Factors affecting replication and cross-species transmission of feline immunodeficiency virus

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Abstract

In order to successfully invade a new species, lentiviruses must overcome restriction factors, dominant blocks to replication, and be able to make use of available host factors such as entry receptors for replication. Human immunodeficiency virus (HIV-1) infection is blocked at a post-entry stage by rhesus macaque TRIM5α, an effect that is enhanced by host factor cyclophilin A (CypA), but largely evades restriction by the human TRIM5α variant. HIV-1 replication is also inhibited by simian APOBEC3G but is able to evade restriction by human APOBEC3G by inducing its degradation through expression of accessory protein Vif. Viral entry and tissue tropism are determined by an interaction of the viral Env glycoprotein with a cell surface receptor and a seven transmembrane domain co-receptor. The feline immunodeficiency virus (FIV) infects diverse felid species including the African lion, where infection has likely been endemic since at least the late Pleistocene, and the domestic cat, a more recent host. For domestic cat strains of FIV (FIV-Fca), entry is mediated by host proteins CD134 and CXCR4, but the identity of receptors in non-domestic strains of FIV is unknown.

This thesis demonstrates that two strains of FIV isolated from lions (FIV-Ple subtypes B and E) differ in their receptor tropism and that subtype E shares entry receptors with FIV-Fca. The findings suggest that alternative receptor usage is a strategy employed by FIV in this species and has implications for the disputed pathology and tissue tropism of infection in African lions. Next, we tested the hypothesis that species which have harboured lentiviral infection for a long time are better able to prevent viral replication than recent hosts. Whilst we found that TRIM5α is non-functional in all felid species tested, evidence of potent APOBEC3 activity was found and, in lion cells, potently restricts production of infectious FIV. Moreover, lion primary T-cells prevent

replication of diverse FIV strains and restrict primate lentiviruses at post-entry stages, suggesting that co-evolution with lentiviruses has driven the selection of broad-ranging restriction factors. Structural and biophysical analyses suggest that whilst FIV's interaction with CypA appears to be conserved in affinity with HIV-1, the interaction does not appear to be crucial for replication and, in the absence of restriction by TRIM5α, the role of the capsid-CypA interaction is discussed. Overall the study explains the permissivity of domestic cat cells to retroviral infection and identifies FIV infection of lions as an example of host adaptation driven by current lentiviral infection.

to Rosie

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List of Abbreviations

Abbreviation	*Meaning*
A3	APOBEC3
Ab	Antibody
agm	African green monkey (*Chlorocebus sabaeus*)
AIDS	Acquired immunodeficiency syndrome
APOBEC	Apolipoprotein B mRNA-editing catalytic polypeptide
BIV	Bovine immunodeficiency virus
bp	Base pairs
CA	Capsid
CAEV	Caprine arthritic encephalitis virus
CA^N	Capsid N-terminal domain
CD	Catalytic domain
cDNA	Complementary deoxyribonucleic acid
CHAPS	3-((3-Cholamidopropyl)dimethylammonio)-1-propanesulfonate
ConA	Concanavalin A
cpz	Chimpazee (*Pan troglodytes*)
CRD	Cysteine-rich domain
CRF	Circulating recombinant form
CsA	Cyclosporine A
CypA	Cyclophilin A
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucelic acid
DPI	Days post-infection
DTT	Dithiothreitol
DU	dUTPase (deoxyuracil triphosphate metabolising enzyme)
EDTA	Ethylenediaminetetraacetic acid
EIAV	Equine infectious anaemia virus
ELISA	Enzyme-linked immunosorbent assay
fe	Domestic cat (*Felis catus domesticus*)
FeLV	Feline leukaemia virus

FFV	Feline foamy virus
FIV	Feline immunodeficiency virus
FIV-Fca	Domestic cat (*Felis catus*) strains of FIV
FIV-Oma	Pallas cat (*Octocolobus manul*) strains of FIV
FIV-Pco	Puma (*Puma concolor*) strains of FIV
FIV-Ple	Lion (*Panthera leo*) strains of FIV
FPLC	Fast protein liquid chromatography
Fv	Friend virus susceptibility locus
FW	Far western blot
GFP	Green fluorescent protein
GL8	Glasgow 8 FIV strain
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HERV	Human endogenous retrovirus
HIV	Human immunodeficiency virus
hr	Hours
hu	Human
IgG	Immunoglobulin class G
IC_{50}	Half maximal inhibitory concentration
IL-2	Interleukin 2
IN	Integrase
IPTG	Isopropyl ß-D-1-thiogalactopyranoside
ITC	Isothermal titration calorimetry
IU	Infectious unit
Kd	Dissociation constant
li	Lion (*Panthera leo*)
LNP	Lion cell non-permissivity
LTR	Long terminal repeat
luc	Firefly luciferase
MA	Matrix
MG132	N-(benzyloxycarbonyl)leucinylleucinylleucinal
MHC	Major histocompatability complex
min	Minutes
MLV	Murine leukaemia virus
MoMLV	Moloney murine leukaemia virus

MVV	Maedi-visna virus
my	Million years
mya	Million years before present
Ni-NTA	Nickel nitrilotriacetic acid
NMR	Nuclear magnetic resonance
omk	Owl monkey (*Aotus trivirgatus*)
ORF	Open reading frame
PAGE	Poly acrylimide gel electrophoresis
PBMC	Peripheral blood mononuclear cell
PBS	Primer binding site
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PIC	Preintegration complex
Polybrene	Hexadimethrine bromide
PR	Protease
PTT	Polypurine tract
R5	HIV-1 strains using co-receptor CCR5
R5X4	HIV-1 strains using co-receptors CCR5 and CXCR4
rb	Rabbit (*Oryctolagus cuniculus*)
RBCC	RING, B-box, coilied coil domain of TRIM proteins
rh	Rhesus macaque (*Macaca mulatta*)
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RT	Reverse transcriptase
SDS	Sodium dodecyl sulphate
SIV	Simian immunodeficiency virus
sm	Sooty mangabey (*Cercocebus atys*)
ss	Single-stranded
SU	Subunit of Env, gp120
TCypA	Cyclophilin domain of TRIMCyp
TIP47	Tail-interacting protein 47
TM	Transmembrane domain of Env, gp41

TNC	Chicken tenascin-C
TRIM	Tripartite motif protein
TRIMCyp	TRIM5-CypA fusion
Tris	tris(hydroxymethyl)aminomethane
UV	Ultra-violet
v/v	Volume to volume ratio
VSV-G	Vesicular stomatitis virus G-glycoprotein
w/v	weight to volume ratio
X4	HIV-1 strains using co-receptor CXCR4
xg	Times gravitational force

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Chapter 1. Introduction

1.1 Preface

Investigating the mechanisms by which viruses appropriate host cell machinery to replicate themselves is crucial to our understanding of infections and their associated pathologies. In conjunction with studies of host immunity, such research can reveal the co-evolutionary battles between host and parasite and expose the interactions important for viral infection and suggest potential strategies of viral disruption. Lentiviruses are widespread mammalian pathogens and include the human and feline immunodeficiency viruses (HIV and FIV) which cause AIDS in their respective hosts. Humans and cat lentiviruses are proposed to have invaded their hosts after comparatively recent cross-species transmission events: within the past 150 years for humans and since the domestication event that gave rise to domestic cats. However, replication of these viruses is blocked in the cells of other species by a suite of proteins termed 'restriction factors.' Evolution of such restriction factors is proposed to have been driven by previous or ongoing encounters with pathogens. In this thesis, a comparison is made between infections in domestic cats and African lions, where FIV is thought to have been endemic for hundreds of thousands, if not millions of years. The study investigates the characteristics of FIV strains circulating in these species and asks what changes long-term lentiviral infection has brought about in host and pathogen with particular reference to restriction factors.

1.2 Retroviruses

Viruses are obligate intracellular parasites, reliant on host cell machinery to carry out the instructions encoded within their genomes. They must use the available host factors in a precise and controlled order to carry out each sequential stage of the viral lifecycle. Several replication strategies are available to viruses with respect to the nature of their genomes and the manner of their replication, all of which must ultimately allow the expression of viral proteins through messenger RNA (Baltimore 1971). The *Retroviridae* virus family is characterised by a replication strategy of positive sense RNA genome that is reverse transcribed by viral enzyme reverse transcriptase (RT) into a double stranded DNA copy which is inserted into the host genomic DNA from which mRNA transcripts and novel genomic RNA transcripts can be derived. Members of the family are broadly categorised into simple or complex retroviruses, with simple viruses generally bearing three open reading frames (ORFs), *gag* (which encodes the major structural proteins), *pol* (encoding RT and other proteins necessary for replication) and *env* (encoding the envelope glycoprotein responsible for entry into cells). The simple retroviruses are subdivided into genera *Alpha-, Beta-* and *Gammaretrovirus,* whist the complex viruses are divided into the *Spuma-* and *Lentivirus* genera both of which possess a number of smaller 'accessory' genes (Figure 1-1).

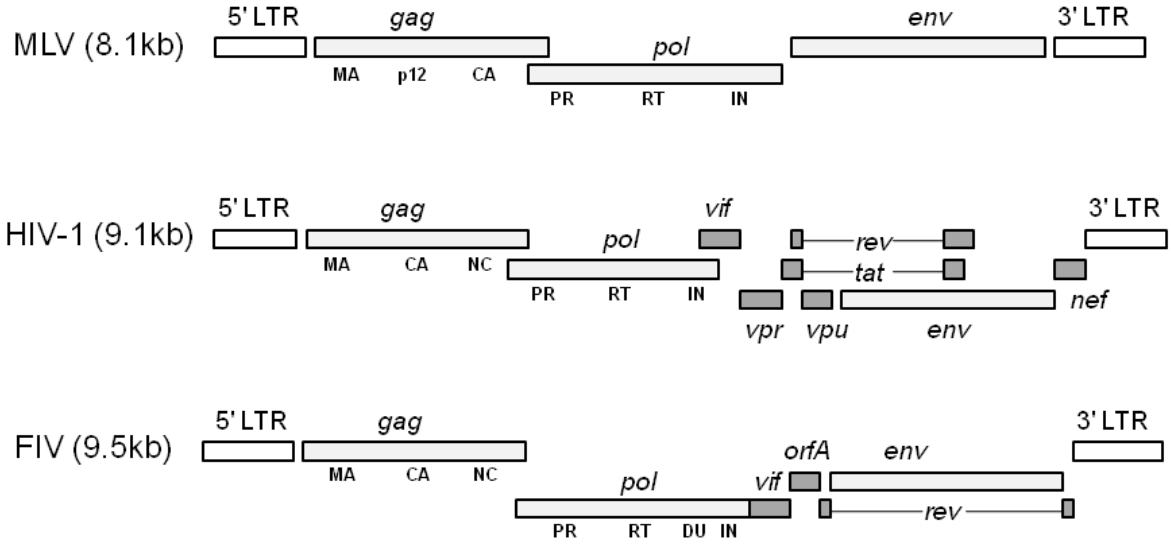


Figure 1-1 Genomic organisation of simple and complex retroviruses. Murine leukaemia virus (MLV) is a simple retrovirus belonging to the *Gammaretrovirus* genus and possesses three open reading frames, as well as 5' and 3' long terminal repeats (LTRs). Human immunodeficiency virus type I (HIV-1) belongs to the complex *Lentivirus* genus and has an additional suite of accessory genes. Some of these genes are shared by the feline immunodeficiency virus (FIV), which has an open reading frame termed *orfA* which encodes a protein which bears similarities to HIV-1 Vpr (Gemeniano et al. 2003). The gag-pol open reading frames are produced as polyproteins which are cleaved by viral protease into mature peptides matrix, MA; capsid, CA; nucleocapsid, NC; protease, PR; reverse transcriptase, RT; dUTPase, DU; and integrase, IN.

The first demonstration of the infectious property of lentiviruses was the transmission of equine anaemia in serum filtrate, now known to bear equine infectious anaemia virus (EIAV; Vallee and Carre 1904). In Icelandic sheep, the maedi visna disease was introduced in the mid-twentieth century and the spread of the disease was shown to be caused by a transmissible virus (Sigurdsson, 1953), later demonstrated to be the prototypic lentivirus, maedi visna virus (MVV; Haase and Varmus 1973). The viruses were given the prefix *lenti-* (*lentus* meaning slow in Latin) due to the long incubation period for pathological manifestations to occur. Symptoms of MVV infection include pulmonary infection and neurologic disease (*meadi,* laboured breathing, *visna,*

wasting in Icelandic) but arise at least two years after infection. This slow progression of disease is a common feature of the lentiviruses but tissue tropism and the associated pathology is diverse (Narayan and Clements 1989). The field rose to prominence as a research subject with the discovery of human immunodeficiency virus type I (HIV-1) which infects and depletes CD4+ T-cells, compromising cellular immunity and causing acquired immunodeficiency syndrome (AIDS) (Barre-Sinoussi et al. 1983; Gallo et al. 1984). The *Lentivirus* genus is known to infect diverse mammalian orders including cattle (bovine immunodeficiency virus; BIV), goats (caprine arthritic encephalitis virus; CAEV), horses (EIAV) and primates (simian and human immunodeficiency viruses; SIV and HIV). However, the discovery of feline immunodeficiency virus (FIV; Pedersen et al. 1987b) was followed by the finding that, uniquely amongst non-primate lentiviruses, it causes an AIDS-like syndrome characterised by recurrent gingivitis-stomatitis, cachexia, neuropathology and an increased incidence of tumour development (Torten et al. 1991; Ackley et al. 1990; Yamamoto et al. 1989; Sparger et al. 1989; Pedersen et al. 1987a; Hosie et al. 1989; Pedersen 1993; Callanan et al. 1992; Callanan et al. 1996). FIV infects an estimated 10% of domestic cats in the UK and species-specific strains of FIV have now been identified in at least 15 non-domestic felid and hyenid species (Brown et al. 1994; Troyer et al. 2004; Barr et al. 1995; Carpenter et al. 1996; Hofmann-Lehmann et al. 1996; Troyer et al. 2005). As well as being a major veterinary pathogen, FIV has attracted considerable attention as a model for investigating common principles of AIDS progression and in the development of vaccines for the prevention of lentiviral infection. On the 25th anniversary of the description of AIDS, a co-discoverer of HIV declared "we can safely say that we know as much about HIV as we do of any pathogen and about AIDS as we do any human disease" (Gallo 2006). Despite this, the virus is now responsible for a global pandemic infecting 40.3 million people, with at least two-thirds of cases occurring in the developing world

(World Health Organisation 2005). It is therefore of necessity and substantial value to investigate the mechanisms by which lentiviruses replicate and interact with their hosts.

The lentiviral lifecycle is superficially simple: particles invade the cell, reverse transcribe and integrate their genomes, translate their proteins and bud from the cell surface. However, the lifecycle is dependent on a vastly complex array of interactions with host proteins. For instance, in July 2009, the HIV-1 Human Protein Interaction Database lists 1,737 entries for Env alone (Fu et al. 2009), a number that will no doubt grow substantially. What's more, the information to carry out these functions is encoded by a genome of only 9,000-10,000 base pairs (bp), resulting in gene products that must carry out multiple functions. For the most part, host-virus interactions promote the viral lifecycle, but it is becoming increasingly clear that an additional suite of host factors that evolved specifically to interfere with retroviral replication must also be overcome for successful replication. These agents are known as 'restriction factors' and are considered as part of the innate immune system or as members of a separate 'intrinsic immune system' (Bieniasz 2004). This separate classification is justified by their constitutive expression in a wide range of host cells and their absence of requirement for pathogen stimulation or intercellular communication. To date, the main restriction factors of retroviral replication fall into three classes, the post-entry restriction factors (including murine *Fv*1 and mammalian TRIM5α), hypermutation-inducing restriction factors (the APOBEC3 family of proteins) and inhibitors of budding (tetherin). A variety of counter-measures have been devised on the part of the virus, including host-specific escape mutants and the generation of entire new genes that disrupt the activities of the restriction factors.

1.3 Cross-species transmission of lentiviruses in the primates and felids

In order to successfully infect a new species, a lentivirus requires a transmission event (zoonosis) and subsequent acquisition of adaptations that facilitate replication and transmission in the new host. There are currently 36 primate species known to be infected with lentiviruses, confined to Africa and Asia (Santiago et al. 2005). The causes of AIDS, HIV-1 and HIV-2, were introduced to humans as multiple cross-species transmissions from nonhuman primates. The chimpanzee (*Pan troglodytes*) simian immunodeficiency virus (SIVcpz) crossed into humans on at least three separate occasions during the 19th and 20th centuries, giving rise to HIV-1 groups M (major), O (outlier) and N (non-M, non-O) (Keele et al. 2006; Gao et al. 1999). HIV-2, originating from SIVsm of sooty mangabeys (*Cercocebus atys*), is thought to have been transmitted to humans on at least eight occasions, giving groups A to H (Damond et al. 2004). However, six of the groups have only been identified as individual infections, with only groups A and B showing evidence of human-human transmission (Damond et al. 2004) and even these groups remain largely confined to West Africa. Additionally, a virus has been identified in a Cameroonian woman that resembles SIV strains circulating in gorilla populations (Plantier et al. 2009). Thus it seems likely that lentiviruses have crossed from apes and monkeys into humans on numerous occasions, no doubt many or most of these unnoticed. Only one transmission event, from chimpanzees to humans around 1904 and giving rise to HIV-1 group M, has given rise to the global pandemic (Keele et al. 2006; Worobey et al. 2008). The comparative rarity of successful zoonosis events in the primates implies that species-specific blocks to replication (be they dominant host blocks or the inability to use host factors in the new host) must be overcome for successful transmission.

Of the 37 known species of felids, 21 species have been shown to harbour antibodies reactive to FIV and many of these species harbour viral sequences consistent with species-specific strains (VandeWoude and Apetrei 2006; Troyer et al. 2008). Unlike the primate lentiviruses, prevalence of FIV is worldwide, with infections occurring in both old and new world species. However, not all branches of felids are infected which suggests that virus colonised these hosts after the modern felid species radiation in the late Miocene ~10.8 million years ago[1] (mya; Johnson et al. 2006). The co-occurrence of diverse primate, ungulate and feline lentiviruses in Africa, and the paucity of FIV in Asia (only one endemic Asian species has tested FIV seropositive, the Mongolian species Pallas cat (Barr et al. 1995)), has lead to the hypothesis that FIV originated in Africa and was subsequently disseminated worldwide (Troyer et al. 2008; Pecon-Slattery et al. 2008b). Unlike most felid taxa, all six species belonging to the *Panthera* genus are infected with FIV, and in lions, six highly divergent viral subtypes (A to F) are circulating (Pecon-Slattery et al. 2008b). These observations are consistent with an ancient infection in this species. The range of modern lions is confined to the African continent and a relic population in the Gir forest of India. However, fossil evidence points to a much larger former distribution, including Asia and North America. The virus is proposed to have colonised the *Panthera* lineage before spreading to America through movement of species across the Bering Straits. Two opportunities for this dissemination have arisen: during periods of low sea level dated to 4.5 mya that allowed the movement of ocelot lineage members that subsequently diverged into cheetahs and pumas, and a more recent event in the late Pliocene/early Pleistocene (3.6 – 2.5 mya) that permitted the movement of lions into North America (Troyer et al. 2008; Johnson et al. 2006). The low sequence diversity of FIV in cheetahs points to a relatively recent introduction of FIV, probably since the extirpation of North American cheetah subspecies 10,000 years ago (Pecon-

[1] N.B. A phylogeny of the *Feliformia* is presented in Appendix 4.

Slattery et al. 2008b; Menotti-Raymond and O'Brien 1993), making it unlikely that the ocelot lineage was infected with lentivirus at the time of migration to North America. It is therefore possible that lions, where infection is more ancient, were vehicle for FIV dissemination into the new world during their migration in the late Pliocene/early Pleistocene. Analysis of mitochondrial and genomic DNA markers in African lions suggests episodes of population fragmentation occurred during the Pleistocene (1.8 mya - 10,000 years before present; Antunes et al. 2008). Geographical concordance between FIV-Ple subtypes and hypothesised historical sub-population ranges is consistent with an invasion of FIV-Ple that pre-dates the expansion of modern lion populations estimated between 324,000–169,000 years before present (Antunes et al. 2008). Diversity of FIV in the North American species puma (also known as cougar or mountain lion; *Puma concolor* with FIV strain FIV-Pco) is also high with substantial divergence between the two clades A and B (Troyer et al. 2005), pointing to this species as an early host for new world colonisation for FIV. In contrast, the closest relative of the domestic cat, *Felis sylevstris,* the wildcat (Driscoll et al. 2007), is free from FIV infection, suggesting that the zoonosis into domestic cats took place after their domestication, estimated at 10,000 years ago from archaeological evidence (Vigne et al. 2004). Thus in a similar manner to the primates, lentiviral infection is still spreading through the taxa, and comparisons between the transmissions of feline and primate lentiviruses have yielded some interesting insights into the relationship between host and pathogen. In the primates, cross-species transmissions tend to occur primarily between closely related species, so that the phylogenies of host and virus closely resemble each other (VandeWoude and Apetrei 2006). In contrast, zoonoses in the felids frequently take place between more distantly related species, resulting in discordant host and virus phylogenies (VandeWoude and Apetrei 2006). Such transmission has given rise to a phylogeographic distribution in the felids, where viral strains are more likely to

resemble those endemic within the same geographical area than those present in closely related host species.

1.4 Lentiviral entry

1.4.1 Receptors for HIV-1 entry

The Env-receptor interaction is a critical determinant for cell tropism and pathogenesis for lentiviral infection. For HIV-1, an interaction with surface-expressed CD4 allows targeting of helper T-cells and cells of the macrophage/monocyte lineage whilst further subpopulation specificity is provided by the differential affinities for chemokine co-receptors, CXCR4 and CCR5. CD4 is a member of the immunoglobulin superfamily that is expressed on the surface of T-helper cells as a transmembrane glycoprotein. Its *in vivo* role is to act, along with the variable T-cell receptor (TCR), as a ligand for major histocompatability complex (MHC) class II, whilst cytotoxic T-cells, which express CD8, interact with MHC class I. MHC class II is expressed on the surface of antigen presenting cells and displays protease-digested oligopeptides in its peptide-binding groove. In the event that antigen presented by MHC class II molecules is recognised by TCR of a given T-helper cell, activation of that T-cell will typically result leading to an antigen-specific clonal expansion. CD4 aids in this process by amplifying the intracellular signal generated by TCR by recruitment of membrane-anchored tyrosine phosphatase Ick, initiating a downstream signalling pathway and resulting in the activation of transcription factors such as NFκB and NFAT which stimulate transcription of T-cell activation genes such as interleukin-2 (Janeway et al. 2001; reviewed in Delon and Germain 2000). The chemokine receptors (CCRs) are G-protein coupled receptors that belong to the 7-transmembrane domain super-family (7TM). CCRs are responsible for the binding of soluble ligands (chemokines) which effect intracellular responses in the target cell (for review see Rot and von

Andrian 2004). Many chemokines act as chemo-attractants for specific cell types, and upon binding initiate intracellular signalling that typically results in a directional kinetic response. Members of the chemokine receptor family bind the various chemokines with differential specificity and chemokines are often promiscuous in their ligand specificity, resulting in various responses such as proliferation, adhesion, differentiation or barrier migration depending on the nature and context of the interaction (Rot and von Andrian 2004).

1.4.2 Molecular mechanism of HIV-1 entry

The majority of research into the mechanisms of lentiviral entry has been performed in the primate lentiviruses (HIV-1/SIVmac), but it is increasingly becoming clear that attributes of HIV attachment and fusion hold true for FIV entry. Moreover, gp120 and gp41 bear structural homology to viruses as distant as influenza A haemagglutanin HA1 and HA2 glycoproteins, suggesting a convergent or conserved mechanism for viral penetration (Weissenhorn et al. 1997). The HIV particle supports several envelope 'spikes' protruding from the surface, each consisting of an exposed trimer of gp120 subunits (SU) anchored to the membrane through a non-covalent interaction with trimeric gp41 transmembrane glycoprotein (TM), in such a manner that gp41 is generally sequestered by gp120 (Weiss et al. 1990; Kowalski et al. 1987). gp120 is a large, multiply-glycosylated protein, comprising several conserved domains (C1-C5), interspersed with variable domains (V1-V5) bounded by intramolecular disulphide bonds, creating loop structures (Starcich et al. 1986; Leonard et al. 1990). Being exposed to the adaptive immune system, antibodies are generated to gp120, particularly to V2 and V3 (Moore et al. 1994), some of which are capable of neutralising infection (Karlsson Hedestam et al. 2008). A considerable degree of sequence variation is tolerated in the variable loops, allowing the expansion of mutants that escape antibody neutralisation but

preserve fusogenic activity (Karlsson Hedestam et al. 2008). Although regions within V2 and V3 do play a part in receptor attachment (Wyatt et al. 1995; Trkola et al. 1996), a major function of the variable loops appears to be to provide a largely irrelevant antibody binding surface, whilst protecting conserved domains critical for entry mechanisms.

Entry to cells expressing co-receptor alone can be promoted using soluble CD4 (Moore et al. 1990). Antibodies directed to co-receptor do not normally inhibit infection, but after incubation with soluble CD4, entry is rendered antibody-sensitive, suggesting that the initial attachment step of Env to CD4 promotes a conformational change that primes the virus for co-receptor attachment (Salzwedel et al. 2000). This conformation change, elucidated by differential sensitivity of V3 to proteases, exposes motifs within the V3 loop which are able to interact with cognate chemokine receptors (Sattentau and Moore 1991). In turn, the Env-chemokine receptor interaction allows gp41 to insert its hydrophobic N-terminus termed the 'fusion peptide' into the cell membrane with the simultaneous dissociation of gp120 (Sodroski 1999). Trimers of gp41 are then thought to bring about the fusion of the two membranes by conformational rearrangement from a prehairpin state to a final six α-helical hairpin structure (Gallo et al. 2001; Weissenhorn et al. 1997). Once the membranes are fused, the viral contents are deposited within the cytoplasm and the intracellular stages of the lifecycle begin.

1.4.3 Variable tropism of HIV-1

HIV-1 strains have long been known to preferentially target either macrophages (MΦ-tropic) or T-lymphocytes (T-tropic) (Collman et al. 1989). For the most part, this specificity can be explained by the preferential use of CCR5 or CXCR4 (R5 and X4 HIV-1 strains), since CXCR4 is widely expressed in helper T-cells, whereas CCR5 is found in the monocyte lineage. Further T-cell subpopulation

tropism can also be explained by CCR use: the memory (CD45RA+) T-cell population expresses CCR5 and accordingly is targeted by R5 isolates. Likewise, infection of other cell types is dependent on alternative chemokine tropism: invasion of the central nervous system is associated with use of D6, expressed widely on astrocytes (Neil et al. 2005) and CCR3-using Envs have been isolated from neural tissue and macrophages (Aasa-Chapman et al. 2006; Peters et al. 2004). However, not all specificity can be explained by CCR expression given that some macrophage-tropic strains use CXCR4 (Simmons et al. 1998), and some R5-using strains can only poorly infect macrophages (Peters et al. 2006). This apparent anomaly can be explained by the ability to use low levels of CD4 and CCR efficiently, perhaps marked by an increased affinity for the receptors (Gray et al. 2005; Peters et al. 2006). Thus for HIV-1, cell tropism is a function of both co-receptor identity and the nature of the CD4 interaction, with variations in the ability to bind its two cell-surface receptors providing substantial subpopulation tropism.

Studies of HIV-infection in humans have shown that tropism usually, but not always, progresses from R5 to X4 or dual-tropic R5X4 over the course of infection, a change that is accompanied by mutations in the V3 loop and an increase in cytotoxicity (Page et al. 1992; Chavda et al. 1994; Fenyo et al. 1988; Connor et al. 1997). Individuals with a CCR5 mutation that prevents R5 infection (CCR5-Δ32) are largely resistant to infection with HIV, despite expressing normal CXCR4 (Dean et al. 1996; Liu et al. 1996). These data imply that replication of X4 variants is somehow restricted and early stages of infection are performed by R5 strains. The use of R5 by early variants appears to be a conserved mechanism in primates, with SIV strains from sooty mangabeys and rhesus macaques as well as HIV-1 and HIV-2 all employing the same strategy (Chen et al. 1998). The precise nature of the CXCR4 restriction is poorly defined, but is proposed to be a series of barriers to viral transmission

at the mucosal surface and sub-mucosa that preferentially select R5 variants over X4, known as the gatekeeper hypothesis (Margolis and Shattock 2006). The expansion of X4 or R5X4 viruses in the later stages of infection is proposed to require a certain level of immune depletion, as a conserved gp120 neutralisation epitope is protected in R5 HIV-1 variants but not in X4 and R5X4 variants (Lusso et al. 2005). Thus changes in tropism are a major aspect of AIDS pathology and represent a mechanism of ensuring primate lentivirus survival through successful transmission and subsequent invasion of tissues.

1.4.4 Receptors for FIV-Fca entry

Although highly divergent at the primary protein sequence level, FIV Env is thought to share a similar domain architecture to HIV-1, with comparable variable loops defined by disulphide bridges (Pancino et al. 1993). In contrast to HIV-1, FIV uses CD134, a member of the tumour necrosis factor receptor family, as a primary receptor for entry (Shimojima et al. 2004). CD134, also referred to as OX40, is widely expressed on activated rat and human CD4+ T-cells (Mallett et al. 1990), where its activation is required for activated T-cell survival and expansion (Song et al. 2005b; Gramaglia et al. 1998; Pippig et al. 1999; for a review of CD134 function see Croft et al. 2009). CD134 is also found at low levels on CD8+ T-cells, macrophages and activated B-cells in humans (Baum et al. 1994). In domestic cats, CD134 is present on mitogen-stimulated T-cells *in vitro* (Shimojima et al. 2004; de Parseval et al. 2004) as well as CD4+ T-cells *in vivo* (Joshi et al. 2005) but absent from CD8+ T-cells (de Parseval et al. 2004). Accordingly, FIV replicates predominantly in activated CD4+ T-cells but during late stages of infection, an expansion of tropism to B-cells, macrophages and CD8+ T-cells is observed (Willett and Hosie 2008; de Parseval et al. 2004). FIV Env is able to use feline but not human CD134 for entry, a fact that has been exploited for the mapping of receptor domain usage by FIV Env.

A crystal structure for complexed human CD134-CD134L has been solved (Compaan and Hymowitz 2006) and reveals the presence of three cysteine-rich domains (CRDs), extracellular pseudo-repeats of a six-cysteine motif, capable of making three disulphide bridges. The presence of these domains gives the protein an elongated shape, stabilised by a ladder of disulphide bonds (Bodmer et al. 2002). A homology-based structure for feline CD134 was predicted using MODELLER (Eswar et al. 2007) and reveals that the primary Env binding site, known to reside in CRD1 (de Parseval et al. 2005), is orientated similarly to human CD134 (Figure 1-2). Additional contacts with Env are made in CRD2 and are found to be dependent on the CD134 NYE motif substituted for non-permissive SSK in the human form (Willett et al. 2006a).

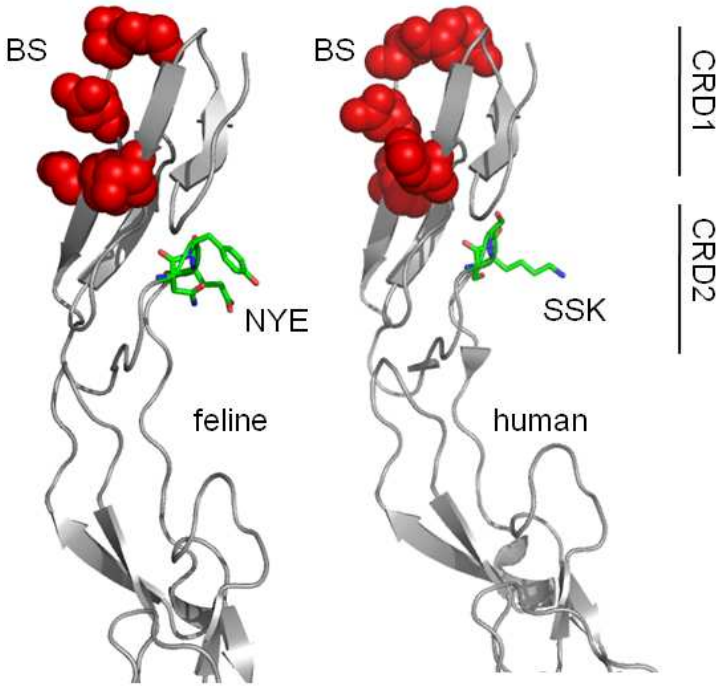


Figure 1-2 Structure of CD134-Env interaction domains. A homology model of feline CD134 based on the human crystal structure (2HEY; Compaan and Hymowitz 2006) reveals the primary binding site (BS) to reside in CRD1. Further Env-receptor interactions are made by 'early' FIV strains in CRD2. Human CRD1 and CRD2 domains are non-functional as receptors for FIV entry. However, 'late' FIV strains that do not

require additional CRD2 interactions are able to use chimeric hCD134/fCRD1 (Willett et al. 2006b; de Parseval et al. 2005).

For FIV strains such as GL8 and CPG41 ('early' or acute stage isolates), entry is contingent on expression of both CD134 and CXCR4. However, other strains circulate with a reduced requirement for the CRD2 domain (Willett et al. 2006b; de Parseval et al. 2005). These strains, such as PPR and B2542 ('late' or chronic stage isolates), are more sensitive to inhibition by soluble CD134L (Willett et al. 2006b) and are able to infect cells expressing a chimeric CD134 that bears the CRD1 domain of feline CD134 in a human CD134 background (hCD134/fCRD1) (de Parseval et al. 2005). The finding recapitulates *in vitro* experiments where it was shown that long term passage of FIV in CD134-negative CrFK cells induces the generation of replication-competent viruses with mutations in the ES(E/D) motif of the V3 loop of Env (Shimojima et al. 1998). For both *in vivo* and *in vitro* isolates, the mutations at this loop involve a charge switch to lysine at one of the glutamates which allows the virus to use CXCR4 directly, with reduced requirement for CD134 (Willett et al. 1997a; Willett et al. 1997b; Willett et al. 2007).

A model of FIV progression is emerging that links the molecular mechanisms of virus-plasma membrane fusion to the broadening of target cell tropism and development of feline AIDS (Willett and Hosie 2008). In the early stages of infection, viral entry is characterised by a more complex Env-receptor interaction involving CD134 CRD1 and CRD2. As with HIV-1, the two-step primary receptor attachment followed by a conformational change allows the sequestration of the relatively invariant CXCR4-binding domain from the humoral immune response until it is required for mediation of entry. In contrast, during the chronic stages of infection, immunity is compromised and correspondingly, viruses have a reduced need for protecting the CXCR4-

interacting domain and isolates become less reliant on CD134. Accompanying the reduced CD134 requirement is a broadening of cell tropism and invasion of new tissue types, reminiscent of the switch from R5 to X4 in HIV-1. Although still dependent on CD134 and CXCR4, late stage FIV infection requires a low complexity and possibly low affinity interaction with CD134, similar to HIV-1 isolates that are able to alter their tropism by altering the nature of the CD4 interaction (Peters et al. 2006; Gray et al. 2005). Substantiation of this model will come from longitudinal studies following the progression and relative prevalence of viral mutants within individual cats, coupled with an understanding of the molecular basis of infection.

1.4.5 Receptor tropism of non-domestic cat FIV

Very little is known about the receptor usage of non-domestic cat lentiviruses. However, domestic cat mitogen-stimulated peripheral blood mononuclear cells (PBMCs) may be infected with FIV isolates from lion (FIV-Ple for *Panthera leo*) and puma (FIV-Pco for *Puma concolor*) (VandeWoude et al. 1997b) but CD134 and CXCR4 are not down-regulated during infection as would be expected if entry was mediated by these receptors (Willett et al. 2006a). Furthermore, infection by FIV-Ple and FIV-Pco cannot be inhibited by CXCR4 inhibitor AMD3100 or by SDF-α, a CXCR4-binding chemokine and a CD134-negative cell line 3201 supports the replication of both viruses but not domestic cat strains of FIV (FIV-Fca) (Smirnova et al. 2005). Taken together, these data suggest that FIV-Ple and FIV-Pco do not require CD134 and CXCR4 as receptor and co-receptor, using in preference an unknown cell surface receptor. Multiple strains of FIV-Pco as well as FIV-Ple B 458 have been used to successfully infect domestic cats (VandeWoude et al. 2003; VandeWoude et al. 1997a; Terwee et al. 2005). The *in vivo* manifestations of these strains are distinct from FIV-Fca infection, characterised by reduced pathogenicity and altered tissue tropism,

with strains such as FIV-Pco-1695 showing a greater propensity to infect intestinal tract mucosa (Terwee et al. 2005). Infected domestic cats were either able to mount a robust humoral and cellular immune response to PBMC-replicating virus (VandeWoude et al. 2003), or were able to prevent PBMC infection and clear pro-viral load three months post-infection (Terwee et al. 2005). It has been suggested that the lengthy period that FIV-Ple and FIV-Pco are proposed to have co-evolved with their hosts has reduced their pathogenicity through host-adaptation [burk] (Carpenter and O'Brien 1995). However, it is possible that differential Env-receptor tropism is at least in part responsible for the altered pathology and, until the identification of the FIV-Ple/Pco receptor/receptors, the relevance of this will remain unknown. Recently, phylogenetic analysis of wild FIV-Ple isolates in Africa revealed that *env* of FIV-Ple subtype E is more closely related to the *envs* of FIV-Fca than to other FIV-Ple subtypes or FIV-Pco (Pecon-Slattery et al. 2008a). The E subtype appears to be the result of a recombination event with other viruses, as trees drawn using the *gag-pol* open reading frame indicate a monophylogenetic relationship with other strains of FIV-Ple. Chapter 3 presents a study of the comparative receptor requirements of FIV-Ple isolates and finds that FIV strains with alternate receptor tropism are circulating in wild lion populations, raising important questions about the evolution and pathogenicity of FIV in this species.

1.5 Post-entry restriction

In order to replicate, lentiviruses are dependent on the presence of compatible host factors, use of which they have specifically evolved to incorporate into their lifecycles. As has been described, infectivity of lentiviruses is conditional on the presence of cognate entry receptors and their absence precludes replication. Thus non-functional receptors, such as human CD134 for FIV, are

thought to provide a substantial barrier to cross-species transmission. The restriction factors provide another barrier to replication that must also be overcome for successful infection and cross-species transmission. This study is primarily concerned with hypermutation inducing enzyme APOBEC3 and post-entry restriction factor TRIM5α and its modulator cyclophilin A.

1.5.1 Murine *Fv*1 system

The first system of retroviral restriction to be described was the murine Friend Virus susceptibility (*Fv*) system (Lilly 1967). Allelic variants of the *Fv*1 gene display strain-specific restriction to the simple retrovirus murine leukaemia virus (MLV). Cells of genotype $Fv1^{n/n}$, present in the NIH strain of mice, are able to restrict strain MLV-B but permit infection by MLV-N while cells bearing $Fv1^{b/b}$, present in BALB/c mice, display the opposite phenotype. The alleles are co-dominant so that $Fv1^{n/b}$ heterozygotes are restrictive to both strains (Pincus et al. 1971). *Fv*1 encodes an endogenous retroviral *gag* gene (Best et al. 1996), prompting speculation that *Fv*1 is able to multimerise with MLV capsid proteins and prevent early stages of infection. However, *Fv*1 is almost entirely unrelated to the MLV capsid gene (Best et al. 1996) and its mode of function remains unknown. Retroviral restriction was thought to be confined to mice until it was shown that non-murine cells of diverse species, including those of human origin, exhibit a phenotype akin to cells bearing $Fv1^{b/b}$: they specifically restrict MLV-N (Towers et al. 2000; Hofmann et al. 1999). This property was historically termed *Ref*1 in humans and *Lv*1 in primates, both now known to be functions of the α-splice variant of host protein TRIM5. Curiously, the viral determinant of N versus B tropism in the *Fv*1 system has been shown to map to capsid residue 110, precisely the same residue that confers MLV-N resistance to TRIM5α restriction in human cells (Towers et al. 2000).

1.5.2 TRIM5α restriction

TRIM5α provides a potent, pre-reverse transcription block to replication. The gene was identified by cDNA screen as the factor responsible for the *Lv*1 restriction phenotype in rhesus macaque cells (Stremlau et al. 2004). TRIM5 belongs to the TRIM (tripartite motif) family of proteins (Reymond et al. 2001), which is found in species extending from the nematode *Caenorhabditis elegans* to primates. A large expansion of *TRIM* genes has occurred, resulting in 68 genes in the human genome including the cluster of closely related *TRIM6/34/5/22* on human chromosome 11 which is found as a syntenic region in many mammalian genomes (Reymond et al. 2001; Sardiello et al. 2008). Of note, the cluster is absent from the chicken genome, suggesting a mammalian origin and its absence from the possum genome indicates that the genes arose sometime after the marsupial/eutherian (placental) mammal split dated to 90-180 mya (Sawyer et al. 2007). In some lineages, *TRIM5* itself is the subject of multiple duplications, with eight predicted tandem copies in the bovine genome and seven in the murine genome (termed *TRIM12* and *TRIM30* in the mouse) (Sawyer et al. 2007; Tareen et al. 2009). It is presumed that the gene duplications are maintained as they may provide restriction factors able to simultaneously combat specific pathogens, although the pathogens that have driven the evolution of each paralogue have in most cases not been identified. In contrast, several taxa possess non-functional pseudogenes of *TRIM5*. Humans, for example have a non-functional inverted duplication of *TRIM5* named *TRIMP1* (Reymond et al. 2001) and the canine *TRIM5* has been disrupted by the insertion of the transcript of an apparently unrelated gene *PNRC1* (Sawyer et al. 2007). Interestingly, in taxa such as the canids which lack TRIM5, positive selection of the *TRIM5* locus seems to have been compensated for by an increase in selection of *TRIM22*, perhaps suggestive of an overlap in antiviral function of these two genes (Sawyer et al. 2007).

The tripartite motif (TRIM) gene family is characterised by the presence of three domains: a RING (really interesting new gene) domain capable of E3 ubiquitin ligation, one or more B-Box domains and a coiled-coil domain responsible for formation of multimers (Reymond et al. 2001). Several members of the gene family (60% of those in humans), including the longest (alpha) isoform of *TRIM5* possess an additional C-terminal B30.2 domain (also known as a PRY-SPRY domain). In some primate species, a retrotransposition event has resulted in the B30.2 domain being replaced by a cyclophilin domain, resulting in a TRIM5-CypA fusion, termed TRIMCyp (Newman et al. 2008; Wilson et al. 2008; Virgen et al. 2008; Sayah et al. 2004). Subcellular localisation of TRIM proteins is found to be diverse with localisation typically nuclear, cytoplasmic or both (Reymond et al. 2001). For TRIM5α, localisation is in the cytoplasm, where it is able to form discrete puncta termed cytoplasmic bodies when over-expressed. However formation of cytoplasmic bodies is not a prerequisite for restriction activity of TRIM5α (Perez-Caballero et al. 2005b), and the biological role of these structures is unknown. Amongst the primate TRIM5α orthologues, an ability to inhibit the replication of a broad range of exogenous and endogenous retroviruses has been documented, including members of the *lenti-*, *beta-*, *gamma-* and *spuma-* retroviruses. (Saenz et al. 2005; Song et al. 2005a; Keckesova et al. 2004; Kaiser et al. 2007; Diehl et al. 2008; Yap et al. 2008). The precise mechanism of the restriction ability of TRIM5α remains the subject of enquiry, but roles for the individual domains have been described, allowing hypotheses for the overall mechanism of restriction to be advanced.

1.5.3 Virus interaction maps to TRIM5 B30.2 or CypA domain

The B30.2 domain is a conserved protein domain found uniquely in vertebrates that possess adaptive immune systems (Rhodes et al. 2005). The domain is a

composite of two more ancient domains: the PRY domain, which contributes the N-terminal 50-60 amino acids, and the SPRY domain, first identified in fungus *Dictyostelium,* which contributes the C-terminal ~140 amino acids. The resulting B30.2 (also referred to as PRY-SPRY domain) is present in more than 150 human proteins in eleven gene families in the human genome including the BTN immunoglobulin receptors and DEAD-box domain proteins (Rhodes et al. 2005; Woo et al. 2006).

The TRIM5α B30.2 domain is thought to engage the incoming viral core in the cytoplasm by recognition of specific features in the capsid N-terminal domain (Owens et al. 2004; Stremlau et al. 2004). An alignment of mammalian TRIM5 B30.2 domains reveals the presence of three areas of variability termed v1 to v3 (Song et al. 2005a). Structures have been solved of the B30.2 domain of TRIM21 (a close relative of the TRIM5/6/32/22 family which is known to bind IgG and is involved in Sjorgen's syndrome (Keeble et al. 2008)) and of a related *Drosophila* protein, GUSTAVUS (Woo et al. 2006), which show the SPRY forming a ß-sandwich pocket formed by the conserved ß-strands, referred to as ß1 to ß13. Protruding from this core structure are the variable loops, forming a surface capable of protein-protein interaction. For TRIM21, and potentially TRIM5, a separate pocket derived from the PRY subdomain is formed and with an exposed surface analogous to TRIM5α v1 (James et al. 2007). Part of this region is lacking in *Macaca nemestrina* TRIM5 isoform TRIM5θ, a deletion which impacts negatively on retroviral restriction (Brennan et al. 2007). Experimental data for TRIM21 suggest the presence of a conformationally rigid binding surface that does not alter its shape upon interaction with its substrate (induced-fit model)(Keeble et al. 2008). Thus in a manner reminiscent of immunoglobulins, a conserved backbone supports variable loops capable of protein-protein interaction.

The variable regions of primate TRIM5α B30.2 exhibit a substantial number of substitutions, insertions and lineage-specific length polymorphisms. The significance of variation in these domains has been highlighted by the finding that the difference in phenotype between rhTRIM5α, which restricts HIV-1, and huTRIM5α, which does not, maps to P332R (P in rh) within the v1 domain (Perez-Caballero et al. 2005a; Stremlau et al. 2005; Yap et al. 2005). Similarly, restriction of MLV-N by huTRIM5α is specific to acidic residues in the v3 loop (E405 and E406) (Perron et al. 2006a). Comparison with TRIM21 reveals that these positions correspond to the points of contact for the protein-protein interaction (James et al. 2007). Length polymorphisms have also been documented: v1 is typically longer in the old-world (catarrhine) monkeys (17 amino acids in squirrel monkey *versus* 46 amino acids in African green monkeys (Song et al. 2005a)), whilst v3 is typically longer in the new world (platyrrhine) monkeys (32 residues in most catarrhines including apes, compared to 41 residues in squirrel monkeys). For the most part, these length polymorphisms are the result of internal tandem duplications. It has been noted that those species with most duplications exhibit the widest repertoires of restriction. For example, African green monkey (agm) TRIM5α, with an expanded v1 domain, is able to target several retroviruses and may have become prevalent due to the selective advantage of increased breadth of capsid targets (Keckesova et al. 2004; Yap et al. 2004). Accordingly, the duplicated sequences in the variable loops are also more likely to have substitutions suggesting that the duplicated sequence may selectively target different capsids (Sawyer et al. 2005).

In some primate species, the B30.2 domain of TRIM5α is replaced by host factor cyclophilin A (CypA), creating fusion protein TRIMCyp. CypA is known to interact with lentiviral capsid (Luban et al. 1993; Franke et al. 1994; Thali et al. 1994) and its association with TRIM5 creates a potent restriction factor capable of targeting lentiviruses whose capsids interact with CypA, such as

HIV-1 and FIV (Sayah et al. 2004; Lin and Emerman 2006). The first TRIMCyp to be described was in the new world species owl monkey (*Aotus trivirgatus*) and formed by a retrotransposition event placing it downstream of exon 7 (Nisole et al. 2004; Sayah et al. 2004). Subsequently, TRIMCyps were found in old world pigtail, rhesus and long-tailed macaque species (*Macaca nemestrina*, *M. mulatta* and *M. fascicularis* respectively) (Newman et al. 2008; Virgen et al. 2008; Wilson et al. 2008). Remarkably, these TRIMCyps were created by an event independent of the owl monkey TRIMCyp as the CypA exon is located downstream of exon 8, and the coding transcript is generated by exon skipping. This indicates that capsid-specific restriction has arisen independently at least four times during mammalian evolution, targeting the same viral determinant. Since lentiviruses are the only retroviruses known to interact with CypA, the independent genesis of TRIMCyps would appear to highlight the strong selective pressure to devise novel restriction factors to circulating lentiviruses. However, no lentiviruses have been discovered in owl monkeys, or indeed any platyrrhine monkey, making the pathogen that drove the evolution of the owl monkey TRIMCyp uncertain. Once fused with the TRIM5 RBCC (the collective term for the RING, B-Box and coiled-coil), the CypA domain of rhesus TRIMCyp appears to have undergone point mutations that allow differential restriction of lentiviruses: tropism has changed from HIV-1-binding to HIV-2-binding through the acquisition of two point mutations that remodel the hydrophobic pocket (Price et al. 2009).

1.5.4 Adaptive evolution of TRIM5α

Evolution at the molecular level is characterised by the generation of mutations that may be deleterious, in which case natural selection will prevent their spread (purifying selection), or advantageous, in which case natural selection will seek to increase their prevalence (Darwinian or positive

selection). In addition, genetic changes may be driven to fixation by random genetic drift. Thus the presence of high levels of mutations between copies of a gene belonging to different species may be the result of very different evolutionary forces. Either 1) changing environmental circumstances may have relaxed the effect of purifying selection allowing new mutations to increase in frequency by genetic drift or 2) positive selection has driven the new mutations to fixation, suggesting a biologically important role. The two scenarios can be differentiated by the calculation of the synonymous (silent) to non-synonymous (amino acid substituting) changes at the nucleic acid level, since synonymous changes are assumed to be neutral with respect to natural selection and therefore give a background rate of the accumulation of changes (Yang 2005). The ratio of non-synonymous to synonymous changes (dN/dS ratio) is therefore an indicator of the nature of the selection that has occurred during evolution, with values >1, 1 and <1 representing positive selection, neutral selection and purifying selection respectively.

Primate TRIM5α has been evolving under episodes of positive selection for at least the past 33 mya (Sawyer et al. 2005). Evidence of particularly strong positive selection is found in the hominid lineage and maps to several residues in the B30.2 domain variable loops and four residues in the coiled coil domain, some displaying a dN/dS ratio as high as 8.8. This analysis highlighted a patch of residues in the v1 loop as under very strong adaptive selection, including the R332P codon that is largely responsible for the change in specificity between hu and rh TRIM5α variants (Perez-Caballero et al. 2005a; Stremlau et al. 2005; Yap et al. 2005). Analysis of the five confirmed protein-coding bovine *TRIM5* paralogues and the murine *TRIM12*/*TRIM30* genes reveals that positive selection of TRIM5α is also present in these species and therefore not unique to the primates (Sawyer et al. 2005; Tareen et al. 2009). The selective pressure also maps to the v1 loop, consistent with the paralogues evolving to target

diverse pathogens. However, the viral targets for most of the genes is unknown, although bovine *TRIM5-3* has been found to restrict retroviral replication in a manner similar to primate TRIM5α, specifically targeting MLV-N (Ylinen et al. 2006; Si et al. 2006). The data imply that antiretroviral activity of TRIM5α is an ancestral phenotype which has been conserved in diverse mammalian taxa. Furthermore, the repeated findings of positive selection in the B30.2 domain are consistent with TRIM5α's critical role in the permissivity of the host to infection by widespread pathogens.

1.5.5 Contribution of RBCC domain to restriction

Target specificity of TRIM5α is provided by the B30.2 domain. However, the RING, B-Box and coiled-coil domains are canonical features of TRIM family proteins, and each domain contributes to TRIM5α-mediated restriction. The coiled coil domain is an approximately 100 residue domain encoded by human TRIM5α exons 2, 3 and 4. Coiled coil domains typically consist of a 7 residue repeat 'abcdefg' where amino acids a and d represent hydrophobic residues responsible for homo-oligomerisation and the other residues are polar (Harbury et al. 1995; Lupas et al. 1991). For TRIM5α, the coiled coil and the downstream linker 2 region between the coiled coil and B30.2 domain mediate oligomerisation (Mische et al. 2005; Javanbakht et al. 2006a; Langelier et al. 2008), although conflicting evidence has been provided as to whether it forms dimers or trimers. Experiments have been confounded by the rapid turnover of TRIM5α and its tendency to form aggregates when purified. These problems have been resolved by the use of chimeric protein with the RING domain of TRIM21 and expression in insect cells to demonstrate that TRIM5α self-associates as a dimer (Langelier et al. 2008). Previous confusing results from cross-linking experiments were obtained due to the anomalously slow migration of TRIM5α dimers by SDS-PAGE. Mutation of hydrophobic residues predicted to

form the surface for self-association dramatically reduces capsid binding (Javanbakht et al. 2006a) and the efficiency of rhTRIM5α to restrict HIV-1 (Javanbakht et al. 2006a; Mische et al. 2005), suggesting that dimerisation of the protein is important for increasing the avidity of the TRIM5α-capsid interaction. Coiled-coil domains expressed alone (and other truncated TRIM5α constructs such as human splice variant TRIM5γ and TRIM5δ) are able to prevent restriction by full length TRIM5α presumably by forming dimers with full length protein in the cytoplasm (Perez-Caballero et al. 2005a).

The RING domain belongs to the E3 ubiquitin ligase class of enzymes which facilitate the transfer of ubiquitin from an E2-conjugating enzyme to a lysine residue of the target protein. TRIM5α is capable of transferring the ubiquitin peptide to itself (auto-ubiquitinylation) which is thought to direct the protein to proteasome-mediated degradation, in turn leading to a high rate of turnover (Diaz-Griffero et al. 2006a; Javanbakht et al. 2005). It appears that incoming retroviral cores that have been bound by TRIM5α are also subject to proteasome degradation, as inhibition of the proteasome with MG132 prolongs the lifetime of cores in the cytoplasm (Campbell et al. 2008) and TRIM5α appears to increase in turnover rate upon inoculation with a restricted virus (Rold and Aiken 2008), suggesting a functional link between proteasome degradation and restriction. However, deletion of the RING domain impairs but does not abolish TRIM5α-mediated restriction and the impairment of restriction could partly be attributed to alteration of subcellular localisation (Javanbakht et al. 2005). Likewise inhibition of the proteasome with MG132 does not restore infectivity (Anderson et al. 2006; Wu et al. 2006), suggesting that although TRIM5α does mediate proteasomal cleavage of the viral core, this is not crucial for its antiviral activity and TRIM5α depends additionally on other mechanisms to prevent retroviral infection.

The B-Box 2 domain is present in the majority of TRIM family proteins and is sometimes accompanied by an adjacent B-Box 1 domain. The domains are unique to the TRIM family but their function is poorly characterised (Reymond et al. 2001). TRIM5α possesses a single B-Box 2 domain which, when deleted, severely impacts on retroviral restriction (Stremlau et al. 2006b; Javanbakht et al. 2005). Homology modelling with a TRIM29 B-Box 2 structure reveals it to be a globular structure with a highly charged surface (Diaz-Griffero et al. 2007). Mutation of some of these residues (such as rhTRIM5α R119) results in the loss of antiretroviral activity, despite retaining the ability to self-associate and interact with capsid, suggesting that the B-Box is involved in an effector function of TRIM5α-mediated restriction (Diaz-Griffero et al. 2007). The nature of this function is unknown but may involve the recruitment of other cellular proteins. It also appears that the charged surface is involved in modulating the stability of the protein in the cytoplasm and in the formation of higher-order complexes of TRIM5α (Diaz-Griffero et al. 2007; Li and Sodroski 2008), although, again the mechanism regulating its longevity is unknown but independent of proteasome degradation (Diaz-Griffero et al. 2007).

In studying the contributions of the individual domains of TRIM5α, several non-mutually exclusive hypotheses for the mechanism of TRIM5α antiviral activity have been advanced. The simplest model is that incoming cores are bound by TRIM5α, which is poly-ubiquitinylated and with a high level of turnover, resulting in degradation of viral proteins. Evidence for this model comes from the finding that under normal conditions, retroviral cores are degraded by the proteasome before they have the chance to reverse transcribe (Wu et al. 2006; Anderson et al. 2006). Although this method of restriction may represent the likely fate of restricted viruses *in vivo,* the persistence of restriction after addition of MG132 suggests that other mechanisms are operating (Campbell et al. 2008). The nature of this block is unknown, although interference with the

uncoating process has been suggested (Stremlau et al. 2006b). Uncoating is a poorly defined stage thought to involve the dissassembly of the capsid core and a restructuring of the viral proteins in order for reverse transription of the viral RNA genome to occur. Evidence that the process occurs is provided by findings that CA protein is present only at very low levels after reverse transcription (Fassati and Goff 2001) and electron microscopy shows cores disintegrating soon after fusion (Grewe et al. 1990). Furthermore, restriction factor TRIMCyp is only able to restict HIV-1 in the first 30-60 minutes post-entry (Perez-Caballero et al. 2005b). It is thought that uncoating is a temporally regulated event that must proceed at the correct speed to allow association with cellular factors whilst retaining the ability to perform necessary viral functions, notably reverse transcription (Forshey et al. 2002). By following the levels of multimeric or soluble capsid post infection, it was suggested that TRIM5α promotes the rapid conversion of HIV-1 and MLV-N capsid structures to the soluble (disassembled) form (Perron et al. 2006b; Stremlau et al. 2006b). In a similar manner CypA, which has been hypothesised to aid uncoating by performing a proline isomerisation reaction, is able to act as a restriction factor for HIV-1 when multimerised (Yap et al. 2007; Javanbakht et al. 2007). This supports the accelerated uncoating hypothesis but disruption of other essential activities such as formation of the reverse transcription complex and translocation across the nuclear membrane has not been ruled out. Indeed, the accelerated uncoating hypothesis does not explain the effect of mutations of charged residues in the B-box which ablate restriction. Nor does it explain the low levels of retroviral restriction by hu or rhTRIM5α when exogenously expressed in dog cells(Berube et al. 2007). These findings suggest that TRIM5α binding to viral cores alone is insufficient for full levels of restriction and the protein may recruit other cellular factors that are required for optimal levels of restriction.

1.6 The role of Cyclophilin A in lentiviral replication

1.6.1 Cyclophilin A and its inhibitors

The cyclophilins are a large protein family found in both prokaryotes and eukaryotes. The family are members of the peptidyl proline isomerases superfamily (immunophilins), a name which reflects their ability to catalyse *cis-trans* isomerisation around peptidyl-proline bonds (Gothel and Marahiel 1999). The 18 kDa human protein CypA was discovered as the intracellular receptor of immunosuppressive drug cyclosporine A (CsA) (Takahashi et al. 1989). There are at least eight Cyp genes expressed in humans, with many other mammalian forms also found, all with sequence identity of >50% (Gothel and Marahiel 1999), but with variable subcellular localisation. For instance CypB is found exclusively in the endoplasmic reticulum (Price et al. 1991), whilst CypA is found at high levels in the cytoplasm (3-7µg per mg total protein (Ryffel et al. 1991)). CsA is a cyclic peptide isolated from the fungus *Tolypocladium inflatum* and is widely used in immune suppression for recipients of allografts. CsA exerts its immunosuppressive effect by binding CypA with a high affinity (IC_{50} ~6nM (Liu et al. 1991)). Once bound, the complex creates a novel binding surface which is able to bind and inhibit calcineurin, a serine/threonine kinase capable of dephosphorylating, and hence activating, transcription factor NF-AT (nuclear factor of activated T-cells), which is required for IL-2 expression associated with activated T-cells (for review of CsA's role in T-cell activation see Clardy 1995). Under CsA inhibition, T-cell receptor (TCR)-mediated activation and subsequent expansion of T-cell clones, which rely on this signalling pathway, are therefore inhibited, seriously compromising cellular immunity. Non-immunosuppressive analogues of CsA have been manufactured, notably NIM811 and Debio-025, which retain

their ability to bind CypA, but do not provide the calcineurin binding surface (Rosenwirth et al. 1994; Ptak et al. 2008).

1.6.2 CypA is a cofactor of HIV-1 replication

CypA was first associated with lentiviral replication when it was found to be packaged into HIV-1 virions via an association with unprocessed Gag polyprotein (Luban et al. 1993; Franke et al. 1994; Thali et al. 1994). These studies found that disruption of the interaction with CsA prevented CypA incorporation into virions and impacted negatively on HIV-1 replication. However it is during the early stages of viral infection, not incorporation, that CypA impacts on the viral lifecycle (Towers et al. 2003). CypA binds to incoming viral cores via an interaction between the catalytic hydrophobic pocket of the enzyme and an exposed proline-rich loop between CA helices 4 and 5 on the external surface of capsid N-terminal domain (Gamble et al. 1996). This loop appears to be confined to the lentiviruses as it is absent in other groups of retroviruses. However, whilst the CA-CypA interaction has been demonstrated for HIV-1, SIVagm and FIV (Lin and Emerman 2006; Diaz-Griffero et al. 2006b; Zhang et al. 2006) the interaction is not a feature of all lentiviruses: SIVmac, HIV-2 and EIAV do not bind CypA (Lin and Emerman 2006; Yoo et al. 1997; Braaten et al. 1996).

HIV-1 CA exists as a mixture of *cis* and *trans* isomers around the G89-P90 peptide bond with 14% of molecules in the *cis* and the remainder in the *trans* conformation (Gitti et al. 1996). Most peptide linkages are made in the *trans* conformation during translation, but peptidyl-proline linkages can be made in *cis* or *trans,* and the activity of peptidyl-proline isomerase (PPI) enzymes is thought to aid proper folding (Gothel and Marahiel 1999). Isomer heterogeneity is therefore rare in folded proteins making HIV-1 CA an unusual case (Fischer et al. 1998). It has been shown that CypA catalyses the inter-conversion of the CA

G89-P90 *cis* and *trans* isomers, increasing isomerisation by about 100-fold but maintaining the same *cis:trans* ratio in a CsA-inhibitable manner (Bosco et al. 2002). The finding supports the idea that CypA performs a catalytic role promoting the dissociation of the capsid core upon infection of a target cell. HIV-1 CA mutants G89V/A and P90V/A result in a 30-150-fold reduction in CypA binding affinity (Yoo et al. 1997) and display a similar phenotype to wildtype virus in the presence of CsA, with reduced infectivity in human cells.

1.6.3 CypA can have alternative effects on lentiviral replication

The effects of CypA on the replication of lentiviruses are not identical in different contexts. CsA treatment results in an *increase* in HIV-1 infectivity upon entry to rhesus macaque cells, but only in the presence of TRIM5α, suggesting that CypA promotes an isomerisation that increases the susceptibility of HIV to post-entry restriction (Keckesova et al. 2006; Berthoux et al. 2005; Stremlau et al. 2006a). Conversely, a reduction in infectivity of HIV-1 in human TE671 cells treated with CsA remains even after knockdown of TRIM5α (Keckesova et al. 2006; Sokolskaja et al. 2006). This phenotype is cell-type specific as TE671 sub-clone 17H1, selected for loss of MLV-N restriction, is permissive for HIV-1 in a CypA-independent manner but maintains wildtype expression levels of TRIM5α, suggesting that in humans, CypA protects HIV from an unknown restriction factor (Sayah and Luban 2004). Long-term passage of HIV-1 in the presence of CsA results in the out-growth of mutants A92E and G94D (Aberham et al. 1996). Infectivity of HIV-1 bearing these mutations is subsequently dependent on the presence of CsA in HeLa but not TE671 cells (Hatziioannou et al. 2005; Sokolskaja et al. 2006). Recently it has been shown that the CsA dependence in HeLa cells is due to high CypA expression levels in HeLa cells compared to TE671, but interestingly the HIV A92E and G94D mutants do not differ in their affinity for CypA (Ylinen et al. 2009). The finding

supports the notion of CypA playing a role in uncoating but that the mutants arising from CsA inhibition differ in their sensitivity to CypA-aided uncoating, rather than their affinity for CypA. Also arising from this study is the suggestion that the CA-CypA affinity appears to be maintained within a narrow range of values (all studies to date report Kd values within 5 – 15 μM (Yoo et al. 1997; Price et al. 2009; Ylinen et al. 2009)) even for the CsA inhibition-dependent mutants. The reasons for this are unclear, but may involve maintaining the correct level of isomerase activity to uncoat the virus in the appropriate manner. However research in the field has been hampered by the absence of CypA mutants that bind CA but do not perform the isomerase activity. Mutating the G89-P90 bond that prevents isomerisation also prevents CypA binding (Yoo et al. 1997), making it difficult to distinguish between the relative contributions of binding and isomerisation.

In some primate species, cyclophilin replaces the B30.2 domain of TRIM5α, creating TRIMCyp (Wilson et al. 2008; Virgen et al. 2008; Sayah et al. 2004; Newman et al. 2008). The CypA domain of the new world primate owl monkey (omk) TRIMCyp binds to a similar repertoire of viruses as human cytosolic CypA, and accordingly restricts HIV-1 and FIV but does not restrict HIV-2 or SIVmac (Sayah et al. 2004; Virgen et al. 2008; Wilson et al. 2008). Newly discovered forms of TRIMCyp, created by an independent retrotransposition event in the *Macaca* genus (rh), have an altered capsid specificity, specifically targeting HIV-2 but no longer restricting HIV-1 (Wilson et al. 2008; Virgen et al. 2008). This switch in specificity is attributable to two substitutions, D66N and R69H. These mutations cause a comparatively large (~16Å) rearrangement of a ten-residue loop at the binding surface of Cyp, changing from a ‘closed fist’ to ‘open palm’ conformation and permitting micromolar binding to HIV-2 CA (Price et al. 2009). Curiously, FIV is restricted by both rh and omkTRIMCyp (Virgen et al. 2008; Wilson et al. 2008; Saenz et al. 2005). So far FIV appears to

be unique in this property suggesting an alternative CypA binding mechanism and making an interesting comparison with the primate lentiviruses, where binding is exclusive to either omkTRIMCyp/CypA or to the structurally modified rhTRIMCyp. This issue is addressed in Chapter 5, where biophysical techniques confirm the dual-specificity of FIV and a crystal structural of FIV CA reveals structural differences to the primate lentiviruses.

1.7 Induction of hypermutation by APOBEC3

1.7.1 Discovery of human APOBEC3G

The discovery of the role of APOBEC enzymes in the lifecycle of retroviruses stems from work on viral infectivity factor (Vif) in HIV-1. Vif is a highly basic protein of 22-29kDa found in all lentiviruses with the exception of equine infectious anaemia virus (EIAV; Kawakami et al 1987). Early work on the gene (known at that time as gene 'A' or *sor*) revealed that Vif was required for replication in certain 'non-permissive' cell lines including macrophages, monocyte and some T-cell derived cells (e.g. CEM, H9 and HUT78) but dispensable in other 'permissive' cell lines such as T-cell lines CEM-SS, Jurkat and non-lymphoid cells such as HeLa (von Schwedler et al. 1993; Gabuzda et al. 1992). It was shown that Vif is packaged into virus particles where it is required for the formation of phenotypically normal virions (von Schwedler et al. 1993; Ohagen and Gabuzda 2000). Furthermore it was shown that packaging of Vif into virus particles is essential for overcoming the block in non-permissive cells (von Schwedler et al. 1993). Thus the phenotype of Δ*vif* viruses is dependent on the permissivity of the virus producing cells: Δ*vif* viruses produced in permissive cells are infectious for a single cycle regardless of the permissiveness of the target cells, whilst Δ*vif* viruses produced in non-permissive cells are uninfectious regardless of the nature of the target cells.

The factor responsible for the differential permissivity between CEM and CEM-SS cells was isolated by subtractive cloning coupled with a cDNA screen (Sheehy et al. 2002). The gene identified was APOBEC3G (huA3G), a member of the cytidine deaminase enzyme family. There are 12 members of the gene family in the human genome residing in four gene clusters: APOBEC1 and AID are separated by 1 megabase on chromosome 12, A2 resides alone on chromosome 6, an expanded cluster of seven A3 genes (A, B, C, DE, F, G and H) is located on chromosome 22, and A4 is located on chromosome 1. The family takes its name after the apolipoprotein B mRNA-editing catalytic polypeptide 1 (APOBEC1), for which only a single role has been described: to deaminate a single nucleoside of *APOB* mRNA transcripts at position C6666 to generate a truncated protein (Teng et al. 1993). The B-cell expressed protein AID (activation-induced deaminase) is responsible for the generation of somatic hypermutation leading to the diversification of antibody (Martin et al. 2002; Di Noia et al. 2007) and also triggers antibody class switching, both operating at the DNA level (Muramatsu et al. 1999). The identification of huA3G as the factor responsible for the non-permissivity of certain cell lines therefore presented a potential mechanism for its activity: host mutation of viral nucleic acids.

1.7.2 Antiviral activity of human APOBEC3G

The cytidine deaminases all bear a catalytic domain (CD) that comprises a Zn-coordinating H/C-X-E-X_{23-28}-P-C-X_2-C motif which is thought to generate zinc hydroxide that is able to deaminate the pyrimidine ring of cytidine by nucleophilic attack resulting in a uracil residue (Harris and Liddament 2004). Several of the huA3 genes (A3B, DE, F and G) possess two CDs: an N-terminal domain (CD1; also known as the pseudocatalytic domain) that is catalytically inactive, and a catalytically active C-terminal domain (CD2; Huthoff and Malim

2007). The domains have distinct functions and antiviral activity of huA3G is dependent upon both the domains.

The huA3G CD2 deaminase specifically targets cytosine residues of single stranded (ss) minus sense DNA but is catalytically inactive on HIV-1 genomic RNA, double stranded DNA and RNA-DNA heteroduplexes (Yu et al. 2004b). Crystal and NMR structures of CD2 reveal a positively charged nucleic acid binding groove that permits interaction with the negatively charged single-stranded DNA backbone and brings the target cytosine into close proximity with the Zn-coordinating catalytic domain (Holden et al. 2008; Chen et al. 2008). The deamination activity of CD2 converts cytosine to uracil in the minus strand DNA. Subsequent reverse transcription results in a guanosine to adenosine transition in the coding strand for the most part of the HIV genome (the exceptions being the U3 region of the 5' LTR and the primer binding sites which exist as single stranded DNA flaps after reverse transcription and accumulate cytosine to thymine mutations (Yu et al. 2004b)). The mutagenesis proceeds from 3' to 5' end of the ssDNA, following the generation of single stranded DNA and digestion of template RNA by reverse transcriptase and RNaseH respectively (Chelico et al. 2006). The sites of mutation are not completely random. Firstly, gradients of A3G-induced mutations exist, increasing in the 5' to the 3' end of the positive sense genome which is thought to reflect the time that single stranded DNA is exposed to huA3G (Yu et al. 2004b; Suspene et al. 2006). These gradients reach their peak just 5' of the polypurine tracts which are the origins of reverse transcription (Suspene et al. 2006; Yu et al. 2004b). Additionally, mutational hotspots for APOBECs have been identified: most prevalently for huA3G, the GG dinucleotide (substrate residue underlined) is most commonly mutated, and in particular in the TGGG tetranucleotide (Yu et al. 2004b; Bishop et al. 2004b; Suspene et al. 2006; Harris et al. 2003). Interestingly, in protein-coding regions, this mutation

frequently results in a TGG to TAG change which alters a tryptophan codon to a stop codon, potentially causing a damaging truncating mutation to the viral protein. Thus the activity of APOBEC3G against HIV-1 Δ*vif* is thought to be the introduction of mutations (hypermutation) to such a level (as many as 10% of guanosines may be mutated to adenosines) that the virus is impaired in its ability to produce infectious progeny virions. When the level of hypermutation renders the virus unable to faithfully replicate and therefore unable to sustain infection it is said that the error catastrophe threshold has been surpassed.

1.7.3 Encapsidation of human APOBEC3G

The fact that the permissivity of the virus producing cell rather than the target cell is the determinant for viral replication suggests that APOBEC3G present in the target cell is not sufficient for inhibition of viral replication. Indeed it has been shown that huA3G is encapsidated into HIV-1 Δ*vif* and this ability is likely to depend on interaction between huA3G CD1 and both viral Gag p55 nucleocapsid region and viral RNA (Mangeat et al. 2003; Khan et al. 2005). The precise mechanisms and relative contributions of each of these interactions is currently under study but is thought to involve multimers of huA3G forming complexes with viral RNA, cellular RNA species 7SL and p55 polyprotein which are then packaged into budding virus particles (Svarovskaia et al. 2004; Wang et al. 2007). Final levels of huA3G are dependent on the levels of expression in the virus producing cell, calculated at 7 enzyme copies per virion when produced in PBMCs but capable of much higher levels of incorporation when overexpressed *in vitro* (Xu et al. 2007). Interestingly, even in the presence of Vif, huA3G particles are thought to be packaged into HIV-1 particles with 0.3 to 0.8 molecules per virion (Nowarski et al. 2008), and low levels of G to A mutations are observed in high passage number HIV-1 cultures (Zhang et al. 2003). As with many pre-integration stages of the retroviral lifecycle,

interactions of huA3G with the reverse transcription complex in the target cell are much less well understood, but presumably require interactions that prevent disassociation of huA3G from the target nucleic acids and allow the transition from RNA binding to single-stranded DNA cytidine deamination.

It has been reported that induced hypermutation is not sufficient to explain the antiviral activity of huA3G and huA3F, giving rise to the possibility of a deamination-independent role of APOBECs on viral replication. Mutation of the conserved zinc coordinating histidine and cysteine residues or the catalytic glutamic acid residues of CD2 abolishes deaminase activity but does not fully destroy antiviral activity (Newman et al. 2005). Recently, a likely mechanism for deaminase-independent activity has been proposed which involves the inhibition of the progression of the reverse transcriptase enzyme along the template RNA in the newly infected cell (Bishop et al. 2008). However the relative contributions of the deaminase-dependent and -independent roles of huA3G are not fully clear and may have different relative contributions depending on the cell type in question (Thielen et al. 2007).

1.7.4 Antiviral activity of other human APOBEC3 genes

The presence of an expanded gene family of APOBEC3 genes raises the possibility that other members of the family possess antiviral activity. Several studies have now been performed confirming that this is indeed the case and anti-lentiviral activity has been associated with several of the two-domain APOBECS, namely 3B (Bishop et al. 2004a), 3DE (Dang et al. 2006), 3F (Zheng et al. 2004) and anti-SIVmac activity has been associated with the one-domain A3C (Yu et al. 2004a; Bishop et al. 2004a). Of these, the gene with largest impact on HIV-1 replication is huA3F which is widely co-expressed with huA3G in non-permissive cells (Wiegand et al. 2004; Liddament et al. 2004; Zheng et al. 2004). Strong evidence of the importance of huA3F in HIV-1 replication is

that, like huA3G, HIV-1 Vif counteracts its activity and its preferred mutational hotspot (WGAA where W is A or T) is detectable in clinical isolates of HIV-1 (Fitzgibbon et al. 1993). However further analysis reveals that huA3G's preferred substrate (GG dinucleotide) is more frequently mutated than huA3F's (GA), suggesting that the role of huA3G is greater in the biological context of HIV-1 replication (Zennou and Bieniasz 2006). Interestingly, the antiretroviral activity of huA3B is resistant to HIV-1 Vif in tissue culture experiments but expression is very low in CD4+ T-cells, making it unlikely to severely hinder HIV-1 replication (Bogerd et al. 2007).

Also of significance is the breadth of targets that APOBECs are able to deaminate whilst being able to distinguish self from non-self. Of the human A3 genes, all seven have now been associated with antiretroviral or antiretroelement activity with individual enzymes such as huA3F able to deaminate targets as diverse as HIV-1 (Wiegand et al. 2004), AluI, a transposable element present in multiple copies in the human genome (Muckenfuss et al. 2006), Ty1, a yeast transposable element (Schumacher et al. 2005) and hepadnavirus hepatitis B virus (Rosler et al. 2005), all of whose lifecycles involve RNA intermediates. This suggests conserved mechanisms for interaction and deamination of target nucleic acids.

1.7.5 The role of Vif in overcoming APOBEC3G and 3F restriction

HIV-1 viral infectivity factor (Vif) is a small accessory protein of 23kDa which allows the virus to replicate almost unaffected by the presence of huA3G and huA3F. Vif acts to decrease the levels of huA3G and huA3F protein levels dramatically in T-cells, reducing the half-life of APOBECs from hours to as little as 5 minutes (Stopak et al. 2003; Marin et al. 2003; Marin et al. 2003; Conticello et al. 2003). Crucial to this activity are three interactions made by Vif: 1) the Vif motif SLQ(Y/F)LA which resembles mammalian BC-box domain

present in SOCS-box proteins and recruits the cellular factor elongin C, an E3 ubiquitin ligase (Mehle et al. 2004b; Yu et al. 2003) 2) a non-canonical zinc-coordinating HCCH motif which interacts with cullin5 (Yu et al. 2004c; Mehle et al. 2004a) and 3) the N-terminal YRHHY and DRMR domains that interact with huA3G and huA3F respectively (Russell and Pathak 2007). The interactions result in the recruitment of a ubiquitylation complex comprising Vif/elonginC/elonginB/APOBEC3/cullin5/Rbx1 that polyubiquitylates the target APOBEC and induces proteasome-mediated destruction (Marin et al. 2003; Sheehy et al. 2003; Yu et al. 2003).

Vif proteins exhibit a certain level of species specificity. For example, Vif from HIV-1 is unable to counteract the A3G from African green monkeys (agmA3G). This specificity was found to map to the Vif-A3G interaction site: in huA3G, 128DPD130 is responsible for the interaction with Vif DRMR motif whilst in agmA3G, a D128K substitution allows the APOBEC to escape Vif-mediated destruction (Bogerd et al. 2004; Mangeat et al. 2004). However, SIVagm overcomes this mutation as its Vif is able to bind agmA3G and mark it for proteasome destruction. Thus the site of interaction between Vif and APOBEC represents a potential arms-race battle ground in the host-virus relationship.

1.7.6 Positive selection of APOBECs

In a similar manner to TRIM5α, primate APOBEC3 genes have been exposed to periods of intense positive selection over the past 33 mya, indicating the importance of these genes in protecting against retroviral infection (Sawyer et al. 2004; OhAinle et al. 2006). Residues under positive selection in A3G map to the catalytic domain, pseudocatalytic domain and to the Vif-interacting domain, but evidence of ancient adaptive selection is also found outside these regions (Sawyer et al. 2004). This is interpreted as the results of previous antagonistic host-virus relationships with now-extinct retroviruses. However

the targets of previous episodes of positive selection are unknown and are likely to be extinct, save for any sequences remaining as endogenised genomes. Interestingly, a human endogenous retrovirus, HERV-K, was found to be targeted by huA3F, and to a lesser extent huA3G, but not by any TRIM5α orthologues examined (Lee and Bieniasz 2007). High expression of A3 is found not only in T-lymphocytes, the target of current lentiviral infections, but also in testis and ovary tissue, organs that must be infected by endogenous retroviruses in order to ensure their propagation (Jarmuz et al. 2002; Sheehy et al. 2002). Thus it seems likely that the APOBEC family targets both horizontally-transmitted and endogenous retroviruses, and the diverse nature of the targets goes some way to explaining the maintenance of multiple A3 gene copies.

1.7.7 An expanded cluster of feline APOBEC3 genes

Recently an analysis of feline APOBECs in the nascent cat genome located the genomic positions of APOBEC1, 2, 3 and 4 (Munk et al. 2008). Interestingly, four APOBEC3 genes were found in an expanded cluster on domestic cat chromosome B4, comprising three copies of APOBEC3C (feA3Ca, feA3Cb and feA3Cc) and one copy of APOBEC3H. All the feline A3 genes identified possess a single catalytic domain. Comparisons with other felids revealed that the duplications of feA3C occurred in two successive events, one duplication of the ancestral feA3C which generated feA3Cc took place after the divergence of the *Felis* and *Panthera* lineages about 10.8 mya and a subsequent duplication of the same ancestral gene generating feA3Ca and feA3Cb took place after the divergence with pumas about 6.7 mya (Johnson et al. 2006; Munk et al. 2008). Of particular interest was the finding that the A3C locus underwent independent duplication events in other lineages: in the *Panthera* lineage a duplication gave rise to two A3C genes in the modern lion (liA3C1 and liA3C2)

and in lynxes, three duplications gave rise to four current A3C genes. Analysis of the felid A3C genes revealed strong positive selection, suggesting strong selection of new variants that confer benefits to the host, selection presumably imposed by pathogens. In contrast the A3H gene was present as a single copy in all felids examined and is not under positive selection, leading the authors to suggest its viral target may be an endogenous retrovirus. Interestingly a read-through transcript comprising components of feA3Ca, 3Cb and 3H, generating a two-domain protein was detected in domestic cats and was also found in lions, tigers, pumas and lynxes and referred to as APOBEC3CH.

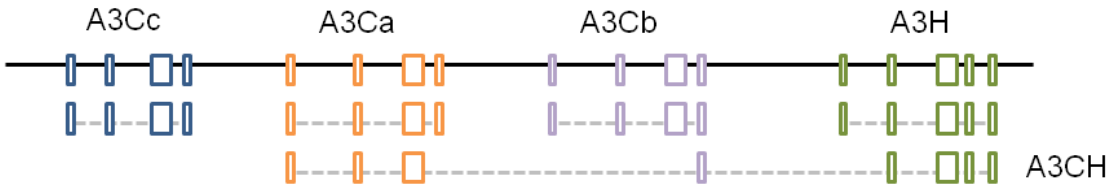


Figure 1-3 Representation of feline APOBEC3 genomic locus and transcripts. The feline APOBEC3 genes reside in a cluster extending ~30kb on domestic cat chromosome B4 (black line). Previously described transcripts (Munk et al. 2008) are represented by dashed grey lines. Readthrough transcript A3CH is also shown and incorporates exons 1-3 from A3Ca, an exon 4 variant from A3Cb that lacks the terminal stop codon and exons 2-5 of A3H.

Munk et al. (2008) tested the antiviral activity of domestic cat APOBEC3 genes against feline retroviruses and found that while single-domain protein feA3C was able to restrict Feline Foamy Virus (FFV), none of the single domain proteins were active against FIV (Munk et al. 2008; Lochelt et al. 2005). In contrast, the two-domain A3CH potently inhibited infectious FIV virus production, suggesting that, like human A3G, two domains are required for virion packaging and deaminase activity.

1.7.8 Vif of Feline Immunodeficiency Virus

Like HIV, the FIV genome bears a *vif* gene but homology at the protein level is minimal save the BC-box SLQ(Y/F)LA motif. The presence of this motif suggests that like HIV-1, FIV recruits the elonginC ubiquitylation complex to induce degradation of A3. Conflicting data exist for the subcellular localisation of FIV Vif. Initially, it was reported Vif localises predominantly to the nucleus (Chatterji et al. 2000), unlike HIV Vif which localises to the cytoplasm (Goncalves et al. 1994). Furthermore, Vif was not detected in FIV particles, suggestive perhaps of an alternative mechanism of action. However, these data are potentially explained by problems in exogenous Vif expression as the protein has a high turnover and nuclear export of transcripts is inefficient for HIV-1 Vif (Nguyen et al. 2004). Indeed a further study has shown that a eukaryotic codon-optimised Vif derived from FIV-Oma (from Pallas cat) invariably and uniquely localises to the cytoplasm (Paul et al. 2007).

1.8 Scope of this thesis

This thesis compares the host and viral factors that affect replication and cross-species transmission of lentiviruses in domestic and non-domestic cats. Specific attention is paid to the domestic cat and African lion, as these species are thought to represent recent and ancient lentiviral infections respectively. It is anticipated that antagonistic relationships between host and virus will have promoted changes in lion factors that interact with viral proteins. In particular, attention is given to the restriction factors which, in primates, are under positive selection driven by retroviral infection, and the hypothesis that lentiviral restriction will be more potent in the lion is tested.

- Chapter 3 explores recent findings that FIV strains with highly divergent *env* genes are present in wild African lion populations. The study finds

that the diversity within env corresponds to alternative receptor usage with implications for the evolution and pathology of infection in this species.

- Chapter 4 investigates the post-entry restriction factor TRIM5 in cats and finds that a mutation that ablates function is present in the felids. Further analysis reveals that the mutation is widespread amongst the Feliformia, dating the time of mutation to just after the Caniformia-Feliformia split, with implications for cross-species transmission and control of lentiviral infection in the carnivores.

- In Chapter 5, the lentiviral co-factor cyclophilin A is examined in the context of FIV replication. Tissue culture experiments and biophysical assays reveal the interaction is important for replication and occurs with a conserved binding affinity. Furthermore, a crystal structure is solved for FIV capsid and feline CypA, the first reported structures for both these proteins, and provides insight into the mechanism of the CA-CypA interaction.

- Finally in Chapter 6, the ability of lentiviruses to infect domestic cat and lion cells is investigated and a post-entry block to replication in lion cells is described. Investigation of APOBEC3 genes from both species finds that these restriction factors are active in felids, particularly in lions. The study provides evidence that is in concordance with theories of host-virus antagonistic relationships and shows evidence that a lack of TRIM5α can be compensated for by other post-entry restriction mechanisms.

Chapter 2. Materials and Methods

2.1 Molecular cloning

2.1.1 Nucleic acid extraction and PCR

Total RNA was prepared from domestic cat-derived cell lines CrFK and Mya-1 as well as non-domestic cat T-cells by homogenising 10^6 cells using QIAshredder Spin Columns (Qiagen, Crawley, UK) and extraction with RNeasy Mini Spin Columns (Qiagen) in a UV-treated laminar flow hood. RNA was eluted in RNase-free water (Invitrogen, Paisley, UK) prior to quantification of RNA by spectroscopy at 260 nm and storage at -80 °C. First strand cDNA synthesis was performed using 1 µg RNA with an oligo d(T) primer and AffinityScript Reverse Transcriptase (Stratagene, Stockport, UK), according to the manufacturer's instructions before downstream applications. DNA was prepared from tissue culture samples by harvesting from 10^6 cells with DNeasy Blood and Tissue kit (Qiagen). DNA extractions from non-domestic Feliform species used 50-100 mg brain tissue or 1 ml blood and were performed using the same kit using the appropriate protocol. All DNA preparations were eluted with EB buffer and stored at -20 °C.

Polymerase chain reaction (PCR) was performed using Phusion HotStart (Finnzymes, Hitchin, UK) polymerase. Reactions were set up on ice in a PCR

clean room UV-treated laminar flow hood. Reaction mix and cycling parameters were typically as per manufacturer's instructions although optimisation of annealing temperature was occasionally necessary by performing multiple reactions as a gradient. Standard conditions were HF buffer 4 µl, dNTPs 200 µM each, primers 0.5 µM each, DMSO 3% v/v, 0.02 U/µl Phusion, 50-200 ng template DNA. For FIV-Ple B 458 *env,* Phusion proved unsuccessful and KOD HotStart (Novagen, Nottingham, UK) was used instead. Lyophilised primers were ordered from MWG Operon and were resuspended in sterile distilled water to a stock concentration of 100 pmol/µl and to working stock concentrations of 10 pmol/µl. Primers were stored at -20 °C. A list of primers used is displayed in Appendix 1 and buffers and solutions is displayed in Appendix 2.

2.1.2 Cloning techniques

For molecular cloning, PCR products were purified by running on a 0.8-1.5% agarose gel, made with TBE buffer. DNA bands were observed on a UV transilluminator and excised using a sterile scalpel. Gel extraction was performed using QIAquick Gel Extraction Minicolumns (Qiagen) and DNA was eluted in 35-50 µl EB. Purified PCR products and vectors were digested using restriction endonucleases at 1 U/µl at 37 °C for 1 hr 30 min in 20 µl reactions with the buffer recommended by the manufacturer (Invitrogen). Digested DNA was purified using QIAquick PCR purification kit (Qiagen) and ligated using T4 DNA ligase at 1 U/µl at 15 °C overnight. DNA was transformed into DH5α chemically competent *Escherichia coli* (Invitrogen) using the heat shock protocol according to the manufacturer and plated onto agar plates bearing ampicillin or kanamycin at 50 µg/ml (Sigma-Aldrich, Dorset, UK). Colonies were picked and grown in 5 ml LB nutrient broth at 37 °C on an orbital shaker set to 200 rpm. When cloning *env* genes, an incubation temperature of 30 °C was

used to aid plasmid stability. Plasmid DNA was extracted from cultures using QIAprep Spin Miniprep kit (Qiagen) and digested as previous. Clones bearing inserts were identified by agarose gel electrophoresis and a large-scale DNA prep was performed by inoculating 500 ml LB flasks with bacterial clones. DNA was prepared from overnight cultures using PureLink HiPure Filter Plasmid Maxiprep Kit (Invitrogen). Pelleted DNA was resuspended in TE buffer and stored at 4 °C. Glycerol stocks of clones were made by diluting cultures to a final concentration 15% glycerol (v/v).

2.1.3 Sequencing of DNA

Chain termination sequencing reactions were set up in house using BigDye v1.1 and thermally cycled according to manufacturer's instructions (Applied Biosystems, Warrington, UK). Reactions were precipitated using 70% ethanol, resuspeded in 20 μl HiDi (Applied Biosystems) and ran on an ABI 7000 genetic analyser using the E80 protocol (Applied Biosystems). Sequence traces were analysed in DNA Dynamo (Blue Tractor Software, UK).

2.1.4 FIV Envelopes

FIV-Ple E Sangre *env* gene was PCR amplified using primers (LLV-E 5' Sal and LLV-E 3' Not) directed to published FIV-Ple subtype E 1027 sequence (Genbank FJ22537). Template cDNA was obtained from total cellular RNA of Mya-1 infected with FIV-Ple E Sangre following first strand cDNA synthesis. FIV-Ple B 458 *env* was amplified from viral supernatant pelleted in an SW28 ultracentrifuge rotor at 28,000 rpm in a Beckman L8-70 ultracentrifuge. Viral membrane and protein cores were disrupted using 0.1% Triton X-100 PBS and RNA was extracted using RNeasy miniprep kit (Qiagen) and reverse transcribed as above. PCR amplification was performed using primers (LLV-B 5' Sal and LLV-B 3' Not) directed to published FIV-Ple B sequence (Genbank EU117991).

Amplicons were cloned into eukaryotic expression vector VR1012 using SalI and NotI digestion sites. Cloned VR1012-*env* plasmids were used for the generation of HIV luciferase pseudotypes and were assayed onto target cells in 96 well plates as described below.

2.1.5 CD134 and CXCR4

Feline variants of CD134 and CXCR4 have been cloned and described previously (Shimojima et al. 2004; Willett et al. 1997b). Lion homologues of CD134 and CXCR4 were amplified from Angola-1 cDNA using primers directed to the feline homologues (feCD134 F, feCD134 R, feCXCR4 F and feCXCR4 R; Genbank accession numbers AB1288982 and U63558 respectively) and the products were cloned into pCR 2.1-TOPO vector (TOPO TA cloning kit, Invitrogen) and sequenced. Lion CXCR4 and CD134 sequences were deposited in Genbank with accession numbers EU915482 and EU915483 respectively. For expression in eukaryotic cells, fragments were then sub-cloned into pDONAI (Takara, Lonza Ltd, Wokingham, UK) using BamHI and SalI restriction sites.

2.1.6 TRIM5

Forward primers directed to conserved regions of the RBCC domain (Ts2, feT5fwd and Ts3) and reverse primers from non-variable regions within the B30.2 domain (Ts12, feT5rev and Ts16) were used in RT-PCR reactions using cDNA from domestic cat cell lines CrFK and Mya-1. Products were obtained for the Ts3-Ts12 and feT5fwd-feT5rev RT-PCR reactions. These amplicons were then sequenced and used to probe the nascent cat genome project database (Pontius and O'Brien 2007) using the NCBI BLAST tool (Altschul et al. 1990). The BLAST search returned sequences of whole genome shotgun (WGS) fragments bearing exact matches to exon 2 (GenBank accession number AANG01555594) and exon 8 (AANG01581224) that had been assembled to chromosome D1 of the

cat genome. From these fragments, PCR primers directed to the 5' end (wam4) and 3' end (wam13) of the open reading frame were designed, and the full length product was sequenced. Cloning primers wam4e and wam13c were used to amplify RT-PCR products from Mya-1 template cDNA and the amplicon was cloned into expression vector CXCR using EcoRI and Csp45I to produce feCTCR. Correct insertion of feTRIM5 was verified by cycle sequencing. Rapid amplification of cDNA ends (RACE) was performed using 5'/3' Second Generation RACE Kit (Roche) using cDNA from Mya-1 cells according to manufacturer's instructions. A PCR-based mutagenesis strategy was employed to introduce a stop codon at proline 306 of huTRIM5α to generate huTRIM5 P306STOP. Forward and reverse mutagenic primers (huT5stopF and shuT5stopR) were used with flanking CXCR specific primers (GT282 and GT283) in two individual reactions. The resulting products were mixed 1:1 and used as a template for a reaction using the flanking primers. The product was cloned into CXCR using EcoRI and Csp45I restriction sites. The sequence of feline TRIM5 was deposited in Genbank (Accession number: GQ183880). A separate TRIM5 exon 8 PCR was also used in order to screen other carnivore species for the sequence of the TRIM5 B30.2 domain. Degenerate primers (gex8 feT5 f and gex8T5 r) were designed that allowed amplification from genomic DNA from divergent carnivore species, cat and dog. Expected sizes of amplicon were 525bp for dog and 536bp for cat and sequencing was performed directly from PCR products.

2.1.7 Capsid and cyclophilin A

N-terminal capsid domain (CA^N) was amplified from a molecular clone of FIV-Fca GL8 and from cDNA from Mya-1 cells infected with FIV-Ple B 458. The region amplified spans residues 136-273 of GL8 *gag*, and is analogous to the monomeric HIV-1 CA domain whose structure has been solved previously (Yoo

et al. 1997). For FIV-Fca, the expressed protein has a molecular weight of 15 kDa and is expressed with a C-terminal His tag, giving a total mass of 16.4 kDa. Primers used were G8 CAN5' Nde, G8 CAN 3'Mlu, LLV CA 5' Nde, and LLV CA 3' Mlu. For putative feline cyclophilin A genes encoded by Genbank contigs AANG01610851 and AC235013, Mya-1 RNA was treated with DNase I on the Qiagen RNeasy column prior to elution as indicated by the manufacturer and subsequent amplification. Differentiation between the two sequences was performed by using alternative 5' primers: feCypA R69 5' amplifies the sequence present in AANG01610851, whilst feCypA C69 5' amplifies the ORF present in Genbank: AC235013. 3' primer for both sequences was feCypA R/C 3'. Once the nature of the CypA expressed in T-cell was identified as AANG01610851, cDNA from Mya-1 and Angola-1 was used as a template for PCR using primers feCypA Nde 5' and feCypA Mlu 3'. Fragments were cloned into prokaryotic expression vector pOPTH.

2.1.8 APOBEC3 and *vif*

Feline and lion APOBECs were amplified from cDNA derived from Mya-1 and Angola-1 T-cells respectively. Primers were based on published domestic cat sequences single domain proteins feA3H (EU109281; primers feA3H F and feA3H R) and feA3Ca (AY971954; primers feA3C F and feA3C R). For read-through transcripts, forward primer feA3C F was used in conjunction with feA3H R. The amplicons were cloned into VR1012 using SalI and NotI restriction sites introduced by the primers. FIV-Fca *vif* was cloned from a molecular clone of strain GL8 using primers GL8 Vif F and GL8 Vif R, whilst FIV-Ple *vifs* were cloned from proviral DNA isolated from infected Mya-1 cells. Primers used were LLV-E Vif F and LLV-E Vif R for FIV-Ple E and for FIV-Ple B, sufficient homology to the E strain was present to use the LLV-E Vif F forward primer with strain

specific reverse primer LLV-B Vif R. Clones were sequenced as described above.

2.2 Cells and viruses

2.2.1 Adherent cells

Adherent cell lines were grown in disposable plastic tissue culture dishes (Corning, Ewloe, UK) and maintained in Dulbecco's modification of Eagle's medium (DMEM; Invitrogen) supplemented with 10% foetal bovine serum, 2 mM glutamine, 0.11 mg/ml sodium pyruvate, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Each cell line has certain growth characteristics and so were split when confluent rather than at a regular time point. When confluent, the monolayer was washed with PBS, and dissociated from the culture plate by incubation with 0.5-2ml 0.05% trypsin (Invitrogen) for a few seconds or minutes at 37°C. Trypsin activity was stopped by suspension in complete DMEM. Cells were then pelleted at 1000 rpm (~400 xg) in a benchtop centrifuge before resuspension in complete DMEM and seeding of new cultures at the required density. Where required, culture medium was supplemented by selection antibiotics G418 (400µg/ml; Invitrogen) or puromycin (1.25 µg/ml; Sigma Aldrich).

2.2.2 Suspension cells

Cells in suspension were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% foetal bovine serum, 2 mM glutamine, 0.11 mg/ml sodium pyruvate, 50 µM ß-mercaptoethanol, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Cells were passaged by centrifugation at 1000 rpm and resuspension in complete RPMI at the required density. Selection antibiotics were used as for adherent cells. Cell lines frequently used in this study the

canine lymphocytic leukaemia (CLL) and feline MCC lines (Cheney et al. 1990). CLL was generated by prolonged in vitro culture of leukaemic domestic dog PBMCs; the established line is $CD3^+$ $CD4^-$ $CD8^-$ $CD134^-$. When stably transduced with FIV primary receptor CD134, these cells can support replication of FIV to high titre (Willett et al. 2006a). MCC is a $CD134^-$ domestic cat cell line amenable to ectopic expression of CD134 variants to dissect the role of CD134 on FIV entry (Willett et al. 2006a; Willett et al. 2006b).

2.2.3 Primary T-cells

Domestic cat primary T-cell line Mya-1 (Miyazawa et al. 1989) was cultured in RPMI 1640 medium supplemented as above. A source of interleukin-2 (IL-2) was provided by further supplementing the medium with previously frozen conditioned medium from a murine cell line (L2.3) transfected with a human IL-2 expression construct (1:50 dilution, equivalent to 100 U/ml of recombinant human IL-2; provided by T. Miyazawa). African lion, European wildcat, puma, tiger, Amur leopard and cheetah peripheral blood mononuclear cells (PBMCs) were cultured as above and T-cells were expanded by stimulation with concanavalin A (2µg/ml; Sigma Aldrich) periodically. Blood samples from which PBMCs were obtained were kindly provided by Sarah Cleaveland, University of Glasgow, UK and John Lewis, ALTA Amur Leopard Conservation. PBMCs were also expanded from a seronegative Angolan lion and were periodically frozen down to generate a large back stock and referred to as Angola-1. Lion samples were collected by Pieter Kat and Rodrigo Serra of the Investigacao Veterinaria Independente, Lisbon, Portugal. Cells were grown in a cyclical manner, with a blasting stage following ConA stimulation in which polarised T-cells were apparent. During this stage, cells were grown at high density in order to promote cell-cell interactions, but large clumps were broken up in order to prevent isolation from nutrients. Blasts were preferentially selected for by

centrifugation at 800 rpm without brake, and resuspended in the same volume of medium. When a sufficient density of cells was obtained, cells were either split 1 in 2 or expanded to a larger tissue culture flask. During this phase, culture typically existed as single cell blasts until next ConA stimulation or in the case of Mya-1, sufficient density was obtained for clumping to recommence.

Cell line	Species	Adherent or Suspension	Reference
3201	Cat	S	(Hardy, Jr. et al. 1980)
AH927	Cat	S	Nelson-Rees, WA
Angola-1	Lion	S	This study
CLL	Dog	S	(Willett et al. 2006a)
CrFK	Cat	A	(Crandell et al. 1973)
HEK 293T	Human	A	(Graham et al. 1977)
HeLa	Human	A	
HT1080	Human	A	(Rasheed et al. 1974)
MCC	Cat	S	(Cheney et al. 1990)
MDTF	Mouse	A	(Lander and Chattopadhyay 1984)
Mv1 Lu CCL 64	Mink	A	(Henderson et al. 1974)
Mya-1	Cat	S	(Miyazawa et al. 1989)
NP2	Human	A	(Soda et al. 1999)
TE671	Human	A	(Stratton et al. 1989)

Table 2.A Cell lines used in this study

2.2.4 Replication-competent FIV

Supernatant containing clonal FIV was produced by transfection of 293T cells with full length FIV molecular clones using Superfect transfection reagent.

Strains used in this study include Glasgow 8 (GL8), a field isolate from the UK (Hosie and Jarrett 1990), B2542, a clade B USA isolate (Diehl et al. 1995) and PPR, a clade A USA isolate from a cat in the symptomatic stage of infection (Phillips et al. 1990). Three days post-transfection, 293T cells were co-cultivated with Mya-1 cells and FIV replication was monitored by p24 ELISA. 293T cells were removed and infected cultures were expanded by addition of fresh Mya-1 cells. Supernatant was collected, filtered at 0.45µm and frozen in 1ml aliquots at -80 °C until required.

FIV-Ple was isolated from lion PBMCs prepared from whole blood of an FIV-positive lion sampled in the Moremi reserve in Botswana (Sangre). Sangre was an eight-year old male lion in good health at the time of sampling and was previously confirmed as seropositive by western blot. In order to expand the viral stock from Sangre, concanavalin-A-stimulated PBMCs were co-cultivated with Mya-1 cells and confirmation of replication-competent virus was sought by reverse transcriptase assay (see 2.3.2). Aliquots of viral supernatant were stored and frozen at -80 °C for future use and infected cells were pelleted and harvested for total cellular RNA.

2.2.5 Tissue culture infection with replication competent FIV

Infections were performed by incubating target cells with viral inocula. For adherent lines, cells were seeded at low density ($1x10^5$ cells per well in a 6 well plate) and left to adhere overnight. Standard conditions for suspension cells were: $1x10^6$ Mya-1/primary PBMC or $5x10^5$ CLL-CD134 in a 5 ml tube (Falcon). Cells were then incubated with viral inoculum (100-500µl) diluted 1 in 2 with complete medium as appropriate at 37 °C for 3 hours. Cells were washed twice with PBS and resuspended in complete medium. Cell-free aliquots were taken periodically and frozen at -80 °C until required for viral quantification by p24 ELISA or reverse transcriptase assay.

2.2.6 Drugs and inhibitors

Inhibitors of cyclophilin, cyclosporine A (CsA; Sigma), NIM811 (a kind gift of Prof. Anthony Page, University of Glasgow) and Debio-025 (obtained from Debiopharm) were diluted to 5 mM working stock in ethanol or DMSO and stored at -20 °C until required. Toxicity was measured by incubating cell lines Mya-1 and CLL with CsA, NIM811 and Debio-025 at 2 µM for five days before enumerating live cells using a haemocytometer with Trypan blue (Invitrogen) dye exclusion as a marker for viability. The effect of the drugs on the replication of FIV in canine and feline cells was assayed by incubating 10^6 CLL or Mya-1 with FIV-Fca GL8 for three hours before washing twice with PBS and resuspension in 1 ml RPMI, supplemented with IL-2 in the case of Mya-1, with CypA inhibitors at 2 µM or an equivalent volume of solvent.

Proteasome inhibitor MG132 (N-(benzyloxycarbonyl)leucinylleucinylleucinal) and As_2O_3 were obtained from Sigma. MG132 was prepared by dissolving in ethanol at a stock concentration of 1 mg/ml and stored at -20 °C. As_2O_3 was prepared as (Berthoux et al. 2003) by initially dissolving in 1 M NaCl before neutralisation with HCl to final pH 7.0. The solution was diluted further to a concentration of 1 mM with PBS, filter sterilised at 0.2 µm before storage at -20 °C. 1 x 10^5 cells were incubated with MG132 or As_2O_3 for 3 hr before aspiration of the drug and washing with PBS. Cells were reseeded in 100 µl replete RPMI with IL-2 and challenged with pseudotype.

AMD3100 (Donzella et al. 1998), a bicyclam inhibitor of the FIV Env-CXCR4 interaction (Egberink et al. 1999; Richardson et al. 1999), was prepared for tissue culture by dissolving in PBS and filter sterilising at 0.2µm and stored at -20 °C. Inhibition of Env-CD134 interaction was performed using trimeric sFc-TNC-CD134L. The preparation and purification of this construct was performed previously (Willett et al. 2009). The construct consists of a haemagglutinin

signal peptide, the hinge and Fc domain of human IgG1 (residues 104–330 of GenBank: P01857), a linker with sequence RSPQPQPKPQPKPEPEGSLH, the trimerisation domain of chicken tenascin-C (TNC), a linker with sequence GTGGGSGGRGLQ, and the extracellular domain (residues 52–183) of feline CD134L. The construct had previously been transfected into 293T cells using Superfect (Qiagen) by other lab members and supernatant harvested after three days and concentrated 10-fold using an Amicon 10kDa molecular weight cut off centrifuge spin-concentrator (Millipore) and stored at -80 °C before use.

2.2.7 Stable transduction of cell lines

In order to create cell lines stably expressing TRIM5, pseudotyped MLV infectious particles were produced by transfecting HEK293T cells with plasmids CMVi, a cytomegalovirus promoter-driven MLV gag-pol expression vector, pMDG bearing pantropic vesicular stomatitis virus G-glycoprotein (VSV-G) envelope and CTCR which encodes a dsRed reporter gene (Clontech, Wokingham, UK) and the desired TRIM5 under the control of the cytomegalovirus promoter as previously described (Besnier et al. 2002), except that Superfect (Qiagen) transfection agent was used according to manufacturer's instruction. Supernatant was harvested 3 days post-transfection, filtered at 0.45µm and used to transduce target cell lines plated at $5x10^4$ cells per well in 6-well plates. Successful transduction was confirmed by enumerating RFP-expressing cells on a Beckman Coulter EPICS MCS-XL flow cytometer. Data were analyzed using EXPO 32 ADC Analysis (Advanced Cytometry Systems).

Lion CXCR4 was expressed as a fusion protein with enhanced green fluorescent protein as has previously been described for feCXCR4 (Willett et al. 2002). For stable dual expression of CXCR4 and CD134 in the NP2 cell line, cells were first transduced with pDONAI vectors bearing lion or domestic cat CXCR4-eGFP

fusion proteins and stable transductants were transduced again with lion or domestic cat CD134 and immuno-enriched by staining firstly with 7D6 anti-feline CD134 (AbD Serotec, Oxford, U.K.) then goat-anti-mouse IgG-coated microbeads (Miltenyi Biotec Ltd., Bisley, U.K.) followed by enrichment on MS columns using an OctoMACS™ separator (Miltenyi Biotec Ltd.). Eluted cells were maintained in G418 supplemented medium and expanded in culture for use in pseudotype infection assays.

2.2.8 Pseudotype production

Retroviral particles capable of single round infection, which deliver a marker gene to the target cell, were used to analyse viral and cellular determinants of infection. The constructs allow the generation of retrovirus particles that bear reporter genes that allow the efficiency of transduction to be assayed. Pseudotypes in this study bore either luciferase (luc) or green fluorescent protein (GFP) and transduction was quantified using alternative techniques. The use of vesicular stomatitis virus G-glycoprotein (VSV-G) allows efficient entry to mammalian cells and obviates the effects of differential expression of receptors. Pseudotypes are denoted VIRUS-reporter(ENVELOPE). For example HIV-luc(FIV) indicates an HIV particle, bearing a luciferase gene, pseudotyped with FIV Env.

HIV-luc(FIV) vectors were prepared in HEK293T cells by two-plasmid co-transfection. The day before transfection, $4x10^6$ 293T cells were seeded in 10 cm poly D-lysine coated plates (BD). To transfect, 10 µl Superfect transfection reagent (Qiagen) was mixed with 7.5 µg pNL4-3-Luc-E^-R^-, an HIV-1 NL4-3 strain-based vector with firefly luciferase inserted into the *nef* gene and defective *env* and *vpr* genes (Connor et al. 1995), and 7.5 µg VR1012-*env*, a mammalian expression plasmid bearing FIV *env* in 300 µl serum-free DMEM. Complexes were allowed to form at room temperature for 15 minutes before

addition of 3 ml complete DMEM and incubation on 293T cells at 37 °C for 3 hours. Cells were then washed with PBS to reduce toxic effect and prevent any transfection complexes affecting downstream procedures. 10 ml fresh, complete DMEM was added to cells and supernatant was harvested after 72 hours incubation at 37 °C. Supernatant was filtered at 0.45µm, aliquoted and frozen at -80 °C.

For other retroviral vectors, a three-plasmid co-transfection protocol was used. Plasmids expressing a gag-pol, a viral vector and pMDG were transfected into 293T cells at a ratio of 1:1:1 (5 µg each). The protocol follows that of (Besnier et al. 2002) except that Superfect (Qiagen) was usually used instead of Fugene-6, as higher titres were given by the former. Plasmids used for production of VSV-G pseudotyped N- and B-tropic MLV, HIV-1, HIV-2, SIVmac and FIV are given in Table 2.B. Supernatant was collected after three days, filtered at 0.45µm and stored at -80 °C. When produced in the presence of drugs, NIM811 and cyclosporine A, pseudotypes were pelleted in a Beckman ultracentrifuge in an SW40 rotor over a 2 ml 20% sucrose cushion to remove drugs. The pellet was resuspended in 1 ml DMEM (10-fold concentration) before use.

Plasmid name	Derived from	Function	Reporter	Reference
pCIG3N	MLV-N	gag-pol		(Bock et al. 2000)
pCIG3B	MLV-B	gag-pol		"
CNCG	MLV	packaged sequence	GFP	"
CNCR	MLV	packaged sequence	dsRed	"
p8.91	HIV-1	gag-pol (ΔVif)		(Naldini et al. 1996)

p8.2	HIV-1	gag-pol (+Vif)		"
SIN CSGW	HIV-1	packaged sequence	GFP	"
SIN CSRW	HIV-1	packaged sequence	dsRed	"
pNL4-3-Luc-E$^-$R$^-$	HIV-1	gag-pol and packaged sequence	luciferase	(Connor et al. 1995)
SIV4+	SIV mac	gag-pol (ΔVif)		(Negre et al. 2000)
SIV3+	SIVmac	gag-pol (+Vif)		"
SIV eGFP	SIV mac	packaged sequence	GFP	"
pSVRΔNBDM	HIV-2	gag-pol		(Griffin et al. 2001)
pSVRΔNB-GFP-ΔH	HIV-2	packaged sequence		"
FP93	FIV	gag-pol		(Poeschla et al. 1998)
GinSin	FIV	packaged sequence	GFP	"
pMDG	VSV	envelope		(Yee et al. 1994)

Table 2.B Plasmids used for pseudotype production. Packaged sequence denotes plasmids that encode an RNA genome that is packaged within pseudotypes.

2.2.9 Pseudotype assay

For luciferase reporter gene assays, $5x10^4$ target cells were seeded in 50 µl complete medium in a CulturPlate-96 assay plate (Perkin-Elmer, Waltham, MA, USA). For adherent cell lines, the cells were allowed to settle overnight whilst suspension cells were used immediately. 50µl supernatant containing the pseudotype was added to wells and each condition was repeated in triplicate. Expression of luciferase was quantified after 48-72 hours by addition of 100µl

Steadylite HTS luciferase substrate (Perkin-Elmer) and measurement by single photon counting on a MicroBeta luminometer (Perkin-Elmer).

When using GFP or dsRed reporter genes, target cells were seeded at $1x10^5$ cells per well in a 6 well plate and allowed to adhere overnight. Supernatant was thawed and titrated onto target cells in the presence of hexadimethrine bromide (polybrene; Sigma Aldrich) dissolved in PBS at 5 µg/ml. After 48-72 hr, GFP expressing cells were harvested and pelleted in 5 ml flow cytometry tubes (Falcon). Cells were resuspended in 0.5-1 ml PBA buffer and kept on ice. GFP-positive cells were enumerated by observing 10,000 events on a Beckman Coulter EPICS MCS-XL flow cytometer. Gates were adjusted so that negative control untransduced cells gave 0% infection. Data were analyzed using EXPO 32 ADC Analysis (Advanced Cytometry Systems). Where necessary, multiplicity of infection (moi) was subsequently calculated using the formula moi = -lnP[0] where P[0] is the proportion of uninfected cells.

A modified protocol was employed for the production of FIV-GFP(VSV) pseudotypes in the presence of APOBEC proteins. 293T cells were plated at 2 x 10^5 cells per well in a 24 well plate and allowed to adhere overnight at 37 °C in 500 µl replete medium. The next day, transfections were performed using Superfect (Qiagen) transfection reagent with a modified protocol. 0.3 µg each of pMDG, GinSin, FP93, 0.5 µg VR1012-A3 and, where indicated, 0.5 µg VR1012-Vif or empty vector were mixed in 20µl serum free DMEM before addition of 2 µl Superfect. Transfection complexes were allowed to form for 15 minutes before the addition of 50 µl replete DMEM and dropwise addition to the 293T cells. Medium was not changed after this stage. Supernatant was collected three days post transfection and centrifuged at 5,000 xg to remove cellular debris. The titre of infectious virus was assayed by plating onto 293T or CrFK

cells in the presence of polybrene (5 µg/ml) and GFP expression monitored after three days by flow cytometry.

2.2.10 RNA interference

Small hairpin (sh) RNAs against domestic cat TRIM5 were designed using shRNA Sequence Designer (Clontech). The chosen target sequences for constructs feT5sh1 and feT5sh2 were GCATCACTGCAAAGACCAA and CTGTAAGACGGATGGGAAG respectively, or a firefly luciferase sequence for shLuc negative control. Top and bottom strand 60bp oligonucleotides were designed using Clontech online shRNA Sequence Designer and synthesised by Eurofins MWG Operon. Nucleotides were annealed by heating to 95 °C and cooling slowly to room temperature. Annealed oligonucleotides were cloned into pSIREN Retro-Q (Clontech) using BamHI and EcoRI restriction sites according to manufacturer's instructions. Infectious MLV particles were generated by co-transfecting pSIREN Retro-Q feT5sh1, feT5sh2 or shLuc with CMVi and pMDG as above. Supernatant was used to transduce CrFK cells expressing feline CD134 (Shimojima et al. 2004), which were exposed to puromycin selection 2 days post transduction. Knockdown of endogenous TRIM5 in these cells was monitored by RT-PCR on serial dilutions of cDNA using primers wam4 and wam13c. RNA was prepared and reverse transcribed as above using 1µg total RNA isolated from 10^6 cells. Reaction conditions were 98 °C 60s, then 30 cycles of 98 °C 10s, 67 °C 30s, 72 °C 30s then 72 °C 2mins on 1µl target cDNA with Phusion High-Fidelity DNA Polymerase (New England Biolabs), using the reaction components described above (Section 2.1.1). Agarose gel electrophoresis images were scanned using an Epson 3200 Perfection flat-bed scanner; contrast and brightness were adjusted in Adobe Photoshop.

2.3 Quantitative techniques and immunoloblotting

2.3.1 p24 ELISA

For monitoring the levels of infection in replication-competent FIV assays, cell-free supernatant from infected cells was collected periodically and stored in a 96 well plate at -80 °C until required. Viral replication was measured by enzyme-linked immunosorbent assay (ELISA) of FIV p24 ELISA by coating immunoabsorbant 96-well plates (Greiner Bio One, Stonehouse, UK) with 100µl anti-p24 antibody vpg-62 diluted 1 in 500 with serum free DMEM and incubated at 4 °C overnight. Plates were blocked with 125µl 2% milk powder (ASDA) dissolved in PBS for 2h before washing with 0.2% Triton X-100 PBS. Alternatively pre-coated ELISA plates (Pet-check anti-FIV IDEXX, Chalfont St Peter, UK) were used. Antigen capture was performed using 100µl thawed cell culture supernatant with 10µl 0.5% Triton X-100 dissolved in PBS to release viral cores at room temperature for 30min. Plates were washed with ELISA wash buffer according to manufacturer's instructions and 100µl anti-FIV HRPO conjugate Ab (IDEXX) was added before development with HRPO substrate (IDEXX). Plates were read on an Ascent Multiscan plate reader (Labsystems) at 450nm.

2.3.2 Quantification of reverse transcriptase activity

In cases when p24 ELISA was not appropriate, for example when monitoring non-domestic cat strains of FIV for which no commercial antibodies are available, reverse transcription activity was assayed using a Lenti-RT nonisotopic RT assay kit (Cavidi Technology, Uppsala, Sweden). The technique uses sample dilution buffer (SDB) to release RT which is quantified by its ability to reverse transcribe a poly(rA) template using an oligo(dT) primer and incorporation of nucleotide analogue, 5-bromo-deoxyuridine-5′-triphosphate

(BrdUTP) during incubation at 33 °C. The reaction is stopped by washing in ELISA wash buffer or with wash buffer supplied with the kit and BrdU levels quantitated using an anti-BrdU Ab and chromogenic detection by alkaline phosphate-conjugated secondary Ab using para-nitrophenyl phosphate substrate followed by reading on an Ascent Multiscan plate reader at 405 nm.

2.3.3 Real-time PCR

TRIM5 knockdown was quantified using real-time PCR with primers feT5 TaqMan 5' and feT5 TaqMan 3' and fluorescently-labelled feT5 Probe 5' 3'. RNA was prepared from CrFKs or CrFKs transduced with feT5sh1 and feT5sh2 and 2µg was used in reverse transcription using Roche High Fidelity Transcriptor with an oligo d(T) primer in a 20 µl reaction according to manufacturer's instructions. 2 µl of the resulting cDNA was mixed with primer at 0.24 pM and probe at 0.12 pM and was used in a reaction with 2x Universal TaqMan Mastermix (Applied Biosystems) in a 50 µl reaction. Cycling conditions were 95 °C 5 min, followed by 40 cycles of 95 °C 15s, 55 °C 60s on an ABI 7500 thermal cycler (Applied Biosystems). Data were analysed using Sequence Detection Software v1.4 (Applied Biosystems).

2.3.4 SDS-PAGE & Immunoblotting

For the analysis of seroprevalence in wild lions, FIV-Ple 458-infected Mya-1 supernatant was filtered at 0.45 µm and virus was pelleted in an SW28 ultracentrifuge rotor (Beckman, UK) at 28,000 rpm. The viral pellet was resuspended in SDS loading buffer. For Ranbp2 western blot, 1,000 293T cells were lysed directly with SDS-PAGE loading buffer. Proteins were boiled for 5 min before being run on Tris-Acetate minigels (Invitrogen). Antigens were transferred to nitrocellulose membrane using rapid dry blotting system (iBlot, Invitrogen) and blotted overnight in Western Blocking Buffer. Antigens were

detected using lion sera from either field isolates or pooled sera from FIV-seronegative or seropositive domestic cats. For Ranbp2, a rabbit monoclonal Ab was used (AbCam ab2938) at 1:1000 dilution. Bound antibody was detected using biotinylated goat anti-cat IgG for FIV-Ple antigens or anti-rabbit IgG for Ranbp2 (Vector laboratories) followed by chromogenic development by the Vectastain® ABC system (Avidin: Biotinylated enzyme Complex) and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) substrate.

2.3.5 Far western blot

For far western blotting (also known as overlay assay), HeLa and CrFK cell lysates were fractionated by incubation with CHAPS lysis buffer on ice for 10 min to lyse cell membranes before centrifugation in a benchtop centrifuge for 10 min at 13k rpm (~16,000 xg). Supernatant (cytoplasmic extract) was removed and diluted in 2x SDS-PAGE loading buffer. The pellets (nuclei) were resuspended by dropwise addition of 1x SDS-PAGE loading buffer without bromophenol blue, with vigourous vortexing after the addition of each drop, before dilution in 2x SDS-PAGE buffer corrected for levels of bromophenol blue. For 293T cells, 1,000 cells were lysed directly with 1x SDS-PAGE loading buffer. Proteins were subjected to SDS-PAGE and transferred to nitrocellulose membranes as above and incubated overnight in FW blocking buffer at 4 °C in the absence of harsh detergent to promote protein refolding. Far western protocol follows (Einarson et al. 2007), but uses immunodetection in place of autoradiography. 30-90 µg recombinant CA or CA^N was diluted in FW interaction buffer and allowed to bind at 4 °C with gentle shaking. The membranes were washed three times for 10 min each with FW wash buffer 1 before a final wash with FW wash buffer 2. A western blot for capsid was then performed using a mixture of monoclonal mouse anti-FIV CA antibodies AE11, DF3 and DF10

(1:2000 each). Chromogenic detection was via the Vectastain® ABC system as above.

2.4 Biophysical and structural techniques

2.4.1 Recombinant protein purification

Expression, purification and biophysical techniques were performed at the MRC Laboratory of Molecular Biology, Cambridge in collaboration with Dr Leo James with guidance from Amanda Price. His-tagged proteins were expressed in 2l *E. coli* cultures grown in TY broth at 37 °C by induction with 0.1 mM IPTG. Cells were lysed on ice with Bug Buster (Novagen) with 3 min sonication and proteins were bound to Ni-NTA beads (Qiagen), washed with Beads Wash Buffer (BWB) and eluted in BWB + 300 mM imidazol (Figure 2-1).

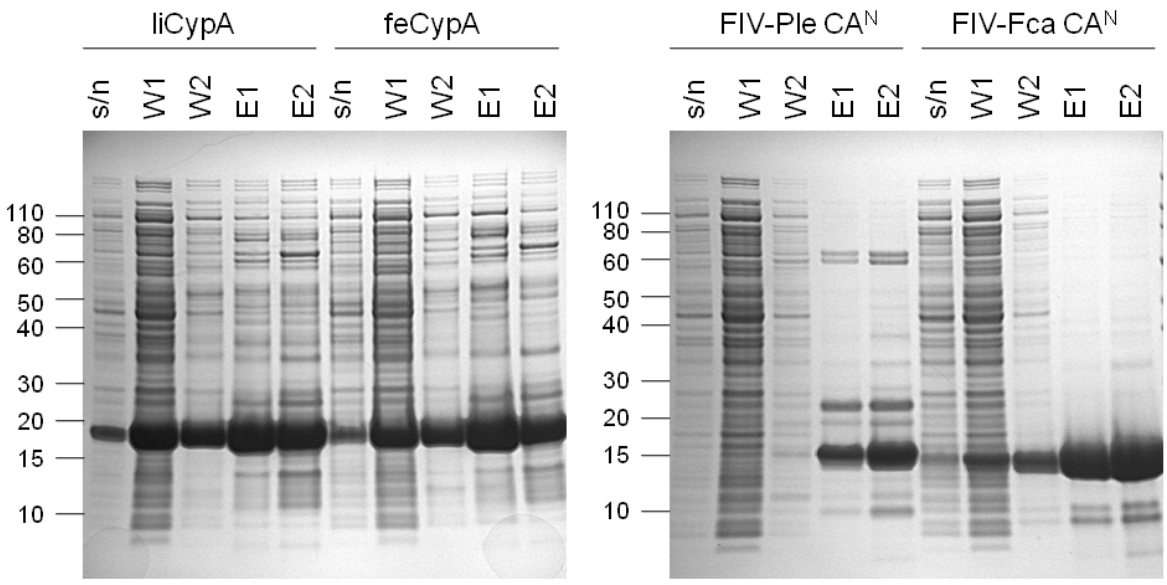


Figure 2-1 Ni-affinity purification of His-tagged proteins. CypA variants from lions and domestic cats as well as FIV-Fca and FIV-Ple capsid N-terminal domains (CA^N) were cloned and expressed as His-tagged recombinant proteins. Ni-affinity chromatography was used to purify protein from bacterial lysate (s/n). Beads were washed twice (W1 and W2) before a two-stage elution (E1 and E2). 10µl aliquots from each stage were analysed by SDS-PAGE.

Eluted proteins were pooled and loaded onto a gel filtration column (GE Healthcare) and collected in 4ml fractions on an Akta FPLC System (GE Healthcare) (Figure 2-2). Sample buffer was 20mM Tris pH 7.4, 100mM NaCl, 1mM DTT for all proteins. For capsid and mutants of capsid, an additional Q ion-exchange chromatography (GE Healthcare) chromatography step was performed in order to remove low molecular weight contaminants (Figure 2-3). Protein was eluted by increasing salt concentration, using 10% buffer B (75mM Tris pH 8.0, 1M NaCl). Pure protein preparations were pooled, desalted and concentrated using Amicon centrifuge concentrators (Millipore). Protein aliquots were snap-frozen in liquid nitrogen and stored at -80 °C prior to use.

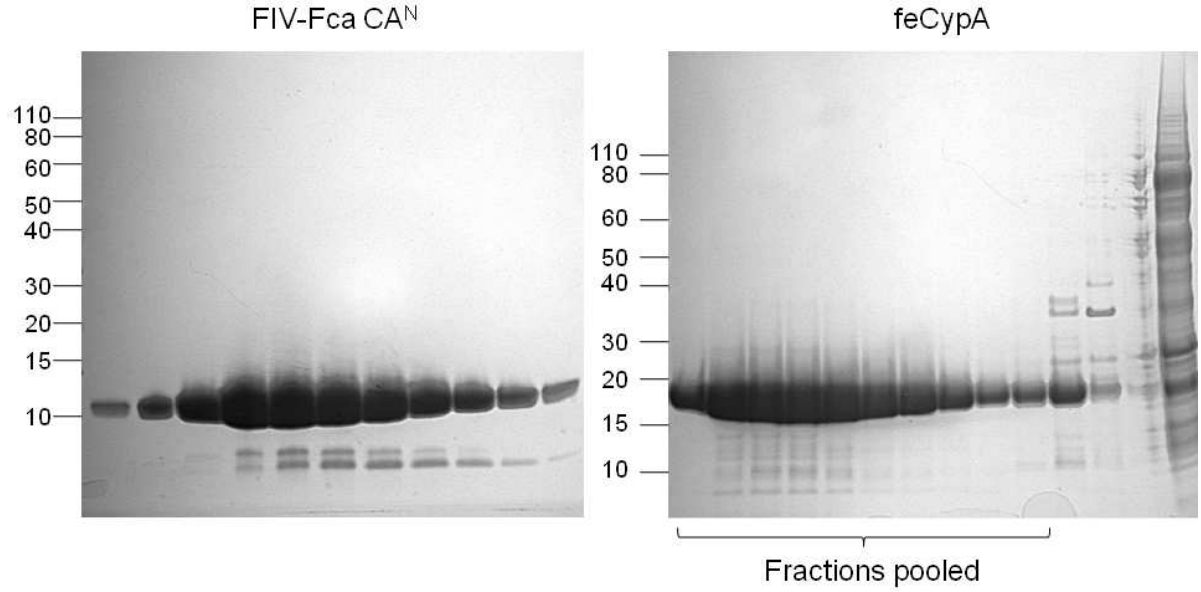


Figure 2-2 Gel filtration of recombinant proteins. CypA and capsids were purified by gel filtration and fractions were run on an SDS-PAGE gel to assess protein purity. Shown are FIV-Fca CA^N and feCypA as representative proteins. CypA was deemed sufficiently pure after this stage and fractions were pooled, concentrated and frozen for future use.

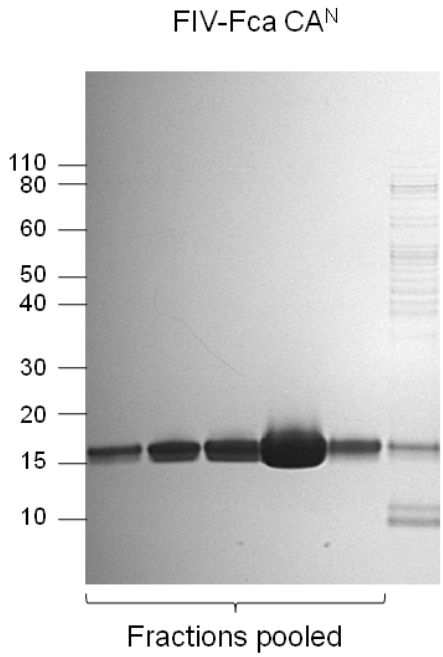


Figure 2-3 Ion exchange chromatography for FIV-Fca CA^N. Protein was eluted by increasing the proportion of buffer B. Lanes 1-5 are taken from fractions eluted with 10% B, whilst the final fraction, bearing contaminants, was eluted with 50% B. The process was repeated for all capsid variants and mutants. Pure proteins were pooled, concentrated and snap-frozen before further use.

2.4.2 Isothermal Titration Calorimetry

ITC is a technique that measures the change in heat associated with the interaction of two molecular species (technique reviewed in Pierce et al. 1999). The apparatus consists of a reference cell and a reaction cell pre-loaded with one of the interacting samples (species A) into which species B is repeatedly injected. Changes in enthalpy in the reaction cell associated with each injection of B produce a change in the power supplied to the reference cell in order to keep the temperature in the cells the same. Thus, the raw data produced from each experiment is a time-dependent series of spikes measured in µcal/s, with negative values indicating an exothermic reaction. Typically, the first few injections of species B will result in all B being bound, giving several large spikes. As the fraction of unbound species A decreases,

subsequent injections will result in smaller changes in enthalpy until no binding is observed. Integration of the spikes allows the calculation of the total change in heat for each injection and curve fitting, along with accurate measurements of the concentrations of the species, allows the calculation of molar stoichiometry (N), dissociation constant (Kd) and the molar change in enthalpy (ΔH). The dissociation constant, Kd, is a measure of the strength of interaction and has a value in molar concentration that corresponds to the concentration at which half of the binding sites are occupied, so that lower values represent stronger binding. ΔH has a value in cal/mol, reflecting the change in heat associated with one mole of interactant binding its ligand. The change in heat reflects the preference of the ligand for its binding partner over the solvent, with negative values indicating enthalpically favoured interactions. In the context of protein-protein interactions, hydrogen-bonding, burial of hydrophobic solvent-exposed surfaces and Van der Waals can all contribute to the ΔH.

For the experiments in this study an ITC200 (MicroCal) machine was used, previously calibrated with EDTA $CaCl_2$ according to manufacturer's instructions. Cell and syringe samples were dialysed overnight at 4 °C into the same beaker of potassium phosphate buffer (20 mM $K_xH_xPO_4$, 20 mM NaCl, 5 mM ß-mercaptoethanol, pH 7.0). Proteins were then diluted to 600 µM for syringe sample and 60 µM for cell sample and re-quantified by measuring absorbance at 280 nm. ITC was performed at 10 °C.

2.4.3 X-ray crystallography

Initial crystal screens varying in buffer constitution were performed in 200 nl droplets comprising 100 nl protein and 100 nl reservoir buffer in sitting drop vapour diffusion 96-well plates. Reservoir buffers and protein were dispensed by Screenmaker 96+8 robot (Innovadyne). Proteins were at a typical

concentration of 12 mg/ml. Domestic cat CypA and CypA-peptide complexes were dialysed into 20 mM HEPES, 20 mM NaCl, 1 mM ß-mercaptoethanol, pH 7.0, whilst FIV CA^N and CA^N –CypA complexes were dialysed into 20 mM Tris, pH 7.4. Screens were set up in 15x 96-well plates and yielded several potential hits (Figure 2-4). Conditions were further optimised with focus screens, varying in the relative concentrations of cryoprotectants PEG 4000 and glycerol, and by the use of additive screens (Hampton Research). The final conditions for FIV CA^N were 0.16 M ammonium sulphate, 0.08 M sodium acetate trihydrate, 20% w/v PEG 4000 and 20% v/v glycerol.

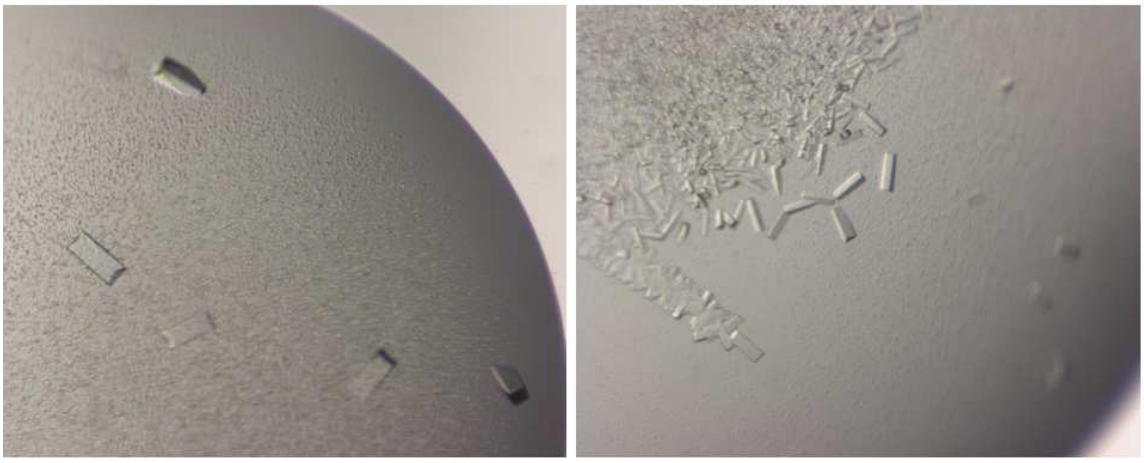


Figure 2-4 FIV capsid N-terminal domain crystals. From a 15x 96 well plate screen, crystals were found in conditions shown (left). An additive screen was performed (right) but did not yield larger crystals. Final conditions excluded additives.

For CypA and CypA-peptide complexes, crystals were obtained in 2.4M ammonium phosphate, 0.1M Tris, pH8.5. CA^N-CypA complex screens were set up and yielded crystals in the same conditions as CypA and CA. However, these

were found to consist of single protein species. Data were collected on a Rigaku rotating anode X-ray set with Mar research image plate and solved by Dr. Leo James. Figures were generated by manipulation of pdb files in PyMol. Superimposition of structures was performed using EBI DaliLite server (http://www.ebi.ac.uk/DaliLite) and aligned pdb files were viewed in PyMol.

2.5 *In silico* techniques

2.5.1 Phylogenetic analysis

Amino acid sequences of full length TRIM open reading frames and viral FIV-Ple E Sangre, FIV-Ple B 1027 and FIV-Fca GL8 sequences were aligned using Clustal W (Thompson et al. 1994) with manual adjustment in Bioedit (Hall 1999). *env* and *gag* alignments were created using multiple alignments of FIV sequences available in Genbank. The codon-optimised nucleic acid sequences were used to construct trees using the Neighbour-Joining method in Mega 4.0 software (Tamura et al. 2007; Saitou and Nei 1987) with 1000 replicates to allow calculation of bootstrap values. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset, meaning for TRIM5 that only the RBCC domain was used in the analysis. For viral sequences, almost identical trees were obtained using Maximum Likelihood method (not shown), suggesting that the trees shown represent a robust fit. N-linked glycosylation sites were predicted using NetNGlyc online tool (http://www.cbs.dtu.dk/services/NetNGlyc) to locate NX(S/T) motifs, where X is any amino acid except proline. Routine manipulation of sequences, primer design and analysis of sequencing runs was performed with DNADynamo (BlueTractor Software).

2.5.2 Graphs and statistics

All graphs were drawn with SigmaPlot 8.0 and, where shown, error bars represent the standard error. Unpaired two-tailed student's t-tests were performed using the same software.

2.5.3 Homology modelling

Templates for a homology model of feline A3H were sought using HHpred software (Soding et al. 2005) to identify the human A2 (2NYT; Prochnow et al. 2007) and A3G CD2 (3E1U; Holden et al. 2008) structures as sharing the highest degree of homology with the query protein. Conservation of secondary structures was confirmed using the Ali2D software on the Max Planck Bioinformatics Server (http://toolkit.tuebingen.mpg.de), which predicted a shared secondary structure with A3G CD2. Subsequently, MODELLER 9v3 (Eswar et al. 2007) was used to create a 3-dimensional model of feline A3H using both A2 and A3G CD2 crystal structures as templates. For feline CD134, the crystal structure of the human homologue (2HEY; Compaan and Hymowitz 2006) was used as a template. Predicted structures were superimposed on A3G CD2 crystal structure using Dailite and structures were viewed in PyMol.

Chapter 3. Receptor tropism of feline immunodeficiency virus isolates from African lions.

3.1 Summary

The feline immunodeficiency virus (FIV) is a major pathogen of domestic cats but also infects non-domestic species including the African lion (*Panthera leo*). Recent analysis of the proviral genome of two African lion FIV (FIV-Ple) field isolates belonging to subtypes B and E suggests that the *env* open reading frame of the E subtype shares greater homology with FIV strains that infect domestic cats (FIV-Fca) than to other strains of FIV-Ple (Pecon-Slattery et al. 2008a). In this study we ask whether this shared identity between FIV-Fca and FIV-Ple E corresponds with shared receptor tropism. Despite only 53% amino acid identity in Env SU, FIV-Ple E is found to share use of CD134 as the primary receptor for attachment. Furthermore, infection by FIV-Ple E is also CXCR4-dependent and can be inhibited by CXCR4 antagonist AMD3100 and soluble trimeric CD134-ligand. We also confirm previous studies which have suggested that FIV-Ple B uses an alternative entry receptor (Smirnova et al. 2005; Willett et al. 2006a): productive infections of the B subtype could be established in CD134 negative lines, whilst replication of the E subtype was dependent on ectopic expression of feline CD134. The shared usage of CD134 and CXCR4 in

FIV-Fca and FIV-Ple E suggests that a relative of FIV-Fca has recombined with African lion strains of FIV, preserving its use of receptor. The findings give an indication of the *in vivo* cell tropism of FIV-Ple E and have implications for future research into the pathogenicity of FIV in wild lion populations.

3.2 Results

In the light of findings that lentiviruses with divergent *env* genes circulate in African lion populations (Pecon-Slattery et al. 2008a), it was attempted to assess whether diversity between FIV-Ple subtypes B and E extended to alternative receptor usage.

3.2.1 Sera from wild lions show immunoreactivity to FIV-Ple

A representative of FIV-Ple subtype B was available (FIV-Ple B 458), but not a subtype E virus. A panel of lion sera was therefore obtained from wild lions in the Moremi reserve in the Okavango Delta, and screened for seroreactivity to viral antigens. Proteins of pelleted FIV-Ple 458 (B subtype) were run on an SDS-PAGE gel, transferred to a nitrocellulose membrane and blotted using the sera of 27 wild lions (Figure 3-1). As controls, pooled sera from uninfected and FIV-Fca-infected cats were also included in the assay. Immunoreactivity to the principal determinant p24 is found in 24 of the 27 serum isolates (89%), in line with previous estimates of populations east of the Kalahari desert (Kalahari game reserve 71% positive (Ramsauer et al. 2007); Okavango Delta 96% (Roelke et al. 2009); Serengeti ~100% positive (Brown et al. 1994)). Unlike typical immune responses to FIV-Fca and HIV-1, previous studies have found only immunoreactivity in FIV-Ple infections to the proteins derived from the Gag polyprotein. Here we find evidence of a humoral response to Env in 13 out of 24 (54%) of the seropositive lions. The specificity of the immunoreactivity to Env is revealed by the fact that serum from an infected domestic cat does not

cross-react with FIV-Ple B Env. Thus, rather than suggesting that only a proportion of lions mount a humoral response against Env, the absence of Env reactivity in certain wild lions may best be explained by the presence of multiple subtypes of FIV-Ple in the sample. Indeed, the serum from the lion Sangre (Figure 3-1, Lane 25), who we later show to be infected with an E-subtype strain, only weakly reacts to the FIV-Ple B 458 Env antigen.

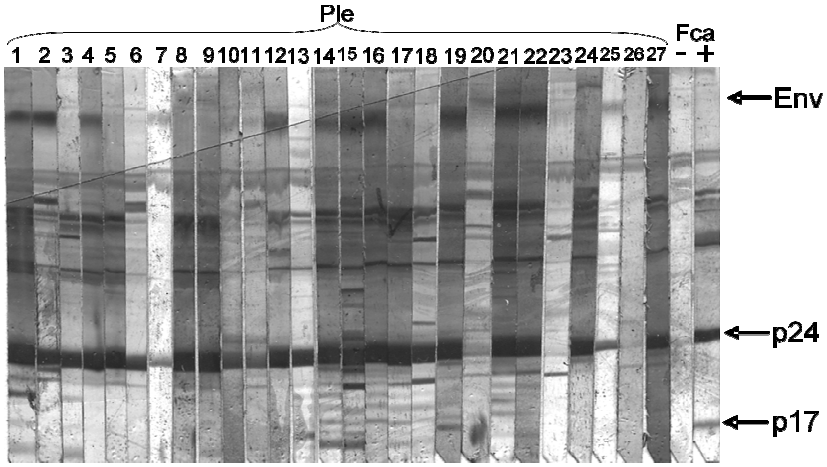


Figure 3-1 Immunoreactivity of sera from lions from the Moremi reserve to FIV-Ple B 458. Sera from wild 27 lions (1-27), pooled uninfected domestic cat serum (-) and pooled FIV-infected cat serum (+) were used to probe a membrane bearing FIV-Ple B 458 virus subjected to SDS-PAGE. Bands corresponding to the principal reactive species p24 reveal that all lions were seropositive except animals 20, 23 and 26. Serum of the lion Sangre, from whom replication-competent isolate FIV-Ple E was derived, is lane 25. Blot performed by BJW.

3.2.2 Virus isolated from African lion Sangre belongs to subtype E

PBMCs were isolated from 15 seropositive isolates and two of these, Chianti and Sangre, yielded replication competent virus. Viral nucleic acid was isolated from Sangre by co-culturing infected PBMCs with Mya-1 feline T-cells and harvesting total cellular RNA. Primers directed to published FIV-Ple E 1027 sequence (Pecon-Slattery et al. 2008a) were used to amplify the Env gene of

Sangre by RT-PCR and the amplicon was cloned into VR1012 using SalI and NotI digestion sites. The Env of FIV-Ple B 458 (Brown et al. 1994) was also cloned and used as a representative of FIV-Ple B subtype. Analysis of the sequences reveals that FIV-Ple E Sangre shares 88% amino acid identity with the FIV-Ple E 1027 isolate used for the genomic sequence of FIV-Ple E subtype (Pecon-Slattery et al. 2008a) and 53% identity with FIV-Fca GL8. A Neighbour-Joining tree was constructed by aligning the entire *gag* and *env* genes from several published FIV isolates available in the NCBI GenBank (Figure 3-2). In a similar finding to (Pecon-Slattery et al. 2008a), phylogenetic relationships for the two genes show substantial differences, suggesting alternative evolutionary histories for the *gag* and *env* genes, and highlighting the relevance of inter-genomic recombination events in the evolution of FIV. The *env* gene of the virus isolated from Sangre is found to cluster closely with FIV-Ple E 1027 (E subtype), and distally from other FIV-Ple strains B and C, confirming its identity as a subtype E virus. More widely, the *env* gene phylogeny forms two distinct groupings: those related to the puma group, including FIV-Ple B and FIV-Oma and those related to the domestic cat group, including both FIV-Ple E 1027 and FIV-Ple E Sangre.

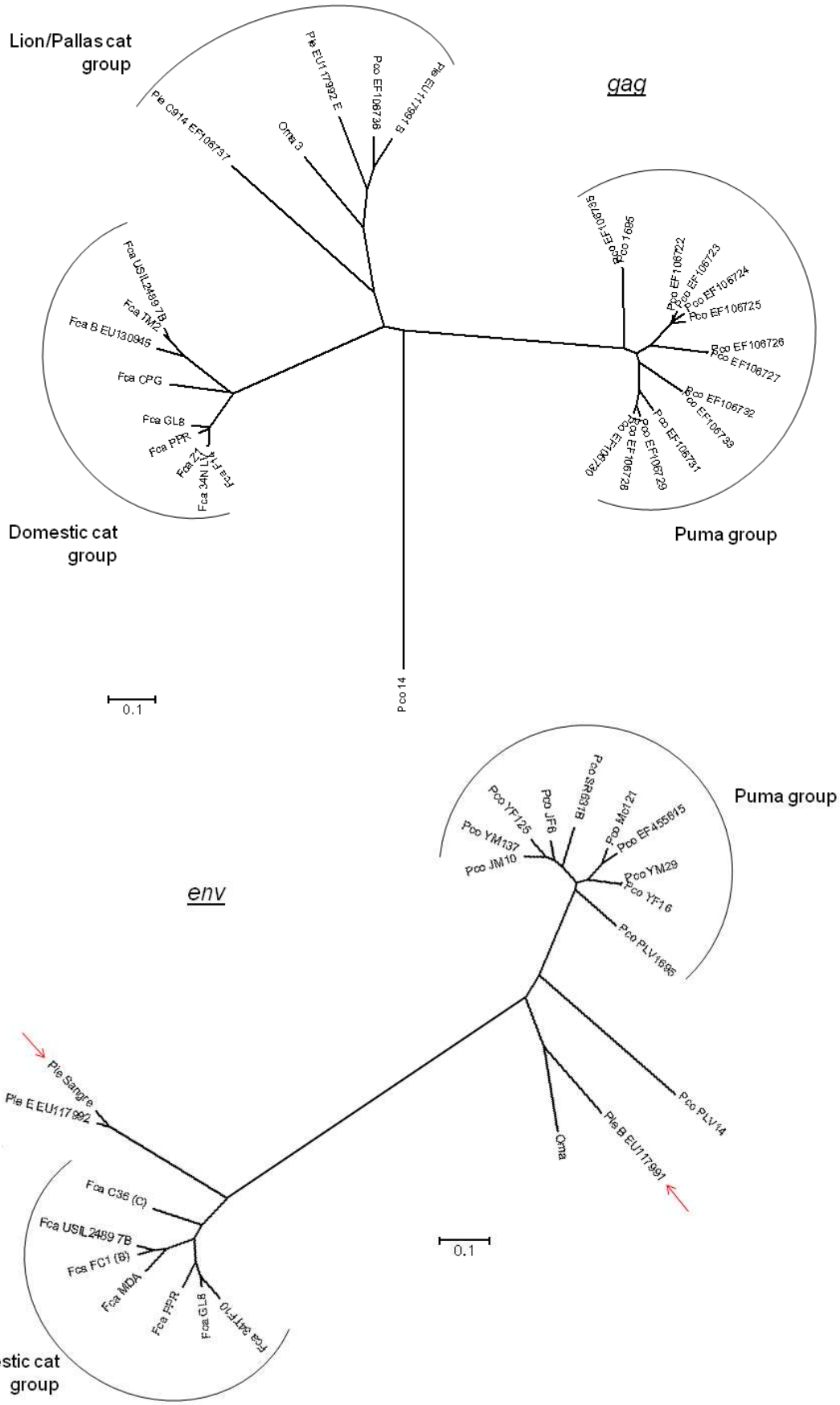

Lion/Pallas cat group
gag
Ple C914 EF106737
Cma 3
Ple EU117992 E
Pco EF106736
Ple EU117991 B
Fca USIL2489 7B
Fca TM2
Fca B EU130945
Fca CPG
Fca GL8
Fca PPR
Fca Z1
Fca 34N FIV
Fca FIV
Domestic cat group
Pco 1695
Pco EF106735
Pco EF106722
Pco EF106723
Pco EF106724
Pco EF106725
Pco EF106726
Pco EF106727
Pco EF106732
Pco EF106733
Pco EF106731
Pco EF106730
Pco EF106728
Pco EF106729
Puma group
0.1
Pco 14
Puma group
env
Pco SR6378
Pco YF125
Pco JF6
Pco M6121
Pco EF455615
Pco YM137
Pco JM10
Pco YM29
Pco YF16
Pco PLV1695
Ple Sangre
Ple E EU117992
Fca C36 (C)
Fca USIL2489 7B
Fca FC1 (6)
Fca MDA
Fca PPR
Fca GL8
Fca 34TFV10
Domestic cat group
Cma
Ple B EU117991
Pco PLV14
0.1

Figure 3-2 Phylogenies of FIV gag and env reveal contrasting evolutionary relationships. Neighbour-Joining trees were constructed using full coding regions of published FIV sequences available in Genbank as well as FIV-Ple E Sangre *env*, with bootstrap of 1000 iterations. When phylogenetic analysis is performed using the *gag* gene, the tree bears four discernable groups: the puma, domestic cat, lion/Pallas cat and outlier FIV-Pco 14. In contrast, when a tree is drawn with *env*, FIV-Ple E groups with FIV-Fca, whilst FIV-Oma and FIV-Ple B 458 (EU117991) form a monophyletic group and bear more similarity to FIV-Pco. FIV-Ple E Sangre clusters closely with published FIV-Ple E 1027 (EU117992), confirming its identity as a lion clade E virus.

Alignment of FIV-Ple E Sangre, FIV-Ple B 458 and FIV-Fca GL8 Envs (Figure 3-3) reveals that FIV-Ple E Sangre is a chimeric *env,* bearing homology with FIV-Ple B 458 in the N-terminus of SU and the C-terminus of TM. The central region more closely resembles FIV-Fca GL8, with 58% identity in this region, compared to 33% in the FIV-Ple B 458 homology region. Furthermore, the FIV-Ple strains share an 82 residue N-terminal sequence not present in the domestic cat strains. The functional significance of this is unknown but may represent extra signal peptide/leader sequence. Several other important structural features are also conserved between FIV-Fca and FIV-Ple E Sangre including all 7 predicted disulphide bonds of GL8 and 17 out of 18 predicted N-linked glycosylation sites. The ES(E/D) motif present at the crown of the V3 loop is commonly found in field isolates of FIV-Fca but is often lost during cell culture adaptation (Figure 3-4). The presence of the ES(E/D) motif correlates with use of CD134 attachment receptor and an alteration of charge at one of the glutamate residues by a conversion to lysine reduces the requirement for CD134, allowing the virus to utilise CXCR4 directly for entry (Shimojima et al. 1998; Willett et al. 1997b; Willett et al. 1997a; Willett et al. 2007). Interestingly the FIV-Ple E Sangre Env lacks the ES(E/D) motif but instead bears a TAT motif at this location.

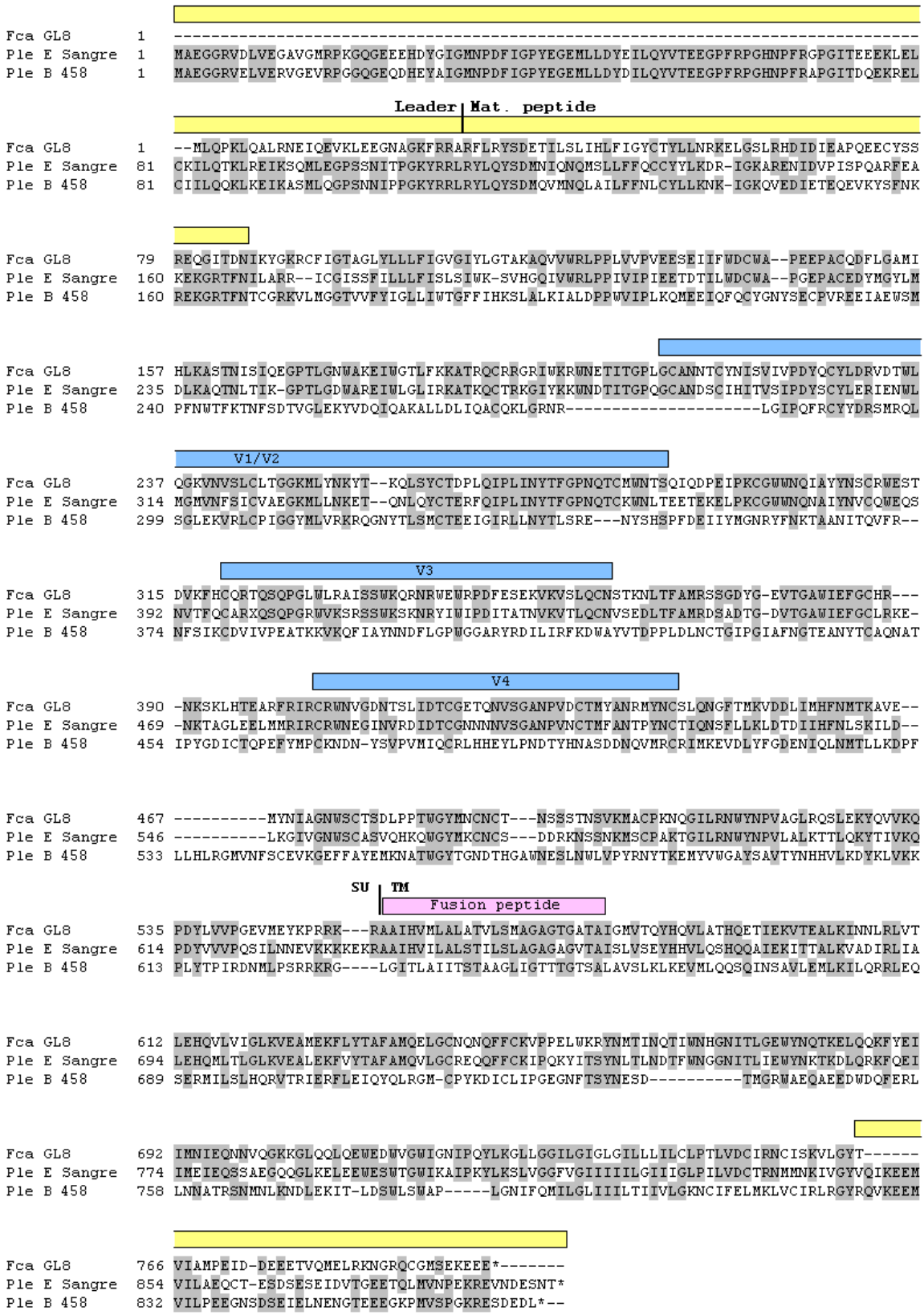


Figure 3-3 Env alignment of FIV-Ple E Sangre with FIV-Ple B 1027 and FIV-Fca GL8. Amino acid residues were aligned using ClustalW and reveal the N and C termini of the FIV-Ple strains are conserved (yellow bars), while the central section of FIV-Ple E

Sangre more closely resembles FIV-Fca GL8, indicative of a recombination event between FIV-Ple and a relative of FIV-Fca. The SU/TM and the leader sequence/mature peptide cleavage sites are indicated in bold, the three major variable loops (Pancino et al. 1993) are shown in blue and the TM fusion peptide is shown in pink.

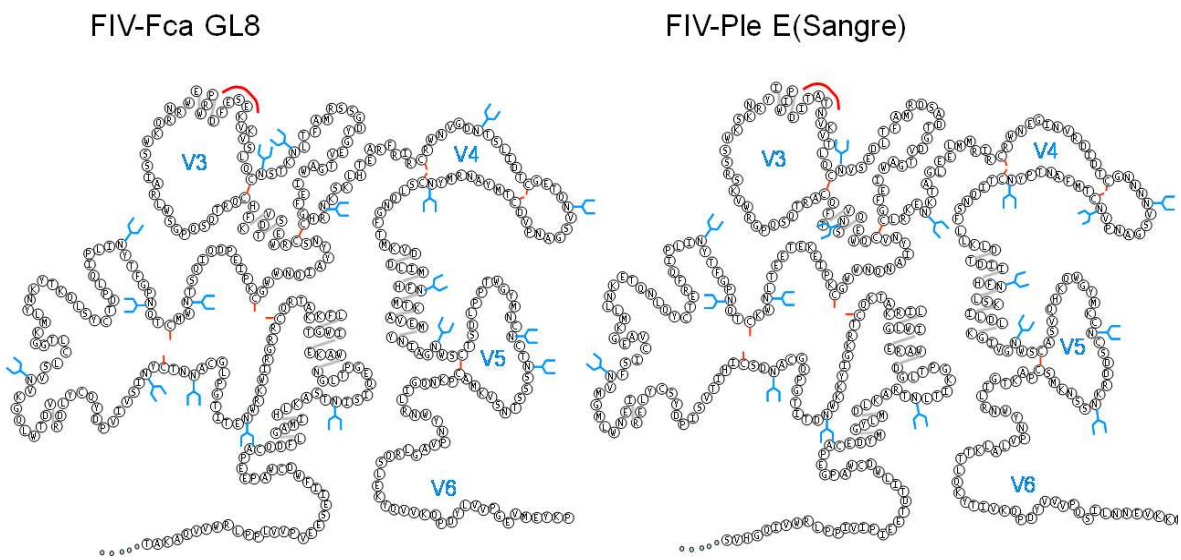


Figure 3-4 Schematic secondary structure model of FIV-Fca GL8 and FIV-Ple E Sangre Env glycoproteins. The two Envs share overall amino acid identity of 53% and are conserved in several key structural areas including cysteine disulphide bridges (red) and predicted N-glycosylation sites (blue Y-shapes). The CD134-interaction domain ES(E/D) in FIV-Fca GL8 and the divergent equivalent in FIV-Ple E Sangre are marked in red. Variable loop architecture (Brown et al. 1994) is denoted.

3.2.3 FIV-Ple E Sangre shares receptor tropism with FIV-Fca

Replication of FIV-Fca in canine chronic lymphocytic leukaemia cells (CLL) is CD134-dependent (Willett et al. 2006a). In order to ascertain whether isolates of FIV-Ple recapitulate this phenotype, CLL cells or CLL cells transduced with feline CD134 were challenged with FIV-Ple E Sangre, FIV-Ple B 458 or FIV-Fca GL8. For FIV-Fca GL8 and FIV-Ple E Sangre high titres of replicating virus were obtained only in cells transduced with CD134, whereas FIV-Ple B 458, which is known to replicate in 3201 cells which lack CD134 (Smirnova et al. 2005;

Willett and Hosie 2008), was able to replicate in the absence or presence of CD134 (Figure 3-5). This provides evidence that the lion E subtype of FIV uses CD134 as an entry receptor, and confirms that CD134 is not required for the replication of the FIV-Ple B subtype.

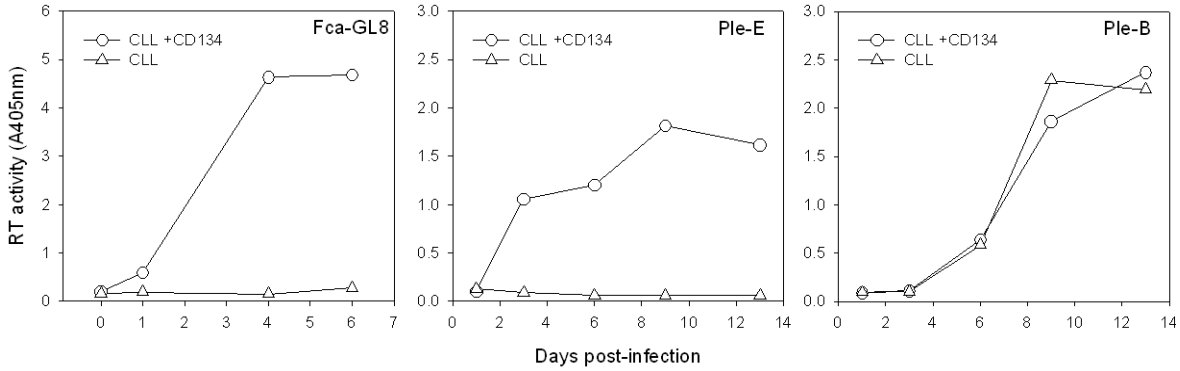


Figure 3-5 Growth of FIV-Ple E Sangre is CD134-dependent in CLL cells. Cells were infected with isolates of FIV-Fca GL8, Ple E and Ple- B subtypes and culture supernatant aliquots were taken periodically and stored at -80^{o}C for subsequent analysis of reverse transcriptase activity. Whilst FIV-Ple B 458 was able to replicate in CLL cells devoid of CD134, replication of FIV-Fca GL8 and FIV-Ple E Sangre was CD134-dependent, providing evidence that FIV strains circulating in wild lion populations share CD134 as an entry receptor with FIV-Fca.

3.2.4 Tropism of FIV-Ple pseudotypes

To further characterise the attachment receptors of FIV-Ple, HIV-1 luciferase pseudotyped particles were generated and their receptor tropism was assessed on MCC cells transduced with human, feline or chimeric CD134. Human CD134 is a non-functional receptor for FIV but FIV-Fca strains such as PPR and B2542 need only to interact with cysteine-rich domain 1 (CRD1) of feline CD134 and are therefore able to use chimeric feline-human CD134 (fCRD1/hCD134) (de Parseval et al. 2005). Other strains such as GL8 and CPG41, typical 'early' isolates, require additional determinants in CRD2 and therefore cannot use the

chimeric receptor (Willett et al. 2006b). In domestic cat FIV strains that attain entry without feline CRD2 requirement (such as PPR and B2542), infection is readily inhibited by soluble trimeric feline CD134-ligand (sFc-TNC-CD134L) perhaps suggesting a weaker interaction between Env and CD134 (Willett et al. 2007; Willett et al. 2009). Like FIV-Fca PPR, FIV-Ple E Sangre infection was also found to be readily inhibited by low concentrations of CD134L (Figure 3-6; 46% reduction in infectivity was seen at 2µg/ml sfeCD134L for FIV-Ple E Sangre compared to 21% for PPR and no decrease for GL8). Additionally, FIV-Ple E Sangre entry could be inhibited by AMD3100, a small molecule inhibitor of CXCR4-dependent entry in FIV-Fca, indicating that FIV-Ple E shares both entry receptor and co-receptor with FIV-Fca. Conversely entry of FIV-Ple B was unaffected, confirming its independence of CXCR4 and CD134.

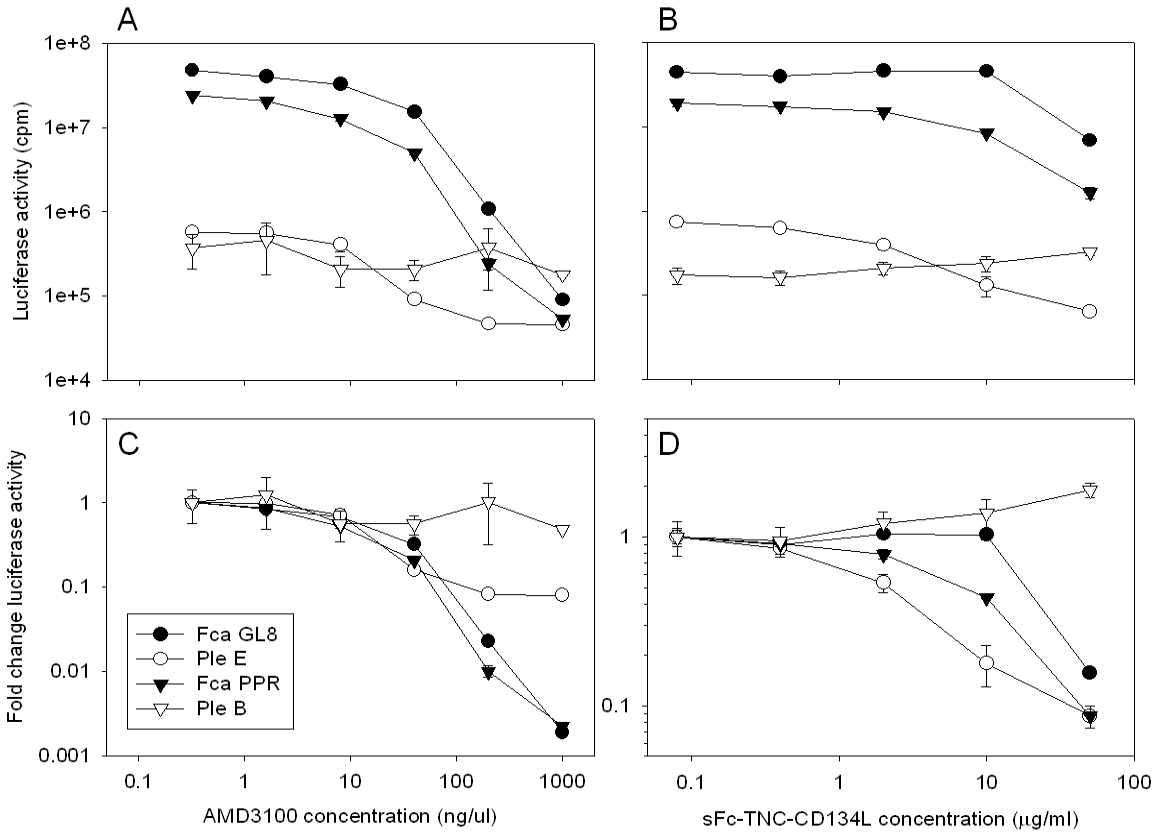


Figure 3-6 Like FIV-Fca, infection by FIV-Ple E can be inhibited with ligands of the receptor and co-receptor. Mya-1 cat T-cells were infected with HIV(luc) pseudotypes bearing envelopes of domestic cat FIV-Fca strains GL8 and PPR as well as FIV-Ple E

Sangre and B 458 in the presence of small molecule CXCR4 inhibitor AMD3100 (panels A and C) or soluble trimeric CD134L (sFc-TNC-CD134L; panels B and D) drawn as raw luciferase readings (A and B) or fold change in luciferase activity (C and D). In common with domestic cat strains of FIV such as PPR, FIV-Ple E Sangre was readily inhibited by CD134L and was also sensitive to AMD3100. In contrast, FIV-Ple B was insensitive to both these inhibitors.

In domestic cat strains of FIV, a high degree of susceptibility to blocking by sCD134L is associated with a reduced requirement for the CRD2 domain of CD134 (Willett et al. 2006b). In order to test whether these features are associated in FIV-Ple infection, the virus was titrated onto a panel of cells expressing human, feline and chimeric fCRD1/hCD134. The co-occurence of high susceptibility to sCD134L with reduced requirement for CRD2 was not found to be the case for FIV-Ple E as entry was not supported by MCC cells bearing fCRD1/hCD134 (Figure 3-7). This finding suggests that FIV-Ple E makes more complex interactions with its receptor, as has been postulated for 'early' domestic cat stains such as GL8. FIV-Ple E Sangre is the first example of an Env displaying these properties and demonstrates that having both a weak interaction with the receptor but a more complex CRD2-dependent interaction are not properties that are mutually exclusive to an individual envelope glycoprotein.

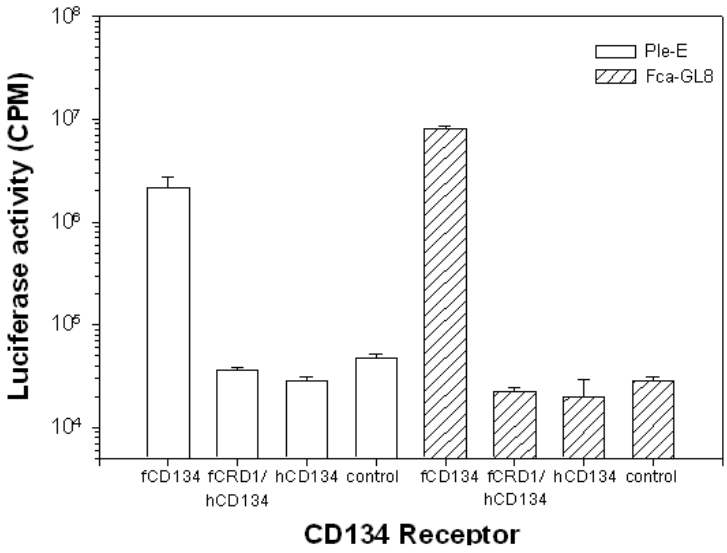


Figure 3-7 CD134 domain requirement for FIV-Ple E Sangre. As has been observed for certain strains of FIV-Fca such as GL8 (Willett et al. 2006b), FIV-Ple E is unable to use chimeric feline-human CD134, indicating that FIV-Ple E requires CD134 determinants in both CRD1 and CRD2.

The CD134 variants from domestic cat and lion were sequenced and aligned to human CD134 (Figure 3-8). Lion CD134 was deposited in Genbank (accession EU915483). Conservation between the homologues is high, with variable residues largely confined to the cleaved leader sequence. The only mutations within the mature peptide are within CRD1, namely K42R and G59S. Interestingly, residue 59 is one of the sites responsible for the differential permissivity between human and feline CD134, and forms part of the primary Env binding site (de Parseval et al. 2005), raising the possibility that one or both of these mutations could alter the Env binding site and have a profound effect on viral entry.

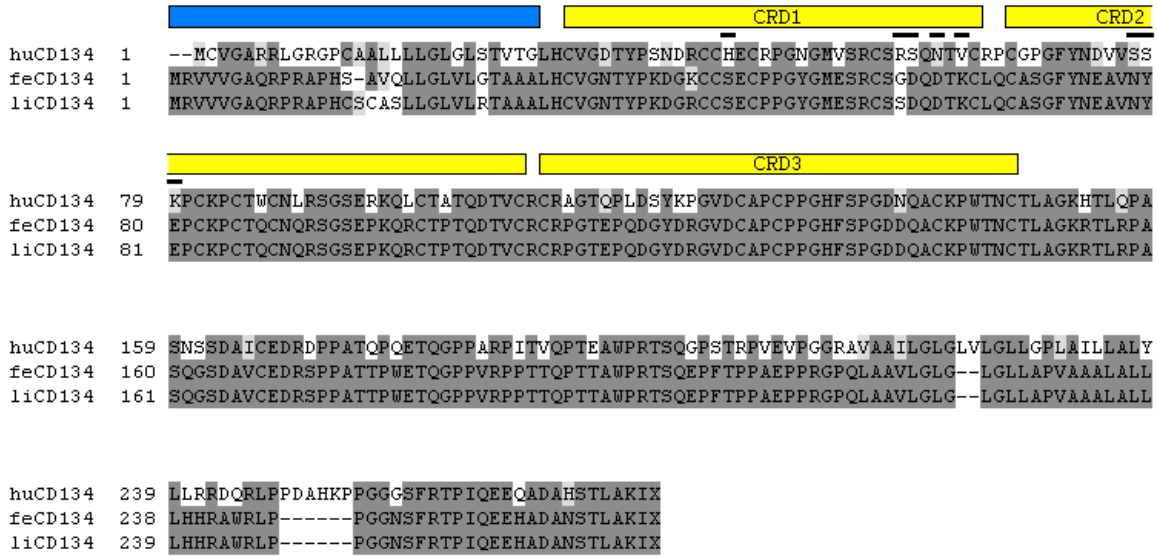


Figure 3-8 Alignment of CD134 homologues. Lion and feline CD134 possess high levels of homology throughout the protein and differences between the homologues reside in the cleaved leader sequence (blue bar) and in cysteine rich domain 1 (CRD1). Residues critical for the FIV Env-receptor interaction that are non-permissive in the human (hu) homologue are marked with a black line and overlap the G59S mutation between cat and lion. Residues that are identical between at least two of the homologues are shaded dark grey and conserved residues light grey.

To further characterise the relative requirements of receptor and co-receptor, plasmids encoding the lion and feline CD134 receptors were used to transduce CXCR4-negative human NP2 cells. The resulting cells were then further transduced with lion CXCR4. The presence of the mutations within lion CRD1 could potentially affect the Env-CD134 interaction. Entry for FIV-Ple E Sangre was dependent on the presence of both CD134 and CXCR4 but, despite the differences in sequence, without preference for the species-specific CD134 variants (

Figure **3-9**).

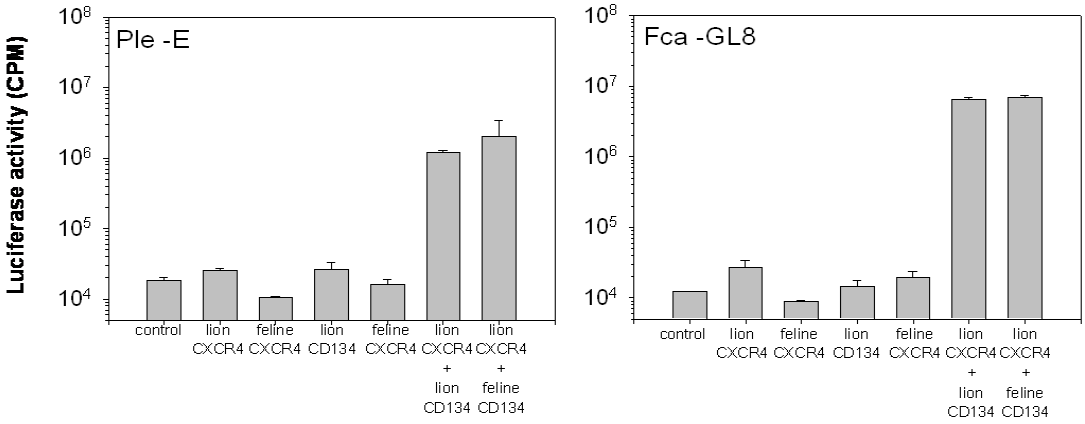


Figure 3-9 CD134 and CXCR4 are obligatory factors for FIV-Ple E entry. Expression of lion or feline CD134 or CXCR4 alone is insufficient for entry for both domestic cat GL8 and lion subtype E FIV variants. The viruses display no substantial preference for lion or feline CD134.

To show that the FIV-Ple Env is capable of mediating membrane fusion, AH927 cells stably expressing CXCR4 alone or CXCR4 and feline CD134 were transfected with plasmids bearing the *env* genes of FIV-Fca GL8 or FIV-Ple E Sangre. Transfection with either *env* resulted in the formation of large syncytia in AH927-fCXCR4-fCD134 (Figure 3-10) while AH927-fCXCR4 cells did not significantly differ from mock transfected cells, indicating that FIV-Ple E Sangre is able to mediate membrane fusion in a CD134-dependent manner.

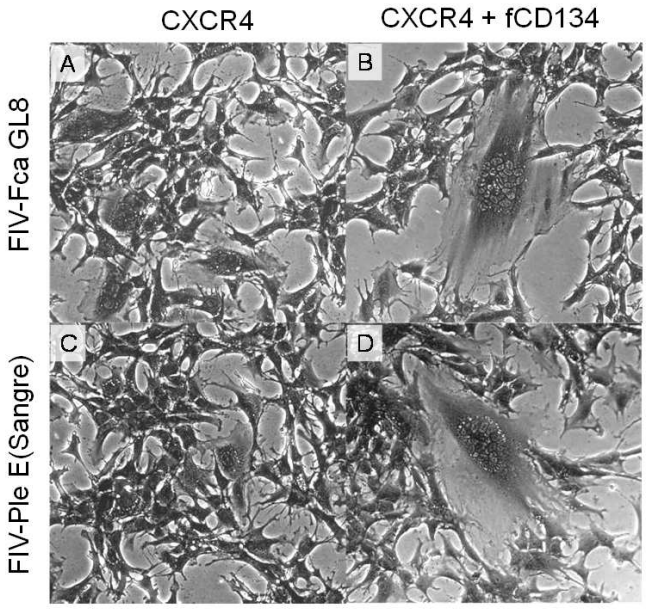


Figure 3-10 FIV-Ple E Sangre is able to mediate CD134-dependent syncytium formation. Feline AH927 cells transduced with feline CXCR4 alone or in combination with feline CD134 were transfected with *env* genes of FIV-Fca GL8 or FIV-Ple E Sangre. Monolayers were fixed and stained 48 hours post transfection. Large syncytia were present in AH927-fCXCR4-fCD134 cells transfected with either *env.*

To investigate whether the ability of FIV-Ple to replicate in the absence of CD134 extends to FIV-Pco, CrFKs transduced with either feline or human CD134 were challenged with FIV-Pco or with FIV-Fca B2542. As with FIV-Ple B 458, replication of FIV-Pco was independent of CD134 expression whilst FIV-Fca only replicated in cells expressing feline CD134.

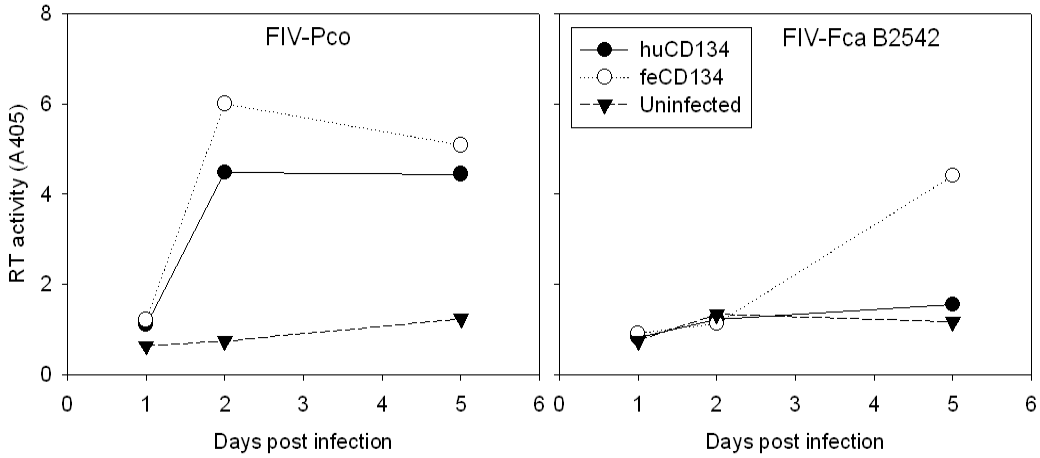


Figure 3-11 FIV-Pco is able to replicate in CrFK independently of CD134. Human CD134 is a non-functional receptor for FIV infection (de Parseval et al. 2005). Here an FIV-Pco primary isolate was able to replicate in CrFKs in the presence of feline (fe) or human (hu) CD134, whilst FIV-Fca was only able to replicate in the presence of feline CD134, suggesting the puma virus uses an alternative receptor expressed in CrFKs.

3.3 Discussion

This study provides evidence that lion lentiviruses belonging to subtype E share usage of CD134 and CXCR4 as attachment receptors for viral entry with domestic cat variants of FIV. Phylogenetic analysis of the lentiviruses reveals that FIV-Ple is a monophyletic strain of FIV related to lentiviruses from puma (FIV-Pco) and Pallas cat (FIV-Oma) when analysis is performed over the *gag* open reading frame. However, using the *env* gene, it is shown that whilst FIV-Ple B remains clustered with FIV-Oma and FIV-Pco, the E subtype is more closely related to FIV-Fca (Pecon-Slattery et al. 2008a). The results are suggestive of a recombination event, but given the relatively high sequence divergence between FIV-Ple E and FIV-Fca, either between circulating lion strains and an ancient ancestor of FIV-Fca, or a more recent recombination event with an FIV strain that resembles FIV-Fca circulating in lions or another species that is yet to be identified. Recombination in lentiviruses is not without

precedent: an increasing number of HIV-1 variants arising from presumed recombination events are being discovered, with 43 circulating recombinant forms (CRFs) listed in the HIV sequence database in July 2009 (http://www.hiv.lanl.gov/content/sequence/HIV/CRFs/CRFs.html).

Recombination has also been shown to be widespread in the evolution of the primate lentiviruses, with the ancestors of both HIV-1 and HIV-2 themselves mosaics of the six major SIV lineages (Salemi et al. 2003). Evidence exists of recombination in FIV *env* and *gag-pol* in *Felis catus* domestic and wild populations (Carpenter et al. 1998; Hayward and Rodrigo 2008). Recombination is therefore likely to be an important mechanism of generating diversity in lentiviruses that otherwise replicate in a clonal manner, lacking the ability to exchange genetic material.

3.3.1 Potential implications of receptor use on FIV-Ple pathogenicity

The virus-receptor interaction is a critical determinant of the host cell population targeted and therefore a significant factor in the cytopathicity of retroviral infection. HIV-1 uses CD4 and chemokine receptors CXCR4 or CCR5 to preferentially infect diverse cell populations (Edinger et al. 1997; Dragic et al. 1996; Feng et al. 1996). In a similar manner FIV-Fca uses CD134 and CXCR4 to target activated T-cells, and changes in receptor tropism correlate with infection of other tissues and associated changes in pathogenicity (Willett et al. 2006b; Willett et al. 2006a; de Parseval et al. 2005). Studies of FIV-Ple infection have found evidence of CD4+ T-cell depletion in both wild (Roelke et al. 2009; Roelke et al. 2006) and captive lions (Bull et al. 2003). However the pathogenicity of FIV-Ple in lions is uncertain, with several studies suggesting that the presumed age of the lentiviral infection in lions has permitted adaptation to the host leading to attenuated pathogenicity, with findings of normal lifespans and fecundity in infected individuals and no association with

other disease correlates (Carpenter and O'Brien 1995; Hofmann-Lehmann et al. 1996). In the most complete analysis of FIV-Ple pathology to date, Roelke et al. (2009) suggested that Botswanan and Serengeti lion populations suffer the typical manifestations of immunodeficiency syndromes including increased levels of lymphadenopathy, oral papilloma lesions and gingivitis as well as chronic inflammatory responses and abnormal red blood cell parameters. However, the finding that circulating subtypes of FIV-Ple use diverse attachment receptors has direct implications for the cytopathology of FIV-Ple, since the E subtype is presumably able to infect and deplete activated T-cells in a manner reminiscent of domestic cat lentiviral infections. Future studies of FIV-Ple infection in wild populations should attempt to correlate receptor usage with pathological manifestations of the immunodeficiency syndrome, as the use of alternate entry receptors may go some way to accounting for the discrepancies in the findings of recent studies. As for the identity of the FIV-Ple B subtype receptor, it is tempting to speculate that it is a more widely expressed cell surface molecule, given that high titres of replicating virus can be obtained in canine CLL cells, (Figure 3-5) and that infection is supported by 3201 cells (Smirnova et al. 2005). Additionally, a strain of North American puma virus FIV-Pco, which is proposed to be a distant relative of FIV-Ple (Pecon-Slattery et al. 2008a), is able to replicate to high titres in CrFK cells that lacked feline CD134 (Figure 3-11). For FIV-Fca, replication in CrFKs that lack feline CD134 requires a period of adaptation which corresponds to mutations in *env* that allow CD134-independent entry (Siebelink et al. 1995; Verschoor et al. 1995). In contrast, FIV-Pco replicates to high titres by day 2 post infection, suggesting that FIV-Pco is able to use an alternative receptor expressed on CrFK without a prior adaptation phase (Figure 3-11). Given the divergent origins of these cell lines (CLL is a naive canine CD3+ T-cell line (Willett et al. 2006a), 3201 is a feline lymphosarcoma cell line (Hardy, Jr. et al. 1980) and CrFK is a feline kidney derived epithelial cell line (Crandell et al.

1973)), it appears likely that the receptor or receptors for these viruses is widely expressed. The ability to replicate in these cells is therefore perhaps indicative of a group of FIV strains including FIV-Ple B, FIV-Pco and FIV-Oma, which share usage of an alternative and perhaps broadly expressed receptor or receptors. Experimental infection of domestic cats with FIV-Ple B 458 and FIV-Pco 1697 showed that these viruses are able to replicate in T-cells, causing acute lymphopoenia (VandeWoude et al. 2003; VandeWoude et al. 1997a; Terwee et al. 2005) but can be confined to gut-associated lymphoid tissue when inoculation is oronasal (Terwee et al. 2005). In the light of the findings presented here, a comparison of tissue tropism of FIV-Ple E and B strains in domestic cats would be of substantial interest, as it may yield insights into the cellular targets and pathology induced by the alternative Env tropism.

3.3.2 Comparison of FIV-Ple E and FIV-Fca receptor usage.

Although FIV-Ple E was found to share receptor tropism for CD134 and CXCR4 with FIV-Fca, it did not fall into the like typical 'early' or 'late' classifications of FIV-Fca. Current understanding of FIV-Fca tropism suggests that viruses isolated from the early stages of infection such as GL8 and CPG41 require a more complex interaction with main receptor CD134 involving determinants in both CRD1 and CRD2 (de Parseval et al. 2005; Willett et al. 2006b). Studies of the mechanics of FIV entry, and comparisons with HIV-1 entry, suggest that an initial interaction between the SU V3 loop and CD134 result in a conformational change that exposes the relatively invariant CXCR4-interacting domain which promotes co-receptor binding followed by TM-mediated fusion with the cell membrane. The two-step receptor, co-receptor binding mechanism is thought to sequester the invariant CXCR4-interacting domain providing cover from humoral immunity. Although the host will typically generate antibodies directed to the variable loops, neutralisation is presumably quickly overcome

by expansion of escape mutations in these regions. Late stage viruses such as PPR and B2542, which are relieved of their CRD2 requirement, are possibly permitted to circulate by the absence of full levels of host immunity, and display a broader cell tropism (Willett and Hosie 2008). This transition is accompanied by an increase in sensitivity to blocking activity of CD134L (Willett et al. 2009; Willett et al. 2007). It is therefore of interest that FIV-Ple E Sangre behaves like early FIV-Fca strains in that it requires components of feline CRD2 for entry but like late FIV-Fca strains, receptor attachment is readily blocked by low concentrations of CD134L. FIV-Ple E Sangre therefore represents the first example of a viral Env simultaneously displaying both these properties. The relevance of these findings is unknown, but may be indicative of a different mechanism of viral entry or of a host-specific adaptation. Also unknown is the stage of virus that FIV-Ple E Sangre represents; although the host was healthy at the time of sampling, Sangre was 8 years old and from a population with high seroprevalence, making an early stage infection unlikely. Further research should establish whether the phenotype that FIV-Ple E Sangre displays is typical amongst subtype E-infected lions and if and how receptor usage and sensitivity to blocking changes during the course of infection.

3.4 Conclusions

All six member species of *Panthera* lineage bear species-specific strains of FIV, whilst in contrast, the *Felis* lineage harbours only two seropositive species (*F. catus* and the Jungle cat, *F. chaus*) (Pecon-Slattery et al. 2008b). The distinct pattern of infection in these taxa is evidence of an ancient infection in the *Panthera,* with evidence suggesting infection before the end of the Pliocene (2.53 mya) that has subsequently spread into other species. Consistent with the age of the infection, the diversity of FIV in free-ranging lions is large [burk] and this study reveals that the diversity extends to use of alternate attachment

receptors. Learning the identity of the attachment receptor of other FIV-Ple subtypes such as subtype B will provide an important insight into the evolution of the lentiviruses. Furthermore if the infection of alternative cell populations is demonstrated for FIV-Ple B, there are likely to be diverse pathogenic manifestations attributable to the use of distinct attachment receptors, with implications for the management of free-ranging and captive breeding lion populations.

The domestic cat separated from its closest relatives about 10,000 years ago and the absence of FIV in the other *Felis* species suggests that the zoonosis of the virus into domestic cats took place after this divergence. Thus the two species represent an interesting comparison between an ancient and a relatively new host for lentiviral infection. Models of pathogen infection suggest that attenuation of viruses over time will occur to a limited extent given certain criteria (May and Anderson 1979). Consistent with a certain degree of attenuation, seroprevalences in lions can approach 100% and it is apparent that lions are able to survive and breed given the continued existence of these populations. Nevertheless, evidence exists for the continued manifestations of immunodeficiency in lion lentiviral infections. Our knowledge of primate intrinsic immunity suggests that in past infections the host gained immunity that prevents retroviral infection, rather than viruses persisting as asymptomatic infections. Chapter 6 will probe the ability of lions to restrict lentiviral infection and show that lion T-cells possess a broad anti-retroviral post-entry restriction mechanism. Furthermore the nature of the APOBEC3 genes in this species is examined and shown to provide a potent block to lentiviral replication, suggestive of an ongoing struggle between host and parasite.

Chapter 4. An investigation of antiretroviral factor TRIM5 in Feliformia

4.1 Summary

TRIM5α is an intracellular restriction factor that interferes with or 'restricts' incoming retrovirus particles in the cytoplasm and prevents efficient reverse transcription and integration (Stremlau et al. 2004). The gene was originally described in primates, where it is proposed to provide a block to cross-species transmission of retroviruses. The protein interacts with incoming retroviral cores in the cytoplasm via an interaction between variable regions in the C-terminal B30.2 domain and the capsid core (Stremlau et al. 2005). In a manner similar to the restriction provided by murine factor $Fv1^{b/b}$, TRIM5α variants from most species are able to restrict the N-tropic strain of MLV but not the B-tropic strain, and the viral determinant of restriction maps to the exposed surface of capsid (Bishop et al. 2001; Cowan et al. 2002; Kozak and Chakraborti 1996). TRIM5α variants from non-primate species such as cows (*Bos taurus*) (Ylinen et al. 2006; Si et al. 2006) and rabbit (*Oryctolagus cuniculus*) (Schaller et al. 2007) have also been shown to posses TRIM5α with antiretroviral properties, suggestive of a mammalian TRIM5α common ancestor with antiretroviral properties. In dogs, TRIM5 has been shown to be disrupted by the insertion of an unrelated gene PNRC1 and the genomic remnants of the gene

are redundantly pseudogenised (Sawyer et al. 2007). However, the nature of TRIM5 in cats is unknown, except that domestic cat cell line CrFK shows none of the characteristics of TRIM5α restriction suggestive of a TRIM5α-null genotype (Towers et al. 2000). For this reason CrFKs are commonly used for the exogenous expression and assay of other mammalian TRIM5α proteins.

This chapter presents a study of domestic cat TRIM5 and shows that while transcripts can be amplified, the encoded protein is truncated early in the B30.2 domain. Further investigation reveals that all feliform species tested possess the truncating mutation, but it is absent from caniform sequences, giving an approximate date for the disruption event. Lacking a B30.2 domain is found to render the gene non-functional as an anti-retroviral agent and the truncated protein is able to act as a dominant negative against human TRIM5α suggesting that the feline TRIM5 retains sufficient homology in the coiled-coil and downstream linker regions to the human orthologue in order to multimerise and disrupt TRIM5α activity. This finding indicates that caution should be taken when expressing exogenous TRIM5 genes in cat cells. The results explain the absence of post-entry restriction in the felids and have implications for cross-species transmission of lentiviruses and the development of animal models for HIV-1 infection.

4.2 Results

4.2.1 Domestic cat cells lack TRIM5α-like restriction

It has previously been reported that, unlike other mammals, cells derived from domestic cats lack a post-entry restriction phenotype typical of TRIM5α (Towers et al. 2000). To confirm these findings, the domestic cat cell line CrFK was challenged with N- or B- tropic MLV that had previously been titrated and normalised on MDTF (*Mus dunni* tail fibroblast) cells, which lack restriction

activity (Bock et al. 2000). Additionally, to confirm the ability of exogenously expressed TRIM5α to mediate restriction in cat cells, CrFK cells transduced with huTRIM5α were also assayed (Figure 4-1). As has previously been reported, cat cells lack the ability to restrict N-tropic MLV, suggestive of an absence of functional TRIM5α. However, unlike canine D17 cells which support restriction by exogenously expressed TRIM5α genes weakly (Berube et al. 2007), CrFK allows potent restriction of MLV-N by the human variant, suggesting that the correct cellular environment and any potential cofactors of restriction are present in domestic cats.

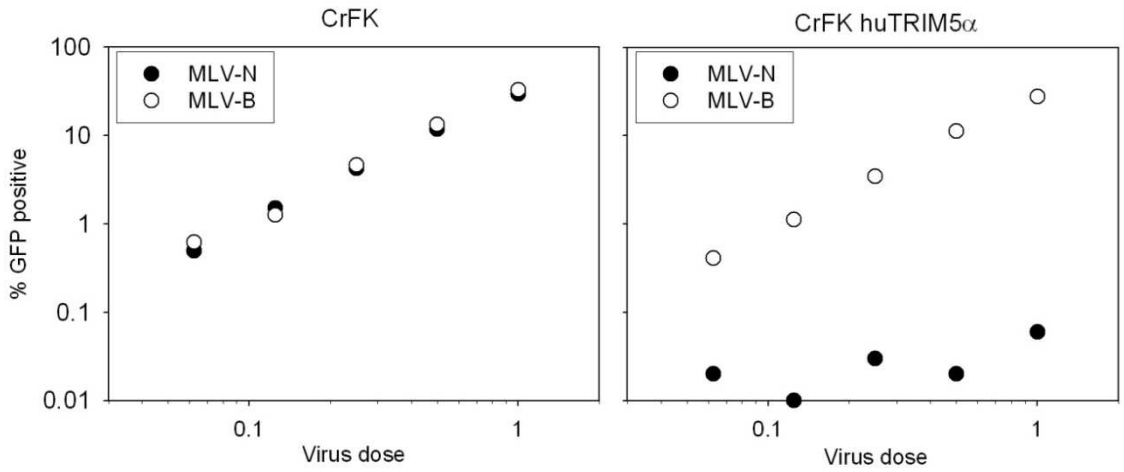


Figure 4-1 Cat cells lack MLV-N-specific post-entry restriction. Domestic cat cells were infected with MDTF-normalised doses of VSV-G pseudotyped N- or B-tropic MLV bearing a GFP reporter gene. CrFK cells were equally susceptible to N- and B-tropic strains indicating a lack of TRIM5α-like post-entry restriction. However, when transduced with huTRIM5α, a potent block to N-MLV is observed, indicating that cat cells are able to support the activity of the restriction factor.

4.2.2 TRIM5 of domestic cats is truncated

Using internal primers directed to huTRIM5α, an amplicon was identified from Mya-1 cDNA which was used to mine the nascent cat genome project (Pontius

and O'Brien 2007) for homologous sequences. This strategy was used rather than performing a cross-species BLAST search with non-feline TRIM5 sequences because of the large number of TRIM family members, and the danger that in an incomplete genome project, an incorrect match may have been identified. Contigs encoding exons 2 and 8 were found in the database, allowing the design of primers to the full-length open reading frame. The resulting TRIM5 transcript from domestic cat T-cells (Mya-1) was cloned and sequenced and deposited in Genbank (Accession number GQ183880). The cat TRIM5 was found to share substantial homology to hu and rabbit (rb) TRIM5α; however the 5' end of exon 8 bears a stop mutation at residue 299. The resulting protein thus encodes only the RBCC domain and does not possess any of the B30.2 variable regions that are known to be responsible for capsid recognition (Figure 4-2). Sequence downstream of the stop codon at position 299 bears multiple frameshift and stop codons, suggesting that lack of protein-level expression has permitted the accumulation of such errors.

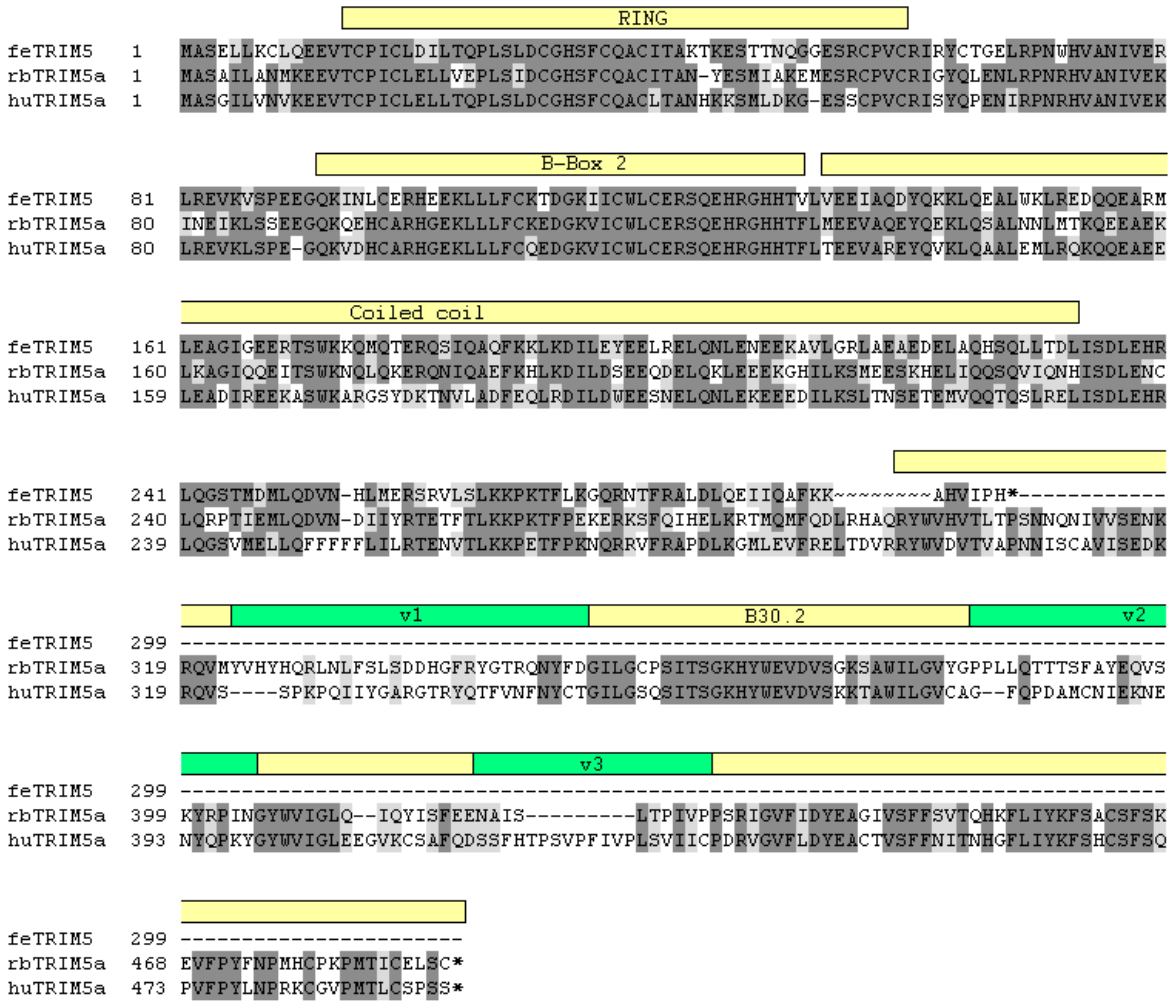


Figure 4-2 Domestic cat TRIM5 is truncated but shows considerable conservation when aligned to TRIM5α orthologues. The amino acid sequence of feTRIM5 was aligned to human and rabbit TRIM5α, revealing substantial conservation in the RBCC domain (RBCC identical residues: fe-hu 59%; fe-rb 59%; hu-rb 60%). The feTRIM5 transcript bears a stop codon at position 299, before the B30.2 domain variable region v1, and encodes a truncated protein. Domain architecture is indicated with yellow bars; variable regions of B30.2 are labelled green; dark grey, conserved residue; light grey, conserved substitution.

4.2.3 Cat TRIM5 is a true orthologue of primate TRIM5α.

Confirmation was sought that the gene identified is a true orthologue of primate TRIM5 and not a closely related TRIM gene. Firstly, using areas of homology to other mammalian genes, a map of the cat chromosome D1 region

was established. This placed TRIM5 amongst its closest relatives: TRIM6, TRIM22 and TRIM34 (Figure 4-3A). As with other mammalian species the domestic cat TRIM gene cluster is flanked by olfactory genes. Haplotypes that are present in multiple species without karyotypic rearrangements are known as regions of conserved synteny. In this case synteny has been conserved between primates (human chr. 11, rhesus chr. 14) and carnivores (cat chromosome D1, dog chromosome 21). The presence of conserved synteny suggests that the gene identified is a true TRIM5 orthologue. However, it is noteworthy that the cat, cow and dog TRIM5 genes are orientated oppositely with respect to human and rhesus macaque TRIM5, suggesting an inversion event has occurred. The timing and relevance of this event are unknown but may for example affect interactions with enhancer/suppressor elements and therefore expression levels. Secondly, a Neighbour-Joining tree was drawn by aligning full length TRIM open reading frames (Figure 4-3B). This reveals feTRIM5 to be monophyletic with TRIM5 variants from other mammalian species and the position of feTRIM5 reflects the established evolution of the mammals. Importantly, feTRIM5 clusters distantly from other closely related TRIM genes such as TRIM6, TRIM22 and TRIM34, thus confirming the transcript identified as a true orthologue of TRIM5.

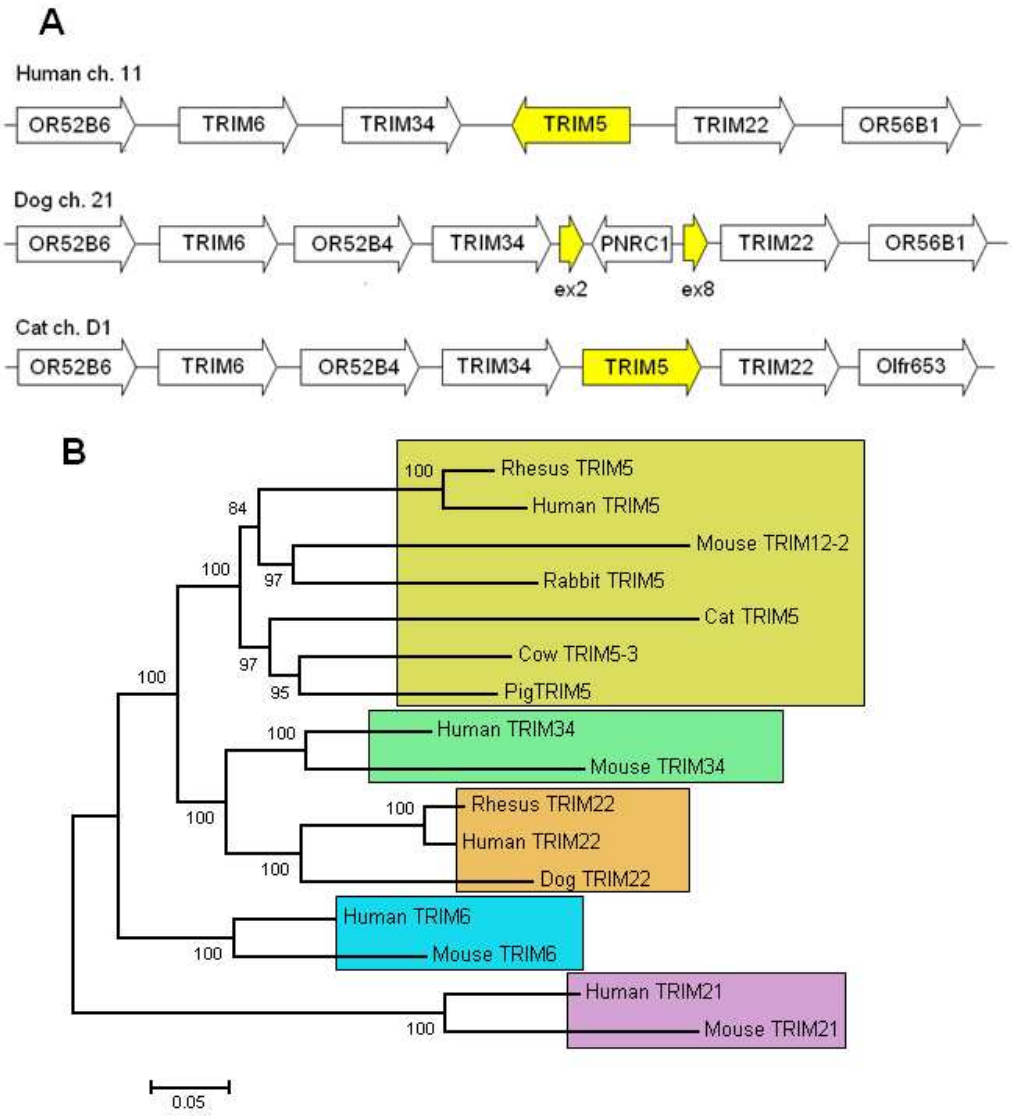


Figure 4-3 Conserved synteny and phylogenetic clustering indicate that cat TRIM5 is a true TRIM5 orthologue. A) Paralogues TRIM6/34/5/22 are found in a cluster in human, dog and cat genomes. In all cases, the cluster is surrounded by olfactory genes. The human TRIM5 is in the opposite orientation to the dog and cat orthologues. Exons 2 and 8 of the pseudogenised dog TRIM5 are shown. Other genes in cat, as well as dog TRIM6 are predicted genes or regions of homology only and may not be expressed or functional. B) A neighbour-joining phylogeny was constructed using amino acid sequence of several TRIM5 orthologues as well as the closely related TRIM34 and TRIM22. Domestic cat TRIM5 is monophyletic with TRIM5 from other species, supported by high bootstrap values from 1,000 iterations. The tree was routed on outlier TRIM21.

4.2.4 Exon structure of cat TRIM5

Primate and non-primate TRIM5 orthologues are known to span eight exons (Nisole et al. 2005), with the largest being exons 2 (which includes the start codon ATG, the RING domain and the B-Box2 domain) and 8 (which codes for almost the entire B30.2 domain). The cat TRIM5 gene was found to share seven of the eight exons present in huTRIM5α, skipping the 27 nucleotide exon 7 and thus moving directly (but remaining in frame) from exon 6 to exon 8. This is a transcript that has not been described in humans. However, two novel isoforms of TRIM5 were found in the macaque species *Macaca nemestrina*: TRIM5η (eta) bears a 2bp deletion between exons 6 and 7 resulting in a frameshift and truncation, resulting in a protein of 300 residues, comparable in size to cat TRIM5; TRIM5θ (theta) skips exon 7, like the cat TRIM5, but remains in frame for the entire exon 8, resulting in a protein of 486 residues (Brennan et al. 2007). Interestingly a TRIM5α transcript was not identified in this species, and the isoforms identified appeared to inhibit viral entry poorly and correspondingly, this species can be inoculated with lentiviruses including HIV-1 (Agy et al. 1992) and HIV-2 (McClure et al. 2000). The role of these alternative isoforms is not clear, but the authors speculate that the deletion of exon 7 impacts on the stability of an α-helix thought to stabilise the N-terminal B30.2 domain, and thus reduces the antiretroviral activity of the protein. By analogy with the TRIM21 crystal structure (Keeble et al. 2008), the exon 7 region may compromise the PRY subdomain scaffold structure which supports the v1 domain critical for lentiviral restriction (Perez-Caballero et al. 2005a; Stremlau et al. 2005; Yap et al. 2005).

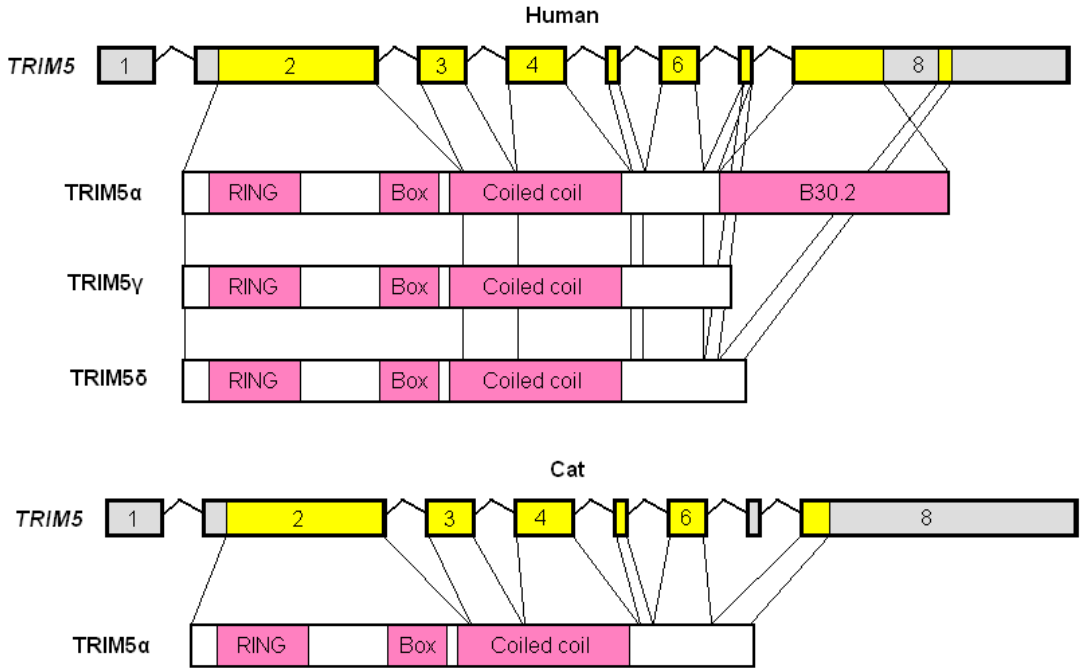


Figure 4-4 Exon structure of cat TRIM5. Transcripts encoding anti-retroviral human TRIM5α are comprised of eight genomic exons. Isoforms TRIM5γ and TRIM5δ encode variants that lack the C-terminal B30.2 domain, with TRIM5γ simply lacking the final exon 8, resulting in a stop mutation beyond the splice donor of exon 6 and TRIM5δ using an alternative splice acceptor in exon 8. Domestic cat TRIM5 is encoded by exons 2 to 8 but skipping the 27 nucleotide exon 7.

Although this study found transcripts of a similar size in diverse Feliform species, it is of course possible that a transcript that encodes all eight exons is expressed in cat cells but was not detected in this study. TRIM5 isoforms such as huTRIM5δ use an alternative splice acceptor in exon 8, downstream of the B30.2-encoding region. To rule out this possibility in domestic cats, 3' RACE was performed, which allows the amplification of the 3' terminus in an unbiased fashion (Frohman et al. 1988). No evidence for an alternative transcript was found, with sequences corresponding only to the transcript previously identified.

4.2.5 Evaluating the antiviral activity of cat TRIM5

Primate TRIM5 is able to interact with incoming retroviral cores through an interaction between its B30.2 domain and viral capsid protein (Mische et al. 2005; Sebastian and Luban 2005; Stremlau et al. 2006b). Given that domestic cat TRIM5 lacks a B30.2 domain, it was predicted that the gene product would be unable to restrict retroviruses. To test the ability of feTRIM5 to restrict retroviral infection, a murine cell line MDTF was stably transduced with huTRIM5α or feTRIM5. The MDTF cell line was chosen since, like CrFK, it lacks a post-entry restriction phenotype (Bock et al. 2000; Hatziioannou et al. 2004; Towers et al. 2000). Cells were challenged with dilution series of N or B tropic MLV, as well as HIV-1 and SIVmac VSV-G pseudotyped particles, bearing a green fluorescent protein (GFP) marker gene and expression of GFP was monitored by flow cytometry (Figure 4-5). Human TRIM5α restricted MLV-N and SIVmac, whilst cat TRIM5 restricted none of the virus particles tested. Thus, we can confirm feTRIM5 to be a non-functional restriction factor and furthermore we can ascribe the lack of post-entry restriction in domestic cat cells to the truncation of TRIM5.

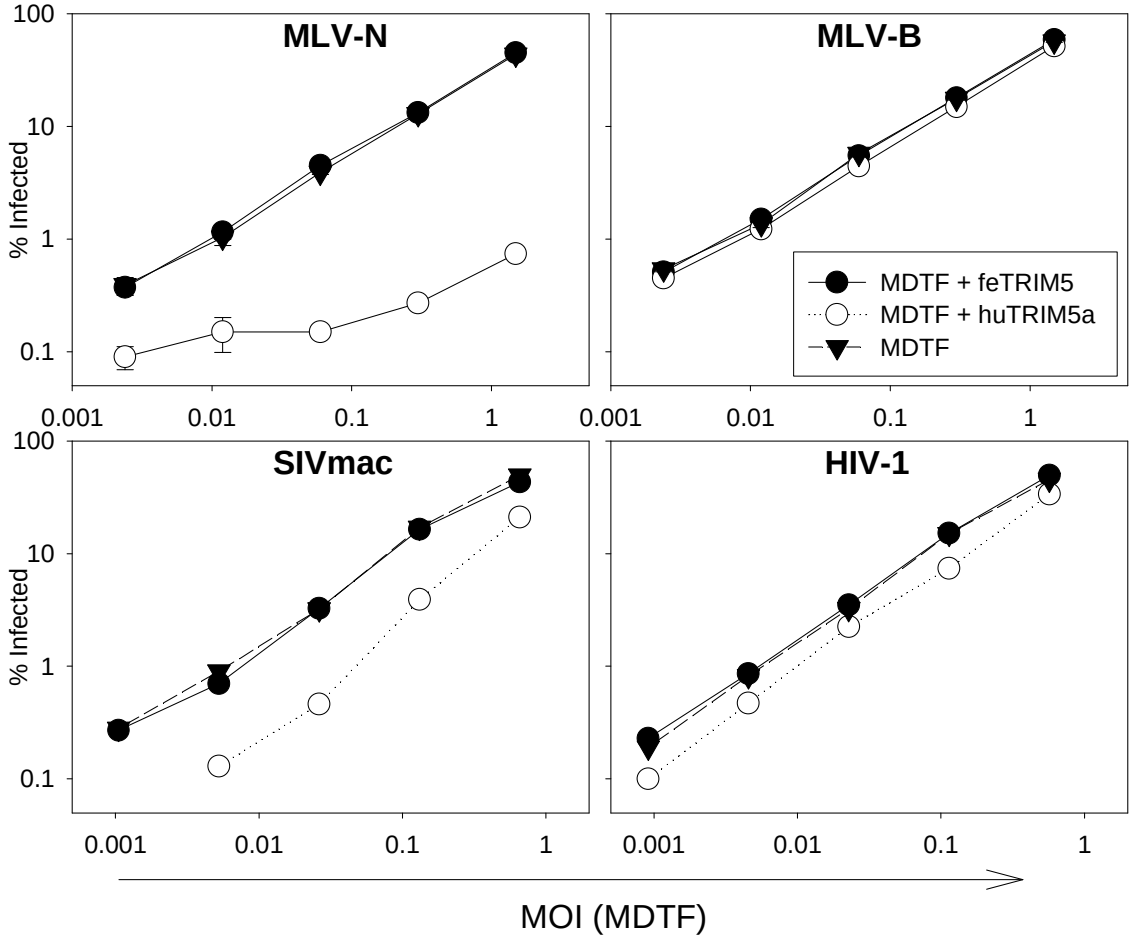


Figure 4-5 Domestic cat TRIM5 is a non-functional restriction factor. Permissive MDTF cells were transduced with domestic cat TRIM5 or human TRIM5α. Intact huTRIM5α specifically recognises and restricts N-tropic but not B-tropic MLV through an interaction of its B30.2 domain and the virus capsid. Domestic cat TRIM5, which lacks a B30.2 domain is unable to restrict MLV-N.

4.2.6 Cat TRIM5 disrupts endogenous human TRIM5α activity

In primates, alternative splice variants TRIM5γ and TRIM5δ lack a B30.2 domain and lose their ability to restrict retroviruses. Moreover, these isoforms have a dominant negative effect, disrupting TRIM5α restriction by forming multimers with full-length TRIM5α (Mische et al. 2005; Perez-Caballero et al. 2005a). To investigate whether the cat TRIM5 is able to perform this dominant negative activity, human TE671 cells which express endogenous levels of TRIM5α and

potently restrict MLV-N (Keckesova et al. 2004) were transduced with cat TRIM5 or a human TRIM5α mutated to bear a stop codon at the same location as the cat transcript, huTRIM5 P306STOP. Cells were then challenged with moi-normalised N- or B-tropic MLV.

Both cat TRIM5 and huTRIM5 P306STOP were found to have a dominant negative effect against endogenous TRIM5α as they were able to rescue titres of MLV-N. This suggests that feTRIM5 localises to the cytoplasm where it is able to form dimers with huTRIM5α to prevent full levels of restriction. The domain responsible for TRIM5 self interaction is the coiled coil and linker 2 region (Javanbakht et al. 2005; Perez-Caballero et al. 2005a). This suggests that sufficient homology exists in these regions between human and feline variants of TRIM5 to allow the formation of higher order complexes. The data also provide evidence that like human TRIM5, cat TRIM5 localises to the cytoplasm. Interestingly, feTRIM5 was able to provide higher levels of dominant negative activity than huTRIM5 P306STOP despite higher levels of expression of the latter. However further attempts to characterise this discrepancy by transient expression of different ratios of the two genes in conjunction with full-length TRIM5α proved unsuccessful (data not shown).

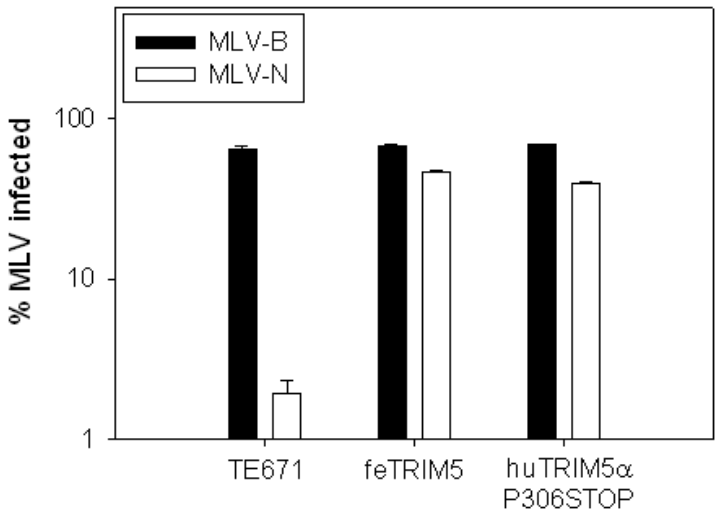


Figure 4-6 Dominant negative activity of feTRIM5. In a similar manner to human isoforms TRIM5γ and δ which also lack a B30.2 domain, feTRIM5 is able to act as a dominant negative against TRIM5α, suggesting that feTRIM5 localises to the cytoplasm and shares sufficient homology with the human variant to interact and form non-antiviral dimers.

4.2.7 Knock-down of endogenous TRIM5 in cat cells

To demonstrate that endogenous TRIM5 in cat cells does not contribute an antiviral phenotype, TRIM5 knockdown CrFK cell lines were made by transducing cells with small-hairpin RNA constructs directed to feline TRIM5. Cells were also transduced with feline CD134 to allow replication of primary FIV isolates. Confirmation of the knockdown was sought by RT-PCR with feTRIM5-specific primers wam4 and wam13 with 2-fold serial dilutions of cDNA as template, which showed a four- to eight-fold reduction in TRIM5 expression. No difference in the ability to replicate in the cells was associated with knockdown of TRIM5, suggesting that TRIM5 does not impact on FIV replication.

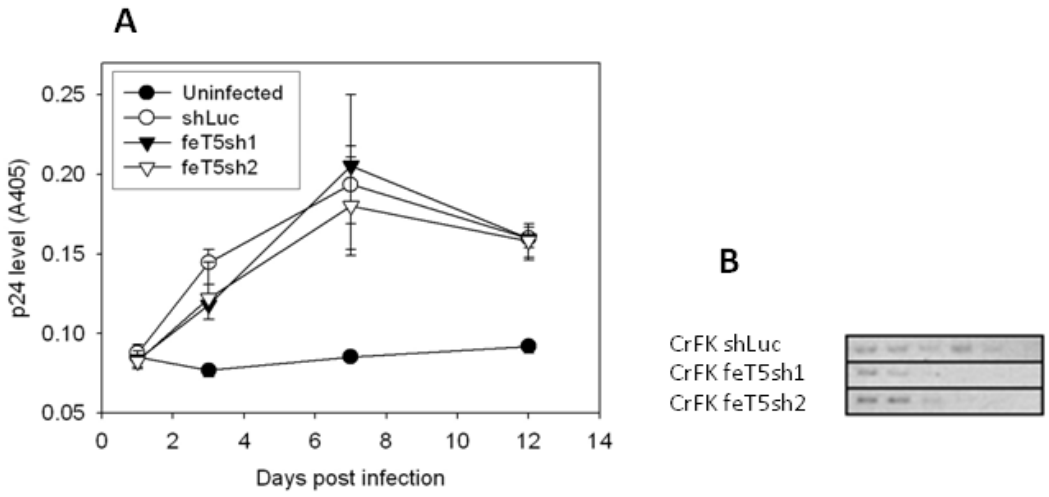


Figure 4-7 Knockdown of TRIM5 in cat cells. Small-hairpin RNAs directed to cat TRIM5 (feT5sh1 and 2) or negative control gene luciferase (shLuc) were transduced into CrFK cells expressing feline CD134. The resulting cells were infected with FIV GL8 and aliquots taken periodically and stored at -80 °C for subsequent p24 ELISA (A). Data are plotted as the mean of three individual wells +/- range. No substantial effect of presence of feT5sh1 or 2 was observed on the replication of FIV. Levels of TRIM5 RNA were measured by serial dilution RT-PCR (B) and appear to show a reduction in expression levels. However subsequent real-time RT-PCR analysis suggested that the transcript levels were not reduced significantly, making the findings questionable.

Further confirmation of effective knockdown was sought using real-time RT-PCR, and, in contradiction to Figure 4-7B, showed that TRIM5 levels were not reduced substantially in either the feT5sh1 or sh2 transduced cells, using either the same or fresh RNA samples (data not shown). The experiment could not be repeated due to time constraints and therefore remains inconclusive.

4.2.8 Sensitivity of FIV to TRIM5α restriction

In order to test whether TRIM5α from other mammalian species is able to restrict the growth of replication-competent FIV *in vitro,* CrFK CD134 cells expressing various mammalian TRIM genes were challenged with FIV molecular

clone CPG41. Cell-free aliquots were stored at -80 °C and examined for p24 levels by ELISA (Figure 4-8). The results imply that conserved lentiviral structures are present in FIV CA that permit recognition and restriction by diverse TRIM5 and TRIMCyp homologues.

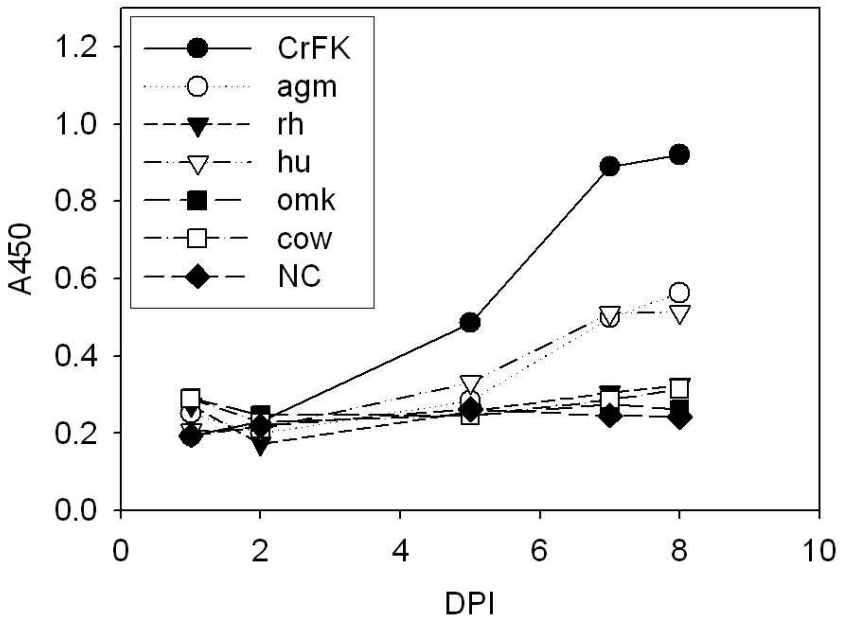


Figure 4-8 FIV replication can be inhibited by diverse TRIM5 and TRIMCyp orthologues. CrFK CD134 cells expressing various TRIM5α variants were tested for their ability to support FIV replication. All TRIM5α variants prevented replication to some degree but owl monkey TRIMCyp (omk), cow and rhesus macaque (rh) TRIM5α prevented significant levels of replication. NC, uninfected negative control; CrFK, untransduced control.

4.2.9 Evolutionary analysis of TRIM5 in the Feliformia

In order to invade a new species, retroviruses must be able to evade destruction by host immune responses. The lack of antiretroviral TRIM5 in cats would therefore have implications for cross-species transmission if the disabling mutation was found to be conserved between species. An analysis of TRIM5 from various Feliform species was therefore conducted in order to determine the prevalence of the mutation. First, we compared TRIM5

expression and identity between members of the Carnivora. PCR using primers spanning all coding exons was performed using template cDNA from domestic cat cell lines and PBMCs from diverse felids (Figure 4-9). In all cases a product of about 901bp indicates that expression of the transcript is maintained in all felid species tested.

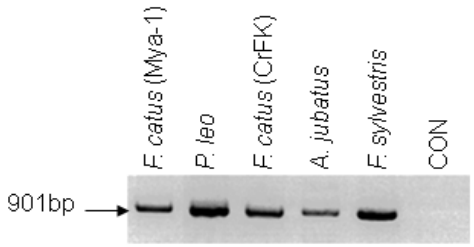


Figure 4-9 TRIM5 expression is maintained in Felids. RT-PCR reveals that a TRIM5 transcript of the same size is expressed in PBMCs from diverse felids and transcripts are present in commonly used domestic cat cell lines CrFK and Mya-1.

Next, genomic DNA was prepared from several species (Table 4.A) and a section of exon 8 was amplified with degenerate primers (gex8T5 5' and gex8T5 3') designed to amplify from both dog and cat DNA. Template DNA obtained from two species (white tailed mongoose and African civet) did not yield PCR products, possibly due to degradation of brain samples prior to extraction, but may be indicative of mutations in these species that do not permit amplification with the degenerate primers.

Common name	***Latin name***	***Family***	***Tissue***	***PCR product***
Domestic cat	*Felis catus domesticus*	Felidae - Domestic cat lineage	Mya-1 T-cell line	Y
Snow leopard	*Uncia uncia*	Felidae – Panthera	Culture PBMC	Y
African lion	*Panthera leo*	Felidae - Panthera	Culture PBMC (Angola-1)	Y

Cheetah	*Acinonyx jubatus*	Felidae - Puma lineage	Culture PBMC	Y
Hyena	*Crocuta crocuta*	Hyenidae	PBMC pellet	Y
European wildcat	*Felis sylevstris*	Felidae – Domestic cat lineage	Culture PBMC	Y
White tailed mongoose	*Ichneumia albicauda*	Herpestidae	Brain	N
African civet	*Civettictis civetta*	Viverridae	Brain (hippocampus)	N
Madagascan fossa	*Cryptoprotca ferox*	Eupleridae	Whole blood	Y
Mink	*Mustela lutreola*	Mustelidae	Mv1 Lu CCL64 cell line	Y
Domestic dog	*Canis lupus familiaris*	Canidae	CLL cell line	Y

Table 4.A Carnivoran samples for genomic DNA extraction. Exon 8 of *TRIM5* was analysed by degenerate primer PCR using template genomic DNA extracted from the above tissues and cell cultures. Expected product sizes were 525bp for dog and 536bp for cat and amplicons were sequenced directly from gel purified PCR product.

Sequencing of successfully amplified exon 8 fragments revealed that remarkably, all Feliform species bear the same stop codon as was found in domestic cat (Figure 4-10A). However, the stop codon was absent from the caniform species mink and dog. A phylogenetic tree of the exon 8 fragments (Figure 4-10B) is consistent with established carnivore phylogeny (Figure 4-10C), confirming the origin of the genomic DNA and obviating the possibility of PCR contamination. The sequences derived from the analysis can be found in Appendix 3.

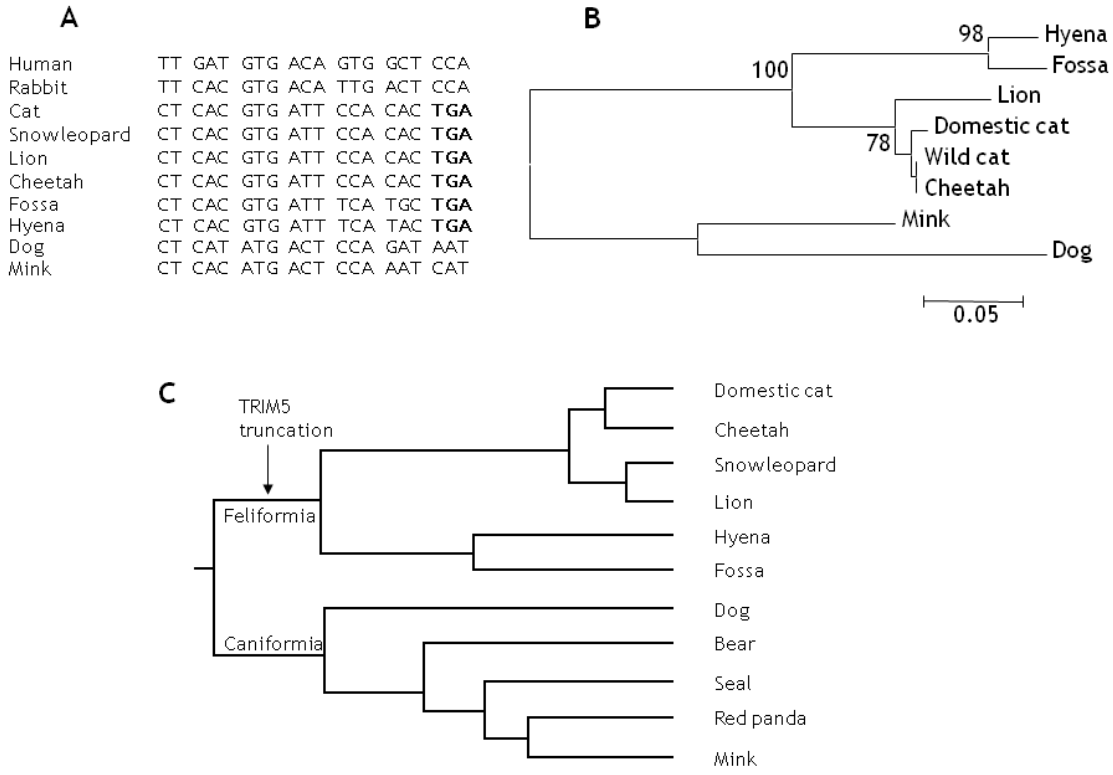


Figure 4-10 TRIM5 truncation is conserved in all feliform species analysed. A) The 5' end of TRIM5 exon 8 was sequenced from genomic DNA. The location of the stop codon (bold) in felids means that the B30.2 is truncated before any of the conserved structural motifs or variable regions. Spaces reflect codon usage except for the dog exon 8 pseudogene sequence where it is preserved for clarity. B) A Neighbour-Joining tree of a 100bp region of TRIM5 exon 8 is consistent with established carnivoran evolution, confirming the identity of amplicons. Sequences used are presented in Appendix 3. Numbers reflect bootstrap values after 1000 iterations and distance is given as base substitutions per site. C) Established phylogenetic tree of the carnivores (Johnson et al. 2006; Gaubert and Cordeiro-Estrela 2006) indicates that truncation of TRIM5 took place before the split of the Felidae and Hyenidae lineages. An independent TRIM5 disruption is proposed to have taken place on the canid lineage after the felid-canid split 53.8 myr.

The presence of the truncation in all Feliform species, but its absence in the dog TRIM5 pseudogene, dictates that the disruption event must have occurred after the Feliform-Caniform split, estimated at 53.8 mya (Bininda-Emonds et al. 1999; Gaubert and Cordeiro-Estrela 2006). However, the presence of the

disrupted gene in fossa and hyena implies that the event took place before the Felidae diverged from the Hyaenidae and Eupleridae 47 mya (Bininda-Emonds et al. 1999). Thus, two independent mutations which disable TRIM5 restriction have occurred during carnivoran natural history. Although the truncation of TRIM5 is compatible with observations that cat cells lack restriction activity, other possibilities such as read-through transcripts or the splicing of another gene to the 3' end of TRIM5 cannot be ruled out. Given the recent findings that fusions between TRIM5 and CypA have arisen at least twice in the primates (Sayah et al. 2004; Virgen et al. 2008; Wilson et al. 2008; Newman et al. 2008; Liao et al. 2007), 3' primers directed to feline CypA were used in conjunction with various 5' TRIM5 primers in RT-PCR reactions, but no product was detected. All published sequence downstream of feTRIM5 was examined for cyclophilin exons using BLAST, with no positive matches. Additionally, 3' primers directed to TRIM22 were used in conjunction with TRIM5 5' primers to detect for potential read-through transcripts, with no products detected. Finally rapid amplification of cDNA ends (3' and 5' RACE) was performed to sequence the transcript ends without bias. No transcript other than the one already described was found.

4.3 Discussion

There is evidence of strong positive selection in primate TRIM5α B30.2 domain, driven by a genetic conflict or 'arms race' between restriction factor and virus within the past 33 myr (Sawyer et al. 2005; Liu et al. 2005). The resulting orthologues are highly divergent in the variable domains of B30.2 and each have specific repertoires of restricted virus (Ohkura et al. 2006; Stremlau et al. 2005; Nakayama et al. 2005). Non-primate species also possess antiretroviral TRIM5α orthologues including cows (Ylinen et al. 2006; Si et al. 2006) and rabbits (Schaller et al. 2007) which also show evidence of positive selection (Si

et al. 2006). Thus it is likely that ancestral mammalian TRIM5α, as well as the ancestral carnivoran TRIM5α, possessed antiviral activity and positive selection of TRIM5α driven by host-virus interactions may be a feature common amongst mammals. Therefore the findings that the Feliformia and at least one taxon in the Caniformia have maintained seemingly deleterious TRIM5 alleles are surprising and in direct contrast to non-carnivoran mammals, where TRIM5α evolution is characterised by strong positive selection and gene duplications.

4.3.1 Implications of TRIM5 truncation for viral transmission and replication

It is thought that escaping TRIM5α restriction may be a widespread requirement for retroviral zoonosis and population invasion in diverse mammalian species. Thus the loss of antiretroviral TRIM5 in carnivorans has implications for the evolution and cross-species transmission of retroviruses. Dogs are currently thought to be free of exogenous retroviral infection but high titres of replicating FIV can be obtained in canine cells which express the FIV receptor feline CD134 (Willett et al. 2006a), suggestive of a lack of intracellular defence (and potential vulnerability) to this pathogen. In the primates, molecular phylogenies of SIV from divergent species broadly reflect phylogenies of the host, suggesting that cross-species transmission is relatively rare and predominantly occurs between closely related species (VandeWoude and Apetrei 2006). In the felids, cross-species transmission of FIV also appears to be relatively rare given the high levels of viral sequence divergence within infected species (VandeWoude and Apetrei 2006; Pecon-Slattery et al. 2008a). However when cross-species transmission events do take place they frequently occur between distantly related species (Troyer et al. 2005). Accordingly, lentiviral evolution in the felids appears to more closely reflect current or historical geographical overlaps of species' ranges rather than the phylogeny of

the host, most notably evidenced by a partition of phylogeny between old world and new world FIV strains which is not recapitulated in phylogenies of the host (Pecon-Slattery et al. 2008a; Pecon-Slattery et al. 2008b; Troyer et al. 2005). For example, cross-species transmission of FIV is hypothesised to have taken place between the distantly related lion and Asian Pallas cat (*Otocolubus manul*) sometime during the Pleistocene, when the range of lions extended to Asia (Pecon-Slattery et al. 2008a). Additionally, the FIV strains infecting cheetahs and leopards are closely related, despite the host species belonging to divergent lineages of the felids: cheetahs belong to the Felis lineage, whilst leopards belong to the Panthera lineage (Johnson et al. 2006; Troyer et al. 2005). In addition to these ancient cross-genus transmissions, recent zoonoses of FIV have also been documented: from captive snow leopard to tiger (Troyer et al. 2005); from domestic cat to puma (Carpenter et al. 1996) and Japanese Tsushima leopard cat (Nishimura et al. 1999); and multiple transmissions of FIV between free-ranging pumas, Florida panthers and bobcats have been reported (Franklin et al. 2007; Miller et al. 2006). Furthermore, experiments have shown that domestic cats can be infected with FIV strains from highly divergent species including lions and pumas (VandeWoude et al. 1997a; Terwee et al. 2005; VandeWoude et al. 2003). The last common ancestor of these three species was at the late Miocene ~10 mya during the radiation of the modern felid genera. The ability of viruses to jump between relatively unrelated species and the number of recent transmissions point to a lack of host defence to FIV and is in contrast to the primates, where it has proven difficult to develop a non-human model for HIV-1 infection in all species but our closest relative, the chimpanzee. Thus the relative rarity of cross-species transmission in the felids appears to owe more to lack of inter-species contact than to robust defences.

The absence of antiretroviral TRIM5α in the felids may have effects on lentiviral evolution in this taxon. It is noteworthy that FIV is particularly sensitive to TRIM5α and rhTRIMCyp and omkTRIMCyp, with several studies reporting high levels of FIV restriction (Schaller et al. 2007; Saenz et al. 2005; Virgen et al. 2008). Thus the absence of TRIM5α or TRIMCyp may have permitted the evolution of FIV towards structural optima that would otherwise be strongly restricted. This hypothesis is consistent with studies of emergence of resistance to antiretroviral drugs, where mutants that escape the effects of the drug are usually less fit than susceptible strains, when replication is compared in the absence of the drug (for a review of fitness effects of drug resistance see Martinez-Picado and Martinez 2008). Although compensatory mutations allowing greater fitness eventually do usually occur, viruses often revert to wildtype once the drug treatment is ceased (Svedhem et al. 2002; Olivares et al. 2004). This comparison is an interesting one and future work detailing the fitness costs associated with escape from restriction factors would be informative. Conversely, experiments detailing the nature of lentiviral mutants that arise in long term culture where restriction is relieved may also help illuminate this aspect of the host-virus relationship.

4.3.2 Possible causes of TRIM5 truncation

Mammalian *TRIM5* has been shown to be highly variable in both molecular sequence and in copy number, with as many as eight copies in the cow genome. However, the evolution of non-antiviral TRIM5 alleles has now been documented in several cases such as: dogs, where it is disrupted by the insertion of *PNRC1,* preventing expression at the RNA level (Sawyer et al. 2007); humans, where a rare, dominant negative allele encoding a TRIM5α transcript truncated at the B30.2 v1 variable loop is present in West African populations (Torimiro et al. 2009); *Macaca nemestrina* where atypical isoforms

have replaced TRIM5α (Brennan et al. 2007); and the Feliformia where RNA expression is maintained but a stop mutation prevents antiretroviral activity. However, the selective pressure that has caused the maintenance of inactive *TRIM5* alleles such as these is unclear.

One possibility is the acquisition of a novel function of a truncated TRIM5 in cats. In this study, transcripts of the gene were detected in cDNA derived from domestic cat, wildcat, lion and cheetah, indicating maintenance of TRIM5 gene expression. Thus felid TRIM5 may have an alternative role that does not involve the B30.2 domain. This is plausible since many TRIM genes are expressed without an additional C-terminal domain, either through alternative splicing such as TRIM5δ and γ, or the absence of a B30.2 domain from the gene structure, such as TRIM19/PML, and have diverse or unknown functions (Sardiello et al. 2008). Indeed, the evolution of novel B30.2-lacking isoforms appears to be ongoing in the primates, with the discovery of TRIM5η and θ which appear to be unique to *Macaca nemestrina* (Brennan et al. 2007) and TRIM5 R332X in humans (Torimiro et al. 2009). Knowledge of the function of these truncated variants may go some way towards explaining the cause of multiple independent short *TRIM5* isoforms in mammals. A second possible explanation for loss of active full length TRIM5α is an unknown cost associated with its expression. Although no evidence for this exists, a parallel can be drawn to human APOBEC3H, where destabilising mutations have arisen at least twice, potentially to limit exposure of cellular nucleic acids to a hypermutation-inducing enzyme (OhAinle et al. 2008). A potential cost associated with TRIM5α expression is the non-specific targeting of cellular proteins, although this remains speculation. Finally, it is presumed that the evolution of restriction factors is sculpted by previous encounters with retroviruses, thus an alternative explanation for the absence of TRIM5α restriction is that carnivorans were relatively unaffected by retroviruses. It is

noteworthy therefore that carnivorans appear to have suffered relatively little endogenous retrovirus activity compared to primates: there are 13,000 LTR/ERV lineage-specific sequences in the dog genome compared to 133,000 in the human and 470,000 in the mouse genomes (Lindblad-Toh et al. 2005) (the annotated cat genome sequence awaits publication). Thus a period of low retroviral activity early in Carnivoran evolution may have reduced the pressure to maintain anti-retroviral TRIM5α and allowed the fixation of non-reversible disrupting alleles. Although there are now several cases of loss of antiviral activity, the nature of the evolutionary forces responsible may be different in each case, and are difficult to ascertain without knowledge of the pathogens that have historically affected each taxon or the function of the novel gene products.

Canine cells support only low levels of restriction by exogenously expressed TRIM5α (Berube et al. 2007), suggesting that either a required co-factor of TRIM5α restriction is absent or that an endogenous dog protein is exerting a dominant negative effect. Since canine *TRIM5* is known to be disrupted and not transcribed (Sawyer et al. 2007), the former explanation seems more likely. In contrast, feline cells, in which truncated TRIM5 is expressed and capable of dominant negative activity, readily support TRIM5α restriction (Saenz et al. 2005). Presumably, the high levels of expression associated with eukaryotic expression vectors saturate the endogenous levels of truncated TRIM5, obscuring any dominant negative effect. The ability to host exogenous TRIM5α suggests that cats have maintained the as-yet-unidentified co-factors necessary for restriction, whilst dogs have not. Thus it remains possible that other post-entry restriction factors are operating in cat cells that use the same pathways. Interestingly, TRIM22, a gene known to be upregulated upon interferon stimulation (Rajsbaum et al. 2008), is under adaptive selection in the

Carnivorae (Sawyer et al. 2007), suggestive of a possible role in antiviral activity.

4.4 Conclusions

The lack of TRIM5α-mediated post-entry restriction in the carnivorans contrasts strongly to the primates where TRIM5α can reduce retroviral infectivity by several orders of magnitude in non-permissive cells. This is thought to provide a barrier to transmission that must be overcome for successful invasion. Since primates and carnivorans are currently affected by closely related lentiviruses that infect and deplete similar cell populations, insight may be gained from direct comparisons between these taxa into the comparative role of TRIM5α and other restriction factors in the evolution and cross-species transmission of lentiviruses. This will be particularly powerful upon the completion of the cat genome project as the nature and extent of endogenised retroviruses in the felids as well as the host defences may be more fully analysed. Of particular interest is the *APOBEC3* gene family which has recently been shown to be expanded and active in felids (Munk et al. 2008), and tetherin which appears increasingly to be a potent block to a broad range of enveloped viruses that must be overcome for successful replication (Neil et al. 2008; Jouvenet et al. 2009). Thus the nature of these restriction factors and the extent to which they may compensate for TRIM5α are worthy of future study.

Chapter 5. The role of Cyclophilin A in the replication of feline immunodeficiency virus

5.1 Summary

Full levels of infectivity for HIV-1 are dependent on the host factor cyclophilin A (CypA), a peptidyl-proline isomerase that interacts with an exposed proline-rich loop in capsid (CA) and catalyses *cis-trans* isomerisation of the G89-P90 bond. Previous studies have shown that CypA is also bound by FIV (Lin and Emerman 2006), but does not interact with HIV-2, SIVmac or EIAV (Yoo et al. 1997; Braaten et al. 1996), suggesting that the interaction is widely conserved but not absolutely required. The role of the CA-CypA interaction is not fully defined but may promote the viral stage known as uncoating – a post-entry restructuring of viral components that promotes reverse transcription and entry to the nucleus. For primate lentiviruses, the affinity of the CA-CypA interaction appears to be maintained within a narrow window, even in mutants that are able to replicate under conditions of CypA inhibition (Yoo et al. 1997; Ylinen et al. 2009). To investigate the effects of CA-CypA inhibition for FIV, replicating virus and pseudotyped FIV particles are used to demonstrate that like HIV-1, inhibition of CypA prevents full levels of infectivity and that CypA is required during post-entry stages of the lifecycle. We next ask whether the affinity of the CA-CypA interaction is conserved between primate and feline

lentiviruses, and find that for a domestic cat strain of FIV, binding affinity to its cognate CypA is almost identical to that reported for HIV-1. Examination of a non-domestic strain of FIV isolated from lions (FIV-Ple, also known as LLV) possesses a higher affinity for CypA.

CypA can also play a part in post-entry restriction: rhesus macaque TRIM5α restricts HIV-1 more readily when CypA is present, suggesting that structural changes induced by the isomerase activity are better recognised by the restriction factor (Keckesova et al. 2006; Berthoux et al. 2005; Stremlau et al. 2006a). To assess the sensitivity of FIV to promotion of TRIM5α restriction by CypA, FIV pseudotypes were used to demonstrate a cell type-dependent decrease in infectivity associated with CypA inhibition in human cells. However, TRIM5α restriction of FIV is only weakly enhanced by CypA inhibition and TRIM5 is non-functional in cat cells, indicating that FIV does not use the CA-CypA interaction to protect from TRIM5α, and suggesting, more generally, that lentiviral CA-CypA interaction is maintained even in the absence of TRIM5.

TRIM5-CypA fusion proteins in rhesus macaques (rh) and owl monkeys (omk) are able to strongly restrict lentiviral replication (Wilson et al. 2008; Sayah et al. 2004; Newman et al. 2008; Virgen et al. 2008). Mutations in the rhTRIMCyp cyclophilin domain D66N and R69H remodel the interacting surface by causing a change from 'open palm' to 'closed fist' conformation, resulting in a change in specificity from viruses that interact with CypA (e.g. HIV-1) to viruses that do not (e.g. HIV-2; Price et al. 2009). Uniquely, FIV is restricted strongly by both rh and omk variants, suggesting an alternative binding mechanism for this virus. ITC was performed to confirm that FIV capsid N-terminal domain (CA^N) is able to bind the mutant rhTRIMCyp cyclophilin domain. To investigate how FIV is able to interact with both Cyp variants, crystal structures of FIV CA^N were solved and showed that FIV has a shortened proline rich loop that is displaced

relative to the HIV-1 loop by ~15Å and hence is unaffected by the change from 'closed fist' to 'open palm' conformation. We next asked how FIV is able to interact with CypA at a similar affinity to HIV-1, given that its proline-rich loop is substantially shorter and its binding surface is therefore reduced. Structure-guided models were used to predict that FIV CA R89 makes stabilising hydrophobic and cation-π interactions with the CypA hydrophobic pocket. In support of this model, mutagenesis of this residue negatively impacts binding affinity. In contrast, mutation of P90 was found to completely ablate CypA binding in findings reminiscent of HIV-1. However, unlike its primate counterpart, the P90A mutant is found to be non-functional in tissue culture assays, suggesting critical structural significance. Finally, an alternative potential binding partner, Ranbp2, located at the nuclear pore is identified that bears a cyclophilin domain. FIV CA is found to interact with this protein in immunoblotting assays, and the potential for future work on this binding partner is discussed.

The study finds that diverse lentiviruses have a conserved interaction with CypA that is beneficial to both HIV and FIV in cells of their respective hosts. The affinity of the interaction is maintained within a narrow range despite the highly divergent structure of the proline-rich loops. FIV is proposed to bind cyclophilin in a manner that allows promiscuous binding to both rhTRIMCyp and cytosolic free CypA. This mechanism is supported by structural and biophysical data.

5.2 Results

5.2.1 Identification of feline CypA

An analysis of the nascent cat genome project (Pontius and O'Brien 2007) revealed the presence of two regions with high CypA homology, present in BAC

clones/contigs AC235013 and AANG01610851. To establish whether the genes are expressed in cat cells, total RNA was extracted from Mya-1 cells and RT-PCR using primers specific to each CypA was performed. Products were cloned into prokaryotic expression vector pOPTH and sequencing confirmed 100% identity to published genomic sequences. Interestingly, the AC235013 sequence bears an arginine to cysteine substitution at position 69; this position is known to switch the specificity of rhesus TRIMCyp restriction from HIV-1 to HIV-2 (Wilson et al. 2008; Virgen et al. 2008). However, when RNA preps were DNase treated before reverse transcription, amplification of the gene encoded by AC235013 was no longer successful, suggesting that previous amplification was from contaminating genomic DNA. In contrast, amplification of AANG01610851 CypA (bearing R69) was DNase resistant, indicating that the latter is expressed in cat cells, confirming it as the biologically relevant version.

An alignment of feline and human (NM_021130) CypA reveals 97% identity between the homologues (Figure 5-1), suggestive of highly conserved structure and function. In contrast, FIV and HIV-1 CA share only partial homology with 29% identical residues, reducing to 23% in the N-terminal domain (CA^N) (Figure 5-2A). The sequence of the proline-rich loop is highly divergent, with the FIV loop shorter by four residues. Given this high level of sequence divergence, the finding that both capsids bind the relatively invariant CypA (Lin and Emerman 2006) is therefore interesting as it suggests that either the capsids share conservation of structure despite divergent primary sequences, or that the capsids use alternative mechanisms to interact with CypA. Amongst strains of FIV, homology of CA^N is substantially higher at 53% identical and 70% conserved residues (Figure 5-2B). Conservation is almost total within the proposed proline-rich loop, suggesting a biologically important and conserved interaction.

Figure 5-1 Human and feline CypA share 97% identical residues. Beta strands are denoted by arrows and helices are denoted by cylinders. Residues F60, M61, F113 and L122 which create the substrate-binding hydrophobic pocket are marked by black bars. Large functional differences between the CypA homologues are unlikely given the high levels of identity. Identical residues in grey.

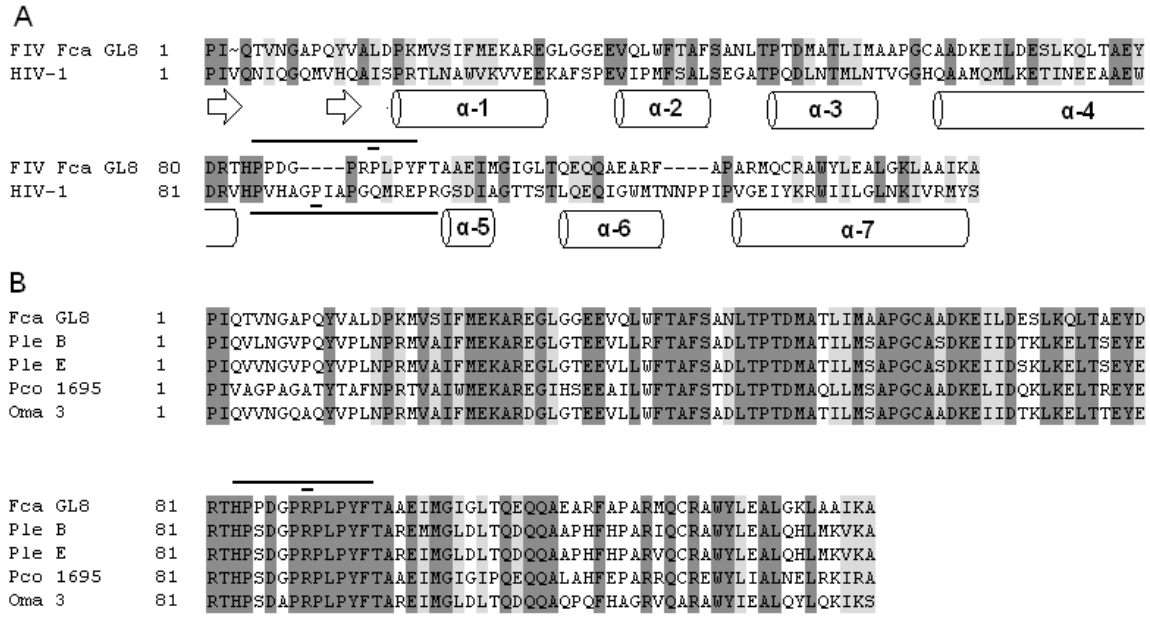


Figure 5-2 Alignment of lentiviral capsid sequences. A) FIV and HIV-1 CA N-terminal domains show substantial variation (23% identical residues, 43% conserved). Secondary structures are shown for HIV-1 CA, with arrows representing beta strands and cylinders representing alpha-helices (numbered for HIV-1). The proline-rich loop between helices 4 and 5 is present in both sequences (single bar) but is highly divergent in both its length and primary sequence. The nature of the isomeric peptidyl-proline bond (double bar) is also divergent, with FIV possessing RP compared to HIV-1's GP. B) Capsid sequences from FIV strains isolated from diverse host species show substantial

conservation throughout CA and show high levels of conservation in the proline-rich loop. P90, shown to be crucial for CypA interaction and proposed site for *cis-trans* isomerisation (Lin and Emerman 2006) is indicated with a double bar.

5.2.2 Biological effects of FIV CA-CypA interaction

The inhibitor of the CA-CypA interaction, cyclosporine A (CsA), is an immunosuppressant that interferes with the intracellular T-cell activation pathway. CsA analogues NIM811 and Debio-025 were designed as non-immunosuppressive CsA analogues that do not provide the calcineurin binding surface when complexed with CypA. The drugs were assayed for their toxic effect on activated feline T-cells Mya-1 (Miyazawa et al. 1989) and on naive CD3+ T-cell line CLL (Willett et al. 2006a) (Figure 5-3). Mya-1 cell viability was found to be substantially reduced after 5 days incubation with 2μM CsA, whereas NIM811 and Debio-025 showed a reduced toxic effect. CLL cells were largely unaffected by the presence of the drugs, as is expected given their lack of mitogen stimulation and activation.

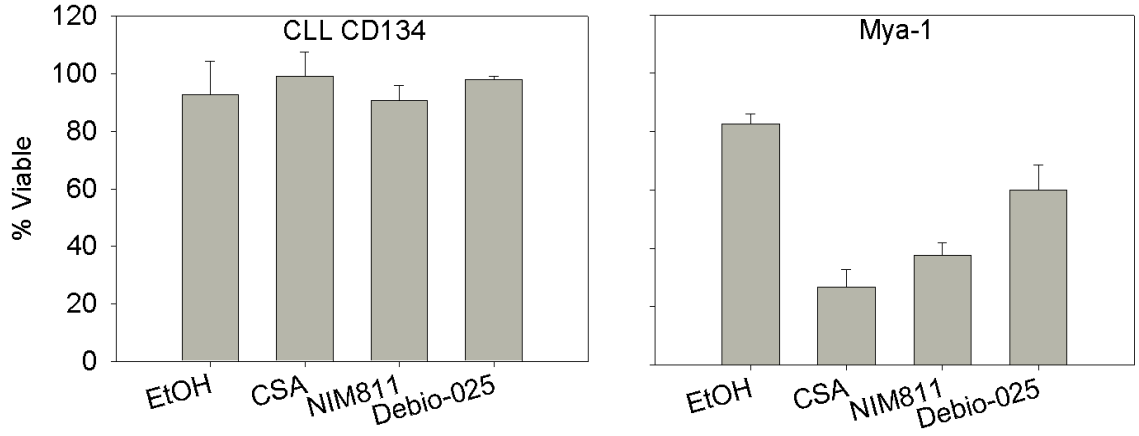


Figure 5-3 Relative toxicity of CypA-binding drugs. Cell viability was measured after 5 days incubation in 2μM drug or solvent only (ethanol). For stimulated T-cell line Mya-1, CsA was found to exert a 67% reduction on the percentage of viable cells, compared to a 27% reduction by non-immunosuppressive Debio-025. In contrast, CLL cells

transduced with feline CD134 were found to be unaffected by any of the drugs, indicative of their naive state.

The ability of FIV to replicate in the presence of CsA and its analogues was assayed by challenging CLL CD134 and Mya-1 with FIV-Fca GL8 (Figure 5-4). In both CLL and Mya-1, a reduction in FIV replication was seen upon the addition of inhibitors, suggesting that the CA-CypA interaction is important for replication of FIV. Inhibition of replication was more pronounced in Mya-1, in particular with CsA, an effect presumably due to the toxicity effects of the inhibitors on Mya-1 cell viability.

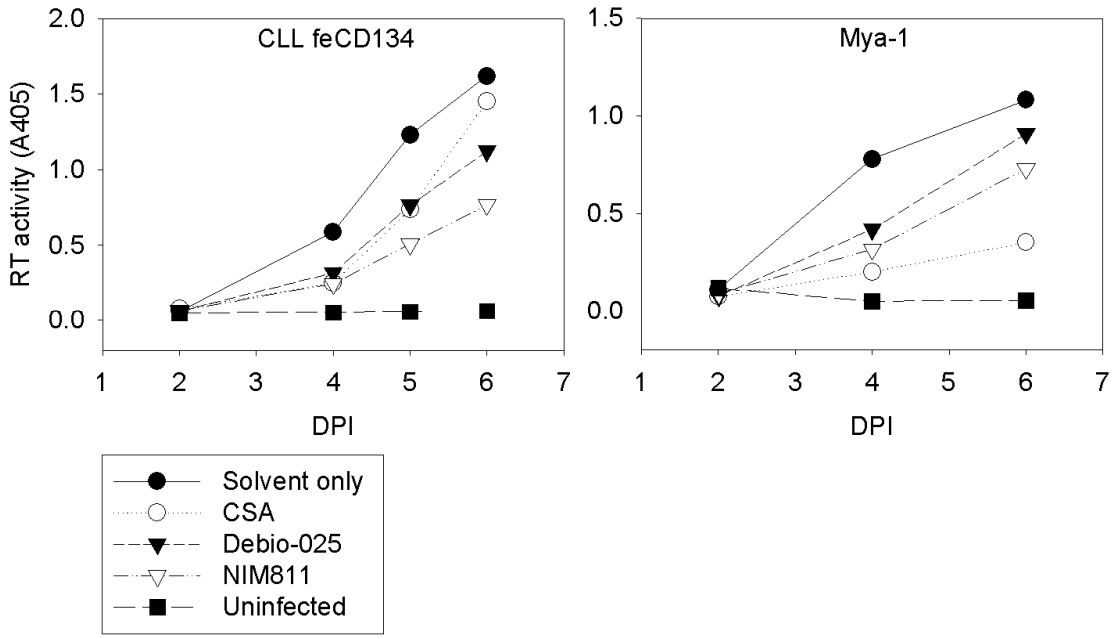


Figure 5-4 Inhibitors of the CA-CypA interaction impair FIV replication. CLL CD134 and Mya-1 cells were challenged with FIV-Fca GL8 and incubated with CsA and its analogues NIM811 and Debio-025 at 2μM. Supernatant aliquots were frozen and subsequently analysed for reverse transcriptase activity. A repeatable decrease in RT activity was observed in the presence of the inhibitors, suggesting that the CA-CypA interaction is important for replication of FIV.

For HIV-1, the effects of the CA-CypA interaction are manifest during the early post-entry stage of replication (Towers et al. 2003). To establish at which

stage of replication the CA-CypA interaction is important in the FIV lifecycle, the drugs were added to cells transfected with FIV-GFP(VSV)-encoding plasmids (virus production) or to target cells (virus entry). When added during virus production, supernatant was subsequently pelleted through a 20% sucrose cushion to remove traces of the inhibitor (Figure 5-5). A small non-significant increase in titre (two-tailed *t*-test; $p = 0.094$ for control vs 2μM CsA) was observed when inhibitors were added during virus production. A similar effect is seen in HIV-1 production in human cells, with a ~10% increase in viral titre reported by Towers et al. (2003). However, during viral entry, a 13-27% decrease in titre was observed at 0.5μM inhibitor that was significant (two-tailed *t*-test; CsA $p = 0.012$; NIM811 $p = 0.018$; Debio-025 $p = 0.0008$ for control vs 0.5μM inhibitor). Although small in magnitude, repeated over several cycles of infection, this decrease in titre could substantially reduce the titre of replicating virus and is likely to be the cause of the reduced replication of FIV in Figure 5-4. We can therefore conclude that CypA acts as a cofactor for viral replication in cells of its host and that CypA exerts its effect post entry, early in the lifecycle. The findings are reminiscent of HIV-1, but of a much lower magnitude: HIV-1 pseudotype infectivity is reduced approximately three-fold during early replication stages of infection in human Jurkat and TE671 cells (Towers et al. 2003; Hatziioannou et al. 2005). However, for HIV-1, CsA treatment is proposed to increase susceptibility to an unidentified restriction factor in human cells (Keckesova et al. 2006; Sokolskaja et al. 2006) so much of the decrease in infectivity may be attributed to this factor.

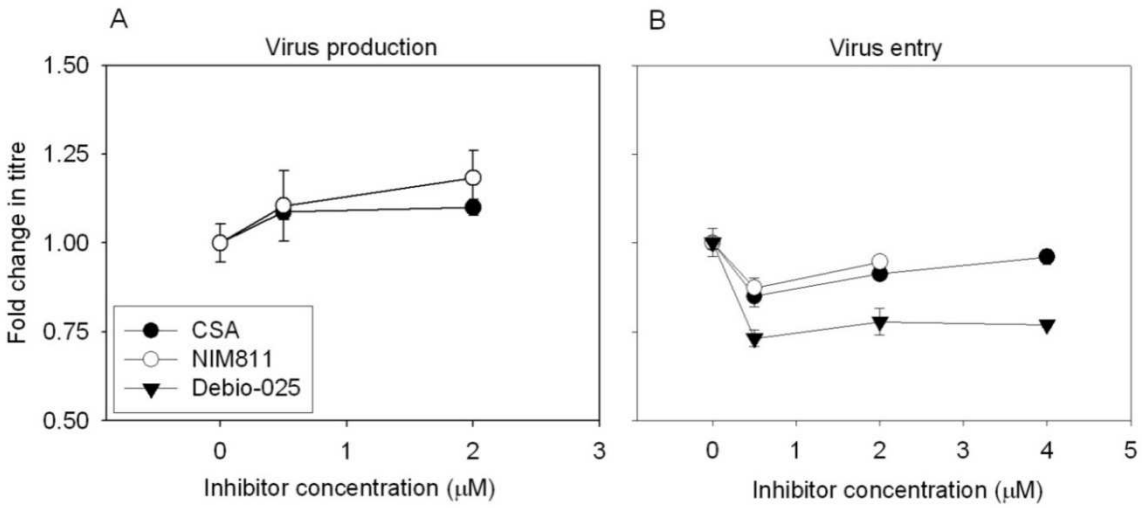


Figure 5-5 Effect of CypA inhibitors on FIV pseudotype production and entry. A) 293T cells, transfected with FP93, GinSin and VSV-G expressor MDG were incubated with CypA inhibitors. Viral supernatant was pelleted over a sucrose cushion to remove inhibitors and titrated onto CrFKs at an moi of ~0.1. A small but non-significant increase in titre was observed for both CsA and NIM811. B) FIV-GFP(VSV) pseudotypes were titrated onto CrFKs pre-incubated with CypA inhibitors at an moi of ~0.1. A significant decrease in titre was seen for 0.5μM of all the drugs, an effect that seemed to diminish as concentrations increased further. A toxic effect of NIM811 on CrFK cells was observed at 4 μM and no data was recorded for these samples. n = 3.

Restriction of HIV-1 by rhesus macaque TRIM5α is enhanced by the presence of CypA, suggesting that CypA perfoms a catalytic role that promotes the TRIM5α-CA interaction (Keckesova et al. 2006; Berthoux et al. 2005; Stremlau et al. 2006a). In contrast, in humans cells it is probable that CypA both aids in uncoating and protects HIV-1 from an unidentified post-entry restriction factor (Keckesova et al. 2006; Sokolskaja et al. 2006). To assess whether CsA affects huTRIM5α-mediated or other restrictions of FIV in human cells, FIV-GFP(VSV) pseudotypes were titrated onto human TE671 and HeLa cells in the presence of CsA or solvent only (Figure 5-6). In TE671 cells, no change in FIV infectivity was observed, suggesting that in these cells FIV is not targeted by the unidentified human factor, nor does the CypA-CA interaction alter the huTRIM5α-mediated

restriction of FIV. However, in HeLa cells, which are known to express much higher levels of CypA, a much lower level of infection is achieved, which reduces further on the addition of CsA. Although the causes of these cell type-specific responses are unknown, by analogy with HIV they suggest a role for CypA in either uncoating or protection from restriction factors.

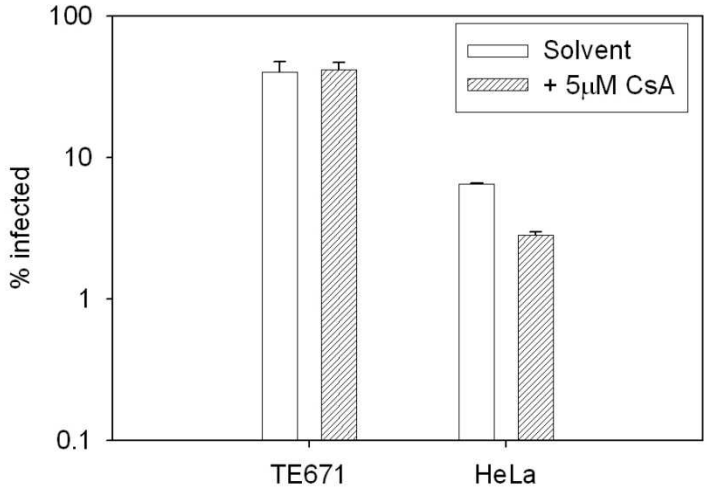


Figure 5-6 Differing effects of CsA treatment on FIV infectivity in human cells. TE671 and HeLa cells, which express low and high levels of CypA respectively, were infected with FIV (VSV)-pseudotyped particles in the presence or absence of CsA. The results suggest that in HeLa cells, CypA aids in FIV infectivity, possibly by aiding uncoating or protecting from a restriction factor such as TRIM5α.

To investigate whether restriction of FIV by TRIM5α can be modulated by the CA-CypA interaction, CrFK cells transduced with huTRIM5α were challenged with FIV-luc(VSV) pseudotypes (Figure 5-7). An approximately 10-fold reduction in titre is observed in cells expressing huTRIM5α, and a reduction in titre associated with addition of CsA is more pronounced in the presence of TRIM5α. However the magnitude of the change is small, and given that TRIM5 is non-functional as a restriction factor in cat cells (Chapter 4; McEwan et al. 2009), the FIV CA-CypA interaction is unlikely to have evolved for this purpose.

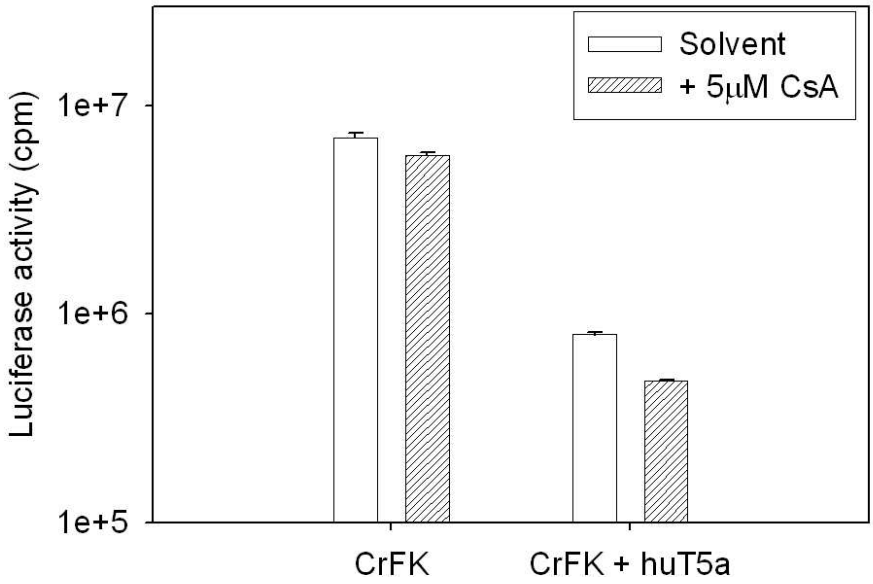


Figure 5-7 Effect of CsA and TRIM5α on FIV infectivity. CrFK cells transduced with human TRIM5α were challenged with FIV-luc(VSV) pseudotypes. TRIM5α reduces FIV infectivity by an order of magnitude. CsA has a greater effect on the infectivity in the presence of TRIM5α, suggesting that isomerase activity of CypA may protect FIV slightly from TRIM5α-mediated restriction. However, FIV has evolved in the absence of functional TRIM5α (Chapter 4; McEwan et al. 2009), making deliberate evasion of restriction by this mechanism an unlikely biological scenario.

5.2.3 ITC reveals lentiviral CA-CypA affinity to be conserved

To investigate the binding properties of FIV capsid for CypA, the CA^N of FIV-Fca and FIV-Ple E Sangre as well as domestic cat and lion CypA genes were cloned into prokaryotic expression vector pOPTH and recombinant His-tagged proteins were purified by Ni-affinity chromatography. Further purification of proteins was performed by ion exchange chromatography and, for CA^N constructs, subsequent gel filtration (see Materials and Methods). The proteins were prepared for isothermal titration calorimetry assays that measure the change in enthalpy associated with intermolecular interactions when CypA is titrated into CA^N (Figure 5-8 and Table 5.A). The analysis reveals that FIV-Fca CA^N binds its cognate CypA partner with a stoichiometry of 1:1 and a Kd of 6.2 μM, a

figure that compares closely to HIV-1 CA-CypA interaction of 5.3 μM. FIV-Ple CA^N was found to bind lion CypA with a slightly higher affinity, at 2.2 μM. Changing the species of the CypA had little effect on the Kd, suggesting that as predicted, the species-specific differences between feline and human CypA have little effect on the interaction.

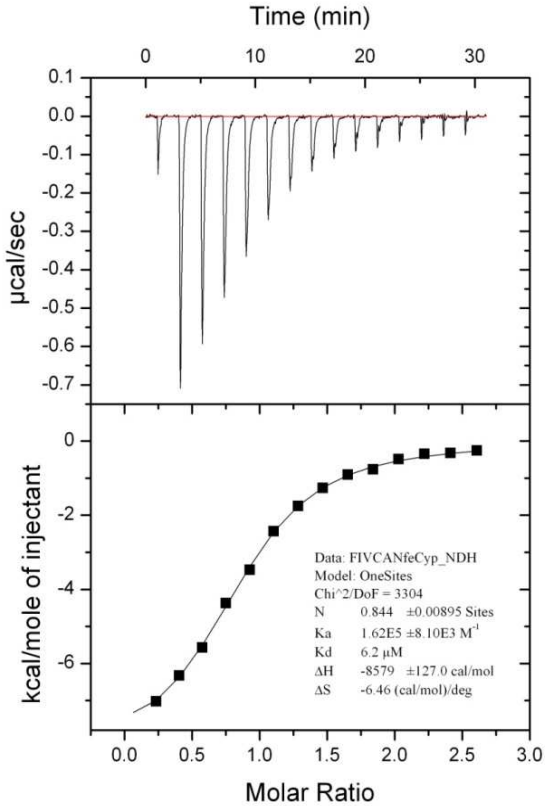


Figure 5-8 Isothermal titration calorimetry reveals FIV to bind CypA. Example trace of ITC shows heat associated with multiple injections of CA^N into cell bearing cat CypA. The reaction has a stoichiometry (N) of approximately 1:1, Kd of 6.2μM and is exothermic, ΔH = -8579 cal/mol.

	Cat CypA	Lion CypA	Human CypA
FIV-Fca	6.2	7.8	7.2
FIV-Ple	1.9	2.2	3.0
HIV-1	n.d.	n.d.	5.3

Table 5.A Binding constants for capsid and CypA variants. Various CA^N domains were assessed for their ability to bind CypA. Shown are Kd values in μM. FIV-Ple is found to have the highest binding affinity at 2.2μM for its cognate CypA, whilst FIV-Fca

and HIV-1 Kd values are very similar. Changing the CypA variant had little effect on the affinity of the interaction, and all interactions were within a narrow range of 2-8 μM.

5.2.4 Structural analysis of feline cyclophilin A

The similarity of binding constants between human and feline CypA are in accordance with the high levels of similarity between these proteins. A crystal structure of feCypA was solved and reveals almost total conservation of structure around the hydrophobic pocket (Figure 5-9). The four amino acid substitutions between the species are found on the distal surfaces.

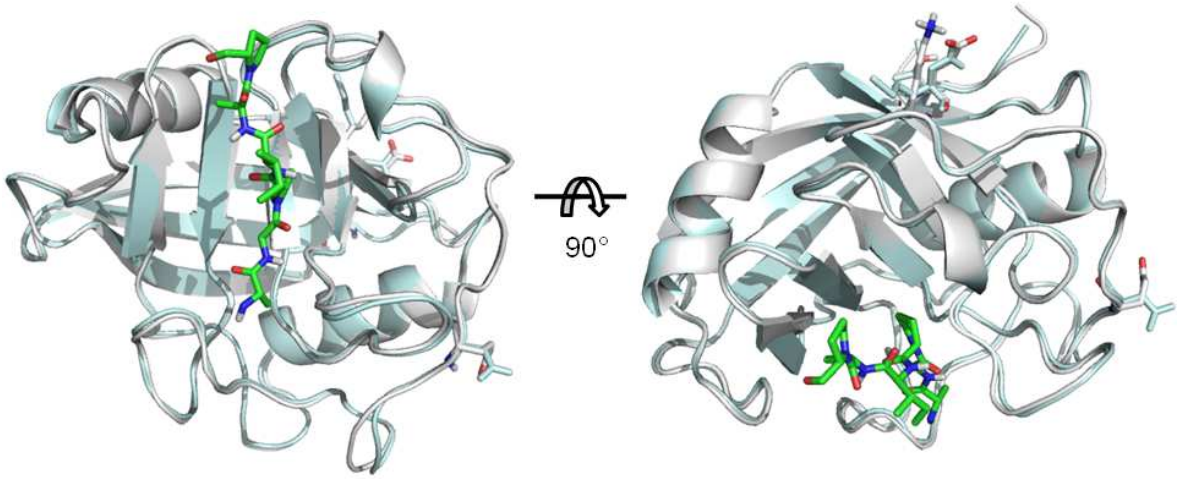


Figure 5-9 Superimposed structures of human and feline cyclophilin A. A crystal structure of feline CypA (light blue) was solved and superimposed on published human CypA (white) structure bound to HIV-1 capsid (proline-rich loop AGPIAP motif shown in green; PDB: 1AK4). Around the hydrophobic pocket, which interacts with HIV-1, structural conservation is high. A rotated view (right) shows feline mutations I5, D23, D84 and M131 (sticks) to be on the posterior and lateral surfaces, making substantial differences in biological activities of human and feline CypA unlikely.

5.2.5 FIV interacts with both CypA and rhesus TRIMCyp

The cyclophilin domain of rhesus macaque TRIMCyp bears two point mutants relative to wildtype CypA that alter its specificity from HIV-1 CA binding to

HIV-2 binding (Price et al. 2009). Substitutions D66N and R69H result in a 15Å remodelling of a 10-residue lobe to give the closed fist conformation and precluding HIV-1 CA binding. FIV is unique in that it can be restricted by both owl monkey TRIMCyp, whose CypA domain resembles wildtype CypA, and rhTRIMCyp. To formally demonstrate FIV CA-rhTRIMCyp binding, ITC was performed using CA^N and the Cyp domain of rhTRIMCyp and, as predicted an interaction was observed. However, suggestions that FIV's high sensitivity to rhTRIMCyp restriction may be due to high binding affinities were unfounded as the Kd was found to be lower than for feCypA (Table 5.B), suggesting that the sensitivity is achieved through other means.

	Cat CypA	**rhTCyp**
FIV-Fca	6.2	10.9
FIV-Ple	1.9	7.3

Table 5.B. Binding constants for FIV CA^N-rhesus TRIMCyp. FIV is particularly sensitive to restriction by rhesus macaque TRIMCyp (Virgen et al. 2008), but the sensitivity is not conferred by a higher affinity interaction between CA and rhTRIMCyp compared to CypA.

5.2.6 Crystal structure of FIV capsid

Despite being highly divergent at the protein level, HIV-1 and FIV both interact with CypA at affinities within a small range of Kd values. Especially interesting is the conformation of the highly divergent predicted proline-rich loop raising interesting questions about the comparative structures. Do FIV and HIV-1 share a similar structure in order to interact with CypA or is binding by FIV achieved via a different mechanism? And what is the basis of FIV's dual specificity for CypA and rhTCypA? In order to address these questions, a crystal structure of FIV CA^N was sought and solved to a resolution of 2.3Å.

The structure of FIV CA^N is the first published to date. Comparison of the structure with HIV-1 capsid (1AK4; Gamble et al. 1996) reveals a remarkably conserved wedge-shaped structure, with the proline-rich loop extending outwards between helices 4 and 5 (Figure 5-10). The α-helices display a strikingly similar orientation in most regions of the protein, representing conserved exposed regions of capsid core meta-structure. One particular region of dissimilarity is in the FIV region corresponding to HIV-1 helices 5 and 6 which appear to be fused creating a longer kinked C-terminal helix. The α-6 α-7 linker region in HIV-1 bears a proline-proline dipeptide that re-orientates the strand the downwards into the α-6 helix. This linker region, including the PP dipeptide, is missing in FIV, resulting in the enlarged fusion helix.

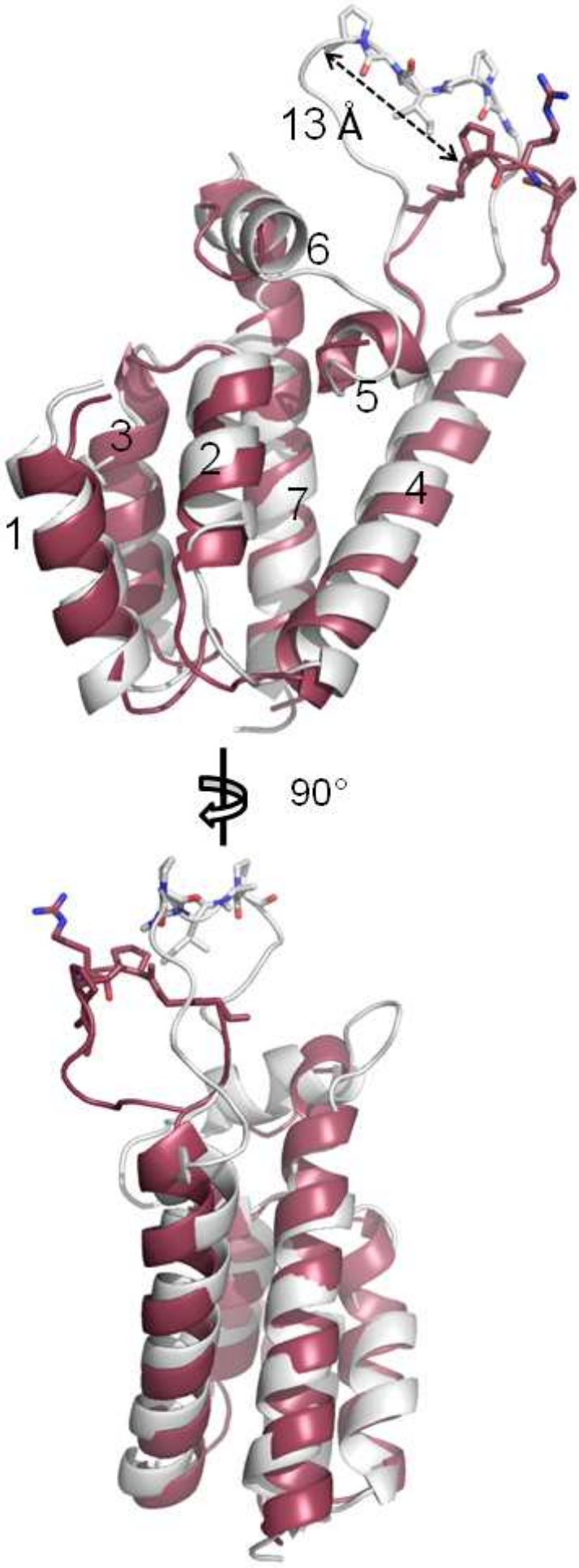

13 Å
6
5
3
2
7
4
1
90°

Figure 5-10 Superimposed structure of FIV and HIV-1 capsid N-terminal domains. Despite only 23% identical residues, FIV (dark red) and HIV-1 (1AK4) (grey) capsids share remarkable structural conservation, characterised by a wedge-shaped structure comprising the major helices 1, 2, 3, 4 and 7 (HIV-1 numbering) with a proline-rich loop extending outwards between helices 4 and 5. The side chains in the crest of the proline-rich loops are shown as sticks (FIV: 87GPRPL91 , HIV-1: 89GPIAP93, isomerised bond underlined) and reveal FIV to have a divergent loop that is displaced by ~13Å. A side view (below) clearly shows the fusion of helices 6 and 7 that creates the larger FIV N-terminal α-helix.

5.2.7 Proline-rich loop mutants identify important residues in the CA-CypA interaction

As is evident from the primary sequence alignment, the proline-rich loop for FIV is considerably shorter than in HIV-1, raising interesting questions about how the shorter FIV loop is still able to bind CypA. To address this issue, two alternative methods were employed. Firstly, it was attempted to crystallise CA^N-CypA complexes. CA^N and huCypA as well as CA^N and rhTCypA were mixed at 1:1 molar ratio and run on a gel filtration column to purify the larger, complexed species. However, crystallisation of the complex was unsuccessful with only uncomplexed CA^N or CypA crystals forming. Secondly, it was attempted to use 4-mer or 5-mer oligopeptides corresponding to the proline-rich loop of FIV CA with feCypA to grow complexed crystals. However, initial ITC experiments to confirm binding of the peptides were unable to show an interaction. A crystal screen was performed nonetheless and yielded crystals for the 4-mer peptide. Subsequent X-ray diffraction revealed that the peptide had not bound, with the symmetric unit comprising only feCypA.

A model of the FIV CA-CypA interaction was therefore made by superimposing the CA structure on HIV-1-CypA structure. The model suggests that the short FIV proline-rich loop has a reduced region of contact with CypA, not extending

across the entire hydrophobic pocket. In HIV-1, the G89-P90 bond exists as *cis-trans* isomers. However in FIV, the corresponding peptide bond is R89-P90, suggesting a functional significance to the change. Arginine commonly contributes to intermolecular protein-protein interactions through H-bonding, the hydrophobic effect and by cation-π interactions with aromatic side chains (Crowley and Golovin 2005). A structure-guided model (Figure 5-11) predicts that R89 comes into close proximity with CypA residues F60 and F113, suggesting that cation-π interactions with these residues may contribute substantially to the affinity of the interaction.

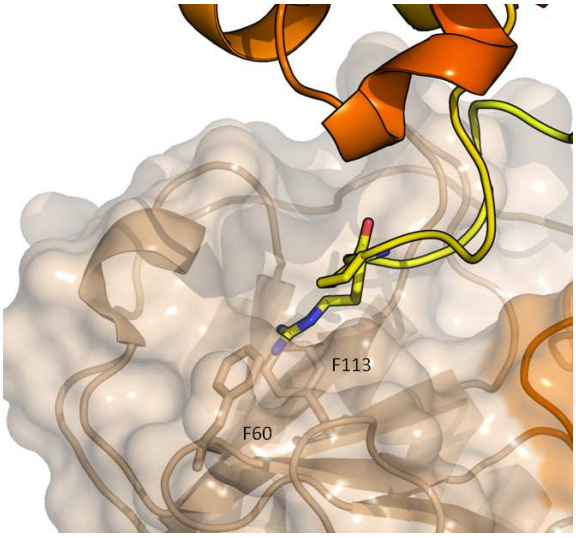


Figure 5-11 Structure-guided model of FIV CA-CypA interaction. FIV is proposed to bury R89 deep within the hydrophobic pocket, potentially forming cation-π interactions acting to increase the binding affinity of the interaction.

To test the hypothesis that R89 contributes to the FIV CA-CypA affinity, the R89 residue was mutated to the HIV-1 glycine by PCR-based mutagenesis and the protein was expressed and purified according to the protocol established for wildtype FIV CA^N. Assessment of this mutant's ability to interact with CypA was performed by ITC and displayed a 4-fold reduction in binding affinity, indicating that this residue contributes to full levels of interaction with CypA (Table 5.C). The finding is therefore in accordance with the model of FIV CA-

CypA interaction, and provides some insight as to why FIV and HIV-1 diverge at this residue. In contrast, mutating the P90 residue to alanine was found to prevent detectable interaction with CypA, indicating the structural importance of this residue. A P90A mutant of replication-competent FIV molecular clone, as well as pseudotyped mutant was also generated but unlike HIV-1 where mutating P90 prevents the CypA interaction but allows infection, the FIV P90A mutants were found to be non-functional. This finding has been repeated by others (G. Towers, personal communication) but is in contradiction to Lin and Emerman (2007) who were able to produce viable pseudotype with the P90A mutation. The discrepancy is potentially explained by the use of alternative strains of FIV (Lin and Emerman used 34TF10, whereas this study used GL8) but suggests that in the GL8 background, P90 is structurally integral to capsid's function.

	Kd (μM)
FIV-Fca WT	5
FIV-Fca R89G	21
FIV-Fca P90A	None detected

Table 5.C Binding affinity for FIV CA mutants with cat CypA. CA^N mutants R89G, which is hypothesised to contribute to the binding of the CypA catalytic domain through cation-π interactions, and P90A, which is proposed to be the site of *cis-trans* isomerase activity, were assayed for their ability to bind cat CypA by ITC. As predicted, R89 contributes to the strength of interaction with Cyp as mutation to the corresponding HIV-1 residue glycine reduces binding affinity substantially. Mutation of P90 prevents detectable interaction.

Structure-guided models were used to assess how FIV is able to interact with both wildtype CypA and rhTRIMCyp. The model suggests that that the shorter FIV proline-rich loop is deflected away from the 66-73 loop by a kink generated by proline 85, so that when bound to rhTRIMCyp with its 'closed fist' conformation, the 66-73 loop does not interfere with binding (Figure 5-12).

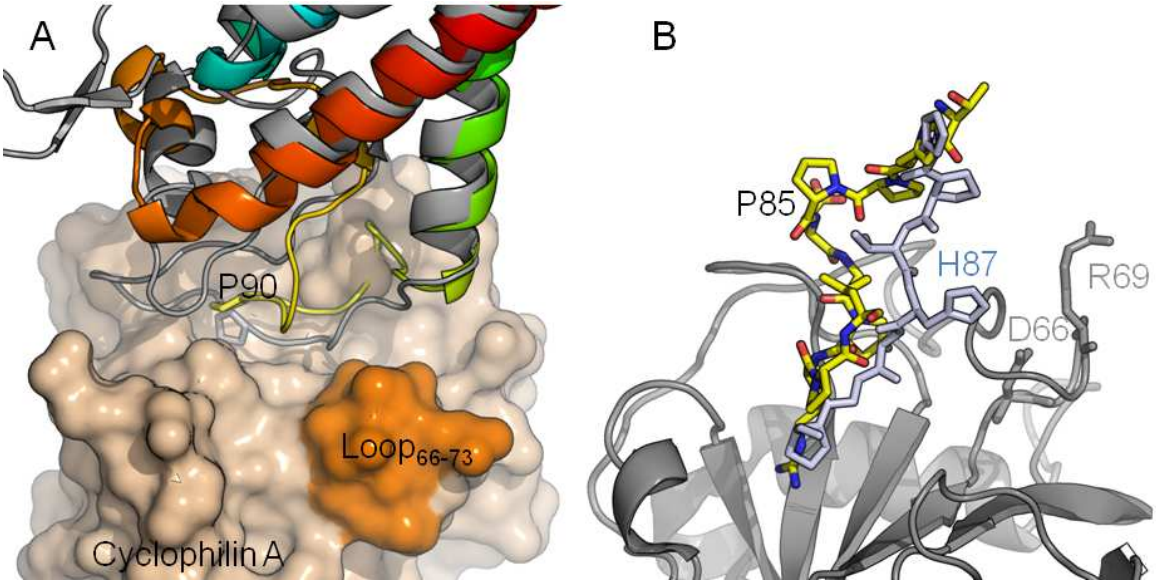


Figure 5-12 Molecular mechanism of FIV CA-CypA interaction. A) FIV is independent of the CypA 66-73 loop (dark orange), which is rotated inwards towards the catalytic domain in rhTRIMCyp, due to a kink in the proline-rich loop at P85 that deflects the backbone away from this site. HIV grey, FIV rainbow, CypA orange. B) An enlarged view shows that FIV P85 (yellow) is considerably further from the 66-73 loop (grey) than HIV H87 (blue), which is displaced by the rhTRIMCyp D66N mutation.

5.2.8 Potential roles of the CA-CypA interaction

This study shows that whilst FIV's proline-rich loop is highly conserved, disruption of the interaction only impacts marginally on infectivity of the virus. However, mutation of P90 ablates binding and appears to prevent replication. This effect is probably best explained by structural abnormalities in the mutant protein that prevent proper function, rather than by functional differences of CypA binding. Curiously, an alignment of diverse lentiviruses capsids reveals that a proline-rich loop is present not only in those viruses with confirmed CypA binding but also those confirmed as non-binding. It is also noteworthy that naturally occurring HIV-1 isolates are found which are insensitive to CsA, again suggesting that the interaction is dispensable (Chatterji et al. 2005; Ikeda et al. 2004).

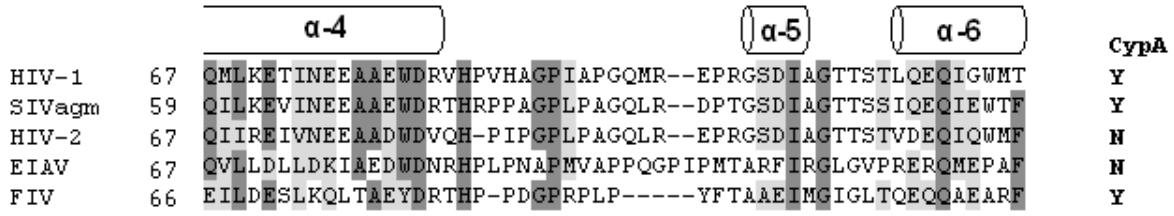


Figure 5-13 The presence of a proline-rich loop is not a feature unique to viruses which bind CypA. Alignment of amino acid sequences to HIV-1 shows that a proline-rich region is present in HIV-2 which does not bind CypA and is even present in EIAV. Y or N indicates studies demonstrating CypA binding or its absence; HIV-1 domain architecture is shown.

These observations support the hypothesis that the primary function of the proline-rich loop is to interact with an alternative protein that resembles CypA, as this would explain the close resemblance to the CypA-binding loop in viruses such as HIV-2. A search was therefore carried out for genes which contain a CypA domain, and that might be important for post-entry stages of the lifecycle. Most notably, a protein was identified in a BLAST search of human RefSeq protein sequences that bears a domain closely related to CypA, named Ranbp2 or Nup358. Ranbp2 is a large (358 kDa) protein located at the nuclear membrane that is involved in conversion of RanGTP to RanGDP, a process important for translocation of cellular proteins across the nuclear membrane. Consistent with a possible role in the lentiviral lifecycle, the protein has been identified in three out of four whole genome siRNA screens as a necessary cofactor for HIV replication (Bushman et al. 2009; Konig et al. 2008; Brass et al. 2008; Ranbp2 was not identified in Zhou et al. 2008). An obvious potential role of Ranpb2 is interaction with capsid present in the RT-complex to facilitate entry to the nucleus, a requirement for replication in non-dividing cells. However, being a major component of the nuclear pore complex, it is unclear whether knockdown of Ranbp2 specifically prevents HIV-1 nuclear import or simply structurally compromises the nuclear pore.

A method known as far-western blot (or overlay assay) was optimised based on an established protocol (Einarson et al. 2007) that allows identification of cellular binding partners for specific proteins. In this case the technique uses recombinant CA protein to probe renatured SDS-PAGE proteins displayed on a nitrocellulose membrane and subsequent immunoblotting for CA. Using recombinant GST-tagged FIV capsid (entire protein) to probe membranes bearing HeLa and CrFK nuclear and cytoplasmic fractions, a series of bands could be detected that are likely to represent cellular binding partners for FIV capsid (Figure 5-14). Several of these bands are conserved in size between cat and human cells, most notably the bands at ~55 and ~65 kDa, whilst others appear to be species- and subcellular location-specific. In cat cells these were CrFK nucleus-specific bands at ~350 kDa and ~170 kDa and CrFK nucleus and cytoplasmic band at ~110 kDa. In human cells, these were a cytoplasm-specific band at ~100 kDa, and predominantly cytoplasm-specific band at ~130 kDa.

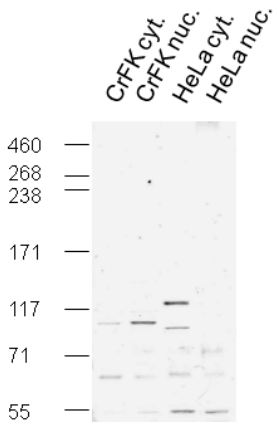


Figure 5-14 Far-western blot using full length FIV capsid. Probing fractionated cell lysates with FIV capsid demonstrates that the far-western technique can identify potential protein binding partners in CrFK (cat) and HeLa (human) cells. Some proteins identified have conserved size between the species, for example the bands present at ~55 and 65 kDa, whilst others appear species-specific but may represent species-specific size variations or isoforms of same protein.

The N-terminal domain of CA bears the proline-rich loop responsible for interacting with CypA. It was therefore decided to use CA^N, in order to identify bands which only interact with this domain (Figure 5-15). Human cell lysate was found to interact with FIV CA^N at ~130 kDa and a doublet at ~65 kDa. The bands at 55 and 110 kDa were no longer present, perhaps indicative of a CA C-terminal-specific interaction. The human cell lysate was also probed with α-Ranbp2 polyclonal antibody and found to identify the same bands at ~130 kDa and a doublet at 65 kDa. The data therefore represent strong evidence that FIV CA can interact with Ranbp2. However, bands at the expected Ranbp2 size of 358 kDa were faint or not identified. This suggests that the protein is perhaps poorly transferred to the membrane in its full size and the bands indentified are likely to be either Ranbp2 isoforms or products of proteolysis. The P90A mutant, previously shown to be non-functional for replication and unable to bind CypA, was found to identify the same bands. The result is surprising given the hypothesised lack of interaction for this protein. One possible explanation for this binding is the large excess of CA used to probe the membrane and the non-stringent binding conditions may have allowed complete saturation of the sites available for binding on the membrane. Further optimisation of this technique may allow detection of bands of differential intensity that better reflect the binding affinity.

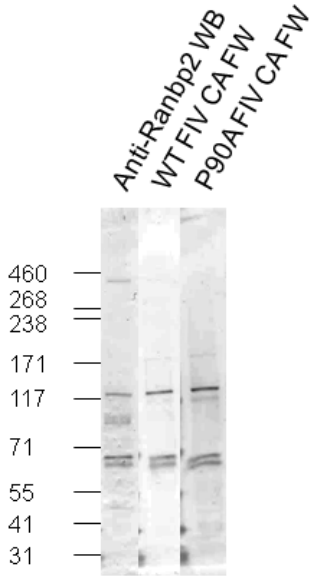


Figure 5-15 FIV capsid and α-Ranbp2 antibodies interact with proteins of the same size. Western blot (WB) on 293T cell lysate with α-human Ranbp2 identifies a band at ~130 kDa and a doublet at ~65 kDa, when probed with an α-rabbit IgG secondary Ab. These bands are of exactly the same size as those identified by FIV CA^N by far western blot (FW) with detection by mouse α-CA and α-mouse IgG secondary. Contrary to expectation, the FIV CA mutant P90A was also found to interact with proteins of the same size.

5.3 Discussion

This study demonstrates that FIV, like the primate lentiviruses, interacts with and is modulated by host factor cyclophilin A. Like HIV-1 in cells of its own host, disruption of the interaction with CsA or its non-immunosuppressive analogues prevents full levels of infectivity in both replicating viruses and in post-entry stages of pseudotype infection. FIV also displays a cell-type dependent CypA dependence in human cells. The precise nature of this relationship is unclear but suggests that FIV is sensitive to cytosolic levels of CypA given that HeLa cells have been shown to express high levels of CypA (Ylinen et al. 2009). Possible mechanisms of CypA's positive role in FIV replication include aiding uncoating, as has been hypothesised for HIV-1, or preventing restriction by other cellular factors. Sensitivity of FIV to huTRIM5α

is modestly increased by treatment with CsA, suggesting that in this system CypA can protect against restriction. In contrast restriction of HIV-1 by rhTRIM5α is promoted by CypA (Keckesova et al. 2006; Berthoux et al. 2005; Stremlau et al. 2006a). The best current model of the effects of CypA on lentiviral infectivity suggests that CypA performs a catalytic isomerisation that alters capsid conformation which subsequently alters the ability of other cellular restriction factors to recognise and destroy incoming capsid (Towers 2007; Luban 2007). This suggests that in the context of TRIM5α, where binding is mediated by variable domains in the B30.2 domain, its ability to bind and restrict can be modulated by CypA (potentially increasing rhTRIM5α's affinity for HIV-1; decreasing huTRIM5α's affinity for FIV). Although CypA has been shown to perform such a catalytic role on CA *in vitro*, the *in vivo* relevance of isomerisation has yet to be formally demonstrated. Other hypotheses, such as generation of a novel CA-CypA complexed surface that is subsequently recognised by restriction factors, or CypA-dependent recruitment of cellular machinery cannot be ruled out. Study in this field is impeded by the absence of CypA mutants that bind CA but do not perform the catalytic function, and similarly, functional CA mutants that bind CypA but cannot be isomerised. Thus like some of the primate lentiviruses, infectivity of FIV is influenced by the CA-CypA interaction but its precise role remains unclear.

Unlike the surfaces of restriction factors that interact with pathogens, CypA is remarkably conserved. Its ubiquitous expression and relative invariance suggest a critical house-keeping role, although its function is elusive. Yeast mutants lacking all twelve cyclophilin genes remain viable, although display retarded growth (Dolinski et al. 1997). Moreover, the contribution of each knockout gene is additive, suggesting that the cyclophilins do not complement each other's function and that each cyclophilin has a specific role, rather than each contributing to a protein folding phenotype. Roles for individual proteins have

been ascribed to several cyclophilins such as signal transduction (Duina et al. 1996) and rhodopsin folding (review Gothel and Marahiel 1999; Stamnes et al. 1991) but CypA has no precise role defined to date. Nevertheless, it is likely that CypA is constrained by cellular tasks and is unable to undergo the high levels of positive selection that interaction with pathogens usually entails. However, the TRIMCyp fusion protein is presumably relieved of its cellular role and, in rhesus macaques, has acquired mutations that remodel the binding surface and promote HIV-2 restriction (Wilson et al. 2008; Virgen et al. 2008; Price et al. 2009). The ability of FIV to bind both Cyp variants demonstrates that binding to CypA and rhTRIMCyp are not properties mutually exclusive to an individual virus.

It is becoming clear that lentiviruses maintain the affinity of the CA-CypA interaction within a narrow range. It would be expected that HIV-1 growing in the absence of CypA (e.g. under CsA inhibition) would acquire mutations to increase the affinity in order to maintain a given level of CA-Cyp interactions. However, the mutants arising possess Kd values within the same low micromolar range but may be more sensitive to the activity of CypA, perhaps by being more liable to uncoating by low levels of peptidyl-proline isomerisation. The finding that FIV binds CypA with almost identical affinity as HIV-1 is surprising given the evolutionary distances and scarce amino acid identity between the viruses and implies that binding to CypA is very tightly regulated on the part of the virus. Moreover it appears that viruses can employ a CypA-dependent or –independent strategy with no more than a few point mutations. HIV-2 can switch to CypA binding with a single mutation at A88 (Price et al. 2009) and HIV-1 CsA-independent strains are circulating in humans at 4-7% of strains in the Los Alamos database (Chatterji et al. 2005). These findings were the rationale for investigating other proteins with cyclophilin domains which raises the potential lentiviral co-factor Ranbp2. Future work

will attempt to demonstrate a functional association between capsid and this protein. It is of note that Ranbp2 interacts with microtubules in the cytoplasm and since lentiviral particles are known to use the microtubules for transport towards the nucleus (Arhel et al. 2006; McDonald et al. 2002), this suggests a potential role of the Ranbp2 Cyp domain in permitting docking of the RT complex at the nuclear pore before transport across the nuclear membrane. If nuclear entry is found to be dependent on Ranbp2, there are likely to be implications for strategies to disrupt the HIV lifecycle through cyclophilin inhibitors. For example, it has been proposed to make artificial restriction factors involving CypA, such as Fv1Cyp and huTRIM5-CypA fusion protein. However, given the ease with which lentiviruses are able to dispense with the CypA interaction the restriction factors are unlikely to be robust against escape mutants. The cyclophilin domain of Ranbp2 is fairly divergent from cytosolic CypA, with the human versions sharing 66% identity, potentially making it possible to maintain Ranbp2 binding whist switching on or off CypA binding. Thus, if the CA-Ranbp2 interaction is proven to be required for infection of non-dividing cells, inhibition of this interaction or restriction factors that mimic the Ranbp2 Cyp domain are more likely to be successful.

Chapter 6. Characterisation of intrinsic immunity to lentiviruses in African lion cells.

6.1 Summary

The feline immunodeficiency virus infects numerous Feliform species as species-specific viral strains. In each host, the virus has undergone considerable selection to allow successful replication and population invasion. In contrast, hosts will attempt to limit infection, and genetic variants that confer resistance to viral replication will be selected, creating a molecular arms race between host and pathogen. In order to assess the impact of such antagonistic relationships in the felids, a comparison was made between a relatively recent host of the virus, the domestic cat (<10,000 years infected) and a more ancient host, the African lion which is likely to have been infected with lentiviruses since the early Pleistocene (Antunes et al. 2008). Lion cells support very low levels of replication of both domestic cat and lion variants of FIV. In contrast, domestic cat cells support high titre replication of all viruses assayed. The results are interpreted as evidence of the existence of a block to lentiviral replication in lion cells relative to the novel host, the domestic cat. This block is termed lion cell non-permissivity (LNP) and is further explored in this study. LNP is found to be a dominant post-entry block to replication that targets both feline and primate lentiviruses. TRIM5α is truncated in lion cells,

eliminating this protein as a candidate for LNP. Moreover, treatment of lion cells with proteasome inhibitor MG312 does not affect the level of restriction, nor does treatment with As_2O_3, a drug that disrupts sub-cellular localisation and antiviral activity of TRIM proteins (Zhu et al. 1997; Anderson et al. 2006). Furthermore, unlike TRIM5α restriction, LNP does not target the N-terminal domain of capsid. The identity of the cause of LNP is unknown but does not act in a manner similar to previous well characterised post-entry restriction factors.

Subsequently, a comparative analysis of the APOBEC3 (A3) family of restriction factors is performed between lions and domestic cats. In domestic cats, read-through fusion protein A3CH is a potent restriction factor whilst single domain proteins A3C and A3H are inactive against FIV (Munk et al. 2008). In lions, A3CH retains this antiviral activity but surprisingly, FIV replication is also blocked by single domain protein, lion A3H. This result is of interest as for most A3 proteins, two domains are required for antiviral activity with one domain specialising in RNA binding and the other in single-stranded (ss) DNA deamination. A homology model is used to predict the residues in lion A3 that potentially permit RNA binding and dimerisation. The study shows that lions possess a strong intrinsic immune protection from lentiviruses and provide evidence of evolution of host restriction in the felids in response to ongoing infections.

6.2 Results

6.2.1 Lion T-cells poorly support the replication of FIV-Fca

To investigate the ability of host cells to support the replication of FIV, concanavalin A stimulated, IL-2 dependent T-cells were challenged with four viral strains, two lion derived strains (FIV-Ple E Sangre and Ple B 458) as well as

two molecular clones of domestic cat FIV (FIV-Fca GL8 and Petaluma). The lion cells used in this experiment are derived from an uninfected Angolan lion, whilst Mya-1 T-cells (Miyazawa et al. 1989) were used for domestic cat. Puma T-cells were derived from a specific-pathogen free North American puma (*Puma concolor*). Frozen viral supernatant was examined for reverse transcriptase activity as a marker of retroviral replication. Cells from domestic cat supported replication of all viruses tested, whist Angolan lion and Puma cells supported only very low titres of FIV-Fca but did permit FIV-Ple B replication (Figure 6-1). Interestingly, the lion cells used in this experiment were unable to support the growth of FIV-Ple E, a strain circulating in southern African lions (Pecon-Slattery et al. 2008a). The findings are reminiscent of VandeWoude et al. (1997) who found that lion cells poorly supported the replication of FIV-Fca. However, these findings go further as they suggest that even host-adapted strains FIV-Ple B 458 and FIV-Ple E Sangre are poorly infectious in lion cells.

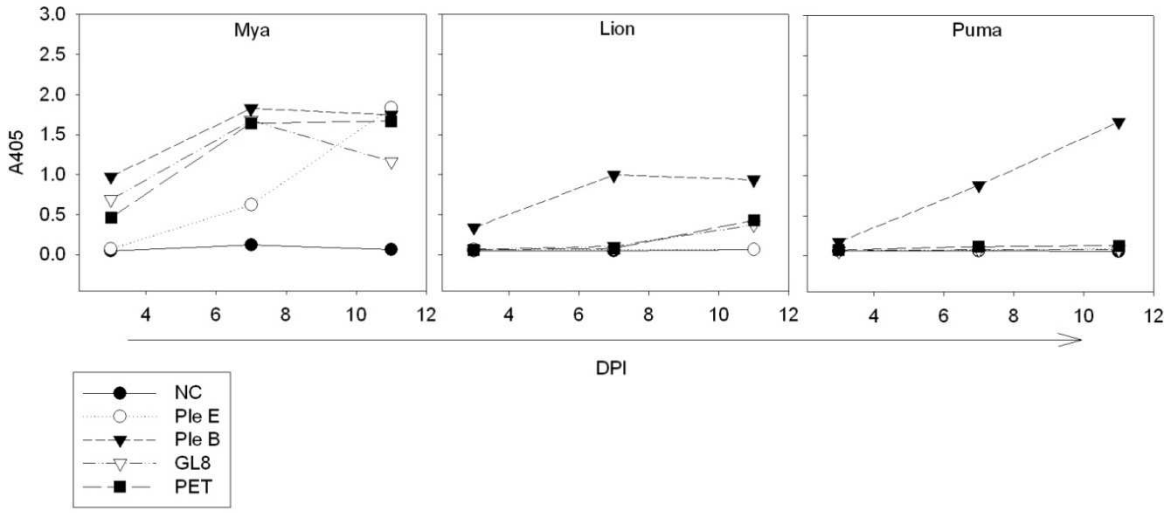


Figure 6-1 Host permissivity to FIV replication. FIV is able to replicate to high titres in cells of the domestic cat, whereas Angolan lion and Puma cells poorly support growth of domestic cat strains of FIV (GL8 and Petaluma) and only support growth of lion-derived FIV in a strain-dependent manner.

The data imply that either the viruses are unable to make use of the lion/puma cell host machinery or there exists a dominant block to replication in these cells. Given that lions are the natural host of FIV-Ple, the former case is unlikely since considerable host-adaptation will have taken place in the time since lentiviruses entered the species, estimated between 169,000 and 3.6 mya (Antunes et al. 2008; Pecon-Slattery et al. 2008b). Furthermore FIV-Ple strains are able to replicate to high titres in domestic cat cells, a non-native host, implying that the ability to replicate to high titre is a factor that is host- but not virus-dependent. The only strain that produced a positive RT result in lion cells at seven days post-infection was FIV-Ple B, a strain that uses non-CD134, non-CXCR4 entry receptors (Chapter 3; McEwan et al. 2008). This raised the possibility that mutations in either of these receptors are responsible for the non-permissive phenotype in lion cells.

6.2.2 Contribution of receptor variation to LNP

To test the hypothesis that LNP is due to lion mutations in CD134 or CXCR4, variants of the receptor and co-receptor from cats and lions were cloned and sequenced. Analysis of the sequences reveals that lion and feline CD134 share 97% amino acid identity with a cluster of amino acid mutations in the predicted signal peptide region and a G59S point mutation in the predicted Env binding site, cysteine-rich domain 1 (CRD1), which could potentially alter Env binding and entry. An alignment of feline and lion CD134 has been presented previously in Figure 3-8**.** A comparison of the lion and feline CXCR4 seven-transmembrane domain protein shows 99% amino acid identity with only a single amino acid change, a serine to asparagine substitution in extracellular loop 2 (ECL2), creating a potential N-glycosylation site in lion CXCR4 at position N193. Whether this site is in reality glycosylated in lion CXCR4 has not been studied but any of these interspecific polymorphisms could potentially alter the Env-

receptor interaction and be responsible for LNP. CD134 and CXCR4 of each species were therefore stably expressed in all four combinations on NP2 cells and challenged with HIV-luc particles pseudotyped with a panel of FIV Env glycoproteins (Figure 6-2). Permissivity of the cell lines was not substantially altered by changing the combinations of receptor and co-receptor, suggesting that domestic cat and lion CD134 and CXCR4 support infection of FIV at similar levels. These data indicate that LNP cannot be explained by differences in entry receptors as lion CD134 and CXCR4 support levels of infection directly comparable to those of the domestic cat for a range of FIV envelopes.

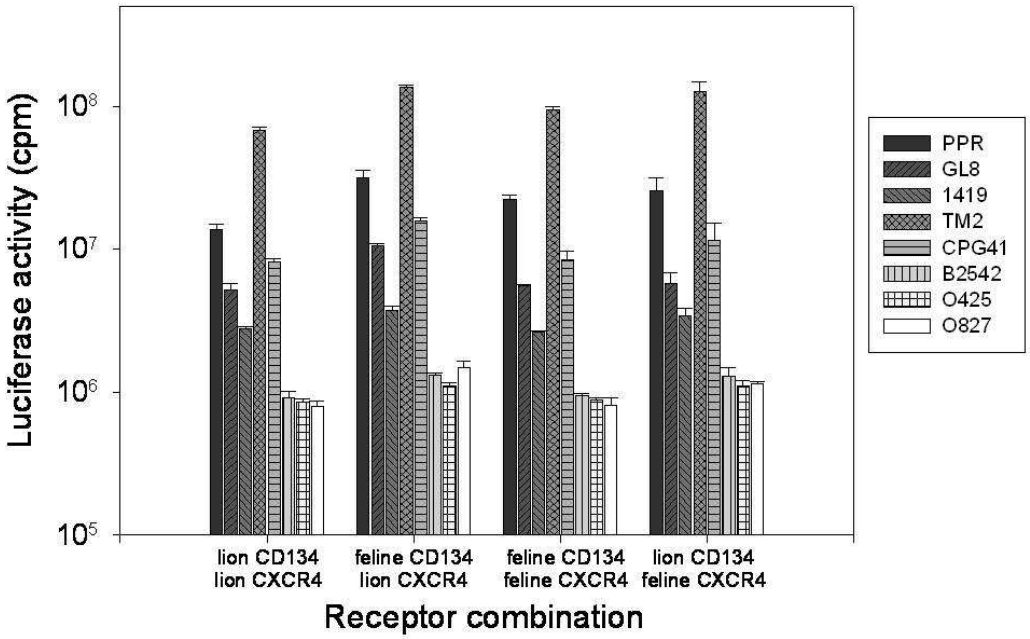


Figure 6-2 FIV usage of lion CD134 and CXCR4. Cat and lion FIV entry receptor CD134 and co-receptor CXCR4 from domestic cats and lions were expressed in all four combinations on NP2 cells and challenged with panel of HIV-luc(FIV) pseudotypes using various Env isolates from domestic cats. Levels of entry were similar regardless of receptor combination indicating that lion cell non-permissivity is not due to differences in efficiency of the entry receptors between cats and lion.

6.2.3 LNP operates post-entry against primate lentiviruses.

The finding that species-specific polymorphisms in receptor and co-receptor do not account for the differing levels of infectivity suggests that receptor binding

and entry are not the stages responsible for LNP. However, it remains possible that receptor expression levels differ between the species or access to receptors is somehow impeded in lion cells. To obviate this problem, pan-tropic VSV-G-pseudotyped particles were used in preference to FIV Env for the remainder of this study. In order to assess the breadth of LNP lentiviral targets, lion cells and domestic cat cells were challenged with primate lentivirus pseudotypes. Lion T-cells were found to permit much lower levels of infection compared to domestic cat cells. Furthermore a differential level of restriction was found between the viruses used: HIV-1 was restricted at a low level, whilst SIV from rhesus macaques (SIVmac) was restricted at much higher levels, a difference that was found to be repeatable and statistically significant (Figure 6-3).

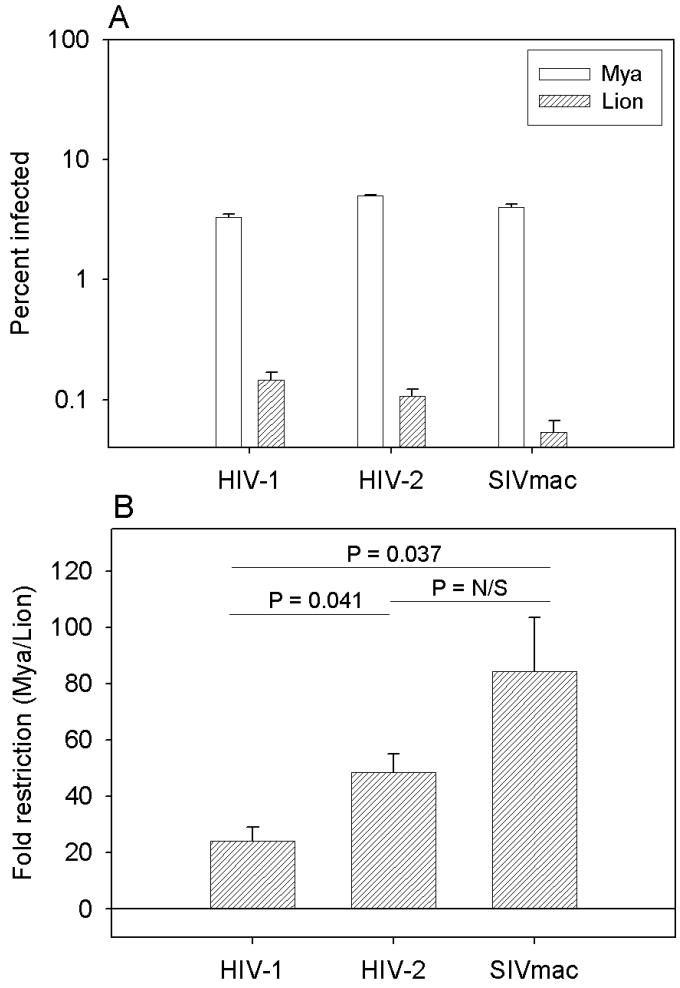
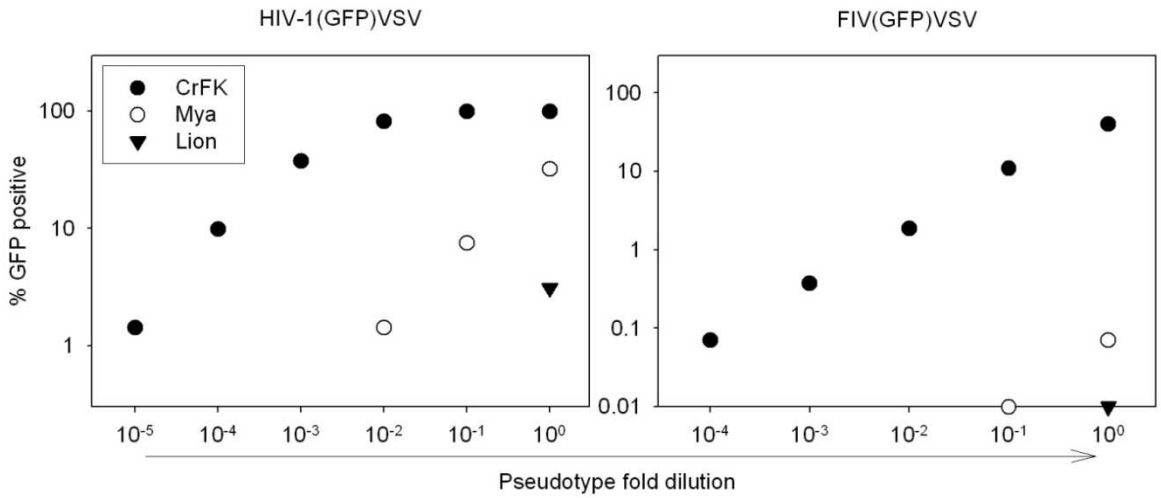


Figure 6-3 Lion cells differentially restrict primate lentiviruses. A) GFP-bearing VSV-G-pseudotyped particles of primate lentiviruses display a reduced titre on lion

compared to domestic cat T-cells. B) Expressed as fold restriction, HIV-1 is restricted about 20-fold whilst SIVmac is restricted about 80-fold and HIV-2 is restricted at an intermediate level. There is a significant difference between levels of restriction between HIV-1 and SIVmac (unpaired two-tailed t-test, n=3).

To assess the relative ability of T-cells to support lentiviral infection, pseudotypes were titrated onto permissive CrFK as well as Mya-1 and Angola-1 cells (Figure 6-4). Mya-1 and Angola-1 cells supported levels of infection far below CrFKs. For FIV, the levels of infection achieved in lion cells were too low for meaningful interpretation. Optimisation of the pseudotype production protocol by altering the ratio of plasmids transfected, use of phenol red-free medium and transfection reagent all allowed marginal increases in titre, but titres were always in the region of 10^5 infectious units per ml. Titres of HIV-1 were much higher, possibly due to the fact that pseudotypes were produced in human cells.



Cell line	HIV moi	FIV moi
CrFK	931.4	1.12
Mya	0.83	0.09
Lion	0.03	0.01

Figure 6-4 Titration of HIV and FIV pseudotypes. 10-fold serial dilutions of pseudotype were titrated onto CrFK, Mya-1 and Angola-1 lions T-cells and GFP positive cells were enumerated. Multiplicities of infection (number of infectious units per cell) were calculated for undiluted supernatant and show that CrFKs are ~1,000 times more

susceptible to HIV infection than Mya-1. Lion cells display a further ~25-fold restriction to HIV compared to Mya-1 cells.

The results imply that LNP operates post-entry and has a broad specificity of infection. The difference in restriction levels between SIVmac and HIV-1 allows further dissection of the LNP phenotype.

6.2.4 Mapping the viral determinants of LNP

Given that post-entry restriction factors such as Fv1 and TRIM5α are known to target incoming viral capsids, it was proposed that LNP might also employ this strategy. Although it is known that lions lack a functional TRIM5α (McEwan et al. 2009; Chapter 4) and Fv1 is specific to mice (Best et al. 1996), it was hypothesised that LNP may share its target in common with these post-entry restriction mechanisms. In order to test this hypothesis, a chimeric HIV-1-SIVmac construct was used, HIV S/H CA (Owens et al. 2003). HIV S/H CA is an HIV-1 gag-pol expressor with the N-terminal 146 amino acids of capsid replaced with that of SIVmac. It was found that using this construct gave no significant increase in levels of restriction relative to HIV-1, indicating that LNP does not target the N-terminal domain of capsid. As a control, the chimeric and wildtype viruses were assayed against puma cells but found to lack the LNP phenotype, providing evidence that LNP is specific to the *Panthera* lineage.

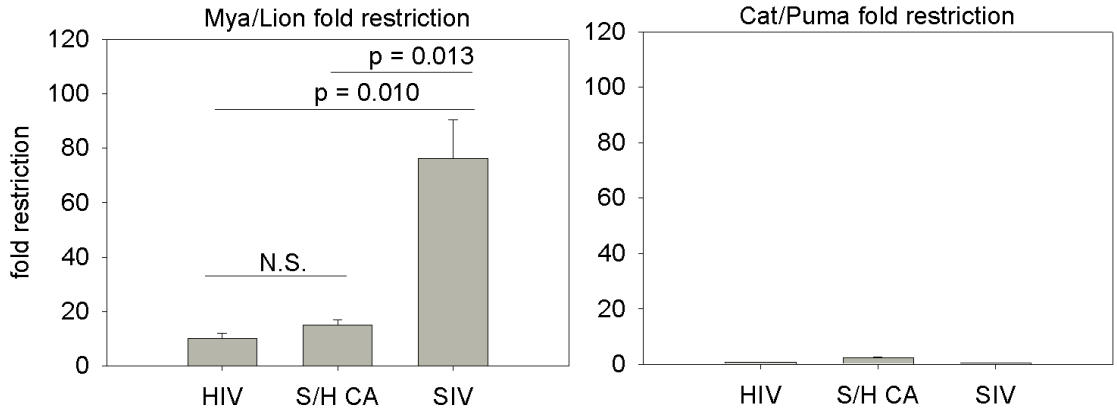


Figure 6-5 Chimeric lentiviral capsid is not targeted by LNP. HIV-1 and chimeric S/H capsids were restricted only mildly, whilst SIV capsid was restricted strongly. Puma cells were used as a comparison and show no restriction of pseudotypes when compared to domestic cats.

6.2.5 Sensitivity of LNP to proteasome inhibitor and arsenic compounds

MG132 is a protease inhibitor that relieves the block to reverse transcription imposed by TRIM5α on restricted lentiviruses (Anderson et al. 2006; Wu et al. 2006). Another drug, As_2O_3, has been shown to rescue restriction of HIV-1 by TRIM5α (Berthoux et al. 2003; Saenz et al. 2005). The activity of As_2O_3 is poorly defined but may exert its effects by disrupting the formation of cytosolic protein concentrations known as cytoplasmic bodies, which contain TRIM family proteins such as TRIM5α (Campbell et al. 2007) and PML (also known as TRIM19) (Reymond et al. 2001; Zhu et al. 1997). Furthermore, the drug has been shown to disrupt an unidentified restriction phenotype against HIV-1 in human dendritic cells (Pion et al. 2007). However, no effect was observed when lion and cat cells were treated with the drugs (Figure 6-6), suggesting that the restriction is not caused by proteasome-dependent pathways or arsenic-sensitive proteins.

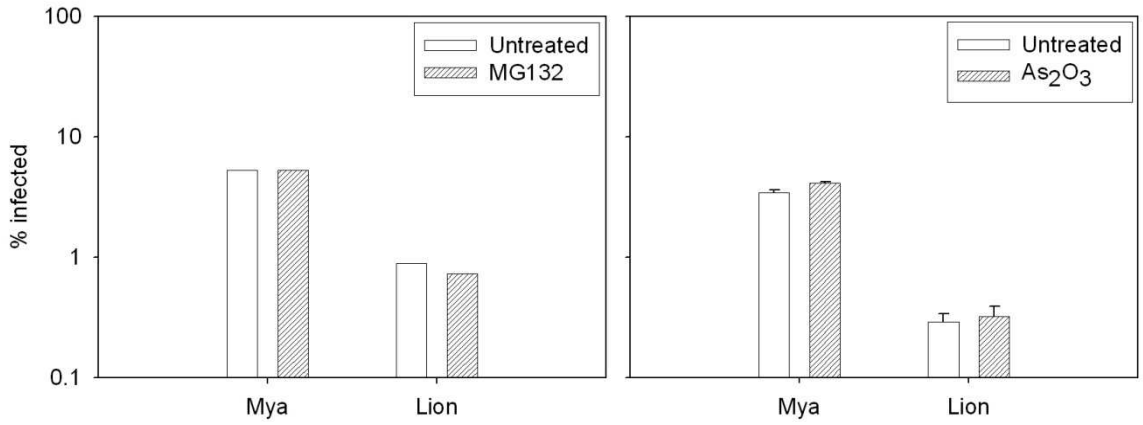


Figure 6-6 Protease inhibitor and arsenic trioxide do not relieve LNP. Cat and lion T-cells were incubated with MG132 (1 μg/ml) or As_2O_3 (2 μM) prior to infection. No substantial change was observed in the restriction of HIV-1. For As_2O_3 experiment, n =3.

6.2.6 Analysis of feline APOBEC3 genes

It has previously been reported that the domestic cat genome bears an expanded cluster of APOBEC3 genes (Munk et al. 2008). In order to test whether extended periods of endemic lentiviral infection have altered the restrictive ability of lion APOBECs, A3C, A3H and A3CH were amplified from lion and domestic cat cDNA and cloned into eukaryotic expression vector VR1012. The cloned genes were sequenced and found to bear 100% identity to published sequences, except lion A3CH, as this is the first published sequence for this gene. The lion A3C transcript cloned was found to encode A3C1 variant as described previously (Munk et al. 2008). Homology between feline A3H and the huA3G catalytically active CD2 is low but the Zn-coordinating motif is present in both (Figure 6-7). Restriction by human A3G is contingent on both RNA-binding and catalytic functions, and accordingly it has previously been reported that domestic cat A3C and A3H are inactive against FIV, whilst two-domain read-through protein A3CH is highly active.

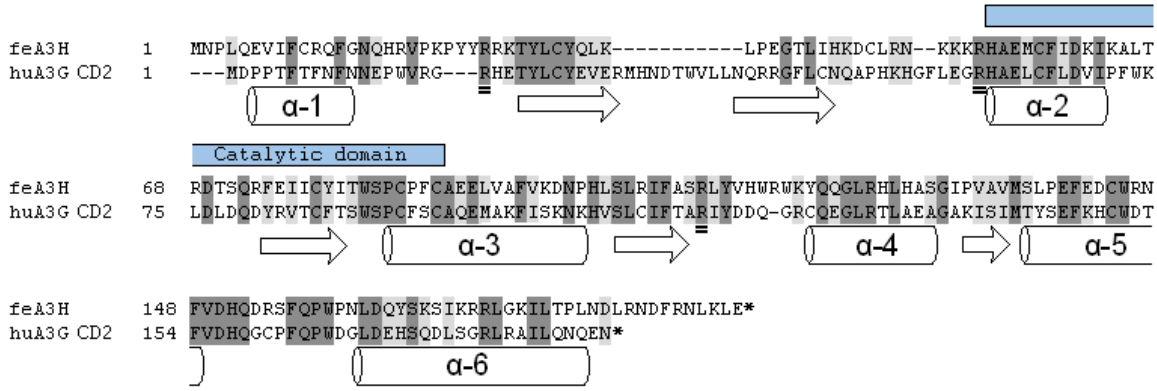


Figure 6-7 Alignment of feline A3H and human A3G C-terminal catalytic domain. The domains share only 33% sequence identity, but share the Zn-coordinating H/C-X-E-X_{23-28}-P-C-X_2-C motif. The three arginine residues of huA3G predicted to form the charged single-stranded DNA binding groove (Chen et al. 2008) are marked with double black bars and are conserved in the feline variant.

To assess the impact of the felid A3 genes on the infectivity of FIV, VR1012-A3 plasmids were co-transfected with FIVΔVif-GFP(VSV)-encoding plasmids FP93, GinSin and MDG. The viral titre was then assayed by plating supernatant onto fresh target 293T cells (Figure 6-8). 0.25μg of plasmids encoding read-through transcript A3CH from both lions and cats was sufficient to completely abolish the production of infectious FIV virions. The feline single domain A3C and A3H were unable to restrict virus, suggesting that in domestic cats fusion of the two domains is necessary to produce a functional restriction factor. In contrast, the lion A3H protein was able to restrict the production of infectious virions, despite being a single domain protein, raising questions concerning the mechanism of restriction for the single-domain restriction factor.

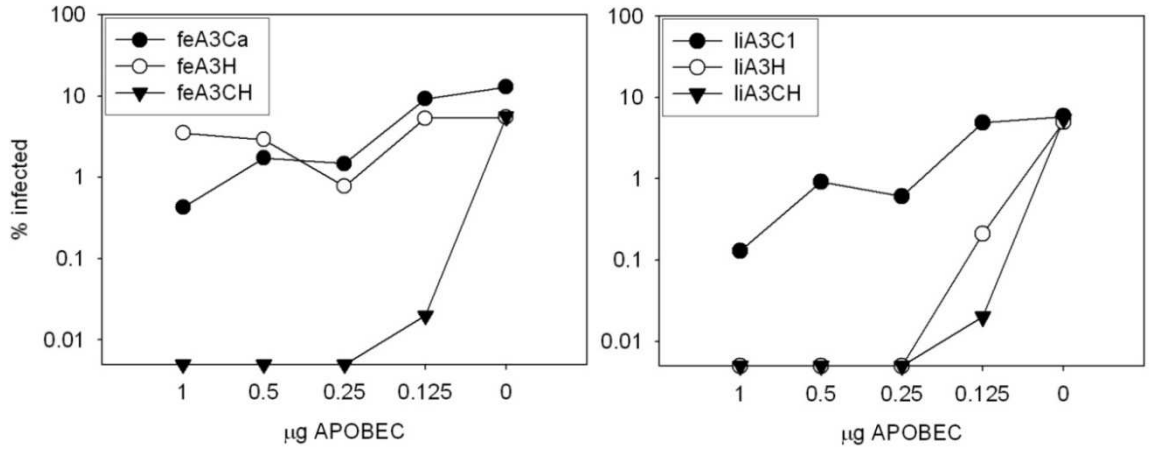


Figure 6-8 Titrations of feline and lion APOBECs. 293T cells were transfected with plasmids encoding FIV-GFP(VSV) pseudotype and VR1012-A3, normalised with empty VR1012 vector. Pseudotype was harvested, plated onto fresh 293T cells and GFP expression examined. Read-through transcripts from both species prevented production of infectious virus above 0.25µg plasmid. A strong restriction was also provided by lion, but not feline, single domain protein A3H.

For both HIV-1 and FIV, the APOBEC restriction is largely overcome by the expression of accessory protein Vif (Munk et al. 2008; Sheehy et al. 2002). To assess the ability of Vifs to counteract the restriction phenotype, genes were cloned from FIV-Ple strains B and E and were co-transfected into viral producing cells. Given the ability of FIV-Ple B to replicate in lion cells, it was expected that this Vif variant may provide the strongest rescue of titre. However, very little Vif-dependent rescue was observed against any of the A3s, with the exception of liA3C1 (Figure 6-9). It is possible that this poor Vif activity is due to poor or mis-located expression (see 6.3 Discussion), making it difficult to draw robust conclusions from these data.

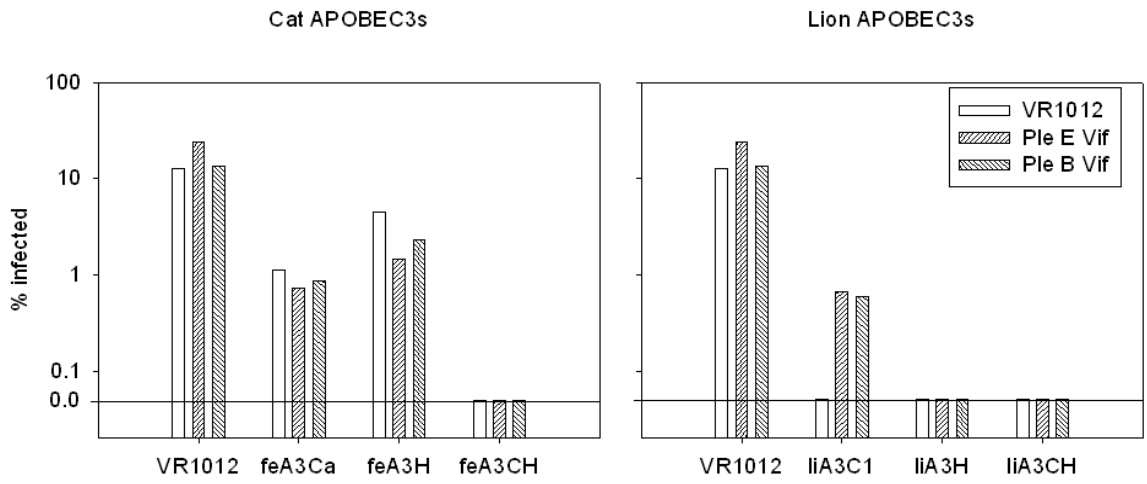


Figure 6-9 Lentiviral restriction imparted by feline and lion APOBEC3 genes. A strong restriction phenotype is associated with the lion A3s as well as feline A3CH. With the potential exception of liA3C1, Vifs were unable to overcome the restriction. However, this may owe more to problems with expression than biological activity.

6.2.7 Structural comparisons of feline APOBEC3H

In human cells, the antiviral APOBEC3s are two-domain proteins with both RNA binding ability (associated with CD1) and ssDNA deaminase activity (associated with CD2). It was therefore surprising to find antiviral activity associated with the lion single domain proteins. Structural studies of human A3G CD2 have revealed the presence of a positively charged groove that permits interaction with the negatively charged nucleic acid backbone (Holden et al. 2008; Chen et al. 2008). In the absence of a DNA-A3G complex structure, the precise orientation of the nucleic acid across the groove is not clear and remains controversial (Bransteitter et al. 2009) but is thought to bring the catalysed base into the proximity of the catalytically active Zn^{2+} ion (Furukawa et al. 2009; Chen et al. 2008; Holden et al. 2008).

Domestic cat A3H only poorly restricts FIV, whilst the lion variant potently prevents the production of infectious virions. Recent work in our lab suggests that the puma A3H variant also possesses antiviral activity. The ability of these

non-domestic cat variants to restrict is unusual, given that the proteins are single domain cytidine deaminases. To investigate possible mechanisms of action, an alignment of feline and lion A3H variants was made (Figure 6-10). Residues shared by lion and puma variants but absent in the feline A3H were identified (V7D, H43D, E51K, A65S, G140S, P106R/H (R in lion; H in puma), A179P, cat numbering). Four of the seven substitutions replace neutral residues with charged ones or switch the charge, suggesting a possible role in alteration of protein-protein or protein-nucleic acid interactions.

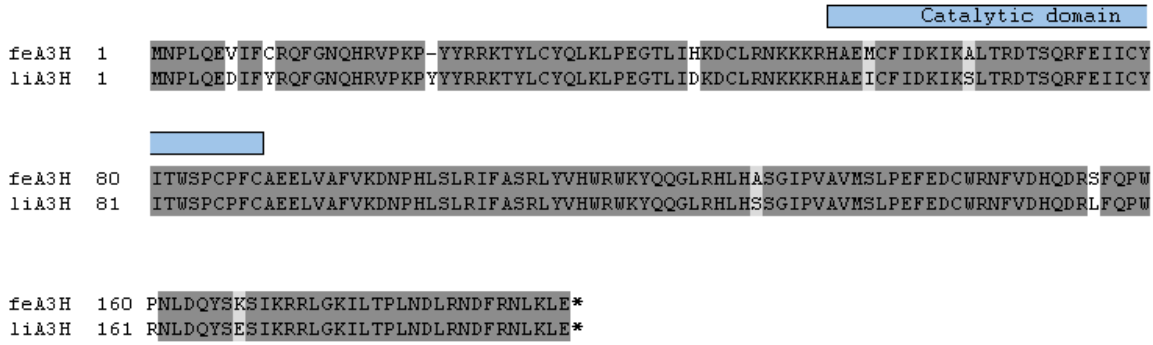


Figure 6-10 Alignment of cat and lion A3H. Whilst feline A3H is unable to restrict FIV, the lion version restricts almost as well as the two-domain A3CH. Possible causes of the large change in phenotype are the two substitutions in the catalytic domain as well as four charge switch substitutions.

Next a homology model of feline A3H was created using the two APOBEC crystal structures solved to date, huAPOBEC2 and huAPOBEC3G CD2 domain (PDB: 2NYT and 3E1U respectively) (Prochnow et al. 2007; Holden et al. 2008). The model predicts the conservation of the prototypic APOBEC structure: a central ß-sheet surrounded by six α-helices (Figure 6-11). APOBEC3G CD2 structurally differs from A2 primarily in its longer loops that protrude from the structure between conserved ß-strands and α-helices (Holden et al. 2008). Superimposed structures of feline A3H on human A3G CD2 suggest that again the main structural difference is the length and orientation of the loops, with

the feline variant possessing shorter loop-3 and a deflected loop-1. Moreover, the model suggests that charge-switch mutations V7D, E51K and P160R/H are located on the surface of the protein predicted to interact with viral single-stranded DNA (Furukawa et al. 2009; Chen et al. 2008; Bransteitter et al. 2009). Of particular interest is the P160R/H mutation that varies between all three species. The residue is neutral in cats but positively charged in both lions and pumas. Analogy with recent human A3G-ssDNA binding models (Chen et al. 2008; Furukawa et al. 2009), suggests that this residue has the potential to make electrostatic interactions with the negatively charged nucleic acid backbone, potentially increasing the affinity of binding in the non-domestic species.

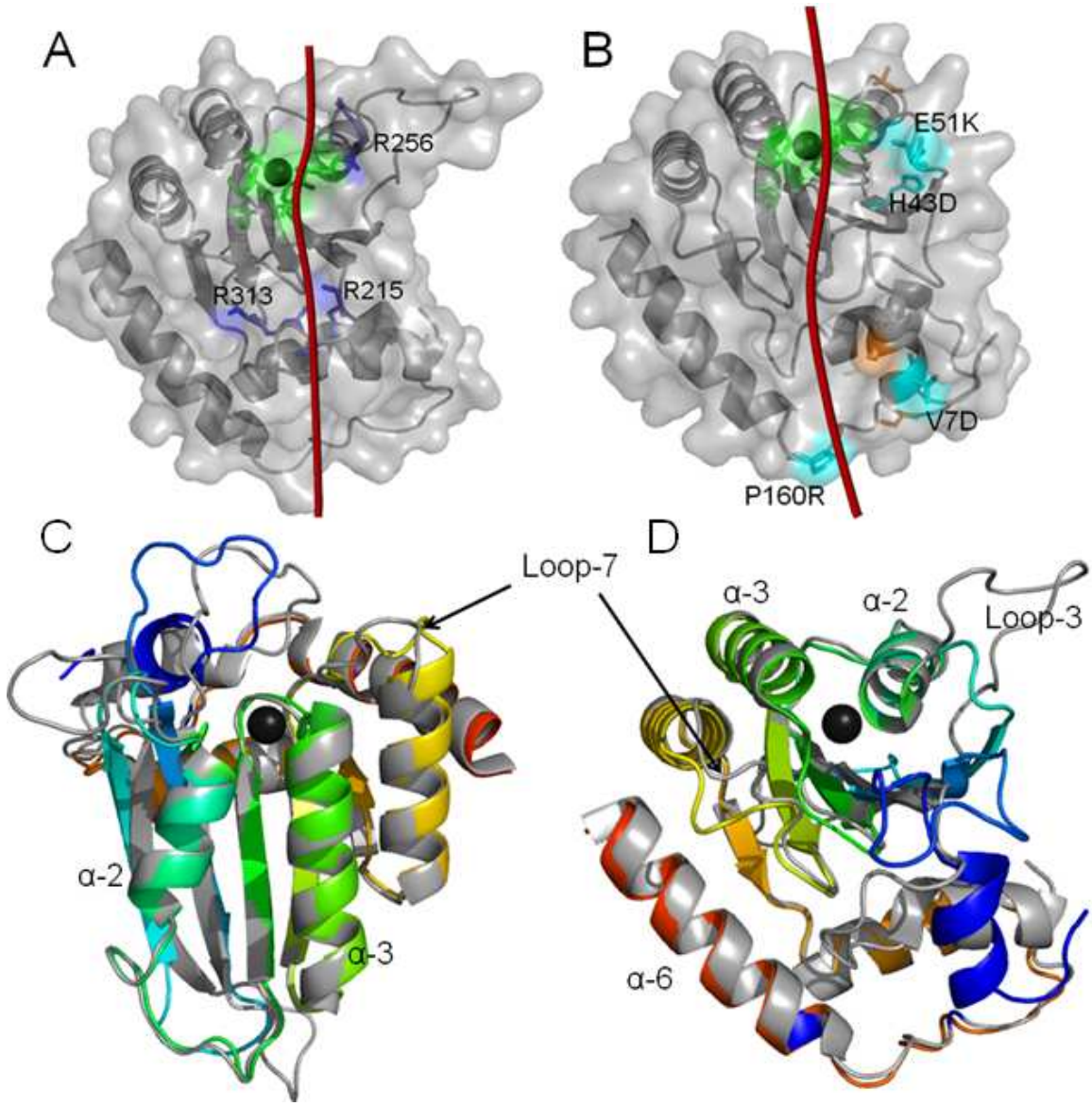


Figure 6-11 Predicted APOBEC-ssDNA catalytic complex. A) Human APOBEC3G catalytically active CD2 domain (3E1U) interacts with single-stranded DNA via interactions between the sugar-phosphate backbone and positively charged arginine residues (blue) (Chen et al. 2008; Bransteitter et al. 2009). Zn-coordinating residues at the catalytic site are shown in green; Zn^{2+} ion, black sphere; DNA binding location, red (as predicted by Chen et al. 2008; Furukawa et al. 2009). B) Homology model of feline APOBEC3H suggests a similar tertiary structure. Substitutions between feline and lion homologue are shown in cyan (charge switches) or orange (neutral changes). C) shows the homology model of feA3H (rainbow; N-terminus blue, C-terminus red) superimposed on huA3G CD2 (grey) rotated to view the catalytic site. The proteins are predicted to share substantial structural homology around the catalytic site between α-helices 2 and 3. (D) shows that substantial variation between human and feline

homologues is present within the loop domains. Loop-3, known to make contact with nucleic acid bases (Bransteitter et al. 2009), is considerably shorter in the feline version but loop-7, involved in dimerisation in CD1 (Huthoff et al. 2009), is conserved.

Recently, a homology model of a human single domain protein A3C, that is active against SIVmac but not HIV-1, has been presented (Stauch et al. 2009). Activity for this protein is dependent on RNA binding which is mediated by a novel substrate binding pocket and subsequent dimerisation performed by exposed aromatic residues that permit packaging into SIVΔVif particles. The catalytic site is therefore unoccupied by RNA and free to bind ssDNA once reverse transcription is under way in the target cell. Human A3G also forms RNA-dependent dimers: CD1 domain residues R24 and R30 interact with RNA (Huthoff et al. 2009) which subsequently promotes intermolecular interactions via aromatic residues Y124, F126 and W127 present in CD1 loop-7 region (Huthoff et al. 2009; Gooch and Cullen 2008). However unlike the single domain A3C, dimerisation is not necessary for huA3G antiviral function (Opi et al. 2006).

The mode of activity observed for liA3H suggests that virion incorporation takes place, given that the restriction observed is post-entry, making RNA-dependent dimerisation a likely mechanism for this protein. A comparison with A3C also shows that of the four residues known to comprise the novel RNA-binding pocket (K22, T92, R122 and N177), two are identical (R122 and T92), one is conserved (K22R), making it possible that RNA binding for liA3H takes place at this alternative location. Inspection of the feline sequence also reveals a patch of aromatic residues in loop-7 including Y122 and W115, leading to the hypothesis that feline A3H exists as a dimer through interactions at this surface. A homology model (Figure 6-12) also predicts adjacent W117 at the crest of α-helix 4 to be exposed and may also contribute to intermolecular interactions. This loop is absolutely conserved between feline and lion

homologues. Thus we hypothesise that anti-viral function of liA3H is dependent on an RNA-binding step with subsequent dimerisation that permits packaging into lentiviral particles.

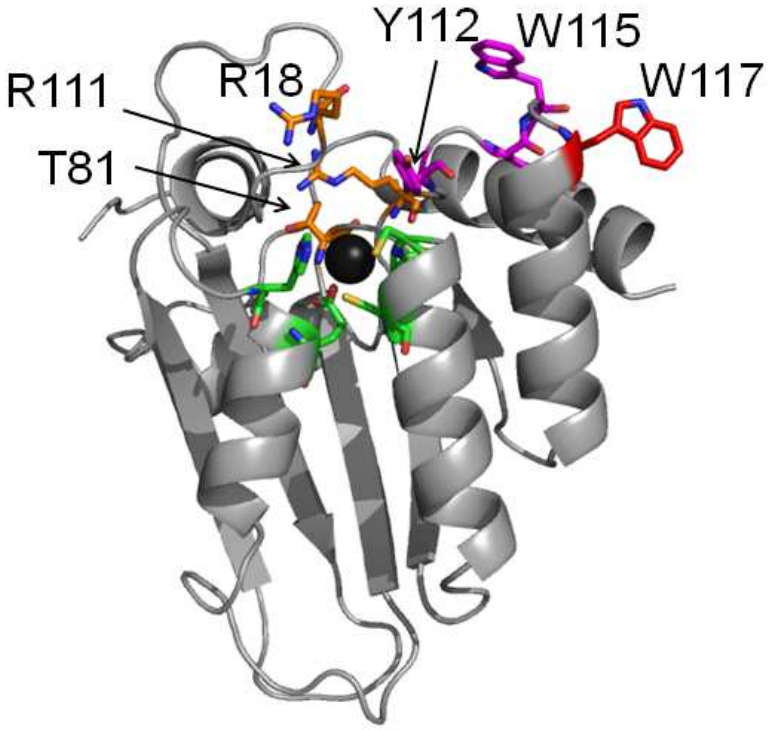


Figure 6-12 Feline APOBEC3H potential dimerisation and RNA-binding domains. The human single domain protein A3C binds RNA at an arginine-rich pocket (Stauch et al. 2009) that promotes dimerisation via aromatic residues in loop 7 (Bransteitter et al. 2009). The homology model shows that feA3H bears partial conservation to huA3C at a potential RNA-binding domain (orange). Furthermore, aromatic residues (Y112, W115, magenta) are present in loop-7. An additional aromatic residue W117 (red) is exposed that may also contribute to the interaction. The Zn-coordinating residues of the catalytic domain are shown in green.

The models presented highlight residues that may be of importance in contributing to the difference in anti-viral activity between the feline and lion homologues, by remodelling the charges in the ssDNA binding groove, affecting RNA binding and promoting assembly of dimers. These features belong separately to the CD1 and CD2 of human A3G, potentially making felid A3H an

interesting case as it is predicted to combine these functions in a single domain protein. Future work should evaluate how A3H impacts on viral infectivity alone and in concert with A3C – both as the fusion protein and individual proteins.

6.3 Discussion

Studies of host-retrovirus interactions in primates have suggested that genes which provide resistance to circulating or endogenous pathogens will be selected for, and, given time, will confer host resistance to the pathogen. It is likely that APOBEC proteins have had a part to play in this process. For example the human endogenous retrovirus, HERV-K, is present in multiple copies in the human genome as the relic of an infection. Some of these insertions have occurred comparatively recently, since the divergence with chimpanzees (Belshaw et al. 2005). The sequences show large numbers of G to A base substitutions, evidence of APOBEC-mediated antiviral activity (Lee et al. 2008; Perez-Caballero et al. 2008). Furthermore, a reconstructed consensus HERV-K is strongly restricted by huA3F, suggesting a causal role of APOBECs in the demise of this parasite (Lee and Bieniasz 2007). It is therefore of interest that lions, which are thought to have harboured lentiviruses for many hundreds of thousands of years, display a very different permissiveness compared to a fairly recent host, the domestic cat.

It has previously been suggested that a certain amount of host adaptation has taken place in lion FIV strains that reduces pathogenicity and therefore prolongs the lifetime of infection[burk] (Carpenter and O'Brien 1995). However, the findings presented in this study show that both FIV-Fca and FIV-Ple, which replicates to a high titre in domestic cat cells, are poorly infectious in lion cells but conversely, these strains are replication-competent in feline cells. The simplest explanation for the findings is that lion cells actively

prevent FIV replication, rather that the virus attenuating to preserve the health of its host. Indeed a lentiviral post-entry restriction (LNP) was described that prevents post-entry stages of human, feline and simian lentiviral infections. The factor or factors responsible for LNP remain unidentified. Experiments to test whether lion A3 proteins could block replication at a post-entry stage were performed, but no evidence of a post-entry APOBEC-mediated block was found (data not shown). However, a post-entry APOBEC block cannot be ruled out until the ability of lion APOBECs to perform RNA-directed mutagenesis has been studied. Another potential candidate for LNP is TRIM22, a protein known to be under positive selection in the Carnivorae, possibly complementing the absence of TRIM5α (Sawyer et al. 2007). Despite repeated attempts to clone the TRIM22 gene from cat and lion cDNA, this study was unable to examine the role of this gene. However, the finding that LNP does not target the N-terminal domain of capsid makes TRIM22 an unlikely candidate, since TRIM5α is known to interact with this viral domain. Furthermore, no effect on LNP was observed by treating cells with arsenic trioxide, a compound that is known to disrupt TRIM protein activity (Zhu et al. 1997; Campbell et al. 2007; Saenz et al. 2005). An unidentified block to HIV-1 post-entry stages has been described in Mya-1 domestic cat T-cells (Munk et al. 2007). It is possible that the restriction described is performed by the same factor as LNP, given that both act at the same stage in the lifecycle. This study used Mya-1 as a permissive cell line relative to lion cells, but in reality both species may be restrictive with more active variants present in lion cells. Interestingly, the FIV-Ple B strain was able to replicate in lion cells (albeit at a lower level than in cat cells), suggesting that FIV-Ple B is able to neutralise or escape LNP. Given that FIV-Ple B and E share considerable levels of identity across the genome with the exception of *env* (Pecon-Slattery et al. 2008a), it is possible that resistance is somehow provided by this same gene product, for example by employing divergent entry pathways or actively overcoming

restriction. This is not without precedence, since HIV-2 uses its Env glycoprotein to fulfil the role of HIV-1 Vpu in overcoming tetherin restriction (Bour et al. 1996; Jia et al. 2009). Like the discovery of alternate entry receptors, the differential permissivity of lion cells to the B and E clade viruses has implications for the pathology and management of FIV in free-ranging lions. However, further questions need to be answered before the implications become clear: for instance, does the differential *in vitro* permissivity correlate with *in vivo* viral titre? And is the differential permissivity specific to the Angolan lions from which the T-cells in this study were derived or is it widespread amongst lion populations? Furthermore, despite evidence of T-lymphocyte depletion (Roelke et al. 2009), the tissue tropism of FIV-Ple has not formally been demonstrated and replication may be more efficient in other cell populations.

It has been reported that lion populations in the west of southern Africa do not harbour FIV (Antunes et al. 2008). This is in stark contrast to eastern populations where seropositivity can approach 100% in adult lions (Ramsauer et al. 2007; Roelke et al. 2009; Brown et al. 1994). The absence of FIV in western areas has been explained by the lower population density (Antunes et al. 2008). However, several felid species that exist at low population densities are known to bear FIV, such as Pallas cats (Barr et al. 1995) and pumas (reviewed in Poss et al. 2008). With the source of the lion cells used in this study, Angola, being on the west coast of southern Africa, it is possible that LNP is present in the localised lion population and has contributed to the absence of FIV west of the Kalahari. If confirmed, the lion would provide the first extant example of a host acquiring resistance to its own lentivirus and would be of substantial interest in the study of host-parasite ecology. On a molecular level, isolating the factor responsible for LNP is of substantial interest given the broad specificity of its activity. A potential strategy for its isolation is a cDNA library

screen: expressing lion cDNA clones in permissive cells and screening for antiviral activity. The technique is limited as it would not identify the cause if LNP were a function of two or more proteins or a recessive property, but with previous restriction phenotypes (rhesus macaque *Lv1* and non-permissivity of CEM cells) the technique has proved successful and lead to the isolation of TRIM5α and huA3G (Stremlau et al. 2004; Sheehy et al. 2002).

A single domain APOBEC presents problems for the host: RNA-binding and RNA-directed catalytic activity allows virion packaging but risks targeting self RNA whilst ssDNA-binding and -deamination prevents virion incorporation. Two strategies to overcome this problem have been described for APOBEC proteins so far: the possession of an additional RNA-binding pseudocatalytic domain, such as huA3G CD1 (Huthoff and Malim 2007), or the presence of an additional RNA binding pocket for single domain proteins, such as huA3C (Stauch et al. 2009). These strategies permit both encapsidation and deaminase activities without high levels of host mRNA mutation. A previous study of domestic cat APOBECs found little or no anti-lentiviral activity associated with the single domain proteins (Munk et al. 2008). Like human A3 proteins, two covalently-joined domains in the form of read-through protein A3CH, were found to be required, presumably with each domain specialising separately in RNA binding and ssDNA deamination (Munk et al. 2008). Whilst we repeated the finding that feline single domain A3 proteins are poorly active against FIV, this study presents the single domain lion APOBEC3H protein capable of potent antiviral activity. The lion A3H protein is therefore of considerable interest as it prompts questions as to the mechanism of action for a single domain cytidine deaminase. Here, we have proposed a model for the activity of A3H which shares conserved residues with the A3C RNA-binding pocket and a potential dimerisation domain. Future work should use such structural information to define which mutations are responsible for rendering the lion and puma A3H

variants more potent against FIV compared to the domestic cat variant and the mechanism of antiviral activity should be addressed. Although RNA-binding and dimerisation are the most attractive hypotheses for liA3H activity, other mechanisms such as the formation of A3C/A3H heterodimers to allow RNA binding and deaminase activity are also potential *in vivo* methods of overcoming the problems associated with single domain proteins. Also possible is the presence of deaminase-independent activity as has been described for A3G (Newman et al. 2005; Bishop et al. 2008). This issue could be resolved by introducing mutations into the catalytic domain of liA3H in order to determine the relative contributions of deamination and deaminase-independent restriction.

Human APOBEC3 activity is overcome by HIV-1 Vif protein (Sheehy et al. 2002). Whilst this study was able to demonstrate some Vif-dependent rescue of viral infectivity in the context of lion A3C1 restriction, no rescue was observed against other A3 proteins. It is likely that this inability of Vifs to counteract APOBECs is due to poor expression or mis-localisation of Vif. The problems are overcome by codon optimisation to alter the third base in each codon to better reflect mammalian codon usage. Doing so has been shown to improve mRNA nuclear export (Nguyen et al. 2004) and restore FIV Vif localisation to the cytoplasm (Paul et al. 2007). Time constraints prevented the construction of such codon optimised Vifs but future work will better assess the ability of FIV strains to overcome APOBEC-mediated restriction.

Although APOBECs are unlikely to be the cause of post-entry LNP in lion cells, it remains possible that the block to replicating FIV is partially due to A3 proteins. This issue could be addressed by recovering viral sequences from infected cells in order to quantify the extent of G to A substitutions. Additionally, it remains possible that the felid APOBECs have alternative or

additional viral targets: potent restriction of FFV and partial restriction of FeLV by feline APOBECs has been observed (Munk et al. 2008; Lochelt et al. 2005). However, FIV is likely to be the principal driver of positive selection in the lions given the pathogenicity and prevalence of FIV in wild lion populations and the apparent absence of FeLV, RD114 and FFV in this species (Antunes et al. 2008; Roelke et al. 2009). Furthermore, the very fact that lion APOBECs have evolved to potently restrict FIV is in itself further evidence that FIV is a major target of restriction, making lions an ideal species for the study of ongoing arms race between host and lentivirus and potentially giving a glimpse of the future for lentiviral infections in other species.

Chapter 7. Concluding Remarks

'A slow sort of country,' said the Queen. 'Now here, you see, it takes all the running you can do, to keep in the same place. If you want to get somewhere else, you must run at least twice as fast as that!'

- *Alice in Wonderland*, Lewis Carroll

The words of the Red Queen have been used to describe situations in biology involving a continuous arms race between conflicting species, where an advantage for one species is quickly counteracted by a reactionary change in the other (Van Valen 1973). During retroviral infection, natural selection in the parasite acts to increase transmission whilst selection in the host acts to suppress replication. An antagonistic relationship therefore develops that drives innovation and selection of measures that promote the cause of each genetic entity. Accordingly, positive selection is apparent in host gene products that provide resistance to infection providing evidence of ongoing and historical conflicts. In contrast, the high mutation rate on the part of the retroviruses obscures the effects of historical conflicts but provides an efficient means of isolating important functions by examining variable and constant regions as well as monitoring escape mutants. Despite the ability to rapidly

evolve resistance to host defences, the vast majority of retroviruses are extinct, as evidenced by the large number of endogenous retroviral sequences present in mammalian genomes (8% of human genomic DNA), suggesting that the host usually wins out in these battles when played out over evolutionary time.

The lentivirus is a comparatively new type of retrovirus, the oldest mammalian encounter dated to 7-12 mya (Katzourakis et al. 2007; Gilbert et al. 2009), and is still spreading through the taxa, arriving in humans within the past 150 years (Worobey et al. 2008). Few endogenous lentiviruses have been found, which has lead some commentators to suggest that their tropism precludes entry to the germline (Stoye 2006). While this may be a contributing factor, it seems likely that simply not enough time has elapsed for substantial levels of endogenisation. Our scope for investigation is also limited to the species for which bioinformatic datasets are available. Two endogenisation events have been described which occurred in the *Leporidae* (rabbits and hares) and the prosimian species grey mouse lemur (Gifford et al. 2008; Katzourakis et al. 2007). However, no replication-competent lentivirus has been isolated from the entire *Lagomorpha* order or from any prosimian species, suggesting that the viruses that gave rise to these endogenous sequences, and their close relatives, are likely to be extinct. We therefore have an idea of the timescale required for a species to clear a lentiviral infection: less than 12-7 my in rabbits and less than 4.3 my in lemurs (Katzourakis et al. 2007; Gifford et al. 2008; Gilbert et al. 2009; van der Loo et al. 2009). The means by which these hosts cleared there infections is unknown but is likely to have involved multiple factors acting at diverse stages in the lentiviral lifecycle. The discovery of an active TRIM5α in rabbits (Schaller et al. 2007) points to the involvement of this protein but restriction of the endogenous RELIK lentivirus has not to date been demonstrated.

In this study we have identified felid species, the domestic cat and African lion, which represent recent and anciently infected hosts. The presumed age of infection in the lions, greater than 324,000 years since the genetic coalescence of modern lions (Antunes et al. 2008) and up to the earliest time of global dissemination of the virus, 3.6 mya (Troyer et al. 2008; Pecon-Slattery et al. 2008b), puts it within an order of magnitude of the lifetime of previous, extinct lentiviral infections, making an ideal host in which to examine the changes in the host brought about by long-term lentiviral infection. The post-entry restriction factor TRIM5α was found to be truncated and non-functional in all felids examined, indicative of a disrupting mutation early in Feliform evolution. Nonetheless, we found that lion cells potently hindered the replication of FIV and primate lentiviruses. This is in direct contrast to the domestic cat, whose cells support FIV replication to a high titre. We found evidence that the APOBEC3 class of restriction factors is active in the felids, and in lions potently restricts FIV and found evidence of a post-entry block to a broad range of viruses.

Lentiviruses, almost by definition, should replicate in the cells of their natural host, meaning that restriction factors must largely be overcome. What therefore is the role of restriction factors? It has been proposed that their primary role is to prevent cross-species transmission by restricting viruses before they can establish themselves in a novel host (Cullen 2006). Whilst restriction of viruses undergoing zoonosis undoubtedly takes place, it is probable that host restriction factors are under selective pressure to target endemic pathogens, even if they contribute only minimally to control of infection. Recent evidence implies that even a small decrease in viral titre can make large differences to pathology. For example, the murine APOBEC3 locus exerts a minor effect on Friend Virus 3 (FV3) replication *in vitro* yet appears to contribute to its clearance by a neutralising antibody response (reviewed in

Ross 2009; Santiago et al. 2008). As has been noted above, the neutralisation of human APOBEC3G is not complete, with low levels of packaged restriction factor and induced mutation observable in clinical HIV-1 isolates (Nowarski et al. 2008; Zhang et al. 2003), suggesting that even this low level of activity could aid in the antiviral response. Also in humans, TRIM5α restricts HIV-1 at a low level (30% reduction; this study, Figure 4-5). Eight non-synonymous TRIM5α polymorphisms have been identified (Javanbakht et al. 2006b) and one of these, R136Q, effects a very modest increase in HIV-1 restriction *in vitro*, yet contributes strongly to survival in AIDS patients after X4 switching, raising percent survival at 3 years post switch from 19% to 45% for RQ heterozygotes and 70% for QQ homozygotes (van Manen et al. 2008). Conversely, mutation H43Y, which impairs TRIM5α E3-ligase activity (Sawyer et al. 2006) accelerates disease progression in humans, with 100% progression to AIDS observed in YY homozygotes within seven years of seroconversion compared to 55% for HH homozygotes (van Manen et al. 2008). Thus it seems probable that even the modest restriction provided by TRIM5α against HIV-1 contributes substantially to limiting the pathology of infection. Furthermore, many of these restriction factors can be up-regulated by interferon, which contributes further to levels of restriction (Rajsbaum et al. 2008; Carthagena et al. 2008; Vetter et al. 2009). Thus those restriction factor alleles conferring even minor increases in restriction are likely to be preferentially inherited and the cumulative effect of many such restrictions may eventually lead to a non-permissive phenotype. A by-product of this process is the potent restriction of retroviruses from other species that share structural features or similar replication strategies, but have not been in locked-step coevolution, leading to a powerful barrier to cross-species transmission. These data imply that the many log restrictions that researchers in the field are accustomed to are probably not the phenotypes on which natural selection routinely works but effects of using viruses and

restriction factors that have not been involved in coevolutionary host-parasite relationships.

The forces that drove the disruption of TRIM5α in both the *Caniformia* and *Feliformia* are unclear. Red Queen situations are characterised by Darwinian struggles between competing biological entities but an alternative view of evolution, termed the 'Court Jester model,' recognises that abiotic factors also influence evolution, speciation and extinction (Benton 2009). Such factors have no doubt impinged on felid and lentiviral evolution, for example in the oceanographic events that permitted felid and FIV migration into North America leading to their subsequent isolation and diversification. Furthermore, certain attributes of modern felids, such as the low genetic diversity in modern cheetah populations (Menotti-Raymond and O'Brien 1993) that has led to high levels of susceptibility to viral infection (e.g. to Feline infectious peritonitis virus; Heeney et al. 1990) cannot be ascribed to the Red Queen hypothesis alone. In this instance, it is likely that a combination of climatic changes and hunting by humans lead to the expunging of genetic diversity during the extinction of the Pleistocene megafauna ~12,000 years before present (Menotti-Raymond and O'Brien 1993; Barnosky et al. 2004). Thus changes in abiotic factors may have had a role to play, for example by genetic isolation of ancestral feliform and caniform species, in sculpting the genetic constitution of modern species. From a Red Queen point of view, however, a potential biotic change that may have lead to the truncation of TRIM5α is a temporary dearth in retroviral infection. Assessing the validity of such hypotheses is impossible but is supported by low levels of taxon-specific ERV sequences and by a paucity of retroviruses in other carnivoran species such as the dog. The loss of functional TRIM5α appears to have rendered domestic cat cells exceptionally permissive to infection, with diverse retroviral pseudotypes able to infect cell lines and replication-competent retroviruses RD114, FIV, FeLV

and FFV all circulating in this species. This permissiveness is not present in lions however, and the lack of TRIM5α restriction appears to be compensated for by an alternative potent post-entry restriction. Along with puma cells, they can prevent the replication of FIV and display potent APOBEC3 activity. These findings are in support of the Red Queen hypothesis and suggest that resistance to infection is developing in these species via a combination of post-entry and APOBEC3-mediated restriction. Although it is probable that FIV-Ple overcomes such restrictions to a sufficient degree in order to replicate and be transmitted, as with murine FV3 and human HIV-1 infection, the restrictive phenotype is likely to play a considerable part in controlling the course of infection and pathology. It may be no coincidence, therefore, that conflicting conclusions about the pathology of FIV infection of African lions have been drawn over the past fifteen years (Carpenter and O'Brien 1995; Roelke et al. 2009; Roelke et al. 2006; Hofmann-Lehmann et al. 1996). The alternative receptor tropisms present in lion FIV isolates may also have confounded these studies as the subtypes are likely to alter in tissue tropism and presumably cytopathology. Future research should further elucidate the prevalence, causes and *in vivo* effects of restriction in lions. Comparisons with other species that display evidence of historic lentiviral infection such as rabbits and Madagascan lemurs may expose the processes involved in species-level clearance of lentiviral infection.

Appendix 1: Primers

Name	Sequence (5' to 3')
feT5fwd	TGTGTGCCGAATCAGGTACT
feT5rev	TCTTCTGCAGCATGTCCATC
gex8 feT5 f	ATCCCTYTYACAGKGTCACA
gex8 feT5 r	MATGAARAGAAYKTATAGATGAGAAACC
	WHERE M=A/C, K=G/T R=G/A AND Y=C/T
huT5stopF	GTTGATGTGACAGTGGCTTGAAACAACATTTCATG
huT5stopR	CATGAAATGTTGTTTCAAGCCACTGTCACATCAAC
Ts12	CCCAGTAATGTTTCCCTGA
Ts16	CATAGTCTAGGAAAACTCCAACACG
Ts2	TGTGGCCACAGCTTCTGCCAAG
Ts3	CATGTGGCCAACATAGTGGAG
wam13c	TCATATTTTCGAATCAGTGTGGAATCACGTGAGC
wam4e2	CAGGGAATTCGCCACCATGGGATTTGTCCTGTCA
feCXCR4 F	GCGAATTCACCATGGACGGGTTTCGTATATAC
feCXCR4 R	CGGTGGATCCGAGGAGTGAAAACTTGAAGA
feCD134 F	CGATCTAGAATGAGGGTGGTTGTGGGGGCT
feCD134 R	ATGGGATCCCGCTGGGGACCCCTGGGGGGCTC
LLV-E 5' Not	ACTGGTCGACACCATGGCAGAAGGAGGAAGAGT
LLV-E 3' Sal	ACAGCGGCCGCTTAGGTATTAGACTCATCAT
LLV-B 5' Sal	ACTGGTCGACACCATGGCGGAAGGAGGAAG
LLV-B 3' Not	ACAGCGGCCGCTCAGACTACTCCTAGAAACT
LLV-E Vif F	ACTGGTCGACACCATGAGTGGTGAAGATATAAGTCAGG
LLV-E Vif R	ACAGCGGCCGCCTAGCCACCTTTCCCTATTAAATATAG
LLV-B Vif R	ACAGCGGCCGCCTACCACATGCTTTCATAGCTAATCTT
LiA3C1 F	ACTGGTCGACACCATGGAGCCCTGGCGCCCCAGCCCAAGAAACCCAATGC
LiA3C1 R	ACAGCGGCCGCTCACCTAAGGATTTCTTGAAGCTCTGCAGCC
LiA3C2 F	ACTGGTCGACACCATGGAGCCCTGGCGCCCCAGCCCAAGAAACCCAATGG
LiA3C2 R	AS LIA3C1-R
LiA3H F	ACTGGTCGACACCATGAATCCACTACAAGAAGACATATTC
LiA3H R	ACAGCGGCCGCTCATTCAAGTTTCAAATTTCTGAAATCATTC
FeA3Ca F	AS LIA3C2-F
FeA3Ca R	AS LIA3C1-R
FeA3H F	ACTGGTCGACACCATGAATCCACTACAGGAAGTCATATTC
FeA3H R	ACAGCGGCCGCTCATTCAAGTTTCAAATTTCTGAAGTCATTC
feCypC69 5' BamHI	CACGGGATCCCGCACTATGTTCTTTGACA
feCypR69 5' BamHI	CTCCGGATCCTTAACCATGGTCAACCCCAT

feCypC/R 3' SalI	GCTAGTCGAGTTAGATTTGTTCACAGTC
feCypA R69 5' Nde	AACATATGGTCAACCCCATCGTG
feCypA C69 5' Nde	AACATATGTTCTTTGACATTACCAT
feCypA R/C 3' Mlu	AAACGCGTTTAGATTTGTCCACAGTCA
GL8 gag F	GCCACCAGGGTGCGCTGCA
GL8 gag R	GCCCATGGATTATTTGGATCT
GL8 G87A	GGTAATGGTCTAGGTGCATCA
GL8 P90A	GGTAATGCTCTAGGACCATCA
LLVE CA 5' nde#2	AACCATATGCCTATACAAGTGGTAAATGGAGTACC
LLVE CA 3' mlu#2	TGCACGCGTGCTTTTTGCTTTTACCTTCATCAAA
feT5TaqMan 5'	GCTGTTGACCGACCTCATCT
feT5TaqMan 3'	AAGACCCTGCTCCTTTCCAT
feT5 Probe	FAM-CAACGATGGACATGCTGCAGGA-TAMRA
wam13	TCAGTGTGGAATCACGTGAGC
wam4	CTGCTATGGCTTCTGAACTCCTG
G8 CAN5' Nde	ACCATATGCCTATTCAAACAGTAAATGGA
G8 CANe' Mlu	TGACGCGTCTAGCTTTTTGCTTTTACCTTC

Appendix 2: Buffers and solutions

Beads wash buffer: 50 mM Tris, 300 mM NaCl, 1 mM DTT, pH 8.0

CHAPS lysis buffer: 30 mM Tris-Cl, 150 mM NaCl, 1% CHAPS w/v, pH 7.5

EB: 10 mM Tris-Cl, pH 8.5

FW basic buffer: 20 mM HEPES, 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1% Triton X-100, pH 7.5

FW blocking buffer: 5% nonfat dry milk in far-Western basic buffer

FW wash buffer 1: 0.2% Triton X-100, PBS

FW wash buffer 2: 0.2% Triton X-100, 100 mM KCl, PBS

LB: 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

SDB: 10 mM HEPES, 4 mM $MnCl_2$, 50 µg/ml dextran sulphate, 1% Triton X-100 v/v, pH 7.6

SDS-PAGE loading buffer: 0.25 M Tris-HCl, 2.5% SDS w/v, 10% glycerol v/v, 1% ß-mercaptoethanol v/v, 0.02% bromophenol blue, pH 6.8

TBE (10x): Tris base 890 mM, Boric acid 890 mM, EDTA 2 mM

TE: 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.6

TY broth: 5% Tryptone-peptone w/v, 3% yeast extract w/v, 10 mM $CaCl_2$

Appendix 3: Carnivoran TRIM5 exon 8 sequences

The 5' end of *TRIM5* exon 8 was PCR amplified from genomic DNA isolated from tissue samples described in Table 4.A using primers gex8 feT5 f and gex8 feT5 r. The first 100 bp of exon 8 are given and the truncating stop codon, where present, is in bold.

Mya-1 cells, domestic cat, *Felis catus domesticus*

CTCACGTGATTCCACAC**TGA**CCCCAAAATACGACATATTTTGGCATTTCTACGGGATGA
AGACCAGTGAGATATATGTGCTGGTGGAAATCAAATAATGC

Hyena, *Crocuta crocuta*

CTCACGTGATTTCATAC**TGA**CCCCCGGATACAACATATTTTGGCAGTTCTACAGAATGA
AGACAAGTGAGATATGTATGCTGTTGGAAGTCAAATAATGC

Cheetah, *Acinonyx jubatus*

CTCACGTGATTCCACAC**TGA**CCCCAAAATACGACATATTTTGGCATTTCTACGGGACGA
AGACCAGTGAGATATATGTGCTGGTGGAAATCAAATAATG

European wildcat, *Felis catus sylvestris*

CTCACGTGATTCCACAC**TGA**CCCCAAAATACGACATATTTTGGCATTTCTACGGGACGA
AGACCAGTGAGATATATGTGCTGGTGGAAATCAAATAATG

Lion, *Panthera leo*

CTCACGTGATTCCACAC**TGA**CCCCAAAATACGACGTATTTTGGCATTTCTACGGGACGA
AGACCAATGAGAGATATGTGCTGTTGGAAATCATATAATG

Mink Mv1 Lu CCL64 cell line, *Mustela lutreola*

CTCACATGACTCCAAATCATCTCCAAAATACAACATATACTCCCCGTTTCTACAGATAAA
AGAGAAATGAGATGTATGCGCTGTTGGAAACCGAATAATG

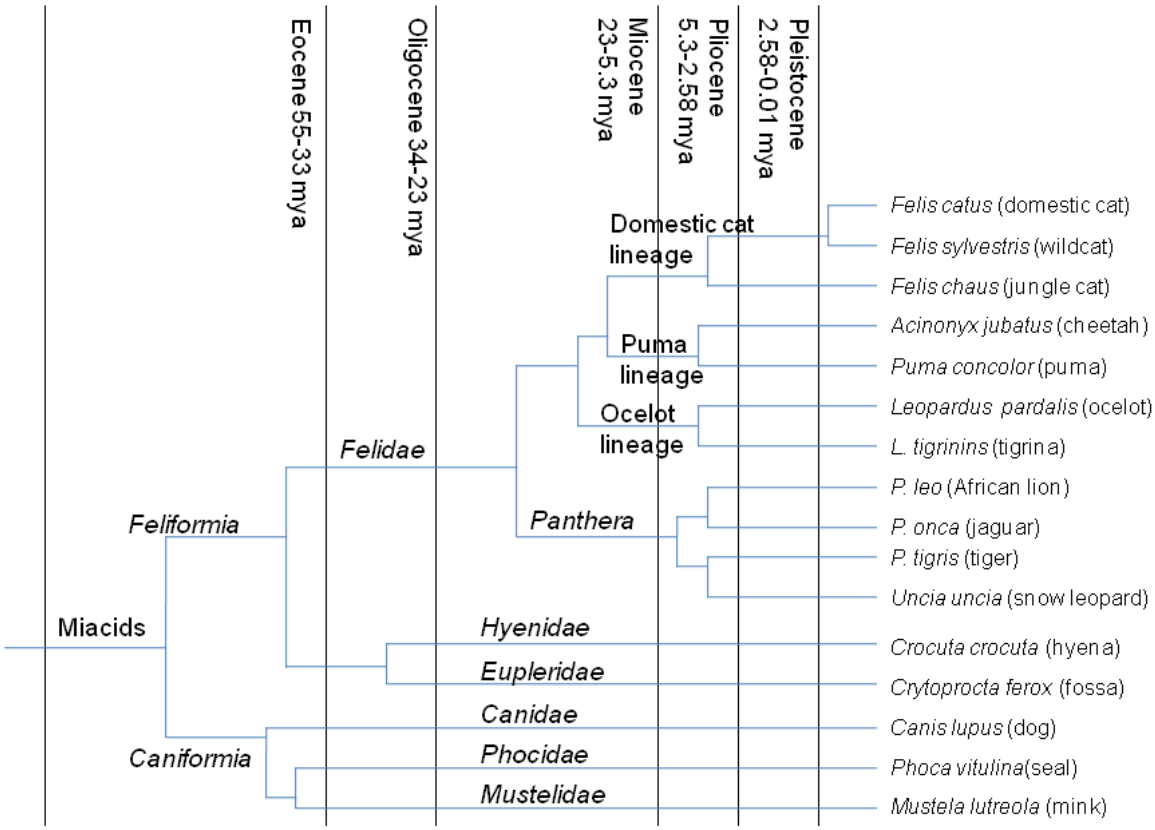
Fossa, *Cryptoprocta ferox*

CTCACGTGATTTCATGC**TGA**CCCCCAAATACAACATATTTTGGCAATTCTACAGAATGA
AGACAAGTGTGATATGTATGCTGTTGGAAGTCAAATAATGC

Dog, *Canis lupus domesticus*

CTCATATGACTCCAGATAATGCCAAAAATAACAACATATGCTATTTCTACAGATCAAAGA
CAAATGAGATATGTGTTTCATTGGAAACTAGATAATGCAT

Appendix 4: Phylogeny of the *Carnivora*



Schematic *Carnivora* phylogeny, based on (Johnson et al. 2006) and (Bininda-Emonds et al. 1999). Progenitor miacids diverged into *Caniformia* and *Feliformia* in the Eocene. The late Miocene radiation has given rise to the modern *Felidae* genera, including *Panthera* and *Felis*.

Publications arising from this work

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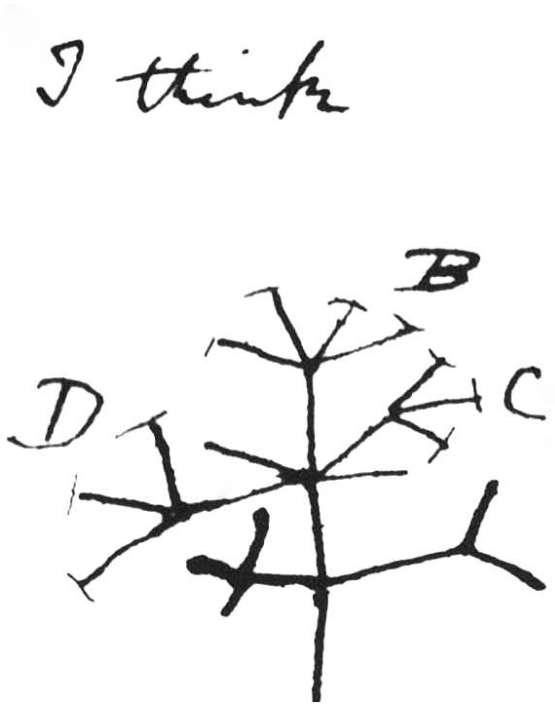

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