Feline restriction factors to lentiviral replication

Isabelle Dietrich

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MRC-University of Glasgow Centre for Virus Research
Institute of Infection, Immunity and Inflammation
College of Medical, Veterinary and Life Sciences
University of Glasgow

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Author's declaration

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

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Abstract

Strong adaptive evolutionary forces shape the interactions between pathogens and their hosts and typically lead to a stable co-existence. In this process of co-evolution, mammals have developed restriction factors that limit retrovirus infectivity, replication or assembly and narrow the spectrum of potential host species. These restriction factors are either constitutively expressed, such as APOBEC3 proteins, cytidine deaminases that interfere with reverse transcription, or form part of the type I interferon-induced innate immunity, such as TRIM5, a member of the tripartite motif protein family that induces degradation of retroviral capsid, blocks reverse transcription, or tetherin (BST-2, CD317), which inhibits release of nascent viral particles from infected cells. Conversely, viruses have evolved antagonists of restriction factors or proteins that limit IFN-induced gene expression, thus evading immune surveillance. The interaction between host and viral components is delicately balanced and has a significant impact on disease outcome.

Feline immunodeficiency virus (FIV), a lentivirus closely related to human immunodeficiency virus (HIV), is a recent introduction into domestic cats and causes an immunodeficiency syndrome analogous to human AIDS. Interestingly, non-domestic cats such as lion or pumas have co-existed with lentiviruses for prolonged periods of time and FIV infections are largely benign. Although plasma viral and proviral loads are high in both domestic and non-domestic cats, *in vitro* studies have shown that FIV infection of non-domestic cat T lymphocytes is significantly less efficient than that of domestic cat T cells. Thus, this thesis tests the hypothesis that the differential disease outcome of FIV infections in felids is caused by differences in lentiviral restriction factor activities or their sensitivities to FIV restriction factor antagonists.

Data presented in this study show for the first time that feline APOBEC3 proteins are expressed in tissues and cell types relevant for FIV infection. The APOBEC3 proteins A3H and A3CH exhibited a high antiviral activity against FIV lacking the APOBEC3 antagonist Vif in single-cycle replication assays, with no difference in activity being detected between domestic and non-domestic cat proteins. However, domestic cat A3CH was significantly more sensitive to antagonism by FIV Vif than lion or puma A3CH, which would allow efficient viral

replication in domestic cat T lymphocytes and subsequently lead to T cell loss and immunodeficiency.

Furthermore, this thesis provides evidence that felid tetherins can prevent FIV particle release from producer cells in single-cycle replication assays; however, stable expression of domestic and non-domestic cat tetherins in feline cell lines did not abrogate FIV replication. Indeed, syncytium formation indicative of viral cell-to-cell spread was significantly enhanced in type I interferon-treated feline cells infected with CD134-independent strains of FIV which often arise in chronic (late) stages of FIV infections *in vivo*.

Finally, this work reports the generation of a synthetic domestic cat TRIM5α-cyclophilin A fusion protein which was highly efficient at preventing FIV pseudotype and productive infection. This novel feline restriction factor represents a potent antiviral defence agent with very low potential for toxicity and could in future be used in gene therapy approaches to treat FIV-infected cats.

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List of Abbreviations

Abbreviation	Meaning
3‘ PPT	3’ poly purine tract
3’ LTR	3’ long-terminal repeat
5’ LTR	5’ long-terminal repeat
A3	APOBEC3
Agm	African green monkey (*Chlorocebus sabaeus*)
AIDS	Aquired immunodeficiency syndrome
AP	Alkaline phosphatase
AP-1	Activator protein-1
APC	Antigen-presenting cell
APOBEC3	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3
ATF2	Activating transcription factor 2
BIV	Bovine immunodeficiency virus
bp	Base pair
CA	Capsid

CAEV	Caprine arthritis/encephalitis virus
CA^N	N-terminal domain of the capsid
CC domain	Coiled-coil
CD	Catalytic domain
cDC	Conventional dendritic cell
cDNA	Complementary deoxyribonucleic acid
CLL cells	Canine chronic lymphocytic leukaemia cells
Clu	Dog (*Canis lupus familiaris*)
CMV	Cytomegalovirus
CON	Control
CP domain	Cytoplasmic domain
CpG	Cytidine-phosphate-guanosine
cPPT	Central poly purine tract
CrFK cells	Crandell feline kidney cells
CRM-1	Cellular exportin-1
CsA	Cyclosporin A
CTL	Cytotoxic T lymphocyte
CTS	Central termination sequence
CypA	Cyclophilin A
DAPI	4',6'-diamidino-2-phenylindole
DC	Dendritic cell

DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-grabbing non-integrin
DC-SIGNR	Dendritic cell-specific intercellular adhesion molecule-grabbing non-integrin-related
DMEM	Dulbecco's modification of Eagle's medium
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
dsRNA	Double-stranded ribonucleic acid
dUTPase	Deoxyuridine triphosphate nucleotidohydrolase
EIAV	Equine infectious anemia virus
Env	Envelope glycoprotein
ER	Endoplasmic reticulum
ESCRT	Endosomal sorting complex required for transport
FBS	Fetal bovine serum
Fca/Fe	Domestic cat (*Felis catus*)
FeFV	Feline foamy virus
FeLV	Feline leukaemia virus
FFU	Focus forming units
FITC	Fluorescein isothiocyanate
FIV	Feline immunodeficiency virus
FIV-Aju	FIV of cheetahs (*Acinonyx jubatus*)
FIV-Ccr	FIV of spotted hyenas (*Crocuta crocuta*)

FIV-Fca	FIV of domestic cats (*Felis catus*)
FIV-Hya	FIV of jaguarundi (*Herpailurus yagouaroundi*)
FIV-Lpa	FIV of ocelots (*Leopardis pardalis*)
FIV-Lru	FIV of bobcats (*Lynx rufus*)
FIV-Oma	FIV of Pallas's cats (*Otocolobus manul*)
FIV-Pco	FIV of North American pumas (*Puma concolor*)
FIV-Ple	FIV of lions (*Panthera leo*)
FIV-Ppa	FIV of leopards (*Panthera pardus*)
Gag	Group-specific antigen
GAS	Interferon-gamma-activated site
gDNA	Genomic deoxyribonucleic acid
GFP	Green fluorescent protein
GPI	Glycosylphophatidylinositol
HA	Haemagglutinin
HERV	Human endogenous retrovirus
HFV	Human foamy virus
HIV-2	Human immunodeficiency virus type 2
Hsa/Hu	Human (*Homo sapiens*)
I domain	Gag-Gag interaction domain
IFITM	Interferon-induced transmembrane (protein)
IFN	Interferon

IFNAR	Type I interferon receptor/Interferon-alpha receptor
IKKε	IkappaB kinase epsilon
IL-2	Interleukin-2
ILT7	Receptor immunoglobulin-like transcript 7
IN	Integrase
Inr	Initiator
IP	Internal promoter
IPC	Type I interferon-producing cell
IRAK1	Interleukin-1 receptor-associated kinase 1
IRF	Interferon regulatory factor
ISGF3	Interferon-stimulated gene factor 3
ISP-1	Interferon-beta promoter stimulator-1
ISRE	Interferon-stimulated response element
JAK	Janus activated kinase
L domain	Late domain
LB	Lysogeny broth
LEDGF/p75	Lens epithelium-derived growth factor/transcriptional co-activator p75
LNC	Lymph node cell
Lv1	Lentivirus susceptibility factor 1
M domain	Membrane binding domain

MA	Matrix
MHC	Major histocompatibility complex
MLV	Murine leukaemia virus
MLV-N	Murine leukaemia virus N-strain
MMTV	Mouse mammary tumour virus
mtDNA	Mitochondrial deoxyribonucleic acid
MYA	Million years ago
NC	Nucleocapsid
Nef	Negative factor
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLS	Nuclear localisation signal
NPC	Nuclear pore complex
nt	Nucleotide
Om	Owl monkey (*Aotus trivirgatus*)
OMK cells	Owl monkey kidney cells
ORF	Open reading frame
OWM	Old World monkey
p.i.	Post-infection
PAMP	Pathogen-associated molecular pattern

PBMC	Peripheral blood mononuclear cell
PBS	Primer binding site/Phosphate-buffered saline
Pco	Puma (*Puma concolor*)
pCTL	Precursor cytotoxic T lymphocyte
pDC	Plasmacytoid dendritic cell
PDC	Pseudocatalytic domain
PHA	Phytohaemagglutinin
PIC	Pre-integration complex
PKR	Protein kinase R
Ple	Lion (*Panthera leo*)
pNP	Para-nitrophenol
pNPP	Para-nitrophenylphosphate
Pol/pol	Polymerase
PPR	Pattern recognition receptor
PPT	Poly purine tract
PR	Protease
$Pr55^{Gag}$	Gag precursor polyprotein
P-TEFb	Positive transcription elongation factor B
RBCC domain	RING-B-box2-coiled-coiled domain
Ref1	Resistance factor 1
Rev	Regulator of virus gene expression

Rh	Rhesus macaque (*Macaca mulatta*)
RIG-I	Retinoic acid-inducible gene I
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RRE	Rev Responsive Element
rRNA	Ribosomal ribonucleic acid
RT	Reverse transcriptase
RTC	Reverse transcription complex
RT-qPCR	Quantitative real-time reverse transcription PCR
SCF	Skp1-cullin1-F-box
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIV	Simian immunodeficiency virus
SIVagm	SIV of African green monkeys (*Chlorocebus sabaeus*)
SIVagm.Sab	SIV of sabaeus monkeys (*Chlorocebus sabaeus*)
SIVagm.Tan	SIV of Tantalus monkeys (*Chlorocebus tantalus*)
SIVagm.Ver	SIV of vervet monkeys (*Chlorocebus pygerythrus*)
SIVcpz	SIV of chimpanzees (*Pan troglodytes*)
SIVgsn	SIV of greater spot-nosed monkeys (*Cercopithecus nictitans*)
SIVmac	SIV of rhesus macaques (*Macaca mulatta*)
SIVmon	SIV of Mona monkeys (*Cercopithecus mona*)
SIVmus	SIV of mustached monkeys (*Cercopithecus cephus*)

SIVsmm	SIV of sooty mangabeys (*Cercocebus atys*)
SIVsyk	SIV of Sykes' monkeys (*Cercopithecus albogularis*)
SP1	Specificity Protein 1
ssRNA	Single-stranded RNA
STAT1	Signal transducer and activator of transcription 1
SU	Surface
TAR	Transactivation response element
Tat	Transactivator of transcription
TBK-1	Tank-binding kinase 1
TGN	Trans-Golgi network
THN	Tetherin
TLR	Toll-like receptor
TM	Transmembrane
TNFα	Tumour necrosis factor alpha
TNFR/NGFR	Tumour necrosis factor receptor/nerve growth factor receptor
TNPO3	Transportin-3
TRAF6	Tumor necrosis factor receptor-associated factor 6
Tregs	T lymphocytes with regulatory function
TRIF	Toll/interleukin-1 receptor domain-containing adaptor inducing interferon-beta
TRIM	Tri-partite motif

TRIMCyp	TRIM5α-cyclophilin A
$tRNA^{Lys}$	Lysine transfer ribonucleic acid
TYK2	Tyrosine kinase 2
UNG	Uracil N-glycosidase
UTR	Untranslated region
Vif	Virus infectivity factor
VLPs	Virus-like particles
Vpr	Viral protein R
Vpu	Viral protein U
VS	Virological synapse
VSV-G	Vesicular stomatitis virus glycoprotein
βTrcP	Beta-transducin repeat-containing protein

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1 Introduction

1.1 Retroviruses

Viruses are obligate intracellular pathogens that are dependent on the cellular transcriptional machinery to replicate their genomes and on a wide array of host factors to express viral proteins and to assemble viral particles (virions). Virions are the extracellular forms of viruses that are suited for transmission to target cells. They are 15 to 400 nm in size and contain the viral nucleic acid enclosed in a capsid. In the case of enveloped viruses the capsid is surrounded by the inner shell of the particle, located just underneath the lipid membrane (Gomez and Hope, 2005).

Viruses can employ different kinds of replication strategies dependent on the nature of their genomes (single or double-stranded RNA or DNA) and the mode of replication. However, all viral proteins are expressed through messenger RNA (Baltimore, 1971). Retroviruses are enveloped viruses whose genetic information is encoded on two single-stranded positive sense RNA molecules. Members of the *Retroviridae* virus family differ from other positive-stranded RNA viruses by using a replication strategy termed reverse transcription (Abbink and Berkhout, 2007). Reverse transcription is a process catalysed by the viral enzyme reverse transcriptase (RT) that converts the viral RNA genome into a double-stranded DNA copy which is inserted into the host genomic DNA as a provirus. Integrated proviral DNA then serves as a template for the transcription of viral genes (Nisole and Saib, 2004; Wu, 2004).

1.1.1 Overview

Retroviruses are pathogens of vertebrates that often possess a high degree of specificity for their respective hosts. We distinguish between the two subfamilies *Orthoretrovirinae* and *Spumaretrovirinae*. They are further categorised into simple and complex retroviruses according to the complexity of their genomes. Simple retroviruses possess the three open reading frames (ORFs) *gag* (group-specific antigen), *pol* (polymerase) and *env* (envelope). *Gag* encodes

the structural proteins, *pol* RT and other enzymes indispensable for replication and *env* the envelope glycoprotein (Env) responsible for cell entry and as such a major determinant for host cell tropism. This group is subdivided into the genera *Alpha-*, *Beta-* and *Gammaretrovirus*. Complex retroviruses encode a variable number of additional regulatory and accessory genes. This group is subdivided into the genera *Deltaretrovirus*, *Lentivirus* and *Spumavirus*.

The infectious property of retroviruses was first discovered in 1904 by Vallée and Carré, who showed that equine infectious anemia virus (EIAV) could be transmitted between horses with a filtrate (Vallee and Carre, 1904). EIAV is transmitted by blood-feeding insects, and clinical signs include recurrent febrile episodes, anaemia, thrombocytopenia, oedema, diarrhoea, and lethargy (Leroux et al., 2004; Craigo et al., 2005). The first oncogenic retrovirus, avian leukosis virus (ALV), was described in 1908 (Ellermann and Bang, 1908). Another oncovirus of the *Alpharetrovirus* genus is the Rous sarcoma virus, the causative agent of sarcomas in chickens, which was discovered in 1911 by Rous (Rous, 1911). In 1936, the mouse mammary tumour virus (MMTV), a member of the *Betaretrovirus* genus, was the first virus shown to cause tumour formation in mammals. It can be transmitted vertically from mothers with breast cancer to pups in the form of a DNA provirus integrated in the DNA of milk lymphocytes (Bittner, 1942). *Gammaretroviruses* include murine leukaemia virus (MLV) and feline leukaemia virus (FeLV). Clinical signs of infection are diverse and include immunosuppression and both proliferative and non-proliferative haematopoeitic disorders.

The first human retrovirus to be isolated was human T lymphotropic virus type 1 (HTLV-1), a deltaretrovirus, which causes leukaemias and lymphomas in mature T cells (Poiesz et al., 1981). Human immunodeficiency virus type 1 (HIV-1) was discovered by Montagnier and Gallo in 1983 (Barre-Sinoussi et al., 1983; Gallo et al., 1984). Human immunodeficiency virus type 2 (HIV-2) was first described in Senegal, West Africa, in 1985 (Barin et al., 1985). Due to the definition of HIV as the cause of human acquired immunodeficiency syndrome (AIDS) retrovirology became the most intensively studied field in virology. HIV is considered pandemic by the World Health Organization, infecting 33.4 million people with approximately 2.7 million new infections and 2 million deaths per year (World Health Organization, 2008). FIV was discovered in 1987 (Pedersen et al., 1987). FIV infection of domestic cats causes an AIDS-like

syndrome characterised by recurrent gingivitis-stomatitis, cachexia, neuropathology and increased incidence of tumour development (Pedersen et al., 1987; Hosie et al., 1989; Sparger et al., 1989; Yamamoto et al., 1989; Ackley et al., 1990; Torten et al., 1991; Callanan et al., 1992; Callanan et al., 1996). FIV infects approximately 0.5 million cats in the UK alone and is therefore an important veterinary pathogen. Moreover, FIV is a valuable animal model for HIV infection in humans for investigating common principles of AIDS progression and is used for the development of vaccines for the prevention of lentiviral infection. Other lentiviruses of veterinary importance infect cattle (bovine immunodeficiency virus; BIV), goats (caprine arthritis/encephalitis virus; CAEV), horses (EIAV) and non-human primates (simian immunodeficiency virus; SIV).

1.1.2 Genome organisation

As mentioned above, the genome of simple retroviruses, such as FeLV, contains the three ORFs *gag*, *pol* and *env* (Fig. 1-1). These functional genes are flanked N-terminally by the 5' long-terminal repeat (5' LTR) and C-terminally by the 3' long-terminal repeat (3' LTR). The LTRs contain transcription and regulatory elements (Pecon-Slattery et al., 2008a). Signals for gene expression found in LTRs include enhancer, promoter, transcription initiation, transcription termination and polyadenylation signals. Both the host cell transcriptional machinery and virus-encoded proteins, which enhance or modulate LTR activity and subsequently the expression of viral RNA and proteins, interact with the LTRs (Krebs et al., 2001). *Gag* encodes for the capsid proteins matrix (MA), capsid (CA) and nucleocapsid (NC). *Pol* encodes for the viral enzymes protease (PR), reverse transcriptase (RT) and integrase (IN), and *env* encodes for the envelope glycoprotein (Env), which consists of surface (SU) and transmembrane (TM) regions.

Complex retroviruses, such as the feline foamy virus (FeFV), HIV or FIV possess additional regulatory and accessory genes. The regulatory proteins help achieve optimal viral replicative efficiency (Emerman and Malim, 1998) and the accessory proteins ensure viral persistence, replication, dissemination and transmission by supporting the evasion from and the manipulation of the host

adaptive and innate immunity or by antagonising host restriction factors (Malim and Emerman, 2008).

FeFV is a member of the genus *Spumavirus* and encodes for three additional genes (Winkler et al., 1997). The two *bel* genes are located between *env* and the 3' LTR of the FeFV provirus (Winkler et al., 1997). The Bel1/Tas protein of foamy viruses is a transactivator of LTR-directed gene expression (Mergia et al., 1990; Keller et al., 1991); (Rethwilm et al., 1991; Keller et al., 1992; Lee et al., 1992; Lochelt et al., 1993; Zou and Luciw, 1996; Winkler et al., 1997) and absolutely required for viral gene expression (Lochelt et al., 1991; Baunach et al., 1993; Yu and Linial, 1993; Winkler et al., 1997; Wagner et al., 2000). Bel2 and a protein called Bet are essential for efficient virus production (Alke et al., 2001; Lochelt et al., 2005; Munk et al., 2008; Perkovic et al., 2009). Bet is generated by a splice event fusing the N-terminal shared domain of *bel1* to the coding sequence of *bel2* (Muranyi and Flugel, 1991; Bodem et al., 1998). Bel2 is fully contained in Bet. Both *bel* and *bet* genes are expressed from an internal promoter (IP) located between *env* and the 3' LTR (Lochelt et al., 1993; Lochelt, 2003). A third ORF corresponding to the human foamy virus (HFV) *bel3* gene (Weissenberger and Flugel, 1994) is not present in FeFV (Winkler et al., 1997).

HIV-1 possesses the most complex genome amongst the retroviruses. It encodes for the six regulatory/accessory proteins Tat, Rev, Vpr, Vif, Vpu and Nef (Emerman and Malim, 1998). Tat, Rev and Vpr are of importance to achieve an optimal viral replication efficiency (Emerman and Malim, 1998). Tat (transactivator of transcription) regulates high-level HIV-1 transcription from proviral DNA, which enables the virus to sustain persistent infections *in vivo* despite a very short half-life of infected cells (Wei et al., 1995; Perelson et al., 1996). Rev (regulator of virus gene expression) (Zou et al., 1997; Lockridge et al., 1999; Miller et al., 2000) is a critical factor for virus replication (Pecon-Slattery et al., 2008a) as it permits nuclear export of the unspliced viral transcripts in infected cells (Malim et al., 1989c; Pollard and Malim, 1998). Vpr (viral protein R) acts in a complementary (but mechanistically distinct) fashion to Rev and facilitates nuclear import of viral DNA (Emerman and Malim, 1998). Another function of Vpr is to delay or arrest infected cells in the G2 phase of the cell cycle. Because the HIV-1 LTR is more active in G2 than in any other phase (Goh et al., 1998) transcription is maximised which can be an important

selective advantage for the virus. The *vif* (virus infectivity factor) gene is present in all known lentiviruses except for EIAV. Vif enhances viral infectivity (Fisher et al., 1987; Strebel et al., 1987; Gabuzda et al., 1992; Kishi et al., 1992; Von Schwedler et al., 1993; Kao et al., 2003) and is essential for productive infection in cells such as primary peripheral blood lymphocytes and macrophages and in some immortalised T-cell lines (Gabuzda et al., 1992; Von Schwedler et al., 1993). Vpu (viral protein U) and Nef (negative factor) down-regulate the primary receptor for HIV-1, CD4. CD4 binds to nascently synthesised HIV-1 Env protein in the endoplasmic reticulum (ER) (Crise et al., 1990). Vpu also binds to CD4 in the ER and targets it for proteolysis (Schubert et al., 1998), thereby decreasing the ability of Env to transit to the cell surface (Willey et al., 1992b).

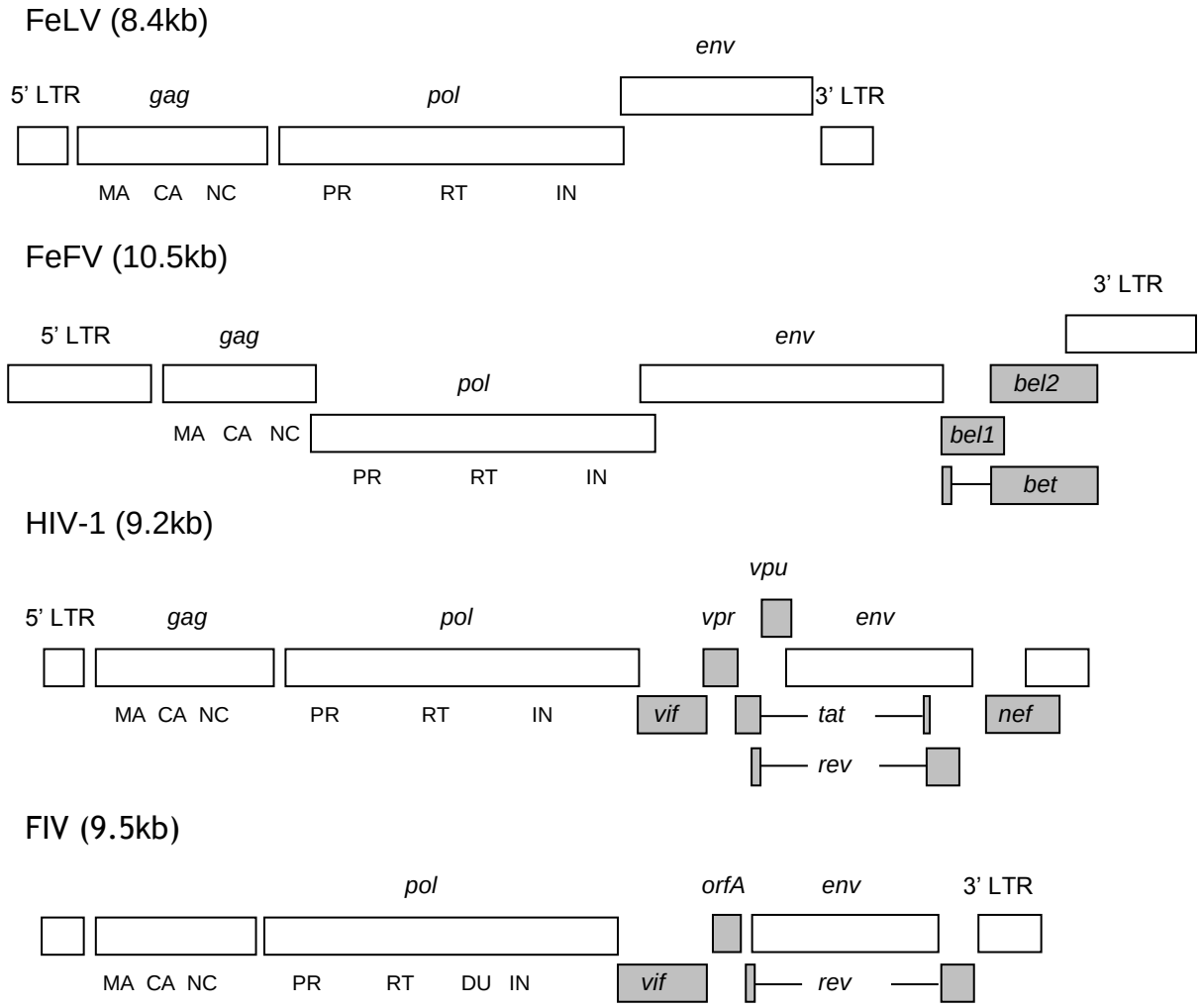


Figure 1-1 Genome organisation of simple and complex retroviruses. Feline leukaemia virus (FeLV) is a simple retrovirus belonging to the *Gammaretrovirus* genus and possesses three open reading frames, as well as 5' and 3' long

terminal repeats (LTRs). Feline foamy virus (FeFV), human immunodeficiency virus type I (HIV-1) and feline immunodeficiency virus (FIV) belong to the complex retroviruses and have an additional suite of accessory genes. Gag, group-specific antigen; pol, polymerase; env, envelope glycoprotein; MA, matrix; CA, capsid; NC, nucleocapsid; PR, protease; RT, reverse transcriptase; DU, dUTPase; IN, integrase; nef, negative factor; orf2, open reading frame A; rev, regulator of virus gene expression; tat, transactivator of transcription; vif, virus infectivity factor; vpu, viral protein U.

In contrast to Vpu, Nef acts to remove CD4 that is already located on the cell surface by endocytosis (Greenberg et al., 1997; Le Gall et al., 1998; Piguet et al., 1998). The rationale for this is that CD4-Env interactions on the cell surface could reduce virion release or inhibit the incorporation of Env into virions, which is prevented by Nef-initiated CD4 endocytosis (Lama et al., 1999; Ross et al., 1999). Both Vpu and Nef also downregulate the expression of the major histocompatibility complex I (Schwartz et al., 1996; Kerkau et al., 1997; Collins et al., 1998; Roeth and Collins, 2006), which potentially helps to avoid host immune surveillance.

Tat, Vpr, Vpu and Nef are absent from the FIV genome. However, it encodes for the proteins OrfA (also called Orf2) and deoxyuridine triphosphate nucleotidohydrolase (dUTPase). OrfA is a transactivator necessary for productive FIV replication in primary T lymphocytes as well as in some feline T cell lines (Phillips et al., 1990; Sparger et al., 1992; Tomonaga et al., 1993; Sparger et al., 1994; Waters et al., 1996; de Parseval and Elder, 1999). *OrfA* deletion mutants have been associated with decreased viral replication and pathogenicity (Sparger et al., 1994; Inoshima et al., 1996; Inoshima et al., 1998b; Norway et al., 2001; Gemeniano et al., 2003; Gemeniano et al., 2004). In addition, it has been shown that OrfA induces G2 cell cycle arrest (Gemeniano et al., 2004) and down-regulates cell surface expression of the FIV primary binding receptor, CD134 (Hong et al., 2010). Studies also suggested that OrfA can affect later steps of virus replication, such as particle formation or release, and possibly early steps, such as binding and entry (Gemeniano et al., 2003). Overall, it seems that OrfA is exhibiting multiple functions homologous to HIV Vpr, Vpu, Tat, or Nef (Malim and Emerman, 2008). *dUTPase* is encoded specifically in non-primate lentivirus genomes and forms part of the *pol* gene (Payne and Elder, 2001). dUTPase

prevents uracil misincorporations during viral replication (Elder et al., 1998; Inoshima et al., 1998a) and dUTPase-defective FIV cannot replicate in feline macrophages (Lerner et al., 1995).

1.1.3 Replication cycle

In general, the retrovirus life cycle can be divided into early and late stages. The early stages comprise the attachment of virions to the target cell and their fusion with the target cell membrane, uncoating of the viral core and reverse transcription of viral RNA into DNA, nuclear import of the viral DNA and its integration into the target cellular genome. The late stages of the retrovirus life cycle include virus replication, assembly and egress.

Because the HIV-1 life cycle (Fig. 1-2) has been studied in far greater detail than the FIV life cycle, all life cycle stages mentioned in this chapter refer to HIV-1 rather than FIV, unless otherwise stated.

1.1.3.1 Cellular entry

HIV transmission between individuals occurs mainly through exchange of blood or other bodily fluids but vertical transmission through breast milk has also been described. FIV is transmitted between cats through deep bite wounds and scratches caused by fighting. HIV targets T lymphocytes, macrophages and, to some extent, dendritic cells (DCs). Its cell tropism is determined by the cell surface receptors required for target cell attachment and entry. HIV uses CD4 as its primary cell entry receptor (Dalgleish et al., 1984; Klatzmann et al., 1984; Maddon et al., 1986; Clapham and McKnight, 2001; Clapham and McKnight, 2002) and the chemokine receptors CCR5 (Alkhatib et al., 1996; Deng et al., 1996; Dragic et al., 1996; Trkola et al., 1996) and CXCR4 (Choe et al., 1996; Feng et al., 1996; Oberlin et al., 1996; Bleul et al., 1997) as co-receptors. Co-receptor usage is strain-dependent. R5 strains of HIV-1 use CCR5 and are able to enter macrophages, DCs and T lymphocytes, whereas X4 strains of HIV-1 use CXCR4 and can infect $CD4^+$ T lymphocytes only (Doms and Trono, 2000). In early stages of infection, CCR5-tropic HIV-1 strains dominate and infect CCR5-expressing

immune cells of the mucosa (de Roda Husman and Schuitemaker, 1998; Clapham and McKnight, 2002); (Miller and Shattock, 2003; Davis and Doms, 2004; Moore et al., 2004; Wilflingseder et al., 2005). Mucosal macrophages and DCs migrate to draining lymph nodes where they infect susceptible T lymphocytes (Clapham and McKnight, 2002; Davis and Doms, 2004; Moore et al., 2004; Wilflingseder et al., 2005). Mucosal DCs can capture viral particles via interactions between HIV-1 Env and the C-type mannose binding lectins DC-SIGN (DC-specific intercellular adhesion molecule-grabbing non-integrin) and DC-SIGNR (DC-SIGN-related) (Geijtenbeek et al., 2000; Pohlmann et al., 2001) and promote HIV-1 internalisation, allowing the virus to remain infectious until it can be presented to T lymphocytes (van Kooyk and Geijtenbeek, 2003). HIV-1 is capable of switching its co-receptors *in vivo*. In about 50% of untreated HIV-1 infections the evolution of R5X4- and X4-tropic viral variants can be observed (Connor et al., 1997). This is accompanied by a change in target cell tropism from macrophages to T lymphocytes and the progression to AIDS (Nielsen et al., 1993). R4-tropic viruses display a broader T lymphocyte tropism than R5-tropic viruses (Bleul et al., 1997; Clapham and McKnight, 2002). In addition to CCR5 and CXCR4, HIV-2 can also use CCR1, CCR2b and CCR3 as co-receptors *in vitro* (Maddon et al., 1986; Sattentau et al., 1988; Alkhatib et al., 1996; Deng et al., 1996; Clapham and McKnight, 2002). Both HIV-1 and HIV-2 can use the chemokine receptor D6 to infect astrocytes in the brain (Neil et al., 2005).

FIV and HIV display a similar cell tropism; however, because feline CD4 is expressed only in T lymphocytes but is absent from macrophages or DCs (Ackley et al., 1990), CD4 was excluded as primary receptor for FIV (Willett et al., 1991; Hosie et al., 1993; Norimine et al., 1993). Instead, primary strains of FIV use the feline homologue of OX40 (CD134), a member of the tumour necrosis factor receptor/nerve growth factor receptor (TNFR/NGFR) superfamily (Shimojima et al., 2004). CD134 is expressed in activated $CD4^+$ T lymphocytes (Paterson et al., 1987; Mallett et al., 1990; de Parseval et al., 2004; Joshi et al., 2005) and at low levels in activated $CD8^+$ T lymphocytes (Baum et al., 1994; Al Shamkhani et al., 1996), macrophages and activated B cells (Durkop et al., 1995). CD134-dependent infection requires co-expression of CXCR4 (Shimojima et al., 2004). Interestingly, during late (chronic) stages of FIV infections, CD134-independent strains of FIV can arise that can enter the cell via CXCR4 only, enabling them to

also target $CD8^+$ T lymphocytes and B cells (Willett et al., 1997a; Willett et al., 1997b; Hosie et al., 1998).

The adsorption of HIV-1 to target cells prior to receptor engagement is aided by host cell surface molecules like heparin sulphate proteoglycan (Mondor et al., 1998), LFA-1 (Fortin et al., 1998) and nucleolin (Nisole et al., 1999). The retrovirus-receptor interactions that lead to cell entry require a series of steps that have been particularly well studied for HIV-1 (Nisole and Saib, 2004). HIV-1 envelope glycoproteins are organised into trimeric spikes displayed on the surface of the viral particle. Each spike comprises a trimer of three transmembrane gp41 (TM) subunits non-covalently bound to a trimer of three exterior gp120 (SU) subunits (Kowalski et al., 1987). Gp120 consists of variable (V1-V5) and conserved regions (Starcich et al., 1986). V1 to V4 form surface-exposed loops, and the conserved regions are involved in interactions with gp41 and the viral receptors (Starcich et al., 1986).

In a first step, the HIV-1 Env gp120 subunit engages CD4. The binding reaction induces a conformational change in gp120, namely the repositioning of V1/V2 and V3, which exposes the co-receptor binding site (Kwong et al., 1998; Rizzuto et al., 1998; Doms, 2000; Rizzuto and Sodroski, 2000). Co-receptor binding in turn leads to a conformational rearrangement of gp41, which triggers fusion of Env with the host cell membrane and entry of the viral core into the host cell cytoplasm (Kwong et al., 1998). In addition to receptor-mediated cell entry, HIV-1 uses clathrin-mediated endocytosis (McClure et al., 1988; Marsh and Pelchen-Matthews, 2000; Fredericksen et al., 2002; Vidricaire et al., 2003) (Daecke et al., 2005; Wei et al., 2005) for cell entry; however, particles entering by endocytosis are not infectious as they are degraded by the proteasome (Schwartz et al., 1998).

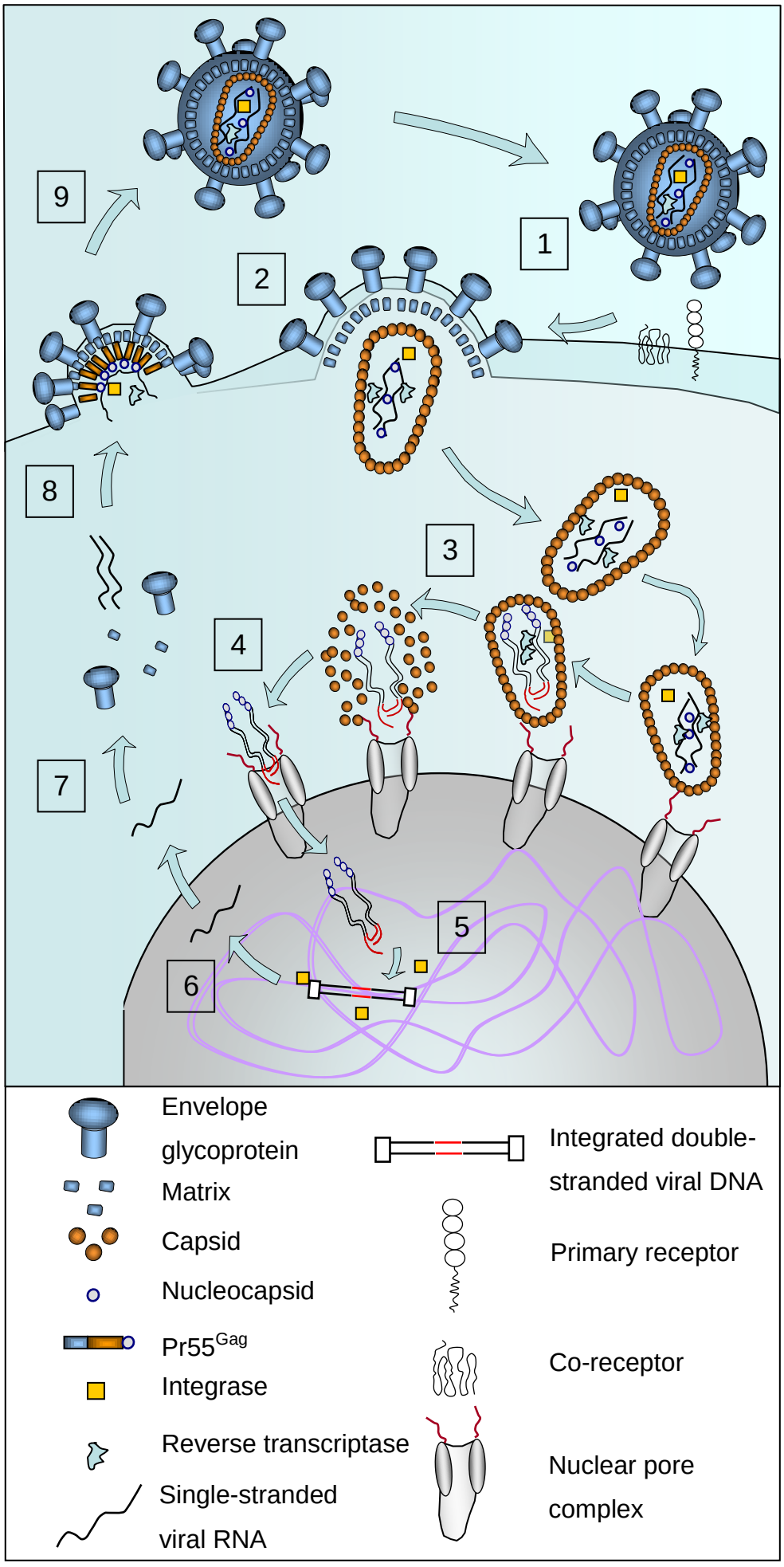

9
1
2
8
3
4
7
5
6
Envelope glycoprotein
Matrix
Capsid
Nucleocapsid
Pr55Gag
Integrase
Reverse transcriptase
Single-stranded viral RNA
Integrated double-stranded viral DNA
Primary receptor
Co-receptor
Nuclear pore complex

Figure 1-2 HIV-1 replication cycle. (1) Virions bind to the primary cell-entry receptor CD4 (CD134 for FIV) on the surface of target cells. Receptor binding causes a conformational change in the viral envelope glycoprotein (Env) that enables co-receptor binding. The main co-receptors for HIV-1 are the chemokine receptors CCR5 and CXCR4 (FIV uses CXCR4 only). (2) Virions enter the target cell by fusion between viral and cellular membranes. Viral cores are released into the cytoplasm. (3) The viral cores interact with the cellular microtubule and actin network (not shown) and are rapidly routed to the nuclear membrane where they bind to nuclear pore complexes. Within intact cores reverse transcription complexes (RTCs) are formed. These are HIV-1 complexes that undergo reverse transcription, the conversion of the single-stranded, positive-sense RNA viral genome into double-stranded DNA (Arhel, 2010). Upon completion of reverse transcription, uncoating of viral cores (the loss of capsid proteins) is initiated and RTCs mature into pre-integration complexes (PICs). (4) Nuclear localisation signals in integrase (IN), matrix (MA) and HIV-1 viral protein R (Vpr) and the central DNA flap in the viral cDNA enable PICs to enter the nucleus in the absence of cell division. (5) IN catalyses the integration of the viral cDNA into the host cell genome. (6) The integrated viral cDNA is transcribed along with host genes and newly synthesised viral RNA is exported into the cytoplasm with the help of the regulator of viral gene expression protein (Rev). (7) Viral proteins are translated in the cytoplasm and are, together with viral RNA, transported to lipid rafts in the host cell plasma membrane, where (8) assembly of viral particles and their budding from the host cell occur. (9) The nascent viral particles then mature and are now infectious for target cells. Figure adapted from Arhel et al. (2007).

1.1.3.2 Uncoating and reverse transcription

Once inside the cytoplasm, viral particles undergo uncoating and reverse transcribe their single-stranded, positive sense RNA genomes into double-stranded cDNA capable of integrating into the host genome. In order to be transported from the plasma membrane to the nuclear membrane viral particles interact with cellular cytoskeleton components such as cortical actin (Bukrinskaya et al., 1998; Ploubidou and Way, 2001) or the microtubule network (McDonald et al., 2002).

There has been a lot of controversy about the timing of uncoating (the rapid disassembly of the viral capsid) and reverse transcription (Arhel, 2010). It has been thought previously that capsid disassembly and loss of CA occurs shortly after cell entry (Dvorin and Malim, 2003; Bukrinsky, 2004; Lehmann-Che and Saib, 2004; Suzuki and Craigie, 2007) and that complete uncoating is required for reverse transcription complex (RTC) formation and the initiation of reverse transcription (Mortuza et al., 2004). RTCs are viral complexes containing MA, PR, RT, IN, Vif, Vpr and two copies of the single-stranded plus-sense RNA genome as well as numerous cellular proteins (Bukrinsky et al., 1993b; Peterlin and Trono, 2003), in which reverse transcription occurs (Telesnitsky and Goff, 1997; Basu et al., 2008). However, uncoating may be a gradual process which is still ongoing while reverse transcription has already been initiated (Warrilow et al., 2009; Hulme et al., 2011). In this scenario molecular rearrangements during reverse transcription may trigger conformational changes and a stepwise disassembly of the viral capsid (Arhel, 2010). Current data suggest a third model that proposes that uncoating occurs in close proximity to nuclear pores in the nuclear membrane upon completion of reverse transcription (Dismuke and Aiken, 2006; Arhel et al., 2007; Schaller et al., 2011). The role of an intact capsid structure may be to concentrate RT enzyme near the target RNA (Klarmann et al., 1993). While it was thought originally that uncoating was a prerequisite for the initiation of reverse transcription, this third model implies that completion of reverse transcription is essential for uncoating to occur (Arhel, 2010).

The regulation of the timing of reverse transcription and uncoating is crucial for successful nuclear import (Forshey et al., 2002; Dismuke and Aiken, 2006; Iordanskiy et al., 2006; Arhel et al., 2007; Yamashita et al., 2007). In the process of reverse transcription the enzyme reverse transcriptase (RT) catalyses the conversion of single-stranded plus-sense RNA to double-stranded DNA. The first step of reverse transcription is the synthesis of minus-strand strong-stop DNA at the primer binding site (PBS) at the 5' end of the RNA genome using a lysine tRNA ($tRNA^{Lys}$; contained within the virion) as a primer (Wain-Hobson et al., 1985; Guyader et al., 1987). The minus-strand strong-stop DNA is then transferred to the 3' end of the RNA genome and anneals to a complementary sequence in the R (repeated) region of the LTR and the synthesis of the minus-strand DNA is completed. RNase H activity of reverse transcriptase degrades the RNA matrix. Plus-strand DNA synthesis is initiated from two RNA regions that are

resistant to RNase H digestion because they contain a unique poly purine tract (PPT) sequence (Charneau et al., 1992; Charneau et al., 1994; Telesnitsky and Goff, 1997). Lentiviruses initiate plus-strand DNA synthesis from the central and 3' PPT (cPPT and 3' PPT, respectively). DNA is copied into the PBS of the lysine tRNA molecule, and the tRNA is degraded by RNase H. The PBS on the plus-strand DNA is now exposed so that plus-strand DNA can anneal to the PBS at the 3' end of the minus-strand DNA in a second strand transfer event. This strand transfer leads to the formation of a circular structure. Both plus-sense and minus-sense DNA syntheses are completed at the central termination sequence (CTS) in the centre of the genome. The downstream strand is displaced over 99 nucleotides creating the central DNA flap (Charneau et al., 1994). Reverse transcription generates pre-integration complexes (PICs) which contain MA, PR, RT, IN and Vpr (Farnet and Haseltine, 1991; Bukrinsky et al., 1993b; Miller et al., 1997; Iordanskiy et al., 2006; Suzuki and Craigie, 2007) but are lacking CA proteins (Farnet and Haseltine, 1991; Miller et al., 1997). PICs also contain many cellular factors such as the high mobility group protein HMG-I (Y) (Miller et al., 1997) and LEDGF/p75 (lens epithelium-derived growth factor/transcriptional co-activator p75) (Llano et al., 2004; Maertens et al., 2004) which may aid the association of the PIC with chromatin or integration (Miller et al., 1997; Ciuffi et al., 2005; Emiliani et al., 2005).

1.1.3.3 Nuclear import and integration

The next step in the retrovirus life cycle is nuclear import and integration of the viral DNA into the host cell genome. Nuclear import of most retroviruses is cell cycle-dependent and requires the breakdown of the nuclear membrane during mitosis (Roe et al., 1993; Lewis and Emerman, 1994). Lentiviruses have the unique ability to replicate in metabolically active non-dividing cells (Gartner et al., 1986; Weinberg et al., 1991; Lewis et al., 1992) such as terminally differentiated macrophages or quiescent T lymphocytes as a result of active nuclear import of their genomes across the nuclear membrane (Bukrinsky et al., 1992). Nuclear pore complexes (NPCs) are known to mediate transport of macromolecules of up to 39 nm in diameter (Pante and Kann, 2002; Tran and Wente, 2006) from the cytoplasm into the nucleoplasm through a central nuclear

pore channel (Fried and Kutay, 2003). Curiously, the stokes diameter of the HIV-1 PIC has been determined to be 56 nm (Yamashita and Emerman, 2004) so that passive transport through nuclear pores cannot take place. Instead, nuclear localisation signals (NLS) in IN (Gallay et al., 1997; Bouyac-Bertoia et al., 2001), MA (Bukrinsky et al., 1993a; Bukrinsky et al., 1993b; Von Schwedler et al., 1994; Dubrovsky et al., 1995; Jenkins et al., 1998; Peterlin and Trono, 2003) and Vpr (Heinzinger et al., 1994; Fouchier et al., 1998; Popov et al., 1998; Vodicka et al., 1998; de Noronha et al., 2001; Le Rouzic et al., 2002) enable signal-mediated nuclear import via interactions with nucleocytoplasmic shuttling receptors of the karyopherin ß family. The nuclear impact factors transportin-3 (TNPO3) (Christ et al., 2008; Goff, 2008; Lee et al., 2010), importins α, ß (Fried and Kutay, 2003) and 7 (Zaitseva et al., 2009) as well as nucleoporins (Woodward et al., 2009) are required for infection of both dividing and non-dividing cells.

Furthermore, the central DNA flap in the viral cDNA has been shown to enhance nuclear localisation of HIV-1 PICs (Charneau et al., 1992; Hungnes et al., 1992; Charneau et al., 1994; De Rijck et al., 2005). Stable integration of viral cDNA into the host cell genome is catalysed by IN. It was previously thought that HIV-1 integration would occur at random sites. Recently however, it has been shown that integration is influenced by chromatin structure (Pryciak et al., 1992) and that HIV-1 prefers gene-rich regions of the genome and actively transcribed genes (Schroder et al., 2002; Wu et al., 2003).

1.1.3.4 Transcription and translation

Once integration of HIV-1 into the host genome has occurred, the provirus behaves like any human gene and transcription is initiated at the 5' end of the proviral genomic DNA and terminated at its 3' end (Jones and Peterlin, 1994). In the early stages of viral transcription, host RNA polymerase II interacts with cellular transcription factors and the proviral LTR. The LTR contains promoter elements and enhancer sequences. The core promoter contains the initiator (Inr) and a TATA box (Berkhout and Jeang, 1992; Jones and Peterlin, 1994). Upstream of Inr are three Specificity Protein 1 (SP1) transcription factor binding sites (Berkhout and Jeang, 1992; Jones and Peterlin, 1994). Together, these elements position RNA polymerase II on the proviral DNA to initiate transcription. Further

upstream is the enhancer, which can bind the transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and nuclear factor of activated T cells (NFAT) (Jones and Peterlin, 1994), which ensure that virus replication proceeds at high levels. Downstream of Inr lays a regulatory element known as transactivation response (TAR) element. Low levels of basal transcription drive the synthesis of HIV-1 transcription factor Tat transcripts. Tat is then translated in the cytoplasm and shuttled back into the nucleus with the help of its NLS. In a second phase of viral transcription, Tat and its cellular co-factor, positive transcription elongation factor B (P-TEFb), interact with TAR to allow RNA polymerase II to generate full-length viral transcripts (Berkhout et al., 1989; Dingwall et al., 1989; Wei et al., 1998; Isel and Karn, 1999; Kao et al., 2003; Raha et al., 2005) and to increase transcription from the HIV-1 LTR several hundred-fold (Taube et al., 1999).

FIV lacks a *tat* gene and TAR but it encodes for OrfA, which transactivates transcription at low levels and is essential for productive FIV replication in primary T lymphocytes (Phillips et al., 1990; Sparger et al., 1992; Tomonaga et al., 1993; Sparger et al., 1994; de Parseval and Elder, 1999).

During HIV-1 transcription, three classes of transcripts are synthesised that give rise to approximately 30 different viral transcripts generated by alternative splicing (Bohne et al., 2005). These include unspliced, 9-kb mRNA species that encode for the Gag/Pol polyprotein (Kim et al., 1989; Arrigo et al., 1990; Schwartz et al., 1990; Neumann et al., 1994), singly spliced, 4-kb mRNAs that encode Vif, Vpr, Vpu and Env (Arrigo et al., 1990), and multiply-spliced mRNA species that encode for Tat, Rev and Nef (Robert-Guroff et al., 1990; Schwartz et al., 1990). Nuclear export of intron-containing mRNAs is inhibited by cellular commitment factors in the nucleus (Legrain and Rosbash, 1989; Cullen, 2003b) and HIV-1 is dependent on Rev to transport unspliced and singly spliced RNA species out of the nucleus (Malim et al., 1989a; Malim et al., 1989c). Rev binds directly to a RNA secondary structure present within the 3' end intron region of all incompletely spliced HIV-1 mRNAs called RRE (Rev Responsive Element) (Chang and Sharp, 1989; Malim et al., 1989b; Zapp and Green, 1989; Hope et al., 1990). Rev multimerises upon RRE binding which initiates a complex with the cellular exportin-1 (CRM-1) and the GTPase Ran (Fornerod et al., 1997; Askjaer et al., 1998). CRM-1 targets the complex to the nuclear pore (Fornerod et al., 1997; Neville et al., 1997; Cullen, 2003a). After translocation to the

cytoplasm, Ran-GTP is converted into Ran-GDP, and Ran, exportin-1 and Rev dissociate from the viral RNA. Rev is then recycled back into the nucleus by importin ß from which it dissociates in the nucleoplasm through interaction with Ran-GTP (Henderson and Percipalle, 1997; Askjaer et al., 1998; Fischer et al., 1999; Cullen, 2003a). Incompletely spliced viral RNA is now available for splicing and translation on ribosomes or packaging into virions.

1.1.3.5 Particle assembly and egress

The retrovirus life cycle is completed with the assembly of viral particles and their release from the producer cell. Virion assembly is driven by retroviral Gag precursor polyprotein ($Pr55^{Gag}$) which is translated on the cytoplasm on free polysomes (Spearman, 2006). In fact, HIV-1 $Pr55^{Gag}$ expression alone is sufficient for the production of virus-like particles (VLPs) that are morphologically indistinguishable from immature viral particles (Gheysen et al., 1989).

The HIV-1 $Pr55^{Gag}$ contains three major domains that play a critical role in the assembly process: a membrane binding domain (M), a Gag-Gag interaction domain (I) and a late domain (L). The M domain lays within MA and is important for targeting of $Pr55^{Gag}$ to the inner leaflet of the plasma membrane and for the recruitment of viral envelope glycoproteins into virions (Freed and Martin, 1995; Freed and Martin, 1996; Freed, 1998). HIV-1 Env precursors are synthesised on endoplasmic reticulum-bound ribosomes and transported to the plasma membrane through the secretory pathway (Spearman, 2006). In the trans-Golgi network, the Env precursor gp160 is proteolytically cleaved into gp41 (TM) and gp120 (SU) (Decroly et al., 1994) and Env trimers are incorporated into the forming viral particle. The membrane-targeting activity of the M domain in MA relies on myristoylation of the MA N-terminus and a patch of basic amino acids (Yuan et al., 1993; Freed et al., 1994; Spearman et al., 1994; Zhou et al., 1994; Hill et al., 1996; Dalton et al., 2007).

The I domain is located in the C-terminus of CA and the N-terminus of the NC region of $Pr55^{Gag}$ and mediates $Pr55^{Gag}$ monomer interactions/multimerisation, RNA binding and formation of preassembled virion complexes (Rein et al., 1998; Burniston et al., 1999; Cimarelli et al., 2000) (Sandefur et al., 2000; Derdowski et al., 2004). Encapsidation of full-length HIV-

1 RNA depends on binding of NC to the RNA packaging signal Ψ (Aldovini and Young, 1990; South and Summers, 1993; Dannull et al., 1994; Tsukahara et al., 1996; Dorman and Lever, 2000) and coincides with packaging of Vif (Liu et al., 1995; Karczewski and Strebel, 1996). $Pr55^{Gag}$ multimerisation and RNA recruitment is followed by the formation of Gag/Gag-Pol complexes, which are also targeted to the plasma membrane.

The L domain is on the N-terminus of HIV-1 p6 (FIV p2) and is essential for viral budding. A PTAP (Pro-Thr-Ala-Pro) motif in p6 recruits the cellular protein Tsg101 (Garrus et al., 2001; Martin-Serrano et al., 2001; VerPlank et al., 2001; Carter, 2002; Freed, 2003). P6 binding to Tsg101 recruits the endosomal sorting complex required for transport (ESCRT) machinery (Babst et al., 2000; Katzmann et al., 2001; Bache et al., 2003; Pornillos et al., 2003). This machinery, which is normally involved in intracellular sorting of membrane proteins and membrane scission, is hijacked by retroviruses to exit infected cells (Gomez and Hope, 2005). P6 also recruits HIV-1 Vpr into the viral particle.

HIV-1 buds from lipid rafts in the plasma membrane. These are detergent-resistant plasma membrane regions enriched in glycosphingolipids, sphingomyelin, cholesterol and glycosylphophatidylinositol (GPI)-linked proteins (Brown, 2002). Release of HIV-1 from infected cells can be directional with virus being released to sites of cell-cell contact (termed infectious synapses; (Jolly et al., 2004)) to facilitate infection of target cells (McDonald et al., 2003). After budding of the immature virion, the radially arranged $Pr55^{Gag}$ molecules within the virions are dismantled and viral protease (PR) cleaves $Pr55^{Gag}$ into MA, which coats the inner leaflet of the viral membrane; CA, which forms a cone-shaped core that contains two copies of genomic viral RNA; and NC, which is bound to genomic RNA, to create a mature, infectious viral particle.

1.2 Immunology of FIV infections of the domestic cat

FIV infection of domestic cats (*Felis catus*) leads to the development of a severe immunodeficiency similar to AIDS in humans. Like HIV, FIV targets and depletes $CD4^+$ T lymphocytes and causes an inversion of the $CD4^+/CD8^+$ ratio. Therefore, FIV infections are not only studied as an important global veterinary pathogen but also serve as a small animal model for HIV infections. FIV was isolated in

1986 from a domestic cat exhibiting multiple opportunistic infections and AIDS-like signs (Pedersen et al., 1987).

1.2.1 Course of FIV infections

The course of disease in FIV and HIV infections is strikingly similar (Fig. 1-3). It can be divided into three stages: a relatively short acute phase (four to eight weeks), a prolonged period of clinical latency (several years) and a terminal phase (several months for FIV; two to three years for HIV).

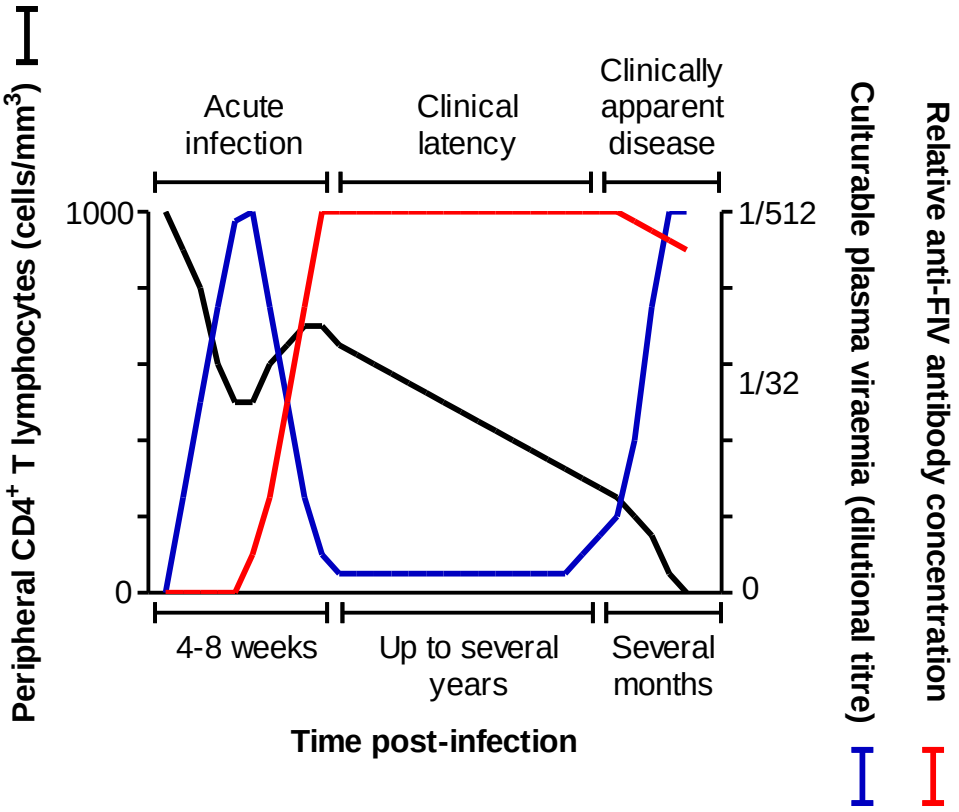


Figure 1-3 Schematic course of a FIV infection. The graph shows infection stage-dependent changes in peripheral $CD4^+$ T lymphocyte counts, plasma viraemia and anti-FIV antibody concentration. Figure adapted from Weiss (1993).

Following infection of a cat with FIV the primary stage of disease is characterised by increasing viral loads associated with mild signs such as febrile episodes, weight loss, lymphadenopathy and neutropenia. Because the virus targets $CD4^+$ T lymphocytes the number of T lymphocytes in the peripheral blood

sharply decreases. This acute phase is followed by a period of clinical latency in which cats are usually asymptomatic. Virus production in the peripheral blood is limited by the onset of robust humoural and cellular antiviral immune responses (see Section 1.2.2) (English et al., 1993; Dean et al., 1996). However, the number of $CD4^+$ T lymphocytes gradually declines (Yamamoto et al., 1988; English et al., 1994; Bendinelli et al., 1995). Once it falls below a certain threshold the feline immune system becomes severely impaired and cats suffer from chronic upper respiratory tract and intestinal infections, skin and ear diseases, neurological signs and secondary infections. Plasma viral loads increase (Goto et al., 2002). Moreover, the broadened FIV cell tropism in later disease stages (B and $CD8^+$ T lymphocytes as well as $CD4^+$ T lymphocytes and macrophages can be infected; [Novotney et al., 1990; English et al., 1993; Dean et al., 1996]) contributes to the immunodeficiency.

Early after infection virus can be detected in the central nervous system, thymus and mesenteric lymph nodes (Toyosaki et al., 1993; Beebe et al., 1994; Dua et al., 1994; Rogers and Hoover, 1998; Obert and Hoover, 2002). Virus replication in peripheral blood mononuclear cells (PBMCs) can be observed several days post-infection (p.i.) (English et al., 1993; Lawson et al., 1993; Dean et al., 1996; Obert and Hoover, 2002). In later stages of the disease FIV replicates mainly in lymphoid tissue; however, infected cells can also be found in the bone marrow, intestine, kidneys and liver (Beebe et al., 1994; Park et al., 1995; Bishop et al., 1996; Rogers and Hoover, 1998; Obert and Hoover, 2000a; Obert and Hoover, 2000b).

1.2.2 Humoural and cellular antiviral immune responses

The transition between acute infection and clinical latency is mediated by substantial humoural and cell-mediated antiviral immune responses (Bendinelli et al., 1995; Burkhard and Dean, 2003). Cats raise antibodies against Gag and Env epitopes from as early as two to four weeks p.i., which persist throughout the course of infection (Yamamoto et al., 1988; Egberink et al., 1992; English et al., 1994). Neutralising antibodies directed against the V3 loop in Env are produced but their presence does not correlate with virus clearance (Lombardi et al., 1993; Tozzini et al., 1993). However, the absence of detectable titres of

neutralising antibodies leads to a more rapid progression to immunodeficiency (Hohdatsu et al., 1993).

The early stages of infection are accompanied by a rapid expansion of circulating $CD8^+$ T lymphocytes (Ackley et al., 1990; Tompkins et al., 1991; Willett et al., 1993; English et al., 1994). These $CD8^+$ T lymphocytes mediate both cytotoxic (Song et al., 1992; Song et al., 1995; Beatty et al., 1996; Burkhard et al., 2001; Flynn et al., 2002) and non-cytotoxic antiviral activities (Hohdatsu et al., 1993; Bucci et al., 1998; Gebhard et al., 1999) that result in a reduction of viraemia (Flynn et al., 1995; Beatty et al., 1996). Anti-FIV Gag-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) have been detected in peripheral blood from two weeks p.i. to about 21 weeks p.i. (Beatty et al., 1996). In the asymptomatic phase of disease classic CTLs disappear from the peripheral blood but anti-FIV Gag and anti-FIV Env precursor CTLs (pCTLs) are present in lymph nodes (Beatty et al., 1996; Flynn et al., 2002) and spleen (Song et al., 1992; Song et al., 1995; Flynn et al., 2002). In addition to cytotoxic $CD8^+$ T cells, non-cytotoxic $CD8^+$ T lymphocytes can be found in blood, lymph nodes and thymus that suppress viral replication in $CD4^+$ T lymphocytes in a non-cytotoxic, non-MHC (major histocompatibility complex) restricted manner (Bucci et al., 1998; Hohdatsu et al., 1998; Hohdatsu et al., 2000; Flynn et al., 2002). Their activity can be either contact-dependent (Bucci et al., 1998; Gebhard et al., 1999) or contact-independent (Hohdatsu et al., 1993; Levy et al., 1996; Choi et al., 2000). Contact-independent, non-cytotoxic $CD8^+$ T cell activity involves the secretion of soluble factors such as chemokines (Hohdatsu et al., 1993; Levy et al., 1996; Flynn et al., 1999; Choi et al., 2000).

1.2.3 FIV-induced immune dysregulation

Despite these early and robust antiviral immune responses virus cannot be cleared and infected cats will develop a progressive immunodeficiency. The immunodeficiency is first characterised by the loss of response to viral antigens and recall antigens (anergy), followed by the loss of mitogen responses (Clerici et al., 1989; Barlough et al., 1991; Torten et al., 1991) and cell-mediated immune responses to secondary pathogens (Davidson et al., 1993; Dean et al., 1998). While the occurrence of secondary infection can be attributed to a

decline in $CD4^+$ T lymphocyte numbers and the resulting decrease in pro-inflammatory cytokines such as interleukin-2 (IL-2) and IFN-γ, an immune dysfunction develops early in infection before $CD4^+$ T cells numbers begin to decline (Clerici et al., 1989; Barlough et al., 1991; Torten et al., 1991; Davidson et al., 1993). This immune dysfunction may be induced by a range of mechanisms (reviewed in: Tompkins and Tompkins [2008] and Burkhard and Dean [2003]) such as cytokine dysregulation (Clerici and Shearer, 1993), immunologic anergy and increased apoptosis (programmed cell death) (Miedema, 1992) as well as an inappropriate activation of immune regulatory cells (Ascher and Sheppard, 1990).

PBMCs of FIV-infected cats show an altered cytokine production profile in response to mitogen stimulation (Lawrence et al., 1995). Elevated levels of IL-10, IL-6, TNFα (tumor necrosis factor alpha) and IFN-γ and decreased levels of IL-2 and IL-12 were found in macrophages (Ritchey et al., 2001; Avery and Hoover, 2004) and lymph nodes (Levy et al., 1998; Levy et al., 2004) of FIV-infected cats. The increased IL-10 to IL-12 ratio in infected cats (IL-10 is known to suppress IL-12 production by DCs) limits their ability to mount a primary immune response to protozoal (Levy et al., 1998; Levy et al., 2004) and bacterial (Dean et al., 1998) pathogens.

Immunologic anergy and apoptosis of $CD4^+$ T lymphocytes in lymphoid tissues contribute to the progressive loss of T cell immune function observed in of FIV-infected cats (Guiot et al., 1993; Sarli et al., 1998). Immunologic anergy is defined as inability of $CD4^+$ T lymphocytes to produce IL-2 and proliferate in response to recall antigens (Tompkins and Tompkins, 2008). Lack of IL-2 drives stimulated cells into a programmed cell death. In T lymphocytes apoptosis is induced by the engagement of the cell surface receptors CD28 or CTLA4 with the co-stimulatory molecules B7.1 (CD80) and B7.2 (CD86). Members of the B7 family are usually found on professional antigen-presenting cells (APCs). CD28 is constitutively expressed on T lymphocytes, and CTLA4 expression is upregulated upon T cell activation. Interaction of B7 with CD28 on T lymphocytes stimulates IL-2 production and T cell proliferation, whereas interaction of B7 with CTLA4 suppresses IL-2 production (Bluestone, 1997). Because the affinity of B7 for CTLA4 is several logs higher than for CD28, termination of a response would dominate over T cell activation. In FIV infections, however, B7 is also expressed on activated $CD4^+$ and $CD8^+$ T lymphocytes due to chronic antigenic stimulation

(Ranheim and Kipps, 1994; Folzenlogen et al., 1997). $B7^+CTLA4^+$ T cells were shown to be anergic and to undergo spontaneous apoptosis in lymph nodes and PBMCs of FIV-positive cats (Tompkins et al., 2002). The B7-CTLA4 induced downregulation of IL-2 production contributes to the cytokine dysregulation described earlier.

Inappropriate activation of immune regulatory cells has also been described in FIV-infected cats. $CD4^+$ T lymphocytes with regulatory function (Tregs) are thymus-derived and develop during T cell maturation (Bluestone and Abbas, 2003). They are long-lived and eliminate autoimmune cells. Tregs are usually anergic (Maloy and Powrie, 2001). Upon activation, however, they migrate to peripheral lymphoid tissues (Bluestone and Abbas, 2003), inhibit the proliferation of activated $CD4^+$ and $CD8^+$ T lymphocytes and induce their apoptosis (Thornton and Shevach, 1998; Shevach et al., 2001), which could prevent an effective immune response to FIV and secondary pathogens.

1.3 FIV infections of non-domestic felids

FIV is endemic in nine free-ranging species of *Felidae* which have been shown to harbour antibodies reactive to FIV (Troyer et al., 2005). Species-specific FIV strains have been described for the domestic cat (*Felis catus*), African lions (*Panthera leo*), North American pumas (*Puma concolor*), Pallas's cats (*Otocolobus manul*), bobcats (*Lynx rufus*), jaguarundi (*Herpailurus yagouaroundi*), cheetahs (*Acinonyx jubatus*), leopards (*Panthera pardus*), ocelots (*Leopardis pardalis*) and spotted hyenas (*Crocuta crocuta*). These FIV strains are termed FIV-Fca, FIV-Ple (or lion lentivirus; LLV), FIV-Pco (or puma lentivirus; PLV), FIV-Oma, FIV-Lru, FIV-Hya, FIV-Aju, FIV-Ppa, FIV-Lpa and FIV-Ccr, respectively (Rigby et al., 1993; Brown et al., 1994; Langley et al., 1994; Carpenter et al., 1996; Barr et al., 1997; Carpenter et al., 1998; Nishimura et al., 1999; VandeWoude and Apetrei, 2006; Troyer et al., 2008).

Interestingly, while FIV infection in domestic cats leads to the development of a severe immunodeficiency, FIV infection of non-domestic cats is usually not associated with disease (Brown et al., 1994; Langley et al., 1994; Osborne et al., 1994; Carpenter and Obrien, 1995; Barr et al., 1997; Packer et

al., 1999). This resembles the correlation between pathogenic HIV infections in humans and asymptomatic SIV infections in non-human primates.

1.3.1 Seroprevalence of FIV in the *Felidae* and *Hyaenidae*

The determination of the seroprevalence of FIVs in non-domestic cats relies greatly on domestic cat-, lion- and puma-based Western blot analyses (VandeWoude et al., 2002; VandeWoude et al., 2003; Troyer et al., 2005). Anti-FIV antibodies in other species can be detected using a multistrain-antigen Western blot approach (Ostrowski et al., 2003; Troyer et al., 2005). The seroprevalence of FIVs varies by species and geographic location (reviewed in: VandeWoude and Apetrei [2006]). Table 1-1 summarises the results of several studies that have investigated FIV seroprevalence and pathogenicity in free-ranging animals from the nine species of *Felidae* and from spotted hyenas in which species-specific FIVs have been identified.

In summary, African lions and leopards, pumas and Pallas' cats show very high seroprevalence rates. Interestingly, FIV infection is more prevalent in African and American populations than in Asian populations. It can be assumed that population densities in Asia are too low to support effective intra-species transmission. Cross-species FIV transmission events are rare and have been documented primarily for captive non-domestic felids (Carpenter et al., 1996; Nishimura et al., 1999; Troyer et al., 2005). This suggests that the artificially close contact in captivity settings contributed to the opportunity for cross-species transmission events to occur, strengthening the concept of species-specific FIV strains.

Table 1-1 FIV prevalence in *Felidae* and *Hyenidae* species (adapted from VandeWoude and Apetrei [2006]).

Common name (species)	Geographic location	Virus	Seroprevalence (%)	Pathogenicity	References
Domestic cat (*Felis catus*)	Worldwide	FIV-Fca	1-35	AIDS	(Sukura et al., 1992; Peri et al., 1994; Lin et al., 1995; Carpenter et al., 1998; Dorny et al., 2002; Lee et al., 2002; Maruyama et al., 2003; Troyer et al., 2005)
Lion (*Panthera leo*)	Africa (Serengeti, Ngorongor Crater, Lake Manyara, Kruger National Park) Namibia Botswana Asia	FIV-Ple (LLV)	80-90 0 26 0	No association with increased morbidity in Serengeti lions	(Olmsted et al., 1992a; Spencer et al., 1992; Brown et al., 1994; Carpenter et al., 1996; Hofmann-Lehmann et al., 1996; Troyer et al., 2004) (Lutz et al., 1992; Brown et al., 1994) (Osofsky et al., 1996) (Lutz et al., 1992; Spencer et al., 1992; Brown et al., 1994)
Puma, mountain lion or cougar (*Puma concolor*)	North, Central and South America Wyoming, Montana	FIV-Pco (PLV)	25 58	Possible $CD4^+$ T lymphocyte depletion; no association with increased morbidity in Midwestern cougars	(Olmsted et al., 1992b; Langley et al., 1994; Carpenter et al., 1996; Troyer et al., 2005) (Biek et al., 2003)

	Washington Florida (Florida panther)		25 15-76		(Evermann et al., 1997) (Miller et al., 2006)
Pallas cat (*Otocolobus manus*)	Mongolia	FIV-Oma	>80	None detected	(Barr et al., 1997; Troyer et al., 2005)
Bobcat (*Lynx rufus*)	California	FIV-Lru	0-30	None detected	(Riley et al., 2004; Troyer et al., 2005)
Jaguarundi (*Herpailurus yagouaroundi*)	Central/South America	FIV-Hya	Ca. 20	None detected	(Langley et al., 1994; Troyer et al., 2005)
Cheetah (*Acinonyx jubatus*)	Africa	FIV-Aju	<10	None detected	(Olmsted et al., 1992b; Osofsky et al., 1996; Munson et al., 2004; Troyer et al., 2005)
Leopard (*Panthera pardus*)	Botswana, Africa Asia	FIV-Ppa	16, ca. 50 0	None detected	(Osofsky et al., 1996; Troyer et al., 2005) (Troyer et al., 2005)
Ocelot (*Leopardus pardalis*)	Central/South America	FIV-Lpa	10	None detected	(Langley et al., 1994; Troyer et al., 2005)
Spotted hyena (*Crocuta crocuta*)	Masai Mara National Reserve (Serengeti)	FIV-Ccr	10-30	None detected	(Harrison et al., 2004; Troyer et al., 2005)

1.3.2 Pathogenicity of FIV in non-domestic cats

The most striking observation to be made from the studies of FIV infections in non-domestic felids is that FIV seems to be apathogenic and infections are mostly benign (Lutz et al., 1992; Carpenter and Obrien, 1995; Packer et al., 1999). This is in stark contrast to FIV infections in domestic cats, which lead to the development of immunodeficiency.

FIV infections of lions and pumas have been examined both on a systemic and on a cellular basis. The findings have been compared to those in domestic cats in order to determine differences in viral cell and tissue tropism, viral pathogenicity and host immune responses to FIV infection. FIV-seropositive lion and puma populations were monitored for a potential impact of FIV infection on survival, fecundity or susceptibility to secondary infections (Hofmann-Lehmann et al., 1996; Biek et al., 2006a; Biek et al., 2006b). In free-ranging animals, no correlation between FIV infection and a reduction in fitness was found (Biek et al., 2006b; Biek et al., 2006c). However, proviral and plasma viral loads of FIV-Pco infected pumas were comparable with those of FIV-positive domestic cats (Poss et al., 2008). Additionally, there are several reports that FIV infection causes $CD4^+$ T lymphocyte depletion in pumas and to a larger extent in lions (Roelke et al., 2006; Roelke et al., 2009). In a population of Botswana lions, a decreased $CD4^+/CD8^+$ ratio correlated with lymphadenopathy, gingivitis and papillomas (Roelke et al., 2009).

Alterations in T cell numbers may not result in terminal-stage feline AIDS but could render these animals susceptible to opportunistic infections. A canine distemper virus (CDV) outbreak has been reported in FIV-positive (but not FIV-negative) lion populations in the Serengeti in 1994 which caused significant mortality (Roelke-Parker et al., 2010). Moreover, a number of FIV-positive Florida panthers died in a recent FeLV outbreak (M. Cunningham, unpublished data).

1.3.3 Infectivity of lion and puma lentiviruses for domestic cats

Experimental infection of domestic cats with either FIV-Ple or FIV-Pco leads to

the development of persistent cell-associated viraemia, transient plasma viraemia and lymphadenopathy, and seroconversion in the absence of disease (VandeWoude et al., 1997a; VandeWoude et al., 1997b; VandeWoude et al., 2002; VandeWoude et al., 2003; TerWee et al., 2005). Moreover, $CD4^+/CD8^+$ ratios were unaltered compared to uninfected control animals (VandeWoude et al., 1997a). These findings indicate that immunodeficiency in domestic cats is not primarily caused by a direct viral cytopathic effect (TerWee et al., 2005). However, it should be noted that the FIV-Ple and FIV-Pco isolates used in these studies had been propagated and amplified in the feline lymphoma cell line 3201 for a considerable length of time prior to inoculation into cats, which could have resulted in their attenuation. Remarkably, infection of domestic cats with non-pathogenic FIV-Pco prevented $CD4^+$ T lymphocyte depletion upon subsequent superinfection with virulent FIV-Fca (VandeWoude et al., 2002; TerWee et al., 2008). This resistance to superinfection was not mediated by acquired immunity but correlated with an increase in IFN-γ expression (TerWee et al., 2008).

1.3.4 Growth of lion and puma lentiviruses in feline cells

To assess the cell tropism of FIV-Ple and FIV-Pco *in vitro*, feline cell lines and primary cells such as feline lymphoma 3201 cells, a feline adherent lymph node cell (LNC) line as well as cat, lion and puma PBMCs were infected with these viruses (VandeWoude et al., 1997b). Cell-free FIV-Ple was able to infect cat PBMCs and to a lesser extent lion PBMCs, but not LNCs. Cell-free FIV-Pco did not infect cat or puma PBMCs. Cell-associated FIV-Pco (PBMCs from a seropositive puma from British Columbia) caused a low-titre infection in cat PBMCs and a high-titre infection in puma PBMCs. All three viruses replicated best in 3201 cells (VandeWoude et al., 1997b). Interestingly, infection of cat 3201 cells and PBMCs with FIV-Pco imparted resistance to FIV-Fca superinfection (VandeWoude et al., 2002).

Similar infection assays were performed in our laboratory. The IL-2 dependent feline T cell line Mya-1 (Miyazawa et al., 1989) and primary lion T lymphocytes were infected with different strains of FIV-Fca, FIV-Ple and FIV-Pco (Fig. 1-4).

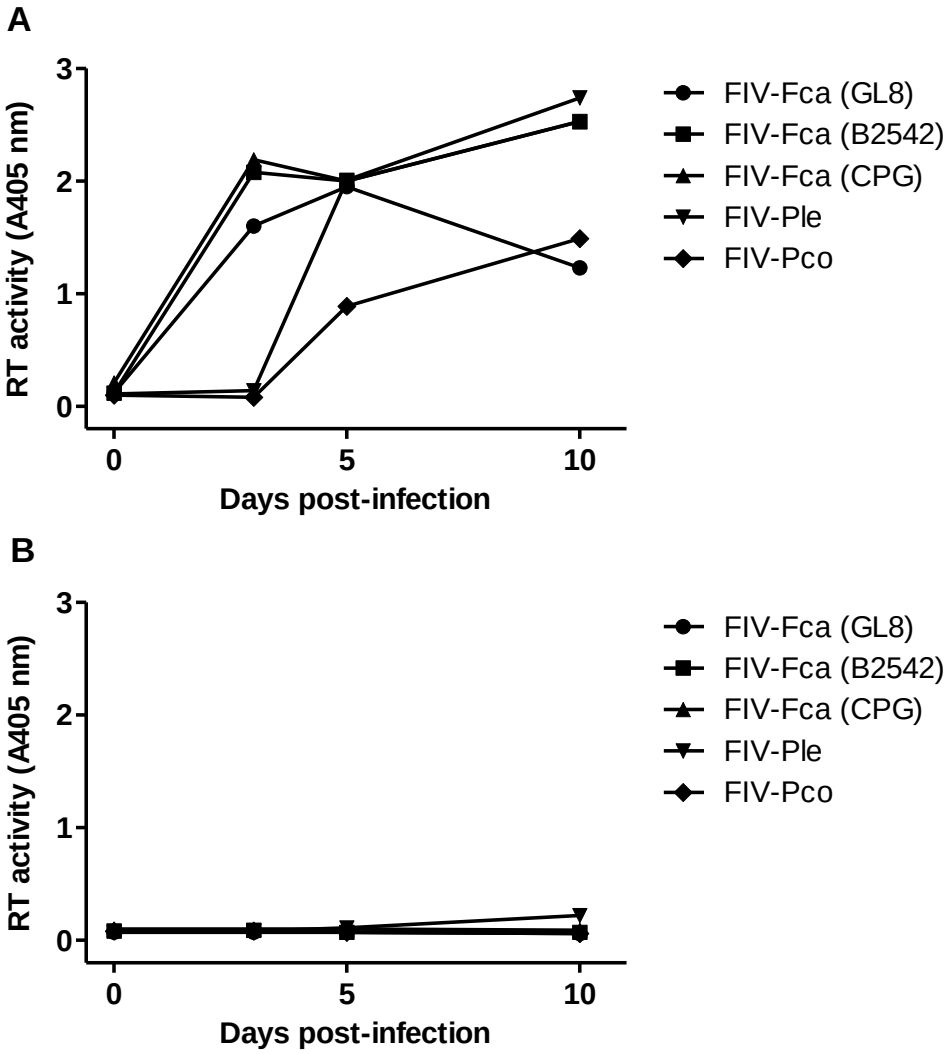


Figure 1-4 FIV cell tropism. The IL-2 dependent feline T cell line Mya-1 (A) and primary T lymphocytes obtained from an Angolan lion (B) were infected with divergent FIV-Fca strains GL8, B2542 and CPG, FIV-Ple and FIV-Pco. Virus production was determined on day 0, 3, 5 and 10 post-infection using a lentiviral RT assay. Whereas virus grew readily in Mya-1 cells, lion T lymphocytes were resistant to virus infection.

Curiously, we observed that all viruses grew readily in Mya-1 cells, whereas lion T lymphocytes were resistant to infection (unpublished data). The inability of different FIVs (including FIV-Ple) to productively infect lion T lymphocytes is somewhat surprising given the fact that FIV-Ple infected lions show a high viraemia (Poss et al., 2008). Because the FIV-Ple isolate used in our study was obtained from groups that had passaged the virus for a long period of time in

3201 cells (VandeWoude et al., 1997a; VandeWoude et al., 1997b; VandeWoude et al., 2002; VandeWoude et al., 2003; TerWee et al., 2005), it is possible that the virus was attenuated. It would be interesting to determine if primary isolates of FIV-Ple can indeed infect lion T lymphocytes. This would point towards a selective restriction of FIV-Fca growth in lion T cells. In fact, the study presented here aims at elucidating the molecular bases for the differential disease outcome seen in FIV infections of domestic and non-domestic cats and the differences in the susceptibility of cat and lion T lymphocytes to lentiviral infection.

1.4 Co-evolution between the *Felidae* and FIV

The apparent lack of pathogenicity of FIV in non-domestic cats may be a consequence of prolonged periods of host-virus co-evolution that resulted either in an increased ability of the non-domestic cat immune systems to restrict FIV replication or in a decreased virulence of non-domestic cat FIV strains, or both. In contrast, FIV may be a relatively recent introduction into the domestic cat lineage resulting in the absence of host-virus co-adaptation and in a fatal disease outcome. Evidence for this hypothesis emerges from studies on the evolutionary history of the *Felidae* and on FIV evolution dynamics in feline populations.

1.4.1 Evolutionary history of the *Felidae*

Modern felids arose from a common ancestor during the late Miocene (ca. 10.8 MYA) in Asia (Johnson et al., 2006). They diverged within a relatively short period of time (10.8 MYA to 6.2 MYA) into eight distinct evolutionary lineages comprising a total of 38 feline species (Johnson et al., 2006). The radiation of modern felids began with the divergence of the *Panthera* lineage followed by the split of the bay cat (9.4 MYA), the caracal (8.5 MYA) and the ocelot (8 MYA) lineages from the ancestor of more recent lineages. The divergence of the lynx lineage (7.2 MYA) was closely followed by the emergence of the puma lineage (6.7 MYA) and the more recently derived leopard and domestic cat lineages (6.2 MYA) (Johnson et al., 2006). Within-lineage species differentiations took place

during the late Miocene/early Pliocene (6.4 MYA to 2.9 MYA) and during the late Pliocene/Pleistocene (3.1 MYA to 0.7 MYA).

Of the feline species relevant to this study the puma is the oldest species and arose approximately 4.5 MYA (Pecon-Slattery et al., 2004; Johnson et al., 2006). African lion species arose 2 MYA and the domestic cat evolved as a unique felid lineage circa 0.99 MYA (Johnson et al., 2006). Lineage and species differentiation events were facilitated by multiple intercontinental migration movements starting from Asia. Early migration of a progenitor of the caracal lineage to Africa occurred around 8.5 MYA to 5.6 MYA. Another migration event translocated a common ancestor of the ocelot, lynx, puma, leopard and domestic cat lineages across the then present Bering Strait land bridge to North America (8.5 MYA to 8 MYA) (Johnson et al., 2006). The leopard and domestic cat lineages either diverged from ancestors that had remained in Asia or from North American ancestors that had crossed the Bering Strait land bridge back to Asia. Additionally, Asiatic *Panthera* species migrated to America (jaguars, lions) and to Africa (lions, leopards) during the late Pliocene/early Pleistocene (Johnson et al., 2006).

The recent evolutionary history and phylogeography of North American pumas and African lions have been studied in detail. North American pumas were extirpated from North America during the last ice age of the late Pleistocene but re-emerged from Brazil about 10,000 to 12,000 years ago (Culver et al., 2000). At present, they inhabit Western continental regions of the United States of America including Arizona, Texas, Colorado and Wyoming (Carpenter et al., 1996; Culver et al., 2000; Biek et al., 2006a). The most recent ancestor of modern lion populations arose about 325,000 years ago from Eastern and Southern African lions and subsequently migrated into Central and North Africa and into Eurasia about 100,000 years ago (Antunes et al., 2008). These population expansions were followed by population reductions. At present, there are eleven lion populations in East Africa (Uganda, Kenia, Serengeti National Park, Ngorongoro Crater) and South Africa (Kruger National Park, Botswana, Namibia) and in Asia (Gir Forest, India) (Fig. 1-5) (Antunes et al., 2008). Interestingly, the Serengeti National Park accommodates three distinct lion populations that admixed recently.

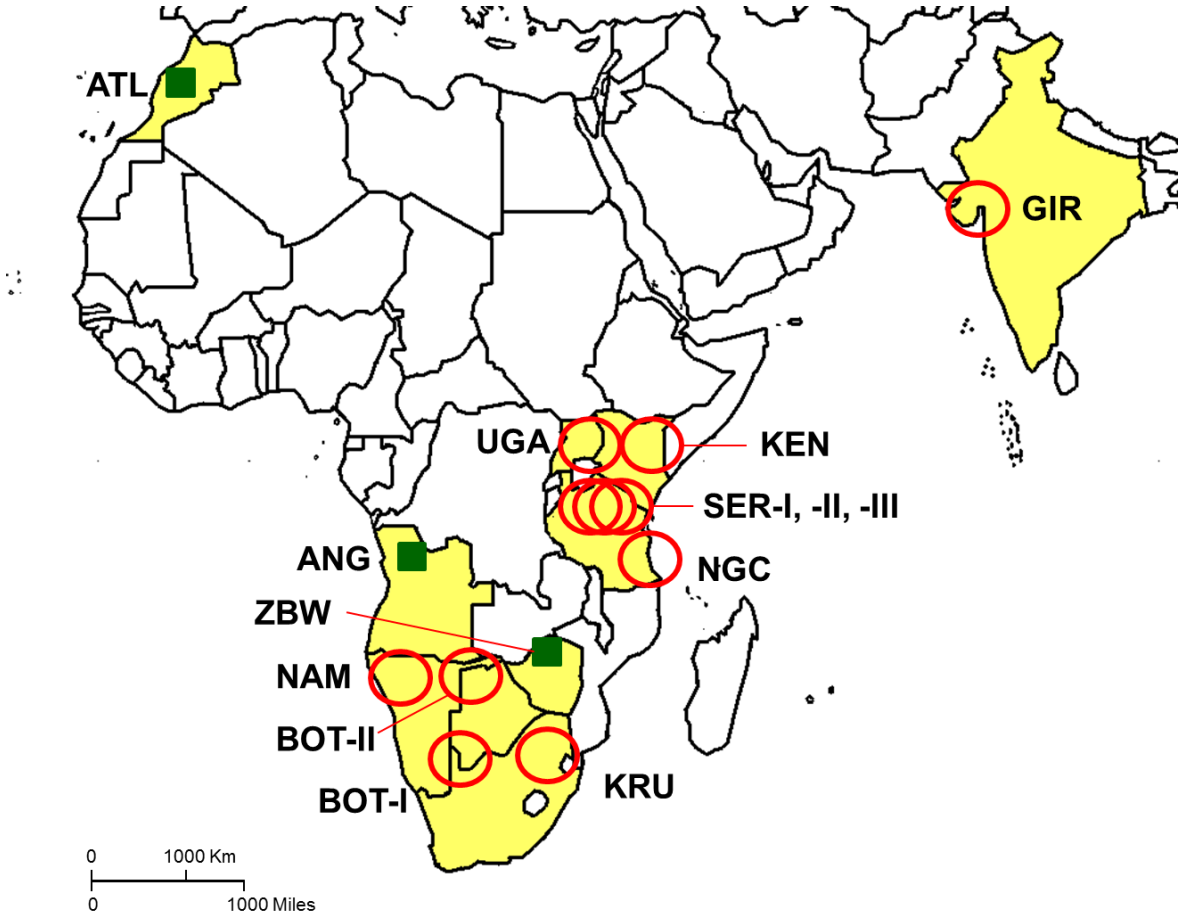


Figure 1-5 Geographic location of extant lion (*Panthera leo*) populations. Analysis of microsatellite and sequencing data of 357 lions from Africa and the Gir forest in India revealed the existence of eleven distinct lion populations that cluster geographically (Antunes et al., 2008). These lion population (red circles) can be found in East Africa (Uganda, UGA; Kenia, KEN; Serengeti National Park, SER; Ngorongoro Crater, NGC), in South Africa (Kruger National Park, KRU; Botswana, BOT; Namibia, NAM) and in India (Gir Forest, GIR). Botswana and the Serengeti National Park accommodate two or three distinct lion populations (BOT-I and BOT-II, and SER-I, SER-II and SER-III, respectively). Furthermore, Antunes et al. (2008) included lions from Zimbabwe (ZBW), Angola (ANG) and the Atlas mountains in Morocco (ATL) into their study (green squares). These lions were representative of isolated, endangered or depleted populations.

1.4.2 Phylogeny of FIV and FIV population dynamics in the *Felidae*

Based on the widespread occurrence of FIV and other lentiviruses in Africa together with large FIV interspecies divergences, which point towards a long FIV residence time within these species, an ancestral origin of FIV in Africa followed by interspecies transmission events and a global dissemination of FIV has been proposed (Pecon-Slattery et al., 2008b). The global dissemination of FIV from Africa occurred as a consequence of felid transcontinental migration movements into Eurasia and the Americas. Such a migration event across the Bering Strait land bridge took place around 4.5 MYA and lead to the introduction of FIV into seven species of the ocelot lineage, into cheetahs, pumas and jaguarundi (puma lineage) and into four modern species of the lynx lineage (Johnson et al., 2006). Subsequently, FIV was disseminated from North America to Central and South America (Johnson et al., 2006).

Phylogenetic analyses of the genetic variation in the conserved *pol* and *gag* genes and the very diverse *env* gene of FIV demonstrated that FIV lineages are highly divergent but are monophyletic and species-specific (Phillips et al., 1990; Olmsted et al., 1992b; Carpenter et al., 1996; Carpenter et al., 1998; Troyer et al., 2005). FIV-Pco can be divided into two major clades (A and B) and eight distinct viral lineages (Biek et al., 2006a). The two clades are highly divergent and appear to be paraphyletic as a consequence of two separate introductions of FIV into the puma lineage (Troyer et al., 2008). Clade A viruses can be found in Florida and California and clade B viruses in other parts of North America, Central America and Brazil. FIV-Pco lineages lack broad geographic associations consistent with recent and frequent FIV-Pco transmission events between puma populations in different locations (Carpenter et al., 1996).

FIV-Ple diverges into six clades (A-F) that are endemic in distinct geographic areas (see Fig. 1-5) (Brown et al., 1994; Troyer et al., 2004; O'Brien et al., 2006; Antunes et al., 2008). Subtype A is present in the Serengeti National Park, the Kruger National Park and Botswana. Subtype B is endemic in lion populations in Uganda, the Serengeti National Park, the Ngorongoro Crater and Botswana. Subtypes C and D can be found in the Serengeti and in the Kruger National Parks, respectively. Subtype E is present in Botswana and subtype F in Kenya (Brown et al., 1994; Troyer et al., 2004; O'Brien et al., 2006; Antunes et

al., 2008). The three lion populations in the Gir forest in India, in southern Botswana and in Namibia are not infected with FIV (Brown et al., 1994; O'Brien et al., 2006). Reasons for the absence of FIV in these populations may be their physical separation from infected populations (for example by the Kalahari desert) or that FIV transmission cannot occur due to too low population densities.

FIV-Fca falls into the five clades A-E with a majority of viruses belonging to either clade A or B (Sodora et al., 1994; Kakinuma et al., 1995; Sodora et al., 1995; Pecoraro et al., 1996; Elder et al., 1998). Clade A and B viruses show a worldwide distribution; however, clade A viruses are predominant in the western part of the United States of America, northern Japan, Germany and South Africa, whereas clade B viral isolates are predominant in eastern Japan, Italy, Portugal and in eastern and central parts of the United States of America (Sodora et al., 1994; Nishimura et al., 1998). Clades C, D and E viruses are rarer. Clade C viruses are present in northern Taiwan (Sodora et al., 1994; Inada et al., 1997; Uema et al., 1999), clade D isolates in western Japan (Nishimura et al., 1998) and clade E viruses in Argentina (Pecoraro et al., 1996).

In general, pathogenic lentiviral infections are characterised by rapid virus evolution, high intrahost diversity and positive selection on several virus genes (Coffin, 1995; Crandall et al., 1999; Rambaut et al., 2004). In non-pathogenic lentivirus infections, however, there is purifying selection on the virus, which usually shows a slower rate of evolution and high interhost diversity (Coffin, 1995; Crandall et al., 1999; Poss et al., 2008). Phylogenetic analyses of the FIV-Fca, FIV-Ple and FIV-Pco genome sequences showed a greater nucleotide diversity within FIV-Pco and FIV-Ple than within FIV-Fca (Brown et al., 1994; Burkala and Poss, 2007). The divergence between FIV-Pco clades A and B is high (20-27%) and comparable to that observed between FIV-Ple clades and significantly higher than that between FIV-Fca clades (Poss et al., 2008). The sequence variation between FIV-Pco lineages within FIV-Pco clades A and B is also very high (up to 24% in clade B) and comparable to the interclade diversity seen for domestic cat FIV clades (13-17%) (Poss et al., 2008). However, the intrahost diversity of FIV-Pco per gene in pumas is lower than 1%. The genome diversity within and between FIV-Ple clades is large, too. In contrast, FIV-Fca isolates exhibit only minimal interclade and intraclade genetic variation (Poss et al., 2008).

Furthermore, FIV-Pco shows slower evolutionary rates compared to FIV-Fca or HIV-1 and purifying selection is the predominant force changing the gene sequences (Poss et al., 2008); (Biek et al., 2003). However, a significant degree of positive selection can be seen in the FIV-Fca genome (Burkala and Poss, 2007). These findings suggest that FIV infection of pumas was the most ancient event (Pecon-Slattery et al., 2008b; Poss et al., 2008), followed by FIV transmission to lions (Brown et al., 1994; Carpenter and Obrien, 1995). The low degree of FIV-Fca divergence points towards a recent emergence in combination with rapid viral diversification within the domestic cat (Pecon-Slattery et al., 2008b). It is very likely that the extended periods of endemic lentiviral infection in pumas and lions have improved the restrictive ability of the puma and lion immune system to FIV infection and reduced the pathogenicity of FIV-Pco and FIV-Ple (Carpenter and Obrien, 1995; Burkala and Poss, 2007). In contrast, the relationship between the domestic cat and FIV-Fca seems to be unbalanced, thus allowing disease to progress.

1.5 Antiretroviral intrinsic immunity

The reduced pathogenicity of FIV infections in non-domestic felids combined with the restriction of FIV replication seen in lion T lymphocytes can best be explained by the presence of potent antiretroviral immune effector molecules in these cells that inhibit FIV growth at a post-entry stage of the lentiviral life cycle. The mammalian intrinsic immunity, a specific arm of the innate immunity, is the first line of defence against pathogens (Bieniasz, 2004; Goff, 2004). The intrinsic immunity acts intracellularly and comprises so-called restriction factors. Restriction factors are proteins that block viral replication at different stages of the viral life cycle. They are either constitutively expressed or their expression can be upregulated by pattern recognition receptor (PPR)-mediated innate immune signalling cascades, which upon detection of viral genomes initiate the transcription of genes encoding for restriction factors, among others.. The cell-type and species-specific expression and activity of restriction factors control the viral host spectrum and may impose a barrier to cross-species transmission events (Troyer et al., 2008). Well-studied examples of such host restriction factors are TRIM5α, an E3 ubiquitin ligase that binds

incoming retroviral capsids in the cytoplasm via its C-terminal PRY/SPRY (B30.2) domain and targets them for proteasomal degradation (Reymond et al., 2001; Stremlau et al., 2004), and APOBEC3 proteins, cytidine deaminases that induce hypermutations and impair viral reverse transcription (Teng et al., 1993; Sheehy et al., 2002; Mangeat et al., 2003; Zhang et al., 2003). Tetherin (BST-2, CD317) is a transmembrane protein that potently inhibits the release of nascent retrovirus particles in single-cycle replication assays (Neil et al., 2008; Van Damme et al., 2008). In order to efficiently replicate and evade immune surveillance, retroviruses have to overcome this line of defence and, thus, have evolved proteins that antagonise the actions of restriction factors or mechanisms to avoid them. Lentiviral Vif proteins (Sheehy et al., 2002; Sheehy et al., 2003; Munk et al., 2008) and spumaviral Bet proteins (Lochelt et al., 2005; Russell et al., 2005; Perkovic et al., 2009) counteract APOBEC3 proteins whereas HIV-1 and certain SIV Vpus, HIV-2 and SIV Envs, and SIV Nefs counteract tetherins (Neil et al., 2007; Neil et al., 2008; Van Damme et al., 2008; Gupta et al., 2009b; Jia et al., 2009; Le Tortorec and Neil, 2009; Sauter et al., 2009; Zhang et al., 2009a; Yang et al., 2010). The permanent conflict between host restriction factors and viral restriction factor antagonists has led to a rapid positive selection on both groups of proteins (Sawyer et al., 2004; McNatt et al., 2009; Ortiz et al., 2009).

1.5.1 Innate interferon response

Restriction factors are effector molecules of the interferon immune signalling pathways. Interferon signalling is triggered by pattern recognition. Several families of pattern recognition receptors are involved in the detection of pathogen-associated molecular patterns (PAMPs) such as the viral genome (Barbalat et al., 2011). Members of the Toll-like receptor (TLR) family have been identified as sensors of viral nucleic acid. Signalling through TLRs results in the production of type I IFNs (especially IFN-α and IFN-ß) and inflammatory cytokines such as IL-6 and IL-12, and stimulates DC maturation and the establishment of antiviral innate immunity (reviewed in: (Medzhitov and Janeway, 1997; Akira et al., 2001; Janeway and Medzhitov, 2002; Takeda et al., 2003; Iwasaki and Medzhitov, 2004)). A second group of viral nucleic acid sensors are cytosolic retinoic acid-inducible gene I (RIG-I)-like RNA helicases (Yoneyama

et al., 2004; Yoneyama and Fujita, 2007). The expression of these two classes of PPRs is cell-type specific, and they induce IFN production via distinct signalling pathways.

TLRs that detect the presence of viruses include TLR3, TLR7 and TLR9, all of which can be found exclusively in endosomal compartments. TLR3 is localised in intracellular vesicles in conventional DCs (cDCs) (Matsumoto et al., 2003; Iwasaki and Medzhitov, 2004; Sousa, 2004) and responds to viral double-stranded RNA (dsRNA) (Alexopoulou et al., 2001). TLR3-dependent immune signalling is initiated by phagocytosis of apoptotic bodies of infected or dsRNA-containing cells by cDCs, which induces the maturation of these cDCs and type I IFN production (Schulz et al., 2005). DC maturation and secreted IFN promote cross-priming of T lymphocytes leading to antigen-specific $CD4^+$ and $CD8^+$ T cell responses (Schulz et al., 2005). TLR7 and TLR9 are highly expressed in plasmacytoid DCs (pDCs) (Iwasaki and Medzhitov, 2004; Sousa, 2004). They recognise nucleic acids of viruses that are taken up by pDCs and are subjected to proteolytic degradation in the endosomal compartment. TLR7 can be activated by single-stranded RNA (ssRNA) rich in guanosine or uridine from viruses such as HIV and influenza (Diebold et al., 2004; Heil et al., 2004; Lund et al., 2004). TLR9 recognises viral single-stranded DNA (dsDNA) containing unmethylated cytidine-phosphate-guanosine (CpG) DNA motifs (Hemmi et al., 2003). TLR7 and TLR9-dependent signalling in pDCs leads to the synthesis of large amounts of type I IFN, in particular of IFN-α (Liu, 2005). Upon agonist binding, TLRs signal via adaptor molecules to phosphorylate and activate latent transcription factors which then translocate to the nucleus to regulate the expression of IFNs. TLR3 signals via TRIF (Toll/IL-1 receptor domain-containing adaptor inducing IFN-ß) (Yamamoto et al., 2002; Yamamoto et al., 2003), which interacts with the kinases TBK-1 (Tank-binding kinase 1) and IKKε (IkappaB kinase epsilon) to phosphorylate the transcription factors IRF3 (IFN-regulatory factor 3) and IRF7 (Fitzgerald et al., 2003). This signalling cascade leads primarily to the production of IFN-ß. Alternatively, TRIF can activate the transcription factors ATF2 (activating transcription factor 2)/c-Jun and NF-κB, which stimulates production of type I interferon and inflammatory cytokines (Gohda et al., 2004). In pDCs, TLR7 and TLR9 signal via the adaptor molecule MyD88 (myeloid differentiation primary response gene 88), the kinase IRAK1 (IL-1 receptor-associated kinase 1) and TRAF6 (TNF receptor-associated factor 6) to

phosphorylate the transcription factor IRF7 (Hoshino et al., 2002; Hemmi et al., 2003; Akira and Takeda, 2004; Honda et al., 2004; Kawai et al., 2004; Uematsu and Akira, 2007). IRF7 induces type I IFN production in pDCs. In cell types other than pDCs this pathway is non-functional and TLR7/TLR9 stimulation couples TRAF6 to the activation of ATF2/c-Jun and NF-κB (Akira and Takeda, 2004). In this case, expression of cytokines such as IL-6, IL-12 and TNF is triggered (Akira and Takeda, 2004).

RIG-I-like RNA helicases are cytosolic PPRs, which detect dsRNA produced during viral replication. RIG-I itself is expressed in cDCs, fibroblasts and epithelial cells (Kato et al., 2005; Wilkins and Gale, 2010) and can signal for IRF3, IRF7 (Fitzgerald et al., 2003) and NF-κB activation (Balachandran et al., 2004), which leads to the induction of type I IFNs (Yoneyama et al., 2004; Onoguchi et al., 2007). ISP-1 (IFN-ß promoter stimulator-1) serves as adaptor mediating between RIG-I and the transcription factors (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005).

Subsequently, secreted IFNs bind and activate the type I IFN receptor (IFNAR), which is a common cell-surface receptor. IFNAR is composed of two distinct chains, IFNAR1 and IFNAR2, which are associated with the Janus activated kinases (JAKs) TYK2 (tyrosine kinase 2) and JAK1, respectively (Silvennoinen et al., 1993). Binding of IFNs to IFNAR induces the JAK-STAT signalling pathway (Fu et al., 1992; Schindler et al., 1992; Silvennoinen et al., 1993) through JAK-mediated tyrosine phosphorylation of the transcription factors STAT1 (signal transducer and activator of transcription 1) and STAT2. This then leads to the formation of STAT1/STAT2/IRF9 complexes, which are also known as ISGF3 (IFN-stimulated gene factor 3) complexes (Levy et al., 1988; Levy et al., 1989; Kessler et al., 1990). These complexes translocate to the nucleus and bind to IFN-stimulated response elements (ISREs) (Israel et al., 1986; Levy et al., 1988) present in promoters of certain genes (so-called IFN-stimulated genes, ISGs) to initiate their transcription (Der et al., 1998). ISGs have antiviral, antiproliferative or immunomodulatory properties. Additionally, type I and type II (IFN-γ) IFNs can cause the formation of STAT homodimers or heterodimers, which can also enter the nucleus to stimulate transcription of genes containing GAS (IFN-γ-activated site) elements in their promoters.

Among the hundreds of ISGs that have been identified (Der et al., 1998) are the host restriction factors whose expression leads to the induction of a

heightened antiviral state. TRIM, APOBEC3 and tetherin genes contain ISREs in their promoters (Asaoka et al., 2005; Tanaka et al., 2006; Carthagena et al., 2009). TRIM5α is expressed in the absence of IFNs but their presence increases TRIM5α expression (Asaoka et al., 2005; Sakuma et al., 2007; Carthagena et al., 2008). Type I IFNs induce APOBEC3s in resting T lymphocytes, macrophages and DCs (Chen et al., 2006; Peng et al., 2006; Wang et al., 2008). Tetherin is constitutively expressed in pDCs (Blasius et al., 2006). In $CD4^+$ T lymphocytes and macrophages, tetherin expression is upregulated by cellular activation and maturation but further enhanced by type I IFNs (Neil et al., 2008; Miyagi et al., 2009).

1.5.2 TRIM5α

TRIM5α is the longest (alpha) isoform of the host protein TRIM5, a member of the tri-partite motif (TRIM) family of proteins (Reymond et al., 2001; Stremlau et al., 2004). This family is large, with at least 68 intact members in the human genome, which are involved in diverse cellular processes such as cell proliferation, differentiation, development, oncogenesis or apoptosis (Nisole et al., 2005). Some TRIM proteins, including TRIM1 (Yap et al., 2004), TRIM5α (Hatziioannou et al., 2004b; Keckesova et al., 2004; Perron et al., 2004; Stremlau et al., 2004; Yap et al., 2004), TRIM19 (Turelli et al., 2001) and TRIM22 (Tissot and Mechti, 1995) form part of the host antiretroviral intrinsic immunity. TRIM5α was identified as the factor responsible for the previously reported Lv1 (lentivirus susceptibility factor 1) and Ref1 (resistance factor 1) antiretroviral activities in rhesus monkey and human cells, respectively (Hatziioannou et al., 2004b; Keckesova et al., 2004; Perron et al., 2004; Stremlau et al., 2004; Yap et al., 2004). It mediates a species-specific, early post-entry block to retroviral infection.

1.5.2.1 Domain structure of TRIM proteins

TRIM proteins typically comprise a RING domain with E3-ubiquitin ligase activity capable of auto-ubiquitination, a B-box 2 domain and a coiled-coil domain

necessary for TRIM oligomerisation, referred to collectively as the RBCC (Reymond et al., 2001). Some TRIM proteins, including TRIM5α (Fig. 1-6), possess a C-terminal B30.2 (PRY/SPRY) domain that is thought to mediate binding of TRIM proteins to the incoming capsid of restriction-sensitive retroviruses (Mische et al., 2005; Sebastian and Luban, 2005; Stremlau et al., 2006).

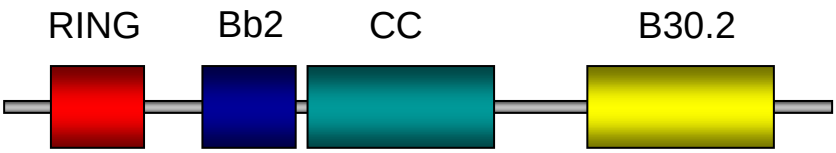


Figure 1-6 Schematic representation of the TRIM5α domain topology. Members of the TRIM family comprise an N-terminal RING domain (RING) with E3-ubiquitin ligase activity, followed by a B-box 2 (Bb2) and a coiled-coil (CC) domain important for protein multimerisation. TRIM5α also possesses a C-terminal B30.2 (PRY/SPRY) domain (B30.2) involved in the binding of retroviral capsid.

1.5.2.2 Mechanism of the antiretroviral activity of TRIM5α

TRIM5α leads to a block in reverse transcription in most non-permissive cells (Stremlau et al., 2004); Keckesova et al., 2004). Evidence suggests that TRIM5α binds directly to the retroviral capsid lattice in the cytoplasm with the help of its B30.2 (PRY/SPRY) domain (Mische et al., 2005; Sebastian and Luban, 2005; Stremlau et al., 2006). The RING domain then catalyses the ubiquitination of the capsid/TRIM5α complex and targets it for a rapid proteasome-mediated degradation so that the virus cannot complete reverse transcription (Diaz-Griffero et al., 2006a; Towers, 2007). While inhibition of the proteasome prevents degradation of the viral core and enables reverse transcription to proceed, virus remains non-infectious and the process of infection does not complete (Anderson et al., 2006; Wu et al., 2006; Campbell et al., 2008), indicating an additional proteasome-independent antiviral function for TRIM5α. Accelerated uncoating of the viral capsid from the incoming virion may underlie

this proteasome-independent restriction activity (Stremlau et al., 2006; Perron et al., 2007).

1.5.2.3 Specificity of the TRIM5α-capsid interaction

Human and non-human primate TRIM5α variants display significant differences in their specificity to lentiviral capsids which govern the species tropism of lentiviruses and prevent their cross-species transmission. Non-human primates can be grouped into apes, Old World monkeys such as guenons (*Cercopithecus* spp.), macaques (*Macaca* spp.), members of the genus *Chlorocebus* and white-eyelid mangabeys (*Cercocebus* spp.), and New World monkeys such as squirrel monkeys (*Saimiri* spp.) and night or owl monkeys (*Aotus* spp.).

Human TRIM5α (huTRIM5α) potently inhibits pre-integration stages of murine leukaemia virus N-strain (MLV-N) replication (Hatziioannou et al., 2004b; Perron et al., 2004; Yap et al., 2004) but shows only weak antiviral activity against HIV-1 and SIV of Old World rhesus macaques (*Macaca mulatta*; SIVmac) (Stremlau et al., 2004; Perez-Caballero et al., 2005; Sawyer et al., 2005; Stremlau et al., 2005; Yap et al., 2005). HIV-2 replication is moderately reduced in human cells (Ylinen et al., 2005). In contrast, Old World rhesus macaque and African green monkey (*Chlorocebus sabaeus*) TRIM5α (rhTRIM5α and agmTRIM5α, respectively) restrict HIV-1 and HIV-2 infectivity, but not that of MLV-N (Hatziioannou et al., 2004b; Keckesova et al., 2004; Stremlau et al., 2004; Yap et al., 2004; Ylinen et al., 2005). Moreover, agmTRIM5α is able to inhibit SIVmac, whereas its rhesus macaque homologue is not (Hatziioannou et al., 2004b; Keckesova et al., 2004; Stremlau et al., 2004; Nakayama et al., 2005). Most New World monkey cells block SIVmac, but not HIV-1 or HIV-2 (Hofmann et al., 1999; Ylinen et al., 2005). Owl monkey (*Aotus trivirgatus*) kidney (OMK) cells are unusual among New World monkey cells in that they allow SIVmac infection but restrict HIV-1 infection (Hofmann et al., 1999).

The antiviral specificity of TRIM5α has been mapped to residues in the B30.2 (PRY/SPRY) domain that are involved in lentiviral capsid recognition (Stremlau et al., 2004; Nakayama et al., 2005; Perez-Caballero et al., 2005; Sawyer et al., 2005; Sebastian and Luban, 2005; Song et al., 2005; Stremlau et al., 2005; Yap et al., 2005; Perron et al., 2006; Kono et al., 2009). Interestingly,

introduction of certain point mutations into the B30.2 (PRY/SPRY) domain of TRIM5αs can change their antiviral properties and their lentiviral specificities. For example, mutation of arginine residue 332 in the B30.2 (PRY/SPRY) domain of huTRIM5α to a proline present in the rhTRIM5α (Arg332Pro) enables the human variant to restrict HIV-1 (Stremlau et al., 2005; Yap et al., 2005). Similarly, 17 amino acid residues and a 20 amino acid duplication in agmTRIM5α are essential for its ability to inhibit SIVmac (Nakayama et al., 2005).

The retroviral determinants for susceptibility to TRIM5α lie within the N-terminal domain of the capsid (CA^N) as demonstrated by site-directed mutagenesis and the use of HIV-1/SIV capsid chimeras (Owens et al., 2003; Hatziioannou et al., 2004b; Owens et al., 2004; Song et al., 2007; Kratovac et al., 2008). Lentiviral capsid proteins are well conserved in terms of both amino acid sequence and structure. Non-conserved residues have been found to cluster into surface-exposed loops of CA^N (Owens et al., 2003; Hatziioannou et al., 2004b) and are the targets for inhibition by TRIM5α variants from different primates. One of these loops constitutes the cyclophilin A-CA binding site (Hatziioannou et al., 2004b) (see Section 1.5.3). Replacement of HIV-1 CA by SIVmac CA rendered HIV-1 sensitive to huTRIM5α and New World squirrel monkey TRIM5α (Owens et al., 2003; Hatziioannou et al., 2004b) and resistant to rhTRIM5α and owl monkey TRIM5α (Owens et al., 2003). Conversely, replacement of the SIVmac CA by that of HIV-1 caused SIVmac to become moderately sensitive to huTRIM5α, very sensitive to rhTRIM5α and owl monkey TRIM5α and resistant to squirrel monkey TRIM5α (Owens et al., 2003).

1.5.2.4 TRIM5α in the *Felidae*

In a previous study we have shown that domestic cat TRIM5α (feTRIM5α) bears a stop codon in its B30.2 (PRY/SPRY) domain (McEwan et al., 2009). This premature stop codon leads to a truncation of feTRIM5α, which ablates its antiviral activity. Interestingly, this truncation was found to be conserved among members of the *Feliformia* (McEwan et al., 2009). Because feline cells display a TRIM5α-null phenotype they are sensitive to infection by lentiviral pseudotype particles that express the vesicular stomatitis virus glycoprotein (VSV-G) to enable cell entry. It should be noted that FIV is highly sensitive to restriction by

TRIM5α proteins from rhesus macaques, African green monkeys and to some degree from humans (Saenz et al., 2005). These findings suggest that HIV-1 and FIV capsids pose similar targets for TRIM5α (Diaz-Griffero et al., 2007).

1.5.3 The role of cyclophilin A in retroviral replication

Another host factor, which has been implicated in early post-entry events and impacts on retroviral infectivity, is cyclophilin A (CypA). CypA is a 18-kDa, abundantly expressed cytosolic petidyl-prolyl isomerase. It is packaged into HIV-1 virions in producer cells via an interaction with unprocessed Gag polyprotein, $Pr55^{Gag}$ (Luban et al., 1993; Franke et al., 1994; Thali et al., 1994). Target cell CypA also interacts with incoming HIV-1 capsid. In human cells, the specific binding of CypA to HIV-1 CA, especially that in target cells, is required for viral infectivity and disruption of the CypA-CA interaction impairs HIV-1 replication (Braaten et al., 1996a; Braaten et al., 1996b; Yin et al., 1998; Sokolskaja et al., 2004; Hatziioannou et al., 2005). Paradoxically, in Old World monkey cells, disruption of the CypA-CA interaction leads to an enhancement of HIV-1 infectivity and it is believed that CypA binding to HIV-1 CA renders HIV-1 more susceptible to Old World monkey TRIM5αs (Berthoux et al., 2005; Keckesova et al., 2006; Sokolskaja et al., 2006; Stremlau et al., 2006).

1.5.3.1 Cyclophilin A-capsid interactions

CypA is recruited to capsids of HIV-1, SIV of African green monkeys (SIVagm) and FIV (Luban et al., 1993; Franke et al., 1994; Thali et al., 1994; Lin and Emerman, 2006; Zhang et al., 2006); Diaz-Griffero et al., 2006b). In contrast, the capsids of most HIV-2 strains, SIVmac and MLV-N do not bind CypA (Franke et al., 1994,Braaten, 1996 #841; Yoo et al., 1997; Lin and Emerman, 2006). CypA binds to viral capsids via an interaction between its catalytic hydrophobic pocket, which contains aromatic residues, and a nine amino acid long, surface-exposed proline-rich loop between alpha-helices 4 and 5 of CA^N (Luban et al., 1993; Franke et al., 1994; Thali et al., 1994; Gamble et al., 1996). Upon capsid binding, CypA catalyses the *cis-trans* isomerisation around the Gly89-Pro90

peptide bond within the CA^N proline-rich loop (Bosco et al., 2002; Bosco and Kern, 2004). The CypA-CA interaction can be experimentally disrupted by mutational inactivation of the CypA binding site within CA^N (Gly89Ala/Val, Pro90Ala) (Luban et al., 1993; Thali et al., 1994), by the immunosuppressive drug cyclosporine A (CsA) or non-immunosuppressive CsA analogues that compete with CA for CypA binding (Takahashi et al., 1989; Rosenwirth et al., 1994; Ptak et al., 2008), or by knock-down of CypA using RNA interference (RNAi) (Berthoux et al., 2005; Keckesova et al., 2006).

As mentioned earlier, prevention of CypA binding to CA^N in target cells impacts negatively on HIV-1 infectivity in human cells and promotes HIV-1 growth in Old World monkey cells. However, disruption of the CypA-CA interaction has no impact on SIVmac because its capsid does not bind CypA (Towers et al., 2003; Berthoux et al., 2005; Keckesova et al., 2006).

1.5.3.2 Impact of cyclophilin A on capsid stability during viral uncoating

The Gly89-Pro90 peptide bond in HIV-1 CA exists as a mixture of *cis* and *trans* isomers with 14% of CA molecules being in the *cis* and the remainder in the *trans* conformation (Gitti et al., 1996). CypA catalyses this *cis-trans* isomerisation (Bosco et al., 2002; Bosco and Kern, 2004). Alteration of the Gly89-Pro90 bond can induce conformational changes within and adjacent to the proline-rich, CypA-binding loop in HIV-1 CA^N (Bosco et al., 2002; Bosco and Kern, 2004), which influence capsid stability. It has therefore been hypothesised that CypA may be critical for the temporal regulation of HIV-1 capsid disassembly (Braaten et al., 1996a; Braaten et al., 1996b; Bosco et al., 2002; Howard et al., 2003; Bosco and Kern, 2004; Luban, 2007; Li et al., 2009; Ylinen et al., 2009).

As discussed earlier, the regulation of the timing of uncoating is crucial for reverse transcription and successful nuclear import of viral cDNA. If uncoating is delayed or prevented, the PIC will fail to enter the nucleus. If instead uncoating is accelerated, reverse transcription will be inhibited. In fact, a decreased stability of HIV-1 CA in certain CA mutants was associated with defects in infectivity (Franke et al., 1994; Forshey et al., 2002; Brun et al., 2008). It is unclear whether the enzymatic activity of CypA is essential for capsid

stability or if simple binding of CypA to HIV-1 CA^N is sufficient to promote HIV-1 infectivity (Luban, 2007).

1.5.3.3 Modulation of Old World monkey TRIM5α activity by the cyclophilin A-capsid interaction

The impact of CypA on HIV-1 CA stability explains the reduced HIV-1 infectivity in human cells upon inhibition of the CypA-CA interaction. Importantly, the positive effect of CypA on HIV-1 replication is independent of huTRIM5α (Keckesova et al., 2006; Sokolskaja et al., 2006). In the absence of CypA binding to CA^N, the susceptibility of HIV-1 to huTRIM5α was unaltered and RNAi-mediated knock-down of huTRIM5α did not rescue HIV-1 infectivity (Keckesova et al., 2006; Sokolskaja et al., 2006).

In contrast, CypA has been shown to impact on Old World monkey (OWM) TRIM5α-mediated HIV-1 restriction (Berthoux et al., 2005; Keckesova et al., 2006; Sokolskaja et al., 2006; Lin and Emerman, 2008). Prevention of CypA binding to HIV-1 CA^N in OWM cells relieves the restriction of HIV-1 by OWM TRIM5αs (Berthoux et al., 2005; Keckesova et al., 2006; Stremlau et al., 2006). In fact, HIV-1 infectivity increased to the same extent that it increased upon knock-down of OWM TRIM5αs (Berthoux et al., 2005). Furthermore, CsA treatment did not lead to enhanced HIV-1 replication in OWM cells lacking OWM TRIM5αs (Keckesova et al., 2006). These data indicate that in OWM cells CypA is required for OWM TRIM5α activity, but to date the exact mechanism of the CA-CypA-TRIM5α interplay is not known. It has been suggested that structural changes induced by *cis-trans* isomerisation of the HIV-1 CA^N Gly89-Pro90 bond are better recognised by OWM TRIM5α (Berthoux et al., 2005; Keckesova et al., 2006; Stremlau et al., 2006). Alternatively, CypA binding to CA^N in the absence of catalysis may be sufficient to promote TRIM5α activity. Binding of CypA might lead to conformational changes in CA^N that could expose the TRIM5α binding site (Berthoux et al., 2005).

1.5.3.4 Cyclophilin A and FIV

It has been shown that FIV capsid binds to CypA (Lin and Emerman, 2006). A previous study in our group demonstrated that FIV CA^N binds to feline CypA with almost the same affinity with which HIV-1 CA^N binds to human CypA (dissociation constants of 6.2 μM and 5.3 μM, respectively) and that inhibition of the CypA-CA interaction reduces FIV infectivity in feline cells (McEwan, 2009). McEwan (2009) further solved the crystal structures of FIV CA^N and feline CypA and used structure-guided models to hypothesise that binding between the two proteins is most likely mediated via a proline-rich loop in FIV CA^N and a hydrophobic pocket in feline CypA. Mutagenesis of amino residues arginine 89 (Arg89) and Pro90 in FIV CA^N ablated capsid binding to CypA. While in HIV-1 CA^N the Gly89-Pro90 bond exists as *cis-trans* isomers, the corresponding Arg89-Pro90 bond in FIV CA^N does not (Leo James, personal communication). Thus, *cis-trans* isomerisation around this bond cannot contribute to the effect of feline CypA on FIV core stability or the timing of FIV uncoating. Given the fact that feTRIM5α is non-functional, it can be excluded that the interaction between feline CypA and FIV CA protects FIV from feTRIM5α. It remains unclear what causes the reduction in FIV infectivity seen upon CypA inhibition.

1.5.4 TRIM5-cyclophilin A fusion proteins

Cells of the New World owl monkey *Aotus trivirgatus* restrict HIV-1 but not SIVmac, and the antiviral activity of owl monkey TRIM5α is overcome by interruption of CypA-CA binding (Towers et al., 2003; Nisole et al., 2004; Sayah et al., 2004). Both observations are explained by the discovery that owl monkey cells express a CypA-TRIM5α fusion protein (omTRIMCyp) (Nisole et al., 2004; Sayah et al., 2004). The CypA ORF replaces the B30.2 (PRY/SPRY) domain of TRIM5α and no owl monkey TRIM5α transcript could be detected in OMK cells (Nisole et al., 2004; Sayah et al., 2004). The CypA domain of omTRIMCyp specifically targets HIV-1 CA and enables the TRIM5α RING domain to ubiquitinate CA, which leads to its proteasomal degradation and an accelerated uncoating of the virus. HIV-1 determinants for sensitivity to omTRIMCyp map to the CypA binding site (Kootstra et al., 2003; Towers et al., 2003; Berthoux et

al., 2004; Hatziioannou et al., 2004a) so that disruption of the CypA-CA interaction affects both CypA and TRIMCyp (Sayah et al., 2004). In addition to HIV-1, omTRIMCyp inhibits SIVagm and FIV infection, whereas SIVmac is not restricted (Diaz-Griffero et al., 2006b). OmTRIMCyp has arisen by retrotransposon-mediated insertion of a processed *CypA* pseudogene within intron 7 of the *TRIM5* gene (Fig. 1-7B) (Nisole et al., 2004; Brennan et al., 2008; Newman et al., 2008; Wilson et al., 2008; Ylinen et al., 2010). The *CypA* insertion generated a 15-nt duplication of the insertion site sequence (target site duplication; TSD) (Fig. 1-7). The *CypA* sequence does not contain any introns and is flanked by 5' untranslated region (UTR), 3' UTR and poly-A tail sequences. The 5' UTR sequence of CypA contributes eleven amino acids to omTRIMCyp, which link the TRIM5α RBCC domain and the CypA domain (not shown) (Nisole et al., 2004).

Soon after the isolation and characterisation of omTRIMCyp, a TRIMCyp protein was found in about 17% of rhesus macaques (*Macaca mulatta*) (Newman 2008), which has arisen independently of omTRIMCyp (Newman et al., 2008; Wilson et al., 2008). In contrast to omTRIMCyp the rhesus TRIMCyp (rhTRIMCyp) is expressed by trans-splicing of a *CypA* pseudogene, which lies downstream of the *rhTRIM5* gene locus, onto the rhTRIM5α RBCC transcript (Fig. 1-7). All rhesus macaques which express rhTRIMCyp are either heterozygous or homozygous for a G-to-T substitution in the 3' splice acceptor site upstream of exon 7 of the *TRIM5α* gene (Newman et al., 2008). This splice site mutation (AG to AU) reduces (in the case of heterozygote animals) or prevents (in the case of homozygote animals) the splicing of exon 6 of the *TRIM5α* gene onto exon 7 and thereby the expression of full-length *TRIM5α*. Instead, a splice acceptor site immediately upstream of *CypA* is used to generate rhTRIMCyp. In heterozygous animals, RhTRIMCyp is expressed alongside a full-length TRIM5α and both proteins show different spectra of antiviral specificities (Wilson et al., 2008). Whereas rhTRIM5α can restrict M and O group HIV-1, HIV-2 and FIV, rhTRIMCyp cannot inhibit M group HIV-1. The activity of rhTRIMCyp is abolished upon prevention of the CypA-CA interaction. It is assumed that at endogenous expression levels both factors restrict in a co-dominant way (Wilson et al., 2008). *CypA-TRIM5* gene fusions have further been detected in pig-tailed macaques (*Macaca nemestrina*) (Brennan et al., 2008; Newman et al., 2008; Virgen et al., 2008; Ylinen et al., 2010) and in crab-eating macaques (*Macaca fascicularis*) (Ylinen et al., 2010).

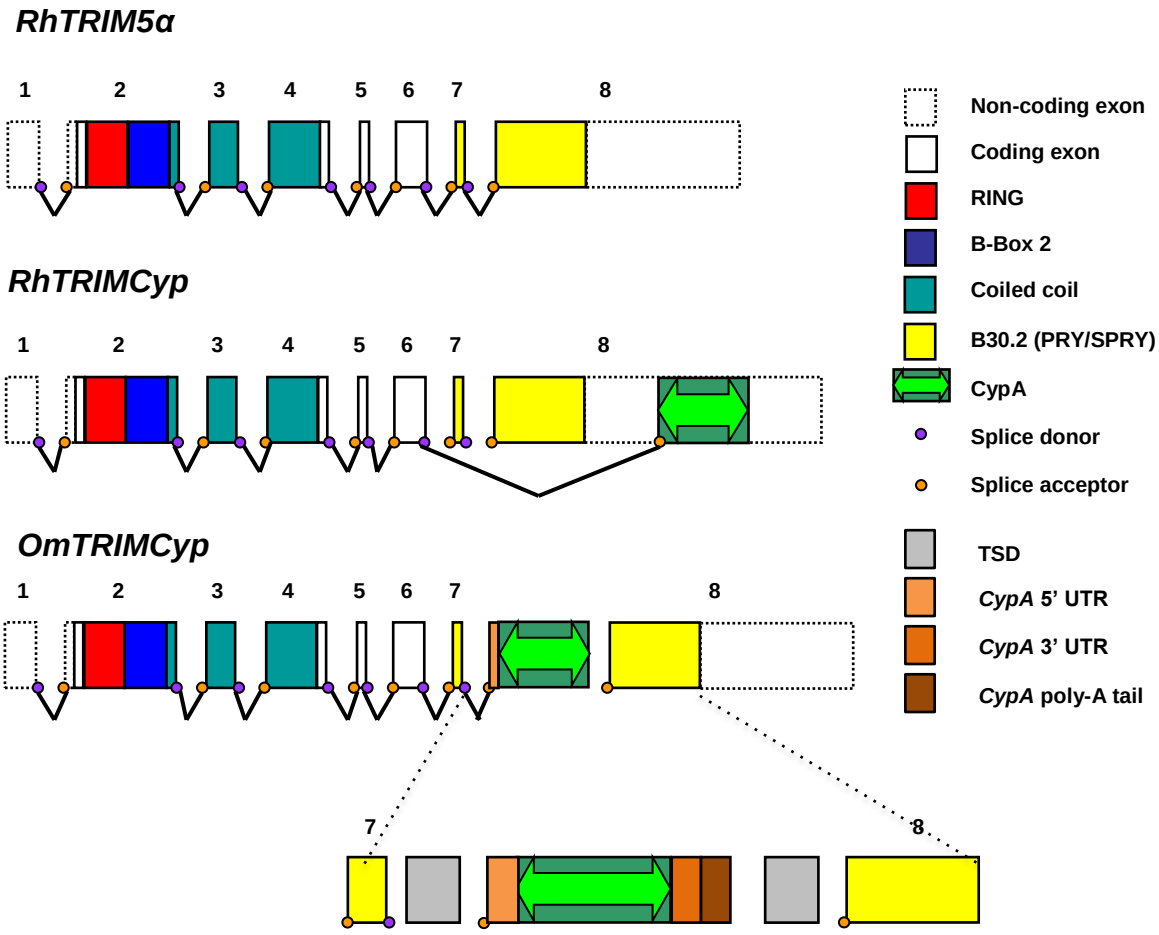


Figure 1-7 Exon organisation and splicing of *TRIM5* and *TRIMCyp* variants. Non-coding exons in the *TRIM5* and *TRIMCyp* genes are marked by a dotted line and coding exons by a solid line. The colour coding indicates which of the coding exons encode for the RING, B-box 2, coiled-coil and B30.2 (PRY/SPRY) domains in rhesus macaque (*Macaca mulatta*) TRIM5α and the CypA domains in rhesus macaque and owl monkey (*Aotus trivirgatus*) TRIMCyps (rhTRIMCyp and omTRIMCyp, respectively). In rhesus macaques, a *CypA* pseudogene is located downstream of the *rhTRIM5* gene locus. A percentage of animals is either heterozygous or homozygous for a splice acceptor site mutation immediately upstream of *TRIM5* exon 7. In these animals, a CypA mRNA is trans-spliced directly onto the rhTRIM5α RBCC transcript (encoded by exons 2 to 6) to give rise to rhTRIMCyp. OmTRIMCyp CypA is encoded by a CypA cDNA located within intron 7 of the *omTRIM5* gene. The CypA cDNA is flanked by a duplicated insertion site sequence (target site duplication; TSD), a fragment of the *CypA* 5' untranslated region (UTR), and the *CypA* 3' UTR and polyadenylation (CypA poly-A tail) sequences. The *CypA* 5' UTR contributes eleven amino acids to

omTRIMCyp. The CypA transcript is spliced onto the transcript encoded by exons 2 to 7 and replaces the B30.2 (PRY/SPRY) domain of omTRIM5α in the mature omTRIMCyp protein. In contrast to rhesus macaques, owl monkeys do not express a TRIM5α with intact B30.2 (PRY/SPRY) domain.

1.5.5 APOBEC3 proteins

The antiviral activity of APOBEC3 (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like) proteins was discovered through the study of the HIV-1 accessory protein Vif (viral infectivity factor) (Wolf and Goff, 2008), which was shown to be dispensable for viral replication in certain permissive cell lines such as CEM-SS and SupT1, but required in non-permissive cells such as primary $CD4^+$ T cells, monocyte-derived macrophages, and some T cell leukaemia lines such as CEM (Fisher et al., 1987; Strebel et al., 1987; Gabuzda et al., 1992; Sakai et al., 1993; Sova and Volsky, 1993). The human APOBEC3G protein (huA3G; initially called CEM-15) was identified as the responsible cellular factor whose expression renders human cells non-permissive for infection by HIV-1 strains devoid of the *vif* gene, but not by *vif*-proficient HIV-1 strains (Sheehy et al., 2002).

1.5.5.1 Domain structure of APOBEC proteins

HuA3G belongs to a large family of cytidine deaminases (reviewed in Harris and Liddament [2004], Aguiar and Peterlin [2008], Goila-Gaur and Strebel [2008]) that catalyse the hydrolysis of cytidines to uracils. A3 proteins and other APOBECs possess a characteristic domain structure. A short alpha-helical domain is followed by a catalytic domain (CD), a short linker peptide and a pseudocatalytic domain (PCD) (Fig. 1-8) (Jarmuz et al., 2002). In certain A3 proteins, such as the human A3 proteins A3B, A3DE, A3F and A3G, the entire unit is duplicated and adapts the form helix 1-CD1-linker 1-PCD1-helix 2-CD2-linker 2-PCD2 (Jarmuz et al., 2002). Each catalytic domain contains the conserved His/Cys-X-Glu-X_{23-28}-Pro-Cys-X_{2-4}-Cys motif in which the histidine and cysteine residues coordinate zinc ions and the glutamate residue is required for proton shuttling during the deamination reaction (Harris et al., 2002; Jarmuz et al., 2002). In double-deamination domain APOBECs one catalytic domain is generally

catalytically active whereas the second catalytic domain is involved in nucleic acid binding and virus encapsidation (Hache et al., 2005; Navarro et al., 2005; Newman et al., 2005; Iwatani et al., 2006; Opi et al., 2006).

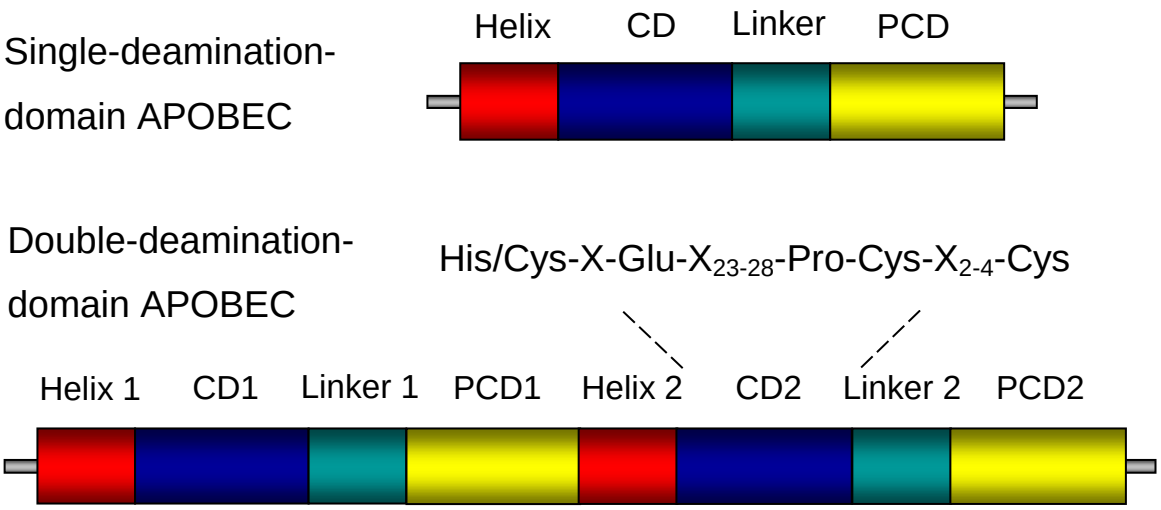


Figure 1-8 Schematic representation of the APOBEC domain topology. The domain organisation of single-deamination-domain APOBECs and double-deamination-domain APOBECs is shown. Members of the APOBEC family comprise an N-terminal alpha-helix followed by a catalytic domain (CD), a short linker peptide and a pseudocatalytic domain (PCD). In double-deamination-domain APOBECs this domain structure is duplicated. Each catalytic domain contains the conserved cytidine-deamination motif His/Cys-X-Glu-X_{23-28}-Pro-Cys-X_{2-4}-Cys. Figure adapted from Harris and Liddament (2004).

1.5.5.2 Mechanism of the antiretroviral activity of APOBEC3 proteins

In order to carry out its antiviral activity, huA3G has to be packaged into Vif-deficient virions as they are formed in producer cells (Sheehy et al., 2002; Harris et al., 2003; Lecossier et al., 2003; Mangeat et al., 2003; Zhang et al., 2003). The N-terminal, catalytically inactive catalytic domain of huA3G is able to interact with viral RNA (Jarmuz et al., 2002; Yu et al., 2004a; Khan et al., 2005; Iwatani et al., 2006; Khan et al., 2007; Tian et al., 2007; Wang et al., 2007) and the NC domain of $Pr55^{Gag}$ (Alce and Popik, 2004; Cen et al., 2004; Schafer et al.,

2004; Zennou et al., 2004). HuA3G is then carried to the target cell, where, upon initiation of reverse transcription, it deaminates cytidine residues in nascent retroviral minus-strand cDNA to uracils. Subsequently, the uracils function as a template for the incorporation of plus-strand adenines resulting in guanine to adenine hypermutations in the viral genome that critically affect viability and infectivity of the virus (Harris et al., 2003; Zhang et al., 2003; Bishop et al., 2004; Liddament et al., 2004; Zheng et al., 2004).

Recent studies propose that, in addition to deamination, deamination-independent mechanisms of huA3G to inhibit viral replication exist (Shindo et al., 2003; Newman et al., 2005; Bishop et al., 2006; Guo et al., 2006; Opi et al., 2006; Guo et al., 2007; Holmes et al., 2007; Iwatani et al., 2007; Li et al., 2007; Yang et al., 2007). These affect multiple reverse transcriptase-mediated DNA elongation reactions during reverse transcription and collectively impair the accumulation of reverse transcription products (Mangeat et al., 2003; Guo et al., 2006; Guo et al., 2007; Iwatani et al., 2007; Li et al., 2007; Luo et al., 2007; Mbisa et al., 2007).

1.5.5.3 APOBEC3-Vif interactions

The primary role of HIV-1 Vif is to prevent huA3G incorporation into virions by targeting A3G for proteasome-mediated degradation (Conticello et al., 2003; Marin et al., 2003; Sheehy et al., 2003; Stopak et al., 2003; Liu et al., 2004; Mehle et al., 2004a; Mehle et al., 2004b). Vif bridges an interaction between huA3G and a ubiquitin E3 ligase complex consisting of elongins B and C, cullin 5 and ring-box-1 (Yu et al., 2003; Mehle et al., 2004a; Yu et al., 2004b; Bergeron et al., 2010). The binding of HIV-1 Vif to huA3G is dependent on the presence of an Asp-Pro-Asp motif at amino acid sequence positions 128 to 130 in A3G (Bogerd et al., 2004; Mangeat et al., 2004; Schrofelbauer et al., 2004; Xu et al., 2004). A positively charged Asp-Arg-Met-Arg motif (residues 14-17) and a hydrophobic motif between residues 40-44 in HIV-1 Vif appear to be important for the interaction with huA3G (Mangeat et al., 2004; Xu et al., 2004; Navarro et al., 2005; Mehle et al., 2007). A highly conserved, hydrophobic, zinc-coordinating His-X_5-Cys-X_{17-18}-Cys-X_{3-5}-His (HCCH) motif and a conserved Ser-Leu-Gln-Tyr/Phe-Leu-Ala (SLQ) motif at positions 144-149 of HIV-1 Vif are essential for interaction

of Vif with the ubiquitin ligase complex (Yu et al., 2003; Liu et al., 2004; Mehle et al., 2004b; Mehle et al., 2006; Xiao et al., 2006). Recently, the cellular transcription factor CBFβ has been shown to be associated with the E3 ubiquitin ligase complex (Hultquist et al., 2012; Jager et al., 2012; Zhang et al., 2012). CBFβ is required for HIV-1 and SIV Vif folding and stability, which is crucial for A3G degradation (Hultquist et al., 2012; Jager et al., 2012).

The interaction between A3 proteins and Vifs is species-specific and partly determines the host range of a virus (Hatziioannou et al., 2006). HIV-1 and SIVagm Vif can inhibit the A3G proteins of their respective host species, but not that of other species (Simon et al., 1995; Simon et al., 1998; Mariani et al., 2003). SIVmac Vif acts more broadly and can neutralise not only rhesus macaque A3G (rhA3G) but also African green monkey A3G (agmA3G) and huA3G (Mariani et al., 2003). HuA3G can block replication of Vif-deficient HIV-1, HIV-2 (Ribeiro et al., 2005), EIAV (Mangeat et al., 2003), MLV (Harris et al., 2003; Bishop et al., 2004; Kobayashi et al., 2004) and foamy virus (Delebecque et al., 2006).

1.5.5.4 APOBEC3 proteins in the *Felidae*

Several *APOBEC3* genes have recently been identified and characterised in the genome of domestic cats (Munk et al., 2008). The *A3* gene locus encodes three highly similar *A3C* (*A3Z2*) genes and an *A3H* (*A3Z3*) gene. Additionally, a fifth transcript, which is generated by read-through alternative splicing, encodes the protein A3CH (A3Z2-Z3) (Munk et al., 2008; Zielonka et al., 2010). Domestic cat A3 proteins are overcome by FIV Vif and the FeFV Bet protein (Lochelt et al., 2005; Munk et al., 2008; Stern et al., 2010; Zielonka et al., 2010). Similar to HIV-1 Vif, FIV Vif recruits an E3 ubiquitin ligase complex consisting of elongin B, elongin C and cullin 5 to induce proteasomal degradation of A3 proteins (Wang et al., 2011). However, in contrast to HIV-1 Vif, FIV Vif does not possess the zinc-coordinating HCCH motif or the SLQ motif to interact with the E3 ubiquitin ligase complex (Wang et al., 2011), and it has yet to be determined which amino acid residues mediate the binding of FIV Vif to cullin 5. In addition to FIV Vif, domestic cat A3 proteins are also overcome by SIVmac Vif, but not by HIV-1 Vif (Stern et al., 2010).

The feline A3 proteins display different degrees of activity against feline retroviruses. Feline A3C proteins inhibit the replication of Bet-deficient FeFV (Lochelt et al., 2005) but do not restrict Vif-deficient FIV or FeLV. In contrast, feline A3H and A3CH proteins are active against Vif-deficient FIV as well as FeLV but not against Bet-deficient FeFV (Lochelt et al., 2005; Munk et al., 2008).

The antiviral activities of A3 proteins from non-domestic cat resemble those of domestic cats (Zielonka et al., 2010). Lion, puma, tiger (*Panthera tigris*) and lynx (*Lynx lynx*) A3H and A3CH isoforms block replication of Vif-deficient FIV, whereas non-domestic cat A3C proteins are sensitive to FIV lacking the Vif protein. FIV Vif induces degradation of all wild cat A3C, A3H and A3CH proteins.

1.5.6 Tetherin

In addition to the early post-entry blocks, restriction factors such as tetherin contribute to a late block to retroviral replication in that they prevent the release of mature enveloped viral particles from the membranes of infected cells. Tetherin (also called HM1.24/BST-2/CD317) was originally identified as a bone marrow stromal cell surface antigen selectively expressed on terminally differentiated normal and neoplastic human B cells and corresponding cell lines (Goto et al., 1994; Ishikawa et al., 1995). The antiviral activity of tetherin was not discovered until 2008, when it was noted that its cell-type specific expression matched closely the dependency of HIV-1 on the accessory protein Vpu for virus release from certain human cell lines (Strebel et al., 1989; Terwilliger et al., 1989; Klimkait et al., 1990; Varthakavi et al., 2003; Neil et al., 2008; Van Damme et al., 2008). Tetherin is constitutively expressed in human cell lines such HeLa cells (Gottlinger et al., 1993), several cancer cell lines (Ohtomo et al., 1999), B cells, T cells, monocytes, macrophages and plasmacytoid dendritic cells (Blasius et al., 2006; Miyagi et al., 2009), and its expression is induced or enhanced by type I and type II interferons in cell lines such as HOS, 293T, HT1080 cells (Neil et al., 2006; Neil et al., 2007; Neil et al., 2008; Van Damme et al., 2008; Miyagi et al., 2009). IFN treatment renders cell lines Vpu-dependent that do not normally require Vpu for efficient virus release (Neil et al., 2007).

1.5.6.1 Domain structure of tetherins and intracellular localisation and trafficking

Several studies have shown that tetherins are novel type II transmembrane proteins with a molecular weight of 30-36 kDa (Ishikawa et al., 1995; Ohtomo et al., 1999; Kupzig et al., 2003). They harbour an N-terminal cytoplasmic tail, followed by a transmembrane domain, an extracellular parallel, dimeric, alpha-helical coiled-coil domain and a C-terminal GPI anchor (Fig. 1-9) (Ishikawa et al., 1995; Ohtomo et al., 1999; Kupzig et al., 2003; Rollason et al., 2007; Hinz et al., 2010).

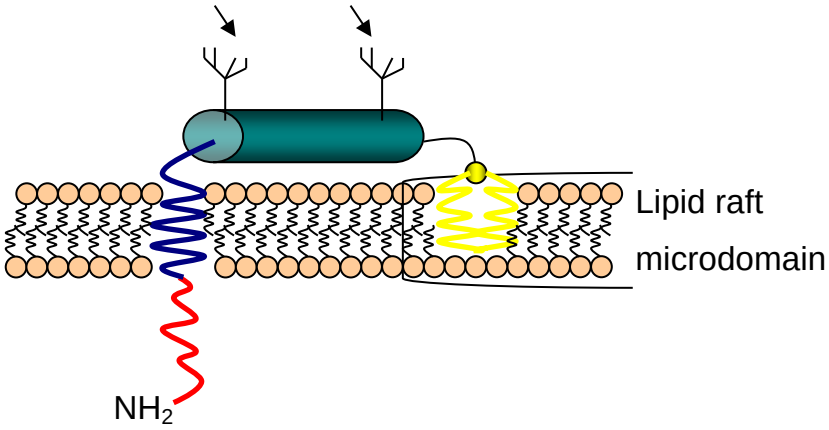


Figure 1-9 Schematic representation of the tetherin domain topology. Tetherins harbour an N-terminal cytoplasmic domain (red), a transmembrane domain (blue), an extracellular alpha-helical coiled-coil domain (cyan) and a C-terminal GPI anchor (yellow). The positions of two putative N-linked glycosylation sites (arrows) in the extracellular domain are highlighted. The GPI-modification causes tetherin to partition into lipid rafts in the plasma membrane.

Two potential N-linked glycosylation sites and three conserved cysteine residues are present in the extracellular domain (Ishikawa et al., 1995; Ohtomo et al., 1999; Kupzig et al., 2003). Heterogeneous glycosylation of tetherin has been shown to be essential for efficient secretion and folding (Andrew et al., 2009; Goffinet et al., 2009; Kaletsky et al., 2009; McNatt et al., 2009; Miyagi et al., 2009; Perez-Caballero et al., 2009). The cysteines take part in intra- and intermolecular disulfide bond formation and enable the homodimerisation of tetherin (Ohtomo et al., 1999; Kupzig et al., 2003; Perez-Caballero et al., 2009). The GPI-modification causes tetherin to partition into and cross-link cholesterol-

and sphingolipid-rich microdomains in the plasma membrane (Simons and Ikonen, 1997; Simons and Toomre, 2000; Kupzig et al., 2003). Tetherin cycles between these lipid rafts on the cell surface and an intracellular pool where it localises predominantly to the Golgi apparatus, the trans-Golgi network (TGN) and recycling endosomes (Kupzig et al., 2003). Internalisation from the plasma membrane is mediated by clathrin-dependent endocytosis (Rollason et al., 2007) (Masuyama et al., 2009).

1.5.6.2 Mechanism of the antiviral activity of tetherin

Tetherin causes the retention of fully formed mature virions on the surface of cells infected with Vpu-deficient HIV-1 (Neil et al., 2008; Van Damme et al., 2008). At the expense of particle release, virions accumulate at the cell surface and a fraction of them are endocytosed via a clathrin-dependent mechanism and degraded (Neil et al., 2006; Neil et al., 2007). Current models predict that tetherin is present at sites of particle assembly in the cell membrane and is incorporated into virions (Perez-Caballero et al., 2009; Fitzpatrick et al., 2010). Presumably, one end of tetherin embeds in the lipid bilayer of the cell and the other in that of the virion, so that cell-surface tetherin homodimerises with virion-associated tetherin via disulfide bonds or via coiled-coil regions in the extracellular domain (Fitzpatrick et al., 2010). Thus, virions remain bound to the cell surface and are cross-linked to each other by tetherin. In addition to lentiviruses, tetherin blocks virion release from members of the alpharetrovirus, betaretrovirus, deltaretrovirus, spumaretrovirus, arenavirus (Lassa) and filovirus (Ebola, Marburg) families (Jouvenet et al., 2009; Kaletsky et al., 2009; Sakuma et al., 2009).

1.5.6.3 Viral tetherin antagonists

HIV-1 Vpu is an integral class I membrane phosphoprotein (Cohen et al., 1988) that promotes virion release from HIV-1 infected human cells that express tetherin (Klimkait et al., 1990; Neil et al., 2006; Neil et al., 2008; Van Damme et al., 2008). It has been shown to colocalise with tetherin (Neil et al., 2008; Van

Damme et al., 2008) and to reduce its cell-surface expression by targeting it for degradation (Van Damme et al., 2008; Douglas et al., 2009; Goffinet et al., 2009; Mitchell et al., 2009; Miyagi et al., 2009). A well-studied role of Vpu is to mediate the proteasomal degradation of the HIV-1 receptor CD4 in the ER through the recruitment of the ß-transducin repeat-containing protein (ßTrCP) subunit of the Skp1-cullin1-F-box (SCF) ubiquitin ligase complex (Willey et al., 1992a; Bour et al., 1995; Margottin et al., 1998). ßTrCP is also involved in the antagonism of tetherin because disruption of the interaction between ßTrCP and the ßTrCP binding motif in the cytoplasmic domain of Vpu reduces the capacity of Vpu to promote virus release (Douglas et al., 2009; Mangeat et al., 2009; Mitchell et al., 2009). Vpu serves as an adapter between ßTrCP and tetherin. Tetherin and Vpu bind to each other through their transmembrane domains (Iwabu et al., 2009; Rong et al., 2009). It seems that Vpu sequesters tetherin within the endolysosomal system either within the TGN after it has been synthesised or within recycling endosomes after natural endocytosis of tetherin from the cell surface has occurred (Dube et al., 2009; Mitchell et al., 2009). This intracellular sequestration is followed by partial lysosomal degradation of both tetherin and Vpu.

Vpu is only encoded by a unique lineage of primate lentiviruses that include HIV-1 and the SIVs of chimpanzees (*Pan troglodytes*) (Cohen et al., 1988), Mona monkeys (*Cercopithecus mona*), mustached monkeys (*C. cephus*) and greater spot-nosed monkeys (*C. nictitans*); SIVcpz, SIVmon, SIVmus and SIVgsn, respectively (Courgnaud et al., 2003). SIVmon, SIVmus and SIVgsn Vpu counteract tetherins of their respective host species as well as macaque tetherins, but, with the exception of SIVgsn, not human tetherin (huTHN) (Sauter et al., 2009; Yang et al., 2010). Accordingly, non-human, non-chimpanzee tetherins are usually insensitive to antagonism by HIV-1 Vpu (Goffinet et al., 2009; Gupta et al., 2009a; Jia et al., 2009; McNatt et al., 2009; Sauter et al., 2009; Zhang et al., 2009a). SIVcpz is the immediate precursor of HIV-1 and its Vpu shares a common ancestry with SIVmon/mus/gsn Vpu (Sauter et al., 2009). However, SIVcpz Vpu is non-functional against both chimpanzee tetherin (cpzTHN) and huTHN. Instead, in SIVcpz the accessory protein Nef has adopted a Vpu-like function. It is likely that, after cross-species transmission from chimpanzees to humans, HIV-1 Vpu has adapted to counteract huTHN, because huTHN is resistant to Nef due to a deletion in its cytoplasmic tail

(Sauter et al., 2009; Zhang et al., 2009a). Species-specific tetherin antagonism by Nef is also conserved in SIVs of sooty mangabeys, rhesus macaques and African green monkeys, SIVsmm, SIVmac and SIVagm, respectively. Like Vpu, Nef also induces cell-surface downregulation of monkey tetherins (Jia et al., 2009). Additionally to Vpu and Nef, the HIV-2 and SIVagm.Tan (SIVagm of the Tantalus monkey, *Chlorocebus tantalus*) envelope glycoproteins possess anti-tetherin activities (Abada et al., 2005; Gupta et al., 2009b; Le Tortorec and Neil, 2009).

1.6 Scope of the thesis

FIV infection of domestic cats leads to the development of a fatal immunodeficiency syndrome similar to AIDS in humans; however, FIV infection of non-domestic cats is usually benign. It is likely that the relatively short period of time for which FIV has been circling in the domestic cat population was insufficient for host-virus co-adaptation to occur, leading to immunodeficiency. Non-domestic felids, on the other hand, have co-existed with lentiviruses for millions of years. Hence, non-domestic cat FIV strains could either be less virulent or less pathogenic, or spreading in species with genetic adaptations that confer efficient immunological defense to viral infection.

In fact, although FIV is able to replicate in both domestic and non-domestic cats, *in vitro* studies have indicated that FIV infection of non-domestic cat T lymphocytes is significantly less efficient than that of domestic cat T cells. Thus, this thesis tests the hypothesis that non-domestic cat restriction factors are either more potent antagonists of lentiviral replication than domestic cat restriction factors or that their activity is less sensitive to viral counteraction.

In this thesis, the activities of domestic and non-domestic cat restriction factors against lentiviruses are compared. Specific attention is further paid to the characterisation of viral accessory proteins and their ability to overcome the feline restriction factors. Finally, the potential of feline restriction factors for use in anti-retroviral therapies is explored.

- Chapter 3 describes the generation of a synthetic feline-specific TRIM5α-cyclophilin A fusion protein (feTRIMCyp). The study finds that feTRIMCyp is highly efficient at blocking lentiviral infection, an activity that can be

reversed by cyclosporine A and its derivatives. FeTRIMCyp and FIV infection of the cat offers a unique opportunity to evaluate TRIMCyp-based approaches to genetic therapy for FIV and HIV infections and the treatment of AIDS.

- In Chapter 4 domestic and non-domestic cat APOBEC3 proteins are characterised and their activities against FIV and other lentiviruses are compared. No significant differences in the antiviral potencies of these restriction factors against FIV lacking the viral APOBEC3 antagonist Vif are detected. Further analysis focuses on *APOBEC3* expression levels in different feline cell lines, primary cells and tissues. Finally, insights in the evolution of lion APOBEC3 proteins in the presence or absence of FIV infection are provided. Surprisingly, no indication for viral selection pressure on the evolution of lion APOBEC3 proteins is found. Consequently, the activities of FIV Vif proteins against feline APOBEC3 proteins are determined. Here, cat APOBEC3CH is significantly more sensitive to FIV containing Vif compared to non-domestic cat APOBEC3CH proteins.

- In Chapter 5 felid homologues of tetherin are characterised and their effects on the replication of FIV are investigated. Domestic cat tetherin displays potent inhibition of FIV and HIV-1 particle release and no tetherin antagonist is found in the FIV genome. However, when stably expressed in feline cell lines, tetherin cannot abrogate the replication of FIV. In fact, tetherin expression is found to promote cell-to-cell spread of cell culture-adapted, CD134-independent FIV strains. The study further focuses on the characterisation of non-domestic cat tetherins and their activity against feline retroviruses. No significant differences in the potencies of domestic and non-domestic cat paralogues are found.

2 Materials and Methods

2.1 Molecular cloning

2.1.1 Nucleic acid extraction

Total RNA or genomic DNA were isolated from human, feline and canine cell lines, primary cells or cat tissue samples by homogenising $1x10^6$ cells using QIAshredder Spin Columns and RNAeasy Mini Spin Columns (Qiagen, Crawley, UK) or the QIAamp DNA Mini and Blood Mini kit (Qiagen), respectively. Nucleic acids were eluted in RNase-free water (Life Technologies, Paisley, UK), quantified by spectroscopy at 260 nm and stored at -80°C until further use.

2.1.2 cDNA synthesis

First strand cDNA synthesis was performed using 1 µg total RNA and oligo(dT) primers and the Transcriptor High Fidelity cDNA Synthesis kit (Roche Applied Science, Roche Diagnostics Ltd., Burgess Hill, UK) according to manufacturer's instructions. CDNA was stored at -20°C until further use.

2.1.3 PCR methods

Transcripts of interest were amplified by polymerase chain reaction (PCR) from 2 µl cDNA using Phusion High-Fidelity DNA polymerase (Finnzymes, Hitchin, UK). Cycling parameters were set as per manufacturer's instructions. The standard reaction mix composition used was 5x HF buffer 4 µl, dNTPs 200 µM each, gene-specific primers 0.5 µM each, DMSO 3% v/v, Phusion High-Fidelity DNA polymerase 0.02 U/µl.

PCR amplification from plasmid DNA was performed using 25-200 ng template DNA. PCR amplification from genomic DNA or bacterial colony suspensions (see below) was carried out using 100 ng genomic DNA or 2 µl of bacterial colony suspension as template and GoTaq Flexi DNA polymerase

(Promega, Southampton, UK). Cycling parameters were adjusted as per manufacturer's instructions. The standard reaction mix composition used was 5x Green GoTaq Flexi Buffer 10 µl, dNTPs 200 µM each, gene-specific primers 0.2 µM each, $MgCl_2$ Solution 1.5 mM, GoTaq DNA polymerase 1.25 U/µl. Annealing temperature optimisation using temperature gradients was performed when necessary.

Lyophilised primers were ordered from Eurofins MWG Operon (Ebersberg, Germany), resuspended in sterile distilled water to a stock concentration of 100 pmol/µl and to a working stock concentration of 10 pmol/µl. Lyophilised real-time PCR primers were resuspended in sterile distilled water to a stock concentration of 100 pmol/µl and to a working stock concentration of 20 pmol/µl. Lyophilised real-time PCR probes were diluted in qPCR Probe dilution buffer (Eurofins MWG Operon) to a stock concentration of 100 pmol/µl and to a working stock concentration of 5 pmol/µl. Primers and probes were stored at -20°C. A list of primers and probes used and a list of buffers and solutions are displayed in the Appendix.

2.1.4 Cloning techniques

PCR products were analysed and purified using agarose gel electrophoresis. TBE buffer containing 1% agarose was dissolved in a microwave and allowed to cool to 50°C. Ethidium bromide was added and the gel was cast into an electrophoresis chamber. It was immersed in TBE buffer prior to use. The DNA was loaded in 1x DNA loading buffer and fragments were separated at 80 V for 40 minutes. 0.5 µg of 1 kb DNA ladder (New England Biolabs, Hitchin, UK) was loaded for product size estimation. DNA products were observed on a UV transilluminator and excised from the gel using a sterile scalpel. Gel extraction was performed using the QIAquick Gel Extraction kit (Qiagen) and PCR products were eluted in 35 µl distilled water.

For molecular cloning, purified PCR products and vectors were digested with restriction endonucleases (New England Biolabs; Life Technologies, Paisley, UK) at a concentration of 1 U/µl for 2 hours at 37°C with the buffers recommended by the manufacturers. Digested DNA was purified using the QIAquick PCR Purification kit (Qiagen) and eluted in 35 µl distilled water.

Digested vector DNA was purified by agarose gel electrophoresis prior to further purification using the QIAquick Gel Extraction kit (Qiagen). Digested vector DNA was eluted in 35 µl distilled water. Digested PCR product was ligated into digested vector DNA using T4 DNA polymerase (New England Biolabs) at 1 U/µl overnight at 14°C. 30% of the ligation mix was transformed into 35 µl chemically competent *Escherichia coli* (New England Biolabs; Life Technologies) using the respective heat shock protocols supplied by the manufacturers. Transformed bacteria were plated onto Luberia Bertani (LB) broth agar plates containing 50 µg/ml ampicillin or kanamycin (Sigma-Aldrich, Dorset, UK) and grown overnight at 30°C (applies to FIV envelope glycoproteins) or 37°C.

Colonies were screened for the presence of the insert-vector combination of interest by colony PCR or by restriction digestion. For colony PCR, colonies were picked and resuspended in 20 µl distilled water. 2 µl of the bacterial colony suspensions and GoTaq Flexi DNA polymerase (Promega) were used per PCR reaction as described above. Gene-specific or vector-specific primers were designed to detect the presence or absence of the desired insert in the bacterial clones. Alternatively, colonies were grown overnight in 5 ml LB broth at 30°C or 37°C in an orbital shaker set to 200 rpm. Plasmid DNA was extracted from the cultures using the QIAprep Spin Miniprep kit (Qiagen) and eluted in 35 µl distilled water. Plasmid DNA was subjected to restriction digestion and agarose gel electrophoresis as previous. Glycerol stocks of clones of interest were made by diluting cultures to a final glycerol concentration of 25% followed by storage at -80°C.

Large-scale plasmid DNA preparations were performed by inoculating 200 ml LB broth containing appropriate antibiotics with bacterial clones. Cultures were grown overnight at 30°C or 37°C and plasmid DNA was isolated using the PureLink HiPure Filter Plasmid Maxiprep kit (Life Technologies). Maxiprep DNA was resuspended in sterile TE buffer and stored at 4°C.

2.1.5 DNA mutations

Site-directed mutagenesis PCRs were carried out to insert nucleotide or amino acid codon changes into plasmid DNA. The QuikChange Site-Directed Mutagenesis kit (Agilent Technologies, Wokingham, UK) was used for plasmids of up to 8 kb in length and the QuikChange XL Site-Directed Mutagenesis Kit (Agilent Technologies) for plasmids over 8 kb in length according to the manufacturer's instructions. The standard reaction mix composition used was 10x reaction buffer 5 µl, dNTP mix 1 µl, gene-specific primers 125 ng each, plasmid DNA template 25 ng, *PfuTurbo* DNA polymerase 2.5 U/µl. Deviating from the instructions, mutated and *Dpn*I-digested DNA contained in 50 µl reaction mix was added to 50 µl of distilled water. The DNA was then precipitated with 200 µl ethanol (100%) containing sodium acetate at a concentration of 15 mM for 30 minutes at – 80°C. The DNA was pelleted at 14,000 rpm (Eppendorf FA-45-18-11 rotor) for 20 minutes and the DNA pellet was resuspended in 10 µl distilled water. The DNA was then transformed into chemically competent *Escherichia coli* strain DH5α as described above. Colonies were picked from the LB agar plates, plasmid DNA was prepared (QIAprep Spin Miniprep kit) and analysed by DNA sequencing.

2.1.6 DNA sequencing

Chain termination sequencing reactions were set up in house using BigDye v1.1 (Applied Biosystems, Life Technologies, Paisley, UK) and thermally cycled according to manufacturer's instructions. Template-specific primers were used at a final concentration of 0.16 µM. PCR reactions were ethanol-precipitated and the DNA pellets were resuspended in 20 µl HiDi (Applied Biosystems). Sequencing reactions were run on an ABI 3700 automated capillary array sequencer (Applied Biosystems) and sequence traces were analysed using the DNADynamo software (Blue Tractor Software Limited, Llanfairfechan, UK).

2.2 Cells and viruses

2.2.1 Culture of cell lines and primary cells

Adherent cells were maintained in Dulbecco's modification of Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM glutamine and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin) at 37°C and 5% CO_2. Suspension cells were grown in RPMI 1640, supplemented with 10% FCS, 2 mM glutamine, antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin), 10% HEPES, 1 mM sodium pyruvate and 0.05 mM 2-mercaptoethanol at 37°C and 5% CO_2. Cells were grown in disposable plastic tissue culture flasks (Corning, Ewloe, UK) and passaged when confluent. When confluent, medium was removed from the cells and the cell monolayer was washed with PBS. Cells were dissociated from the flask by incubation with 0.05% trypsin (Life Technologies) in PBS for 5 minutes at room temperature. Trypsin activity was stopped by resuspension of the cells in complete DMEM. Cells were pelleted at 1,000 rpm (Eppendorf A-4-62 rotor) for 5 minutes and the cell pellet was resuspended in complete DMEM. New cultures were seeded at the desired density.

The feline T-cell line Mya-1 and primary T lymphocytes were maintained in complete RPMI containing conditioned medium from a murine cell line (L2.3) transfected with a human IL-2 expression construct (equivalent to 100 U/ml of recombinant human IL-2). Primary T lymphocytes were expanded from PBMCs by stimulation with concanavalin A (2 µg/ml; Sigma-Aldrich). Cells were passaged at the required density by centrifugation at 800 rpm (Eppendorf A-4-62 rotor) for 5 minutes and resuspension in complete RPMI.

Cells used throughout the study are HEK-293T cells (human embryonic kidney cells) (Graham et al., 1977), the feline cell lines 3201 (thymic lymphosarcoma cells) (Snyder et al., 1978), AH927 (fibroblasts) (Rasheed and Gardner, 1980), Crandell feline kidney (CrFK; clone ID10; kidney epithelioid cells) (Crandell et al., 1973), FEA (foetal embryo fibroblast-like cells) (Jarrett et al., 1973) and Mya-1 (IL-2 dependent T cell line) (Miyazawa et al., 1989). Additionally, CLL cells (canine chronic lymphocytic leukaemia cells) (Willett et al., 2006), primary feline macrophages and primary lion T lymphocytes were used.

2.2.2 Infection of cells with replication-competent viruses

Infection of cells with replication-competent FIVs was performed by incubating target cells with viral inocula. Supernatant containing clonal FIV was produced by transfection of 293T cells with full-length molecular clones of different FIV strains using SuperFect transfection reagent (see below). Three days post-transfection, supernatant from 293T cells was harvested, cleared by centrifugation at 2,000 rpm (Eppendorf A-4-62 rotor) for 10 minutes and incubated with Mya-1 cells. FIV replication was confirmed using a lentivirus reverse transcriptase activity assay (see below). Supernatant from infected Mya-1 culture was then harvested and cleared. Virus stocks were stored at -80°C.

Prior to infection, adherent cells were seeded at a density of $2x10^5$ cells per well in 6-well plates and left to adhere overnight. Cells were incubated with 100 µl viral inoculum for 2 hours at 37°C, washed with PBS and cultured in complete DMEM. Typically $2x10^6$ suspension cells per well of a 6-well plate were transferred to Falcon tubes (BD Biosciences, Oxford, UK) and pelleted. They were resuspended in 0.9 ml complete RPMI and incubated with 100 µl viral inoculum for 2 hours at 37°C, washed with PBS and cultured in 6-well plates in complete RPMI. To monitor virus growth, 100 µl aliquots of cell-free supernatant were collected on a daily basis and stored at -80°C for quantification by reverse transcriptase activity assays.

2.2.3 Stable transduction of cells

For stable expression, transgenes were inserted into the mammalian expression vector pDON-AI-2neo (Takara Bio Europe S.A.S./Clontech, Saint-Germain-en-Laye, France). Retroviral vector transduction was performed to integrate the vector construct into CrFK, FEA or CLL cells. MLV pseudotypes containing the vector construct to be transduced were produced by seeding $1x10^6$ 293T cells in 100-mm cell culture dishes overnight and transfecting them with pCIG3N (MLV-N Gag-Pol) (Bock et al., 2000), pMDG (VSV-G) (Yee et al., 1994) and the transgene-pDON-AI-2neo construct. 72 hours post-transfection, MLV pseudotypes were harvested, cleared by centrifugation at 2,000 rpm (Eppendorf A-4-62 rotor) for 10 minutes and used to infect target cells. For transduction, target cells were

seeded in T-25 flasks 16 hours prior to transduction at a low density. Target cells were incubated with the pseudotypes for 2 to 4 hours. Cells were then washed in PBS and resuspended in complete cell culture media. 48 hours post-transduction, stably-transduced cells were selected in geneticin (G418; 800 µg/ml) (Life Technologies). After selection the geneticin concentration was reduced to 400 µg/ml. Certain stable cell lines used in this study, especially those expressing CD134, were created using the vector pDsRed2 (Takara Bio Europe/Clontech) instead of p-DON-AI-2 Neo. These cell lines were selected using puromycin (1 µg/ml) (Life Technologies).

2.2.4 Transient transfection of cells

293T cells were seeded in 12-well plates at a density of $1x10^5$ cells per well or in 100-mm culture dishes at a density of $1x10^6$ cells per dish and allowed to adhere overnight. Cells were then transfected using SuperFect (Qiagen) according to the manufacturer's recommendations. The medium of transfected cells was removed 4 hours post-transfection. The cell monolayer was washed with PBS and cells were grown in complete DMEM for 48 to 72 hours at 37°C.

2.2.5 Pseudotype production and pseudotype assay

In this study pseudotype assays were performed to investigate the effect of feline restriction factors on lentiviral egress from producer cells or on early post-entry processes in target cells in the absence of viral spread. Pseudotypes were produced by three-vector transfection of 293T cells. Equal amounts of a vector encoding a lentiviral Gag-Pol protein, a marker protein and an Env protein were transfected. The *gag-pol*-expression construct is known as the packaging vector. In producer cells, *gag* and *pol* are transcribed and their mRNAs are translated. Env is produced from the *env*-expressing vector. Gag, Pol and Env assemble into VLPs. The marker gene-expressing plasmid contains the RNA packaging signal Ψ. In producer cells, the marker gene is transcribed and marker gene transcripts are translated. Marker gene vector mRNA is also packaged into VLPs. In target cells, the marker gene is reverse transcribed into DNA by RT contained in viral

particles. The marker gene then integrates in the host genome with the help of VLP-contained lentiviral integrase and is stably expressed. In contrast, viral particles do not contain Gag-Pol or Env-encoding RNAs so that in target cells only Gag-Pol and Env proteins are present. Thus, in pseudotype assays only single-cycle lentiviral replication occurs. In pseudotyped viral particles the lentiviral Env protein is replaced by a foreign envelope glycoprotein. In this study the vesicular stomatitis virus G glycoprotein (VSV-G) was used as a surrogate Env. VSV-G enables high-titre VLP production and exhibits a broad cell tropism as it binds to phospholipids in plasma membranes (Burns et al., 1993; Yee et al., 1994). VSV-G pseudotyped viral particles enter target cells by clathrin-mediated endocytosis instead of receptor-mediated uptake (Matlin et al., 1982).

HIV-1 pseudotypes were prepared using the HIV-1-derived packaging plasmids p8.91 (expressing Gag-Pol, Tat and Rev) or p8.2 (expressing Gag-Pol, Tat, Rev, Vpr, Vpu, Vif and Nef) (Zufferey et al., 1997), SIN CSGW (GFP-encoding HIV-1 genome) (Naldini et al., 1996), and pMDG (VSV-G) (Naldini et al., 1996). SIV pseudotypes were produced using the SIVmac-derived vectors SIV4+ (expressing Gag-Pol, Tat, Rev) or SIV3+ (expressing Gag-Pol, Tat, Rev, Vif, Vpx and Vpr) (Negre et al., 2000), SIV-GFP (GFP) (Negre et al., 2000) and pMDG (VSV-G). FIV pseudotypes were prepared using the FIV-based vectors FP93 (Gag-Pol) (Poeschla et al., 1998; Saenz and Poeschla, 2004), pGinSin (GFP) (Poeschla et al., 1998) and pMDG (VSV-G).

In this study pseudotypes were often produced in the presence of restriction factor expression plasmids. In this case, $1x10^5$ 293T cells per well in 12-well plates or $1x10^6$ 293T cells in 100-mm culture dishes were left to adhere overnight at 37°C. Transfections in 12-well plates were performed using 600 ng of each of the respective three lentiviral vectors described above and 100 ng (tetherin) or 200 ng (APOBEC3 proteins) restriction factor expression plasmid. The eukaryotic expression vector VR1012 (Vical Inc., San Diego, U.S.A.) was chosen in these experiments because it enables CMV promoter-driven, high-level expression of restriction factors in 293T cells. Transfections in 100-mm culture dishes were carried out using five times the amount of plasmid DNA used for 12-well plates. Unless otherwise stated, APOBEC3 activity assays were performed in 12-well plates with or without 500 ng FIV Vif expression construct. Tetherin activity assays were performed in 100-mm culture dishes using 750 ng tetherin expression plasmid in the presence or absence of 250 ng FIV OrfA expression

plasmid 1S-5RL (Pistello et al., 2003), 3 µg FIV Env expression plasmids or a replication-defective molecular clone of FIV (CMVG8MΔ*pol*4).

48 hours post-transfection, fresh 293T cells (target cells) were seeded in 12-well plates at a density of $2.5x10^4$ cells per well and left to adhere overnight at 37°C. 72 hours post-transfection, the medium was removed from target cells and 1 ml pseudotype-containing supernatant from transfected 293T cells was used to transduce target cells. Remaining supernatant was stored at -80°C until further use. Transfected 293T cells were washed with PBS, harvested, pelleted and stored at -20°C for immunoblotting. 72 hours post-transduction, the medium was removed from target cells and the cells were resuspended in 1 ml PBS-BSA-azide in 5-ml round-bottom Falcon tubes (BD Biosciences). The cells were then analysed for marker gene expression by flow cytometry using a BD Accuri C6 cytometer (BD Biosciences).

2.3 Quantitative techniques and immunoblotting

2.3.1 Quantification of reverse transcriptase activity

Reverse transcriptase activity in supernatants of virus-producing cells was assayed using a Lenti-RT (for FIV) or C-type RT (for FeLV and RD114) non-isotopic RT assay kit (Cavidi Technology, Uppsala, Sweden). The procedure consists of a DNA synthesis and a DNA quantification step. A reaction mixture is added to a 96-well plate that is coated with a RNA template. The reaction mixture contains a primer and nucleotides (BrdUTP) both of which are used by RT in the sample to synthesise a DNA strand. After incubation at 33°C the plates are washed using the provided wash buffer and tracer reagent is added which contains alkaline phosphatase (AP)-conjugated anti-BrdU antibodies. These bind to the double-stranded DNA/RNA molecule. Plates are again washed and the product is quantified by addition of the chromogenic AP substrate *para*-Nitrophenylphosphate (pNPP). PNPP is converted into yellow *para*-Nitrophenol (pNP). AP activity is proportional to the RT activity present in the sample. 10 µl of sample were used for RT assay and absorbance of pNP at 405 nm was determined using a spectrophotometer.

2.3.2 Real-time PCR

Real-time PCR was carried out on 1 µl cDNA template. Cycling parameters were 1 cycle of 50°C for 2 minutes; 1 cycle of 95°C for 10 minutes; 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The standard reaction mix composition used was 2x Universal TaqMan Mastermix (Applied Biosystems) 25 µl, gene-specific primers 0.9 µM each, dual-labelled (5' FAM, 3' TAMRA) probe 225 nM. Serially diluted (10^9–10^0 copies/µl) plasmid DNA was used as standard for real-time PCR. 18S ribosomal RNA (rRNA) was used as housekeeping gene. Reactions were set up in MicroAmp Optical 96-well plates (Applied Biosystems) and results were analysed on an Applied Biosystems 7500 real-time PCR system. ΔC_T values (where "C_T" represents "threshold cycle") were calculated by subtracting the mean C_T for 18S rRNA from the C_T for the transcript of interest.

2.3.3 SDS-PAGE and immunoblotting

Cells were pelleted and lysed in CHAPS lysis buffer supplemented with protease inhibitor cocktail (Complete protease inhibitor; Roche). Protein concentration of lysates was determined in 96-well plates using Coomassie Blue Bradford Assay Reagent (Thermo Scientific, Fisher Scientific, Loughborough, UK). Absorbance of samples was measured at 650 nm in a spectrophotometer. Virions in cleared cell culture supernatants were pelleted by ultracentrifugation at 28,000 rpm for 2 hours at 4°C in a Beckman L8-70M ultracentrifuge (SW-41 rotor; Beckman Coulter Limited, High Wycombe, UK). Cell lysates and corresponding pelleted virions were combined with protein loading dye and separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% Bis-Tris SDS-PAGE gels (Life Technologies) in NuPAGE MES SDS Running buffer (Life Technologies). Separated proteins were then transferred onto nitrocellulose membranes using the iBlot system (Life Technologies) and blocked with 5% dried milk powder in PBS containing 0.1% (vol/vol) Tween-20 for 1 hour at room temperature. Membranes were incubated with the appropriate primary antibody diluted to 1 µg/ml in PBS-0.1% Tween-20-5% dried milk powder for 1 hour at room temperature. Membranes were washed with PBS-0.1% Tween-20 three times for 5 minutes. Primary antibodies were detected with biotinylated anti-

mouse/rabbit/goat IgG secondary antibodies (Vector Laboratories, Peterborough, UK) at a dilution of 1:1,000 in PBS–0.1% Tween-20 for 1 hour at room temperature and subsequent chromogenic development using a Vectastain ABC system (Vector Laboratories) and 5-bromo-4-chloro-3-indolyl phosphate-nitroblue tetrazolium (BCIP/NBT; Vector Laboratories) as a substrate.

2.4 *In silico* techniques

2.4.1 Use of databases

Nucleotide and protein sequences were retrieved from Genbank (http://www.ncbi.nlm.nih.gov/genbank) and aligned using ClustalW2 (http://www.ebi.ac.uk/tools/msa/clustalw2/) (Larkin et al., 2007; Goujon et al., 2010). Nucleotide and protein sequence similarity searches were carried out using Blast (http://blast.ncbi.nlm.nih.gov/blast.cgi) (Altschul et al., 1990). The annotation of the 1.9x domestic cat (*Felis catus*) genome (GARField, http://lgd.abcc.ncifcrf.gov/cgi-bin/gbrowse/cat/) was used to identify genes of interest.

2.4.2 Nucleotide and protein sequence alignments

Nucleotide and protein sequence alignments and analyses of sequences obtained by in-house sequencing were performed using DNADynamo (Blue Tractor Software Limited).

2.4.3 Graphs and statistics

Graphs were prepared and statistical analyses (unpaired, two-tailed student's t-test) were carried out using GraphPad Prism 5 software (GraphPad Software, La Jolla, U.S.A.). Where shown, error bars represent standard errors.

3 Generation of a synthetic feline TRIM5α-cyclophilin A fusion protein with potent antilentiviral properties

3.1 Summary

Primate TRIM5α provides an early, post-entry block to lentiviral replication. It contains a C-terminal B30.2 (PRY/SPRY) domain, which mediates binding to lentiviral capsids, and an N-terminal RING domain, which poly-ubiquitinates capsid proteins and targets them for proteasomal degradation. This results in a premature uncoating of the virus and the inhibition of reverse transcription. Feline (domestic cat) TRIM5α (feTRIM5α), however, bears a truncation in its B30.2 (PRY/SPRY) domain, which ablates its binding to lentiviral capsids and its antiviral function (McEwan et al., 2009). Interestingly, this truncation was found to be conserved among members of the *Feliformia* (McEwan et al., 2009) and, hence, cannot account for the block to FIV infection that we observed in lion T lymphocytes (see Fig. 1-4). However, the feTRIM5α RBCC (RING-B-box2-coiled-coiled) domain is abundantly expressed in feline cells (McEwan et al., 2009) and little is known about its biological role.

FIV is potently restricted by TRIM5α proteins from rhesus macaques and African green monkeys (Saenz et al., 2005) and by TRIM5α-cyclophilin A fusion proteins (TRIMCyps) of New World owl monkeys (Diaz-Griffero et al., 2006b) and of certain Old World macaques (Brennan et al., 2008; Newman et al., 2008; Virgen et al., 2008; Wilson et al., 2008). Moreover, it was shown that high-titre FIV replication in feline cells is dependent upon the interaction between cyclophilin A (CypA) and the FIV capsid (McEwan, 2009). The molecular basis for this dependency remains poorly understood but it has been hypothesised that CypA may regulate the timing of uncoating by stabilising capsid-capsid interactions. Although FIV is restricted by primate TRIMCyps and its capsid is able to interact with both primate TRIM5α and primate and feline CypA, no evidence of a feline TRIMCyp was found in domestic or non-domestic felids (McEwan, 2009).

In this study a synthetic feline TRIM5α-cyclophilin A fusion protein (feTRIMCyp) was therefore generated by fusing feline CypA (feCypA) to the feTRIM5α RBCC and its antilentiviral activity was tested. As expected, feTRIMCyp was highly efficient at preventing infection with both HIV-1 and FIV-based

pseudotypes, and feTRIMCyp-expressing cells resisted productive infection with either FIV-Fca or FIV-Pco. The restriction of FIV infection by feTRIMCyp was reversed by the cyclosporine A (CsA) derivatives NIM811 and Debio-025 indicating that the feCypA domain mediates binding to FIV capsid and enables FIV restriction by the feTRIM5α RBCC domain present in feTRIMCyp.

This feline-specific TRIMCyp represents an effective antiviral defence agent with very low potential for toxicity and could be used in the treatment of FIV-positive cats. FeTRIMCyp and FIV infections of the domestic cat further offer a unique opportunity to evaluate TRIMCyp-based approaches to genetic therapy for HIV infection and the treatment of AIDS in humans (Neagu et al., 2009).

3.2 Materials and Methods

3.2.1 Generation of feTRIMCyp

As the feTRIM5α RBCC is encoded by exons 2 to 6 of *feTRIM5*, the start codon of *feCypA* was fused to the last codon of exon 6 to generate feTRIMCyp. The feline TRIM5α RBCC was reamplified from feT5-CXCR (McEwan, 2009) using primers feT5a-1 (5'-GCGGATCCATGGCTTCTGAACTCCTGAAAT-3') (*BamH*I restriction site underlined) and feT5a-2 (5'-CACGATGGGGTTGACCATTTTTTTAAAGGCTTGTATTAT-3'). FeCypA was amplified from cDNA derived from Mya-1 cells using primers directed to the predicted feCypA (GenBank AANG01610851), fCypA R69 5' Nde (5'-AACATATGGTCAACCCCATCGTG-3') (*Nde*I restriction site underlined) and feCypA 3' Mlu (5'-AAACGCGTTTAGATTTGTCCACAGTCA-3') (*Mlu*I restriction site underlined). The amplicon was cloned into the prokaryotic expression vector pOPTH using *Nde*I and *Mlu*I restriction sites and subsequently reamplified using primers feCypA-1 (5'-ATAATACAAGCCTTTAAAAAAATGGTCAACCCCATCGTG-3') and feCypA-2 (5'-GCGTCGACTTAGATTTGTCCACAGTCAGC-3') (*Sal*I restriction site underlined). The feTRIM5α RBCC and feCypA amplification products were annealed and used as templates to generate a feline *TRIMCyp* gene fusion by reamplification with feT5a-1 and feCypA-2.

3.2.2 Stable expression of feTRIMCyp

FeTRIMCyp DNA was cloned into *BamH*I and *Sal*I sites of the retroviral vector pDON-AI-2neo (Takara Bio Europe S.A.S.), and the nucleic acid sequence of the feTRIMCyp fusion was confirmed by DNA sequencing (GenBank accession number HM246715). CrFK cells were transduced with MLV(VSV-G) pseudotypes bearing pDON-AI-2neo or the feTRIMCyp-pDON-AI-2neo construct to create stable cell lines.

3.2.3 Molecular cloning of feline CD134

Feline CD134 (feCD134) was amplified from cDNA derived from Mya-1 cells using primers directed to the predicted feCD134 transcript (GenBank AB128982), feCD134-Fwd (5'-TTGGATCCAGGATGAGGGTGGTTGTGGGGGCT-3') (*BamH*I restriction site underlined) and feCD134-Rev (5'-AAGAATTCTCAGATCTTGGCCAGG GTGGAGT-3') (*EcoR*I restriction site underlined). The amplicon was cloned into the eukaryotic expression vector pDsRed2 (Takara Bio Europe/Clontech) using *BamH*I and *EcoR*I restriction sites.

3.2.4 Inhibitors of the CypA-capsid interaction

The CypA antagonists cyclosporine A (CsA; Sigma-Aldrich) and its derivatives NIM811 (Novartis, Basel, Switzerland) and Debio-025 (Debiopharm S.A., Lausanne, Switzerland) were diluted to 5 mM working stock in ethanol or DMSO and stored at -20°C until further use. In order to inhibit the interaction between the CypA domain of feTRIMCyp and lentiviral capsids, control or feTRIMCyp-expressing CrFK cells were pre-treated with CypA antagonists for 1 hour at 37°C. In lentiviral pseudotype cell entry assays and in FIV replication assays these drugs were used at final concentrations of 2 μM or 2.5 μM. In some experiments CypA antagonists were titrated to final concentrations of 0 μM, 0.25 μM, 0.5 μM, 1 μM and 2 μM.

3.3 Results

3.3.1 Generation of a synthetic feline TRIM5α-cyclophilin A fusion protein (feTRIMCyp)

To assess whether a synthetic TRIMCyp of feline origin would display the potent lentiviral restriction activity observed in primate TRIMCyps, feline TRIM5α and feline CypA were fused experimentally (Fig. 3-1). FeTRIM5α lacks a full-length B30.2 (PRY/SPRY) domain due to a premature stop codon in the exon homologous to human *TRIM5* exon 8 (McEwan, 2009). As the feTRIM5α RBCC domain is encoded by exons 2 to 6, the start codon of *feCypA* was fused to the last codon of exon 6 of *feTRIM5*. Thus, the synthetic feline *TRIMCyp* was designed to mimic the naturally occurring *TRIMCyp* of rhesus macaques (*rhTRIMCyp*). The *feTRIMCyp* transgene was then cloned into the retroviral vector pDON-AI-2neo, transduced into CrFK cells and stably selected.

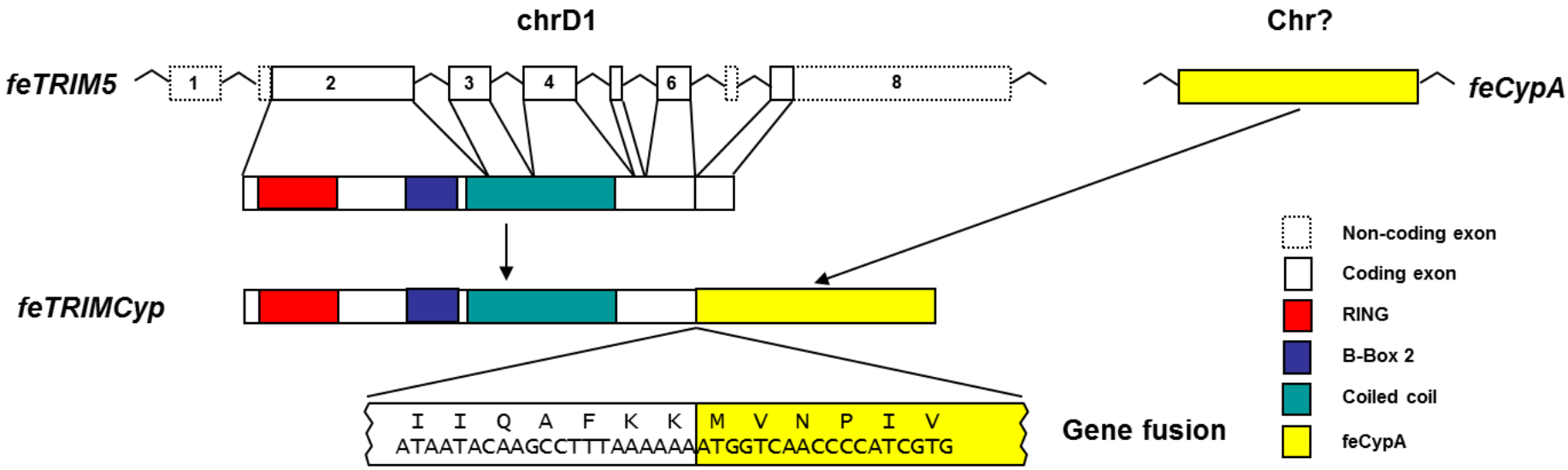


Figure 3-1 Scheme for the generation of a synthetic feline *TRIM5-cyclophilin A* gene fusion (*feTRIMCyp*). Because *feTRIM5* bears a premature stop codon in the gene region homologous to human *TRIM5* exon 8, the start codon of feline *CypA* was fused to the last codon of exon 6 of *feTRIM5*. The nucleotide and amino acid sequence of the fusion site is shown. Non-coding exons of *feTRIM5* are marked by a dotted line and coding exons by a solid line. The colour coding indicates which of the coding exons encode for the feTRIM5α RING, B-box 2 and coiled-coil domains and the feCypA domain.

3.3.2 Specific restriction of lentiviral replication by feTRIMCyp

In primate TRIMCyp proteins the CypA domain is responsible for binding of the protein to retroviral capsids known to interact with cyclophilins, such as HIV-1 and FIV CA. The TRIM5α RING domain of TRIMCyp then ubiquitinates capsid proteins and targets them for rapid proteasomal degradation. This leads to a premature uncoating of the virus, a block to reverse transcription and the inhibition of viral DNA integration into the host genome. To test whether feTRIMCyp could exert a similar early post-entry, reverse transcription block to lentiviral replication, FIV(VSV-G), HIV-1(VSV-G) and SIVmac(VSV-G) pseudotypes containing a green fluorescent protein (GFP) marker gene were titrated onto control or feTRIMCyp-expressing CrFK cells (Fig. 3-2). In this assay the activity of feTRIMCyp is inversely proportional to the number of GFP-positive cells.

In CrFK cells expressing feTRIMCyp, infection with FIV(VSV-G) (Fig. 3-2A) and HIV-1(VSV-G) (Fig. 3-2B) pseudotypes was blocked completely, while SIVmac(VSV-G) pseudotypes infected feTRIMCyp-expressing CrFK cells with similar efficiency to that of control cells (Fig. 3-2C). These data indicate that feTRIMCyp possesses potent antilentiviral activity and that this activity is specific to lentiviruses whose capsids are able to interact with CypA. Because pseudotypes undergo a single cycle of viral entry and gene expression and thus distinguish entry from productive infection, it can be concluded that viral replication was targeted at an early stage of the viral life cycle.

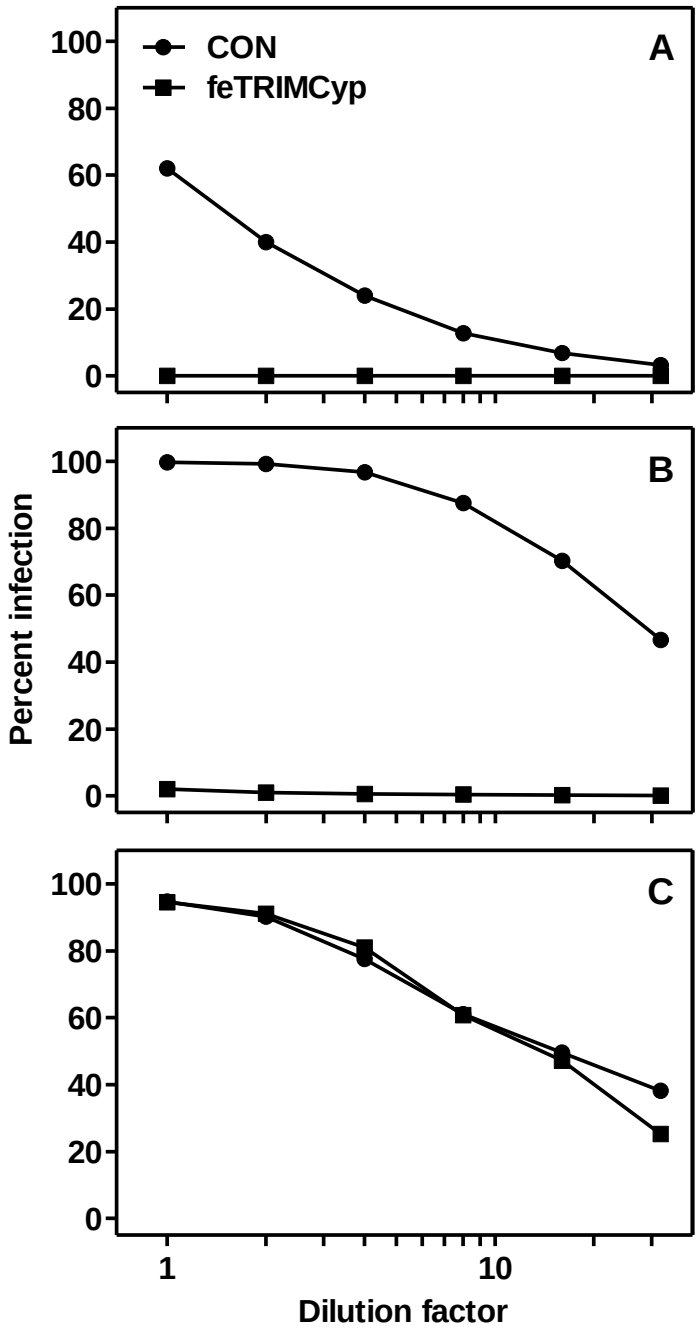


Figure 3-2 Specific inhibition of lentiviral infection at an early post-entry step by feTRIMCyp. CrFK cells stably transduced with vector only (CON, circles) or a vector bearing feline TRIMCyp (feTRIMCyp, squares) were infected with two-fold serial dilutions of (A) FIV(VSV-G), (B) HIV-1(VSV-G) or (C) SIVmac(VSV-G) pseudotypes containing GFP as a marker gene. Infection was assessed 72 hours post-infection by flow cytometry. Points represent the mean of duplicate infections assayed in duplicate (*n*=2).

3.3.3 Reversal of the antilentiviral activity of feTRIMCyp by CypA antagonists

The mechanism of the inhibitory effect of feTRIMCyp was further confirmed by the addition of specific antagonists of the CypA-capsid interaction. Control or feTRIMCyp-expressing CrFK cells were pre-treated with 2 μM CsA or its non-immunosuppressive derivatives NIM811 or Debio-025 and infected with FIV(VSV-G) and HIV-1(VSV-G) pseudotypes as before (Fig. 3-3). While CsA reversed the inhibition of HIV-1(VSV-G) pseudotype infection of target cells by feTRIMCyp modestly (Fig. 3-3D), it was unable to reverse the inhibition of FIV(VSV-G) pseudotype infection (Fig. 3-3A). NIM811 was slightly more effective and reversed the inhibition of FIV(VSV-G) modestly (Fig. 3-3B) and of HIV-1(VSV-G) completely (Fig. 3-3E). Finally, Debio-025 was able to block the inhibitory activity of feTRIMCyp against both FIV(VSV-G) (Fig. 3-3C) and HIV-1(VSV-G) (Fig. 3-3F) pseudotype infection. These data indicate that the restriction of FIV by feTRIMCyp is less sensitive to antagonism by CsA and NIM811 than that of HIV-1. Debio-025 is the most potent of the tested CypA antagonists at the concentration used.

Titrating the CypA antagonists confirmed the differential sensitivities of FIV(VSV-G) and HIV-1(VSV-G) pseudotypes to reversal of the feTRIMCyp restriction (Fig. 3-4). Restriction of HIV-1(VSV-G) was readily reversed by NIM811 and Debio-025 (Fig. 3-4B,C), while Debio-025 alone reversed the restriction of FIV(VSV-G) to near control levels of infection (Fig. 3-4C). CsA displayed a low potency at all concentrations used (Fig. 3-4A). These findings suggest that the antilentiviral activity of feTRIMCyp against FIV is extremely potent, and that the FIV CA-feTRIMCyp interaction is less sensitive to disruption by CypA antagonists than the HIV-1 CA-feTRIMCyp interaction.

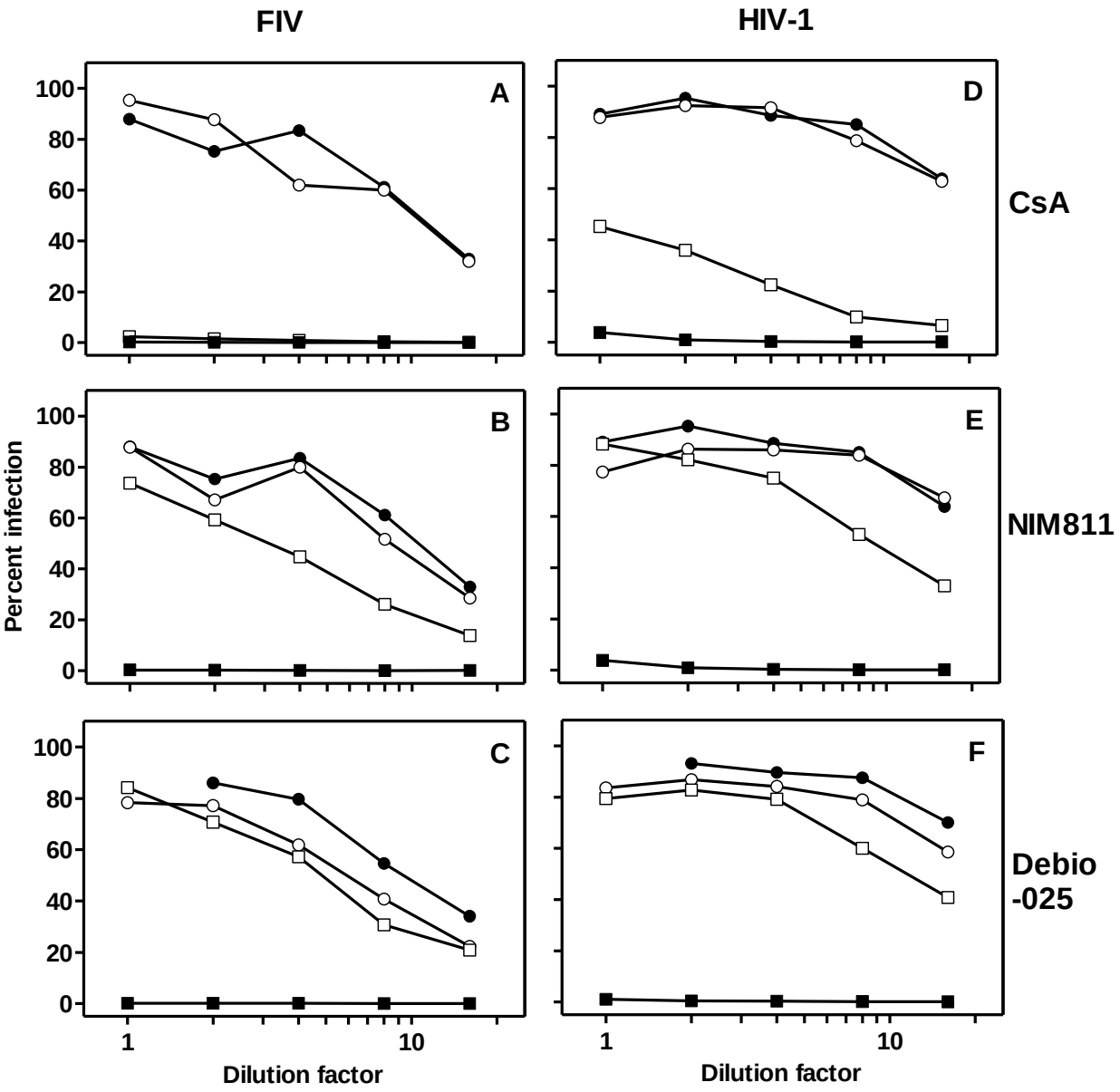


Figure 3-3 Reversal of feTRIMCyp-mediated lentiviral restriction by CypA antagonists. CrFK cells stably transduced with vector only (circles) or a vector bearing feTRIMCyp (squares) were infected with a two-fold serial dilution of FIV(VSV-G) (A-C) or HIV-1(VSV-G) (D-F) pseudotypes containing GFP as a marker gene. Infections were performed in the presence of 2 μM of the CypA antagonists CsA (A,D), NIM811 (B,E), or Debio-025 (C,F) (open symbols) or in the presence of their respective solvents DMSO (CsA and NIM811) or ethanol (Debio-025) (filled symbols). Infection was assessed 72 hours post-infection by flow cytometry. Points represent the mean of duplicate infections assayed in duplicate (*n*=2).

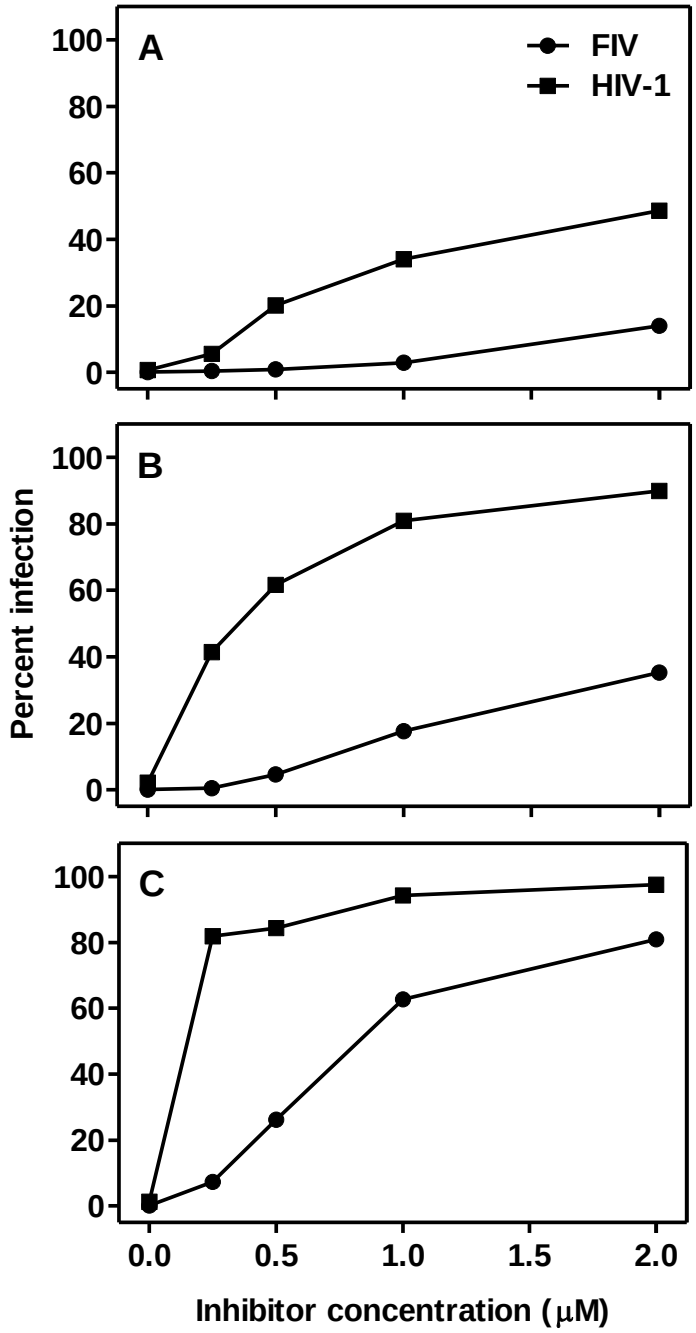


Figure 3-4 Sensitivity of FIV and HIV-1 to reversal of feTRIMCyp restriction by CypA analogues. CrFK cells stably expressing feTRIMCyp were incubated with CsA (A), NIM811 (B) and Debio-025 (C) at final concentrations of 0, 0.25, 0.5, 1.0, or 2.0 μM prior to infection with FIV(VSV-G) (circles) or HIV-1(VSV-G) (squares) pseudotypes containing GFP as a marker gene. Infection was assessed 72 hours post-infection by flow cytometry. Points represent the mean of duplicate infections assayed in duplicate (*n*=2).

3.3.4 Inhibition of productive lentiviral infection by feTRIMCyp and its reversal by CypA antagonists

Next, the ability of feTRIMCyp to inhibit productive infection with replication-competent feline lentiviruses was investigated. CrFK cells support the replication of cell culture-adapted, CD134-independent feline lentiviruses such as the FIV-Fca strain Petaluma isolate F14 or FIV-Pco isolate CoLV. CrFK cells stably transduced with vector only or a vector bearing feTRIMCyp were infected with F14 and CoLV (Fig. 3-5). Expression of feTRIMCyp rendered CrFK cells refractory to the replication of both viruses, while they replicated efficiently in control cells.

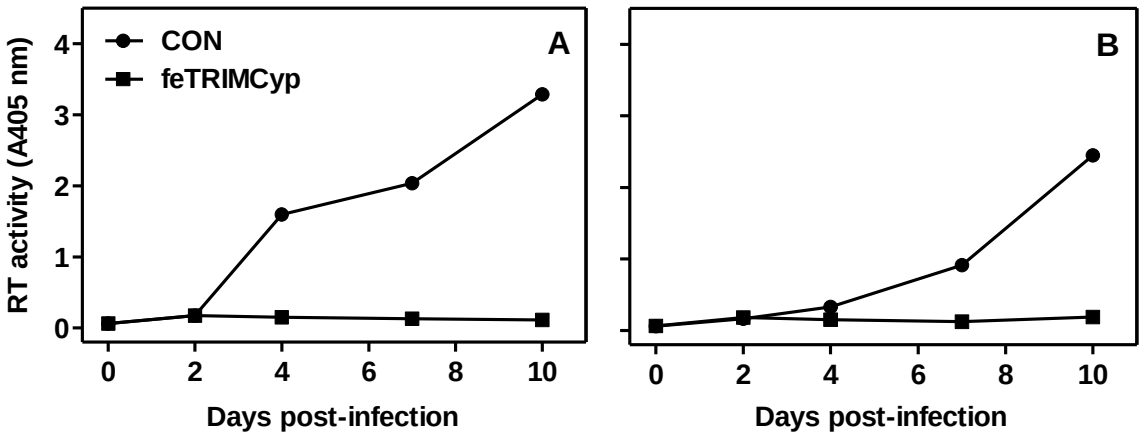
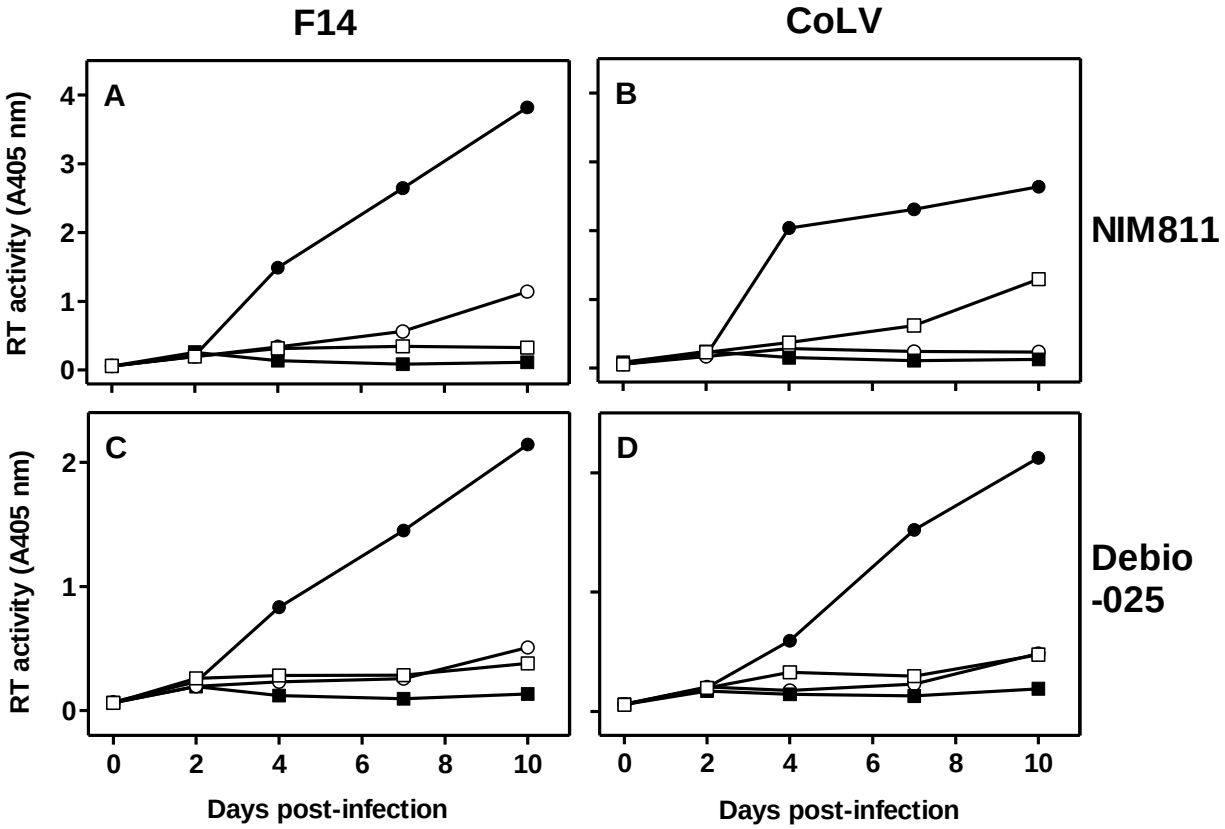


Figure 3-5 Inhibition of productive replication of cell culture-adapted, CD134-independent FIV strains by feTRIMCyp. CrFK cells stably transduced with vector only (circles) or a vector bearing feTRIMCyp (squares) were infected with FIV-Fca Petaluma strain F14 (A) or FIV-Pco strain CoLV (B). Cell culture supernatants were collected on days 0, 2, 4, 7 and 10 post-infection and assayed for RT activity by non-isotopic Lenti-RT assay. Points represent the mean of duplicate infections assayed in duplicate (*n*=2).

The infection assays were then repeated in the presence of 2.5 μM NIM811 and Debio-025 or their solvents (DMSO or ethanol, respectively) to determine whether CypA antagonists could reverse the inhibitory effect of feTRIMCyp on productive viral replication (Fig. 3-6). Both NIM811 and Debio-025 blocked replication with FIV-Fca and FIV-Pco in control cells (Fig. 3-6A-D), indicating an important role for CypA in the replication of these viruses in CrFK cells and,

consistent with previous studies, suggesting a role for CypA in the replication of FIV (Lin and Emerman, 2006). In contrast, both Debio-025 (Fig. 3-6B,D) and NIM811 (Fig. 3-6A,C) countered the inhibition of viral growth by feTRIMCyp to a small extent. Moreover, whereas FIV-Fca replication in control cells was accompanied by the formation of prominent syncytia (Fig. 3-6E), no syncytia were observed in feTRIMCyp-expressing cells infected with FIV-Fca (Fig. 3-6G). Treatment of control cells with 2.5 μM Debio-025 prior to infection with FIV-Fca prevented viral growth and syncytium formation (Fig. 3-6F). However, pre-treatment of feTRIMCyp-expressing cells with Debio-025 restored viral growth to a level at which small syncytia could be visualised (Fig. 3-6H, arrows) and RT activity could be detected (Fig. 3-6B).



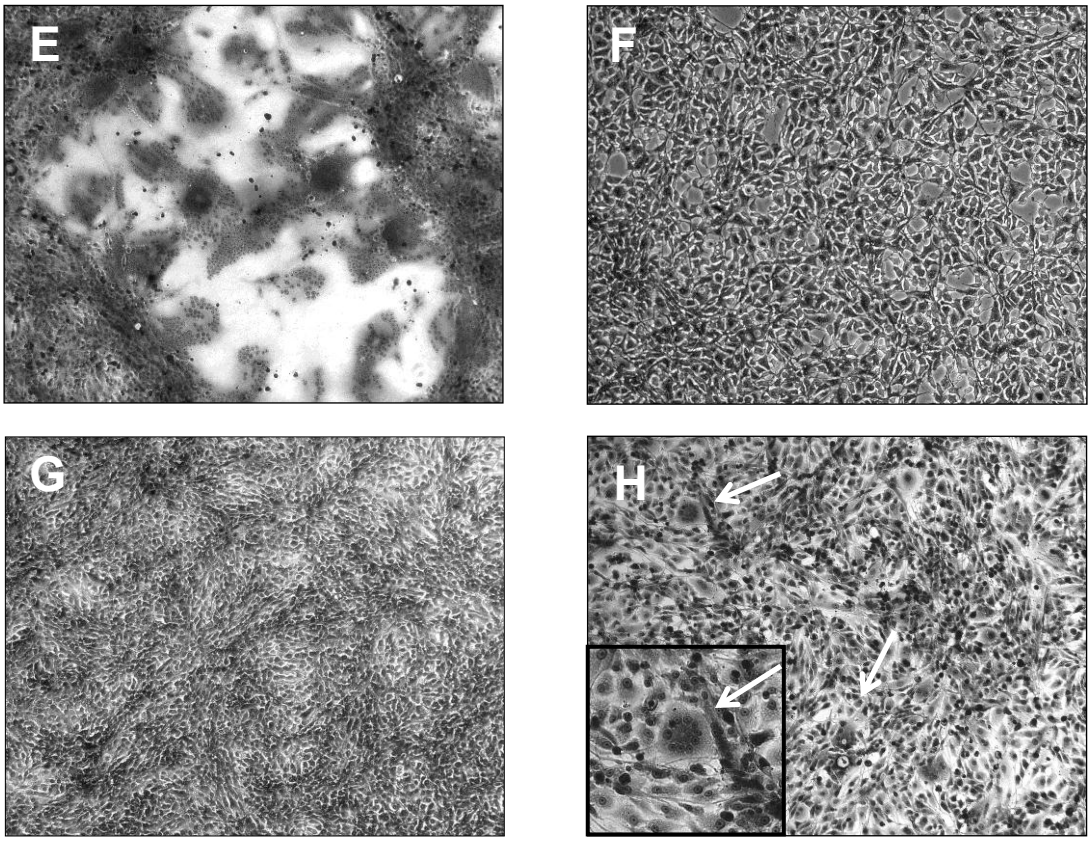


Figure 3-6 Reversal of feTRIMCyp-mediated restriction of productive FIV infection by CypA antagonists. CrFK cells stably transduced with vector only (circles) or a vector bearing feTRIMCyp (squares) were infected with FIV-Fca Petaluma strain F14 (A,B) or FIV-Pco strain CoLV (C,D). Infections were performed in the presence of 2.5 μM of the CypA antagonists NIM811 (A,C), or Debio-025 (B,D) (open symbols) or in the presence of their respective solvents DMSO (NIM811) or ethanol (Debio-025) (filled symbols). Cell culture supernatants were collected on days 0, 2, 4, 7 and 10 post-infection and assayed for RT activity by non-isotopic Lenti-RT assay. Points represent the mean of duplicate infections assayed in duplicate (*n*=2). (E-H) Syncytium formation in CrFK cells infected with FIV-Fca. Cells expressing vector only (E,F) or feTRIMCyp (G,H) were infected with FIV-Fca in the presence of 2.5 μM Debio-025 (F,H) or its solvent ethanol (E,G). They were fixed and stained 10 days post-infection with 1.0% methylene blue-0.2% basic fuchsin in methanol. The arrows indicate small syncytia, magnified in the inset (H).

It needs to be considered that, where endogenous CypA and ectopically expressed feTRIMCyp are coexpressed, the CypA antagonists prevent binding of both proteins to FIV CA. While disruption of the interaction between endogenous CypA and FIV CA would impact negatively on viral replication, blocking of binding of feTRIMCyp to FIV CA would rescue viral growth. It appears that, at a CypA antagonist concentration of 2.5 µM, the activity of feTRIMCyp is inhibited while binding of endogenous CypA to FIV CA continues to occur at low levels so that in total viral replication is favoured. To gain a better understanding of the dynamics between inhibition and restoration of viral infection in the presence of endogenous and exogenous CypA and CypA antagonists, the viral replication assay was repeated in the presence of a reduced antagonist concentration of 2 µM (Fig. 3-7).

While growth of FIV-Pco in control cells was suppressed efficiently by both NIM811 (Fig. 3-7E) and Debio-025 (Fig. 3-7F), growth of FIV-Fca was reduced modestly (Fig. 3-7B,C). In feTRIMCyp-expressing cells FIV-Fca replication was partially restored by 2 µM NIM811 and completely by Debio-025 (Fig. 3-7B,C), with viral replication accompanied by prominent syncytium formation. Notably, the CypA antagonists did not restore FIV-Pco growth in the presence of feTRIMCyp.

It can be concluded that the effect of CypA antagonists on the interaction between endogenous and exogenous CypA with FIV CA was concentration-dependent. Lower CypA antagonist concentrations were needed to overcome the inhibitory activity of feTRIMCyp than were necessary to fully block binding of endogenous CypA to FIV CA. Again, Debio-025 was more potent than NIM811. Differences between FIV strains were observed which seem to have a different degree of dependence on endogenous CypA. 2 µM Debio-025 restored FIV-Fca F14 growth in feTRIMCyp-expressing cells which indicates that the number of endogenous CypA molecules binding to FIV CA in these cells was sufficiently high to support F14 growth. In contrast, endogenous CypA was insufficient to promote FIV-Pco CoLV replication. Thus, FIV strains may have adapted to the CypA levels in their natural target cells so that these levels are optimal for their growth.

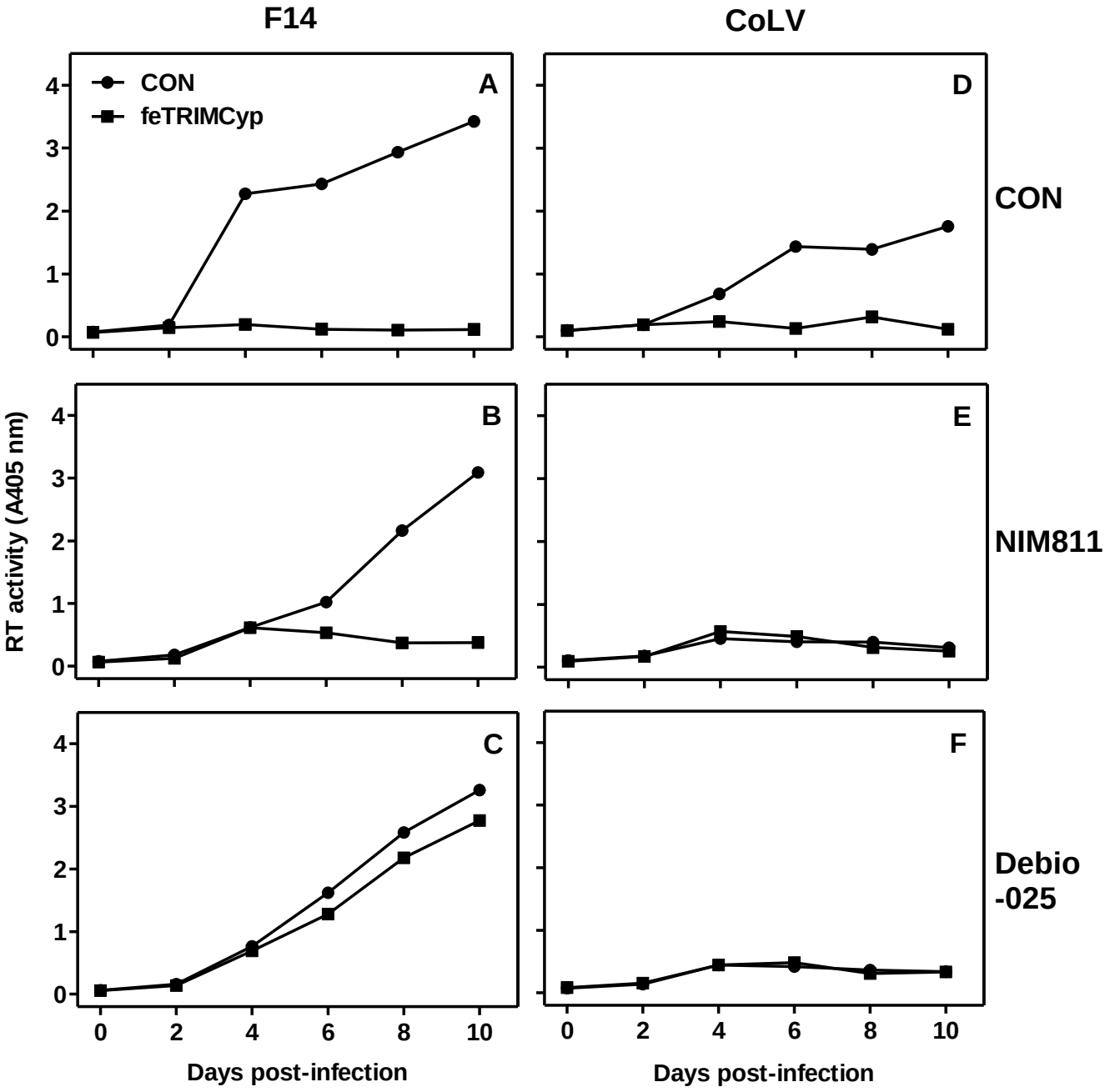


Figure 3-7 Restoration of viral replication in feTRIMCyp-expressing CrFK cells by 2.0 μM NIM811 and Debio-025. CrFK cells stably transduced with vector only (circles) or a vector bearing feTRIMCyp (squares) were infected with FIV-Fca Petaluma strain F14 (A-C) or FIV-Pco strain CoLV (D-F). Infections were performed in the presence of 2 μM of the CypA antagonists NIM811 (B,E), or Debio-025 (C,F) (open symbols) or in their absence (A) (filled symbols). Cell culture supernatants were collected on days 0, 2, 4, 6, 8 and 10 post-infection and assayed for RT activity by non-isotopic Lenti-RT assay. Points represent the mean of duplicate infections assayed in duplicate (*n*=2).

In addition to cell culture-adapted, CD134-independent feline lentiviruses the activity of feTRIMCyp against the primary, CD134-dependent FIV-Fca strains Glasgow 8 (GL8) and PPR was assessed. CrFK cells stably expressing vector only or feTRIMCyp were transduced with the retroviral vector pDsRed2 or a feline CD134-pDsRed2 construct and stably selected. All four cell lines were infected with GL8 and PPR (Fig. 3-8). Replication of GL8 (Fig. 3-8A) and PPR (Fig. 3-8B) occurred only in the presence of feline CD134 and was completely blocked by the feline TRIMCyp fusion protein.

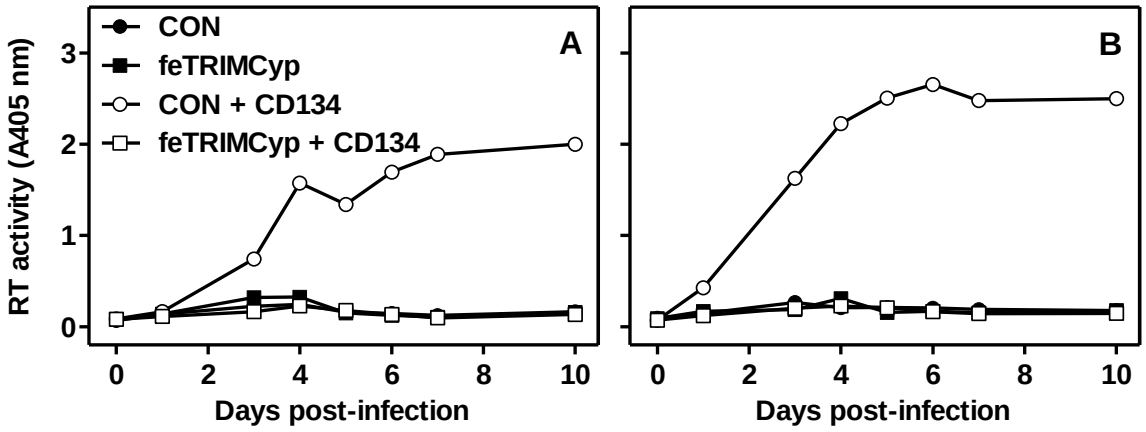


Figure 3-8 Inhibition of productive replication of primary, CD134-dependent FIV strains by feTRIMCyp. CrFK cells stably transduced with vector only (circles) or a vector bearing feTRIMCyp (squares) were retransduced with the retroviral vector pDsRed2 (filled symbols) or a feline CD134-pDsRed2 construct (open symbols) and infected with FIV-Fca strain GL8 (A) or FIV-Fca strain PPR (B). Cell culture supernatants were collected on days 0, 1, 3-7 and 10 post-infection and assayed for RT activity by non-isotopic Lenti-RT assay. Points represent the mean of duplicate infections assayed in duplicate (*n*=2).

3.4 Discussion

In this study a synthetic feline TRIM5α-cyclophilin A fusion protein (feTRIMCyp) was created based on the naturally occurring TRIMCyp of Old World rhesus monkeys (*M. mulatta*). FeTRIMCyp exhibited a robust antilentiviral activity confirming that the feTRIM5α RBCC retains full ability to restrict FIV and HIV-1 and simply lacks a capsid-targeting B30.2 (PRY/SPRY) domain. The inability of

feTRIM5α to bind lentiviral capsids is compensated for by the feCypA domain of feTRIMCyp. FeTRIMCyp potently blocked infection with FIV(VSV-G) and HIV-1 (VSV-G) pseudotypes at an early post-entry, pre-reverse transcription step (Fig. 3-2). In contrast, SIVmac pseudotype replication was unaffected by feTRIMCyp because of the inability of SIVmac CA to interact with cyclophilins (Franke et al., 1994; Braaten et al., 1996b; Lin and Emerman, 2006) (Fig. 3-2). Furthermore, feTRIMCyp completely ablated *in vitro* growth of all tested cell-culture adapted, CD134-independent (Fig. 3-5) and primary, CD134-dependent strains of FIV (Fig. 3-8).

The specificity of the feTRIMCyp-capsid interaction is dependent on its feCypA domain; thus, this interaction, like the interaction between endogenous feCypA and lentiviral capsid, was sensitive to disruption by CsA and its derivatives NIM811 and Debio-025. In control cells, CypA antagonists blocked the replication of FIV(VSV-G) and HIV-1(VSV-G) pseudotypes (Fig. 3-3) and of replication-competent FIV-Fca and FIV-Pco viral strains (Fig. 3-6 and 3-7). Interestingly, the CypA antagonists displayed different degrees of potency to inhibit lentiviral replication with CsA having the lowest and Debio-025 having the highest activity at the antagonist concentrations used. These findings indicate that the CypA antagonists bind CypA with different affinities. In fact, the K*i* values (a measure of the binding affinity of an inhibitor) of CsA, NIM811 and Debio-025 for human CypA inhibition have been measured to be 9.8, 2.1 and 0.35 nM, respectively (Ptak et al., 2008). Binding affinities of the CypA antagonists to feCypA have not been determined. In feTRIMCyp-expressing cells, the antilentiviral activity of feTRIMCyp was modestly reversed by Debio-025 and partially by NIM811, but not by CsA (Fig. 3-4, 3-6 and 3-7). As mentioned before, in cells stably expressing feTRIMCyp, endogenous and exogenous feCypA are co-expressed and influence viral replication in opposite manners. Whereas the presence of endogenous feCypA is essential for viral replication (McEwan, 2009), feTRIMCyp acts as a viral restriction factor. CypA antagonists negatively affect the activities of both forms of feCypA. Hence, the overall effect of CypA antagonists on viral replication is determined by the affinity of feCypA for the respective lentiviral capsid and on the dependence of the virus on endogenous feCypA. Indeed, it was observed that HIV-1 replication was more susceptible to CsA and its derivatives than FIV replication (Fig. 3-3 and 3-4). Because the binding affinities of FIV and HIV-1 CA for feline and human CypA lie within the

same range (McEwan, 2009) it is unlikely that FIV CA binding to feCypA and feTRIMCyp is stronger than that of HIV-1 CA. Thus, it can be concluded that HIV-1 replication is dependent on higher endogenous feCypA levels than FIV replication. Moreover, 2 μM NIM811 and Debio-025 inhibited FIV-Pco CoLV growth in control cells completely and FIV-Fca F14 growth only modestly (Fig. 3-7). In feTRIMCyp-expressing cells infected with FIV-Fca F14, Debio-025 was able to overcome feTRIMCyp and to restore infection to control cell levels while the drug showed no activity against feTRIMCyp in FIV-Pco CoLV-infected cells. Again, because the FIV CA proline-rich feCypA binding loop is conserved across different FIV strains (McEwan, 2009) the more likely explanation for these results is that FIV-Pco strain CoLV requires higher endogenous feCypA levels for its replication than FIV-Fca F14.

Although this study has confirmed the requirement of feCypA for efficient FIV replication, the mechanism by which feCypA promotes FIV growth is still unknown. The FIV feCypA binding site within FIV CA^N differs from the HIV-1 CypA binding site in that the proline-rich loop is considerably shorter which results in a reduced region of contact with CypA (McEwan, 2009). Furthermore, the HIV-1 CA^N Gly89-Pro90 bond exists as *cis-trans* isomers. *Cis-trans* isomerisation is mediated by human CypA and it has been proposed that this enzymatic reaction in combination with structural changes in HIV-1 CA contributes to capsid stability which prevents premature uncoating of viral particles (Braaten et al., 1996a; Braaten et al., 1996b; Bosco et al., 2002; Howard et al., 2003; Bosco and Kern, 2004; Luban, 2007; Li et al., 2009; Ylinen et al., 2009). In FIV CA^N, however, the glycine in position 89 is replaced by an arginine (Arg89) and the Arg89-Pro90 bond is not *cis-trans* isomerised by feCypA (Leo James, personal communication). The glycine to arginine change is structurally important as Arg89 comes in close proximity with feCypA residues Phe60 and Phe113. Cation-π interactions between these residues may contribute to the affinity of the interaction between FIV CA^N and feCypA (McEwan, 2009). In the absence of feCypA enzymatic activity simple binding to FIV CA^N may be sufficient for promoting FIV infectivity (Luban, 2007).

Additionally, the data presented here indicate that the feTRIM5α RBCC domain possesses the ability to target lentiviral capsids for proteasome-mediated degradation. Although feTRIM5α lacks a capsid-binding B30.2 (PRY/SPRY) domain and therefore an antiviral activity, it is abundantly

expressed in feline cells. Recent studies have provided evidence that human TRIM5α is involved in innate immune signalling (Pertel et al., 2011; Tareen and Emerman, 2011). TRIM5α bound to the lentiviral capsid lattice has been shown to interact with the heteromeric, ubiquitin-conjugating enzyme UBC13–UEV1A (UBE2N-UBE2V1) and to synthesise unattached K63-linked ubiquitin chains that activate the TAK1 protein kinase complex and stimulate AP-1 (activator protein-1) and NF-κB signalling (Pertel et al., 2011). Thus, TRIM5α is both an antiretroviral restriction factor and a pattern recognition receptor for the viral capsid lattice. Because of the inability of feTRIM5α to bind lentiviral capsids, it is important to note that human TRIM5α also signals in the absence of capsid but that binding to capsid greatly enhances signalling and expression of inflammatory chemokines and cytokines (Pertel et al., 2011). Capsid-independent signalling is mediated by the TRIM5α RING domain rather than the B30.2 (PRY-SPRY) domain and activates AP-1 and NF-κB (Pertel et al., 2011; Tareen and Emerman, 2011). Thus, a critical role in immune signalling may explain why the expression of the TRIM5α RBCC domain remains high in felids.

Given the high potency of feTRIMCyp to block lentiviral replication, feTRIMCyp would be an excellent candidate for use in gene therapy in FIV-infected cats. Treatment of the lentivirus-infected host using feTRIMCyp may be achieved by transduction of bone marrow-derived haematopoietic progenitor stem cells and repopulation of the host immune system following bone marrow ablation. However, transduction of peripheral blood-derived $CD4^+$ T cells and *ex vivo* expansion of the transduced cells prior to repopulation of the host immune system may be sufficient to provide a pool of FIV-resistant T lymphocytes to overcome the immunodeficiency associated with feline AIDS. Accordingly, in addition to the successful *in vitro* studies described here *in vivo* analyses should be performed to examine the effects of feTRIMCyp expression on the function and development of feline haematopoietic stem cells and T lymphocytes and to exclude any detrimental effects that may manifest following ectopic expression of feTRIMCyp in primary cells.

An insight into the likely therapeutic utility of TRIMCyp fusions in humans has been provided by Neagu et al. (2009) in their study on the effect of stable expression of a synthetic human TRIMCyp on human T cell function. Human $CD4^+$ T lymphocytes stably transduced with human TRIMCyp (hT5Cyp) proliferated at the same rate, produced similar levels of IL-2 and expressed comparable levels

of cell surface CD4, CXCR4 and MHC class I as cells transduced with an empty vector (Neagu et al., 2009). Human $CD4^+$ T cells stably expressing hT5Cyp were then transplanted into $Rag2^{-/-}/\gamma c^{-/-}$ mice, a mouse strain that lacks B, T and NK cells and which does not reject xenografts (Mazurier et al., 1999). Following challenge of the engrafted mice with HIV-1 they resisted infection, as evidenced by reduced plasma viral load and maintenance of $CD4^+$ T lymphocyte numbers in peripheral blood and in lymphoid tissues (Neagu et al., 2009). $Rag2^{-/-}/\gamma c^{-/-}$ mice were also transplanted with hT5Cyp-transduced $CD34^+$ haematopoietic progenitor cells. The mean viraemia following HIV-1 challenge was less than 30% of that observed in mice reconstituted with a non-functional TRIMCyp construct (HT5CypHis126Gln) (Neagu et al., 2009). Thus, initial studies with a synthetic human TRIMCyp offer great hope for the use of TRIMCyp as an approach to gene therapy for lentiviral infections. TRIMCyp-based lentiviral gene therapies have significant advantages over other approaches. By targeting viral entry the virus is denied the opportunity to replicate and thus, escape mutants cannot be generated. Moreover, as TRIMCyp does not target the function of an endogenously expressed molecule, it is unlikely to have side effects that are detrimental to the host. In addition, the generation of synthetic TRIMCyp fusion proteins using endogenous TRIM5α and CypA circumvents the potential pitfall of the host mounting an immune response against xenoantigens.

4 Investigation into the role of APOBEC3 cytidine deaminases in the *Felidae*

4.1 Summary

APOBEC3 (A3) proteins are cellular cytidine deaminases that catalyse cytidine-to-uracil changes in nascent viral minus-strand cDNA during reverse transcription. Viral uracil-containing cDNAs that escape degradation by host DNA repair enzymes give rise to viral cDNAs containing guanine-to-adenine hypermutations (Harris et al., 2003; Lecossier et al., 2003; Mangeat et al., 2003; Mariani et al., 2003; Zhang et al., 2003; Bishop et al., 2004; Liddament et al., 2004). A low number of proviruses will survive; however, the hypermutations lead to an increased number of alternate splicing and premature termination events of the viral transcripts. Non-functional proteins are produced and the virions are defective and non-infective (Zheng and Peterlin, 2005). Deamination-independent mechanisms of A3 proteins to block viral replication have also been reported. It has been shown that both wild-type forms and catalytically inactive mutants of human APOBEC3G and APOBEC3F interfere with individual steps of reverse transcription, such as minus- and plus-strand DNA synthesis, and cDNA transfer and elongation, which consequently leads to a reduction of viral cDNA transcript levels being produced (Newman et al., 2005; Holmes et al., 2007; Iwatani et al., 2007). Human, simian and feline A3s are overcome and targeted for proteasomal degradation by lentiviral Vif proteins in a species-specific manner (Conticello et al., 2003; Marin et al., 2003; Sheehy et al., 2003; Stopak et al., 2003; Yu et al., 2003; Mehle et al., 2004b; Munk et al., 2008; Stern et al., 2010; Zielonka et al., 2010). Feline A3C proteins are counteracted by the FeFV Bet protein (Lochelt et al., 2005; Munk et al., 2008).

A3 proteins belong to a large family of cytidine deaminases that have arisen to protect vertebrate genomes from mobile endogenous retroelements, the ancestors of modern exogenous retroviruses (Mangeat and Trono, 2005; Goila-Gaur and Strebel, 2008). During their evolution *A3* genes have undergone a series of gene duplications and positive selection (Sawyer et al., 2004; Zhang and Webb, 2004; Conticello, 2008). A3s are unique in their ability to restrict retroviruses and their presence is limited to placental mammals (Conticello, 2008). All species possess two ancestral *A3* genes from which different

repertoires of *A3* genes have evolved through gene duplications and fusions, ranging from only one double deaminase domain *A3* gene in rodents, pigs and cattle to seven single and double catalytic domain *A3* genes in primates (Conticello et al., 2005; Munk et al., 2008). The domestic cat genome possesses four single deaminase domain *A3* genes that give rise to a fifth, double catalytic domain A3 protein (A3CH) by read-through alternative splicing using exons from both *A3C* and *A3H* genes (Munk et al., 2008).

In the absence of a functional TRIM5α in felids and no evidence for a naturally occurring feline TRIMCyp, differences in the antiretroviral activities of A3 proteins may be responsible for the inability of FIV to grow efficiently in lion T lymphocytes (see Fig. 1-4) and to establish immunodeficiency in lions. Studies on the phylogeny of feline lentiviruses and felids have indicated that lions have been harbouring lentiviruses for a considerably longer time than domestic cats (Johnson et al., 2006; Antunes et al., 2008; Poss et al., 2008). Thus, we hypothesise that lion A3 proteins are more potent against FIV than cat A3s, or, alternatively, that the anti-A3 activity of FIV-Ple Vif has decreased over time resulting in host-virus adaptation and a lower pathogenicity of FIV infections in lions.

In this study lion A3 cDNAs were amplified, cloned and characterised. The organisation of the lion *A3C* gene locus was determined and found to resemble that of domestic cats. Furthermore, the activities of lion A3 proteins against FIV lacking Vif (FIVΔ*vif*) and FIV harbouring Vifs of different feline lentiviruses were compared to those of domestic cats and pumas in single-cycle replication assays. While all felid A3C isoforms showed only modest activity against FIVΔ*vif* and FIV wild-type, all felid A3H and A3CH isoforms restricted FIVΔ*vif* completely. Differences were observed between the activities of cat and lion A3CH (FcaA3CH and PleA3CH, respectively) against FIV containing FIV-Fca Vif and FIV-Pco Vif, with PleA3CH being more potent than FcaA3CH. It should be noted that the expression of particular FIV Vifs in 293T cells, which were used in A3 activity assays, was inefficient so that the activity of FIV-Ple E Vif and FIV-Oma Vif against feline A3s could not be studied.

Furthermore, expression levels of domestic cat and lion *A3* genes in several cat cell lines and cat tissues as well as primary lion PBMCs were quantified. Domestic cat and lion *A3C* and *A3H* genes were expressed modestly and their expression was upregulated by interferons only in certain cell lines.

Interestingly, *A3C* gene expression was higher than that of *A3H* in cat cell lines and tissues, whereas the opposite trend was found in lion PBMCs. *FcaA3CH* and *PleA3CH* expression levels were low in all samples tested.

Surprisingly, an investigation into the long-term evolution of lion A3 proteins in the presence or absence of FIV infection revealed no evidence for selection pressure by FIV on A3 proteins.

The data presented here confirm the important role of A3 proteins in the protection of mammalian genomes against DNA mutators and support the hypothesis of A3-FIV Vif coadaptation in lions that may prevent immunodeficiency from occurring.

4.2 Materials and Methods

4.2.1 Amplification and molecular cloning of feline APOBEC3 cDNAs

Cat (*Felis catus*, Fca), lion (*Panthera leo*, Ple) and puma (*Puma concolor*, Pco) A3s were amplified from cDNA derived from Mya-1 cells, Angolan lion PBMCs or puma PBMCs using primers directed to their predicted sequences. FcaA3Ca and FcaA3H (GenBank AY971954, EU011792) (Munk et al., 2008) were amplified using the primer pairs FeA3Ca F (5'-ACTGGTCGACACCATGGAGCCCTGGCGCCCCAGCCCA AGAAACCCAATGG-3') and FeA3Ca R (5'-ACAGCGGCCGCTCACCTAAGGATTTCTTGA AGCTCTGCAGCC-3'), and FeA3H F (5'-ACTGGTCGACACCATGAATCCACTACAGGAA GTCATATTC-3') and FeA3H R (5'-ACAGCGGCCGCTCATTCAAGTTTCAAATTTCTGAA GTCATTC-3'), respectively. The FcaA3H cDNA sequence obtained differed in two positions from the GenBank entry (G193T and G231C), resulting in two changes at the amino acid level (Ala65Ser and Met77Ile). These changes may represent nucleotide polymorphisms in the different cell lines used for amplification (CrFK compared to Mya-1 cells). FcaA3CH (GenBank EF173021) (Munk et al., 2008) was amplified using primers FeA3Ca F and FeA3H R. PleA3C1 and PleA3H (GenBank EU007543, EU007549) were obtained using primers LiA3C1 F (5'-ACTGGTCGACACCATGGAGCCCTGGCGCCCCAGCCCAAGAAACCCAATGC-3') and LiA3C1 R (5'-ACAGCGGCCGCTCACCTAAGGATTTCTTGAAGCTCTGCAGCC-3'), and

LiA3H F (5'-ACTGGTCGACACCATGAATCCACTACAAGAAGACATATTC-3') and LiA3H R (5'-ACAGCGGCCGCTCATTCAAGTTTCAAATTTCTGAAATCATTC-3'), respectively. PleA3CH (GenBank GU097662) (Zielonka et al., 2010) was amplified using primers LiA3C2 F (5'-ACTGGTCGACACCATGGAGCCCTGGCGCCCCAGCCCAAGAAACC CAATGG-3') and LiA3H R. PcoA3C and PcoA3H (GenBank EU007545, EU007552) were amplified using primers LiA3C2 F and LiA3C1 R, and primers LiA3H F and LiA3H R, respectively. PcoA3CH (GenBank GU097659) (Zielonka et al., 2010) was amplified using primers LiA3C2 F and LiA3H R. All forward primers contained *Sal*I restriction sites and all reverse primers *Not*I restriction sites (restriction sites underlined). The amplicons were cloned into the eukaryotic expression vector VR1012 (Vical Inc.) using *Sal*I and *Not*I sites.

4.2.2 Characterisation of the lion *A3C* gene locus

The domestic cat genome contains an *A3* gene locus (GenBank EU109281) on chromosome B4 in which three *A3C* genes (*A3C*c, *A3C*a and *A3C*b), which have arisen by two gene duplication events and thus share high nucleotide similarity, are aligned in a head-to-tail orientation (Munk et al., 2008). The *A3C* genes consist of four exons (E1-E4) and three introns (I1-I3). *A3C*c and *A3C*a and *A3C*b, respectively, are separated from each other by a stretch of genomic sequence. Downstream of *A3C*b lies *A3H*. In order to determine the number and order of *PleA3C* genes in the lion genome primers were designed based on the published sequences of PleA3C1 and PleA3C2 (GenBank EU007544) and fragments between exons were amplified using GoTaq Flexi DNA polymerase (Promega). Domestic cat *A3C*c can be distinguished from *A3C*a and *A3C*b by shorter I1 and I2. Primers used to amplify the fragment between *PleA3C* E1 and E2 were LiA3C-Ex1-Fwd (5'-ATGGAGCCCTGGCGCCCCA-3') and LiA3C-Ex2-Rev (5'-CCGAAAAACTCCCCAGTC GCTGTCATT-3') (primers were designed to bind to both *PleA3C*1 and *PleA3C*2). Primers used to amplify the fragment between E2 and E3 were LiA3C-Ex2-Fwd-1 (5'-CCTTCCATTTCCAGTTTCCA-3') and LiA3C-Ex3-Rev-1 (5'-GTCACGGAACCAAAAG AGGA-3') (primer pair designed to bind specifically to *PleA3C*1) and LiA3C-Ex2-Fwd-2 (5'-TGGGCGGAAACTCTGTTATC-3') and LiA3C-Ex3-Rev-2 (5'-ACGGATACTG GTTACGGAAC-3') (primer pair designed to bind specifically to *PleA3C*2). Alternative reverse primers used to bind to E3 were LiA3C-Ex3-Rev-3 (5'-

TATTCATCACGGCATGGATAC-3') (specific to *PleA3C*1) and LiA3C-Ex3-Rev-4 (5'-TCCTCATCACGGTACGGATAC-3') (specific to *PleA3C*2). Fragments between E3 of one *PleA3C* gene and E1 of the following *PleA3C* gene were amplified using primer pairs LiA3C-Ex3-Fwd-1 (5'-GTATCCATGCCGTGATGAATA-3') and LiA3C-Ex1-Rev-1 (5'-TGGGGCGCCAGGGCTCCAT-3') (specific to *PleA3C*1) or LiA3C-Ex3-Fwd-2 (5'-GTATCCGTACCGTGATGAGGA-3') and LiA3C-Ex1-Rev-1 (specific to *PleA3C*2). Amplified gene fragments were cloned into the vector pCR2.1-TOPO (Life Technologies) and sequenced. In this study a previously uncharacterised PleA3C was discovered which differs from PleA3C1 in one nucleotide position in E3 (A499G) and in four nucleotides in E4 (G515A, A539G, A561G, G575A). This new PleA3C was named PleA3C3, amplified with primers LiA3C1 F and LiA3C3-Rev (5'-ACAGCGGCCGCTTACCTAAGGATTTCCTG-3') (*Not*I restriction site underlined) and cloned into VR1012 (Vical Inc.) using *Sal*I and *Not*I restriction sites.

4.2.3 Amplification and molecular cloning of FIV Vifs

All FIV Vifs used in this study were tagged with a C-terminal haemagglutinin (HA) tag. FIV-Fca Vif was amplified from the GL8Mya molecular clone (Hosie et al., 2002) of FIV-Fca isolate GL8 using primers GL8 Vif F (5'-ACTGGTCGACACCATGAG TGACGAAGATTGGCAGG-3') and GL8 Vif Rev HA (5'-ACAGCGGCCGCTCA**AGCGTAA TCTGGAACATCGTATGGGTA**TAGTTTTCCCGACCATAACAG-3'). FIV-Ple E Vif was amplified from cDNA derived from Mya-1 cells infected with a primary isolate of FIV-Ple E obtained from serum of a wild, 8 year old male lion (Sangre) from the Moremi reserve in the Okavango Delta, Botswana (McEwan, 2009). Primers were designed based on the available FIV-Ple E 1027 sequence (GenBank EU117992) (Pecon-Slattery et al., 2008a): LLV-E Vif F (5'-ACTGGTCGACACCATGAGTGGTGAA GATATAAGTCAGG-3') and LLV-E Vif Rev HA (5'-ACAGCGGCCGCTCA**AGCGTAATCT GGAACATCGTATGGGTA**GCCACCTTTCCCTATTAAATATAG-3'). FIV-Pco Vif was amplified from cDNA derived from Mya-1 cells infected with FIV-Pco strain PLV-14 (GenBank U03982) using primers PLV Vif F (5'-ACTGGTCGACACCATGGCTTCAA TCAGACAGACAGAACAG-3') and PLV Vif Rev HA (5'-ACAGCGGCCGCCTCA**AGCGTAA**

TCTGGAACATCGTATGGGTAGAATGATAATGTTAAATCCATCCA-3'). FIV-Oma Vif was amplified from FIV-Oma molecular clone pOma3 (GenBank AY713445) using primers FIV-Oma Vif F (5'-ACTGGTCGACACCATGAGTGGTGAAGAGGATTGGCAGGT AAG-3') and Oma3 Vif Rev HA (5'-ACAGCGGCCGCTCA**AGCGTAATCTGGAACATCGT ATGGGTA**ACTCTTCATCCGATATAACACTTCATAGGGTACA-3'). All forward primers contained *Sal*I restriction sites and all reverse primers *Not*I restriction sites (restriction sites underlined). The HA tag coding sequence in the reverse primers is marked in bold. The amplicons were cloned into the eukaryotic expression vector VR1012 (Vical Inc.) using *Sal*I and *Not*I sites.

FIV-Fca GL8 Vif was also cloned into the FIV-Fca Gag-Pol-expressing construct FP93 (Saenz and Poeschla, 2004) in order to express *vif in cis* to other viral genes and to achieve higher *vif* expression levels in 293T cells. FP93 is based on the FIV-Fca strain Petaluma (molecular clone 34TF10) (GenBank M25381) (Talbott et al., 1989) and bears deletions in *vif*, *orf2* and *env*, among others. To delete large parts of *vif*, a unique *Sal*I restriction site near the 5' end of *vif* has been introduced into FP93 (nucleotide positions 5390-5395) (Saenz and Poeschla, 2004). FP93 also possesses a unique *Bcl*I restriction site between nucleotides 6782 and 6787. Untagged FIV-Fca GL8 Vif was amplified with primers GL8Mya Vif-Fwd (5'-ACTGGTCGACATTGGCAGGTAAGTAGAAGACT-3') (*Sal*I restriction site underlined) and GL8Mya Vif-Rev (5'-ACATGATCAGTGGGATTTGTA ATGGGTCTGTAC-3') (*Bcl*I restriction site underlined). FP93 was propagated in *Escherichia coli* SCS110 cells (*dam* methylation-negative; Stratagene, LaJolla, U.S.A.). GL8 Vif was then cloned into FP93 using *Sal*I and *Bcl*I restriction sites. The construct was sequenced using primers GL8MyaVifSeq-Fwd (5'-GTGTCTTAGGAACTCACCTCCA-3'), GL8MyaVifSeq-F_1 (5'-TGAGACTATAACAGGAC CATTAG-3') and GL8MyaVifSeq-Rev (5'-ATCTCTAGTATGAAAGCTCCAT-3'). Site-directed mutagenesis was performed to delete one nucleotide near the 5' end of *vif* to shift the *vif* sequence after the *Sal*I restriction site into frame with the *vif* start codon. Mutagenesis primers used were GL8MVifMut-Fwd (5'-CCTGAAGGGGATGAGTGATCGACATTGGCAGGTAAGTAG-3') and GL8MVifMut-Rev (5'-CTACTTACCTGCCAATGTCGATCACTCATCCCCTTCAGG-3'). The new construct (GL8Mya Vif-FP93) expresses FIV-Fca GL8 Vif in the background of FIV-Fca Petaluma 34TF10 *gag* and *pol*. Vif bears two amino acid changes compared to the original GL8Mya Vif (Glu4Arg, Asp5His).

4.2.4 Quantification of feline APOBEC3 mRNAs by quantitative real-time reverse transcription PCR

Quantitative real-time reverse transcription PCR (RT-qPCR) was employed to determine the expression levels of domestic cat and lion *A3* genes in the feline cells lines FEA, CrFK, AH927 and 3201, in Mya-1 cells, in primary lion PBMCs and in different cat tissues. Lion full blood samples for isolation of PBMCs were obtained from Bojnice Zoo, Slovakia. Cat tissues included bone marrow, lymph nodes, ovarian tissue, spleen, thymus and tonsils, which were isolated from three FIV-negative cats that had been enrolled in a previous study (Kraase et al., 2010). Before RNA isolation, feline cell lines and lion PBMCs were either left untreated or were stimulated with 10^3 IU/ml recombinant feline IFN-α (R&D Systems Europe Ltd, Abingdon, UK) or 10^3 IU/ml recombinant feline IFN-ω (Virbac Limited, Bury St Edmonds, UK), respectively, for 24 hours. All RNAs were treated with DNAse I (Life Technologies) prior to cDNA synthesis. 1 µg or 450 ng of domestic cat cell line and lion PBMC or domestic cat tissue RNA, respectively, was used for cDNA synthesis.

All three FcaA3C isoforms were detected using primers qPCR-FcA3C-Fwd (5'-GGACAGGATAGATCCTAACACC-3') and qPCR-FcA3C-Rev (5'-CCACTTGGAAGCAG AGATAAC-3') and probe qPCR-FeA3C-Pro (5'FAM-TTCCACTTTCCAAACCTGCTCTATG CTTCT-3'TAMRA). FcaA3H was amplified with primers qPCR-FeA3H-Fwd (5'-CAAGATCAAGGCACTGACGC-3') and qPCR-FeLiA3H-Rev (5'-ACAAACGCAACCAGTTC C-3') and probe qPCR-FeLiA3H-Pr (5'FAM-CGAAATCATCTGCTATATCACATGGAGCCC CT-3'TAMRA). FcaA3CH and PleA3CH were detected using primers qPCR-FcA3CH-Fwd (5'-TCCTGGCTGCAAAGCTTCAAG-3') and qPCR-FcA3CH-Rev (5'-TCTGGGCAAGAGGAAGGAAACC-3') and probe qPCR-FeLiA3CH-P (5'FAM-CAGGAGGTGACAGAGCCTGGGATAAACACCAGA-3'TAMRA). All three PleA3C isoforms were detected using primers qPCR-LiA3C-Fwd (5'-GATCCTAAGACCTTCCA TTTCC-3') and qPCR-LiA3C-Rev (5'-ACCTTGTTCCGAAAAACTCC-3') and probe qPCR-LiA3C-Pro (5'FAM-GTTTCCAAACCTGCGCTACGCTTCT-3'TAMRA). PleA3H was amplified with primers qPCR-LiA3H-Fwd (5'-CAAGATCAAGTCACTGACGC-3') and qPCR-FeLiA3H-Rev and probe qPCR-FeLiA3H-Pr. Domestic cat 18S rRNA was used as housekeeping gene and was amplified with primers rDNA 343F cat (5'-CCATTCGAACGTCTGCCCTA-3') and rDNA 409 R (5'-TCACCCGTGGTCACCATG-3') and probe rDNA 370Pcat (5'FAM-CGATGGTAGTCGCCGTGCCTA-3'TAMRA).

Because specific A3C and A3H primer and probe sets could not be designed, A3CH copy numbers were subtracted from A3C and A3H copy numbers. With the help of the standard curve equations copy numbers were then converted back into C_T values (where "C_T" represents threshold cycle) and expression levels between *A3* genes in different samples were compared using the ΔC_T method. ΔC_T values were calculated by subtracting the mean C_T for the abundant 18S rRNA from the C_T for A3 mRNAs.

4.2.5 Genetic APOBEC3 diversity in African and Asian lion populations

A total of eight lion whole blood samples were obtained from Angola (kindly provided by Pieter Kat and Rodrigo Serra of the Investigacao Veterinaria Independente, Lisbon, Portugal), from the Moremi reserve in the Okavango Delta (Botswana), from the Serengeti National Park (Tanzania; kindly provided by Sarah Cleaveland, University of Glasgow, UK), from Lahore Zoo and Lahore Safari Park (Pakistan) and from Bristol Zoo (UK). Genomic DNA was prepared using the PAXgene Blood DNA kit (Qiagen) or the QIAamp DNA Mini and Blood Mini kit (Qiagen).

To determine the geographical origin of the lions from which the samples had been taken, the genetic variation of their 12S-16S mitochondrial DNA (mtDNA) genes was investigated. Fragments of the 12S-16S mtDNA genes were amplified using GoTaq Flexi DNA polymerase (Promega) and primer pair 12S-UP-F (5'-AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT-3') and PAN-16S-Cy-R (5'-CAGAACTCAGATCACGTAG-3') or L12S-Cy-F (5'-CTTAAGTGACTAGCCCCTA-3') and PAN-16S-Cy-R (Antunes et al., 2008). Cycling parameters were 1 cycle of 94 °C for 3 minutes; 1 touch-down cycle of 94 °C for 30 seconds, 52 °C for 1 minute decreased by 1 °C in the next cycle for 10 cycles, 72 °C for 2 minutes; then 35 cycles of 94 °C for 30 seconds, 52 °C for 1 minute, 72 °C for 2 minutes; then 1 cycle of 72 °C for 5 minutes. The fragments were cloned into pCR2.1-TOPO (Life Technologies) and five clones of each fragment per animal were sequenced. The sequences were aligned to twelve lion 12S-16S mtDNA gene sequences (GenBank FJ151641-FJ151652) corresponding to different haplotypes specific for the

geographic location of African and Asian lion population habitats (Antunes et al., 2008).

PleA3C genes were amplified, cloned and sequenced as described in section 4.2.3. Sequences were compared to the sequences of PleA3C1 (GenBank EU007543), PleA3C2 (EU007544) and PleA3C3 (this study). *PleA3H* genes were amplified using primers LiA3H F and LiA3H R (see section 4.2.1), cloned into pCR2.1-TOPO and sequenced. Sequences were compared to that of PleA3H (GenBank EU007549).

4.3 Results

4.3.1 Amplification of feline APOBEC3 cDNAs

In addition to the well-characterised domestic cat APOBEC3 transcripts (Munk et al., 2008), puma and lion A3C and A3H transcripts were amplified from cDNA derived from puma and Angolan lion PBMC RNA, respectively, using primers directed against their predicted sequences (GenBank EU007545 and EU007552, or EU007543 and EU007549, respectively) (Fig. 4-1). Amplification of PleA3C2 cDNA (GenBank EU007544) was attempted, but proved unsuccessful due to the high nucleotide similarity to PleA3C1. Puma and lion A3CH transcripts (PcoA3CH and PleA3CH) were amplified using primers LiA3C2 F and LiA3H R. Their sequences were determined and were found to resemble the recently published sequences (GenBank GU097659 and GU097662, respectively) (Zielonka et al., 2010). Domestic cat, lion and puma A3C amplicons were cloned into the eukaryotic expression vector VR1012 (Vical Inc.).

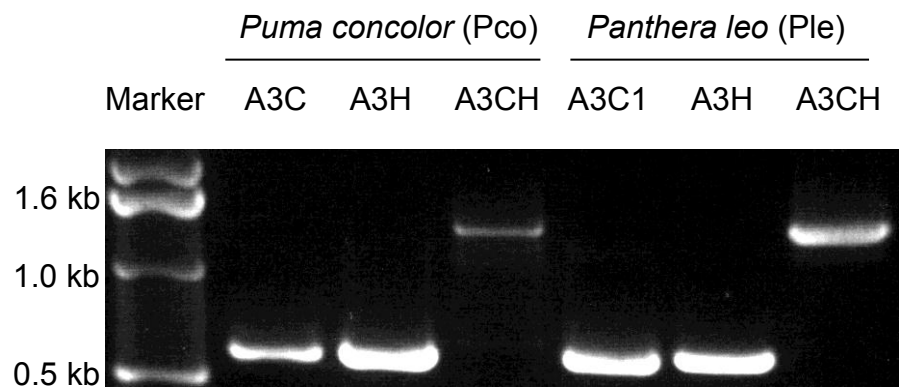
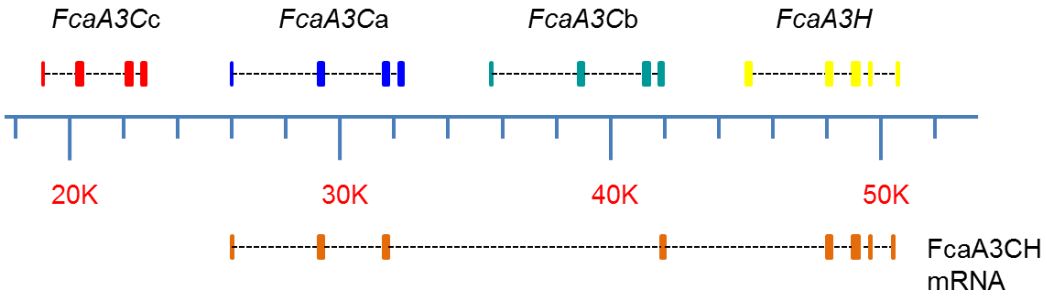


Figure 4-1 Amplification of puma and lion A3 transcripts. Puma and lion A3 transcripts were amplified from puma or lion PBMC-derived cDNA using primers directed against their predicted sequences. PcoA3C, PcoA3H, PleA3C1 and PleA3H transcripts encode for single deaminase domain A3s, whereas PcoA3CH and PleA3CH give rise to double deaminase domain A3s.

4.3.2 Genomic organisation of the lion *A3* gene locus

The domestic cat *A3* gene locus (GenBank EU109281) on chromosome B4 spans 32 kb and contains three *A3C* (*A3C*c, *A3C*a and *A3C*b) and one *A3H* gene, which are aligned in a head-to-tail orientation (Munk et al., 2008) (Fig. 4-2A). *A3C*c is the most ancient of the *A3C* genes and duplicated to give rise to an *A3C*a/b precursor gene that further duplicated to create *A3C*a and *A3C*b (Munk et al., 2008). Due to the common origin of the three domestic cat *A3C* genes, they share the same exon-intron structure and their nucleotide sequences are highly similar (Munk et al., 2008). The *A3C* genes each consist of four exons (E1-E4) and three introns (I1-I3) (Fig. 4-2B). A3Cc possesses 97.8% and 97.1% nucleotide identity with A3Ca and A3Cb cDNAs, respectively, whereas there is 98.8% identity between A3Ca and A3Cb cDNAs. This results in 96.3 to 96.5% amino acid sequence identity between the various A3C proteins (Munk et al., 2008).

A



B

	E1	I1	E2	I2	E3	I3	E4	I
5' ⟶ *FcaA3Cc*	26	1268	157	1615	280	234	116	3088
FcaA3Ca	26	3220	157	2429	280	230	116	3079
FcaA3Cb	26	3216	157	2474	280	230	116	⟶ 3'

Figure 4-2 Schematic representation of the domestic cat *A3* gene orientation and their exon/intron organisation. (A) The domestic cat *A3* gene locus is situated on chromosome B4 and spans 32 kb. It contains the three *A3C* genes *A3C*c, *A3C*a and *A3C*b as well as an *A3H* gene. A fifth A3 protein, FcaA3CH, is expressed by read-through alternative splicing using exons of *FcaA3C*a, *FcaA3C*b and *FcaA3H*. (B) Each *FcaA3C* gene consists of four exons (E1-E4) and three introns (I1-I3) of indicated lengths (nt).

In order to determine the number and order of *PleA3C* genes in the lion genome primers were designed that bound specifically within the exon regions of either PleA3C1 or PleA3C2 (GenBank EU007543 and EU007544, respectively). PleA3C1 and PleA3C2 showed 97% nucleotide and 94% amino acid identity. Fragments between exons E1 and E2, E2 and E3, E3 and E1 of the following *PleA3C* gene were amplified from lion genomic DNA, cloned and sequenced (Fig. 4-3).

Domestic cat *A3C*c can be distinguished from *A3C*a and *A3C*b by shorter I1 and I2 (Fig. 4-2B). Thus, if the lion genome contained an *A3C* gene that resembled the oldest domestic cat *A3C* gene (*A3Cc*), the shorter intron sequences between E1 and E2 and between E2 and E3 could be used to identify its presence. Also, possible amplification of longer fragments between E1 and E2, and E2 and E3 would indicate the existence of at least one other *A3C* gene in

the lion genome analogous to *FcaA3C*a/b. Finally, successful product amplification between E3 of both the lion *A3C* genes that resembled *FcaA3C*c and *FcaA3C*a/b and E1 of any *PleA3C* gene would confirm the presence of a third *PleA3C* gene and would provide an insight into the order in which the lion *A3C* genes are arranged in the lion *A3C* gene locus.

Firstly, the genomic DNA (gDNA) fragment between E1 and E2 of both *PleA3C*1 and *PleA3C*2 was amplified using primers LiA3C-Ex1-Fwd and LiA3C-Ex2-Rev. Only one fragment of about 1.3 kb in length was obtained (Fig. 4-3A), similar to the short intron I1 of *FcaA3C*c. A fragment of 3.2 kb in length resembling the longer intron I1 of *FcaA3C*a or *FcaA3C*b could not be amplified despite repeated attempts at further optimisation of the PCR.

Next, the gDNA fragment between E2 and E3 of either *PleA3C*1 (primers LiA3C-Ex2-Fwd-1 and LiA3C-Ex3-Rev-1) or *PleA3C*2 (primers LiA3C-Ex2-Fwd-2 and LiA3C-Ex3-Rev-2) was amplified. Both PCR reactions yielded two fragments of approximately 1.6 kb and 2.5 kb in length (Fig. 4-3B), indicating that the primer combinations used could not discriminate between *PleA3C*1 and *PleA3C*2, but that there are two species of I2, similar to *FcaA3Cc* and *FcaA3C*a/b I2. The PCR was repeated using primer sets LiA3C-Ex2-Fwd-1/LiA3C-Ex3-Rev-3 and LiA3C-Ex2-Fwd-2/LiA3C-Ex3-Rev-4 that were specific for *PleA3C*1 and *PleA3C*2, respectively (Fig. 4-3C). The first primer pair enabled amplification of a 2.5 kb fragment, which confirms that *PleA3C*1 is homologous to *FcaA3C*a/b. Use of the second primer pair led to amplification of a 1.6 kb fragment, which indicates that *PleA3C*2 is homologous to *FcaA3Cc*. Furthermore, the gDNA fragment between E3 of one *PleA3C* gene and E1 of the one downstream was amplified using forward primers LiA3C-Ex3-Fwd-1 (specific for *PleA3C*1) or LiA3C-Ex3-Fwd-2 (specific for *PleA3C*2) and reverse primer LiA3C-Ex1-Rev-1. In both cases, PCR products of 3.5 kb were obtained (Fig. 4-3D), showing that both genes lay upstream of a distinct *PleA3C* gene. However, upon sequencing of the *PleA3C*1 E3-E1 fragment multiple nucleotides were identified in the E4 region that differed from the E4 sequence of *PleA3C*1. Thus, not *PleA3C*1 but a similar *PleA3C* gene is situated upstream of another *PleA3C* gene. The newly identified *PleA3C* was termed *PleA3C*3 and the corresponding cDNA was amplified using specific primers LiA3C1 F and LiA3C3-Rev (Fig. 4-3E), cloned into VR1012 (Vical Inc.) and sequenced. PleA3C3 differs from PleA3C1 at two nucleotide positions in E3 (C412G, A499G) and in four nucleotides in E4 (G515A, A539G, A561G, G575A),

which result in the amino acid changes Arg138Gly, Arg167Gly, Arg172Lys and Asp180Gly.

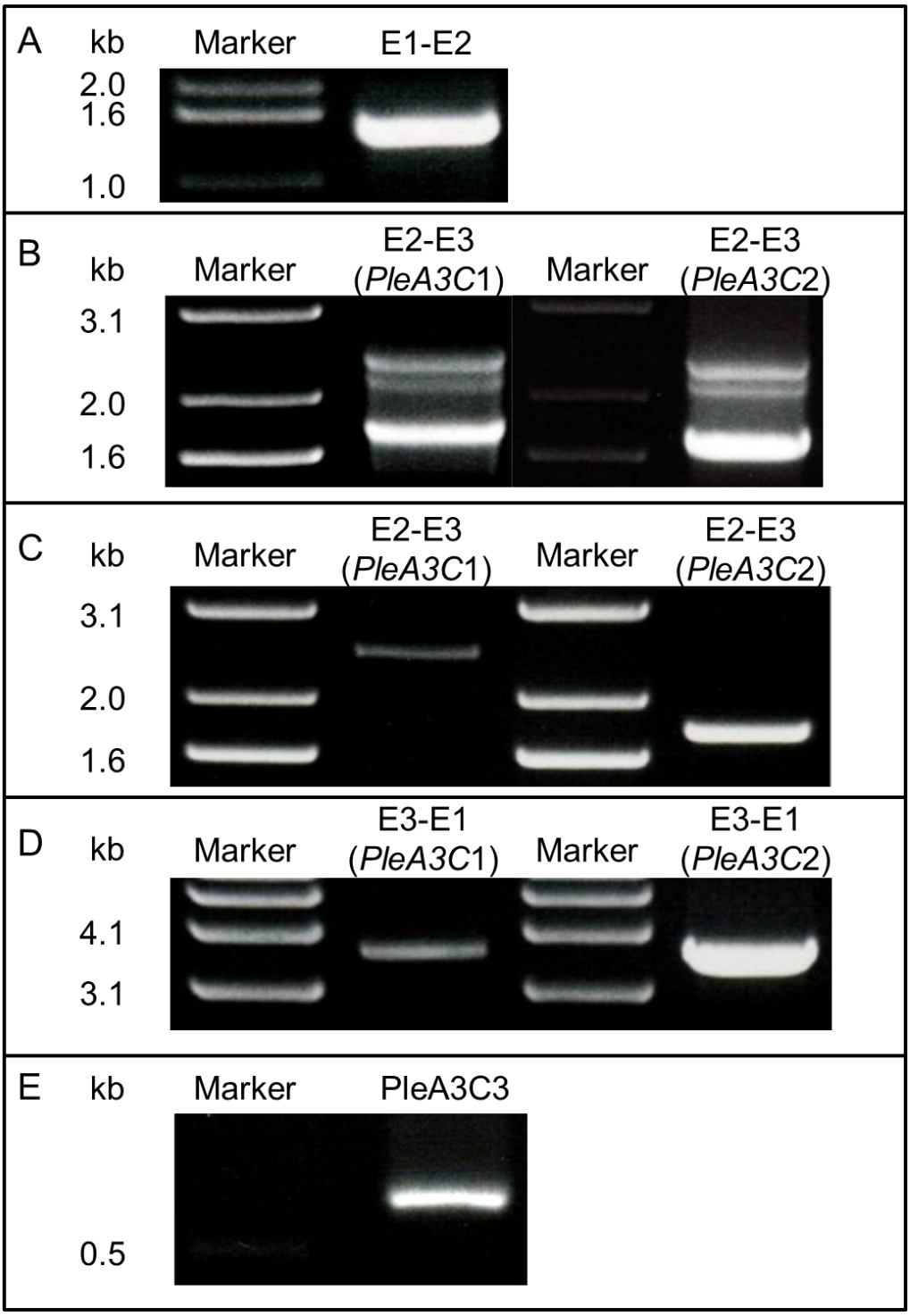


Figure 4-3 Identification of lion *A3C* genes by intron lengths and determination of *A3C* gene order within the lion *A3* gene locus. PleA3C genomic DNA fragments between E1 and E2 (A), E2 and E3 (B,C) and E3 and the E1 of the following *PleA3C* gene (D) were amplified with specific primers based on

the published PleA3C1 or PleA3C2 cDNA sequence as indicated. (E) The PleA3C3 transcript was amplified from Angolan lion PBMC-derived cDNA.

This study provides evidence for the presence of three *PleA3C* genes in the lion genome. Because both *PleA3C*2 and *PleA3C*3 lay upstream of a *PleA3C* and because *PleA3C*3 was similar to *PleA3C*1 in its intron lengths and exon sequences, the most likely order of the three genes in the lion genome is 5'-*PleA3C*2-*PleA3C*3-*PleA3C*1-3' (Fig. 4-4). It can be assumed that these genes have arisen in a comparable fashion to the domestic cat *A3C* genes, namely from an ancestral *A3C* gene undergoing two sequential gene duplication events.

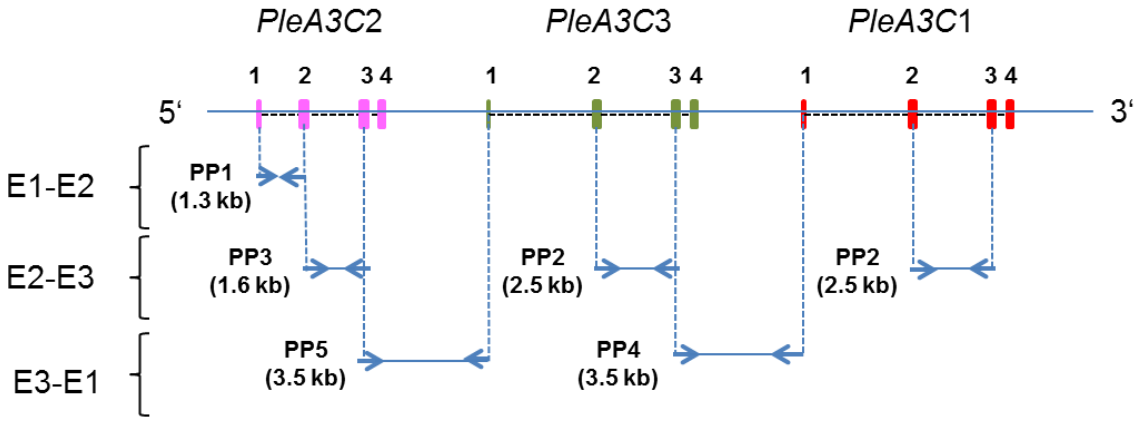


Figure 4-4 Schematic representation of the lion *A3C* gene locus as determined by PCR amplification of genomic DNA fragments and binding sites of primers used in the study. According to data obtained by PCR amplification of genomic intron and exon sequences within the lion *A3* gene locus, the lion genome contains three *A3C* genes that are aligned in a head-to-tail fashion in the order 5'-*PleA3C*2-*PleA3C*3-*PleA3C*1-3'. PleA3C1 and PleA3C2 cDNA sequences have been previously described (GenBank EU007543 and EU007544, respectively). *PleA3C*3 has been identified in this study. The binding sites of primers used in this study are indicated. Primer pair (PP) 1 consisted of primers LiA3C-Ex1-Fwd and LiA3C-Ex2-Rev and was used to amplify a 1.3 kb genomic DNA (gDNA) fragment between PleA3C3 exons 1 and 2 (E1-E2) (Fig. 4-3A). PP2 (LiA3C-Ex2-Fwd-1/LiA3C-Ex3-Rev-3) mediated amplification of a 2.5 kb gDNA fragment between exons 2 and 3 (E2-E3) of PleA3C3 and PleA3C1 (Fig. 4-3C). PP3 (LiA3C-Ex2-Fwd-2/LiA3C-Ex3-Rev-4) bound specifically to *PleA3C*2

exons 2 and 3 (E2-E3) and PCR amplification yielded a 1.6 kb gDNA fragment (Fig. 4-3C). To amplify the gDNA fragment between exon 3 of *PleA3C*2 and exon 1 of *PleA3C*3 (E3-E1) or exon 3 of *PleA3C*3 and exon 1 of *PleA3C*1 (E3-E1), PP5 (LiA3C-Ex3-Fwd-2/LiA3C-Ex1-Rev-1) or PP4 (LiA3C-Ex3-Fwd-1/LiA3C-Ex1-Rev-1) were used. Both PCR fragments obtained were 3.5 kb in length (Fig. 4-3D).

4.3.3 Antilentiviral activity of feline APOBEC3 proteins

In order to identify possible differences in the anti-lentiviral activities of domestic cat and non-domestic cat A3 proteins that may offer an explanation for the resistance of lion T lymphocytes to FIV infection, lentiviral single-cycle replication assays in the presence of A3 proteins were performed. Wild-type or *vif*-deleted, GFP-labelled HIV-1, SIVmac and FIV-Fca pseudotypes were produced in cells co-expressing either empty VR1012 vector or domestic cat A3 proteins (FcaA3Ca, FcaA3H, FcaA3CH), lion A3 proteins (PleA3C1, PleA3C3, PleA3H, PleA3CH) or puma A3 proteins (PcoA3C, PcoA3H, PcoA3CH). Pseudotypes were then used to infect target cells. Activity of A3s was assessed by flow cytometry and assumed to be inversely proportional to the percentage of transduced, GFP-expressing cells.

Firstly, the activities of domestic cat and lion A3 proteins against HIV-1Δ*vif*, HIV-1 wild-type, SIVmacΔ*vif* and SIVmac wild-type were investigated (Fig. 4-5). Whereas FcaA3Ca and PleA3C1 showed no activity against HIV-1Δ*vif* and very little activity against HIV-1, FcaA3H, FcaA3CH, PleA3H and PleA3CH were able to strongly inhibit replication of HIV-1Δ*vif* and HIV-1 (Fig. 4-5A,B). A similar trend was observed for SIVΔ*vif* and SIV, against which FcaA3Ca and PleA3C1 were slightly more potent than against HIV-1Δ*vif* and HIV-1 (Fig. 4-5C,D). These data indicate that domestic cat and lion A3 proteins are functional restriction factors, which block the replication of primate lentiviruses and are not counteracted by the HIV-1 or SIVmac Vif proteins.

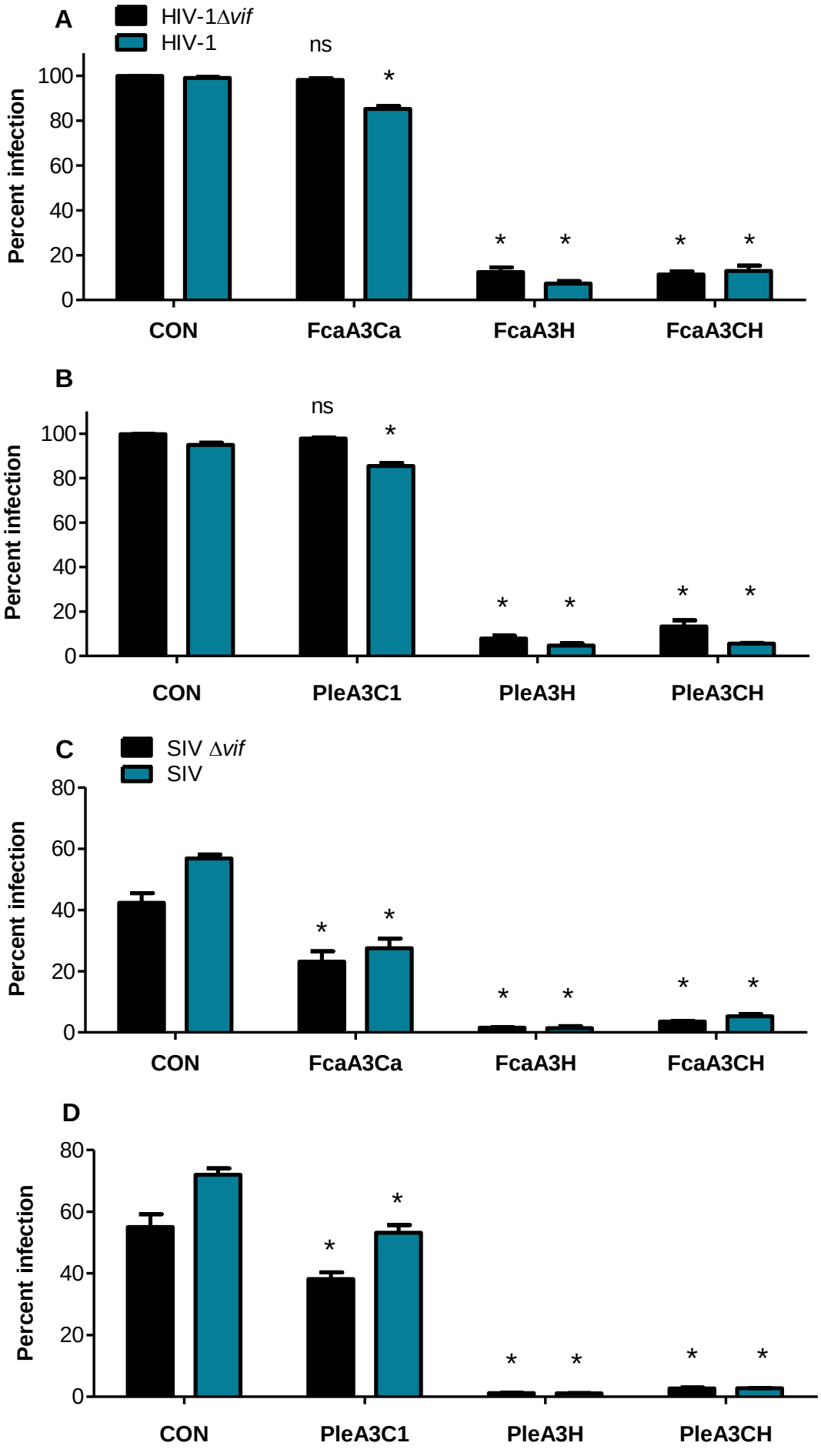
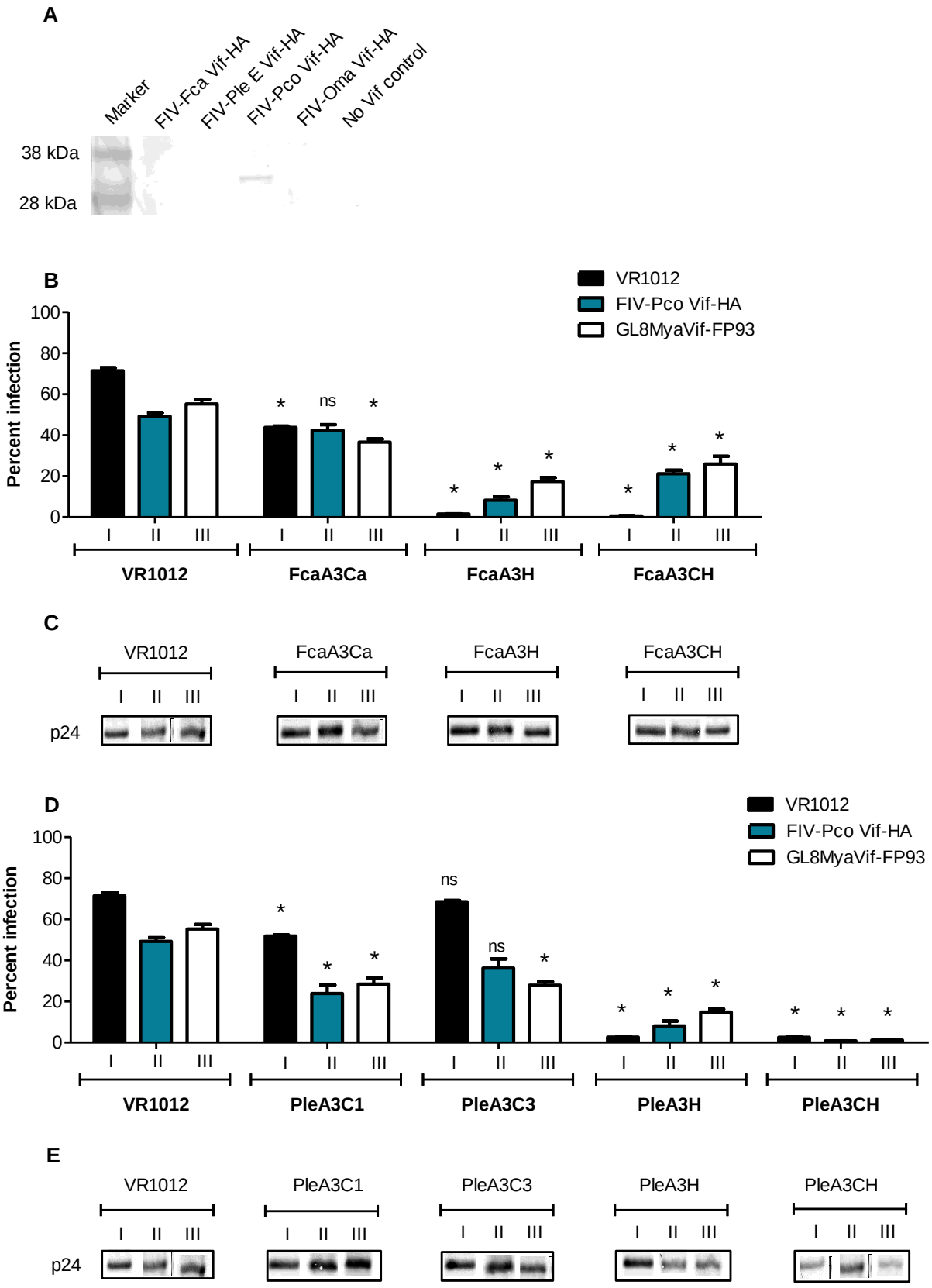

A
HIV-1Δvif
HIV-1
ns
*
Percent infection
100
80
60
40
20
0
CON
FcaA3Ca
FcaA3H
FcaA3CH
B
ns
*
Percent infection
CON
PleA3C1
PleA3H
PleA3CH
C
SIV Δvif
SIV
Percent infection
CON
FcaA3Ca
FcaA3H
FcaA3CH
D
Percent infection
CON
PleA3C1
PleA3H
PleA3CH

Figure 4-5 Domestic cat and lion A3 proteins block primate lentivirus pseudotype infection. VSV-G-pseudotyped, GFP-expressing HIV-1 (A,B) and SIVmac (C,D) wild-type and *vif*-deleted particles were produced in 293T cells co-transfected with empty vector only (CON), domestic cat (FcaA3) (A,C) or lion (PleA3) (B,D) A3 expression constructs. Pseudotype-containing cell supernatant was then used to transduce 293T target cells and activity of A3 proteins was assessed by measuring GFP-expression in target cells by flow cytometry. Single-cycle replication assays were performed in triplicate (*n*=3; results shown as mean ± S.E.). Statistically significant differences relative to the empty vector only control are indicated by asterisks (*, P<0.05, Dunnet's t-test), non-significant differences by 'ns'.

Next, the activity of domestic cat, lion and puma A3 proteins against FIVΔ*vif* and FIVΔ*vif* provided with either vector only (CON), FIV-Fca GL8Mya Vif-HA, FIV-Ple E Vif-HA, FIV-Pco Vif-HA or FIV-Oma Vif-HA *in trans* was studied (Fig. 4-6B,D,F). Firstly, the expression levels of the FIV Vif proteins in 293T cells were assessed (Fig. 4-6A). For that, $2x10^6$ cells in 100-mm cell culture dishes were transfected with 5 µg of each FIV Vif-HA expression construct and cell lysates were blotted with an anti-HA antibody. Immunoblotting confirmed that only FIV-Pco Vif was expressed in 293T cells at detectable levels, whereas all other FIV Vifs were not expressed at detectable levels and could not be used in A3 activity assays. Thus, FIV-Fca GL8Mya Vif was then also expressed *in cis* to FIV-Fca Petaluma *gag* and *pol* (GL8Mya Vif-FP93). Expression of GL8Mya Vif in the FP93 backbone could not be assessed by immunoblotting due to the lack of appropriate antibodies. However, because expression of lentiviral Vif protein is Rev-dependent (Garrett et al., 1991; Schwartz et al., 1991), it can be assumed that expression of FIV *vif* *in cis* to other FIV genes, as opposed to *vif* expression *in trans*, improved Vif protein expression to a level where an anti-A3 effect, if present, could be observed.

FIV p24 Gag expression in producer cells was investigated to rule out a negative impact of A3 or Vif expression on particle production (Fig. 4-6C,E,G).


A
Marker
FIV-Fca Vif-HA
FIV-Ple E Vif-HA
FIV-Pco Vif-HA
FIV-Oma Vif-HA
No Vif control
38 kDa
28 kDa
B
VR1012
FIV-Pco Vif-HA
GL8MyaVif-FP93
Percent infection
100
80
60
40
20
0
ns
I
II
III
VR1012
FcaA3Ca
FcaA3H
FcaA3CH
C
VR1012
FcaA3Ca
FcaA3H
FcaA3CH
p24
D
VR1012
FIV-Pco Vif-HA
GL8MyaVif-FP93
Percent infection
ns
VR1012
PleA3C1
PleA3C3
PleA3H
PleA3CH
E
VR1012
PleA3C1
PleA3C3
PleA3H
PleA3CH
p24

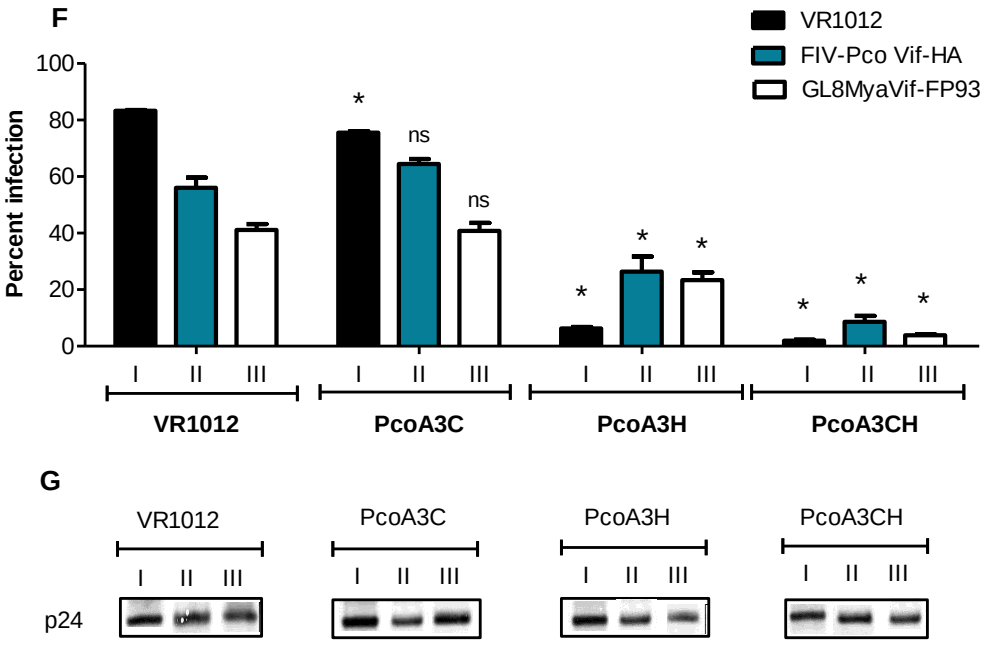


Figure 4-6 Activities of domestic cat, lion and puma A3 proteins against FIV-Fca pseudotype infection in the presence and absence of FIV Vifs. VSV-G-pseudotyped, GFP-bearing FIV-FcaΔ*vif* particles were produced in 293T cells co-transfected with empty vector only (CON), domestic cat (FcaA3) (B), lion (PleA3) (D) or puma (PcoA3) (F) A3 protein expression vectors in the presence or absence of different FIV Vif-HA expression constructs. Pseudotype-containing cell supernatant was then used to transduce 293T target cells and activity of A3 proteins was assessed by measuring GFP-expression in target cells by flow cytometry. Single-cycle replication assays were performed in triplicate (*n*=3; results shown as mean ± S.E.). Statistically significant differences relative to the empty vector only control are indicated by asterisks (*, P<0.05, Dunnet's t-test), non-significant differences by 'ns'. (A) Expression of FIV Vifs in 2x10^6 293T cells transfected with 5 µg of either of the FIV-Vif expression constructs was assessed by immunoblotting using a rabbit anti-HA antibody (Sigma-Aldrich). (C,E,G) FIV-Fca Gag (p24) expression in transfected 293T producer cells was assessed by immunoblotting using mouse anti-FIV p24 antibody (vpg50) to exclude a negative effect of A3 expression on Gag production.

As observed for primate lentivirus pseudotypes, feline A3C isoforms could limit FIVΔ*vif* pseudotype infectivity of target cells only modestly, if at all (Fig. 4-6B,D,F). Co-expression of different FIV Vifs did not result in an increase of

pseudotype infectivity. In contrast, feline A3H and A3CH proteins were able to block FIVΔ*vif* replication significantly (Fig. 4-6B,D,F). FcaA3H and FcaA3CH reduced FIVΔ*vif* infectivity 47- and 119-fold, PleA3H and PleA3CH 27- and 28-fold, and PcoA3H and PcoA3CH 13.2- and 42-fold, respectively. Interestingly, FIV-Pco Vif and GL8Mya Vif expressed *in cis* with FIV-Fca Petaluma *gag* and *pol* (GL8Mya Vif-FP93) were able to antagonise feline A3H and A3CH proteins to different degrees. FcaA3CH was more sensitive to counteraction by FIV-Pco and FIV-Fca Vif than FcaA3H (Fig. 4-6B). FIV(VSV-G) pseudotype infectivity in the presence of PleA3H was rescued slightly when either of the two Vifs was co-expressed. However, Vif expression had no negative effect on the activity of PleA3CH and even augmented restriction (Fig. 4-6D). Finally, PcoA3H and PcoA3CH were equally sensitive to the Vif proteins (Fig. 4-6F).

The data presented here indicate that domestic cat, lion and puma A3H and A3CH proteins potently restricted not only primate but also feline lentivirus replication in single-cycle replication assays. However, in contrast to primate lentivirus Vif proteins, which do not show any activity against feline A3s, FIV Vifs were able to overcome feline A3 proteins in an A3-specific, non-species specific manner. Due to the lack of expression of some FIV Vifs in 293T cells and the low expression of others it was difficult to evaluate their maximum potency against feline A3 proteins. Because the activity of FIV-Pco Vif and FIV-Fca GL8 Vif in FP93 rescued viral infectivity to a significant degree, future work should include the insertion of FIV-Ple Vif and FIV-Oma Vif into the FP93 backbone for use of these constructs in A3 activity assays.

4.3.4 Quantification of feline APOBEC3 expression

This study has shown that feline A3 proteins function as anti-lentiviral restriction factors *in vitro*. However, information about their expression *in vivo*, which may give an insight into their role in limiting viral infection, is sparse. Thus, RT-qPCR was performed to generate feline *A3* expression profiles in domestic cat cell lines and tissues and in lion PBMCs. Moreover, the effect of type I interferon on A3 expression was determined.

Cat and lion *A3C* and *A3H* were modestly expressed in all samples tested (Fig. 4-7A-C). Cat and lion *A3CH*, however, were expressed only at low levels in

most samples and were not expressed in three domestic cat tissue samples (Fig. 4-7B). Interestingly, in cat cell lines and tissues *FcaA3C* expression was higher than *FcaA3H* expression whereas the opposite trend was observed in lion PBMCs. Interferon treatment upregulated *A3* expression in 3201, CrFK and Mya-1 cells, but had no effect on *A3* expression in AH927 or FEA cells (Fig. 4-7A).

The data indicate that *A3* genes have a broad expression profile in domestic cat cells and tissues. Importantly, expression levels of *A3H* and *A3CH*, whose gene products have been shown to possess the highest anti-lentiviral activity (Fig. 4-5 and 4-6), were lower than that of the three *A3C* isoforms together. However, a small number of A3H and A3CH proteins per cell may suffice to elicit a potent antiviral response.

Moreover, expression levels of lion *A3* genes were comparable between PBMCs of African and Asian lion origin, indicating that *A3* expression did not increase in the process of long-term host-FIV co-evolution that took place in African, but not in Asian lion populations.

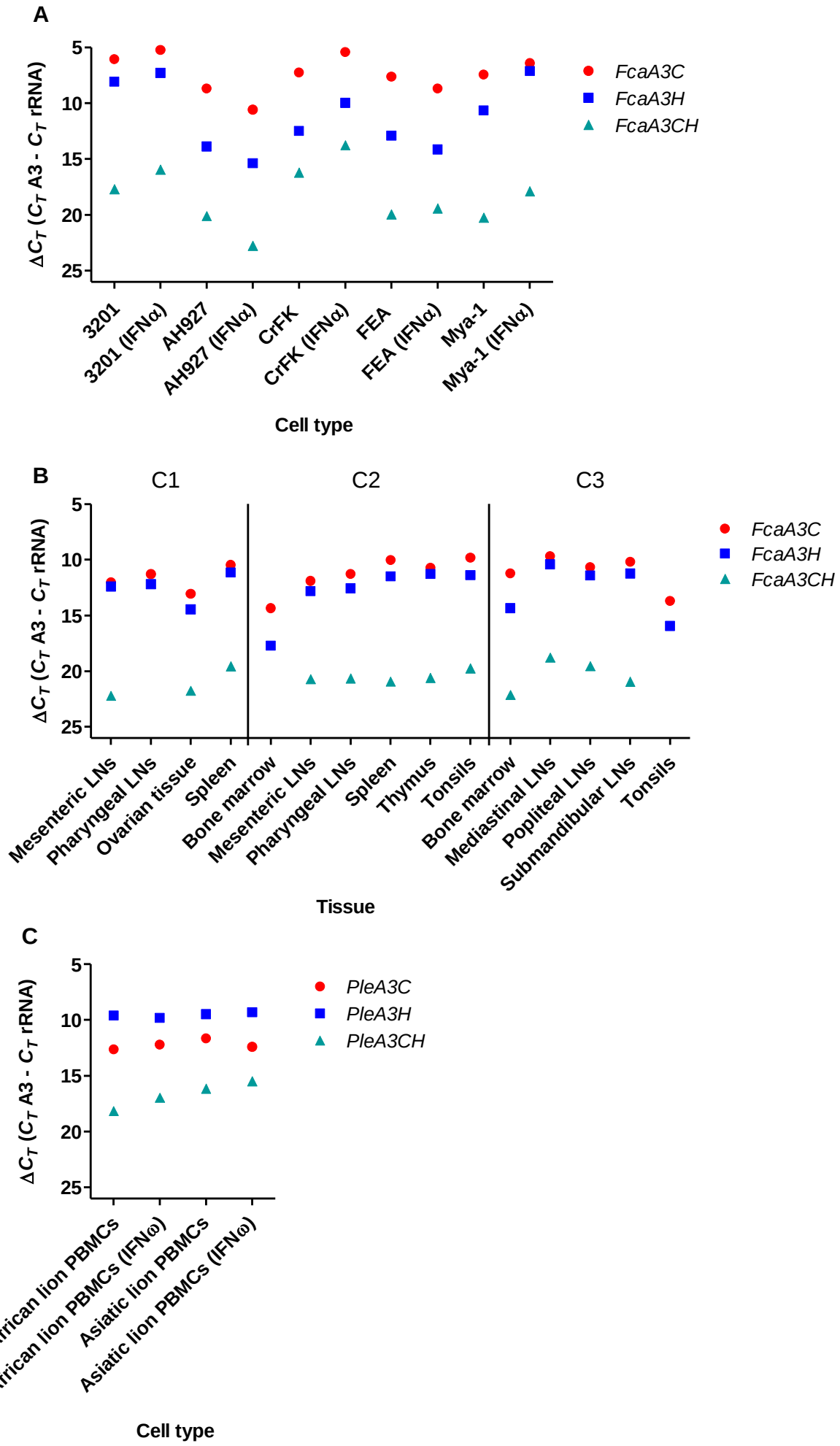

A
ΔC_T (C_T A3 - C_T rRNA)
5
10
15
20
25
3201
3201 (IFNα)
AH927
AH927 (IFNα)
CrFK
CrFK (IFNα)
FEA
FEA (IFNα)
Mya-1
Mya-1 (IFNα)
Cell type
FcaA3C
FcaA3H
FcaA3CH
B
C1
C2
C3
Mesenteric LNs
Pharyngeal LNs
Ovarian tissue
Spleen
Bone marrow
Mesenteric LNs
Pharyngeal LNs
Spleen
Thymus
Tonsils
Bone marrow
Mediastinal LNs
Popliteal LNs
Submandibular LNs
Tonsils
Tissue
C
PleA3C
PleA3H
PleA3CH
African lion PBMCs
African lion PBMCs (IFNω)
Asiatic lion PBMCs
Asiatic lion PBMCs (IFNω)
Cell type

Figure 4-7 Quantification of domestic cat and lion mRNA by real-time reverse transcription PCR and effect of interferon treatment. *A3* expression in the domestic cat cell lines 3201, AH927, CrFK (ID10) and FEA as well as in IL-2-dependent $CD4^+$ T cells (Mya-1) (A), in tissues of three FIV-negative cats (B; cats labelled C1-C3) and in African and Asian lion PBMCs (C) was quantified by RT-qPCR. Domestic cat cell lines were cultured overnight with or without IFN-α at 10^3 IU/ml prior to RNA extraction, cDNA preparation, and A3 cDNA quantification. Lion PBMCs were cultured overnight with or without IFN-ω at 10^3 IU/ml prior to RNA extraction, cDNA preparation, and A3 cDNA quantification. Real-time PCR was performed using 1 µl cDNA (1/20) or standard plasmid DNA once in triplicates (n=1). C_T values (where "C_T" represents threshold cycle) for A3C, A3H and A3CH transcripts were determined and converted into copy numbers with the help of the standard curve equation. Because A3C and A3H primer and probe sets non-specific and could also detect A3CH transcripts, A3CH copy numbers were subtracted from A3C and A3H copy numbers. Copy numbers were then converted back into C_T values. Results are expressed as ΔC_T values, which were calculated by subtracting the mean C_T for the abundant 18S rRNA from the C_T for A3 mRNAs.

4.3.5 Analysis of APOBEC3 diversity in African and Asian lion populations

Currently, a total of eleven distinct lion populations exist across Africa and in the Gir forest in India (see Fig. 1-5). Of these lion populations, only the three populations in the Gir forest in India, in southern Botswana and in Namibia are FIV-negative (Brown et al., 1994; O'Brien et al., 2006), whereas in other lion populations in Africa the FIV seroprevalence can be as high as 90% (see Table 1-1). In order to investigate whether long-term FIV prevalence in African lions and host-virus co-evolution has led to an adaptive evolution of A3 proteins that may have altered their anti-lentiviral activity, the genetic diversity of African lion A3s was compared to that of Asian lion A3s.

Antunes et al. (2008) found that maternal inherited (mitochondrial DNA, mtDNA) sequence variation in lions is generally low. Only twelve 12S-16S mtDNA haplotypes (H1-H12) were identified, which cluster into four distinct mtDNA

lineages (I-IV). Interestingly, their pairwise genetic distances among the eleven lion populations showed a significant relationship with geographic distances between lion populations (Antunes et al., 2008). MtDNA lineage I is present in East Africa and includes haplotype 4 (H4) which can be found exclusively in Kenya (KEN) (Fig. 1-5). MtDNA lineage II comprises haplotypes H1 to H3 which are present in the southern African lion populations in northern Botswana (BOT-II), Kruger National Park in South Africa and Mozambique (KRU) and Namibia (NAM), respectively. MtDNA lineage III includes haplotypes present in northern and central African (H5 and H6) and in Asian lion populations (H7 and H8). H5 and H6 can be found in Morocco (ATL), Angola (ANG) and Zimbabwe (ZBW), and H7 and H8 can be found solely in the Gir forest in India (GIR) (Fig. 1-5). Finally, mtDNA lineage IV is predominant in southern and East Africa and comprises haplotypes H9 to H12. H9 is limited to BOT-II and H10 to KRU. H11 is the most prevalent mtDNA haplotype and exists in lion populations in southern Botswana (BOT-I), KRU, in the Ngorongoro Crater in Tanzania (NGC), in the Serengeti National Park in Tanzania (SER-I, SER-II, SER-III) and in Uganda (UGA) (Fig. 1-5). H12 can be found in SER-I. Hence, mtDNA haplotypes can be used to identify geographic origins of individual lions.

In this study mtDNA haplotypes of eight lion samples were determined in order to link possible *A3* gene sequence variations with geographic origin of lions. It was of particular importance to distinguish African from Asian lion samples, as cross-breeding regularly takes place in captive settings, which leads to the generation of genetic hybrids. Table 4-1 summarises mtDNA haplotypes and geographic origins of the samples used. One sample was of Angolan origin (ANG; H5; “ANG1”). Three samples possessed mtDNA haplotype H1 and hence originated from Northern Botswana (BOT-II; “Chianti”, “Clairette”, “Krystal”). Two samples mapped to haplotypes H2 and thus, originated from lions from the Kruger National Park (KRU; “KRUA1”, “KruA4”). One sample came from the Serengeti National Park (SER; H11; “SER 156.04”). Finally, one sample was of Asian origin (GIR; H7; “BRISTOL 9971”). Thus, lion samples from different African and Asian lion populations were used to investigate the diversity of lion *A3* genes.

Table 4-1 Analysis of geographic origins of lion whole blood samples by mtDNA haplotyping. Genomic DNA from eight lion whole blood samples obtained from different sources was isolated. In order to determine the geographic origin of the lions, a 1882-nt fragment of 12S-16S mtDNA was amplified from the gDNA, cloned and sequenced. This fragment contains variable sites, which allows classification of sequences into twelve lion mtDNA haplotypes (H1-H12) and four mtDNA lineages (I-IV) (Antunes et al., 2008). Haplotype and lineage affiliation correlates with geographic origin of samples (see Fig. 1-5). Samples used in this study originated from Angola (ANG), northern Botswana (BOT-II), Kruger National Park (KRU), the Serengeti National Park (SER) and the Gir forest (GIR).

Sample name	**Source**	**MtDNA haplotype**	**MtDNA lineage**	**Geographic origin**
ANG1	Angola	H5	III	ANG
Chianti	Moremi reserve, Okavango Delta, Botswana	H1	II	BOT-II
Clairette				
Krystal				
KRUA1	Lahore Zoo, Pakistan	H2	II	KRU
KRUA4	Lahore Safari Park, Pakistan			
SER 156.04	Serengeti National Park, Gol Kopjes, Shinyanga, Tanzania	H11	IV	SER
BRISTOL 9971	Bristol Zoo, UK	H7	III	GIR

A3C gene fragments from individual lion gDNAs were amplified, cloned and sequenced as described in Section 1.3.2. Additionally, the full-length *PleA3H* gene was amplified, cloned and sequenced. Sequences were aligned to the known PleA3C1 (GenBank EU007545), PleA3C2 (GenBank EU007544), PleA3C3 (this study) and PleA3H (GenBank EU007552) cDNA sequences. The reference

sequences had originally been obtained from an Angolan lion (H5) (Munk et al., 2008). Unexpectedly, none of the tested lion samples contained any nucleotide changes in the PleA3C or PleA3H coding region compared to the reference sequences. Thus, it can be concluded that FIV, and in particular the FIV Vif protein, did not exert a selection pressure on A3 proteins during extended periods of host-virus co-evolution.

4.4 Discussion

The data presented here suggest that the number and nature of A3 transcripts is conserved between domestic and non-domestic cat genomes and that A3 genes are broadly expressed in various feline cell types and tissues. Feline A3H and A3CH proteins were shown to be potent anti-FIV restriction factors in the absence of the FIV Vif protein. In contrast, feline A3C proteins displayed only a low antiviral activity. Although FIV Vif is an A3 antagonist, no evidence was found to support the hypothesis that Vif has shaped the functions of A3 proteins during long-term host-virus co-adaptation.

The activities of feline A3 proteins and their susceptibility to FIV Vifs have also been investigated in other recent studies (Munk et al., 2008; Stern et al., 2010; Zielonka and Munk, 2011). In agreement with our findings, feline A3C isoforms did not reduce the infectivity of FIVΔ*vif* or FIV. However, because the A3 activity assay used in this study relies heavily on mutation of the reporter protein GFP by deamination of GFP minus-strand cDNA during reverse transcription, deamination events outwith the cDNA sequence that encodes for the fluorophore active site or deamination-independent A3 activities may have been missed.

Production of FIVΔ*vif*(VSV-G) pseudotypes in the presence of FcaA3H or FcaA3CH led to an inhibition of viral replication by five- or ten-fold, respectively, and was accompanied by an increased G-to-A mutation rate of viral genomes (Munk et al., 2008). Likewise, A3H and A3CH proteins of non-domestic felids decreased FIV infectivity in the absence of Vif (Zielonka and Munk, 2011). In contrast, in this study and in the study by Stern et al. (2010) significantly higher fold reductions in FIV infectivity by feline A3H and A3CH proteins were

observed that could be explained by differences in A3 expression from the various expression constructs used (data not shown).

Notably, FIV-Fca Vif not only suppressed the activities of domestic cat A3s and induced their depletion from producer cells, but also counteracted lion and puma A3 proteins (Zielonka and Munk, 2011). In comparison, our results indicate that there are indeed differences in the sensitivity of feline A3 proteins to FIV Vifs (Fig. 4-6B,D,F). Whereas the antiviral activity of FcaA3CH was partially overcome by FIV Vifs, Vif proteins were less potent against FcaA3H. Similarly, Vif modestly counteracted PleA3H, PcoA3H and PcoA3CH. Paradoxically, FIV Vifs enhanced the PleA3CH-mediated reduction of FIVΔ*vif* infectivity. The inability of FIV Vifs to suppress PleA3CH may be caused by impaired Vif-PleA3CH binding, which has been shown to be essential for Vif-mediated A3 degradation (Stern et al., 2010). Future research should thus focus on the identification of amino acid residues that are essential for the FIV Vif-feline A3 interaction. Importantly, the resistance of PleA3CH to FIV Vif may contribute to the inability of FIV to replicate in lion T lymphocytes. Other feline A3 proteins do not seem to play a major role in limiting FIV replication and interspecies transmission.

No differences in activity against feline A3 proteins were observed between FIV-Pco Vif (in VR1012) and GL8Mya Vif (in FP93), which suggests that FIV Vif is not specific for A3 proteins of a particular felid species. In contrast, HIV-1 Vif degrades human A3G but not non-human primate A3Gs (Marin et al., 2003). However, FIV-Fca Vif is non-functional against human A3G (Munk et al., 2008; Zielonka et al., 2010). The expression of FIV-Ple E Vif and FIV-Oma Vif was attempted but proved unsuccessful (Fig. 4-6G). Codon usage optimisation of these FIV Vifs could be performed to improve their expression in 293T cells.

The data presented here show that feline A3H and A3CH proteins possess a broad anti-lentiviral activity that extends to HIV-1 and SIVmac (Fig. 4-5). In agreement with the work of Stern et al. (2010), HIV-1 Vif was non-functional against feline A3 proteins and even augmented domestic cat A3C-mediated restriction (Fig. 4-5A). Surprisingly, Stern et al. (2010) reported that SIVmac Vif counteracts FcaA3CH while increasing the restriction of SIV by FcaA3Ca and FcaA3H. In contrast, in this study no evidence for activity of SIV Vif against FcaA3CH was found (Fig. 4-5C). However, our data confirm the augmentation of feline A3H activity by SIV Vif. No differences between the potencies of domestic and non-domestic cat A3s against primate lentiviruses were detected.

Because A3 and Vif proteins are highly overexpressed in the *in vitro* single-cycle replication assays commonly used to determine their activities, the results obtained may not reflect the situation in natural lentiviral target cells. To gain a better insight into the role of feline A3 proteins in FIV restriction it was important therefore to link *in vitro* A3 restriction potentials with A3 expression levels in relevant feline cell lines and tissues.

A recent study has investigated the human A3 mRNA expression profile in human lymphocytes (T cell lines and leukocytes) and tissues (Refsland et al., 2010). The data showed that multiple *A3* genes were expressed constitutively in most cell types and tissues and that the expression of some of them could be enhanced by T-cell activation and interferon treatment. As would be expected, genes encoding human A3G and A3F, the cytidine deaminases known to inhibit HIV-1 replication (Sheehy et al., 2002; Liddament et al., 2004; Zheng et al., 2004), were highly expressed in non-permissive cell lines such as CEM and H9, but not in permissive cell lines like CEM-SS or SupT1 (Refsland et al., 2010). High *A3G* expression levels were also found in un-stimulated primary PBMCs. In comparison, *A3F* expression levels were approximately 10-fold lower in these cells (Koning et al., 2009; Refsland et al., 2010). IL-2 and mitogen (phytohaemagglutinin, PHA) stimulation of the $CD4^+$ T lymphocyte fraction led to upregulation of *A3G*, but not *A3F* expression (Refsland et al., 2010). Treatment of PBMCs or $CD4^+$ T cells with leukocyte interferon resulted in an upregulation of *A3G* expression in PBMCs, but not in $CD4^+$ T lymphocytes. Other recent reports have shown that *A3* expression is only IFN-responsive in $CD14^+$ phagocytic cells such as monocytes and macrophages (Peng et al., 2006; Koning et al., 2009).

Lastly, *A3G* and *A3F* expression was very low in tissues such as brain, heart, kidney, skeletal muscles or small intestine and low in bone marrow, liver, thymus and tonsils. Tissues with high *A3G* and *A3F* expression were lung, lymph node, ovary and spleen (Koning et al., 2009; Refsland et al., 2010). With the exception of ovarian tissue, high *A3G* and *A3F* expression levels correlated with high expression levels of the lymphocyte marker CD3 suggesting that the lymphocyte content of a particular tissue was a major determinant of the *A3G*/*A3F* expression level (Koning et al., 2009).

In the current study, differences between the *FcaA3C*, *FcaA3H* and *FcaA3CH* expression levels in feline cell lines were observed. Feline thymic lymphosarcoma cells (3201) and kidney epitheliod cells (CrFK) showed higher

basal expression levels of all domestic cat *A3* genes than fibroblasts (AH927), foetal embryo fibroblast-like cells (FEA) and feline T lymphocytes (Mya-1) (Fig. 4-7A). *A3* gene expression was inducible by IFN-α treatment in 3201, CrFK and Mya-1 cells. In all cell lines expression of *FcaA3C* was higher than that of *FcaA3H* and significantly higher than that of *FcaA3CH*. Expression of *PleA3C*, *PleA3H* and *PleA3CH* was comparable between un-stimulated and un-stimulated, IFN-ω-treated African and Asian lion PBMCs (Fig. 4-7C). However, *PleA3H* expression was higher than *PleA3C* and significantly higher than *PleA3CH* expression.

One possible explanation why *FcaA3C* expression in domestic cat cells was higher than that of *FcaA3H*, while *PleA3H* was expressed at higher levels in lion PBMCs than *PleA3C* may be that domestic cat *FcaA3C* expression has responded to the presence of other retroviruses that have a significantly higher prevalence in domestic cats than in lions, such as feline foamy virus (FeFV) (Lutz et al., 1994). Indeed, FcaA3Ca has been shown to possess potent antiviral activity against FeFVΔ*bet* (Lochelt et al., 2005).

A3 expression levels in eleven different tissues from three FIV-negative cats were quantified. In accordance with A3 expression data in human tissues (Refsland et al., 2010), bone marrow and tonsils had lower A3 mRNA contents than different species of lymph nodes, spleen and thymus. *A3* expression in ovarian tissue closely resembled those in bone marrow and tonsils and was, therefore, lower than expected. Apart from ovaries all tissues used in this study were lymphoid tissues and thus rich in lymphocytes, which should have high *A3* gene expression levels. It can be assumed that, similar to the study by Koning et al. (2009), there are differences in the lymphocyte content of these tissues that may explain the observed variation in *A3* gene expression.

A3 proteins are single-stranded DNA-editing enzymes that protect hosts from viral infection. Lentiviral Vif proteins, in return, target A3 proteins for ubiquitination and proteasome-mediated degradation. The Vif-A3 interaction is mostly species-specific as Vif of a particular lentivirus will only counteract A3 proteins of the lentivirus' host species, but not that of other hosts. Thus, to constantly escape from or enhance interactions, respectively, both *A3* and *vif* genes are expected to engage in adaptive co-evolution (Zhang and Webb, 2004), a concept known as the red queen hypothesis (Van Valen, 1973).

Indeed, multiple *A3* and lentiviral *vif* genes have been shown to be under positive (diversifying) selection (Yang et al., 2003; Sawyer et al., 2004; Zhang

and Webb, 2004; Munk et al., 2008; Compton et al., 2012). In felids, *A3C* genes were found to be under positive selection, whereas *A3H* genes evolve under negative (purifying) selection (Munk et al., 2008). It was proposed that feline *A3C* genes may be under high selective pressure from FeFV, given that FcaA3C proteins possess potent anti-FeFV activity (Lochelt et al., 2005; Munk et al., 2008). In contrast, feline A3H genes do not evolve adaptively, although feline A3H proteins can counteract FeLV and FIV (Munk et al., 2008).

Here, we tested the hypothesis that the long-term presence of FIV in African lion populations has driven adaptive evolution of African lion *A3* genes and has shaped the activities of lion A3 proteins through the continuous activity of Vif. For that, fragments of *A3C* and *A3H* genes were amplified from different African lion gDNAs and their sequences were compared to that of Asiatic lion *A3C* and *A3H* genes. Currently, only one Asian lion population exists, namely in the Gir forest in India, which is FIV-negative (Lutz et al., 1992; Spencer et al., 1992; Brown et al., 1994) and can thus serve as a control for A3 evolution in the absence of FIV Vif. Unexpectedly, no differences in the sequences of African and Asiatic lion *A3C* and *A3H* gene exons were identified, indicating that FIV Vif has not been a driving force in feline *A3* gene evolution.

One reason for the apparent lack of Vif selection pressure on African lion *A3* genes may be that FIV-positive African and FIV-negative Asian lion populations have not been separated from each other for sufficient amounts of time in order for adaptive evolution to take place. The most recent ancestor of modern lion populations dates back to only 325,000 years ago. Starting from Eastern and Southern Africa, lions migrated into Central and North Africa and finally into Eurasia about 100,000 years ago (Antunes et al., 2008). Unfortunately, it is not clear when lions first became infected with FIV.

Interestingly, other groups have investigated the impact of HIV-1 Vif on human *A3G* evolution and found that, although *A3G* has been subject to strong diversifying selection throughout primate evolution, the selection pressure driving this evolution predates the emergence of modern lentiviruses about 1 MYA (Sawyer et al., 2004; Zhang and Webb, 2004). This indicates the presence of more ancient genetic conflicts involving *A3G* (Sawyer et al., 2004). Such conflicts may have taken place not only in lymphocytes but also in the germline and may have involved LTR-bearing human endogenous retroviruses (HERVs) or retrovirus-like eukaryotic mobile elements (Sawyer et al., 2004). Furthermore,

in agreement with our findings, Zhang and Webb (2004) found no difference in selective pressure on A3G between hosts and non-hosts of HIV-1 or SIV (Zhang and Webb, 2004), demonstrating again that lentiviral Vif proteins are not likely to have a major impact on A3 selection.

Conversely, A3 proteins may well be the driving force for the positive selection of lentiviral Vifs, especially given the high mutation rate of lentiviruses that would allow for rapid adaptation (Sawyer et al., 2004; Zielonka et al., 2010; Compton et al., 2012).

It is plausible that positive evolution of feline *A3C* genes is also not influenced by FIV Vif, especially given its low anti-FIV activity, and that it may predate the presence of FIV. Similar to human *A3G*, feline *A3H* may be under negative selection because A3H may protect against endogenous retroelements (Munk et al., 2008). Alternatively, the current A3H amino acid sequence and activity may be optimal to target highly conserved viral structures of FeLV or FIV. Although A3H alone has only intermediate activity against lentiviruses, its combination with exons of *A3C* genes has created a potent lentiviral restriction factor (A3CH). Lastly, apart from innate immune defence, A3H may also be required for other, currently unknown processes.

5 The role of BST-2/tetherin in feline retrovirus infection

5.1 Summary

The findings presented in Chapter 4 indicate that, with the exception of PleA3CH, which potently restricted FIV even in the presence of FIV Vif, feline A3 proteins do not contribute significantly to the prevention of FIV replication and its interspecies transmission.

In addition to TRIM5α and A3 proteins a third restriction factor against HIV-1 has been characterised. BST-2/tetherin/CD317 exerts a late block to retroviral replication in that it prevents the release of mature enveloped viral particles from membranes of viral producer cells, an activity that is counteracted by Vpu of HIV-1 and certain SIVs, HIV-2 and SIV Envs, and SIV Nef proteins (Neil et al., 2007; Neil et al., 2008; Van Damme et al., 2008; Gupta et al., 2009b; Jia et al., 2009; Le Tortorec and Neil, 2009; Perez-Caballero et al., 2009; Sauter et al., 2009; Zhang et al., 2009b; Goffinet et al., 2010; Yang et al., 2010). Tetherin is a specific cell surface marker of type I IFN-producing plasmacytoid dendritic cells (pDCs); however, its expression can be upregulated in many different cell types by stimulation with type I IFN and IFN-γ (Blasius et al., 2006). The antiviral activity of tetherin has been studied almost exclusively in single-cycle replication assays using lentiviral pseudotypes but information on its potency to block viral spread is sparse.

In this study a gene encoding the feline homologue of tetherin was identified in the domestic cat genome. Alignment of the coding sequence of this feline tetherin with cDNAs of other bona fide tetherins revealed significant homology between them on both nucleotide and amino acid levels. Interestingly though, the feline tetherin cDNA was predicted to lack the otherwise conserved initiation codon. Hence, the full-length protein was likely to possess a shorter N-terminus than other tetherins. However, because the tetherin 5' region was still encoded in the domestic cat genome and would contain a double tyrosine motif shown to be important for tetherin endocytosis from the cell surface (Rollason et al., 2007; Iwabu et al., 2009; Masuyama et al., 2009) we initially decided to amplify the feline tetherin cDNA with a forward primer that contained an initiation codon followed by the tetherin 5' sequence naturally present in the domestic cat genome and feline tetherin cDNA. The protein encoded by this 561

nt long feline tetherin cDNA is referred to as ”FcaTHN” in this thesis. Later during the course of this study the 504 nt long feline tetherin cDNA, whose translation is initiated at a downstream ATG, was amplified and characterised. To our current knowledge, this cDNA encodes for the wild-type feline tetherin, which is referred to as “FcaTHN-WT”. The term “feline tetherin” is used when reference is made to both FcaTHN and FcaTHN-WT.

Feline tetherin was found to be expressed in many feline cell lines, and expression was IFN-inducible. Like human tetherin, FcaTHN displayed potent inhibition of FIV and HIV-1 particle release; however, this activity resisted antagonism by either HIV-1 Vpu or the FIV Env and OrfA proteins. Interestingly, stable expression of FcaTHN in feline cell lines did not abrogate the replication of FIV. Indeed, syncytium formation was significantly enhanced in FcaTHN-expressing cells and in type I interferon-treated CrFK cells infected with cell culture-adapted, CD134-independent strains of FIV. Thus, while tetherin may prevent the release of nascent viral particles, its expression seems to facilitate viral transmission by inducing a shift from a cell-free to a cell-to-cell transmission mode.

In the second part of the study, the activity of FcaTHN against other feline retroviruses was determined. Most notably, the replication of RD114, an endogenous feline retrovirus, was severely affected by FcaTHN. Furthermore, tetherins of non-domestic felids were characterised and their potential to limit retroviral replication was tested. Lion and puma tetherins (PleTHN and PcoTHN, respectively) were amplified using the same strategy as described for FcaTHN and resembled FcaTHN in their inability to block viral spread.

Finally, the activity of FcaTHN-WT to limit FIV particle release in the presence or absence of different FIV Envs and to prevent FIV spread was assessed. FcaTHN-WT was slightly more potent in blocking FIV particle release than FcaTHN, but again, this activity was not overcome by FIV Envs. Importantly, like FcaTHN, FcaTHN-WT was not able to control FIV spreading infection.

In summary, these data suggest that while felid tetherins prevent particle release, they do not play a significant role in the suppression of retroviral replication *in vivo*.

5.2 Materials and Methods

5.2.1 Amplification, molecular cloning and stable expression of feline and human tetherin cDNAs

The genomic sequence of the prospective domestic cat homologue of tetherin was identified by blasting the sequences of human (HsaTHN; GenBank NM_004335) and dog (*Canis lupus familiaris*) tetherins (CluTHN; GenBank XM_847295, XM_860510) against the 2X domestic cat genome using megaBLAST (Zhang et al., 2000). A candidate domestic cat tetherin gene was identified on *Felis catus* c430601298.contig1 (GenBank ACBE01053987). This gene showed a high degree of nucleotide and amino acid homology to other known tetherin, but was lacking an otherwise conserved initiation codon at the 5' end of its first coding exon. To determine the initiation site of the coding sequence of feline tetherin, 5'RACE PCR was performed using the 5'/3' RACE kit (Roche Applied Science) and gene-specific primers FcTHN-SP1 (5'-GAAGCCAACAGGGTTACCAA-3') and FcTHN-SP2 (5'-GACACCGTGACACTCCTCCT-3') according to manufacturer's instructions. A feline tetherin cDNA fragment (referred to as FcTHN-WT; 504 nt; DDBJ [Data Base of Japan] AB564550), that would encode for a tetherin with a shortened N-terminus compared to other tetherins, was identified as full-length transcript and amplified using primers FcTHN-delCT-Fwd (5'-ACTGGTCGACACCATGGTGCCAGGTCGGAGTCTT-3') (*Sal*I restriction site underlined) and FcTHN-Rev-1. A longer cDNA containing an engineered 5' initiation codon and the naturally present feline tetherin 5' cDNA sequence (FcaTHN; 561 nt; GenBank HM461970/NM_001243085) was amplified from Mya-1-derived cDNA with primers FcTHN-Fwd-2 (5'-ATCGGTCGACACCATGGCACCTGCTTTTTTACCAC-3') (*Sal*I restriction site underlined) and FcTHN-Rev-1 (5'-ACAGCGGCCGCTCAGGCCAGCAGAGCAACGAA-3') (*Not*I restriction site underlined). The sequence of FcaTHN has been deposited in GenBank under accession number HM461970. The same primer set as for FcaTHN was used to amplify lion tetherin (PleTHN) from Angolan lion PBMC cDNA and puma tetherin (PcoTHN) from puma PBMC-derived cDNA. HsaTHN was amplified from 293T cell-derived cDNA using primers HuTHN-Fwd-1 (5'-ACTGGTCGACACCATGGCATCTACTTCGTAT-3') (*Sal*I restriction site

underlined) and HuTHN-Rev-1 (5'-ACAGCGGCCGCTCACTGCAGCAGAGCGCTG-3') (*Not*I restriction site underlined).

All tetherin cDNAs were cloned into VR1012 (Vical Inc.) using *Sal*I and *Not*I restriction sites to generate constructs FcaTHN-VR1012, PleTHN-VR1012, PcoTHN-VR1012, HsaTHN-VR1012 and FcaTHN-WT-VR1012.

Furthermore, site-directed mutagenesis was performed to delete a putative endocytosis motif at the N-terminus of FcaTHN. At first the mutation Tyr8Ala was created using FcaTHN-VR1012 as template and mutagenesis primers FcTHN-Y8A-Fwd (5'-GCACCTGCTTTTTACCACGCGTGGCCTGTGCCCAGGAC -3') and FcTHN-Y8A-Rev (5'-GTCCTGGGCACAGGCCACGCGTGGTAAAAAGCAGGTGC-3'). Another mutation (Tyr6Ala) was then introduced into FcaTHN-Y8A-VR1012 using mutagenesis primers FcTHN-Y6/8A-Fwd (5'-GCACCTGCTTTTGCGCACGCGTGGCCT GTGCCCAGGAC-3') and FcTHN-Y6/8A-Rev (5'-GTCCTGGGCACAGGCCACGCGTGCG CAAAAGCAGGTGC-3') to generate construct FcaTHN-Y6/8A-VR1012.

For stable expression in CrFK, FEA and canine CLL cells, all tetherins were cloned into pDON-AI-2neo (Takara Bio Europe S.A.S./Clontech) using *Not*I and *BamH*I restriction sites. For confocal microscopy experiments, FcaTHN was labeled with an internal haemagglutinin (HA) tag by amplification of two fragments by the use of primer pair FcTHN-Fwd-3 (5'-ATCGGCGGCCGCATGGCAC CTGCTTTTTACCAC-3') (*Not*I restriction site underlined) and FcTHN-HA-Rev-1 (5'-GACGTAGTCTGGGACGTCGTATGGGTATTCCTTTTTCTTGCTCGAG-3') and primer pair FcTHN-HA-Fwd-1 (5'-TACCCATACGACGTCCCAGACTACGTCGTCGCGTCTGCCA GCTCCTTGA-3') and FcTHN-Rev-2 (5'-ACAGGATCCTCAGGCCAGCAGAGCAACGAAG-3') (*BamH*I restriction site underlined), with the construct FcaTHN-VR1012 used as template. The products were then purified, combined, and used as a template for a second round of amplification using primers FcTHN-Fwd-3 and FcTHN-Rev-2. The product (FcaTHN-HA) was then cloned into pDON-AI-2 Neo using *Not*I and *BamH*I restriction sites and transduced into CRFK cells.

5.2.2 Quantification of domestic cat tetherin mRNA by real-time reverse transcription PCR

Quantitative real-time reverse transcription PCR (RT-qPCR) was performed to determine the expression levels of the domestic cat tetherin gene (*THN*) in the

feline cells lines FEA, CrFK, AH927 and 3201, in Mya-1 cells and in primary feline macrophages. Before RNA isolation, feline cell lines and macrophages were either left untreated or were stimulated with 10^3 IU/ml feline IFN-α, feline IFN-γ (both from R&D Systems Europe Ltd, Abingdon, UK) or recombinant feline IFN-ω (Virbac Limited, Bury St Edmonds, UK), respectively, for 24 hours. All RNAs were treated with DNAse I (Life Technologies) prior to cDNA synthesis.

Feline tetherin mRNA was detected using primers qPCR-FcTHN-Fwd (5'-GAGAAGGCCCAGAGCCAGGAG-3') and qPCR-FcTHN-Rev (5'-GCAACGAAGGCCAGGA GCAG-3') and probe qPCR-FcTHN-Pro (5'FAM-TGCAGAACGCTTCGGTGGAGGTGGAA AGACTGAGAAA-3'TAMRA). Domestic cat 18S rRNA was used as housekeeping gene and was amplified with primers rDNA 343F cat (5'-CCATTCGAACGTCTGCCCTA-3') and rDNA 409 R (5'-TCACCCGTGGTCACCATG-3') and probe rDNA 370Pcat (5'FAM-CGATGGTAGTCGCCGTGCCTA-3'TAMRA). Feline tetherin gene expression levels were analysed using the ΔC_T method. ΔC_T values were calculated by subtracting the mean C_T for the abundant 18S rRNA from the C_T for feline tetherin mRNA.

5.2.3 Amplification, molecular cloning and detection of lentiviral tetherin antagonists

In order to identify putative lentiviral tetherin antagonists, FIV OrfA (Orf2), various FIV envelope glycoproteins (Envs) and HIV-1 Vpu were amplified, cloned and expressed in conjunction with HIV-1(VSV-G) and FIV(VSV-G) pseudotypes in the presence or absence of feline tetherins in single-cycle replication assays as described under 2.2.5.

HIV-1 NL-43 Vpu in pCDNA3.1 (Life Technologies) was a kind gift of Greg Towers, University College London, UK. FIV-Fca GL8 OrfA was amplified from GL8Mya molecular clone (Hosie et al., 2002) using primers GL8 orf2 Fwd (5'-ACTGGTCGACACCATGGAAGAAATAATAGTATTATTC-3') (*Sal*I restriction site underlined) and GL8 orf2 Rev (5'-ACAGCGGCCGCCTAAGCAGTACGATGGATAATGTA -3') (*Not*I restriction site underlined) and cloned into VR1012. In addition, codon-optimised FIV OrfA was expressed from construct 1S-5RL (kindly provided by Mauro Pistello, Università di Pisa, Italy). FIV-Fca GL8 Env (GenBank X69496) was amplified from GL8Mya molecular clone using primers GL8 Env-Fwd (5'-ACTGGTC

GACACCATGAATGAAGAAGGGCCACTA-3') (*Sal*I restriction site underlined) and GL8 Env-Rev (5'- ACAGCGGCCGCTCATTCCTCCTCTTTTTCAGA-3') (*Not*I restriction site underlined). C8.1 (CrFK-adapted GL8) Env was amplified using the same primer set. FIV-Fca strain Petaluma clone F14 and clone 34TF10 Envs (GenBank NC_001482) were amplified with primers PET Env-Fwd (5'-ACTGGTCGACACCATG GCAGAAGGATTTGCAGCCA) (*Sal*I restriction site underlined) and GL8 Env-Rev. All FIV-Fca Env cDNAs were cloned into VR1012 using the *Sal*I and *Not*I restriction sites. Furthermore, FIV-Fca Petaluma KKS Env (Env of *in vivo* readapted FIV-Fca Petaluma; [Bendinelli et al., 2001; Pistello et al., 2003]) was expressed from the mammalian expression vector pEE14 (Lonza Biologics, Slough, UK). The construct pEE14-Env (Pistello et al., 2010) was a kind gift of Mauro Pistello, Università di Pisa, Italy. FIV-Ple E Env was amplified from cDNA derived from Mya-1 cells infected with a primary isolate of FIV-Ple E obtained from serum of a wild, 8 year old male lion (Sangre) from the Moremi reserve in the Okavango Delta, Botswana (McEwan, 2009). Primers were designed based on the available FIV-Ple E 1027 sequence (GenBank EU117992) (Pecon-Slattery et al., 2008a), LLV-E Env-Fwd (5'-ACTGGTCGACACCATGGCAGAAGGAGGAAGAGTA -3') (*Sal*I restriction site underlined) and LLV-E Env-Rev (5'-ACAGCGGCCGCTTAGGTATTAGACTCATCATTCA C-3') (*Not*I restriction site underlined). FIV-Ple B Env was amplified from cDNA derived from Mya-1 cells infected with a FIV-Ple B 458 using primers directed to the published FIV-Ple B sequence (GenBank EU117991), LLV-B Env-Fwd (5'-ACTGGTCGACACCATGGCGGAAGGAGGAAGAGTA-3') (*Sal*I restriction site underlined) and LLV-B Env-Rev (5'-ACAGCGGCCGCTCAAAGATCCTCATCAGACTCCC T-3') (*Not*I restriction site underlined). Both Env cDNAs were cloned into VR1012.

FIV-Fca GL8 Env and OrfA were also expressed from CT5G8MΔ*pol*4, a derivative of pCT5b (Poeschla et al., 1998) in which a *Pac*I-*Nde*I fragment was exchanged with the equivalent fragment from the GL8 Mya molecular clone. The resulting clone, CT5-G8M, was then modified by deleting a fragment of *pol* by *Pac*I/*SanD*I digestion followed by treatment with the Klenow fragment of DNA polymerase I prior to religation. CT5G8MΔ*pol*4 thus produces all FIV-encoded proteins under the control of a cytomegalovirus (CMV) promoter but is not replication-competent.

Immunoblotting was performed to confirm expression of FcaTHN-HA and FIV-Fca Envs. FcaTHN-HA was detected using a rabbit anti-HA antibody (Sigma-

Aldrich). FIV-Fca Env expression was verified using mouse anti-FIV Env antibody (vpg71.2).

5.2.4 Confocal microscopy

CrFK cells stably expressing FcaTHN-HA were seeded onto 13 mm-diameter glass coverslips in 24-well plates and incubated overnight. Cells were then infected with FIV-Fca F14 for 72 hours. Cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 15 minutes and neutralised with 0.1 M glycine in PBS for 5 minutes. Where appropriate, cells were then permeabilised with 0.2% triton-X100 in PBS for 20 minutes. Cells were blocked by incubation with 2% FCS in PBS for 20 minutes before incubation with a mixture of primary antibodies comprising mouse anti-FIV Env (vpg71.2) and rabbit anti-HA (Sigma-Aldrich) antibodies in 1% FBS in PBS for 1 hour at room temperature. Cells were then washed three times with PBS, incubating for 5 minutes with each wash. Secondary antibodies were then added (Alexa-Fluor 594-conjugated F(ab')2 fragment of goat anti-rabbit IgG [Life Technologies/Molecular Probes] and fluorescein isothiocyanate [FITC]-conjugated F(ab')2 fragment of goat anti-mouse IgG [Abd Serotec, Oxford, UK]) and incubated in 1% FBS in PBS for 45 minutes at room temperature. Cells were washed three times with PBS, incubating for 5 minutes with each wash, removed from the 24-well plate, mounted onto glass slides in mounting medium containing DAPI (4',6'-diamidino-2-phenylindole; Vectashield, Vector Laboratories). Slides were analysed on a Leica TCS SP2 confocal microscope.

5.2.5 Electron microscopy

Electron microscopy was performed with the help of David Bhella, University of Glasgow. Monolayer cultures of FIV-infected CrFK cells were fixed *in situ* for 2 hours at room temperature with 4% paraformaldehyde–0.1 M sodium phosphate buffer (pH 7) and stored at 4°C in 0.2% paraformaldehyde. Prior to processing for electron microscopy, the cells were exposed to 2.5% glutaraldehyde overnight at 4°C. The cells were then scraped and pelleted by centrifugation followed by 1% osmium tetroxide fixation. Fixed cells were then resuspended

and pelleted through 1% SeaPlaque agarose (Lonza, Slough, UK). The cell pellets were dehydrated through a graded alcohol series and embedded in EPON 812 resin. Sections approximately 120 nm in thickness were cut with an UC6 ultramicrotome (Leica Microsystems, Germany) and stained with saturated uranyl acetate (in 50% ethanol) and Reynolds lead citrate (Reynolds, 1963). Sections were observed and photographed with JEOL 1200 EX and JEOL JEM-2200FS transmission electron microscopes, and images were recorded on a Gatan Ultrascan camera.

5.2.6 Feline foamy virus replication assays

Feline foamy virus (FeFV) molecular clones were kind gifts of Martin Löchelt, German Cancer Research Center, Heidelberg, Germany. Wild-type FeFV was expressed from construct pCF-7 (Schwantes et al., 2002). FeFV devoid of *bel2* and *bet* (FeFVΔ*bel*/*bet*) was expressed from construct pCF-7-BBtr (Alke et al., 2001). Single-cycle replication assays were performed in 293T cells by co-transfecting FcaTHN and FeFV expression constructs in molar ratios of 0:1, 0.1:1, 0.25:1, 0.5:1, 1:1 and 2:1. Transfections were set up in 60-mm cell culture dishes using constant amounts of FeFV expression plasmids (5 µg DNA) and increasing amounts of FcaTHN-VR1012 (0 µg, 0.2 µg, 0.5 µg, 1 µg, 2 µg or 4 µg DNA). Where appropriate, the total amount of transfected DNA was adjusted to 9 µg using VR1012. FeFV titres were determined 48 hours post-transfection by titration of cell supernatants onto FeFAB reporter cells in 24-well plates (Zemba et al., 2000). FeFAB cells are genetically modified CrFK cells, which stably carry the ß-galactosidase gene (*lacZ*) under transcriptional control of the FeFV LTR promoter in their genomes. Upon transduction of these cells with *bel*1-expressing FeFV vectors, Bel1 transactivates the FeFV LTR promoter which leads to expression of *lacZ*. *Lac*Z expression in cell nuclei was visualised 72 hours post-transduction by LacZ staining and light microscopy. Briefly, cell medium was removed, cells were washed with 1 mM $MgCl_2$ in PBS and fixed with 1% formaldehyde/0.2% glutaraldehyde/1 mM $MgCl_2$ in PBS for 5 minutes. Cells were then stained with 4 mM $K_4[Fe(CN)_6]$ (II)/4 mM $K_3[Fe(CN)_6]$ (III)/4 mg/ml X-Gal/1 mM $MgCl_2$ in PBS overnight at 37°C. Staining solution was removed and the

staining reaction was stopped with ddH_2O. FeFV titres were expressed as focus forming units per ml of cell supernatant (FFU/ml).

5.3 Results

5.3.1 Identification of a feline homologue of BST-2/tetherin

A genomic sequence with significant homology to canine (*Canis lupus familiaris*) (GenBank XM_860510) and human (GenBank NM_004335) BST-2/tetherin was identified by screening the 2X domestic cat genome. The predicted associated cDNA was 504 nt in length. However, the genomic sequence upstream of the putative start codon shared high similarity with 5' termini of known tetherin cDNAs but was missing a thymidine in its start codon. Because of this high similarity and because only the longer cDNA (561 nt) would encode for a double tyrosine motif important for tetherin endocytosis from the cell surface (Rollason et al., 2007; Iwabu et al., 2009; Masuyama et al., 2009) we initially decided to amplify the longer cDNA of domestic cat tetherin (FcaTHN) and engineered the forward primer to contain a complete start codon.

FcaTHN (GenBank HM461970) possesses 65% nucleotide and 38% amino acid sequence identity to human tetherin (HsaTHN), and 79% nucleotide and 57% amino acid sequence identity to canine tetherin (Fig. 5-1). Secondary structure prediction softwares NetSurfP (Petersen et al., 2009) and TMHMM Server 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/) indicated that the 184 amino acid long protein contained an N-terminal cytoplasmic domain, followed by a transmembrane domain, an extracellular loop with three conserved cysteines (C59, C69, and C97) involved in tetherin dimerisation via disulfide bond formation, and a coiled-coil domain. The big-π predictor software (Eisenhaber et al., 2000) revealed a potential attachment site for a glycosylphosphatidylinositol (GPI) anchor at serine 161 followed by a C-terminal hydrophobic domain (Fig. 5-1). Thus, FcaTHN adopts the same protein topology described for other tetherins (Ishikawa et al., 1995; Ohtomo et al., 1999; Kupzig et al., 2003).

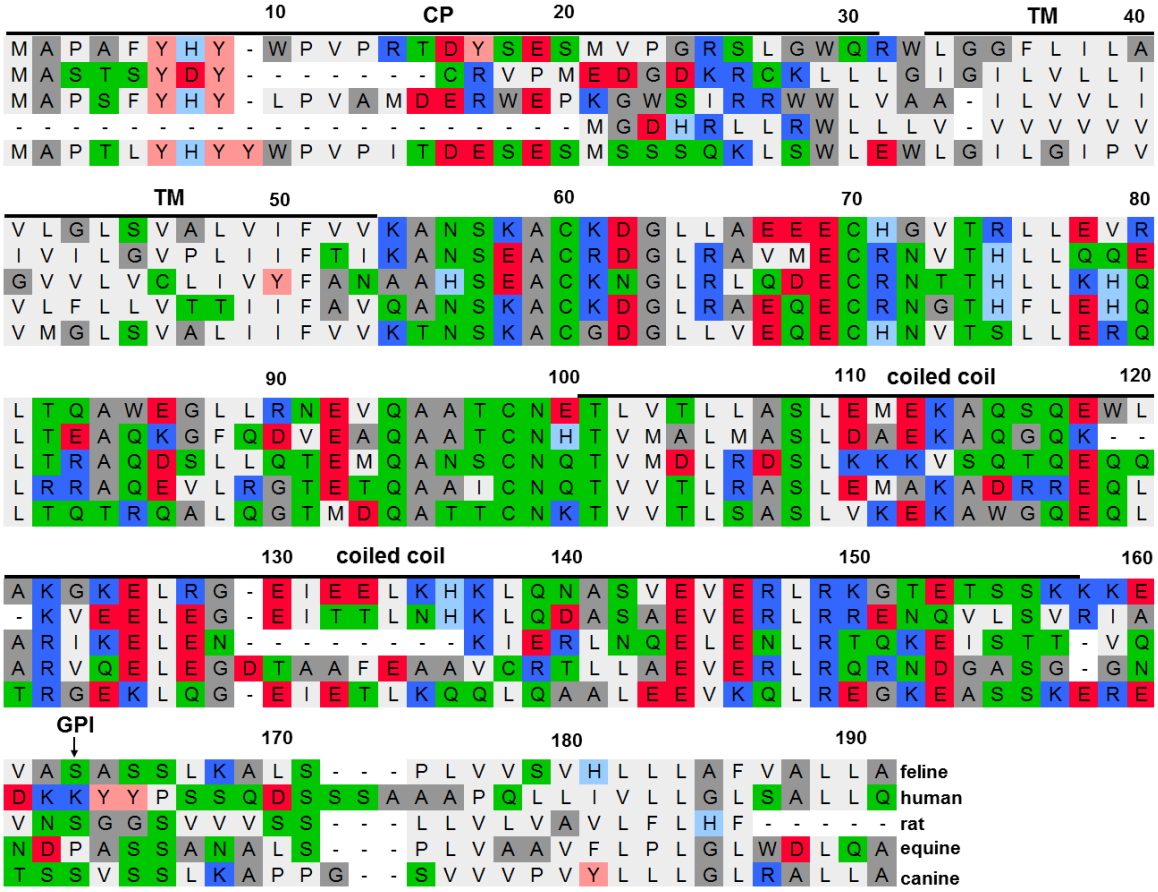
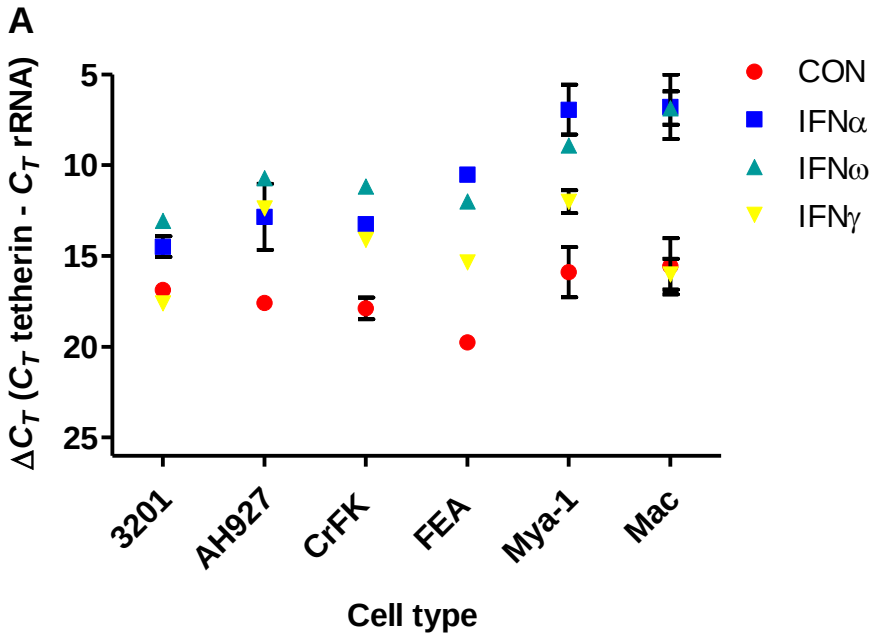


Figure 5-1 Identification of a feline homologue of tetherin. Predicted amino acid sequences of feline (FcaTHN), human, murine (rat) (GenBank NM_198134), equine (GenBank XM_001915091) and canine tetherins were aligned. Black bars denote N-terminal cytoplasmic domain (CP), transmembrane (TM) and extracellular, α-helical coiled-coil regions (coiled coil). An arrow marks a predicted site for glycosylphosphatidylinositol (GPI) anchor attachment. Amino acid residues are colour-coded as follows: light gray, hydrophobic FILMPV; dark gray, amphiphilic AGW; green, hydrophilic neutral CNQST; light blue, slightly basic H; blue, basic KR; pink, slightly acidic Y; red, acidic DE.

In the mouse, tetherin is expressed constitutively in B cells and plasmacytoid dendritic cells, however, expression may be upregulated in many cell types following exposure to both type I (Ohtomo et al., 1999; Blasius et al., 2006; Neil et al., 2007; Kawai et al., 2008; Miyagi et al., 2009) and type II (Blasius et al., 2006) interferons. Four feline cell lines, 3201, AH927, CrFK and FEA, and two primary cell cultures (IL-2-dependent $CD4^+$ T cells [Mya-1] and monocyte-derived macrophages) were treated with type I (α and ω) and type II (γ) interferons and

tetherin mRNA expression levels were examined by RT-qPCR (Fig. 5-2A). The cell lines showed various levels of basal tetherin mRNA expression, with the lowest levels in FEA cells. 3201, AH927 and CRFK cells expressed broadly similar levels of tetherin. The highest level of basal expression was seen with the IL-2-dependent $CD4^+$ T Mya-1 cell line. Treatment of feline cells with the type I interferons IFN-α and IFN-ω increased tetherin expression for all of the cell types examined. A similar increase in tetherin expression was also noted with the AH927, CrFK and FEA cells treated with IFN-γ. A more modest induction of tetherin expression was observed with 3201 cells following treatment with IFN- α and IFN-ω, while IFN-γ did not have a significant effect on 3201 tetherin expression. Mya-1 cells and monocyte-derived macrophages responded to IFN-α and IFN-ω treatment by increasing expression of tetherin, whereas IFN-γ had a modest effect on $CD4^+$ T cells and no effect on macrophages. Treatment of AH927, CrFK, FEA and Mya-1 cells with INF-ω for either 48 or 72 hours led to a significant upregulation of tetherin expression within 24 hours post-stimulation (Fig. 5-2B). Therefore, as with previous observations with human tetherin, feline tetherin expression may be induced by both type I and type II interferons and is expressed on the known *in vivo* cellular targets for FIV, activated T cells and macrophages.



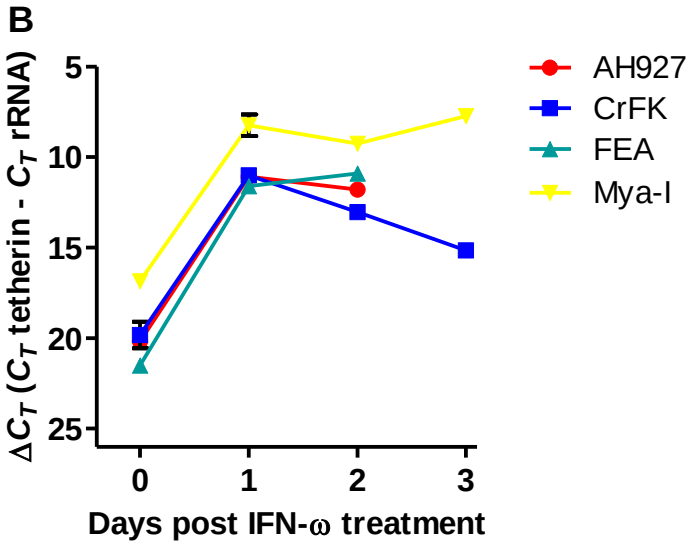


Figure 5-2 Quantification of feline tetherin mRNA by real-time reverse transcription PCR and effect of interferon treatment on tetherin expression. (A) IL-2-dependent $CD4^+$ T cells (Mya-1), macrophages (Mac), and the feline cell lines 3201, AH927, CrFK and FEA were cultured overnight in the presence or absence of feline IFN-α, IFN-ω, and IFN-γ at 10^3 IU/ml prior to RNA extraction, cDNA preparation, and tetherin cDNA quantification by RT-qPCR. (B) AH927, CrFK, FEA and Mya-1 cells were stimulated with 10^3 IU/ml IFN-ω and feline tetherin gene expression was monitored over 48 (AH927 and FEA cells) or 72 hours (CrFK and Mya-1 cells) by RT-qPCR. Results are expressed as mean ΔC_T values ± S.E. as determined in three independent experiments (n=3), with three replicate experiments per analysis.

5.3.2 Inhibition of lentiviral particle release by FcaTHN

In order to assess the ability of FcaTHN to prevent lentiviral particle release from infected cells, single-cycle replication assays were performed using FIVΔ*vif*(VSV-G), HIV-1(VSV-G) or HIV-1Δ*vpu*(VSV-G) pseudotypes in the presence or absence of FcaTHN (Fig. 5-3). FcaTHN reduced the titre of the FIVΔ*vif*(VSV-G) pseudotypes significantly (Fig. 5-3A), suggesting inhibition of particle release from the transfected cells. In agreement with this notion, viral p24 released into the culture supernatant was reduced by tetherin expression, whereas virus

production in the transfected cells was largely unaffected (Fig. 5-3D). Similar activity of FcaTHN against HIV-1 lacking a functional Vpu (HIV-1Δ*vpu*) was detected (Fig. 5-3B). Furthermore, FcaTHN prevented the release of wild-type HIV-1 encoding an intact *vpu* open reading frame (Fig. 5-3C) indicating that, unlike human tetherin, the activity of FcaTHN was not overcome by HIV-1 Vpu.

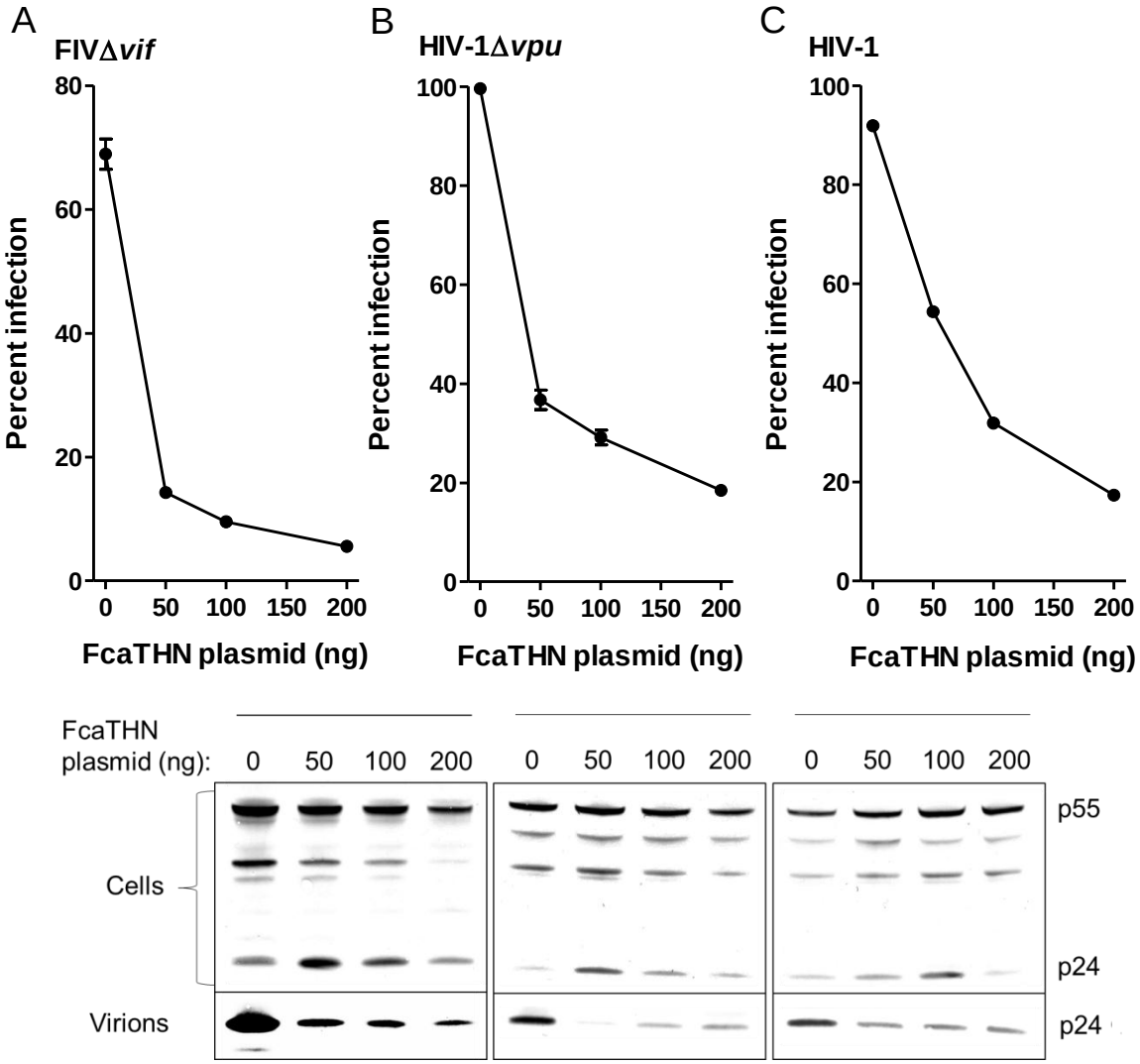


Figure 5-3 FcaTHN restricts FIV and HIV-1 particle release and is not overcome by the HIV-1 accessory protein Vpu. VSV-G-pseudotyped, GFP-expressing FIVΔ*vif* (A), HIV-1Δ*vpu* (B) or HIV-1 (C) particles were produced in 293T cells co-transfected with empty vector only (VR1012) or indicated amounts of FcaTHN expression vector. Pseudotype-containing cell supernatant was then used to transduce 293T target cells and activity of FcaTHN was assessed by measuring GFP-expression in target cells by flow cytometry. Single-cycle

replication assays were performed in triplicate (*n*=3; results shown as mean ± S.E.). (D) FIV and HIV-1 Gag (p24) expression in lysates and in pelleted virions from supernatants of transfected 293T producer cells were assessed by immunoblotting using mouse anti-FIV p24 (vpg50) and 183-H12-5C anti-HIV-1 CA (NIH AIDS Research and Reference Reagent Program) antibodies, respectively.

5.3.3 Characterisation of putative tetherin antagonists in the FIV genome

In the absence of an open reading frame with significant homology to HIV-1 *vpu* in the FIV genome, we asked whether the FIV *env* or *orfA* gene products were able to counteract the activity of domestic cat tetherin (FcaTHN), in analogy to the activities ascribed to HIV-2 Env and the SIV Nef and Env proteins (Neil et al., 2007; Gupta et al., 2009b; Jia et al., 2009; Le Tortorec and Neil, 2009; Zhang et al., 2009a). Co-expression of a replication-defective molecular clone of FIV (CT5G8MΔ*pol*4) in which expression of the entire FIV molecular clone is enhanced by a cytomegalovirus (CMV) promoter, thus increasing expression of all FIV proteins, had no effect on the ability of FcaTHN to counteract FIV release from transfected cells (Fig. 5-4A). Similarly, co-transfection of codon-optimised FIV OrfA expression vector 1S-5RL (Pistello et al., 2003), or of a GL8 *orfA* expressed from VR1012 (data not shown), had no effect on the activity of domestic cat tetherin against the release of FIVΔ*vif* from transfected cells (Fig. 5-4B). Thus, no evidence for a tetherin-counteracting activity was found in the FIV genome.

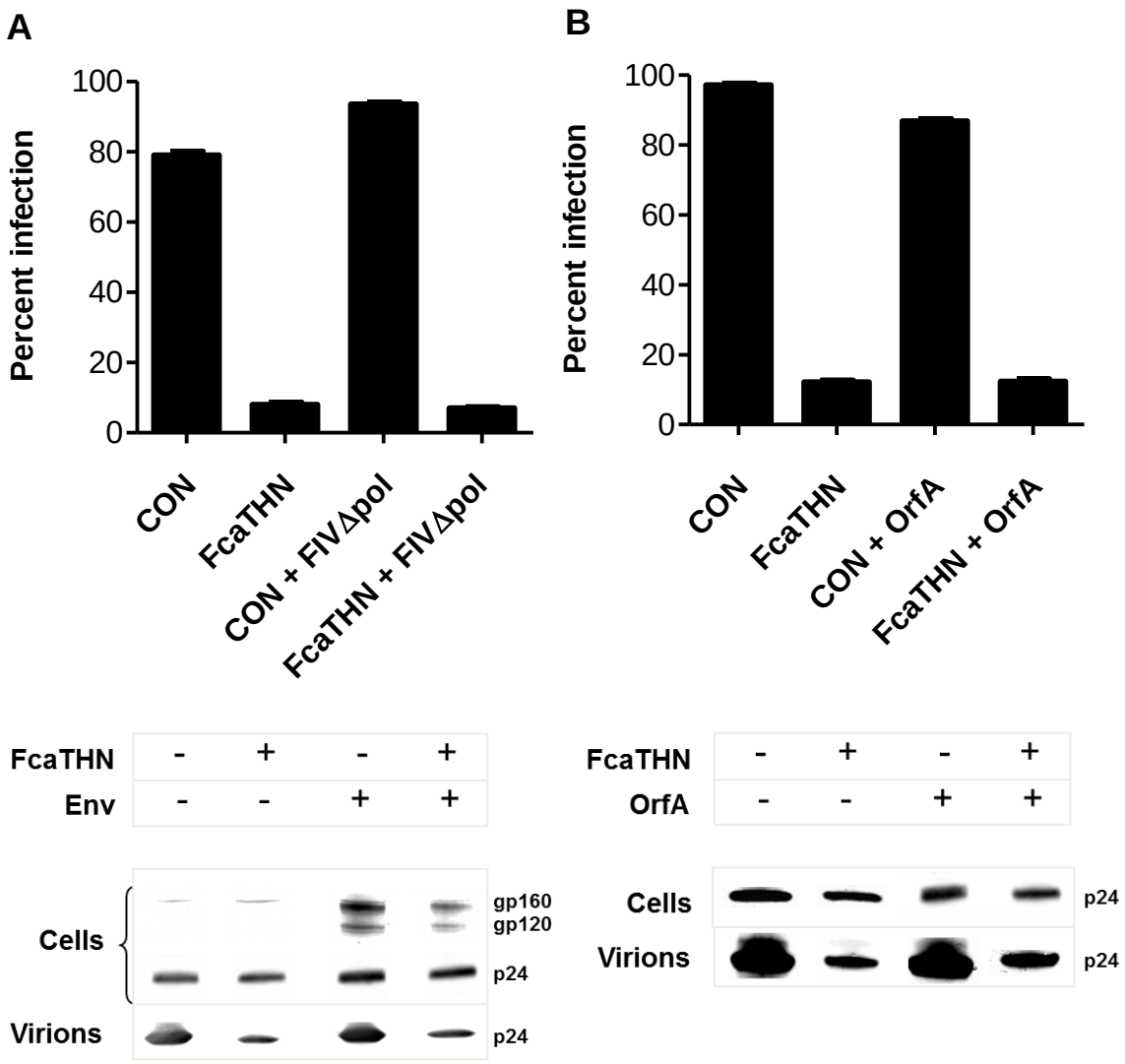


Figure 5-4 Absence of a feline tetherin countermeasure in the FIV genome. VSV-G-pseudotyped, GFP-expressing FIVΔ*vif* particles were produced in 293T cells co-transfected with empty vector only (CON) or FcaTHN expression vector (FcaTHN) in the presence or absence of (A) a replication-defective molecular clone of FIV, CT5G8MΔ*pol*4 (+ FIVΔ*pol*) or (B) a codon-optimised FIV OrfA (+ OrfA). Pseudotype-containing cell supernatant was used to transduce 293T target cells and activity of FcaTHN was assessed by measuring GFP-expression in target cells by flow cytometry. Single-cycle replication assays were performed in triplicates (*n*=3; results shown as mean ± S.E.). Expression of FIV Env from CT5G8MΔ*pol*4 was confirmed by immunoblotting with mouse anti-FIV Env antibody (vpg71.2). FIV Gag (p24) expression in lysates and in pelleted virions from supernatants of transfected 293T producer cells was assessed by immunoblotting using mouse anti-FIV p24 (vpg50) antibody.

5.3.4 Effect of FcaTHN on replication of cell culture-adapted, CD134-independent strains of FIV

Given the high potency of FcaTHN in preventing the release of FIV in a single-cycle replication assays and the absence of tetherin-counteracting activity in the FIV genome, it was of interest to investigate the ability of FcaTHN to inhibit spreading infection of FIV. Thus, CrFK cells stably expressing FcaTHN were infected with cell culture-adapted, CD134-independent strains of FIV-Fca (F14; Fig. 5-5A) and FIV-Pco (CoLV; Fig. 5-5B), and virus spread was monitored by a reverse transcriptase activity assay of the culture supernatant. Interestingly, in contrast to the marked inhibitory effect of tetherin on lentiviral pseudotype release, ectopic expression of tetherin did not inhibit virus production from FIV-infected CrFK cells. In fact, syncytium formation following FIV-Fca F14 infection was enhanced significantly in the tetherin-expressing cells (Fig. 5-5D) compared with control cells (Fig. 5-5C).

To confirm tetherin expression in the stably transduced CrFK cells, RT-qPCR was performed. This analysis indicated that the cells expressed abundant tetherin mRNA (ΔC_T = 8.95 ± 0.25) compared with the vector-only containing control cells (ΔC_T = 16.68 ± 0.34). Thus, the stably transduced cells achieved levels of tetherin expression that exceeded those attained following IFN-ω treatment of control CrFK cells (ΔC_T = 11.17 ± 0.17) (Fig. 5-2A). In comparison, when 293T cells were transfected transiently with either vector only or FcaTHN (see Fig. 5-3 and 5-4), expression increased from a ΔC_T value of 23.43 ± 0.43 (CON) to 5.97 ± 0.39 (FcaTHN), representing an order of magnitude higher than the level achieved in stably transduced CrFK.

This observation suggests that, although functional levels of tetherin are expressed, available tetherin molecules on the cell surface of infected cells become limited as viral particle production increases. The antiviral tethering activity of FcaTHN is thus overcome by high viral loads and viral replication is able to continue. The findings presented here are also consistent with the presence of trapped virions at the cell surface promoting cell-to-cell spread, a phenomenon proposed recently for HIV-1 (Jolly et al., 2010).

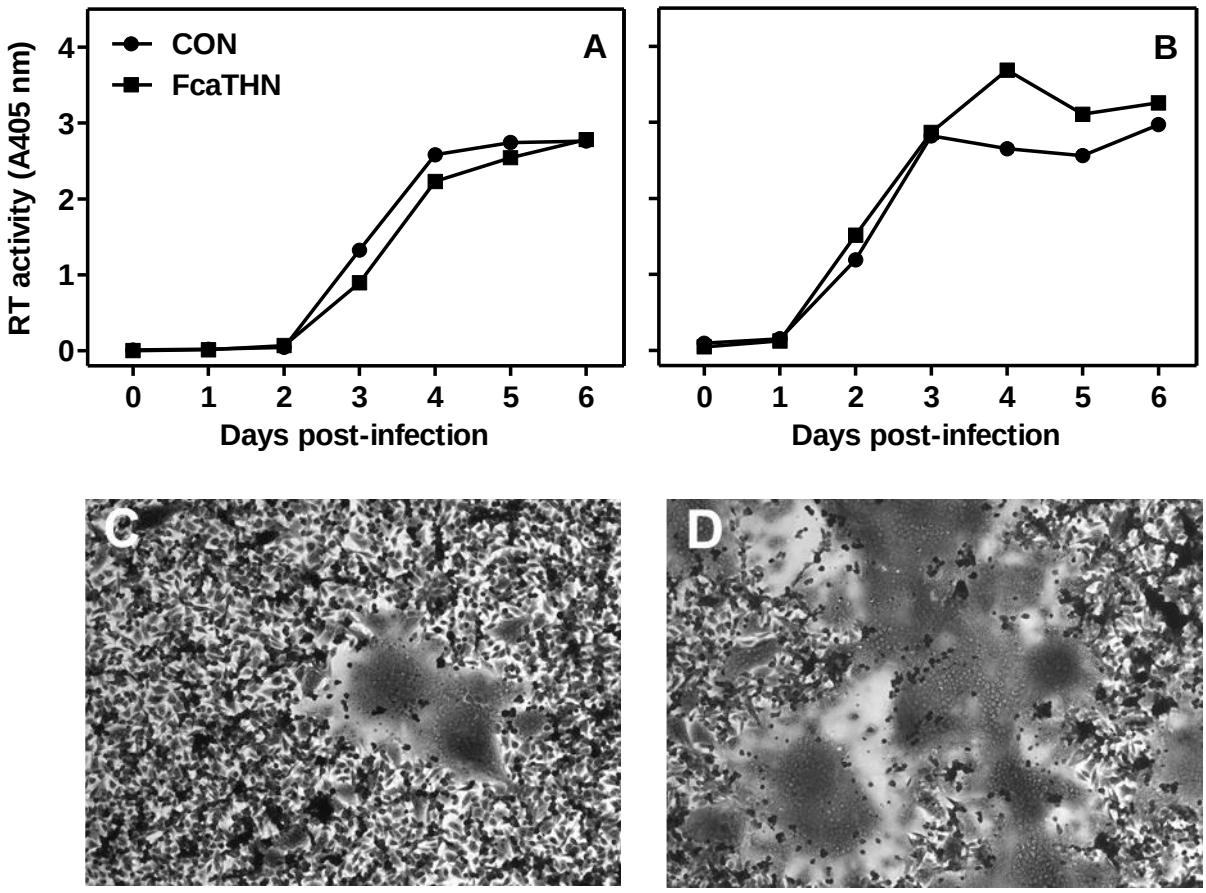
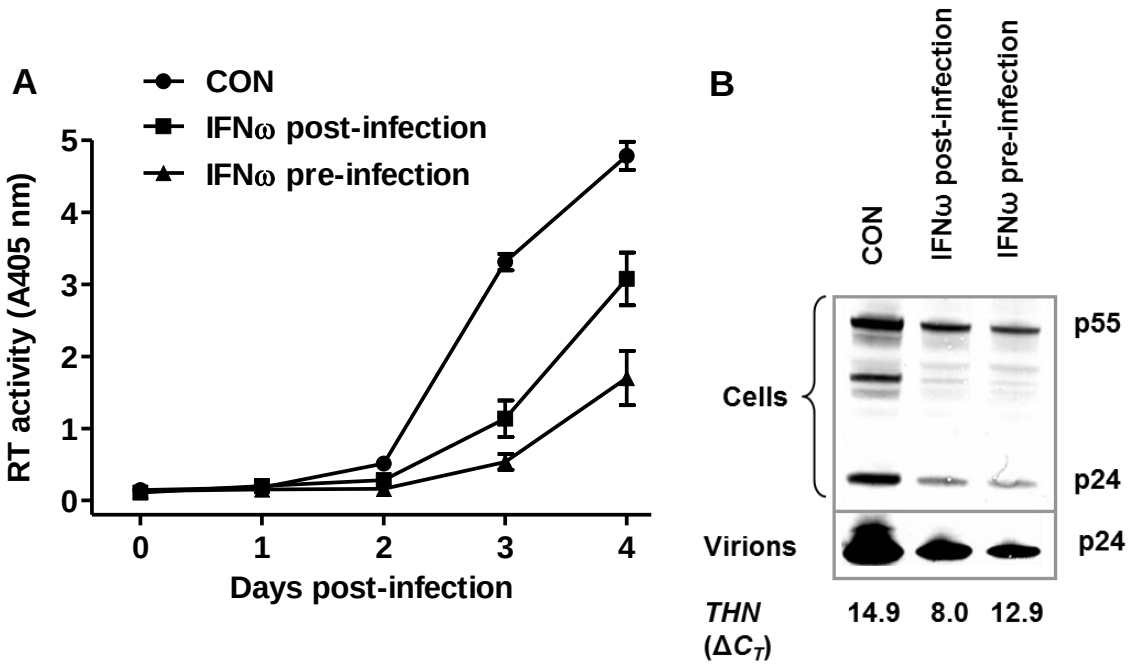


Figure 5-5 Effect of stable expression of FcaTHN on replication of cell culture-adapted strains of FIV. CrFK cells were stably transduced with an empty retroviral vector (CON) or a retroviral vector bearing domestic cat tetherin (FcaTHN). Cells were infected with the cell culture-adapted, CD134-independent strains of FIV-Fca (F14; A) or FIV-Pco (CoLV; B), and virus replication was monitored by RT assay of the supernatant (means [*n*=2]). (C,D) Representative fields (light microscopy) displaying enhanced syncytium formation in tetherin-expressing cells (D) following FIV-Fca F14 infection compared with control cells (C). Cell monolayers were fixed and stained six days post-infection with 1.0% methylene blue-0.2% basic fuchsin in methanol.

5.3.5 Effect of interferon-ω on FIV production and syncytium formation

Enhanced syncytium formation following increased tetherin expression would be detrimental to the host and counterintuitive with respect to the idea of a role

for tetherin in restricting viral growth. We therefore asked whether such a scenario would occur following upregulation of endogenously expressed tetherin by interferon. CrFK cells were either left untreated or were treated with IFN-ω either 24 hours prior to infection or 24 hours post-infection with FIV-Fca strain F14. As expected, treatment of cells with IFN-ω either pre- or post-infection suppressed virus production (Fig. 5-6), which is consistent with the pleiotropic antiviral activities of type I interferons inhibiting viral growth.



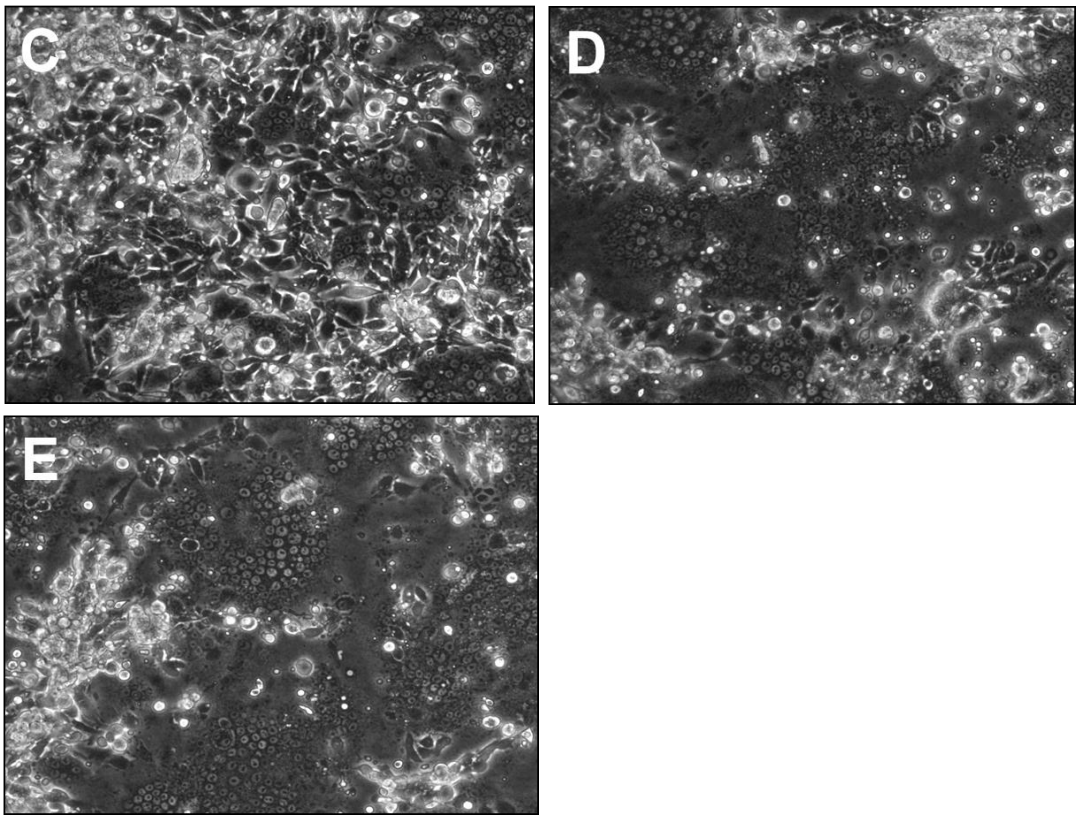


Figure 5-6 Effect of IFN-ω on FIV production and syncytium formation. (A) CrFK cells were infected with FIV-Fca F14, and virus production was monitored by RT assays. Cells were left untreated (CON), were pre-treated with 10^3 IU/ml IFN-ω 24 hours prior to infection or 24 hours post-infection. (B) FIV-Fca Gag expression (p24) was monitored in lysates of infected cells and in culture supernatants at day four post-infection by immunoblotting using mouse anti-FIV p24 antibody (vpg50). Induction of tetherin expression was confirmed by RT-qPCR (*THN* ΔC_T). (C-E) Representative images (phase-contrast microscopy) of syncytium formation at day three post-infection. (C) Untreated and FIV-Fca F14-infected CrFK cells. (D) FIV-Fca F14-infected CrFK cells treated with IFN-ω 24 hours post-infection. (E) CrFK cells treated with IFN-ω 24 hours pre-infection with FIV-Fca F14.

As expected, the reduction in virus replication was more pronounced in cells pre-treated with IFN-ω compared to cells stimulated post-infection (Fig. 5-6A). IFN-ω led to a decrease in viral production within cells, which also resulted in a reduction of particle release into the cell culture supernatant (Fig. 5-6B). As qRT-PCR analysis of tetherin transcripts confirmed that tetherin expression had been induced following IFN stimulation (Fig. 5-6B), syncytium formation in the presence of IFN-ω was examined. Indeed, a marked enhancement of syncytium formation was noted in F14-infected CrFK cells treated with IFN-ω (Fig. 5-6D,E), irrespective of whether the interferon was added before or after viral infection. These data suggest that the results observed in cells stably expressing tetherin are recapitulated in cells in which tetherin expression is induced by IFN-ω.

5.3.6 Effect of FcaTHN on replication of a primary, CD134-dependent strain of FIV

It is theoretically possible that the stably modified cell lines expressed only low levels of tetherin on the cell surface, which were insufficient to block viral release and consequently spreading FIV infection. To control for this possibility, we took advantage of the fact that the primary FIV-Fca isolate GL8 cannot replicate in CrFK cells in the absence of the FIV receptor CD134. Transfection of CrFK cells with GL8 therefore does not lead to a spreading infection but allows particle release into the cell culture supernatant to occur.

CrFK cells or CrFK cells stably expressing FcaTHN were transfected with either FIV-Fca GL8 (Fig. 5-7A) or FIV-Fca F14 as control (Fig. 5-7B). In CrFK cells expressing FcaTHN, release of GL8 was blocked, whereas viral particles were released in control cells (Fig. 5-7A), which is consistent with tetherin preventing virus release. As reported above, spreading infection of CD134-independent FIV-Fca F14 was not inhibited by tetherin expression in these cells (Fig. 5-7B), but was accompanied by enhanced syncytium formation in FcaTHN-expressing cells (Fig. 5-7D) as compared to control cells (Fig. 5-7C).

To test the effect of FcaTHN on spreading infection of CD134-dependent FIV-Fca GL8, control or FcaTHN-expressing CrFK cells were transduced with a vector expressing CD134. CD134 expression rendered CrFK cells permissive for GL8 infection, whether or not tetherin was co-expressed (Fig. 5-7E). Tetherin expression reduced replication by about two-fold, leading to a delay of approximately one day in achieving peak virus loads, but tetherin was unable to block replication in a manner analogous to that seen with feTRIMCyp (Fig. 3-8). This effect was confirmed by analysis of the degree of syncytium formation at the end of the experiment (day 6). While the absolute numbers of syncytia in the GL8-infected cells were similar, the syncytia in the tetherin-expressing cells were smaller (Fig. 5-7F), which is consistent with a partial retardation of viral growth.

Thus, two contrasting outcomes of spreading FIV infection in the presence of FcaTHN were observed. Whereas GL8 replication was partially retarded and syncytium formation was proportionally reduced, F14 infection was unaffected and syncytium formation was enhanced.

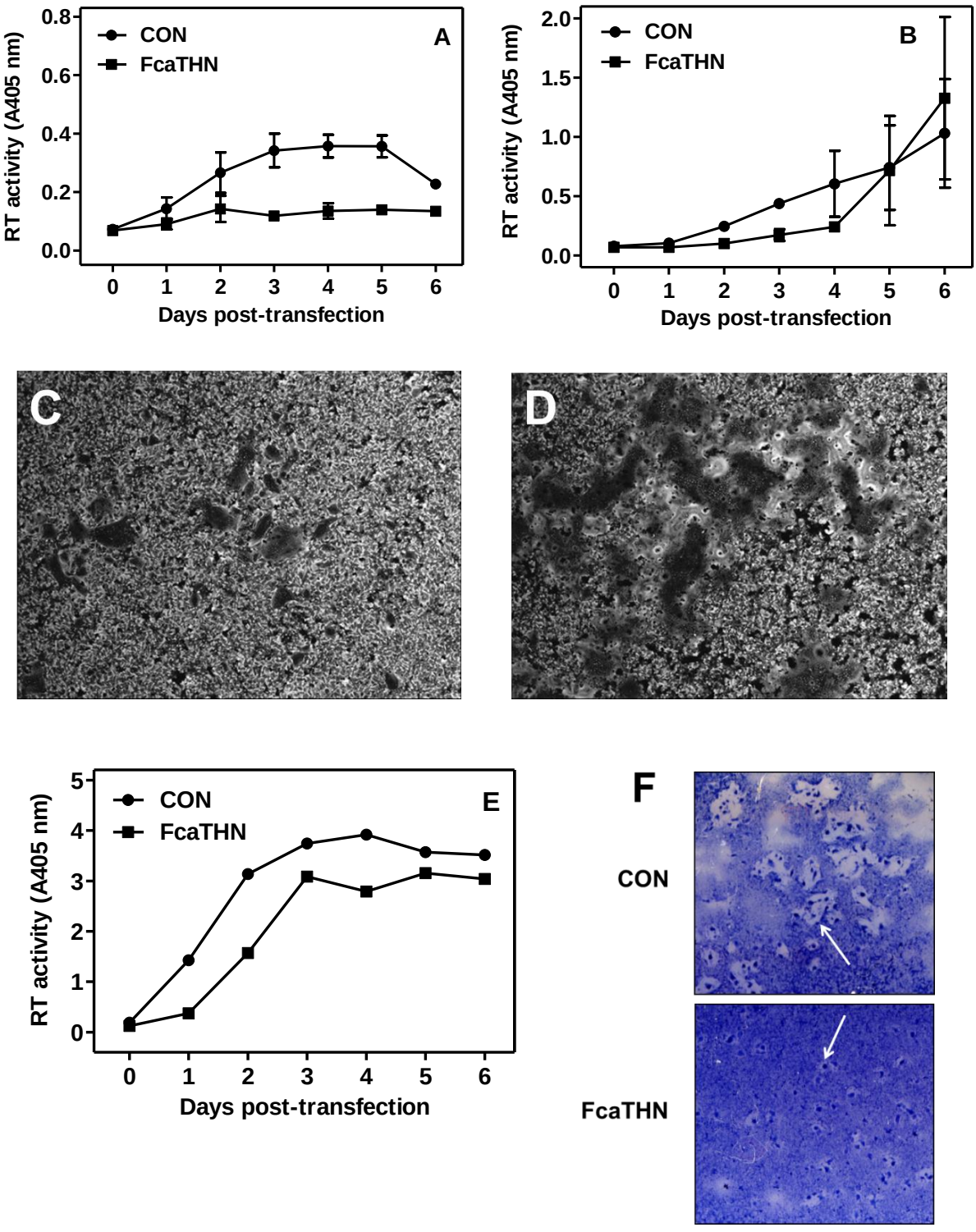


Figure 5-7 Effect of stable expression of FcaTHN on release and spread of a primary strain of FIV. CrFK cells stably transduced with an empty retroviral vector (CON) or a retroviral vector bearing domestic cat tetherin (FcaTHN) were transfected with molecular clones of FIV-Fca GL8 (A) or FIV-Fca F14 (B). Virus release into the cell culture supernatant was monitored by RT assays (means ± S.E. [*n*=3]). (C,D) Representative fields (light microscopy) displaying enhanced

syncytium formation in tetherin-expressing cells (D) following FIV-Fca F14 transfection compared with control cells (C). Cell monolayers were fixed and stained six days post-infection with 1.0% methylene blue-0.2% basic fuchsin in methanol. (E,F) Control or FcaTHN-expressing CrFK cells were transduced with CD134-pDsRed2 and infected with FIV-Fca GL8. Virus release was monitored by RT assays of supernatant (means [*n*=2]) (E), while syncytium formation was examined macroscopically following staining (as described above). Representative syncytia are indicated (white arrows) (F).

5.3.7 Intracellular localisation of FcaTHN

In order to examine the cellular localisation of FIV and domestic cat tetherin (FcaTHN) in infected cells, CrFK cells were stably transduced with a retroviral vector expressing FcaTHN tagged in the extracellular domain with an internal HA tag. Confocal analysis of Env expression on intact (non-permeabilised) cells demonstrated enrichment of Env staining along the entire perimeter of syncytia (Fig. 5-8A). While tetherin expression was detected with the majority of single cells, where expression appeared punctate (Fig. 5-8B), expression on the perimeter of syncytia followed a pattern similar to that seen with Env, and in several regions, marked overlap of Env and tetherin was noted (Fig. 5-8C). In permeabilised cells, Env expression was diffuse throughout the cytoplasm of the cells (Fig. 5-8D) whereas tetherin expression appeared largely punctate (Fig. 5-8E). It was notable that, in some Env-expressing cells, tetherin appeared to be concentrated at the periphery of the cell (Fig. 5-8F, lower left), whereas in cells lacking Env, tetherin appeared to be perinuclear (Fig. 5-8E, top centre). Occasionally, syncytia contained regions of co-localisation of tetherin and Env, possibly indicating the presence of intracellular bodies rich in both Env and tetherin (Fig. 5-8F, inset).

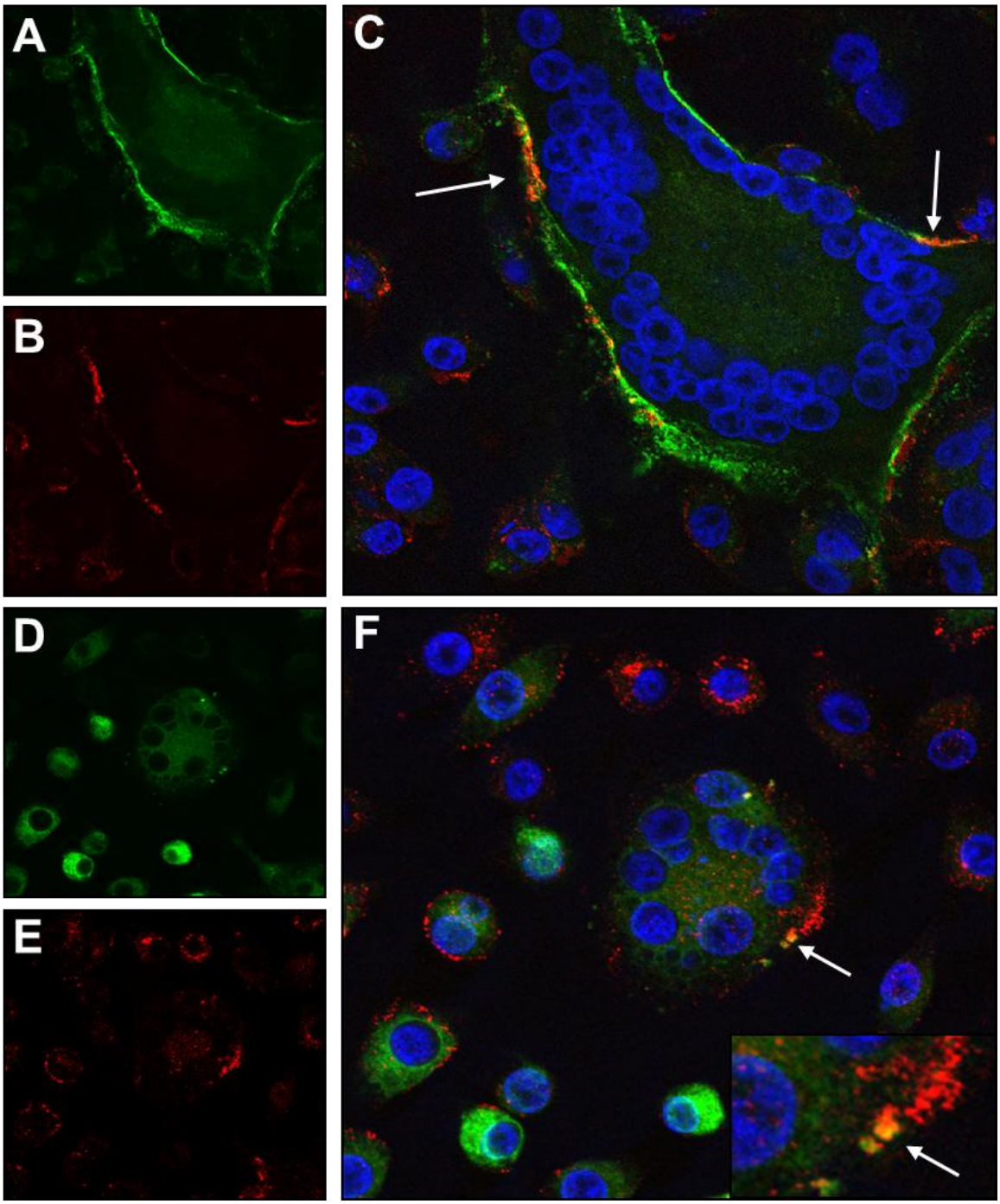


Figure 5-8 Intracellular localisation of FcaTHN. CrFK cells stably expressing FcaTHN incorporating an internal HA tag (FcaTHN-HA) were infected with FIV-Fca F14, fixed and stained for expression of FIV-Fca Env (FITC [green]) (A,D) or FcaTHN-HA (Alexa Fluor 594 [red]) (B,E). In merged images (C,F), nuclei were also visualised with DAPI (blue). Cells were stained either in intact form (A-C) or following detergent permeabilisation (D-F). Images are representative of at least five separate fields; arrows indicate regions where Env and tetherin expression coincided.

5.3.8 Electron microscopic analysis of tetherin-mediated FIV particle retention

FIV-Fca F14-infected tetherin-expressing CrFK cells were analysed by electron microscopy for evidence of the retention of virus particles on the cell surface (electron microscopy was performed with the help of David Bhella, University of Glasgow). Infected cells produced abundant microvilli in the presence or absence of tetherin (Fig. 5-9A) with morphologies similar to that described previously for HeLa cells (Fisher and Cooper, 1967). While 0.1-μm-diameter particles were noted in the regions rich in microvilli, the resolution of the CrFK-derived images made it difficult to distinguish conclusively electron-dense virus-like particles from a cross-section through the tip of a microvillus (Fig. 5-9A). Previous reports have described mouse mammary tumour virus budding from the tip of microvilli in CrFK cells (Lasfargues et al., 1976). However, aggregates of 0.1-μm-diameter particles aligned on the surface of cells were unique to the F14-infected tetherin-expressing cells (Fig. 5-9B,C), a feature that could not be identified in F14-infected control cells. The uniformity and alignment of these particles on the cell surface were consistent with the presence of trapped virions.

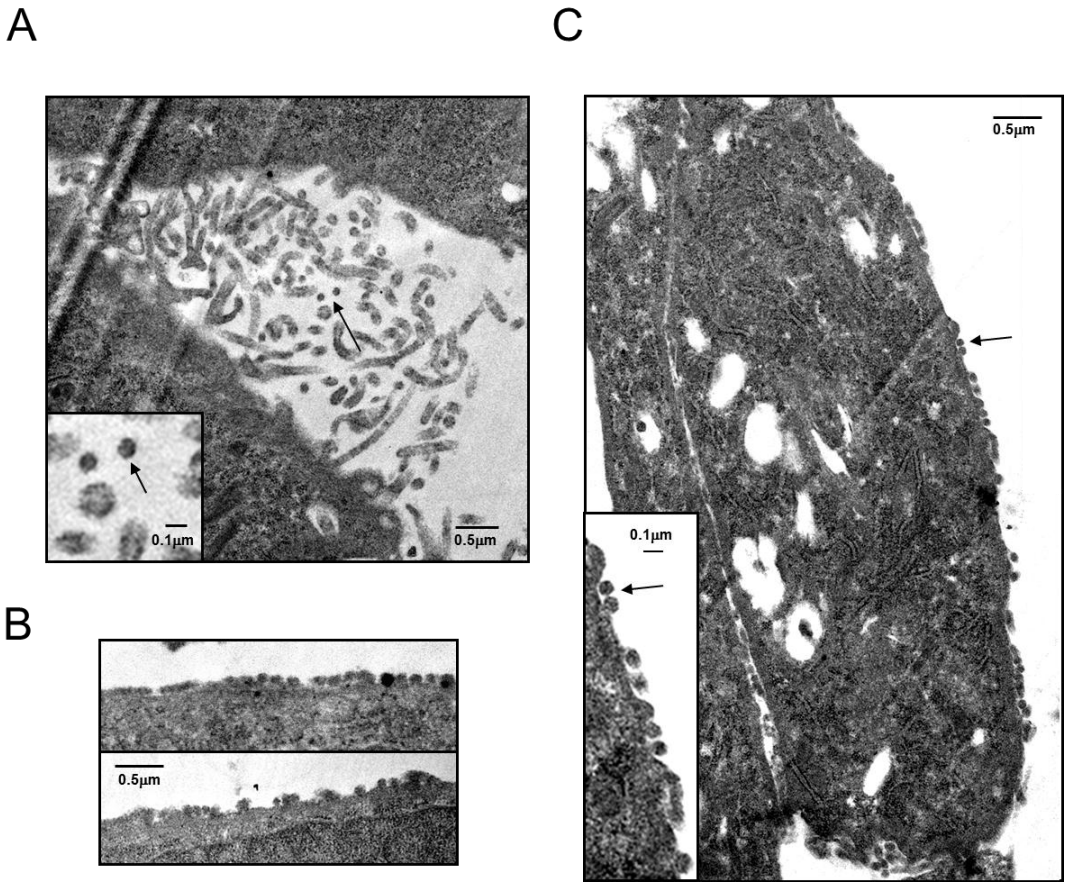


Figure 5-9 Electron microscopy of CrFK cells infected with FIV. (A) Microvillus-rich regions in FIV-Fca F14-infected control cells with occasional 0.1-µm-diameter particles (inset, two particles [arrow] adjacent to a cross-section of a microvillus for comparison). (B,C) Aggregates of 0.1-µm-diameter particles aligned on the surface of FcaTHN-expressing cells infected with F14. Arrows indicate 0.1-µm-diameter particles associated with the cell surface.

5.3.9 Activity spectrum of FcaTHN

It has been shown that human tetherin does not only limit the release of HIV-1 particles from infected cells but also blocks virion release from members of the alpharetrovirus, betaretrovirus, deltaretrovirus, spumaretrovirus, arenavirus (Lassa) and filovirus (Ebola, Marburg) families (Neil et al., 2008; Van Damme et al., 2008; Jouvenet et al., 2009; Kaletsky et al., 2009; Sakuma et al., 2009). Thus, human tetherin exhibits a broad spectrum activity and specificity.

The data presented here show that domestic cat tetherin (FcaTHN) prevents FIV, HIV-1 and SIV particle release from viral producer cells. However, expression of tetherin has little effect on FIV spread. In order to characterise its activity spectrum, control and tetherin-expressing cells were transfected with molecular clones of the gammaretroviruses FeLV-A and RD114 (Fig. 5-10) and viral growth in the presence or absence of FcaTHN was assessed by C-type RT assays. Furthermore, infections of the same cell lines with the spumaretrovirus FeFV (Fig. 5-11) were performed and FeFV growth was monitored by titration onto FeFAB reporter cells and LacZ staining.

Firstly, CrFK (Fig. 5-10A) and FEA (Fig. 5-10B) cells stably transduced with an empty vector control or a vector encoding FcaTHN were transfected with a FeLV-A molecular clone. As observed for FIV-Fca GL8 (Fig. 5-7E), in the presence of tetherin, FeLV-A growth was initially reduced by up to two-fold resulting in a growth delay of about 24 hours in CrFK cells and 36 hours in FEA cells compared to the control. However, peak viral loads were comparable in the presence and absence of FcaTHN, indicating that FcaTHN is unable to exert a potent block to FeLV-A spread. Next, the stably transduced CrFK (Fig. 5-10C) and FEA (Fig. 5-10D) cells were transfected with a molecular clone of the endogenous feline

gammaretrovirus RD114. RD114 did not grow in FEA cells but, notably, tetherin expression prevented RD114 replication in CrFK cells completely.

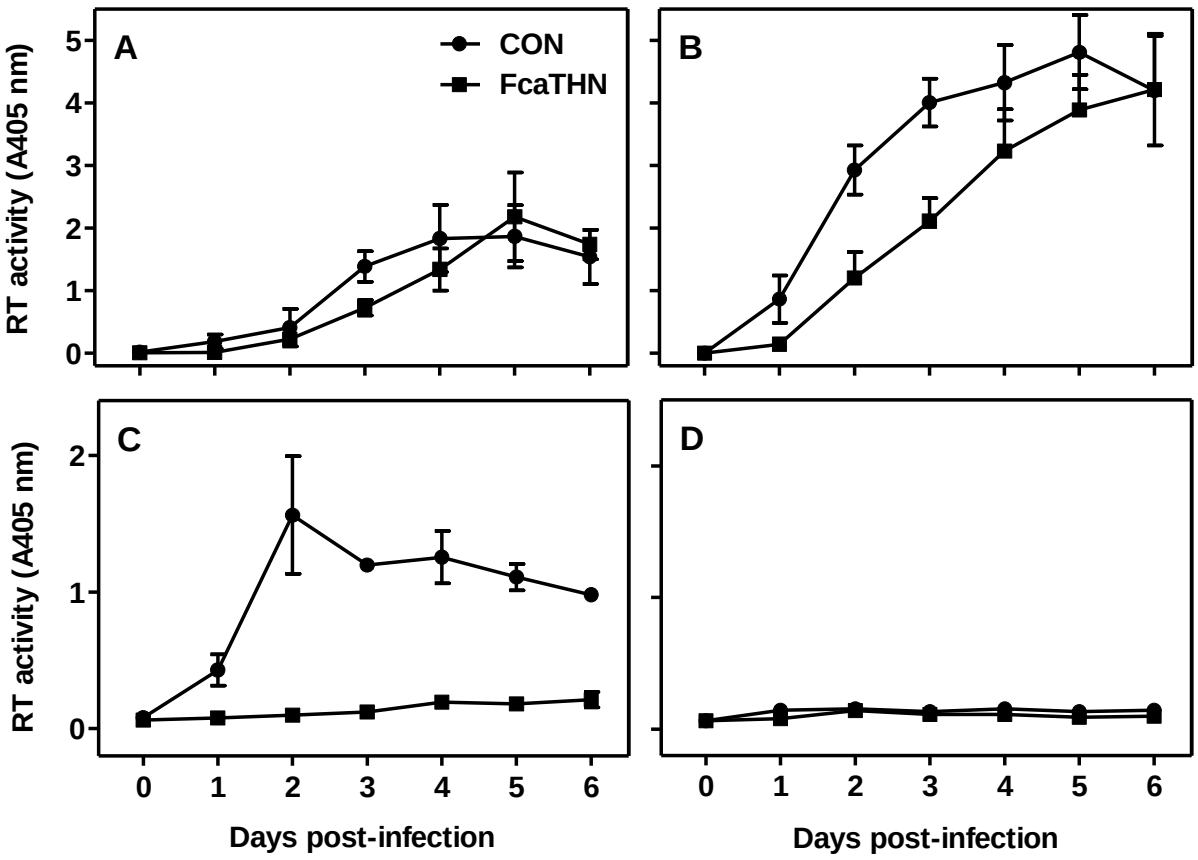


Figure 5-10 Effect of stable expression of FcaTHN on replication of gammaretroviruses. CrFK (A,C) or FEA (B,D) cells stably transduced with an empty retroviral vector (CON) or a retroviral vector bearing domestic cat tetherin (FcaTHN) were transfected with molecular clones of FeLV-A (A,B) or RD114 (C,D). Virus release into the cell culture supernatant was monitored by RT assays (means ± S.E. [*n*=3]).

Finally, the ability of FcaTHN to prevent spumaretroviral release and spread was investigated (Fig. 5-11). FeFVΔ*bel*/*bet* (devoid of *bel*2 and *bet*) and a wild-type FeFV molecular clone were transfected into 293T cells in the presence or absence of FcaTHN (Fig. 5-11A). Whereas the amount of virus-encoding plasmid, that was transfected, was kept constant, the amount of FcaTHN-encoding plasmid was varied. Virus-containing supernatants were titrated onto FeFAB reporter cells, which express *lacZ* in the presence of FeFV Bel1 (Zemba et al., 2000). Bel1 is a transactivator of the FeFV LTR, which is stably integrated into

the genome of the reporter cells and drives expression of *lacZ*. Viral titres were determined by LacZ staining.

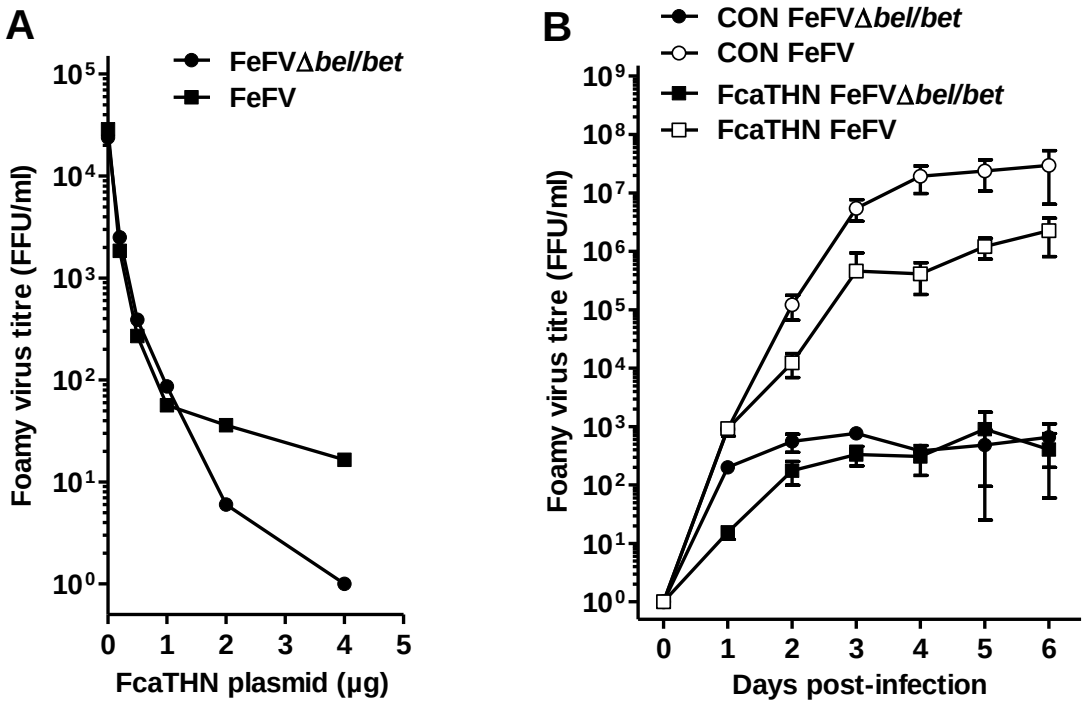


Figure 5-11 Effect of FcaTHN on release and spread of FeFV. (A) FeFVΔ*bel/bet* and FeFV wild-type particles were produced in 293T cells co-transfected with empty vector only (VR1012) or FcaTHN expression vector. Whereas the amount of transfected virus-encoding plasmid DNA was kept constant at 5 µg, the amount of FcaTHN expression plasmid DNA was varied. 48 hours post-transfection, cell supernatant was titrated onto FeFAB reporter cells and the activity of FcaTHN was assessed by measuring FeFV Bel1-driven *lacZ* expression in target cells by LacZ staining. Viral titres are expressed as mean focus forming units per ml cell supernatant (FFU/ml) from duplicate wells (*n*=1). (B) FeFV*bel/bet* and FeFV wild-type virus stocks were prepared by transfection of 293T cells with respective molecular clones. 100 µl of virus stocks were used to transduce CrFK cells stably expressing a control (CON) or domestic cat tetherin (FcaTHN). Viral growth was monitored by titration of cell supernatants onto FeFAB reporter cells and LacZ staining. Viral titres are expressed as mean FFU/ml ± S.E. from triplicate wells (*n*=1).

FcaTHN blocked FeFV particle release in a dose-dependent manner. When low amounts of FcaTHN plasmid DNA were co-transfected, FcaTHN prevented release of FeFVΔ*bel*/*bet* and wild-type FeFV to a comparable degree; however, at high amounts of FcaTHN wild-type FeFV titres remained up to one log higher than FeFVΔ*bel*/*bet* titres. The data points towards a role of FeFV Bet, which has previously been shown to prevent dimerisation of feline A3C proteins and their incorporation into viral particles (Lochelt et al., 2005), in FcaTHN antagonism. Therefore, single-cycle replication assays using FeFVΔ*bel*/*bet* in the presence of FcaTHN and FeFV Bet were performed. Bet expression did not rescue FeFVΔ*bel*/*bet* titres if tetherin was co-expressed (data not shown). So far, the product of *bel*2 has not been identified.

In order to investigate the activity of FcaTHN to block not only FeFV release but also spread, control CrFK cells or CrFK cells stably expressing FcaTHN were infected with FeFVΔ*bel*/*bet* or FeFV wild-type (Fig. 5-11B). Independent of tetherin expression, titres of FeFVΔ*bel*/*bet* were about 4.5 logs lower than that of FeFV. Indeed, the *bel*2/*bet* mutation has been shown to result in reduced particle release and possibly in reduced infectivity of viral particles (Alke et al., 2001). Tetherin expression reduced titres of FeFVΔ*bel*/*bet* in the first 72 hours of infection by a maximum of one log. At later time points, FcaTHN had no effect on FeFVΔ*bel*/*bet* replication. In contrast, in the presence of FcaTHN a constant decrease in wild-type FeFV titres of up to 1.5 logs was observed. Thus, domestic cat tetherin efficiently blocks FeFV particle release and partly limits FeFV spread.

5.3.10 Identification and characterisation of non-domestic cat tetherins

In addition to domestic cat tetherin (FcaTHN), non-domestic cat tetherin homologues were identified and characterised, and their antiviral activities were compared to that of FcaTHN. African lion tetherin (PleTHN) shares 95% nucleotide and 89% amino acid identity with FcaTHN (Fig. 5-12). Puma tetherin (PcoTHN) possesses 97% and 94% nucleotide and amino acid identity, respectively, with FcaTHN. Both PleTHN and PcoTHN adopt the same protein topology as described for FcaTHN (see Fig. 5-1). The double tyrosine endocytosis

motif at amino acid positions 6 and 8, the three cysteines involved in tetherin dimerisation at positions 59, 69 and 97 and the GPI anchor attachment site at serine 161 are conserved among felid tetherins.

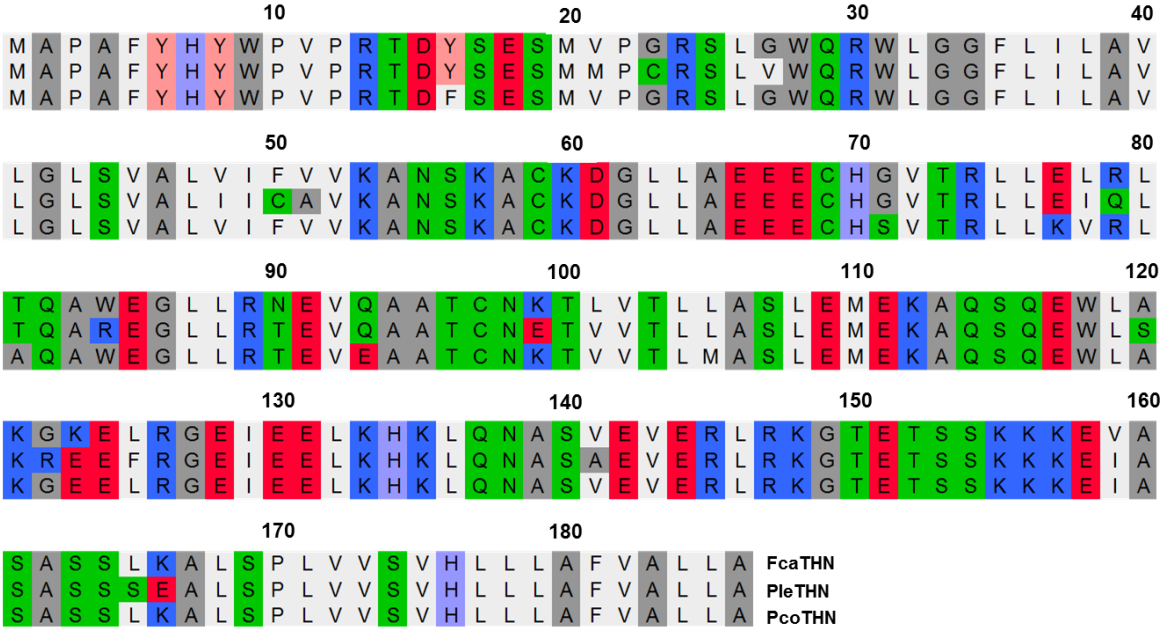


Figure 5-12 Identification of non-domestic cat homologues of tetherin. Alignment of the amino acid sequences of feline (FcaTHN), lion (PleTHN) and puma (PcoTHN) tetherins. Amino acid residues are colour-coded as follows: light gray, hydrophobic FILMPV; dark gray, amphiphilic AGW; green, hydrophilic neutral CNQST; light blue, slightly basic H; blue, basic KR; pink, slightly acidic Y; red, acidic DE.

To assess the potential of felid tetherins to limit viral particle release from infected cells, single-cycle replication assays were performed using FIVΔ*vif*(VSV-G) pseudotypes in the presence or absence of FcaTHN, PleTHN or PcoTHN (Fig. 5-13). Human tetherin (HsaTHN) was used as positive control for restriction of FIV particle release (Jouvenet et al., 2009). Pseudotypes were produced in the presence or absence of FIV Envs to determine whether they are able to counteract non-domestic cat tetherins. As shown for FcaTHN and HsaTHN, PleTHN and PcoTHN potently restricted FIVΔ*vif*(VSV-G) pseudotype release from 293T cells. Neither FIV-Fca GL8 Env, nor FIV-Ple B or FIV-Ple E Envs antagonised the felid tetherins or HsaTHN.

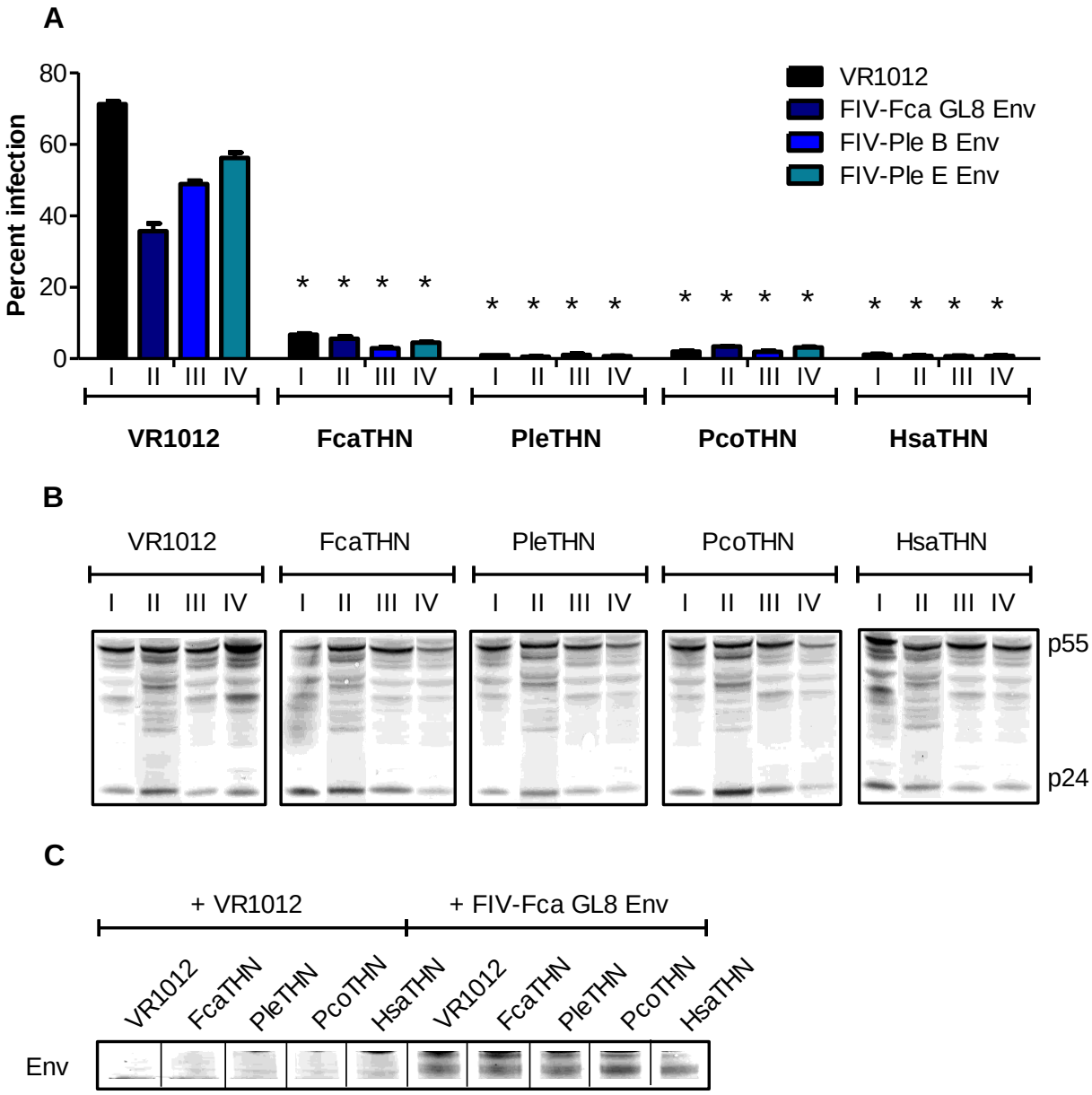


Figure 5-13 Felid tetherins restrict FIV particle release and are not overcome by FIV Envs. (A) VSV-G-pseudotyped, GFP-expressing FIVΔ*vif* particles were produced in 293T cells co-transfected with empty vector only (CON) or tetherin (FcaTHN, PleTHN, PcoTHN, HsaTHN) expression vectors. Assays were performed in the presence of empty vector only (CON) or FIV-Fca GL8 Env, FIV-Ple B Env or FIV-Ple E Env. Pseudotype-containing cell supernatant was then used to transduce 293T target cells and tetherin activity was assessed by measuring GFP-expression in target cells by flow cytometry. Single-cycle replication assays were performed in triplicate (*n*=3; results shown as mean ± S.E.). Statistically significant differences relative to the empty vector only control are indicated by asterisks (*, P<0.05, Dunnet's t-test). (B) FIV Gag (p24) and Gag

precursor polyprotein $Pr55^{Gag}$ (p55) expression in lysates of transfected 293T producer cells was assessed by immunoblotting using mouse anti-FIV p24 (vpg50) antibody. (C) FIV-Fca GL8 Env expression in lysates of transfected 293T producer cells was confirmed by immunoblotting using mouse anti-FIV Env (vpg71.2) antibody.

Because of the clear difference seen between the ability of FcaTHN to prevent FIV particle release and spread, CrFK and canine CLL cells stably expressing lion or puma tetherins were infected with different strains of FIV and virus growth was monitored (Fig. 5-14). CrFK and CLL cells stably transduced with a construct bearing a FcaTHN mutant lacking the double tyrosine motif (FcaTHN-Y6/8A) or HsaTHN were also included in the study.

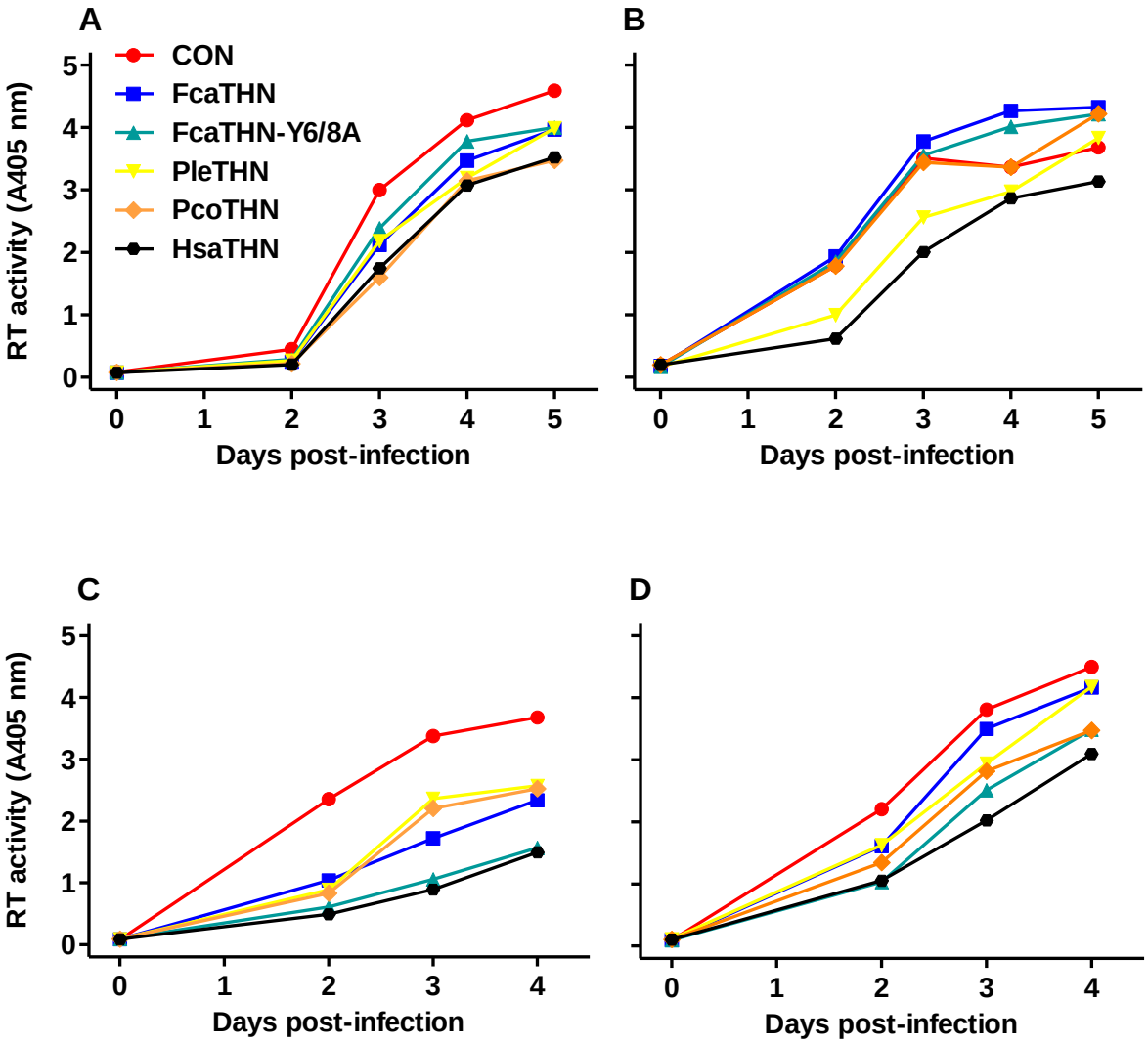
All CrFK cell lines were first infected with the cell culture-adapted, CD134-independent FIV strains FIV-Fca F14 (Fig. 5-14A) and FIV-Pco CoLV (Fig. 5-14B). In agreement with our findings for domestic cat tetherin (FcaTHN; see Fig. 5-5), none of the tetherins was able to prevent productive FIV replication, with PleTHN and HsaTHN being the most effective tetherins at limiting viral F14 and CoLV spread. CoLV growth was in fact enhanced by expression of FcaTHN, FcaTHN-Y6/8A and PcoTHN compared to the empty vector control (CON) (Fig.5-14B). This enhancement is especially noteworthy in the case of FcaTHN-Y6/8A because it indicates that prevention of tetherin endocytosis from the cell surface and hence increased tetherin surface expression did not increase the antiviral activity of FcaTHN.

Moreover, tetherin-expressing cells were transduced with a construct encoding feline CD134 and infected with the primary, CD134-dependent strains of FIV-Fca GL8 (Fig. 5-14C) and PPR (Fig. 5-14D). Interestingly, similar to our data obtained for FcaTHN (Fig. 5-7E) all tetherins modestly limited FIV-Fca GL8 growth. This effect was less pronounced for PPR. Expression of FcaTHN-Y6/8A and HsaTHN led to the highest reduction in viral growth.

Next, CrFK cells stably transduced with felid and human tetherins and CD134 were infected with FIV-Ple E, which, like FIV-Fca, uses CD134 as primary receptor (McEwan, 2009). FIV-Ple E growth was not affected by tetherin expression with the exception of HsaTHN, which reduced viral growth significantly (Fig.5-14E).

Lastly, FIV-Fca GL8 (Fig. 5-15F) and FIV-Ple E (Fig. 5-15G) growth was also assessed in canine CLL cells stably expressing tetherins and CD134. Again, none of the felid tetherins were able to prevent viral spread. These data indicate that the inability of felid tetherins to restrict FIV spreading replication was not limited to CrFK cells and seemed to be cell type-unspecific.

Infections of both CrFK and CLL cells with FIV-Ple B were attempted, but the virus grew only to very low titres, insufficient to draw reliable conclusions about the antiviral activities of tetherins.



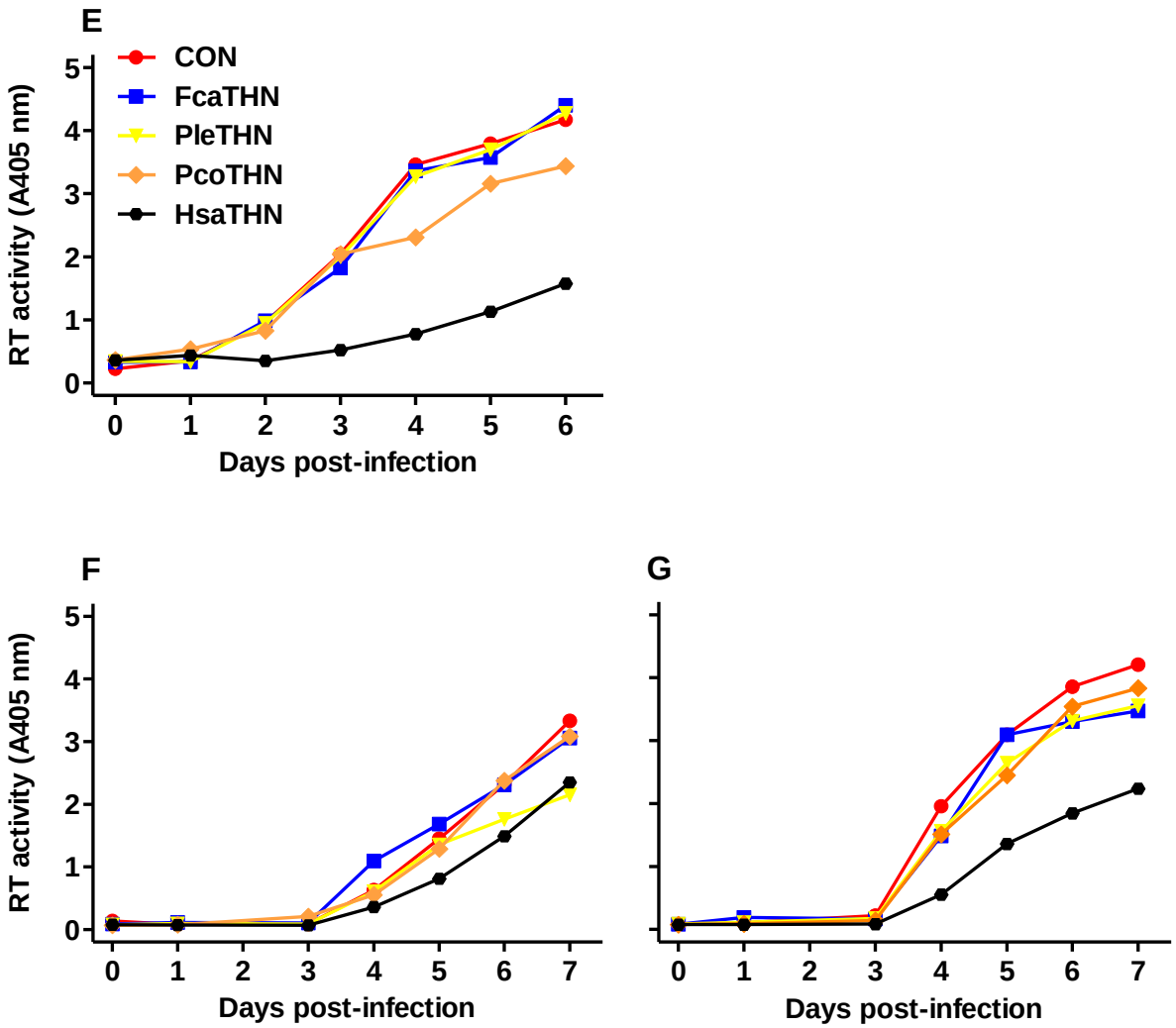


Figure 5-14 Effect of stable expression of felid tetherins on replication of cell culture-adapted and primary strains of FIV. CrFK cells (A-E) and canine CLL cells (F,G) were stably transduced with an empty retroviral vector (CON) or retroviral vectors bearing domestic cat (FcaTHN), lion (PleTHN), puma (PcoTHN) or human (HsaTHN) tetherin or a FcaTHN mutant lacking its N-terminal double tyrosine motif (FcaTHN-Y6/8A). To enable growth of CD134-dependent FIV strains, cells were further transduced with the construct CD134-pDsRed2 (C-G). Cells were infected with the cell culture-adapted, CD134-independent strains of FIV-Fca (F14; A) or FIV-Pco (CoLV; B), with the primary FIV-Fca strains GL8 (C,F) and PPR (D) or with FIV-Ple E (E,G), and virus replication was monitored by RT assay of the supernatant (means [*n*=2]).

5.3.11 Characterisation of the feline tetherin N-terminal region

In this study a domestic cat homologue of feline tetherin was identified and characterised. Its genome locus and the sequence of the corresponding cDNA were analysed. This cDNA was predicted to be 504 nt in length; however, the genomic sequence upstream of its start codon shared significant homology with known tetherins. Interestingly, the upstream sequence encoded for the double tyrosine motif involved in tetherin endocytosis from the cell surface and tetherin turn-over in cells, but was missing the thymidine in its start codon. Thus, a form of tetherin 561 nt in length was amplified from Mya-1 and CrFK cDNA with a forward primer containing an intact start codon. This tetherin (FcaTHN) was used throughout this study.

Recently, two groups have independently performed 5' RACE (rapid amplification of cDNA ends) PCR to characterise tetherin's 5' terminus and identified the shorter cDNA starting from the downstream, intact start codon as the coding sequence (Fukuma et al., 2011; Celestino et al., 2012). In agreement with our observations, Fukuma et al. (2011) found that domestic cat tetherin, which was termed FcaTHN-WT in this study, was indeed IFN-inducible and was able to restrict RD114 particle release. Surprisingly, Celestino et al. (2012) found that, although both forms of tetherin were able to dimerise and were correctly expressed on the cell surface, neither of them prevented FIV wild-type particle release in single-cycle replication assays. However, FIVΔ*env* particle release was blocked by the short form of tetherin and rescued by expression of FIV Env *in trans*, identifying FIV Env as an antagonist of domestic cat tetherin.

Due to the conflicting data on FIV Env antagonism of feline tetherin, 5'RACE PCR was performed. Moreover, the downstream start codon was verified for both domestic cat tetherin and lion and puma tetherins. The upstream sequence resembling the 5' end of known tetherin cDNAs formed part of the domestic cat tetherin transcript, but was lacking an ATG start codon. Then the domestic cat tetherin cDNA (501 nt), which encodes for a protein with a shorter cytoplasmic tail (wild-type domestic cat tetherin; FcaTHN-WT) than FcaTHN (Fig. 5-15), was amplified and cloned.

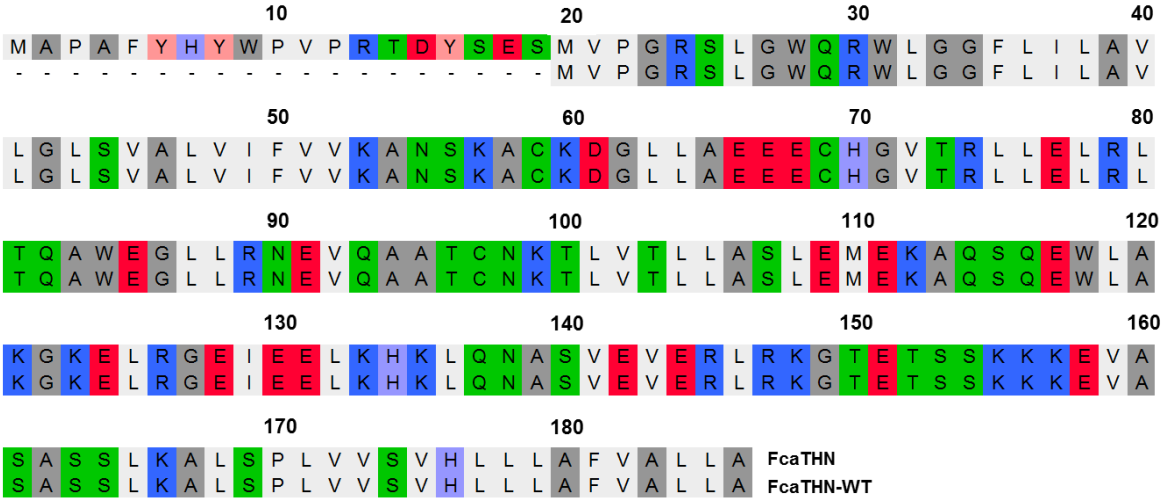


Figure 5-15 Amino acid sequence alignment of domestic cat tetherins. A 561 nt long domestic cat tetherin cDNA was amplified from Mya-1 and CrFK cell cDNA with a forward primer engineered to contain a functional start codon. This cDNA (FcaTHN) encodes for a tetherin with a cytoplasmic tail that resembles those of other known tetherins (see Fig. 5-1). A 501 nt long domestic cat tetherin cDNA was amplified from Mya-1 and CrFK cell cDNA that encodes for a tetherin with a shortened cytoplasmic tail (FcaTHN-WT). Amino acid residues are colour-coded as follows: light gray, hydrophobic FILMPV; dark gray, amphiphilic AGW; green, hydrophilic neutral CNQST; light blue, slightly basic H; blue, basic KR; pink, slightly acidic Y; red, acidic DE.

5.3.12 Comparison of FcaTHN and FcaTHN-WT antiviral activities

In order to identify possible differences in the abilities of FcaTHN and FcaTHN-WT to limit FIV particle release from producer cells, single-cycle replication assays were performed in the presence or absence of both proteins (Fig. 5-16). Both FcaTHN and FcaTHN-WT were able to block FIV(VSV-G) pseudotype release from 293T cells (Fig. 5-16A). In agreement with the findings of Celestino and co-workers, restriction by FcaTHN-WT was more pronounced than restriction by FcaTHN (Celestino et al., 2012).

Next, the replication assays were repeated in the presence of the FIV-Fca GL8, C8.1 (CrFK-adapted GL8), Petaluma clone F14 and 34TF10 Envs provided *in trans* or in the presence of KKS Env (Env of *in vivo* readapted FIV-Fca Petaluma) expressed from construct pEE14-Env. The latter construct is identical to that

used by Celestino et al. (2012). Expression of all Envs was confirmed by immunoblotting (Fig. 5-16B). In stark contrast to the observations by Celestino et al. (2012), neither FcaTHN nor FcaTHN-WT was counteracted by FIV Envs, confirming that FIV Env is not a functional tetherin antagonist.

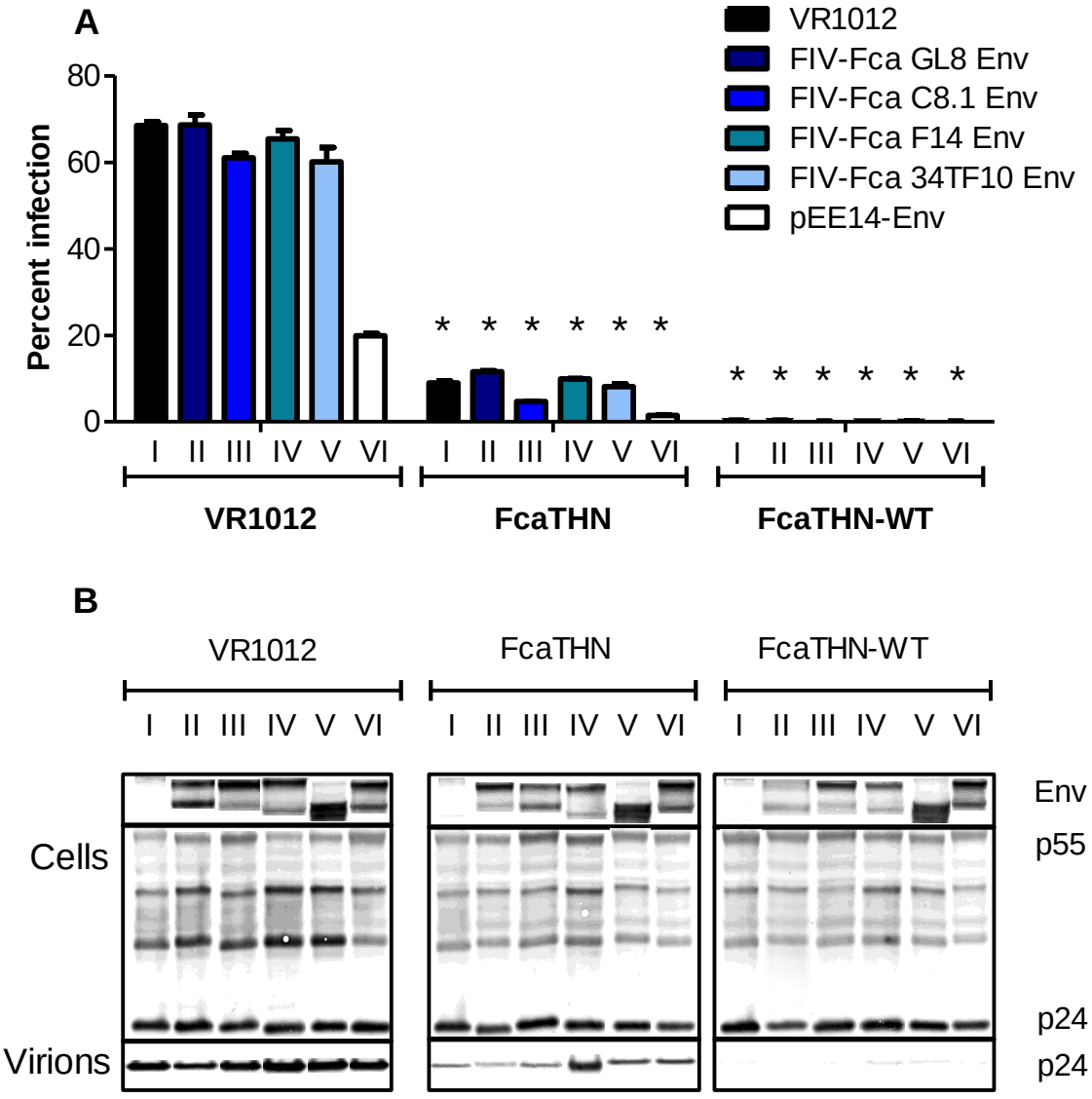


Figure 5-16 FcaTHN and FcaTHN-WT restrict FIV particle release and are not overcome by FIV Envs. (A) VSV-G-pseudotyped, GFP-expressing FIVΔ*vif* particles were produced in 293T cells co-transfected with empty vector only (CON) or tetherin (FcaTHN, FcaTHN-WT) expression vectors. Assays were performed in the presence of empty vector only (CON) or envelope glycoproteins (Envs) of FIV-Fca GL8, C8.1, Petaluma clones F14 and 34TF10 or Petaluma clone KKS Env expressed from construct pEE14-Env. Pseudotype-containing cell supernatant was then used to transduce 293T target cells and tetherin activity was assessed by measuring GFP-expression in target cells by flow cytometry. Single-cycle

replication assays were performed in triplicate (*n*=3; results shown as mean ± S.E.). Statistically significant differences relative to the empty vector only control are indicated by asterisks (*, P<0.05, Dunnet's t-test). (B) FIV Gag (p24) and Gag precursor polyprotein $Pr55^{Gag}$ (p55) expression in lysates and p24 expression in pelleted virions from supernatants of transfected 293T producer cells were assessed by immunoblotting using mouse anti-FIV p24 (vpg50) antibody. FIV Env expression in cell lysates was confirmed by immunoblotting using mouse anti-FIV Env (vpg71.2) antibody.

Furthermore, it was of interest to assess whether FcaTHN-WT, unlike FcaTHN, would be able to prevent FIV spreading infection. For this, CrFK cells stably expressing FcaTHN or FcaTHN-WT were infected with cell culture-adapted, CD134-independent strains of FIV-Fca (F14; Fig. 5-17A) and FIV-Pco (CoLV; Fig. 5-17B), and virus spread was monitored by reverse transcriptase activity assay of the culture supernatant. Importantly, ectopic expression of FcaTHN-WT did not inhibit virus production from FIV-infected CrFK cells. These data validate the findings obtained with FcaTHN and indicate that its prolonged N-terminus did not interfere with its ability to limit FIV spread (compare Fig. 5-5).

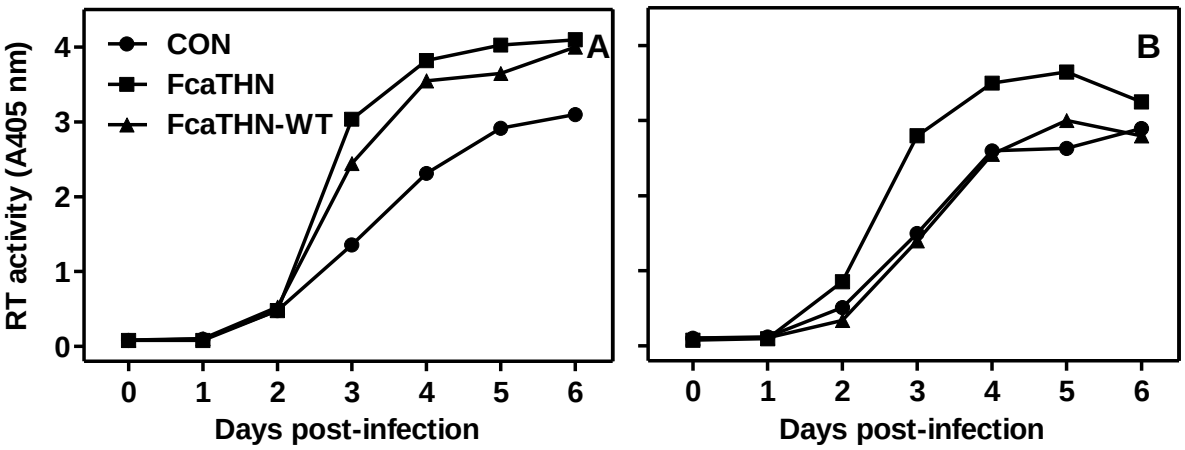


Figure 5-17 Effect of stable expression of FcaTHN and FcaTHN-WT on replication of cell culture-adapted strains of FIV. CrFK cells were stably transduced with an empty retroviral vector (CON) or a retroviral vector bearing either FcaTHN or FcaTHN-WT. Cells were infected with the cell culture-adapted, CD134-independent strains of FIV-Fca (F14; A) or FIV-Pco (CoLV; B), and virus replication was monitored by RT assay of the supernatant (means [*n*=2]).

5.4 Discussion

In addition to FIV, domestic cats harbour gammaretroviruses such as exogenous and endogenous feline leukaemia viruses (FeLVs) or RD114, as well as the spumaretrovirus feline foamy virus (FeFV) (Reeves and O'Brien, 1984). Whereas FIV is highly prevalent in felids with 21 of the 37 known species of felids harbouring antibodies to FIV (VandeWoude and Apetrei, 2006; Troyer et al., 2008), gammaretroviruses are, with the exception of sporadic cross-species transmission events, restricted to domestic cats (Benveniste and Todaro, 1975; Reeves and O'Brien, 1984). This suggests that they entered the *Felis* genus after it diverged from the main lineage of the *Felidae* approximately 6.2 MYA (Johnson et al., 2006). The high abundance of different retroviruses in cats necessitates the presence of potent and broadly specific anti-retroviral restriction factors. However, as discussed in previous chapters, feline TRIM5α is non-functional (McEwan et al., 2009) and feline A3 proteins are, at least partially, overcome by FIV Vif and FeFV Bet proteins (Lochelt et al., 2005; Munk et al., 2008; Stern et al., 2010; Zielonka et al., 2010).

In this study a homologue of tetherin has been identified in the domestic cat genome. Its coding sequence and predicted protein structure shared high similarity with known tetherins (Fig. 5-1). For the main part of this work, a protein termed FcaTHN with an N-terminus, which resembled that of other tetherins, was used. During the course of the project, however, it was discovered that wild-type domestic cat tetherin (FcaTHN-WT) most likely possesses a shorter cytoplasmic tail than FcaTHN. Consequently, the activities of both proteins to limit FIV release in single-cycle replication assays and to block FIV spreading infection were compared and were found to be similar.

Feline tetherin has been shown to be IFN-inducible in different feline cell lines, Mya-1 T lymphocytes and primary macrophages (Fig. 5-2). In agreement with studies on human tetherin, FcaTHN prevented FIV(VSV-G) and HIV-1(VSV-G) pseudotype release from infected cells in a dose-dependent manner (Fig. 5-3).

Primate lentiviruses have evolved diverse mechanisms to evade the antiviral activities of tetherin. Pandemic HIV-1 M strain isolates as well as SIVs of Mona monkeys (*Cercopithecus mona*), mustached monkeys (*C. cephus*) and greater spot-nosed monkeys (*C. nictitans*) (SIVmon, SIVmus and SIVgsn, respectively) encode Vpu proteins with potent anti-tetherin activities

(Courgnaud et al., 2003; Neil et al., 2007; Neil et al., 2008; Sauter et al., 2009; Yang et al., 2010). In contrast, SIV of chimpanzees (*Pan troglodytes*; SIVcpz), the nearest ancestor of HIV-1, uses Nef to counteract tetherin (Sauter et al., 2009), a feature it shares with SIVagm of African green monkeys (*Chlorocebus sabaeus*), SIVmac of rhesus macaques (*Macaca mulatta*), SIVsmm of sooty mangabeys (*Cercocebus atys*) and SIVsyk of Sykes' monkeys (*Cercopithecus albogularis*) (Jia et al., 2009; Sauter et al., 2009; Zhang et al., 2009a). Furthermore, HIV-2 and SIVagm.Tan (SIV of Tantalus monkeys [*Chlorocebus tantalus*]) Envs antagonise tetherins (Abada et al., 2005; Gupta et al., 2009b; Le Tortorec and Neil, 2009). This diversity of tetherin countermeasures points towards an ongoing struggle between hosts and lentiviruses. In search of a tetherin antagonist encoded in the FIV genome, FIV pseudotypes were produced in the presence of domestic cat tetherin (FcaTHN) and either FIV OrfA or FIV Envs (Fig. 5-4) neither of which rescued FIV particle release from producer cells.

In contrast to particle release, tetherin was unable to limit spreading infection of cell culture-adapted, CD134-independent strains of FIV (Fig. 5.5 and 5-14A). Indeed, tetherin expression promoted enhanced syncytium formation in CrFK cells by a mechanism that is likely to involve trapped virions on the cell surface. Rather than being released into the cell culture supernatant, their enrichment on the cell surface may trigger cell-to-cell spread, leading to syncytium formation. Interestingly, enhanced syncytium formation was also observed upon infection of IFN-treated CrFK cells with the CD134-independent F14 strain of FIV-Fca (Fig. 5-6). These data indicate that IFN stimulation upregulated tetherin expression favouring cell-to-cell rather than cell-free virus transmission and leading to syncytium formation.

There is evidence that suggests that Vpu-deficient HIV-1 can replicate in cell culture with the same kinetics as wild-type virus (Strebel et al., 1989; Terwilliger et al., 1989; Klimkait et al., 1990) by shifting from a cell-free to a cell-to-cell mode of transmission. As a consequence of this shift, viral replication was not inhibited, in direct contrast to the effect of tetherin on viral release. Furthermore, it has recently been shown that in T lymphocytes infected with Vpu-defective HIV-1, but not wild-type HIV-1, virus envelope glycoproteins accumulated on the cell surface due to the action of tetherin, promoting the formation of virological synapses (VS) and direct cell-to-cell spread of virions (Jolly et al., 2010). Depletion of tetherin using small interfering RNAs (siRNAs)

impaired cell-to-cell transmission (Jolly et al., 2010). Strikingly, prior to the discovery of tetherin, studies reported an increase in the size of syncytia in HeLa-CD4$^+$ cells transfected with HIV-1 devoid of Vpu (Klimkait et al., 1990), while enhanced cell-to-cell spread and the formation of giant syncytia in Jurkat cells infected with a mutant virus bearing a frameshift in *vpu* were noted (Gummuluru et al., 2000).

What is the significance of the enhanced syncytium formation seen with cell culture-adapted strains of FIV? Cell culture-adapted strains of FIV are similar to, and are frequently derived from, CD134-independent strains of FIV that arise during chronic infection *in vivo*. They may be considered to be analogous to the X4 strains of HIV-1 that emerge in the later stages of infection. Initially, these viruses were thought to be the consequence of cell culture adaptation, a selection for adaptation for growth in CD134-negative cell lines such as CrFK. Indeed, FIV-Fca Petaluma clones F14 and 34TF10, the prototypic molecular clones of FIV, bear mutations in their V3 loops consistent with CrFK adaptation and CD134-independent infection. However, it has also been shown that CD134-independent viral variants of primary isolates of FIV-Fca can arise, either during culture in CD134-positive peripheral blood mononuclear cells or *in vivo* in infected cats (Kraase et al., 2010). Thus, it appears that there is selective pressure *in vivo* for the generation of CD134-independent strains of virus. Such selective pressure may be exerted either by the humoural immune response (Willett et al., 2010) or indeed by tetherin, both of which impact on virus-receptor interaction and receptor usage. While IFN-induced tetherin expression may prevent particle release from infected cells, it may also, as an unintended consequence of its antiviral activity, enable efficient viral cell-to-cell spread.

It is noteworthy that infection of CrFK cells stably expressing FcaTHN with the primary, CD134-dependent GL8 strain of FIV-Fca led to a modest viral growth delay and a proportionate reduction in the size, but not number of syncytia that were formed (Fig. 5-7 and 5-14C).

How can the discrepancy between enhanced syncytium formation seen with F14 and reduced syncytium formation observed with GL8 be explained? It is likely that the switch to CD134-independence facilitates a more efficient cell-cell fusion mediated by use of the abundant co-receptor CXCR4 alone. Assuming that CD134-dependent strains of FIV rely more heavily on cell-free transmission than CD134-independent strains, it is notable that a mutant of FcaTHN that lacks

the N-terminal double tyrosine motif involved in tetherin recycling from the cell surface (FcaTHN-Y6/8A) reduced GL8 replication significantly (Fig. 5-14C). Deletion of this double tyrosine motif has been shown to increase tetherin surface expression (Hauser et al., 2010). It is therefore likely that FcaTHN-Y6/8 is more effective at inhibiting particle release than FcaTHN, which leads to the observed reduction in GL8 growth in FcaTHN-Y6/8-expressing cells.

In addition to FIV, the ability of tetherin to limit gammaretroviral and spumaretroviral release and spread was investigated (Fig. 5-10 and 5-11). FcaTHN was able to reduce FeLV-A growth modestly. Moreover, RD114 replication was completely blocked by tetherin. The high potency of tetherin to block RD114 spread may be facilitated by low viral loads or a strong dependence of RD114 on cell-free transmission. FeFV release was inhibited and its spreading replication in tetherin-expressing cells was reduced in comparison to control cells. Although RD114 and FeFV infect cats without causing apparent disease, their expression and replication could sequester available tetherin and limit its activity against FeLV and FIV, thereby potentially contributing to disease progression.

In order to compare tetherin sequences and antiviral activities within the *Felidae*, lion and puma tetherin homologues were amplified, cloned and expressed transiently in single-cycle replication assays or stably for use in productive retroviral infection studies. Both tetherins resemble FcaTHN with respect to sequence, predicted structure and their ability to restrict viral particle release and spread (Fig. 5-12 to 5-14).

The final part of this study focused on the characterisation of the 5' terminus of domestic cat tetherin. In recent publications, a tetherin cDNA encoding for a protein with a shortened N-terminal cytoplasmic tail (see Fig. 5-15) was identified as the naturally occurring homologue of domestic cat tetherin (Fukuma et al., 2011; Celestino et al., 2012). In disagreement with our data, Celestino et al. (2012) found that FIV-Fca Petaluma Env is a potent antagonist of cat tetherin. To rule out differences in activity and susceptibility to FIV Envs between the tetherin used in this study (FcaTHN) and the tetherin used by other groups (here called FcaTHN-WT), single-cycle replication assays were repeated in the presence or absence of different FIV-Fca Envs (Fig. 5-16). Under our experimental conditions we found no evidence of an activity of FIV Envs against either FcaTHN or FcaTHN-WT and were thus unable to confirm the key finding of

Celestino and co-workers (Celestino et al., 2012). Interestingly, FcaTHN-WT restricted FIV(VSV-G) pseudotype release more potently than FcaTHN. As discussed above, deletion of tetherin's N-terminal double tyrosine motif leads to enhanced tetherin surface expression (Hauser et al., 2010) and possibly to a more efficient restriction of particle release. However, despite its higher activity in single-cycle replication assays, FcaTHN-WT was, like FcaTHN, unable to prevent FIV spreading infection of cell culture-adapted, CD134-independent strains of FIV (Fig. 5-17). As discussed above, these viruses are capable of cell-to-cell spread, thereby overcoming the action of tetherin. It can be assumed that FcaTHN-WT will behave like the tetherin mutant FcaTHN-Y6/8A in retroviral growth assays with primary, CD134-dependent strains of FIV (see Fig. 5-14C,D).

In this study we showed that felid tetherins inhibit virus release but not spreading infection. Thus, there may be other roles for tetherin that do not involve the inhibition of viral growth, in particular that of a regulator of innate immunity. Tetherin has been identified as a specific marker of type I IFN-producing cells (IPCs) or plasmacytoid dendritic cells (pDCs) (Blasius et al., 2006). These cells circulate through the blood and infiltrate lymph nodes that drain sites of infection. While they were initially thought to have a primary role in immunomodulation resulting from the secretion of large amounts of type I interferon, subsequent studies indicated that pDCs were capable of presenting antigen to both $CD4^+$ and $CD8^+$ T cells and might have a potential role in antigen capture, processing, and presentation to T cells at sites of infection and in lymph nodes (reviewed in (Villadangos and Young, 2008)]).

Viruses trigger Toll-like receptor (TLR) 7/9-induced production of type I IFN and proinflammatory cytokines that activate antiviral intrinsic, innate and adaptive immune responses (Colonna et al., 2004; Liu, 2005). A chronic activation of pDCs and continuous IFN production caused by lentivirus infection leads to immune dysregulation, T cell anergy and apoptosis (Tompkins and Tompkins, 2008). Tetherin has been shown to interact with the orphan receptor immunoglobulin-like transcript 7 (ILT7), which is expressed exclusively on pDCs (Cao et al., 2009). This interaction induces a negative feedback loop on the production of type I IFN and proinflammatory cytokine production and adjusts the magnitude of immune activation upon viral infection (Cao et al., 2009).

By trapping virions on the cell surface tetherin may mark infected cells for destruction by antibody-dependent cellular cytotoxicity. Moreover, virions

tethered on the surface of dendritic cells may facilitate interactions with B cells bearing compatible surface immunoglobulin. Finally, tetherin is known to target virions for endocytosis and degradation, and internalised virions may be processed for antigen presentation in conjunction with MHC (Major histocompatibility complex) class II to $CD4^+$ T lymphocytes.

The elucidation of the role of feline tetherin in controlling replication of feline retroviruses *in vivo* and in regulating the antiviral immune response may lead to the development of promising new approaches for the prevention and treatment of viral infections.

6 Concluding remarks

Throughout the course of evolution, lentiviruses have invaded a broad range of mammalian species. However, their host spectrum is limited and successful viral transmission events are rare, even between closely related host species. Cross-species transmission is dependent on the compatibility between the virus and host factors that are either beneficial for viral replication (viral host dependency factors) or that protect the host from viral infection (restriction factors). Thus, transmission often requires adaptive changes in the viral genome to occur. Newly established lentiviral infections are characterised by high viral virulence leading to apparent pathology and rapid genetic diversification of the virus in the new host. During prolonged periods of host-virus co-evolution, the host-virus relationship eventually becomes balanced resulting in reduced viral virulence and/or improved host antiviral immune responses.

The HIV-1 M (main) group pandemic in the human population started in the early 20th century and was caused by a single successful zoonotic transmission event of SIVcpz of West African chimpanzees to humans (Gao et al., 1999; Korber et al., 2000). This transmission was, among others, facilitated by molecular and functional changes in the SIV accessory proteins Nef, Vif and Vpu (Mangeat et al., 2004; Schrofelbauer et al., 2004; Sauter et al., 2009), known restriction factor antagonists. Because of the as yet unbalanced host-virus relationship, HIV-1 infection in the susceptible human population leads to a fatal immunodeficiency syndrome. In contrast, natural SIV infections of non-human primates such as sooty mangabeys, African green monkeys, mandrills, chimpanzees and others are mostly benign due to host-virus adaptation (Silvestri et al., 2007; Pandrea et al., 2008).

The non-primate lentivirus FIV infects a wide range of non-domestic felids as well as domestic cats. Similar to lentivirus infections in primates, a difference in the pathogenicity of FIV infections can be observed between non-domestic cats and domestic cats. FIV has been endemic in non-domestic cats such as pumas or lions for hundreds of thousand or maybe several million years (Antunes et al., 2008; Pecon-Slattery et al., 2008b; Poss et al., 2008; Troyer et al., 2008) and infection is not usually associated with disease. However, FIV is a relatively recent introduction into domestic cats and infection leads to the development of feline AIDS.

Restriction factors form part of the mammalian antiviral intrinsic immunity, a form of cellular innate immunity that allows for a rapid restriction of viral infection and replication. It has been shown that the genes encoding the well-known restriction factors TRIM5α, APOBEC3G and tetherin evolve unusually fast (Sawyer et al., 2004; Sawyer et al., 2007; McNatt et al., 2009). Their diversifying selection is undoubtedly driven by frequent encounters with infectious agents such as viruses. Alterations in the specificity and potency of restriction factors, in return, necessitate adaptive changes in the virus genome to occur in order to ensure sufficiently high levels of viral replication.

Because of the apparent importance of restriction factors in limiting host susceptibility to viral infection and in preventing cross-species transmission events we hypothesised that differences in restriction factor activities may account for the differential disease outcome seen between FIV infections of non-domestic and domestic cats. These differences may have arisen as a consequence of long-term co-adaptation between non-domestic cat restriction factors and FIV restriction factor antagonists on one side and the lack of adaptation between restriction factors of domestic cats and their only recently acquired lentivirus FIV-Fca on the other side.

In this study felid restriction factors were characterised and their activities against FIV strains of non-domestic and domestic cats were compared. As previously shown, feline TRIM5α bears a deletion in its B30.2 (PRY/SPRY) domain essential for lentiviral capsid binding and hence does not contribute to FIV restriction in felids (McEwan et al., 2009). Here, a synthetic domestic cat TRIM5α-cyclophilin A fusion (feTRIMCyp) was generated based on naturally occurring simian TRIMCyps. FeTRIMCyp was able to potently restrict FIV(VSV-G) pseudotype and FIV productive infection, indicating that the feline TRIM5α RBCC (RING-B-box-coiled-coil) domain has indeed retained its functionality in the absence of capsid binding.

No significant differences in the activities of domestic cat, lion and puma APOBEC3C (A3C), APOBEC3H (A3H) and APOBEC3CH (A3CH) proteins against FIVΔ*vif*(VSV-G) were detected. However, especially feline A3CH proteins differed in their susceptibility to FIV Vifs. Whereas cat A3CH was potently counteracted by FIV-Fca Vif and FIV-Pco Vif, lion and puma A3CH proteins were largely resistant to these FIV Vifs. These differences in the susceptibility of A3 proteins

to FIV Vifs may be a result of disparate degrees of host-virus co-adaptation that has taken place between domestic or non-domestic cats and FIV.

Felid tetherins were shown to block FIV(VSV-G) pseudotype particle release from transfected cells efficiently. Unexpectedly, they were unable to restrict FIV spreading infection. In fact, we found that domestic cat tetherin enhanced syncytium formation in CrFK cells upon infection with the cell culture-adapted, CD134-independent FIV-Fca strain F14. These data indicate that tetherins may force lentiviruses to change their mode of replication from cell-free to cell-to-cell transmission in order to circumvent the block to particle release posed by tetherin. As an unintended consequence of restriction, viral propagation may thus be facilitated.

Differences in the feline APOBEC3-FIV Vif interactions seem to offer the most likely explanation for the sensitive phenotype of Mya-1 T cells and the restrictive phenotype of primary lion T lymphocytes with respect to FIV infection described in Section 1.3.4. It has been shown that A3 genes have been evolving under diversifying selection since long before the emergence of modern day retroviruses (Sawyer et al., 2004; Zhang and Webb, 2004). Here, we compared the coding sequences of A3C and A3H isoforms of African lions, in which FIV-Ple is endemic, and Asian lions, which are FIV sero-negative. In agreement with the ancient origin of cytidine deaminases, we found no evidence to suggest that FIV Vif poses a selective pressure on A3 genes. Thus, it appears more likely that lentiviral Vif proteins have to adapt to A3 proteins for the virus to be able to successfully replicate in a new host species. This adaptation may then lead to the observed host species-specificity of lentiviral Vifs (Mariani et al., 2003; Hatziioannou et al., 2006). Given that cat, lion and puma A3 proteins were equally potent at restricting FIVΔ*vif* infection in single-cycle replication assays and that FIV Vif was able to counteract cat A3H and A3CH, but not lion A3 proteins, it would be justified to propose a scenario in which A3 proteins generally possess a high anti-lentiviral activity in the absence of Vif. In contrast, Vif proteins may be very potent in a new host but over time adapt to allow some degree of restriction to occur. Incomplete neutralisation of A3 antiviral activity by Vif may be beneficial for the virus because a low, optimal level of viral genome mutation could enable viral escape from host immune defences (Simon et al., 2005; Harris, 2008; Jern et al., 2009; Kim et al., 2010). Notably, within human and non-human primate populations, some degree of variation exists in

A3 coding sequences, activity and expression levels that can modulate natural lentivirus infections (Jin et al., 2005; Biasin et al., 2007; Land et al., 2008; Ulenga et al., 2008; Zielonka et al., 2010; Cagliani et al., 2011; Compton et al., 2012). In principle, increased A3 activity and expression correlated with a lower viral load and preserved $CD4^+$ T cell numbers, but not necessarily with higher levels of A3-mediated hypermutations in proviral sequences *in vivo* (Jin et al., 2005; Pace et al., 2006; Land et al., 2008; Mussil et al., 2011; Kourteva et al., 2012). This indicates that deaminase-independent rather than deaminase-dependent A3 activities may be responsible for the inverse relationship between A3 expression levels and viral loads (Kourteva et al., 2012).

Conversely, there is natural variation in Vif function in infected individuals that can result in partial neutralisation of A3 protein activity, thereby promoting viral sequence diversification, or host control of viral replication (Alexander et al., 2002; Farrow et al., 2005; Simon et al., 2005). Interestingly, inactivating mutations in the form of premature stop codons in the Vif coding sequence occur at higher frequencies in individuals with lower viral loads (long-term non-progressors or patients receiving anti-retroviral treatment) than in patients with high viral loads, where viral replication selects for active *vif* alleles (Simon et al., 2005). Again, these observations point towards a direct correlation between A3 activity, viral loads and levels of *vif* hypermutation. There are also polymorphisms in non-hypermutated *vif* sequences, but these are rarely associated with a reduction in Vif function (Pace et al., 2006).

A recent study has investigated SIVagm.Ver (SIVagm of vervet monkeys [*Chlorocebus pygerythrus*]) *vif* evolution in individual infected sabaeus monkeys (*C. sabaeus*) harbouring either of two naturally occurring APOBEC3G (A3G) variants, Asp130 or Asp130His (Compton et al., 2012). Variant Asp130 was sensitive to all SIVagm Vifs, whereas mutant Asp130His was resistant to SIVagm.Ver and SIVagm.Tan (Compton et al., 2012). During the course of infection an adaptation of SIVagm.Ver to Vif-resistant A3G Asp130His was observed. The Vif adaptation enhanced viral infectivity significantly compared to an unadapted control. All virus isolates obtained from the sabaeus monkey expressing the A3G Asp130His variant shared a common mutation in Vif (Tyr46Cys) that is not present in naturally adapted SIVagm.Sab (SIVagm of sabaeus monkeys) Vif (Compton et al., 2012). These findings demonstrate that Vif evolution is driven by naturally occurring A3 variants. Vif adaptation can take

place very rapidly and can occur via different evolutionary routes (Compton et al., 2012). Overall, the A3-Vif relationship seems to be delicately balanced and may be a crucial determinant of the outcome of lentiviral infections.

Genome-wide screens have been performed to identify additional host factors that act as inhibitors of lentiviral replication. Particular attention has been paid to interferon-stimulated genes (ISGs). ISGs are genes whose expression is upregulated upon induction of the innate type I interferon response (see Section 1.5.1) and whose products exhibit antiviral, anti-proliferative or immunomodulatory properties. HIV-1 single-stranded RNA has been shown to induce IFN-α production in plasmacytoid dendritic cells (pDCs) (Heil et al., 2004; Beignon et al., 2005; Meier et al., 2007) and expression of several hundred ISGs in primary human PBMCs and macrophages (Woelk et al., 2004; Solis et al., 2011; Berg et al., 2012). However, the validation of ISG product antiviral activity has proven difficult due to their high number, their possibly selective expression in certain cell types and the fact that they may inhibit viral replication at different stages of the viral life cycle. In addition to TRIM5α, APOBEC3 proteins and tetherin, ISG products with confirmed anti-HIV-1 activity are IFITM (interferon-induced transmembrane) proteins, IRF1 (interferon regulatory factor 1), ISG15, PKR (protein kinase R) and TRIM22, among others. IFITM proteins interfere with HIV-1 cellular entry and affect viral Gag expression (Lu et al., 2011). IRF1 is a transcription factor of the interferon pathway that regulates expression of ISGs (Schoggins et al., 2011). ISG15 is an ubiquitin-like molecule that inhibits HIV-1 release by blocking the ubiquitination process that normally enhances HIV-1 Gag-mediated release of infectious virus (Okumura et al., 2006). PKR reduces HIV-1 production by suppressing protein translation (Nagai et al., 1997) and TRIM22 disrupts Gag assembly (Barr et al., 2008). The apparent wealth of (potentially yet undiscovered) ISGs with antiviral function raises the possibility that restriction factors other than APOBEC3 proteins are, at least in part, responsible for the inability of FIV to infect lion T lymphocytes.

So far, this study has taken into account two explanations for the observed difference in disease outcome following FIV infection of domestic and non-domestic cats, namely higher antiviral activities of non-domestic cat restriction factors compared to domestic cat restriction factors and reduced pathogenicity of non-domestic cat FIVs compared to FIV-Fca, both a consequence of prolonged periods of host-virus co-evolution in wild cats.

An intriguing third possible explanation has emerged in recent years and looks at differences in overall levels of immune activation between host species in which lentiviral infection is pathogenic and those in which it is non-pathogenic. A feature that distinguishes pathogenic lentiviral infections (HIV-1 infection of humans and FIV infection of domestic cats) from benign lentiviral infections (SIV infection of non-human primates) is the high level of chronic immune activation, which is positively associated with disease progression (Giorgi et al., 1999; Sousa et al., 2002; Silvestri, 2005). In contrast, limited immune activation, in particular during chronic stages of infection, seems to be the key factor protecting natural SIV hosts from simian AIDS (Silvestri, 2005).

The immune dysfunction seen in HIV-1 infected patients and FIV-infected cats comprises cytokine dysregulation (Clerici and Shearer, 1993), immunologic anergy and increased apoptosis of $CD4^+$ T lymphocytes (Miedema, 1992), and inappropriate activation of immune regulatory cells (Ascher and Sheppard, 1990) (see Section 1.2.3). Because the high state of T cell activation is associated with enhanced levels of viral replication, viral loads are usually high in treatment-naïve patients and an indicator for progression to AIDS. Exceptions are long-term non-progressors who show low levels of immune activation, preserved numbers of $CD4^+$ T cells and can control viral replication (Silvestri et al., 2007). Immune activation also induces ISGs and leads to the expression of high levels of restriction factors. However, high-level ISG transcription is positively correlated with viral burden (Sedaghat et al., 2008; Rotger et al., 2010), indicating that restriction factors cannot prevent viral replication *in vivo*. Notably, elevated tetherin expression in untreated patients was reported to contribute to disease progression (Mous et al., 2012). In agreement with our findings, tetherin was unable to control viral replication by cell-to-cell spread (Coleman et al., 2011). Indeed, accumulation of tethered virus particles on the cell surface may also enhance levels of immune activation (Tokarev et al., 2009).

Curiously, in natural, non-pathogenic lentiviral infections (SIV infections of sooty mangabeys [SIVsmm] and African green monkeys [SIVagm]) the levels of viral replication and $CD4^+$ T cell depletion during acute infection are similar to that of HIV-1 infection of humans (Gordon et al., 2007; Pandrea et al., 2007). In fact, one study reported that 10-15% of SIV-infected sooty mangabeys showed significant T cell depletion, but these animals remained completely asymptomatic for more than five years (Milush et al., 2007). In addition,

humoural and cellular immune responses are either similar or weaker in natural SIV infections (that excludes experimental infection of rhesus macaques with SIVmac) compared to pathogenic lentiviral infections (Hirsch, 2004; Dunham et al., 2006). However, T lymphocyte proliferation and turnover rates are normal, immune cell functions are preserved and levels of pro-inflammatory cytokines are not elevated (Kirchhoff, 2009). Thus, despite high viral loads and some degree of $CD4^+$ T cell loss, simian SIV hosts can prevent immune hyperactivation and its deleterious effects. It is important to note that type I interferon production was shown to be transiently induced in pDCs from sooty mangabeys and African green monkeys during early stages of infection (Diop et al., 2008). However, this interferon response quickly diminished and T cell activation levels and expression levels of ISGs returned to normal (Bosinger et al., 2009; Jacquelin et al., 2009).

FIV replicates to high titres in domestic cats and elevated viral loads are associated with progression to feline AIDS (Diehl et al., 1996; Goto et al., 2002). FIV-Pco proviral and plasma viral loads in naturally infected pumas were reported to be comparable to FIV-Fca viral loads in domestic cats (Blake et al., 2006). These findings indicate that the benign nature of FIV infection in pumas is not due to an efficient control of virus replication (Blake et al., 2006). Moreover, $CD4^+$ T cell depletion occurred in a proportion of FIV–infected lions and pumas, but was not associated with severe clinical disease (Roelke et al., 2006).

Immune activation is not only dependent on host factors but is also influenced by viral determinants. Specifically, the ability of viruses to reduce the level of T cell activation and depletion and to suppress innate immune responses may help the host to prevent chronic immune hyperactivation.

In this regard, the differential expression and activity of the two lentiviral proteins Vpu and Nef in HIV-1 and SIVsmm/SIVagm are of particular importance. As discussed in Section 1.5.6.3, HIV-1 belongs to a primate lentiviral lineage that has acquired *vpu* (Cohen et al., 1988; Courgnaud et al., 2003; Sauter et al., 2009) in addition to *nef*, which is present in all primate lentiviral lineages (Sauter et al., 2009). SIVsmm, SIVagm and other non-human primate lentiviruses use Nef to counteract tetherin (Jia et al., 2009; Sauter et al., 2009; Zhang et al., 2009a). The switch of pandemic HIV-1 group M strain from Nef to Vpu to antagonise tetherin was most likely forced by a deletion in the cytoplasmic domain of human tetherin that has been shown to confer resistance to Nef

(Sauter et al., 2009; Zhang et al., 2009a). In primate lentiviruses that do not encode *vpu*, Nef is multifunctional and acts as a regulator of host immune activation (Arien and Verhasselt, 2008; Kirchhoff et al., 2008; Kirchhoff, 2009). It down-regulates CD3, a component of the T cell receptor (TCR) complex (Schindler et al., 2006). This blocks activation, migration and apoptosis of infected T lymphocytes, and prevents formation of the immunological synapse between infected $CD4^+$ T lymphocytes and DCs or macrophages (Kirchhoff, 2009). Additionally, it reduces cell surface expression of CD4, CD28 and CXCR4 (T cell surface glycoproteins involved in T cell activation, the induction of cell proliferation and cytokine production), and down-modulates major histocompatibility complex class I (MHC I) (Bell et al., 2001; Swigut et al., 2001; Schindler et al., 2006; Kirchhoff, 2009).

The presence of a functional Nef is not always sufficient to prevent chronic immune activation. Whereas SIVsmm is non-pathogenic in sooty mangabeys, it is highly virulent in rhesus macaques (Silvestri, 2005). Hence it seems that the lack of host adaptation to a virus results in disease progression. In HIV-1 the acquisition of *vpu* has resulted in the loss of the protective function of Nef. In fact, HIV-1 Nef is a virulence factor (Kirchhoff et al., 1995) because it increases the responsiveness of infected T lymphocytes to TCR-mediated stimulation and enables effective viral transcription and production (Schrager and Marsh, 1999; Wang et al., 2000; Fortin et al., 2004; Fenard et al., 2005).

These data indicate that lentiviruses play an important role in the regulation of host immune responses. Although FIVs neither encode for *vpu* nor *nef*, there are differences in the levels of immune activation that FIVs of domestic and non-domestic cats trigger in their respective hosts. What causes these differences is incompletely understood and requires further investigation. Overall, the clinical outcome of lentiviral infections appears to be determined by a complex interplay between host and viral factors.

The observation that in natural SIV hosts efficient viral replication and depletion of infected $CD4^+$ T lymphocytes generally does not correlate with a negative disease outcome poses a serious challenge to any vaccine development efforts that aim at controlling viral replication. It seems that the disease outcome of newly established lentivirus infections is rather related to the nature and magnitude of the host response to infection (Silvestri et al., 2007). However, if viral replication was blocked early in infection, induction of chronic

immune hyperactivation could be prevented. Further research should thus focus on the development or improvement of therapies that avert immune hyperactivation and progression to immunodeficiency.

APPENDIX 1 List of Primers

Primer name	Primer sequence (5‘-3‘)
Chapter 3	
feT5a-1	GCGGATCCATGGCTTCTGAACTCCTGAAAT
feT5a-2	CACGATGGGGTTGACCATTTTTTTAAAGGCTTGTATTAT
fCypA R69 5’ Nde	AACATATGGTCAACCCCATCGTG
feCypA 3’ Mlu	AAACGCGTTTAGATTTGTCCACAGTCA
feCypA-1	ATAATACAAGCCTTTAAAAAAATGGTCAACCCCATCGTG
feCypA-2	GCGTCGACTTAGATTTGTCCACAGTCAGC
feCD134-Fwd	TTGGATCCAGGATGAGGGTGGTTGTGGGGGCT
feCD134-Rev	AAGAATTCTCAGATCTTGGCCAGGGTGGAGT
Chapter 4	
FeA3Ca F	ACTGGTCGACACCATGGAGCCCTGGCGCCCCAGCCCAAGAA ACCCAATGG
FeA3Ca R	ACAGCGGCCGCTCACCTAAGGATTTCTTGAAGCTCTGCAGCC
FeA3H F	ACTGGTCGACACCATGAATCCACTACAGGAAGTCATATTC
FeA3H R	ACAGCGGCCGCTCATTCAAGTTTCAAATTTCTGAAGTCATTC
LiA3C1 F	ACTGGTCGACACCATGGAGCCCTGGCGCCCCAGCCCAAGAA ACCCAATGC
LiA3C1 R	ACAGCGGCCGCTCACCTAAGGATTTCTTGAAGCTCTGCAGCC
LiA3H F	ACTGGTCGACACCATGAATCCACTACAAGAAGACATATTC
LiA3H R	ACAGCGGCCGCTCATTCAAGTTTCAAATTTCTGAAATCATTC

LiA3C2 F	ACTGGTCGACACCATGGAGCCCTGGCGCCCCAGCCCAAGAA ACCCAATGG
LiA3C-Ex1-Fwd	ATGGAGCCCTGGCGCCCCA
LiA3C-Ex2-Rev	CCGAAAAACTCCCCAGTCGCTGTCATT
LiA3C-Ex2-Fwd-1	CCTTCCATTTCCAGTTTCCA
LiA3C-Ex3-Rev-1	GTCACGGAACCAAAAGAGGA
LiA3C-Ex2-Fwd-2	TGGGCGGAAACTCTGTTATC
LiA3C-Ex3-Rev-2	ACGGATACTGGTTACGGAAC
LiA3C-Ex3-Rev-3	TATTCATCACGGCATGGATAC
LiA3C-Ex3-Rev-4	TCCTCATCACGGTACGGATAC
LiA3C-Ex3-Fwd-1	GTATCCATGCCGTGATGAATA
LiA3C-Ex1-Rev-1	TGGGGCGCCAGGGCTCCAT
LiA3C-Ex3-Fwd-2	GTATCCGTACCGTGATGAGGA
GL8 Vif F	ACTGGTCGACACCATGAGTGACGAAGATTGGCAGG
GL8 Vif Rev HA	ACAGCGGCCGCTCAAGCGTAATCTGGAACATCGTATGGGTA TAGTTTTCCCGACCATAACAG
LLV-E Vif F	ACTGGTCGACACCATGAGTGGTGAAGATATAAGTCAGG
LLV-E Vif Rev HA	ACAGCGGCCGCTCAAGCGTAATCTGGAACATCGTATGGGTA GCCACCTTTCCCTATTAAATATAG
PLV Vif F	ACTGGTCGACACCATGGCTTCAATCAGACAGACAGAACAG
PLV Vif Rev HA	ACAGCGGCCGCCTCAAGCGTAATCTGGAACATCGTATGGGT AGAATGATAATGTTAAATCCATCCA
FIV-Oma Vif F	ACTGGTCGACACCATGAGTGGTGAAGAGGATTGGCAGGTAA G
Oma3 Vif Rev HA	ACAGCGGCCGCTCAAGCGTAATCTGGAACATCGTATGGGTA ACTCTTCATCCGATATAACACTTCATAGGGTACA

GL8Mya Vif-Fwd	ACTGGTCGACATTGGCAGGTAAGTAGAAGACT
GL8Mya Vif-Rev	ACATGATCAGTGGGATTTGTAATGGGTCTGTAC
GL8MyaVifSeq-Fwd	GTGTCTTAGGAACTCACCTCCA
GL8MyaVifSeq-F_1	TGAGACTATAACAGGACCATTAG
GL8MyaVifSeq-Rev	ATCTCTAGTATGAAAGCTCCAT
GL8MVifMut-Fwd	CCTGAAGGGGATGAGTGATCGACATTGGCAGGTAAGTAG
GL8MVifMut-Rev	CTACTTACCTGCCAATGTCGATCACTCATCCCCTTCAGG
qPCR-FcA3C-Fwd	GGACAGGATAGATCCTAACACC
qPCR-FcA3C-Rev	CCACTTGGAAGCAGAGATAAC
qPCR-FeA3C-Pro	FAM-TTCCACTTTCCAAACCTGCTCTATGCTTCT-TAMRA
qPCR-FeA3H-Fwd	CAAGATCAAGGCACTGACGC
qPCR-FeLiA3H-Rev	ACAAACGCAACCAGTTCC
qPCR-FeLiA3H-Pr	FAM-CGAAATCATCTGCTATATCACATGGAGCCCCT-TAMRA
qPCR-FcA3CH-Fwd	TCCTGGCTGCAAAGCTTCAAG
qPCR-FcA3CH-Rev	TCTGGGCAAGAGGAAGGAAACC
qPCR-FeLiA3CH-P	FAM-CAGGAGGTGACAGAGCCTGGGATAAACACCAGA-TAMRA
qPCR-LiA3C-Fwd	GATCCTAAGACCTTCCATTTCC
qPCR-LiA3C-Rev	ACCTTGTTCCGAAAAACTCC
qPCR-LiA3C-Pro	FAM-GTTTCCAAACCTGCGCTACGCTTCT-TAMRA
qPCR-LiA3H-Fwd	CAAGATCAAGTCACTGACGC
rDNA 343F cat	CCATTCGAACGTCTGCCCTA

rDNA 409 R	TCACCCGTGGTCACCATG
rDNA 370P cat	FAM-CGATGGTAGTCGCCGTGCCTA-TAMRA
12S-UP-F	AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT
PAN-16S-Cy-R	CAGAACTCAGATCACGTAG
L12S-Cy-F	CTTAAGTGACTAGCCCCTA

Chapter 5

FcTHN-Fwd-2	ATCGGTCGACACCATGGCACCTGCTTTTTTACCAC
FcTHN-Rev-1	ACAGCGGCCGCTCAGGCCAGCAGAGCAACGAA
HuTHN-Fwd-1	ACTGGTCGACACCATGGCATCTACTTCGTAT
HuTHN-Rev-1	ACAGCGGCCGCTCACTGCAGCAGAGCGCTG
FcTHN-SP1	GAAGCCAACAGGGTTACCAA
FcTHN-SP2	GACACCGTGACACTCCTCCT
FcTHN-delCT-Fwd	ACTGGTCGACACCATGGTGCCAGGTCGGAGTCTT
FcTHN-Y8A-Fwd	GCACCTGCTTTTTACCACGCGTGGCCTGTGCCCAGGAC
FcTHN-Y8A-Rev	GTCCTGGGCACAGGCCACGCGTGGTAAAAAGCAGGTGC
FcTHN-Y6/8A-Fwd	GCACCTGCTTTTGCGCACGCGTGGCCTGTGCCCAGGAC
FcTHN-Y6/8A-Rev	GTCCTGGGCACAGGCCACGCGTGCGCAAAAGCAGGTGC
FcTHN-Fwd-3	ATCGGCGGCCGCATGGCACCTGCTTTTTACCAC
FcTHN-HA-Rev-1	ACGTAGTCTGGGACGTCGTATGGGTATTCCTTTTTCTTGCTCGAG
FcTHN-HA-Fwd-1	TACCCATACGACGTCCCAGACTACGTCGTCGCGTCTGCCAGCTCCTTGA

FcTHN-Rev-2	ACAGGATCCTCAGGCCAGCAGAGCAACGAAG
qPCR-FcTHN-Fwd	GAGAAGGCCCAGAGCCAGGAG
qPCR-FcTHN-Rev	GCAACGAAGGCCAGGAGCAG
qPCR-FcTHN-Pro	FAM-TGCAGAACGCTTCGGTGGAGGTGGAAAGACTGAGAAA-TAMRA
rDNA 343F cat	CCATTCGAACGTCTGCCCTA
rDNA 409 R	TCACCCGTGGTCACCATG
rDNA 370Pcat	FAM-CGATGGTAGTCGCCGTGCCTA-TAMRA
GL8 orf2 Fwd	ACTGGTCGACACCATGGAAGAAATAATAGTATTATTC
GL8 orf2 Rev	ACAGCGGCCGCCTAAGCAGTACGATGGATAATGTA
GL8 Env-Fwd	ACTGGTCGACACCATGAATGAAGAAGGGCCACTA
GL8 Env-Rev	ACAGCGGCCGCTCATTCCTCCTCTTTTTCAGA
PET Env-Fwd	ACTGGTCGACACCATGGCAGAAGGATTTGCAGCCA
LLV-E Env-Fwd	ACTGGTCGACACCATGGCAGAAGGAGGAAGAGTA
LLV-E Env-Rev	ACAGCGGCCGCTTAGGTATTAGACTCATCATTCAC
LLV-B Env-Fwd	ACTGGTCGACACCATGGCGGAAGGAGGAAGAGTA
LLV-B Env-Rev	ACAGCGGCCGCTCAAAGATCCTCATCAGACTCCCT

APPENDIX 2: List of Buffers and Solutions

Name	Composition
CHAPS lysis buffer	30 mM Tris-HCl, 150 mM NaCl, 1% CHAPS (w/v), pH 7.5
DNA loading dye (6x)	30% glycerol (v/v), 0.25% bromophenol blue (w/v), 0.25% xylene cyanol FF (w/v)
LB agar	10% Bacto-Tryptone (w/v), 5% yeast extract (w/v), 85 mM NaCl, 1.5% (w/v) agar, pH 7.5
LB broth	10% Bacto-Tryptone (w/v), 5% yeast extract (w/v), 85 mM NaCl, pH 7.5
PBS	137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4
PBS-BSA-azide	137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, 1% bovine serum albumin (w/v), 0.1% sodium azide (w/v), pH 7.4
SDS-PAGE loading dye	0.25 M Tris-HCl, 2.5% SDS (w/v), 10% glycerol (v/v), 1% β-mercaptoethanol (v/v), 0.02% bromophenol blue, pH 6.8
TBE buffer (10x)	890 mM Tris base, 890 mM Boric acid, 2mM EDTA
TE	10 mM Tris-HCl, 0.1 mM EDTA, pH 7.6

Publications arising from this work

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