre-cast gradient PAGE gel and stained with Coomassie blue and then silver stain.

5.12 Transcriptional signature of SIVmon Vpr

To attempt to characterise the transcriptional signature activated by SIVmon Vpr transfection HEK293T cells were transfected with plasmids expressing SIVmon Vpr, its S40N mutant, the SIVgsn Vpr or an empty vector (pcDNA.3). Cells were harvested for RNA extraction at 48hrs. 12 samples were analysed, 3 replicates each of 4 conditions (SIVmon Vpr, SIVgsn Vpr, SIVmon S40N Vpr, and pcDNA.3). One sample of the SIVgsn Vpr was degraded and excluded. All samples were labeled for gene expression microarrays with fluorophores Cy3 and Cy5 and array images were acquired with Agilent's dual-laser microarray scanner G2565BA. The Agilp Agilent Feature Extraction software was used to extract raw data from the scanner output file and log2-transform median Cy3 and Cy5 signal intensities. These were normalised and only gene symbol–annotated probes expressed above background negative-control levels in at least one sample were analysed.

An initial comparison of SIVmon Vpr to empty vector showed only 126 genes with a >2 fold change in expression (figure 5.12A). This is unlikely to be compatible with a significant activation of NFκB signalling pathways which are central to the regulation of many thousands of genes by many more than 2 fold. Of these only 102 were annotated and of these 102 only 52 showed increased expression (30 had a refseq ID), the remaining 50 showed reduced expression (of which 29 had a refseq ID). These 59 genes were analyzed using gene ontology enrichment analysis, pathway analysis, Transcription binding factor site analysis and Ingenuity pathway analysis (see chapter 2) none of which showed any evidence of NFκB regulatory gene enrichment.

The experiment lacked a technical positive control but more significantly the plasmids were transfected at a dose (16.7ng) that likely only stimulated a small percentage of the cells (figure 5.12B). Early experiments had showed a maximal activation of NFκB signal at low doses with a co-transfected plasmid and so these doses were used in the array. Whilst when using a luciferase assay even a small amount of secreted luciferase may be detected if a gene expression change is seen in only a small number of cells then the change in mRNA signal may be lost in background noise. This hypothesis was tested by transfecting cells with a titration of GFP under control of the same promoter in the same plasmid vector and showing that at the dose used in the array experiment only <20% of cells were likely to be transfected. At the time when the array results were analysed further data on the phenotype of HIV-1M Vpr including nuclear localisation and the effect on co-transfected plasmids called into question the validity of using SIVmon Vpr as an experimental tool to identify the binding partner for HIV-

1 M and so considering the lack of clarity regarding a hypothesis another array was not attempted.

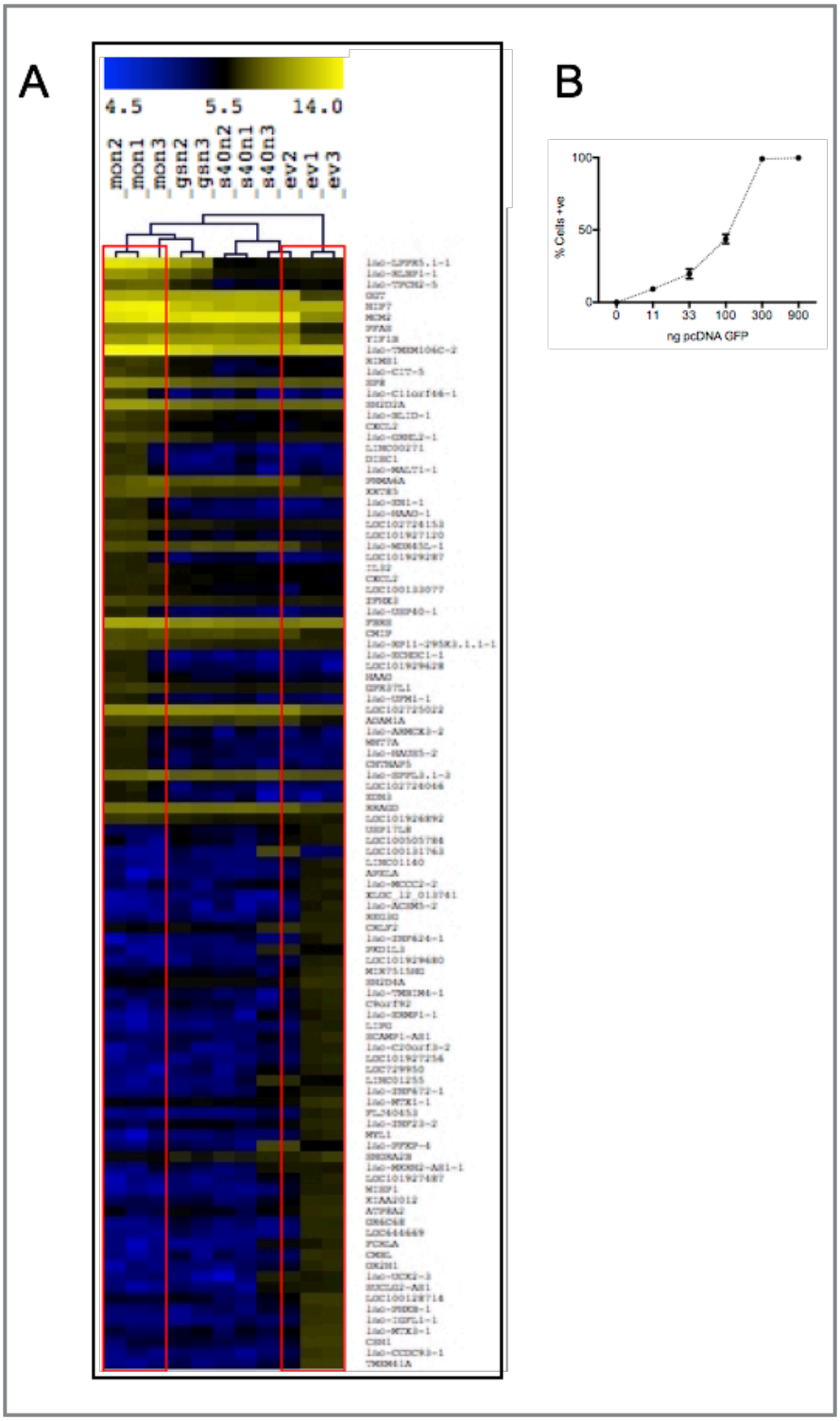

Figure 5.12 Comparison of relative gene expression changes following transfection of SIVmon Vpr (A)126 genes with a >2 fold change in expression in HEK293T cells following SIVmon Vpr transfection compared to empty vector. (B) % cells transfected at different doses of the expression vector used to express SIVmon Vpr.

6 Chapter 6: Discussion

The evasion of host cellular innate immune responses is a requirement that shapes the structure and lifecycle of all viruses. The investigation of the wide variety of strategies that viruses employ to achieve this improves both clinical understanding and treatment of agents of disease, as well as enabling deeper understanding of cellular immune processes since, through a process of real world combinatorial optimisation, viruses have a deep "understanding" of our immune system and how to subvert it.

All primate lentiviruses encode the Vpr accessory protein and, while a number of binding partners and *in vivo* and *in vitro* phentotypes have been observed, an explanation for the universal conservation of Vpr remains elusive. It has proved hard to reconcile, for example, the most biochemically investigated property of Vpr, that it causes G2 arrest (44,438,585–588), with the observation that it enhances a spreading infection in non-dividing cells (428,465,589).

Data from our lab (figure 3.1, Jane Rasaiyaah, unpublished, personal communication) indicate that Vpr is able to rescue a spreading infection in macrophages from the addition of cGAMP, the second messenger in the cGAS mediated cytosolic DNA sensing pathway. It may be that inadvertent activation of this or a related pathway in published experiments accounts for previous published observations that Vpr is able to improve infection efficiency (428,589,590). It was therefore hypothesised that Vpr was antagonising this pathway. Vpr, like other accessory proteins is able to recruit cellular ubiquitin ligases and is packaged inside the virion, both features that would be consistent with it functioning as an early antagonist of innate immune signalling or pattern recognition. An experimental transfection assay was developed to test this.

The cGAS/STING mediated cytosolic DNA sensing pathway was reconstituted in HEK293T cells and was shown to activate NFκB and IRF3 sensing using luciferase reporters and qRT-PCR of endogenous genes. Initial experiments were undertaken with a Vpr from the HIV-1 M YU2 used in the spreading infection experiment. The *Vpr* gene was codon-optimised using a proprietary algorithm for protein expression optimization from Blue Heron Bio and expressed relatively weakly. It was observed to specifically antagonise NFκB without affecting IRF3 signalling which appeared to indicate a specificity of effect (Fig 3.4). Additionally, loss of stimulatory molecules on immunoblot was observed and interpreted as Vpr induced possible degradation. This was a plausible hypothesis in view of published observations that Vpr is able to degrade host proteins SLX4 and UNG (44,384,387,438). However subsequent titration experiments with a re-codon-optimised Vpr gene showed that this was not the case. The much

higher sensitivity of IRF3 sensitive endogenous and reporter genes to activation by low doses of transfected cGAS and STING (or to cGAS and STING reduced to a low dose by Vpr) disguised the fact that the effect on IRF3 signalling was identical to the effect on NFκB signalling. Loss of both signals was subsequently shown to be due to suppression of expression from the co-transfected stimulatory molecules (Figure 4.3).

6.1 Function and Mechanism of HIV-1 M Vpr

There are three main findings of this thesis which have implications both for the potential function and mechanism of Vpr in vivo and for future experimental work.:

1) **Antagonism of expression from co-transfected plasmids:** The observation that ectopically expressed Vpr inhibits expression from all tested co-transfected plasmids encoding a variety of gene products (GFP, cGAS, IKK, TKB1, STING, MAVS, TNFα, renilla luciferase, firefly luciferase and SIV Vpr molecules). This antagonism correlates with localisation to the nuclear rim: breaking nuclear rim localisation breaks the inhibitor activity consistent with an effect regulating nuclear transport.

2) **Antagonism of NFκB activation by TNFα at an integrated reporter:** The observation that HIV-1 M Vpr inhibits TNFα mediated NFκB signalling from an integrated luciferase reporter and that this inhibition is rescued in the same way as the inhibition of expression from transfected plasmids by the Q65R and F34I /P35N mutagenized Vpr.

3) **Activation of NFκB signaling by SIVmon Vpr:** the observation that one of the two sequenced Vprs from the SIVmon lentivirus is able to stimulate NFκB signalling and that this appear to be dependent on the formation of intranuclear puncta.

It may prove possible to reconcile these findings with a single hypothesis – that Vpr regulates nuclear transport. This, in turn, may give insight into both the *in vivo* function of Vpr and mechanisms of nuclear import.

6.1.1 Antagonism of expression from co-transfected plasmids

The effect of Vpr on transcription does not seem to be promoter specific. Vpr antagonises expression of various genes, encoded by various expression plasmids with varied promoters with sensitivities to different transfection factors. Detailed descriptions of the transcriptional regulation of all the promoters used are not available but transcription factor binding information has been demonstrated experimentally and inferred for most of these promoters

and indicates a wide range of transcription factors. An attempt to investigate the effect of co-transfected Vpr on a luciferase construct with an HIV-1 M LTR was considered but previous use of similar constructs requires the co-expression of Tat the expression of which would be suppressed by Vpr (591,592). The range of transcription factors acting at these promoters of diverse origins makes it seem unlikely that the effect of Vpr is due to degradation or inhibition of a specific transcription factor or factors and there was no evidence of a global impairment of transcription (by either objective measurements like Live/Dead stain and trypan blue uptake or more subjective observations like media colour or cell appearance under the microscope). Additionally, the lack of effect on baseline transcription of an integrated reporter of NFκB compared to the >10 fold effect on a co-transfected reporter (fig 4.7) provides evidence that the inhibition of expression is unique to co-transfected DNA.

Remarkably little is known about cellular or nuclear entry of co-transfected DNA despite chemical transfection of nucleic acid into cultured mammalian cells having been first reported over 50 years ago (593,594). A number of different chemical methods have been used for transfection including DEAE-dextran (a cationic polymer), calcium phosphate and synthetic cationic lipids. All chemical transfection methods involve complexes of positively charged chemicals with negatively charged nucleic acids. The negative cell membrane then attracts these positive complexes but precisely how they traverse the membrane is not known. Endocytosis and phagocytosis may be required. Intranuclear localisation is required for transfected DNA to be expressed but this mechanism is also not known (595). This may be due to the proprietary nature of many of the current technologies. The FuGENE® HD transfection reagent (Promega) was used in these assays which has a proprietary non-liposomal formulation. It is hypothesized that the ability of Vpr to block expression from transfected plasmids relates interference with nuclear entry.

6.1.2 Antagonism of NFκB activation by TNFα at an integrated reporter

These effects on transcription would ideally be reconciled with the effect on TNFα signalling at an integrated reporter. The only transfected plasmid in the experiments in figure 4.10 is the Vpr. It is assumed that, since the cells are identical to the other assays, that the previous observations regarding lack of toxicity hold true (and indeed subjective observations showed no indication of toxicity) and so this appears to be a true effect of Vpr on signalling. Importantly the phenotype is abrogated in the same way by the mutants Q65R and F34I/ P35N as the effect on expression from transfected plasmids. This is perhaps the most tantalising finding with relevance to *in vivo* studies indicating a true specificity of effect on NFκB signalling that

appears to be independent of toxicity and transfection. It is possible that there is a common mechanism for inhibiting nuclear import of transfected DNA.

6.1.3 Reconciling the effects on co-transfected plasmids and TNFα signalling

Following observations that Vpr localises to the nucleus, much of the literature describes Vpr as being a regulator of nuclear import of the "pre-integration complex". It has been proposed that it does this in an importin independent way (405), an importin dependent way (488) or in an importin-β independent way (479). Vpr has also been implicated in import of proviral DNA into the nucleus of macrophages using biochemical techniques (596).

A model is proposed here where the defining selection force on many aspects of the viral life cycle is avoidance and suppression of innate immunity. Increasingly the viral capsid core is believed to have a vital role in these functions, concealing reverse transcription, and the nascent genome produced, in a closed environment, shielding it from host factors such as cGAS and TREX1 (see 1.10.1.2). The main evidence for this role of the capsid core comes from the observation that cytosolic DNA sensors are not activated by viral cDNA (170,597) unless there are capsid mutations which disrupt the interaction of capsid with cellular factors (165).

As evidence mounts that the capsid core remains intact until arrival at the nuclear pore the role of Vpr should also be reconsidered. Rather than being part of a "pre-integration complex" it may be that it has a highly local and specific effect at the NPC during or following uncoating. Three proteins involved in nuclear import, transportin-3 (TNPO3), Nup153 and Nup358, have been identified as co-factors for HIV-1 infection in genome wide small interfering RNA (siRNA) or yeast two-hybrid screens (333,598,599) but the mechanism by which the HIV capsid core or pre-integration complex engages these proteins remains unclear. Intriguingly both CA and Vpr have cyclophilin binding domains (165,411,502,503). CypA is has been shown to catalyse the cis/trans isomerisation of the Gly89-Pro90 peptide bond of CA (600) and while the exact effect that this has on the stability of the core has not been elucidated, CypA knockdown, deletion and inhibition all partially restrict infection (506,601). Vpr has also been shown to bind cyclophilin *in vitro* but the link between CypA binding and any of the many observed phenotypes of Vpr remains to be determined. While Vpr and CA have cyclophilin binding domains, Nup358 has a cyclophilin homology domain which has been shown in some studies to interact with CA and induce isomerisation and thus been linked to uncoating (602). Other studies have shown that the Nup358 cyclophilin homology domain is not a requirement for infectivity although this study was in mice and it's not clear that co-factor usage is the same in

mouse cells (603). A 2011 study determined that Vpr CypA binding was stronger than that of CA and CypA (503). Whilst it is not possible at this stage to form a fully coherent hypothesis about the mechanism of Vpr function there are several observations consistent with a role in nuclear transport including the localisation to the nuclear rim, the interactions with known nuclear import pathways and the cyclophillin binding. These all provide further avenues for investigation.

6.2 Future work on HIV-1 M Vpr

An important consideration in the planning and interpretation of future experiments is that Vpr may have an extremely localized effect on a cell. It may be that Vpr works in small doses at highly specific regions of the nuclear rim proximate to an infecting virion core; so its localisation all around the nuclear rim is only seen when it is present in large quantities following over expression. It is possible to imagine a scenario where virion associated Vpr is released in a 'cloud' at the nuclear pore, and prevents signal transduction in close proximity to that particle at the nuclear rim but has no effect on overall nuclear transduction. In this case it would be extremely hard to measure a signal (or loss of signal) that would not be lost in noise. A hint at this idea comes from observations from our lab (unpublished) that whilst Vpr is able to rescue from addition of soluble cGAMP it does not seem to be able to rescue infected cells from capsid mutants believed to trigger DNA sensing (P90A, N74D).

Considering the effect on all tested co-transfected plasmids observed in these studies and in these cells it might be expected that only a limited amount more information could be gained from future co-transfection work that would provide insight into Vpr function.

6.2.1 Transfection Assays

Further investigation of Vpr function would benefit from approaches more closely related to *in vivo* function but stimulated by the exciting finding that HIV-1 M Vpr does seem able to modulate an integrated NFκB reporter following activation mediated by TNFα. I would like to characterize in detail the effect of HIV-1 M Vpr and a series of mutants (described in table 4.2) on the stimulatory effect of a variety of added, soluble stimuli on a range of integrated reporters in transfectable cells lines. Integrated reporters in HEK293T cells could be tested using a variety of stimuli including RNA viruses, IFN-β and cGAMP.

6.2.2 Vpr expressing lentiviral vectors

To investigate the effect of Vpr on primary cells or non-transfectable cell lines it may be possible to make vesicular stomatitis virus glycoprotein (VSV G) pseudotyped lentiviral vectors

containing a genome encoding Vpr driven by a strong promoter. This process may be complicated by the effect of Vpr on co-transfected plasmids as it would be expressed from the genome construct to be packaged in the vector.

VLPs bearing Vpr could be used to take the same approach as was used to investigate the mechanism of action of Vpx (368,369). The effect on a variety of integrated reporters using a variety of stimuli in a range of less transfectable cell lines (pIFIT1-GLuc THP-1 reporter cells for example) and possibly primary cells could be studied. This approach would have the advantage of being quantifiable in terms of the numbers of cells infected with VLPs and might this make experiments examining effects on phosphorylation or translocation of transcription factors or other signalling molecules possible.

It would also be possible to assess the effect of transfected Vpr on vector transduction – whether Vpr blocks a transduced genome expression in the same way as it blocks expression from transfected DNA for example. As well as giving insight into the function of Vpr this approach might also contribute understanding to mechanisms of nuclear import in transfection.

These experiments would help to inform other studies using full length virus in primary cells including CD4+ T cells and monocyte derived macrophages. Previously published experiments have not focussed on the ability of Vpr to rescue infection from innate immune stimuli. An approach where cells are activated with a range of PAMPS or immune stimuli and Vpr is tested for an ability to recue infection could be followed by genetic depletion studies to identify the partner molecules following an unbiased approach using SILAC.

6.2.3 Microscopy

Optimisation of confocal microscopy of stained DNA plasmids might enable detection of differences in localisation that could be quantified. Use of the existing automated platform microscopes is complicated by the need to determine whether a stained plasmid is intra-nuclear or merely “in front of” or “behind” the nucleus. Automated high resolution microscopy platforms were tried including the WiScan Hermes and the Perkin Elmer Operetta but these both proved unable to resolve localisation of stained DNA. In order to measure whether Vpr had an effect on the physical localisation of transfected DNA a number of variables in this experiment would need to be optimised including use of confocal microscopy, Vpr expression labelling and changes in dose and intensity of plasmid dyes in order to get useful information about the effect of Vpr.

Whilst the observations of stained plasmid after transfection (figure 4.9) were not able to resolve a difference in the intracellular position of stained plasmid when expressed with either HIV-1 M Vpr or empty vector they did imply that every cell contained plasmid although not every cell was expressing GFP. This is consistent with the notion that transfection efficiency may have nuclear entry as the rate limiting step. A technique like fluorescence *in situ* hybridization (FISH), using a labelled probe to identify nuclear plasmid might give greater resolution.

6.2.4 Reporter plasmid circularisation assay

Biochemical techniques might prove more successful. A linearised plasmid with a promoter separated from a reporter would be expected to be circularised on entry to the nucleus by the cellular non-homologous end joining pathway. A reduction in re-circularised plasmids and thus reporter expression on co-transfection of Vpr would provide evidence of a block to nuclear entry of transfected DNA.

6.2.5 Effects on NFκB subunit localisation

It has not proved possible to observe effects on NFκB subunit translocation or phosphorylation in HEK293T cells using either immunoblotting or confocal microscopy. This is possibly due to difficulty in timing these experiments: transfected cells do not seem to have NFκB stimulation activated simultaneously. A luciferase assay works because of the relatively stable nature of luciferase which therefore accumulates but the translocation of NFκB subunits may be transient and thus impossible to capture by fixing cells at a particular time point (either in a lysate or on a microscope slide) when those cells are asynchronously expressing the stimulating protein. Luciferase assays are further complicated by the fact that it is not possible to tell how many cells are taking part in the experiment except by indirect means. If only a small number of cells are taking part then it may be that effects on activation of signalling molecules are lost in background noise as is hypothesised to have been the case with the micro-array investigation of SIVmon Vpr.

6.2.6 Future species specificity characterisation

A panel of Vprs representing all six major primate lentiviral lineages was constructed and tested against a variety of expression vectors encoding differing signalling molecules and luciferase reporters with differing promoters. It was observed that there was a conserved pattern of inhibition (or lack of inhibition) across all pathway stimulating molecules (cGAS/STING, MAVS, IKK and TBK-1) apart from TNFα signalling which was unaffected. The

significance of this being an added rather than a transfected molecule did not become apparent immediately.

These experiments were designed to identify the level in the pathway at which Vpr is acting and, while with hindsight they were unable to do this, they are not uninformative, acting essentially as replicates of a single experiment. The differential phenotype observed between each species' Vpr is not of course related to specific degradation of signalling molecules but reduced expression from the transfected plasmids however the same approximate pattern is seen across the Vpr panel with each stimulatory molecule.

The high sequence variability of Vpr together with the lack of a binary phenotype mean it is not possible to make inferences from examination of any single primate Vpr. An alignment shows there to be no specific region or site, no deletion or insertion that confers or abrogates the phenotype.

The analysis is further complicated by the number of plasmids in the experiments in figures 3.6-3.14 that are affected. At the doses of Vpr and the renilla luciferase reporter that were used, the effects on renilla luciferase expression were small but present nonetheless present. As well as the loss of expression of the molecules which are activating NFκB signalling there is also a direct effect on the NFκB sensitive luciferase reporter itself as can be seen in figure 4.6.

Further insights into species specificity will be gained using non-human primate cell lines. African green monkeys (Chlorocebus) are the only genus of non-human primates naturally infected with SIV for which laboratory cell lines are readily available and commonly used and as such provide an opportunity for molecular investigation of this phenotype. Accordingly, an attempt was made to reconstitute the signalling pathway described in figure 3.2 in 4 separate cell lines derived from African green monkeys (Vero, BSC-1, COS-1, MA104). Despite attempts with a matrix of different stimulatory molecules, transfection reagents and reporter genes no luciferase or PCR signal could be activated against which to test an antagonism by Vpr. Part of this lack of signal was attributable to lower transfection efficiency of AGM cell lines than the HEK293T cells. Transfection of the AGM cell lines with a plasmid encoding GFP indicated an 10-20 fold reduced expression of GFP compared with HEK293T as indicated by both mean fluorescence intensity and transfected cell number. But it is also possible that the lack of detectable signal was due to incompatibility of human innate immune signalling proteins with other pathway molecules in AGM cells although these proteins are generally well conserved. Members of pathways involved in detection and restriction of microorganism infection are likely to be under strong selection pressure and it may be that the ~30 million

year evolutionary distance between African green monkeys and humans would prevent interaction between human and monkey pathway molecules (604). At the time that these studies were being performed a complete genome of an African green monkey species was not available but had it been possible to identify the binding partner or partner of HIV-1 M Vpr then it would have been possible to sequence and clone pathway members from these cells. Although it is now understood that the tested innate immune molecules were not being specifically degraded this approach could still be used in order to test species specificity following the identification of a putative nuclear rim binding partner. This will be facilitated by the publication of a complete genome from *Chlorocebus aethiops* has now been published (604).

If AGM cell lines prove sufficiently transfectable then a transfection assay using costitutively expressed luciferase reporter constructs could be used to determine differential antagonism of expression by SIVagm Vpr and HIV-1 M Vpr. It might also prove possible to visualise FLAG tagged Vpr at the nuclear rim using confocal microscopy and assess species specific nuclear localisation of HIV and SIVagm in AGM cells and human cells. Since the SIVagm Vprs tested (SIVver, SIVtan) fail to antagonise expression from co-transfected plasmids in human cells it could be hypothesised that they would not be expected to localise to the nuclear envelope. Conversely if this localisation of Vpr is linked to an evolved function of Vpr then it would be expected to be observed in AGM cells. Further mutational analyses could be combined with biased and unbiased immunoprecipitation approaches to try to identify a binding partner in cell lines of green monkey origin.

6.3 SIVmon Vpr

Studies on the SIVmon Vpr were undertaken to provide insight into both the function and mechanism of HIV-1 M Vpr, and also to characterize the NFκB signalling pathway. It was hypothesised that it would show the same intracellular localisation, and similar responsiveness to co-factor depletion and that this might enable determination of a binding partner for HIV-1 M Vpr. Using the HIV-1 M Vpr itself was complicated by the requirement for overexpression of stimulatory molecules none of which it seemed to associate with.

SIVmon Vpr was observed to activate NFκB dependent luciferase expression >10 fold whilst suppressing expression from an ISG56 promoter and a HSV thymidine kinase promoter >10 fold. These effects are unique to the SIVmon AY340701. The only other SIVmon sequence (AJ549283) and the other Vprs from the mona monkey group (SIVgsn and SIVmus) are inactive for signalling. This would seem to make it unlikely that the observed effect has been selected for – there is no reason to suppose that SIVmon Vpr has been under a selection

pressure in humans or other apes to activate NFκB signalling. Although SIVcpz is likely a recombinant of SIVrcm and SIVgsn (from the mona group) the Vpr in SIVcpz appears to have greater sequence homology with SIVrcm. It is therefore hypothesised that SIVmon Vpr may provide a useful experimental reagent rather than providing insight into the *in vivo* effects of SIVmon Vpr in its host species.

The activation of luciferase expression from an NFκB sensitive promoter is abrogated by an S40N mutant which, in a structural alignment, appears analogous to the residue of the P35N cyclophilin binding mutant in HIV-1 M Vpr. This initially appeared to bolster the hypothesis that SIVmon was acting at the same site in the pathway. However, despite a small but significant effect observed following depletion of CypA, overexpression and depletion studies using Nup358cyp, CypA and Nup358 were inconclusive. Immunoprecipitation experiments also failed to produce a binding partner using a biased overexpression approach.

Confocal microscopy showed a clear difference in localisation of SIVmon compared to HIV-1 M Vpr and the SIVmon S40N mutant and SIVgsn. SIVmon Vpr forms discrete intranuclear puncta or dots. This localisation pattern resembles that of promyelocytic leukaemia protein nuclear bodies (PML-NBs). These are nuclear structures which have been implicated in a range of nuclear functions including the activation of NFκB signalling pathways via the DNA damage response (DDR) pathway (605,606): following DNA damage, DNA double strand breaks (DSBs) increase the number and size of PML-NBs. They have been proposed to function as DNA damage sensors (607) and both DNA repair and checkpoint proteins dynamically co-localise with PML-NBs potentially enabling them to function as catalytic surfaces (605).Several kinases which trigger NFκB activation following DNA damage localise to the PML-NBs including ataxia telangiectasia-mutated protein kinase (ATM) and Rad3 related kinase (ATR) (608,609).

As discussed in section 1.9.2.5, NFκB acts as a cellular stress sensor. It can be activated by a wide range of cellular stresses (610,611) but the details of the signalling pathways which activate NFκB following activation of the DNA damage response remain poorly defined. In all NFκB signalling pathways the activating stimulus activates an IKK complex (IKKβ, IKKα, and NEMO), which phosphorylates IκB proteins. Phosphorylation of IκB leads to its ubiquitination and proteasomal degradation, freeing NF-κB/Rel subunit complexes. These subunits undergo further modification by phosphorylation, acetylation and ubiquitination then translocate to the nucleus to induce target gene expression. Activation of NFκB by the DNA damage response requires activation of ATM and nuclear accumulation of the adaptor protein NFκB essential modulator (NEMO) in PML-NBs (611,612). Here NEMO associates in a complex with ATM

and subsequently translocates to the cytoplasm allowing activation of the IκB kinase (IKK) complex. This in turn mediates phosphorylation and subsequent proteasomal degradation of IkB and NFkB subunit release (613).

Localisation of SIVmon Vpr to PML-NBs could be confirmed using microscopy in HELA cells expressing PML-YFP. If it is shown that there is co-localisation then the studies of the DNA damage response and PML dependent NFκB activation provide a useful starting point for potential SIVmon VPr interaction.

The low doses used for these confocal microscopy pictures were the same as activated the maximum amount of expression from a co-transfected reporter. Addition of more plasmid reduced image quality due to excessive staining in cells. Additionally, the signal from the reporter plasmid was diminished at higher doses (see figure 5.2 and 5.5). If SIVmon Vpr is directly binding NFκB promoters then it may be that it activates in a non-linear fashion. When an integrated reporter was tested the NFκB response is dose dependent. Remarkably little is known about the mechanics of transfection but it is believed that DNA forms complexes with transfection reagents such that each transfected cell gets all co-transfected plasmids. Therefore the mathematical relationship between the number of SIVmon Vpr molecules expressed “per reporter” may be very different between integrated vs co-transfected reporters.

The relatively low dose of Vpr (16.7ng – a number arrived at by using a three fold dilution series starting at 150ng)) that maximally activated a co-transfected NFκB reporter gene was used for confocal microscopy and also in the expression array in figure 5.12. Following the lack of changes in gene expression seen with the array it was subsequently determined that while a dose of 16.7ng maximally activated a co-transfected reporter it in fact meant that <20% of cells were transfected – few enough that even large changes in gene expression might not have been detected. Repetition of this array with a positive control (say TNFα stimulation) and a higher dose of Vpr ensuring transfection of close to 100% of cells (150-300ng) would be the first step in determining the gene expression profile change induced by SIVmon Vpr. Following this, unbiased quantitative mass spectrometry using SILAC may be used to identify a binding partner.

6.4 Conclusion

This thesis presents evidence for two effects of HIV-1 M Vpr. Firstly, it inhibits expression from co-transfected plasmids in HEK293T cells, independent of promoter or the gene being expressed (likely by inhibiting plasmid nuclear transport). Secondly it inhibits NFκB signalling from an integrated reporter in HEK293T cells following addition of soluble TNFα. Both effects are dependent on nuclear rim localisation and are partially abrogated by Q65R mutants and completely abrogated by the double mutant F34I/P35N. They may stem from an interaction which implicates Vpr as having a role in innate immune signalling. Work to further characterise species specificity and the phenotype outside of a transfection assay may provide insights into the function of Vpr *in vivo* and provide 'drugable' targets in this universally conserved lentiviral accessory protein.

7 Appendix of tables

Table 7.1 Plasmids used in this study

Plasmid name	Description
IFNβ-LUC	IFNβ promoter fused with firefly luciferase, AmpR, obtained from A. Bowie
ISG 56 LUC	ISG-56 promoter fused with firefly luciferase, AmpR, obtained from A. Bowie
ISRE-LUC	ISRE responsive promoter fused with firefly luciferase, AmpR, obtained from A. Bowie (Trinity College Dublin)
M5P Ren LUC	MLV LTR promoter fused with renilla luciferase obtained from R.Sumner
NF-κB-LUC	NF-κB responsive promoter fused with firefly luciferase, AmpR (Promega)
pcDNA +/- FLAG HIV-1 M CH77 Vpr	Accession No JN944909 (535) constructed by C. van Tulleken
pcDNA +/- FLAG HIV-1 M SUMA Vpr	Accession No JN944928 (535) constructed by C. van Tulleken
pcDNA +/- FLAG HIV-1 M SUMA Vpr F34I P35N	Accession No JN944928 (535) bearing F34I P35N mutations
pcDNA +/- FLAG HIV-1 M SUMA Vpr Q65R	Accession No JN944928 (535) bearing Q65R mutation
pcDNA +/- FLAG HIV-1 M WITO Vpr	Accession No AY835451(614) constructed by C. van Tulleken
pcDNA +/- FLAG HIV-1 M YU2(a+b) Vpr	Accession No M93258 (615) constructed by C. van Tulleken
pcDNA +/- FLAG HIV-1 N Vpr	Accession No AY532635 (616) constructed by C. van Tulleken
pcDNA +/- FLAG HIV-1 O Vpr	Accession No AB485666 constructed by C. van Tulleken

pcDNA +/- FLAG HIV-2 A Vpr	Accession No PJK7312AS constructed by C. van Tulleken
pcDNA +/- FLAG SIVcpz*Pts*1 Vpr	Accession No AF447763 (38) constructed by C. van Tulleken
pcDNA +/- FLAG SIVcpz*Pts*2 Vpr	Accession No DQ374658 (38) constructed by C. van Tulleken
pcDNA +/- FLAG SIVcpz*Ptt*1 Vpr	Accession No: DQ373063 (617) constructed by C. van Tulleken
pcDNA +/- FLAG SIVcpz*Ptt*2 Vpr	Accession no. X52154 (39) constructed by C. van Tulleken
pcDNA +/- FLAG SIVdeb Vpr	Accession No AY523866 (618) constructed by C. van Tulleken
pcDNA +/- FLAG SIVden Vpr	Accession No AJ580407 (619) constructed by C. van Tulleken
pcDNA +/- FLAG SIVgor Vpr	Accession No FJ424865(620) constructed by C. van Tulleken
pcDNA +/- FLAG SIVgsn Vpr	Accession No AF468658 (32) constructed by C. van Tulleken
pcDNA +/- FLAG SIVlst Vpr	Accession No AF188115 constructed by C. van Tulleken
pcDNA +/- FLAG SIVmon1 Vpr	Accession No AY340701 (32) constructed by C. van Tulleken
pcDNA +/- FLAG SIVmon1 Vpr S40N	Accession No AY340701 (32) bearing S40N mutation
pcDNA +/- FLAG SIVmon2 Vpr	Accession No AJ549283 (619) constructed by C. van Tulleken
pcDNA +/- FLAG SIVmus Vpr	Accession No AY340700 (32) constructed by C. van Tulleken
pcDNA +/- FLAG SIVolc Vpr	SIVolc Vpr Accession No FM165200 constructed by C. van Tulleken
pcDNA +/- FLAG SIVrcm Vpr	Accession No AF3496801(542) constructed by C. van Tulleken
pcDNA +/- FLAG SIVsmm Vpr	SIVsmm Vpr Accession No AF077017 constructed by C. van Tulleken
pcDNA +/- FLAG SIVsyk Vpr	Accession No AY523867 (618) constructed by C. van Tulleken

pcDNA +/- FLAG SIVtal Vpr	Accession No AY655744 (621) constructed by C. van Tulleken
pcDNA +/- FLAG SIVtan Vpr	Accession No U58991(425) constructed by C. van Tulleken
pcDNA +/- FLAG SIVver Vpr	Accession No DJ048201 (622) constructed by C. van Tulleken
pcDNA3 MAVS	MAVS with no tag constructed by C.P. Tan
pcDNA3.1 FLAG	CMV promoter, SV40 origin for episomal replication, Ampicillin resistance gene and pUC origin for selection and maintenance in *E. coli. (*Thermo-Fisher), FLAG epitope tag inserted by C.P.Tan.
pcDNA3.1 FLAG IKKβ	IKKβ with N-terminal FLAG tag, AmpR. via R. Sumner (623)
pcDNA3.1 FLAG TBK1	TBK1 with N-terminal HA tag, AmpR, constructed by C. van Tulleken
pcDNA3.1 FLAG TNFα	TNFα with N-terminal FLAG tag from Made by C.P. Tan
pcDNA3.1 FLAG-TRAF3	TRAF-3 with N-terminal FLAG tag, AmpR, constructed by R. Sumner
pcDNA3.1 GFP	GFP untagged. Constructed by C.P.Tan
pcDNA3.1 HA cGAS	Human cGAS with N-terminal FLAG tag, AmpR. Made by C.P.Tan
pcDNA3.1 HA STING	Human STING with N-terminal FLAG tag, AmpR. Made by C.P.Tan
pCSGW	pHR'SIN-cPPT-SGW, self-inactivating HIV-1-derived vector encoding GFP from an SFFV promoter
pDUAL SFXU Luc	HIV-1 vector construct with a Ubiquitin B promoter fused with Renilla luciferase constructed by David Stirling/Yasu Takeuchi
pGL4 CMV LUC	CMV promoter fused with firefly luciferase obtained from R.Sumner
pGL4 NFκB LUC	5 copies of an NF-κB response element in promoter fused to firefly luciferase (Promega).

pGL4 SV40 Ren LUC	SV40 promoter fused with Renilla luciferase obtained from R.Sumner
PLG4 CMV Ren LUC	CMV promoter fused with Renilla luciferase obtained from R.Sumner
pMD.G	Encodes envelope protein G of the vesicular stomatitis virus under control of CMV promoter (511)
SFFV LUC	MLV LTR promoter fused with renilla luciferase obtained from R.Sumner
TK-Renilla-LUC	TK promoter fused with renilla luciferase, AmpR, (Promega)

Table 7.2 Primers for qRT-PCR

Primer	Sequence 5'-3'
Human GAPDH FWD	5' ACCCAGAAGACTGTGGATGG 3'
Human GAPDH REV	5' TTCTAGACGGCAGGTCAGGT 3'
Human CCL-5 FWD	5' CCCAGCAGTCGTCTTTGTCA 3'
Human CCL-5 REV	5' TCCCGAACCCATTTCTTCTCT 3'
Human IFIT-2 FWD	5' GGAGGGAGAAAACTCCTTGGA 3'
Human IFIT-2 REV	5' GGCCAGTAGGTTGCACATTGT 3'
Human CXCL-10 FWD	5′-GAACTGTACGCTGTACCTGCA-3′
Human CXCL-10 REV	5′-TTGATGGCCTTCGATTCTGGA-3′
Human ISG-56 FWD	5′-CCTGGAGTACTATGAGCGGGC-3'
Human ISG-56 REV	5′-TGGGTGCCTAAGGACCTTGTC-3'
Human STING FWD	5′- AACACCGGTCTAGGAAGCAG-3′
Human STING REV	5′-CATATTTGGAGCGGTGACCT-3′
Human cGAS FWD	5' GGGAGCCCTGCTGTAACACTTCTTAT 3'
Human cGAS REV	5' CCTTTGCATGCTTGGGTACAAGGT 3'

Table 7.3 SDM primer sequences

Vpr Mutation	Plasmid	Sequence 5'-3'
SIVmon S40N FWD	pcDNA3	CATCCTGTACCGGCGGTACAGAGAGGGCTG
SIVmon S40N REV	pcDNA3	CAGCCCTCTCTGTACCGCCGGTACAGGATG
HIV-1 M Q65R FWD	pcDNA3	GTACCGGCACTACAGCGAGGGCTGCTACCAC
HIV-1 M Q65R REV	pcDNA3	GTGGTAGCAGCCCTCGCTGTAGTGCCGGTAC
HIV-1 M F34I P35N	pcDNA3	CAGTAAGACATTTCTCGAGACCCTGGCTTC
HIV-1 M F34I P35N	pcDNA3	GAAGCCAGGGTCTCGAGAAATGTCTTACTG

Table 7.4 Antibodies used in this study

Antibody	Source	Dilution
Rabbit-anti-β-actin	Sigma (A2066)	1:1000
Mouse-anti-FLAG	Sigma (F1804)	1:1000
Rabbit-anti-HA	Sigma (H6908)	1:1000
Mouse-anti-HA	Cambridge Bioscience (MMS-101P)	1:200
Rabbit-anti-VCP	Ab109240 Abcam®	1:1000
Mouse-anti-Actin	Ab6276 Abcam®	1:20,000
Rabbit-anti-Nup358	C288 Catavas.E	1:2000
Mouse-anti-STING	Ab92605	1:1000

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