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# Cellular Restriction Factors: Exploiting the Body's Antiviral Proteins to Combat HIV-1/AIDS

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## 1. Introduction

### 1.1 Overview of HIV-1/AIDS therapies

In 1983, when researchers first isolated HIV-1 from an AIDS patient, few imagined that it foretold a worldwide pandemic (Broder & Gallo, 1984, Barre-Sinoussi et al., 1983). More than 25 years later, 65 million people have been infected with HIV-1; nearly half of these people have died of AIDS, and despite many scientific advances we are still without an efficacious vaccine (Merson, 2006). The majority of HIV-1 infections and deaths have occurred in developing countries, with sub-Saharan Africa accounting for over 38 million HIV-1 infections alone. Sadly, the number of new infections currently exceeds our ability to treat everyone infected with the virus, and in the hardest-hit countries the social and economic backlash has been profound.

After HIV-1 was isolated, a blood test to screen patients and the blood supply quickly followed, as did research on its structure and pathogenesis. Many assumed that a vaccine would be available in a few years, and excitement increased further with the licensing of the first effective drug against HIV-1, zidovudine (AZT) (Fischl et al., 1987). However, researchers soon discovered that the virus was highly resilient, and HIV-1 quickly developed resistance to AZT (Poli et al., 1989, Richman et al., 1994). Over the next few years, a number of new antiretroviral drugs were developed that attacked the virus in different ways, and it was at this time that a new approach to therapy ensued. Highly active antiretroviral therapy (HAART) combined three or more different drugs to reduce HIV-1 replication, and significantly improved the prognosis of HIV-1-infected individuals (Richman et al., 2009). However, HAART was not a cure, and many patients were resistant to at least one of the antiretroviral drugs. In addition, the drugs were highly toxic, making adherence to treatment difficult.

During this time, the vaccine field was also hard at work, trying to develop a safe and effective HIV-1 vaccine. Most initial vaccine approaches focused on the HIV-1 envelope protein (gp120), and aimed to induce an antibody response to gp120. AIDSVAX was the first vaccine candidate of this type, and was developed by a U.S. pharmaceutical company called VaxGen (Flynn et al., 2005, Pitisuttithum et al., 2006). An alternative approach, developed by Merck, aimed to induce a T-cell response to HIV-1 using a recombinant adenovirus vector expressing HIV-1 Gag, Pol and Nef proteins (Shiver et al., 2002). Unfortunately, results from

both of these trials were disappointing, and neither approach provided protection from HIV-1 infection (Buchbinder et al., 2008, McElrath et al., 2008). Moreover, the Merck vaccine actually seemed to suppress the immune response, due to pre-existing immunity to the adenovirus vector (Priddy et al., 2008, Roberts et al., 2006).

The third and largest trial was recently performed in Thailand, and aimed to induce both a T-cell and antibody response to HIV-1. In the study, 16,000 Thai men and women received either placebo or vaccine injections, and were subsequently monitored for HIV-1 infection over a 3 year period (Rerks-Ngarm et al., 2009). The vaccine group received injections of a recombinant canarypox vector vaccine ALVAC-HIV (Sanofi Pasteur), plus booster injections of a recombinant gp120 subunit vaccine AIDSVAX B/E (Global Solutions for Infectious Diseases). Results from this trial showed a modest benefit among vaccine recipients, with a vaccine efficacy of 26-30%. However, vaccination did not affect the levels of viremia or CD4+ T cell counts of infected individuals, and many were disappointed with the results (Rerks-Ngarm et al., 2009).

Over the last decade, several host proteins have been identified that are capable of inhibiting HIV-1 replication (Figure 1). These so-called ‘cellular-restriction factors’ are a new arm of the innate immune system, and inhibit stages of the HIV-1 lifecycle that are not targeted by current AIDS therapies. Our understanding of how cellular restriction factors target HIV-1 replication is far from complete, but research in this area may provide a new avenue for future AIDS therapies (Barr, 2010).

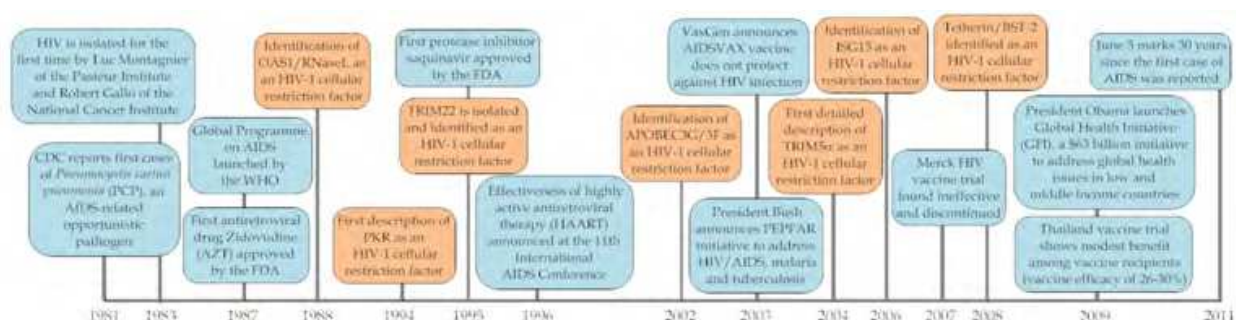


Fig. 1. HIV-1/ AIDS timeline. Timeline documenting major events in the HIV-1/ AIDS pandemic, as well as the identification of several key HIV-1 cellular restriction factors.

## 1.2 Cellular restriction factors

Cellular restriction factors are host proteins that can inhibit specific steps in the lifecycle of a virus. The concept of cellular restriction factors first emerged in the 1970's, when researchers identified strains of inbred mice that were resistant to Friend murine leukemia virus (MLV)-induced leukemia (Lilly, 1967, Pincus et al., 1971). Interestingly, these studies showed that mice with certain 'Friend virus susceptibility' (Fv) loci, could inhibit MLV replication *in vitro* and were subsequently resistant to leukemia. The *Fv1* and *Fv4* genes were particularly interesting, and were later shown to encode host proteins that resembled virus components. The *Fv1* gene encoded a protein that was similar to an endogenous retroviral Gag protein, and was shown to inhibit a post-entry stage of MLV replication (Ikeda et al., 1985). In contrast, the *Fv4* gene encoded a protein that resembled *env* (envelope) sequences in a specific strain of MLV, which obstructed binding of wild-type MLV to target cells (Pryciak & Varmus, 1992).

Since these discoveries, several new cellular restriction factors have been identified, including a number of factors that restrict HIV-1 replication (Chakrabarti & Simon, 2010). Many of these restriction factors are up-regulated in response to type I interferons (IFNs), which are typically activated in the presence of viruses by pattern recognition receptors (PRRs), such as Toll-like receptors or retinoic acid induced gene (RIG)-like receptors (Kumagai et al., 2008). Following secretion, type I IFNs bind to the interferon  $\alpha/\beta$  receptor (IFNAR) on the cell surface and induce signalling through the Janus Kinases/Signal Transducers and Activators of Transcription (Jak/Stat) pathway. This leads to the activation of hundreds of IFN-responsive genes that restrict viral replication, including cellular restriction factors (Baum & Garcia-Sastre, 2010) (Figure 2).

Type I IFNs potently inhibit early and late stages of the HIV-1 lifecycle, and systemic administration of IFN $\alpha$  reduces HIV-1 plasma viremia *in vivo* (Meylan et al., 1993, Tavel et al., 2010). During viral infection, plasmacytoid dendritic cells (pDCs) are the main producers of IFN $\alpha$ , however the capacity of pDCs to produce IFN $\alpha$  is impaired during acute HIV-1 infection, and this DC subtype appears to be depleted in chronic HIV-1 infection (Borrow & Bhardwaj, 2008, Soumelis et al., 2001). In addition, the HIV-1 accessory proteins Vpr and Vif can degrade interferon regulatory factor 3 (IRF-3), which plays a critical role in type I IFN induction (Okumura et al., 2008). Since cellular restriction factors are the 'effector' proteins of the IFN response, differences in the ability of pDCs to produce IFN $\alpha$  may contribute to differences in HIV-1 replication and disease progression among patients. Understanding the molecular mechanisms behind these HIV-1 restriction factors, may lead to the development of drugs that mimic or promote their activities.

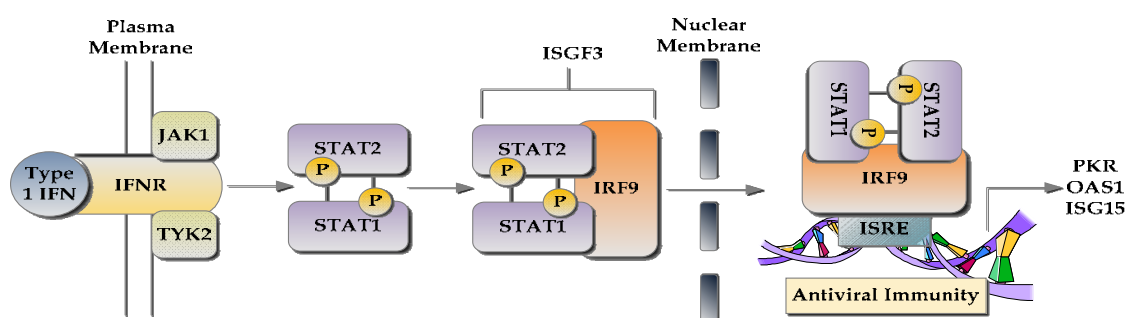


Fig. 2. The interferon response. Interferon (IFN) binds to the interferon receptor at the plasma membrane, activating the Janus Kinases/Signal Transducers and Activators of Transcription (Jak/Stat) pathway. This results in the up-regulation of hundreds of IFN-responsive genes, including cellular restriction factors that prevent HIV-1 replication.

### 1.3 The HIV-1 lifecycle

The HIV-1 lifecycle offers a multitude of steps that can be targeted by cellular HIV-1 restriction factors (Figure 3). The lifecycle begins when the HIV-1 envelope protein (gp120) binds to the host cell via its CD4 receptor, and following a conformational change, it binds to either the CXCR4 or CCR5 chemokine co-receptor (Deng et al., 1996). This interaction facilitates viral and cell membrane fusion, which is followed by the release of the viral core into the cell cytoplasm. In the cytoplasm, the HIV-1 capsid protein is lost in a process called uncoating, and the single-stranded RNA genome is reverse-transcribed into double-stranded cDNA. Reverse transcription is carried out by the virion-associated reverse

transcriptase enzyme, and precedes the formation of the multimeric pre-integration complex. This complex, which consists of both host and viral proteins, is transported along microtubules to the nucleus, where the HIV-1 integrase enzyme facilitates integration of the viral cDNA into the host genome. Following integration, HIV-1 transcription occurs from the 5' long terminal repeat (LTR) promoter, and leads to the synthesis of spliced HIV-1 RNA and unspliced HIV-1 genomic RNA. The RNA is exported into the cytoplasm where the main structural protein of HIV-1, the Gag polyprotein, is translated along with other viral proteins. The Gag protein oligomerizes and traffics to the cell membrane, where it forms higher-order structures and assembles into virions with other viral proteins. Cellular proteins are also involved in assembly, particularly Tsg101 and AIP1/ALIX, which participate in the budding and release of immature, non-infectious virions from the cell membrane (Garrus et al., 2001, Strack et al., 2003). As budding occurs, the Gag protein is cleaved into its four structural domains (matrix, capsid, nucleocapsid and p6) by the viral protease, generating mature infectious viral particles that are released from the cell membrane (Ganser-Pornillos et al., 2008, Ono, 2009). The following sections review the main HIV-1 cellular restriction factors in the order of their point of attack in the lifecycle, beginning with capsid uncoating and ending with viral release.

## 2. Lifecycle target: HIV-1 capsid uncoating

### 2.1 TRIM5 $\alpha$

#### 2.1.1 TRIM5 $\alpha$ : History and background

For years, researchers have been aware of a barrier to HIV-1 infection in Old world monkey (OWM) cells, however they have only recently begun to understand the nature of it. Early in the AIDS epidemic, the discovery that the host-range of HIV-1 was limited to humans and apes suggested that other primates may have an internal mechanism to combat HIV-1 infection (Alter et al., 1984, Gajdusek et al., 1985, Lusso et al., 1988). A large number of mammalian cell lines were tested for susceptibility to HIV-1 infection, including cells derived from humans, OWMs (monkeys of African and Asian origin) and New world monkeys (NWM) (monkeys of Central and South American origin) (Hofmann et al., 1999). Interestingly, HIV-1 replication was blocked in most OWM-derived cell lines and one species of NWM, the Owl monkey. Because of its initial definition as a genetic barrier to lentiviral infection, the restriction factor was originally named lentivirus susceptibility factor-1 (Lv-1) (Cowan et al., 2002).

TRIM5 $\alpha$  was not identified as the protein responsible for the OWM block until it was isolated during a cDNA screen of HIV-1 resistant rhesus macaque cells. In the study, a cDNA library was created from HIV-1 resistant rhesus macaque cells and the cDNA clones from this library were transduced into an HIV-1 sensitive human cell line (HeLa) (Stremlau et al., 2004). The human cells were then challenged with HIV-1 and screened for resistant clones, which identified the rhesus orthologue of TRIM5 $\alpha$  (rhTRIM5 $\alpha$ ). Excitingly, around the same time, TRIM5 $\alpha$  was also linked to the HIV-1 block in Owl monkey cells; however, the Owl monkey version of *TRIM5a* encoded a TRIM5 $\alpha$ -cyclophilin A fusion protein (Sayah et al., 2004). Cyclophilin A (CypA) was previously shown to bind to the HIV-1 capsid protein and promote HIV-1 replication in human cells; however, in Owl monkey cells CypA seemed to restrict HIV-1 (Luban, 2007, Sokolskaja & Luban, 2006). The discovery of a TRIM5 $\alpha$ -CypA fusion protein explained these results and indicated that CypA may target TRIM5 $\alpha$ -CypA to incoming HIV-1 capsid proteins.



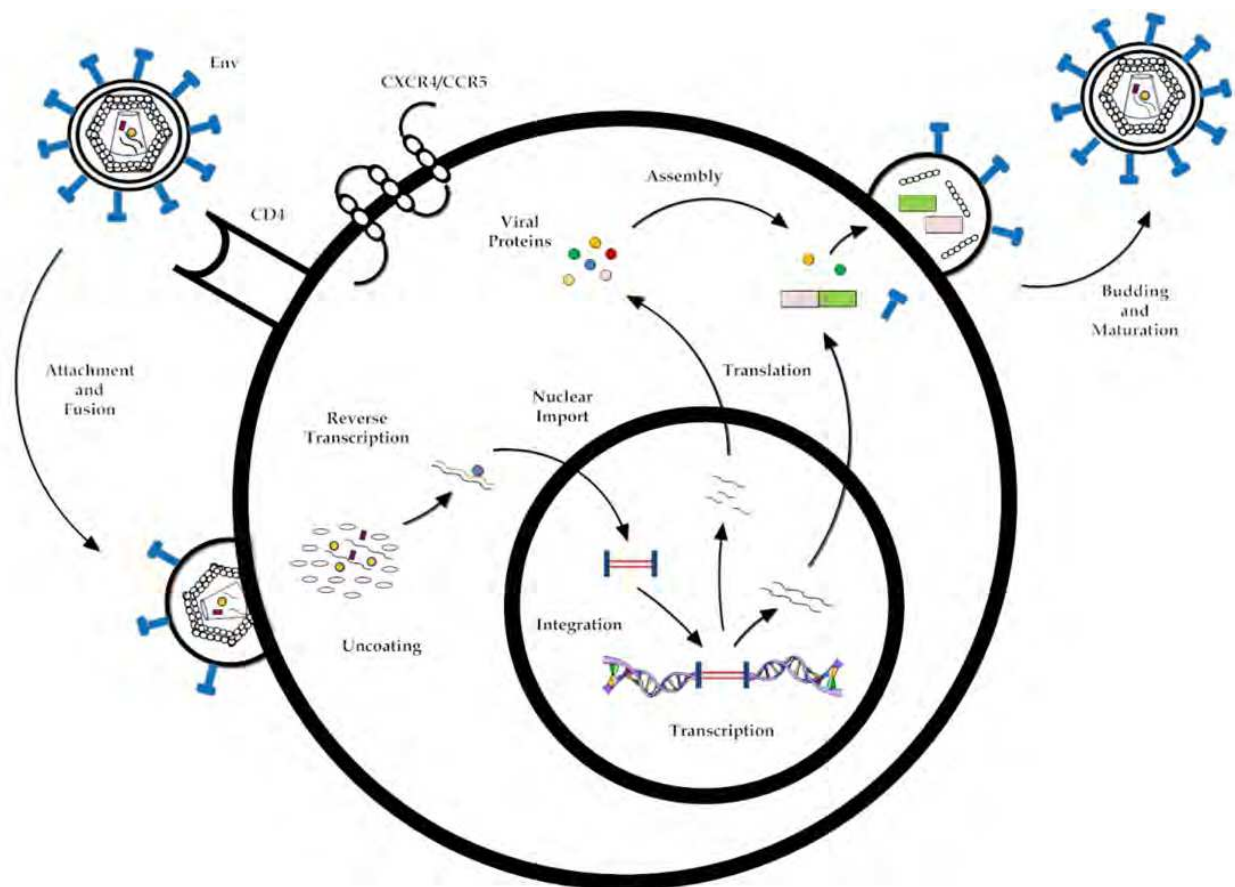


Fig. 3. The HIV-1 lifecycle. The HIV-1 envelope protein (Env) binds to the CD4<sup>+</sup> receptor and CXCR4/CCR5 co-receptor on the host cell. The viral core is released into the cytoplasm, where the RNA genome is reverse-transcribed into double-stranded cDNA. The cDNA is imported into the nucleus, where it integrates into the host genome. The viral genes are transcribed and the RNA is exported to the cytoplasm, where it is translated into protein. The viral proteins traffic to the membrane where they assemble and bud out of the host cell.

### 2.1.2 TRIM5α: Structure and function

TRIM5α belongs to the tripartite motif-containing (TRIM) family of proteins, of which there are currently 77 identified members (Nisole et al., 2005). Although the *TRIM5* gene gives rise to several isoforms through differential splicing, TRIM5α is the only isoform with potent anti-HIV-1 activity. All TRIM proteins have a conserved RBCC motif, which consists of a RING domain, one or two B-box domains and a predicted coiled-coil region. The majority of TRIM proteins, including TRIM5α, have a C-terminal B30.2 domain. The Really Interesting New Gene (RING) domain has intrinsic E3 ligase activity, and together with an E1 activating enzyme and an E2 conjugating enzyme it can transfer ubiquitin or ubiquitin-like molecules to target proteins (Ozato et al., 2008). This modification can alter a protein's half-life, subcellular localization or interaction with other proteins. Importantly, two RING domain cysteine residues (C15 and C18) are essential for the E3 ligase activity of the RING domain (C15 and C18). These two residues are also required by rhTRIM5α for restricting HIV-1 replication (Diaz-Griffero et al., 2006).

The function of the B-box domain remains largely uncharacterized; however, it is an interesting domain because it is unique to TRIM proteins. Deletion of the B-box domain of TRIM5 $\alpha$  eliminates the ability of TRIM5 $\alpha$  to restrict HIV-1, suggesting that this domain is critical for TRIM5 $\alpha$ -mediated restriction (Stremlau et al., 2004, Javanbakht et al., 2005, Li & Sodroski, 2008, Perez-Caballero et al., 2005). In addition, it was recently shown that the B-box domain promotes HIV-1 capsid binding by mediating higher-order self-association (Li & Sodroski, 2008). The coiled-coil region is involved in protein-protein interactions and more specifically, it is thought to mediate TRIM5 $\alpha$  oligomer formation. It has been proposed that oligomer formation is important for positioning the B30.2 domain of TRIM5 $\alpha$  for optimal HIV-1 capsid binding and accordingly, TRIM5 $\alpha$  coiled-coil mutants fail to restrict HIV-1 (Perez-Caballero et al., 2005, Javanbakht et al., 2006). Finally, the specificity and interspecies variability of TRIM5 $\alpha$  is found in the B30.2 domain. Sequence analysis has shown a significant amount of interspecies variability within the B30.2 domain of both NWM and OWM, especially on several variable loops that are thought to form the binding surface for HIV-1 capsid recognition (Ohkura et al., 2006, Woo et al., 2006, Yao et al., 2006).

### 2.1.3 TRIM5 $\alpha$ -mediated HIV-1 restriction

To date, rhTRIM5 $\alpha$  is the earliest-acting HIV-1 restriction factor and targets virus replication immediately after HIV-1 entry into target cells. Several studies have shown that rhTRIM5 $\alpha$  blocks reverse transcription and nuclear import of viral cDNA. The mechanisms underlying this restriction are thought to include: sequestration of the virus core in the cytoplasm, modification of the virus core leading to degradation, or interference in the trafficking of the preintegration complex (Bieniasz, 2004, Chatterji et al., 2006, Stremlau et al., 2006). The most favoured mechanism involves rhTRIM5 $\alpha$  binding to the viral core and disrupting the normal uncoating process of the core (Figure 4). This binding involves the recognition of specific sequences

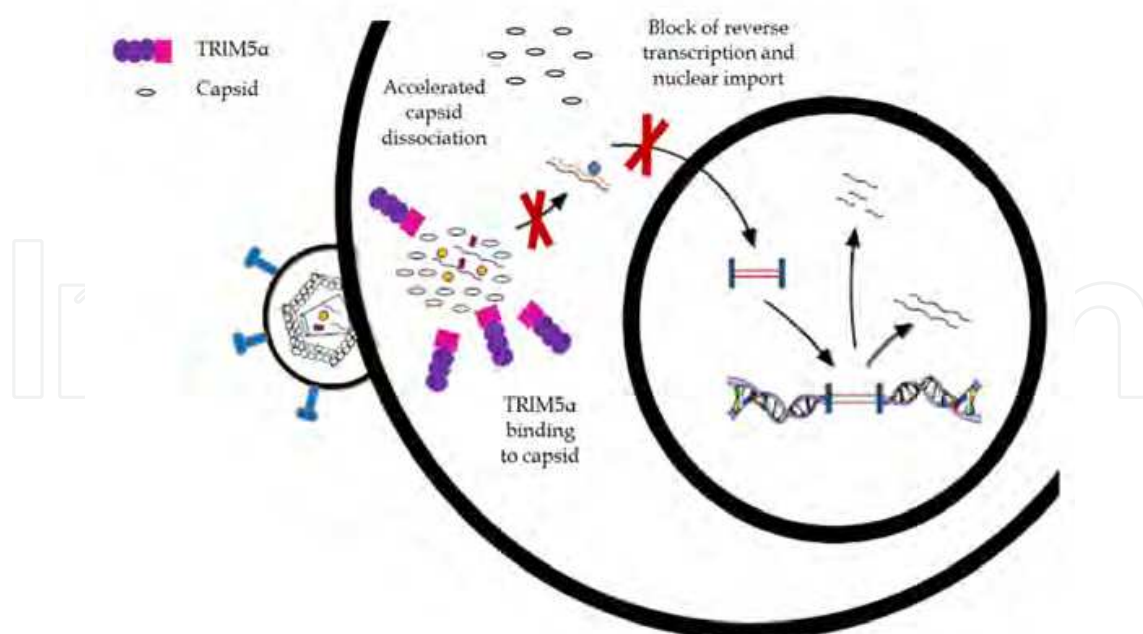


Fig. 4. TRIM5 $\alpha$ -mediated HIV-1 restriction. RhTRIM5 $\alpha$  binds to incoming HIV-1 capsid proteins via its B30.2 domain, causing them to rapidly dissociate and prematurely disassemble. This leads to a block in HIV-1 reverse transcription and inhibits nuclear import of viral cDNA, restricting further propagation of the virus.

on the HIV-1 capsid by the B30.2 domain of rhTRIM5 $\alpha$  and subsequent block at the level of reverse transcription. Interestingly, human TRIM5 $\alpha$  (huTRIM5 $\alpha$ ) only modestly inhibits HIV-1 replication and substitution of a single amino acid (R332) in its B30.2 domain enables it to restrict HIV-1 as potently as rhTRIM5 $\alpha$  (Yap et al., 2005).

### 3. Lifecycle target: HIV-1 reverse transcription

#### 3.1 APOBEC3

##### 3.1.1 APOBEC3: Structure and function

The human apolipoprotein B mRNA-editing catalytic polypeptide-like 3 (APOBEC3) family is part of a larger family of APOBEC cytidine deaminases, capable of converting cytosine to uracil in RNA or DNA. The APOBEC3 family is found on chromosome 22 and contains seven members (A-H), which are believed to be the result of multiple duplication events (Conticello et al., 2005). Interestingly, APOBEC3 proteins appear to be under positive selective pressure, possibly as a defence against endogenous retroelements, and have been shown to have antiviral activity against a variety of viruses, including murine leukemia virus, human T-lymphotropic virus, simian immunodeficiency virus and the recently discovered Xenotropic Murine leukemia virus-Related Virus (XMRV) (Sawyer et al., 2004, Sawyer et al., 2004, Groom et al., 2010, Groom et al., 2010, Aguiar & Peterlin, 2008, Niewiadomska & Yu, 2009). In addition, all seven members have been implicated in HIV-1 restriction, however APOBEC3F/G are the best studied and appear to be the most potent restrictors (Hultquist & Harris, 2009).

Before APOBEC3 proteins were specifically identified, it was discovered that HIV-1 clones with the accessory protein Vif deleted were capable of replicating in certain cell lines. These cells were termed “permissive cells”, and included HeLa, HEK 293T, SupT1 and CEM-SS lines. In other cells, such as primary human T-lymphocytes or macrophages, or the H9 and CEM T cell lines, virions produced from Vif-deficient strains were up to 1,000 times less infectious compared to virions from wild-type strains (Gabuzda et al., 1992). Cell fusion experiments revealed that this “non-permissive” phenotype was dominant, and comparison of the related CEM T and CEM-SS cell lines revealed a 1.5 kb cDNA segment expressed in CEM T cells that was not produced in CEM-SS cells (Madani & Kabat, 1998, Sheehy et al., 2002, Simon et al., 1998). This protein, termed CEM15 and later renamed APOBEC3G (A3G), was shown to be suppressed by Vif, thus resulting in productive infection of non-permissive cells with wild-type HIV-1 (Sheehy et al., 2002).

##### 3.1.2 APOBEC3-mediated HIV-1 restriction

In the absence of Vif, A3G is packaged into newly formed virions and blocks HIV-1 replication after infection of a new cell. Two mechanisms of HIV-1 inhibition have been reported for A3G: 1) the production of hyper-mutated viral DNA and 2) decreased accumulation of viral DNA (Figure 5) (Anderson & Hope, 2008, Bishop et al., 2008, Lecossier et al., 2003, Mangeat et al., 2003, Simon & Malim, 1996). During reverse transcription, A3G induces cytidine deamination (C $\rightarrow$ U mutations) in the negative strand of newly synthesized viral cDNA. The latter results in G $\rightarrow$ A hyper-mutated viral DNA and increases the probability of producing premature stop codons or mutated, non-functional viral proteins. Interestingly, cytidine deamination also recruits cellular uracil-DNA glycosylases that cleave off the uracil side chain as part of the base-excision repair pathway. The resulting abasic site may then prevent plus-strand DNA synthesis or lead to degradation of viral DNA by endonucleases (Klarmann et al., 2003, Yang et al., 2007). However, there is some controversy over the degree to which APOBEC3-mediated cytidine deamination contributes to reduced



accumulation of HIV-1 DNA, and it has been reported that the HIV-1 accessory protein Vpu induces the degradation of certain cellular uracil-DNA glycosylases (Schrofelbauer et al., 2005). Furthermore, loss of certain uracil-DNA glycosylases does not appear to affect APOBEC3 restriction of HIV-1, and similar results have been obtained for A3G restriction of other viruses (Kaiser & Emerman, 2006, Nguyen et al., 2007). Nevertheless, this does not rule out the possible involvement of other glycosylases, and additional studies will be required to fully elucidate the effects of APOBEC3 cytidine deaminase activity on HIV-1 replication.

Cytidine deaminase mutants of both A3F and A3G still retain a degree of anti-HIV-1 activity, and both proteins have been shown to affect the initiation of reverse transcription by interfering with tRNA<sub>3</sub><sup>Lys</sup> binding to viral RNA. In addition, A3G has been shown to inhibit both plus and minus strand transfer RNA integration and DNA elongation during reverse transcription (Hultquist & Harris, 2009). Interestingly, endogenous A3G in resting naive and memory CD4<sup>+</sup> T cells may inhibit newly infecting virus, contributing to the known resistance of quiescent T cells to HIV-1 infection (Chiu et al., 2005, Muckenfuss et al., 2006). In this model, A3G can exist in two forms: an inactive high molecular mass complex (HMM) in activated T cells or an enzymatically active low molecular mass form (LMM) in resting T cells. Since A3G can interfere with multiple steps of reverse transcription, this hypothesis is consistent with infection of resting T cells, in which cDNA synthesis is initiated, but the viral genome is not completely reverse transcribed.

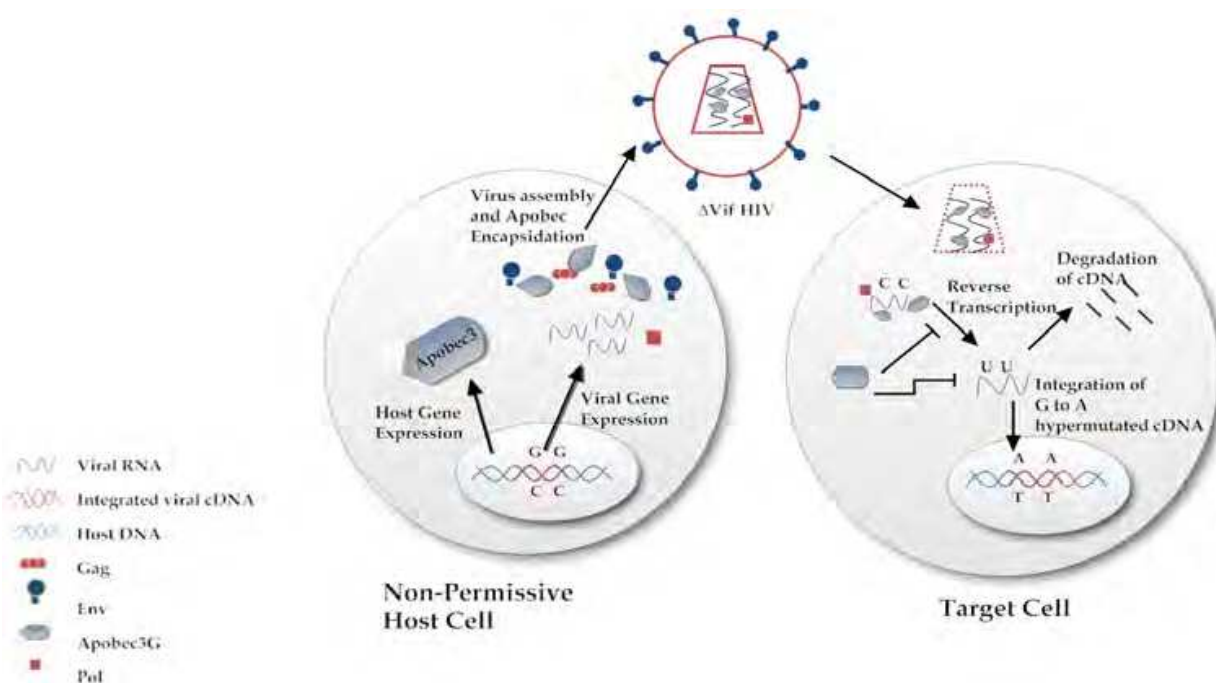


Fig. 5. APOBEC3G-mediated restriction of Vif-deficient HIV-1. APOBEC3G (A3G) is packaged into newly formed virions and interferes with viral replication upon infection of new cells. A3G directly interferes with reverse transcription, resulting in decreased amounts of viral cDNA. In addition, A3G acts as a cytidine deaminase, inducing C→U mutations in minus-strand viral cDNA. This leads to base excision by cellular uracil-DNA glycosylases or uracil bases are replaced with thymine, resulting in extensive G→A hypermutations in the viral genome.

In recent years, the resting T cell theory has received criticism, mainly because several results in the original work were not repeatable. However, it should be noted that many results were still repeatable including the presence of LMM/HMM in resting/activated T cells and the enzymatically active nature of LMM forms (Chiu et al., 2005). Furthermore, it was observed that factors such as IL-2 and IL-15 both increase susceptibility of resting T cells to HIV-1 infection and induce a shift of A3G organization from LMM to HMM forms, suggesting that these results may warrant further investigation. In addition, several APOBEC3 proteins appear to have cytidine deaminase-independent antiviral activity against Hepatitis B virus, Adeno-associated virus, and a number of retroelements, further supporting the existence of a cytidine deaminase-independent antiviral mechanism (Bogerd et al., 2006, Chen et al., 2006, Stenglein & Harris, 2006, Turelli et al., 2004). As such, the extent to which cytidine deaminase-dependent or -independent functions contribute to the antiviral activity of APOBEC3 proteins against HIV-1 requires further elucidation.

### 3.1.3 Countermeasures to APOBEC3-mediated HIV-1 restriction

Vif restores HIV-1 infectivity by inducing the degradation of APOBEC3 proteins (Conticello et al., 2003, Marin et al., 2003, Sheehy et al., 2003). Vif and APOBEC3 proteins physically interact with each other, and these interactions are specific, however they differ depending on the APOBEC3 member being targeted (Niewiadomska & Yu, 2009). Vif also contains several conserved elements that allow it to form a complex with certain cellular proteins. Specifically, the SLQxLA motif in Vif interacts with ElonginC, allowing the recruitment of ElonginB and Cul5, which bind Vif through another conserved motif. Rbx1 is also recruited to the complex, creating an E3 ligase capable of polyubiquitinating APOBEC3 proteins and targeting them for 26S proteasomal degradation (Figure 6) (Kobayashi et al., 2005, Mehle et al., 2004,

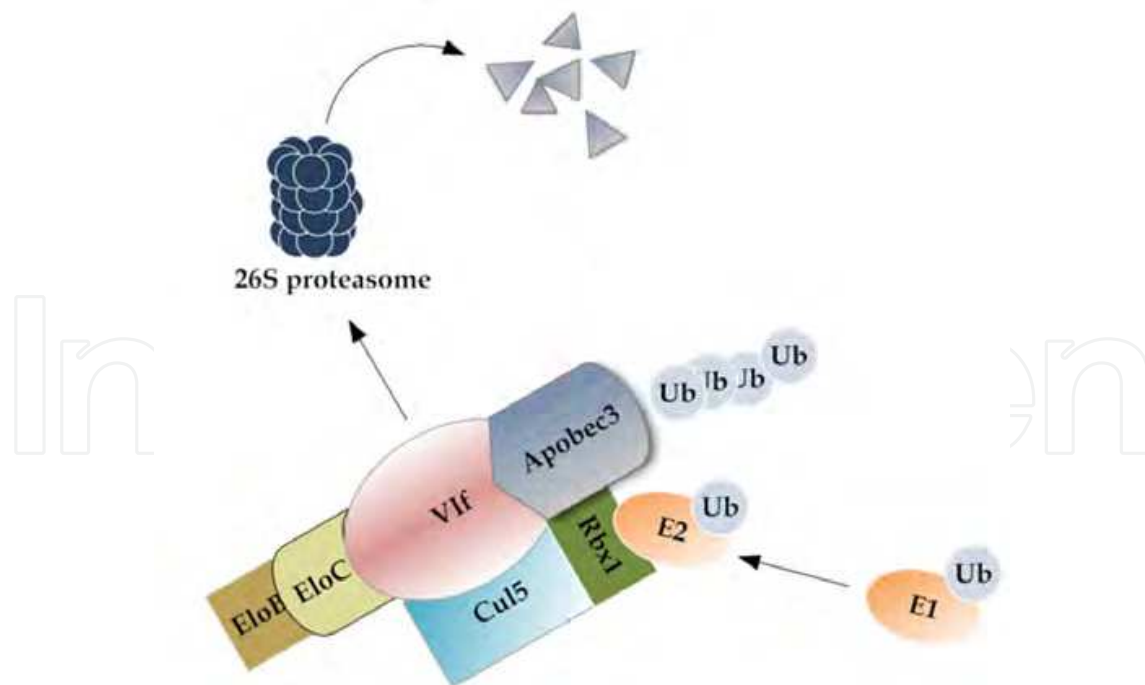


Fig. 6. Vif-mediated degradation of APOBEC3. Vif interacts with cellular factors to create a Skp1-cullin-F-box (SCF)-like complex that polyubiquitinates and degrades APOBEC3 molecules. Vif binds Cullin5 (Cul5) through two conserved motifs and other cellular factors, forming a scaffold for other E3 ligase components.

Yu et al., 2003). Interestingly, one report suggests that Vif instead of A3G is actually polyubiquitinated, possibly to serve as a way to transport A3G to the 26S proteasome for degradation (Dang et al., 2008). However, mutated and modified forms of Vif that are still capable of degrading A3G are unable to restore viral infectivity (Mehle et al., 2004, Kao et al., 2007). Thus, Vif may also inhibit A3G through degradation-independent mechanisms. Possible theories include a competitive inhibition model, where Vif binds to a common target, preventing A3G packaging, or that Vif inactivates A3G by inducing the formation of high molecular mass complexes (Goila-Gaur & Strebel, 2008, Goila-Gaur et al., 2008).

### 3.1.4 APOBEC3: Effects on HIV-1 replication *in vivo*

The extent to which APOBEC3 proteins are functional during HIV-1 infection *in vivo* is a highly contested topic. One report has observed the presence of extensive G→A hypermutation in virus samples collected from one HIV-1 long-term non-progressor (Wang et al., 2003)(Kao et al., 2007)(Kao et al., 2007)(Kao et al., 2007)(Kao et al., 2007)(Kao et al., 2007)(Kao et al., 2007)(Kao et al., 2007). Although this may suggest a role for APOBEC3 in controlling infection, it appears to be the only reported case, and thus the effects of APOBEC3 may have been secondary to some other mechanism of control. Still, it is possible that APOBEC3 proteins are more functional in certain patients/infections than in others. There are reports of both significant correlations and lack of correlation, between hypermutation and reduced viral load/higher CD4+ cell counts (Land et al., 2008, Piantadosi et al., 2009, Ulena et al., 2008). Though hypermutation-independent mechanisms may exist, other groups have shown a negative correlation between A3G mRNA expression levels and HIV-1 viremia, and a positive correlation between A3G mRNA levels and CD4+ cell counts (Ulena et al., 2008, Vazquez-Perez et al., 2009). Furthermore, it was shown that A3F/G mRNA levels post-infection are higher in patients with low viral set points than in patients with high viral set points, and higher in seronegative patients compared to healthy controls. Nevertheless, another group observed no correlation between A3F/G mRNA levels and viremia or CD4+ cell counts (Cho et al., 2006). Although conflicting reports exist, these may, in part, be explained by varying levels of APOBEC3 mRNA between donors (Koning et al., 2009). More detailed studies into both APOBEC3 expression and activity at the host level, in addition to correlation with disease progression, will be required to further elucidate its relationship to HIV-1 infection.

## 4. Lifecycle target: HIV-1 RNA

### 4.1 PKR

#### 4.1.1 PKR: Structure and function

Protein kinase R (PKR) is constitutively expressed in human cells as an inactive monomer. In the presence of double-stranded RNA (dsRNA), which forms the genetic material of some viruses, PKR forms a dimer and phosphorylates itself to become active (Dey et al., 2005, Garcia et al., 2007). Activation of PKR is also possible through type I IFN signalling, and PKR is upregulated in the presence of IFNs. PKR contains a N-terminal dsRNA binding domain (dsRBD), which is made up of two dsRBD motifs and can bind to dsRNA as short as 30bp (Figure 1) (Lemaire et al., 2008). Dimerization relies strongly on the dsRBD, and studies deleting this domain show impaired dimerization as well as lack of PKR activation (Cosentino et al., 1995). In addition to its dsRBD, PKR contains a C-terminal kinase domain that has intrinsic phosphotransferase activity. This function hinges on a key lysine residue at position 296, without which the kinase domain is inactive (Sadler et al., 2009).

#### 4.1.2 PKR-mediated inhibition of HIV-1 transcription

Upon activation, PKR inhibits HIV-1 replication through a number of different mechanisms. One mechanism involves phosphorylation of the enzyme RNA Helicase A (RHA), which has been shown to enhance HIV-1 transcriptional activity and binds to the HIV-1 trans-activation response (TAR) element (Fujii et al., 2001, Jeang & Yedavalli, 2006). The HIV-1 TAR element is required for trans-activation of the viral promoter and binds to the viral trans-activator of transcription (Tat) protein. This interaction greatly increases transcription of viral genes from the HIV-1 promoter, and results in the production of many full-length HIV-1 transcripts. The TAR element is an unusual stem-loop RNA structure that is located at the 5' end of all HIV-1 mRNAs (Adelson et al., 1999, Nagai et al., 1997). PKR recognizes TAR RNA as dsRNA, and it binds to the upper bulge of the lower stem-loop structure using both of its dsRNA binding motifs (Carpick et al., 1997, Kim et al., 2006).

Like PKR, RHA contains a dsRBD with two dsRNA binding motifs, which are required for TAR recognition and binding (Fujii et al., 2001). A lysine residue at position 236 of RHA is essential for TAR binding (Fujii et al., 2001, Jeang & Yedavalli, 2006). Recently, PKR has been shown to phosphorylate the dsRBD of RHA; a modification that depends on lysine 296 of the PKR protein (Sadler et al., 2009). Phosphorylation of RHA by PKR seems to inhibit RHA-TAR binding, which in turn decreases the levels of HIV-1 mRNA transcripts (Sadler et al., 2009).

#### 4.1.3 PKR-mediated inhibition of HIV-1 translation

Upon activation, PKR inhibits HIV-1 replication through a number of different mechanisms. The most widely researched pathway occurs through PKR-mediated phosphorylation of the alpha subunit of the eukaryotic initiation factor 2 (eIF2 $\alpha$ ), a key regulator of protein synthesis (Figure 7) (Dey et al., 2005, Rojas et al., 2010). In eukaryotic cells, eIF2 $\alpha$  bound to GTP mediates the formation of a trimeric complex with methionine transfer RNA (met-tRNA). This complex leads to met-tRNA binding to the 40S ribosomal subunit, and allows for the initiation of translation (Rojas et al., 2010, Nallagatla et al., 2011). However, phosphorylation of eIF2 $\alpha$  by PKR prevents the eIF2 $\alpha$ -GTP interaction and eliminates the formation of the trimeric complex, thus inhibiting translation of all mRNA, including viral mRNA (Nallagatla et al., 2011).

The HIV-1 trans-activation response (TAR) element is required for trans-activation of the viral promoter and binds to the viral trans-activator of transcription (Tat) protein. This interaction greatly increases viral gene expression from the HIV-1 promoter by inducing chromatin remodelling and by recruiting elongation-competent transcriptional complexes onto the viral LTR. The TAR element is an unusual stem-loop RNA structure that is located at the 5' end of all HIV-1 mRNAs (Adelson et al., 1999, Nagai et al., 1997). PKR recognizes TAR RNA as dsRNA, and it binds to the upper bulge of the lower stem-loop structure using both of its dsRNA binding motifs (Carpick et al., 1997, Kim et al., 2006). This leads to activation of PKR and phosphorylation of eIF2 $\alpha$ , which subsequently inhibits protein translation (Nallagatla et al., 2011, Roy et al., 1991).

#### 4.1.4 Countermeasures to PKR-mediated HIV-1 restriction

Although *in vitro* studies have shown that PKR can inhibit HIV-1 replication, *in vivo* models of infection fail to exhibit viral restriction. Interestingly, low levels of dsRNA have been shown to have beneficial effects on PKR activation; however, higher levels of dsRNA



(greater than a 1:1 ratio of dsRNA:PKR) may actually inhibit PKR activity (Chu et al., 1998, Hunter et al., 1975). During the initial stages of HIV-1 infection, low levels of TAR RNA (dsRNA) are produced and this leads to PKR activation (Lemaire et al., 2008). Conversely, in later stages of viral replication (when Tat-TAR binding enhances transcription), much more TAR RNA (dsRNA) is generated, which seems to inhibit PKR activity (Lemaire et al., 2008, Hunter et al., 1975, Clerzius et al., 2011, Manche et al., 1992). It has been proposed that high concentrations of dsRNA cause PKR to bind dsRNA as a monomer, which inhibits PKR dimerization and subsequent activation (Manche et al., 1992, Cole, 2007).

PKR activation is also interrupted by the HIV-1 Tat protein. Tat binds to the HIV-1 TAR element and in doing so, masks TAR recognition by PKR and inhibits PKR activation (Cai et al., 2000). Furthermore, Tat binds to PKR directly and therefore competes for PKR binding with eIF2 $\alpha$  (Brand et al., 1997). There is a high degree of sequence homology between the Tat and eIF2 $\alpha$  binding sites in PKR, which leads to a decrease in eIF2 $\alpha$  phosphorylation (Cai et al., 2000, Brand et al., 1997). In addition, Tat binding to PKR inhibits autophosphorylation, possibly by inhibiting PKR dimerization, which is necessary for its antiviral activity (Cai et al., 2000, Brand et al., 1997). There is also evidence that phosphorylation of Tat by PKR at several key amino acids (S62, T64, S68) actually enhances Tat's ability to initiate transcription; however, the precise mechanism of transcriptional enhancement is not yet clear (Endo-Munoz et al., 2005).

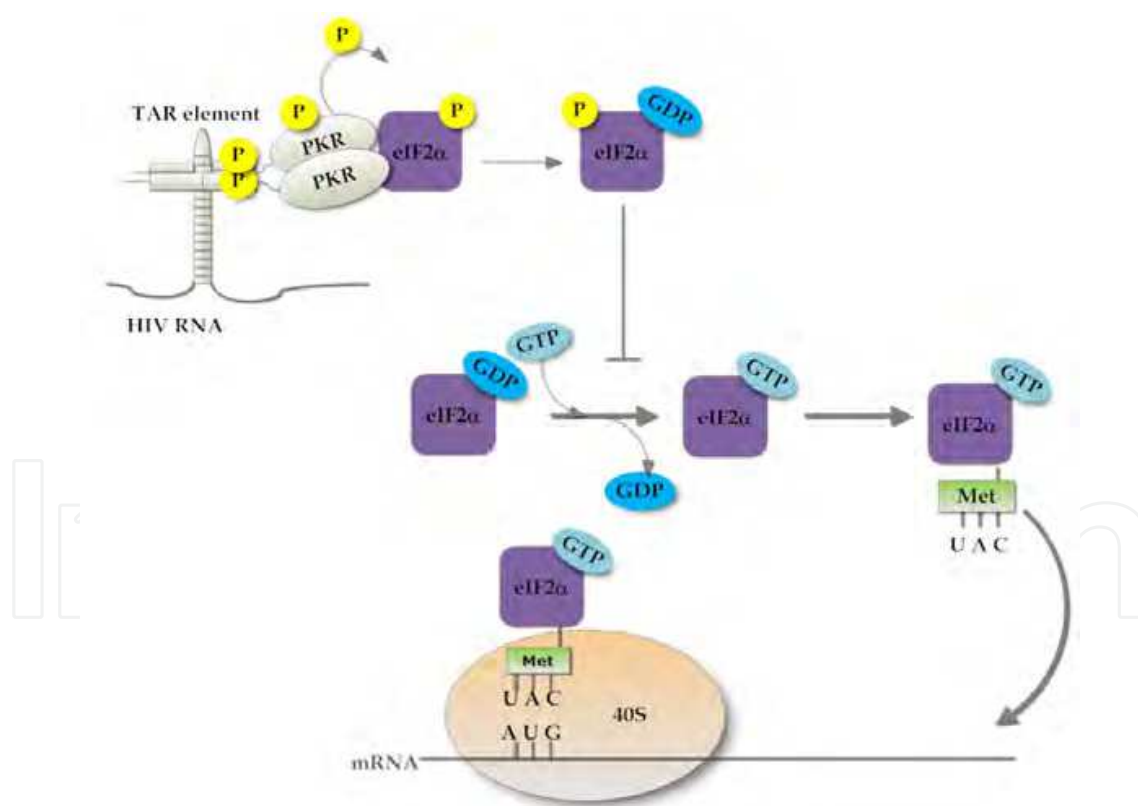


Fig. 7. PKR-mediated inhibition of HIV-1 protein translation. PKR recognizes the HIV-1 TAR element (dsRNA) and dimerizes and phosphorylates itself to become active. Active PKR phosphorylates eIF2 $\alpha$ , preventing guanine nucleotide exchange and eIF2 $\alpha$  activation. Inactive eIF2 $\alpha$  is unable to transfer methionine-tRNA to the 40S ribosome for mRNA translation, and protein translation is inhibited.

## 4.2 TRIM22

### 4.2.1 TRIM22: Structure and function

Tripartite motif-containing protein 22 (TRIM22) was originally isolated during a search for IFN-induced genes in Daudi cells, and is located at chromosomal position 11p15, immediately adjacent to the TRIM5 $\alpha$  gene (Tissot & Mechti, 1995). Similar to TRIM5 $\alpha$ , TRIM22 belongs to the TRIM family of proteins and is upregulated in response to Type I and Type II IFNs (Bouazzaoui et al., 2006). TRIM22 expression is altered by multiple cytokines and viral antigens/infections, including Hepatitis B virus, encephalomyocarditis virus, and HIV-1 (Gao et al., 2009, Gao et al., 2009, Eldin et al., 2009). TRIM22 may also play a role in cellular processes such as cell differentiation/proliferation, as it is a known p53 target gene and has been suggested to have potential anti-proliferative functions (Obad et al., 2004, Obad et al., 2007).

To date, several studies have addressed the effect of TRIM22 on HIV-1 replication. TRIM22 expression was first shown to reduce HIV-1 transcription from a luciferase reporter construct under control of the HIV-1 long terminal repeat promoter (LTR) (Tissot & Mechti, 1995). Although this report did not follow up these observations in the context of full-length, replication competent HIV-1, it was fundamental in identifying TRIM22 as a potential HIV-1 restriction factor. Eleven years later, TRIM22 expression was shown to be increased in response to *ex vivo* HIV-1 infection of primary monocyte-derived macrophages, a biological target of HIV-1 (Bouazzaoui et al., 2006). In addition, overexpression of TRIM22 restricted HIV-1 infection by 70-90% and prevented the formation of syncytia. In 2008, TRIM22 was confirmed to be a potent effector of the IFN response against HIV-1 infection, and two different methods of TRIM22-mediated late-stage HIV-1 restriction were observed: one dependent on, and a second independent of, the HIV-1 Gag polyprotein (Barr et al., 2008).

### 4.2.2 TRIM22-mediated inhibition of HIV-1 transcription

It has since been confirmed that TRIM22 is capable of restricting HIV-1 mediated transcription (Kajaste-Rudnitski et al., 2011). Different clones of the U937 promonocytic cell line have previously been identified to be either permissive or nonpermissive to HIV-1 replication (Franzoso et al., 1994). Examination of multiple IFN-induced restriction factors revealed that only TRIM22 was present in nonpermissive clones and absent in permissive clones. LTR-driven transcription between permissive and nonpermissive clones was examined using a reporter construct expressing luciferase under control of the HIV-1 LTR. Basal transcription levels were decreased 7-10 fold in nonpermissive clones, but recovered to levels observed in permissive cells when shRNA was used to knockdown TRIM22 expression. Furthermore, LTR-driven transcription was decreased in permissive cells transduced with TRIM22, suggesting that the constitutive expression of TRIM22 is responsible for the restrictive phenotype observed in nonpermissive clones (Kajaste-Rudnitski et al., 2011). Reduced LTR-driven luciferase expression and HIV-1 replication were also observed in A3.01 cells (T cell line) expressing TRIM22, further supporting the effects of TRIM22 on HIV-1 infection in critical cell targets (Kajaste-Rudnitski et al., 2011).

TRIM22 appears to strongly target basal transcription from the HIV-1 LTR. In further experiments using LTR-driven luciferase constructs, TRIM22 had no effect on transcription when cells were transfected with a plasmid encoding the HIV-1 Tat protein (Kajaste-Rudnitski et al., 2011). Although statistical significance was not reached, this may be due to the effects of exogenously provided Tat masking the efficacy of TRIM22. It should also be

noted that all direct evidence to date of TRIM22 inhibiting HIV-1 transcription has been through the use of LTR-driven reporter constructs. It will be important to test the effects of TRIM22 on replication-competent HIV-1, which will provide a more natural scenario of virus transcription, Tat-induction, and possible effects of other HIV-1 accessory proteins.

#### 4.2.3 TRIM22-mediated effects on HIV-1 replication *in vivo*

Interestingly, there is evidence to support a role for TRIM22 as an anti-HIV effector *in vivo*. A study monitoring gene expression in high-risk HIV-1 negative individuals detected a positive correlation between TRIM22 expression and increased control of HIV-1 infection (Singh et al., 2011). It was observed that IFN $\beta$  and TRIM22 levels in peripheral blood mononuclear cells (PBMCs) were increased in patients after HIV-1 infection. In addition, infected patients expressing higher TRIM22 levels exhibited significantly lower viral loads and significantly higher CD4 $^{+}$  T cell counts, suggesting that TRIM22 may play a role in controlling HIV-1 infection. Surprisingly, a significant inverse correlation was observed between the closely related, IFN-inducible TRIM5 $\alpha$  protein and IFN $\beta$  expression (Singh et al., 2011). TRIM22 and TRIM5 $\alpha$  have been under positive selection episodically for approximately 23 million years; however, these two genes have evolved in a mutually exclusive manner, with only one being selected for in a given primate lineage (Sawyer et al., 2007). Since human TRIM5 $\alpha$  has little to no inhibitory effect on HIV-1 replication compared to the potent inhibitory effects of rhesus TRIM5 $\alpha$ , it is possible that human TRIM22 has evolved to compensate for the loss of antiretroviral activity of human TRIM5 $\alpha$ .

### 5. Lifecycle target: HIV-1 protein

#### 5.1 OAS1/RNaseL

##### 5.1.1 OAS1/RNaseL-mediated inhibition of HIV-1 translation

Similar to PKR, 2'5' oligoadenylate synthetase 1 (OAS1) senses viral infection by recognizing dsRNA, and is constitutively expressed in an inactive monomeric form (Sadler et al., 2009). However, unlike PKR, OAS1 recognizes dsRNA in the absence of a dsRBD. Exactly how OAS1 recognizes dsRNA without this domain remains unclear (Kodym et al., 2009, Marie et al., 1990, Sadler & Williams, 2008). Once OAS1 is activated by dsRNA, it forms a tetramer, which converts ATP molecules into 2'5' oligoadenylates (2-5A) (Marie et al., 1990, Hovanessian, 2007). These 2-5As are strong inducers of an enzyme called RNaseL. By binding to the N-terminus of RNaseL, 2-5As activate the ribonuclease activity of RNaseL, which then degrades single-stranded RNA (ssRNA) by cleaving the phosphodiester bonds of uracil rich sequences to produce products with 3' monophosphate and 5' hydroxyl termini (Chakrabarti et al., 2011, Malathi et al., 2007). This leads to a reduction in mRNA translation and induces the RIG-I and/or MDA5 pathways, which are positive regulators of interferon signalling and the antiviral response (Figure 8) (Malathi et al., 2007).

The HIV-1 TAR element (dsRNA) is sufficient for OAS1 recognition and activation, and OAS1-TAR binding leads to 2-5A production, RNaseL recognition, cleavage of HIV-1 transcripts and inhibition of protein translation (Maitra et al., 1994). Interestingly, Jurkat T cells that overexpress RNaseL show a substantial decrease in HIV-1 mRNA production, as well as a 1000-fold decrease in HIV-1 replication two weeks post-infection (Maitra & Silverman, 1998). In addition, overexpression of RNaseL leads to accelerated HIV-induced apoptotic cell death, possibly through Fas-Fas ligand-mediated signalling (Maitra & Silverman, 1998). In contrast, cells devoid of RNaseL are unable to restrict HIV-1 replication, highlighting the importance of this pathway in cellular restriction of virus replication.

### 5.1.2 Countermeasures to OAS1/RNaseL-mediated HIV-1 restriction

Overexpression studies have shown that OAS1/RNaseL-mediated HIV-1 restriction is possible; however, when RNaseL is expressed at biologically relevant levels, HIV-1 can inhibit this pathway. The HIV-1 Tat protein sequesters the TAR element *in vivo*, thus preventing OAS1-TAR binding and RNaseL activation (Schroder et al., 1990b). It is possible that this pathway is responsible for the low levels of mRNA production during the initial stages of HIV-1 infection; however, as Tat expression increases, activation of the OAS1/RNaseL pathway decreases and the production of HIV-1 mRNA rises significantly. In addition, cells that contain latent virus may be kept under control by the OAS1/RNaseL pathway. Nevertheless, the ability of cells to endocytose Tat from other apoptotic cells may lead to trans-activation of the TAR element and HIV-1 mRNA production in these latently infected cells (Schroder et al., 1990b, Frankel & Pabo, 1988).

Although PKR and OAS1/RNaseL are potent HIV-1 restriction factors *in vitro*, the HIV-1 Tat protein is an effective viral countermeasure *in vivo*. Research on these restriction factors however, does provide insight into future therapeutics that may target the HIV-1 Tat protein, or overpower Tat for TAR binding. *In vitro* studies have already shown that shRNA directed against Tat or a TAR RNA decoy can provide long-term inhibition of HIV-1 replication; however, these studies must still be confirmed *in vivo* (Li et al., 2005).

## 5.2 TRIM22

### 5.2.1 TRIM22-mediated HIV-1 restriction

Interestingly, TRIM22 appears to restrict HIV-1 replication by multiple mechanisms. TRIM22 has been shown to be an integral part of IFN $\beta$ -mediated HIV-1 restriction, and its expression can restrict HIV-1 replication in several transformed cell lines. In cell lines such as human osteosarcoma (HOS) and HeLa, TRIM22 restricts the release of virus, but has little to no effect on intracellular levels of the HIV-1 structural protein Gag. Conversely, in the osteosarcoma cell lines U2OS and 143B, TRIM22 expression not only restricts the release of virus, but also prevents the intracellular accumulation of Gag protein (Barr et al., 2008). The presence of different mechanisms in different cell lines, and the fact that multiple localizations for TRIM22 have been observed, suggests that there are many complex details of TRIM22 function that remain to be discovered (Figure 9).

Further investigation of the mechanism of TRIM22-induced restriction in HOS cells revealed that TRIM22 likely interferes with intracellular trafficking of the HIV-1 Gag protein (Barr et al., 2008). Of note, Gag is both necessary and sufficient for budding and release of virus particles. This property allows Gag, in the absence of other viral proteins, to assemble and bud from the cell membrane, resulting in the production of non-infectious, virus-like particles (VLP). Importantly, TRIM22 expression was shown to inhibit the release of VLPs and prevent accumulation of Gag at the cell membrane, a step that is critical for virus assembly. These effects were dependent on the E3 ubiquitin ligase activity of TRIM22.

## 5.3 TRIM5 $\alpha$

### 5.3.1 TRIM5 $\alpha$ -mediated HIV-1 restriction

RhTRIM5 $\alpha$  was originally shown to target incoming HIV-1 capsid proteins, thus inhibiting early stages of HIV-1 replication (see Section 2.1). For this reason, it was initially assumed that rhTRIM5 $\alpha$  didn't affect late stages of HIV-1 replication. However, subsequent research showed that it could also restrict HIV-1 through rapid degradation of the Gag polyprotein, the main structural component of HIV-1 (Sakuma et al., 2007). Treatment of cells



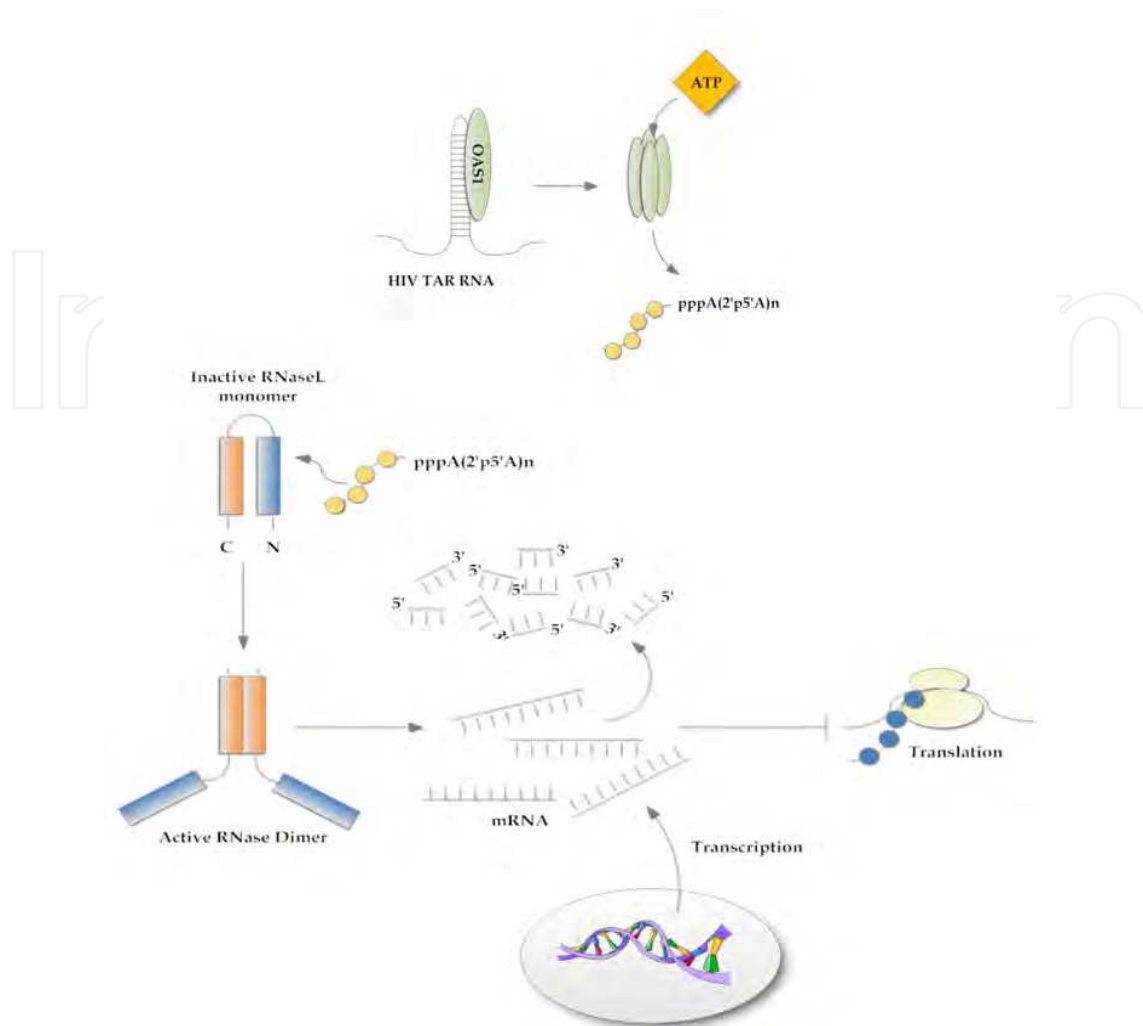


Fig. 8. OAS1/RNaseL-mediated inhibition of HIV-1 protein translation. Following recognition of the HIV-1 TAR element (dsRNA), OAS1 forms a tetramer whose catalytic activity turns ATP molecules into 2'5'oligoadenylates (2-5As). The 2-5As activate the RNaseL enzyme, which leads to its dimerization and stimulates it to cleave ssRNA (such as mRNA). Cleavage of mRNA by RNaseL results in the inhibition of protein translation, including the translation of viral proteins.

with the proteasome inhibitors MG132 and MG115 did not restore HIV-1 Gag protein stability, suggesting that late restriction by rhTRIM5 $\alpha$  occurs independently of the ubiquitin/proteasome system. Interestingly, similar to early-stage restriction, the human orthologue of rhTRIM5 $\alpha$  did not restrict late stages of HIV-1 replication (Sakuma et al., 2007, Sakuma et al., 2007, Sakuma et al., 2010).

Unlike early-stage rhTRIM5 $\alpha$ -mediated restriction, the B30.2 domain was dispensable for Gag degradation. However, two amino acids in the coiled-coil domain (M133 and T146) and the E3 ligase activity of rhTRIM5 $\alpha$  were required for late-stage restriction (Sakuma et al., 2010). It is possible that rhTRIM5 $\alpha$  acts synergistically with other TRIM proteins or cell proteases to degrade the Gag polyprotein. For example, TRIM22 has been shown to affect late stages of HIV-1 replication and thus may be involved in rhTRIM5 $\alpha$ -mediated restriction (Barr et al., 2008). Additional research will determine if other TRIM proteins are involved and help define the exact mechanism of late-stage rhTRIM5 $\alpha$ -mediated restriction.

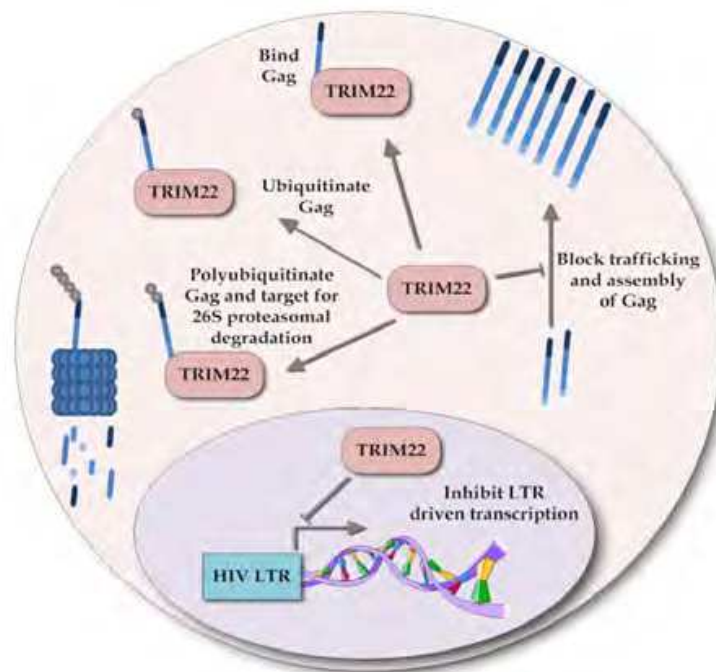


Fig. 9. Possible mechanisms of TRIM22-mediated HIV-1 restriction. TRIM22 prevents accumulation of the Gag polyprotein at the plasma membrane, and as such may bind directly to Gag. Alternatively, TRIM22 may mono-ubiquitinate or polyubiquitinate Gag. Experiments using LTR-luciferase reporter constructs have also shown that TRIM22 restricts transcription from the 5' HIV-1 LTR.

## 6. Lifecycle target: HIV-1 budding

### 6.1 ISG15

Interferon-stimulated gene 15 (ISG15) is a ubiquitin-like protein (Ubl) that was first discovered in 1979, and is highly induced in the presence of IFN- $\alpha/\beta$  (Herrmann et al., 2007, Farrell et al., 1979). The ISG15 protein is composed of two ubiquitin-like domains that can modify substrate proteins similarly to ubiquitin (Jeon et al., 2010). In addition, the C-terminus of ISG15 contains the Gly-Gly motif which is required for ISG15 conjugation to target proteins. ISG15ylation requires the aid of an E1 activating protein, an E2 conjugating protein, and an E3 ligase protein. First, the ISG15-specific E1 activating protein, Ube1L, uses ATP to adenylate the Gly-Gly motif of ISG15. Ube1L then forms a thioester bond between its catalytic cysteine residue and the C-terminal Gly residue of ISG15. With the help of the E2 conjugating protein, Ubch8, and a substrate-specific E3 ligase, ISG15 forms a covalent bond with the  $\epsilon$ -NH<sub>2</sub> of a substrate lysine residue (reviewed in (Kerscher et al., 2006)). Importantly, ISG15 is conjugated to both viral and host proteins, and can have an antiviral effect by altering the activity of substrate proteins required for viral propagation (Harty et al., 2009, Shi et al., 2010).

ISG15ylation has been implicated in restriction of HIV-1 replication at the budding stage of the HIV-1 lifecycle (Okumura et al., 2006, Pincetic et al., 2010). The HIV-1 Gag protein contains a late-budding or L domain that has a PTAP motif, and can interact with endosomal sorting complex required for transport (ESCRT)-I. Specifically, tumour susceptibility gene 101 (TSG101), a component of ESCRT-I, interacts with the PTAP motif on

the HIV-1 Gag protein, and subsequently recruits ESCRT-II and ESCRT-III (VerPlank et al., 2001). ESCRT-III promotes viral budding and the recruitment of vacuolar protein sorting (Vps4), an ATPase that releases ESCRT factors from the membrane (reviewed in (Usami et al., 2009)(Williams & Urbe, 2007)). Interestingly, ISG15 has been shown to interrupt the interaction between TSG101 and the HIV-1 Gag protein; however, neither TSG101 nor HIV-1 Gag are directly modified with ISG15 (Okumura et al., 2006). ISG15 was also shown to interfere with the recruitment of Vps4 to the HIV-1 budding complex; however, the mechanism of this interruption has not yet been characterized. It is possible that charged multi-vesicular body protein CHMP-5, a component of ESCRT-III, prevents the recruitment of Vps4 as it was shown to be ISG15ylated (Pincetic et al., 2010). Further characterization of ISG15-mediated HIV-1 restriction is required to understand the antiviral effects of ISG15 on HIV-1 budding.

## **7. Lifecycle target: HIV-1 release**

### **7.1 Tetherin**

#### **7.1.1 Tetherin: History and structure**

For the past two decades, scientists have known that the HIV-1 Vpu protein is required for efficient release of virus particles (Gottlinger et al., 1993). HIV-1 particles lacking Vpu (HIVΔVpu) cannot release properly from certain cells; however, until recently the cause of this phenotype was unknown (Varthakavi et al., 2003). Tetherin (also known as BST-2 and CD317) was first suggested to be an antiviral protein in 2006, when it was shown to target the K5 protein of Kaposi's sarcoma-associated herpes virus (Bartee et al., 2006). A few years later, tetherin was identified as the causative agent of the HIVΔVpu phenotype when it was shown to inhibit the release of HIVΔVpu particles at the cell membrane of certain restrictive cells such as the HeLa cell line (Neil et al., 2008).

Tetherin is an interferon-induced, transmembrane protein that contains a short cytoplasmic N-terminus, a transmembrane region, an ectodomain, and a C-terminal glycosylphosphatidylinositol (GPI) anchor (Kupzig et al., 2003). Both the transmembrane region and the ectodomain are made from a single alpha helix, and the ectodomain contains an additional coiled-coil region. Tetherin exists as a homodimer, which is formed by disulphide bridges between the coiled-coil ectodomain regions of two tetherin proteins. Importantly, tetherin dimerization has been shown to be crucial for HIVΔVpu restriction (Andrew et al., 2009).

#### **7.1.2 Tetherin-mediated restriction of HIV-1 release**

Currently, the precise mechanism of tetherin-induced HIVΔVpu restriction is uncertain. Among the proposed models, two aspects seem to be consistent: 1) tetherin proteins form homodimers via the coiled-coil regions in their ectodomains and 2) the N- and C-terminus of tetherin are incorporated into the cell and/or viral membrane (Perez-Caballero et al., 2009). Tetherin homodimers localize to the cell membrane where they associate with HIV-1 Gag oligomers on lipid rafts (where budding of the virus occurs) (Neil et al., 2008, Nguyen & Hildreth, 2000). Details of the tethering mechanism underlying restriction are poorly understood. One favourable hypothesis involves the C-terminal GPI being anchored to the cell membrane and the N-terminal transmembrane region being associated with the Gag oligomers of the budding virus. As budding occurs, the cell membrane-bound C-terminus tethers the budding virus to the cell via the virion-bound N-terminus (Perez-Caballero et al.,

2009). Conversely, the N-terminal transmembrane region of tetherin may anchor to the cell membrane whereas the GPI terminus may associate with the budding virus. Another plausible hypothesis is that one tetherin molecule binds to the cell membrane and another tetherin molecule binds to the budding virus. In this case, HIV-1 release would be inhibited by the interaction between coiled-coil regions in the tetherin dimer (Perez-Caballero et al., 2009) (Figure 10).

In spite of tetherin-induced restriction of HIV $\Delta$ Vpu virus, trapping virus at the membrane is not sufficient to prevent cell-to-cell transmission of HIV-1 (Casartelli et al., 2010, Kuhl et al., 2010). This binding leads to efficient cell-to-cell transmission of the virus. Interestingly, tetherin has also been shown to prevent HIV-1 transmission through this route. Specifically, tetherin appears to link budding HIV-1 particles together in a chain-like fashion, tethering them to the cell membrane in viral aggregates (Hammonds et al., 2010). The formation of these aggregates prevents HIV-1 transmission through the virological synapse, possibly because the aggregates cannot fuse properly to the target cells (Casartelli et al., 2010). It is possible that tetherin is incorporated into the budding virions and that this causes abnormal virus fusion to the target cell; however, more studies are needed to confirm the presence of tetherin in HIV-1 particles and further define its role in HIV-1 restriction at the virological synapse. Taken as a whole, current research suggests that tetherin may have two roles in HIV-1 restriction: tethering virus particles to the cell membrane and preventing cell-to-cell transmission of HIV-1 to uninfected target cells.

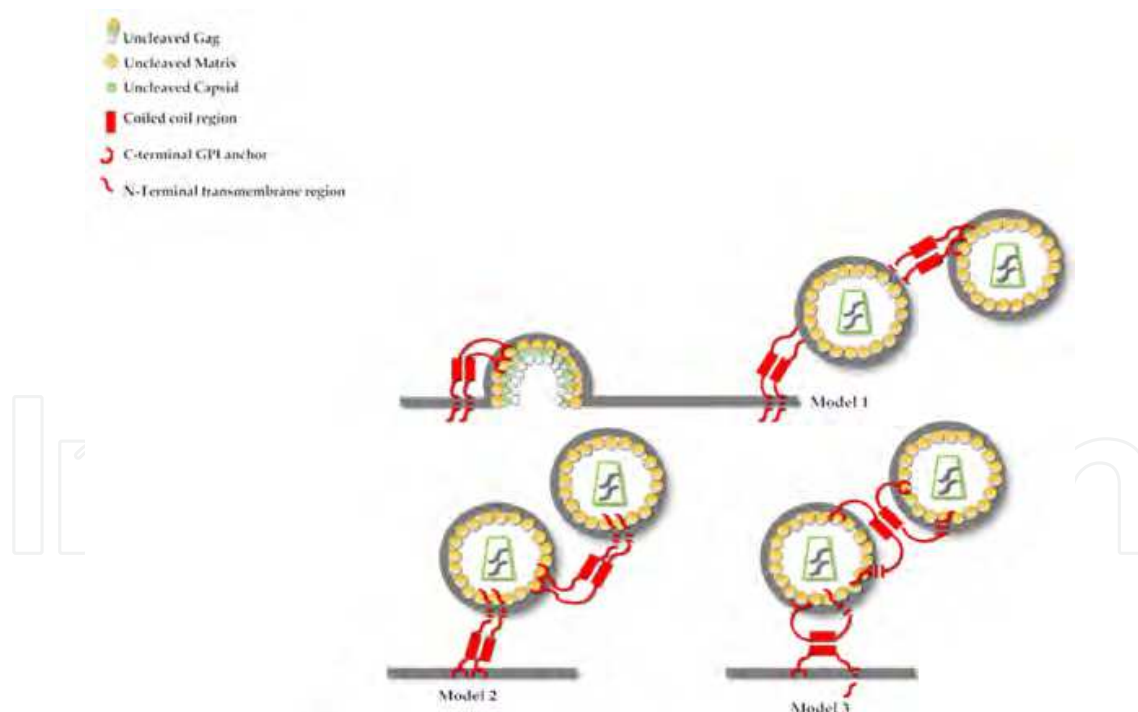


Fig. 10. Tetherin-induced inhibition of HIV-1 particle release. Each model centres on the dimerization of two tetherin molecules. In model 1, the N-terminal transmembrane regions of the tetherin dimer anchor to the cell surface and the C-terminal GPI domains associate with the budding virus. Model 2 is the opposite of model 1. In model 3, each tetherin molecule of the dimer associates with either only the budding virus or only the cell membrane, and HIV-1 restriction depends on the interaction between the coiled-coil regions.



### 7.1.3 Countermeasures to tetherin-mediated HIV-1 restriction

The HIV-1 Vpu protein has been shown to degrade tetherin, thus abolishing its anti-HIV-1 effects. Specifically, the transmembrane domain of Vpu can bind to tetherin, and this domain is necessary for Vpu localization to the cell membrane and subsequent association with tetherin (Kobayashi et al., 2011, Skasko et al., 2011, Vigan & Neil, 2010). Interestingly, mutating a single amino acid in the Vpu transmembrane domain (A18H) traps Vpu in the endoplasmic reticulum, where it is unable to translocate to the cell membrane or degrade tetherin (Skasko et al., 2011). Furthermore, it has recently been shown that four amino acids in the Vpu transmembrane domain (I34, L37, L41, and T45) are necessary for Vpu interaction with and antagonism of tetherin (Kobayashi et al., 2011). Mutational experiments with tetherin show that its transmembrane domain is also important for Vpu-tetherin interactions. Given this data, it is likely that Vpu and tetherin interact through their respective transmembrane domains and thus, that these domains are critical for Vpu-mediated tetherin degradation.

There are currently two major hypotheses for the mechanism of Vpu-mediated tetherin degradation. The first hypothesis involves tetherin degradation at a post-translational step, as there is no decrease in tetherin transcript levels in the presence of Vpu, but there is a decrease in protein expression (Douglas et al., 2009, Mangeat et al., 2009). It is possible that this degradation is mediated by Vpu binding to  $\beta$ -transducin repeat-containing protein ( $\beta$ -TrCP), which is a substrate adaptor for a multi-subunit E3 ligase complex and is able to interact with Vpu through its C-terminus. The consequence of Vpu binding to the  $\beta$ -TrCP-E3 ligase complex is the ubiquitination of cell surface proteins, including tetherin, on lysine residues at positions 18 and/or 21 (Mangeat et al., 2009, Guatelli, 2009, Iwabu et al., 2009, Pardieu et al., 2010). Tetherin ubiquitination leads to its endocytosis from the cell membrane and degradation through either the proteasomal or lysosomal degradation pathways (Douglas et al., 2009, Mitchell et al., 2009, Van Damme et al., 2008).

Tetherin degradation by Vpu and the  $\beta$ -TrCP-E3 ligase complex however, is insufficient to explain one interesting finding: Vpu constructs that contain mutations in the motif that recognizes  $\beta$ -TrCP can still partially, or in some cases totally, overcome tetherin-mediated HIV-1 restriction (Douglas et al., 2009, Mangeat et al., 2009, Mangeat et al., 2009, Mitchell et al., 2009, Miyagi et al., 2009). Thus, a second hypothesis has been proposed that involves tetherin degradation in late endosomal compartments. It has previously been shown that Vpu is distributed throughout the trans-golgi network, and that it can modulate tetherin cell surface expression by sequestering it intracellularly. Sequestration of tetherin prevents its anterograde trafficking to the cell membrane and subsequently delivers it to late endosomal compartments (Dube et al., 2010, Dube et al., 2010, Hauser et al., 2010). Of note, the specifics of this mechanism of Vpu-mediated degradation are still largely uncharacterized and further studies are needed to elucidate the details of this mechanism. However, taken together, current research suggests that there may be two mechanisms by which Vpu counteracts the antiviral activity of tetherin.

## 8. Conclusion

### 8.1 Pharmaceutical approach

In the future, it is probable that new HIV-1 therapies will be developed based on the actions of cellular restriction factors. Currently, many studies are focused on defining the molecular mechanisms of these factors; however, it is still unclear how this information will be used to

create effective therapies. In the short-term, drug-based therapies are the most likely to be successful, and due to the constant development of HIV-1 resistance, new drugs are always needed. To date, there are 32 antiretroviral drugs approved by the FDA, and none of these drugs target the same steps in the HIV-1 lifecycle as cellular restriction factors. This makes restriction factors excellent candidates for drug design, specifically proteins such as TRIM22 or ISG15, which do not appear to be directly targeted by any HIV-1 proteins. With the development of any new HIV-1 drug, resistance is always a concern; however, identifying new stages of the HIV-1 lifecycle to antagonize may reduce viral replication enough to prevent escape mutants.

Alternatively, drugs targeting cellular restriction factor antagonists could be developed. For example, the HIV-1 Vif protein antagonizes APOBEC3 by marking it for proteasomal degradation (Yu et al., 2003). Inhibiting the interaction between APOBEC3 and Vif may prevent this degradation, and many studies have focused on identifying important interacting regions on both proteins (Chen et al., 2009, Huthoff & Malim, 2007, Yamashita et al., 2008). Unfortunately, many of the interacting regions on Vif differ depending on the specific APOBEC3 protein it is interacting with, and it is likely that three-dimensional structures of APOBEC3 proteins bound to Vif will be needed to effectively target this interaction (Russell et al., 2009, Tian et al., 2006). Despite these challenges, one small molecule antagonist of Vif was recently identified (RN-18) and shown to decrease levels of Vif protein *in vitro* (Nathans et al., 2008). It will be interesting to follow-up this research *in vivo*, and learn whether RN-18 has an effect on HIV-1 replication in infected individuals. Finally, another option to shield APOBEC3 from Vif involves designing a molecule that binds to APOBEC3 and prevents this interaction; however, this molecule would also have to preserve APOBEC3's antiviral function (Albin & Harris, 2010).

The HIV-1 Tat protein is another attractive drug target, since it is essential for HIV-1 replication and antagonizes two HIV-1 restriction factors (PKR and OAS1). For unknown reasons, Tat hasn't received much attention as a potential drug target, possibly because its actions are hard to re-create *in vitro*. However, inhibition of Tat potentially inhibits HIV-1 replication, and further research in this area is certainly warranted. Drugs that target the HIV-1 Vpu protein could also be considered; however, because Vpu is not critical for HIV-1 replication *in vivo* it may not be an ideal candidate (Friborg et al., 1995, Terwilliger et al., 1989). Conversely, drugs that mimic the effects of tetherin, but are resistant to Vpu, may successfully reduce HIV-1 replication. In fact, an artificial Vpu-resistant tetherin protein was recently engineered; however, it has not yet been tested in clinical trials (Perez-Caballero et al., 2009). Inhibiting the action of host proteins that assist HIV-1 replication is another possibility. For example, cyclophilin A (CypA) is required for HIV-1 replication, and without Cyp A HIV-1 virions are not infectious (Sokolskaja & Luban, 2006, Thali et al., 1994). Small molecule inhibitors targeting Cyp A may block HIV-1 replication at the uncoating stage, and targeting a host protein avoids the problem of viral resistance. However, HIV-1 propagation in the absence of Cyp A may allow HIV-1 variants to evolve that no longer require Cyp A for replication.

## 8.2 Gene therapy approach

An alternative approach to HIV-1 therapy involves using cellular restriction factors in conjunction with gene therapy. In this approach, DNA encoding one or more cellular restriction factors is inserted into target cells to interfere with HIV-1 infection or replication.

One advantage of this approach is that cellular restriction factors are naturally expressed in human cells, and as such may be less toxic or immunogenic *in vivo* (Barr, 2010). Since there are no known viral countermeasures to TRIM22, Rhesus TRIM5 $\alpha$  or ISG15, these proteins are currently the best candidates for gene therapy. Another possibility involves using molecular engineering to create modified restriction factors that are resistant to viral antagonists, making them more suitable candidates for gene therapy. For example, a human protein modeled after the TRIM5 $\alpha$ -CypA fusion protein in Owl monkeys was recently engineered, and shown to block HIV-1 replication in primary CD4<sup>+</sup> T-cells and macrophages (Neagu et al., 2009). In addition, mice engrafted with inhibitor-expressing CD4<sup>+</sup> T-cells had decreased viremia and increased levels of CD4<sup>+</sup> T-cells. It is possible that this human TRIM5 $\alpha$ -CypA protein could be used for gene therapy, and it is likely that it will be tested clinically in the near future.

Since many cellular restriction factors are IFN-inducible, they are not constitutively expressed in cells. As such, it is desirable to employ a gene therapy approach that mimics this pattern of expression. One interesting strategy involves creating a construct that contains restriction factor genes under the control of the HIV-1 LTR promoter. In this strategy, target cells are preloaded with the construct, and when HIV-1 infects these cells, Tat expression activates transcription of the LTR-fused restriction factor genes. Restriction factor expression reduces HIV-1 replication in infected cells, limiting further propagation of the virus. Notably, this approach has been successfully tested *in vitro* using the restriction factors PKR, OAS1 and ISG15; however, more experiments are needed to validate this strategy *in vivo*, and to test various construct delivery methods (Muto et al., 1999, Schroder et al., 1990a, Su et al., 1995). Gene therapy continues to be a promising approach for the treatment of HIV/AIDS; however, several problems need to be addressed before this technology can be fully realized. Some of these issues include, but are not limited to, increasing the stability of DNA and longevity of target cells, avoiding adverse immune responses, and targeting specific cells or tissues.

### 8.3 Additional approaches

#### 8.3.1 Nanotechnology

Nanotechnology is revolutionizing many areas of medicine, particularly in the realm of drug delivery. With nanotechnology, it is now possible to target drugs to specific cells or tissues, a method that could be used to direct antiretroviral drugs to CD4<sup>+</sup> T-cells and macrophages (Farokhzad, 2008, Farokhzad & Langer, 2009). In addition, targeted antiviral delivery to the brain or other organs could ensure that drugs reach latent HIV-1 reservoirs (Vyas et al., 2006, Vyas et al., 2006, Amiji et al., 2006). The development of controlled-release delivery systems could also allow antiretroviral drugs to be released over longer times, and enhance their half-lives. For example, a new anti-HIV-1 drug called Rilpivirine was recently administered to dogs and mice in nanosuspensions (Baert et al., 2009). This resulted in the sustained release of the drug over 3 months in dogs and 3 weeks in mice, compared to a half-life of 38 hours for free drug. Importantly, this type of drug delivery system could have major implications in reducing antiretroviral toxicity and improving drug adherence. Thus, nanotechnology should be considered in the development of new antiretroviral drugs, including drugs that mimic the effects of cellular restriction factors.

In addition to improving antiretroviral therapies, there are ongoing efforts to apply nanotechnology to gene therapy. Early attempts in gene therapy for HIV/AIDS have used viral vectors as gene delivery systems, with some encouraging results (Li et al., 2005, Morris

& Rossi, 2006, Morris & Rossi, 2006, Lee et al., 2005, Lee et al., 2005). However, the use of viral vectors for gene delivery poses several potential problems such as toxicity, immunogenicity, and insertion mutagenesis. As such, the use of non-viral vectors for gene delivery must be further explored, and nanotechnology is one promising option (Lundin et al., 2009, Mintzer & Simanek, 2009). One example is the use of RNA interference (RNAi) for HIV/AIDS therapies. RNAi may have therapeutic potential in the treatment of HIV/AIDS; however, delivery of siRNA to specific cells continues to be a problem (Haasnoot et al., 2007, Haasnoot et al., 2007, Whitehead et al., 2009, Whitehead et al., 2009, Berkhout & ter Brake, 2009). Nanosuspensions of siRNA are currently being tested in humans for cancer treatment, and have recently entered Phase I clinical trials (Davis, 2009). If this technique is successful, it could be applied to cellular restriction factor-based HIV/AIDS gene therapy. For example, nanosuspensions of siRNA could be targeted to HIV-1-infected cells to knockdown the viral mRNA of restriction factor antagonists, such as Vif, Tat and Vpu. This would increase the antiviral activity of restriction factors, specifically reducing HIV-1 replication in infected cells. Alternatively, DNA from one or more cellular restriction factors could be delivered to HIV-1 infected cells using nanotechnology platforms. This may provide a safe and effective way to deliver cellular restriction factor genes to HIV-1 infected cells.

### 8.3.2 Zinc finger nucleases

Zinc finger nucleases (ZFN) have recently emerged as an important technology for gene modification, and there are several potential applications for ZFNs in HIV/AIDS therapy. ZFNs function by inducing a double-stranded break in a specific DNA sequence and generate the desired gene modification during DNA repair (Urnov et al., 2010). One of the main advantages of ZFNs is that the changes they make are both permanent and heritable, eliminating the need for persistent therapeutic intervention. For HIV-1, most ZFN research has focused on the manipulation of the human CCR5 gene, which encodes one of HIV-1's co-receptors and is required for viral entry into the host cell. Deletion of a 32-bp region from this gene (CCR5 $\Delta$ 32) results in a non-functional receptor, and people with this mutation are resistant to HIV-1 infection (Huang et al., 1996). Thus far, ZFN researchers have succeeded in deleting the 32-bp region from the human CCR5 gene, both in primary CD4<sup>+</sup> T-cells and hematopoietic stem cells (Bobis-Wozowicz et al., 2011, Lei et al., 2011, Perez et al., 2008). Furthermore, there are two Phase I clinical trials in progress testing the efficacy of *ex vivo* expansion and infusion of these modified cells in HIV-1 infected individuals (Urnov et al., 2010).

In addition to gene deletion, ZFNs have also been used successfully for gene correction (allele editing) and gene addition (Urnov et al., 2005, Urnov et al., 2005, Moehle et al., 2007). Both gene correction and addition may be useful for cellular restriction factor-based HIV-1 therapies; however, to date this has never been experimentally tested. For example, the addition of one or more cellular restriction factor genes to HIV-1 target cells may produce a 'super-restrictive' phenotype, whereby cells with multiple genes express higher levels of restriction factor proteins, thus increasing their capacity to fight HIV-1 infection. Several HIV-1 restriction factors have been shown to be more effective restrictors when expressed at higher levels. For example, higher expression of TRIM22 was recently shown to be correlated with lower levels of viremia and higher CD4<sup>+</sup> T-cell counts in HIV-1-infected individuals (Singh et al., 2011). Another possibility involves adding cellular restriction factor



genes to hematopoietic stem cells, allowing the generation of ‘super-restrictive’ cells in all blood lineages (including macrophages and dendritic cells, which are often the first cells to encounter HIV-1 *in vivo*). However, many more studies need to be performed, particularly to identify any deleterious effects caused by amplified expression of cellular restriction factors.

### 8.3.3 Next-generation sequencing

Next-generation sequencing is another new and exciting technology that has potential applications in HIV/AIDS therapy. With this calibre of sequencing, it is now possible to read hundreds of DNA samples simultaneously, an approach that has helped researchers identify polymorphisms in different human genes. It is well known that people differ significantly in their susceptibility to HIV-1 infection and disease progression to AIDS, and polymorphisms in cellular restriction factors may contribute to these differences (Ball et al., 2007, Beyrer et al., 1999, Cao et al., 1995). For example, there is research suggesting that various polymorphisms in the TRIM5 $\alpha$  and APOBEC3 genes contribute to HIV-1 disease progression; however, due to confounding reports further research is needed in this area (van Manen et al., 2008, van Manen et al., 2008, An et al., 2009, Goldschmidt et al., 2006, Harari et al., 2009, Valcke et al., 2006). In addition, polymorphisms in other cellular restriction factors may influence the clinical course of HIV-1 infection, but many of these factors have never been tested. In the future, it may be possible to generate an individual’s cellular restriction factor polymorphism “blueprint” (Barr, 2010). This blueprint could help predict a person’s susceptibility to HIV-1 infection and progression to AIDS, and may potentially lead to a more personalized HIV-1 treatment regime. Alternatively, with the advent of ZFN technology it may also be possible to “edit” multiple restriction factor genes (change disadvantageous polymorphisms to advantageous polymorphisms) to create an optimal cellular restriction factor blueprint *in vivo*, and better equip individuals to fight HIV-1 infection.

## 9. References

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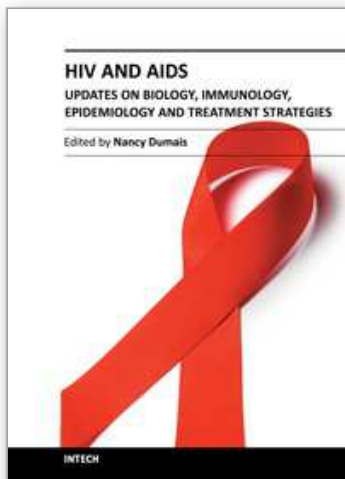
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The continuing AIDS pandemic reminds us that despite the unrelenting quest for knowledge since the early 1980s, we have much to learn about HIV and AIDS. This terrible syndrome represents one of the greatest challenges for science and medicine. The purpose of this book is to aid clinicians, provide a source of inspiration for researchers, and serve as a guide for graduate students in their continued search for a cure of HIV. The first part of this book, “From the laboratory to the clinic,” and the second part, “From the clinic to the patients,” represent the unique but intertwined mission of this work: to provide basic and clinical knowledge on HIV/AIDS.

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