Lentiviral host restriction and viral countermeasures:

The *vif* gene of maedi-visna virus

Harpa Lind Björnsdóttir

Thesis for the degree of Master of Science
University of Iceland
Faculty of medicine
School of Health Sciences

HÁSKÓLI ÍSLANDS

Lentiveiruhindrun og varnir veira gegn hindrun:
vif *gen mæði-visnuveiru*

Harpa Lind Björnsdóttir

Ritgerð til meistaragráðu í líf- og læknavísindum

Umsjónarkennari: Valgerður Andrésdóttir

Meistaranámsnefnd: Ólafur Andrésson og Vilhjálmur Svansson

Læknadeild

Heilbrigðisvísindasvið Háskóla Íslands

Júní 2012

Lentiviral host restriction and viral countermeasures:
The vif *gene of maedi visna virus*

Harpa Lind Björnsdóttir

Thesis for the degree of Master of Science

Supervisor: Valgerður Andrésdóttir

Masters committee: Ólafur Andrésson and Vilhjálmur Svansson

Faculty of Medicine

School of Health Sciences

June 2012

Prentun: Leturprent 2012

Reykjavík, Ísland 2012

Ágrip

Mæði-visnuveira (MVV) er lentiveira af flokki retróveira (Retroviridae). Veiran sýkir kindur og veldur lungnabólgu (mæði) og heilabólgu (visnu). Dæmi um aðrar lentiveirur eru eyðniveirurnar í mönnum (HIV-1 og HIV-2) og köttum (FIV), smitandi liða- og heilabólga í geitum (CAEV) og smitandi blóðleysi í hestum (EIAV).

Á síðustu árum hefur komið sífellt betur í ljós að stöðugt vopnakapphlaup er á milli lífvera og veira. Lífverur hafa komið sér upp ýmis konar vörnum gegn veirum og veirur hafa svarað með þróun fjölbreyttra aðferða til að komast fram hjá vörnum hýsilfrumna sinna.

Lentiveirur hafa próteinið Vif (virion infectivity factor) sem hefur það velþekkta hlutverk að leiða til niðurbrots APOBEC3 (A3) próteina hýsilfrumna, en þau eru cytósín afamínasar sem valda miklum fjölda G-A stökkbreytinga í +DNA veirunnar ef Vif prótein er ekki virkt. HIV-1 Vif binst við Cullin 5 (Cul5) sem er hýsilprótein og kallar að önnur prótein sem tilheyra Cul5 E3 úbíkítín lígasaflóka. Þessi flóki fjölúbíkínierar A3 prótein sem leiðir til þess að þau eru brotin niður í próteasómi frumunnar. MVV bindur Cullin 2 (Cul2) vel en Cul5 verr, auk þess að vera ekki með varðveitt Cul5 set.

Við fundum hugsanlegt Cul2 bindiset í MVV Vif og gerðum stökkbreytingar á því bæði í klónaðri veiru og einnig í táknabestuðu *vif* plasmíði til tjáningar í spendýrafrumum í eins hrings sýkingarkerfi með kinda A3.

Niðurstöður leiddu í ljós að veira með stökkbreytinguna vex hægar og verr en villigerð veiru. Þegar niðurbrot á A3 var skoðað kom í ljós að A3 var aðeins brotið niður að hluta til með Vif sem hafði stökkbreytingar í þessu hugsanlega Cul2 seti.

Þetta bendir til að ætlaða Cul2 setið sé ekki notað einvörðungu, og ef til vill sé Cul5 notað til að mynda E3 ubikítín lígasaflóka þegar Cul2 binding er eyðilögð. Niðurstöðurnar benda til þess að Vif prótein lentiveira hafi að nokkru leyti þróast óháð til að nota mismunandi leiðir við niðurbrot A3 próteina.

Niðurstöður úr rannsóknum á Keldum benda til þess að Vif próteinið í MVV gegni fleiri hlutverkum en að hindra A3.

CA-Vif veira er mæði-visnuveira með tvær stökkbreytingar, L120R í hylkispróteini (CA) og P205S í Vif sem valda því að veiran vex illa í fósturliðþelsfrumum (FOS) og átfrumum. Veiran vex hinsvegar ágætlega í kindaæðaflækjufrumum (SCP). Ef veirur með aðra hvora stökkbreytinguna en ekki báðar voru ræktaðar í FOS eða átfrumum kom þessi svipgerð ekki fram. Þetta bendir til þess að einhver tengsl séu á milli hylkis og Vif próteins.

Til að kanna hvort þessi hindrun á veirufjölgun í FOS frumum væri í víxlritun, voru gerðar sýkingartilraunir með CA-Vif veirur með uppruna úr annað hvort kindaæðaflækjufrumum eða FOS frumum og sýni tekin á fyrstu klukkustundunum eftir sýkingu. Niðurstöður sýndu að aðalmunur á CA-Vif og villigerð af MVV kom fram eftir 24 tíma sýkingu , þ.e.a.s. eftir að víxlritun er lokið. Þetta bendir til þess að hindrun verði ekki við víxlritastig veirunar, en gæti einnig bent til að í FOS frumum væri hindri sem hefur áhrif á hylkið á einhverju stigi eftir víxlritun, og að Vif verji veiruna fyrir þessum hindra.

Abstract

Maedi-visna virus (MVV) belongs to the lentivirus subgroup of retroviruses (Retroviridae). MVV infects sheep and causes slowly progressive pneumonia (maedi) and encephalomyelitis (visna). Examples of other lentiviruses are the immunodeficiency viruses of humans (HIV-1 and HIV-2) and cats (FIV), caprine arthritis encephalitis virus (CAEV) and equine infectious anemia virus (EIAV).

Recently a constant arms race between host organisms and viruses has become apparent. Organisms have evolved a variety of mechanisms to fight viral infections while the viruses have developed means of counteracting those defenses in various ways.

Lentiviruses have the protein Vif (virion infectivity factor) whose well known role is to lead to degradation of the host cell APOBEC3 (A3) proteins, but they are cytosine deaminases that deaminate cyrosine in the viral –DNA strand, thus causing G-A hypermutations in the viral +strand DNA if the Vif protein is defective or missing. HIV-1 Vif binds to Cullin 5 (Cul5), a host protein, and recruits other host proteins to form a Cul5 E3 ubiquitin ligase complex. This complex polyubiquitinates A3 proteins, leading to their degradation by the cellular proteasome. MVV Vif binds Cullin 2 (Cul2) more strongly than Cul5, and does not have a conserved Cul5 binding site.

We found a putative Cul2 binding site in MVV Vif and mutated it both in a molecularly cloned virus and in a *vif* plasmid codonoptimized for expression in mammalian cells. The cloned Vif was tested in single-cycle infectivity assays with ovine A3.

The results show that a virus with mutations grows slower than a wild type MVV. When A3 degradation was analyzed we saw that A3 is only partially degraded if Vif has the mutation in the putative Cul2 binding site.

This indicates that the putative Cul2 site is not used solely, and possibly Cul5 is used to form an E3 ubiquitin ligase complex when Cul2 binding is disrupted. The results indicate that the lentiviral Vif proteins have evolved diverse ways to degrade A3 proteins.

Results from research done at Keldur implies that the MVV Vif protein has other roles than counteracting A3.

CA-Vif virus has two mutations, L120R in CA and P205S in Vif that cause the virus to replicate poorly in fetal ovine synovial (FOS) cells and macrophages while replication in sheep choroid plexus (SCP) cells is not inhibited. This phenotype is not seen in a virus with either mutation alone. This implies that some connection exists between capsid and the Vif protein of MVV.

To test whether the CA-Vif mutant had a block before or after reverse transcription, CA-Vif virus obtained from either SCP or FOS cells was compared to a wild type virus in FOS or SPC cells for the first hours after infection. The results show that the main difference between CA-Vif and the wild type virus was seen after 24 hour infection, i.e. after reverse transcription is completed. This implies that the block is not at the reverse transcription stage, but could also imply that FOS cells have a host restriction factor that affects the capsid at some stage after reverse transcription and that Vif protects the virus from this restriction factor.

Acknowledgements

I would sincerely like to thank my supervisor Valgerður Andrésdóttir for guiding and supporting me with this project. I would also like to thank the members of my M.Sc. committee, Ólafur S. Andrésson and Vilhjálmur Svansson for their help with the thesis. I would like to thank Stefán Ragnar Jónsson for his help with the HIV-infectivity assay and Birkir Þór Bragason for his help with real-time PCR and for reagents.

I am thankful to the administration at the Institute for Experimental Pathology, University of Iceland, Keldur where the research was carried out.

The project was financially supported by the Research Fund of the University of Iceland and the Icelandic Research Council. I would like to thank the organizing committee at the 23rd Workshop on Retroviral Pathogenesis and the travel grants of the University of Iceland Research Fund and the Association of Academics' Educational fund of for financial support to travel to the meeting in the autumn of 2011.

I thank my fellow students and co-workers for their valuable support and good advice. Finally I would like to thank my family and friends for their patience and support through this work.

Table of contents

List of figures

List of tables

Abbreviations

APOBEC	Apolipoprotein B mRNA editing enzyme catalytic polypeptide-like
BIV	Bovine immunodeficiency virus
BLAST	Basic Local Alignment Search Tool
bp	base pairs
CA	Capsid protein
CAEV	Caprine Arthritis Encephalitis virus
DMEM	Dulbecco's modification of Eagles' medium
DNA	Deoxyribonucleic acid
eGFP	Enhanced green fluorescent protein
EIAV	Equine infectious anaemia virus
Env	Envelope protein
FACS	Fluorescence-activated cell sorting
FIV	Feline immunodeficiency virus
FOS	Fetal ovine synovial cells
HIV	Human immunodeficiency virus
IN	Integrase
kb	Kilo base
LTR	Long terminal repeats
MA	Matrix protein
mRNA	Messenger RNA
MVV	Maedi-visna virus
NC	Nucleocapsid protein
nt	Nucleotide
PBS	Primer binding site
PCR	Polymerase chain reaction
PIC	Preintegration complex
Pol	Polymerase
PR	Protease
RNA	Ribonucleic acid
RT	Reverse transcriptase
SIV	Simian immunodeficiency virus
SU	Surface protein
Tat	Trans-activation protein

TM	Trans membrane protein
TRIM	Tripartite motif
Vif	Virion infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U
VSV-G	Vesicular stomatitis virus protein G

1 Introduction

1.1 History of maedi-visna virus

Maedi-visna virus (MVV) is a lentivirus of sheep first found in Iceland after import of German sheep of the Karakul breed in 1933. The sheep were quarantined and afterwards distributed to various places in Iceland (Sigurdsson, 1954). Soon Icelandic sheep started showing signs of new diseases. Two of those diseases, pneumonia and a central nervous system disease, mæði and visna respectively, were later found to be caused by the same previously unknown virus, named maedi-visna virus.

Visna caused damage to the myelin sheeth of neurons in the central nervous system, spinal cord and cerebellum which led to widespread paralysis in the affected sheep. Maedi caused progressive respiratory distress and enlargement of lungs and lungnodes in affected sheep. Both diseases usually ended in death (Sigurðsson, 1958).

Björn Sigurðsson, a medical doctor by training, made observations on the progress of visna and maedi in affected sheep and published a definition of a new kind of a disease, a slow disease where a long time passes after the infecting agent enters the body until the first sign of disease, the disease persists for a long time and leads to a serious disease or death. Each disease only affects one species and tissue changes can only be found in one type of tissue or organ (Sigurdsson, 1954; Sigurðsson, 1958).

A previously unknown virus was cultured from the brains of sheep with encephalitis in 1957-1958 and it was also shown that a virus caused the pulmonary disease maedi (referenced in (Guðnadóttir, 1977)).

Visna and maedi were eventually shown to be caused by the same virus (Gudnadóttir and Pálsson, 1965). The diseases were finally eradicated in Iceland in 1965 after the slaughter of all affected sheep and all sheep from the same area as affected sheep were found in.

1.2 Retroviruses

Maedi-visna virus belongs to retroviruses, the *retroviridae* family. All retroviruses have two single-stranded (+) RNA molecules which are reverse transcribed into double stranded DNA and integrated into the genome of the host cell. All retroviruses have the structural genes *gag*, *pol* and *env*. Their genome size is 7-12 kilobases (kb) in length (Lin and Thormar, 1970; Vogt, 1997).

The *retroviridae* family is divided into three main groups, *oncoviridae* or oncoviruses, *spumaviridae* spuma- or foamyviruses and *lentivirinae* or lentiviruses (Figure 1).

Oncoviruses are tumour causing viruses that can be divided into 5 subdivisions

Alpha α, simple very host specific tumour causing viruses that are mostly found in birds.

Beta β, simple oncogenic retroviruses that cause tumours in a variety of species.

Delta δ, complex retroviruses, causing leukaemias in a number of species.

Gamma γ, the largest subgroup containing a number of endo- and exogenic viruses, simple viruses in a variety of species.

Epsilon ε, a group of simple oncoviruses, found mainly in fish and not well studied.

Spumaviruses are widespread but not known to cause disease, and are unconventional retroviruses since they do not cleave the Gag polyprotein into smaller subunits, the NC part of the Gag protein does not contain a metal-binding site and reverse transcription is almost completed before budding of the virus from the producing cell.

Of all retroviruses lentiviruses are the most complex, both in structure and genome.

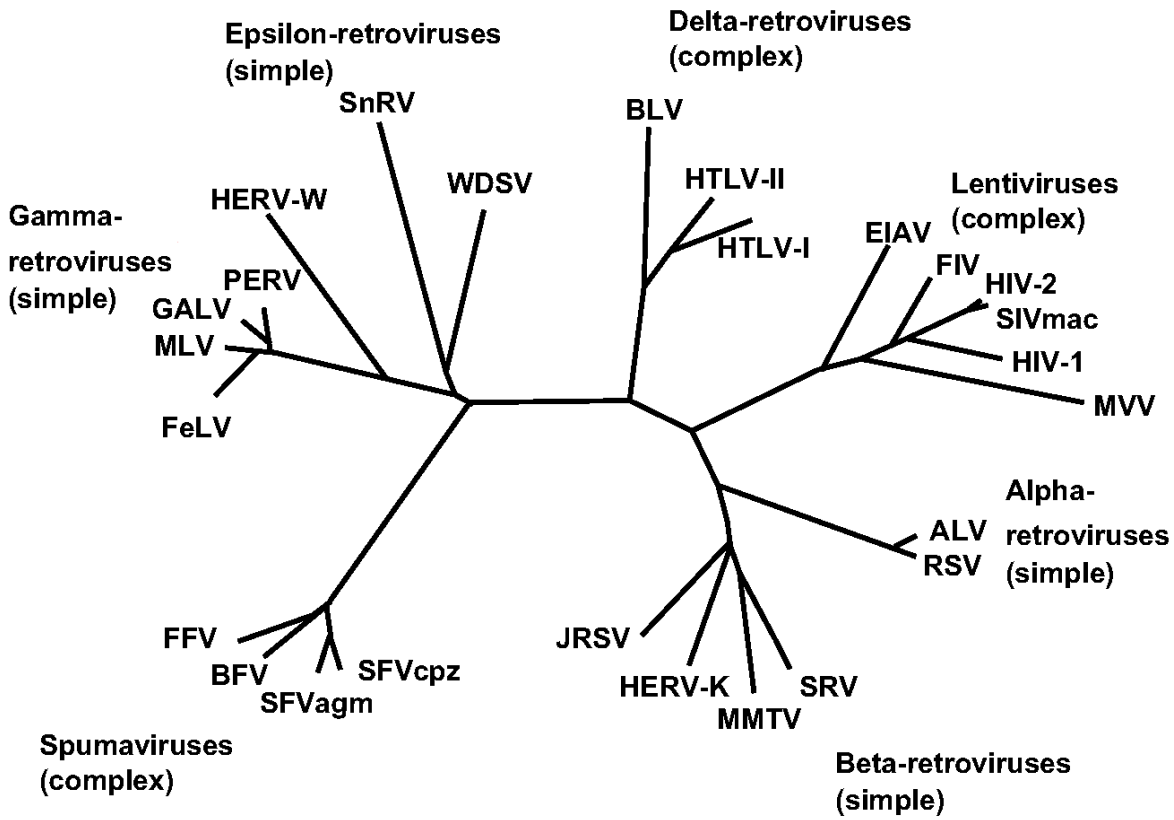


Figure 1. Phylogeny of retroviruses. Unrooted phylogenic tree of retroviruses based on the homology of *pol* sequences. Figure from (Weiss, 2006).

All retroviruses have spherical virions, 80-130 nm in diameter with a lipid bilayer envelope containing glycoproteins encoded by *env*. Inside of the envelope is a matrix, then a capsid, and inside it a nucleoprotein core that contains a dimeric single stranded RNA that is associated with several viral proteins.

The viral RNA is reverse transcribed into double stranded linear DNA with long terminal repeats (LTRs) derived from the unique sequences at each end of the genomic RNA. The double stranded DNA is then integrated into the host cell genome by the integrase. The LTRs provide signals for cellular factors that control synthesis and processing of viral RNA.

Virion proteins:

Gag proteins: Matrix (MA), is 15-20 kDa, located between the membrane and capsid. In mammalian retroviruses the matrix is modified at the N-terminus by myristylation. Capsid (CA) protein is 24-30 kDa and the major structural component of the capsid. Nucleocapsid (NC) is 10-15 kDa with a cysteine-histidine motif that resembles metal-binding domains of cellular proteins that interact with nucleic acids. NC has high affinity for the viral RNA genome. In many retroviruses additional proteins are derived from the Gag polyprotein.

Pol proteins: Protease (PR) is 10-15 kDa and mediates cleavage of Gag and Pol polyproteins during virion assembly and maturation. The reverse transcriptase (RT) is a RNA-dependent DNA polymerase encoded by *pol* in all replication-competent retroviruses. Each virion contains 10-20 molecules of RT. A separate domain [attached to the RT] functions as a ribonuclease (RNase-H) specific for RNA-DNA hybrids. Integrase (IN) is derived from the C-terminal of Pol.

Env polyproteins are post-translationally modified in the endoplasmic reticulum by cleavages and glycosylation to yield surface (SU) and transmembrane (TM) domains. The surface domain is located on the external surface of membrane and binds the virion to cell receptors. The TM is embedded in the lipid bilayer and anchors the SU to the membrane. TM mediates the fusion of viral and cell membranes during entry. The glycoproteins are also the main targets of the antiviral immune responses of the host cell. In some retroviruses the glycoproteins control some cytopathic events, such as syncytium formation *in vitro*.

Viral RNA

The two singlestranded virus RNAs both have positive polarity in respect to translation. In each virion two copies of RNA are packaged, but only one provirus is produced from each infectious virus particle. The two RNAs appear to interact near the 5’ ends, either through hydrogen bonds formed by molecules aligned in the same polarity or through short antiparallel alignments. Host cell enzymes carry out posttranscriptional modifications of the viral RNA, the 5’ end has a cap structure, the 3’ end a 100-200 base poly-A tail and there are also internal methylations of the RNA. These modifications mimic cellular mRNA.

The virion RNA has a short repeated sequence (R) at each end that is 15-80 bases, depending on the retrovirus. In reverse transcription this sequence provides a means to transfer newly initiated DNA strands from the 5’ end to the 3’ of the viral RNA. Only the 5’ end R is capped and only the 3’ end R has a poly-A tail. U5 is 80-100 bases and found between R and a pairing sequence site for the tRNA that functions as a primer for viral DNA synthesis. The U5 has structural features that are important for initiation of reverse transcription. Reverse transcription can be seen in Figure 2 and with a detailed description in text accompanying the figure (Basu et al., 2008; Telesnitsky and Goff, 1997). A specific tRNA bound near the 5’ end initiates DNA synthesis. A 16-19 base sequence at the 3’ end of the tRNA is hydrogen bonded to the complimentary sequence, the primer binding site (PBS). Each group of related retroviruses contains a specific tRNA primer obtained from the producer cell. Following the PBS site there is an untranslated leader (L) sequence, 150-200 bases, that precedes the initiation codon of *gag*. Packaging (Ψ) or encapsidation (EN) sequence element within L play a role in assembling viral RNA into virions. At the 3’ end the polypurine tract (PPT) is about 15 bases and rich in purines. Its role is to function as a RNA primer to initiate synthesis of plus-strand DNA. U3 is 200-

1000 bases between PPT and R at the 3' end. U3 has promoter elements that control transcriptional initiation of integrated provirus by cellular RNA polymerase II. The LTRs are produced during viral DNA synthesis and contain U3 juxtaposed to R-U5. Members of closely related retroviruses show relatively high sequence variation in U3 (Luciw and Leung, 1992).

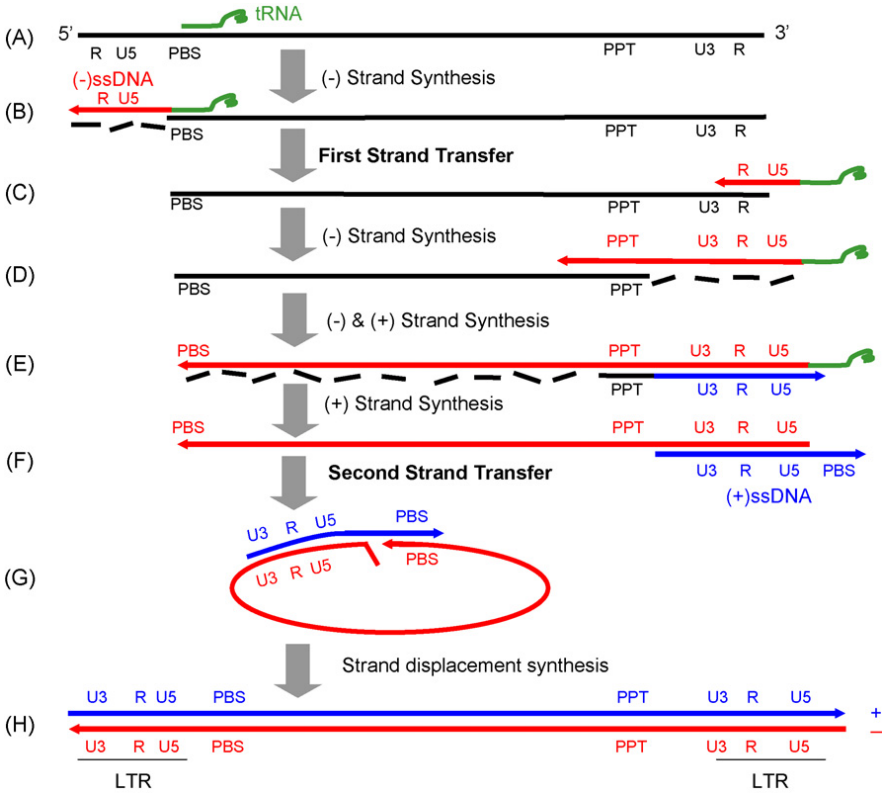


Figure 2. Reverse transcription. A) Reverse transcription initiates from the 3'-end of a primer tRNA (green) that partially anneals to the primer binding site (PBS) of the genomic RNA (black). B) The primer extends over the 5'-end U5 and R regions of genomic RNA, generating a minus strand strong stop DNA (-ssDNA, red). The 5'-end of the genomic RNA is degraded by the RNase H activity of the RT. C) After translocation of the –ssDNA to the 3'-end of the genomic RNA the R of the –ssDNA anneals to the 3'-R region of the genomic RNA to allow continued minus strand synthesis. Annealing is facilitated by the viral nucleocapsid protein. D) Minus strand DNA synthesis resumes and RNase H digests the template RNA strand partly but not completely until it reaches the short polypurine tract (PPT). E) While –ssDNA synthesis continues into the PBS region plus strand synthesis (blue) is started from the PPT fragment(s) of genomic RNA that acts as a primer for plus strand synthesis. DNA is synthesized until a portion of the tRNA primer has been copied, creating a copy of the PBS at the plus strand strong stop DNA (+ssDNA). RNase H removes the rest of the genomic RNA. F) Primer tRNA is removed by RNase H. G) Complementary copies of PBS sequences at the 3'-ends of +ssDNA and the newly formed minus strand DNA base pair. H) After second strand transfer, minus and plus strand DNA synthesis continues, with each strand using the other as a template until the double stranded DNA is fully synthesized with long terminal repeats (LTRs) at the ends. Figure from (Basu et al., 2008).

High level of mutations in retroviruses is caused by the high level of errors in the reverse transcription process, averaging 0.1-1 mutation per replication circle, which is about a million times higher than the error rate in DNA replication. Since retroviruses have two strands of RNA they can recombine, but only if the producer cell has produced two different kinds of viral RNA, since

recombination is only observed after the RNA has been encapsidated. Recombination during minus-strand DNA synthesis allows for repair of single-stranded breaks (Temin, 1992).

Retroviruses all have common steps in their replicate pathways, and they are: entrance, reverse transcription, integration, transcription, splicing, transport, translation and budding (Figure 3).

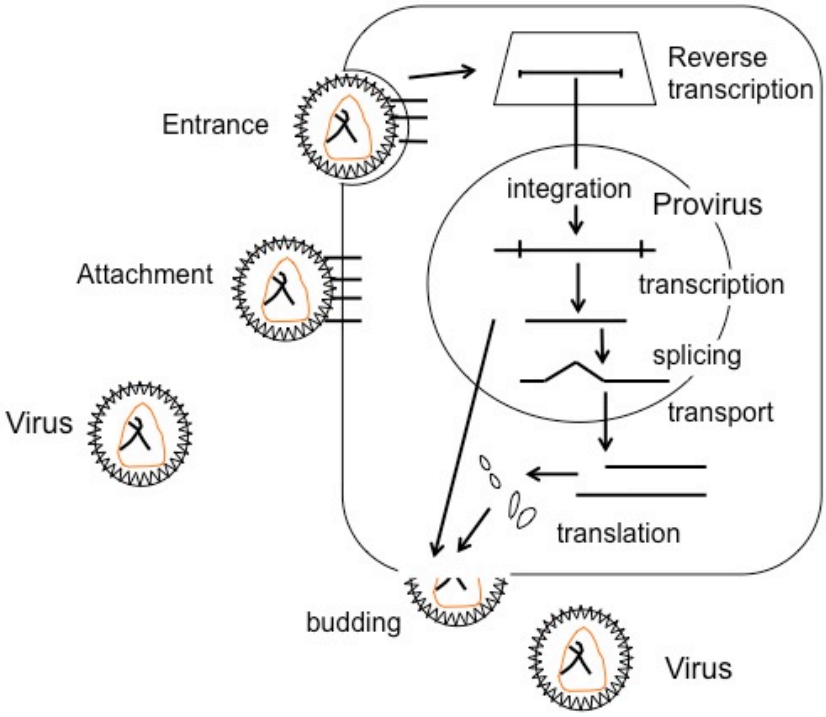


Figure 3. The infection cycle of simple retroviruses. Virus attaches to the cellular membrane and enters the cell. Reverse transcription happens after entry and before integration of DNA into cellular DNA. The provirus is then transcribed and RNA spliced and transported to the cytoplasm for translation and assembly of virions that bud out of the membrane, taking part of it as envelope. Mature virions can start the cycle again. Figure based on (Temin, 1992).

Since retroviruses form a provirus and are integrated into the cellular genome they can become latent, going from a replicating state to non-replicating state and back again, and therefore they can maintain a particular sequence for a long time (Temin, 1992).

1.3 Replication cycle

All retroviruses share certain steps in their replication cycle. The replication cycle is divided into early and late stages. The early stage starts with the virion binding to a receptor on the host cell. The attachment and the viral entry following attachment is mostly mediated by the Env glycoproteins. The membranes of the virion and the host cell fuse together and the core is released into the cytoplasm. The reverse transcriptase and integrase are associated with the viral RNA in the nucleoprotein complex, where reverse transcription takes place. The newly synthesized DNA in the nucleoprotein complex is then transported into the nucleus, where the viral DNA is covalently linked to the host DNA by the integrase. The integrated viral DNA is called a provirus.

The late stage starts with the transcription of the provirus and ends with the release of mature virions capable of initiating a replication cycle. Viral transcripts have a 5’ end methylated cap and a 3’

end poly-A tail, so the transcripts resemble eukaryotic mRNA. Both full-length and spliced RNAs are then transported to the cytoplasm and translated on polysomes into polyproteins. The full-length transcripts also interact with the polyproteins and are assembled into immature virus particles. The virus acquires its envelope by budding through the cell plasma membrane, acquiring a lipid bilayer membrane with virus glycoproteins. The maturation steps involve processing of assembled polyproteins by the viral protease to yield fully infectious virions (Luciw and Leung, 1992). The early and late stages of the HIV-1 replication cycle can be seen in Figure 4.

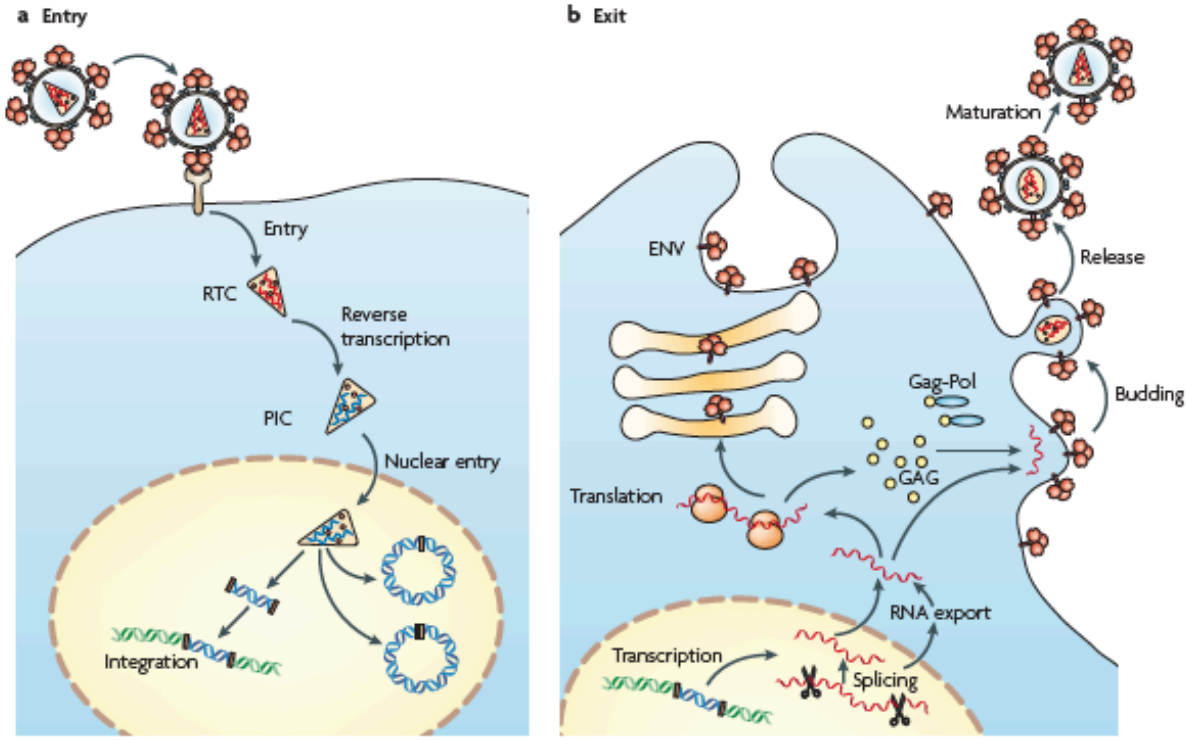


Figure 4. Lentiviral replication cycle. The early a) and late b) events of the HIV-1 replication cycle. Figure from (Goff, 2007).

1.4 Lentiviruses and their genomes

Lentiviruses are a complex form of retroviruses and are found in a number of mammalian species. Maedi-visna virus is part of the subfamily *lentivirinae* of the *retroviridae* family. “Lenti” means “slow”, indicating the slow progression of diseases caused by lentiviruses. MVV shares the group of lentivirus with human immunodeficiency virus-1 (HIV-1), human immunodeficiency virus-2 (HIV-2), the simian immunodeficiency viruses (SIVs), feline immunodeficiency virus (FIV), caprine arthritis-encephalitis virus (CAEV), equine infectious anaemia virus (EIAV), and bovine immunodeficiency virus (BIV) (Haase, 1975). MVV, CAEV, BIV and EIAV primarily infect macrophages but FIV, SIV and HIV infect both macrophages and T lymphocytes (Gonda, 1994). Viral proteins are expressed from either full length or from spliced mRNAs.

The nucleotide sequence of MVV showed a strong correlation to HIV-1 and was used to support the placement of HIV-1 in the *lentiviridae* group of retroviruses (Sonigo et al., 1985).

HIV-1 and HIV-2 specifically infect cells that express the HLA class II receptor CD4 on their cell surface. Those cells are CD4+ lymphocytes, cells of the monocyte-macrophage lineage and microglia cells in the brain. CD4 is the primary receptor for HIV-1 but a co-receptor is needed. HIV-1 strains can be classified as M-tropic or T-tropic, infecting primary monocytes/macrophages on one hand or T cell lines or primary T-lymphocytes on the other. M- and T-tropic strains use different chemokine co-receptors, CCR5 for M-tropic HIV-1 and some HIV-2 or SIV isolates. CXCR4 is the co-receptor of T-tropic HIV-1, but can also act as the primary receptor of some strains of HIV-2 without CD4. FIV also uses CXCR4 without CD4.

Additional chemokine receptors support entry by more restricted groups of primary HIV-1 isolates. HIV-1 entry is initiated by high-affinity interaction between the viral glycoprotein gp120 and CD4. CD4 binding induces conformational changes in gp120 that allow for generation of a high-affinity binding site for the functionally relevant chemokine co-receptors. During primary infection and the asymptomatic phase of HIV infection blood isolates are usually CCR5 dependent, non-syncytium inducing (NSI) and M-tropic. Later T-tropic, syncytia-inducing (SI) strains that use CXCR4 emerge, associated with accelerated disease progression. This evolution of co-receptor use coincides with the viral phenotypic switch from NSI to SI and progression to AIDS.

Individuals that show resistance to HIV infection have a homozygous deletion in CCR5 that leads to truncated protein not expressed at the cell surface (Tang et al., 1999).

During virus assembly the HIV-1 Gag polyprotein interacts with the cellular molecular chaperone cyclophilin A (CypA) and forms a complex in the virion core required for maturation of the virion, as virions without this complex are not infectious. CypA destabilizes the direct Gag-Gag interaction of core and facilitates the uncoating process.

Unlike retroviruses that require cell division and breakdown of the nuclear envelope to enter the nucleus the lentiviral preintegration complex (PIC) is actively imported into the nucleus in interphase (Tang et al., 1999).

The capsid protein of HIV-1 seems to be directly involved in some steps of infection, as mutations in CA can render mutants unable to infect non-dividing cells, but able to infect dividing cells. Results show that CA can affect a post-nuclear entry step since the nuclear entry of PIC is not the limiting step of infection for those mutants (Yamashita et al., 2007).

1.4.1 The regulatory and accessory genes and proteins of human immunodeficiency virus (HIV)

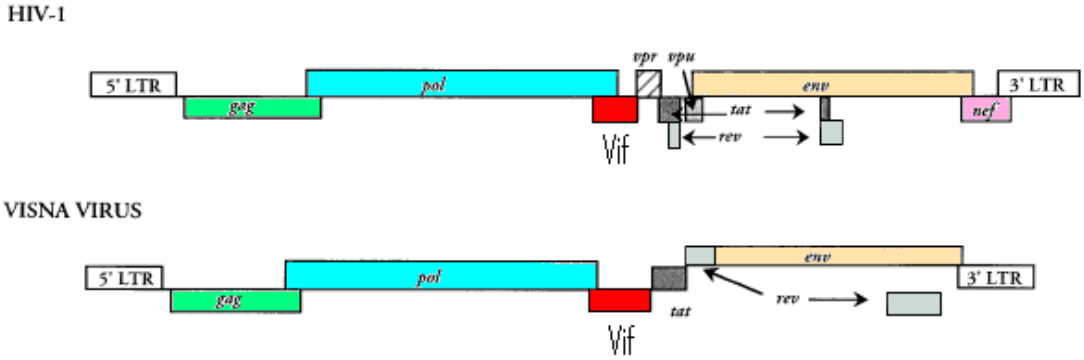


Figure 5. Genomic structure of HIV-1 and MVV. Figure modified from (Clements and Zink, 1996).

The genomic structure of HIV-1 and MVV can be seen in Figure 5. The regulatory genes of HIV are *tat* and *rev* and both are primarily found in the cell nucleus. The Tat protein is 14 kDa and stimulates transcription by binding TAR sequence in viral RNA to facilitate initiation and elongation of viral transcription. The Rev protein is 19 kDa and regulates viral mRNA production by binding RRE and facilitating nuclear export of spliced or unspliced viral RNAs. Tat and Rev can also be found in all non-primate lentiviruses.

The accessory genes of HIV are *nef*, *vif*, *vpr*, *vpu* and *vpx*.

The Nef protein is 27 kDa and is found in the cytoplasm and plasma membrane. It is pleiotropic and can increase or decrease virus replication, reduces expression of MHC class I, the CD4 and CD8 receptors. Nef affects T-cell activation and enhances virion infectivity.

The Vif protein is a highly basic, contains 192 amino acid, is 23 kDa, and primarily found in the cytoplasm. Vif's main role is to increase virus infectivity by affecting virion assembly and/or viral DNA synthesis. Vif will be discussed further in chapter 1.9.

The Vpr protein is 15 kDa and found in the virion. It causes G^2 arrest of the cell and facilitates nuclear entry of the preintegration complex. Reviewed in (Flint et al., 2004).

Vpu is only present in HIV-1. The protein is 16 kDa and is an integral cell membrane protein. It affects virus release by mediating the degradation of tetherin, which is a host factor that interferes with the detachment of virus particles from infected cells (Neil et al., 2008).

vpx is only found in SIV and HIV-2 and may be a duplication of *vpr*. The Vpx protein is 15 kDa and counteracts the newly discovered SAMHD1 protein which is a restriction factor expressed in macrophages and dendritic cells (Hrecka et al., 2011; Laguette et al., 2011).

1.5 Maedi-visna virus

1.5.1 Structure

The MVV virion is about 100 nm in diameter and enveloped by a lipid bilayer derived from the host cell by the budding of the virion. The envelope is covered with about 9 nm long spikes that consist of

surface (gp135) and trans-membrane (gp44) glycoproteins. Matrix (p16) proteins form an inner shell under the envelope, and inside the matrix is a 40 nm virion core shaped like a cone (Pépin et al., 1998; Thormar, 1961; Thormar and Cruickshank, 1965). The core is made up of the capsid (CA, p25) and inside it are nucleocapsid proteins (NC, p14) bound to the two single stranded viral RNAs (9,2 kb), and cellular $tRNA^{lys}$, protease (PR, p11), reverse transcriptase (RT, p66/p51) and integrase (IN, p34) Figure 6 (Thormar and Cruickshank, 1965).

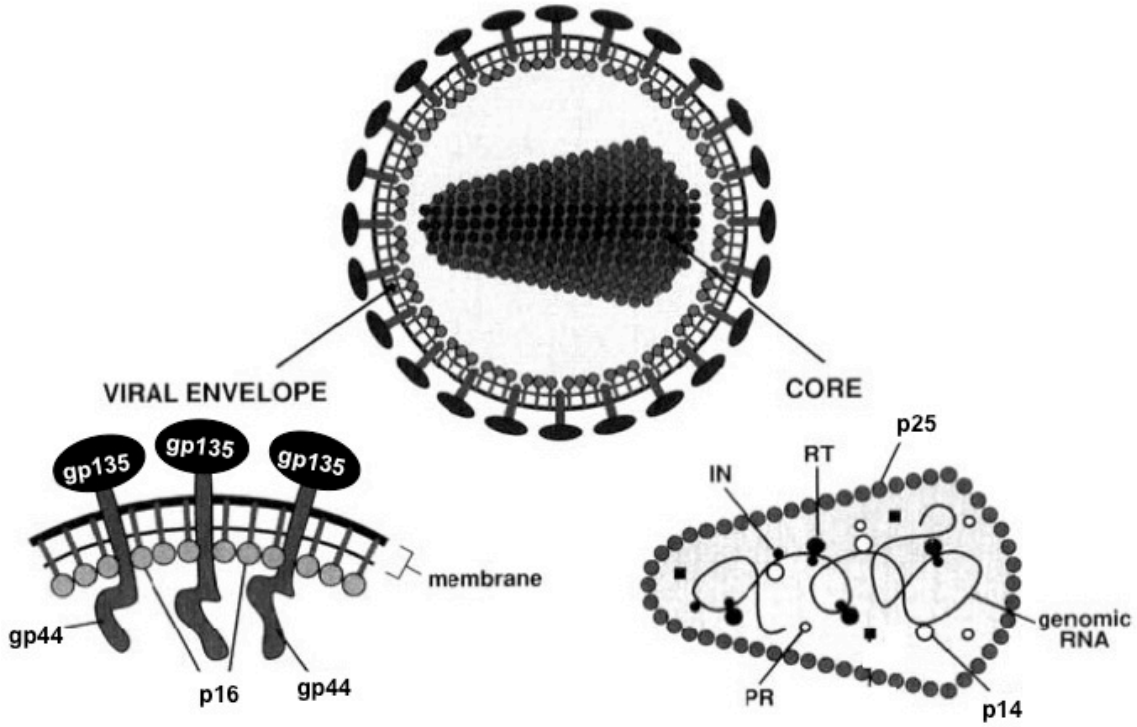


Figure 6. Structure of MVV. The figure shows the locations of MVV structural proteins, figure modified from (Gonda, 1994).

1.5.2 Genes and proteins

The nucleotide sequence of MVV is 9202 bp (Sonigo et al., 1985). Like all retroviruses MVV has three structural genes, organized from 5´to 3´, *gag*, *pol* and *env* that encode for the necessary structural proteins and enzymes required for replication. The enzymes are the reverse transcriptase, which is a heterodimer that displays RNAse H activity, dUTPase, which primate lentiviruses lack, integrase and protease. Long terminal repeats (LTRs) that flank the proviral DNA provide signals needed for transcription, integration and polyadenylation of viral RNA. The genomic structure of MVV can be seen in Figure 5.

Lentiviruses also code for various numbers of regulatory and auxilliary genes located in open reading frames (ORFs) between *pol* and *env*, and at the 3´end of the *env* gene. MVV has only the three regulatory and auxiliary genes *vif*, *tat* and *rev*, but HIV-1 also has the genes *vpr*, *vpu* and *nef* (Figure 5) (Narayan and Clements, 1989; Pépin et al., 1998).

1.5.2.1 The regulatory and accessory genes of maedi-visna virus (MVV)

The Rev protein is derived from 1.4 kb mRNA, leading to a 167 amino acid protein that is 19 kDa. It is an early gene and Rev plays an important part in transportation of unspliced mRNA from the

nucleus to cytoplasm with its nuclear export signals. Its regulatory role is exerted by a Rev responsive element (RRE) in the *env* RNA.

Tat is translated from 1.7 kb mRNA that encodes a 10 kDa protein (Pépin et al., 1998). MVV and CAEV Tat seem to have a similar function as the Vpr protein has in HIV-1 and the small ruminant lentivirus Tats are not transactivators of transcripition like the Tat of HIV-1. Tat localizes to the nucleus of transfected cells and is incorporated into the viral particle. MVV Tat induces G_2 arrest (Ólafsdóttir, 2009; Villet et al., 2003a; Villet et al., 2003b).

Vif will be discussed further in chapter 1.9.

1.6 Retroviral host defences in mammals

Hosts to viral infection have a variety of ways to protect themselves against retroviruses such as the innate immune system with interferon responses against viral infection and natural killer cells that attack infected cells, and the acquired immune system, with B-cells, cytotoxic T-cells and antibody responses. But mammals have also evolved specific restriction factors against retroviruses that can restrict some viruses that have already entered a cell from replicating, and are therefore a part of the immune system of hosts. These restriction factors have been called intrinsic immunity. These factors are often broad-spectrum and can work against a number of related viruses.

Some of those defenses include the APOBEC3 proteins, TRIM5α, tetherin and the newly discovered SAMHD1 (Emerman, 2006; Harris and Liddament, 2004; Kuhl et al., 2011; Laguette et al., 2011). Unknown host defences are a tantalising subject for reasearch and possible targets for viral drug therapies.

Retroviruses have been with mammals for a very long time. Over time the cells of mammals have evolved defenses against the viruses, the cellular host restriction factors. In response the viruses have evolved a variety of different ways to circumvent the host restriction factors, but those defenses are normally specific for the restriction factor of their own host, and don't work against related defenses of other host species and thus restrict the virus host range.

1.7 The APOBEC family

HIV-1 viruses lacking a functional *vif* gene were known to be severely restricted in replication in some cell lines, termed non-permissive, while not in others, called permissive. Heterokaryon analysis of permissive and non-permissive cells fused together showed that the non-permissive phenotype was dominant, suggesting that non-permissive cells expressed an unknown host restriction factor that could restrict HIV-1 without *vif* (Madani and Kabat, 1998). The unknown host restriction factor causing hypermutations in proviral DNA was identified as CEM15 (Sheehy et al., 2002), now known as APOBEC3G (A3G). This protein is a member of the APOBEC family of cytosine deaminases that include AID, APOBEC1, APOBEC2, seven APOBEC3 genes and APOBEC4 in humans (Reviewed in (Goila-Gaur and Strebel, 2008)). The family includes many proteins and they are numerous in mammals, although the number of genes is different between species. This chapter will only deal with the APOBEC3 genes. All the A3 genes are found between CBX6 and CBX7, on chromosome 22 in humans and they are highly conserved among vertebrates. The APOBEC3 genes of sequenced

mammals (Figure 7) have been duplicated and lost numeruous times leading to a great diversity in number of A3 genes in mammals, the mouse having only a single A3 gene while humans have seven (LaRue et al., 2008). The locus seems to have been under positive selection for a very long time.

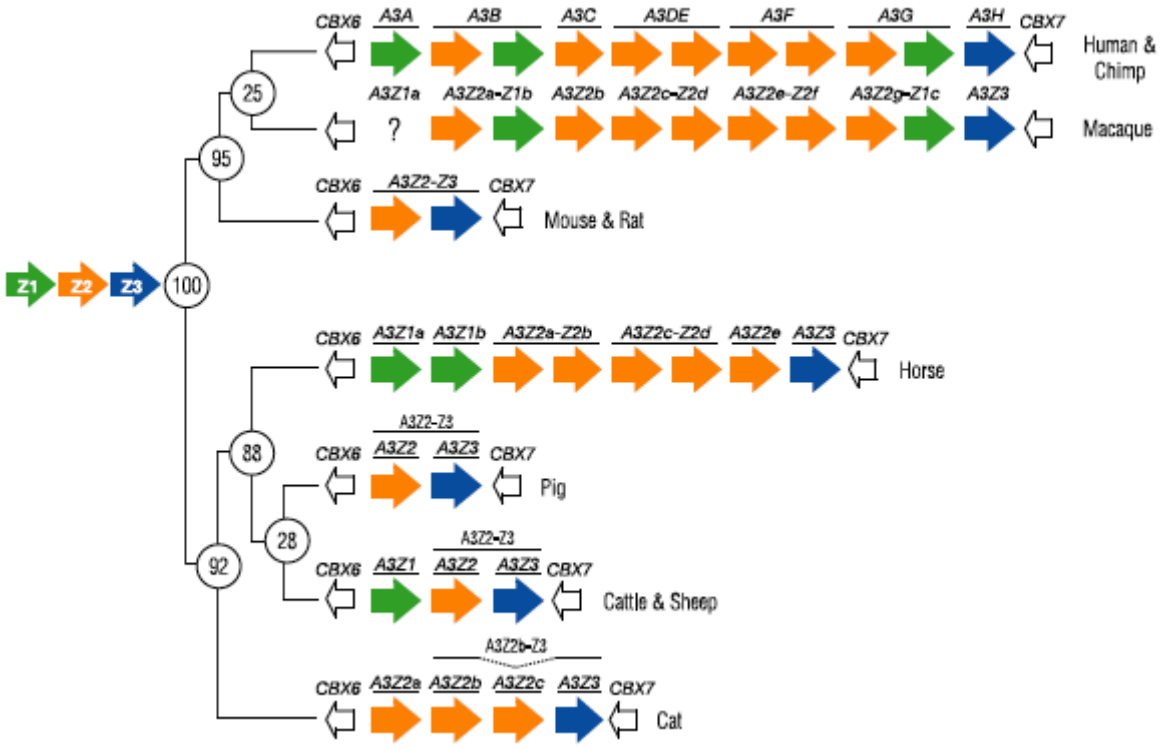


Figure 7. The APOBEC3 genes from mammals that have been sequenced. Numbers at the phylogenetic branch points indicate the approximate time in millions of years since the divergence of the ancestors of the present day species. Figure from (LaRue et al., 2009).

All known A3 genes have either a single zinc-binding domain (the catalytically active domains of three types, Z1, Z2 and Z3), or two zinc-binding domains with any of the three types (LaRue et al., 2009). Generally only one domain is catalytically active in A3s with two zinc-binding domains and the other one is involved in binding nucleic acids and in virus encapsidation (Reviewed in (Goila-Gaur and Strebel, 2008)). Nonprimate APOBEC3 genes and proteins were suggested to be named after the zinc-binding domains with an added prefix of A3 for APOBEC3 to guard against false implications of orthology between certain human A3 genes and those found in other mammals. (LaRue et al., 2009). This naming convention is used in Figure 7 and will be used in this thesis.

The A3 proteins are cytosine deaminases that target the viral single stranded cDNA synthesized during reverse transcription. A3 proteins work by being packaged into Vif deficient viral particles in non-permissive cells. The virions then bud from the cell. When the new virus infects another cell the A3 protein causes G-A hypermutations in viral DNA (Mangeat et al., 2003), leading to destruction of viral gene function. An overview of the effect of APOBEC3G on HIV-1 with or without vif can be seen in Figure 8.

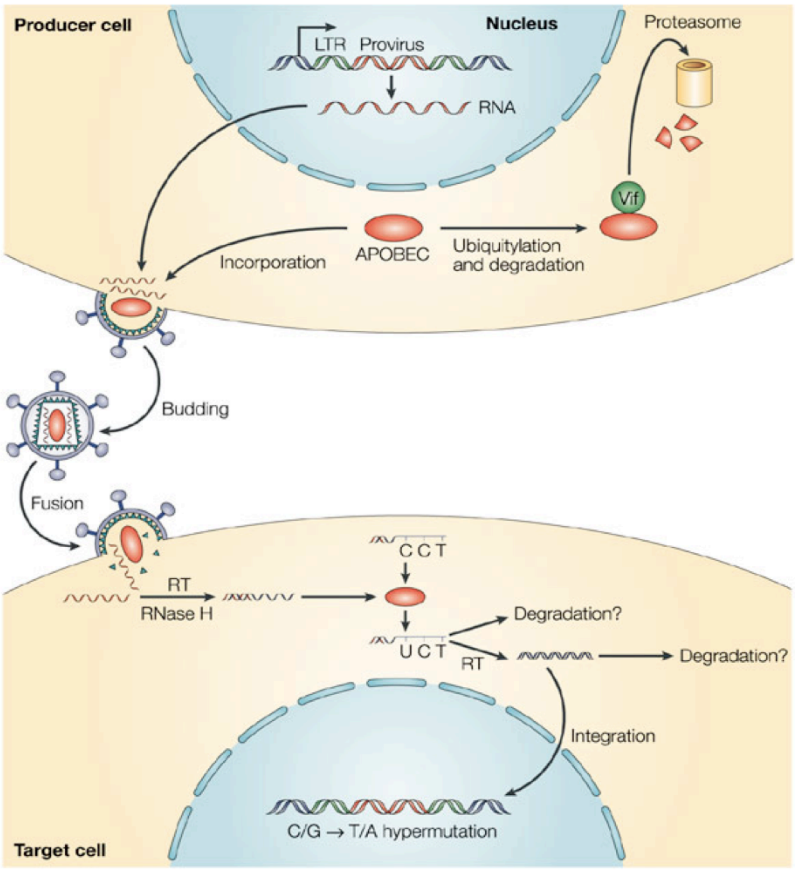


Figure 8. The effect of APOBEC3G on HIV-1. If HIV-1 virus with Vif infects a producer cell, Vif binds to A3G and targets it for proteosomal degradation. If the HIV-1 virus is Vif-deficient, A3G is packaged into budding virions, and when the virus reaches a target cell A3G deaminates cytosine residues into uracil during reverse transcription of the viral RNA into the first DNA strand, yielding C/G to T/A hypermutations that affect virus viability. Figure from (Harris and Liddament, 2004).

Not all A3 proteins have antiviral activity (Table 1), human A3G and A3F are the most potent, and human A3B even limits infectivity of HIV-1 in the presence of Vif, and A3F is also resistant to Vif, although not as strongly as A3B (as reviewed in (Harris and Liddament, 2004; Huthoff and Towers, 2008; Takeuchi and Matano, 2008)). The different A3s show differences in which cytosine they preferentely deaminate, A3G shows a nearly exclusive preference for 5'-C<u>C</u>, A3F and A3B for 5'-T<u>C</u>, the underlined residue preferenced for deamination, as reviewed in (Harris and Liddament, 2004). On the other hand, if a virus has Vif then it binds to a cullin-RING E3 ligase complex, taking the place of a protein with a SOCS-box, and binds to A3, and causes it to be polyubiquitinated, and therefore degraded in the proteasome.

A single amino acid at residue 128 in human A3G controls the switch of species specificity from sensitivity to HIV-1 Vif and insensitivity to SIV_{agm} Vif to insensitivity to HIV-1 Vif and sensitivity to SIV_{agm} Vif (Schröfelbauer et al., 2004). HIV-1 Vif strongly counteracts both human A3G and A3F (Sheehy et al., 2002; Wiegand et al., 2004; Zheng et al., 2004) although transiently expressed A3F might only show antiviral effects, but not stably expressed A3F because of different levels of expression. A3F might be catalytically less active than A3G. Vif also inhibits packaging of A3G more

potently than A3F, suggesting that Vif can inhibit A3G packaging via degradation-dependent and degradation-independent mechanisms, but only prevent A3F packaging via a degradation-dependent process (Miyagi et al., 2010).

Table 1. Antiviral activity against HIV-1 and sensitivity to Vif of human A3s.

A3	Antiviral activity against HIV-1	Sensitivity to Vif
A3A	No	
A3B	Yes	No
A3C	Weak. Active against SIV	
A3DE	Yes. Active against SIV_{agm}, SIV_{mac}	Yes
A3F	Strong	Partly resistant. Sensitive to some HIV-1 Vif alleles
A3G	Strong	Sensitive to most HIV-1 Vif alleles
A3H A3H-hapI A3H-hapII A3H-hapIII A3H-hapIV	Yes Yes Yes No No	 No. SIV Vif sensitivity Yes. SIV Vif sensitivity

Human A3F is also incorporated into virions and has deaminating antiviral activity against HIV-1. HIV-1 and HIV-2 Vif proteins suppress A3F (Wiegand et al., 2004). A3DE was also reported to inhibit the replication of HIV-1, SIV_{agm} and SIV_{mac} and to be encapsidated but it was also polyubiquitinated by a Cul5 E3 ubiquitin ligase, mediated by Vif. Sheep (*Ovis aries*) and cattle (*Bos taurus*) each have three A3 genes, A3Z1, A3Z2 and A3Z3 and pigs (*Sus scrofa*) have two, A2Z2 and A3Z3, having lost the A3Z1 gene. All species additionally code for a double-domain protein (LaRue et al., 2008). The double domain artiodactyl A3 proteins of sheep, cattle and pigs named OaA3Z2-Z3, BtA3Z2-Z3 and SsA3Z2-Z3 respectively, are fully resistant to HIV-1 Vif and cause G-A mutations. The deaminase activity of these proteins lies in the N-terminal Z domain (Z2) but not in the C-terminal domain as in several human double domain A3 proteins (Jónsson et al., 2006). Human A3G can inhibit porcine endogenous retrovirus (PERV) zoonosis from pig to human cells in a deamination-independent way while porcine A3 is inactive against it (Jónsson et al., 2007).

The Vif proteins of each lentivirus are normally very constricted in their ability to counteract A3 proteins, since Vif proteins usually only work against the A3 proteins of their host species. MVV Vif strangely has very broad activity against A3 proteins with a Z3 type domain, not only degrading ovine A3 but also the A3s of cows and cats, and partially degrading human and rhesus A3s (LaRue et al., 2010).

Human A3H proteins have 4 haplotypes, but only two that have any antiviral activity (Li et al., 2010), haplotypes I and II with haplotype II having the highest antiviral activity and sensitivity to Vif (reviewed in (Kitamura et al., 2011)). The human A3H is truncated because of a premature stop codon but when expressed at the full length it blocks HIV-1 and SIV (Dang et al., 2008; Harari et al., 2009) and is counteracted by Vif if it is of haplotype II (Binka et al., 2012; LaRue et al., 2010). A3H of haplotype I is not expressed as highly as haplotype II but when expression is increased it has

comparable antiviral activity to haplotype II but is not sensitive to Vif (Zhen et al., 2010) except for HIV-2 Vif that neutralizes both haplotypes (Li et al., 2010).

Human A3G and A3F have strong antiviral activity against HIV-1, and A3B and A3DE where antiviral activity can be seen by overexpression in single-cycle infectivity assays. All of those A3s, except for A3B seem to be Vif sensitive, as reviewed in (Albin and Harris, 2010; Goila-Gaur and Strebel, 2008), and A3H is also Vif sensitive (Binka et al., 2012; LaRue et al., 2010). Both A3C and A3A seem unlikely to have any antiviral activity *in vivo*, A3C has only showed weak antiviral activity, while A3A needs to fuse to a helper protein to shuttle it into the viral core. Strangely, A3C is active against SIV. Sometimes other effects than deamination effects of A3 proteins are reported, but those might simply be caused by overexpression of A3, as reviewed in (Albin and Harris, 2010; Goila-Gaur and Strebel, 2008).

1.8 The TRIM family

The *Fv1* gene of mice is known to encode a restriction factor that caused post-entry, pre-integration restriction of murine leukemia virus (MuLV). The inhibitor was saturable with inactive virion particles and the target of restriction is the capsid protein. The protein is expressed at low levels, possibly explaining why its restriction is saturable with high levels of incoming virions. The protein has 60% homology to the capsid-like domain of the ERV-L family of endogenous retroviral Gag proteins (reviewed in (Bieniasz, 2003)). Residue 110 in the MuLV capsid controls restriction, both for Fv1 and Ref1, in human cells. The HIV-1 target of Lv1 is also found in the capsid, as reviewed in (Goff, 2004). The tripartite motif protein 5α (TRIM5α), formerly known as Ref1 in humans and Lv1 in primates, is part of the tripartite motif family that includes about 70 genes in humans. The role of most of them is unknown. Orthologs are found in many mammals. TRIM5α proteins show a related restriction to Fv1.

The structure of the tripartite motif consists of a RING domain, either one or two B-boxes and coiled coil (RBCC) domains that are characteristic for the TRIM family. The RING domain has E3 ubiquitin ligase activity. The coiled coil area causes the binding of TRIM into homo- or heterotrimers with related TRIM enzymes. The C-terminal PRY/SPRY domain recognizes molecular patterns of virus capsids entering a cell, and leads the RING-Bbox area of TRIM5 to viral particles and inactivates them. Differential splicing of mRNA leads to different TRIM5 isomers but only TRIM5 with PRY/SPRY has viral restriction activity (Huthoff and Towers, 2008). This domain should be called B30.2, as it is a SPRY domain with an incorporation of a PRY domain, leading to different structure and function, and it seems to have been selected for after the emergence of the adaptive immune system. The B30.2 domain seems to have a broad role in innate immune recognition of retroviruses (Rhodes et al., 2005). TRIM5α_{rh} shows potent restriction of HIV-1 (Keckesova et al., 2004; Stremlau et al., 2004) while the human TRIM5α doesn't work against HIV-1. There is evidence supporting that TRIM5α causes fast uncoating of the core of a virion (Figure 9), disrupting the reverse transcription and causing restriction in the beginning of the viral replication cycle (reviewed in (Emerman, 2006)).

TRIM5α attacks the CA of the viral capsid, but the SPRY area only recognizes polymers of CA, like in the complex surface of multimerized CA (Sebastian and Luban, 2005). It acts as a pattern recognition receptor specific for the retroviral capsid (Pertel et al., 2011).

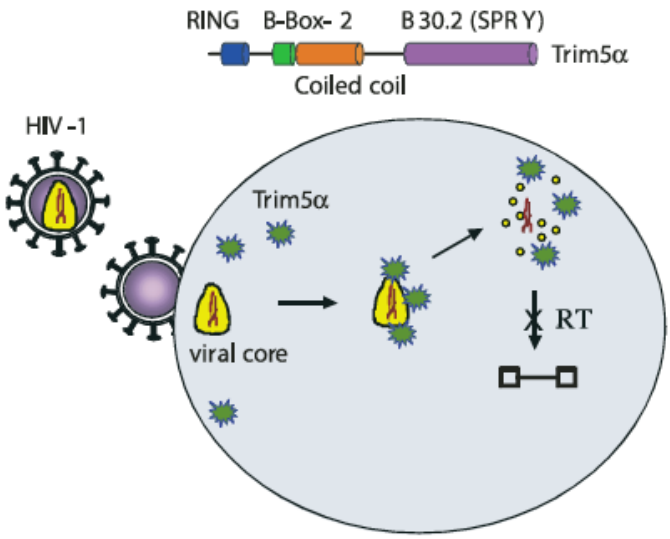


Figure 9. Structure of TRIM5α and proposed restriction activity against HIV-1. From the left: HIV-1 virion, virion envelope fuses with cell membrane. Capsid (yellow) enters cytosol, TRIM5α (green) binds to CA and leads to instability and breakdown of the core, inhibiting reverse transcription of viral RNA. Figure from (Emerman, 2006).

TRIM5α proteins from Old World monkeys can inhibit HIV-1 by binding specifically to the capsid, but human TRIM5α binds HIV-1 capsid more weakly, which explains the lower potency in blocking HIV-1 infection. Most New World monkey TRIM5α are unable to block HIV-1 infection and the protein doesn't associate with the capsid of HIV-1. Owl monkey TRIM5 is the exception, with a fusion protein of TRIM5-cyclophilin A (TRIMCyp) that can inhibit HIV-1, but the effect can be inhibited by cyclosporine, a protein known to inhibit cyclophilin A-HIV-1 capsid interaction (Stremlau et al., 2004). The coiled-coil and B30.2 domains are needed to associate with CA-NC complexes for restriction of HIV-1 by Old world monkey TRIM5α. The different levels of HIV-1 capsid interaction with TRIM5s from different species correlate with the ability of those TRIM5s to restrict HIV-1. Inhibiting the proteasome does not significantly abrogate restriction by TRIM5α_{rh} of HIV-1 (Campbell et al., 2008; Stremlau et al., 2006). HIV-1 can associate with high levels of TRIM5α_{rh} called cytoplasmic bodies. These cytoplasmic bodies contain ubiquitin but high concentrations were not found. Possible explanation for the lack of ubiquitination of the capsid observed could be that the TRIM5α is inserted or bound to the capsid, ubiquitinated and the TRIM5α degraded by the proteasome, putting strain on the capsid, causing it to fall apart (Campbell et al., 2008). That requires intact activity of self-ubiquitination of the molecule, possibly to remove TRIM5α when it forms hexagonal structures on the capsid surface (Lienlaf et al., 2011). Engagement of TRIM5α proteins by retrovirus cores from viruses that are susceptible to restriction from the TRIM5α causes the proteasomal-dependent degradation of the TRIM5α (Rold and Aiken, 2008). Cyclophilin A (CypA) binding to HIV-1 CA that can assist in HIV-1 replication in certain cells also leads to susceptibility to TRIM5α from Old world monkeys, possibly because of conformational changes caused by CypA binding CA in two places, between helices 4 and 5 and the helices 6 and 7. This restriction affects replication mostly after reverse transcription. The HIV-1 capsid is susceptible to both CypA-independent and CypA-dependent TRIM5α restriction (Lin and Emerman,

2008). TRIM5α$_{rh}$ and TRIM5α from two subspecies of African green monkeys, the tantalus and pygerythrus, forms trimers with the coiled-coil and B30.2 domains that are likely inserted into the capsid of incoming viral cores (Mische et al., 2005). Replacing arginine at residue 332 with proline in human TRIM5α confers ability to restrict HIV-1, as can be seen in Rhesus macaque and African green monkeys, but their TRIM5αs have proline at that residue and are resistant to HIV-1 infection (Yap et al., 2005).

In a mature HIV-1 virion the capsid forms a core shaped like a cone, surrounding the viral RNA and associated proteins. The CA is proteolytically cleaved from the Pr55^{Gag} polyprotein upon maturation. CA is composed of the N-terminal domain spanning residues 1-145 and the C-terminal domain with residues 151-231. The N-terminal domain functions in viral maturation and incorporating CypA to an exposed loop while the C-terminal domain contributes to Gag-Gag interactions (reviewed by (Freed, 1998)). The CypA protein binds to the loop between alpha helices 4 and 5 of N-terminal domain of CA (Bukovsky et al., 1997; Gamble et al., 1996) early after viral entry (Lin and Emerman, 2008).

TRIM5α and Fv1 operate independently and can compete for viral cores when co-expressed in human cells. Virus restricted by TRIM5α does not reverse transcribe so TRIM5α needs to recognize the capsid before reverse transcription, but Fv1 restricted virus is reverse transcribed normally, although the virions are eventually noninfectious. Fv1 recognized virus particles seem to be protected from TRIM5α (Passerini et al., 2006).

1.9 The ubiquitin-proteasome system

The eukaryotic cell often uses an ubiquitin-proteasome system to break down unneeded and misfolded proteins. This system consists of a few different steps: An E1 ubiquitin activating enzyme that activates ubiquitin, a small 8.5 kDa protein that consists of 76 amino acids. Ubiquitin has several highly conserved lysine residues, K48, K63, K6, K11, K27, K29 and K33. An E2 ubiquitin conjugating enzyme binds to the C-terminus of ubiquitin. The E3 ubiquitin ligase binds to both the substrate and E2, and catalyses the attachment of ubiquitin to the substrate from E2. Different signals can be given for which lysine residue is used to connect to the substrate and the number of ubiquitin conjugated. A poly-ubiquitin chain with at least four linked K48 residues used to give a signal for the breakdown of the protein in the 26S proteasome, which consists of a 20S center and two 19S ends. The proteasome can be found both in the nucleus and the cytosol and it breaks down proteins by proteolysis, driven by ATP hydrolysis. The attachment of ubiquitins also costs ATP (reviewed in (Deshaies and Joazeiro, 2009)).

Many proteins are known to have a SOCS-box (Suppressor of Cytokine signaling) domain at the C-terminal end. This domain seems to be involved in targeting proteins for ubiquitination through the so-called ECS-type (Elongin C-cullin-SOCS-box) E3 ubiquitin ligase family. The SOCS-box also involves a sub-domain called a BC-box. HIV-1 Vif mimics SOCS-box proteins, not by amino acid sequence, but by folding, and the BC-box is highly similar to the highly conserved SLQXLA sequence (Stanley et al., 2008).

Three of four HIV-1 accessory proteins interact with the cullin-RING finger E3 ubiquitin ligases (CRLs) of their host cells to induce polyubiquitination and degradation of cellular proteins.

The HIV-1 Vpr has been shown to interact with a $CRL4^{DCAF1}$ E3 ubiquitination ligase assembled from cullin 4A (CUL4A), RBX1, DDB1 (DNA damage-binding protein 1) and DCAF1 (DDB1- and CUL4-associated factor 1) leading to the polyubiquitination and proteosomal-dependent degradation of uracil-DNA glycosylase-2 (UNG2), that removes uracil lesions from DNA in the base excision repair pathway. The effect of UNG2 on HIV-1 infection is unclear but it has been suggested that it acts as an antiviral host cell factor (Ahn et al., 2010). HIV-2 or SIV Vpx (Sharova et al., 2008) counteracts the cellular host factor SAM domain HD domain-containing protein 1 (SAMHD1) that inhibits HIV-1 infection of dendritic cells and lessens infection of macrophages by preventing efficient viral cDNA synthesis. Vpx targets SAMHD1 for proteosomal degradation with a $CRL4^{DCAF1}$ E3 ubiquitin ligase with DDB1 (Ahn et al., 2012; Hrecka et al., 2011; Laguette et al., 2011).

1.10 Vif (virion infectivity factor)

The auxilliary genes of lentiviruses often protect the virus against host restriction factors. One of these genes is *vif* (virion infectivity factor), which is found in all lentiviruses except EIAV. The amino acid sequence similarity of Vif from different lentiviruses is rather limited, except for the highly conserved SLQXLA region in the C-terminal half, which is necessary for Vif function. The SLQXLA region is at amino acids 145-151 in HIV-1 and at 174-179 in MVV (Oberste and Gonda, 1992).

Cells can be designated permissive or non-permissive for growth of Δ*vif* lentiviruses. Δ*vif* viruses can grow in permissive cells but not in non-permissive cells. This is because of difference in expression of A3 genes. Vif is essential for viral replication in non-permissive cells (von Schwedler et al., 1993). In non-permissive cells Vif is degraded rapidly by cellular proteases. High levels of Vif can inhibit viral infectivity independently of cell-type and is associated with accumulation of Gag-processing intermediates (Akari et al., 2004).

Δ*vif* MVV (Kv1772 Δ*vif*) grows poorly in SCP or macrophages (which are the natural target cells of the virus) and it leads to high levels of A->G mutations. MVV needs functional Vif for *in vivo* infections and infecting non-permissive cells *in vitro* (Kristbjörnsdóttir et al., 2004). CAEV Vif expressed in goat cells can increase infectivity of Vif defective HIV-1 (Seroude et al., 2002). Vif is relatively unstable with a half-life of about 30 min and is degraded by cellular proteasomes (reviewed in (Goila-Gaur and Strebel, 2008)). A substantial fraction of Vif is membrane associated and colocalizes with HIV-1 Gag (Tang et al., 1999).

HIV-1 Vif tends to form multimers and the PPLP domain is necessary for this. Vif mutants without the PPLP domain cannot rescue infectivity of *vif*-defective HIV-1 in non-permissive H9 T-cells (Yang et al., 2001) although that is probably because of A3 proteins.

The main function of Vif is to counteract the cytosine deaminase APOBEC3 (A3) proteins of host cells that would otherwise cause G-A hypermutations in viral DNA. Vif works by sending the A3 protein to the proteasome to be broken down (Figure 8). This is achieved by recruiting an E3 ubiquitin ligase to the A3 proteins and thereby inducing their degradation in the proteasome. E3 ubiquitin ligases with HIV-1 Vif are composed of Vif - Elongin C- Elongin B - Cullin 5 - Rbx2 (Cul5 ubiquitin ligase) (Yu et al.,

2003) where the viral BC-box (SLQYLA sequence) binds to the ubiquitin ligase and the cullin box of Vif is critical for interaction between Vif and Cul5 (Figure 10). The cullin box for cellular proteins consists of ΦXXΦXXXXXΦXXXΦ amino acids where Φ denotes a hydrophobic amino acid (Figure 10B) (Stanley et al., 2008). The primate lentiviral Vif proteins assemble with a Cul5-E3 ligase through an $Hx_5Cx_{17-18}Cx_{3-5}H$ zink-binding motif (Luo et al., 2005). The SOCS-box motif of HIV-1 Vif (Figure 10B) mediates interaction with Elongin C and is necessary but not sufficient for interaction with a Cul5 ubiquitin ligase. Residues upstream of the BC-box are necessary for binding with Cul5 but not Elongin C and might have something to do with the preferred recruitment of Cul5 over Cul2 for HIV-1 Vif (Yu et al., 2004). Once the BC-box of vif has bound to Elongin C the PPLP motif in the cullin box interacts with Elongin B, and this interaction, with the interaction of HCCH motif with Cul5 is necessary for the assembly of a functional Cul5 ubiquitin ligase (Bergeron et al., 2010). Both HIV-1 Vif and SIV Vif recruit the transcription cofactor CBF-β to the Cul5 ubiquitin ligase to break down A3G and CBF-β seems to stabilize Vif or be required for folding of Vif in cells and be required for binding A3G (Jäger et al., 2012). Further research has shown that both human isotypes of CBF-β stabilize vif and increase viral infectivity, and that CBF-β leads to increased breakdown of all human A3s that are sensitive to HIV-1 vif, A3C, A3DE, A3F, A3G and A3H haplotype II, and lessens viral packaging of A3DE, A3F, A3G and A3H while increasing viral infectivity. This also happens with SIV Vif so CBF-β interaction seems to be conserved and essential for Vif function (Hultquist et al., 2012). Deletions of the BC-box or the PPLP motif of HIV-1 can lead to a dominant negative effect of A3G breakdown, probably because of competitive binding to Cul5 E3 ubiquitin ligases or A3G (Walker et al., 2010).

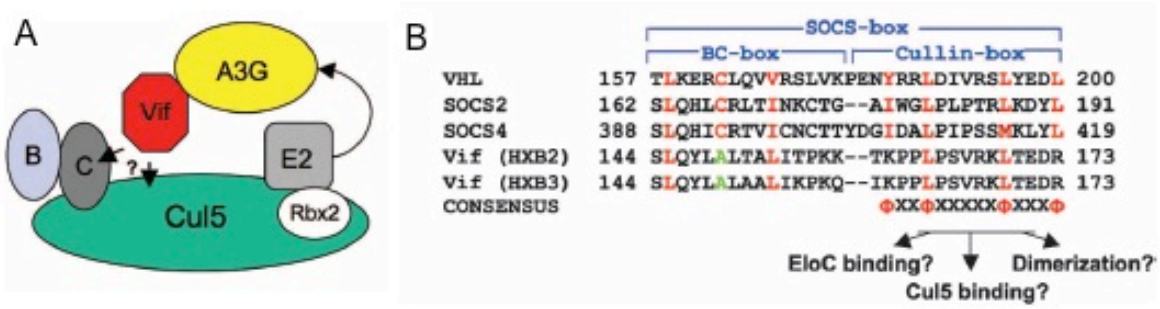


Figure 10. Vif-Cul5 E3 ubiquitin ligase and sequence alignment of SOCS-box proteins. A) Schematic depiction of the E3 ubiquitin ligase Vif forms with Elongin C, Elongin B, Cul5 and Rbx2 that polyubiquitinates A3G. B) Alignment of HIV-1 Vif SOCS-box with cellular SOCS-box protein sequences shows conserved hydrophobic amino acids in red. An alanine in Vif that replaces a conserved cysteine in other SOCS-box proteins is green. Figure from (Stanley et al., 2008).

FIV Vif also forms a Cul5 ubiquitin ligase although it lacks the HCCH motif, using the BC-box to interact with ElonginC and recruiting ElonginB and Cul5. FIV Vif means of interaction with Cul5 are unknown (Wang et al., 2011).

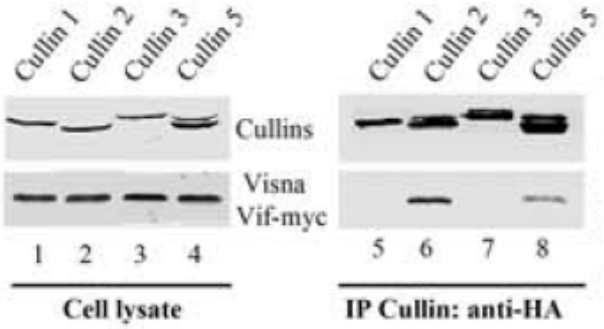


Figure 11. MVV Vif interacts with Cul2 but only weakly with Cul5. Immunoprecipitation of cell lysates from 293T cells cotransfected with different cullins and MVV vif. Figure from (Luo et al., 2005), supplementary data.

The MVV Vif also lacks this HCCH motif and there is evidence to suggest that MVV Vif interacts with Cul2 rather than Cul5 (Luo et al., 2005)(Figure 11). This suggests that MVV Vif hijacks Cul2 and composes Cul2 ubiquitin ligase with Vif - Elongin C - Elongin B - Cullin 2 - Rbx-1 to degrade A3 proteins. Cellular proteins that bind Cul2 have a cullin box with ΦPXXΦXXXΦ amino acid sequence (Mahrour et al., 2008). A putative effect of MVV Vif in a Cul2 ubiquitin ligase can be seen in Figure 12a where Vif binds to OaA3, EloCB complex and Cul2, leading to polyubiquitination of OaA3. Effects of two mutations can also been seen, SLQ-AAA mutation in the BC-box that disrupts binding to the EloCB complex and leads to no ubiquitination of OaA3 (Figure 12b) and then the expected effect of disrupting a putative Cul2 binding se by IP-AA mutations, expected to disrupt binding of Vif to Cul2 and therefore lead to no ubiquitination of OaA3 (Figure 12c).

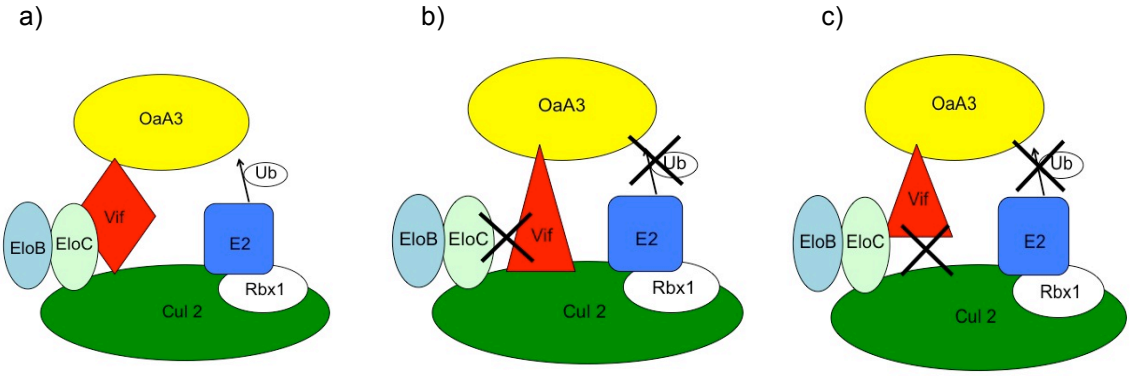


Figure 12. Putative effect of MVV Vif in a Cul2 ubiquitin ligase complex. a) effect of WT Vif b) effect of $Vif_{SLQ-AAA}$ c) effect of Vif_{IP-AA}.

Neutralization of A3 proteins by Vif may not always be complete, and this could lead to hypermutations in the viral genome which might serve as a reservoir of genetic diversity and contributing to drug resistance and viral immune evation in HIV-1 (Albin and Harris, 2010).

Several lines of evidence indicate that A3 degradation is not the sole activity of Vif. Vif deficient HIV-1 had reduced ability for reverse transcription and Vif associated with viral RNA in infected cells and *in vitro* (Dettenhofer et al., 2000). HIV-1 Vif also acts early after infection as a part of the reverse transcription complex (RTC) as an accessory factor for reverse transcription and viral infectivity (Carr et al., 2008). Interaction between HIV-1 Vif and the $Pr55^{Gag}$ precursor protein was observed, but no

interaction between Vif and the mature capsid protein (Bouyac et al., 1997). However, HIV-1 Vif has been found to increase the stability of virion cores (Öhagen and Gabuzda, 2000).

CA(L120R)-Vif(P205S) is a Kv1772 (wt) strain of MVV with two mutations that cause attenuated growth in macrophages, T1277G and C5578T that lead to a L120R mutation in CA and P205S in Vif (Figure 13) This virus strain will be called CA-Vif hereafter.

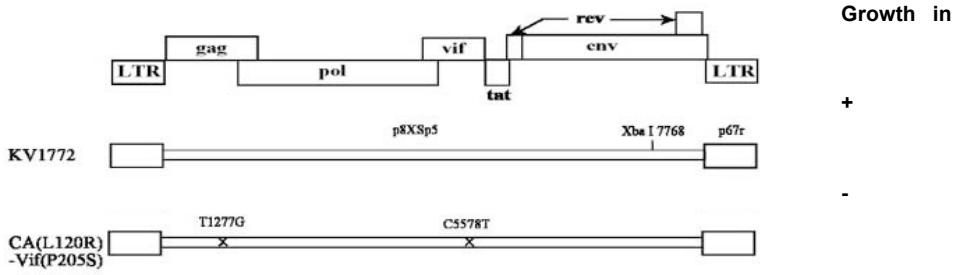


Figure 13. Mutations in CA-Vif compared to kv1772 (wt) and difference in growth in macrophages. Figure from (Gudmundsson et al., 2005).

When Kv1772 viruses, wt or with either CA or Vif mutations and CA-Vif virus were used to infect sheep choroid plexus cells and macrophages, the CA mutation affected replication in macrophages somewhat but the Vif mutation did not affect growth in macrophages. The CA-Vif virus had very reduced growth in macrophages compared to Kv1772 (Figure 14a). None of the mutations affected growth in sheep choroid plexus cells (Figure 14b) (Gudmundsson et al., 2005). There were no G-A mutations associated with the replication of CA-Vif in non-permissive cells (macrophages and fetal ovine synovial (FOS) cells), indicating that this mutation in Vif describes a different function of Vif than causing A3 degradation. The cell-dependent effect of these double mutations suggests the involvement of a host factor either in the replication of the CA-Vif mutant in sheep choroid plexus cells or a restriction factor that is present in macrophages and fetal ovine synovial (FOS) cells.

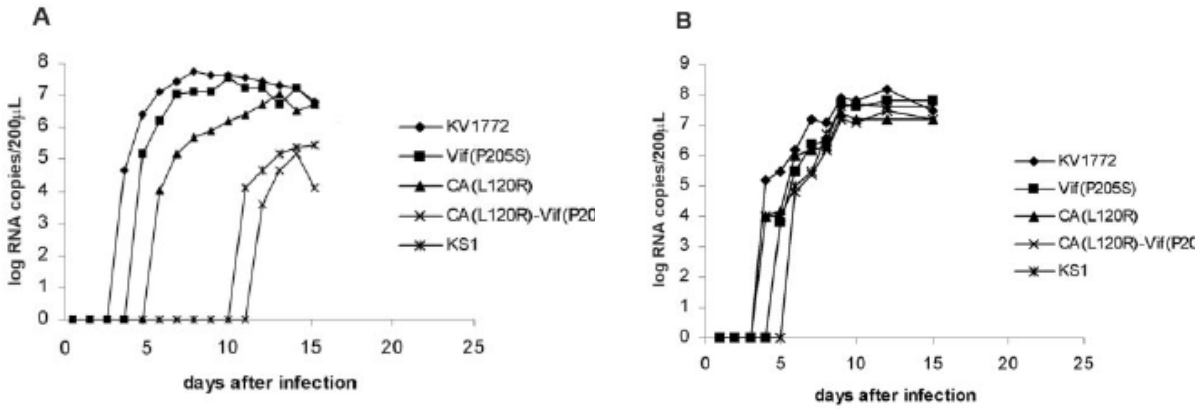


Figure 14. Replication kinetics of MVV with different varieties of vif. Growth in blood-derived macrophages a) and sheep choroid plexus cells b) measured by TaqMan-based real-time PCR. Figure from (Gudmundsson et al., 2005).

2 Aims

The long term aim of the study is to discover new strategies of antiviral defence and viral counterdefence

The specific aims of the study were twofold:

-To find out why the CA-Vif mutant (Figure 13) of MVV has attenuated replication in FOS cells compared to SCP cells, and investigate whether the attenuation is at the stage of reverse transcription.

-To investigate whether a putative Cul2 binding site (Figure 15) was necessary for breakdown of OaA3Z2-Z3 and efficient replication in vitro in the presence of OaA3Z2-Z3.

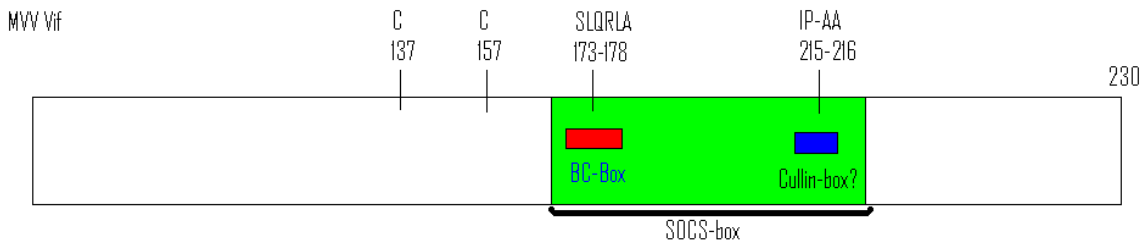


Figure 15. Putative Cul2 site in Cullin-box and mutations to it.

3 Materials and methods

3.1 Molecular clones

The molecular clone kv1772 (formerly **Kv1772, kv72/67**) is derived from a plaque-purified biological clone of visna virus strain Kv1772, selected for neurovirolence by serial passages of strain K1514 in sheep (Andrésson et al., 1993). The proviral DNA is kept in two subclones, p8XSp5-RK1 and p67f in pBluescript II SK+ plasmids (Stratagene).

p8XSp5-RK1: 5' visna molecular clone with a *Stu*I site in a 296 bp sheep genomic flanking sequence from the 5' long terminal repeat of the virus through to the *Xba*I site at position 7768 (Skraban et al., 1999).

p67f: 3' visna molecular clone with kv1772 sequence from the *Xba*I site at position 7768 to the end of the LTR.

CA-Vif: p8XSp5-RK1 plasmid with C5578T and T1277G substitutions. This leads to **L120R** in **CA** and **P205S** amino acid substitution in Vif (Gudmundsson et al., 2005).

When designing the IP-AA mutation in the *vif* gene, visna molecular clone p8 was used. This is a pUC19 plasmid with a kv1772 sequence from 4587 (*BamH*I) to 6392 (*Hinc*II) from 8XSp5.

3.1.1 Primers

Mutagenic primers were designed from kv1772 sequences and sequences for codonoptimized vif. Other primers were previously designed by others. All primers used are listed in Appendix I.

3.1.2 Construction of kv1772 clones with IP-AA mutation in the *vif* gene

A clone with IP-AA amino acid substitutions in the vif gene was constructed.

kv1772*vif gene*

Atgttaagttcataccgccaccaaaagaaatacaaaaagaataaagcaagggagataggaccccagttgccacta
tgggcatggaaagaaacagcatttagtataaatcaggaaccctattggtatagtaccataaggctacaagggttg
atgtggaataaaagagggcataaacttatgtttgtaaaagaaaaccaagggtatgagtattgggaaacatcagga
aaacagtggaaaatggagataagacgagatttggatctgatagcccaaataaattttagaaatgcatggcaatat
aaaagccagggagaatggaaaacaataggggtctggtatgaatcaccaggggattacaagggaaaagagaatcag
ttttggttccattggagaatagctctctgcagctgtaacaaaacaaggtgggatatacgggaattcatgataggg
aagcataggtgggatttatgtaaatcgtgtatacaaggggagatagttaagaatacaaatccaagaagcttacaa
cgcttagctttattgcacctagcaaaagaccatgtatttcaagtaatgccattgtggagagccaggagagtcaca
gtgcagaagtttccatggtgtcgtagcccaatgggttacacg**atacct**tggtctctgcaggaatgctgggaaatg
gaatccatctttgaataa

kv1772vifAA gene

Atgttaagttcataccgccaccaaaagaaatacaaaaagaataaagcaagggagataggaccccagttgccacta
tgggcatggaaagaaacagcatttagtataaatcaggaaccctattggtatagtaccataaggctacaagggttg

atgtggaataaaagagggcataaacttatgtttgtaaaagaaaaccaagggtatgagtattgggaaacatcagga
aaacagtggaaaatggagataagacgagatttggatctgatagcccaaataaattttagaaatgcatggcaatat
aaaagccagggagaatggaaaacaataggggtctggtatgaatcaccaggggattacaagggaaaagagaatcag
ttttggttccattggagaatagctctctgcagctgtaacaaaacaaggtgggatatacgggaattcatgatagggg
aagcataggtgggatttatgtaaatcgtgtatacaaggggagatagttaagaatacaaatccaagaagcttacaa
cgcttagctttattgcacctagcaaaagaccatgtatttcaagtaatgccattgtggagagccaggagagtcaca
gtgcagaagtttccatggtgtcgtagcccaatgggttacacg**gcagct**tggtctctgcaggaatgctgggaaatg
gaatccatctttgaataa

In vitro site-directed mutagenesis was performed with Phusion site-directed mutagenesis method. This method is performed by using Phusion® Hot Start high-fidelty (HF) DNA polymerase that has proofreading activity (3' → 5' exonuclease activity). Two long oligonucleotide primers (24-30 nt) that both contain the desired mutations (1772VifAA forw and 1772VifAA rev) in the center of the sequence and have a Tm of 62-69°C calculated by the nearest-neighbor method anneal to the same sequence on opposite strands of the plasmid were used with non-mutagenic primers (V5094 and V-5951) about 500 bp away in a PCR reaction with molecular clone p8.

Temperature cycling:

1) 98°C for 30 sec 2) 98°C for 10 sec 3) 65°C for 30 sec 4) 72°C for 2 min and 15 sec 5) 72°C for 10 min 6) 4°C on hold. Steps 2-5 were repeated 24 times.

Extension of the primers generates two PCR bands containing the mutation (Figure 16). The bands were run on agarose gel and then gel extracted. Both bands were used as template for PCR with the non-mutagenic primers. Band and p8 were cut with the restriction enzymes *Nco*I and *Bgl*II and ligated together. Then p8XSp5-RK1 and p8 with mutation were cut with *Mlu*I and *Bgl*II and the mutated part of p8 inserted into p8XSp5-RK1 by ligation. Mutated p8XSp5-RK1 and p67f were both cut with the restriction enzyme *Xba*I and ligated together. The ligation mixture was transfected into FOS cells with Lipofectamine 2000 and kept for several days for virus to be released into the medium.

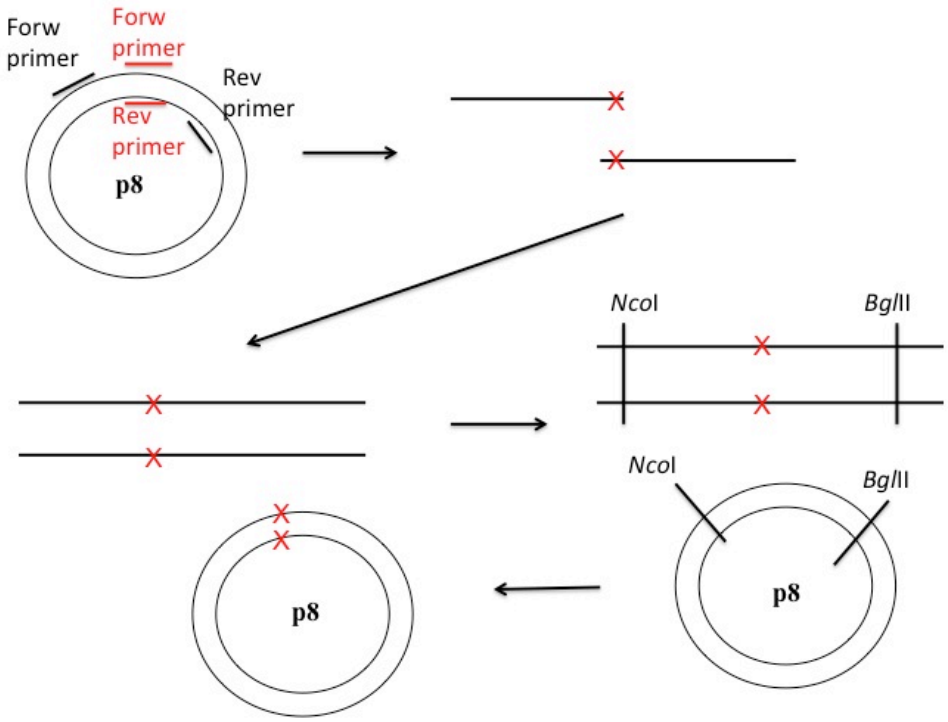


Figure 16. Site-directed mutagenesis. Mutagenic primers and mutations are shown in red. Phusion site-directed mutagenesis was done by two pcr runs on p8, each with one mutagenic primer and one normal. Gel extracted bands were used in a pcr with two normal primers to yield a double stranded band of DNA with mutation. The gel extracted dsDNA band and unmutated p8 were then cut with *Nco*I and *Bgl*II and ligated together to yield mutated p8.

3.1.2.1 Restriction enzyme digestion

P8 and a mutated PCR band between the primers V5094 and V-5951 were both digested with the restriction enzymes *Nco*I and *Bgl*II. The digestion was kept at 37°C for 3 hours and inactivated at 65°C for 20 min.

3.1.2.2 Ligation

The restriction enzyme digested p8 (2 μl) and mutated PCR band (6 μl) were mixed in a 10 μl ligation mixture with 1X T4 ligase buffer and 5 u T4 ligase (Fermentas, EL0011) and kept at 16°C overnight, and then the ligation mixture was used in heat-shock transformation of bacterial cells.

3.1.3 Codonoptimized clones

Vif: codonoptimized wt MVV vif in pVR1012 with either a myc or HA tag.

Vif_{SLQ}: codonoptimized MVV vif in pVR1012 with SLQ-AAA mutation, with either a myc or HA tag.

$Vif_{IP\text{-}AA}$: codonoptimized MVV vif in pVR1012 with IP-AA mutation, with either a myc or HA tag. Primers containing mutations (VifAAforw and VifAArev) and other non-mutagenic primers (vifopti1forw and vifopti2rev) used are found in Appendix I.

3.2 Cells and cell cultures

The cells used in this study were monolayers of **sheep choriod plexus** (**SCP**) cells, **fetal ovine synovial** (**FOS**) cells and **293T (human embryonic kidney)** cells.

When cells were not in use they were kept in liquid nitrogen tanks in aliquots for T75 culture flasks. To take cells from tanks first 10% growth medium fitting for the cell type was put into a new culture flask and then the cells were removed from the tank and gently thawed in a 37°C water bath. Thawed cells were immediately placed in a culture flask and the flask gently swirled and placed in a 37°C cell incubator.

To freeze cells from a T75 culture, the cells were trypsinized and loosened from the flask with serum-free medium, then the cells were placed in a sterile tube fitting for the total volume with a small amount of serum added (ca 1% of total volume), and centrifuged at 1000 rpm for 10 min at room temperature in a Sorvall table top centrifuge. The supernatant was gently poured off and the cells were resuspended in serum (lamb or fetal calf serum) and 10% dimethyl sulfoxide (DMSO), 1 ml in total for cells from each T75 culture flask. The cell mixture was then placed in 1.0 ml CryoTubes™ vials (Nunc) and kept in a refrigerator for 30 minutes, then placed in a small Styrofoam box filled up with cotton wool and placed in a -80°C freezer for at least a day before placing cells in liquid nitrogen tanks.

3.2.1 Cell culture of SCP and FOS cells

Monolayers of SCP and FOS cells were grown at 37°C in humidified atmosphere of 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) from Invitrogen supplemented with 2 mM glutamine, 100 IU/ml streptomycin, 100 IU/ml penicillin, and either 10% (growth medium) or 1% (maintenance medium) lamb serum.

3.2.2 Cell culture of 293T cells

Monolayers of 293T cells were grown at 37°C in humidified atmosphere of 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) from Invitrogen supplemented with 2 mM glutamine, 100 IU/ml streptomycin, 100 IU/ml penicillin, and either 10% (growth medium) or 1% (maintenance medium) fetal calf serum.

3.2.3 Transfection and inoculation of the virus

Transfection of DNA clones was performed using either liposome-mediated transfection or non-lipid cationic polymer mediated transfection.

3.2.3.1 Liposome-mediatated transfection

Transfection of DNA clones was performed by using Lipofectamine™ 2000 (Invitrogen). FOS, SCP or 293T cells were cultured in T25 flasks or 6-well plates (Nunc) until 90-100% confluent. Maintenance medium without antibiotics was added before transfection. Before transfection ligation mixtures were incubated at 60°C for 20 min to sterilize them. For transfection in T25 flasks 6 μg of DNA was used. The DNA was added to 500 μl of serum free Optimem medium and incubated for 5 min at room temperature. After incubation the diluted DNA was combined with Lipofectamine™ 2000 diluted in Optimem for a total volume of 1 ml and incubated for 30 min at room temperature. The mixture was

added to the cell medium and incubated at 37°C in humidified atmosphere of 5% CO_2. The next day the medium was removed and maintenance medium with antibiotics was added.

3.2.3.2 Non-lipid cationic polymer mediated transfection

Transfection of DNA clones was performed by using TransPass™ COS/293 Transfection reagent (New England Biolabs). 293T cells were cultured in 6-well plates (Nunc) until 50% confluent. DMEM medium without antibiotics and 5% fetal calf serum was added before transfection. Before transfection ligation mixtures were incubated at 72°C for 14 min to sterilize them. Up to 3 µg of DNA were added to 250 µl of serum free DMEM without antibiotics and 6 µl of TransPass and incubated for 20-30 min at room temperature. The mixture was added to the cell medium and incubated at 37°C in humidified atmospere of 5% CO_2. Medium was not changed for 48-72 h.

3.2.3.3 Inoculation/infection with virus

Inoculation with virus was done in 100% confluent FOS or SCP cells in T25 flasks, 12- or 24-well plates. The growth medium was removed, cells rinsed with PBS and 1-5 ml of maintenance medium added. Virus was then added and the flask or plate gently moved to disperse virus. Cells were incubated at 37°C in humidified atmospere of 5% CO_2. Maintenance medium was changed the day after.

3.2.3.4 Titration of virus from supernatant

Titrations were performed with monolayers of SCP or FOS cells grown in 96-well flat-bottomed tissue culture plates from NUNC. Samples were thawed and tenfold dilutions were made with 20 µl of supernatant and 180 µl of DMEM maintenance medium. One hundred µl of each dilution were added to quadruplicate wells of the tissue culture plates. Control wells recived 100 µl of maintenance medium. All wells contained 100 µl of maintenance medium before addition of virus dilutions. Plates were incubated at 37°C in humidified atmosphere of 5% CO_2 for 4 weeks. Cytopathic effects were monitored after 7, 14, 21 and 28 days with a light microscope. Calculations of tissue culture infectivity dose ($TCID_{50}$) titer were done by the Reed-Muench method, as reviewed in (Flint et al., 2004).

3.2.4 Green fluorescence microscopy

A DM IL LED inverted microscope (Leica) with a DFC425 C color microscope camera and a mercury-vapour lamp were used to inspect cells transfected with enhanced green fluorescence protein (eGFP), which has a major excitation peak at 490 nm and an emission peak at 509 nm. Cells were inspected by eye in the microscope and pictures taken when wished.

3.2.5 HIV-infectivity assay

Human 293T cells cultured in Dulbecco modified Eagle medium (DMEM) were supplemented with 10% fetal bovine serum (FBS) and 0.5% penicillin-streptomycin. Cells were transfected (Lipofectamine™ 2000; Invitrogen) at 90% confluence with 1 µg HIV-green fluorescent protein (GFP) cocktail [0.44 µg of CS-CG (Miyoshi et al., 1998), 0.28 µg pRK5/Pack1 (Gag-Pol), 0.14 µg pRK5/rev,

0.14 μg pMDG (vesicular stomatitis virus G protein [VSV-G]-Env)], 100 ng of codon-optimized *vif* or empty vector, and 250 ng of A3 or empty vector. All experiments were performed in triplicate.

After 48-h supernatants containing virus were harvested and any remaining producer cells were removed by purification by centrifugation. Producer cells were processed for Western analysis to measure protein expression levels. Purified supernatants were placed on fresh 293T target cells. Target cells were harvested and infectivity (GFP) was measured by flow cytometry (FACS Aria II) after 72 h.

3.2.5.1 Fluorescence-activated cell sorting (FACS)

Target cells to be measured by FACS were trypsinized with 250 µl of trypsin and then the trypsin was inactivated by 2 ml of medium. Cells in medium (800 µl) were placed in a sterile 1.5 ml eppendorf tube, then centrifuged at 3000 rpm at 4°C for 2 min, supernatants poured off and cells were washed twice with ice-cold PBS. Finally cells were gently resuspended in 250 µl of ice-cold PBS and kept on ice under aluminum sheet to protect them from light. 250 µl of fixative (Appendix III) was mixed with cells and kept on ice under aluminum sheet for 20 min, then spun for 1 min and the supernatant removed and the cells gently resuspended in 500 µl of ice-cold PBS. The cells were analyzed with an FACS Aria II flow cytometer (Matís).

3.3 DNA and RNA methods

3.3.1 DNA quantification

DNA samples were measured in a NanoDrop® ND-1000 Spectrophotometer from NanoDrop Technologies Inc. according to protocol.

3.3.2 Isolation of DNA

Viral DNA was isolated from infected cells by removing the medium from the cells, washing them with PBS and placing Cell lysis solution (Gentra puregene kit) with proteinase K (290 µl Cell lysis solution and 10 µl proteinase K for each well in a 12-well plate, half for 24-well plate) on the cells, then pipetting the cells up and down several times and placing the lysed solution in 1.5 ml Eppendorf tubes. Tubes were kept at room temperature for at least 24 h. Then 100 µl Protein Precipitation was placed on the lysed solution and vortexed vigorously for 20 sec. The mix was then centrifuged at 16000 x *g* for 1 min. 300 µl of isopropanol was placed in a clean 1.5 ml Eppendorf tube and supernatant from centrifuged mix poured carefully into it and mixed by inverting the tube 50 times. The mix was then centrifuged at same speed for the same time as before, and the supernatant poured off and the tube drained by inverting on clean absorbent paper. 300 µl of 70% ethanol was used to clean the DNA pellet by inverting several times, then centrifuged again. The supernatant was carefully poured off and the tube drained on clean absorbent paper and then allowed to airdry for 15 min. 50 µl of DNA Hydrating Solution were added to the pellet and vortexed for 5 sec at maximum speed. DNA was dissolved by incubating at 65°C, then incubated overnight with gentle shaking.

3.3.3 Isolation of viral mRNA and cDNA preparation

Supernatants from cells were removed, centrifuged to get rid of all possible cell debris and then frozen at -80°C in single use aliquots. When infection of cells with virus was being investigated over time equal amounts of medium were added to cells after each sample was taken. Cell free supernatants from cells infected with virus were taken and centrifuged at 20800 x*g* for 1 h at 4°C. Supernatant was removed and pelleted virus resuspended in 10 µl of sterilized TNE (Appendix III). 9 µl of resuspended virus were placed in a PCR-tube with 2 µl of V-1818 XbaI primer (20 µM) and 1.5 µl DEPC-treated water.

Temperature cycling:

1) 65°C for 5 min 2) cooled down to 22°C for 7 min

Then a mix from RevertAid™ first strand synthesis kit (Fermentas) was placed in the tube, 1X reverse transcriptase buffer, 4 µM dNTP, 200 u RevertAid™ M-MuLV reverse transcriptase and 20 u RiboLock™ RNase Inhibitor to a final volume of 20 µl.

Temperature cycling:

1) 42°C for 60 min 2) 70°C for 10 min 3) cooled to 4°C

Samples were kept in a refrigerator and used in real time PCR the same day.

3.3.4 Real-time PCR

Quantitative PCR measures the amount of nucleic acid target after each cycle of amplification. TaqMan method was used. One probe is labelled with different fluorochromes at each termini, reporter dye (FAM) and quencher dye that quenches the reported dye. During PCR amplification the probe is degraded by the 5'-3' exonuclease activity of Taq DNA polymerase, causing physical separation of the dyes so the signal of the reporter dye is no longer quenced and can be detected (Holland et al., 1991).

Quantitative measures of DNA were performed using the StepOne™ system (Applied Biosystems). In a 20 µl reaction mixture 5 µl of DNA(10 ng/µl) or cDNA samples were used, 1X buffer, 200 µM dNTP, 0.9 µM v1636 forward primer, 0.9 µM v-1719 reverse primer, 0.25 µM V1665 TaqMan probe, 4 mM $MgCl_2$, 1x reference dye for quantitative PCR (Sigma, R4526) and 1.25 U of Amplitaq Gold polymerase (Roche) or with 1X TaqMan® Gene Expression Master Mix (Applied Biosystems), 0.9 µM v1636 forward primer, 0.9 µM v-1719 reverse primer, 0.25 µM V1665 TaqMan probe and water. Instrument settings for ROX were used for the measurement of the reference dye. Primers used in real-time PCR are V1636 and V-1719 and the probe is V1665 TaqMan.

Temperature cycling: 1) 95°C for 10 min 2) 95°C for 15 sec 3) 57°C for 60 sec, measurement of DNA. 4) Steps 2-3 repeated 39 times.

A standard curve was made with ten-fold dilutions (10^7 – 10^2) of p8XSp5-RK1 cut with *Xba*I. Samples were measured in duplicate and standards in duplicate or triplicate.

3.3.5 PCR methods

Polymerase chain reactions were performed in 20-50 µl reaction volumes containing templates (DNA/cDNA), 1x buffer, 200 µM dNTPs, 1 µM of each primer and 1 U of Phusion hot start high-fidelity polymeras (Finnzymes).

Program: 1) 98°C for 30 sec 2) 98°C for 10 sec 3) 55°C for 30 sec 4) 72°C for 30 sec 5) 72°C for 10 min 6) 4°C on hold. Steps 2-5 were repeated 24-39 times.

3.3.6 Agarose gel electrophosesis of DNA

PCR products and DNA were run on an agarose gel. Agarose gel electrophoresis was carried out in 1% agarose (Agarose, for routine use, Sigma) dissolved in 0.5x Tris borate-EDTA (TBE, Appendix III). Agarose was melted in the buffer until boiling, then cooled (50-60°C) and 1 drop of ethidium bromide (Sigma) added. Before electrophoresis samples were mixed with 10x restriction buffer (Appendix III). Electrophoresis was carried out at 70-75V for 40-45 min. Size of products was estimated by comparison to a 2-log DNA ladder from New England Biolabs.

3.3.7 Gel extraction

Gel extraction was done with QIAquick gel extration kit (Qiagen). DNA bands were visualized under UV light and excised out of electophoresis gels with a sterile knife and placed in 2 ml eppendorf tubes. Bands were weighted and mixed with 3x volume of buffer QG, then incubated at 50°C for 10 min. 1x volume of isopropanol was added and the mixture vortexed, then placed onto a QIAquick spin column and centrifuged at 17,900 x g for 1 min. Flow-through was discarded and 750 µl of buffer PE was placed onto column, centrifuged at 17,900 x g for 1 min. Flow-through was discarded and the column was centrifuged again, and flow-through was discarded again. The column was placed onto a 1.5 ml eppendorf tube and 30 µl of buffer EB added to the center of the membrane of the column. Allowed to stand for 1 min, then centrifuged. The column was discarded.

3.3.8 Transformation of bacterial cells

Transformation was done in chemically competent DH5α (*E. coli*) cells.

3.3.8.1 Heat-shock transformation

DH5α cells (100 µl) taken from -80°C and placed onto ice, 1-5 µl of ligation mix or plasmid placed on cells and kept on ice for 10 min. Cells were heat-shocked at 42°C for 1 min, and then placed on ice for 2 min. Mixed with 200 µl SOC medium (Appendix III) and incubated on a shaker for 1 hour at 37°C. The transformation mixture was spread on LB agar (Appendix III) plates with 100 µg/ml ampicillin or kanamycin and incubated for 16-18 hours at 30°C or 37°C.

3.3.8.2 Isolation of plasmid

Plasmids were isolated with miniprep (QIAprep spin miniprep kit, Qiagen) or midiprep (PureLink HiPure plasmid midiprep kit, Invitrogen) kits . A 2 ml culture was grown overnight for minipreps, and for 6-8 hours, then added to 25 ml of media and grown overnight for midipreps. The plasmid isolation the

following day was done according to the manufacturer's instruction. Plasmid DNA was collected in 30 μl EB buffer for miniprep and 200 μl of TE buffer for midiprep.

3.3.9 DNA sequencing methods

The DNA plasmids or PCR products were sequenced by using BigDye Terminator v3.1 Cycle Sequencing Kit and sequencing capillary electrophoresis was carried out in an ABI PRISM 310 Genetic Analyzer from Applied Biosystems. PCR products were gel extracted to get clean bands for use in sequencing reaction.

3.3.9.1 Sequencing reaction

Sequencing mixture:

Plasmid DNA (200-500 ng) or PCR products (5-20 ng), 1 μl BigDye, 1.5 μl 5X buffer, 0.16 μM primer and dH_2O to 10 μl final volume. A variety of primers were used for sequencing.

PCR for cycle sequencing:

1) 95°C for 5 min 2) 95°C for 20 sec 3) different annealing temperature according to the Tm of the primer for 15 sec 4) 60°C for 4 min 5) 4°C on hold. Steps 2-4 were repeated 29 times.

The PCR product was precipitated in 75% isopropanol for 15 min, centrifuged at 20800 x g for 30 min and washed in 75% isopropanol and centifuged at the same speed for 5 min. The isopropanol was discarded and the pellet dried at 90°C for 1 min and dissolved in 15 µl of Hi-Di™ Formamide (Applied Biosystems). The solution was heated for 2 min at 95°C and loaded onto instrument.

The sequencing results were analysed using Sequencer 4.9 from Gene Codes Corporation and the results used for Basic Local Alignment Search Tool (BLAST), nucleotide blast, FASTA sequences were used to search for highly similar sequences in other databases.

3.4 Protein methods

3.4.1 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Producer cell lysates from triplicate experiments of HIV-infectivity assay were harvested, pooled and resuspended in 2x Laemmli sample buffer (Appendix III) and heated for 10 min at 98°C and then spun for 2 min. The proteins from a fraction of each sample were run on a 12% SDS-PAGE gel in the Mini-protean II system (Bio-Rad) under reducing conditions. Size of proteins was estimated using either PageRuler Prestained molecular mass marker (Fermentas, #SM0671), Spectra™ Multicolor Broad Range Protein Ladder (Fermentas, #SM 1841) or SuperSignal Molecular Weight Protein Ladder (Pierce, #84785). The proteins were visualized using Western blot on polyvinylidene difluoride (PVDF) membrane (Millipore).

3.4.1.1 Western blotting

Protein was transferred to a PVDF membrane by wet transfer for 1 h in a transfer buffer (Appendix III). After transfer membranes were incubated in TBS-T (Appendix III) with 5% milk proteins for 30 min to 1 h. The membranes were washed with TBS-T (5x for 5 min) and incubated with a primary antibody, either anti-c-myc or anti-HA (Sigma-Aldrich) for detection at 4°C over night, washed again and then

incubated with alkaline phosphatase (AP)-conjugated secondary antibody or horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 1 h.

Membranes incubated with alkaline phosphatase (AP)-conjugated secondary antibody were developed with BCIP/NBT from Roche (Appendix III) diluted 1:50 in alkaline phosphatase buffer (Appendix III) until bands were visible.

Two different methods of chemiluminescence development were used.

A) Membranes incubated with horseradish peroxidase (HRP)-conjugated secondary antibody were developed by either mixing 2 ml of Lumigen™ PS-3 detection reagent solution A and 50 µl of Lumigen™ PS-3 detection reagent solution B from the ECL Plus Western Blotting Detection System from Amersham (GE Healthcare) and placing on membrane for 5 min, then the membrane was placed inside smoothened out shrink wrap and placed in developing cassette. In a darkroom an x-ray film (Amersham Hyperfilm™ ECL High performance chemiluminescence film from GE Healthcare) was placed on membrane for about 10 sec, then developed using Kodak X-OMAT EX II developer and replenisher.

B) Or by mixing 1:1 ml of SuperSignal® West Pico Stable Peroxide Solution with SuperSignal® West Pico Luminol/Enhancer Solution (SuperSignal® West Pico Chemiluminescent Substrate, #34077 from Pierce), incubating mixture on membrane for 5 min and then the membrane was placed inside smoothened out shrink wrap and placed in developing cassette, then inside a Chemiluminescence imaging system, the GeneGnome5 From Syngene Bio Imaging and the system allowed to automatically choose the exposure time for each membrane. Accompanying software was used to measure chemiluminescence and calculate amount of protein.

4 Results

4.1 Experimental infections of cells with CA-Vif virus

To find out why the CA-Vif mutant of MVV has attenuated replication in FOS cells compared to SCP cells, experimental infections were performed. Monolayers of sheep choroid plexus (SCP) or fetal ovine synovial (FOS) cells in 24 well plates were infected with virus derived from the kv1772 molecular clone (Andrésson et al., 1993) and CA-Vif viruses grown in SCP or FOS cells in equal amounts based on virus titer. kv1772 virus inactivated at 70°C for 15 min was used as a negative control. Samples were taken at different timepoints for 39 hours. DNA was isolated and quantitative real-time PCR performed. The results are shown in Figure 17 for 24 hours and Figure 18 for 39 hours.

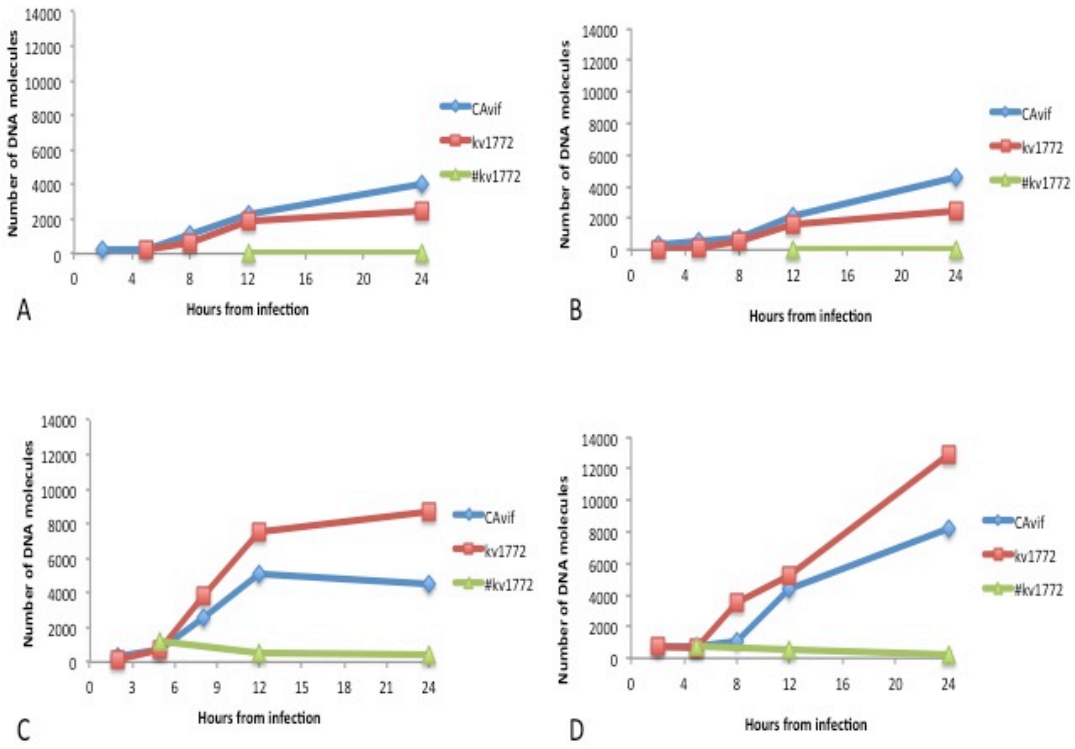


Figure 17. Experimental infections with CA-Vif virus, Kv1772 virus and control for 24 hours. A) Virus from SCP cells used to infect SCP cells B) Virus from SCP cells used to infect FOS cells C) Virus from FOS cells used to infect SCP cells D) Virus from FOS cells used to infect FOS cells.

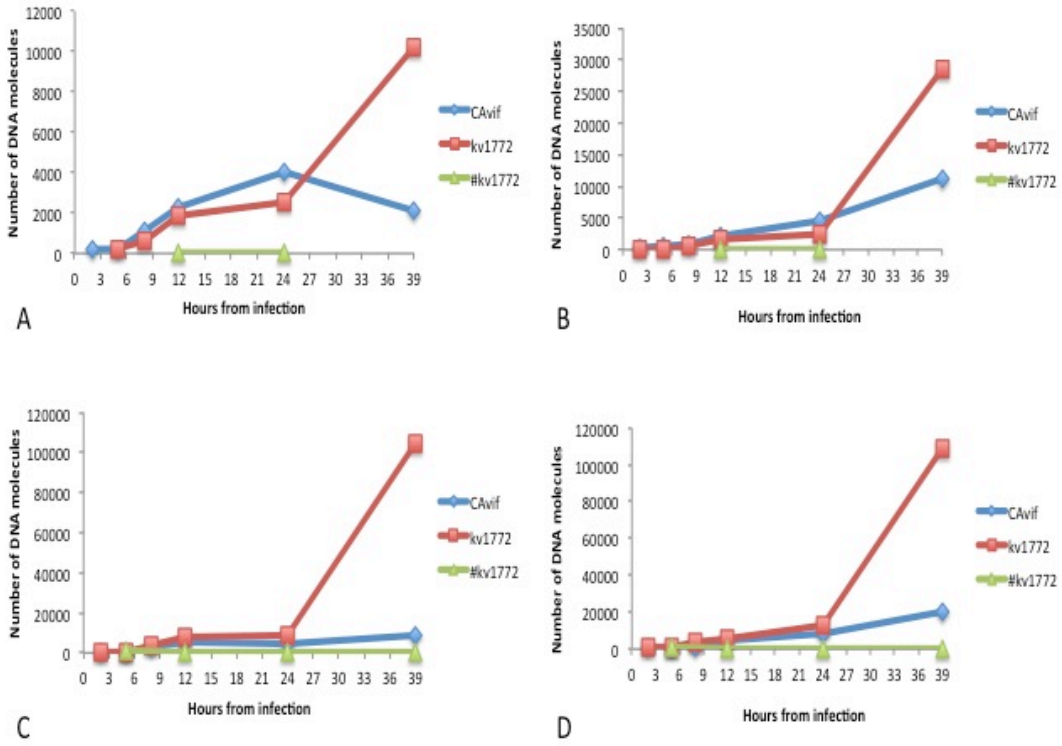


Figure 18. Experimental infections with CA-Vif virus, Kv1772 virus and control for 39 hours. A) Virus from SCP cells used to infect SCP cells. B) Virus from SCP cells used to infect FOS cells C) Virus from FOS cells used to infect SCP cells D) Virus from FOS cells used to infect FOS cells.

To see if the *vif* gene of CA-Vif virus produced in FOS cells had reverted back to the wt state DNA from infection over four days was sequenced to see if mutations had appeared (Appendix IV). No revertants were found.

Difference in viral replication can only be seen after 24 hours, showing that the inhibition is not at the stage of viral reverse transcription.

4.2 A putative Cul2 binding site

4.2.1 Construction of VifAA mutant virus and codonoptimized plasmid

The *vif* gene in kv1772 spans nt 4966 to 5657 in the genome (Andrésson et al., 1993). Since MVV Vif does not seem to bind Cul5 to form a Cul5 E3-ubiquitin ligase but Cul2 instead ((Luo et al., 2005; Mahrour et al., 2008; Stanley et al., 2008) a search for the consensus Cul2 binding sequence of ΦPXXΦXXXΦ, where Φ denotes a hydrophobic amino acid and X denotes any amino acid led to one site in the *vif* gene (Figure 19) near the C-terminus of Vif. The amino acids chosen for mutagenesis to find out if this putative Cul2 binding site was a Cul2 binding site were the 215IP216 and those were changed to two alanines.

```
Cul2 consensus sequence     ΦPXXΦXXXΦ

MVV Vif                 215 IPWSLQECW 223
```

Figure 19. Part of MVV Vif aligned to the Cul2 consensus sequence.

The kv1772 molecular clone (Andrésson et al., 1993) was mutated with Phusion-site directed mutagenesis of plasmids and the resulting p8XSp5-RK1 with the IP-AA mutations was sequenced and verified (Appendix IV).

Vif is notoriously hard to express in vitro without *tat* and *rev* so HIV-1 Vif has been codonoptimized and a cytomegalovirus (CMV) promoter used for expression in human cells. This removed the blockage of expression, mostly due to enhanced steady levels of mRNA (Nguyen et al., 2004). MVV Vif has also been codonoptimized and cloned in a VR1012 plasmid containing a CMV promoter (LaRue et al., 2010). The IP-AA mutation was put in codonoptimized MVV *vif* in pVR1012 and sequenced to confirm the mutation (Appendix IV).

4.2.2 Experimental infections of cells with VifAA virus

To see if the IP-AA mutation had any effect on the growth of the virus *in vivo*, first SCP cells were transfected with VifAA plasmids and virus in the supernatant collected and titrated. Then the virus was used in infections of SCP cells and compared to kv1772. The cells were observed in a microscope and supernatant was collected every 24 hours for 15 days. After the experimental period virus particles were lysed and cDNA made and analysed by quantitative real-time PCR. Replication of the mutated virus was delayed by two to three days and never reached as high titer as the wild type virus (Figure 20). DNA from infection of SCP cells over 18 days was sequenced and no reversion mutations were seen (Appendix IV).

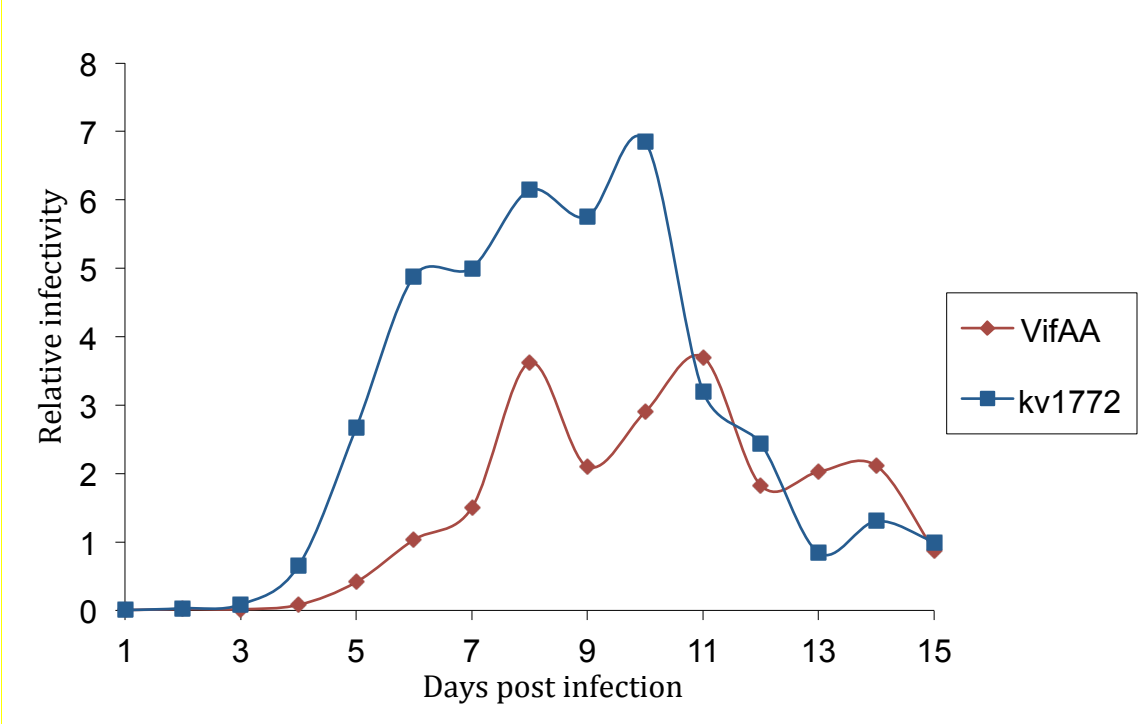


Figure 20. Kinetics of Kv1772-vif IP-AA replication compared to parent Kv1772 in SCP cells as measured by Taqman-based real-time PCR.

4.2.3 Single-cycle infectivity assays

4.2.3.1 Analysis of the effect of different levels of Vif on cells

The reported detrimental effects of large amounts of HIV-1 Vif on cells (Akari et al., 2004) resulted in an interest to find out if large amounts of MVV Vif had detrimental effects in cells. Human 293T cells were transfected with HIV-1 GFP plasmid and different amounts of codonoptimized *vif* expression plasmid, and observed in a fluorescence microscope and images taken after 48 and 72 hours transfections (Figure 21, Figure 22).

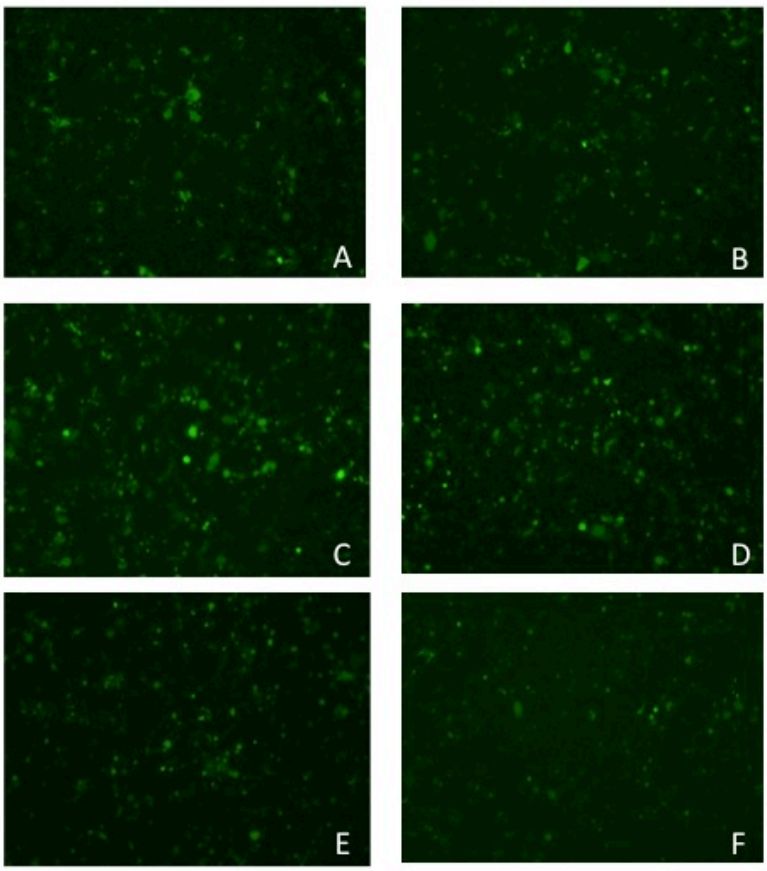


Figure 21. Different levels of vif 48-hours after transfection. A) 1000 ng empty vector B) 20 ng *vif* plasmid C) 50 ng *vif* plasmid D) 100 ng *vif* plasmid E) 500 ng *vif* plasmid F) 1000 ng *vif* plasmid

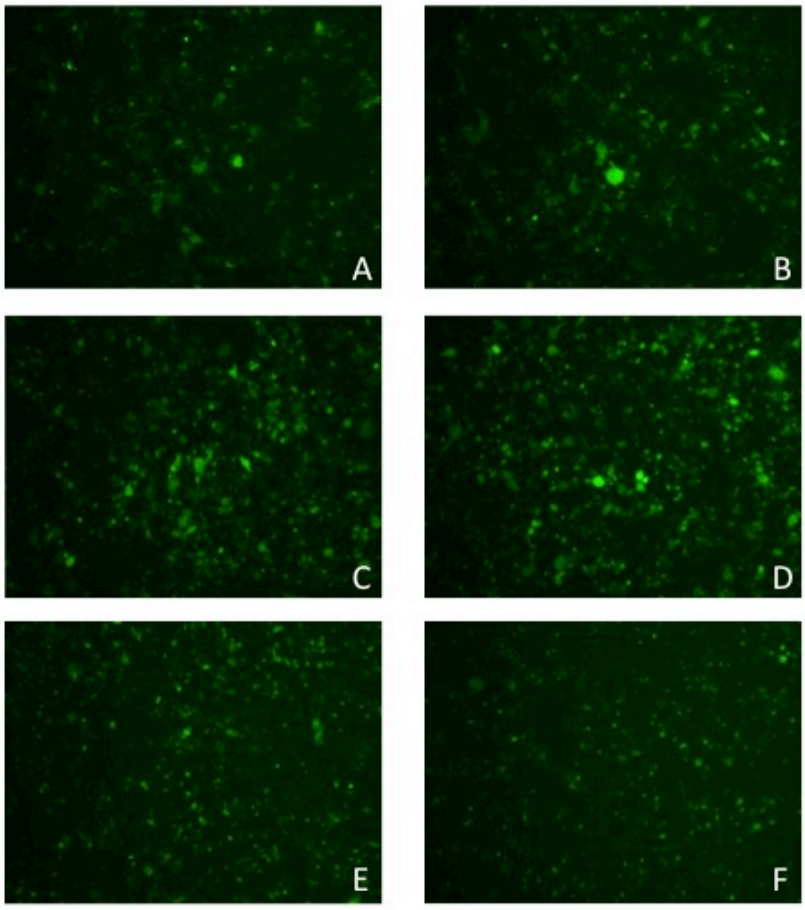


Figure 22. Different levels of vif 72-hours after transfection. A) 1000 ng empty vector B) 20 ng *vif* plasmid C) 50 ng *vif* plasmid D) 100 ng *vif* plasmid E) 500 ng *vif* plasmid F) 1000 ng *vif* plasmid

Increasing amounts of *vif* lead to stronger GFP expression when up to 50-100 ng of *vif* plasmids are used, the transfection is strongest for 50 ng of *vif* plasmid at 48 hours (Figure 21C) and at 100 ng of *vif* plasmid at 72 hours (Figure 22D). Transfection decreases when 500 (Figure 21E and Figure 22E) and 1000 (Figure 21F and Figure 22F) ng of *vif* plasmids are used. In the following experiments 100 ng of *vif* plasmid DNA was used.

4.2.3.2 HIV infectivity assay

Single cycle infectivity assays were performed on 293T cells. Producer cells were transfected with a cocktail of HIV-GFP, HIV Gag-Pol, HIV Rev and VSV-G envelope plasmids, and combinations of OaA3Z2-Z3 and MVV Vif in codonoptimized plasmids. Wt Vif was used as a positive control and a $Vif_{SLQ-AAA}$ mutant which has a mutation in the highly conserved BC-box that interacts with Elongin C and Elongin B heterodimer was used as a negative control. After 48 hours the supernatant was used to infect target cells and producer cells were collected for western blotting. After 72 hours infections were measured by fluorescence activated cell sorting (FACS). Figure 23 shows that whereas wt MVV Vif fully restores infectivity when ovine A3 is present, mutation in the conserved BC box abolishes

infectivity. However, mutation in the putative Cul2 box (IP-AA Vif) does not significantly reduce infectivity.

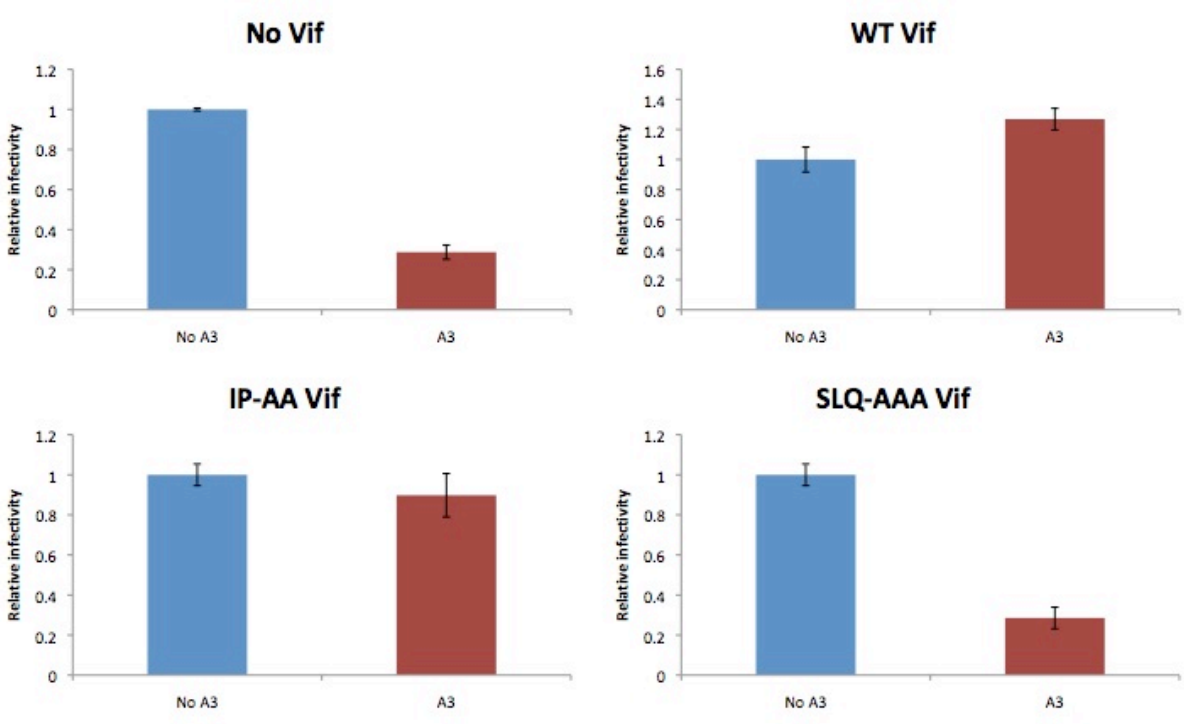


Figure 23. Anti-A3 function of wt Vif and Vif with IP-AA and SLQ-AAA mutations. Relative infectivity of HIV-GFP produced in the presence of various combinations of ovine A3 and wt and mutated MVV Vif. Vif or empty vector without A3 were normalized to 1. Error bars are the standard errors of the mean for three replicates.

Immunoblots for HA-tagged Vif and OaA3Z2Z3 were analysed by chemiluminescense (Figure 24) and the molecular weight (Table 2) and quantity of proteins in bands was measured (Table 3).

Table 2. Molecular weights of proteins measured from immunoblots.

Protein	Molecular weight (kDa)
β-actin	42.2
Average Vif	29.05
Vif_{WT}	28.89
$Vif_{IP\text{-}AA}$	29.43
$Vif_{SLQ\text{-}AAA}$	28.89
A3	43.41

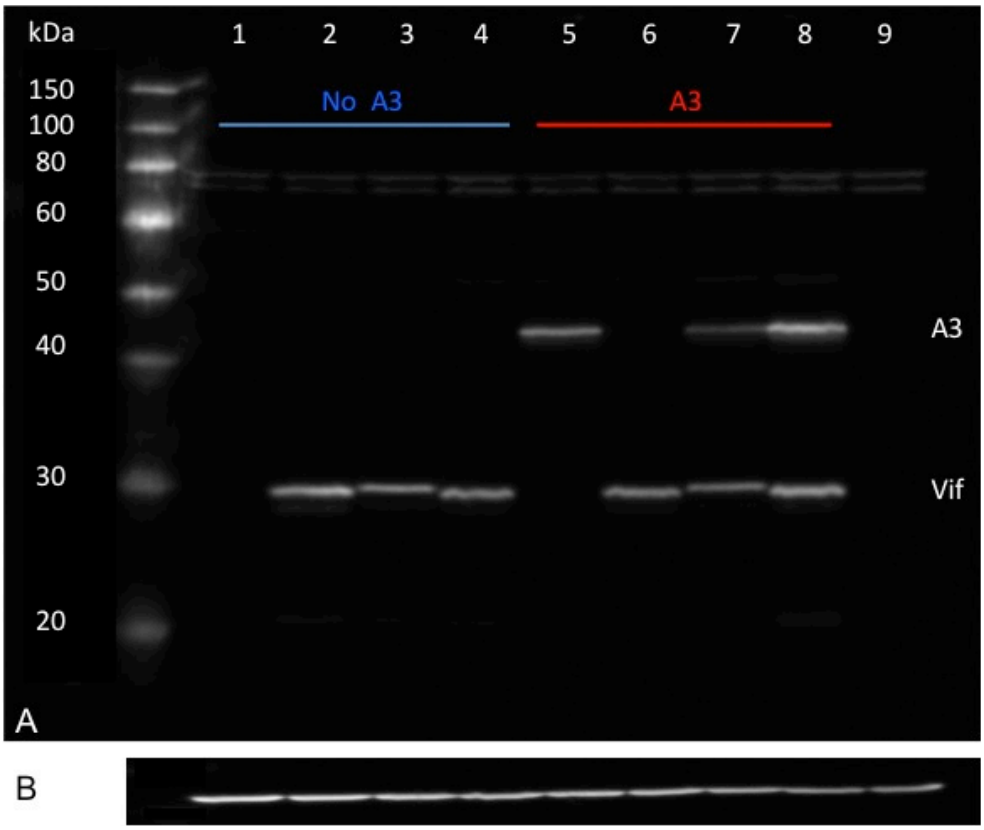


Figure 24. Immunoblot showing ovine APOBEC3-HA and Vif-HA. Immunoblots showing the producer cell levels of A) A3 and Vif proteins. A3 is ~ 45 kDa and Vif ~29 kDa. Lanes: 1) No Vif 2) wt Vif 3) $Vif_{IP\text{-}AA}$ 4) $Vif_{SLQ\text{-}AAA}$ 5) No Vif 6) wt Vif 7) $Vif_{IP\text{-}AA}$ 8) $Vif_{SLQ\text{-}AAA}$ 9) no vector. B) β-actin.

Table 3. Quantity of proteins measured from immunoblots. Quantity of wt Vif in the absence of A3 was defined as 1, and A3 in the absence of Vif was defined as 1. n.d.=not detected

	No A3	Vif with A3	A3 with Vif
No Vif	n.d.	n.d.	1
Vif_{WT}	1	0.63	n.d.
$Vif_{IP\text{-}AA}$	0.61	0.59	0.61
$Vif_{SLQ\text{-}AAA}$	0.67	0.86	1.52

Only partial degradation of A3 can be seen in the western blot (Figure 24), indicating that the mutation had effect on the anti A3 function of Vif. However this effect was not detected in the single cycle infection assay (Figure 23).

5 Discussion

In recent years the evidence for host defenses against retroviruses has shown that mammals have evolved several ways to protect themselves against retroviral infections, including host restriction factors. Retroviruses have on the other hand developed several countermeasures against the host defenses. The overall aim of this study was to investigate the role of Vif and possible interactions with the capsid in MVV and to examine the mechanism used by MVV Vif to eliminate APOBEC3.

5.1 CA-Vif

The molecular clone KS1 of MVV can not be used to infect sheep and grows poorly in macrophages (Torsteinsdóttir et al., 1997). Analysis of the sequence of the molecular clone showed that several mutations were found in KS1 compared to Kv1772, an infectious wt strain of MVV. The phenotype of KS1 could be traced to two mutations, one in CA and another in Vif. A molecular clone with both mutations, L120R in CA and P205 in Vif, was made (Gudmundsson et al., 2005) and will be called CA-Vif. These mutations cause attenuated replication in macrophages and FOS cells but not in SCP cells. This effect is only observed if both mutations are present (Figure 25) (Franzdóttir, 2004; Gudmundsson et al., 2005; Kristbjörnsdóttir et al., 2004).

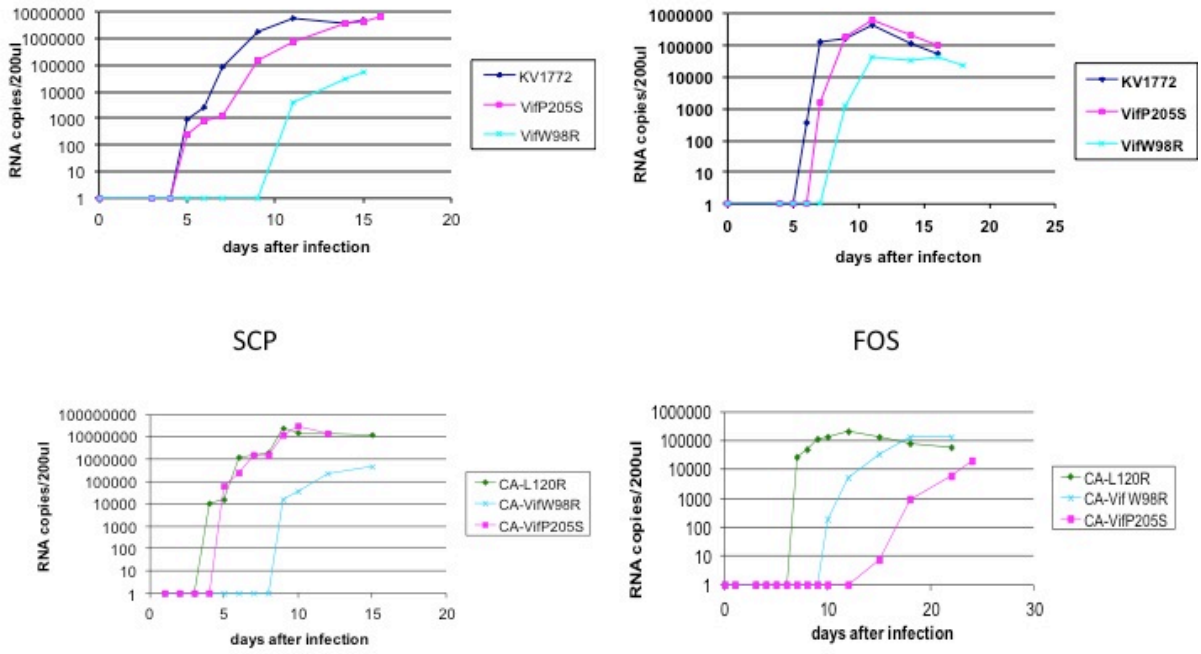


Figure 25. Effects of Vif_{P205S} and Vif_{W98R} mutations with or without CA_{L120R} mutation in FOS and SCP cells. Virus with the W98R mutation in Vif grows poorly in SCP cells but a little better in FOS cells. When combined with the L120R mutation in CA no difference is observed regardless of cell type. Vif_{W98R} virus has the same phenotype as Δ*vif* virus. The P205S mutation in Vif has no effect on virus growth unless combined with the L120R mutation in CA, leading to a strikingly lower growth in FOS cells, but no effect in SCP cells. Figure from (Franzdóttir, 2004).

We hypothesized that this effect was seen because of a previously unknown association between the viral capsid and the Vif protein of MVV that was either being negatively influenced by an unknown

host restriction factor in FOS cells and macrophages that is not expressed in SCP cells, or that some host protein lacking in FOS cells and macrophages but found in SCP cells was needed if this putative association between capsid and Vif was disturbed. The effects of A3 cytosine deaminases were ruled out since G-A hypermutations were not observed (Franzdóttir, 2004; Kristbjörnsdóttir et al., 2004; Ólafsdóttir, 2009).

To determine the stage of the block in the virus replication cycle we analyzed reverse transcription products in the first hours after infection in permissive (SCP) and nonpermissive (FOS) cells. We did infection experiments in FOS and SCP cells for 24 to 39 hours with CA-Vif viruses obtained from either FOS or SCP cells, compared to Kv1772, and with inactivated Kv1772 as a negative control. DNA samples were taken regularly and analyzed by quantitative real-time PCR.

When the growth of viruses produced in SCP cells and used to infect SCP and FOS cells for 24 hours was analyzed, the CA-Vif virus seemed to replicate as well as the wt virus (Figure 17A and B), although a striking difference could be seen after 39 hours in SCP and FOS cells, respectively (Figure 18A and B). When virus produced in FOS cells was used to infect SCP and FOS cells for 24 to 39 there was a slight difference in growth of CA-Vif and wt virus at 24 hours regardless of cell type (Figure 17C and D) but much more striking at 39 hours (Figure 18C and D) where the wt virus was multiplied more than 6 times between 24 and 39 hours, but the levels of CA-Vif were only slightly higher at 39 hours than at 24 hours post infection.

Reverse transcription of MVV is completed 12-24 hours after infection (Guðmundsdóttir, 2010) and it takes similar time for HIV-1 (Butler et al., 2001). There did not seem to be a defect in reverse transcription in the CA-Vif virus for the first 24 hours after infection. This shows that the block is not at the reverse transcription level but must be at some stage after reverse transcription. Although it can not be ruled out that some factor is expressed in SCP cells that is needed for the replication, it seems more likely that a restrictive factor is found in FOS cells, that affects viruses with a lost putative connection between the viral capsid and Vif. It has been shown that the restriction of the CA-Vif mutant is cell-dependent. However, this cell-dependence was not apparent after 39 hours, and in fact as can be seen in Figure 25, there is a lag of one day in the replication of CA-Vif in SCP cells compared to the control strain.

The mutation in amino acid 120 in the capsid corresponds to K131 in helix 7 in the N-terminal domain of HIV-1 CA. In all lentiviruses except for MVV and CAEV this amino acid or a corresponding amino acid is hydrophilic, but leucine is very hydrophobic (von Schwedler et al., 1998). Arginine on the other hand is hydrophilic so this amino acid likely leads to structural changes in the capsid.

Most known CA inhibitors restrict the CA at an early stage in the virus replication cycle prior to reverse transcription. There is one report, however, that describes mutations in HIV CA that can affect a post-nuclear entry step in an unknown cell-dependent way (Yamashita et al., 2007) and it has also been reported that cyclophilin A-dependent TRIM5α restriction mostly acts at a stage after reverse transcription (Lin and Emerman, 2008). We may be on the track of a host restriction factor that restricts CA at a post-reverse transcription step and is counteracted by Vif.

5.2 Cul2

Sheep code for three A3 cytosine deaminase genes and produce four proteins, three single-deaminase domain proteins and one double domain protein (Jónsson et al., 2006; Jónsson et al., 2007; LaRue et al., 2009; LaRue et al., 2008). The Vif protein of MVV has a near complete degradation effect on the OaA3Z2-Z3 double-domain protein (LaRue et al., 2010). HIV-1 Vif degrades A3 proteins by hijacking the cellular ubiquitin-dependent proteasomal degradation system and forming a Cul5 E3 ubiquitin ligase that polyubiquitinates A3 proteins, leading to their degradation by the proteasome (Dang et al., 2006; Luo et al., 2005; Stanley et al., 2008; Yu et al., 2003; Yu et al., 2004).

Since MVV Vif binds with Cul2 in pull-down experiments, but binds more weakly with Cul5 (Figure 11) (Luo et al., 2005) we hypothesized that a putative Cul2 binding site in MVV Vif (Figure 19) was used to form a Cul2 E3 ubiquitin ligase (Mahrour et al., 2008) used to degrade ovine A3 proteins.

We introduced mutations in the putative Cul2 binding site in Vif, both in the infectious clone kv1772 and in an expression vector with codonoptimized *vif*. A Kv1772 virus with IP-AA mutations in the putative Cul2-box (VifAA) used in experimental infections (Figure 20) showed delayed replication kinetics compared to Kv1772 indicating importance of the site for efficient replication of the virus.

Since high amounts of HIV-1 Vif can have negative consequences for infection (Akari et al., 2004) we transfected 293T cells with increasing amounts of codonoptimized wt *vif* plasmids using the producer cell part of the HIV-infectivity assay for 48 to 72 hours. This showed that increasing amounts of *vif* plasmids lead to stronger transfection when up to 50-100 ng of *vif* plasmids are used, the transfection is strongest for 50 ng of *vif* plasmid at 48 hours (Figure 21C) and at 100 ng of *vif* plasmid at 72 hours (Figure 22D). Transfection decreases when 500 (Figure 21E and Figure 22E) and 1000 (Figure 21F and Figure 22F) ng of *vif* plasmid are used. This showed that using 100 ng of *vif* plasmid in transfections for single cycle infectivity assays was safe.

When codonoptimized plasmids of *vif* were used in HIV-infectivity assay (Figure 23) the observed results for no *vif*, wt *vif* and a $Vif_{SLQ-AAA}$ mutant that has a mutation in the highly conserved BC-box that interacts with Elongin C and Elongin B heterodimer, were as expected. However, the IP-AA mutation had no effect on infectivity when ovine A3Z2-Z3 was present in the system. When cell lysates from producer cells were analysed by western blot (Figure 24) the wt Vif seemed to almost fully degrade the A3 protein and the $Vif_{SLQ-AAA}$ mutant did not degrade A3 at all. Vif_{IP-AA} seemed to partially degrade A3.

It is possible that only changing the isoleucine and proline in MVV Vif to double alanine was not enough to disturb the possible binding of Cul2. However, in experiments by *Mahrour et al.* this was enough in all cases to deactivate Cul2 binding (Mahrour et al., 2008).

The *A3* genes of sheep and cattle have all three zinc-binding domains that are found in mammals, indicating that the common ancestor of artiodactyls and primates had all three domains. Most if not all retroelements can be inhibited by at least one A3 protein, and all successful retroelements appear to have evolved strategies to resist restriction by A3 proteins of their hosts (LaRue et al., 2008). MVV and FIV are more closely related than either is to HIV-1 (Gonda, 1994) and MVV and FIV are therefore more likely to have similar ways of counteraction to A3 proteins than using the same counteraction as

HIV-1. Recently FIV Vif has been reported to form a Cul5 E3 ubiquitin ligase complex to ubiquitinate feline A3s, although FIV Vif does not have a known Cul5 binding site (Wang et al., 2011), just like MVV. MVV and FIV Vif share some similarities in amino acid sequence (Figure 26), more than MVV Vif and HIV-1 Vif (Figure 27). It is likely that conserved amino acids have a role in the highly preserved degradation of A3 proteins, like the 173SLQRLA178 sequence in MVV, that has the consensus sequence of T/SLQR/YLA in lentiviral Vif proteins, serving as a BC-box for binding Elongin C and Elongin B heterodimer.

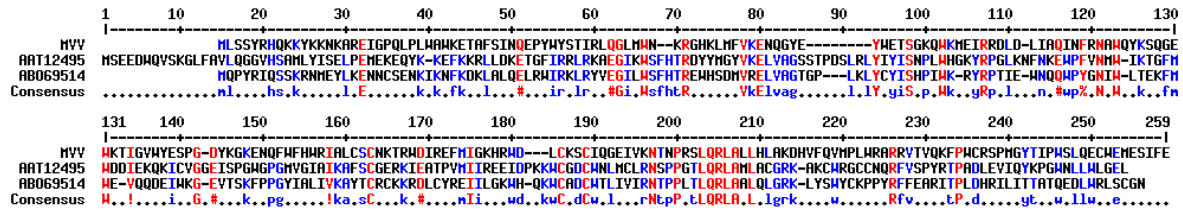


Figure 26. Amino acid alignment of MVV Vif and Vif from two strains of FIV. Some amino acid similarity is found between MVV and FIV Vifs. Conserved amino acids are red. (http://multalin.toulouse.inra.fr/multalin/multalin.html).

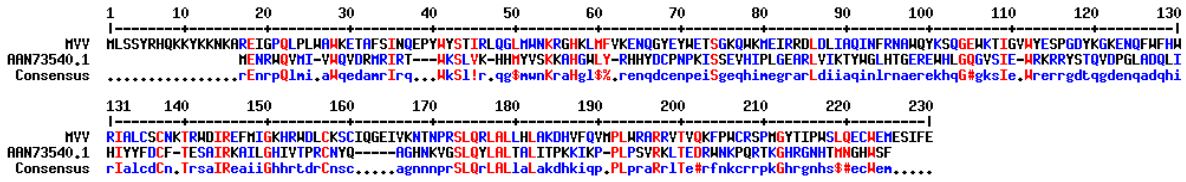


Figure 27. Sequence alignment of MVV and HIV-1 Vif proteins. Conserved amino acids are red. (http://multalin.toulouse.inra.fr/multalin/multalin.html).

Although neither FIV nor MVV Vif have a Cul5 binding site, ΦXXΦXXXXXΦXXXΦ (Stanley et al., 2008) where Φ denotes a hydrophobic amino acid and X denotes any amino acid, it is feasible to speculate whether MVV Vif binds to Cul5 with the same unknown binding site as FIV Vif does. It is also possible that MVV Vif utilizes both Cul2 and Cul5 for the degradation of A3 proteins and the putative Cul2 binding site where the IP-AA mutation was expressed is not enough to stop degradation since Cul5 could bind to Vif at some other site, although the degradation of OaA3Z2-Z3 was not complete.

6 Conclusions

In this study we established that a mutation in MVV Vif that interacts with a mutation in the capsid defines a previously unknown role of Vif at a stage in the viral cycle post transcription. The attenuation seems to be caused by a cellular factor found in FOS cells that acts upon the capsid at some stage after reverse transcription and that Vif has the new role of protection against this restriction factor.

The mutation in the putative Cul2 binding site affects infection efficiency of MVV and may indicate that it is used to bind Cul2, but disrupting Cul2 binding is not enough to stop degradation of OaA3Z2-Z3, since it is partially degraded. Recently it has been shown that FIV Vif uses Cul5 to form an E3 ubiquitin ligase to degrade feline A3s, although no known Cul5 binding site is found in FIV. Some sequence similarity is found between FIV and MVV Vif, and MVV Vif also binds Cul5, although not as strongly as Cul2. Further studies will reveal whether MVV Vif uses Cul5 as well as Cul2. The results indicate that the Vif proteins of the lentiviruses have evolved diverse ways to degrade the A3s of their respective hosts.

References

Ahn, J., Hao, C., Yan, J., Delucia, M., Mehrens, J., Wang, C., Gronenborn, A.M., Skowronski, J., 2012. HIV/Simian Immunodeficiency Virus (SIV) Accessory Virulence Factor Vpx Loads the Host Cell Restriction Factor SAMHD1 onto the E3 Ubiquitin Ligase Complex $CRL4^{DCAF1}$. *J Biol Chem* 287, 12550-12558.

Ahn, J., Vu, T., Novince, Z., Guerrero-Santoro, J., Rapic-Otrin, V., Gronenborn, A.M., 2010. HIV-1 Vpr loads uracil DNA glycosylase-2 onto DCAF1, a substrate recognition subunit of a cullin 4A-ring E3 ubiquitin ligase for proteasome-dependent degradation. *J Biol Chem* 285, 37333-37341.

Akari, H., Fujita, M., Kao, S., Khan, M.A., Shehu-Xhilaga, M., Adachi, A., Strebel, K., 2004. High level expression of human immunodeficiency virus type-1 Vif inhibits viral infectivity by modulating proteolytic processing of the Gag precursor at the p2/nucleocapsid processing site. *J Biol Chem* 279, 12355-12362.

Albin, J.S., Harris, R.S., 2010. Interactions of host APOBEC3 restriction factors with HIV-1 in vivo: implications for therapeutics. *Expert Rev Mol Med* 12, e4.

Andrésson, O.S., Elser, J.E., Tobin, G.J., Greenwood, J.D., Gonda, M.A., Georgsson, G., Andrésdóttir, V., Benediktsdóttir, E., Carlsdottir, H.M., Mäntylä, E.O., 1993. Nucleotide sequence and biological properties of a pathogenic proviral molecular clone of neurovirulent visna virus. *Virology* 193, 89-105.

Basu, V.P., Song, M., Gao, L., Rigby, S.T., Hanson, M.N., Bambara, R.A., 2008. Strand transfer events during HIV-1 reverse transcription. *Virus Res* 134, 19-38.

Bergeron, J.R., Huthoff, H., Veselkov, D.A., Beavil, R.L., Simpson, P.J., Matthews, S.J., Malim, M.H., Sanderson, M.R., 2010. The SOCS-box of HIV-1 Vif interacts with ElonginBC by induced-folding to recruit its Cul5-containing ubiquitin ligase complex. *PLoS Pathog* 6, e1000925.

Bieniasz, P.D., 2003. Restriction factors: a defense against retroviral infection. *Trends Microbiol* 11, 286-291.

Binka, M., Ooms, M., Steward, M., Simon, V., 2012. The activity spectrum of Vif from multiple HIV-1 subtypes against APOBEC3G, APOBEC3F, and APOBEC3H. *J Virol* 86, 49-59.

Bouyac, M., Courcoul, M., Bertoia, G., Baudat, Y., Gabuzda, D., Blanc, D., Chazal, N., Boulanger, P., Sire, J., Vigne, R., Spire, B., 1997. Human immunodeficiency virus type 1 Vif protein binds to the $Pr55^{Gag}$ precursor. *J Virol* 71, 9358-9365.

Bukovsky, A.A., Weimann, A., Accola, M.A., Göttlinger, H.G., 1997. Transfer of the HIV-1 cyclophilin-binding site to simian immunodeficiency virus from *Macaca mulatta* can confer both cyclosporin sensitivity and cyclosporin dependence. *Proc Natl Acad Sci U S A* 94, 10943-10948.

Butler, S.L., Hansen, M.S., Bushman, F.D., 2001. A quantitative assay for HIV DNA integration in vivo. *Nat Med* 7, 631-634.

Campbell, E.M., Perez, O., Anderson, J.L., Hope, T.J., 2008. Visualization of a proteasome-independent intermediate during restriction of HIV-1 by rhesus TRIM5α. *J Cell Biol* 180, 549-561.

Carr, J.M., Coolen, C., Davis, A.J., Burrell, C.J., Li, P., 2008. Human immunodeficiency virus 1 (HIV-1) virion infectivity factor (Vif) is part of reverse transcription complexes and acts as an accessory factor for reverse transcription. *Virology* 372, 147-156.

Clements, J.E., Zink, M.C., 1996. Molecular biology and pathogenesis of animal lentivirus infections. *Clin Microbiol Rev* 9, 100-117.

Dang, Y., Siew, L.M., Wang, X., Han, Y., Lampen, R., Zheng, Y.H., 2008. Human cytidine deaminase APOBEC3H restricts HIV-1 replication. *J Biol Chem* 283, 11606-11614.

Dang, Y., Wang, X., Esselman, W.J., Zheng, Y.H., 2006. Identification of APOBEC3DE as another antiretroviral factor from the human APOBEC family. *J Virol* 80, 10522-10533.

Deshaies, R.J., Joazeiro, C.A., 2009. RING domain E3 ubiquitin ligases. *Annu Rev Biochem* 78, 399-434.

Dettenhofer, M., Cen, S., Carlson, B.A., Kleiman, L., Yu, X.F., 2000. Association of human immunodeficiency virus type 1 Vif with RNA and its role in reverse transcription. *J Virol* 74, 8938-8945.

Emerman, M., 2006. How TRIM5α defends against retroviral invasions. *Proc Natl Acad Sci U S A* 103, 5249-5250.

Flint, S.J., Enquist, L.W., Racaniello, V.R., Skalka, A.M., 2004. Principles of virology, 2. ed. ASM Press, Washington, D.C.

Franzdóttir, S.R., 2004. Stökkbreytigreining Vif próteins mæði-visnuveiru, Læknadeild. Háskóli Íslands, Reykjavík, p. 137.

Freed, E.O., 1998. HIV-1 gag proteins: diverse functions in the virus life cycle. *Virology* 251, 1-15.

Gamble, T.R., Vajdos, F.F., Yoo, S., Worthylake, D.K., Houseweart, M., Sundquist, W.I., Hill, C.P., 1996. Crystal structure of human cyclophilin A bound to the amino-terminal domain of HIV-1 capsid. *Cell* 87, 1285-1294.

Goff, S.P., 2004. HIV: replication trimmed back. *Nature* 427, 791-793.

Goff, S.P., 2007. Host factors exploited by retroviruses. *Nat Rev Microbiol* 5, 253-263.

Goila-Gaur, R., Strebel, K., 2008. HIV-1 Vif, APOBEC, and intrinsic immunity. *Retrovirology* 5, 51.

Gonda, M.A., 1994. Molecular biology and virus-host interactions of lentiviruses. *Ann N Y Acad Sci* 724, 22-42.

Guðmundsdóttir, E.Þ., 2010. Cell tropism of maedi-visna virus, Faculty of medicine. University of Iceland, Reykjavík, p. 69.

Gudmundsson, B., Jónsson, S.R., Ólafsson, O., Agnarsdottir, G., Matthíasdóttir, S., Georgsson, G., Torsteinsdóttir, S., Svansson, V., Kristbjörnsdóttir, H.B., Franzdóttir, S.R., Andrésson, O.S., Andrésdóttir, V., 2005. Simultaneous mutations in CA and Vif of Maedi-Visna virus cause attenuated replication in macrophages and reduced infectivity in vivo. *J Virol* 79, 15038-15042.

Guðnadóttir, M., 1977. Um visnu og þurramæði í sauðfé. Prentsmiðjan Hólar.

Gudnadóttir, M., Pálsson, P.A., 1965. Successful transformation of visna by intrapulmonary inoculation. *J Infect Dis* 115, 217-225.

Haase, A.T., 1975. The slow infection caused by visna virus. *Curr Top Microbiol Immunol* 72, 101-156.

Harari, A., Ooms, M., Mulder, L.C., Simon, V., 2009. Polymorphisms and splice variants influence the antiretroviral activity of human APOBEC3H. *J Virol* 83, 295-303.

Harris, R.S., Liddament, M.T., 2004. Retroviral restriction by APOBEC proteins. *Nat Rev Immunol* 4, 868-877.

Holland, P.M., Abramson, R.D., Watson, R., Gelfand, D.H., 1991. Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase. *Proc Natl Acad Sci U S A* 88, 7276-7280.

Hrecka, K., Hao, C., Gierszewska, M., Swanson, S.K., Kesik-Brodacka, M., Srivastava, S., Florens, L., Washburn, M.P., Skowronski, J., 2011. Vpx relieves inhibition of HIV-1 infection of macrophages mediated by the SAMHD1 protein. *Nature* 474, 658-661.

Hultquist, J.F., Binka, M., Larue, R.S., Simon, V., Harris, R.S., 2012. Vif Proteins of Human and Simian Immunodeficiency Viruses Require Cellular CBFβ To Degrade APOBEC3 Restriction Factors. *J Virol* 86, 2874-2877.

Huthoff, H., Towers, G.J., 2008. Restriction of retroviral replication by APOBEC3G/F and TRIM5α. *Trends Microbiol* 16, 612-619.

Jäger, S., Kim, D.Y., Hultquist, J.F., Shindo, K., LaRue, R.S., Kwon, E., Li, M., Anderson, B.D., Yen, L., Stanley, D., Mahon, C., Kane, J., Franks-Skiba, K., Cimermancic, P., Burlingame, A., Sali, A., Craik, C.S., Harris, R.S., Gross, J.D., Krogan, N.J., 2012. Vif hijacks CBF-β to degrade APOBEC3G and promote HIV-1 infection. *Nature* 481, 371-375.

Jónsson, S.R., Haché, G., Stenglein, M.D., Fahrenkrug, S.C., Andrésdóttir, V., Harris, R.S., 2006. Evolutionarily conserved and non-conserved retrovirus restriction activities of artiodactyl APOBEC3F proteins. *Nucleic Acids Res* 34, 5683-5694.

Jónsson, S.R., LaRue, R.S., Stenglein, M.D., Fahrenkrug, S.C., Andrésdóttir, V., Harris, R.S., 2007. The restriction of zoonotic PERV transmission by human APOBEC3G. *PLoS One* 2, e893.

Keckesova, Z., Ylinen, L.M., Towers, G.J., 2004. The human and African green monkey TRIM5α genes encode Ref1 and Lv1 retroviral restriction factor activities. *Proc Natl Acad Sci U S A* 101, 10780-10785.

Kitamura, S., Ode, H., Iwatani, Y., 2011. Structural Features of Antiviral APOBEC3 Proteins are Linked to Their Functional Activities. *Front Microbiol* 2, 258.

Kristbjörnsdóttir, H.B., Andrésdóttir, V., Svansson, V., Torsteinsdóttir, S., Matthíasdóttir, S., Andrésson, O.S., 2004. The vif gene of maedi-visna virus is essential for infectivity in vivo and in vitro. *Virology* 318, 350-359.

Kuhl, B.D., Cheng, V., Wainberg, M.A., Liang, C., 2011. Tetherin and its viral antagonists. *J Neuroimmune Pharmacol* 6, 188-201.

Laguette, N., Sobhian, B., Casartelli, N., Ringeard, M., Chable-Bessia, C., Ségéral, E., Yatim, A., Emiliani, S., Schwartz, O., Benkirane, M., 2011. SAMHD1 is the dendritic- and myeloid-cell-specific HIV-1 restriction factor counteracted by Vpx. *Nature* 474, 654-657.

LaRue, R.S., Andresdottir, V., Blanchard, Y., Conticello, S.G., Derse, D., Emerman, M., Greene, W.C., Jonsson, S.R., Landau, N.R., Lochelt, M., Malik, H.S., Malim, M.H., Munk, C., O'Brien, S.J., Pathak, V.K., Strebel, K., Wain-Hobson, S., Yu, X.F., Yuhki, N., Harris, R.S., 2009. Guidelines for naming nonprimate APOBEC3 genes and proteins. *J Virol* 83, 494-497.

LaRue, R.S., Jónsson, S.R., Silverstein, K.A., Lajoie, M., Bertrand, D., El-Mabrouk, N., Hötzel, I., Andrésdóttir, V., Smith, T.P., Harris, R.S., 2008. The artiodactyl *APOBEC3* innate immune repertoire shows evidence for a multi-functional domain organization that existed in the ancestor of placental mammals. *BMC molecular biology* 9, 104.

LaRue, R.S., Lengyel, J., Jónsson, S.R., Andrésdóttir, V., Harris, R.S., 2010. Lentiviral Vif Degrades the APOBEC3Z3 Protein of its Mammalian Host and is Capable of Cross-Species Activity. *J Virol* 84, 8193-8201.

Li, M.M., Wu, L.I., Emerman, M., 2010. The range of human APOBEC3H sensitivity to lentiviral Vif proteins. *J Virol* 84, 88-95.

Lienlaf, M., Hayashi, F., Di Nunzio, F., Tochio, N., Kigawa, T., Yokoyama, S., Diaz-Griffero, F., 2011. Contribution of E3-ubiquitin ligase activity to HIV-1 restriction by TRIM5αrh: Structure of the RING domain of TRIM5α. *J Virol* 85, 8725-37.

Lin, F.H., Thormar, H., 1970. Ribonucleic acid-dependent deoxyribonucleic acid polymerase in visna virus. *J Virol* 6, 702-704.

Lin, T.Y., Emerman, M., 2008. Determinants of cyclophilin A-dependent TRIM5α restriction against HIV-1. *Virology* 379, 335-341.

Luciw, P.A., Leung, N.J., 1992. Mechanisms of Retrovirus Replication, in: Levy, J.A. (Ed.), The Retroviridae. Plenum Press, New York.

Luo, K., Xiao, Z., Ehrlich, E., Yu, Y., Liu, B., Zheng, S., Yu, X.F., 2005. Primate lentiviral virion infectivity factors are substrate receptors that assemble with cullin 5-E3 ligase through a HCCH motif to suppress APOBEC3G. *Proc Natl Acad Sci U S A* 102, 11444-11449.

Madani, N., Kabat, D., 1998. An endogenous inhibitor of human immunodeficiency virus in human lymphocytes is overcome by the viral Vif protein. *J Virol* 72, 10251-10255.

Mahrour, N., Redwine, W.B., Florens, L., Swanson, S.K., Martin-Brown, S., Bradford, W.D., Staehling-Hampton, K., Washburn, M.P., Conaway, R.C., Conaway, J.W., 2008. Characterization of Cullin-box sequences that direct recruitment of Cul2-Rbx1 and Cul5-Rbx2 modules to Elongin BC-based ubiquitin ligases. *J Biol Chem* 283, 8005-8013.

Mangeat, B., Turelli, P., Caron, G., Friedli, M., Perrin, L., Trono, D., 2003. Broad antiretroviral defence by human APOBEC3G through lethal editing of nascent reverse transcripts. *Nature* 424, 99-103.

Mische, C.C., Javanbakht, H., Song, B., Diaz-Griffero, F., Stremlau, M., Strack, B., Si, Z., Sodroski, J., 2005. Retroviral restriction factor TRIM5α is a trimer. *J Virol* 79, 14446-14450.

Miyagi, E., Brown, C.R., Opi, S., Khan, M., Goila-Gaur, R., Kao, S., Walker, R.C., Jr., Hirsch, V., Strebel, K., 2010. Stably expressed APOBEC3F has negligible antiviral activity. *J Virol* 84, 11067-11075.

Miyoshi, H., Blomer, U., Takahashi, M., Gage, F.H., Verma, I.M., 1998. Development of a self-inactivating lentivirus vector. *J Virol* 72, 8150-8157.

Narayan, O., Clements, J.E., 1989. Biology and pathogenesis of lentiviruses. *J Gen Virol* 70 (Pt 7), 1617-1639.

Neil, S.J.D., Zang, T., Bieniasz, P.D., 2008. Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. *Nature* 451, 425-430.

Nguyen, K.L., llano, M., Akari, H., Miyagi, E., Poeschla, E.M., Strebel, K., Bour, S., 2004. Codon optimization of the HIV-1 vpu and vif genes stabilizes their mRNA and allows for highly efficient Rev-independent expression. *Virology* 319, 163-175.

Oberste, M.S., Gonda, M.A., 1992. Conservation of amino-acid sequence motifs in lentivirus Vif proteins. *Virus Genes* 6, 95-102.

Öhagen, Å., Gabuzda, D., 2000. Role of Vif in stability of the human immunodeficiency virus type 1 core. *J Virol* 74, 11055-11066.

Ólafsdóttir, K., 2009. Intrinsic antiretroviral defences and viral countermeasures: The *vif* and *tat* genes of maedi-visna virus, Faculty of medicine. University of Iceland, Reykjavík, p. 76.

Passerini, L.D., Keckesova, Z., Towers, G.J., 2006. Retroviral restriction factors Fv1 and TRIM5α act independently and can compete for incoming virus before reverse transcription. *J Virol* 80, 2100-2105.

Pépin, M., Vitu, C., Russo, P., Mornex, J.F., Peterhans, E., 1998. Maedi-visna virus infection in sheep: a review. *Vet Res* 29, 341-367.

Pertel, T., Hausmann, S., Morger, D., Zuger, S., Guerra, J., Lascano, J., Reinhard, C., Santoni, F.A., Uchil, P.D., Chatel, L., Bisiaux, A., Albert, M.L., Strambio-De-Castillia, C., Mothes, W., Pizzato, M., Grütter, M.G., Luban, J., 2011. TRIM5 is an innate immune sensor for the retrovirus capsid lattice. *Nature* 472, 361-365.

Rhodes, D.A., de Bono, B., Trowsdale, J., 2005. Relationship between SPRY and B30.2 protein domains. Evolution of a component of immune defence? *Immunology* 116, 411-417.

Rold, C.J., Aiken, C., 2008. Proteasomal degradation of TRIM5α during retrovirus restriction. *PLoS Pathog* 4, e1000074.

Schröfelbauer, B., Chen, D., Landau, N.R., 2004. A single amino acid of APOBEC3G controls its species-specific interaction with virion infectivity factor (Vif). *Proc Natl Acad Sci U S A* 101, 3927-3932.

Sebastian, S., Luban, J., 2005. TRIM5α selectively binds a restriction-sensitive retroviral capsid. *Retrovirology* 2, 40.

Seroude, V., Audoly, G., Gluschankof, P., Suzan, M., 2002. Viral and cellular specificities of caprine arthritis encephalitis virus Vif protein. *Virology* 292, 156-161.

Sharova, N., Wu, Y., Zhu, X., Stranska, R., Kaushik, R., Sharkey, M., Stevenson, M., 2008. Primate lentiviral Vpx commandeers DDB1 to counteract a macrophage restriction. *PLoS Pathog* 4, e1000057.

Sheehy, A.M., Gaddis, N.C., Choi, J.D., Malim, M.H., 2002. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. *Nature* 418, 646-650.

Sigurdsson, B., 1954. Observations on three slow infections of sheep. *Br Vet J* 110, 255-270.

Sigurðsson, B., 1958. Annarlega hæggengir smitsjúkdómar. *Skírnir* 132, 165-183.

Skraban, R., Matthiasdottir, S., Torsteinsdottir, S., Agnarsdottir, G., Gudmundsson, B., Georgsson, G., Meloen, R.H., Andresson, O.S., Staskus, K.A., Thormar, H., Andresdottir, V., 1999. Naturally occurring mutations within 39 amino acids in the envelope glycoprotein of maedi-visna virus alter the neutralization phenotype. *J Virol* 73, 8064-8072.

Sonigo, P., Alizon, M., Staskus, K., Klatzmann, D., Cole, S., Danos, O., Retzel, E., Tiollais, P., Haase, A., Wain-Hobson, S., 1985. Nucleotide sequence of the visna lentivirus: relationship to the AIDS virus. *Cell* 42, 369-382.

Stanley, B.J., Ehrlich, E.S., Short, L., Yu, Y., Xiao, Z., Yu, X.F., Xiong, Y., 2008. Structural insight into the human immunodeficiency virus Vif SOCS box and its role in human E3 ubiquitin ligase assembly. *J Virol* 82, 8656-8663.

Stremlau, M., Owens, C.M., Perron, M.J., Kiessling, M., Autissier, P., Sodroski, J., 2004. The cytoplasmic body component TRIM5α restricts HIV-1 infection in Old World monkeys. *Nature* 427, 848-853.

Stremlau, M., Perron, M., Lee, M., Li, Y., Song, B., Javanbakht, H., Diaz-Griffero, F., Anderson, D.J., Sundquist, W.I., Sodroski, J., 2006. Specific recognition and accelerated uncoating of retroviral capsids by the TRIM5α restriction factor. *Proc Natl Acad Sci U S A* 103, 5514-5519.

Takeuchi, H., Matano, T., 2008. Host factors involved in resistance to retroviral infection. *Microbiol Immunol* 52, 318-325.

Tang, H., Kuhen, K.L., Wong-Staal, F., 1999. Lentivirus replication and regulation. *Annu Rev Genet* 33, 133-170.

Telesnitsky, A., Goff, S.P., 1997. Reverse transcriptase and the generation of retroviral DNA. Cold Spring Harbor Laboratory Press, New York.

Temin, H.M., 1992. Origin and General Nature of Retroviruses, in: Levy, J.A. (Ed.), The Retroviridae. Plenum Press, New York.

Thormar, H., 1961. An electron microscope study of tissue cultures infected with visna virus. *Virology* 14, 463-475.

Thormar, H., Cruickshank, J.G., 1965. THE STRUCTURE OF VISNA VIRUS STUDIED BY THE NEGATIVE STAINING TECHNIQUE. *Virology* 25, 145-148.

Torsteinsdóttir, S., Agnarsdóttir, G., Matthíasdóttir, S., Rafnar, B., Andrésdóttir, V., Andrésson, Ó.S., Staskus, K., Pétursson, G., Pálsson, P.A., Georgsson, G., 1997. I*n vivo* and *in vitro* infection with two different molecular clones of visna virus. *Virology* 229, 370-380.

Villet, S., Bouzar, B.A., Morin, T., Verdier, G., Legras, C., Chebloune, Y., 2003a. Maedi-visna virus and caprine arthritis encephalitis virus genomes encode a Vpr-like but no Tat protein. *J Virol* 77, 9632-9638.

Villet, S., Faure, C., Bouzar, B.A., Morin, T., Verdier, G., Chebloune, Y., Legras, C., 2003b. Lack of *trans*-activation function for Maedi Visna virus and Caprine arthritis encephalitis virus Tat proteins. *Virology* 307, 317-327.

Vogt, V.M., 1997. Retroviral Virions and Genomes, in: Coffin, J.M., Hughes, S.H., Varmus, H.E. (Eds.), Retroviruses. Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY.

von Schwedler, U., Song, J., Aiken, C., Trono, D., 1993. *vif* is crucial for human immunodeficiency virus type 1 proviral DNA synthesis in infected cells. *J Virol* 67, 4945-4955.

von Schwedler, U.K., Stemmler, T.L., Klishko, V.Y., Li, S., Albertine, K.H., Davis, D.R., Sundquist, W.I., 1998. Proteolytic refolding of the HIV-1 capsid protein amino-terminus facilitates viral core assembly. *EMBO J* 17, 1555-1568.

Walker, R.C., Jr., Khan, M.A., Kao, S., Goila-Gaur, R., Miyagi, E., Strebel, K., 2010. Identification of dominant negative human immunodeficiency virus type 1 Vif mutants that interfere with the functional inactivation of APOBEC3G by virus-encoded Vif. *J Virol* 84, 5201-5211.

Wang, J., Zhang, W., Lv, M., Zuo, T., Kong, W., Yu, X., 2011. Identification of a Cullin5-ElonginB-ElonginC E3 complex in degradation of feline immunodeficiency virus Vif-mediated feline APOBEC3 proteins. *J Virol* 85, 12482-12491.

Weiss, R.A., 2006. The discovery of endogenous retroviruses. *Retrovirology* 3, 67.

Wiegand, H.L., Doehle, B.P., Bogerd, H.P., Cullen, B.R., 2004. A second human antiretroviral factor, APOBEC3F, is suppressed by the HIV-1 and HIV-2 Vif proteins. *EMBO J* 23, 2451-2458.

Yamashita, M., Perez, O., Hope, T.J., Emerman, M., 2007. Evidence for direct involvement of the capsid protein in HIV infection of nondividing cells. *PLoS Pathog* 3, 1502-1510.

Yang, S., Sun, Y., Zhang, H., 2001. The multimerization of human immunodeficiency virus type I Vif protein: a requirement for Vif function in the viral life cycle. *J Biol Chem* 276, 4889-4893.

Yap, M.W., Nisole, S., Stoye, J.P., 2005. A single amino acid change in the SPRY domain of human Trim5α leads to HIV-1 restriction. *Curr Biol* 15, 73-78.

Yu, X., Yu, Y., Liu, B., Luo, K., Kong, W., Mao, P., Yu, X.F., 2003. Induction of APOBEC3G ubiquitination and degradation by an HIV-1 Vif-Cul5-SCF complex. *Science* 302, 1056-1060.

Yu, Y., Xiao, Z., Ehrlich, E.S., Yu, X., Yu, X.F., 2004. Selective assembly of HIV-1 Vif-Cul5-ElonginB-ElonginC E3 ubiquitin ligase complex through a novel SOCS box and upstream cysteines. *Genes Dev* 18, 2867-2872.

Zhen, A., Wang, T., Zhao, K., Xiong, Y., Yu, X.F., 2010. A single amino acid difference in human APOBEC3H variants determines HIV-1 Vif sensitivity. *J Virol* 84, 1902-1911.

Zheng, Y.H., Irwin, D., Kurosu, T., Tokunaga, K., Sata, T., Peterlin, B.M., 2004. Human APOBEC3F is another host factor that blocks human immunodeficiency virus type 1 replication. *J Virol* 78, 6073-6076.

7 Appendix

7.1 Appendix I – primers

Name	Sequence	Tm (°C)	GC%
1772VifAA forw	5'-GCC CAA TGG GTT ACA CGG CAG CTT GGT CTC TGC AG-3'	75.3	60.0
1772VifAA rev	5'-CTG CAG AGA CCA AGC TGC CGT GTA ACC CAT TGG GC-3'	75.3	60.0
V5094	~20 nt from the 5'-end base nr 5094 of MVV		
V-5951	5'-GACAGGCTTATCACAGTTCA-3'	55.3	45.0
vifopti1forw	5'-GCG GTG CTG TTA ACG GTG G-3'	61.0	63.2
vifopti2rev	5'-CAG GGT GTG TCA CAG AGA AGG GG-3'	66.0	60.9
VifAAforw	5'-GGG ATA CAC CGC CGC CTG GAG TCT GCA GG-3'	75.2	69.0
VifAArev	5'-CCT GCA GAC TCC AGG CGG CGG TGT ATC CC-3'	75.2	69.0
V-1818xbal	5'-GCT CTA GAT TAC AAC ATA GGG GGC GCG GA-3'	69.5	55.2
V1636	5'-TAA ATC AAA AGT GTT ATA ATT GTG GGA-3'	55.8	25.9
V-1719	5'-TCC CAC AAT GAT GGC ATA TTA TTC-3'	57.6	37.5
V1665 TaqMan	5'-FAM-CCA GGA CAT CTC GCA AGA CAG TGT AGA CA-BHQ-1-3'	68.1	51.7
V1142	5'-TGA AGA AGC AGA AAG GTG GG-3'	57.3	50.0
V-5789	5'-TAG CCA TCG TTG TAG TCT TTC G-3'	58.4	45.5
V5461	5'-GTT AAG AAT ACA AAT CCA AGA AG-3'	56.0	30.4
V-5951	5'-GAC AGG CTT ATC ACA GTT CA-3'	55.3	45.0

7.2 Appendix II – Nucleotide and amino acid sequences

Codonoptimized Vif

HA-tag
c-myc-tag
720 bases

```
atgctctccagttacaggcaccagaagaaatataaaaagaacaaggcccgggagataggc
ccccagctgccactgtgggcatggaaagaaacagcattctctatcaatcaggagccctac
tggtatagcactattagactgcaggggctgatgtggaacaagcgaggccataagctgatg
tttgtgaaggaaaatcagggctacgaatattgggaaacctcagggaagcagtggaagatg
gaaatcagaagggatctggacctgatcgctcagatcaattttcgcaacgcttggcagtac
aagtcccaggggggaatggaagacaattggggtgtggtacgagagccccggagactacaag
ggaaaagagaaccaattctggttccactggcggattgccctctgcagctgtaacaagacc
aggtgggacatcagagagtttatgattgggaagcacaggtgggacctgtgcaaaagctgc
atccagggcgagattgtcaaaaacactaaccccagaagcctgcagagacttgccctgctt
cacctcgctaaggatcatgtgttccaggtcatgcctctttggagggcacggagggtgact
gtgcaaaagttcccatggtgcaggtctccaatgggatacaccatccctggagtctgcag
                                          gccgcc
gagtgttgggagatggagagcatttttgagtacccatacgacgtcccagattatgcgtag
                                gaacaaaaacttatttctgaagaagatctgtag
```

239 a.a.

```
  1   MLSSYRHQKKYKKNKAREIGPQLPLWAWKETAFSINQEPYWYSTIRLQGLMWNKRGHKLM
 61   FVKENQGYEYWETSGKQWKMEIRRDLDLIAQINFRNAWQYKSQGEWKTIGVWYESPGDYK
121   GKENQFWFHWRIALCSCNKTRWDIREFMIGKHRWDLCKSCIQGEIVKNTNPRSLQRLALL
181   HLAKDHVFQVMPLWRARRVTVQKFPWCRSPMGYTIPWSLQECWEMESIFEYPYDVPDYAstop
                                        AA                EQKLISEEDLstop
```

7.3 Appendix III – Buffers and solutions used

Fixative

2 g paraformaldehyde, 0.5 mM NaOH, 1x PBS, water to 50 ml, at pH 7.4

TNE buffer

0.1% Triton X-100, 10 mM Tris at pH 7.5, 100 mM NaCl and 1 mM EDTA at pH 7.5.

0.5x Tris borate-EDTA (TBE)

0.045 M Tris borate, 0.001 M EDTA

10X Restriction buffer (RSB)

50% glycerol, 15 mM EDTA, 0.25% bromophenol blue

LB medium

1% Tryptone, 0.1% Yeast extract, 1% NaCl

SOC medium

2% Tryptone, 0.5% Yeast extract, 0.05% NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM Glucose

LB agar

1% Tryptone, 0.1% Yeast extract, 1% NaCl, 1.5% Bacto agar

2x Laemmli sample buffer

0.5% 2-mercaptoethanol, 20% glycine, 2% SDS, 0.1% bromophenol blue, 130 mM Tris

Transfer buffer

25 mM Tris, 192 mM glycine, 20% methanol

TBS-T

Tris buffered saline, 0.1% Tween 20

BCIP/NBT

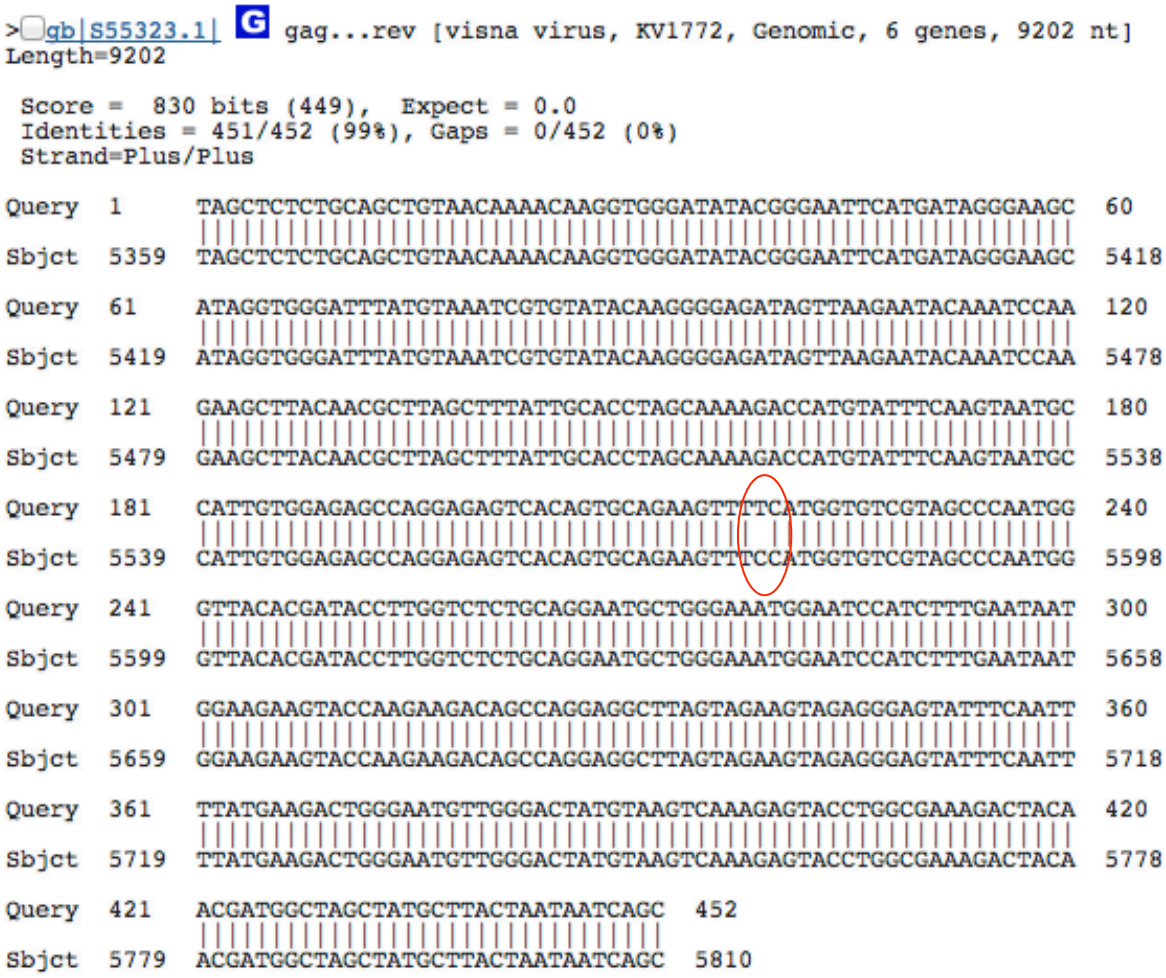
5-bromo-4-chloro-3-indosyl phosphate/Nitro blue tetrazoliumchloride

Alkaline phosphatase buffer

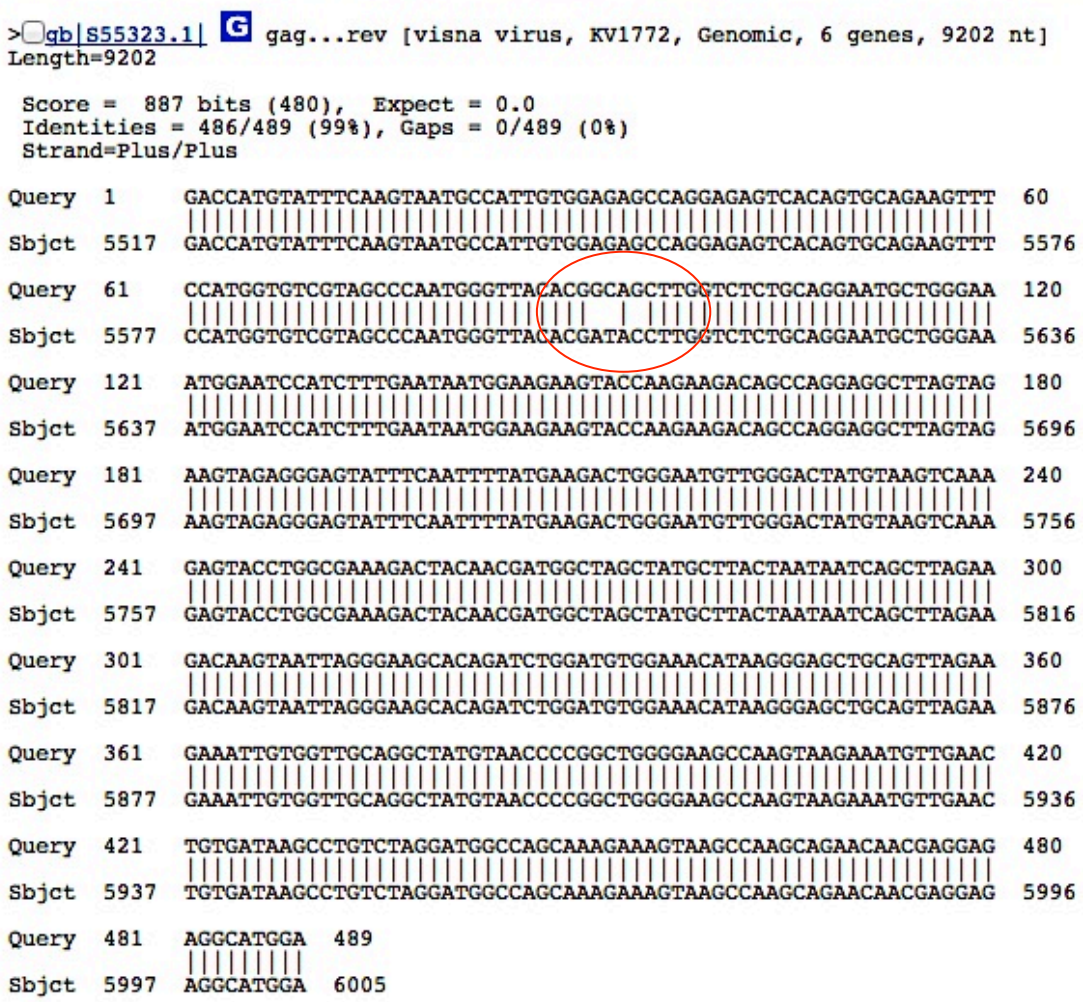
100 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, 0.05% Tween 20, at pH 9.5

7.4 Appendix IV – BLAST alignments of sequences

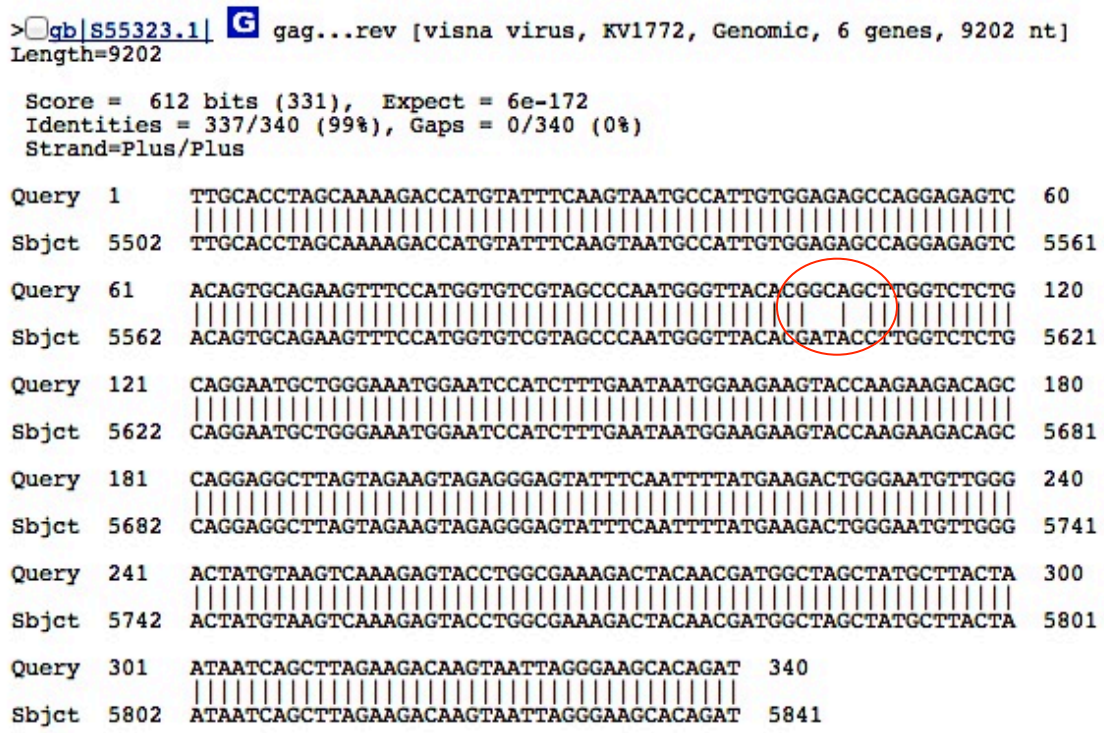
CA-Vif virus grown in SCP cells sequenced and aligned with Kv1772. C5578T mutation circled in red.



p8XSp5-RK1 with IP-AA mutations was sequenced and aligned with kv1772. Mutations in nucleotides 5607, 5608 and 5610 are circled in red. Other nucleotides match Kv1772 strain of MVV perfectly.



VifAA virus from infection of FOS cells was sequenced and aligned with kv1772. Mutations in nucleotides leading to IP-AA are circled in red.



Codonoptimized vif in pVR1012 with IP-AA mutations was sequenced and aligned with codonoptimizes wt vif with HA-tag in pVR1012. Mutations are circled in red, ATCC in subject sequence should line up with GCCG in query sequence.