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HIV-1 Vif Versus the APOBEC3 Cytidine Deaminases: An Intracellular Duel Between Pathogen and Host Restriction Factors

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Abstract

The Vif protein of HIV is essential for the effective propagation of this pathogenic retrovirus *in vivo*. Vif acts by preventing virion encapsidation of two potent antiviral factors, the APOBEC3G and APOBEC3F cytidine deaminases. Decreased encapsidation in part involves Vif-mediated recruitment of a ubiquitin E3 ligase complex that promotes polyubiquitylation and proteasome-mediated degradation of APOBEC3G/F. The resultant decline in intracellular levels of these enzymes leads to decreased encapsidation of APOBECG/F into budding virions. This review discusses recent advances in our understanding of the dynamic interplay of Vif with the antiviral APOBEC3 enzymes.

Keywords

HIV-1; Vif; APOBEC3G; APOBEC3F; non-structural protein

1) Introduction

Human immunodeficiency virus-1 (HIV-1) encodes four accessory proteins—Vif, Vpu, Vpr, and Nef. Early studies indicated that these accessory proteins are not always required for viral replication in cell cultures, but each is important for the success of natural infections. These accessory proteins often function by modulating host immune responses, including countering the intrinsic antiviral effects of host restriction factors (Bieniasz, 2004; Malim and Emerman, 2008). Vif, a cytoplasmic, 23-kDa basic phosphoprotein encoded during late stages of the HIV-1 life cycle, is a notable example. Vif is conserved among all lentiviruses except equine infectious anemia virus, suggesting a prominent role in the life cycle of these retroviruses. Remarkably, its precise function remained mysterious for many years.

2) Early Vif observations

Soon after the discovery of HIV-1, it became apparent that Vif (viral infectivity factor) is required for HIV replication in some but not all cell types (Fisher et al., 1987; Gallo et al.,

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1988; Sodroski et al., 1986; Strebel et al., 1987). Specifically, HIV-1 virions lacking Vif (ΔVif HIV-1) can only spread in so-called "permissive" adherent cell cultures (e.g., HeLa and 293T) and various leukemic T-cell lines (e.g., CEM-SS and SupT1); they fail to spread in "nonpermissive" cells that include physiologically relevant primary CD4 T lymphocytes and macrophages, as well as various other T cell lines like CEM, HUT78 and H9. Of note, ΔVif virions are produced in normal numbers by these nonpermissive cells but the ability of these virions to productively infect the next target cell is greatly compromised (Courcoul et al., 1995; Fan and Peden, 1992; Fouchier et al., 1996; Gaddis et al., 2003; Goncalves et al., 1996; Hoglund et al., 1994; Simm et al., 1995; Simon and Malim, 1996; Simon et al., 1998b; Sova and Volsky, 1993; von Schwedler et al., 1993). Thus, Vif alters the quality but not quantity of HIV-1 virions produced by virus-infected CD4 T cells or macrophages (Gabuzda et al., 1992; Goncalves et al., 1996; Simon et al., 1998a; Simon and Malim, 1996; von Schwedler et al., 1993).

Why does replication of ΔVif HIV-1 in nonpermissive cell lines produces virions that cannot productively infect other cells? Two hypotheses have been offered: (1) the producer cells express an antiviral factor that Vif can restrict or (2) permissive cells express a proviral factor that can replace Vif and facilitate viral production. To determine which hypothesis was correct, heterokaryons were formed between permissive and nonpermissive cells and infected with ΔVif HIV-1. These heterokaryons yielded non-infectious virions, indicating that nonpermissive cells produce an HIV inhibitory factor whose action is circumvented by Vif (Madani and Kabat, 1998; Simon and Malim, 1996).

3) Discovery of A3G

Using a subtractive hybridization approach between the nonpermissive CEM cell line and the closely related but permissive cell line CEM-SS, Sheehy and colleagues identified this inhibitor as CEM-15 (Sheehy et al., 2002), now known as APOBEC3G (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G, or A3G), its expression in permissive cells is sufficient to render these cells nonpermissive. This finding showed that A3G can block HIV-1 replication in the absence of Vif (Fig. 1).

A3G belongs to a family of cytidine deaminases that includes seven family members (A3A–H), all located in a gene cluster on chromosome 22 (Conticello et al., 2005; Jarmuz et al., 2002). Of note, mice contain only a single A3 gene located at a synthetic region on chromosome 15. Forty-five percent of the human genome consists of endogenous mobile genetic elements or retroelements, such as short interspersed nuclear elements or long interspersed nuclear elements, which pose a potential threat to genome integrity. It is thought that the human A3 family arose by gene duplication by unequal crossover due to strong evolutionary pressure to control these elements (Conticello et al., 2003; Sawyer et al., 2004). A3G and A3F are best known for their antiviral activity against HIV-1 (Chiu and Greene, 2008), although A3A, A3B, A3C, A3DE, and A3H can also defend against HIV-1 to some extent (discussed below).

The common characteristic of all these enzymes is the presence of one (A3A, A3C, A3H) or two cytidine deaminase domains (CDAs) (A3B, A3DE, A3F, A3G) (Conticello et al., 2003; Jarmuz et al., 2002). Although the two CDAs are highly similar, they have different functions (Jarmuz et al., 2002). In primates, the N-terminal domain mediates RNA binding, whereas the C-terminal domain mediates sequence specific cytidine deamination of single-stranded DNA (Friew et al., 2009; Gooch and Cullen, 2008; Hache et al., 2005; Iwatani et al., 2006; Li et al., 2004; Navarro et al., 2005).

CDAs are characterized by a conserved zinc-binding motif (C/H)-X-E-$X_{23\sim28}$-P-C-$X_{2\sim4}$-C (Conticello et al., 2005; Jarmuz et al., 2002). This enzymatically active site catalyzes the hydrolytic deamination at the C4 position of 2′-deoxycytidine, resulting in a 2′-deoxyuridine.

During the deamination reaction, the cysteines coordinate a single zinc ion, while the key glutamate is involved in proton shuttling (Betts et al., 1994).

Structural studies of the C-terminal CDA of A3G (Chen et al., 2008; Furukawa et al., 2009; Harjes et al., 2009; Holden et al., 2008; Zhang et al., 2007) and the single domain APOBEC2 protein (Prochnow et al., 2007) revealed that, the CDA consists of five β strands flanked by an α-helix on each side plus appropriate connecting loops.

4) Intravirion Packaging of A3G/F

In nonpermissive cells, inhibition of ΔVif HIV-1 by A3G only occurs during the next round of viral infection, indicating that A3G exerts its antiviral activity in the target cell. A3G is effectively incorporated into budding ΔVif virions, providing a potential mechanism for how A3G influences the infectivity of progeny virions in the next target cell (Gaddis et al., 2003; Sheehy et al., 2002; Suspene et al., 2004) (Fig.1). The amount of A3G molecules incorporated into the budding virus is proportional to its expression level in the virus-producing cell. Virus budded from activated peripheral blood mononuclear cells (PBMCs) contain approximately 7 (± 4) molecules of A3G (Xu et al., 2007); therefore, only a few A3G molecules are necessary to inhibit viral replication. For successful incorporation into ΔVif virions, A3G must interact with the N-terminal region of the nucleocapsid (NC) of Gag (Luo et al., 2004; Schafer et al., 2004; Svarovskaia et al., 2004; Zennou et al., 2004). HIV-1 virus-like particles that lack the nucleocapsid domain fail to package A3G (Schafer et al., 2004; Zennou et al., 2004).

A3G and NC have a strong propensity to bind RNA, which may serve an adaptor for binding, as co-immunoprecipitation of NC and A3G is RNase sensitive (Svarovskaia et al., 2004). Indeed, RNA binding is likely indispensable for the packaging of A3G into virions (Luo et al., 2004; Navarro et al., 2005; Schafer et al., 2004; Svarovskaia et al., 2004; Zennou et al., 2004). Mutations in the N-terminus (amino acids 124–127) of A3G impair its RNA binding properties, thus greatly compromising its encapsidation into budding virions. Such mutants of A3G function as very poor inhibitors of HIV-1 infection (Burnett and Spearman, 2007; Huthoff et al., 2009; Huthoff and Malim, 2007; Navarro et al., 2005). Therefore, A3G takes advantage of the fundamental property of retroviral RNA packaging to be incorporated into the virus (Zennou et al., 2004).

The nature of the RNA bound by A3G for encapsidation is unclear. Genomic HIV-1 RNA (Khan et al., 2007; Khan et al., 2005; Soros et al., 2007) was suggested, as well as 7SL RNA (a component of signal-recognition particles) (Wang et al., 2007). Recent results using an in vitro system showed that any single-stranded RNA (with G residues) can facilitate the formation of an A3G-RNA-NC complex (Bogerd and Cullen, 2008). Cellular A3G is mainly confined to high-molecular-mass (HMM) ribonucleoprotein complexes (Kreisberg et al., 2006; Stopak et al., 2003), which raises the question of how it is packaged into budding virions. Subsequent studies suggested that virion A3G is mainly recruited from the cellular pool of newly synthesized enzyme that is not yet assembled into HMM complexes (Soros et al., 2007). A second study suggested that “old” A3G was as efficiently packaged as “newly” synthesized A3G (Goila-Gaur et al., 2009); however, this study did not distinguish whether the old A3G emanated from a low or high molecular mass pool of A3G. A recent study showed that the N-terminal region of A3G including amino acids Y124 and W127 that are important for RNA binding, also facilitates lipid raft association. This finding reveals an interesting correlation between the ability of A3G to associate with lipid rafts and viral genomic RNA with its ability to be effectively encapsidated into budding virions (Khan et al., 2009).

5) Cytidine deaminase activity of A3G and A3F and inhibition of viral growth

Once incorporated into the virion, A3G is introduced into the next target cell as a result of virion fusion. Within the cell, the enzyme triggers massive deamination converting specific dC residues to dU during synthesis of the minus strand viral DNA (Suspene et al., 2004; Yu et al., 2004a) (Fig.1 point 2). During synthesis of the DNA plus strand, adenosines are incorporated instead of the original guanines, resulting in G-to-A mutation (Harris et al., 2003; Mangeat et al., 2003; Mariani et al., 2003; Zhang et al., 2003). During overexpression of A3G the level of these G-to-A mutations can exceed 10%. Accordingly, this phenomenon is often termed hypermutation. *In vitro* studies have shown that A3G displays a preference for deamination of 5′-C<u>C</u> (on the minus strand) (Bishop et al., 2004a; Harris et al., 2003; Suspene et al., 2004; Yu et al., 2004a), and up to 90% of sites containing the 5′-CC<u>C</u>A consensus sequence can be mutated (Yu et al., 2004a).

G-to-A mutations occur throughout the viral genome but in an unequal distribution (Kijak et al., 2008; Koulinska et al., 2003; Suspene et al., 2006; Suspene et al., 2004; Yu et al., 2004a). Most hypermutations are present in the envelope (Env) and Nef regions and decrease toward the 5′ UTR. This gradient of hypermutation is explained in part by the nature of the reverse transcription reaction. For HIV, this reaction begins with the binding of the $tRNA^{Lys-3}$ to the primer-binding site of the viral RNA. The reverse transcriptase starts to synthesize minus-strand DNA, using the $tRNA^{Lys-3}$ as a primer. The minus-strand strong-stop DNA is then transferred to 3′ of the viral RNA. RNaseH degrades the plus-strand RNA and restores the enzymatic activity of A3G, enabling it to act on the single-strand minus DNA and deaminate dC to dU. Plus-strand synthesis is initiated from two RNaseH-resistant polypurine tracts, cPPT and 3′PPT, which remain associated with the minus-strand cDNA to serve as initiation sites.

The chance that a cytidine on the minus-strand will be deaminated correlates strongly with the time during which these DNA regions remain single stranded (Chelico et al., 2006; Suspene et al., 2006; Yu et al., 2004a). Therefore, the specificity of A3G for single-stranded DNA accounts for the highly polarized (5′→3′) mutational twin gradients within the viral genome, each containing the greatest number of mutations just 5′ to the cPPT and 3′PPT (Suspene et al., 2006). This deamination gradient could be further reinforced by a proposed model in which A3G, slides predominantly in the 3′→5′ direction on its minus strand DNA template (Chelico et al., 2006). This could also contribute to A3G's preference for deaminating the 3′ C in the consensus sequence (5′-CC<u>C</u>A-3′) (Chelico et al., 2006). However, a more recent study suggests that A3G does not translocate on the DNA in a positionally correlated fashion by sliding or microscopic jumping but instead translocates in an uncorrelated fashion by macroscopic jumping or intersegmental transfer (Nowarski et al., 2008).

In rare cases, C→T mutations are detected in the U3 region of the 5′UTR, which reflects the deamination of a cytidine on the single plus strand. Of note, the primer binding site becomes single stranded through the action of RNaseH when it degrades the $tRNA^{Lys-3}$ bound at this site (Yu et al., 2004a).

A3G incorporated into virions resides in the viral core, bound in a ribonucleoprotein complex containing the viral genomic RNA as well as the viral NC, IN and Vpr proteins. A3G assembly with HIV RNA results in an inhibition of its intrinsic deaminase activity (Soros et al., 2007). How, then, is the virion-incorporated A3G ultimately activated so that it mediate mutation of the newly synthesized minus-strand DNA? It appears that the action of the viral RNase H during reverse transcription frees the enzyme from its RNA inhibitor, allowing full expression of its deaminase activity (Soros et al., 2007). These findings highlight a rather unexpected host-pathogen interaction in which the anti-HIV activity of A3G depends on its activation by an HIV enzyme.

Although A3G mediates hypermutation of the viral cDNA, it was also clear that wildtype A3G diminishes the accumulation of early and the late reverse transcription products in newly infected cells in a dose-dependent manner, with the greatest effect observed for late products (Anderson and Hope, 2008; Bishop et al., 2006; Guo et al., 2006; Holmes et al., 2007; Iwatani et al., 2007; Kaiser and Emerman, 2006; Luo et al., 2007; Mangeat et al., 2003; Mbisa et al., 2007). One possible explanation for this finding is that cellular host proteins degrade the hypermutated viral cDNA products. The resulting massive dU mutations could trigger the removal of the uracil in the nascent viral DNA by uracil-DNA-glycosylases (UNG2 and SMUG1), leading to abasic sites, and degradation by apurinic/apyrimidinic endonucleases (Schrofelbauer et al., 2005; Yang et al., 2007). However, more recent studies have shown that cells deficient in UNG2 (Kaiser and Emerman, 2006) or both UNG2 and SMUG1 (Langlois and Neuberger, 2008) do not rescue the accumulation of defective viral DNA.

Thus, it seems that A3G decreases HIV-1 infectivity by inducing hypermutation of the viral genome, where dU-containing nascent viral DNA serves as a template for plus-strand DNA synthesis, resulting in G-to-A mutations and viral error catastrophe, where viral proteins are truncated or rendered nonfunctional. How A3G induces a reduction in viral cDNA products remained an unanswered question.

6) A3G and A3F exert antiviral effects independent of their deaminase activity

Although the antiviral activity of A3 proteins is clearly linked to their cytidine deaminase activity (Mangeat et al., 2003; Shindo et al., 2003; Zhang et al., 2003) (Fig. 2, point 2), increasing evidence suggests that the A3G and A3F proteins also exert antiviral activity independently of cytidine deamination (Fig. 1, point 1). Specifically, mutagenesis of key amino acids at the center of the enzymatically active CDA2 prevents deaminase activity (Navarro et al., 2005; Newman et al., 2005), yet these mutants continue to moderately impair infectivity of HIV-1 and reduce the amount of HIV-1 cDNA produced in new target cells (Newman et al., 2005). Of note, hypermutation levels do not directly correlate with the extent of HIV restriction or the amount of viral cDNA products (Bishop et al., 2006; Langlois and Neuberger, 2008).

Many laboratories have investigated how this deaminase-independent antiviral activity of A3 proteins reduces the amount of viral cDNA. Various, sometimes contradictory, reports have emerged, suggesting that A3G can interfere with primer tRNA annealing, minus- and plus-strand DNA transfer, primer tRNA progression and removal, and DNA elongation (Anderson and Hope, 2008; Bishop et al., 2006; Guo et al., 2006; Guo et al., 2007; Guo et al., 2009; Holmes et al., 2007; Iwatani et al., 2007; Li et al., 2007; Luo et al., 2007; Mangeat et al., 2003; Mariani et al., 2003; Mbisa et al., 2007).

Recently, studies employing a cell-free reverse transcriptase assay, lacking any nucleases, showed that the reduced HIV-1 cDNA levels are due to A3G-mediated inhibition of RT elongation. This finding suggests that A3G can physically impair reverse transcription, further excluding the possibility that increased cDNA degradation accounts for the lower levels of HIV-1 cDNA (Bishop et al., 2008) (Fig. 1, point 1).

Expression of A3G also inhibits viral DNA integration and provirus formation, probably by inducing defects in tRNA cleavage during plus-strand DNA transfer, leading to the formation of aberrant viral DNA ends (Fig. 1, point 3). These aberrant structures presumably interfere with the chromosomal integration of the double-stranded viral DNA required for provirus formation. Of note, cytidine deaminase activity was necessary to inhibit provirus formation, although the mechanism remains unclear (Mbisa et al., 2007). Interestingly A3G, A3F, and noncatalytic A3G mutants interact with components of the HIV-1 preintegration complex,

namely integrase. This interaction might interfere with the structural integrity of this complex, resulting in diminished nuclear import and a lower integration rate (Luo et al., 2007).

In general, most of the cytidine deaminase–independent actions of A3 proteins are dose-dependent, with the greatest effects occurring at higher levels of expression, which may exceed physiological concentrations of enzyme (Holmes et al., 2007; Miyagi et al., 2007; Schumacher et al., 2008). However, in the aforementioned *in vitro* RT study where a hypermutation-independent decrease in viral cDNA synthesis was observed, wildtype A3G was used at levels analogous to that found endogenously (Bishop et al., 2008).

Comparison of noncatalytic mutants of A3G and A3F revealed that both proteins can function as antiviral proteins in the absence of cytidine deaminase activity, although an important portion of their antiviral activity depends on this enzymatic activity Overall, the loss of deaminase activity appears to have a more pronounced effect on the antiviral activity of A3G than A3F (Holmes et al., 2007).

In addition to these described deaminase-independent antiviral activities of A3G, a post-entry restricting activity of low molecular mass A3G was reported in resting CD4 T cells (Chiu et al., 2005). These cells are normally refractory to HIV-1 infection due to a postentry block exerted at or shortly after reverse transcription (Stevenson et al., 1990; Zack et al., 1990). A role for A3G in this post-entry block was suggested by the finding that siRNA-mediated knockdown of A3G in resting CD4 T cells rendered these cells permissive to HIV infection. While a similar postentry restricting function for A3G has also been described in dendritc cells (Pion et al., 2006) and CCR6 expressing memory T cells (Lafferty et al., 2010), the initial results in resting T cells have proven difficult to reproduce (Kamata et al., 2009; Santoni de Sio and Trono, 2009). As such, the mechanisms underlying the nonpermissiveness of resting CD4 T cells to HIV infection remains unclear.

7) Vif-mediated degradation of A3G

A3G can effectively inhibit the spread of ΔVif HIV-1 but not wildtype HIV-1. A principal function of the Vif protein is to circumvent the action of A3G (Fig. 2). After the discovery of A3G, it was observed that Vif reduces A3G incorporation into the budding virion by ~99% (Kao et al., 2003;Mariani et al., 2003;Marin et al., 2003;Mehle et al., 2004b;Sheehy et al., 2003;Stopak et al., 2003), suggesting that Vif inhibits the packaging of A3G into virions. To achieve this, Vif could directly inhibit the encapsidation of A3G or deplete the cellular pool of A3G. While there is evidence for both mechanisms, the depletion of cellular A3G levels has been shown to be paramount. Vif accomplishes this by hijacking a cellular E3 ubiquitin ligase complex. Vif binds to A3G and simultaneously to an E3 ubiqutin ligase complex consisting of cullin5 (Cul5), elonginB, elonginC, and a Ring finger protein (Rbx). Rbx binds to an unknown, E2 ubiquitin-conjugating enzyme (E2). Vif therefore connects the ligase complex and its substrate A3G (Yu et al., 2003). Recruitment of the E3 ubiquitin ligase complex induces the polyubiquitination of A3G and directs it to the 26S proteasome for degradation (Conticello et al., 2003;Kobayashi et al., 2005;Marin et al., 2003;Mehle et al., 2004b;Sheehy et al., 2003;Stopak et al., 2003;Yu et al., 2003) (Fig. 2 point 1 and Fig. 3B).

A recent report identified four critical lysine residues (K297, K301, K303, and K334) in the C-terminal region of A3G that are required for Vif-mediated degradation (Iwatani et al., 2009). Mutation of these residues restored the antiviral function of A3G in the presence of Vif (Iwatani et al., 2009). However, another study showed evidence for the polyubiquitination and degradation of a lysine-free A3G in the presence of HIV-1 Vif (Shao et al, 2010). Other data suggest that the polyubiquitination of Vif is required for A3G degradation, rather than A3G polyubiquitination (Dang et al., 2008b). Therefore, it remains unresolved which residues in A3G, if any, are important for A3G polyubiquitination and proteasomal degradation.

HIV-1 Vif binds the E3 ubiquitin ligase complex through at least two interaction sites: it binds to the elongin C protein through its SOCS box, a $S^{144}LQYLA^{149}$ motif, (Mehle et al., 2004a; Yu et al., 2004b), and to cullin5 through a zinc-binding $H^{108}x_5Cx_{17\text{-}18}Cx_{3\text{-}5}H^{139}$ motif (Luo et al., 2005; Mehle et al., 2006) (Fig. 3B). Interestingly, the SOCS box of Vif is the most conserved region in different Vif proteins (Oberste and Gonda, 1992), indicating that this motif is crucial for the function of Vif. In addition, post-translational modifications may influence Vif's function. Vif's binding to elonginC can be negatively regulated by the phosphorylation of S144 in the SOCS box motif (Mehle et al., 2004a). An S144A mutation that prevents phosphorylation leads to the production of virions with poor infectivity, although this mutant effectively depletes A3G.

The integrity of the zinc-binding motif is also indispensable for Vif function. Any mutations that alter the arrangement of the zinc-binding domain or the spacing within the HCCH motif markedly compromise the ability of Vif to recruit A3G to the E3 ubiquitin ligase (Paul et al., 2006; Xiao et al., 2006). The binding of zinc to the HCCH leads to a conformational change in Vif, rendering it capable of forming high-order protein assemblies, probably by altering the protein conformation to expose protein-protein interaction sites (Giri and Maynard, 2009; Paul et al., 2006). Therefore, chelation of zinc by a cell-permeable zinc chelator inhibits Vif's function in infectivity assays, allowing the virus to become sensitive to the antiviral activity of A3G (Xiao et al., 2007).

8) Vif facilitates HIV-1 in addition to promoting the degradation of APOBEC3

Several mechanisms have been proposed to explain how Vif increases the viral infectivity of HIV-1 besides degrading A3 proteins. For instance, Vif can deplete intracellular A3G by impairing the translation of its mRNA (Kao et al., 2003; Mariani et al., 2003; Stopak et al., 2003). Vif achieves this by binding to the 3′UTR and the 5′UTR of the A3G mRNA (Mercenne et al., 2010) (Fig. 2, point 2).

Vif also directly prevents the encapsidation of A3G, as shown by Vif's ability to inhibit the packaging of the degradation-resistant A3G mutant C97A (Opi et al., 2007) and by the greater reduction of A3G levels in the virion than in the cell (Kao et al., 2007; Mariani et al., 2003; Schrofelbauer et al., 2004) (Fig. 2, point 3). These observations could indicate that Vif competes for the A3G binding site on viral genomic RNA or components of Gag, both of which are needed for A3G encapsidation (Kao et al., 2004; Mariani et al., 2003; Opi et al., 2007).

Furthermore, both Vif and Vpr, another HIV-1 accessory protein, can induce G2 cell-cycle delay by recruiting the same E3 ubiqutin ligase complex that targets A3 proteins for degradation (DeHart et al., 2008; Sakai et al., 2006). To add even more complexity to its mode of action, Vif also induces the degradation of Vpr (Wang et al., 2008) and therefore alters Vpr-induced G2 arrest in HIV-1-infected cells. These findings suggest that Vif alters the activity of A3G by means beyond its degradation in 26S proteasome, although this remains the dominant mechanism by which Vif counters the action of A3G.

9) Interaction between Vif, A3G, and A3F

Because it is difficult to express soluble full-length A3G or Vif at high levels in prokaryotic cells or insect cells, data on the three-dimensional structure of the A3G–Vif interaction are not yet available (Auclair et al., 2007; Iwatani et al., 2006; Reingewertz et al., 2009; Stanley et al., 2008). However, considerable information has emerged from the analysis of mutants in Vif-A3G interaction assays (Fig. 3).

The interaction between A3G and Vif is critically dependent on amino acids 128–130 in A3G (Huthoff and Malim, 2007; Russell et al., 2009b) (Fig. 3B). Their importance is shown by the

fact that the species specificity of HIV/SIV Vif is determined by D128 in A3G. More precisely, the African green monkey (agm) A3G protein contains a lysine (K) in lieu of aspartic acid (D) at position 128 and is not degraded by HIV-1 Vif. Substitution of D for K in agm A3G renders the enzyme sensitive to HIV-1 Vif, while mutation of D to K in human A3G makes it resistant to HIV-1 Vif but sensitive to SIV-1 Vif (Bogerd et al., 2004; Mangeat et al., 2004; Schrofelbauer et al., 2004; Xu et al., 2004). The corresponding sequence in Vif that likely interacts with the D128 region of A3G is $D^{14}RMR^{17}$. Substitution of Vif $D^{14}RMR^{17}$ with $S^{14}ERQ^{17}$ or $S^{14}EMQ^{17}$, the sequence found in agm Vif, allows the mutant HIV-1 Vif to degrade agm A3G but not human A3G. This species specificity was attributed to two positively charged amino acids (15 and 17) in Vif and their interaction with negatively charged D128 in human A3G (Schrofelbauer et al., 2006). Another region in Vif that is essential for the degradation of A3G is facilitated by amino acids 40–44 ($Y^{40}RHHY^{44}$) (Russell and Pathak, 2007; Yamashita et al., 2008). Of note, co-immunopreciptation experiments demonstrated that the $Y^{40}RHHY^{44}$ domain is actually the region necessary for the binding of Vif to A3G, while the $D^{14}RMR^{17}$ domain is more involved in a secondary step necessary for A3G degradation (Russell and Pathak, 2007). Indeed, Vif can bind the D128K A3G mutant but cannot facilitate its degradation (Russell and Pathak, 2007; Xu et al., 2004). Another region important for A3G binding to Vif is the amino acid stretch between 82 and 99 (Santa-Marta et al., 2005).

Other A3 family members besides A3G are also active against HIV-1, as will be discussed below. The most important is A3F, which is expressed in the natural target cells of HIV-1, but is produced at lower levels than A3G and is a less potent inhibitor of HIV-1 (Koning et al., 2009; Liddament et al., 2004; Simon et al., 2005; Xu et al., 2004; Zennou and Bieniasz, 2006). Vif can also degrade A3F, but with lower efficiency (Liddament et al., 2004; Zennou and Bieniasz, 2006).

Vif binds to A3F via the $D^{14}RMR^{17}$ domain, but not the $Y^{40}RHHY^{44}$ domain, which is essential for A3G degradation. Therefore Vif has two different binding sites—the $Y^{40}RHHY^{44}$ site, which is critical for A3G binding, and the $D^{14}RMR^{17}$ site, which is critical for A3F binding (Russell and Pathak, 2007). Interestingly, mutations in the A3G binding domain of Vif increase its sensitivity to A3F. Hence, it seems that Vif evolved to target the more potent A3G, even though Vif can reduce A3F with lower efficiency (Russell et al., 2009b) (Fig. 3A).

Other amino acids involved in the binding of Vif to A3F, but not to A3G, are W11, Q12, 74-76, and W79 (He et al., 2008; Russell and Pathak, 2007; Simon et al., 2005; Tian et al., 2006). In contrast, I9, K22, E45, Y40, and N48 are critical for Vif's ability to degrade A3G, but not A3F (Russell and Pathak, 2007; Simon et al., 2005; Wichroski et al., 2005). The amino acids $Y^{69}xxl^{72}$ (He et al., 2008; Pery et al., 2009; Yamashita et al., 2008) and the residues W5, W21, W38, N48, and W89 are needed to degrade A3G as well as A3F (Tian et al., 2006). Recently two groups identified $W^{21}KSLVK^{26}$ as a novel motif in Vif critical for neutralizing A3G and A3F (Chen et al., 2009; Dang et al., 2009) with K22 and K26 playing especially important roles in this neutralization. However, these groups disagreed about the importance of $S^{23}LV^{25}$ residues for neutralization of A3F and A3G. One group identified SLV as important for both A3G and A3F neutralization (Dang et al., 2009), while the other found only L24 to be important for both A3G and A3F neutralization, and V25 important for the degradation of only A3F (Chen et al., 2009) (Fig. 3A). Recently two additional domains in Vif, $L^{81}GxGxSIEW^{89}$ and $E^{171}DRW^{174}$, were shown to be important for Vif neutralization of A3G and A3F. In the $L^{81}GxGxSIEW^{89}$ domain, residues $S^{86}IEW^{89}$ are involved in Vif binding to A3G, A3F and Cul5; residue G84 is important for Vif binding to both A3G and A3F; and residues L81, G82 are involved in Vif binding to A3F. The $E^{171}DRW^{174}$ domain is critical for Vif neutralization of A3F (Dang et al., 2010).

A3G and A3F also bind to different regions of Vif. A3G binds to the amino acid stretch 128–130 (Huthoff and Malim, 2007; Russell et al., 2009b), whereas A3F binds to amino acids 283–300 (Russell et al., 2009b).

Two additional domains are important for the steady-state levels of Vif and for interactions with tyrosine kinases—the $E^{88}WRKKR^{93}$ site (Fujita et al., 2004; Fujita et al., 2003) and the proline-rich $P^{161}PLP^{164}$ rich region (Donahue et al., 2008) (Fig. 3A). Mutating these sites results in reduced Vif expression (Fujita et al., 2004; Fujita et al., 2003), diminished Vif-Vif multimerization (Yang et al., 2003; Yang et al., 2001), loss of viral infectivity (Yang et al., 2001), and reduced binding to A3G (Donahue et al., 2008) and HIV-1 RT (Kataropoulou et al., 2009). Interestingly, Vif containing mutations or deletions in the PPLP motif (and in the HCCH and SOCS-box motifs) functions as a dominant negative inhibiting wild-type Vif from excluding A3G from virion encapsidation. Such dominant negative versions of Vif decrease virion infectivity (Walker et al. 2010).

10) Other A3 proteins

As noted above, the A3 family is comprised of seven members, A3A–H (Conticello et al., 2005). A3G and A3F have been the most intensively studied members due to their ability to restrict HIV spread. These proteins are over 50% identical, and both use the same conserved amino acids to bind RNA and to facilitate cytidine deamination. Like A3G, A3F acts on the minus-strand DNA, resulting in G-to-A mutation on the plus strand. However, unlike A3G, whose target sequence is CC, the target sequence of A3F is TC. A3F is expressed in the same cells as A3G, but at lower levels, and it is less potent than A3G (Bishop et al., 2004a; Liddament et al., 2004; Wiegand et al., 2004; Zennou and Bieniasz, 2006; Zheng et al., 2004). Nevertheless, G-to-A mutations induced by A3F can be easily identified in HIV-1 isolates from infected individuals (Simon et al., 2005). HIV-1 Vif degrades A3F and A3G by similar mechanisms, although the binding sites for these two proteins differ, and A3F is less sensitive to Vif-mediated proteasomal degradation than A3G (Liddament et al., 2004).

A3B has a moderate activity against HIV-1. A3B is highly similar to A3G and A3F, and like A3G and A3F, it contains two CDAs. It can also bind to the nucleocapsid of HIV-1 Gag and therefore is packaged into the budding virus. Unlike A3G and A3F, A3B is resistant to Vif-induced degradation. However, A3B is expressed at extremely low levels in the natural target cells of HIV-1 and thus is unlikely to exert antiviral effects against HIV-1 under normal conditions (Bishop et al., 2004b; Bogerd et al., 2007; Doehle et al., 2005).

A3DE corresponds to another A3 protein with two CDA domains. It displays moderate activity against HIV-1, but Vif can facilitate its degradation through the 26S proteasome. In the absence of Vif, A3DE is encapsidated into virions and can deaminate CG on the minus-strand DNA, resulting in GC-to-AC mutations on the viral plus strand (Dang et al., 2006).

A3 protein family members containing only a single CDA domain (A3A, A3C, and A3H) may also impair HIV-1. A3C is expressed in CD4 T cells and can introduce limited G-to-A mutations (Bourara et al., 2007). A3A is active against HIV-1 and is expressed at higher levels in monocytes than in macrophages (Peng et al., 2007). It was suspected that A3A is partly responsible for the relative resistance of monocytes to HIV-1 infection, as silencing of A3A increases their susceptibility to infection (Peng et al., 2007). A3H was thought to be unstable due to two different mutations (Dang et al., 2008a; OhAinle et al., 2008; OhAinle et al., 2006). However, the re-mutated stable form of A3H can restrict HIV-1 up to 150-fold. Whether A3H is associated with G-to-A hypermutation is still controversial (Dang et al., 2008a; Harari et al., 2009). Of note, a haplotype of A3H, hapII-RDD, is stably expressed in cells and is active against HIV-1. Although this haplotype is common in people of African descent (Harari et al.,

2009), stable endogenous expression could not be shown in PBMCs, the natural target cells of HIV-1 (Li et al., 2009).

11) Intravirion encapsidation of Vif

Vif is an RNA-binding protein, but it binds RNA in a relatively nonspecific manner. This is illustrated by its effective binding to homopolymeric RNA (Zhang et al., 2000). Importantly, Vif interacts with HIV-1 genomic RNA in the cytoplasm of infected cells (Dettenhofer and Yu, 1999; Khan et al., 2001; Zhang et al., 2000). In this case, Vif binds in a cooperative manner to the 5′-untranslated region of HIV-1 RNA and Gag (Bernacchi et al., 2007). More precisely, it binds with strong affinity to the apical loop of TAR and to a short region in Gag and with lower affinity to the neighboring primer-binding site and the whole leader region (Bernacchi et al., 2007; Henriet et al., 2005). The fact that Vif binds with different affinities to two sites suggests that when Vif is expressed at high levels, it will bind to the whole 5′ UTR region, leading to effective encapsidation into budding virions. Conversely, when Vif is expressed at low levels in the virus-producing cell, only the high-affinity binding sites will be engaged and much less Vif will be present in the virion. Thus, intravirion Vif levels correlate with intracellular expression levels (Simon et al., 1998b).

Vif also interacts with $Pr55^{gag}$ (Simon et al., 1999). Vif was found in a complex together with Gag and the Gag-Pol precursor (Zimmerman et al., 2002). Therefore, Vif's encapsidation into the virus might be mediated by an interaction with genomic RNA and partly by interaction with Gag and Gag-Pol (Bardy et al., 2001; Khan et al., 2001). In addition, a recent report suggests that virion encapsidation of Vif is dependent on Vif binding to A3G and A3F (Yamashita et al. 2010).

It is not entirely clear whether Vif is encapsidated into virions under normal conditions and if so in what amounts. An initial report suggested that 60–100 copies of Vif are incorporated per virion (Liu et al., 1995). Later studies lowered the amount to 20 copies or even none (Camaur and Trono, 1996; Dettenhofer and Yu, 1999; Simon et al., 1998b). These discrepancies were attributed to contamination during the virus preparation with Vif-containing microvesicles or secreted proteins and to nonspecific encapsidation due to overexpression of Vif (which overestimates copy number) or degradation of virion-incorporated Vif (which underestimates copy number), especially as virus-associated Vif is unstable (Henriet et al., 2009; Kao et al., 2003).

12) Naturally occurring variations of Vif, Cullin5, and A3G/F affect the fitness of HIV-1

The finding that ΔVif HIV-1 cannot effectively spread in cultures of CD4 T cells—a natural target of HIV-1 *in vivo*—has been principally attributed to the antiviral action of A3G and A3F proteins. Overexpression of A3G can modestly suppress wildtype HIV-1 (Mangeat et al., 2003; Sheehy et al., 2002; Zhang et al., 2003), probably by overwhelming the intracellular Vif block. These facts make the Vif-APOBEC3 interaction a worthwhile pharmacological target. Although current antiretroviral drugs effectively suppress viral replication and entry, this may not be true of viral escape mutants. Therefore, explorations of new drug targets are constantly needed.

G-to-A hypermutations were first recognized in the *env* gene region during propagation of HIV-1 *in vitro* (Vartanian et al., 1991). Periodic G-to-A hypermutations are easily detectable in clinical samples from HIV-1 infected subjects and can account for >9% of the integrated viral DNA in cells (Gandhi et al., 2008; Kieffer et al., 2005; Land et al., 2008; Simon et al., 2005). Hypermutated virus could not be found in patient plasma, indicating that A3G/F

function *in vivo* gives rise to hypermutated viral genomes that can be integrated but do not produce progeny viruses (Kieffer et al., 2005). Infection studies with ΔVif HIV-1 in CEM cells (which express A3G) and CEM-SS cells (which do not express A3G) show high levels of G-to-A mutations in viral DNA, as expected. However, significantly fewer mutations were detected in intracellular viral RNA, and even fewer in virion RNA (Russell et al., 2009a).

Genetic polymorphisms in A3G, A3F, or cullin5 and natural variations in their levels of expression could also affect HIV-1 pathogenesis. Indeed, in HIV-1-infected patients, A3G mRNA levels correlate inversely with HIV-1 viral loads and positively with CD4 cell counts (Jin et al., 2005), A3G mRNA levels are higher in long-term nonprogressors than in uninfected controls, and the lowest A3G mRNA levels are found in HIV progressors (Jin et al., 2005). Another study has described a significant increase in A3G expression in PBMCs from HIV-1-exposed seronegative individuals compared to HIV-1-seropositive patients or healthy controls (Biasin et al., 2007).

Specific single nucleotide polymorphisms (SNPs) in A3G and cullin5 can affect the rapidity of HIV-1 disease progression. An SNP in A3G (H186R), which is highly abundant in African Americans (37%) but rare in Europeans or European Americans (5%, <3%), is strongly associated with a more rapid decline in the number of CD4 T cells and accelerated progression to AIDS (An et al., 2004; Reddy, 2010). This can also be seen with a codon-changing variant in the fourth exon of A3G and a 3′ extragenetic mutation (Reddy et al., 2010). The cullin5 gene exhibits several different SNPs, which can be classified into two clusters or haplotypes. Interestingly, the two haplotypes produce opposing effects on CD4 T-cell counts during HIV-1 infection; the loss of these cells was delayed by cluster I and accelerated by cluster II. Cullin5 polymorphisms linked to accelerated CD4 T cell decline are associated with increased HIV-1 viral load (An et al., 2007). These findings raise the possibility that the Vif–cullin5 interface might represent a tractable target for small molecule development

Other studies indicate that A3G/F hypermutation of HIV-1 is associated with increased CD4 T-cells count (Land et al., 2008) and a 0.7 $\log^{10}$ reduction in viremia (Pace et al., 2006). However, some studies have not found a correlation between A3G/F mRNA levels or polymorphisms and viral load and/or CD4 T-cell counts (Cho et al., 2006; Do et al., 2005; Gandhi et al., 2008; Piantadosi et al., 2009; Reddy, 2010).

Interestingly, defective Vif alleles that cannot effectively neutralize A3G/F are readily detected in infected patients (Simon et al., 2005). Unopposed but nonlethal A3G/F action could promote viral sequence diversification in these patients. This raises the question of whether these G-to-A mutations might actually be beneficial for the virus, for example by promoting HIV-1 drug escape mutants or using A3G-induced mutations as a means to achieve rapid viral evolution.

Low-level mutation and single nucleotide variations of HIV-1 caused by A3G/F have been identified (Keele et al., 2008), and increased drug resistance to 3TC induced by suboptimal A3G concentrations has been reported (Mulder et al., 2008). Although A3G/F hypermutations can contribute to HIV-1 evolution, mutations introduced by HIV-1 reverse transcriptase are more likely to be important in the evolution of drug-resistance variants (Berkhout and de Ronde, 2004).

13) The Vif – A3G interaction as a pharmacological target

Recently, increased efforts were made to block the A3G interaction with Vif. For example, inhibition of the Vif–Vif interaction with peptides that interfere with the PPLP motif of Vif restored A3G encapsidation in nonpermissive cells and reduced HIV-1 infectivity (Miller et al., 2007; Yang et al., 2003). While peptides will not emerge as viable Vif antagonists, these studies supporting the notion that true Vif antagonists could be an exciting new class of antiviral

drugs. The fact that the membrane-permeable zinc chelator (TPEN) prevents Vif function in nonpermissive cells by inhibiting cullin5 recruitment and A3G degradation (Xiao et al., 2007) provides yet an additional protein-protein interface for pharmaceutical targeting.

Without question, a potent, orally bioavailable small-molecule inhibitor of Vif would be an exciting medicinal lead. We and others have used different approaches to find an inhibitor of the interactions of Vif with A3G, cullin5, and elonginC (Cao et al., 2005; Nowotny et al., 2010)(W. Yonomoto, and W. C. Greene, unpublished data). Using YFP-tagged A3G and HIV-1 vectors with and without Vif, Rana and colleagues screened a library of 30,000 small molecules and identified 66 compounds, which were also scored in a secondary screen. The most promising was RN-18, which enhances Vif degradation in the presence of A3G, increases A3G virion encapsidation, and enhances hypermutation (Nathans et al., 2008). Recently, Cen et al. reported two compounds, IMB-26 and IMB-35, that protect A3G from Vif induced proteasomal degradation and recover A3G virion incorporation in the presence of Vif. These two compounds bind directly to A3G and thereby suppress the Vif/A3G interaction without inhibiting A3G's catalytic activity (Cen et al., 2010).

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Biography

Vitae

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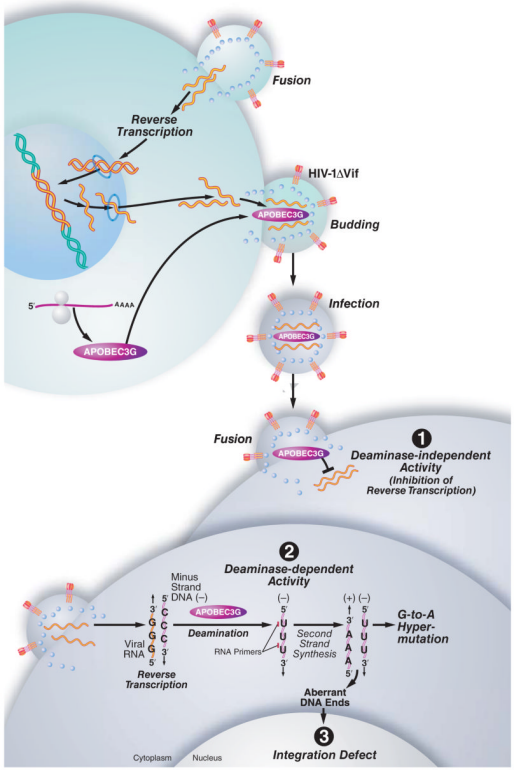

Figure 1. The impact of A3G on the lifecycle of ΔVif HIV-1

In cells infected with ΔVif HIV-1, A3G is not degraded and is effectively incorporated into the budding virus and transferred to the next target cell, where it exerts antiviral effects at multiple levels. (1) A3G can inhibit the elongation of the reverse transcriptase in a deaminase-independent manner. Presumably, A3G binds directly to the viral RNA and thereby impairs the movement of the reverse transcriptase along the RNA template. (2) Most importantly, A3G can trigger massive deamination of dC to dU during synthesis of the viral minus strand. During synthesis of the DNA plus strand, adenosines are incorporated instead of the original guanines, resulting in G-to-A mutation. (3) Finally, A3G also inhibits viral DNA integration and provirus formation, probably by inducing defects in tRNA cleavage during plus-strand DNA transfer, leading to the formation of aberrant viral DNA ends. A3G also interacts with the integrase enzyme of HIV-1, which might interfere with the integrity of the integration complex, resulting in diminished integration rates. Figure was adapted from Chiu et al. 2008 with permission.

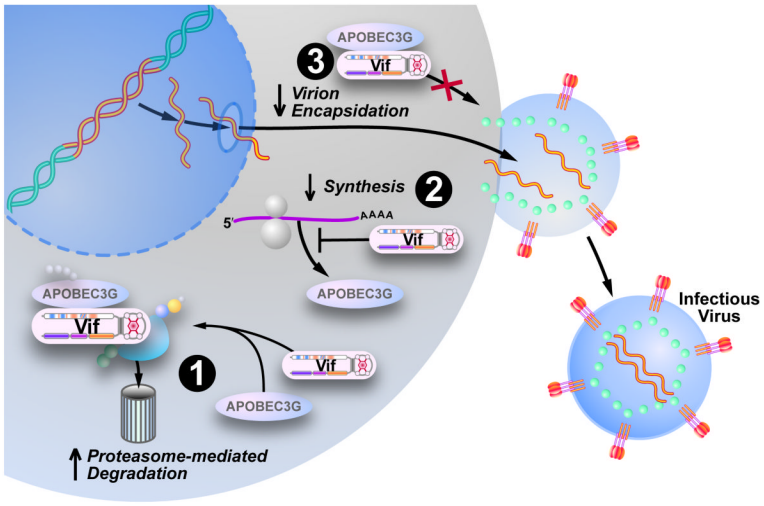

Figure 2. Vif diminishes the intracellular pool of A3G and A3F impairing virion encapsidation

(1) Vif facilitates the degradation of the antiviral proteins A3G and A3F by hijacking the cellular ubiquitin-proteasome pathway. Vif connects the cellular E3 ubiquitin ligase with A3G/A3F, which initiates the polyubiquitination of the A3 proteins, followed by their degradation by the 26S proteasome. (2) Vif also impairs the translation of A3 mRNA and therefore reduces the cellular protein pool. (3) Finally, Vif may also interfere with the encapsidation of A3 proteins into the budding virus in the absence of A3G/F degradation. Adapted, with permission, from Chiu et al. 2008.

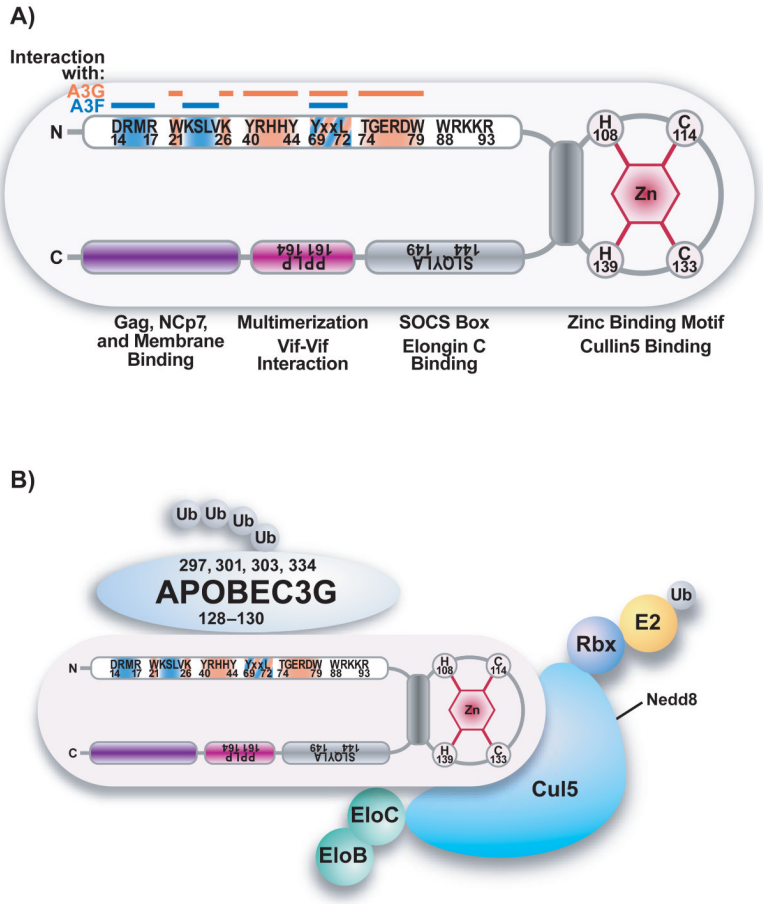

Figure 3. Summary of HIV-1 Vif domain structure and a model depicting the interaction of HIV-1 Vif, cullin5, elongin BC, and A3G

(A) Schematic structure of the Vif domains. The N-terminal A3G binding sites are indicated in orange, A3F binding sites in blue. The structure of the $H^{108}x_5Cx_{17\text{-}18}Cx_{3\text{-}5}H^{139}$ zinc finger motif that connects Vif to cullin5 is shown in gray as well as the viral BC-box ($S^{144}LQYLA^{149}$) that facilitates the binding to elongin C (EloC). Vif multimerization is facilitated by the hydrophobic amino acids $P^{161}PLP^{164}$ (purple). At the C-terminus the Gag, NC, and membrane binding domain is located (indicated in violet). B) Vif initiates the degradation of A3G by binding to the cytidine deaminase and simultaneously to an E3 ubiquitin ligase complex. This complex consists of cullin5 (Cul5), elonginB (EloB), elonginC (EloC), and a ring finger protein (Rbx). Rbx binds to an unknown E2 ubiquitin-conjugating enzyme (E2). Therefore Vif connects the ligase complex and its A3 protein substrates to initiate polyubiquitination and proteasome-mediated degradation of A3 proteins.